Russian Journal of Bioorganic Chemistry, Vol. 23, No. 12, 1997, pp. 857–865. Translated from Bioorganicheskaya Khimiya, Vol. 23, No. 12, 1997, pp. 857–978.

Spectral Properties of Bisbenzimidazole Dyes upon Interaction with DNA

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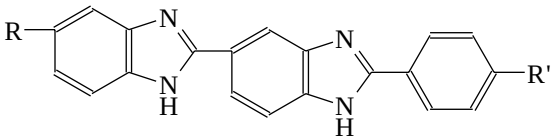
Received October 28, 1996; in final form, June 16, 1997

Abstract—Absorption and fluorescence spectra of a series of bisbenzimidazole dyes were studied in the presence of various concentrations of DNA and in diverse media. Variations in spectral properties of the compounds under study were found to depend on the measurement conditions. The bases for selecting the optimal composition of the media for DNA detection were presented. The modes of substrate–ligand binding and the dynamics of the alterations of fluorescent properties of structurally varying dyes upon their interaction with DNA were considered.

Key words: DNA, bisbenzimidazoles, spectral properties

Fluorescent DNA ligands are currently finding ever-widening application in the development of highly sensitive express methods of diagnostics of radiochemical damages and the prediction of long-term radiation pathology, for express evaluation of the biological contamination of water, for solving the ecological problems connected with the analysis of small doses of gene poisons, etc. Such compounds have been studied and used for practical purposes for several decades both in this country [1–4] and abroad [4–7]. Nowadays, externally binding compounds of the bisbenzimidazole series, such as Hoechst 33258, Hoechst 33342, and others [8–10], are among the most used fluorescent ligands for DNA. The mechanisms of their substrate interaction have been described in detail [11, 12]. However, the search for new dyes that would be more sensitive, specific, and selective to various medium conditions, to the state of cells, and to the conformational features of the nucleic acids binding them is still relevant. In particular, to develop an algorithm of the directed synthesis of new efficient fluorescent ligands for nucleic acids, the study of relations between the spectral properties and structural features of compounds of the same type is needed.

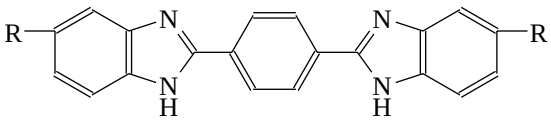
To this end, we investigated five compounds of the bisbenzimidazole series: 2'-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bis(1*H*-benzimidazole) (Hoechst 33258) (**I**); 2'-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bis(1*H*-benzimidazole) (Hoechst 33342) (**II**); 2'-(4-hydroxyphenyl)-5-(1-piperazinyl)-2,5'-bis(1*H*-benzimidazole) (**III**); 1,4-bis[5(6)-(4-methyl-1-piperazinyl)-2-benzimidazolyl]benzene (**IV**); and 1,4-bis[5(6)-(3-dimethylaminopropylcarbamoyl)-2-benzimidazolyl]benzene (**V**).



(**I**) R = –N⟨⟩N–CH_3; R' = –OH

(**II**) R = –N⟨⟩N–CH_3; R' = –O–CH_2–CH_3

(**III**) R = –N⟨⟩NH; R' = –OH



(**IV**) R = –N⟨⟩N–CH_3

(**V**) R = –C(=O)–N(H)–$(CH_2)_3$–N(CH_3)(CH_3)

The absorption and fluorescence excitation and emission spectra of these compounds were studied at various ratios of molar concentrations of DNA and a dye (C_s/C_d) in aqueous media of various compositions. To study the interaction of dyes (**I**)–(**V**) with DNA, we used various buffers, taking into account that the electrostatic (ionic) bonds in the DNA–ligand system weaken in solutions with a high ionic strength [12, 13], and in the presence of 4 M urea at a low ionic strength hydrogen bonds weaken [14, 15]. It is known that a

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Table 1. Wavelengths (nm) of the fluorescence excitation (λ_{ex}) and emission (λ_{em}) maxima and isobestic points (λ_{i1}–λ_{i3}) of the compounds under study depending on the composition of the medium and the presence of DNA*

Parameter	Dye				
	(I)	(II)	(III)	(IV)	(V)**
$(\lambda_{ex}^{0})_{sw}$	267	267	267	260	263
$(\lambda_{ex}^{max})_{sw}$	282	282	282	275	265
$(\lambda_{ex}^{0})_{lw}$	345	350	345	360	327
$(\lambda_{ex}^{max})_{lw}$	353	355	350	370	346
λ_{em}^{0}	497	500	495	553	400
λ_{em}^{max}	455	455	455	500	400
λ_{ex}^{ip}	353	355	350	369	350
λ_{em}^{ip}	458	458	458	504	400
λ_{i1}	350	354	349	367	332
λ_{i2}	383	385	380	395	372
λ_{i3}	363	365	362	379	350

* Indices sw and lw correspond to the SW and LW maxima of fluorescence excitation and absorption of the dyes; indices 0 and max correspond to the values measured in aqueous buffers A–C in the absence of DNA and at the highest ratio of the molar concentrations C_s/C_d (for LW maxima, $C_s/C_d > 100$; for SW maxima, $C_s/C_d > 200$); index ip corresponds to the values measured for compounds (I)–(V) in the absence of DNA in isopropanol (SW maxima of fluorescent excitation and absorption are absent in this solvent).

** The fluorescence emission spectra of compound (V) contain additional maxima at λ_{em} 380, 420, and 450 nm independent of the composition of the medium.

nonpolar environment promotes an increase in the emission of potentially active luminescence probes [7, 13, 16, 17]. Therefore, we studied the spectral properties of compounds (**I**)–(**V**) in the absence of DNA in isopropanol, in which the microenvironment of the dye molecule is the most similar to that in the minor groove of DNA [13, 17] and in the presence of the nonionic surfactant Triton X-100 in various concentrations.

