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Binding of Platinum(II) Intercalation Reagents to Deoxyribonucleic Acid. Dependence on Base-Pair Composition, Nature of the Intercalator, and Ionic Strength†

Mary Howe-Grant‡ and Stephen J. Lippard*

ABSTRACT: The DNA binding of three platinum(II) intercalation reagents has been studied and found to depend upon base composition, the nature of the intercalator, and the ionic strength of the solvent medium. In 0.2 M NaCl, binding data for calf thymus DNA show the association constants to be $\sim 10^4$ M^{-1}. The binding constants decrease in the order [(*o*-phen)Pt(en)]$^{2+}$ ≥ [(terpy)Pt(HET)]$^+$ ≫ [(bipy)Pt(en)]$^{2+}$. The number of available intercalation sites for the doubly charged intercalators is only 70% of the number expected from the nearest-neighbor exclusion model. Binding of [(*o*-phen)Pt(en)]$^{2+}$ and [(terpy)Pt(HET)]$^+$ to various DNAs depends linearly on G·C content. Both reagents exhibit essentially the same degree of G·C specificity. Intercalative binding is a function of ionic strength. Increasing the salt concentration minimizes the importance of metallointercalator charge, and extrapolation to 1 M salt reveals the intercalative abilities, as reflected in binding constants, to be equivalent for [(terpy)Pt(HET)]$^+$ and [(*o*-phen)Pt(en)]$^{2+}$ and about 1 order of magnitude less than that of ethidium.

Platinum(II) complexes containing aromatic ligands coplanar with the metal coordination sphere bind intercalatively to double-stranded DNAs, while noncoplanar-aromatic and planar-nonaromatic complexes do not (Jennette et al., 1974; Howe-Grant et al., 1976; Lippard et al., 1976; Nordén, 1978; Lippard, 1978). Intercalation is a mode of nucleic acid binding for a variety of drugs and antibiotics, many of which are frameshift mutagens or inhibitors of DNA synthesis (Waring, 1968). This binding mode has been postulated for protein–nucleic acid recognition in vivo (Gabbay et al., 1973; Coleman & Armitage, 1978; Maurizot et al., 1978). Because of their high electron density, platinum intercalation reagents have been used to investigate nucleic acid structure and the intercalative binding mode itself. X-ray fiber diffraction studies of DNA containing bound metallointercalators (Bond et al., 1975; Lippard et al., 1976) and a crystal structure determination of the platinum intercalation reagent [(terpy)Pt(HET)]$^+$ [1] (Figure 1) contained within a segment of double-helical DNA (Wang et al., 1978) have helped to clarify the frequency and distribution of bound intercalators at saturation, the total number of sites available to an intercalation reagent along the DNA helix, and the sugar puckering and unwinding angle of DNA in the intercalation complex. The [(terpy)Pt(HET)]$^+$ cation also inhibits genetic recombination in pneumococci (Seto & Tomaz, 1977).

The present study examines several factors involved in the binding of platinum intercalators to DNA. The relative importance of intercalator structure, DNA base composition, and electrostatic charge interactions is elucidated by varying the nature of the metal complex (see Figure 1), the DNA, and the ionic strength of the solvent medium.

Materials and Methods

Metal Complexes. The compounds [(terpy)Pt(HET)]NO_3 (Howe-Grant & Lippard, 1980), [(*o*-phen)Pt(en)]$(NO_3)_2$ (Howe-Grant, 1978), [(bipy)Pt(en)]$(NO_3)_2$ (Erickson, 1969), and [$(py)_2$Pt(en)]$(ClO_4)_2$ (Appleton & Hall, 1971) were prepared by published procedures. Tritiated complexes were synthesized from [^{3}H]-2,2′,2″-terpyridine or [^{3}H]-1,10-phenanthroline supplied by New England Nuclear Co. The radiolabeled ligands were diluted with unlabeled material in ethanol or equimolar ethanol–benzene solutions and isolated after precipitation with water. Purity of the labeled complexes was determined spectrophotometrically.

Concentrations of stock solutions containing metal complexes were determined spectrophotometrically in water or the appropriate buffer on the basis of reported molar extinction coefficients (Howe-Grant, 1978).

Buffers and Other Chemicals. All experiments were carried out at pH 7.5 in buffer 1 (10 mM sodium chloride and 10 mM Tris-HCl), buffer 2 (100 mM sodium chloride and 50 mM Tris-HCl), buffer 3 (200 mM sodium chloride and 50 mM Tris-HCl), buffer 3′ (200 mM sodium chloride, 50 mM Tris-HCl, and 1 mM EDTA), buffer 4 (190 mM sodium chloride and 10 mM Tris-HCl), buffer 5 (5 mM sodium

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[1] Abbreviations used: EtdBr, ethidium bromide; bipy, 2,2′-bipyridine; en, ethylenediamine; *o*-phen, 1,10-phenanthroline; terpy, 2,2′,2″-terpyridine; py, pyridine; HET, 2-hydroxyethanethiolate; EDTA, disodium salt of ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane; *r*, ratio of bound metal to nucleotide concentrations; P_0, concentration of nucleotide; C_B, C_F, C_T, concentrations of bound, free, and total intercalator, respectively; C_{obsd}, observed concentration of free intercalator, equal to C_F provided that no C_D, dimerized free intercalator, is present in solution.

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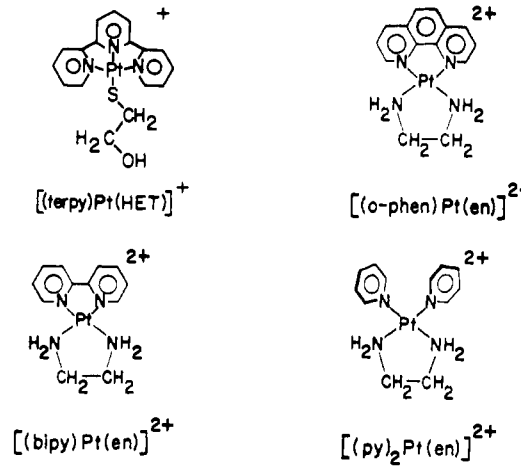

FIGURE 1: Structures and nomenclature of platinum complexes employed in this study.

chloride and 50 mM Tris-HCl), and buffer 6 (50 mM sodium chloride and 50 mM Tris-HCl). All chemicals were reagent grade. Solutions were prepared in deionized, distilled water in sterilized glassware or sterile disposable containers. Ethidium bromide was purchased from Sigma Chemical Co.

Nucleic Acids. Escherichia coli DNA was either isolated from *E. coli* K strain cells (Sigma Chemical Co.) by the method of Marmur (1961) or purchased as Type VIII DNA from Sigma Chemical Co. *Micrococcus lysodeikticus* (*Micrococcus luteus*) DNA and *Clostridium perfringens* DNA (Type XII) were purchased from Research Products, Miles Laboratories, Inc., and Sigma Chemical Co., respectively. Calf thymus DNA (Type I) was purchased (Sigma Chemical Co.) and purified as previously described (Jennette et al., 1974).

