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INVESTIGATION OF DIFFERENTIAL ABSORPTION OF DNA COMPLEXES WITH LIGANDS

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The investigation of complexes of ethidium bromide (EtBr) and methylene blue (MB) with DNA at 0.02 *M* Na^+ ionic strength of solution has been carried out. Absorption and differential absorption spectra of EtBr and MB and their complexes with DNA were obtained. The isobestic point was revealed on the absorption and differential absorption spectra of EtBr and its complexes with DNA, while on analogous spectra of MB and its complexes with DNA there is no isobestic point.

***Keywords*:** EtBr, MB, absorption spectra, differential absorption spectra, isobestic point.

Introduction. The investigations of binding peculiarities of ligands that contain aromatic rings are very important nowadays, because of their pronounced biological activity. Furthermore, these compounds may directly or indirectly bind to DNA and change its functional activity [1, 2]. These observations allow to determine the specificity of ligands to certain sequences of DNA and to detect the mechanisms of these interactions [1–5]. Different informative methods, such as absorption and fluorescence spectroscopy, CD (circular dichroism), NMR (nuclear magnetic resonance) spectroscopy, were used for studying DNA–ligand complex-formation. Above mentioned methods are advantageous, because due to complex-formation the spectral characteristics of ligands are changed both qualitatively and quantitatively [5–7].

Most of ligands, particularly ethidium bromide (EtBr), methylene blue (MB), may bind to DNA by several modes [1–4, 6–15]. However, it is difficult to identify these binding modes only by one method. For example, at EtBr interaction with DNA one binding mode may be hidden under the other one. From this point of view the most effective way of studying DNA–ligand interactions is a comparative investigation of binding by different methods. The purpose of this paper is to analyze qualitatively the absorption spectra and the differential absorption spectra of DNA–EtBr and DNA–MB complexes.

Materials and Methods. In this work ultrapure Calf Thymus DNA (“Sigma”, USA), EtBr (“Sigma”, USA), MB (“Aldrich”, USA), NaCl, Na-citrate,

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EDTA (ethylenediamintetraacetat , "Chemically Pure") were used. The solutions of EtBr and DNA were prepared in 0.1×SSC, containing 0.015 *M* NaCl, 0.0015 *M* Na-citrate, 10^{-5} *M* EDTA (Na^+ concentration is equal to 0.02 *M*). All preparations were used without additional purification. Concentrations of EtBr, MB and DNA were determined spectrophotometrically using the following extinction coefficients: ε_{480}=5800 $M^{-1}cm^{-1}$ for EtBr, ε_{664}=76000 $M^{-1}cm^{-1}$ for MB and ε_{260}=6600 $M^{-1}cm^{-1}$ for DNA. Investigations were carried out at t=25°*C* and pH 7.0.

Spectrophotometric measurements were carried out on single beam spectrophotometer Jenway 6715 UV-VIS (England). Absorption measurement was registered in hermetically closed quartz cuvettes with 1 *cm* optic pathway length with similar optic parameters. The average sensitivity of the equipment in working diapason spectrum was measured without samples equal to $5\cdot10^{-5}$ optic density unit corresponding to $\Delta I/I = 10^{-4}$ change that finally gives the total deviation, which does not exceed 3%. At registration of differential absorption of complexes as a basic line the absorption spectrum of ligand solution in the absence of DNA was taken. At registration of complex absorption, ligand concentration remains constant and concentration ratio r=[DNA]/[ligand] was changed in $2 \le r \le 0.05$ interval (calculated per one base pair of DNA).

The titration of the solutions was performed by micropipette with 10 (l total volume ("Hamilton", USA)). Absorption spectra of the solution of the complexes were registered versus pure EtBr and MB solution in λ=400–600 *nm* and λ=500–800 *nm* wavelength change interval respectively. Moreover, the concentrations of ligands in control and investigated solutions were the same.

Results and Discussion. The quantitative analysis of absorption spectra of DNA–EtBr complexes is based on the determination of EtBr absorption values in completely free and bound states, in certain concentration of DNA. This allows to calculate the proportions of bound and unbound ligand molecules and using Sketchard's transformations to determine the binding constant of these ligands with DNA.

