Medium Effects on the Prototropic Equilibria of Fluorescein Fluoro Derivatives in True and Organized Solution

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The stepwise ionization ($H_3R^+ \rightleftarrows H_2R \rightleftarrows HR^- \rightleftarrows R^{2-}$) of four fluorescein fluoro derivatives was studied by visible spectroscopy. The pK_a values were determined in water, in 50 mass % aqueous ethanol, in oil-in-water microemulsions (benzene + CTAB + pentanol-1 in water with 1.0 M KCl; CTAB = cetyltrimethylammonium bromide), and in reversed ones (water + AOT in *n*-octane; AOT = bis-2-ethylhexylsulphosuccinate or Aerosol OT). The medium effects, ΔpK_a, i.e., changes in pK_a of these dyes on going from water to some other solvent systems, were rationalized by considering the tautomerism, the values of microscopic ionization constants, and the charge types of the acid−base couples. An expressed shift of the tautomeric equilibria of H_2R toward colorless lactone was registered on going from water to both aqueous ethanol and organized solutions. While the monoanions HR^- of 3′,4′,5′,6′-tetrafluoro- and 2,7,3′,4′,5′,6′-hexafluorofluorescein exist in all the systems studied as a tautomer with ionized carboxylic and nonionized hydroxy groups, in the case of 2,4,5,7-tetrafluorofluorescein, the prevalence of another tautomer was observed (COOH and O^- groups). For 2,7-difluorofluorescein (Oregon Green 488), the partial shift of the tautomeric equilibrium of HR^- was registered from (COO^- and OH) in water to (COOH and O^-) in other solvent systems. The data for the dyes located in an AOT-based pseudophase indicate that the interior of the latter exerts essential differentiation of the acid strength of the dyes, probably caused by the peculiarity of dye species location in water pools. While the state of tautomeric equilibria resembles that in nonaqueous media, the absorption maxima of R^{2-} species are close to those in water. Such nonuniform influence displayed by AOT-based water droplets should be taken into account when examining them by using different molecular probes.

Introduction

Fluorinated fluorescein dyes, such as Oregon Green 488 (2,7-difluorofluorescein) and its analogues, being introduced into photophysical, biochemical, and biomedical research within the recent decade,[1] appeared to be a very promising group of fluorescent dyes owing to their high quantum yield and photostability. However, their prototropic equilibria still have not been elucidated in detail.

In this paper, we demonstrate the variability of the pK_a values and the state of tautomeric equilibria of several representatives of this group of dyes on going from water to nonaqueous solvents, which appeared to be also a valuable test subject for validation of the general scheme of protolytic conversions of fluoresceins.[2]

Fluoresceins belong to the most widely used organic dyes, serving as luminophores,[3] molecular probes, bioconjugates, stains, and biologically active substances.[1c,4] These dyes are utilized for photosensibilization of redox processes,[3b,5] in energy transfer and light sensitization studies.[6] They are also used in light-harvesting dendrimers,[7] in biochemistry and medicine as reactants for determination of Zn^{2+},[8] NO,[8e,9] and H_2O_2,[10] in sensor devices for H_2S[11] and pH,[12] in studying carbon nanotubes,[13] for creation of water-soluble fluorescent polymers[14] and new ionic liquids,[4c] and in many other fields.

In addition to common dyes, such as fluorescein, eosin, Rose Bengal B, and so forth, several new groups of hydroxyxanthenes have been proposed nowadays. Some of them are created by using traditional dyes as platforms.[1c,6a,b,8,9a,c,10] A set of phthalic residue-substituted fluorescein derivatives, including the so-called Tokyo Green dyes, were synthesized and studied in order to understand the properties of the hydroxyxanthene fluorophore.[15]

Acid−base properties and tautomerism of hydroxyxanthenes govern their chemical and photophysical behavior.[1a,3,7a,8b,c,9a,12] Therefore, it is not surprising that these properties are permanently studied and reconsidered. So, the protolytic equilibrium of one of the Tokyo Greens was examined by the Alvarez-Pez group.[16] The possibility of adaptation of fluorescein for biochemical studies by tuning the pK_a value was demonstrated using 2,7-diethylfluorescein.[17] Recently, Fontana and associates determined the four pK_a values of 5′-carboxyfluorescein by using different UV−visible (UV−vis) spectroscopic techniques.[18] Zhang et al.[19] reported the pK_a values of stepwise deprotonation of dibenzofluorescein from the cationic to dianionic form (earlier Lee determined the pK_a values of the hydroxy group for this and some related compounds by fluorescence titrations[20]). All these data refer to aqueous solutions.

The aforementioned fluorinated and other substituted fluoresceins are widely used in biochemical and medical research and possess a lot of advantages. However, the acid−base properties of fluorinated fluoresceins and their tautomerism in solutions are poorly studied. Probably the sole exception is the

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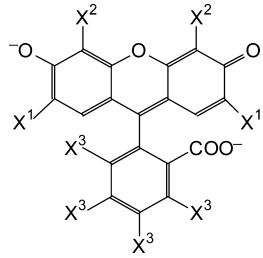
CHART 1: General Structural Formulae of Fluorescein Dyes (I−IV) Dianion, R^{2-} [a]



[a] I (2,7-difluorofluorescein): $X^1 = F$, $X^2 = X^3 = H$; II (2,4,5,7-tetrafluorofluorescein): $X^1 = X^2 = F$, $X^3 = H$; III (3′,4′,5′,6′-tetrafluorofluorescein): $X^1 = X^2 = H$, $X^3 = F$; IV (2,7,3′,4′,5′,6′-hexafluorofluorescein): $X^1 = X^3 = F$, $X^2 = H$.

detailed examination of protolytic reactions of Oregon Green 488 in ground and excited states that was recently undertaken in aqueous solutions.[21]

Meanwhile, it is the influence of the solvent nature and variation of the substituent number and position that allows elucidation of the peculiarities of the scheme of detailed prototropic equilibria. Even for unsubstituted fluorescein, numerously studied in nonaqueous media,[2a,b,22] systematical investigations of acid−base equilibria in water−organic solvents are currently continued.[23]

Even more so, the examination of solvent influence on prototropic equilibria of fluorescein derivatives is worthwhile. Therefore, we have sought to further our previous work on iodo-, bromo-, and chlorofluoresceins with the novel group of dyes. A set of fluorine derivatives of fluorescein described already in 1997[1a] gave us such an opportunity.

These dyes, possessing different functional groups (OH, COOH), ionize in a stepwise manner:

$$H_3R^+ \rightleftarrows H_2R + H^+, \quad K_{a0} \tag{1}$$

$$H_2R \rightleftarrows HR^- + H^+, \quad K_{a1} \tag{2}$$

$$HR^- \rightleftarrows R^{2-} + H^+, \quad K_{a2} \tag{3}$$

The dyes studied here are specified in Chart 1. The R^{2-} species exhibit intensive fluorescence. So, in water (pH 8.5, phosphate buffer), the λ_{max}^{emiss} values are 514, 527, and 527 nm for dyes I, III, and IV, respectively.

The ionization constants were determined by vis-spectroscopic method. In some cases, the stepwise equilibria constants are very close; the absorption spectra of HR^- forms were singled out from the absorption of mixtures of H_2R, HR^-, and R^{2-} using the K_{a1} and K_{a2} values.

The following solvent systems were used as media: (i) water, as a reference solvent (for all the dyes except dye IV, the neutral form of which is sparingly soluble in water); (ii) aqueous ethanol (mass fraction 50%); (iii) benzene-in-water microemulsion (ME), stabilized by cetyltrimethylammonium bromide (CTAB) and 1-pentanol, at high bulk ionic strength; (iv) water-in-*n*-octane ME, stabilized by sodium bis-2-ethylhexylsulphosuccinate (Aerosol OT, AOT).

The reasons for such choice are as follows. Aqueous 50 mass % ethanol is a standard solvent used by us for pK_a determinations of fluorescein dyes.[24] As reduced models of biomembrane microenvironments, we used direct oil-in-water ME (benzene + cetyltrimethylammonium bromide + pentanol-1), with a volume fraction of organic pseudophase equal to 1.3% and bulk ionic strength of 1.00 M (KCl); the latter was applied to essentially screen the interfacial charge of microdroplets. AOT-based reversed ME represents another type of microenvironment, where the solvent properties of "water pools" differ from those of bulk water.[25] We used water-rich reversed ME with a value of the so-called water/surfactant ratio, *W*, equal to 20, because, under these conditions, the water pools are relatively large and the internal water can be considered as a kind of aquatic media.[25] In these two lyophilic colloidal systems, the indices of the so-called "apparent" ionization constants, pK_a^a,[26] were determined instead of common pK_a's.

The analysis and interpretation of acid−base and tautomeric equilibria in different media allows one to rationalize medium effects, i.e., the $\Delta pK_a = pK_a - pK_a(\text{in water})$ quantities, to predict these effects on going from solvent to solvent, and also to tune the pK_a values in desirable directions.

Also, some data for unsubstituted fluorescein, 2,7-dichloro and 3′,4′,5′,6′-tetrachlorofluorescein, as well as of eosin (2,4,5,7-tetrabromofluorescein), obtained earlier, are also used for comparison. The detailed scheme of protolytic equilibria of hydroxyxanthene dyes is given in Chart 2.

