Binding of the Antitumor Drug Platinum Uracil Blue to Closed and Nicked Circular Duplex DNAs†

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ABSTRACT: Platinum uracil blue (PUB), a potent antitumor drug, reacts with closed circular duplex DNA to form a covalent adduct. The course of the reaction has been monitored by a variety of methods including gel electrophoresis, buoyant density centrifugation, and band sedimentation. Electrophoresis in agarose gels of a mixture of closed (I) and nicked (II) DNAs reveals that DNA I is cooperatively excluded from gels over a narrow range of relatively low drug concentrations. DNA II is also excluded following a further fivefold increase in the drug concentration. The reaction leads to precipitate formation at low salt, 0.5 M or less, but the precipitate may be redissolved by increasing the ionic strength to 1.0 M or greater. The reaction is stopped but not reversed by the addition of concentrated NaCl, sodium acetate, or CsCl. The buoyant density in CsCl increases cooperatively as the incubation concentration of PUB is increased. At a formal platinum/nucleotide mole ratio (r_f) of 2.5, the buoyant shift is approximately 50% of the maximum value of 50 mg/mL for both viral PM-2 I and plasmid pSM1 I DNAs. The buoyant band width passes through a maximum over the same interval. The sedimentation coefficient of pSM1 DNA I increases in a complex fashion with PUB addition and eventually reaches a value 30% greater than that of drug-free DNA. The reaction does not involve transfer of uracil to DNA, as indicated by the failure of [^{14}C]uracil-PUB radioactivity to coband with the dense DNA complex following incubation at an r_f of 10. The reaction may be largely reversed by the addition of potassium cyanide, as determined by the recovery of approximately 85% of the maximum buoyant shift. The cyanide-reversed reaction product, which migrates like DNA I in gel electrophoresis, does not contain backbone chain scissions. The reaction with DNA I is relatively rapid, complete gel exclusion being attained by 1 h at 37 °C in 20 mM NaCl for $r_f \geq 2$.

Platinum uracil blue (PUB[1]) is the prototype of a class of reagents formed by the interaction of the well-known antitumor drug *cis*-[Pt(NH_3)$_2$Cl$_2$] and its aqueous substitution products with pyrimidines and pyrimidine-containing polynucleotides (Davidson et al., 1975; Aggarwal et al., 1975; Flynn et al., 1977). PUB and its derivatives possess potent antitumor activity in a variety of standard screening systems and, by comparison with most of the other platinum-containing antitumor drugs, renal toxicity is a less significant side effect (Aggarwal et al., 1975). The platinum blue compounds are potentially useful for a variety of other purposes, including cytological staining. Chromatin, nucleoli, ribosomes, and cell surfaces may all be selectively visualized with proper choice of staining conditions (Wagner et al., 1974; McAllister et al., 1977). A recent report indicating a correlation between cell surface staining by PUB and tumorigenicity of cell types (Aggarwal et al., 1975) has, however, not been confirmed (McAllister et al., 1977).

The formation of PUB involves reaction over a period of several days between *cis*-DDP and uracil. The eventual product is an amorphous powder of reported stoichiometric composition 1Pt:2NH_3:20:1uracil (Davidson et al., 1975). Blue compounds are not obtained using either *trans*-DDP or substituted DDP derivatives, including [(en)PtCl$_2$] (Flynn et al., 1977). Experiments in our laboratory indicate that PUB is most likely an oligomer, of degree of polymerization approximately 20 (Lerner, Bauer, and Lippard, in preparation). PUB is related to a class of platinum amide complexes (Gillard & Wilkinson, 1964; Brown et al., 1969) first termed "Platinblau" (Hofmann & Bugge, 1908).

Although PUB itself has not been structurally characterized, a closely related compound formed by reaction of α-pyridone with *cis*-DDP has been crystallized and its structure determined (Barton et al., 1977). This latter compound possesses an ESR spectrum similar to that of PUB (Lippert, 1977) and quite likely embodies many of the salient structural features. These include: net positive change; mixed platinum oxidation states, formally 3Pt(II):1Pt(III); interactions among platinum residues in a one-dimensional chain; and amidate bridges between adjoining residues. The structure of PUB is likely to resemble that of *cis*-diammineplatinum α-pyridone blue, one possibility for which is shown in Figure 1.

We have previously reported investigations of the binding of a variety of other platinum-containing compounds to DNA (Howe-Grant et al., 1976) and have shown that covalently closed duplex DNA is especially useful as a substrate. This utility arises from two properties, one topological and the other structural. First, changes in duplex winding are accompanied by equal and opposite change in superhelical winding, thereby greatly amplifying the detectibility of small duplex winding changes (Vinograd & Lebowitz, 1966; Fuller, 1971). Second, a small fraction of the bases in highly supercoiled closed circular DNA show enhanced reactivity, owing to reduction in the helix-coil transition temperature resulting from the free energy of superhelix formation (Bauer & Vinograd, 1970; Dean & Lebowitz, 1971; Woodworth-Gutai & Lebowitz, 1976). With the use of both closed and linear DNAs, we were able to group the platinum-containing reagents into four

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[1] Abbreviations are: PUB, platinum uracil blue; DDP, dichlorodiammineplatinum(II); DNA I, covalently closed circular duplex DNA; DNA II, nicked circular duplex DNA; DNA III, linear duplex DNA; EtdBr, ethidium bromide; EDTA, disodium salt of ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; en, ethylenediamine; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

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classes. Class A compounds bind exclusively by intercalation and exhibit competitive inhibition of EtdBr binding (effect upon the binding constant, K, only). Class B reagents bind by both intercalation and covalent interaction, thereby altering both K and the number of sites, n, in competition with EtdBr. Class C compounds exhibit very slow or no binding. This effect can arise from a variety of causes, including the nature and concentration of added salt and the presence of kinetically inert leaving groups on the platinum. Finally, class D reagents bind covalently to DNA without intercalation. This reaction is sometimes followed by DNA backbone chain scissions. In the present investigation we extend these previous studies to include the interaction of PUB with both closed and nicked circular DNAs.

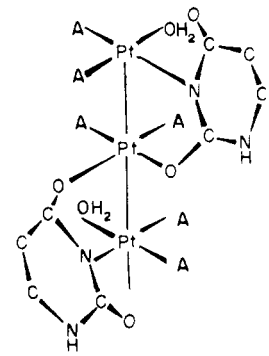

FIGURE 1: A possible oligomeric structure for PUB. A represents the NH_3 group.