The DNAs were phenol-extracted prior to use and shown to have less than 1% protein by the Lowry method (Lowry et al., 1951) using bovine serum albumin as the standard. The melting temperatures of the individual DNAs in buffer 1 were found to be 60.5 (*Cl. perfringens*), 71.5 (*E. coli*), 78.8 (*M. luteus*), and 69.3 °C (calf thymus).

DNA concentrations per nucleotide were determined spectrophotometrically by assuming ϵ for *M. luteus* DNA = 6900 M^{-1} cm^{-1}, for *E. coli* DNA = 6500 M^{-1} cm^{-1}, for *Cl. perfringens* = 6200 M^{-1} cm^{-1}, and for calf thymus DNA = 6600 M^{-1} cm^{-1} (Felsenfeld & Hirschman, 1965).

Pelleting Experiments. Binding studies were carried out by the pelleting method, as described elsewhere (Howe-Grant, 1978; Barton & Lippard, 1979), using either a Beckman Model L-3 50 or L-2 50 preparative ultracentrifuge equipped with a 50.1 swinging bucket rotor at 20 °C or a Beckman airfuge. A temperature of 20–23 °C was maintained in the Beckman airfuge for the last 30 min of each run, and at no time did the measured temperature exceed 30 °C. As shown by control experiments in our laboratory (Barton & Lippard, 1979) and elsewhere (Pohl et al., 1972; Hsieh & Wang, 1975; Lloyd et al., 1968), equilibrium conditions are maintained even under the hydrostatic pressure gradient generated during centrifugation; there is good agreement between results obtained by this and other methods such as equilibrium dialysis.

Liquid Scintillation Counting. Radioactivity was measured in a Searle Analytic Isocap/300 liquid scintillation counter at ambient temperature. Solutions were mixed in Bray's (Bray, 1960) scintillation fluid in Wheaton vials of Vitro "180" low-potassium glass. Samples were run in duplicate, and the resultant counts were normalized to the internal standard, corrected for background, and averaged to yield one number per sample for each counting window. Specific activity curves were prepared for each set of radioactively labeled stock solutions of dye. Each DNA stock solution was also monitored for radioactivity; however, DNA solutions never gave readings above background.

Binding Isotherms. Binding isotherms are presented as Scatchard plots (Scatchard, 1949) of r/C_F vs. r, where r is the ratio of the bound intercalator concentration, C_B, to the total nucleotide concentration, P_0, and C_F is the concentration of free intercalator. C_F was determined directly from the pellet supernatant, either spectrophotometrically, by liquid scintillation counting, or, in the case of $[(terpy)Pt(HET)]^+$, from eq 3, and C_B was then obtained by the difference $C_B = C_T - C_F$. Each C_T was calculated from the dilution factor experienced by an individual stock dye solution where stock concentrations were determined by the same method as the corresponding C_F's. P_0 was determined spectrophotometrically from a sample containing buffer and an aliquot of DNA stock solution but no intercalator.

The observed concentration of free intercalator, C_{obsd}, for $[(terpy)Pt(HET)]^+$ contained a dimer population, C_D, in addition to the monomer (C_F); the dimerization constant for $[(terpy)Pt(HET)]^+$, K_D, is 7 ($\pm$5) $\times$ 10^3 M^{-1} (Jennette et al., 1976). Since the monomer is presumably the only entity that intercalates into DNA, the data were corrected for the dimerization of free dye in solution as follows. The dimerization of any compound may be expressed as shown in eq 1 where C_D and C_F are related to the observed concentration C_{obsd} as

$$K_D = \frac{C_D}{C_F^2} \tag{1}$$

shown by eq 2. Algebraic manipulation of these equations

$$C_{obsd} = 2C_D + C_F \tag{2}$$

gives eq 3, from which C_F may be determined. The uncertainty

$$C_F = \frac{-1 + (1 + 8K_DC_{obsd})^{1/2}}{4K_D} \tag{3}$$

in the value of K_D is of little or no consequence at values of $C_{obsd} \leq 10^{-6}$ M, i.e., at high r/C_F values. At higher values of C_{obsd}, however, this large experimental error can produce an uncertainty in C_F as great as 20% ($C_{obsd} = 10^{-4}$ M). These uncertainties at no time affect the value of r and are present only at the lower r/C_F values of the binding curve.

Control Experiments. Samples of $[^3H][(terpy)Pt(HET)]^+$ and $[^3H][(o\text{-phen})Pt(en)]^{2+}$ in buffer 3′ were passed through a ~3.5-cm column of Bio-Rad AG 50W-X8 cation-exchange resin, previously equilibrated with the buffer, contained in a plastic disposable pipet. Samples were prepared in the presence and absence of DNA under the conditions of the pelleting experiments. Column fractions were monitored both spectrophotometrically and through liquid scintillation counting. In all cases the radioactively labeled material was retained on the column whereas the DNA passed through, indicating that no covalent linkages had formed between the intercalator and the DNA. This experiment was also performed by using $[(bipy)Pt(en)]^{2+}$ with spectrophotometric monitoring of the column fractions. No metal complex was observed in the eluate.

Results

Binding of Metallointercalation Reagents to Calf Thymus DNA in 0.2 M NaCl. The binding isotherms of $[(o\text{-phen})Pt(en)](NO_3)_2$ and $[(terpy)Pt(HET)]NO_3$ to calf thymus DNA in 0.2 M NaCl as determined by pelleting experiments are shown in parts a and b of Figure 2, respectively. The data

Table I: Parameters for the Binding of Intercalation Reagents to Calf Thymus DNA in 0.2 M NaCl As Determined from Pelleting Experiments[a]

reagent	method[b]	buffer	$K_{app} \times 10^{-4}$ (M^{-1})	$\sigma \times 10^{-3}$ (M^{-1})	$K(0) \times 10^{-4}$ (M^{-1})	$\sigma(0) \times 10^{-3}$ (M^{-1})
[((bipy)Pt(en)]$^{2+}$	A	3, 4	1.2 ± 0.5	1.8 ± 0.8	0.63 ± 0.12	2.1 ± 0.4
[(*o*-phen)Pt(en)]$^{2+}$	A	3, 3′	7.2 ◻ 0.8	9.2 ± 1.03	2.7 ± 0.3	9.3 ± 1.0
	B	3	5.0 ± 0.6	7.9 ± 1.0	2.0 ± 0.2	8.1 ± 2.7
[(terpy)Pt(HET)]$^{+}$	A	3, 4	2.8 ± 1.0	6.6 ± 2.4	2.7 ± 0.4	9.7 ± 1.4
	B	3	4.8 ± 0.8	9.4 ± 1.6	2.0 ± 0.2	10 ± 2.2
[$(py)_2$Pt(en)]$^{2+}$	A	4	0.00	0.00	0.00	0.00
ethidium bromide	A	3′	26 ± 5	59 ± 7	14 ± 2	73 ± 9

[a] Symbols are defined in the text. [b] Methods A and B correspond to ultracentrifuge and airfuge pelleting, respectively.

