Isolation of the Products Resulting from the Reaction of *cis* and *trans* Diaminedichloroplatinum [II] with DNA and Chromatin on the Dowex 50 W Column

Ryszard Oliński and Zofia Walter

Department of Biochemistry, Institute of Biochemistry and Biophysics, University of Łódź, Banacha 12/16, Łódź 90-237, Poland

Z. Naturforsch. **39 c**, 1057–1062 (1984); received July 24, 1984

cis and *trans* Diaminedichloroplatinum, DNA, Chromatin, Platinated Derivatives of Nucleic Acid Bases

The production of platinated derivatives of nucleic acid bases resulting from the reaction of *cis* and *trans* DDP with DNA and chromatin was studied.

Bifunctional complex of guanine appeared to be the major product of the interaction of *cis* isomer with both DNA and chromatin, although other bifunctional adducts of A-Pt-G and A-Pt-A were also isolated. The main product of the interaction of *trans* DDP with DNA was a monofunctional adduct of guanine. Small amounts of the bifunctional complexes were also isolated. When ssDNA was incubated with *trans* DDP more bifunctional complexes appeared, what suggests that geometric constrains of double helix prevent formation of these complexes. *Trans* isomer reacts more easily with chromosomal proteins than *cis* DDP does. Therefore after the reaction of *trans* DDP with chromatin less platination occurs on DNA moieties.

Introduction

After the discovery of antitumor properties of *cis* diaminedichloroplatinum (*cis* DDP) several investigations have tried to elucidate its mechanism of action [1, 2].

From most of the studies it appeared that DNA is the essential target for the action of the drug [1]. Though *trans* diaminedichloroplatinum (*trans* DDP) also interacts with DNA it is biologically inactive [1, 2]. It is of particular interest then to compare the interaction of both Pt isomers with DNA.

In the previous paper we described the system which allowed to separate different platinated complexes of nucleic acid bases [3]. The purpose of this work is to compare the effects of both the Pt isomers on the production of the adducts resulting from the interaction of the drugs with DNA and chromatin.

Materials and Methods

DNA was isolated from calf thymus by the method of Zamenhof [4]. The RNA content estimated by the orcynol method [5] as well as the protein content estimated by the method of Lowry *et al.* [6] were lower than 1%. DNA was dissolved in 0.05 M $NaClO_4$, pH 6.4 at a concentration of 1 mg/ml. Nuclei were isolated from calf thymus by the hypotonic shock procedure [7] and further purified by centrifugation through 0.25 M sucrose followed by a 0.5% Triton X-100 wosh. Chromatin was isolated according to the procedure of Spelsberg and Hnilica [8]. Isolated chromatin preparations (1 mg DNA/ml) were rehydrated in 0.05 M $NaClO_4$ – 0.1 mM PMSF, pH 6.4. *cis* DDP was purchased from Sigma Chemical Company and *trans* DDP (NSC 132558) was a product of National Cancer Institute. Both the Pt-isomers were dissolved in 0.05 M $NaClO_4$, pH 6.4 and allowed to hydrolyze for 72 h at 37 °C.

Interaction of the Pt compounds with DNA or chromatin was allowed to proceed at 37 °C for 24 h at Pt/*d*-nucleotide ratios (r_i) of 0.05–0.3. After that time the possible unreacted Pt was dialyzed back. To get rid of the chromosomal proteins the chromatin samples were digested by proteinaze K (0.1 mg/ml) and removed by Zamenhof procedure [4]. The ethanol precipitated DNA was dissolved in 10 mM Tris-HCl, pH 8.1 digested by RN-aze (0.1 mg/ml) and extracted by Zamenhof procedure [4]. The content of RNA in DNA samples obtained in this way, was lower than 1%, and protein impurities were not higher than 3%. DNA was precipitated with 96% ethanol and hydrolized with 0.5 M HCl for

Reprint requests to Dr. R. Oliński.

0341-0382/84/1100-1057 $ 01.30/0

30′ at 100 °C. Control samples were treated in the same way except the addition of Pt compounds.

