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Protolytic Properties of Thiofluorescein and Its Derivatives

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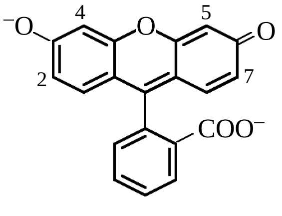
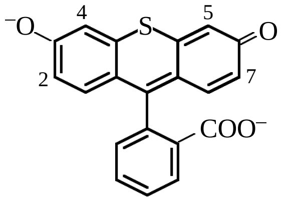
Abstract—Ionization constants of thiofluorescein, 4-nitro-, 4,5-dinitro-, 2,4,5,7-tetrabromo-, and 2,4,5,7-tetraiodothiofluoresceins, and also of 4,5-dibromofluorescein and *N*-ethyl-4,5-dinitroazafluorescein in 50 wt % aqueous ethanol have been determined. The electronic absorption spectra of their ionic and molecular forms have been recorded and analyzed. The behavior of these dyes can be described in terms of the scheme of prototropic equilibria proposed previously for fluorescein and its halo derivatives.

In continuation of our systematic studies on hydroxyxanthene dyes and related compounds [1–7], in the present work we have examined acid–base and tautomeric transformations of thiofluorescein and its derivatives. Although thio analogs of fluorescein dyes were synthesized as early as at the turn of the XIXth century [8–10], their absorption and luminescence spectral properties have become the subject of detailed studies only recently [11]. Taking into account a few quantitative data available on acid–base properties of fluorescein thio analogs, the goal of our study was to fill this gap and compare their pK_a values and tautomerization constants with those found for hydroxyxanthenes.

As reference compounds we used Fluorescein (**I**), 2,4,5,7-tetrabromofluorescein (**II**, Eosin), and 2,4,5,7-tetraiodofluorescein (**III**, Erythrosin). These were compared with thiofluorestcein (**IV**), 2,4,5,7-tetrabromothiofluorescein (**V**), and 2,4,5,7-tetraiodothiofluorescein (**VI**). The structures of dianions derived from compounds **I** and **IV** are shown below.





We recently studied in detail nitro derivatives of fluorescein [6, 7]; therefore, two nitro derivatives of thiofluorescein, 4-nitrothiofluorescein (**VII**) and 4,5-dinitrothiofluorescein (**VIII**) were also included. The properties of the latter were compared with those of both 4,5-dinitrofluorescein (**IX**) and 4,5-dibromofluorescein (**X**), and *N*-ethyl-4,5-dinitroazafluorescein (**XI**). The quinoid molecular form of **XI** may be referred to as 2-(10-ethyl-6-hydroxy-4,5-dinitro-3-oxo-3,10-dihydroacridin-9-yl)benzoic acid. The values of pK_a, corresponding to step ionization equilibria (1)–(3), were determined by spectrophotometry. The pH values were monitored by potentiometry using a glass electrode in an electron transport circuit.

$$\mathrm{H_3R^+ \rightleftarrows H_2R + H^+}, \quad K_{\mathrm{a},0}, \tag{1}$$

$$\mathrm{H_2R \rightleftarrows HR^- + H^+}, \quad K_{\mathrm{a},1}, \tag{2}$$

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

The use of 50 wt % aqueous ethanol instead of water allowed us (as previously [1, 7]) to increase the solubility of neutral forms and improve the resolution of bands corresponding to mono- and dianions ($\mathrm{HR^-}$ and $\mathrm{R^{2-}}$, respectively) of some dyes. The pH values were reduced to infinite dilution in a given solvent ($\mathrm{p}a^*_{\mathrm{H^+}}$) and were estimated by adding a correction to the experimental value ($\mathrm{pH_{instr}}$): $\mathrm{p}a^*_{\mathrm{H^+}} = \mathrm{pH_{instr}} - 0.20$ [12–14]. By analysis of the spectral curves (Fig. 1) and dependences of optical density D upon $\mathrm{p}a^*_{\mathrm{H^+}}$ at a constant ionic strength (Fig. 2) we estimated pK_a values; the thermodynamic values are collected in table.

Figures 3 and 4 show the electronic absorption spectra of ionic and molecular forms of the dyes under study. The molar absorption coefficients ε of $\mathrm{HR^-}$ at various wavelengths were calculated together with p$K_{\mathrm{a},1}$ and p$K_{\mathrm{a},2}$, since the highest yield of $\mathrm{HR^-}$ for

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† Deceased.

