Equilibrium Binding of Benzo[a]pyrene Tetrol to Synthetic Polynucleotides: Sequence Selectivity, Thermodynamic Properties, and Ionic Strength Dependence†

George H. Shimer, Jr., Alan R. Wolfe, and Thomas Meehan*
Division of Toxicology and Department of Pharmacy, University of California, San Francisco, San Francisco, California 94143-0446

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ABSTRACT: We have investigated the equilibrium binding of racemic 7*r*,8*t*,9*t*,10*c*-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene to the double-stranded, synthetic polynucleotides poly[d(A-T)], poly[d(G-C)], and poly[d(G-m^{5}C)] at low binding ratios. Difference absorption spectroscopy shows a 10-nm red shift for binding to poly[d(A-T)] and an 11-nm red shift for binding to either poly[d(G-C)] or poly[d(G-m^{5}C)]. The value of $\Delta\epsilon$ for binding is approximately the same for all three hydrocarbon–polynucleotide complexes. Binding of this neutral polycyclic aromatic hydrocarbon derivative to these polynucleotides is dependent upon ionic strength and temperature. Analysis of complex formation employing polyelectrolyte theory shows a greater release of counterions associated with binding to poly[d(A-T)] than with the other two polynucleotides (0.5 and ca. 0.36, respectively). Thus, sequence-selective binding of this hydrocarbon in DNA would be expected to change depending on salt concentration. The temperature dependence of binding was studied at 100 mM Na^+ where the equilibrium binding constants for poly[d(A-T)] and poly[d(G-m^{5}C)] are roughly equivalent and 6-fold greater than the binding affinity for poly[d(G-C)]. The binding to poly[d(A-T)] and poly[d(G-C)] is characterized by a $\Delta H^\circ = -7.0$ kcal/mol, and the large difference in affinity constants arises from differences in negative entropic contributions. Formation of hydrocarbon–poly[d(G-m^{5}C)] complexes is accompanied by a $\Delta H = -9.1$ kcal/mol. However, the affinity for poly[d(G-m^{5}C)] is the same as that for poly[d(A-T)] due to the much more negative entropy associated with binding to poly[d(G-m^{5}C)].

Benzo[*a*]pyrene (BP)[1] is an important environmental procarcinogen that requires metabolic activation in order to alkylate DNA (Heidelberger, 1975). The activated form of the procarcinogen has been identified as 7*r*,8*t*-dihydroxy-9*t*,10*t*-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (*anti*-BPDE) (Sims et al., 1974; Huberman et al., 1976; King et al., 1976; Koreeda et al., 1976; Meehan et al., 1976; Weinstein et al., 1976). The principal lesion formed is an adduct with the exocyclic amino group of guanine (Straub et al., 1977). The adducts lead to a variety of mutations (Wei et al., 1984; Burgess et al., 1985).

Prior to alkylation, *anti*-BPDE forms a physical complex with the DNA, which would be expected to affect the course of the covalent binding reaction. Many BP derivatives and other polycyclic aromatic hydrocarbons form similar physical complexes with DNA (Craig & Isenberg, 1970; Abramovich et al., 1985). The principal binding mode results from intercalation of the planar hydrocarbons between the stacked base pairs as is evident from the unwinding of supercoiled DNA in the presence of BPDE (Meehan et al., 1982), the red shift of the absorption maximum of the bound hydrocarbon (Geacintov et al., 1981; MacLeod & Selkirk, 1982), and the linear dichroism of hydrocarbons physically bound to flow-oriented DNA (Geacintov et al., 1984). However, there have been several reports of weaker complexes that are external to the helix (Ibanez et al., 1980; Chen, 1983, 1984). Bulky alkyl substituents have been shown to reduce the binding affinity of BP derivatives to DNA (Paulius et al., 1986) and to reduce their carcinogenicity (Harvey et al., 1985).

Since random-sequence DNA contains a variety of possible intercalation sites, it is important to examine the effect of sequence on equilibrium binding. One approach to this problem is to utilize synthetic polynucleotides with regular or alternating base sequences. Several groups have examined physical binding of uncharged intercalators to synthetic polynucleotides and report preferential binding to poly[d(A-T)] relative to poly[d(G-C)], with little binding to double-stranded homopolymers. These results were obtained with (±)-7*r*,8*t*-dihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (Yang et al., 1983), pyrene (Chen, 1983), 7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydroxybenzo[*a*]pyrene (Chen, 1984), and *anti*-BPDE (Geacintov, 1987). The latter group also reported the preferential binding of *anti*-BPDE to the 5-methylcytosine derivative of poly[d(G-C)].

In order to identify those parameters responsible for sequence-selective binding, we have examined the temperature and ionic strength dependencies of *trans*-tetrol complex formation with poly[d(A-T)], poly[d(G-C)], and poly[d(G-m^{5}C)]. We have found that the variation in physical binding affinities results from sequence-dependent effects on both the enthalpy and entropy associated with intercalation as well as ionic strength.

Experimental Procedures

Chemicals. (±)-*anti*-BPDE was synthesized as described previously (Meehan et al., 1982) and was hydrolyzed to its 7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene derivative by mild acid treatment. The resultant *cis*- and *trans*-tetrols were separated by reverse-phase, high-performance liquid chromatography (Yang et al., 1977). Acid hy-

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[1] Abbreviations: BP, benzo[*a*]pyrene; (±)-*anti*-BPDE, (±)-7*r*,8*t*-dihydroxy-9*t*,10*t*-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; *trans*-tetrol, racemic 7*r*,8*t*,9*t*,10*c*-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; *cis*-tetrol, racemic 7*r*,8*t*,9*t*,10*t*-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; EDTA, ethylenediaminetetraacetic acid.

