Reaction of Nucleic Acids with *cis*-Diamminedichloroplatinum(II): Interstrand Cross-Links†

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ABSTRACT: In the reaction of *cis*-diamminedichloroplatinum(II) (*cis*-DDP) with double-helical (dC-dG)$_4$·(dC-dG)$_4$ or (dC-dG)$_5$·(dC-dG)$_5$, intrastrand and interstrand cross-links between two guanine residues are formed. This is shown by gel electrophoresis in denaturing conditions of the reaction products and by high-performance liquid chromatography (HPLC) analysis of the products digested with nuclease P1. In the reaction of *cis*-DDP and poly(dG-dC)·poly(dG-dC), at relatively low levels of platination, it is mainly interstrand cross-links between two guanine residues that are formed. This is shown by HPLC analysis of the nuclease P1 digest and by gel electrophoresis in denaturing and nondenaturing conditions of the platinated polymer after cleavage with the restriction enzyme *Hha*I. Moreover, the antibodies to platinated poly(dG-dC)·poly(dG-dC) cross-react with the interstrand cross-linked (dC-dG)$_4$ or (dC-dG)$_5$ but not with the intrastrand cross-linked (dC-dG)$_4$ or (dC-dG)$_5$. These antibodies cross-react with platinated natural DNA. The amount of interstrand cross-links deduced from radioimmunoassays (0.5% of the total bound platinum) is lower than that (2%) deduced by gel electrophoresis in denaturing conditions of a platinated DNA restriction fragment. By gel electrophoresis, it is also shown that in vitro the isomer *trans*-DDP is more efficient in forming interstrand cross-links than *cis*-DDP.

Numerous studies support the concept that DNA is the likely target for the antitumor drug *cis*-diamminedichloroplatinum(II) (Roberts & Thomson, 1979; Roberts & Pera, 1983; Pinto & Lippard, 1985). Most of the adducts formed in the reaction of *cis*-DDP[1] and DNA have been identified (Eastman, 1986; Fichtinger-Schepman et al., 1985; Johnson et al., 1985). Two major adducts arise from an intrastrand cross-link between two adjacent guanine residues and between adjacent adenine and guanine residues. Minor adducts arise from intrastrand cross-links between two guanine residues separated by one base and from interstrand cross-links between two guanine residues. It is not yet known which adduct(s) is (are) responsible for the antitumor activity of *cis*-DDP.

We are interested in the interstrand cross-links for two main reasons. One reason is that the role of these adducts in the antitumor activity of *cis*-DDP is still open to question. Some results show a correlation between interstrand cross-linking and cytotoxicity, but other results seem to exclude the importance of the interstrand cross-links [general review, Pinto and Lippard (1985)]. On the other hand, recent experiments on Fanconi's anemia cells suggest that the interstrand cross-links are potentially lethal lesions (Plooy et al., 1985). The second reason is that, from a physicochemical point of view, the binding of *cis*-DDP to nucleic acids might help in the characterization of the dynamic properties of nucleic acids. The formation of interstrand cross-links implies a distortion of the double helix.

A physicochemical study of the interstrand cross-links in platinated DNA is difficult because they are relatively rare, representing about 1% of the total platination (Roberts & Friedlos, 1982; Eastman, 1985). Moreover, such a study is hampered by the presence of the other adducts that are preferentially formed. We show here that, in the reaction of *cis*-DDP and (dC-dG)$_n$·(dC-dG)$_n$ oligonucleotides, intrastrand and interstrand cross-links are formed while, in the reaction with poly(dG-dC)·poly(dG-dC), mainly interstrand cross-links are formed. The recognition of platinated natural DNA and of platinated oligonucleotides containing interstrand cross-links by the antibodies to platinated poly(dG-dC)·poly(dG-dC) suggests the formation of the same interstrand adduct in these platinated nucleic acids.

Materials and Methods

The oligonucleotides 5′-pd(CGCGCGCG) and 5′-pd(CGCGCGCGCG) from Pharmacia, Uppsala, were incubated with alkaline phosphatase and then purified by HPLC on a C_{18} column. Synthetic polydeoxynucleotides from Pharmacia or Boehringer, Mannheim, were treated twice with phenol and then precipitated with ethanol. The DNA restriction fragment (1100 base pairs) from *Cebus* (Malfoy et al., 1986) and pBR322 DNA were a gift of Dr B. Malfoy. The DNA molecular weight marker III was from Boehringer, Mannheim.

The reaction between platinum derivatives (a gift of Dr. J. L. Butour, Toulouse) and polynucleotides or DNA was done in 10 mM $NaClO_4$ and 1 mM phosphate buffer, pH 7.5, at 37 °C during 24 h. The synthesis and purification of bis(deoxyguanosine)platinum have been performed as previously described (Chu et al., 1978).

