Equilibrium Binding of Derivatives of the Carcinogen, Benzo(a)pyrene, to DNA

THERMODYNAMIC ANALYSIS*

(Received for publication, April 4, 1986)

Michael C. MacLeod, Bettye Smith, and John McClay

From the Science Park-Research Division, University of Texas System Cancer Center, Smithville, Texas 78957

The physical binding of polycyclic aromatic hydrocarbon derivatives which are ultimate carcinogens to DNA may play a role in the formation of covalent DNA adducts by these compounds or in the detoxification of the compounds via DNA-catalyzed hydrolysis. Previous studies of DNA-binding interactions of derivatives of benzo(a)pyrene (BP) have been confined to low r values (r = ligands bound/base pair). We have now applied the Scatchard formalism (as modified to include neighbor exclusion) to the spectrophotometric determination of the binding of two derivatives of BP, trans-9,10-dihydroxydihydro-BP and 7r, 8t-dihydroxy-7t,8t,10t-oxy-7,8,9,10-tetrahydro-BP, to double-stranded DNA at reasonably high r values. Exclusion parameters, binding constants, and thermodynamic parameters are all within the ranges found for other intercalants. Although these ligands are uncharged, the binding exhibits significant ionic strength dependence which can be rationalized (partially) by polyelectrolyte theory. Using the measured ionic strength dependence, a thermodynamic association constant, independent of ionic interactions, can be calculated which is very close to the calculated thermodynamic association constants for ethidium and proflavine.

For over 20 years it has been known that the polynuclear aromatic hydrocarbon carcinogen benzo(a)pyrene (BP), binds noncovalently to DNA. More recently it has been shown that a number of metabolites of BP also bind noncovalently to DNA. Previous studies have been confined to studies at relatively high ratios of DNA to intercalant. In the present work, we have exploited the physical binding of BPDE-I to DNA in vitro shows a high degree of stereoselectivity, with the (+)-enantiomer forming 4–5 times the level of covalent adducts as the (-)-enantiomer. The stereoselectivity of covalent binding has suggested the possibility that noncovalent intercalation complexes are precursors to the final covalent adducts. In addition, it has become clear that DNA catalyzes the detoxification of BPDE-I in vitro and that the exocyclic amino moeity of deoxyguanosine is involved in this hydrolysis reaction.

Over a wide range of DNA concentrations and solvent conditions catalyzed hydrolysis is the predominant reaction in aqueous solution and, therefore, is the major factor limiting covalent binding. Intercalation complexes may also be involved in this process. Thus, a thorough description of the equilibrium binding of BP metabolites to DNA is necessary for a better understanding of the factors which control covalent adduct formation.

Many heterocyclic dye molecules and antibiotics are known to intercalate in DNA, and the equilibrium parameters have been well studied in some cases. Many of the well-studied intercalants (e.g., ethidium bromide, proflavine, actinomycin) contain at least one positively charged moiety which contributes strongly to the binding equilibrium. Thus, analysis of potential ionic strength dependence mediated by changes in DNA conformation and flexibility is difficult. Intercalation of actinomycin, which is uncharged, has been studied but is complicated by an apparent cooperativity at low binding ratios. In addition, interactions between the cyclic peptide rings of actinomycin and DNA contribute appreciably to the binding. Thus, studies of the binding of BP metabolites to DNA could contribute significantly to our understanding of intercalation. The molecules are uncharged, eliminating charge-charge interactions, and the longest dimension of the intercalant is similar in size to the distance across the helix in an intercalation site, eliminating secondary interactions such as are found with actinomycin.

Previous studies of intercalation of BP metabolites have been confined to studies at relatively high ratios of DNA to hydrocarbon, due primarily to the relatively poor solubilities of the compounds in aqueous media. Solubility can be improved by increasing concentrations of organic solvent, but this generally results in a decrease in the apparent association constant and may introduce alterations in DNA conformation. Thus, very low r values (r = molecules of hydrocarbon bound per base pair) have routinely been used, and no information is available on heterogeneity of or interaction between intercalation sites. In the present work, we have exploited the better than average solubility and relatively high affinity of several bay region derivatives of BP to study DNA-binding parameters at reasonably high r values.