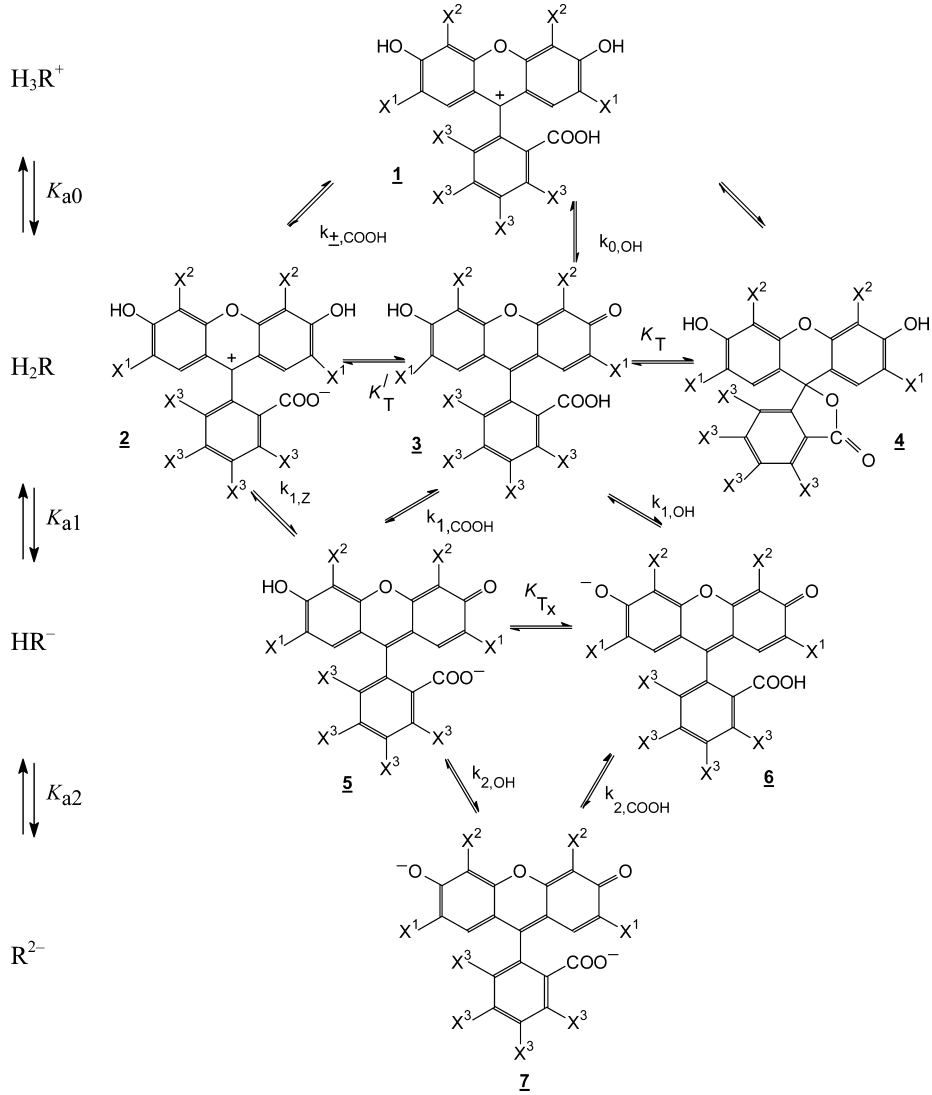
This scheme was discussed in previous publications.[2,22,24] Probably, the first steps toward the understanding such a complicated equilibrium were made as early as 1909.[27] The main difference between fluorescein dyes and other phthaleins[28] consists in the absence of anions−lactones. The latter were detected only in the case of nitro derivatives of fluorescein.[29]

Experimental Section

Materials. The synthesis of 2,7-difluorofluorescein (I), 2,4,5,7-tetrafluorofluorescein (II), 3′,4′,5′,6′-tetrafluorofluorescein (III), and 2,7,3′,4′,5′,6′-hexafluorofluorescein (IV) was described previously.[1a] The IR spectra of molecular species can be found in the Supporting Information; the intense band 1728−1732 cm^{-1} (C═O stretching vibration of the lactone ring) of the samples of dye I give evidence for the presence of tautomer **4**. In the case of dye IV, the band 1720 cm^{-1} can be ascribed to the C═O of the carboxylic group of tautomer **3**, while the band 1775 cm^{-1} is poorly expressed. However, after treating with dimethyl sulfoxide (DMSO) and further drying under vacuum, the sample becomes colorless, and the intensity of the first band dramatically decreases, while that of 1775 cm^{-1} increases strongly, indicating the conversion into the lactone **4**. The samples of *N,N*′-di-*n*-octadecylrhodamine (chloride) and 2,6-diphenyl-4-(2,4,6-triphenylpyridinium-1) phenolate were gifts of Dr. V. I. Alekseeva, Research Institute of Organic Intermediates and Dyes, Moscow, Russia, and Professor Dr. C. Reichardt, Philipps University of Marburg, Germany, respectively. The sample of bromophenol blue was of USSR origin (Minkhimprom).

CTAB (Sigma-Aldrich, purity 99%) and AOT (Sigma, 99%) were used as commercially obtained. Potassium and sodium chloride, potassium bromide, benzene, pentanol-1, aqueous hydrochloric, phosphoric and acetic acids, and borax were of analytical grade. Ethanol was purified according to standard procedure; the absence of aldehydes was checked by UV spectroscopy. Standard aqueous solutions of NaOH were prepared using CO_2-free water and kept protected from the atmosphere. High-quality *n*-octane was washed successively with sulfuric acid, solution of alkali, and distilled water and, after drying with burnt K_2CO_3, distilled under atmospheric pressure (t = 125.5−126 °C).

CHART 2: Protolytic equilibria of the fluorescein fluoroderivatives[a]



[a] $K_T = [\mathbf{4}]/[\mathbf{3}]$; $K'_T = [\mathbf{2}]/[\mathbf{3}]$; $K''_T = K_T/K'_T = [\mathbf{4}]/[\mathbf{2}]$; $K_{T_x} = [\mathbf{6}]/[\mathbf{5}]$; $k_{\pm,\,COOH} = a_{H^+}a_{\mathbf{2}}/a_{\mathbf{1}}$; $k_{0,OH} = a_{H^+}a_{\mathbf{3}}/a_{\mathbf{1}}$; $k_{1,Z} = a_{H^+}a_{\mathbf{5}}/a_{\mathbf{2}}$; $k_{1,COOH} = a_{H^+}a_{\mathbf{5}}/a_{\mathbf{3}}$; $k_{1,OH} = a_{H^+}a_{\mathbf{6}}/a_{\mathbf{3}}$; $k_{2,OH} = a_{H^+}a_{\mathbf{7}}/a_{\mathbf{5}}$; $k_{2,COOH} = a_{H^+}a_{\mathbf{7}}/a_{\mathbf{6}}$.

Apparatus. The vis absorption spectra were measured with the SP-46 and Hitachi U-3210 spectrophotometers. IR spectra of solid samples of the dyes in KBr pellets were obtained using the Spectrum 100 Perkin-Elmer apparatus. pH measurements were performed at 25.0 ± 0.1 °C with a standard deviation of ±(0.01−0.02), using a potentiometer P 37−1 and a pH-meter pH-121, equipped with an ESL-63-07 glass electrode and a Ag/AgCl reference electrode in a cell with liquid junction (1.00 M KCl). The cell calibration was performed using standard buffers (pH 1.68, 4.01, 6.86, and 9.18 at 25.0 °C).

Procedure. Stock solutions of dyes were prepared using water as the solvent, with addition of calculated amounts of NaOH. For the experiments in 50 mass % ethanol, the stock solutions of dyes were prepared using 95.6 mass % ethanol as a solvent. The concentration of dyes in working solutions was within the range $(0.2-5.9) \times 10^{-5}$ M.

In water and in 50 mass % aqueous ethanol, the ionic strength of acetate and phosphate buffer solutions, as well as of HCl solutions was maintained constant (I = 0.05 M) by NaCl additives. In experiments with 50 mass % aqueous ethanol, the cell with liquid junction was calibrated using the above standard aqueous buffers. The instrumental pH values of working solutions were then converted into $pa^*_{H^+}$ values according to the relation $pa^*_{H^+} = pH - 0.20$.[2a,24,30] The $pa^*_{H^+}$ values are the pH values in this mixed solvent, standardized with respect to a hypothetical 1 M lyonium solution, which possesses properties of infinitely diluted solution. For the calculation of the thermodynamic values of pK_a, the Debye−Hückel equation (second approach) for ionic activity coefficients was applied. The ionic parameter was assumed to be equal to 0.5 nm, and activity coefficients of molecules were assumed to be unity.

Stock ME of benzene in water, stabilized by CTAB and pentanol-1, was prepared in the following way:[31] Pentanol-1 (2.3 cm^3) was added to 1.64 g of cationic surfactant. Hydrocarbon (0.43 cm^3) was dissolved in the obtained mixture and then 5.42 cm^3 of water was added to solution. After mixing, a transparent ME is formed. The molar ratio of the components is as follows: CTAB/pentanol-1/benzene = 1:4:1. The volume fraction of the dispersed phase was φ = 1.3%. Suitable pH values of the working solutions used for vis spectroscopic pK^a_a determinations were provided with acetate, phosphate, and borate buffers. Hydrochloric acid was used to prepare solutions having pH ≤ 3.5. In acidic media (pH ≤ 1.0) the scale $pH_c = -\log c_{HCl}$ was used in calculations. The ionic strength of ME bulk phase was kept constant (1.00 M) by adding KCl and taking into account the contribution of the buffer mixtures.

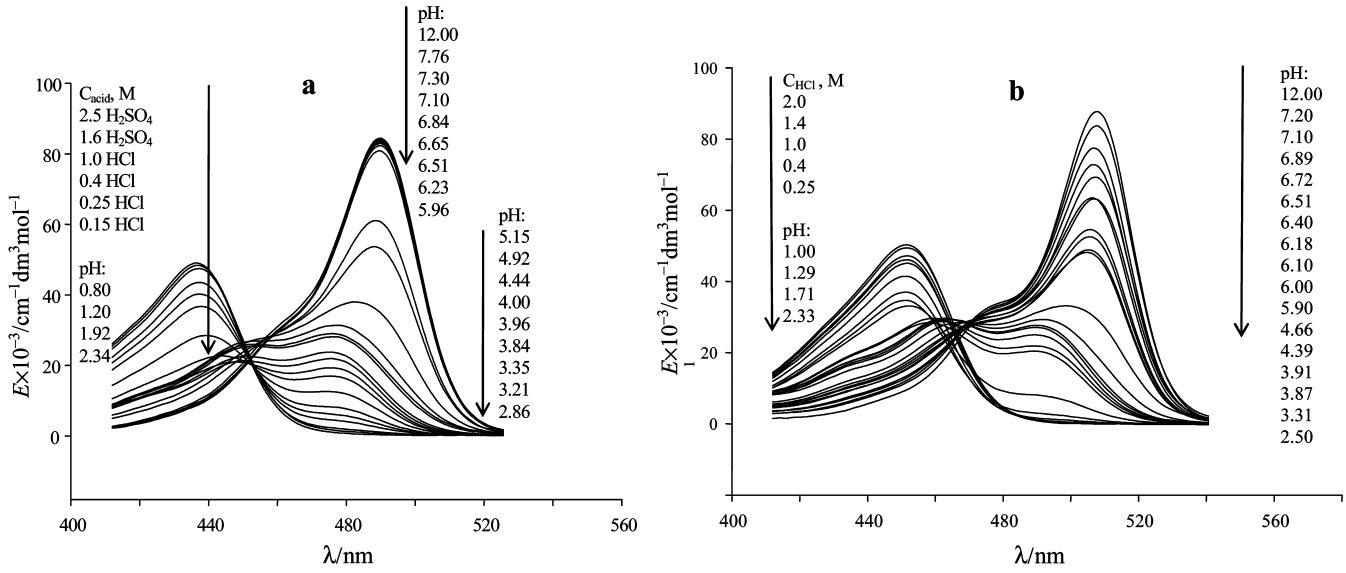


Figure 1. Absorption spectra of 2,7-difluorofluorescein, 1.39×10^{-5} M (a) and 3′,4′,5′,6′-tetrafluorofluorescein, 1.12×10^{-5} M (b) in water at various pH values, $I = 0.05$ M (NaCl + buffer or HCl), except solutions with pH ≤ 1.3, $t = 25$ °C.

