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Intrastrand cross-links are not formed in the reaction between transplatin and native DNA: relation with the clinical inefficiency of transplatin

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ABSTRACT

The reaction between *trans*-diamminedichloroplatinum(II) and single-stranded oligonucleotides containing the sequence d(GXG) (X being an adenine, cytosine or thymine residue) yields *trans*-{Pt(NH$_3$)$_2$[d(GXG)-G*N7*,G*N7*]} intrastrand cross-links. These cross-links do not prevent the pairing of the platinated oligonucleotides with their complementary strands but they decrease the thermal stability of the duplexes. The thermal stability is not much affected by the chemical nature of the X residue and its complementary base. By gel electrophoresis, it is shown that the *trans*- {Pt(NH$_3$)$_2$[d(GTG)-*GN7*,G*N7*]} cross-link bends the DNA double helix (26°) and unwinds it (45°). The pairing of the platinated oligonucleotides with their complementary strands promotes the rearrangement of the 1,3-intrastrand cross-links into interstrand cross-links. At a given temperature, the nature of the X residue, its complementary base and of the base pairs adjacent to the adducts do not dramatically affect the rate of the reaction. To know whether *trans*-{Pt(NH$_3$)$_2$[d(GXG)-G*N7*,G*N7*]} cross-links do not rearrange in some sequences, the location of these adducts was searched in double-stranded DNA after reaction with *trans*-diamminedichloroplatinum(II) by means of the 3′–5′ exonuclease activity of T4 DNA polymerase. At low level of platination, *trans*-{Pt(NH$_3$)$_2$[d(GXG)-G*N7*, G*N7*]} cross-links were not detected. Monofunctional adducts and interstrand cross-links were mainly formed. These results are discussed in relation with the clinical inefficiency of *trans*-diamminedichloroplatinum(II).

INTRODUCTION

cis-Diamminedichloroplatinum(II) (*cis*-DDP) is a potent antitumor agent widely used in clinical treatment of cancers. It is generally accepted that the antitumor activity of the drug is related to its binding to cellular DNA and formation of bifunctional lesions. *trans*-Diamminedichloroplatinum(II) (*trans*-DDP, transplatin), the stereoisomer of *cis*-DDP, forms also bifunctional lesions but is clinically ineffective (general reviews, 1–4). In the *in vitro* reaction with DNA, the two isomers present similarities and differences. The exchange of chloro groups of the two isomers is rate-limiting in the initial attack of DNA. Their preferred site of initial binding is the N7 atom of guanine (G) residues. Among the differences, one is that stereochemical limitations preclude *trans*-DDP forming intrastrand cross-links between adjacent base residues (general review, 5). The most prevalent bifunctional adducts are intrastrand cross-links between two G residues or between G and cytosine (C) residues separated in both cases by at least one residue and interstrand cross-links between complementary G and C residues (6–10). Another difference concerns the rate of closure of the monofunctional adducts to bifunctional adducts. The interstrand cross-linking reaction is slower with the *trans* isomer than with the *cis* isomer (10). As concerns the intrastrand cross-links, the results are contradictory. Bancroft *et al.* (9) find that the rate is of the same order of magnitude for the two isomers; Eastman *et al.* (8) report that the rate is much slower for the *trans* isomer than for the *cis* isomer.

It is generally accepted that in conditions close to the physiological conditions, the bifunctional lesions in *cis*- or *trans*-DDP-modified DNA are stable . However, one exception concerns the *trans*-{Pt(NH$_3$)$_2$[d(GXG)-G*N7*,G*N7*]} intrastrand cross-links [(G1,G3)-cross-links], where X stands for adenine (A), thymine (T) or C residue. Within single-stranded oligonucleotides in which the 5′ residue adjacent to the adduct is a C residue, the (G1,G3)-intrastrand cross-link rearranges into the 1,4-*trans*-{Pt(NH$_3$)$_2$[d(CGXG)-C*N3*,G*N7*]} intrastrand cross-link (11,12). The *trans*-{Pt(NH$_3$)$_2$[d(GXG)-G*N7*,G*N7*]} cross-links in the sequences d(PyGXGPy) (Py stands for pyrimidine residue) rearrange into interstrand cross-links as soon as the platinated oligonucleotides are hybridized with their complementary strands (13).

The linkage isomerization reaction promoted by the double helix might be related to the clinical inefficiency of *trans*-DDP. Moreover, this reaction seems to be a promising tool in the antisense strategy (14,15) to irreversibly cross-link the oligonucleotides to their targets (13). Our purpose was to better characterize this reaction. In the present work, we show that the *trans*-{Pt(NH$_3$)$_2$[d(GXG)-G*N7*,G*N7*]} intrastrand cross-links do not prevent the pairing of the platinated oligonucleotides with their complementary strands but they decrease the thermal stability of the duplexes. Considering the *trans*-{Pt(NH$_3$)$_2$-[d(GTG)-*GN7*,G*N7*]} cross-link, it bends the double helix and

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unwinds it. The rearrangement of the *trans*-{$Pt(NH_3)_2$[d(GXG)-G*N7*,G*N7*]} intrastrand cross-links into interstrand cross-links within duplexes containing a single adduct occurs whatever the chemical nature [purine (Pu) or pyrimidine] of the base residues adjacent to the adduct. This result is apparently in contradiction with the high percentage of (G,G)-cross-links in double-stranded DNA after modification with *trans*-DDP (7–9). On the one hand, we find that in several sequences, the (G1,G3)-intrastrand cross-links rearrange into interstrand cross-links and, on the other hand, in *trans*-DDP-modified DNA the percentage of (G,G)-intrastrand cross-links is much larger than the percentage of interstrand cross-links (7–10). To know whether the (G,G)-intrastrand cross-links are stable in some sequences, the location of the intrastrand cross-links in *trans*-DDP-modified DNA restriction fragments was searched by means of the 3′–5′ exonuclease activity of T4 DNA polymerase. At low level of platination, (G1,G3)-, (G1,G4)- and (C1,G4)-intrastrand cross-links were hardly detected which excludes that these intrastrand cross-links represent the major adducts.

