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INTERACTION OF POLY(I) WITH ETHIDIUM BROMIDE

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The interaction of ethidium bromide (EtBr) with polyinosine (poly(I)) has been studied by absorption and fluorescence spectroscopy methods. Our investigations have shown that titration experiments within raising of poly(I) concentration the maximum of absorption in visible region decreases and is shifted toward longer wavelength region. According to the obtained results it is assumed that poly(I) in physiological conditions exists both in single- and double-stranded helices formed contemporaneously. The studies are relevant to understand the polyinosine structure in aqueous-salt solution.

Keywords: poly(I), ethidium bromide, complex-formation, absorption and fluorescence spectra.

Introduction. In recent years, more information has been obtained about the structural peculiarities of helical polynucleotides. It has been revealed that polynucleotides in aqueous solutions and in the membranes may form not only single-stranded structures but also double- and four-stranded structures [1–4]. Several homopolymers, such as poly(I), have biological activity and have practical uses. Poly(I) can induce immune response, particularly single-stranded poly(I) may incite plasminogen and tissue factor production [5]. It binds to cellular receptors and antibodies, thus promoting immune response.

Polyinosinic acid is a synthetic homopolynucleotide, which may exist in different conformations [6]. At low ionic strength, in the absence of magnesium ions, it forms single-stranded structure [6–10]. Simultaneously, at high ionic strength it forms multi-stranded helix [6–13]. By the method of circular dichroism four-stranded conformation of poly(I) was discovered [13]. From this point of view, the structure of polyriboinosinic acid (poly(I)) and possible changes in it are less observed. It is well known that poly(I) in aqueous solution forms either single- or double-helical or four-stranded (quadruplex) structures [1, 4]. Investigations have shown that the pH value, the concentration of alkali ions and temperature of medium are the key factors, which determine poly(I) structure. At room temperature, neutral pH and 0.1 *M* NaCl conditions almost do not form four-stranded structures [4], but a further increase of NaCl up to 1 *M* causes poly(I) to reversibly transit to four-stranded structure.

It is known that during the interaction of double-stranded nucleic acids and synthetic polynucleotides with ethidium bromide (EtBr) in the case of maximal saturation

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one molecule of ligand binds to 4 nitrogenous bases [14–16]. Consequently, through the investigation of synthetic poly(I) interaction with EtBr and by determining the changes in values of thermodynamic parameters for these interaction one may anticipate, which structure will generally form polyriboinosinic acid in physiological conditions.

Materials and Methods. Poly(I), EtBr, NaCl and Tris buffer (all from "Sigma", USA) were used. Concentrations of EtBr and poly(I) were determined spectrophotometrically, using the following extinction coefficients: $\varepsilon_{480} = 5800\ M^{-1}cm^{-1}$ for EtBr; $\varepsilon_{260} = 5000\ M^{-1}cm^{-1}$ for poly(I). The interaction of poly(I) with EtBr were investigated in buffer, which contained 0.1 *M* NaCl and 0.01 *M* Tris, at pH=7.5 and in the temperature interval T=290.15–310.15 *K*. In the mentioned conditions poly(I) did not form four-stranded structure [4].

Absorption spectra were obtained measuring on double beam spectrophotometer PYE Unicam-SP8-100 (England). Fluorescence spectra were obtained on FluoroMax (USA). The excitation wavelength was λ=510 *nm*.

EtBr molecules in solution may form self-assemblies, i.e. dimmers or excimers, so for this reason during quantitative study of the interactions between nucleic acids and EtBr, it is important to carry out the experiments at such EtBr concentrations where the process of self-assembly may be ignored. Calculations have shown that if EtBr concentration is $C_0 \leq 1.4 \cdot 10^{-4}\ M$, then one may ignore self-assembly phenomenon in solution [16].

Results and Discussion. Poly(I) interaction with EtBr was investigated by spectrophotometric and fluorometric methods. Figs. 1 and 2 illustrate the obtained absorption and fluorescence spectra for EtBr and poly(I)–EtBr complexes. It is important to mention that EtBr concentration during titration experiments remains constant.

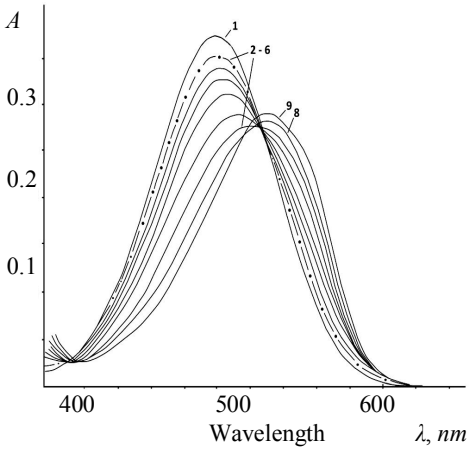

Fig. 1. Change of EtBr absorption spectra due to complex-formation with poly(I) at 300.15 *K*. [Poly(I)] in buffer was continuously growing: C_p=0 (1); $8 \cdot 10^{-4}\ M$ (6); $2.3 \cdot 10^{-3}\ M$ (9). [EtBr] was kept constant during titration, $C_0 = 1.36 \cdot 10^{-4}\ M$.

