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Adducts of the Antitumor Drug *cis*-Diamminedichloroplatinum(II) with DNA: Formation, Identification, and Quantitation†

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ABSTRACT: Salmon sperm DNA, treated with the antitumor agent *cis*-diamminedichloroplatinum(II) (*cis*-DDP), was enzymatically degraded to (oligo)nucleotides. Four Pt-containing products were identified by ^{1}H NMR after preparative chromatography on a diethylaminoethyl-Sephacel column at pH 8.8. In all identified adducts, comprising approximately 90% of the total Pt in the DNA, Pt was linked to the N7 atoms of the nucleobases guanine and adenine. The two major adducts were *cis*-Pt$(NH_3)_2$d(pGpG) and *cis*-Pt$(NH_3)_2$d(pApG), both derived from intrastrand cross-links of *cis*-DDP on neighboring nucleobases. Only the d(pApG) but not the d(pGpA) adduct could be detected. Two minor adducts were Pt$(NH_3)_3$dGMP, resulting from monofunctionally bound *cis*-DDP to guanine, and *cis*-Pt$(NH_3)_2$d$(GMP)_2$, originating from interstrand cross-links on two guanines as well as from intrastrand cross-links on two guanines separated by one or more bases. For analytical purposes we developed an improved method to determine *cis*-DDP adducts. Routinely, 40-μg samples of enzymatically degraded *cis*-DDP-treated DNA are now analyzed by separation of the mononucleotides and Pt-containing (oligo)nucleotides on the anion-exchange column Mono Q (FPLC) at pH 8.8 (completed within 14 min) and subsequent determination of the Pt content in the collected fractions by atomic absorption spectroscopy. The method was used to optimize the digestion conditions for *cis*-DDP-treated DNA. In kinetic studies on the formation of the various adducts, a clear preference of the Pt compound to react with guanines occurring in the base sequence d(pGpG) was established.

The antitumor drug *cis*-diamminedichloroplatinum(II) (*cis*-DDP)[1] is a frequently used chemotherapeutic agent; its working mechanism, however, is not fully clarified yet [for reviews, see Marcelis & Reedijk (1983) and Lippard (1982)]. It is generally accepted now that the antineoplastic activity of the drug is based on its interaction with cellular DNA leading to the formation of various types of adducts. In Figure 1 the hitherto known *cis*-DDP-DNA adducts are schematically depicted. Apart from these adducts, DNA–protein cross-links are formed by *cis*-DDP (Plooy et al., 1984), but these adducts are outside the scope of this study. Recently, we (Fichtinger-Schepman et al., 1982) and other investigators (Johnson, 1982; Eastman, 1983) reported methods to inventory the adducts in DNA by degrading *cis*-DDP-treated DNA, followed by chromatography of the resulting products.

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[1] Abbreviations: *cis*-DDP, *cis*-diamminedichloroplatinum(II); *cis*-DDP-DNA, salmon sperm DNA modified with *cis*-DDP in vitro; r_b, number of Pt atoms bound per nucleotide; r_t, total input drug–nucleotide ratio in solution; AAS, atomic absorption spectroscopy; ^{1}H NMR, proton nuclear magnetic resonance; FPLC, fast protein liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl.

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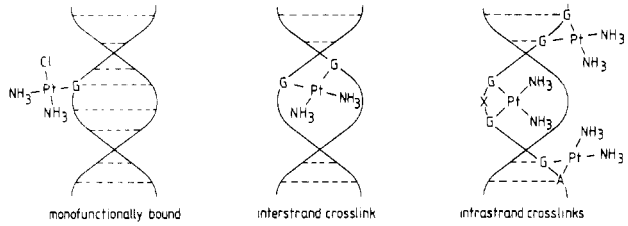

FIGURE 1: Schematic representation of the hitherto known adducts formed in DNA upon treatment with *cis*-DDP.

In this paper we present a preparative separation, by anion-exchange chromatography, of the mononucleotides and Pt-containing (oligo)nucleotides resulting from enzymatic digestion of *cis*-DDP-DNA, followed by the identification of the major *cis*-DDP adducts by ^{1}H NMR. Furthermore, for analytical purposes, we developed an improved separation method based on superior chromatographic materials (FPLC), which allowed the rapid analysis of digests of Pt-containing DNA samples. This method was applied in the cochromatographic identification of adducts. In addition, it was used in experiments aimed at the optimization of the conditions for the enzymatic degradation of *cis*-DDP-treated DNA and in studies on the kinetics of the formation of *cis*-DDP adducts in DNA in vitro.

Materials and Methods

cis-DDP Binding to DNA, dGMP, and dGuo. The treatment of salmon sperm DNA (Millipore Corp.) with *cis*-DDP and the subsequent inactivation of monofunctionally bound *cis*-DDP with NH_4HCO_3 (Fichtinger-Schepman et al., 1984b) were performed as described before (Fichtinger-Schepman et al., 1982), except that *cis*-DDP was now dissolved in water instead of in dimethyl sulfoxide, to prevent possible substitution and isomerization reactions (Kerrison & Sadler, 1977). In all cases the DNA concentration was 0.5 mg/mL and the *cis*-DDP concentration 10 or 25 μg/mL, resulting in a total input drug–nucleotide ratio in the solution (r_t) of 0.022 or 0.055.

$Pt(NH_3)_3$dGMP was synthesized from 2′-deoxyguanosine 5′-monophosphate (Na salt; Sigma) and *cis*-DDP in a 1:1 ratio as described before (Fichtinger-Schepman et al., 1984b). Subsequent dephosphorylation with alkaline phosphatase, type III [EC 3.1.3.1; Sigma; suspension in 2.5 M $(NH_4)_2SO_4$] yielded $Pt(NH_3)_3$dGuo.

