Interstrand Cross-Links and Sequence Specificity in the Reaction of *cis*-Dichloro(ethylenediamine)platinum(II) with DNA†

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ABSTRACT: Characterization of the adducts produced in DNA by the cancer chemotherapeutic drug *cis*-diamminedichloroplatinum(II) and a radiolabeled analogue, [^{3}H]-*cis*-dichloro(ethylenediamine)platinum(II) ([^{3}H]-*cis*-DEP) was recently reported [Eastman, A. (1983) *Biochemistry 22*, 3927]. Both drugs reacted at identical sites in DNA, most of which produced intrastrand cross-links. DNA-interstrand cross-links, which represent less than 1% of total platination, have now been characterized. DNA containing interstrand cross-links was enriched for on the basis of its renaturability after boiling. This DNA was digested to deoxyribonucleosides, and the adducts were separated by high-pressure liquid chromatography. A cross-link between two deoxyguanosines was observed to be the most prominent adduct. It is proposed that the major sequence in which this cross-link occurs is 5′-CG-3′. DNA that was incubated with [^{3}H]-*cis*-DEP for 1 h showed low levels of interstrand cross-links. After removal of unreacted drug, their frequency increased significantly over 6 h with a maximum occurring at about 12 h. A similar phenomenon was seen in the case of intrastrand cross-links that contained adenine, in particular when the cross-link was between the terminal bases in an ANG trinucleotide sequence (N is any nucleotide). The primary site of reaction is at guanine, with a slow subsequent cross-link to the adenine. A model is presented that is consistent with the observation that adenine is always at the 5′ terminus of these adducts. The proportion of adducts at ANG sequences also increased at elevated temperatures. This is discussed with regard to potential significance during hyperthermia treatment of patients. Possible distortions in DNA resulting from platination were also investigated. Individual *cis*-DEP adducts were not recognized by single strand specific S_1 nuclease, demonstrating minimal denaturation of the DNA helix. Previous studies showing that these adducts are susceptible to S_1 nuclease digestion were performed at high levels of modification, conditions under which the concerted effect of several adducts would have produced a digestible site.

The interaction of the cancer chemotherapeutic agent *cis*-diamminedichloroplatinum(II) (*cis*-DDP)[1] with DNA has been implicated as essential to the toxic action of the drug [reviewed in Roberts & Thomson (1979)]. Until recently *cis*-DDP-induced DNA-interstrand cross-links and DNA–protein cross-links were studied, not because they represented the major adducts in DNA but rather that techniques were available for their measurement. Accordingly, in many experiments, the production of DNA-interstrand cross-links was correlated with cell toxicity (Zwelling et al., 1979; Erickson et al., 1981) even though other experiments suggested that more significant lesions existed (Filipski et al., 1980; Strandberg et al., 1982).

Analysis of the relative frequency of interstrand cross-links led to the conclusion that they represented less than 1% of the platination of DNA (Roberts & Friedlos, 1981; Pera et al., 1981; Eastman, 1982a). As an alternative lesion, the formation of DNA-intrastrand cross-links between two neighboring guanine bases has been inferred from 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).

In the past 2 years a number of investigators have reported characterization of DNA intrastrand cross-links as a result of either enzyme digestion (Eastman, 1983; Fichtinger-Schepman et al., 1982) or acid depurination (Johnson, 1982; Rahn, 1984) of platinated DNA and separation of the products by HPLC. The studies from this laboratory (Eastman, 1983) were made possible by the synthesis of a radiolabeled analogue of *cis*-DDP. This analogue, [^{3}H]-*cis*-dichloro(ethylenediamine)platinum(II) ([^{3}H]-*cis*-DEP), was used as a sensitive monitor of platination of DNA. After platination, DNA was enzymatically digested, and the products were separated by ion-suppression reverse-phase HPLC. The results demonstrated that the major adduct in DNA was an intrastrand cross-link between two neighboring guanines although cross-links could also form in AG, GNG, and ANG sequence (N is any nucleotide).

A number of questions were raised by the preceding studies and are addressed in this paper. In particular, an interstrand cross-link was reported but at levels too low to be characterized. Additional studies presented here relate to the kinetics of adduct formation, the influence of temperature, and the sequence specificity of platinated sites in DNA.