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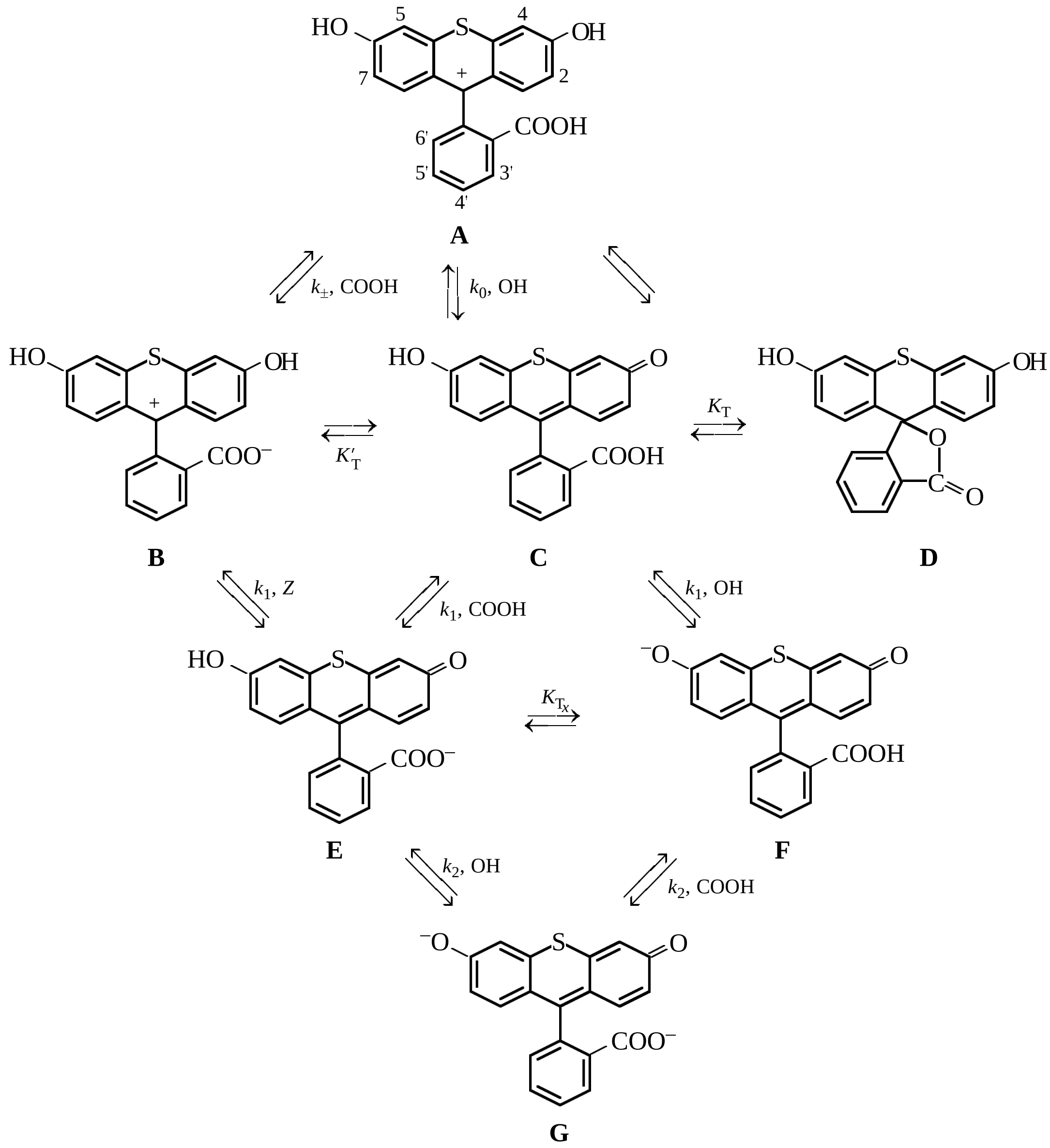


K_T = [**D**]/[**C**], K_T' = [**B**]/[**C**], K_T'' = K_T/K_T' = [**D**]/[**B**], K_{T_x} = [**F**]/[**E**], $k_{\pm,\mathrm{COOH}} = a^*_{\mathrm{H}^+}a_{\mathbf{B}}/a_{\mathbf{A}}$, $k_{0,\mathrm{OH}} = a^*_{\mathrm{H}^+}a_{\mathbf{C}}/a_{\mathbf{A}}$, $k_{1,Z} = a^*_{\mathrm{H}^+}a_{\mathbf{D}}/a_{\mathbf{B}}$, $k_{1,\mathrm{COOH}} = a^*_{\mathrm{H}^+}a_{\mathbf{E}}/a_{\mathbf{C}}$, $k_{1,\mathrm{OH}} = a^*_{\mathrm{H}^+}a_{\mathbf{F}}/a_{\mathbf{C}}$, $k_{2,\mathrm{OH}} = a^*_{\mathrm{H}^+}a_{\mathbf{G}}/a_{\mathbf{E}}$, $k_{2,\mathrm{COOH}} = a^*_{\mathrm{H}^+}a_{\mathbf{G}}/a_{\mathbf{F}}$.

some dyes in solution did not attain 50%, so that direct registration of the corresponding spectrum was impossible. As with hydroxyxanthene dyes, a sharp reduction in the absorption intensity was observed in going from anionic and cationic species to neutral. These data suggest formation of lactones as colorless neutral species in which the spiro carbon atom has sp^3 hybridization rather than sp^2 (as in the dye). Depending on the nature and number of substituents, the spectra of monoanions HR^- are either similar to or different from the spectra of dianions R^{2-}. Therefore, for thiofluorescein dyes we propose the same scheme of protolytic transformations as that given in [1–5] for fluorescein derivatives.

