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Binding of Platinum and Palladium Metallointercalation Reagents and Antitumor Drugs to Closed and Open DNAs†

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ABSTRACT: The interaction of platinum and palladium complexes with closed and nicked circular and linear DNAs was investigated by a variety of methods. Cationic metal complexes containing flat, aromatic ligands, such as 2,2′,2″-terpyridine, *o*-phenanthroline, and 2,2′-bipyridine, interfere with the usual fluorescence enhancement of ethidium bromide by competing for intercalation sites on calf-thymus DNA. Metal complexes having kinetically exchangable ligands, including the antitumor drugs *cis*-$[(NH_3)_2PtCl_2]$ and $[(en)PtCl_2]$, inhibit noncompetitively the DNA-associated ethidium fluorescence enhancement by binding covalently to the bases and blocking potential intercalation sites. Only the metallointercalators were capable of altering the DNA duplex winding, as judged by the effects of these reagents upon the electrophoretic mobility and sedimentation behavior of PM-2 DNAs. Long-term ($t > 120$ h) interactions of metal complexes with PM-2 DNAs I, I_0, and II, corresponding to superhelical, closed relaxed, and nicked circles, respectively, showed that covalent binding occurs the most readily to DNA I, possibly because of the presence of underwound duplex regions in this tightly wound superhelical DNA. The active antitumor drugs *cis*-$[(NH_3)_2PtCl_2]$ and $[(en)PtCl_2]$ bind covalently to DNA I under conditions where the inactive *trans*-$[(NH_3)_2PtCl_2]$ does not. Most of the complexes studied were capable of producing chain scissions in PM-2 DNA I. Exceptions are the kinetically inert complexes $[(bipy)Pt(en)]^{2+}$ and $[(terpy)Pt(SCH_2CH_2OH)]^+$, suggesting that covalent binding might be a prerequisite for nicking.

Recent studies in our laboratories have demonstrated that the $[(terpy^1)Pt(HET)]^+$ monocation (Figure 1) binds to double-stranded DNAs by intercalation with a duplex unwinding angle comparable to that of ethidium bromide (EtdBr) (Jennette et al., 1974). The electron-dense platinum atoms in this complex, intercalatively bound to calf-thymus DNA, were subsequently detected by fiber x-ray diffraction techniques at a distribution along the helix axis that accords with the nearest neighbor exclusion binding model (Bond et al., 1975). The present study was undertaken to determine the effects of charge, number of aromatic ligands, number of chelate rings, and choice of additional ligands on the intercalative binding of metallointercalation reagents to DNA. Moreover, since it has been suggested (Thomson et al., 1972) that the mechanism of action of the platinum antitumor drugs (Rosenberg and Van Camp, 1970; Cleare, 1974) might involve intercalative binding to DNA, several compounds in this class were included in the investigation.

We have also examined the nature of the nonintercalative interaction of several metal complexes with DNA by monitoring the changes in electrophoretic mobility of both closed and nicked circular PM-2 DNAs that had been incubated with reagent. A growing body of evidence has suggested that the

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[1] Abbreviations are: EtdBr, ethidium bromide; r_f, formal ratio of metal to nucleotide concentrations; Tris, tris(hydroxymethyl)aminomethane; EDTA, disodium salt of ethylenediaminetetraacetic acid; $^{24}\sigma_0$, number of superhelical turns per ten base pairs in a native closed DNA, assuming an EtdBr unwinding angle of 24°; terpy, 2,2′,2″-terpyridine; bipy, 2,2′-bipyridine; *o*-phen, *ortho*-phenanthroline; en, ethylenediamine; dien, diethylenetriamine; HET, 2-hydroxyethanethiolato; AET, 2-aminoethanethiolato; CMT, carboethoxymethanethiolato; and cys, cysteinato; uv, ultraviolet.

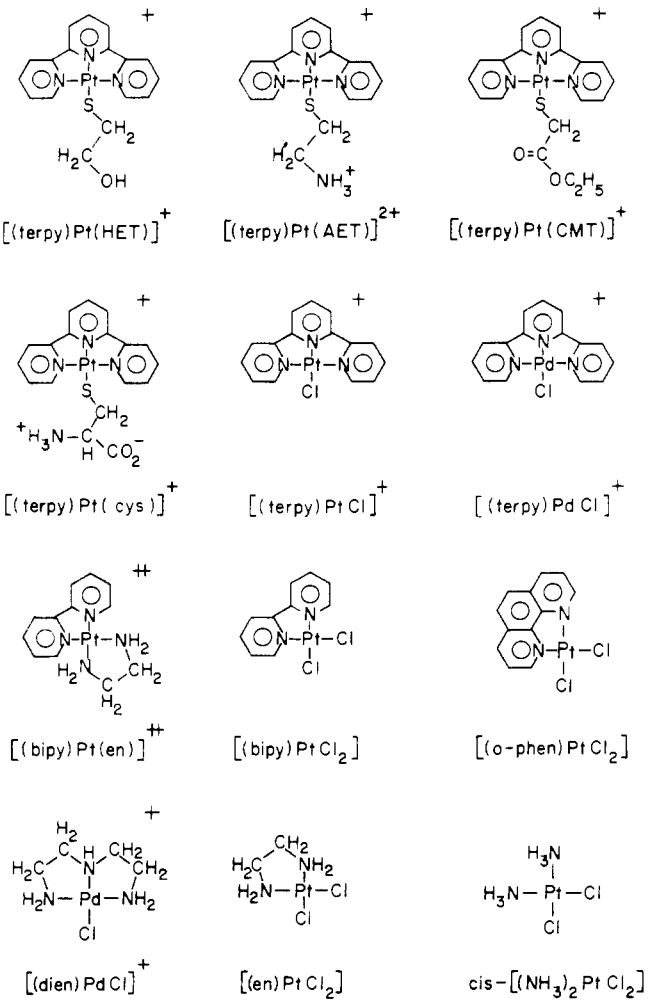

FIGURE 1: Structures and nomenclature of the metal complexes. The trans isomer of $[(NH_3)_2PtCl_2]$ is not shown.

