EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 35, No. 6, 527-533, December 2003

The binding of ethidium bromide with DNA: interaction with single- and double-stranded structures

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Accepted 24 November 2003

Abbreviations: AMD, Actinomycin D; DAPP, 3,8-diamino-6-phenylphenantridine; ds-DNA, double stranded DNA; EtBr, ethidium bromide; NFC, non-fluorescence complexes; ss-DNA, single stranded DNA

Abstract

The pH-induced helix-coil transition of DNA and its complexes with EtBr is carried out at acidic pH in a wide interval of change of concentration ratio of EtBr/DNA. The binding isotherms of EtBr on double and single-stranded DNA at pH = 7.0 and pH = 3.0 (t = 25°C) are obtained by absorption and fluorimetric methods. Binding constants (K) and number of bases (n), corresponding to one binding site were determined. Non fluorescent "strong" complex with ds-DNA at pH = 7.0 and t = 25°C as well as "strong" and "weak" complexes with ss-DNA at pH = 3.0 and t = 25°C are revealed.

Keywords: absorption isotherms in Scatchard`s coordinates; acidic values of pH; constants and number site sizes; pH-induced denaturation; ss- and ds-DNA

Introduction

The existing data on interaction of EtBr with DNA specifies that this ligand can form several types of complexes with ds-DNA and ss-DNA (Karapetian *et al*., 1996, Vardevanyan *et al*., 2000). The conformation of ss-DNA basically is defined by intermolecular interactions between nitrogenous bases (stacking interaction, hydrogen bounds). Intermolecular binding in ss-DNA are weaker, than in ds-molecules (Veselkov *et al*., 1997). It is necessary to note also, that the research of behaviour of ss-DNA in a solution is connected with certain difficulties. It is connected with occurrence of intermolecular as well as intramolecular binding in diluted solutions that carry in essential contribution to the enthalpy of intermolecular binding at t = 25°C (Seanger, 1987; Graves, 1993). The earlier reported data showed a high affinity and selective binding of the antibiotic actinomycin D (AMD) with ss-DNA. Ethidium Bromide (EtBr) also shows binding specificity with ss-oligonucleotide sequences (Veselkov *et al*., 1997, Vardevanyan *et al*., 2000). It is possible to assume that the selectivity complex formation of ligands with certain single-stranded sequences plays an important role in functioning of biologically active substances in processes connected with transcription and replication of DNA.

However, the binding of EtBr with ss-DNA is not clearly understood. Moreover, the possibility of interaction of EtBr with ss-DNA can occur by several modes (Tishchenko *et al*., 1996; Borisova *et al*, 1998). Existing experimental data for complex formation of EtBr with ss-polynucleotides are relevant to conditions, not excluding presence of ds-sites in DNA (Borisova *et al*, 1998).

Recently we obtained isotherms of EtBr adsorption on native and denatured poly(dA)-poly(dT) in the temperature interval of 20-70°C. On the basis of data obtained, a model can be postulated in which the binding of EtBr with ss-polynucleotides at 70°C may cause stacking contacts between bases. The experimental data obtained specifies the presence of several ways of EtBr binding with ss-nucleic acids at high temperatures. On the other hand, the data available in the literature shows that an interaction of EtBr with ss-nucleic acids is basically carried out with synthetic polynucleotides. From this point of view the data of EtBr binding with heterogeneous ss-DNA can be very interesting and valuable. In this work, the investigation of behaviours of ss-DNA was carried out at t = 25°C and pH = 3.0 while the existence of ds-sites in DNA and its complexes with ligand is excluded.

Materials and Methods

Materials

Ultrapure Calf Thymus DNA and EtBr ("Serva", Germany), HCl were used in this work. All preparations were used without additional purification. Concentration of used preparations was determined by absorption spectroscopy, using the values of molar extinction for calf thymus DNA $\varepsilon_{260} = 6400\ M^{-1}cm^{-1}$ and EtBr- $\varepsilon_{480} = 5600\ M^{-1}cm^{-1}$, respectively.

Spectrophotometric measurements

Fluorimetric and Spectrophotometric measurements were carried out on fluorimeter FluoroMax TM (France) and on spectrophotometers Pye Unicam-SP8-100 (England) and Specord M-400 (Germany).

