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STUDY OF THE INTERACTION OF SINGLE-STRANDED POLYRIBOADENYLIC ACID WITH ETHIDIUM BROMIDE AND METHYLENE BLUE

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The study of the interaction of phenothiazine dye methylene blue (MB) and phenantridine dye ethidium bromide (EtBr) with synthetic single-stranded (ss)-poly(rA) has been carried out at the ionic strength of the solution 0.1 *mol*, in wide interval of ligand/phosphate ratio change by the method of absorption spectroscopy. The binding curves of MB and EtBr with ss-poly(rA) were obtained. MB was shown to interact with this polynucleotide cooperatively, while for EtBr the cooperativity was not revealed. The binding parameters of MB and EtBr with ss-poly(rA) were determined – association constant *K* and base number *n* per binding site. It was shown that both ligands bind to ss-poly(rA) at least by two modes, though the affinity of MB to the polynucleotide is higher by an order, than that of EtBr.

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***Keywords*:** methylene blue, ethidium bromide, ss-poly(rA), absorption spectra, Scatchard's curve, binding constant.

Introduction. Studies on the interaction of biologically active compounds with nucleic acids are actual topic to date and are directed to understanding of molecular aspects of such interactions, as well as for elaboration of effective and non-toxic therapeutic measures in application goals. Nucleic acids (NA) present a special importance as targets for multiple ligands, since the nucleic acid polymorphism *in vivo* plays the certain role for elaboration of new, specific therapeutic agents to NA. From this point of view, small molecule design, interacting with non-canonic structures of NA nowadays is an active sphere [1, 2].

Among different small molecules phenothiazine compound methylene blue (MB) and phenantridine compound ethidium bromide (EtBr) present a special interest. MB is a photosensibilizer and widely is used as an optic probe in many biological systems [3–9]. MB is often used as an electrochemical indicator for DNA-biosensors [10–15]. EtBr is a phenantridine dye – analogous to MB. EtBr molecule is flat and similar to DNA base pair. Due to its chemical structure, this ligand can intercalate (or be inserted) into single-stranded (ss-), double-stranded (ds-) as well as three-stranded (ts-) or four-stranded (fs-) structures of NA. Binding immediately to DNA, EtBr inhibits DNA-polymerase. There were carried out studies on animals

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to estimate EtBr as potential anti-tumor chmeitherapeutic agent and it was shown that EtBr acts as toxin for topoisomerase I and for several anti-tumor preparations [16, 17].

Among ss-NA polyriboadenylic acid – poly(rA) has a special biological value. It plays an important role in mRNA functioning and gene expression. It was established that poly(rA) exists as a single wrapped helix, stabilized by pair stacking interactions between neighboring bases at physiological pH and temperature [18–21]. Though, poly(rA) is flanking with many mRNAs and is a special target for numerous ligands [4].

Taking into account the important biological value of poly(rA), in this work we are aimed at studying the affinity of two aromatic compounds – classical intercalator EtBr and non-classical intercalator MB toward ss-poly(rA) on the basis of absorption spectra and binding curves in Scatchard's coordinates.

Materials and Methods. In this work ultra-pure synthetic polynucleotide poly(rA) (P9403), MB, EtBr ("Sigma", USA), bistilled water, NaCl, Na-citrate, Na_2EDTA (content in working interval was 10^{-5} *M*) were used in experiments. Preparation concentrations were determined spectrophotometrically, using the following extinction coefficients: ε_{665}=76000 $M^{-1}cm^{-1}$ for MB [4], ε_{480}=5800 $M^{-1}cm^{-1}$ for EtBr [16], ε_{257}=10500 $M^{-1}cm^{-1}$ for poly(rA) [22]. Average molecular mass of ss-polynucleotide was 800÷1000 *kDa*. All experiments were carried out in 0.5×SSC (1×SSC contains 0.15 *M* NaCl, 0.015 *M* Na-citrate), ionic strength – ~0.1 *M*, pH≈7.0.

