THERMODYNAMICS OF BERENIL BINDING TO POLY[d(AT)]·POLY[d(AT)] AND POLY[d(A)]·POLY[d(T)] DUPLEXES[#]

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[#]This paper is dedicated to Prof. Dr. Davorin Dolar on occasion of his 80th birthday.

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Abstract

Thermodynamics of berenil binding to poly[d(AT)]·poly[d(AT)] and poly[d(A)]·poly[d(T)] duplexes in neutral buffer solutions was studied using a combination of calorimetric and spectroscopic techniques. By titration calorimetry we found that at 25 °C the enthalpies of berenil binding to poly[d(AT)]·poly[d(AT)] and poly[d(A)]·poly[d(T)] are –20.4 and 10.3 kJ/mol of bound drug, respectively. Our CD results show that berenil binding to both duplexes is at low berenil/DNA ratios slightly positively cooperative whereas at higher binding densities it is characterized by a constant affinity and a binding site size, n, of 4 base pairs/berenil molecule. Berenil binding to both polynucleotides was also followed by performing UV titrations of berenil solutions with DNA solutions at 25 °C and by measuring the UV melting curves at different berenil/DNA molar ratios. From Scatchard plots the best fit of experimental data with the predictions of the neighbor exclusion model was obtained with $K = 1.3\cdot10^7$ and $n = 3.5$ for poly[d(AT)]·poly[d(AT)] and $K = 1.5\cdot10^7$ and n = 3.8 for poly[d(A)]·poly[d(T)]. UV melting experiments showed for both polynucleotides that above their saturation with bound berenil they exhibit monophasic melting accompanied by a large increase in T_m, whereas at low drug/base pair ratios their melting is biphasic. Berenil binding constants determined at 25 °C using the T_m data and the neighbor exclusion model are $K = 2.3\cdot10^7$ for poly[d(AT)]·poly[d(AT)] and $K = 7.4\cdot10^6$ for poly[d(A)]·poly[d(T)]. From these K values the standard free energies of binding were determined and combined with the measured enthalpies of binding to obtain the corresponding entropies of binding. The resulting thermodynamic binding profiles show that berenil binding to poly[d(AT)]·poly[d(AT)] is governed by about equal enthalpic and entropic forces whereas its binding to poly[d(A)]·poly[d(T)] is overwhelmingly entropy driven.

Introduction

Berenil (Fig. 1) is an antitrypanosomal drug[1] which has been found to bind reversibly to double helical DNA predominantly at AT-rich domains.[2-4] It has been shown that bound berenil does not cause unwinding of closed circular DNA. This indicates that it does not intercalate but rather binds in the minor groove of the double helix[5]. The interaction of berenil with DNA has been confirmed by footprinting studies which have revealed that strong binding takes place only when the binding sequences are at least three AT base pairs long.[6,7] Molecular modeling studies have indicated that due to its concave shape berenil molecule is well accommodated by the minor groove at AT sequences where it forms favorable directed hydrogen bonds with the bases.[7-10]

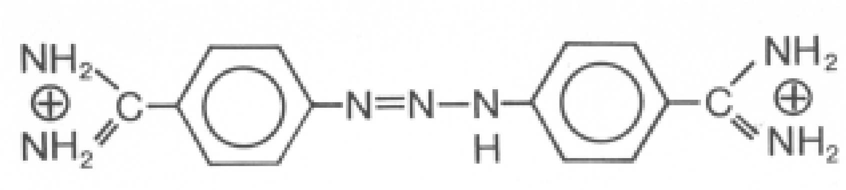

Figure 1: Structural formula of berenil.

Recent X-ray study of berenil complex with d(CGCGAAATTTCGCG)$_2$ oligonucleotide has shown that in such A3T3 complex berenil binds symmetrically in the 5'-AATT region of the minor groove with both amidinium groups hydrogen bonded to O-2 atoms of thymines at each end of the binding site.[12]

Several studies on the drug-DNA interactions have shown that for a thorough understanding of the binding process both thermodynamic and structural studies are necessary.[13-16] Only such complementary, parallel studies can provide data required to define the nature and the relative importance of the forces that stabilize the DNA structure and the structure of the corresponding drug/DNA complexes.[17] Recently, three thermodynamic studies on berenil binding to the poly[d(AT)]·poly[d(AT)] alternating copolymer and the poly[d(A)]·poly[d(T)] homopolymer have been reported. In the first one Barceló and Portugal[18] claim that upon berenil binding the CD spectra displayed by poly[d(AT)]·poly[d(AT)] resemble those obtained with poly[d(A)]·poly[d(T)]. They ascribe this resemblance to the conformational changes of poly[d(AT)]·poly[d(AT)] caused by the bound berenil. From the DSC data they estimated the enthalpies of berenil binding to both duplexes. The value of -65 kJ/mol b.d. they report for berenil binding to homopolymer in 50 mM NaCl at pH = 7.0 is substantially more exothermic than the corresponding value of -7 kJ/mol b.d. obtained for the alternating copolymer. For these rather unexpected results no explanation has been suggested.

In the second study Schmitz and Hübner[19] report complete thermodynamic binding profiles for berenil binding to poly[d(AT)]·poly[d(AT)] and poly[d(A)]·poly[d(T)]. The enthalpies of binding they determined from DSC data in 10 mM NaCl solutions at pH = 7.0 are -29 kJ/mol b.d. for alternating copolymer and -25 kJ/mol b.d. for homopolymer.

