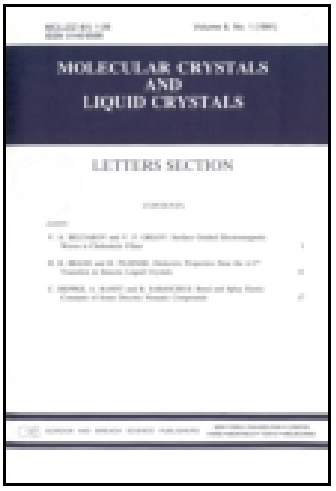

Molecular Crystals and Liquid Crystals
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gmcl20

Peculiarities of the Binding of Some Small Ligands to DNA

V. Gumenyuk [a], S. Kutovyy [a], T. Sych [a], R. Savchuk [a] & N. Bashmakova [a]

[a] Taras Shevchenko National University of Kyiv, 60, Volodymyrs'ka Str., Kyiv, 01601, Ukraine

Published online: 28 Mar 2014.

To cite this article: V. Gumenyuk, S. Kutovyy, T. Sych, R. Savchuk & N. Bashmakova (2014) Peculiarities of the Binding of Some Small Ligands to DNA, Molecular Crystals and Liquid Crystals, 589:1, 242-250, DOI: 10.1080/15421406.2013.872856

To link to this article: http://dx.doi.org/10.1080/15421406.2013.872856

Mol. Cryst. Liq. Cryst., Vol. 589: pp. 242–250, 2014

ISSN: 1542-1406 print/1563-5287 online
DOI: 10.1080/15421406.2013.872856

Peculiarities of the Binding of Some Small Ligands to DNA

V. GUMENYUK, S. KUTOVYY,* T. SYCH, R. SAVCHUK, AND N. BASHMAKOVA

Taras Shevchenko National University of Kyiv, 60, Volodymyrs 'ka Str., Kyiv, 01601, Ukraine

The comparative study of the interaction of ethidium bromide, acridine orange, and isoquinoline alkaloids such as sanguinarine and berberine with DNA in aqueous solutions using the optical spectroscopy methods is presented. The dependences of the spectral characteristics of ligands on the concentration ratio P/D between the DNA base pairs and ligands molecules in solutions are considered. The character of binding is found to depend on P/D. The parameters of the binding with DNA are determined for the external binding and the intercalation, by using the modified Scatchard and McGhee–von Hippel equations. The influence of a ligand form on binding ways is shown.

Keywords DNA; ligand; binding equations; intercalation; external binding

Introduction

Studying the interaction between small ligands and DNA is very important for the creation of effective low-toxic antineoplastic preparations on the basis of natural alkaloids. Alkaloids are topical for such preparations because of their property to be selectively accumulated in tumor cells and their capability to form complexes with nucleic acids, by blocking the processes of transcription and replication of the latter.

This work continues our researches [1] aimed at studying the interaction between DNA and small ligands (alkaloids). Now, one object is an isoquinoline alkaloid sanguinarine (Sa, benzophenantridine group), others are ethidium bromide (EtBr, phenantridines), acridine orange (AO, pyridines). We also used some of our previous results [1] on an alkaloid berberine (Be, protoberberines) for comparison. The structural formula of sanguinarine is $C_{20}H_{15}NO$, acridine orange – $C_{17}H_{19}N_3$, ethidium bromide - $C_{21}H_{20}BrN_3$, and berberine – $C_{20}H_{19}NO_5$. (Fig. 1). Sanguinarine is known [2] to exist in aqueous solutions in two forms, imine (cation, SaI, pH < 6) and alkanolamine (neutral, SaII, pH > 8.5). Sanguinarine and acridine molecules are flat (all rings are in plane), berberine and ethidium are not (some rings are out of plane); which is of importance for our analysis.

