Proc. Nat. Acad. Sci. USA
Vol. 71, No. 10, pp. 3839–3843, October 1974

Metallointercalation Reagents. 2-Hydroxyethanethiolato(2,2′,2″-terpyridine)-platinum(II) Monocation Binds Strongly to DNA By Intercalation

(metal binding/supercoiled DNA/viscosity/thermal denaturation/fluorescence)

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Communicated by Ronald Breslow, June 26, 1974

ABSTRACT **The red, cationic complex 2-hydroxyethanethiolato(2,2′,2″-terpyridine)platinum(II), [(terpy)-Pt(SCH₂CH₂OH)]⁺, binds strongly to DNA by a mechanism involving intercalation. By means of fluorescence spectroscopy, the platinum complex was shown to inhibit competitively the binding of the intercalating dye ethidium bromide to calf thymus DNA. This platinum complex increases the viscosity of calf thymus DNA, raises the melting temperature by up to 5°, and exhibits induced circular dichroism when bound to the DNA. The closed circular viral DNA from bacteriophage PM2 is unwound by the complex in a manner that is similar to that of ethidium bromide and with an unwinding angle that appears to be slightly less than that of ethidium. Studies of the related complex [(terpy)PtCl]⁺, which has a substitutionally more labile chloride ligand, suggest that it also intercalates, especially at [DNA-P]:[Pt] ratios greater than 2. The potential utility of these new metallointercalation reagents as heavy atom probes in fiber diffraction or electron microscopic studies of the interaction process is discussed.**

There have been extensive studies of the binding of metals to DNA, RNA, and their constituents (1–3). In an attempt to achieve base specificity, several complexes of the polarizable class b (or "soft") metals (4, 5) were used with the aim of providing fluorescent or heavy metal electron microscope labels for thiolated bases (6). During the course of this work, [(terpy)-PtCl]⁺, where terpy is 2,2′,2″-terpyridine, (PtTC, Fig. 1) was found to shift the 335-nm band of 4-thiouridine in native *Escherichia coli* tRNA (K. W. Jennette, unpublished results), conditions under which the sterically less cumbersome mercury complex, *p*-chloromercuribenzoate (PCMB), does not bind (6). In view of the remarkable similarity between the molecular geometries of the PtTC cation and the acridine dyes, the result was rationalized by proposing that intercalation of the platinum complex sufficiently disturbed the tertiary structure of the tRNA in the region of the 4-thiouridine to permit the formation of a Pt-S bond.

Intercalation as a mode of binding was first introduced by Lerman (7) to explain the stable complex formed between DNA and planar aromatic dyes. Dyes that intercalate usually show spectral shifts, exhibit induced circular dichroism spectra, increase the viscosity and reduce the sedimentation coefficient of the DNA, alter the extent of supercoiling in closed circular duplex DNA, and increase the length of the DNA (8). Experiments of this kind were, therefore, carried out on PtTC to determine whether it binds by an intercalative mode to DNA.§ Although preliminary studies suggested intercalation, complications (*vide infra*) were introduced by the substitutionally labile chloride ion in the complex, which may permit covalent binding to the DNA. This problem was largely circumvented by the design and synthesis of the related cation, [(terpy)Pt(SCH₂CH₂OH)]⁺ (PtTS, Fig. 1), in which the rate of substitution of the Pt-S bond is expected to be slower (9) than the Pt-Cl bond in PtTC. The present report describes several lines of evidence that PtTS binds strongly to DNA by intercalation.

