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Chemical Reactivity of Monofunctional Platinum-DNA Adducts†

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ABSTRACT: Complexes formed in vitro between *cis*- or *trans*-$PtCl_2(NH_3)_2$ (DDP) and DNA were found to contain monofunctional adducts that reacted with exogenous guanosine. [^{14}C]Guo bound irreversibly to *cis*- and *trans*-DDP-DNA complexes to form bis-Gua adducts. The reaction was first order with respect to the concentration of both [^{14}C]Guo and platinum-DNA complex, but the rate of the reaction varied nonlinearly as a function of the level of platinum binding on DNA. The reaction between [^{14}C]Guo and these platinum-DNA complexes was used to probe the concentration and stability of the monofunctional adducts and to investigate their chemistry in situ. The concentration of monofunctional adducts was highest immediately after reaction of DDP with DNA for 2 h at 37 °C, at which time they represented greater than 15% of the *cis*-DDP-DNA lesions and on the order of 80% of the *trans*-DDP-DNA lesions. The *cis*-DDP-DNA complex reacted with [^{14}C]Guo by two kinetically distinct processes, indicating two types of reactive adducts. The most reactive adduct represented 5% of the platinum lesions. These monofunctional adducts disappeared during the incubation of the platinum-DNA complexes in the absence of drug, probably as a result of chelation to DNA. The half-lives of this chelation at 37 °C, 10 mM $NaClO_4$, were 15 and 30 h for the cis and trans complexes, respectively. Monofunctional adducts were formed on Gua bases in DNA. Hydrolysis was not a rate-limiting step for the reaction between [^{14}C]Guo and the platinum-DNA complex, which suggests that the platinum atom did not possess an inner shell chloride ligand. The stability of these monofunctional adducts and the products formed by their chelation with DNA depend on DNA secondary structure.

Fixation of *cis*-$PtCl_2(NH_3)_2$ on DNA is believed to be responsible for the antitumor activity and other biological effects of this drug (Roberts & Pera, 1983). The trans isomer also reacts with cellular DNA (Pascoe & Roberts, 1974), but it is not antitumoral (Connors et al., 1972; Cleare & Hoeschele, 1973). The majority of the *cis*-DDP[1] molecules rapidly chelate

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with DNA in vitro to form bis-purine adducts (Eastman, 1983; Fichtinger-Schepman et al., 1982, 1985; Johnson et al., 1985). However, there is evidence that some of the DNA lesions formed by *cis*- and *trans*-DDP are made in two steps with different kinetics. In particular, a small number of lesions continue to slowly form bifunctional adducts in the absence of drug. The number of DNA–DNA interstrand cross-links increases during several hours after the removal of *cis*- or *trans*-DDP from mammalian cells (Zwelling et al., 1979a). In addition, posttreatment formation of inter- and intrastrand cross-links in *cis*-DDP–DNA complexes has been observed in vitro (Filipski et al., 1980; Roberts & Friedlos, 1982; Eastman, 1985).

This delayed reaction seems to play an important role in the biological activity of *cis*-DDP. Thiourea reacts with monofunctional platinum–DNA adducts in mammalian cells and prevents the formation of interstrand cross-links. The addition of thiourea to cultured cells immediately after treatment by *cis*-DDP also blocks the mutagenicity and the toxicity of this drug. After several hours incubation in the absence of *cis*-DDP, thiourea no longer inhibits these phenomena (Zwelling et al., 1979b; Bradley et al., 1982). These results indicate that *cis*-DDP initially forms monofunctional adducts that are potential genotoxic lesions and that the biologically active bifunctional adducts are subsequently formed by a slow reaction. Hence, it is important to understand the chemistry of this slow reaction in order to explain the biological effects of *cis*-DDP.

In this study we have trapped the reactive monofunctional adducts by fixing radioactive guanosine on the platinum–DNA complex in vitro. The first part of this paper reports the kinetics of the reaction between Guo and *cis*- or *trans*-DDP–DNA and identifies the products. In the second part, the reaction with [^{14}C]Guo is used to investigate the chemistry of these monofunctional adducts as they chelate with DNA.

