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Differential pulse voltammetric studies of ethidium bromide binding to DNA

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Abstract

The interaction of ethidium bromide (EtBr) with calf thymus DNA is investigated electrochemically with the use of differential pulse voltammetry (DPV) at two different ionic strengths of a solution (0.154 M and 0.02 M [Na^+], pH 7.0). It is revealed that EtBr binds with DNA in more than one way. The appropriate values of constants (K) and number site sizes (n) of EtBr binding to DNA are determined. The values of binding constants are equal to $1.9 \cdot 10^6$ and $5.6 \cdot 10^5$ M^{-1}, and number site sizes to 9 and 3.6 for strong interactions at ionic strengths of solutions 0.02 and 0.154 M Na^+ at 28 °C, respectively. For a weaker interaction, these parameters are equal to $7 \cdot 10^4$ and $8 \cdot 10^4$ M^{-1} and 1.5 and 1 at the mentioned ionic strengths of solutions, respectively. Thus, EtBr interacts with DNA in more than one way—intercalative and electrostatic at low ionic strength, and semi-intercalative and electrostatic at a higher strength of the solution. These results are in good accordance with the ones obtained by spectroscopic (absorption and fluorimetric) methods.

Keywords: DNA; EtBr; Differential pulse voltammetry; Binding constant; Number site sizes; Intercalation

1. Introduction

The study of interaction of various low weight substances–ligands with DNA is urgent, as being bound in various ways (covalent, non-covalent, intercalative or external, electrostatic binding); ligands can change conformations of macromolecules in different ways. The formation of various complexes of DNA with ligands may lead to changes of its structure and functional activity. The study of DNA–ligand complexes may serve as a basis for getting new preparations specifically interacting with certain sequences of DNA [1–3]. From this point of view, the classic intercalator and fluorescent dye ethidium bromide (EtBr) is suitable for such an investigation, as it binds with DNA in several ways [4–8]. EtBr being the stabilizer of double-stranded (ds) structure of DNA specifically binds with single-stranded (ss) oligonucleotides and polynucleotides as well [9–11]. The specificity of binding to ss- or ds-sites of DNA may play an important role in functioning of biologically active low weight substances as well as DNA in different processes of cell cycle [9].

The EtBr binding to DNA is studied by various methods (absorption, fluorescent, NMR) (see for example [5,6,11]). Recently, many authors have used electrochemical methods to study the interaction of small molecules with DNA [12–20]. The electrochemical investigations of DNA binding molecule interactions can provide a useful complement to spectroscopic methods (for non-absorbing species). Electrochemical methods differ by their sensitivity, simplicity and versatility and yield in information about the mechanism of binding. Electrochemical investigations of DNA binding molecule interactions based on

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different electrochemical activities of free and bound DNA molecules were first applied by Berg and Eckardt [21].

Recently, there has been a tremendous progress towards the development of electrochemical DNA biosensors. Planar aromatic organic compounds, which also include EtBr, are redox-active markers used in DNA hybridization biosensors. The identification and quantitative research of various mechanisms of low weight molecule interaction with DNA is useful at a choice of a marker and definition of conditions of their application in DNA biosensors [22–27].

Interaction of EtBr with DNA has also been investigated by a method of voltammetry with wall-jet flow system [28] and by using alternating current voltammetry [29,30]. Recently, a more sensitive and accessible method of differential pulse voltammetry has been applied to survey other ligands complex-formation with DNA [15,16]. As a result, it was supposed that the use of DPV will allow to reveal different ways of ligand binding to biopolymers and estimate the parameters of interaction, at multimodal ligand EtBr with DNA complex-formation, in particular.

The purpose of the present work is the study of different modes of EtBr binding to DNA by the method of the DPV and the determination of K and n (K—binding constant, n—number of bases corresponding to one site of binding), as well as the comparison of the obtained data with the results received by spectroscopic methods.