To isolate cytosine derivative, pyrimidine nucleotides were hydrolized for 2 h with 5 M $HClO_4$ at 100 °C then neutralized with NaOH. The Dowex 50 W × 2 200–400 mesh (Fluka) columns were applied to separate the bases and derivatives. The bases were located on a column (20 × 1 cm) and eluted by the gradient of 0.75–3 M HCl at a flow rate of 0.5 ml/min. Five ml fractions were collected. After evaporation and mineralization of samples, platinum concentrations were measured by the stannous chloride method, determining absorption at 405 nm [9]. The exact amounts of Pt and bases were calculated from standard curves and Pt/base ratios (r_b) were counted. The estimation of amino acids was done with ninhydrine reagent, determining absorption at 570 nm.

Single stranded DNA (ssDNA) was obtained by heating DNA samples in 0.05 M $NaClO_4$ at 100 °C for 15 min followed by quick cooling in an ice bath.

Results

In the previous paper the method which enables to isolate and characterize the different products of nucleic acid bases platination was presented [3]. This method was employed here for the separation of the products resulting from the reaction of *cis*- and *trans*-DDP on DNA ("naked" DNA) and DNA complexed with chromosomal proteins (chromatin). To test the resolution ability of the Dowex column all the platinated adducts described in the previous paper [3], as well as their standards were applied to the column. Results of the experiment are shown in Fig. 1. Good resolution of all the products was obtained although some overlapping of the peak containing A-Pt-G complex with peak of A/N7/PtA/N7 was observed.

Isolation of the products resulting from the reaction of Pt compounds with DNA

A) Reaction of *cis* DDP with DNA

Control DNA hydrolized with HCl was eluted from the Dowex column in three separate peaks (Fig. 2). Basing on UV spectra and position of elution they were identified as follows I-pyrimidine nucleotides, II-guanine, III-adenine.

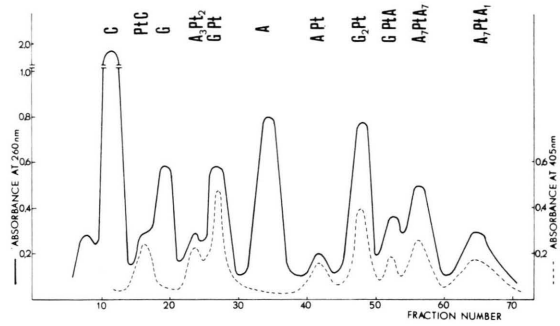

Fig. 1. Dowex 50 W chromatography of the products resulting from the reaction of *cis* DDP with equimolar *d*-guanosine, *d*-adenosine and *d*-cytidine. Additional mixed complex of A-Pt-G was loaded on the column (for details see the previous paper [3]).

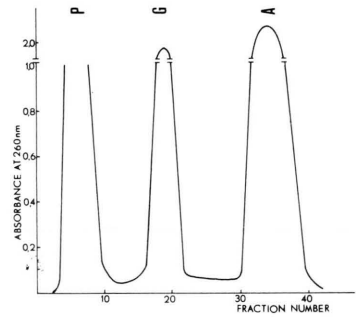

Fig. 2. Dowex 50 W chromatography of control DNA.

After DNA was incubated with different concentrations of *cis* DDP a variety of new peaks associated with Pt presence appeared (Fig. 3). At low r_i value ($r_i = 0.05$) one distinctive and one small peak appeared (Fig. 3A). They co-chromatographed with previously described G-Pt-G and G-Pt-A complexes [3].

Both the calculated r_b values and UV spectra proved them to be these complexes. At r_i value 0.1 two additional peaks appeared (Fig. 3B). The positions of elution, UV spectra and calculated r_b values pointed out that both possible bifunctional complexes of adenine were eluted in these peaks. However, there was higher amount of the chelate *via* both N7 atoms. In high r_i value ($r_i = 0.3$) apart from the above characterized adducts, monofunctional adduct of guanine was separated. The appearance of the platinated bases was associated with simultaneous disappearance of unmodified bases mainly guanine. At r_i 0.05 about 25% of guanine disap-