All measurements were carried out in hermetic closed quartz cuvetts, with length of an optical way 1 cm. pH-titration was carried out on ionomer-universal EV-74 (USSR). A solution of DNA was titrated by 0.2N HCl: each time 1 μl of acid was added, mixed, then the value of pH was registered which is varied between ± 0.02 units. The titration of EtBr solution with DNA is carried out by micropipette with volume of 10 μl ("Hamilton"). All measurements were conducted at room temperature (t = 25°C), at ionic strength of $\mu = 2.0\times10^{-2}$ M [Na^+].

Data analysis

Spectrophotometric method of study of pH-induced helix-coil transition of DNA/EtBr complexes, as in case of thermo-induced transition, is based on measurement of absorption spectra at λ=260 nm with change of pH in the range of acidic values (Vardevanyan *et al.*, 2001). For construction of helix-coil transition curves, the value of a degree of helixity-1-ϑ was used, appropriate to the given values of pH:

$$1-\vartheta = \frac{A_{pH}-A_{helix}}{A_{coil}-A_{helix}} \qquad (1)$$

where A_{pH} is the optical density of samples at the given value of pH, A_{helix} and A_{coil} -the optical densities of samples at completely helix and coil conditions respectively. The values of pH corresponding to a point and interval of transition were determined by the same way as in the case of thermal denaturation. Quantity of bound molecules of EtBr with DNA was determined from the results of absorption and fluorimetric titration, as described in works (Borisova *et al.*, 1998).

Adsorption isotherms were constructed in Scatchard's coordinates r/C_f~r, where r is the number of bases complexed with EtBr, C_f are the free (unbound) molecules of ligand in solution.

The binding curves of EtBr with DNA were constructed by formula:

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

where K is binding constant, n - number of bases, corresponding to one binding site (Vardevanyan *et al.*, 2000).

Results

Earlier, we carried out studies of EtBr interaction with ss-polynucleotides by denaturation of poly(dA)-poly(dT) at t = 70°C and pH = 7.0, $\mu = 2\times10^{-2}$ M Na^+ (Vardevanyan *et al.*, 2000). However, a similar study on the complexes of EtBr with ss-DNA is complicated, as it is impossible to remain the complete isolation of system in these conditions. Therefore the addition of EtBr can result in formation of ds-sites, (as EtBr is the stabilizer of ds-DNA) that may distort the real situation.

To reveal the conditions, at which the complexes of DNA-EtBr in an interval of change of r_b-$0 \le r_b \le 0.5$, (r_b = EtBr/DNA) are in ss-condition at t = 25°C, we investigated the helix-coil transition induced by the change of environmental pH to the acidic range. On Figure 1, the curves of transition of DNA itself (1) and its complexes with EtBr (2-5) are given. As indicated from the given Figure, the width of an interval of transition pH of complexes increased at low concentrations of EtBr, and shows the tendency to decrease when achieving certain value of ligand concentration. It indicates that during the denaturation of DNA there is a redistribution of ligand molecules from denatured (ssDNA) on native (not denatured yet-dsDNA) sites, as it takes place at thermo-induced transition (Vardevanyan *et al.*, 2001). It is necessary also to note that the curves of transition of complexes are shifted in

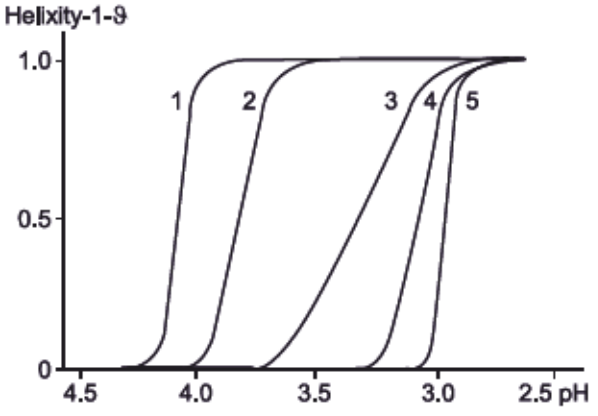


Figure 1. The pH-induced helix-coil transition of DNA itself (1) and its complexes with EtBr (2-5) in the interval of changes of r_b -$0 \le r_b \le 0.5$, at $\mu_{Na^+} = 2\cdot10^{-2}$ M.

