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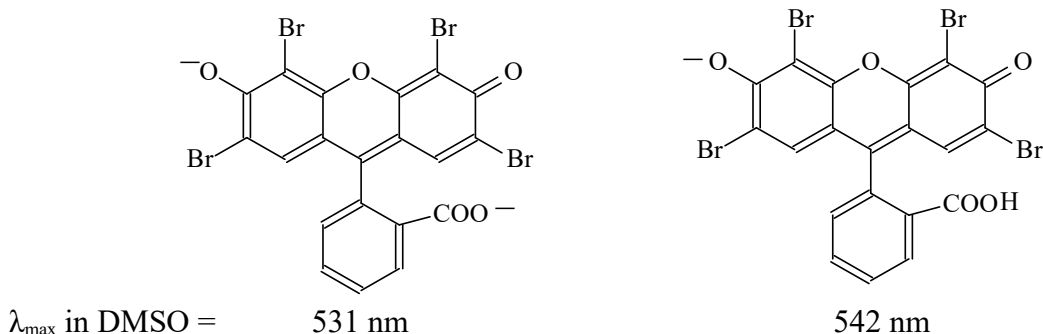
TRANSMITTANCE OF ELECTRONIC EFFECTS IN THE FLUORESCEIN MOLECULE: NITRO AND AMINO GROUPS IN THE PHTHALIC ACID RESIDUE**E. G. Moskaeva^a, K. I. Ostrovskiy^b, S.V. Shekhovtsov^c, N.O. Mchedlov-Petrosyan^d***V.N. Karazin Kharkiv National University, School of Chemistry, 4 Svobody sqr., 61022 Kharkiv, Ukraine*a) ✉ holdhelena@gmail.comb) ✉ gori2obr@gmail.comc) ✉ shekhovtsov@karazin.uad) ✉ mchedlov@karazin.uaID <https://orcid.org/0000-0002-5936-5923>ID <https://orcid.org/0000-0003-1180-0081>ID <https://orcid.org/0000-0002-1519-1669>ID <https://orcid.org/0000-0001-6853-8411>

This paper is aimed to estimate the transmittance of the electronic effects in the fluorescein molecule between the phthalic acid residue and the xanthene moiety. Despite the almost orthogonal orientation of the latter with respect to the rest of the molecule, some influence of substituents in this 9-aryl ring on the dissociation of the hydroxyl group of the hydroxyxanthene cannot be ruled out. In order to reveal this (possible) effect, we blocked the carboxylic group via esterification. The reason of using dimethyl sulfoxide as solvent was the high Hammett's "rho" constant for phenolic group. The pK_a values of eight methyl or ethyl esters of 3'-, 4'-, and 5'-nitro and amino fluoresceins were determined in benzoate and salicylate buffer solutions using the spectrophotometric method. For nitro derivatives, the dissociation constants of the cationic forms of the dyes were also determined in diluted *p*-toluenesulfonic acid. The study reveals increasing in the pK_a by 0.2–0.3 units in the case of amino derivatives, while the nitro group decreases the pK_as by 0.2–0.8. Also, the position of the substituent is of importance; the 5'-substituents display the least influence. In addition, the pK_a values of methyl and ethyl esters of eosin (2,4,5,7-tetrabromofluorescein) were determined in order to clarify the influence of the alkyl group. The absorption maxima and molar absorptivities of the anions, as well as the pK_as in DMSO coincide within the margin of errors.

Keywords: fluorescein esters; 3'-, 4'-, and 5'-nitro and amino derivatives; spectrophotometry; pK_a values; transmittance of electronic effects.

