Kinetic Investigation of the DNA Platination Reaction: Evidence for a Transient Adduct between Deoxyribonucleic Acid and *cis*-Platinum(II)†

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ABSTRACT: Kinetics of the synthesis of adducts between salmon testis DNA and platinum(II) compounds were measured by their effects on DNA synthesis, circular dichroism, and ethidium bromide dependent fluorescence. Transient incorporation of [^{14}C]cyanide into DNA adducts of *cis*-diammineaquochloroplatinum(II) and respectively *cis*-diamminediaquoplatinum(II) compounds but not of *trans*-diammineaquochloroplatinum(II) was observed. A minimal kinetic scheme is derived, in which a transient monodentate DNA–platinum(II) adduct is formed in a bimolecular reaction between DNA and aquated platinum(II) compounds. Second-order rate constants are 2000–3000 M^{-1} min^{-1} for *cis*-diamminediaquoplatinum(II) and 280–400 M^{-1} min^{-1} for *cis*- and *trans*-diammineaquochloroplatinum(II), respectively. The dependence of pseudo-first-order rate constants is not linear for high concentrations of DNA, suggesting competitive formation of more than one primary adduct. The monodentate adducts inhibit DNA polymerase catalyzed DNA synthesis. The bimolecular reaction is followed by a rearrangement (rate constant 0.22 min^{-1}) that gives rise to most of the decrease in the fluorescence intensity and that depends on the state of aquation of the DNA-bound platinum(II) complex. By exchange of coordinated water with a second nucleotide, the monodentate adduct can form cross-links in a reaction joining the rearrangement. Adducts containing a chloro group liberate it by hydrolysis prior to cross-linking. In the case of the *trans*-platinum(II) adduct, the hydrolysis is aided by the trans effect of the bound first nucleotide. The reaction sequence is concluded by a final rearrangement (rate constant 0.02–0.05 min^{-1}) that gives rise to characteristic changes in the spectra of circular dichroism. *cis*- and *trans*-platinum(II)–DNA adducts depart from a common reaction path after formation of the monodentate adduct.

Since its discovery by Rosenberg (Rosenberg et al., 1965, 1969), *cis*-diamminedichloroplatinum(II) has been introduced as a potent therapeutical drug against human testicular and ovarian cancers (Loehrer & Einhorn, 1984). The stereoisomer *trans*-diamminedichloroplatinum(II) is clinically unsuccessful and has less profound antiproliferative and mutagenic effects than the cis isomer (Roberts & Thomson, 1979). Because of severe side effects of the drug, new *cis*-platinum(II) compounds are synthesized worldwide and assayed for improved therapeutical utility.

Biological and molecular evidence favors the interaction of *cis*-platinum(II) with DNA to be responsible for its antineoplastic activity [Pinto and Lippard (1985b) and references cited therein]. Structural analysis of end products revealed that preferred attachment and cross-linking occurred at $(dG)_{n\geq 2}$ segments of DNA (Eastman, 1985; Fichtinger-Schepman et al., 1985). The trans isomer was also incorporated and cross-linked, though with less specificity, to sites of the type d(GpNpG), N = A and C (Pinto & Lippard, 1985a). Inhibition of DNA replication due to template-primer inactivation by platinum(II) compounds was suggested to be responsible for their cytotoxicity (Roberts & Pera, 1983). Catalysis by DNA polymerases was shown to be inhibited (Harder et al., 1976). In the case of DNA polymerase I (*Escherichia coli*), polymerization was shown to be preferentially interrupted at the nucleotide positions mentioned above (Pinto & Lippard, 1985a). The idea was formulated by these scientists that the different antitumor and cytotoxic effects of *cis*- vs. *trans*-platinum(II) are mediated by a faster repair of *trans*- than of *cis*-platinum(II) DNA lesions (Ciccarelli et al., 1985).

With one exception (Johnson et al., 1980) previous investigations did not aim at the kinetics of DNA–platinum(II) interactions. One particular question is whether the reaction route to the known end products involves long-living intermediates. They are likely to be recognized by repair enzymes and to be eliminated or to react spontaneously with a variety of biomolecules, which they encounter during their life span. We report here a kinetic analysis of reactions between DNA and platinum(II) compounds together with an observation of a transient adduct.

Experimental Procedures

Materials

cis- and *trans*-diamminedichloroplatinum(II) and *cis*-diammine(1,1-cyclobutanedicarboxylato)platinum(II) were gifts of Degussa, Frankfurt. Meso and racemate forms of *cis*-[1,2-bis(4-fluorophenyl)ethylenediamine]platinum(II) as sulfates were gifts of Dr. Jennerwein, Regensburg. These compounds, in particular the racemate form, had antitumor activities in hormone-dependent breast cancer cell lines (H. Schönenberger, Regensburg, personal communication).

Salmon testis DNA, ethidium bromide, and $AgNO_3$ were purchased from Sigma, $K^{14}CN$ or $Na^{14}CN$ (60 Ci/mol) and deoxy[*methyl*-^{3}H]thymidine 5′-triphosphate (47 Ci/mmol) were from Amersham, and all other nucleotides were from Boehringer, Mannheim. *Escherichia coli* DNA polymerase I was the same preparation described by Muise and Holler (1985). All other reagents of highest available purity were

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obtained from Merck, Darmstadt.

cis-Diamminediaquoplatinum(II) was prepared by 20-h hydrolysis of the dichloro compound (0.05 mM, pH 5.5, 10 mM KNO_3) at room temperature in the dark. The equilibrium constant for hydrolysis of the second chloro ligand from the platinum(II) complex is 0.4 mM (25 °C; Aprile & Martin, 1962), on the basis of which a fraction of 6% is calculated to remain as a monochloro complex. We have also prepared the diaquo complex by reaction of the dichloro compound with $AgNO_3$ (Scovell & O'Connor, 1977). Results with both preparations were identical.

cis-Diamminemonochloromonoaquoplatinum(II) was prepared by 20-h hydrolysis of the dichloro compound (1 mM, pH 5.5, 10 mM KNO_3) at room temperature in the dark. The equilibrium constant for hydrolysis of the first chloro ligand from the complex is 3.3 mM (Aprile & Martin, 1962), on the basis of which a fraction of less than 20% is calculated to remain as dichloro complex. *trans*-Diamminemonochloromonoaquoplatinum(II) (0.05 mM) was prepared by hydrolysis as in the other cases. The equilibrium constant is 0.32 mM for the first and 0.02 mM for the second chloro ligand (Aprile & Martin, 1962), corresponding to 12% dichloro complex. In accordance with the observation by Johnson et al. (1980), the diaquo complex could not be prepared by the $AgNO_3$ method. Only the first chloro group was replaced by water as estimated by the amount of Ag^+ ions that were titratable after ligand exchange.

