# Characterization of the Adducts Produced in DNA by *cis*-Diamminedichloroplatinum(II) and *cis*-Dichloro(ethylenediamine)platinum(II)†

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ABSTRACT: A radiolabeled analogue of the cancer chemotherapeutic drug *cis*-diamminedichloroplatinum(II) (*cis*-DDP) has been used to determine the sites of platination in DNA. This drug, [$^3$H]-*cis*-dichloro(ethylenediamine)platinum(II) (*cis*-DEP), was incubated with DNA, defined nucleic acid heteropolymers, and dinucleoside monophosphates. The products were enzymatically digested to deoxyribonucleosides or oligonucleotides and separated by high-pressure liquid chromatography. The identity of the adducts was confirmed after removal of the drug with 1 M thiourea and analysis of the constituent nucleotides. At low levels of modification of DNA, greater than 50% of the lesions were attributed to an intrastrand cross-link between two neighboring guanines, enzymatic removal of the phosphate between the two nucleosides being inhibited by the complex. At higher levels of modification, these sites became saturated, and pronounced reaction occurred at several other sites. One of these represented an intrastrand cross-link between a neighboring adenine and guanine. Reaction was also demonstrated between two guanines separated by a third base, the latter being removed during digestion. This was a relatively minor adduct. More frequent was an intrastrand cross-link between adenine and guanine separated by a third base. In this case, the third base was retained during digestion. These trinucleotides were shown to contain either adenine, cytosine, guanine, or thymine as their middle base. A specific orientation in the DNA was also observed with adenine always at the 5′ terminus. An additional, more hydrophilic adduct was identified by denaturation studies as an interstrand cross-link, but it represented a maximum of 1% of the total platination. A small proportion of monofunctional adducts, predominantly deoxyguanosine dependent, were also detected. These reacted with protein during digestion and chromatographed as the protein–Pt–nucleoside complex. These monofunctional adducts arose preferentially during short incubation of drug and DNA, but the majority of adducts appeared to arise by direct bifunctional attack. At high levels of DNA modification, it was also possible to characterize the interaction of *cis*-DDP with DNA as the adducts were detectable by ultraviolet absorbance. Adducts were obtained at identical sites in DNA with both *cis*-DDP and *cis*-DEP.

The interaction of the cancer chemotherapeutic agent *cis*-diamminedichloroplatinum(II) (*cis*-DDP)[1] with its putative target DNA has been studied by a variety of techniques including alkaline elution (Zwelling et al., 1979a; Strandberg et al., 1982), DNA renaturation (Harder, 1975; Eastman, 1982a), and DNA sedimentation (Pera et al., 1981). These techniques all depend upon the capacity of *cis*-DDP to form either DNA-interstrand cross-links or DNA–protein cross-links. However, these cross-links may represent less than 1% of the platination of DNA (Eastman, 1982a; Roberts & Friedlos, 1981). Although the formation of interstrand cross-links has frequently been correlated with drug toxicity (Zwelling et al., 1979a), considerable evidence has been presented that does not support such a correlation (Filipski et al., 1980; Strandberg et al., 1982). The most frequently implicated alternative lesion is a DNA-intrastrand cross-link between two neighboring guanine bases. The formation of such a lesion has been inferred from studies on inhibition of restriction endonucleases (Kelman & Buchbinder, 1978; Ushay et al., 1981) or inhibition of exonuclease cleavage at sites identified by DNA sequencing gel electrophoresis (Royer-Pokora et al., 1981; Tullius & Lippard, 1981).

An alternative technique for analyzing DNA-binding drugs is to digest the modified DNA to deoxyribonucleosides and separate the products by chromatography. Such an approach has routinely been used in studies of the interaction of chemical carcinogens with DNA (Baird & Brookes, 1973; Eastman et al., 1981). A high-pressure liquid chromatography system is now available for separating *cis*-DDP-modified deoxyribonucleosides (Eastman, 1982b), and this present report demonstrates the feasibility of using this technique to characterize the interaction of platinum coordination complexes with DNA. This study has further been facilitated by synthesis of a radiolabeled derivative, [$^3$H]-*cis*-dichloro(ethylenediamine)-platinum(II) (*cis*-DEP). This analogue only differs from *cis*-DDP in that it possesses a two-carbon bridge between the two amines. However, *cis*-DEP is also an effective antitumor agent that demonstrates a similar profile of sensitivity and resistance to that observed for *cis*-DDP in several murine leukemia L1210 cell lines (Eastman & Bresnick, 1981). Additionally, in experiments not presented, *cis*-DEP has been reacted with deoxyribonucleosides, and adducts were obtained that were identical with those characterized for *cis*-DDP interaction (Eastman, 1982b).

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[1] Abbreviations: *cis*-DDP, *cis*-diamminedichloroplatinum(II); *cis*-DEP, *cis*-dichloro(ethylenediamine)platinum(II); HPLC, high-pressure liquid chromatography; A, adenosine; C, cytidine; G, guanosine; T, thymidine; d, deoxy; p, phosphate. Hence, dApdG is deoxyadenylyl(3′–5′)deoxyguanosine (referred to subsequently as adenine guanine dinucleoside monophosphate) and lacks a 5′-phosphate. All oligonucleotide sequences are presented in the 5′→3′ orientation.