The scheme was interpreted on the basis of the previously verified concept, according to which the electronic absorption bands of structures differing only by the 2'-substituent are similar (structures **A** and **B**, **C** and **E**, and **F** and **G**, respectively). When the absorption bands are sufficiently narrow (structures **F** and **G**), dissociation of the carboxy group (COOH → COO⁻) is accompanied by a blue shift by several nanometers. In this respect, the spectra of mono- and di-

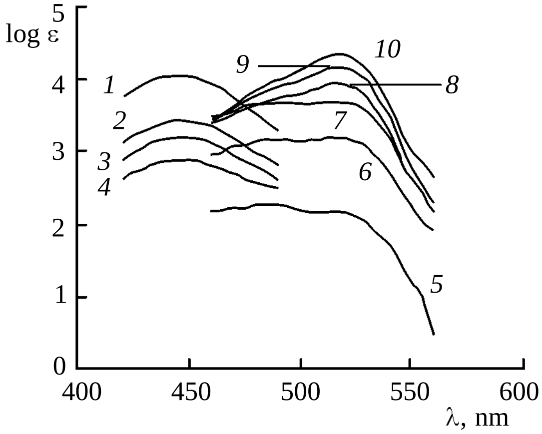


Fig. 1. Electronic absorption spectra of thiofluorescein (**IV**) in 50% aqueous ethanol at different acidities: (*1*) 2–4 M H_2SO_4 (cation H_3R^+), (*2*) $pa^*_{H^+}$ 1.63, (*3*) $pa^*_{H^+}$ 1.93, (*4*) $pa^*_{H^+}$ 2.32, (*5*) neutral form H_2R, (*6*) $pa^*_{H^+}$ 6.33, (*7*) monoanion HR^- [calculated by Eq. (10)], (*8*) $pa^*_{H^+}$ 7.19, (*9*) $pa^*_{H^+}$ 7.66, and (*10*) $pa^*_{H^+}$ 10–12 (dianion R^{2-}).

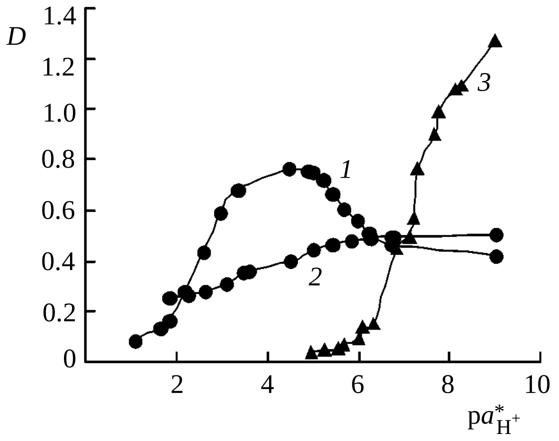
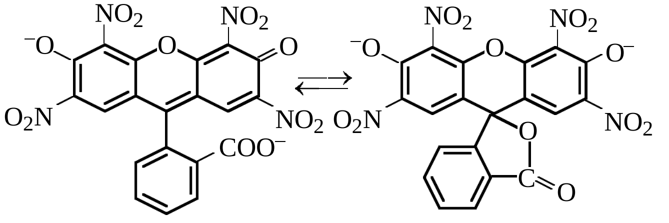


Fig. 2. Plots of optical density (*D*) of dye solutions in 50% aqueous ethanol vs. $pa^*_{H^+}$; ionic strength 0.05 M: (*1*) compound **V**, λ 550 nm; (*2*) compound **XI**, λ 450 nm; and (*3*) compound **IV**, λ 540 nm.

anions of compounds **V**, **VI**, **VIII**, and **XI** (Figs. 3, 4) almost do not differ from those of anions derived from Eosin (**II**), Erythrosin (**III**), and Bengal Rose B [1–5]. Mutual arrangement of bands corresponding to the mono- and dianions of thiofluorescein (**IV**) (Fig. 1) strongly resembles the spectral pattern observed for Fluorescein (**I**) [1–4]. Even for compound **I** zwitter-ionic species **B** is observed only in aqueous solution or in aqueous–organic mixtures containing a lot of water [1–4]. As concerns our systems, tautomer **B** can be excluded from consideration; we presume that only neutral quinoid structure **C** (as colored form) coexists with colorless lactone **D**, i.e., $K_T' \ll 1$.

It seemed important to elucidate whether mono- and dianions **H** and **I** having lactone structure exist. As shown previously [7], such structures are typical of nitrofluoresceins with nitro groups in positions *2* and *7*, e.g., for 2,4,5,7-tetranitrofluorescein (**XII**):



G **I**

Structure **H** is analogous to **I**, but one of the hydroxy groups is not ionized. Due to the presence of the electron-acceptor nitro group the effective positive charge on C^9 increases. Lactone anions were also observed for phenolphthalein and its derivatives [15–17]; this is explained, among other factors, by reduced steric hindrance to formation of tetrahedral configuration with its center on C^9. The molar absorption coefficients at the absorption maxima (ε_{max}) of structures **G** and **F** of fluorescein (**I**) and its halo derivatives in water and aqueous–alcoholic media range from 88×10^3 to 112×10^3 $l\,mol^{-1}\,cm^{-1}$ [1–3]. The $\varepsilon_{max}(R^{2-})$ value of 4,5-dibromofluorescein (**X**) is 72.5×10^3 $l\,mol^{-1}\,cm^{-1}$, whereas the corresponding values for thiofluorescein (**IV**) and *N*-ethyl-4,5-dinitroazafluorescein are as low as 22×10^3 and 42×10^3 $l\,mol^{-1}\,cm^{-1}$, respectively. On the average, $\varepsilon_{max}(R^{2-})$ values for sulfur-containing compounds are lower by a factor of 2.3 than those for their oxygen analogs.

