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# Joint interaction of ethidium bromide and methylene blue with DNA. The effect of ionic strength on binding thermodynamic parameters

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Large amount of data of experimental and theoretical studies have shown that ethidium bromide (EtBr) and methylene blue (MB) may bind to nucleic acids via three modes: intercalation between two adjacent base pairs, insertion into the plane between neighboring bases in the same strand (semi-intercalation), and outside binding with negatively charged backbone phosphate groups. The aim of the given research is to examine the behavior of these two ligands at both separate and joint DNA binding. The obtained experimental data show that the effect of simultaneous binding of EtBr and MB on double-stranded DNA has a non-additive effect of separate binding. The analyses of the melting thermodynamic parameters of DNA complexes with two bound ligands suggest competitive mechanism of interaction.

**Keywords:** DNA; melting thermodynamic parameters; ethidium bromide; methylene blue; competitive mechanism



## Introduction

The complex-formation of DNA with different biologically active small molecules (ligands) plays an important role in many intracellular processes. It is assumed that the majority of ligands binding to DNA show biological activity, which may influence various processes that occur in a cell (Chaires, 1998; Lane & Jenkins, 2000; Neible & Waving, 1993; Sibirtsev, 2005). Detailed information about the binding structure and thermodynamic properties of drug–DNA complexes is very important for rational design of more effective chemotherapeutic compounds in the treatment of different diseases, including cancer (Sibirtsev, 2005). DNA binding properties of a great variety of substances including dyes, drugs, and antibiotics have been studied, as a result of which it becomes possible to suggest a more plausible model of interaction and possible mechanisms of biological activity of some ligands (Chaires, 1998; Hajian, Shams, & Mohagheghian, 2009; Lane & Jenkins, 2000; Neible & Waving, 1993; Sibirtsev, 2005; Vardevanyan et al., 2008). Detailed thermodynamic and structural description of binding of the known ligands with the macromolecular target will provide basis for modifying the effectiveness and the activity of the chemotherapeutic agent (Grigoryan & Karapetyan, 2015). One of the promising ways to solve this problem is to construct a compound consisting of several subunits, each of which has specific structural and thermodynamic characteristics at binding to substrate. It is reasonable to expect more increased affinity and specificity to substrate than in the case of earlier used compounds. One of the possible ways to construct such heterofunctional compound is the knowledge of the biological influence of simultaneous actions of the subunits on the substrate. Nowadays, many studies have shown that a large number of biologically active small molecules in cell bind to DNA in a variety of ways one of which is the intercalation (Chaires, 1998; Hajian et al., 2009; Lane & Jenkins, 2000). It was shown that at small values of drug/DNA ratio ($r$), the majority of these ligands form the well-described intercalation mode of binding (Lane & Jenkins, 2000). For a long time, the prominent representative of the intercalating ligands – ethidium bromide (EtBr) and methylene blue (MB) – has been the object of intensive experimental and theoretical investigations in our laboratory. Therefore, in the given study, we choose these two well-known ligands for experimental characterization of their simultaneous binding influence on DNA thermodynamic parameters. The obtained experimental results provided clear evidence that the thermodynamic parameters of EtBr and MB at the complex-formation with DNA are non-additive of that of these ligands at separate binding with the biopolymer. This approach is in accordance with one of the important conclusions of the recent theoretical

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observation of the helix-coil transition (Badasyan et al., 2014; Ghaderian, 2012; Morozov, Badasyan, Grigoryan, Sahakyan, & Mamasakhlisov, 2004) of DNA complexes with two different ligands having different binding parameters (Karapetian et al., 2015). Besides, it was shown that the number of binding sites for EtBr (more probable intercalation sites) was independent of ionic strength of solution and, more important, the competition between EtBr and MB for a binding site was in favor of EtBr: EtBr displaced MB to bind to DNA.