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## Materials and Methods

Calf thymus DNA, deoxynucleosides, dinucleoside monophosphates, and all enzymes were obtained from Sigma Chemical Co., St. Louis, MO. Defined heteroduplexes were purchased from P-L Biochemicals, Milwaukee, WI. Authentic *cis*-DEP and *cis*-DDP were purchased from Alfa Ventron, Danvers, MA. Thiourea (Baker, Phillipsburg, NJ) was deionized over AG 501-X8 (Bio-Rad, Richmond, CA) prior to use.

Synthesis of [$^3$H]-*cis*-DEP was adapted from Robins (1973). Aminoacetonitrile (Aldrich Chemical Co., Milwaukee, WI) was reduced to [$^3$H]ethylenediamine with tritium by Amersham Corp., Arlington Heights, IL, and supplied as a crude preparation. It was purified by passage through a Whatman SCX 10 column attached to a Varian Model 5000 HPLC eluted with water during application of the sample. Repeat 0.5-mL aliquots were injected until up to 5 mL (10 mCi) of sample had been applied. After a 10-mL wash in water, the ethylenediamine was eluted in 0.5 M NaCl. This technique had the additional advantage of concentrating the sample.

[$^3$H]Ethylenediamine in 0.5 M NaCl was incubated for 15 min with 1 mg/mL potassium tetrachloroplatinate(II) (Aldrich Chemical Co.) on a boiling water bath. On cooling, 0.5-mL aliquots were passed through the same column as above and eluted with water. Dichloro(ethylenediamine)platinum(II) eluted as a discrete peak 2 mL after the column void volume. Unreacted $PtCl_4$ eluted at the void volume. About 70% of the radioactivity was recovered within the *cis*-DEP peak. This was stored at 4 °C in 0.15 M NaCl.

The identity of [$^3$H]-*cis*-DEP was confirmed by incubation overnight at 37 °C in 0.02 M $NaClO_4$ with 1 mM deoxyguanosine and then chromatographed by HPLC as recently detailed (Eastman, 1982b). At least 90% of the tritium cochromatographed with authentic bifunctional standard derived from deoxyguanosine and *cis*-DEP. The specific radioactivity of the drug was obtained by measuring its ability to cause inhibition of growth of murine leukemia L1210 cells as compared to that observed for authentic *cis*-DEP (Eastman & Bresnick, 1981). The specific activity was calculated to be 16 Ci/mmol.

Aliquots of [$^3$H]-*cis*-DEP were diluted with 5 mg/mL authentic *cis*-DEP in 0.1 N HCl at 80 °C. Crystallization was achieved by cooling to 4 °C. A slight reduction in the specific activity was obtained, but repeated recrystallization resulted in a constant specific activity. Several reactions with the recrystallized *cis*-DEP and DNA were performed, and identical results were obtained with those presented below. The impurities therefore did not appear to react with DNA. For all the experiments presented here, the drug was used after only HPLC purification. This was advantageous as recrystallization resulted in a very low specific activity.

*Reaction of cis-DEP with Nucleic Acid Components.* Nucleic acids (100 μg) were incubated with [$^3$H]-*cis*-DEP in either 0.02 or 0.1 M $NaClO_4$, pH 5.5. The reaction products in calf thymus DNA were independent of the salt used, but the higher salt ensured the double-stranded character of the defined heteroduplexes. Incubations were usually performed for 16 h at 37 °C. DNA was precipitated with 2 volumes of ethanol–2% potassium acetate, washed in 70% ethanol–potassium acetate, dried under nitrogen, resuspended in 20 mM sodium acetate–50 mM NaCl–1 mM $ZnSO_4$–10 mM $MgCl_2$, pH 4.6, and digested to deoxyribonucleosides and oligonucleotides sequentially at 37 °C for 4 h with 200 Kunitz units of deoxyribonuclease I (bovine pancreas) and for 16 h with 1000 units of $S_1$ nuclease. One-tenth volume of 1.0 M Tris, pH 9, and 5 units of alkaline phosphatase were added, and incubation was continued for a further 4 h. Aliquots (100 μL) were then injected onto an Altex Ultrasphere ODS column and eluted with 0–30% methanol in 0.1 M aqueous ammonium acetate, pH 5.5, as previously detailed (Eastman, 1982b). During an additional 5 min, a 30–100% methanol in ammonium acetate gradient was applied. Flow rate was 1 mL/min and monitored with an ultraviolet ($A_{254}$) detector. Fractions, 0.5 mL, were collected, and tritium was assessed by liquid scintillation counting.

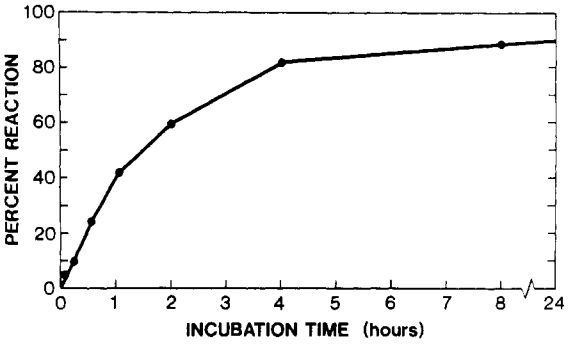


FIGURE 1: Kinetics of reaction of [$^3$H]-*cis*-DEP with DNA at a molar ratio of 0.01 Pt/nucleotide. The extent of reaction was determined from the decrease in ethanol-soluble radioactivity at each time point.

