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Ionization and Tautomerism of Fluorescein, Rhodamine B, N,N-Diethylrhodol and Related Dyes in Mixed and Nonaqueous Solvents

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ABSTRACT

The protolytic equilibria of Fluorescein, Rhodamine B and of asymmetrical amino-oxyanthene dye, N,N-diethylrhodol (a 'hybrid' of Rhodamine B and Fluorescein) were studied in aqueous DMSO and EtOH (91 wt% of organic cosolvent). The pK_a *values of these dyes, as well as of related substances were determined. On the basis of the visible absorption spectra in various solvents conclusions are made about the tautomerism in the dye molecule. The values of the tautomeric equilibrium constants and of the microscopic ionization constants were obtained. Some new data on the tautomerism of oxyxanthene monoanions in methanol are presented.*

1 INTRODUCTION

Aminoxanthenes (rhodamines, pyronines) as well as oxyxanthenes (Fluorescein and its derivatives) are widely used as luminophores, laser dyes and analytical reagents.[1,2] Their protolytic equilibria in water and in non-aqueous solvents has been studied.[3–18] The asymmetrical amino-

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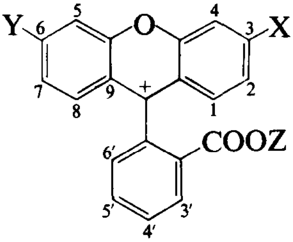
oxyxanthene dyes (rhodols), also possess valuable optical properties,[19–22] but only a few studies have been reported on their protolytic behaviour in solutions.[23,24] In the present paper we report the results of an investigation of the ionization and tautomerism of N,N-diethylrhodol [6-diethylamino-3-oxo-9(2-carboxyphenyl)xanthene] in water–dimethylsulphoxide and water–ethanol mixtures. The results are compared with the data for Fluorescein and Rhodamine B. Some new data on the tautomerism of oxyanthene anions in methanol are also reported. The different types of xanthene dyes in their cationic forms are given below as derivatives of the 9-arylxanthene carbocation:



Fluorescein: X = Y = OH, Z = H; Rhodamine B: X = Y = NEt_2, Z = H; N,N-diethylrhodol: X = OH, Y = NEt_2, Z = H; ethyl ester of N,N-diethylrhodol: X = OH, Y = NEt_2, Z = Et. We regard the positions 3- and 6- as equivalent, because the 9-aryl substituent is at an angle of ~90° to the plane of the xanthene nucleus; the charge in the xanthene chromophore is always strongly delocalized.

In a previous study[24] on the equilibria of rhodol and N,N-diethylrhodol in methanol and aqueous acetone, an attempt was made to ascertain if the relationships reported for Fluoresceins and Rhodamines are valid for the asymmetrical amino-oxyxanthenes. We used 91·3 wt% DMSO and 91·4 wt% EtOH as solvents, because some xanthene dyes have already been studied in these media.[18,25] We also attempted to evaluate the relationships of the nature of the solvents on the protolytic equilibria of xanthene dyes.

2 EXPERIMENTAL

2.1 Materials

N,N-diethylrhodol was prepared as described in the literature.[22,24] The ethyl ester of N,N-diethylrhodol was obtained by esterification of rhodol with ethanol in the presence of H_2SO_4, using the conditions described for the preparation of the ethyl ester of Fluorescein.[26] The dye thus obtained was identified as 2'-carbethoxy-N,N-diethylrhodol using IR and ^{1}NMR and elemental analysis. No decolorization (lactone formation occurred in any solvent, which is evidence for the esterification of the carboxylic group. The purity was also checked chromatographically (Silufol plates). A chromatographically pure sample of Rhodamine B chloride[8,11] was used. Rhodamine 3B was obtained from Lambda Physics. The samples of oxyxanthenes were purified as described previously.[9,10,12,17,18] Buffer acids, used to give the required pa_H^* values (salicylic, benzoic, diethylbarbituric), were purified by recrystallization. Glacial acetic acid, as well as perchloric and hydrochloric acids (all of analytical grade) were used as such. Standard aqueous solutions of sodium hydroxide were prepared using CO_2-free water, DMSO was purified using alkali and then zeolites (NaA; 4 Å). The water content was estimated by titration according to Fischer's method. Ethanol was purified by a standard procedure; the azeotropic mixture was used to prepare the working solutions. Methanol, acetone and 1,4-dioxane were purified by standard methods, the latter solvent being freed of peroxides with KOH and Na.

2.2 Measurements

The absorption spectra in the visible region were measured by using an SP-46 spectrophotometer (of USSR origin). A series of solutions were prepared with constant dye concentrations (as a rule, $\sim 1 \times 10^{-5}$ M). The buffers were made directly by mixing various amounts of buffer acids (HA) and a constant volume of NaOH stock solutions; thus the ionic strength was supposed to be constant and equal to $[Na^+]$: 0·01 M in 91·3% DMSO and 0·0025 M or 0·002 M in 91·4% EtOH. The exact contents of the organic cosolvents were determined by weighting, and are always expressed in wt%. While measuring the spectra of the neutral form of N,N-diethylrhodol (HR) in aqueous DMSO and dioxane, and of Fluorescein (H_2R) in 91·4% EtOH, the dye concentration was being increased. The pa_H^* values of buffer mixtures were calculated using the pK_a values of buffer acids (pK_{HA}):[17,18]

$$pa_H^* = pK_{HA} + \log\{[A^-]/[HA]\} + \log f_1 \qquad (1)$$

where A^- is the buffer acid anion, f_1 is the activity coefficient of the monocharged ion and a_H^* denotes the hydrogen ion activity standardized to infinite dilution in the mixed solvent.[27] The equilibrium concentration

$[A^-]$ is equated with the NaOH analytical concentration; these interrelations are valid only if $[Na^+] \gg [H^+]$. The pK_{HA} values in 91·3% DMSO had been determined previously using the indicator method (sulphonephthalein series) through the procedure of overlapping (5·22, 8·05, 8·54 and 10·77 for salicylic, benzoic, acetic and diethylbarbituric acids respectively);[18] in 91·4% EtOH the corresponding values, obtained in a similar manner, are: 5·58, 7·50, 7·61 and 10·79.[25] In more acid pa_H^* areas (<3·5), dilute mineral acids (HCl and $HClO_4$) were used instead of buffers:

$$pa_H^* = -\log [H^+] - \log f_1 \qquad (2)$$

The details can be found in a previous paper.[24] The reduction of all pK_A values to zero ionic strength was performed by means of the Debye–Hückel equation for ionic activity coefficients (the ionic parameter was taken to be equal to 5 Å). All the pa_H^* and pK_a values are given on a molar scale.

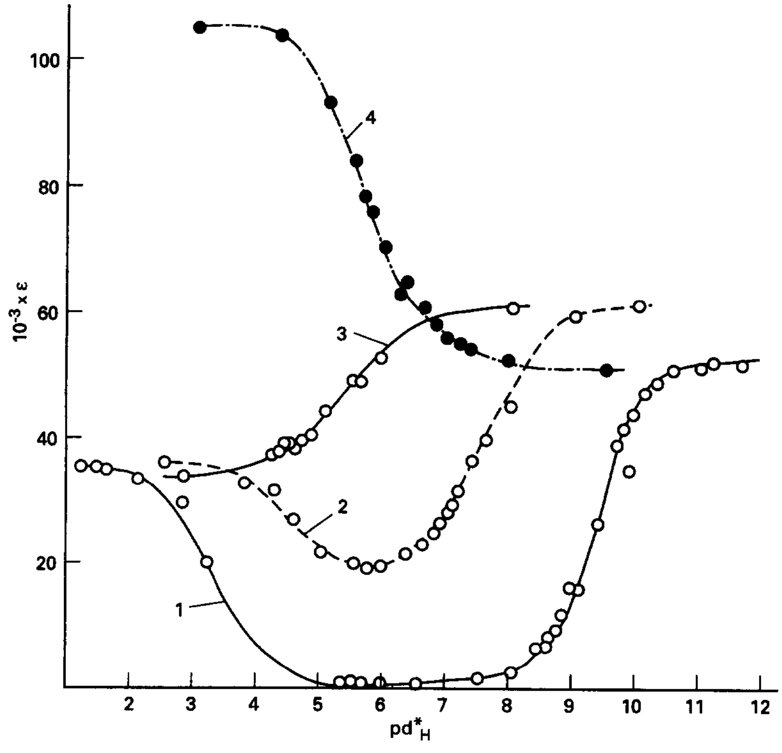


Fig. 1. Typical relationships of ε with pa_H^*; N,N-diethylrhodol, 520 nm: 1, in 91·3 wt% DMSO; 2, in 91·4 wt% EtOH; 3, 2'-carbethoxy-N,N-diethylrhodol in 91·4 wt% EtOH, 520 nm; 4, Rhodamine B in 91·4 wt% EtOH, 560 nm.