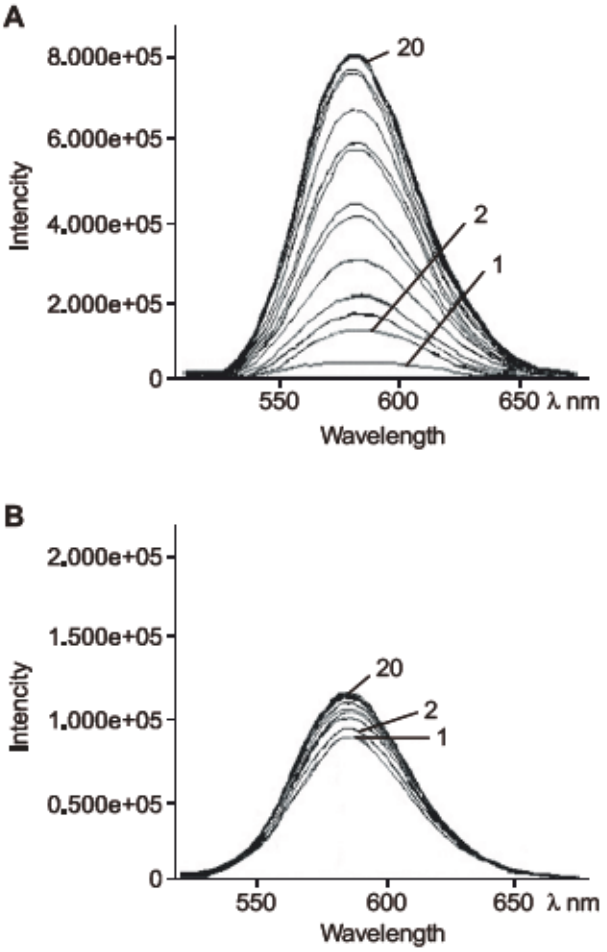


Figure 2. A. The fluorescence spectra of EtBr (1) and its complexes with ds-DNA (2-15) at pH = 7.0; t = 25°C; $\mu_{Na+} = 2 \cdot 10^{-2}$ M. The intensity of fluorescence spectra of complexes increase significantly in comparison with the intensity of EtBr spectrum. B. The fluorescence spectra of EtBr (1) and its complexes with ss-DNA (2-15) at pH = 3.0; t = 25°C; $\mu_{Na+} = 2 \cdot 10^{-2}$ M. The intensity of fluorescence spectra of complexes increase insignificantly in comparison with the intensity of EtBr spectrum.