These values, as well as those reported by Barceló and Portugal,[18] are highly questionable since they were determined from small differences between the helix to coil transition enthalpies of the drug-bound and drug-free duplexes.[20] From Scatchard plots they concluded that berenil binds to both polynucleotides in two distinct modes, one at low and one at high drug/base pair molar ratio. They claim that at saturation berenil occupies approximately three base pairs of either of the host duplexes. This result is consistent with the values for the corresponding berenil binding site sizes reported in the literature[18,19,21] as well as in this study. By contrast, the sizes of the low drug/base pair binding sites they obtained from Scatchard plots are unreasonably large. The reason for this is very likely an improper choice of the wavelength at which the spectral binding curves were constructed and/or an inappropriate fitting procedure used for fitting these binding curves.[21] By measuring the UV melting curves and applying the theoretical treatment of Crothers[22] they determined the apparent binding constants, K, for both polynucleotides at different concentrations of added NaCl. From the log$K$ vs. log$[Na^+]$ plots they showed that the dicationic berenil binds to both host duplexes in such a manner that the predicted release of two counterions is not achieved.[19] According to their results the association of berenil with poly[d(AT)]·poly[d(AT)] and poly[d(A)]·poly[d(T)] is followed by a release of 1.0 and 1.4 counterions, respectively. They ascribe the larger number of released counterions observed with poly[d(A)]·poly[d(T)] duplex to stronger electrostatic interactions between berenil and homopolymer. Finally, by combining the standard free energies of binding, ΔG_B^0, obtained from K values determined for high drug/base pair binding modes with the uncertain enthalpies of binding determined indirectly from DSC measurements they obtained the corresponding standard entropies of binding, ΔS_B^0, which are also rather unsafe.

Recently, Breslauer et al.[21] published a paper on berenil binding to poly[d(AT)]·poly[d(AT)] and poly[d(A)]·poly[d(T)] in which they report the appearance of two distinctive binding sites on each of the measured duplexes, one of about 11 and one of about 4 base pairs/bound berenil. They suggest that the smaller binding site reflects a specific mixed binding mode in which berenil binds to a specific DNA site via minor groove binding and intercalation. For the binding event associated with the larger binding site they suggest that it may reflect an allosteric conformational change in the host duplex induced by the bound berenil. They also report binding constants for high drug/base pair binding mode obtained from UV melting curves and the corresponding enthalpies of binding obtained from calorimetric titrations. Their K values are for about one to two orders of magnitude higher than those reported by Schmitz and Hübner[19] while their enthalpies of binding are significantly lower.

The sets of thermodynamic quantities that describe binding of berenil to poly[d(AT)]·poly[d(AT)] and poly[d(A)]·poly[d(T)] duplexes (Barceló and Portugal[18], Schmitz and Hübner[19] Breslauer et al.[21]) differ significantly from each other. Apparently, there is yet no relevant thermodynamic information pertinent to the putative berenil/poly[d(AT)]·poly[d(AT)] and berenil/poly[d(A)]·poly[d(T)] complexes. For this reason, we carried out the work presented here in which we combined several spectroscopic and calorimetric equilibrium binding studies with a goal to understand in more detail the mechanism that governs binding of berenil to DNA. In the work reported here titration calorimetry and UV-spectroscopy were used to elucidate directly the thermodynamics of berenil binding to poly[d(AT)]·poly[d(AT)] and poly[d(A)]·poly[d(T)]. Our results reveal that, in contrast to previous studies of the same system, berenil binding to poly[d(AT)]·poly[d(AT)] is governed by about the same enthalpic and entropic forces whereas binding to poly[d(A)]·poly[d(T)] is overwhelmingly entropy driven.[15]

Experimental

Materials. Synthetic polynucleotides, poly[d(AT)]·poly[d(AT)] and poly[d(A)]·poly[d(T)] employed in this work were purchased from Pharmacia LKB, Biotechnology (Uppsala, Sweden). Berenil was obtained from Sigma (St. Louis, U.S.A.) and used without further purification.[21] Stock solution of the DNA polymers and berenil were prepared in a buffer solution consisting of 0.1 mM monobasic Na phosphate, 10 mM dibasic Na phosphate and 1 mM disodium EDTA. The pH of these solutions was 7.70 and the total sodium ion concentration was calculated to be 22.1 mM.

Concentrations of DNA solutions expressed in moles of phosphates were determined at 25 °C spectrophotometrically using the following extinction coefficients:[19,23] ε_{260} of poly[d(AT)]·poly[d(AT)] = 6650 $M^{-1}cm^{-1}$ and ε_{260} of poly[d(A)]·poly[d(T)] = 6500 $M^{-1}cm^{-1}$. The concentration of berenil solutions was also determined spectrophotometrically using the extinction coefficient ε_{370} = 36900 $M^{-1}cm^{-1}$.

Due to the low solubility of berenil in phosphate buffer we performed all calorimetric titrations in buffer solution that consisted of 10 mM sodium cacodylate, 100 mM NaCl, and 1mM Na_2EDTA and was adjusted to pH 6.9. In this buffer berenil obeys Beer's law below 1.5 mM, which implies that below this concentration berenil does not show any noticeable association.