covalent interaction of platinum-containing drugs with DNA is related to their antitumor behavior (Harder and Rosenberg, 1970; Howle and Gale, 1970; Munchausen, 1974). The closed DNA assay employed in the present study is extremely powerful for the detection of covalent binding and is especially sensitive to nicking, the introduction of chain scissions in the DNA sugar-phosphate backbone. During the course of our work, a study of the sedimentation of closed PM-2 DNAs through a solvent containing *cis*- and *trans*-diammineplatinum(II) complexes was reported (Wakelin, 1974). This investigation, in which no effect of the drugs upon the DNA was detected, was limited to short incubation periods.

Materials and Methods

Metal Complexes. The compounds [(terpy)Pt(HET)](NO_3), [(terpy)Pt(AET)]$(PF_6)_2$, and [(terpy)Pt(CMT)](PF_6) were prepared as described by Jennette et al. (1976). The complex [(terpy)Pt(cys)]$(PF_6)\cdot 2H_2O$ was synthesized by an analogous procedure in which a 1.5-fold excess of an aqueous cysteine solution was added dropwise with stirring under nitrogen to an aqueous solution of $[(terpy)Pt(H_2O)]^{2+}$. The pH of the reaction mixture was adjusted to approximately 6 and the red complex precipitated with acetone. Subsequent dissolution in a minimum quantity of water followed by addition of excess sodium hexafluorophosphate and recrystallization from 1:1 water–ethanol yielded the desired product.

Analysis. Calcd for $PtC_{18}N_4H_{21}SO_4PF_6$: C, 29.64; H, 2.90; N, 7.68. Found: C, 29.55; H, 2.73; N, 7.59. Standard procedures were used to prepare [(terpy)PtCl]Cl·$2H_2O$, [(terpy)PdCl]Cl·$2H_2O$ (Intille, 1970), [(bipy)$PtCl_2$] (Morgan and Burstall, 1934), [(bipy)Pt(en)]I_2 (Watt and Upchurch, 1966), [(bipy)Pt(en)]$(NO_3)_2$ (Erickson, 1969), *cis*- and *trans*-$[(NH_3)_2PtCl_2]$ (Kauffman and Cowan, 1963), [(en)-$PtCl_2$] (Johnson, 1966), [(*o*-phen)$PtCl_2$] (Watt and Cuddeback, 1971), and [(dien)PdCl]Cl (Basolo et al., 1960).

Buffers and Other Chemicals. Experiments were carried out in: buffer 1, 1 mM sodium phosphate, 3 mM sodium chloride, pH 6.8; buffer 2, 50 mM Tris-HCl, 0.1 M sodium chloride, pH 7.5; buffer 3, 50 mM Tris-HCl, 0.2 M sodium chloride, pH 7.5; buffer 4, 20 mM sodium acetate, 10 mM Tris-acetate, 0.1 mM EDTA, pH 8.1; and buffer 5, 40 mM Tris-acetate, 20 mM sodium acetate, 4 mM EDTA, pH 8.1. EtdBr was purchased from the Sigma Chemical Company and optical grade CsCl from the Harshaw Chemical Company. All other chemicals were reagent grade. Stock solutions of metal complexes and of EtdBr used in all binding experiments were prepared in deionized distilled water, stored in the cold, and used within a week.

Source of Nucleic Acids. Calf-thymus DNA (type I) was purchased from the Sigma Chemical Company and was purified as described previously (Jennette et al., 1974). The native, covalently closed circular DNA from bacteriophage PM-2 (DNA I) was obtained by the procedure of Espejo and Canelo (1968a,b). Nicked circular PM-2 DNA (DNA II) was isolated as upper band material in a CsCl–EtdBr density gradient (Radloff et al., 1967) from a sample of DNA I which underwent a relatively small degree of conversion upon storage, presumably by the action of incompletely removed endonuclease. Relaxed, covalently closed circular PM-2 DNA (DNA I_0) was produced by incubation of DNA I with the nicking–closing enzyme isolated from HeLa cell nuclei in 0.2 M NaCl, 0.01 M Tris-HCl, 0.1 mM EDTA, pH 7.4 at 37 °C (Vosberg et al., 1975). Alternatively, PM-2 DNA I_0 was prepared by incubation of DNA I with the nicking–closing enzyme isolated from vaccinia virus (Ressner, Kates, and Bauer, in preparation) in buffer 4.

Gel Electrophoresis. The electrophoresis of PM-2 DNA samples was carried out on 1% agarose gels in buffer 5. Gels were poured into glass tubing of 6 mm internal diameter and cut to 10 cm with a razor blade or, alternatively, were poured into a slab apparatus to form a slab gel 10 × 14 × 0.35 cm. The cylinder gels were run at 100 V for 2 h, with thermostating at 25 °C and recirculation of buffer between reservoirs. The slab gels were run at 50 V for 4 h at ambient temperature without buffer recirculation. At the conclusion of an electrophoretic experiment, the gels were soaked for 1 h in buffer 5 containing EtdBr at a concentration of 0.5 μg/ml and photographed with Polaroid 55 P/N film using illumination from below with a Mineralight short-wave uv lamp supplied by Ultraviolet Products, San Gabriel, Calif. The resulting negatives were scanned with a Joyce-Loebl recording microdensitometer. The relative areas under the resulting scans, as measured with a Neumonics curve tracer, were used to estimate the relative amounts of each component. We recognize that this procedure introduces in principle a minor error in the higher concentration ranges (J. Vinograd, private communication), but all experiments reported here were performed under conditions such that the peak areas were linearly related to the amounts of DNA.

