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ANALYSIS OF FLUORESCENCE SPECTRA OF COMPLEXES OF METHYLENE BLUE WITH DNA

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The study of methylene blue (MB) binding to DNA has been carried out by fluorescence spectroscopy method. It was shown that on the fluorescence spectra of MB and its complexes with DNA two peaks exist at λ=583 nm and λ=682 nm wavelengths. Peak at λ=583 nm enhances with increasing of DNA concentration, but at λ=682 nm it decreases. The comparison of obtained data with the results received for DNA-EtBrcomplexes shows that MB binds to DNA by semi-intercalative and electrostatic modes, despite the fact that this ligand is an intercalator.

DNA – methylene blue – fluorescence spectrum – binding mode – intercalation – semi-intercalation – electrostatic binding

Կատարվել է ԴՆԹ-ի հետ մեթիլենկապույտի (ՄԿ) կապման ուսումնասիրություն ֆլյուրեսցենտային սպեկտրասկոպիայի մեթոդով: Ցույց է տրվել, որ ՄԿ-ի և ԴՆԹ-ի հետ նրա կոմպլեքսների ֆլյուորեսցենտային սպեկտրների վրա առկա է երկու գագաթ՝ λ=583 նմ և λ=682 նմ ալիքի երկարությունների դեպքում: λ=583 նմ ալիքի երկարության դեպքում գագաթն աճում է ԴՆԹ-ի կոն-ցենտրացիայի մեծացմանը զուգընթաց, իսկ λ=682 նմ դեպքում՝ այն նվազում է: Ստացված տվյալների համեմատումը ԴՆԹ-ԷԲ կոմպլեքսների համար ստացված արդյունքների հետ ցույց է տալիս, որ ՄԿ-ն ԴՆԹ-ի հետ կապվում է կիսաինտերկալյացիոն և էլեկտրաստատիկ եղանակներով՝ չնայած այն հանգամանքին, որ այս լիգանդը ինտերկալյատոր է:

ԴՆԹ – մեթիլենկապույտ – ֆլյուորեսցենտային սպեկտր – կապման եղանակ – ինտերկալյացիա – կիսաինտերկալյացիա – էլեկտրաստատիկ կապում

Проведено исследование связывания метиленового синего (МС) с ДНК методом флуоресцентной спектроскопии. Показано, что на спектрах флуоресценции МС и его комплексов с ДНК проявляются два пика: при λ=583 нм и λ=682 нм. Пик при λ=583 нм возрастает по мере увеличения концентрации ДНК, а при λ=682 нм – уменьшается. Сравнение полученных данных с результатами, полученными для ДНК-БЭ комплексов, указывает, что МС связывается с ДНК полуинтеркаляционным и электростатическими способами, несмотря на то что этот лиганд является интеркалятором.

ДНК – метиленовый синий – спектрофлуоресценции – способ связывания – интеркаляция – полуинтеркаляция – электростатическое связывание

DNA in the cell is surrounded by different non organic and organic molecules that may form complexes with it at functioning. From this point of view the studies of DNA complexes with different compounds (ligands) have a large interest since they permit revealing different aspects of the molecular mechanism of the effect of

biologically active compounds, including drug compounds, on DNA structure and function [8, 12].

According to the interaction type ligands are divided into intercalators (ethidium bromide (EtBr), methylene blue (MB) etc.) and groove binding ligands (Hoechst 33258, netropsin etc.). Intercalators are inserted into the plane of nucleotide pairs and invoke structural reconstructions in DNA which may be reflected on spectral characteristics at complex-formation process with DNA [1, 3-5, 7-13].

At the investigations of complex-formation of different ligands with DNA fluorescence spectroscopy is one of more informative methods which allow to carry out measurements at significantly low concentrations of reagents. Moreover in the case of several ligands, binding to DNA, this method permits carrying out both qualitative and quantitative analyses and determining different characteristics of interaction [1,5,9]. Besides it was revealed that in the case of some ligands fluorescence is registered at the binding by only one mode (intercalation or groove binding) which is important for finding out the binding mechanisms of low-molecular compounds with DNA [1,2,5,6,9,14-17]. Among ligands binding to DNA and having an applicative value MB is important. Literature data indicate that MB is an intercalator and has an analogous to EtBr structure. It may interact with DNA by different mechanisms depending on its nucleotide sequence, solution ionic strength, MB/DNA concentration ratio [4,7,10,11]. From this point of view to reveal the binding mechanisms of this ligand with DNA, a comparison of several characteristics of DNA-MB complexes with analogous characteristics of well-known models, particularly of DNA-EtBr complexes, may become one of applied approaches.

The aim of presented work is the study of MB binding with DNA by fluorescence spectroscopy method and the comparison of obtained results with the data obtained for DNA-EtBr complexes.