2. Materials and methods

2.1. Reagents

Calf thymus DNA was purchased from "Sigma" (USA), EtBr from "Serva" (Germany), and NaOH, methanol and acetone (ultrapure) from "Fluka". All preparations were used without further purification. The physiological solution of sodium chloride 0.9% w/w (0.154 M NaCl) for internal injections (PharmaTech CJSC, Armenia) was used as a background electrolyte and solvent for DNA and EtBr. For preparation of DNA and EtBr solutions in background electrolyte with ionic strength of 0.02 M NaCl, the physiological solution was diluted by deionized water (H_2O Economy, LLC, Armenia-US JC) with R 16 MΩ. Solutions of DNA and EtBr were made in the two above-mentioned solutions 0.02 M and 0.154 M sodium chloride. They were deoxygenated via purging with N_2 gas for barboting of electrolyte solutions.

The concentrations of stock solutions of DNA and EtBr were determined by absorption spectroscopy, using values of molar extinction for DNA (per one nucleotide) $\varepsilon_{260}=6000\ M^{-1}\ cm^{-1}$ and for EtBr $\varepsilon_{480}=5600\ M^{-1}\ cm^{-1}$, respectively [11].

2.2. Apparatus

Voltammetric measurements of solutions were carried out through a three-electrode system (3C cell stand) electrochemical analyzer BAS 100 B/w (Bioanalytical Systems Inc., US) with the computer management of a special program "BAS". The glassy carbon electrode (GCE) was used as a working electrode, while the Ag/AgCl (in 3.0 M NaCl) electrode and Pt wire were used as reference and auxiliary electrodes in this experiment, respectively. The experiments were carried out in an electrochemical cell with thermostatic water shirt connected with circulating thermostat (all electrodes, circulating electrochemical cells from Bioanalytical Systems Inc., US). The temperature in the electrochemical cell was established with accuracy of ±0.1 °C. The work of the electrochemical analysis system was checked up with the help of a running solution $Fe(CN)_6^{-3}$, for which calibration linear correlation coefficient of 0.9991 was obtained.

The diagrams of functions, correlation equations and coefficients of correlation were received with the help of the program Excel (Microsoft). Adsorption isotherms and the thermodynamic parameters of EtBr binding to DNA were obtained with the help of the mathematical program SigmaPlot (version 4).

2.3. Procedure of DNA–EtBr complex-formation studies

To carry out experiments, two solutions of DNA with ionic strengths 0.02 M and 0.154 M [Na^+] were used. The value of pH=7.0 was controlled with the help of ionomer-universal EV-74 (USSR) in all the experiments.

Before each experiment, the GCE was thoroughly serially washed out for 8–10 times with methanol, acetone and deionized water, and then polished with 0.05 μm alumina powder (Bioanalytical Systems Inc., US). The polished electrode was serially washed out for 8–10 times with methanol, deionized water and acetone. Then this was rinsed in deionized water and activated by the method described [26]. The activation of GCE electrode was done by applying potential 1.20 V (electrode of comparison Ag/AgCl (in 3.0 M NaCl)) in a mixed aqua solution 0.5 M NaOH for 10 min. After the activation, GCE was washed out with deionized water and by background electrolyte. The above-mentioned solution was mixed with the help of a magnetic mixer at a speed of 700 ppm, using Teflon-coated magnetic stirring bar. Solutions were previously barbotated by gas nitrogen for 60 min to remove the dissolved oxygen.

Cyclic volammetric experiments were performed with sweep rate of 50 mV s^{-1} and in a potential range from −300 to 1150 mV as well as from −1000 to 1150 mV.

Differential pulse voltammograms of EtBr solutions (in the presence and absence of DNA) were carried out in a potential range from 300 to 1150 mV. The value at characteristic potential of oxidation 700±20 mV was used for voltammetric titration, which depended on the concentration of electrochemically active free (unbound with DNA) molecules of EtBr. Before each titration of EtBr by

DNA, the dependence of value limiting the current on initial concentration of EtBr and calibration plot was checked. The values of a limiting current were calculated by automatic subtraction of a background current. DPV was registered after 20–25 min of the input of each amount of titrant in a mixing solution. The total volume of an entered solution of titrant increased the volume of the initially titrated solution by not more than 8%. It should be noted that DNA and its complexes with EtBr in the investigated range of potentials do not show registered electrochemical activity (explanation in the text). The concentration of free molecules of EtBr in the initial solution as well as in the process of titration by DNA was determined from DPV, using calibration dependences of anodic currents peak magnitudes on ligand concentration.