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d(TGTGT):   5′  TCTCTCCTCTGTGTCTCCTTCT
d(ACACA):         AGAGGAGACACAGAGGAAGAAG  5′

d(TGAGT):   5′  TCTCTCCTCTGAGTCTCCTTCT
d(ACTCA):         AGAGGAGACTCAGAGGAAGAAG  5′
d(ACCCA):         AGAGGAGACCCAGAGGAAGAAG  5′

d(TGCGT):   5′  TCTCTCCTCTGCGTCTCCTTCT
d(ACGCA):         AGAGGAGACGCAGAGGAAGA  5′

d(AGAGA):   5′  CTTCTCCTCAGAGACTCCTT
d(TCTCT):       GAAGAGGAGTCTCTGAGGAA  5′

d(GGTGT):   5′  CTTCTCCTCGGTGTCTCCTT
d(CCACA):         AGAGGAGCCACAGAGGAAGA  5′
d(CCTCA):         AGAGGAGCCTCAGAGGAAGA  5′

d(TGTTGT):  5′  CTTCTCCTCTGTTGTCTCCTT
d(ACAACA):        AGAGGAGACAACAGAGGAAGA  5′

d(TG[pO(CH2)3Op]GT): 5′ TCTCCTCTG[pO(CH2)3Op]GTCTCCTTCT
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Figure 1. Sequences of the oligodeoxyribonucleotides and their abbreviations.

MATERIALS AND METHODS

Materials

The oligodeoxyribonucleotides from Institut Pasteur (Paris) were purified by strong anion exchange chromatography (Pharmacia monoQ column) on a Pharmacia FPLC system with 10 mM NaOH, 0.2–0.7 M NaCl gradient. The oligonucleotide containing the sequence d(TG[pO(CH_2)$_3$Op]GT) was from Genset (Paris); purity was >95% as assessed by gel electrophoresis. The oligonucleotides, which are referred to by their central sequences, are listed in Figure 1. pSP73KB and pTG132 plasmids were prepared as previously described (16,17). Plasmid pAG22 was obtained by inserting a $d(AG)_{22}$ synthetic oligonucleotide at the *Sma*I site of pUC18 plasmid. T4 DNA polymerase, endonuclease P1, alkaline phosphatase and T4 polynucleotide kinase were purchased from New England BioLabs. The radioactive products were from Amersham. All chemicals were from Merck, except *trans*-DDP, which was from Johnson-Mattey (UK).

Platination

The oligonucleotides containing the *trans*-{$Pt(NH_3)_2$[d(GXG)-G*N7*,G*N7*]} cross-links were prepared as previously described (13,18). In a typical synthesis, the single-stranded (ss) oligonucleotides (c = 30 μM) were incubated with 1.1 equivalent of *trans*-DDP, in 10 mM $NaClO_4$, 4 mM acetate buffer, pH 3.6, at 37°C during 24 h [in the case of d(AGAGA), no acetate buffer was added and the pH of the solution was adjusted to 3.1 with HNO_3 (Ultrapure)]. Then, monofunctional adducts were removed by treatment with thiourea 10 mM, for 10 min at 37°C (7,18). The oligonucleotides containing the (G,G)-intrastrand cross-links were purified by FPLC with a 0.2–0.7 M NaCl gradient. For d(AG*AG*A) and d(GG*TG*T) [* indicates the site of platination], a second purification was done by C_{18}-reverse phase HPLC. The nature of the adduct was verified as previously described (11,13), either by HPLC analysis of the digests after enzymatic hydrolysis with endonuclease P1 and then with alkaline phosphatase, or by the non-reactivity of dimethylsulfate (DMS) at the G residues involved in the cross-link.

The oligonucleotides containing the monofunctional adduct *trans*-{$Pt(NH_3)_2$(dG)Cl}$^+$ were obtained by reacting the oligonucleotides (c = 30 μM) with the monoaquamonochloro species generated by allowing *trans*-DDP to react with $AgNO_3$ at a platinum/oligonucleotide molar ratio equal to 5, in 10 mM $NaClO_4$, 4 mM acetate buffer, pH 3.6, at 37°C for 30 min (10). The platinated oligonucleotides were purified by FPLC. Purity was ~90% as assessed by gel electrophoresis. It was verified that in these oligonucleotides containing a monofunctional single adduct, all the G residues were approximately equally reactive with DMS.

The DNA restriction fragments [(*Nde*I–*Xho*I) 164 base pairs (bp) from plasmid pSP73KB, (*Eco*RI–*Hind*III) 104 bp from plasmid pAG22 and (*Eco*RI–*Bam*HI) 160 bp from plasmid pTG132] were reacted in 10 mM $NaClO_4$ with *trans*-DDP at an input molar ratio drug-to-nucleotide residue equal to 0.005, during 24 h at 37°C and then incubated in 10 mM thiourea at 37°C for 10 min and finally precipitated twice with ethanol. *cis*-DDP-modified DNA was prepared in the same conditions but it was not incubated in thiourea. The efficiency of platination was controlled by determining the percentages of interstrand cross-links by means of denaturing 1% agarose, 30 mM NaOH gel electrophoresis (16). The amounts of bound platinum residues were determined by atomic absorption by Pr G. Natile (Bari, Italy).