Materials and Methods

cis-DDP, *trans*-DDP, and *cis*-[$PtCl(NH_3)_2$(dGuo)]Cl were prepared as previously reported (Kauffman & Cowan, 1963; Dhara, 1970; Johnson et al., 1985). Platinum–DNA complexes were prepared by mixing fresh solutions of DDP and Worthington salmon sperm DNA in 10^{-2} M $NaClO_4$ (Fluka) at 37 °C. In some experiments, the reaction was allowed to proceed for 24 h before adding [^{14}C]Guo. In order to study the disappearance of monofunctional adducts, unreacted DDP was removed after 2-h reaction by ethanol precipitation (Johnson & Butour, 1981), and the washed precipitate was resuspended in 10^{-2} M $NaClO_4$ at 37 °C and incubated for various times before addition of radioactive Guo. The r_i or r_b values of these solutions were determined by flameless atomic absorption.

Radioactive Guo, 0.25 mg/mL in 10^{-2} M $NaClO_4$, was prepared from guanosine purchased from Sigma and [2-^{14}C]Guo from Commissariat à l'Energie Atomique (Saclay, France), final sp act. 1 Ci/mol. Platinum–DNA complex was mixed with radioactive Guo at the desired concentrations, and triplicate aliquots were removed at various times. These were precipitated with 10% ice-cold trichloroacetic acid (Prolabo) and filtered on glass-fiber disks (Whatman GF/C), which were counted in Beckman Ready-Solve EP scintillation fluid. Initial reaction rates were determined from the slope of the linear plot of the acid-precipitable radioactivity vs. time during the first 4 h of the reaction for *cis*-DDP–DNA and during the first 90 min for the trans complex.

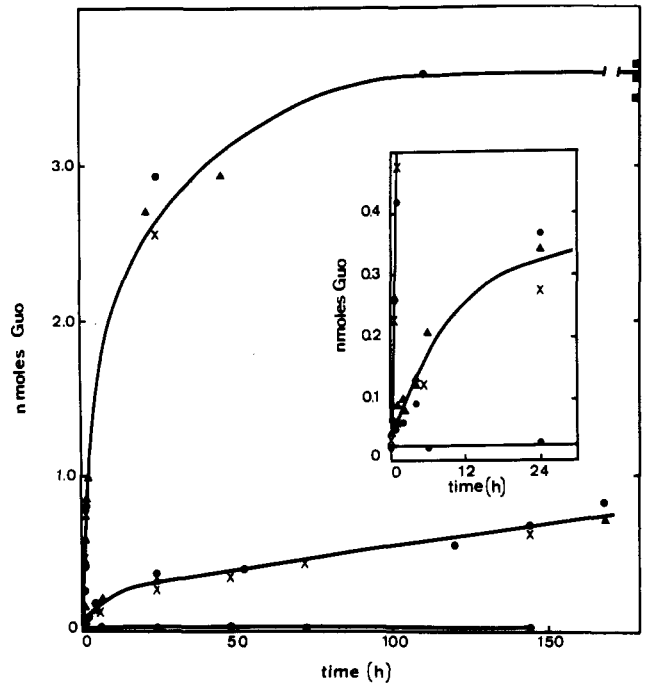

FIGURE 1: Binding of [^{14}C]Guo to *trans*-DDP–DNA (upper curve), *cis*-DDP–DNA (middle curve), or DNA alone (lower curve). DNA was reacted with *cis*- or *trans*-DDP for 24 h at 37 °C, r_b = 0.1 or r_b = 0. Radioactive Guo and 10 mM $NaClO_4$ were then added to give final concentrations of 2.9×10^{-4} M Guo and 3.3×10^{-4} M DNA. The reaction was followed by measuring the acid-precipitable Guo in 0.5-mL aliquots after various times at 37 °C. Different symbols are from independant experiments. Saturation binding for reaction with 2-fold excess Guo (■).

Acid hydrolysis and electrophoresis of the platinum–DNA complexes were performed by previously published methods (Johnson, 1982; Johnson et al., 1985). Chromatography was performed with an Excellulose GF-5 desalting gel (Pierce Chemical Co.) in a 1 × 20 cm column eluted with 10 mM $NaClO_4$.

Results

cis- or *trans*-DDP was reacted with DNA for 24 h at 37 °C, r_b = 0.1, at which time all of the platinum is bound on the DNA. These platinum–DNA complexes were subsequently incubated at 37 °C with radioactive Guo, aliquots were taken at various times, and acid-precipitable radioactivity was determined. Figure 1 shows the kinetics of the fixation of [^{14}C]Guo to these complexes. No Guo precipitated with DNA that had not reacted with DDP. In contrast, Guo continued to bind to *cis*-DDP–DNA for at least 1 week, at which time 5 Guo per 100 platinum atoms had fixed on the platinum–DNA complex. Guo reacted more rapidly with *trans*-DDP–DNA, and the level of binding reached a plateau of 0.22 Guo/Pt after 100 h of reaction.

