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Extraordinary Character of the Solvent Influence on Protolytic Equilibria: Inversion of the Fluorescein Ionization Constants in H_2O–DMSO Mixtures

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The protolytic equilibria of fluorescein and sulfonefluorescein in the H_2O–DMSO system are studied spectrophotometrically. The pK_a values, as well as the absorption spectra, attributed to individual ionic (molecular) forms of the dyes, are determined. The pa_H^* scales used in pK_a calculations [at DMSO contents (wt.%) of 21.6, 42.2, 60.3, 81.4 and 91.3] were estimated by the indicator method (sulfonephthalein series) through the overlapping procedure.

The character of the solvent influence on the fluorescein stepwise ionization ($H_3R^+ \rightleftarrows H_2R \rightleftarrows HR^- \rightleftarrows R^{2-}$; K_{a0}, K_{a1} and K_{a2}, respectively) results in extraordinary interrelations between the K_a constants, up to inversion: $pK_{a1} > pK_{a2}$. The K_{a1}/K_{a2} ratio changes gradually from 224 in H_2O to 0.045 in 91% DMSO; meanwhile the K_{a0}/K_{a1} ratio increases from 204 to 7×10^{10}.

The anomalous character of the fluorescein pK_a dependence on the content of DMSO (up to $pK_{a1} > pK_{a2}$) is explained through the tautomeric equilibria shift and the nature of the functional groups. The tautomerization constants of the fluorescein neutral form are given as obtained on the basis of the extrathermodynamic assumption proposed previously (N. O. Mchedlov-Petrossyan, *Zh. Anal. Khim.*, 1979, **34**, 1055). This allowed calculation of the ionization microconstants (k), which were found to be in agreement with pK_a values of model compounds.

The stepwise ionization of acids in solutions is one of the most thoroughly studied chemical equilibrium processes. The interrelations among K_{a1}, K_{a2}, ..., K_{an} values vary over a vast range; there are a lot of cases when the ionization constants of neighbouring steps differ by less than one order of magnitude ($pK_{a(m+1)} - pK_{am} < 1$). However, one viewpoint still dominates according to which the following interrelation is maintained:

$$pK_{am} < pK_{a(m+1)} \quad (1)$$

that is, the splitting off of each proton is more difficult than for the previous one. Such notions are based on Bjerrum's classical electrostatic model and other considerations, summarized by Bell.[1] Polyprotic acids are usually weaker when ionizing in the second step than in the first, and so on ($pK_{a1} < pK_{a2} < pK_{a3}$ ...). Familiar exceptions are not numerous; for aqueous solutions they include, in particular, *p*-dihydroxyglutacondianil,[2] diazonium ion,[3] some complexes of rare-earth metals with gluconic acid,[4] the aqua-complex of bivalent mercury.[5] In dimethyl sulfoxide (DMSO), inversion of the constants is found for tetra(*tert*-butyl)phthalocyanine, $pK_{a1} = 12.48$, $pK_{a2} = 11.75$;[6] in cyclohexylamine, for the formation of the Cs salt of 9,9′-bifluorenyl, $pK_1 \approx pK_2$.[7] Other experimental data contrary to the above interrelation [eqn. (1)] are few and not always reliable.[8–11] Determination of the overlapping pK_a values is often complicated by experimental difficulties, which can deform the true interrelation of the constants. However, if certain structural changes are possible in a molecule (besides simple dissociation), eqn. (1) can be violated. Systematic studies of the protolytic equilibria of multifunctional organic compounds, carried out in our laboratory, show that the tautomeric equilibria shift caused by organic solvents can result in K_a values becoming essentially closer. Inversion may then occur ($pK_{a1} > pK_{a2}$).

This problem is of great importance for understanding the properties of polyfunctional analytical organic reagents. At fluorescein ionization

$$H_3R^+ \rightleftarrows H_2R + H^+; \quad K_{a0} \quad (I)$$

$$H_2R \rightleftarrows HR^- + H^+; \quad K_{a1} \quad (II)$$

$$HR^- \rightleftarrows R^{2-} + H^+; \quad K_{a2} \quad (III)$$

the tendency of the value pK_{a2}–pK_{a1} to fall was observed in water–organic media as compared with aqueous solutions.[12,13] In some mixtures of water with acetone,[13,14] as well as in 91 wt.% DMSO,[15] even the inversion $pK_{a1} > pK_{a2}$ was observed. It seemed of interest to follow the gradual change in the K_{a1}/K_{a2} ratio during the transfer from water to DMSO and to clarify if the model suggested for the interpretation of such a rare ratio of K_a values of stepwise ionization[12–14] is universal.

Experimental

Apparatus and Reagents

The spectra of dye solutions were measured with apparatus SP-46 (USSR) and Specord M-40. Fluorescein (Minkhimprom, USSR) and sulfonefluorescein (prepared according to Orndorff and Vose[16]) were purified by reprecipitation from aqueous solution (pH 11–12) with hydrochloric acid, as well as chromatographically. The purity was checked by means of fluorescence and excitation spectra as well as chromatographically (Silufol plates). For purified fluorescein in dilute (0.01–0.001 mol dm^{-3}) aqueous alkali, $\lambda_{max} = 491$ nm, $\varepsilon_{max} = 88 \times 10^3$; for sulfonefluorescein under the same conditions, $\lambda_{max} = 495$ nm and $\varepsilon_{max} = 84 \times 10^3$. As will be shown these spectral characteristics are related to the dianions (R^{2-}). Indicators bromophenol blue, bromocresol green and bromothymol blue were commercial products of reagent grade and were used as such. The molar absorptivities of their yellow (HR^-) and blue (R^{2-}) forms are in agreement with literature values. Buffer acids (salicylic,

benzoic, diethylbarbituric) were purified by recrystallization. Glacial acetic acid, as well as the mineral acids HCl and $HClO_4$ (all of analytical grade), were used without additional purification. Standard aqueous solutions of NaOH were prepared using CO_2-free water. DMSO was purified by the standard procedure using alkali and then zeolites (NaA; 4 Å). The water content was estimated by titration according to Fischer's method.