2.4. Determination of EtBr binding parameters with DNA

Isotherms of EtBr adsorption on DNA were constructed in Scatchard's coordinate (r/C_f on r, $r=C_b/C_p$, where C_b—concentration of bound with DNA molecules of ligand, and $C_b=C_0-C_f$, where C_0—total concentration of EtBr, C_f—concentration of free molecules of ligand in a solution and C_p—concentration of phosphate groups of DNA). The experimental binding curves were analyzed by the method of regression analysis of theoretical curves obtained from Crothers' equation [31]:

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

and transformed by Arakelyan et al. for a case of two types of ligand–DNA interaction [32]:

$$r/C_f = K[1-(2n-1)r] \quad (2)$$

The meanings of K and n were determined from adsorption isotherms according to Eq. (2).

3. Results

For the specification of the terms for DPV measurements, we obtained cyclic voltammogram of EtBr solution (in the absence and presence of DNA), given in Fig. 1. The peaks of irreversible oxidation of EtBr were revealed at 685±10 mV, the reduction at −550±10 mV, which is in accordance with the data of work [28,33]. The differences in the electrochemical behaviors of the EtBr molecules in the absence and presence of DNA (Fig. 1, curves 2 and 3) were quite obvious. The anodic peak of oxidation, which corresponded to the process of electrochemical oxidation of NH_2 groups of EtBr, was chosen to obtain more reproduced results [28]. In this range of potentials, DNA is electrochemically inactive and peaks of potential appropriate to guanine and adenine are observed at significantly higher values [34].

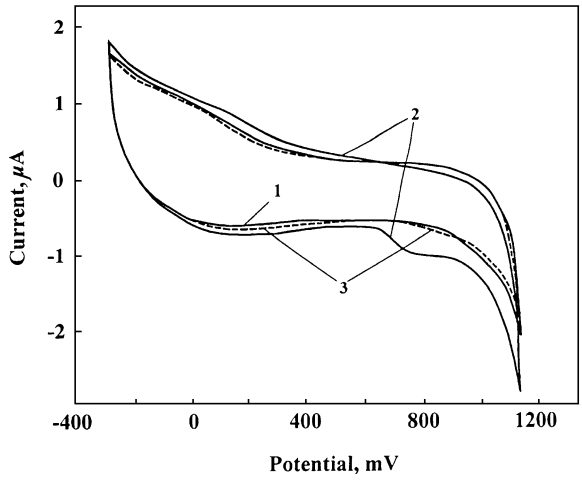

Fig. 1. Cyclic voltammograms of $2.77\cdot10^{-4}$ M DNA (1), $3.301\cdot10^{-6}$ M EtBr in the absence (2) and presence of $2.774\cdot10^{-4}$ M DNA (3), respectively. Ionic strength of solution $\mu_{Na^+}=0.154$ M, potential-scan rate: 100 mV s^{-1}, initial potential 1150 mV.

As it follows from Fig. 1, the characteristic peak of EtBr at 685±10 mV is sensitive to DNA entered in an EtBr solution and, at 80 multiple surpluses of the latter, the peak becomes non-detected (curve 3). Near to potential 685±10 mV DNA and the molecules of EtBr bound with it are electrochemically inactive (curves 1 and 3, respectively). With lower concentrations of ligand, we used the method of the DPV, in order to more accurately determine the EtBr–DNA binding parameters.

DPV measurements of EtBr solutions in the presence and absence of DNA were carried out in a potential range from 300 to 1000 mV and it was revealed that the anodic peak of EtBr oxidation corresponded to 730±30 mV, which is appropriate to the potential of EtBr oxidation in two solutions with ionic strengths 0.02 M and 0.154 M [Na^+]. It should be noted that the peak of anodic oxidation of EtBr, shifted to more positive potentials up to 60 mV, depends on the concentration of the added DNA. A similar experimental fact was observed in the work [15]. As it is shown in Fig. 2 (see curves 1 and 11), the essential difference in electrochemical activity of free and bound EtBr at 730±30 mV allows to carry out a reliable quantitative analysis of voltammetric measurements, particularly by the method of DPV.

