Reevaluation of Interaction of *cis*-Dichloro(ethylenediamine)platinum(II) with DNA†

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ABSTRACT: Intrastrand cross-links represent the majority of modifications in DNA resulting from interaction with the cancer chemotherapeutic drug *cis*-diamminedichloroplatinum(II) (*cis*-DDP). These adducts were recently characterized although several discrepancies remained to be resolved. In these studies, [^{3}H]-*cis*-dichloro(ethylenediamine)platinum(II) (*cis*-DEP) was used because of the convenience of the radiolabel; this analogue produces adducts at identical sites in DNA as *cis*-DDP. Both drugs platinate the following sequences in DNA: GG, 65%; AG, 25%; GNG, 6%. The adduct at AG sequences invariably has adenine on the 5′-terminus of the dimer. The present enzyme digestion protocol included P_1 nuclease, which produced complete digestion rather than as previously reported. The frequency of platination at GG was too high to be explained by an initial monofunctional platination at any guanine. However, direct bifunctional attack preferentially at GG was obviated because monofunctional adducts could be trapped with thiourea at short time periods. After short incubations, with *cis*-DEP and removal of unreacted drug, the monofunctional adducts slowly rearranged to bifunctional adducts. It is suggested that this evolution of adducts may result from the drug "walking" along the double helix, a phenomenon that does not appear to occur in single-stranded DNA.

The cancer chemotherapeutic drug *cis*-diamminedichloroplatinum(II) (*cis*-DDP)[1] is generally considered to elicit its antineoplastic activity as a result of interaction with DNA [reviewed in Roberts & Thompson (1979)]. The majority of damage in DNA is intrastrand cross-links. Less than 1% of platination appears to be involved in DNA interstrand cross-links (Roberts & Friedlos, 1981; Eastman, 1982a), and a small, but undetermined, quantity of DNA–protein cross-links occur. These bifunctional reactions are critical to the toxic action of the drug because monofunctional analogues show no therapeutic activity. In addition, the trans isomer, which also reacts bifunctionally with DNA, is therapeutically inactive. The trans isomer produces both DNA interstrand cross-links and DNA–protein cross-links but cannot form an intrastrand cross-link between two neighboring bases on a single strand. This has implicated intrastrand cross-links as important to the activity of *cis*-DDP. The formation of DNA intrastrand cross-links has previously been suggested by studies on inhibition of restriction endonucleases (Kelman & Buchbinder, 1978; Ushay et al., 1981) and by inhibition of exonuclease cleavage at guanine–guanine sequences in defined DNA templates (Royer-Pokora et al., 1981; Tullius & Lippard, 1981).

Two research groups have provided most of the direct characterization of these intrastrand cross-links in DNA. This has been achieved by enzyme digestion of platinated DNA and chromatographic separation of the products. The studies from this laboratory (Eastman, 1983, 1985) were made possible by the synthesis of a radiolabeled analogue of *cis*-DDP, [^{3}H]-*cis*-dichloro(ethylenediamine)platinum(II) ([^{3}H]-*cis*-DEP). After platination, DNA was enzymatically digested, and the products were separated by HPLC. Subsequent studies with nonradioactive *cis*-DDP confirmed that this drug reacted with DNA in a manner identical with that for *cis*-DEP. Fichtinger-Schepman et al. (1982, 1985) used nonradioactive *cis*-DDP and, after enzyme digestion, separated the products by anion-exchange chromatography with further characterization by nuclear magnetic resonance spectrometry. The major adduct in DNA is an intrastrand cross-link between two neighboring guanines. Other adducts contain adenine, which is almost invariably on the 5′-end of a platinated sequence. A discrepancy between the reports is whether these sequences are AG (Fichtinger-Schepman et al., 1985) or ANG (N is any nucleotide; Eastman, 1985). A further discrepancy in the reports relates to the kinetics of formation of adducts. Eastman (1985) identified monofunctional adducts at short incubation times that rearrange to bifunctional adducts with increased time while Fichtinger-Schepman et al. (1985) failed to detect significant levels of monofunctional adducts at any time. An explanation for these discrepancies is presented here as well as further analysis of the kinetics of formation of the bifunctional adducts.