Assuming that the low $\varepsilon_{max}(R^{2-})$ values observed for some dyes result from the presence of the colorless lactone dianion in appreciabe amounts, it would be reasonable to expect a notable change of $\varepsilon_{max}(R^{2-})$ on variation of the solvent, as was the case of compound **XII** [7]. Therefore, the spectra of dianions R^{2-} of dyes **IV**, **VIII**, and **XI** were measured not only in 50% ethanol, but also in water, 80% ethanol, 80% acetone, a micellar solution of cetyltrimethylammonium bromide (0.003 M), and in the same solution containing 4 mol/l of KCl. In all cases the spectra change insignificantly; in particular, some variation of λ_{max} was observed, but no appreciable change in the absorption intensity was detected. Therefore, in contrast to fluorescein dyes having nitro groups in

Thermodynamic ionization constants of xanthene dyes in 50 wt % aqueous ethanol (25°C)

Compound	p$K_{a,0}$	p$K_{a,1}$	p$K_{a,2}$
Fluorescein (**I**) [1]	0.94	6.82	7.66
2,4,5,7-Tetrabromofluorescein (**II**) [1]	–	3.46	5.98
2,4,5,7-Tetraiodofluorescein (**III**) [1]	–	3.2[a]	6.0[a]
Thiofluorescein (**IV**)	0.92 ± 0.01	6.85 ± 0.12	7.88 ± 0.06
2,4,5,7-Tetrabromothiofluorescein (**V**)	–	2.56 ± 0.02	6.20 ± 0.05
2,4,5,7-Tetraiodothiofluorescein (**VI**)	–	2.12 ± 0.03	6.37 ± 0.08
4,5-Dibromofluorescein (**X**)	–	5.85 ± 0.06	6.24 ± 0.04
4,5-Dinitrofluorescein (**IX**) [7]	–	5.36 ± 0.16	5.0 ± 0.2
4-Nitrothiofluorescein (**VII**)	–	5.80 ± 0.07	6.81 ± 0.04
4,5-Dinitrothiofluorescein (**VIII**)	–	4.99 ± 0.14	5.69 ± 0.05
N-Ethyl-4,5-dinitroazafluorescein (**XI**)	–	3.54 ± 0.02	5.87 ± 0.06

[a] In 40% ethanol [1].

positions *2* and *7*, we excluded from consideration lactone anions of compounds **IV**–**XI** like we did before for **I**–**III**. The existence of colorless carbinol dianions (products of addition of OH^- and H^+ ions to the central carbon atom and ionized hydroxt group, respectively) also seems to be improbable, for the activities of water in the above media are strongly different and $\varepsilon_{max}(R^{2-})$ values change only slightly. Presumably, the reduction in ε_{max} of dianions of thiofluoresceins **IV**, **VII**, and **VIII**, as compared to oxygen analogs, is explained by distortion of bond angles in the thiopyran ring and of the planar structure of the xanthene fragment with increase in the heteroatom radii; as a result, the conjugation therein becomes weaker.

Let us consider the effects of substituents and heteroatom on the position of the long-wave absorption maximum in the spectra of quinoid dianions **G** derived from dyes **I**–**XI**. Replacement of the oxygen atom by sulfur in the series of 4,5-nitro derivatives leads to a 19-nm red shift of the absorption maximum, while replacement by nitrogen atom with a substituent, to a blue shift of an almost the same magnitude (–18 nm). This pattern may be explained by reduction of the donor effect of the heteroatom bridge (M) in the series S > O > N–Et, which weakens polarization in the short conjugation system $^{-}$O–C=C–C–M– of compounds **IX**, **VIII**, and **XI** in the ground state. In keeping with the classical chromaticity theory [18], electron transition in the above system should be hindered, so that it shifts to the blue region. An analogous blue shift upon replacement of oxygen by NH group (–62 nm) is observed for the dye couple Pyronin–Acridine Orange [18]. Replacement of the oxygen atom by sulfur in unsubstituted Fluorescein (**I**) and Eosin (**II**) also induces a red shift of the long-wave absorption maximum (by 22 and 16 nm, respectively). Presumably, participation of diffuse 3*p* (and even 3*d*) orbitals of the sulfur atom in the formation of molecular orbitals of Thiofluorescein (**IV**) reduces the probability of electron transitions in the short conjugation chain including the heteroatom. In this case the chromophore structure less corresponds to the "branched" conjugation chain model but approaches the state of a simple "long" conjugation chain passing through the bridgehead carbon atom, which is typical of phenolphthalein (λ_{max} 560 nm). In such a way we can rationalize the red shift of λ_{max} in going from Fluorescein to its sulfur analog.

