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cis- and *trans*-Diamminedichloroplatinum(II)-Mediated Cross-Linking of Chromosomal Non-Histone Proteins to DNA in HeLa Cells†

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ABSTRACT: The cross-linking of chromosomal non-histone proteins to DNA in isolated nuclei or intact HeLa cells exposed to different concentrations of *cis*- and *trans*-diamminedichloroplatinum(II) (*cis*- and *trans*-DDP) for various time intervals was investigated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunochemical methods. Both the *cis*- and the *trans*-DDP cross-linked significant numbers of chromosomal non-histone proteins to the DNA. The quantity and the types of the cross-linked proteins depended on the time of incubation as well as on the concentrations of the drugs. The immunochemical techniques revealed that both the 0.35 M sodium chloride insoluble and soluble chromosomal non-histone proteins were cross-linked to the DNA by both isomers. The action of *cis*- and *trans*-DDP was reversed and/or blocked by thiourea or 2-mercaptoethanol. Pretreatment of isolated nuclei or chromatin with iodoacetamide or *N*-ethylmaleimide did not prevent the DNA–protein cross-linking.

Since Rosenberg et al. (1965) reported the growth-inhibitory effects of the platinum complexes in bacteria, several investigators have tested this class of compounds for their antitumor activity (Roberts & Thomson, 1979). Because of its clinical applications in human cancer chemotherapy, *cis*-diamminedichloroplatinum(II) (*cis*-DDP)[1] and its biologically inactive isomer *trans*-diamminedichloroplatinum(II) (*trans*-DDP) have been subjected to intensive investigations. Cellular DNA is the principal target of this drug, and the interaction is of a bifunctional nature in a manner analogous to bifunctional alkylating agents. However, there is disagreement as to whether the critical lesion is an inter- or intrastrand DNA cross-linking (Roberts & Thomson, 1979; Shooter et al., 1972; Pascoe & Roberts, 1974). While extensive work has been done on the interaction of *cis*-DDP with DNA (Zwelling & Kohn, 1980), very little is known about the interaction of *cis*-DDP with proteins. Recent literature indicates that in addition to intra- and interstrand cross-linking of DNA, the platinum coordination complexes could also form covalent protein–DNA cross-links (Zwelling et al., 1979; Lippard, 1982; Filipski et al., 1983; Banjar et al., 1983). In this paper, we used SDS–polyacrylamide gel electrophoresis and immunochemical methods to investigate the nature of the protein–DNA cross-links in isolated nuclei or intact live HeLa cells exposed for various times and to different concentrations of *cis*- as well as *trans*-DDP. Our results show that both isomers cross-linked significant numbers of chromosomal non-histone proteins to DNA in isolated nuclei or intact cells and that the quantity and the quality of the cross-linked proteins is time as well as concentration dependent. Furthermore, the immunochemical methods showed that both the 0.35 M NaCl insoluble and soluble chromosomal non-histone proteins cross-linked to the DNA, suggesting that both types of proteins are closely associated with the DNA and within the cross-linking distance of the two isomers. Thus, in addition to its importance in human cancer chemotherapy, *cis*-DDP and its biologically inactive isomer can be used to probe chromatin structure.

Materials and Methods

Cell Culture. HeLa cells (S3 strain) were maintained in suspension cultures in Eagle's (1959) minimum essential medium as modified by W. Joklik and supplemented with 3.5% each of calf and fetal calf serum.

Nuclei Isolation. Cells in cultures were harvested by centrifugation at 1200 rpm for 10 min. Nuclei were isolated by homogenizing the cells in 0.25 M sucrose/0.01 M Tris-HCl, pH 7.5, and centrifuging at 660*g* for 10 min. The crude nuclei were further purified by homogenization in 2.2 M sucrose/0.01 M Tris-HCl/5 mM $MgCl_2$, pH 7.5, and centrifugation at 110000*g* for 1 h. Finally, nuclear pellets were homogenized in 0.25 M sucrose/0.01 M Tris-HCl 0.5% (v/v) Triton X-100, pH 7.5, until dispersed, and nuclei were sedimented by centrifugation at 1000*g* for 10 min. Triton X-100 was removed by washing the nuclei in 0.25 M sucrose/0.01 Tris-HCl, pH 7.5, followed by centrifugation at 1000*g* for 10 min. All solutions used for the isolation of nuclei contained 0.1 mM phenylmethanesulfonyl fluoride (PMSF) and were used at 0–4 °C.

Drug Treatment. cis-DDP and *trans*-DDP (Sigma) were dissolved in water with gentle heating and stirring and used within 2 h. Suspension of cells (1×10^8 cells) in fresh serum-free medium or corresponding amounts of nuclei in 1 mM

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[1] Abbreviations: *cis*- and *trans*-DDP, *cis*- and *trans*-diamminedichloroplatinum(II); SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PAP, peroxidase–antiperoxidase; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane.

