

Article

Protolytic Equilibria in Organized Solutions: Ionization and Tautomerism of Fluorescein Dyes and Related Indicators in Cetyltrimethylammonium Chloride Micellar Solutions at High Ionic Strength of the Bulk Phase

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Abstract: Ionic equilibrium of 22 hydroxyxanthenes, including halogen and nitro derivatives of fluorescein, and their thio- and aza analogues, were studied spectrophotometrically in micellar solutions of cetyltrimethylammonium chloride at ionic strength of the bulk phase 4.0 M KCl. This micellar pseudophase is characterized by the electrostatic surface potential of +(15–16) mV and the E_T^N value of 0.623. In the case of dyes bearing the COOH group, colorless lactone is the predominant tautomer of the molecular form H_2R . A new classification of fluoresceins is developed. The dyes were divided into four groups based on the nature of tautomerism of the anions. In the case of the fluorescein type, the monoanions HR^- exist predominantly as “carboxylate” tautomers, with ionized carboxylic and non-ionized hydroxylic group. For the dyes of the eosin type, the situation is opposite, while for the intervening type of compounds, the concentrations of the two tautomers are comparable. Dyes capable of forming lactone anions HR^- were classified as the fourth type. For some of them, even the dianion R^{2-} exists as a lactone. The relationship between the stepwise ionization constants, K_{a1}/K_{a2} , varies from 1.3 to 1.07×10^5 and is determined by the state of tautomeric equilibrium of molecules and ions.

Keywords: fluorescein; xanthene dyes; cationic surfactant micelles; absorption spectra; apparent ionization constants; tautomerism of molecules and anions



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1. Introduction

Micellar surfactant solutions in water are widely used in photochemistry, chromatography, chemical analysis, micellar catalysis, nanoparticle synthesis, etc. Such systems are a popular and very promising type of liquid media. According to Shinoda, they are called organized solutions [1]. It can be even considered that this terminology goes back to the works of Hartley [2].

From colloidal point of view, surfactant micelles, drops of microemulsions (including reverse ones), macromolecules of polymers, aggregates formed by dendrimers, calixarenes, cyclodextrins, etc., are lyophilic, or thermodynamically stable reversible ultramicroheterogeneous dispersions with extremely high specific surface area. Such organized solutions, and among them first of all micelles of self-assembled surfactants, are reduced models of more complicated systems studied in biochemistry and biophysics. Therefore, reactions occurring on the micellar interfaces are of considerable interest from both applied and fundamental points of view [3–6]. In particular, the Stern region of ionic surfactant micelles represents a unique combination of water, high concentration of organic and inorganic electrolytes, hydrocarbon chains, and relatively high electrical charge [6,7]. Variations in the properties of micellar pseudophases can be achieved by modifying solutions of self-assembled surfactants using various additives [8].

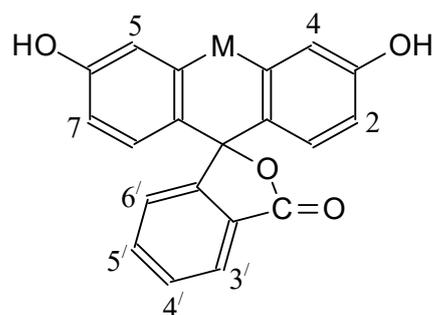
The properties of surfactant micelles are often studied by means of pK_a —probes, i.e., acid-base indicators whose protolytic equilibrium markedly changes when linked by micelles [8–12]. As a rule, such indicators are located in the interfacial layer of surfactant micelles [7–13]. Usually, such a study of the micellar pseudophase is carried out under conditions of extreme dilution of indicators. As a result, there is no more than one indicator molecule (ion) per micelle, which makes it possible to minimize the distortion of the properties of micelles.

Along with other pK_a —probes, dyes of the fluorescein series are used to understand micellar solutions and related systems [7,8,12–17]. Their fluorescence can be used as an additional source of information on the properties of surfactant micelles [18,19].

Fluorescein dyes belong to the most popular molecular probes for biochemical and biomedical research [20–23]. A number of new compounds of this family are proposed for such purpose [24,25] and for creating sensor systems [26,27].

Accordingly, new works appear on the protolytic and spectral properties of both previously known [28–32] and new [25,33–35] or previously little studied [36–42] fluorescein compounds, including data analysis in the gas phase [43–45].

In this paper, we present the results of a spectrophotometric study of fluorescein dyes in cetyltrimethylammonium-based micelles at high ionic strength of the bulk (aqueous) phase. Most of the dyes selected for this study are able to form lactonic molecular structure (Scheme 1):



Scheme 1. Molecular structure of fluorescein and related dyes; M = O; S; NC_2H_5 .

When the nature of the solvent changes, the colorless lactone transforms to two other molecular tautomers, while the protons addition or release lead to the formation of colored ions. Besides, for some of the fluorescein dyes anions can also form lactones. The stepwise acid-base equilibrium is characterized by three constants, K_{a0} , K_{a1} , and K_{a2} , Equations (1)–(3).



It is of interest to study such dye + micelle systems, which are of practical importance and at the same time allow us to expand our knowledge of the properties of dyes. The most widely studied and applied are fluorescein and its halogen derivatives [14,16–23,26–28,30–32,39]. Some new properties of nitro derivatives [15,29,37,38,46,47] and thio analogues of fluorescein [48] have been also described. Besides, these representatives of fluorescein group have been left poorly studied up to now, the more so, that recently a new kind of tautomerism has been revealed for nitro derivatives: the chain-ring tautomerism of anions [47].

In the present study, ionization and tautomerism of a set of hydroxyxanthene dyes, halogen and nitro derivatives of fluorescein, their thio and aza analogues, as well as of some related compounds, are studied in the micellar solutions of cetyltrimethylammonium chloride, CTAC, at ionic strength of the bulk phase 4.00 M KCl. Surfactant concentration was as a rule 0.003 M.

Our goal was to consider a significant number of various compounds belonging to the same class of dyes under identical experimental conditions. As a result, it became possible to classify protolytic equilibria of these dyes from a unified standpoint. On the other hand, the study of these compounds in micellar solutions allows observing the medium influence on the ionization as well as on tautomerism. Micelles are known as reduced models of more complicated organized systems including biological ones. Thus, it is possible to predict the behavior of a variety of fluorescein dyes in such systems.

The key characteristic of the acid-base reaction $\text{HB}^z \rightleftharpoons \text{H}^+ + \text{B}^{z-1}$ in micellar media is the so-called "apparent" $\text{p}K_a$ value, designated as $\text{p}K_a^a$, Equation (4). For the acid-base couple of an indicator $\text{HB}^z/\text{B}^{z-1}$ completely embedded in micellar pseudophase, the $\text{p}K_a^a$ value can be determined using the pH values in the bulk (aqueous) phase, pH_w , and the ratio of equilibrium concentrations of the equilibrium forms of the indicator obtained by a spectroscopic method.

$$\text{p}K_a^a = \text{pH}_w + \log \frac{[\text{HB}^z]_m}{[\text{B}^{z-1}]_m} \quad (4)$$

According to the electrostatic theory, the $\text{p}K_a^a$ value of an indicator completely bound to ionic micelles depends on the electrostatic potential Ψ at the location of the indicator in the micelle, Equation (5). As a rule, the Stern layer is considered the most reliable locus of acid-base indicators.

$$\text{p}K_a^a = \text{p}K_a^w + \log \frac{{}^w\gamma_B^m}{{}^w\gamma_{\text{HB}}^m} - \frac{\Psi F}{2.303RT} \quad (5)$$

Here $\text{p}K_a^w$ is the thermodynamic $\text{p}K_a$ value in water, ${}^w\gamma_i^m$ are the transfer activity coefficients of the corresponding species from water to the pseudophase, Ψ is the electrical potential of the Stern layer, F is the Faraday constant, R is the gas constant, and T is the absolute temperature. The $\text{p}K_a^a$ values of indicators are used for evaluation of the interfacial electrical potentials of surfactant micelles and related objects [8–12,16,17]. The first two terms of the right-hand side are usually equated to the $\text{p}K_a^a$ value of the same indicator in micelles of non-ionic surfactants, where Ψ approaches zero. The $\text{p}K_a^a$ values of the indicators in surfactants micellar systems can correlate with their partition constants between water phase and mitochondria, which is important for understanding the protonophoric mechanism in biomembranes [49].

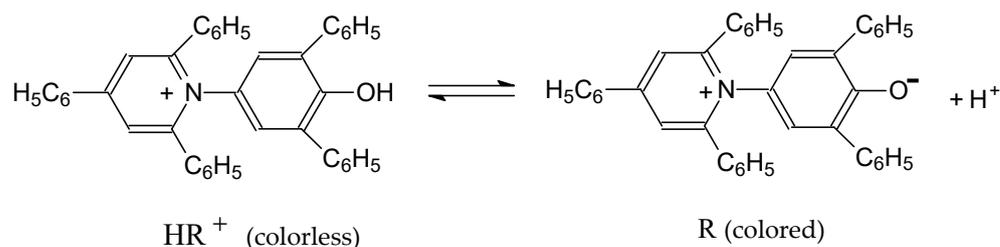
The reasons for the choosing a CTA^+ -based system and high ionic strength of the bulk are as follows:

- (i). Ion exchange between Cl^- ions and components of buffer solutions is negligible;
- (ii). The Cl^- concentrations in the bulk and in the Stern region are coming closer;
- (iii). The Ψ value is getting extremely low;
- (iv). Using of high enough HCl concentrations is possible under condition of constant ionic strength (HCl + KCl);
- (v). In contrast to micelles of non-ionic surfactants (where $\Psi \rightarrow 0$), where compounds of various hydrophobicity can more or less deeply sink in the polyoxyethylene "mantle", in the micelles of chosen type compounds are localized in more compact Stern region, thus being in relatively identical conditions;
- (vi). Our preliminary experiments for several fluorescein derivatives showed that the neutral dye molecules are completely bound by micelle [7,37]. The same is true for dye anions, despite screening of the surface charge;
- (vii). The $\text{p}K_a^w$ s of nitrofluoresceins are rather low (even in 50 mass % ethanol some of $\text{p}K_a$ values are quite small [47]), and while studying them in solutions of cationic surfactants at low bulk ionic strength the $\text{p}K_a^a$ values would have been extremely low and practically unavailable.
- (viii). Analogous systems with 4.00 M NaBr or KBr turned out to be less stable over time and with small temperature fluctuations.

Some data characterizing the CTAC–4.00 M KCl system have already been obtained in this laboratory. Using the indicators *n*-decylfluorescein and *N,N'*-di-*n*-octadecyl rhodamine,

the Ψ value was estimated as $+(15-16)$ mV [8]. It means that the $\Psi F/2.303RT$ value is about 0.26 and the difference between pK_a^a and pK_a^w is caused mainly by the $\log(\gamma_B/\gamma_{HB})$ item. A much higher Ψ value obtained using 2,6-dinitro-4-*n*-dodecyl phenol was explained by assuming some specific interactions [50].

The charge-transfer absorption band of the standard solvatochromic dye 2,6-diphenyl-4-(2,4,6-triphenylpyridinium-1-yl) phenolate, or Reichardt's dye [51], exhibits a maximum at $\lambda_{max} = 562$ nm [8]. Therefore, the normalized polarity parameter, E_T^N , equals 0.623. This means that the micellar interface is much less polar than that of the *n*-dodecyltrimethylammonium chloride (DTAC) micelles in the presence of 4 M NaCl, where λ_{max} is 537 nm and $E_T^N = 0.652$ [52]. This dye is also an acid-base indicator [52], Scheme 2.



