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ETHIDIUM BROMIDE BINDING TO POLY(G): DEPENDENCE ON Na^+-CONCENTRATION

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In the current work the investigation of interaction between polyguanylic acid and ethidium bromide (EtBr) at different ionic strengths and at three different temperatures has been carried out. Based on absorption spectra of formed complexes, adsorption isotherms were constructed and binding constants were determined. Thermodynamic parameters of this interaction were determined as well. Obtained data show that in four-stranded structure, the "classical" intercalation becomes impossible and the only possible binding mode remains semi-intercalation. It was also shown that thermodynamic parameters of interaction by semi-intercalation mode do not strongly depend on the ionic strength of solution.

Keywords: EtBr–$[poly(G)]_4$ complexes, ionic strength of solution, semi-intercalation.

Introduction. Literature data indicate that single-stranded poly(G) *in vitro* forms four-stranded (fs) structure [1–5]. In some works it was shown, that fs structures may form also *in vivo*, particularly at the telomeric regions, where a large number of guanine bases are contained [6–8]. A considerable amount of literature was published about the fs structures of guanine-rich nucleic acids and every new study consolidates the idea that guanine-rich regions demonstrate an exceptional structural polymorphism. However, little attention has been devoted to the structure-formation of synthetic polyguanylic acid (poly(G)) in aqueous solution. Poly(G) in solution may form single- and double-stranded (ds) helical structures, as well as fs structures, which is called G-quadruplex [7]. In fs structure of poly(G), separate areas of chain can be arranged towards one another in different ways [2], and their structure greatly depends on the concentration of univalent ions in the environment [5, 6, 8, 9].

In recent years several studies have shown that the fs structures can be used as important targets for anticancer therapies, due to their biological significance [6, 7, 10]. From this point of view, the binding of low-molecular compounds, which interact with ds helical nucleic acids with intercalative or non-intercalative binding modes on G-quadruplexes, has been carried out. It was shown that classic intercalator ethidium bromide (EtBr) molecules may interact with G-quadruplexes as well [11]. Moreover, in the case of EtBr binding constant values are bigger then the values of

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those for G-quadruplex interaction with antitumorale drugs netropsin and distamycin A that interact with DNA via non-intercalative binding mechanism, and binding constant values are smaller by almost three orders ($\approx 8\cdot10^4\ M^{-1}$). Due to this fact, a great exothermic binding enthalpy is registered (≈ -10.8 *kcal /mol*) [12].

Quite recently, the poly(G) structure has been investigated by electrophoresis methods, which is remarkably susceptible to conformational states of nucleic acids. The studies were carried out at several ionic strengths and it has been revealed, that if ionic strength is greater than 0.01 *M*, poly(G) forms mainly fs structure [13]. Although in some studies there was mentioned the formation of fs structures, mono-, di- and trimetric structures are, apparently, possible.

It is apparent that besides guanines' biological importance in RNA chain, they also play an important role in the formation of fs structure. In this context, it is worthwhile to explore the peculiarities of biologically active compounds binding to fs structures of synthetic poly(G) at different ionic strength conditions.

Therefore, the investigation of EtBr complex-formation with poly(G) particularly determining the changes of thermodynamic parameters due to binding processes, and by evaluating the dependence of those parameters on the ionic strength of solution, we may assume how the chains may flex in aqueous solution at different ionic strengths. Our earlier study [14] has been dedicated to the complex-formation of EtBr with $[poly(G)]_4$ under physiological ionic strength conditions, so, the main goal of this paper was to examine the same phenomenon at several ionic strengths.