## Results

*Kinetics of Reaction.* Calf thymus DNA was incubated with [$^3$H]-*cis*-DEP for various time periods and then precipitated with ethanol. The radioactivity in both supernatant and DNA pellet was measured and the percentage reaction calculated (Figure 1). Reaction was rapid with the majority of platination occurring in the first 4 h. Maximum reaction occurred between 8 and 24 h. Subsequent incubations were therefore performed for 16 h.

*Separation of Reaction Products in DNA.* DNA was incubated with various concentrations of [$^3$H]-*cis*-DEP for 16 h, ethanol precipitated, and enzymatically digested to deoxyribonucleosides. Separation by HPLC gave a variety of radioactive peaks (Figure 2). At low levels of platination (0.01 Pt per nucleotide) about 50% of the radiolabel was recovered in a single peak. At 0.001 Pt per nucleotide, the same adduct profile was obtained (not shown). At higher levels of platination, this preferred site was saturated, and pronounced reaction occurred at five other sites. In addition, two early peaks appeared relatively unaffected by the different drug doses while one minor peak disappeared at high dose. For reference, these adducts have been numbered 1–9.

*Reaction of cis-DEP with Dinucleosides.* It has frequently been proposed that neighboring guanines could be cross-linked by *cis*-DDP to form an intrastrand cross-link. Various dinucleoside monophosphates were therefore incubated with a 20% molar equiv of [$^3$H]-*cis*-DEP. It was not possible to readily separate the unreacted drug from the nucleosides. The whole incubation was therefore diluted with sodium acetate buffer and digested with $S_1$ nuclease and alkaline phosphatase. The products were then separated by HPLC. The ultraviolet trace confirmed complete digestion of unmodified dinucleosides in each case. Guanine guanine dinucleoside produced one major peak that cochromatographed with the major adduct detected in DNA (Figure 3). As will be shown below, this adduct has retained the phosphate between the two nucleosides, the presence of the drug apparently inhibiting its digestion. A similar situation arose when adenine guanine dinucleoside was used, demonstrating reaction with both bases and inhib-

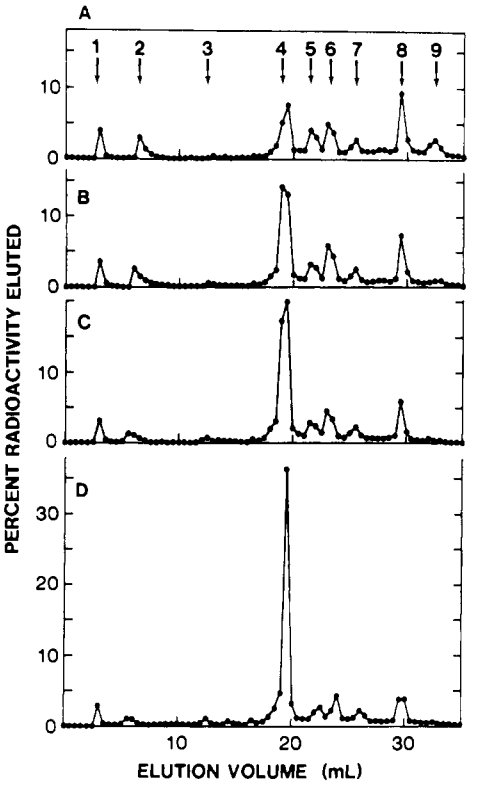


FIGURE 2: HPLC separations of the enzyme digestion products of [$^3$H]-*cis*-DEP-modified DNA at molar ratios of (A) 0.1 Pt/nucleotide, (B) 0.05 Pt/nucleotide, (C) 0.02 Pt/nucleotide, and (D) 0.01 Pt/nucleotide. The numbers 1–9 identify the radioactive adducts for reference in the text.

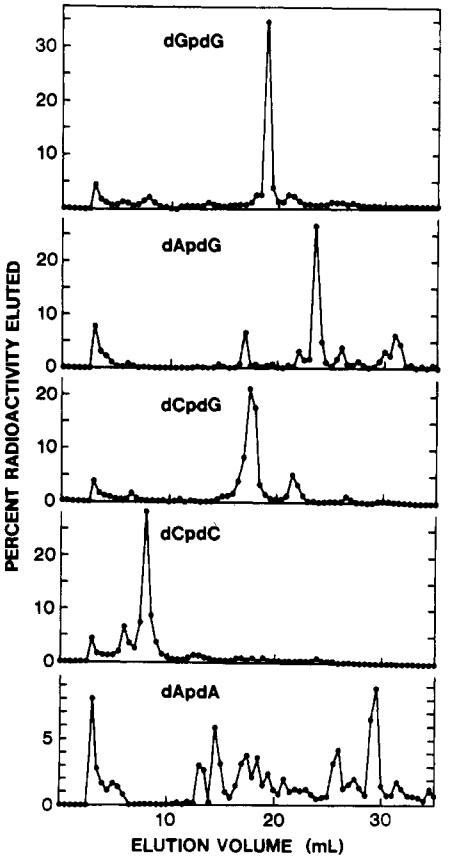


FIGURE 3: HPLC separations of the enzyme digestion products of [$^3$H]-*cis*-DEP-modified dinucleoside monophosphates.

ition of cleavage of the phosphodiester bond. Cytosine guanine dinucleoside gave a major adduct that cochromatographed with