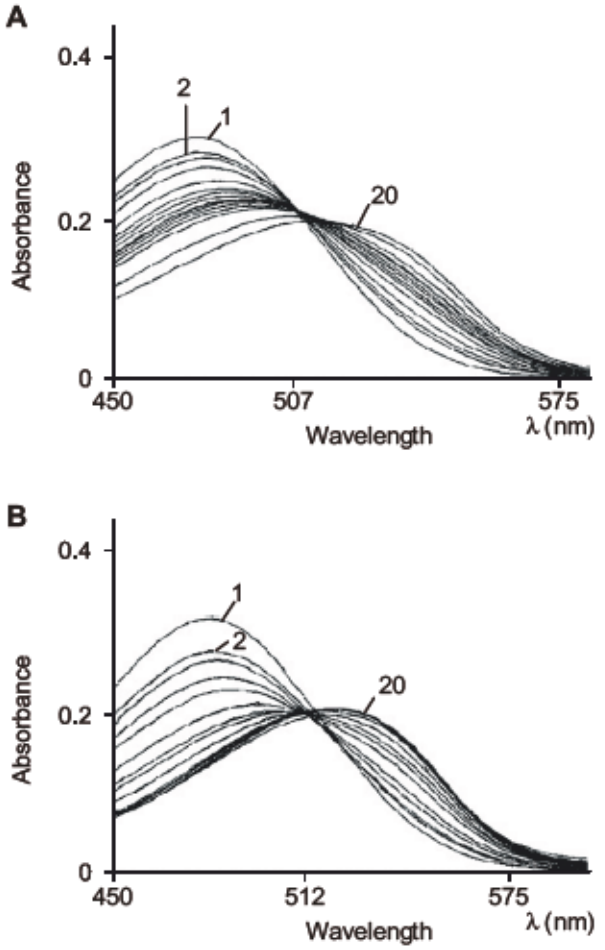


Figure 3. A. The absorption spectra of EtBr (1) and its complexes with ss-DNA (2-15) at λ = 400-600 nm, pH = 3.0, t = 25°C, $\mu_{Na+} = 2 \cdot 10^{-2}$ M. The absorption spectra of complexes decrease in maxima and are shifted in the range of high wavelengths in comparison with those of EtBr itself. There is the isosbestic point on spectra at 507 nm, indicating that, in solution, the EtBr molecules are in two spectrophotometrically diferent conditions bound and unbound. B. The absorption spectra of EtBr (1) and its complexes with ds-DNA (2-15) at λ = 400-600 nm, pH = 7.0, t = 25°C, $\mu_{Na+} = 2 \cdot 10^{-2}$ M. The absorption spectra of complexes decrease in maxima and are shifted in the range of high wavelengths in comparison with those of EtBr itself. There is the isosbestic point on spectra at λ = 512 nm, indicating that, in solution, the EtBr molecules are in two spectrophotometrically different conditions-bound and unbound.

the range of low values of pH in comparison with the curve of DNA itself, that shows a stabilizing effect of EtBr on ds-structure of DNA (see the works (Cantor and Schimmel, 1980; Antonyan *et al.*, 2001; Vardevanyan *et al.*, 2001). Thus, complete denaturation of DNA and its complexes with EtBr occurs at pH = 3.0 and t = 25°C.

For the estimation of EtBr interaction with DNA the suitable values are the binding constant (K) and number of binding site (n). Determination of these parameters allows an adequate description of various ligands influence on conformation and conformational transitions of DNA.

It was recently shown that EtBr could form at least three types of complexes with ds-DNA and two types with ss-DNA (Karapetian *et al.*, 1996). The strong way of binding of EtBr with ds-DNA corresponds to intercalating mechanism of interaction and is characterized by high quantum yield of fluorescence (Borisova *et al.*, 1998, Vardevanyan *et al.*, 2002). On the other hand, Borisova *et al.* (1998) have shown that, except of strong fluorescent complexes, EtBr forms also strong non-fluorescence complexes (NFC).

With the purpose of finding out the contribution of NFC in binding parameters of EtBr with ss- and ds-DNA, we carried out the spectroscopic (absorption

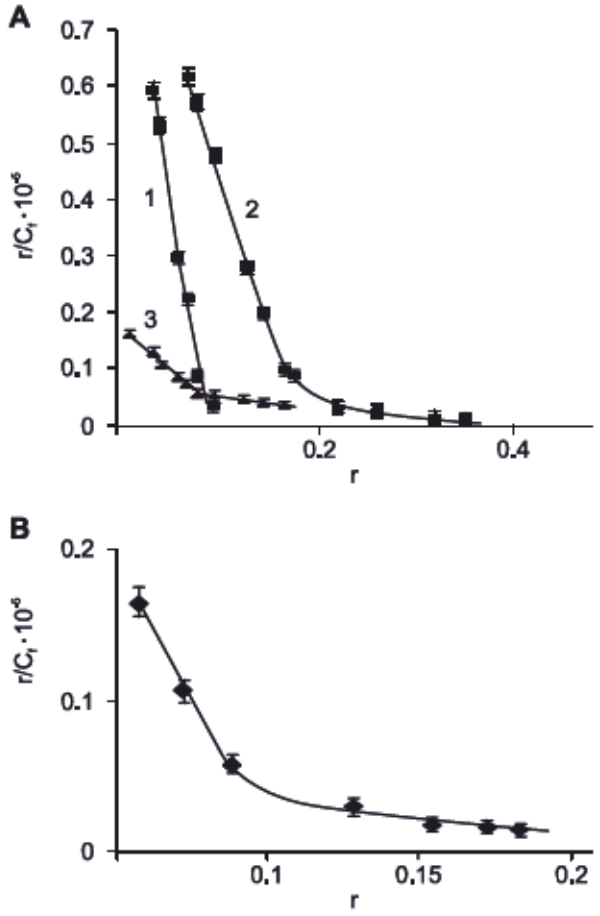


Figure 4. A. The binding curves of EtBr with ds-DNA in Scatchard's coordinates, obtained from fluorimetric (1) and absorption spectra (2). Curve 3 is obtained by subtraction of curve 1 from curve 2 and corresponds to nonfluorescent complexes of EtBr with DNA at pH = 7.0, t = 25°C, $\mu_{Na+} = 2 \cdot 10^{-2}$ M. B. The binding curves of EtBr with ss-DNA in Scatchard's coordinates, obtained at pH = 3.0, t = 25°C, $\mu_{Na+} = 2 \cdot 10^{-2}$ M.