Table II: Binding Parameters for Metallointercalators with DNAs of Varying G·C Content[a]

		[(*o*-phen)Pt(en)]$^{2+}$				[(terpy)Pt(HET)]$^{+}$			
DNA	% G·C	$K_{app} \times 10^{-4}$ (M^{-1})	$\sigma \times 10^{-4}$ (M^{-1})	$K(0) \times 10^{-4}$ (M^{-1})	$\sigma(0) \times 10^{-4}$ (M^{-1})	$K_{app} \times 10^{-4}$ (M^{-1})	$\sigma \times 10^{-4}$ (M^{-1})	$K(0) \times 10^{-4}$ (M^{-1})	$\sigma(0) \times 10^{-4}$ (M^{-1})
Cl. perfringens	30	9.7 ± 1.2	1.44	4.1 ± 0.4	1.52	5.9 ± 0.6	0.89	2.2 ± 0.2	0.91
calf thymus	42	16 ± 3	2.02	8.0 ± 0.6	2.31	8.5 ± 0.4	1.24	3.2 ± 0.2	1.25
E. coli	51	18 ± 3	2.41	9.8 ± 1.2	2.93	10 ± 2	1.55	4.2 ± 0.5	1.63
M. luteus	72	22 ± 2	3.57	12.2 ± 2	4.39	10 ± 3	2.34	4.2 ± 0.5	2.52

[a] Data were taken in buffer 2 at 22 °C in the airfuge. C_F was determined by liquid scintillation counting. Terms are defined in the text.

were fit both to the Scatchard equation (Scatchard, 1949), eq 4, and, as shown in the figures, to a statistically weighted

$$r/C_F = (n - r)K_{app} \quad (4)$$

binding equation derived from the neighbor exclusion binding model (Crothers, 1968) modified to accommodate intercalation into fewer than the number of theoretically available sites (Lawrence & Daune, 1976), eq 5. The binding constant of

$$r/C_F = K(0)(a - 2n'r)^{n'}/[2[a - (n' - 1)2r]^{(n'-1)}] \quad (5)$$

eq 4 is the apparent binding constant, K_{app}, of the reagent to the DNA molecule whereas the binding constant $K(0)$ of eq 5 is the intrinsic binding constant for an isolated potential binding site. The symbol n (eq 4) is r_{max}, the maximum number of sites on the DNA which are available to the intercalator, and n' (eq 5) is the minimum number of base pairs separating a bound dye from another potential binding site on the DNA. In the nearest-neighbor excluded-site model of intercalative binding, n' is taken to be 2, i.e., binding is restricted to every other base pair, corresponding to an r_{max} = 0.25. The parameter a of eq 5 is the fraction of the theoretically available sites which are experimentally available to the intercalator. The binding parameters of the Scatchard analysis were determined by a best-fit linear least-squares program run on a Texas Instruments SR-52 pocket calculator; the parameters of eq 5 were determined by using an iterative, nonlinear least-squares program run on an IBM 360-91 computer. The two data treatments may be compared by examining r/C_F in the limit when r approaches zero, sometimes referred to as the affinity of binding (Müller & Crothers, 1975) and designated σ (for the Scatchard analysis) or $\sigma(0)$ (for the excluded-site analysis). Equations 6 and 7 define these parameters. Since they both correspond to the y intercept of the binding isotherm, they should be in close agreement with one another. The binding of [(bipy)Pt(en)]$(NO_3)_2$, [$(py)_2$Pt(en)]$(ClO_4)_2$, and ethidium bromide to calf thymus DNA was also determined by pelleting in 0.2 M NaCl. The binding parameters for all compounds are summarized in Table I.

$$\sigma = \lim_{r \to 0} \left(\frac{r}{C_F} \right) = (K_{app})n \quad (6)$$

$$\sigma(0) = \lim_{r \to 0} \left(\frac{r}{C_F} \right) = \frac{K(0)a}{2} \quad (7)$$

The K_{app} of (26 ± 5) × 10^4 M^{-1} obtained for EtdBr at 20 °C is lower than the average K_{Etd} of (33 ± 3) × 10^4 M^{-1} determined from fluorescence measurements at 25 °C (Howe-Grant et al., 1976). The $K(0)$ value of 14 × 10^4 M^{-1}, however, is in good agreement with a fluorescence value determined in buffer 3 at 21 °C, 13 × 10^4 M^{-1} (Reinhardt & Krugh, 1978). Since the determination of C_F by electronic absorption spectroscopy in the pelleting method is not as sensitive at low r values as is the fluorescence determination method for C_B, the pelleting values are deemed acceptable. The complex [$(py)_2$Pt(en)]$^{2+}$ exhibited no evidence of binding to the DNA under the conditions of the pelleting experiment, in agreement with a previous report (Lippard et al., 1976).

Effect of G·C Content on Metallointercalator Binding to DNA. The binding of [^{3}H][(*o*-phen)Pt(en)]$(NO_3)_2$ and [^{3}H][(terpy)Pt(HET)]NO_3 to DNAs of various G·C content was studied in buffer 2 at 22 °C by pelleting in the airfuge. The results are summarized in Table II. The binding isotherms of [^{3}H][(*o*-phen)Pt(en)]$(NO_3)_2$ to the various DNAs are shown in Figure 3. The degree of binding of each platinum intercalator varies with the DNA. Both reagents exhibit G·C specificity, binding most strongly to *M. luteus* DNA (72% G·C) and most weakly to *Cl. perfringens* DNA (30% G·C). Moreover, as shown in Figure 4, the binding affinity, σ, increases linearly as a function of the G·C content of the DNA for both of the metallointercalation reagents.

The dependence upon G·C content of a reagent's DNA binding can be discussed in terms of α, defined (Müller & Crothers, 1975) as the ratio of binding affinity to DNA_x, σ_x, to the binding affinity to DNA_y, σ_y (eq 8). The experimentally

$$\alpha = \sigma(DNA_x)/\sigma(DNA_y) \quad (8)$$

determined value of α may then be compared to the corresponding ratio of probability of occurrence of a G·C-containing site or a G·C base pair. An α value of 1 would, of course, denote no specificity in binding.

