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A dibasic acid with reversed order of the stepwise ionization constants: 2,7-dichlorofluorescein in the ternary solvent mixture benzene–ethanol–water

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ABSTRACT: The ionization constants of 2,7-dichlorofluorescein were determined and the molecular structures of the equilibrium forms were established in the ternary solvent system benzene–ethanol–water (mass ratio 47:47:6) by using visible spectroscopy. In this solvent mixture with relative dielectric constant $\varepsilon_r = 12.8$ and normalized Reichardt's parameter $E_T^N = 0.587$, an inversion of the stepwise ionization constants of the dye, K_{a1} and K_{a2}, occurs. The K_{a1}/K_{a2} ratio changes from 16 in water and 4.8 in 50 mass % aqueous ethanol to 0.1 in the ternary solvent system with ionic strength of $0.002\,\mathrm{mol\,dm^{-3}}$, where $\mathrm{p}K_{a1} = 9.23 \pm 0.08$ and $\mathrm{p}K_{a2} = 8.22 \pm 0.08$ (25 °C). Such an extraordinary interrelation between the K_a constants is in line with the molecular structure of ionic and nonionic species of 2,7-dichlorofluorescein deduced from their vis absorption spectra, as well as with the expressed salt effects and the probable ion association. The dianion R^{2-} (**5** in Scheme 1) possesses a band with $\lambda_{max} = 512\,\mathrm{nm}$ and a molar absorptivity of $E = 84.3 \times 10^3\,\mathrm{cm^{-1}\,mol^{-1}\,dm^3}$. The tautomeric equilibrium of the neutral form, H_2R, is strongly shifted toward the colorless lactone (**2** in Scheme 1). The (very intensive) absorption band of the HR^- species singled out from the vis spectra at different acidity, is red-shifted by $\Delta\lambda \approx 20\,\mathrm{nm}$ against the band of the dianion R^{2-}. Hence, the monoanion HR^- is found to be completely converted into the 'phenolate' tautomer **4** with groups —COOH and —O^-, while in aqueous solutions the 'carboxylate' tautomer **3** with groups —COO^- and —OH predominates. In 50 mass % aqueous ethanol, the two tautomers **3** and **4** exist in commensurable concentrations. Emission and fluorescence excitation spectra and fluorescence quantum yield of the dianion R^{2-} in the ternary solvent mixture were determined. Copyright © 2006 John Wiley & Sons, Ltd.

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KEYWORDS: 2,7-dichlorofluorescein; visible absorption; protolytic equilibrium; ternary solvent mixture; ionization constants; tautomerism

INTRODUCTION

Dibasic acids are usually weaker when ionizing in solutions in the second step than in the first, that is, $\mathrm{p}K_{a1} < \mathrm{p}K_{a2}$.[1] However, there are some organic substances, which demonstrate regular deviations from this rule. For example, in a series of solvents, fluorescein manifests itself as such an extraordinary acid.[2]

The $\mathrm{p}K_{a1}$ and $\mathrm{p}K_{a2}$ values of 2,7-dichlorofluorescein in different media, including micellar solutions of colloidal surfactants, are in some cases approaching one another.[2a,b,3] Now we report on the results of visible spectroscopic studies of 2,7-dichlorofluorescein in the ternary solvent mixture benzene–ethanol–water (mass ratio 47:47:6), at low ionic strength, under gradual changes in the acidity created with buffer systems. In this solvent mixture, a markedly expressed inversion of the stepwise ionization constants ($\mathrm{p}K_{a1} > \mathrm{p}K_{a2}$) occurs, thus demonstrating the role of solvent in governing protolytic equilibria.

Fluorescein dyes are widely used owing to their unique spectral properties.[4] Though 2,7-dichlorofluorescein was numerously utilized in photophysical studies,[5] as an acid–base fluorescent indicator,[6] as adsorption indicator,[7] as a reactant in free radical chemistry,[8] and as a probe for examination of micellar media,[9] the constitution of the ionic and nonionic species of this dye in solutions is studied in less detail as compared with unsubstituted fluorescein, 2,4,5,7-tetrabromofluorescein (eosin), 2,4,5,7-tetraiodofluorescein (erythrosin), and 2,4,5,7-

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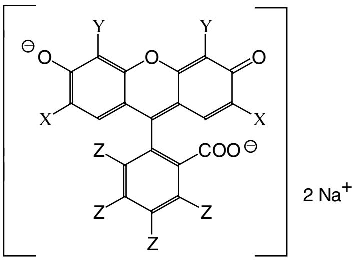
tetraiodo-3′,4′, 5′,6′-tetrachlorofluorescein (Rose Bengal B). All these dyes are presented below in form of their disodium salts, Na_2R.

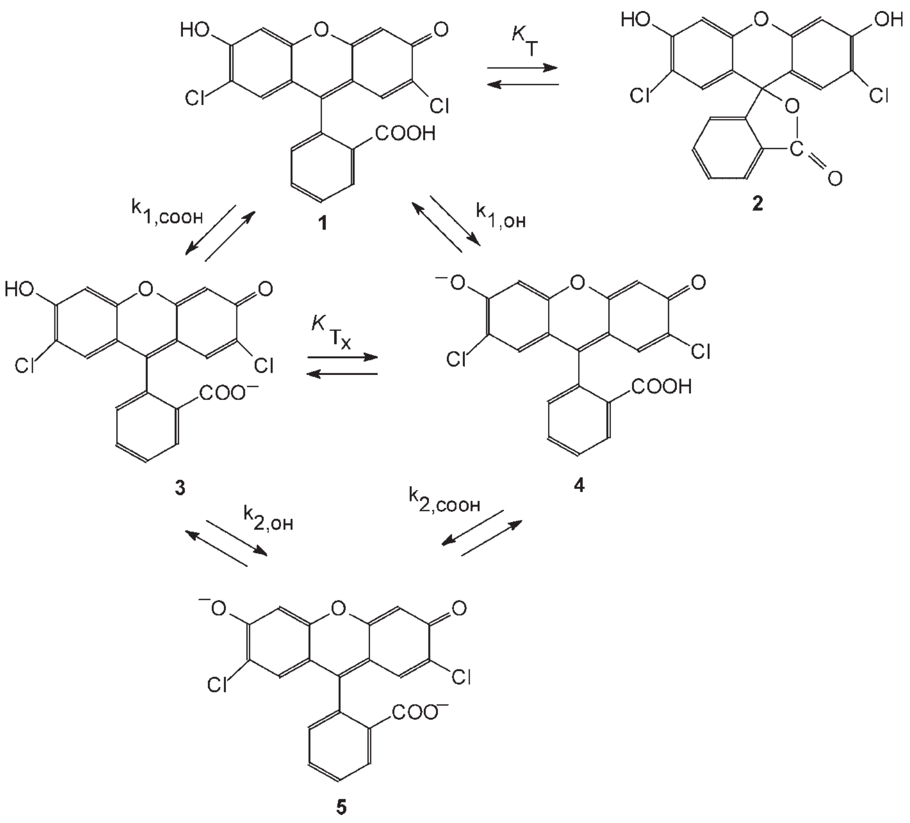


Fluorescein: X=Y=Z=H; 2,7-dichlorofluorescein: X=Cl, Y=Z=H; eosin: X=Y=Br, Z=H; erythrosin: X=Y=I, Z=H; Rose Bengal B: X=Y=I, Z=Cl.