Linkage isomerization reaction

The time dependencies of the disappearance of the (G,G)-intrastrand cross-links within double-stranded (ds) oligonucleotides were measured in 200 mM $NaClO_4$, 5 mM phosphate buffer, pH 7.5 (13). The duplexes were formed by incubation of the platinated oligonucleotides (2 μM) with their complementary strands for 45 min at 5°C. At various times, aliquots were removed and analyzed on a 24% polyacrylamide gel electrophoresis under denaturing conditions. Quantitation of the bands intensity was done on a Molecular Dynamics PhosphorImager using ImageQuant software version 3.3 for data processing.

T4 DNA polymerase

The oligonucleotides (0.15 nmole nucleotide residues) and plasmid restriction fragments (~40 ng) were incubated at 37°C for 30 min with the enzyme (0.3–3 U) in 50 mM NaCl, 10 mM Tris–HCl pH 7.9, 10 mM $MgCl_2$, 1 mM DTT, conditions for the

3′–5′ exonuclease activity of the enzyme. In parallel, Maxam–Gilbert sequencing reactions were carried out as described (19).

Melting curves

The thermal stability of the duplexes was determined by measuring the absorbance at 260 nm as a function of temperature using a Kontron Uvikon 810 spectrophotometer. The temperature increase was 1 °C/min. Melting temperatures were obtained from the midpoint of the sigmoidal plots of the absorbance versus temperature, with an estimated accuracy of ±0.5 °C.

Ligation and electrophoresis

Oligonucleotides of various lengths [from 20 to 24mer: d(CTTCTCCTCTGTGTCTCCTT),d(TCTCTCCTCTGTGTCTCTTC), d(TCTCTCCTCTGTGTCTCCTTCT), d(CTCTCTTCTGTGTCTTCTTCTTC) and d(CTTATCTTCTGTGTCTTCTATCTC)], unplatinated or containing a single *trans*-{$Pt(NH_3)_2$[d(GTG)-G*N7*,G*N7*]} cross-link, were ^{32}P 5′-end phosphorylated with polynucleotide kinase and then allowed to anneal with their 5′-end phosphorylated complementary strand at 0 °C for 1 h (20). The duplexes were incubated with T4 DNA ligase in 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 10 mM Tris–HCl, pH 7.5 at 12 °C for 1.2 h. After ligation, half of each platinated sample was treated with 0.3 M NaCN, overnight at 50 °C. The migrations of the ligated products were examined on 8% native polyacrylamide electrophoresis gels as previously described (20–23).

RESULTS

Thermal stability of the platinated duplexes

Two independent reports have shown that two oligonucleotides containing a single *trans*-{$Pt(NH_3)_2$[d(GNG)-G*N7*,G*N7*]} intrastrand cross-link, N being A or T, form duplexes with their complementary strands but the thermal stability of the duplexes was differently affected by the adducts. As compared with the corresponding unplatinated duplexes, the *trans*-{$Pt(NH_3)_2$[d(GTG)-G*N7*,G*N7*]} adduct lowers the T_m (24) while the *trans*-{$Pt(NH_3)_2$[d(GAG)- G*N7*,G*N7*]} adduct does not change it (18). Since our aim is to study the rearrangement of the *trans*-{$Pt(NH_3)_2$[d(GNG)-G*N7*,G*N7*]} cross-links in duplexes, the thermal stability of several duplexes has been investigated. For all the unplatinated and platinated duplexes, the melting curves presented a cooperative profile. The T_m values and the hyperchromicities at 260 nm are given in Table 1. All the platinated duplexes melt at lower temperatures than the corresponding unplatinated duplexes. Their T_m are in the same temperature range whatever the nature of the intervening base between the two chelated G residues and its complementary base (a mismatch or not). These results are confirmed by the study of d(TG*[pO(CH_2)$_3$Op]G*T).d(ACTCA) in which the intervening nucleotide residue has been replaced by a propylene residue. The T_m is slightly lower than the T_m of the 'complete' duplex. The conclusion of this study is that oligonucleotides containing *trans*-{$Pt(NH_3)_2$[d(GNG)-G*N7*,G*N7*]} cross-links form duplexes with their complementary strands but the platinated duplexes are thermally less stable than the corresponding unplatinated duplexes. On the other hand, we found that T_m increased with an increase of $NaClO_4$ concentration (~17 °C for a factor of 10 in concentration).

Table 1. Melting temperatures (T_m) of the duplexes[a] in which the top strand contains a single *trans*-{$Pt(NH_3)_2$[d(GXG)-G*N7*,G*N7*]} cross-link (+Pt) or not (–Pt)

	–Pt		+Pt	
	T_m (°C)	H (%)[b]	T_m (°C)	H (%)[b]
d(TGTGT).d(ACACA)	44	14	25.5	11
d(TGCGT).d(ACGCA)	48	18	24.5	15
d(TGAGT).d(ACTCA)	44	18	27	15
d(TGAGT).d(ACACA)	37.5	15	26.5	10
d(TGAGT).d(ACCCA)	38	15	26	13
d(AGAGA).d(TCTCT)	44	25	28	16
d(TG[pO(CH_2)$_3$Op]GT). d(ACTCA)	nd[c]	nd[c]	23.5	14

[a]The concentrations of duplexes were 3.6 μM. Solvent: 10 mM $NaClO_4$, 3 mM Tris–HCl, 0.2 mM EDTA, pH 7.5.
[b]Hyperchromicity at 260 nm.
[c]Not determined.