## Materials and methods

Calf thymus DNA was purchased from Sigma (USA), the concentration of which was determined according to the absorbance at 260 nm using $\varepsilon_{DNA} = 6600$ L mol$^{-1}$ cm$^{-1}$, MB was purchased from Aldrich (USA), and EtBr from Serva (Germany). EtBr and MB concentrations were measured using the following molar extinction coefficients of $\varepsilon_{480} = 5800$ L mol$^{-1}$ cm$^{-1}$ and $\varepsilon_{664} = 76{,}000$ L mol$^{-1}$ cm$^{-1}$, respectively. All chemicals were of analytical reagent grade and were used without further purification.

Stock solutions of DNA in $6.0 \cdot 10^{-5}$ M phosphate (P) were made in sodium citrate buffer which contains 150 mM NaCl and 15 mM Na–citrate. In each set of experiments, the ligand solution of the known concentration was added in the appropriate ionic strength buffer and the initial polynucleotide solution sample of the given concentration. UV–visible absorbance studies were carried out on Pye Unicam SP 8-100 spectrophotometer (England). Cells with optical pathway length of 10 mm and Teflon stoppers were used and the solution in cells was heated by SPX 876 S2 Temperature Program Controller (England).

For the identical results during the melting of complexes EtBr–DNA–MB, the concentrations of each ligand at certain $r$ were equal to half of the concentration corresponding to the ligands at separate binding. All the experiments were performed at pH 6.8. Double-distilled water was used.

## Results and discussion

Among the ligands interacting with DNA and substantially affecting the structural and functional characteristics of the biopolymer, EtBr is of particular interest inhibiting *in vivo* and *in vitro* replication and transcription (Borisova, Scholkina, Surovaya, & Karapetyan, 1998; Karapetian et al., 1996; Vardevanyan, Antonyan, Manukyan, & Karapetyan, 2001). This ligand is a prominent representative of the intercalators and is an appropriate substance for simulation of the molecular mechanisms of interaction of variety of biologically active molecules with DNA.

Recently, we have theoretically observed conformational transitions in DNA–ligand complexes, which allow the existence of different binding parameters of the ligand (multimodal ligands) to different DNA conformations (Karapetian et al., 1996). The comparison of the theory and experiment in the case of the helix-coil transition of the complexes of DNA with EtBr revealed at least three types of complexes with double-stranded DNA (ds-DNA). Our UV–vis and fluorimetric spectroscopic investigations confirm this outcome. The results of these experiments showed that EtBr may bind to ds-DNA in several ways forming two strong complexes – fluorescent (intercalation (Vardevanyan, Antonyan, Parsadanyan, Davtyan, & Karapetyan, 2003)); non-fluorescent (strong external-semi-intercalation mode (Borisova et al., 1998; Monaco, 2007; Vardevanyan et al., 2003; Vardevanyan et al., 2013)) and one weak – electrostatic (external). It is important to notice that the intercalation mode of binding is independent of the ionic strength, pH, and other environmental conditions which make it a good informative model for investigation of other DNA–ligand complexes (Vardevanyan et al., 2001; Vardevanyan et al., 2013). In order to reveal the distinctive characteristics of the simultaneous binding of MB and EtBr to DNA, we compared the thermodynamic parameters of helix-coil transition of the complexes of these ligands at their separate and simultaneous binding with the polynucleotide, employing the melting method in the ultraviolet region of light. By applying this method, we obtained thermodynamic parameters of melting of the complexes of EtBr–DNA–MB at two ionic strengths of solution ($\mu_1 = 2$ mM $Na^+$ and $\mu_2 = 20$ mM $Na^+$) varying the concentration of these two ligands in the $0 \le r \le .33$ region ($r$ = [ligand]/DNA b. p.) at $\lambda = 260$ nm wavelength. The melting curves have been obtained (curves are not represented here). The pure DNA (DNA in the absence of the ligand) showed a well-marked cooperative transition with the melting temperatures ($T_0$) 56 °C and 70 °C at $\mu_1$ and $\mu_2$, respectively. The melting curves of DNA–ligand complexes were shifted to high temperatures at all ligand and $Na^+$ concentrations pointing to the stabilizing effect of the ligands on helix structure of DNA at both ionic strengths. The values of the melting temperatures – $T_m$ were determined. The dependences of inverse transition point $\delta(1/T_m) = 1/T_0 - 1/T_m$ on $r$ were plotted.