The meso and respective racemate forms of *cis*-[1,2-bis(4-fluorophenyl)ethylenediamine]platinum(II) were sulfato complexes. Sulfato ligands are rapidly exchanged with water in agreement with identical results for either fresh solutions (1 mM) or solutions that were kept in the dark for 20 h at room temperature (pH 5.5, 10 mM KNO_3).

Salmon testis DNA was activated by limited digestion with DNase I according to Loeb (1969). The degree of digestion was measured as acid-soluble nucleotides with reference to total DNA in A_{260} units as described. Commercial "native" DNA contained 2% acid-soluble nucleotides. Unless mentioned otherwise, results were independent of the degree of activation (2–15% acid-soluble nucleotides). Before submission to the reaction with platinum compounds, DNA was purified by phenol extraction in the presence of 10 mM Tris-HCl[1] (pH 8) and 10 mM $MgCl_2$ and by subsequent repeated precipitation by ethanol (2 volumes of ethanol and 0.1 M NaCl). The last precipitate was dissolved and dialyzed (two to three changes) against a solution (pH 5.5) containing 10 mM KNO_3. DNA solutions were chloride-free by argentometric determination. Concentrations of DNA were determined photometrically (6360 M^{-1} cm^{-1} at 258-nm wavelength) in terms of nucleotides. Template-primer activities of DNA were routinely measured by the DNA polymerase standard assay in the presence of 0.5 unit of *E. coli* DNA polymerase I.

Methods

DNA Polymerase Standard Assay. The DNA polymerase standard assay was carried out as described (Muise & Holler, 1985) in a 150-μL volume containing 50 mM MOPS buffer (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 3 mM EDTA, 3 mM 2-mercaptoethanol, 33 μM dATP, 33 μM dCTP, 33 μM dGTP, 3 μM [^{3}H]dTTP (1 Ci/mmol), 30 μg of activated DNA (or platinated activated DNA), 80 μg of bovine serum albumin, and DNA polymerase I (0.02–0.4 unit). After 30-min incubation at 37 °C, 2 mL of 10% (v/v) saturated cold trichloroacetic acid was added, and the precipitate was collected on GF/C filters for radioactivity counting in toluene scintillation cocktail. One unit of DNA polymerase activity is defined as the amount of enzyme catalyzing the incorporation of 1 nmol of mononucleotide under assay conditions.

Inhibition of DNA Synthesis. Appropriate amounts of temperature-equilibrated stock solutions of 10 mM KNO_3 (pH 5.5), activated DNA, and platinum(II) compound were mixed in an Eppendorf cup at 37 °C to give a final volume of 200–500 μL. Reactions were carried out in a water bath at 37 ± 2 °C in the dark. Samples (10 μL) were drawn at time intervals and pipetted immediately into a chemical quenching mixture (90 μL) containing 83 mM MOPS buffer (pH 7.5), 83 mM KCl, 17 mM $MgCl_2$, 5 mM EDTA, 4 mM NaCN, and 0.9 mg/mL bovine serum albumin. The mixture was incubated 30 min at room temperature and then cooled on ice. After addition of a deoxyribonucleoside 5′-triphosphate mixture [40 μL, final concentrations 1 μM of each dATP, dCTP, and dGTP and 0.3 μM [^{3}H]dTTP (1 mCi/mmol)], 10 μL (0.5 unit) of *E. coli* DNA polymerase I was added, and DNA synthesis was allowed for 15 min at 37 °C. The amount of radioactivity incorporated into DNA was measured as in the standard polymerase assay. It was verified in control experiments that DNA template-primer activities were preserved throughout the experiments, that platination of DNA was immediately arrested upon addition of cyanide solution, and that this had no effect on template-primer activity of free or platinated DNA even after prolonged incubation time (1–3 h) with the chemical quencher.

Ethidium Bromide Fluorescence Assay. Platination of DNA was followed by the decrease of fluorescence intensity (546-nm excitation wavelength and 590-nm emission wavelength) in the presence of 10–50 μM ethidium bromide (10 mM KNO_3, pH 5.5, 37 °C). The method was similar to that of Howe-Grant et al. (1976) and of Butour and Macquet (1977) except that ethidium bromide was present during platination and 0.4 M salt was omitted during measurements. We could not find any discrepancy with results of controls, in which the dye had been omitted during the platination reaction and had been added only together with NaCl for the purpose of fluorescence measurement. Our method was precise as well as convenient and allowed a continuous measurement in a Perkin-Elmer MPF-2A spectrofluorometer equipped with a thermostated cell holder (10 × 10 mm quartz cells at 37 ± 0.2 °C) and with a BBC SE120 recorder. DNA concentrations varied between 0.1 and 2 mM at a ratio of 0.05–0.15 platinum(II) per DNA nucleotide (mol/mol).

Circular Dichroism. The method was based on results of circular dichroic measurements by Macquet and Butour (1978). A marked increase in ellipticity at 280-nm wavelength was reported for the cis isomer in contrast to a relatively small decrease for the trans isomer of diamminedichloroplatinum(II)–DNA adducts at ratios of 0.1–0.15 platinum (II) per DNA nucleotide (mol/mol). Kinetics of the reactions were followed in a Jasco J-500A spectropolarimeter equipped with a Jasco DP-500N data processor and thermostated (37 ± 0.2 °C) 1- and 2-cm quartz cells. Instrument settings were 0.1–1.0 mdeg/cm sensitivity and 0.1–0.5 cm/min chart speed.

[^{14}C]Cyanide Labeling of Platinum(II)–DNA. A typical platination mixture (1–2 mL) contained 0.25–0.5 mM DNA, 0.025–0.05 mM platinum(II) compound, and 10 mM KNO_3 (pH 5.5) and was incubated at 37 °C. At various time points,

[1] Abbreviations: Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; MOPS, 4-morpholinepropanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; dATP, 2′-deoxyadenosine 5′-triphosphate; dCTP, 2′-deoxycytosine 5′-triphosphate; dGTP, 2′-deoxyguanosine 5′-triphosphate; dTTP, 2′-deoxythymidine 5′-triphosphate.

