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Spectral Properties of the Prototropic Forms of Fluorescein in Aqueous Solution

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The commonly used fluorescent probe, fluorescein, can exist in seven prototropic forms. We have used global analysis procedures to reanalyze the absorption data of Diehl and Horchak-Morris (*Talanta* **34**, 739–741, 1987) in terms of five alternative ionization models. We identify the forms of fluorescein present in aqueous solution and the pK_a of each ionisation transition. The pK_a values of the neutral xanthene, carboxylic acid, and cationic xanthene groups are 6.3, 3.1–3.4, and 3.1–3.4, respectively, and the pK_a value of lactonization is 2.4. As a consequence, the neutral form of fluorescein is a mixture of the lactone (70%), zwitterionic (15%), and quinoid (15%) forms. A knowledge of the forms present in solution permits the characterization of their spectral properties. It is shown that the quinoid and monoanion forms have similar absorption spectra, while the zwitterion spectrum is similar to that of the cation but blue-shifted by 3 nm. The emission spectra of the monoanion and quinoid forms are also identified and shown to be similar but not identical. A model for the excited-state reactions of fluorescein is presented.

KEY WORDS: Fluorescein; ionization.

INTRODUCTION

The popularity of fluorescein as a fluorescent probe is associated with its high quantum yield and its absorption and emission in the visible region of the spectrum. These properties are of great significance to biologists who require a probe which can sense biochemical reactions at low concentrations yet possesses an emission in the visible region of the spectrum well removed from the intrinsic background fluorescence of biological samples. Unfortunately, fluorescein possesses three disadvantages—it is prone to photodecomposition, particularly in the presence of oxygen[1]; it has a relatively short lifetime, which limits its use in fluorescence anisotropy studies; and more importantly, it possesses complex prototropic equilibria, which makes its spectral properties particularly sensitive to pH. While the pH sensitivity of fluorescein and its derivatives can be put to practical use, it is important to understand the physicochemical basis of its emission characteristics.

The multiple prototropic forms of fluorescein arise from the presence of the xanthene and benzoic acid moieties which can exist in multiple ionization states. The presence of the benzoate carboxyl group also makes it possible for the molecule to form a lactone. Zanker and Peter[2] identified seven prototropic forms of fluorescein based on absorption spectra in dioxane/water mixtures at various hydrogen ion concentrations (Fig. 1). The work was subsequently extended by Lindqvist,[1] who measured the variation in absorbance with pH in aqueous solution. These measurements have since been reproduced a number of times, and in all cases the data have been analyzed in terms of the reaction scheme shown as Model A in Fig. 2. Although the results obtained have been consistent—three transitions corresponding to pK_a values of 2.2, 4.4, and 6.3—there are

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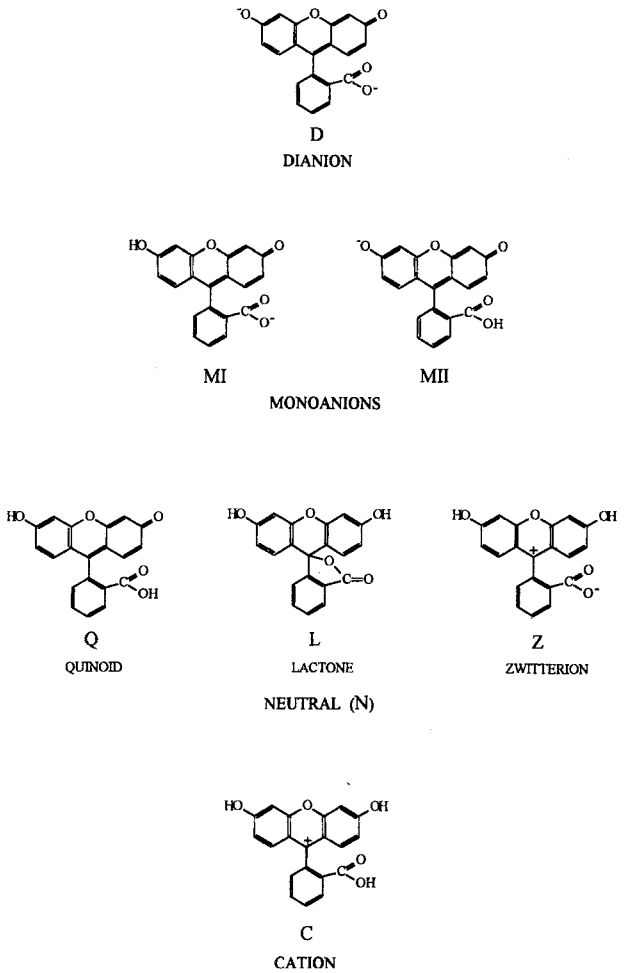

Fig. 1. Prototropic forms of fluorescein.

differences in the interpretations concerning the nature of the neutral species present in aqueous solution. This is sometimes regarded as a single neutral species (quinoid[3,4] or lactone[5]) or as a mixture of two (quinoid and lactone[1]) or three (quinoid, lactone, and zwitterion[6,7]) neutral forms.