Alkaloids Sa and Be are components of some antineoplastic preparations; EtBr is a mutagen and the well-known fluorescent tag of nucleic acids, AO is a nucleic acid binding dye; in addition, latter two are model samples for investigations of the binding of small ligands with nucleic acids. The interaction between the considered ligands and DNA was studied in a great number of works (see reviews [3–5] and cited works). All of them

*Corresponding author, S. Kutovyy. E-mail: sangulaire@gmail.com

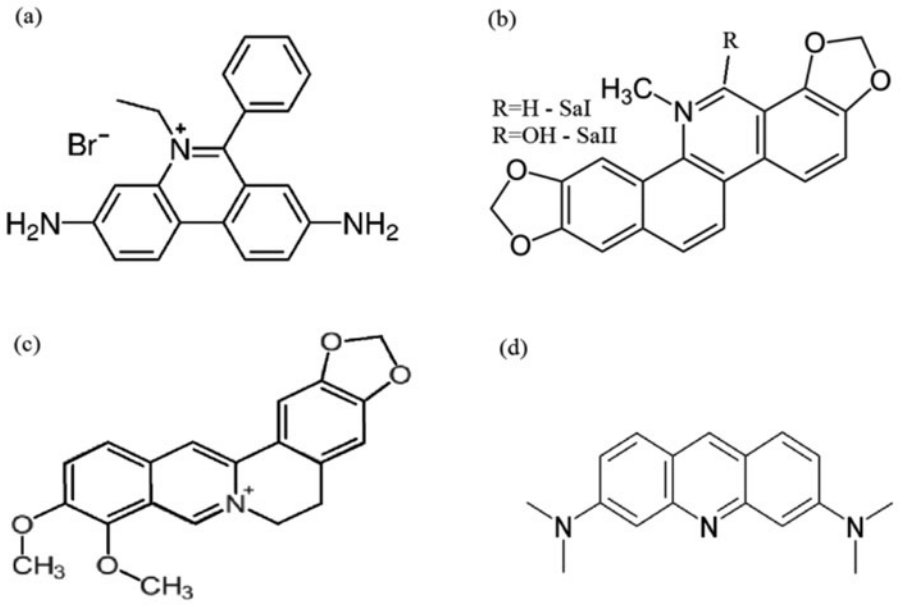

Figure 1. Formula structures of ethidium bromide (a), sanguinarine (b), berberine (c), and acridine orange (d).

interact with DNA as intercalates, full or partial. Usually the binding processes between ligands and DNA were analyzed and the binding constants were determined using the Scatchard equation or, what is more properly, the McGhee–von Hippel equation. In both cases, however, the Scatchard coordinates were used. But the usage of these coordinates has significant deficiencies [1, 6]. In addition, in spite of a great number of works on the considered samples, some peculiarities in their optical spectra, and, correspondingly, in binding curves were not reported up to now.

In this work, we obtained the dependences of the spectral characteristics of EtBr, AO, Sa and Be on the ratio (P/D) between the number of DNA base pairs and the number of ligand molecules. These characteristics were used to determine the binding parameters using, as we believe, a proper system of modified Scatchard and McGhee–von Hippel equations for the independent variable.

Experimental Specimens and Technique

We used dyes sanguinarine chloride, berberine chloride, ethidium bromide, and acridine orange fabricated in the form of microcrystalline powders (supplied by the Institute of Molecular Biology and Genetics of the NAS of Ukraine). The latter were dissolved in water for injections at a required temperature within the interval 20–70 °C. The concentrations of experimental ligands ranged from 6 to 50 μM. We used the DNA of calf thymus (DNA CT, "Serva", Heidelberg, Germany). The average molar mass of a nucleotide pair was about 650 Da. When measuring the concentration dependence for the solutions ligand+DNA, the concentration of a ligand remained constant, but the concentration of DNA varied. The ratio between the molar concentrations of DNA and ligands (P/D) was expressed in terms of the number of nucleotide pairs per one ligand molecule.

The absorption spectra were registered on a Specord UV VIS spectrophotometer in the range of 200–700 nm. The spectral resolution was 1 nm. Fluorescence spectra (PL) were obtained with the use of a Cary Eclipse fluorometer in the range of 300–800 nm. The spectral width of a slit for fluorescence measurements was 5 nm.

Calculation of Binding Parameters.

