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ORIGINAL PAPER

Spectroscopic Study of the Interaction of Styrylcyanine Dyes Sbo, Sil and Their Derivatives with Bovine Serum Albumin

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Abstract The spectral-luminescent characteristics of newly synthesized styrylcyanine dyes on the base of dyes Sbo ((E)-2-(4-(dimethylamino)styryl)-3-methylbenzo[d]oxazol-3-ium iodide) and Sil ((E)-2-(4-(dimethylamino)styryl)-1,3,3-trimethyl-3H-indolium perchlorate) in aqueous solutions without and in the presence of bovine serum albumin (BSA) were studied. It was established that the absorption spectra of dyes Tol-6, Dbo-10 and Dil-10 with increasing amount of BSA appear new bands with λ_{max}=505 nm, λ_{max}=512 nm and λ_{max}=566 nm, respectively, whose intensity increases in proportion to the amount of albumin. The intensity of the glow of the main band of fluorescence in the presence of BSA sharply increases. The binding constant (K) and the number of binding sites (N) of studied dyes with BSA were determined. The dependence of binding constants with BSA on the dipole moment of dye molecules was determined, which indicates that besides electrostatic forces of attraction between molecules styrylcyanine dyes with BSA, hydrophobic interactions are essential.

Keywords Styrylcyanine dyes · Fluorescence · Electronic absorption spectra · Bovine serum albumin · Binding constant

Introduction

Importance of the study of spectral luminescence, photophysical and photochemical properties of organic dyes in recent years has increased dramatically [1, 2]. This is due to the fact that organic dyes are widely used in various branches of science and technology. Depending on the application scope of fluorophores, their defining properties are different—the quantum yield [3] and photostability [4], pH dependence [5, 6], fluorescence lifetime [7]. Currently, there is a large number of organic dyes of various classes, which are used in photodynamic therapy [8], tissue optics [9], analysis of cells [10], etc. The probes and labels for fluorescence measurements in vivo, of great interest are those dyes, absorption and fluorescence spectra which are in the red and near infrared spectral region [11–13].This is due to the fact that in this region of the spectrum the self-absorption and fluorescence of biological objects is minimal. In addition, the availability of diode lasers as cheap sources of light in this spectral range also promotes the use of these dyes [14]. Substantial interest in this plan represent styrylcyanine dyes, which represent a subclass of polymethyne dyes. Earlier the interaction of rhodamine [12] squaraine [13] and styrylcyanine dyes on the base of F-dye (4 - (4 - (dimethylamino)styryl)-1-methylpyridinium iodide) with BSA and deoxyribonucleic acid [15] was studied. This paper is a logical extension of previous work and present the results on the interaction between newly synthesized monomeric and homodimer styrylcyanine dyes on the base of dye Sbo and Sil with BSA by molecular absorption and fluorescence spectroscopy methods.

Materials and Experimental Method

Structural formulas of the studied dyes are listed in Table 1. Synthesis of the studied dyes was implemented by the Institute of Molecular Biology NAS of Ukraine, according to the methods described in [16, 17]. Electronic absorption

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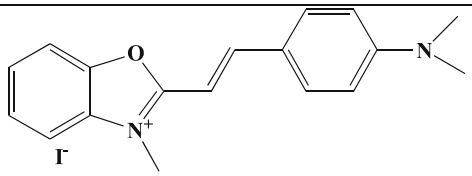
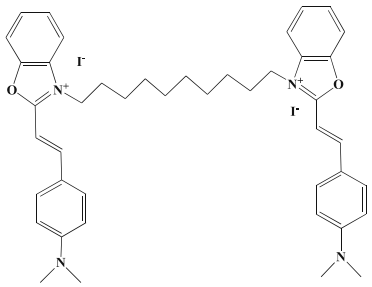
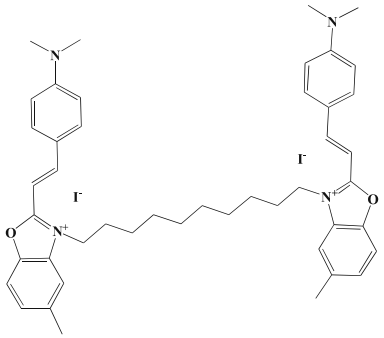
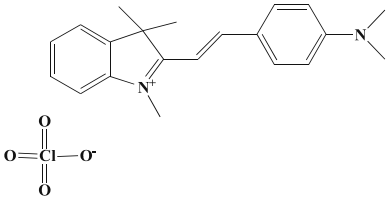
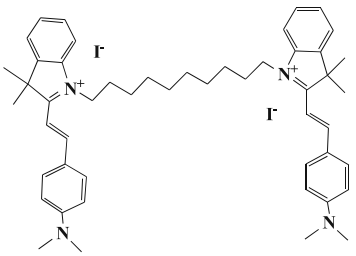
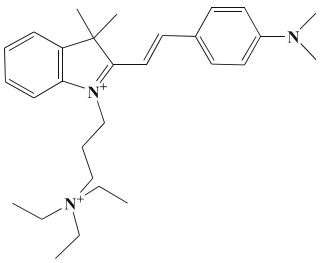
Table 1 Structural formulas of the studied dyes