Table III displays both the experimentally determined α values for [(*o*-phen)Pt(en)]$^{2+}$ and [(terpy)Pt(HET)]$^{+}$ binding to the various DNAs and those calculated from the G·C mole fraction ratios for these DNAs. Although the binding affinity (σ) to each DNA is greater for the dispositive cation [(*o*-phen)Pt(en)]$^{2+}$, the specificities (α) of both intercalators are

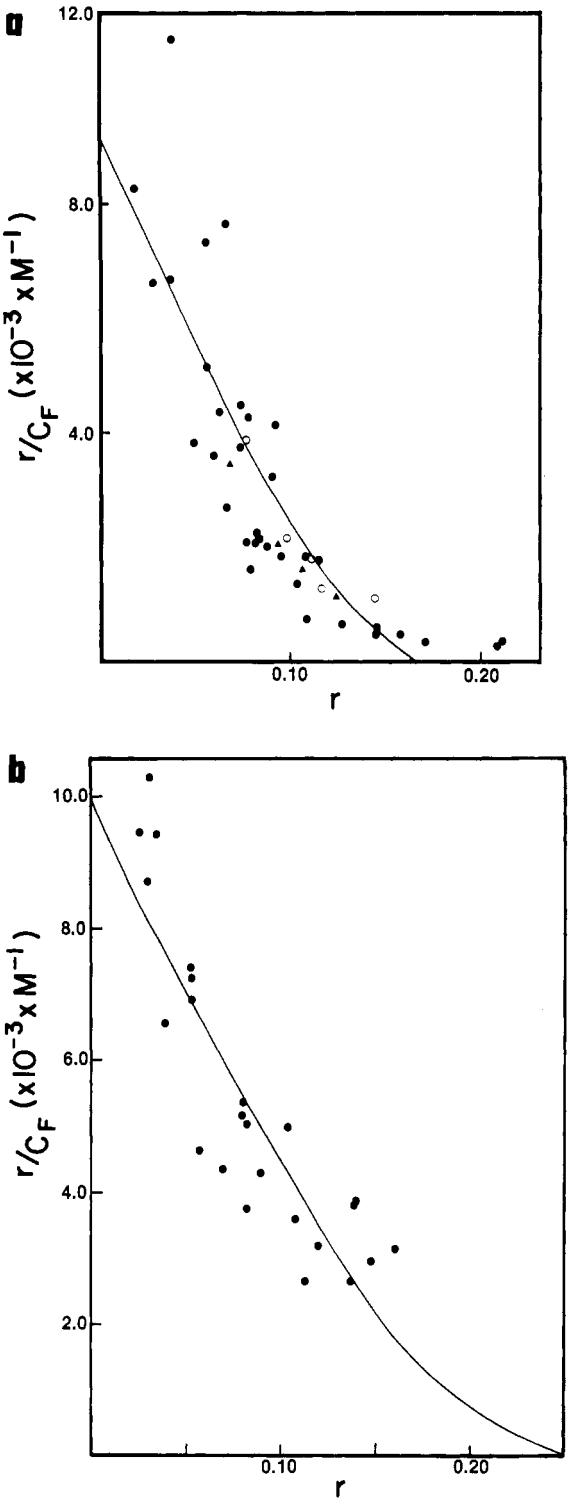

FIGURE 2: (a) Binding of [(*o*-phen)Pt(en)]$^{2+}$ to calf thymus DNA in 0.2 M NaCl at 20 °C. The $[Pt]_{total}$ ranged from 900 to 7.80 μM whereas P_0 varied from 3.98 × 10^{-4} to 0.43 × 10^{-4} M. C_F was determined spectroscopically in buffer 3 (●) or 3′ (▲) or by liquid scintillation counting in buffer 3′ (○) after pelleting in the ultracentrifuge. Data are fit (solid line) to eq 5 in the text with $n' = 2$, $K(0) = (2.7 \pm 0.3) \times 10^4$ M^{-1}, $a = 0.69 \pm 0.03$, and a correlation factor of 0.87. Scatchard analysis gave $K_{app} = (7.2 \pm 0.8) \times 10^4$ M^{-1}, $n = 0.129$, and a correlation of 0.70 for $r < 0.14$. (b) Binding of [^{3}H][(terpy)Pt(HET)]$^+$ to calf thymus DNA in 0.2 M NaCl at 22 °C. The $[Pt]_{total}$ ranged from 105 to 5.17 μM whereas P_0 varied from 1.20 × 10^{-4} to 0.89 × 10^{-4} M in buffer 3. C_{obsd} was determined by liquid scintillation counting after pelleting in the airfuge. Data are fit (solid line) to eq 5 in the text, with $n' = 2$, $K(0) = (2.0 \pm 0.2) \times 10^4$ M^{-1}, $a = 0.99 \pm 0.1$, and a correlation factor of 0.89. Scatchard analysis gave $K_{app} = (4.8 \pm 0.8) \times 10^4$ M^{-1}, $n = 0.197$, and a correlation coefficient of 0.75.

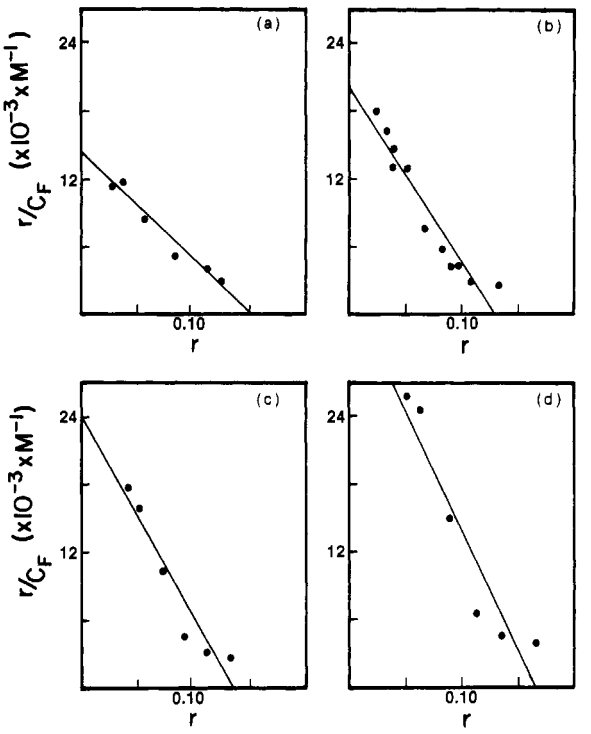

FIGURE 3: Binding of [^{3}H][(*o*-phen)Pt(en)]$^{2+}$ to DNAs of varying G·C content in 0.10 M NaCl at 22 °C. C_F was determined by liquid scintillation counting after pelleting in the airfuge. Data shown are fit to eq 4 (see Table II for binding parameters). (a) *Cl. perfringens* DNA: $[Pt]_{total}$ varied from 67.3 to 8.3 μM with $P_0 = 1.90 \times 10^{-4}$ M. (b) Calf thymus DNA: $[Pt]_{total}$ varied from 71.0 to 1.7 μM with $P_0 = 2.13 \times 10^{-4}$ to 0.55×10^{-4} M. (c) *E. coli* DNA: $[Pt]_{total}$ varied from 71.0 to 8.7 μM with $P_0 = 1.55 \times 10^{-4}$ M. (d) *M. luteus* DNA: $[Pt]_{total}$ varied from 60.6 to 7.4 μM with $P_0 = 1.10 \times 10^{-4}$ M.

