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МОЛЕКУЛЯРНА БІОФІЗИКА

THERMODYNAMICAL ANALYSIS BY ^{1}H NMR OF THE COMPLEXATION OF ETHIDIUM BROMIDE TO A DNA OCTAMER, 5′-d(GpApCpApTpGpTpC), IN AQUEOUS SOLUTION

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The self-association of the self-complementary deoxyoctanucleotide, 5′-d(GpApCpApTpGpTpC), and its complexation with the phenanthridinium drug, ethidium bromide (EB), has been studied by 1 D and 2D 500/600 MHz NMR spectroscopy. 2D homonuclear correlation PMR spectroscopy (TOCSY and NOESY) was used for complete assignment of the non-exchangeable protons of the molecules and for qualitative determination of the preferred binding site(s) of the ligand with the oligonucleotide chain. An NMR analysis has been developed for determining the thermodynamical parameters of self-association of the DNA octamer and its multicomponent equilibrium of complex formation with EB in solution. Quantitative determination of the equilibrium constants and thermodynamic parameters (free energy, enthalpy and entropy) of duplex formation of the octamer and its complexation with EB is based on investigation of the dependence of proton chemical shifts of the molecules on temperature and on concentration. The experimental results were interpreted In terms of complexes containing different numbers of drug molecules in the octamer duplex (1:2, 2:2, 3:2 and 4:2 complexes). It is also found that (i) the relative amount of each molecular complex depends on the ratio of the initial concentrations of the octamer and drug and on the temperature of solution and (ii) successive binding of drug molecules to the octamer duplex is anti-cooperative.

KEY WORDS: deoxyoctanucleotide, ethidium bromide, intercalation, thermodynamic parameters, NMR spectroscopy

INTRODUCTION

The thermodynamical stability of oligonucleotide duplexes depends substantially on the length of the oligomer but also on base content and nucleotide sequence in the chain [1-6]. It has been shown [3-6] that even minor changes in nucleotide sequence lead to appreciable differences in the values of thermodynamical parameters of duplex formation of oligonucleotides. It has also been found that intramolecular interactions in single strands of DNA at room temperature make a significant contribution (≈40%) to the enthalpy (ΔH) of duplex formation [7]. At the same time, there is a substantial influence of the 'free ends' (*i.e.* instability of the terminal base pairs [5, 6]) on the value of ΔH of duplex formation in short oligonucleotide sequences, as well as on the thermodynamics of their binding with aromatic drug molecules [8, 9]. Thus the enthalpies of duplex formation for self-complementary deoxytetranucleotides [5, 6] are somewhat lower than those calculated for 'ideal' duplexes [10]. however, the absolute values of ΔH for complexation of the intercalating drug, ethidium bromide (EB), with tetramers [8, 9] are higher than ΔH for binding of EB with longer deoxyoligonucleotides and macromolecular DNA [11,12,13]. Previous work [8, 9, 14] on the complexation of EB with DNA tetramers has shown that EB binds preferentially to the pyrimidine-purine (*pyr-pur*) sites of the tetranucleotide duplexes and that EB intercalates from the minor groove of the double helix.

In this work we report an NMR (500/600 MHz) study of the self-association of a self-complementary deoxyoligonucleotide, 5′-d(GpApCpApTpGpTpC), and its complexation with the trypanocidal drug, (EB), in aqueous salt solution. The DNA octamer has two *pyr-pur* (CpA and TpG) sites in the middle of the sequence, expected to be the sites of preferential binding of EB, whereas at the ends of the octamer duplex there are *pur-pur* and *pyr-pyr* sequences with less affinity of drug complexation. It is expected that the association constant of EB binding to different sites of the DNA octamer will be substantially different and the influence of the terminal nucleotides will be considerably lower in the double-helical structure of the octamer than for the deoxytetranucleotide duplexes. An NMR analysis developed in previous work [8, 9, 14] for complexation of intercalating drugs with self- complementary tetramers included formation of only 1:2 and 2:2 complexes, *i.e.* one and two drug molecules intercalated into the tetranucleotide duplex according to the "excluded neighbour" model [15]. The octamer has four possible sites of intercalative binding with EB and so a new model, which takes into account the 1:2, 2:2, 3:2 and 4-2 complexes of the drug with the octamer, needs to be developed as an extension of the approach considered earlier [8, 9, 14]. Quantitative determination of the thermodynamic parameters of self-association of the octamer and its complexation with EB is based on investigation of the dependence of proton chemical shifts of the molecules on concentration and temperature [8, 9].