For the definition of the terms of titration (optimal concentration of free EtBr and the interval of a ligand/DNA concentrations ratio change) the calibration curve—the linear dependence of DPV anodic peak current on the concentration of EtBr in an interval from $2.5\cdot10^{-7}$ M up to $2.0\cdot10^{-5}$ M. In the above-mentioned conditions is kept the linear dependence of DPV peak current force on the concentration of free molecules of EtBr with rather high coefficient of correlation. The equations of linear regression are the following:

$$i_{pa}(\mu A) = 3.293\cdot10^{-2} + 6.082\cdot10^{4}[\mathrm{EtBr}],\ R^2 = 0.9851 \quad (3)$$

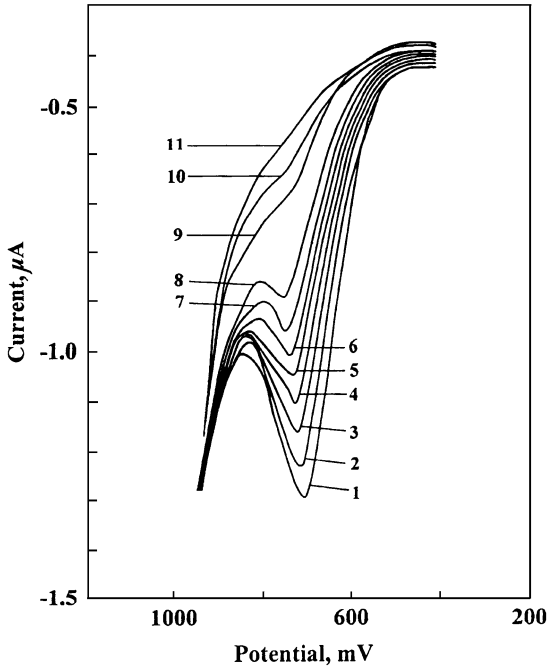

Fig. 2. Differential pulse voltammograms $1.162 \cdot 10^{-5}$ M EtBr in the absence (1) and presence (2) of $8.17 \cdot 10^{-6}$ M, (3) $1.218 \cdot 10^{-5}$ M, (4) $1.612 \cdot 10^{-5}$ M, (5) $2.001 \cdot 10^{-5}$ M, (6) $2.390 \cdot 10^{-5}$ M, (7) $2.775 \cdot 10^{-5}$ M, (8) $3.526 \cdot 10^{-5}$ M, (9) $6.022 \cdot 10^{-5}$ M, (10) $9.874 \cdot 10^{-5}$ M, (11) $1.531 \cdot 10^{-4}$ M DNA. Scan rate 20 mV s^{-1}. Initial $E = 300$ mV and pulse amplitude 100 mV. Ionic strength of solution $\mu_{Na^+} = 0.02$ M.

At ionic strength of a solution 0.02 M [Na^+] and

$$i_{pa}(\mu A) = 5.5876 \cdot 10^{-2} + 7.846 \cdot 10^{4}[\text{EtBr}],\ R^2 = 0.9869 \quad (4)$$

at ionic strength of a solution 0.154 M [Na^+].

The titration of EtBr solutions could be accordingly carried out with initial concentration equal to $4.94 \cdot 10^{-6}$ M at ionic strength of 0.154 M and $\sim 1.15 \cdot 10^{-5}$ M at ionic strength of 0.02 M. The titration of EtBr solutions was carried out by DNA solution with initial concentration of $1.24 \cdot 10^{-3}$ M to achieve a residual concentration of free EtBr below one percent of its initial one.