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drolysis of *anti*-BPDE results in a 5:1 ratio of *trans*- to *cis*-tetrol, and therefore *trans*-tetrol was used in these studies. No experiments have been performed to compare *trans*- and *cis*-tetrol binding.

Poly[d(G-C)], poly[d(G-m^{5}C)], and poly[d(A-T)] were purchased from Pharmacia P-L Biochemicals (Piscataway, NJ) and were used without additional purification. All other chemicals used were of analytical or spectral quality.

Concentration Determinations. Concentrations of (±)-*anti*-BPDE, *trans*-tetrol, and polynucleotides were determined by absorption spectroscopy. Extinction coefficients (M^{-1} cm^{-1}) were determined for the two BP derivatives and are as follows: (±)-*anti*-BPDE in tetrahydrofuran, ϵ_{346nm} = 46 600; *trans*-tetrol in methanol, ϵ_{343nm} = 44 400. Values for the polynucleotides, obtained from the literature, were as follows: poly[d(G-C)], ϵ_{256nm} = 8400 (in bases) (Wells et al., 1970); poly[d(G-m^{5}C)], ϵ_{256nm} = 7000 (Gill et al., 1974); poly[d(A-T)], ϵ_{260nm} = 7000 (Wells et al., 1970).

Physical Binding of trans-Tetrol to Polynucleotides. Difference absorption spectra resulting from the physical binding of *trans*-tetrol to polynucleotides were measured directly through the use of tandem cuvettes with a Cary 118 spectrophotometer. Each chamber of the tandem cuvettes has a path length of 0.437 cm. The temperature was maintained by using a circulating water bath and was monitored by immersion of a thermocouple into the rear compartment of the sample cuvette. All binding experiments were performed in 5 mM sodium phosphate, pH 7.5, and 0.1 mM EDTA (buffer A), and NaCl was added to give the indicated Na^+ concentration.

Titrations were performed by adding 1.0 mL of *trans*-tetrol (~2.5 μM) in buffer to the front chambers and 1.0 mL of buffer to the rear chambers of both the sample and reference cuvettes. The *trans*-tetrol in the sample cuvette was titrated with a concentrated solution (ca. 25 mM in bases) of the desired polynucleotide, and the same amount of polynucleotide was added to the rear chamber (buffer only) of the reference cuvette to correct for light scattering by the polynucleotide in the 310–400-nm wavelength region. The *trans*-tetrol in the reference cuvette was diluted with an identical volume of buffer. Use of this method results in the direct measurement of the physically bound *trans*-tetrol, and the measured difference absorption is defined as (Herskovits, 1967)

$$\Delta A = \Delta\epsilon C_B \ell \quad (1)$$

where ΔA is the observed difference absorption, C_B is the concentration of the bound *trans*-tetrol, and $\Delta\epsilon$ is the extinction coefficient of the bound *trans*-tetrol minus that of the free *trans*-tetrol ($\epsilon_B - \epsilon_F$).

Data Analysis. The binding of *trans*-tetrol to DNA can be described by the mass action expression

$$K = \frac{C_B}{C_F(nC_{N^0} - C_B)} \quad (2)$$

where C_B and C_F are concentrations of bound and free *trans*-tetrol, respectively, C_{N^0} is the total concentration of polynucleotide in base pairs, and n is the number of binding sites per base pair. We have analyzed our data using a variant of the Benesi–Hildebrand equation for low binding ratios (i.e., $C_B/C_{N^0} \ll n$) as presented in Bloomfield et al. (1974). This method should be suitable for analysis since in our experiments the value of C_B/C_{N^0} never exceeded 0.015.

The affinity constant (K') is defined as $K' = nK$, assuming identical and independent binding sites. Substituting into eq 2 and assuming $C_B/C_{N^0} \ll n$, the mass action equation reduces to

$$K' = C_B/C_F C_{N^0} \quad (3)$$

Substitution of eq 1 into eq 3 followed by rearrangement yields the equation

$$\frac{C_{N^0}C_T}{\Delta A} = \frac{C_{N^0}}{\ell\Delta\epsilon} + \frac{1}{K'\ell\Delta\epsilon} \quad (4)$$

where C_T is the total *trans*-tetrol concentration and the other terms are the same as described above. Plots of absorbance versus C_{N^0} were used to determine $\Delta\epsilon$ and K'. $\Delta\epsilon$ was obtained from the slope of the linear least-squares fit to the data, and K' was calculated by extrapolation to the ordinate intercept.