Deoxyribonuclease I, P1 nuclease, and alkaline phosphatase were purchased from Boehringer, and T_4 polynucleotide kinase and the restriction enzymes were from Biolabs, Beverley, MA. Enzyme buffers were those recommended by the suppliers. Ultrapure agarose was from Bethesda Research Laboratories,

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[1] Abbreviations: *cis*- or *trans*-DDP, *cis*- or *trans*-diamminedichloroplatinum(II); dien-Pt, chlorodiethylenetriamineplatinum(II); r_b, molar ratio of platinum residues per nucleotide; HPLC, high-performance liquid chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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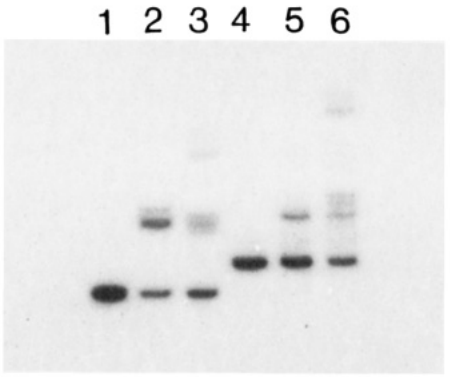


FIGURE 1: Autoradiogram of platinated $(dC\text{-}dG)_4$ and $(dC\text{-}dG)_5$ electrophoresed on a denaturing polyacrylamide gel. $(dC\text{-}dG)_4$, lane 1; $(dC\text{-}dG)_4$ reacted either with dien-Pt, lane 2, or with *cis*-DDP, lane 3; $(dC\text{-}dG)_5$, lane 4; $(dC\text{-}dG)_5$ reacted either with dien-Pt, lane 5, or with *cis*-DDP, lane 6. The reactions were carried out in 50 mM $NaClO_4$ at 37 °C for 3 days. Electrophoresis was performed on a 24% polyacrylamide gel containing 7 M urea for 2 h at 50 V/cm in a buffer containing 90 mM Tris-HCl, pH 7.5, 90 mM boric acid, and 1 mM EDTA.

and electrophoresis-grade acrylamide and bis(acrylamide) were from Bio-Rad, Munich.

The 5′ end labeling of oligonucleotides with [γ-^{32}p]ATP (Amersham, Les Ulis) and polyacrylamide gel electrophoresis were performed as described by Banaszuk et al. (1983). Alkaline agarose gel electrophoresis of platinated restriction fragments was carried out as described (Maniatis et al., 1982).

The antibodies were elicited in rabbits immunized with a poly(dG-dC)·poly(dG-dC) modified by *cis*-DDP at a $r_b = 0.03$ following the procedure of Malfoy et al. (1982). The results were obtained with the antisera of the bleeding a week after the first booster. Radioimmunoassays were performed as previously described (Malfoy et al., 1982). Poly(dG-dC)·poly(dG-dC) was labeled by nick translation with [α-^{32}P]dGTP (111 TBq/mmol) or [8-^{3}H]dGTP (200–700 GBq/mmol) from Amersham and DNA polymerase from Boehringer.

A RPC-5 column (a gift of Dr. B. Barbier) and a C_{18} column (Lichrosorb RP-18 from Merck, Darmstadt) were attached to a Hitachi Model 655 HPLC. In all the experiments, the flow rate was 1 mL/min and the detection was monitored at 260 nm.

RESULTS

Platinated $(dC\text{-}dG)_4$ and $(dC\text{-}dG)_5$. We were interested to know whether intrastrand and/or interstrand cross-links were formed in the reaction of *cis*-DDP and the oligonucleotides $(dC\text{-}dG)_4$ and $(dC\text{-}dG)_5$. The reactions were performed at constant oligonucleotide concentration (0.5 mM expressed in nucleotide residues) and at a molar ratio of oligonucleotide over *cis*-DDP equal to 30 in order to avoid the binding of several platinum residues to one oligonucleotide. After incubation of the reaction mixtures for 3 days, the products of the reaction were analyzed by gel electrophoresis in denaturing conditions and by HPLC.

Electrophoresis. At the end of the platination reaction, the oligonucleotides were 5′ end labeled with polynucleotide kinase and [γ-^{32}P]ATP. Figure 1 shows the gel electrophoresis pattern in which three main spots are detected (lanes 3 and 6). One spot (bottom of the gel) migrates as the unmodified oligonucleotide (lanes 1 and 4); the intermediate spot (several bands) migrates as the oligonucleotides modified by the monofunctional platinum derivative chlorodiethylenetriamine-platinum(II) (lanes 2 and 5); the last spot migrates more slowly. These results suggest that the intermediate spots

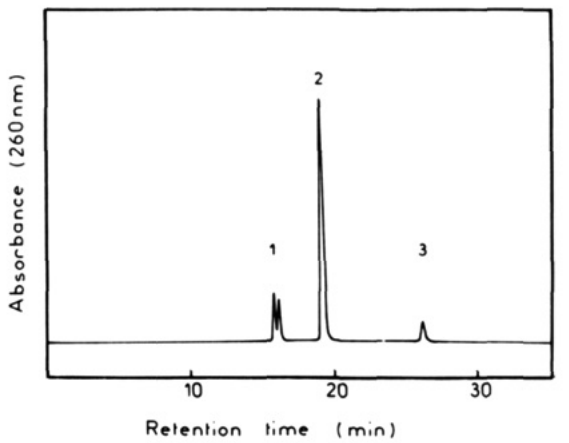


FIGURE 2: HPLC separation of the products resulting from the reaction of $(dC\text{-}dG)_4$ and *cis*-DDP. Separation was done on a RPC-5 column with a 40-min linear gradient of 0–20% buffer B (buffer A = 5 mM Tris, pH 11; buffer B = 0.2 M $NaClO_4$ and 5 mM Tris, pH 11).