It is difficult to use the absorption properties of fluorescein to determine the forms present in aqueous solution due to the overlapping absorption spectra involved and to the relatively small differences in the pK_a values of the ionizable groups. The fluorescence properties of fluorescein are further complicated by the presence of excited-state reactions, which allow some of the species to interconvert during the lifetime of the excited state.[3,6,8,9]

Presented here is a reanalysis of the absorption data obtained by Diehl and Horchak-Morris[10] in terms of more complex models which allow the identification of

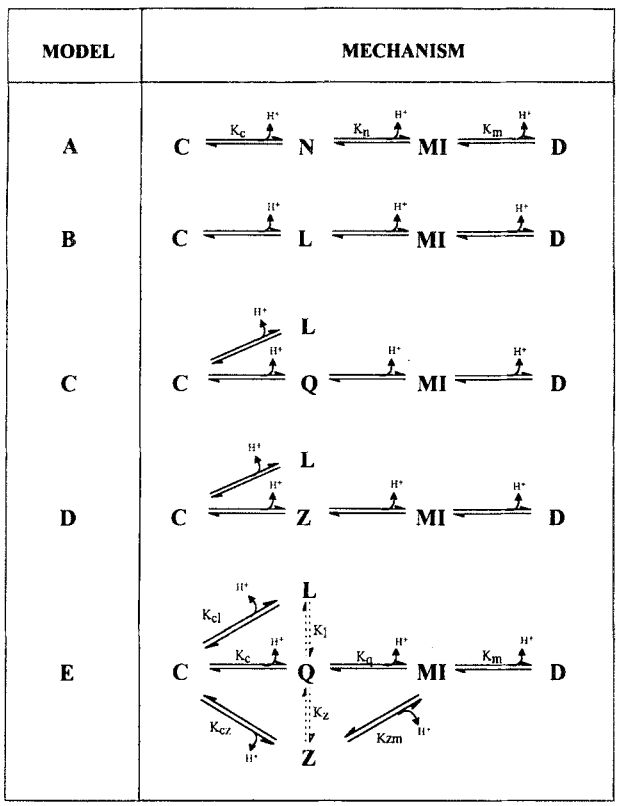

Fig. 2. Models (A–E) used to analyze the absorption titration data of fluorescein. Abbreviations are defined in Fig. 1.

the different forms of fluorescein present in aqueous solution. This identification permits the characterization of the absorption and fluorescent properties of the different forms.

EXPERIMENTAL

Fluorescein. Disodium fluorescein was from Sigma and was shown to be pure by thin-layer chromatography using a chloroform:methanol:water (65:25:4) solvent system. Concentrated stock solutions were stored in methanol at −20°C in the dark. Fluorescein concentrations were determined spectrophotometrically after dilution in 0.01 *M* NaOH using an extinction coefficient of 88,000 M^{-1} cm^{-1} at 490 nm.[1,11–15]

Solutions. Dilute buffers at low ionic strength were used to minimize the effects of salt[3] and buffer concentration[9] on the spectral properties of fluorescein. The solutions used were 0.01 *M* NaOH, 5 m*M* sodium phosphate (pH 6.4 and 6.0), 5 m*M* potassium hydrogen

phthalate (pH 5.5, 4.5, 3.3), 5 m*M* KCl (pH 2.5), and 5 m*M* KCl (pH 1.6).

Absorption and Fluorescence Spectra. Absorbance spectra were measured on a Cary 5 UV-VIS spectrophotometer. Corrected fluorescence spectra were measured on a SPEX Fluorolog-τ2. The fluorescein concentrations were less than 10 μM and less than 900 nM for the absorbance and fluorescence measurements, respectively, and were within the linear range. All spectroscopic measurements were performed at 20°C.

Fluorescence Lifetimes. Lifetime measurements were performed by the phase method on a SPEX Fluorolog-τ2 at 20°C using a glycogen suspension as the reference lifetime. Typically, 8–12 frequencies were scanned from 10 to 200 Mhz and the phase and modulation data analyzed using Globals Unlimited software. The fluorescein concentration used was 1 μM. Preliminary measurements of the dianion lifetime revealed that there was a concentration-dependent increase in the measured lifetime at concentrations higher than 2 μM, which was probably a consequence of the reabsorption and subsequent reemission of the fluorescent light.[16,17] In all cases, the data could be adequately fitted to a single discrete lifetime.

Analysis of Absorbance Titration. Diehl and Horchak-Morris obtained absorption data at five wavelengths for the pH titration of fluorescein at constant ionic strength (0.1 M KCl).[10] These data were reanalyzed according to the models shown in Fig. 2. The total fluorescein concentration of 24.07 μM was altered to a value of 21.58 μM to be consistent with previously published values of 88,000 M^{-1} cm^{-1} for the extinction coefficient of the dianion at 490 nm.

Model A has been used previously to interpret absorption spectra of fluorescein. In this model, the neutral form is considered a distinct species with its own unique absorbance. In other models, the neutral form is regarded as one, two, or three distinct species. The following assumptions are made in these analyses.

(i) The state of ionization of the xanthene group is the main determinate of the absorption properties of the molecule in the visible region of the spectrum. The spectral properties of various fluorescein analogues are consistent with the notion that the state of ionization of the carboxyl group does not significantly affect the absorption of the molecule.[5–7] As a consequence, the absorption spectra of the monoanion MI (Fig. 1) and the neutral quinoid species Q (Fig. 1) are assumed to be identical, as are the absorption spectra of the cation and zwitterion species.

(ii) The lactone form is colorless in the visible region of the spectrum. Inspection of the lactone structure reveals that lactonization destroys the resonance structure of the xanthene group. The colorless nature of the lactone has been previously observed in dioxane.[1,2]

(iii) The lactone form of fluorescein is one of the neutral species present in solution. Fluorescein shows a characteristic decrease in absorbance in the pH region where the neutral form exists. This behavior is not observed with fluorescein analogues which are incapable of forming the lactone.[5–7]

(iv) The lactone species is formed from the cation. Inspection of possible mechanisms of lactone formation indicate that the most likely mechanism is from a cation (or zwitterion) intermediate.

(v) The monoanion form MII (Fig. 1) is not present in aqueous solution. Previous analyses according to Model A indicate that the relative acidities of the xanthene and benzoic acid groups differ by two orders of magnitude and indicate that the MII species is a minor component.