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potassium phosphate buffer, pH 7.5, were incubated with continuous shaking at 37 °C with the above drugs at different concentrations and for various time intervals. The reaction was quenched by raising the chloride concentration to 0.2 M and making the incubation mixtures 5 mM thiourea by addition of concentrated NaCl and thiourea solutions. In some experiments, the reaction mixtures were made 1 M thiourea before addition of the drugs. In other experiments, after the initial incubation, the reaction mixtures were made 1 M thiourea and incubated for an additional 2 h at 37 °C. Controls were treated with the same solvents without drugs. After incubation, nonreacted drugs were removed by centrifugation at 3000 rpm for 15 min.

To determine the involvement of sulfhydryl groups in the DNA–protein cross-linking, isolated HeLa nuclei were first incubated in 2 mM Tris-HCl/10 mM iodoacetic acid buffer, pH 8.0, at 37 °C for 1 h, in 10 mM Tris-HCl/10 mM iodoacetamide buffer, pH 8.0, at room temperature for 2 h, or in 0.1 M potassium phosphate/10 M *N*-ethylmaleimide buffer, pH 7.0, at room temperature for 2 h. After incubation, the mixtures were centrifuged at 3000 rpm at 15 min, resuspended in 1 mM potassium phosphate buffer, pH 7.5, and centrifuged again. The washed nuclear pellets were then reacted with 1 mM *cis*-DDP/1 mM potassium phosphate buffer, pH 7.5, by incubation at 37 °C for 1 h. After incubation, the nuclei were processed as described.

Isolation of Proteins Cross-Linked to DNA. Controls and treated cells or nuclei were homogenized gently in 2% SDS/50 mM Tris-HCl/5 mM thiourea/0.1 mM PMSF, pH 8, and stirred overnight. The thiourea was incorporated into the lysing solution to prevent side-chain reactions of monofunctional platinum–DNA adducts (Eastman, 1983). In some experiments, thiourea was omitted. The un-cross-linked proteins were then removed by centrifugation at 110000*g* for 18 h. This step was repeated, and the resulting DNA pellets were then hydrated, extensively sheared by sonication (Glass et al., 1980), and digested (1 h, 4 °C) with 40 μg of DNase I Worthington Biochemical Corp., Freehold, NJ) per mg of DNA. The released proteins were solubilized for gel electrophoresis.

Polyacrylamide Gel Electrophoresis. Samples were mixed with 0.9 volume of solubilizing solution [0.139 M Tris/4.44% SDS/22.2% (w/v) glycerol/25 μg/mL pyronin Y] and with 0.1 volume of 2-mercaptoethanol. The samples were boiled for 5 min, resonicated, and then electrophoresed under conditions described by Laemmli (1970) in a 3% stacking gel and 7.5% running gel. The gels were stained with Coomassie Brilliant Blue.

Immunochemical Detection of Cross-Linked Antigens. Identification of immunoreactive proteins cross-linked to DNA was accomplished by the method of Glass et al. (1980). Briefly, proteins separated with SDS–PAGE were transferred to nitrocellulose sheets as described by Towbin et al. (1979) with some modifications. The nitrocellulose sheets were then incubated with the appropriate antiserum, and the antigen–antibody complexes were visualized by the peroxidase–antiperoxidase (PAP) procedure (Sternberger, 1974).

Two antisera, raised in New Zealand white rabbits following the schedule described by Chytil & Spelsberg (1971), were used in our study. One antiserum was raised to the 0.35 M NaCl extract of HeLa nuclei and the other one to the residual, 0.35 M NaCl washed nuclei.

Results

cis-DDP Cross-Linking of Nuclear Proteins to DNA. As described under Materials and Methods, purified nuclei or intact live HeLa cells were incubated with *cis*-DDP at a final concentration of 2 mM for 2 h at 37 °C. After incubation, cells or isolated nuclei were dissolved in 2% buffered SDS, and the unbound proteins were removed by extensive ultracentrifugation. The DNA pellets were then processed as described under Materials and Methods, and the cross-linked proteins were analyzed by SDS–polyacrylamide gel electrophoresis. As can be seen in Figure 1, a considerable number of nuclear proteins (as detected by Coomassie Brilliant Blue staining) became cross-linked (since they resisted repeated extraction with 2% SDS) to DNA when isolated nuclei (lane 2) or intact cells (lane 1) were treated with *cis*-DDP. Although the Coomassie Brilliant Blue stained gels showed both qualitative and quantitative differences between the intact cells and isolated nuclei, the immunodetection of antigens with antisera to 0.35 M NaCl nuclear wash and 0.35 M NaCl nuclear residue revealed only changes in the quantities of cross-linked proteins (Figure 1, lanes 1 and 2 in panels B and C). No antigens or non-histone proteins (Coomassie Brilliant Blue staining) could be detected in samples containing an equivalent amount of DNA from untreated controls (Figure 1, lanes 3). However, there were differences between cross-linked antigens detectable by the two antisera, although some antigens could be detected by both.