Figure 1 shows the dependences of $\delta(1/T_m)$ on $r$ in the case of separate (curves 1 and 2) and simultaneous binding of EtBr and MB to DNA $\mu_2 = 20$ mM $Na^+$ (curve 3). The addition of the curves (1) and (2) of Figure 1 is presented by curve 4 which monotonically increases like the behavior of corresponding curve of EtBr–DNA complexes (see the curves 2 and 4, Figure 1). By contrast, the curve of $\delta(1/T_m)$ dependence on $r$ corresponding to the simultaneous binding of both ligands to

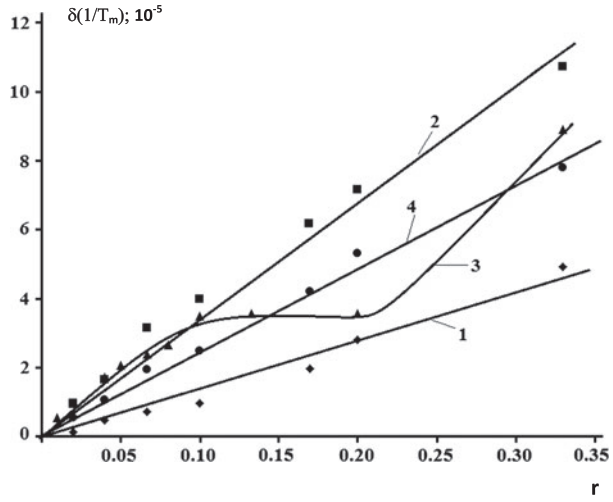


Figure 1. Dependences of $\delta(1/T_m)$ on $r$ of DNA–MB (1); DNA–EtBr (2); MB–DNA–EtBr (3) complexes. The simple mathematical addition of curves (1 and 2) gives curve 4. Ionic strength was equal to $\mu_2$ = 20 mM $Na^+$.

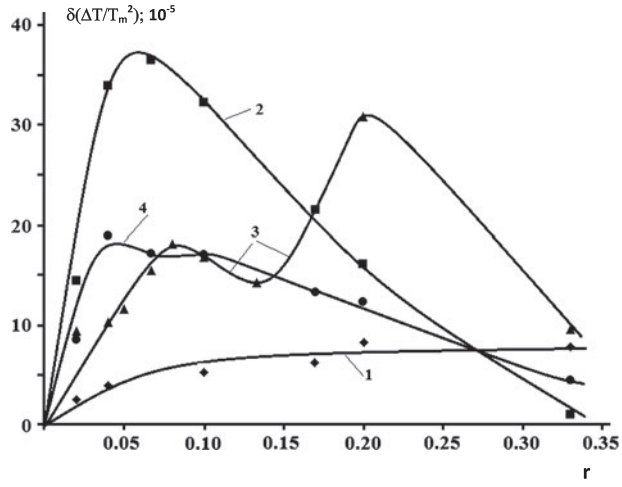


Figure 2. Dependences of $\delta(\Delta T/T_m^2)$ on $r$ of the DNA–MB (1), DNA–EtBr (2), MB–DNA–EtBr complexes at 20 mM $Na^+$. The simple mathematical addition of curves (1 and 2) gives curve 4.

