In Vivo Effects of *cis*- and *trans*-Diamminedichloroplatinum(II) on SV40 Chromosomes: Differential Repair, DNA–Protein Cross-Linking, and Inhibition of Replication†

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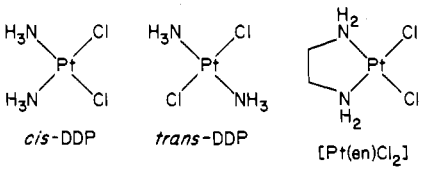
ABSTRACT: The mechanism of action of the antitumor drug *cis*-diamminedichloroplatinum(II), *cis*-DDP, was investigated by using the ~5200 base pair (bp) chromosome of simian virus 40 (SV40) as an in vivo chromatin model. Comparative studies were also carried out with the clinically ineffective isomer *trans*-DDP. Although 14 times more *trans*- than *cis*-DDP in the culture medium is required to inhibit SV40 DNA replication in SV40-infected green monkey CV-1 cells, the two isomers are equally effective at inhibiting replication when equimolar amounts are bound to SV40 DNA in vivo. Since both isomers are transported into CV-1 cells at similar rates, differential uptake cannot account for the greater ability of *cis*-DDP to inhibit SV40 DNA replication. Rather, this result is explained by the finding that *cis*-DDP–DNA adducts accumulate continuously over the incubation period, whereas *trans*-DDP binding to DNA reaches a maximum at 6 h and thereafter decreases dramatically. We suggest that the different accumulation behavior of *cis*-DDP and *trans*-DDP on DNA is due to their differential repair in CV-1 cells. A variety of non-histone proteins, including SV40 capsid proteins but virtually no histones, are cross-linked to SV40 DNA in vivo by either *cis*- or *trans*-DDP. More DNA–protein cross-links are formed by *trans*-DDP than by *cis*-DDP at equivalent amounts of DNA-bound platinum. Since the *cis*-DDP analogue dichloro(ethylenediamine)platinum(II) inhibits SV40 DNA replication as efficiently as *cis*- and *trans*-DDP but does not form DNA–protein cross-links, platinum-mediated DNA–protein cross-linking is not directly responsible for the inhibition of DNA replication. We discuss these findings in relation to the known cytotoxic and antitumor properties of *cis*-DDP.

cis-DDP[1] is a clinically important antitumor drug used alone and in combination chemotherapy for the treatment of several types of human cancers (Prestayko et al., 1980; Loeher & Einhorn, 1984). The stereoisomer of this inorganic Pt(II) complex, *trans*-DDP, is clinically ineffective and less cytotoxic



and mutagenic than *cis*-DDP (Roberts & Thompson, 1979). Elucidation of the mechanism of cytotoxicity of *cis*-DDP [for a recent review, see Pinto & Lippard (1985a)] is important not only for the understanding of its antitumor activity but also for the development of second generation *cis*-DDP analogues, since clinical applications of *cis*-DDP are often limited by severe side effects (Loehrer & Einhorn, 1984).

The cytotoxic properties of *cis*-DDP are most likely a consequence of interactions with its major intracellular target, DNA. Treatment with *cis*-DDP of human cells in culture (Harder & Rosenberg, 1970) or tumor cells in vivo (Howle & Gale, 1970) inhibits DNA synthesis and cell division. Since the clinically ineffective isomer *trans*-DDP is also a cytotoxic inhibitor of DNA synthesis, albeit a weaker one than *cis*-DDP, the specific mechanism of *cis*-DDP antitumor activity is not clear. Several types of platinum–DNA adducts have been identified in cultured mammalian cells following treatment with either *cis*- or *trans*-DDP, and considerable controversy exists over whether the inter- or intrastrand DNA cross-link or the DNA–protein cross-link is the "critical" lesion resulting in arrest of DNA synthesis [for reviews, see Zwelling (1983) and Pinto & Lippard (1985a)]. The major DNA adduct produced by *cis*-DDP in vivo is the intrastrand DNA cross-link formed by bifunctional coordination of Pt to the N(7) positions of two adjacent guanosines (Pinto & Lippard, 1985a). The three-dimensional structure of this adduct has recently been determined by X-ray crystallography (Sherman et al., 1985). Since *trans*-DDP is stereochemically incapable of forming such a structure, this 1,2-intrastrand cross-link could be responsible for differences in biological activity between the two isomers (Plooy et al., 1984; Pinto & Lippard, 1985a).

Little information exists on the interactions of *cis*- and *trans*-DDP with chromosomes in vivo. Both *cis*- and *trans*-DDP form cross-links between nuclear DNA and non-histone chromosomal proteins (Banjar et al., 1984), but neither the identity of the cross-linked proteins nor the effects of such

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[1] Abbreviations: DDP, diamminedichloroplatinum(II); en, ethylenediamine; SV40, simian virus 40; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; RNase A, bovine pancreatic ribonuclease A; D/N, measured drug-to-nucleotide ratio; AAS, atomic absorption spectroscopy.

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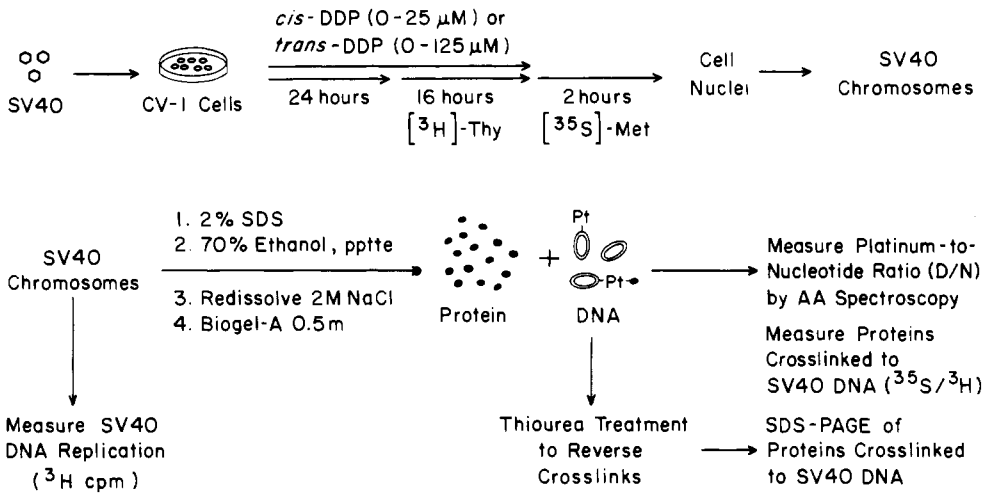

FIGURE 1: Protocol for SV40 experiments.

cross-links on chromatin function are known.

