Russian Journal of Bioorganic Chemistry, Vol. 27, No. 1, 2001, pp. 57–65. Translated from Bioorganicheskaya Khimiya, Vol. 27, No. 1, 2001, pp. 64–73.

Comparative Study of DNA-Specific Dyes of the Indole and Benzimidazole Series

V. S. Sibirtsev*, A. V. Garabadzhiu, and S. D. Ivanov*[1]**

**Central Research Institute of Roentgenology and Radiology, Russian Ministry of Public Health, Leningradskaya ul. 70/4, Pesochny-2, St. Petersburg, 189646 Russia*

***St. Petersburg State Technological Institute, St. Petersburg, Russia*

Received May 12, 1999; in final form, September 8, 2000

Abstract—Various models of complex formation of low-molecular ligands with DNA are considered. Using the Scatchard model, parameters of binding of fluorescent monophenylindole, monophenylbenzimidazole, and bisbenzimidazole dyes with calf thymus DNA were evaluated. By means of graphic (nonparametric) and correlation analysis, various spectral and complexation properties of these dyes in the presence of DNA are compared.

Key words: DNA, complex formation, benzimidazoles, phenylindoles

INTRODUCTION

Lately, biochemistry and molecular biology have intensely studied the mechanisms of interaction with DNA of various DNA-specific ligands possessing fluorescent properties. These studies have not only a theoretical but also an applied importance since the range of potential applications of such compounds is large. They can be used in diagnostistics—in assessing the quantitative and qualitative changes in the structure of nucleic acids at different states of the organism [1, 2], for the biotesting of components of the environment [3], as vectors specific to certain DNA fragments [4, 5], and directly as drugs [6–8].

For integral characterization of interaction in the DNA–ligand systems, the following approach is currently most widespread. Suppose, a polymer contains F binding sites for ligand, and all of them are spectrally equivalent. Assume also that binding to each site and the ensuing alteration of the spectral parameters do not depend on the interaction with the neighboring binding sites. Assume the following designations: $m = C_L^f$, molar concentration of the free ligand in solution; $r = C_L^b/C_D$, the amount of the bound ligand per unity of the substrate concentration (here, C_D is the overall molar concentration of nucleotides in the system; C_L^b, molar concentration of the bound ligand in solution); $n = r_{max}$, the maximum possible number of ligand molecules that can be bound to one DNA molecule with the total number of nucleotides taken into account (this value is also referred to as the number of binding sites per polynucleotide's base pair). It should especially be noted that values $h = C_D^b/C_L^b$ (reflecting the number of polynucleotide basepairs really bound by a ligand molecule) and $q = 1/n = [(C_D^b + C_D^f)/C_L^b]_{min}$ (where C_D^b and C_D^f are molar concentrations of ligand-bound and free nucleotides in the system; it reflects the average number of basepairs in the polynucleotide that on binding a ligand molecule become inaccessible for other molecules of the ligand) are not to be identified since the q quantity would comprise both h basepairs really bound by one molecule of ligand and a number of basepairs with which the ligand, owing to the specific character of its interaction with the polynucleotide, cannot in principle bind. For example, for such a well-known intercalator as ethidium bromide upon its binding to various polynucleotides the h quantity will always be equal to 2 bp (this can be illustrated by X-ray analysis) whereas q will be more than or equal to 2 bp depending on the kind of the polynucleotide (as an example, the higher the proportion of A · T pairs and superspiralization degree, the larger will be q upon ethidium bromide binding [10]).

At $n = 1$, equation of the Langmuir isotherm [11] will be correct for the system under consideration:

$$K = r/(m - mr). \quad (1)$$

At the same time, for $n < 1$ (which takes place in real DNA–ligand systems), Scatchard [12] proposed to transform equation (1) into:

$$r/m = Kn - Kr. \quad (2)$$

[1]To whom correspondence should be addressed; phone: +7 (812) 596-6703, 599-5573.

Here, r and $n - r$ can be interpreted as fractions of the occupied and free binding sites (each h bp long) on the polymer under consideration, and K as an empirical constant with the value inverse to the concentration of the free ligand in the system, when it occupies half of the potential binding sites on the substrate. The K value is often referred to as binding constant, but by analogy with the Langmuir model we will call it adsorption constant to avoid mixing with the thermodynamic constant of stability of complex formed by a ligand with h bp (a binding site) on a polynucleotide as it took place in [9, 13]. Some researchers [14, 15] interpreted this quantity as the thermodynamic constant of stability of complex formed by a single molecule of dye with the whole of a polynucleotide molecule rather than with a binding site on it. However, such interpretation would only be correct when C_D in formula (2) was determined as the concentration of DNA molecules rather than nucleotides, which is not easy.

There also exist other models developing this approach. Thus, if to assume the presence in system G of types of complexes with differing values of K_i and n_i, then, generalizing for this case the conclusions made for equation (2), the following expression can be obtained [11]:

$$r = \sum [n_i K_i m/(1 + K_i m)]; \quad i = 1–G. \qquad (3)$$

It was noted [16] that in process of binding of extended ligands $h > 1$ to DNA several microforms emerge, in some of which ligands are situated in such a way that the lattice cannot bind more ligands whereas in others there still remains some place for additional molecules. All these microforms are in an equilibrium with the free ligand. On the ligand binding to the polynucleotide it is continuously redistributed until a terminal state is set which can be characterized as the saturation of the polynucleotide with the ligand. To evaluate the binding characteristics in this case, the following equation was proposed [17]:

$$r/m = KL[L/(L + r)]^{q-1}, \quad \text{where} \quad L = 1 - rq, \qquad (4)$$

and in the case of G types of complexes with differing K_i and n_i values, the equation system as follows:

$$r_i/m = K_i L^{q_i}/(L + \sum r_i)^{q_i - 1}, \qquad (5)$$

$$\text{where} \quad L = 1 - \sum r_i q_i, \quad i = 1–G.$$

Mutual influence of ligands can be expressed through the cooperativity parameter w, which allows equation (4) to be written with cooperative effects taken into account [18]:

$$r/m = KL\left(\frac{Lrw + L + r - R}{Lr(w-1)}\right)^{q-1}\left(\frac{L - r + R}{2L}\right)^2, \qquad (6)$$

$$\text{where} \quad R = \sqrt{(L-r)^2 + 4wrq}.$$

At $w = 1$ (no interaction between ligands), equation (6) is reduced to equation (4). When $w < 1$ (anticooperative binding), antagonism on binding of ligands to polynucleotide is observed. When $w > 1$ (cooperative binding), sorption of a ligand on the substrate facilitates the addition of another ligand. As an increase in q results in anticooperativity traits on binding, at $w > 1$ and $q > 1$ the curve described by equation (6) apparently results from a compromise between influences of these two factors.