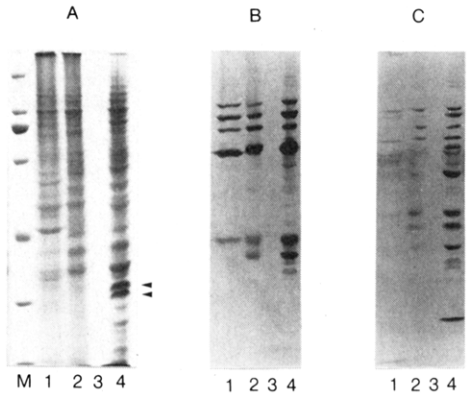

FIGURE 1: Cross-linking of chromosomal non-histone proteins to DNA by treatment with 2 mM *cis*-DDP for 2 h. (A) Coomassie Brilliant Blue stained gels containing non-histone proteins that sedimented with the DNA in 2% SDS (see Materials and Methods for details). The individual lanes are (M) molecular weight standards (myosin, M_r 200 000; β-galactosidase, M_r 116 000; phosphorylase B, M_r 94 000; bovine serum albumin, M_r 68 000; ovalbumin; M_r 43 000; carbonic anhydrase, M_r 32 000), (1) 60 μg of DNA pellet from intact cells incubated with *cis*-DDP, (2) 60 μg of DNA pellet from *cis*-DDP-treated isolated nuclei, (3) 60 μg of DNA from untreated control cells, and (4) total HeLa nuclear proteins (20 μg as DNA of untreated nuclei dissolved directly in the electrophoresis buffer). The position of H1 histone is indicated by the arrows in lane A-4. (B) Duplicated gel as in (A) but transferred to a nitrocellulose sheet, which was incubated with antiserum to 0.35 M NaCl soluble proteins of isolated HeLa nuclei and immunochemically stained with peroxidase–antiperoxidase. The molecular weight standards were omitted. (C) Duplicate gels as in (B) incubated with antiserum to 0.35 M NaCl insoluble residue of HeLa cell nuclei. Lanes 1–4 are the same as described in (A).

Because of the low concentration of acrylamide in the gels (7.5%), the core histones are not represented in the electrophoretic patterns shown here. Only the H1 histones, due to their anomalous electrophoretic migration, are detectable (arrows in Figure 1A, lane 4). It is noteworthy that very little, if any, DNA–H1 cross-linking can be detected either in the Coomassie Brilliant Blue stained gels or in the immunotransfers (Figure 1 and the subsequent figures).

Kinetics of the Protein–DNA Cross-Linking by cis-DDP. Effects of the incubation time as well as the concentration of *cis*-DDP on the protein–DNA cross-linking were also tested.

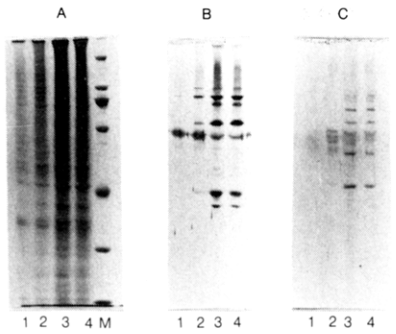

FIGURE 2: Effects of *cis*-DDP concentration on DNA–protein cross-linking. Live HeLa cells were incubated with indicated *cis*-DDP concentrations for 1 h. Each experimental point represents the same number of HeLa cells. (A) Coomassie Brilliant Blue staining of proteins sedimented with DNA in 2% SDS. Each lane contains protein equivalent to 60 μg of DNA in the pellet. (Lanes 1–4) DNAs from cells incubated with 0.2, 0.5, 2.0, and 1.0 mM *cis*-DDP, respectively. (Lane M) Molecular weight standards (as in Figure 1). (B) Duplicate gel transferred to a nitrocellulose sheet, incubated with antiserum to the 0.35 M NaCl HeLa nuclear extract and developed with peroxidase–antiperoxidase staining. (C) Duplicate gel incubated with antiserum to the 0.35 M NaCl HeLa nuclear residue. Lanes 1–4 are as indicated in (A).