We have used the small circular chromosome of SV40 (Tooze, 1980) as an in vivo chromatin model to characterize the adducts formed by *cis*- and *trans*-DDP with chromosomal DNA and to assess the effects of such adducts on DNA replication. SV40 proliferates within the nucleus of infected green monkey cells as a minichromosome containing, in particular, host histones. Apart from initiation of DNA replication, which requires the participation of the virus-encoded large T antigen, SV40 DNA replication and other aspects of SV40 metabolism are carried out by the host cell enzymatic machinery. Earlier work showed that treatment with *cis*-DDP inhibits SV40 virus infectivity and replication in green monkey cells (Kutinova et al., 1972a,b). Here, we report mechanistic studies of the inhibition of SV40 replication following treatment with either *cis*- or *trans*-DDP.

Experimental Procedures

The experimental protocol is depicted schematically in Figure 1.

Platinum Treatment of SV40-Infected Cells. Confluent monolayers of African green monkey kidney cells (CV-1) in 15-cm culture plates (Nunc) were infected with SV40 (strain 777) at 5 plaque-forming units/cell as described (Varshavsky et al., 1978). Immediately following infection, either *cis*- or *trans*-DDP, freshly dissolved in 0.15 M NaCl, was added to cells in 25 mL of medium containing 2% calf serum. Final concentrations of *cis*-DDP ranged from 0 to 25 μM and of *trans*-DDP from 0 to 125 μM. At 24 h postinfection, [*methyl*-^{3}H]thymidine (New England Nuclear, ~80 Ci/mmol) was added to a final concentration of 5 μCi/mL. Following an additional 16 h, cell monolayers were rinsed with 150 mM NaCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.0, and proteins were labeled for 2 h with 250 μCi/plate L-[^{35}S]methionine (New England Nuclear, ~1000 Ci/mmol) in 3 mL of methionine-free MEM medium (GIBCO). Cells were harvested, nuclei were isolated, and SV40 chromosomes were extracted and purified by sedimentation through a 5–30% sucrose gradient as described previously (Varshavsky et al., 1978). The yields of SV40 chromosomes were nearly equal for untreated control and for *cis*- or *trans*-DDP-treated samples. SV40 DNA replication was measured by determining the incorporation of [*methyl*-^{3}H]thymidine into SV40 DNA.

Isolation of SV40 DNA from SV40 Chromosomes. SDS was added to the peak fractions from the sucrose gradient to a final concentration of 2%, and DNA was precipitated with 70% ethanol. SV40 DNA was redissolved in 2.0 M NaCl, 10 mM Tris-HCl, and 1 mM Na_2EDTA, pH 8.0, and chromatographed through a Bio-Gel A-0.5m (100–200-mesh) (Bio-Rad) column (20 cm × 1 cm) eluted with the same buffer. DNA eluted in the void volume and was precipitated with 70% ethanol. Separation between ^{3}H-labeled DNA and dissociated ^{35}S-labeled proteins was monitored by measuring ^{3}H and ^{35}S radioactivity in the column fractions.

Determination of Platinum in SV40 DNA. Column-purified DNA was redissolved in TE buffer (1 mM Na_2EDTA, 10 mM Tris-HCl, pH 8.0), an aliquot was removed for liquid scintillation counting, and the remainder was analyzed for Pt by electrothermal AAS with a Varian Model AA-1475 spectrometer equipped with deuterium background correction and a CRA-90 furnace. The ratio of Pt bound per nucleotide (D/N) was calculated from the specific radioactivity of [^{3}H]DNA (~100 000–150 000 cpm/μg of DNA), after the DNA concentration was determined by the diaminobenzoic acid method (Ciccarelli & Wetterhahn, 1982). In control experiments, column-purified DNA was digested with RNase A (50 μg/mL, 25 °C, pH 6, 2 h) and proteinase K (50 μg/mL in TE buffer, 37 °C, 2 h) and repeatedly extracted with 24:1 chloroform/isoamyl alcohol prior to Pt analysis, which resulted in a 6–9% decrease in D/N.

Measurement of Protein Associated with SV40 DNA. An aliquot of column-purified DNA was analyzed by liquid scintillation counting, and the relative amount of protein cross-linked to SV40 DNA was determined by measuring ^{35}S/^{3}H. Controls (no Pt treatment) consistently had only background levels of ^{35}S associated with the DNA. To determine whether any free proteins cofractionated with the SV40 DNA, it was centrifuged to equilibrium in a CsCl density gradient (Varshavsky et al., 1979) and the DNA peak banding at 1.68–1.71 g/cm^3 was isolated. The ^{35}S/^{3}H ratio decreased by less than 10% and the D/N by less than 7% following this procedure.

Electrophoretic Analysis of Proteins Cross-Linked to SV40 DNA. Column-purified, ethanol-precipitated DNA was dissolved in the reversal/loading buffer (100 mM thiourea, 1 mM 2-mercaptoethanol, 2% SDS, 1 mM Na_2EDTA, 15% glycerol, 0.1 mg/mL bromophenol blue, 0.8 M Tris-HCl, pH 6.8) and heated at 90 °C for 30 min. Proteins were electrophoresed on 12% polyacrylamide separating gels [0.4% bis(acrylamide)] with 5% polyacrylamide stacking gels (0.25% bisacrylamide) (Laemmli, 1970). Fluorography of the dried gels (Laskey & Mills, 1975) was carried out with Kodak X-Omat films at −70 °C.

