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BINDING MECHANISMS OF METHYLENE BLUE AND HOECHST 33258 WITH DNA

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In this work the data of methylene blue (MB) and Hoechst 33258 interaction with DNA are presented. It has been shown that at 0.002 *M* ionic strength of solution MB is bound to double-stranded DNA by three modes: intercalation, semi-intercalation and electrostatic. It was revealed that at higher ionic strengths of solution complete intercalation of MB becomes thermodynamically unacceptable. It was shown that Hoechst 33258 was bound to DNA by two types – "strong" and "weak". It was also shown that the "strong" type of Hoechst 33258 binding depends on solution ionic strength.

***Keywords*:** DNA ligand, Hoechst 33258, methylene blue, intercalation, semi-intercalation.

Introduction. Studies of non-covalent binding of low molecular compounds (ligands) to DNA represent a certain interest, because the most important biological processes of cell life are controlled by DNA itself. Moreover, DNA may be subjected to different conformational changes being in surrounding of different molecules [1–3]. Particularly, at functioning DNA may be in both double-stranded (ds) and single-stranded (ss) states, with which different intracellular compounds surrounding DNA or penetrating from external medium may bind [1–3]. These compounds mainly are bound non-covalently, forming slowly dissociating complexes and significantly influence cellular processes. According to the main mechanism of DNA interaction with ligands, they are divided into intercalators and groove binding compounds. Despite this, most of ligands are bound to DNA by more than one mode, moreover, depending on medium factors ligands may be bound with DNA by different mechanisms [1–4]. In the present work some generalizations of MB and Hoechst 33258 (H33258) binding modes to DNA are presented.

Interaction of MB with DNA. One of the compounds that directly is bound to DNA and has wide application in medicine is methylene blue (MB). This ligand is a photosensitizer and is widely used in photodynamic therapy [5–8]. Interaction of this ligand to DNA is studied comprehensively, but hitherto MB interaction mechanisms to DNA are not thoroughly investigated and many questions remain

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discursive. Particularly, spectroscopic investigations showed that MB is bound to DNA AT and GC regions by different modes [8]. Studies showed that the binding of MB with GC regions of decameric DNA occurs by intercalation mechanism, because it is energetically more favorable. Simultaneously, the researchers consider a specific interaction of MB with AT sequences of DNA at high ionic strengths of solution on the similarity of groove-binding ligands (netropsin, H33258, etc.) [10–15]. At the same time, literature data indicate that MB may be bound with DNA via several modes, depending on the nucleotide sequences and ionic strength of the solution [6].

We have investigated the peculiarities of MB binding to DNA by several methods, such as UV melting, absorption and fluorescence spectroscopies. Spectroscopic studies elicit that ligand absorbs light at long-wavelength region (λ=668 *nm*) and fluoresces at the same wavelength region (the intensity of fluorescence is registered at 500–1000 *nm* region). This can be explained by the fact that the excitation energy of the electrons as a result of transition to unexcited stationary state is mainly emitted as a light, with small losses. Based on the absorption and fluorescence spectra, binding curves in Scatchard's coordinates were obtained for MB–DNA interaction according to Eq. (2) (Fig. 1) [16].

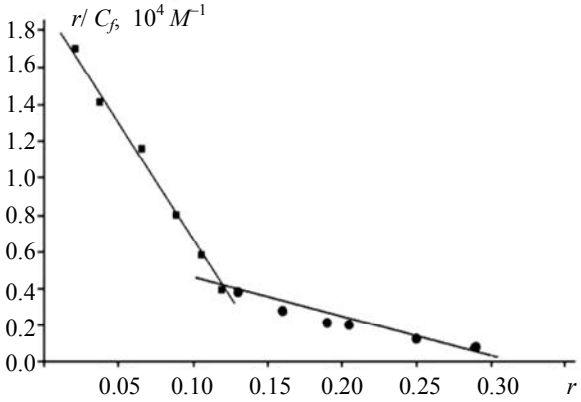

Fig. 1. Binding curves of MB to DNA presented in Scatchard coordinates, derived from fluoresence and absorption spectra, at pH 7.0, $t = 25^{o}C$, at ionic strength of solution $2{\cdot}10^{-2} M$.