FIGURE 3: Schematic representation of an intrastrand cross-link (top) and an interstrand cross-link (bottom) in $(dC\text{-}dG)_4$.

correspond to a single-stranded oligonucleotide with an intrastrand cross-link, $(dC\text{-}dG)_4Pt$ and $(dC\text{-}dG)_5Pt$, respectively, and the slowest spot corresponds to two oligonucleotides cross-linked by platinum, $[(dC\text{-}dG)_4]_2Pt$ and $[(dC\text{-}dG)_5]_2Pt$, respectively.

The products of the reaction of *cis*-DDP and $(dC\text{-}dG)_4$ were analyzed also by HPLC on a RPC-5 column (Pearson et al., 1971). An elution profile for the reaction of *cis*-DDP and $(dC\text{-}dG)_4$ carried out in 50 mM $NaClO_4$ and at 37 °C is shown in Figure 2. Peak 2 (t_R = 19 min) cochromatographs with the unmodified oligonucleotide. Two sets of experiments were performed with the two peaks at t_R = 16 min, called peaks 1, and with peak 3 (t_R = 27 min).

In the first experiment, the products in peaks 1 and 3 were analyzed by gel electrophoresis in denaturing conditions. Peak 1 comigrates with the intermediate spot in Figure 1 (intrastrand adduct), and peak 3 comigrates with the slowest spot (interstrand adduct).

In the second experiment, the oligonucleotides in peaks 1 and 3 were digested with the endonuclease P1 and then treated with alkaline phosphatase (Fichtinger-Schepman et al., 1985; Eastman, 1986). The digestion products were analyzed by HPLC on a reverse-phase C_{18} column. The elution profiles of peak 1 and peak 3 digests were similar, but the areas of the peaks were different. There were three peaks which cochromatographed with deoxycytidine, deoxyguanosine, and bis(deoxyguanosine)platinum, respectively. The areas of the peaks were about in the ratio 2/1/1 and 4/3/1 for peak 1 and peak 3 digests, respectively (results not shown). These values suggest that peak 1 corresponds to $(dC\text{-}dG)_4Pt$ and peak 3 to $[(dC\text{-}dG)_4]_2Pt$ in which the cross-links are between two guanine residues (Figure 3).

In our experimental conditions, the unplatinated oligonucleotides form double-stranded helices [the T_m of $(dC\text{-}dG)_4\cdot(dC\text{-}dG)_4$ is 63 °C in 20 mM $NaClO_4$ and 5 mM Tris-HCl, pH 7.5]. The formation of interstrand adducts between two double-stranded helices is unlikely since the relative percentages of interstrand and intrastrand adducts were the same when the concentration of the reagents in the

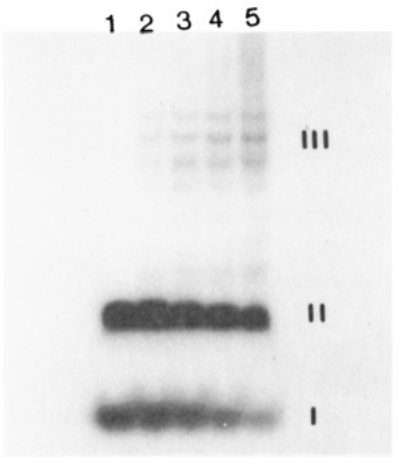


FIGURE 4: Autoradiogram of platinated poly(dG-dC)·poly(dG-dC) digested by *Hha*I endonuclease electrophoresed on a nondenaturing polyacrylamide gel. Lanes 1, 2, 3, 4, and 5, r_b = 0.00, 0.01, 0.02, 0.03, and 0.05, respectively. Electrophoresis was performed on a 20% polyacrylamide gel for 4 h at 15 V/cm in the same buffer as in Figure 1.

reaction mixture was decreased by a factor of 2. Nevertheless, these relative percentages depended upon the experimental conditions. Most interstrand cross-links occurred under conditions that favored double-stranded oligonucleotides (results not shown).

Platinated Poly(dG-dC)·Poly(dG-dC). The next point was to determine the adducts in platinated poly(dG-dC)·poly(dG-dC). The reaction of *cis*-DDP and poly(dG-dC)·poly(dG-dC) was carried out in 10 mM $NaClO_4$ and 5 mM Tris-HCl, pH 7.5, at 37 °C for 24 h.

In a first experiment, the platinated poly(dG-dC)·poly(dG-dC) at a molar ratio r_b (platinum residues over nucleotide residues) equal to 0.03 was cleaved by deoxyribonuclease I and P1 nuclease and then treated with alkaline phosphatase (Fichtinger-Schepman et al., 1985; Eastman, 1986). The HPLC elution profile of the digest presents three peaks which cochromatographed with deoxycytidine, deoxyguanosine, and bis(deoxyguanosine)platinum (not shown).

