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# INVESTIGATION OF ACIDIC DENATURATION OF ETHIDIUM BROMIDE COMPLEXES WITH DNA

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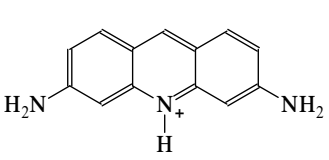
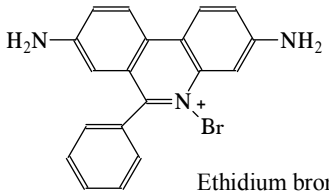
In the present work the temperature-induced and the acidic denaturation of ethidium bromide (EtBr) complexes with DNA have been carried out. The curves of helix-coil transition of both DNA and its complexes with EtBr in $0 \le r \le 0.5$ ($r$ = [ligand] / [DNA]) were obtained. It has been shown that with enhancement of EtBr concentration the transition point is shifted to the "high values" for both factors invoking this transition. It was also revealed that EtBr interacts with DNA by several modes being in non protonated and protonated states, moreover ligand binding mechanisms to DNA are analogous.

***Keywords***: DNA, EtBr, thermo-induced denaturation, acidic denaturation, transition point, transition interval width.

**Introduction.** To understand the interaction mechanisms of ligands with DNA the investigation of structure and stability of their complexes in different conditions of solution (ionic strength, pH, presence of mixtures of different compounds etc.) is necessary, since the major part of ligands may bind to DNA by several modes. Particularly, acridine or phenanthridine dyes for instance acridine orange (AO), proflavine (Pf), ethidium bromide (EtBr) (see Scheme) that are mutagen and cancerogeneous may show bacteriastatic properties depending on DNA binding mode [1–9].




Proflavine




Ethidium bromide

Scheme.

Biological activity of these compounds is connected firstly with binding intercalation mode that results in structural reconstructions of DNA double helix and inhibition of its functions [1–9]. Formation of different types of EtBr complexes with DNA has been discussed in [2–4], where it was shown that depending on both

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ionic strength and concentration ratio between DNA and EtBr $r$ ($r$=[EtBr]/[DNA]) different types of complexes may be formed. Particularly, at low ratios mainly the intercalation type is formed, at high ratios – the external type of complexes [2, 3]. It was shown that intercalative type of interaction significantly influences on DNA structure [5, 6]. For example, EtBr binding by intercalation type invokes DNA B-form untwisting approximately by 20° and double helix duplex lengthening [5]. Such changes in DNA structure result differing of thermodynamic characteristics and helix-coil transition parameters at free DNA melting from those at ligand complex with DNA.

It was shown that functioning of DNA may be in not only double-stranded (ds), but also single-stranded (ss) states. Consequently, two types of conformation transitions in DNA with destruction or without destruction of ds-structure may be observed [2]. Moreover the transition from one conformation state into another occurs in certain, non zero interval at changing of some external parameter effecting on DNA (temperature, pH, ionic strength, etc.) [2]. So, different molecules may have a certain affect on these transitions. Particularly EtBr, actinomycin (AMD), Pf, Hoechst 33258 (H33258), methylene blue (MB) bind not only *in viv*o, but also *in vitro* and affect on DNA functional activity [2, 3, 10–16]. At interaction of these ligands with DNA the shift of transition point takes place and simultaneously transition interval width is changed depending on their concentration [2, 3].

The aim of present work was the investigation of helix-coil transition parameter change of DNA and its complexes with EtBr at acidic denaturation, and the comparison of obtained data with analogous magnitudes obtained at temperature melting of DNA–EtBr complexes when pH≈7.0, as well as the effect of protonation on EtBr binding peculiarities with DNA.

**Materials and Methods.** The following preparations were used in this work: Calf Thymus DNA (“Sigma”, USA), ethidium bromide (“Serva”, Germany), NaCl, Na-citrate (triple-substituted), HCl (v.p.), ethylenediaminetetraacetate (EDTA, v.p.). All preparations were used without additional purification. DNA and EtBr concentrations were determined by absorption method, using the following extinction coefficients: DNA – $\varepsilon_{260}$=6600 $mol^{-1}\cdot L\cdot cm^{-1}$, EtBr – $\varepsilon_{480}$=5800 $mol^{-1}\cdot L\cdot cm^{-1}$. Experiments were carried out at 0.02 $M$ $Na^+$ concentration. Spectrophotometric measurements were carried out on PYE Unicam-SP8-100 spectrophotometer (England). Quartz cuvettes with 3 $mL$ volume and 1 $cm$ optic pathway length were used for spectrophotometric measurements. pH titration was carried out on ionomer-universal ЭВ-74 with ЭСЛ 63-07 measuring electrode (USSR). The solution of DNA and its complexes with EtBr was titrated by 0.2 $N$ HCl: each time 2 $\mu L$ of acid was added, the solution was mixed by magnetic stirrer, after what pH value was registered. The error of pH measurements does not exceed ±0.02.