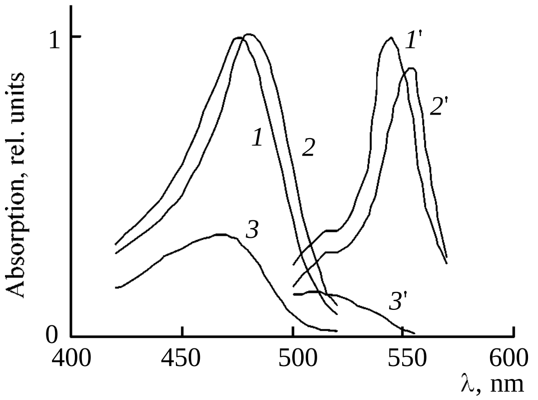


Fig. 3. Absorption spectra of (*1*, *1'*) dianions R^{2-}, (*2*, *2'*) monoanions HR^-, and (*3*, *3'*) neutral forms H_2R of compounds (*1*–*3*) **XI** and (*1'*–*3'*) in 50% aqueous ethanol; ionic strength 0.05 M.

The opposite shifts of λ_{max} caused by successive introduction of one or two nitro groups into positions *4* and *5* of thiofluorescein may be explained in terms of different orientations of the nitro groups relative to the xanthene fragment. According to the results of

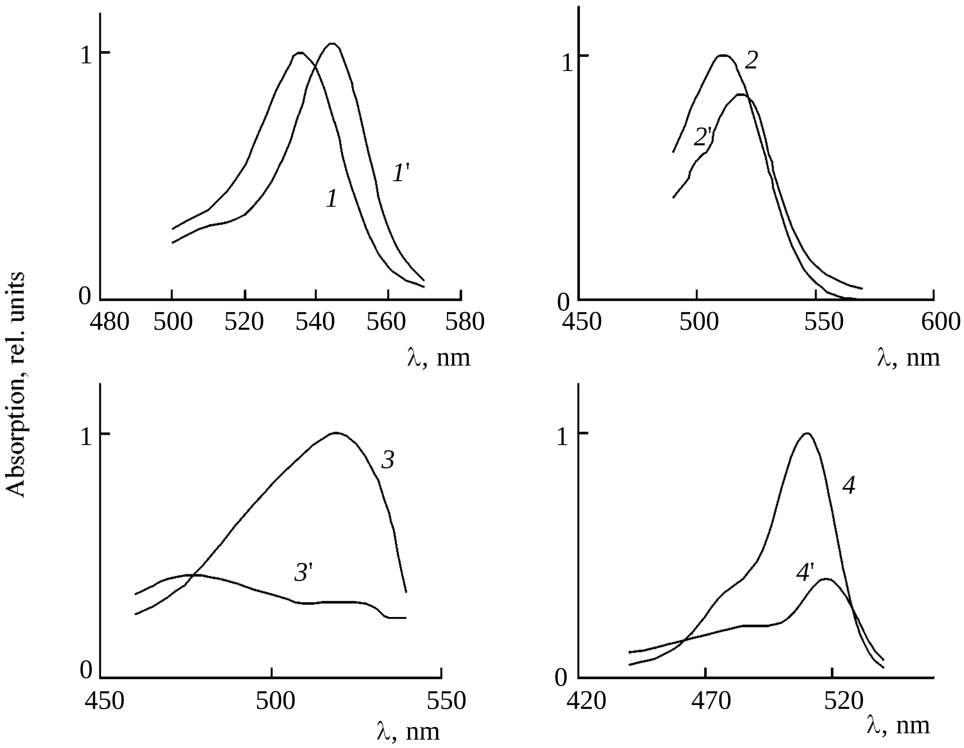


Fig. 4. Absorption spectra of (*1–4*) dianions R^{2-} and (*1'–4'*) monoanions HR^- of compounds (*1, 1'*) **V**, (*2, 2'*) **VIII**, (*3, 3'*) **VII**, and (*4, 4'*) **X** in 50% aqueous ethanol; ionic strength 0.05 M.

our quantum-chemical calculations, both nitro groups in 4,5-dinitrothiofluorescein (**VIII**), as well as in oxygen analog **IX** [7], are almost orthogonal to the xanthene core (presumably, because of mutual repulsion of the oxygen atoms), which leads to a small blue shift (–5 nm). By contrast, the only nitro group in mononitro derivative **VII** is only slightly turned relative to the thioxanthene fragment (through an angle of 14°) and is involved in the conjugation system, giving rise to a red shift typical of nitrophenoxide ions, though its magnitude is much smaller (Δλ 3 nm).

Successive introduction of bromine atoms into positions *4, 5* and *2, 7* of Fluorescein induces long-wave shifts of the absorption maxima by 15 and 10 nm per each pair of bromine atoms, respectively [7]. The shift produced by four halogen atoms in tetrabromothiofluorescein **V** relative to thiofluorescein **IV** is somewhat smaller (+21 nm), as compared to the couple Fluorescein–Eosin (+25 nm) [7].

The shifts of long-wave absorption maxima observed for singly charged phenoxide-like quinoid anions **F** of 4,5-dinitro derivatives **IX**, **VIII**, and **XI** and tetrabromo compounds **V** and **X** on heteroatom replacement are almost the same as for the corresponding dianions: **IX** → **VIII**: +17 nm; **IX** → **XI**: –23 nm; **X** → **V**, +16 nm. It seems unreasonable to analyze the effect of successive introduction of substituents in the case of monoanions, for monoanions of unsubstituted compounds **I** and **IV** exist mainly as structure **E** (see below) with a different chromophore. The contribution of structure **E** to nitro- and dibromo-substituted derivatives **VII** and **X** is also significant, which makes such analysis quite incorrect.

