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Ionization and Tautomerism of Chloro-Derivatives of Fluorescein in Water and Aqueous Acetone

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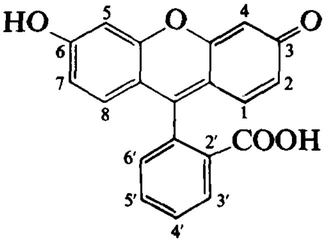
ABSTRACT

The pK_a *values of 2,7-dichlorofluorescein and 3′,4′,5′,6′-tetrachlorofluorescein in water and in aqueous acetone (to 90 wt%* $(CH_3)_2CO$*) were determined. On the basis of the visible absorption spectra of the dyes, conclusions are made about the tautomerism of the dyes and their monoanions. Values of the tautomeric equilibrium constants and the microconstants of ionization were calculated. An equilibrium scheme is proposed, which adequately explains the influence of solvents on the* pK_a *values.*

1 INTRODUCTION

Fluorescein dyes are widely used in analytical chemistry, biochemistry, photochemistry, and laserochemistry because of their unique optical properties.[1–3] Information on the equilibria parameters in dye solutions would enable more rational use to be made of these compounds. Structural variations of such dyes (typified by the neutral tautomeric configuration shown) have been studied in water and in non-aqueous solutions, with



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respect to unsubstituted fluorescein and to its 2,4,5,7-tetrabromo and -tetraiodo derivatives (eosin and erythrosin, respectively).[3-13] The behaviour of other representatives of this series has received less attention; and, in particular, 2,7-dichlorofluorescein (DCF) and 3′,4′,5′,6′-tetrachlorofluorescein (TCF), both of which are industrial products with practical utilization, have not been studied sufficiently. This present study is concerned with an investigation of the protolytic equilibria in solution of the above chloro-derivatives.

The functional-group ionization, as well as the tautomeric equilibria, is essentially different for the unsubstituted dye and its 2,4,5,7-tetrahalogen derivatives. It is therefore of interest to clarify the nature of the influence of the introduction of only two halogen atoms into the resorcinol rings, as well as to ascertain the effect of substitution only in the phthalic acid residue. To obtain a more complete picture of these effects, the equilibria were studied in water and also in water—acetone mixtures containing the organic cosolvent up to 90 wt%.

2 EXPERIMENTAL

2.1 Materials

DCF and TCF (VEB Apolda) were purified by reprecipitation from aqueous solution with hydrochloric acid and then by chromatography. Their purity was checked by means of spectrophotometry and chromatography (Silufol plates).

Twice-distilled water was used for preparing all solutions. In determining pK_a values of the dyes in water, suitable pH values were obtained with analytical-grade reagents: hydrochloric acid, potassium hydroxide, acetic acid, sodium hydrophosphate, borax, and sodium chloride (the last-named for adjusting to constant ionic strength). Chemically pure acetone was distilled from potassium permanganate and then from potassium carbonate. The water content was estimated by titration in pyridine according to K. Fischer's method. To create the required pH values, analytical-grade perchloric acid, sodium hydroxide, salycilic acid, benzoiacid, acetic acid, and diethylbarbituric acid (all of analytical grade) were used.

2.2 Measurements

The pH values of solutions were checked by means of potentiometry by using cells with a liquid junction, glass electrode, and a silver–silver chloride reference electrode, according to the compensation scheme on a potentiometer P 363/3 and pH meter–millivoltmeter pH-121. All solutions were prepared and pH measurements performed at 25·0 ± 0·1°C. Spectra of dye solutions were measured by using SP-26 and SP-46 spectrophotometers (of USSR origin). All spectral measurements were performed at 25 ± 1°C.

In investigating DCF in aqueous solutions, the absorption spectra were measured at 400–520 nm (21 different wavelengths were used as analytical positions in pK_a calculations) for dye solutions having pH values ranging from 1·2 to 9·0 at constant ionic strength ($I = 0{\cdot}05\,\text{M}$) and also for dye solutions with hydrochloric acid concentration ranging from 0·2 to 10·0 M (where the principle of constant ionic strength was not adhered to). Some 33 spectra of different solutions were measured in all. In most of the solutions investigated, the dye concentration was 2×10^{-5} M, excluding solutions having a pH of less than 3·2, where it was 8×10^{-6} M because of the low solubility of the neutral form. In investigating TCF in aqueous solutions, the absorption spectra were measured in the same wavelength interval of 400–520 nm (within which 19 wavelengths were chosen as analytical positions) for dye solutions with pH values ranging from 1·10 to 12·6 at $I = 0{\cdot}05\,\text{M}$, and also for dye solutions with a hydrochloric acid concentration ranging from 0·4 to 4·5 M (I not constant; see above); 28 spectra were measured. The dye concentration was 1×10^{-5} M in most cases and 4×10^{-6} M in acidic solutions because of the dye solubility.

For studies of the dye equilibria in water–acetone mixtures, solutions were prepared by the volume method, and the acetone content was determined by weighing (water contamination in acetone taken into account). Various pH values of solutions were achieved by using perchloric acid and sodium hydroxide, and principally through buffers (salycilate, benzoate, acetate, and diethylbarbiturate), pH values (in the scale of pa_{H}^{*}[14]) being calculated on the basis of pK_{HA} values of buffer acids:

$$\text{pa}_{\text{H}}^{*} = \text{p}K_{\text{HA}} + \log\frac{[\text{A}^-]}{[\text{HA}]} + \log f_1$$

where [A$^-$] and [HA] are equilibrium concentrations, f_1 the activity coefficient of the single-charged ion (calculated by using the Debye–Huckel equation; the ionic parameter å equals 5). The value [A$^-$]/[HA] may be calculated by using the analytical concentrations (c) as $c_{\text{NaOH}}/(c_{\text{HA}} - c_{\text{NaOH}})$, but only if $[\text{Na}^+] \gg [\text{H}^+]$.

The most reliable values were selected as a result of comparative analysis of the data published by other authors:[15–19] in 8 wt% acetone, the pK_a of the salycilic, benzoic, acetic, and diethylbarbituric acids equals 3·16, 4·46, 4·96, and 8·18, respectively; in 90 wt% acetone, the values were 7·22, 9·75, 10·27, and 12·82, respectively. In 16% acetone, the pK_a value of acetic acid is 5·16, in 52% 6·55, in 73% 8·01. In 52% acetone, the pK_a value of salycilic acid is 4·28 and that of the diethylbarbituric acid is 9·58.