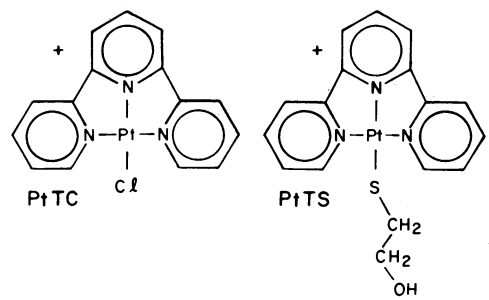

FIG. 1. The structures of chloroterpyridineplatinum(II), PtTC, and 2-hydroxyethanethiolatoterpyridineplatinum(II), Pt-TS. The ultraviolet (UV)-visible spectral maxima and their molar extinction coefficients ±2% (M^{-1} cm^{-1}, in parentheses) are, for PtTC in buffer 2: 343 (11,300), 327 (12,600), 278 (25,100), and 248 (28,800) nm, and for PtTS in buffer 3, 475 (875), 342 (12,500), 327 (10,700), 311 (10,500), 278 (19,300), and 242 (28,700) nm. These values apply to concentrations of 15 μM or less. Above this concentration, both compounds deviate from Beer's law, and may associate in solution. PtTC shows spectral changes with the chloride content of the buffer.

MATERIALS AND METHODS

Unless stated otherwise, all experiments were carried out in: (*a*) Buffer 1, 1 mM sodium phosphate, pH 6.8, 3 mM sodium chloride; (*b*) Buffer 2, 50 mM Tris·HCl, pH 7.5, 0.1 M sodium chloride; or (*c*) Buffer 3, 50 mM Tris·HCl, pH 7.5, 0.2 M sodium chloride.

Ethidium bromide (EthBr) hemihydrate was purchased from Sigma Chemical Co., and its purity was checked by thin-layer chromatography [ε at 480 nm, 5570 M^{-1} cm^{-1}; literature (10), 5600 M^{-1} cm^{-1} in pure water]. Calf thymus

Abbreviations: terpy, 2,2′,2″-terpyridine; PtTS, [(terpy)Pt-(SCH₂CH₂OH)]⁺; PtTC, [(terpy)PtCl]⁺; EthBr, ethidium bromide; UV, ultraviolet; T_m, melting temperature.

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§ The affinity of square platinum(II) complexes such as $Pt(NH_3)_2Cl_2$ for DNA has been postulated by Williams (R.J.P. Williams in *Coordination Complexes in Cancer Chemotherapy*, summaries of papers presented at the VII International Chemotherapy Congress, Prague, 1971, p. 18) to involve intercalation as the intermediate of attack.

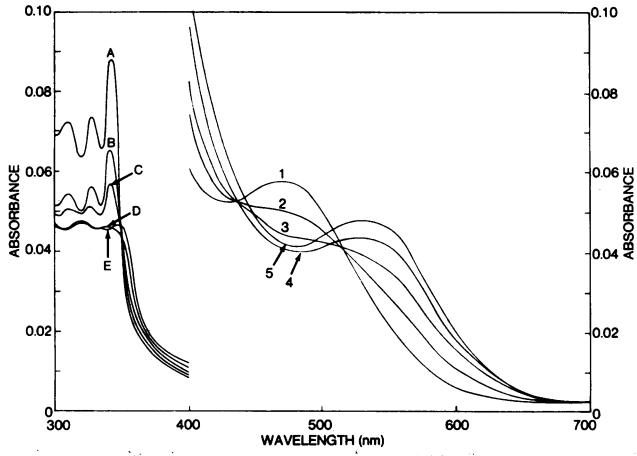

FIG. 2. Electronic spectra of PtTS in the absence and in the presence of various amounts of calf thymus DNA in buffer 1. In curves A through E the concentration of PtTS is 6.97 μM and the DNA-P concentrations are 0 (A), 17 μM (B), 34 μM (C), 146 μM (D), and 303 μM (E). In curves 1 through 5 the concentration of PtTS is 70.4 μM and the DNA-P concentrations are 0 (1), 97.7 μM (2), 189 μM (3), 356 μM (4), and 700 μM (5).