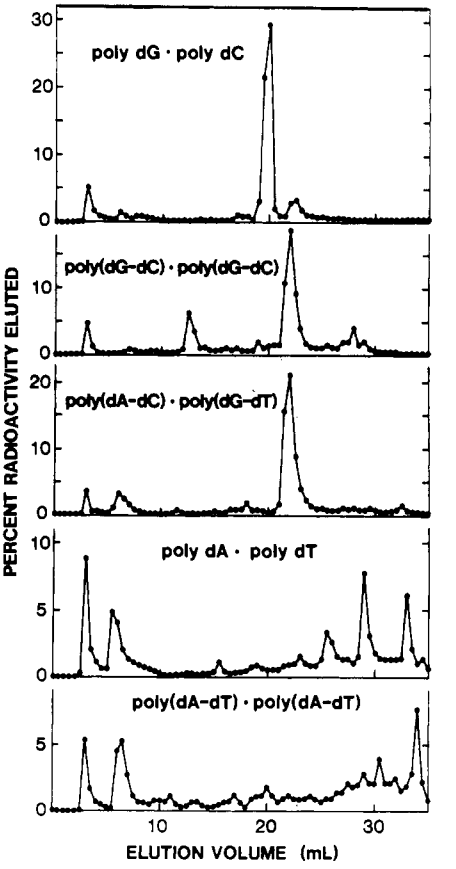


FIGURE 4: HPLC separations of the enzyme digestion products of [$^3$H]-*cis*-DEP-modified defined heteropolymers of DNA.

the previously identified guanosine–platinum adduct and a minor adduct that cochromatographed with diguanosine–platinum, probably arising from a cross-link between two separate dinucleoside molecules. It can be concluded that no cross-link occurred between cytosine and guanine, and the nuclease therefore cleaved the phosphodiester bond. Cytosine cytosine dinucleoside gave a cytosine–platinum adduct while adenine adenine dinucleoside produced many peaks of which the major one may have arisen from two neighboring adenines. However, the complexity of this profile probably reflects reaction at both N(1) and N(7) of each adenine.

*Reaction of cis-DEP with Defined Heteropolymers of DNA.* Heteroduplexes were incubated with [$^3$H]-*cis*-DEP (0.01 Pt per nucleotide), precipitated with ethanol, enzymatically digested, and separated by HPLC (Figure 4). Poly(dG)·poly(dC) gave one major peak that represented the cross-link between two neighboring guanines. Both poly(dG-dC)·poly(dG-dC) and poly(dG-dT)·poly(dA-dC) produced a major peak that cochromatographed with a diguanosine–platinum standard. This presumably arose from a cross-link between two guanines separated by a third base as has previously been proposed (Marcelis et al., 1982). Multiple adducts resulted from platination of either poly(dA)·poly(dT) or poly(dA-dT)·poly(dA-dT). In the former case, one adduct might represent an intrastrand cross-link between two neighboring adenines, but as with the adenine dinucleoside, a complex profile of adducts was obtained.

*Identification of DNA-Interstrand Cross-Links.* It has previously been reported that interstrand cross-links are rare events in DNA, occurring at a frequency of less than 1% of total platination (Roberts & Friedlos, 1981; Eastman, 1982a). An attempt was made to determine whether any of the adducts observed represented interstrand cross-links. DNA modified

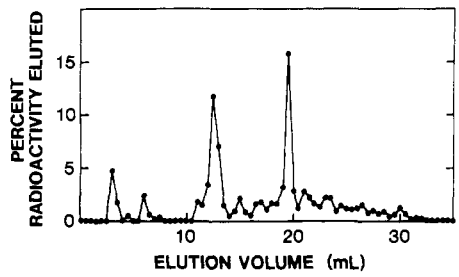


FIGURE 5: HPLC separation of the enzyme digestion products of [$^3$H]-*cis*-DEP-modified DNA that has been enriched for DNA interstrand cross-links by denaturation–renaturation and passage through a nitrocellulose filter.

with [$^3$H]-*cis*-DEP at low level was sheared by 10 passes through a 25-gauge needle, alkali denatured, renatured, and passed through a nitrocellulose filter as previously described (Eastman, 1982a). Single-stranded DNA binds to these filters while double-stranded DNA resulting from the presence of an interstrand cross-link passes through. This DNA was precipitated with ethanol in the presence of 200 $\mu$g of unmodified DNA. The resulting precipitate was enzymatically digested and separated by HPLC. The contribution from one adduct was markedly enhanced by this procedure (Figure 5). This was adduct 3 as defined in Figure 2, in which it was present at about 1% of the total platination. It therefore seems likely that this represents the only interstrand cross-link that forms.

*Identification of Monofunctional Platination in DNA.* Bifunctional platinum drugs may also form monofunctional adducts. These may be, in part, precursors to the bifunctional adducts. Thiourea was selected as a means of saturating these monofunctional forms as it has previously been reported as capable of preventing cross-linking but not reversing them (Zwelling et al., 1979b). Initially, deoxyribonucleosides were incubated with *cis*-DEP and subsequently at varying concentrations of thiourea for 1 h at 25 °C. The products were then separated by HPLC. At 100 mM thiourea, some reversal of platination was observed. At 10 mM thiourea, complete saturation of monofunctional adducts was obtained with no apparent reversal of platination. The thiourea–Pt–nucleoside adducts were all slightly more hydrophilic (eluted earlier) than their Pt–nucleoside precursors.

