Determination of Platinum Binding Bases in Oligonucleotides. Application of Exonuclease Digestion Method

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The cytotoxicity of the antitumor complex, cis-dichlorodiammine Pt(II), (cis-DDP), is thought to be caused by the binding of cis-DDP with DNA. Studies on the interaction of cis-DDP with DNA and its constituents show a selective binding to the guanine base, whose N(7) is the kinetically favored site [1]. Recently, attention has been focused on intrastrand platinum binding between two adjacent guanine bases [2]. Such a platinum binding mode has been proposed on the basis of H-NMR studies of Pt-modified oligonucleotides [3]. We present direct evidence for such an intrastrand crosslinking.

In the present paper, enzymatic digestion using the snake venom phosphodiesterase (VPD) and calf spleen phosphodiesterase (SPD) was employed for the determination of platinum binding base in oligonucleotides. The products formed by the enzymatic digestion were separated and identified by means of HPLC. The reaction mixture of cis-DDP with self-complementary deoxyribotetranucleoside tetraphosphate, d[(pGpGpCpC)]$_2$ and [d(pCpCpGpG)]$_2$, was used as a substrate solution. Three hundred microliters of [d(pGpGpCpC)]$_2$ solution was allowed to react with 12 μl of 2 m*M* cis-DDP at 37 °C for 15 h in aqueous solution, and the reaction mixture was then treated with VPD [4].* It is known that VPD has relatively low substrate specificity and digests oligonucleotide exonucleolytically from the 3′-OH end. Figure 1-a shows the chromatogram of the reaction mixture prior to VPD digestion. The peak at t_R = 21.0 min (t_R retention time) is assignable to the

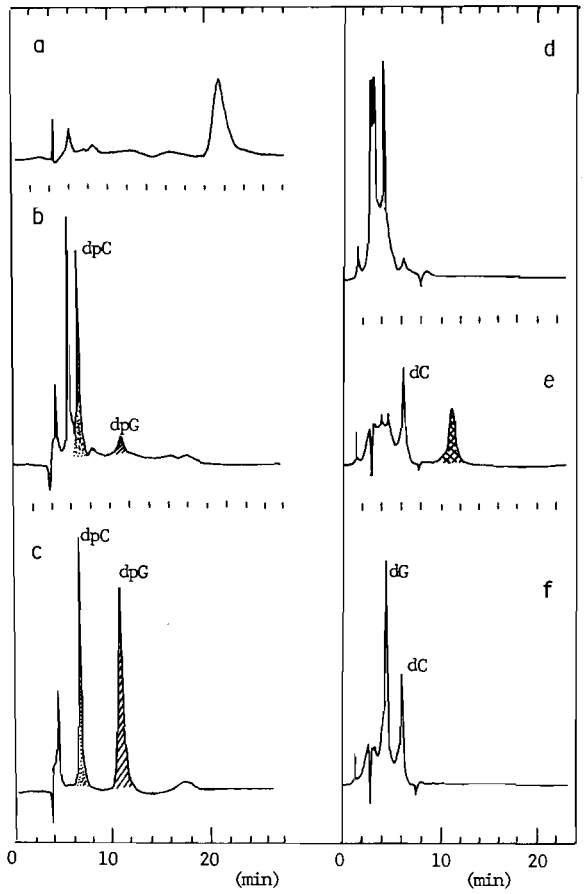

Fig. 1. High performance liquid chromatograms of the reaction mixture of cis-Pt$(NH_3)_2Cl_2$ with [d(pGpGpCpC)]$_2$ and its enzymatic digestion products. Experimental conditions of HPLC. a, b, and c: column, Partisil 10-SAX (0.4 × 30 cm); Detector, UV at 260 nm; eluant, 0.05 *M* KH_2PO_4 (1.0 ml/min). d, e, and f: TSK-Gel IEX 530 K (weak cation exchange column); Detector, UV at 260 nm; eluant, 0.05 *M* KH_2PO_4 (1.0 ml/min). a) Reaction mixture of cis-Pt$(NH_3)_2Cl_2$ with [d(pGpGpCpC)]$_2$; b) VPD digestion products of the reaction mixture; c) VPD digestion products of unplatinated [d(pGpGpCpC)]$_2$; d) VPD digestion products of the reaction mixture; e) VPD and alkaline phosphatase digestion products of the reaction mixture; f) VPD and alkaline phosphatase digestion products of unplatinated [d(pGpGpCpC)]$_2$.

platinated d(pGpGpCpC) because unplatinated [d(pGpGpCpC)]$_2$ was not eluted under the conditions used. The peak area accounted for approximately 80% of the total optical density at 260 nm. VPD digestion of the reaction mixture was allowed until disappearance of the peak of t_R = 21.0 min and appearance of new peaks at t_R = 5.7, 6.7, and 11.2 min (see Fig. 1-b). The peak at t_R = 6.7 and 11.2 min was assigned to deoxycytidine-5′-monophosphate (dpC) and deoxyguanosine-5′-monophosphate (dpG), respectively, by comparison with the standard

*A 5.0 $A_{260\ nm}$ unit of [d(pGpGpCpC)]$_2$ was dissolved in 1.75 ml of distilled deionized water. The molar extinction coefficient of [d(pGpGpCpC)]$_2$ is thought to be approximately 28500 [5, 6]. The molar ratio of Pt/base is about 0.2 under the conditions used. The concentration of [d(pGpGpCpC)]$_2$ was also verified by quantitative analysis of the VPD digestion products. VPD digestion was performed in a buffer that contained 0.1 *M* Tris-HCl, pH 8.9, 1 m*M* $MgCl_2$, 1 unit of VPD (Worthington Co. LTD) in a volume of 100 μl of the reaction mixture at 37 °C for 30 min. The VPD digestion goes essentially to completion within 20 min.