Table 1. Experimental values of K obtained from binding curves of EtBr with ds- and ss-DNA at $\mu = 2.0 \cdot 10^{-2}$ M [Na^+], t = 25°C.

pH = 7.0	pH = 3.0
$K_s'' = 130 \cdot 10^4$ $M^{-1} \pm 0.10$	$K_s' = 14 \cdot 10^4$ $M^{-1} \pm 0.10$
$K_s^f = 100 \cdot 10^4$ $M^{-1} \pm 0.18$	
$K_s^{nf} = 30 \cdot 10^4$ $M^{-1} \pm 0.20$	
$K_w = 3.7 \cdot 10^4$ $M^{-1} \pm 0.15$	$K_w' = 2.3 \cdot 10^4$ $M^{-1} \pm 0.15$

K_s"-the binding constant of EtBr with ds-DNA for "strong" complex received from absorption analysis. K_w"-the binding constant of EtBr with ds-DNA for "weak" complex received from absorption analysis. K_s^f-the binding constant of EtBr with ds-DNA for "strong" complex received from fluorescent analysis. K_s^{nf}-the binding constant of EtBr with ds-DNA for strong nonfluorescent complex received from absorption analysis. Ks'-the binding constant of EtBr with ss-DNA for "strong" complex received from absorption analysis. K_w'-the binding constant of EtBr with ss-DNA for "weak" complex received from absorption analysis. "s"-"strong", "w"-"weak".

Table 2. Experimental values of n, obtained from binding curves EtBr with ds- and ss-DNA at $\mu = 2.0 \cdot 10^{-2}$ M [Na^+], t = 25°C.

pH=7.0	pH=3.0
$n_s'' = 9.0$	$n_s' = 8.0$
$n_s^F = 11.0$	$n_w' = 3.0$
$n_s^{nf} = 8.0$	
$n_w'' = 3.0$	
$n_w^{nf} = 3.0$	

n_s"-the value of n for "strong" complex of EtBr with ds-DNA. n_s^f-the value of n for "strong" fluorescent complex of EtBr with ds-DNA. n_s^{nf}-the value of n for "strong" nonfluorescent complex of EtBr with ds-DNA. n_s'-the value of n for "strong" complex of EtBr with ss-DNA. n_w^{nf}-the value of n for "weak" nonfluorescent complex of EtBr with ds-DNA. n_w-the value of n for "weak" complex of EtBr with ds-DNA. n_w'-the value of n for weak complex of EtBr with ss-DNA. "s"-strong, "w"-weak.

and fluorimetric) study of complexes at pH = 7.0 and pH = 3.0, at t = 25°C ($\mu = 2.0 \cdot 10^{-2}$ M Na^+). In Figure 2, the spectra of fluorescence of EtBr complexes with ds-DNA (A) at pH=7.0 and with ss-DNA (B) at pH = 3.0, t = 25°C are given. As shown in Figure 2, the intensity of fluorescence of complexes EtBr-ds-DNA at pH = 7.0 and t = 25°C monotonously increases with decreasing of r_b since more quantity of unbound ligand molecules intercalated between base pairs. On the other hand, possibility of the redistribution of bound non-intercalated molecules in intercalating sites is not excluded. Since saturation is observed on fluorescence spectra with a certain value of r_b ($r_b \leq 0.1$), this may provide an evidence for the discontinuation of ligand molecule redistribution in intercalated sites. Figure 2B shows spectra of fluorescence of EtBr-ss-DNA complexes at pH = 3.0 and t = 25°C. The intensity of complex fluorescence depending on r_b change altered insignificantly. In all probability, the quenching of EtBr fluorescence takes place, because the complete intercalation in ss-condition is impossible and ligand molecules become accessible for the molecules of water-active quenchers of fluorescence (Lakovich, 1985). For confirmation of the fact of binding of EtBr with ss-DNA, we carried out also spectrophotometric measurements of complexes in the same conditions, as in fluorimetric titration.

In Figure 3, the spectra of absorption of EtBr and its complexes with ss-DNA (A) at pH=3.0 and ds-DNA (B) at pH = 7.0, t = 25°C, are given. As shown in Figure 3, the absorption spectra of complexes depending on r_b change decrease in maxima and are

shifted in the range of high wavelengths in comparison with those of EtBr itself. Moreover, the isosbestic point was observed at $\lambda = 507$ nm indicating that the ligand molecules are in two spectrophotometrically distinguished condition - bound and free - as it occurs at EtBr interaction with ds-DNA (Figure 3B). Based on these data, it is possible to suggest that EtBr interact also with ss-DNA. This interaction, however, fails to be subjected to a quantitative estimation due to suppression of fluorescence.