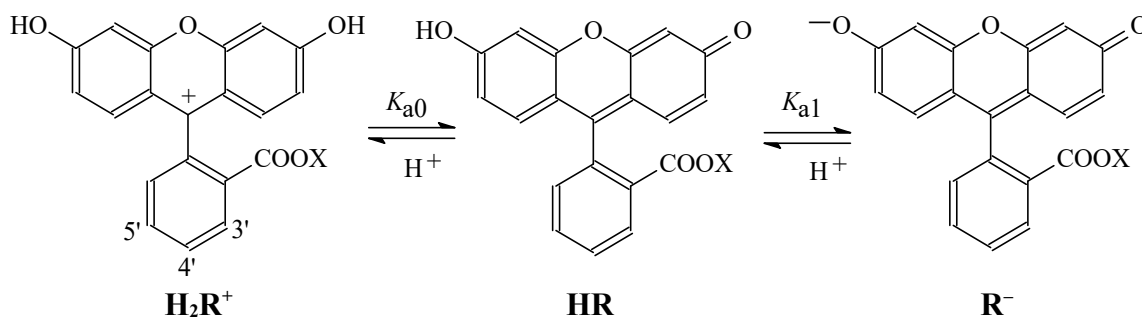
Introduction

Fluorescein and its derivatives belong to most popular organic dyes in many fields of chemistry and related sciences [1–3]. Despite the huge number of publications devoted to these compounds, there are still many unsolved problems. One of such problems is the influence of the substituents in the arene cycle on the xanthene portion. It is generally known that this cycle, i.e., the residue of the phthalic acid, is rotated by 60–90° relative to the xanthene plane [4, 5]. Hence, relatively small electronic interactions between these parts of the molecule should be expected. However, it is firmly proved that the protonation of the COO⁻ group in the dianion of eosin (2,4,5,7-tetrabromofluorescein) and other dyes of this type leads to a bathochromic shift, as it is exemplified in Scheme 1.

**Scheme 1.** Ions R²⁻ and HR⁻ anions of eosin

In this work, we examined the influence of the substituents in the phthalic residue on the dissociation of the hydroxy group of the compound. This can shed some light upon the transmittance of the electronic effects between the two parts of the fluorescein molecule. In order to avoid

difficulties connected with interpretation of lactone formation and complicated overlapping equilibria, we decided to examine the methyl (ethyl) esters of fluorescein, Scheme 2.



Scheme 2. Stepwise dissociation of the esters of nitro and amino fluoresceins; X = CH₃ or C₂H₅. The NO₂ or NH₂ groups occupy positions 3', 4', or 5'.

As substituents which are known to display opposite electronic effects, the nitro and amino groups were chosen. The Hammett σ_{para} constants are +0.78 and -0.66, whereas the σ_{meta} values are +0.71 and -0.16, respectively [6]. In the case of fluorescein series, the 4'-substituents are in the *para* position to the nodal C₉ atom. As a solvent appropriate for the declared goal, dimethyl sulfoxide (DMSO) was used. In this solvent, the Hammett ρ constant for phenols is 4.29, while in water $\rho = 2.11$ [7]. The pK_{a1} values were obtained for all the dyes under study, while those of pK_{a0} were determined only for nitro derivatives, because in the case of the amino fluoresceins, the protonation of the NH₂ group should also be taken into account.

Experimental

Materials

The nitro and amino derivatives were synthesized and identified in this Laboratory; the procedures will be published elsewhere. Dimethyl sulfoxide was purified by freezing and distilled under vacuum; water content around 0.02 % as determined by coulometric Karl Fischer method. Benzoic and salicylic acids were purified by sublimation. Potassium benzoate and sodium hydrosalicylate were purified by re-crystallization. *p*-Toluenesulfonic acid monohydrate was re-crystallized from acetonitrile. Diazabicyclo[5.4.0]undec-7-ene (DBU) was used as purchased from Merck.