Materials and Methods. In the experiments poly(G) ("Sigma", USA), ethidium bromide ("Serva", Germany), NaCl and Tris ("Sigma", USA) were used. The concentration of poly(G) and EtBr were determined by spectrophotometric measurement with the following extinction coefficients: ε_{260}=9900 $M^{-1}cm^{-1}$ for poly(G) and ε_{480}=5800 $M^{-1}cm^{-1}$ for EtBr respectively. The studies of EtBr absorption on poly(G) were performed at three different ionic strength conditions: 0.11, 0.31 and 0.51 *M* It is important to emphasize that in mentioned external conditions poly(G) mainly has fs structure. Further in the text for polyoxyriboguanine $[poly(G)]_4$ abbreviation will be used.

Absorption spectra were obtained by measuring on double beam spectrophotometer PYE Unicam SP8-100 (England).

Low-molecular compounds, which contain planar cyclic groups (e.g. EtBr), may form self-assemblies in aqueous solution. Particularly in the case of quantitative study of interactions of these molecules with nucleic acids, it is important to carry on the experiments at concentration, at which we can ignore the process of dimerization. It is known, that at 0.1 *M* Na^+ ionic strength and at 300.15*K* temperature dimerization constant (K_D) for EtBr is 70 M^{-1} [15]. The process of dimerization may be described by the equation $2C_M \Leftrightarrow C_D$, where C_M and C_D are concentrations of monomers and dimmers respectively, and $C_D=K_DC^2_M$. If concentration of EtBr in solution is C_0, then

$$C_0 = C_M + 2C_D \quad \text{or} \quad C_0 = C_M + 2K_DC_M^2 . \tag{1}$$

Inserting values of C_0 and K_D in Eq. (1) the value of C_M could be obtained. In our investigations maximum concentration of EtBr was $C_0 \le 1.4\cdot10^{-4}\ M$ and

$C_D<C_0 \cdot 0.2\%$, so, consequently in the case of mentioned EtBr concentrations the process of self-assembly may be ignored.

Results and Discussion. EtBr interaction with $[poly(G)]_4$ at mentioned ionic strengths of solution was studied by spectrophotometric method. The absorption spectra obtained at the above mentioned ionic strengths and temperatures were the same as it was in [14], in which spectra were registered at 0.11 *M* ionic strength of solution (hence, the spectra are not provided). The changes in EtBr absorption spectra due to interaction with $[poly(G)]_4$ were evaluated. It is known that nucleic acid does not absorb visible light, accordingly, the changes in absorption spectra at visible wavelengths are caused by the formation of EtBr–$[poly(G)]_4$ complexes at the mentioned ionic strengths.

In order to elucidate what conformational changes undertake fs $[poly(G)]_4$ in EtBr complexes, the dependence of absorption at 480 *nm* wavelength (A_{480}) on poly(G) concentration was measured. The measuring was performed for three different ionic strengths of solution (Fig. 1). It is apparent that at the same changes of relative concentration ratio, alongside with an increase of ionic strength A_{480} the value decreases. Consequently, at different ionic strengths of solution, when C_p/C_0 is the same, poly(G)'s conformational change due to EtBr binding is less.

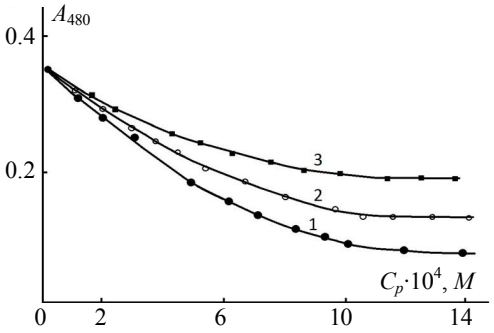

Fig. 1. Change of EtBr absorption at λ=480 *nm* due to the interaction with $[poly(G)]_4$ at 300.15 *K* at different ionic strength, *M* Na^+:
1 – 0.11; 2 – 0.31; 3 – 0.51.
During titration EtBr concentration kept constant ($C_p=1.4 \cdot 10^{-4}$ *M*).