The initial rate of this reaction as a function of the concentration of each reactant is shown in Figure 2. Double-logarithmic plots of these data revealed that the reaction was first order with respect to [^{14}C]Guo and *cis*- or *trans*-DDP–DNA.

The initial rate of this reaction varied with r_b in a more complex manner (Figure 3). The rate of the reaction between Guo and *cis*-DDP–DNA increased with r_b and approached a plateau at higher levels of platinum fixation. In contrast, the

[1] Abbreviations: DDP, $PtCl_2(NH_3)_2$; r_b, number of bound platinum atoms per nucleotide; r_i, initial number of platinum atoms per nucleotide; en, ethylenediamine; DDP–DNA, complex formed between DDP and DNA.

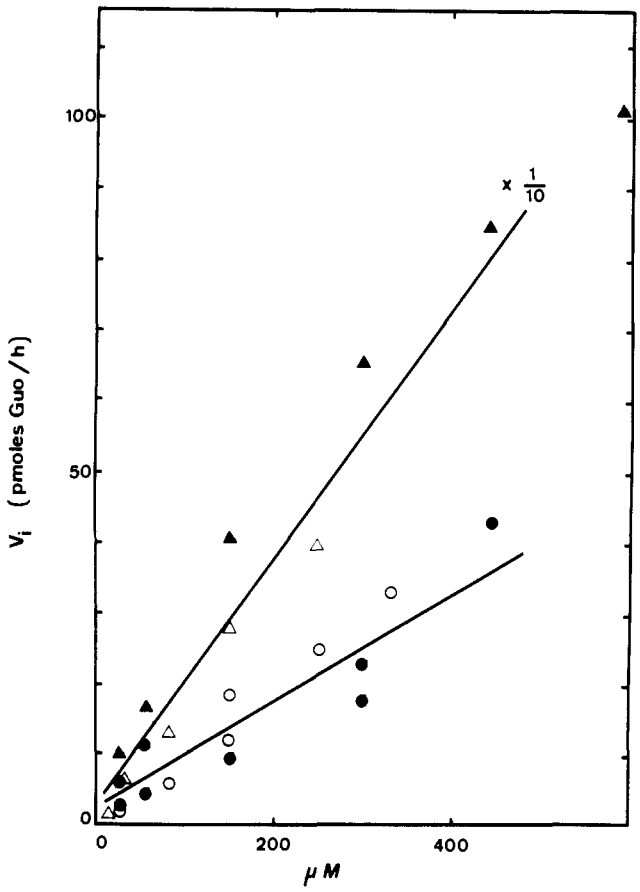

FIGURE 2: Initial rates of [^{14}C]Guo binding to *cis*-DDP–DNA (●, ○) or *trans*-DDP–DNA (▲, △) as a function of the concentration of platinum–DNA complex (open symbols) or [^{14}C]Guo (closed symbols). Reaction conditions were the same as in Figure 1.

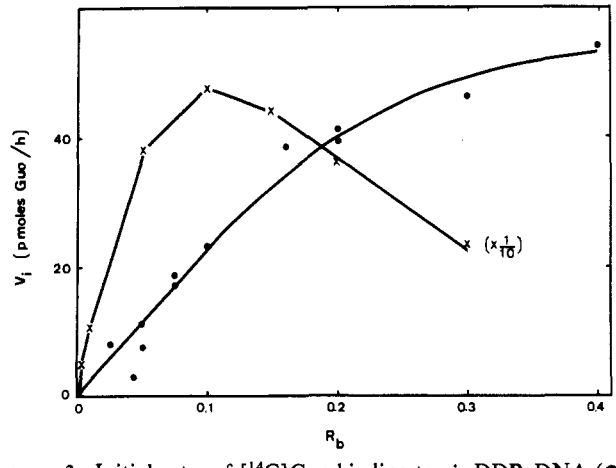

FIGURE 3: Initial rates of [^{14}C]Guo binding to *cis*-DDP–DNA (●) and *trans*-DDP–DNA × $^1/_{10}$ (×) as a function of r_b. Reaction conditions were the same as in Figure 1.

initial rate for the reaction between Guo and *trans*-DDP–DNA increased until r_b = 0.1 and decreased at higher levels of platinum binding. The reaction rate was proportional to r_b for the cis complex, $r_b \leq 0.1$, and for the trans complex, $r_b \leq 0.05$. The concentrations of [^{14}C]Guo that was bound to these platinum–DNA complexes after 24-h reaction showed the same variations with r_b as the initial rates. Hence, the changes in the initial rates that are shown in Figure 3 appear to be the result of different concentrations of reactive platinum–DNA lesions as a function of r_b.