DNA purchased from Sigma (type I) was dissolved in buffer 2 in the presence of ethylenediaminetetraacetate (EDTA), centrifuged, precipitated with two volumes of ethanol at 0°, then redissolved and dialyzed into the desired buffer. The A_{260}/A_{280} ratio of 1.9 indicated that the DNA was reasonably free of protein contamination (11). Concentrations of DNA were computed by assuming that 20.2 A_{260} units = 1 mg/ml of DNA (12) and $\epsilon(P)_{260}$ = 6600 $M(P)^{-1}$ cm^{-1} (13). Growth and harvesting of the PM2 virus and extraction of the DNA were carried out as described by Espejo and Canelo (14, 15). Closed circular PM2 DNA of low superhelix density was prepared by incubating viral PM2 I DNA in the presence of an enzyme activity prepared from a crude extract of HeLa cells, and which was a gift from Dr. L. Grossman.

Complexes. $[(terpy)PtCl]Cl \cdot 2H_2O$ was synthesized according to the procedures of Morgan and Burstall (16) and Intille (17). $[(terpy)Pt(SCH_2CH_2OH)]NO_3$ was prepared from PtTC in aqueous solution by first converting the latter

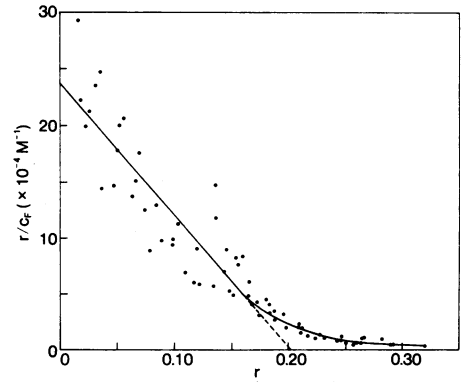

FIG. 3. Scatchard plot (λ = 342 nm) for PtTS binding to calf thymus DNA in buffer 1. The dye concentration ranged from 2.17 μM to 93.6 μM and the DNA-P concentration varied from 8.56 μM to 661 μM. The straight line drawn was determined by a least-squares fit of the data points for which $r < 0.17$. The apparent binding constant $K = 1.2 \pm 0.2 \times 10^6$, and $n = 0.20$. c_F is the concentration of free PtTS.

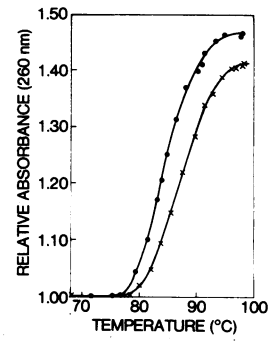

FIG. 4. Thermal denaturation of calf thymus DNA ([DNA-P] = 85 μM) in buffer 2 in the presence (×) and absence (●) of 3.5 μM PtTS.

to the nitrate by adding two equivalents of silver nitrate, then allowing the nitrate complex to react with a 2-fold excess of 2-mercaptoethanol under a nitrogen atmosphere. The red complex that formed was immediately precipitated out of solution with acetone. Analysis: Calculated weight percents for $[(terpy)Pt(SCH_2CH_2OH)](NO_3)$, $C_{17}H_{16}N_4O_4SPt$: C, 35.98; H, 2.84; N, 9.87; S, 5.65. Found: C, 35.84; H, 2.77; N, 9.77; S, 5.63. Infrared and proton nuclear magnetic resonance spectral studies of the PtTS cation are consistent with the structure shown in Fig. 1, in which the hydroxyethyl arm is not coordinated. Stock solutions of PtTC and PtTS were prepared in deionized, distilled water and used within 1 week.

Spectrophotometric Measurements were made with a Cary 17 or Cary 118C recording spectrophotometer. For titration experiments, aliquots of concentrated DNA stock solutions were dispensed through Corning disposable (0.5% accuracy) or Pederson Lang-Levy (1% accuracy) micropipets to a known volume of the PtTC or PtTS solution. The sample was maintained at 25°. Thermal denaturation studies were conducted in a Cary 17 spectrophotometer supplied with a thermostated cell holder. The temperature was measured with a thermocouple and Leeds and Northrup potentiometer. Circular dichroism measurements were made on a Jasco J-40 recording spectropolarimeter. Fluorescence was measured with a Perkin–Elmer MPF-3L spectrofluorimeter with the cell thermostated at 25°. Fluorescence lifetime measurements were performed on an instrument similar to that described by Tao (18).