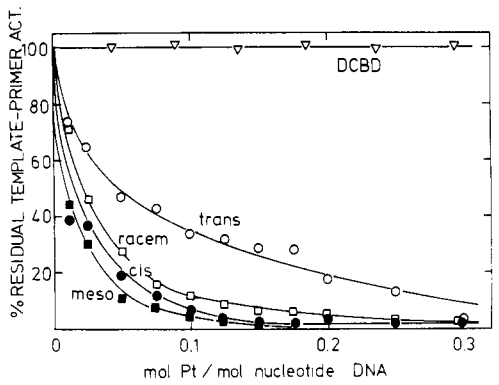

FIGURE 1: Residual template-primer activity for *E. coli* DNA polymerase I as a function of the ratio of platinum(II) incorporated at equilibrium. Samples (100 μL) containing 0.05–1.8 mM platinum(II) compounds and 6 mM activated salmon testis DNA were incubated for 20 h. Aliquots of 15 μL were assayed by the standard polymerase assay. cis = *cis*-diamminedichloroplatinum(II), trans = *trans*-diamminedichloroplatinum(II), meso = *cis*-[1,2-bis(4-fluorophenyl)-ethylenediamine]platinum(II), racem = *cis*-[1,2-bis(4-fluorophenyl)ethylenediamine]platinum(II), and DCBD = *cis*-diammine-(1,1-cyclobutanedicarboxylato)platinum(II).

50–100-μL samples were drawn and immediately mixed with 10–20 μL of freshly prepared solution containing 60 mM $Na^{14}CN$ [(2–10) × 10^6 cpm/μmol] (pH 7–8). The mixture was incubated for 30 min at 37 °C. The samples were then precipitated on ice with cold 10% saturated trichloroacetic acid and prepared for radioactive counting as described for the standard polymerase assay. Support of precipitation by addition of carrier DNA did not increase the yield in precipitable radioactivity but rather enhanced background and was omitted.

Flameless Atomic Absorption Spectrophotometry. Of a typical reaction mixture containing 1 mM DNA and platinum(II) compounds, 10–500 μL was mixed with NH_4Cl (2 M) to give a final concentration of 200 mM Cl^- ions. After 30 min at room temperature, 20 μL of carrier DNA (50 A_{260} units/mL) was added and total DNA precipitated in the presence of 700 μL of 10% saturated trichloroacetic acid. After centrifugation, precipitates were washed twice with 500 μL of cold ethanol, dried in vacuo, and resuspended in 125 μL of distilled water. These samples were analyzed by flameless atomic absorption spectroscopy with reference to a platinum standard (Sigma). Atomic peak heights were recorded at 265.9-nm wavelength in a Model 460 Perkin-Elmer instrument equipped with a graphite furnace. Results by this method were used as controls over the amount of platinum(II) incorporated into DNA after equilibration of the reaction and also to verify that the level of inhibition of DNA synthesis due to bimolecular adduct formation was linearly interrelated with the level of platinum(II) incorporated. We found that in all equilibrated reaction mixtures more than 90% of the total platinum(II) had been incorporated into DNA.

RESULTS

Three Expressions of the DNA–Platinum Reaction: DNA Polymerase Dependent DNA Synthesis, Circular Dichroism, and Ethidium Bromide Dependent Fluorescence. Formation of DNA–platinum(II) adducts was accompanied by physical and biochemical changes, among which are circular dichroism, ethidium bromide dependent fluorescence, and DNA polymerase catalyzed DNA synthesis. Kinetics expressed the same or complementary additional elementary reactions, albeit with different characteristic amplitudes as seen below.

The effect on DNA polymerase catalyzed DNA synthesis was inhibition of template-primer activity. Equilibrium levels of inhibition distinquished between *cis*- and *trans*-platinum(II) compounds and varied with the ratio of platinum incorporated (Figure 1). Levels did not depend on the concentration of DNA (0.1–5 mM) but responded to the degree of DNA activation of DNase I digestion (lower levels of residual template-primer activity at higher degrees of activation). The data in Figure 1 refer to 2% acid-soluble nucleotides after DNase I digestion. The nature of the leaving ligand was not important provided the half-life of its hydrolysis from the platinum compound was short compared with the incubation time; a very slow aquation of *cis*-diammine(1,1-cyclobutanedicarboxylato)platinum(II) has been reported (Knox et al., 1986).

Table I: Rate Constants for Reaction of DNA with Platinum(II) Compounds of the Bimolecular Reaction (k_1), the Fast Rearrangement (k_f), and the Slow Rearrangement (k_2)[a]

platinum(II) complexes	k_1 (M^{-1} min^{-1})[e]	k_f (min^{-1})	k_2 (min^{-1})
cis-diamminemonochloromono-aquo	260[b] (100)		
trans-diamminemonochloro-monoaquo	280[b] (100)		
cis-diamminediaquo	2000[b] (75)	0.22[c]	0.020[b,c]
	3000[d] (25)		0.017[d]
racemate *cis*-1,2-bis(4-fluoro-phenyl)ethylenediamine as sulfate	500[b] (90)	0.23[c]	0.05[b]
			0.04[c]

[a] In the presence of 10 mM KNO_3, pH 5.5, 37 °C. [b] Inhibition of DNA synthesis. [c] Ethidium bromide dependent fluorescence. [d] Circular dichroism at 280-nm wavelength. [e] Values in parentheses are the amplitudes of the bimolecular reactions in percent for the total reaction amplitude. Standard deviations were of the order of 10%.

From an analysis of inhibition kinetics shown in Figure 2 the following results were obtained. (1) The kinetics of diaquo but not of monoaquo compounds were biphasic and followed the superposition of two exponential decays (Figure 2B). (2) The rate constants of the fast but not of the slow exponential as well as the rate constants for monoaquated *cis*- and *trans*-diamminechloroplatinum(II) depended linearly on the concentration of DNA below 1 mM and tended to level off at higher concentrations (inset in Figure 2B). Rate constants were not sensitive to changed relative levels of starting concentrations of [platinum(II)]/[DNA] $\lesssim$ 0.2 at constant concentration of DNA (not shown). This behavior is consistent with the rapid reactions corresponding to the bimolecular attack of platinum on DNA (the observed rate constants were of pseudo first order), and with the slow, second reaction being a subsequent rearrangement of the primary adduct. This last point will be elaborated later. A reason for the nonlinearity of the concentration dependencies will be assessed under Discussion. (3) The rate constants and reaction amplitudes reflected the nature of both the conserved and leaving ligands (Table I; inset in Figure 2B).