For this reason, through the entire process of poly(I)'s titration experimental solution was added, which also contained such amount of EtBr that its total concentration in the sample remained constant. As it can be seen from Fig. 1 within raising of poly(I) concentration (C_p) wavelength corresponding to the maximum of absorption decreases and shifts towards longer wavelength region. Moreover, at certain interval of changing relation concentration of C_0/C_p, two isosbestic points were registered at 390 and 510 *nm* wavelengths, which were caused by complex-formation of poly(I) with EtBr (C_0 is total concentration of EtBr in solution). In order to have constant isosbestic point(s) different binding centers with the same quantity were required. Let's consider that one binding mode is strong and the other is weak. The presence of isosbestic

points in certain interval of change of C_0/C_p ratio is due to the fact that EtBr molecules in solution are in two spectrophotometrically different forms: (i) free and (ii) bound to the mentioned binding centers. These two forms have different absorption spectra, but in isosbestic point they have the same absorption value. Probably at 0.1 *M* ionic strength poly(I) forms single- and double-stranded structures and moreover when C_0/C_p ratio has greater values two isosbestic points are formed and in all probability this phenomenon is caused by saturation of all binding centers. Using the first order approximation one may consider that absorption spectra of bound EtBr molecules are the average spectrum for EtBr molecules which are bound to different binding centers. During the decrease of C_0/C_p ratio the shift of curve's maximum to longer wavelength region increases.

With further decrease of C_0/C_p ratio the binding equilibrium between poly(I) and two different structures break and due to that the absorption spectra deviate from isosbestic point (Fig. 1). From such values of C_0/C_p ratio the absorption spectra may change as a result of free EtBr binding and also as a result of weakly and strongly bound EtBr molecule redistribution. At high concentrations of poly(I) almost all EtBr molecules are in bound state and further changes (through poly(I) continuous increment) in absorption spectra are conditioned only by redistribution of EtBr molecules, which are bound by different binding centers. From certain values of C_0/C_p ratio the absorption value of solution varies insignificantly after further increase of poly(I) concentration.

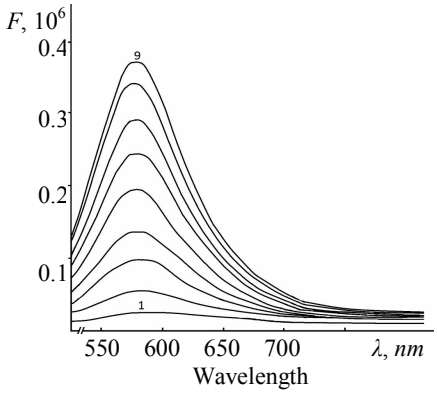

Fig. 2. Change of intensity (*F*) of EtBr fluorescence spectra due to binding with poly(I) at 300.15 *K*. [EtBr] was kept constant during titration: C_0=4.2·10^{-5} *M*. [Poly(I)] in buffer varies from 0 (1) to 6.8·10^{-4} *M* (9).

There are 3 types of characteristic spectra presented in Fig. 1: (i) free EtBr spectrum (curve 1); (ii) EtBr is bound with all binding centers almost with the same quantity when $C_0/C_p \geq 6$ (curves 2–6); (iii) EtBr generally binds to strong binding centers when $C_0/C_p \geq 17$ (curves 8–9). This phenomenon is due to the fact that when $C_0/C_p \geq 17$ concentration of polynucleotide prevails ligand's concentration and for that reason binding sites are plentifully presented. In these conditions ligand molecules bind to macromolecules only by one binding mode. It is well known that EtBr generally binds to double-stranded polynucleotide via intercalation mechanism, when there is an abundance of binding sites on it. Consequently, we may assume that in such conditions EtBr binds to poly(I) generally via intercalation binding.

It has been shown earlier that EtBr, depending on the ionic strength of medium, binds both to single- and to double-stranded DNA via several binding modes [17]. Moreover, in the case of DNA, when medium has physiological ionic strength conditions from a certain value of change in C_0/C_p ratio absorption curves do not pass through isosbestic point and also absorption maximum at 520 *nm* increases [14–17]. There is a good correlation between the obtained data for EtBr with DNA and EtBr with poly(I)

interactions. This is testified by fluorometric spectra obtained for DNA–EtBr and poly(I)–EtBr complexes, which have the same behavior of change (see Fig. 2 and [17]).

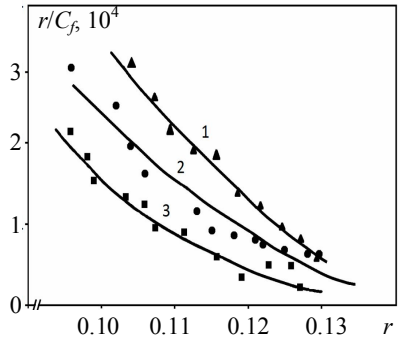

Fig. 3. Binding isotherms of EtBr with poly(I) at 290.15 (1), 300.15 (2) and 310.15 *K* (3) temperatures.