3 RESULTS AND DISCUSSION

3.1 Determination of ionization constants

The pK_{a0} and pK_{a1} values of N,N-diethylrhodol

$$H_2R^+ \rightleftharpoons HR + H^+, \qquad K_{a0} \qquad (3)$$

$$HR^+ \rightleftharpoons R^- + H^+, \qquad K_{a1} \qquad (4)$$

as well as the pK_{a0} value of its ethyl ester and of Rhodamine B

$$HR^+ \rightleftharpoons R + H^+, \qquad K_{a0} \qquad (5)$$

were estimated from the ε − pa_H^* curves (Fig. 1); 32 and 18 working solutions with various pa_H^* in aqueous DMSO and EtOH respectively

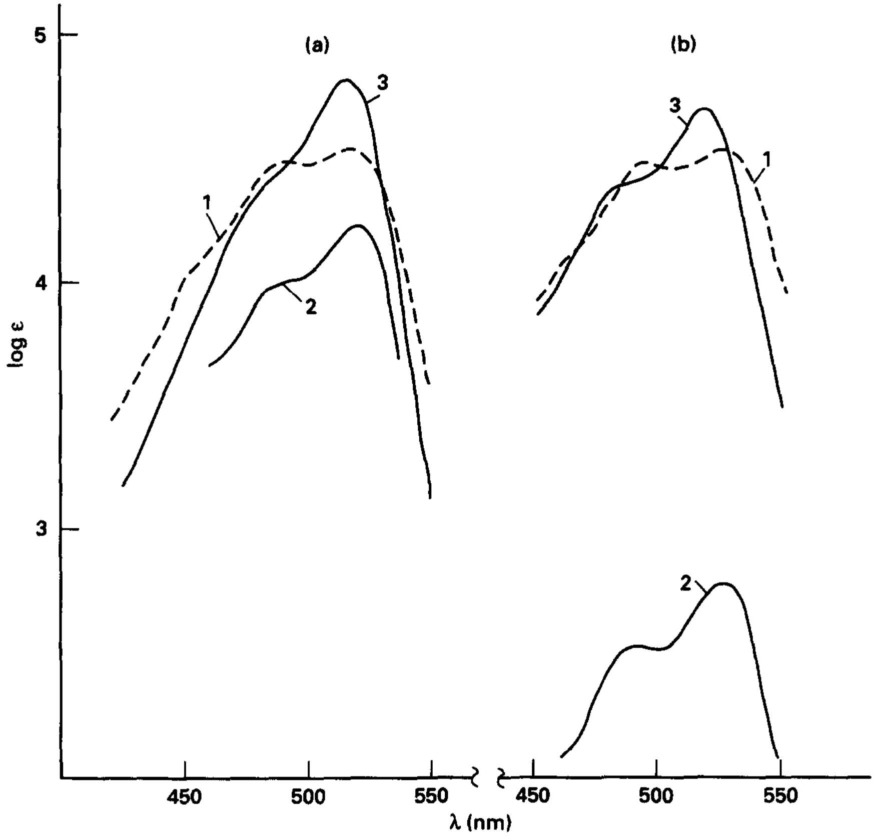


Fig. 2. Absorption spectra of various forms of N,N-diethylrhodol in: (a) 91·4 wt% EtOH; (b) in 91·3 wt% DMSO; 1, H_2R^+ (XIV); 2, HR (XV $\rightleftharpoons$ XVI $\rightleftharpoons$ XVII); 3, R (XVIII).

were used in the two-step ionization studies (eqns (3), (4)) and 7–13 various pa_H^* values in simpler cases (eqn (5)). While in the case of aqueous DMSO the pK_{a0} and pK_{a1} values can be estimated, regarding the ionization steps as practically isolated, the iterative procedure for the joint calculation of K_{a0}, K_{a1} and ε_{HR}[28] is used in the case of aqueous EtOH starting with the lowest ε value as a first ε_{HR} approximation. The ε_{HR} values at the wavelength used in the pK_a calculations (9λ values from 495 nm to 540 nm in aqueous DMSO and 6λ values from 505 nm to 520 nm in aqueous EtOH), as well as in the whole visible region, were estimated using the following equation:

$$\varepsilon_{HR} = \varepsilon + a_H^*(K_{a0})^{-1}(\varepsilon - \varepsilon_{H_2R^+}) + (a_H^*)^{-1}K_{a1}(\varepsilon - \varepsilon_{R^-}) \tag{6}$$

The spectra of the neutral forms thus obtained are given in Fig. 2, together with the ionic species spectra; the last-named are easily measurable directly in appropriate pa_H^* regions. The data obtained with the help of different buffer systems agree satisfactorily; for example, the values $pK_{a1} = 9{\cdot}49 \pm 0{\cdot}07$ and $9{\cdot}43 \pm 0{\cdot}19$ are found for N,N-diethylrhodol in 91·3% DMSO by using benzoate and diethylbarbiturate buffers respectively. The pK_{a0} value in aqueous DMSO was determined in HCl solutions on the ε_{HR} in salicylate buffers. In 91·4% EtOH, the pK_{a1} and pK_{a0} values were obtained principally in acetate and salicylate buffers respectively; the last-named buffer system was used in both mixed solvents for the determination of pK_{a0} values, describing the equilibria (5). Some spectra were measured in dilute NaOH, but in the case of

TABLE 1

Thermodynamic Ionization Constants and Molar Absorptivities of Xanthene Dyes in 91·4 wt% EtOH, 20°C

	Fluorescein	*Rhodamine B*	*N,N-Diethylrhodol*	*2'-Carbethoxydiethylrhodol*
pK_{a0}	1·21 ± 0·09[a]	5·79 ± 0·05	4·45 ± 0·04	5·15 ± 0·07
pK_{a1}	8·42 ± 0·05	—	7·61 ± 0·02	—
pK_{a2}	9·41 ± 0·17	—	—	—
λ_{max} (nm) ($\varepsilon_{max} \times 10^{-3}$)				
Cation	445 (61·85)[a]	555 (114·5)	518 (36·7)	520 (33·6)
Neutral	455 (0·541)	545 (101·1)	520 (17·4)	525 (64·0)
Mono-anion	455 (30·0)	—	515 (66·5)	—
Dianion	498 (89·8)	—	—	—

[a] 90·7 wt% EtOH.

TABLE 2
Thermodynamic Ionization Constants and Molar Absorptivities of Xanthene Dyes in 91·3 wt% DMSO, 25°C

	Fluorescein[15,18]	*Rhodamine B*[15,16]	*N,N-Diethylrhodol*	*2′-Carbethoxydiethylrhodol*
pK_{a0}	−1·51 ± 0·03	5·60 ± 0·02	3·23 ± 0·02	5·65 ± 0·15
pK_{a1}	10·33 ± 0·02	—	9·46 ± 0·13	—
pK_{a2}	8·98 ± 0·17	—	—	—
λ_{max} (nm) ($\varepsilon_{max} \times 10^{-3}$)				
Cation	450 (58·2)	565 (108)	525 (35·9)	525 (37·0)
Neutral	460 (0·051)	505 (1·80)	527 (0·603)	530 (53·0)
Mono-anion	460 (30)	—	515 (53·1)	—
Dianion	513 (98·3)	—	—	—

the ethyl ester of N,N-diethylrhodol, the appropriate buffers with high pa_H^* were used instead of alkali to avoid hydrolysis of the COOEt group.

The results are given in Tables 1 and 2. The pK_a (eqn (7)) and values for Fluorescein in 91% DMSO have been previously determined;[18] the data in 91% EtOH[25] were obtained in a similar manner (Tables 1 and 2, Fig. 3).

$$H_3R^+ \overset{K_{a0}}{\rightleftharpoons} H_2R \overset{K_{a1}}{\rightleftharpoons} HR^- \overset{K_{a2}}{\rightleftharpoons} R^{2-} \tag{7}$$

The data for the aqueous solutions are given in Table 3. The pK_a of N,N-diethylrhodol in water was obtained according to the procedure described previously;[17] in this case the pH values were measured with the help of a glass electrode in a cell with a liquid junction.

Attribution of the pK_a values cannot be made without a knowledge of the tautomeric interrelations.[9–12,17,18,24]

3.2 Tautomeric equilibria of Rhodamine B

For Rhodamine B and other rhodamines, Scheme 1 is valid.[4,6,11,13,14] Here the cations H_2R^{2+} and H_3R^{3+} (with protonated nitrogen atoms) are not shown, because they exist only in strongly acidic media.[3,8] The dimers $(HR^+)_2$, R_2 and HR_2^+ occur principally in water at high concentrations of the dye,[3,8,25] and are not typical for organic solvents.

The similarity of the spectra for HR^+ and R in water (Table 3) is usually explained in terms of the similarity of the bands of the cation **I** and the zwitter-ion **II**. The lowering of $\varepsilon_{max}(R)$ in non-aqueous solvents is