Titration calorimetry. The enthalpies of berenil binding to poly[d(AT)]·poly[d(AT)] and poly[d(A)]·poly[d(T)] were determined by measuring the heat effects accompanying successive additions of berenil titrant solution to the DNA solution. These calorimetric titrations were performed at 25 °C in the Thermometric 2277 microcalorimeter equipped with a 4 mL insertion titration vessel. 20 μL aliquots of berenil solution in cacodylic buffer (~1.3 mM) freshly prepared for each experiment were added to 2 mL of DNA solution in the same buffer (~0.2 mM in base pairs) through a 250 μL syringe operated with a plunger driven by a stepping motor. The reference cell of the microcalorimeter was filled with distilled water and the instrument was calibrated by means of a known electrical pulse. 10 injections of 20 μL were added in a single experiment. The area under the peak that follows each injection is proportional to the resulting heat of the ligand-DNA interaction. The measured heat effects were around 500 μJ while the accompanying heats of ligand and DNA dilution were of the order of the experimental error (≈ ±20 μJ) and were neglected. The enthalpies of berenil binding to both polynucleotides, ΔH_B, expressed per mole of bound berenil were obtained from

$$\Delta H_B = \frac{q}{\Delta V c_B}, \tag{1}$$

where q is the measured heat effect, ΔV is the volume of the added berenil solution and c_B is berenil molar concentration. All calorimetric measurements were performed at the calorimeter amplifier set to 10 μW. The performance of the calorimeter was checked at the same experimental conditions chemically by measuring the heat of dilution of aqueous sucrose solutions. Comparison of experimentally determined values with those obtained from literature[24] showed an agreement within ± 2%.

Circular Dichroism (CD) Spectropolarimetry. CD spectra were measured in an AVIV Model 62A DS spectropolarimeter (Aviv Associates, Lakewood, N.J.) equipped with a thermoelectrically controlled cell holder and a cuvette of 1 cm path length. CD titrations were conducted at 25 °C by incrementally injecting 2.5 - 20 μL aliquots of 0.6 mM berenil solution into a 2 ml of 50 μM (in base pairs) duplex solution in the same buffer. After each injection CD spectrum was recorded between 460 nm and 220 nm. and normalized to 1 M (base pair) duplex concentration and 1 cm optical path length (Fig. 3B, C).[25-27]

UV absorbance spectroscopy. The UV-absorbance measurements were performed in a thermoelectrically controlled Cary 1 spectrophotometer. To access the effect of the bound berenil on the thermal stability of the two duplexes UV melting experiments were conducted in the absence and presence of berenil. Absorbance at 260 nm was measured as a function of temperature at the heating rate of 0.5 °C/min. The concentration of each polynucleotide was around 40 μM in base pairs while concentration of berenil varied from 0 to about 10 μM.

Optical titrations in which 10 μL aliquots of concentrated DNA stock solution were added incrementally to a berenil solution until no further change in berenil absorbance was observed were performed in buffer solutions at 25 °C. The absorbance spectra were corrected for small dilution effects so that they refer to a constant concentration of berenil in the measured solutions. Below the DNA saturation with the bound berenil such constant molarity of berenil, c_0, can be expressed as:

$$c_0 = c + rc_D\,, \tag{2}$$

where c is the molarity of the free berenil in solution, r denotes the number of moles of berenil bound per mole of DNA base pairs and c_D is the DNA concentration in the solution expressed as molarity of base pairs. If at given wavelength both free and bound ligand obey Beer's law over the experimental concentration range, if the extinction coefficient of the bound drug remains constant with varying r, and if the polymer does not absorb at this wavelength, the measured absorbance, A, can be throughout the titration experiment expressed as:

$$A = \left(\frac{c}{c_0} \right) A_f + \left(\frac{r c_D}{c_0} \right) A_b , \tag{3}$$

where A_f and A_b are the absorbances of the free and bound berenil at molarity c_0, respectively. Since our measurements have shown that these conditions were fulfilled (Fig. 2) it follows from eqs. 2 and 3 that

$$r = \frac{c_0}{c_D} \left(\frac{A_f - A}{A_f - A_b} \right) \quad \text{and} \quad c = c_0 \left(\frac{A - A_b}{A_f - A_b} \right). \tag{4}$$

As can be seen from Fig. 2 the absorption spectra of solutions containing constant concentration of berenil and varying concentrations of poly[d(AT)]·poly[d(AT)] or poly[d(A)]·poly[d(T)] show a progressive shift in berenil spectrum from a peak at around 370 nm for a free drug to a peak at around 380 nm for the fully bound drug. The biggest change in berenil's absorbance occurs around 352 nm, and for this reason the absorbance measurements at this wavelength were used to calculate r and c from eq. 4.

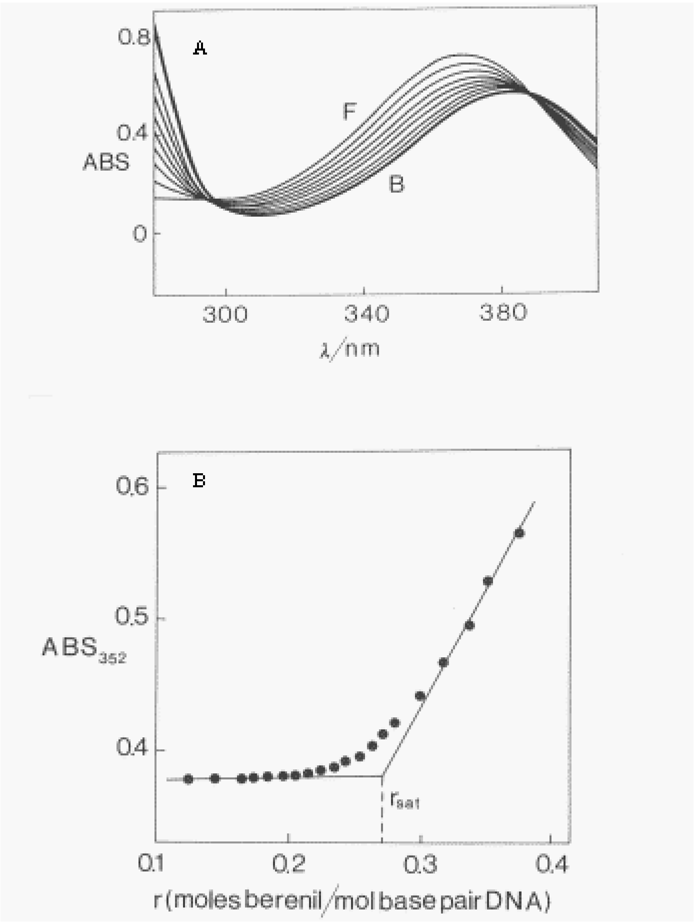

Figure 2: Panel A - UV-absorbance spectra at constant total berenil concentration of 19.9·μM as a function of the drug/DNA ratio varying between 0.1 and 0.4 together with the spectra of free berenil (F - no DNA present) and fully bound berenil (B – excess of DNA). Panel B - UV titration curve constructed at 352 nm from which the binding site size, r_{sat} is estimated. Almost identical absorption spectra and UV titration curve were determined with berenil binding to poly[d(A)]·poly[d(T)].