Particularly, 2,7-dichlorofluorescein possesses properties which are very promising from some viewpoints. For instance, the intramolecular quenching of fluorescence by heavy atoms is in 2,7-dichlorofluorescein dianion R^{2-} not so expressed as in the cases of 2,4,5,7-tetrabromo (or -iodo) derivatives.[4a] The pK_a values are lower than those of fluorescein, and this ensures the existence of the dye in form of the intensively fluorescing dianion R^{2-} in media of such kinds in which fluorescein is not yet completely dissociated ($HR^- \rightleftarrows R^{2-}+H^+$). This is of special significance for the application of these dyes as tracers in natural water systems[10] and in other unbuffered systems, as well as in biological systems within the 'physiological' pH region, etc. In addition, 2,7-dichlorofluorescein is more photostable as compared with the unsubstituted luminophore. Recently, protolytic equilibria in water of another dihalogen derivative of fluorescein, 2,7-difluorofluorescein, was studied in detail both in the ground and excited states.[11] Hence, we decided to elucidate the acid–base and tautomeric equilibria of 2,7-dichlorofluorescein in a new type of media, namely, in a ternary solvent mixture, containing benzene, ethanol, and water.

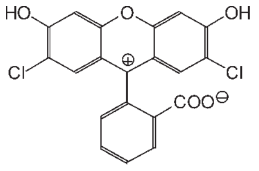
The most typical species of 2,7-dichlorofluorescein are presented in Scheme 1. The negative 'phenolate' charge of the dianion R^{2-} (**5**) is delocalized within the xanthene moiety. Interestingly, some salts of 2,7-dichlorofluorescein, for example, Ag_2R, CdR, etc., behave in the solid state as semiconductors.[12]

The neutral form H_2R exists in solutions as an equilibrium mixture of two tautomers, quinonoid **1** and lactonic **2**, the latter being colorless due to sp^3-hybridization of the central carbon atom C-9.[2,3,8b,d] Contrary to fluorescein, the zwitterionic structure **6** is less typical for 2,7-dichlorofluorescein due to an increase in



Scheme 1. Protolytic conversions of 2,7-dichlorofluorescein in solution: $K_T=[\mathbf{2}]/[\mathbf{1}]$, $K_{T_x}=[\mathbf{4}]/[\mathbf{3}]$, $k_{1,COOH}=a^*_{H^+}a_3/a_1$; $k_{1,OH}=a^*_{H^+}a_4/a_1$; $k_{2,OH}=a^*_{H^+}a_5/a_3$; $k_{2,COOH}=a^*_{H^+}a_5/a_4$ (possible ion association of dye anions with Na^+ cations not shown).

the acidic strength of the hydroxy groups resulting from *ortho*-chloro substitution.[2b,d,3]



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This highly polar zwitterionic tautomer of dichloro-substituted fluorescein exists only in extremely small amounts even in water,[3a,d,11] evidently due to the marked strengthening of acidity of the OH groups by the *ortho*-chlorine substituents. Therefore, this structure is not included in Scheme 1.

Less clear and much poorer studied is the structure of monoanion HR^-, which can exist as 'carboxylate' (**3**) and 'phenolate' (**4**) tautomer. In the case of fluorescein, the 'carboxylate' tautomer **3** predominates in all solvent systems studied,[2,3,4a,13] while in the case of eosin, erythrosin, Rose Bengal B, and other 2,4,5,7-tetrahalogen derivatives the monoanion HR^- exists as 'phenolate' tautomer **4**.[2a,b,d,3] The relative population of the aforesaid HR^- tautomers of 2,7-dichlorofluorescein gradually changes along with alterations in the nature of the solvent, that is, pure and mixed solvents. On transferring from water to organic solvents, the tautomeric equilibrium ($\mathbf{3} \rightleftarrows \mathbf{4}$) shifts toward the right.[2a,b,3] However, some authors take into account only species **3** in any solvent system.[9d]

Versatile applications of hydroxyxanthene dyes often imply the use of organic solvents.[2d,4a,9b,d,g,14] However, all the studies were performed either in pure organic solvents or in binary water-organic solvent mixtures. Some of the effects registered can be explained in terms of preferential solvation. Therefore, we decided to study the protolytic equilibrium of 2,7-dichlorofluorescein in a ternary solvent mixture containing water, ethanol, and benzene, namely, in the azeotropic mixture benzene–ethanol–water, with mass ratio 47:47:6. In this complicated solvent system with density $\rho = 0.8415\ \mathrm{g\,cm^{-3}}$ (25 °C), the normalized Reichardt parameter, E_T^N, equals to 0.587[15] and the relative dielectric constant, ε_r, is 12.8. According to our expectation, preferential solvation of the different species or even of different portions of the bulky dye species must be expressed to a greater extent. Moreover, such a solvent mixture providing the presence of hydrocarbons together with water can be considered as a (reduced) model of organized solutions or lyophilic microheterogeneous systems. In addition, information concerning ionic equilibria and spectra in ternary solvent systems is sparse.

In this solvent mixture, acid–base equilibria of some organic acids and indicator dyes have been recently studied in this laboratory.[15] The pH scale was established earlier;[16] the indices of proton activity designated as $\mathrm{p}a^*_{\mathrm{H}^+}$ correspond to the hypothetical standard state in the given solvent with activity equal to unity and with properties corresponding to infinite dilution.[16,17]

The aim of the present work was to run the visible absorption spectra of 2,7-dichlorofluorescein in the ternary solvent mixture in a wide acidity range, to determine the ionization constants of the dye, to analyze the spectra of single forms, and thus to obtain a better insight into the molecular structure and properties of this luminophore in solutions. Simultaneously, comparative studies of protolytic equilibria of the dye in a more common solvent, 50 mass % aqueous ethanol ($\varepsilon_r = 49.0$), were performed.