* This work was supported by Grant CA 35881 from the National Cancer Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: BP, benzo(a)pyrene; BPDE-I, 7r, 8t-dihydroxy-9t,10t-oxy-7,8,9,10-tetrahydro-BP; BP-7,8-diol, trans-7,8-dihydroxy-7,8-dihydro-BP; BP-9,10-diol, trans-9,10-dihydroxy-9,10-dihydro-BP; BP-tetrols, 7,8,9,10-tetrahydroxytetrahydro-BP.

2 M. C. MacLeod, unpublished observations.
MATERIALS AND METHODS

Chemicals

trans-7,8-Dihydroxy-7,8-dihydro-BP (BP-7,8-diol), trans-9,10-dihydroxy-9,10-dihydro-BP (BP-9,10-diol), and 7,8-dihydroxy-9,10-xyloxy-7,8,9,10-tetrahydro-BP (BPDE-I) were obtained from the Chemical Carcinogen Reference Compound Repository, Division of Cancer Cause and Prevention, National Cancer Institute and were found to be >98% pure by high pressure liquid chromatography analysis. Concentrated stocks were prepared in absolute ethanol and stored at -20 °C. 7,8,9-Tetrahydroxynitrohydro-BP (BP-tetrols) were prepared by diluting BPDE-I 20-fold with 20 mM Tris, pH 7.4, and allowing hydrolysis to proceed overnight at room temperature. Concentrations were determined by absorbance spectroscopy using the molar extinction coefficients given in Table I, supplied by the Repository. High molecular weight salmon DNA was extensively purified as previously described (10). DNA concentrations are expressed in mol of base pairs/liter, determined spectrophotometrically using c_{600} = 15,200. BPDE-I and BP-7,8-diol are potent carcinogens and must be handled carefully. Wherever possible, disposable labware was used, and all vessels contacting the carcinogens were decontaminated by treatment with dilute H_{2}SO_{4}. All waste was disposed of with the laboratory's radioactive waste.

Spectrophotometric Binding Isotherms

All experiments with BP-9,10-diol were carried out in 10 mM Tris, pH 7.4, as supporting buffer with additions as noted. To minimize the effects of BPDE-I hydrolysis, which is acid catalyzed (22), measurements of BPDE-I binding were carried out in 10 mM Tris, pH 9.0. Binding measurements were made by mixing aqueous DNA stocks with ethanolic BP-9,10-diol or BPDE-I stocks at various ratios and recording the absorbance spectrum on a Hewlett-Packard 8456 spectrophotometer. All solutions were adjusted to contain 5.0% (v/v) ethanol, and all spectra were adjusted to zero at 400 nm, outside of the hydrocarbon absorption bands. To correct for the appearance of hydrolysis products of BPDE-I, the spectrophotometer was programmed to record spectra at 2-s intervals beginning 6 s after mixing, and the spectra were extrapolated to zero time. Previous work has suggested that the time constant for intercalation of BPDE-I is less than 5 ms (6, 11). Thus, at each time point used the system is expected to be close to equilibrium, and any errors associated with this assumption should be well within experimental error. As shown in Fig. 1, addition of DNA to a solution of BP-9,10-diol produces a red-shift in the absorbance spectrum, exhibiting an isosbestic point at 349 nm. Total hydrocarbon concentration can be obtained from the absorbance at the isosbestic point. To determine the concentration of intercalated hydrocarbon, an extinction coefficient for the complex at the shifted wavelength (354 nm) was needed. As shown in the inset to Fig. 1, absorbance at 354 nm was determined as a function of DNA concentration at constant BP-9,10-diol concentration and extrapolated to infinite DNA concentration (i.e. complete binding) in a double reciprocal plot. As was first shown by Benesi and Hildebrand (23), the y-intercept of such a plot gives the reciprocal of the absorbance of the bound complex; since the total concentration (and hence the bound concentration) is known, the extinction coefficient can be calculated. The relevant molar extinction coefficients at the red-shifted wavelength of the intercalation complexes determined in this way are given in Table I.

