Induction and Repair of DNA Cross-Links in Chinese Hamster Ovary Cells Treated with Various Platinum Coordination Compounds in Relation to Platinum Binding to DNA, Cytotoxicity, Mutagenicity, and Antitumor Activity

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ABSTRACT

Several effects of four diamminechloroplatinum compounds (II and IV) in Chinese hamster ovary cells were studied. The two cis-compounds [cis-diamminedichloroplatinum(II) and cis-diamminetetrachloroplatinum(IV)] are known to possess antitumor activity, whereas the two trans-stereoisomers [trans-diamminedichloroplatinum(II) and trans-diamminetetrachloroplatinum(IV)] are inactive.

When the effects of the cis- and trans-platinum compounds were compared after treatments that resulted in the binding of equal amounts of platinum to the DNA of the cells, the following differences were found: (a) the cis-platinum adducts gave a much higher cytotoxicity; (b) only the cis-platinum-DNA complexes were strongly mutagenic (forward mutations at the hypoxanthine-guanine phosphoribosyltransferase locus); (c) the cis-platinum adducts induced more sister chromatid exchanges; (d) the cis compounds initially induced fewer DNA-protein cross-links (Factors 5 to 8), but these cis-platinum cross-links were much more persistent; (e) for both cis complexes, the amount of DNA interstrand cross-links passed through a maximum between 6 and 12 hr after treatment, and the cross-links were repaired slowly. One trans-compound [trans-diamminetetrachloroplatinum(IV)] resembled the cis complexes with respect to the overall kinetics of formation and disappearance of this type of lesion, but the repair went faster. For the other trans compound [trans-diamminedichloroplatinum(II)], the highest number of cross-links was detected directly after the treatment of the cells, and they were rapidly eliminated.

Neither the number of platinum-DNA lesions as such nor the initial amount of DNA interstrand cross-links could be related to the (geno)toxic effects of the compounds. However, as the slow repair of the cis-platinum-induced interstrand and DNA-protein cross-links leads to a certain persistency of these adducts, the unrepairled lesions might be responsible for cytotoxicity, mutagenicity, and antitumor activity. This indicates discriminating properties of the repair systems for certain cis- or trans-platinum-DNA adducts. The sister chromatid exchange induction seems to be related to the persistent DNA interstrand cross-links.

INTRODUCTION

cis\(^3\)\(^\dagger\) has been found to be superior to other available antitumor drugs for the treatment of some types of cancer (Ref. 27, 2, Clinical Studies). The stereoisomer of this coordination complex, tll, does not show comparable cytostatic properties. In clinical chemotherapy, cll shows severe side effects (27); this has stimulated the search for more selective congeners. Specific attention has been paid to the elucidation of the working mechanism of cll at the molecular level (27, 30). For review of the literature on cll and related platinum compounds, see the studies of Roberts and Thomson (30) and Prestayko et al. (27) and their cited literature.

Upon entering the cell, the platinum compounds will become hydrolyzed (13), and then they can react with DNA as well as with RNA and proteins. DNA, however, is believed to be the principal site of action (21). Within DNA, the main targets are the nucleobases, especially guanine (18). Only at high exposure levels are adducts of adenine and cytosine formed with Pt but, so far, no complexes with thymine have been detected (20).

Two types of platinum DNA binding have been found: (a) monofunctional; and (b) bifunctional. The monofunctional binding is unlikely to be responsible for the observed cytostatic effects, since the trans compounds are at least as effective as the cis compounds in forming monofunctional Pt-DNA adducts (21).

Bifunctional binding results in chelation and various kinds of cross-linking, presumably by a 2-step reaction of which the last step is rate limiting (17). The formation in vitro of a chelate with one guanine base by binding of cll at the N(7)-O(6) positions has been reported (6), but there are no reports of chelation in vivo.

In mammalian cells, the platinum compounds can form DNA-protein cross-links (39), of which the biological importance is not yet known. Intrastrand cross-links are the major type of bifunctional lesions induced by cll in DNA in vitro (9). They have been detected between 2 adjacent guanines (9), 2 adjacent adenines, adjacent adenosine and cytosine (31), and between 2 guanines separated by one (4, 17) or more bases (1). Also, in Escherichia coli, intrastrand cross-links have been found; there, they can lead to base-pair substitution (4). In mammalian cells, DNA intrastrand cross-links are, therefore, likely to be induced. These lesions might be responsible for antitumor activity, since they are not formed, at least not in vitro, by the noncytostatic trans compounds (9, 32). Interstrand DNA cross-links are formed by both cis and trans compounds, in vivo as well as in vitro (39). In mammalian cells, the cis compounds show a delayed formation of these cross-links, while the trans-DNA-interstrand cross-links are almost immediately formed (40, 41). For some other cytostatic agents, e.g., for cyclophosphamides (10), the formation of

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Received July 20, 1983; accepted December 27, 1983.
\(1\) Supported by the Queen Wilhelmina Fund, the Netherlands, Project MBL 79-1.

