Acid-Base Equilibria of Fluorescein and 2′,7′-Dichlorofluorescein in Their Ground and Fluorescent States

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Publication costs assisted by National Science Foundation Undergraduate Research Participation Program

The acidic forms of dichlorofluorescein are stronger acids than the corresponding forms of fluorescein. For the cationic and monoanionic acids, the difference in pK is 1.7; for the neutral acids, it is 0.9. The anionic acids of fluorescein and dichlorofluorescein are both slightly weaker in their first excited singlet than in their ground states, the difference in pK being −0.2. The fluorescence spectra of both dyes were measured in aqueous solutions, ranging from 8 m $HClO_4$ to a pH of 12. The emission spectra of the dianonic, monoanonic, and cationic forms have been estimated. The neutral acids appear to be nonfluorescent.

Introduction

As Zanker and Peter[2a] pointed out, fluorescein in aqueous solutions occurs in cationic, C, neutral, N, monoanionic, A, and dianionic, D, forms, the relative concentrations depending on the pH of the solutions. Neutral fluorescein in dry dioxane exists as a lactone, L, but in aqueous solutions the dominant neutral form is a quinoid, Q.[2b] The fundamental equilibria are as follows

$$K_1' = \frac{[Q][H^+]}{[C]}$$

$$K_2' = \frac{[A][H^+]}{[Q]}$$

$$K_3' = \frac{[D][H^+]}{[A]}$$

$$K_I = \frac{[Q]}{[L]} = \frac{[Q]}{[N] - [Q]}$$

The isomeric equilibrium in aqueous solutions cannot be determined from available spectroscopic data. Accordingly, the equilibrium constants that can be derived from absorption spectra are K_3', K_2, and K_1, where $K_1 = K_1'[1 + K_I)/K_I$ and $K_2 = K_2'K_I/(1 + K_I)$.

It is impossible to obtain aqueous solutions which contain only the monoanionic or the neutral form of fluorescein; also the absorption spectra of the several species overlap seriously. However, it is possible to evaluate K_1, K_2, and K_3' from spectroscopic data by a simple iterative process.[2b]

If, during the lifetime of fluorescence, molecules in their first-excited singlet state attain acid–base equilibrium it should be possible to evaluate their equilibrium constants from the results of quantitative measurements of fluorescence spectra, corresponding to different values of pH. However, as is discussed in a following section, this is practicable only for K_3'*. Uncertainties in regard to the fluorescence and the isomeric equilibrium of the neutral dye, difficulties due to overlap of the fluorescence spectra of the several forms, and nonnegligible corrections necessitated by fluorescence quenching and wavelength shifts observed in concentrated $HClO_4$, render estimates of K_2* and K_1* too unreliable to be of real value.

Experimental Methods and Materials

Materials. CH_3COONa, CH_3COOH, Na_2HPO_4, KH_2PO_4, and 95% C_2H_5OH were reagent grade and were used without further purification. The $HClO_4$ used in the fluorescein measurements was of reagent grade, but that used with dichlorofluorescein was Baker's Ultrex. Perylene and fluorescein were purified chromatographically.[3] Our sample of fluorescein, dissolved in 0.010 m NaOH, had an extinction coefficient at λ 490 nm of 87,600, in good agreement with 88,800 reported by Lindquist.[2b] The 2′,7′-dichlorofluorescein had an extinction coefficient of 101,000 at 503 nm in 0.010 m NaOH and was used without further purification.

Apparatus. Absorption measurements were made with a Cary Model 14 spectrophotometer. Fluorescence intensities were measured relative to a perylene standard (10^{-5} m in 95% C_2H_5OH) with a modified Farrand fluorometer, a double-beam instrument, used as a null device. The exciting light was predominantly of 365 nm, isolated from a GE-H85A3 Hg lamp by a Corning 7-60 filter. The intensities of the fluorescent and compensating light were measured with RCA 1P28PM tubes. The fluorescent light passed through a grating monochromator before striking the PM tube.