Determination of Thermodynamic Parameters. The temperature dependence of binding was used to estimate the enthalpy and entropy parameters for the formation of *trans*-tetrol–polynucleotide complexes. All experiments were performed in buffer A with [Na^+] = 100 mM. The experiments were carried out in two ways. In the first method, the solution of *trans*-tetrol was titrated with polynucleotide, as described above, at the desired temperature. In the second method, a constant ratio of *trans*-tetrol and polynucleotide was mixed in the tandem cuvettes at 25 °C, and the difference spectrum was recorded. The temperature was then adjusted as necessary, and spectra were collected at several temperatures. The value for C_B was determined from eq 1 by using a $\Delta\epsilon$ value determined at 25 °C (assuming that $\Delta\epsilon$ for binding is independent of temperature), and K' was determined from eq 3. Comparison of the two methods is presented under Results. To assure that the binding reactions are reversible, following all experiments at temperatures other than 25 °C, the temperature was subsequently adjusted to 25 °C and spectra were collected. Only those experiments where the spectra coincided with titration experiments originally performed at 25 °C were used in determining the thermodynamic parameters of physical binding.

Linear least-squares analysis of ln K' vs 1/[T (K)] plots was used to determine the enthalpy from the van't Hoff relationship

$$\ln K' = (1/T)\frac{\Delta H^\circ}{R} - \frac{\Delta S^\circ}{R} \quad (5)$$

Salt Dependence of trans-Tetrol Binding. The dependence of intercalative binding on salt concentration was determined by mixing the *trans*-tetrol and the polynucleotide at the lowest Na^+ concentration (8.7 mM) studied. The Na^+ concentration was increased by adding aliquots of 5 M NaCl to each chamber in the tandem cuvettes. It was assumed that $\Delta\epsilon$ for binding remained constant as a function of salt since the absorption spectrum of 2.5 μM *trans*-tetrol remained unchanged over the 8.7–400 mM [Na^+] range employed in these experiments (data not shown).

RESULTS

Difference Absorption Spectra. Panels A, B, and C of Figure 1 show the difference absorption spectra obtained upon titrating a solution of *trans*-tetrol with poly[d(A-T)], poly[d(G-C)], and poly[d(G-m^{5}C)], respectively. Since the polynucleotides have no absorption bands in the 320–400-nm wavelength region, the difference spectrum reflects only changes that occur in the absorption of *trans*-tetrol upon binding to the polynucleotide. The poly[d(A-T)]–*trans*-tetrol complex has a long-wavelength maximum centered at 353 nm resulting from a red shift of the 343-nm absorption maximum of *trans*-tetrol upon intercalation. The isosbestic point at 348 nm indicates that the poly[d(A-T)]–*trans*-tetrol interaction can be analyzed as a two-state system. The three minima at 343, 326, and 312 nm correspond in wavelength with the

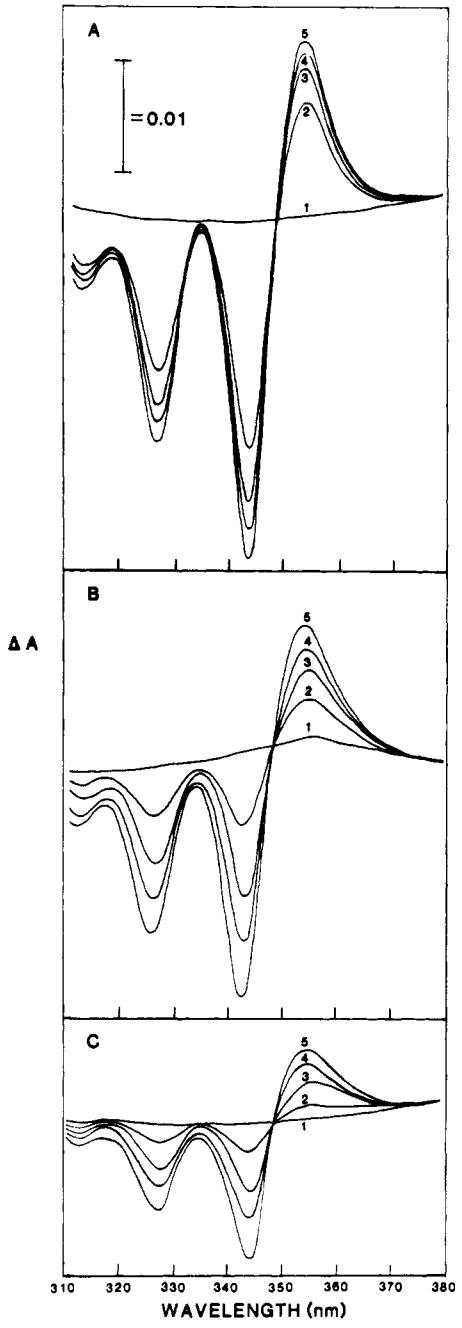

FIGURE 1: Difference absorption spectra obtained from titrations of *trans*-tetrol with poly[d(A-T)], poly[d(G-C)], and poly[d(G-m^{5}C)] in buffer A ([Na^+] = 8.7 mM) at 25 °C. (A) Poly[d(A-T)] (μM base pairs/μM *trans*-tetrol): 0/2.65 (1); 69.9/2.64 (2); 139/2.63 (3); 209/2.62 (4); 346/2.61 (5). (B) Poly[d(G-C)]: 0/2.21 (1); 40.1/2.19 (2); 119/2.18 (3); 185/2.17 (4); 313/2.14 (5). (C) Poly[d(G-m^{5}C)]: 0/2.60 (1); 18.4/2.59 (2); 67.4/2.57 (3); 91.7/2.57 (4); 116/2.56 (5).

absorption peaks of *trans*-tetrol in aqueous solution, and all three peaks decrease in absorption due to the red shift and hypochromicity associated with intercalation (Philpott, 1970).