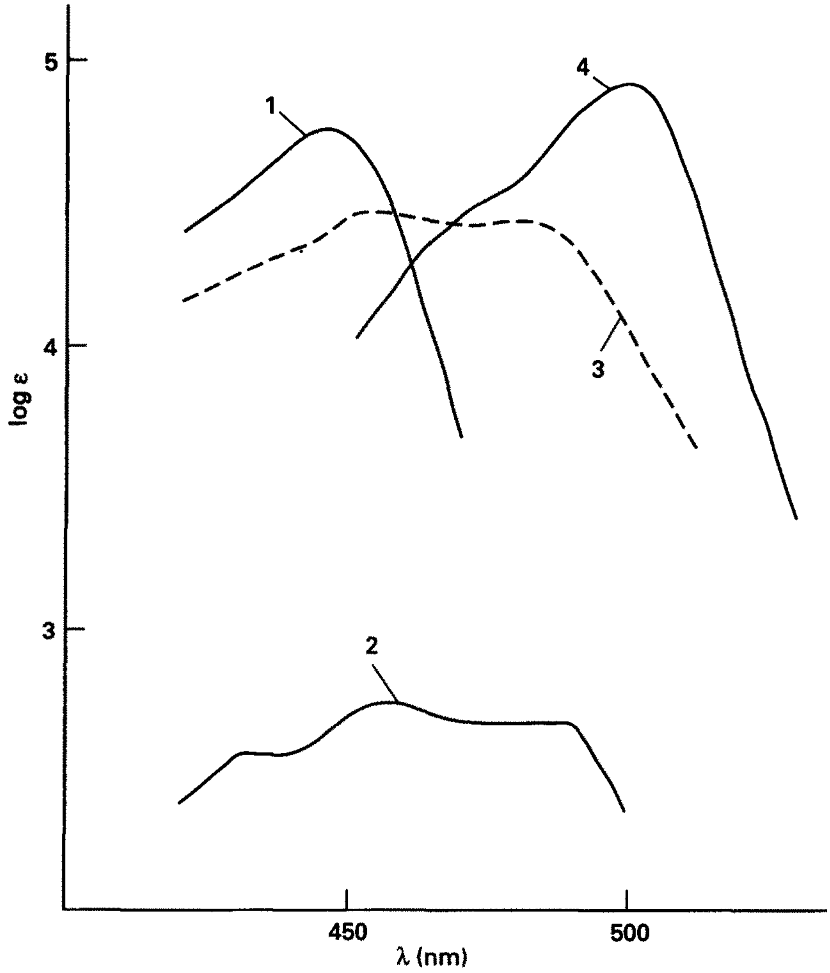
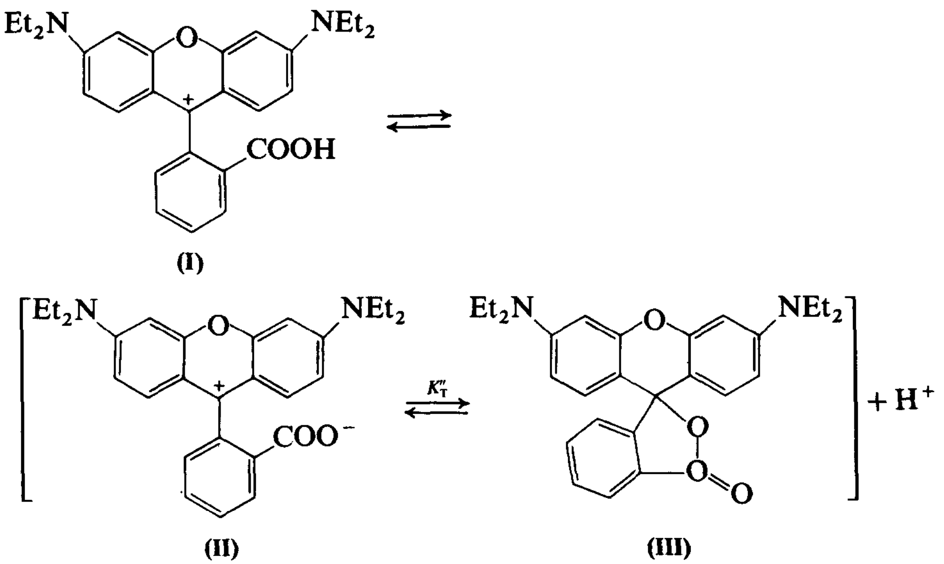


Fig. 3. Absorption spectra of various forms of Fluorescein in 91 wt% EtOH: 1, H_3R^+ (IV); 2, H_2R (VI $\rightleftharpoons$ VIII); 3, HR^- (VII); 4, R^{2-} (X).

the result of the transformation of **II** into the colourless lactone **III**. The tautomeric equilibrium (**II** $\rightleftharpoons$ **III**) has been discussed in several previous publications.[4,6,11,13,14] It was concluded that H-bonds are of great significance in the stabilization of the zwitter-ion **II**. The spectra of the neutral form R, measured in various solvents, after 'removing' traces of cationic absorption (HR^+, **I**) may be used for the calculation of K''_T:

$$\varepsilon_R = \varepsilon_{II}\alpha_{II}; \qquad K''_T = [III]/[II] = \alpha_{II}^{-1} - 1 \tag{8}$$

The values of ε_{max}(R) in solvents where the amount of lactone, α_{III}, is negligible, can be used as ε_{max}(**II**) in other solvents to calculate α_{II},[4,6] but there are reasons to equate the ε_{max}(**II**) in each solvent to the ε_{max}(**I**) in



Scheme 1. Protolytic equilibria of Rhodamine B.

the same solvent, assuming that the ionization of the carboxylic group (COOH → COO⁻) is observable only from the λ_{max} values (see Tables 1–3, Fig. 4). In mixtures of water and aprotic solvents containing very small amounts of water, the configuration and λ_{max} values of R differ from those of HR^+ to such an extent that only rough estimations of α_{II} are possible.[13–16]

TABLE 3

Thermodynamic Ionization Constants and Molar Absorptivities of Xanthene Dyes in Water, 20–25°C

	Fluorescein[9]	*Rhodamine B*[8,13,25]	*N,N-Diethylrhodol*
pK_{a0}	2·14 ± 0·01	3·22 ± 0·02	3·08 ± 0·12
pK_{a1}	4·45 ± 0·02	—	5·83 ± 0·01
pK_{a2}	6·80 ± 0·01	—	—
λ_{max} (nm) ($\varepsilon_{max} \times 10^{-3}$)			
Cation	437 (54·3)	557 (108)	520 (22·6)
Neutral	437 (13·9)[b]	554 (108)	515–520 (23·5)
Mono-anion	454–474 (32·7–33·8)	—	520 (51·5)
Dianion	491 (88)	—	—

[a] Shoulder: 490 (21·25).

[b] Shoulder: 470–485 (4–3·1).

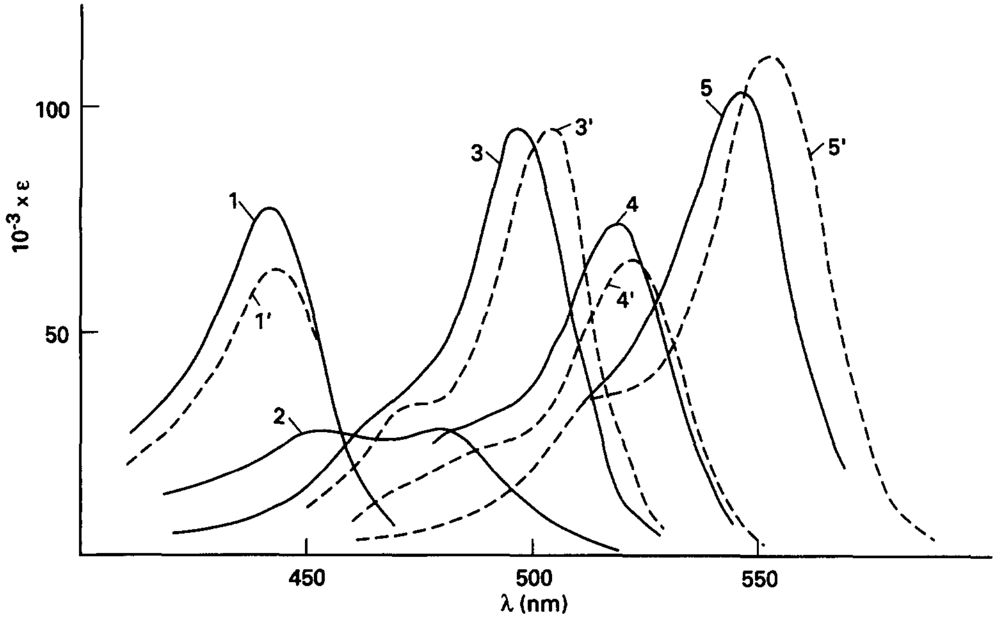


Fig. 4. Absorption spectra in methanol: 1, H_3R^+ of Fluorescein (IV); 1', H_2R^+ of ethyl fluorescein (XII); 2, HR^- of Fluorescein (VIII), 3, R^{2-} of Fluorescein (X); 3', R^- of ethyl fluorescein (XIII); 4, R^- of N,N-diethylrhodol (XVIII); 4', R of 2'-carbethoxy-N,N-diethylrhodol (XX); 5, R of Rhodamine B (II; ≈10% as III); 5', HR^+ of Rhodamine B (I).

The pK_{a0} values may be presented as a sum of two quantities:

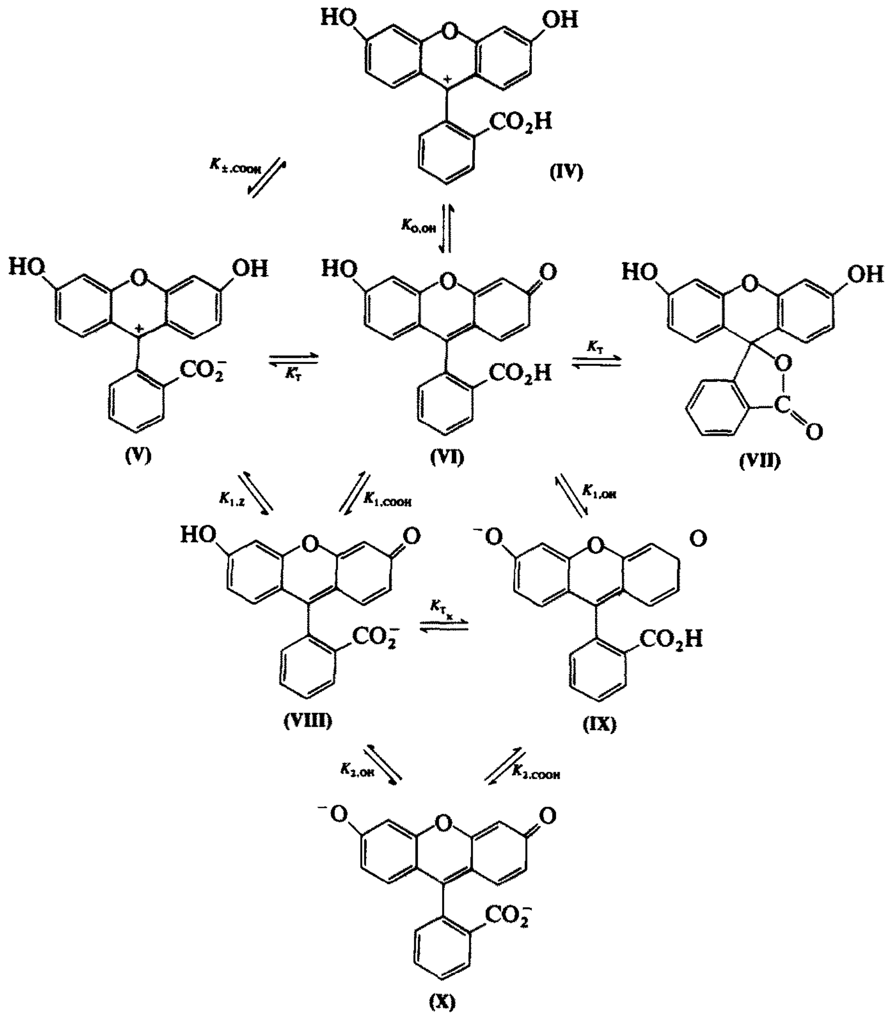
$$pK_{a0} = pK_{\pm,\mathrm{COOH}} - \log(1 + K''_{\mathrm{T}}) \qquad (9)$$

$k_{\pm,\mathrm{COOH}} = \alpha_{\mathrm{H}}^{*}(\mathrm{II})/(\mathrm{I})$. In 91% DMSO, $\alpha_{\mathrm{II}} = 0{\cdot}017$, $K''_{\mathrm{T}} = 59$,[15,16,25] $pk_{\pm,\mathrm{COOH}} = 7{\cdot}38$ and in 91% EtOH $\alpha_{\mathrm{II}} = 0{\cdot}88$, $K''_{\mathrm{T}} = 0{\cdot}1$, $pK_{\pm,\mathrm{COOH}} = 5{\cdot}84$.

3.3 Protolytic equilibria of Fluorescein and its derivatives

In the case of Fluorescein, the protolytic equilibria are more complicated (Scheme 2).[5,7,9,10,12,15,17,18] The cation H_3R^+ exists as **IV**, the monoanion HR^- in the case of the unsubstituted Fluorescein in all solvents yet studied exists as **VIII**, and for the dianion R^{2-} structure **X** is typical. The zwitter-ionic (**V**), quinoide (**VI**) and colourless lactonic (**VII**) tautomers are possible for the neutral form H_2R. The main assumption used for the calculation of the fractions of **V**, **VI** and, finally, of **VII**

$$\alpha_{\mathrm{V}} + \alpha_{\mathrm{VI}} + \alpha_{\mathrm{VII}} = 1 \qquad (10)$$



Scheme 2. Protolytic equilibria of oxyxanthene dyes.

involved the relatively small influence of the ionization of the carboxylic group on the absorption band in the visible region. So, the spectra of the **V** and **VI** tautomers may be modelled with the spectra of the ions **IV** and **VIII** respectively,[9,10,12,17,18] see Fig. 3.

The data in Figs 4 and 5 help to form an understanding of the influence of the 2'-substituent on the absorption of xanthene dyes. On replacing COOH by COOEt, the λ_{max} of the cationic chromophore changes slightly (compare the spectra of species **I** and **IV** with the spectra of the cations of Rhodamine 3B (**XI**) and ethyl fluorescein (**XII**)

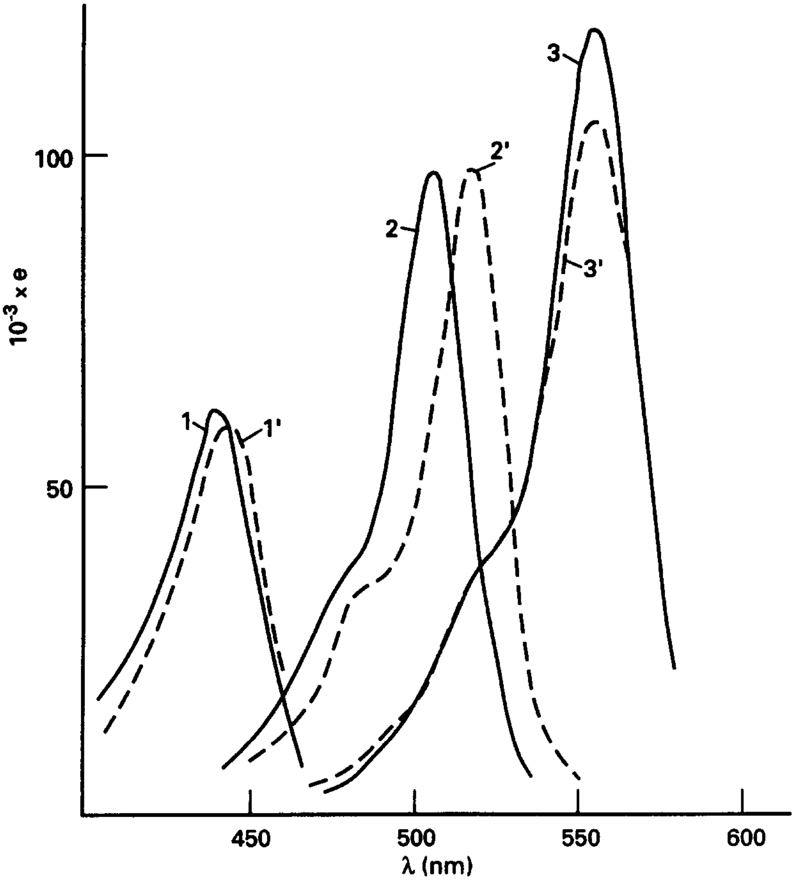
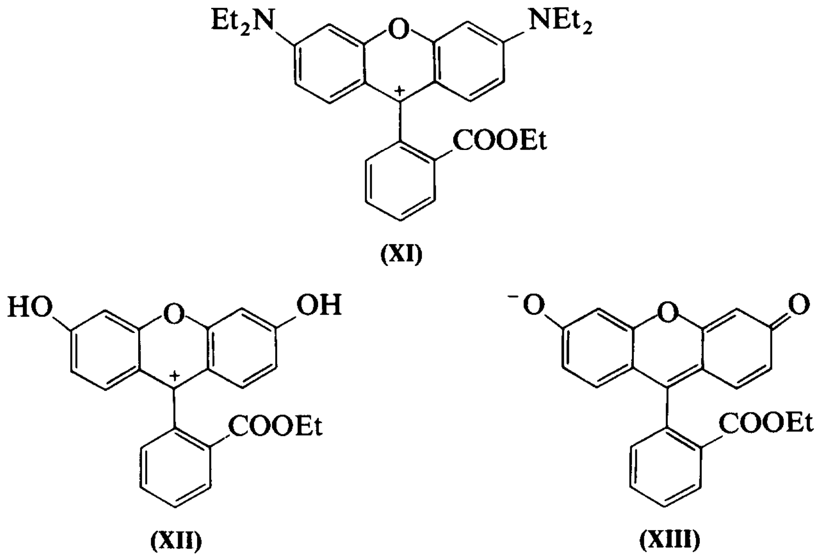


Fig. 5. Absorption spectra of ionic species in 90 wt% acetone: 1, Fluorescein, H_3R^+ (IV); 2, Fluorescein, R^{2-} (X); 3, Rhodamine B, HR^+ (I); 1', ethyl fluorescein, H_2R^+ (XI); 2', ethyl fluorescein, R^- (XIII); 3', Rhodamine 3B, R^+ (XI).

(Scheme 3), respectively). The satisfactory coincidence of the spectra of the fluorescein cation H_3R^+ (**IV**) with those of model compounds, e.g. **XII**, can be observed in various solvents;[12,18,25] an example of the poorest relationship is presented in Fig. 4.

Ionization of the carboxylic group of Rhodamine B (**I** → **II**) leads to a blue shift[3,6,8,11,13,14] (in MeOH: 7 nm, Fig. 4; see also Tables 1–3). Replacing COOH (or COOEt) by COO^- also has some influence on the absorption of anionic oxyxanthene chromogens, resulting in hypsochromic shifts. The absorption band of the anion of ethyl fluorescein (**XIII**) is shifted markedly compared to that of the Fluorescein dianion R^{2-} (**X**), Figs 4 and 5. The same shift (10 nm in MeOH) is registered in the case of the R^- anion of ethyl eosin and the R^{2-} anion of eosin.[12] Thus, the absence of a band width $\lambda_{max} > \lambda_{max}(R^{2-})$ in the



(XI)

(XII)

(XIII)

Scheme 3

spectrum of the HR$^-$ form of Fluorescein (Fig. 3) indicates that the structure **IX** is unlikely.

In water, for Fluorescein K_T = [VII]/[VI] = 6·04, K'_T = [V]/[VI] = 1·96, K''_T = [VII]/[V] = K_T/K'_T = 3·08.[9,17,18] With a small addition of an organic cosolvent, the fraction of the zwitter-ion, α_V, sharply decreases, and the equilibria **VI** $\rightleftharpoons$ **VIII** shifts towards the lactone. The latter tautomer is especially stable in solvents which are unable to be donors of H-bonds.[15,18,24,25] In 91·4% EtOH (Fig. 3), α_{VI} = 0·018, α_{VII} = 0·982 and K_T = 54·4.