Materials and Methods

DNA, Platinum Uracil Blue and Other Chemicals. PM-2 DNA was isolated from bacteriophage-infected Bal-31, a marine pseudomonad, as described by Salditt et al. (1972). ^{3}H-labeled PM-2 DNA was prepared similarly, except that the bacteria were grown in modified BAL synthetic media (Franklin et al., 1969) containing one-fourth the normal amount of yeast extract. Samples of PM-2 DNA of altered extent of supercoiling were prepared by treatment of DNA I with nicking–closing enzyme in the presence of EtdBr and were a gift from Dr. R. L. Burke. Plasmid pSM1 DNA (Mickel & Bauer, 1976) was grown and isolated from *E. coli* strain W677. All DNA concentrations were determined from absorbance readings at 260 nm using a molar extinction coefficient of 6600. Platinum uracil blue was purchased from Matthey Bishop or, alternatively, was synthesized as described by Davidson et al. (1975). ^{14}C-labeled uracil was used to prepare samples of this compound, as described by Lerner (1976). Stock solutions of PUB were prepared by dissolving weighed amounts in deionized water before addition to reaction mixtures containing DNA and were allowed to stand for a minimum of 1 h before use. As will be reported elsewhere (Lerner, Bauer, and Lippard, in preparation), the spectrum of PUB in water changes rapidly over this time period and exhibits only relatively small changes thereafter. Concentrations of commercial PUB were checked from the absorbance at 675 nm measured immediately after dissolving the compound, using a formal extinction coefficient of 430 per equivalent platinum (Lerner, 1976). EtdBr was purchased from Sigma and optical grade CsCl from the Harshaw Chemical Co. Restriction endonuclease *Eco*RI was obtained from Dr. Geoffrey Childs and was used in reaction mixtures containing 50 mM NaCl, 10 mM $MgCl_2$, 0.1 M Tris. All other chemicals were reagent grade.

Gel Electrophoresis. Closed circular DNA containing a small amount of nicked circular DNA was allowed to react with PUB at various formal ratios, r_f, equivalents platinum per mole DNA phosphate. Incubation was conducted in a thermostated Lauda circulating bath at 37 °C in various concentrations of NaCl, 1.5 mM Tris, 0.15 mM EDTA. Electrophoresis of reacted samples was carried out in 1% agarose slab gels prepared in 40 mM Tris–acetate, 20 mM NaOAc, and 2 mM EDTA (E buffer), for 4 h at ambient temperature with recirculation of buffer. After 4 h the gels were stained for 1 h in E buffer containing 0.5 μg/mL EtdBr. The gels were photographed and the photographs analyzed as described previously (Howe-Grant et al., 1976).

Preparative Buoyant Equilibrium Sedimentation. Incubation conditions for mixtures of PUB and DNA were as described above for gel electrophoresis with the exception that larger quantities of DNA were used and the NaCl concentration was 30 mM. The formation of a visible precipitate in the incubation tube required a minimum DNA concentration of approximately 2.5 μg/mL. Precipitates, which rapidly settled to the tube bottom, were either separated from the supernatant with a capillary pipet or else temporarily resuspended with a vortex mixer before transfer to centrifuge tubes. The DNA used was [^{3}H]PM-2 DNA I at a specific activity of 470 cpm/μg and at a concentration of 40 μg/mL. The PUB was ^{14}C-labeled at a specific activity of 326 cpm/μg. Where necessary, radioactive DNA was diluted with unlabeled DNA so as to avoid substantial ^{3}H spillover into the ^{14}C channel. Polyallomer centrifuge tubes were filled to 3.0 mL with the above samples suspended in CsCl of initial density 1.72 g/mL and the tubes were topped up with light mineral oil. Centrifugation was at 44 000 rpm, 20 °C, in a SW50.1 rotor. Platinum reagents were observed to bind to cellulose nitrate centrifuge tubes, resulting in significant losses at low concentrations. Following attainment of equilibrium the gradients were fractionated from the bottom, collecting 10-drop fractions. The first five out of every group of six fractions were dripped onto filter papers and dried for 20 min with a 250-W infrared heat lamp before transfer into scintillation fluid composed of 18 mM PPO and 0.14 mM POPOP in toluene and then counted with a Searle Mark II liquid scintillation counter. Every sixth fraction was collected in a conical polyallomer tube and the refractive index immediately measured to calculate the solution density. All data were fully corrected for spillover and quenching.

Analytical Equilibrium Buoyant Density and Sedimentation Velocity Determinations. pSM1 DNA I was incubated with PUB for indicated times at 37 °C in 50 mM NaCl, 10 mM Tris, 0.15 mM EDTA (pH 7.7). Reactions were terminated by diluting the contents of each incubation tube tenfold with a CsCl solution of density 1.71 g/mL. Diluted solutions were transferred to centrifuge cells which were spun in an AnG-Ti or AnF-Ti rotor. For equilibrium buoyant density determinations, a rotor speed of 44 000 rpm and a temperature of 25 °C were used. A 0.2-μg portion of PM-2 DNA II was added as a marker to each solution following the addition of CsCl. After 24 h, equilibrium band positions were determined by absorbance scans at 265.4 nm. Sedimentation coefficients were determined by band sedimentation (Vinograd et al., 1963), using 20-μL reacted sample and a sedimentation solvent containing CsCl at a density of 1.30 g/mL, 20 °C, and 40 000 rpm. Sedimentation coefficients were corrected according to the method of Bruner & Vinograd (1965). Reversibility of binding was studied by incubating previously reacted mixtures of DNA and PUB for up to one additional hour in a medium containing various concentrations of added KCN. Following incubation, solutions were first dialyzed in order to remove unreacted PUB and KCN and then transferred into centrifuge

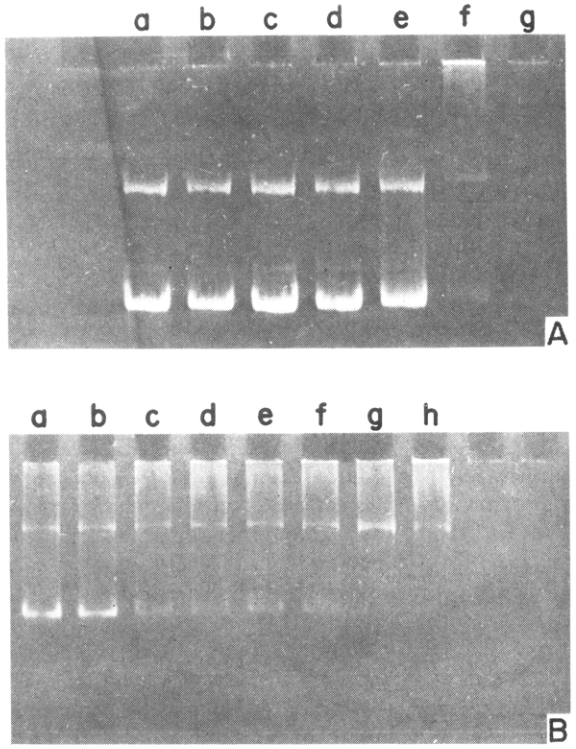

FIGURE 2: Gel electrophoresis in 1% agarose of PM-2 DNAs I and II in the presence of increasing formal ratios of PUB to DNA nucleotide (r_f). Samples containing 70% PM-2 DNA I at an absorbance (260 nm) of 0.10 were incubated at the indicated r_f at 37 °C for 1 h in 0.02 M NaCl, 0.01 M Tris. Aliquots containing 0.1 μg of total DNA were then added to each channel and subjected to electrophoresis for 4 h at 5 V/cm (50 mA). (A) Series in which r_f was increased logarithmically. The intermediate band corresponds to a small (<5%) amount of linear PM-2 DNA III. The r_f values are: (a) 0; (b) 10^{-3}; (c) 10^{-2}; (d) 10^{-1}; (e) 1; (f) 10; (g) 100. (B) Expanded series over the region of exclusion of DNA I from the gel. The r_f values are: (a) 0.6; (b) 1.0; (c) 1.6; (d) 2.0; (e) 2.5; (f) 3.0; (g) 3.5; (h) 4.0.