![FIG. 1. Isosbestic point for BP-9,10-diol-DNA complexes. Absorbance spectra are plotted for aqueous solutions of 4.0 μM BP-9,10-diol containing 0, 60.5, 121, 242, and 485 μM DNA; an isosbestic point is apparent at about 349 nm and a red-shifted local maximum at about 354 nm. In the inset, the reciprocal [DNA] is plotted against the reciprocal absorbance at 354 nm after subtracting the A_{max} in the absence of DNA (n = 5; replicates differed by less than 5%).](image1)

TABLE I

| Compound        | \(λ_{\text{max}}\) | \(ε_{\text{max}}\) | \(ε_{\text{m}}\) | \(ε_{\text{b}}\) | \(δ_{\text{m}}\) | \(δ_{\text{b}}\) |
|-----------------|------------------|------------------|----------------|----------------|----------------|----------------|
| BP-7,8-diol     | 345              | 44.9             | 20.4           | 7.3            | 32.9           | 17.5           |
| BP-9,10-diol    | 345              | 44.9             | 20.4           | 7.3            | 32.9           | 17.5           |
| BPDE-I          | 345              | 44.9             | 20.4           | 7.3            | 32.9           | 17.5           |

\(λ_{\text{max}}\) is the position of the longest wavelength absorption peak of the compound in ethanol; \(ε_{\text{max}}\) is the molar extinction coefficient at \(λ_{\text{max}}\); \(ε_{\text{m}}\) is the molar extinction coefficient at the wavelength which is isosbestic for DNA complexes with the indicated compound. \(ε_{\text{b}}\) is the molar extinction coefficient of bound molecules at the red-shifted wavelength, determined from Benesi-Hildebrand plots. \(δ_{\text{m}}\) is the molar extinction coefficient of free molecules at the red-shifted wavelength, determined by direct measurement.

Equilibrium Dialysis

Disposable dialysis chambers were constructed from 14-ml polypropylene centrifuge tubes and caps (available from Sarstedt, Princeton, NJ, catalog numbers 57.527 and 65.793) similar to the method previously described for smaller chambers (24). The caps, which hold a maximum volume of approximately 2 ml, served as the actual chambers, while a 1.5-cm band cut from the top of the centrifuge tube was used to secure the dialysis membrane and to connect the two half-cells. Plastic parts were rinsed well with methanol followed by ether and air dried before use to remove fluorescent impurities. Although previous workers have described adsorption of hydrocarbons on plastic surfaces (25), we found that when BP-tetrols were diluted (concentration range, 1-9 μM) into either glass tubes or the caps used for dialysis, equal concentrations were found spectrophotometrically in either type of vessel whether measured immediately after mixing, or after 15 min, 4, 24, or 48 h at room temperature (data not shown), indicating that adsorption to the cells was not a problem. Unless otherwise indicated, dialysis membranes were Spectrapor (molecular weight cutoff, 12,000-14,000); they were boiled for 5-10 min in 10 mM EDTA and washed extensively with deionized water prior to use. Measurements were made in 20 mM Tris, pH 7.4. DNA stocks and buffers were passed through a reverse-phase cartridge (SepPak C-18, Waters Associates) prior to use to remove trace impurities which could interfere with fluorescence measurements. In a typical experiment, side I was loaded with 1.8 ml of a solution containing the desired concentration of DNA plus hydrocarbon (usually at a concentration of 3 μM, final ethanol concentration of 0.5%), and a square of dialysis membrane was secured over the chamber. Side II was filled with 1.8 ml of 20 mM Tris, pH 7.4, containing the same concentration of hydrocarbon and ethanol. Side I was then inverted and secured over side II. Completed chambers were placed on their sides in a standard tissue culture roller bottle and rotated at about 0.5 rpm for 40-48 h. Identical results were obtained when dialysis was continued for 60-64 h, indicating that equilibrium was attained in 40 h. In most experiments the roller apparatus was exposed to ambient conditions (22°C) and was located adjacent to the analytical equipment to minimize temperature differentials between attainment of equilibrium and the analysis conditions. In one experiment, dialysis was conducted in a cold room (4°C), and fluorescence measurements were made in a thermostatted cell held at 4°C.