Most of the dyes studied in the present work have two maxima of fluorescent excitation, long-wave (LW) and short-wave (SW), and one emission maximum. The maxima in the excitation spectra of the dyes coincide by their position with the maxima in the absorption spectra under the same conditions (Table 1). For compounds (**I**)–(**III**), with the identical aromatic phenylbisbenzimidazole fragment, positionally identical SW excitation (and, correspondingly, absorption) maxima were also observed both in the free and DNA-bound states. At the same time, the position of the LW excitation maxima is even affected by the replacement of the hydroxyl with an ethoxy group in the terminal phenyl ring of compounds (**I**) and (**II**). Hence, the SW band in the excitation (and, respectively, absorption) spectra of the compounds studied appears mainly to be due to the structure of the chromophore groups in the nucleus of the molecule, whereas the LW band appears to be due to the overall structure of the dye molecule, including the terminal group features. This is consistent with the fact that a more developed system of conjugated bonds results in a bathochromic shift of the absorption band [19]. The occurrence of LW and SW excitation (and, respectively absorption) maxima was also shown for different DNA-binding compounds of the monophenylbenzimidazole and monophenylindole series [13, 20], netropsin, distamycin A [21], and ethidium bromide [7].

From the absorption spectra of dyes (**IV**) and (**V**) with the addition of calf thymus DNA in the low-salt buffer A[2] (Fig. 1), one can see that, when the concentration ratio C_s/C_d increased from 0 to 200, there was a bathochromic shift of the absorption maximum (λ_a) by 5–15 nm. With increasing DNA concentration, the absorption of the solution first decreased (down to the molar ratio $C_s/C_d \cong 10$) and then began increasing to reveal two isobestic points in the spectra, λ_{i1} and λ_{i2}, respectively. The absorption spectra of compounds (**I**)–(**III**) were similar to that of dye (**IV**) (see Table 1 and [13]). For all the dyes studied, we observed a sequence $\lambda_{i2} > \lambda_a^{max} > \lambda_{i1} > \lambda_a^{0}$ (where λ_a^{max} and λ_a^{0}, nm are the wavelengths of the LW absorption maxima at a ratio of molar concentrations $C_s/C_d > 10$ and in the absence of DNA, respectively). The ambiguous character of the change of the absorption along with the occurrence of two isobestic points indicates, as shown in [13, 14], that the compounds, under the given conditions, possess two types of binding to the polynucleotide. Thus, as the DNA concentration increases, the type of the ligand–substrate interaction changes. When the concentration ratio $C_s/C_d < 10$, one interaction type prevails, which corresponds to λ_{i1}, whereas the C_s/C_d value exceeding 10 provides another type of binding, corresponding to λ_{i2}.

As mentioned above, in addition to the LW maximum, all compounds studied have a second, SW maximum in the absorption spectra. This is seen in Fig. 2, where the absorption and fluorescence spectra of dyes (**I**)–(**V**) in the low-salt buffer A in the absence of DNA are given. However, the position of the lower SW maximum coincides with the position of the DNA absorption (260–280 nm), which produces a strong masking effect. At the same time, DNA does not display this effect on the fluorescence excitation spectra of the dyes, because the polynucleotide essentially does not fluoresce in an unbound state at room temperature. Therefore, the fluorescence excitation spectra of the com-

[2] For the buffer compositions, see the Experimental section.

pounds under study [e.g., dyes (**IV**) and (**V**)] in the low-salt buffer A in the presence of calf thymus DNA (Fig. 3) exhibit two maxima. When the concentration ratio C_s/C_d changes, the behavior of these maxima differs. The intensity of emission upon the LW excitation of compounds (**I**)–(**IV**) increased progressivelly with C_s/C_d increasing from 0 to 200. At the same time, when C_s/C_d > 100, the emission of the dyes began to decrease, with the DNA concentration increasing at SW excitation (Fig. 3). An exception was compound (**V**), for which, at C_s/C_d > 160, the emission began to decrease at both SW and LW excitations (the absorption of the compound remained constant). In addition, for dye (**V**) when C_s/C_d > 160, an additional SW excitation maximum appeared at λ_{ex} 280 nm.

The fluorescence emission spectra of the dyes in the presence of DNA in the low-salt buffer A upon LW excitation (Fig. 4) show that a single emission maximum is displayed under these conditions for compound (**IV**). Note that for dyes (**I**)–(**IV**) the spectra are similar (see Table 1 and [13]). When the ratio C_s/C_d increased in a range of 0–30, the wavelength of the fluorescence emission maximum of compounds (**I**)–(**IV**) underwent a significant hypsofluoric shift (≅50 nm). Upon further increase of the ratio C_s/C_d, the maximum position remained constant. In the presence of DNA, the emission spectra of compound (**V**) have a fine structure with maxima at λ_{em} 380, 400, 420, and 450 nm. The positions of these maxima did not vary with an increase in the ratio C_s/C_d in the system. The values of the Stokes shifts for compounds (**I**)–(**V**) decreased when C_s/C_d increased except for the SW excitation of compound (**V**) (see Table 1).

The character of the changes in the spectra of compound (**I**) in the presence of DNA in buffers B[3] (with urea) and C (of high ionic strength) (Fig. 5) was on the whole similar to that observed in the low-salt buffer A (Fig. 2). However, from the data given in Fig. 5 and Table 2, one can see that, in buffers B and C at an increasing concentration of DNA, the changes in the spectral characteristics of the dye are less significant than in buffer A. Moreover, in buffer C, a monotonous decrease of the absorption of compound (**I**) occurred in the whole range of the concentration ratios C_s/C_d at the same bathochromic shift of the absorption maximum as in buffer A. In this case, the spectrum has a single isobestic point at λ_{i3} intermediate between the wavelengths of the two isobestic points λ_{i1} and λ_{i2} characteristic of this compound in the low-salt buffers A and B. A similar regularity of the variations of the spectral characteristics in these buffers was observed for dyes (**II**)–(**V**) (see Tables 1, 2).