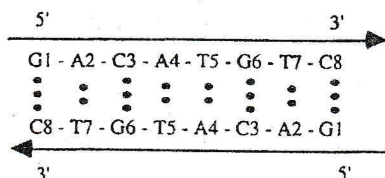
MATERIALS AND METHODS

The deoxyoctanucleotide 5' -d(GpApCpApTpGpTpC) was synthesized by the "Oswel DNA service" (University of Southampton). Ethidium bromide was purchased from the Sigma Chemical Company and used without further purification. The samples were lyophilized from 99.95% D_2O and redissolved in deuteriated 0.1 mol·l^{-1} phosphate buffer, pD 7.15, containing 10^{-4} mol·l^{-1} EDTA. The concentration of drug stock solution was measured spectrophotometrically using the molar absorption coefficient ε=5860 mol·l^{-1}·cm^{-1} (λ=480 nm) [16]. The method for the preparation of solutions and conditions of the NMR experiments were described earlier [8, 14]. 500 MHz ^{1}H-NMR spectra were recorded on a JEOL GSX 500 spectrometer. Measurements as a function of octamer concentration were made at three temperatures (298, 308 and 318 K) in standard 5 mm NMR tubes using a minimum volume of 0.5 ml of solution. Measurements as a function of temperature were made at constant concentration in the temperature range 278 to 358 K. Chemical shifts were measured relative to an internal reference tetramethylammonium bromide (TMA) and then recalculated, with respect to DSS (sodium 2,2-dimethyl-2-silapentane-5-sulphonate), *i.e.* $\delta_{DSS}=\delta_{TMA}+3.178$ (ppm). Two-dimensional homonuclear TOCSY and NOESY experiments were carried out on a 600 MHz Bruker AMX spectrometer. The sample temperature was regulated using either Bruker or JEOL variable temperature control units, as appropriate.

RESULTS AND DISCUSSION

Thermodynamics of self-association of the octamer.

Experimental observations. NMR assignment of all the non-exchangeable protons spectrum of the deoxyoligonucleotide 5′-d(GpApCpApTpGpTpC) (I) was made using homonuclear 2-D TOCSY and 2-D NOESY measurements and is in good agreement with the previous assignment for this octamer under similar experimental conditions [16]. There are only very small changes of proton chemical of I shifts with concentration in the range of 0.06 to 4.2 mmol·l^{-1} at two temperatures (298 and 308 K), most of them being within the limits of accuracy of the experimental measurements ($\Delta\delta=(1\text{-}2)\times10^{-3}$ ppm). Such behaviour most likely results from a relatively high equilibrium constant for duplex formation of the octamer so that at both temperatures, 298 and 308K, practically all the oligonucleotide is in the duplex form in solution and, correspondingly, the contribution of the single-stranded form to the observed proton chemical shift δ is negligible. It should be noted that, in order to observe NMR signals of sufficient intensity for reliable quantitative analysis it is necessary to use relatively high concentrations of oligonucleotide which favour duplex formation. At the same time, in order to obtain accurate estimates for association constants, experiments should be carried out at concentrations of the molecules that are near the reciprocal of the association constants [8]. Taking all these factors into account it follows that it is very difficult to use the concentration dependence of proton NMR chemical shifts to analyse duplex formation of the self-complementary octamer 5′- d(GACATGTC) as done previously [5, 6] for the deoxytetranucleotides in solution with self-association constants in the range (10^4 -10^5 l·mol^{-1}).



I. Schematic representation of the duplex of the octamer , 5′- d(GpApCpApTpGpTpC). Dotted lines represent hydrogen bonds between bases in the double-helical structure.

For molecules with relatively large self-association constants (*i.e.* $K > 10^5$ l·mol^{-1}) it is feasible to use the temperature dependence of proton chemical shifts in order to determine the equilibrium constant and thermodynamical parameters of self-association. In contrast to the curves observed for deoxytetranucleotides [5, 6] the temperature dependence of a number of proton chemical shifts of the octamer shown in Fig. 1 has a pronounced sigmoidal-shape which is characteristic of melting of a duplex.

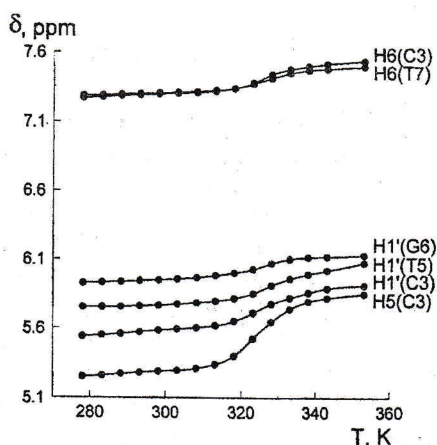


Fig. 1. Experimental temperature dependence of some of the non- exchangeable protons of the deoxyoctanucleotide, d(GACATGTC), at constant concentration (N_0=1.89 mmol·l^{-1}).