Distortion of the DNA double helix induced by the *trans*-{$Pt(NH_3)_2$[d(GTG)-G*N7*,G*N7*]} intrastrand cross-link

Two independent works, based on gel electrophoresis experiments, have led to quite different conclusions concerning the distortions induced in duplexes by the *trans*-{$Pt(NH_3)_2$ [d(GTG)-G*N7*,G*N7*]} intrastrand cross-link [hinge joint (22,25), bend (24)]. Some structural features of *cis*- or *trans*-DDP modified oligonucleotides such as bending of the double helix longitudinal axis and variation from the canonical twist of B-DNA have been determined from electrophoretic mobility of their multimers on polyacrylamide gels (26,27). We have applied this technique to characterize again the conformational change of the DNA double helix induced by the *trans*-{$Pt(NH_3)_2$[d(GTG)-G*N7*,G*N7*]} intrastrand cross-link. Autoradiograms of 8% native electrophoresis gels corresponding to the ligation products of 20–23 bp duplexes of central sequence d(TGTGT).d(ACACA) are presented in Figure 2A. Retardation in mobility of the platinated oligonucleotides occurs and is maximum for multimers of 22 and 23 bp duplexes (the bands whose migration is hardly affected by cyanide treatment correspond to circles). The variation of the K factor (mobility of the unplatinated multimers relative to the linear ones) as a function of the multimer length is shown in Figure 2B. The K factors corresponding to 140 bp fragments are deduced from those curves and plotted as a function of the interadduct distance (insert of Fig. 2B). The parabolic profile is characteristic for bending. As previously described (22,28,29 and references therein), the value of the bend angle can be obtained from the empirical formula:

$$K-1 = (9.6 \times 10^{-5} L^2 - 0.47)(RC)^2 \quad \mathbf{I}$$

where L is the length of a particular multimer which has the relative mobility K and RC is the curvature relative to a DNA bending induced by a tract of six A residues. For the 130, 140 and 150 bp fragments corresponding to 22- and 23mer d(TG*TG*T).d(ACACA) duplexes (Fig. 2B), equation **I** leads to a relative curvature of 0.72. The average bend angle is ~26° taking 18° for the absolute curvature of the A_6 tract (29).

The maximum retardation observed for the platinated 22 and 23 bp multimers shows that the canonical 10.5 bp per turn repeat