Increasing the concentration of $NaClO_4$ inhibited the reaction (Figure 4). The initial rate decreased by 50% on

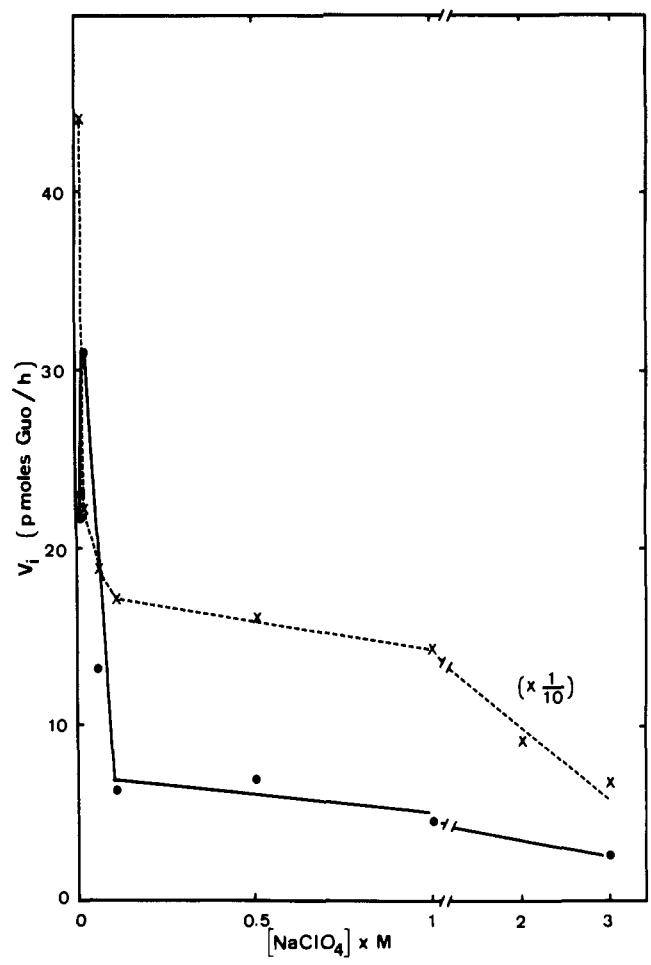

FIGURE 4: Initial rates for the reaction of [^{14}C]Guo with platinum–DNA complexes, r_b = 0.1, as a function of $NaClO_4$ concentration. Formation of platinum–DNA complexes and conditions for the reaction with [^{14}C]Guo were the same as in Figure 1. $NaClO_4$ concentration was adjusted prior to addition of [^{14}C]Guo. *cis*-DDP–DNA (●); *trans*-DDP–DNA × $^1/_{10}$ (×).

increasing $NaClO_4$ from 20 to 50 mM for *cis*-DDP–DNA and from 10 to 30 mM for *trans*-DDP–DNA. Higher salt concentrations decreased the reaction rate more slowly. The quantity of [^{14}C]Guo that was bound to the platinum–DNA complex after 24- and 48-h reaction decreased proportionally to the inhibition of the initial reaction rate. Hence, adding $NaClO_4$ appears to decrease the number of binding sites in the platinum–DNA complex. Lowering $NaClO_4$ from 17 to 7 mM also inhibited the reaction between Guo and *cis*-DDP–DNA. However, this inhibition of the initial rate was not accompanied by lower levels of Guo binding at later times. Apparently, low salt concentrations kinetically inhibit the reaction between Guo and *cis*-DDP–DNA without decreasing the number of reactive sites. A similar inhibition at low salt concentrations was not observed for the trans complex.