Procedure

The pK_a values of the fluorescein derivatives were determined in DMSO at 25 °C using spectrometric measurements. The acidity of the solvents in buffer solutions was characterized by the $p\alpha_{H^+}^*$ values. These values were calculated using the pK_{HA} values of the buffer acids and the formation constants of the HA₂⁻ ions, $K_{HA_2^-}^f$ (Eq. 1) [8].

$$(a_{H^+}^*)^2 f_1^2 c_s - a_{H^+}^* f_1 K_{HA} [c_a + c_s + K_{HA_2^-}^f (c_a - c_s)^2] + K_{HA}^2 c_a = 0 \quad (1)$$

Here c_a and c_s are molar concentrations of the acid and salt, respectively; f_1 stands for activity coefficient of a single-charged ion. Normally, the c_s value was fixed, while that of c_a was variable. As result, at $c_a > c_s$ thus estimated $p\alpha_{H^+}^*$ values were lower than those calculated without taking into account the homoassociation process. The results were opposite at $c_a < c_s$. The pK_{HA} values of the salicylic and benzoic acids used in the calculations are 6.80 and 11.10, respectively, $K_{HA_2^-}^f = 30 \text{ M}^{-1}$ and 60 M^{-1} , respectively [9]. All salts were considered as completely dissociated. The f_1 values were calculated using the Debye-Hückel second approach law. The possibility of heteroassociation between the dyes and buffer compounds was ignored. High acidities were created by the *p*-toluenesulfonic acid.

The pK_a values of the dyes were obtained by processing the absorption spectra at different $p\alpha_{H^+}^*$ values. The dye concentrations were two orders of magnitude lower than those of buffer components. The spectra of the forms R⁻ and H₂R⁺ were measured in 0.02 M DBU solution and *p*-toluenesulfonic acid solutions at concentration of about 0.2 M, respectively. The spectra that coincide in a salicylate buffer solution, in pure salicylic and benzoic acids with concentrations of 0.02 and 0.21 M,

respectively, can be considered as first approximation as the spectra of the HR forms. The problem of the HR spectra will be discussed below. The absorption spectra are typified in Figures 1–3.

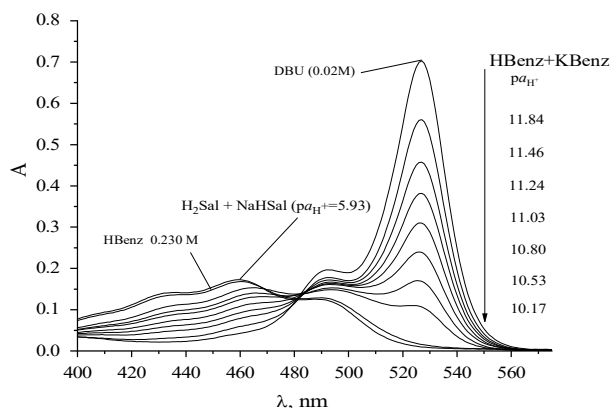


Figure 1. Absorption spectra of ethyl ester of 4'-aminofluorescein in DMSO at different $pK_{aH^+}^*$ values.

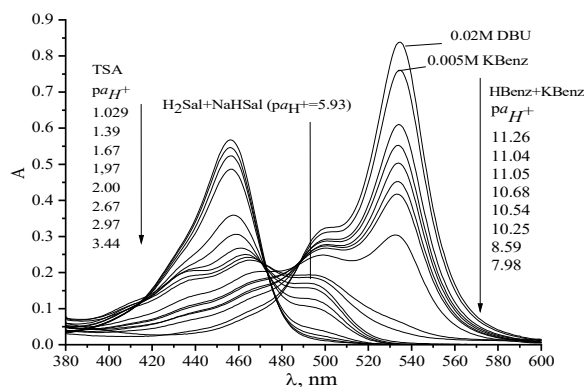


Figure 2. Absorption spectra of ethyl ester of 5'-nitrofluorescein in DMSO at different $pK_{aH^+}^*$ values.

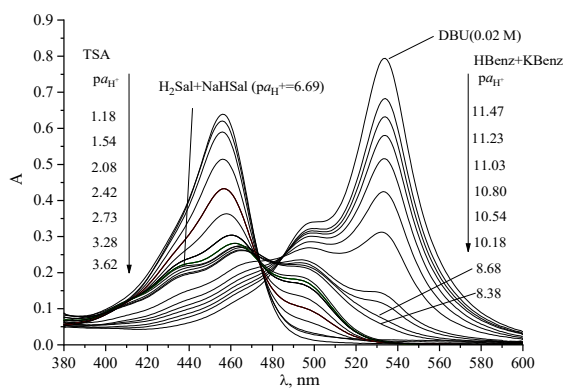


Figure 3. Absorption spectra of methyl ester of 4'-nitrofluorescein in DMSO at different $pK_{aH^+}^*$ values.