Scheme 2. Acid-base equilibrium of 2,6-diphenyl-4-(2,4,6-triphenylpyridinium-1-yl) phenolate.

The pK_a^a values of this dye in different micellar systems can be found in several articles [8,52–54]. In the CTAC–4.0 M KCl and DTAC–4.0 M NaCl systems, $pK_a^a = 8.60 \pm 0.02$ and 8.82 ± 0.03 , respectively [8,52]. In addition, the pK_a^a values in the CTAC–4.0 M KCl system were determined for six fluoresceins [7,37], eight sulfonephthalein dyes [8], and three dinitrophenols [55]. These data will be presented below and compared with the new results obtained in this study.

2. Experimental Section

Cetyltrimethylammonium (=n-hexadecyltrimethylammonium) bromide, CTAB, was from Sigma (St. Louis, MO, USA, 99% purity). KCl, HCl, H₂SO₄, H₃PO₄, and glacial acetic acid were of analytical grade, KCl of high quality was additionally purified through re-crystallization. Na₂B₄O₇ × 10H₂O sample was obtained by means of re-crystallization of borax. Organic solvents were purified using accepted procedures. Acetate and phosphate buffer solutions were obtained by mixing required amounts of stock acid solutions and standard sodium hydroxide solution, prepared from the saturated alkali solution using CO₂-free water. Borate buffer solutions were prepared by dilution of standard borax buffers, with adding required amounts of HCl or NaOH solutions.

The samples of 4,5-dinitrofluorescein, 4,5-dinitro-2,7-dibromofluorescein, 4,5-dibromo-2,7-dinitrofluorescein, 4,5-dibromo-2-nitrofluorescein, 2,4,5,7-tetranitrofluorescein thiofluorescein, 2,4,5,7-tetrabromothiofluorescein, 4-nitrothiofluorescein, 4,5-dinitrothiofluorescein, 4,5-dibromo fluorescein, 3',4',5',6'-tetrabromofluorescein, and 4,5-dinitro-N-ethylazafluorescein were used in previous studies [15,37,38,47,48]. They were synthesized in the laboratory of Prof. A.V. Eltsov, Saint-Petersburg State Institute of Technology, Russia and transferred to us by Dr. Denis V. Samoylov. Ethyl fluorescein, n-hexadecyl fluorescein, and n-decyl eosin were synthesized in the Research Institute of Organic Intermediates and Dyes, Russia and donated to us by Dr. V.I. Alekseeva. 6-Hydroxy-9-phenylfluorone was prepared from resorcinol and benzotrichloride. The above dyes were characterized by ¹H and ¹³C NMR spectroscopy in (CD₃)₂SO (Bruker AM-500 spectrometer (Billerica, MA, USA), 500 and 50 MHz, respectively) and elemental analysis; their purity was also confirmed using TLC (Silufol plates) [47,48].

Absorption spectra were run on a SP-46 apparatus (LOMO company, Russia). The pH values (± 0.02 units) were determined at 25.0 ± 0.1 °C in a cell with liquid junction (1 M KCl) with an ESL-63-07 glass electrode (ZIP, Gomel, Belarus) and an Ag/AgCl reference

electrode. Standard buffers with pH 1.68, 4.01, 6.86 and 9.18, and dilute HCl solutions were used for cell calibration.

3. Results

3.1. Determination of the Apparent Ionization Constants

The pK_a values of the dyes in micellar media in the presence of 4.00 M KCl were evaluated as a rule at CTAB concentration of 0.003 M. Potassium chloride was introduced into each working solution in the form of a separate weighed sample. In the presence of 4.00 M KCl, the CTAB micelles completely convert into those of CTAC [7]. Stock solutions of the dyes were prepared by dissolution in the CTAB micellar solution and then diluted down to the working solutions. The sequence of mixing of components was as follows: KCl, water, buffer components, surfactant stock solution containing the corresponding dye. The buffer concentrations were as a rule around 0.01 M. The pH values of acetate and phosphate buffers in water and in 4.00 M KCl as a rule stay practically unchanged after CTAB adding. The largest deviations did not exceed 0.07 units. The working concentrations of dyes were around 10^{-5} M. In some cases, higher concentrations were also used. The absorption spectra of the dyes at different pHs are exemplified in Figure 1. In some cases, measurements were carried out at different concentrations of dyes, and the A values were then adjusted to a single concentration.

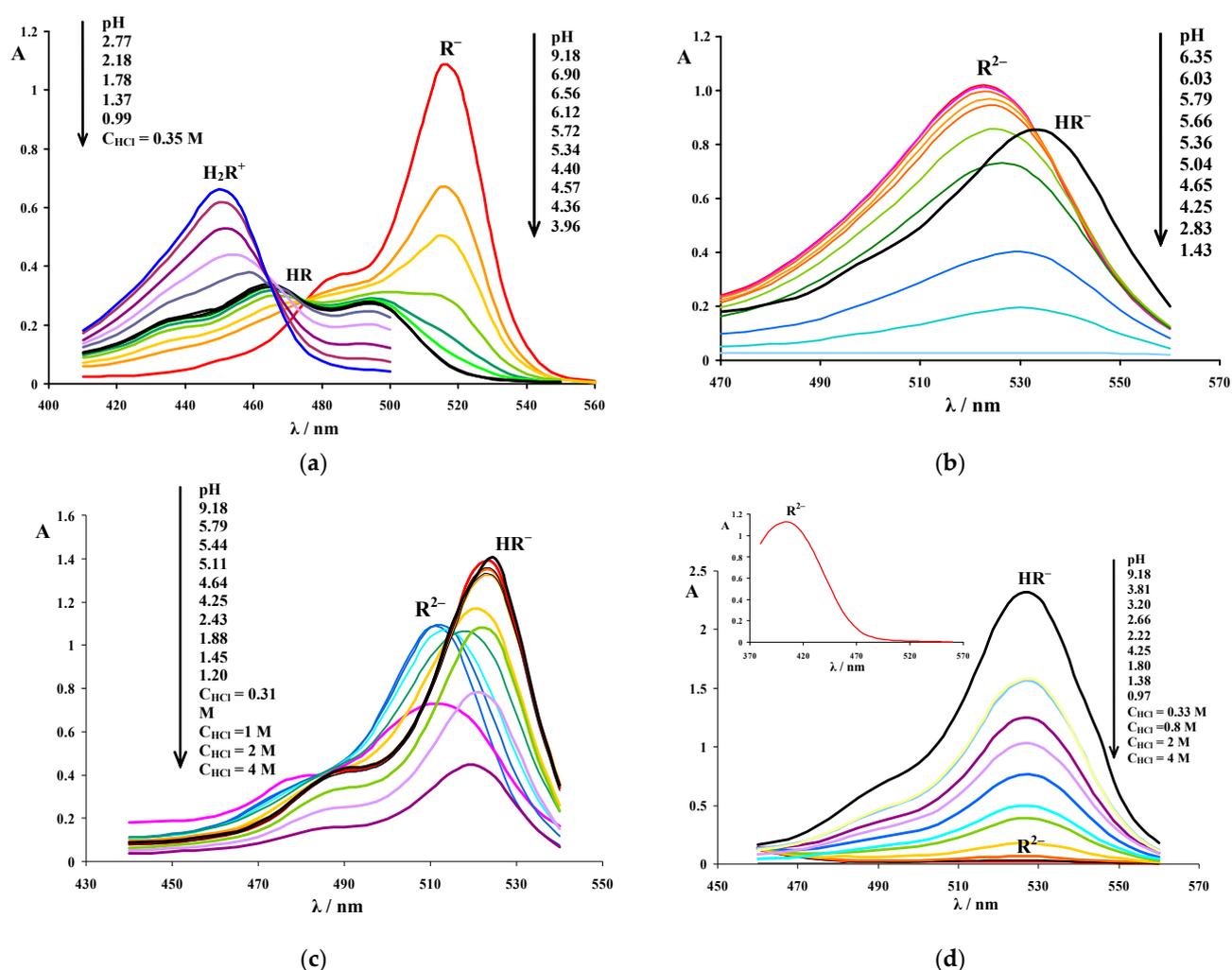


Figure 1. Absorption spectra of dyes at different pH values: 6-hydroxy-9-phenylfluorone (a); 4,5-dinitrothiofluorescein (b); 4,5-dinitro-2,7-dibromofluorescein (c); and 2,4,5,7-tetranitrofluorescein (d). The insert in Figure 1d shows the absorption band of the dianion of 2,4,5,7-tetranitrofluorescein that practically does not absorb light at $\lambda > 500$ nm.

The stepwise ionization constants were calculated by using the dependences A vs. pH at fixed selected wave length and constant total dye concentration and optical path length (Equation (6)):

$$A = \frac{A_{H_3R^+}h^3 + A_{H_2R}h^2K_{a0}^a + A_{HR^-}hK_{a0}^aK_{a1}^a + A_{R^{2-}}K_{a0}^aK_{a1}^aK_{a2}^a}{h^3 + h^2K_{a0}^a + hK_{a0}^aK_{a1}^a + K_{a0}^aK_{a1}^aK_{a2}^a} \quad (6)$$

Here A stands for the absorbance at the current pH value; $h \equiv 10^{-\text{pH}}$; $A_{R^{2-}}$, A_{HR^-} , A_{H_2R} , and $A_{H_3R^+}$ are absorbances under conditions of complete conversion of the dye into the corresponding form.

Representative dependences of A versus pH are depicted in Figure 2. The repeatability of A measurements for two or more independent experiments was about ± 0.01 . The A_{HR^-} values and, in some cases, A_{H_2R} values are unavailable for direct measurements and are to be calculated jointly with the $\text{p}K_a^a$ values. The values of $A_{R^{2-}}$, $A_{H_3R^+}$ and (as the first iteration) A_{H_2R} were obtained directly at suitable pH, in NaOH and HCl (or H_2SO_4) solutions, respectively. For the majority of the dyes the calculations can be simplified due to negligible equilibrium concentrations of H_3R^+ ions at working pH values. The data were processed by using the CLINP program [15,39,40,56]. Normally, 15 working solutions with various pH values and 20 analytical wave lengths were used for determining the $\text{p}K_a^a$ values of “overlapping” equilibria, Equations (4) and (5). The same procedure was used by us earlier for data processing in different systems [15,37,39–41].

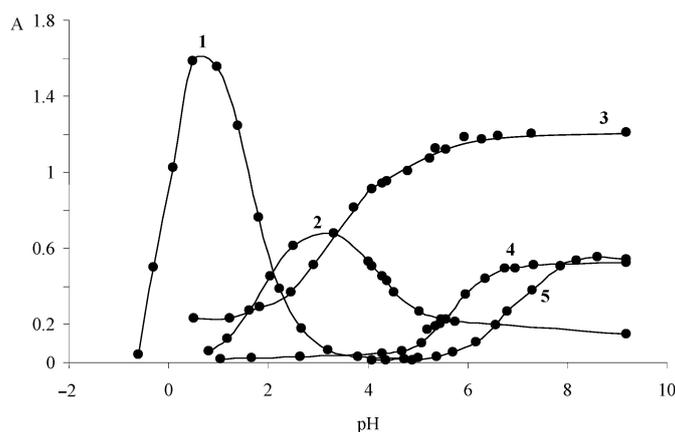


Figure 2. Absorbance dependence vs. pH: 2,4,5,7-tetranitrofluorescein, $\lambda = 525$ nm (1); 4,5-dibromo-2,7-dinitrofluorescein, $\lambda = 555$ nm (2); 4,5-dinitro-*N*-ethylazafuorescein, $\lambda = 455$ nm (3); 4,5-dibromofluorescein, $\lambda = 495$ nm (4); 3',4',5',6'-tetrabromofluorescein, $\lambda = 495$ nm (5).