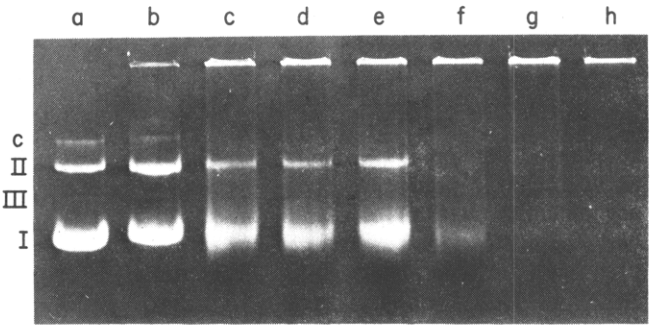

FIGURE 3: Gel electrophoresis in 1% agarose of pSM1 DNAs I and II as a function of increasing incubation r_f. Samples of 20 μL containing 96% DNA I at a total DNA absorbance of 0.56 (260 nm) were incubated at 37 °C for 2 h in 0.03 M NaCl, 0.01 M Tris, 0.15 mM EDTA. The entire incubation solution, containing 0.08 μg of DNA, was then added to each channel and subjected to electrophoresis for 4 h at 5 V/cm (50 mA). The bands labeled III and C correspond to contaminants of pSM1 linear DNA and of *E. coli* chromosomal DNA, respectively. The r_f values are: (a) 0.0; (b) 0.5; (c) 1.0; (d) 1.25; (e) 1.5; (f) 2.0; (g) 2.25; (h) 3.0.

cells for equilibrium buoyant density determinations. In some experiments the dialysis was omitted, with no measurable effect upon the results.

Results

Gel Electrophoresis Assay of Binding of PUB to Closed and Nicked Circular DNAs. The binding of PUB to PM-2 DNAs I and II was investigated by addition of the reagent to mixtures containing 70% DNA I and 30% DNA II, followed by incubation at 37 °C for 1 h in 0.02 M NaCl, 0.01 M Tris (pH 8.3). Subsequent to incubation, aliquots containing 0.1 μg of DNA were removed and analyzed by gel electrophoresis. Figure 2 presents the results of a series of such incubations over the range $10^{-3} \leq r_f \leq 100$, Figure 2A, and over the expanded range $0.6 \leq r_f < 5.0$, Figure 2B. The electrophoretic behavior of both DNA components is unaltered at all values of $r_f \leq 1$. In the region $1 \leq r_f \leq 4$ the band corresponding to DNA I is progressively diminished in amount, although unaltered in mobility. Over this same region DNA I appears in the upper portion of the gel and migrates very slowly if at all. No species of intermediate mobility were detected as a result of the reaction of PUB with DNA I, and both the amount and mobility of the DNA II were unaltered over this range of r_f values. At $r_f > 4$ the original band of DNA I is entirely shifted to the top of the gel and some reaction is also apparent with DNA II. At $r_f = 10$ a substantial reduction in the amount of DNA II is evident, and the mobility of the remaining nicked material is somewhat reduced. At $r_f = 100$ all DNA species fail to enter the gel. In no case was there any apparent conversion of closed to nicked DNA.

The results of a similar incubation series with pSM1 DNA, except that incubation was for 2 h at 37 °C, 30 mM NaCl, 1.5 mM Tris, are shown in Figure 3. The general features of the reaction are similar to those with PM-2 DNA at 1-h incubation (see above), except that all characteristic transitions are shifted to lower r_f values. Thus, the appearance of nonmigrating DNA is first apparent at $r_f = 0.5$ instead of at $1.6 < r_f < 2$, and all DNA I is transferred to the gel top by $r_f = 2$. The loss of DNA II is also complete by $r_f = 2$, compared with the previous $r_f = 10$. The extended incubation time thus appears to affect DNA II more markedly than DNA I, suggesting that the rate-limiting step is a nucleation phenomenon. The kinetics of the reaction are explored in greater detail below. The evidence of Figures 2 and 3 shows clearly that the reaction of PUB with both DNAs I and II is highly cooperative and results in the formation of a nonmigrating species. DNA I is in both cases more highly reactive than DNA II, although both species are eventually completely excluded from the gel. PM-2 and pSM1 DNAs appear to exhibit similar behavior. The former has a molecular weight of 6.6×10^6, whereas the latter is 3.7×10^6. The respective base compositions (G + C) of these DNAs are 42% and 48%, and their superhelix densities (tertiary turns per ten base pairs) are −0.114 and −0.098.

The Buoyant Behavior of pSM1 DNA I following Incubation with PUB. Samples containing >95% pSM1 DNA I were incubated with PUB in 0.05 M NaCl, 0.01 M Tris (pH 7.7) for 1 h, followed by the addition of stock CsCl of density 1.710 g/mL. The increase in salt concentration prevents further reaction (see below) but is not expected to remove covalently bound reagent. Following CsCl addition PM-2 DNA II (θ = 1.694 g/mL) was added as a density marker and the samples were centrifuged for 24 h at 44 000 rpm, 25 °C. Figure 4 presents a series of analytical scans at increasing values of r_f: 0, 0.5, 1.5, 2.5, and 4.5. The buoyant density of the marker did not appear to be influenced by the presence of excess unbound reagent, which redistributes in increasing concentration toward the cell bottom. The width of the marker band, a sensitive inc of reaction, was constant throughout.

Both the buoyant density and the width of the pSM1 DNA I band are, however, markedly altered by the prior incubation with PUB. The magnitude of these effects is shown in Figure 5, in which the buoyant density with respect to PM-2 II (A) and the width of the buoyant band at $1/\sqrt{2}$ of the maximum

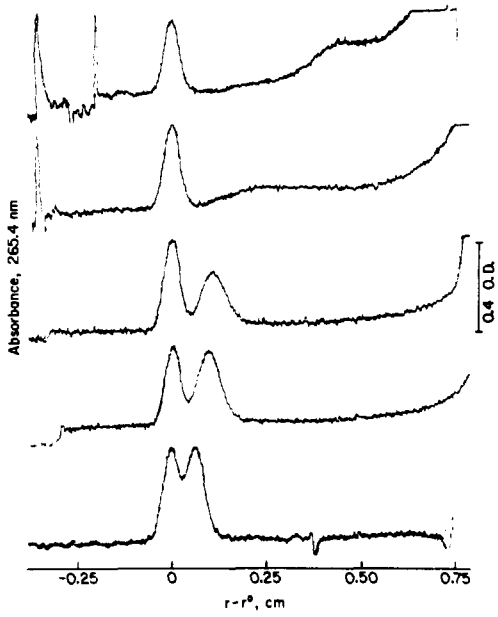

FIGURE 4: Photoelectric scanner recordings from the analytical ultracentrifuge of pSM1 DNA I which had been preincubated with PUB. The band centered at $r = r^\circ$ represents unreacted PM-2 DNA II, 0.2 μg, added subsequent to CsCl addition. Samples were incubated for 1 h at 37 °C in 0.05 M NaCl, 0.01 M Tris, and the reaction stopped by the introduction of CsCl of density 1.71 g/mL. Each centrifuge cell contains 0.2 μg of reacted pSM1 DNA, and the densities were adjusted to 1.7176 g/mL before the run. The pSM1 DNA was 96% DNA I. From bottom to top, the samples represent untreated control and r_f values of 0.5, 1.5, 2.5, and 4.5. Centrifugation was for 24 h at 44 000 rpm, 25 °C. The absorbance indicated is for a 1.2-cm optical path.