Materials and Methods

Salmon testes DNA and all enzymes were obtained from Sigma Chemical Co., St. Louis, MO. Sephadex G-50 was purchased from Pharmacia, Piscataway, NJ. Unlabeled *cis*-DEP was purchased from Alfa Ventron, Danvers, MA. The preparation of [^{3}H]-*cis*-DEP was as previously detailed (Eastman, 1983). Thiourea was deionized over AG 501-X8 (Bio-Rad, Richmond, CA) prior to use. The majority of HPLC standards were prepared and characterized by NMR as previously described (Eastman, 1982b). Adducts at GG and AG sequences were prepared from dinucleosides (Sigma Chemical Co.), while the adduct at AGN was obtained by enzyme digestion of platinated DNA (Eastman, 1983).

In most experiments, 100 μg of DNA was incubated with [^{3}H]-*cis*-DEP in 20 mM $NaClO_4$, pH 5.5, for 16 h at 37 °C. The platinated DNA was precipitated with ethanol, redissolved

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[1] Abbreviations: *cis*-DDP, *cis*-diamminedichloroplatinum(II); *cis*-DEP, *cis*-dichloro(ethylenediamine)platinum(II); HPLC, high-pressure liquid chromatography; Tris, tris(hydroxymethyl)aminomethane.

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in 50 mM sodium acetate and 10 mM $MgCl_2$, pH 5.5, and digested to deoxyribonucleosides sequentially at 37 °C for 4 h with 200 Kunitz units of deoxyribonuclease I (bovine pancreas) and for 16 h with 20 μg of P_1 nuclease (stock solution 2 mg/mL, stored at −20 °C in 10 mM sodium acetate and 50% glycerol, pH 5.5). 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 (10–100 μL) were injected onto an Altex Ultrasphere ODS column and eluted with a 30-min linear gradient of 0–30% methanol in aqueous ammonium acetate, pH 5.5. During an additional 5 min, the gradient was 30–100%, and finally, the column was eluted with 100% methanol for 5 min. The concentration of ammonium acetate was varied according to the particular experiment. The flow rate was 1 mL/min and monitored with an ultraviolet (A_{254}) detector. Fractions, 0.5 mL, were collected, and liquid scintillation was counted.

In experiments requiring short incubation times, platination of the DNA was terminated by a 1-min centrifugation of 100-μL aliquots through a 1-mL Sephadex G-50 column that had previously been equilibrated with either 20 mM $NaClO_4$, pH 5.5, if the DNA was to be postincubated, or 50 mM sodium acetate and 10 mM $MgCl_2$, pH 5.5, if the DNA was to be digested. A comparison of ethanol precipitation and gel filtration demonstrated that both techniques produced identical adduct profiles, thereby showing no significant carryover of unbound drug.

Results

Ion-Suppression Reverse-Phase HPLC. The separation of *cis*-DDP adducts by reverse-phase chromatography is dependent upon the amount of salt in the aqueous phase (Eastman, 1982b). The adducts are positively charged and interact with the residual negative charges on the column. This interaction can be overcome in 0.1 M ammonium acetate, pH 5.5. At lower concentrations of cation, the adducts elute later and produce broader peaks. It has now been found that the change in elution position with the concentration of cation is diagnostic for the relative charge on the adduct. Reference adducts were separated at a variety of salt concentrations (Figure 1A). Monofunctional adducts, e.g., dA–Pt and dG–Pt, have a single positive charge and show a modest shift in elution position. Bifunctional adducts, e.g., dA–Pt–dG and dG–Pt–dG have two positive charges and show a dramatic shift in elution position. Bifunctional adducts between neighboring bases have two positive charges from the platinum–base complex and one negative charge from the phosphate for a net one positive charge. Their change in elution position parallels that of monofunctional adducts. Platinated trinucleotides have a net zero charge and demonstrate no alterations in elution position. These various effects on elution position have been used to facilitate resolution and characterization of the adducts.