The difference absorption specta of poly[d(G-C)]- and poly[d(G-m^{5}C)]-*trans*-tetrol complexes display a red-shifted long-wavelength absorption maximum at 354 nm. Both of these polynucleotide–*trans*-tetrol difference absorption spectra have an isosbestic point at 348 nm. The three minima are located at the same wavelengths and follow the same patterns as those observed for the poly[d(A-T)]–*trans*-tetrol complexes.

Figure 2 displays the results from the titration experiments of *trans*-tetrol with the polynucleotides in the form of Benesi–Hildebrand plots. The values of ΔA used for eq 4 are

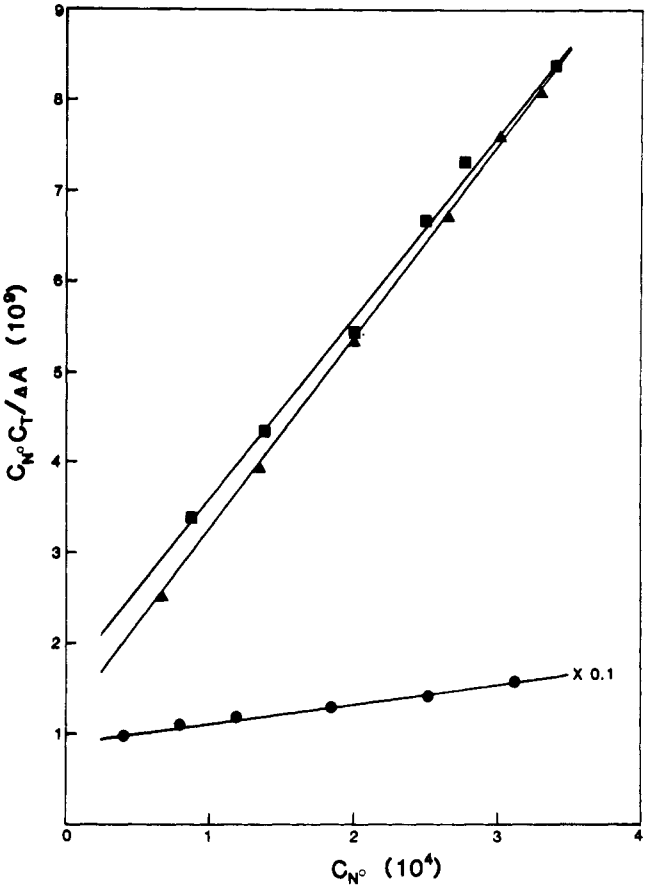

FIGURE 2: Benesi–Hildebrand plot (eq 4) of equilibrium binding of *trans*-tetrol to polynucleotides as measured in Figure 1. *trans*-Tetrol binding to poly[d(A-T)] (▲), poly[d(G-C)] (●), and poly[d(G-m^{5}C)] (■). Data were fit by linear least-squares regression, and correlation coefficients were greater than 0.99.

Table I: Binding Parameters[a]

	$\Delta\epsilon$ (10^{-4})[b] (M^{-1} cm^{-1})	K' (10^{-3}) (M^{-1})
poly[d(A-T)]	4.7	18.8 ± 0.7
poly[d(G-C)]	4.6	2.4 ± 0.3
poly[d(G-m^{5}C)]	5.0	12.6 ± 0.4

[a] Determined from Figure 2; 5 mM sodium phosphate, pH 7.5, 0.1 mM EDTA [b] Determined from $\Delta A_{max} - \Delta A_{343}$.

ΔA(maximum) – ΔA_{343nm}, where ΔA(maximum) is the change in absorption at the long-wavelength peak. This difference value was used because small, observable drifts in the base line of the spectrophotometer over the course of an experiment would lead to appreciable error. Because of this problem, this difference value gave the most consistent results. Values of $\Delta\epsilon$ and K' were derived from linear least-squares regression of the data to eq 4 (correlation coefficients > 0.99). The results are presented in Table I. The K' values determined for intercalative binding of *trans*-tetrol to poly[d(A-T)] and poly[d(G-m^{5}C)] are almost 8-fold and 5-fold greater, respectively, than for binding to poly[d(G-C)].

There is very little variation in the value of $\Delta\epsilon$ determined with the three polynucleotides. Since the wavelength shift and $\Delta\epsilon$ are theoretically related (Marcus, 1965) and the *trans*-tetrol–polynucleotide complexes are all similarly red shifted, it is not surprising that the values for $\Delta\epsilon$ are equivalent.

Thermodynamics of Intercalation. The temperature dependence of intercalation has been used to derive the enthalpy of *trans*-tetrol binding to the three polynucleotides studied. The difference absorption spectra presented in panels A–C of Figure 3 maintain their peak positions and isosbestic points,

Table II: Thermodynamics of Intercalation

DNA	compd	[M^+] (mM)	K^a (10^{-3} M^{-1})	$\delta \log K'/\delta \log [M^+]$	$\Delta H°$	$\Delta S°$ [cal/(deg·mol)]	ref
poly[d(A-T)]	*trans*-tetrol	100	5.4	−0.50	−7.0	−6.4	this work
poly[d(G-C)]	*trans*-tetrol	100	1.2	−0.39	−6.9	−9.1	this work
poly[d(G-m^{5}C)]	*trans*-tetrol	100	5.5	−0.35	−9.1	−13.4	this work
calf thymus	BP-9,10-diol[b]	8	8.5	−0.38	−10.2	−16.1	MacLeod et al., 1987
calf thymus	(±)-*anti*-BPDE	8	20		−8.5	−8.9	MacLeod et al., 1987
calf thymus	pyrene	10	9.5	−(0.3 to 0.5)	−8.1		Nelson & DeVoe, 1984
calf thymus	ethidium	1000	14		−8.4	−9.2	Bresloff & Crothers, 1975

[a] Calculated from the $\Delta H°$ and $\Delta S°$ at 25 °C by employing the relationship $-\ln K = (\Delta H - T\Delta S)/RT$. [b] BP-9,10-diol: *trans*-9,10-dihydroxy-9,10-dihydrobenzo[*a*]pyrene.