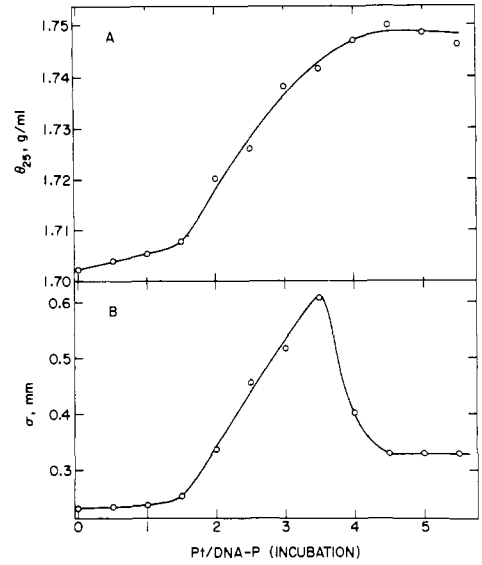

FIGURE 5: Variation in (A) the buoyant density, θ, and (B) the bandwidth at $1/\sqrt{2}$ of the maximum height, σ, of a series of pSM1 DNA I samples treated as described in the legend to Figure 4.

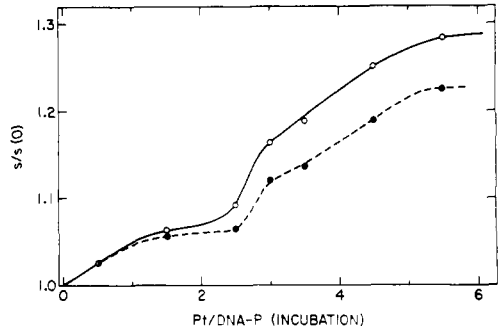

FIGURE 6: Variation in the sedimentation coefficient of pSM1 DNA I with PUB incubation r_f. The same pSM1 DNA sample was employed (96% DNA I) as in Figures 3 and 4. Incubation conditions were as described in the legend to Figure 4. Following incubation, samples were chilled in ice and immediately placed in the sample wells of band-forming centerpieces. The amount of pSM1 DNA added was 0.4 μg and layering onto the sedimentation solvent of CsCl, 1.30 g/mL, was accomplished within 15 min. Plotted is the ratio of $s_{20,w}$ for each treated sample to that of unreacted pSM1 DNA I, for which $s_{20,w} = 23.21 \pm 0.11$ S. The Bruner-Vinograd (1965) correction was employed to calculate $s_{20,w}$, using the buoyant density of untreated DNA (●) or the actual measured values, Figure 5 (○).

height above the interpolated reagent baseline (B) are plotted as a function of the incubation r_f. The buoyant density is relatively constant, as is the bandwidth, at values of $r_f < 1.5$. Here θ increases from the initial value of 1.7016 to 1.7078 g/mL, while σ increases hardly at all. In the region $1.5 < r_f < 4$, however, θ increases greatly and cooperatively, eventually reaching a value of approximately 1.75 g/mL. At $r_f > 4$ no further increases in the buoyant density take place. The behavior of σ over the same region is similar but more complex. The bandwidth also increases sharply over the region $1.5 < r_f < 3.5$, but this increase is followed by a decrease to a new plateau level at $3.5 < r_f \lesssim 4$. Above $r_f = 4.5$ the width also remains constant at a value (0.33 mm) approximately 57% greater than the initial value (0.21 mm) but at only about 54% of the maximum (0.61 mm) obtained at $r_f = 3.5$.

Several conclusions can be drawn from these experiments, some of which will be discussed in greater detail below. (1) The magnitude of the buoyant increment indicates that a considerable extent of binding has occurred. We infer this binding to be covalent since any ionic interactions would be reversed under the high salt conditions employed. (2) The binding is cooperative on the r_f scale. (3) The DNA becomes saturated at high r_f values. (4) The intermediate stages of the binding, $1.5 < r_f < 4$, are accompanied by considerable density heterogeneity as shown by the large increase in the band widths (an increase in DNA molecular weight per se would shapen the bands). (5) As the binding sites become saturated the density heterogeneity abruptly diminishes. (6) Finally, the relative elevation in σ at high r_f suggests that, even at saturation, some heterogeneity in binding persists among the family of DNA molecules.

The Effect of PUB upon the Sedimentation Velocity of pSM1 DNA I. Following 1-h incubation under the same conditions described in the previous section, the reactions were stopped by adding an equal volume of CsCl of density 1.25 g/mL. Aliquots containing 0.4 μg of PUB-reacted pSM1 DNA I were then placed in the sample wells of 12-mm band-forming centerpieces and layered onto a sedimentation solvent of CsCl, 1.30 g/mL, 20 °C. The time lapse between termination of incubation at 37 °C and the layering was never greater than 15 min. The sedimentation coefficients were determined at each r_f and converted to apparent $s_{20,w}$ values using the Bruner-Vinograd (1965) correction. Figure 6 presents the ratio between these apparent $s_{20,w}$ coefficients and the value at $r_f = 0$. The Bruner-Vinograd correction was applied in two ways: employing the initial buoyant density, θ_0, throughout (filled circles) and taking into account the measured density increments, θ, of Figure 5A (open circles). The curves are of similar shape regardless of the type of correction used.

The sedimentation velocity profiles, which are sensitive to molecular shape, are clearly more complex for this closed circular DNA than are the buoyant density curves, which depend upon the extent of binding. The velocity ratio increments

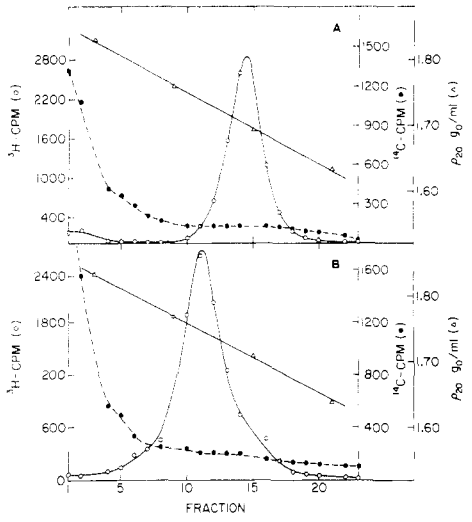

FIGURE 7: Equilibrium buoyant banding of [^{3}H]PM-2 DNA I following reaction with [^{14}C]PUB. The initial r_f was 8.0 and the incubation solvent was 0.03 M NaCl, 1.5 mM Tris, 0.15 mM EDTA. (A) The reaction mixture contained 6.3 μg of DNA in 0.5 mL. DNA and PUB were mixed; then 2.5 mL of stock CsCl solution was added with no incubation and the final density adjusted to 1.736 g/mL. (B) The reaction mixture contained 12.6 μg of DNA in 1.0 mL. Incubation was for 2 h at 37 °C, following which the precipitated material was removed as described in the text, taken up in CsCl, and the final density adjusted to 1.736 g/mL. Centrifugation in both cases was for 36 h at 41 000 rpm in an SW50.1 rotor at 20 °C.