Platinated nucleosides obtained from the digestion of DNA were incubated with 10 mM thiourea for 1 h and separated by HPLC. No thiourea-modified adducts were obtained, suggesting that no monofunctional adducts existed. It was considered possible that monofunctional adducts present in DNA produced bifunctional adducts during subsequent processing. Platinated DNA was therefore incubated with 10 mM thiourea for 1 h. The DNA was reprecipitated with ethanol, then digested, and chromatographed as usual. A large peak, 25% of the total, was observed that eluted at 15 mL, the same position as a deoxyguanosine–Pt–thiourea standard. However, a 30% reduction was seen in all the other adducts, suggesting that some reversal of cross-links had occurred. In addition, adduct 2 completely disappeared. Although the contribution from the interstrand cross-link, adduct 3, was small, it appeared to be resistant to this concentration of thiourea. The thiourea concentration was reduced to 1 mM at which 5% reversal of each adduct was observed but 50% disappearance of adduct 2.

Thiourea, therefore, exhibits complex interactions with the various adducts, having different effects when the adducts are in DNA than as its digestion products. Adduct 2 appeared to be monofunctional in DNA but bifunctional after digestion. In one experiment, a very short incubation was performed with alkaline phosphatase, and an adduct eluted from HPLC at the position of the deoxyguanosine–Pt standard. On continued digestion, this disappeared to produce adduct 2. Interestingly, alkaline phosphatase itself eluted at this same position. It can be concluded that monofunctional products react with the enzyme presumably through sulfhydryl groups and can be recovered after HPLC as the protein–Pt–nucleoside complex.

It has frequently been reported that interstrand cross-linking increases with time even after the drug has been removed (Zwelling et al., 1979a; Strandberg et al., 1982). This is presumed to arise from initial formation of monofunctional adducts that subsequently produce cross-links. DNA incubated with [$^3$H]-*cis*-DEP for 1 h resulted in 20% of the radioactivity being recovered as adduct 2. When the same DNA was incubated for a further 24 h in the absence of drug, the contribution from this adduct was reduced to 3% with a concomitant increase in the bifunctional adducts, particularly adduct 8. This supports the concept that monofunctional reaction is the preliminary step. However, in most cases the second step must be very rapid when it is considered that 80% of the reaction products are already bifunctional after a 1-h incubation.

*Analysis of Oligonucleotides after Removal of cis-DEP.* At high levels of platination of DNA, the adducts were detectable by ultraviolet absorbtion. The profile obtained after modification of DNA at 0.1 Pt per nucleotide is presented in Figure 6A. The four major peaks, in order of elution, represent deoxycytidine, deoxyguanosine, thymidine, and deoxyadenosine. Superimposed upon this is the radioactive profile resulting from [$^3$H]-*cis*-DEP. Each adduct was collected after HPLC, lyophilized, and incubated in 1 M thiourea for 24 h at 37 °C to remove the Pt (Filipski et al., 1979). The products were again separated by HPLC. The results obtained for adduct 8 are presented in Figure 6B. The large initial peak was thiourea. The second peak was thiourea-modified Pt. The two late eluting peaks represented oligonucleotides. Each of these was lyophilized, redissolved in sodium acetate buffer, pH 4.6, and digested with $S_1$ nuclease. The products separated by HPLC were dGMP, dAMP, and deoxyadenosine (Figure 6C) and dTMP, dGMP, and deoxyadenosine (Figure 6D). These identities were confirmed by digestion with alkaline phosphatase which gave deoxyguanosine and deoxyadenosine (Figure 6E) or deoxyguanosine, thymidine, and deoxyadenosine (Figure 6F). The relative peak heights were also consistent with the molar ratios proposed below. In Figure 6C–F the small peak at 5 mL was $S_1$ nuclease, and in Figure 6E,F, the peak at 6 mL was alkaline phosphatase.

This analysis of adduct 8 has led to the following assignment of its structure. There are in fact two adducts, each of which was isolated as a trinucleotide. In both cases, $S_1$ nuclease digestion demonstrated that deoxyadenosine must be on the 5′ end as it lacks a phosphate. As *cis*-DEP does not react with thymidine, it probably represents the middle nucleotide; if it were on the 3′ end, it would have been removed during digestion of DNA. The sequence of this trinucleotide is therefore ATG, and the drug must cross-link the deoxyadenosine and deoxyguanosine. Similarly, the other trinucleotide is AAG, with the drug cross-linking the terminal deoxyadenosine and deoxyguanosine. The alternate possibility, that is, an AGA sequence with the two deoxyadenosines cross-linked, seems highly unlikely as deoxyguanosine is much the preferred site of platination.

*Assignment of Structures for cis-DEP Adducts in DNA. Adduct 1.* This elutes at the void volume of the column.