Model and analysis. The experimental results for the octamer (Fig. 1) have been analysed using the "monomer-duplex" model of molecular association, where the chemical shift of the *i*-th proton at temperature *T* can be presented in the form:

$$\delta_i(T) = f_m(T)\,\delta_{mi}(T) + f_d(T)\,\delta_{di}(T). \qquad (1)$$

In equation (1) $\delta_{mi}(T)$, $\delta_{di}(T)$ and $f_m(T)$, $f_d(T)$ are the proton

chemical shifts and equilibrium mole fractions (where $f_m(T) + f_d(T)=1$) of the octamer at temperature T in the monomer and duplex forms, respectively. In the analysis of the temperature dependence of chemical shifts it was assumed that δ_{mi} and δ_{di} are monotonic functions of temperature due to changes in intramolecular base stacking in the different conformational states of the octamer in solution; linear and quadratic approximations for $\delta_m(T)$ and $\delta_d(T)$, respectively, were used in the calculations [2, 5, 6, 18]. It is expected that application of parametric regression equations would be a suitable computational method to describe the temperature dependence of mole fractions. As the experimental temperature dependence of chemical shifts has sigmoidal character (Fig. 1), the equation for the mole fraction $f_d(T)$ has the form [8, 9] used to describe the ''helix-coil'' transition

$$f_d(T) = f_d(T_0)[1+S(T_0)]/[1+S(T)], \qquad (2)$$

where $S(T) = (T/\theta)^b$, θ and b are the parameters of the regression equations for mole fraction $f_d(T)$; $f_d(T_0)$ is the mole fraction at T=298 K. Note that the constant θ of the regression equation (2) has a definite physical meaning. The value of θ corresponds to the melting temperature T_m of the duplex, *i.e.* the temperature when the mole fraction of the duplex decreases to half the content compared with its value at low temperature (*ca.*273 K). Equilibrium constants of duplex formation, $K(T)$, at different temperatures were determined from calculated values of mole fractions using the mass law equation and the mass conservation law for this reaction. Equilibrium constants $K(T)$, in turn, were expressed in terms of the thermodynamical parameters ΔH^0 and ΔS^0

$$K(T) = exp\left(\frac{\Delta S^0}{R} - \frac{\Delta H^0}{RT}\right) \qquad (3)$$

assuming that values of ΔH^0 and ΔS^0 do not depend substantially on temperature in the range studied. It should be noted that use of model summarised in equation (1) assumes that the molecules are in fast exchange on the NMR time scale. The numerical procedure of minimisation was described earlier [5, 6, 19]. The calculated values of θ (*i.e.* T_m), K(298 K), ΔH and ΔS and ΔG (298 K) are listed in Table 1 for each proton studied. The mean values of these parameters for twelve protons are, respectively, K=830 (±190)×10³ l·mol⁻¹ at T=298 K; T_m=321 (±5) K; ΔH=-410(±38) kJ/(mol duplex); ΔS=-1.26(±0.13) kJ/(mol duplex·K). Calculations have shown that the parameters determined using all the experimental points and those at higher temperatures only (T>308 K) are the same within the error limits.

Table 1 . Calculated values of equilibrium constant K (l·mol^{-1} at T=298 K and thermodynamic parameters ΔH^0, ΔG^0 (kJ/(mol duplex)) and ΔS^0 (kJ/(mol duplex·K)) of the self-association of the deoxyoligonucleotide 5'- d(GpApCpApTpGpTpC) [a].

proton	K,10³	T_m, K	$-\Delta H^0$	$-\Delta S^0_{298}$	$-\Delta G^0_{298}$
H8(A2)	477	300	466	1.46	32.4
H8(A4)	679	324	410	1.26	33.3
H8(G1)	982	327	440	1.36	34.2
H8(G6)	997	324	422	1.30	34.2
H6(C8)	625	320	434	1.35	33.1
H2(A2)	997	320	284	0.84	34.2
H1'(T7)	986	319	454	1.41	34.2
H1'(G6)	972	330	398	1.22	34.1
H1'(T5)	434	326	350	1.07	32.1
H1'(C3)	976	323	432	1.33	34.2
H5(C3)	801	324	380	1.16	33.7
H5(C8)	998	319	448	1.39	34.2
mean values	830(±190)	321(±5)	410(±38)	1.26(±0.13)	33.7(± 0.6)

[a] In 0.1 mol·l⁻¹ phosphate buffer, pD 7.15.