Materials and Methods

Salmon testes DNA, calf thymus DNA, and all enzymes were obtained from Sigma Chemical Co., St. Louis, MO. Sephadex G-50 was purchased from Pharmacia, Piscataway, NJ. Rat liver mRNA was purified by standard procedures in the laboratory of Dr. Bresnick. Unlabeled *cis*-DEP was purchased from Alfa Ventron, Danvers, MA. The preparation of [^{3}H]-*cis*-DEP was as previously detailed (Eastman, 1983).

The method for platination of DNA, enzyme digestion, and HPLC separation have been described previously (Eastman,

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[1] Abbreviations: *cis*-DDP, *cis*-diamminedichloroplatinum(II); *cis*-DEP, *cis*-dichloro(ethylenediamine)platinum(II); HPLC, high-pressure liquid chromatography. All oligonucleotide sequences are presented in the 5′ → 3′ direction.

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1983). In most experiments 100 μg of nucleic acid was incubated with [^{3}H]-*cis*-DEP in 0.02 M $NaClO_4$, pH 5.5, for 16 h at 37 °C. The platinated DNA was precipitated with ethanol, redissolved in S_1 nuclease buffer, and digested to deoxyribonucleosides. Optimization of the digestion procedure is described under Results. Aliquots (100 μL) were injected onto an Altex Ultrasphere ODS column and eluted with a gradient of 0.1 M aqueous ammonium acetate in methanol (Eastman, 1983).

In experiments involving interstrand cross-links the DNA was first sheared by 10 passes through a 25-gauge needle. To enrich for interstrand cross-links, 1 mL of platinated DNA was placed in a boiling water bath for 15 min, a condition under which the adducts were stable, and then rapidly cooled on ice. S_1 nuclease (100 or 1000 units) was added, and single-stranded DNA was digested for 1 h at 37 °C. Residual double-stranded DNA that contained the interstrand cross-links was precipitated by the addition of 90 μL of concentrated perchloric acid (70–72%). The precipitate was allowed to form on ice for 30 min and then collected by centrifugation at 15000*g* for 5 min in an Eppendorf microfuge. The supernatant was removed and the pellet hydrolyzed in 0.6 N perchloric acid at 80 °C for 30 min. The A_{260} and radioactivity of both supernatant and hydrolyzed pellet were used as a measure of DNA and drug. When the specific adducts enriched by denaturation and renaturation were analyzed, the acid-insoluble pellet was washed with 70% ethanol, digested to deoxyribonucleosides, and separated by HPLC.

In several experiments platination of the DNA was terminated after 1 h by a 1-min centrifugation of 100-μL aliquots through a 1-mL Sephadex G-50 column that had previously been equilibrated with 0.02 M $NaClO_4$.

Results

Separation of Platinated Products in DNA. Upon interaction of DNA with [^{3}H]-*cis*-DEP followed by enzyme digestion and HPLC separation, a profile of adducts is obtained (Figure 1A). The peaks have previously been characterized (Eastman, 1983) and are numbered for reference. The major adduct, peak 2, represents an intrastrand cross-link between two neighboring guanines. Peak 4 represents an intrastrand cross-link between neighboring adenine and guanine. Peaks 3, 5, and 6 are derived from intrastrand cross-links between the terminal bases in trinucleotide sequences. Peak 3 is dG–Pt–dG derived from a GNG sequence while peaks 5 and 6 are platinated ANG sequences.

Several differences from the previous report are worth mentioning. In the present experiments the synthesis of [^{3}H]-*cis*-DEP has provided better preparations which is thought to result from a better custom synthesis of [^{3}H]-ethylenediamine provided by Amersham Corp. This is reflected in the cleaner adduct profiles obtained. In particular the radioactivity eluting at the void volume is usually negligible, while the frequency of monofunctional platination is reduced (peak 1). The latter is probably due to impurities that react with the alkaline phosphatase and therefore eluted with it. As previously reported, however, at high levels of modification of DNA, monofunctional adducts do occur that react with alkaline phosphatase and chromatograph as peak 1.

The enzyme digestion has been better characterized. It is not necessary to include deoxyribonuclease in these digestions. S_1 nuclease (1000 units) is capable of digesting 100 μg of double-stranded DNA completely in 16 h. This enzyme is single strand specific at short time periods, but it also nicks double-stranded DNA, leading to accelerated digestion of total DNA with time. It is important not to add too much S_1 nuclease because the cross-link between two neighboring guanines (peak 2) can be cleaved to give dG–Pt–dG (peak 3). Similarly, platinated ANG sequences may be cleaved to dA–Pt–dG. The acceptable range for S_1 nuclease is 200–2000 units for a typical digestion containing 100 μg of DNA. With regard to alkaline phosphatase, less than 0.2 unit resulted in incomplete digestion. This was most noticeable as early elution of the ANG adduct due to retention of the 5′-phosphate group. Routinely 1 unit of alkaline phosphatase is used although up to 10 units did not alter the adduct profile.