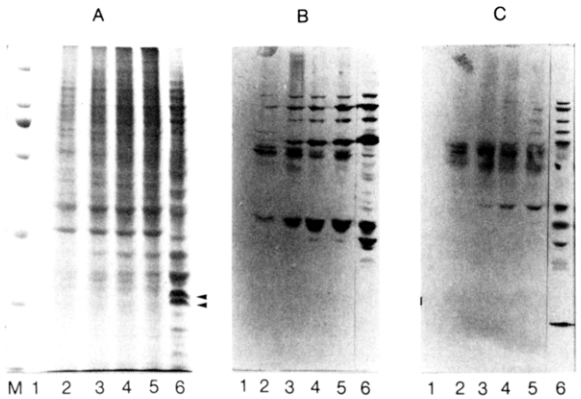

FIGURE 3: Effects of the incubation time on DNA–protein cross-linking by *cis*-DDP. Equal numbers of HeLa cells were incubated with 1 mM *cis*-DDP for the indicated time intervals, and their DNA was analyzed for associated proteins. (A) Coomassie Brilliant Blue staining of proteins equivalent to 60 μg of DNA in the pellets. (Lane M) Molecular weight standards as indicated in Figure 1; (lane 1) DNA from untreated cells; (lanes 2–5) DNA from cells incubated with 1 mM *cis*-DDP for 15, 30, 60, and 120 min, respectively; (lane 6) total proteins of HeLa nuclei (20 μg of DNA equivalent). Arrows in lane A-6 indicate the position of H1 histones. (B and C) Duplicate gels transferred to nitrocellulose sheets, incubated with antiserum to 0.35 M NaCl nuclear extract (B) or residue (C) and developed with peroxidase–antiperoxidase staining. Lanes 1–6 are the same as in (A).

Intact HeLa cells were exposed to various concentrations of *cis*-DDP for 1 h or to 1 mM *cis*-DDP for different time intervals. The cross-linked proteins were then isolated and analyzed by both the SDS–PAGE and the immunotransfer method. As can be seen in Figure 2, the Coomassie Brilliant Blue as well as the immunochemical staining showed that the amount of proteins and/or antigens cross-linked to the DNA increased with the *cis*-DDP concentration. The Coomassie Brilliant Blue staining in Figure 3A shows that the quantity of proteins cross-linked to the DNA also increases with the time of incubation with *cis*-DDP. This was also confirmed by the immunochemical staining of the cross-linked protein antigens transferred to nitrocellulose sheets (Figure 3B,C).

Cross-Linking of Chromosomal Proteins to DNA by trans-DDP. The protein–DNA cross-linking by the biologically inactive isomer *trans*-DDP was also examined. Purified

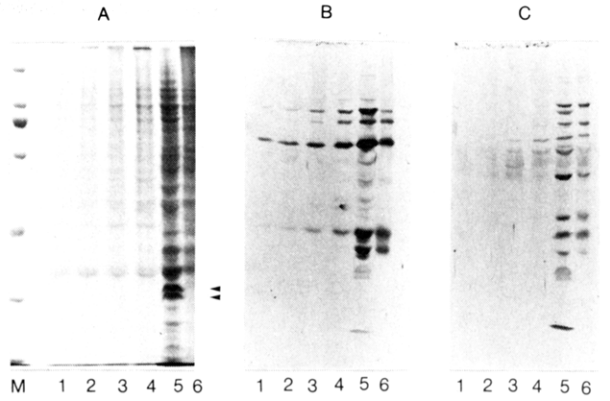

FIGURE 4: *trans*-DDP DNA–protein cross-linking in HeLa cells. Equal numbers of HeLa cells were incubated with 1 mM *trans*-DDP for the indicated time intervals and analyzed as described under Materials and Methods. (A) Coomassie Brilliant Blue stained gels containing DNA-cross-linked proteins. (Lane M) molecular weight standards (as described in Figure 1); (Lanes 1–4) DNA (100 μg) from cells incubated with 1 mM *trans*-DDP for 15, 30, 60, and 120 min respectively; (lane 5) total proteins of isolated HeLa nuclei (20 μg of DNA equivalent); (lane 6) DNA (100 μg) from nuclei incubated with 2 mM *trans*-DDP for 2 h. Arrows in lane A-6 indicate the position of H1 histones. (B and C) Duplicated gels transferred to nitrocellulose sheets, incubated with antiserum to 0.35 M HeLa nuclear extract (B) or nuclear residue (C) and developed with peroxidase–antiperoxidase staining.

nuclei or intact HeLa cells were treated with *trans*-DDP and the cross-linked proteins analyzed by SDS–PAGE and by immunotransfers. The Coomassie Brilliant Blue staining (Figure 4A) showed again that many chromosomal proteins became cross-linked to the DNA by incubation of intact HeLa cells (lanes 1–4) or isolated nuclei (lane 6) with *trans*-DDP. Most antigens that can be detected with both antisera (Figure 4B,C) were cross-linked to the DNA. Again, both the immunological and the Coomassie Brilliant Blue staining showed little difference between the cross-linking patterns of intact cells or isolated nuclei, and again, there were some differences between cross-linked proteins detectable by the two antisera. The experiments also showed that the amount of proteins and/or antigens cross-linked to DNA increased with the incubation time. Comparison of corresponding lanes in Figures 3 and 4 shows that essentially the same antigens became cross-linked by both isomers. However, it was necessary to load nearly twice as much of the *trans*-DDP-treated samples (DNA) on each gel to match the staining intensity of the *cis*-DDP samples.