Several experiments were performed to test the stability of the products formed in this reaction. Measurement of the r_b before and after the reaction showed that the fixation of Guo did not remove platinum from the polynucleotide. The quantity of acid-precipitable [^{14}C]Guo was not diminished by the addition of 3 M $NaClO_4$, which denatures DNA (Johnson & Schleich, 1974) and might therefore be expected to disrupt reversible binding. Finally, radioactive Guo comigrated with platinum and DNA when the reaction mixture was chromatographed on an Excellulose GF-5 column or subjected to paper electrophoresis. These results show that the [^{14}C]Guo bound irreversibly to the platinum–DNA complex.

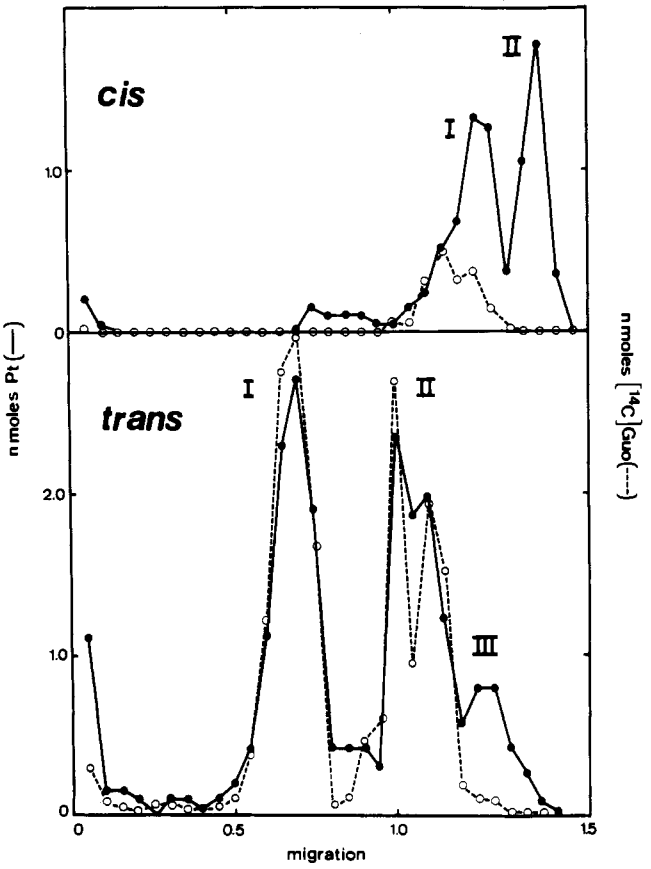

FIGURE 5: Electrophoresis profile of the complex formed between *cis*- or *trans*-DDP–DNA and radioactive Guo. Hydrolyzed samples were placed at the origin, and the abscissa is the distance migrated toward the cathode relative to guanine, which migrated 20 cm in these experiments.

In order to identify the products of the reaction, DDP was added to DNA at $r_i = 0.25$ in 10 mM $NaClO_4$, and after 2 h at 37 °C the platinum–DNA complex was precipitated to remove the unreacted DDP. Under these conditions, 40% of the *cis*-DDP or 80% of the *trans*-DDP was bound to the polynucleotide. The resulting complexes were resuspended in 10 mM $NaClO_4$ and reacted with radioactive Guo for 3 days at 37 °C under reaction conditions that were the same as in Figure 1. These solutions were then dialyzed to remove unreacted Guo, and the products of the reaction between Guo and the platinum–DNA complex were identified by depurination and electrophoresis at acid pH (Johnson, 1982; Johnson et al., 1985). The resulting electrophoresis profiles (Figure 5) show that the radioactive Guo comigrated with previously observed platinum–DNA adducts. For *cis*-DDP–DNA, peak I has been identified as *cis*-$[Pt(NH_3)_2(Gua)_2]^{2+}$. About half of these adducts contained a radioactive ligand. In contrast, no radioactivity comigrated with peak II, *cis*-$[Pt(NH_3)_2(Gua)(Ade)]^{2+}$. For the trans complex, peak I has been identified as *trans*-$[Pt(NH_3)_2(Gua)_2]^{2+}$. Figure 5 shows that after reaction with radioactive Guo all of these bis-Gua adducts contained one radioactive ligand. The remaining *trans*-DDP–DNA adducts have not been identified.