Considering rate constants of the linear portion of the concentration dependencies (at low DNA concentrations in Figures 2B and 3C), the bulky groups of the racemate of [1,2-bis(4-fluorophenyl)ethylenediamine]platinum(II), a bidentate conserved ligand, retarded the bimolecular reaction by a factor of 4 in comparison with the amino groups in *cis*-diamminediaquoplatinum(II) (the sulfato groups of the racemate are supposed to be immediately exchanged in solution by water). Rate constants for the second exponential (the supposed rearrangement) were enhanced in the presence of the bulky ligands (Table I). The chloro leaving group lowered the value of the bimolecular rate constant by a factor of 7 when monoaquated diamminechloroplatinumn(II) was compared

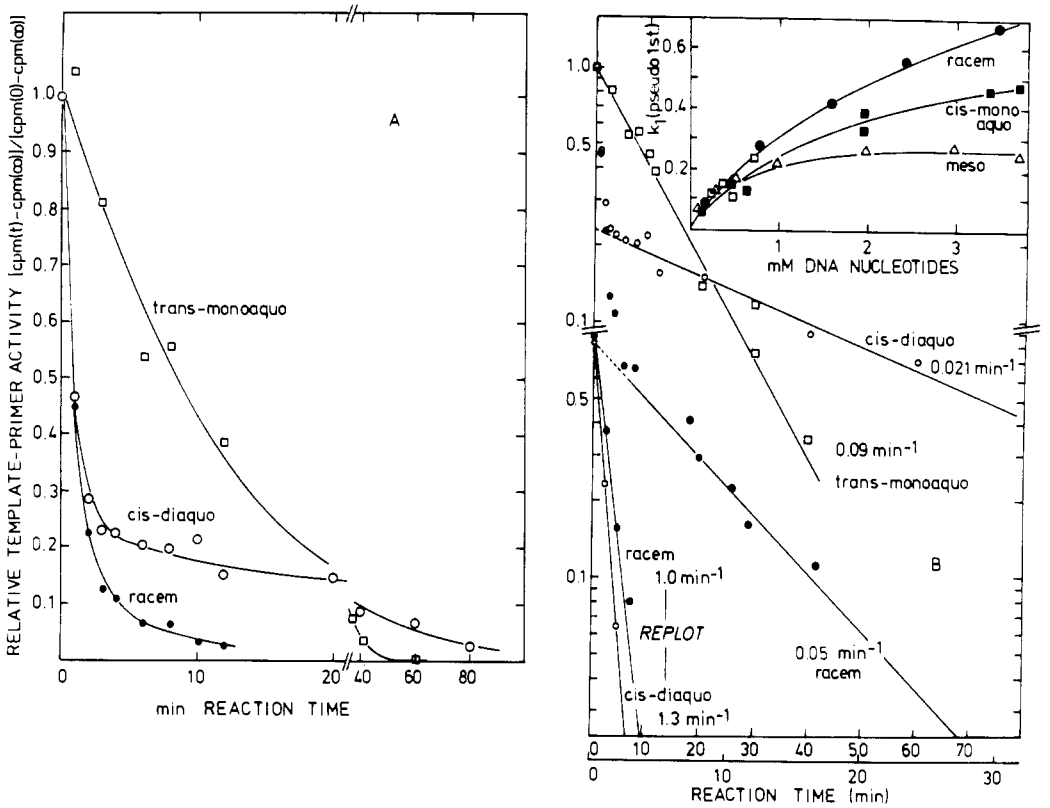

FIGURE 2: Inhibition of *E. coli* DNA polymerase catalyzed DNA synthesis by platinum(II) compounds. (Panel A) The reaction mixture (10 mM KNO_3, pH 5.5, 37 °C) contained 0.5 mM (in terms of nucleotides) DNase I activated salmon testis DNA and 0.02 mM *cis*-diamminediaquoplatinum(II) (○), 0.5 mM DNA and 0.025 mM *trans*-diamminemonochloromonoaquoplatinum(II) (□), or 3.7 mM DNA and 0.15 mM racemate of *cis*-[1,2-bis(4-fluorophenyl)ethylenediamine]platinum(II) (●). Samples were analyzed by the special DNA polymerase assay described under Methods. (Panel B) Semilogarithmic plots were analyzed in terms of two superimposed exponentials, the method of which is shown under Figure 3. Replots for the first exponential are shown in the lower left corner. Pseudo-first-order rate constants obtained from the replots are shown in the inset of panel B and in Figure 3 as a function of DNA concentration. Observed rate constants are indicated. Pseudo-first-order rate constants are given in min^{-1} in panel B inset. The lower time scale in panel B belongs to the replot.

with the diamminediaquoplatinum(II) complex. The stereogeometry of *cis*- and *trans*-diammineplatinum(II) did not have an effect on the magnitude of the bimolecular rate constant (Table I).

The effect of ionic strength was probed in the case of monoaquated *cis*-diamminechloroplatinum(II) by addition of 100, 200, and 400 μM KNO_3 to a reaction mixture containing 2 mM DNA and 0.08 mM platinum(II) complex. Values of rate constants attained a plateau value of 0.20 min^{-1} above 100 mM salt concentration corresponding to 60% of the original activity in the absence of added salt.

Kinetics in the case of *cis*-diamminediaquoplatinum(II) were also measured via circular dichroism at 280-nm wavelength. As in the inhibition of DNA synthesis, they were biphasic displaying rate constants of the first but not of the second exponential to be dependent on the concentration of DNA (Figure 3). Their values were in accord with those in the above inhibition experiments (Table I). The relative magnitudes of the reaction amplitudes were inverse; however, the second exponential contributed 75% of the total change in ellipticity. This high proportion is reasonable for the first exponential to resemble the bimolecular attack and the second exponential the subsequent conformational rearrangement involving DNA secondary structure.