with increasing r_f may, however, be resolved into three major regions: an initial increase from 1.0 to 1.06 as r_f increases from 0 to 1.5; a short plateau in the vicinity of $r_f = 2$; and a second larger, complex, and cooperative increase to a ratio of 1.29 which appears to terminate at about $r_f = 5$. These regions correspond approximately, but not exactly, to those described above for the buoyant shifts. The sedimentation experiments thus confirm that the DNA I–PUB complex is not dissociated by high salt concentrations and support the conclusion that the extent of binding is considerable and approaches saturation at the higher r_f values following 1-h incubation in low salt. The results of Figure 6 further demonstrate that the DNA I–PUB complex behaves as a soluble species in high salt, even though it fails to enter agarose gels (low salt) following similar incubation. This general solubility behavior is discussed further below. Figures 5 and 6 taken together strongly suggest that the binding is cooperative at the single molecule level and is not an adventitious correlate of a precipitation phenomenon. In comparable experiments (not shown), no effects upon the sedimentation velocity of pSM1 DNA II were observed over this range of incubation conditions.

The failure to observe an initial, characteristic diminution in the ratio $s/s(0)$ suggests that the velocity effects are not due simply to an unwinding of the DNA duplex in this negatively superhelical DNA. The occurrence of unwinding at $r_f < 0.5$ cannot, however, be ruled out because of insufficient data in this region. The observed increases in $s/s(0)$ might be ascribed to (1) an increase in the mass per unit length due to platinum binding; (2) a compaction of the DNA due to the formation of regions of diminished base pairing; or (3) the generation of positive superhelical turns. The general hydrodynamic behavior of closed DNA following reaction with platinum compounds is under investigation and will be reported in greater detail elsewhere.

Buoyant Density of Complexes between [^{3}H]PM-2 DNA I and [^{14}C]PUB. The preceding experiments were reported as a function of r_f, the formal mole ratio of platinum to DNA nucleotide. In an attempt to estimate r_b, the corresponding ratio of moles of platinum bound per mole of nucleotide, [^{14}C]PUB was incubated with [^{3}H]PM-2 DNA I. Following incubation the resulting precipitate was pelleted by centrifugation at 3000 rpm for 5 min in a Sorvall SS34 rotor, and the supernatant was removed with a Pasteur pipet. The pellet was then taken up in CsCl and the resulting mixture subjected to preparative ultracentrifugation. Figure 7 shows the results of such an experiment, with incubation for 2 h at $r_f = 8$ in 30 mM NaCl, 1.5 mM Tris, 0.15 mM EDTA, 37 °C. Panel A shows the profile of the buoyant DNA and of the [^{14}C]PUB following addition of all reagents but with no incubation. The measured solution density at band center was 1.698 g/mL, comparable to the reported buoyant density of 1.694 g/mL for unreacted PM-2 DNA. Panel B shows the corresponding buoyant profile following the 2-h incubation. The solution density at band center is 1.750 g/mL. When corrected for pressure effects according to Hearst et al. (1961), this corresponds to a buoyant density of 1.738 g/mL. This buoyant increment, 44 mg/mL, is consistent with that obtained for pSM1 DNA I at high r_f (cf. Figure 5A). The redistribution pattern of the [^{14}C]PUB is, however, essentially the same as in panel A. In particular, no radioactive uracil is associated with the shifted DNA band. This result may indicate that the uracil–platinum bond is broken in the process of DNA binding. Alternatively, the reactive species might be a component of PUB containing platinum not linked covalently to uracil. This question is under further investigation. A number of comparable experiments were performed over the range $0 \leq r_f \leq 8$, except that the entire reaction mixture was added directly to a CsCl solution of the appropriate density. The absolute buoyant shifts, corrected for pressure effects (Hearst et al., 1961), are indistinguishable from those of Figure 5A. In no case was [^{14}C]PUB associated with the [^{3}H]DNA band, even at the highest r_f levels examined.

In a complementary set of experiments the precipitated DNA–PUB complex was separated from the supernatant by low-speed centrifugation, following which CsCl was added to the supernatant and equilibrium buoyant banding performed as in Figure 7. The results showed that, under the reaction conditions described above, very little DNA remains unprecipitated. The buoyant profile of the small amount of DNA which remained in the soluble phase was broader and approximately 10 mg/mL less dense than the precipitate. This result suggests that the soluble DNA fraction is unsaturated by PUB and that distribution of occupied sites among DNA molecules is relatively broad.

Ionic Conditions for Formation and Resolubilization of Precipitates Formed between PUB and PM-2 DNA I. The results of the gel electrophoresis experiments, Figures 2 and 3, suggest that DNA I forms an insoluble complex with PUB at the ionic strengths (20–30 mM) used in those incubations. The buoyant (Figures 4, 5, and 7) and sedimentation velocity (Figure 6) results indicate, however, that the complex is soluble at relatively high ionic strengths. Two sets of experiments were performed to delimit the precipitation/solubilization regions. The results are diagrammed in Figure 8, which shows the experimental regions defining the dependence of precipitate formation and resolubilization upon ionic strength. In these experiments 15 μg of PM-2 DNA I was incubated with PUB at $r_f = 8$ for 2 h at 37 °C. Panel A indicates the results which are obtained by increasing the ionic strength of the incubation medium. Precipitate formation was clearly indicated by the appearance of a blue-gray solid at the bottom of the tube; the loss of [^{3}H]DNA from the supernatant was confirmed by scintillation counting. At ionic strengths below about 0.3 M all the DNA precipitates; between 0.3 M and 0.6 M increasing

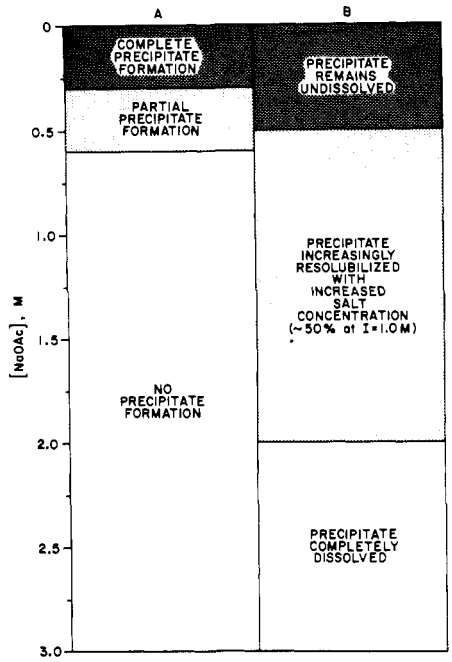

FIGURE 8: Effect of incubation salt concentration upon precipitation of PM-2 DNA I by PUB. Solutions containing 15 μg of DNA were incubated at an r_f of 8.0 for 2 h at 37 °C. (A) Incubations were conducted at the indicated salt concentrations. (B) Incubations were conducted in the presence of 0.03 M NaCl, and solid sodium acetate was then added to obtain the indicated final salt concentration. The extent of resolubilization was determined visually following vigorous shaking for 2 min at room temperature.