Spectrophotometric measurements of the samples were carried out on UV-VIS Perkin Elmer Lambda 365 spectrophotometer (USA) in quartz cuvettes with optic pathway length 1 *cm*. All experiments were carried out at room temperature ~ 22°C.

At spectrophotometric titration the ligand concentration remained constant, macromolecule concentration was increased in interval $0 \le r \le 20$ (r=P/D, where P is phosphate group concentration of polynucleotides, D is ligand concentration). Absorption changes at λ_{max} (λ_{max}=665 *nm* for MB and λ_{max}=480 *nm* for EtBr) were registered at each ratio up to such values of P/D, at which the spectra changes were practically unregistrable. Spectral changes, observed at absorption changes, were used to calculate the binding constants in Scatchard's coordinates (dependence of r/C_f on r). Scatchard's curve in the case of MB was analyzed for the cooperative binding by the equation of McGheee and von Hippel [4].

$$\frac{r}{C_f} = K(1-nr)\left[\frac{(2\omega+1)(1-nr)+r-R}{2(\omega-1)(1-nr)}\right]\cdot\left[\frac{1-(n+1)r+R}{2(1-nr)}\right]^2,$$

$$R = \left([1-(n+1)r]^2 + 4\omega r(1-nr)\right)^{\frac{1}{2}}, \quad (1)$$

where C_f is concentration of ligand free molecules, r is number of bases, complexed with ligand molecules, K is binding constant, n is number of base pairs, per binding site, ω is cooperativity coefficient. In the case of EtBr, the analysis of the binding curves was carried out for non-cooperative binding by the Eq. (2) [16]

$$\frac{r}{C_f} = K(1-nr)\cdot\left[\frac{1-nr}{1-(n-1)r}\right]^{n-1}, \quad (2)$$

which was linearized by Eq. (3):

$$\frac{r}{c_f} = K(1 - (2n - 1)r). \quad (3)$$

Results and Discussion. The absorption spectra of MB (1) and its complexes with ss-poly(rA) (2–23) are presented in Fig. 1. As it is obvious, the absorption spectra of MB in visible region (500–750 *nm*) significantly decrease at sufficiently low concentrations of the polynucleotide, then at further enhancement of ss-poly(rA) concentration the absorption spectra of the ligand decrease a little. It indicates the strong the strong intermolecular interaction due to the effective suoerposition of π-electronic clouds of MB and ss-poly(rA) leading to occurrence of hypochromic and bathochromic effects. From the presented Fig. 1 it is also obvious that in the absorption spectra of the complexes MB–ss-poly(rA) an isosbestic or pseudo-isosbestic point is not formed, as it was in the case of ds-NA [23]. The significant hypochromic effect of the complexes along with concentration enhancement of ss-poly(rA) in the solution, indicates the high affinity of this ligand toward ss-structure of the polynucleotide.

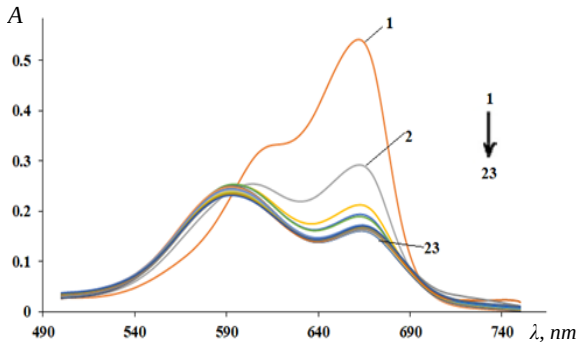

Fig.1. Absorption spectra of MB (curve 1) and its complexes with ss-poly(rA) (curves 2–23). Concentration ratio – *r* = ligand/phosphate = 1/20.