Procedure

The ionization constants were evaluated spectrophotometrically at 25 °C in dilute acid solutions ($HClO_4$, HCl), as well as in buffers (salicylate, benzoate, acetate and diethylbarbiturate). The latter were prepared by mixing NaOH stock solutions with buffer acids. The absorption spectra were measured at 400–550 nm; the pK_a values of fluorescein and sulfonefluorescein were obtained from analysis of ε–pa_H^* curves (Fig. 1). The dye concentration was usually 1×10^{-5} mol dm^{-3} (but while determining ε_{H_2R} and pK_{a0} of fluorescein it was increased to 10^{-3} mol dm^{-3}). For each DMSO content, 30–40 working solutions in the case of fluorescein and *ca.* 20 in the case of sulfonefluorescein were used. As a rule, the ionic strength was 0.01 mol dm^{-3} while estimating pK_{a1} and pK_{a2} values of fluorescein and sulfonefluorescein (as well as the pK_{a2} values of sulfonephthalein), and 0.01–0.03 while estimating pK_{a0} of fluorescein. Even the value of $pK_{a0} = -0.5$ was possible to determine at $[H^+] \leqslant 0.1$ mol dm^{-3}, since $\varepsilon_{H_3R^+} \gg \varepsilon_{H_2R}$ at high DMSO concentrations. While working with high pa_H^* values certain test experiments were carried out in nitrogen atmosphere.

The pK_a values of buffer acids (pK_{HA}) at each DMSO content were determined with the indicator method (using the sulfonephthalein series) through the procedure of overlapping, starting with measurements in solutions of completely dissociated acids ($HClO_4$, HCl). The measurements were carried out using the dye concentrations $(1.5–2) \times 10^{-5}$ mol dm^{-3} in the spectral region near λ_{max} of R^{2-} (± 20 nm); $\varepsilon_{R^{2-}} \gg \varepsilon_{HR^-}$. As a rule, nine λ values were used. The measurements were made at, on average, ten different C_{HA}/C_{NaOH} ratios. The pa_H^* values of buffer mixtures were calculated with the help of pK_{HA} values. In the present study a_H^* denotes the proton activity standardized to infinite dilution in the corresponding mixed solvent.[17]

All the dye solutions obey Beer's law within the concentration range used.

The reduction of all pK_a values to zero ionic strength is performed by means of the Debye–Hückel equation for ionic activity coefficients (the ionic parameter was taken to be equal to 5 Å). The details of the experiment can be found in ref. 18.

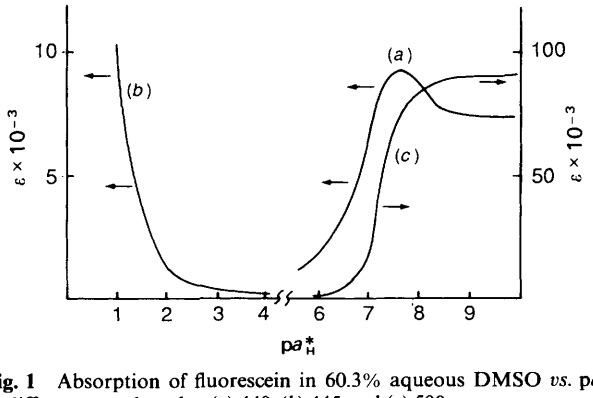


Fig. 1 Absorption of fluorescein in 60.3% aqueous DMSO *vs.* pa_H^* at different wavelengths: (*a*) 440, (*b*) 445 and (*c*) 500 nm

Results

pa_H^* Scale Estimation

The pa_H^* scale[17,19] at each DMSO content was stated with the help of the pK_a values of buffer acids, HA [pK_a (buffer acid) = pK_{HA}]:

$$pa_H^* = pK_{HA} + \log \frac{[A^-]}{[HA]} + \log f_1 \qquad (2)$$

where A^- is the buffer acid anion ($[A^-]$ is equated with the NaOH analytical concentration; C_{NaOH} = const. = 0.01 mol dm^{-3}), and f_1 is the activity coefficient of the monocharged ion. The pK_{HA} values were determined *via* the indicator method through the procedure of overlapping, starting with measurements in solutions of mineral acids; $[HA] = C_{HA} - C_{NaOH}$. The ionic strength of buffer solutions may be equated with $[A^-]$. Such an approach to $[A^-]$ and pa_H^* calculations is correct only in the case where $[Na^+] \gg [H^+]$. Otherwise (*e.g.* salicylate buffers at low or medium DMSO contents) somewhat more complicated calculations, using the electroneutrality principle, must be made.

The pK_{a2} values of bromophenol blue ($HR^- \rightleftarrows R^{2-} + H^+$; yellow → blue) were determined in dilute ($\leqslant 0.01$ mol dm^{-3}) solutions of hydrochloric acid. The pa_H^* values were calculated as

$$pa_H^* = -\log[H^+] - \log f_1 \qquad (3)$$

Equilibrium hydrogen ion concentrations, $[H^+]$, were calculated from HCl analytical concentrations, taking into account the additional H^+ ions, which originate from the indicator acid (H_2R).

$$pK_{a2} = pa_H^* + \log \frac{\varepsilon_{R^{2-}} - \varepsilon}{\varepsilon - \varepsilon_{HR^-}} + \log f_1 - \log f_2 \qquad (4)$$

where f_2 is the activity coefficient of the R^{2-} ion, ε is the apparent molar absorptivity value at the working pa_H^*. Similar pK_{a2} values were obtained while carrying out experiments with solutions of $HClO_4$, the latter being known as a much stronger acid than HCl. This confirms the idea of practically complete HCl dissociation in the solvent systems under study and agrees with the pK_a(HCl) values available in the literature (2.10 in DMSO, and 0.8 in 85% DMSO).[20]

The $[HR^-]/[R^{2-}]$ values for bromophenol blue were measured in salicylate buffer solutions, and the pa_H^* values of the latter were calculated, eqn. (4). This, in turn, allows pK_{HA} values of salicylic acid to be obtained, eqn. (2). The pK_{a2} values of bromocresol green were found while carrying out measurements in salicylate buffers; with the help of these pK_{a2} values the pK_{HA} values of benzoic and acetic acids were determined. Going on with this procedure, the pK_{a2} values of bromothymol blue and pK_{HA} values of diethylbarbituric acid were obtained.

The homoconjugation process ($HA + A^- \rightleftarrows HA_2^-$) does not seem to be of great significance at H_2O contents in DMSO of 10 wt.% or more.[21–23] The ion pairing (*e.g.* Na^+A^- formation) is not characteristic for DMSO,[24] and less so for DMSO–water mixtures.

The error of the relative pK_a values of the indicators was within $\pm$(0.01–0.05) (Table 1).