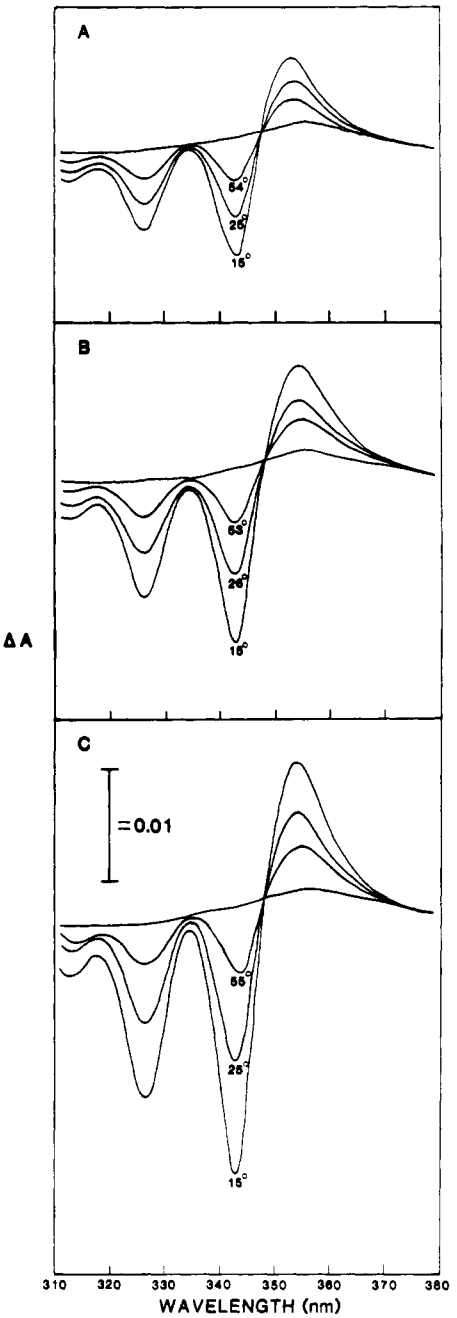

FIGURE 3: Temperature dependence of difference absorption spectra for *trans*-tetrol–polynucleotide interactions in buffer A with [Na^+] = 100 mM. Spectra were collected at constant [polynucleotide]/[*trans*-tetrol] ratios, and the temperature was varied. The concentration ratios are as follows: (A) poly[d(A-T)], 132/1.58; (B) poly[d(G-C)], 326/3.14; (C) poly[d(G-m^{5}C)], 166/1.35.

over the temperature range studied, at the same wavelengths as those observed for the titration experiments performed at 25 °C (panels A–C of Figure 1). This indicates that, although

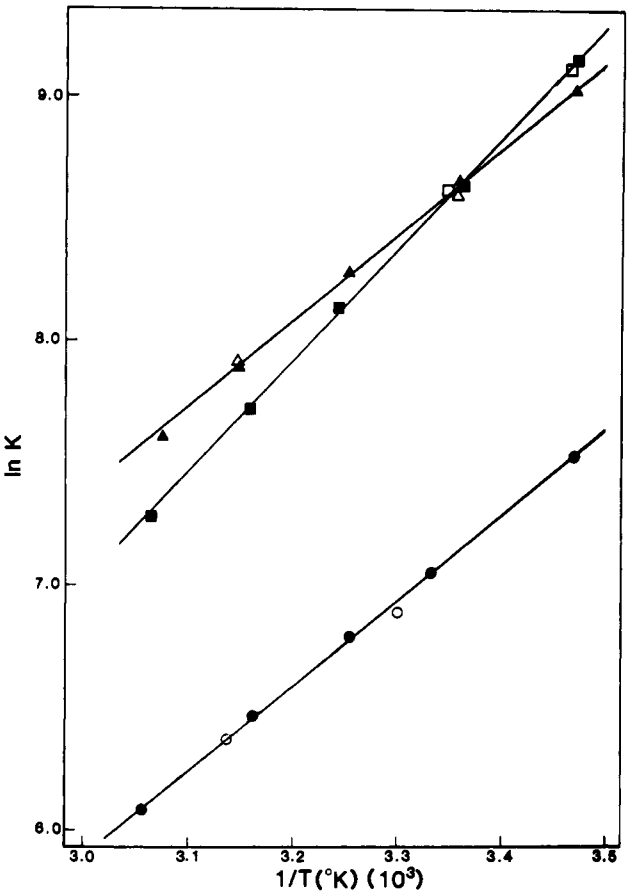

FIGURE 4: van't Hoff plot for *trans*-tetrol–polynucleotide binding in buffer A with [Na^+] = 100 mM. Equilibrium binding constants were determined from titrations at the desired temperature (open symbols) and from varying temperature at a constant [polynucleotide]/[*trans*-tetrol] ratio (solid symbols) as shown in Figure 3.