Ethidium bromide dependent fluorescence (Howe-Grant et al., 1976; Butour & Macquet, 1977) was employed for further characterization. Kinetics in Figure 4 show the effects of the state of aquation of diamminedichloroplatinum(II). Those for the diaquo complex were measured in detail. They were found to follow a time dependence of two superimposed exponentials

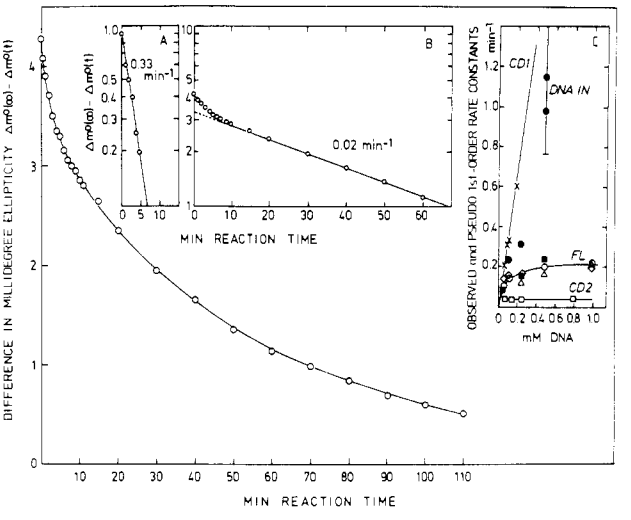

FIGURE 3: Changes in ellipticity during reaction of DNA with *cis*-diamminediaquoplatinum(II). The reaction mixture (2 mL) contained 0.13 mM salmon testis DNA, 0.02 mM *cis*-diamminediaquoplatinum(II) (as nitrate), and 10 mM KNO_3 (pH 5.5, 37 °C). Circular dichroism was continuously measured at 280-nm wavelength (main panel). (Panel A) Replot of the difference obtained after subtraction of the second slow exponential (k_{obsd} = 0.02 min^{-1}) from the ellipticity in panel B. (Panel C) Dependence of pseudo-first-order rate constants [k_1(ps)] from the fast exponential (CD1) and of first-order rate constants from the slow exponential (CD2) as a function of DNA nucleotide concentration. Also shown are pseudo-first-order rate constants measured by the DNA synthesis inhibition assay (DNA IN) and observed rate constants from the fast exponential of the ethidium bromide dependent fluorescence assay (FL, the various symbols refer to DNA with various levels of activation), both for 0.05 mol of *cis*-diamminediaquoplatinum(II)/mol of DNA nucleotides.

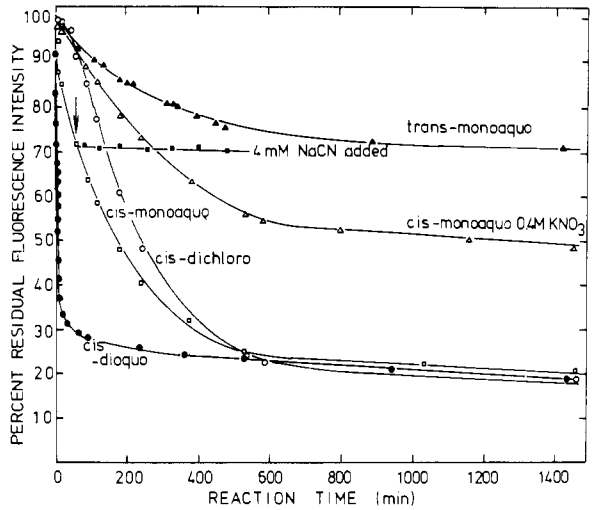

FIGURE 4: Decrease in ethidium bromide dependent fluorescence intensity during reaction of DNA with platinum(II) compounds. The reaction mixture (10 mM KNO_3, pH 5.5, 37 °C) contained 13 μM ethidium bromide, 0.14 mM salmon testis DNA, and 0.02 mM *cis*-diamminediaquoplatinum(II) (●), 0.02 mM *cis*-diamminemonochloromonoaquoplatinum(II) (□), 0.02 mM *cis*-diamminedichloroplatinum(II) (○), or 0.02 mM *cis*-diamminemonoaquomonochloroplatinum(II) in the presence of 0.4 mM KNO_3 (△). A solution of concentrated NaCN has been added at the position of the arrow giving a final concentration of 4 mM NaCN. (▲) Refers to 0.21 mM DNA, 0.048 mM *trans*-diamminemonochloromonoaquoplatinum(II), and 67 μM ethidium bromide.

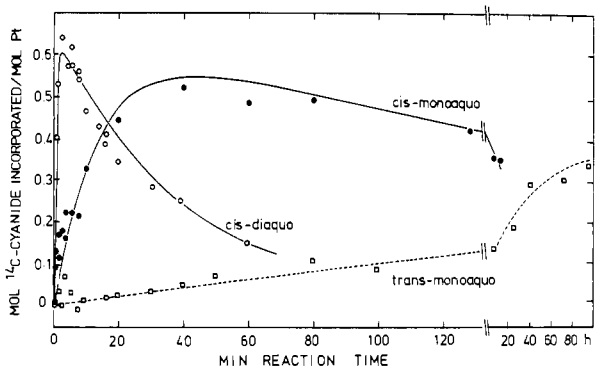

FIGURE 5: Transient monodentate adducts of the reaction between DNA and platinum(II) compounds. The reaction mixture (10 mM KNO_3, pH 5.5, 37 °C) contained 0.025 mM *cis*-diamminediaquoplatinum(II) and 0.25 mM salmon testis DNA (○), 0.05 mM *cis*-diamminemonochloromonoaquoplatinum(II) and 0.5 mM DNA (●), or 0.05 mM *trans*-diamminemonochlormonoaquoplatinum(II) and 0.5 mM DNA (□). Solid curves were calculated with the equation and rate constants described in the text.

(not shown) similarly as by the foregoing methods. The value of the rate constant of the fast exponential increased linearly with varied low concentrations of DNA approaching a limiting value k_f at high concentrations (Figure 3C). The value of the rate constant for the second exponential did not depend on DNA concentration and was of the size of the first-order rate constants measured by the foregoing methods (k_2 in Table I).

The new kinetic result obtained by the fluorescence method was that, in contrast to the previous methods, the value of the fast exponential approached a concentration-independent value k_f (Figure 3C). Furthermore, the kinetics measured for the [1,2-bis(4-fluorophenyl)ethylenediamine]platinum(II) compounds followed the same limiting value k_f at variance with those measured by the inhibition method (Table I). Because of both the concentration independence and the insensitivity against structural variations of the conserved ligand groups, we favor the assumption that k_f refers to another conformational rearrangement of the DNA–platinum(II) adduct that precedes the rearrangement already discovered with the inhibition and circular dichroism methods. It should be stressed that the observed concentration independence was not an artifact due to the presence of ethidium bromide, because the same kinetics were measured for platination in the absence of the dye and for quenching of the reaction by addition of cyanide and then the dye and performance of the fluorescence measurement. In the plateau region of the concentration dependence, more than 75% of the overall fluorescence decrease was generated, indicating that this new conformational rearrangement contributed most of the reaction amplitude.