Measurement of Pt Associated with Uninfected CV-1 Cells

and Cellular DNA. For platinum uptake analysis, monolayers of CV-1 cells in 10-cm plates (Nunc) in medium containing 2% serum (Varshavsky et al., 1978) were treated with 1, 5, or 10 μM *cis*- or *trans*-DDP for 48 h. CV-1 cells were treated with 10 μM *cis*- or *trans*-DDP for 0–48 h in experiments to analyze platinum binding to DNA. Following Pt treatment, cells were washed twice in 5 mL of 0.15 M NaCl and 5 mM Tris-HCl, pH 7.5 at 4 °C, followed by a 30-min incubation at 37 °C in 5 mL of the Pt-free medium and two additional 5-mL washes at 4 °C. For Pt uptake experiments, washed cells were scraped with a rubber policeman into 1 mL of H_2O and lyophilized to dryness. Dried cell pellets were treated with 6 M HNO_3 at 60 °C for 24 h, and the platinum content of the sample was determined by AAS as described above. Additional plates at the same cell density were used for cell counting with a hemacytometer ($\sim 5 \times 10^6$ cells/plate). For DNA binding experiments, nuclei from washed cells were sequentially digested with RNase A and proteinase K and extracted with chloroform/isoamyl alcohol as described above. Purified cellular DNA was treated with 6 M HNO_3 at 60 °C for 24 h and analyzed for Pt by AAS.

Results

Effects of cis- and trans-DDP on SV40 Replication in Vivo. Figure 2A,C shows the replication of SV40 DNA in CV-1 cells following treatment with either *cis*- or *trans*-DDP, measured by the incorporation of [^{3}H]thymidine. While SV40 DNA replication is nearly completely inhibited by 25 μM *cis*-DDP, *trans*-DDP at this concentration has no effect (Figure 2A). Only at concentrations greater than 25 μM does *trans*-DDP inhibit SV40 DNA replication (Figure 2A). A structural analogue of *cis*-DDP, [Pt(en)Cl_2], inhibits SV40 DNA replication with approximately the same efficiency as *cis*-DDP (data not shown). The concentration of Pt in the culture medium resulting in 50% inhibition of SV40 DNA replication is approximately 5 μM for *cis*-DDP and 70 μM for *trans*-DDP, i.e., 14 times more *trans*-DDP in the medium is necessary to produce the same effect on DNA replication. On the other hand, when inhibition of SV40 DNA replication is examined as a function of the amount of Pt bound to DNA, expressed as the drug to nucleotide ratio (D/N), little difference is observed in the effects of *cis*- and *trans*-DDP (Figure 2C). Replication of SV40 DNA is inhibited by 50% by either *cis*- or *trans*-DDP at D/N $\sim 4 \times 10^{-4}$. Identical results were obtained for [Pt(en)Cl_2] (data not shown). This D/N ratio corresponds to approximately four Pt atoms per 5243-bp SV40 duplex. In fact, an average of one Pt/SV40 duplex inhibits DNA replication by approximately 20%, while an average of 20–25 Pt/SV40 is necessary to inhibit DNA replication by more than 80% (Figure 2). Thus, although equal amounts of *cis*- and *trans*-DDP bound to SV40 DNA have similar effects on DNA replication, the Pt concentrations in the culture medium necessary to produce equivalent amounts of the two isomers bound to DNA differ greatly (Figure 2B).

To address further this dramatic difference, we determined (i) cellular uptake of Pt into uninfected CV-1 cells 42 h after addition of *cis*- or *trans*-DDP, the time point at which SV40 DNA replication is measured in infected cells (Figure 3A), and (ii) the binding of Pt to cellular DNA as a function of time (Figure 3B). Equal amounts of *cis*- and *trans*-DDP are found in CV-1 cells after 42 h of incubation with either 1, 5, or 10 μM Pt (Figure 3A). Thus, the fact that *cis*-DDP inhibits SV40 DNA replication at these extracellular concentrations (Figure 2A), while *trans*-DDP does not, cannot be due to differences in the total amount of Pt in the cells. Total levels of Pt in the cell nuclei were also similar for the two isomers

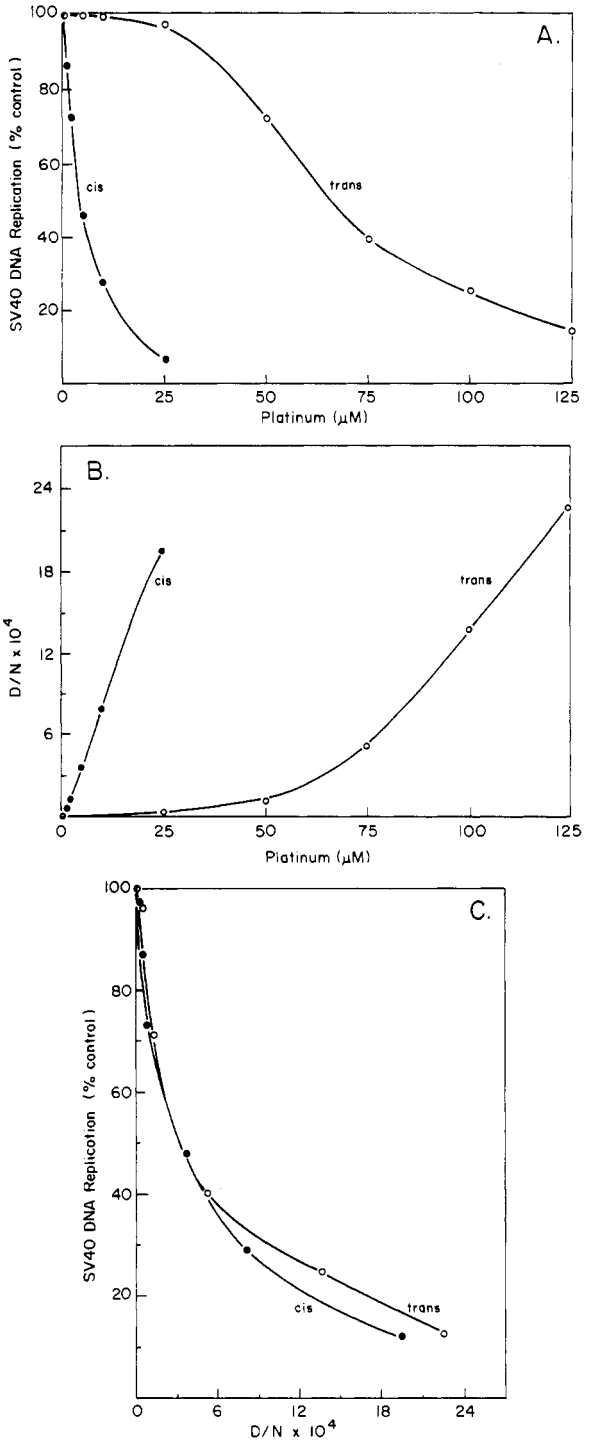

FIGURE 2: SV40 DNA replication in CV-1 cells as a function of platinum concentration in the medium (panel A) or platinum-to-nucleotide ratio, D/N (panel C). In panel B, D/N is plotted as a function of platinum concentration in the medium. SV40-infected cells were treated with *cis*-DDP (●) or *trans*-DDP (○) at the indicated concentrations. SV40 DNA replication relative to control (untreated) cells was measured by [^{3}H]thymidine incorporation, and Pt in isolated SV40 chromosomes was measured by AAS (see Experimental Procedures). The data shown are from a representative experiment. Experiments were carried out in quadruplicate.