Thus the main adduct arises from a cross-link between two guanine residues. These cross-links can be intrastrand and/or interstrand cross-links. To answer to this question, the following experiment was done. Platinated and unplatinated [^{32}P]poly(dG-dC)·poly(dG-dC) were cleaved by the restriction enzyme *Hha*I (recognition site GCGC) and the fragments separated by gel electrophoresis (Figure 4). Complete digestion of unplatinated poly(dG-dC)·poly(dG-dC) gives two fragments which comigrate with $(dC\text{-}dG)_2$ and $(dC\text{-}dG)_3$ (labeled I and II, respectively).

The platinated samples (r_b = 0.01–0.03) are cleaved in five main fragments. Two of them comigrate with $(dC\text{-}dG)_2$ and $(dC\text{-}dG)_3$, respectively, and the three other fragments (labeled III) migrate more slowly. For the sample r_b = 0.05, the same pattern is observed with, in addition, a smear of larger molecular weight fragments.

The three fragments (III) from the digestion of the platinated sample (r_b = 0.03) were purified by HPLC on a RPC-5 column. In 7 M urea, the electrophoretic migrations of these fragments before and after treatment with NaCN were compared [NaCN is known to remove bound platinum from nucleic acids (Bauer et al., 1978)]. As shown in Figure 5, removal of platinum modifies the electrophoretic pattern with the fragments migrating much faster than before. For comparison, the migrations of $[(dC\text{-}dG)_5]_2Pt$ before and after treatment with NaCN (lanes 3 and 4) and of $(dC\text{-}dG)_n$ (lanes 5 and 6) are also shown. These results strongly suggest that, in platinated poly(dG-dC)·poly(dG-dC), interstrand cross-links are formed.

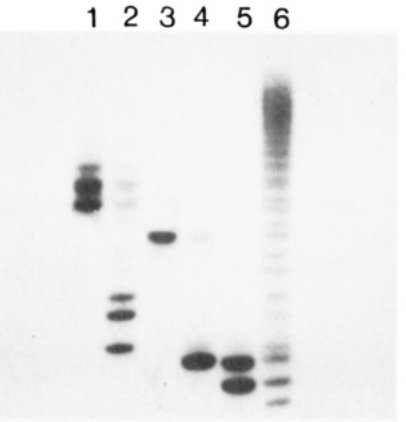
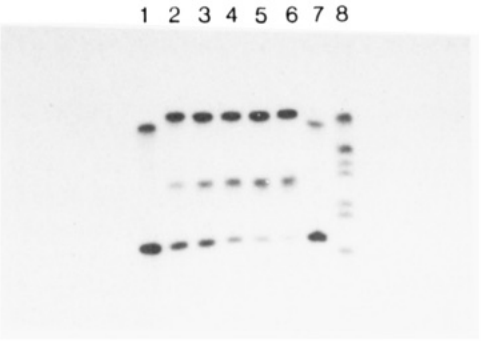


FIGURE 5: Autoradiogram of platinated *Hha*I fragments and of $[(dC\text{-}dG)_5]_2Pt$ before and after NaCN treatment, electrophoresed on a denaturing polyacrylamide gel. Lanes 1 and 2, purified fragments after digestion of platinated poly(dG-dC)·poly(dG-dC) (r_b = 0.03) by *Hha*I, before and after incubation in 0.2 M NaCN, respectively; lanes 3 and 4, $[(dC\text{-}dG)_5]_2Pt$ before and after incubation in 0.2 M NaCN, respectively; lane 5, mixture of unplatinated $(dC\text{-}dG)_5$ and $(dC\text{-}dG)_4$; lane 6, $(dC\text{-}dG)_n$ (n = 3, 4, 5, ...) mixture produced by *Hha*I degradation of [^{32}P]poly(dG-dC)·poly(dG-dC). Same experimental conditions as in Figure 1.



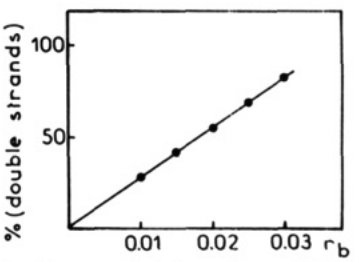


FIGURE 6: Autoradiogram of platinated natural DNA electrophoresed on a denaturing agarose gel. The two pBR322 DNA restriction fragments (622 and 3741 base pairs) were reacted with *cis*-DDP at r_b = 0.00 (lane 1), 0.01 (lane 2), 0.015 (lane 3), 0.02 (lane 4), 0.025 (lane 5), and 0.03 (lane 6). Lane 7 corresponds to DNA modified with dien-Pt, r_b = 0.03. Lane 8 corresponds to DNA molecular weight marker III: 564, 831, 983, 1330, 1584, and 1904 bases, respectively. The samples were analyzed on a 3% agarose gel in a buffer containing 30 mM NaOH and 1 mM EDTA at 5 V/cm over 6 h.