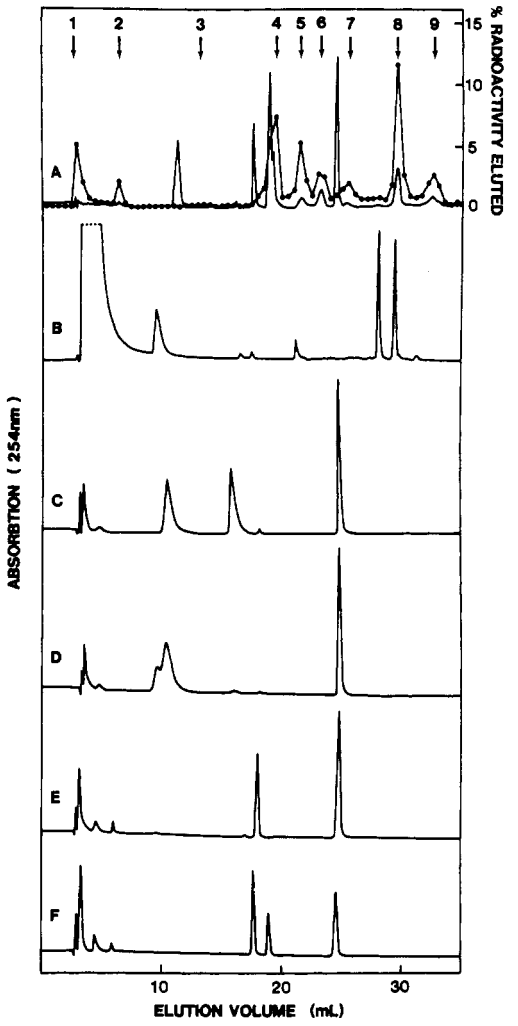


FIGURE 6: Characterization of adduct 8. (A) DNA was modified with [$^3$H]-*cis*-DEP at a molar ratio of 0.1 Pt/nucleotide, digested, and separated by HPLC. The ultraviolet trace and radioactivity are presented. (B) Adduct 8 was incubated with 1 M thiourea and rechromatographed by HPLC. (C) The peak eluting at 29 mL was digested with $S_1$ nuclease and rechromatographed. (D) The peak eluting at 30 mL was digested with $S_1$ nuclease and rechromatographed. (E) Profile C after digestion with alkaline phosphatase. (F) Profile D after digestion with alkaline phosphatase. The identity of each peak is described under Results.

Thiourea released no detectable nucleosides. It may be attributed to a contaminant in the radiolabeled drug as several preparations produced lower amounts of this adduct.

*Adduct 2.* This represents a cross-link between a nucleoside and protein. Incubation with thiourea resulted in about 98% deoxyguanosine and 2% deoxyadenosine. This arises from monofunctional platination of DNA.

*Adduct 3.* Characterized above as the DNA interstrand cross-link, it is produced in too low a proportion to permit determination of its constituent parts, but its prevalence in a poly(dG-dC)·poly(dG-dC) substrate suggests it may arise from a cross-link between cytosine and guanine. However, it did not arise in either poly(dG-dT)·poly(dA-dC) or poly(dG)·poly(dC) which would suggest an important influence from neighboring bases.

*Adduct 4.* This adduct arose in heteropolymers and the dinucleoside that contained neighboring guanines. Incubation with 1 M thiourea gave a new peak that cochromatographed with unmodified dGpdG. This was confirmed with $S_1$ nuclease digestion which gave dGMP and deoxyguanosine. Subsequent alkaline phosphatase digestion gave only deoxyguanosine.

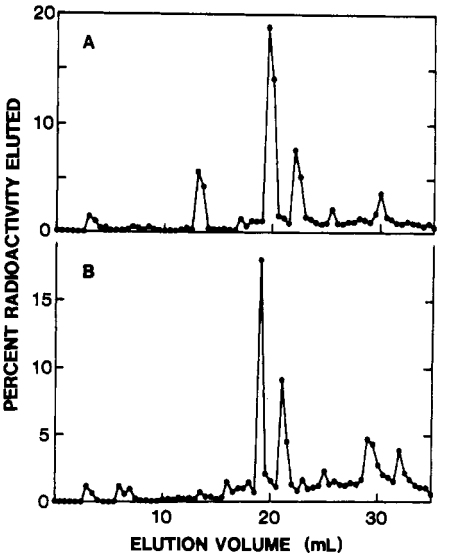


FIGURE 7: HPLC separations of the enzyme digestion products of [$^3$H]-*cis*-DEP-modified single-stranded DNA at molar ratios of (A) 0.01 Pt/nucleotide and (B) 0.1 Pt/nucleotide.

*Adduct 5.* This adduct cochromatographed with a synthesized standard of two deoxyguanosines cross-linked by *cis*-DEP. Incubation with 1 M thiourea produced only deoxyguanosine. It therefore represents a cross-link between two deoxyguanosines at a distance on a strand, presumably separated by a third base as it was the major adduct in alternating poly(dG-dC) and poly(dG-dT) templates (Figure 4).

*Adduct 6.* This adduct cochromatographed with platinated dApdG, and after removal of drug by thiourea the product chromatographed as unplatinated dApdG. Digestion with $S_1$ nuclease gave two major peaks, dGMP and deoxyadenosine, and two minor peaks, dAMP and deoxyguanosine. It therefore contains the same adduct in two different orientations in the DNA. About 90% of the adduct is platination of a dApdG sequence while 10% arose from dGpdA.

*Adduct 7.* Two oligonucleotides were obtained after removal of Pt by thiourea. The major peak gave dCMP, dGMP, and deoxyadenosine upon $S_1$ nuclease digestion and probably represents platination of an ACG sequence. The minor peak gave dGMP and deoxyadenosine only, but the former was present at twice the concentration of the latter. This would appear to represent platination of an AGG sequence.

*Adduct 8.* As discussed above, this represents platination of ATG and AAG sequences in DNA.

*Adduct 9.* This adduct only appeared at high levels of platination. Incubation with thiourea produced multiple peaks, and all possible nucleotides were represented. This is probably derived from neighboring platinations on DNA and does not represent any novel adducts.