Data Analysis

McGhee-von Hippel—The well-known nonlinearity of Scatchard plots (26) obtained for ligand binding to DNA (14-21) has been
explained by an excluded site model, in which a ligand bound at one site prevents subsequent binding at neighboring sites. A general solution to the problem was derived by McGhee and von Hippel (27), and we have analyzed our data according to their equation,

\[
r = K'_i(1 - nr) \left( \frac{1 - nr}{1 - (n - 1)r} \right)^{-1}
\]

where \(K_i\) is the intrinsic association constant, \(r\) is the binding density (moles of hydrocarbon bound/mol of base pairs), \(c_r\) is the free hydrocarbon concentration, and \(n\) is the number of base pairs which become inaccessible for further binding due to complex formation. We determined \(c_r\), the concentration of bound hydrocarbon, from the measured absorbance at the red-shifted wavelength; \(c_b\) the total hydrocarbon concentration, from the absorbance at the isobestic point; and \(c_r = c_i - c_b\); \(r\) is simply \(c_r\) divided by the DNA concentration. In a typical experiment, 25–30 experimental points were fit to Equation 1 by nonlinear least-squares regression using the approach of Duggleby (28) adapted to an IBM 9000 computer.

Benesi-Hildebrand—If excluded site effects are neglected (i.e., \(n = 1\)) then Equation 1 reduces to the classical Scatchard equation, \(r/c_r = K_i(1 - r)\). At sufficiently low \(r\) values, where excluded site effects are negligible, the last term in the Scatchard equation approaches 1 and the binding can be approximated by

\[
r = K_i\frac{c_i}{c_r} = K_i \frac{K_D}{1 + K_D}
\]

and it is easily shown that \(c/c_r\) is proportional to \(A_{obs}/A_{nw}\) where "iso" indicates the isobestic wavelength for the complex and "shift" indicates the red-shifted wavelength characteristic of the complex. Data collected at \(r\) values less than 0.025 were fit to Equation 3 by nonlinear regression.

Equilibrium Dialysis—For equilibrium dialysis experiments carried out at low \(r\) values, it is useful to transform Equation 2 into the linear form

\[
\frac{c_i}{c_r} = 1 + K_D.
\]

At equilibrium, \(c_r\) must be the same on both sides of the dialysis chamber and can be determined spectrophotometrically on side II (i.e., the chamber lacking DNA). \(c_r\) on side I can be determined by two methods: 1) directly from the absorbance at the isobestic point on side I; 2) by summation of \(c_r\) determined on side II and \(c_i\) determined from the absorbance on side I at the red-shifted wavelength, characteristic of the intercalation complex. It can easily be shown that if a class of secondary binding sites exists, \(K_i\) determined by method 1 will be the sum of the intrinsic association constants for the two classes of sites. If it is assumed that ligands bound at the secondary sites do not exhibit a red-shift, then the existence of such sites will produce a higher value for \(K_i\) by method 1 than is obtained by method 2. Clearly, only secondary sites with a high intrinsic association constant will be detected. We estimate that a class of sites with association constant 10-fold lower than the association constant for intercalative binding should be easily demonstrable by this method.

It has been found that the fluorescence quantum yield of BP-tetrols intercalated in DNA is virtually zero (3). However, a second class of binding sites has been suggested based on equilibrium dialysis (3) at which bound BP-tetrols are thought to have fluorescence parameters equivalent to free BP-tetrols. If the association constant for such a class of sites is called \(K'\), then at low \(r\) values (\(c_r' + c_b)/c_r' = 1 + K'D\) where \(c_r'\) is the concentration of ligand bound to the secondary sites. Assuming equal fluorescence yields, the ratio \(c_r' + c_b/c_r'\) is given by the ratio of steady state fluorescence in the dialysis chamber containing DNA (side I) to the fluorescence on side II. Thus, the slope of the plot of this fluorescence ratio (side I/side II) versus DNA concentration should give \(K'\) for the putative external binding site.