(data not shown). A time-course analysis of Pt binding to cellular DNA at 10 μM extracellular Pt, however, reveals striking differences between *cis*- and *trans*-DDP (Figure 3B). The amount of *cis*-DDP bound to DNA increases steadily from 0 to 48 h (Figure 3A). This kinetic pattern was also observed for the [Pt(en)Cl_2] analogue (data not shown). By contrast, the amount of *trans*-DDP bound to DNA increases more

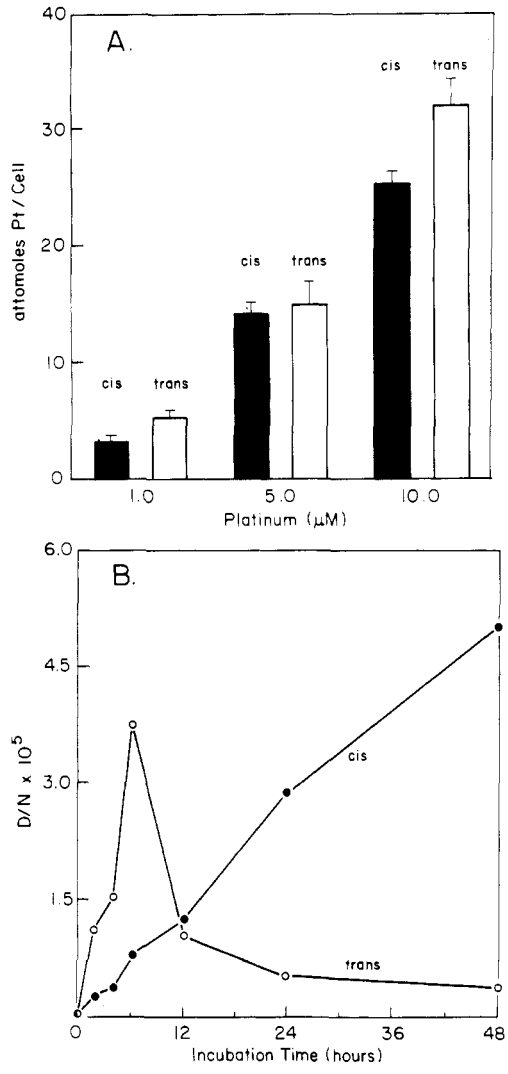

FIGURE 3: Uptake of platinum by CV-1 cells (A) and time course of platinum binding to DNA of uninfected CV-1 cells (B). Uptake of *cis*-DDP (closed bars) and *trans*-DDP (open bars) at the indicated concentrations 48 h following addition of the compounds was measured by platinum AAS of cellular acid digests as described in the text. Over the time course of 0–48 h, cells were treated with 10 μM *cis*-DDP (●) or *trans*-DDP (○), and cellular DNA was purified and analyzed by platinum AAS as described in the text. Error bars in (A) indicate the mean values of triplicate analyses. The time-course measurements were performed in quadruplicate, and data from one representative experiment are shown in (B).

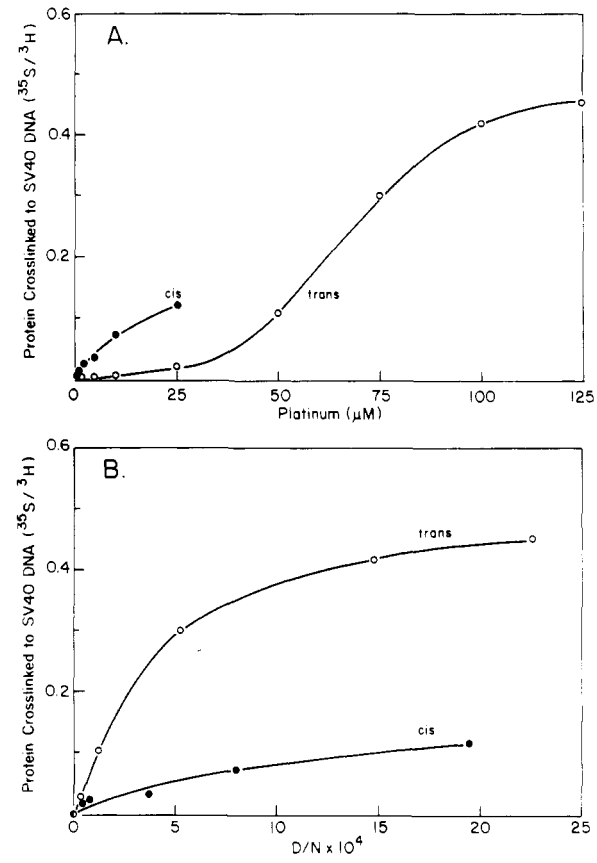

FIGURE 4: Relative amount of protein cross-linked to SV40 DNA in CV-1 cells as a function of platinum concentration in the medium (A) or platinum to nucleotide ratio on the SV40 DNA (B). SV40-infected cells were treated with *cis*-DDP (●) or *trans*-DDP (○) at the indicated concentrations, and double-labeled SV40 chromosomes (see Experimental Procedures) were isolated at 42 h. DNA was purified and analyzed for platinum as described in the text. DNA–protein cross-links relative to control (untreated) cells are expressed as $^{35}S/^{3}H$. Data from one representative experiment are shown. Experiments were carried out in quadruplicate.

rapidly at early time points, reaches a maximum at 6 h, but then rapidly diminishes in the next 6 h, and continues to decrease through 48 h (Figure 3B). An approximately 11-fold difference in the amount of *cis*- vs. *trans*-DDP bound to cellular DNA is observed after 42 h of treatment with equal extracellular concentrations (10 μM) of the two isomers. The levels of Pt bound to cellular DNA are similar to those bound to SV40 DNA following treatment with equal Pt concentrations in the culture medium (data not shown).