\(2\) The abbreviations used are: cll, cis-diamminedichloroplatinum(II); cIV, cis-diamminetetrachloroplatinum(IV); tll, trans-diamminedichloroplatinum(II); tIV, trans-diamminetetrachloroplatinum(IV); CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; SCE, sister chromatid exchange; dThd, thymidine; Pt-DNA adduct, platinum-DNA adduct.
interstrand cross-links is closely related to the cytotoxicity but, in the case of the platinum compounds, a clear relationship has not been demonstrated (24, 39).

In the present study, an attempt is made to relate the effects of different platinum compounds to the lesions they form in CHO cells. In particular, the relation between the formation and repair of DNA interstrand and DNA-protein cross-links and the induced genotoxic and cytotoxic effects is investigated. For this purpose, 4 platinum compounds (cii, cIV, tl, and tIV), which are known to react differently with DNA in vitro (34) and in Salmonella typhimurium (5, 19), have been studied. Previously, we reported (2, 25, 26) some other effects of these platinum compounds in CHO cells: (a) induction of micronuclei and unscheduled DNA synthesis; (b) the inhibition of DNA synthesis; and (c) the absence of induction of single-strand breaks.

**MATERIALS AND METHODS**

**Compounds.** The compounds used were cII, cIV, tII, and tIV. The platinum compounds were synthesized and kindly provided by the group of Professor Dr. J. Reedijk (State University, Leiden, Netherlands). Solutions of the compounds were freshly prepared in dimethylsulfoxide. Dilutions were made in Ham’s F-10 medium (Flow Laboratories, Cambridge, MA) supplemented with 7.5% Newborn calf serum (Flow), with final concentration of dimethyl sulfoxide <1%. Treatment of the cells was performed in the dark for 1 hr at 37° in a humidified atmosphere containing 5% CO2.

**Cell Cultures.** Monolayers of CHO cells were grown in flasks (75 sq cm; Costar, Cambridge, MA) in Ham’s F-10 medium, supplemented with 15% newborn calf serum, 1 mM l-glutamine (BDH, Poole, United Kingdom), penicillin (100 units/ml; Gist-Brocades NV, Delft, Netherlands), and streptomycin (100 µg/ml; Gist-Brocades, NV) at 37° in a 5% CO2 incubator (Heraeus, Hanau, Federal Republic of Germany). The cultures were handled in a laminar downflow system (Microflow Pathfinder, Ltd.; Fleet, United Kingdom) under yellow light (filtered T. L. light; wavelength, >525 nm; Philips, Eindhoven, Netherlands) and checked periodically for possible Mycoplasma contamination (23).

**Cell Survival.** Cells were trypsinized, counted in a hemocytometer (AI 134; Analysis Instruments, AB, Stockholm, Sweden), serially diluted, and subcultured in 6-cm Petri dishes (Greiner GmbH and Co., KG, Nörtingen, Federal Republic of Germany) at a density of 500 to 10,000 cells/plate. At 4 hr after seeding of the cells, the medium was replaced by medium containing 7.5% newborn calf serum and Pt-compound at the desired concentration. After 1 hr at 37°, the platinum-containing medium was removed, and the cells were washed twice with PBS (8.1 mM Na2HPO4, 1.5 mM KH2PO4, 0.14 mM NaCl, and 2.6 mM KCl), and fresh medium was added. After 7 days of culturing, the cells were stained with 1% methylene blue, and the colony-forming ability of the treated cells was determined.

**Platinum Determinations.** DNA was isolated according to the method of Kirby and Cooke (14) and dissolved in 0.01 N phosphate buffer, pH 7.4. The DNA concentration was measured spectrophotometrically (E = 6600/m, and the amount of platinum in the DNA samples was determined with a Perkin-Elmer atomic absorption spectrophotometer, Model 4000, equipped with a HGA 300 graphite furnace and an AS-40 automatic sampler. K3PtCl4 solutions, supplemented with heat-denatured salmon sperm DNA (500 µg/ml; Millipore Corp., Freehold, NJ), were used for calibration.

**Induction of SCEs.** CHO cells (7.105) were seeded in 50 sq cm tissue culture flasks. At 4 hr after seeding, cells were exposed for 1 hr to medium containing the compound and were then washed with PBS. Subsequently, the cells were allowed to grow for 10 days in medium without hypoxanthine (cells were subcultured every 2 to 3 days during this period). Mutants resistant to 6-thioguanine (Sigma Chemical Co., St. Louis, MO), at a concentration of 5 µg/ml, were selected by plating 106 cells (ten 9-cm Petri dishes) in medium without hypoxanthine. At the same time, the plating efficiency of the cells was determined by plating 500 cells in normal medium. After 7 days of culturing, the colonies were stained and counted.

**Induction of SCEs.** CHO cells (7.105) were seeded in 50 sq cm tissue culture flasks. At 4 hr after seeding, they were treated with the platinum compound for 1 hr, followed by a 24-hr incubation in the dark in medium containing 5-bromodeoxyuridine (5 µg/ml; Sigma). At 2 hr before collecting of the cells, colchicine (final concentration, 1 µg/ml; Sigma) was added. Cells were processed as air-dried preparations and were stained differentially with Hoechst 33258 (Riedel-de Haen AG, Hannover, Federal Republic of Germany) and Giemsa (Hopkins and Williams R66 solution; BDH, Poole, United Kingdom). In each preparation, 25 to 50 nuclei (haploid chromosome number is 21) were scored for SCEs; these were expressed per chromosome.