Concentration Dependence of the Protein–DNA Cross-Linking by trans-DDP. Intact HeLa cells were exposed to various concentrations of *trans*-DDP for 1 h at 37 °C. The cross-linked proteins and/or antigens were then analyzed as previously described. Both the Coomassie Brilliant Blue and the immunochemical staining (Figure 5) show that the quantity of the proteins and/or antigens cross-linked to DNA increased with the concentration of *trans*-DDP, i.e., situation already observed for the cis isomer. Again there were no significant qualitative differences between the cross-linking patterns of *cis*- or *trans*-DDP.

Thiourea Reverses the Action of cis- and trans-DDP. The ability to reverse and/or block the formation of protein–DNA cross-links by the two isomers was tested by using thiourea (Figure 6). The incubation mixtures of HeLa cells with the above drugs were made 1 M thiourea before (to block the reaction) or after (to reverse the reaction) the addition of the drugs. In the later case, the reaction mixtures were incubated for an additional 2 h (see Materials and Methods). As can

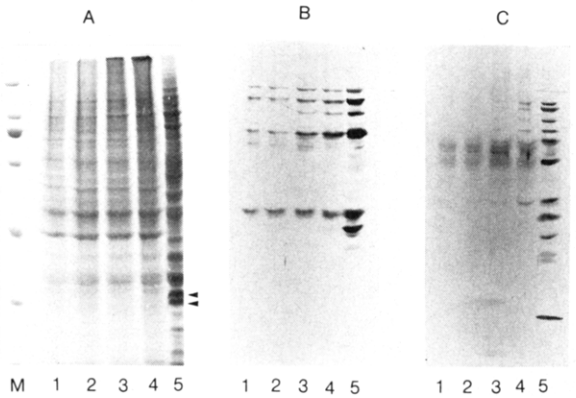

FIGURE 5: Effects of the *trans*-DDP concentration on DNA protein cross-linking in HeLa cells. Equal numbers of HeLa cells were incubated with the indicated concentrations of *trans*-DDP for 1 h. (A) Coomassie Brilliant Blue stained gels of DNA containing the cross-linked proteins. (Lane M) Molecular weight standards (as indicated in Figure 1); (lanes 2–4) 100 μg of DNA from cells incubated with 0.2, 0.5, 1.0, and 2.0 mM *trans*-DDP, respectively; (lane 5) total HeLa nuclear proteins (20 μg of DNA equivalent). Arrows in lane A-5 indicate the position of H1 histones. (B and C) Duplicate gels transferred to nitrocellulose sheets, incubated with antiserum to the 0.35 M NaCl nuclear extract (B) or nuclear residue (C) and developed with peroxidase–antiperoxidase staining.