EXPERIMENTAL

Absorption spectra of dye solutions were run using an SP-46 apparatus. Emission and excitation spectra were obtained with a Hitachi F-4010 spectrometer, $\mathrm{p}a^*_{\mathrm{H}^+}$ measurements were performed on a P 363-3 potentiometer and a pH-121 pH-meter equipped with an ESL-43-07 glass electrode and a Ag/AgCl reference electrode in a cell with liquid junction $1.0\ \mathrm{mol\,dm^{-3}}$ KCl in water. The dye sample used was described in previous studies.[2a,b,3] Solvents were purified according to standard procedures. Suitable $\mathrm{p}a^*_{\mathrm{H}^+}$ values of working solutions were provided with buffers made up with analytical-grade acids: acetic and phosphoric in aqueous ethanol, salicylic, benzoic, and 5,5-diethylbarbituric in ternary solvent mixtures, and with hydrochloric acid in both systems. All solutions were prepared and $\mathrm{p}a^*_{\mathrm{H}^+}$ measurements performed at 25.0 ± 0.1 °C. Buffer solutions were prepared by mixing stock solutions of the corresponding acids with aliquots of aqueous NaOH. Standard aqueous solutions of NaOH were prepared using CO_2-free water and kept protected from the atmosphere. In the ternary solvent mixture, the initial (analytical) concentration of alkali was $0.005\ \mathrm{mol\,dm^{-3}}$. In 50% ethanol, the ionic strength of acetate and phosphate buffer solutions, as well as of HCl solutions was maintained constant ($0.05\ \mathrm{mol\,dm^{-3}}$) by NaCl additives. In the ternary solvent mixture, the working dye concentrations were as a rule *ca.* $2 \times 10^{-5}\ \mathrm{mol\,dm^{-3}}$, only at $\mathrm{p}a^*_{\mathrm{H}^+} < 7$ they were near $10^{-4}\ \mathrm{mol\,dm^{-3}}$. In 50% ethanol, the concentrations were within the range from 7.68×10^{-6} to $3.07 \times 10^{-5}\ \mathrm{mol\,dm^{-3}}$. The dye solutions obey Lambert–Beer's law. All the spectrophotometric experiments were performed at 25 ± 1 °C. The optical path length was 1 and 5 cm. All concentrations and activities are expressed in molar scale ($\mathrm{mol\,dm^{-3}}$). Emission, fluorescence excitation, and quantum yields, φ, were measured by V. V. Moroz in the laboratory of Professor A. O. Doroshenko, V. N. Karazin Kharkov National University; in these experiments, the dye concentration was $1.47 \times 10^{-6}\ \mathrm{mol\,dm^{-3}}$. Fluorescein in aqueous carbonate buffer was used as a standard for the φ determination.

RESULTS AND DISCUSSION

$pa^*_{H^+}$ scale estimation

In the ternary solvent mixture, the $pa^*_{H^+}$ scale was stated with the help of the pK_a values of buffer acids, HX, by using mixtures of HX and NaOH:

$$pa^*_{H^+} = pK^0_{aHX} + \log\frac{[X^-]}{[HX]} + \log f_1 \qquad (1)$$

Here f_1 is the activity coefficient of a single-charged ion; $[Na^+] \gg [H^+]$. The activity coefficients of neutral molecules were taken to be unity. In the ternary solvent mixture with $\varepsilon_r = 12.8$, an incomplete dissociation of salts NaX must be taken into account. In fact, the indices of dissociation constants, pK_{dis}, are equal to 3.46–3.48.[16] Hence, the equilibrium concentrations $[X^-]$ must be calculated by using the mass action law [Eqn (2)], material balance, and electroneutrality principle, and applying an iterative procedure.

$$K_{dis} = [Na^+][X^-]f_1^2[NaX]^{-1} \qquad (2)$$

The ionic activity coefficients were calculated by using the Debye–Hückel equation (second approach), with $A = 7.75\,mol^{-1/2}\,dm^{3/2}$, $B = 0.814 \times 10^{10}\,m^{-1}\,mol^{-1/2}\,dm^{3/2}$, and ionic parameter $= 5 \times 10^{-10}\,m$. After the fourth iteration the equilibrium concentrations stay unchanged. Then, the $pa^*_{H^+}$ values of working buffer mixtures with constant initial concentration of NaOH ($0.005\,mol\,dm^{-3}$) and varying concentrations of HX ($0.007–0.02\,mol\,dm^{-3}$) were calculated according to Eqn (1). The ionic strength was estimated as $0.00199\,mol\,dm^{-3}$, $f_1 = 0.509$.

The thermodynamic pK^0_{aHX} values in the ternary solvent mixture in molar scale of concentrations are as follows: 7.60 (salicylic acid), 9.26 (benzoic acid), 9.35 (acetic acid), and 11.97 (5,5-diethylbarbituric acid).[15] Note, that these values, especially those of carboxylic acids, are relatively close to the corresponding values 7.9, 9.4, 9.7, and 12.7 in anhydrous methanol,[3a] a solvent with a much higher ε_r value (32). This observation reflects the fact that the proton in the ternary solvent mixture is still preferably hydrated. Hence, the contribution to the pK_a values, caused by the proton-exchange process [H^+ (hydrated) $\rightarrow H^+$ (solvated)], is insignificant.

Besides, the $pa^*_{H^+}$ values were checked in aforementioned cell with liquid junction, standardized using buffer solutions in the ternary solvent mixtures as recommended by Aleksandrov.[16] Solutions with $pa^*_{H^+} = 6.83$ ($0.0084\,mol\,dm^{-3}$ salicylic acid + $0.0084\,mol\,dm^{-3}$ lithium salicylate) and $pa^*_{H^+} = 8.48$ ($0.0084\,mol\,dm^{-3}$ benzoic acid + $0.0084\,mol\,dm^{-3}$ lithium benzoate) were used for cell calibration.[16] These results of potentiometric measurements confirm the calculated $pa^*_{H^+}$ values. The $pa^*_{H^+}$ values in the acidic region, below 4, were created with HCl solutions ($pK_a = 3.1$).[16]

In experiments with 50 mass % aqueous ethanol, the cell with liquid junction was calibrated using standard aqueous buffers (pH 4.01, 6.86, and 9.18). The instrumental pH values of working solutions were then converted into values according to the relation: $pa^*_{H^+} = pH - 0.20$.[17]

Determination of ionization constants

Representative $pa^*_{H^+}$-dependences of the vis absorption spectra of 2,7-dichlorofluorescein are depicted in Figs. 1 and 2; initial experimental data are available as Electronic Supplementary Material. The curves of absorbance, *A*, *versus* $pa^*_{H^+}$, are typified in Fig. 3. Here, the absorbances ≤ 1.2 are measured directly with dye concentrations of $2 \times 10^{-5}–10^{-4}\,mol\,dm^{-3}$ and path length 5 cm, while other data are obtained with dye concentration of $2.12 \times 10^{-5}\,mol\,dm^{-3}$ and path length 1 cm and recalculated to 5 cm.

2,7-Dichlorofluorescein is a dibasic acid (H_2R), which is able to protonation in acidic media:

$$H_3R^+ \rightleftarrows H_2R + H^+; \quad K_{a0} \qquad (3)$$

$$H_2R \rightleftarrows HR^- + H^+; \quad K_{a1} \qquad (4)$$

$$HR^- \rightleftarrows R^{2-} + H^+; \quad K_{a2} \qquad (5)$$

In aqueous solutions, $pK_{a0} = 0.35$, $pK_{a1} = 4.00$, and $pK_{a2} = 5.19$; the band with $\lambda_{max} = 450\,nm$ was attributed to cations H_3R^+, having a structure analogous to that of **6**, but with COOH group instead of COO^-.[3d] In the ternary solvent mixture studied, the first equilibrium does not