The experiments with reversed ME were performed at AOT and water concentrations of 0.05 and 1.00 M, respectively. Hence, the value of the W ratio was equal to 1.00:0.05 = 20, and the volume fraction of dispersed water was $\varphi = 1.8\%$. The aqueous buffer solutions were prepared with $I = 0.05$ M (NaCl + buffer), and the pH values were determined by using a glass electrode before mixing with AOT and *n*-octane.

Liquid−liquid distribution (solvent extraction) was examined at 25 °C, using equal (25 cm^3) *n*-octane and aqueous solutions of dyes. The pH values of aqueous phase were created by HCl and NaOH addition. The systems were equilibrated by 3 min sonication, stored in the dark (special experiments demonstrated that the equilibrium was certainly reached after 30 min), and then the aqueous phase was analyzed.

The value of the electrostatic surface potential of CTAB-stabilized oil-in-water microdroplets was estimated using *N*,*N*′-di-*n*-octadecylrhodamine.[26,32] The Reichardt's polarity parameter, E_T^N, of the same interface was determined as described earlier.[26]

All the solutions were prepared and measurements were done at 25 °C, except the fluorescence measurements, which were made at room temperature (about 20 °C). The absorption spectra were measured against solvent blanks, in cells with absorbing layer lengths of 1 and 5 cm.

Results

Determination of Ionization Constants. Representative pH-dependences of absorption spectra are depicted in Figures 1 and 2.

At a fixed wavelength, the dependence of molar absorptivity, E versus pH (or $pa_{H^+}^*$ in the case of aqueous ethanol) can be described by eq 4.[33]

$$E = \frac{E_{H_3R^+}h^3 + E_{H_2R}h^2K_{a0} + E_{HR^-}hK_{a0}K_{a1} + E_{R^{2-}}K_{a0}K_{a1}K_{a2}}{h^3 + h^2K_{a0} + hK_{a0}K_{a1} + K_{a0}K_{a1}K_{a2}} \quad (4)$$

Here E is the molar absorptivity at the current pH value, $E_{R^{2-}}$, E_{HR^-}, E_{H_2R}, and $E_{H_3R^+}$ are molar absorptivities under conditions of complete conversion of the dye into the corresponding form, $h = 10^{-pH}$.

The stepwise ionization constants were calculated conjointly with the E_{HR^-} and E_{H_2R} values. The spectra of R^{2-} ions were measured directly at pH 9−12, while those of H_3R^+ at the appropriate concentration of HCl. The criterion of complete conversion of the dyes into the cationic forms was the constancy of the spectrum under acidity variation. However, equilibrium (1) is strongly shifted toward the low pH values, and it was studied quantitatively only in three systems; in AOT-based reversed ME, too strong acidification of the "water pools" resulted in turbidity. Therefore, the simplified eq 5 was used for treating the majority of the systems under study:[2b,24,29]

$$E = \frac{E_{H_2R}h^2 + E_{HR^-}hK_{a1} + E_{R^{2-}}K_{a1}K_{a2}}{h^2 + hK_{a1} + K_{a1}K_{a2}} \quad (5)$$

The data were processed by using the CLINP program.[34] As a rule, 15−20 working solutions with various pH values and 20 analytical wavelengths were used for determining the K_{a1} and K_{a2} values. A complete set of initial experimental data used for calculations is compiled in the Supporting Information. Some of the E versus pH dependences are typified in Figure 3.

After making corrections for ionic activity coefficients, the ionization constants thus obtained in water and aqueous ethanol at $I = 0.05$ M are presented in Tables 1−4. Hence, these pK_a values are thermodynamic ones.

In organized solutions, the nature of the experimentally determined ionization constants differs from that in true solutions, i.e., in water and aqueous ethanol. Indeed, the key characteristic of a pH-dependent indicator dye H_jR^z, dissolved in aqueous micellar solutions of colloidal surfactants and other organized media of such a kind, is the so-called apparent ionization constant as defined by eq 6:[22c,24b,26,29]

$$pK_a^a = pH + \log\frac{[H_jR^z]}{[H_{j-1}R^{z-1}]} \quad (6)$$

The ratio of equilibrium concentrations of H_jR^z and $H_{j-1}R^{z-1}$ of a dye is determined vis-spectroscopically; the pH values utilized in calculations characterize only the bulk (aqueous)

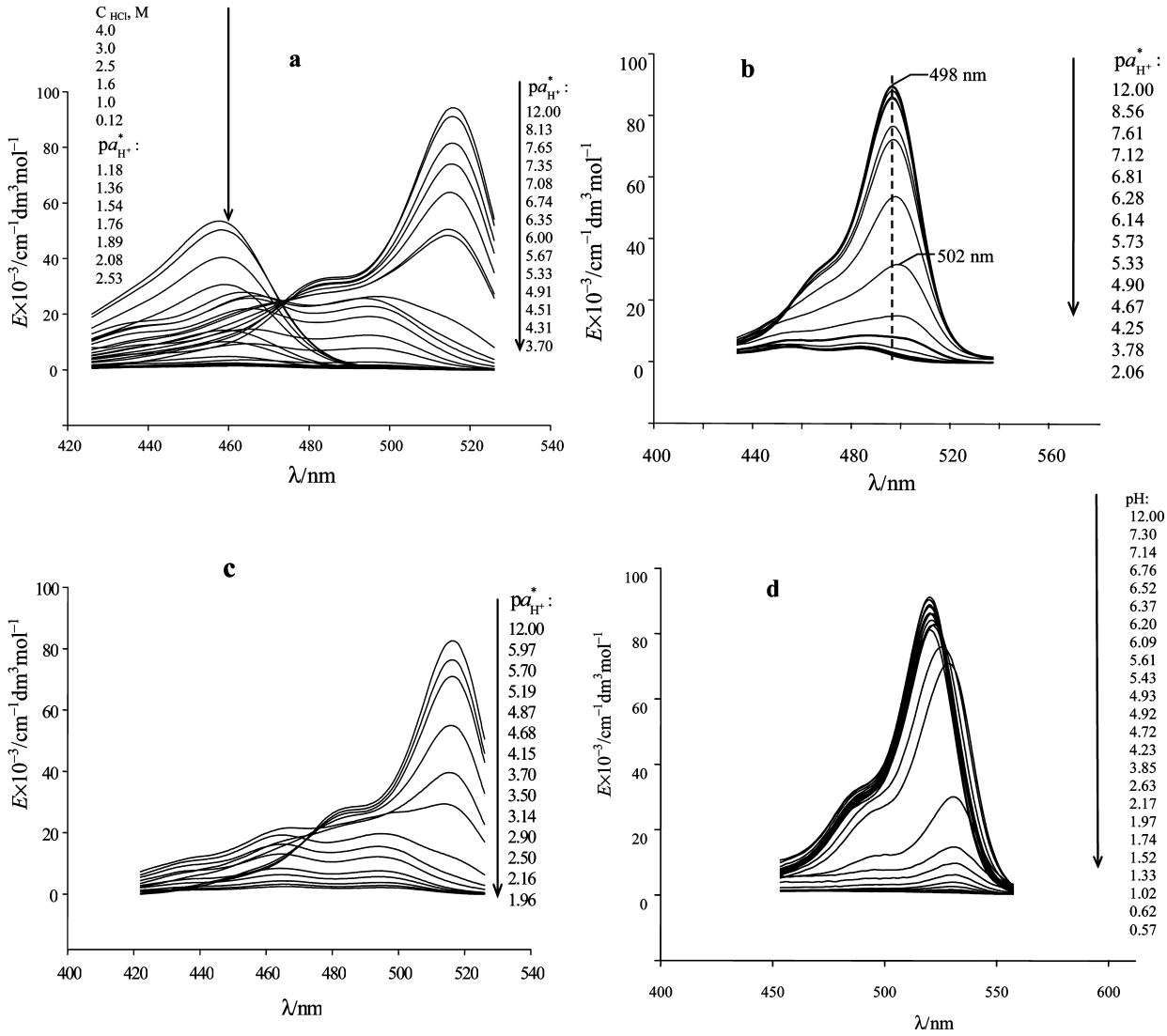


Figure 2. Absorption spectra of 3′,4′,5′,6′-tetrafluorofluorescein, 1.19×10^{-5} M (a), 2,7-difluorofluorescein, 2.22×10^{-5} M (b), and 2,7,3′,4′,5′,6′-hexafluorofluorescein, 2.83×10^{-5} M (c) in 50 mass % aqueous ethanol, $I = 0.05$ M (NaCl + buffer or HCl), except solutions with pH ≤ 1.3; 2,4,5,7-tetrafluorofluorescein, 2.76×10^{-5} M (d) in direct ME benzene−pentanol-1−CTAB−water, $I = 1.0$ M KCl at various $pa^*_{H^+}$ or pH values; $t = 25$ °C.

phase and are as a rule measured with a glass electrode, following the approved procedure.[22c,24b,26] Hence, we deal with a specific kind of two-phase equilibrium, because the dye species are completely or, at least, partly fixed in the pseudophase. In our case, the bands of R^{2-} dianions of the dyes in CTAB-based ME are 10−12 nm red-shifted as compared with aqueous solutions (Tables 1−4), the spectra of HR^- monoanions also undergo changes, and the solubility of H_2R molecules increases on going from water to direct cationic ME. The spectral shifts did not change along with further increase in φ, so the dye species can be considered as practically completely bound by the microdroplets.