Acidic denaturation of DNA–EtBr complexes is based on measurement of DNA absorption spectra at $\lambda$=260 $nm$ and pH changing (to acidic side). At titration of complexes by HCl solution when $\lambda$=260 $nm$ the contribution of acid absorption in overall value of this parameter was insignificant. Helix-coil transition curves were constructed as at temperature melting.

At temperature melting the heating of solutions of preparations was performed by ultra thermostat PYE Unicam-SP8-100 spectrophotometer (England) with the help of SP 876 Series 2 with $0.25^0 C/min$ speed. During the melting

process absorption values of complexes were displayed on PC monitor with program elaborated in LabVIEW programming medium.

For construction of melting curves the part of undisturbed for given temperature base pairs ($1-\vartheta$) is used, which is bound to UV-absorption via following relation:

$$1-\vartheta = \frac{A - A_{hel}}{A_{coil} - A_{hel}},$$

where $A_{hel}$ and $A_{coil}$ are DNA optic absorption in fully helical and fully coil states respectively; $A$ is the sample absorption at given temperature or pH. Helix-coil transition curves are characterized by two parameters: by the point ($pH_m$ at acidic and $T_m$ at temperature denaturation), when 50% of molecules are in coil-like (helical) state and the transition width ($\Delta$pH or $\Delta T$) determining as a tangent line through the point corresponding to the transition.

**Results and Discussion.** The investigations of ligands interaction with DNA indicate that depending on concentration they may bind to DNA by different mechanisms, moreover the displaying of these mechanisms for several ligands depends on solution ionic strength and for several – not. Particularly, in the case of intercalator MB as well as non intercalator H33258 the dependence of binding mechanism displaying on solution ionic strength is obtained, while for EtBr such dependence is not revealed. This is indicated by theoretical and experimental results of analysis of parameters at temperature melting of DNA and its complexes with EtBr at pH≈7.0 [2]. This fact may be conditioned by independence of EtBr binding mechanisms with DNA on concentration of ions. From this point of view the question about hydrogen ion affect on interaction peculiarities of ligands with DNA becomes important. Moreover in this case hydrogen ions may have two values – play the role of cations on the one hand and protonate DNA functional groups and ligand molecules on the other hand. For this aim the studies of EtBr interaction with DNA by acidic denaturation method at 0.02 *M* ionic strength of solution have been carried out.

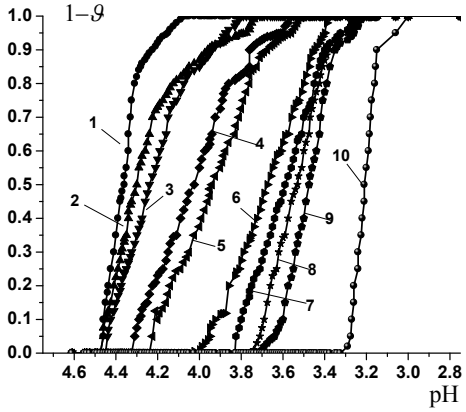


Fig. 1. Helix-coil transition curves of pure DNA (1) and its complexes with EtBr (2–10) in 2.5≤pH≤7.0 interval at 0.02 *M* of $Na^+$ concentrations, $t$=25ºC and the following values of $r$: 1 – 0; 2 – 0.01; 3 – 0.013; 4 – 0.02; 5 – 0.025; 6 – 0.04; 7 – 0.067; 8 – 0.10; 9 – 0.20; 10 – 0.33.