For Fluorescein, the following interrelations between the macroscopic (K_a) and microscopic (k) ionization constants are as follows:

$$\begin{aligned} pK_{a0} &= pk_{0,OH} - \log(1 + K_T + K'_T) \\ &= pk_{\pm,COOH} - \log(1 + K''_T + (K'_T)^{-1}) \qquad (11) \end{aligned}$$

$$\begin{aligned} pK_{a1} &= pk_{1,COOH} + \log(1 + K_T + K'_T) \\ &= pk_{1,Z} + \log(1 + K''_T + (K'_T)^{-1}) \qquad (12) \end{aligned}$$

$$pK_{a2} = pk_{2,OH} \text{ (here } \alpha_{VIII} \gg \alpha_{IX}) \qquad (13)$$

$k_{0,OH} = a^*_H$(VI)/(IV), $k_{\pm,COOH} = a^*_H$(V)/(IV), $k_{1,COOH} = a^*_H$(VIII)/(VI), $k_{1,Z} = a^*_H$(VIII)/(V), $k_{2,OH} = a^*_H$(X)/(VIII).

Contrary to this, the **IX** type structure is prevalent for the monoanions of 2,4,5,7-tetrahalogeno-derivatives of Fluorescein (Eosin, Erythrosin, Rose Bengal), which is due to the increase in the acidity of the OH groups:

$$K_{Tx} = [IX]/[VIII] = k_{1,OH}/k_{1,COOH} = k_{2,OH}/k_{2,COOH} \qquad (14)$$

here $k_{1,OH} = a^*_H(IX)/(VI)$, $k_{2,COOH} = a^*_H(X)/(IX)$. It is evident that the increased acidity of the hydroxy-groups, occurring as a result of substitution in the 2-, 4-, 5- and 7-positions, leads to a shift of the tautomeric equilibria ($V \rightleftharpoons VI$) toward the right.

In a previous paper,[17] a marked shift of the tautomeric equilibria (VIII → IX) was described for 2,7-dichlorofluorescein comparing water and aqueous acetone; this is related to the more apparent effects of the solvent for carboxylic acids compared to phenols ($\Delta pk_{COOH} > \Delta pk_{OH}$).[12,17,18,27,29] The bands of the IX species are red shifted compared to dianionic spectra of the corresponding dye,[5,7,10,12,15,17,18] just as the anionic spectra of ethyl fluorescein (XIII) are shifted against the R^{2-} spectra of Fluorescein. These shifts are in agreement with quantum-chemical calculations.[30] In Fig. 6 the spectra of the HR^- ions of 3',4',5',6'-tetrachlorofluorescein, 2,7-dichlorofluorescein and Rose Bengal A in methanol are presented; the ε_{HR} values, as a rule unavailable from direct measurements, are calculated by eqn (15) from equilibrium data at $pK_{a1} \leq pa^*_H \leq pK_{a2}$:[12,25]

$$\varepsilon_{HR^-} = \varepsilon + a^*_H(K_{a1})^{-1}(\varepsilon - \varepsilon_{H_2R}) + (a^*_H)^{-1}K_{a2}(\varepsilon - \varepsilon_{R^{2-}}) \qquad (15)$$

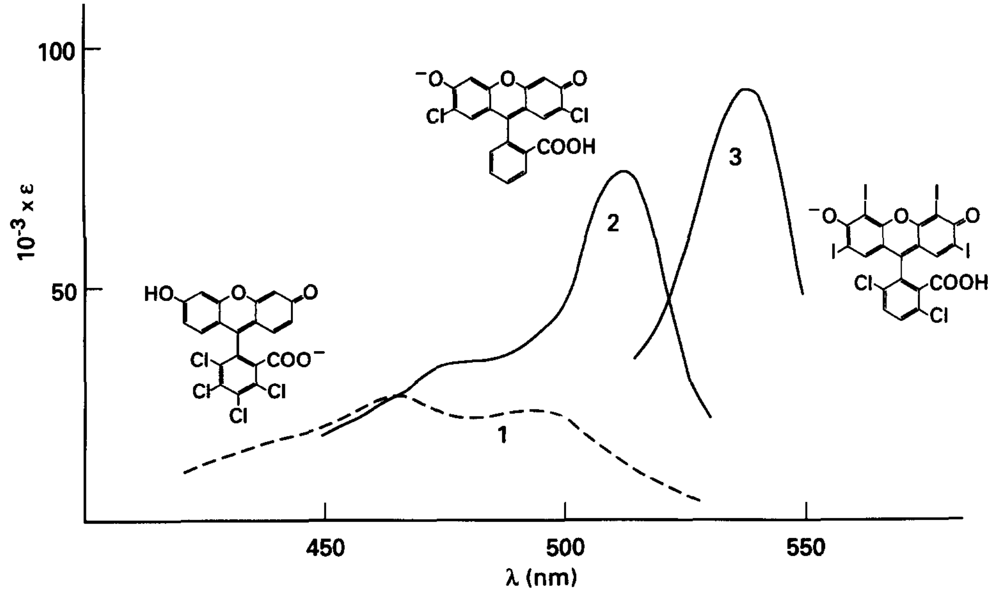


Fig. 6. Absorption spectra of mono-anions HR^- in methanol: 1, 3',4',5',6'-tetrachlorofluorescein; 2, 2,7-dichlorofluorescein; 3, Rose Bengal A.

TABLE 4
Spectral Characteristics of Oxyanthene Anions in MeOH and Approximate Values of the Tautomerization Constant: $K_{T_X} = [IX]/[VIII]$

Dye	λ_{max}(*nm*) ($\varepsilon_{max} \times 10^{-3}$)		$\approx K_{T_X}$
	R^{2-} (X)	HR^-	
Fluorescein[12]	495 (94·0)[a]	450 ± 5 (27·3)	0·05
3',4',5',6'-Tetrachlorofluorescein	518 (90·0)	465 (26·6)	0·05
2,7-Dichlorofluorescein[12]	508 (83·3)	512 (74·6)	5
Eosin[12]	521 (98·0)[b]	527–530 (81·3)	10^3
Erythrosin	530 (103·2)	535–537 (79·0)	5×10^2
Rose Bengal A	530 (97·3)	537 (92·0)	5×10^2
Rose Bengal B[12]	557 (97·4)	558–562 (92·5)	10^2

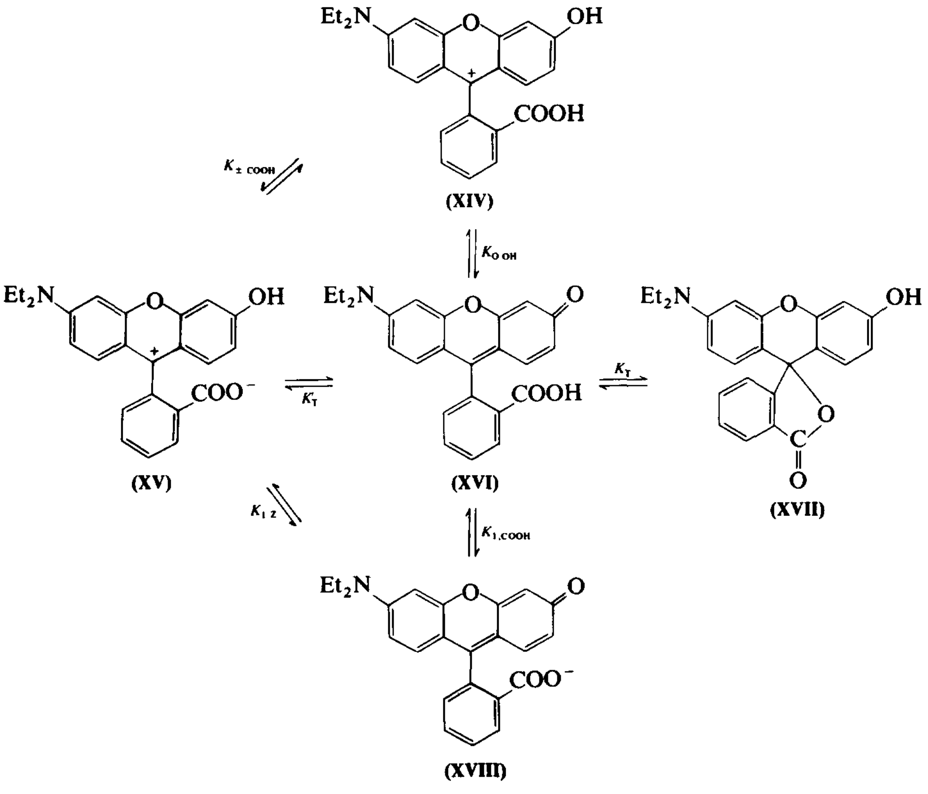
[a] For R^- (**XIII**) of ethyl fluorescein: 505 (95·7).
[b] For R^- of ethyl eosin: 531 (104).

with the help of pK_a values in MeOH (pK_{a1} and pK_{a2} = 9·60 and 10·39, 9·6 and 9·8, 6·40 and 9·6 respectively, at 20°C), previously obtained for the above three dyes.[12] The λ_{max} (HR^-, R^{2-}) values are dependent upon the substituents (Table 4), but the configurations of the R^{2-} spectra are always analogous to those of the fluorescein dianion (Fig. 4). The configuration and ε_{max} values in the case of the HR^- ion of 3',4',5',6'-tetrachlorofluorescein are much closer to the monoanionic absorption of fluorescein (**VIII** species, see Fig. 4), while in the case of 2,7-dichlorofluorescein and Rose Bengal A, the HR^- spectra are typical for a 'phenolate' chromophore (i.e. tautomers of type **IX**). In cases where the mono-anionic tautomer of type **IX** (e.g. for Eosin, Erythrosin, Rose Bengal) the K_{a2}s are equal to $k_{2,COOH}$, and pK_{a1} can be expressed as p$k_{1,OH}$ + log (1 + K_T).