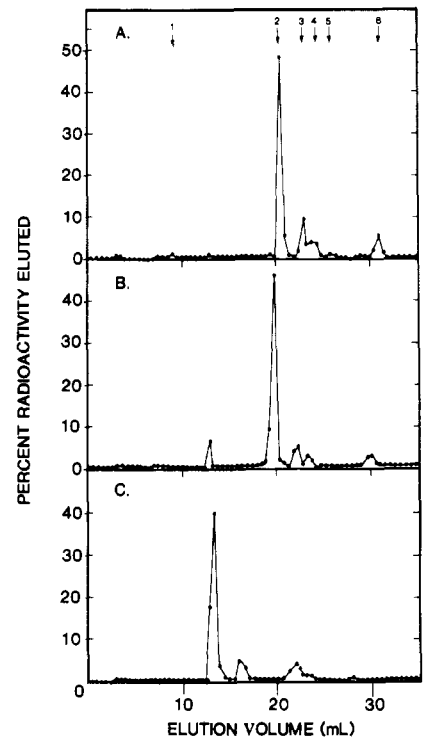

FIGURE 1: HPLC separation of the enzyme digestion products of [^{3}H]-*cis*-DEP-modified (A) salmon sperm DNA, (B) calf thymus DNA with trace contamination of RNA, and (C) rat liver mRNA. The numbers 1–6 refer to the radioactive adducts in DNA. Peak 1 derives from monofunctional platination. Peak 2 is platinated dGpdG. Peak 3 is dG–Pt–dG mainly derived from platinated trinucleotide GNG. Peak 4 is platinated dApdG. Peak 5 is platinated trinucleotides ACG and AGG. Peak 6 is platinated trinucleotides AAG and ATG.

Characterization of Interstrand Cross-Links. In the previous publication (Eastman, 1983) a single interstrand cross-link was reported. This became relatively rare at higher levels of platination in contradiction to other experiments in which the renaturable fraction of DNA correlated with the level of platination (Eastman, 1982a). These observations can now be explained. The adduct previously thought to be an interstrand cross-link is in fact due to contaminating RNA. Figure 1 contrasts an HPLC separation of the products obtained from this contaminated DNA with that from purified DNA and from RNA. The major RNA adduct cochromatographed with the adduct previously considered to be the interstrand cross-link. This error arose from the use of nitrocellulose filters to enrich for double-stranded DNA after denaturation and renaturation. The RNA was poorly retained under those conditions and therefore became the most predominant adduct. In several experiments the RNA adducts have been removed by passage of the enzyme digest through a column of Affigel 601 (Bio-Rad, Richmond, CA), a boronate gel that removes ribonucleosides because of their vicinal diols. A profile is then obtained that is identical with that with purified DNA. Salmon testes DNA is now routinely used since it contains negligible RNA contamination.

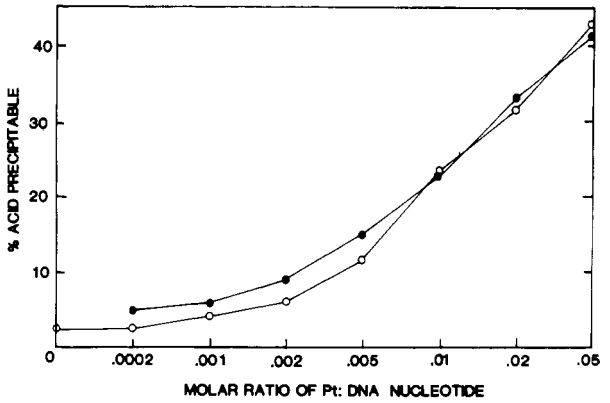

FIGURE 2: Formation of *cis*-DEP-induced interstrand cross-links in DNA at different levels of platination. DNA was incubated with [^{3}H]-*cis*-DEP at various molar ratios for 16 h, ethanol precipitated, and redissolved. The DNA was denatured by boiling, renatured, and digested with 1000 units of S_1 nuclease for 1 h. The undigested DNA was acid precipitated, and the radioactivity (●) and A_{260} (○) associated with both the pellet and supernatant were determined.