the absorption spectrum of *trans*-tetrol broadens slightly upon going from 15 to 55 °C (data not shown), there is no significant change in $\Delta\epsilon$ for binding and that the intercalation of *trans*-tetrol as a function of temperature can be analyzed as a two-state system. The validity of this assumption has also been ascertained by using two methods of determining K' at a given temperature. Figure 4 is the van't Hoff plot for intercalation of *trans*-tetrol into the three polynucleotides. The open symbols represent experiments where K' was determined from eq 4, and the solid symbols are determined from eq 3 where it has been assumed that $\Delta\epsilon$ is independent of temperature. There is no significant difference observed between these two methods. Similar results have also been reported for the temperature dependence of daunomycin interactions with calf thymus DNA (Chaires, 1985).

With all three polynucleotides, intercalation is favored by a negative enthalpic contribution and is opposed by the decrease in entropy. A comparison of our results with those

obtained for other intercalators is presented in Table II.

Ionic Strength Dependence of Intercalation. The effect of NaCl concentration on the physical binding of *trans*-tetrol to poly[d(A-T)], poly[d(G-C)], and poly[d(G-m^{5}C)] has been investigated. The difference absorption spectra over the entire NaCl concentration range studied have identical absorption extrema and isosbestic wavelengths as observed for the titration of *trans*-tetrol with the polynucleotides at low salt (Figure 1) and therefore have not been included. Taken together with the observation that the absorption spectrum of 2.5 μM *trans*-tetrol remains unchanged with added NaCl up to 400 mM, it is reasonable to assume that $\Delta\epsilon$ for binding will remain constant as a function of added salt. Therefore, at the low values of C_B/C_{N^0} employed in these experiments K' was calculated by using eq 3.

The polyelectrolyte theory of Manning (1978) and Record (1978) was originally developed to interpret the effects of counterion concentration on ligand binding to polyelectrolytes. In this theory, the polynucleotide is modeled as a linear array of negative charges on the phosphates and the axial distance between phosphates determines the extent of counterion condensation on the polynucleotide. This theory has been modified to account for the additional counterion release arising from structural changes of the polynucleotide which accompany intercalation (Wilson & Lopp, 1979; Friedman & Manning, 1984).

Wilson and Lopp (1979), using the classical thermodynamic formalism of Record, model intercalation into DNA as a two-step process in which the DNA conformation changes to that of the bound structure with a concomitant release of counterions followed by ligand binding. If the intercalator is charged, this second step would also result in the release of counterions from the polynucleotide due to ion pair formation. The dependence of K' on counterion (M^+) concentration is

$$\frac{\delta \log K'}{\delta \log [M^+]} = -2N(\psi - \psi^*) - m'\psi^* \qquad (6)$$

where N is the number of base pairs affected by the conformational change and m' is the number of ion pairs formed between the ligand and the DNA. ψ and ψ^* are the fractions of counterions condensed per phosphate in the unbound and bound conformations, respectively. This extension of polyelectrolyte theory explains why the intercalation of neutral molecules is dependent on counterion concentration. As the simplest model, the change in DNA conformation with intercalation can be viewed as an increase in length of the DNA with a corresponding increase in the phosphate–phosphate axial separation. For a neutral intercalator, the theoretical value of $\delta \log K'/\delta \log [M^+]$ is calculated to be −0.24 (Wilson & Lopp, 1979).

The ionic strength dependence of K' for intercalation of *trans*-tetrol into the three polynucleotides is presented in Figure 5. All three log–log plots are straight lines with correlation coefficients greater than 0.99. The values for $\delta \log K'/\delta \log [M^+]$ are −0.39 and −0.35 for poly[d(G-C)] and poly[d(G-m^{5}C)], respectively, and the value for poly[d(A-T)] is significantly greater at −0.50. These values are larger than the theoretical value by approximately 50–100%.

Discussion

The work presented here demonstrates that *trans*-tetrol intercalates into different sequences of DNA with varying affinities. The use of alternating-sequence purine–pyrimidine polynucleotides allowed us to systematically investigate the parameters governing intercalation of *trans*-tetrol. The K' for

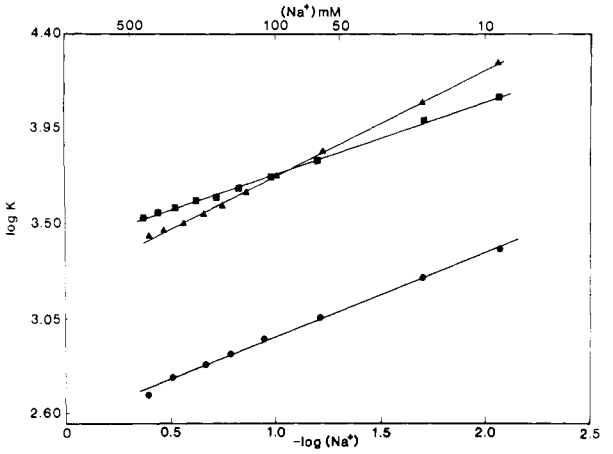

FIGURE 5: log K' vs −log sodium ion concentration for binding of *trans*-tetrol to poly[d(A-T)] (▲), poly[d(G-C)] (●), and poly[d(G-m^{5}C)] (■) in buffer A. Equilibrium binding constants were calculated by using eq 3, assuming $\Delta\epsilon$ to be independent of ionic strength. Lines are from linear least-squares regressions, and correlation coefficients were ≥0.99.

association with poly[d(A-T)], poly[d(G-C)], and poly[d(G-m^{5}C)] is a function of both ionic strength and temperature. In general, intercalation of this neutral hydrocarbon is accompanied by a release of thermodynamically bound counterions from the DNA, is driven by a favorable change in the enthalpy of the interaction, and is opposed by a decrease in entropy.