**RESULTS**

Analysis of Hydrocarbon: DNA Binding Parameters—For our initial studies we chose BP-9,10-diol as a model ligand since it is chemically unreactive and previous studies (at low \(r\) values) had demonstrated an apparent association constant similar to that of BPDE-I. Preliminary studies demonstrated that under our solvent conditions the absorbance of aqueous BP-9,10-diol solutions followed Beer’s law up to at least 50 \(\mu M\). Over this concentration range no changes in absorbance spectra or in fluorescence emission spectra were noted, suggesting that self-association is negligible. Binding to DNA was measured at two DNA concentrations (76 and 144 \(\mu M\)) and at a series of BP-9,10-diol concentrations from 5 to 80 \(\mu M\). At 24 °C, this gave \(r\) values ranging from about 0.01 to 0.16. In Fig. 2 we plot \(r\) versus \(r/c_r\) for one such experiment; the solid line is the computer-generated best fit to Equation 1. Data from 4 experiments gave 3.3 ± 0.1 for the exclusion parameter and 8.9 ± 0.2 \(\times 10^3\) liters/mol for the intrinsic binding constant. This binding constant is on the same order of magnitude as previous estimates of binding constants for several related derivatives of BP obtained at low \(r\) values (3–7, 10–12). A direct comparison with a binding isotherm obtained at low \(r\) values for BP-9,10-diol by the Benesi-Hildebrand approach is given in Fig. 3; the estimated associated constant is 8.2 ± 0.4 \(\times 10^3\) liters/mol, in reasonably good agreement with the value obtained from the McGhee-von Hippel analysis.

To apply the same kind of analysis to the ultimate carcinogen, BPDE-I, analyses were carried out at pH 9.0 to slow down the rate of hydrolysis, and the residual time dependence due to hydrolysis was eliminated by extrapolating to zero time. The actual changes in the absorbance in the first 20 s after mixing the BPDE-I and DNA were relatively small such that linear extrapolation introduced little or no error. The binding data obtained at 24 °C for BPDE-I were fit satisfactorily by Equation 1 giving the following binding parameters.

\[
K_i = 22.2 ± 4.6 \times 10^3\ \text{liters/mol, } n = 2.4 ± 0.5
\]

Again the apparent \(K_i\) was similar to values obtained at low \(r\) values, and the exclusion parameter was between 2 and 3.

Equilibrium Dialysis Studies of Secondary Binding Sites—In the above analysis we have assumed that there is no

![Fig. 2. Scatchard plot for BP-9,10-diol-DNA binding. As described under “Experimental Procedures," \(r\) and \(c_r\) were determined spectrophotometrically for mixtures containing 76 or 144 \(\mu M\) DNA and from 5–80 \(\mu M\) BP-9,10-diol at 24 °C. Individual points from one representative experiment are plotted; the solid line represents the least squares best fit of the data to Equation 1.](image-url)
significant binding to secondary nonintercalative sites in the concentration range studied. Since there is one report (3) of a nonintercalative DNA binding site for BP-tetrols, we have attempted to verify this assumption by equilibrium dialysis techniques for several nonreactive BP derivatives. Equilibrium dialysis data were obtained in three ways as detailed under "Materials and Methods": 1) absorbance at the red-shifted wavelength characteristic of intercalation complexes; 2) absorbance at an isosbestic wavelength; 3) fluorescence. In each case, an apparent association constant can be obtained (at low r values) as the slope of a linear plot of the chosen parameter versus DNA concentration. In the case of measurements at the isosbestic wavelength, this apparent association constant, \( K^* \), should be the sum of the \( K_i \) values for all classes of binding sites. In the case of fluorescence measurements, intercalated molecules are not detected (due to a quantum yield close to zero (3)), and the apparent association constant, \( K^* \), should be specific for the putative secondary binding sites.

The results of typical equilibrium dialysis determinations of the apparent association constant are shown in Fig. 4. For BP-9,10-diol, absorbance measurements specific for the intercalation complex \( A_{260} \) and at the isosbestic point \( A_{290} \) yield linear plots (Fig. 4). The values for \( K_i \) and \( K^* \) (Table II) determined by both methods are not significantly different from each other. The lower values obtained by this method compared to the McGhee-von Hippel or Benesi-Hildebrand approaches (see above) are due to a higher ionic strength in the supporting buffer for the equilibrium dialysis studies. Ionic strength is shown below to reduce the apparent \( K_i \). Similar determinations for BP-tetrols (Fig. 4) again yield similar linear plots, and the values for \( K_i \) and \( K^* \) (Table II) do not differ significantly. Within the limits of the technique, these studies provide no suggestion of a class of secondary binding sites.