№	Dye	Structural formula
1	Sbo	
2	Dbo-10	
3	Tol-6	
4	Sil	
5	Dil-10	
6	S-3	

spectra were measured on a Specord 50 SA (Analytikjena, Germany) which allows measurements with an accuracy (+/− 0.003 D) and resolution (0.3 nm) in the range of 190–1,100 nm. Fluorescence spectra were measured on a home-made fluorescence measurement setup, assembled on the basis of monochromator MDR-12 (LOMO, Russia) with photomultiplier tube FEU-100 (Russia). High brightness LED's were used as an excitation source.

Was used the BSA ("Medpreparat", Konotop, Ukraine) as the protein. The bidistilled water as a solvent was used. Preparation of initial solutions of dyes was spent by a volume-weight method. Accurate samples of dyes were weighed on microanalytical balance AB-135-S/FACT (Mettler Toledo, Switzerland) with an accuracy of 0.01 mg to get the initial solutions with a given concentration. Working concentration of 10^{-5}–10^{-6} M solutions were prepared by diluting the stock solution. The magnitude of systematic error associated with inaccurate calibration and distributions with different wettability of the walls dimensional dish does not exceed 1%. The concentration of BSA (p) is defined by formula: $p = 1.45 \times D_{280} - 0.74 \times D_{260}$ (in mg/ml), where D_{280} and D_{260} - optical density of BSA solution at the wavelengths of the absorption at 280 nm and 260 nm [18]. To determine the binding constants (K) and the number of binding sites (N) studied dyes with BSA fluorescence titration by the method of Scatchard was carried out [19].

Titration of the dye with BSA was carried out at constant dye concentration by serial dilution of the initial concentration of BSA. Titration of BSA and the water stain was performed at a constant concentration of BSA, by adding a sample volume of 3 ml BSA solution for 20, 40, 60, 80 and 100 μl of the initial aqueous dye. The values of K and N are determined based on the results of titrations and measurements of fluorescence spectra by the method described in [18, 20]. All measurements for calculating the binding constants (K) of the dye with the protein and the number of binding sites (N) was determined by fluorescence intensity of aqueous solutions of the maximum emission band. All measurements were performed at room temperature (297 K). For ease of comparison, the presented absorption and fluorescence spectra were normalized to unity. Quantum-chemical calculations were performed using program package MOPAC 2009 [21], semiempirical AM1 method with a standard set of parameters [22]. Conformational search and geometry optimization of molecules were performed using restricted Hartree-Fock method and Polak-Ribier algorithm with 0.001 kcal/(Å × mole) accuracy.

Results and Discussion

The concentration dependence of absorption and fluorescence spectra of dyes in water was studied. It was established that in the concentration range 10^{-5}–10^{-6}M the form of absorption and fluorescence spectra of the studied dyes remains constant, and they refer to the monomeric form. The quantum yield of aqueous solutions of the studied dyes is very low and approximately equals to 0.01–0.02. Based on experimental measurements data for aqueous solutions of the studied dyes, according to calculation procedures [13] the main spectral-luminescent characteristics of dyes in monomeric form were determined: extinction coefficient (ε), oscillator strength (f_e), radiative lifetime of the excited state (τ), the frequency of purely electronic transition ($\nu_{0\text{-}0}$) and Stokes shift (SS), which are shown in Table 2. Styryl dyes Sbo and Sil is monomeric ones and differ from each other by the presence in indoline fragment of the dye molecule oxygen atom and a methyl group, respectively. In addition, the dye Sbo anion acts as a iodine atom, while the dye Sil perchlorate. Dyes Dbo-10,