Enzymatic Degradations. Routinely, enzymatic digestions of *cis*-DDP-DNA were carried out as follows: the samples (250 μg) were dissolved in 200 μL of buffer A [10 mM Tris-HCl, pH 7.2, containing 0.1 mM of the disodium salt of ethylenediaminetetraacetate (EDTA) and 4 mM $MgCl_2$]. After addition of 4.8 μL of 10 mM $ZnSO_4$ and NaN_3 to a final concentration of 0.02%, the mixtures were incubated for 16 h at 37 °C in the presence of 12 units of deoxyribonuclease I (EC 3.1.21.1; Sigma) and 20 μL of nuclease P1 (EC 3.1.4.-; Boehringer; dissolved in 30 mM sodium acetate, pH 5.3, at a concentration of 1 mg/mL). Finally, the digests (total volume 235 μL) were heated for 3 min at 100 °C and centrifuged for 10 min in an Eppendorf centrifuge.

To digest 200 mg of *cis*-DDP-treated DNA, 5000 units of deoxyribonuclease I and, initially, 1.5 mL of nuclease P1 were added to the DNA solution (33.7 mL) in water, containing 2 mL of the 10 times concentrated buffer A (see above), 480 μL of 10 mM $ZnSO_4$, and 650 μL of 0.65% NaN_3. After 20 h at 37 °C, the solution was concentrated by evaporation under diminished pressure to 24 mL, which raised the salt concentrations to those in buffer A. However, to obtain maximal digestion, more nuclease P1 was required: an additional 2.7 mL of the enzyme stock had to be used, which was added in six portions. After each addition, the mixture was incubated for 8 or 16 h at 37 °C, followed by FPLC analysis. Finally, the mixture was heated for 3 min at 100 °C.

Anion-Exchange Chromatography on DEAE-Sephacel. The 200-mg *cis*-DDP-DNA digest was diluted with water to 100 mL, adjusted to pH 8.8, and put on top of a 200-mL DEAE-Sephacel (Pharmacia) column, equilibrated in 5 mM Tris-HCl, pH 8.8. Elution was performed with the same buffer. Fractions of 10 mL were collected at a flow rate of 37.5 mL/h. From fractions 11–81, a 0–350 mM linear NaCl gradient (2 × 400 mL) in the buffer was applied, followed by elution with 1 M NaCl in the buffer.

Rechromatography at pH 7.5 of the *cis*-$Pt(NH_3)_2$-d(pApG)-containing fractions, after desalting on a Sephadex G-25 column (eluted with 0.02 M triethylammonium hydrogen carbonate) and lyophilization, was performed on a 45-mL DEAE-Sephacel column—equilibrated in 5 mM Tris-HCl, pH 7.5—by using a 0–150 mM linear NaCl gradient (2 × 60 mL) in the buffer. Fractions of 1.5 mL were collected at a flow rate of 10 mL/h.

Fast Protein Liquid Chromatography (FPLC) on Mono Q. For chromatography on the anion-exchange column Mono Q (Pharmacia), an FPLC system (Pharmacia) equipped with a Pye Unicam variable-wavelength detector and a Spectra-Physics SP 4100 computing integrator was used. The Mono Q column was equilibrated in buffer B (12.5 mM Tris-HCl, pH 8.8). The elution of *cis*-DDP adducts and unmodified nucleotides from the column was effected by admixing increasing concentrations of buffer C (buffer B with 1 M NaCl) to buffer B, as follows: 1-min isocratic elution with 5% C, then 5–7.5% C in 3 min, 3.5-min isocratic elution at 7.5% C, 7.5–10% C in 3 min, and finally 10–50% C in 2 min, at a flow rate of 1 mL/min. Then, the column was washed with 100% C for 2.5 min followed by buffer B for 2.5 min at a flow rate of 1.75 mL/min.

Platinum Determinations. The amount of DNA-bound platinum was determined from atomic absorption spectroscopy (AAS) and UV-spectroscopy data (Fichtinger-Schepman et al., 1982).

Nuclear Magnetic Resonance (NMR). ^{1}H NMR spectra of *cis*-DDP adducts were recorded on a Bruker WM300 spectrometer after desalting of the samples on Sephadex G-25 (eluted with 0.02 M triethylammonium hydrogen carbonate), lyophilization, and dissolution in 99.95% D_2O. Tetramethylammonium nitrate was used as an internal reference. If necessary, selective irradiation was used to reduce the HDO signal.

Results

Preparative Chromatography of the cis-DDP-DNA Adducts. In order to obtain sufficient amounts of *cis*-DDP-DNA adducts for ^{1}H NMR analysis, 200 mg of salmon sperm DNA was treated with *cis*-DDP (r_t = 0.055) for 5 h at 50 °C in the dark, followed by treatment with NH_4HCO_3 to inactivate the monofunctionally bound platinum (Fichtinger-Schepman et al., 1984b). After the reaction, the DNA contained 64 atoms of Pt/1000 nucleotides (r_b = 0.064). It was enzymatically degraded to mononucleotides and Pt-containing (oligo)nucleotides, which were separated on a DEAE-Sephacel column at pH 8.8. A relatively high pH was chosen, because under these conditions guanine residues involved in the Pt binding are largely deprotonated at N1 (see Figure 2; Marcelis et al., 1982; Lippert, 1981), which facilitates the separation of the various products. In the eluate fractions, the presence of

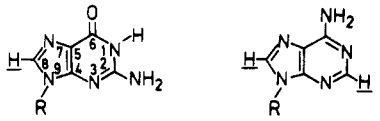

FIGURE 2: Structure and numbering scheme of the nucleobases guanine (left) and adenine (right). The nonexchangeable protons (H8 of guanine; H2 and H8 of adenine) are underlined.