Note, that in the case of 6-hydroxy-9-phenylfluorone (Figure 1a) and ethyl fluorescein and other esters of fluorescein and eosin, the $\text{p}K_{a0}^a$ and $\text{p}K_{a1}^a$ refer to the dissociation of H_2R^+ and HR , respectively.

The $\text{p}K_a^a$ values of fluorescein dyes determined in the course of the present study are collected in Table 1. This Table also contains previously published data for some other compounds that relate to the same experimental conditions. The uncertainty of the $\text{p}K_a^a$ values is characterized as the half-width of the confidence interval with a confidence level of 0.95.

Table 1. The pK_a^a values of hydroxyxanthene dyes in CTAC micellar solutions, 4.00 M KCl; 25 °C.

Dye	pK_{a0}^a	pK_{a1}^a	pK_{a2}^a
Fluorescein ^a	0.60 ± 0.10	6.41 ± 0.10	7.17 ± 0.06
Thiofluorescein	0.55 ± 0.09	7.67 ± 0.06	7.77 ± 0.12
Sulfonefluorescein ^a	—	2.33 ± 0.05	7.00 ± 0.01
6-Hydroxy-9-phenylfluorone	1.98 ± 0.04	6.67 ± 0.03	—
Ethyl fluorescein	1.86 ± 0.02	6.59 ± 0.03	—
<i>n</i> -Decyl fluorescein ^b	2.13 ± 0.01	6.61 ± 0.07	—
<i>n</i> -Hexadecyl fluorescein	1.58 ± 0.04	7.06 ± 0.09	—
2,4,5,7-Tetrabromofluorescein (eosin) ^a	—	1.83 ± 0.07	5.76 ± 0.06
2,4,5,7-Tetrabromothiofluorescein	—	1.69 ± 0.02	6.07 ± 0.03
Ethyl eosin ^a	—	1.11 ± 0.03	—
<i>n</i> -Decyl eosin ^b	—	1.18 ± 0.05	—
3',4',5',6'-Tetrabromofluorescein	— ^c	6.66 ± 0.03	7.27 ± 0.02
2,7-Dichlorofluorescein ^a	<−0.50 ^d	5.50 ± 0.05	5.79 ± 0.06
4,5-Dibromofluorescein	—	5.34 ± 0.05	6.00 ± 0.04
4,5-Dinitrofluorescein	—	4.04 ± 0.02	4.92 ± 0.03
4,5-Dinitro- <i>N</i> -ethylazafluorescein	—	3.10 ± 0.01	5.21 ± 0.02
4,5-Dinitrothiofluorescein	—	4.88 ± 0.02	4.91 ± 0.04
4-Nitrothiofluorescein	—	5.74 ± 0.04	6.56 ± 0.02
4,5-Dinitro-2,7-dibromofluorescein	—	−0.4 ± 0.2 ^c	4.63 ± 0.06
4,5-Dibromo-2-nitrofluorescein	—	3.92 ± 0.02	5.50 ± 0.02
4,5-Dibromo-2,7-dinitrofluorescein	—	1.83 ± 0.02	4.35 ± 0.02
2,4,5,7-Tetranitrofluorescein ^e	—	0.16 ± 0.03	1.45 ± 0.02
Bromophenol blue ^b	—	—	3.86 ± 0.03
Bromocresol green ^b	—	—	5.13 ± 0.01
Bromocresol purple ^b	—	—	7.02 ± 0.02
Bromothymol blue ^b	—	—	8.26 ± 0.01
Phenol red ^b	—	—	8.71 ± 0.03
<i>ortho</i> -Cresol red ^b	—	—	9.46 ± 0.01
<i>meta</i> -Cresol purple ^b	—	—	9.72 ± 0.03
Thymol blue ^b	—	1.25 ± 0.04	10.47 ± 0.02
2,4-Dinitrophenol ($c_{surf} = 0.01$ M) ^f	—	3.57 ± 0.03	—
2,5-Dinitrophenol ($c_{surf} = 0.01$ M) ^f	—	4.60 ± 0.05	—
2,6-Dinitrophenol ($c_{surf} = 0.01$ M) ^f	—	2.51 ± 0.02	—
2,6-Dinitro-4- <i>n</i> -dodecylphenol ^g	—	2.20 ± 0.02	—
2,6-Diphenyl-4-(2,4,6-triphenylpyridinium-1-yl) phenolate ^b	8.60 ± 0.02	—	—
<i>N,N'</i> -Di- <i>n</i> -octadecyl rhodamine ($c_{surf} = 0.01$ M) ^b	3.94 ± 0.09	—	—

Note. ^a From ref. [7]. ^b From ref. [8]. ^c The spectrum at 2.0 M HCl shows no signs of the cation H_3R^+ of the dye. ^d Concentration scale of pH. ^e From ref. [37]. ^f From ref. [55]. ^g From ref. [50].

Kibblewhite et al. [13] reported the pK_a^a values for lipoidal fluorescein and eosin dyes in *n*-dodecyltrimethylammonium bromide micelles at NaBr concentration of 4.0 M. For 5'-(*N*-octadecanoyl)amino fluorescein, $pK_{a1}^a = 5.66$ and $pK_{a2}^a = 7.47$; for 5'-(*N*-hexadecanoyl)amino eosin, the corresponding values are 2.32 and 4.53, the uncertainty is within $\pm(0.04-0.08)$. In DTAC micellar solutions at low ionic strength of the bulk, the values are 1.52–1.95 units lower [13].

3.2. Absorption Spectra of Ionic and Molecular Forms

Having the K_{a1}^a and K_{a2}^a values, it was possible to calculate the absorbances of HR^- at various wavelengths, and in such a way obtaining the spectra of these species (Equation (7)):

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

As a rule, the interval $pK_{a1}^a \leq pH \leq pK_{a2}^a$ was used in this case. The refinement of A_{H_2R} values was carried out at $pK_{a0}^a < pH < pK_{a1}^a$ with the help of Equation (8):

$$A_{H_2R} = A + (A - A_{H_3R^+})h(K_{a0}^a)^{-1} + (A - A_{HR^-})h^{-1}K_{a1}^a + (A - A_{R^{2-}})h^{-2}K_{a1}^aK_{a2}^a \quad (8)$$

Despite the predominance of the neutral form within a wide pH range, this procedure was necessary for avoiding any influence of traces of intensely colored ions, HR^- , R^{2-} , and in several cases H_3R^+ on the spectra of the neutral forms. For the dyes with free carboxylic group, the absorptivity of the molecular form is negligible as compared with that of ions.

The spectra of ionic forms, which are singled out using Equation (7), are presented for some fluorescein dyes in Figure 3. The λ_{max} values of spectra of the anions of several dyes are summarized in Table 2. It also includes the data obtained by us earlier for some of the dyes [7].

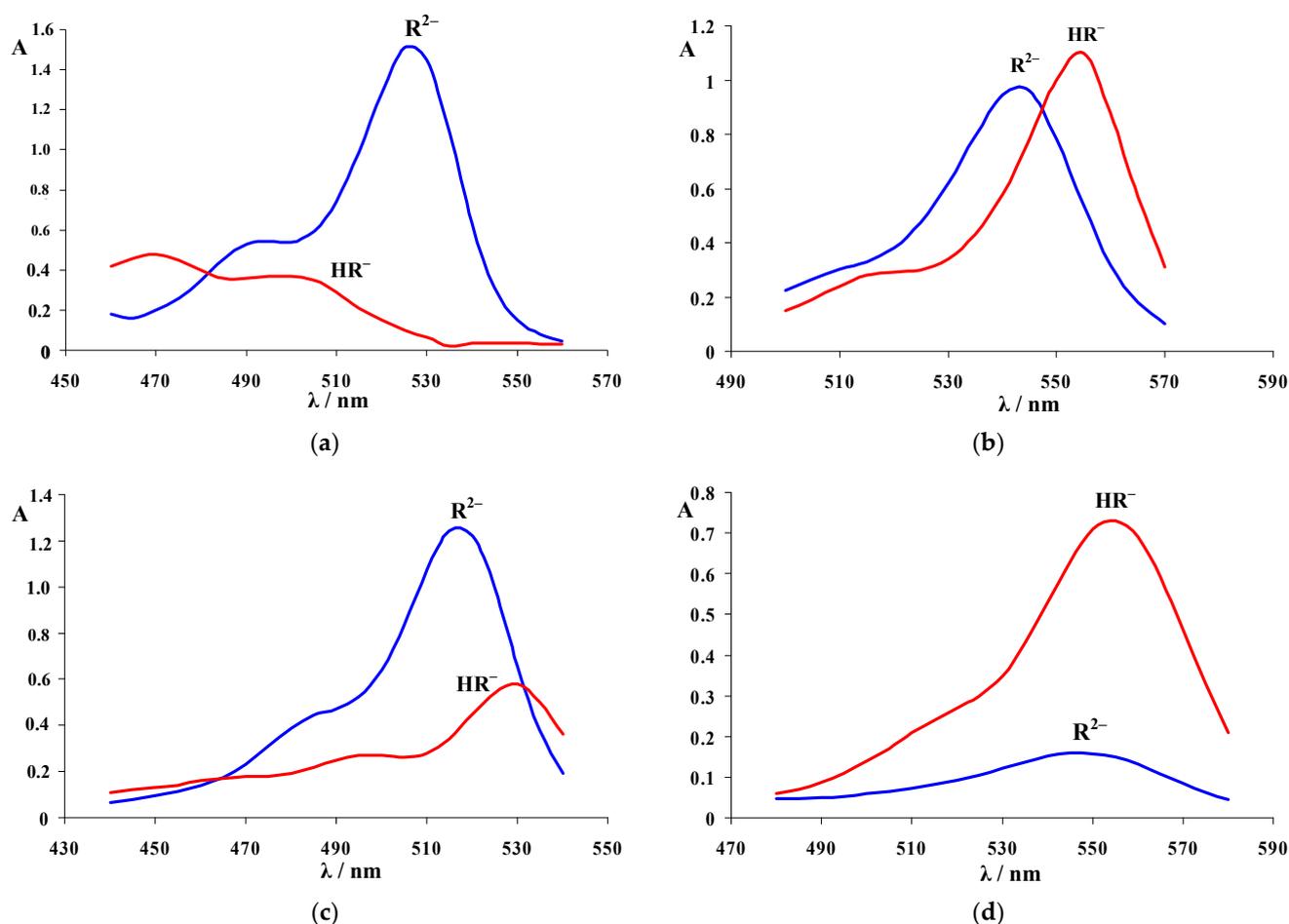


Figure 3. Absorption spectra of dye anions: 3',4',5',6'-tetrabromofluorescein (a); 2,4,5,7-tetrabromothiofluorescein (b); 4,5-dibromofluorescein (c); 4,5-dibromo-2,7-dinitrofluorescein (d).

The absorption maxima of anions are bathochromically shifted compared to those in water by ca. 10–20 nm. The sole exception is the dianion R^{2-} 2,4,5,7-tetranitrofluorescein, which exists in form of a lactone (see below).

The introduction of two halogen atoms in the chromophoric system of fluorescein R^{2-} ion results in a bathochromic shift of 15–17 nm; 2,4,5,7-substitution leads to a 26 nm-shift. In the case of thiofluorescein and thioeosin, the corresponding shift is 19 nm. In contrast, the introduction of two nitro groups at 4 and 5 positions show negligible effect for both fluorescein and thiofluorescein. However, the effect of 2,7-dinitro substitution is much more pronounced, as is seen from the comparison of 4,5-dinitro-2,7-dibromo- and 4,5-dibromo-

2,7-dinitrofluoresceins. As it will be shown below, the tendency to lactone formation by anions is also more expressed in the second case.