G-quadruplexes demonstrate a specific binding with counterions (particularly with Na^+ and K^+), which penetrate into inner side of fs structure and stabilize G-quadruplex [5]. Such changes are caused by structural variations in $[poly(G)]_4$. Increasing of ionic strength may cause conversion in the chain of $[poly(G)]_4$ or structural changes in folding of fs conformation. Particularly, considering the fact that in fs state the electronegative charge density is regulated by the amount of phosphate groups, so, this structure may be formed only at high ionic strength conditions. The increasing of ionic strength of solution leads to forming more compact structure of $[poly(G)]_4$. The last one will significantly effect on the ligand binding. This phenomenon has major contribution in decreasing of absorbance at 480 *nm* wavelength of EtBr–$[poly(G)]_4$ complexes, while ionic strength of solution was increased.

The most important parameters describing complex-formation process are the binding constant (K) and the parameter, which defines the stoichiometry of complex (n), when binding sites are saturated. These two parameters were determined from the absorption spectra. The concentration values of free (C_f) and bound ligand (C_b) molecules are:

$$C_f = C_0(A - A_b)/(A_f - A_b), \quad C_b = C_0 - C_f, \tag{1}$$

where A_f and A_b are the absorption values at maximum wavelength (λ_{max}=480 *nm* for EtBr) of free and bound ligand molecules respectively, and A refer to the absorption of EtBr–[poly(G)]$_4$ complexes at intermediate state. The value of A_b was calculated from the linear extrapolation, when $1/C_p \to 0$: $A = f(1/C_p)$.

Values of C_f and C_b have been obtained from Eq. (1) and the binding isotherm was built. The isotherm was constructed using Scatchard coordinates (the dependence of r/C_f on r), where $r = C_b/C_p$. Using Scatchard coordinates the binding isotherms were built for three ionic strengths at different temperatures by equation, describing the adsorption of low-molecular compounds on nucleic acids [14, 15].

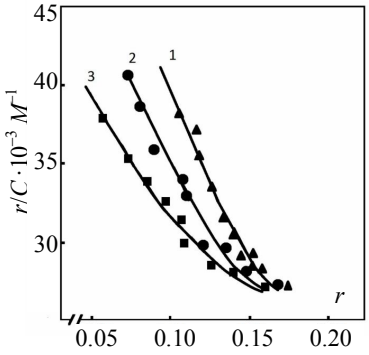

Fig. 2. Binding isotherms built using the obtained data from spectrophotometrical titration of EtBr with [poly(G)]$_4$ at 300.15 *K* at different ionic strength, *M* Na^+: 1 – 0.11; 2 – 0.31; 3 – 0.51.

Binding isotherms determined for EtBr–[poly(G)]$_4$ complexes for three ionic strengths at 300.15 *K* temperature are illustrated in Fig. 2 (we also obtained binding isotherms for the cases of three different temperatures: 300.15, 310.15 and 320.15 *K*). The theoretical curve (solid line) was built through the experimental points and implementing the linear least squares approach. *K* for three different ionic strengths and temperature were computed from the above-stated theoretical curve (see Table).

Using the determined values of *K* the dependence of ln*K* vs 1/*T* was built (Fig. 3). Through the experimental points a line was built, applying the method of linear least squares and ΔS and ΔH values were calculated (see Table).

Some thermodynamic parameters of EtBr–[poly(G)]$_4$ interactions

Ionic strength, *M*	*T*, *K*	*K*, $\cdot 10^{-4}$ M^{-1}	ln*K*	$-\Delta G$, *kcal/mol*	$-\Delta H$, *kcal/mol*	ΔS, *cal/mol·K*
0.11	300.15	5.2	10.86	6.47	2.29	13.9
	310.15	4.8	10.78	6.64		
	320.15	4.1	10.62	6.75		
0.31	300.15	4.65	10.75	6.40	2.18	14.1
	310.15	4.2	10.65	6.55		
	320.15	3.7	10.52	6.68		
0.51	300.15	4.25	10.66	6.35	2.14	14.0
	310.15	3.8	10.55	6.49		
	320.15	3.4	10.43	6.63		