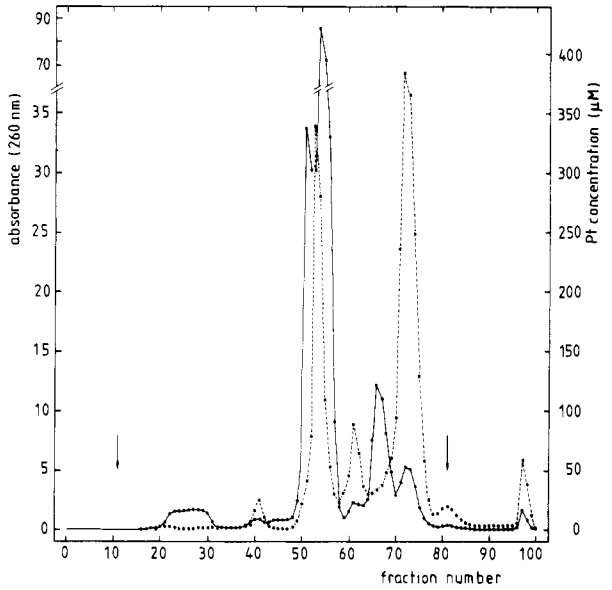

FIGURE 3: DEAE-Sephacel elution pattern of 200 mg of enzymatically digested salmon sperm DNA, which had been treated with *cis*-DDP at r_t = 0.055 for 5 h at 50 °C in the dark. Arrows indicate the start and end of the gradient elution (0–350 mM NaCl) in 5 mM Tris-HCl, pH 8.8. Finally, the NaCl concentration was raised to 1 M. Fractions of 10 mL were collected in which the absorbance at 260 nm (—) and the Pt concentration in μM (---) were measured. For the identity of the components of each peak, see Table I.

nucleotides (A_{260nm}) as well as platinum (atomic absorption spectroscopy) was determined (Figure 3). The elution position of the four common deoxymononucleotides, eluted from the column in the major UV-absorbing peaks, could be established by means of cochromatography of an aliquot from the appropriate peak fractions with reference compounds in the FPLC system described below (see Table I). The fractions containing Pt adducts were pooled per peak. This material was used for the analysis by ^{1}H NMR spectroscopy, after further purification and desalting on a Sephadex G-25 column. Only the material eluted around fraction 53 of the DEAE-Sephacel column (Figure 3) required additional purification by means of ion-exchange chromatography. To separate this Pt adduct from the accompanying mononucleotides, the material was chromatographed on a second DEAE-Sephacel column, now at pH 7.5.

Identification of the cis-DDP-DNA Adducts. The identification by ^{1}H NMR spectroscopy was based on monitoring of the nonexchangeable base protons of guanine (H8) and adenine (H2 and H8) (see Figure 2). In general, the chemical shifts of these protons move downfield upon binding of the Pt compound to the nucleobases. The pH dependence of the chemical shifts reveals whether or not the N7 position of the bases can still be protonated at low pH; platination at this site excludes protonation. In addition, upon platination at N7 a lowering of the pK_a value of the N1 deprotonation should be observed. Further evidence for assignment of the adducts was obtained by mixing them with the synthetic analogues to achieve identical chemical and physical conditions and, therefore, overlapping ^{1}H NMR spectra. The following adducts were identified.

Table I: Preparative (DEAE-Sephacel) and Analytical (FPLC-Mono Q) Chromatography of Enzymatically Digested *cis*-DDP-DNA (r_b = 0.064)[a] at pH 8.8

	DEAE-Sephacel			Mono Q		
(oligo)nucleotide	fraction no.[b]	%[c]	N^d	retention time (min)	%[c]	N^d
dCMP	51			5.0		
dAMP	54			5.8		
TMP	54			6.4		
dGMP	66			12.0		
$Pt(NH_3)_3$dGMP[e]	41	2	0.001	1.7	3	0.002
cis-$Pt(NH_3)_2$-d(pApG)	53	28	0.018	3.5	23	0.015
cis-$Pt(NH_3)_2$-d(pGpG)	72	50	0.032	7.8	47	0.030
cis-$Pt(NH_3)_2$-$d(GMP)_2$	61	10	0.007	9.7	8	0.005

[a] Number of Pt atoms bound per nucleotide in salmon sperm DNA treated at r_t = 0.055 for 5 h at 50 °C. [b] The fraction of the eluate containing the top of the eluted peak. [c] The quantity of the adduct expressed as percentage of the total amount of platinum eluted from the column. [d] Number of *cis*-DDP adducts of this type that occur per nucleotide in *cis*-DDP-treated DNA. [e] Identified in the DEAE-Sephacel fractions as $Pt(NH_3)_3$dGuo.