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(2) (a) V. Zanker and W. Peter, *Chem. Ber.*, **91**, 572 (1958); (b) L. Lindquist, *Arkiv. Kemi.*, **16**, 79 (1960).

(3) (a) L. Koch, *J. Ass. Offic. Agr. Chem.*, **39**, 397 (1956); (b) R. Sangster and J. Irvine, *J. Chem. Phys.*, **24**, 697 (1956).

The fluorescence intensities of the solutions were normalized by dividing them by their absorbance (measured in a 100-mm silica cell) at 366 nm. For the concentrations of dye used, Beer's law is valid for the entire pH range. Scattering of the exciting light from a water-filled cell was not detectable under our experimental conditions. The fluorometer was stable within $\pm 1\%$ during the 40-min periods required to complete a run.

Preparation of Solutions. Solutions were prepared by dissolving a weighed amount of the purified dye in 0.10 *m* NaOH and then neutralizing the excess base with $HClO_4$. In preliminary experiments, fluorescein was dissolved by heating it in distilled water. However, fluorescein in hot aqueous solutions proved to be unstable, and this procedure was discontinued.

For solutions of pH <3, the hydrogen ion concentration was fixed by the addition of an appropriate amount of concentrated $HClO_4$. For pH $\geq$3, either an acetate or phosphate buffer was used. The total (acid and salt) concentration was 0.10 *m* for acetate and 0.025 *m* for phosphate buffers. All solutions were air-saturated. pH values greater than 2 were measured with a Beckman pH meter; more acidic solutions were titrated with a standard NaOH solution.

Ground-State Equilibria. Figure 1 consists of plots of the molar, decadic extinction coefficient, ϵ (for 10^{-6} *m* dichlorofluorescein), against wave number, $\bar{\nu}$, at several typical values of pH. The absorption maxima (*cf.* ref 2b, pp 42, 43) of the cation, neutral molecule, monoanion, and dianion are at 448, 456, 460 and 485 (double maxima), and 502 nm, respectively. In Figure 2, log $(\epsilon - \epsilon_i)/(\epsilon_j - \epsilon)$ is plotted against pH. The straight lines were drawn with slopes of 1 to correspond to the sets of experimental points. The values of ϵ_i and ϵ_j, which correspond to each of the several forms of the dye, were taken from the absorption spectra but have been slightly corrected by an iterative process for the presence of small amounts of other forms.[2b] The corresponding values of pK_3', pK_2, and pK_1 are, respectively, 4.95, 3.50, and 0.47. These values are based on the assumption that the activity coefficients of the reactants are each equal to 1. The correction for K_1 should be relatively small, but the correct constants corresponding to K_2 and K_3' must be smaller than those listed.

Our absorption data for fluorescein are in good agreement with those published by Lindquist.[2b] Accordingly, we have omitted our absorption spectra and have accepted his values for the equilibrium constants (see Table II).

Aqueous solutions of fluorescein, at pH 1.0, 3.3, and 5.5, conform to Beer's law in the concentration range 10^{-6} to 10^{-5} *m*.[2b] We obtained similar results for dichlorofluorescein at pH 0.0, 4.6, and 12.0 and between 5×10^{-7} and 5×10^{-6} *m* at a pH of 2.0. Presumably dimerization in those solutions is of negligible importance. However, in the pH range 1 to 3 the solubility of dichlorofluorescein is less than 10^{-5} *m*.

In strongly acid solutions, the absorption maximum is shifted to shorter wavelengths by increasing concentrations of $HClO_4$ or H_2SO_4, but not of HNO_3 (see Table I).