The K_{T_X} estimations in MeOH with the help of eqn (14) confirm this conclusion (Table 4; the pK_{a1} and pK_{a2} values of Erythrosin in methanol are equal to 6·55 and 9·55). The p$k_{1,COOH}$ values of Fluorescein (8·9) and 3',4',5',6'-tetrachlorofluorescein (8·1) (as well as the p$k_{1,OH}$ values of 2-,4-,5- and 7-halogeno-derivatives (5·6–6·2; and in the case of 2,7-dichlorofluorescein: ≈8·2), which are necessary for calculations of K_{T_X}, are obtained by using the pK_{a1} and K_T values of the corresponding dyes; the K_{a2}values corresponded with the $k_{2,OH}$ values of the first two dyes and with $k_{2,COOH}$ of the other dyes. The pK_{a1} value of ethyl fluorescein and of 6-hydroxy-9-phenylfluoron on MeOH (9·96, 10·25) was used as that of p$k_{1,OH}$ of Fluorescein and 3',4',5',6'-tetrachlorofluorescein, while the p$k_{1,COOH}$ values of Eosin, Erythrosin, Rose Bengal A and 2,7-dichlorofluorescein were equated with the $pk_{1,COOH}$ values of the unsubstituted dye. The approximate values of $pk_{2,COOH}$ for the latter compounds and for 3',4',5',6'-tetrachlorofluorescein, as well as the $pk_{2,OH}$ of 2,4,5,7-tetrachlorosubstituted dyes and 2,7-dichlorofluorescein were obtained from the corresponding $pk_{1,COOH}$ and $pk_{1,OH}$ values, taking into account the influence of the additional negative charge (which leads, in MeOH, to an increase in the pk_2 values of ~1–1·5 units compared with the corresponding pk_1 value[12,29]).

3.4 Tautomerism of N,N-Diethylrhodol

Proceeding from Schemes 1 and 2 and considering the structure of N,N-diethylrhodol, the protolytic interconversions shown in Scheme 4, can be considered.[24] Strongly acidic media, where protonation of the diethylaminogroup may occur, are not studied here. The tautomerization constants, describing the equilibria between the zwitter-ion **XV**, quinoid **XVI** and lactone **XVII**, are expressed with the same symbols as in



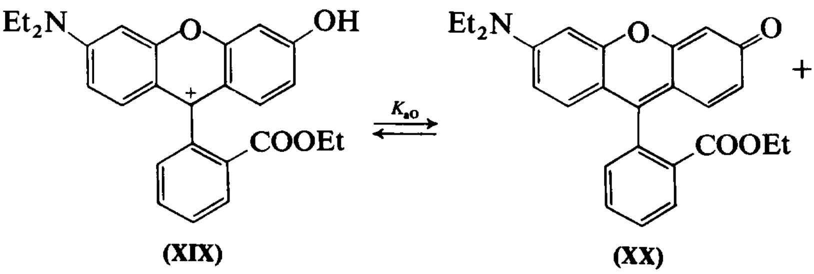
Scheme 4. Protolytic equilibria of N,N-diethylrhodol.

Schemes 1 and 2: K_T = [XVII]/[XVI], K'_T = [XV]/[XVI], K''_T = [XVII]/[XV] = K_T/K'_T. The decrease in the molar absorptivities of the neutral form (HR) in organic solvents compared to aqueous solutions (Tables 1–3, Fig. 2) indicates the partial inter-conversion of HR into the colourless lactone **XVII**, but the structure of the coloured molecules (**XV**, or **XVI**, or both) is less understandable.

The application of the approach, used in the case of Rhodamine B and Fluorescein, presupposes modelling the spectra of species **XV** and **XVI** through the spectra of ions **XIV** and **XVIII** respectively (in corresponding solvents). However the closeness of the λ_{max} values of the cations and anions (Tables 1–3, Fig. 2) characteristic for rhodols (contrary to Fluorescein, Tables 1–3, Figs 3–5) presents some difficulty. For the unsubstituted rhodol [6-amino-3-oxo-9(2-carboxyphenyl)xanthene] the ε_{max} values of the coloured species are also close; for example, it is impossible to obtain the pK_a values in water.[24,25]

Although for the relatively broad bands of chromophores of type **VIII** (Figs 3 and 4) replacement of COO^- by COOEt or H has little influence on the spectra,[12,15,17,25] in the case of the narrow bands of the symmetrical negatively charged chromophores (**X**; the oxygen atoms in the 3 and 6 positions are then equal) such a replacement leads to a marked bathochromic shift (Figs 4 and 5), typical for the oxyxanthene series[5,7,10,12,15,17,18] (see also Table 4). The bands of the symmetrical cationic chromophores of rhodamines also display bathochromic shifts as a result of COO^- replacement by COOH (see above).

The character of the anionic band of N,N-diethylrhodol (species **XVIII**; Figs 2 and 4) allows the classification of this chromophore as type **X** (or **XIII**) rather than **VIII**. In terms of traditional colour theory[1] it can be interpreted as a result of the high electron–donor properties of the NEt_2 group. Therefore, a bathochromic shift can be anticipated as a result of protonation of the carboxylate group (COO^- → COOH) of species **XVIII**. This is confirmed by the spectra of species **XX**, Scheme 5 (see Figs 4, 7 and 8, Tables 1 and 2).



Scheme 5. Protolytic equilibria of the ethyl ester of N,N-diethylrhodol.

The data obtained using methanol as solvent may be compared with those of Hammond.[20] In 99% MeOH with 0·01M NaOH for Fluorescein (R^{2-}, X) N,N-diethylrhodol (R^-, **XVIII**) and Rhodamine B (R, principally **II** and, perhaps, ≈10% of the colourless lactone **III**) the values for λ_{max}, nm ($10^{-3} \times \varepsilon_{max}$) are reported as 497 (84), 517 (39) and 545 (114) respectively.[20] Our data, with 10^{-4}–10^{-2}M MeONa in MeOH are 495 (94), 520 (74) and 545 (100).[12,24,25] The most marked discrepancy is for N,N-diethylrhodol and is hard to explain. For N,N-dimethylrhodol in MeOH

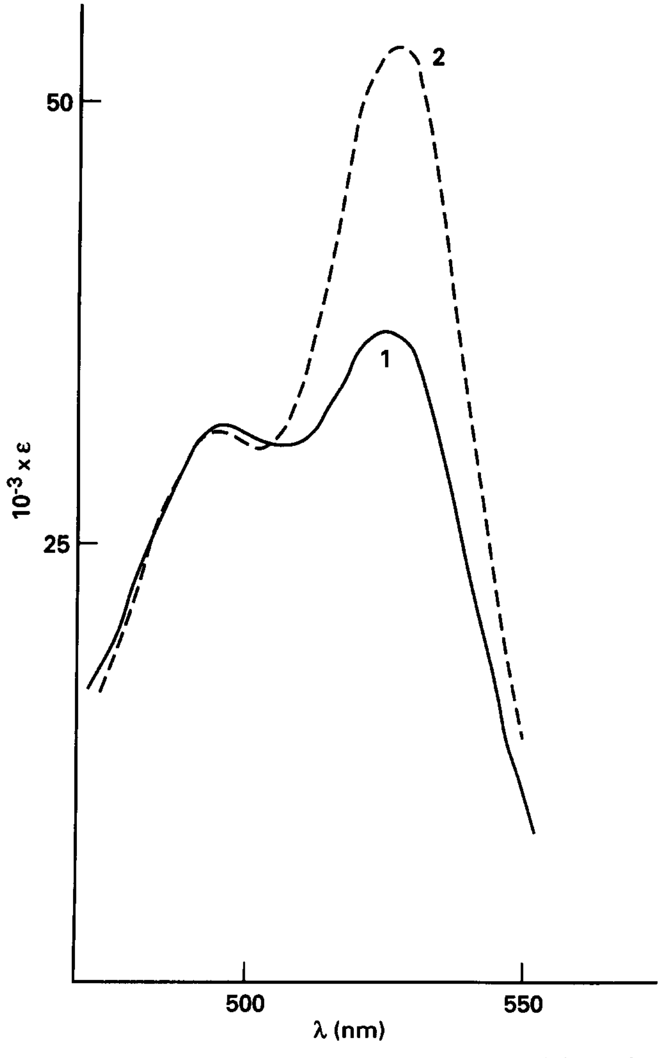


Fig. 7. Absorption spectra of 2'-carbethoxy-N,N-diethylrhodol in 91·3 wt% DMSO: 1, cation HR^+ (**XIX**); 2, neutral quinoid R (**XX**).

without any addition of alkali the λ_{max} and ε_{max} values are reported as 511 nm and 60×10^3.[22] Possibly, the partial interconversion into the lactone, as well as ionization of COOH group, may occur in neutral alcohol; for the similar dye, containing C_6H_5 instead of C_6H_4COOH, the molar absorptivity is higher ($\varepsilon_{max} = 76 \times 10^3$)[22] and the band is shifted towards the red (528 nm).[22]

On the whole we can conclude that when modelling the band of the **XVI** species with that of **XVIII**, it is necessary to shift the band.

