Detection and quantification of adducts formed upon interaction of diamminedichloroplatinum (II) with DNA, by anion-exchange chromatography after enzymatic degradation+

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ABSTRACT A method has been developed to determine the adducts formed upon interaction of cis- and trans-diamminedichloroplatinum(II) (cis- and trans-DDP) with DNA. After 5 h at 50 °C in the dark, the amount of cis-DDP bound to salmon sperm DNA was larger than the amount of the trans-isomer. After enzymatic degradation with deoxyribonucleases to nucleotides and Pt-containing (oligo)nucleotides, the various products were separated by DEAE chromatography and analyzed for Pt by flameless AAS. Indications were obtained for the presence of nucleotides containing monofunctionally bound Pt and of adducts originating from interstrand DNA crosslinks. DEAE chromatography of digests of cis-DDP-treated DNA yielded a product with overall charge -1, which was identified with NMR and CD as cis-[$Pt(NH_3)_2$-d(pGpG)], the oligonucleotide derived from intrastrand crosslinks between two adjacent guanines. Another major peak contained Pt-oligonucleotides with overall charge -2, which could be derived from intrastrand crosslinks between two guanines at sites with pGpXpG (X=T,C,A or G) base sequences.

INTRODUCTION

During the last decade, cis-diamminedichloroplatinum (II) (cis-DDP) has proven to be an useful anti-tumor drug, especially when used in appropiate combination chemotherapy (for a review see ref. 1).

The main target of this drug is generally accepted to be the chromosomal DNA in the cells, to which DDP binds preferentially at the N7 atom of guanine[2,3]. In the reactions, DDP can form adducts with one or two DNA bases, in the latter case the Pt-compound may link either two bases in opposite strands (interstrand crosslink) or two bases in the same strand (intrastrand crosslink). Trans-DDP, the stereoisomer of cis-DDP, shows no anti-tumor activity. Nevertheless, this compound is also able to bind monofunctionally and bifunctionally to DNA. For cis-DDP, the formation of intrastrand crosslinks between two adjacent guanines has been reported[4,5,6]. On the basis of results from mutation studies in Escherichia coli cells, which revealed that the base sequences pGpApG and pGpCpG are hotspots for cis-DDP-induced base-pair substitutions, the interaction between cis-DDP and two guanines separated by a third base has been

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proposed[7,8]. Recently, it has been proven by NMR that this type of crosslink can be formed in vitro in d(GpCpG)[9].

The consequences of the formation of the various Pt-DNA adducts in the cell are still uncertain. The intrastrand crosslinks between two adjacent guanines have been suggested to be responsible for the anti-tumor activity of the cis-DDP compound[10,11], because this type of adduct can be formed only by the cis- and not by the trans-compound. This is not a strong argument, as the mutagenic intrastrand crosslinks between two guanines in pGpApG and pGpCpG base sequences are also induced by cis-DDP only[7]. Other investigators believe that interstrand crosslinks by cis-DDP are responsible for cell killing[12] and cytotoxicity[13,14]. On the other hand, Meyn et al.[15] suggested that interstrand crosslinks induced in mammalian cells by cis-DDP are not the only lesions responsible for cytotoxicity. Also for bacteriophages the frequency of interstrand crosslinks has been reported to be too low to account for the phage killing[16].

To study the contribution of each lesion to the observed effects on the cellular level, it will be necessary to be able to detect and quantify the various adducts formed in DNA upon treatment with DDP. Recently, Johnson has described a method to characterize the Pt-adducts by electrophoresis of the reaction products obtained after partial acid hydrolysis of DNA that has been treated with Pt-compounds[17]. However, this procedure - based on depurination of the DNA - does not distinguish between inter- and intra-strand crosslinks and does not give information about the base sequences in which the intrastrand crosslinks were formed. In other attemps to elucidate the nature of the lesions, enzymes have been used, such as restriction enzymes[5,10,18] and exonuclease III[11]. The enzymatic activities are supposed to be unable to attack sites where the Pt-binding has distorted the configuration of the DNA, so probably base sequences containing intrastrand crosslinks remain intact.