Table 2. The λ_{\max} values of hydroxyxanthene dyes anions in CTAC micellar solutions; 4.0 M KCl.

Dye	λ_{\max} , nm	
	HR	R [−]
6-Hydroxy-9-phenylfluorone	465, 495	517
Ethyl fluorescein	465, 495	517
<i>n</i> -Decyl fluorescein	465, 490–495	516.5
Ethyl eosin	485.5	542.5
	HR [−]	R ^{2−}
Fluorescein	455, 480	500
Sulfonefluorescein	460, 490	512
Thiofluorescein	500, 520	524
3',4',5',6'-Tetrabromofluorescein	470, 495–500	526.5
2,7-Dichlorofluorescein	525	515
4,5-Dibromofluorescein	495–500, 530	517
2,4,5,7-Tetrabromofluorescein (eosin)	538–540	526.5
2,4,5,7-Tetrabromothiofluorescein	555	543
4,5-Dinitrofluorescein	510	502
4,5-Dinitro- <i>N</i> -ethylazafluorescein	495	483.5
4,5-Dinitrothiofluorescein	535	522.5
4,5-Dinitro-2,7-dibromofluorescein	525	511
4,5-Dibromo-2-nitrofluorescein	535	527
4,5-Dibromo-2,7-dinitrofluorescein	555	547
2,4,5,7-Tetranitrofluorescein	525	405

The expressed drop of the molar absorptivity of molecules in the case of dyes with free COOH group indicates a pronounced displacement of the tautomeric equilibrium towards the colorless lactone H₂L as a result of binding by micelles. In accordance with the degree of conversion to lactone, the compounds can be arranged in the following row: eosin < 4,5-dibromofluorescein < 2,7-dichlorofluorescein < fluorescein < nitro- and bromonitro derivatives. The λ_{\max} values of the forms H₂R, in fact, those of the H₂Q tautomers, are 465 and 490 nm for fluorescein, 470 and 495 nm for 3',4',5',6'-tetrabromofluorescein, 470 and 500 nm for 4,5-dibromofluorescein, and 475 and 505 nm for 2,7-dichlorofluorescein. For eosin, the main molecular absorption maximum corresponds to 480 nm, while for thioeosin it is 510 nm. Similar are the λ_{\max} values for nitro derivatives, but the molar absorptivity is very low.

Among the different dye forms studied in this paper, only cations of fluorescein, ethyl fluorescein, 6-hydroxy-9-phenylfluorone, and thiofluorescein can be not bound by the cationic micelles. However, the λ_{\max} values of the cations of the first three dyes are 444, 447, and 448 nm, respectively, while that of *n*-decyl fluorescein, firmly bound to micelles, equals to 448 nm. For thiofluorescein cation, λ_{\max} = 452 nm; for this dye, the absorption bands of anionic forms are also shifted bathochromically as compared with those of fluorescein.

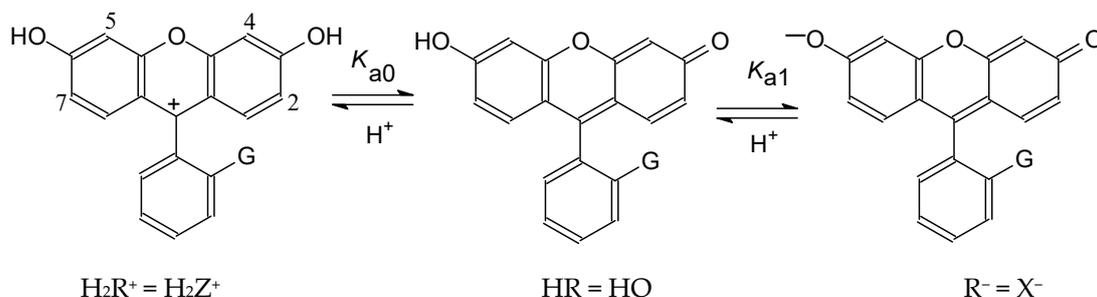
4. Discussion

4.1. Ionization of Fluoresceins with Blocked Carboxylic Group and Similar Compounds

Following conclusions were made basing on the spectra of the molecular and ionic forms of the dyes.

First, let us consider the absorption bands of dyes unable to lactone formation (Scheme 3). To this group belong compounds with a carboxylic group blocked by esterification, namely, ethylfluorescein, *n*-decyl fluorescein, *n*-hexadecyl fluorescein, ethyl eosin, and *n*-decyleosin; a dye without the carboxylic group, i.e., 6-hydroxy-9-phenylfluorone; and sulfonefluorescein, a compound bearing the SO₃H group instead of COOH. In the last

case, the formation of an intramolecular ester called “sultone cycle”, is not typical at least in the presence of water. In Figure 1a, the spectra of 6-hydroxy-9-phenylfluorone cation H_2R^+ , neutral form HR, and anion R^- are shown. They correspond to the H_2Z^+ , HQ, and X^- types of bands. In this paper, we use the symbols Z, Q, and X in order to distinguish between three states of the xanthene portion, namely, cationic, neutral, or quinonoidal, and anionic, respectively. Of course, this does not reflect the real distribution of the electronic density. In particular, the 3- and 6-oxygen atoms in the X^- structure are equal.



Scheme 3. Ionization scheme of fluorescein dyes unable to lactone formation; G = H; $COOC_2H_5$; $COOC_{10}H_{21}$; $COOC_{16}H_{33}$; SO_3H (SO_3^- in solution); for ethyl eosin and *n*-decyl eosin, Br atoms are in 2,4,5, and 7 positions.

In the case of alkyl fluorescein, alkyl eosin, and some other model compounds the ionization occurs in two steps. However, the formation of cations of eosin dyes occurs in very acidic media and was not studied here. In the case of sulfonefluorescein, $G = SO_3^-$ and all the three species have one positive charge less.

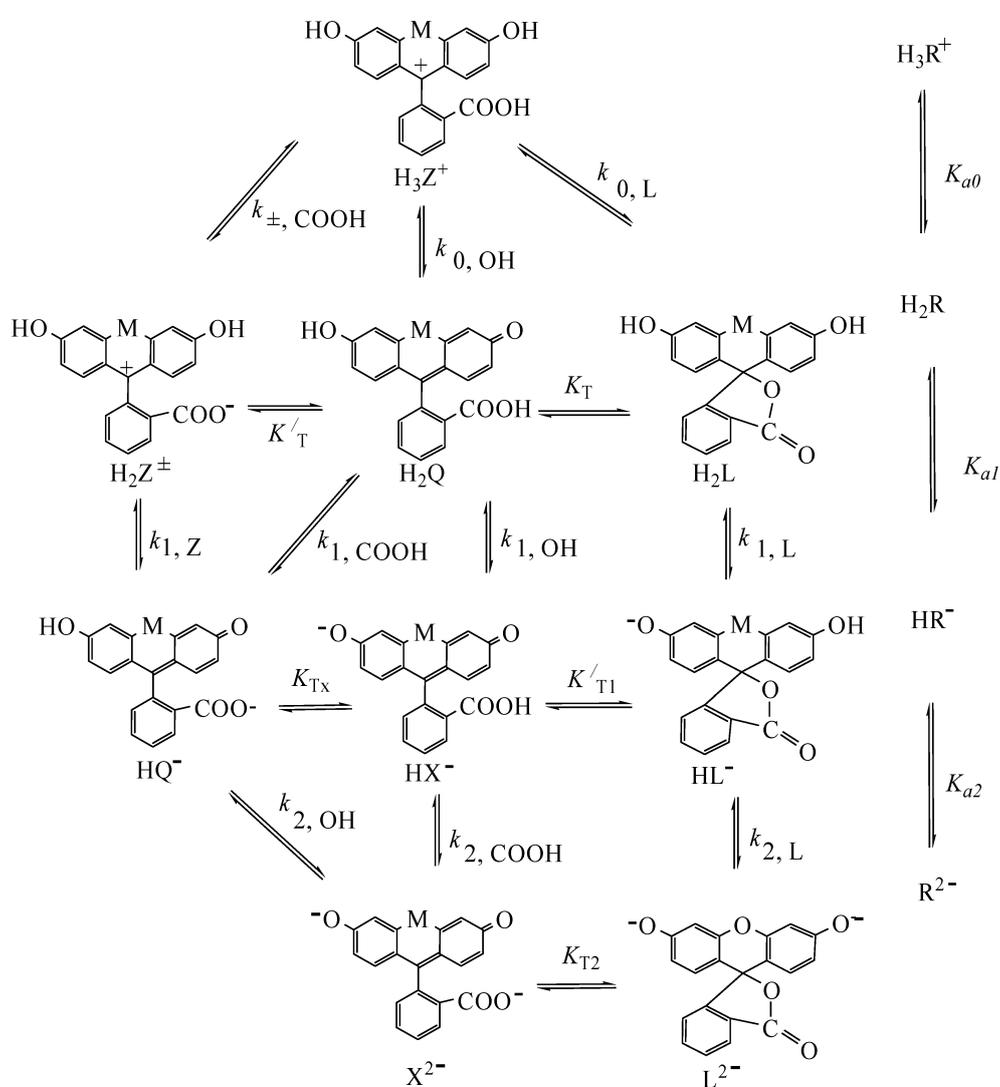
The spectra of the quinonoid molecule HQ exhibit two main maxima around 465 and 495 nm (Figure 1a). The ratios of the maximal absorbances of the molecule and anion, $A_{\max}(HQ)/A_{\max}(X^-)$ or, in fact, $A_{\max}(HR)/A_{\max}(R^-)$ for 6-hydroxy-9-phenylfluorone are 0.305 and 0.253. For ethyl fluorescein, the $A_{\max}(HQ)/A_{\max}(X^-)$ values are 0.335 and 0.290, whereas for *n*-decyl fluorescein, 0.294 and 0.260. The relative absorption of the molecular form is somewhat lower for ethyl eosin: $A_{\max}(HQ)/A_{\max}(X^-) = 0.222$.

For these dyes, the pK_{a0}^a and pK_{a1}^a values correspond to the transitions $H_2Z^+ \rightarrow HQ$ and $HQ \rightarrow X^-$, respectively. For ethyl eosin and *n*-decyl eosin, the H_2Z^+ cations appear in acidic region and their spectra were not observable. In the case of sulfonefluorescein, the molecular and ionic structures are H_2Z^{\pm} , HQ^- , and X^{2-} , owing to the presence of additional SO_3^- group. In this case, the transitions refer to pK_{a1}^a and pK_{a2}^a , respectively.

4.2. Detailed Scheme of Protolytic Equilibria in the Micellar Pseudophase

Much more complicated is the equilibrium in the case of the main group of fluorescein dyes with the free COOH group. Basing on a series of our previous studies [7,15,31,37–42,47,48,57], we propose the following generalized scheme of a detailed protolytic equilibrium (Scheme 4). The symbols H_3R^+ , H_2R , HR^- , and R^{2-} designate the stoichiometric composition irrespective of the molecular (ionic) structure. Molecules and anions may be equilibrium mixtures of different tautomers depending on the specificity of each of the dyes. The possible tautomers of the neutral form H_2R are well known. It deals about the zwitter-ion H_2Z^{\pm} , quinonoid H_2Q , and colorless lactone H_2L . Their structures in solid state [37,58,59] and solution [13,16,17,28–30,32,36,60,61] are well documented by different authors. The H_2Z^{\pm} tautomer was observed mainly for fluorescein in water [7,57,61]. The fraction of the H_2Q tautomer decreases while that of H_2L increases on going from water to organic solvents [13,30,37,39–41,57,60] or different kinds of organized solutions, such as micellar solutions of colloidal surfactants [7,13,16,17,39], direct or reversed microemulsions [15,39,62], solutions of dendrimers [63], calixarenes [64], and cyclodextrins [65]. Monoanions HR^- of HQ^- type are usually observed for fluorescein and its derivatives bearing halogen atoms in the phthalic acid residue [15,62]. Contrary to it, the same substituents in the xanthene moiety favor formation of the HX^- tautomer [13,15–17,29,30,37,39–41,50,57,62,63].