The character of spectral changes during step ionization and the pK_a values given in the table suggest exclusive similarity of thiofluorescein (**IV**) and Fluorescein (**I**), thioeosin (**V**) and Eosin (**II**), and thioerythrosin (**VI**) and erythrosin (**III**), respectively. A more detailed interpretation of the properties of these and other compounds requires a detailed scheme of protolytic equilibria, from which Eqs. (4)–(6) can be deduced. These equations interrelate pK_a, pk, K_T, K'_T, K''_T, and K_{T_x} values [1–4,7].

$$\mathrm{p}K_{a,0} = \mathrm{p}k_{0,\mathrm{OH}} - \log(1 + K_T + K'_T) =$$

$$= \mathrm{p}k_{\pm,\mathrm{COOH}} - \log[1 + K''_T + (K'_T)^{-1}], \tag{4}$$

$$\mathrm{p}K_{a,1} = \mathrm{p}k_{1,\mathrm{COOH}} + \log(1 + K_T + K'_T) - \log(1 + K_{T_x})$$

$$= \mathrm{p}k_{1,Z} + \log[1 + K''_T + (K'_T)^{-1}] - \log(1 + K_{T_x})$$

$$= \mathrm{p}k_{1,\mathrm{OH}} + \log(1 + K_T + K'_T) - \log(1 + \bar{K}^{-1}_{T_x}), \tag{5}$$

$$\mathrm{p}K_{a,2} = \mathrm{p}k_{2,\mathrm{COOH}} + \log(1 + \bar{K}^{-1}_{T_x}) = \mathrm{p}k_{2,\mathrm{OH}} + \log(1 + K_{T_x}). \tag{6}$$

The relations are simplified to some extent on the assumption that $K'_T \ll 1$ or $K_T \gg K'_T$. On the basis of the electronic absorption spectra we can estimate tautomerization constants for molecular forms of thiofluoresceins. The quantity $\varepsilon_{max}(H_2R)$ can be regarded as a criterion of the degree of transformation of neutral dye into colorless lactone **D**. Then, in keeping with the K_T values, compounds **IV**–**VI** rank as follows: **IV** > **V** > **VI**. This series is the same as that observed for hydroxy xanthenes **I**–**III**. Insofar as the equilibrium is strongly displaced toward the lactone form, we failed to determine $\varepsilon_{max}(H_2R)$ for compound **VIII** with acceptable accuracy (as previously [7] for xanthene analog **IX**). The presence of only one nitro group notably increases the fraction of quinoid form **C** of compound **VII**. The effect of replacement of of the oxygen or sulfur atom by NEt is even stronger, even though molecule **XI** has two nitro groups; here, the K_T value is likely to be smaller than unity.

Monoanion HR^- of thiofluorescein (**IV**) exists as structure **E** (Fig. 1), and monoanions of compounds **V**, **VI**, **VIII**, **IX**, and **XI** have structure **F** (Figs. 3, 4). The λ_{max} values of ions HR^- and R^{2-} derived from thioeosin (**V**) are 545 and 536 nm, respectively. The corresponding values for thioerythrosin (**VI**) are 555 and 544 nm. Hence, $pK_{a,2}$ corresponds to $pk_{2,COOH}$ within experimental error not only for Eosin (**II**) and Erythrosin (**III**), but also for compounds **V**, **VI**, **VIII**, **IX**, and **XI**. That the $pk_{2,COOH}$ values of the nitro derivatives are lower than those of eosin (6.0) and thioeosin (6.2) can be explained by increase of the effective positive charge on the central carbon atom as a result of the strong electron-acceptor effect of the nitro groups. Despite the obvious coplanarity of the phthalic moiety and the xanthene core, the positively charged C^9 atom could affect the acidity of the carboxy group [7]. However, such effect is observed only for compound **IX** which was studied previously, and it is strongly reduced because of replacement of the oxygen atom by sulfur or the NC_2H_5 group. Comparison of the $pK_{a,2}$ values of compounds **V** and **II**, as well as of **VI** and **III**, leads us to conclude that the carboxy group becomes somewhat less acidic in going from fluorescein dyes to thiofluorescein analogs.

The $pK_{a,2}$ value for thiofluorescein (**IV**), as well as for Fluorescein (**I**), coincides with $pk_{2,OH}$; these values (7.88 and 7.66) differ by 0.2 log units. Assessment of K_T for compound **IV** on the assumption that ε_{max} for structures **C** and **E** are similar gives a value of 24 against $K_T = 30$ for compound **I** [1]. Then $pk_{0,OH}$ and $pk_{1,COOH}$ are equal to 2.32 and 5.45 for **IV**, while they are 2.43 and 5.33, respectively, for **I** [1]. Thus, the similar pK_a values of thiofluorescein (**IV**) and fluorescein (**I**) are not the result of mutual compensation of the differences in K_T and p*k* but of the coincidence of each of these parameters for both dyes. Further on, by comparing $pk_{2,OH}$ of **I** and $pk_{1,OH}$ of 6-hydroxy-9-phenyl-3*H*-xanthen-3-one in 50% ethanol (7.66 and 6.80 [19], respectively) we can estimate the effect of additional negative charge in the nonconjugated fragment of the molecule according to the Bjerrum–Kirkwood–Westheimer relation [4]. Then $pk_{\pm,COOH}$ and $pk_{1,Z}$ for compound **IV** can be estimated roughly at 4.6 and 3.2, respectively. Using Eq. (7) which follows from Eqs. (4)–(6), the tautomeric equilibrium constant **C** $\rightleftarrows$ **B** can be found.