Table I: Shift of Absorption Maxima in Acid Solutions

[Acid], *m*	λ_{max}, nm, fluorescein	λ_{max}, nm, dichlorofluorescein
$HClO_4$		
0.50	436	448
1.00	436	448
1.70	436	
2.15		449
2.50	435	
3.00	434	446
4.00	434	
5.40		445
7.40	431	443
11.7		441
H_2SO_4		
10.0 *m*	433	444
HNO_3		
7.0 *m*	436	

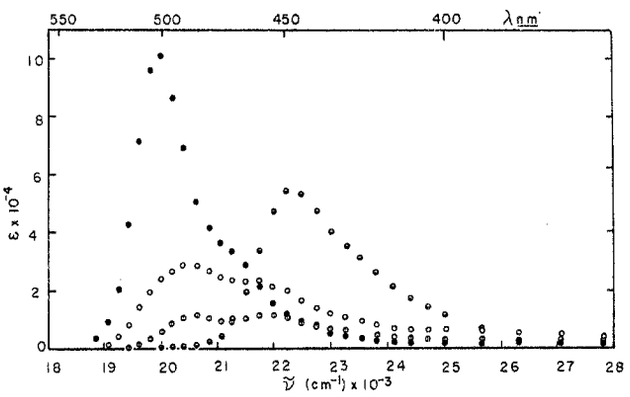

Figure 1. Absorption spectra of dichlorofluorescein: ●, pH 12.0; ○, pH 3.89; ◐, pH 2.05; ◒, pH −0.33.

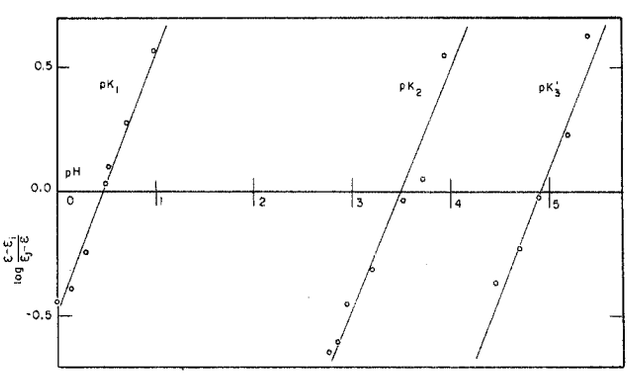

Figure 2. Graphical determination of the pK_i for dichlorofluorescein.

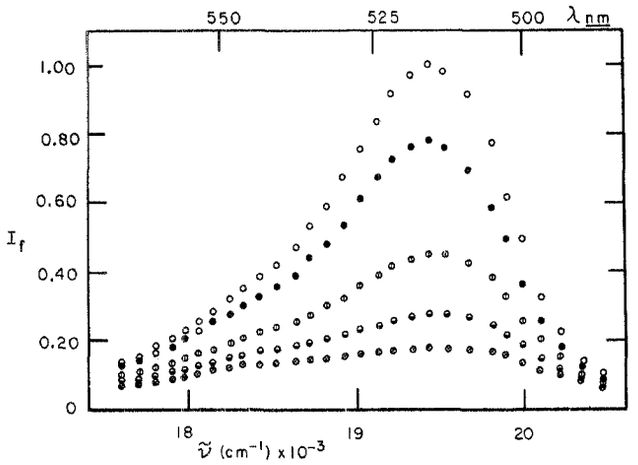

Figure 3. Emission spectra of fluorescein in basic and weakly acidic solutions. I_f, normalized to maximum for pH 11.7: ○, pH 11.7; ●, pH 7.40; ⊕, pH 6.70; ◒, pH 6.0; ⊗, pH 2.75.