The spectral data obtained for the dyes studied in the absence of DNA in isopropanol are presented in Fig. 6. Comparison of these results (see Figs. 1–4, 6 and Tables 1, 2) demonstrates that the spectral characteristics of the LW range of absorption and fluorescence of dyes (**I**)–(**V**) in isopropanol (dielectric constant χ = 20.1) are closer to the data obtained in the low-salt buffer A (χ = 78) at high values of C_s/C_d than to the data obtained in this buffer in the absence of DNA. In the SW range, the absorption and fluorescence excitation maxima of the compounds disappeared in the alcohol medium.

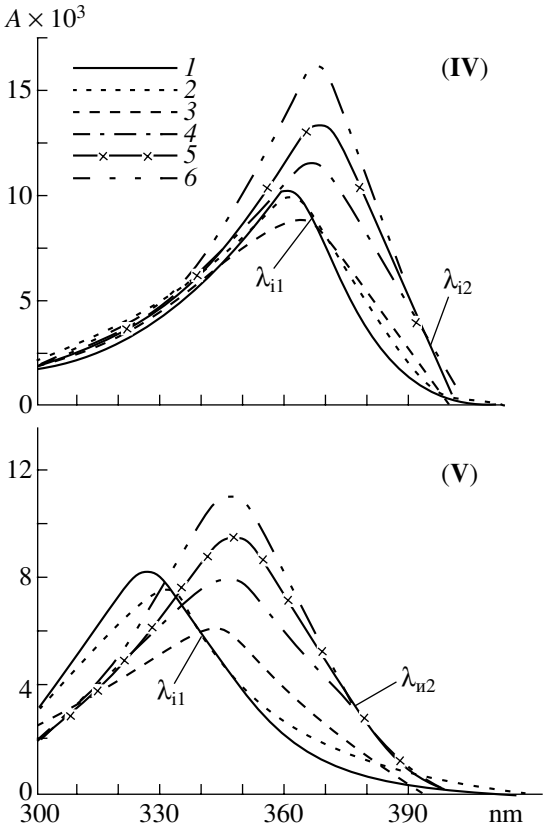

Fig. 1. Absorption spectra of compounds (**IV**) and (**V**) in the presence of DNA in the low-salt buffer A (see Experimental section) at various C_s/C_d ratios: (*1*) 0, (*2*) 3.5, (*3*) 10, (*4*) 50, (*5*) 100, and (*6*) 200. λ_{i1} and λ_{i2} indicates the positions of the isobestic points in the spectra of the compounds studied.

A nonionic surfactant was similar to isopropanol in affecting the spectra of the dyes. As an example, we give (Fig. 7) the absorption and fluorescence spectra of compound (**I**) in the low-salt buffer A in the presence of a nonionic surfactant in various concentrations. With an increasing concentration of the nonionic surfactant, a monotonous increase in the intensity occured only for the LW excitation (and, correspondingly, absorption) maximum of the dye accompanied by a bathochromic shift similar to that observed for the DNA–ligand system. In the absorption spectrum of compound (**I**) in the presence of Triton X-100 (T), an isobestic point appeared at λ_{i4} 346 nm. The position of the maximum and the fluorescence intensity in the emission spectra at

[3]For the buffer compositions, see the Experimental section.

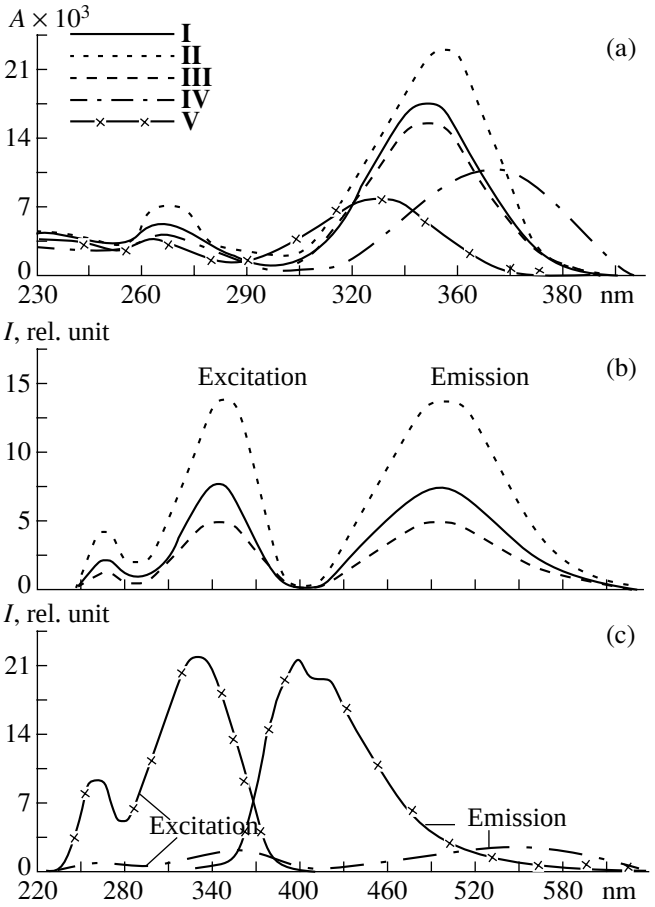

Fig. 2. Spectra of absorption (a), fluorescence excitation (b), and emission (c) of compounds studied in the low-salt buffer A in the absence of DNA. Curves **I**–**V** corresponds to compounds (**I**)–(**V**).