We have developed a method to determine the adducts formed upon interaction of DDP with DNA, based on enzymatic degradation of the DDP-treated DNA. In this method, the nucleotides and the Pt-containing (oligo)nucleotides obtained are separated chromatographically and characterized by comparison with reference Pt-adducts. In our studies, DDP-treated DNA was degraded with deoxyribonuclease I and nuclease P1, which normally digest to mononucleotides but were expected to be impeded in the breakdown at or near sites where platinum is bound. The present paper describes the first results of this investigation.

MATERIALS AND METHODS

Treatment of DNA with Platinum Compounds

Salmon sperm deoxyribonucleic acid (Millipore Corporation) was dissolved in 10 mM sodium phosphate, pH 7.0, to a final concentration of 0.37 mg/ml (assuming $\epsilon_{260\ nm}$ = 6,600 $l.mole^{-1}.cm^{-1}$). Solutions of cis- and trans-DDP (gifts from Department of Chemistry, Gorlaeus Laboratories, State University Leiden, The Netherlands) were freshly prepared in dimethylsulfoxide (2.5 mg/ml). Prewarmed 10 ml portions of the DNA solution were incubated with the Pt-compounds for 5 h at 50 °C in the dark. To stop the reaction, 2 ml of 0.5 M NH_4HCO_3 was added, followed by dialysis for 18 h at room temperature against 0.1 M NH_4HCO_3. By this procedure the remaining coordination site of monofunctionally bound platinum is likely to become occupied by NH_3. Finally, the solutions were dialyzed against distilled water for 24 h at 0 °C, or 8 h at room temperature, and evaporated to dryness.

Enzymatic Degradations

The DDP-treated DNA samples (approximately 3.7 mg) were resuspended in 2.6 ml buffer (10 mM TRIS/HCl pH 7.2 containing 0.1 mM EDTA and 4 mM $MgCl_2$) and incubated for 65 h at 37 °C with 105 units deoxyribonuclease I (EC 3.1.21.1; Sigma). Subsequently, the DNA solutions were concentrated by evaporation to 200 µl and, after addition of 400 µl 30 mM sodium acetate pH 5.3 and 24 µl 10 mM $ZnSO_4$, incubated at 37 °C with 9 µl nuclease P1 (EC 3.1.4.-; Boehringer). After 24 h, a second portion of the enzym was added and the incubation continued for another 24 h. The nuclease P1 was dissolved in 30 mM sodium acetate pH 5.3 at a concentration of 1 mg/ml. To calculate the amounts of nucleotides present in the digests, a value of $\epsilon_{260\ nm}$ = 11,000 $l.mol^{-1}.cm^{-1}$ was used. For the preparation of nucleosides, 100 µl samples of nucleotide solutions were incubated for 24 h at 37 °C with 0.9 units alkaline phosphatase, type III (EC 3.1.3.1; Sigma; suspension in 2.5 M $(NH_4)_2SO_4$), after addition of 1 µl 1 M TRIS/HCl pH 9.0.

Anion-Exchange (DEAE) Chromatography

Samples of the digests of DDP-treated DNA (125 µl, originating from approximately 0.7 mg DNA) were diluted with distilled water (250 µl) and put on top of 5 ml DEAE-Sephacel (Pharmacia) columns, equilibrated in 5 mM TRIS/HCl pH 7.5. The columns were briefly eluted with the starting buffer and then a 0-150 mM linear NaCl gradient (2 x 60 ml) in the TRIS buffer was applied, followed by elution with 1 M NaCl in the same buffer. Fractions of 2.5 ml were collected at a flow rate of 20 ml/h.

Platinum Determinations

The amounts of platinum in the adduct-containing samples were determined by atomic absorption spectroscopy (AAS) by using a Perkin Elmer model 4000 atomic absorption spectrophotometer equipped with a HGA-500 graphite furnace and an AS-400 autosampling system. K_2PtCl_6 solutions were used for calibration.