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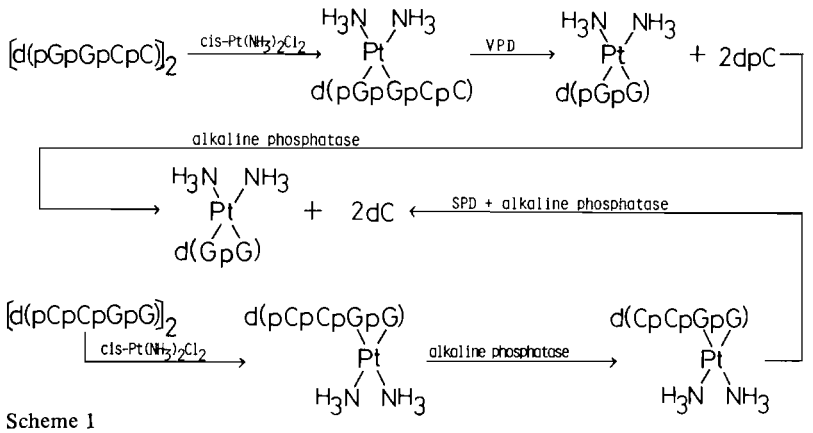

Scheme 1

materials. It is noteworthy that the peak of dpG is markedly small when compared with that of dpC. Figure 1-c shows the chromatogram of the VPD digestion products of the unplatinated $[d(pGpGpCpC)]_2$. The peak area of dpC in Fig. 1-b was approximately 90% of the corresponding one in Fig. 1-c, suggesting that the cytosine base in $[d(pGpGpCpC)]_2$ does not participate in binding with the platinum atom. On the contrary, the peak area of dpG is less than 10% of the corresponding one in Fig. 1-c. This strongly suggests that cis-DDP selectively binds to the guanine bases in $[d(pGpGpCpC)]_2$. This is also supported by the fact that the peak at t_R = 5.7 min in Fig. 1-b is identical with that of cis-$Pt(NH_3)_2$(d(pGpG)) in the retention time, in which cis-$Pt(NH_3)_2$(d(pGpG)) was formed from the reaction of cis-DDP with d(pGpG).* Chottard *et al.* have reported that the reaction of d(pGpG) with $[cis\text{-}Pt(NH_3)_2(OH_2)_2](NO_3)_2$ gave a single monomeric product having intrastrand crosslinking between adjacent guanines by the cis-$Pt(NH_3)_2^{2+}$ moiety [5]. In order to gain further evidence, the VPD digestion products of the platinated d(pGpGpCpC) were treated with alkaline phosphatase. Figures 1-d and -e show the chromatograms of the VPD digestion products and their hydrolytic products by alkaline phosphatase. The peak at t_R = 4.0 min in Fig. 1-d, assigned to cis-$Pt(NH_3)_2$(d(pGpG)), disappears after treatment with alkaline phosphatase, and a new peak appears at t_R = 11.4 min (Fig. 1-e). This peak was identical with that of cis-$Pt(NH_3)_2$(d(GpG)) in the retention time, in which cis-$Pt(NH_3)_2$(d(GpG)) is the complex with an intrastrand crosslink between two guanine bases through N(7)–N(7) sites [6].** These results unequivocally indicate that cis-$Pt(NH_3)_2^{2+}$ is chelated to the N(7) sites of the adjacent guanine bases in d(pGpGpCpC). The peak height of mononucleosides, produced by treatment with VPD and alkaline phosphatase, also supports this conclusion (Figs. 1-e and -f).

In order to determine the effect of the terminal phosphate on the complex formation reaction, cis-DDP was allowed to react with $[d(pCpCpGpG)]_2$† and the resulting reaction mixture was treated with alkaline phosphatase and SPD.†† It gave cis-$Pt(NH_3)_2$(d(GpG)) and dC as the digestion products, and the chromatogram obtained eventually was identical with that of Fig. 1-e. This means that cis-DDP also shows selective binding to the adjacent guanine bases in $[d(pCpCpGpG)]_2$. The terminal phosphate does not seem to affect selective platinum binding to the adjacent guanine bases. The results are summarized in Scheme 1.

In conclusion, cis-DDP reacts with $[d(pGpGpCpC)]_2$ and $[d(pCpCpGpG)]_2$ to form a complex with an intrastrand crosslink between the adjacent guanine bases through N(7)–N(7) sites, and the reaction is extremely selective. VPD, SPD and alkaline phosphatase are very useful in order to prove the binding of cis-DDP with oligonucleotide. The method described has the following advantages: (1) the experimental procedure is very simple, (2) the experiment can be run using a minute amount of sample, and (3) quantitative treatment is possible.

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*The reaction solution of cis-DDP with d(pGpG) was measured by HPLC.

**We have isolated cis-$Pt(NH_3)_2$(d(GpG)) from the reaction solution of cis-DDP with d(GpG). The platinum binding at N(7)–N(7) of the guanine bases has been confirmed by the pH dependence of N(1) proton, using UV difference spectral analysis and atomic absorption spectroscopy [7].

†Experimental conditions are the same as in ref. 4.

††To a volume of 100 μl of the reaction mixture, 10 μl of 1.0 *M* Tris-HCl, pH 8.9 and 10 units of alkaline phosphatase (Boehringer Mannheim, Grade 1), were added, and it was then incubated at 37 °C for 30 min. After adjusting to pH 6 by adding acetic acid, 10 μl of SPD (Boehringer Mannheim) was added and it was then incubated at 37 °C for 2 days. SPD is an enzyme that degrades oligonucleotides exonucleolytically from the 5′-OH end.

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