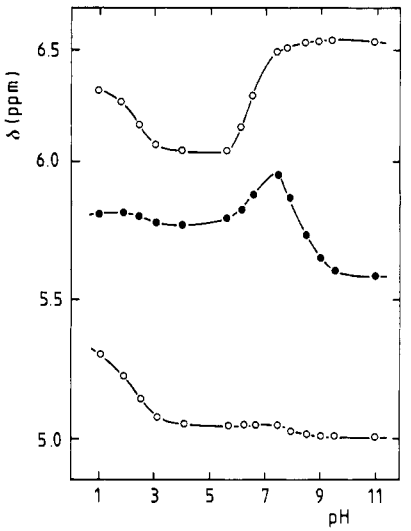

FIGURE 4: Chemical shifts (δ) of the nonexchangeable base protons of *cis*-$Pt(NH_3)_2$d(pApG) in ^{1}H NMR spectra recorded at various pH values. (●) H8 of guanine; (○) proton signals of adenine; the upfield signal originates from the H8, the downfield one is H2.

(*1*) *cis-Pt(NH₃)₂d(pApG).* The Pt adduct eluted around fraction 53 of the DEAE-Sephacel column at pH 8.8 showed an r_b value of 0.55 after the additional DEAE-chromatography at pH 7.5 and the subsequent desalting on Sephadex G-25. The structure of this product was proven to be *cis*-Pt-$(NH_3)_2$d(pApG), with Pt attached to both adenine and guanine, from the pH dependency of the base proton signals in the ^{1}H NMR data, as shown in Figure 4. The presence of N7-platinated guanine was demonstrated by the H8 proton signal showing a lowered pK_a of 8.5 for the N1 deprotonation and no N7 protonation at low pH values (Marcelis et al., 1982). The two other signals shown in Figure 4 are in agreement with what would be expected for the base protons of N7-platinated adenine, with an apparent pK_a of 2.5 for the N1 protonation (Den Hartog et al., 1984) and no N7 protonation at low pH. The integrated intensities of all these protons are in agreement with a 1:1 ratio of the two nucleobases in this Pt adduct. Because of the large influence of the phosphate protonation at pH 6 on the adenine H8 resonance compared to the effect seen in the guanine signal, it was

concluded that the platinum was bound to d(pApG) rather than to d(pGpA). Additional information was obtained from the position and the splitting pattern of both H1 resonances (data not shown), which confirmed this conclusion and, furthermore, excluded the presence of an adduct formed by binding of the platinum compound to the monomers AMP and GMP via their N7 atoms. Finally, after the isolated adduct was mixed with synthetic *cis*-$Pt(NH_3)_2$d(pApG), overlapping spectra were obtained.

(*2*) *cis-Pt(NH₃)₂d(pGpG).* The largest Pt-containing peak, eluted around fraction 72 of the DEAE-Sephacel column, resulted in two UV-absorbing peaks upon desalting on Sephadex G-25. The major peak (r_b = 0.52) contained *cis*-$Pt(NH_3)_2$d(pGpG) as was deduced from the ^{1}H NMR spectra, which were in full agreement with our previous data on this adduct (Fichtinger-Schepman et al., 1982). The minor adduct appeared to be *cis*-$Pt(NH_3)_2$d(GpG), which must have been formed by dephosphorylation during storage of the main product after DEAE chromatography. The identity of the latter adduct was unambiguously proven by the ^{1}H NMR spectra of a mixture of this adduct and the reaction product of *cis*-DDP and synthetic d(GpG).

(*3*) *cis-Pt(NH₃)₂d(GMP)₂.* The presence of this minor adduct (r_b = 0.53) in the peak eluted around fraction 61 was proven by studying the ^{1}H NMR spectra of a mixture of this adduct with synthetic *cis*-$Pt(NH_3)_2$d$(GMP)_2$ at various pH values. The results clearly showed the presence of only one H8 and one H1′ signal.

(*4*) *Pt(NH₃)₃dGMP.* A small Pt-containing peak was eluted at low salt concentration around fraction 41 (Figure 3). On the basis of the retention time, this Pt adduct was expected to be $Pt(NH_3)_3$dGMP; however, the base and H1′ proton resonances in the NMR spectra and those of the admixed synthetic $Pt(NH_3)_3$dGMP did not coincide. Instead, the product was found to be $Pt(NH_3)_3$dGuo, because no differences could be observed in the NMR spectra between the isolated compound and synthetic $Pt(NH_3)_3$dGuo. Apparently, complete dephosphorylation of $Pt(NH_3)_3$dGMP to $Pt(NH_3)_3$dGuo occurred after the isolation from the DEAE-Sephacel column.

(*5*) *Additional Pt Adducts.* The small Pt-containing peak eluted around fraction 81 could be separated into three different peaks by using gel filtration on Sephadex G-25. The amounts were too small for ^{1}H NMR analysis, but the high r_b values (>0.8) suggest that these peaks comprise Pt-containing oligonucleotides not susceptible to further enzymatic degradation due to their high Pt content. The same holds for the Pt-containing peak eluted with 1 M NaCl (fraction 97), which has not been analyzed further.

Analytical Separation of cis-DDP-DNA Adducts by FPLC. In addition to the preparative separation of the adducts, a more refined analytical method was developed to be used for kinetic studies on adduct formation and in studies on the effects of experimental conditions. Very satisfactory results were obtained with regard to resolution, rapidity, and reproducibility, with an FPLC system based on a Mono Q anion-exchange column and programmed gradient elution. An example, obtained with a small sample (45 μg) of the same digest as was used in Figure 3, is shown in Figure 5. A comparison of the two elution profiles demonstrates the superior separation in the FPLC system, which is reached within 14 min. For the identification of the peaks, authentic samples of deoxymononucleotides were chromatographed, as well as aliquots of the peaks eluted from the DEAE-Sephacel column (Table I). Thus, it could be established that the peak eluted from the