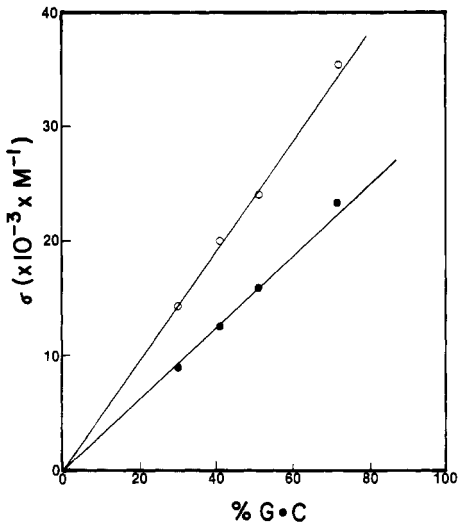

FIGURE 4: Dependence of the binding affinity, σ, of two platinum intercalation reagents on the G·C content of the DNA. Data for [^{3}H][(*o*-phen)Pt(en)]$^{2+}$ (○) and [^{3}H][(terpy)Pt(HET)]$^+$ (●) are for binding at 22 °C in buffer 2, as summarized in Table II.

nearly the same and almost identical with the calculated G·C mole fraction ratios. The [(terpy)Pt(HET)]$^+$ cation exhibits a slightly higher specificity toward *M. luteus* DNA than does [(*o*-phen)Pt(en)]$^{2+}$, but since α_{obsd} is greater than α_{calcd} for both reagents, the significance of this result is obscure.

Effect of Ionic Strength on Metallointercalator Binding. The binding of [(*o*-phen)Pt(en)]$(NO_3)_2$ and [(terpy)Pt(HET)]NO_3 to calf thymus DNA at various concentrations of sodium chloride was investigated at 20 °C by pelleting in the ultracentrifuge. Data were fit to eq 4 and 5, and the

Table III: Binding Specificities (α) for [(*o*-phen)Pt(en)]$^{2+}$ and [(terpy)Pt(HET)]$^{+}$ to DNAs of Varying G·C Content[a]

DNA	% G·C	[(*o*-phen)-Pt(en)]$^{2+}$ α_{obsd}	[(*o*-phen)-Pt(en)]$^{2+}$ α_{calcd}	[(terpy)-Pt(HET)]$^{+}$ α_{obsd}	[(terpy)-Pt(HET)]$^{+}$ α_{calcd}
Cl. perfringens	30				
calf thymus	42	1.40	1.40	1.39	1.40
E. coli	51	1.68	1.70	1.71	1.70
M. luteus	72	2.49	2.40	2.62	2.40

[a] Terms are defined in the text. Data to compute α are taken from Table II. The ratio α_{obsd} is based on the equation $\alpha = \sigma_x/\sigma_{Cl.\ perfringens}$. Calculated values ($\alpha_{calcd}$) are the ratio G·C mole fraction of DNA_x to G·C mole fraction of *Cl. perfringens* DNA.

binding parameters are summarized in Table IV. A decrease in ionic strength increases the affinity of binding for each of the metallointercalators. This increase, however, is greater for the divalent phenanthroline complex than for the monopositive terpyridine derivative.

The dependence of the apparent binding constant, K_{app}, upon the ionic strength of the solvent medium expressed as total positive ion concentration, $[M^+]$, is shown in Figure 5. The nature of this relationship has been stated (Record et al., 1976) in terms of the number of monovalent solvent counterions, $m'\psi$, released from a polynucleotide upon binding of a ligand to a site containing m' phosphate residues (eq 9), where ψ is the

$$\log K_{app} = -m'\psi \log [M^+] + \log K_0 \qquad (9)$$

extent of counterion binding per phosphate residue before ligand association. The ψ value is inversely proportional to the distance between phosphates and has a value of 0.88 for normal double-stranded DNA. K_0 is the value of K_{app} at 1 M positive-ion concentration and is assumed to be free of electrostatic components. The slopes of these lines thus yield information regarding the electrostatic contributions to the binding whereas the y intercepts determine the extent of the nonelectrostatic interactions. Also shown in Figure 5 are data corresponding to ethidium–DNA binding from fluorescence studies at 23 °C (LePecq & Paoletti, 1967). The results indicate that [(*o*-phen)Pt(en)]$^{2+}$ replaces 2.2 counterions in a minimum of 2.5 phosphate residues upon binding to DNA whereas [(terpy)Pt(HET)]$^{+}$ replaces 1.5 counterions and ethidium replaces 1.0 counterion in a minimum of 1.75 and 1.2 phosphate residues, respectively. Extrapolation to 1 M positive-ion concentration yields K_0 values that are essentially the same for each of the metallointercalation reagents, 3.5×10^3 M^{-1}, and 1 order of magnitude less than the K_0 for ethidium, 7.4×10^4 M^{-1}.