**Measurements of DNA Interstrand Cross-Links.** The alkaline elution method of Kohn et al. (15), as modified by van der Schans et al. (37), was adapted to allow measurement of DNA cross-links as follows. CHO cells (106) were seeded in 6-cm Petri dishes. After 6 hr, 0.1 ml of [14C]-dThd (0.25 µC/ml; specific activity, 56.5 Ci/mol; Amersham, Buckinghamshire, United Kingdom) was added to the control cells, and 0.1 ml [3H]-dThd (2.0 Ci/ml; specific activity, 24 Ci/mmol; Amersham) was added to the cells to be treated. Incubation was continued for 16 hr at 37°. After this labeling, the cells were washed twice with PBS. The control cells were given fresh medium and incubated further, while the [3H]dThd-labeled cells were treated for 1 hr with the platinum compound. Thereafter, the cells were washed, given fresh medium, and "postincubated" for the desired time. Subsequently, the cells were placed on ice, medium was removed, and 1 ml of ice-cold PBS was added. At 0.5 to 1 hr later, the cells were irradiated with 6 Gy of X-rays (Philips MG321, 300 kV, 10 mA, 1.5 mm copper filter, 4.5 Gy/min; Hamburg, Federal Republic of Germany) or with 9 Gy of 60Co- y-rays (Gammacell 200; dose rate, 1.1 Gy/sec; Atomic Energy of Canada, Ltd., Canada) at 0°. Control and treated cells were irradiated simultaneously. After irradiation, the cells were kept at 0° and scraped off from the Petri dishes with a rubber policeman. Control and treated cells were mixed. The combined cell suspension was layered on a polyvinyl chloride filter (25-mm diameter, Millipore) and alkaline elution was performed as described (37), with the exception that eleven 1.5-hr fractions (of 3.3 ml) were collected instead of fifteen 1-hr fractions (of 2.4 ml). The procedure includes a treatment with proteinase K (no. 24568; Merck, Darmstadt, Federal Republic of Germany) to remove proteins possibly cross-linked to DNA.

**Measurement of DNA Repair.** The alkaline elution pattern of the eluted DNA was analyzed by scintillation counting. The results were expressed as the relative amount of radioactivity still retained by the filter at the moment the fraction was collected. These percentages of DNA not eluted were plotted (on a logarithmic scale) as a function of elution volume. The amount of breaks (in arbitrary units) was calculated from the averaged slope over the first 5 fractions of the elution pattern of 14C]-dThd-labeled irradiated control cells, from which the slopes of the unirradiated controls were subtracted (Chart 1). From the 54C]-elution pattern the amount of cross-links present in the DNA of the treated [3H]dThd-labeled cells was calculated according to the method described below. In this method, the value for C0--m must be known; this value is obtained from the experimentally determined elution profiles, in the way schematically shown on Chart 1. (m4 is defined as the molecular weight of single-strand DNA fragments passing through the filter at the 50% elution point of control DNA; see "Appendix.")

In correctly performed experiments, only a very small proportion of

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Calculation of the Number of DNA Interstrand Cross-Links. Although alkaline elution is a method to measure DNA single-strand breaks, it can be used to detect the presence of DNA interstrand cross-links by studying the effect of the induction of a known number of single-strand breaks by X- or γ-rays. When interstrand cross-links are present, interconnected single-stranded DNA fragments will not separate upon the alkaline denaturation, which results in a slower elution and consequently, in a seemingly smaller number of single-strand breaks. Ewig and Kohn (7, 8, 15) have described a method to calculate the number of DNA interstrand cross-links from alkaline elution profiles. This resulted in a curve (Chart A-1, Curve c), which was used for the calculation of the amount of cross-links from the elution pattern found for the irradiated, treated (cross-linked) cells, 3H-labeled. Then, the value of x/p was obtained by fitting this curve to the following equation, which relates the amount of cross-links found after application of proteinase K (DNA interstrand cross-links), the amount of γ-ray-induced breaks masked by DNA-protein cross-links remains. Kohn et al. (15, 16) have found a linear relationship between the amount of DNA-protein cross-links thus measured and the dose of several compounds used. Although the relation between the absolute amount of DNA-protein cross-links and the amount of masked radiation-induced single-strand breaks is unknown, this relationship has been considered to be 1:1. This permitted us to express the DNA-protein cross-links as Gy-equivalents or breaks per dalton of DNA (1 Gy induces 300 breaks/10^{12} dalton DNA; see above). If the relationship were different, the absolute amount of DNA-protein cross-links would change, but not the conclusions obtained in this study.

