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The Chemical and Biological Effects of *cis*-Dichlorodiammineplatinum (II), an Antitumor Agent, on DNA

[*trans*-dichlorodiammineplatinum (II)/DNA crosslinking/transformation]

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ABSTRACT ***cis*-Dichlorodiammineplatinum (II) binds irreversibly to the bases in DNA; the amount of platinum complex bound can be determined from changes in the ultraviolet absorption spectrum. As the ratio of platinum to phosphate is increased, an increasing inactivation of bacterial transforming DNA is observed. At a ratio that corresponds to spectrometric saturation, transforming activity is inactivated $>10^5$-fold. The *trans* isomer of the platinum complex, which is not effective against tumors, induces a similar inactivation of transforming DNA but with half the efficiency, indicating a different mode of binding. The sensitivity to inactivation by *cis* isomer varies slightly with the genetic marker assayed but is not dependent on the excision repair system. Uptake of DNA by competent cells is unaffected by bound platinum complex; however, integration of platinum-bound transforming DNA into the host genome decreases as the mole fraction of platinum increases. This loss of integration parallels the decreased transforming activity of the DNA. Although the drug induces interstrand crosslinks in DNA *in vitro*, these crosslinks are relatively rare events and cannot account for the observed inactivation.**

cis-Dichlorodiammineplatinum (II) (*cis*-DDP), *cis*-$PtCl_2(NH_3)_2$, was the first inorganic antitumor drug submitted for clinical trials (1, 2). Since the simplicity of molecular geometry and functional groups allows a vast number of possible derivatives, this square planar platinum complex may represent an entire series of pharmacologically active compounds. In spite of extensive investigation, however, the biological mode of action of platinum drugs is uncertain. In addition to causing regression of certain tumors, *cis*-$PtCl_2(NH_3)_2$ has been reported to cause filamentous growth in *Escherichia coli* (3), induce prophage from lysogenic strains of bacteria (4), selectively inhibit DNA synthesis in human amnion AV_3 cells (5), and induce interstrand crosslinks in the DNA of treated HeLa cells (6). Early studies indicated that those complexes which induced filamentous growth actually attacked and remained bound to the DNA (7). Recently evidence has been presented for a direct attack of the platinum on the bases in DNA (8, 9). Nevertheless, the exact effect of bound platinum complex on DNA has not been defined. The following experiments assess the effect of bound *cis*-$PtCl_2(NH_3)_2$ on the biological activity of DNA and attempt to relate the biological effect to chemical changes in the DNA.

One initial problem is that observable phenomena arising from treatment of cells with platinum complex may be the result of reaction of the complex with some cellular component other than DNA. For certain bacteria this problem can be overcome by treating DNA *in vitro* and returning it to the cell. Thus, the biological activity of platinum-bound DNA has been studied by using it for transformation of *Haemophilus influenzae*.

MATERIALS AND METHODS

Preparation and Treatment of DNA. Calf thymus DNA was obtained from Worthington Biochemicals. DNA from *Escherichia coli*, *Micrococcus luteus*, and *H. influenzae* (containing genetic markers conferring resistance to 200 μg/ml of streptomycin and 25 μg/ml of novobiocin) was purified according to the procedure of Marmur (10). For ^{32}P or ^{3}H labeling of *H. influenzae* DNA, cells were grown in MI_c-cit medium (11) supplemented with 0.01 μCi/ml of $^{32}PO_4$ or 12μ Ci/ml of [^{3}H]-dThd. ^{32}P-labeled DNA was isolated as previously described (12).

DNA was diluted in 0.01 M phosphate buffer, pH 7, and the molar concentration was determined spectroscopically. An aliquot of *cis*-$PtCl_2(NH_3)_2$ dissolved to 1 mg/ml in 50 mM NaCl was added, and the mixture was incubated at about 40° in the absence of light for 2 days, or until no further change could be detected in the absorption spectrum. The extent of reaction was determined by scanning the spectrum from 360 to 210 nm on a dual-beam Beckman DK-1A recording spectrophotometer.

Transformation. The biological activity of transforming DNA was assayed on two strains of *H. influenzae:* strain Rd, wild type; and Rd(DB112)UV^s, a transformant of strain Rd which contains the *uvrI* mutation and lacks activity of the specific UV endonuclease (13). Cells were made competent, and transformation was measured according to the procedure of Spencer and Herriott (14).