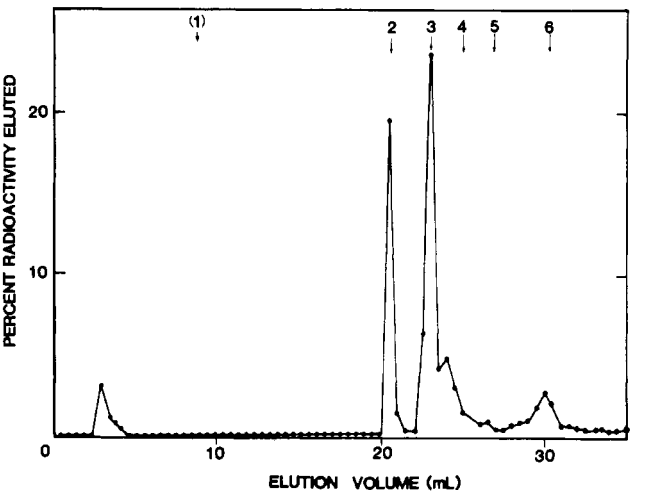

FIGURE 3: HPLC separation of the enzyme digestion products of [^{3}H]-*cis*-DEP-modified DNA that has been enriched for DNA interstrand cross-links by denaturation–renaturation and digestion with S_1 nuclease.

DNA containing interstrand cross-links can also be enriched after denaturation and renaturation by digesting for 1 h with S_1 nuclease, at which time the enzyme reacts specifically with single-stranded DNA. The double-stranded DNA that contains interstrand cross-links is then precipitated with acid. DNA that had been incubated with various concentrations of [^{3}H]-*cis*-DEP was subjected to this enrichment procedure. The percentage of DNA that was acid precipitable increased in proportion to the level of platination up to a molar ratio of 0.01 (Figure 2). Considering that only about 1% of platination produces an interstrand cross-link and that only 20% of the DNA was cross-linked at a moar ratio of 0.01, this is consistent with one interstrand cross-link every 2000 bases. This value is close to the average size of 3000 base pairs (6000 bases) for DNA sheared as in these studies (Eastman, 1982a). At higher levels of platination, there is also digestion of portions of DNA containing two or more close platinations, thereby resulting in smaller DNA (discussed below).

At the lowest levels of plantation, the renatured strands would contain the fewest additional adducts, that is, those from intrastrand cross-links. DNA that had been incubated with only radiotracer quantities of [^{3}H]-*cis*-DEP was therefore denatured, renatured, digested for 1 h with S_1 nuclease, and acid precipitated. This procedure remmoved 95% of the DNA and accordingly 95% of the intrastrand cross-links. The remainder of the DNA should contain all of the interstrand cross-links but only 5% of other adducts. The pellet was washed with ethanol and digested to deoxyribonucleosides and platinated products. These were separated by HPLC. The adduct derived from a cross-link between two deoxyguanosines became the most pronounced under these conditions (Figure 3). This therefore represents the major interstrand cross-link. Unfortunately, in its digested form it is identical with the intrastrand cross-link derived from platination at GNG sequences. These two lesions cannot therefore be discriminated by this system. The possibility of other minor interstrand cross-links cannot be completely excluded.

Kinetics of Formation of DNA Cross-Links. In cells, an increase in interstrand cross-links has routinely been observed even after removal of the drug from the culture medium (Zwelling et al., 1979; Pera et al., 1981; Strandberg et al., 1982). This is presumed to result from initial formation of a monofunctional adduct that slowly produces the cross-link. An alternate explanation is that intracellular drug could contribute to continued platination of DNA after the change of culture medium (Pera et al., 1981). Previous experiments in which purified DNA was platinated for 1 h, ethanol precipitated, and then incubated again in the absence of free drug failed to show such an increase in interstrand cross-links (Eastman, 1983). Although it is now known that RNA adducts were being studied in those experiments, it is still possible that the manipulations required in ethanol precipitation and redissolution of the DNA would have driven the formation of interstrand cross-links to completion. These experiments were therefore repeated but, instead of precipitation of the platinated DNA after 1 h, the sample was centrifuged through a 1-mL Sephadex G-50 column equilibrated with 0.02 M $NaClO_4$. By this means the unreacted *cis*-DEP was removed in 1 min. An aliquot of this DNA was immediately denatured and renatured while the remainder was incubated for longer time periods and then subjected to denaturation and renaturation. The samples were diluted with S_1 nuclease buffer, digested for 1 h with S_1 nuclease, and acid precipitated. After 1-h platination of DNA at an input ratio of 1 Pt/50 nucleotides, 3% of the DNA was acid precipitable after S_1 nuclease digestion. This value increased to 14% by 24 h after platination. The major increase occurred within the first 6 h, which agrees with the previous observations made in cells.