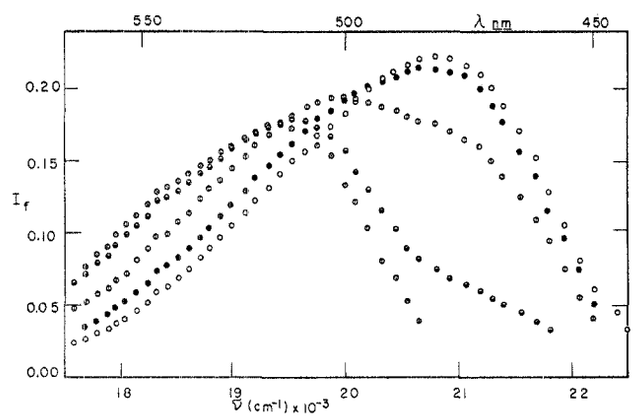

Figure 4. Emission spectra of fluorescein in acidic solutions. I_f normalized to maximum for pH 11.7, see Figure 3: ○, [HClO$_4$] 7.4 *m*; ●, [HClO$_4$] 3.0 *m*; ⊕, [HClO$_4$] 1.7 *m*; ◒, [HClO$_4$] 0.5 *m*; ⊗, pH 2.80.

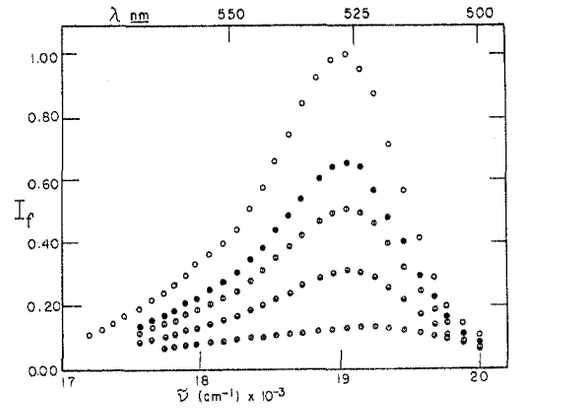

Figure 5. Emission spectra of dichlorofluorescein in basic and weakly acidic solutions. I_f normalized to maximum for pH 12.0: ○, pH 12.0; ●, pH 5.45; ⊕, pH 5.19; ◒, pH 4.69; ⊗, [HClO$_4$] 0.50 *m*.

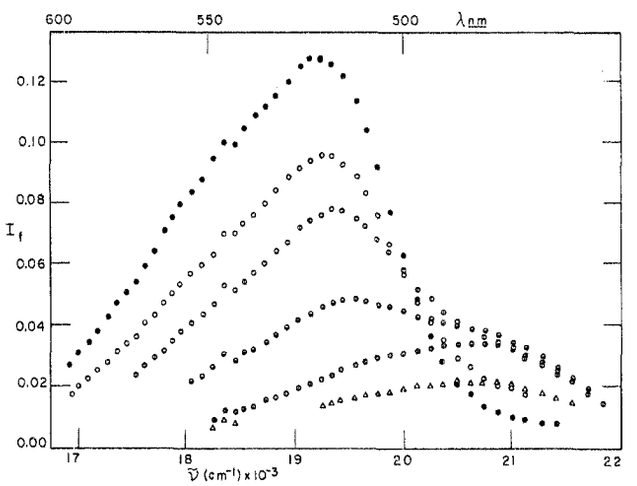

Figure 6. Emission spectra of dichlorofluorescein in acidic solutions. I_f normalized to maximum for pH 12.0, see Figure 5: ○, 1.09 *m*; ●, 3.24 *m*; ⊕, 4.34 *m*; ◒, 5.4 *m*; ⊗, 7.55 *m*; △, 11.7 *m* ($zm \equiv$ [HClO$_4$]).

Florescence as a Function of pH. The normalized (see the section of Experimental Methods) values of the intensities of fluorescence, I_f, vary with wavelength and pH but are independent of intensity of exciting light and concentration of the dye. The fluorometer readings, upon which the I_f values are based, were not corrected for variation with wavelength of the sensitivity of the photomultiplier or of the transmissivity of the optical system. In Figures 3–6, values of I_f, for various typical acidic and basic solutions, are plotted against $\tilde{\nu}$ (cm^{-1}). Rozwadowski[4] published qualitatively similar data for fluorescein.