The absorption spectra of EtBr (curve 1) and its complexes with ss-poly(rA) (curves 2–21) are presented in the Fig. 2. It is obvious that the spectra exhibit a weak hypochromism and little bathochromic shift. Though, in the absorption spectra a pseudo-isosbestic point is practically formed. It indicates that the ligand molecules are in spectroscopically registerable free and bound states. It should be mentioned that poly(rA) in the solution at physiological values of pH is in ss-state with high stacking degree between neighboring bases [4], and EtBr may bind to not only ds-, but also ss-NA by intercalation mechanism. Obviously, the binding to ss-poly-nucleotides by complete intercalation is practically impossible, though, the semi-intercalation is not excluded [24].

In the bound state EtBr molecules have other optic characteristics, compared to the free one. Proceeding from this we assume that chromophore part of the ligand molecule is in interaction with monomeric rings of the polynucleotide, due to which the pseudoisosbestic point is formed in the absorption spectra of the complexes. It is contributed by the fact that hydrophobic shifting of the ligand chromophore groups from the polar water medium to much less polar one that is to the space between neighboring adenillic residues of ss-poly(rA) is more beneficial. Meanwhile, it

cannot be excluded the electrostatic interaction of positive charged MB and EtBr molecules with negative phosphate groups of the polynucleotide. The contribution of this binding mode into absorption of the formed complexes is irrelevant.

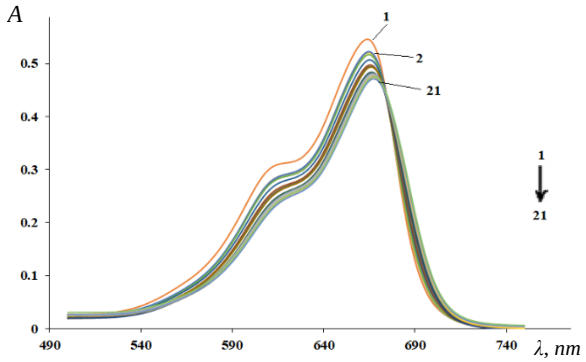

Fig. 2. Absorption spectra of EtBr (curve 1) and its complexes with ss-poly(rA) (curves 2–21). Concentration ratio – r = ligand/phosphate = 1/20.

Spectrophotometric titration data were analyzed by Scatchard's curves [4, 16]. Scatchard's curve (dependence of r/C_f on r) reflects the cooperative binding of MB with ss-poly(rA), since the curve is bulging at sufficiently low values of r, and at higher values of this variable, the curve sharply decreases, then weakly alters at higher values of r (Fig. 3). This curve was analyzed by the Eq. (1) to estimate the binding parameters – binding constant K and number of bases n per binding site [4]. Values of the binding parameters of MB to ss-poly(rA) are presented in Table.

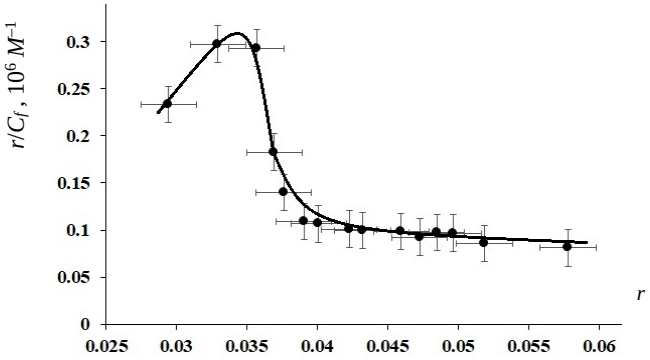

Fig. 3. Binding curve of MB to ss-poly(rA) in Scatchard's coordinates (dependence of r/C_f on r).