DNA Uptake and Integration. Uptake was assayed by methods previously described (15). Integration was measured by the method of Muhammed and Setlow (16), which is based on the finding that labeled competent cells release DNA fragments into the medium equivalent to the amount of transforming DNA integrated (17).

Crosslinks. ^{3}H-Labeled DNA was treated with *cis*-$PtCl_2(NH_3)_2$, heat denatured, and centrifuged in CsCl as previously described (12). The fraction of radioactivity corresponding to denatured DNA was taken as a measure of the fraction of single-stranded DNA in the preparation.

The denatured DNA was also digested to completion with SI endonuclease from *Aspergillus oryzae*, which is specific for

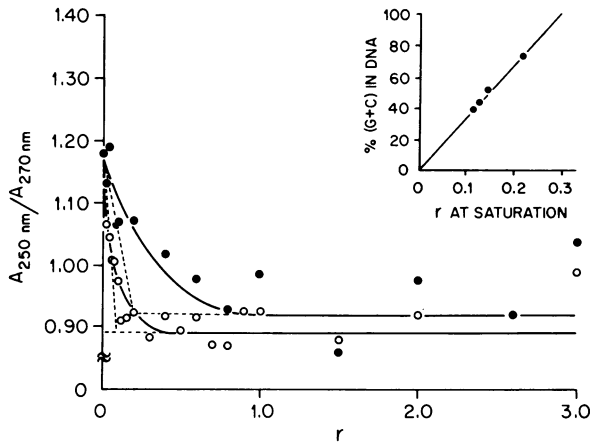

FIG. 1. Spectrometric titrations of *H. influenzae* DNA: *cis*-$PtCl_2(NH_3)_2$ (O), *trans*-$PtCl_2(NH_3)_2$ (●). Similar curves were obtained for titrations of *E. coli*, *M. luteus*, and calf thymus DNAs with *cis*-$PtCl_2(NH_3)_2$. Insert: Relationship between G+C content of DNA (23, 24) and *r* at saturation with *cis*-$PtCl_2(NH_3)_2$.

single-stranded regions in DNA (18) and thus can provide another measure of the fraction of single-stranded DNA. Enzyme was prepared and reacted according to the method of Sutton (19), except that the NaCl concentration in the reaction mix was 0.2 M.

RESULTS

Spectrophotometric Titration. The absorption maximum of DNA is shifted to longer wavelength by reaction with platinum compounds. The ratio of the absorbance at 250 nm relative to that at 270 nm for DNA samples treated with *cis*- and *trans*-$PtCl_2(NH_3)_2$ is plotted against *r*, the mole ratio of added platinum complex to DNA phosphate, as shown in Fig. 1. As the concentration of platinum complex is increased, the curves level off and can be resolved into the sum of two straight lines, as indicated by the dashed lines in Fig. 1. The value of *r* necessary to saturate the available binding sites was taken as the intersection of these lines. For example, the binding capacity of *H. influenzae* DNA for *cis*-$PtCl_2(NH_3)_2$ is 12 platinum complexes per 100 nucleotides or 50 base pairs. Although the shapes of the curves obtained for titrations with the *cis* and *trans* isomers are similar, the decrease in the ratio of absorbances is less rapid for the *trans* isomer. The binding capacity of *H. influenzae* DNA for the *trans* isomer is 22 platinum complexes per 100 nucleotides. Thus, at saturation nearly twice as much *trans*-$PtCl_2(NH_3)_2$ is bound as *cis*-$PtCl_2(NH_3)_2$. One possible explanation of this phenomenon resides in hydrolysis data, which indicate that while both chloride ligands are removed during aquation of *cis*-$PtCl_2(NH_3)_2$, only one chloride is removed during aquation of *trans*-$PtCl_2(NH_3)_2$ (20). On the basis of these data, one might then predict monodentate binding of the *trans* isomer as opposed to bidentate binding of the *cis* isomer.