Experimental points, obtained from the analysis of fluorimetric and absorption data, coincide with each other, so, we present a summarized curve. Isotherm curve at low values of r, where r =[ligand] / [DNA], is linear until r=0.14, and at r >0.14 the second linear region is formed on the r/C_f dependence curve on r. The performance of two distinct linear regions on the curve indicates the existence of at least two binding modes for MB with DNA. Moreover, one of them is "strong" and is characterized by a $K \approx 6.5{\cdot}10^5\ M^{-1}$ binding constant and by a number of residues, corresponding to one binding site $n \approx 4$. The second one, "weaker" mode, has binding constant $1.5{\cdot}10^5\ M^{-1}$ and $n \approx 2$. However, no specificity to any DNA sequences, particularly to AT sequences is mentioned in [8]. Simple and informative method for identifying the specificity of DNA–ligand binding is UV melting method, due to which we have studied DNA–MB complexes and from the curves (curves are not presented) melting parameters such as T_m and ΔT were obtained for DNA and MB–DNA complexes. Using the obtained parameters we built the dependence of $\delta\Delta T$ on r at 0.002 M (curve 1) and 0.02 M (curve 2) ionic strength conditions (Fig. 2, a).

The dependence of $\delta\Delta T$ on r has bell-like shape, because it increases at low ligand concentrations and passing the plateau it decreases. Dependence curves of $\delta\Delta T$ on r (Fig. 2, a) cannot be explained in term of MB specificity to AT sequences of DNA, since in this case these curves should have negative values, as it is obtained in case of H33258 to DNA (Fig. 3, curve 1). The increase of the dependence of $\delta\Delta T$ on r at low MB concentrations is due to the fact that during melting process redistribution of bound molecules takes place, from denatured regions to non-denatured regions, as it is illustrated for EtBr–DNA complexes [17, 18]. Bell-like change of $\delta\Delta T$ dependence on r is usually observed in the case of intercalators, which also is bound to DNA via other binding modes (multimodal ligands) [17–19]. Based on this we assume that the obtained melting data for DNA–MB complexes confirm the conclusion that MB at low ionic strength of solution is bound to DNA via intercalative binding mode. It can be seen from Fig. 2, a, that curve 2 increases at low ligand concentrations, then it reaches a plateau at $r > 0.1$. This indicates the fact that at μ=0.02 *M* MB is bound to DNA by single or at least two binding modes.

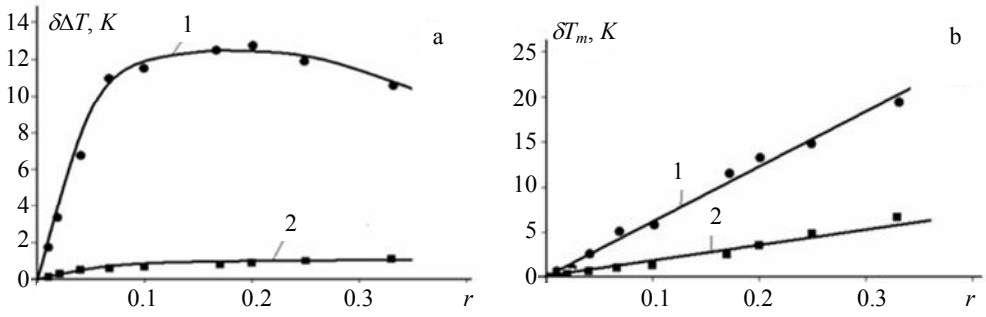

Fig. 2. Dependence of $\delta\Delta T$ (a) and δT_m (b) on r for DNA–MB complexes. Curve 1 was obtained at 0.002 *M*, curve 2 was obtained at 0.02 *M* ionic strength of solution [9].