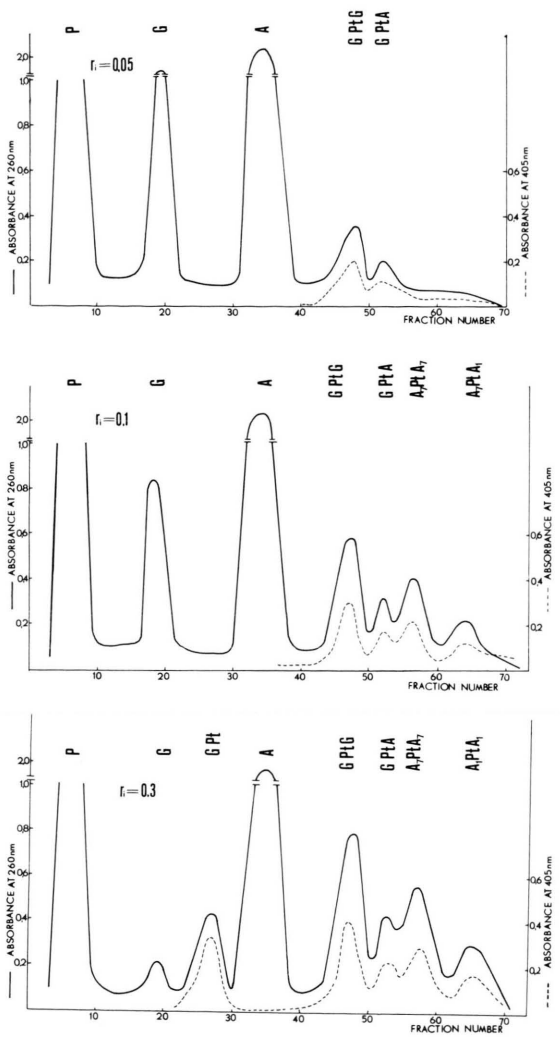

Fig. 3. Dowex 50 W chromatography of the products resulting from the reaction of DNA with *cis* DDP at indicated Pt/nucleotide ratios (r_i).

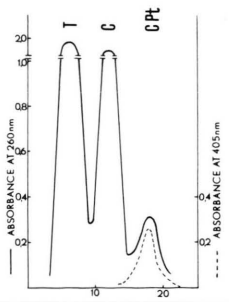

Fig. 4. Dowex 50 W rechromatography of pyrimidine nucleotides (eluted in peak I, Fig. 3), after the reaction of DNA with *cis* DDP at $r_i = 0.3$.

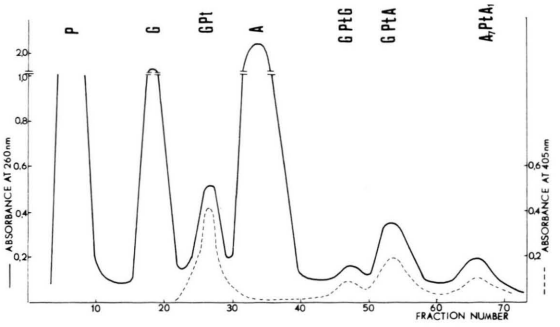

Fig. 5. Dowex 50 W chromatography of the products resulting from the reaction of DNA with *trans* DDP at Pt/nucleotide ratio $|r_i| = 0.1$.

peared from the standard peak and at $r_i = 0.3$ the peak disappeared almost completely. At $r_i = 0.3$ the peak of unmodified adenine also significantly decreased. The platinated derivative of cytosine was isolated only after incubation of *cis* DDP with DNA at high r_i value ($r_i = 0.3$). The samples of pyrimidine nucleotides (peak I in Fig. 2) were evaporated and hydrolized with 5 M $HClO_4$. After neutralization the samples were rechromatographed on the Dowex column.

Thymine and cytosine were detected during the rechromatography of pyrimidines from control DNA. When DNA had reacted with *cis* DDP a small additional peak eluted after cytosine standard appeared (Fig. 4). The position of elution, UV spectra and r_b value proved it to be the monofunctional complex of cytosine and Pt.

B) Reaction of *trans* DDP with DNA

Comparative studies were performed after the reaction of *trans* DDP with DNA at $r_i = 0.1$.

A different picture emerged from Fig. 5.