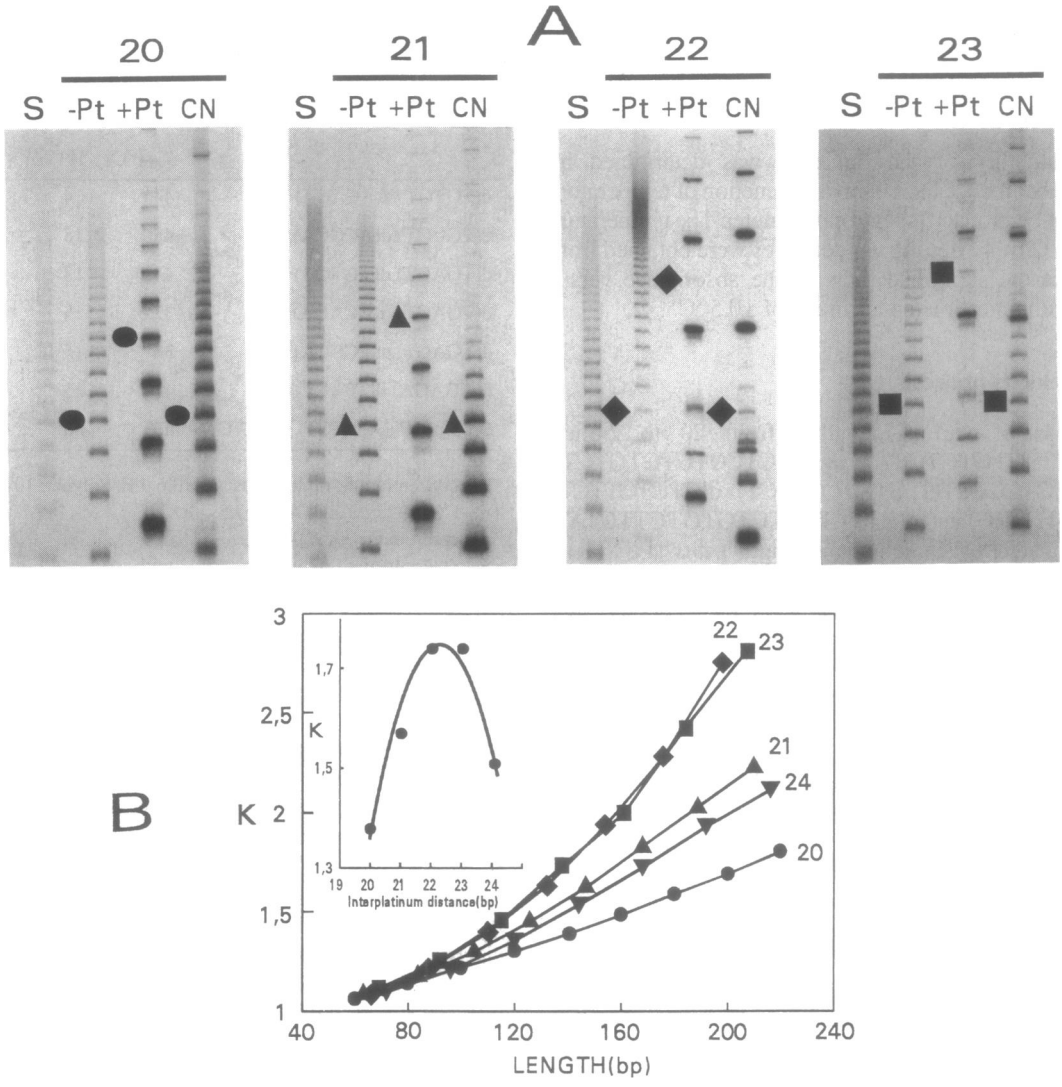

Figure 2. (**A**) Autoradiograms of 8% native polyacrylamide gels of the ligation products of the 20–23mer oligonucleotides of central sequence d(TGTGT).d(ACACA), unplatinated (lanes –Pt) or containing a *trans*-{$Pt(NH_3)_2$[d(GTG)-G*N7*,G*N7*]} cross-link (lanes +Pt). In order to condense the figure, the bottom of the gels are not shown and autoradiogram of 24mer ligation products is omitted. In lanes CN, the platinated samples were treated with 0.3 M NaCN at 50°C overnight. The symbols ●, △,◆, and ■ indicate respectively the 120, 126, 132 and 138 bp (platinated or not) multimers. Lanes S, migration of multimers of a double-stranded unplatinated 15mer oligonucleotide. (**B**) Variation of the relative mobility K versus length for the multimers of 20–24mer oligonucleotides containing a *trans*-{$Pt(NH_3)_2$[d(GTG)-G*N7*,G*N7*]} cross-link. Insert, plot showing the relative mobility K versus interplatinum distance (bp) for the 140 bp platinated fragments. The curve represents the best fit of these experimental points to the equation $K = ax^2 + bx + c$ (25), where x is the interplatinum distance (bp).

(B-DNA) is not conserved in platinated duplexes as a consequence of DNA unwinding. The maximum of the curve (Fig. 2) leads to an helical repeat of 22.3 bp which corresponds to a difference with the helical repeat of B-DNA of [22.3 – (2 × 10.5)] = 1.3. This allows the deduction that DNA unwinding due to one *trans*-{$Pt(NH_3)_2$[d(GTG)-G*N7*,G*N7*]} intrastrand cross-link is ~45°.

Rearrangement of the *trans*-{$Pt(NH_3)_2$[d(GXG)-G*N7*,G*N7*]} intrastrand cross-links into interstrand cross-links

Recently, we have shown (13) that the rearrangement of the 1,3-intrastrand cross-links into interstrand cross-links occurs in the duplexes d(PyG*XG*Py).d(PuCYCPu) [Y stands for A, T or C] formed by pairing the platinated oligonucleotides d(PyG*XG*Py) with their complementary strands. We now extend this observation to other duplexes in which Pu residues are adjacent to the intrastrand cross-links.

The results relative to the two duplexes d(GG*TG*T).d(ACACC) and d(GG*TG*T).d(ACTCC), which differ only by the base complementary to the one between the two chelated G, are shown in Figure 3. The duplexes were incubated in 200 mM $NaClO_4$ and at neutral pH. At various times, aliquots were withdrawn and analyzed by gel electrophoresis under denaturing conditions. In both cases, as a function of the incubation time, the intensity of the band corresponding to the starting product decreases as a new band [in the case of d(GG*TG*T).d(ACTCC)] or two close bands [in the case of d(GG*TG*T).d(ACACC)] appear. Because of the large differences in the migration of the products and as anticipated from our previous results (13), one can state that the intrastrand cross-links rearrange into interstrand cross-links (the

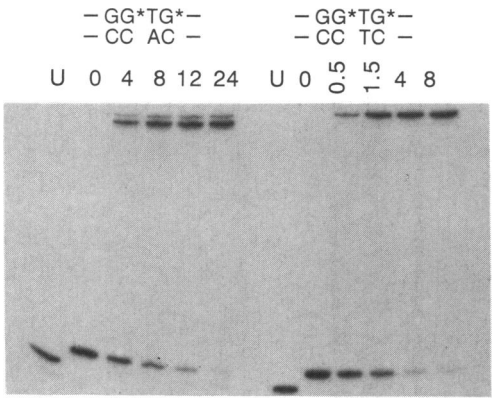

Figure 3. Instability of *trans*-{Pt(NH$_3$)$_2$[d(GTG)-G*N7*,G*N7*]} cross-links within the duplexes d(GG*TG*T).d(ACACC) (left) and d(GG*TG*T).d(ACTCC) (right). Autoradiogram of a denaturing 24% polyacrylamide gel. The 5′ end of the d(GGTGT) strand was labeled with ^{32}P. The duplexes were incubated at 37°C in 200 mM $NaClO_4$, 5 mM phosphate buffer, pH 7.5. Incubation times are indicated in hours above the lanes. Lanes U refer to the unplatinated d(GGTGT) oligonucleotide.

results are independent of the duplex concentration in the range 2–30 μM). Only one interstrand cross-link is formed in d(GGTGT).d(ACTCC) and two in d(GGTGT).d(ACACC) [the ratio of the bands intensity is ~80/20]. The plots of the intrastrand cross-link percentages (logarithmic scale) as a function of time were roughly linear (not shown). At 37°C, the half-lives of the intrastrand cross-links within d(GG*TG*T). d(ACTCC) and d(GG*TG*T).d(ACACC) are 1.2 and 4 h, respectively.