To determine the binding parameters, we used our program BindFit. This program allows the operation with direct experimental data without any coordinate transformation known as "linearization". It allowed us to improve the accuracy of the parameter determination for the processes described by nonlinear plots even after the linearization. The main principle of processing the data with this program is the approximation of experimental points by some binding equations. One of the classical binding equations is the Scatchard equation [7] $\frac{v}{c_f} = K\,(1 - v)$. Here, v is the ratio between the concentration of bound ligands c_b and the total concentration of binding sites N, c_f is the concentration of free ligands, and K is the association constant. For some reasons, this dependence can be nonlinear, in particular, if a ligand molecule occupies more than one binding site (namely, n sites) in the DNA matrix. Then the equation looks like $\frac{v}{c_f} = K \cdot (1 - nv)$, but its use is not always proper. Using the probability theory methods, McGhee and von Hippel [8] developed Scatchard's approach, by extending it onto the case $n > 1$. In addition, they determined properly the number of empty binding sites. For the non-cooperative and cooperative bindings, the McGhee–von Hippel equations are as follows:

$$\frac{v}{c_f} = K\,(1 - nv) \cdot \left(\frac{1 - nv}{1 - (n-1)\,v}\right)^{n-1}, \tag{1}$$

$$\frac{v}{c_f} = K\,(1 - nv) \cdot \left(\frac{(2\omega - 1)\,(1 - nv) + v - R}{2\,(\omega - 1)\,(1 - nv)}\right)^{n-1} \cdot \left(\frac{1 - (n+1)\,v + R}{2\,(1 - nv)}\right)^{2}, \tag{2}$$

where ω is the parameter of cooperativity, $R = \sqrt{(1 - (n+1)\,v)^2 + 4\omega v\,(1 - nv)}$.

Modified equations (see [1] for details)**.**

One type of binding sites. The basic McGhee–von Hippel equations were transformed from their original form (1, 2) to that including only the variables directly related to the experiment. As a rule, what is experimentally measured is the dependence of the certain optical parameter of a solution on the concentration ratio between the dissolved components; normally, it is the ratio between the total concentration of binding sites to the total concentration of ligands, N/c (here, $N/c \equiv$ P/D). It is the concentrations of components rather than those of bound and free ligands that are known. Therefore, such variables as the total concentrations of ligands, c, and binding sites, N, would be more expedient for the computerized processing. In this case, there remains only one unknown variable in the equation, which can be determined numerically. After a number of simple transformations, the McGhee–von Hippel equations for the non-cooperative and cooperative bindings are reduced to the expressions, which include the variable c_b:

$$K \cdot (c - c_b) \cdot (N - nc_b) \cdot \left(\frac{N - nc_b}{N - (n-1)\,c_b}\right)^{n-1} - c_b = 0, \tag{3}$$

$$K \cdot (c - c_b) \cdot (N - nc_b) \cdot \left(\frac{(2\omega - 1)(N - nc_b) + c_b - R'}{2(\omega - 1)(N - nc_b)} \right)^{n-1} \cdot \left(\frac{N - (n+1)c_b + R'}{2(N - nc_b)} \right)^2 - c_b = 0, \quad (4)$$

$$R' = \sqrt{(N - (n+1)c_b)^2 + 4\omega c_b (N - nc_b)}.$$

These equations were implemented in the program and solved numerically. This enabled us to operate with the quantity c_b as with the function $c_b = c_b(N,c;K,n)$.

Two types of binding sites. In this case, the binding processes are described by a system of two equations, which must take into account whether the processes of binding of the ligands that occupy one binding site (i.e., the base pair and phosphates) are interdependent or not. If the parameter N stands for the concentration of DNA base pairs, then $2N$ binding sites correspond to the first type of binding (with a phosphate), and N binding sites do to the second type (intercalation).

For our experimental data, the best approximation results were obtained with the use of a system of modified Scatchard (for external binding) and McGhee–von Hippel (for intercalation) equations. The system describes two interdependent processes of binding of the ligands that occupy one binding site. The intercalation into the interval between the base pairs is allowed only if both phosphates in this interval are not connected with ligands and *vice versa*, i.e. the binding with phosphates is possible only if no ligand has intercalated into the corresponding interval. In addition, the process of direct transition by bound ligands from sites of the first type onto sites of the second type is impossible, and there must be not less than $n-1$ free intervals between two intercalated ligands. This model brings about the following system of equations:

$$\begin{cases} c_b^{(1)} = K_1\left(c - c_b^{(1)} - c_b^{(2)}\right) \cdot \left(2N - c_b^{(1)}\right) \cdot \left(1 - \frac{c_b^{(2)}}{N}\right) \\ c_b^{(2)} = K_2\left(c - c_b^{(1)} - c_b^{(2)}\right) \cdot \left(N - nc_b^{(2)}\right) \cdot \left(\frac{N - nc_b^{(2)}}{N - (n-1)c_b^{(2)}}\right)^{n-1} \cdot \left(1 - \frac{c_b^{(1)}}{2N}\right)^2, \end{cases} \quad (5)$$

where $c_b{}^{(1)}$ and $c_b{}^{(2)}$ are the concentrations of ligands bound to sites of the first and second types, K_1 and K_2 are the binding constants for external binding and intercalation, respectively. It is important that an additional multiplier emerges in the equations for independent processes; we mention also some important restrictions given in [1]. In addition, the program took pH of a solution (that is, the concentration of ions H^+) into account; this gave an opportunity to allow for the number of external binding sites more properly (not only $2N$). In general, the given equations have a number of solutions. However, it turned out that only one of them satisfies the required restrictions in the working range of parameters.

Experimental Results

The interaction of small ligands with DNA is characterized by such phenomena in absorption and fluorescence spectra as the hypochromism in absorption bands; the red and blue shifts of maxima in the absorption and fluorescence spectra, respectively; and the variation of the fluorescence quantum yield. These and other manifestations of the binding of the

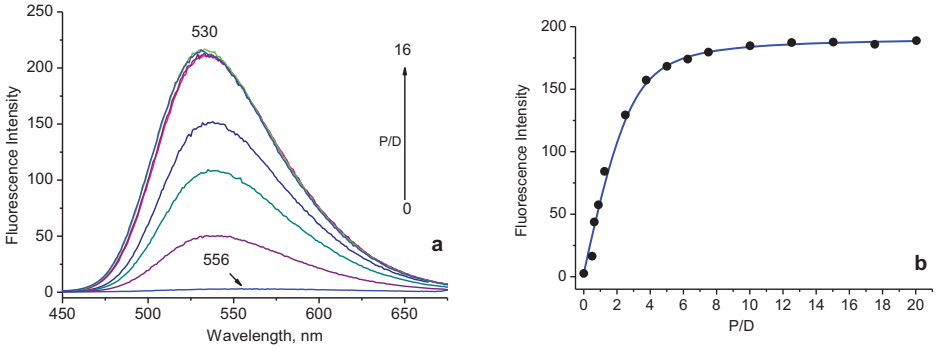

Figure 2. Fluorescence spectra (a) and binding curve (b) for berberine ($c_{Be} = 41$ μM, pH$\sim$6.9).

considered specimens with DNA were observed in optical spectra. The binding parameters were determined by us on the base of fluorescence spectra. Therefore, we describe only them here in brief.

To describe the binding processes most properly, system (5) of the dependent Scatchard and McGhee–von Hippel equations for two types of binding sites (with negligible influence of an external binding in some cases) with regard for the pH-values was used. In some cases, the McGhee–von Hippel equations for the non-cooperative and cooperative bindings were used.

The binding parameters taken from other works and given below for a comparison were obtained for DNA of calf thymus as well.

Berberine. The fluorescence spectrum of berberine has one band with a maximum at about 556 nm [1], Fig. 2a. The fluorescence quantum yield of berberine is very low. The fluorescence spectra of the complex Be-DNA are characterized by a very considerable amplification of their intensity (up to 200 times) and a blue shift of the PL maximum (up to 26 nm). On the base of these results, the binding curve, i.e., the P/D-dependence of I_{max}, was obtained (Fig. 2b; points are the experimental values, and the full line is a result of the approximation).

In the approximation of experimental data, the modified McGhee–von Hippel equations (3) and (4) were used. The best results were obtained, if the binding was considered to be cooperative, but the degree of cooperativity was small. The obtained values were $K_2 = (5.2 \pm 0.2)\cdot 10^4\ M^{-1}$, $n = 1.9 \pm 0.1$, $\omega = 1.3 \pm 0.2$. A small cooperativity can testify to a certain untwisting of the DNA helix at intercalation sites.