We originally intended to measure the concentration of the monofunctional adducts by using the total radioactive Guo attached to the platinum–DNA complex. However, the reaction with [^{14}C]Guo (Figure 1) did not appear to be sufficiently rapid to quantitatively label these lesions. Therefore, the initial rate of [^{14}C]Guo fixation (which is proportional to the initial concentration of reactive sites on DNA) was used to measure the disappearance of the monofunctional adduct

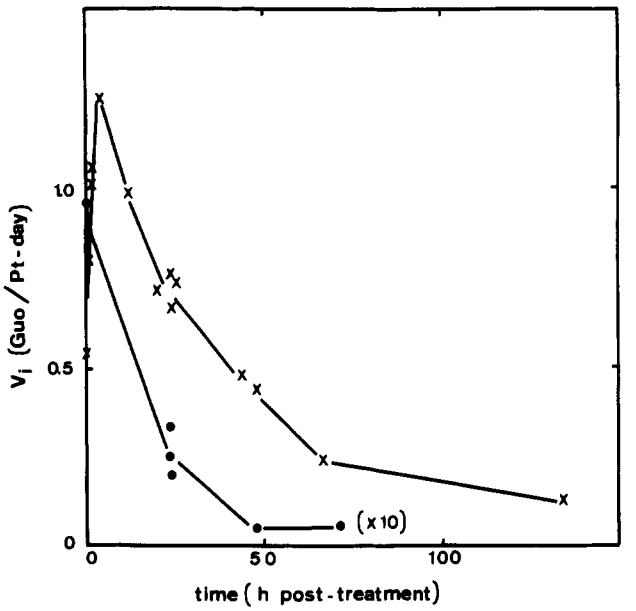

FIGURE 6: Initial rates of fixation of [^{14}C]Guo to *cis*-DDP–DNA × 10 (●) or *trans*-DDP–DNA (×) as a function of time after the fixation of DDP on DNA. DNA and [^{14}C]Guo concentrations were the same as in Figure 1.

during incubation of the platinum–DNA complex in the absence of drug. DNA was reacted with *cis*- or *trans*-DDP for 2 h at 37 °C and then precipitated to remove the unreacted platinum compound. This complex was resuspended in 10 mM $NaClO_4$ and kept at 37 °C. At various times, an aliquot was reacted with radioactive Guo and the initial rate of Guo fixation was determined as above. These experiments were performed at various r_b values between 0.04 and 0.1, and the reaction velocity was normalized to the total concentration of platinum bound to the DNA (Figure 6).

For the *cis*-DDP–DNA complex, the reaction rate decreased during 48-h posttreatment incubation at which time the initial fast reaction (Figure 1) had entirely disappeared. The rates of Guo fixation to *cis*-DDP–DNA after 48 and 72 h in the absence of *cis*-DDP were identical. These results indicate that there are two classes of reactive *cis*-DDP–DNA adducts, one of which disappeared with a half-life of 15 h and the other which was stable at least for several days. The rate of reaction of Guo with *trans*-DDP–DNA increased during the first few hours of posttreatment incubation and thereafter decreased with a half-life of 30 h. In these experiments, the final level of Guo on the *trans*-DDP–DNA complex did not increase with posttreatment incubation, indicating that the increased rate of binding immediately after fixation of the drug to DNA was not due to the creation of additional binding sites. Rather, it appears to be due to an increase in their availability to the exogenous Guo, perhaps as a result of conformational changes brought about by the initial chelation of the monofunctional adduct.

The maximum level of [^{14}C]Guo binding to both platinum–DNA complexes decreased throughout posttreatment incubation. The Guo binding was highest when radioactive ligand was added immediately after fixation of *cis*- or *trans*-DDP on the DNA. For the *trans*-DDP–DNA complex, the maximum ratio of Guo/Pt that could be achieved under these conditions was 0.7 ± 0.1, and for the cis complex, 15 ± 5% of the platinum atoms reacted with radioactive Guo.