TABLE 1

Thermodynamic pK_a Values of 3′,4′,5′,6′-Tetrachlorofluorescein, 25°C

Acetone, wt%	D^a	I	n^b	m^c	pK_{a0}	pK_{a1}	pK_{a2}
0	78·5	0·05	28	19	$-0{\cdot}29 \pm 0{\cdot}06$	$3{\cdot}65 \pm 0{\cdot}05$	$6{\cdot}55 \pm 0{\cdot}02$
8	74·1	$1{\cdot}7 \times 10^{-3}$	19	18	—	$4{\cdot}37 \pm 0{\cdot}06$	$6{\cdot}56 \pm 0{\cdot}03$
52	46·9	$3{\cdot}74 \times 10^{-3}$	15	5	—	$7{\cdot}17 \pm 0{\cdot}08$	$7{\cdot}22 \pm 0{\cdot}08$
90	24·0	3×10^{-4}	23	21	—	$10{\cdot}9 \pm 0{\cdot}4$	$11{\cdot}57 \pm 0{\cdot}07$

[a] D = Dielectric constant.

[b] n = Number of working solutions.

[c] m = Number of analytical positions (wavelengths).

TABLE 2

Thermodynamic pK_a Values of 2,7-Dichlorofluorescein, 25°C

Acetone, wt%	D	I	n	m	pK_{a0}	pK_{a1}	pK_{a2}
0	78·5	0·05	32	21	$0{\cdot}35 \pm 0{\cdot}05$	$4{\cdot}00 \pm 0{\cdot}10$	$5{\cdot}19 \pm 0{\cdot}06$
16	69·4	$4{\cdot}84 \times 10^{-3}$	10	4	—	$5{\cdot}0 \pm 0{\cdot}6$	$5{\cdot}5 \pm 0{\cdot}3$
33[a]	59·1	0·05	9	28	—	$6{\cdot}03 \pm 0{\cdot}04$	$6{\cdot}40 \pm 0{\cdot}04$
52	46·9	$4{\cdot}84 \times 10^{-3}$	7	3	—	$7{\cdot}0 \pm 0{\cdot}1$	$7{\cdot}8 \pm 0{\cdot}2$
73	33·9	$5{\cdot}2 \times 10^{-3}$	7	6	—	$8{\cdot}6 \pm 0{\cdot}2$	$8{\cdot}4 \pm 0{\cdot}1$
90	24·0	$1{\cdot}76 \times 10^{-3}$	28	14	—	$10{\cdot}3 \pm 0{\cdot}3$	$10{\cdot}4 \pm 0{\cdot}2$

[a] Additional series ($n = 21$, $m = 4$, 21°C, $D = 60$) gives: $5{\cdot}9 \pm 0{\cdot}2$, $6{\cdot}28 \pm 0{\cdot}07$.

In some cases, the pH values of solutions were checked potentiometrically by using published values of δ ($pa_H^* = pH - \delta$).[19] Ionic strengths values and other experimental parameters are reported in Tables 1 and 2.

3 RESULTS AND DISCUSSION

3.1 Calculation of pK_a values of dyes in aqueous solutions

Calculation of pK_{a0}, pK_{a1}, and pK_{a2} values corresponding to the stepwise ionization of the dyes in solution, namely:

$$\begin{aligned} &H_3R^+ \rightleftharpoons H_2R + H^+, && K_{a0} \\ &H_2R \rightleftharpoons HR^- + H^+, && K_{a1} \\ &HR^- \rightleftharpoons R^{2-} + H^+, && K_{a2} \end{aligned}$$

was performed with a personal computer ISKRA-1030 by the program CLINP, created by Yu. V. Kholin. The algorithm of calculation assumes utilization of the whole of the experimental data.[20]

Spectra of dye solutions at different pH values, together with spectra of H_3R^+- and R^{2-}-forms, and calculated spectra of HR^--forms for both dyes are shown in Figs 1 and 2. Typical plots of molar absorptivities against pH are shown in Fig. 3. The relationships are governed by eqn (1):

$$\varepsilon = \frac{\varepsilon_{H_2R} \times 10^{pK_{a1} - pH} + \varepsilon_{HR^-} + \varepsilon_{R^{2-}} \times 10^{pH - pK_{a2}}}{10^{pK_{a1} - pH} + 1 + 10^{pH - pK_{a2}}} \qquad (1)$$

where ε_{H_2R}, ε_{HR^-}, and $\varepsilon_{R^{2-}}$ are the molar absorptivities of the corresponding forms. When pK_a values of the dyes in aqueous solution were calculated, an assumption was made regarding the negligible difference between the spectra of the cationic and neutral forms in solutions of ionic strength 0·05 M and above. For joint calculation of all pK_a values, and spectra of H_2R- and HR^--forms, the measured pH values of solutions with $I = 0{\cdot}05$ were used as well as the H_0 values for concentrated hydrochloric acid solutions taken from the literature.[21] The thermodynamic pK_a values were obtained by using activity coefficients of single- and double-charged ions, calculated according to Davies's formula ($I = 0{\cdot}05$: $f_1 = 0{\cdot}82, f_2 = 0{\cdot}45$). In the case of TCF, p$K_a$ values were calculated jointly with the spectra of H_2R-, HR^-- and H_3R^+-forms.

The DCP pK_a values in water, as obtained by us, roughly agree with the data of other authors. Thus, pK_{a0}, pK_{a1}, and pK_{a2} values estimated from the plots of absorbances against pH have been reported[24] as 0·6, 3·9, and 5·1, respectively, although full details were not given. Other reported values of

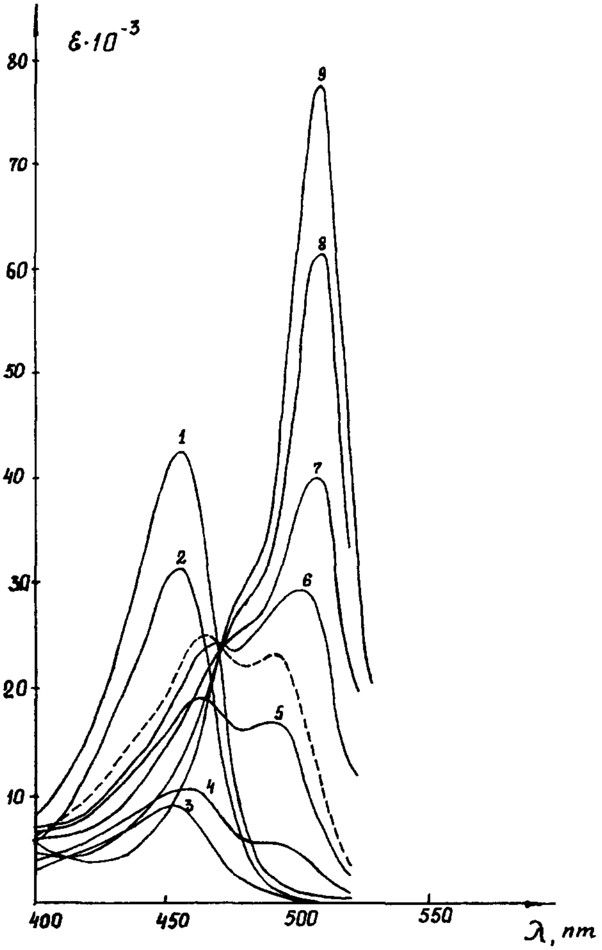


Fig. 1. Absorption spectra of TCF in water: 1—4·35M HCl (spectrum of H_3R^+ form), 2—1·82M HCl, 3–8—pH = 1·97, 2·96, 3·98, 5·73, 6·12, 6·73, respectively; 9—pH > 9 (spectrum of R^{2-} form); dashed line—spectrum of HR^- form, calculated jointly with pK_a values.