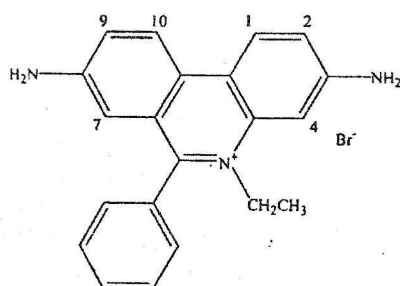
Magnitudes of the thermodynamical parameters. Each of the thermodynamical parameters (ΔH, ΔS and ΔG) of duplex formation of the octamer is summation of different kinds of physical interactions [20, 21] such as: 1) molecular interactions (hydrogen bonding, hydrophobic, van der Waals and electrostatic interactions); 2) conformational alterations in the oligonucleotide; 3) changes in hydration and release of the solvent counterion or proton in the self-association of oligonucleotide chains. The magnitudes of the contributions of each of the above

factors to the measured thermodynamical parameters are not completely understood [20], although experimental results [22] and theoretical analysis [23] confirm that hydrogen bonding and stacking interactions of the bases give comparable contributions to the stability of the double helix.

The magnitudes of the thermodynamic parameters for duplex formation of the deoxyoctanucleotide d(GpApCpApTpGpTpC) are in good agreement calculated relative to one base pair) with the corresponding ΔH and ΔS values for dimerisation reactions of self-complementary hexamers in solution [2, 17]. The enthalpy of duplex formation of the octamer is also consistent, within error limits, with the corresponding theoretical value of ΔH determined by the nearest-neighbour model [10]. The ΔH value found for the octamer in this work is more than double ($\approx$ 2.4 - 2.8) the values found for analogous deoxytetranucleotide sequences measured at the same solution conditions [5, 6]; the difference probably reflects a substantially larger influence of the melting of terminal base pairs in the tetramer compared with the octamer and the greater probability of complementary strands ''sliding'' relative to each other in short tetranucleotide duplexes so that in equilibrium there may be different helical structures in solution [5, 6] . A similar relation for ΔH values for duplex formation is observed between deoxytetranucleotides [5, 6] and dinucleotides [24] *i. e.* double the length of the oligonucleotide sequence leads to an approximately 2.5 times increase of the absolute values of enthalpy. It is likely that some contribution to the enthalpy of duplex formation in longer oligonucleotide sequences is given by such factors as stronger stacking interactions and smaller conformational alterations in the chain due to the effect of 'free ends'.

Complexation of EB with 5'-d(GpApCpApTpGpTpC)

Experimental observations. The structural formula of ethidium bromide, EB , (II) shows the atom positions of the aromatic protons having different NMR resonance peaks used in the drug binding studies. Signal assignment of all the non-exchangeable protons in the PMR spectrum of EB was obtained previously using both 2D homonuclear COSY and NOESY experiments [24]. Only a few intermolecular cross-peaks between EB and the octamer protons were observed in the 2-D NOESY spectra of mixed solutions obtained at different mixing times (τ_{ml}=90 ms, τ_{m2}=200 ms), a situation which is similar to that found previously for EB binding to self-complementary deoxytetranucleotides [8, 14].



II. Structural formula of the phenantridinium drug, ethidium bromide, (EB) with numbering of atom positions for protons used in complexation studies.

The 2-D NOE spectrum of EB with the octamer d(GACATGTC) exhibits intermolecular cross peaks of relatively small intensities between the *ortho* protons of the EB phenyl ring and H1' , deoxyribose protons of cytosine (C3) and thymine (T5) providing qualitative evidence about preferential binding of EB to the pyrimidine-purine [5'-d(T-G) and 5'-d(C-A)] sites of the octamer from the minor groove of the duplex as found [14], for example, for d(TGCA). In order to estimate quantitatively the complexation of EB with the octamer, the chemical shift dependence of the six protons of the EB chromophore was measured as a function of both the concentration of the octamer (at three temperatures, 298, 308 and 318 K) (an example is given at 318 K in Fig. 2) and temperature (Fig. 3). The temperature dependence of EB proton chemical shifts

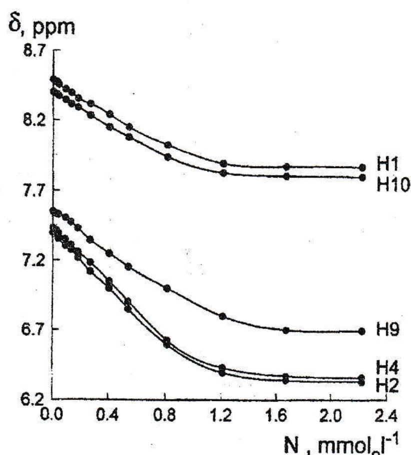


Fig. 2. Dependence of some of the EB proton chemical shifts on concentration of the octamer, d(GACATGTC), at constant drug concentration D_0=1.15 mmol·l^{-1} in solution, T=318 K.