Note that the values $K = 3.54\cdot 10^4\ M^{-1}$and $n\sim 2$ were obtained in work [9] on the basis of analysis of other equations (but in the Scatchard coordinates).

The values $K = 3.8\cdot 10^4\ M^{-1}$ and $n\sim 2$ presented in [5] were obtained with the use of the McGhee–von Hippel equation for the non-cooperative binding.

Ethidium Bromide. The PL spectrum of the EtBr water solution has one band with a maximum at about 614 nm. In the presence of DNA, the PL intensity of EtBr increases by more than 10 times with the DNA concentration in a solution. A blue-shift up to 14 nm was also observed (Fig. 3a). The binding curve (the P/D-dependence of I_{max}) is presented in Fig. 3b.

The binding curve for the EtBr+DNA solution has the s-like form in the range of small P/D. This form can be a result of the presence of another type of ligands in a solution; the latter can be ions H^+ or aggregates of EtBr. With adding of NaCl to a solution, the s-like

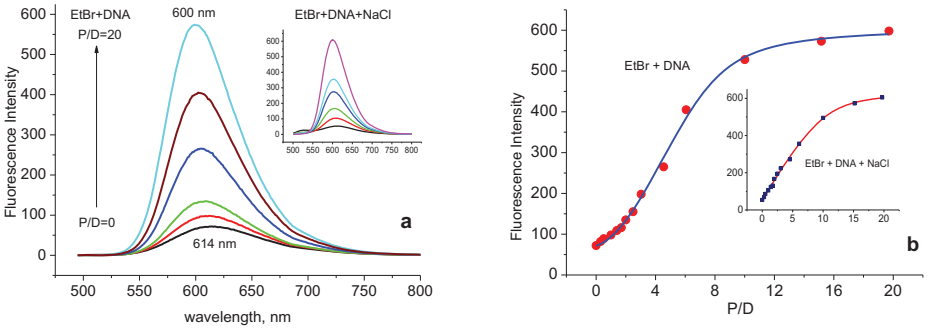

Figure 3. Fluorescence spectra (a) and binding curves (b) for ethidium bromide ($c_{EtBr} = 6.6\ \mu M$, $c_{NaCl} = 0.1$ M, pH~7.0).

form of the binding curve vanishes, probably due to the elimination of additional types of ligands from binding processes. The monotonous elevation of the binding curve means that EtBr binds only in one way, namely through the intercalation.

Based on experimental data, the following values of binding parameters were calculated with the use of the system of dependent Scatchard and McGhee–von Hippel equations: $K_1 = (7.4 \pm 1.5)\cdot 10^2\ M^{-1}$, $K_2 = (1.2 \pm 0.3)\cdot 10^5\ M^{-1}$, $n = 5.7 \pm 1.1$ (two types of binding); $K_2 = (3{,}8 \pm 0.9)\cdot 10^6\ M^{-1}$, $n = 6.6 \pm 1.2$ (one type of binding).

The values of intercalation binding constants $K = 1.5\cdot 10^5\ M^{-1}$ (without NaCl) and $K = 3\cdot 10^6\ M^{-1}$ (with NaCl) were obtained in work [10] on the basis of analysis of the Scatchard equation, which are in good agreement with our results. In [13], the value $K = 6.58\cdot 10^4\ M^{-1}$ was obtained from the UV-spectroscopy data.

Sanguinarine. As was mentioned above, the sanguinarine molecule can exist in the imine (at pH <6) or alkanolamine (at pH > 8.5) form. In our experiments, pH was about 7.6, so that both forms are available. So, the fluorescence spectrum consists of two bands with the maxima at 587 nm (SaI) and 419 nm (SaII), Fig. 4, which different excitation spectra correspond to.