Bifunctional complex of Pt and guanines – the main product of the reaction with *cis* isomer, was represented in only minute quantities. Three other peaks associated with Pt presence represented monofunctional adduct of guanine (the main product) mixed bifunctional complex of adenine-guanine (probably at the presence of N7 adenine N7 adenine complex) and N7–N1 bifunctional adduct of adenines.

However, when ssDNA was incubated with *trans* DDP all the possible bifunctional complexes ap-

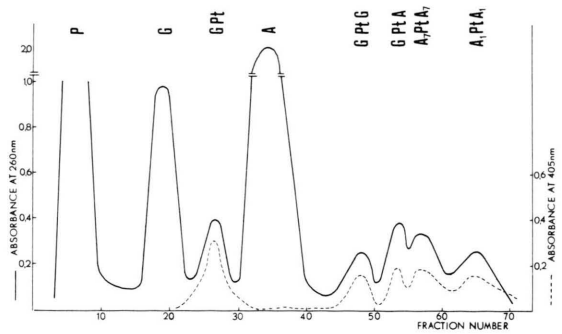

Fig. 6. Dowex 50 W chromatography of the products resulting from the reaction of ssDNA with *trans* DDP at Pt/nucleotide ratio $|r_i| = 0.1$.

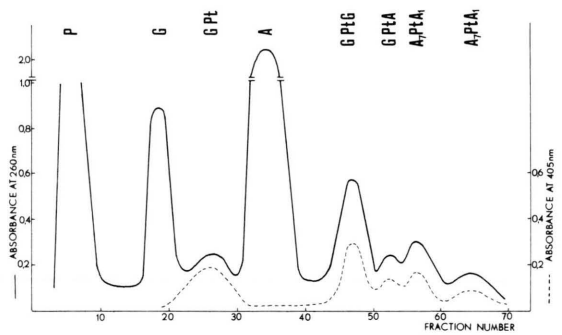

Fig. 7. Dowex 50 W chromatography of the products resulting from the reaction of chromatin with *cis* DDP at Pt/nucleotide ratio $|r_i| = 0.1$.

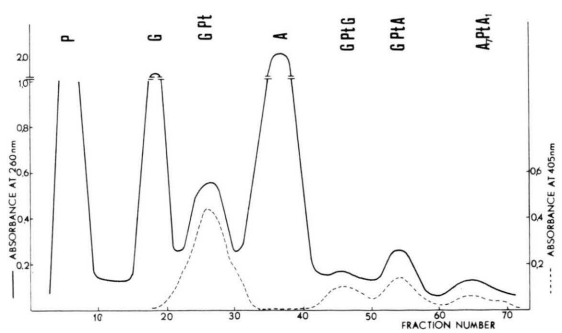

Fig. 8. Dowex 50 W chromatography of the products resulting from the reaction of chromatin with *trans* DDP at Pt/nucleotide ratio $|r_i| = 0.1$.

peared at the same quantities (Fig. 6), what suggests that geometric constrains of double helix prevent formation of some bifunctional complexes. No platinated cytosine was detected even after DNA incubation with *trans* DDP in r_i value > 0.1.

Isolation of the product resulting from the reaction of Pt compounds with chromatin

In order to be as close as possible to conditions prevailing in the living cell the comparative studies were done with chromatin. Samples of chromatin were incubated for 24 h with *cis*- or *trans* DDP, then the possible unreacted drug was dialyzed back. To get rid of the protein the chromatin samples were digested by proteinase K and removed by Zamenhof procedure as described in Materials and Methods. DNA was ethanol precipitated and hydrolized with 0.5 M HCl. Control samples were treated in the same way except the addition of the drug.

Reaction of cis DDP with chromatin

When chromatographed on the Dowex column chromatin treated with *cis* DDP yielded similar picture to that of the platinated DNA.

However, smaller amounts of bifunctional complexes of adenine were eluted and in the place where monofunctional complex of guanine was eluted a small peak appeared (Fig. 7). It is likely to represent the complex between guanine and amino acids arising from DNA-protein crosslinks.

Reaction of trans DDP with chromatin

After the *trans* isomer reacted with chromatin one major and three smaller peaks with Pt were detected (Fig. 8).