In parallel studies, DNA platinated as above was digested to deoxyribonucleosides, and the products were separated by HPLC. The adduct profiles obtained by this technique were the same as previously observed after ethanol precipitation (Eastman, 1983); that is, after a 1-h incubation of [^{3}H]-*cis*-DEP with DNA, about 10% of the radioactivity was recovered in an adduct derived from monofunctional platination. When the same DNA was incubated for a further 16 h in the absence of drug, the adduct profile resembled that in Figure 1A; that is, the monofunctional adduct was markedly reduced with a concomitant increase in the bifunctional adducts. The latter appeared to be specific for those adducts previously identified as containing adenine, which suggests that the initial monofunctional platination occurs on a guanine and that the second, slower reaction is with the adenine. Although the interstrand cross-links also increased during this period, their rarity precluded any increase in the amount of dG–Pt–dG which is derived predominantly from intrastrand cross-links in GNG sequences.

Effect of Temperature on Platination of DNA. The slow formation of interstrand cross-links and adducts in ANG sequences is presumably due to a major conformational change

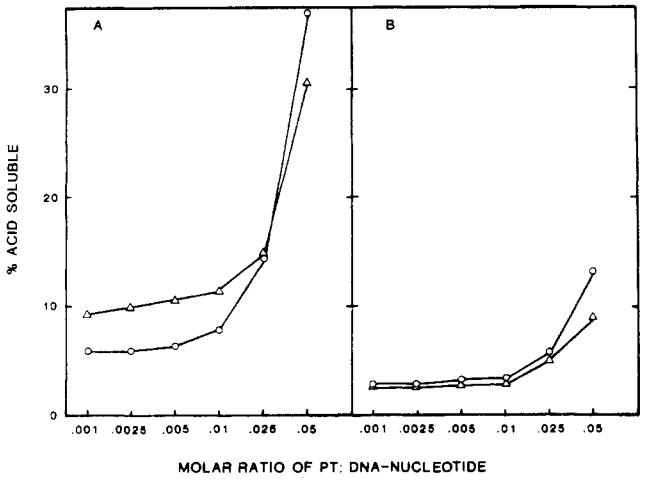

FIGURE 4: S_1 nuclease digestibility of DNA modified to various extents by *cis*-DEP. [^{3}H]-*cis*-DEP was incubated with DNA at the indicated molar ratio for 16 h, then ethanol precipitated, redissolved, and digested with (A) 1000 units of S_1 nuclease or (B) 100 units of S_1 nuclease. After 60 min, DNA was precipitated with perchloric acid, and the percentage of DNA (Δ) or Pt (O) released into the supernatant was calculated from assay of A_{260} and radioactivity.

that would be required to accommodate this type of lesion in the DNA helix. It was therefore of interest to determine the effect of temperature on the adduct profiles. DNA was incubated for 16 h with [^{3}H]-*cis*-DEP at temperatures from 4 to 50 °C. Although greater than 90% reaction of *cis*-DEP occurred at 37 and 50 °C, only 65% reaction occurred at 20 °C and 10% at 4 °C. On analyzing the adduct profile after HPLC, the most pronounced effect was indeed in the contribution from adducts at ANG sequences (peak 6) which increased from 3.3% of the total adducts at 4 °C to 11.5% at 50 °C. At 37 °C, these adducts consistently represent 7–8% of the total. No other significant differences were observed at the various temperatures.

S_1 Nuclease Sensitivity of Platinated DNA. In these investigations of interstrand cross-links, S_1 nuclease has been used in the digestion of platinated DNA, implying that interstrand cross-links are resistant to S_1 nuclease digestion. Apparently, these cross-links do not create sufficient distortion of the helix to provide a substrate for this nuclease. These studies have therefore been extended to determine whether any of the intrastrand cross-links create sufficient distortion to be digested from double-stranded DNA by S_1 nuclease.