We have analyzed our data using a two-state model considering only intercalated *trans*-tetrol and that free in solution. For positively charged intercalators, such as proflavin, there is evidence for both intercalative and outside binding sites (Li & Crothers, 1969; Ramstein et al., 1980). Ibanez et al. (1980) reported the presence of both types of binding for tetrol to calf thymus DNA. MacLeod et al. (1987) have performed analogous experiments and conclude that dissociation of tetrol from DNA occurred as the temperature of dialysis (4 °C) was raised to 25 °C for the fluorescence measurements made by Ibanez et al. (1980) and this artifact is responsible for the putative external binding. In support of this, we find that the association constant decreases by 2.4- to 3.4-fold, depending on the polynucleotide, upon going from 4 to 25 °C in 5 mM sodium phosphate, pH 7.5, 0.1 mM EDTA, and 100 mM NaCl. Therefore, although the difference absorption method employed here will measure only the intercalated *trans*-tetrol, the determination of free *trans*-tetrol from $C_F = C_T - C_B$ used in our calculations should be accurate. In addition, it is worthwhile to note that the possible presence of an outside binding site is of more importance than just for accuracy in these and other spectroscopic studies of intercalation. The covalent adducts of the parent compound, (±)-*anti*-BPDE, with DNA are reported to be external to the helix (Geacintov, 1987) although noncovalently bound BPDE does intercalate (Meehan et al., 1982). On the basis of this information, Chen (1983) has proposed that an external equilibrium binding site for BPDE is involved with adduct formation. However, whether or not equilibrium binding at an external or intercalation site leads directly to adduct formation is still undecided and will be difficult to determine (Geacintov, 1986).

The thermodynamic parameters for the binding of *trans*-tetrol are compared to those of several other intercalators in Table II. The values of ΔH° for binding of *trans*-tetrol to poly[d(A-T)] and poly[d(G-C)] are the same within experimental error. The roughly 4.5-fold increase in $K'_{25°C}$ for binding to poly[d(A-T)] relative to poly[d(G-C)] at 100 mM

Na^+ is seen to arise from significant differences in $\Delta S°$ for these two systems. The reason for this difference is $\Delta S°$ is not readily apparent, but a contributing factor may be the greater counterion release associated with binding to poly[d(A-T)]. $\Delta H°$ of binding is 2 kcal/mol more favorable for binding to poly[d(G-m^5C)] relative to both poly[d(A-T)] and poly[d(G-C)]. The methyl group at the 5-position of cytosine increases the surface area of the base pair and possibly leads to a greater van der Waals stacking interaction between the hydrocarbon and the adjacent base pairs, which would result in a larger negative value for the enthalpy. However, the binding affinity is not significantly greater because the observed difference in $\Delta H°$ is opposed by the much more negative entropy associated with *trans*-tetrol binding to poly[d(G-m^5C)]. The values for standard enthalpies and entropies are within the ranges reported for other intercalators. As shown in Table II, the binding of the charged intercalator ethidium bromide at high ionic strength, where contributions from ion pair formation should be negligible, is characterized by thermodynamic parameters similar to those for neutral intercalators.

The physical binding of *trans*-tetrol to all three polynucleotides is dependent on the ionic strength of the solution. Analysis of the results using polyelectrolyte theory shows that *trans*-tetrol binding to poly[d(G-C)] and poly[d(G-m^5C)] is accompanied by the release of 0.35 and 0.39 thermodynamically bound counterions, respectively. Investigations with other uncharged intercalators and calf thymus DNA have resulted in similar values. MacLeod et al. (1987) have reported that $\delta \log K'/\delta \log [Na^+]$ equals −0.38 for *trans*-9,10-dihydroxy-9,10-dihydroBP binding to DNA, and we (Wolfe et al., 1987) have reported values of −0.35 and −0.37 for *trans*-tetrol binding to native and denatured DNA, respectively. In contrast to the linear slopes obtained with these experiments, the ionic strength dependence of pyrene binding to calf thymus DNA resulted in a nonconstant slope varying from −0.3 at low NaCl concentrations to −0.5 at the highest (700 mM) NaCl concentration employed (Nelson & DeVoe, 1984). For poly[d(A-T)]–*trans*-tetrol complexes, we observe $\delta \log K'/\delta \log [Na^+]$ to be equal to −0.5, which is significantly larger than we found for poly[d(G-C)] and poly[d(G-m^5C)]. The larger value of $\delta \log K'/\delta \log [M^+]$ observed for poly[d(A-T)] compared with the other two polynucleotides may be partially due to differences in the extent of counterion condensation on the different polynucleotides. Blake and Haydock (1979) reported 40% more ions released (in a thermodynamic sense) per nucleotide denatured in A-T rich regions than in G-C rich regions upon melting λ DNA.