Adsorption effects do not have an essential influence on the results of measurements. The above-mentioned linear dependence of the peak current on the concentration of EtBr ((3) and (4)) testifies the absence of adsorption effects connected with EtBr. Simultaneously, the additional peaks of potentials connected with adsorption effect of EtBr on an electrode in its cyclic voltammogram are not observed either in the presence or in the absence of DNA in a solution [35]. It also specifies the absence of adsorption effects connected with both EtBr and DNA (see Fig. 1). With an excess of EtBr in relation to DNA in a solution, the linear dependence of the current on concentration of EtBr is observed. Thus, the relation $di_p/d[\text{EtBr}]$ with ±10% accuracy coincides with that received in the absence of DNA. Once again, it shows the insignificant small contribution of DNA adsorption to electrode processes.

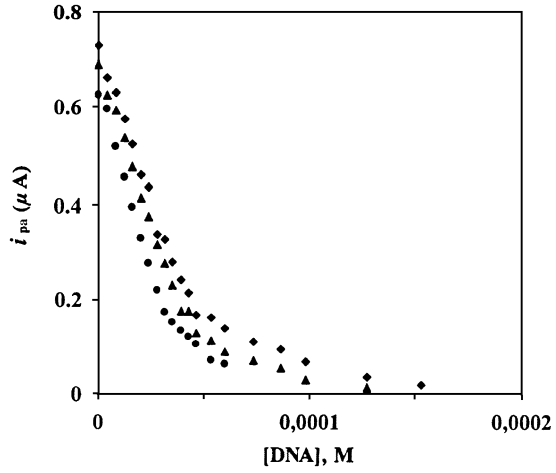

Fig. 3. Relationship between the DPV peak currents and the concentration of DNA at starting concentrations (♦) $1.189 \cdot 10^{-5}$ M, (▲) $1.162 \cdot 10^{-5}$ M and (●) $1.137 \cdot 10^{-5}$ M EtBr. Other conditions were the same as in Fig. 2.

By using the data of differential pulse voltammograms of titrating solutions (which example was given in Fig. 2), titrating curves were built (see Fig. 3). The obtained results indicate that it is possible to find out interaction of EtBr with DNA by the method of DPV as well as to carry out a quantitative analysis and to determine parameters of interaction—K and n. From the titration curves using the equations of linear regression ((3) and (4)), the experimental values of r/C_f and r are obtained and the binding isotherms are built in these coordinates (Figs. 4–6). Figs. 4–6 show

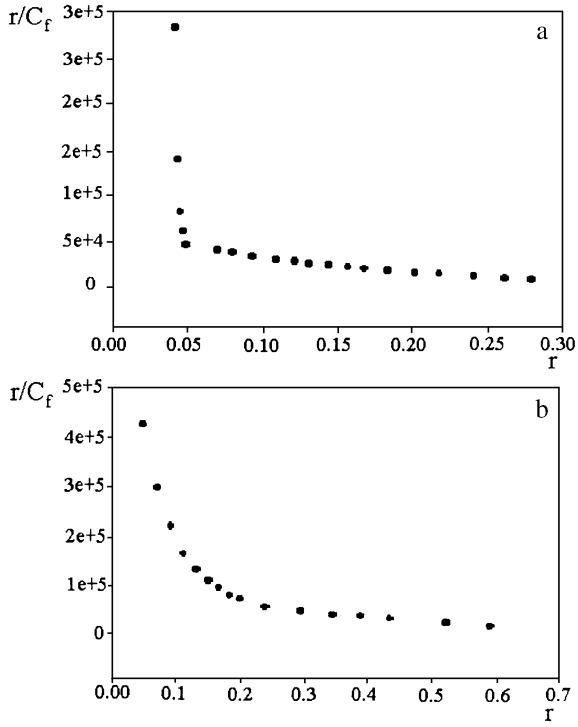

Fig. 4. EtBr binding curves to DNA obtained at ionic strengths of solution $\mu_{Na^+} = 0.02$ M (a) and $\mu_{Na^+} = 0.154$ M (b) by Crothers' equation. Curves consist of two parts, corresponding to two types of binding.