The major peak was eluted near the place of monofunctional adduct of Pt and guanine (Fig. 8). It also exhibits UV characteristic similar to that of the adduct. Yet the presence of amino acid detected in the peak suggests that it represents the complex of guanine, Pt and amino acid(s) arising from DNA-protein crosslinks. In the three other peaks G-Pt-G, A-Pt-G and A-Pt-A complexes were eluted but the amount of these complexes was lower when compared with *trans* Pt treated DNA.

Discussion

Since the discovery of antitumor activity of *cis* DDP a great number of papers have been published in order to explain the mechanism of its activity [1, 2]. It is believed that DNA is a primary target for Pt antitumor drug. Though that *trans*

DDP also interacts with DNA it is biologically inactive [1, 2]. It is of particular interest then to compare the interaction of both Pt isomers with DNA.

In the previous paper we described the system which allowed to separate different platinated complexes of nucleic acid bases [3]. This system was applied here to isolate the adducts resulting from the interaction of both Pt isomers with DNA. As the presence of chromosomal proteins in cell nuclei may modify interaction of Pt complexes with DNA target we also used this method to separate the reaction product of Pt complexes with chromatin. From the studies presented above following conclusions may be drawn:

1. In low r_i values ($r_i \leqq 0.1$) *cis* DDP was bound mostly to guanine moieties and the major product of the reaction was the bifunctional complex of guanine. It was associated with lowering of the peak in which unmodified guanine was eluted (Fig. 3). It is in a good agreement with Housier *et al.* [10]. Basing on the data from Tb^{+3} fluorescence they proposed that 55–60% of the Pt binding occurs through intrastrand cross-linking of guanines [10].

There is a little chance for GpG sequences to occur in calf thymus DNA [11] and therefore the bifunctional complexes must represent crosslinks between neighboring guanines as well as links between guanines separated by the third base. Crosslinks between the adjacent guanines have been suggested by Stone *et al.* [12, 13], on the basis of the experiments on the interaction of *cis* DDP with poly (dG) and poly (dC). Recently detailed NMR studies of the chelate formed upon reaction of d(GpG) have been completed [14–16]. From the same studies intrastrand crosslinks between two guanines separated by the third base appeared to be possible [14–16]. More definitive evidence for this type of binding modes comes from the experiments in which DNA was treated with *cis* DDP [17–19]. In the studies presented here the presence of bifunctional complexes of adenine was detected, as well.

These kinds of adducts were proposed previously basing on CD and NMR studies [20]. In addition to chelation *via* both N7 atoms binding to N1 was also observed for the small amount of the products [21]. It is in an excellent agreement with our experiments (Fig. 3).

2. Monofunctional adduct of guanine was the main product of the interaction of *trans* DDP with DNA. Bifunctional complex of this base was represented only in minute quantities. Two other bifunctional complexes of A-Pt-G and A(N7)-A(N7) were also present (Fig. 5). In the case of *trans* isomer where Cl atoms are 4.6 Å apart, the formation of intrastrand crosslinks between the adjacent bases in double helical DNA is unlikely. The bifunctional complexes should, therefore, represent interstrand crosslinks or intrastrand links between nonadjacent bases (the bases separated by the third base). The appearance of these links requires local denaturation of double helix. It was shown that *trans* isomer, alike *cis* DDP unwinds and shortens the DNA duplex [22, 23].

Shortening of DNA might be a result of intrastrand crosslinks [22]. Higher frequency of G-A and A-A bifunctional complexes observed in this study may be a result of greater unstability of A-T rich regions upon initial reaction of double stranded DNA with *trans* DDP [24]. When ssDNA reacted with the drug more bifunctional complexes appeared (Fig. 6) what pointed out that geometrical constrains prevent formation of the adducts.

3. It seems that *trans* isomer reacts more easily with chromosomal proteins than *cis* DDP does. Thus after the reaction of *trans* DDP with chromatin less platination occurs on DNA moieties.

Other studies also reported that DNA-protein and protein-protein crosslinks were evidenced in much higher proportion with the *trans* isomer [25, 26].