Comparison of pK_{HA} and pK_{a2} Values with other Data

The pK_a values for salicylic acid at DMSO contents of 20, 40, 60 and 80 wt.% as calculated [eqn. (2)] using the pa_H^* values of standard buffer solutions [19] coincide with our data (Table 1). The pK_{HA} values in 35 vol.% and 65 vol.% DMSO[25] are somewhat (by 0.2–0.3 units) lower than ours; the data for 95

Table 1 Thermodynamic pK_a values of indicators and buffer acids

acid	DMSO content (wt.%) 0	21.6	42.2	60.3	81.4	91.3
bromophenol blue	4.20	4.17	4.24	4.26	4.59	4.94
salicylic acid	3.00	3.16	3.29	3.58	4.46	5.22
bromocresol green	4.90	4.96	5.07	5.22	5.61	6.27
benzoic acid	4.20	4.52	4.90	5.55	6.47	8.05
acetic acid	4.76	—	—	—	7.27	8.54
bromothymol blue	7.30	7.39	7.84	8.06	8.36	9.36
diethylbarbituric acid	7.98	7.85	8.33	8.62	9.26	10.77
relative permittivity	78	77	76	72	64	56

vol.% DMSO ($pK_{HA} = 5.64$)[25] and pure DMSO ($pK_{HA} = 6.8$)[21] do not contradict our results.

At DMSO contents over 60%, deviation of our results from those of other authors is observed for benzoic and acetic acids. For instance, in 80% DMSO for benzoic acid the following pK_{HA} values are available: 7.30,[26] 7.24 (ionic strength = 0.5),[23] 7.19 (calculated from the pa_H^* values of standard buffers),[19] 7.34,[27] 7.22;[28] our value, 6.47 in 81.4% DMSO (Table 1). Further, the pK_{HA} values for acetic acid in 80% DMSO are as follows: 8.10,[26] 8.02,[29] 7.70 (80 vol.% DMSO),[30] 8.00,[31] 8.02;[32] our value, 7.27 in 81.4% DMSO. The literature data cited are obtained from e.m.f. measurements of cells with, as well as without, a liquid junction, and also from indicator methods or conductometric measurements.

The pK_{a2} values for bromophenol blue coincide with previously published data[25] just as was the case with salicylic acid. The same is valid for bromocresol green up to 80% of DMSO;[25,31,33] in 95 vol.% DMSO[25] and in the pure solvent[21] pK_{a2} is 6.36 and 7.30, respectively. However, while the pK_{a2} for the indicator in 80% DMSO[31,33] is higher than our value in 81.4% DMSO only by 0.06–0.12 units, the pK_{HA} value for acetic acid in these studies[31,32] is 0.7 units higher than ours. Naturally, the apparent pK_{a2} value for bromothymol blue in 80% DMSO, determined in acetate buffer,[31] is also higher than ours (in 81.4% DMSO) by 0.6 units. Meanwhile, our data for bromothymol blue (Table 1) agree better with the pK_{a2} values in 95 vol.% DMSO (9.90)[25] and in 100% DMSO (11.3).[21]

The fact that indicator measurements in both salicylate and benzoate (acetate) buffers were carried out by us with the same dye (bromocresol green) can be regarded as a guarantee of the correctness of the pK_{HA} difference for the pair of buffer acids. Considering the agreement of our results for the pK_{HA} of salicylic acid with other data,[19,25] the discrepancy between the pK_{HA} of benzoic (acetic) acid, obtained in the cited works, and our data is hard to understand.

The results obtained with help of cells without a liquid junction using the Ag/AgCl electrode as reference can be somewhat erroneous owing to difficulties in accounting for the formation of complex particles (*e.g.* $AgCl_2^-$).[28,29] That is why it is important to underline that when using the cell without a liquid junction consisting of quinhydrone and Tl(Hg)/TlBr electrodes (in the latter case the complexation is insignificant), the value $pK_{HA} = 8.37$ is obtained for benzoic acid in 93 wt.% DMSO,[34] which is close to our data (Table 1).

Electrochemical experiments with buffer solutions in 91.3% DMSO were carried out in our laboratory[18] using glass electrodes in cells with a liquid junction. The cell was calibrated with help of aqueous standard buffers; according to the well known approach,[19,35] the pa_H^* values are connected with the electrochemically obtained pH ones through the equation $pa_H^* = pH - \delta$. While using the published δ values for H_2O–DMSO mixtures[19,36] (confirmed by our experiments with HCl solutions, $\delta = 0.68 \pm 0.02$), the pa_H^* obtained for benzoate and acetate buffers lead to pK_{HA} values [eqn. (2)] which are close to the data mentioned above;[19,26–28] *e.g.* for benzoic acid in 91.3% DMSO $pK_{HA} = 8.69$. However, some doubts arise about the constancy of δ values within a wide pa_H^* range in the solvent under study.

Calculation of pK_a Values of Fluorescein and Sulfonefluorescein in H_2O–DMSO Mixtures

The general formula describing the ε–pa_H^* dependence for fluorescein[37] is simplified in the case of H_2O–DMSO mixtures owing to the strong shift of the H_3R^+ dissociation equilibria to the acidic region. In the case under study, the relationships at a fixed wavelength and constant ionic strength are governed by eqn. (5):

$$\varepsilon = \frac{(\varepsilon_{H_2R} \times 10^{pK_{a1} - pa_{H^*}}) + \varepsilon_{HR^-} + (\varepsilon_{R^{2-}} \times 10^{pa_{H^*} - pK_{a2}})}{1 + 10^{pK_{a1} - pa_{H^*}} + 10^{pa_{H^*} - pK_{a2}}} \quad (5)$$

where ε_{H_2R}, ε_{HR^-} and $\varepsilon_{R^{2-}}$ are the molar absorptivities of the corresponding forms at the λ values chosen. ε denotes the apparent value at the current pa_H^*. Some examples of ε dependences on pa_H^* are given in Fig. 1.

The spectra of the fluorescein neutral forms were measured in salicylate buffers (Fig. 2). The R^{2-} spectra were measured in (1×10^{-2})–(1×10^{-4}) mol dm^{-3} NaOH solutions (Fig. 3).