High-Performance Liquid Chromatography (HPLC)

For HPLC a Beckman 421 system, equipped with gradient pumps and a 165 variable-wavelength detector, was used. Elution of nucleosides was effected in 10 min at a flow rate of 1 ml/min on a reversed phase column (Lichrosorb RP 18-5) by applying a linear gradient of 15 to 40% methanol in water.

Nuclear Magnetic Resonance (NMR) and Circular Dichroism (CD)

^{1}H-NMR spectra were measured at 300 MHz on a Bruker WM-300 spectrometer connected to an Aspect 2000 computer. CD spectra were recorded with a CNRS Roussel-Jouan III dichrograph (Jobin Yvon, France).

RESULTS AND DISCUSSION

Platinum Binding to DNA

A solution of salmon sperm DNA was treated with various concentrations of cis- and trans-DDP for 5 h at 50 °C in the dark. After dialysis, the DDP-treated DNAs were digested with deoxyribonuclease I and nuclease P1 to (oligo)nucleotides. To determine the amounts of Pt-atoms bound to the DNA, samples of the mixtures were analyzed by AAS. In Figure 1 the number of Pt-atoms bound per nucleotide (r_b) is indicated as a function of the concentration of the Pt-compound in the incubation mixture. The curves show linear dose-effect relationships for both cis- and trans-DDP. At equal concentration, approximately twice the amount of cis-DDP is bound to the DNA in comparison with trans-DDP. Similar results were obtained when the Pt-DNA samples were analyzed before the enzymatic digestion (data not shown).

Separation and Identification of the Pt-Adducts

Anion-exchange chromatography was utilized to separate the various Pt-adducts obtained after enzymatic degradation of DDP-treated DNA on the basis of differences in the overall charges of the Pt-containing (oligo)nucleotides. Table 1 lists the normally-occurring nucleotides and possible Pt-adducts, which may be present in the digested Pt-DNA. The overall charges of the products are indicated at pH 7.5, at which pH the

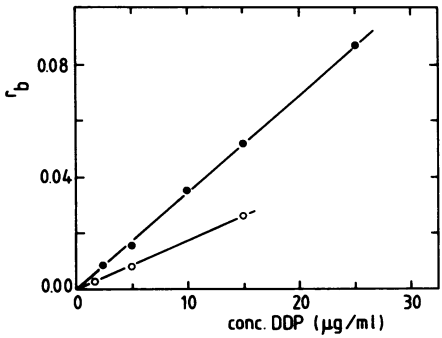

Figure 1. Relationship between the number of Pt-atoms bound per nucleotide (r_b) in DDP-treated DNA and the amount of DDP in the incubation mixture. The amount of nucleotides was calculated from the absorbance (260 nm). Platinum was measured by AAS.
——●—— cis-DDP;
——○—— trans-DDP.

net charges result from the positive charge of the Pt-compound (+2) and the negative charges of the phosphate groups. At this pH the significant decrease in the pKa value of the N1 of guanine, occurring after binding of DDP to guanine[6,9,19], does not influence the overall charges of the Pt-adducts.

Figures 2A and 2B show the elution patterns of DEAE - anion exchange - columns, loaded with digested DNA samples obtained after incubation of DNA with cis- and trans-DDP, respectively. The eluate fractions were monitored for A260 nm and Pt-content.

The main UV-absorbing peaks in the elution profiles (IIA and IIB) are composed of the free nucleotides, as was proven by HPLC after further enzymatic degradation to nucleosides. Peak IIA contains dpT and dpC and peak IIB dpA and dpG, all having an overall charge of -2. The UV-absorbing peak 0 in Figures 2A and 2B appears to be composed of zero-charged pro-

Table 1. Overall charge of possible (oligo)nucleotides, expected after enzymatic degradation of DDP-treated DNA

Pt-binding in DNA	(Oligo)nucleotide	Overall charge at pH 7.5
None	dpB[a]	-2
Monofunctional	$Pt(NH_3)_3$dpB	0
Bifunctional		
- interstrand crosslink	$Pt(NH_3)_2(dpB)_2$	-2
- intrastrand crosslink	$Pt(NH_3)_2$d(pBpB)	-1
	$Pt(NH_3)_2$d(pBpBpB)	-2

[a] B = nucleobase (T,C,A or G).