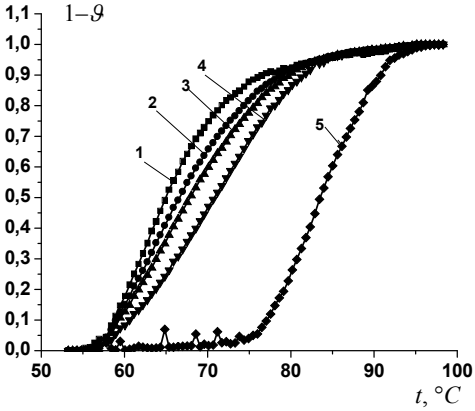


Fig. 2. Helix-coil transition curves of pure DNA (1) and its complexes with EtBr (2–5) at temperature denaturation, 0.02 *M* of $Na^+$ concentration and the following values of $r$: 1 – 0; 2 – 0.02; 3 – 0.04; 4 – 0.1 and 5 – 0.33.

On Fig. 1 the melting curves of DNA (1) and its complexes with EtBr (2–10) at mentioned values of $r$ and increasing of $[H^+]$ concentration are represented. To compare the behavior of transition curves the temperature melting curves of DNA (1) and its complexes with EtBr (2–5) are presented on Fig. 2 in $0< r \leq 0.33$ interval of change. It is obvious from Fig. 1, that the curves of complexes are shifted to the higher values of $[H^+]$. Moreover the slope on curves of DNA–EtBr complexes decreases at relatively low values of $r$ (curves 2–10 on Fig. 1), but at $r >0.25$ the slope of curves approaches the analogous curve of pure DNA (curve 5 on Fig. 1). The comparison of transition curves obtained at acidic and temperature denaturation reveals one-direction affect of both factors on helix-coil transition parameters, despite the ions of hydrogen apart from being a denaturizing object are also the protonating factor of DNA functional groups and EtBr.

Based on helix-coil transition curves the changes of transition parameters $\delta pH_m$ and $\delta\Delta pH$ (where $\delta pH_m = pH_0 - pH_m$, $\delta\Delta pH = \Delta pH_m - \Delta pH_0$, $pH_0$ and $pH_m$ are the points, $\Delta pH_0$ and $\Delta pH_m$ are the helix-coil transition interval width of DNA and DNA–EtBr complexes respectively) on ligand concentration by analogy to temperature melting were obtained. The dependencies of $\delta\Delta pH$ and $\delta pH_m$ changes in $0 \leq r \leq 0.33$ interval are presented on Fig 3. As it is obvious from presented Figure, the dependence of $\delta\Delta pH$ on $r$ has a bell-like shape as in the case of thermo-induced transition (Fig. 4).

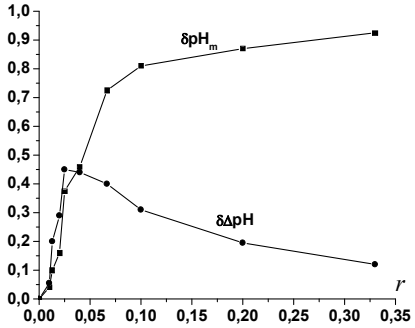


Fig. 3. $\delta\Delta pH$ (1) and $\delta pH_m$ (2) dependence curves on $r$ in $0 \leq r \leq 0.35$ interval at $t$=25°C, 0.02 $M$ $Na^+$. The curves of $\delta pH_m$ and $\delta\Delta pH$ changes are obtained from the curves represented on Fig. 1.

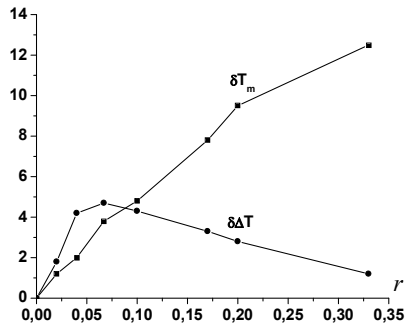


Fig. 4. The curves of $\delta T_m$ and $\delta\Delta T$ dependencies on $r$ in $0 \leq r \leq 0.35$ interval at 0.02 $M$ $Na^+$. The curves of $\delta\Delta T_m$ и $\delta\Delta T$ changes on $r$ are obtained from the curves represented on Fig. 2.