Calculation of the Number of DNA-Protein Cross-Links. DNA-protein cross-links can be measured with the alkaline elution method by omitting proteolytic digestion of the cell lysate with proteinase K. Then, the undegraded DNA-protein cross-links will, together with the DNA interstrand cross-links, reduce the elution rate of the labeled DNA on the filters. The calculation of the amount of both types of cross-links simultaneously from the elution patterns is identical to the calculation of the amount of DNA interstrand cross-links alone. After subtracting the amount of cross-links found after application of proteinase K (DNA interstrand cross-links), the amount of γ-ray-induced breaks masked by DNA-protein cross-links remains. Ewig and Kohn (7, 8) have described a method to calculate the number of DNA interstrand cross-links from alkaline elution profiles. This resulted in a curve (Chart A-1, Curve c), which was used for the calculation of the amount of cross-links from the elution pattern found for the irradiated control cells. The x/p value of the cross-linked samples should not be too small, otherwise, the determination of x/p becomes exceedingly inaccurate.

\[ x/p \]

The DNA isolated from the untreated, unirradiated control cells should be eluted. For this reason, control experiments showing more than 10% elution over the first 9 fractions were discarded.

The limitations of this method to calculate DNA interstrand cross-links are: (a) that the number of cross-links should not surpass the amount of irradiation-induced breaks; and (b) that corrections have to be made for the elution pattern of untreated, unirradiated control DNA from cells. These corrections are negligible, however, when only a small proportion of the control DNA is eluted, as was the case in our experiments that passed the <10% criterion mentioned above.

With the new method, the absolute number of cross-links is changed in comparison to the method by Ewig and Kohn (7, 8). In this particular case, however, qualitatively identical conclusions would have been drawn from our results when their method had been used.

In theory, single-strand breaks may be masked not only by interstrand cross-links but also by cross-links that connect 2 distant nucleotides within one DNA strand. It is not known whether such "long-distance" intrastand cross-links are really formed to a significant extent. However, it appears highly improbable that these connections might reduce the elution of the DNA in the alkaline elution procedure, in view of the fact that the average distance between 2 γ-ray-induced breaks amounts to 1 to 2 \times 10^6 nucleotides, which is many orders of magnitude larger than the distance expected to be bridged by long-distance cross-links.

Effects of Platinum-induced DNA Cross-Links in CHO Cells

**Chart 1.** Model for the calculation of DNA cross-links from the elution pattern, obtained by the alkaline elution method. Abecasis, fraction number; ordinate, relative amount of 3H- and 14C-radioactivity remaining on the filter (logarithmic scale). p, 14C elution pattern of the irradiated, untreated control cells; t, 14C labeled; R, 14C pattern of the irradiated, untreated control cells; c, 3H labeled; H, 14C labeled; c', 3H labeled. In Curve R, the elution volume is determined at which 50% of the radioactivity (equals DNA) from the untreated, irradiated control cells has passed through the filter (the slope of this line is a measure for the single-strand breaks induced by the radiation). Then, Cr الكتابة، is read from Curve c, by estimation of the proportion of the cross-linked DNA eluted at this volume. C0 الكتابة، is a measure for the number of cross-links (x) relative to the number of radiation-induced breaks (p), as is described in the "Appendix." The value for x/p is obtained with the help of Curve c in "Appendix," Chart A-1, where C0 الكتابة، has been plotted as a function of x/p. In the accepted experiments, Curve r remained above 90%, and no corrections of Curves R and c had to be applied. The C0 الكتابة، value of the cross-linked samples should not be too small, otherwise, the determination of x/p becomes exceedingly inaccurate.

**Chart 2.** Survival of CHO cells as a function of the dose of the palladium compounds. Data represent the averages of at least 3 independent experiments, with duplicates for each dose. Bars, S.D. From the linear part of the graphs, the D0 values of the platinum compounds have been calculated. @, cll (D0 = 8 μM); △, c1V (D0 = 8 μM); O, till (D0 = 275 μM); □, tIV (D0 = 125 μM).
RESULTS

The effects of the 4 platinum compounds on survival of CHO cells is shown in Chart 2. In agreement with previous observations (25), the semilogarithmic survival graphs are straight lines with a slight shoulder. From the linear part of the graphs, the D0 values (increase in dose resulting in a decrease in survival to 37% of the initial value) given in Table 1 were calculated. The 2 cis compounds (cI and cIV) are highly cytotoxic, both with a D0 of 8 μM, whereas the trans compounds, tII and tIV, show a much lower toxicity (16- and 34-fold, respectively). These results compare reasonably well with earlier data obtained under somewhat different experimental conditions (2).

When the amount of platinum adducts formed (i.e., the amount of platinum bound to DNA) was determined as function of the treatment concentration, the differences between the 4 compounds were less pronounced. All 4 gave an incorporation of platinum that was proportional to dose (not shown). The amounts of platinum bound to DNA per μM of agent present during treatment differed less than one order of magnitude (see Table 2 under Pt-DNA adducts). According to this criterion, cI was the most active compound; cIV was distinctly less reactive (about 3.5-fold) and was clearly surpassed by tIV. These results, in combination with the survival data, indicate that, on the average, lesions caused by the cis compounds are more lethal than those induced by the trans analogues, and that cIV lesions are more lethal than cII adducts, as is illustrated by Chart 3A, where survival is plotted as a function of DNA-bound platinum.