The calculation methods were partly described earlier,[12,14,15,38,39] and have certain elements in common with those reflected in well known monographs.[40,41]

To determine the pK_{a1} and pK_{a2} values the following algorithm was used, based on a knowledge of the visible spectra of fluorescein ionic forms. A pa_H^* interval was singled out where the bands of the R^{2-} ions (first of all a narrow band with $\lambda_{max} \geqslant 495$ nm) are absent from the visible spectra; owing to the low H_2R spectral intensity in the presence of organic co-solvents ($\varepsilon_{H_2R} \ll \varepsilon_{HR^-}$), the ε–λ curves reflect only the monoanion absorption (two bands of approximately equal intensity, $\lambda_{max} \approx 450$–$460$ nm and *ca.* 480 nm) differing in ε values in accordance with $[HR^-]$ variations resulting from pa_H^* changes. Considering the equilibrium eqn. (II) as isolated, K_{a1} can be evaluated. Then a higher pa_H^* interval was used for calculation of K_{a2} according to eqn. (5), with the

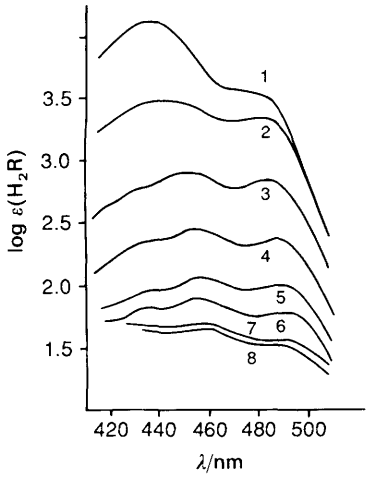


Fig. 2 Absorption spectra of the neutral form, H_2R, of fluorescein at various DMSO contents (wt.%): 1, 0; 2, 21.6; 3, 42.2; 4, 60.3; 5, 72.0; 6, 81.4; 7, 94.2; 8, 96.2

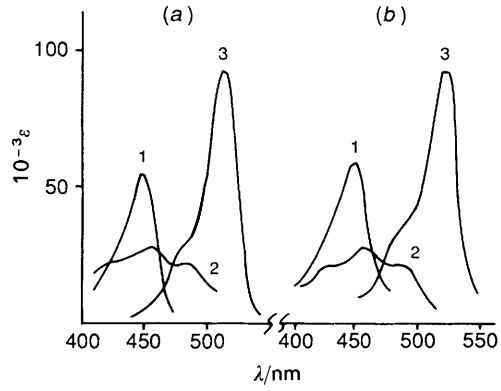


Fig. 3 Absorption spectra of ionic forms of fluorescein (*a*) and sulfonefluorescein (b) in 91.3 wt.% DMSO. (*a*) 1, cation H_3R^+ (**I**); 2, monoanion HR^- (**V**); 3, dianion R^{2-} (**VI**). (*b*) 1, zwitterion (**VII**); 2, monoanion HR^- (**VIII**); 3, dianion R^{2-} (**IX**)

help of K_{a1} values obtained as mentioned above. In calculating K_{a1} the λ region near λ_{max} of HR^- was used, while in calculating K_{a2} the λ region near λ_{max} of the dianion was used. The ε_{HR^-} values, obtained earlier in water–organic mixtures, were used as a first approximation [these values are known to be slightly affected by the solvent composition;[12–14,18,38] *e.g.* $\varepsilon_{HR^-} = (28–33) \times 10^3$ at $\lambda_{max} = 450–460$ nm]. Having the above K_{a1} and K_{a2} values, it became possible to calculate [through eqn. (6), resulting fron eqn. (5)] more precise ε_{HR^-} values (which practically coincide with those accepted initially).

$$\varepsilon_{HR^-} = \varepsilon + a_H^*(K_{a1})^{-1}(\varepsilon - \varepsilon_{H_2R}) + (a_H^*)^{-1}K_{a2}(\varepsilon - \varepsilon_{R^{2-}}) \quad (6)$$

As a rule, each K_{a1} value was calculated using the data within a pa_H^* interval of at least one unit, while for K_{a2} data within 0.3–1 pa_H^* units were used. The described sequence of K_{a1}, K_{a2} and E_{HR^-} calculations [using eqn. (5) and (6)] was repeated several times, but the second iteration proved to be sufficient.

Calculation of the K_{a1} and K_{a2} constants (jointly with ε_{HR^-}) was also performed using two programs, created by Dr. A. A. Bugaevsky and Dr Yu. V. Kholin, namely SOLEX, adjusted for spectrophotometry (at fixed analytical wavelength) and CLINP (the algorithm assumes utilization of the whole of the experimental data at various pa_H^* and λ). Both programs are based on the idea of finding the 'best values' of the unknown parameters (K_{a1}, K_{a2}, ε_{HR^-}), using the iterative method of Gauss–Newton.[42] The results of such calculations are in good agreement with those obtained by the iteration procedure described above. The calculations were performed with a personal computer ISKRA-1030.

The pK_{a0} values in water–DMSO mixtures were calculated according to the following formula:

$$pK_{a0} = -\log C_{HCl} + \log\left(\frac{\varepsilon - \varepsilon_{H_2R}}{\varepsilon_{H_3R^+} - \varepsilon}\right) \quad (7)$$

($f_{H^+} = f_{H_3R^+}$). The f_{H_2R} values were considered to be equal to 1. The cation molar absorptivities (Fig. 3) were obtained as described below. In the case of pK_{a0}, calculations were conducted at wavelength near the λ_{max} of H_3R^+. In 21.6% DMSO, the pK_{a0} value of fluorescein was also determined in salicylate buffers.

In order to compare our methods of calculation with those of other authors we have also determined the pK_a and ε values of fluorescein in aqueous solution (ionic strength 0.1), using the experimental data of Diehl and Horchak-Morris.[43] In water, all of the three ionization steps [eqn. (2)–(4)] should be treated together. For instance, the utilization of data at $\lambda = 437$ nm (40 pH values)[43] made it possible to obtain the following values: $pK_{a0} = 2.19 \pm 0.03$, $pK_{a1} = 4.39 \pm 0.08$ and $pK_{a2} = 6.30 \pm 0.04$ (SOLEX program). These coincide with literature values, 2.18, 4.40 and 6.36, respectively,[43] as well as with pK_a values published by us earlier, 2.27, 4.32 and 6.50.[44] The 10^{-3} ε values of H_3R^+, H_2R and HR^- at 437 nm as obtained by us from Diehl's experimental data and pK_a values are as follows: 49.4, 11.6 and 22.0 (the results of the original study are 49.4, 12.05 and 21.6, respectively).[43]