For HR absorption of N,N-diethylrhodol at a fixed wavelength, eqn (16) is valid:

$$\varepsilon_{HR} = \varepsilon_{XV}\alpha_{XV} + \varepsilon_{XVI}\alpha_{XVI} \tag{16}$$

$$1 = \alpha_{XV} + \alpha_{XVI} + \alpha_{XVII} \tag{17}$$

In 91% DMSO, as in 90% Me_2CO,[24] the curves ($\varepsilon/\varepsilon_{max}$–$\lambda$) for HR and R^- superpose after a corresponding shift of λ_{max}; this permits us to consider that $\alpha_{XVI} \gg \alpha_{XV}$; $\alpha_{XVI} = \varepsilon_{max}(HR)/\varepsilon_{max}(R^-) = 0{\cdot}0114$; $\alpha_{XVII} = 0{\cdot}9886$.

In 91% EtOH, the coexistence of **XV** and **XVI** tautomers is more probable (Figs 2 and 8): the specific bathochromic shift of the HR band relative to the R^- spectra is accompanied by additional (compared with the anionic spectra) absorption in the region 480–490 nm. Utilizing eqn (16) at λ = 490 nm and 520 nm, and using the $\varepsilon_{H_2R^+}$ values as ε_{XV} and ε_{R^-} (after shifting the R^- band toward the red on 5 nm) as ε_{XVI}, we obtain the following results: $\alpha_{XV} = 0{\cdot}188$, $\alpha_{XVI} = 0{\cdot}158$, $\alpha_{XVII} = 0{\cdot}654$.

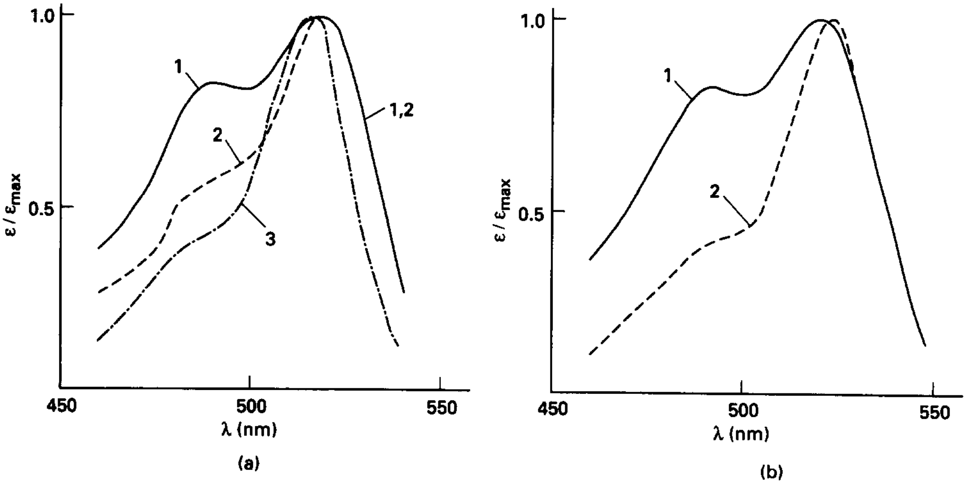


Fig. 8. Normalized spectral curves of (a) N,N-diethylrhodol and (b) of its ethyl ester in 91·4 wt% EtOH: (a) 1, H_2R^+ (**XIV**): 2, HR; 3, R^- (**XVIII**); (b) 1, HR^+ (**XIX**); 2, R (**XX**).

Without shifting the R^- band α_{XV} is found to be ~30% lower, and α_{XVI} is much higher. This has only minor effects on the pk values (±0·1 units). While modelling the spectra of the zwitter-ionic tautomer **XIV** through the spectra of **XV**, the cationic bands were not shifted; we consider the **XV** chromophore to belong to an asymmetrical type (see above). Notwithstanding the good agreement of the λ_{max} values of **I** and **XI**, **IV** and **XII**, **XIV** and **XIX** respectively, the ε_{max} values of the corresponding species differ sometimes by 10–15%, even if highly pure samples are used (Figs 4 and 5, Tables 1 and 2). Possibly, this reflects the peculiarities of the influence of the bulky COOEt substituent on the absorption; this was the reason for the use of the spectra of **XIV** and of **XVIII** (but not those of **XIX** and **XX**) for α_{XV} and α_{XVI} estimations.

3.5 Microscopic ionization constants of N,N-diethylrhodol

It can be derived from Scheme 4, that:

$$\begin{aligned} \mathrm{p}K_{a0} &= \mathrm{p}k_{\pm,\mathrm{COOH}} - \log\,(1 + K''_\mathrm{T} + (K'_\mathrm{T})^{-1}) \\ &= \mathrm{p}k_{\mathrm{O,OH}} - \log\,(1 + K_\mathrm{T} + K'_\mathrm{T}) \end{aligned} \tag{18}$$

$$\begin{aligned} \mathrm{p}K_{a1} &= \mathrm{p}k_{1,\mathrm{Z}} + \log\,(1 + K''_\mathrm{T} + (K'_\mathrm{T})^{-1}) \\ &= \mathrm{p}k_{1,\mathrm{COOH}} + \log\,(1 + K_\mathrm{T} + K'_\mathrm{T}) \end{aligned} \tag{19}$$

here $k_{\pm,\mathrm{COOH}} = a^*_\mathrm{H}(\mathbf{XV})/(\mathbf{XIV})$, $k_{\mathrm{O,OH}} = a^*_\mathrm{H}(\mathbf{XVI})/(\mathbf{XIV})$, $k_{1,\mathrm{Z}} = a^*_\mathrm{H}(\mathbf{XVIII})/(\mathbf{XV})$, $k_{1,\mathrm{COOH}} = a^*_\mathrm{H}(\mathbf{XVIII})/(\mathbf{XVI})$, $K_\mathrm{T} = \alpha_{\mathbf{XVII}}/\alpha_{\mathbf{XVI}}$, $K'_\mathrm{T} = \alpha_{\mathbf{XV}}/\alpha_{\mathbf{XVI}}$, $K''_\mathrm{T} = \alpha_{\mathbf{XVII}}/\alpha_{\mathbf{XV}} = K_\mathrm{T}/K'_\mathrm{T}$.

Equations (18) and (19) can be used for pk estimation. In 91% DMSO: $\alpha_{XV} \ll \alpha_{XVI}$, $K_\mathrm{T} = 87$, $K'_\mathrm{T} \ll K_\mathrm{T}$, $\mathrm{p}k_{\mathrm{O,OH}} = 5{\cdot}17$, $\mathrm{p}k_{1,\mathrm{COOH}} = 7{\cdot}52$. These values may be compared with $\mathrm{p}k_{\mathrm{O,OH}}$ (= $\mathrm{p}K_{a0}$) of the ethyl ester (Scheme 5, $\mathrm{p}K_{a0} = 5{\cdot}65$) and $\mathrm{p}k_{1,\mathrm{COOH}}$ of Fluorescein (7·56[18]). Taking into account the errors of the pK_a and α estimations, as well as the approximate nature of such modelling of pk, the coincidence may be considered satisfactory. Of course the modelling of pk within the conjugated system is impossible: the $\mathrm{p}k_{\mathrm{O,OH}}$ value of the 'hybrid' dye is 3 units higher than those of Fluorescein ($\mathrm{p}k_{\mathrm{O,OH}} = 2{\cdot}26$[18]), which underlines the high electronodonor properties of the NEt_2 group compared with the OH group (see Schemes 2 and 4).

On the other hand, it is evident from Scheme 4 that

$$K'_\mathrm{T} = [\mathbf{XV}]/[\mathbf{XVI}] = k_{\pm,\mathrm{COOH}}/k_{\mathrm{O,OH}} \tag{20}$$

If the $\mathrm{p}k_{\pm,\mathrm{COOH}}$ value of N,N-diethylrhodol can roughly be equated with the same value of Rhodamine B in 91% DMSO (7·38[15,16]), the value

$K'_T \sim 6 \times 10^{-3}$ can be obtained, which confirms the negligibility of the concentration of zwitter-ions compared with those of quinoid configurations in 91% DMSO.

In 91% EtOH: $K_T = 4{\cdot}13$, $K'_T = 1{\cdot}19$, $K''_T = 3{\cdot}48$, $pk_{\pm,COOH} = 5{\cdot}17$ (for Rhodamine B: 5·84), $pk_{O,OH} = 5{\cdot}25$ (for the ester, Scheme 5: 5·15), $pk_{1,COOH} = 6{\cdot}81$ (for Fluorescein: 6·68), $pk_{1,Z} = 6{\cdot}89$. The differences ($pk_{1,COOH} - pk_{\pm,COOH}$) and ($pk_{1,Z} - pk_{O,OH}$) are equal to 1·6 pK_a units, which is in accord with the semi-quantitative Bjerrum–Kirkwood–Weztheimer approach,[29] describing the influence of additional charge upon the pK_a value for the given functional group.

Alternatively, on the basis of the $pk_{\pm,COOH}$ Rhodamine B and of the $pk_{1,COOH}$ of Fluorescein, it is possible to estimate the tautomerization constants of N,N-diethylrhodol using eqns (18) and (19): $K_T = 7{\cdot}1$, $K'_T = 0{\cdot}4$, $K''_T = 18$. Both methods for α estimation (with the help of modelling of ε values of tautomers and of their pk values) are based upon the extrathermodynamic assumptions, and their results may differ somewhat (the accumulation of errors during pK_a and ε evaluation must also be taken into account). An accurate coincidence of pk value with those of model compounds is not always evident; for example, the $pk_{\pm,COOH}$ values of xanthene dyes may to some extent depend on the effective charge on the C_9 atom.