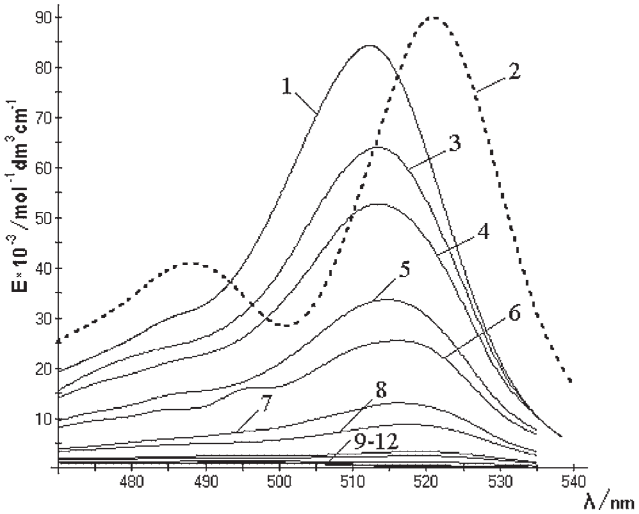

Figure 1. Vis absorption spectra of 2,7-dichlorofluorescein in the ternary solvent mixture: spectrum of R^{2-}, $0.01\,mol\,dm^{-3}$ NaOH (1); spectrum of HR^-, singled out from experimental data using Eqn (7) (2); spectra at $pa^*_{H^+} = 8.96$ (3), 8.78 (4), 8.61 (5), 8.48 (6), 8.18 (7), 8.03 (8), 7.83 (9), 7.48 (10), 7.12 (11), and 6.75 (12)

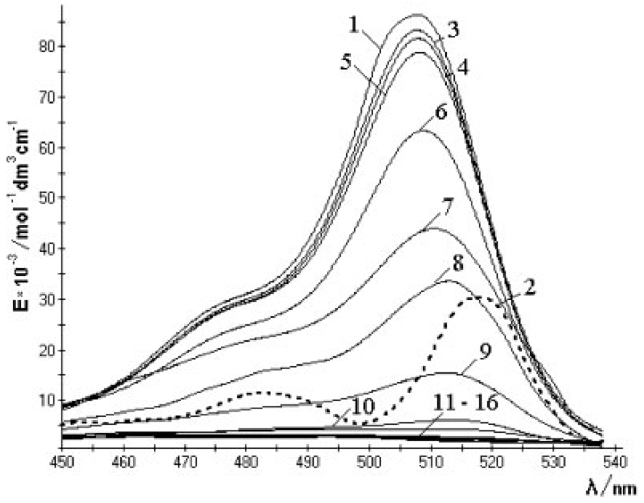

Figure 2. Vis absorption spectra of 2,7-dichlorofluorescein in 50 mass % aqueous ethanol: spectrum of R^{2-}, 0.01 mol dm^{-3} NaOH (1); spectrum of HR^-, singled out from experimental data using Eqn (7) (2); spectra at pa^*_{H+} = 7.68 (3), 7.37 (4), 7.02 (5), 6.42 (6), 5.90 (7), 5.62 (8), 5.44 (9), 4.98 (10), and 4.64–2.06 (11–16)

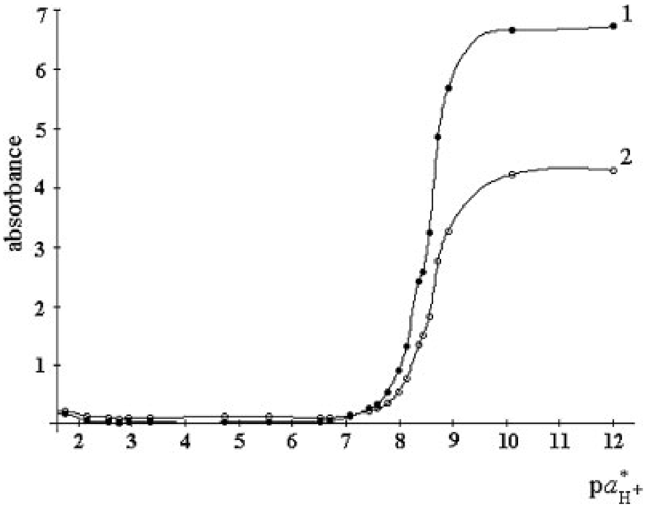

Figure 3. Dependence of absorbance of 2,7-dichlorofluorescein in the ternary solvent mixture *versus* pa^*_{H+}: $\lambda = 520$ nm (1) and $\lambda = 495$ nm (2); salicylate, benzoate and 5,5-diethylbarbiturate buffer solutions, and diluted HCl were used. The *A* values are obtained at dye concentration of 2.12×10^{-5} mol dm^{-3} and optical path length 5 cm, or converted to this conditions from other concentrations and path lengths

manifest itself within the pa^*_{H+} range studied: no spectral changes were registered up to $pa^*_{H+} = 3$ (Fig. 3, see also Electronic Supplementary Material), and no evidence of the appearance of cationic species was observed. At higher acidity (HCl, H_2SO_4), the solutions became turbid. At fixed λ the dependence of molar absorptivity *E* (or absorbance *A* at constant dye concentration and optical cell) *versus* pa^*_{H+} can be described by Eqn (6):[2b,c,3]

$$E = \frac{E_{H_2R}(a^*_{H^+})^2 + E_{HR^-}a^*_{H^+}K_{a1} + E_{R^{2-}}K_{a1}K_{a2}}{(a^*_{H^+})^2 + a^*_{H^+}K_{a1} + K_{a1}K_{a2}} \qquad (6)$$

In this case only the E_{H_2R} (±3%) and $E_{R^{2-}}$ (±1%) values can be measured directly at the appropriate acidity. The band of dianion **5** is observed at $\lambda_{max} = 512$ nm, with a molar absorptivity, $E_{R^{2-}}$, equal to 84.34×10^3 dm^3 mol^{-1} cm^{-1} (in water: 502 nm and 75.02×10^3 dm^3 mol^{-1} cm^{-1}, correspondingly). The spectrum at $pa^*_{H+} = 3 - 6$ was taken as the H_2R spectrum. For the pK_{a1} and pK_{a2} determination the CLINP program[18] was used. The data for 13 working solutions with various pa^*_{H+} and 8 wavelength within the range from $\lambda = 490$ to 525 nm were utilized in the calculations to give as result: $pK_{a1} = 9.23 \pm 0.08$ and $pK_{a2} = 8.22 \pm 0.08$. During the primary analysis of the data, we have not taken into account the possible (or, more precise, probable) formation of ionic associates of Na^+ with the dye anions. This phenomenon will be considered in the final part of this paper. Owing to the low dye concentration (2×10^{-5} mol dm^{-3}) within the region of its transformation into anions, its influence on the equilibrium state of $Na^+ + X^-$ association is negligible.

The study in 50 mass % aqueous ethanol was made in a similar manner. The indices of ionization constants at ionic strength 0.05 mol dm^{-3} (NaCl + buffer) are as follows: $pK_{a1} = 5.51 \pm 0.08$ and $pK_{a2} = 5.88 \pm 0.05$. As ion association in this solvent with $\varepsilon_r = 49.0$ is negligible, the thermodynamic values of 5.67 and 6.35, correspondingly, were calculated using the Debye–Hückel equation for ionic activity coefficients ($A = 1.034$ mol$^{-1/2}$ dm$^{3/2}$, $B = 0.416 \times 10^{10}$ m^{-1} mol$^{-1/2}$ dm$^{3/2}$, ionic parameter = 5×10^{-10} m).