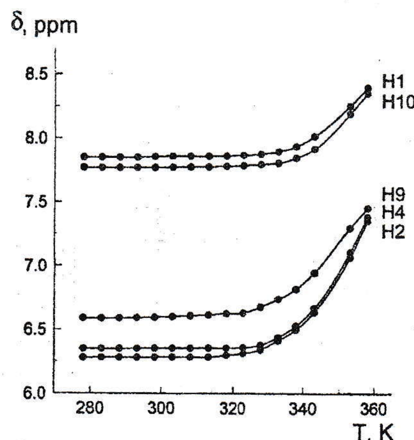
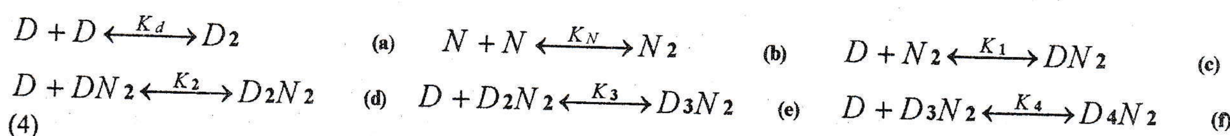


Fig. 3. Experimental temperature dependence of some of the EB proton chemical shifts in solution of the drug with the octamer (D_0=1.15mmol·l^{-1}, N_0=1.21 mmol·l^{-1}).

at a constant ratio of drug to nucleotide in Fig. 3 has the form of those observed for ''helix-coil'' transition in macromolecular DNA; in the presence of EB the melting temperature T_m of the octamer duplex is increased *ca.* 23-24 K (T_m *ca.* 344-345 K).

Model of complexation and analysis. As the relative content of the duplex form of the octamer is overwhelmingly predominant in solution at moderate temperatures, it was considered that the basic scheme for molecular complexation could neglect binding of EB with the single-stranded form. Taking into consideration the "excluded neighbour'' model [25] for the binding of intercalators to DNA duplexes, there are four accessible sites of intercalative binding of EB with the octamer. Hence the basic model had to take into account the 1:2, 2:2, 3:2 and 4:2 complexes of the drug with the octamer, which are characterised by the binding constants K_1, K_2, K_3 and K_4, respectively. A schematic representation of examples of the different complexes of EB with the octamer are summarised in Fig. 4. Previous work [8, 14] on the complexation of EB with DNA tetramers showed that the preferred sites of drug binding are *pyr-pur* base sequences in the chain. As there are two *pyr-pur* binding sequences in the octamer, it is likely that EB preferentially forms complexes with these sites in the duplex, *i.e.* K_1 and K_2 values are expected to be higher than K_3 and K_4 binding constants, because the latter will include reactions with *pur-pur* or *pyr-pyr* sequences in the octamer duplex.

The following equilibrium reactions, including self-association of drug D (K_d) and nucleotide N (K_N), were considered for quantitative analysis of chemical shifts resulting from drug-octamer complexation:



$$D + D \xleftrightarrow{K_d} D_2 \quad \text{(a)} \qquad N + N \xleftrightarrow{K_N} N_2 \quad \text{(b)} \qquad D + N_2 \xleftrightarrow{K_1} DN_2 \quad \text{(c)}$$

$$D + DN_2 \xleftrightarrow{K_2} D_2N_2 \quad \text{(d)} \qquad D + D_2N_2 \xleftrightarrow{K_3} D_3N_2 \quad \text{(e)} \qquad D + D_3N_2 \xleftrightarrow{K_4} D_4N_2 \quad \text{(f)}$$

(4)

Chemical shifts were calculated using an additive model [8, 14]:

$$\delta = \delta_m f_m + \delta_d f_d + \sum_{i=1}^{4} \delta_i f_i \qquad (5)$$

where δ_m, δ_d, δ_1 -δ_4 are chemical shifts of EB in the monomer , dimer, 1 :2 (DN_2), 2:2 (D_2N_2), 3:2 (D_3N_2) and 4:2 (D_4N_2) complexes with the octamer duplex; f_m, f_d, f_1-f_4 are mole fractions of EB in the monomer dimer and in the abovementioned complexes, respectively. The validity of such a model assumes fast exchange between interacting molecules.

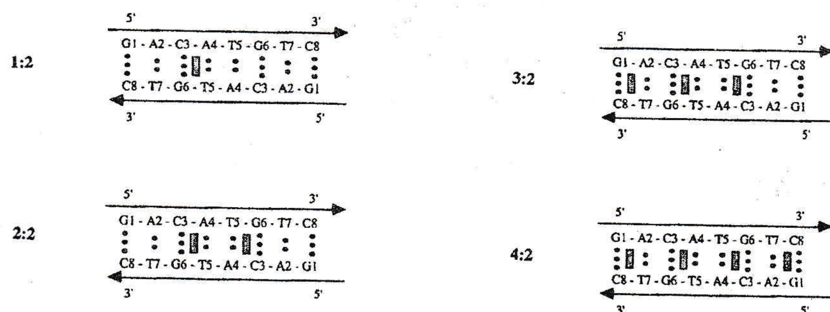


Fig. 4. Schematic representation of examples of intercalated complexes of EB (shaded rectangles) with the octanucleotide duplex at different drug: oligonucleotide ratios.