Direct tests for binding at a putative external binding site (3) by fluorescence measurements were carried out for BP-9,10-diol and BP-tetrols at 21 °C. As shown in Fig. 4, the fluorescence ratios were close to 1.0 at all DNA concentra-

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**Equilibrium Binding of Benzo(a)pyrene Diol Epoxide to DNA**

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**Fig. 3.** Benesi-Hildebrand plot for BP-9,10-diol-DNA binding. The fraction of added hydrocarbon bound \( f_a = c_o/c_i \) was determined spectrophotometrically as described under "Experimental Procedures" using the equation,

\[
f_a = \frac{A_{260} - A_{290}}{A_{260} - A_{290}}
\]

in mixtures containing 4.0 \( \mu \text{M} \) BP-9,10-diol and the indicated concentration of DNA. Means ± S.D. (n = 3) from a representative experiment are plotted; the solid line represents the least squares best fit of the data to Equation 3.

**Fig. 4.** Equilibrium dialysis analyses of association constants. As described under "Experimental Procedures," the ratio \( c_o/c_i \) was determined spectrophotometrically in equilibrium dialysis experiments with BP-9,10-diol (O, C) or BP-tetrols (A, D) and the indicated concentration of DNA. Data used for the determinations were collected at the isosbestic wavelength (O, D) or at the red-shifted wavelength (O, A). In the same experiment, external binding was determined from fluorescence measurements for BP-9,10-diol (O) or BP-tetrols (C). Individual points from a representative experiment are plotted along with the best fit straight lines determined from linear regression.

**Table II**

| Compound  | \( 10^{-9} \times K_i \) | \( 10^{-9} \times K^* \) | \( 10^{-9} \times K' \) |
|-----------|------------------------|------------------------|------------------------|
|           | M\(^{-1}\)              | M\(^{-1}\)              | M\(^{-1}\)              |
| BP-7,8-diol| 14.64 ± 0.57           | 18.53 ± 0.27           | 0.33 ± 0.29             |
| BP-9,10-diol| 6.86 ± 0.17           | 6.55 ± 0.22           | 0.04 ± 0.07             |
| BP-tetrols| 2.73 ± 0.45           | 2.56 ± 0.31           | 0 ± 0.06               |

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Table III

| Temperature (°C) | Binding Constant (M⁻¹) |
|------------------|-------------------------|
| 21              | 1.06 ± 0.03             |
| 24              | 1.04 ± 0.06             |
| 27              | 0.95 ± 0.02             |
| 30              | 1.21 ± 0.05             |
| 33              | 1.31 ± 0.11             |

*Equilibrium dialysis with 3 μM BP-tetrols was performed as described in a cold room at 4 °C. The solutions in each half-cell at equilibrium were separated and kept on ice. Fluorescence was measured in a thermostatted cell held at 4 °C. The solutions and the fluorimeter cell were then allowed to come to room temperature, and the fluorescence was again measured. Means and standard deviations are pooled data from 2 separate experiments, with a total of 6 determinations for each experimental point.