The thermodynamic pK_{a1} values were calculated using Equation 2 at a fixed wavelength, optical path length, and constant dye concentration:

$$pK_{a1} = pK_{aH^+}^* + \log \frac{A_{R^-} - A}{A - A_{HR}} - \log f_1 \quad (2)$$

Here A_{R^-} and A_{HR} are absorbances of the solution under complete transformation into the corresponding form, A is the absorbance at the current $p\alpha_{H^+}^*$ value. Three wavelengths around the λ_{max} of the R^- ion were used as analytical positions. For the determination of the thermodynamic pK_{a0} values in solutions of *p*-toluenesulfonic acid, TSA (Equation 3), the wavelengths within the range of 450–460 nm were used. The latter was considered as completely dissociated; c_{H^+} is equated to the analytical concentration of the acid.

$$pK_{a0} = -\log c_{H^+} + \log \frac{A_{HR} - A}{A - A_{H_2R^+}} \quad (3)$$

In the case of the eosin esters (Figures 4 and 5), the pK_{a1} values are substantially lower and were also determined in the *p*-toluenesulfonic acid solutions, Eq. 4.

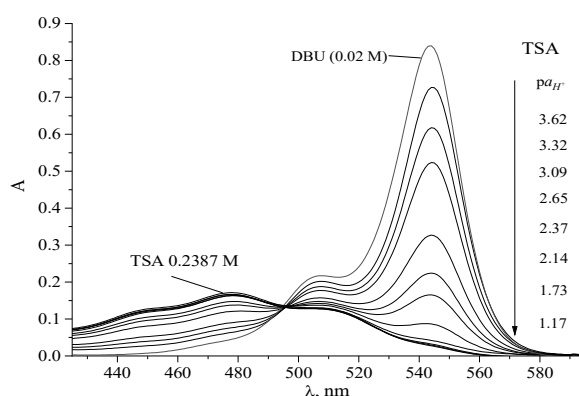


Figure 4. Absorption spectra of methyl ester of eosin in DMSO at different concentrations of *p*-toluenesulfonic acid; $p\alpha_{H^+}^* = -\log c_{H^+} - \log f_1$. The values below $p\alpha_{H^+}^*$ 2.37 should be considered as conventional ones because of relatively high ionic strength. The spectra in 0.239 M acid and 0.02 M DBU correspond to the neutral form, HR, and to the anion, R^- , respectively.

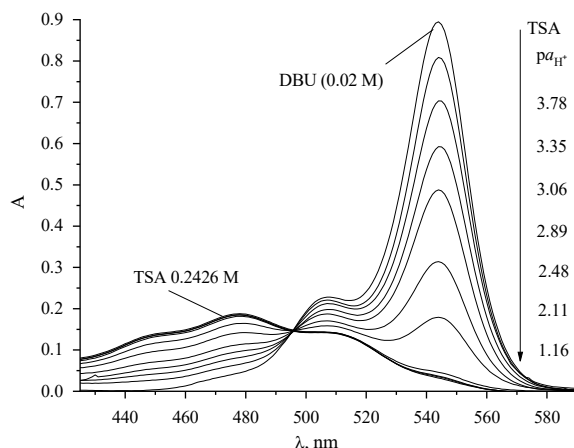


Figure 5. Absorption spectra of methyl ester of eosin in DMSO at different concentrations of *p*-toluenesulfonic acid; $p\alpha_{H^+}^* = -\log c_{H^+} - \log f_1$. The values below $p\alpha_{H^+}^*$ 2.89 should be considered as conventional ones because of relatively high ionic strength. The spectra in 0.243 M acid and 0.02 M DBU correspond to the neutral form, HR, and to the anion, R^- , respectively.