The above approach was further developed in the Crothers allosteric model [7, 19, 20]. It suggests that each basepair of the polynucleotide exists in one of two forms (or conformations) capable of mutual transitions with the equilibrium constant s and cooperativity parameter σ. The value of the latter reflects hindrances on transition of a basepair from form 1 to form 2, which emerge within the substrate molecule that is ligand-free and is preferentially in form 1. The ligand can bind both forms of the substrate with the absorption constants K_1 and K_2 and the numbers of binding sites n_1 and n_2, respectively. By initially binding with substrate in form 1, the ligand can transform it in a local area to form 2. Therefore, the ratio between forms 2 and 1 in the polynucleotide changes from $\sigma^2 s$ (in a free state) to s (upon complete binding of form 1 by the ligand). A similar transition of DNA from A (or Z) conformation to B conformation induced by such classic outer-binding dyes as netropsin, dystamycin A, DAPI (4',6-diamidino-2-phenylindole), etc. is considered in [19, 21, 22].

One of generally accepted models for determining the adsorption constants and the number of binding sites, as well as revealing the cooperative and anticooperative character of the interaction in the DNA–ligand system is also the Zasedatelev–Gurskii model [11, 17, 23]. Additional conditions can also be introduced into these models for certain systems. For example, in [24] a model is considered that takes into consideration the intercalation of the dyes under study, their outer cooperative binding with negatively charged phosphate groups of DNA, the influence of a sodium ion competing for the binding sites of the dye with phosphate groups of the polynucleotide, and possible dimerization of the free ligand.

Thus, the current theory seems to encompass almost all acceptable variants of the interaction of ligands between themselves and with the polynucleotide. However, the practical use of such models is associated with a number of difficulties caused by their considerable dependence on the quality of the experimental data and the necessity of employment of a rather intricate special mathematical and program apparatus. As a result, the excessive sophistication of the model not accompanied with a reliable and sound method of computation of its parameters may worsen its adequacy, which took place, for instance, in [24]. At the same time, the effects described by the above models contribute perceptible distortions into the Scatchard equation only at a suffi-

ciently high overall ligand concentration and low ratios of the DNA and ligand concentrations in the system [9, 25]. Moreover, the statistical effects described by equation (4) can take place only in systems with rather homogeneous substrates (e.g., in the case of poly-AT, but to a much lower extent with DNA of living organisms) or with low specific ligands (in particular, with intercalators but not with outer-binding compounds). All this results in that the Scatchard equation (2) remains by far a widely distributed model in computing parameters of formation of complexes involving DNA-binding compounds [9, 10, 13, 25–27].

RESULTS AND DISCUSSION

As an object of study, we chose five outer-binding fluorescent DNA-specific dyes of the monophenylbenzimidazole and monophenylindole series, henceforth referred to as monoderivatives since each of them contains only one benzimidazole or indole fragment capable of active fluorescence:

5(6)-amino-2-(4-aminophenyl)benzimidazole (**I**),

5(6)-amidino-2-(4-aminophenyl)benzimidazole (**II**),

5(6)-amidino-2-(4-nitrophenyl)benzimidazole (**III**),

4',6-diamidino-2-phenylindole (DAPI) (**IV**),

6-(2-imidazoline-2-yl)-2-[4-(2-imidazoline-2-yl)phenyl]indole (DIPI) (**V**),

as well as five ligands of the bisbenzimidazole series, which will be called bisderivatives since each of them contains two benzimidazole fragments capable of active fluorescence:

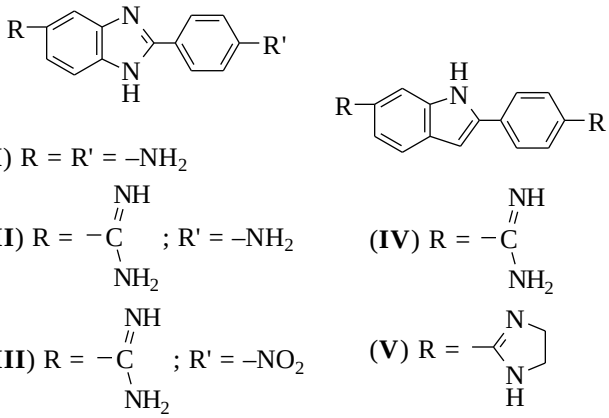
2-[2-(4-hydroxyphenyl)benzimidazole-5(6)-yl]-5(6)-(4-methylpiperazine-1-yl)benzimidazole (Hoechst-33258) (**VI**),

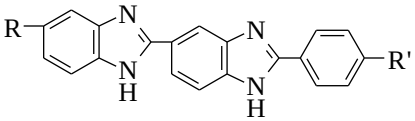
2-[2-(4-hydroxyphenyl)benzimidazole-5(6)-yl]-5(6)-(piperazine-1-yl)benzimidazole (**VII**),

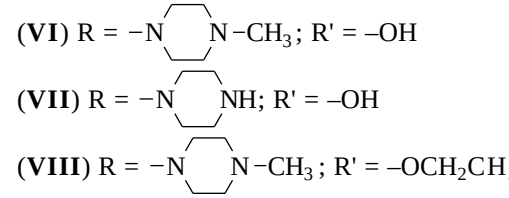
2-[2-(4-ethoxyphenyl)benzimidazole-5(6)-yl]-5(6)-(4-methylpiperazine-1-yl)benzimidazole (Hoechst-33342) (**VIII**),

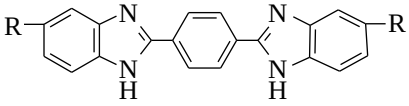
1,4-di[5(6)-(4-methylpiperazine-1-yl)benzimidazole-2-yl]benzene (**IX**),

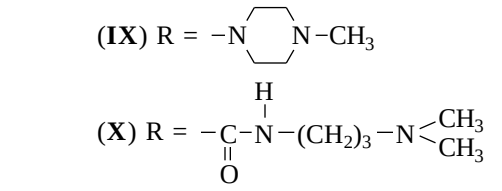
1,4-di[5(6)-(3-dimethylaminopropylcarbamoyl)benzimidazole-2-yl]benzene (**X**).