Summarizing, the differences reported in these studies concerning the occurrence of various platinated derivatives of nucleic acid bases after the *trans* and *cis* DDP reacted with DNA and chromatin, may be very helpful in explanation of various biological activities of these compounds.

Acknowledgements

The authors are indebted to Dr. L. S. Hnilica from Vanderbilt University for the generous gift of *trans* DDP. These investigations were partly supported by grant R.III.13.1.

[1] J. J. Roberts and A. J. Thompson, Prog. Nucl. Acid Res. Mol. Biol. **22,** 71–133 (1978).
[2] J. Drobnik, Can. Chem. Pharmacol. **10,** 145–149 (1983).
[3] R. Oliński and Z. Walter, Z. Naturforsch. **39c,** 1052–1056 (1984).
[4] S. Zamenhof, Biochem. Prep. **6,** 8–12 (1958).
[5] G. Ceriotti, J. Biol. Chem. **214,** 59–70 (1955).
[6] O. H. Lowry, N. J. Rosenbrough, A. L. Farr, and R. J. Randal, J. Biol. Chem. **193,** 265–275 (1951).
[7] J. A. Wilhelm, A. T. Anserin, A. W. Johnson, and L. S. Hnilica, Biochim. Biophys. Acta **272,** 220–230 (1972).
[8] T. C. Spelsberg and L. S. Hnilica, Biochim. Biophys. Acta **228,** 202–211 (1971).
[9] Z. Marczenko, Spectrophotometric determination of Elements, Ellis Horwood Chichester, 1976.
[10] C. Houssier, M. N. Macquet, and E. Fredericq, Biochim. Biophys. Acta **739,** 312–316 (1983).
[11] J. Josse, A. D. Kaiser, and A. Kornberg, J. Biol. Chem. **236,** 864–875 (1961).
[12] P. J. Stone, A. D. Kelman, F. N. Sinex, M. M. Bhargara, and H. O. Halvorson, J. Mol. Biol. **104,** 793–804 (1976).
[13] A. D. Kelman, H. J. Peresie, and P. J. Stone, J. Clin. Hematol. Oncol. **7,** 440–451 (1976).
[14] J. H. J. den Hartog, C. Altona, J. H. van Boorn, A. T. M. Marcelis, G. A. van der Marel, L. J. Rinkel, G. Wille-Hazeleger, and J. Reedijk, Eur. J. Biochem. **134,** 485–495 (1983).
[15] A. T. M. Marcelis, J. H. J. den Hartog, G. A. van der Marel, G. Wille, and J. Reedijk, Eur. J. Biochem. **135,** 343–349 (1983).
[16] A. T. M. Marcelis, J. H. J. den Hartog, and J. Reedijk, J. Am. Chem. Soc. **104,** 2664–2665 (1982).
[17] J. Brouwer, P. van de Putte, A. M. J. Fichtinger-Schepman, and J. Reedijk, Proc. Natl. Acad. Sci. USA **78,** 7010–7014 (1981).
[18] A. M. J. Fichtinger-Schepman, P. H. M. Lohman, and J. Reedijk, Nucleic Acid Res. **10,** 5345–5356 (1982).
[19] A. Eastman, Biochemistry **22,** 3927–3933 (1983).
[20] T. A. G. Roos, A. J. Thompson, and S. Mansy, J. Am. Chem. Soc. **96,** 6484–6487 (1974).
[21] J. C. Chottard, J. P. Girault, G. Chottard, J. Y. Lallemand, and D. Mansny, J. Am. Chem. Soc. **102,** 5565–5567 (1980).
[22] C. M. Markel and S. J. Lippard, Cold. Spring Harb. Symp. Quant. Biol. **XLVII,** 355–360 (1983).
[23] J. L. Butour and J. P. Macquet, Biochim. Biophys. Acta **653,** 305–315 (1981).
[24] R. C. Srivastava, J. Froehlich, and G. L. Eichhorn, Biochimie **60,** 879–890 (1978).
[25] L. A. Zwelling, T. Anderson, and K. W. Kohn, Cancer Res. **39,** 365–369 (1979).
[26] S. J. Lippard and J. D. Hoeschele, Proc. Natl. Acad. Sci. USA **76,** 6091–6095 (1979).