Viscosity Measurements were carried out in duplicate with a Zimm–Crothers low-shear viscometer (Spinco Division of Beckman Instruments, Inc.) at 25°. Solutions were passed through 0.45 μm Millipore filters prior to use.

Ultracentrifugation experiments were conducted with a Beckman model E analytical ultracentrifuge equipped with photoelectric scanner, multiplex accessory, and mirror optics. All experiments were carried out at 20° in 0.2 M NaCl, 4 mM Tris·HCl, pH 7.5, with 50% D_2O added in band sedimentation experiments. The sedimentation coefficients reported here, s_{20}, refer to the sodium salt of the complexed or free DNA and are not corrected for solution density and viscosity.

RESULTS

Optical Properties of PtTS with DNA. When calf thymus DNA is added to a solution of PtTS in buffer 1, changes occur in the UV-visible spectra (Fig. 2). A Scatchard plot (19) of the

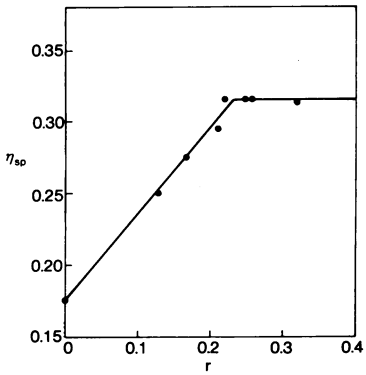

FIG. 5. Specific viscosity of calf thymus DNA at 25° as a function of r ([bound PtTS]/[DNA-P]) in buffer 1. The concentration of [DNA-P] was 75.5 μM and the total [PtTS] varied from 0 to 96.4 μM. Values for r were computed from the binding isotherm shown in Fig. 3 by the method described in ref. 35.

data (Fig. 3) shows curvature at large values of r (the ratio of bound dye to DNA-P), behavior typical for the binding of intercalating dyes to DNA, especially at low ionic strength (8). Analysis of the binding isotherm (20) shows $K = 1.2 \pm 0.2 \times 10^6\ M^{-1}$ ($n = 0.20$), where n is the apparent maximum number of bound dye molecules per nucleotide and K is the apparent association constant. At high ionic strength (buffer 3) the spectral changes are too small to provide meaningful data.

The binding of PtTC and PtTS to calf thymus DNA is reversible, as shown by the complete loss of the dye absorption bands in the 300- to 400-nm region (Fig. 2) after passing the DNA–dye complex through Bio-Rad cation exchange resin AG 50W-X8 or after dialysis against 2 M urea, 0.2 M magnesium sulfate, 0.1 M Tris·HCl, pH 8.0.

Circular dichroism spectra of 30–300 μM solutions of PtTS dissolved in buffer 1 or 3 in the presence of about 350 μM calf thymus DNA showed three positive bands at 352 nm, 420 nm, and about 530 nm.

Thermal Denaturation of DNA. The effect of PtTS on the melting temperature (T_m) of calf thymus DNA in buffer 2 is shown in Fig. 4. The T_m increases from 84.4° to 87.8° upon addition of the platinum complex, indicating stabilization of the duplex structure. Thermal denaturation studies in low salt (buffer 1), at DNA-P and PtTS concentrations identical to those cited in Fig. 4, showed T_m to increase by 5.0°, whereas ΔT_m was only about 2.5° in buffer 3 (it was not possible to observe the completion of the transition in this high salt buffer).