Scheme 1.

For these ten compounds, differing in the character of not only their terminal groups [27] but also the core of the molecule, potentially capable of active fluorescence, parameters of complex formation K and n with calf thymus DNA were computed using the Scatchard model. The features of the computation method are given below.

1. The dye under study was titrated with different amounts of the polynucleotide. To prevent complications, the overall concentration of the dye in solution was maintained constant and rather low (C_L 6.42×10^{-7} M) whereas the ratio of molar concentrations DNA/dye were set within a C_D/C_L range of 100–200. To avoid dilution effects, the mixture of buffer, dye, and DNA in concentration ensuring the C_D/C_L ratio equal to 200 was supplemented with aliquots of a solution containing buffer with the dye in the same concentration (C_L).

2. On the basis of fluorescence intensities of the dye in the presence of a given amount of DNA (I_i), the value of maximal fluorescence intensity of a given dye in the studied system (I_{max}) was computed. This value was determined by extrapolation of the upper linear portion of the plot of dependence of I value on the ratio of concentrations C_L/C_D, obtained in the course of fluorescent titration of the dye, to the zero value of C_L/C_D as is shown in Fig. 1a. It should be noted that this graph serves only for the illustration and choice of the number of points (in this case, $Q = 4$) in the upper portion of the

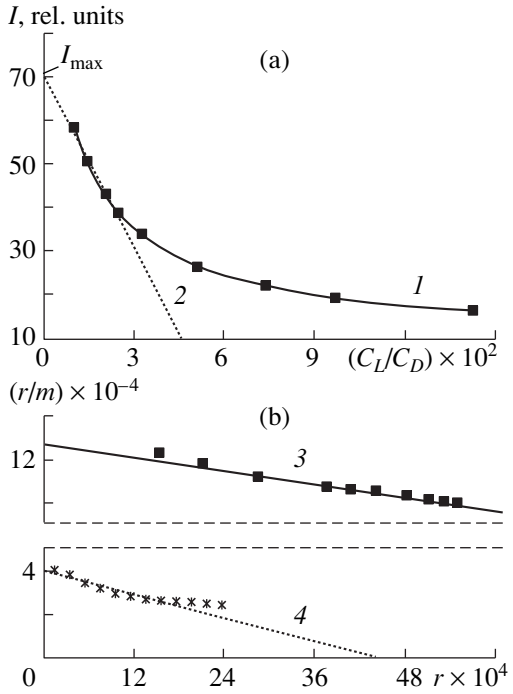


Fig. 1. Computation of parameters of complex formation of fluorophores with DNA; (a) determination of the maximum possible fluorescence intensity (I_{max}) of dye (**IV**) (DAPI) in the presence of DNA; (b) presentation of the computed and experimental data obtained for compounds (**IV**) and (**VI**) (Hoechst-33258) according to Scatchard.

Curve *1* shows dependence obtained after global cubic sline-interpolation of experimental data for compound (**IV**); line *2* is obtained by the linear extrapolation of the upper portion of curve *1* to the value of C_L/C_D equal to 0 (a); curves *3* and *4* correspond to the characteristic equation of the Scatchard model upon binding of compounds (**IV**) and (**VI**) to DNA (b).

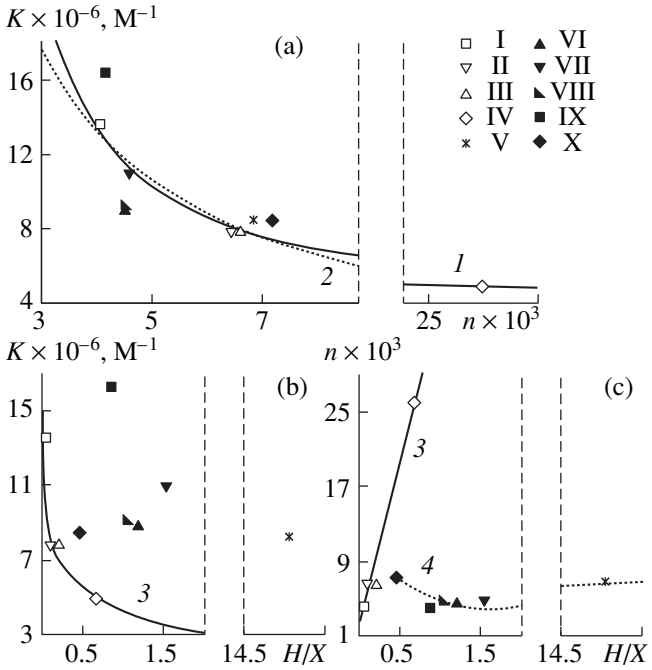


Fig. 2. Interdependences between parameters of complex formation of dyes (**I**)–(**X**) in the presence of DNA, computed on the basis of the Scatchard model (*K* and *n*), and the value of the ratio of contributions to formation of a specific fluorescent ligand–polynucleotide complex of hydrogen and ion bonds (*H*/*X*).