It should be noted, that the total bromide concentration in our experiments was 0.016 M, and thus the Br^-/Cl^- ratio in the CTAB-based ME is 1:62. Under such conditions, the surfactant is in fact not CTAB, but cetyltrimethylammonium chloride (CTAC). Indeed, the Br^-/Cl^- exchange constant in cetyltrimethylammonium micelles is around 0.2−0.37.[22c,26] In addition, we studied the standard indicator bromophenol blue in the CTAB-based ME, at pH around 2. It was revealed that the values of the indicator ratio (yellow/blue) at KCl concentration of 1.00 M and in mixed 0.95 M KCl + 0.05 M KBr background coincide. Consequently, such excess of chloride ions ensure the complete enough displacement of Br^- ions from the pseudophase, and hereafter we consider the oil-in-water ME as CTAC-stabilized.

Although the indices of ionization constants determined in AOT-based reversed ME are also specified as pK^a_a, their nature is somewhat different. The pH values refer to the bulk aqueous buffer solutions, which are then dispersed and scattered in the form of "water pools". Additionally, the locus of the dye species is *a priori* not so clear.

Resolving of Absorption Spectra of H_2R and HR^- Species. The molar absorptivities of H_2R and HR^- forms were obtained together with ionization constants. For final refinement, the E_{HR^-} values were then recalculated within the whole visible region, using the pH range of maximal yield of the sought species in solutions ($pK_{a1} \le pH \le pK_{a2}$):

$$E_{HR^-} = E + h(K_{a1})^{-1}(E - E_{H_2R}) + h^{-1}K_{a2}(E - E_{R^{2-}}) \quad (7)$$

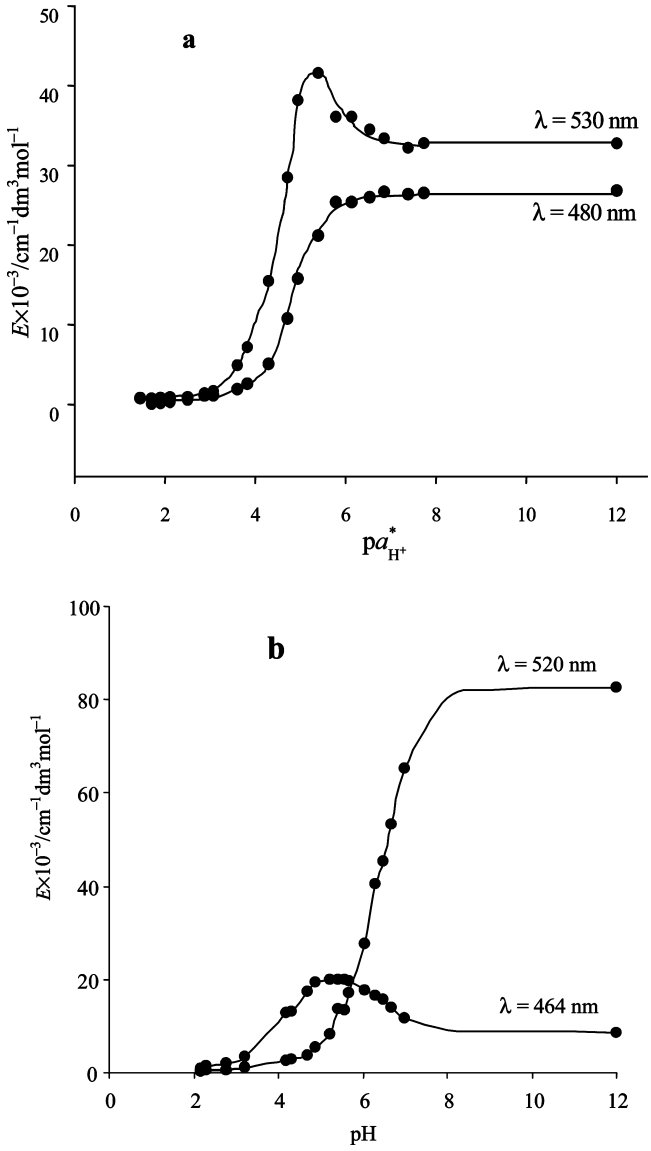


Figure 3. Absorbance of the dyes versus $pa^*_{H^+}$ or pH in 50 mass % aqueous ethanol, $I = 0.05$ M (NaCl + buffer or HCl), except solutions with pH ≤ 1.3 (2,4,5,7-tetrafluorofluorescein, 9.94×10^{-6} M (a)), in direct ME benzene−pentanol-1−CTAB−water, $I = 1.0$ M KCl (3′,4′,5′,6′-tetrafluorofluorescein, 8.00×10^{-6} M (b)). The wavelengths are indicated near the curves; $t = 25$ °C.

In most cases, the spectra of molecular forms of H_2R were determined directly within the pH region of predominance of these species. For dyes I and III in aqueous solutions, the E_{H_2R} values were calculated conjointly with other parameters. In nonaqueous systems, these values for all the dyes under study are low owing to increase in the fraction of the lactonic tautomer **4**. Therefore, small variations in the E_{H_2R} values do not influence the calculated pK_{a1} and pK_{a2} values. However, even traces of intensely colored species (HR^- and H_3R^+) may strongly influence the spectra measured in the pH region of H_2R predominance. In order to remove the ionic contributions, the following E_{H_2R} verification should be carried out:

$$E_{H_2R} = E + h(K_{a0})^{-1}(E - E_{H_3R^+}) + h^{-1}K_{a1}(E - E_{HR^-}) \quad (8)$$

Thus singled out absorption spectra of neutral and ionic species of various fluorescein dyes in different media are presented in Figure 4. The spectra of the rest of the systems studied are available in the Supporting Information.

The numerical characterization of absorption bands is collected in Tables 1−4.

The thermodynamic pK_a values for dye I in water agree with those reported by Alvarez-Pez and co-workers spectrophotometrically:[21] 1.016 ± 0.007, 3.610 ± 0.010, and 4.688 ± 0.004. In their experiments, the ionic strength was $I \leq 0.1$ M.[35]

Discussion

Medium Effects on Going from Water to Aqueous Ethanol. When going from water to 50 mass % aqueous ethanol, the increase in the thermodynamic pK_{a1} and pK_{a2} values was registered, while the pK_{a0} values decreased. The medium effect, ΔpK_a, i.e., the difference between the pK_a in the given medium and that in water, pK_a^w, can be expressed in terms of activity coefficients of transfer of the corresponding *i* species from water to the given solvent, ${}^w\gamma_i^s$ (eq 9).

$$\Delta pK_a = pK_a - pK_a^w = \log {}^w\gamma^s_{H^+} + \log \frac{{}^w\gamma^s_{H_{j-1}R^{z-1}}}{{}^w\gamma^s_{H_jR^z}} \quad (9)$$

However, the forms H_2R and HR^- are in fact equilibrium mixtures of tautomers. Hence, as it was already demonstrated within the course of study of different fluorescein dyes in different organic and water−organic solvents,[2,22,24,29,33,39] the interpretation of the pK_a values demands the elucidation of the tautomeric equilibria state (Chart 2). The latter can be deduced from the absorption spectra of molecular and ionic species, with understanding that the lactone **4** is colorless owing to the sp^3-hybridization of the central carbon atom (see Introduction).

The Main Extrathermodynamic Assumption. Our approach is based on the well-known negligibility of the influence of carboxylic group ionization on the principal band in the visible. Therefore, it becomes possible to consider the vis-spectra of **1** (the H_3R^+ cation) and **5** species as spectra of the zwitterionic **2** and quinonoidal **3** tautomers, respectively.[2,22,24,29,33] For example, the absorption band of an HR^- monoanion of fluorescein, existing as **5** tautomer, practically coincides with those of neutral species of ethylfluorescein and 6-hydroxy-9-phenylfluorone (HR, Chart 3), respectively, with $COOC_2H_5$ and H instead of the COO^- group.[22b−d,33]

On the other hand, the anions **6** and **7** possess similar absorption spectra, but the long-wavelength bands are sharp, and in this case it is possible to observe a distinct small blue shift on going from mono- to dianion. For example, in 50% aqueous ethanol, λ_{max} values for fluorescein dianion (**7**) and 6-hydroxy-9-phenylfluorone monoanion (R^-, Chart 3) are equal to 495 and 503 nm, respectively. Such a shift is also predicted by quantum-chemical calculations.[36]

This regularity has been numerously observed with eosin, erythrosin, Rose Bengal, and other 2,4,5,7-tetrahalogen derivatives of fluorescein in different solvents.[2a,22a,c,d,33] Hence, if there is a band in the HR^- spectrum, which is red-shifted against that of R^{2-}, this can be considered as a criterion of tautomer **6** existence.

Taking into account that the lactone does not contribute to the absorption in the visible, it can be written for the given wavelength:

$$E_2\alpha_2 + E_3\alpha_3 = E_{H_2R} \quad (10)$$

TABLE 1: Parameters of Ionic Equilibria of 2,7-Difluorofluorescein in Various Systems (25 °C)

				λ_{max}/nm ($E_{max} \times 10^{-3}$/cm^{-1} M^{-1})			
system	pK_{a0}	pK_{a1}	pK_{a2}	H_3R^+	H_2R	HR^-	R^{2-}
water, $I \rightarrow 0$	1.03 ± 0.01	3.82 ± 0.02	5.07 ± 0.01	436 (48.4)	450 (20.1); 476 (20.9)	452 (29.6); 472 (31.0)	490 (82.4)
50 mass % ethanol, $I \rightarrow 0$	—[a]	5.54 ± 0.02	6.12 ± 0.03	—	454 (4.8); 482 (4.3)	482 (29.1); 502 (37.2)	498 (88.6)
direct CTAC-based ME, 1.0 M KCl[b]	—[a]	4.65 ± 0.03	5.22 ± 0.04	—	460 (5.83); 492 (5.37)	510 (62.32)	502 (95.29)
reversed AOT-based ME, 0.05 M NaCl[b,c]	—[a,d]	4.43 ± 0.05	5.68 ± 0.04	—	464 (5.8); 484 (6.2)	496 (42.4)	492 (68.4)

[a] The pK_{a0} value is markedly shifted toward the acidic region as compared with the "aqueous" value. [b] "Apparent" pK_a^a values instead of pK_a [c] Ionic strength in water pools. [d] In the acidic region, the solutions became turbid.