Finally, thermal stability of the drug-free and drug-bound duplexes was estimated from the corresponding UV-melting curves which were measured in buffer solutions with and without added NaCl at λ = 260 nm, at the temperature scanning rate of 0.5·°C/min and at the hold time of 2 minutes.

Results and discussion

Binding of berenil to the two duplexes was characterized by measuring the berenil-induced CD spectra in the wavelength range between 220 and 460 nm (Fig. 3A). The induced CD signals resulting from the ligand-duplex complexation were used to monitor the berenil-DNA binding modes. To see these CD spectral alternations more clearly the measured CD spectra are presented also in a form of difference spectra in which the DNA spectral contributions are subtracted out (Fig. 3B).

Inspection of CD spectra presented in Fig. 3A reveals the absence of discrete isoelliptic points for both berenil-DNA complexes while the corresponding single wavelength CD titration curves show two inflection points (Fig. 3C). The one at $r \approx 0.09$ corresponds to about 11 base pairs/bound berenil and the other at $r \approx 0.25$ to 4 base pairs/bound berenil. These values agree very well with the corresponding binding site sizes determined from CD titrations by Breslauer et al.[21] Evidently, our CD binding isotherms measured at 390 nm (Fig 3C) show that interaction of berenil with either of the two duplexes displays at low r values a positive cooperativity which is according to the small changes of the $\partial(\Delta[\theta])/\partial r$ slopes rather weak. This observation may be explained in terms of the allosteric binding model according to which the initial binding of berenil molecules (at $r < 0.1$) within the all AT minor groove induces conversion of the host duplex into a new conformation which binds berenil more strongly than the initial duplex conformation. The suggested explanation is consistent with the results of crystallographic studies of berenil complexed to the oligonucleotides which have shown that binding of berenil to AT regions in DNA results in slight widening of the minor groove and consequently in more effective binding.[12] These studies have also shown that bound berenil overlaps four AT base pairs which corresponds to the inflection point observed at $r \approx 0.25$ on the measured CD titration curves. As can be seen from Fig 3 our CD data do not allow one to resolve unambiguously the molar ellipticities to the different bound forms of berenil. Therefore they cannot be used to construct Scatchard plots from which one could obtain the corresponding binding constants. In principle, the

CD titration curves could be discussed in terms of a simple binding model which assumes the duplex molecules to have two independent binding sites, each with its characteristic binding constant K_1 and K_2. Unfortunately, the K_1 and K_2 values can be estimated from the model analysis of the measured CD titration curves only if the molar ellipticities of drug complexes with the first and the second binding site differ substantially,[25-27] a condition that is not fulfilled for the studied berenil-DNA complexes.

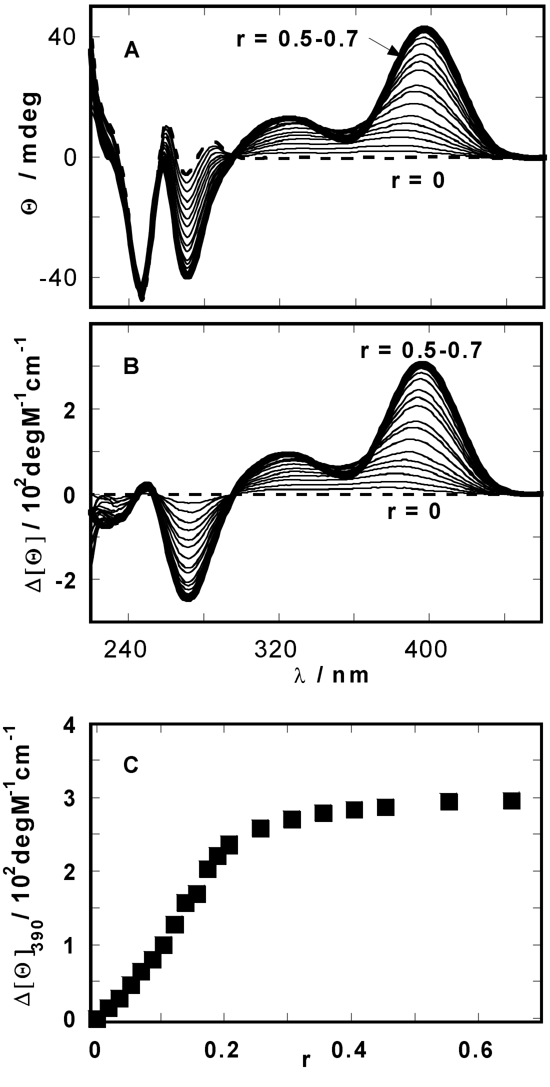

Figure 3: Bound berenil induced CD spectra (panel A), the corresponding molar CD difference spectra (panel B) and the binding isotherm at 390 nm (panel C) measured for berenil binding to poly[d(A)]·poly[d(T)] at *r* varying between 0 and 0.7. With berenil binding to poly[d(AT)]·poly[d(AT)] almost identical CD results were obtained.