It has been previously reported that monofunctional adducts that chelate slowly on DNA react primarly with adenine (Eastman, 1985; Johnson et al., 1985). In order to determine

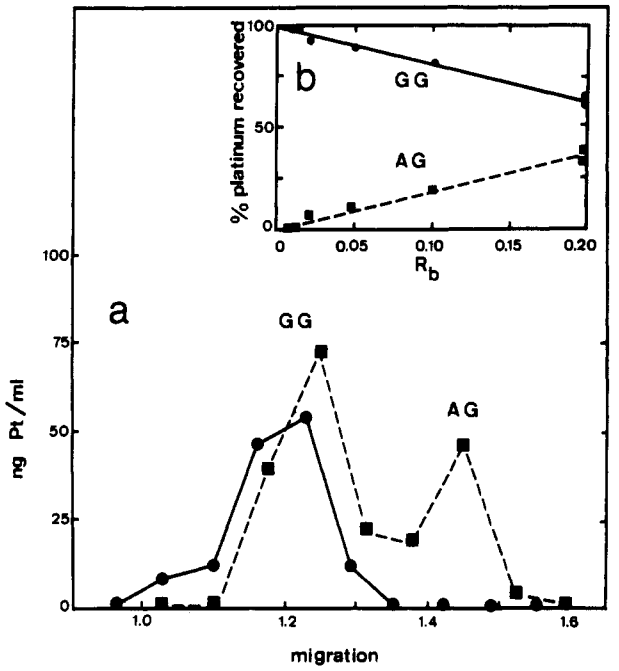

FIGURE 7: (a) Electrophoresis profile of the products of the reaction between *cis*-[PtCl(NH_3)$_2$(dGuo)]Cl and DNA. r_b = 0.01 (●); r_b = 0.2 × $^1/_{10}$ (■). Electrophoresis conditions were the same as in Figure 5. (b) Relative concentrations of the products of this reaction as a function of r_b.

whether this specificity is due to constraints placed on the monoadduct by DNA, we have examined the reaction of *cis*-[PtCl(NH_3)$_2$(dGuo)]Cl (I) with DNA. The products of this reaction were determined by acid hydrolysis and electrophoresis (Figure 7a). For r_b = 0.01, compound I bound exclusively to Gua residues on DNA. At higher r_b values, Ade–Gua adducts appeared, which accounted for 20 and 35% of the products at r_b = 0.1 and 0.2, respectively (Figure 7b).

Discussion

Several techniques have previously been used to measure monofunctional platinum–DNA adducts. Fichtinger-Schepman and collaborators (Fichtinger-Schepman et al., 1982, 1985) trapped the monofunctional adducts formed by *cis*-DDP and DNA by reacting them with NH_4HCO_3 and found that these adducts represent 5% of the platinum–DNA lesions. Eastman (1983) has reported that 10–20% of the adducts formed between $PtCl_2$(en) and DNA after 1 h at 37 °C were able to react with proteins and concluded that these represented monofunctional adducts. Isolation of the monofunctional adduct by acid hydrolysis and electrophoresis showed that 10% of the platinum–DNA lesions are monofunctional after 2-h reaction of *cis*-DDP and DNA at 37 °C, r_b = 0.04 (Johnson et al., 1985).

The present results, based on [^{14}C]Guo binding to *cis*-DDP–DNA, are consistent with these data. The reaction between *cis*-DDP–DNA and Guo did not reach a plateau during the measurement (Figure 1), and 15% of the platinum–DNA adducts that bound [^{14}C]Guo must be considered as a lower limit for the number of binding sites. The *cis*-DDP–DNA complexes reacted with [^{14}C]Guo by two kinetically distinct processes. After 24-h reaction between *cis*-DDP and DNA, the fast reacting species represented 2% of the bound platinum (Figure 1). Immediately after a 2-h reaction between *cis*-DDP and DNA, this fast reacting lesion should account for about 5% of the platinum–DNA lesions (Figure 6). Judging from the initial rates of Guo binding and chelation (Figures 1 and 6), the maximum observed Guo fixation probably underestimates the initial concentration of monofunctional adducts by 10–20%. Taking this correction into account, 80% of the *trans*-DDP–DNA adducts were able to bind radioactive Guo after 2-h reaction between DDP and DNA.

The formation of [$Pt(NH_3)_2(Gua)_2]^{2+}$ during the reaction between [^{14}C]Guo and the *cis*-DDP–DNA complex (Figure 5) indicates that the platinum atom was attached to a Gua base on DNA prior to reacting with exogenous Guo, in agreement with previous work (Fichtinger-Schepman et al., 1982, 1985; Eastman, 1983, 1985; Olinsky & Walters, 1984; Rahn, 1984; Johnson et al., 1985). In contrast, no radioactivity comigrated with *cis*-[$Pt(NH_3)_2$(Gua)(Ade)]$^{2+}$ after the reaction of [^{14}C]Guo with *cis*-DDP–DNA. This result shows that *cis*-DDP does not bind monofunctionally on an Ade in DNA. Rather the AG adducts in DNA are formed by the reaction of a monofunctional Pt–Gua adduct with an adenine base.