Table 2 Spectral-luminescent characteristics of aqueous solutions of the studied dyes

Dye	$\lambda^{abs.}_{max}$, (nm)	$\lambda^{fl.}_{max.}$, (nm)	ε, (l × mole^{-1} × cm^{-1})	f_e	τ, (ns)	$\nu_{0\text{-}0}$ (cm^{-1})	SS (cm^{-1})
Sbo	488	556	37,400	0.77	5.0	18,870	2,506
Sbo + БСА	488	553	33,900	0.72	5.4	18,900	2,408
Dbo-10	467	570	9,700	0.12	0.3	19,490	3,869
Dbo-10 + БСА	512	552	5,400	0.10	0.5	18,520	3,160
Tol-6	466	553	25,800	0.40	8.8	19,270	3,376
Tol-6 + БСА	505	560	11,100	0.23	8.0	17,540	1,944
Sil	535	596	21,300	0.45	0.1	17,100	1,910
Sil + БСА	535	600	20,000	0.41	0.2	17,180	2,024
Dil-10	512	600	6,000	0.08	0.5	17,580	2,865
Dil-10 + БСА	566	600	11,250	0.30	0.1	17,000	1,032
S-3	561	600	58,100	0.81	6.3	17,210	1,160
S-3 + БСА	564	598	55,300	1.0	4.7	17,180	1,008

Tol-6, and Dil-10 is the homodimers containing two molecules of monomeric dyes Sbo and Sil, respectively, are covalently linked by the polymethylene chain. Dye Tol-6 differs from the dye molecule Dbo-10 by the fact that methyl groups are attached to the main chromophore. Dye S-3 differs from dye-Sil by presence of effector group in the chromophore. As can be seen from Table 2 the increase in length binding leads to a hypsochromic shift of the absorption bands, a decrease in the extinction coefficient of dye Dbo-10 is approximately four times.

Input of the methyl groups in the chromophore dimer Tol-6 did not affect the position of maximum absorption, but lead to a decrease in the extinction coefficient of 1.5 and a hypsochromic shift of fluorescence spectra on 17 nm. Similar phenomena were also observed for the derivatives of the dye Sil. When covalent dye Sil polymethylene chain n=10 (dye Dil-10), the value of the extinction coefficient decreased by approximately 6 times, whereas the introduction of the effector group (dye S-3) lead to an increase in contrast extinction ratio which is approximately two times. The position of maximum absorption of homodimer Dil-10 shifts hypsochromically in relation to the dye Sil at 23 nm, while in relation to the S-3 for 49 nm there is bathochromic shift. The peak position of fluorescence spectra of dyes Sil, Dil-10 and S-3 remain constant.

The interaction of dyes Sbo, Dbo-10, Tol-6, Sil, Dil-10 and S-3 with BSA was studied. The form of absorption and fluorescence spectra of aqueous solutions of dyes Sbo, Sil and S-3 in the presence of BSA remains constant. The intensity of the dye absorption at the same time slightly decreases and the emission intensity of fluorescence increases (Table 3). The most interesting pattern is observed in the absorption spectra of dyes Tol-6, Dil-10 and Dbo-10. In dye Tol-6 at increase BSA concentrations, at a constant dye concentration, along with the absorption band of monomers with λ_{max}=466 nm a new band with λ_{max}= 505 nm appers, the intensity, of which, with increasing content of BSA in solution, increases and decreases the intensity of the band of monomers (Fig. 1a). A similar pattern is observed in the absorption spectra of the dye

Table 3 Binding parameters of the studied dyes with BSA

Dye	Concentration, M		K, M^{-1}	N	$\frac{I_{BSA}}{I_{water}}$
	dye	BSA			
Sbo	2.5×10^{-5}	3.0×10^{-5}	3.8×10^{3}	15.0	1.30
Dbo-10	2.5×10^{-5}	4.4×10^{-5}	3.0×10^{4}	0.11	20.6
TOL-6	1.0×10^{-5}	4.1×10^{-5}	6.0×10^{5}	0.40	11.9
Sil	2.5×10^{-5}	2.2×10^{-6}	1.7×10^{5}	5.45	5.20
Dil-10	2.0×10^{-5}	2.2×10^{-5}	8.2×10^{2}	55.0	44.7
S-3	1.0×10^{-5}	6.5×10^{-5}	7.6×10^{5}	0.30	6.20