The equilibrium constants K_i (i= 1 - 4) were expressed in terms of the corresponding parameters ΔH_i^0, ΔS_i^0 using relations equiva-lent to (3). The values of δ_m and δ_d , as well as ΔH_d^0 , ΔS_d^0 were determined previously [24] from investigations of EB self-association using the dependence on concentration of drug proton chemical shifts in the same solvent system (0.1 mol·l^{-1} phosphate buffer, pD 7.1). Taking into consideration the mass law equations for reactions (4) and the mass conservation law [8, 14] , the observed chemical shift in equation (5) is a function of the following twelve parameters: δ_1 -δ_4, ΔH_1^0 -ΔH_4^0, ΔS_1^0 - ΔS_4^0. The parameters of the model were determined by minimising the quadratic discrepancy function, Δ, between the experimental and calculated values of chemical shifts for the six aromatic protons of the EB chromophore; the detailed computational procedure is described in previous work [8, 19, 25, 26]. In the calculations the data on the temperature and concentration (at three different temperatures) dependence of EB proton chemical shifts in the mixed solutions have been processed jointly.

It is reasonable to solve such a multiparametric problem by the method of successive complication of the model [8, 14]. The calculated equilibrium constants and thermodynamical parameters for different complexation reactions (1:2, 2:2, 3:2 and 4:2) are summarised in Table 2. It is seen from Table 2 that $K_1 > K_2 >> K_3 > K_4$ *i.e.* formation of the 1:2 and 2:2 complexes of EB with the duplex is preferred to the 3:2 and 4:2 complexes. The results also show that successive binding of drug molecules to the octamer duplex is anti-cooperative. The relatively high magnitudes of K_1 and K_2 are consistent with complexation of the first and the second drug

molecules being preferred to the *pyr-pur* sites [d(CA) and d(TG)] of the octamer duplex. The probability of binding for the first EB molecule (*i.e.* formation of the 1 :2 complex, DN_2) when there are two preferred binding sites available should be about double that for the second drug molecule, which has access to only one such site. This is in agreement with the values of association constants K_1 and K_2 determined in the present work. The probability of complexation of EB to *pur-pur* d(GA) or *pyr-pyr* d(TC) sites (*i.e.* formation of 3:2 (D_3N_2) and 4:2 (D_4N_2) complexes) was found to be much lower than binding of drug to sites with alternating base sequence in the chain in agreement with previous work [3, 12, 27].

Table 2. Equilibrium constants K_i at T=298 K and thermodynamic parameters of EB complexation with the octamer 5′ d(GpApCpApTpGpTpC) [a].

Complex, K_i	K_i, 10^3 ($l \cdot mol^{-1}$)	$-\Delta G^0_{298}$ (kJ/mol)	$-\Delta H^0$ (kJ/mol)	$-\Delta S^0_{298}$ (J/(mol·K))
DN_2 K_1	718(±65)	33.4(±3.3)	47.8(±2.5)	48.4(±7.5)
D_2N_2 K_2	315(±41)	31.4(±4.1)	75.9(±3.6)	149(±26)
D_3N_2 K_3	47(±7)	26.6(±4.0)	99.8(±3.6)	245(±43)
D_4N_2 K_4	18(±4)	24.3(±5.4)	86.3(±4.2)	208(±51)

[a] In 0.1 $mol \cdot l^{-1}$ phosphate buffer, pD 7.15.

Properties of the complexation equilbrium. The analysis of the dynamic equilibrium in solution is important for determining the contribution of each type of complex to the experimentally-observed proton chemical shift and for reliable determination of the thermodynamic parameters of formation of different complexes [8, 9, 14]. Using the values of the equilibrium constants (Table 2) the relative content of each of the molecular complexes in solution has been calculated as a function of r (=N_2/D_0, the ratio of initial concentrations of the octamer duplex and drug) at different temperatures. An example is shown in Fig. 5 (at 298 K), where it can be seen that the contribution of different types of complexes to the general equilibrium in solution is determined not only by the values of the equilibrium reaction constants, but also by the value of r, as shown previously for drug binding with self-complementary and non-selfcomplementary deoxytetranucleotides [8, 14, 27]. Characteristic maximums of the concentration curves for 4:2, 3:2 and 2:2 complexes are observed at r values corresponding approximately to the stoichiometric relations of octamer duplex and drug, *i.e.* r= *ca.*0.25 for D_4N_2, r= *ca.*0.33 for D_3N_2 and r= *ca.*0.5 for D_2N_2 complexes. At small r values (r<0.4), when the drug concentration is higher than the concentration of the octamer in solution, the relative amount of the 2:2, 3:2 and 4:2 complexes are greater than the 1:2 complex in solution, whereas at $r \geq 1$ the fraction of the 1 :2 complex of the drug with the duplex becomes predominant. It should be noted that it would be incorrect to assume that the 1:2 complex predominates for drug-octamer solutions at different concentration ratios; at r=1 calculations show that only about 40 % of the drug (D) is in the 1:2 complex whereas about 50 % of the drug is in the 2:2 complex. Similarly for solutions made up at 1:2 drug-octamer concentration ratios (*i.e.* r=2) there is still a significant amount of drug in the 2:2 complex (*ca.*30 %) compared with *ca.*70 % in the 1 :2 complex at 298 K.