Our absorption spectroscopic studies have shown that as a result of EtBr binding to DNA the decreasing of absorption spectra maximums as well as the shift to longer wavelengths take place at λ_{max} (λ_{max}=480 *nm* for EtBr). Particularly, in the case of EtBr with DNA concentration enhancement at constant ligand concentration at λ=535 *nm* peaks on the absorption spectra of complexes appear (Fig. 1, a). This is caused by the fact that free and bound ligand molecules with DNA have different absorption maximum (the values of ε of free and bound EtBr molecules differ). The peculiarities of EtBr absorption spectra at binding to DNA are conditioned by the saturation of EtBr binding sites on DNA at its relatively low concentrations. The saturation occurs by the binding sites corresponding to all binding modes and as a consequence, no free binding sites remain. Because of saturation of binding sites of DNA, maximum absorption at λ=480 *nm* is reduced and the shift towards longer wavelengths region is observed. With DNA concentration increase in the solution more free molecules of EtBr turn to be in bound state and at $C_{DNA}>C_{EtBr}$, whenalmost all molecules of ligand are in bound state the peaks appear only at λ=520 *nm*. With further increasing of DNA concentration, the redistribution of bound ligand molecules by all modes to free intercalation binding sites on DNA takes place, because intercalation is the main

binding mode for EtBr and it is characterized by bigger binding constant [11]. Moreover, on the absorption spectra the isobestic point is detected at λ=510 *nm*. The isobestic point appears, when there are two spectrophotometrically different forms of ligand molecules in medium, and these forms have the same absorption at certain wavelength. Isobestic point disappears when $C_{DNA}>>C_{EtBr}$, and the absorption values of complexes are not changed at λ=520 *nm*.

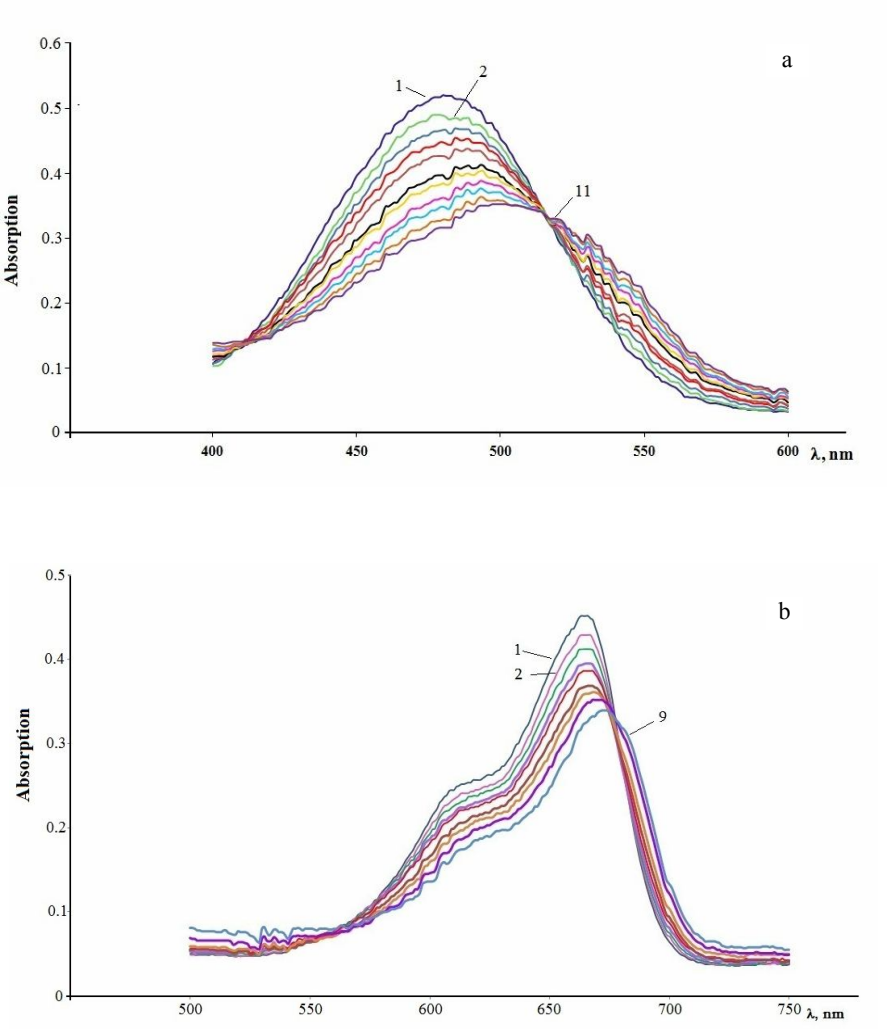

Fig. 1. Absorption spectra: a) EtBr (1) and its complexes with DNA (2–11); b) MB (1) and DNA–MB complexes (2–9).