Band Sedimentation. Analytical band sedimentation velocity experiments were conducted in a sedimentation solvent

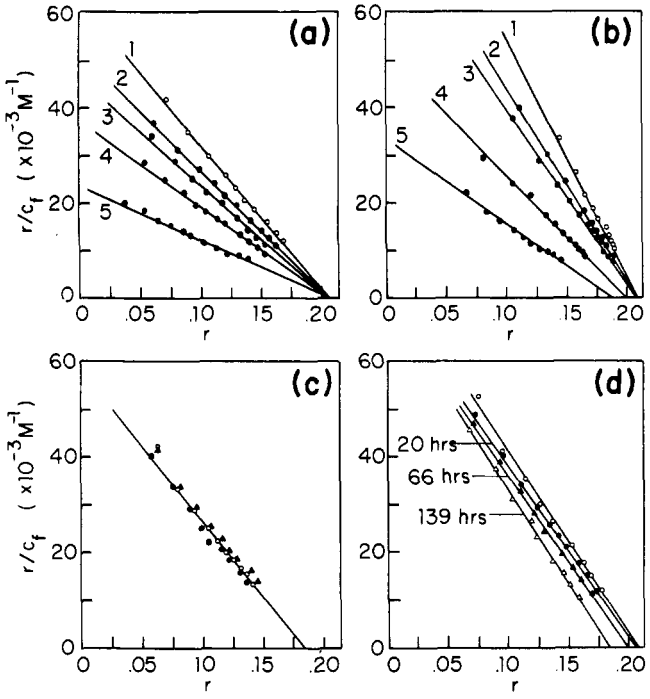

FIGURE 2: Fluorescence Scatchard plots of the binding of EtdBr to calf-thymus DNA in the absence (○) and the presence (●) of various metal complexes. Four types of behavior were observed, designated A, B, C, and D (see text), and these are depicted in a-d, respectively. (a) [(terpy)Pt(HET)]$^+$ in buffer 3; r_f increases in the order 0, 0.19, 0.31, 0.77, 1.9 for lines 1-5, respectively. The nucleotide concentration was 8.4 μM and the EtdBr concentration varied from 2.3 to 18 μM. (b) [(terpy)PtCl]$^+$ in buffer 2; r_f increases in the order 0, 0.19, 0.38, 1.0, 1.9 for lines 1-5, respectively. The nucleotide concentration was 5.8 μM and the EtdBr concentration varied from 5.2 to 20 μM. (c) *cis*-[$(NH_3)_2PtCl_2$] in buffer 3; the r_f values were 0.17 (▲) and 2.5 (●). The nucleotide concentration was 8.5 μM and the EtdBr concentration varied from 2.0 to 12 μM. (d) *cis*-[$(NH_3)_2PtCl_2$] in buffer 3 at r_f of 0.83. Incubation times are shown. The nucleotide concentration was 10.5 μM and the EtdBr concentration varied from 2.2 to 17 μM.

of 0.2 M Nacl-50% D_2O as described previously (Jennette et al., 1974).

Measurement of Binding Curves and of Fluorescence Lifetimes. Spectrophotometric measurements were performed with a Cary 118C recording spectrophotometer thermostated at 25 °C. Fluorescence measurements were made with a Perkin-Elmer MPF-2A spectrofluorimeter supplied with a Hammamatsu R446F photomultiplier and a thermostated cell accessory. The sample was excited at 540 nm and its emission observed at 590 or 600 nm. All measurements were made at 25 °C. Fluorescence lifetime experiments were conducted with a single photon counting device in line with a Hewlett-Packard 9830A desktop computer by a method similar to that described by Tao (1969).

Results

The Effect of Metal Complexes on the Binding of Ethidium to DNA. Fluorescence Scatchard plots for the binding of EtdBr to calf-thymus DNA in the presence of varying concentrations of metal complex were obtained as described previously (Le-Pecq and Paoletti, 1967; Jennette et al., 1974). Equation 1 expresses the binding of EtdBr to DNA in the presence of a competing metal complex (M).

$$r_{Etd}/c_{Etd} = (n - r_{Etd})[K_{Etd}/(1 + K_M c_M)] \quad (1)$$

Here r_{Etd} is the ratio of bound ethidium to total nucleotide concentration (P_0), c_{Etd} is the concentration of free ethidium,

TABLE I: The Effect of Metal Complexes upon the Fluorescence of Ethidium in the Presence of Calf-Thymus DNA in Buffer 3 at 25 °C.

Complex	r_f	Behavioral Type[a]	$K_M \times 10^{-5}$	τ_{Etd} (ns)[b]
[(terpy)Pt(HET)]$^+$	0.22-2.0	A	1.2	22
[(terpy)Pt(AET)]$^{2+}$	0.15-2.0	A	4.3	21
[(terpy)Pt(cys)]$^+$	0.20-2.0	A	1.0	20
[(terpy)Pt(CMT)]$^+$	0.20-2.0	A, B	0.5	19
[(terpy)PtCl]$^+$	0.19-2.0	B	1.3[c]	18
[(terpy)PdCl]$^+$	0.10-1.5	B	1.9[c]	18
[(bipy)Pt(en)]$^{2+}$	1.6-3.2	A	0.1	21
cis-[$(NH_3)_2PtCl_2$]	0.17-2.5	C[d]		
[(en)$PtCl_2$]	0.19-3.3	C[d]		
[(dien)PdCl]$^+$	0.26-1.25	C		