TABLE 2: Parameters of Ionic Equilibria of 2,4,5,7-Tetrafluorofluorescein in Various Systems (25 °C)[a]

			λ_{max}/nm ($E_{max} \times 10^{-3}$/cm^{-1} M^{-1})		
system	pK_{a1}	pK_{a2}	H_2R	HR^-	R^{2-}
water, $I \rightarrow 0$	3.65 ± 0.05	4.12 ± 0.04	470 (5.6); 496 (4.9)	514 (52.4)	508 (78.1)
50 mass % ethanol, $I \rightarrow 0$	4.94 ± 0.01	5.63 ± 0.03	470 (0.9); 498 (0.7)	522 (83.8)	514 (89.7)
direct CTAC-based ME, 1.0 M KCl[b]	2.85 ± 0.04	4.33 ± 0.02	484 (1.23)	528 (80.62)	520 (91.13)

[a] The pK_{a0} values of this dye are in the strongly acidic region. [b] "Apparent" pK_a^a values instead of pK_a.

TABLE 3: Parameters of Ionic Equilibria of 3′,4′,5′,6′-Tetrafluorofluorescein in Various Systems (25 °C)

				λ_{max}/nm ($E_{max} \times 10^{-3}$/cm^{-1} M^{-1})			
system	pK_{a0}	pK_{a1}	pK_{a2}	H_3R^+	H_2R	HR^-	R^{2-}
water, $I \rightarrow 0$	1.22 ± 0.11	4.02 ± 0.24	6.41 ± 0.03	452 (49.1)	460 (26.9); 488 (20.8)	464 (29.5); 492 (29.8)	508 (85.6)
50 mass % ethanol, $I \rightarrow 0$	−0.05[a]	4.82 ± 0.02	7.30 ± 0.01	458 (53.5)	466 (2.2); 494 (1.7)	466 (34.7); 494 (28.6)	514 (93.0)
direct CTAC-based ME, 1.0 M KCl[b]	—[c]	4.20 ± 0.02	6.45 ± 0.01	—	460 (0.83); 500 (0.40)	464 (23.05); 496 (19.12)	520 (82.57)
reversed AOT-based ME, 0.05 M NaCl[b,d]	—[e]	4.12 ± 0.02	7.18 ± 0.02	—	466 (14.8); 492 (11.2)	466 (29.4); 500 (29.9)	510 (83.7)

[a] In $pH_c = -\log C_{HCl}$ scale of acidity. [b] "Apparent" pK_a^a values instead of pK_a. [c] Shifted toward the acidic region. [d] Ionic strength in water pools. [e] In the acidic region, the solutions became turbid.

TABLE 4: Parameters of Ionic Equilibria of 2,7,3′,4′,5′,6′-Hexafluorofluorescein in Various Systems (25 °C)[a,b]

			λ_{max}/nm ($E_{max} \times 10^{-3}$/cm^{-1} M^{-1})		
system	pK_{a1}	pK_{a2}	H_2R	HR^-	R^{2-}
50 mass % ethanol, $I \rightarrow 0$	3.68 ± 0.02	5.40 ± 0.01	466 (2.5); 494 (2.2)	466 (20.1); 494 (17.8)	518 (81.7)
direct CTAC-based ME, 1.0 M KCl[c]	2.88 ± 0.03	4.31 ± 0.03	472 (2.55); 500 (2.15)	468 (24.72); 496 (24.50)	520 (88.02)
reversed AOT-based ME, 0.05 M NaCl[c,d]	2.24 ± 0.02	3.66 ± 0.02	470 (3.4); 494 (3.6)	466 (30.6); 494 (30.4)	510 (82.6)

[a] The solubility of neutral species, H_2R, of this dye in water is too small to obtain exact pK_a^w values. [b] The pK_{a0} values of this dye are in the strongly acidic region. [c] "Apparent" pK_a^a values instead of pK_a. [d] Ionic strength in water pools.

Here α represents the fractions of corresponding tautomers in the equilibrium mixture of H_2R brutto-formula. Evidently, $\alpha_2 + \alpha_3 + \alpha_4 = 1$ and $K_T = \alpha_4/\alpha_3$, $K'_T = \alpha_2/\alpha_3$. For unsubstituted fluorescein in aqueous solutions, the fractions were found to be $\alpha_2 = 0.218$, $\alpha_3 = 0.111$, $\alpha_4 = 0.671$.[37] Such approach was also used for 2,7-difluorofluorescein.[35] Accordingly, for the monoanion absorptivity at a fixed wavelength, eq 11 is valid.

$$E_5\alpha_5 + E_6\alpha_6 = E_{HR^-}; \quad K_{T_x} = \alpha_6/\alpha_5 \qquad (11)$$

Tautomerism of Molecules and Ions in Water and in Aqueous Ethanol. In aqueous media, the neutral species of dye IV is not soluble enough to determine the H_2R spectra and pK_a. In the case of dyes I and III (Figure 1, Tables 1 and 3), the monoanionic species HR^- exist predominantly as the carboxylate tautomer **5**; there is no sign of a band with $\lambda_{max} > \lambda_{max}$(of R^{2-}).

In contrast, the HR^- ion of dye II in water (Table 2) exhibits a spectrum rather similar to that of R^{2-}, which gives evidence for the existence of phenolate tautomer **6**. However, the E_{max} value of HR^- is 1.5 times lower than that of R^{2-}. This can be a result of incomplete shift of the tautomeric equilibrium (**5** ⇄ **6**) toward the right. The fractions of the two monoanionic tautomers were estimated by equating the E_{max} value of tautomer **6** to that of R^{2-} and the molar absorptivity of tautomer **5** at 514 nm to that of the HR^- of dye III: $\alpha_5 = 0.35$, $\alpha_6 = 0.65$.

In 50% aqueous ethanol, the HR^- form of dye III (Figure 4a, Table 3) exists as **5**, and that of dye II (Table 2) exists as **6** tautomer. In the last case, the E_{max} value of HR^- monoanion is

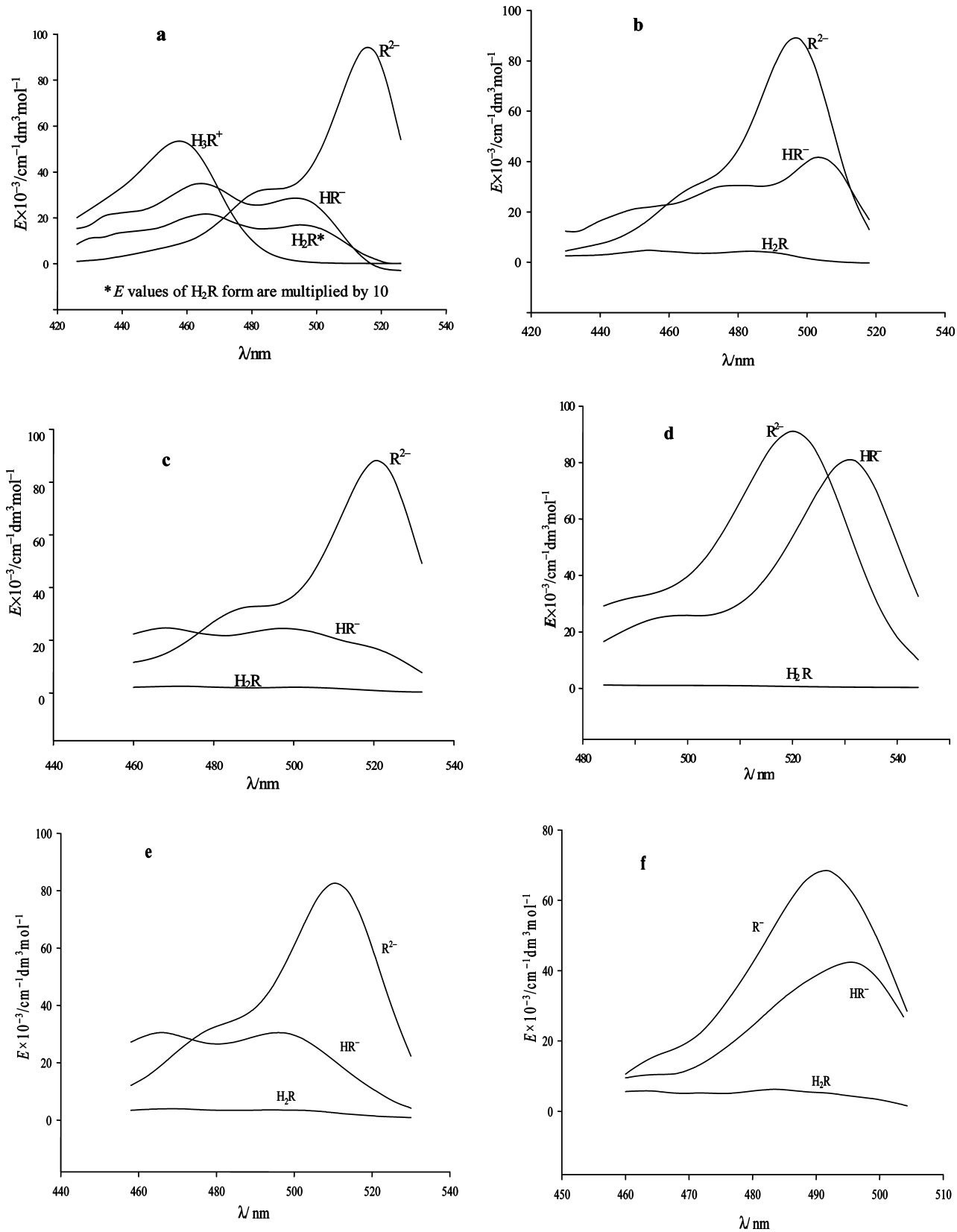
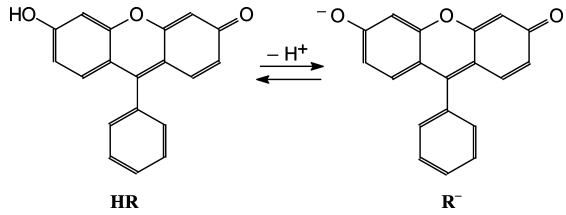


Figure 4. Absorption spectra of neutral and ionic forms of the fluorescein fluoroderivatives in 50 mass % aqueous ethanol, $I = 0.05$ M (NaCl + buffer or HCl), except the solution of H_3R^+ species (3′,4′,5′,6′-tetrafluorofluorescein (a), 2,7-difluorofluorescein (b)), in direct ME benzene−pentanol-1−CTAB−water, $I = 1.0$ M KCl (2,7,3′,4′,5′,6′-hexafluorofluorescein (c), 2,4,5,7-tetrafluorofluorescein (d)), and in AOT-based reversed ME, $I =$ 0.05 M (NaCl + buffer or HCl) (2,7,3′,4′,5′,6′-hexafluorofluorescein (e), 2,7-difluorofluorescein (f)); $t = 25$ °C.

only 7% lower than that of R^{2-} species, and this difference is too small to be confidently attributed to the presence of the carboxylate tautomer **5**.

