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In Search for the “Phenolate” Monoanion of Fluorescein in Solution

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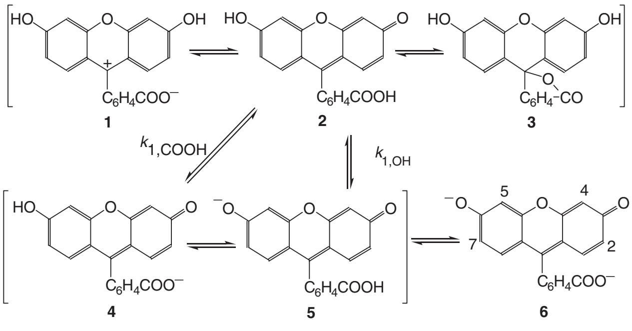
Fluorescein is exclusively popular owing to bright fluorescence of its R^{2-} dianion. While the structure of the latter and tautomerism of the H_2R form are well established, only the species with COO^- and OH groups has been supposed for HR^- up to now. Using absorption, emission, and excitation at different pH in non-HBD solvents, DMSO, acetonitrile, and acetone, we reveal that 10–12% of HR^- ions nevertheless exist as “phenolate” monoanion with nonionized COOH group.

Fluorescein and its derivatives belong to the most popular and abundant dyes owing to their unique bright fluorescence, which is pH-dependent.[1,2] These dyes are triprotic acids: $H_3R^+ \rightleftharpoons H_2R \rightleftharpoons HR^- \rightleftharpoons R^{2-}$, with acidity constants K_{a0}, K_{a1}, and K_{a2}, respectively. The spectra of molecular (H_2R) and ionic forms and the pK_as are well documented. For fluorescein in aqueous solutions, the thermodynamic values are as follows: $pK_{a0} = 2.14$, $pK_{a1} = 4.45$, and $pK_{a2} = 6.80$.[3] The possible structures of molecules and anions are given in Scheme 1.[3,4]

The visible absorption spectra of the dianionic species, R^{2-} (**6**), exhibit a principal band with ε_{max} (of R^{2-}) $\approx 10^5\,M^{-1}\,cm^{-1}$; the position of the maximum varies along with substituents and solvent nature.[1–4] The only exceptions are derivatives with NO_2 groups in the xanthene moiety, which are able to form anion–lactones (not shown here).[5] The similarity of visible absorption bands of the zwitterion **1** and quinoid **2** to those of the cation (not shown) and monoanion **4**, respectively, was proven using model substances.[3,4,6] Lactonic tautomer **3** is colorless due to sp^3 hybridization of the central C atom. As early as 1979, we estimated the fractions of the fluorescein tautomers ($\alpha_1 = 0.22$, $\alpha_2 = 0.11$, and $\alpha_3 = 0.67$) and the microscopic constants, k, in aqueous solutions.[3]

The tautomerism of fluorescein monoanion HR^- is still not elucidated definitely. Until 2006,[7] only the species with ionized carboxylic and nonionized phenol group was supposed. The visible spectrum of this “carboxylate” tautomer **4**, with maximal absorptivity around $\varepsilon = (0.2\text{–}0.3) \times 10^5\,M^{-1}\,cm^{-1}$, differs significantly from that of dianion **6** (Figure 1).



Scheme 1. Protolytic equilibrium of fluorescein.

Contrary to fluorescein, in the case of eosin and other 2,4,5,7-tetrahalogenated derivatives, the “phenolate” tautomer **5** predominates, with an intense band slightly red-shifted against that of dianion **6** (Figure 1).[4,5] Such red shift results from disappearance of the negative charge in 2′ position on going from **6** to **5** ($COO^- + H^+ \rightarrow COOH$).[7] The comparison of both absorption and emission spectra of fluorescein dianion, R^{2-}, with those of R^- anions of 6-hydroxy-9-phenylfluorone and ethylfluorescein, support this statement.[3,7,8] The results of quantum-chemical calculations[9] agree with this regularity.