Platinated DNA. We have titrated the amount of interstrand cross-links by analysis of the electrophoretic mobility of a platinated restriction fragment DNA at basic pH. pBR322 DNA was first cleaved with the restriction enzyme *Sal*I, 5′-end labeled with polynucleotide kinase and [γ-^{32}P]-ATP and then cleaved into two fragments (622 and 3741 base pairs, respectively) with the restriction enzyme *Hin*dIII. The fragments were reacted with *cis*-DDP and then analyzed by gel electrophoresis. Two bands appear with the unplatinated sample and three bands with the platinated samples (Figure

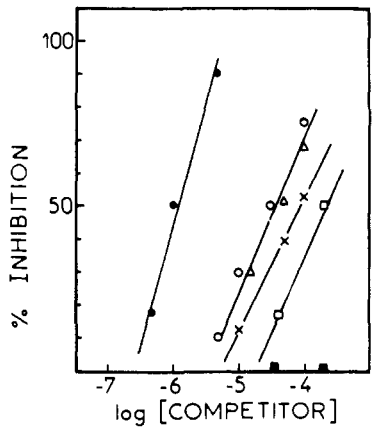


FIGURE 7: Inhibition of tracer–antibody binding by various platinated DNAs in competitive radioimmunoassay. The tracer was platinated [^{3}H]poly(dG-dC)·poly(dG-dC) at r_b = 0.02; antiserum dilution was 1/500; medium was 0.15 M $NaClO_4$, 5 mM Tris-HCl buffer, pH 7.5, and 1 mM EDTA; temperature was 4 °C. Competitors: (●) platinated poly(dG-dC)·poly(dG-dC) at r_b = 0.02; (○) purified fragments of platinated poly(dG-dC)·poly(dG-dC) digested with *Hha*I; (△) $[(dC\text{-}dG)_5]_2Pt$; (□) $[(dC\text{-}dG)_4]_2Pt$; (×) platinated pBR322 DNA or restriction fragment from *Cebus* (r_b = 0.1); (■) $(dC\text{-}dG)_5$, $(dC\text{-}dG)_5Pt$, $(dC\text{-}dG)_4$, or $(dC\text{-}dG)_4Pt$. No competition has been detected with native or denatured DNA, platinated poly(dA-dC)·poly(dG-dT), platinated poly(dG)·poly(dC), platinated poly(dA-dG)·poly(dC-dT), or nDNA or poly(dG-dC)·poly(dG-dC) modified by *trans*-DDP at an r_b = 0.10.

6). The intensity of the intermediate band increases as r_b increases whereas the intensity of the lower band decreases. We explain these results in the following way. In the reaction of *cis*-DDP and DNA, intrastrand and interstrand cross-links are formed. The lower band is the single-stranded fragment (622 bases). The intrastrand cross-links hardly change the migration of the fragment (compare to the DNA modified by dien-Pt, lane 7). The intermediate band represents the two complementary fragments cross-linked by at least one platinum residue (interstrand adduct). From the relative intensities of the lower and intermediate bands, one can calculate that at least 2% of the bound platinum form interstrand cross-links at 37 °C.

The upper band (Figure 6) migrates slower than the single-stranded fragment (3741 bases) modified by dien-Pt, and thus the two complementary (3741 bases) fragments are cross-linked by at least one platinum residue. Assuming the same distribution for the potential interstrand sites in the small and large DNA restriction fragments, this result confirms that the interstrand adducts represent about 2% of the total adducts.

The ability of *trans*-DDP to form interstrand cross-links has been also studied by gel electrophoresis at basic pH. At 37 °C and at an r_b = 0.01, no single stranded fragment (622 bases) has been detected whereas with *cis*-DDP the ratio of single-stranded fragments over interstrand cross-linked fragments is equal to 75% (results not shown).

Antibodies to Platinated Poly(dG-dC)·Poly(dG-dC). The interstrand cross-links in platinated oligonucleotides and in platinated natural DNA have been compared by means of antibodies to platinated poly(dG-dC)·poly(dG-dC) elicited in rabbits immunized with platinated poly(dG-dC)·poly(dG-dC) (r_b = 0.03). Radioimmunoassays show that the antibodies bind to platinated poly(dG-dC)·poly(dG-dC) and with less affinity to $[(dC\text{-}dG)_5]_2Pt$ (Figure 7). They do not bind to the intrastrand adducts $(dC\text{-}dG)_5Pt$ and $(dC\text{-}dG)_4Pt$. They recognize equally well the fragments from platinated poly(dG-dC)·poly(dG-dC) cleaved by *Hha*I and $[(dC\text{-}dG)_5]_2Pt$. These results confirm that, in the reaction of *cis*-DDP and poly(dG-dC)·poly(dG-dC), interstrand adducts are formed.

The antibodies do not cross-react with several double-stranded polynucleotides such as platinated poly(dG)·poly(dC), poly(dA-dC)·poly(dG-dT), and poly(dA-dG)·poly(dC-dT) (Figure 7). On the other hand, they cross-react with platinated natural DNA. In DNA the interstrand cross-links are not only formed in $(CG)_n$ or $(GC)_n$ sequences. Two platinated samples (r_b = 0.1), pBR322 DNA and a DNA restriction fragment from *Cebus* DNA (1100 base pairs) that is not cleaved by either *Hha*I (recognition site GCGC) or *Fnu*DII (recognition site CGCG), are recognized by the antibodies equally well (Figure 7).

The antibodies do not cross-react with poly(dG-dC)·poly(dG-dC) or natural DNA modified by *trans*-DDP.

Discussion

In the reaction of *cis*-DDP and DNA, intrastrand and interstrand cross-links are formed. A purpose of this work was to find a model system for the interstrand cross-links.