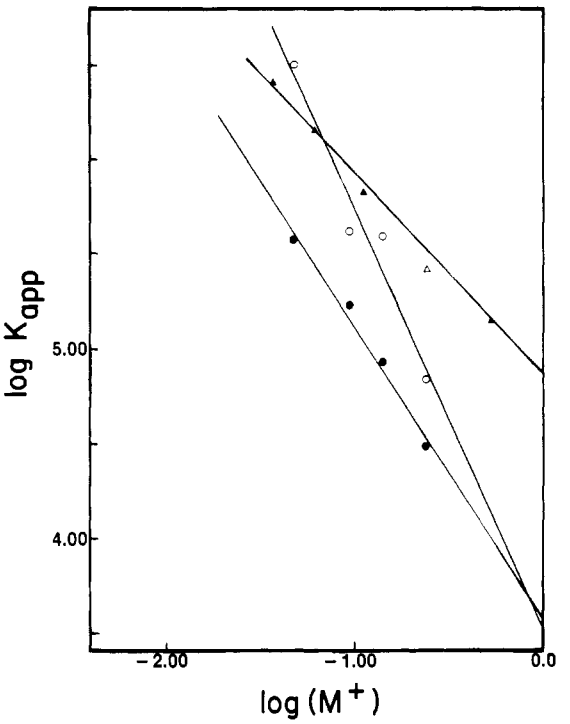

FIGURE 5: Apparent binding constant as a function of ionic strength, log K_{app} vs. log $[M^+]$, for [(*o*-phen)Pt(en)]$^{2+}$ (○), [(terpy)Pt(HET)]$^{+}$ (●), and Etd^{+} (▲) binding to calf thymus DNA. Points correspond to data in Table IV for the metallointercalators at 20 °C and are taken from the literature (LePecq & Paoletti, 1967) for ethidium at 23 °C. The total positive ion concentration, $[M^+]$, is the summation of the $[Na^+]$ and [Tris-acid$^+$] where [Tris-acid] = 0.043 M for all buffers with 0.05 M Tris-HCl, pH 7.5, since pK_a = 8.3 = p([Tris-base]/[Tris-acid]) + pH. A linear least-squares fit to eq 9 gave $-m'\psi$ = 2.21, $K_0 = 3.4 \times 10^3$ M^{-1}, and a correlation factor of 0.943 for [(*o*-phen)Pt(en)]$^{2+}$, $-m'\psi$ = 1.54, $K_0 = 3.7 \times 10^3$ M^{-1}, and a correlation factor = 0.985 for [(terpy)Pt(HET)]$^{+}$, and $-m'\psi$ = 1.05, $K_0 = 7.4 \times 10^4$ M^{-1}, and a correlation coefficient = 0.997 for Etd^{+}.

Discussion

G·C Specificities. Both [(*o*-phen)Pt(en)]$^{2+}$ and [(terpy)-Pt(HET)]$^{+}$ exhibit a definite G·C base-pair specificity in binding to DNA. The majority of intercalation reagents show G·C specificity in DNA binding (Müller & Crothers, 1975) and even those that do not, such as ethidium (LePecq & Paoletti, 1967), may exhibit a C·G sequence preference in binding (Kastrup et al., 1978; Reinhardt & Krugh, 1978). Differences between G·C and A·T base pairs include the overall dipole moment and the number of hydrogen bonds, both of which are greater for the G·C pair (DeVoe & Tinoco, 1962). These factors combine to make the G·C base pair more polar and more highly polarizable. More polarizable intercalators, those having electronic absorption bands at longer wavelengths, are more G·C specific in binding DNA (Müller et al., 1975; Müller & Crothers, 1975).

The linear dependence of the metallointercalator binding affinities on DNA G·C content (Figure 4) indicates that the presence of a single G·C base pair is all that is required for preferred binding to an intercalation site; i.e., a (G·C)–(A·T) site is as attractive as a (G·C)–(G·C) site. Such a result

Table IV: Binding Parameters of [(*o*-phen)Pt(en)]$^{2+}$ and [(terpy)Pt(HET)]$^{+}$ to Calf Thymus DNA at Various Concentrations of Sodium Chloride[a]

	[(*o*-phen)Pt(en)]$^{2+}$				[(terpy)Pt(HET)]$^{+}$			
$[Na^+]$ (M)	$K_{app} \times 10^{-5}$ (M^{-1})	$\sigma \times 10^{-4}$ (M^{-1})	$K(0) \times 10^{-5}$ (M^{-1})	$\sigma(0) \times 10^{-4}$ (M^{-1})	$K_{app} \times 10^{-5}$ (M^{-1})	$\sigma \times 10^{-4}$ (M^{-1})	$K(0) \times 10^{-5}$ (M^{-1})	$\sigma(0) \times 10^{-4}$ (M^{-1})
0.200	0.7 ± 0.1	0.9 ± 0.1	0.3 ± 0.0	0.9 ± 0.1	0.3 ± 0.0	0.7 ± 0.0	0.3 ± 0.0	1.0 ± 0.1
0.100	3.9 ± 1.0	4.3 ± 1.1	1.8 ± 0.1	4.7 ± 0.6	0.85 ± 0.2	1.2 ± 0.3	0.3 ± 0.0	1.2 ± 0.2
0.050	4.2 ± 0.8	5.8 ± 1.2	2.4 ± 0.1	7.3 ± 1.1	1.7 ± 0.6	2.9 ± 0.9	0.7 ± 0.1	3.0 ± 0.4
0.005	31.5 ± 10.0	42 ± 12	3.4 ± 3.8	16.5 ± 29	3.7 ± 1.0	6.3 ± 1.3	1.9 ± 0.2	7.3 ± 1.4

[a] Data were taken at 20 °C in the ultracentrifuge, and all buffers contained 0.05 M Tris-HCl at pH 7.5 in addition to the designated sodium chloride concentration. Terms are defined in the text.

Table V: Ten DNA Intercalation Sites and Their Frequencies of Occurrence[a]

site[b]	no.	calf thymus DNA	*E. coli* DNA	*M. luteus* DNA
A·T / A·T	1	17.6	14.5	3.6
A·T / T·A	2	5.3	5.1	1.1
T·A / A·T	3	7.3	6.8	2.2
G·C / G·C	4	10.4	11.2	22.5
C·G / G·C	5	4.4	8.3	12.1
G·C / C·G	6	1.6	6.7	13.9
C·G / A·T	7	10.8	10.9	11.3
G·C / A·T	8	13.9	11.0	9.9
C·G / T·A	9	13.1	11.1	12.8
G·C / T·A	10	15.6	14.2	10.6

[a] All frequencies are reported in terms of 100 possible sites and are based on nearest-neighbor sequence analyses (Josse et al., 1961). [b] Arrows indicate the 5′ to 3′ polarity of the DNA, e.g., A·T / T·A indicates A(3′,5′)T.

signifies that the specificity is limited to one side of the G·C base pair (Müller & Crothers, 1975), e.g., the 5′ side of G. The metallointercalators must either selectively recognize one side of a G·C base pair during binding (a kinetic preference) or experience a sufficiently stronger interaction when bound to the preferred side of a G·C pair to yield a significant increase in the binding constant (a thermodynamic preference). It is likely that differences in the two sides of a G·C pair are brought about by the intercalation process itself, since there is considerable evidence from crystal structure determinations of dinucleoside monophosphate intercalation complexes that sugar conformations which are normally C2′ endo in uncomplexed DNA remain C2′ endo at the 3′ end of the chain but become C3′ endo at the 5′ end (Sobell et al., 1976, 1977; Wang et al., 1978). An alternation in sugar puckering was also found (Bond et al., 1975) to be a good model for interpreting [(terpy)Pt(HET)]$^+$ intercalated DNA X-ray fiber diffraction patterns.

The differences in the x intercepts of the 0.2 M NaCl binding isotherms of the intercalators with calf thymus DNA (Figure 2) suggest differences in binding mode. The data show that only 69% of the intercalation sites available according to the excluded-site model are experimentally available to [(*o*-phen)Pt(en)]$^{2+}$ whereas 100% of these sites are available to [(terpy)Pt(HET)]$^+$. In an attempt to account for the lesser binding of the dicationic complex, the frequencies of occurrence of the 10 normal DNA intercalation sites were investigated. Table V lists the intercalation sites and their frequencies of occurrence for calf thymus, *E. coli*, and *M. luteus* DNA (Josse et al., 1961). Sites 1–3 contain no G·C base pairs whereas sites 4–10 do. As may be seen from Table VI, which summarizes the number of sites in a variety of categories, the type of site on calf thymus DNA closest to the experimental value of $a = 0.69$ contains at least one G·C base pair and has 69.8 sites/100. These data support a rather selective binding mechanism for this complex whereby the dication recognizes and binds only those sites containing a G·C base pair.