Materials and methods. Calf thymus DNA (ultrapure) “Sigma” (USA), MB “Aldrich” (USA), EtBr “Serva” (Germany), NaCl, Na-citrate (ultrapure) were used in this work. All preparations were used without further purification. Concentrations of used preparations were determined by absorption spectroscopy method, using the following extinction coefficients: ε_{260}=6600 $M^{-1}cm^{-1}$ for DNA, ε_{664}=76000 $M^{-1}cm^{-1}$ for MB, ε_{480}=5800 $M^{-1}cm^{-1}$ for EtBr. The investigations were carried out at 0.02 M Na^+ ionic strength of the solution and 25^0C.

Concentrations of used preparations were measured on spectrophotometer UV VIS Unicam SP8-100 (England), fluorescence spectra were registered on spectrofluorometer Varian Cary Eclips Fluorescence Spectrophotometer (Australia) using quartz cuvettes with 3 ml volume with 1 cm optic pathway length. Titration of the solutions of the studying samples was carried out by micropipette with 10μl volume from “Hamilton” (USA).

To obtain fluorescence spectra the concentration of MB was remained constant in the solution, DNA concentrationincreases by the titration process. Fluorescence spectra of MB and its complexes with DNA were measured in $500\leq\lambda\leq800$ nm wavelength interval at λ=290 nm excitation wavelength. MB fluorescence maximums correspond to λ=583 and λ=682 nm. Fluorescence spectra of EtBr and its complexes with DNA were measured in $400\leq\lambda\leq700$ nm wavelength interval at λ=480 nm excitation wavelength. EtBr fluorescence maximum corresponds to λ=590 nm. During titration MB fluorescence maximums increase at λ=583 nm and decrease at λ=682 nm with DNA concentration enhancement. In the case of EtBrfluorescence maximums increase at λ=590 nm up to boundary values of r, then the fluorescence intensity of DNA-EtBr complexes remains constant despite decreasing of the values of r.

Results and Discussion. For revealing of MB binding mechanisms with DNA the fluorescence spectra of MB and its complexes with DNA in $500\leq\lambda\leq800$ nm wavelength change interval have been obtained at excitation wavelength λ=290 nm

and presented on Figure 1. It is obvious from fig.1 that two peaks are shown on fluorescence spectra. In average at analogous investigations the excitation is carried out at wavelength near to the emission longest wavelength [4].

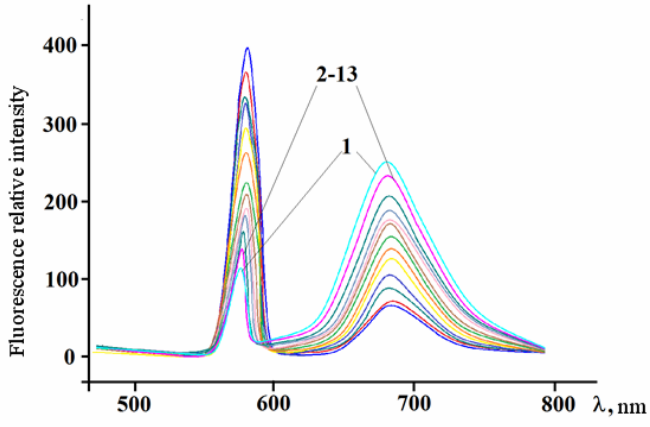

Figure 1. Fluorescence intensity of pure MB (1) and DNA-MB complexes (2-13). The curve 1 corresponds to fluorescence intensity spectrum of pure MB. The curves 2-13 correspond to fluorescence intensity spectra of DNA-MB complexes with the following concentrations – 3.2; 6.8; 9.6; 11.2; 12.8; 16.0; 20.8; 24.0; 32.0; 35.2; 40.0 and 48.0 μM/l respectively. MB initial concentration was 1.6 μM/l; pH=7.0, t=25^{0}C, μ=2.0·10^{-2} M, λ_{ex}=290 nm.

Particularly at MB excitation by the wavelength corresponding to the absorption maximum (λ=664 nm), the fluorescence peak is registered at λ=682 nm. Moreover at excitation by λ=290 nm, with the peak at λ=682nm one more shorter wavelength peak is performed at λ=583 nm, despite the fluorescence spectra of organic compounds are single-band if there is no excimerization process [5,9]. Peaks displaying at the excimerization are shifted to the longer wavelengths compared with the main peak. At the same time, the excimerization usually takes place at either relative high concentrations of ligand (C>10-4mol/l) or in the presence of matrix (for example DNA) at lower concentrations of ligand (C>10-5mol/l). At the chosen MB concentrations (1.6 μM/l) dimerization or excimeri-zation are not obtained in the presence or absence of DNA (long wavelength peaks are absent); consequently the presence of two emission peaks indicates the peculiarities of MB fluorescence spectrum. During the experiment with DNA concentration increasing the fluorescence intensity of complexes decreases compared with MB fluorescence intensity λ=682 nm [14]. This fact means that with DNA concentration increasing in the solution a quenching of MB fluorescence is observed at λ=682 nm [4]. In the present work it was shown that MB fluorescence quenching at complex-formation with DNA is conditioned by ligand molecule intercalation into macromolecule double-stranded structure, as a consequence of which, most probably, a quenching of MB fluorescence by DNA molecules takes place. To reveal MB binding mode to DNA MB fluorescence quenching was determined by the formula of Stern-Volmer [4].