Comparison of our results obtained in H_2O–DMSO mixtures with other authors' data is impossible because of the absence of the latter. However, in general, a worse coincidence than obtained in water can be expected between various authors' results in mixtures rich in organic cosolvant. For instance, in water–dioxane systems, the thermodynamic pK_{a1} and pK_{a2} values also became much closer than in pure water. At a 1,4-dioxane content of 64 wt.%, pK_{a1} and pK_{a2} are equal to 9.45 ± 0.09 and 9.53 ± 0.22, respectively, as obtained spectrophotometrically in buffer solutions[45] through the iterative procedure of calculation described above. For pa_H^* scale estimation both indicator and pH-potentiometric methods were used.[45] In contrast, in the case of structurally similar 5-iodoacetamidofluorescein in 60% dioxane, studied by Drummond and collaborators,[46] $pK_{a2} - pK_{a1} \approx 2\ pK_a$ units, but the equilibria (II) and (III) were treated as isolated,[46] and no buffer solutions were used for adjusting pH, but only NaOH and HCl; pH values were checked electrochemically.[46]

The fluorescein thermodynamic pK_a values obtained at various DMSO contents are recorded in Table 2. The sulfonefluorescein pK_a (Table 3) were determined *via* a similar procedure. However, owing to great differences between pK_{a1} and pK_{a2}, the calculations are considerably simplified as

Table 2 Thermodynamic pK_a and other parameter values of fluorescein

DMSO (wt.%)	pK_{a0}	pK_{a1}	pK_{a2}	$\lambda_{max}(R^{2-})$ /nm	$\varepsilon_{max}(H_2R) \times 10^{-3}$	K_T	α_{III}	$pk_{0,OH}$	$pk_{1,COOH}$
0[a]	2.14 ± 0.01	4.45 ± 0.02	6.80 ± 0.01	491	13.9[b]	[c]	0.11	3.10	3.49
21.6	1.44 ± 0.07	5.50 ± 0.05	7.05 ± 0.12	495	3.18	8.43	0.106	2.41	4.53
42.2	1.07 ± 0.04	6.09 ± 0.06	7.08 ± 0.13	498	0.821	35.5	0.027	2.52	4.64
60.3	0.32 ± 0.03	7.21 ± 0.15	7.13 ± 0.16	503	0.287	104	0.010	2.34	5.18
72.0	—	—	—	—	0.122	245	4.1×10^{-3}	—	—
81.4	-0.37 ± 0.04	8.67 ± 0.03	7.46 ± 0.15	509	0.078	384	2.6×10^{-3}	2.21	6.09
91.3	-0.51 ± 0.03	10.33 ± 0.02	8.98 ± 0.17	513	0.051	587	1.7×10^{-3}	2.26	7.56
94.2	-0.50 ± 0.15	—	—	—	0.048	624	1.6×10^{-3}	—	—
96.2	-0.36 ± 0.11	—	—	—	0.044	681	1.5×10^{-3}	—	—
100	—	—	—	520[d]	—	303[e]	—	—	—

[a] The parameters in water are given as reported in ref. 47. [b] In this case, $\lambda_{max} = 437$ nm; at different DMSO contents, 450–460 nm and 480–490 nm. [c] **III** is also present: $\alpha_{II} = 0.218$, $\alpha_{III} = 0.111$, $\alpha_{IV} = 0.671$. [d] According to ref. 50. [e] According to ref. 49.

Table 3 Thermodynamic pK_a values and band maxima positions of ionic and molecular forms of sulfonefluorescein

DMSO (wt.%)	pK_{a1}	pK_{a2}	λ_{max}/nm		
			H_2R	HR^-	R^{2-}
0[a]	3.22 ± 0.11	6.76 ± 0.03	440	455; 480	495
21.6	2.89 ± 0.05	6.83 ± 0.03	443	455; 485	500
42.2	2.78 ± 0.05	6.90 ± 0.03	445	455; 485	505
60.3	2.66 ± 0.11	7.12 ± 0.05	447	455; 485	508
81.4	2.52 ± 0.10	7.64 ± 0.05	447	457; 487	515
91.3	3.16 ± 0.07	9.04 ± 0.04	450	460; 490	520

[a] pK_a values in water as reported in ref. 48.

compared with fluorescein. The spectra of H_2R, HR^- and R^{2-} forms were measured in concentrated HCl (H_2SO_4) solutions ($\geq$0.1 mol dm^{-3}), in salicylate buffers, and in dilute NaOH, respectively. The pK_{a1} values of sulfonefluorescein were determined in HCl solutions, and the pK_{a2} values in benzoate and diethylbarbiturate buffers. While the di- and mono-anion spectra of both xanthene dyes are similar (Fig. 3), the sulfonefluorescein neutral form possesses spectra nearly equal to those of the fluorescein cation, and the pa_H^* region of low absorption intensity is absent (Fig. 3). The transformation of H_2R into H_3R^+ is undetectable, and it was impossible to calculate the pK_{a0} values in the case of sulfonefluorescein.

Absorption Spectra of Ionic and Molecular Forms of the Dyes

For fluorescein (as well as for sulfonefluorescein), stable $\varepsilon_{R^{2-}}$ values in dilute NaOH were obtained (Fig. 3). However, it should be taken into account that the reaction (IV)

$$R^{2-} + OH^- \rightleftarrows ROH^{3-} \quad \text{(IV)}$$

became perceptible in the case of bromophenol blue and bromocresol green, and owing to this fact the $\varepsilon_{R^{2-}}$ of these sulfonephthaleins was evaluated in diethylbarbiturate buffers. (Note that the pa_H^* scale extent increases significantly in the H_2O–DMSO system as compared with pure water; so, the ionic product of the solvent, $[H^+] \times [OH^-]$, is *ca.* $10^{-18.4}$ in 80% DMSO.[31])

The fluorescein cation spectra (Fig. 3) can be measured only at high ionic strength (specifically at high DMSO content) because of the strongly acidic character of H_3R^+ species: $C_{HCl} \geq 1$ mol dm^{-3} in water and $C_{H_2SO_4} = 1.8$ mol dm^{-3} in 91% DMSO.

Evidently, the K_{a1} and K_{a2} closeness results in the inaccessibility of HR^- spectra for direct measurements. The calculations were carried out through eqn. (6), using the previously obtained K_{a1}, K_{a2}, ε_{H_2R} and $\varepsilon_{R^{2-}}$ values; the pa_H^* values (corresponding to the ε values) are chosen in the area of maximum output of monoanions. The procedure was performed over the whole λ region used. The results agree satisfactorily with those obtained using the CLINP program (which presupposes utilization of the whole pa_H^* and λ range and allows ε_{HR^-} values to be obtained).