DNA differs considerably from their separate effects on the melting of DNA. The dependence of $\delta(1/T_m)$ on $r$ for joint binding of the ligands with DNA (curve 3 on Figure 1) may be conditionally divided into three areas: the first $0 < r \leq .1$ area where the $\delta(1/T_m)$ monotonously increases, the second – reaches plateau ($.1 < r < .2$), and the third area at $r \geq .2$ where it sharply increases. Interestingly, the value of $\delta(1/T_m)$ of EtBr–DNA–MB complexes at $\mu_2$ is considerably lower than that of EtBr–DNA complexes (see curve 3 with curve 2, Figure 1) in addition to the corresponding curves of EtBr–DNA and MB–DNA complexes (curve 4, Figure 1). Most probably, this is the effect of decreased stabilization at the interaction of these two ligands with ds-structure of DNA. Presumably at joint binding, both ligands mutually weaken the stabilizing influence of each other on ds-structure of DNA. This assumption is confirmed by the experimental results represented in Figures 2, where subjections of the change in melting width $\delta(\Delta T/T_m^2)$ on $r$ are plotted at $\mu_2$ ionic strength. Curve 2 (Figure 2) corresponds to EtBr–DNA complexes and curve 1 shows the dependence of $\delta(\Delta T/T_m^2)$ on MB–DNA complexes. Figure 2 shows that curves increase at low r values and reach their maximum values at $r \approx .1$. This thermodynamic parameter of melting undergoes sharper decreasing at further enhancement of $r$ for EtBr–DNA complexes (Karapetian, Vardevanian, Terzikian, & Frank-Kamenetskii, 1990; Karapetian et al., 1996; Monaco, 2007; Vardevanyan et al., 2003; Vardevanyan et al., 2013; Wadkins, Jares-Erijman, Klement, Rüdiger, & Jovin, 1996). It is known that at low concentrations of EtBr, the main mode of interaction is insertion of the planar phenanthridium ring between adjacent nucleotide pairs of DNA, which primarily depends on hydrophobic transfer of the ligand from the solution into the surrounding bases (Chaires, 1998). This type of interaction is referred to intercalation. Binding of the ligand (particularly EtBr or MB) is expected to shift the melting temperature to higher values (curves 1 and 2, Figure 1) since the dyes stabilize the helix form of DNA (Borisova et al., 1998; Karapetian et al., 1990; Vardevanyan et al., 2001). Taking into account that these ligands do not exhibit base specificity to any DNA nucleotide (Wadkins et al., 1996), the melting of the ligand–DNA complexes may be considered to occur in a manner of generally similar to that of free DNA.

From the proposed point of view, the ligand molecules dissociated from denatured sites are redistributed and intercalated to the still native regions additionally stabilizing them. When the concentration of EtBr is higher than is necessary for the saturation of ds-DNA binding sites, dissociated ligands interact with the denatured regions of DNA, decreasing the melting width $\Delta T$ (Karapetian et al., 1996). Therefore, $\delta(\Delta T/T_m^2)$ decreases (curve 2, Figure 2 at $r > .125$). We assume that the plateau in the case of the melting of MB–DNA complexes (curve 1, Figure 2) is the result of the existence of a weak type of binding of this ligand to DNA (may be weak binding at either grooves of ds-DNA or, most probably, binding by the electrostatic outside interaction to DNA backbone phosphate groups).

The quite different shape and size of dependence of $\delta(\Delta T/T_m^2)$ curve obtained in the case of joint binding of EtBr and MB to DNA at $\mu_2$ = 20 mM $Na^+$ (curve 3, Figure 2) provide the information on the probable sequences of events accompanying the thermal

denaturation of the MB–DNA–EtBr complexes which are quite different from that of separate binding of these ligands to DNA. The analyses of the obtained data and the comparison of those with the ones existing in the literature thermodynamic characteristics of the complexes enable us to conclude that:

First: Similar to the change in melting temperature, the curve of dependence of $\delta(\Delta T/T_m^2)$ on $r$ corresponding to EtBr–DNA–MB complexes is not the simple addition of the curves of $\delta(\Delta T/T_m^2)$ obtained for EtBr–DNA and MB–DNA complexes (compare curve 1 and 2, with curves 3 and 4, Figure 2). These data clearly show that the stabilization effect of one of the ligands at separate binding differs greatly from that of joint interaction with ds-DNA and are in agreement with the above-mentioned conclusion of decrease in stability of DNA helix-structure.