[a] Behavioral types are defined in the text and in the legend to Figure 2. [b] Fluorescence lifetime of ethidium in the presence of DNA and metal reagent at the upper r_f limit. τ_{Etd} in the presence of DNA alone is 21 ns. Standard deviations in these numbers at ±3 ns. [c] Buffer 2. [d] Type D behavior is observed following incubation for long time periods (see Figure 2d).

n is the maximum value of r_{Etd}, K_{Etd} and K_M are the intrinsic binding constants for Etd$^+$ and M, respectively, and c_M is the concentration of free metal complex. Using fluorescence to determine r_{Etd}, binding isotherms were determined in the presence of the metal complexes depicted in Figure 1 and the corresponding Scatchard plots constructed. Four classes of behavior could be distinguished. As shown in Figure 2a, competitive inhibition of ethidium binding (type A behavior) produces a Scatchard plot in which the slope decreases in the presence of increasing amounts of metal complex, with no change in the intercept on the abscissa (n). In type B behavior, illustrated in Figure 2b, both the slope and the intercept change. Two additional behavioral classes are those in which metal complexes lack effect on the fluorescence Scatchard plot of EtdBr (type C behavior, Figure 2c) and those that shift only the n value but not the slope (type D behavior, Figure 2d). The results for all the substances examined are summarized in Table I.

For metal compounds exhibiting type A behavior, K_M can be determined from a plot of K_{obsd}^{-1} vs. c_M, as in eq 2

$$K_{obsd} = K_{Etd}/(1 + K_M c_M) \quad (2)$$

provided that c_M is constant for a given ethidium binding isotherm. This condition was checked for type A complexes in the following manner. The binding of a metal complex to DNA in the presence of ethidium is expressed as eq 3

$$r_M/c_M = (n - r_M)[K_M/(1 + K_{Etd}c_{Etd})] \quad (3)$$

where r_M is the ratio of bound metal per nucleotide. Algebraic manipulation of eq 1 and 3 yields eq 4, where r_M is the only unknown.

$$(n - r_{Etd})K_{Etd}(c_{Etd}/r_{Etd}) - 1 = r_M[(1 + K_{Etd}c_{Etd})/(n - r_M)] \quad (4)$$

Determination of r_M by eq 4 for each data point in the titration enables the computation of c_M according to eq 5.

$$c_M = [M]_{tot} - r_M P_0 \quad (5)$$

Since the two sides of eq 4 are equal to the product $K_M c_M$, the value of K_M for each point may also be calculated. Table II

TABLE II: Sample Calculation of Parameters for Fluorescence Scatchard Plot of Calf-Thymus DNA with Ethidium in the Presence of $[(terpy)Pt(cys)]^+$, $r_f = 0.66$.

$[Etd]_{tot}$ $\times 10^6$ (M)	c_{Etd} $\times 10^6$ (M)	r_{Etd}	r_{Etd}/c_{Etd} $\times 10^{-3}$ (M^{-1})	$[Pt]_{tot}$ $\times 10^6$ (M)	c_{Pt} $\times 10^6$ (M)	K_{Pt} $\times 10^4$ (M^{-1})
2.213	1.765	0.059	33.2	5.018	4.73	7.6
3.308	2.723	0.077	28.2	5.000	4.73	8.4
4.395	3.696	0.092	24.9	4.982	4.75	8.2
5.474	4.692	0.103	22.0	4.964	4.75	8.6
6.545	5.704	0.112	19.6	4.946	4.74	9.6
7.609	6.699	0.121	18.1	4.928	4.76	8.5
8.665	7.703	0.128	16.7	4.911	4.76	8.1
10.75	9.720	0.139	14.3	4.876	4.75	7.9
12.81	11.74	0.145	12.3	4.842	4.71	9.4
14.85	13.73	0.153	11.1	4.808	4.71	8.0

TABLE III: Winding of PM-2 I_0 DNA by Metal Complexes.

Reagent	Concn $\mu g/ml$[a]	$s(I_0)/s(II)$	Method S,[b] Winding	Method G,[c] Winding
None		1.000		
$[(terpy)Pt(HET)]^+$	7.0	1.397	+	+
$[(terpy)Pt(AET)]^{2+}$	9.9	1.313	+	NA
$[(terpy)Pt(cys)]^+$	8.3	1.319	+	+
$[(terpy)Pt(CMT)]^+$	8.8	1.314	+	NA
$1[(terpy)PtCl]^+$	6.6	1.340	+	NA
$[(terpy)PdCl]^+$	5.8	1.373	+	NA
$[(bipy)Pt(en)]^{2+}$	43.4	1.249	+	+
$[(bipy)PtCl_2]$	Sat.	1.390	+	NA
$[(o\text{-phen})PtCl_2]$	Sat.	1.155	±	±
$cis\text{-}[(NH_3)_2PtCl_2]$	3.9	1.000	−	−
$[(en)PtCl_2]$	4.0	1.000	−	−
$[(dien)PdCl]^+$	3.6	1.000	−	−

[a] All are 11-13 μM, $r_f \sim 0.84$, except for the bipy and *o*-phen derivatives. Sat., saturated. [b] Sedimentation solvent 0.2 M NaCl, 50% D_2O, 20 °C. [c] Buffer 5, 25 °C, cylinder gels; NA, not attempted.

presents sample calculations of this kind for the titration of DNA with EtdBr in the presence of $[(terpy)Pt(cys)]^+$. Values for c_M are seen to be constant to within 3% for each binding isotherm. The binding constants, K_M, determined by this method agree with the values obtained graphically from eq 2 to within experimental error.

Binding constants for compounds exhibiting type B behavior were estimated for the binding isotherms having a common intercept on the abscissa (Figure 2b). These K_M values are also included in Table I.