$$pK_{a1} = p\alpha_{H^+}^* + \log \frac{A_{R^-} - A}{A - A_{HR}} - \log f_1 \quad (4)$$

The isosbestic point is well expressed in the spectra of eosin esters; the same is the case with the equilibrium of 3'-, 4'-, and 5'-substituted fluorescein esters in the acidic region. On the contrary, this point is clearly blurred when moving from benzoate buffers to the HR absorption curve. The reason can be heteroassociations of the HR molecules with the benzoate ions. As a result, the molecular

spectrum of the given dye in the presence of the $C_6H_5COO^-$ ions somewhat differs from that in benzoic and salicylic acids and salicylate buffer solutions. At the lowest $p\alpha_{H^+}^*$ in the benzoate buffers, the A^- ions are almost completely transformed to the complex HA_2^- ; the latter is much less pronounced H-bonds acceptor. Therefore, the absorption curve does not cross the isosbestic point.

Note, that for the sulfonefluorescein, a dye with the SO_3^- group instead of $COOC_2H_5$, the spectrum of the monoanion, HR^- , in DMSO also does not cross the isosbestic point in the spectra in *p*-aminobenzoate buffer solutions, where the equilibrium between the anions HR^- and R^{2-} takes place [10]. At the same time, in 50 mas % aqueous ethanol, where the heteroassociation processes are improbable, the isosbestic point in the spectra of the fluorescein ethyl ester at different $p\alpha_{H^+}^*$ values is well expressed [11].

Two experiments were made to confirm our above explanation. The measurements with benzoate buffers in DMSO in the presence of 1 and 10 vol % water were performed. Whereas introduction of 1 vol % displays practically no influence on the spectra, in the presence of 10 vol % a distinct isosbestic point is observed (Figure 6).

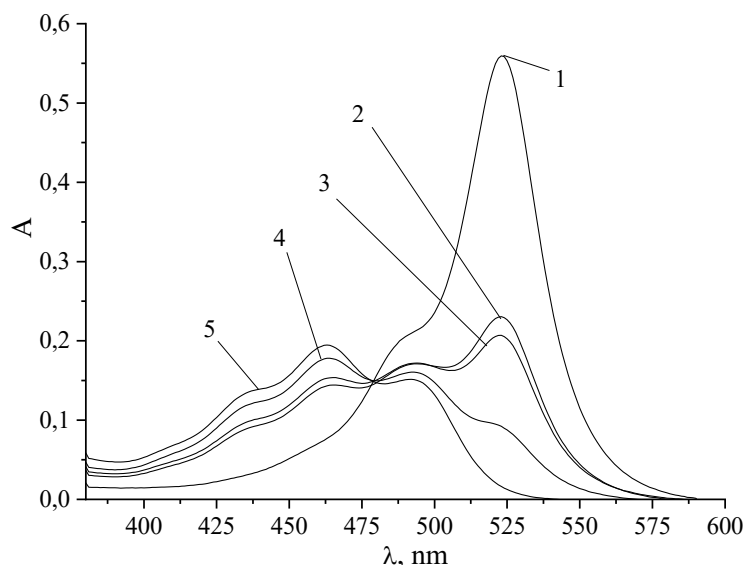


Figure 6. Absorption of 4'-nitrofluorescein methyl ester in benzoate and salicylate buffer solutions in DMSO with 20 vol % water: 1 – 0.005 M KBenz, 2 – 0.005 M HBenz + 0.005 M KBenz, 3 – 0.0075 M HBenz + 0.005 M KBenz, 4 – 0.020 M HBenz + 0.005 M KBenz, 5 – 0.001 M H₂Sal + 0.005M NaHSal.

In any case, the absorption of the neutral form at λ_{max} of the anion is negligible even if the spectrum in salicylate buffer solution is displaced to cross the isosbestic point in benzoate buffers. Variation in the absorbance of HR at absorption maximum of R^- results in pK_a changes of approximately about 0.04 units. Ultimately, one must take into account that the pK_a value of the benzoic acid in DMSO (11.1) is determined with uncertainty of ± 0.1 . Additional digits in the pK_{a1} values in Table 1 primarily reflect the accuracy of the spectral data.