The (**4** $\rightleftharpoons$ **5**) equilibrium state in various media can be predicted by the relation $\alpha_5/\alpha_4 = k_{1,OH}/k_{1,COOH}$, by taking into account that the decrease in acidic strength of carboxylic acids on going from water to nonaqueous media, especially to non-hydrogen bond donor (non-HBD) solvents, is known to be more expressed than that of phenols.[4,10,11] This tendency for fluorescein is indicated by quantum-chemical calculations,[12] though the α_5 values are overestimated as compared with the experimental data.[3,4,7] For 2,4,5,7-tetrahalogenated derivatives, $pk_{1,COOH}$ is essentially higher than $pk_{1,OH}$ already in aqueous solutions,[4] and thus $\alpha_5 \gg \alpha_4$. Contrary to it, for fluorescein the $pk_{1,COOH} = 3.5$ value in water is much lower than $pk_{1,OH}$ (≈ 6.3),[3,4,7] and in the up to now studied solvents the difference stays substantial.[3,4,7]

So, a question arises: in what media the tautomer **5** of fluorescein can be observed? The existence of the “phenolate” tautomer **5** of fluorescein can be directly proved only by detection of a band with $\lambda_{max} > \lambda_{max}$ (of R^{2-}). Unfortunately, in solvents where the α_5 value is expected to be high enough, K_{a1} and K_{a2} approach each other up to inversion, very largely due to the sharp increase in the α_3 value.[4,11] Therefore, the concentration of HR^- is very small, the spectra reflect the conversion of H_2R directly into R^{2-},[11,13] and observation of HR^- spectrum is hindered. Despite this difficulty, we detected the band of the desired tautomer **5** of fluorescein in DMSO, within a narrow pH range.[7] Now we present a conclusive evidence for the existence of **5** in a set of solvents.

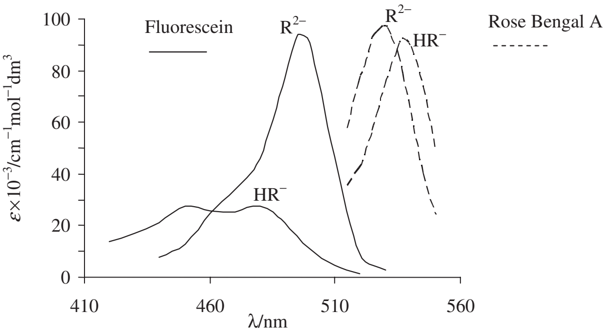


Figure 1. Absorption spectra of mono- and dianions of fluorescein and 2,4,5,7-tetraiodo-3′,6′-dichlorofluorescein (Rose Bengal A) in methanol.

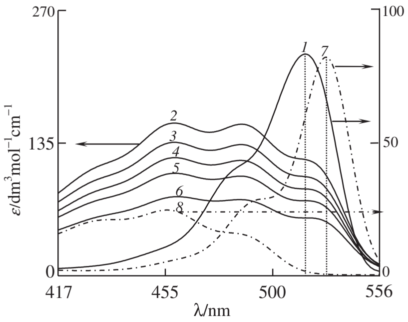


Figure 2. Absorption spectra in acetone with 5 mol % DMSO: 1: fluorescein, R^{2-} (in 1 M DBU, $\lambda_{max} = 515$ nm), 2–6: fluorescein in benzoate buffer solutions, pH 14.2, 13.9, 13.5, 13.2, and 12.9, respectively; 7: ethylfluorescein, R^- (in 1 M DBU, $\lambda_{max} = 526$ nm), 8: ethylfluorescein, HR (in salicylate buffer solution, pH ≤ 9).

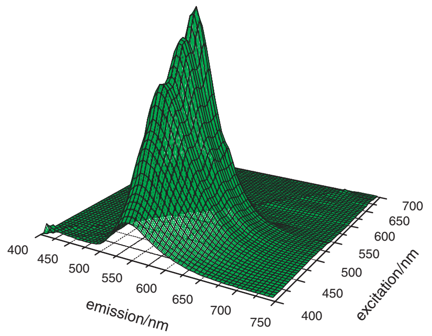


Figure 3. The three-dimensional fluorescence spectrum of HR^- form of fluorescein in a benzoate buffer (pH 20.8) in AN; λ_{max} (abs.): 465, 500, 537 nm; λ_{max} (excit.): 472, 504, 533 nm; λ_{max} (emiss.): 553 nm.