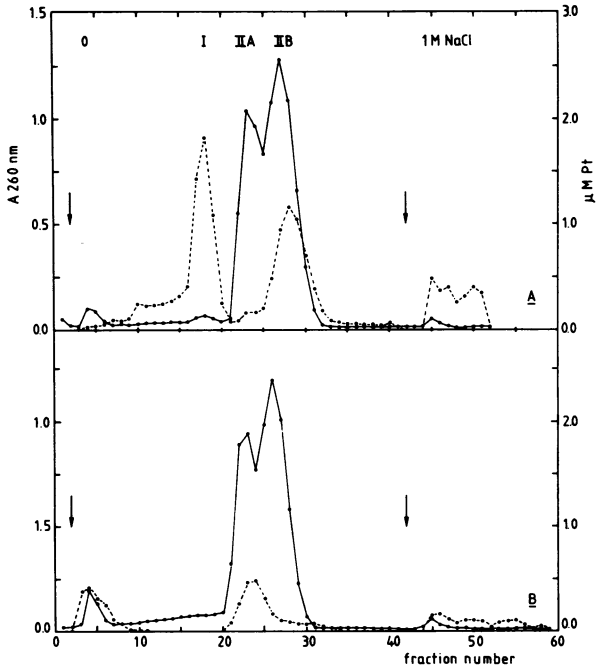

Figure 2. DEAE elution patterns of digests of DDP-treated DNA. A: cis-DDP, 5µg/ml; B: trans-DDP, 5µg/ml; ——— absorbance (260 nm); -------- Pt-concentration (µM). Arrows indicate start and end of gradient elution (0-0.15 M NaCl). Finally, the NaCl concentration in the eluens was raised to 1 M.

ducts like dT, dC, dA and dG as has been confirmed by HPLC. Apparently, these mononucleosides are by-products formed during the enzymatic degradation to nucleotides of the Pt-DNA samples.

In addition to the mononucleosides with overall charge zero, peak 0 of the digest of trans-DDP-treated DNA contains platinum, as was measured by AAS (see Figure 2B). Probably, this platinum is present in adducts with an overall charge of 0, presumably trans-$[Pt(NH_3)_3dpB]$ (cf. Table 1) in which the third NH_3 group originates from NH_4HCO_3, used to stop the reaction between DDP and DNA. These adducts are almost absent in the digest of DNA treated with cis-DDP under the conditions used (Figure 2A).

Peak I, obtained after chromatography of the digest of cis-DDP-treated DNA, has a very high Pt-content in relation to the A260 nm. From the ratio, it can be calculated that possibly a Pt-oligonucleotide with 1 Pt-atom per 2 nucleotides is present. The nature of this product could be elucidated by NMR and CD studies as cis-$[Pt(NH_3)_2d(pGpG)]$, which has an

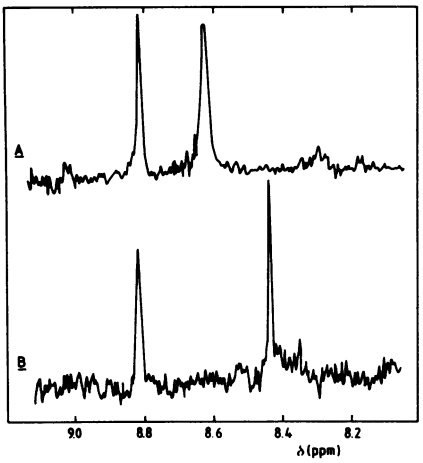

Figure 3. Low-field parts of the 300 MHz ^{1}H-NMR spectra of the material eluted in DEAE peak I during chromatography of digests of cis-DDP-treated DNA. Recorded in D_2O (15 °C) at pH* 7.4 (A) and pH* 1.4 (B). (pH* is the uncorrected pH meter reading in D_2O solution).