Evidence has been provided by Mansy and others (8, 9, 21) that *cis*-$PtCl_2(NH_3)_2$ binds to adenosine, cytidine, and guanosine but not to either thymidine or uridine. In addition, Sinex and Stone (22) have reported a hypochromic shift and possible helix compression for *M. luteus* DNA when titrated with *cis*-$PtCl_2(NH_3)_2$. This helix compression is attributed to enhanced interstrand crosslinking due to the high probability of GpC base sequences. Thus, an attempt was made to correlate the

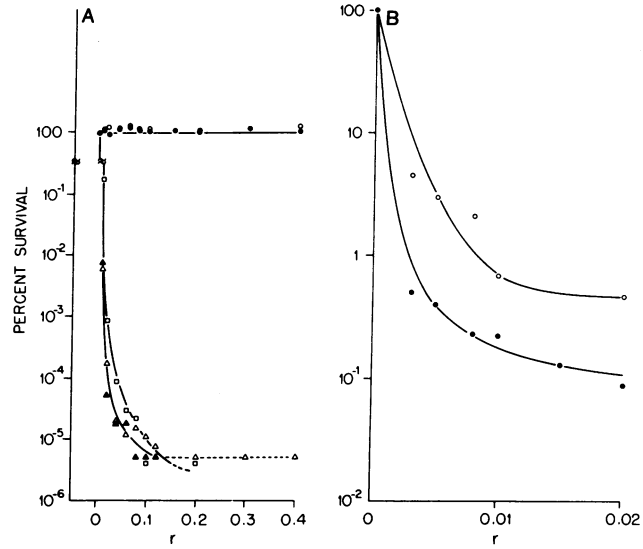

FIG. 2. Inactivation of transforming DNA as a function of mole ratio $PtCl_2(NH_3)_2$ to DNA phosphate. (A) Transformation to streptomycin resistance (200 μg/ml) of Rd (△) and *uvr1* (▲) strains and total number of cells plated without antibiotic (●) using *cis*-$PtCl_2(NH_3)_2$-treated DNA. Similar curves were obtained for transformation to novobiocin resistance for Rd using *cis*-$PtCl_2(NH_3)_2$-treated DNA at these doses. The plateau beyond $r = 0.12$ represents spontaneous mutation to antibiotic. Transformation to streptomycin resistance of Rd (□) and total number of cells plated without antibiotic (O) using *trans*-$PtCl_2(NH_3)_2$-treated DNA. (B) Transformation to two different levels of novobiocin resistance for Rd cells at low doses of *cis*-$PtCl_2(NH_3)_2$: N25, (●); N2.5, (O). Note: Scale of x-axis is not the same as in Fig. 2A.

number of platinum molecules bound with the G+C content of various DNAs. The empirical saturation points for four different DNAs (Insert, Fig. 1) define a linear plot, indicating that *r* at saturation with *cis*-$PtCl_2(NH_3)_2$ increases with G+C content. This result is consistent with the assumption that there is little or no binding of *cis*-$PtCl_2(NH_3)_2$ to thymine residues in DNA. A maximal value of three platinum complexes per 10 nucleotides is suggested by extrapolation to a G+C content of 100%. The saturating concentration of *cis*-$PtCl_2(NH_3)_2$ can now be predicted for other DNAs by the use of this plot.

Biological Inactivation. Fig. 2A shows the percent of transformants and total viable cells in a population of competent wild-type or *uvr1* cells after treatment with platinum-bound DNA. Since the viability in the absence of antibiotic is unaffected both at low *r* values where all the added platinum compound is bound to DNA and at high *r* values where there are both bound and free platinum complexes, the free platinum complex does not affect cell survival at these doses, e.g., up to three times the amount bound. Transformation, however, decreases dramatically for both wild-type and *uvr1* recipients as a function of concentration of either isomer. As *r* approaches the saturation value, the number of detectable antibiotic-resistant cells approaches the level of spontaneous mutation to antibiotic resistance. Thus, no significant biological activity is observed for DNA that has been saturated with either *cis*- or *trans*-$PtCl_2(NH_3)_2$. The biologically inactivating dose can be approximated from these curves as the mole ratio of complex that results in 37% survival or one lethal event per piece of