Designations: symbols I–X correspond to the experimental data for compounds (**I**)–(**X**). Curves *1* and *2* show dependences, significant for compounds (**I**)–(**X**), of the type: $K = 4.72 \times 10^6 + 139.6/n^2$, $R = 0.850$, $p < 0.01$, $\varepsilon = 10.8\%$ and $K = 5.29 \times 10^4/n$, $R = 0.815$, $p < 0.01$, $\varepsilon = 17.6\%$, respectively (a); curve *3* shows dependences, significant for compounds (**I**)–(**IV**), of the type: $K = 4.29 \times 10^6 - 1.74 \times 10^6 \ln(H/X)$, $R = -0.987$, $p < 0.05$, $\varepsilon = 8.4\%$ (b), and $n = 2.75 \times 10^{-3} + 0.0342H/X$, $R = 0.985$, $p < 0.05$, $\varepsilon = 16.2\%$ (c); curve *4* shows the dependence obtained after smoothing the data for compounds (**V**)–(**X**) (c); ε is the relative mistake of approximation (see formula (10)).

plot $Y = f(X)$ (where $Y = I$ and $X = C_L/C_D$) for their further approximation by the least square method by the dependence:

$$Y = a_0 + a_1 X, \tag{7}$$

where

$$a_1 = \frac{\sum X_i \sum Y_i - Q\sum (X_i Y_i)}{(\sum X_i)^2 - Q\sum X_i^2},$$
$$a_0 = 1/Q(\sum Y_i - a_1 \sum X_i), \quad i = 1\text{–}Q. \tag{8}$$

The accuracy of the I_{max} value proves thereby much higher than upon its purely graphic determination, which in turn affects the accuracy of the complexation parameters determined using this value.

3. By choosing the required number of approximation points, for the corresponding $C_{D,i}$ value it was calculated:

$$(C_L^b)_i = C_L(I_{max} - I_i)/(I_{max} - I_0), \tag{9}$$

where I_0 is the fluorescence intensity of the dye in the absence of DNA. Then, assuming $X_i = r_i = (C_L^b)_i/C_{D,i}$ at $Y_i = r_i/m_i = X_i/(C_L - (C_L^b)_i)$ and computing a_0 and a_1 by formulas (8), $K = -a_1$ and $n = -a_0/a_1$ were determined.

As is seen from Fig. 1b, the experimental data are in good agreement with the computed ones for compounds (**IV**) and (**VI**), chosen as typical representatives of the mono- and bisderivatives. This is also confirmed by the fact that the relative error of approximation computed by formula:

$$\varepsilon = 100/Q\sum (Y_{t,i} - Y_{e,i})/Y_{e,i}, \tag{10}$$

(where $Y_{t,i}$ and $Y_{e,i}$ are theoretical (*t*), computed by equation (7), and experimental (*e*) values of parameter *Y* [28]), did not exceed 3% for all dyes studied in this work.

Spectral and complex forming properties of the studied dyes*

Parameter	(**I**)	(**II**)	(**III**)	(**IV**)	(**V**)	(**VI**)	(**VII**)	(**VIII**)	(**IX**)	(**X**)
$K \times 10^{-6}$, M^{-1}	13.60	7.76	7.88	4.90	8.42	8.90	10.90	9.03	16.30	8.44
$n \times 10^{3}$	4.06	6.48	6.62	26.00	6.85	4.55	4.61	4.57	4.15	7.18
$K/n \times 10^{-9}$, M^{-1}	3.35	1.20	1.19	0.19	1.23	1.96	2.36	1.98	3.93	1.18
H, %	0	8	15	37	90	51	57	48	44	29
X, %	94	86	79	56	6	43	37	45	50	65
H/X	0	0.09	0.19	0.66	15.00	1.19	1.07	1.54	0.88	0.45
Φ	0.55	21.60	23.40	27.10	35.40	41.60	39.10	39.60	38.80	13.80
$\eta_{10} \times 10^{-6}$, rel. units	–1.56	0.72	0.43	4.67	3.59	4.07	3.57	2.45	1.34	4.31
$\eta_{100} \times 10^{-6}$, rel. units	–0.39	0.39	0.23	0.50	0.59	1.69	2.85	1.18	0.44	3.52
λ_{ex}, nm	330	340	340	350	367	353	350	355	370	350
λ_{em}, nm	450	455	455	455	452	455	455	455	500	400

* K, the Scatchard constant, with value inverse to the free dye concentration in the system, when it occupies half of the potential binding sites on DNA; n, maximum possible number of molecules of ligand that can be bound by one DNA molecule calculated per overall basepairs in it; H and X, relative contributions of the hydrogen and ion bonds, respectively, to formation of specific actively fluorescing complex DNA–dye; η_{10} and η_{100}, coefficients of fluorescent sensitivity, reflecting the increase in the dye fluorescence intensity correlated with the increase in the DNA concentration (C_D) at 1 M and ratios of molar concentrations DNA/dye (C_D/C_L) equal to 10 and 100, respectively; $\Phi = \phi_{max}/\phi_0$, ratio of quantum yields of the dye in the presence of the saturating amount of DNA and in the absence of the polynucleotide; λ_{ex} and λ_{em}, wavelengths of maxima of luminescent excitation and emission of the dye in the visible area.

After computing, the data gained along with other complexation and spectral properties of compounds (**I**)–(**X**) described by us in [27, 29, 30] were collected in a table and compared with the chemical structure of the dyes under study. As is seen, the values of the parameters did not differ statistically significantly for mono- and bisderivatives studied in this work.

Dependence between parameters K and n computed using the Scatchard model was also determined unambiguously for all compounds studied (see Fig. 2a). As the specificity of the compounds toward the substrate (which can be relatively expressed by value $q = 1/n$) rose, the affinity of dyes to DNA (expressed by K) increased the more, the less was the n value.

As for the q value, it should be noted once again that by definition it only reflects the overall number of basepairs amounting on average per molecule of the dye upon maximal filling of the polynucleotide, including sites both binding and non-binding with the ligand [11, 13]. The h value (the number of basepairs bound by one ligand molecule), determined from the X-ray study data, equals for compound (**IV**) to 3 bp [26] and for compound (**VI**) to 4 bp [6]. In addition, it looks peculiar that while in [26] the K and n values obtained for compound (**IV**) are similar to those presented in this paper, the respective values for a number of compounds in [9, 25] proved quite different. This can be explained by the fact that in the latter case data of spectrophotometric rather than fluorimetric titration were employed, which were affected by not only strong specific but also weak nonspecific type of DNA–ligand binding.