overall charge of -1, in agreement with the position of the peak in the gradient. The NMR spectra indicate an almost pure guanine-containing product, in view of the presence of ^{1}H-resonances specific for Pt-guanines and the absence of signals specific for the other nucleobases. The chemical shifts (δ) of the H8 protons of the guanines (Figure 3), measured both at pH* 7.4 and pH* 1.4, fully agree with the data reported by Girault *et al.* for synthetic cis-$[Pt(NH_3)_2d(pGpG)]$[20], in which the platinum is coordinated by both guanines at N7. Moreover, the CD spectra (Figure 4) recorded at pH 9.0 and 11.0 confirm the identity of the isolated species in peak I[20]. A Pt-containing peak at this position is exclusively present

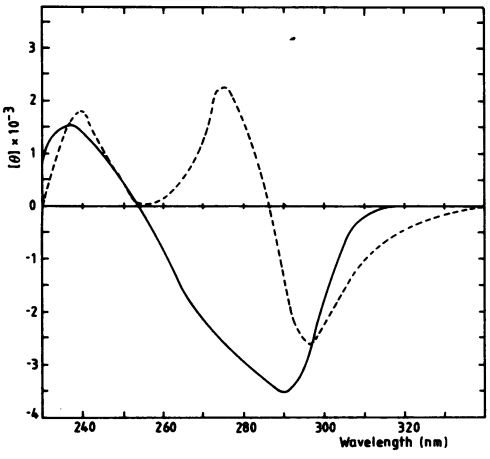

Figure 4. Circular dichroism spectra of material eluted in DEAE peak I during chromatography of digests of cis-DDP-treated DNA, identified as cis-$[Pt(NH_3)_2d(pGpG)]$. [θ] in deg.cm^2.dmol^{-1} per nucleotide residue. Recorded at pH 11.0 (——) and pH 9.0 (- - - -).

in the digest of cis-DDP-treated DNA, demonstrating that the formation of intrastrand crosslinks between two adjacent guanines is only occurring in DNA upon treatment with cis-DDP.

Also in the region of peak II, substantial amounts of platinum are eluted (Figure 2). The Pt-adducts derived from interstrand crosslinks and from the expected intrastrand crosslinks at pGpXpG (X=T,C,A or G) base sequences, are supposed to have an overall charge of -2 (cf.Table 1) and are therefore assumed to elute in this region, together with the -2 charged free nucleotides. The Pt-containing material eluted in peak IIA is present in digests of both cis- and trans-DDP-treated DNA, but is more abundant in the latter. It may contain Pt-adducts derived from interstrand crosslinks, because the product cis-$[Pt(NH_3)_2(dpG)_2]$, which we prepared by reaction of cis-DDP with dpG, co-chromatographs with dpT and dpC in peak IIA. Peak IIB has a high Pt-content only in the case of cis-DDP-treated DNA. Because of the specificity for cis-DDP, this material may represent the Pt-crosslinks on pGpApG and pGpCpG base sequences, which are hotspots for base-pair substitutions in E.coli cells[7,8]. At higher temperature, these intrastrand crosslinks are the most important lesions in the cis-DDP-induced mutagenesis[8], whereas trans-DDP is not mutagenic for E.coli.

Relatively small amounts of platinum are eluted at the higher NaCl concentrations (Figure 2). The possible nature of this material will be discussed below.

From Figure 2 the conclusion can be drawn that the different elution profiles, obtained after chromatography of the digests of cis- and trans-DDP-treated DNA's, reflect the presence of different lesions in those DNA's. Under the used conditions, the trans-compound (Figure 2B) binds monofunctionally to the DNA (Pt-adduct eluted in peak 0), as well as bifunctionally under the formation of interstrand crosslinks (Pt-adduct in peak IIA). The cis-compound, however, forms mainly intrastrand crosslinks between two adjacent guanines (Figure 2A, peak I) and possibly on pGpXpG (X = T,C,A or G) base sequences (peak IIB). In addition, small amounts of interstrand crosslinks (Pt-adducts eluted in peak IIA) are possibly formed.