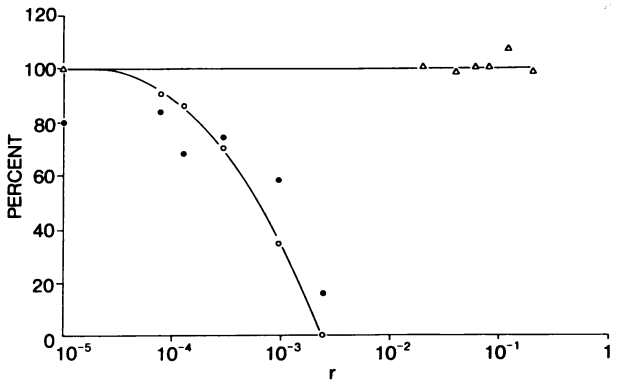

FIG. 3. Uptake and integration of *cis*-$PtCl_2(NH_3)_2$-treated transforming DNA by competent Rd cells. Uptake of ^{32}P-labeled DNA (△) and transformation to streptomycin resistance (○) and integration (●) of nonlabeled DNA as a function of *r*. Transformation to streptomycin resistance for the ^{32}P-labeled DNA corresponded to Fig. 2.

transforming DNA (25): e.g., for *cis*-$PtCl_2(NH_3)_2$, $r = 0.001$; for *trans*-$PtCl_2(NH_3)_2$, $r = 0.002$. These values indicate that both isomers are lethal at 1% of saturation.

The effect of *cis*-$PtCl_2(NH_3)_2$ on transforming ability at very low *r* values is shown in Fig. 2B. The marker conferring resistance to 25 μg/ml of novobiocin, N25, is a complex base sequence consisting of two lower level markers (26). It is nearly 10 times more sensitive to bound platinum complex early in the curve than the low level (2.5 μg/ml) marker, N2.5, assayed. This enhanced sensitivity of the complex marker is analogous to that observed for ultraviolet-damaged DNA (26). The sensitivity of Rd and *uvr1* to streptomycin resistance at these doses of complex is only about twice that shown for the low level novobiocin marker. Since transforming DNA does not appear more sensitive when assayed on *uvr1*, either transformation is inactivated prior to repair processes, or the UV endonuclease in which *uvr1* is deficient has no effect on the platinum-induced lesion.

Although both isomers of the platinum complex inactivate transforming DNA, only the *cis* isomer is useful as an antitumor drug. Therefore, the mechanism of inactivation was studied using *cis*-$PtCl_2(NH_3)_2$ as the model.

Uptake and Integration of Platinum-Bound DNA. Fig. 3 shows that whereas uptake into competent cells is unaffected by bound platinum complex at *r* values that produced considerable inactivation, integration is significantly affected even when there is only 1 *cis*-$PtCl_2(NH_3)_2$ molecule per 1000 nucleotides. The loss of integration appears to parallel the loss of transforming ability.

The Primary Interaction. There are two possibilities for the primary interaction between *cis*-$PtCl_2(NH_3)_2$ and DNA. It is usually assumed that the conversion of the compound to *cis*-diaquodiammineplatinum (II) is the initial step in the reaction sequence and that the diaquo form of the complex is the reactive intermediate (8). Ligand exchange, however, is a well-known reaction of transition metals. Thus, the possibility exists that the bases in DNA react in a nucleophilic fashion with the platinum complex, driving in and displacing the chloride ligand. If DNA displaces the chloride ligand, formation of the diaquo complex would not be essential to

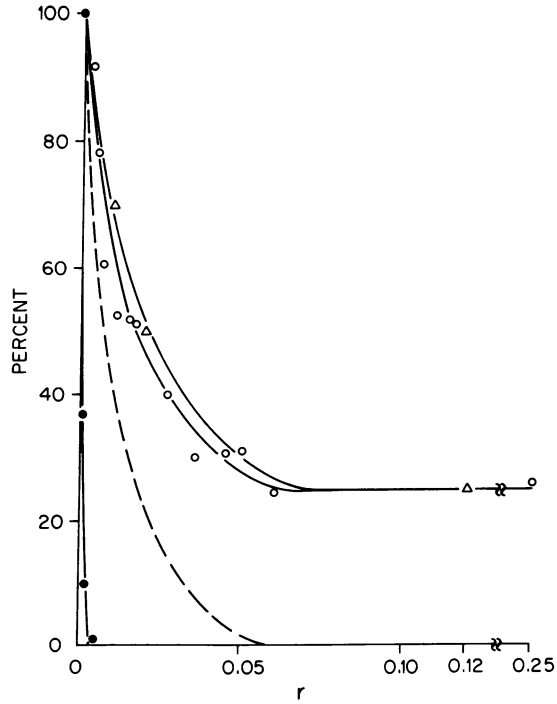

FIG. 4. *cis*-$PtCl_2(NH_3)_2$-induced crosslinks. Transformation of Rd cells to streptomycin resistance (●) and percent single-stranded DNA after heat denaturation as a function of *r* assayed by equilibrium sedimentation (△) and SI endonuclease digestion (○). The broken line represents the average percent single-stranded DNA with the plateau level (25%) subtracted.