In the case of dye I in 50% ethanol (Figure 4b, Table 1), contrary to aqueous solution, a clear indication of tautomer **6** becomes evident. The fractions of tautomers **5** and **6** were

CHART 3: Neutral Molecule and Monoanion of 6-Hydroxy-9-phenylfluorone



estimated as described previously:[2b,22d] $\alpha_5 = 0.62$ and $\alpha_6 = 0.38$, respectively. However, this is not the case with dye IV (Table 4), where the introduction of four fluorine atoms into the phthalic residue destabilizes the phenolate tautomer **6** and thus results in the carboxylate tautomer **5** predominance.

The fractions of the neutral form of dye III in various solvents were estimated by equating the E_2 and E_3 values to $E_{H_3R^+}$ and E_{HR^-} values; for this dye, they are in fact E_1 and E_5, respectively. However, no distinct signs of zwitterionic species **2** were detected in the H_2R spectra even in water, contrary to the case of 3′,4′,5′,6′-tetrachlorofluorescein.[38] Thus, only the α_3 values were calculated (Table 5).

The same algorithm was utilized for dye I in water and for dye IV in aqueous ethanol. However, for dye I in nonaqueous media and for dye II in any solvent, there is no internal standard for the E_3 value. Therefore, the maximal E_{HR^-} value of dye III (in water) and an average value between that of dyes III and IV in 50% ethanol were used. Thus obtained α_3 values are compiled in Table 5.

Any reliable evidence for zwitterion **2** existence in aqueous solutions of fluorinated fluoresceins is absent. Even more so, it was not found in aqueous ethanol. Indeed, on going from water to a water−organic media, the fraction of the zwitterion **2** is known to decrease.[2,22,24,33,37] The neutral species of 2,7-difluorofluorescein and 2,4,5,7-tetrafluorofluorescein appear in the form of equilibrium mixtures (**3** ⇄ **4**). Naturally, introduction of halogen atoms in ortho position to hydroxy groups destabilize the zwitterions **2**. The tautomeric equilibrium (**3** ⇄ **4**) is strongly shifted toward the right.

The Data for Cationic MEs. The quantities given in Table 5 were estimated in the same way; the monoanions of dyes III and IV are of **5** type, while, in the case of 2,7-derivative, the fractions of **5** and **6** are commensurable: 0.37 and 0.63, respectively. Note that, for 2,7-dichlorofluorescein in the same colloid system, the shift of the (**5** ⇄ **6**) equilibrium toward the right is much more expressed.[24b] The band of the HR^- ion of dye II is red-shifted against that of R^{2-}; the E_{max} of the monoanion is only 15% lower as compared with that of dianion, which can be a result either of the existence of a small fraction of **5** tautomer or of peculiarity of the tautomer **6** long-wavelength band.

Concluding this analysis, it should be mentioned that the character of tautomerism of dye III is similar to that of 3′,4′5′,6′-tetrachloro- and 3′,4′5′,6′-tetrabromofluorescein.[22d,24b,37] The sole exception is the high fraction of the tautomer **3** and the absence of essential amounts of tautomer **2** in aqueous solutions of the fluorine derivative. The nature of this phenomenon cannot now be specified; the pK_{a0} and pK_{a1} values of dye III in water are excluded from further discussion. On the other hand, for dyes with halogen substituents in resorcin rings, the shift of the tautomeric equilibrium (**5** ⇄ **6**) toward the right is sometimes less expressed in the case of fluoro derivatives as compared with bromo and iodo derivatives.

Having the α values, it is possible to evaluate the so-called microscopic ionization constants of the dyes in various media.

Microscopic Ionization Constants. The relationship between the experimentally determined pK_a values and the indices of the microscopic ionization constants, p*k*, can be easily derived from the detailed scheme of protolytic equilibria (Chart 2):

$$pK_{a0} = pk_{0,OH} + \log \alpha_3 \quad (12)$$

$$pK_{a1} = pk_{1,COOH} - \log \alpha_3 + \log \alpha_5 = pk_{1,OH} - \log \alpha_3 + \log \alpha_6 \quad (13)$$

$$pK_{a2} = pk_{2,COOH} - \log \alpha_6 = pk_{2,OH} - \log \alpha_5 \quad (14)$$

Utilizing these equations and the α values estimated above, we managed to evaluate the p*k* quantities, which are gathered in Table 5. Just these microscopic ionization constants should be used to rationalize the substituent and solvent effects.

The introduction of two fluorine atoms in the 2 and 7 positions of the unsubstituted dye decreases the $pk_{1,OH}$ value by 1.8 units in 50% aqueous ethanol, while four F atoms in the 2,4,5, and 7 positions decrease it by 3.2−3.4 units in water and 50% ethanol. On going from 2,7-difluoro- to 2,4,5,7-tetrafluorofluorescein, the $pk_{1,OH}$ value drops by 1.6−2.6 units in 50% ethanol and in cationic ME.

TABLE 5: The Fractions of Tautomer 3 and Indices of Microscopic Ionization Constants in Various Media

medium	substituted fluorescein	α_3	$pk_{0,OH}$	$pk_{1,OH}$	$pk_{2,OH}$	$pk_{1,COOH}$	$pk_{2,COOH}$
water	unsubstituted[a]	0.11	3.10	6.3	6.80	3.49	—
	III (3′,4′,5′,6′-tetrafluoro-)	(0.8)	(1.3)	—	6.41	(3.9)	—
	I (2,7-difluoro-)	0.68	1.20	4.8[b]	5.07	3.65	—
	II (2,4,5,7-tetrafluoro-)	0.181	—	3.09	3.66	3.36	3.93
50% ethanol	unsubstituted[a]	0.032	2.43	6.8	7.66	5.32	—
	(III) 3′,4′,5′,6′-tetrafluoro-	0.0616	1.16	—	7.30	3.61	—
	(IV) 2,7,3′,4′,5′,6′-hexafluoro-	0.124	—	—	5.40	2.77	—
	(I) 2,7-difluoro-	0.177	—	5.00	5.70	5.21	5.91
	(II) 2,4,5,7-tetrafluoro-	0.0312	—	3.43	—	—	5.63
CTA-ME, 1 M KCl	unsubstituted[a]	0.052	≈1.2	—	6.50	4.56	—
	(III) 3′,4′,5′,6′-tetrafluoro-	0.0285	—	—	6.45	2.65	—
	(IV) 2,7,3′,4′,5′,6′-hexafluoro-	0.0953	—		4.31	1.86	—
	(I) 2,7-difluoro-	0.244	—	4.24	4.78	4.47	4.78
	(II) 2,4,5,7-tetrafluoro-	0.0515	—	1.56	—	—	4.33

[a] The data for unsubstituted fluorescein are taken from refs 24b, 37, and 38. [b] The value of the methyl ester of 4′-carboxy Pennsylvania Green is from ref 1d.

The $pk_{2,OH}$ value of the parent dye fluorescein decreases by 1.7−2.0 units after introducing two fluorine atoms in the 2 and 7 positions, while 3′,4,′5′,6′-substitution results in a much smaller decrease, 0.5 or even less, probably due to the inductive effect.

The $pk_{2,OH}$ values are 0.5−0.9 units higher as compared with the corresponding $pk_{1,OH}$ values of the dyes with the same substituents. Accordingly, the $pk_{2,COOH}$ values are 0.3−0.7 units higher than the $pk_{1,COOH}$ values.

On going from 2,7-difluoro to 2,4,5,7-tetrafluoro derivative, the $pk_{1,COOH}$ decreases by 0.3−0.5 units due to the inductive effect of the remote F-substituents. In contrast, the $pk_{1,COOH}$ value of dye IV in the media studied is 2.4−2.6 units lower as compared with that of dye I, because four F atoms are introduced directly in the phthalic acid moiety. In 50% aqueous ethanol and in cationic ME, the $pk_{1,COOH}$ value of dye III is 1.7−2.0 units lower as compared with that of fluorescein, but in water the corresponding difference is much smaller (Table 5). This fact, together with the state of the tautomeric equilibria of the molecular species of dye III, is the sole point that remains unclear in the above discussion.

The $pk_{0,OH}$ value of fluorescein in water drops from 3.10 to 1.20 as a result of the introduction of two F atoms in the 2 and 7 positions. Somewhat surprisingly, the same effect displays four fluorine atoms in the phthalic acid residue. In 50% aqueous ethanol, the difference becomes smaller: $pk_{0,OH} = 2.43$ for fluorescein and 1.16 for the 3′,4′,5′,6′-tetrafluoro derivative (III).

Medium Effects for Microscopic Ionization Constants. Now it becomes clear that the changes in the pK_a values declared by eq 9 can be rationalized by using eqs 12−14 and separately estimating the Δpk and α values. So, the shift of the tautomeric equilibrium of the neutral species toward the lactone **4** results in pK_{a1} increase. On the other hand, the character of medium effects for microscopic ionization constants, Δpk, can be used for confirmation of the adequacy of the general equilibrium scheme. Indeed, on addition of ethanol to water, the strength of cationic acids can even increase, while the Δpks of neutral and anionic acids are as a rule positive. Normally, the Δpk_{COOH} values are higher as compared with Δpk_{OH}.[2b,22d,24a,30] In the case under study, the $\Delta pk_{0,OH} = -0.67$ value was observed for fluorescein, while the $\Delta pk_{1,OH}$ and $\Delta pk_{2,OH}$ values of some dyes (Table 5) vary within the ranges of 0.34−0.50 and 0.63−0.89, correspondingly. The $\Delta pk_{1,COOH}$ and $\Delta pk_{2,COOH}$ values vary from 1.56 to 1.83. Hence, the Δpk values seem to be reliable.