In the case of [(terpy)Pt(HET)]$^+$, which exhibits about the same G·C specificity as [(*o*-phen)Pt(en)]$^{2+}$ but has all neighbor

Table VI: Summary of Intercalation Sites and Their Corresponding Specificities[a]

site type	frequency of occurrence: calf thymus DNA	*E. coli* DNA[b]	*M. luteus* DNA[c]
(G·C)–(G·C)	16.4	26.2 (1.60)	48.5 (2.96)
(G·C)–(X·Y)	69.8	73.4 (1.05)	93.2 (1.34)
5′ side of G	38.7	41.5 (1.07)	58.7 (1.51)
3′ side of G	41.5	43.1 (1.04)	56.9 (1.37)
C(3′,5′)purine	15.2	19.2 (1.25)	23.4 (1.52)
purine(3′,5′)C	17.2	20.9 (1.22)	24.5 (1.42)
ρ[d](G·C)–(X·Y)	66.4	76.0 (1.14)	92.2 (1.39)

[a] Site summations are given in terms of the frequencies reported in Table V. Specificities, α's, are calculated on the basis of data for calf thymus DNA and are given in parentheses. [b] The observed values of α_{CT}^{EC} are 1.19 [[(*o*-phen)Pt(en)]$^{2+}$] and 1.25 [[(terpy)Pt(HET)]$^+$]. [c] The observed values of α_{CT}^{ML} are 1.77 [[(*o*-phen)Pt(en)]$^{2+}$] and 1.89 [[(terpy)Pt(HET)]$^+$]. [d] Probability of a G·C-containing intercalation site based on the G·C percentage composition and assuming a random distribution.

excluded sites experimentally available, hydrogen bonding of the nonplanar mercaptoethanol tail might contribute to stabilization in sites with no G·C base pair. This stabilization would render the [(terpy)Pt(HET)]$^+$ complex less selective than [(*o*-phen)Pt(en)]$^{2+}$ at low r values. [(terpy)Pt(HET)]$^+$ binds to double-stranded RNAs with no G·C base pairs, such as poly(A)·poly(U), and appears to do so cooperatively and as dimers (Barton & Lippard, 1979).

Included in Table VI are a variety of types of intercalation sites and their corresponding calculated specificities based on the data given for calf thymus DNA. The observed (Table II) binding specificities based on σ for calf thymus DNA, α_{CT}, are 1.19 (*E. coli*) and 1.77 (*M. luteus*) for [(*o*-phen)Pt(en)]$^{2+}$ and 1.25 (*E. coli*) and 1.89 (*M. luteus*) for [(terpy)Pt(HET)]$^+$. Although α_{CT} values from ratios of percentage G·C content, 1.21 (*E. coli*) and 1.71 (*M. luteus*), give the best agreement to the observed data, it is interesting to compare the numbers of Table VI since intercalation involves a site defined not by one base pair but by two. The observed α_{CT} values are much lower than those calculated from the $(G{\cdot}C)^2$ sites and, for those sites containing at least one G·C base pair, come closest to the α values of sites which have a sequence of C(3′,5′)purine with $\alpha^{EC} = 1.25$ and $\alpha^{ML} = 1.52$. This pyrimidine–purine sequence is seen in the majority of crystal structures involving dinucleosides and intercalation reagents (Sobell et al., 1976, 1977; Wang et al., 1978), and it includes the reported sequence d(CpG) preferred for ethidium binding (Kastrup et al., 1978; Reinhardt & Krugh, 1978). Intercalator preference for pyrimidine–purine sequences has been attributed to the relative enhancement of stacking interactions upon insertion of the reagent since the normal base-pair overlap in the double helix is less in a pyrimidine–purine sequence than in a purine–pyrimidine one (Pack & Loew, 1978). The enhanced stacking interactions in an intercalated complex are especially evident in the [(terpy)Pt(HET)]$^+$ d(CpG) crystal where all three pyridine rings are intercalated between the DNA base pairs (Wang et al., 1978).

Neither sites which allow binding only to the 3′ side of G nor those allowing binding to the 5′ side give α values as close to the observed, although the latter, which would include the CpG pyrimidine–purine sequence, is closer. The reasons behind a preference in binding to one side of a G·C base pair remain unclear.

Effect of Metallointercalator Charge and Structure on Binding to Calf Thymus DNA. For a given DNA and ionic strength, the structure of a metallointercalator influences its

binding as previously shown by studies of the inhibition of the fluorescence of ethidium bound to calf thymus DNA (Howe-Grant et al., 1976; Howe-Grant, 1978) and by alteration of the duplex winding angle of closed circular PM-2 DNA (Howe-Grant et al., 1976; Lippard et al., 1976). Terpyridine complexes were found to produce a larger effect in both these cases than either [(*o*-phen)Pt(en)]$^{2+}$ or [(bipy)Pt(en)]$^{2+}$, and these results were interpreted to mean that the number of planar aromatic rings of a complex and/or the presence of the nonplanar HET tail were more important to intercalative ability than an increase in charge on the complex ion. The present study shows, however, that the binding constants of the platinum intercalation reagents in 0.2 M NaCl with calf thymus DNA decrease in the order Etd$^+$ > [(*o*-phen)Pt(en)]$^{2+}$ > [(terpy)Pt(HET)]$^+$ > [(bipy)Pt(en)]$^{2+}$; the ordering of the binding affinities is Etd > [(terpy)Pt(HET)]$^+$ ~ [(*o*-phen)Pt(en)]$^{2+}$ > [(bipy)Pt(en)]$^{2+}$, with close agreement between values of σ and $\sigma(0)$. These results suggest that increasing the charge on an intercalator is about as effective as removing a nonplanar, hydrogen-bonding tail and one of the chelate rings.

