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STUDY OF PECULIARITIES OF DIFFERENTIAL ABSORPTION SPECTRA OF COMPLEXES OF ACRIDINE ORANGE, ETHIDIUM BROMIDE AND METHYLENE BLUE WITH DNA

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Interaction of intercalators: ethidium bromide (EtBr), methylene blue (MB) and acridine orange (AO), with DNA at 0.02 *M* ionic strength of the solution has been studied by differential spectroscopy method. It was revealed that EtBr binds to DNA by intercalation, semi-intercalation and electrostatic modes; MB by semi-intercalation and electrostatic modes and AO by intercalation and electrostatic modes. It was shown that the simultaneous performance of intercalation and semi-intercalation modes condition the formation of a real isosbestic point (IP) in the spectra of EtBr–DNA complexes. In the absence of intercalation at MB interaction with DNA a pseudo-IP in differential absorption spectra of those complexes is formed. In the spectra of AO–DNA complexes both IP and pseudo-IP are not formed, despite the fact that this ligand binds to DNA by intercalation mode.

***Keywords*:** DNA, ethidium bromide, methylene blue, acridine orange, differential spectra, isosbestic point, pseudoisosbestic point.

Introduction. Studies of binding peculiarities of ligands, comprising a group of aromatic rings with DNA have practical importance since most of these compounds show a pronounced biological activity. This property of ligands appeared due to its ability to bind to DNA immediately or in mediated way and to condition its functional activity [1–4]. From this point of view such studies permit revealing of specificity of ligands to certain sequences of DNA or mechanism of these interactions as well [5–8].

Different methods are used for studying ligand binding with DNA; among them spectral methods (absorption, fluorescence, CD (circular dichroism), NMR (nuclear-magnetic resonant) spectroscopies) are sufficiently informative. The study of complex-formation of most of ligands with DNA by aforementioned methods is based on the fact that binding with biomacromolecules (DNA) the spectral characteristics of these ligands undergo quantitative and qualitative changes [4, 5]. It is important to mention as well that ligands may bind to DNA by several modes, one or several of which may seem to be the main one. For instance, in the case of ethidium bromide (EtBr) interaction with DNA one of the binding modes, semi-intercalation is masked as intercalation [8], moreover, these concealed modes may influence the system spectral characteristics as well, consequently for revelation of spectral peculiarities of DNA–ligand complexes the differential methods are

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informative, since in the last case such small changes in the measuring characteristics of the sample at low concentrations of preparations may be recorded [9], which are not practically revealed by absorption spectroscopy.

The aim of this research is the comparative study of differential spectra peculiarities of absorption of complexes of EtBr, methylene blue (MB) and acridine orange (AO) with DNA.

Materials and Methods. Calf Thymus ultrapure DNA – D-1501 ("Sigma", USA), EtBr ("Serva", Germany), MB, AO ("Sigma", USA), NaCl, Na-citrate, EDTA (chemically pure) were used in experiments. EtBr and DNA solutions were prepared in 0.1×SSC, containing 0.015 *M* NaCl and 0.0015 *M* Na-citrate, 10^{-5} *M* EDTA. To establish the respective ionic strength of the solution at 0.02 *M*, 1×SSC was diluted by double distilled water. All preparations were used without further purification. Concentrations of DNA, EtBr, MB and AO were determined spectrophotometrically using the following extinction coefficients: ε_{260}=6600 $M^{-1}cm^{-1}$ for DNA CT, ε_{480}=5600 $M^{-1}cm^{-1}$ for EtBr, ε_{664}=76000 $M^{-1}cm^{-1}$ for MB and ε_{490}=35000 $M^{-1}cm^{-1}$for AO. Experiments were carried out at t=25°*C* and pH 7.0.

Spectrophotometric measurements were carried out on double-beam spectrophotometer UV-VIS Unicam-SP8-100 (UK) and single-beam spectrophotometer UV-VIS JENWAY 6715. Absorption measurements were carried out in quartz cuvettes with 1 *cm* optic pathway length and similar optic parameters. Spectrophotometric titration of solutions was carried out by micropipette with 10 μL total volume ("Hamilton", USA).