It is possible that the relative quenching of the fluorescence of bound Etd^+ by a metal complex takes place by a mechanism not involving the blocking of intercalative binding sites. This possibility was of special concern for the $[(terpy)Pt(SR)]^{n+}$ compounds since these latter substances have electronic absorption bands in the visible region of the spectrum. In order to evaluate this possibility, the fluorescence lifetime (τ) of ethidium bound to DNA was measured in the presence of each complex. The results, included in Table I, showed no significant effect. Moreover, none of the metal complexes exhibited any measurable fluorescence in the presence of DNA under the experimental conditions employed, and none quenched the fluorescence intensity of free ethidium.

The fluorescence parameters k_b and k_0 (LePecq and Paoletti, 1967) and the Scatchard binding isotherms were determined by a least-squares fit using an IBM 360-91 computer. The value of k_b was found to be constant over the course of an experiment, so that no modification of the basic equations (Hinton and Bode, 1975) was required.

Unless otherwise noted, all fluorescence competition experiments were carried out in buffer 3. Since Tris is known to ligate transition metal ions (Allen et al., 1967), a control experiment was performed in 1 mM phosphate, 0.2 M NaCl, pH 7.5. For $[(terpy)Pt(HET)]^+$, as well as for all compounds exhibiting type C behavior, the results were indistinguishable from those shown in Table I and Figure 2.

Assay for Duplex Winding. The ability of a given metal complex to alter the DNA duplex winding angle was determined by two methods. In method S, a mixture of PM-2 DNAs I_0 (closed in 0.2 M NaCl at 37 °C) and II was sedimented at 20 °C through a solvent consisting of 0.2 M NaCl, 50% D_2O, and the test reagent. As had been shown previously in the case of $[(terpy)Pt(HET)]^+$ (Jennette et al., 1974), DNAs I_0 and II separate under such conditions only if the test reagent alters the DNA duplex winding. In method G, a mixture of PM-2 DNAs I_0 (closed in buffer 4 at 25 °C) and II was layered onto a 25-mm, 1% agarose cylinder gel that had been prepared with buffer containing the indicated concentration of test reagent. No test reagent was added to either reservoir, but electrophoresis was terminated before the advancing DNA bands encountered the retreating (in the case of cationic metal complexes) reagent front (Espejo and Lebowitz, 1976). Electrophoresis for 3 h at 8 V/cm was found to be adequate for this purpose. Experiments in which EtdBr was added to a

gel at a concentration of 12 μM established that complete separation of DNAs I_0 and II occurs under these conditions, whereas the two components comigrate in the absence of this dye.

The results obtained with both methods are shown in Table III. In all cases the two methods provide consistent indications of winding. Method G, however, has the advantages of greater simplicity and, according to preliminary indications, of higher sensitivity. In Table III, a value of 1.0 for the sedimentation coefficient ratio, $s(I_0)/s(II)$, indicates that DNAs I_0 and II do not separate in the presence of the indicated concentration of the metal complex. The sedimentation coefficient of negatively supercoiled PM-2 DNA varies in a complex way with the extent of supercoiling (Upholt et al., 1971). A systematic study of the variation of the sedimentation coefficient ratio with reagent bound would therefore be required to compare the molar unwinding angles of these complexes. In general, however, a sedimentation coefficient ratio in excess of 1.3 may be taken as a clear indication that the reagent unwinds the DNA. Lower values, lying between 1.1 and 1.3, probably indicate that the reagent unwinds the DNA but that, owing to the limited solubility of the compound in this relatively high salt buffer, a comparatively small extent of binding has taken place.

Gel Assay for Covalent Interactions of Metal Complexes with PM-2 DNA. Solutions of PM-2 DNA containing approximately 90% closed circular molecules were incubated with various metal complexes, all at values of $r_f \sim 0.84$, and aliquots were withdrawn for analysis by agarose gel electrophoresis. Photographs of representative cylinder gels, following incubation of a mixture of PM-2 DNAs I and II in buffer 3 at 23 °C, are displayed in Figure 3A after 24 h and in Figure 3B after 160 h, respectively. Two reagents, $[(terpy)Pt(HET)]^+$ and $[(dien)PdCl]^+$, produce no alteration in the mobility of either the rapidly (I), intermediate (III), or slowly (II) migrating PM-2 DNAs. (The linear DNA III was present as a minor contaminant in this preparation and is of undetermined origin.) Incubation with $[(en)PtCl_2]$ results in the transfer of all closed DNA to the nicked band after 24 h, with little or no accompanying alteration in the mobility of the linear species. The complex *cis*-$[(NH_3)_2PtCl_2]$ clearly exhibits covalent binding to DNA I after 24 h, as shown by the greatly reduced mobility of this band. This reaction does not appear to be a simple chain scission, however, since the closed band does not comigrate with the still slower nicked species (II). Following 160 h incubation, extensive nicking has apparently occurred, and Figure 3B shows complete transfer of the closed to the nicked band in the case of this reagent. The complex $[(terpy)PtCl]^+$ shows similar covalent binding to the closed DNA, but here the reduction in mobility occurs over a much longer time interval. Data recorded at still later times show that this compound, too, probably causes DNA chain scissions.