At the same time, as exemplified by the dependence of the K and n values on the ratio of contributions of hydrogen and ionic bonds (H/X) to the formation of the specific fluorescent DNA–ligand complex (Figs. 2b, 2c), differences in the character of interaction of mono- and bisderivatives of benzimidazole and phenylindole with the substrate are evident. Thus, whereas for monoderivatives (**I**)–(**IV**) the K value diminished significantly with an increase in H (relative contribution of hydrogen bonds to the formation of the specific fluorescent complex DNA–ligand) and n value rose, for bisderivatives (**VI**)–(**X**) and dye (**V**) nothing of the kind was observed.

Moreover, for monoderivatives (**I**)–(**IV**) a statistically significant decrease in the ratio of quantum yields ($\Phi = \varphi_{max}/\varphi_0$) was noted with an increase in ratio K/n (R –0.982, $p < 0.05$, where R is the correlation coefficient and p is its confidence level) both in the presence of the saturating amount of DNA and in the absence of the polynucleotide. At the same time, for bisderivatives (**VI**)–(**X**) and compound (**V**) the interrelation between these parameters (Φ and K/n) was absent even by the Spirman range correlation criterion (see Fig. 3a). In Fig. 3b, illustrating the dependence of Φ on H/X, the differences between the above groups of compounds (**I**)–(**IV**) and (**V**)–(**X**) are perceptible but much less distinct than in the previous case. Finally, for the case of dependence of Φ on the fluorescent sensitivity coefficients η_{10} and η_{100} (reflecting the value of increase in

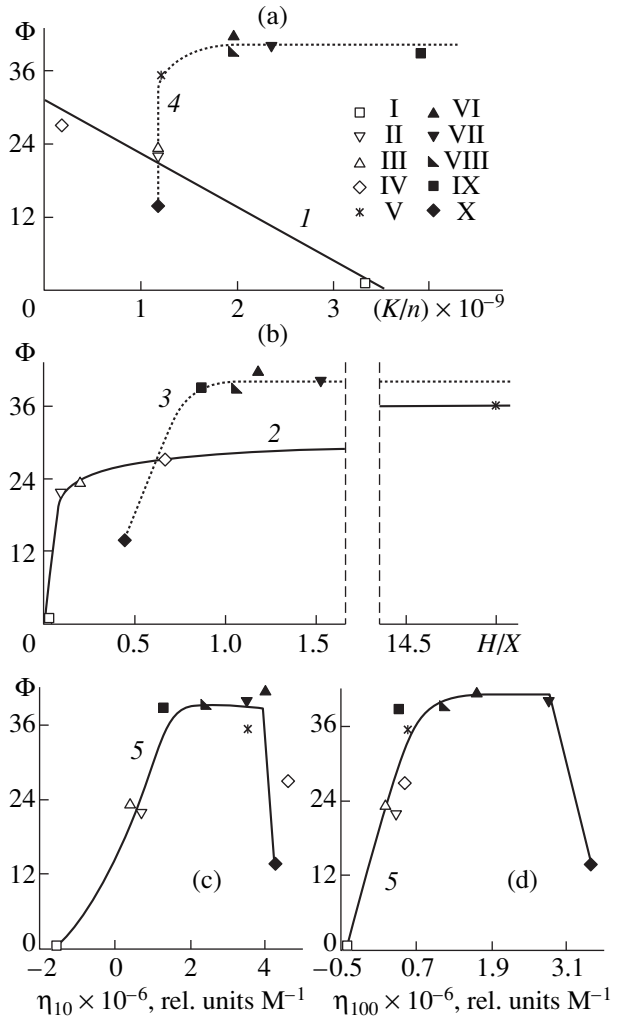


Fig. 3. Dependence of the ratio of quantum yields of dyes (**I**)–(**X**) in the presence of the saturating amount of calf thymus DNA and in its absence (Φ) on parameters of complex formation *K*/*n* (a), *H*/*X* (b), and coefficients of fluorescent sensitivity (η) upon molar concentrations ratio DNA/dye 10 (c) and 100 (d).

Designations: symbols I–X correspond to the experimental data for compounds (**I**)–(**X**). Curve *1* (a) shows dependence, significant for compounds (**I**)–(**IV**), of the type: $\Phi = 31.24 - 8.82 \times 10^{-9}\ K/n$, $R = -0.982$, $p < 0.05$; curves *2*–*5* show dependences obtained after smoothing the data for compounds (**I**)–(**V**), (**VI**)–(**X**), (**V**)–(**X**), and (**I**)–(**X**), respectively.

the fluorescence intensity of the dye upon an increase in C_D by 1 M and ratios of molar concentrations C_D/C_L 10 and 100, respectively), data for all compounds studied fit a plot (see Figs. 3c, 3d). The ratios of concentrations DNA/dye were chosen basing on the character of dependences of η on C_D/C_L (presented by us for the compounds studied in [27, 30]) as most suitable for reflecting the typical values of fluorescence coefficients of the dyes under consideration in the area of small and great C_D/C_L.

On the other hand, upon considering the dependence of the fluorescent sensitivity coefficients η_{10} and η_{100} on the complexation parameters *H*/*X*, *K*, and *n* (Fig. 4), the differences between their characters for the mono- and bis-derivatives (with which compound (**V**) borders by the totality of its spectral and complex formation properties) are very distinct. Thus, for monoderivatives (**I**)–(**IV**) a statistically significant correlation takes place between η_{10} and *H*/*X* (R 0.969, $p < 0.05$); 1/*K* (R 0.994, $p < 0.01$) and ln(*n*) (R 0.992; $p < 0.01$). In addition, for compounds (**I**)–(**V**) a dependence was noted of η_{100} on ln(*H*/*X*) (R 0.966, $p < 0.05$); K^2 (R –0.911, $p < 0.05$) and e^{-n} (R –0.949, $p < 0.05$). At the same time, for bisderivatives (**VI**)–(**X**) and compound (**V**) we could only note the presence of a significant correlation η_{10} on *K* (R –0.815, $p < 0.05$) and e^{-n} (R –0.854, $p < 0.05$); besides, for compounds (**VI**)–(**X**) there was a dependence of η_{100} on e^{-n} (R –0.883, $p < 0.05$) (see Figs. 4c, 4d).