0·47, 3·50, and 4·95, obtained spectrophotometrically,[25] are based on the assumption that activity coefficients of ions are equal to 1 and the I value was not kept constant. The pK_{a1} and the pK_{a2} values, estimated by taking account of f, have been reported as 3·69 and 4·71,[26] but the equilibria were considered in isolation. Other reported pK_{a0}, pK_{a1}, and pK_{a2} values (2·28 ± 0·08, 4·7 ± 0·3, and 12·1 ± 0·9)[27] are probably erroneous.

3.2 Calculation of pK_a values in mixtures H_2O–$(CH_3)_2CO$

An illustration of the relationship between pa_H^* and spectra is shown in Fig. 4. The results are presented in Tables 1 and 2. Calculations were performed

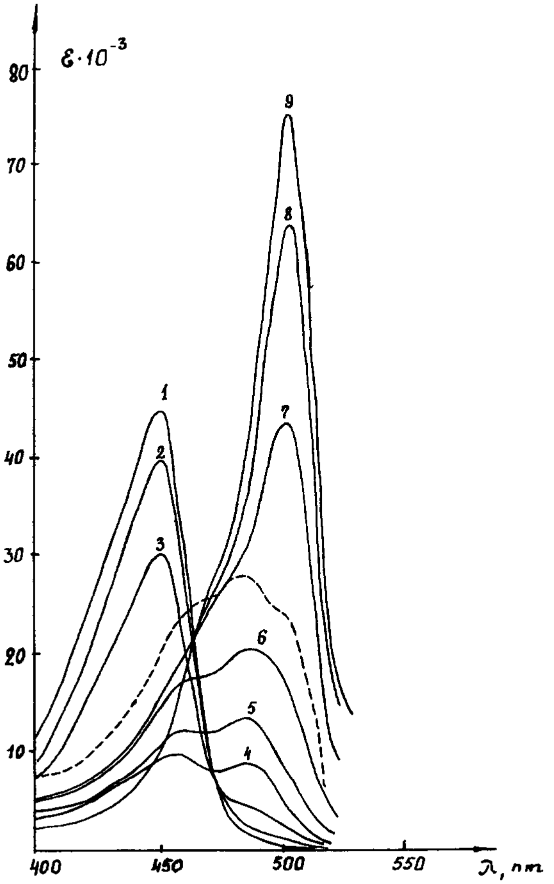


Fig. 2. Absorption spectra of DCF in water: 1—4·53M HCl (spectrum of H_3R^+ form); 2—1·82M HCl; 3—0·34M HCl; 4–8—pH = 1·97, 3·37, 3·97, 4·77, 5·62, respectively; 9—pH > 7 (spectrum of R^{2-} form); dashed line—spectrum of HR^- form, calculated jointly with pK_a values.

by using the CLINP program. Other methods give similar results. The SOLEX program,[28] adjusted to spectrophotometry, has been used previously,[23] as has the successive-approximation method (the K_{a1}, K_{a2} and ε_{HR^-} calculations made by using eqn (1))[22] or similar, based on equilibria overlapping.[29,30] In general, the data thus obtained are similar, but sometimes, especially for DCF, differences are quite marked (Table 1).

3.3 Tautomeric equilibria in solutions: background

An interpretation of the pK_a values is not possible without full understanding of the tautomeric equilibria involved. The protolytic

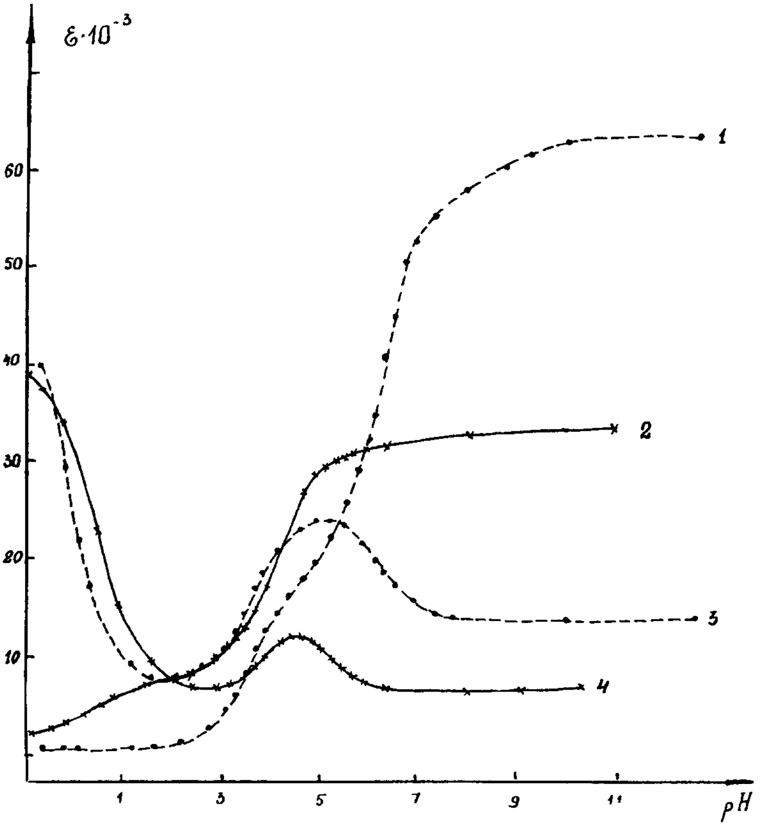


Fig. 3. Dependences of ε upon pH: 1—TCF, 500 nm; 2—DCF, 480 nm; 3—TCF, 460 nm; 4—DCF, 440 nm; solvent—water.

transformations of oxyxanthene dyes, using for unsubstituted fluorescein as an example, are shown in Scheme 1.

An analysis of the different stages of the ionization has been made in several previous investigations.[3-5,9-13,22,29-31] It has been shown that, for fluorescein, these transformations are as follows: I→(II⇌III⇌IV)→V→VII, but that, for 2,4,5,7-tetrahalogene substituted dyes (such as eosin and others), the transformations are: I→(III⇌IV)→VI→VII. The existence of the tautomers II, III, and IV in the solid state has been demonstrated by using IR- and mass-spectrometry.[6] The main assumptions on which estimations of the tautomerization constants are based are as follows:

(i) the lactone (IV) is colourless, owing to sp^3-hybridization of the central carbon atom, and

(ii) the absorption bands of the species differing only in the state of the carboxyl group (i.e., COOH or COO^- as in I and II; III and V; VI and VII) are similar.