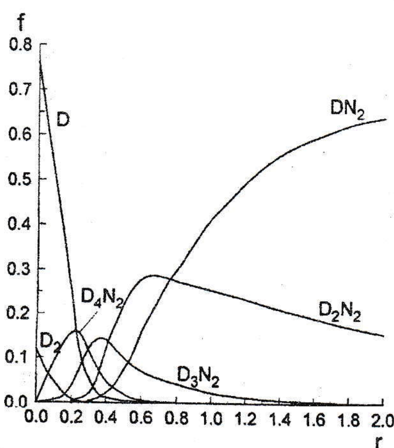


Fig. 5. Calculated relative content (f) of the drug (D) and the different complexes with the octamer 5′-d(GpApCpApTpGpTpC) in solution as a function of r (= N_2/D_0, the ratio of initial concentrations of octamer duplex and drug) at T=298 K.

The calculated temperature dependence of the mole fractions of different types of EB-octamer complexes are shown in Fig. 6 for r *ca.*0.8. It is seen that at lower temperatures (< 320 K) practically all the drug is in the complexed state but at higher temperatures the concentration of the 'free' drug (D) increases as the drug-octamer complexes disassociate. The temperature dependence of the mole fractions of the 1:2, 2:2 and 3 :2 complexes of EB with the octamer duplex are typical for "melting curves" of double-helical oligonucleotides. (The mole fraction of the 4:2 complex is negligible at the conditions of the experiment (r=*ca.*0.8), as seen in Fig. 5). The value of the melting temperature T_m determined directly from the experimental curves (Fig. 3, *ca.*345 K) is significantly smaller (~ 11 K) than the calculated T_m for the 1:2 complex, which is the most stable complex in solution for the octamer studied. The calculations have also shown that successive binding of the drug to the octamer duplex decreases the melting temperatures of the complexes being formed (*i.e.* T_m *ca.*344 K for 2:2 and T_m *ca.*338 K for 3:2 complexes). It follows that successive intercalative binding of EB to the octamer duplex destabilises the helical structure, presumably due to unwinding of the helix by the drug in such a relatively short oligonucleotide sequence. It should be emphasized that observed melting curves for a multicomponent systems are averaged

values and without a quantitative analysis of the complex equilibrium it is impossible to differentiate the contribution of each type of complex.

Magnitudes of the thermodynamical parameters of complexation of EB with the octamer I. The thermodynamical parameters summarised in Table 2 show that all the reactions of complex formation of EB with the octamer duplex are exothermic. Hopkins *et al* [13] suggested that enthalpy changes for binding of aromatic drugs with double-helical DNA is the sum of at least six components, including: 1) diminution of the stacking interactions of the bases caused by formation of the cavity for the drug molecule to occupy; 2) stacking interactions of the bases in the cavity with drug chromophore; 3) electrostatic interactions between the positively-charged drug cations and the negatively-charged phosphates of nucleotides; 4) alteration of solvation due to complex formation ; 5) different arrangements of the intercalator in the complex ; 6) specific hydrogen bonds which drug molecules form in the complex. Some components of enthalpy change (1), 4), 5)) may adopt positive values [13, 28]. Positive entropy contributions are determined, in the main by hydrophobic interactions due to transfer of the drug molecule from solvent to the intercalation site.

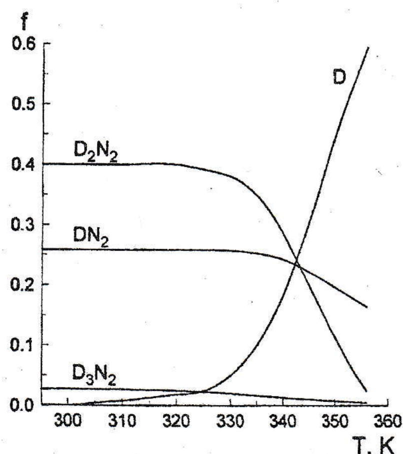


Fig. 6. Calculated temperature dependence of the relative content, f (*T*), of different complexes of EB with the octanucleotide, d(GACATGTC), in solution at D_0=1.15 mmol·l^{-1} and N_0=1.21 mmol·l^{-1}.