The pk_{a1} values of N,N-diethylrhodol in 91% EtOH and 91% DMSO differ markedly (7·61 and 9·46, respectively). The values, attributed to the carboxylic group, are much closer to each other ($pk_{1,COOH} = 7{\cdot}52$ and 6·81), in the same way as the pK_a values of benzoic acid in these solvents (8·05 and 7·50).

A similar picture was also observed on comparing data in MeOH and in 90% Me_2CO ($pK_{a1} = 9{\cdot}4$ and 11·41[24]). The $pk_{1,COOH}$ values are closer to each other (8·8 and 9·46[24]); the pK_a values of benzoic acid in these two solvents are 9·4 and 9·75, respectively. The coincidence of the $pk_{\pm,COOH}$, $pk_{1,COOH}$ and $pk_{O,OH}$ values of N,N-diethylrhodol in MeOpH and of the $pk_{1,COOH}$ and $pk_{O,OH}$ in 90% Me_2CO on the one hand and of the values $pk_{\pm,COOH}$ of Rhodamine B, $pk_{1,COOH}$ of Fluorescein and pK_{a0} of 2'-carbethoxy N,N-diethylrhodol on the other is satisfactory.[24]

3.6 Medium effects upon tautomerization and ionization of N,N-diethylrhodol

As was stated for water–dioxane mixtures,[23] the tendency to lactone formation increases in the sequence: Rhodamine < Rhodol < Fluorescein. However, a quantitative comparison is hindered, because in the case of Rhodamine only the zwitter-ion (**II**) coexists with the lactone (**III**) (quinoid structures of type **VI** or **XVI** are impossible), and in the case of rhodol, and especially Fluorescein, the zwitter-ion fractions in most organic solvents are low. For both Schemes 2 and 4, eqn (21) is valid:

$$K''_{\mathrm{T}} = K_{\mathrm{T}} \cdot k_{\mathrm{O,OH}}/k_{\pm,\mathrm{COOH}} \tag{21}$$

Using the $k_{\pm,\mathrm{COOH}}$ of Rhodamine B, instead of the corresponding values of N,N-diethylrhodol and Fluorescein in solvents where these constants for the latter two dyes are unavailable, as well as the K_{T} and $k_{\mathrm{O,OH}}$ values obtained above, it is possible to assess approximate K''_{T} values (Table 5). In each solvent studied the K''_{T} values showed the following tendency:

Rhodamine B < N,N-diethylrhodol < Fluorescein

Comparison of the K''_{T} values in various solvents with their dielectric constants (D) and Dimroth–Reichardt parameters ($E_{\mathrm{T}}(30)$, $E_{\mathrm{T}}^{\mathrm{N}}$)[31] underlines the prominent role of the H-bonding ability of the solvent, as has been previously noted for rhodamines.[4,6,13,14] Therefore, the internal factor causing zwitter-ion stabilization, i.e. substitution of OH groups by NEt_2 groups, can be explained as a result of lowering of the effective charge on the C_9 atom (due to the stronger electron-odonor properties of the diethylamino group), while the external factor, i.e. solvent nature, is connected with the solvation of carboxylate groups (COO^-) due to H-bonding.

The K_{T} values of N,N-diethylrhodol are always lower than those of Fluorescein (Table 6). For the 'hybrid' dye, a correlation between log K_{T} and $E_{\mathrm{T}}^{\mathrm{N}}$ can be found:

$$\log K_{\mathrm{T}} = 6{\cdot}047 - 7{\cdot}879E_{\mathrm{T}}^{\mathrm{N}} \tag{22}$$

($E_{\mathrm{T}}^{\mathrm{N}}$: 0·50–0·76, $n = 5$, $r = -0{\cdot}956$, standard deviation: 0·22).

TABLE 5

K''_{T} Values of Various Xanthene Dyes, Describing the Tautomeric Equilibria: Zwitter-ion $\rightleftharpoons$ Lactone, 20–25°C

Solvent	*D*	$E_{\mathrm{T}}^{\mathrm{N}}$	K''_{T}		
			Rhodamine B	*N,N-Diethylrhodol*	*Fluorescein*
MeOH[a]	32	0·762	0·1	0·8	~10^4
91·4% EtOH	28·5	0·67	0·1	3·5–18	~4 × 10^4
90% Me_2CO[b]	24	0·57	17	~2 × 10^3	~10^7
91·3% DMSO	56	0·50	59[c]	~1 × 10^4	~10^8
H_2O	78	1·000	~0·005–0·01[d]	<1	3[e]

[a] From Refs 12 and 24.
[b] From Refs 11 and 24.
[c] From Ref. 15.
[d] From Refs 11, 13 and 14.
[e] From Refs 9 and 18.

TABLE 6

Log K_T Values for N,N-Diethylrhodol and Fluorescein in Various Solvents, 20–25°C

Solvent	*m.f.*[a]	*D*	E_T^N	*Log* K_T	
				N,N-Diethylrhodol	*Fluorescein*
91·3% DMSO	0·708	56	0·50	1·94	2·77[b]
90% Me_2CO	0·736	24	0·57	1·95[c,d]	3·28[c]
64% dioxane	0·267	24	0·64	1·86	2·24[e]
91·4% EtOH	0·806	28·5	0·67	1·616	1·74
MeOH	1·000	32	0·762	0·114	1·73[f]

[a] Molar fraction.

[b] From Refs 15 and 18.

[c] From Ref. 24.

[d] The value of log K_T = 2·20 was obtained with the help of eqns (18) and (9) by using the values $pk_{\pm,COOH}$ of Rhodamine B (7·7) and $pk_{1,COOH}$ Fluorescein (9·2).[24]

[e] From Ref. 25.

The spectra of the neutral form (HR) of N,N-diethylrhodol in water is close to those of the cationic form (Table 3), and the hypothesis of predomination of the **XV** form over the **XVI** and **XVII** forms in aqueous solution is supported by the closeness of the pK_{a0} value to those of Rhodamine B (Table 3), the neutral form of which also exists in water mainly as a zwitter-ion.[3,4,6,11,13,14] The pK_{a1} value of diethylrhodol (Table 3) is then close to the $pk_{1,Z}$ value (Scheme 4); taking into account the character of influence of the charge in the 2' position on the p*k* of OH group (see above) and using the $pk_{1,COOH}$ value of Fluorescein in water (3·50[9,18]) as the $pk_{1,COOH}$ of diethylrhodol, one can evaluate, using eqn (20) and a similar expression (23)

$$K_T = k_{1,COOH}/k_{1,Z} \tag{23}$$

that in water, for the 'hybrid' dye (Scheme 4), K_T ~ 10^2. The same estimations givn, for MeOH and 91% EtOH: K_T = (1·6–0·2), while the value for 91% DMSO and 90% Me_2CO: ~(0·01–0·08). The shift of the equilibrium (**X** **XVI**) toward the right with weakening of the H-bonding ability of the media is evident.

The adequacy of the proposed ionization scheme (Scheme 4) is confirmed with the character of the medium effects, $\Delta pK_a = pK_a - pK_a$ (in H_2O). So, the medium effects while transferring from water to 91% EtOH are: ΔpK_{a0} = 1·37, ΔpK_{a1} = 1·78. At the same time the $\Delta pK_{\pm,COOH}$ value is markedly higher than $\Delta pK_{1,Z}$ (2·1 and 1·1, respectively). Such a tendency is in agreement with the nature of ionizing groups (COOH and OH) and charge types of the acid–base couple species (A^+, $B^\pm$ and $A^\pm$, B^-, respectively).[27] The Δpk of N,N-diethylrhodol is difficult to calculate in 91% DMSO, because the accurate α_{XV} value is unavailable; but some additional increase in pK_{a1} and the fall in pK_{a0} is connected with a rise in α_{XVII} (see eqns (18) and (19)). As a result, the ΔpK_{a0} and ΔpK_{a1} values are equal to 0·15 and 3·63, respectively.

4 CONCLUSIONS

The tautomeric equilibrium of the neutral form (H_2R) of Fluorescein is strongly shifted toward the colourless lactone in 91·4% EtOH compared with aqueous solutions; the ratio of the lactone and quinoid forms is found to be 98 : 2, while the zwitter-ion (whose proportion in water is com mensurable with those of the other two tautomers) practically disappears.

The data for a series of oxyxanthenes in MeOH illustrate the effect of substituents on the tautomerism of the monoanions: spectral data as well as approximate values of tautomerization constants, estimated with the help of microscopic ionization constants, prove that the HR^- species with an ionized hydroxy group and an unionized carboxylic group do exist in the case of five 2,4,6,7- and 2,7-halogeno-derivatives, while the reverse situation (groups OH and COO^-) is observed for the unsubstituted Fluorescein and for 3',4',5',6'-tetrachlorofluorescein.

In solvents of various nature and at varying pa^*_H, the N,N-diethylrhodol may exist as a cation, anion and three neutral tautomers, viz. zwitter-ion, quinoid and colourless lactone. This is confirmed with the help of electronic absorption spectra; the values of the microscopic ionization constants are in relatively satisfactory agreement with those of Rhodamine B, Fluorescein and the ethyl ester of N,N-diethylrhodol.

The proportion of the tautomers of the 'hybrid' amino-oxyanthene dye depend upon the nature of the solvent. H-bonds stabilize the zwitter-ion, which predominates in water, while in 91·3% DMSO its extent is negligible compared with that of the quinoid tautomer, but the lactone form predominates. The lactone is especially stable in aprotic solvents. The ratio of the zwitter-ion : lactone for the xanthene dyes follow the sequence : Rhodamine B > N,N-diethylrhodol > Fluorescein in all the solvents studied.

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