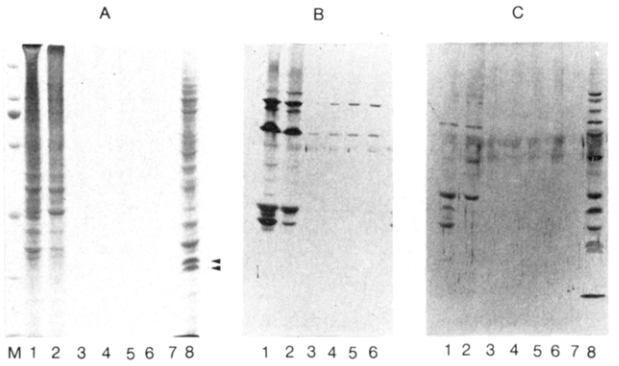

FIGURE 6: Effects of thiourea on the *cis*- or *trans*-DDP-mediated DNA–protein cross-linking in HeLa cells. Equal numbers of HeLa cells were incubated and treated as shown. (A) Coomassie Brilliant Blue stained gels. (Lane M) Molecular weight standards (see Figure 1); (lane 1) HeLa cells incubated with 1 mM *cis*-DDP for 1 h, 60 μg of DNA, no thiourea; (lane 2) conditions as in lane 1 with 5 mM thiourea in the 2% SDS-solubilizing buffer; (lane 3) same as in lane 1, except the culture was made 1 M in respect to thiourea before adding the *cis*-DDP and the incubation time was 1 h; (lane 4) *trans*-DDP incubated with cells under conditions described in lane 3 (1 M thiourea); (lane 5) cells were first incubated in 1 mM *cis*-DDP for 1 h, then thiourea was added to the culture (1 M final concentration), and the incubation was continued for 2 h before solubilizing the cells; (lane 6) the same experiment as in lane 5 with *trans*-DDP; (lane 7) untreated control cells (60 μg of DNA); (lane 8) total proteins of isolated HeLa nuclei (20 μg of DNA equivalent). Arrows in lane A-8 indicate the position of H1 histones. (B and C) Duplicate gels transferred to nitrocellulose sheets, incubated with antiserum to the 0.35 M NaCl nuclear extract (B) or nuclear residue (C) and developed with peroxidase–antiperoxidase staining.

be seen in Figure 6, thiourea reversed and/or blocked the protein–DNA cross-linking by either of the two isomers. Two antigenic bands, which were detectable with the antiserum to 0.35 M NaCl extract of HeLa nuclei, were partially resistant to the action of thiourea. The experiment also shows that addition of thiourea, final concentration of 5 mM, to the 2% buffered SDS dissociating solution somewhat decreased the cross-linking efficiency of *cis*-DDP, lane 2, without changing the number of cross-linked antigens (compare Figure 6B,C, lanes 1 and 2). Apparently, thiourea, even at only 5 mM concentration, was able to partially reverse the *cis*-DDP-facilitated DNA–protein cross-links. Some of the cross-linked antigens seem to be more sensitive to the action of thiourea than others (e.g., antigens of approximately M_r 40K–46K in Figure 6B,C, approximately M_r 96K in Figure 6B, and approximately M_r 48K in Figure 6C). 2-Mercaptoethanol, in 5% concentration, completely reversed or prevented the cross-linking. Incubation of isolated nuclei with 10 mM iodoacetamide, iodoacetic acid, or *N*-ethylmaleimide somewhat weakened, but did not prevent, the DNA–protein cross-linking (data not shown). Our results also indicate that the observed cross-links are formed between DNA and adjacent proteins and do not involve reactions of monofunctional DNA–platinum adducts with cellular proteins following the disruption of nuclear morphology.

Discussion

The nuclei in eukaryotic cells contain complexes of DNA with histones and a considerable number of chromosomal non-histone proteins. Studies with platinum-labeled *cis*- and *trans*-DDP showed that both isomers enter the cell and bind to the DNA with equal efficiency (Johnson et al., 1980). The interaction of *cis*- and *trans*-DDP with DNA has been the subject of several investigations, and it is now generally accepted that DNA is the principal target for the two isomers (Roberts & Thomson, 1979). However, the specific nature of the cytotoxic lesion caused by *cis*-DDP remains to be resolved. Studies with deoxyribonucleosides (Eastman, 1982) showed that monofunctional platination occurred at N(7) of guanosine, N(3) of cytidine, N(1) of adenosine, and N(7) of adenosine. Bifunctional platination occurred with adenosine N(7) to N(7), guanosine N(7) to N(7), and adenosine N(1) to N(7). Also, mixed bifunctional products N(7) of guanosine to N(1) of adenosine and N(7) of adenosine to N(7) of guanosine were formed. DNA interstrand cross-links have been reported by several investigators (Roberts & Thomson, 1979), and their formation seems to correlate with the cytotoxicity (Zwelling et al., 1979, 1981). Intrastrand cross-linking of DNA has also been reported (Kelman et al., 1972). Due to their bifunctionality, the platinum coordination complexes could also form covalent protein–DNA cross-links in a manner analogous to bifunctional alkylating agents. Indeed, recent literature indicates that *cis*- and *trans*-DDP can form protein–DNA cross-links (Zwelling et al., 1979, 1981; Lippard, 1982, Filipski et al., 1983; Banjar et al., 1983). However, the nature of the cross-linked proteins has not been investigated. In our studies, we have applied a sensitive immunochemical method, combined with the high-resolution power of SDS–polyacrylamide gel electrophoresis, to investigate the nature of these protein–DNA cross-links. Our results show that treatment of HeLa cells or pure isolated nuclei with *cis*- or *trans*-DDP resulted in cross-linking of several proteins to the DNA. The protein patterns cross-linked by either of the two isomers were very similar. However, at equal concentrations and for the same periods of incubation, *cis*-DDP was a more efficient protein–DNA cross-linker than the trans isomer. That not all the nuclear proteins were cross-linked to DNA indicates selectivity of the cross-linking mechanism. There was little qualitative difference in the cross-linking patterns obtained by incubation of intact live cells or isolated nuclei. Hence, the isolation of nuclei did not change significantly the relationships between the cross-linkable chromosomal proteins and DNA, indicating that many nuclear proteins are associated with the DNA in intact live cells.