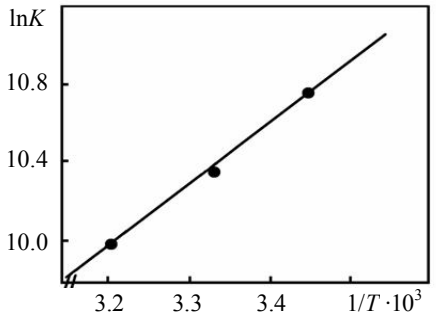

Fig. 4. Dependence of ln*K* on 1/*T* for EtBr–poly(I) complexes.

Characteristic quantitative parameters for simultaneous complex-formation of EtBr with strong and weak binding centers are binding constant (*K*) and average number of nucleotides bound with one EtBr molecule (bound per one EtBr molecule) when binding is saturated (*n*). These parameters were determined from the binding curve, which has been built using absorption spectra. From the absorption spectra by using Eq. (1) the concentrations of free (C_f) and bound (C_b):

$$C_f = \frac{A - A_b}{A_f - A_b} C_0, \; C_b = C_0 - C_f, \tag{1}$$

where A_f is the absorption value of free EtBr; A_b is the absorption value for bound EtBr to strong and weak binding sites (at λ=480 *nm*) and A is EtBr's absorption at intermediate states. A_b was determined by the linear extrapolation of $A = f(1/C_p)$ dependence when $(1/C_p) \to 0$.

Using values of C_f and C_b calculated from Eq. (1), binding curve in Scatchard coordinates (dependence of r/C_f from r) was built, where $r = C_b / C_p$. This curve can be described by Eq. (2), which quite precisely describes the adsorption of low-molecular compounds on nucleic acids [18]:

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

Fig. 3 illustrates binding isotherms for EtBr binding to poly(I) at 290.15, 300.15, 310.15 *K* built via Eq. (1). Solid line is a theoretical line built on the experimental points via Eq. (2) and by the method of linear least square, from which the values of *K* and *n* were determined. Calculations revealed that for poly(I)–EtBr complexes at T_1=290.15 *K* temperature $K_1 \approx 4.8 \cdot 10^4\ M^{-1}$ and $n \approx 6$, while at T_2=300.15 *K*; $K_1 \approx 3.2 \cdot 10^4\ M^{-1}$, $n \approx 6$ and at T_3=310.15 *K*; $K_1 \approx 3.2 \cdot 10^4\ M^{-1}$, $n \approx 6$. Applying determined values of *K* and using Eq. (3) the change of Gibbs free energy of complex-formation may be calculated:

$$\Delta G = -RT \ln K, \tag{3}$$

where R is the universal gas constant and T is absolute temperature. The change of entropy (ΔS) and enthalpy (ΔH) may be calculated via Eq. (4)

$$\Delta G = \Delta H - T\Delta S. \quad (4)$$

Modifying the last equation and taking into account Eq. (3) it may be presented in this way

$$\ln K = -\frac{\Delta H}{R} \cdot \frac{1}{T} + \frac{\Delta S}{R}. \quad (5)$$

According to Eq. (5), if dependence of ln$K$ on $1/T$ is linear, then the angle tangent of this line with abscissa axis is equal to $-\Delta H/R$ value, while the intersection point of the line with ordinate axis is $\Delta S/R$ value. Using experimentally obtained values of K (see Eq. (3)) a dependence curve of ln$K$ on $1/T$ was built (Fig. 4). By the method of linear least square, a line was built on the experimental points from which the values of ΔH and ΔS were determined. Calculations have revealed that $\Delta H \approx -6.9$ *kcal*/*mol* and $\Delta S \approx -2.3$ *cal*/(*mol*·*K*). Considering the data mentioned in literature [15, 16] and according to our studies we may state, that the value of ΔH in the case of EtBr–poly(I) is approximately as much as it is in the case of intercalative binding of EtBr to single-stranded and double-stranded nucleic acids. Thus, at mentioned conditions, EtBr generally binds to different structures of poly(I) via intercalative binding mode.

Studies have shown that for EtBr interactions with double-stranded polynucleotides $n=4$ [14, 16], while in case of EtBr interactions with single-stranded polynucleotides $n=8$ [3, 15]. If the solution simultaneously contains these two types of structures, then according to our calculations $n=6$. Thus, in all probability at the studied conditions the concentration of single- and double-stranded forms of poly(I) (by the concentration of inosine) are equal to each other.

According to other works [3, 15, 16], the change of entropy for EtBr complex-formation with single-stranded polynucleotides is $\Delta S<0$, while in case of double-stranded polynucleotides $\Delta S>0$. These values are greater then the order of the same value obtained in our study for poly(I)–EtBr complexes, which is $\Delta S=-2.3$ *cal*/*mol*·*K*. Probably this is caused by the fact that as a result of one EtBr molecule binding to single-stranded poly(I) a higher change of entropy (in absolute value) is needed and due to that the change of entropy is negative.

To conclude, we assume that in conditions of 0.1 M ionic strength, neutral pH and 290.15–310.15 K temperature, approximately half of poly(I) molecules form double-stranded helical structure.

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