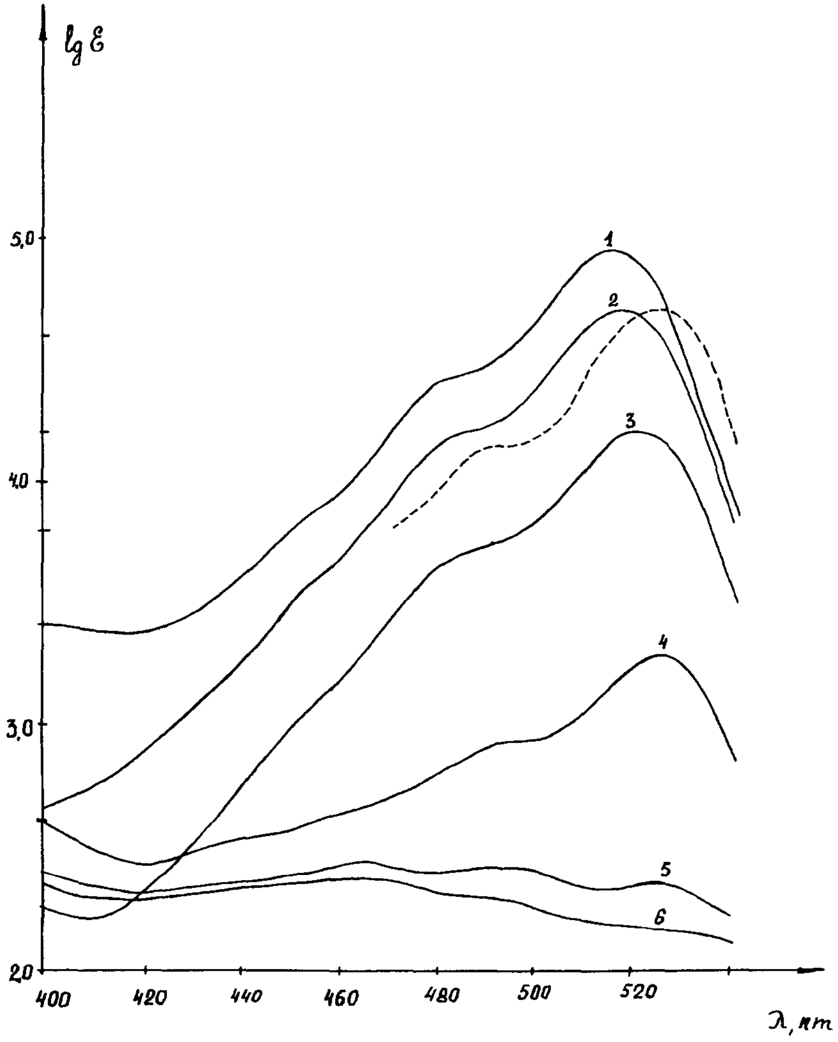
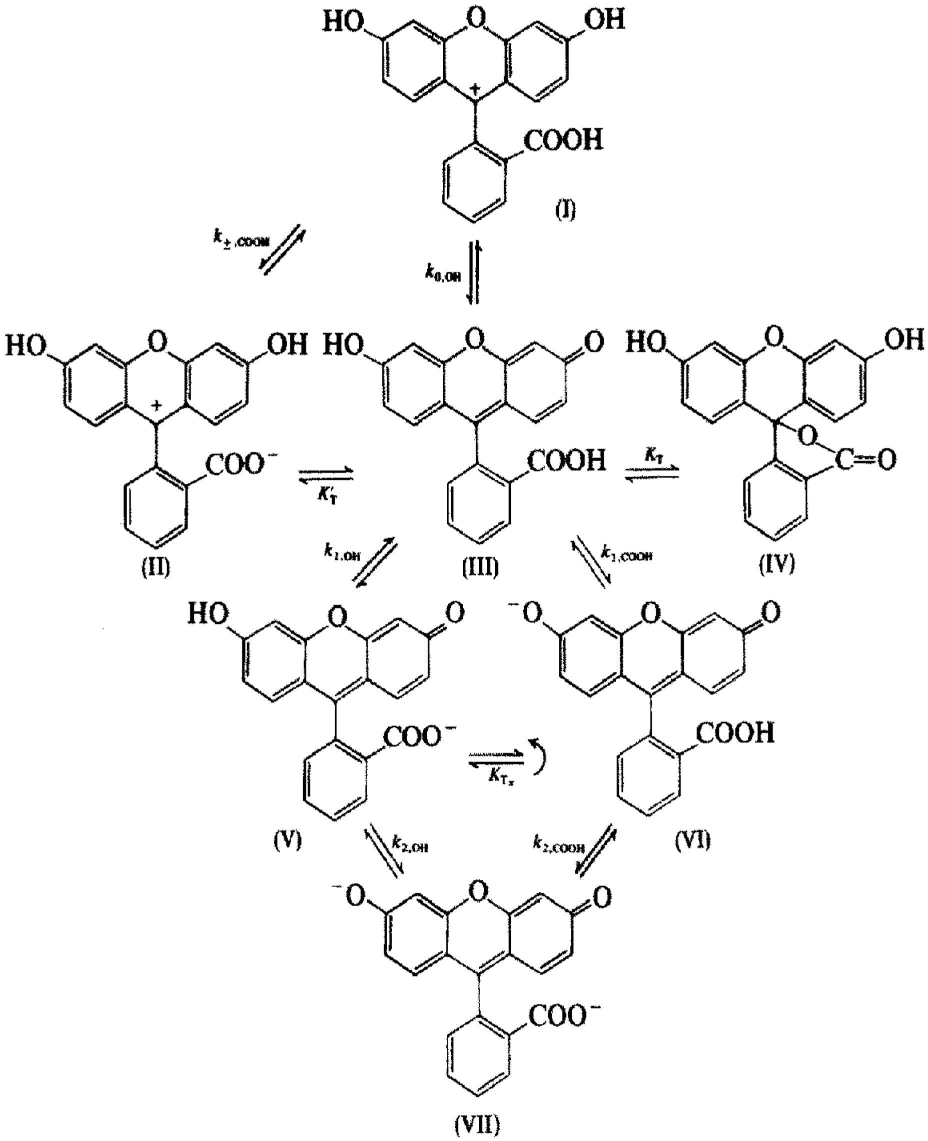


Fig. 4. Absorption spectra of DCF in 90 wt% acetone: 1—pH > 12·8 (R^{2-}); 2–6—pa_H^* = 9·89, 9·62, 8·70, 7·08, 5·65, respectively; dashed line: HR^-.