After reaction of *cis*-DDP and $(dC\text{-}dG)_4$·$(dC\text{-}dG)_4$, $(dC\text{-}dG)_5$·$(dC\text{-}dG)_5$, or poly(dG-dC)·poly(dG-dC), the platinated products were cleaved with P1 nuclease. HPLC analysis of the digests shows that the main adduct is a cross-link between two guanine residues in agreement with previous results (Eastman, 1983; Rahmouni et al., 1985). These cross-links can be intrastrand and/or interstrand.

The products after reaction of *cis*-DDP and the oligonucleotides are separated by gel electrophoresis in denaturing conditions as three main spots. One of these spots comigrates with the unplatinated oligonucleotide, one comigrates with the oligonucleotide modified by the monofunctional platinum derivative dien-Pt (in these spots several bands are present), and one migrates more slowly.

The binding of *cis*-DDP to an oligonucleotide can modify the electrophoretic migration by a charge effect and a conformational effect. The charge effect (a platinum residue bears two positive charges) depends upon the degree of polymerization of the oligonucleotide, decreasing as the degree of polymerization increases. The charge effect might be modulated to some extent by the location of the bound platinum since along the unplatinated oligonucleotide, the electrostatic potential is not uniform (Lavery & Pullman, 1981). An intrastrand cross-link changes the torsion angles of at least three nucleotide residues (den Hartog et al., 1983, 1985). Depending upon the location of the cross-link in the chain, the shape of the oligonucleotide can be significantly different.

It is difficult to evaluate the relative importance of these effects. The study of the oligonucleotides platinated with dien-Pt was expected to reveal mainly the charge effect. In fact, for example, $(dC\text{-}dG)_4$dien-Pt migrates much slower than unplatinated $(dC\text{-}dG)_5$. On the other hand, the presence of two close bands for $(dC\text{-}dG)_4$dien-Pt could reflect a conformational change and/or the importance of the location of dien-Pt along the oligonucleotide on the charge effect. Assuming that the charge effect plays a major role, the comigration of $(dC\text{-}dG)_4$dien-Pt [or $(dC\text{-}dG)_5$dien-Pt] and one of the products of the reaction of $(dC\text{-}dG)_4$ [or $(dC\text{-}dG)_5$] with *cis*-DDP suggests strongly the presence of an intrastrand cross-link in platinated $(dC\text{-}dG_4)$ [or $(dC\text{-}dG)_5$]. This is also supported by the relative proportions of deoxyguanosine, deoxycytidine, and bis(deoxyguanosine)platinum in these platinated oligonucleotides.

The slowest band in the pattern of the gel electrophoresis (Figure 1) cannot be due to the binding of two platinum residues to a single-stranded oligonucleotide. The relative proportions of deoxyguanosine, deoxycytidine, and bis(deoxyguanosine)platinum after P1 nuclease digestion of the oli-

gonucleotide excludes such an hypothesis. The proportions agree with an interstrand adduct. We conclude that intrastrand and interstrand cross-links are formed in the reaction of *cis*-DDP and the $(dC\text{-}dG)_n$ (n = 4 or 5) double helices.

In platinated poly(dG-dC)·poly(dG-dC), only cross-links between two guanine residues have been found. To determine whether the cross-links are intrastrand or interstrand, we have studied by gel electrophoresis the migration of the fragments obtained by digestion of platinated poly(dG-dC)·poly(dG-dC) with the restriction enzyme *Hha*I (recognition site GCGC). In nondenaturing conditions, only five main bands are detected. Two of them are also found with the unplatinated polymer digested with *Hha*I. The three other bands are close and migrate slightly less than $[(dC\text{-}dG)_5]_2Pt$. After incubation of these three bands with NaCN to remove the bound platinum (Bauer et al., 1978), by gel electrophoresis in denaturing conditions three bands are detected which migrate much faster than the platinated products. The comparison between these results and those with $[(dC\text{-}dG)_5]_2Pt$ strongly suggests that interstrand cross-links are formed in the reaction of *cis*-DDP and poly(dG-dC)·poly(dG-dC). The presence of intrastrand cross-links cannot be excluded but seems unlikely. If the distortions induced in the double helix by the intrastrand and the interstrand cross-links are not the same, the fragments not cleaved by *Hha*I should be of different length and thus should be detected by gel electrophoresis. The nature of the cross-link is confirmed by means of the antibodies to platinated poly(dG-dC)·poly(dG-dC). We had previously reported that the platinated polymer (r_b = 0.10) was a good immunogen in rabbits (Malinge et al., 1984). We now show that even platinated poly(dG-dC)·poly(dG-dC) (r_b = 0.03) injected in rabbits induces the synthesis of antibodies. These antibodies bind to platinated poly(dG-dC)·poly(dG-dC), to platinated *Hha*I fragments, to $[(dC\text{-}dG)_5]_2Pt$, and to $[(dC\text{-}dG)_4]_2Pt$ but do not cross-react with $(dC\text{-}dG)_4Pt$ and $(dC\text{-}dG)_5Pt$. The conclusion is that mainly interstrand cross-links are formed in the reaction of *cis*-DDP and poly(dG-dC)·poly(dG-dC).