Also, with regard to the ability to induce mutations at the hypoxanthine-guanine phosphoribosyltransferase locus of CHO cells, the cis compounds appeared much more effective than the trans isomers. When compared on the basis of the number of mutants induced per amount of Pt-DNA adduct present in the cell (Chart 3B), the 2 cis compounds were of almost equal potency, and about 40 times as active as the 2 trans complexes. When the mutation induction was plotted as a function of survival (data not shown), cII and cIV were still more mutagenic than tII and tIV; in this comparison, under equitoxic conditions, cII appeared to be as mutagenic as cIV.

The induction of SCEs is another indicator for the occurrence of DNA damage. In CHO cells, these chromosomal abnormalities are readily induced by the 2 cis compounds, and rather poorly by their trans analogues. When expressed as a function of the number of Pt-DNA adducts, as is done in Chart 3C, cII and cIV are virtually indistinguishable, and about 9 times as effective as tII, while the SCE induction by tIV is almost negligible. Evidently, the lesions in DNA caused by the cis compounds are much more harmful, also with respect to the induction of SCEs.

The occurrence of interstrand cross-links in the DNA of CHO cells treated with any of the 4 agents was determined at different moments after the treatment to study both the induction and repair of these lesions. At the various moments after induction (after repair periods ranging between 0 and 48 hr), the number of DNA interstrand cross-links was, for all 4 compounds, approximately proportional to the concentration of the agent during treatment, up to a certain number of cross-links. Beyond this value, the induction leveled off. In Chart 4, an illustration of this observation is given for 4 concentrations of cII, after 0, 6, or 24 hr of posttreatment incubation of the cells at 37°. An interesting phenomenon can be seen in this graph, namely, that during the first 6 hr of this incubation, a strong increase in the number of cross-links takes place. Apparently, additional cross-links are formed, although the agent has been removed, presumably because adducts with monofunctionally bound platinum, which still possess one Pt—Cl bond, become activated and react with a neighboring DNA strand. This observation is in agreement with the results of Roberts and Friedlos (28).

A comparison of the 4 compounds with respect to the kinetics of formation or repair of cross-links is shown in Chart 6A, where the number of DNA interstrand cross-links determined in cells treated with equitoxic dosages of cII, cIV, tII, or tIV is plotted against the length of the posttreatment incubation. Striking dif-

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Table 1

Cytotoxicity and induction and repair of DNA interstrand and DNA-protein cross-links in CHO cells treated with platinum compounds, at a D0 dose increment

<table>
<thead>
<tr>
<th>Compound</th>
<th>Symbol</th>
<th>D0 (μM)</th>
<th>DNA interstrand-cross links/</th>
<th>DNA-protein cross-links/cell</th>
<th>Pt-DNA adducts/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 hr</td>
<td>6 hr</td>
<td>24 hr</td>
<td>48 hr</td>
</tr>
<tr>
<td>cis-Pt(NH2)Cl2</td>
<td>cII</td>
<td>8</td>
<td>448</td>
<td>1008</td>
<td>592</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>31.8</td>
<td></td>
<td>31.8</td>
</tr>
<tr>
<td>cis-Pt(NH2)Cl2</td>
<td>cIV</td>
<td>8</td>
<td>128</td>
<td>484</td>
<td>272</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>63.6</td>
<td></td>
<td>63.6</td>
</tr>
<tr>
<td>trans-Pt(NH2)Cl2</td>
<td>tII</td>
<td>275</td>
<td>1040</td>
<td>347</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2277</td>
<td></td>
<td>2277</td>
</tr>
<tr>
<td>trans-Pt(NH2)Cl2</td>
<td>tIV</td>
<td>125</td>
<td>150</td>
<td>244</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2644</td>
<td></td>
<td>2644</td>
</tr>
</tbody>
</table>

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Table 2

Amount of DNA interstrand and DNA-protein cross-links and total Pt-DNA adducts per cell, per 1 μM drug, directly after the treatment of the cells (t = 0)

The amounts of DNA cross-links and the total amounts of Pt-DNA adducts were derived from measurements directly after a 1-hr treatment of the cells (t = 0) with approximately equitoxic dosages of the 4 platinum compounds, at 37°. From the results, the number of these lesions per cell, per 1 μM dose increase, were calculated.

<table>
<thead>
<tr>
<th>Compound</th>
<th>DNA interstrand-cross links</th>
<th>DNA-protein cross-links</th>
<th>Pt-DNA adducts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Per cell</td>
<td>%</td>
<td>Per chromosome</td>
</tr>
<tr>
<td>cII</td>
<td>56</td>
<td>0.11</td>
<td>70</td>
</tr>
<tr>
<td>cIV</td>
<td>16</td>
<td>0.11</td>
<td>56</td>
</tr>
<tr>
<td>tII</td>
<td>3.8</td>
<td>0.05</td>
<td>8.3</td>
</tr>
<tr>
<td>tIV</td>
<td>1.2</td>
<td>0.004</td>
<td>21.2</td>
</tr>
</tbody>
</table>

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* DNA interstrand and DNA-protein cross-links were calculated per cell (DNA content of the cell, 6 x 10⁶) and were expressed as the percentage of the total Pt-DNA adducts per cell; in addition, the number of these lesions per chromosome (2N = 42) is given.