*Reaction of [$^3$H]-cis-DEP with Single-Stranded DNA.* The observation that the adducts formed in a specific orientation in double-stranded DNA made the study of single-stranded DNA of interest. Calf thymus DNA was placed in a boiling water bath for 15 min, rapidly cooled, and incubated with [$^3$H]-*cis*-DEP at molar ratios of 0.01 Pt/nucleotide and 0.1 Pt/nucleotide. After ethanol precipitation, the DNA was digested, and the adducts were separated by HPLC (Figure 7). The same adducts were detected as with double-stranded DNA, but the relative proportions were different. At the lower level of modification, the intrastrand cross-link between two guanines represented only 33% of the total, with both the interstrand cross-link and the crosslink between two distant guanines showing a marked increase. The higher proportion

of interstrand cross-links may be indicative of geometric constraints in double-stranded DNA that are not so prevalent in the single-stranded form. At higher levels of modification, the other adducts became more apparent although the adduct derived from the interstrand cross-link disappeared. These adducts were purified, Pt was removed with 1 M thiourea, and the oligonucleotides were further analyzed by HPLC. Adduct 6 appeared to contain about 70% dApdG and 30% dGpdA. This was a slight increase in the latter compared to that detected in double-stranded DNA. However, adducts 7 and 8 were almost exclusively trinucleotides with a 5′-terminal adenine as found with double-stranded DNA. There was only a very small contribution from guanine as the 5′-terminal base.

*Reaction of cis-DDP with DNA.* The ability of ultraviolet absorbance to identify the adducts produced at high levels of DNA modification has permitted the application of the techniques to a study of the reaction of *cis*-DDP with DNA. *cis*-DDP was incubated with DNA at a molar ratio of 0.1 Pt/nucleotide, ethanol precipitated, enzymatically digested, and chromatographed by HPLC. An identical profile of absorbance peaks was observed to that for *cis*-DEP. The difference in structure of the drugs appeared not to affect the elution positions, perhaps because an ion-suppression chromatographic system was used (Eastman, 1982b). These adducts were purified and lyophilized, and the Pt was removed with thiourea. Analysis of the resulting nucleosides and oligonucleotides confirmed that *cis*-DDP and *cis*-DEP produced adducts at identical sites in DNA.

## Discussion

For a number of years *cis*-DDP induced DNA-interstrand cross-links, and DNA–protein cross-links have been extensively studied because techniques have been available for their assay. However, it has always been known that such lesions represent a minor fraction of the total platination of DNA. A complete analysis of all the platinum adducts in DNA has been long overdue. Such an analysis is presented here. It has been achieved by applying techniques that have routinely been used in studying the interaction of chemical carcinogens with DNA. Platinated DNA was enzymatically digested to deoxyribonucleosides, and the adducts were separated by reverse-phase HPLC. The success of this approach has depended upon several criteria. A radiolabeled analogue of *cis*-DDP was chosen so that adducts could readily be discriminated from unmodified nucleosides and also be detected at low levels of DNA modification. The choice of enzymes was also critical. A variety of enzyme digestions were attempted. Each gave a slightly different profile. In particular, digestion with a crude acid phosphatase preparation (Sigma Chemical Co.) removed the phosphate from the adduct formed between two neighboring guanines. The enzymes chosen gave the most reliable adduct profiles and permitted resolution of different types of intrastrand cross-links, both in dinucleoside monophosphate and in trinucleotide sequences.

A recent report (Fichtinger-Schepman et al., 1982) has used a similar approach to characterize the reaction of *cis*-DDP with DNA. However, they chose to digest only to deoxynucleotides and then separated by ion-exchange chromatography. This only permitted separation into groups of adducts with the same charge and did not provide the resolution presented here.

At low levels of platination of DNA by [$^3$H]-*cis*-DEP, greater than 50% of the adducts were a cross-link between neighboring guanines. If it is assumed that the first step in the formation of this cross-link is a monofunctional reaction with guanine, then, considering there is only a 25% chance that a nearest neighbor is guanine, no more than 25% of the total adducts should be this lesion. This suggests that much of the reaction may indeed be a direct bifunctional attack at preferred sites in DNA. Only after these sites became saturated did pronounced reaction occur at other sites, although some reaction occurred at all possible sites even at very low levels of modification.

A significant amount of reaction occurred between adenine and guanine separated by a third base (adducts 7 and 8). Platination of a trinucleotide was previously demonstrated when guanine was present on each end (Marcelis et al., 1982). However, this was a relatively minor adduct in these studies (adduct 5). It was of particular interest that when present adenine was always at the 5′ end of the trinucleotide (adducts 7 and 8) and most frequently at the 5′ end of dinucleoside monophosphates (adduct 6). There appeared to be a very specific orientation of the drug in DNA that was thought to arise from structural limitations of the double helix. All of these intrastrand cross-links must cause considerable buckling of the DNA strand, and the ability of the helix to accommodate certain of these torsions could control the observed orientations of the platinated sequences. However, the adducts obtained in single-stranded DNA were, for the most part, identical with those in double-stranded DNA. Therefore, the observed orientations of the adducts must be the result of other limitations.