In the absence of precise knowledge of the reagent binding constants under these experimental conditions, it is not possible to calculate the extent of binding to DNA I (r_f values were always in the range 1–2 during incubation). For those compounds showing types A, B, or D behavior, it might be anticipated that some EtdBr binding sites would be blocked, reducing the extent of staining of the corresponding electrophoretic bands with this latter dye. Such an effect would bring about an apparent loss of DNA as measured by the procedure used here (see Materials and Methods). Experimentally, such an apparent loss of material was observed for two reagents only: $[(en)PtCl_2]$ and *cis*-$[(NH_3)_2PtCl_2]$. In both cases the apparent amount of material migrating as closed DNA was

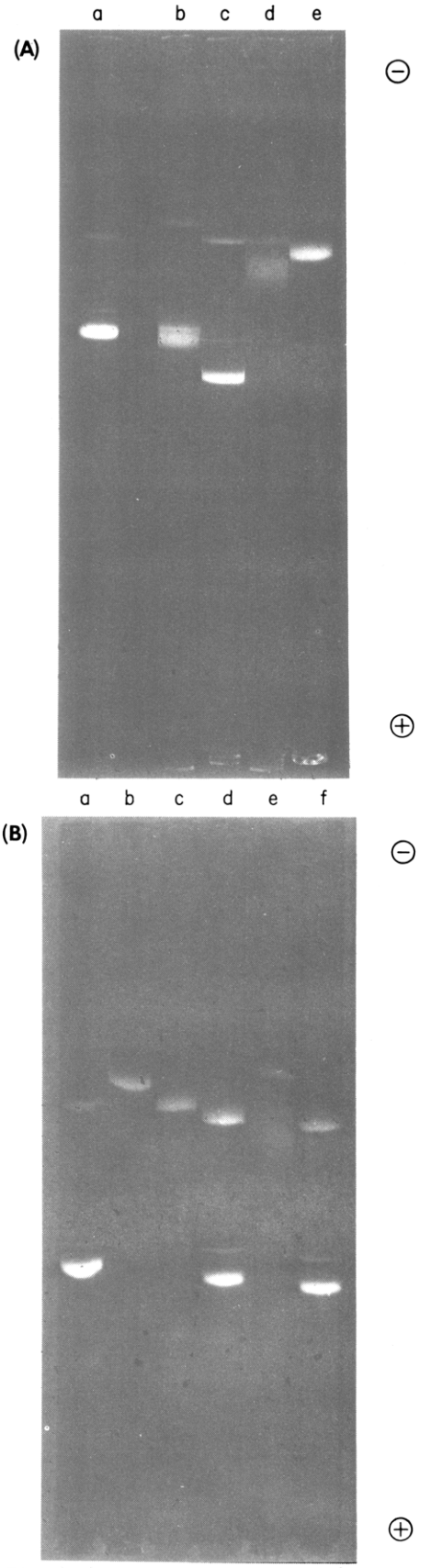

FIGURE 3: Electrophoresis in 1% agarose gels of PM-2 DNAs following incubation with selected metal complexes at an $r_f \sim 0.84$ in buffer 3 at 23 °C. (A) Twenty-four-hour incubation with: (a) no metal complexes, electrophoresis for 2 h at 100 V, employing samples of 20 μl volume containing 0.1 μg of DNA; (b) $[(terpy)PtCl]^+$; (c) $[(dien)PdCl]^+$; (d) *cis*-$[(NH_3)_2PtCl_2]$; and (e) $[(en)PtCl_2]$. In b–e electrophoresis was for 3 h at 100 V. (B) Incubation (160 h) with: (a) $[(terpy)Pt(HET)]^+$; (b) $[(en)PtCl_2]$; (c) *cis*-$[(NH_3)_2PtCl_2]$; (d) $[(dien)PdCl]^+$; (e) $[(terpy)PtCl]^+$; and (f) no metal complex. Electrophoresis was for 4 h at 100 V.

TABLE IV: Interactions of PM-2 DNAs with Metal Complexes as Assayed by Agarose Gel Electrophoresis.[a]

Complex	Covalent Binding				Nicking[e]		
	I[b]	I[c]	I_0[b]	II[d]	I[b]	I[c]	I_0[b]
[(terpy)Pt(HET)]$^+$	−	−	−	−	−	−	−
[(terpy)PtCl]$^+$	+	+	−	−	+	+	+
[(terpy)PdCl]$^+$	NA	+	NA	NA	NA	−	NA
[(dien)PdCl]$^+$	−	−	−	−	−	±	−
[(en)$PtCl_2$]	+	+	+	−	+	+	+
cis-[$(NH_3)_2PtCl_2$]	+	+	−	−	+	+	+
trans-[$(NH_3)_2PtCl_2$]	NA	−	NA	NA	NA	−	NA
[(bipy)Pt(en)]$^{2+}$	NA	−	NA	NA	NA	−	NA
[(*o*-phen)$PtCl_2$]	NA	−	NA	NA	NA	±	NA

[a] The DNA concentration was 15.2 μM nucleotide, and $r_f \sim 0.84$. NA, not attempted. [b] Buffer 3, 20 °C, 140-h incubation. [c] Buffer 4, 23 °C, 130-h incubation. [d] Identical results in both buffer systems. [e] Conversion to II at a rate in excess of that observed for reagent-free DNA.

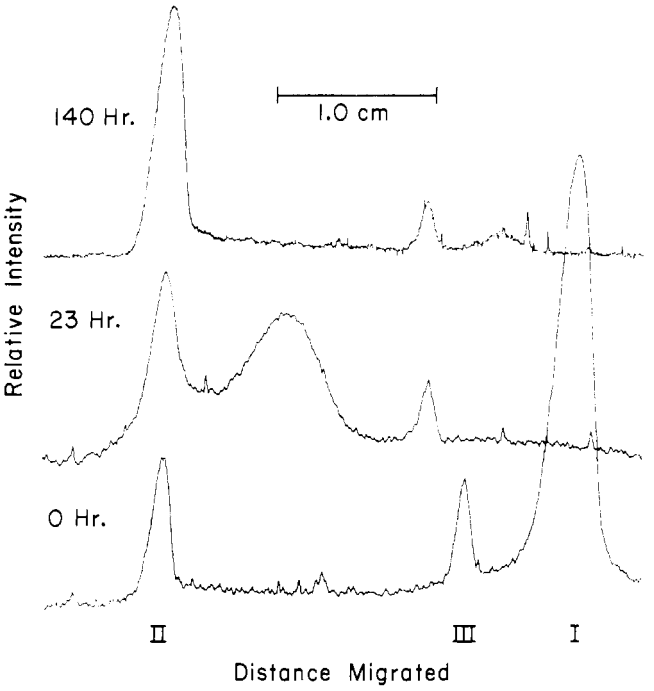

FIGURE 4: Microdensitometer traces of electrophoresis gels of PM-2 DNAs stained with EtdBr, following incubation with *cis*-[$(NH_3)_2PtCl_2$] under conditions given in the legend of Figure 3.