Our calorimetric titrations have shown for both polynucleotides (Fig 4) that heat effects accompanying each injection of berenil solution into DNA solution can be detected only below $r = 0.25$. The binding enthalpies, ΔH_B, were obtained as an average of the first two almost identical peaks determined at low r values at which practically all of the added berenil should be bound considering the polynucleotide concentrations and values of berenil binding constants.

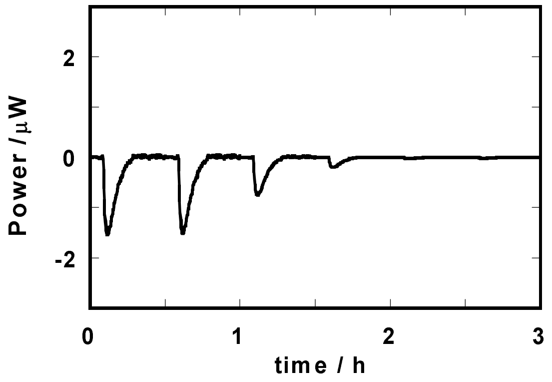

Figure 4: Calorimetric titration at 25 °C presenting the heat effects measured at successive injections of berenil solution into the poly[d(AT)]·poly[d(AT)] solution in the same solvent.

The ΔH_B value of –20.4 kJ/mol b.d. obtained for berenil binding to poly[d(AT)]·poly[d(AT)] is very close to the ΔH_B value of –20.1 kJ/mol b.d. determined from calorimetric titrations by Breslauer et al.[21] By contrast, the enthalpy of berenil binding to the homoplymer poly[d(A)]·poly[d(T)] determined in this work is 10.3 kJ/mol b.d. and differs even in sign from the corresponding value of –9.2 kJ/mol b.d. reported by Breslauer et al.[21] We have no explanation for this discrepancy. All we can say is that we repeated the titrations of poly[d(A)]·poly[d(T)] many times and that we always used freshly prepared berenil solutions. The ΔH_B values obtained from these experiments were always endothermic and varied within ±10% of the reported value. It should be noted, however, that similar differences in association enthalpies for ligand binding to poly[d(AT)]·poly[d(AT)] versus poly[d(A)]·poly[d(T)] have been observed with some other minor groove binders.[15,23,28-30] For example, for daunomycin binding to the alternating and homopolymeric AT duplexes the reported ΔH_B values determined calorimetrically are –25.6 kJ/mol b.d. and 14.8 kJ/mol b.d., respectively.[30] It has been proposed that in its drug-free state poly[d(A)]·poly[d(T)] assumes a non-B conformation which is substantially more hydrated than the standard B-form double helix of poly[d(AT)]·poly[d(AT)]. Furthermore, it has been suggested that upon drug binding this altered conformation of poly[d(A)]·poly[d(T)] is converted to normal B conformation. In line with this feature, the thermodynamics for drug binding to the poly[d(A)]·poly[d(T)] duplex at 25 °C has been shown to be overwhelmingly entropy driven compared to the poly[d(AT)]·poly[d(AT)] duplex. This difference has been rationalized by proposing that drug binding to more hydrated poly[d(A)]·poly[d(T)] results in larger quantity of the displaced water and is therefore reflected in less exothermic or even endothermic enthalpies of binding and in much more positive entropies of binding.[15] The proposed explanation has been supported by several volumetric studies which clearly show that ligand binding to the homopolymeric duplex is accompanied by a significantly larger increase in volume than binding to the heteropolymeric duplex.[31-33]

Berenil binding to the two duplexes was followed also by means of UV titrations in which absorbance of the solution with constant berenil concentration was measured at 352 nm and 25 °C as a function of the drug/base pair molar ratio, r. Saturation value of this ratio, r_{sat}, which corresponds to a point at which the added DNA is just saturated with the bound berenil (Fig. 2), is related to the binding site size or so called "site exclusion parameter" n as $n = 1/r_{\mathrm{sat}}$. The stoichiometric ratios for the berenil-DNA complexes and the corresponding site exclusion parameters were determined for each duplex from the intersection of the two straight-line portions of the titration curve. The resulting n values of 3.7 for poly[d(AT)]·poly[d(AT)] and 3.8 for poly[d(A)]·poly[d(T)] are very reproducible and in good agreement with the values obtained from footprinting,[6,7] molecular modeling[7-10] and X-ray structural[7,11,12] studies. They agree well also with the n values obtained from the inflection points of the corresponding CD titration curves observed at high drug /DNA densities. Inspection of UV titration curves also shows that each of them is characterized by a single inflection point. Evidently, from these curves no cooperativity in berenil binding at low r values can be detected. For this reason we tried to interpret berenil binding to the host duplexes in terms of the Scatchard[34] plot, r/c vs. r, in which (Fig. 5) r and c values were obtained from the absorbance measurements (eqs. 3 and 4).