DNA was incubated for 16 h with various concentrations of [^{3}H]-*cis*-DEP. The platinated DNA was precipitated with ethanol, redissolved in 1 mL of S_1 nuclease buffer, and digested with either 100 or 1000 units S_1 nuclease for 1 h at 37 °C. Incubations were terminated by precipitating undigested DNA with perchloric acid. Both the supernatant and pellet were assayed for nucleic acid (A_{260}) and drug (radioactivity).

It is evident that the interaction of *cis*-DEP with DNA led to the formation of single-stranded regions that could be digested by S_1 nuclease (Figure 4). However, such regions were only detected at a level of modification greater than 1 Pt/100 nucleotides. Superimposed upon this in Figure 4 is the amount of [^{3}H]-*cis*-DEP-derived adducts that have been released by S_1 nuclease digestion. It can be seen that the release of adducts closely paralleled the digestion of DNA, demonstrating that there is no preferential digestion of platinum adducts as would be expected if they created single-stranded regions. Only at the highest level of modification (1 Pt/20 nucleotides) did the release of radioactivity exceed the release of nucleotides. This would be expected in those areas of DNA that are heavily platinated and therefore single stranded by virtue of the concerted effect of several adducts.

To determine whether minor adducts were removed by S_1 nuclease at low levels of modification, the platinated DNA was enzymatically digested and separated by HPLC. After incubation under conditions in which 6% of the total DNA was digested by S_1 nuclease (i.e., DNA modified at 1 Pt/1000 nucleotides and incubated for 60 min with 1000 units of S_1 nuclease), the adduct profile was identical with that shown in Figure 1A. This confirmed that no minor adducts were preferentially released by S_1 nuclease digestions.

Discussion

The major products resulting from the interactions of *cis*-DEP with DNA are intrastrand cross-links. These predominantly involve cross-links with the N(7) position of neighboring guanines although adenine occasionally contributes to cross-linking. Additionally, intrastrand cross-links can form between the terminal bases of a trinucleotide. An intriguing observation that was made in the previous studies was that when adenine was platinated in a trinucleotide sequence, the adenine was always at the 5′ end (Eastman, 1983). In the case of adenine in a platinated dinucleotide, a 90% preference was noted for the 5′ end. Other studies also support this sequence preference (Dewan, 1984; Wing et al., 1984). Information to help explain this directional specificity is presented here. When DNA is platinated for 1 h and the adduct profile analyzed, it is evident that the adenine-containing trinucleotide adduct is underrepresented compared to DNA platinated for longer periods. However, after this 1-h platination period, a significant quantity of monofunctional adducts is seen that arise predominately from platinated deoxyguanosine (Eastman, 1983). During extended incubation, these monofunctional adducts can form the adenine-containing adducts. Guanine is therefore the primary site of reaction while reaction with adenine is relatively slow. The reason for the directional specificity becomes apparent upon consideration of a trinucleotide sequence as presented in Figure 5A. Three guanines are shown on one strand for clarity with the central guanine platinated at its N(7). The Pt is in the major groove, directly above the next base in a 5′ direction but well away from the adjoining base in a 3′ direction. This is also seen in Figure 5B where a side view of the helix demonstrates that a cis ligand (here drawn in the chloride form) tries to occupy the same space as the 5′-neighboring base. It is therefore evident that a much greater probability occurs for cross-linking to the next base in a 5′ direction, which if adenine would explain the observed directional preference. In many cases, the next 5′ base will be neither guanine nor adenine. In this situation, the neighboring base would not be a substrate for cross-linking but would be displaced into the minor groove. Accordingly the next base in a 5′ direction could become a candidate for cross-linking. This explanation is consistent with the 5′ location of adenine in the trinucleotide adducts. It should be mentioned that if the neighboring base is adenine, then two options are in fact available. Occasionally a cross-link would form yielding platinated AG, while on other occasions, the neighboring adenine may be perturbed, which would lead to cross-links in AAG sequence. Perhaps the trinucleotide adduct is preferred over the dinucleotide form, with the latter occurring only in the absence of another appropriate base.