The Relation between the Tautomerization Constant and the Microscopic Ionization Constants. This can be derived from the scheme given in Chart 2.

$$\log K_{T_x} = pk_{2,COOH} - pk_{2,OH} = pk_{1,COOH} - pk_{1,OH} \quad (15)$$

This equation is of key character for understanding the tautomerism of fluorescein dyes. The introduction of halogen atoms into the xanthene moiety increases the acidity of hydroxy groups, thus decreasing $pk_{1,OH}$ and $pk_{2,OH}$ and increasing the K_{T_x}values. Therefore, the monoanions of eosin and erythrosin always exist as tautomers of **6** type, while in the case of fluorescein, the monoanion **5** predominates. The fluorine atoms exhibit somewhat smaller effect, and in the case of dye II in water, the admixture of tautomer **5** cannot be excluded (see above), and the $K_{T_x} = \alpha_6/\alpha_5 = 1.9$ value seems to be reliable. For 2,7-dichlorofluorescein in water, the HR^- species exists mainly as tautomer **5**;[38] the same picture was observed for dye I. The state of tautomeric equilibria is somewhat changed on going from water to organic solvents, because of different medium effects for carboxylic acids and phenols. Indeed, owing to more expressed increase in pk_{COOH} values as compared with that in pk_{OH}, the (**5** ⇄ **6**) equilibrium shift toward the right takes place in line with eq 15; $K_{T_x} = 0.61$. Moreover, even for the unsubstituted fluorescein, we have recently fixed the appearance of the extremely small amount of tautomer **6** in DMSO, at appropriate acidity.[39]

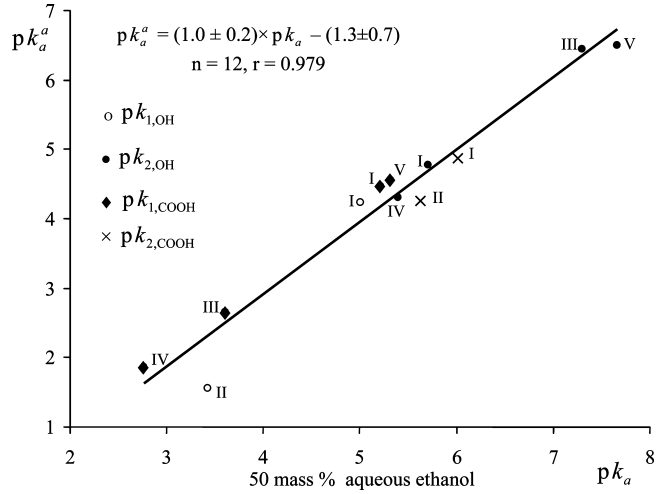


Figure 5. The dependence of pK_a^a values of fluorinated fluoresceins in direct ME benzene−pentanol-1−CTAB−water at high bulk ionic strength (1.0 M KCl) versus pK_a in 50 mass % aqueous ethanol (V = unsubstituted fluorescein); $t = 25$ °C. For macroscopic constants, the corresponding correlation is as follows: $pK_a^a = (1.0 \pm 0.2) \times pK_a - (0.6 \pm 0.5)$; $n = 10$, $r = 0.938$.

On the other hand, the increase in the acidity of carboxylic group, i.e., the pk_{COOH} decrease, as a result of replacing hydrogen atoms by halogens in the phthalic residue, stabilizes the monoanionic tautomer of **5** type. The predominance of the latter in nonaqueous media under study in the case of the dye IV, despite the shift of the tautomeric equilibrium toward the phenolate tautomer **6** for dye I, demonstrates that the strengthening of the acidity of COOH group overcomes the effect of 2,7-substitution. This effect was first demonstrated in the present study.

Medium Effects in CTAC-Stabilized Benzene-in-Water MEs. The positions of absorption maxima of dye species and the state of tautomeric equilibria in these ME resemble in some features the data obtained in aqueous ethanol. According to the electrostatic model, the apparent value, pK_a^a, under conditions of complete binding of the indicator couple by pseudophase depends on the electrostatic surface potential (electrical potential of the Stern layer), Ψ, of the micelle or microdroplet:[24b,26,31,32]

$$pK_a^a = pK_a^w + \log({}^w\gamma^m_{H_{j-1}R^{z-1}}/{}^w\gamma^m_{H_jR^z}) - \Psi F/(RT \ln 10) \quad (16)$$

Here, ${}^w\gamma_i^m$ is the transfer activity coefficient of the corresponding species from water to the pseudophase, F is the Faraday constant, R is the gas constant, and T is the absolute temperature.

All the pK_a^a values in cationic ME are lower than the corresponding pK_a's in 50 mass % ethanol; the same is the case with the indices of microscopic ionization constants. The plot of pK_a^a versus pK_a has a lower correlation coefficient (0.938) as compared with that of the pk^a versus pk dependence (0.979, see Figure 5).

The probable reason is the interference of the ionization and tautomerism. Therefore, the microscopic ionization constants are used in eq 17:

$$\mathrm{p}k(\text{in 50\% } C_2H_5OH) - \mathrm{p}k^{a}(\text{in ME}) = \log {}^{w}\gamma^{s}_{H^+} + \log \frac{{}^{w}\gamma^{s}_{H_{j-1}R^{z-1}}}{{}^{w}\gamma^{s}_{H_jR^z}} - \log \frac{{}^{w}\gamma^{m}_{H_{j-1}R^{z-1}}}{{}^{w}\gamma^{m}_{H_jR^z}} + \Psi F/(RT \ln 10) \quad (17)$$

The mean value of $\{\mathrm{p}k(\text{in 50\% } C_2H_5OH) - \mathrm{p}k^{a}(\text{in ME})\}$ is 1.04 ($n = 12$). According to the so-called tetraphenylborate assumption, the $\log {}^{w}\gamma^{s}_{H^+}$ in 50 mass % aqueous ethanol is negative (−0.67).[40] The $\Psi = +53$ mV value was determined using *N,N′*-di-*n*-octadecylrhodamine as the interfacial indicator, following the procedure described earlier.[32] Thus, the following relation can be obtained:

$$\log \frac{{}^{w}\gamma^{s}_{H_{j-1}R^{z-1}}}{{}^{w}\gamma^{s}_{H_jR^z}} - \log \frac{{}^{w}\gamma^{m}_{H_{j-1}R^{z-1}}}{{}^{w}\gamma^{m}_{H_jR^z}} = 0.82 \quad (18)$$

Within the framework of the extrathermodynamic assumptions utilized, a conclusion can be made about the somewhat more "water-like" nature of the interface of the CTAC-stabilized microdroplets as compared with 50 mass % aqueous ethanol.

On the other hand, for the latter mixed solvent, the E^{N}_{T} value equals 0.748,[41] while the value $\lambda_{max} = 539$ nm, determined by us for the Reichardt's solvatochromic indicator, 2,6-diphenyl-4-(2,4,6-triphenylpyridinium-1) phenolate, corresponds to an $E^{N}_{T} = 0.689$ value. This is close to the data obtained for other cationic surfactant-based MEs and micelles.[26] It is generally accepted that this indicator is also embedded into the interfacial pseudophase/water region. Hence, judging by the E^{N}_{T} parameter, this surface layer is less polar (and thus less water-like) as compared with the above water−ethanol mixture.

The Character of Location of the Dye Species in AOT-Stabilized Water-in-Octane MEs. The protolytic behavior of the dyes in AOT-stabilized water pools, dispersed in *n*-octane ($W = 20$), is of especial interest. It allows one to shed light on the properties of liquid aqueous media under conditions, "when is water not water".[25b]

Strictly speaking, the $\mathrm{p}K^{a}_{a}$ values are hardly comparable with those in "normal" (bulk) water, $\mathrm{p}K^{w}_{a}$, because the Gibbs energy of proton transfer from bulk water to the water pools is unknown.

Another problem is connected with the locus of the dye species. In order to clarify the partition of dyes in the colloidal system, we studied their distribution between bulk water and *n*-octane. At high pH values of the aqueous phase, the extraction of Na_2R species appeared to be negligible, in line with the idea of R^{2-} location within the dispersed pseudophase of reversed ME. Indeed, in the AOT-stabilized ME, the λ_{max} values of the R^{2-} species of the dyes practically coincide with those in normal water (Tables 1, 3, and 4).

However, although the sodium salts of the dyes are octane-insoluble, the anionic species can be situated either within the pools or in the ionic layer of AOT molecules. And really, the fluorescence intensity of R^{2-} differs principally, thus giving evidence for another, probably more rigid, microenvironments of the dyes as compared with pure water. The decrease in emission intensity was observed along with *W* decrease ($W =$ 15, 10, and 5), which is probably caused by fluorescence quenching owing to high local concentration of Na^+.[41] In Figure 6, a typical example is given. For dyes I, III, and IV, the ratio of emission intensities in reversed ME ($W = 20$) and in water are equal to 1.40, 1.77, and 1.47, respectively.

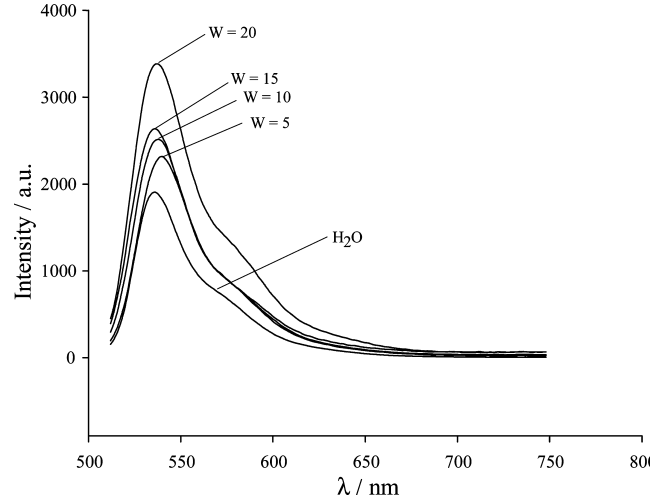


Figure 6. Fluorescence spectra of R^{2-} form of 3′,4′,5′,6′-tetrafluorofluorescein in AOT-based reversed ME at various *W* values; the initial pH value of the aqueous phase equals 12 (diluted NaOH), $I = 0.05$ M (NaCl + NaOH), and *t* around 20 °C.

Note, that in these experiments the concentrations of the dyes used were the same as those in $\mathrm{p}K^{a}_{a}$ determination (for the aforementioned dyes, the initial concentration in dispersed water was 1.7×10^{-4}, 5.61×10^{-4}, and 1.74×10^{-3} M, respectively). Therefore, the λ_{max} of fluorescence spectra in water are 2−7 nm higher than in extremely diluted dye solutions in the same solvent.