Reaction of cis-DEP with Double-Stranded DNA. DNA was incubated with various concentrations of [^{3}H]-*cis*-DEP, and the adducts were separated by HPLC (Figure 1B,C). With 0.02 M ammonium acetate in the eluent, the major adducts are all resolved. Although at 0.1 M ammonium acetate the AG and dG–Pt–dG adducts coelute, this condition discriminates incompletely digested adducts that elute around 30 mL and become significant at high levels of modification. At very low levels of platination, only three adducts were detected. The percentages recovered sum to about 92% (Table I). The remainder represents a smear of radioactivity and does not represent any specific peaks. Individual adducts after purification by HPLC also produce this smear.

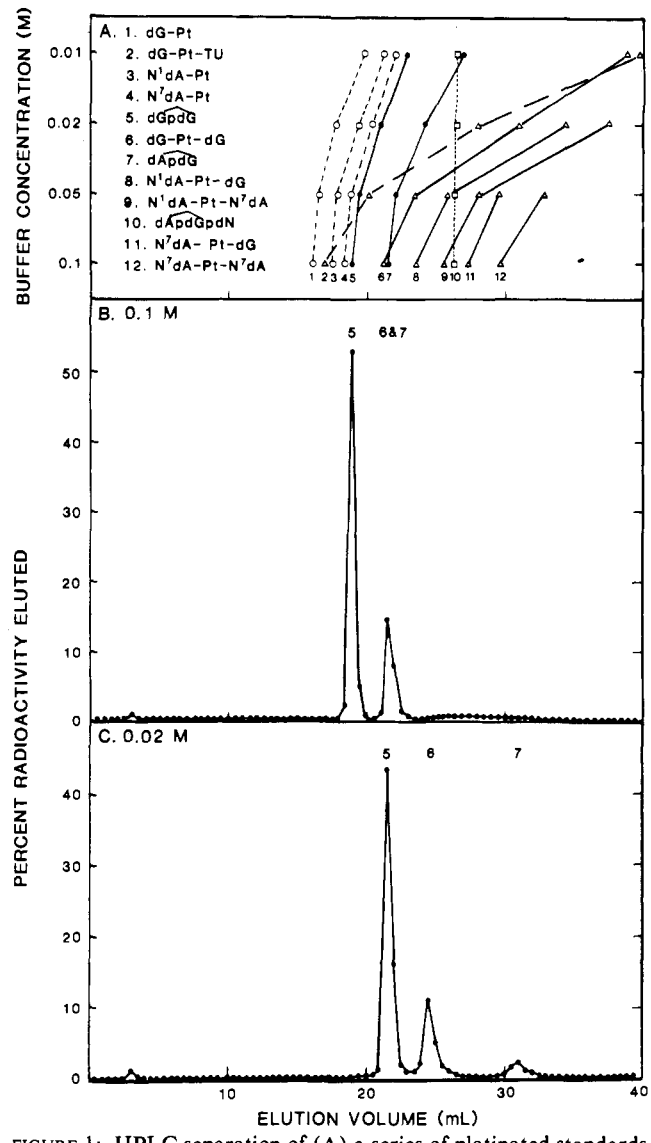

FIGURE 1: HPLC separation of (A) a series of platinated standards and (B and C) the enzyme digestion products of [^{3}H]-*cis*-DEP-modified double-stranded DNA. The elution position of the standards is shown for four concentrations of ammonium acetate in the HPLC eluting buffer. The concentration of ammonium acetate was (B) 0.1 and (C) 0.02 M. DNA was modified with *cis*-DEP at a Pt:DNA ratio of 0.002.

Table I: Effect of Amount of Platination on Percent of Each Adduct Detected after Reaction of [^{3}H]-*cis*-DEP with Double-Stranded DNA

input ratio, Pt:DNA	% radioactivity eluted			
	dGpdG	dApdG	dG–Pt–dG	undigested
0.002	63	22	7	0
0.01	62	21	7	0
0.02	57	24	7	2
0.05	42	32	6	6
0.1	27	33	6	14

At higher levels of platination, the percentage contributed by the adduct at GG is reduced as this sequence becomes saturated in DNA. Both adducts at AG and incomplete digestion products increase. The latter becomes prevalent because digestion is inhibited by close sites of platination. It should be emphasized that characterization of these adducts was also performed by purification of adducts, removing the platinum with thiourea and rechromatographing the products as detailed previously (Eastman, 1983).