Values of K and n of the complexes of MB and EtBr with ss-poly(rA) (s is strong, w is weak)

Ligand	K_s, 10^6 M^{-1}	K_w, 10^6 M^{-1}	n_s	n_w
MB	5.5±1.0	0.13±0.6	25.0±1.0	8.0±0.5
EtBr	0.36±0.5	0.0086±0.0005	8.0±1.0	3.0±0.5

Analogous analysis of the absorption spectra at the interaction of EtBr with ss-poly(rA) gives non-linear curve of Scatchard, which sharply decreases at low values of r, but at higher values of this variable the curve decreases a little (Fig. 4). Non-linear curves in Scatchard's coordinates indicate that the interaction is anti-cooperative and realized by the binding site exclusion principle [2] and analyzed by the Eq. (2) or the binding by more, than one mode occurs with different binding constants [24]. Because both MB and EtBr in the solution are in cationic form and poly(rA) is a poly-anion, the one of the binding modes should be electrostatic. This mode is weaker and characterized by low values of the binding constant – K_w. At the same time the linear part of the binding curves, corresponding to low values of r, has a high slope and characterizes stronger binding of both ligands to ss-polynucleotide. Analysis of this region by Eq. (1) in the case of MB and by Eq. (3) (linearized curve 2) [24] gives high values of K_s (see Table).

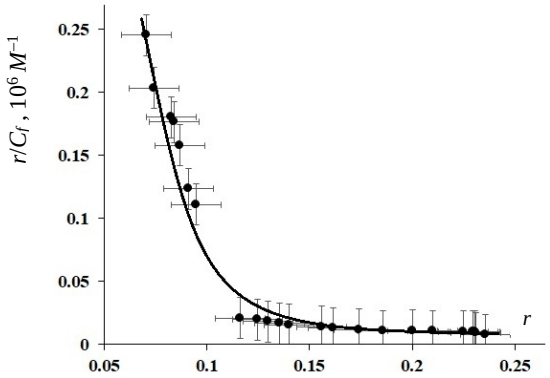

Fig. 4. Binding curve of EtBr with ss-poly(rA) in Scatchard's coordinates (dependence of r/C_f on r).

Therefore, non-linearitiy of the binding curves of MB and EtBr with ss-poly(rA) in Scatchard's coordinates is conditioned by at least two binding modes of these ligands with ss-polynucleotide.

From the table data it is revealed that the binding constant of MB with ss-poly(rA) by the strong mode is higher by more, than an order, than the binding constant of EtBr with this polynucleotide. In the case of MB the value of K_s is higher than K_w by 1.5 times. In the case of EtBr the ratio $K_s/K_w \approx 40$. The obtained data are practically in good correspondence to literature results [4, 16, 24]. Moreover, the data, obtained for the complexes of EtBr with ss-poly(rA) are in good accordance to the analogous results, referring to the complexes of EtBr with ds- and ss-DNA.

At the same time the high value for n_s was unexpected at MB interaction with ss-poly(rA). Relevantly higher value was obtained for n_w as well, while in the case of EtBr the values of n_s and n_w are in good accordance to analogous ones for the complexes of EtBr with ds- and ss-DNA.

High values of K at MB binding to ss-poly(rA) resulted from the cooperative interaction and reflect the high affinity of this ligand to polyadenilic acid in ss-state. In the case of EtBr as well the binding constant value by the strong mode is high, though, its affinity to this polynucleotide is less pronounced, as compared to MB. These arguments are maintained by the values of n as well: for MB on this

polynucleotide there is more confined number of adsorption centers with high affinity to them, while for EtBr the number of these centers is less confined. Practically, the polynucleotide structure is more available for semi-intercalation of EtBr, than for MB, although the latter binds with much higher affinity with these centers.

Conclusion. Based on the obtained data we conclude that the semi-intercalation is the main binding mode of MB and EtBr to ss-poly(rA). Another important conclusion is that MB shows high affinity to ss-poly(rA) with binding constant value order about ~10^6 M^{-1}. Meanwhile, the interaction of MB with this polynucleotide is cooperative, which is resulted from the conformational change of the polynucleotide structure, which in turn leads to high values of K and n [4]. Analogous effect for EtBr is absent, which indicates that the structure of the polynucleotide is more available for this ligand and the binding centers are independent on each other, than for MB.