Visible absorption spectra and molecular structure of different ionic and nonionic species

Having the pK_{a1} and pK_{a2} values, it was then possible to calculate the molar absorptivities of HR^- at various wavelengths by Eqn (7), which can be derived from Eqn (6):

$$E_{HR^-} = E + (E - E_{H_2R})a^*_{H^+}K_{a1}^{-1} + (E - E_{R^{2-}}) \times (a^*_{H^+})^{-1}K_{a2} \qquad (7)$$

In the ternary solvent mixture, the E_{HR^-} values are estimated with a confidence interval ±6%. Note that even at optimal pa^*_{H+} the fraction of the dye existing as HR^- in the ternary solvent mixture is only 14% (Fig. 4). The monoanionic spectrum obtained in such a way (Figs. 1 and 5c, Table 1) manifests itself as a typical one for 'phenolate' species **4**.[2a,b,d,3,18a]

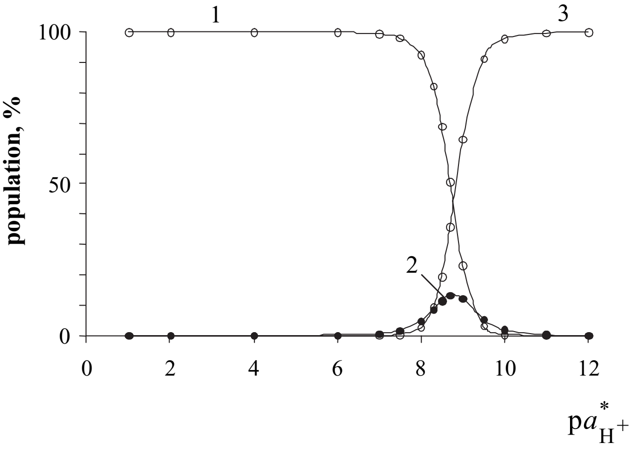

Figure 4. Population of 2,7-dichlorofluorescein species H_2R (1), HR^- (2) and R^{2-} (3) *versus* $pa^*_{H^+}$ in the ternary solvent mixture at an ionic strength of 0.002 mol dm^{-3}, as calculated using the values $pK_{a1} = 9.23$ and $pK_{a2} = 8.22$

The band of HR^- is red-shifted by $\Delta\lambda = 8$ nm as compared to that of R^{2-}; this proves the protonation of the carboxylate ($COO^- \rightarrow COOH$).[2a,b,d,3,18a] The shoulder at $\lambda \approx 490$ nm is typical for the absorption of ionized hydroxyxanthene nuclei[2a,b,d,3,18a,19] and is attributed to the vibrational structure of the main band. Attribution of this shoulder to the principle band of species **3** is unlikely because the integral absorption of species of the types **4** and **5** must be nearly equal. In addition, the E_{max} value of species **3** in the region of 490 nm is known to be *ca*. 30×10^3 cm^{-1} mol^{-1} dm^3 as estimated in water, under conditions of the predominance of **3** (Fig. 5a, Table 1). Therefore, the E_{max} (HR^-) value of 40×10^3 cm^{-1} mol^{-1} dm^3 at $\lambda = 490$ nm is too high to be explained by the existence of tautomer **3**, because the E_{max} value of HR^- at the principal maxima at $\lambda = 520$ nm is even somewhat higher than that of R^{2-} at 512 nm (Figs. 1 and 4a, Table 1). Hence, the value of the (carboxylate phenolate) equilibrium constant, $K_{T_x} = [\mathbf{4}]/[\mathbf{3}]$, is rather high.

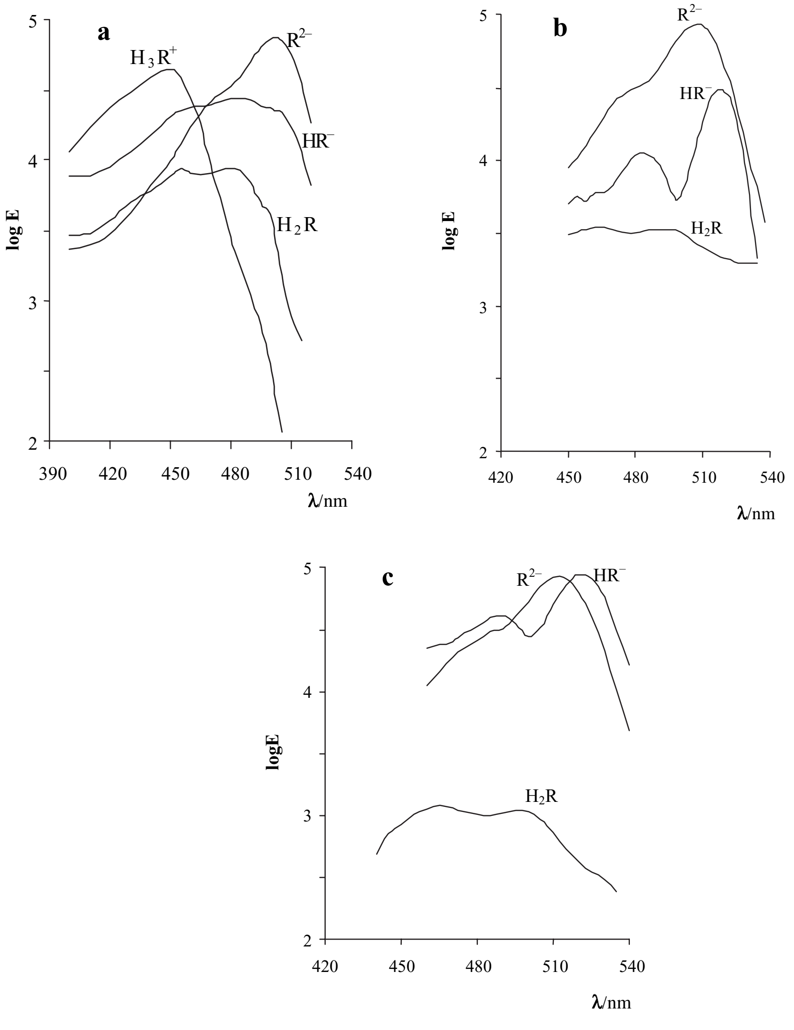

Figure 5. Vis absorption spectra of ionic and nonionic species of 2,7-dichlorofluorescein (in logarithmic scale): (a) in water, (b) in 50 mass % aqueous ethanol, and (c) in the solvent mixture benzene–ethanol–water, with mass ratio 47:47:6 (c)

Table 1. Vis absorption maxima and molar absorptivities of 2,7-dichlorofluorescein species in the ternary solvent mixture ethanol–benzene–water (mass ratio 47:47:6), in 50 mass % aqueous ethanol, and in water

	λ_{max}/nm ($10^{-3} \times E_{max}$/cm^{-1} mol^{-1} dm^3)		
Species	Ternary mixture	50 mass % aqueous ethanol	Water[a]
R^{2-}	512 (84.3)	506 (85.7)	502 (75.02)
HR^-	490 (40.3), 520 (89.3)	482 (12.0), 518 (30.2)	465–470 (24.1), 490 (28.3)
H_2R	465 (1.19), 495 (1.0)	466 (3.45), 494 (3.39)	460 (8.96), 485 (8.70)

[a] From Ref. 3d.