Using analytical electron microscopy, Khan & Sadler (1978) studied the distribution of platinum in HeLa cell sections after treatment with *cis*-DDP. Their results showed

that the nucleolus and the inner side of the nuclear double membrane were the primary targets for the drug. Very little platinum was found in the cytoplasm. This observation may explain the surprising efficiency of *cis*-DDP in forming the DNA–protein cross-links.

The immunochemical localization of the cross-linked antigens not only confirmed the Coomassie Brilliant Blue staining results but also distinuished between the weakly and tightly DNA-bound chromosomal proteins (i.e., antisera to the nuclear 0.35 M NaCl wash and residue). Our results show that the 0.35 M NaCl insoluble and the 0.35 M NaCl soluble chromosomal proteins are closely associated with the DNA and within the cross-linking distance of the two isomers.

Roberts & Friedlos (1981) showed that the extent of DNA interstrand cross-linking and the total binding of platinum to the DNA increased linearly with dose over a wide range of concentrations of *cis*-DDP in Chinese hamster cells. Our results indicate that the quantity (but not the numbers) of antigens that sediment with the DNA and can be detected by our antisera also increased with time or with concentration of the cross-linking agent. We interpret this for the cross-linking to be selective for proteins either associated with or close to the DNA, i.e., within the relative short cross-linking distance of the platinum coordination complexes. Random associations such as those resulting from entrapment of proteins within the growing network of cross-linked DNA would be expected to show a time- or concentration-dependent increase in the qualitative complexity of the DNA-associated polypeptides. The interpretations of our results are based on the assumption of simple DNA–protein cross-links. The possible presence of protein–protein–DNA trimers or, perhaps, even polymers was not addressed in our experiments. However, the relative simplicity of the electrophoretic patterns shown for the individual cross-linked antigens, which did not change qualitatively with time or increasing concentrations of the platinum complexes, argues against a significant presence of such trimers or polymers in the incubated cells or nuclei. Indeed, the main advantage of using the immunochemical detection of cross-linked proteins is that each antigen can be followed even when complexed with other macromolecules and, thus, migrating at a different position.

DNA–protein cross-links have been detected by a number of investigators using different techniques. Reduction in alkalin elution rates, which were reversed by incubation of the cell lysates with proteinase K, were reported when L1210 mouse leukemia cells were treated with *cis*- or *trans*-DDP (Zwelling et al., 1979). The binding of *cis*- and *trans*-DDP to isolated nucleosomes cores was studied by Lippard & Hoeschele (1979). *cis*-DDP was found to react mainly with DNA and to produce histone–DNA cross-links, but only at relatively high doses and long incubation periods. On the other hand, *trans*-DDP produced extensive histone–histone and histone–DNA cross-linking. Contrary to these findings, we could not detect significant amounts of histone–DNA cross-links. Recently, Filipski et al. (1983) reported that treatment of mouse L1210 nuclei with *cis*-DDP resulted in cross-linking non-histone proteins to DNA to the extent that their nuclear morphology was retained even in the presence of SDS. Histones were not cross-linked to this nuclear structure and were extractable with SDS. The bifunctional platinum compounds have high affinities for sulfur atoms within proteins and amino acids (Morris & Gale, 1973; Odenheimer & Wolf, 1982). Among histones, only H3 contains sulfhydryl residues but these are buried and, hence, not reactive (McGhee & Felsenfeld, 1980).

Among the sulfur-containing compounds, thiourea has been reported to be the most effective reagent in reversing the binding of *cis* and *trans*-DDP to DNA (Filipski et al., 1979). Cyanide ion has also been employed to remove platinum from biopolymers (Baner et al., 1978) and was successfully used to identify protein–protein cross-links formed by *trans*-DDP in isolated nucleosome cores (Lippard & Hoeschele, 1979). Thiourea, in our experiments, reversed and/or blocked the protein–DNA cross-linking by *cis*- and *trans*-DDP. As suggested by Eastman (1983), we also used this compound to prevent the side reaction of monofunctional platinum–DNA adducts by incorporating 5 or 10 mM thiourea into the SDS-solubilizing solution (cross-linking of cytoplasmic proteins to the DNA during the solubilization and ultracentrifugation steps). Thiourea was also used by Filipski et al. (1983) to reverse the DNA–protein cross-links formed by treatment of mouse L1210 nuclei with *cis*- and *trans*-DDP. However, if active sulfhydryl groups were the principal target of the observed DNA–protein cross-linking, preincubation of isolated nuclei or chromatin with 10 mM concentrations of several sulfhydryl reactive agents should have significantly decreased or abolished this reaction. Since the preincubation of nuclei had only a small effect on the cross-linking, it appears that other reactive protein groups may be involved in the descried phenomenon. Indeed, Odenheimer & Wolf (1982) reported that the nitrogen atoms within proteins and amino acids can also participate in the binding of *cis*-DDP. This type of binding could account for the cross-linking of histone to DNA seen by other investigators in isolated nucleosome cores.