Quantification of the Pt-Adducts

To study the amounts of the various adducts formed by the biologically more important cis-DDP as a function of the concentration of the compound during the treatment, DNA samples were incubated with cis-DDP at 2.5, 5, 10, 15 and 25 µg/ml, digested and analyzed. The DEAE elution patterns of

the digests after treatment with cis-DDP at 10 and 25 µg/ml are depicted in Figures 5A and 5B, respectively. A comparison of these profiles with that obtained after treatment with 5 µg/ml (Figure 2A) shows a relatively strong increase in the peak eluted with high NaCl concentration (1 M NaCl peak). This material must comprise Pt-oligonucleotides having a charge more negative than -2; probably they result from incomplete enzymatic degradation of the DDP-treated DNA, due to the presence of two or more Pt-atoms close to each other in the DNA.

In Table 2, the amounts of cis-DDP bound in the various adducts are listed as percentages of the total amount of platinum eluted from the DEAE column. Generally, the recovery of the platinum after chromatography was

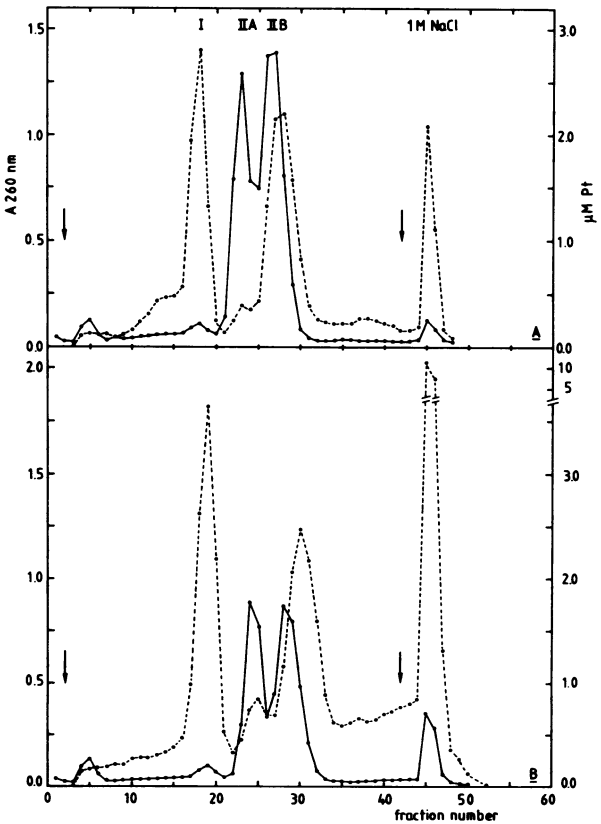

Figure 5. DEAE elution patterns of digests of cis-DDP-treated DNA. A: cis-DDP, 10 µg/ml; B: cis-DDP, 25 µg/ml; ——— absorbance (260 nm); ------ Pt-concentration (µM). See legend to Figure 2 for further details.

Table 2. Quantitative analysis of Pt-adducts after DEAE chromatography

cis-DDP	Peak I		Peak IIA		Peak IIB		1 M NaCl peak	
(μg/ml)	Pt(%)[a]	freq[b].	Pt(%)	freq.	Pt(%)	freq.	Pt(%)	freq.
2.5	38	3.3	3	0.3	29	2.5	10	0.9
5.0	34	5.4	3	0.5	33	5.2	17	2.7
10.0	28	9.9	4	1.4	35	12.4	14	5.0
15.0	30	15.8	nd[c]	nd[c]	27	14.2	19	10.0
25.0	20	17.4	4	3.5	21	18.3	37	32.2

[a] Percentage of the total amount of platinum eluted from the DEAE column.
[b] Frequency of the occurrence of the adducts eluted in this peak, expressed in Pt-atoms per 1000 nucleotides in the DDP-treated DNA.
[c] Not determined.

high (92 ± 11%). The fact that so much of the platinum was recovered in the form of adducts indicates that there was no substantial disruption of the bonds between platinum and DNA during the enzymatic digestion. In order to quantify the amounts of the various adducts in the DDP-treated DNA, the frequencies of their occurrence per 1000 nucleotides were calculated. This calculation was based on the r_b value of the material subjected to DEAE chromatography (Figure 1) and the percentages of platinum recovered in the various peaks. The results are listed in Table 2 and visualized in Figure 6.