The bell-like shape of $\delta\Delta T$ dependence on $r$ ($\delta\Delta T_m = \Delta T_m - \Delta T_0$, where $\Delta T_m$ and $\Delta T_0$ are the helix-coil transition interval width of DNA–EtBr complexes and pure DNA respectively) is conditioned by the fact that at ligand low concentrations ($0<r\leq 0.1$) $\delta\Delta T$ increases and passing through the weakly expressed maximum ($0.05<r\leq 0.1$) decreases. Moreover, at $r>0.25$ $\delta\Delta T$ tends to zero, i.e. $\Delta T \approx \Delta T_0$ [6]. The increasing of $\delta\Delta T$ dependence on $r$ at ligand low concentrations is the consequence of redistribution of EtBr molecules from denaturized to still non denaturized regions of DNA during the melting process. Though in these conditions EtBr binds to DNA mainly by intercalation mode and stabilizes ds-structure [3]. At higher concentrations all preferable sites on ds-DNA for

intercalation of ligand molecules are saturated and the redistribution during the transition process becomes impossible as a result of what $\delta\Delta T$ comes out on plateau. It was shown in [3, 4], that EtBr may bind to DNA by semi-intercalation mode, at which ligand molecules bind to one of its chains. Moreover, if at intercalation of one ligand molecule into DNA the saturation is reached at $n\approx5$–6 b.p., at semi-intercalation one ligand molecule may bind maximally with two bases, which corresponds to $r\approx0.25$. Consequently transition interval width starts decreasing tending to zero.

Another parameter – the dependence of the melting temperature change $\delta T_m$ increases in $0<r<0.33$ interval. This indicates that besides intercalation and semi-intercalation, positively charged EtBr molecules bind with negatively charged phosphate groups by electrostatic mode as well, which results in additional stabilization of ds-DNA.

At acidic denaturation the dependence of $\delta pH_m$ increases in $0<r\leq0.1$ interval, while at $r>0.1$ the increasing of this parameter is insignificant. Most probably at binding of hydrogen ions to negative phosphate groups the electrostatic binding of EtBr molecules is interrupted. At the same time the behavior of $\delta\Delta$pH dependence curve corresponds to the behaviour of $\delta\Delta T$ curve on $r$. This may be the consequence of the fact that at acidic denaturation and EtBr low concentrations the increasing of $\delta\Delta$pH is conditioned by stabilizing effect of this ligand on DNA ds-structure, i.e. in these conditions EtBr molecules intercalate into ds-DNA. At further increasing of ligand concentration δΔpH passes through weakly expressed maximum ($0.05 \leq r \leq 0.1$), so, compared to thermo-induced transition, in this case the maximum appears at relatively low concentrations of ligand and after their saturation EtBr starts binding to DNA by other mode. Probably the protonation of ligand molecules that takes place at pH≈5.5 (EtBr aminogroups are in completely protonated state [8]) results in becoming of binding sites for EtBr molecule intercalation more complicated at binding to DNA. It should be mentioned that in these conditions DNA is in ds-state in complex with EtBr up to pH≈3.0 as it is obvious from Fig. 1, i.e. the intercalation sites on macromolecule are non destroyed [5, 7].

As it was mentioned above, EtBr may bind with DNA by semi-intercalation mode, and this mode is revealed in both cases ds- and ss-DNA [2, 4], moreover $K_1/K_2\approx2$ (where $K_1$ and $K_2$ are EtBr binding constants to ds- and ss-DNA by semi-intercalation mode respectively). This fact indicates that at semi-intercalation EtBr does not show an expressed specificity to ds- or ss-regions of DNA. In its turn this results in balancing of the melting temperatures of DNA mentioned regions, and in the complex with EtBr it melts as a homogenous system in these conditions, as a consequence of that on the dependence curve of $\delta\Delta T$ on $r$ the weakly expressed maximum is formed. At the same time on the dependence curve of $\delta\Delta$pH on $r$ this maximum is more expressed which may be the result of differing of EtBr binding constants with ds- and ss-regions of DNA by semi-intercalation mode at acidic values of pH of the solution.

With increasing of EtBr concentration the binding sites on ds-regions are saturated, in the result ligand molecules start binding with ss-regions of DNA,

destabilizing it and facilitating the transition, which results in decreasing of $\delta\Delta T$ and $\delta\Delta$pH dependence curves on $r$ tending to zero.

**Conclusion.** The above mentioned data indicate that depending on concentration EtBr binds by different modes with both ds- and ss-structures. The obtained data also indicate that EtBr interacts with DNA by several modes, moreover EtBr binding modes to DNA are similar at both protonated and non protonated states of ligand, since the changes of EtBr binding mechanisms with both ds- and ss-DNA at neutral and acidic values of pH of the solution are not observed.