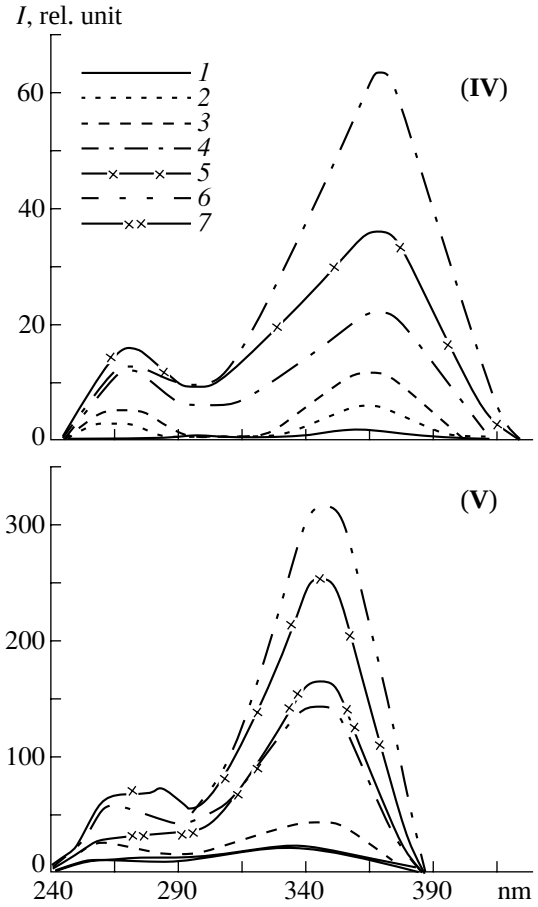

Fig. 3. Spectra of fluorescence excitation of compounds (**IV**) and(**V**) in the presence of DNA in the low-salt buffer A for various ratios of the molar concentrations C_s/C_d for (**IV**): (*1*) 0, (*2*) 3.5, (*3*) 10, (*4*) 50, (*5*) 100, and (*6*) 200; and for (**V**): (*1*) 0, (*2*) 3.5, (*3*) 10, (*4*) 50, (*5*) 100, (*6*) 160, and (*7*) 230.

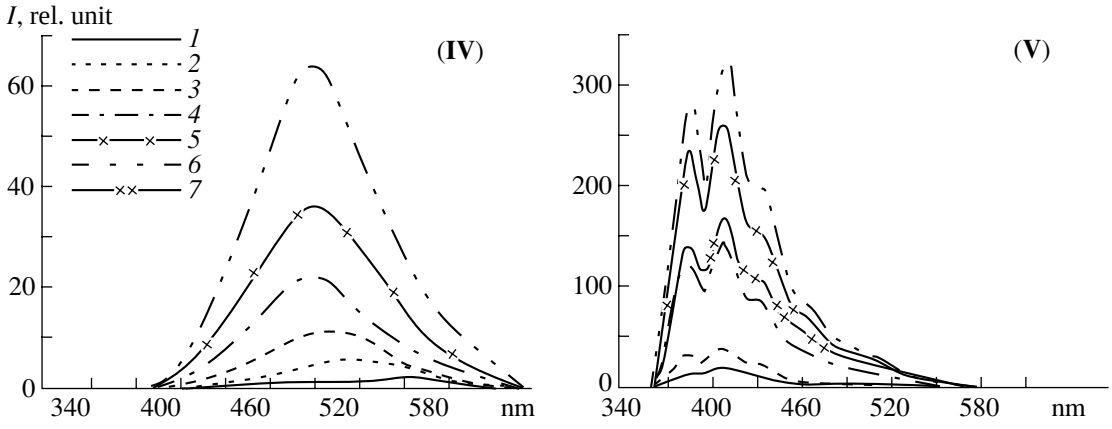

Fig. 4. Spectra of fluorescence emission of compounds (**IV**) and (**V**) in the presence of DNA in the low-salt buffer A. The designations are identical to those in Fig. 3.

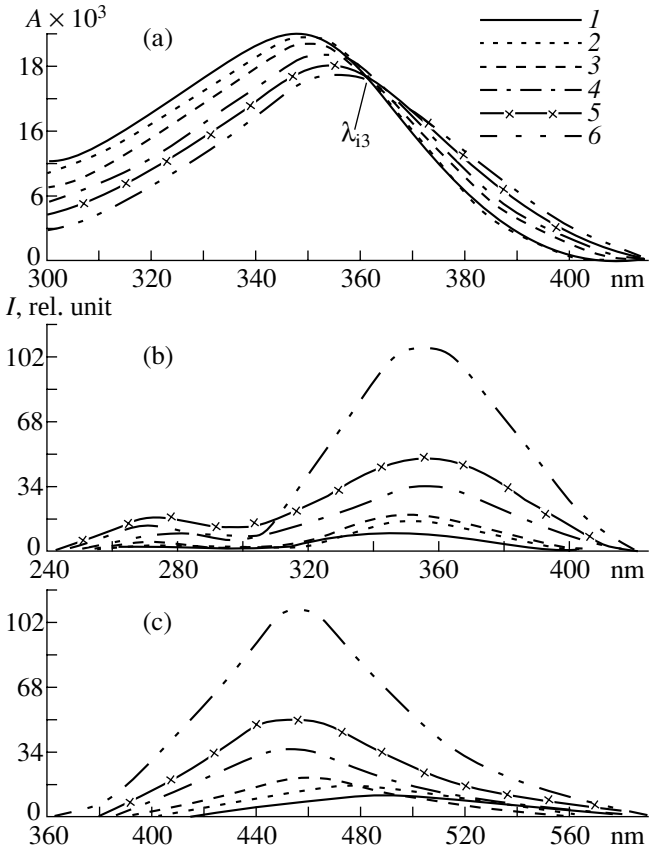

Fig. 5. Spectra of (a) absorption, (b) fluorescence excitation, and (c) emission of compound (**I**) in the presence of DNA in the high-ionic-strength buffer C (see the Experimental section) at various ratios of molar concentrations C_s/C_d: (*1*) 0, (*2*) 3.5, (*3*) 10, (*4*) 50, (*5*) 100, and (*6*) 200.