All of these values are greater than the theoretical value for $\delta \log K'/\delta \log [M^+]$, which is −0.24 (Wilson & Lopp, 1979). The differences observed between experiment and theory may arise from assumptions used in the development of the polyelectrolyte theory (Record, 1978). Variation in the equilibrium constant with ionic strength is assumed to be due to entropy changes arising from counterion release. However, Chaires (1985) reports a strong ionic strength dependence for the enthalpy of intercalation for the cationic drug daunomycin with calf thymus DNA. Whether this dependence occurs in the binding of neutral intercalators is not known. It should be noted that the DNA helix is believed to wind more tightly with increasing strength (Tunis-Schneider & Maestre, 1971; Hanlon et al., 1975). This would result in a decrease in the phosphate–phosphate distances before intercalation, which could effectively increase the value of $\delta \log K'/\delta \log [M^+]$ (see eq 6).

In this and other reports (Chen, 1983; Yang et al., 1983; Geacintov, 1987) on the equilibrium binding of pyrene and BPDE derivatives to synthetic polynucleotides, a large preference for binding to poly[d(A-T)] relative to poly[d(G-C)] has been observed at low ionic strengths. The nonlinearity of Scatchard plots for ligand binding to DNA has been explained by the excluded site model developed by McGhee and Von Hippel (1974). While this model accounts for excluded site size, the large differences observed for equilibrium binding to sequence-specific polynucleotides might also help explain the nonlinearity in Scatchard plots of binding to random sequences of DNA. The binding of *trans*-9,10-dihydroxy-9,10-dihydrobenzo[*a*]pyrene to calf thymus DNA at low ionic strength has a reported excluded site size equal to 3.3 bases (MacLeod et al., 1987), which is significantly larger than the value of 2 found for the well-studied intercalator ethidium bromide. Ignoring the contributions to curvature in Scatchard plots resulting from multiple binding sites with widely varying affinities may give rise to erroneously large values of excluded site size.

In summary, we have observed that intercalation of *trans*-tetrol into poly[d(A-T)], poly[d(G-C)], and poly[d(G-m^5C)] are all characterized by a favorable enthalpy and opposed by a negative entropy contribution to binding. Intercalation into all three polynucleotides is accompanied by the release of thermodynamically bound counterions. On the basis of the larger value of $\delta \log K'/\delta \log [Na^+]$ found for binding of poly[d(A-T)] relative to poly[d(G-C)], the ratio of binding to different sites in random-sequence DNA is probably strongly dependent on ionic strength.

Registry No. *trans*-Tetrol, 62697-19-2; poly[d(A-T)], 26966-61-0; poly[d(G-C)], 36786-90-0; poly[d(G-m^5C)], 51853-63-5.

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Inhibition and Derivatization of the Renal Na,K-ATPase by Dihydro-4,4′-diisothiocyanatostilbene-2,2′-disulfonate†

Carlos H. Pedemonte and Jack H. Kaplan*
Department of Physiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6085
Received April 11, 1988; Revised Manuscript Received June 23, 1988

ABSTRACT: Treatment of purified renal Na,K-ATPase with dihydro-4,4′-diisothiocyanatostilbene-2,2′-disulfonate (H_2DIDS) produces both reversible and irreversible inhibition of the enzyme activity. The reversible inhibition is unaffected by the presence of saturating concentrations of the sodium pump ligands Na^+, K^+, Mg^{2+}, and ATP, while the inactivation is prevented by either ATP or K^+. The kinetics of protection against inactivation indicate that K^+ binds to two sites on the enzyme with very different affinities. Na^+ ions with high affinity facilitate the inactivation by H_2DIDS and prevent the protective effect of K^+ ions. The H_2DIDS-inactivated enzyme no longer exhibits a high-affinity nucleotide binding site, and the covalent binding of fluorescein isothiocyanate is also greatly reduced, but phosphorylation by P_i is unaffected. The kinetics of inactivation by H_2DIDS were first order with respect to time and H_2DIDS concentration. The enzyme is completely inactivated by the covalent binding of one H_2DIDS molecule at pH 9 per enzyme phosphorylation site, or two H_2DIDS molecules at pH 7.2. H_2DIDS binds exclusively to the α-subunit of the Na,K-ATPase, locking the enzyme in an E_2-like conformation. The profile of radioactivity, following trypsinolysis and SDS-PAGE, showed H_2DIDS attachment to a 52-kDa fragment which also contains the ATP binding site. These results suggest that H_2DIDS treatment modifies a specific conformationally sensitive amino acid residue on the α-subunit of the Na,K-ATPase, resulting in the loss of nucleotide binding and enzymatic activity.

The Na,K-ATPase (EC 3.6.1.3) is the plasma membrane enzyme that couples the transmembrane transport of Na^+ and K^+ ions to the hydrolysis of ATP (Glynn & Karlish, 1975). ATP hydrolysis is catalyzed through a reaction cycle involving conformational changes of the protein with Na^+-dependent phosphorylation from ATP and subsequent K^+-dependent breakdown of the phosphoenzyme. In recent years significant progress has been made in linking the biochemical transformations catalyzed by the enzyme to the transmembrane ion movements (Kaplan, 1983, 1985). However, most of the

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