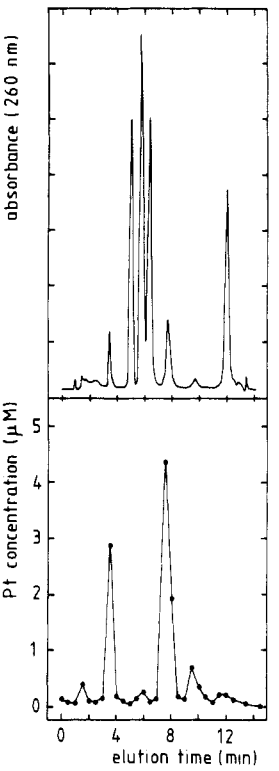

FIGURE 5: Mono Q elution pattern (pH 8.8) of a 45-μg sample of the same *cis*-DDP-DNA digest as used in Figure 3. For experimental conditions, see Materials and Methods. In this figure the upper panel (absorbance at 260 nm in arbitrary units) and the lower panel (Pt concentrations in μM) illustrate the presence of the various digestion products (see Table I).

Mono Q column after 3.5 min corresponds with *cis*-$Pt(NH_3)_2$d(pApG) and that the adducts *cis*-$Pt(NH_3)_2$d(pGpG) and *cis*-$Pt(NH_3)_2$d$(GMP)_2$ are eluted after 7.8 and 9.7 min, respectively. The material eluted from the DEAE-Sephacel column around fraction 41, which had been identified by ^{1}H NMR as $Pt(NH_3)_3$dGuo, was eluted from the Mono Q column after 0.8 min (data not shown). However, in the elution pattern of the digested *cis*-DDP-DNA on the Mono Q column (Figure 5), no such peak was observed, but the latter elution pattern did show a Pt-containing peak—eluted after 1.5 min—that cochromatographed with synthetic $Pt(NH_3)_3$dGMP. This supports our idea that the enzymatic degradation product $Pt(NH_3)_3$dGMP was present in the original digest of *cis*-DDP-DNA but was dephosphorylated during the time between DEAE chromatography and ^{1}H NMR measurements.

Enzymatic Digestion of cis-DDP-DNA. In experiments performed to analyze the enzymatic degradation conditions of *cis*-DDP-DNA, the amount of nuclease P1 required for optimal digestion in an overnight incubation was determined. Samples (250 μg) of a new batch of *cis*-DDP-treated salmon sperm DNA (r_t = 0.055; resulting r_b = 0.060) were digested as described, with various amounts of nuclease P1 (1–20 μL), and chromatographed in the FPLC system. An increase of the amount of nuclease at the upper end of the range did not result in significant changes in the elution pattern, which was comparable to that shown in Figure 5. Evidently, maximal digestion was achieved. With small amounts of nuclease, however, some interesting differences in the elution patterns were obtained. These differences were not seen in the digestion to unmodified nucleotides, which appeared almost complete already when 1 μL was used, as judged from the A_{260nm} pattern. Nor was there much effect on the adducts $Pt(NH_3)_3$dGMP and *cis*-$Pt(NH_3)_2$d(pGpG), which were present

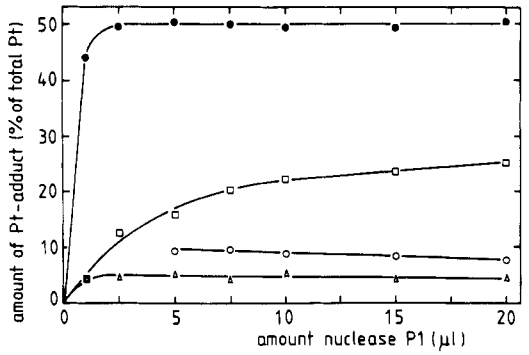

FIGURE 6: Effect of the amount of nuclease P1 on the digestion of *cis*-DDP-DNA. The amounts of *cis*-DDP adducts are given as percentages of total platinum eluted from the Mono Q column after digestion of *cis*-DDP-treated salmon sperm DNA (r_t = 0.055, 5 h at 50 °C) with the indicated amounts of nuclease P1 in the digestion mixture. (●) *cis*-$Pt(NH_3)_2d(pGpG)$; (□) *cis*-$Pt(NH_3)_2d(pApG)$; (○) *cis*-$Pt(NH_3)_2d(GMP)_2$; (△) $Pt(NH_3)_3dGMP$. The adduct *cis*-$Pt(NH_3)_2d(GMP)_2$ could not be determined with acceptable accuracy in digests with less than 5 μL of nuclease P1.

to about the same extent irrespective of the amount of nuclease P1, as can be seen in Figure 6 where the amounts of the four identified *cis*-DDP-DNA adducts are plotted as a function of the amount of nuclease P1 in the incubation mixture. The amounts of the adducts *cis*-$Pt(NH_3)_2d(pApG)$ and *cis*-$Pt(NH_3)_2d(GMP)_2$, on the other hand, showed a strong influence of the amount of nuclease (Figure 6); *cis*-$Pt(NH_3)_2d(pApG)$ was only present as a minor adduct in the samples with little nuclease P1 and its quantity continued to increase with the amount of enzyme. In the first samples *cis*-$Pt(NH_3)_2d(GMP)_2$ could hardly be detected because of the presence of uncompletely digested Pt-containing oligonucleotides eluting in the same part of the elution pattern. When 20 μL of nuclease P1 is used for digestion, the four adducts comprise approximately 88% of the platinum eluted from the Mono Q column (Figure 6).