In the latter case, as shown for rhodamine B (cation and zwitter-ion[5,22,32]), the presence of an additional negative charge in the 2′-position leads to a small hypsochromic shift (from 3 to 15–20 nm, depending on a solvent), which allows an estimation of pK_{a2} to be made for the transition from HR^- to R^{2-} (as for eosin). Examples of HR^- and R^{2-} spectra are represented in Fig. 5. They illustrate two types of the HR^--spectra, corresponding to species V and VI, respectively. For fluorescein and eosin in different solvents (water–acetone,[12,29,33] water–alcohol,[30] water–dioxan,[13] water–dimethyl



Scheme 1

sulphoxide,[31] methanol,[22] micellar solutions of cationic[34] and non-ionic surfactants[23]), absolute values of λ_{max} undergo certain changes, but the spectra type remains unchanged. The assumption noted above regarding the similarity of the absorption bands is confirmed by the electronic spectra of some compounds that may be considered as model ones with respect to fluorescein and eosin. Thus, visible spectra of forms H_2R^+, HR, and R^- of 6-hydroxy-9-phenylfluorone (Scheme 2) are similar to those of forms H_3R^+, HR^-, and R^{2-} of fluorescein[9,11,29,31] and the latter, in turn, are

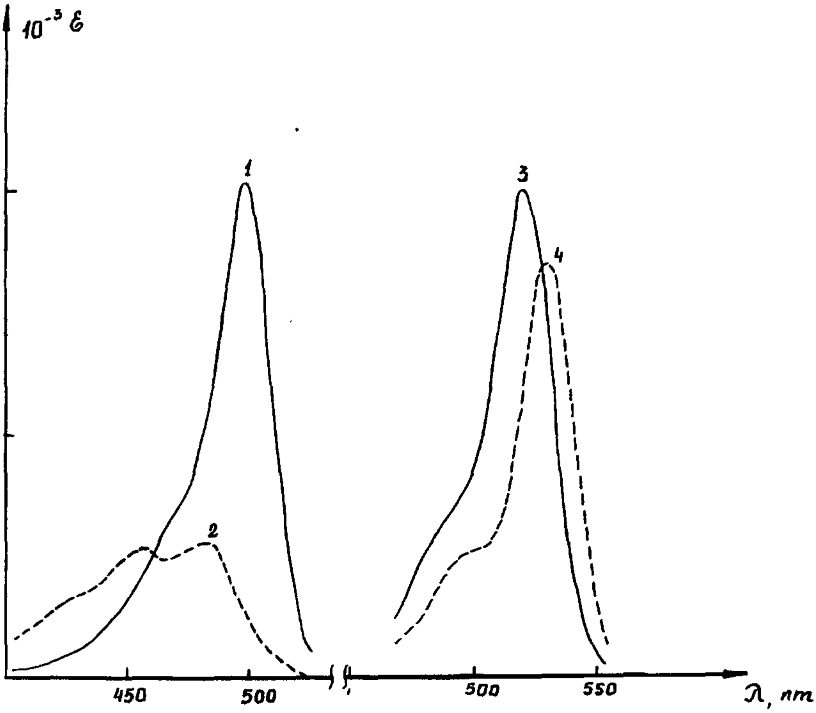
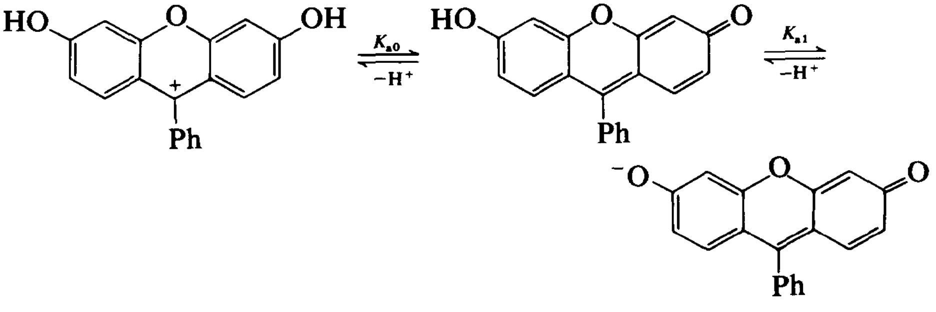
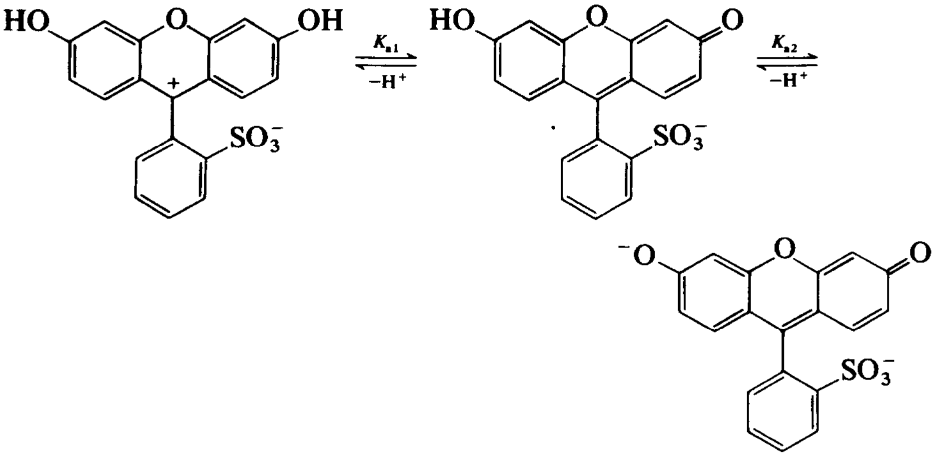


Fig. 5. Absorption spectra of fluorescein (1,2) and eosin (3,4) ions in 52 wt% acetone, $I = 0{\cdot}05$; 1,3—R^{2-}; 2,4—HR^-; spectra taken from previous studies.[12,29]

coincident with the spectra of the corresponding forms of sulphofluorescein (Scheme 3) in water,[34] water–dimethyl sulphoxide mixtures,[31] and other media, demonstrating the insignificant influence of replacement of an SO_3^- group by COOH- or COO^--groups. Ethyleosin in the R^- form, i.e., in which the OH is ionized, has absorption bands (in methanol, water–alcohol, water–acetone and water–dimethyl sulphoxide[22,30,31,33]), analogous to those of the HR^--form of eosin (species VI),



Scheme 2



Scheme 3

and shifted to longer wavelengths as for the band of the R^{2-} form of eosin in corresponding solvents. The data reported for esters of rose bengal B (2,4,5,7-tetraiod-3′,4′,5′,6′-tetrachlorofluorescein),[3,35,36] confirm these relationships.