The values of I_f for fluorescein in acidic solutions exhibit an isobestic point near 508 nm (Figure 4). However, the data show a greater scatter about the isobestic point than is consistent with the estimated uncertainty ($\lesssim 2\%$) of the measurements. This scatter appears to be the result of weak quenching of fluorescence and a concurrent shift of the maximum of fluorescence occurring in concentrated HClO$_4$. Similar quenching, etc., were produced by H_2SO_4 and by the addition of NaClO$_4$ to 1.0 *m* HClO$_4$. The scatter can be reduced to the expected range if the data are corrected, assuming that the wavelength shifts for absorption and emission are identical and that the Stern–Vollmer constant is about 0.025 m^{-1}. For dichlorofluorescein, similar corrections can be made but appear to be even more arbitrary.

The several curves of Figures 3 and 4 can be represented by the sum of the intensities of fluorescence of each species, β_i, weighted by their relative concentrations

$$I_f = \sum_i C_i\beta_i / \sum_i C_i$$

The β_i are the values which I_f would have if the excited

(4) M. Rozwadowski, *Acta Physiol. Pol.*, **20**, 1005 (1961).

dye were all in its ith acid–base form. Inspection of the data for fluorescein in the pH range from 1 to 3 showed that β_N cannot be greater than β_A; either $\beta_N \cong 0$ or $\beta_N \cong \beta_A$. Postulating that $\beta_N = 0$ (for all wavelengths), values of β_C, β_A, and β_D of fluorescein and β_A and β_D of dichlorofluorescein have been estimated and are plotted in Figure 6. The values, particularly of β_D and β_C, are not seriously affected by the assumption that $\beta_N = 0$. However, corrections for quenching, etc., would increase the values of β_C perhaps by as much as 7%.

The quantum yields of fluorescence can be estimated from the areas under the β_i curves, using

$$\varphi_i = \varphi_D \sum_{\bar{\nu}} \beta_i \Delta\bar{\nu} / \sum_{\bar{\nu}} \beta_D \Delta\bar{\nu}$$

We have taken the absolute values of φ_D for fluorescein[5] and dichlorofluorescein[6] as 0.93 and 0.88, respectively. The corresponding values of φ_A and φ_C (fluorescein) and of φ_A (dichlorofluorescein) are 0.26, 0.39, and 0.17, respectively. The values for fluorescein are not directly comparable to those reported by Rozwadowski,[4] since his experimental conditions were different and he used $\varphi_D = 1.00$. Also it should be remembered that our values of I_f were not corrected for the variation of photomultiplier sensitivity with wavelength and that this introduces a corresponding uncertainty in the values of β and φ.

Comparison of the effect of pH on the absorption and emission spectra demonstrates that the protolytic equilibria are different in the ground and excited states. It is possible to evaluate $K_3'^*$ by plotting log $(\beta_D - I_f)/(I_f - \beta_A)$ against pH, as is shown in Figure 8. The straight lines drawn with a slope of unity indicate that $pK_3'^*$ for excited fluorescein and dichlorofluorescein are 6.93 and 5.24, respectively. In obtaining these values, it was assumed that equilibrium between the di- and monoanions was attained in their excited states, but nothing was assumed about the behavior or properties of the neutral and cationic forms.