The ε_{H_2R} values of fluorescein, obtained in salicylate buffers (Fig. 2), were verified with the help of eqn. (8)

$$\varepsilon_{H_2R} = \varepsilon + a_H^*(K_{a0})^{-1}(\varepsilon - \varepsilon_{H_3R^+}) + (a_H^*)^{-1}K_{a1}(\varepsilon - \varepsilon_{HR^-}) + (a_H^*)^{-2}K_{a1}K_{a2}(\varepsilon - \varepsilon_{R^{2-}}) \quad (8)$$

to avoid any influence of traces of intensely coloured ions (H_3R^+, HR^- and R^{2-}) on the spectra of the neutral forms. However, these corrections turned out to be insignificant.

Thus not only the set of K_{a0}, K_{a1} and K_{a2} values (Table 2), but also a series of ε–λ or log ε–λ curves (Fig. 2 and 3), characterising each of the forms (H_3R^+, H_2R, HR^-, R^{2-}) is obtained for every H_2O–DMSO mixture studied.

The same can be said about sulfonefluorescein (Table 3, Fig. 3); however, the ε_{HR^-} calculation through eqn. (6) does not expose any new information, unlike that obtained directly at $pa_H^* \approx 0.5(pK_{a1} + pK_{a2})$.

Discussion

Ratios of Ionization Constants and the Detailed Ionization Scheme

The gradual, regular variation of the K_{a1}/K_{a2} value of fluorescein with increase of DMSO content in the mixed solvent (up to $K_{a1} \ll K_{a2}$) is evident from the data presented in Table 2 and Fig. 4. It is easy to show that even at high DMSO content (when $K_{a1} < K_{a2}$) there exists a pa_H^* region where $[HR^-] \gg [R^{2-}]$, which legitimatizes the K_a calculation procedure used in the present study. Even if the pa_H^* scale is somewhat shifted it cannot cause inversion of the ionization constants.

While up to 60 wt.% DMSO, only the tendency of K_{a1} and K_{a2} to approach one another is seen, in 81% and 91% DMSO the inversion becomes obvious. The K_{a1}/K_{a2} ratio changes from 224 in water to 0.045 in 91% DMSO. Meanwhile, the ratio K_{a0}/K_{a1}, in contrast, increases from 204 to 7×10^{10} see Fig. 4.

No explanation of such anomalous ionization constant ratios can be made without considering the structural formulae describing the ionization processes,[12–15,38,39,45,47,48] see Scheme 1. The H_3R^+ cation exists in solutions as **I**, the neutral form H_2R is a mixture of three tautomers: the zwitterion **II**, quinoid structure **III** and colourless lactone **IV**. HR^- and R^{2-} ions exist as **V** and **VI**.[12–15,38,39,45,47,48] The estimation of tautomer interrelations is impossible without extrathermodynamic assumptions. Attribution of the microconstants, k, is correct so long as the detailed ionization scheme is adequate. In the tautomerism study, the spectra assigned to individual ionic (molecular) forms [see eqn. (6) and (8)], must be used. The principal extrathermodynamic assumptions used are:[12,38,39,46,47,49] (i) the lactone (**IV**) is colourless due to sp^3 hybridization of the central carbon

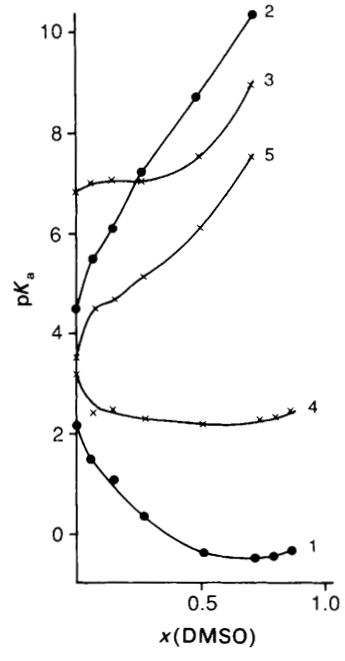
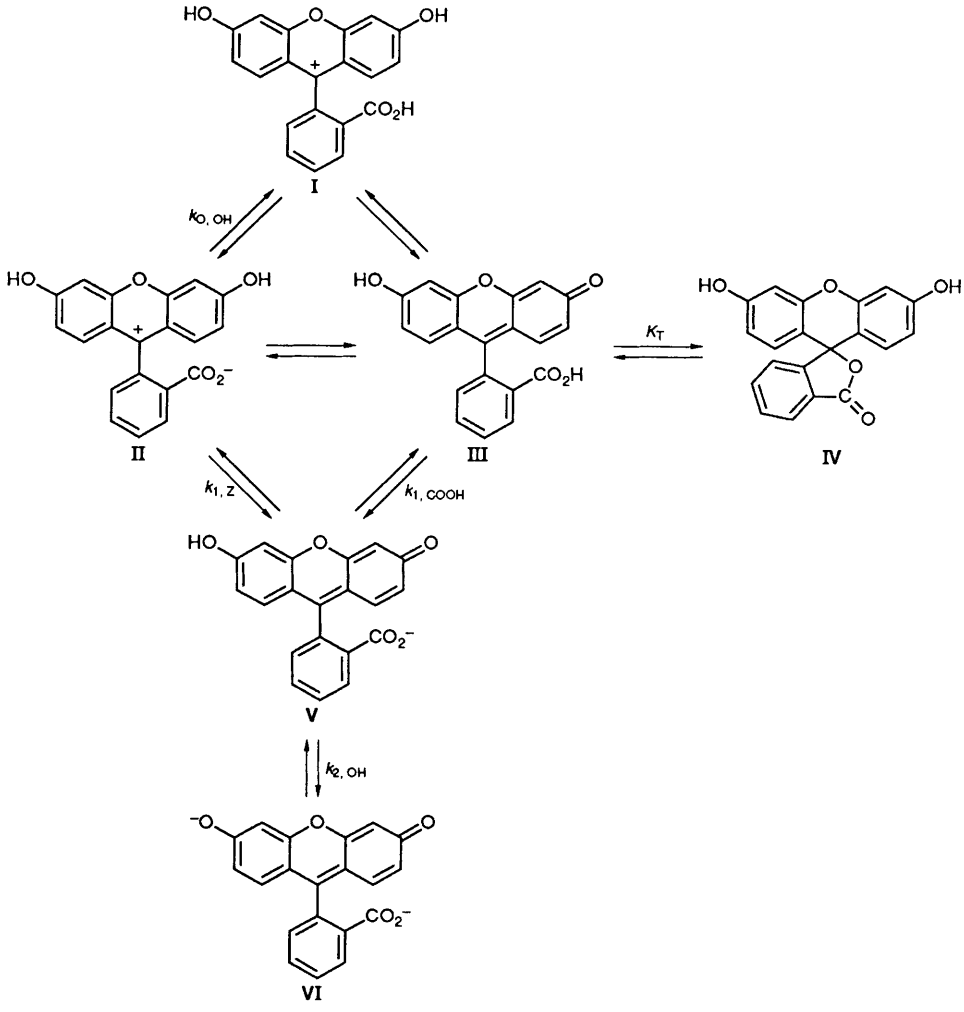