Viscosity Measurements. Both PtTS and PtTC increase the viscosity of calf thymus DNA. The specific viscosity, η_{sp}, of the DNA solution increases with increasing concentration of bound PtTS, and finally reaches a saturation value at r of about 0.23 (Fig. 5). Addition of PtTC increases the intrinsic viscosity of DNA in buffer 2 by a factor of 1.78. In 0.05 M NaCl, 0.05 M Tris·HCl, pH 7.5, there is a 2-fold increase in intrinsic viscosity with added PtTC.

Fluorescence Studies—Competition of PtTC and PtTS with Ethidium Bromide for Binding Sites on DNA. The fluorescence Scatchard plots for the binding of EthBr to calf thymus DNA in the presence of various concentrations of PtTS are shown in Fig. 6a. Control experiments showed that (*i*) no complex

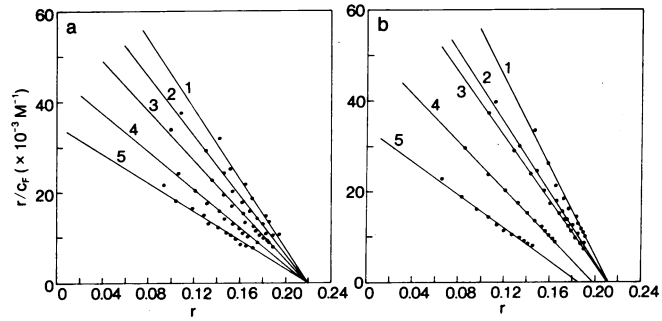

FIG. 6. (a) Fluorescence Scatchard plots of EthBr (concentration 4.9 to 19 μM) bound to calf thymus DNA ([DNA-P] = 3.5 μM) in buffer 3 (line 1) and in the presence of increasing concentrations of PtTS, [DNA-P]/[PtTS] = 4.5 (line 2), 1.8 (line 3), 0.90 (line 4), and 0.45 (line 5). (b) Fluorescence Scatchard plots of EthBr (concentration 5.2 to 20 μM) bound to calf thymus DNA ([DNA-P] = 5.8 μM) in buffer 2 (line 1) and in the presence of increasing concentrations of PtTC, [DNA-P]/[PtTC] = 5.2 (line 2), 2.6 (line 3), 1.0 (line 4), and 0.52 (line 5).

is formed (UV-spectral criterion) between PtTS and EthBr at the concentrations at which these studies were performed, (*ii*) PtTS does not quench the fluorescence of EthBr, (*iii*) PtTS does not itself show appreciable fluorescence in the spectral region studied, either free or bound to DNA, and (*iv*) bound PtTS does not quench bound EthBr, since the fluorescence life-time of bound EthBr was 22.3 ± 0.5 nsec in the absence and 21.8 ± 0.5 nsec in the presence of bound PtTS, under binding conditions similar to those used to obtain line 5 in Fig. 6a. The loss of DNA-bound EthBr fluorescence in the presence of PtTS can, therefore, be ascribed to inhibition of EthBr binding by the platinum complex. As seen from Fig. 6a, the effective number of binding sites, n, remains the same (0.22) but the effective association constant, K', is decreased in the presence of PtTS, i.e., there is competitive inhibition. Treatment of the data (21) gave binding constants of $3.9 \pm 0.3 \times 10^5\ M^{-1}$ for EthBr and $2.0 \pm 0.4 \times 10^5\ M^{-1}$ for PtTS in buffer 3. A literature value for the binding of EthBr to calf thymus DNA, extrapolated to the same ionic strength as buffer 3, is $3.4 \times 10^5\ M^{-1}$ at 23° (21).