If it is assumed that the several species attain protolytic equilibria during the lifetimes of their excited states and that relative concentrations of the isomeric forms of the neutral dyes do not depend on pH, the following equation can be derived by a straightforward steady-state analysis.

$$I_f = \frac{\beta_C[H^+]^3 + \beta_N K_1^*[H^+]^2 + \beta_A K_1^* K_2^*[H^+] + \beta_D K_1^* K_2^* K_3'^*}{[H^+]^3 + K_1^*[H^+]^2 + K_1^* K_2^*[H^+] + K_1^* K_2^* K_3'^*}$$

For acid concentrations greater than 10^{-3} m, terms containing $K_1^* K_2^* K_3'^*$ may be neglected and the equation put into the following simplified form

$$(I_f - \beta_C)[H^+]^2 + (I_f - \beta_N)K_1^*[H^+] + (I_f - \beta_A)K_1^* K_2^* = \rho$$

where ρ is the residual and K_1^*, K_2^* and the β_i are adjustable constants. An attempt was made to evaluate K_1^* and K_2^* with aid of a computer program for a stepwise, multiple regression analysis[7] using values of β_A and β_C from Figure 7 and setting β_N equal either to 0 or to β_A. Although values ($K_1^* = 0.18$ and $K_2^* = 9.0$) obtained in this way (for fluorescein) are compatible with the experimental data, it is doubtful if they have physical significance. Probably, the isomerization reactions are slow, relative to the decay of fluorescence, and are acid–base catalyzed. Furthermore, the values of K_1^* and K_2^*, obtained in this way, depart widely from predictions based upon Förster's cycle.[8,9] If $\beta_N = 0$, the excited neutral form must have a very short life and cannot reach protolytic equilibrium. The values of K_1^* and K_2^* given by the computer analysis are strongly correlated, which introduces an additional uncertainty.

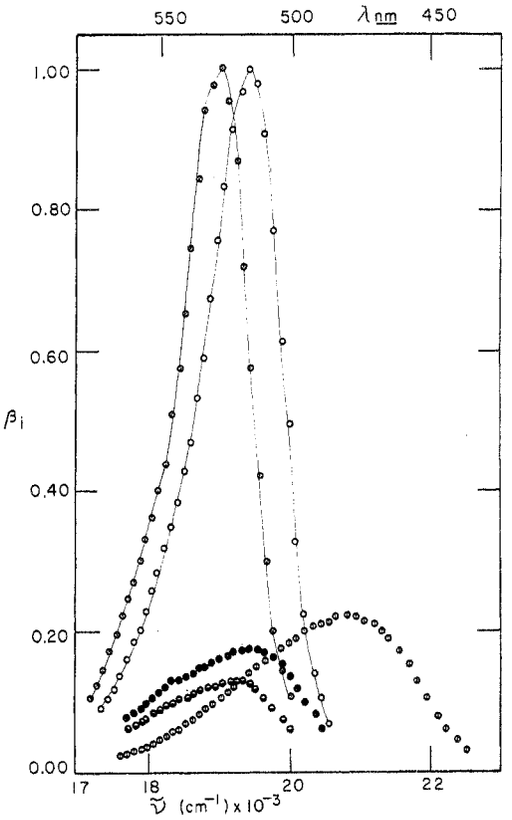

Figure 7. Emission spectra of several acidic forms of fluorescein and dichlorofluorescein: ⊗, β_D; ◒, β_A, dichlorofluorescein; ○, β_D; ●, β_A; ⊙, β_C, fluorescein.

(5) P. Seybold, M. Goutermon, and J. Collis, *Photochem. Photobiol.*, **9**, 229 (1969).

(6) L. Forster and D. Dudley, *J. Phys. Chem.*, **66**, 838 (1962).

(7) IBM System 360, Scientific Subroutine Package 360-CM-03X, Version III, Manual H20-020503, 1968.

(8) A. Weller, *Progr. React. Kinet.*, **1**, 189 (1961).

(9) E. Vander Donckt, *ibid.*, **9**, 273 (1969).