This was already mentioned in earlier publications [60,66–69]. In some rare cases, for instance, in entire dimethyl sulfoxide, the last tautomer was observed even for fluorescein, as admixture to HQ^- [41]. However, both experiments [43–45] and quantum-chemical calculations [42] show that in vacuum the HX^- tautomer predominates. Involving of nitro derivatives in research led to interesting results. In this case, both R^{2-} and HR^- anions may form lactonic anions L^{2-} and HL^- [15,29,37,38,47,48]. The lactonic structure of 2,4,5,7-tetranitrofluorescein dianion R^{2-} in solution was proved using IR- and NMR-spectroscopy [37,38]. Previously, such kind of tautomerism was described only for phenolphthalein and its derivatives [70–73]. The introduction of NO_2 groups in 2, 4, 5, or 7 positions of fluorescein evidently decreases the electronic density on the nodal carbon atom, which results in the last-named effect and in a substantial stabilization of the H_2L tautomer [37,38,47,48]. We do not consider here more complicated equilibria of aminofluoresceins because of the presence of additional acid-base centers [34,35,40]. Fluoro derivatives of fluorescein obey the same regularities as the chloro- and bromofluoresceins [39]. Interesting data were published recently for silicone analogues of fluorescein, dyes with $Si(CH_3)_2$ group instead of the pyrane oxygen [25].



Scheme 4. General scheme of the protolytic equilibrium of fluorescein dyes in solution; M = O; S; NC_2H_5 .

In this study, we have selected a set of dyes for which all the listed types of tautomerism are characteristic to one degree or another (Scheme 4). The constants of the

tautomeric equilibria are as follows:

$$K_T = \frac{[H_2L]}{[H_2Q]}; K'_T = \frac{[H_2Z^\pm]}{[H_2Q]}; K''_T = \frac{[H_2L]}{[H_2Z^\pm]} = K_T(K'_T)^{-1} \quad (9)$$

$$K_{Tx} = \frac{[HX^-]}{[HQ^-]}; K_{T1} = \frac{[HL^-]}{[HQ^-]}; K'_{T1} = \frac{[HL^-]}{[HX^-]}; K_{T2} = \frac{[L^{2-}]}{[X^{2-}]}; \quad (10)$$

Hereafter, we use the equilibrium concentrations instead of activities. Accordingly, the fractions α of the tautomers should be expressed using the above tautomerization constants:

$$\alpha_{H_2Z^\pm} = \frac{1}{1 + K''_T + (K'_T)^{-1}}; \alpha_{H_2Q} = \frac{1}{1 + K_T + K'_T}; \alpha_{H_2L} = \frac{1}{1 + K_T^{-1} + (K'_T)^{-1}} \quad (11)$$

$$\alpha_{HQ^-} = \frac{1}{1 + K_{Tx} + K_{T1}}; \alpha_{HX^-} = \frac{1}{1 + K_{Tx}^{-1} + K'_{T1}}; \alpha_{HL^-} = \frac{1}{1 + K_{T1}^{-1} + (K'_{T1})^{-1}} \quad (12)$$

$$\alpha_{X^{2-}} = \frac{1}{1 + K_{T2}}; \alpha_{L^{2-}} = \frac{1}{1 + K_{T2}^{-1}} \quad (13)$$

The tautomerization constants and correspondingly the α values are in fact extrathermodynamic quantities. However, their estimates may be made using some reliable assumptions concerning the absorption spectra in the visible [7,31,37,40,57,61]. The molar absorptivities at the band maxima of H_2Q should be equated to that of the HQ^- species of the same dyes, for which the tautomers of HX^- and HL^- types are not typical. Otherwise, they may be equated to the molecular forms of corresponding compounds with the carboxylic group being blocked. For example, for the H_2Q tautomer of fluorescein, the anion HR^- of the same dye, the molecule HQ of fluorescein esters, 6-hydroxy-9-phenylfluorone, and the HR^- ion of sulfonefluorescein are such model species. The maximal molar absorptivity of the H_2Z^\pm tautomer should be equated to that of the H_3Z^+ cation. The HR^- spectrum of eosin coincides with the spectrum of the anion R^- of ethyleosin in the micellar media under study that allows supposing a structure of HX^- type [7]. At the same time, the band of R^{2-} of eosin (X^{2-} structure) is hypsochromically shifted by ca. 13 nm, thus exhibiting the role of the carboxylate group COO^- [7]. For R^{2-} of fluorescein (also X^{2-} structure), the corresponding absorption band shift with respect to the maxima of the anions of fluorescein esters and 6-hydroxy-9-phenylfluorone reaches 17 nm (Table 2). Such shifts are of universal character and have been confirmed theoretically [74]. They are also observed for tautomers of the "Z" type; compare the absorption bands of cation of fluorescein esters and 6-hydroxy-9-phenylfluorone, on the one hand, and of the zwitter-ion of sulfonefluorescein. Unfortunately, corresponding model compounds are not available for all the dyes under study. Hence, sufficiently accurate numerical estimates are not always possible, but semi-quantitative evaluations may be made. However, it should be also kept in mind that it is impossible to directly estimate too large or too small values of the tautomerization constants. In addition, the presence of colorless lactones in an equilibrium mixture of tautomers may be surely stated only on substantial decrease in the molar absorptivity.

The so-called microscopic ionization constants are following:

$$k_{\pm,COOH} = h \frac{[H_2Z^\pm]}{[H_3Z^+]}; k_{0,OH} = h \frac{[H_2Q]}{[H_3Z^+]}; \quad (14)$$

$$k_{1,COOH} = h \frac{[HQ^-]}{[H_2Q]}; k_{1,Z} = h \frac{[HQ^-]}{[H_2Z^\pm]}; k_{1,OH} = h \frac{[HX^-]}{[H_2Q]}; k_{1,L} = h \frac{[HL^-]}{[H_2L]}; \quad (15)$$

$$k_{2,\text{OH}} = h \frac{[\text{X}^{2-}]}{[\text{HQ}^-]}; k_{2,\text{COOH}} = h \frac{[\text{X}^{2-}]}{[\text{HX}^-]}; k_{2,\text{L}} = h \frac{[\text{L}^{2-}]}{[\text{HL}^-]} \quad (16)$$

Finally, the experimentally determined $\text{p}K_{\text{a}}$ values can be expressed through the $\text{p}k$ and α in the following manner:

$$\text{p}K_{\text{a}0} = \text{p}k_{\pm,\text{COOH}} + \log \alpha_{\text{H}_2\text{Z}^\pm} = \text{p}k_{0,\text{OH}} + \log \alpha_{\text{H}_2\text{Q}} \quad (17)$$

$$\begin{aligned} \text{p}K_{\text{a}1} &= \text{p}k_{1,\text{COOH}} - \log \alpha_{\text{H}_2\text{Q}} + \log \alpha_{\text{HQ}^-} = \text{p}k_{1,\text{OH}} - \log \alpha_{\text{H}_2\text{Q}} + \log \alpha_{\text{HX}^-} \\ &= \text{p}k_{1,\text{Z}} - \log \alpha_{\text{H}_2\text{Z}^\pm} + \log \alpha_{\text{HQ}^-} = \text{p}k_{1,\text{L}} - \log \alpha_{\text{H}_2\text{L}} + \log \alpha_{\text{HL}^-} \end{aligned} \quad (18)$$

$$\begin{aligned} \text{p}K_{\text{a}2} &= \text{p}k_{2,\text{OH}} - \log \alpha_{\text{HQ}^-} + \log \alpha_{\text{X}^{2-}} = \text{p}k_{2,\text{COOH}} - \log \alpha_{\text{HX}^-} + \log \alpha_{\text{X}^{2-}} \\ &= \text{p}k_{2,\text{L}} - \log \alpha_{\text{HL}^-} + \log \alpha_{\text{L}^{2-}} \end{aligned} \quad (19)$$

Returning to the compounds considered in Section 4.1, it should be noted that in this case $\text{p}K_{\text{a}0} = \text{p}k_{0,\text{OH}}$ and $\text{p}K_{\text{a}1} = \text{p}k_{1,\text{OH}}$. For sulfonefluorescein, $\text{p}K_{\text{a}1} = \text{p}k_{1,\text{Z}}$ and $\text{p}K_{\text{a}2} = \text{p}k_{2,\text{OH}}$.

4.3. Classification of Fluorescein Dyes According to the Type of Tautomerism of Anions

Below we will consider the tautomeric equilibria of anions in the medium under study. As for molecular forms, the lactone predominates. The fraction of quinonoidal tautomer is very small, especially for nitrofluoresceins. The zwitter-ionic tautomer is not detected in the spectra at all; generally speaking, this tautomer was previously observed only for fluorescein in water and aqueous solutions with small additives of organic solvents.

Fluorescein type of the ionic equilibrium: $\text{HQ}^- \rightarrow \text{X}^{2-}$ (Figure 3a). The monoanion HR^- of fluorescein exists in solution as HQ^- tautomer, with two main band maxima, 455 and 480 nm; for the dianion R^{2-} , X^{2-} structure, $\lambda_{\text{max}} = 500$ nm [7]. The $A_{\text{max}}(\text{HR}^-)/A_{\text{max}}(\text{R}^{2-})$ ratios are 0.290 and 0.279, respectively, which is similar to the $A_{\text{max}}(\text{HR}^-)/A_{\text{max}}(\text{R}^-)$ values of the corresponding esters and 6-hydroxy-9-phenylfluorone (see above). For sulfonefluorescein, the monoanionic spectra also exhibits two maxima; $A_{\text{max}}(\text{HR}^-)/A_{\text{max}}(\text{R}^{2-}) = 0.357$ and 0.305. For 3',4',5',6'-tetrabromofluorescein, the corresponding ratios are 0.317 and 0.245. For this group of dyes, $\text{p}K_{\text{a}2}^{\text{a}} = \text{p}k_{2,\text{OH}}$. For the above dyes, $\text{p}K_{\text{a}2}^{\text{a}} = 7.00$ to 7.27. For thiofluorescein this value was somewhat higher, 7.77, while $\text{p}K_{\text{a}2}^{\text{a}} = 6.56$ of 4-nitrothiofluorescein reflects the influence of the substituent NO_2 .

Eosin type of ionic equilibria: $\text{HX}^- \rightarrow \text{X}^{2-}$ (Figure 3b). Another type of monoanions HR^- is the HX^- tautomer, with the principal absorption band shifted bathochromically compared to the X^{2-} band. For these dyes of the eosin type, the $A_{\text{max}}(\text{HR}^-)/A_{\text{max}}(\text{R}^{2-})$ values are 0.944 (eosin); 1.15 (2,4,5,7-tetrabromothiofluorescein); and 1.10 (4,5-dinitro-N-ethylazafluorescein). For 4,5-dinitrofluorescein and 4,5-dinitrothiofluorescein, the $A_{\text{max}}(\text{HR}^-)/A_{\text{max}}(\text{R}^{2-})$ ratio equals to 1.069 and 0.842, respectively. Note, that in the last two cases, the $K_{\text{a}1}^{\text{a}}$ and $K_{\text{a}2}^{\text{a}}$ values are close and the (possible) errors in estimation $A_{\text{max}}(\text{HR}^-)$ value [Equation (7)] may increase. The dye 4,5-dinitro-2,7-dibromofluorescein with $A_{\text{max}}(\text{HR}^-)/A_{\text{max}}(\text{R}^{2-}) = 1.293$ can also be conditionally ranked to the same group. However, the last value substantially exceeds unity and may reflect the presence of some fraction of the R^{2-} ions in the lactonic form L^{2-} .