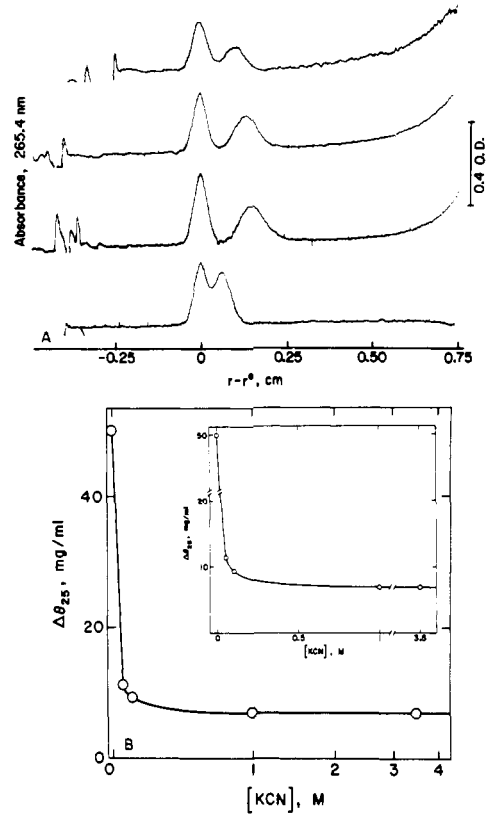

FIGURE 9: Reversibility of the binding of PUB to pSM1 DNA I by the addition of KCN. All incubations were at r_f = 10 for 30 min at 37 °C in 0.05 M NaCl, 0.01 M Tris. Solid KCN was then added to obtain the desired final concentration and the incubation continued for an additional 45 min at 37 °C. The solutions containing 0.2 μg of DNA were then added to stock CsCl; 0.2 μg of marker PM-2 DNA II was added and the final density (25 °C) adjusted to 1.7176 g/mL. Centrifugation was for 24 h at 25 °C, 44 000 rpm. (A) Photoelectric scanner recordings at equilibrium, density increasing to the right. The bottom scan shows an untreated mixture of PM-2 DNA II (left) and pSM1 DNA I (right). The remaining three scans represent, from bottom to top, the results of KCN treatment at concentrations of 0.05, 0.10, and 3.5 M. (B) The buoyant density difference between KCN-reversed, PUB-treated pSM1 DNA I and untreated pSM1 DNA I ($\Delta\theta_{25}$), as a function of KCN concentration. A value of $\Delta\theta$ = 0 would correspond to complete reversibility of the reaction. All buoyant densities were measured as in A, employing unreacted PM-2 DNA II as a marker.

amounts of DNA remain in the supernatant; and above 0.6 M no precipitate forms. Although sodium acetate was used in these experiments, identical results were obtained with CsCl or NaCl.

Panel B of Figure 8 summarizes the results of complementary experiments in which incubation was allowed to proceed for 2 h in 30 mM NaCl, 1 mM Tris, pH 7.7, 37 °C, at r_f = 8. Precipitation is complete under these conditions. Salt was then added and, using the same criteria as above, the concentrations determined under which the precipitate can be redissolved. A minimum ionic strength of 0.5 M is required, as indicated in the figure, whereafter resolubilization proceeds and is complete by about 2.0 M. All buoyant and velocity experiments were conducted above this ionic strength, whereas the gel electrophoresis experiments were performed below an ionic strength of 0.5 M. As pointed out above, bound platinum is not removed from the DNA by increasing the ionic strength into the resolubilization region.

Kinetics of the Binding of PUB to PM-2 DNA I. A mixture containing 86.5% PM-2 DNA I and 13.5% PM-2 DNA II (by electrophoretic analysis on 1% agarose) was incubated with PUB at r_f = 1.3 in the presence of 50 mM NaCl, 10 mM Tris (pH 8.3) at 37 °C for times ranging up to 16 h. Aliquots containing 0.2 μg of DNA were withdrawn at various times and mixed with CsCl of density 1.71 g/mL, following which the buoyant density was determined analytically. The closed DNA clearly reacted to a much greater extent than did the nicked species, confirming the earlier gel results (Figures 2 and 3). The two DNA components cobanded in the absence of reaction; after 1 h the buoyant separation was 4.9 mg/mL; and after 4 h $\Delta\theta$ had increased to 13.0 mg/mL. Only very small changes were observed beyond 4 h, the buoyant separation at 9.5 h being 14.8 mg/mL. The band corresponding to DNA II appeared to be somewhat denser at 9.5 h than at 4 h, but this result was not confirmed by the addition of an unreacted marker DNA.

Reversibility of the Binding of PUB to DNA I by the Addition of KCN. Cyanide ion is known to form the extremely stable ($K \sim 10^{41}$) platinum complex $[Pt(CN)_4]^{2-}$. The reversibility of the reaction was therefore investigated by incubating previously prepared complexes between PUB and pSM1 DNA I in the presence of varying concentrations of KCN. The extent of reversibility was then examined by two methods: measurement of the buoyant density of the complex and gel electrophoresis following removal of excess KCN by dialysis.

Figure 9A shows the results of representative buoyant analyses. In these experiments the initial incubations were for 30 min at 37 °C in 0.05 M NaCl, 0.01 M Tris (pH 7.7). KCN was then added and the incubation continued for an additional 45 min. Aliquots of 50 μL were then mixed with 500 μL of CsCl, 1.71 g/mL, and a marker PM-2 DNA II added. The results shown here correspond to added KCN concentrations of 0, 0.05, 0.10, and 3.5 M. In Figure 9B the buoyant shifts are plotted as a function of the molarity of KCN used in the second incubation. Here a value of $\Delta\theta$ = 0 corresponds to complete

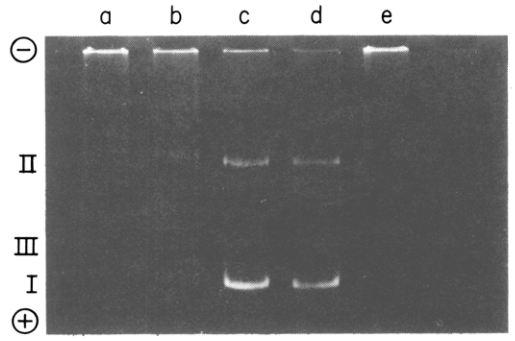

FIGURE 10: Gel electrophoresis in 1% agarose of PM-2 DNA following sequential incubations with PUB and KCN, employing the conditions described in the legend to Figure 9. The samples were initially 87% DNA I, 13% DNA II, and a very small amount of DNA III. Channel e contains DNA which was not treated with KCN following the PUB incubation. Channels a–d show the results of incubation with KCN at concentrations of 5, 10, 50, and 100 mM, respectively. Electrophoresis was for 4 h at 5 V/cm.

reversal of the reaction. Complete reversibility is clearly not attained, the final buoyant density being 6.9 mg/mL greater than that of the untreated pSM1 DNA I. The initial buoyant shift was 49.9 mg/mL; hence 86% of the PUB-induced buoyant shift can be recovered by KCN incubation. Increasing the KCN incubation time, to as long as 4 h, was without further effect upon the buoyant shift.