Discussion

The present work demonstrates the usefulness of the classical Scatchard approach in the analysis of the binding of some derivatives of BP to DNA. The two hydrocarbons which we have analyzed successfully, BPDE-I and BP-9,10-diol, are relatively polar which allowed us to achieve higher concentrations in aqueous solution than could be obtained with the parent hydrocarbon or other less polar derivatives. Indeed, attempts to study the binding of trans-7,8-dihydroxy-7,8-dihydro-BP, a related but less polar derivative, were unsuccessful because concentrations higher than about 10 μM could not be achieved (data not shown). On the other hand, derivatives which are considerably more polar than BP-9,10-diol, for example BP-tetrols, exhibit lower association constants and are also not amenable to this approach. The data obtained for BPDE-I and BP-9,10-diol are consistent with a model in which the major DNA-binding interactions are at intercalation sites, and we have chosen to analyze the binding using the McGhee-von Hippel formalism. Although several alternative explanations for the curvature of the Scatchard plot are possible (27, 32), the data are explained satisfactorily by the combinatorial approach of McGhee-von Hippel, and there is no need to invoke secondary binding modes as a source of the observed curvature. Indeed, the equilibrium dialysis studies failed to provide evidence for any secondary binding sites, at least for the stable BP derivatives which could be studied by this technique. The reactivity of BPDE-I and its low fluorescence quantum yield (5,11) prevents us from studying possible secondary binding sites by the equilibrium dialysis technique. The exclusion parameters determined above, 2.4 for BPDE-I and 3.3 for BP-9,10-diol, are somewhat higher than that determined for the classical intercalant ethidium (n = 2, Refs. 17 and 33), but similar to values for daunomycin (n = 3.5, Ref. 19) and several other anthracycline intercalants (34). Theoretical studies of the energetics of forming an intercalation site in a double-helical lattice (35, 36) predict 3 families of intercalation sites, all with deviations from normal B-DNA geometry extending for one base pair on either side of the intercalant. Interestingly, the unwinding angles for BP derivatives and the anthracyclines (6, 36) are in the range 8–12°, placing them in the class I intercalation site of Miller, while ethidium, with an unwinding angle of 26° would be in class III (36). Whether this apparent correlation between the temperature and the binding of BPDE-I to DNA at pH 9.0 was also determined. The van't Hoff analysis is shown in Fig. 5 (dashed line), and the derived entropy and enthalpy parameters are given in Table IV. As might reasonably be expected, the binding of this ligand is also exothermic and exhibits an unfavorable entropy loss.

Salt Dependence of BP-9,10-diol-DNA Binding—Since BP-9,10-diol is an uncharged ligand, it appeared to be of interest to determine the ionic strength dependence of the apparent binding constant, which should be due primarily to polyelectrolyte effects (29–32). Binding data was obtained at added Na⁺ concentrations of 0.02, 0.113, and 0.198 mM. Due to the decrease in the apparent association constant with increasing ionic strength we could not achieve r values sufficiently high to define the anticooperative part of the McGhee-von Hippel plot. To estimate Kᵣ we assumed that the exclusion parameter, n, is independent of ionic strength as has been found for other intercalants (19). The ionic strength dependence of Kᵣ determined in this way in two experiments with n constrained to be 3.3 is shown in the log-log plot of Fig. 6. A straight line is obtained with a slope of −0.38, in reasonable agreement with the predictions of polyelectrolyte theory (Fig. 6, dotted line; see "Discussion").
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TABLE IV
Thermodynamic parameters

| Compound       | n     | 10^4 x K_{on} | [M^+] | H^o  | S^o  | d(log K)/d(log[M^+]) | ln K^c | Reference |
|----------------|-------|---------------|-------|------|------|--------------------|--------|-----------|
| BP-9,10-diol   | 3.3   | 8.5           | 0.006 | -10.2| -16.1| -0.38              | 7.4    | This work |
| BPDE-I         | 2.4   | 20            | 0.008 | -8.5 | -8.9 | -0.36              | (8.2)  | This work |
| Daunomycin     | 3.5   | 700           | 0.2   | -12.8| -16.2| -0.84              | 12.3   | 19        |
| Actinomycin D  | 270   | 0.2           | -8.7  | -6.7 |      | -0.36              | 13.1   | 32        |
| Proflavine     | 2     | 26            | 0.2   | -9.4 | -10.4| -0.38              | (7.4)  | 16        |
| Ethidium       | 59    | 0.2           | -8.4  | -9.2 |      | -0.38              | (9.0)  | 20        |
| Ethidium       | 2     | 14            | 1.0   | -8.4 | -9.2 | -0.38              | (9.8)  | 17        |

\*The intrinsic association constant, K, was interpolated at 25 °C from the plots in Fig. 5. The thermodynamic definitions ΔG^o = -RT ln K = ΔH^o - TΔS^o gave ΔH^o from the slope of the ln K versus 1/T plot, and ΔS^o was obtained by substitution. Literature values were obtained by similar methods, e.g., entropy unit.