Second: It was shown the existence of two maximums on the curve of dependences of $\delta(\Delta T/T_m^2)$ on the concentration of the ligands, one of which like EtBr–DNA complexes, is located at area of $r \approx .1$ (see the curves 1 and 3, Figure 2) and the other one is at $r \approx .2$ (curve 3, Figure 2) area. These data may indicate that the binding affinity of EtBr is much stronger than that of MB at the ionic strength of $\mu_2 = 20$ mM $Na^+$ and the role of EtBr intercalation is dominant in the stabilization of ds-DNA structure compared to other binding types (groove binding, outside binding with backbone phosphate groups). The appearance of the second maximum on the curve 3, (Figure 1) at $r \approx .2$ area, apparently, indicates the competitive interaction between bound ligands with ds-DNA as a result of which EtBr have much more affinity at binding (about an order of magnitude) (Changlun, Zhou, & Jianmin, 2010; Vardevanyan et al., 2003) than MB, additionally intercalates into the double-helix displacing bound MB molecule. This conclusion is supported by the experimental results of sharp increase in the melting temperatures of EtBr–DNA–MB complexes at this spot (curve 3, Figure 1).

The results of recent thermodynamic analyses of DNA complexes with EtBr and MB obtained (Vardevanyan et al., 2013; Vardevanyan et al., 2013) are in good agreement with those obtained by theoretical, experimental, and modeling investigations, have shown that depending on ionic strength, base composition of polynucleotide, and ligand concentration, there is more than one type of binding mode: intercalation, semi-intercalation and external electrostatic interaction with backbone negatively charged phosphate groups of DNA (Borisova et al., 1998; Changlun et al., 2010; Karapetian et al., 1990; Karapetian et al., 1996; Vardevanyan et al., 2001; Vardevanyan et al., 2003; Vardevanyan et al., 2013; Vardevanyan et al., 2013; Wadkins et al., 1996). This fact further proved the above made conclusion.

Thermodynamic parameters of the melting of EtBr and MB complexes with DNA at $\mu_1 = 2$ mM $Na^+$ are quite different from those at $\mu_2 = 20$ mM $Na^+$. Results of the helix-coil transition for the ligand complexes of both separate binding with DNA (EtBr–DNA and MB–DNA complexes) and simultaneously bound with biopolymer (EtBr–DNA–MB) are represented in Figures 3 and 4. Figure 3 shows that 10-fold decrease in the ionic strength ($\mu_1 = 2$ mM $Na^+$) radically changes the shape and size of the dependence of $\delta(1/T_m)$ on $r$ for both separate (curves 1,2) and simultaneous (curves 3) binding of the ligands with DNA.

Unlike the curves obtained in the case $\mu_2 = 20$ mM $Na^+$, they all have a similar shape. The values of $\delta(1/T_m)$ monotonously increase up to $r \approx .25$, and at further increase in the ligand concentration, they tend to the plateau (curves 1–3, Figure 3). It is interesting that the shape of the added curve of dependences of $\delta(1/T_m)$ on $r$ qualitatively does not differ from that of experimentally obtained curve for simultaneous binding of the ligands to DNA (see curves 4 and 3, Figure 3). The results of the comparison of the experimental values of $\delta(1/T_m)$ obtained for EtBr–DNA and EtBr–DNA–MB complexes show that they do not substantially differ from each other which apparently points to the dominant role of EtBr in the stabilization of ds-structure of DNA at this ionic strength. In a similar way, the decrease in ionic strength leads to the radical changes in the shapes and sizes of the dependences of $\delta(\Delta T/T_m^2)$ on $r$ (curves 1–3, Figure 4). Figure 4 shows that the curves 1–3 have the same bell-like shape for all investigated complexes. The similarity of the shapes of the dependences of $\delta(\Delta T/T_m^2)$ for EtBr–DNA and MB–DNA complexes could be a result of the combination of different binding types of these ligands to DNA: intercalation, semi-intercalation,