Several other platinated duplexes have been studied. The results resemble those shown in Figure 3. Depending upon the nature (Py or Pu) of the residue opposite to the intervening residue X between the two chelated G residues, one or two interstrand cross-links are formed. The $t_{1/2}$ values of the 1,3-intrastrand cross-links are of the same order of magnitude (Table 2), even in d(TG*[pO(CH$_2$)$_3$Op]G*T).d(ACTCA). We conclude that the intervening residue X and the chemical nature of the 5′ and 3′ base pairs adjacent to the intrastrand cross-link do not play a major role in the linkage isomerization reaction. It was verified by Maxam–Gilbert footprinting experiments (13) that the major interstrand cross-links were between the 5′ G and its complementary C residues. The d(TGAGT).d(ACTCA) duplex containing an interstrand cross-link was purified by gel electrophoresis under denaturing conditions and incubated in 200 mM $NaClO_4$ and at neutral pH for 48 h at 37°C. No new products were detected by gel electrophoresis indicating that the rearrangement of the (G1,G3)-intrastrand cross-links into interstrand cross-links can be considered as irreversible.

The disappearances of the intrastrand adducts as a function of time were determined at various temperatures for two duplexes, d(TG*AG*T).d(ACTCA) and d(AG*AG*A).d(TCTCT). The experimental first-order rate constants k_{obs} for these intramolecular reactions were obtained from a non-linear least-squares fit of ln[% intrastrand adduct] versus time. An Eyring plot of temperature dependence of the forward rate constants was used to determine the kinetic activation parameters according to the usual equation (30). The results are summarized in Table 2.

T4 DNA polymerase

A question was to know whether in the reaction between DNA and *trans*-DDP, the intrastrand cross-links are first formed and subsequently rearrange into interstrand cross-links. Recently, we have shown (13) that in the duplexes d(TGXGT).d(ACYCA) containing a single monofunctional adduct *trans*-{Pt(NH$_3$)$_2$(dG)Cl}$^+$, the closure of the monofunctional adducts to bifunctional cross-links yields mainly interstrand cross-links and the reaction is slow. The intrastrand cross-links were hardly detected. The same experiment was repeated with several duplexes (Fig. 1) containing a single monofunctional adduct. The results (not shown) resembled those obtained with the platinated duplexes d(TGXGT).d(ACYCA). The half-lives of the monofunctional adducts were >15 h and the major bifunctional adducts were the interstrand cross-links. The formation of the intrastrand cross-links was a rare event.

Table 2. Rearrangement of the *trans*-{Pt(NH$_3$)$_2$[d(GXG)-G*N7*,G*N7*]} intrastrand cross-links into interstrand cross-links

	T(°C)[a]	$t_{1/2}$(h)[b]	$10^5 \times k_{obs}$ (s^{-1})[c]	$\Delta H^{\neq}$ ($kJ.mol^{-1}$)	$\Delta S^{\neq}$ ($J.mol^{-1}.K^{-1}$)
d(TGAGT).d(ACTCA)	37	6	3.2 ± 0.3		
d(AGAGA).d(TCTCT)	37	1.6	12 ± 1		
d(GGTGT).d(ACACC)	37	4	4.8 ± 0.5		
d(GGTGT).d(ACTCC)	37	1.2	16 ± 1		
d(TGpO(CH$_2$)$_3$OpGT). d(ACTCA)	37	1.5	13 ± 1		
d(TGAGT).d(ACTCA)	30	14.7	1.3 ± 0.1	82 ± 5	–67 ± 15
	40	4.8	4 ± 0.4		
	44	3.3	5.8 ± 0.5		
d(AGAGA).d(TCTCT)	15	21.2	0.91 ± 0.08	79 ± 2	–64 ± 7
	20	8.2	2.3 ± 0.3		
	27	5	3.8 ± 0.4		
	30	3.6	5.35 ± 0.5		

[a]The kinetics were measured at the mentioned temperatures in 200 mM $NaClO_4$, 5 mM phosphate buffer, pH 7.5.
[b]The $t_{1/2}$ values correspond to the times at which half the intrastrand cross-links have disappeared.
[c]The pseudo-first-order rate constants, k_{obs}, were obtained from non-linear least squares fits of ln(% intrastrand cross-link) versus time.

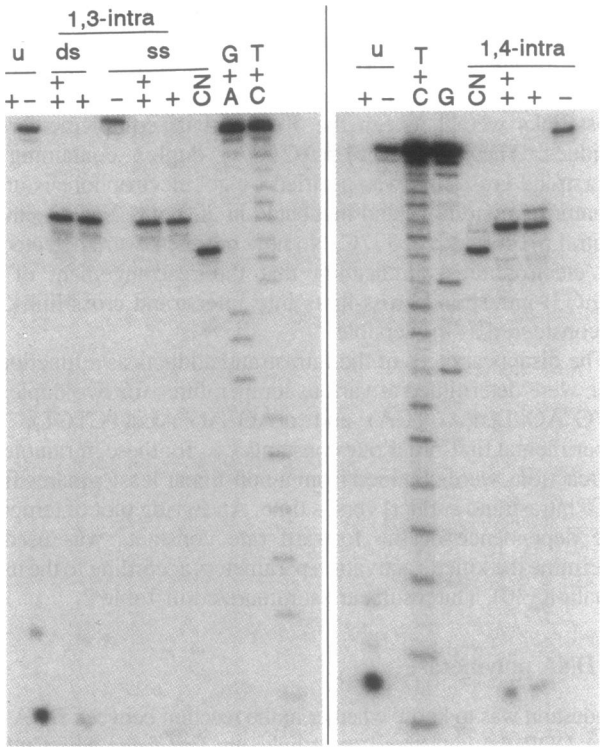

Figure 4. Digestion by T4 DNA polymerase of *trans*-DDP-modified oligonucleotides. ImageQuant scans of denaturing 24% polyacrylamide gels. Lanes u refer to the unplatinated oligonucleotides. Lanes G, G+A and T+C are relative to Maxam–Gilbert sequencing. Lanes 1,3-intra refer to the d(TGAGT) oligonucleotide containing a single *trans*-{$Pt(NH_3)_2$[d(GAG)-G*N7*,G*N7*]} cross-link hybridized with its d(ACTCA) complementary strand (lanes ds) or not (lanes ss); lanes 1,4-intra are relative to the d(TGTTGT) strand bearing a single *trans*-{$Pt(NH_3)_2$[d(GTTG)-G*N7*,G*N7*]} cross-link. The enzymatic reaction (+ and ++ correspond to 1 or 3 U enzyme, respectively) was carried out for 30 min at 37°C on 0.15 nmole of the oligonucleotides. In lanes (–), the samples were not subject to enzyme hydrolysis. The enzymatic digests after incubation in 300 mM NaCN overnight and at 50°C are presented in lanes CN.