Table II: Effect of Amount of Platination on Percent of Each Adduct Detected after Reaction of [^{3}H]-*cis*-DEP with Single-Stranded DNA

input ratio, Pt:DNA	% radioactivity eluted			
	dGpdG	dApdG	dG–Pt–dG	undigested
0.002	38	12	22	13
0.01	35	11	19	13
0.02	31	11	20	15
0.05	27	12	15	20
0.1	21	12	14	23

Reaction of cis-DEP with Single-Stranded DNA. Parallel experiments were performed with single-stranded DNA to determine the contribution of DNA secondary structure to the observed adducts. DNA was denatured in a boiling water bath for 15 min and rapidly cooled on ice prior to platination. The relative proportions of the major adducts were markedly different from that observed in double-stranded DNA (Table II). Platination at both GG and AG sequences was reduced with a concomitant increase in dG–Pt–dG, presumably because of the increased freedom of movement in single-stranded DNA. There was always some incompletely digested DNA. This could not be digested further upon addition of more P_1 nuclease. On purification by HPLC and reversal of platination with thiourea, a large number of peaks were obtained representing a heterogeneous collection of short oligonucleotides.

Kinetics of Formation of DNA Intrastrand Cross-Links. It has been proposed that *cis*-DDP interacts with DNA in a two-step process. In these studies, the intermediate, monofunctional adducts have been trapped with thiourea. Conditions for using thiourea were first characterized. Platinated DNA was incubated at 23 °C with various concentrations of thiourea for up to 16 h. The DNA was precipitated with ethanol and radioactivity associated with supernatent and pellet assayed. In 1 M thiourea, 44% of the radiolabel was removed from DNA by 16 h. The equivalent values for 100 and 10 mM thiourea were 10.1 and 2.1%, respectively. The adducts in this experiment were analyzed after digestion and HPLC separation. A 1-h incubation with 10 mM thiourea caused no significant loss of radioactivity from the DNA, but about 10% eluted as a thiourea-containing adduct at the elution position shown in Figure 1A. A 10-min incubation with thiourea produced none of this adduct. These conditions were therefore used to saturate monofunctional adducts.

DNA was incubated at 37 °C with [^{3}H]-*cis*-DEP for 15–60 min. Unreacted *cis*-DEP was removed by centrifuging the DNA though a 1-mL Sephadex G-50 column. The DNA was further incubated at 37 °C for 0–16 h to permit rearrangement of the adducts and then incubated at 23 °C for 10 min in 10 mM thiourea. The unreacted thiourea was removed by recentrifugation through a Sephadex G-50 column, the DNA was digested, and the adducts were separated by HPLC. At very short time periods, more than 40% of the radioactivity was recovered as a thiourea complex (Table III). During subsequent incubations, the monofunctional adducts were able to rearrange to bifunctional adducts such that no thiourea complexes were observed after 16 h. At short time periods, the adduct at GG was present at 32%, and this increased to a maximum value of about 60% by 16 h. Similarly, the adduct at AG increased markedly from 2 to 20% by 16 h. The dG–Pt–dG adduct contrasted this in that it appeared relatively constant with perhaps a slight decline at longer incubation times.

Sequence Orientation of cis-DEP Adducts. In previous reports a marked sequence orientation has been observed in adducts that contain adenine. The adenine is almost invariably at the 5′-terminus of the platinated sequence. Because it is now realized that the major adenine-containing sequence is AG rather than ANG, the sequence orientation has been reconfirmed. The adducts from DNA platinated at an input ratio of 0.01 were separated by HPLC. The adduct at AG was purified by separation first in 0.1 M ammonium acetate and then in 0.02 M ammonium acetate. The appropriate fractions were lyophilized and redissolved in 1 M thiourea, and platinum was removed by incubation at 37 °C for 16 h. The AG sequence, now without drug, was repurified by HPLC, lyophilized, redissolved, and digested for 1 h with 1 μg of P_1 nuclease. Separation of the digestion products by HPLC showed only deoxyadenosine and dGMP. This demonstrated that all the adenosine was at the 5′-end of the dimer. This experiment was repeated with single-stranded DNA with the same result. The sequence orientation was further tested at an input ratio of 0.1, a condition at which the adduct at AG is the most abundant in double-stranded DNA. Even under these conditions, the sequence orientation was maintained in both double- and single-stranded DNA.