The Scatchard plot for the binding of EthBr to DNA in the presence of PtTC is shown in Fig. 6b. Competitive inhibition is seen for [DNA-P]:[PtTC] ratios > 2. When the platinum concentration is increased beyond this point, the inhibition is noncompetitive, with both n and K' decreasing in value. In the competitive range with buffer 2, the apparent binding constants are $5.1 \pm 0.2 \times 10^5\ M^{-1}$ for EthBr [literature (21) = $5.8 \times 10^5\ M^{-1}$] and $2.3 \pm 0.5 \times 10^5\ M^{-1}$ for PtTC.

Effect of PtTS on Closed-Circular DNA. Closed circular duplex DNA is characterized by the requirement that the total winding of the two strands about one another remain constant in the absence of a chain scission. This requirement may be expressed by the relationship $\alpha = \beta + \tau$, where β is the number of duplex turns, τ is the number of tertiary turns, and α (a constant) is the net interstrand winding (22). Any process, such as intercalative unwinding, which changes the duplex winding in a closed DNA, therefore, alters the number of superhelical turns according to $\Delta\tau = -\Delta\beta$. A relaxed closed DNA ($\tau = 0$) is expected to cosediment with the corresponding nicked circle, which is not subject to the topological constraint. The addition of a duplex unwinding

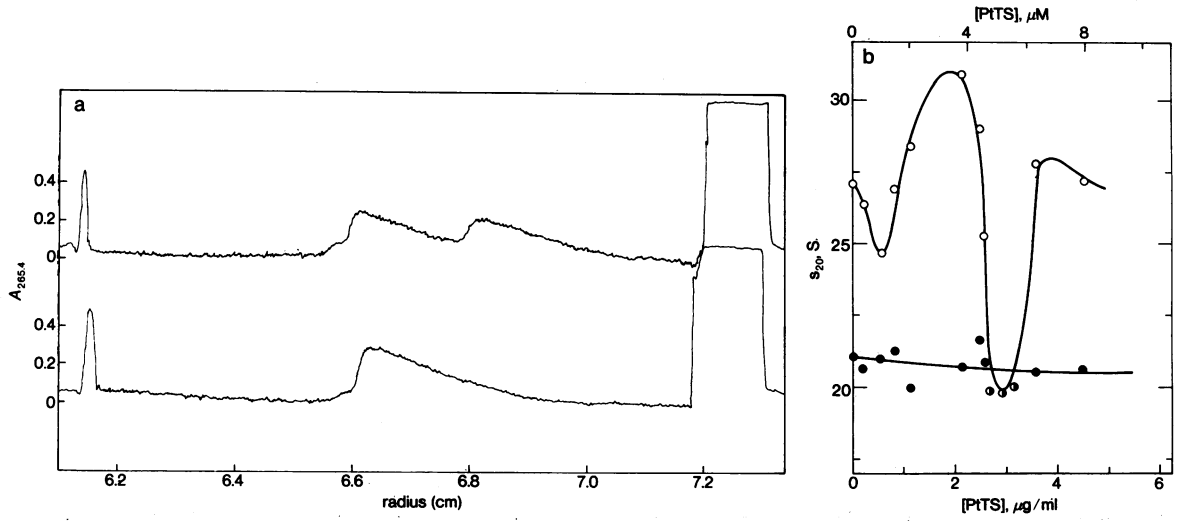

FIG. 7. (a) The band sedimentation behavior of a mixture of 0.4 μg each of closed ($\tau = 0$) and nicked circular PM2 DNAs in the presence (upper tracing) and absence (lower tracing) of 13 μM PtTS. The photoelectric scanner records were made 57 min (upper) and 55 min (lower) after the attainment of full speed, 32,000 rpm, in 50% D_2O, 0.2 M NaCl, 4 mM Tris·HCl, pH 7.5, at 20°. The skewed shape of the bands is due to the presence of a small dependence of sedimentation upon concentration in this solvent system. The small amount of trailing material, present in both samples, represents an uncharacterized impurity. (b) The variation of uncorrected sedimentation coefficient, s_{20}, of PM2 DNAs I (○) and II (●) and a mixture (◑) at 20° in 0.2 M NaCl, 4 mM Tris·HCl, pH 7.5. Boundary sedimentation was used, with a rotor speed of 32,000 rpm, and with an initial DNA concentration of 2.39 μg/ml for each component. The PtTS concentration represents c_T, the formal amount added per unit volume. The bound and free dye concentrations at the principal minimum are approximately 1.3 μM and 3.8 μM, respectively.

reagent results in a reduction in β and, therefore, a twisting of the closed molecule with a consequent increase in sedimentation coefficient.