* Data taken from Chart 3C.
Effects of Platinum-induced DNA Cross-Links in CHO Cells

Chart 3. Amount of platinum bound to the DNA of CHO cells in relation to survival (A), mutagenicity (B) and SCE induction (C). Abscissa, amount of platinum bound to the DNA, measured by atomic absorption spectroscopy, is given as the number of platinum atoms per $10^6$ nucleotides. A, survival (plotted on a logarithmic scale) as a function of Pt-DNA binding; the results of 2 independent experiments have been computed. B, induction of hypoxanthine-guanine phosphoribosyltransferase mutants (per $10^5$ surviving cells) as a function of Pt-DNA binding. The data from 4 separate mutation/survival experiments have been combined. The relation between survival and platinum binding was taken from A. C, SCEs induced per chromosome as a function of Pt-DNA binding. Per dose, the SCEs in 25 nuclei were counted ($n = 21$). The results of 2 separate experiments are given (bars, S.D.). ◆, cIV; ▲, cIV; ○, tIV; □, tIV.

Chart 4. DNA interstrand cross-links induced by cIV in CHO cells, measured by alkaline elution, after a 1-hr treatment at 37°. The amount of cross-links is given as function of the dose of cIV, after various posttreatment incubation periods (at 37°). Data are mean values of 3 or more independent experiments; bars, S.D. (points without bars, values of less than 3 experiments). ◆, directly after the removal of platinum compounds; ■, after 6-hr postincubation; ○, after 24-hr postincubation.

Chart 5. DNA-protein cross-links induced by tIV in CHO cells. The cells were treated for 1 hr at 37°; subsequently, the amount of cross-links (ordinate) was determined by alkaline elution. The DNA-protein cross-links present after different postincubation periods (at 37°) have been plotted as a function of the concentration of the drug. Data are mean values of 3 independent experiments. Bars, S.D. ◆, directly after treatment; ■, after 6-hr postincubation; ○, after 12-hr postincubation.

Interferences are seen, both in the number of cross-links and in the kinetics. The induction of DNA interstrand cross-links by tIV is extremely low, particularly when related to the total number of Pt-DNA adducts which, at equitoxic doses, is by far the highest for this compound (Chart 3A). As for the kinetics, the amount of cross-links induced by both cis compounds reaches a maximum at 6 to 12 hr after the removal of the drug, probably because the continuing formation of cross-links overcompensates for the disappearance caused by repair reactions. Thereafter, repair exceeds formation. After 48 hr, some cis-induced DNA interstrand cross-links are still detectable in the DNA (Chart 6A). Treatment of CHO cells with tIV results in a maximum amount of DNA interstrand cross-links directly after the treatment, followed by a fast repair process. The small amount of cross-links induced by tIV appears to show a slight increase during the first 6 hr after the removal of the drug. The essence of the results shown in Chart 6A has been summarized in Table 1, where effects of the compounds are expressed per dose-increase equal to $D_0$. The conclusions from these data are that: (a) with regard to DNA interstrand cross-links, strong dif-
ferences exist between til and the 2 cis compounds (and, possibly, tIV). Both cis compounds show a delayed formation of these cross-links, whereas til shows a strong immediate cross-link induction and not a trace of delayed formation; tIV appears to resemble the cis compounds but is a much weaker inducer; (b) the Pt(IV) complexes are less efficient cross-linkers than the corresponding Pt(II)-congeners; and (c) cross-links induced by the cis compounds are repaired more slowly than are the trans-induced cross-links.

In DNA-protein cross-link studies, linear relationships were found between the dose of the drugs and the amount of DNA-protein cross-links, detected after various postincubation times. Chart 5 shows such dose-response curves for tIV, which (as til) induces initially a large number of DNA-protein cross-links. In Chart 6A and Table 1, the data of the induction and repair of DNA-protein cross-links are given for all 4 platinum compounds when used at equitoxic doses. All 4 platinum complexes show a maximal amount of these cross-links directly after the removal of the drug, but the number of DNA-protein cross-links induced by the trans compounds is much higher than that caused by the cis isomers. Furthermore, the til- and tIV-induced DNA-protein cross-links are repaired much faster than those induced by cll and cIV. One-half of the initially detected cross-links have disappeared after about 3 to 6 hr in case of the trans compounds; for the cis isomers, this process takes about 24 hr.

Table 1 summarizes the results of the time course experiments on induction and repair of cross-links, after mathematical conversion of the equitoxic doses used to D0. As is indicated in Table 1, an estimate has been made of the cross-links still present per cell at 48 hr after the removal of the drug, as a consequence of a dose increment equal to D0. Table 2 gives a survey of the amounts of cross-links and of total Pt-DNA adducts per cell and per chromosome directly after the treatment of the cells, and the fraction of cross-links in relation to total adducts; in Table 2, the 4 compounds are compared per equal increase of molar dose.