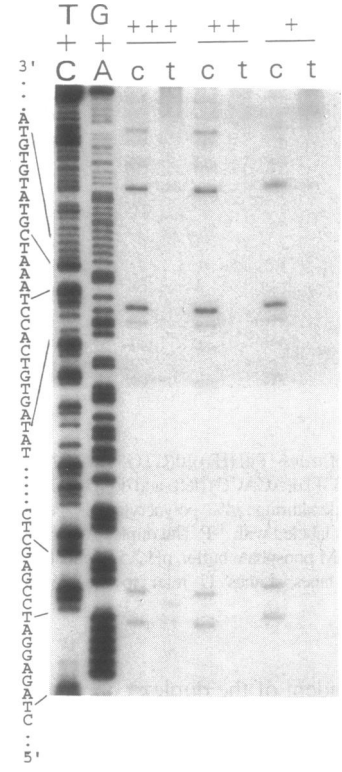

Figure 5. Digestion by T4 DNA polymerase of platinated DNA. Autoradiogram of a denaturing 8% polyacrylamide gel of *cis*- [lanes c] and *trans*-DDP-modified [lanes t] (*NdeI*/*XhoI*) restriction fragments from plasmid pSP73KB. *trans*-DDP-modified fragments were subject to thiourea treatment for 10 min at 37°C prior to ethanol precipitation. Platinated fragments (~40 ng) were treated with increasing quantities of T4 DNA polymerase (lanes +, ++ and +++ correspond to 0.3, 1 and 3 U enzyme, respectively) for 30 min at 37°C. A part of the sequence of the restriction fragment is indicated on the left side of the figure.

The next step was to detect the intrastrand cross-links in *trans*-DDP-modified double-stranded DNA. It is known that *cis*-DDP–DNA bifunctional adducts block the 3′–5′ exonuclease activity of T4 DNA polymerase (31–34) whereas the *trans*-DDP-interstrand cross-links do not block it (10). The results (Fig. 4) show that *trans*-{$Pt(NH_3)_2$[d(GTG)-G*N7*,G*N7*]} and *trans*-{$Pt(NH_3)_2$[d(GTTG)-G*N7*,G*N7*]} intrastrand cross-links within single-stranded and double-stranded oligonucleotides stop the exonucleolytic progression of the enzyme. As revealed by the migration of the digests after incubation overnight at 50°C in 300 mM sodium cyanide (to remove the bound platinum), the enzyme stops one residue before the 3′ platinated G residue in the case of *trans*-{$Pt(NH_3)_2$[d(GTG)-G*N7*,G*N7*]} and at the 3′ platinated G residue in the case of *trans*-{$Pt(NH_3)_2$[d(GTTG)-G*N7*,G*N7*]}. Similar results were obtained with longer oligonucleotides, the lesions being located 30 or 50 residues away from the 3′ end of the oligonucleotides. The enzyme was also stopped by the *trans*-{$Pt(NH_3)_2$[d(CGAG)-C*N3*,G*N7*]} intrastrand cross-link (12).

The mapping experiments were then done on a DNA restriction fragment (164 bp) from plasmid pSP73KB after modification with *cis*- or *trans*-DDP (DNA and the drug were incubated 24 h, at 37°C, at an input molar ratio drug/nucleotide equal to 0.005). Prior to precipitation, the *trans*-DDP-modified DNA was incubated with thiourea (10 mM) during 10 min and at 37°C. Analysis of the samples by atomic absorption indicated that ~80% of the bound platinum residues were removed by the treatment with thiourea. It was verified by gel electrophoresis under denaturing conditions (16) that the interstrand cross-links were formed in the platinated (164 bp) DNA restriction fragment. The percentage of platinum residues involved in the interstrand cross-links was ~15% of the total bound platinum before thiourea treatment. The samples were subjected to the action of T4 DNA polymerase and the digests were analyzed by gel electrophoresis. As expected, the enzyme is blocked at the potential d(GpG) and d(ApG) binding sites in *cis*-DDP-modified DNA (Fig. 5). Less stops are found in *trans*-DDP-modified DNA. There are mainly two bands of weak intensity in the sequence d(CTAGAGCGA). No firm conclusion can be drawn on the nature of the adducts but these results do not support that (G1,G3)-, (G1,G4)- and (C1,G4)-intrastrand cross-links are the major adducts. A few bands of weak intensity (results not shown) were obtained with the two *trans*-DDP-modified restriction fragments [(*Eco*RI–*Hin*dIII) from plasmid pAG22 and (*Eco*RI–*Bam*H1) from plasmid pTG132] containing the sequences $d(AG)_{22}$ and $d(GC)_5(TG)_{28}$, respectively.