Kinetics of Formation of the cis-DDP-DNA Adducts: Effects of Temperature. To study the kinetics of the formation of the various *cis*-DDP-DNA adducts in more detail, salmon sperm DNA was incubated with *cis*-DDP (r_t = 0.022) at 37 and 50 °C. At certain time intervals, aliquots were taken in which the adduct formation was stopped by treatment with NH_4HCO_3. After dialysis and maximal enzymatic digestion, 40 μg of each of the various samples was chromatographed on the Mono Q column and the fractions were analyzed for Pt by means of AAS. In Figure 7 the amounts of the four identified *cis*-DDP adducts in the DNA samples, expressed as percentages of the total Pt eluted from the column, are given as a function of the incubation time. Together, these adducts account for 90–95% of total Pt. In addition, Figure 7 shows the number of each *cis*-DDP adduct per nucleotide in the DNA. The most striking phenomenon seen in this figure is the constancy of the spectrum of the four adducts during the progression of the reaction over a considerable range.

Discussion

In our previously published attempts to detect and quantitate *cis*-DDP-DNA adducts (Fichtinger-Schepman et al., 1982), digests of *cis*-DDP-treated DNA were chromatographed on DEAE-Sephacel columns at pH 7.5, revealing the presence of *cis*-$Pt(NH_3)_2d(pGpG)$, *cis*-$Pt(NH_3)_2d(GMP)_2$, very small amounts of Pt-containing monoadducts, and products tentatively identified as intrastrand cross-links on two guanines in the base sequence d(pGpXpG) (X is thymine, adenine, cytosine, or guanine). Recently, chromatography at higher pH

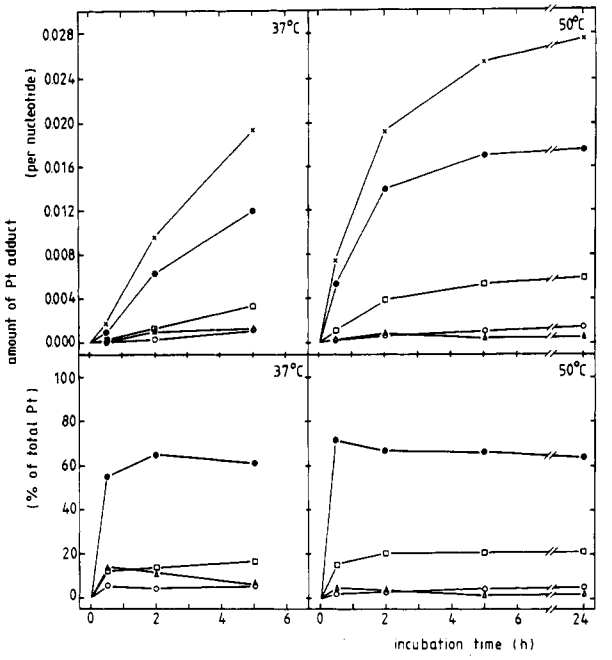

FIGURE 7: Effect of duration and temperature of treatment on the formation of *cis*-DDP-DNA adducts. The panels show the amounts of *cis*-DDP adducts determined in digests of salmon sperm DNA treated with *cis*-DDP (r_t = 0.022) for the periods indicated on the abscissa at 37 or 50 °C. In the upper panels the adducts are given per nucleotide in the *cis*-DDP-treated DNA; in the lower panels the amounts are expressed as percentages of the total Pt eluted from the Mono Q column. (●) cis-$Pt(NH_3)_2d(pGpG)$; (□) *cis*-$Pt(NH_3)_2d(pApG)$; (○) *cis*-$Pt(NH_3)_2d(GMP)_2$; (△) $Pt(NH_3)_3dGMP$; and (×) total platination of the DNA, i.e., the r_b value of the *cis*-DDP-DNA.

values showed remarkable shifts in the elution patterns of the Pt-containing adducts, mainly due to partial deprotonation of the Pt-bound guanine residues. In this way we discovered an additional adduct in the digest: *cis*-$Pt(NH_3)_2d(pApG)$ (Fichtinger-Schepman et al., 1984a), a dinucleotide adduct derived from the intrastrand coordination of *cis*-DDP to the N7 atoms of the two neighboring bases adenine and guanine, as was now proven by ^1H NMR. It is remarkable to note that in the fractions studied, no indications for the presence of *cis*-$Pt(NH_3)_2d(pGpA)$ in significant amounts could be obtained.

The use of the FPLC system makes it possible to perform a rapid analysis on an anion-exchange column at pH values above 7; separation of the various digestion products of *cis*-DDP-DNA at pH 8.8 is now feasible within 14 min with only 5% of the amount of material used in our previous separations on the analytical DEAE-Sephacel columns (Fichtinger-Schepman et al., 1982).

In this way we investigated the degradation conditions for *cis*-DDP-treated DNA, using the Mono Q column for the separation and AAS for the quantitation of the adducts. As can be seen from Figure 6, the extent of degradation of some Pt-containing (oligo)nucleotides is strongly dependent on the amount of nuclease P1 present in the incubation mixture. In particular, much enzyme is required for the *cis*-$Pt(NH_3)_2d(pApG)$ adduct to reach a plateau level. Possibly, the poor susceptibility for enzymatic degradation of oligonucleotides containing this type of Pt adduct reflects a more general problem for enzymes to deal with this type of Pt adducts. If this were true for repair enzymes in the cell, these adducts could be responsible for (at least some of) the observed effects of the antitumor drug. The difficult digestion to *cis*-$Pt(NH_3)_2d(pApG)$ implies that incomplete digestion may occur, yielding *cis*-$Pt(NH_3)_2d(pApG)$-containing oligonucleotides