The above findings provide a simple explanation for the differential inhibition of SV40 DNA replication by *cis*- and *trans*-DDP (Figure 2), namely, that there is preferential repair of *trans*-DDP–DNA adducts (Figure 3B). Thus, *cis*-DDP forms a DNA adduct that accumulates on the DNA and inhibits replication while, at equivalent extracellular doses, *trans*-DDP forms a DNA adduct that is more easily removed by as yet unidentified repair pathway(s), thereby minimizing its effect on DNA replication (Figure 3). Consequently, a 14-fold excess of extracellular *trans*-DDP is required to produce an equivalent effect on SV40 DNA replication (Figure 2).

Pt-Induced in Vivo DNA–Protein Cross-Links. The relative amount of ^{35}S-labeled protein cross-linked to SV40 DNA following in vivo exposure to *cis*- and *trans*-DDP, expressed as $^{35}S/^{3}H$, is shown in Figure 4. For platinum concentrations below 25 μM, low levels ($^{35}S/^{3}H < 0.1$) of DNA–protein cross-links are observed following *cis*-DDP treatment, whereas little or no DNA–protein cross-links are formed by identical *trans*-DDP treatments (Figure 4A). Since concentrations of *cis*-DDP above 25 μM completely inhibit SV40 DNA replication, protein–DNA cross-linking in this range could not be assessed. DNA–protein cross-links induced by *trans*-DDP increase dramatically in the range 25–125 μM, reaching levels 4 times greater than those observed for *cis*-DDP at 25 μM ($^{35}S/^{3}H = 0.1$–0.4). To normalize these data (see Figure 2B), the amounts of protein cross-linked to DNA by the two isomers are plotted as a function of the bound platinum/nucleotide ratio (D/N), in Figure 4B. For equivalent D/N, *trans*-DDP cross-links much greater amounts of protein to DNA than does *cis*-DDP (Figure 4B). At D/N = 0.0004, the level corresponding to 50% inhibition of SV40 DNA replication (Figure 2), approximately 6-fold more protein is cross-linked to DNA by *trans*-DDP than by *cis*-DDP. In addition, for equivalent D/N, the *cis*-DDP analogue [$Pt(en)Cl_2$] does not cross-link proteins to SV40 DNA, even though it inhibits DNA replication as effectively as *cis*-DDP (data not shown). Thus,

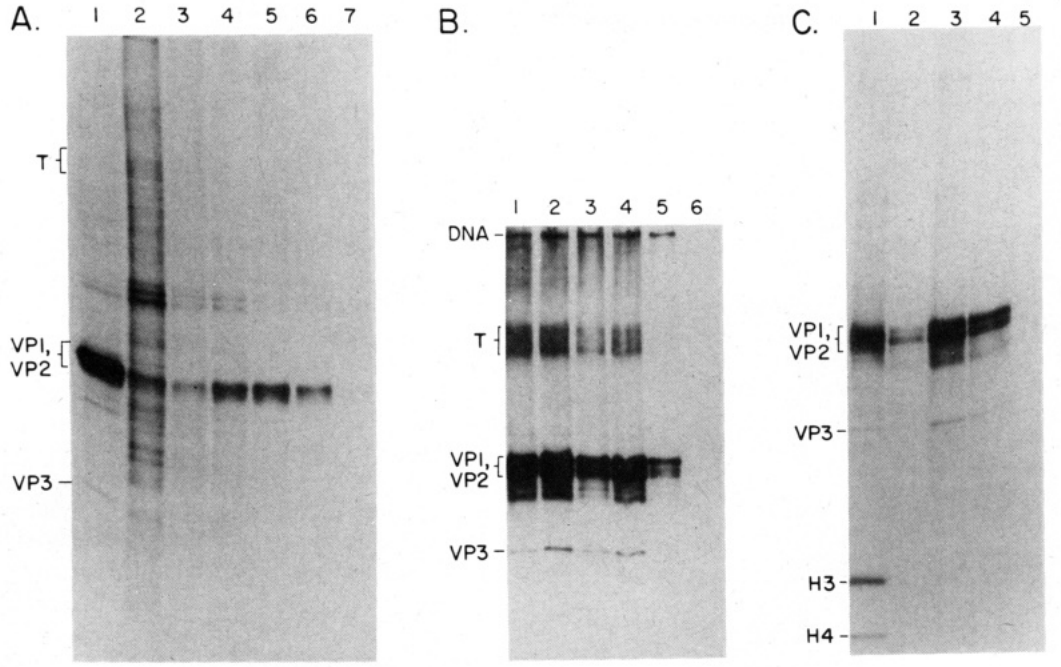

FIGURE 5: SDS–polyacrylamide electrophoresis of ^{35}S-labeled proteins released from SV40 chromosomes after reversal of platinum–DNA–protein cross-links by thiourea treatment. SV40-infected CV-1 cells were treated with *cis*- or *trans*-DDP. After double labeling with ^{3}H and ^{35}S, SV40 chromosomes were isolated at 42 h, postinfection SV40 DNA was purified, and DNA–protein cross-links were reversed by heating with thiourea as described in the text. (A) Proteins cross-linked to SV40 DNA by *trans*-DDP: (lanes 1–7) 125, 100, 75, 50, 25, 10, and 0 μM *trans*-DDP, respectively. (B) Proteins cross-linked to SV40 DNA by *cis*-DDP: (lanes 1–6) 25, 10, 5, 2, 1, and 0 μM *cis*-DDP, respectively. Constant amounts of SV40 DNA were loaded per lane, 1 μg of DNA/lane in (A) and 5 μg of DNA/lane in (B). (C) Proteins cross-linked to 1 μg of DNA from double-labeled SV40 chromosomes incubated in vitro with *cis*-DDP at 37 °C in 10 mM Tris-HCl/1 mM Na_2EDTA, pH 8.0 for 4 h: (lanes 2–5) D/N = 0.005, 0.018, 0.011, and 0, respectively; (lane 1) non-cross-linked proteins of isolated SV40 chromosomes (0.2 μg of SV40 DNA). For (A) and (B), values of D/N can be found in Figure 2. Bands corresponding to the SV40 capsid proteins VP1, VP2, and VP3, to the histones H3 and H4, and also apparently to the SV40 large T antigen are indicated.

platinum-induced formation of DNA–protein cross-links is not directly responsible for inhibition of SV40 DNA replication in vivo.