reduced by about 50% after 24 h incubation under the conditions of Table IV. We cannot definitively establish at present whether this apparent loss of DNA results from inhibition of EtdBr staining or from some other phenomenon.

Figure 4 presents microdensitometer scans of EtdBr-stained gels following 0, 23, and 140 h incubation of the same PM-2 DNA sample (Figure 3) with *cis*-[$(NH_3)_2PtCl_2$]. These scans illustrate (i) the progressive loss with increasing incubation time of material migrating with the mobility of native closed DNA; (ii) the appearance of a relatively broad band at intermediate times, containing material which migrates between the closed and nicked bands; and (iii) the eventual transfer of most of the originally closed DNA to the nicked band. The relatively large width of the intermediate band suggests, on this time scale, some heterogeneity in binding among the population of DNA molecules.

A series of experiments similar to the above was conducted with both native (I) and relaxed (I_0) closed PM-2 DNAs in buffers 3 and 4, the results of which are summarized in Table IV. No evidence for covalent binding of any of the metal complexes with nicked DNAs was detected, as defined by the absence of altered mobility in the gel electrophoresis experiments. This result does not, however, rule out the possibility of covalent binding at relatively low levels. It should be noted, moreover, that the experiments were performed at an r_f of 0.84. Since less than 10% of the DNA is present as II, preferential binding to the closed circles could account for this result. In the case of [(terpy)Pt(HET)]$^+$, as reported earlier (Bond et al., 1975), no nicking or covalent reaction was observed even following very long incubation times. A similar result was obtained for [(bipy)Pt(en)]$^{2+}$, a complex having two chelate rings and not expected to react covalently with the bases. The occurrence of nicking did not appear to depend upon the presence of supercoiling, but the extent of covalent binding was clearly greater (at constant time) for a twisted than for a relaxed closed circular DNA.

Discussion

Intercalative Binding. Platinum and palladium complexes having planar aromatic ligands terpy, *o*-phen, or bipy all competitively inhibit the binding of ethidium to calf-thymus DNA (Figure 2, Table I). These type A complexes also wind PM-2 I_0 DNA, as assayed either by the sedimentation velocity (S) or gel electrophoresis (G) methods (Table III). Such behavior is strongly indicative of intercalation (Jennette et al., 1974). The relatively low solubility of [(*o*-phen)$PtCl_2$] in these solvents hampered study of its binding to DNA, but recent measurements on the more soluble [(*o*-phen)Pt(en)]$^{2+}$ complex clearly established intercalative binding (Lippard et al., 1976). By contrast, complexes in which the ligands lack aromaticity do not bind to DNA by an intercalative mechanism (Tables I and III). Included in this category are the type C complexes *cis*-[$(NH_3)_2PtCl_2$] and [(en)$PtCl_2$], both antitumor drugs. We therefore conclude that intercalation is not a necessary prerequisite for antitumor activity. The fluorescence and gel electrophoresis studies have also identified a third category, the type B complexes, that exhibit both intercalative and covalent interactions with DNA. These complexes all contain a relatively good leaving group (chloride or acetate ion) in the buffers employed. This feature would allow binding of the metal to donor sites on the bases, most likely to the N-7 ring nitrogen of purines.

For complexes showing type A or type B behavior, it is possible to evaluate the apparent DNA binding constants. The results, listed in Table I, suggest that the doubly charged [(terpy)Pt(AET)]$^{2+}$ metallointercalator forms the most stable

adduct with DNA and that the $[(bipy)Pt(en)]^{2+}$ complex, having only two pyridine rings and nonpolar ligands, forms the least stable adduct. The winding results presented in Table III lead to a similar conclusion. Although probably indicative of the relative binding strengths, the K_M values reported in Table I may not correspond to intrinsic binding constants. Since the binding to DNA of both Etd^+ and of $[(terpy)Pt(HET)]^+$ is known (Bauer and Vinograd, 1970; Bond et al., 1975) to be governed by the nearest neighbor exclusion principle (Cairns, 1962), the Scatchard equation used to plot the fluorescence data is inadequate (Crothers, 1968). Moreover, although fluorescence lifetime measurements suggest that Förster type energy transfer does not occur, metal complex quenching of ethidium fluorescence by some mechanism other than occupancy of intercalation sites cannot be absolutely ruled out. Thus, the fluorescence inhibition plots (Figure 2) should be taken only as a useful and qualitative, perhaps semiquantitative, indication of the mode of metal complex binding. Intrinsic binding constants must be evaluated by alternative methods.