The fact that at the mentioned ionic strength $\delta\Delta T$ dependence curve on r increases in $0<r\leq 0.1$ interval shows, that binding sites for MB on DNA are specific and limited. However, when these sites are saturated and there is no second binding mode, the dependence curves of $\delta\Delta T$ on r should reach the plateau, but it is not detected. At the same time, curve 1 and curve 2 (Fig. 2, b) increase throughout the r variation interval ($0 \leq r \leq 0.33$). This is caused by the fact that when intercalative and semi-intercalative binding sites on DNA at μ=0.002 *M* fill up, or specific binding sites at μ = 0.02 *M* conditions become occupied, ligand molecules start to bind electrostatically, which at low ligand concentrations appear insignificantly and become primary binding mode at relatively great values of r. Therefore we assume that at μ = 0.02 *M* MB is bound to DNA at least by two modes, which is reflected on the binding curve presented in Scatchard's coordinates. Based on the facts that MB is intercalator and it does not exhibit any specificity to AT sequences, it can be concluded that at $\mu \geq 0.02$ *M* the specific binding mode for MB to DNA is semi-intercalation.

Thus, the obtained data reveal that binding mode of MB to DNA depends on the molar ratio r. At greater values of r, positively charged MB molecules generally are bound with the phosphate groups of DNA electrostatically, whereas

fluorescence intensity decreases, and hypochromicity is registered on the absorption spectra. At smaller values of r, the second binding mode appears, at which MB molecules intercalate or semi-intercalate into DNA at low ionic strength, and semi-intercalate at relatively high ionic strength conditions.

Interaction of Hoechst 33258 with DNA. Drugs that selectively are bound to certain DNA sequences are of a great interest, because of their high biological activity and practical value, particularly in the field of cancer chemotherapy. A typical representative of this group is H33258, which selectively is bound to AT sequences of minor groove of DNA. The complexes generally stabilize by van der Waals' interactions between the ligand molecules and the walls of minor groove, and also by hydrogen bonds between amide groups of ligand and nitrogenous bases of DNA. In the complex stabilization an essential role plays electronegative charge density in the minor groove of DNA [9–15]. In general, binding specificity of H33258 significantly is caused by the geometric corresponding of the ligand molecule and the minor groove width, which depends on the sequence of DNA [1, 2]. However, the possibility of intercalative binding of H33258 to DNA (mostly at GC-rich regions) cannot be excluded [14, 15]. The fact of specific binding of H33258 with AT sequences of DNA is reflected on the $\delta\Delta T$ dependence on r at μ>0.04 M [4], because the change of melting interval width for DNA–H33258 with an increase of r decreases, acquiring negative values (Fig. 3).

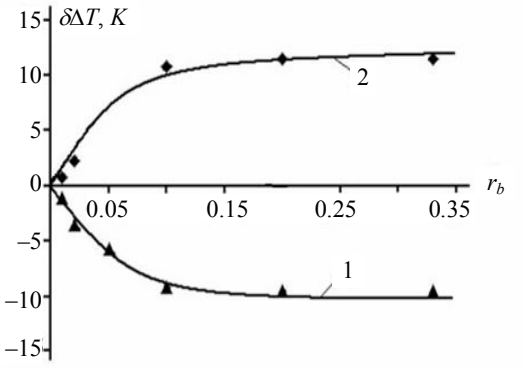

Fig. 3. Dependence curves of $\delta\Delta T$ on r for DNA–H33258 complexes at 0.02 M (1) and 0.002 M (2) ionic strengths of solution.