Results and discussion

The results are presented in Tables 1 and 2. The bathochromic (4 to 9 nm) and hypsochromic (2 to 3 nm) shifts of the absorption bands of the nitro and amino derivatives, respectively, in respect to those of the unsubstituted compound give additional evidence of small but distinct influence of the NO_2 groups.

Table 1. Absorption spectra maxima and dissociation constant indices of the esters of nitro and amino fluoresceins in DMSO

Dye	λ_{\max} , nm/ $E_{\max} \times 10^{-3}$, M ⁻¹ cm ⁻¹		pK _{a0}	pK _{a1}	ΔpK_a
	cation	anion			
Fluorescein ethyl ester	451/48.7	530/101.0	2.74±0.01	10.91±0.08	0
3'-Nitrofluorescein methyl ester	462/50.9	539/96.6	1.99±0.02	10.46±0.07	-0.4
4'-Nitrofluorescein ethyl ester	456/64.1	535/73.4	2.39±0.05	10.63±0.09	-0.3
4'-Nitrofluorescein methyl ester	456/53.3	534/70.4	2.35±0.04	10.63±0.12	-0.3
5'-Nitrofluorescein ethyl ester	457/58.5	534/87.8	2.44±0.08	10.74±0.12	-0.2
3'-Aminofluorescein methyl ester	n.d.	528/109.9	n.d.	11.12±0.07	+0.2
4'-Aminofluorescein ethyl ester	n.d.	527/116.3	n.d.	11.22±0.07	+0.3
5'-Aminofluorescein ethyl ester	n.d.	527/110.8	n.d.	11.10±0.11	+0.2

It can be concluded that the nitro group in the phthalic acid residue displays pK_{a1} shifts up to 0.4 units. The molecular absorption band of the esters is broad and triple-humped; the maxima positions and molar absorptivities are collected in Table 2.

Table 2. Absorption spectra maxima of the neutral forms of the esters of nitro and amino fluoresceins in DMSO

Compound	λ_{\max} , nm ($E_{\max} \times 10^{-3}$, M ⁻¹ cm ⁻¹)		
Fluorescein ethyl ester	437 (17.5)	460 (21.9)	488 (14.9)
3'-Nitrofluorescein methyl ester	442 (18.0)	464 (22.2)	492 (14.6)
4'-Nitrofluorescein ethyl ester	440 (20.2)	463 (25.3)	490 (17.9)
4'-Nitrofluorescein methyl ester	441 (18.4)	463 (22.8)	489 (16.0)
5'-Nitrofluorescein ethyl ester	440 (21.2)	463 (25.8)	491 (17.6)
3'-Aminofluorescein methyl ester	436 (23.6)	459 (29.4)	487 (20.7)
4'-Aminofluorescein ethyl ester	433 (23.8)	459 (29.2)	489 (21.2)
5'-Aminofluorescein ethyl ester	434 (21.6)	458 (27.2)	485 (19.3)
Eosin methyl ester	453 (17.3)	478 (22.8)	504 (17.4)
Eosin ethyl ester	452 (17.2)	478 (22.9)	504 (17.6)

Table 3 additionally demonstrates the similarity of the pK_{a1} values and spectral parameters of the hydroxyxanthene dyes with COOCH₃ and COOC₂H₅ groups in 2' position.

Table 3. Absorption spectra maxima and dissociation constant indices of the eosin esters in DMSO

Dye	pK _{a1}	λ_{\max} (R ⁻), nm	$E_{\max}(R^-) \times 10^{-3}$, M ⁻¹ cm ⁻¹
Eosin methyl ester	2.91±0.04 ^a	544	112.0
Eosin ethyl ester	2.85±0.04 ^b	544	108.5

Note. ^a For calculations, the data at $p\alpha^*_{H^+} = 3.32, 3.09, 2.65,$ and 2.37 were used. ^b For calculations, the data at $p\alpha^*_{H^+} = 3.78, 3.35, 3.06,$ and 2.89 were used.