One of the aims of these studies was to characterize the DNA-interstrand cross-links induced in DNA by *cis*-DEP. Although many studies have detected interstrand cross-links, few studies have provided information as to their chemical structure. This is in part due to their relative rarity, representing in the order of 1% of total platination of DNA (Roberts

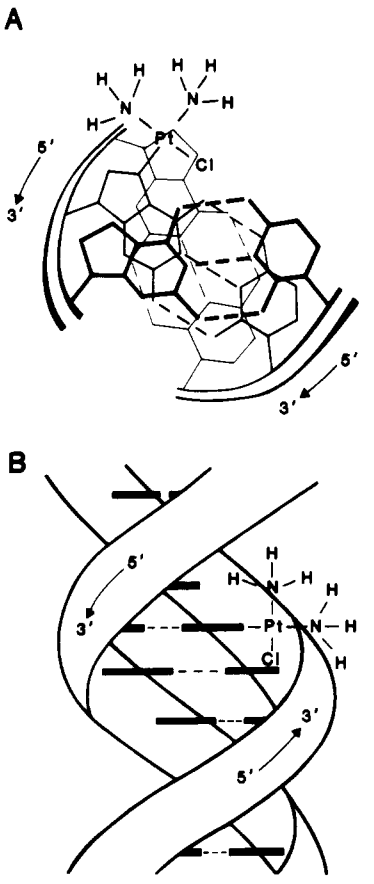

FIGURE 5: Location of monofunctional platination of DNA at N(7) guanine in a GGG sequence as viewed (A) down the helix or (B) from the side. See text for discussion.

& Friedlos, 1981; Pera et al., 1981; Eastman, 1982a,b). Preferential interstrand cross-linking has been demonstrated in DNA rich in guanine and cytosine (Gangull & Theopanides, 1979). This could be caused by cross-links either between a cytosine and guanine as suggested by Harder & Lee (1983) or between two guanines on opposite strands as suggested by Fichtinger-Schepman et al. (1982). The latter view has generally been prevalent because of the preference for reaction of *cis*-DDP with N(7) of guanine in DNA. In addition, at least at the nucleoside level, it was not possible to form bifunctional adducts that contained cytidine (Inagaki & Kidani, 1979; Eastman, 1982b). In the present studies platinated DNA was denatured, renatured, and digested with single strand specific S_1 nuclease to enrich for interstrand cross-links. Subsequent separation of the adducts by HPLC showed enrichment for a bifunctional cross-link between to deoxyguanosines.

This poses the question as to the distance between the initial site of platination and the guanine on the opposite strand. If the adjacent base is required, then interstrand cross-links can form only in CG or GC sequences. These two possibilities are shown in Figure 6A. In this case the platinated guanine is located between two cytosines. The cytosine in the 3′ direction is opposite a guanine whose N(7) is about 7 Å from the Pt. The distance to the N(7) of guanine in the opposite direction is about 11 Å. It is not possible for either of these positions to cross-link while maintaining their normal hydrogen bonding. The discussion presented above suggests that the cytosine in a 5′ direction will be perturbed and displaced into the minor groove. The formerly hydrogen-bonded guanine would then be the most probable base to cross-link. The resulting structure requires that hydrogen bonds are broken for both base pairs

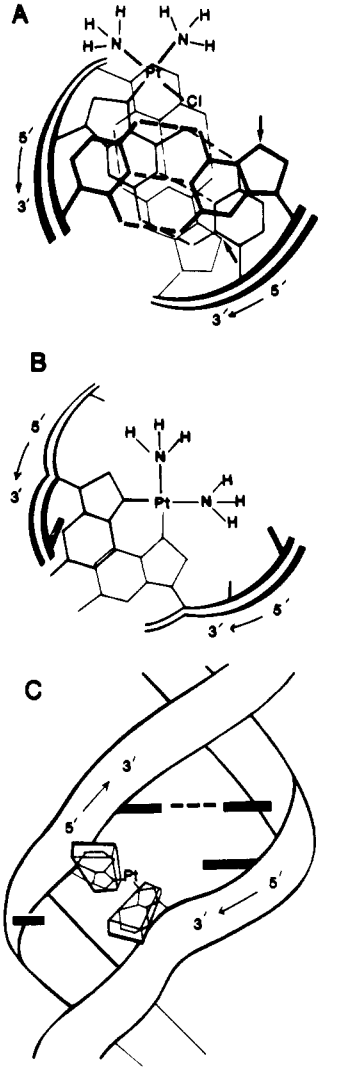

FIGURE 6: Formation and structure of a DNA interstrand cross-link. (A) Location of monofunctional platination of DNA at the N(7) position of guanine in a CGC sequence as viewed down the helix. The arrows indicate the N(7) position of the two guanines on the opposite strand. (B) Possible structure of an interstrand cross-link between the initial site of platination and the N(7) position of an opposite guanine. It is drawn such that the cross-link is in a 5′-CG-3′ orientation. The other bases have been removed for clarity. (C) A side view of the helix shown in (B) with the interstrand cross-link protruding into the minor groove.

involved and that both guanine bases are rotated into the minor groove (Figure 6B). The distance between the glycosidic bonds of the two opposite deoxyribose sugars is decreased from 12 to 10 Å, hence, the kink in the backbone. The two cross-linked bases must also rotate in opposite directions away from the normal plane (Figure 6C). These structures and measurements were obtained by manipulating three-dimensional molecular models.