Fig. 1, b illustrates absorption spectra of MB complexes with DNA. As it is obvious from the Figure, MB has maximum of absorption at λ=664 *nm* with shoulder at $\lambda \approx 610$ *nm*. The absorption of MB is decreased (hypochromic effect) with increasing DNA concentration in medium. Hypochromic effect appears when $C_{DNA}<<C_{MB}$ while the shift of λ_{max} is not practically detected. With DNA concentration enhancement, with hypochromic effect, the shift of λ_{max} towards longer wavelength (~15–16 *nm*) region and pseudoisobestic point appears on absorption

spectra. This shift is common phenomenon for intercalating ligands. The fact that isobestic point is distorted indicates that spectrophotometric characteristics of bound and free molecules of MB differ from each other less, opposite to EtBr. However, the shift of λ_{max} towards longer wavelengths with ~15–16 *nm* on absorption spectra of DNA–MB complexes may be a confirmation of the fact that at high concentrations of DNA the main binding mode for ligands is intercalation.

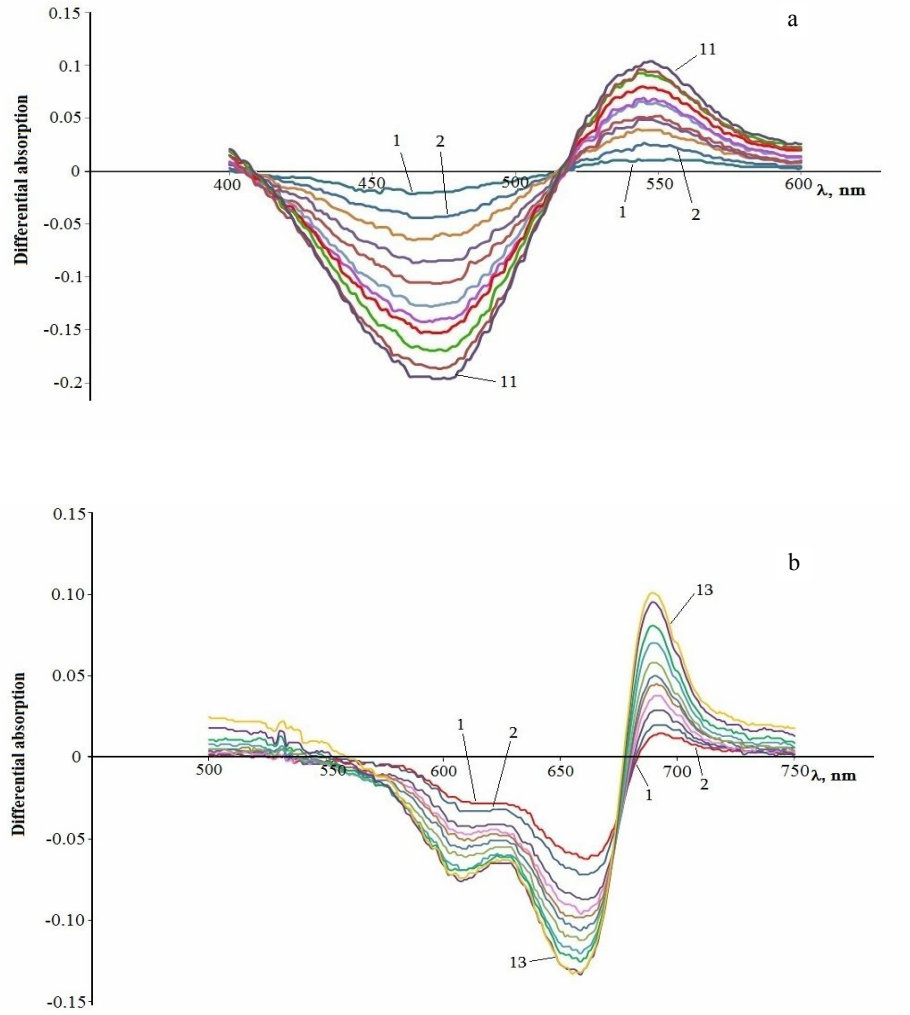

Fig. 2. Differential absorption spectra: a) EtBr (1) and its complexes with DNA (2–11); b) MB (1) and DNA–MB complexes (2–13).

For clarification of this fact methods that are more sensitive should be applied. From this point of view it is important to mention that in some cases the integral curve that describes certain physical process is not enough informative. Particularly, as it was mentioned above, in the case of EtBr, which is known as classical intercalator, the molecules of ligand may bind with DNA via electrostatic and semi-intercalation modes. Moreover, the semi-intercalation mode is hidden under the intercalation one and the experimental revealing of this mode requires

indirect methods of investigation [12]. In this situation the possible solution of this problem is differential study of ligands that bind with DNA via several modes. There are two approaches for differentiating the integral curves: numerical or direct (if it is possible to realize experimentally). Based on this, experimentally we obtain different absorption spectra for EtBr and MB complexes with DNA. In this case the absorption of complexes is registered relative to ligand solution.