When adding DNA, a variation of the fluorescence intensity and the band shift (blue ~20 nm for SaI and red ~30 nm for SaII) were observed in the PL spectra of Sa.Variations of the fluorescence intensity (and optical density [1]) for sanguinarine depend on the

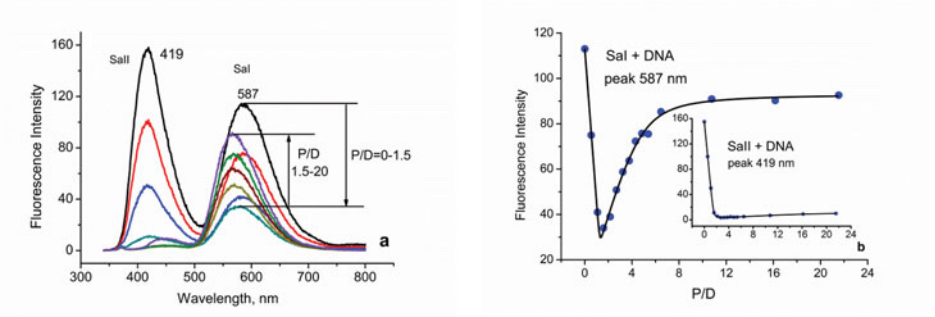

Figure 4. Fluorescence spectra (a) and binding curves (b) for sanguinarine ($c_{Sa} = 23.75\ \mu M$, $c_{SaI} = 16.38\ \mu M$, $c_{SaII} = 7.37\ \mu M$, pH~7.6).

ratio P/D in a nonstandard way. Namely, the corresponding curves have a minimum (see Fig. 4b), and such a behavior is observed for both forms. A similar dependence was found (for SaI) only in work [11] without any explanation.

This "atypical" variation of the optical parameter of ligands can be explained as a manifestation of two types of binding of Sa to DNA: the external one and the intercalation [1]. The minimum in the dependences corresponds to the most compact arrangement of sanguinarine molecules on the DNA matrix, which brings about the hypochromism in the absorption bands and the fluorescence quenching. At small P/D values, the most probable mechanism of binding is the mechanism of external stacking. When the DNA concentration grows, the number of binding sites also increases, and the most probable is the intercalation way of binding.

Since two forms of Sa are available in a solution, the calculation of binding parameters requires us to determine the concentrations of SaI and SaII separately from an initial concentration Sa (23.75 μM). On the base of the fluorescence spectra (the bands 419 and 587 nm), the "concentration" dependence (the curves of a relative content of each form of sanguinarine as a function of pH) was constructed (a similar dependence was presented in [2] on the base of absorption spectra). The determined concentrations of sanguinarine were 16.38 (SaI) and 7.37 (SaII) μM.

Since the dependences of the fluorescence intensity on the DNA concentration in the solutions Sa+DNA are nontrivial, the McGhee–von Hippel equation cannot be applied directly. The relevant experimental results are better described by the system of equations (5), which takes two interdependent processes of ligand binding into account; these are the external binding with phosphates and the intercalation into the DNA double helix.

The calculated binding parameters for SaI and SaII are:

SaI (iminium): $K_1 = (2.5 \pm 0.4)\cdot 10^6\ \mathrm{M}^{-1}$, $K_2 = (26.7 \pm 5.7)\cdot 10^6\ \mathrm{M}^{-1}$, $n = 2.3 \pm 0.1$,

SaII (alkanolamine): $K_1 = (1.8 \pm 0.4)\cdot 10^6\ \mathrm{M}^{-1}$, $K_2 = (4.5 \pm 0.7)\cdot 10^6\ \mathrm{M}^{-1}$, $n = 14.1 \pm 0.3$.

In [5], the presented value of binding constant (DNA of calf thymus) for the imine form of Sa is $0.94\ 10^6\ \mathrm{M}^{-1}$, $n = 1.7$; it was obtained with the use of the McGhee-von Hippel equation for the non-cooperative binding. In [2], the constants determined with the use of the Scatchard equation are $K_2 = 1.5\cdot 10^7\ \mathrm{M}^{-1}$ (in buffer pH = 5.2) and $K_2 = 7.5\cdot 10^6\ \mathrm{M}^{-1}$ (in buffer pH = 10.4). In these experiments, the binding curve of Sa was standard (without a minimum).