Investigations on the ionization of the dyes in dimethyl sulphoxide by IR-spectroscopy,[31] confirmed the lactonic structure of the colourless tautomer of the neutral form (stretching vibration of the C=O group of the lactones in the region 1755–1772 cm^{-1}) and also the existence of the VI-type species as intermediates during the gradual transition from H_2R to R^{2-} (eosin, erythrosin, rose bengal A). For DCF and TCF, the IR-spectra of the HR^- form in dimethyl sulphoxide cannot be observed because of the close values of pK_{a1} and pK_{a2}.

It has been shown that protonation of the neutral forms H_2R of 2,4,5,7-tetrahalogen substituted fluoresceins (resulting in H_3R^+ formation) takes place only in very acidic media (in water $pK_{a0} \approx -2$[5,10]). The tautomeric equilibrium II$\rightleftharpoons$III$\rightleftharpoons$IV is mobile and is easily displaceable by variations in the nature of the solvents. In an organic environment, the proportion of IV increases, for fluorescein, more abruptly than for eosin and erythrosin, and zwitter ions II are apparent mostly for fluorescein in water; the initial addition of organic solvents leads to a considerable decrease in II.

3.4 Tautomeric equilibria of DCF and TCF in water

In aqueous solutions of DCF and TCF, one can observe spectra for monoanions that are characteristic for species V (Scheme 1), whereas there are no evident signs of VI-type bands (Figs 1 and 2).

The tautomeric equilibria constants for the neutral forms of chloroderivatives in aqueous solutions were calculated on the basis of the assumptions (Section 3.3) for spectra of species II–IV. In the case of TCF, this calculation was conducted at nine wavelengths over the range 465–505 nm and in the case of DCF at ten wavelengths over the range 400–470 nm. The tautomeric equilibrium constants can be expressed in the following manner:

$$K_T = [\mathrm{IV}]/[\mathrm{III}];\ K'_T = [\mathrm{II}]/[\mathrm{III}];\ K''_T = [\mathrm{IV}]/[\mathrm{II}] = K_T/K'_T$$

Since the lactone is colourless, then the measured molar absorptivity, ε_{H_2R}, is the sum of two quantities:

$$\varepsilon_{H_2R} = \varepsilon_{II}\alpha + \varepsilon_{III}\beta \qquad (2)$$

where α and β are proportions of the corresponding tautomers in the total amount of the H_2R form, and ε_{II} and ε_{III} are the molar absorptivities of II and III, being equal to the molar absorptivities of H_3R^+ and HR^- forms, respectively (according to the assumptions mentioned above). Then, on the basis of eqn (2), the following equation can be derived:

$$\varepsilon_{H_2R}/\varepsilon_{H_3R^+} = \alpha + \beta\,\varepsilon_{HR^-}/\varepsilon_{H_3R^+} \qquad (3)$$

at every wavelength in the chosen interval. This allows a system of linear equations to be obtained, and α and β values may then be calculated by the least-squares method. Values of the tautomeric constants may be expressed with the fractional values as follows:

$$K_T = (1-\alpha-\beta)/\beta;\ K'_T = \alpha/\beta;\ K''_T = (1-\alpha-\beta)/\alpha$$

Calculated values are presented in Table 3, in which previously calculated data for fluorescein, are also shown. The experimentally determined ionization constants (K_a) may be represented as a combination of the tautomerisation constants (K_T, K'_T, and K''_T) and the ionization constants of the tautomers (k, microconstants), which characterize the

TABLE 3
Values of Tautomerization Constants and Microconstants of Fluorescein Dyes

Constant	*DCF*	*TCF*	*Fluorescein*[9]
K_T	2·00 ± 0·05	28 ± 2	6·09
K'_T	0·14 ± 0·02	4 ± 1	2·0
K''_T	15 ± 2	6 ± 1	3·04
$pk_{0.OH}$	0·85 ± 0·06	(1·24 ± 0·07)	3·10
$pk_{\pm.COOH}$	(1·71 ± 0·08)	0·6 ± 0·1	2·80
$pk_{1.COOH}$	3·5 ± 0·1	(2·12 ± 0·06)	3·49
$pk_{1.z}$	(2·6 ± 0·1)	2·76 ± 0·09	3·79
$pk_{2.OH}$	5·19	6·55	6·80

acidity of the individual functional groups (see Scheme 1). These interrelations are as follows:

$$\begin{aligned} \mathrm{p}K_{\mathrm{a}0} &= \mathrm{p}k_{0,\mathrm{OH}} - \log(1 + K_\mathrm{T} + K'_\mathrm{T}) \\ &= \mathrm{p}k_{\pm,\mathrm{COOH}} - \log(1 + K''_\mathrm{T} + K'_\mathrm{T})^{-1}) \end{aligned} \tag{4}$$

$$\begin{aligned} \mathrm{p}K_{\mathrm{a}1} &= \mathrm{p}k_{1,\mathrm{COOH}} + \log(1 + K_\mathrm{T} + K'_\mathrm{T}) - \log(1 + K_{\mathrm{T}_x}) \\ &= \mathrm{p}k_{1,z} + \log\{1 + K''_\mathrm{T} + (K'_\mathrm{T})^{-1}\} - \log(1 + K_{\mathrm{T}_x}) \\ &= \mathrm{p}k_{1,\mathrm{OH}} + \log(1 + K_\mathrm{T} + K'_\mathrm{T}) - \log(1 + K_{\mathrm{T}_x}^{-1}) \end{aligned} \tag{5}$$

$$\mathrm{p}K_{\mathrm{a}2} = \mathrm{p}k_{2,\mathrm{COOH}} + \log(1 + K_{\mathrm{T}_x}^{-1}) = \mathrm{p}k_{2,\mathrm{OH}} + \log(1 + K_{\mathrm{T}_x}) \tag{6}$$

Estimation of the p*k* values of DCF and TCF calculated according to eqns (4)–(6) ($K_{\mathrm{T}_x} \ll 1$), are presented in Table 3, which also contains the data for fluorescein.