In [14] it was shown that $n \approx 5$ for EtBr binding to [poly(G)]$_4$ interactions and it almost does not depend on the ionic strength of the solution. The data from several studies demonstrate that for EtBr interaction with ds helical nucleic acids

the value of n does not depend on the ionic strength, too [16–18]. One EtBr molecule interacts with a greater number of nitrogenous bases of $[poly(G)]_4$, because polynucleotide's fs structure prevents the intercalation process, and due to it EtBr binds not with 4, but 5 guanines (when binding is saturated).

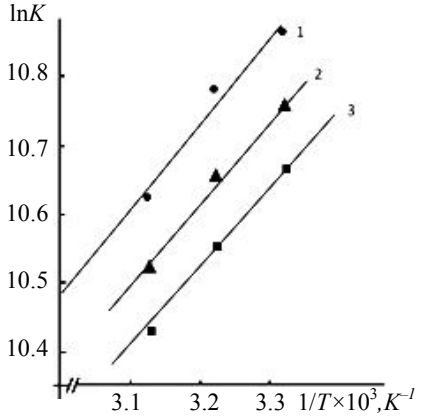

Fig. 3. Dependence of ln$K$ on 1/T, calculated from the adsorption isotherm for EtBr–$[poly(G)]_4$ complexes at three ionic strengths, M: 1 – 0.11; 2 – 0.31; 3 – 0.51.

It is apparent that EtBr molecules during the interaction with $[poly(G)]_4$ do not stack between base pairs, it takes place in the case of ds nucleic acids. This assumption is supported also with the fact that the enthalpy change is much greater for the intercalation process (driving force of intercalation is an enthalpy), since the structure of ds nucleic acid changes remarkably. Particularly, it has been revealed that the nucleic acid helix untwists and it was shown that at the intercalation area a deviation (by 1Å) from the longitudinal axis is registered [19]. From this point of view, there could not be such kind of conformational changes in fs structure, due to the structural peculiarities of this molecule, so, complete intercalation in this case can be excluded. This explanation elucidate, why the enthalpy change reduces. It accentuates the contention that in this case the binding mechanism most probably is semi-intercalation [20–22]. From the data provided in Table it can be seen that the values of ΔG due to binding of EtBr with synthetic ds polyribonucleic acids and fs $[poly(G)]_4$ are very similar, and within the limits of experimental error they can be considered as the same [23]. Calculations show that $\Delta H \approx -2.2$ *kcal*/*mol* and practically do not depend on the ionic strength. Previous studies indicate that for EtBr intercalative interaction with ds helical nucleic acids enthalpy is $\Delta H \approx -(7 \div 8)$ *kcal*/*mol* [17, 23], which is about three times greater than the same value for complex-formation ($\Delta H \approx -2.2$ *kcal*/*mol*) obtained by us. Simultaneously, this research indicates that for the interaction of semi-intercalating anticancer drug mitoxantrone [18, 24] with ds helical nucleic acids $\Delta H \approx -(2.5 \div 3)$ *kcal*/*mol* [25], corresponds with the value derived for EtBr–$[poly(G)]_4$ complex. This value of ΔH is in correspondence to the value of enthalpy changes at EtBr semi-intercalation with ds DNA [20]. Thus, the results allow us to assume that in this case EtBr partially intercalates into fs $[poly(G)]_4$. Summing up, in all probability due to compact folding of fs structures, "classical" intercalation (with an enthalpy change $\Delta H \approx -(7 \div 10)$ *kcal*/*mol*) becomes impossible and the only feasible binding mode becomes semi-intercalation ($\Delta H \approx -(2 \div 3)$ *kcal*/*mol*). The characteristic thermodynamic parameters of semi-intercalation do not strongly depend on the ionic strength of environment, in contrast with the case of intercalative binding.

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