In 50 mass % aqueous ethanol, the E_{HR^-} values are determined with a confidence interval of ±4.5%. In this solvent, the vis spectrum of the 2,7-dichlorofluorescein monoanion (Fig. 4b, Table 1) is of intermediate character; a similar situation was observed for 4,5-dibromofluorescein.[17c] Estimations of K_{T_x} lead to a value of 0.56 (for details see Electronic Supplementary Material).

Such a gradual shift of the tautomeric equilibrium (carboxylate **3** ⇄ phenolate **4**) proves the adequacy of the proposed detailed ionization scheme.

The low E_{max} value of the form H_2R allows to expect that the colored fraction **1** appears as an admixture to the colorless lactone **2**. In order to estimate the corresponding tautomeric equilibrium constant, $K_T = [\mathbf{2}]/[\mathbf{1}]$, extrathermodynamic assumptions must be made, because the molar absorptivity of the quinonoid tautomer **1** is unavailable for direct determination. We assume that the E_{max} (**1**) value is an average between those of 2,4,5,7-tetrabromofluorescein and fluorescein, which can be, in turn, equated to the E_{max} values of neutral molecules of ethyleosin and ethylfluorescein, respectively. These values ($E_{max} \approx 24 \times 10^3$ and $\approx 30 \times 10^3$ cm^{-1} mol^{-1} dm^3, correspondingly) vary somewhat from solvent to solvent. Finally, a value of E_{max} (**1**) $= 27 \times 10^3$ cm^{-1} mol^{-1} dm^3 seems to be reliable. This leads to a K_T value of *ca.* 22.

In 50 mass % aqueous ethanol, the same procedure leads to a value of $K_T = 6.8$.

Microscopic ionization constants

With the designations for the microscopic ionization constants, $k_{1,COOH}$, $k_{1,OH}$, $k_{2,OH}$, and $k_{2,COOH}$, as given in Scheme 1, it is easy to show that:

$$\begin{aligned} pK_{a1} &= pk_{1,COOH} + \log(1 + K_T) - \log(1 + K_{T_x}) \\ &= pk_{1,OH} + \log(1 + K_T) - \log(1 + K_{T_x}^{-1}); \end{aligned} \tag{8}$$

$$\begin{aligned} pK_{a2} &= pk_{2,COOH} + \log(1 + K_{T_x}^{-1}) \\ &= pk_{2,OH} + \log(1 + K_{T_x}) \end{aligned} \tag{9}$$

Using for pK_{a1} and pK_{a2} the expressions with $K_{T_x}^{-1}$ and taking into account that in the ternary solvent mixture $K_{T_x}^{-1} \ll 1$, one can obtain: $pk_{2,COOH} = pK_{a2} = 8.2$ and $pk_{1,OH} = 7.9$. However, in such calculations, the K_T values are estimated not in the activity scale, but in the scale of equilibrium concentrations. Moreover, ion association of the dye with Na^+ cations is also omitted.

In 50% ethanol, $pk_{1,OH} = 5.22$, $pk_{2,OH} = 6.16$, $pk_{1,COOH} = 4.97$, and $pk_{2,COOH} = 5.91$. The last value practically coincides with the pK_{a2} value of eosin in the same solvent (5.98);[17c] for eosin (i.e., 2,4,5,7-tetrabromofluorescein), $pK_{a2} = pk_{2,COOH}$. Furthermore, the $pk_{1,COOH}$ value of 2,7-dichlorofluorescein is close to the analogous value of fluorescein in 50 mass % aqueous ethanol (5.35).[17c]

The peculiarities of protolytic equilibria in the ternary solvent mixture

Electronic spectra of all the three forms of 2,7-dichlorofluorescein, H_2R, HR^-, and R^{2-}, provide information on the peculiarities of their solvation in the ternary solvent mixture. From Table 1 it becomes clear that the positions of the tautomeric equilibria are strongly shifted as compared with the situation in pure water (Fig. 5a) and even with that in 50% ethanol (Fig. 5b). Strong decolorization of the neutral form H_2R testifies the shift **1** → **2**, while the monoanion HR^- is converted from the 'carboxylate' tautomer **3** to the 'phenolate' one, **4**. Such shifts are typical for a transfer from water to non-aqueous media.[3] The difference between λ_{max} (**4**) and λ_{max} (**5**) in solvent systems presented in Table 2 varies from $\Delta\lambda = 4$ to 11 nm.

However, even in systems in which the tautomer **4** manifests itself in a distinct absorption band with λ_{max} (HR^-) > λ_{max} (R^{2-}), the ratio $E_{max}(HR^-)/E_{max}(R^{2-})$ does not reach unity. This ratio is 0.896 in methanol, 0.717 in CTAC micellar system (with 4.0 mol dm^{-3} KCl), and 0.643 in 64% aqueous 1,4-dioxane. In micellar solutions of cetylpyridinium chloride, this ratio equals to 0.51, and in micellar solutions of nonyl phenol −12 and in 90% acetone it is less than 0.5. Hence, in these solvent systems, the conversion of **3** into **4** is incomplete and the fractions of the two species are

Table 2. pK_a values of 2,7-dichlorofluorescein in different solvent systems (20–25 °C)[a]

Solvent	ε_r	E_T^N[b]	pK_{a1}	pK_{a2}	K_{a1}/K_{a2}	$\Delta\lambda_{max}$[c]	$E_{max}(H_2R)$[d]	Reference
Water	78.5	1.00	4.00 ± 0.10	5.19 ± 0.06	15.5	−12	8960	3d
50% ethanol	49.0	0.75	5.67 ± 0.08	6.35 ± 0.05	4.8	+12	3450	This work
33% acetone	59.1	0.79	6.03 ± 0.04	6.40 ± 0.04	2.3	≈+12	952	3d
52% acetone	46.9	0.72	7.0 ± 0.1	7.8 ± 0.2	6.3	≈+5	500	3d
73% acetone	33.8	0.66	8.6 ± 0.2	8.4 ± 0.1	0.63	≈+10	—	3d
90% acetone	24	0.570	10.3 ± 0.3	10.4 ± 0.2	1.3	+10	243	3d
64% 1,4-dioxane	24	0.640	8.05 ± 0.03	8.24 ± 0.12	1.5	+10	609	3e
91.3% DMSO	56	0.50	7.70 ± 0.04	7.77 ± 0.03	1.2	+11	334	2b
Methanol	32.7	0.762	9.6 ± 0.2	9.8 ± 0.2	1.6	+4	932	3a
N,N-dimethylformamide	37	0.386	10.4 ± 0.2	13.2 ± 0.1	631	+5	380	2d
CPC[e] micellar solution, 0.05 mol dm^{-3} KCl	—	0.693[f]	3.58 ± 0.08	3.70 ± 0.12	1.3	+8	2910	3b
CTAC[g] micellar solution, 4.0 mol dm^{-3} KCl	—	0.617	5.50 ± 0.03	5.79 ± 0.05	1.9	+10	822	3f
NP–12[h] micellar solution, 0.05 mol dm^{-3} NaCl	—	0.685	6.08 ± 0.08	5.54 ± 0.07	0.29	+17	720	3c
Ethanol–benzene–water (mass ratio 47:47:6); ionic strength 0.002 mol dm^{-3}	12.8	0.587	9.23 ± 0.08	8.22 ± 0.08	0.098	+8	1190	This work