reaction. Hydrolysis of *cis*-$PtCl_2(NH_3)_2$ to the diaquo form has been shown to be inhibited by addition of chloride ions to the solution (22, 27). When DNA was treated with *cis*-$PtCl_2(NH_3)_2$ in 0.015 M NaCl for over a week, no inactivation of transforming ability was observed. Therefore, DNA does not displace the chloride ligand but reacts instead with the hydrated divalent platinum ion. Further, since inactivation is not observed in the absence of binding, one may conclude that free platinum complex does not affect the transforming ability of DNA.

Interstrand Crosslinks. Originally the pharmacological activity of *cis*-$PtCl_2(NH_3)_2$ was compared to that of the classical bifunctional alkylating agents and was assumed to arise from formation of interstrand crosslinks in DNA (6, 28). This assumption was supported by the fact that although *cis*-$PtCl_2(NH_3)_2$ can induce crosslinks both *in vitro* and *in vivo*, *trans*-$PtCl_2(NH_3)_2$, which is not biologically active as an antitumor agent, induces crosslinks only *in vitro* (6). Recent studies, however, indicate that crosslinks in DNA are not the inactivating lesion for bacteriophage (29).

Platinum-induced interstrand crosslinks cause rapid renaturation after denaturation of the DNA (30). Use has been made of this phenomen on for assay of crosslinks. Fig. 4 shows the biological inactivation and crosslinking induced in DNA by treatment with *cis*-$PtCl_2(NH_3)_2$. The amount of single-stranded DNA after denaturation of the treated DNA was estimated by equilibrium centrifugation and by SI nuclease degradation. Without pretreatment with *cis*-$PtCl_2(NH_3)_2$, about 95% of the denatured DNA was degraded by the enzyme, whereas there was no detectable degradation of untreated native DNA. At saturating *r* values, the fraction of single-stranded DNA

remaining, measured by either equilibrium sedimentation or enzymatic digestion, levels off at 25%. The inability to obtain complete renaturation may reflect a loosened helical structure for the saturated DNA due to inhibition of base pairing by bound platinum molecules. Some support for this hypothesis comes from the fact that undenatured platinum-saturated DNA becomes degradable with SI nuclease, suggesting that the bound complex results in local denatured regions.

In spite of the uncertainties in interpretation of the enzymatic digestion and sedimentation data of Fig. 4, an estimate may be made of the r value (0.012) corresponding to an average of one crosslink per molecule (37% single-stranded DNA) by subtracting the 25% residual single-strand character, as shown by the dashed line, to obtain a plot of the fraction of DNA that was crosslinked. Thus, whereas one platinum per 1000 bases was the biologically inactivating dose for *cis*-$PtCl_2(NH_3)_2$, a crosslink is formed when there are 12 platinum molecules per 1000 bases. It is concluded that interstrand crosslinks are not responsible for most of the biological inactivation observed. Sedimentation of platinum-bound DNA in 5–20% alkaline sucrose gradients in this laboratory provided no evidence for single-strand breaks.

DISCUSSION

These data indicate that the biological inactivation of transforming DNA by bound platinum (II) complexes results from interference with integration of the DNA into the recipient genome, even when the bound platinum molecules are separated by as many as 500 base pairs. The inactivating lesions are not interstrand crosslinks and are apparently not exclusively bidentate interactions. The data suggest that pairing of transforming DNA and recipient DNA may be depressed by local perturbations in donor DNA 3×10^5 daltons apart.

Several lines of evidence support the hypothesis that DNA is the most important cellular target for platinum (II) drugs (5, 29). Since both *cis*- and *trans*-$PtCl_2(NH_3)_2$ inactivate transforming DNA at low doses, it is not clear why the *cis* isomer alone has antitumor activity. It is known that the *trans* isomer hydrolyzes more rapidly (8) and that the hydrolysis product is the reactive intermediate (30). Additionally, our experiments indicate that there may be a different mode of binding for the *trans* isomer. It is possible, therefore, that for the *trans* isomer either the major lethal lesion differs from that induced by the *cis* isomer or that cell death is the result of reaction with some cellular component other than DNA.

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