$$\log K'_T = pk_{0,OH} - pk_{\pm,COOH} = pk_{1,Z} - pk_{1,COOH}. \quad (7)$$

It is important that the value of K'_T estimated as described above (0.005) confirms the permissibility of neglecting the concentration of zwitter ion **B** against the concentration of quinoid form **C**. Naturally, this is even more valid for derivatives in which the thioxanthene core contains substituents enhancing the acidity of the hydroxy group in zwitter-ionic structure rather than in quinoid. In this case K'_T further decreases.

The K_T value for thioeosin (**V**) was estimated at 1.6 against 3.6 for Eosin (**II**). Hence $pk_{1,OH} = 2.14$; this value is somewhat lower than that obtained for the xanthene analog (2.80) [1]. It is difficult to reliably estimate $pk_{1,OH}$ for compounds **VI**, **VIII**, **IX**, and **XI** on the basis of the available data because of the lack of data on ε_{max} of structures **C**, which are necessary for calculation of K_T. We can only propose a plausible explanation of the ratio of $pK_{a,1}$ for compounds **IX**, **VIII**, and **XI**: 5.36, 4.99, and 3.54, respectively. Indeed, $\varepsilon_{max}(H_2R)$ values increase in the same order; therefore, the contribution of the term $\log(1 + K_T)$ to $pK_{a,1}$ decreases.

Presumably, monoanions HR^- derived from compound **VII** and even from **X** are not completely converted into structure **F**. The absorption spectra shown in Fig. 4 indicate the existence of dynamic equilibrium **E** $\rightleftarrows$ **F**. In fact, the value of K_{T_x} can be estimated using Eq. (8) which is readily obtained from Eqs. (4–6).

$$\log K_{T_x} = pk_{1,COOH} - pk_{1,OH} = pk_{2,COOH} - pk_{2,OH}. \quad (8)$$

Assuming the $pk_{1,OH}$ value for 4,5-dibromofluorescein (**X**) to be equal to arithmetic mean of the corresponding values for **I** and **II** [1] (4.80) and $pk_{1,COOH}$ to be the same as for Fluorescein (**I**), K_{T_x} is equal to 3.4 and hence the equilibrium concentration ratio of **E** and **F** is 23 : 77. According to the data for

2,7-dichlorofluorescein [2, 3], organic solvents favor formation of tautomer **F**. A similar effect of the medium is observed for phenols and benzoic acids [1–4]. Probably, in going to systems with a low concentration of water tautomeric equilibrium of monoanions of compounds **VII** and **X** should shift toward tautomer **F**.

Thus the scheme of prototropic equilibria proposed previously for Fluorescein and its halogen derivatives also applies to its thio analogs and nitro derivatives.

EXPERIMENTAL

4-Nitrothiofluorescein (VII). To a solution of 0.35 g of thiofluorescein (**IV**) in 10 ml of sulfuric acid we added at 0°C under vigorous stirring 0.077 ml of nitric acid (d = 1.51) at such a rate that the temperature did not exceed 0–3°C. The cooling bath was then removed, and the mixture was kept for 30 min and poured into 50 ml of water. The precipitate was filtered off and washed with water to pH 3–4. Yield 0.150 g. The product was dissolved in a 0.1 N solution of NaOH, and the solution was filtered and acidified to pH 3–4 with hydrochloric acid. The dye was filtered off, washed with water, and dried in air. It was purified by chromatography on Silufol UV-254 plates using diethyl ether as solvent and diethyl ether–methylene chloride (4 : 1) as eluent. The product was washed off from the plate with ether (R_f 0.72). Orange amorphous powder, soluble in ether, acetone, alcohol, benzene, aqueous alkali. Yield of the chromatographically pure substance <10%. mp 270–273°C. ^{1}H NMR spectrum (DMSO-d_6), δ, ppm: 6.70 d (1H, 2-H, J_{AB} = 8.5 Hz), 6.98 d (1H, 8-H, J_{AB} = 14 Hz), 7.07 d (1H, 7-H, J_{AB} = 14 Hz), 7.05 s (1H, 5-H), 7.75–8.01 m (4H, C_6H_4COO). Found, %: C 61.10; H 2.80; N 3.5. $C_{20}H_{11}NO_6S$. Calculated, %: C 61.07; H 2.82; N 3.56.

The other dyes examined in this work were synthesized, purified, and identified as described in [11, 20]. Chemically pure sodium and potassium chlorides and phosphoric and acetic acids were used; sulfuric acid was of pure grade. Sodium tetraborate decahydrate was purified by recrysallization. Buffer solutions were prepared from a solution of NaOH protected from CO_2. Solutions of ethanol were prepared from distilled 96% azeotrope of high purity; the absence of aldehydes was checked by UV spectroscopy. Acetone of ultrapure grade was used without additional purification. Cetyltrimethylammonium bromide (from Sigma) contained 99% of the main substance.