For the compounds of the eosin type, $\text{p}K_{\text{a}2}^{\text{a}} = \text{p}k_{2,\text{COOH}}$. These values are 5.76 and 6.07 for eosin and thioeosin, respectively; for four 4,5-dinitro derivatives, $\text{p}k_{2,\text{COOH}} = 4.63$ to 5.21, which reflects the inductive effects of two NO_2 groups.

Strictly speaking, the intensities of the HX^- and X^{2-} bands do not have to match exactly. For example, the maximal molar absorptivities of single-charged anions of ethylfluorescein and ethyleosin in various solvents and micellar media are close to that of dianions of fluorescein and eosin, respectively, but some small deviations are still observed [7,16,17,31]. The same was repeatedly observed for the HR^- and R^{2-} ions (in fact, HX^- and X^{2-} tautomers) of eosin and erythrosin in different organic solvents [30,37,39,57] and micellar pseudophases [7,13,15–17].

Intervening type: $(\text{HQ}^- \rightleftharpoons \text{HX}^-) \rightarrow \text{X}^{2-}$ (Figure 3c). More serious deviation of the ratio $A_{\max}(\text{HR}^-)/A_{\max}(\text{R}^{2-})$ from unity was fixed for 4,5-dibromofluorescein (0.466) and earlier [7] for 2,7-dichlorofluorescein (0.717). At the same time, some less intensive absorption corresponding to the HQ^- tautomer is observable (Figure 3c). The transformations of these two compounds are a good illustration of the mobility of tautomeric equilibria. In water, the HQ^- tautomer is predominating for the HR^- ions, and the $\text{p}K_{\text{a}2}$ values at ionic strength of 0.05 M are 4.96 and 4.94, while the thermodynamic are 5.21 and 5.19, respectively [7,62], whereas that of eosin equals to 3.75 [69]. By contrast, in the CTAC micellar system (Table 1) the $\text{p}K_{\text{a}2}^{\text{a}}$ values for the dihalogen derivatives, 5.79 and 6.00, are much closer to the $\text{p}K_{\text{a}2}^{\text{a}} = 5.76$ of eosin. This should be explained by the partial conversion of the “carboxylate” tautomer HQ^- to HX^- . As result, the $\text{p}K_{\text{a}2}^{\text{a}}$ s of the dihalogen derivatives approach $\text{p}k_{2,\text{COOH}}$. Equation (20) demonstrates the factors that determine the position of the tautomeric equilibrium of the monoanion.

$$\log K_{\text{T}x} = \text{p}k_{1,\text{COOH}} - \text{p}k_{1,\text{OH}} = \text{p}k_{2,\text{COOH}} - \text{p}k_{2,\text{OH}} \quad (20)$$

Lactonic anions type: $(\text{HX}^- \rightleftharpoons \text{HL}^-) \rightarrow (\text{X}^{2-} \rightleftharpoons \text{L}^{2-})$ (Figure 3d). Finally, a group of hydroxyxanthene dyes able to formation of lactonic anions should be regarded. Here, besides anions of HX^- and X^{2-} types, the ions HL^- and L^{2-} appear. As it was mentioned above, 4,5-dinitro-2,7-dibromofluorescein may be also considered as a (potential) member of this group. But much more expressed are the $A_{\max}(\text{HR}^-)/A_{\max}(\text{R}^{2-})$ values for 4,5-dibromo-2,7-dinitrofluorescein (4.62) and 4,5-dibromo-2-nitrofluorescein (0.566). In 50 mas % ethanol, the corresponding ratios are 1.52 and 0.35, respectively [47]. For 2,4,5,7-tetranitrofluorescein (Figure 1d), the X^{2-} fraction is negligible and the L^{2-} tautomer predominates; $\lambda_{\max} = 405$ nm, molar absorptivity $E_{\max} = 30 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. The presence of the HX^- tautomer of the monoanion is evident because the value $E_{\max} = 61.9 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ registered at 525 nm [37]. At the same time, the fraction of HL^- is less understandable.

In order to clarify the mobility of the $\text{X}^{2-} \rightleftharpoons \text{L}^{2-}$ tautomeric equilibria, we determined the absorption spectra of the 4,5-dibromo-2,7-dinitrofluorescein dianion R^{2-} in different solvents.

4.4. Chain-Ring Tautomerism of the Dianion of 4,5-dibromo-2,7-dinitrofluorescein

The chain-ring tautomerism of the dianion of 4,5-dibromo-2,7-dinitrofluorescein, $\text{X}^{2-} \rightleftharpoons \text{L}^{2-}$, was additionally studied in H_2O –ethanol and H_2O –acetone mixtures. Some representative spectra are shown in Figure 4. The data clearly demonstrate the dependence of tautomeric equilibrium state on the solvent composition (Scheme 5).

In the CTAC micellar solutions at 4.0 M KCl, the molar maximal absorptivities, E_{\max} , of the R^{2-} and HR^- ions are 17.2×10^3 and $43.3 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, respectively. For the structures of X^{2-} , HX^- , and X^- in this organized solvent, the E_{\max} values are much higher [7]. For the R^{2-} ions of fluorescein, sulfonefluorescein, and eosin $E_{\max} = (85.9, 83.7, \text{ and } 103.4) \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. For HR^- of eosin and R^- of ethyl eosin, these values are 97.6×10^3 and $97.1 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. For these dye anions, the variations of solvent nature [7] result in λ_{\max} changes whereas the alterations of molar absorptivities are much less expressed than that in Figure 4 and should be classified as common solvatochromic effects.

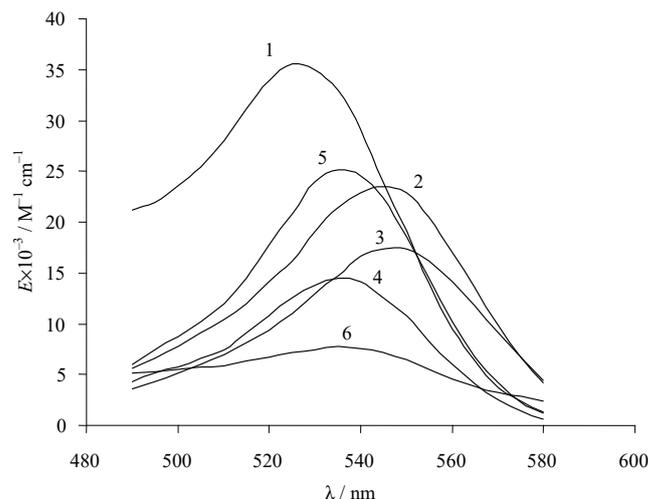
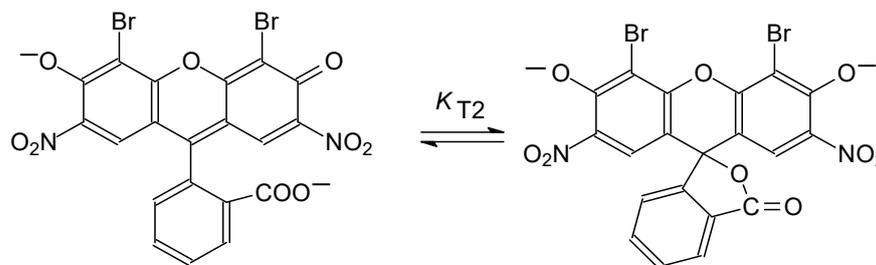


Figure 4. Absorption spectra of the dianion R^{2-} of 4,5-dibromo-2,7-dinitrofluorescein in: water (1); 0.003 M CTAB micellar solution (2); 0.003 M CTAC + 4.0 M KCl solution (3); 50 mass % aqueous ethanol (4); 90 mass % aqueous ethanol (5); and in 90 mass % aqueous acetone (6).



Scheme 5. Tautomeric equilibrium $X^{2-} \rightleftharpoons L^{2-}$ of 4,5-dibromo-2,7-dinitrofluorescein dianion.

It should be noted that it is precisely the pronounced dependence of the molar absorptivity on the solvent nature that convincingly indicates the mobility of the tautomeric equilibrium. The low E_{\max} value itself may be due to the peculiarities of the chromophore system. For instance, the molar absorptivities of HR^- and R^{2-} anions of thiofluorescein in water–ethanol and water–acetone mixtures are ca. 5-fold lower as compared with those of fluorescein and related dyes [48,75], but their dependence on the solvent composition is practically not expressed. For thioeosin, thioerythrosin, and 4,5-dinitrothiofluorescein, the E_{\max} values in 50 mass % ethanol of anions are 2-fold lower than for eosin, erythrosin, and 4,5-dinitrofluorescein, but for all these sulfur-containing dyes, no fundamental changes in the spectra were observed on going to 80% ethanol or acetone, as well as to a CTAC micellar solution with or without 4.0 M KCl [48]. Hence, for these dyes there are no reasons to suspect the substantial fractions of lactonic anions.

In the case of 4,5-dibromo-2,7-dinitrofluorescein anions, the shift of the tautomeric equilibrium is much more expressed. If the value of $(90\text{--}100) \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ is chosen as a tentative standard of molar absorptivity for X^{2-} and HX^- species, then the $\alpha_{X^{2-}}$ and α_{HX^-} values are ≈ 0.2 and ≈ 0.5 , respectively. Accordingly, $\alpha_{L^{2-}} \approx 0.8$ and $\alpha_{HL^-} \approx 0.5$. In general, the expressions for the tautomerization constants of anions look like this:

$$\begin{aligned} \log K_{T1} &= pk_{1,COOH} - pk_{1,L} + \log K_T; \\ \log K'_{T1} &= pk_{1,OH} - pk_{1,L} + \log K_T; \end{aligned} \quad (21)$$

$$\log K_{T2} = pk_{2,OH} - pk_{2,L} + \log K_{T1} = pk_{2,COOH} - pk_{2,L} + \log K'_{T1} \quad (22)$$

Generally speaking, chain-ring tautomeric equilibria in the fluorescein series can be considered as an intramolecular reaction of an anionic carboxylate group with a central carbon atom acting as a Lewis acid.

4.5. Dependence of the Ratio of the Values of the Stepwise Ionization Constants on the Character of Tautomerism

Fluorescein type: The pK_{a2}^a values are equal to $pK_{2,OH}$ and are similar for dyes with identical xanthene moiety, namely, fluorescein, 3',4',5',6'-tetrabromofluorescein, and sulfonefluorescein. The average value is 7.15, while replacing of the oxygen heteroatom by sulfur atom or introduction of a nitro group result in increase and decrease by 0.6 units, respectively. The pK_{a1}^a values are complicated, and thus the difference between the pK_{as} of stepwise dissociation can be represented as follows:

$$pK_{a2}^a - pK_{a1}^a = pK_{2,OH} - pK_{1,COOH} + \log \alpha_{H_2Q} \quad (23)$$

With the exception of sulfonefluorescein, where the neutral form exists as zwitterion, other ($pK_{a2}^a - pK_{a1}^a$) values are very small because of the substantial contribution of the $\log \alpha_{H_2Q}$ item. The increase in the dissociation constant $k_{1,COOH}$ of 3',4',5',6'-tetrabromofluorescein is evidently compensated by the less expressed formation of the lactonic cycle of the neutral molecule.