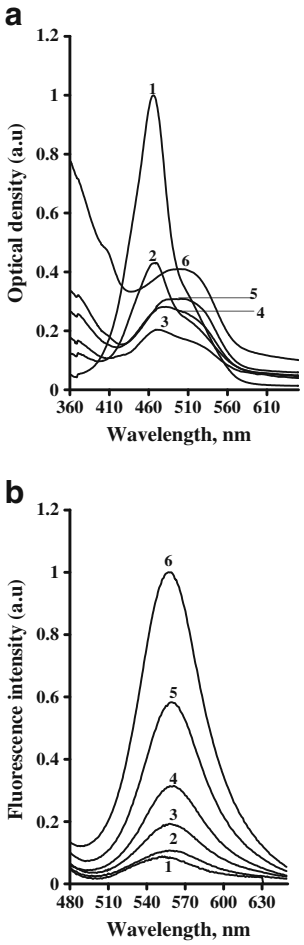


Fig. 1 The spectra of absorption (**a**) and fluorescence (**b**) of aqueous solutions of dye Tol-6 (c=10^{-5} M) with the addition of BSA: 1–0, 2–2×10^{-6}, 3–5×10^{-6}, 4–10^{-5}, 5–2×10^{-5}, 6–4×10^{-5} M

Dbo-10 (Fig. 2a). As shown in Fig. 2a by adding the BSA decrease in the intensity of the absorption of about 10% is observed initially.

Further increase of the concentration of BSA leads to the emergence of a new band with λ_{max}=512 nm (Fig. 2a, curve 2 and 3) the intensity of which increases in proportion to the concentration of BSA. The shape of the absorption spectrum at the same time broadens and the maximum absorption bathochromic shifted by 8 nm. In absorption spectrum of dye Dil-10 reverse situation is observed in comparison with the spectra of Tol-6 and Dbo-10. Here, at a low concentration of BSA in solution, an increase of absorption intensity is observed. Further increase in the BSA leads to a new band from the long waves, with λ_{max}= 566 nm whose intensity increases in proportion to the quantity of BSA (Fig. 3a). In the fluorescence spectra of dyes Tol-6, Dbo-10, and Dil-10 at increase of the concentration of BSA in solution, the shape and position of the bands does not change noticeably, but the intensity at the monomer band increases on the order of magnitude (Figs. 1b-3b).

The phenomena observed in the absorption and fluorescence spectra are a consequence of the interaction of dye

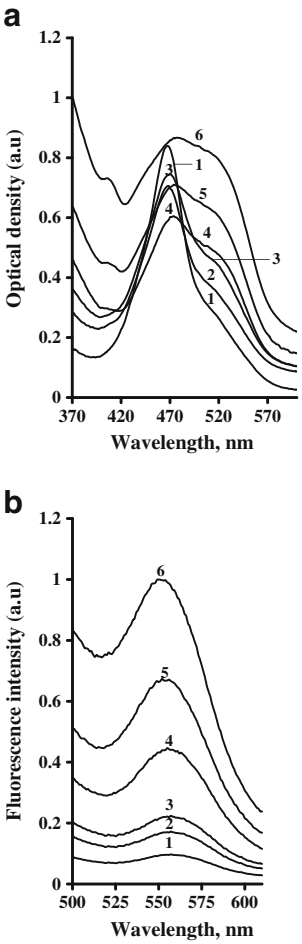


Fig. 2 The spectra of absorption (**a**) and fluorescence (**b**) of aqueous solutions of dye Dbo-10 ($c=10^{-5}$ M) with the addition of BSA: 1–0, 2–2.7×10^{-6}, 3–5.5×10^{-6}, 4–1.1×10^{-5}, 5–2.2×10^{-5}, 6–4.4×10^{-5} M

molecules Dbo-10, Tol-6, and Dil-10 with BSA, which can be explained as follows. The presence of polymethylene chain leads to a rotation of the chromophores, which causes a disturbance of complanarity and the appearance of cis-trans isomerization. Cis-isomers fluoresce usually much weaker than that of the trans-isomers [23]. These differences are amplified as the fact that the length of the π-system along the main axis of the molecule and, consequently, the probability of electron transition of the first ones is much smaller than the latter. Less rigid connection between the chromophores of dyes related to the long polymethylene chain provides an opportunity for interaction with molecules of BSA to preserve its trans-conformation, which leads to increased fluorescence intensity.