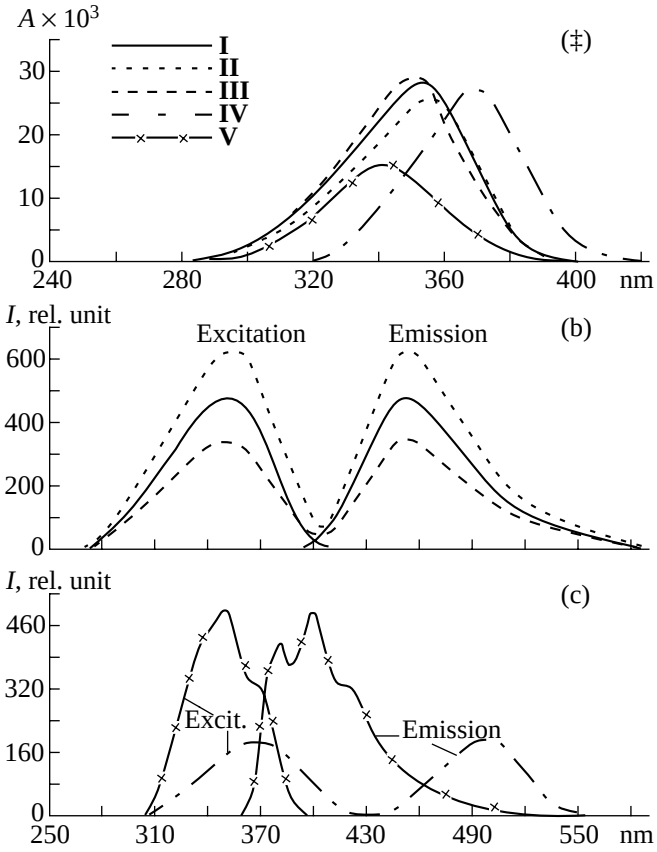

Fig. 6. Spectra of (a) absorption, (b) fluorescence excitation, and (c) emission of compounds studied in the absence of DNA in isopropanol. Curves **I**–**V** correspond to compounds (**I**)–(**V**).

the LW excitation changed with an increasing ratio of C_t/C_d just as in the polynucleotide–ligand system with an increasing ratio of C_s/C_d (see Figs. 5, 7).

Thus, from the practical point of view, to attain the maximum fluorescent sensitivity of a dye (ligand) toward the substrate (DNA) interacting with the dye, one should select an aqueous buffer with the ionic strength sufficient for the formation of hydrogen and ionic bonds between the ligand and DNA. This buffer should not contain impurities that either hinder the formation of these bonds or decrease the dielectric constant of the buffer solution. An increase in the viscosity of the solution also harms the fluorescent sensitivity of the DNA–ligand complexes, which we demonstrated for the 50% sucrose solution [18]. These data (φ_{suc}/φ_0) are listed in Table 2. Furthermore, to select the conditions providing the maximum fluorescent sensitivity of a dye toward polynucleotide, one should take into account the optimal ranges of the temperature and pH of the medium, which must be determined from preliminary investigations for each dye as was demonstrated in [22, 23], and the C_s/C_d ratio (see Fig. 8a and Table 2).

To illustrate the aforesaid, we used parameter η, the fluorescent sensitivity of the compound toward the polynucleotide (see the Experimental section) as a quantitative criterion. This parameter is the most representative for characterizing the ability of a dye to be an efficient fluorophore for the substrate. The values of η and of the relative quantum yield decreased as the ionic strength of the buffer increased and the polarity of the solvent in the alcohol decreased (Table 2). These values for compounds (**I**)–(**V**) also decreased in the presence of a substantial amount of urea (buffer B) and sucrose [18].

This can be accounted for by the fact that, although for all the compounds studied we observed two types of binding to oligonucleotide in low-salt buffers, the variation in the fluorescence in the DNA–dye systems was mainly determined by one of them, the specific one, for which the formation of both hydrogen and ionic bonds is characteristic. Similar results were obtained for Hoechst 33258 dye in [11]. The sum of the relative contributions of the hydrogen (*H*) and electrostatic (*X*) bonds to the formation of a specific fluorescent complex in the DNA–ligand system was close to 100% for

Table 2. Spectral properties of the compounds studied in diverse media and in the presence of various amounts of DNA

Parameter	Dye					Parameter	Dye				
	(**I**)	(**II**)	(**III**)	(**IV**)	(**V**)		(**I**)	(**II**)	(**III**)	(**IV**)	(**V**)
$\varphi_0 \times 10^3$	10.7	15.4	8.1	5.0	66.8	$\varepsilon_{lw} \times 10^{-3}$	27.3	35.5	24.4	17.1	12.5
φ_{max}	0.45	0.60	0.32	0.20	0.92	$\varepsilon_{sw} \times 10^{-3}$	8.1	11.1	6.5	5.6	6.2
$(\varphi_{max}/\varphi_0)_A$	41.60	39.10	39.60	38.80	13.80	$\mu_{lw} \times 10^{-6}$	11.7	21.6	7.8	3.4	33.3
$(\varphi_{max}/\varphi_0)_B$	18.20	18.20	15.30	19.90	9.45	$\mu_{sw} \times 10^{-6}$	3.4	6.5	2.0	1.0	14.5
$(\varphi_{max}/\varphi_0)_C$	21.90	19.30	23.00	17.60	4.71	$\eta_1^A \times 10^{-6}$	4.90	4.23	6.06	2.13	1.16
φ_{ip}/φ_0	38.50	35.42	36.18	35.61	12.60	$\eta_{10}^A \times 10^{-6}$	4.07	3.57	2.45	1.34	4.31
φ_{suc}/φ_0 [18]	7.0	6.4	6.9	8.4	2.0	$\eta_{100}^A \times 10^{-6}$	1.69	2.85	1.18	0.44	3.52
H, %	51	48	57	44	29	$\eta_{10}^B \times 10^{-6}$	1.50	1.47	0.72	0.77	3.63
X, %	43	45	37	50	65	$\eta_{10}^C \times 10^{-6}$	1.23	1.35	1.04	0.39	1.37
H/*X*	1.19	1.07	1.54	0.88	0.45	Ψ	0.82	0.92	1.44	0.51	0.38
E/*R* [18]	5.76	6.04	5.54	4.10	11.80						