At registration of absorptions of complexes the ligand concentration remains constant and DNA concentration raises up to those ligand/DNA ratios, at which changes of differential absorption spectra of the complexes are not practically observed. The absorption spectra of the complexes were recorded versus those of free ligands in the interval of wavelengths λ=400–600 *nm* in the case of EtBr as well as AO and λ=600–700 *nm* in the case of MB.

Results and Discussion. Studies on interaction of some intercalators, such as EtBr, proflavin, AO etc. with DNA by the absorption spectroscopy method are based on the fact that the absorption spectra of the complexes are shifted to longer wavelengths in relation to pure ligand absorption spectrum and decrease in maximums [10–13]. Particularly, at EtBr binding to DNA maximums of the absorption spectra of complexes in the wavelength interval $400 \le \lambda \le 600$ *nm* are significantly shifted to longer wavelengths. Thus, free state EtBr absorption maximum corresponds to λ=480 *nm*, while at entirely bound state with DNA at λ=520 *nm*. Moreover, in the absorption spectra an isosbestic point (IP) appears at λ=510 *nm* [10, 11]. In the absorption spectra there is one more peculiarity: at certain DNA concentrations ($C_{DNA} \gg C_{lig.}$) the absorption spectra of the complexes deviate from IP. Literature data indicate that IP is a criterion of binding intercalation mode of ligands to DNA. From this point of view it may be assumed that this point is one of fundamental characteristics of DNA–ligand systems [13–18]. Consequently, there is a necessity of studying by the experimental methods that permit maintaining IP existence reality in the absorption spectra of DNA–EtBr complexes. Earlier using the elaborated mathematical model, the existence of real IP was proved at 510 *nm*. Moreover, the existence of IP in the absorption spectra of DNA–ligands complexes can be determined experimentally with high precision by DS method, which is more sensitive than absorption spectroscopy by almost 5–6 times [9].

Note that in literature it is accepted to consider that IP is a characteristic of the binding intercalation mode with DNA, our obtained data do not affirm this fact.

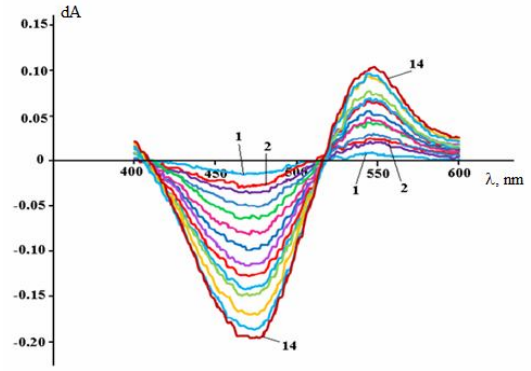

Fig. 1. Differential absorption spectra of EtBr and its complexes with DNA. In the spectra of EtBr–DNA complexes IP appears at λ=510 *nm*. EtBr concentration in solution was equal to $9.0 \cdot 10^{-5}$ *M*, DNA concentration increases up to ligand/DNA=1/30 ratio [10, 11].

That is why we have carried out a comparative study of peculiarities of differential absorption spectra of complexes of AO, EtBr and MB with DNA to understand the effect of binding modes on IP formation in their spectra.

Absorption spectra of the DNA–ligands complexes were recorded versus those of pure ligand solution (differential absorption), its spectra of EtBr and its complexes with DNA were presented in Fig. 1. From here it is obvious that at low DNA concentrations ($C_{DNA} \ll C_{EtBr}$) and λ=480 *nm* negative peaks are observed and at λ=540 *nm* positive peaks are observed (at low concentrations of DNA there are no these peaks in the absorption spectra). This is conditioned by the fact that binding with DNA the total concentration of ligand free molecules decreases. Moreover, the new system is formed in the medium ligand–DNA complexes with other spectral characteristics. It results in appearing of the negative peaks in the differential absorption spectra at such wavelength which corresponds to the free ligand absorption and the positive peaks at longer wavelengths corresponding to absorption of EtBr bound molecules. With DNA concentration increasing the absolute values of negative peaks at λ=480 *nm* decrease, the positive peaks of the same spectrum of DNA–EtBr complexes at λ=540 *nm* increase, while at simple dilution of EtBr solution 0.1×SSC–multiple to dilution at titration by DNA solution, only decreasing of the peaks is observed at λ=480 *nm* (spectra are not presented). The IP at λ=510 *nm* appears in the differential absorption spectra of EtBr–DNA complexes with zero value of absorption. Actually, in the differential spectra of EtBr–DNA complexes (in absolute absorption spectra as well) peaks corresponding to EtBr free molecules appear at λ=480 *nm*, whereas maximums in the spectra of the complexes at λ=540 *nm* correspond to EtBr bound molecules and enhance with increasing of portion of ligand bound molecules. It should be mentioned that at λ>520 *nm* peaks appear in the absorption spectra of complexes that correspond to entirely bound molecules of EtBr in the case of high concentrations of DNA (in these conditions intercalation mode of the binding is performed).