Quantitative measure of the interaction of dye molecules with biomacromolecules is binding parameters: binding constant (K) and the number of binding sites (N) (Table 3).

Study of binding parameters of styrylcyanine dyes showed their strong dependence on the length of the polymethylene chain in homodimer dyes. As can be seen from Table 3 for the monomer Sbo observed a slight increase in fluorescence and a small value of the binding constants of the dye molecules to BSA. Dye S-3 has the highest value of the binding constant to the protein BSA compared with related dyes Sil, Dil-10, which is due to the inclusion of an effector group. At the same time dimeric dyes (Dbo-10 and the Tol-6) of the monomer with the polymethylene chain ($n=10$) showed increased binding affinity to the BSA. For the homodimers, the fluorescence intensity in the presence of BSA, greater than for the monomer Sbo by an order of magnitude. In addition, in this series of dyes Tol-6 and Dbo-10 have the highest binding constant. The same pattern was found for homodimer dyes, dye-based F [15], where they showed a strong dependence of spectral-luminescent properties of the length of the linking group increased binding affinity to BSA and DNA. Large values of binding constants can be explained by the fact that in dyes Dbo-10 and the Tol-6 the heterocycles are at a sufficient distance from each other and a flat trans-conformation is the main form of these dyes. In the presence of protein such chromophores increase the fluorescence by attached to them. The low value of the binding constants of dye Dil-10 can be explained by the structural features of the dye. As noted above in the structure of the dye Dbo-10 and the Tol-6 are oxygen atoms, which have a negative charge, whereas in the dye

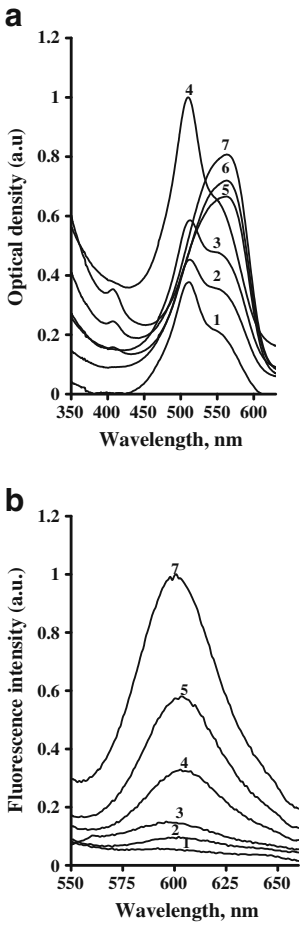


Fig. 3 The spectra of absorption (**a**) and fluorescence (**b**) of aqueous solutions of dye Dil-10 ($c=10^{-5}$ M) with the addition of BSA: 1–0, 2–5×10^{-7}, 3–10^{-6}, 4–2.2×10^{-6}, 5–5×10^{-6}, 6–10^{-5} 7–2.2×10^{-5} M

Dil-10, they are replaced by methyl groups. In the interaction of dye with protein the determining factors are the structure of the dye molecules and the peculiarity of the binding sites of the protein. It is known that negatively charged groups of the first and second domains of BSA are inside the globule, while the positively charged are on the surface of the globule [24]. Dye binding to proteins can occur either due to hydrophobic interactions, or by electrostatic interaction. It should be noted that in non-covalent binding of the dye with the protein the dye itself chooses the binding site and can respond to changes not only in a certain fragment of the protein, but also in other places where the binding sites can appear and it can be reallocated. Active groups of BSA surrounded by hydrophobic regions forming binding sites able to interact with dye molecules. So it is possible to assume that the binding to BSA is due to the forces of electrostatic attraction between the oxygen atoms and positively charged globules on the surface of the protein. Figure 4 shows the fluorescence intensity (F/F_0) on the ratio of dye concentration to the concentration of BSA (C/P). Dye Tol-6, with the binding of the protein BSA ($p=4.1\times10^{-5}$M) enhances the fluorescence intensity (F/F_0) 20 times with equal ratio of the concentration of the dye/protein concentration (C/P) 1:10 (Fig. 4, curve 1). A similar pattern is observed for dye Dil-10 for binding to the protein BSA ($p=2.2\times10^{-5}$M) (Fig. 4, curve 2). Further increase in the quantity of dye leads to a decrease in fluorescence intensity. This picture can be explained by static quenching of fluorescence, which is associated with the formation of non-fluorescent dimers or larger aggregates at a high concentration of dye molecules. Dependence of binding constants on the dipole moment of molecules of styrylcyanine dyes is shown in Fig. 5. The figure shows that there is a clear correlation between the binding constant of styrylcyanine dyes with BSA and their molecular structure, in particular, with its electrostatic field. This confirms a series of experimentally observed regularities, particularly, a growth in a series of Tol-6 and S-3 (arrow 1). Fundamental importance is the growth of the binding constants in the