Five possible ionization models (A–E) are presented in Fig. 2. To illustrate the approach adopted, we describe the analysis of Model E. The concentration of each species at equilibrium is

$$q = cK_c/[H^+] \quad (1)$$

$$l = cK_cK_l/[H^+] \quad (2)$$

$$z = cK_cK_z/[H^+] \quad (3)$$

$$m = cK_cK_q/[H^+]^2 \quad (4)$$

$$d = cK_cK_qK_m/[H^+]^3 \quad (5)$$

The total concentration of fluorescein (C_t) is constant throughout the titration:

$$C_t = d + m + q + l + z + c \quad (6)$$

Substituting Eqs. (1) to (5) into Eq. (6) and solving for c,

$$c = C_t[H^+]^3/([H^+]^3 + [H^+]^2 K_c(1 + K_l + K_z) + [H^+] K_cK_q + K_cK_qK_m) \quad (7)$$

The total absorbance at each wavelength (Abs_λ) is the sum of the absorbances of each species:

$$Abs_{(\lambda)} = \varepsilon_{D(\lambda)} d + \varepsilon_{M(\lambda)} (m + q) + \varepsilon_{C(\lambda)} (c + z) \quad (8)$$

where $\varepsilon_{i(\lambda)}$ is the extinction coefficient of the ith species at each wavelength.

The data were fitted globally according to Eq. (8) by linking the equilibrium constants across five wavelengths using the curve-fitting facility of SigmaPlot. The extinction coefficients at each wavelength and the equilibrium constants were allowed to float during the fitting procedure. The additional equilibrium constants (pK_{cl},

Table I. χ^2 Values Resulting from the Global Analysis of the Absorption Titration Data of Diel and Horchak-Morris [10] According to Models A–E Depicted in Fig. 2

Model	χ^2
A	0.21
B	0.97
C	0.46
D	0.79
E	0.25

pK_{cz}, pK_{zm}) were calculated:

$$pK_{cl} = pK_c - \log(K_l) \quad (9)$$

$$pK_{cz} = pK_c - \log(K_z) \quad (10)$$

$$pK_m = pK_q + \log(K_z) \quad (11)$$

Resolution of Monoanion Absorption Spectrum. Monoanion absorption spectra were resolved from spectra obtained at pH 6.4, 6.0, and 5.5 by subtracting the dianion spectrum obtained in 0.01 *M* NaOH until the ratio of the absorbances obtained at 490, 475, 464, 455, and 437 nm matched the ratio of the extinction coefficients of the monoanion determined from the analysis according to Model E (Table III) at these same wavelengths. The three resolved monoanion spectra were superimposable.

Resolution of Fluorescence Spectra. A single fluorescent species present in solution generally produces excitation and emission spectra which are independent of the respective emission and excitation wavelengths. A solution containing two noninteracting fluorescent species produces excitation and emission spectra which are dependent on the emission/excitation wavelengths. However, if the spectrum of one species is known, subtraction of its contribution should result in the resolution of the spectrum of the unknown species, which should then become wavelength independent. This principle was used to remove the dianion contribution from fluorescein solutions containing multiple species. This was achieved by obtaining the dianion excitation and emission spectra at multiple wavelengths in 0.01 *M* NaOH. These were progressively subtracted from corresponding spectra obtained in the region pH 4.5–6.4 until the resultant normalized spectra were superimposable. These spectra are referred to as dianion-corrected spectra. The amount of the dianion spectra which was required to be subtracted also permitted the estimation of the dianion concentration.

Quantum Yield Calculations. Quantum yields were calculated from emission spectra obtained at 455-nm excitation. The spectra were divided by the absorbance at that wavelength and the quantum yield calculated relative to the dianion (in 0.01 *M* NaOH) by integration assuming that the latter has a quantum yield of 0.93.[13,18] The emission spectra used in these calculations were recorded from 460 to 750 nm.

RESULTS

Analysis of Absorption Titration. The data of Diehl and Horchak-Morris[10] were analyzed according to the models shown in Fig. 2 using the approach described above. The χ^2 values obtained from each analysis are summarized in Table I. The models differ with respect to the nature of the neutral form: in Model A this is regarded as a single species whose extinction coefficient is unknown, whereas in the other models it is assumed that the lactone is one of the neutral species present. For Models B to E, best fits to the titration data at the five wavelengths, as judged by χ^2 values, were obtained with Model E, indicating that the neutral form of fluorescein in solution is a mixture of the three neutral species.

The fit obtained with Model A was similar to that obtained with Model E (Fig. 3). The parameters obtained from these analyses are summarized in Tables II and III. Both models produced similar values for the pK_m and the extinction coefficients of the cation, monoanion, and dianion. Diehl[4] obtained similar parameters in the analysis of the same data using Model A (when corrections are made for the differences in the fluorescein concentration). The similarity of the extinction coefficients indicates that the assumptions regarding the absorbances of the three neutral species made in Model E were valid. If this were not the case, then differences would be expected between the cation and the monoanion extinction coefficients determined according to the different models.

The values obtained for pK_q, pK_c, K_l, and K_z determined from Model E were highly correlated. In contrast, the values of pK_n and pK_c determined from Model A were not correlated. By assuming that the neutral form in Model A is a mixture of the three neutral species and that the lactone has no absorbance, while the zwitterion and quinoid have the same extinction coefficients as the cation and monoanion, respectively, the relative contribution of each species to the neutral form can be determined. The values of K_l and K_z determined in this way are shown in Table II. In addition, the following values can also be determined:

$$K_c^E = K_c^A/(K_l^A + K_z^A + 1) \quad (12)$$

$$K_q^E = K_n^A (K_l^A + K_z^A + 1) \quad (13)$$

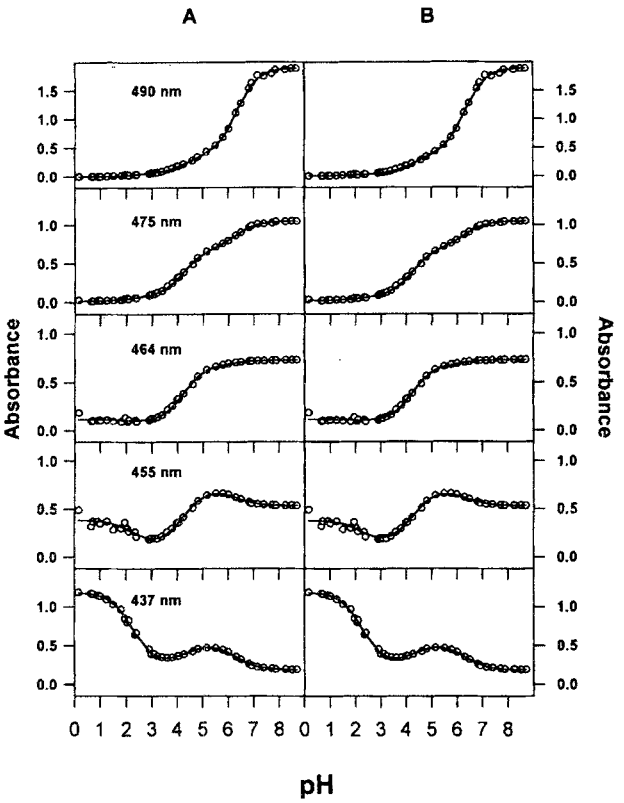

Fig. 3. Analysis of the absorption titration data for fluorescein reported by Diehl and Horchak-Morris[10] (open symbols). The lines represent the best fit obtained by global analysis of the data according to Models A (A) and E (B). The fitted parameters (pK_a values and molar extinction coefficients) are summarized in Tables II and III.

where the superscript A denotes the equilibrium constants determined from Model A and the superscript E the equilibrium constants as defined in Model E. The pK_a values determined in this way are similar to those obtained directly from Model E (Table II). The pK_a values of the cationic xanthene, benzoic acid, and neutral xanthene groups were thus determined to be 3.1–3.4, 3.1–3.4, and 6.3, respectively, while the pK_a for lactonization was 2.4. The pH dependence of the prototropic forms based on these parameters are shown in Fig. 4.

Absorption Spectra. Dianion and cation spectra were obtained in 0.01 *M* NaOH and 1 *M* HCl, respectively. The monoanion absorption spectrum was resolved at pH 5.5–6.4. Under these conditions, the monoanion and dianion should be the only absorbing species present.

Attempts to resolve the monoanion spectrum at pH 4.5 were unsuccessful as would be expected from inspection of Fig. 4, which shows that the zwitterion and quinoid species are also present at this pH. The dianion contribution to this spectrum was removed based on the absorbance ratio at 490 and 500 nm for the monoanion, while the monoanion contribution was removed based on the remaining absorbance at 490 nm. The result was a residual spectrum which resembled the cation spectrum (Fig. 5). The absence of any significant absorbance at wavelengths greater than 475 nm indicates that there was very little contribution from quinoid absorbance to this spectrum. The latter was presumably subtracted with the monoanion contribution, indicating that the quinoid possesses a similar spectrum to the monoanion. The residual spectrum was therefore identified as the zwitterion spectrum.

Further support for the nature of quinoid and zwitterion spectra was obtained by the ability to reconstruct the spectrum obtained at pH 3.3 from monoanion, cation, and zwitterion spectra. At this pH, there should be considerably more quinoid than monoanion. The inability to reconstruct the spectrum solely from the cation and monoanion spectra indicates that the zwitterion and cation spectra, although similar are not identical. These results were combined to produce the absorption spectra and extinction coefficients for the prototropic forms shown in Fig. 5.

Fluorescence Spectra. The excitation and emission spectra of fluorescein in 0.01 *M* NaOH were independent of the emission/excitation wavelengths. The emission spectrum was a mirror image of the excitation spectrum, and the excitation spectrum was superimposable on the absorption spectrum (Fig. 6). In contrast, fluorescence spectra obtained at pH 4.5–6.4 were dependent on the excitation and emission wavelength, indicating the presence of multiple species. In each case spectra independent of the excitation and emission wavelength could be produced by subtraction of the dianion contribution to the fluorescence. An example of the procedure is shown in Fig. 7. The dianion concentration which was required to produce these wavelength-independent spectra corresponded to the dianion concentration which was determined from the absorbance analysis (Table IV). We make the following points.

(i) The dianion-corrected fluorescence spectra resolved at pH values 5.5, 6.0, and 6.4 (Figs. 8A–C) were superimposable. The respective excitation spectra were similar to the monoanion absorption spectrum, an indication that these resolved fluorescence spectra represented the monoanion fluorescence.

(ii) The emission spectrum resolved at pH 4.5 (Fig. 8D) was identical to the monoanion emission spectrum but the excitation spectrum corresponded to the dianion-corrected absorption spectrum at the same pH.

(iii) Fluorescence spectra measured below pH 4.5 were all independent of the excitation and emission

Table II. pK_a Values Determined from Analysis of the Absorption Titration Data According to Models A and E[a]

	pK_m	pK_n	pK_q	pK_c	K_l	K_z	K_{cl}	pK_{cz}	pK_{zm}
Model A	6.31 (± 0.01)	4.23 (± 0.02)	—	2.25 (± 0.02)	—	—	—	—	—
Model E	6.32 (± 0.01)	—	3.27 (± 0.05)	3.20 (± 0.04)	6.6 (± 0.6)	1.5 (± 0.2)	2.38	3.01	3.46
Model A[b]	6.31	—	3.43	3.05	4.30	0.94	2.42	3.08	3.40

[a] The pK_i notation is indicated in Fig. 2. Standard errors are indicated in parentheses.
[b] Values obtained from the analysis according to Model A recalculated according to definitions in Model E (see text).