DISCUSSION

Earlier, we found some similarities in the action of the 4 platinum compounds (25). All inhibit the DNA synthesis in CHO cells (although the recovery of the DNA synthesis is much faster after treatment of the cells with the trans- than with the cis-platinum compounds); they induce micronuclei (2) and cause some repair synthesis (25); with none of the Pt-compounds used, treatments resulted in the induction of single-strand breaks (25). However, in the present study, differences in the action of the Pt-compounds became apparent, now that other biological end points were measured. The 2 cis-platinum compounds (cll and cIV) are very cytotoxic and induce SCEs as well as gene mutations, whereas the trans compounds are much less active in this respect. The cytotoxicity seems to correlate with the other 2 measured biological end points.

The amount of Pt-DNA adducts cannot itself be held responsible for the differences between the cis and trans compounds, since, at equi-effective treatments, much more trans complexes are bound to the DNA than are cis-platinum compounds (Chart 3A). Apparently, one or more very toxic and mutagenic adducts are exclusively or preferentially introduced by the cis compounds. For a complete understanding of the mechanism of action of the platinum compounds, the nature of the various Pt-DNA adducts has to be identified, as well as the cellular responses to these adducts. Presently, only a part of the DNA lesions has been analyzed, and their respective contributions to biological effects have not yet been established. Nevertheless, a few conclusions with regard to the relation between lesions and effects appear warranted.

Both, cis- and trans-platinum compounds can react with DNA either monofunctionally or bifunctionally. According to recent results, initially, the majority of the adducts in the DNA of the

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*a A. C. M. Plooy, unpublished data.
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The formation of "short-distance" DNA intrastrand cross-links, known to be formed in vitro (9) and in bacteria (4) by cis-platinum compounds, appears unlikely for the trans-compounds because of steric reasons (32). This type of cross-link, which connects 2 nucleotides of one DNA strand that are adjacent or are separated by one nucleotide, has been found also in CHO cells after treatment with cll. Their biological importance in mammalian cells has not yet been established. The differences between cis- and trans-platinum compounds with regard to the induction, however, makes these adducts possible candidates for the genotoxic lesions. The steric factors that make the formation of "short-distance" intrastrand cross-links by trans compounds rather unlikely, will not prevent the cross-linking of 2 more remote nucleotides of the same strand. There is no obvious reason to expect that the induction of these "long-distance" intrastrand cross-links should occur preferentially by either the cis or trans compounds and no reason, therefore, to attribute a particular biological significance to these (probably rare) lesions.

According to several authors, DNA interstrand cross-links induced by cll correlate with cytotoxicity. Strandberg et al. (33), however, have reported that the DNA interstrand cross-links alone cannot be responsible for the cytotoxicity. In the present study, the DNA interstrand cross-links appear to be only minor reaction products when measured shortly after treatment (<1% of the total Pt-DNA adducts). However, the 2 cis compounds and possibly the weakly inducing trans compound 1IV show a delayed formation of these lesions, which has also been found by other authors (29, 33, 41). Eventually, the interstrand cross-links appear to be removed from the DNA. Fraval and Roberts (11) demonstrated that this decrease in the amount of cross-links is not due to chemical instability of the cross-links or to the induction of breaks in the DNA, but has to be ascribed to the action of repair systems.

Our results indicate that the cellular repair systems are able to distinguish between DNA interstrand cross-links induced by cis- and trans-platinum compounds, respectively (Table 1; Chart 6). The trans-platinum-induced cross-links are repaired faster; this phenomenon may very well underlie the more rapid recovery from the DNA synthesis inhibition in CHO cells after treatment with a trans-platinum compound. Because of the delayed formation and slow repair, the cis-platinum-induced cross-links show a rather persistent character. Persistent interstrand cross-links can be very noxious for the cells, especially during DNA replication. It is known (12) that cells in G2 phase (the phase preceding the S phase, when DNA is replicated) are more sensitive for cll than are asynchronously growing CHO cells (about 30% S phase) or than are cells half-way through the S phase. Moreover, DNA synthesis has been found to be required to express cll-induced cellular toxicity. These results may be considered to point to the interstrand cross-link as the biologically important DNA lesion. However, in our experiments, the DNA-protein cross-links also showed a persistent character and, therefore, cannot be ruled out as a (cyto)toxic lesion. A similar type of relation has been found for bifunctional alkylating agents (22); the irreparable DNA interstrand as well as DNA-protein cross-links correlated with cytotoxicity.

In our results, the residual amount of persistent cross-links (DNA interstrand as well as DNA-protein) induced by the cis-platinum compounds in the cells correlates not only to cytotoxicity, but also to mutagenicity and SCE induction. In contrast to DNA-protein cross-links, the interstrand cross-links cause a mutilation of the genetic information in both DNA strands at (approximately) the same position. To circumvent this double block on the template, recombination with homologous chromosomes appears to be necessary. Such a process is thought to be involved in the induction of SCEs. It is tempting, therefore, to attribute great importance to the interstrand cross-links in relation to genotoxicity. This way of reasoning is not supported, however, by data of Bradley et al. (3), who found that SCEs are not the result of bifunctional platinum binding.