For DCF, the K_T value can be determined reliably; therefore the $k_{0,\mathrm{OH}}$ and $k_{1,\mathrm{COOH}}$ values can be regarded as the most accurate compared with the other microconstants. In fact, the $\mathrm{p}k_{1,\mathrm{COOH}}$ value is approximately equal to that of fluorescein, but $\mathrm{p}k_{0,\mathrm{OH}}$ is considerably lower because substituent at the 2 and 7 positions influence the ionization of the phenolic group more stronger than that of the carboxylic group, which is more distant from these substituents and which is also removed from the conjugation system (the 9-aryl

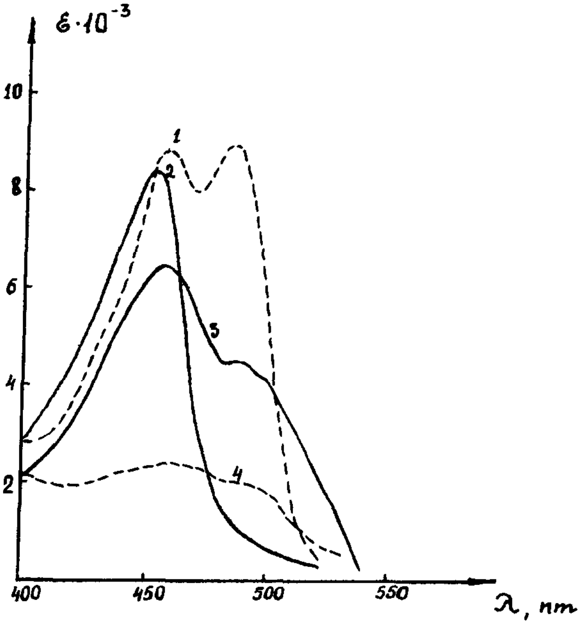


Fig. 6. Absorption spectra of neutral forms of DCF (1,4) and TCF (2,3) in water (1,2) and in 90 wt% acetone (3,4). The intensities of the curves in 90% acetone are multiplied by ten.

substituent is at the angle of ~90° to the plane of the xanthene nucleus[3]). For eosin, the estimation of $pk_{0,OH}$ is about $-1{\cdot}5$.[5]

When one compares the $pk_{2,OH}$ values of fluorescein and its chloro-derivatives, just as for DCF, this value (5·2) is essentially lower than it is for fluorescein (6·8). For TCF, this influence is much less ($pk_{2,OH} = 6{\cdot}6$). The difference between the values of $pk_{1,OH}$ of 6-hydroxy-9-phenylfluorone (see Scheme 2) and those of $pk_{2,OH}$ of fluorescein can be explained in terms of the presence of an additional negative charge[5,22,29,30] and equals 0·5. For eosin, $pk_{1,OH}$ is 2·3, and it is possible to assume that $pk_{2,OH}$ of eosin equals 2·8 (taking into account the previously discussed influence of the charge on the species). Hence we may conclude that the introduction of four bromine atoms into the resorcinol ring lowers $pk_{2,OH}$ by four units, and that the introduction of two chlorine atoms lowers it by 1·6 units. For sulphonphthaleins, a similar influence was observed.[37] The $pk_{2,OH}$ value is less influenced by substituents in the phthalic acid ring, but the four chlorine atoms do exert some influence: $pk_{1,z}$ is 2·76 for TCF, i.e. it is one unit lower than for fluorescein; however, $pk_{\pm,COOH}$ is significantly lower (0·6) than for florescein and DCF (see Table 3).

3.5 Ionization and tautomeric constants in H_2O–acetone mixtures

On passing from water to the water–acetone mixtures, the absorption intensity of the neutral forms (H_2R) is lowered (Fig. 6). This indicates displacement of the tautomeric equilibria towards the formation of the colourless lactone species. For DCF, indications of the tautomer II are absent from the spectra. To evaluate K_T, the ε_{max} (III) value was assumed equal to ε_{max} of the neutral form of ethyleosin, (24×10^3; see Section 3.3). In various water–acetone mixtures (wt%) the following K_T values were calculated: 3·5 (8%), 8·8 (16%), 24·2 (33%), 36·6 (43%), 66·6 (62%), 97·8 (90%), 114 (98%).

At the same time, decrease in the K'_T constants occurs for TCF, which is understandable in view of the highly polar character of the zwitter-ion. The tautomer II is stable only in water-rich media (high D values, as well as H-bond formation with the COO^- group).

For the monoanion HR^- of TCF, the spectrum configuration changes only slightly in comparison with that in aqueous solution. As a rule, its intensity changes insignificantly, and in some cases the amount of the HR^- form is so small that fluctuations in the absorbance intensity of this form may be attributed to errors within the calculation of the pK_{a1}, pK_{a2} and ε_{HR^-} values.

Quite different results are apparent in the case of the HR^- forms of the spectrum of DCF (see Fig. 7). Even at a relatively low acetone content, for

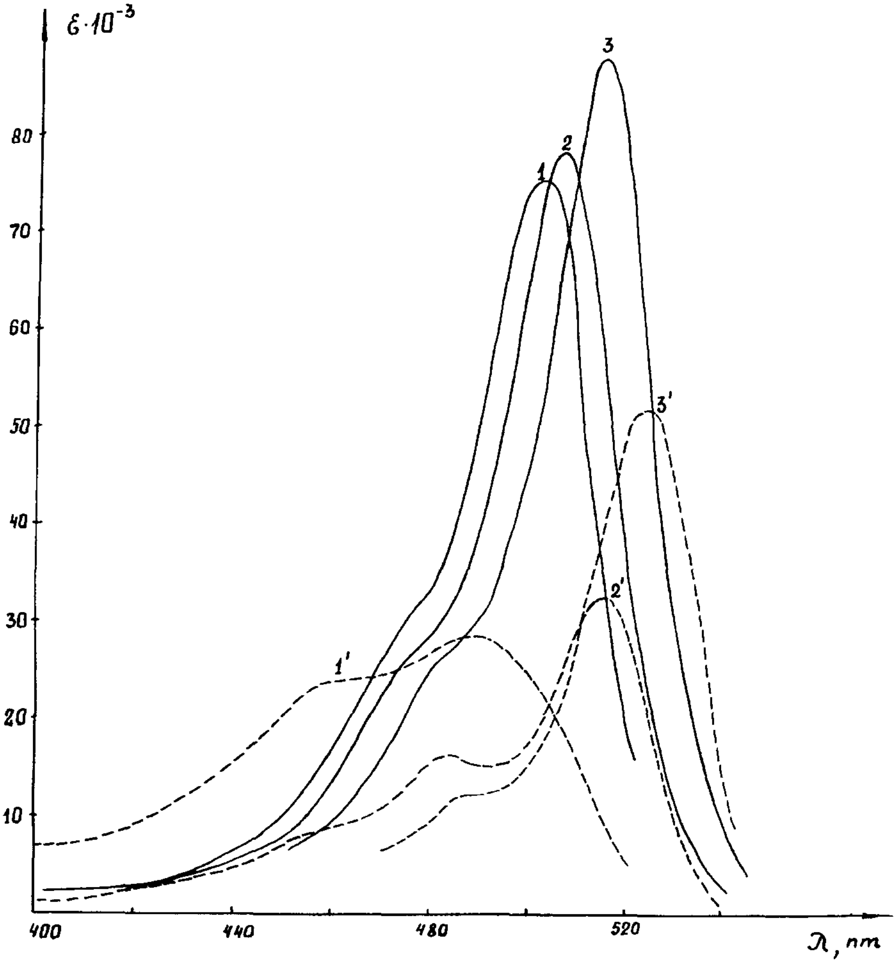


Fig. 7. Absorption spectra of the DCF anions: 1–3—R^{2-}, 1′–3′—HR^-; 1,1′—in water; 2,2′—in 33 wt% acetone; 3,3′—in 90 wt% acetone.