These peculiarities of the differential absorption spectra of EtBr–DNA complexes allow to assume that the maximums in spectra at λ=540 *nm* correspond to EtBr molecules bound to DNA by the intercalation mode. The fact that the absolute values of changes of the peaks of the same spectrum are not similar may serve as a confirmation of the following: at λ=480 *nm* the change is bigger than at λ=540 *nm*. Consequently we assume that the enhancement of absolute values of negative peaks of the differential absorption spectra at λ=480 *nm* reflect EtBr binding to DNA as by intercalation mode, so, by semi-intercalation and electrostatic modes [7].

The differential absorption spectra of MB–DNA complexes presented in Fig. 2 was obtained, it is obvious that in the differential spectra of MB–DNA complexes both negative (at λ=660 *nm*) and positive (at λ=690 *nm*) peaks are revealed.

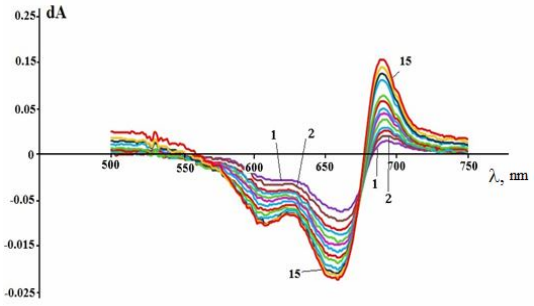

Fig. 2. Differential absorption spectra of MB and its complexes with DNA. [MB] = $6.05 \cdot 10^{-6}M$, [DNA] increases up to ligand/DNA=1/30 ratio [10, 11].

By increasing [DNA] the absolute values of both negative and positive peaks at λ=660 and 690 *nm* correspondingly increase, while in the spectra at simple dilution of MB solution only decreasing of peaks at λ=660 *nm* (negative change) is observed as in the case of EtBr (spectra are not presented). It is conditioned by the fact that the negative peaks correspond to absorption of free ligand molecules, concentration of which decreases with DNA concentration enhancement, the positive peaks correspond ligand bound molecules. It has been revealed from the obtained spectra that absolute values of the peaks at λ=660 and 690 *nm* of the same spectrumless differ from each other, that is why we assume that this ligand probably binds to DNA by two modes.

Note that in the case of MB in spite of EtBr there have not been revealed IP in the differential absorption spectra. This fact is the result of intercalation mode lack of MB binding to DNA, though literature data indicate its existence [19, 20].

In differential absorption spectra of DNA–MB complexes a region is formed, in which all spectra are approaching, here differential absorptions of complexes differ from each other less (but do not have zero value). Consequently it can be assumed that real IP in the spectra of DNA–MB complexes is not formed and this region in differential absorption spectra may be called pseudo-IP. Obtained data can be the result of that DNA binds by two modes at 0.02 *M* ionic strength of solution: semi-intercalation and electrostatic, as it was early revealed by virtue of studies by absorption and fluorescence spectroscopies.