Table III. Extinction Coefficients Determined from Analysis of the Absorption Titration Data According to Models A and E

Model	Species	Extinction coefficient (M^{-1} cm^{-1})[a]				
		490 nm	475 nm	464 nm	455 nm	437 nm
A	D	87,692	48,649	33,799	24,560	8,706
	MI	16,425	31,945	31,562	32,671	24,059
	N	2,695	3,934	4,172	5,887	12,144
	C	34	966	5,329	17,752	54,984
E	D	87,759	48,652	33,813	24,598	8,654
	MI	17,105	32,176	31,524	32,352	24,117
	C	306	1,048	5,267	17,527	55,063

[a] Standard errors of determinations were in the range ± 300–500.

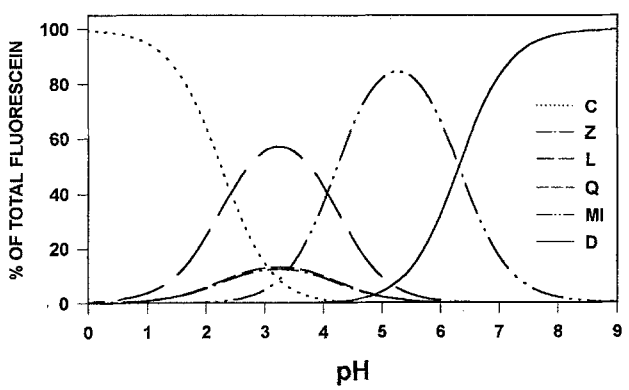

Fig. 4. The pH dependence of the various species of fluorescein in aqueous solution. The data were calculated according to the pK_a values reported for Model E (see Table II and text). Abbreviations are defined in Fig. 1.

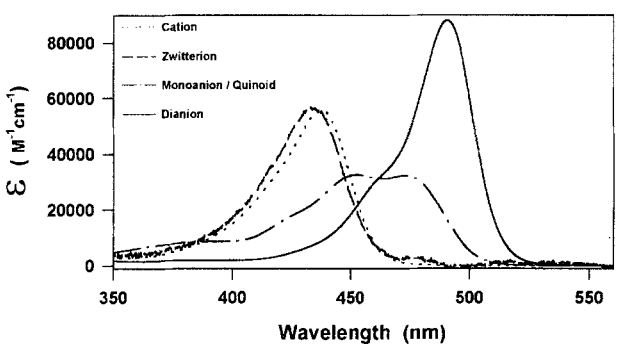

Fig. 5. Absorption spectra of the prototropic forms of fluorescein in aqueous solution. The dianion and cation spectra were obtained in 0.01 *M* NaOH and 1 *M* HCl, respectively. The monoanion and zwitterion spectra were resolved as described in the text. An extinction coefficient of 32,176 M^{-1} cm^{-1} at 475 nm was assumed for the monoanion and 55,000 M^{-1} cm^{-1} at 437 nm for the zwitterion.

wavelength, with the excitation spectra superimposable on the absorption spectra (Figs. 8E–G). Emission spectra obtained under these conditions were similar to the monoanion emission spectra. However, closer inspection revealed subtle differences between the monoanion emission spectrum and the spectra obtained under more acidic conditions, with a transition apparent at about pH 3.5 (Fig. 9). The emission spectrum obtained at these higher acidities was assumed to represent the quinoid emission (see Discussion).

Quantum Yields and Lifetimes. The quantum yields and lifetimes of each fluorescent species of fluorescein in aqueous solution are reported in Table V. For the quinoid, these parameters were measured at pH 1.6, where this form is the only emitting species. The monoanion quantum yield was determined by using the resolved monoanion absorption and emission spectra. In contrast, the lifetime was determined at pH 4.5 at excitation and emission wavelengths of 455 and 550 nm, respectively, where there is negligible (1%) contribution

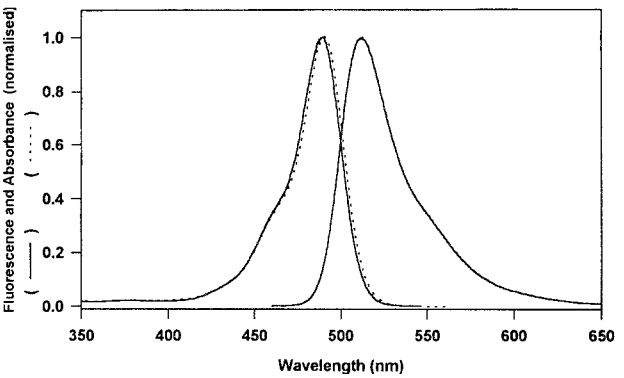

Fig. 6. Normalized fluorescence and absorption spectra for the dianion. Spectra were measured in 0.01 *M* NaOH.

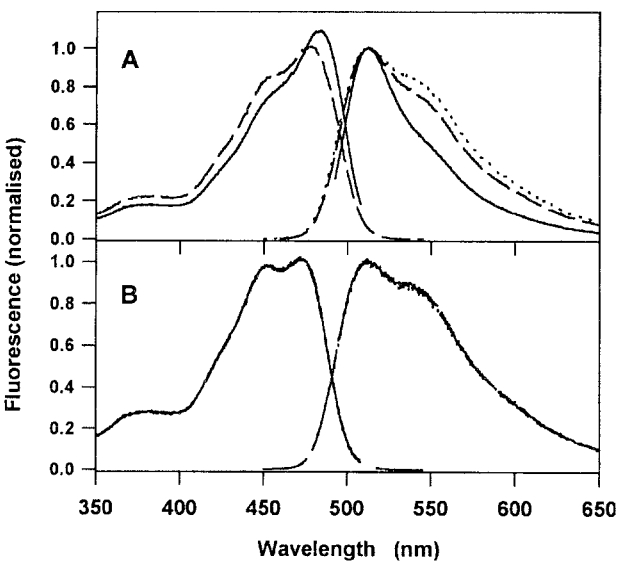

Fig. 7. Resolution of the nondianion spectrum of fluorescein at pH 5.5. The excitation and emission spectra were recorded at several excitation and emission wavelengths (A). Excitation wavelengths for the emission spectra were 420 (····), 455 (– – –) and 490 (—) nm. Emission wavelengths for the excitation spectra were 511 (—) and 550 (– – –) nm. Dianion spectra obtained in 0.01 *M* NaOH under the same conditions were subtracted from the corresponding spectra in A until the resultant spectra were independent of the excitation and emission wavelengths (B). In this illustration, 8.3% of the dianion spectrum was subtracted from the spectra in A to produce the dianion-corrected spectra in B.

from the dianion and the observed emission is due entirely to the monoanion.