We note that the platinated *Hha*I fragments are less recognized than the platinated poly(dG-dC)·poly(dG-dC). This does not necessarily mean that the conformations of both products are different. We are comparing a monovalent antigen to a polyvalent antigen. In addition to the polyelectrolyte effect, the two binding sites of an antibody molecule can bind to two antigenic determinants on the same polymer, which is not possible with the platinated *Hha*I fragments, and thus the antibody-platinated polymer complexes are more stable than the antibody-platinated *Hha*I fragments (Crothers & Metzger, 1972).

The antibodies to platinated poly(dG-dC)·poly(dG-dC) react with platinated natural DNA, which makes valid the choice of the platinated alternating $(dC\text{-}dG)_n$ oligonucleotides as model compounds. However, in natural DNA, interstrand cross-links are not just formed in $(CG)_n$ or $(GC)_n$ sequences since two platinated DNAs, one containing 31 GCGC sequences and 23 CGCG sequences and the other containing none of these sequences, are equally well recognized by the antibodies.

From the radioimmunoassays, the interstrand cross-links represent about 0.5% of the bound platinum, assuming that the neighboring bases of the cross-linked guanine residues do not interfere with the binding of the antibodies. Since the size of the antigenic determinant is unknown, it can only be said that the interstrand cross-links represent 0.5% or more of the bound platinum. A larger value (2%) can be deduced from the study of a platinated DNA restriction fragment by gel electrophoresis in denaturing conditions. This value includes other interstrand cross-links, if present, than the G–G cross-links. Cross-links between G and C residues have been found in platinated poly(dG)·poly(dC) (Harder & Lee, 1983) but not in platinated natural DNA (Eastman, 1985).

Finally, it was found by gel electrophoresis in denaturing conditions that more interstrand cross-links are formed in the in vitro reaction of a DNA restriction fragment with *trans*-DDP than with *cis*-DDP. This agrees with the nitrocellulose filter binding assay and S1 nuclease sensitivity reported by Eastman (1982) [in vivo, *cis*-DDP is more efficient than *trans*-DDP in forming interstrand cross-links (Roberts & Thomson, 1979)]. The conformational changes induced by the binding of *cis*-DDP and *trans*-DDP are different. DNA and poly(dG-dC)·poly(dG-dC) modified by *trans*-DDP are not recognized by antibodies to poly(dG-dC)·poly(dG-dC) modified by *cis*-DDP in agreement with a previous circular dichroism study on platinated poly(dG-m^5dC)·poly(dG-m^5dC) (Rahmouni et al., 1985).

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Registry No. *cis*-DDP, 15663-27-1; *trans*-DDP, 14913-33-8; $(dC\text{-}dG)_4$, 71203-49-1; $(dC\text{-}dG)_5$, 71304-71-7; poly(dG-dC), 36786-90-0.

REFERENCES

Banaszuk, A. M., Deugau, K. V., Sherwood, J., Michalak, M., & Glick, B. R. (1983) *Anal. Biochem. 128*, 281–286.

Bauer, W., Gonias, S. L., Kam, S. K., Wu, K. C., & Lippard, S. J. (1978) *Biochemistry 17*, 1060–1068.

Chu, G. Y. H., Mansy, S., Ducan, R. E., & Tobias, R. S. (1978) *J. Am. Chem. Soc. 100*, 593–606.

Crothers, D. M., & Metzger, H. (1972) *Immunochemistry 9*, 341–357.

Den Hartog, J. H. J., Altona, C., Van Boom, J. H., Marcelis, A. T. M., Van der Marel, G. A., Rinkel, L. J., Wille-Hazeleger, G., & Reedijk, J. (1983) *Eur. J. Biochem. 134*, 485–495.

Den Hartog, J. H. J., Altona, C., Van den Elst, H., Van der Marel, A., & Reedijk, J. (1985) *Inorg. Chem. 24*, 983–985.

Eastman, A. (1982) *Biochem. Biophys. Res. Commun. 105*, 869–875.

Eastman, A. (1983) *Biochemistry 22*, 3927–3933.

Eastman, A. (1985) *Biochemistry 24*, 5027–5032.

Eastman, A. (1986) *Biochemistry 25*, 3912–3915.

Fichtinger-Schepman, A. M. J., Van der Veer, J. L., Den Hartog, J. H. J., Lohman, P. H. M., & Reedijk, J. (1985) *Biochemistry 24*, 707–713.

Harder, H. C., & Lee, C. C. (1983) *Cancer Res. 43*, 4799–4804.

Johnson, N. P., Mazard, A. M., Escalier, J., & Macquet, J. P. (1985) *J. Am. Chem. Soc. 107*, 6376–6380.

Lavery, R., & Pullman, B. (1981) *Nucleic Acids Res. 9*, 3765–3777.

Malfoy, B., Rousseau, N., & Leng, M. (1982) *Biochemistry 21*, 5463–5467.

Malfoy, B., Rousseau, N., Vogt, N., Viegas-Pequignot, E., Dutrillaux, B., & Leng, M. (1986) *Nucleic Acids Res. 14*, 3197–3214.