Acridine Orange. The fluorescence spectra of the AO+DNA water solution at various P/D are presented in Fig. 5a. The spectrum has one band with a maximum at about 528 nm. A small blue-shift ($\sim$2 nm) was observed with the addition of DNA to a solution. In the range of small P/D, the PL intensity of AO changes non-monotonously (Fig. 5b): the binding curve has a minimum at P/D$\sim$0.5. A similar behavior was observed for sanguinarine. The decreasing PL intensity in the range of small P/D indicates another (external) mechanism binding of AO to DNA. This means that AO binds to DNA in two ways – an external binding and the intercalation. In the presence of NaCl (0.1 M), the dependence of the PL intensity on P/D has no minimum: NaCl excludes the external binding sites.

Here, we mention work [12], where the qualitative explanation of different binding processes was done, and this explanation is like to our one given later for sanguinarine [1]. In addition, the binding constants were determined in [12] for the cases without/with NaCl, by using the Scatchard plots, and the constants for intercalation are in good agreement

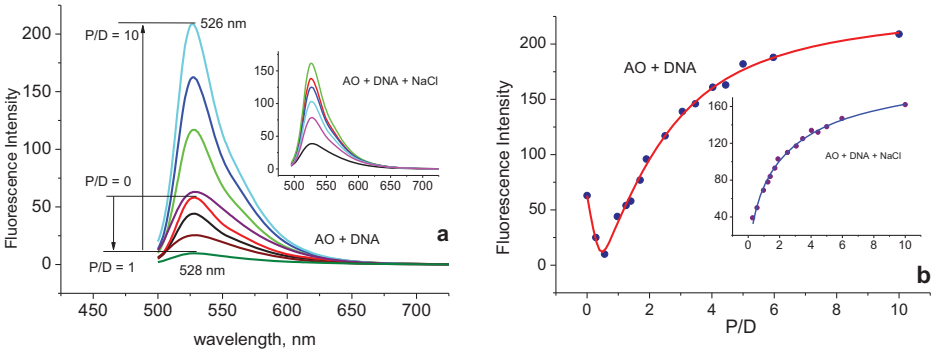

Figure 5. Fluorescence spectra (a) and binding curves (b) for acridine orange ($c_{EtBr} = 6.6$ μM, $c_{NaCl} = 0.1$ M, pH~7.0).

with our results, which were determined from the system of the dependent Scatchard and McGhee–von Hippel equations:

$K_1 = (3.0 \pm 1.0)\cdot 10^5$ M^{-1}, $K_2 = (1.8 \pm 0.2)\cdot 10^6$ M^{-1}, $n = 3.2 \pm 0.3$ (two types of binding, without NaCl); $K_2 = (1.4 \pm 0.1)\cdot 10^5$ M^{-1}, $n = 3.4 \pm 1.1$ (one type of binding, with NaCl).

Results of [12]: $K_2 = 2\cdot 10^6$ M^{-1}, n~2.5 (two types of binding, without NaCl); $K_2 = 1.3\cdot 10^5$ M^{-1}, n~2 (one type of binding, with NaCl). For comparison, the value $K = 2.7\cdot 10^4$ M^{-1} was obtained in [13] from UV-spectroscopy data.

Conclusions

On the base of modified Scatchard and McGhee–von Hippel equations, the binding parameters of ethidium bromide, acridine orange, sanguinarine and berberine have been determined.

Some peculiarities in the optical spectra and, correspondingly, in binding curves were observed, namely, the s-like form of a binding curve of EtBr, the binding curves with minima for acridine and sanguinarine, and some others. This concerns with the binding processes at small (up to 5) P/D, which were investigated insufficiently up to now. Our work, we hope, is a small step forward on this way.

A rather general conclusion can be done about the influence of the form of a ligand on the binding curve, i.e., about the binding mechanisms. If the ligand molecules are flat, they can intercalate into a double helix "more completely'; this means that two binding mechanisms can be realized – the external binding and the intercalation. If a part of the ligand is out of the main plane, then this part is not intercalating, it places into the minor groove and so excludes external binding sites. Here, we have observed the external binding and the intercalation for the flat ligands acridine and sanguinarine and the intercalation only for partially flat ethidium and berberine.

Acknowledgments

The authors are grateful to V. Kosach for the help in the recording of the absorption and fluorescence spectra and L. Zaika (Institute of Molecular Biology and Genetics of the NASU, Kyiv) for the specimens.

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