While the fluorescence intensity decreased in the minutes scale in the case of the diaquoplatinum(II) compounds, that in the case of monoaquated *cis*- and *trans*-diamminechloroplatinum(II) required half-lives of 150–200 min corresponding to rate constants of the order of 0.003 min^{-1} (Figure 4). These reactions were of first order and independent of DNA concentration. Sigmoidal kinetics were observed in the case of *cis*-diamminedichloroplatinum(II) (Figure 4). The rate constants of these reactions were of the order (0.001–0.006 min^{-1}) of those reported for hydrolysis of chloro ligands from platinum(II) compounds [Johnson et al. (1980) and references cited therein; Segal & LePecq, 1985]. The concentration independence and the similarity in the order of magnitudes of rate constants suggested that the observed first-order reactions referred to the hydrolysis as the rate-limiting reaction in these cases of chloro-containing platinum(II) compounds. Indeed, the monoaquated platinum(II) compounds form monodentate DNA adducts at much higher rates than those seen by the fluorescence method (Table I; Figure 4), indicating that this reaction did not perturb fluorescence. With reference to the result with the diaquoplatinum(II) complex, it is concluded that generation of the fluorescence signal required at least the substitution of the second chloro group by water.

The ionic strength dependence of the reactions monitored by the fluorescence method was probed for the diaquo and the aquochloro complexes of *cis*-diammineplatinum(II). In the case of the diaquo complex, values of the rate constant for the rapid exponential fluorescence decay decreased from 100% in the absence to 50% in the presence of 60 mM KNO_3 and to 10% in the presence of 500 mM KNO_3. In the case of the monochloroplatinum(II) compound, the first-order rate constant of the slow reaction (Figure 4) was not affected by the addition of 500 mM KNO_3 to the reaction mixture. However, the reaction amplitude was decreased, whereas no such decrease could be observed in the case of the diaquo complex.

Evidence for Transient Monodentate cis-Platinum(II)–DNA Adduct. In this section it will be shown that the two rearrangement reactions suggested by the above investigation are interconnected by a second coordination reaction of platinum(II) that is already bound to DNA.

A transient monodentate DNA–platinum(II) adduct was resolved by reaction with radioactively labeled cyanide. At various time points, samples were drawn from the DNA platination mixture and reacted with $Na^{14}CN$ (Figure 5). Labeled cyanide that had been reacted with DNA-bound platinum(II) was precipitated together with DNA; free platinum remained soluble. Incorporation of [^{14}C]cyanide into acid-precipitable material was immediate. Prolonged incubation with cyanide did not result in a decrease in radioactivity due to cleavage of DNA–platinum coordination bonds.

Results for *cis*-diamminediaquoplatinum(II) showed a rapid incorporation of radioactivity that passed through a maximum (Figure 5). The time points could be fitted on the basis of the

integrated equation describing a transient formation of a coordination complex between platinum(II) and DNA (for derivation of the equation, see kinetic textbooks):

$$\text{Pt(II)} + \text{DNA} \xrightarrow{k_I} \text{Pt(II)–DNA} \xrightarrow{k_{II}} \text{Pt(II)=DNA} \quad (1)$$

$$\frac{[\text{Pt(II)–DNA}]}{[\text{Pt(II)}]_0} = \frac{k_I(\text{ps})}{k_{II} - k_I(\text{ps})}[\exp[-k_I(\text{ps})t] - \exp(-k_{II}t)] \quad (2)$$

$$k_I(\text{ps}) = k_I[\text{DNA}]_0 \text{ for } [\text{DNA}]_0 \gg [\text{Pt}]_0 \quad (3)$$

where k_I and k_{II} denote corresponding rate constants, $k_I(\text{ps})$ denotes pseudo-first-order rate constant, $[\text{Pt(II)}]_0$ and $[\text{DNA}]_0$ denote total concentrations of platinum(II) compound and of DNA, respectively, Pt(II)–DNA denotes single-coordinated platinum(II)–DNA complex, and Pt(II)=DNA denotes biscoordinated platinum(II)–DNA complex. In deriving eq 1 we assume that only the monodentate DNA–platinum(II) adduct but not the bidentate adduct reacts with cyanide. This seems justified if the remaining leaving ligand in the monodentate adduct is loosely bound water.

The rate constants giving an optimal fit were $k_I = 4000$ M^{-1} min^{-1} and $k_{II} = 0.026$ min^{-1} (Figure 5). The values are in agreement with those for the bimolecular attack (k_1) and for the slow rearrangement (k_2), respectively (Table I). The result implied that either cross-linking (formation of the second coordination) or rearrangement was rate-limiting with respect to each other. However, the rearrangement when observed by circular dichroism could be arrested by addition of cyanide to the DNA platination mixture, indicating that it was not rate limiting.

From the results of several experiments, it was calculated that maximum incorporation of radioactivity corresponded to cyanylation of 50–70% of the total platinum(II) in the reaction mixture assuming mole per mole stoichiometry. Apparently, part of the metal had escaped the reaction with $^{14}CN^-$. Prolonged incubation of the DNA platination mixture (not shown) revealed a second, considerably slower incorporation of radioactivity, the level of which finally remained constant. We assigned this incorporation to the binding of cyanide to monodentate DNA–platinum(II) that could not undergo a second coordination. This implied that *cis*-diamminediaquoplatinum(II) reacted more readily with sites on DNA that were cross-linkable than with sites in which it remained single coordinated.

The same technique was applied to the cis and trans isomers of diamminemonochloromonoaquoplatinum(II) (Figure 5). Reaction with the trans isomer did not exhibit a transient but rather a slow incorporation of radioactivity without a decline, indicating that *trans*-platinum(II) had formed a monodentate adduct of DNA that could not make cross-links during the time of observation. The half-life of the reaction was 150 min, considerably longer than expected on the basis of the results obtained with the inhibition assay of DNA synthesis (half-life less than 10 min, Table I), and comparable with that of hydrolysis of coordinated chloride.

A transient monodentate adduct was observed in the case of *cis*-diamminemonochloromonoaquoplatinum(II) that could react with [^{14}C]cyanide (Figure 5). The time dependence could be fitted on the basis of eq 2 with rate constants k_I = 150 M^{-1} min^{-1} and $k_{II} = 0.0035$ min^{-1}. The values are in accordance with the bimolecular rate constants measured in the DNA synthesis assay (Table I) and with the rate constant for the hydrolysis of the second chloro ligand (see also Figure 4). Thus cross-links were formed with the rate of hydrolysis being limiting. Although this interpretation is tempting, it remains to be shown why, in contrast to the trans isomer, the monodentate, chloro ligand containing intermediate is labeled by [^{14}C]cyanide.