The pH values (pH_{instr}) were determined using a glass electrode in an electron transport circuit which was calibrated against standard aqueous buffers (pH 1.68, 4.01, 6.86, 9.18). Values of $pa^*_{H^+}$ were varied using acetate and phosphate buffers and also solutions of HCl and NaOH. The absorption spectra were measured on an SF-46 spectrophotometer. Values of pK_a were determined by spectrophotometry in 50% C_2H_5OH; cell length l = 1 to 5 cm; concentration $c = (2–6) \times 10^{-5}$ M (25°C). Stock solutions of dyes were prepared using 96% ethanol. For determination of $pK_{a,1}$ and $pK_{a,2}$ optical densities of 15–18 solutions were measured (ionic strength 0.05 M; NaCl and buffer components). Values of pK_a were calculated using Eq. (9) [21] from the dependence of optical density D on $pa^*_{H^+}$, c and l being constant:

$$D = \frac{D_{H_2R} \times 10^{pK_{a,1} - pa^*_{H^+}} + D_{HR^-} + D_{R^{2-}} \times 10^{pa^*_{H^+} - pK_{a,2}}}{10^{pK_{a,1} - pa^*_{H^+}} + 1 + 10^{pa^*_{H^+} - pK_{a,2}}}. \tag{9}$$

Here, D_{H_2R}, D_{HR^-}, and $D_{R^{2-}}$ are the optical densities corresponding to complete transformation into the respective species. When necessary, the measurement was performed at different c and l values, and the results were recalculated to the required conditions. The values of D_{H_2R} and $D_{R^{2-}}$ were measured directly under the conditions ensuring predominant formation of the respective species; the spectrum which did not change throughout a range of 2–3 $pa^*_{H^+}$ units (in phosphate or borate buffer and in dilute solutions of NaOH) was assumed to be the spectrum of dianion. D is the optical dendity at a current $pa^*_{H^+}$ value (as a rule, in solutions of HCl + NaCl and acetate and phosphate buffers). The spectra of neutral forms H_2R in solutions of HCl and H_2SO_4 at $pa^*_{H^+} < 1.3$ were measured at an ionic strength exceeding 0.05 M. Additional calculations with the use of pK_a values found confirmed the negligible contribution of intensely colored ionic species to the absorption spectrum of H_2R. The spectrum corresponding to the cationic form of thiofluorescein (**IV**) was measured only in 4 M H_2SO_4; its pK_{a0} value was determined in solutions of HCl containing NaCl, considering equilibrium (1) separately from equilibria (2) and (3). After determination of interval estimates of ionization constants using the most informative wavelengths (usually, 10–15 values of λ), the overall spectra of HR^- ions were determined from the spectra of solutions with $pa^*_{H^+}$ corresponding to the maximal yield of monoanions. The D_{HR^-} were calculated by Eq. (10):

$$D_{HR^-} = (D - D_{H_2R}) \times 10^{pK_{a,1} - pa^*_{H^+}} + D + (D - D_{R^{2-}}) \times 10^{pa^*_{H^+} - pK_{a,2}}. \qquad (10)$$

The values of $\varepsilon(HR^-)$ were then calculated from the relation $\varepsilon(HR^-) = D_{HR^-}\, c^{-1}\, l^{-1}$. Thermodynamic values of pK_a (see table) were determined using the second Debye–Hückel approximation for activity coefficients of ions, the ionic parameter being assumed equal to 5. The activity coefficients of molecules were taken equal to unity. Therefore, the $pK_{a,1}$ and $pK_{a,2}$ values given in the table exceed those calculated from the experimental data (“mixed” values; Figs. 1, 2) by 0.16 and 0.47 log unit, respectively. Below are given λ_{max}, nm ($\varepsilon_{max} \times 10^{-3}$, l mol^{-1} cm^{-1}), for dyes **I**, **II**, **IV**, **V**, and **VII**–**XI** in 50 wt % aqueous ethanol: **I**: R^{2-}, 495 (88.5); HR^-, 455 (34.3); H_2R, 455 (0.978); H_3R^+, 445 (62.4) [1]; **II**: R^{2-}: 520 (99.6); HR^-, 530 (84.0); H_2R: 480 (5.19) [1]; **IV**: R^{2-}, 517.5 (22); HR^-, 490, 515 (5.0); H_2R, 490 (0.2); H_3R^+, 445 (11.5); **V**: R^{2-}, 536 (46.6); HR^-, 545 (48.0); H_2R, 505 (4.3); **VII**: R^{2-}, 520 (39.4); HR^-, 475 (15.0); H_2R, 475 (1.6); **VIII**: R^{2-}, 512 (48.0); HR^-, 520 (40.0); **IX**, R^{2-}, 493 (76); HR^-, 503 (93) [7]; **X**: R^{2-}, 510 (72.5); HR^-, 515 (32.5); H_2R, 470, 1.7; **XI**: R^{2-}, 475.5 (42.3); HR^-, 480 (42.7); H_2R: 470 (14.3).

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