that are eluted from the column at higher salt concentration than the dinucleotide adduct, or even in the 1 M NaCl fraction. We believe now that at least part of the oligonucleotides assumed to be *cis*-$Pt(NH_3)_2d(pGpXpG)$ in our previous paper (Fichtinger-Schepman et al., 1982) was in fact incompletely digested *cis*-$Pt(NH_3)_2d(pApG)$-containing oligonucleotides. This assumption is supported by our finding that the reference compounds *cis*-$Pt(NH_3)_2d(GpCpG)$ and *cis*-$Pt(NH_3)_2$-d(GpApG)—in both cases with the platinum bound to the N7 of the two guanines—are digested during our routine digestion procedure to *cis*-$Pt(NH_3)_2$dGuodGMP and dCMP and dAMP, respectively (data not shown). These results indicate that the digestion products derived from intrastrand cross-links in DNA on two guanines, separated by a third base (Brouwer et al., 1981; Marcelis et al., 1982), will have the structure *cis*-$Pt(NH_3)_2d(GMP)_2$, which is identical with that of the adduct supposed to result from interstrand cross-links on two guanines. This explains why the percentage of this adduct exceeds the generally assumed frequency of less than 1% for the interstrand cross-links in the total DNA platination (see Table I and Figure 7) (Roberts & Friedlos, 1981). According to this reasoning, the percentages of *cis*-$Pt(NH_3)_2d(GMP)_2$ found after incubation of DNA with *cis*-DDP for 5 h at 50 °C (8 and 4%) should indicate the presence, in the *cis*-DDP-treated DNA, of some 7–3% of the adduct *cis*-$Pt(NH_3)_2$-d(pGpXpG).

Using a similar method, Eastman (1983) demonstrated in digests of DNA treated in vitro with dichloro(ethylenediamine)platinum(II) (DEP)—which should modify the DNA in a fashion analogous to that for *cis*-DDP (Eastman, 1983)—the presence of substantial amounts of adducts assumed to be derived from intrastrand cross-links between adenine and guanine in the base sequence d(ApXpG). Because we do not find any evidence for the presence of *cis*-Pt-$(NH_3)_2d(pApXpG)$ in our *cis*-DDP-DNA digests but instead find large amounts of *cis*-$Pt(NH_3)_2d(pApG)$ (Table I and Figure 7), it might be possible that the described adducts are incompletely digested oligonucleotides with the base sequence ApGpX, in which the adenine and guanine are chelating to the platinum compound.

As can be seen from Figure 7 and Table I, incubation of salmon sperm DNA with *cis*-DDP yielded the same types of adducts, irrespective of the r_t value (0.022 or 0.055) or the temperature (37 or 50 °C) used. The difference in the amount of adduct formed after 5-h incubation at 50 °C at $r_t = 0.022$ and 0.055, respectively, is in perfect agreement with the difference in concentration. The difference between the extent of adduct formation at 37 and 50 °C (Figure 7) can be attributed to the effect of the temperature on the reaction rate, because the amounts of the various adducts formed after 5 h at 37 °C agree with the data obtained after approximately 2 h at 50 °C.

The high proportion of bifunctional Pt adducts from the onset of the reaction and the constancy of their relative amounts (Figure 7) are surprising. The formation of such adducts with nucleobases has been considered to result from a two-step reaction, with the second step to be relatively slow (Marcelis & Reedijk, 1983). As we have applied an experimental procedure that efficiently converts the monofunctional adducts with general formula $Pt(NH_3)_2(X)Cl$ into $Pt(NH_3)_3X$ and thus prevents their further reaction, a substantial fraction of this type of products was expected, at least in the first hours of the reaction of DNA with *cis*-DDP. The results, however, show very small amounts of the monofunctional adduct, and although there is some indication of a shift from mono- to bifunctional adduct with time (in particular at 37 °C), the high proportion of *cis*-$Pt(NH_3)_2d(pGpG)$—which remains virtually constant between 0.5 and 24 h of reaction—makes a relatively slow second step in the chelate formation with DNA rather unlikely.

A second, important conclusion can be drawn from the results shown in Figure 7 with regard to the reactivity of guanines in certain nucleotide sequences. On the basis of a guanine content of the salmon sperm DNA of 20.5% and when a random distribution of the nucleobases in the DNA is assumed, the fraction of the guanines not occurring in a sequence d(pGpG) will be $(0.795)^2$, i.e., 63.2% of the guanines. Thus, if the primary reaction of *cis*-DDP with the guanines in DNA occurred without any preference for particular sequences, not more than 36.8% (100 − 63.2) of the Pt should be able to bind in d(pGpG) sequences. However, the amount of *cis*-Pt-$(NH_3)_2d(pGpG)$ found after treatment of salmon sperm DNA at $r_t = 0.022$ (60–65%) is much higher than the expected value of 36.8%. Consequently, other adducts are much less abundant than would be predicted if all guanines in DNA were equally reactive toward *cis*-DDP. The inevitable conclusion is that *cis*-DDP in its reaction with DNA has a strong preference for guanines that occur in d(pGpG) sequences. In the experiments performed at $r_t = 0.055$, summarized in Table I, the same tendency is seen as in Figure 7, but there only about 50% of the Pt were found in the cross-links on d(pGpG). It is not surprising, however, that in this experiment the fraction *cis*-$Pt(NH_3)_2d(pGpG)$ remained below the value reached at $r_t = 0.022$, if one considers the fact that this 50% corresponds with 32 cross-links on adjacent guanines per 1000 nucleotides ($r_b = 0.064$), which is very close to 33, the maximum attainable number in DNA with a guanine content of 20.5% [36.8% of the guanines are located in d(pGpG) sequences among which $(0.205)^2 \times 100 = 4.2\%$ in d(pGpGpG) sequences. This yields a maximum amount of $0.5 \times (0.368 - 0.042) \times 205$ cross-links/1000 nucleotides]. The values found for the adduct *cis*-$Pt(NH_3)_2d(pApG)$, 20% after prolonged treatment of the DNA at $r_t = 0.022$ and 25% at $r_t = 0.055$, do correspond with the theoretical percentage of 23.5% [$0.295 \times 0.795 \times 100$ of the guanines, provided that in the sequence d(pApGpG) the second G, rather than the A, reacts with the platinum monofunctionally bound to the middle G].