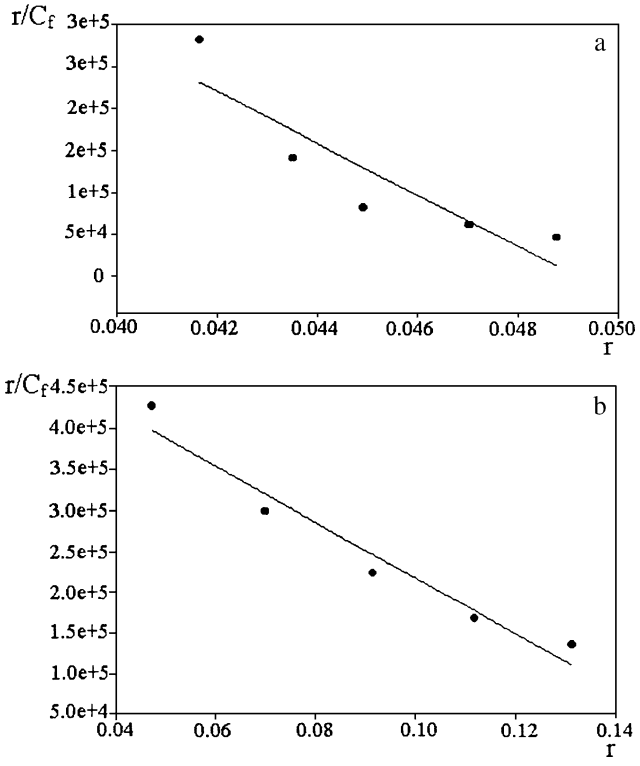

Fig. 5. EtBr binding curves to DNA in Scatchard's coordinates correspond to strong modes and obtained at ionic strengths of solution μ_{Na^+}=0.02 M (a) and μ_{Na^+}=0.154 M (b) by Arakelyan's Eq. (3).

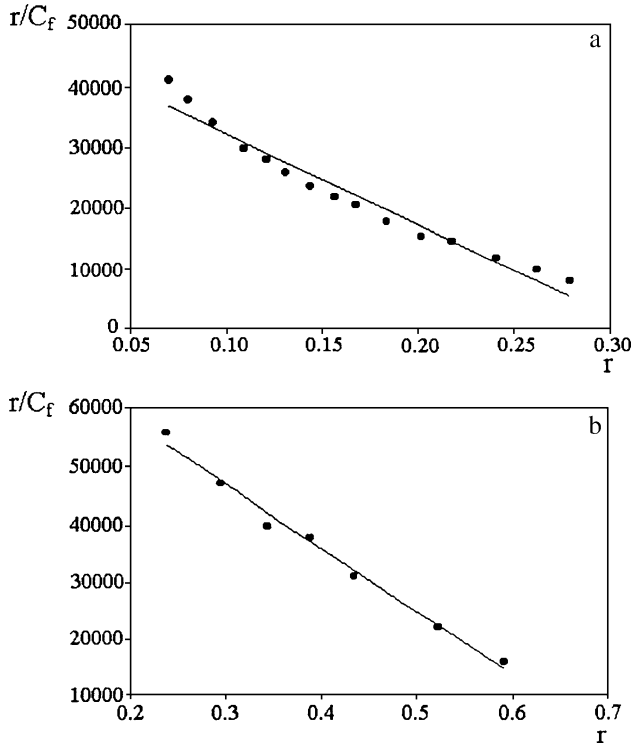

Fig. 6. EtBr binding curves to DNA in Scatchard's coordinates correspond to weak types and obtained at ionic strengths μ_{Na^+}=0.02 M (a) and μ_{Na^+}=0.154 M (b) by Arakelyan's Eq. (3).