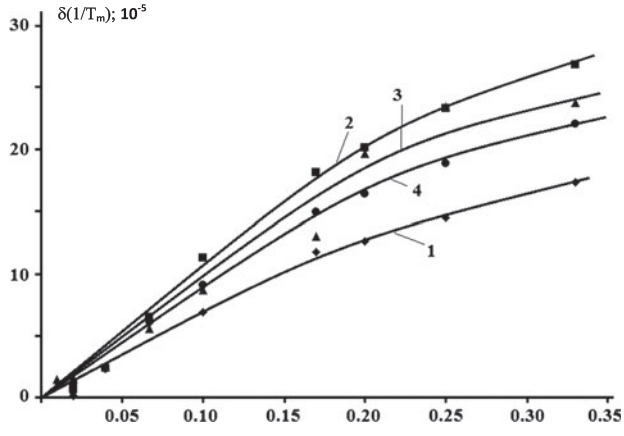


Figure 3. Dependences of $\delta(1/T_m)$ on $r$ of DNA–MB (1); DNA–EtBr (2); MB–DNA–EtBr (3) complexes. The simple mathematical addition of curves 1 and 2 is curve 4. Ionic strength is equal to $\mu = 2$ mM $Na^+$.

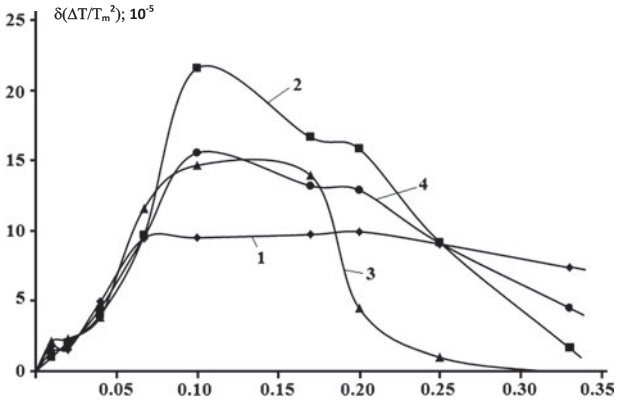


Figure 4. Dependences of $\delta(\Delta T/T_m^2)$ on $r$ of DNA–MB (1), DNA–EtBr (2), MB–DNA–EtBr (3) complexes at 2 mM $Na^+$. The simple mathematical addition of curves 1 and 2 is curve 4.

and external binding to phosphate group which coincides with the recently obtained thermodynamic (Karapetian et al., 1996; Vardevanyan et al., 2003; Vardevanyan et al., 2013) spectroscopic (Changlun et al., 2010; Hossain, Giri, & Kumar, 2008; Nafisi, Saboitry, Keramat, Neault, & Tajmir-Riahi, 2006; Vardevanyan et al., 2013) and modeling studies (Hajian et al., 2009; Hossain et al., 2008; Nafisi et al., 2006; Rohs & Sklenar, 2004; Rohs, Sklenar, Lavery, & Röder, 2000; Tong, Hu, & Wu, 2010).

The comparison of the obtained curves 1, 2, and 3 shows that in contrast with separate binding (curves 1, 2), due to the competition between the bound two ligands which is in favor of EtBr, the curve of $\delta(\Delta T/T_m^2)$ corresponding to the joint binding of the ligands (curve 3) achieves its maximum at $r_b < .125$. Passing the plateau at the area $.125 < r_b < 0.175$, it sharply decreases up to ≈0 showing that the intercalating and semi-intercalating sites are completely saturated by the ligands. In this case, the ligands molecules bind to DNA by electrostatically and dissociated ligands binding to the negatively charged phosphate groups of ds-DNA stabilize the helix structure of the biopolymer, increasing only the melting temperature of the complexes (curve 3, Figure 3) without any considerable change in the melting width of the complexes (curve 3, Figure 4). These data are supported by our experimental results represented by the curve 3, (Figure 3), the melting temperature of the complexes increases without registering any change in width of helix-coil transition.

The obtained experimental results made it possible to conclude that:

- The effect of simultaneous binding of EtBr and MB to DNA is not the simple addition of the effect of the separate binding.
- The number of EtBr binding types to DNA does not depend on ionic strength of solution as well as absence or presence of MB.
- Comparative analyses of the melting thermodynamic parameters of DNA complexes with EtBr and MB made it possible to suggest the competitive mechanism of interaction: EtBr intercalates into two adjacent base pairs of ds-structure displacing MB-bound molecule due to 10-fold higher affinity for EtBr interaction with biopolymer compared to that of MB.

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