Thus, the series of compounds compared in this work, each containing one (monoderivatives) or two (bisderivatives) benzimidazole or indole fragments capable of active fluorescence, differed not so much by the values of their spectral and complex forming parameters (Φ, η, *H*/*X*, *K*, *n*) as by their totality and interrelationship. The most illustrative in this respect, the dependences proved between the fluorescent sensitivity coefficients (η) of compounds (**I**)–(**X**) toward DNA and the parameters of complex formation *K* and *n*, computed on the basis of the Scatchard model (see Figs. 4d, 4e).

It should be noted that compound (**V**) of the monophenylindole series occupies, by the combination of its spectral and complex forming characteristics, an intermediate position between groups of mono- and bisderivatives, apparently, due to the presence in its molecule of three heterocycles within a united conjugated system. This can easily be followed in Figs. 4c–4f. Thus, while upon the ratio of molar concentrations in system C_D/C_L equal to 10, the η, *K*, and *n* values of compound (**V**) were closer to the plot characteristic of bisderivatives (**VI**)–(**X**), at C_D/C_L equal to 100 the values of these parameters were closer to the plot characteristic of monoderivatives (**I**)–(**IV**).

Besides, we should especially note the fact of a statistically significant dependence between the Scatchard constant value and the number of potential binding sites per mononucleotide (*n*): $K = a/n$ or (more reliably) $K = b + c/n^2$ (where *a*, *b*, and *c* are the corresponding empirical constants (see Fig. 2a).

EXPERIMENTAL

Commercial fluorophores (**IV**)–(**VII**) (DAPI, DIPI, Hoechst-33258, and Hoechst-33342, respectively) were obtained from Serva (Germany). Compounds (**I**)–(**III**) and (**VIII**)–(**X**) were synthesized at the Depart-

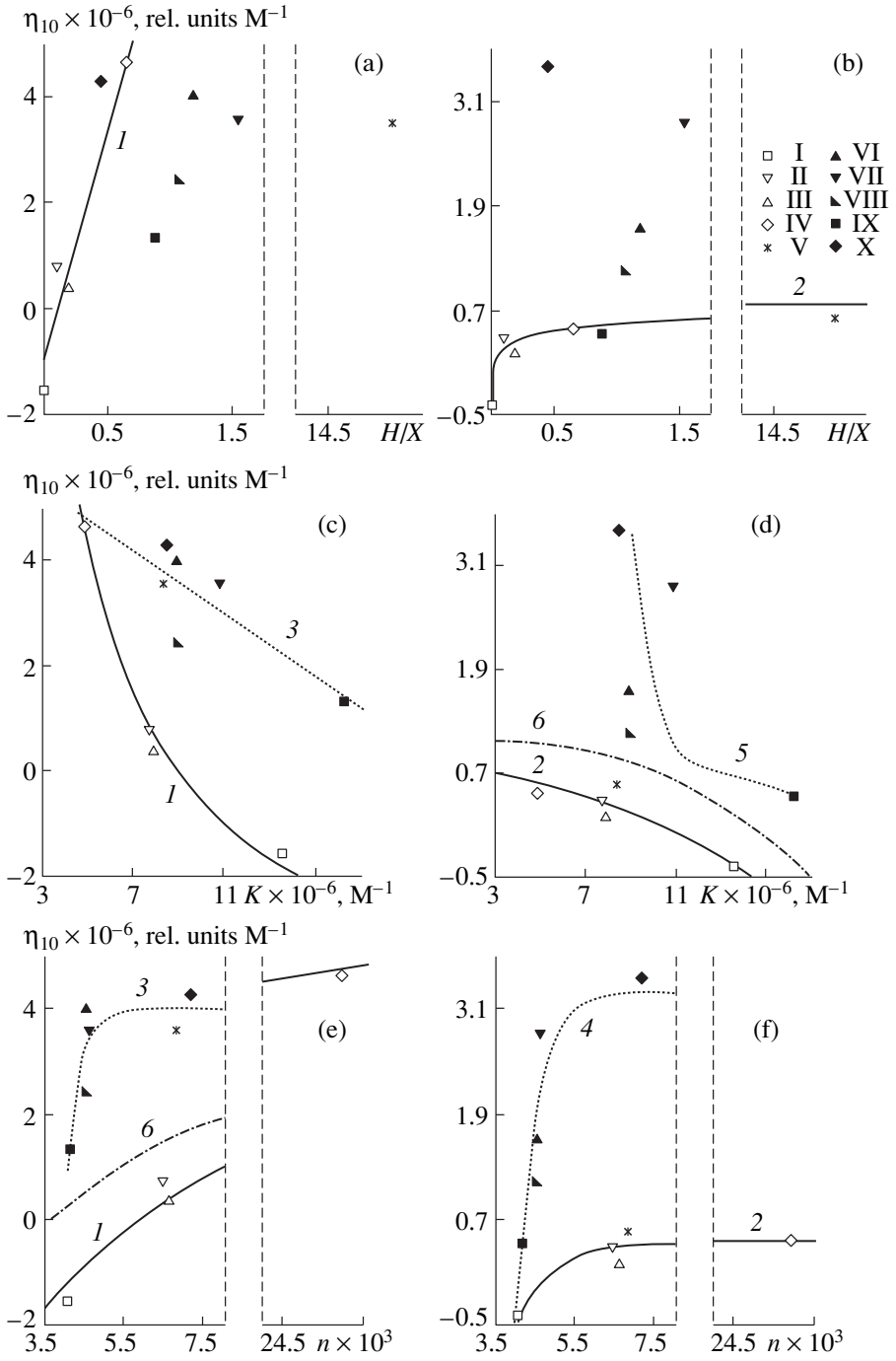


Fig. 4. Dependence of the fluorescent sensitivity coefficients η_{10} and η_{100} of dyes (**I**)–(**X**) on parameters of their complex formation with DNA: *H/X* (a, b), *K* (c, d), and *n* (e, f).