To examine the proteins cross-linked to DNA by *cis*- and *trans*-DDP, the cross-links were reversed by thiourea treatment and the released proteins analyzed by SDS–PAGE (Figures 5 and 6). When constant amounts of DNA were loaded per lane for increasing concentrations of *cis*-DDP (Figure 5B) or *trans*-DDP (Figure 5A), the amount of cross-linked proteins increased with increasing platinum concentration. The major protein species cross-linked to SV40 DNA in vivo by either of the isomers are the viral capsid proteins VP1, VP2, and VP3, as well as large T antigen (Figures 5 and 6). Above 50 μM, *trans*-DDP also cross-links cellular proteins, but these might be cross-linked to a small amount of cellular RNA or DNA present as a contaminant in the SV40 DNA preparation (see below). The only cross-linking selectivity manifested by either *cis*- or *trans*-DDP is their complete failure to cross-link SV40 DNA to histones, the major protein component of the SV40 chromosome (Figures 5 and 6). Since histones have low methionine content and consequently are not well labeled by [^{35}S]methionine, an analogous series of gels was stained with Coomassie blue (data not shown), but again no histones were detected.

SV40 chromosomes were also isolated and incubated with either *cis*-DDP or *trans*-DDP in vitro (Figure 5C and data not shown). Viral proteins are cross-linked to DNA in vitro with increasing D/N (Figure 5C); the results are similar for *cis*- and *trans*-DDP. As in vivo, histones are not cross-linked to SV40 DNA in vitro, even though the in vitro D/N is approximately 100-fold greater than that for the in vivo experiments.

When constant levels of ^{35}S per lane are loaded onto the gels (Figure 6), the protein species cross-linked by *cis*- and *trans*-DDP are similar, even though 5-fold more SV40 DNA per lane is required for the *cis*-DDP-treated samples in order to obtain equal ^{35}S counts as for *trans*–DDP-treated samples. Small amounts of cellular RNA and DNA are routinely present as contaminants in isolated SV40 chromosomes. As seen in preparations from mock-infected CV-1 cells (no SV40 added), some cellular proteins are present in the void volume of the Bio-Gel column (see Experimental Procedures) independent of the presence of SV40 DNA (Figure 6, lanes 3 and 6). These lanes were overloaded 10-fold relative to the other lanes of Figure 6 to achieve equal counts (30 000 ^{35}S cpm per lane). Total SV40 chromosomal proteins present after *cis*-DDP treatment in vivo, but prior to removal of free proteins from DNA, are shown in lane 7 (Figure 6). Similar results were obtained following *trans*-DDP treatments (data not shown).

Discussion

The available evidence strongly suggests that the cytotoxic effect of *cis*-DDP is due to its interaction with DNA [for reviews, see Roberts & Pera (1983) and Pinto & Lippard (1985a)]. Early studies with mammalian cells in culture (Harder & Rosenberg, 1970) and tumor cells in vivo (Howle & Gale, 1970) showed that DNA synthesis was inhibited by *cis*-DDP at subcytotoxic doses having little effect on the synthesis of RNA and proteins. The clinically ineffective stereoisomer, *trans*-DDP, inhibits DNA synthesis in mammalian cells only at much higher doses. The cis isomer is much more mutagenic and cytotoxic than the trans isomer in both procaryotic and eucaryotic cells (Zwelling et al., 1979a,b; Brouwer et al., 1982; Plooy et al., 1984).

Our findings with the SV40 system support the conclusions of previous studies in that *cis*-DDP inhibits DNA replication at substantially lower extracellular doses than does *trans*-DDP.

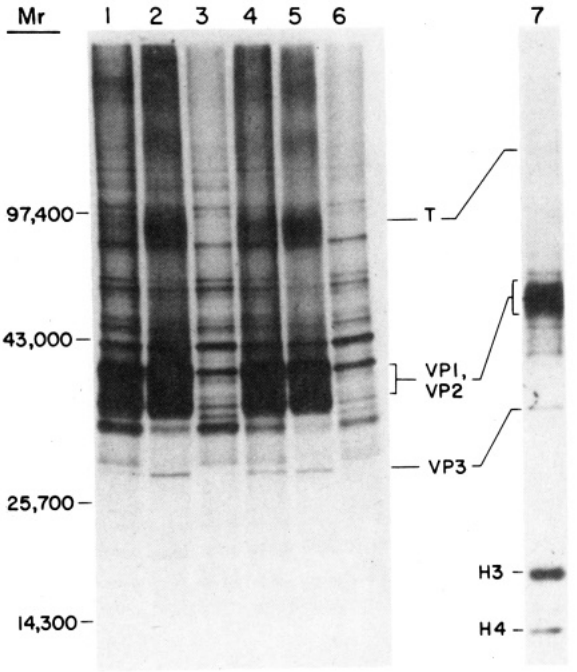

FIGURE 6: SDS–polyacrylamide electrophoresis of ^{35}S-labeled proteins cross-linked to SV40 DNA. The same protocol was used as in Figure 5 except that lanes 1–6 contain equal ^{35}S cpm (~30 000 cpm/lane). (Lanes 1 and 2) 2 μM (D/N = 0.0001) and 5 μM (D/N = 0.0004) *cis*-DDP, respectively; (lanes 4 and 5) 50 μM (D/N = 0.0002) and 100 μM (D/N = 0.0014) *trans*-DDP, respectively. Lane 3 (5 μM *cis*-DDP) and lane 6 (100 μM *trans*-DDP) are from mock SV40 infections (no added virus, see text) of CV-1 cells, followed by platinum treatment and the usual isolation procedure for SV40 chromosomes. The ^{35}S counts in lanes 3 and 6 are amplified 10-fold over normal background levels (2000–3000 cpm). Markings to the left of lane 1 denote Coomassie blue stained molecular weight standards (not shown) in order of decreasing molecular weight: phosphorylase *b*, ovalbumin, α-chymotrypsinogen, and lysozyme. (Lane 7) Non-cross-linked (total) proteins of isolated SV40 chromosomes.