Note: φ are the quantum yields of dyes; the values + for the compounds studied in aqueous buffers A–C and a 50% sucrose solution are designated by indices A, B, C, and suc, respectively; the remaining indices coincide with those in Table 1; *H* and *X* are relative contributions of the hydrogen and ionic bonds to the specific, actively fluorescing complex DNA–dye (see the Experimental section); *E* and *R* are relative contributions of intramolecular donor–acceptor interaction and rotational diffusion to the variation of the fluorescence of a dye molecule in solution upon its interaction with DNA (from [18]); ε (M^{-1} cm^{-1}) and μ (rel. unit M^{-1} cm^{-1}) are the molar coefficients of absorption and fluorescence of the compounds studied in the absence of DNA in the low-salt buffer A at LW and SW excitation; η (rel. unit M^{-1}) are the coefficients of the fluorescent sensitivity of the compounds studied toward DNA (see the Experimental section) in buffers A–C at LW excitation and the ratios of molar concentrations C_s/C_d 1, 10, and 100, respectively; for compound (**V**), as in the case of the ε and μ values, the highest peak registered was used; and $\Psi = \eta_{10}^C / \eta_{10}^B$.

the dyes studied. However, their ratio (*H*/*X*) varied from 1.54 for compound (**III**) to 0.45 for compound (**V**) (Table 2). Accordingly, the ratio of the fluorescent sensitivities η of these dyes changed in solutions with high ionic strength and urea, which inhibit the formation of the ionic and hydrogen bonds in DNA–ligand systems. For compounds (**I**)–(**V**), a linear correlation with coefficient *r* 0.935 ($p < 0.05$) was observed between Ψ values, which reflect the ratio of the η values for a dye at $C_s/C_d = 10$ in buffers C and B, and *H*/*X* (see Table 2 and Fig. 8b).

At low C_s/C_d ratios in the standard low-salt buffer A, a positive linear correlation with the coefficient $r_{H_\eta} = 0.937$ ($p < 0.05$) was also observed between the values of *H* and the fluorescent sensitivity (η) of dyes (**I**)–(**V**) toward the polynucleotide. This fact indicates that, when the polynucleotide is saturated with the dye in a sufficiently high degree ($C_s/C_d = 1$), the hydrogen bond formation has a crucial effect on the fluorescent sensitivity in the DNA–ligand system. However, when the saturation degree of the polynucleotide with a dye decreased, the governing role of the hydrogen component on the η value decreased (r_{H_η} decreased to –0.603 at C_s/C_d 100), and the ionic bonds made a more substantial contribution to the formation of the fluorescent complex of DNA with a dye [r_{H_η} 0.988 for compounds (**I**)–(**III**)]. Similar results were obtained for DNA-binding dyes of the monophenylindole and monophenylbenzimidazole series [13].

Possible causes leading to an increase in the quantum yield of the fluorophores in the absence of DNA and, consequently, to a decrease in their fluorescent sensitivity toward the polynucleotide as the dielectric constant of the medium decreases (in the presence of alcohols and nonionic surfactants) and the solution viscosity increases (in the presence of sucrose) have to do with the general mechanism of fluorescence. As was shown earlier, the character of the spectral properties of compounds similar to those studied in this work are determined by a complex mechanism [18, 24, 25].

On the one hand, this mechanism must take into consideration the known fact that only rigid, planar, and conjugated electron systems can intensively fluoresce. If the structure of a dye does not possess the required rigidity, it will fluoresce weakly in aqueous solutions in the unbound state even if it comprises fragments capable of fluorescing, because in this case, the probability of nonradiative transition from the excited state to the ground state by rotational diffusion is significant. When a specific interaction with polynucleotide occurs or in a

substantially viscous sucrose solution, the fluorophore molecule stabilizes. This excludes the possibility of vibrational motions, and the contribution of the radiative transitions and, therefore, the quantum yield for this compound will increase.

On the other hand, the π-electron system of molecules with an approximately equal rigidity and planarity is particularly affected by electron-accepting terminal substituents. In polar solvents for an unbound dye molecule, these substituents will inhibit the luminescence of the fluorophore molecule. However, upon a specific interaction with a substrate or in a nonpolar solvent, e.g., in isopropanol, when the electron densities of the substituents and the nucleus of the molecule capable of active fluorescence are separated, the compound will regenerate its fluorescent properties [13,16].

The ratio of the contributions of the donor–acceptor and rotation–diffusion factors to the variation of the fluorescence of compounds (**I**)–(**V**) upon their interaction with DNA was determined earlier [18] and, designated as *E*/*R*, is listed in Table 2 in this paper. The ratio *E*/*R* as a function of the zero (φ_0) and maximum (φ_{max}) quantum yields are given in Fig. 8a for the compounds under study in the low-salt buffer A in the presence of DNA. When the *E*/*R* ratio for the dyes increases, the φ_0 values increase insignificantly, whereas the φ_{max} values increase to a greater extent. These data are in agreement with the earlier results for dyes (**I**)–(**V**) [18] and may indicate that the electron-donating factors make a major contribution to the luminescence of the ligands, while the role of the rotation–diffusion factors decreases in the case of the high ratios of concentrations C_s/C_d.

Thus, designing the structure of a potential fluorophore from the point of view of the highest relative quantum yield that may be provided in the presence of DNA, one has to consider both the highest fluorescence intensity that can be provided by the nucleus of the designed compound and the rigidity of the molecule, as well as the presence in the molecule of terminal electron-accepting substituents correlating in their activity with the nucleus.