Table III: Effect of Incubation Time on Percent of Each Adduct Detected after Reaction of [^{3}H]-*cis*-DEP with Double-Stranded DNA[a]

incubation time (min)	postincubation time (h)	% radioactivity eluted			
		dG–Pt–TU	GpdG	dApdG	dG–Pt–dG
15	0	42	32	2	9
30	0	40	36	3	8
60	0	26	45	5	9
60	1	14	54	9	9
60	2	4	57	15	9
60	16	0	60	18	6

[a] All reactions were stopped by removal of unreacted drug, and monofunctional adducts were trapped with thiourea (TU).

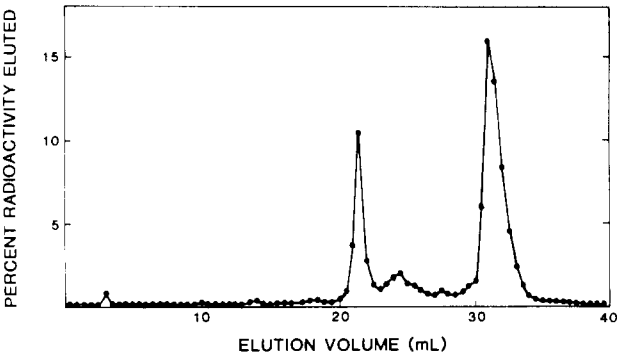

FIGURE 2: HPLC separation of the enzyme digestion products of [^{3}H]-*cis*-DEP-modified DNA that has been enriched for DNA interstrand cross-links by denaturation–renaturation and digestion with S_1 nuclease. The HPLC separation was performed with 0.02 M ammonium acetate in the eluting buffer. The peak eluting at 31 mL represents a markedly enriched dG–Pt–dG adduct.

Confirmation of the Character of Interstrand Cross-Links. Interstrand cross-links were previously characterized as between two deoxyguanosines on opposite strands (Eastman, 1985). This has been reassessed because of the change in digestion protocol reported here. DNA containing interstrand cross-links was enriched by denaturation and renaturation of 500 μg of platinated DNA (input ratio 0.002) followed by 1-h digestion with 1000 units of S_1 nuclease to remove single-stranded DNA. Unplatinated DNA, 100 μg, was added and ethanol-precipitated. The pellet was redissolved, and the denaturation, renaturation, digestion, and precipitation were repeated. The residual DNA was digested, and the adducts were separated by HPLC (Figure 2). About 50% of the radioactivity was detected in an adduct that cochromato-

graphed with dG–Pt–dG under both high- and low-salt elution conditions. This is in agreement with the previous observation that interstrand cross-links form between two deoxyguanosines.

Discussion

There is now complete agreement on the character of the intrastrand cross-links produced in DNA by *cis*-DDP. Although the present work has used [^{3}H]-*cis*-DEP because of the convenience of the radiolabel, this analogue produces adducts at identical sites in DNA as *cis*-DDP. A major discrepancy between the previous work from this laboratory and that of Fichtinger-Schepman et al. (1985) has now been resolved by the use of P_1 nuclease rather than S_1 nuclease to digest the DNA. *cis*-DEP platinated the following sequences: GG, 65%; AG, 25%; GNG, 6%. The formation of DNA interstrand cross-links between two guanines has also been reconfirmed. The adduct at AG demonstrated a marked sequence specificity with the deoxyadenosine always on the 5′-end of the dimer. No new adducts were detected at higher levels of platination; only the addition of incompletely digested DNA was detectable in the HPLC profiles. The difficulty in obtaining complete digestion of the adducts at AG, even when P_1 nuclease was used, might have significance in vivo if the appropriate DNA repair enzymes also exhibit a poor digestion capability. It is interesting that the phosphodiester bond that is difficult to cleave is not adjacent to the adenine but between guanine and the next base in the 3′-direction. This conclusion arises from the previous observation that in platinated AGN sequences the adenine is also at the 5′-terminus. In contrast, the 3′-nucleotide is readily digested out of a platinated GGN sequence.