Except for the histones, the exact identity of the proteins cross-linked to the DNA by the two isomers is still unknown. However, the cross-linking of antigenic proteins to the DNA by *cis*- or *trans*-DDP followed by their immunochemical detection with specific antisera can be used to identify at least some of the individual antigens.

Registry No. *cis*-DDP, 15663-27-1; *trans*-DDP, 14913-33-8.

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Chemically Induced Dynamic Nuclear Polarization Studies of Guanosine in Nucleotides, Dinucleotides, and Oligonucleotides†

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ABSTRACT: The nuclear magnetic resonance (NMR) technique of chemically induced dynamic nuclear polarization (CIDNP) has been used to study the reactions between photoexcited flavins and a wide variety of nucleotides, dinucleotides, and oligonucleotides. The greatest emphasis is placed on the purine nucleosides adenosine (A), inosine (I), and guanosine (G), particularly guanosine. The presence of G suppresses the CIDNP effect for A, although A by itself shows very strong CIDNP [Kaptein, R., Nicolay, K., & Dijkstra, K. (1979) *J. Chem. Soc., Chem. Commun.*, 1092–1094]. Very intense CIDNP signals are observed for the H8 proton in G-containing mononucleotides, but no nuclear polarization is detected for the sugar H1′ proton. In contrast, both H8 and H1′ protons exhibit CIDNP for G in a wide range of dinucleotides and higher oligonucleotides. Several possible mechanisms are analyzed to explain the H1′ polarization, and it is concluded that the sugar H1′ proton probably obtains spin density through interaction with guanine nitrogen 3. The proximity of the H1′ proton to N3 depends explicitly on the glycosidic torsion angle, χ. CIDNP studies of several model compounds in which χ is fixed are consistent with this suggestion. CIDNP for the self-complementary tetramer ApGpCpU was studied as a function of temperature. Strong CIDNP from G is only observed at temperatures above the double-strand melting temperature, suggesting that CIDNP is only detected in single-stranded regions, where the base is accessible to solvent. The use of brominated riboflavin as the photoreagent in place of riboflavin is shown to selectively invert the sign of A, I, and 1-methylguanosine polarization, providing a convenient method for distinguishing the NMR spectra of these residues in complex oligonucleotides.

The conformations of nucleic acids and the forces responsible for maintaining the structures of oligonucleotides provide a basis for understanding their biological function. Nuclear magnetic resonance (NMR)[1] and circular dichroism (CD) have been the primary methods used to determine the conformations of nucleotides, oligonucleotides, and nucleic acids in solution [Sarma (1980) and references cited therein; Davies, 1978a; Johnson, 1978]. Measurements of vicinal coupling constants can provide values (or ranges of values for species equilibrating between conformations) for the backbone torsion angles β, γ, δ, and ϵ but not for the glycosidic torsion angle χ or for the phosphodiester bond angles α and ζ (see G in Figure 1 for notation). At low temperatures, the phosphodiester bond angles are assumed to be predominantly in the $\zeta^{-}\alpha^{-}$ conformation, on the basis of chemical shift calculations and consideration of molecular models [Sarma (1980) and references cited therein].

It is generally assumed that in neutral aqueous solution all naturally occurring nucleic acids exist as an equilibrium mixture of syn ($-90° < \chi < 90°$) and anti ($90° < \chi < 270°$) conformations, with a preference for the anti conformation (Lee et al., 1976). Indirect NMR parameters can be used to estimate χ, such as the chemical shifts of the base and ribose protons as a function of temperature, pH, and various substituents, changes in chemical shifts and relaxation times upon

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[1] Abbreviations: CD, circular dichroism; CIDNP, chemically induced dynamic nuclear polarization; 8BrF, 7-methyl-8-bromo-10-(1-D-ribityl)isoalloxazine; DSS, 3-(trimethylsilyl)-1-propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; NOE, nuclear Overhauser enhancement; NMR, nuclear magnetic resonance. Standard abbreviations are used for nucleic acids (Davies, 1978a; Nishimura, 1978). Nomenclature used for torsion angles in the nucleotides is from Davies (1978b); all references to angles from other sources have been converted to this nomenclature.

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