The frequencies of the occurrence of the various adducts increase linearly with the dose up to a cis-DDP concentration of approximately 10 μg/ml in the incubation mixture with DNA (Figure 1 shows that this concentration results in a binding of 35 Pt-atoms per 1000 nucleotides in the total DNA). Only the adduct derived from interstrand crosslinks (curve with open circles) shows a continuation of the linear increase at higher cis-DDP concentration. The amount of highly-charged Pt-oligonucleotides eluted with 1 M NaCl (curve with open squares) shows a strong increase at cis-DDP concentrations higher than 10 μg/ml, which is presumably due to a reduction in the susceptibility to enzymatic degradation, because of the increased density of bound platinum in the DNA. Below a cis-DDP concentration of 10 μg/ml, the adducts derived from the two types of intrastrand crosslinks are apparently the predominant products (closed circles and open triangles). At higher cis-DDP concentrations, the increase in the

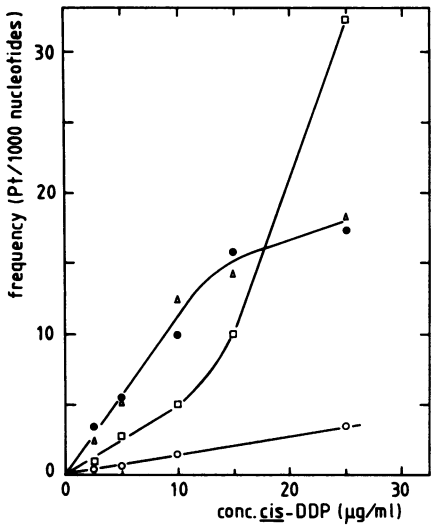

Figure 6. Frequencies of the occurrence, in the original cis-DDP-treated DNA, of the adducts eluted in DEAE peaks in relation to the concentration of cis-DDP in the incubation mixture. The frequencies are expressed in Pt-atoms per 1000 nucleotides.
——●——Peak I (cis-[$Pt(NH_3)_2$d(pGpG)]);
——○——peak IIA (cis-[$Pt(NH_3)_2$(dpG)]);
——△——peak IIB (presumably cis-[$Pt(NH_3)_2$d(pGpXpG)] with X = T,C,A or G);
——□—— 1 M NaCl peak (Pt-containing oligonucleotides with a high negative charge).

amount of these adducts is no longer linear. In part this may be ascribed to the incomplete enzymatic degradation at higher Pt-density; moreover, it is expected that the curves will level off because of the limited availability of suitable base sequences in the DNA. Theoretically, the sequences pGpG and pGpXpG should occur in the DNA with the same frequency (when a random distribution is assumed). Consequently, the fact that the curves for the adducts in the Peaks I and IIB in Figure 6 are almost identical, supports the idea that the Pt-products in peak IIB (Figures 2A and 5) originate from intrastrand crosslinks on pGpXpG base sequences. This would imply that in these experiments there is no clear preference for X = T,C,A or G.

From a previous study[8] we know that the total DNA in E.coli cells, treated in vivo with cis-DDP at a concentration allowing 10% survival, has a r_b value of approximately 0.013. The same value is obtained after in vitro treatment of salmon sperm DNA with cis-DDP at 3.5 µg/ml (Figure 1); in this DNA, the various adducts could well be detected (Figure 6). Therefore, we expect that the Pt-lesions can also be detected and analyzed in in vivo treated DNA. This will be the subject of further investigations.

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