Previous reports emphasized that the high frequency of platination at GG sequences cannot be explained by a random first reaction at any guanine and subsequent cross-linking to a neighboring base if also guanine. Such a model would theoretically result in only 36.8% of the adducts at GG sequences. This suggests that the drug reacts preferentially at GG sequences. To prove this, an attempt was made to isolate monofunctional intermediates of platination at very short time periods. It has been demonstrated that monofunctional adducts can be trapped with ammonium bicarbonate (Fichtinger-Schepman et al., 1984). However, this requires an overnight incubation during which the adducts would likely rearrange. Very low levels of monofunctinal platination were detected by this method (Fichtinger-Schepman et al., 1985). Thiourea, alternately, can trap intermediates almost instantly. After a 15-min incubation with [^{3}H]-*cis*-DEP and stopping of the reaction with thiourea, up to 42% of the adducts were recovered complexed to thiourea. Interestingly, adducts at GG represented close to the theoretical value of 36.8% for the random platination model. Continued incubation after removal of unreacted drug, demonstrated evolution of the adducts until greater than 60% occurred at GG. This observation does not support the hypothesis of direct bifunctional attack preferentially at GG sequences. Two alternate models are possible. The first is that there is a monofunctional attack preferentially at one of the guanines in a GG sequence with slow cross-linking to the neighboring base. This model would require an explanation to discriminate the formation of some bifunctional adducts very rapidly while the remainder take several hours to form. The second model is that reaction with guanine is random but that subsequent evolution of adducts occurs that can result in breaking of the initial complexes; that is, the drug can "walk" along the helix or "jump" between helices. Significant to this model is the observation that such evolution does not occur in single-stranded DNA where the frequency of adducts at GG closely reflects the theoretical value for random reaction. This suggests that drug is unlikely to jump between helices. Rather, it may walk along a helix by a mechanism dependent on the tertiary structure of the double helix. Evidence for such a model can best be obtained with defined-sequence DNA.

Registry No. *cis*-DEP, 14096-51-6.

References

Eastman, A. (1982a) *Biochem. Biophys. Res. Commun. 105*, 869.

Eastman, A. (1982b) *Biochemistry 21*, 6732.

Eastman, A. (1983) *Biochemistry 22*, 3927.

Eastman, A. (1985) *Biochemistry 24*, 5027.

Fichtinger-Schepman, A. M. J., Lohman, P. H. M., & Reedijk, J. (1982) *Nucleic Acids Res. 10*, 5345.

Fichtinger-Schepman, A. M. J., van der Veer, J. L., Lohman, P. H. M., & Reedijk, J. (1984) *J. Inorg. Chem. 21*, 103.

Fichtinger-Schepman, A. M. J., van der Veer, J. L., den Hartog, J. H. J., Lohman, P. H. M., & Reedijk, J. (1985) *Biochemistry 24*, 707.

Kelman, A. D., & Buchbinder, M. (1978) *Biochimie 60*, 893.

Roberts, J. J., & Thomson, A. J. (1979) *Prog. Nucleic Acid Res. Mol. Biol. 22*, 71.

Roberts, J. J., & Friedlos, F. (1981) *Biochim. Biophys. Acta 655*, 146.

Royer-Pokora, B., Gordon, L. K., & Haseltine, W. A. (1981) *Nucleic Acids Res. 9*, 4595.

Tullius, T. D., & Lippard, S. J. (1981) *J. Am. Chem. Soc. 103*, 4620.

Ushay, H. M., Tullius, T. D., & Lippard, S. J. (1981) *Biochemistry 20*, 3744.