instance, 33 wt% (molar fraction 0·13), one can observe the band with $\lambda_{max} > \lambda_{max}(R^{2-})$. On the basis of previous investigations, this band can be assigned to the species type VI. When the acetone content in the mixed solvent is increased, the intensity of the HR^- band, which is shifted bathochromically relative to the R^{2-} band, is increased. In 90 wt% acetone, the ε_{max} of the HR^- form is approximately 60% that of the R^{2-} band. This may indicate the presence of some monoanions of species V type, but it may also be caused by incomplete resolution of the VI and VII spectra, and also by errors in the estimation of the HR^- spectrum because of the small amount of the corresponding species in the solutions. Assuming the V absorptivity at λ_{max}(VI) to be negligible, and also that $\varepsilon_{max}(VI) = \varepsilon_{max}(VII)$, it can be estimated that K_{T_x} is 0·7 in 33% acetone and 1·5 in 90% acetone.

With respect to the spectrum of DCF of the R^{2-} form, on transition from water to 90 wt% acetone there is a marked bathochromic shift, as in the case of TCF, and for fluorescein[12,29,33] and eosin.[12,33] Apparently, the nature of this effect is purely solvatochromic. The character of the spectral changes is analogous to that described[38] for the R^{2-} form of fluorescein in solvents of differing H-bonding capacities.

From Scheme 1, eqn (7) can be derived:

$$\log K_{T_x} = pk_{1,COOH} - pk_{1,OH} = pk_{2,COOH} - pk_{2,OH} \qquad (7)$$

The increase in K_{T_x} value when passing from water to water–acetone solution is clarified if one takes into account that the pK_a values of the carboxylic acids, when transferred from water to organic solvents and, especially to the so-called differentiating solvents (of which acetone is a typical representative) increase more than the pK_a values of phenols under the same conditions, i.e. $\Delta pk_{COOH} > \Delta pk_{OH}$.[39,40] Such a displacement of the tautomeric equilibrium ($V \rightleftharpoons VI$) was only observed for DCF among all the dyes of the fluorescein series investigated. This fact is of significance. To date, it was possible to observe only two extreme cases:

(a) HR^- form as V type (fluorescein, TCF), and
(b) HR^- form as VI type (eosin, erythrosin, rose bengal).

It is evident that in the case of fluorescein and TCF, on passing to organic solvents, even the increased proportion of the VI-type species is too small to be shown by means of spectrophotometry, whereas the monoanion of VI type for the 2,4,5,7-tetrahalogen derivatives is intrinsically predominant in water and increases even further in organic media.

3.6 Medium effects (ΔpK_a) and the equilibria scheme

To verify the above conclusions, the changes in the pK_a values (ΔpK_a, or medium effects) during the change from H_2O to 90 wt% $(CH_3)_2CO$ needs to be examined. For TCF, ΔpK_{a1} is 7·2 and ΔpK_{a2} is 5·0; for DCF, these values are 6·3 and 5·2, respectively. The larger medium effects for pK_{a1} compared with the corresponding effects for the second ionization step are to a certain extent due to the increase in K_T values (see eqn (5)). To interpret the ΔpK_a values, various combinations based on eqns (5) and (6) can be used, for instance:

$$\begin{aligned}\Delta pK_{a2} - \Delta pK_{a1} &= \Delta(pK_{a2} - pK_{a1})\\ &= \Delta pk_{2,OH} - \Delta pk_{1,COOH} - \Delta \log(1 + K_T + K'_T) + 2\Delta \log(1 + K_{T_x})\end{aligned}$$

For TCF the difference $pK_{a2} - pK_{a1}$ in water is 2·9 and in 90 wt% acetone it is 0·7; thus, $\Delta(pK_{a2} - pK_{a1})$ is $-2·2$. For DCF, this value is $-1·1$. For TCF, K_{T_x} is $\ll 1$, and such a ΔpK_a relationship is caused by the Δpk_{COOH} with respect to Δpk_{OH}, as well as to K_T increase. For DCF, the K_T increase (from $\ll 1$ in water to $\geqslant 1·5$ in 90% acetone) contributes to the change in the opposite direction, and this is the reason why the $(pK_{a2} - pK_{a1})$ value decreases less significantly. Data for media with other acetone contents confirm this explanation.

It was shown earlier that for fluorescein in 90 wt% acetone, ΔpK_{a1} is 8·0, and ΔpK_{a2} is 4·4; this leads to inversion of the pK_{a1} and pK_{a2} values (12·5 and 11·2, respectively).[33] For eosin under the same conditions, ΔpK_{a1} is 3·6 and ΔpK_{a2} is 6·2.[33] This results in the acidity differentiating of H_2R and HR^-, instead of inversion of K_{a1} and K_{a2}. It was found that for fluorescein $\Delta pk_{1,COOH}$ is 5·7, and for eosin $\Delta pk_{1,OH}$ is 2·9, which confirms the above conclusions ($\Delta pk_{COOH} > \Delta pk_{OH}$).

On the whole, it can be noted that the medium effects are caused both by the nature of the ionizing groups and by the shift of tautomeric equilibria. Hence the pK_{a0} decrease of all the dyes discussed is undoubtedly conditioned by the rise in K_T and K''_T (eqn (4)).

The relationships examined justify the validity of the proposed equilibrium scheme.

4 CONCLUSIONS

The neutral molecules H_2R of chloro-substituted fluoresceins exist in water as a mixture of three different tautomers: the coloured quinone and zwitterion (the first more characteristic for 2,7-dichlorofluorescein, and the second for 3′,4′,5′,6′-tetrachlorofluorescein), as well as the colourless lactone. The ratio between the equilibria concentrations of tautomers is strongly dependent upon the composition of solvents, in particular, in acetone-rich media, the lactonic particles predominate for both the dyes.

Whereas the monoanions HR^- of 3′,4′,5′,6′-tetrachlorofluorescein at any composition of the mixed solvent (H_2O—$(CH_3)_2CO$) are ionized not through the hydroxy, but through the carboxylic group (as well as the monoanions of the unsubstituted fluorescein), in the case of 2,7-dichlorofluorescein, HR^- ion possesses such a structure only in water. In contrast, with mixtures with a high acetone content, the hydroxy-group is ionized, whereas the carboxylic group is not. Under these conditions, 2,7-dichlorofluorescein becomes similar to the 2,4,5,7-tetrahalogen derivatives of fluorescein in which the monoanions have a similar structure in all solvents.

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