Additional evidence that this is the most probable interstrand cross-link also comes from knowledge of the known trinucleotide sequences in which intrastrand cross-links can form. Almost all platination of ANG sequences occurs at ATG and AAG sequences (Eastman, 1983) as can be seen in Figure 1A (peak 6). Very little occurs at ACG or AGG sequences (peak 5). Obviously, in an AGG sequence the preferred cross-link would be between the two guanines. However, the rarity of platination at ACG sequences could be due to the presence of the guanine opposite the middle cytosine which would preferentially lead to an interstrand cross-link. The resistance of interstrand cross-links to S_1 nuclease also suggests that the

nearest opposite base is the most probable site of reaction.

Although this discussion does not prove the structure of interstrand cross-links beyond the fact that two guanines are involved, it does demonstrate the feasibility of formation of cross-links in CG sequences. It is interesting that CG sequence are rare in DNA and are the major location of 5-methylcytosine. These sequences are presumed to play a role in gene regulation [reviewed in Ehrlich & Wang (1981)].

The formation of all of these adducts will result in some distortion of the helical structure of DNA. These distortions have frequently been reported. Electron microscopy studies of closed-circular viral DNA have demonstrated that platinated strands can be shortened by at least 50% of their original length (Macquet & Butour, 1978; Cohen et al., 1979; Mong et al., 1981). This shortening also produces a change in electrophoretic mobility (Cohen et al., 1979; Mong et al., 1981; Scovell & Kroos, 1982). Circular dichroism and DNA melting curves (Macquet & Butour, 1978), nitrocellulose binding (Eastman, 1982a), single strand specific S_1 nuclease (Mong et al., 1981; Eastman, 1982a; Scovell & Capponi, 1982), and terbium fluorescence (Arquilla et al., 1983) have all been used to demonstrate a localized melting of the DNA helix. In every case these changes have been attributed to DNA-intrastrand cross-links. However, all of these changes were only detectable at very high levels of platination off the DNA. I have previously reported that no S_1 nuclease sensitive sites, or nitrocellulose binding sites, were present in DNA that had been modified at a molar ratio of platinum per nucleotide of less than 1/100 (Eastman, 1982a). This led to the conclusion that localized unwinding was dependent upon the concerted effect of several adducts in close proximity rather than to any individual adduct.

As S_1 nuclease was used in the present experiment to enrich for interstrand cross-links, the study was extended to analyzing the S_1 nuclease sensitivity of platinated double-stranded DNA. The availability of [^{3}H]-*cis*-DEP facilitated assessment of S_1 nuclease sensitivity of individual adducts in DNA. The results presented here demonstrated no preferential removal of any adduct from DNA in support of the contention that several neighboring adducts are required before sufficient distortion occurs to provide a substrate for S_1 nuclease.

Cytotoxicity of *cis*-DDP in Chinese hamster ovary cells is potentiated by hyperthermia although only part could be attributed to an increased interstrand cross-linking (Meyn et al., 1980). It was therefore postulated that formation of intrastrand cross-links at GNG sequences might be involved because the higher temperature would facilitate the necessary local denaturation of the DNA. Studies in *Escherichia coli* found that GNG sequences were hot spots for mutation by *cis*-DDP (Brouwer et al., 1981) and that the frequency of mutation was enhanced disproportionately at higher temperatures (Brouwer et al., 1982). The effect of temperature on the formation of adducts in DNA was therefore investigated. Although an increase was observed in the proportion of intrastrand cross-links at ANG sequences, it seems insufficient to explain the large differences in the mutation rate obtained at different temperatures (Brouwer et al., 1982). It is possible that DNA in the cell exhibits a more profound alteration in structure with increasing temperature or that this phenomenon is related to temperature sensitivity of biological responses such as DNA repair or error-prone replication. Further studies must be directed toward assessing whether particular adducts contribute more significantly to either toxicity or mutagenicity.

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