DISCUSSION

Prototropic Forms in Solution. Previous analyses of the pH dependence of fluorescein absorption have identified three transitions corresponding to pK_a values of 2.2, 4.3, and 6.4. These have generally been interpreted in terms of the reaction scheme shown in Model A (Fig. 2). However, the precise nature of the neutral form has not been unambiguously resolved. In particular, we were concerned that small errors in the determined pK_a values could result in apparent monoanionic and cationic contributions to the neutral form. By making a number of well-founded assumptions it was possible to analyse the absorption titration data according to the more complex models shown in Fig. 2. This analysis indicated that the data required the neutral form of fluorescein to be a mixture of the three neutral species. Although most of the equilibrium constants determined using Model E were highly correlated (as might be expected for such a complex model), similar values for the pK_a's were determined by a more thorough analysis of the parameters obtained from Model A. The present analysis thus shows that the neutral form of fluorescein in solution is a mixture of the three neutral species, with the lactone representing the major proportion (70%) and the quinoid (15%) and zwitterion (15%) representing minor but significant species. Similar breakdowns of the neutral form have also been reported based on a spectral analysis of the neutral form.[6,7] Indeed the spectrum of the neutral form has recently been resolved by analysing a spectral titration according to Model A.[3] Although the neutral form was interpreted as a single quinoid species, inspection of its spectrum reveals a relatively low absorbance (lactone) and apparent cationic (zwitterion) and monoanionic (quinoid) contributions.

Table IV. The Proportion of the Total Fluorescein Present as the Dianion Determined by Analysis of Absorbance and Fluorescence Spectra as Shown in Fig. 7

	Dianion (%)	
pH	Absorbance	Fluorescence
6.4	47	45
6.0	23	24
5.5	8.3	8.3
4.5	1.2	0.7

The present analysis provides the pK_a of each ionizable group of fluorescein. The pK_a values of the cationic xanthene, benzoic acid, and neutral xanthene groups are 3.1–3.4, 3.1–3.4, and 6.3, respectively, while the pK_a value for lactonization is 2.4. The fluorescein analogues 6-hydroxyphenylfluoron (HPF) and sulfonefluorescein show only two transitions corresponding to the cationic and neutral xanthene groups, with pK_a values of 3.1 and 6.3, respectively.[6,7] These agree with the

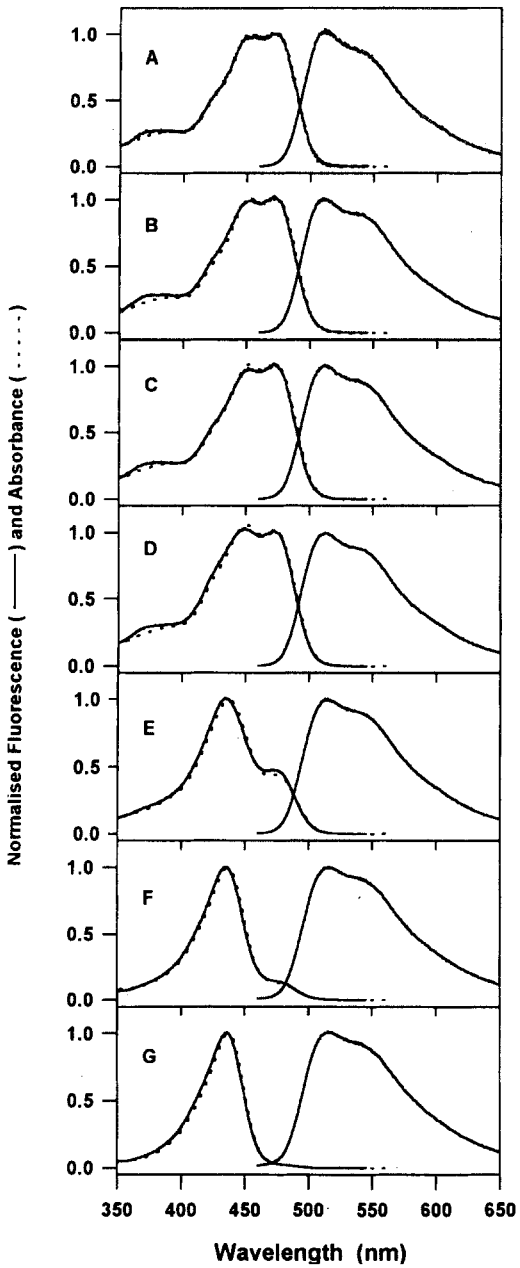

Fig. 8. Absorbance (····) and fluorescence excitation and emission spectra (—) of the nondianionic forms of fluorescein were obtained in solutions with the following pH: (A) 6.4, (B) 6.0, (C) 5.5, (D) 4.5, (E) 3.3, (F) 2.5, and (G) 1.6. Fluorescence spectra were obtained at multiple excitation and emission wavelengths. Dianion-corrected spectra from pH 4.5 to 6.4 were resolved as described in the legend to Fig. 7. The dianion contributions removed to produce these spectra are listed in Table IV.

values obtained in the present analysis for the same groups.