Therefore, the obtained data permit assuming that polyadenilic tails in mRNA can be a good target for the certain ligands, which will allow to control or modulate cellular activity via such compounds. The obtained data also can lie at the basis of design and screening of biologically active compounds, binding to NA.

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Զ. Հ. ՄՈՎՍԻՍՅԱՆ

ՄԻԱՇՂԹԱ ՊՈԼԻՌԻԲՈԱԴԵՆԻԼԱԹԹՎԻ ՓՈԽԱԶԴԵՑՈՒԹՅԱՆ ՈՒՍՈՒՄՆԱՍԻՐՈՒԹՅՈՒՆԸ ԷԹԻԴԻՈՒՄԻ ԲՐՈՄԻԴԻ ԵՎ ՄԵԹԻԼԵՆԱՅԻՆ ԿԱՊՈՒՅՏԻ ՀԵՏ

Ուսումնասիրվել է ֆենոթիազինային ներկանյութ մեթիլենային կապույտի (ՄԿ) և ֆենանտրիդինային ներկանյութ էթիդիումի բրոմիդի (ԷԲ) փոխազդեցությունը սինթետիկ միաշղթա (մշ) պոլինուկլեոտիդ poly(rA)-ի հետ լուծույթի 0,1 *մոլ* իոնական ուժի պայմաններում, լիգանդ/ֆոսֆատ հարաբերության փոփոխության լայն միջակայքում, կլանման սպեկտրոսկոպիայի մեթոդով: Ստացվել են ՄԿ-ի ԷԲ-ի կապման կորերը մշ-poly(rA)-ի հետ: Բացահայտվել է, որ ՄԿ-ն այս պոլինուկլեոտիդի հետ փոխազդում է կոոպերատիվորեն այն դեպքում, երբ ԷԲ-ի դեպքում կոոպերատիվություն չի հայտնաբերվում: Ստացված են ՄԿ-ի և ԷԲ-ի կապման պարամետրերը մշ-poly(rA)-ի հետ՝ կապման հաստատունը *K* և մեկ կապման տեղին ընկնող հիմքերի թիվը *n*: Ցույց է տրվել, որ երկու լիգանդն էլ մշ-poly(rA)-ի հետ կապվում են առնվազն երկու եղանակներով, ընդ որում, ՄԿ-ի խնամակցությունը պոլինուկլեոտիդի նկատմամբ մեկ կարգով ավելի մեծ է, քան ԷԲ-ի խնամակցությունը:

З. О. МОВСИСЯН

ИССЛЕДОВАНИЕ ВЗАИМОДЕЙСТВИЯ ОДНОЦЕПОЧЕЧНОЙ ПОЛИРИБОАДЕНИЛОВОЙ КИСЛОТЫ С БРОМИСТЫМ ЭТИДИЕМ И МЕТИЛЕНОВЫМ СИНИМ

Проведено исследование по взаимодействию фенотиазинового красителя метиленового синего (МС) и фенантридинового красителя бромистого этидия (БЭ) с синтетическим одноцепочечным (оц) полинуклеотидом poly(rA) при ионной силе раствора 0,1 *моль* в широком интервале изменения соотношения лиганд/фосфат методом абсорбционной спектроскопии. Получены кривые связывания МС и БЭ с оц-poly(rA). Выявлено, что МС с этим полинуклеотидом взаимодействует кооперативно, в то время как в случае БЭ кооперативность не обнаруживается. Получены параметры связывания МС и БЭ с оц-poly(rA) – значения констант ассоциации *K* и числа оснований *n*, приходящихся на одно место связывания. Выявлено, что оба лиганда с оц-poly(rA) связываются по крайней мере двумя способами, при этом сродство МС к полинуклеотиду на порядок больше, чем сродство БЭ.