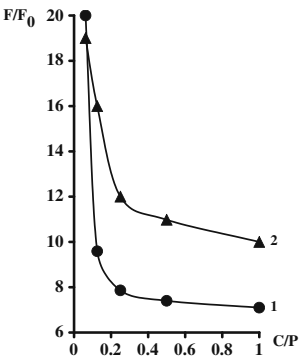


Fig. 4 Relative fluorescence intensity ratios of dye to the concentration of BSA in water: 1-Tol-6, 2-Dil-10

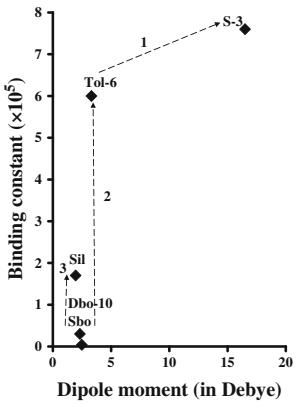


Fig. 5 Dependence of binding constants with BSA on the dipole moment of dye molecules

series Sbo, Dbo-10, Tol-6 (arrow 2). The established pattern suggests that in addition to electrostatic forces of attraction between molecules of styrylcyanine dyes with BSA, hydrophobic interactions are essential. Arrow 3 confirms quite natural growth of the binding constant with increase of the charge of anionic dyes, in particular, the transition from the anion of iodine atoms to perchlorate. A similar pattern was observed in [25] where it was shown that the asymmetric introduction of sulfo groups in polymethynes leads to increased polarity of its molecules, as well as to increase of the quantum yield of molecules in a biological environment.

Thus, it can be affirmed that the basis for the interaction of molecules of styrylcyanine dyes with BSA is electrostatic attraction, combined with hydrophobic interaction. This means that near a positively charged site of the BSA molecule there is a hydrophobic cavity, which can be penetrated by the dye. Similar conclusions were made by authors [26] and [27], who studied non-covalent interactions of symmetrical and asymmetrical squaraine dyes with BSA. The researchers [26] showed that although neither hydrophobic nor electrostatic interaction can not be considered as basic ones in their interaction, hydrophobic dye molecule provide a greater effect on their ability to bind to the protein and binding occurs in the hydrophobic cavity, which is located near the positively charged site. However, in the BSA molecules there are other binding sites, such as uncharged hydrophobic ones.

Electrostatic and hydrophobic nature of binding molecules styrylcyanine dyes suggests that the surface-active agents (detergents) can influence this interaction. At this, part of the non-polar molecules of detergent would be associated with the hydrophobic centres of adsorption of proteins and thereby inhibit the interaction of proteins with the dye molecules [12]. Identification of exact centre to be associated dye is strongly influenced by the number of deputies, and especially its location. Apparently, this is a reason of such significant difference between the values of binding constant obtained for the studied styrylcyanine dyes.

Conclusions

1. The spectral-luminescent characteristics of the new styrylcyanine dyes Sbo, Sil and their derivatives in water were studied. It was established that in the concentration range 10^{-5}–10^{-6} M molecules of studied dyes are in monomeric form. For dye molecules, which are in free state and upon binding to BSA calculated basic spectral-luminescent characteristics.
2. It was established that in the absorption spectra of dyes Tol-6, Dbo-10 and Dil-10 with rising amount of BSA there appear new bands with λ_{max}=505 nm, λ_{max}= 512 nm and λ_{max}=566 nm, respectively, intensity of which increases in proportion to the amount of albumin. The intensity of the glow of the main band of fluorescence in the presence of bovine serum albumin sharply increases.
3. The binding constant (K) and the number of binding sites (N) of the studied dyes with bovine serum albumin were determined. The dependence of the binding constants on the dipole moment of dye molecules, which indicates that besides electrostatic forces of attraction between molecules of styrylcyanine dyes with BSA, the hydrophobic interactions are of essential importance.

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