The pK_a values of 2.2, 4.3, and 6.4 which have previously been reported are seen to represent the cation/lactone, lactone/monoanion, and monoanion/dianion transitions, respectively (Fig. 4). This is a consequence of the lactone representing the major neutral species in solution and of the monoanion/dianion transition essentially being described by a two-state reaction. Indeed, the pK_a for this transition was found to be independent of the model used in the analysis of the absorption titration.

The monoanion species MII was not considered in the present analysis since it was assumed to represent a very minor species. The pK_a calculated for the neutral xanthene and benzoic acid groups supports this assumption: the relevant values differ by 3 pH units, indicating that the MII species should represent about 0.1% of the total monoanion species.

Absorption of the Different Forms. The dianion and cation forms of fluorescein are the only species which can be observed by themselves in the absence of others. This occurs under mildly basic and strongly acidic conditions, respectively. It is difficult to observe the spectra of the other forms due to the relatively small

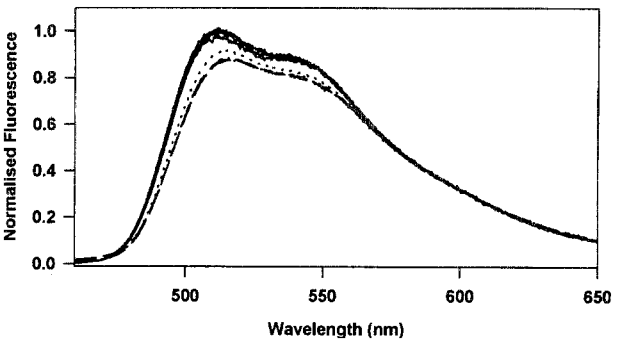

Fig. 9. Nondianion emission spectra. The emission spectra shown in Fig. 8 were normalized at 600 nm: pH 4.5 to 6.4 (—), pH 3.3 (····), and pH 2.5 and 1.6 (– – –).

Table V. Quantum Yields and Lifetimes of the Fluorescent Species of Fluorescein

Species	Quantum yield	Lifetime (ns)
Dianion[a]	0.93	4.06 ± 0.02
Monoanion[b]	0.36	3.37 ± 0.02
Quinoid[c]	0.29	2.97 ± 0.02
Cation[d]	0.9–1.0	3.5–4.4

[a] Quantum yield from Refs. 13 and 18. Lifetime determined in 0.01 *M* NaOH.
[b] Quantum yield determined from resolved monoanion fluorescence and absorption spectra. Lifetime determined at pH 4.5.
[c] Quantum yield and lifetime determined at pH 1.6.
[d] Quantum yield and lifetimes from Refs. 6 and 8.

differences in the pK_a values of the ionizable groups and to the presence of overlapping spectra. The monoanion spectrum was resolved at pH 5.5–6.4 utilizing the extinction coefficients determined in the analysis of the absorption titration. Under these conditions, the monoanion and the dianion are the only absorbing species present. The resolved spectrum is similar to that obtained by Sjoback *et al.* in their analysis of fluorescein spectra according to Model A[3] and to a similar analysis of spectra obtained between pH 5.5 and pH 9 according to a two-state transition.[19]

Resolution of the spectra of the zwitterion and quinoid forms is complicated by the absorption of multiple species under the conditions where these forms exist. However, it was possible to resolve the zwitterion spectrum, which was shown to be similar to the cation spectrum, but blue-shifted by 3 nm. The results were also consistent with the quinoid form, possessing a similar spectrum to the monoanion. The similarity of these absorption spectra validates the assumptions made in the analysis of the absorption titration and is consistent with the observed spectra of fluorescein analogues containing different groups in place of the carboxyl group.[5–7] These indicate that the presence and state of ionization of the carboxyl group should not have major effects on the absorption properties of the molecule.

Dianion and Monoanion Fluorescence. The dianion fluorescence reflects the ground-state concentration of the species at pH values greater than 4.5. The dianion therefore exists as a independent, noninteracting fluorophore. The fluorescence lifetime was 4.06 ns (Table V). Previous determinations of the lifetime range from 3.8 to 4.6 ns.[3,8,14,16,17,20]

The dianion-corrected fluorescence spectra at pH 5.5–6.4 were superimposable. In this pH range, the excitation spectra were similar to the monoanion absorption spectrum, indicating that the observed fluorescence was due to the monoanion. The monoanion fluorescence spectra were similar to those resolved by Sjoback *et al.* by analysis of spectra at pH 5.56 and 6.53 according to a two-state model.[3] Their report of 0.37 for the monoanion quantum yield also agrees with the value of 0.36 determined in the present analysis. Values of 0.25–0.35 have been consistently reported for the monoanion quantum yield but are subject to uncertainties associated with the use of uncorrected spectra,[12] unresolved spectra,[6] or very high concentrations of fluorescein.[21] The problems associated with the latter have been documented previously.[18]

The lifetime of the monoanion was 3.37 ns (Table V). This was measured at pH 4.5, where there was essentially no contribution of the dianion to the total fluorescence intensity, and where the emission was due entirely to the monoanion (see below). A similar value was obtained by Rozwadowski at pH 4.62.[8] A value of 3.0 ns has been reported by Sjoback *et al.*[3] from measurements at pH 5.1 analyzed according to two lifetimes (dianion and monoanion). However, with the phase method, it is difficult to resolve lifetimes which differ by less than a factor of 1.6.[22,23]

Fluorescence of Acidic Solutions. A system containing two noninteracting fluorophores results in fluorescence spectra which are dependent on wavelength but which can be resolved into the constituent spectra contributing to the total fluorescence. In contrast, excited-state reactions in which equilibrium is reached within the lifetime of the excited-state produce fluorescence spectra which are independent of the excitation and emission wavelength. In these cases, the emission spectrum is independent of excitation wavelength since the excited species reach the same equilibrium concentration regardless of which species is excited. The intensity of the emission thus reflects the total number of molecules which are excited at the excitation wavelength and, hence, is proportional to the absorbance at that wavelength. As a consequence, the excitation spectrum of such a system reflects the absorption spectrum and is also independent of emission wavelength.