A convenient method for obtaining of values of K and n is the Scatchard analysis-the construction of dependence of r/C_f on r. The values of r and C_f are determined from spectra of absorption and fluorescence (see section Data analysis). Figure 4A shows the binding curve of EtBr with ds-DNA, received from spectra of fluorescence and absorption. Curve 1, obtained by the Scatchard-analysis of fluorescence spectra of EtBr-ds-DNA complexes, is linear, and corresponds to one, intercalation way of binding (Borisova *et al.*, 1998). Curve 2, obtained from the analysis of absorption spectra, is nonlinear, and indicates that the molecules of EtBr cooperate with ds-DNA by more than one way. It is remarkable that these curves differ: the inclination of a binding curve of fluorescent molecules of EtBr (curve 1) is less than the inclination of a curve received from absorption spectra (curve 2). The subtraction of curve 1 from curve 2 results in a curve of EtBr binding with ds-DNA by the non-fluorescent way (curve 3), also given in Figure 4A. This curve is nonlinear and consists of two linear sites appropriate to strong and weak ways of binding.

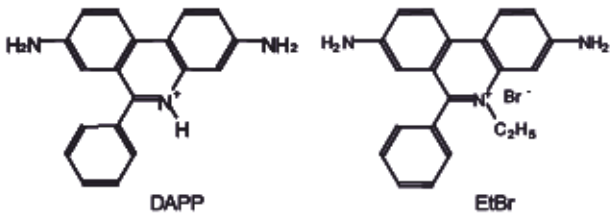
Figure 4B shows the binding curve of EtBr with ss-DNA, received at pH = 3.0, t = 25°C. In the work (Karapetian *et al.*, 1996), theoretical calculations reveal that EtBr can interact with ss-DNA at least by two independent ways. We therefore analyzed the received curve, based on this reason.

Discussion

The effectiveness of most drugs that exert their biological activity by direct binding to DNA is due to their ability to form the covalent and non-covalent complexes with polynucleotide (Haroutiunian *et al.*, 1998). They may locally blockade or initiate replication and transcription of DNA by intercalating and poisoning topoisomerases or by binding in the minor groove and inhibiting transcription (Searcey *et al.*, 1998). The researches on the ligand interaction with DNA at acidic pH draw special interest intriguing fact that protonation specifically affects the structure of biopolymer. It is known, that at acidic pH, the double-stranded DNA is denatured. However, at one-half an equivalent of [H^+] protons bound per Cytosine (C) base residues, there is an extremely sharp transition to the acidic helix form C-H^+-C. If more protons are added, this helix becomes destabilized, and future titration eventually converts it into two single-stranded, fully protonated poly CH^+. The acid double helix of poly(A) also is not a fully protonated form. However, the protons added to poly(A) are not directly involved in base pairing. The acid form becomes stable relative to single strands at degrees of proton uptake far less than one per A residue. The further reduction of pH results in destabilization of these structures and formation of one-stranded helixes with protonated bases AH^+ and CH^+ (Cantor and Schimmel, 1980). It is obvious that the protonation of bases brings about an increase of a total positive charge, resulting in somewhat different interaction between positively charged ligands rather, than at neutral values of pH.

Taking into account the above mentioned, acidic values of pH were chosen as the factor of an environment for research of EtBr interaction with ss-DNA. However, it is necessary to consider pK_a of ligand. It is known that pK_a of analogue of EtBr-3,8-diamino-6-phenylphenantridine (DAPP) remains constant at given ionic strength, and the optical characteristics of this compounds coincide with those for EtBr at neutral, as well as at acidic values of pH, therefore we did not control this parameter (Jones and Wilson, 1981).