In summary, the persistent DNA interstrand cross-links, because of their damage to the DNA as a template, may account for the cytotoxicity, mutagenicity, and presumably also for the antitumor activity of the cis compounds. However, the contribution of DNA-protein and short-distance intrastrand cross-links cannot be excluded. Further research is required to establish, among others, the importance of monofunctionally Pt-DNA adducts and of intrastrand cross-links and their contribution to the observed effects in CHO cells.

ACKNOWLEDGMENTS

The authors wish to thank Dr. F. Berends, Dr. A. M. J. Fichtinger-Schepman, and Dr. G. P. van der Schans for the critical reading of the manuscript and their helpful discussions, Dr. H. P. Liepman for the editorial assistance, and Dr. A. T. M. Marcelis for the synthesis of the platinum compounds.

APPENDIX

Distribution of Molecular Weights in Alkali-denatured DNA Samples with Randomly Induced Single-Strand Breaks and Interstrand Cross-Links.

The elution of DNA single-strand molecules through membrane filters is assumed to depend on strand length. This assumption is supported by the observed dependence of DNA elution on the frequency of single-strand breaks produced by X-rays (8, 15). The distribution of DNA over the fractions eluted from the filter is, therefore, determined by the molecular weight distribution in the DNA sample studied. The distribution in the DNA sample itself is determined by the distribution of the single-strand breaks and the cross-links over the original DNA molecules. Let us consider a homogenous population of double-stranded DNA molecules with molecular weight M which contain, on the average, p breaks per single strand, randomly distributed. In the absence of cross-links, the weight fraction of the single-strand fragments with a molecular weight between m and m + dm is given by:

\[ C_m = \frac{p^2 \cdot M^2 \cdot m \cdot e^{-mM}}{\alpha m^2} \text{dm} \quad (A) \]

provided \( M \) is large compared to \( m \) (i.e., \( m < 1 \)) (36). Next, we assume that the original DNA contained \( x \) randomly distributed cross-links/molecule. If we consider one cross-link to consist of 2 "links", there are \( x \) such links per original single strand with molecular weight M. This implies that in a population of single-strand fragments with molecular weight \( m \), a fraction \( e^{-mM} \) does not contain a link. Consequently, the weight fraction of fragments with a molecular weight between \( m \) and \( m + dm \) that contain one or more links, is:

\[ X_m = \frac{p^2 \cdot M^2 \cdot \text{e}^{-mM} \cdot (1 - \text{e}^{-mM}) \text{dm}}{\alpha m^2} \quad (B) \]

We define \( m_b \) as the molecular weight of single-strand DNA fragments that are passing through the filter at the elution curve of non-cross-linked DNA, where 50% of the material has been eluted. For a random distribution of the breaks over the molecule, the number average molecular weight of the fragments \( m_b \) is equal to \( m_b = \frac{1}{2} \cdot 1.68 \). As \( m_b = \frac{M}{p + 1} \), we get:

\[ m_b = \frac{M}{p + 1} = 1.68 \quad (C) \]

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20.5% of the links in X are connected to other small fragments, and the products remain smaller than m<, so that X<, are replaced by 0.205X (20.5 x x) as a function of the cross-linking material with m > mx, are expected to be eluted at 50% point. Calculation of the sum of X< in 0.795x and X< (with 0.5x) for various values of x/x between 0 and 2 yields a corrected version of X< in Chart A-1. In an analogous manner, Curve B can be constructed upwards by adding X< (0.205x). In this way, the distance between the 2 graphs can be reduced (from 4.0% at x/x = 0.2 to 2.0% at 50%, 10.5% at x/x = 1.0, as well as 2.0, 4.5%).

According to the same approach, the range between the 2 curves can be narrowed further, by subdividing the material with m < mx, into more weight categories. Subdivision into 4 classes reduces the above-mentioned values to 0.9, 1.7, 2.3, and 2.3%, respectively. Line c in Chart A-1 is constructed on the basis of these corrections as a fair approximation of the real curve (in the range x/x between 0 to 2, this curve does not deviate more than 0.8% from the more easily constructed graph that is obtained by substituting 0.85x for x in Equation E. If a reconstruction of Curve c is desired, the following calibration points can be used: x/x = 0.2, 42.4%: 0.5, 33.7%; 1.0, 24.0%; 1.5, 17.8%; and 2.0, 13.7%). More refined calculations are without meaning because, in this approach, the possibility is ignored that more than 2 fragments are linked together when multiple links per fragment are present, which will lead to inaccuracies in particular at higher values of x/x.

For comparison, the curve according to the approach of Ewig and Kohn (8, 15) has been drawn in Chart A-1. It is clear that the latter will result in an underestimate of the number of cross-links of at least a factor of 2.

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Induction and Repair of DNA Cross-Links in Chinese Hamster Ovary Cells Treated with Various Platinum Coordination Compounds in Relation to Platinum Binding to DNA, Cytotoxicity, Mutagenicity, and Antitumor Activity

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Cancer Res 1984;44:2043-2051.