The pK_{a0} values of the nitro derivatives are 0.30–0.75 units lower than that of ethyl fluorescein. The NO₂ group in the position 4' displays a somewhat larger effect, 0.35–0.39 units, as compared with the pK_{a0} shift of 0.30 in the case of 5'-nitrofluorescein ethyl ester. This is in line with the relation between the σ_{para} and σ_{meta} constants of the nitro group. Here, the terms *para* and *meta* indicate the position of the substituents in respect to the nodal carbon atom C₉. The last is in direct polar conjugation with both quinone and OH groups. The distinct difference between the influence of the substitution in the positions 3' and 5', 0.75 and 0.30 units, respectively, reflects the specificity of the *ortho*-effect in the respect to the carbalkoxy group.

Similar effects are observed for the pK_{a1} of 3', 4', and 5' nitro derivatives: $\Delta pK_{a1} = -0.4; -0.3;$ and $-0.2,$ respectively (Table 1). In contrast, the ΔpK_{a1} values of the corresponding amino derivatives are equal to +0.2; +0.3; and +0.2, respectively. Therefore, the effects of the 4' and 5' substitution in the 9-aryl portion are for the hydroxy group in the xanthene moiety on average an order of magnitude lower than those for the *para* and *meta* substitutions for phenols as predicted by the $\rho\sigma$ product. Of course, such estimates are only conventional.

The effect of 3'-substitution is obviously caused by the neighboring carbalkoxy group. It deals about the *ortho* effect, which is formed both by electronic and steric factors.

On the other hand, the corresponding effects in aqueous solution should be predicted to be $4.29/2.11 = 2.03$ times lower than those determined in the present work in DMSO.

The above-mentioned heteroassociation leads to some “drift” of the pK_{a1} along with the absorbance dependence vs. $pa^*_{H^+}$. While the association of R^- with HBenZ causes the apparent strengthening of the dye acidity, the analogous interaction of HR with $Benz^-$ leads to an opposite result. Indeed, within the $pa^*_{H^+}$ decreasing by 1.2–1.5 units an average decrease in the pK_{a1} values of 0.24 units is observed for all the dyes.

Since the pK_{a1} values are somewhat distorted by heteroassociation and their accuracy is limited by that of the pK_{HA} of the benzoic acid, we additionally compared the spectrophotometric data for different dyes at the same $pa^*_{H^+}$ values. In Table 4, the ratio of the absorbance of the anion and that at a given $pa^*_{H^+}$ value is presented.

Table 4. Ratios of absorbances at a given $pa^*_{H^+}$ and of the anion

Compound	$A/A(R^-)$ at λ_{max} of the anion	
	$pa^*_{H^+} = 11.04 \pm 0.01$	$pa^*_{H^+} = 10.54 \pm 0.01$
3'-Nitrofluorescein methyl ester	0.805	0.615
4'-Nitrofluorescein methyl ester	0.731	0.534
4'-Nitrofluorescein ethyl ester	0.731	0.533
5'-Nitrofluorescein ethyl ester	0.659	0.498
Fluorescein ethyl ester	0.601	0.378
3'-Aminofluorescein methyl ester	0.491	0.290
4'-Aminofluorescein ethyl ester	0.443	0.243
5'-Aminofluorescein ethyl ester	0.494	0.283

An inspection of Table 4 confirms the above observations: the 4'-substitution has a somewhat stronger effect than the influence of the 5'-derivatives. Introduction of the NO_2 group in 3' position reduces pK_{a1} even more.

Conclusions

The introduction of nitro and amino groups into the phthalic acid residue affects the protolytic properties of the functional groups in the xanthene part of the fluorescein molecule in DMSO. The decrease in the pK_a caused by the NO_2 group and increase with the introduction of the NH_2 group is somewhat more pronounced with the substitution at the 4'- compared to the 5'-position. For the nitro derivatives, the decrease in the pK_{a0} value is more pronounced than that in the case of pK_{a1} . Here the impact sequence is as follows: $3' > 4' > 5'$.