Table 1
Thermodynamic parameters of EtBr binding to DNA obtained at ionic strengths of solution μ_{Na^+}=0.02 M and μ_{Na^+}=0.154 M

μ_{Na^+}	K, M^{-1}		n	
	K_1	K_2	n_1	n_2
0.02 M	$1.9\cdot10^6$ M^{-1}	$7.0\cdot10^4$ M^{-1}	9	1.5
0.154 M	$5.6\cdot10^5$ M^{-1}	$8.0\cdot10^4$ M^{-1}	3.6	1.0

Temperature is equal to 28 °C.

that there are at least two types of binding. For the analysis of experimental data on ligand adsorption on macromolecules, it is important to achieve a state when all sites on a macromolecule are completely filled with ligands. However, in some cases, due to a weak binding of ligand to a macromolecule, it is not possible to achieve a completely bound state [31,36,37]. For this reason, it is expedient to estimate the parameters of adsorption in a small-filling domain, where only a small amount of ligand is bound to DNA. In the work [33], the isotherm of ligand adsorption on macromolecules is obtained at small filling. The process of adsorption is considered as a quasichemical reaction of ligand binding from solution to free sites available on the macromolecule. Thus, the macromolecule is represented as a one-dimensional crystal with N sites of binding, while the ligand, having much smaller linear sizes, occupies n sites on macromolecule. At small fillings, an isotherm obtained in the work [32] is linear (see Eq. (2)). For this reason, the parameters of binding corresponding to two types are received according to Eq. (2), which are presented in Table 1. For comparison, in Table 2 are given the values of K and n, corresponding to intercalative and semi-intercalative complexes of EtBr binding to ds-DNA, obtained by absorption and fluorimetric experiments.

4. Discussion

The theoretical and numerous experimental studies on interaction of EtBr with DNA have revealed that this ligand can bind with polynucleotides in several ways (see for

Table 2
Values of constants and site sizes of EtBr binding to DNA by intercalating and semi-intercalating modes at ionic strengths of solution μ_{Na^+}=0.02 M

K_1^a, M^{-1}	K_2^a, M^{-1}	n_1^a	n_2^a
Strong intercalating mode of EtBr binding to DNA			
$1.3\cdot10^6$ M^{-1}	$3.7\cdot10^4$ M^{-1}	11	1.5
Strong semi-intercalating mode of EtBr binding to DNA			
$3.0\cdot10^5$ M^{-1}	$2.3\cdot10^4$ M^{-1}	4	1.5

Temperature is equal to 28 °C.
Reliability of estimated parameters—$P<0.0002$.
K_1—binding constant by strong mode; K_2—binding constant by weak mode; n_1—number site size, corresponding to one binding site by strong mode; n_2—number site size, corresponding to one binding site by weak mode.
[a] Data obtained from [30].

example [4,14]). Thus, it is shown that, at comparatively low concentrations, EtBr molecules intercalate in a plane between base pairs of DNA with a binding constant of $K=10^6-10^7$ M^{-1} and a number of site sizes—$n\approx 10$ b.p. [4,14,38]. Spectrophotometric (absorption and fluorimetric) experiments have revealed that intercalative EtBr may also form semi-intercalative complexes with the binding constant of $K=10^5-10^6$ M^{-1} and a number of site sizes—$n\approx 4$ b.p. [5]. However, it is necessary to note that the semi-intercalative method of EtBr binding is revealed from the comparison of absorption and fluorimetric data [5,11]. Simultaneously, DPV has allowed to find out the different types of binding during one titration. The results of research have revealed that molecules of EtBr interact with DNA in at least two independent types—strong and weak, as $K_s/K_w\approx 30$ at ionic strength of $\mu_{Na^+}=0.02$ M and $K_s/K_w\approx 10$ at $\mu_{Na^+}=0.154$ M, where K_s and K_w are binding constants of EtBr to DNA in strong and weak ways, respectively. Thus, it is obvious that the strong type corresponding to the intercalative one depends on the ionic strength of the solution. The authors of the work [39] concluded that the change of ionic strength of a solution does not influence the intercalative type of binding. However, a certain dependence of intercalation on ionic strength is observed, as in this way the molecules of a ligand bind by stacking interactions with aromatic groups of the DNA bases. Alongside with hydrophobic forces, the stacking ones, having an electrostatic nature [40], are stabilized by van der Waals' binds as well. Hence, the ionic strength of a solution, though in a weak manner, influences the intercalation. Moreover, as it follows from the results [41], the binding constant of EtBr molecules to DNA in an intercalation way decreases with the increase of the ionic strength of the solution. Taking this into account, we assume that intercalative EtBr–DNA complexes appear only at low ionic strengths of a solution ($\mu\leq 0.154$ M Na^+). Taking into account the above-mentioned, it is possible to conclude that the strong way of EtBr binding to DNA is realized for the account of intercalation of molecules of ligand in a plane between base pairs of DNA. The other criterion of ligand binding to DNA by the given mode may also serve the parameter of stoichiometry of saturation—n. As Table 1 shows, ionic strength $\mu_{Na^+}=0.02$ M $n\approx 10$ reflected the binding of EtBr molecules to DNA in an intercalative way and is coordinated with the model of exclusive sites of binding [42]. For comparison in Table 2, the analogue values of parameters of EtBr binding to ds-DNA obtained from fluorimetric experiments are given. The good coincides of obtained results by two different methods is apparent. It is necessary to mention that fluorimetric methods registered only the intercalative type of binding, as at complete intercalation EtBr molecules are shielded from aqua molecules—active quenchers of fluorescence and the intensity of EtBr fluorescence increases twice as high [43].