In the second set of experiments, samples of PM-2 DNA treated as above were first subjected to dialysis to remove KCN and excess PUB. The DNA was then analyzed by agarose gel electrophoresis as shown in Figure 3. These results are presented in Figure 10 for KCN concentrations of 0, 5, 10, 50, and 100 mM. The reversibility is essentially complete for both components by 100 mM, in agreement with the buoyant results of Figure 9B. No significant nicking of the closed DNA is associated with either the reaction with PUB or the subsequent reversal with KCN.

Finally, experiments were performed to investigate whether or not the residual buoyant shift following cyanide treatment is associated with the presence of supercoiling in the DNA. Two samples of closed PM-2 DNA, of different extents of supercoiling, were incubated with PUB at $r_f = 1.3$ for 30 min at 37 °C in 50 mM NaCl, 10 mM Tris. KCN was then added to a final concentration of 1.0 M and the incubation continued for 45 min at 37 °C, following which the buoyant densities were measured as described for pSM1 DNA I (Figure 9) employing *M. luteus* DNA as a marker ($\theta = 1.7527$ g/mL; Bauer & Vinograd, 1968). For the highly supercoiled sample, superhelix density −0.162 turns per ten base pairs, the buoyant density following cyanide treatment was 8.1 mg/mL greater than that of the untreated DNA. For the less supercoiled sample, −0.088 turns per ten base pairs, the corresponding final buoyant shift was 5.1 mg/mL. For pSM1 DNA I, having −0.098 turns for ten base pairs, the buoyant increment following incubation at 1 M KCN was 7.0 mg/mL. These results suggest a possible correlation between the superhelix density and the extent of the residual buoyant shift. This effect is most likely due to the greater extent of initial reaction in the more highly supercoiled DNAs.

Discussion

Buoyant Density Increments Associated with PUB Binding to DNA. The results reported here demonstrate that large increases in the buoyant density ($\Delta\theta$) accompany the binding of PUB to DNA I. In the case of pSM1 DNA, Figure 5, $\Delta\theta$ approaches 50 mg/mL in CsCl as r_f increases. This shift is due primarily to the binding of the dense platinum moiety, since uracil is absent from the platinum–DNA complex (cf. Figure 7). In the event that disruption of base pairing accompanies PUB binding, the associated DNA denaturation will also contribute to the buoyant increment. This contribution is expected to be relatively small, however, since even fully heat denatured DNA exhibits a buoyant increment of only 15 mg/mL (Vinograd & Hearst, 1962). Similar large increases in the buoyant density in Cs_2SO_4 have been shown to result from the long term incubation of both *trans*- and *cis*-DDP with duplex DNAs from various sources (Stone et al., 1976) and with synthetic polydeoxyribonucleotides (Stone et al., 1976; Amacher & Lieberman, 1977).

Since the nature of the PUB–DNA complex is unknown, it is difficult to relate quantitatively the buoyant increment to the extent of binding. For example, we obtain from an analysis of the results of Stone et al. (1976) the estimate $d\theta/dr_b = 2.14$ g/mL for the binding of *cis*-DDP to calf thymus DNA. The maximum possible value of this derivative in the absence of major contributions from DNA structural changes is, however, only 1.34 g/mL (Bauer & Vinograd, 1969). Such a large discrepancy suggests either that the extent of binding of *cis*-DDP to DNA was seriously underestimated in that study or that the secondary structure of the complex is unlike that of either native or denatured DNA. Knowledge of r_b, the amount of bound platinum per nucleotide, would greatly facilitate analysis of the buoyant density results. An attempt to measure this quantity using a radiolabeled uracil was unsuccessful (vide infra). Further studies are in progress.

The buoyant density of native DNA (θ_0) may be written in terms of the partial specific volumes of anhydrous, neutral polymer ($\bar{v}_D$) and of water ($\bar{v}_H$); and of the preferential hydration (Γ_0') in grams water per gram of DNA (Hearst & Vinograd, 1961).

$$\theta_0 = \frac{1 + \Gamma_0'}{\bar{v}_D + \Gamma_0'\bar{v}_H} \tag{1}$$

Addition of PUB, with corresponding partial specific volume $\bar{v}_b$ and at weight binding ratio r_b', results in an increase in the buoyant density according to (Bauer & Vinograd, 1969)

$$\theta = \frac{1 + \Gamma' + r_b'}{\bar{v}_D + \Gamma'\bar{v}_H + r_b'\bar{v}_b} \tag{2}$$

In the present case platinum is neither bound nor released in the course of centrifugation, but the DNA band is shifted to a region of reduced water activity. This shift results in reduced water binding, and we assume that $\Gamma' = \Gamma_0' + kr_b'$. In the event that $r_b' \ll 1 + \Gamma_0'$, this assumption may be combined with eq 1 and 2 to obtain

$$r_{b2}/r_{b1} = (\theta_1/\theta_2)(\Delta\theta_2/\Delta\theta_1) \tag{3}$$

where $\Delta\theta = \theta - \theta_0$ and the subscripts 1 and 2 refer to any two DNA species.

In the absence of knowledge of $\bar{v}_b$, or of some calibration value of r_b', it is not possible to calculate the binding ratio absolutely. Equation 3 may, however, be used to relate the extent of binding at any r_f to the maximum value of r_b, taken to be that beyond which no further buoyant shift results from an increase in r_f. From the data in Figure 5, r_b changes from zero to 13% of saturation as r_f increases from zero to 1.5; upon increasing r_f from 1.5 to 3.0, the corresponding change in r_b is an additional 60% of the saturation value; finally, r_b increases by approximately 20% as r_f is increased from 3.0 to 4.5. The maximum in the buoyant band width, Figure 5B, is at r_b equal to 80% of saturation. The extent of reversibility by KCN addition, Figure 9, may be calculated similarly. The limiting

buoyant density of the CN^--reversed complex is 6.9 mg/mL greater than that of the untreated DNA; application of eq 3 leads to the result that 14% of the reagent remains bound irreversibly.

Enhanced Reactivity of PUB with Superhelical DNA. The reactivity of DNA I with PUB is clearly greater than that of DNA II, as demonstrated both by gel exclusion and by changes in the buoyant density. This enhancement of binding is not simply a kinetic phenomenon, at least in the region of low to moderate r_f, since the PUB-induced buoyant separation between PM-2 DNAs I and II approaches a constant value at very long incubation times. In this particular case, with incubation at $r_f = 1.3$, the limiting buoyant separation of 14.8 mg/mL corresponds to about 29% of the saturation value shown by pSM1 DNA I. The very slight buoyant shift exhibited by DNA II suggests that only a small amount of reagent is bound under these conditions; Figure 2 shows that all PM-2 DNA I, but no PM-2 DNA II, is excluded from agarose gels at this incubation r_f.