\*d(log K)/d(log[M^+]) was given by the slope of the plot shown in Fig. 6 or a similar plot.

\*Calculation of ln K^c is described under “Discussion.” Figures in parentheses were calculated using the following assumed values of Δr for BPDE-I; daunomycin, -0.38; and ethidium, -0.84 (as determined for daunomycin); proflavine, -1.68 (twice the value for daunomycin due to the double positive charge in proflavine).

\*These values were obtained with a synthetic oligonucleotide (dCA_G·dCT_G). All other values are for double-stranded random-sequence DNA.

exclusion parameter and the unwinding angle has physical significance remains to be determined.

Thermodynamic parameters obtained for binding of the BP derivatives to DNA are compared in Table IV to representative published parameters for other intercalating drugs. Data for the cationic drugs was obtained in most cases from experiments done in the presence of 0.2 M monovalent salt to partially suppress contributions from charge-charge interactions. The values of the standard enthalpy and entropy changes for hydrocarbon binding are all within the ranges established for the cationic drugs, although it must be noted that the measurements were done at very different ionic strengths. Both enthalpy and entropy have been reported to be strong functions of ionic strength over the relevant range for the cationic drug daunomycin (37). Whether a strong ionic strength dependence exists for the uncharged hydrocarbons is not known. To circumvent ionic strength dependence, we have corrected our data and literature data to a standard state in which all reactants have unit concentration as suggested by Record et al. (30) and more recently by Chaires et al. (19). The equation used, adapted from the above references, is

\[ \ln K^c = \ln K^c_0 + \xi^{-1} \ln(\gamma_+ \cdot b) + \Delta r \cdot \ln[M^+] \]

where Δr is the apparent ion release upon binding one ligand and is defined experimentally by the slope of the log K versus log[M^+] plot (Fig. 6). γ_+ is the mean activity coefficient of the Na^+ ion; ξ and δ are constants for B-DNA (ξ = 4.2, δ = 0.56); K^c_0 is the thermodynamic association constant. The value for ln K^c_0 given in Table IV for BP-9,10-diol is the average of determinations at several ionic strengths, all of which agreed very well; ln K^c_0 was also calculated for BPDE-I by assuming the same value of Δr as was found for BP-9,10-diol. The thermodynamic constants for the BP derivatives are much lower than that for daunomycin, reflecting a free energy difference of about 3 kcal/mol. When we calculated ln K^c_0 for several other intercalants from published data, we found values similar to the BP derivatives for ethidium and proflavine (Table IV) whereas actinomycin D was similar to daunomycin. Although it is difficult to draw conclusions based on such limited data, it is possible that the higher values for daunomycin and actinomycin reflect the sum of two components of the binding interaction: stacking interactions between the planar intercalating moiety and interactions in the grooves of the helix with either the cyclic peptides of the actinomycin molecule or with the amino sugar of the daunomycin molecule. The BP derivatives and the smaller proflavine and ethidium ions would lack the latter interactions. In any case, after appropriate correction is made for ionic effects it is clear that the strength of the intercalation of BPDE-I and BP-9,10-diol into DNA is comparable to the analogous interaction of several other well-studied intercalants.

The ionic strength dependence found for BP-9,10-diol, Δr = -0.38, is very close to the reported dependence for another uncharged ligand, actinomycin D (Δr = -0.36; Ref. 32). This effect arises in part from the lengthening of the DNA which occurs upon formation of an intercalation site, lowering the average charge density of the helix and in effect releasing condensed counterions (31, 32). Our experimental value is somewhat higher than the predictions of polyelectrolyte theory as recently formulated by Friedman and Manning (Δr = -0.24; Ref. 32). Although again the data base is limited and the difference may simply reflect experimental error, further refinement of the theory may be necessary. Chaires (37) has noted an even larger discrepancy between experiment and theory with regard to the ionic strength dependence of the standard enthalpy of daunomycin:DNA binding.

Acknowledgments—We thank Tim Lohman and Andy Butler for a
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