Malinge, J. M., Ptak, M., & Leng, M. (1984) *Nucleic Acids Res. 12*, 5767–5778.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) in *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor

Laboratory Press, Cold Spring Harbor, NY.
Pearson, R. L., Wiess, J. F., & Kelmers, A. D. (1971) *Biochim. Biophys. Acta 228*, 770-774.
Pinto, A. L., & Lippard, S. J. (1985) *Biochim. Biophys. Acta 780*, 167-180.
Plooy, A. C. M., Van Dijk, M., Berends, F., & Lohman, P. H. M. (1985) *Cancer Res. 45*, 4178-4184.
Rahmouni, A., Malinge, J. M., Schwartz, A., & Leng, M. (1985) *J. Biomol. Struct. Dyn. 3*, 363-375.
Roberts, J. J., & Thomson, A. J. (1979) *Prog. Nucleic Acid Res. Mol. Biol. 22*, 71-112.
Roberts, J. J., & Friedlos, F. (1982) *Chem.-Biol. Interact. 39*, 181-189.
Roberts, J. J., & Pera, M. F. (1983) in *Molecular Aspects of Anti-Cancer Drug Action* (Neidle, S., & Waring, M. J., Eds.) pp 183-231, Macmillan, London.

Footprinting DNA-Protein Complexes in Situ following Gel Retardation Assays Using 1,10-Phenanthroline-Copper Ion: *Escherichia coli* RNA Polymerase-*lac* Promoter Complexes†

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ABSTRACT: Protein-DNA complexes isolated in gel retardation assays can be digested within the acrylamide matrix by the nuclease activity of 1,10-phenanthroline-copper ion (OP-Cu). When the oligonucleotide products are eluted and analyzed on a sequencing gel, a footprint of the DNA-protein complex is obtained. Therefore, any protein-DNA complex isolated by the widely used gel retardation technique can be defined in terms of sequence-specific interactions by this simple methodology. The binding of the *lac* repressor and *Escherichia coli* RNA polymerase to an *Eco*RI fragment containing the *lac* control region has been studied by the combined gel retardation-1,10-phenanthroline-copper ion footprinting procedure. Footprints of *lac* repressor binding correspond to those obtained in solution with OP-Cu and DNase I and verify the experimental procedures. In studying *E. coli* RNA polymerase-promoter complexes, we have found that magnesium ion is required to form single-stranded DNA structures characteristic of kinetically competent open transcription complexes.

Gel retardation assays are a practical and powerful method for studying DNA-protein interactions (Garner & Revzin, 1981, 1982, 1986; Revzin et al., 1986; Fried & Crothers, 1981; Crothers, 1987). In this paper, we extend the utility of this technique by demonstrating that protein-DNA complexes can be footprinted within the gel matrix using the nuclease activity of 1,10-phenanthroline-copper ion (Sigman et al., 1979; Marshall et al., 1981; Sigman, 1986). It is therefore possible to define rapidly and accurately the DNA sequences directly interacting with protein in any complex that can be detected by the retardation assay.

Using the *lac* repressor-operator as a model system, we demonstrate that footprints obtained by this technique are consistent with those obtained in solution with DNase I (Galas & Schmitz, 1978). The advantages of combining gel retardation and OP-Cu[1] footprinting in a single procedure are illustrated by studying the interaction of the *lac* UV5 promoter with *Escherichia coli* RNA polymerase (Spassky & Sigman, 1985; Spassky, 1986). Using this method, we demonstrate that magnesium ion is essential for the formation of the kinetically competent open complex.

Experimental Procedures

Materials

The following reagents were purchased from the indicated suppliers: *E. coli* RNA Polymerase (Pharmacia); 1,10-phenanthroline and 2,9-dimethyl-1,10-phenanthroline (G. F. Smith); cupric sulfate (Mallinckrodt); 3-mercaptopropionic acid (Aldrich). The *Eco*RI fragment containing the *lac* L8-UV5 promoter was isolated and uniquely labeled as previously described (Spassky & Sigman, 1985, Kuwabara et al., 1986). *lac* repressor was a generous gift of Dr. Phillip Pjura.

Methods

Gel Retardation Assays. Slab gels were used for all gel retardation assays. The general conditions of Fried and Crothers (1981) were used for experiments with the *lac* repressor and of Straney and Crothers (1985) for gel retardation assays with *E. coli* RNA polymerase.

Footprinting in Solution. 5'-Labeled L8-UV5 186-bp DNA (10^5 cpm; 0.03 pmol) was incubated with 50 ng of *lac* repressor in 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, and 0.2 mg/mL BSA in 10 μL for 10 min at 37 °C. For DNase I footprinting, 1 μL of 40 units/mL DNase I was added and incubated for 2.0 min at 37 °C. Digests were stopped by addition of a 10-μL aliquot of the digestion solution to 10 μL of a mixture composed of the following: 300 mg of sucrose, 840 mg of urea, 40 μL of 0.5 M EDTA, 100 μL of 0.1% bromophenol blue, and 100 μL of 0.1% xylene cyanol. In order to obtain 10 μL of a homogeneous mixture, the latter was liquified by heating

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[1] Abbreviations: OP, 1,10-phenanthroline; 2,9-dimethyl-OP, 2,9-dimethyl-1,10-phenanthroline; MPA, 3-mercaptopropionic acid; OP-Cu, 1,10-phenanthroline-copper ion complex; ApA, adenylyl(3'-5')adenosine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid.

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