At $\mu_{Na^+}=0.154$ M, the values of n ($n=3.6$) coincide with the one corresponding to semi-intercalation way of binding revealed by absorption method ($n=4$). The value of K is also in a good conformity with the similar parameter of semi-intercalating complexes of EtBr (see Tables 1 and 2). Proceeding from this, we propose that at low ionic strengths of a solution the basic type of EtBr binding to DNA is intercalation, while the semi-intercalative type of interaction prevails at higher ionic strengths. At high ionic strengths of solutions, the DNA molecules become more compact and the complete intercalation of EtBr molecules on a plane of base pairs becomes more difficult, as it should be accompanied by unwinding of DNA helix. As it is shown [40], at high ionic strengths of solutions, the molecules of hydrate environment of DNA "sew" the edges of the sugar–phosphate skeleton on the part of a large groove, in the result of which the molecule as a whole becomes more compact and unwinding. Therefore, the complete intercalation of EtBr molecules becomes energetically non-beneficial. As it is shown [11] for semi-intercalative type of binding, such restrictions become insignificant and the phenanthridine ring of EtBr molecules is built on the part of a narrow groove in a plane between the neighboring bases of DNA, which remains accessible to the molecules of ligand at high ionic strengths of a solution. The obtained results are in good conformity with the theoretical generalized data [4], where it is shown that EtBr forms two types of strong complexes with ds-DNA.

It is known that EtBr may interact with DNA also by electrostatic mechanism [4,43]. The weaker type of EtBr binding to DNA revealed at high concentration of EtBr and at ionic strength $\mu_{Na^+}=0.02$ M and at $\mu_{Na^+}=0.154$ M corresponds to this mechanism, and the values of K and n, obtained in these conditions, almost coincide. However, it is necessary to note that at ionic strength $\mu_{Na^+}=0.02$ M the weak way of binding is shown at lower concentrations of the ligand, than at ionic strength $\mu_{Na^+}=0.154$ M. It is probably caused by stronger shielding of negatively charged phosphate groups of DNA by cations of electrolyte at high ionic strengths, so higher concentrations of ligand are required for electrostatic binding. Therefore, the value of n obtained at $\mu_{Na^+}=0.154$ M is less than at $\mu_{Na^+}=0.02$ M, while the binding constants at both ionic strengths of a solution have almost the same meaning.

Thus, generalizing the data obtained, it is possible to conclude that molecules of EtBr interact with DNA in several ways, which were suggested on the basis of theoretical calculations [4], and is experimentally proven by the results of absorption as well as electrochemical methods. The comparison of results obtained by different methods allows to conclude that the method of differential pulse voltammetry is a successful and informative supplement to widely applied spectral methods when studying the DNA–ligand complex-formation. Moreover, the method of the DPV is more productive, as it allows to reveal the interaction of ligands with DNA in more than one mode.

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