The fluorescence properties of fluorescein in acidic solutions reflect those expected for a system in an excited state equilibrium. It has been observed that at a pH between 1 and 4 the emission spectrum remains constant, while the excitation spectrum varies significantly and reflects the absorption spectrum.[3,8] This has been interpreted in terms of excited-state proton transfer reactions. Rozwadowski[8] also considered excited-state lactonization reactions. However, Martin and Lindqvist have argued that such reactions would not be energetically feasible in the excited state.[6]

The present results confirm that the nondianionic absorbing species of fluorescein are in excited-state equilibrium under acidic conditions. However, at pH 4.5 the dianion exists as an independent, noninteracting species, whereas the other species present at this pH (zwitterion, quinoid, and monoanion) are in an excited-state equilibrium. At this pH the equilibrium favors the excited monoanion species, that is, there is a net loss of a proton from the excited neutral quinoid and zwitterion species to form the excited monoanion species. It has been postulated that excited-state proton transfers involving the cationic group occur via water, while those involving the xanthene group require a proton acceptor/donor.[3,9] The present results support a different mechanistic route for excited-state proton transfers involving the cationic and

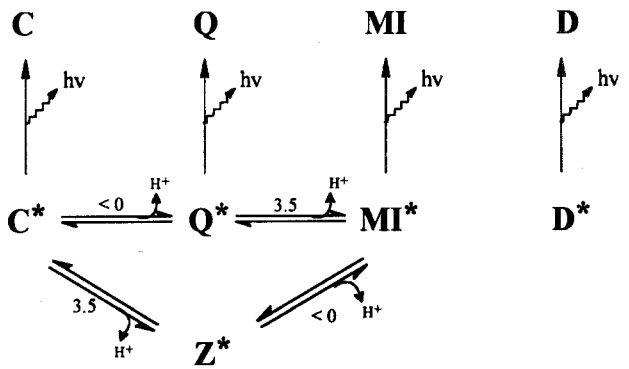

Fig. 10. Excited-state reactions of fluorescein. Estimates of the pK_a values are as indicated (see text).

benzoic acid groups and those involving the xanthene group.

Based on theoretical calculations, the pK_a of the monoanion-to-dianion equilibrium in the excited state is 5.5–6.0.[3,6] The absence of this reaction has been ascribed to the low concentrations of protons and hydroxide ions at conditions close to neutrality, where this transition is favored.[8,21] It has been reported that excited-state equilibrium can be achieved for the monoanion and dianion in the presence of high buffer concentrations (1 *M*) due to buffer-mediated excited-state proton transfers.[9] However, our analysis of fluorescence spectra under similar conditions reveals that such a system is not in an excited-state equilibrium (data not shown).

The emission spectrum of fluorescein in acidic conditions has been assumed to be identical to the monoanion emission spectrum.[3,6] This has been interpreted as arising from excited-state proton transfer from the nonfluorescent neutral species to form the monoanion or from the neutral quinoid species possessing an emission spectrum identical to that of the monoanion.[3,6,9] The latter interpretation was based on the similarity of the emission spectra of the neutral form of the analogue HPF with the monoanion emission spectrum. The present results show that although the emission spectra appear similar under acidic conditions, there are subtle differences, with a transition apparent at pH 3.5 (Fig. 9). Given the results with HPF and the similarity of this transition to the pK_a of the benzoic acid group, the emission at pH 1.5–2.5 was ascribed to the quionid species and the transition to the pK_a of the benzoic acid group for the molecule in the excited state.

The lifetime of the quinoid species is 3.0 ns (Table V), which compares with previous values of 3.2 and 3.1 ns reported at pH 1.9–2.1 and 3.17, respectively.[3,8] The quinoid quantum yield of 0.29 (Table V) compares with the value of 0.20–0.25 reported by Martin and Lindqvist[6] and the value of 0.28 reported by Rozwadowski[8] at pH 1.9 (The latter was reported relative to the dianion quantum yield).

The cation emission spectrum was not analyzed in the present study. Previous measurements show that the cation emission begins to appear under more acidic conditions (1 *M* HCl or H_2SO_4) and is observed in isolation only at much higher acid concentrations. This emission spectrum contains a peak at 470–480 nm and is a mirror image of the cation absorption spectrum.[3,6,8] The pK_a for the excited-state cation group is thus less than zero. In contrast to the benzoic acid group, the cationic xanthene group becomes a much stronger acid in the excited state. A consequence of this is that no emission would be expected from the zwitterion since the differences in the excited-state pK_a of the cation and benzoic acid groups would prevent significant concentrations of the zwitterion existing in the excited state.

Based on these results, a model for the excited-state reactions of fluorescein is shown in Fig. 10. This model is qualitatively similar to that proposed by Martin and Lindqvist [6]. However, they assumed that the pK_a value of 4.4 obtained from previous analyses according to Model A represented the carboxyl group and assumed that this was not altered in the excited state (the present results validate the latter assumption).

In some respects the fluorescence properties of fluorescein are less complicated than its absorption properties. The dianion exists as an independent noninteracting fluorophore, whereas the other absorbing species are in an excited-state equilibrium. The increase in acidity of the cationic xanthene group results in the absence of any fluorescence from the zwitterion and permits the measurement of the monoanion, quinoid, and cation fluorescence emission. Use was made of this property in measuring the quantum yield and lifetime of the quinoid species and the lifetime of the monoanion. The rationale for this is that for the system to be in excited-state equilibrium, the proton transfers have to occur on a time scale faster than the lifetime of the emitting species and hence the emission should be identical to that if the emitting species had been excited on its own in the first place.

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