Eosin type: The pK_{a2}^a values are equal to $pK_{2,COOH}$. They are similar to eosin and thioeosin, but are ca. a unity lower for 4,5-dinitro derivatives. This reflects strong inductive effect of nitro groups. By contrast, the pK_{a1}^a values are quite different within this group of dyes. The $pK_{1,OH}$ value of eosin and, probably, for thioeosin should be equated to the pK_{a1}^a of ethyl eosin or n-decyl eosin, which are 1.11 and 1.18, respectively. On the other hand, the comparison of the pK_{as} of ethyl fluorescein, sulfonefluorescein, ethyl eosin, and 4,5-dinitrosulfonefluorescein in water [7,76] allows concluding that two NO_2 groups in ortho-position to the OH group display ca. the same effect as four Br atoms. Analogous conclusion may be made when comparing the pK_{a2}^a values of 3,3',5,5'-tetrabromo sulfonephthalein (indicator bromophenol blue) and 3,3'-dinitro-sulfonephthalein (nitrophenol violet) both in water and dimethyl sulfoxide [77]. Therefore, the dramatic increase in the pK_{a1}^a and decrease in the ($pK_{a2}^a - pK_{a1}^a$) of 4,5-dinitro derivatives (Table 3) as compared with eosin dyes is caused by the expressed shift of the tautomeric equilibrium $H_2Q \rightleftharpoons H_2L$ toward the right.

$$pK_{a2}^a - pK_{a1}^a = pK_{2,COOH} - pK_{1,OH} + \log \alpha_{H_2Q} \quad (24)$$

Table 3. Main types of tautomerism of fluorescein anions.

Type of Tautomerism. Predominating Forms of Anions	Dye	pK_{a1}^a	pK_{a2}^a	$pK_{a2}^a - pK_{a1}^a$
Fluorescein type $HQ^- \rightarrow X^{2-}$	Sulfonefluorescein	2.33	7.00	4.67 ^a
	Fluorescein	6.41	7.17	0.76
	3',4',5',6'-Tetrabromofluorescein	6.66	7.27	0.61
	Thiofluorescein	7.67	7.77	0.10
	4-Nitrothiofluorescein	5.74	6.56	0.82
Intervening type $(HQ^- \rightleftharpoons HX^-) \rightarrow X^{2-}$	2,7-Dichlorofluorescein	5.50	5.79	0.29
	4,5-Dibromofluorescein	5.34	6.00	0.66
Eosin type $HX^- \rightarrow X^{2-}$	Eosin	1.83	5.76	3.93
	Thioeosin	1.69	6.07	4.38
	4,5-Dinitro-N-ethylazafluorescein	3.10	5.21	2.11
	4,5-Dinitrofluorescein	4.04	4.92	0.88
	4,5-Dinitrothiofluorescein	4.88	4.91	0.03
	4,5-Dinitro-2,7-dibromofluorescein ^b	-0.40	4.63	5.03
Lactoid anions type $(HX^- \rightleftharpoons HL^-) \rightarrow (X^{2-} \rightleftharpoons L^{2-})$	4,5-Dinitro-2,7-dibromofluorescein ^b	-0.40	4.63	5.03
	4,5-Dibromo-2,7-dinitrofluorescein	1.83	4.35	2.52
	4,5-Dibromo-2-nitrofluorescein	3.92	5.50	1.58
	2,4,5,7-Tetranitrofluorescein	0.16	1.45	1.29

Note. ^a In this case, the pK_{a1}^a value corresponds to the equilibrium $H_2Z^{\pm} \rightleftharpoons HQ^- + H^+$; $pK_{a2}^a - pK_{a1}^a = pK_{2,OH} - pK_{1,Z}$. ^b This compound can be attributed to both groups of dyes.

As result, the negative contribution of the term $\log \alpha_{\text{H}_2\text{Q}}$ is substantial. For example, the $\alpha_{\text{H}_2\text{Q}} = 0.036$ value was estimated for the dye 4,5-dinitrofluorescein, which allows to estimate the value $\text{pk}_{1,\text{OH}} = 2.6$. 4,5-dinitro-2,7-dibromofluorescein is an exception, because of the presence of both two nitro groups and two bromine atoms, which results in dramatic drop of the $\text{pk}_{1,\text{OH}}$ value and hence increasing in $\text{pK}_{\text{a}2}^{\text{a}} - \text{pK}_{\text{a}1}^{\text{a}}$. Besides, in the case of this dye, the intensity of the absorption band of R^{2-} is ca. 30% lower as compared with that of the monoanion HR^- . Therefore, this dye may be (partly) referred to the lactoid anions type (Table 3).

Intervening type: For dihalogen derivatives, the concentrations of tautomers HQ^- and HX^- are commensurable, and the pk_{OH} values lower than those of fluorescein.

$$\begin{aligned} \text{pK}_{\text{a}2}^{\text{a}} - \text{pK}_{\text{a}1}^{\text{a}} &= \text{pk}_{2,\text{OH}} - \text{pk}_{1,\text{COOH}} + \alpha_{\text{H}_2\text{Q}} - 2 \log \alpha_{\text{HQ}^-} \\ &= \text{pk}_{2,\text{COOH}} - \text{pk}_{1,\text{OH}} + \log \alpha_{\text{H}_2\text{Q}} - 2 \log \alpha_{\text{HX}^-} \end{aligned} \quad (25)$$

As result, the $\text{pK}_{\text{a}1}^{\text{a}}$ and $\text{pK}_{\text{a}2}^{\text{a}}$ values approach each other, although the $\alpha_{\text{H}_2\text{Q}}$ values are not as small as for the un-substituted fluorescein. First of all, the thermodynamic $\text{pK}_{\text{a}1}$ values of 4,5-dibromo- und 2,7-dichlorofluorescein in water are equal to 4.32 and 4.00 [7,62], and ongoing to CTAC media (Table 1) they increase by 1.0–1.5 units largely thanks to the pronounced shift of the tautomeric equilibrium $\text{H}_2\text{Q} \rightleftharpoons \text{H}_2\text{L}$ toward the right. By contrast, the $\text{pK}_{\text{a}1}^{\text{a}}$ of eosin even drops on going to CTAB medium (1.83) to water, where $\text{pK}_{\text{a}1} = 2.81$ [69]. This is due to a well-known regularity: for the molecular form of eosin, the transition from water to non-aqueous media weakly shifts the position of the tautomeric equilibrium [7,57].

For 4,5-dibromofluorescein, the above assumptions about the absorption spectra of tautomers allow us to estimate the values $\alpha_{\text{H}_2\text{Q}} = 0.067$ and $\alpha_{\text{HX}^-} = 0.47$. Then $\text{pk}_{1,\text{OH}} = 4.5$; $\text{pk}_{1,\text{COOH}} = 4.45$; $\text{pk}_{2,\text{OH}} = 5.72$; $\text{pk}_{2,\text{COOH}} = 5.67$. These, as well as the above data, allow us to compose the following series (Table 4).

Table 4. Indices of the ionization constant of the OH group in the fluorescein derivative series.

Dye	$\text{pk}_{1,\text{OH}}$
Ethyl fluorescein; <i>n</i> -decyl fluorescein; ^a 6-hydroxy-9-phenylfluorone; <i>n</i> -hexadecyl fluorescein	6.59; 6.61; 6.67; 7.06
4,5-Dibromofluorescein; 2,7-dichlorofluorescein	4.5; 4.1
4,5-Dinitrofluorescein	2.6
Eosin; ^b ethyl eosin ^b	1.40; 1.11
4,5-Dibromo-2,7-dinitrofluorescein; 4,5-dinitro-2,7-dibromofluorescein	below 0

Note. ^a From ref. [8]. ^b From ref. [7].

Thus arranged data clearly demonstrate the huge effect displayed by the nitro groups.

Data in Table 4 may be supplemented with $\text{pk}_{2,\text{OH}}$ values 7.00–7.27 of fluorescein, sulfonefluorescein, and 3',4',5',6'-tetrabromofluorescein, on the one hand, and 5.14–5.72 of 2,7-dichloro- and 4,5-dibromofluorescein. As can be seen, these $\text{pk}_{2,\text{OH}}$ values are always more or less higher than the corresponding $\text{pk}_{1,\text{OH}}$ s. The effect may be semi-quantitatively explained by the Bjerrum–Kirkwood–Westheimer equation [57]

$$\Delta \text{pk}_{\text{a}}^{\text{el}} = \frac{e^2 N_{\text{A}}}{4\pi \times 8.854 \times 10^{-12} \times 2.303 R T r \epsilon_{\text{eff}}} = \frac{24.7}{r \epsilon_{\text{eff}}} \quad (26)$$

Here, e , N_{A} , R , and T have their usual meanings, r is the distance between the negatively charged group and the ionizing group (here expressed in nm; $T = 298.15$ K), ϵ_{eff} is the effective relative permittivity of the space permeated by the electric field lines. For more details, see the book by Vereshchagin [78].

Lactonic anions type: Most complicated is the equilibrium scheme in the case of the dyes inclined to formation of anionic lactones. Introduction of nitro groups in 2- and 7-positions favors the lactone formation owing to the decrease in the electronic density at the nodal C₉. However, the resulting effects are not so obvious. For example, in the case of 2,4,5,7-tetranitrofluorescein the dianion R²⁻ exists almost in the form of a lactone. Note, that the last is not colorless but yellow, owing to nitrophenolate absorption.

Let us consider the protolytic equilibrium of 4,5-dibromo-2,7-dinitrofluorescein in more detail. Tamburello-Luca et al. [46] studied the ionization of this dye on the water/air interface using the surface second harmonic generation; the values $pK_{a1} = 4.0$ and $pK_{a2} = 4.2$ were reported. These authors considered only the deeply colored anionic structures HX^- and X^{2-} , ignoring the possibility of their lactonization. However, this was before the appearance of our publications [47,48].

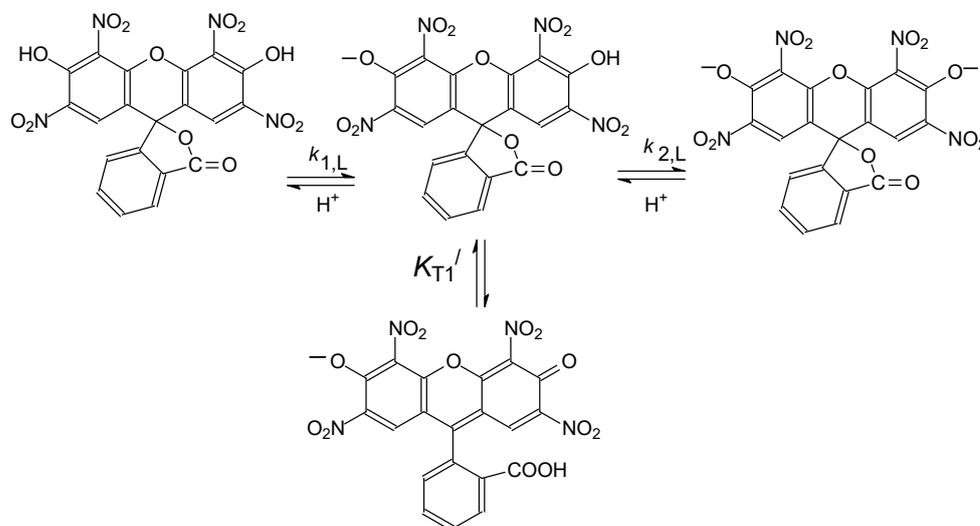
Using our values determined in CTAC micellar solution, $pK_{a1}^a = 1.83$ and $pK_{a2}^a = 4.35$ (Table 1), and the above estimated fractions of the tautomers, the following indices of microscopic ionization constants can be calculated: $pk_{1,L} = 1.5$, $pk_{2,L} = 4.1$, and $pk_{2,COOH} = 4.05$. The last value is in line with the aforementioned inductive effect of the NO₂ group, because the conjugation between the xanthenes moiety and phthalic acid residue is absent. In 50 mass % aqueous ethanol [47], the corresponding values are as follows: $pk_{1,L} = 3.5$, $pk_{2,L} = 5.1$, and $pk_{2,COOH} = 5.3$.