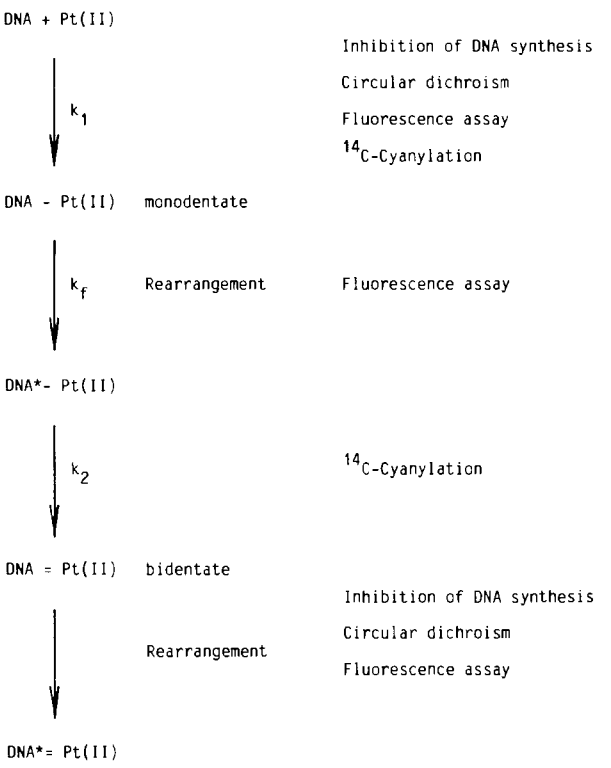

FIGURE 6: Kinetic scheme of the reaction between DNA and *cis*-diamminediaquoplatinum(II). The kinetic techniques are indicated.

Discussion

The results for the reaction of *cis*-diamminediaquoplatinum(II) with DNA are summarized in Figure 6. DNA and the aquated complex form a monodentate adduct via a bimolecular attack, which is resolved by all three methods, the inhibition of polymerase-dependent DNA synthesis, circular dichroism, and ethidium-dependent fluorescence.

The bimolecular reaction is followed by a first rearrangement (k_f in Table I), which is only detected by the fluorescence method. The rearrangement contributes most of the overall reaction amplitude.

The rearrangement is joined by fixation of the second coordinate of bound platinum(II) that simultaneously becomes inaccessible for the reaction with ^{14}C-labeled cyanide (Figure 5). Fixation of the second coordinate on DNA is accompanied by a second, presumably rapid rearrangement of the bidentate DNA–platinum(II) adduct. This arrangement was resolved by all three techniques and contributed most of the reaction amplitude measured by circular dichroism.

Monoaquated diamminechloroplatinum(II) also forms a monodentate DNA adduct. The cis and trans isomers react with the same rates (Table I). Synthesis of this adduct is not monitored by the fluorescence method, presumably because the bimolecular attack as in the case of the diaquoplatinum(II) compounds does not perturb the fluorescence of DNA-bound ethidium bromide. Perturbation of the fluorescence and subsequent ligation of platinum(II) within the primary adduct requires previous hydrolysis of the remaining chloride from the adduct. From the time of hydrolysis, the reaction paths of the *trans*- and *cis*-platinum(II) adducts depart from each other. This is seen by the difference in the overall fluorescence reaction signal (Figure 4) and by the different results in the cyanylation assay (Figure 5) and is known from the different

kinds of products formed during intramolecular cross-linking of DNA [Pinto and Lippard (1985a,b) and references cited therein].

The following observations are of interest. (1) The rate constants for the synthesis of the monodentate platinum(II) adducts depended on the structures of both the conserved and the second leaving groups (Table I). The conserved 1,2-bis-(4-fluorophenyl)ethylenediamine ligand was bulky compared to ammine groups and retarded the reaction, suggesting steric hindrance to inhibit the attack of DNA. The rate of diammineaquochloroplatinum(II) was slower than that with the diaquo complex because of electronic and entropic disadvantages exerted by the remaining chloro ligand. (2) The monodentate adducts were inhibitory for DNA polymerase catalyzed DNA synthesis. In contrast, monofunctional chloro-(diethylenetriamine)platinum(II) has been reported not to inhibit DNA synthesis (Pinto & Lippard, 1985a,b). (3) The dependence of rate constants for the fast exponential measured by the inhibition method did not follow linearity at high concentrations of DNA contrary to the expected behavior of pseudo-first-order reactions (Figure 2B). They followed a tendency to approach a limiting value, the magnitude of which depended on the nature of both conserved and leaving ligands. The nonlinearity of this kind could be the result of a switch from rate limitation by the second-order attack of platinum(II) at low DNA concentrations to rate limitation by a first-order rearrangement of a primary platinum(II)–DNA adduct at high concentrations. It is possible that an increasing portion of platinum(II) binds rapidly and reversibly to phosphate groups when the concentration of DNA is raised. Because these phosphate-directed adducts are instable, they could rearrange to yield base-coordinated adducts. The formation of phosphatoplatinum(II) complexes has been reported (Segal & LePecq, 1985; Bose et al., 1985). (4) The observed effect of ionic strength was to inhibit rates of adduct formation primarily in the case of the bivalently charged diaquoplatinum(II) complex and less in the case of the monovalently charged aquochloroplatinum(II) complex. The correlation between the number of charges of the attacking platinum(II) complexes and the rate of inhibition suggested that attractive electrostatic forces were involved during the reaction that were masked by the electrostatic shielding of added salt. (5) Hydrolysis of the second chloro ligand in free *trans*-diamminedichloroplatinum(II) has been reported to be very slow if not thermodynamically impossible at 10 μM drug concentration (Aprile & Martin, 1962; Johnson et al., 1980; our own results). Yet in the monodentate DNA adduct, this chloride hydrolyzed as fast as the chloride from the DNA adduct of the cis isomer (Figures 4 and 5). We refer this to the trans effect by the bound nucleotide (Howe-Grant & Lippard, 1980).