In contrast to our results, Johnson & Butour (1981) found that the reaction rates of the binding of aquated *cis*-DDP to the DNAs from *Micrococcus lysodeikticus* and *Clostridium perfringens* were proportional to their G but not to their pGpG contents. Although they did not study the formed adducts, they concluded that the Pt compound reacts with all guanines with equal probability. Further work in our laboratories is aimed at a better understanding of this pronounced preference of *cis*-DDP to react with guanine located in a pGpG sequence.

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Registry No. *cis*-$Pt(NH_3)_2d(pGpG)$, 94234-93-2; *cis*-Pt-$(NH_3)_2d(pApG)$, 94234-94-3; $Pt(NH_3)_3dGMP$, 91514-24-8; *cis*-$Pt(NH_3)_2d(GMP)_2$, 87414-18-4.

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Toroidal Condensation of Z DNA and Identification of an Intermediate in the B to Z Transition of Poly(dG-m^{5}dC)·Poly(dG-m^{5}dC)†

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ABSTRACT: Using a combination of spectroscopic techniques, quasi-elastic laser light scattering (QLS), and electron microscopy (EM), we have been able to show that the B to Z transition of poly(dG-m^{5}dC)·poly(dG-m^{5}dC) is accompanied by extensive condensation of the DNA in both low and high ionic strength buffers. At low concentrations of NaCl (2 mM Na^+), an intermediate rodlike form, which exhibits a circular dichroism (CD) spectrum characteristic of an equimolar mixture of B and Z forms, is observed. This is produced by the orderly self-association of about four molecules of the polymer after prolonged incubation of a concentrated solution at 4 °C. On addition of 5 μM $Co(NH_3)_6^{3+}$, the CD spectrum of the intermediate changes to that of the Z form, which is visualized as a dense population of discrete toroids on an EM grid stained with uranyl acetate. On the other hand, addition of NaCl to a solution of poly(dG-m^{5}dC)·poly(dG-m^{5}dC) in the absence of any multivalent ion condenses the polymer to toroidal structures at the midpoint (0.75 M NaCl) of the B to Z transition. Further addition of NaCl unfolds these toroids to rodlike structures, which show characteristic Z-form CD spectra. These results show that Z DNA can take up a variety of tertiary structural forms and indicate that its inverted CD spectrum is due to its left-handed helical sense rather than to differential scattering artifacts.

Alternating purine–pyrimidine sequences of the type poly(dG-dC)·poly(dG-dC) and its substituted derivatives are known to undergo a transition from a right-handed B to a left-handed Z conformation under a variety of conditions (Pohl & Jovin, 1972; Wang et al., 1981; Zacharias et al., 1982; Dickerson et al., 1982; Behe & Felsenfeld, 1981; Revet et al., 1983; Moller et al., 1984). Various spectral studies have confirmed the essential identity of the structures observed in poly(dG-dC)·poly(dG-dC) at high salt (Thamann et al., 1981; Patel et al., 1979) and in fibers (Arnott et al., 1980) as well as in single crystals of alternating deoxyguanosine–deoxycytidine oligonucleotides (Wang et al., 1979; Crawford et al., 1980; Drew et al., 1980). The inversion of the circular dichroism (CD) spectrum accompanying the B to Z transition is similar for poly(dG-dC)·poly(dG-dC), poly(dG-m^{5}dC)·poly(dG-m^{5}dC), and other substituted derivatives. Recently, there has been considerable interest in the B to Z transition of poly(dG-m^{5}dC)·poly(dG-m^{5}dC) (Zimmerman, 1982; Rich et al., 1984) since the methylation of DNA is known to play an important role in gene regulation (Doerfler, 1983) and since the B to Z transition of this polymer occurs at physiological or lower salt conditions, especially in the presence of the ubiquitous cellular components, the polyamines (Behe & Felsenfeld, 1981). Additionally, C-5-methylation of cytosine has been shown to stabilize the Z form, perhaps by increased hydrophobic interactions (Fujii et al., 1982).

A variety of trivalent cations that effect the B to Z transition in poly(dG-m^{5}dC)·poly(dG-m^{5}dC) are known to induce the collapse of native DNA's into compact structural forms that are visualized by EM as toroids, folded fiber rods, and spheroids (Gosule & Schellman, 1976, 1978; Chattoraj et al., 1978; Eikbush & Moudrianakis, 1978; Widom & Baldwin, 1980; Allison et al., 1981). Even monovalent cations can cause extensive association or condensation of Z DNA (Castleman & Erlanger, 1983; Revet et al., 1983). The toroidal condensation of DNA by polyamines and other cations is an in vitro model system to study the energetic and kinetic factors involved in the organization of DNA in virus capsids, since toruslike

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