It is of common knowledge that for statistical reasons the difference $pk_{2,L} - pk_{1,L}$ should include the log 4 contribution. In addition, the abovementioned Δpk_a^{el} should be taken into account:

$$pk_{2,L} - pk_{1,L} = 0.602 + \frac{24.7}{re_{eff}} \quad (27)$$

Therefore, the last-named value in CTAC micelles may be estimated as $\Delta pk_a^{el} = 4.1 - 1.5 - 0.602 = 2.0$. In 50 mass % ethanol, the corresponding value is equal to 1.0.

Now consider also the protolytic equilibrium of 2,4,5,7-tetranitrofluorescein. From the molar absorptivities given in Section 4.3, the $\alpha_{HX^-} = 0.65$ and $\alpha_{HL^-} = 0.35$ values can be estimated as for 4,5-dibromo-2,7-dinitrofluorescein was done. This leads to the values $pk_{1,L} = 0.62$, $pk_{2,L} = 0.99$ (Scheme 6). In 50 mass % aqueous ethanol [47], the corresponding values equal to $pk_{1,L} = 1.6$ and $pk_{2,L} = 2.1$. Batistela et al. [29] reported for 2,4,5,7-tetranitrofluorescein in water the values $pK_{a1} = 0.38$ and $pK_{a1} = 2.48$. Using these data and their $E_{max} = 25 \times 10^3$ value for the HR^- form, we have calculated approximate estimates of $pk_{1,L}$ and $pk_{2,L}$ to be 0.9 and 1.9, respectively. Therefore, the difference here is also very small.



Scheme 6. Acid-base ionization and tautomerism of 2,4,5,7-tetranitrofluorescein.

In the micellar system, $pK_{2,L} - pK_{1,L} = 0.37$, and after subtracting the statistical difference of 0.602 we receive even a negative value of -0.23 , which seems to contradict the physical meaning. Indeed, the ΔpK_a^{el} must be substantially positive. In 50 mass % ethanol the situation is similar. Possible ionic association in the Stern layer can further stabilize the dianion, but such effects are less likely in aqueous ethanol. Probably, in this case we are dealing with an increased stabilization of the dianion-lactone structure due to the maximum delocalization of negative charges on nitro groups. Such effects are known for compounds of porphyrin series where they may cause even an inversion of the stepwise dissociation constants [79,80]. Hence, the advantage of maximum charge delocalization in the aromatic system outweighs the electrostatic factor described by Equation (26).

Note, that in the lactonic structure L^{2-} , which absolutely dominates in the case of the tetranitrofluorescein dianion R^{2-} , the conjugated aromatic system is split into two almost isolated rings. The character of the tautomerism of 2,4,5,7-tetranitrofluorescein anions is rather instructively. Indeed, for the monoanion HR^- of the dye, the HX^- structure is preferable. This is an argument in favor of the importance of the symmetry of the chromophore system for the stabilization of the corresponding tautomer.

Lactone formation is much more expressed for phthalein dyes. Indeed, in the case of the un-substituted phenolphthalein in water with 8 mass % ethanol, the tautomer of HL^- type predominates whereas the fraction of the L^{2-} tautomer is about 0.54 [81]. Again, the thermodynamic values of $pK_{1,L} = 9.3$ and $pK_{2,L} = 10.3$ are very close [72,81]. For chloro-, bromo- and nitro-derivatives of phenolphthalein, the tendency of anions to form lactone is even stronger [71].

4.6. Solvation Properties of the CTAC–4.0 M KCl System

Table 1 contains 45 pK_a^a values, which characterize the protolytic equilibria of 22 fluorescein dyes. In addition, there are pK_a^a s of eight sulfonephthalein indicators, four dinitrophenols, 2,6-diphenyl-4-(2,4,6-triphenylpyridinium-1-yl) phenolate, and N,N' -di-*n*-octadecyl rhodamine. These 59 values cover the range from -0.5 to 10.47 . To compare the solvation properties of the CTAC + 4.0 M KCl system with those of some non-aqueous solvents, we chose a 1:1 mixture of water and ethanol (by mass). This (or similar) mixed solvent is often used not only in our studies [39,40,47,48], but also in works of other authors [28,82].

The polarity of the above mixed solvent is much higher in respect to the micellar pseudophase studied in this paper; the corresponding E_T^N values are 0.75 [83] and 0.623 (see above). Consequently, the Stern layer in this micellar system is somewhat less polar with respect to cationic micelles at a low bulk ionic strength. The reason probably lies in the weaker hydration of the Stern layer in the presence of 4.0 M KCl. For the last-named cationic micelles at low ionic strength, $E_T^N = 0.69$ – 0.70 [8]. At the same time, the pK_a values in 50 mass % ethanol are always higher as compared with the pK_a^a s of the same dyes. The correlation is depicted in Figure 5.

However, a more detailed analysis requires taking into account the differences in the type of these dissociation constants. Indeed, for the pK_a values in the solvent “s” the following expression is valid:

$$pK_a^s = pK_a^w + \log {}^w\gamma_{H^+}^s + \log \frac{{}^w\gamma_B^s}{{}^w\gamma_{HB}^s} \quad (28)$$

Here ${}^w\gamma_{H^+}^s$ stands for activity coefficient of proton transfer from water to the given solvent; for 50 mass % ethanol, $\log {}^w\gamma_{H^+}^s = -0.66$ [84]. In the case of the pK_a^a values, there is no such contribution, since the pH values are determined in the bulk phase and not in the pseudophase. On the other hand, the indices of the apparent constants contain the item $-\Psi F/2.303RT$. For the colloidal system under study, it was estimated above as -0.26 . Strictly speaking, both ${}^w\gamma_{H^+}^s$ and Ψ values are extra-thermodynamic quantities, but using them shows that the ${}^w\gamma_B^m/{}^w\gamma_{HB}^m$ and ${}^w\gamma_B^s/{}^w\gamma_{HB}^s$ values approach each other.

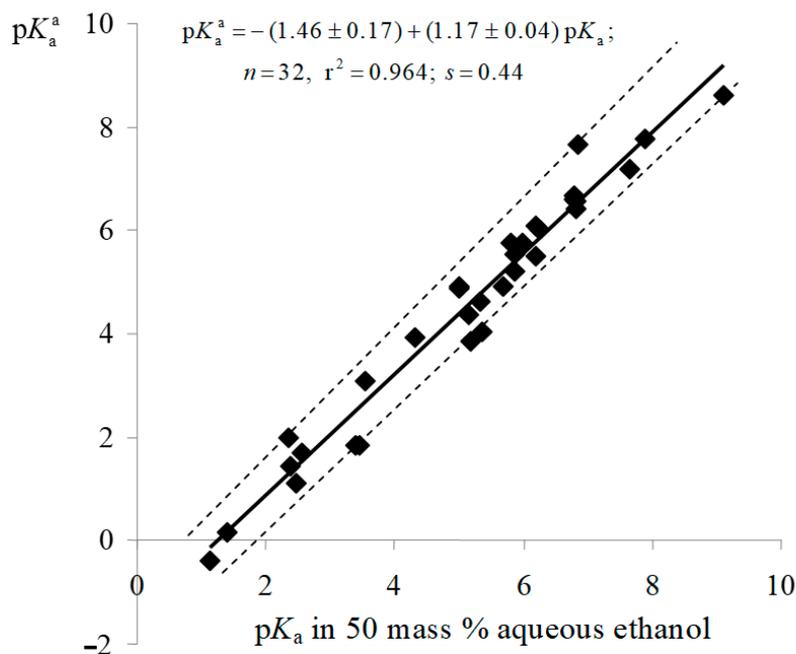


Figure 5. Dependence of pK_{a1}^a and pK_{a2}^a values of fluorescein dyes, pK_{a0}^a of 2,6-diphenyl-4-(2,4,6-triphenylpyridinium-1-yl) phenol, and pK_{a2}^a of bromophenol blue on their pK_a values in 50 mass % aqueous ethanol.

Electrostatic interactions between the cationic head groups of the surfactant and dye anions may additionally stabilize the latter. However, as was shown earlier [7], such interactions should not be considered as neutralization of negative charges. Rather, they should be viewed as the formation of “loose” ionic associates that (latent) influence the ${}^w\gamma_i^m$ values.

Finally, it should be noted that these transfer activity coefficients include the influence of the shift of the tautomeric equilibrium (if any) on going from one medium to another. For the fluoresceins with free carboxylic group, these equilibria were considered above.

5. Conclusions

In this paper, visible spectra and apparent ionization constants of a set of fluorescein dyes and their analogues in CTAC micelles in the presence of 4.0 M KCl are reported. Neutral forms of the dyes exist as colorless lactone H_2L with very small fraction of the quinonoidal H_2Q tautomer. Basing on the character of the absorption bands of monoanions HR^- , which were singled out of the spectral data, conclusions about their structure in solution are made.

As a result, the most detailed classification of tautomerism of fluorescein dyes known to date is proposed. The dyes with free COOH group can be divided into four groups.

First, for dyes of the “fluorescein type”, fluorescein, sulfonefluorescein, 3',4',5',6'-tetrabromofluorescein, thiofluorescein, 4-nitrothiofluorescein, the monoanion exists in solution as a “carboxylate” tautomer HQ^- , with non-ionized OH group and COO^- (or SO_3^-) in the phthalic residue.

Second, for the “eosin type” (eosin, thioeosin, 4,5-dinitro-*N*-ethylazafluorescein, 4,5-dinitrofluorescein, 4,5-dinitrothiofluorescein) a tautomer with ionized hydroxyl group and non-ionized COOH group, HX^- predominates.

Third, for dyes with two halogen atoms in the xanthene portion, 2,7-dichloro- and 4,5-dibromofluoresceins, the concentrations of HQ^- and HX^- tautomers are comparable. This is an “intervening type” of fluorescein dyes.

For all the three groups of dyes, the dianion R^{2-} exists as a deeply colored dye X^{2-} with ionized xanthene moiety and COO^- group.

Fourth group consists of dyes whose anions tend to form lactones HL^- and L^{2-} along with intensively colored tautomers. These are following compounds: 4,5-dibromo-2,7-dinitrofluorescein, 4,5-dibromo-2-iodofluorescein, 2,4,5,7-tetranitrofluorescein, and 4,5-dinitro-2,7-dibromo fluorescein. The last can also be partially attributed to the “eosin type”. For 2,4,5,7-tetranitrofluorescein, the L^{2-} tautomer predominates. Despite its lactonic structure, it is not colorless but yellow owing to the “nitrophenolate” absorption.

Lactone formation is impossible for dyes with esterified carboxylic groups and related compounds. Their ionization constants are also included in the discussion of the general scheme of protolytic equilibrium. It should be also kept in mind that it is impossible to directly estimate too large or too small values of the tautomerization constants. In addition, the presence of colorless lactones in an equilibrium mixture of tautomers may be surely stated only on substantial decrease in the molar absorptivity on going from one solvent to another.

Equations are proposed that allow to explain the difference between the indices of the stepwise ionization of the dyes, $pK_{a2}^a - pK_{a1}^a$, and to predict the state of the tautomeric equilibrium of anions.

Finally, a correlation between the apparent pK_{a1}^a and pK_{a2}^a values and the corresponding pK_a s in 50 mass % aqueous ethanol is obtained.

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