Our results are compared with those of Johnson et al. (1980), who measured kinetics of incorporation of radioactive ^{195m}Pt into DNA. Rate constants for bimolecular reactions were 8580, 19.2, and 57 M^{-1} min^{-1} for *cis*-diaquo, *cis*-monochloromonoaquo, and *trans*-monochloromonoaquo derivatives, respectively, of diamminedichloroplatinum(II). While the value for the diaquo complex is in the range of our value, those for the other complexes are lower by a factor of 5–10. The discrepancy may be due, in part, to their reaction conditions as are temperature (25 °C), salt ($NaClO_4$), and, in the case of the trans isomer, pH value (pH 7.5).

Our present techniques did not provide information about the nature of residues that coordinated with platinum. For example, replacement of chloride by water was suggested on the basis of comparative values of rate constants and other criteria. In the case of DNA, several possible ligand residues were known from the analysis of end products, among which purines (guanine preferred over adenine) and in particular $(dG)_{n\geq 2}$ (via N7 cross-links) were favored in the case of *cis*-platinum and d(GpNpG) (N = A and C; via N7 cross-links between dG) in the case of *trans*-platinum [Pinto and Lippard (1985a,b) and references cited therein]. Segments of oligo(dG) are better targets than isolated dG residues for a bimolecular attack because of an elevated local "concentration" of guanine residues. This is in accordance with the observed abundance of cross-links within these nucleotide stretches, and we have interpreted our results of cyanylation accordingly.

The nature of the rearrangement reactions is speculative. The second one (described by rate constant k_2) is probably a conformation change as indicated by the relatively large effect on ellipticity and might be ultimately related to the reported shortening of DNA (Macquet & Butour, 1978a,b). The first rearrangement (described by k_f) could be involved as a reaction promoting direct or induced steric hindrance between coordinated platinum(II) and intercalated ethidium bromide.

The conditions of our experiments are not physiological (pH 5.5, low ionic strength, and absence of chloride, magnesium ions, and other competing reactants). Results by Johnson et al. (1980) and results in Figure 4 show, for instance, that under conditions of pH 7 and elevated salt concentrations the rate of bimolecular attack is probably slower than under our conditions. Preliminary results indicate that magnesium ions also tend to depress the rate of the attack (unpublished results). Since the magnitude of such effects on the values of rate constants cannot be estimated at present, the level of accumulation of intermediates remains unpredicted under in vivo conditions. Nevertheless, our results reveal that the reaction between DNA and platinum(II) may be described by a sequence of intermediates until stable products are formed. The biochemical fate not only of the end products but also of intermediates could be important for understanding the mechanism and the design of platinum(II) antitumor drugs.

Registry No. *cis*-Diamminemonochloromonoaquo Pt(II) complex, 53861-42-0; *trans*-diamminemonochloromonoaquo Pt(II) complex, 44046-05-1; *cis*-diamminediaquo Pt(II) complex, 20115-64-4; *cis*-1,2-bis(4-fluorophenyl)ethylenediamine sulfate, 105856-34-6; platinum, 7440-06-4.

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Specific Activation of Transcription Initiation by the Sequence-Specific DNA-Binding Agents Distamycin A and Netropsin†

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ABSTRACT: A series of promoters with nine base-pair substitutions in the spacer DNA separating the −10 and −35 regions was used to demonstrate that *Escherichia coli* RNA polymerase is sensitive to events affecting the spacer DNA—a region not directly contacted by the enzyme. The drugs distamycin A and netropsin specifically enhanced the rate of functional complex formation at a promoter bearing a substitution of nonalternating A-T base pairs. The effect is exerted at an early step in the RNA polymerase–promoter interaction. We hypothesize that a drug-induced structural alteration in the spacer DNA occurs, similar to that normally resulting from RNA polymerase binding. These findings are relevant to an understanding of potential mechanisms of transcription activation.

Escherichia coli promoters have two regions of DNA that are contacted by RNA polymerase in a functional or "open" complex. These stretches are referred to as the −10 and −35 regions to indicate their distances upstream from the start site of transcription (Siebenlist et al., 1980; Hawley & McClure, 1983b; von Hippel et al., 1984; McClure et al., 1985). The contacted regions are optimally separated by a spacer of 17 base pairs. We recently conducted an investigation of the kinetics of open complex formation between RNA polymerase and a series of promoter variants bearing block substitutions in their spacer DNA (see Figure 1). We concluded that the sequence of the spacer DNA can influence the kinetics of open complex formation even though the spacer is not contacted by RNA polymerase in the substituted region (Auble et al., 1986). This effect was ascribed to an altered DNA structure adopted by the substituted spacers (Pulleyblank et al., 1985; Kohwi-Shigematsu et al., 1985) that affected the interaction of RNA polymerase with promoter variants S(CC) and S(GG): evidence has accrued from NMR studies (Sarma et al., 1986) that stretches of DNA consisting of nonalternating G-C base pairs are in the A instead of B form.

The availability of promoters with spacers of defined sequence allowed the targeting of the sequence-selective drugs distamycin A, netropsin, and actinomycin D to this region. Since the binding of each drug alters the structure of the DNA (Kopka et al., 1985; Neidle & Abraham, 1985; Klevit et al., 1986), we hoped to thus alter the spacer structure without interfering with the contacts in the −10 and −35 regions. Actinomycin D (see Figure 2) is an antibiotic inhibitor of RNA synthesis that is specific for G-C-containing DNA. It contains two cyclic polypeptides and a phenoxazone ring system that intercalates between adjacent base pairs of the preferred sequence dGpdC; binding causes unwinding of the DNA helix by about 26° per actinomycin D molecule bound (Sobell, 1973; Van Dyke et al., 1982; Lane et al., 1983; Neidle & Abraham, 1985).

Distamycin A and netropsin (Figure 2) are structurally similar, with distamycin A containing three methylpyrrolecarboxamide rings, while netropsin has two such rings and a guanidinium group in place of the third (Zimmer, 1975). They have very similar specificities for A-T-rich regions of double-helical DNA (Van Dyke et al., 1982; Lane et al., 1983) and apparently a preference for regions with stretches of nonalternating A-T base pairs (Wahnert et al., 1975; Zakrzewska et al., 1983). They bind in the minor groove of B-DNA through hydrogen bonding and hydrophobic interactions, covering four to five base pairs (Van Dyke et al., 1982; Kopka et al., 1985). The binding of netropsin widens the minor groove by several angstroms, and the helical axis of the DNA is bent by 8° per molecule bound (Kopka et al., 1985). Distamycin A apparently introduces similar alterations in the DNA structure (Klevit et al., 1986).

We observe promoter-specific effects that are consistent with the known binding specificities and affinities of each of the

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