The reason of this decrease is that with binding of H33258 molecules to AT sequences the melting temperature of it increases, while the same parameter for GC-sequences practically remains constant. As follows from the presented Fig. 3, at 0.002 M ionic strength conditions the $\delta\Delta T$ on r dependence (curve 2) grows until $0 < r \le 0.1$, then with a further increase of r this curve practically remains unchanged. Simultaneously, from Fig. 3 it can be seen that curve 1 decreases in $0 \le r \le 0.1$ interval and with growth of r it does not change. This fact indicates the existence of two specific binding sites on DNA for H33258 at the mentioned ionic strength conditions. One of them is saturated when $r \approx 0.1$ (AT-specific regions at 0.02 M and intercalation sites at 0.002 M), the second type of interaction practically is not saturated up to $0.1 \le r \le 0.35$. In the latter case, the ligand molecule electrostatically is bound to the phosphate groups of DNA. These sites are not limited up to the saturation stoichiometry 1:1 value, and are binding centers both at 0.002 and 0.02 M ionic strength conditions [4, 14, 15].

The existence of two types of binding sites is confirmed with the curve obtained from the absorption and fluorescence spectra of H33258 and its complexes with DNA (Fig. 4). Experimental points, derived from the analysis of fluorimetric and absorption data (at small values of r) coincide with each other, thus, we provide a generalized curve, which is non-linear and is composed from two linear regions.

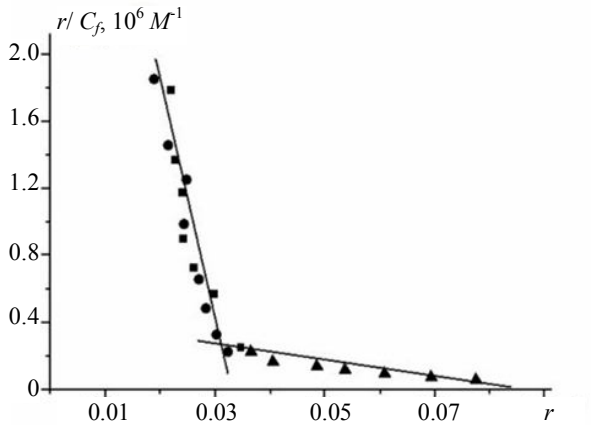

Fig. 4. Binding curves of H33258 to DNA presented in Scatchard coordinates, derived from fluoresence and absorption spectra at pH 7.0, t=25°C, μ= $2\cdot10^{-2}$ M. Circle points were derived from the fluorescence measurements, squares and triangles were obtained from the absorption measurements.

This points out on the existence of at least two binding modes of H33258 with DNA. One of these binding modes is strong and it corresponds to specific binding of ligand molecules to AT sequences at μ=0.02 M, and the second one is weaker electrostatic binding mode of H33258 to the phosphate groups of DNA [4]. The binding curves obtained from the analysis of fluorescence and absorption spectra in $0 < r \le 0.035$ range are linear and practically coincide. So, fluorimetric and absorption methods reveal the same type of binding for H33258 (as it is in case of MB) with DNA. At μ >0.04 this mode corresponds to AT-specific binding [4]. Second region, which reveals at $r > 0.035$ values, is the result of a weaker binding mode which stands for electrostatic mechanism [14, 15].

Binding parameters K and n were obtained from the adsorption curves, and it was revealed from these parameters that K_s (binding constant of strong binding mode), obtained from the fluorimetric and absorption studies, coincide with each other ($K_s \approx 4.5\cdot10^7\ M^{-1}$). The value of binding constant for H33258 weak binding to DNA ($K_w \approx 2\cdot10^5\ M^{-1}$) is almost two order smaller than binding constant of strong binding. The n_s ($n_s \approx 9$), obtained from the fluorimetric and absorption data for H33258 binding to DNA corresponded to "strong" binding mode, indicates the fact that the number of strong binding sites is more limited, in contrast with the same parameter of weak binding ($n_w \approx 4$).

Conclusion. The obtained data reveal that MB and H33258 may be bound to DNA by several modes. Performance of one or other binding type of MB and H33258 depend on solution ionic strength and in the case of H33258 on DNA nucleotide sequence as well. Furthermore, the possibility of H33258 binding to DNA by intercalation (predominantly in GC-rich regions) is not excluded [15].

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