EXPERIMENTAL

Dyes (**I**) and (**II**) (Hoechst 33258 and Hoechst 33342, respectively) were purchased from Serva (Germany). Compounds (**III**)–(**V**) were synthesized in the Department of Molecular Biotechnology, St. Petersburg Technological Institute [26, 27]. DNA from calf thymus (Serva, Germany) (58% AT pairs, average molecular mass per nucleotide of 326 Da, molar absorbance ε_{260} of 6600 M^{-1} cm^{-1}) was used as a substrate. To homogenize and decrease the light scattering, DNA was preliminarily sonicated in a UZDN-2 device (Russia) for 15 s at 0.3 A and a resonance frequency of 22 kHz (average molecular mass of the DNA was 3500 Da).

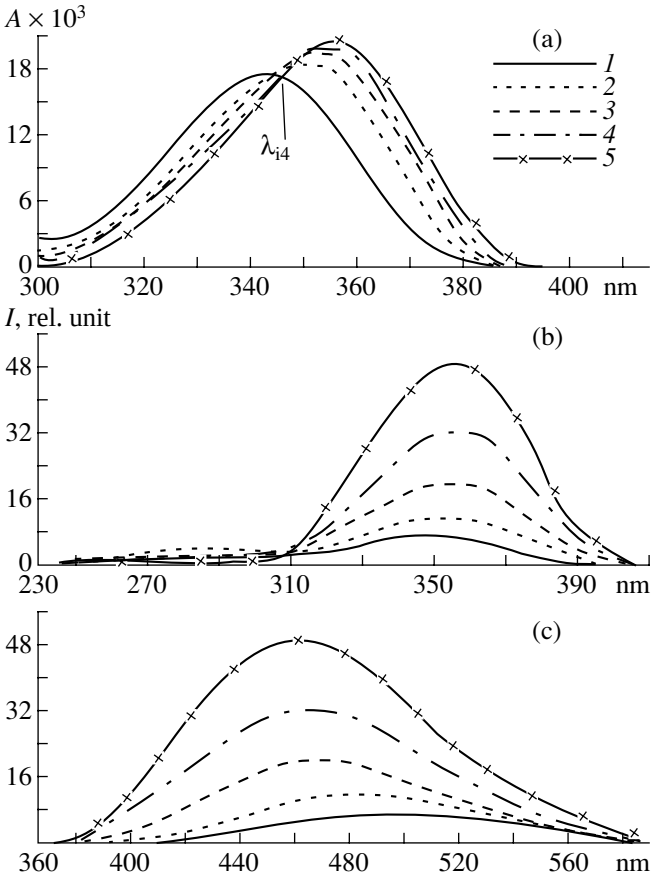

Fig. 7. Spectra of (a) absorption, (b) fluorescence excitation, and (c) emission of compound (**I**) in the absence of DNA in the low-salt buffer A with addition of Triton X-100 at various ratios of molar concentrations C_t/C_d: (*1*) 0, (*2*) 3.4, (*3*) 10, (*4*) 20, and (*5*) 50.

The substances were analyzed in the following buffers: (A) 0.01 M NaCl, 0.01 M Na_2-EDTA, and 0.01 M Tris-HCl (pH 7.4); (B) buffer A containing additionally 4 M urea (pH 7.6); and (C) 2 M NaCl, 0.1 M Na_2-EDTA, and 0.01 M Tris-HCl (pH 8.0).

The spectral characteristics of compounds (**I**)–(**V**) were also determined in an isopropanol solution and low-salt buffer A with addition of the nonionic surfactant Triton X-100 (up to a final ratio of the molar concentrations $C_t/C_d = 50$).

All measurements were carried out at the dye constant concentration $C_d = 6.4 \times 10^{-7}$ M and various concentrations of the polynucleotide at 20–25°C. To avoid the effect of dilution, we added aliquots of the solution containing a buffer, a dye, and DNA in the concentration providing $C_s/C_d = 200$ to a blank solution containing the buffer and the dye. The absorption spectra of the solutions were recorded on a Beckman Model 35 spectrophotometer (Austria). The fluorescence of the samples was analyzed on a Hitachi Model 850 spectrofluorimeter (Japan). The fluorescence spectra were