Fig. 7a shows the results of the addition of PtTS to a mixture of relaxed closed ($\tau = 0$) and nicked circular PM2 DNAs. The molecules cosediment in the absence of the metal complex, whereas the ratio of sedimentation coefficients is 1.31 in the presence of 13 μM PtTS. Alteration of the duplex winding by PtTS was investigated in greater detail using a mixture of nicked (form II) and closed (form I) circular PM2 DNAs, Fig. 7b. The sedimentation coefficient of PM2 DNA (I) first decreases to a secondary minimum (1.0 μM), increases to maximum (3.4 μM), passes through a primary minimum (5.1 μM) and finally increases to a sloping plateau (>6 μM). This curve is very similar in shape to that produced by the binding of EthBr to the same DNA (23). The addition of PtTS has very little effect upon the sedimentation coefficient of PM2 DNA (II), as is also the case for EthBr. No conversion of I to II was observed during these experiments, indicating that PtTS does not produce chain scissions.

DISCUSSION

Studies of the equilibrium binding of PtTS to calf thymus DNA by observing the competitive inhibition of EthBr fluorescence in buffer 3 are consistent with an intercalative mode of binding, which has already been demonstrated for ethidium (8, 10, 21). The apparent association constant is quite close to the corresponding value for EthBr. Since the inhibition is competitive, it is reasonable to assume that n is also 0.22 for PtTS. This value is in accord with the corresponding estimate of $n = 0.20$ obtained spectrophotometrically in buffer 1 (Fig. 3). In the case of EthBr, the value of the maximum site ratio appears to be independent of ionic strength (21). The fact that PtTS exhibits an induced circular dichroism spectrum in the presence of calf thymus DNA is also consistent with intercalative binding.

As shown in Fig. 4, PtTS increases the melting temperature of calf thymus DNA in buffer 2. Application of a statistical thermodynamic model (ref. 24, Eq. 30) to the data of Fig. 4 predicts a value of 0.055 for $r_{helix} - r_{coil}$, the difference between the extent of binding for helix and coil. If one assumes that all of the PtTS is bound (K for buffer 2 is not yet available), r would be 0.041. A similar calculation for data in buffer 3 having $r = 0.032$ gave $r_{helix} - r_{coil} = 0.038$. Thus, within the limits of the model and the accuracy of the experimental data, the increase in T_m with added PtTS is in accord with theoretical predictions for substantially greater binding of the dye to duplex versus random coil DNA, as expected for intercalation.

Hydrodynamic measurements provide additional evidence for intercalation. From the plot of specific viscosity, η_{sp}, versus r (Fig. 5) it may be seen that the viscosity of the DNA increases to a maximum in the vicinity of 0.23 moles of PtTS bound per DNA phosphate, in approximate agreement with the spectral data (Fig. 3). This result is consistent with intercalative binding of PtTS to the DNA, since lengthening of the DNA would increase its viscosity. Direct evidence for lengthening comes from preliminary electron microscope studies (with M. Beer and M. Cole) which show that PtTS increases the length of bacteriophage T7 DNA by about 20–30%.