We have studied the binding of another intercalator AO with DNA by differential spectroscopy method, also. The respective spectra were presented in Fig. 3. It is necessary to note that the differential absorption spectra of AO–DNA, in spite of EtBr– and MB–DNA complexes, consist of one negative peak at low concentrations of DNA. These peaks decrease with DNA concentration enhancement. At DNA concentration further increasing both negative (at λ=490 *nm*) and positive (at $\lambda\approx$505 *nm*) peaks are formed in the spectra and their absolute values enhance with DNA concentration increasing. For obviousness of this effect the differential absorption spectra of AO–DNA complexes were divided into two parts: the spectra of complexes (1–10) are presented in Fig. 3, A at relatively low concentrations of DNA, the spectra (11–23) are presented in Fig. 3, B at high concentrations of DNA. Increasing of absolute values of the negative peaks at λ=490 *nm* (curves 1–10) is the result of decreasing the free AO molecule concentration by complex-formation with DNA compared with the control. Moreover, at further increasing of DNA concentration the reversible phenomenon is

observed, i.e. the absolute values of negative peaks start decreasing, along with this at λ~505 *nm* positive peaks appear (curves 11–23). Another peculiarity of the differential absorption spectra of AO–DNA complexes is that the isosbestic or pseudo-IPs are not formed. These spectral peculiarities of AO–DNA complexes may be explained based on the fact that in the case of this ligand the main binding mode is intercalation which becomes prevailing especially at DNA high concentrations and positive peaks at $\lambda\approx505$ *nm* as well as hyperchromism effect correspond to this mode. This effect is conditioned by insertion of aromatic chromophore part of AO molecule into the plain of DNA base pairs orienting parallelly to these pairs (intercalation). Analogous effect was obtained in the case of EtBr. Binding intercalation mode of AO to DNA is indicated as well by enhancing of fluorescence intensity of the complexes compared with that of free MB, which is a consequence of the fact that this ligand does not bind to DNA by intercalation mode.

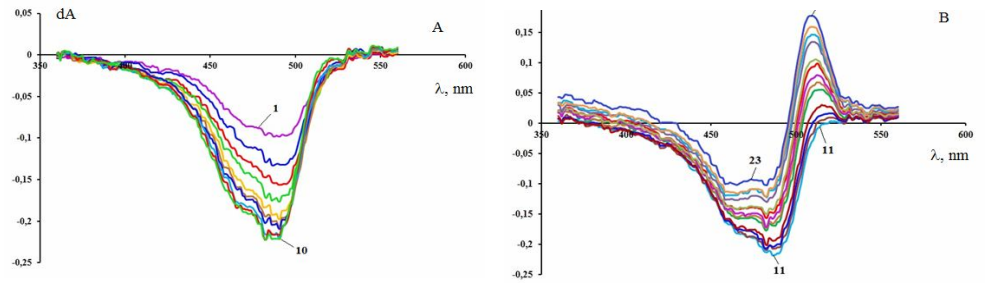

Fig. 3. Differential absorption spectra of AO and its complexes with DNA (curves 1–13). [AO] =1.5·10^{-6} *M*, [DNA] increases up to ligand/DNA=1/30 ratio. A – part of the spectra (1–10) decreases with [DNA] enhancement (at all modes of binding); B – part of the spectra (11–23) increases with [DNA] enhancement (at intercalation mode of binding of AO with DNA).

Another mode, most apparently, corresponds to the external binding of this ligand to DNA. The most probable one is electrostatic mode since AO in solutions is cationic intercalator. This mode corresponds to external binding of this ligand to DNA phosphate groups, which occurs in the cases of EtBr and MB. However, the possibility of stacking-like orientation of ligand chromophore rings to each other without interaction between themselves (lack of dimerization) is not excluded. It may lead to relevant decreasing of absorption at λ=490 *nm* (curves 1–10, Fig. 3, A).

Conclusion. Thus, based on the obtained data, it can be concluded that the presence of IP in differential absorption spectra (also absolute) indicates that there are initial and final forms of the same compound that have optic characteristics significantly different from each other, besides there is an intermediate form as well at which ligand bound and free molecules have similar absorption. These two states may be realized due to free, intercalated and semi-intercalated molecules correspondingly in the case of EtBr, since the latter being in bound state possess some degree of freedom as well. In the absence of total intercalation in the case of MB, bound and free molecules of ligand have close optic characteristics, which conditions the lack of real IP. In the case of AO two states exist – intercalated (with low degree of freedom) and free (molecules bound by electrostatic mode can be considered as being in free state), in consequence of which the intermediate form is absent and the isosbestic or pseudo-IP is not formed in differential absorption spectra of AO–DNA complexes.

The obtained data also indicate that the presence of IP probably is the result of simultaneous performance of several binding modes, moreover, this point is not intercalation criterion of ligand molecules into DNA.

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