Fig. 4 Dependences of pK_a values on the mole fraction of DMSO: 1, pK_{a0}; 2, pK_{a1}; 3, pK_{a2}; 4, $pk_{0,OH}$; 5, $pk_{1,COOH}$



Scheme 1 Protolytic equilibria of fluorescein

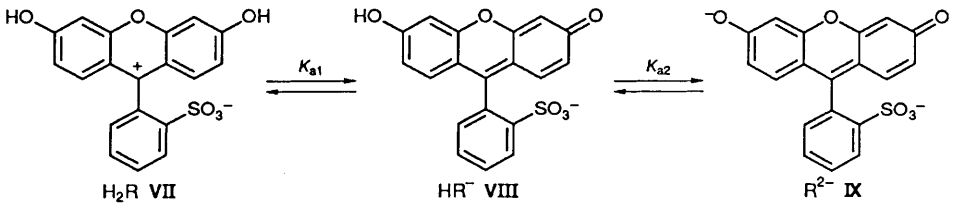
atom; (ii) the substituent in the 2′ position influences the absorption band in the visible region, but only slightly (hence the spectra of **II** and **III** must be close to those of **I** and **V**, respectively). The latter is confirmed in our study by the similarity of the R^{2-} spectra of fluorescein and sulfonefluoroscein (Fig. 3); the ionization scheme of sulfonefluorescein is given in Scheme 2.

The spectra of the various forms are either measured directly, or in the case of HR^-, obtained together with the pK_a (Fig. 3). Relatively small ε_{max} changes, observed for ionic forms at various DMSO contents [for instance, $\varepsilon_{max}(R^{2-})$ in water is 88×10^3, in 91% DMSO, 98.3×10^3, and in pure DMSO,[50] 113×10^3], are of solvatochromic nature, *cf.* $\lambda_{max}(R^{2-})$ shifts.[50]

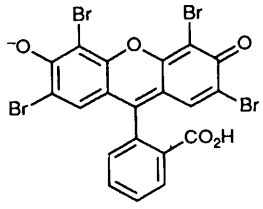
The solvent composition seems to affect the H_3R^+ spectra still less (*e.g.* in water, $\lambda_{max} = 437$ nm, $\varepsilon_{max} = 54.3 \times 10^3$; in 91% DMSO, $\lambda_{max} = 450$ nm and $\varepsilon_{max} = 58.2 \times 10^3$). The spectrum of the fluorescein HR^- form differs from that of R^{2-}, and practically coincides with that of the corresponding sulfonefluorescein. This proves the existence of HR^- as **V** (it is self-evident, that the acidity of SO_3H group of sulfonefluorescein is much higher than that of the OH group of the dye). Contrary to this, in the case of eosin (2,4,5,7-tetrabromofluorescein) the monoanion HR^- is ionized through the oxy-group, while the COOH group remains undissociated, see Scheme 3. [In various solvents the HR^- ion spectrum of eosin is somewhat shifted (3–15 nm) to the red as compared to the R^{2-} band, $\varepsilon_{max}(HR^-) \approx 0.9 \times \varepsilon_{max}(R^{2-})$.]

The fluorescein HR^- spectrum is practically independent of the DMSO content. However, in the case of the H_2R form, the absorptivity drops by two orders of magnitude from 21.6 wt.% to 91.3 wt.% DMSO (Fig. 2). This effect is in contrast to that mentioned above for fluorescein ions; in the case of transfer of fluorescein molecules from water to alcohols, acetone and dioxane, interconversion into the lactone structure usually occurred.[12–15,38,39,45,46,49]

The IR spectra also confirm the lactone predominance over other tautomers in slightly coloured solutions: the



Scheme 2 Ionization of sulfonefluorescein



Scheme 3 Eosin monoanion (HR^-)

marked band at 1755 cm^{-1} of the fluorescein neutral form in DMSO is assigned to the C=O stretching vibrations of the lactone.[15,49]

Determination of Tautomeric Equilibrium Constants

Considering the fluorescein neutral form H_2R as a mixture of three tautomers (**II** ⇄ **III** ⇄ **IV**), eqn. (9) follows:

$$\varepsilon_{H_2R} = \varepsilon_{II}\alpha_{II} + \varepsilon_{III}\alpha_{III} + \varepsilon_{IV} \quad (9)$$

where

$$1 = \alpha_{II} + \alpha_{III} + \alpha_{IV} \quad (10)$$

α are the fractions of the tautomers. According to the assumption (ii) made above, the $\varepsilon_{H_3R^+}$ and ε_{HR^-} values can be used as ε_{II} and ε_{III}, respectively; carrying out the calculations at several wavelengths, α values can be obtained.[38,39,47] In aqueous solution, the H_2R tautomer fractions are as follows:[47] $\alpha_{II} = 0.218 \pm 0.009$; $\alpha_{III} = 0.111 \pm 0.001$; $\alpha_{IV} = 0.671 \pm 0.009$.

The very first DMSO additives cause the disappearance of tautomer **II** (the sharp drop in intensity of the band at *ca.* 440 nm, corresponding to the zwitterionic structure), while the equilibrium **III** ⇄ **IV** is displaced to the right, which may be due to strong decoloration. Even at a DMSO content of 21.6 wt.%, a reliable estimation of α_{II} is impossible; $\alpha_{II} \ll \alpha_{III}$. Thus to determine the α_{III} values at various DMSO contents, the following simplified expression may be used:

$$\alpha_{III} = \varepsilon_{H_2R}/\varepsilon_{III} \quad (11)$$

The wavelengths used here correspond to λ_{max} of **III** and **V** (450–460 nm); ε_{III} is set equal to ε_V (*i.e.* ε_{HR^-}). The mean value for ε_{max} of HR^- forms of fluorescein (as well as of sulfonefluorescein) throughout the series is 30×10^3. The results of the α_{III} calculation are given in Table 2. The constant of the tautomeric equilibrium **III** ⇄ **IV**, K_T, is connected with α_{III} in the following manner:

$$K_T = \alpha_{III}^{-1} - 1 \quad (12)$$

provided that $\alpha_{III} \gg \alpha_{II}$.