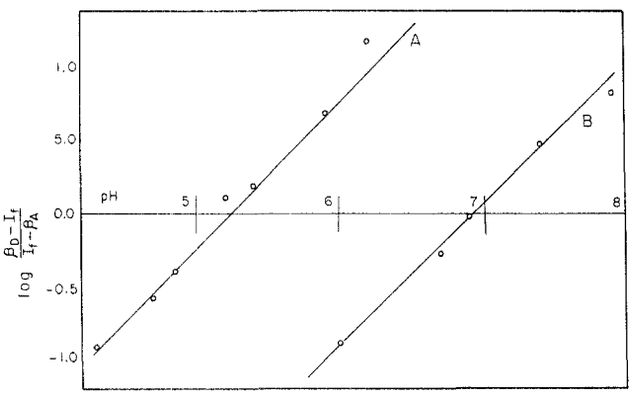

Figure 8. Graphical determination of K_3'*: A, dichlorofluorescein; B, fluorescein.

Discussion

The pK_i for fluorescein and dichlorofluorescein are listed in Table II. The ground-state fluorescein values are quoted from Lindquist.[2b] The others are from Figures 2 and 8.

Table II: Values of pK_i

	pK_1	pK_2	pK_3'	pK_3'*	pK_3' − pK'*	0.625 $\Delta\tilde{\nu}/T$[a]
Fluorescein	2.2	4.4	6.7	6.9	−0.2	0.1
Dichlorofluorescein	0.5	3.5	5.0	5.2	−0.2	0.3

[a] See ref 8.

Consistent with Förster's cycle,[8,9] excitation has only a small effect on pK_3'. In computing $\Delta\tilde{\nu}$, we have neglected the absorption maximum at 2.20×10^4 cm^{-1} (Figure 1 and Figure 7, ref 2) and the emission maximum at 1.83×10^4 cm^{-1} (Figures 4 and 6). These secondary maxima which occur at the same frequencies for fluorescein and dichlorofluorescein may be due to the presence of small amounts of persistent impurities. This view is supported by the fact that the fluorescein samples used in the present study and by Lindquist[2] and Rozwadowski[4] were purified in the same way.[3]

Substitution of chlorine atoms in the 2′ and 7′ positions renders the ground-state species and the excited monoanion stronger acids. The difference, 1.7, is the same for pK_1, pK_3', and pK_3'*. For pK_2, it is 0.9.

The present data support Rozwadowski's[4] opinion (based on Zanker and Peter's[2b] evidence) that neutral fluorescein is practically nonfluorescent. This is in contradiction to the view of Stoughton and Rollefson[10] and of Umberger and La Mer[11] that the neutral rather than the monoionic form is fluorescent. Their opinion was based on the observation that the Stern–Vollmer constant, for the quenching of fluorescein by I^-, shows no significant dependence on ionic strength. A possible interpretation of this apparent contradiction is that small changes in fluorescence intensity due to salt effects on the quenching constant are approximately canceled by corresponding effects on the K_i and K_i*.

Lumry and Georghiou[12] measured the mean lifetime of fluorescence of fluorescein in solutions containing, severally, 7.0 *m* $HClO_4$, 0.20 *m* $HClO_4$, and a buffer having a pH of 2.73. Their results in general agree with those published by Rozwadowski.[4] Their measurements, which were made with a modified Bennet-type apparatus,[13] showed no significant departure from simple exponential decay. This latter result indicates that, at each of these acidities, there is only a single fluorescent species present.

Acknowledgments. The authors wish to thank L. Lindquist for reading the section on the ground-state equilibria, D. Coffey for advice and assistance in setting up the computer analysis, and R. Lumry and Georghiou for making the fluorescence lifetime measurements. We are also grateful for financial assistance from the Research Corporation and from the National Science Foundation Undergraduate Research Participation Program.

(10) R. Stoughton and G. Rollefson, *J. Amer. Chem. Soc.*, **61**, 2634 (1939).

(11) J. Umberger and V. La Mer, *ibid.*, **67**, 1099 (1945).

(12) R. Lumry and S. Georghiou, unpublished work, 1969.

(13) W. Ware, "Creation and Detection of the Excited State," A. Lamola, Ed., Marcel Decker, New York, N. Y., 1970.