These studies have also identified an adduct arising from an interstrand cross-link. Its presence at about 1% of the total platination is consistent with its reported frequency in DNA. It therefore probably represents the only interstrand cross-link that forms. It has recently been reported that interstrand cross-links can form between poly(dC) and poly(dG) (Lee & Harder, 1982). Also, preferential interstrand cross-linking occurred in DNA rich in guanine and cytosine (Gangull & Theophanides, 1979). This adduct occurred on a poly(dG-dC)·poly(dG-dC) template (Figure 4), and together with its relative hydrophilicity, this would support the contention that a cytosine–guanine cross-link is involved. However, this identification can not be confirmed at present, particularly as it was absent in both poly(dG)·poly(dC) and poly(dA-dC)·poly(dG-dT).

The results obtained also confirmed that *cis*-DEP modified DNA in a fashion analogous to that for *cis*-DDP, the parent drug that is clinically efficacious. The availability of the radiolabeled analogue will therefore facilitate studies of the adducts that occur in cell systems and can thereby help elucidate the mechanism of action of platinum complexes.

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# 13-*cis*-Retinoic Acid Metabolism in Vivo. The Major Tissue Metabolites in the Rat Have the All-Trans Configuration†

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ABSTRACT: The liver and intestinal metabolites of orally dosed 13-*cis*-[11-$^3$H]retinoic acid were analyzed in normal and 13-*cis*-retinoic acid treated rats 3 h after administration of the radiolabeled retinoid. *all-trans*-Retinoic acid was identified as a liver and intestinal mucosa metabolite in normal rats given physiological doses of 13-*cis*-[$^3$H]retinoic acid. *all-trans*-Retinoyl glucuronide was identified as the most abundant radiolabeled metabolite in mucosa and a prominent liver metabolite under the same conditions. Thus, the major 13-*cis*-retinoic acid metabolites retained in liver and mucosa, two retinoid target tissues, had the all-trans configuration. These data indicate that the isomerization of 13-*cis*-retinoic acid to *all-trans*-retinoic acid and the subsequent conversion to *all-trans*-retinoyl glucuronide are central events in the in vivo metabolism of 13-*cis*-retinoic acid in the rat. Moreover, the *all-trans*-retinoic acid detected in vivo could account for a significant fraction of the physiological activity of 13-*cis*-retinoic acid. The tissue disposition and metabolism of orally dosed 13-*cis*-[$^3$H]retinoic acid are modulated by retinoid treatment. Chronic 13-*cis*-retinoic acid treatment apparently increased the intestinal accumulation of *all-trans*-retinoic acid, *all-trans*-retinoyl glucuronide, and 13-*cis*-retinoyl glucuronide. The liver concentrations of tritiated *all-trans*-retinoic acid and *all-trans*-retinoyl glucuronide were also elevated in 13-*cis*-retinoic acid treated rats.

*all-trans*-Retinoic acid (*all-trans*-RA),[1] a normal intermediate in retinol metabolism (McCormick & Napoli, 1982), is at least an order of magnitude more potent than retinol in the maintenance of epithelial differentiation in cultured trachea (Sporn et al., 1976) and prostate (Lasnitzki & Goodman, 1974), and in the induction of embryonal carcinoma cell (Strickland & Mahdavi, 1978; Strickland, 1978; Jetten et al., 1979) and S91 melanoma cell (Lotan et al., 1980) differentiation in culture. These data argue that *all-trans*-RA, or a further metabolite, is the form of retinol active in directing differentiation. 13-*cis*-Retinoic acid (13-*cis*-RA) has been reported as an in vivo metabolite of *all-trans*-RA by several laboratories (Napoli & McCormick, 1981; Sundaresan & Bhat, 1982; Zile et al., 1982a) and is a physiological retinol metabolite in the rat.[2] 13-*cis*-RA is equipotent to *trans*-RA in promoting growth (Zile & DeLuca, 1968), maintaining differentiation in tracheal organ cultures (Frolik et al., 1980), and inducing embryonal carcinoma cell differentiation (Strickland, 1978). Moreover, 13-*cis*-RA has potent chemopreventive activity in urinary bladder (Becci et al., 1978, 1981; Thompson et al., 1981), respiratory tract (Port et al., 1975), and skin (Mayer et al., 1978; Verma et al., 1979). Thus, 13-*cis*-RA may be a physiologically important metabolite of retinol.

Limited information is available concerning the in vivo metabolism of 13-*cis*-RA. The cis isomer is rapidly metabolized to 13-*cis*-4-hydroxyretinoic acid and 13-*cis*-4-ketoretinoic acid in vivo (Frolik et al., 1979; Vane & Buggé, 1981) and in vitro (Frolik et al., 1979; Vane et al., 1982). These C(4) oxidized metabolites are less active than the parent retinoid in maintaining epithelial differentiation in cultured trachea (Frolik et al., 1979). Further metabolism of 13-*cis*-4-keto-RA in vitro produced the 2-hydroxy and 3-hydroxy derivatives

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[1] Abbreviations: HPLC, high-performance liquid chromatography; RP-HPLC, reverse-phase high-performance liquid chromatography; NP-HPLC, normal-phase HPLC; *trans*-RA, *all-trans*-retinoic acid; 13-*cis*-RA, 13-*cis*-retinoic acid; 5,6-epoxy-RA, 5,6-epoxyretinoic acid; 4-hydroxy-RA, 4-hydroxyretinoic acid; 4-keto-RA, 4-ketoretinoic acid; 13-*cis*-5,6-epoxy-RA, 13-*cis*-5,6-epoxyretinoic acid; 13-*cis*-4-keto-RA, 13-*cis*-4-ketoretinoic acid; UDPGA, uridine-5′-diphosphoglucuronic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

[2] J. L. Napoli, unpublished results.

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