Designations for symbols are the same as in Fig. 3. Curve *1* shows dependence, significant for compounds (**I**)–(**IV**), of the type: $\eta_{10} = -9.67 \times 10^5 + 8.60 \times 10^6 H/X$, $R = 0.969$, $p < 0.05$ (a); $\eta_{10} = -5.38 \times 10^6 + 4.83 \times 10^{13}/K$, $R = 0.994$, $p < 0.01$ (c); and $\eta_{10} = -5.73 \times 10^6 + 3.23 \times 10^6 \ln(1000n)$, $R = 0.992$, $p < 0.01$ (e); curve *2* shows the dependence, significant for compounds (**I**)–(**V**), of the type: $\eta_{100} = 5.49 \times 10^5 + 1.21 \times 10^5 \ln(H/X)$, $R = 0.966$, $p < 0.05 < 0.05$ (b); $\eta_{100} = 7.31 \times 10^5 - 5.80 \times 10^{-9} K^2$, $R = -0.911$, $p < 0.05$ (d); and $\eta_{100} = 4.78 \times 10^5 - 5.06 \times 10^7 \exp(-1000n)$, $R = -0.949$, $p < 0.05$ (f); curve *3* shows dependences, significant for compounds (**V**)–(**X**), of the type: $\eta_{10} = 6.31 \times 10^6 - 0.30K$, $R = -0.815$, $p < 0.05$ (c) and $\eta_{10} = 4.02 \times 10^6 - 6.67 \times 10^{11} \exp(-3000n)$, $R = -0.854$, $p < 0.05$ (e); curve *4* shows dependence, significant for compounds (**VI**)–(**X**), of the type: $\eta_{100} = 3.35 \times 10^6 - 1.25 \times 10^{10} \exp(-2000n)$, $R = -0.883$, $p < 0.05$ (e); curve *5* shows dependence obtained after smoothing the data for compound (**VI**)–(**X**) (d); curve *6* shows the boundary between the areas of parameter values for mono- and bisderivatives (d, e).

ments of Organic Chemistry and Molecular Biotechnology, St. Petersburg Technological Institute, using protocols described in [31–33].

As a substrate, calf thymus DNA was used (58% A · T-pairs, average molecular mass 326 Da per mononucleotide, molar absorption coefficient ε_{260} 6600 M^{-1} cm^{-1}), prepared by dissolution of the dry preparation from Serva (Germany) in distilled water and treated (for homogenization and reduction of light scattering) with ultrasound on a UZDN-2 instrument (Russia) for 15 s at a current of 0.3 A at a resonance frequency of 22 kHz, after which the average mass of the molecule was 3500 Da.

Titration was performed at a constant concentration of the dye C_L 6.42×10^{-7} M and different concentrations of the polynucleotide (in a range of C_D/C_L 100–200) at 20–25°C in buffer of the following composition: 0.01 M NaCl, 0.01 M Na_2-EDTA, and 0.01 M Tris (pH 7.4).

Fluorescence was analyzed on a spectrofluorimeter Hitachi model 850 (Japan) (slots of monochromators of excitation and emission 5 nm, response time 2 s, photoelectromultiplier normal amplification). Excitation spectra were registered at a wavelength corresponding to the highest maximum of emission, and emission spectra at a wavelength corresponding to the highest maximum of excitation of the compound studied in the visible range (see table).

Quantum yields of fluorescence (φ) of dyes were determined by the relative method using as a standard a quinine sulfate solution in 1 M sulfuric acid (φ 0.55). The sensitivity coefficients (η_S) were computed by the formula:

$$\eta_S = (I_{k+1} - I_{k-1})/2C_L, \qquad (11)$$

where I_{k+1} and I_{k-1} are fluorescence intensities of the dye (rel. units) at the molar concentrations ratios C_D/C_L equal to $k + 1$ and $k - 1$. The H and X values of compounds (**I**)–(**X**) were computed as described in [30].

Coefficient of the pair correlation for functions of the type $Y = a_0 + a_1 f(X)$ was computed by the formula [28]:

$$R = \frac{\sum(y_i z_i) - (\sum y_i \sum z_i)^2/Q}{\sqrt{\sum y_i^2 - (\sum y_i)^2/Q} \times \sqrt{\sum z_i^2 - (\sum z_i)^2/Q}}, \qquad (12)$$

where $z = f(X)$ and Q is the number of compounds in a selection. It was then checked for the confidence by criterion $|R| > t_\alpha/\sqrt{t_\alpha^2 + Q - 2}$, where t_α is the table value of the Student criterion for the significance level α and the number of freedom degrees $Q - 2$.

The ordinal correlation procedure according to Spirman [34] consisted in the following: let it be known that in an excerpt under study quantity X_i occupies B_i and quantity Y_i occupies D_i place; then if

$$\sqrt{Q-1}[1 - 6/(Q^3 - Q)]\sum(B_i - D_i)^2 > u_\alpha,$$

where u_α is the table value of the La Place function ($u_{0.1}$ 0.2533, $u_{0.05}$ 0.125, $u_{0.01}$ 0.025, $u_{0.001}$ 0.0025), the hypothesis of independence of parameters Y and X is rejected with the significance level α.

To plot the curves in Figs. 1–4, in the case of the absence of the statistically significant functional dependences between parameters under study, we used, with slight modifications, the procedure of smoothing of the experimental data by global cubic β-splines described in [35, 36].