The value $K_T^{-1} = 3.3 \times 10^{-3}$ for fluorescein in DMSO given in the literature[49] differs from our data (Table 2). The deviation is caused, from our point of view, by the presence of some HR^- ions while measuring the H_2R electronic absorption spectrum in the cited work.[49]

As to the nature of the solvent influence on the tautomerism (in terms of selective solvation *etc.*), this is a subject for separate discussion; the H-bonds are known to affect significantly the equilibrium **III** ⇄ **IV**.[15,39,49] Here, we have only to mention the linear correlation between log K_T and the Dimroth–Reichardt parameter $E_T(30)$, as well as E_T^N, the latter is known to reflect both the polarity and H-bonding ablity of the solvent:

$$\log K_T = 5.09 - 4.33E_T^N \quad (13)$$

0–96.2 wt.% DMSO; $n = 9$, $r = -0.993$. (The E_T^N values for H_2O–DMSO mixtures are taken from some recent studies.[51,52])

Medium Effects and Inversion of Ionization Constants

It is easy to show that for pK_{a0} and pK_{a1}:

$$pK_{a0} = pk_{0,OH} + \log \alpha_{III} \quad (14)$$

$$pK_{a1} = pk_{1,COOH} - \log \alpha_{III} \quad (15)$$

$$pK_{a2} = pk_{2,OH} \quad (16)$$

where k are ionization microconstants; $k_{0,OH} = [H^+][III]/[I]$, $k_{1,COOH} = [H^+][V]/[III]$, $k_{2,OH} = [H^+][VI]/[V]$, see Scheme 1. The $pk_{0,OH}$ and $pk_{1,COOH}$ values are presented in Table 2. Owing to additional treatments, the error in pk estimations is somewhat higher than pK_a standard deviations (Table 2).

The medium effect, ΔpK_a [*i.e.* pK_a(in the given solvent) − pK_a(in water)], for the ionization process of an acid, HB, can be expressed through the transfer activity coefficients (γ) in the following manner:

$$\Delta pK_a = \log \gamma_H + \log \gamma_B - \log \gamma_{HB} \quad (17)$$

The high basicity of water–DMSO mixtures [according to various extrathermodynamic approaches, $\log \gamma_H$ is always negative,[53–55] reaching −(1.6–5.7) in pure DMSO[18,53–57]] results in relatively low pK_a and pk values of the dyes under study compared with those in methanol[38] and in aqueous acetone.[13] However, this factor cannot, of course, influence the ratios K_{a0}/K_{a1} and K_{a1}/K_{a2}.

According to eqn. (14)–(17):

$$\Delta pK_{a0} = \Delta pk_{0,OH} + \Delta \log \alpha_{III} \quad (18)$$

$$\Delta pK_{a1} = \Delta pk_{1,COOH} - \Delta \log \alpha_{III} \quad (19)$$

$$\Delta pK_{a2} = \Delta pk_{2,OH} \quad (20)$$

where $\Delta pk_{0,OH} = \log \gamma_H + \log(\gamma_{III}/\gamma_I)$, $\Delta pk_{1,COOH} = \log \gamma_H + \log(\gamma_V/\gamma_{III})$, $\Delta pk_{2,OH} = \log \gamma_H + \log(\gamma_{VI}/\gamma_V)$.

For pK_{a0}, pK_{a1} and K_{a2} differences:

$$\Delta(pK_{a1} - pK_{a0}) = \Delta pk_{1,COOH} - \Delta pk_{0,OH} - 2\Delta \log \alpha_{III} \quad (21)$$

$$\Delta(pK_{a2} - pK_{a1}) = \Delta pk_{2,OH}/\Delta pk_{1,COOH} + \Delta \log \alpha_{III} \quad (22)$$

Thus, in the case of pK_{a1}, the medium effect consists of changes in the carboxyl group pk, and a component from the tautomeric equilibrium shift. The same approach can be applied to interpret the medium effect for pK_{a0}. Since pK_a values of cationic acids ($pk_{0,OH}$) change comparatively slightly on transfer from water to organic solvents,[17,54] and medium effects for carboxylic acids ($pk_{1,COOH}$) are usually higher than for phenols ($pK_{a2} = pk_{2,OH}$)[25,58,59] (see Fig. 4), the resulting changes in the ratios K_{a1}/K_{a2} and K_{a0}/K_{a1} can be rationalised, especially considering the significant α_{III} decrease, the latter contributing to the pK_{a1} growth and pK_{a0} lowering [see eqn. (21) and (22)].

The correctness of the obtained pK_{a2} values of fluorescein is confirmed by their closeness to those of sulfonefluorescein (Scheme 2, Table 3). As the latter is unable to undergo any tautomerization, its K_{a1} value can be considered a model for the ionization constant $k_{1,Z}$ of the zwitterionic tautomer of fluorescein (**II**); therefore, the pK_{a1} values of sulfonefluorescein when compared with the $pk_{1,COOH}$ values of fluorescein (Fig. 4) confirm that [**II**] ≪ [**III**] (see Scheme 1) at medium and high DMSO contents.

The solvent composition dependences of the $pk_{1,COOH}$ of fluorescein and the pK_a ($=pK_{HA}$) of benzoic acid are similar (Tables 1 and 2). The dependence of the $pk_{0,OH}$ of fluorescein and the pK_{a1} ($=pk_{1,Z}$) of sulfonefluorescein on DMSO content is in accordance with that for other cationic[60,61] as well as zwitterionic[62] acids. All this confirms the validity of the above extrathermodynamic assumptions made for α estimation.

Therefore, in some cases, consideration of organic acid tautomerism is necessary in order to reationalise medium effects and inversion phenomena. Note the similarity of the effects discussed and those registered for fluorescein ionization in aqueous solutions of some non-ionic polymers.[37] In the latter case, however, the effects are less evident and inversion of K_{a1} and K_{a2} is not reached.

Conclusion

The results obtained enlarge to a certain degree the existing knowledge about acid–base equilibria, in particular, the inversion of ionization constants, studied through solvent variation. The inversion of the experimentally obtained apparent constants of fluorescein, K_{a1} and K_{a2}, is a result of the shift of the tautomeric equilibrium, as well as of the chemical nature of the ionizing groups (COOH, OH).

The universal nature of the explanation in terms of medium effects for oxyxanthene (fluorescein) dyes and the adequacy of the detailed ionization scheme for these substances are established. Within this reagent series in various solvents many systems with pK_a inversion are expected.

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