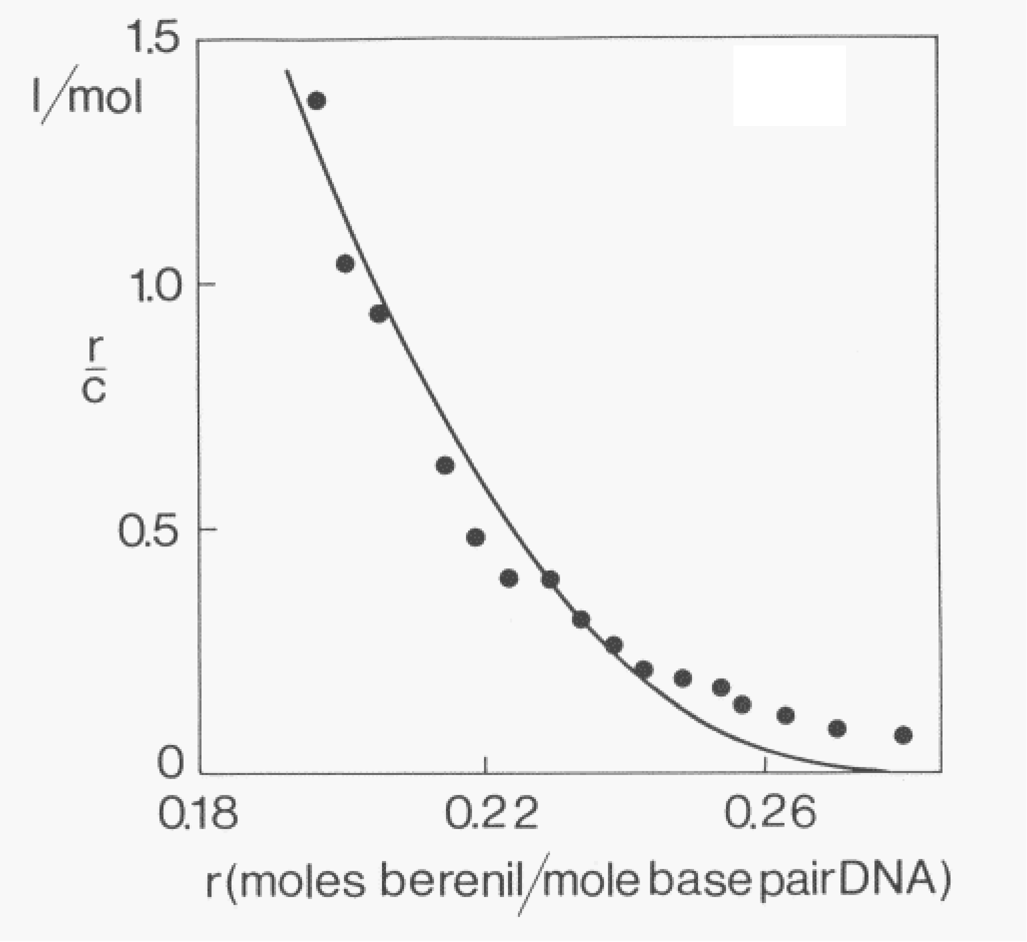

Figure 5: Scatchard plot for binding of berenil to poly[d(AT)]·poly[d(AT)]. Full line represents the best fit of the model function (eq. 5) to experimental data (●). Similar plot was obtained also for berenil binding to poly[d(A)]·poly[d(T)].

Experimental data were analyzed in terms of the McGhee-von Hippel's theory which, provided that there is no cooperativity in the ligand binding to the DNA lattice, describes the binding process as:[35]

$$\frac{r}{c} = K(1 - nr)\left[\frac{1 - nr}{1 - (n - 1)r}\right]^{n-1}, \tag{5}$$

where *K* is the isolated site binding constant and n is the exclusion parameter. Parts of titration curves refering to the *r* values below the DNA saturation were converted to Scatchard plots (Fig. 5) using the eq. 4. The solid lines in these plots were calculated from eq. 5 using a nonlinear least-square fitting of eq. 5 to the experimental *r/c* vs. *r* data. The best fit was obtained with $K = 1.3{\cdot}10^7$ and $n = 3.5$ for poly[d(AT)]·poly[d(AT)] and with $K = 1.5{\cdot}10^7$ and $n = 3.8$ for poly[d(A)]·poly[d(T)]. It should be noted that these *n* values agree well with those obtained at high *r* values from CD and UV titration curves.

The berenil-DNA duplex binding constants were estimated also from the measured UV-melting curves (Fig 6).