Binding constants for the dipositive metallointercalators determined by pelleting experiments (Table I) agree with the corresponding K_M values determined from a Scatchard analysis of ethidium fluorescence inhibition data. The K_{app} for [(terpy)Pt(HET)]$^+$ is only about one-third the value of the corresponding K_M, however. The K_M values for these compounds are 5×10^4 M^{-1} for [(*o*-phen)Pt(en)]$^{2+}$ (Howe-Grant, 1978), 1×10^4 M^{-1} for [(bipy)Pt(en)]$^{2+}$, and 1.2×10^5 M^{-1} for [(terpy)Pt(HET)]$^+$ (Howe-Grant et al., 1976). The discrepancy in the observed ordering of the binding constants determined by the two methods is due to differences in the binding isotherms revealed by the pelleting method and not apparent when examining ethidium fluorescence Scatchard plots. Whereas the binding isotherms of the three metallointercalators with calf thymus DNA in 0.2 M NaCl have essentially the same shape as that of ethidium with DNA, the curves progressively shift to the left as one moves from Etd$^+$ and [(terpy)Pt(HET)]$^+$ to [(*o*-phen)Pt(en)]$^{2+}$ and [(bipy)Pt(en)]$^{2+}$. Determination of K_M by ethidium fluorescence inhibition was based on the assumption that r_{max} is the same for ethidium and any complex exhibiting competitive inhibition. If, however, some of the intercalation sites on the DNA available to ethidium, $n = 0.23$, are not available to a metallointercalator, such as [(*o*-phen)Pt(en)]$^{2+}$ where $n = 0.13$, the inhibition will be effective toward only ~60% of the total ethidium sites and the corresponding K_{Pt} could be low by as much as 40%.

The unavailability of sites on the DNA to [(*o*-phen)Pt(en)]$^{2+}$ and [(bipy)Pt(en)]$^{2+}$ as well as other differences in relative binding constants and in the pelleting isotherms might result from charge and structural differences between [(terpy)Pt(HET)]$^+$ and the dipositive intercalators. The major differences between [(terpy)Pt(HET)]$^+$ and the dicationic complexes studied are their charge, occupancy of three platinum coordination sites for the terpy vs. two sites for *o*-phen and bipy, the existence of a polar Pt–S bond along with three Pt–N bonds vs. four Pt–N bonds, and the presence of a nonplanar hydrogen-bonding hydroxyethanethiolate tail vs. a pseudoplanar ethylenediamine chelate ring. Of these four differences, the charge and the hydrogen-bonding tail are probably the most important. Charge effects could reduce the number of intercalators bound at saturation. Moreover, the potential of the nonplanar mercaptoethanol tail of [(terpy)Pt(HET)]$^+$ for hydrogen bonding to the DNA backbone either in the major groove, as observed in the crystal structure of the intercalated deoxydinucleoside monophosphate complex (Wang et al., 1978), or in the minor groove of the DNA (Bond et al., 1975) could possibly serve as an anchor, stabilizing intercalation into non-G·C-containing sites. Certainly, for the thiolatoterpyridineplatinum reagents studied by inhibition of ethidium binding, the K_M values increase as the electropositive nature of the nonplanar tail increases (Howe-Grant et al., 1976). The extent of this interaction is discussed for [(terpy)Pt(HET)]$^+$ under Electrostatic Considerations.

Table VII: Thermodynamic Data for the Binding of Intercalation Reagents with Calf Thymus DNA

reagent	$-\Delta G^\circ$ (kcal/mol)	K_0 (M^{-1})	T (°C)
[(*o*-phen)Pt(en)]$^{2+}$	4.7	3.4×10^3	20
[(terpy)Pt(HET)]$^+$	4.8	3.7×10^3	20
Etd$^+$	6.6	7.4×10^4	23

Electrostatic Considerations. The dipositive metallointercalator, [(*o*-phen)Pt(en)]$^{2+}$, is shown by the number of monovalent counterions (2.2) released from DNA upon binding to have a larger electrostatic interaction than either monovalent cation [(terpy)Pt(HET)]$^+$ or Etd$^+$. Moreover, although both carry the same charge, [(terpy)Pt(HET)]$^+$ releases 1.5 counterions compared to only 1.0 for the Etd$^+$ cation. This additional counterion release could arise from hydrogen bonding of the mercaptoethanol tail to an adjacent-site phosphate group.

Both metallointercalation reagents have the same affinity for calf thymus DNA at high ionic strength where the electrostatic considerations are removed. This result shows that the terpyridine and *o*-phenanthroline ring systems are equivalent in terms of their ability to interact with DNA through stacking with the DNA base pairs. The [(terpy)Pt(HET)]$^+$ cation intercalates symmetrically into d(CpG)$_2$ with the three pyridine rings stacking with DNA base pairs above and below the metal complex (Wang et al., 1978). A similar configuration with the phenanthroline ring system totally inserted between the base pairs would produce equivalent binding. This head-on configuration for [(*o*-phen)Pt(en)]$^{2+}$, however, differs from the configuration for 3,8-dimethyl-*N*-methylphenanthrolinium intercalation postulated from NMR data (Reuben et al., 1976).

Neither of the metallointercalators has the intercalative affinity of ethidium as evidenced by the extrapolated values of K_0 for calf thymus DNA. The difference in binding abilities at high ionic strength, K_0 of Etd$^+$ being ~20 times that of the metallointercalators, is partially attributable to the lack of G·C specificity of the Etd$^+$ which binds equally well to DNAs of differing G·C content. Determination of K_0 for the intercalators allows the calculation of ΔG° for the intermolecular stacking interactions alone. Table VII summarizes these data which are on the same order as calculated total interaction energies for ethidium (Pack & Loew, 1978).

The strength of the electrostatic interaction of [(*o*-phen)Pt(en)]$^{2+}$ with DNA is so large at low ionic strengths that methods such as the pelleting one, which depend upon monitoring the concentrations of free dye and determining that of bound reagent by difference, cannot be used to study binding. For example, in a low-salt phosphate buffer with [M$^+$] ~ 4.5 μM, K_{app} is calculated to be in the order of 10^8 which means that even at low r values, e.g., $r = 0.02$, C_F would be on the order of 10^{-1} μM. Attempts to obtain binding parameters for [(*o*-phen)Pt(en)]$^{2+}$ with calf thymus DNA at this ionic strength led to nonreproducible data. All data ob-

tained, however, indicated a high degree of cooperative binding at $r < \sim 0.10$. Cooperativity could occur at low ionic strength and not be observed at higher salt concentrations for, with a high degree of external DNA–dye association, the change in sugar conformation thought to occur upon intercalation might induce additional intercalative binding by a complex presumably already aligned along the DNA backbone. Cooperativity at low r would also be consistent with fiber diffraction data obtained for [(*o*-phen)Pt(en)]$^{2+}$ and [(bipy)Pt(en)]$^{2+}$ and calf thymus DNA (Lippard et al., 1976). These fibers, especially those containing [(bipy)Pt(en)]$^{2+}$, appeared highly crystalline which could be attributed either to cooperativity in binding or to long runs of G·C base pair containing sites.

In conclusion, the strength of the metallointercalator–DNA interaction depends upon the structural characteristics of the intercalating complex, the composition of the DNA, and the ionic strength of the medium. Each of these factors, together with the shape of the binding isotherm, must be considered when comparing binding parameters or attempting to order intercalative abilities of the various reagents.

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