Although the variations in the pK_a values in DMSO are only in the range of 0.2–0.8, they convincingly prove the transmission of the electronic effects despite the almost perpendicular orientation of the phthalic residue to the xanthene fragment. In water, the effects are expectedly less.

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О. Г. Москаєва, К.І. Островський, С.В. Шеховцов, М.О. Мchedlov-Петросян. Провідність електронних ефектів в молекулі флуоресцеїну: нітро- та аміногрупи у залишку фталевої кислоти.

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Метою цієї роботи є оцінка передачі електронних ефектів між залишком фталевої кислоти та ксантеновою частиною в молекулі флуоресцеїну. Незважаючи на майже ортогональну орієнтацію цих фрагментів, не можна виключити певного впливу замісників у 9-арильному кільці на дисоціацію гідроксильної групи гідроксиксантену. Щоб виявити цей (можливий) ефект, ми заблокували карбоксильну групу шляхом естерифікації. Причиною використання диметилсульфоксиду як розчинника була висока константа Гаммета, «ро», для фенольної групи. Значення pK_a восьми метилових або етилових естерів 3'-, 4'- і 5'-нітро- та амінофлуоресцеїнів визначали в бензоатних і саліцилатних буферних розчинах спектрофотометричним методом. Константи дисоціації катіонних форм нітропохідних визначали в розведених *p*-толуолсульфоновій кислоті. Значення pK_a збільшуються на 0,2–0,3 одиниці у випадку амінопохідних, тоді як для нітропохідних pK_a зменшується на 0,2–0,8. Для замісників у положенні 5' відмічено найменший вплив. Для уточнення впливу алкільної групи були отримані значення pK_a метилових та етилових естерів еозину (2,4,5,7-тетрабромфлуоресцеїну). Максимуми поглинання і молярні коефіцієнти поглинання аніонів, а також pK_a в ДМСО збігаються в межах довірчого інтервалу.

Ключові слова: естери флуоресцеїну; 3'-, 4'- і 5'-нітро- та амінопохідні; спектрофотометрія; значення pK_a ; передача електронних ефектів.

Е. Г. Москаева, К. И. Островский, С.В. Шеховцов, Н. О. Мchedlov-Петросян. Проводимость электронных эффектов в молекуле флуоресцеина: нитро- и аминогруппы в остатке фталевой кислоты.

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Целью этой работы является оценка передачи электронных эффектов между остатком фталевой кислоты и ксантеновой частью в молекуле флуоресцеина. Несмотря на почти ортогональную ориентацию этих фрагментов, нельзя исключить определённого влияния заместителей в 9-арильном кольце на диссоциацию гидроксильной группы гидроксиксантена. Чтобы выявить этот (возможный) эффект, мы

заблокировали карбоксильную группу путём образования сложных эфиров. Причиной использования в качестве растворителя диметилсульфоксида была высокая константа Гаммета «ро» для фенольной группы. Значения pK_a восьми метиловых или этиловых сложных эфиров 3'-, 4'- и 5'-нитро- и аминофлуоресцеинов определяли в бензоатных и салицилатных буферных растворах при помощи спектрофотометрического метода. Константы диссоциации катионных форм нитропроизводных определяли в разбавленных растворах *l*-толуолсульфоновой кислоты. Значения pK_a увеличиваются на 0.2–0.3 единицы в случае аминопроизводных, тогда как в случае нитропроизводных pK_a уменьшаются на 0.2–0.8. Для заместителей в положении 5' отмечено наименьшее влияние. Для уточнения влияния алкильной группы были получены значения pK_a метиловых и этиловых эфиров эозина (2,4,5,7-тетрабромфлуоресцеина). Максимумы поглощения и коэффициенты молярного поглощения анионов, а также pK_a в ДМСО совпадают в пределах доверительного интервала.

Ключевые слова: сложные эфиры флуоресцеина; 3'-, 4'- и 5'-нитро- и аминопроизводные; спектрофотометрия; значения pK_a ; передача электронных эффектов.

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