REFERENCES

1. Ivanov, S.D., Kovan'ko, E.G., Remizova, I.V., and Stefanenko, F.A., *Radiobiologicheskie podkhody k diagnostike luchevykh porazhenii* (Radiobiological Approaches to Diagnosis of Radiational Lesions), Komar, E.V., Ed., Leningrad: LGIUV, 1987, pp. 69–75.
2. Ivanov, S.D., Postradiation Reactions of DNA Nucleotides of Blood Leukocytes: Detections, Rules, and Diagnostic Values, *Doctoral (Biol.) Dissertation*, Leningrad: TsNIRRI, 1992.
3. Monger, B.C. and Landry, M.R., *Appl. Environ. Microbiol.*, 1993, vol. 59, pp. 905–911.
4. Nel, D., Cooper, R., and Martin, R.F., Abstracts pf Papers, *Int. Congr. Radiation Research 1895–1995*, Wuerzburg, 1995, vol. 1, p. 199.
5. Nievergelt-Egido, M.C., Weinreich, R., and Larsson, B., Abstracts pf Papers, *Int. Congr. Radiation Research 1895–1995*, Wuerzburg, 1995,vol. 1, p. 429.
6. Pjura, P.E., Grzeskowiuk, K., and Dickerson, R.E., *J. Mol. Biol.*, 1987, vol. 197, pp. 257–271.
7. Wilson, F.W., Tanious, A.T., Barton, H.J., Jones, P.L., Fox, K., Wydra, R.L., and Strekowski, L., *Biochemistry*, 1990, vol. 29, pp. 8452–8461.
8. Martin, R.F. and Denison, L., *Int. J. Radiat. Oncology Biol. Phys.*, 1992, vol. 23, pp. 579–584.
9. Kolosova, O.Yu., Fluorescent DNA Probes of the Imidazole Series, *Cand. Sci. (Chem.) Dissertation*, Leningrad, 1991.
10. Morgan, A.R., Lee, J.S., Pulleyblank, D.E., Murray, N.L., and Evans, D.H., *Nucleic Acids Res.*, 1979, vol. 7, pp. 547–571.
11. Zasedatelev, A.S., Gurskii, G.V., and Vol'kenshtein, M.V., *Mol. Biol.* (Moscow), 1971, vol. 5, pp. 245–251.
12. Scatchard, G., *Ann. N. Y. Acad. Sci.*, 1949, vol. 51, pp. 660–672.
13. Roslov, A.A., Study on Specific DNA Ligands of the Imidazole Series, *Cand. Sci. (Chem.) Dissertation*, Leningrad, 1989.
14. Borisova, O.F., Golova, Yu.B., Gottikh, B.P., Zibrov, A.S., Il'icheva, I.A., Lysov, Yu.P., Mamaeva, O.K., Chernov, B.K., Chernyi, A.A., Shchelkina, A.K., and Florent'ev, V.L., *Mol. Biol.* (Moscow), 1989, vol. 23, pp. 1535–1552.

15. Borisova, O.F., Shchelkina, A.K., Karapetyan, A.T., and Surovaya, A.N., *Mol. Biol.* (Moscow), 1998, vol. 32, pp. 855–862.
16. Crothers, D.M., *Biopolymers*, 1968, vol. 6, pp. 575–584.
17. Gurskii, G.V., Zasedatelev, A.S., and Wolkenstein, M.V., *Mol. Biol.* (Moscow), 1972, vol. 6, pp. 479–490.
18. McGhee, J.D. and van Hippel, P.H., *J. Mol. Biol.*, 1974, vol. 86, pp. 469–489.
19. Hogan, M., Dattagupta, N., and Crothers, D.M., *Nature*, 1979, vol. 278, pp. 521–524.
20. Dattagupta, N., Hogan, M., and Crothers, D.M., *Biochemistry*, 1980, vol. 19, pp. 5998–6005.
21. Zimmer, C. and Wahnert, U., *Prog. Biophys. Mol. Biol.*, 1986, vol. 47, pp. 31–112.
22. Eriksson, S., Kim, S.K., Kubista, M., and Norden, B., *Biochemistry*, 1993, vol. 32, pp. 2987–2998.
23. Nechipurenko, Yu.D., Zasedatelev, A.S., and Gurskii, G.V., *Mol. Biol.* (Moscow), 1984, vol. 18, pp. 798–812.
24. Kruglova, E.B. and Zinenko, T.L., *Mol. Biol.*, 1993, vol. 27, pp. 655–665.
25. Garabadzhiu, A.V., Benzimidazole Derivatives as DNA Complexons, *Doctoral (Chem.) Dissertation*, S. Petersburg, 1994.
26. Barcellona, M.L., Favilla, R., von Berger, J., Avitahile, M., Ragusa, N., and Masotti, L., *Arch. Biochem. Biophys.*, 1986, vol. 250, pp. 48–53.
27. Sibirtsev, V.S., Garabadzhiu, A.V., and Ivanov, S.D., *Bioorg. Khim.*, 1994, vol. 20, pp. 650–668.
28. Dubrov, A.M., Mkhataryan, V.S., Troshin, L.I., and Maslenchenko, I.V., *Matematiko-statisticheskii analiz na programmiruemykh mikrokal'kulyatorakh* (Mathematical and Statistical Analysis on Programmable Microcalculators), Moscow: Finansy i Statistika, 1991.
29. Sibirtsev, V.S., Garabadzhiu, A.V., and Ivanov, S.D., *Bioorg. Khim.*, 1995, vol. 21, pp. 731–736.
30. Sibirtsev, V.S., Garabadzhiu, A.V., and Ivanov, S.D., *Bioorg. Khim.*, 1997, vol. 23, pp. 969–978.
31. Sokolova, N.Yu., Kuznetsov, V.A., Garabadzhiu, A.V., Ginzburg, O.F., Dobrynin, Ya.V., Nikolaeva, T.G., Fin'ko, V.E., and Ivanova, T.P., *Khim.–Farm. Zh.*, 1990, vol. 24, pp. 31–33.
32. Preston, I., De Winter, W., and Hofferbert, W.I., *J. Heterocycl. Chem.*, 1969, vol. 6, pp. 119–121.
33. Sklyarova, I.V., Kuznetsov, V.A., Sokolova, N.Yu., Garabadzhiu, A.V., Ginzburg, A.V., Dobrynin, Ya.V., Nikolaeva, T.G., and Fin'ko, V.E., *Khim.–Farm. Zh.*, 1989, vol. 22, pp. 697–699.
34. *Ammeraal L. Programming Principles in Computer Graphics*, N.Y.: Wiley, 1986.
35. Shikin, E.V., Boreskov, A.V., and Zaitsev, A.A., *Nachala komp'yuternoi grafiki* (ABC of Computer Graphics), Moscow: Dialog-MIFI, 1993.
36. Korn, G.A. and Korn, T.M., *Mathematical Handbook for Scientists and Engineers. Definitions, Theorems and Formulas for Reference and Review*, N.Y.: McGraw-Hill, 1968.