In organic solvents studied, the cation H_3R^+ appears in HCl or $HClO_4$ solutions. The neutral form predominates at much higher pHs (e.g., in salicylate buffers), and there are wide pH windows where the H_2R spectrum stays unchanged. Tautomer **1** disappears, the colorless tautomer **3** predominates,[4,11] and the fraction of **2** drops (e.g., up to $\alpha_2 = 3.2 \times 10^{-3}$ in DMSO).[7] This allows observing the morphology of HR^- spectrum even if only 1% of the dye is converted into the monoanion. Indeed, a pH range exists, where any traces of the intense band of R^{2-} species cannot be observed, while the predominating H_2R species is practically colorless. Such studies were performed in dry non-HBD solvents: DMSO, acetonitrile (AN), and acetone (AC).[6] Here, in addition to the typical spectrum of **4**, we observed distinct absorption with $\lambda_{max} > \lambda_{max}$ (of R^{2-}) (Figure 2). Accordingly, in emission and fluorescence excitation spectra, corresponding bands were registered (Figure 3 and Table 1). On further pH increase, the band becomes more intense, with $\lambda_{max} = \lambda_{max}$ (of R^{2-}).

For the absorptivity of the monoanion, the relation ($\varepsilon_{HR^-} = \varepsilon_4\alpha_4 + \varepsilon_5\alpha_5$) is valid. The ε_4 and ε_5 values are equated respectively to those of molecular and anionic species of ethyl fluorescein, a model compound with blocked carboxylic group (Figure 2), in the given solvent; wavelength around 460 and 530 nm were used as analytical positions.[6] In all the solvents studied, thus calculated α_5 is around 0.1–0.12.

From Scheme 1, it can be easily derived that $pk_{1,COOH} = pK_{a1} + \log\alpha_2 - \log\alpha_4$ and $pk_{1,OH} = pK_{a1} + \log\alpha_2 - \log\alpha_5$.

Table 1. The long-wavelength maxima (nm) of fluorescein R^{2-} species and of traces of HR^- species (registered as small admixture to H_2R); 23–25 °C[a]

Kind of spectra	λ_{max} of R^{2-}/λ_{max} of HR^-			
	DMSO	AN	AC	AN–AC–DMSO 2:2:1 (vol:vol:vol)
Absorption	520/525	514/537	515/534	510/536
Excitation	525/528	517/533	518/—	517/540
Emission	543/549	535/553	536/551	539/564

[a] The R^{2-} spectra are determined in KOH solutions (with addition of 18-crown-6), and those of of HR^- in benzoate buffers; 23–25 °C.

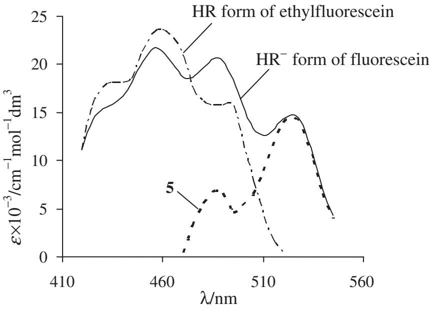


Figure 4. Absorption spectra in DMSO: quinonoidal neutral species of ethylfluorescein, fluorescein monoanion (**4** + **5**), and the desired tautomer **5** ($\varepsilon \times 0.125$), singled out from the HR^- spectrum by subtracting of [ε (of HR of ethylfluorescein) $\times$ 0.875)].

Having the thermodynamic value of fluorescein in DMSO, $pK_{a1} = 13.0 \pm 0.1$, and the $\alpha_2 = 3.2 \times 10^{-3}$, $\alpha_4 = 0.875$, and $\alpha_5 = 0.125$ values,[6] one can obtain $pk_{1,COOH} = 10.6$ and $pk_{1,OH} = 11.4$. While the first value is, as usual,[3,4] somewhat smaller than the pK_a of benzoic acid (11.1),[10] the second one is close to the value (11.6 ± 0.1) of ethylfluorescein,[6] a dye bearing COOEt group instead of COOH.

The restored spectrum of HR^- form of fluorescein in DMSO is compared in Figure 4 with that of HR molecules of ethyl fluorescein.

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