[a] Thermodynamic values, pK_a^0, unless otherwise indicated; in the case of micellar solutions: 'apparent' pK_a^a values.
[b] Values for organic solvents from Ref. [20]
[c] $\Delta\lambda_{max}/\text{nm} = \lambda_{max}(HR^-) - \lambda_{max}(R^{2-})$.
[d] cm^{-1} mol^{-1} dm^3.
[e] Cetylpyridinium chloride.
[f] NaCl instead of KCl.
[g] Cetyl-trimethylammonium chloride.
[h] Nonyl phenol −12 (nonionic surfactant).

commensurable, while in the ternary solvent mixture benzene–ethanol–water, the 'phenolate' tautomer **4** predominates. Note, that this solvent mixture possesses the lowest dielectric constant ($\varepsilon_r = 12.8$) among all the solvents compared in Table 2. It can be easy shown that the following expression is valid:

$$\log K_{T_x} = pk_{1,COOH} - pk_{1,OH} = pk_{2,COOH} - pk_{2,OH} \qquad (10)$$

Hence, the increase in the K_{T_x} value in going from water to the ternary solvent mixture is in accord with the more expressed decrease of the acid strength of carboxylic acids as compared with that of phenols. This effect is well documented for organic solvents including binary solvent mixtures.[17a]

On the other hand, the shift of the equilibria $\mathbf{1} \rightarrow \mathbf{2}$ toward the right in the ternary solvent mixture is not so pronounced as in *N,N*-dimethylformamide, 91.3% aqueous DMSO, and 90% aqueous acetone, where K_T equals to *ca*. 70–100. If we take the decrease in E_{max} of the molecular form H_2R as a criterion of this shift, we can conclude that the ability of solvent molecules to be donors of hydrogen bonds is much more decisive than the ε_r value (Table 2). Especially, the data give evidence for a preferential solvation of the dye molecules with ethanol as compared with benzene in the ternary solvent mixture. Interestingly, the changes in log K_T values are in general parallel to the E_T^N variations, though the linear correlation [Eqn (11)] is much poorer than those for fluorescein and eosin:[2d]

$$\log K_T = 3.08 - 2.48\, E_T^N; \quad (r = -0.77;\ n = 13, \text{data from Table 2}) \qquad (11)$$

In Fig. 6, the emission and the fluorescence excitation spectra of the R^{2-} dianion measured in water, in aqueous ethanol with 6 mass % of H_2O, and in the ternary solvent mixture benzene–ethanol–water with 6 mass % H_2O are presented.

The data allow to conclude that replacement of 47 mass % of benzene by 47 mass % of ethanol displays only a very small blue shift ($\Delta\lambda_{max} \approx 2$ nm), while the absorption, emission, and excitation spectra in water and 94% C_2H_5OH differ markedly ($\Delta\lambda_{max} \approx 7$–9 nm). In turn, the λ_{max} value of the absorption band of the R^{2-} species in absolute ethanol is with 511 nm practically the same as in 94 mass % aqueous ethanol, while in benzene with an excess of (KOH+18–crown–6), $\lambda_{max} = 522$ nm. Hence, the dianion R^{2-} is solvated preferably by the ethanol molecules. The quantum yield, φ, of R^{2-} in the ternary solvent mixture is with 0.421 somewhat lower than those in water (0.454) and 94% ethanol (0.439).

The K_{a1}/K_{a2} ratios of 2,7-dichlorofluorescein in different solvent systems, including micellar media, are compiled in Table 2. In all organic solvents, the

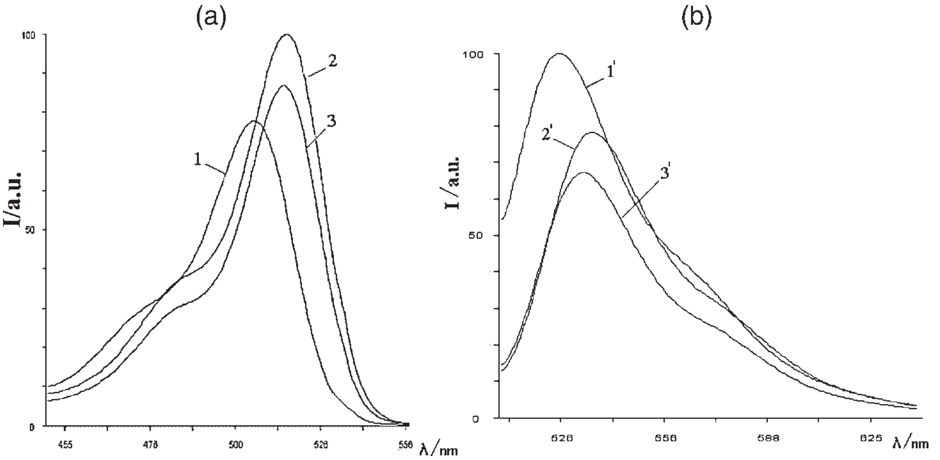

Figure 6. (a) Fluorescence excitation (λ (emission) = 530 nm) and (b) emission (λ (excitation) = 490 nm) spectra of the R^{2-} ion in water (1,1′), in the ternary solvent mixture benzene–ethanol–water with mass ratio 47:47:6 (2,2′), and in 94 mass % ethanol (3,3′); NaOH concentration 0.005 mol dm^{-3}; slits band pass 3 mm

constants of stepwise ionization approach one another, as compared with the situation in water. It can be seen that in the ternary solvent mixture studied in the present work, at an ionic strength of 0.002 mol dm^{-3}, the inversion is most expressed: the K_{a1}/K_{a2} decreases from 15.5 in water to 0.1 under the aforesaid conditions. It can be stated that all these peculiarities of solvation cause the extraordinary character of ionization of the dye: strong inversion of the stepwise ionization constants. For instance, the increase in the K_T value leads to an additional rise in the pK_{a1} values of these dyes;[2,3] see Eqn (8). The difference between the pK_{a1} and pK_{a2} values can be expressed in different manners, as given in Eqn (12):

$$\begin{aligned} \mathrm{p}K_{a1} - \mathrm{p}K_{a2} &= \mathrm{p}k_{1,\mathrm{COOH}} - \mathrm{p}k_{2,\mathrm{OH}} + \log(1 + K_\mathrm{T}) \\ &\quad - 2\log(1 + K_{\mathrm{T_x}}) \\ &= \mathrm{p}k_{1,\mathrm{OH}} - \mathrm{p}k_{2,\mathrm{COOH}} + \log(1 + K_\mathrm{T}) \\ &\quad - 2\log(1 + K_{\mathrm{T_x}}^{-1}) \\ &= \mathrm{p}k_{1,\mathrm{COOH}} - \mathrm{p}k_{2,\mathrm{COOH}} + \log(1 + K_\mathrm{T}) \\ &\quad - \log(2 + K_{\mathrm{T_x}} + K_{\mathrm{T_x}}^{-1}) \\ &= \mathrm{p}k_{1,\mathrm{OH}} - \mathrm{p}k_{2,\mathrm{OH}} + \log(1 + K_\mathrm{T}) \\ &\quad - \log(2 + K_{\mathrm{T_x}} + K_{\mathrm{T_x}}^{-1}) \end{aligned} \tag{12}$$

Moreover, in the ternary solvent mixture with an ε_r value of 12.8, some additional processes can take place.[21] For example, we have not taken into account the possible association of dye anions with Na^+ ions.