At equitoxic doses, those producing the same degree of inhibition of DNA replication, the amount of Pt associated with SV40 DNA is the same for the two isomers, as well as for the related compound [Pt(en)Cl_2]. Approximately four platinum atoms are bound to the SV40 chromosome (D/N = 4×10^{-4}) when either isomer inhibits replication by 50%. These data agree with Salles et al. (1983), who reported 50% inhibition of cellular DNA replication in mouse L1210 cells for both *cis*- and *trans*-DDP at D/N = 2×10^{-4}.

Previous in vitro studies have also measured the levels of *cis*- and *trans*-DDP required to inhibit DNA replication (Harder et al., 1976; Johnson et al., 1978). In general, these in vitro replication systems require approximately 10-fold more Pt bound per DNA nucleotide to inhibit replication by 50% compared with the in vivo systems. In addition, several of the in vitro studies required much higher levels of *trans*-DDP bound to DNA to inhibit replication to the same extent as *cis*-DDP, also in contrast to our in vivo findings.

It has been postulated that certain "critical" Pt–DNA lesions, different for *cis*- and *trans*-DDP, are required to inhibit DNA replication (Zwelling, 1983; Pinto & Lippard, 1985a). Our findings support the hypothesis that different Pt–DNA lesions occur for *cis*- and *trans*-DDP, but also point to *differential repair* of these lesions by the cell as being responsible for the greatly increased cytotoxicity of the cis vs. the trans isomer. Specifically, analysis of the effects of *cis*-DDP, *trans*-DDP, and [Pt(en)Cl_2] on SV40 DNA replication in vivo suggests that, although equal amounts of the three molecules bound to DNA inhibit replication to the same extent, much higher extracellular doses of *trans*-DDP are required to produce such binding levels because the adduct of *trans*-DDP with DNA is more efficiently removed by cellular repair systems. The 1,2-intrastrand cross-link formed by *cis*-DDP, but not by *trans*-DDP due to stereochemical restrictions (Lippard 1982), might be responsible for accumulation of *cis*-DDP on DNA and, ultimately, inhibition of DNA replication. Studies by Scovell & Kroos (1982a,b) on SV40 DNA in vitro suggest that this adduct forms, in particular, at oligo(dG·dC) stretches within the origin of SV40 DNA replication. Structural perturbation of the DNA due to this adduct (Kozelka et al., 1985) may be inefficiently recognized by cellular repair systems. Other adducts, such as interstrand cross-links, various forms of intrastrand cross-links, and monoadducts, are also formed by both *cis*- and *trans*-DDP (Reedijk et al., 1983; Zwelling, 1983; Pinto & Lippard, 1985b). The ability of cells to repair any or all of these adducts, the mechanism of differential repair of *trans*-DDP–DNA adducts, and the exact relationship of these repair events to the lower cytotoxicity of *trans*- relative to *cis*-DDP remain to be established.

It was previously shown that certain *cis*- and *trans*-DDP adducts with DNA are differentially repaired in mammalian cells. Zwelling et al. (1979a,b) observed maximum formation of DNA–protein and DNA interstrand cross-links in Chinese hamster V79 cells and mouse L1210 cells 6 h after transient exposure to *trans*-DDP and 12 h following transient exposure to *cis*-DDP. Repair of these adducts occurred over the next 24 h. Studies with hamster CHO cells revealed that *trans*-DDP-induced cross-links were repaired faster, while *cis*-DDP-induced cross-links were both delayed in formation and more resistant to repair (Plooy et al., 1984). These results are not directly comparable to our findings, however, since they employed an alkaline elution technique, which can only measure interstrand and DNA–protein cross-links. These cross-links comprise only a minor fraction (1–2%) of total platinum–DNA adducts formed in vivo (Reedijk et al., 1983). Our analysis measures *total* platinum–DNA adducts (expressed as D/N) for either *cis*- or *trans*-DDP. The alkaline elution technique (Kohn et all., 1976) cannot detect intrastrand cross-links and monoadducts, which are the major adducts formed in vivo.

Other studies of the repair of *cis*-DDP adducts with DNA in CHO cells revealed that caffeine inhibits excision repair, which otherwise permits DNA replication to proceed past a DNA–platinum lesion (van der Berg & Roberts, 1976; Fraval & Roberts, 1978, 1979). In addition, a thymine dimer/excision repair-deficient cell line from a patient with *Xeroderma pigmentosum* was more sensitive to *cis*-DDP at various D/N ratios than were normal human cells (Fraval et al., 1978). These findings all point to repair as being an important aspect of *cis*-DDP cytotoxicity, as we have found in the present investigation of SV40 chromosomes.

Our results further indicate that DNA–protein cross-links formed by both *cis*- and *trans*-DDP are not directly responsible for inhibition of DNA replication. Indeed, the structural analogue of *cis*-DDP, [Pt(en)Cl_2], does not cross-link proteins to SV40 DNA in vivo yet inhibits SV40 DNA replication to the same extent as *cis*-DDP. Perhaps protein–DNA cross-linking requires loss of ammonia from *cis*-DDP (Barton & Lippard, 1980), which is not possible for [Pt(en)Cl_2]. Much higher amounts of protein are cross-linked to SV40 DNA following *trans*- vs. *cis*-DDP treatment, even though the extent of inhibition of DNA replication is similar for the two isomers at equivalent D/N. The fact that DNA–protein cross-linking is not responsible for the inhibition of SV40 DNA replication

is analogous to findings reported by others (Zwelling et al., 1979a,b; Zwelling, 1983), although Plooy et al. (1984) have suggested that DNA–protein as well as interstrand cross-links, which were persistent (not repaired), may be responsible for cytotoxicity. Since *trans*-DDP forms a larger number of DNA–protein cross-links than *cis*-DDP (per Pt bound) and [Pt(en)Cl_2] forms none, it is unlikely that such cross-links constitute the "critical lesions" responsible for inhibition of DNA replication.