The results shown in Fig. 7b, together with the above measured binding constant for PtTS to DNA, may be used to compare the apparent unwinding angle, ϕ, of PtTS with that of EthBr. This unwinding angle is computed from the relationship (25) $\tau = \tau_0 + (10\beta^\circ/\pi)\phi r$, where τ_0 is the number of superhelical turns when $r = 0$, ϕ is the unwinding angle in radians, and $\beta^0 = (10^{-1}) \times$ (the number of nucleotide pairs). At the principal minimum, $r = r_c$, in Fig. 7b, $\tau = 0$ and $\phi r_c = -(\pi\tau_0/10\beta^0)$. By comparison with the corresponding experiment conducted with EthBr, $(\phi r_c)_{PtTS} = (\phi r_c)_{EthBr}$. Using the

value $K = 2.0 \times 10^5\ M^{-1}$ measured for calf thymus DNA at 25° in buffer 3, we estimate that, for PtTS, 0.088 ($n = 0.20$) < r_c < 0.095 ($n = 0.22$) as compared to the value of 0.071 for EthBr under these experimental conditions (26). This suggests that the PtTS unwinding angle is approximately 70–80% of the value for EthBr, generally taken to be 12° (27). Alternatively, the apparent difference might indicate that 20–30% of the PtTS is bound in a nonunwinding mode in this solvent, or might simply reflect uncertainty in the values of the binding parameters.

The fact that the PtTS cation (Fig. 1) is a good metallointercalation reagent depends upon several features that deserve comment. First, a planar aromatic ligand of appropriate size is required. The terpyridine ligand is ideally suited, being stereochemically very similar to the intercalating part of the ethidium cation. The presence of the platinum(II) atom probably enhances the intermolecular stacking forces that stabilize the intercalative binding mode, since the related d^8 palladium(II) complexes [(terpy)PdCl]$^+$ (28) and bis(9-benzyl-6-mercaptopurine)palladium(II) (29) both exhibit close (about 3.2–3.5 Å)intermolecular parallel ring contacts in their crystalline state as determined by x-ray diffraction studies.¶ This feature distinguishes complexes such as PtTS from other metallointercalation reagents that might be designed by covalently attaching a metal atom to EthBr or a related organic dye. A further point is the relative inertness of the PtTS complex. The tridentate terpy ligand is kinetically inert to substitution, as apparently is the Pt-S bond under the conditions of the present study. Thus PtTS is a metal complex that does not readily bond covalently either to the bases or to the phosphate backbone of DNA, as do many metal ions and complexes (1–3). The PtTC cation, however, has a substitutionally more labile leaving group in the chloride ion. At [DNA-P): [PtTC) ratios < 2, the fluorescence studies provide clear evidence for non-competitive inhibition of EthBr binding, with chloride ion probably being displaced from the coordination sphere of the platinum atom.

The binding isotherms of numerous planar aromatic dyes that intercalate into the DNA duplex reveal that the apparent number of binding sites is consistently smaller than the number of base pairs (8). The PtTS and PtTC complexes behave similarly. To explain this behavior, Crothers has proposed a "neighbor exclusion" model which requires every other inter-base pair site to contain bound dye at saturation (20). The complex of PtTS intercalated into DNA should enable us to test this hypothesis experimentally using fiber x-ray diffraction or high resolution scanning (31) or dark field (32) electron microscopy in which the heavy platinum atoms would serve as marker groups (33). If the markers are evenly spaced at the correct distance and frequency along the polymer backbone, the neighbor exclusion model would be verified. Additional questions concerning the orientation of the intercalated dye with respect to the polymer backbone (34) might also be answered by physical studies using suitably designed metallointercalation reagents.

We are grateful to Drs. C. R. Cantor and I. B. Weinstein for their advice, and to Mr. Robert Fairclough for assistance with the fluorescence lifetime measurements. This work was supported at Columbia University by grants from the National Science Foundation (GP-40936) and the National Cancer Institute (CA-15826) and at S.U.N.Y., Stony Brook, by Grant no. GM-21176 from the National Institute of General Medical Sciences, U.S. Public Health Service.

¶ Square d^8 complexes have been widely studied and are known to form one-dimensional columnar stacks in their solid lattices (30).

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