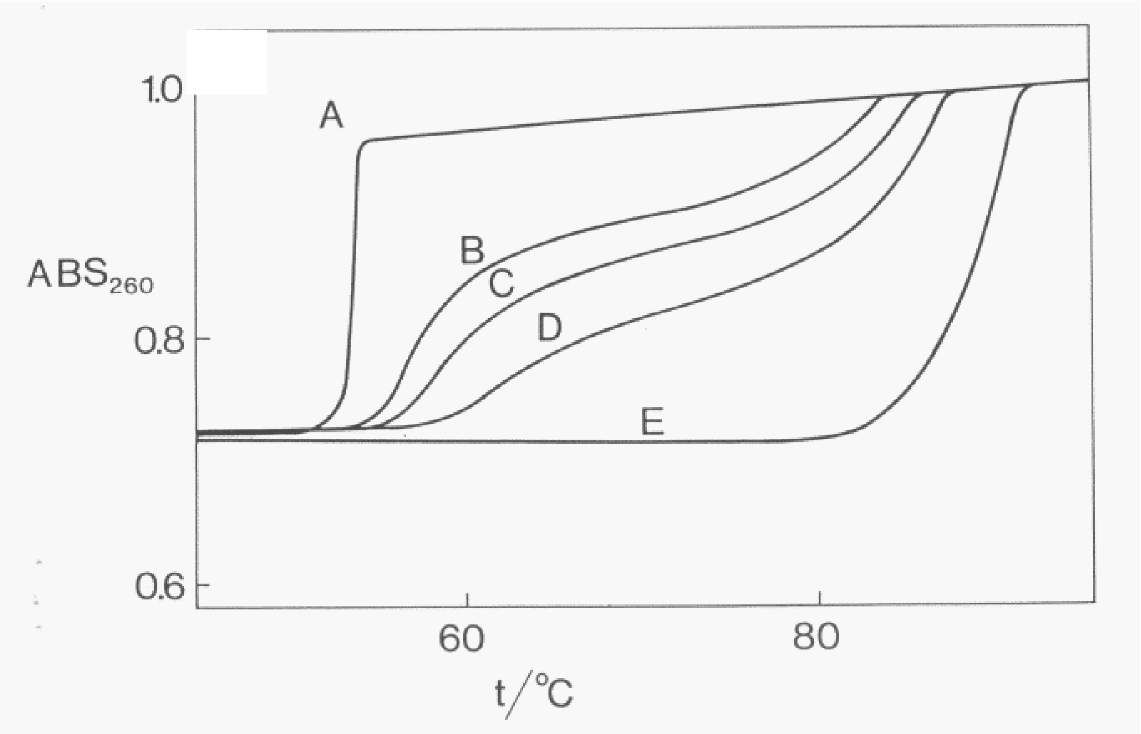

Figure 6: UV-melting curves of ligand free poly[d(A)]·poly[d(T)] (curve A (r = 0.000)), poly[d(A)]·poly[d(T)] partialy saturated with berenil (curves: B (r = 0.063), C (r = 0.083), D (r = 0.125)) and poly[d(A)]·poly[d(T)] completely saturated with berenil (curve E (r = 0.250)). These UV-melting curves are very close to those measured with berenil binding to poly[d(AT)]·poly[d(AT)] (not shown here).

Using the theoretical treatment of Crothers[22] and assuming that berenil binds only to separate, noninteracting sites on the DNA lattice of the initial duplex state, one obtains that at saturation

$$\frac{1}{T_m^f} - \frac{1}{T_m^b} = \frac{R}{n\Delta H_{h\rightarrow c}} \ln\left(1 + K_{app}\, c\right), \tag{6}$$

where T_m^f and T_m^b are the measured melting temperatures of the berenil-free and berenil-bound duplexes at which one half of the DNA molecules undergoes helix-coil transition, n is the site exclusion parameter expressed as the number of base pairs covered by each bound berenil molecule, $\Delta H_{h\rightarrow c}$ is the helix-coil transition enthalpy per base pair of the drug free duplex, K_{app} is the apparent drug-duplex binding constant at T_m^b, c represents the concentration of the free berenil at T_m^b and R is the gas constant. K_{app} values derived at the melting temperatures of the drug-bound complexes were extrapolated to 25 ^{o}C to obtain the corresponding binding constants, K, at this temperature (Table 1). According to van't Hoff relation

$$\ln K = \ln K_{app} - \frac{\Delta H_B}{R}\left(\frac{1}{298.15} - \frac{1}{T_m^b}\right), \tag{7}$$

where ΔH_B is the enthalpy od berenil binding to DNA duplex determined from calorimetric titration experiments and assumed to be independent of temperature and concentration (then $\Delta H_B = \Delta H_B^0$). This assumption seems to be supported by a good agreement between K values derived from ΔT_m data and from classic binding isotherms observed with some other ligand/DNA systems.[23,36-39] Thus, by employing eqs. 6 and 7 the following berenil binding constants at 25 °C were obtained: $K = 2.6\cdot10^7$ for poly[d(AT)]·poly[d(AT)] and $K = 6.8\cdot10^7$ for poly[d(A)]·poly[d(T)]. Since these values appear to be more reliable than those obtained from Scatchard plots they were used to determine the thermodynamic binding profiles for berenil binding to poly[d(AT)]·poly[d(AT)] and poly[d(A)]·poly[d(T)] at 25 °C (Table 1), by combining the general thermodynamic relations $\Delta G_B^0 = -RT \ln K$ and $\Delta G_B^0 = \Delta H_B^0 - T\Delta S_B^0$ with the experimental and $\Delta H_B = \Delta H_B^0$ values obtained from the corresponding calorimetric titrations.

Association of doubly charged berenil molecules to highly charged DNA polymers obviously involves electrostatic interactions that are typical of strong polyelectrolyte solutions. The relative importance of these interactions in determining binding of berenil cations to DNA is reflected in the salt dependence of the berenil binding constant. This dependence may be interpreted in terms of the electrostatic theory of polyelectrolyte solutions based on the infinite line charge model.[40] The model considers the polyion as a linear array of univalent charges of average spacing, *b*, characterized with a dimensionless charge density parameter, ξ, defined as:

$$\xi = \frac{e_0^2}{\varepsilon kTb}, \tag{8}$$

where e_0 is the proton charge, ε the solvent dielectric constant, k the Boltzmann constant and T the absolute temperature. It has been shown that the fraction of counterions bound to the polyion, Ψ, is determined by the direct condensation of counterions on the polyion[40] and by the screening effect of counterions and coions on the remaining unneutralized charges of the polyion.[31] It is given as:

$$\psi = 1 - \frac{1}{2\xi} \tag{9}$$

and for the B form of DNA in aqueous solutions at 25 °C its value is $\Psi = 0.88$.[42] Record et al.[41,42] have shown that when a charged ligand is added to a polyelectrolyte solution its binding constant, *K*, depends on the total counterion concentration, $[M^+]$, as:

$$\frac{\partial \log K}{\partial \log [M^+]} = -z\psi, \tag{10}$$

where z is the charge on the ligand molecule and the product $z\Psi$ is the number of counterions released when such ligand molecule binds to the polynucleotide. Thus, the theoretical slope of the log$K$ vs. log$[M^+]$ relation for singly charged ligands is equal to -0.88 while for doubly charged ligands this value becomes -1.76. Binding constants for the association of berenil to both polynucleotides at several salt concentrations were determined from the corresponding T_m measurements as described earlier. For both duplexes linear log$K$ vs. log$[Na^+]$ plots (not shown here) were obtained with the slopes presented in Table 1. Comparison of these results with those predicted by the theory indicates that in both complexes one of the berenil charges is less strongly involved in binding than the other and therefore less than 1.76 moles of Na$^+$ ions per mol of bound berenil are released to the solvent.