Our results also show that both *cis*- and *trans*-DDP-induced DNA–protein cross-links formed in vivo contain almost exclusively non-histone proteins, even though histones are the major protein component of the SV40 chromosome (Elder et al., 1981). Other studies using HeLa cells in vivo, or isolated nuclei in vitro, have also reported cross-links only to non-histone proteins (Filipski et al., 1983; Banjar et al., 1984). An earlier study by Lippard & Hoeschele (1979) reported histone cross-links in isolated nucleosomes in vitro, but only at long incubation times and relatively high platinum concentrations.

Our findings resolve the controversy over which of the two isomers, *cis*- or *trans*-DDP, is the more efficient cross-linker of proteins to DNA. We showed that at equivalent, noncytotoxic doses *cis*-DDP cross-links more protein to DNA than the trans isomer [see also Banjar et al. (1984)], but this difference occurs because much more *cis*-DDP is bound to DNA, presumably because of the ongoing differential repair. When extracellular platinum concentrations are chosen to give equivalent D/N, i.e., equitoxic concentrations, the trans isomer is the more efficient DNA–protein cross-linker. The highly preferential cross-linking of non-histone proteins to DNA in vivo by both *cis*- and *trans*-DDP and the ease of reversing the platinum cross-links with thiourea or cyanide ion could be exploited to probe the in vivo distribution of specific chromosomal proteins on DNA.

In conclusion, our results suggest the following rationale for the enhanced biological activity of *cis*-DDP over *trans*-DDP. Both isomers are equally efficient at entering mammalian cells and diffusing into nuclei. Hydrolysis of the chlorides followed by monofunctional and bifunctional binding to DNA probably occurs with similar efficiency for both isomers. Several types of DNA adducts are formed, most of which are recognized and repaired by cellular repair systems. Certain major platinum-mediated DNA adducts, such as the 1,2-intrastrand cross-link between adjacent guanosines (only formed by *cis*-DDP), are not efficiently recognized or repaired and thus accumulate in the DNA. Accumulation of intrastrand cross-links by *cis*-DDP, and of minor adducts by both *cis*- and *trans*-DDP, would reach a critical level and arrest DNA replication, but at significantly different extracellular doses for the two isomers. Arrest of DNA replication is probably responsible for cytotoxicity, and hence antitumor activity. Our findings are consistent with the idea that the preferential toxicity of *cis*-DDP for tumor vs. normal cells may be the result of differential cellular repair processes. This hypothesis can be experimentally tested.

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Registry No. *cis*-DDP, 15663-27-1; *trans*-DDP, 14913-33-8; Pt, 7440-06-4; dichloro(ethylenediamine)platinum(II), 14096-51-6.

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Modification of Proteins by Mono(ADP-ribosylation) in Vivo†

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ABSTRACT: We have pursued the detection of in vivo modified, ADP-ribosylated proteins containing N-glycosylic linkages to arginine. ADP-ribosylated histone, elongation factor 2, and transducin, containing the different known ADP-ribosylated amino acids (arginine, diphthamide, and cysteine, respectively), were employed as model conjugates to establish conditions for the selective detection of adenosine(5′)diphosphoribose (ADP-ribose) residues bound to arginine. We report here the detection and quantification of protein-bound ADP-ribose residues in adult rat liver with linkages characteristic of arginine. These mono(ADP-ribose) residues were present in vivo at a level of 31.8 pmol/mg of protein which is 400-fold higher than polymeric ADP-ribose residues. A minor fraction (23%) of the ADP-ribose residues detected were bound via a second, more labile linkage with chemical properties very similar to those described for carboxylate ester linked ADP-ribose.

Both prokaryotic and eukaryotic cells, and some bacterial viruses as well, possess enzyme activities which catalyze the release of nicotinamide from NAD^+ and transfer of the remaining adenosine(5′)diphosphoribose (ADP-ribose)[1] moiety to specific proteins. Certain bacterial toxins represent a group of such mono(ADP-ribosyl)transferases whose physiological role is fairly well understood [reviewed by Vaughan & Moss (1981)]. For example, diphtheria toxin (Collier, 1967; Honjo et al., 1968; Gill et al., 1969) and *Pseudomonas* exotoxin A (Iglewski & Kabat, 1975) inhibit protein synthesis in vertebrate cells by catalyzing the ADP-ribosylation of elongation factor 2 (EF-2). Cholera toxin (Gill, 1975; Moss et al., 1976; Cassel & Pfeuffer, 1978), *Escherichia coli* heat-labile enterotoxin (Moss & Richardson, 1978; Gill & Richardson, 1980), and islet-activating protein (one of the pertussis toxins) (Katada & Ui, 1982a; Bokoch et al., 1983) irreversibly activate adenylate cyclase in animal cells by catalyzing the ADP-ribosylation of GTP-binding regulatory proteins of the cyclase system. Diphtheria (Van Ness et al., 1980), cholera (Moss & Vaughan, 1977), and pertussis (West et al., 1985) toxins modify proteins in vivo by catalyzing the ADP-ribosylation of either diphthamide (a hypermodified histidine), arginine, or cysteine residues, respectively.

In addition to bacterial toxins, animal cells themselves possess mono(ADP-ribosyl)transferase activities. The presence of such enzymes has been demonstrated in avian (Moss & Vaughan, 1978) and human (Moss & Stanley, 1981a) erythrocytes, rat (Moss & Stanley, 1981b; Richter et al., 1983) and bovine (Iglewski et al., 1984) liver, rat testis (Momii & Koide, 1982), rat and bovine thyroid cells (DeWolf et al., 1981; Kohn, 1978), rabbit skeletal muscle (Soman et al., 1983), and polyoma virus transformed baby hamster kidney cells (Lee &

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[1] Abbreviations: ADP-ribose, adenosine(5′)diphosphoribose; MOPS, 4-morpholinepropanesulfonic acid; etheno(ADP-ribose), 1,N^6-etheno-adenosine(5′)diphosphoribose; HPLC, high-performance liquid chromatography; EF-2, eukaryotic elongation factor 2; buffer A, 50 mM MOPS, 6 M guanidinium chloride, and 10 mM EDTA, pH 4.0 (5 °C); buffer B, 100 mM ammonium acetate, 6 M guanidinium chloride, and 10 mM EDTA, pH 9.4 (5 °C); EDTA, ethylenediaminetetraacetic acid; DHB, dihydroxyboronyl; Tris, tris(hydroxymethyl)aminomethane; Cl_3CCOOH, trichloroacetic acid; kDa, kilodaltons.

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