Covalent Binding and Nicking. Although the relatively high chloride concentration in the buffers used in most of these studies reduces the covalent reaction rates of all compounds investigated, incubation for long time intervals and/or use of excess reagent enabled this mode of binding to be detected. The noncompetitive inhibition of Etd^+-associated fluorescence by high concentrations of $[(terpy)PtCl]^+$ in the presence of calf-thymus DNA was observed previously and interpreted in terms of covalent binding to the bases (Jennette et al., 1974). The present results support this interpretation. The metal complex $[(terpy)PdCl]^+$ similarly exhibits class B behavior. By contrast, $[(dien)PdCl]^+$ had no apparent effect on ethidium binding. It therefore appears that intercalation can facilitate the covalent binding of the appropriate metal complexes to DNA. Although the antitumor drugs *cis*-$[(NH_3)_2PtCl_2]$ and $[(en)PtCl_2]$ exhibited class C behavior under standard experimental conditions, incubation for long time intervals with DNA prior to titration with ethidium produced a systematic reduction in the abscissa intercept of the fluorescence Scatchard plots (Figure 2d). The fact that the slopes of the lines remain unaltered may be interpreted in the framework of the Scatchard model to indicate that these nonintercalating class D complexes can block intercalation sites for ethidium. Such blockage might occur by covalent binding to the bases with disruption of the DNA duplex. Since the titrations are carried out in buffer containing 0.2 M NaCl, the complexes are in the chloro, rather than the aquo, form. The rate of substitution is therefore relatively slow. The ability of the terpyridine ligand to labilize chloride ion (Basolo et al., 1960) and/or to effect intercalation accounts for the more rapid covalent binding of $[(terpy)PtCl]^+$ and $[(terpy)PdCl]^+$ in chloride-containing buffers.

The results with PM-2 DNAs show that some metal complexes exhibit one or both of two additional modes of interaction that appear to be independent of intercalation: covalent binding without chain scission and nicking (conversion of closed to open DNAs). As shown in Figure 4 for the reaction of *cis*-$[(NH_3)_2PtCl_2]$ with PM-2 DNA I, covalent binding clearly precedes nicking, at least for this reagent. Since tightly wound superhelical DNA probably contains duplex regions that are relatively destabilized (Bauer and Vinograd, 1970; Dean and Lebowitz, 1971; Jacob et al., 1974; Bartok and Denhardt, 1976), the initial sites of covalent binding might be associated with these regions of the molecule. As more platinum-base binding occurs, the DNA becomes increasingly susceptible to backbone chain scissions. The data represented in Table IV illustrate analogous results for the interactions of $[(terpy)PtCl]^+$ and $[(en)PtCl_2]$ with DNA I. In the case of DNA I_0, nicking was observed for all three reagents, even though covalent binding was detected in buffer 3 only in the case of $[(en)PtCl_2]$. This result reflects a reduced rate of binding to DNA I_0 compared with I in the presence of a constant rate of nicking. It should be noted that the assay employed is much more sensitive to the detection of nicking than of covalent binding of reagent. Covalent binding of $[(dien)PdCl]^+$ to PM-2 DNAs either does not occur or is reversed under the electrophoresis conditions employed in the gel assay. Reversal would be consistent with the expected kinetic lability of the covalent bonds of palladium to the bases. It is clear, however, that no nicking occurs in this case. The kinetically inert, class A metallointercalators $[(terpy)Pt(HET)]^+$ and $[(bipy)Pt(en)]^{2+}$ show no interactions under the conditions of Table IV. These results are in complete accord with those of the fluorescence inhibition experiments.

The data of Table IV provide no indication that the antitumor drugs *cis*-$[(NH_3)_2PtCl_2]$ and $[(en)PtCl_2]$ bind covalently to nicked DNA, at least in the presence of a tenfold excess of closed DNA and at an $r_f \sim 0.84$. The occurrence in DNA I of locally destabilized duplex region(s) (see next section) would permit intrastrand cross-linking with these cis-substitutionally labile platinum drugs. The coordination of donor atoms on exposed bases to square platinum(II) complexes would further destabilize the duplex. Intrastrand cross-links would be very effective at disrupting ethidium intercalation sites and could account for the observed loss in gel electrophoresis of ethidium stained DNA in the presence of covalently bound *cis*-$[(NH_3)_2PtCl_2]$ and $[(en)PtCl_2]$. It is noteworthy that the *trans*-$[(NH_3)_2PtCl_2]$ complex does not bind covalently to DNA I and produces no chain scissions. This complex is not an active antitumor drug and, for geometric reasons, is less likely to coordinate to adjacent bases on the same strand.

Interaction of Drugs with Closed DNAs. The experiments presented here demonstrate that superhelical DNA is especially useful for detecting a variety of modes of binding of metal complexes to DNA. Although we have not conducted a systematic investigation of the variation of binding type and affinity with superhelix density, a comparison of the results with native I and relaxed I_0 closed PM-2 DNAs suggests that the relatively enhanced binding of most reagents depends upon the presence of at least some topological underwinding. PM-2 DNA I, with $^{24}\sigma_0 = -0.106$, is the most highly supercoiled naturally occurring DNA that has yet been reported. It has been suggested (Dean and Lebowitz, 1971; Kato et al., 1973; Jacob et al., 1974) that DNAs of high superhelix density contain altered duplex regions, possibly of a hairpin structure. More recent experiments employing the *Neurospora crassa* single-strand specific endonuclease (Bartok and Denhardt, 1976) show that if any such regions exist they must be essentially randomly distributed, at least in ϕX174 RF DNA. It is likely that closed DNA of high superhelix density contains either potential or actual regions of lowered duplex winding that at least partially relieve torsional stress on the duplex, as might be expected from free energy considerations. A reduced local duplex winding would expose donor sites on the bases that are normally involved in base pairing for covalent reaction with the metal complexes employed in the present study. This mechanism might be generally applicable for the covalent binding of platinum drugs to internal base sites on double-stranded DNA. Such binding could be both cooperative and a strong function of the extent of supercoiling.

Acknowledgment

We thank Engelhard Industries for a loan of K_2PtCl_4, R. Fairclough and Dr. G. Schuster for assistance with fluorescence measurements, and J. Gruskin, J. Lehmann, and M. Pleatman for technical help.

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