Both PM-2 and pSM1 DNAs I are relatively highly supercoiled, the respective superhelix densities being −0.114 (Upholt et al., 1971; as corrected according to Wang, 1974) and −0.098 (Mickel et al., 1977). Such DNAs are known to display enhanced reactivity toward a variety of reagents which bind to the bases. These include formaldehyde (Dean & Lebowitz, 1971), methymercuric hydroxide (Beerman & Lebowitz, 1973), carbodiimides (Pulleyblank & Morgan, 1975; Lebowitz et al., 1976, 1977), and alkali (Vinograd et al., 1968; Wang, 1974). Our results are therefore consistent with a mode of binding in which accessibility of the bases is a prerequisite for PUB binding. The greater extent of reaction of PUB with DNA I is ascribed to the fraction of bases rendered more accessible in this supercoiled molecule because of bending and torsional stress on the duplex. The amount of the preferential binding should therefore increase with the degree of supercoiling, but experiments to test this point have not yet been performed. The suggestion of Burness et al. (1977) that the primary site of PUB binding is by ionic association with the phosphate residues is not consistent with our results. The suggestion of phosphate binding was based upon experiments in which the conductivity of solutions of calf thymus DNA was measured as a function of added PUB and which were carried out under conditions (deionized water) in which the DNA was certainly denatured. Their observation that the conductivity of PUB solutions is reduced in the presence of DNA might well be explained by removal of PUB by precipitation.

Nature of the Binding of PUB to DNA. The binding of PUB to DNA most likely involves a covalent attachment, the site of which is currently under investigation. This conclusion is supported by (1) the occurrence of a large buoyant density increment in concentrated CsCl; (2) the nearly complete reversibility of the buoyant shift with cyanide ion; (3) the kinetic dependence of the buoyant density changes, reflecting typical platinum(II) to ligand binding properties; and (4) the altered electrophoretic mobility under conditions in which ionically bound reagent would be removed. The observation that [^{14}C]uracil is not associated with the platinum–DNA complex, Figure 7, implies either that the uracil is lost upon binding or that a nonuracil containing component of PUB is the reactive species. In oligomeric structures such as that of Figure 1, the most reactive site for DNA binding is at a terminal platinum atom which has two amine ligands, one donor atom from uracil, and probably a water or hydroxide oxygen atom in the fourth coordination position. Platinum atoms internal to the chain lack a good leaving group such as water and are therefore less reactive. Transfer of a terminal platinum atom from PUB to DNA could result in cleavage of the bond to uracil, which would then remain attached to the penultimate platinum in binding to the DNA. The alternative explanation, that the reactive species is a component of PUB lacking uracil-bound platinum, cannot be ruled out at present. The nature of PUB and related platinum blues in solution is complex and not yet fully understood (Lerner, Bauer, & Lippard, in preparation; Barton & Lippard, unpublished results). It is possible that species such as $[(NH_3)_2Pt(OH)(OH_2)]^+$ exist in aqueous solutions of PUB and are responsible for the reaction with DNA.

The cooperativeness of the binding of PUB to DNA (Figures 2, 3, and 5) is noteworthy. This property undoubtedly originates, at least in part, from the free energy released upon binding to DNA I. Another cause may be the ionic nature of PUB, which is known (Burness et al., 1977; Lerner, Bauer, & Lippard, in preparation) to be a polycation in aqueous solution. It is not surprising that there is a strong interaction of this polycation with polyanionic DNA, leading to precipitation at low ionic strengths (see Figure 8). Knowledge of r_b should provide further insight into the nature of the PUB–DNA binding interaction, and studies to obtain this information are in progress.

Finally, the failure of cyanide to restore fully the DNA to its initial buoyant density deserves some comment. The stability of the $[Pt(CN)_4]^{2-}$ complex is sufficiently great that if any residual platinum were bound to DNA it would most likely require four donor atoms from the bases to protect against cyanide reactivity. Such a situation could only occur if ammonia as well as uracil were released from a fraction of PUB upon DNA binding. This possibility is not wholly unreasonable since aqueous solutions of PUB are known to lose ammonia in the absence of DNA (Barton & Lippard, submitted for publication).

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Kinetic Studies of *Escherichia coli* Transfer RNA (Uracil-5-)-methyltransferase†

Lee Shugart

ABSTRACT: The kinetic mechanism of a semipurified tRNA (uracil-5-)-methyltransferase (EC 2.1.1.35) preparation obtained from *Escherichia coli* has been studied at pH 9.0 in the presence and absence of products. The initial velocity and product inhibition patterns are consistent with a random order of addition of adenosylmethionine and transfer RNA to separate and independent binding sites on the enzyme. Values have been determined for the Michaelis and product inhibitor constants.

An important step(s) in the maturation of transfer RNA[1] (Smith, 1976; Altman, 1975) in prokaryotic organisms such as *Escherichia coli* involves a class of enzymes (Fleissner & Borek, 1962) designated tRNA methyltransferases. Their function is to bring about the substitution of methyl groups onto specific nucleotides (Kerr & Borek, 1973), utilizing as substrates AdoMet and precursor tRNA of the same size as mature tRNA (Davis & Nierlich, 1974).

A considerable body of experimental evidence suggests that methylation of tRNA results in a large number of diverse structural entities which can modulate various biochemical activities of the cell (Starr & Sell, 1969; Borek, 1970; Söll, 1971; Hall, 1971; Kerr & Borek, 1972, 1973; Nau, 1976; Salvatore et al., 1977).

Although the properties of several tRNA methyltransferases isolated from *E. coli* have been reported (Hurwitz et al., 1969a,b; Salvatore et al., 1977), information about the kinetic mechanisms of these enzymes is limited. In this paper, initial velocity measurements, both in the presence and in the absence of products (Cleland, 1970), have been determined in an effort to understand the kinetic mechanism of the enzyme rTase (EC 2.1.1.35) from *E. coli*.

Experimental Procedure

Materials

All chemicals were reagent grade. [^{14}C]CH_3AdoMet (specific activity, 56 Ci/mol) was obtained from Amersham/Searle, and AdoHcy was from Sigma Chemical Co. Aminex A-6, Cellex-D, and Bio-Gel HTP were obtained from Bio-Rad Laboratories. Sephadex G-200 was obtained from Pharmacia Fine Chemicals. An ultrafiltration apparatus, Model 202, was purchased from Amicon Corp.

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[1] Abbreviations used: $tRNA_n$, normal transfer RNA; $tRNA_{md}$, tRNA isolated from *rel*$^-$ mutant of *E. coli* after methionine starvation, a mixture of normal and methyl-deficient species [since relative proportions of two species can vary, see Shugart (1973) for discussion]; AdoMet, adenosylmethionine; AdoHcy, adenosylhomocysteine; A_{260} unit, that amount of tRNA in 1 mL which possesses an absorbance of 1 when measured with a 1-cm optical path at a wavelength of 260 nm; rTase, tRNA (uracil-5-)-methyltransferase; AdoHcyase, adenosylhomocysteine nucleosidase; DEAE-cellulose, *O*-(diethylaminoethyl)cellulose.

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