DISCUSSION

In the reaction between *trans*-DDP and single-stranded oligonucleotides containing a single sequence d(GXG), 1,3-intrastrand cross-links are formed. The T_m of several platinated duplexes containing a *trans*-{$Pt(NH_3)_2$[d(GXG)-G*N7*,G*N7*]} intrastrand cross-link are in the same range of temperature, even when X is replaced by a propylene bridge. However the T_m are lower than those of the corresponding unplatinated duplexes (Table 1). We conclude that the intervening base residue between the two chelated G residues and its complementary base do not participate strongly to the stability of the duplexes. This conclusion is in disagreement with a report on the duplex d(CCTCG*AG*TCTCC).d(GGAGACTCGAGG) containing a single *trans*-{$Pt(NH_3)_2$[d(CGAG)-G*N7*,G*N7*]} intrastrand cross-link which shows that the platinated and the unplatinated duplexes melt at the same temperature (18). The duplexes were prepared by heating the solutions at 85°C and allowing them to cool slowly to 0°C. In these conditions, it seems likely that during the cooling, most of the 1,3-intrastrand cross-links were transformed into interstrand cross-links.

The duplexes containing a single *trans*-{$Pt(NH_3)_2$[d(GTG)-G*N7*,G*N7*]} intrastrand cross-link were polymerized and the products analyzed by gel electrophoresis. The extent of DNA bending and unwinding produced by the adducts was revealed by the gel mobility shifts of the platinated multimers relative to unplatinated controls and calculated according to the procedure used previously for platinum(II) adducts (22,23,25). The *trans*-{$Pt(NH_3)_2$[d(GTG)-G*N7*,G*N7*]} intrastrand cross-link bends the double helix by ~26° and unwinds it by ~45°. These results disagree with some data in the literature (22,25), likely for the same reason above discussed in the case of the thermal stability.

The kinetics of the rearrangement of the 1,3-intrastrand cross-links into interstrand cross-links were investigated in several duplexes to determine whether the linkage isomerization reaction depends upon the chemical nature of the (X·Y) base pair and of the base pairs adjacent to the adduct. The results, summarized in Tables 1 and 2 of our previous paper (13), show that the half-lives of the 1,3-intrastrand cross-links are comprised between 1.2 and 6 h whatever the nature of X (Pu or Py) and of the base pairs adjacent to the adduct, Y being a Py residue. In all the cases, the product of the reaction is an interstrand cross-link between the platinated 5′ G residue and its complementary C residue. When Y is a Pu residue, $t_{1/2}$ are longer (4–24 h) and two interstrand cross-links are formed (the major one being between the platinated 5′ G and its complementary C residue).

The rate of the rearrangement depends upon temperature which allows deduction of the activation parameters. The values of these parameters do not prove either a reaction proceeding through solvent-associated intermediate (35) or a reaction resulting from a direct nucleophilic attack of the C residue complementary to the platinated 5′ G residue on the platinum residue. The latter mechanism requires that the C residue comes close to the platinum residue along a line perpendicular to the platinum square plane. The distortions induced in the double-helix by the 1,3-intrastrand cross-link are not yet well-characterized. A NMR study of d(CCTCG*AG*TCTCC). d(GGAGACTCGAGG) has been done (18) but the sample was heated and it seems likely that the 1,3-intrastrand cross-links are in part transformed into interstrand cross-links. Chemical probes suggest a local distortion >4 bp including the 3 bp at the level of the adduct and the 5′ bp adjacent to the adduct in the 22mer duplex d(TG*TG*T). d(ACACA) (24). This local distortion and the bending of the double helix could allow the C residue to be in the position required for the reaction.

To determine whether the rearrangement of the (G1,G3)-intrastrand cross-links is prevented by some sequence effects, we searched the presence of these adducts in double-stranded DNA after modification with *trans*-DDP by means of the exonuclease activity of the T4 DNA polymerase. The enzyme is stopped by (G1,G3)-, (G1,G4)- and (C1,G4)-intrastrand cross-links in single-stranded and double-stranded oligonucleotides (Fig. 5; ref. 12) but not by the interstrand cross-links (10). Several *trans*-DDP-modified DNA restriction fragments were digested by T4 DNA polymerase. Very few stops were detected which lead us to exclude (G1,G3)-, (G1,G4)- and (C1,G4)-intrastrand cross-links as the major adducts. Supporting this finding is the fact that in several duplexes containing a monofunctional adduct *trans*-{$Pt(NH_3)_2(dG)Cl\}^+$ within the d(GXG) sequences, the closure of the monofunctional adducts to bifunctional cross-links yields mainly interstrand cross-links. There was no evidence for a two-step reaction in which first the 1,3-intrastrand cross-links are formed and subsequently rearrange into interstrand cross-links. The formation of the 1,3-intrastrand cross-links by closure of the monofunctional adducts is a rare event. It is important to recall that a similar result was obtained with *cis*-DDP-monofunctional adducts within d(GTG).d(CAC) sequences; the $t_{1/2}$ for closure to intrastrand cross-links was >20 h (36,37).

In conclusion, all these results are consistent with the finding that after 24 h of reaction between DNA restriction fragments and *trans*-DDP, the major adducts are monofunctional adducts and the bifunctional adducts are mainly interstrand cross-links. The apparent discrepancy on the nature of the adducts (this work; 7,9) might originate from the different levels of *trans*-DDP bound to DNA. It is tempting to speculate that *trans*-DDP has no antitumor activity because it cannot form bifunctional adducts. *In vivo*, the drug-to-nucleotide ratio is low and the closure of the monofunctional adducts to bifunctional cross-links is expected to yield mainly interstrand cross-links. In fact, the interstrand cross-links are not formed because the rate of the cross-linking reaction is slow (10) and the monofunctional adducts can be trapped by compounds such as glutathione (7–9).

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