The absolute values of entropy and enthalpy changes for 1 :2 complexes of EB with the octamer duplex are substantially smaller than the ΔH and ΔS values for other complexes (2:2, 3:2 and 4:2), indicating that hydrophobic interactions play a significant role in the binding of EB to the double-helical deoxyoctanucleotide in aqueous solution. It is seen from Table 2 that successive binding of EB molecules to the octamer duplex occurs with more favourable enthalpy and less favourable entropy compared with 1:2 complex formation. These results are consistent with those found for deoxytetranucleotides [9] and may be interpreted as arising from 1) increased van der Waals interactions (a decrease of the distance between adjacent base pairs has been found in the 2:2 complex compared with the 1:2 complex of EB with d(TGCA) [14], containing the same preferred binding sites for the drug (d(T-G) and d(C-A) sites) as in the octamer studied); 2) an increase of the rigidity of the double-helical structure due to intercalation of different numbers (2-4) of drug molecules, accompanied by a decrease of entropy in view of limiting the number of accessible conformation states [29, 30]; 3) and arising from a combination of ion, and water interactions [20].

The values of the thermodynamic parameters obtained for the 1:2 and 2:2 complexes of EB with the octamer duplex, which predominate in solution at $r \geq 1$ (Fig. 5), are in good agreement with calorimetric results for EB binding to oligonucleotide duplexes of comparable length [11, 12,31]. Previously it had been found from NMR data at the same experimental conditions [9] that the values of enthalpy changes on binding of EB to relatively short deoxytetranucleotide sequences were substantially larger (in absolute value) than those determined for macromolecular DNA [12, 13] and longer oligonucleotide duplexes [11, 12] The observed differences [9] may be explained by the following: 1) - the contribution of hydrophobic effects would be different on binding of the drug to macromolecular DNA compared with short oligonucleotides; 2) - the conformational transitions on drug binding to short duplexes, due to the influence of "free ends" in such molecules, is quite different from DNA macromolecules where their influence can be neglected. The results obtained in this work confirm these assumptions; the influence of "free ends" is expected to be much smaller for the octamer duplex compared with deoxytetranucleotides and the thermodynamic parameters for EB complexation with the octamer (Table 2) are in good agreement with the results published previously for relatively long oligonucleotide duplexes [11,12]. Additional support to confirm these assumptions are the much higher ΔH values for complexation of the drug with terminal sites of the octamer duplex (*i.e.* 3:2 and 4:2 complexes in Table 2) which may be explained in terms of the higher conformational freedom of the nucleotides situated at the ends of the chain.

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ТЕРМОДИНАМІЧНИЙ АНАЛІЗ КОМПЛЕКСОУТВОРЕННЯ БРОМИСТОГО ЕТИДІЮ З ОКТАМЕРОМ ДНК 5′-d(GpApCpApTpGpTpC) У ВОДНОМУ РОЗЧИНІ МЕТОДОМ ^{1}H-ЯМР СПЕКТРОСКОПІЇ

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Методами 1М- и 2М-ЯМР спектроскопії досліджена самоасоціація самокомплементарного дезоксиоктануклеотиду 5′-(GpApCpApTpGpTpC) та його комплексоутворення з фенантридиновым барвником бромистим етидієм (ЕБ). Гомоядерна кореляційна ПМР спектроскопія 2М-TOCSY та 2М-NOESY використовані для повного віднесення сигналів необмінних протонів молекул і якісного визначення міст переважного з'вязування ліганду з олігонуклеотидною послідовністю. Розроблено методику визначення термодинамічних параметрів самоасоціації октамеру ДНК та аналізу складной рівноваги у водному розчині при його комплексоутворенні з бромистим этидієм на основі даних ЯМР. Розрахунок констант рівноваги та термодинамічних параметрів (вільної енергії, ентальпії та ентропії) утворення дуплексу октамеру та реакції комплексоутворення з ЕБ обгрунтован на дослідженні злежностей протоних хімічних зсувів молекул від температури і концентрації. Експериментальні результати оброблені за допомогою моделі, яка враховує утворення комплексів з різним числом інтеркальованих у дуплекс молекул фарбнику (1:2, 2:2, 3:2 та 4:2 комплекси). Показано, що відносний склад молекулярних комплексів кожного типу залежить від співвідношення початкових концентрацій октамеру та барвника і температури розчину; послідовне вбудовування молекул барвника у октамерний дуплекс відбувається анти-кооперативно.

КЛЮЧОВІ СЛОВА: дезоксиоктануклеотид, бромистий етидій, інтеркаляція, термодинамічни параметри, ЯМР-спектроскопія