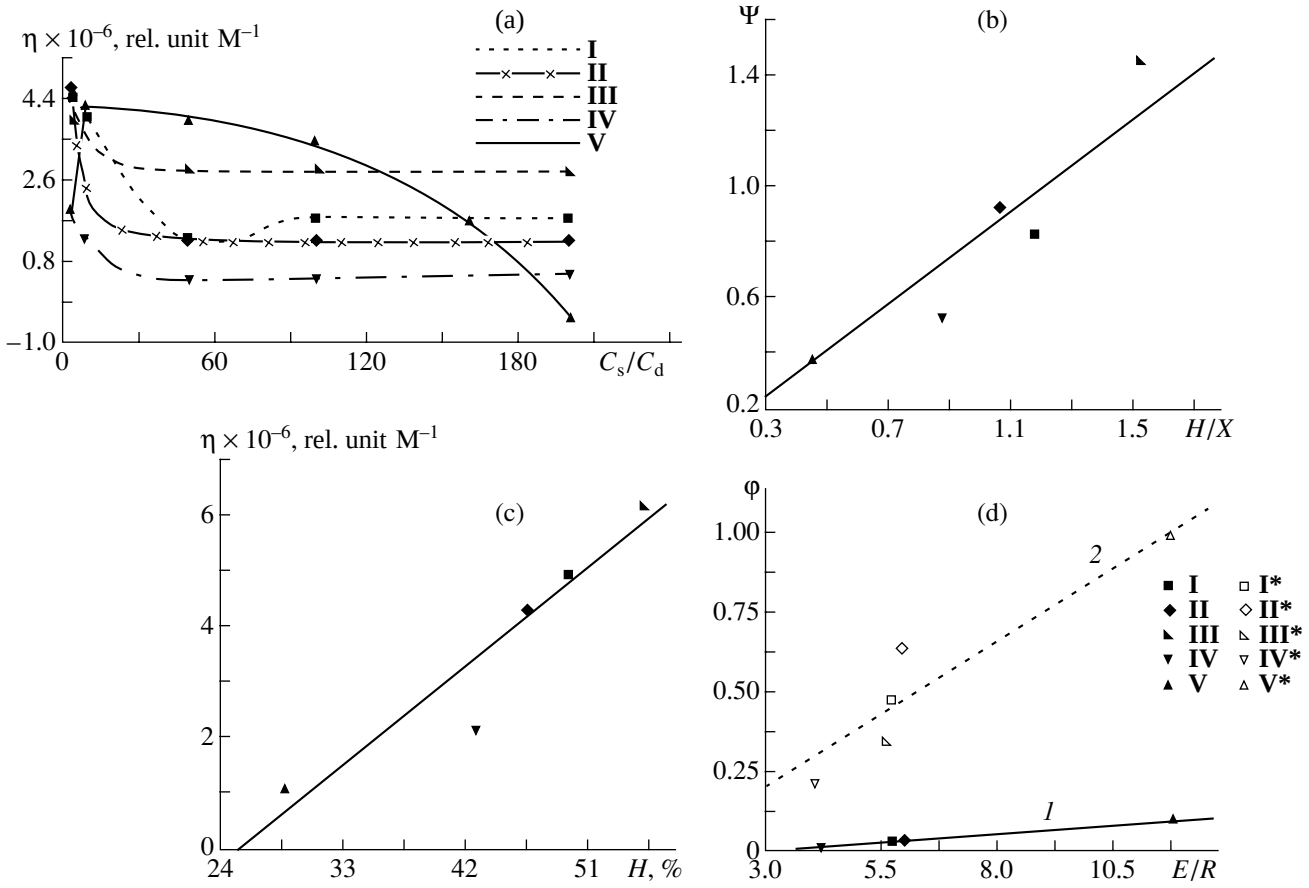

Fig. 8. (a) Coefficients of the fluorescent sensitivity (η of compounds (**I**)–(**V**) (curves (**I**–**V**) vs. the C_s/C_d ratio); (b) the ratio of the coefficients of the fluorescent sensitivity of the dyes studied at C_s/C_d 10 in buffers with a high ionic strength and with urea ($\Psi = \eta^C_{10}/\eta^B_{10}$, see Table 2) vs. the ratio of contributions (*H*/*X*) of the hydrogen and ionic bonds to the formation of the specific ligand–polynucleotide fluorescent complex; (c) coefficients of the fluorescent sensitivity (η) of compounds (**I**)–(**V**) at the concentration ratio C_s/C_d 1 vs. the relative contribution of hydrogen bonds (*H*) to formation of the specific ligand–polynucleotide fluorescent complex; (d) The zero (φ_0, curve *1*) and maximum (φ_{max}, curve *2*) quantum yields of the compounds studied vs. *E*/*R* (see Table 2). The data in (a), (c), (d) were obtained in the low-salt buffer A. Points represent the experimental data.

recorded under the following conditions: the bandwidth was 3 nm for the excitation and emission, the scan rate was 120 nm/min, the response time was 2 s, and the photomultiplier attenuation was normal. Excitation spectra were recorded at the wavelength corresponding to the highest emission maximum, and the emission spectra were recorded at the wavelength corresponding to the maximum of the LW peak of excitation of the compound for each ratio of the concentrations C_s/C_d in the system.

The zero quantum yields of fluorescence (φ_0) of the dyes were determined relative to the standard quinine sulfate solution in 1 M H_2SO_4 (φ_0 = 0.55) [16]. Since the absorption and fluorescence spectra for compounds (**I**)–(**V**) were not overlapped, the relative quantum yields φ_{DNA}/φ_0 and φ_{ip}/φ_0 were calculated from the maximum absorption and fluorescence of the compounds under appropriate conditions using equation

$$\varphi_2/\varphi_1 = (I_2A_1)/(I_1A_2),$$

where I_1, I_2 and A_1, A_2 are the values of the fluorescence intensity and absorbance of the compounds in states 1 and 2 [18].

The values of the relative quantum yields φ_{suc}/φ_0 and of the ratios of contributions of intramolecular donor–acceptor interaction and rotational diffusion to the variation of the fluorescence of compounds (**I**)–(**V**) upon their specific interaction with DNA (*E*/*R*) were taken from [18]. The values of molar absorbance and fluorescence were determined as $\varepsilon = A/C_d$ and $\mu = I/C_d$. Standard cells with an optical pathlength of 1 cm were used.

The relative contributions of the hydrogen and electrostatic bonds to the formation of an intensively fluorescing complex of dyes with the polynucleotide (*H* and *X*, respectively) were calculated from equations

$$X = \frac{(\varphi_{max}/\varphi_0)_B - 1}{(\varphi_{max}/\varphi_0)_A - 1}100\%,$$

$$H = \frac{(\varphi_{max}/\varphi_0)_C - 1}{(\varphi_{max}/\varphi_0)_A - 1}100\%,$$

where $(\varphi_{max}/\varphi_0)_A$, $(\varphi_{max}/\varphi_0)_B$, and $(\varphi_{max}/\varphi_0)_C$ are the highest relative quantum yields in the presence of DNA for a given dye in buffers A, B, and C, respectively [13].

The coefficients of sensitivity (η_S) reflecting the value of the increment in the fluorescence intensity of the dye per mole of DNA at the concentration ratio $C_s/C_d = S$ were calculated according to the equation

$$\eta_S = (I_{S+1} - I_{S-1})/(2C_d),$$

where I_{S+1} and I_{S-1} are the fluorescence intensities of the dye in relative units at the concentration ratios $C_s/C_d = S + 1$ and $C_s/C_d = S - 1$, respectively.

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