During the experiment, when DNA concentration is growing, differential absorptions of complexes were registered, since during the binding with DNA, the whole concentration of free ligand molecules decreases, consequently the new system of DNA–ligand complex is formed in the solution, which has different spectral characteristics. Hereupon, there are negative peaks at λ_{max} and positive peaks at longer wavelengths on different absorption spectra.

Fig. 2, a illustrates differential absorption spectra of EtBr and its complexes with DNA. As it may be seen from the Figure, there are negative peaks at λ=480 *nm* and positive ones at λ=540 *nm* when $C_{DNA} << C_{EtBr}$ (these peaks are missing on absorption spectra (see Fig. 1, a)). Increasing DNA concentration, absolute values of negative and positive peaks at λ=480 *nm* and 540 *nm* are respectively increased. When we simply dilute solution of EtBr with DNA solution there is only one peak at λ=480 *nm*, which has negative change, and there is no peak at λ=540 *nm* (spectra are not presented). Besides that, the isobestic point appears on differential absorption spectra of EtBr at λ=515 *nm* with zero absorption value. On differential absorption spectra, λ_{max} is 540 *nm* while the isobestic point practically is not shifted in contrast with absorption spectra. Moreover, EtBr absorption maximum (Fig. 1, a) and the negative peaks of DNA–EtBr complexes are revealed at 480 *nm*.

Most probably in case of differential absorption, maximums at 540 *nm* are caused by intercalated molecules of EtBr. From this point of view, the increasing of absolute values of negative peaks on differential absorption spectra at λ=480 *nm* may reflect the binding of EtBr to DNA by all possible modes (in case of EtBr: intercalation, semi-intercalation and electrostatic mode) [12]. Fig. 2, b shows that in case of DNA–MB complexes there are negative peaks at λ=660 *nm* and positive ones at λ=690 *nm*. Increasing DNA concentration, absolute values of negative and positive peaks at λ=660 *nm* and 690 *nm* are enhanced respectively, while at simple dilution of MB solution decreasing of peaks are observed on spectra at 660 *nm* peaks (negative changes) as in case of EtBr (spectra are not presented). From the obtained spectra is revealed that absolute values of peaks at λ=660 *nm* and 690 *nm* slightly differ from each other, whereas in case of EtBr these values differ a lot. In all probability, this is caused by the fact that negative peaks correspond to absorption of free molecules of ligand and concentration of ligand free molecules is decreased during titration with DNA. Positive peaks correspond to absorption of ligand molecules that are bound to DNA with certain mode. From this point of view, it may be assumed that binding mode in this case is intercalation (hypochromic effect is caused by occurrence of additional stacking interactions between DNA bases and aromatic rings of ligand) [6, 15, 16]. Based on this, we assume that a little difference of absolute values of peaks in case of MB is conditioned by two modes of binding [3], and in case of EtBr, as it has been already mentioned, the difference

of absolute values of peaks is quite significant and it is probably caused by the fact that ligand bind to DNA via several modes.

It is necessary to note, that unlike DNA–EtBr's case no isobestic point was detected on DNA–MB differential absorption spectra. This may be the result of the fact that MB is not completely intercalated. The confirmation of this assumption may be the fact that there is no isobestic point on the absorption spectra of non-intercalating DNA ligands (such as Hoechst 33258, which localizes in the minor groove of DNA), too [5]. On the other hand, literature data indicate the intercalative mode of MB binding with DNA (see [3, 4, 8, 9]). Thus, we assume that positive peaks on differential absorption spectra of DNA–MB complexes are the result of semi-intercalation of MB into DNA, while in the case of EtBr there is a complete intercalation and spectrophotometric characteristics of bound molecules of ligand significantly differ from spectrophotometric characteristics of unbound molecules. Simultaneously, the presence of isobestic point indicates the fact that there is an intermediate state, at which free and bound molecules of EtBr have the same absorption. Semi-intercalated molecules that are bound and have some degree of freedom at the same time may be in this intermediate state. From this point of view, we may explain the vanishing of isobestic point in conditions of low concentrations of DNA, when EtBr binds with DNA mainly with intercalative mode.

Conclusion. Thus, based on analyses we conclude that spectral characterristics of MB and EtBr depend on their binding mechanisms. It was shown that via differential absorption spectra of DNA–ligand complexes, different binding modes may be identified and relying on the absorption and differential absorption spectra qualitative analyses may be realized. Besides, the fraction of differently bound ligand molecules may be calculated. This, in its turn, will allow to determine the number of binding sites and binding constant. Another important conclusion is that contemporaneous usage of aforementioned two approaches allow to reveal some peculiarities of DNA–ligand interactions, which cannot be revealed by using only one of them.

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