In addition, the ability of the neutral dye molecules to be dissolved in the bulk *n*-octane phase must be clarified. Our experiments revealed, that at pH values of 1.5−2.0, corresponding to the maximal conversion of the dyes into the H_2R forms in water, the concentration of dye I in aqueous phase decreases only by ca. 0.5%, while other dyes are concentrated in the water/*n*-octane interface region. Thus, the possibility of transfer of neutral species of the fluorinated fluoresceins from microdroplets into the *n*-octane phase of the colloidal system is doubtful. On the other hand, the molar absorptivities of molecular forms of dyes I and III are substantially lower in the reversed ME than in pure water, thus indicating the shift of the tautomeric equilibrium toward the colorless lactone (Tables 1, 3, and 4). This indicates the specific properties of water pools as compared with "normal" water.

It is more difficult to examine the partition of monoanionic species in the reversed ME by modeling it by direct extraction experiments. However, the lack of both Na_2R and H_2R extraction into *n*-octane allows deducing the similar effect for the NaHR forms. In the reversed ME, the HR^- ions of dyes III and IV exist in the form of **5** tautomers, as in other solvent systems studied. However, for dye I, the (**5** ⇄ **6**) equilibrium shift toward the right is distinctly stronger as compared with aqueous solutions (Table 1).

The Structure of AOT-Stabilized Water Pools. The number of publications devoted to the structure and properties of the AOT-based reversed ME is huge. According to the recognized viewpoint, the water pool inside AOT-stabilized ME is nonuniform.[42−47] For example, both Raman[44] and infrared[45]

spectra allow one to distinguish two water domains[44] or "regions".[45] On the basis of NMR spectra, Maitra[43] says there are "three distinct states of water - bound water, trapped water, and apparently free water". Oliveira et al.[47] also state that the dispersed water can be represented by three populations: "trapped water molecules, localized up to 0.1 nm from the AOT interface, an intermediate layer of bound water, localized from 0.3 to 0.6 nm from the AOT interface, and the free or bulk water, localized at least 0.6 nm away from the AOT interface". Microcalorimetric investigation of AOT-solubilized water pools in *n*-octane also allow one to reveal three states of dissolved water.[46]

In *iso*-octane and *n*-heptane, at AOT concentrations of 0.025−0.5 M and *W* values around 20, the reported hydrodynamic radii of the microdroplets are 3.27,[48] 3.4,[49] 3.6,[50] 4.0,[51] 4.5,[25a] and 5.0.[52] Maitra[43] reported the values of 4.4 and 4.6 nm in *iso*-octane and cyclohexane, respectively. In these solvents, the aggregation numbers of AOT are 302 and 257, while the radii of the water core are 3.5 and 3.3 nm, respectively.[43] This agrees in outline with the data of Menger et al. for *n*-octane[53] and *n*-heptane.[54]

Thus, the volume of the water pool is at least 150 nm^3, while that of apparently free water is ca. 80−100 nm^3. However, not only bound and trapped, but also the apparently bulk water inside water pools exhibits some peculiarities. So, using a highly charged decavanadate anion as a probe,[25b] it was recently shown that some properties of water in the middle of polar core of the large reversed ME differ from those of bulk water; in particular, a gradient of pH exists inside such water pools of ME.[25b] Earlier it was shown that compressibility parameters of water, confined in the pools, never reach the properties of common bulk water.[55]

Taking into account the AOT and fluorofluorescein working concentrations and the aggregation numbers of the surfactant (about 250−300), one can calculate that there is one dye molecule for every 80−100 water pools. Using the data reported by Flamigni,[56] the fluorescein dianion R^{2-} goes in a $0.95 \times 1.1 \times 0.5$ $nm^3 = 0.52$ nm^3 cage. Dutt[57] gives the value of 0.64 nm for the axial length. The size of the fluorinated fluoresceins ions (molecules) is somewhat larger. However, under the conditions of our experiments, the isolated dye occupies in any case less than 1% of the total volume of water pool. We assume that the presence of dyes and buffer components does not affect the size of microdroplets.

Medium Effects and Tautomerism in AOT-Stabilized Water-in-Octane MEs. In fact, the emission intensity of R^{2-} dianions and the tautomerism of H_2R molecules and (in the cases of I) HR^- monoanions demonstrate the specificity of the interior of nanosized droplets. Taking into account modern concepts of AOT-stabilized reversed ME structure, two limiting models can be proposed for rationalizing the results obtained. They are as follows: (i) the dye species are situated within the central region of water microdroplet, and the data presented give evidence for specific properties of the apparently bulk water; (ii) the dye locus is close to the interfacial water/AOT layer.

The first model seems to be more realistic for anionic species, taking into account the expressed negative interfacial potential.[48] However, it is known that, despite electrostatic repulsion between AOT head groups and negatively charged species, the latter spend significant time penetrating into the hydrophobic portion of the reversed MEs.[25a] Moreover, such effect was recently obtained for fluorescein dianion R^{2-} by means of ^1H NMR spectroscopy.[58] Even more so, the same can be expected for neutral species.

Therefore, a complicated picture of dye partitioning within the microdroplets can be assumed, which is intermediate between the two limiting models, and comparison between the microscopic ionization constants of the dyes is hindered. In any case, at $W = 20$, the ΔpK_a^a values of the dyes vary from 0.1 to 0.8, thus demonstrating differentiating influence of the microdroplets, despite high water contents. The components of buffer mixtures probably stay within the pools; for example, the partition constant of acetic acid between water and *n*-hexane is 1.43×10^{-3}.[59]

The influence of four fluorine atoms in the phthalic acid residue on the ionization of the OH group of the xanthene moiety demonstrates the $pk_{2,OH}$ values of dyes III and IV: 7.18 and 3.66, respectively. For these dyes, $\alpha_3 = 0.44$, $\alpha_4 = 0.56$, $pk_{1,COOH} = 3.76$, and $\alpha_3 = 0.115$, $\alpha_4 = 0.885$, $pk_{1,COOH} = 1.30$, respectively. For dye I, the fractions of tautomers are $\alpha_3 = 0.20$, $\alpha_4 = 0.80$, $\alpha_5 = 0.40$ and $\alpha_6 = 0.60$; consequently, $pk_{1,OH} = 3.95$, $pk_{1,COOH} = 4.12$ and $pk_{2,OH} = 5.28$, $pk_{2,COOH} = 5.46$. A detailed comparison of these data is difficult, because of probable multiplicity of positions of dyes and even of different species of the fixed dye within the microdroplets.

This feature should be taken into account when examining the AOT-based reversed MEs by using versatile molecular probes.

Conclusions

The pK_{a1} and pK_{a2} values of water-soluble fluorofluoresceins grow on adding 50 mass % ethanol and, in the case of 2,7-difluoro and 3′,4′,5′,6′-tetrafluorofluoresceins, also on going from water to direct CTAC-based ME at high bulk ionic strength and to AOT-based reversed ME. In contrast, the pK_{a0} values decrease on going from water to both types of organized solutions. For more detailed characterization of the different changes in ionization constants, the state of the movable tautomeric equilibria must be taken into account.

While cations H_3R^+ (**1**) and dianions R^{2-} (**7**) always exist as carbocationic and quinonoidal intensively fluorescing species, respectively, three tautomers of neutral forms, H_2R, and two tautomers of monoanions, HR^-, can appear in solution, depending on substituent number and position, as well as on solvent nature.

An expressed shift of the tautomeric equilibria of H_2R from quinonoid molecule (**3**) toward colorless lactone (**4**), was registered on going from aqueous solutions to both aqueous ethanol and ME.

Though the shift of the tautomeric equilibrium (**5** ⇄ **6**) toward the right is sometimes less expressed in the case of fluoro derivatives as compared with bromo and iodo derivatives, the monoanion HR^- of 2,4,5,7-tetrafluorofluorescein exists mainly as a tautomer (**6**) with an ionized hydroxy group and nonionized COOH group, such as the 2,4,5,7-tetrabromo (or iodo) derivatives of fluorescein. In contrast, in the monoanion of 3′,4′,5′,6′-tetrafluorofluorescein, the state of these two functional groups is reversed (carboxylate tautomer of **5** type predominates), as usual for fluorescein and its 3′,4′,5′,6′-tetrachloro (or bromo) derivative. In the case of 2,7-difluorofluorescein, an incomplete shift of the tautomeric equilibrium **5** ⇄ **6** toward the right was observed on going from water to water-containing organic media and both direct and reversed ME. This is in line with the medium effects for phenolic and carboxylic groups, and is confirmed by the microscopic ionization constants calculated using some extrathermodynamic assumptions.

However, in the case of 2,7,3′,4′,5′,6′-hexafluorofluorescein. the strengthening of the acidity of COOH group overcomes the

effect of 2,7-substitution and thus prevents the aforementioned tautomeric shift. This effect, although predictable for fluorescein dyes, was first experimentally revealed in the present study.

The analysis of microscopic ionization constants of the fluorofluoresceins, made by using the so-called tetraphenylborate assumptions for transfer activity coefficients, allows preliminary conclusion that the surface of the CTAC-stabilized microdroplets is more "water-like" as compared with 50 mass % aqueous ethanol. On the other hand, judging by the E_T^N parameter, this interface is less polar (and thus less water-like) as compared with the above water−ethanol mixture.

The results obtained with fluorofluoresceins located in an AOT-based pseudophase indicate that the interior of the latter exerts essential differentiation: the ΔpK_a values vary from 0.1 to 0.8 on going from water to MEs. Such an influence can be caused both by different location of dye species (in the center of water pools, in the hydrophobic portion of the droplets, etc.) and by the specificity of this kind of microenvironment. While the state of tautomeric equilibria of molecules and ions resembles that in nonaqueous media, the positions of absorption maxima of dianionic species, R^{2-}, are close to those in water. Such nonuniform medium effects displayed by AOT-based water-in-oil nanosized droplets should be taken into account when examining them by using different molecular probes.

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Supporting Information Available: The initial experimental data for calculations of pK_a and molar absorptivities of neutral and anionic forms of the dyes are presented in 15 tables. The mixed pK_a values of the dyes in water and 50 mass % aqueous ethanol and the apparent pK_a^a values in ME for each of the solvent systems are compiled in 4 tables. Examples of absorption spectra of the dyes under changing acidity and some typical dependences of molar absorptivities versus pH, as well as emission spectra in AOT-stabilized reversed MEs and IR spectra of solid samples of the dyes are depicted in 31 figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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