The kinetics of the reaction between DDP–DNA and [^{14}C]Guo gives evidence concerning the chemical nature of the monofunctional platinum–DNA adduct in situ. If this adduct contained a Cl^- ligand, then the reaction rate would be described by the general rate law for substitution reactions of Pt(II) chloroamines (Gray, 1962):

$$V_i = [\text{Pt–DNA*}](k_1 + k_2[\text{Guo}])$$

In this equation, [Pt–DNA*] is the initial concentration of reactive DDP–DNA adducts, k_1 is the first-order rate constant for hydrolysis of the chloroamine, and k_2 is the rate constant for the second-order reaction between the adduct and [^{14}C]-Guo. The plot of initial rate as a function of [^{14}C]Guo concentration passes through the origin (Figure 2), indicating that the monofunctional adduct does not have an inner shell chloride.

Several results show that the chemistry of these monofunctional adducts depends on DNA secondary structure. For example, the effect of low salt concentrations on the number of monofunctional platinum–DNA adducts (Figure 4) is correlated with the variation of the melting temperature of DNA with ionic strength (Marmur & Doty, 1962). Stabilizing the DNA appears to decrease the accessibility of the monofunctional adduct to [^{14}C]Guo and/or favor its chelation with DNA. Second, a compound that chemically resembles the monoadduct, *cis*-[PtCl(NH_3)$_2$(dGuo)]Cl, reacts primarily with guanine bases on DNA (Figure 7). In contrast, it has recently been reported that the monofunctional platinum–DNA adduct that undergoes a slow chelation with DNA reacts primarily with adenine (Eastman, 1985; Johnson et al., 1985). Hence, the products formed by chelation of the monofunctional adduct with DNA are not only a consequence of its reactivity but also depend in part on its placement in DNA.

These results show that the stability of the monofunctional adducts and the products formed by their chelation on DNA depends in part on topological constraints placed by the polynucleotide on the platinum atom and the incoming ligand. It is possible that in vivo the maximum concentration of monofunctional adducts would be found in relatively unstable double-stranded DNA such as the replicating region of the genome. The role of DNA secondary structure in this reaction clearly merits further study.

The majority of the DNA lesions formed by *cis*-DDP do not react with exogenous Guo. In these adducts, *cis*-DDP has chelated with adjacent purine bases on the same strand of DNA (Fichtinger-Schepman et al., 1982, 1985; Eastman, 1983). Because of the orientation of the labile ligands,

trans–DDP cannot chelate adjacent nucleobases, and after 2-h contact with DNA, 80% of the platinum–DNA lesions formed by *trans*-DDP are monofunctional adducts. The greater concentration of protein–DNA cross-links observed after treatment of mammalian cells (Zwelling et al., 1979a) and nucleosomes in vitro (Lippard & Hoeschele, 1979) with *trans*-DDP compared to *cis*-DDP is probably a consequence of the greater concentration of stable monofunctional adducts formed in the *trans*-DDP–DNA complex.

In contrast with the rapid chelation of *cis*-DDP on adjacent nucleobases, the monofunctional adducts observed in these experiments disappear with half-lives of 15 and 30 h for *cis*- and *trans*-DDP–DNA, respectively (Figure 6). The formation of interstrand DNA–DNA cross-links (Zwelling et al., 1979a,b; Roberts & Friedlos, 1982) and non-nearest-neighbor intrastrand cross-links (Eastman, 1985) and the disappearance of the monofunctional adducts (Figure 6) all occur simultaneously. The monofunctional adduct does not possess an inner shell chloride ligand. Therefore, the slow kinetics of disappearance of these monofunctional adducts in Figure 6 is not due to the slow kinetics of aquation. Rather, it appears to be a consequence of the chelation of monofunctional adducts with non-nearest-neighbor purine bases. Hence, the stability of the monofunctional adducts observed in these experiment is probably the result of their separation from potential nucleophilic ligands, which is maintained by DNA secondary structure.

Registry No. *cis*-DDP, 15663-27-1; Guo, 118-00-3; *cis*-[PtCl-$(NH_3)_2$(dGuo)]Cl, 98064-87-0; *trans*-DDP, 14913-33-8.

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