Table 1: Thermodynamic parameters determined for the interactions of berenil with poly[d(AT)]·poly[d(AT)] and poly[d(A)]·poly[d(T)] at $[Na^+]$ = 22.1 mM and 25 °C from UV titrations (exclusion parameter n), UV melting curves (T_m^f, T_m^b, apparent equilibrium constant K) and calorimetric titrations (ΔH_B^0).

	poly[d(AT)]·poly[d(AT)]	poly[d(A)]·poly[d(T)]
$\Delta H_{h\rightarrow c}$[α] (kJ/mol$_{\mathrm{b.p.}}$)	30.5	34.3
T_m^f (°C)	47.8	54.2
T_m^b (°C)	80.3	87.4
n (base pair/drug)	3.7 (3.5)[β]	3.8 (3.8)[β]
K	$2.3\cdot10^7$ ($1.3\cdot10^7$)[β]	$7.4\cdot10^6$ ($1.5\cdot10^7$)[β]
ΔG_B^0 (kJ/mol$_{\mathrm{b.d.}}$)	-42.0	-39.2
ΔH_B^0 (kJ/mol$_{\mathrm{b.d.}}$)	-20.4	10.3
$T\Delta S_B^0$ (kJ/mol$_{\mathrm{b.d.}}$)	21.6	49.5
$\partial\log K/\partial\log[Na^+]$	-1.26	-1.33

[α] Enthalpy of melting of a single base pair, $\Delta H_{h\rightarrow c}$, was obtained from the literature.[19]
[β] For comparison the values obtained from Scatchard plots (Fig. 5, eq. 5) are presented in the round brackets.

As presented in Table 1, our comparative study of berenil binding to poly[d(AT)]·poly[d(AT)] and poly[d(A)]·poly[d(T)] shows that berenil exhibits similar binding affinity (ΔG_B^0) for complexation to the alternating copolymer duplex and the homopolymer duplex. It also shows that berenil binding to poly[d(AT)]·poly[d(AT)] is about equally driven by enthalpic and entropic forces, whereas binding to poly[d(A)]·poly[d(T)] is overwhelmingly entropy driven. In other words, despite of different structures and different modes of binding enthalpy-entropy compensations cause berenil to exhibit similar affinity when it complexes with the two all-AT polynucleotides. Similar results have been obtained from binding studies of some other minor groove binders and intercalators and, as already mentioned, interpreted in terms of an altered and more hydrated B conformation of the homopolymer duplex.[15,23] It has been suggested that the enthalpy-entropy compensations observed when drugs bind to the all-AT homopolymer result from the drug induced melting of the polymer hydration domains at levels beyond the minor groove coupled with the release of displaced water to the bulk medium. The first effect accounts for the observed decrease in the exothermic enthalpy of binding and the second for the observed increase in the corresponding entropy of binding.

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Povzetek

Termodinamiko vezave berenila na poly[d(AT)]·poly[d(AT)] in poly[(dA)]·poly[(dT)] dvojni vijačnici v nevtralnih puferskih raztopinah smo raziskovali s kombinacijo kalorimetričnih in spektroskopskih metod. S titracijsko mikrokalorimetrijo smo določili entalpiji vezanja berenila na DNK, ki znašata za poly[d(AT)]·poly[d(AT)] –20,4 kJ/mol, za poly[d(A)]·poly[d(T)] pa 10,3 kJ/mol. Rezultati merjanja CD spektrov kompleksov berenil/DNK kažejo, da je vezanje berenila pri nizkih razmerjih berenil/DNK nekoliko kooperativno, pri visokih gostotah vezanja, kjer se veže ena molekula berenila na 4 AT bazne pare, pa postane konstanta vezanja od gostote vezanja neodvisna. Vezanje berenila smo pri 25 ^{o}C spremljali tudi s pomočjo UV titracij berenila z DNK in s pomočjo merjenja UV talilnih krivulj pri različnih razmerjih berenil/DNK. Iz Scatchardovih diagramov smo s prilagajanjem modelne funkcije eksperimentalni določili $K = 1{,}3\cdot10^{7}$ in $n = 3{,}5$ za poly[d(AT)]·poly[d(AT)] and $K = 1{,}5\cdot10^{7}$ in $n = 3{,}8$ za poly[d(A)]·poly[d(T)]. UV talilne krivulje so pokazale, da je za oba polinukleotida nasičena z vezanim berenilom, taljene enofazno in ga spremlja veliko povišanje T_m. V primeru razmerij berenil/DNK manjših od ustreznega razmerja pri nasičenju pa postane taljenje dvofazno. Konstanti vezanja določeni pri 25 ^{o}C na osnovi izmerjenih T_m vrednosti in modela, ki predpostavlja medsebojno

neodvisnost veznih mest sta $K = 2{,}3 \cdot 10^7$ za poly[d(AT)]·poly[d(AT)] in $K = 7{,}4 \cdot 10^6$ za poly[d(A)]·poly[d(T)]. Iz teh *K* vrednosti sta bili določeni standardni prosti entalpiji vezanja berenila na oba polinukleotida in njih kombinacija z izmerjenima entalpijama vezanja je dala ustrezni entropiji vezanja. Tako dobljena termodinamska profila vezanja kažeta, da je vezanje berenila na poly[d(AT)]·poly[d(AT)] s približno enako težo določeno z entalpijskim in entropijskim prispevkom, vezanje na poly[d(A)]·poly[d(T)] pa je izrazito entropijsko voden proces.