The analysis of binding curve of EtBr with ds-DNA, given on Figure 4A reveals that this ligand binds with ds-DNA at least by three ways. It specifies that the binding curves of EtBr with ds-DNA received from fluorescence and absorption spectra are differed (curves 1 and 2). This distinction is caused by the presence of non-fluorescent molecules of EtBr on ds-DNA. From binding curves 1 and 2 the values of K are acquired by two independent methods, generalized in table 1. The values of K_s obtained from curve 1 and 2 (Figure 4A) differ whilst $K_s^f < K_s$ (where K_s^f is binding constant of fluorescent molecules of EtBr, K_s-constant of binding of EtBr with ds-DNA, obtained by absorption method). This distinction is due to as they can bind with ds-DNA by two various ways. Values of K_s, obtained from a binding curve 2 (Figure 4A), corresponds to strong fluorescent and strong non fluorescent (semi-intercalating) types of EtBr binding with ds-DNA, since K_s^{nf}, acquired from a binding curve 3 (Figure 4A), coincides with a differ-



ence K_S-K_S^f. The analysis of binding curves of EtBr with ss-DNA reveals that this ligand binds with ss-DNA at least in two ways, one of which is strong, $K_S'/K_W' \sim 10$ (K_S' corresponds to the "strong" binding constant with ss-DNA, K_W' the same parameter corresponding to the weak interaction) as it was predicted in the work (Karapetian *et al.*, 1996) and, on the other hand, the value of K_S^{nf} practically coincides with one received at t = 70°C (Vardevanyan *et al.*, 2000). It specifies that this type of binding has an intercalation nature, though the mechanisms of influence of pH as well as temperature essentially differ from each other. Moreover, the value of K_S^{nf}, appropriate to non-fluorescent strong complex of EtBr with ds-DNA coincides with those received for complexes of EtBr-ss-DNA ($K_S^{nf}/K_S' \approx 2$). The values of K_W^{nf} and K_W' also are in good accordance (see Table 1). In the work (Wadkins *et al.*, 1996) is shown that actinomycin D (AMD) interacts with ss-polynucleotides. Moreover, aromatic ring of AMD is partially inserted in a plane between the neighbor bases, *i.e.* semi-intercalated in a molecule of ss-DNA. In this work is shown also that AMD interacts with ss-DNA by several ways. The work (Karapetian *et al.*, 1996) reveals the possibility of binding of EtBr and AMD with DNA by several types and the thermodynamic parameters of binding of these ligands with ds- and ss-DNA practically coincide. Therefore, EtBr, as well as AMD, will bind with ss-DNA in more than one way, where a "strong" way is of an intercalative nature.

Thus, the obtained values of K revealed, that EtBr may interact both with ds- and ss-sites of DNA (Antonyan *et al.*, 2001; Vardevanyan *et al.*, 2001). It is reasonable to assume that a non-fluorescent strong complex both with ds-, and ss-DNA is formed by partial intercalation of EtBr molecules into a plane of the neighbor bases of one strands of DNA. Most probably, the phenantridine ring of EtBr inserted in one of ds-DNA grooves (Karapetyan *et al.*, 2001), and becomes accessible to molecules of water - intensive quenchers of fluorescence (Lakovich, 1985).

The acquired values of a number of base pairs - n reveal, that the n_S^f for fluorescent molecules of EtBr exceeds the value of n_S, appropriate to a "strong" mode of EtBr binding with ds-DNA and that received from absorption analysis (Table 2). It specifies that while intercalating the number of binding sites is limited, than in the other ways of interaction. The value of n_S, obtained for interaction of EtBr with ss-DNA, coincides with this parameter obtained for non-fluorescent molecules of EtBr on ds-DNA that indicates the similarity of binding sites. The values of n_W are identical for "weak" binding type both for ds- and ss-DNA, as they correspond to electrostatic (weak) type of interaction with phosphate groups of DNA (Table 2). The value of n_W is half that of the n_S and corresponds to electrostatic mechanism of interaction of positive charged molecules of EtBr with negatively charged phosphate groups of DNA. This fact indicates that those electrostatic binding sites are more accessible for molecules of ligand. From this point of view it is necessary to note that phosphate groups of DNA at pH = 3.0 are protonated particularly and in neutral conditions these groups are nearly fully disprotonated (Seanger, 1987). Therefore, the possibility of electrostatic interaction between ligand molecules and DNA can not be ruled out.

Thus, on the basis of the obtained results it is possible to suggest that strong non-fluorescence complexes of EtBr with ds-DNA correspond to strong semi-intercalating complexes with ss-DNA. It is necessary also to note that NFC plays an important role in stabilizing of ds-structure of DNA, that was not taken into account in earlier works. On the other hand, the binding of EtBr with ss-DNA does not depend on factors of the environment (temperature, pH).

The presented data can be useful in understanding of mechanisms of interaction of various drugs with DNA in processes of metabolism of a cell and the organisms as a whole, and also by development of new medicinal preparations.

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