$$\frac{F_0}{F} = 1 + K_{SV}\left[DNA\right] \qquad (1)$$

where F0 and F are MB fluorescence intensities in the absence and in the presence of DNA quencher respectively, KSV – Stern-Volmer quenching constant. The dependence curve of F_0/F on DNA increasing concentration is presented on Figure 2. It is obvious from Figure 2, that F_0/F curve shows a linear dependence on DNA increasing concentration and the quenching constant – K_{SV}=4.2·10^4 l/mol. This is a consequence of

the fact that the decreasing of intensities of complexes compared with MB analogous value takes place at λ=682 nm. Despite the fact that this result is in good coincidence with literature data, MB fluorescence quenching by DNA at λ=682 nm may be the result of significant contribution of electrostatic mode at high ratios of MB/DNA which results in fluorescence quenching. At the same time the increasing of MB fluorescence intensity takes place at λ=583 nm at DNA concentration enhancement in the solution which is the result of the binding intercalative mechanism performance. Moreover the quenching observed at λ=682 nm may occur in the case when ligand molecules are not fully screened from water molecules (quenchers). Consequently, we assume that semi-intercalation is the most probable binding mechanism.

The maintenance of this may be the experimental fact that in the case of EtBr the fluorescence intensity enhances about 20-30 times at the intercalation into DNA when λ=590 nm [14]. It is necessary to mention that in the case of EtBr only one peak is formed on fluorescence spectra the amplitude of which increases with DNA concentration enhancement in the solution and starting from certain ratios of EtBr/DNA (r≥2, where r=[EtBr]/[DNA]) reaches the saturation [14]. The other important peculiarity is that the fluorescence spectra of EtBr and its complexes with DNA are registered at λ=590 nm when the excitation wavelength is – λ=480 nm, while MB absorbs and fluoresces in the 600≤λ≤700 nm interval (λ=664 nm and λ=682 nm respectively). Fluorescence intensity increasing is conditioned by the intercalation of EtBr molecules into the plane of DNA bases [5,9]. This is also indicated by the fact that in intercalated state ligand molecules are screened from water molecules that are fluorescence active quenchers [9]. Beginning from r≥0.2 values the fluorescence intensity practically does not change, which means that all ligand molecules are in intercalated state [14].

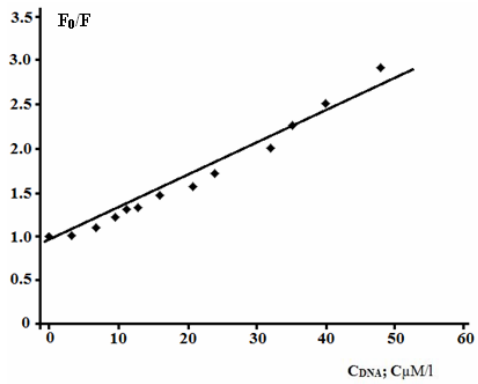

Fig. 2. The dependence curve of MB fluorescence quenching by DNA molecules. MB concentration is 1.6 μM/l, pH=7.0, t=25^{0}C, μ=2.0·10^{-2} M.

It was shown in [14,17] that EtBr binds to double-stranded DNA by three modes – intercalative, semi-intercalative and electrostatic. It should be noted that the semi-intercalative mode is revealed by the comparison of binding curves in Scatchard's coordinates obtained from fluorometric and absorption data analysis. It was revealed that EtBr binding curves with DNA, constructed according to fluorometric and absorption data, differ at low ratios of EtBr/DNA [14]. In this work it was shown that besides intercalative (fluorescence) one, EtBr forms semi-intercalative (non-fluorescence) complex which is hidden under the intercalative complexes when investigations of EtBr interaction with DNA are carried out by only one (absorption or fluorescence) method. In the case of MB the comparative investigation of these

methods was carried out in [16]. From the obtained in this work data it was shown that at low ratios of MB/DNA the binding curves received from fluorometric and absorption studies coincide with each other [16].

Therefore the obtained data indicate that the binding peculiarities of ligands-intercalators with DNA are reflected on spectral characteristics of its complexes with DNA. On the other hand by the comparison of absorption and fluorescence spectra of EtBr and MB complexes with DNA the mechanisms of their binding may be revealed.

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