Influence of ionic association: indirect estimates

The K_a values of the dye, determined in the manner described above, are mixed ones, because we use the proton activity and the ratio of equilibrium concentrations, determined using a conventional spectroscopic procedure. Vis spectra hardly allow to distinguish between dye anions and ion pairs, etc. Therefore, we use the term ‘ionization constants,’ in order to underline that the equilibria between dye species in their different ionization state, that is, H_2R, HR^-, and R^{2-} are described. In case of dye anions strongly associated with Na^+ cations, these constants can even be named as ‘observed’ (or ‘apparent’) ones, K_{a1}^{obs} and K_{a2}^{obs}. In this case, more general expressions, Eqns (13) and (14) can be proposed for the true thermodynamic dissociation constants, which more precisely describe the equilibria shown by Eqns (4) and (5), respectively:

$$\mathrm{p}K_{a1}^{0} = \mathrm{p}K_{a1}^{\mathrm{obs}} + \log\{1 + [\mathrm{Na}^+] f_1^2 K_{\mathrm{dis1}}^{-1}\} - \log f_1 \tag{13}$$

$$\begin{aligned} \mathrm{p}K_{a2}^{0} &= \mathrm{p}K_{a2}^{\mathrm{obs}} \\ &\quad + \log \frac{1 + [\mathrm{Na}^+] f_2 K_{\mathrm{dis2}}^{-1} + [\mathrm{Na}^+]^2 f_1^2 f_2 K_{\mathrm{dis2}}^{-1} K_{\mathrm{dis3}}^{-1}}{1 + [\mathrm{Na}^+] f_1^2 K_{\mathrm{dis1}}^{-1}} \\ &\quad - \log f_2 + \log f_1 \end{aligned} \tag{14}$$

where f_2 is the activity coefficient of double-charged ion, R^{2-}, and K_{dis1}, K_{dis2}, and K_{dis3} refer to the dissociation of the associated species Na^+HR^-, Na^+R^{2-}, and $(Na^+)_2R^{2-}$, correspondingly. At a first approximation, it is reasonable to assume that $K_{\mathrm{dis1}} = K_{\mathrm{dis3}} = K_{\mathrm{dis}}$ for NaX salts of buffer systems, that is, $\approx 3 \times 10^{-4}$. In this case, $\mathrm{p}K_{a1}^{0} = 9.93$, and the thermodynamic value of $\mathrm{p}k_{1,\mathrm{OH}}^{0}$ equals to 8.6.

More difficult is it to propose a justified approximation for the K_{dis2} quantity. Direct conductivity estimations are doubtful due to the expected low value of the constant, the situation being similar to that for tetraethylammonium succinate in 2-propanol and *tert*-butanol, described by Kolthoff and Chantooni.[22] In addition, such a procedure is hindered by the possible hydrolysis of dye anions. If the K_{dis2} value is equal to *ca.* 3×10^{-5}, *ca.* 10^{-5}, and *ca.* 3×10^{-6}, then $\mathrm{p}K^0_{a2} = 9.7$, 10.2, and 10.7, correspondingly. Hence, in all cases the inversion of the experimentally determined stepwise ionization constants (actually, K^{obs}_{a1} and K^{obs}_{a2}) is strongly caused by both ion association and low values of activity coefficients. The f_1 and f_2 values are here estimated by using the Debye–Hückel equation (second approach). From this viewpoint, the extraordinarily strong inversion is of 'virtual' kind.

In all solvents yet studied, the thermodynamic $\mathrm{p}K_a$ value of benzoic acid and the $\mathrm{p}K_{a2}$ value of eosin, being actually $\mathrm{p}k_{2,\mathrm{COOH}}$, are relatively close. The difference varies from 0.45 to −0.25.[2d] In the ternary solvent mixture benzene–ethanol–water, the $\mathrm{p}K^0_a$ value for benzoic acid is 9.26; if the $\mathrm{p}K^0_{a2}$ ($=\mathrm{p}k_{2,\mathrm{COOH}}$) value of 2,7-dichlorofluorescein is the same, then within the framework of ionic association [Eqn (14)] the K_{dis2} value must be *ca.* 1×10^{-4}.

In 50 mass % aqueous ethanol ($\varepsilon_r = 49.0$), the thermodynamic values of ionization constants, determined vis-spectroscopically, can be regarded as identical with common dissociation constants.

In fact, the result of this study demonstrates the possibilities of governing the acid–base properties of a solute by a ε_r value of the solvent.

CONCLUSIONS

We have demonstrated that in the ternary solvent mixture benzene–ethanol–water (mass ratio 47:47:6), an inversion of the stepwise ionization constants of 2,7-dichlorofluorescein, K_{a1} and K_{a2}, occurs. At an ionic strength of $0.002\ \mathrm{mol\ dm^{-3}}$, created by buffer solutions, the following values were calculated from spectrophotometric data: $\mathrm{p}K_{a1} = 9.23 \pm 0.08$ and $\mathrm{p}K_{a2} = 8.22 \pm 0.08$. This is the most drastic case of an inversion among those measured for the dye in other thirteen solvent systems.

The analysis of the vis absorption spectra allowed to make conclusions about the tautomerism of nonionic and ionic species. The maximal fraction of 2,7-dichlorofluorescein, which can exist as monoanion HR^-, is less than 14%. However, having the values of ionization constants, it became possible to evaluate the molar absorptivities of the form HR^- within a wide spectral range. The vis absorption band of the monoanion thus obtained was red-shifted by $\Delta\lambda \approx 20$ nm as compared with the spectrum of the dianion, R^{2-}. This proves, that the monoanion HR^- is practically completely converted into the 'phenolate' tautomer with groups —COOH and —O^-, while in aqueous solutions the 'carboxylate' tautomer with groups —COO^- and —OH predominates.

The tautomeric equilibrium of the neutral form, $\mathrm{H_2R}$, is strongly shifted towards the colorless lactone as compared with the corresponding equilibrium in aqueous solution. Absorption, emission, and fluorescence excitation spectra of the dianion R^{2-} in a ternary solvent mixture are discussed and a quantum yield of 0.421 is determined.

In 50 mass % aqueous ethanol, the indices of thermodynamic ionization constants, identical with common dissociation constants, are 5.67 ± 0.08 and 6.35 ± 0.05. In this solvent, the position of the tautomeric equilibria of $\mathrm{H_2R}$ and HR^- species is of intermediate character between those found in pure water and in the benzene–ethanol–water mixture.

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