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REFERENCES


1. **Ruiz R., Garcia B., Ruisi G., Silvestri A., Barone G.** Computational Study of the Interaction of Proflavine with $d(ATATATATAT)_2$ and $d(GCGCGCGCGC)_2$. // Journal of Molecular Structure: THEOCHEM, 2009, № 915, p. 86–92.
2. **Vardevanyan P.O., Antonyan A.P., Manukyan G.A., Karapetyan A.T.** Study of Ethidium Bromide Interaction Peruliarities with DNA. // Exp. and Mol. Medicine, 2001, v. 33, № 4, p. 205–208.
3. **Vardevanyan P.O., Antonyan A.P., Parsadanyan M.A., Shahinyan M.A., Hambardzumyan L.A., Torosyan M.A., Karapetian A.T.** The Influence of GC/AT Composition on Intercalating and Semi-intercalating Binding of Ethidium Bromide to DNA. // J. Braz. Chem. Soc., 2012, v. 23, № 11, p. 2016–2020.
4. **Veselkov A.N., Baranovsky S.F., Dymant L.N., Petrenko N.V., Veselkov D.A., Tucker A., Davis D.B.** Complex-Formation of Ethidium Bromide with Single-Stranded Non-Complementary Dezoksitetranukleotid 5'-d(ApApGpC). // J. Molecular Biology, 1997, v. 31, № 2, p. 263–273.
5. **Saenger V.** Principles of Structural Organization of Nucleic Acids. M.: Mir, 1987, 584 p. (in Russian).
6. **Lane A.N., Jenkins T.C.** Thermodynamics of Nucleic Acids and Their Interactions with Ligands. // Q. Rev. Biophys., 2000, v. 33, № 3, p. 255–306.
7. **Jones R.L., Wilson W.D.** Effect of Ionic Strength on the $pK_a$ of Ligands Bound to DNA. // Biopolymers, 1981, № 20, p. 141–154.
8. **Brana M.F., Cacho M., Gradillas A., de Pascual-Teresa B.** Ramos a Intercalators as Anticancer Drugs. // Curr. Pharm. Des., 2001, № 7, p. 1745–1780.
9. **Graves D.E., Velea L.M.** Intercalative Binding of Small Molecules to Nucleic Acids. // Curr. Org. Chem., 2000, № 4, p. 915–929.
10. **Zipper H., Brunner H., Bernhangen J., Vitzthum F.** Investigations on DNA Intercalation and Surface Binding by SYBR Green I, Its Structure Determination and Methodological Implications. // Nucleic Acids Res., 2004, № 32, p. 103.
11. **Biver T., Biasi A.D., Secco F., Venturini M., Yarmoluk S.** Cyanine Dyes as Intercalating Agents: Kinetic and Thermodynamic Studies on the DNA/Cyan40 and DNA/CCyan2 Systems. // J. Biophys., 2005, № 89, p. 374–383.
12. **Wu J., Du F., Zhang P., Khan I.A., Chen J., Liang Y.** Thermodynamics of the Interaction of Aluminum Ions with DNA: Implications for the Biological Function of Aluminum. // J. Inorg. Biochem., 2005, № 99, p. 1145–1154.
13. **Khrebtova A.S., Dukhopelnikov E.V., Berezniak E.G., Gladkovskaya N.A., Zinchenko A. V.** // Biofizichny Visnik, 2010, № 25 (2), p. 26–33.
14. **Bai Y., Ge Q., Wang J., Li T., Liu Q., Lu Z.** Investigation of DNA–Protein Sequence-Specific Interactions with a ds-DNA Array. // Molecules, 2005, № 10, p. 417–426.
15. **Arya D.P., Willis B.** Reaching Into the Major Groove of B-DNA: Synthesis and Nucleic Acid Binding of a Neomycin-Hoechsts 33258 Conjugate. // J. Am. Chem. Soc., 2003, № 125, p. 12398–12399.
16. **Trieb M., Rauch C., Wibowo F.R., Wellenzohn B., Liedl K.R.** Cooperative Effects on the Formation of Intercalation Sites. // Nucleic Acids Res., 2004, № 32, p. 4696–4703.