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# Effects of *cis*-Diamminedichloroplatinum (CDDP) on HeLa Cell Non-Histone Nuclear Proteins

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***Summary.*** *Two-dimensional isoelectric focusing and sodium dodecyl sulfate (SDS) gel electrophoretic analyses of successive salt extracts of purified HeLa cell nuclei were used to study the effects of cis-diamminedichloroplatinum (CDDP) (cisplatin) on the synthesis and extractability of nuclear non-histone proteins. Nuclei were extracted sequentially with 0.025 M NaCl–0.075 M EDTA, 0.01 M Tris, and 0.6 M NaCl. Each fraction contained 100–400 polypeptide spots, only a few of which were affected by a 3.5-h CDDP pretreatment of the cells. The biosynthesis and/or metabolism of four polypeptide spots was significantly affected by the CDDP treatment. These polypeptides included: (a) 36K/5.8 (designated by MW/PI) in the 0.025 M NaCl–0.075 M EDTA extract, which decreased in intensity with treatment at 10 μg CDDP/ml; (b) polypeptide spots 50K/6.0 and 45K/5.3 in the Tris extract, which increased in intensity over a range of CDDP concentrations of 0–5 μg CDDP/ml; and (c) a polypeptide complex at 110K/7.7 in the 0.6 M Na Cl extract, which decreased in intensity at CDDP concentrations of 0–5 μg CDDP/ml. Scanning densitometry of the protein spots of the 0.6 M NaCl extract demonstrated that the 110K/7.7 complex decreased to half its intensity compared with non-drug-treated controls at a CDDP concentration of 0.9 μg CDDP/ml. We have found that high-resolution two-dimensional gel electrophoretic analysis of nuclear proteins is a valuable technique for studying the effects of cytotoxic agents on the synthesis and/or extractability of specific cellular proteins.*

## Introduction

CDDP is an effective antitumor agent used clinically for the treatment of head and neck cancer and testicular and ovarian carcinomas. The mechanism of action of CDDP is generally thought to involve its interaction with cellular DNA. CDDP forms intra- and interstrand crosslinks with DNA, as documented by Roberts and Thomson [13], and unwinds superhelical PM-2 DNA, as shown by Mong et al. in our own laboratory [10].

Several reports have implicated CDDP- protein binding as a possible mechanism of activity and/or toxicity of CDDP. Crosslinks between DNA and protein have been detected by Kohn and Ewig [6] using the alkaline elution assay method. Lippard and Hoeschele [8] have reported that histones are crosslinked in isolated nucleosome cores by *cis*- and *trans*-DDP. CDDP and its analogs have been shown by Aull et al. [2] and Friedman et al. [4] to inhibit several enzymes, particularly glyceraldehyde-3-phosphate dehydrogenase and malic dehydrogenase. The interaction of CDDP with protein sulfhydryl groups in the kidney is a likely explanation of CDDP-induced renal damage. Sulfhydryl compounds are capable of reducing the nephrotoxicity of the drug as reported by Borch and Pleasants [3] and Levi et al. [7]. In addition, CDDP has been shown to specifically bind to methionine residues in proteins [9].

We have used high-resolution two-dimensional gel electrophoresis to investigate effects on the synthesis and extractability of nuclear proteins of HeLa cells after CDDP treatment. We believe the systems employed may be of value in the elucidation of the effects of various cytotoxic agents on specific cellular proteins.

## Materials and Methods

*Materials.* Leupeptin was obtained from the Peptide Institute, Osaka, Japan. EnHance was from New England Nuclear, Boston, MA, USA. L-$^{35}$S-Methionine had a specific activity of 1,200 Ci/mmole and was supplied by Amersham, Arlington Heights, IL, USA. CDDP was obtained from Bristol Laboratories, Syracuse, NY, USA. All other reagents were of the highest purity obtainable.

*Cell and Culture Techniques.* HeLa cells were maintained in monolayer culture on Eagle's MEM with 1% penicillin-streptomycin, 1% glutamine, and 10% calf serum. Cells were grown in T-75 flasks (Falcon) in a 5% $CO_2$ humidified incubator at 37° C.

*Drug Treatment and Radiolabeling.* CDDP dissolved in deionized distilled water was added to the growth medium at a final concentration of 0–10 μg CDDP/ml. Ninety minutes after addition of CDDP, the medium was replaced with methionine-free MEM containing 20–40 μCi L-$^{35}$S-methionine/ml and incubation was continued for a further 2 h. The monolayer was rinsed twice with unlabeled medium without calf serum and the cells were released by brief trypsinization (approximately 1 min). The cell suspensions were cooled on ice and then centrifuged (900 *g* for 5 min) and washed twice in cold medium.

*Isolation of Nuclei.* HeLa cells (5–10 × $10^6$ cells) were homogenized in 0.5% Nonidet P-40/12 m*M* $MgCl_2$/3 m*M*

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*Abbreviations used in this paper are:* CDDP, *cis*-diamminedichloroplatinum (II); SDS, sodium dodecyl sulfate

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$CaCl_2$/10 m*M* Tris-HCl, pH 8.0/0.25 *M* sucrose. The homogenate was centrifuged at 1,000 *g* for 10 min. The crude nuclear pellet was resuspended by gentle homogenization in 2.2 *M* sucrose/12 m*M* $MgCl_2$/3 m*M* $CaCl_2$/10 m*M* Tris-Hcl, pH 8.0 and centrifuged at 53,000 *g* for 60 min, followed by resuspension in homogenization buffer without Nonidet P-40 and centrifugation at 1,000 *g* for 5 min.

The nuclei thus obtained were morphologically statisfactory by light microscopic analysis. Phenylmethylsulfonyl fluoride and leupeptin were added to all solutions at final concentrations of 1.0 m*M* and 0.1 m*M*, respectively, to inhibit proteases.

*Nuclear Extraction.* Nuclei were extracted sequentially with 0.075 *M* NaCl/0.025 *M* EDTA, pH 8.0, 10 m*M* Tris-HCl, pH 8.0, and 0.6 *M* NaCl, 10 m*M* Tris-HCl, pH 8.0. The nuclei were gently resuspended by homogenization in 1.5 ml of the above buffers, then placed on ice for 20 min with intermittent mixing and centrifuged at 12,000 *g* for 10 min. Each extraction was performed twice.

Each extract was dialyzed for a minimum of 10 h against two changes of 1.0 m*M* phenylmethylsulfonyl fluoride/1.0 m*M* phenylmethylsulfonyl fluoride/1.0 m*M* Tris-HCl, pH 8.0, lyophilized, and subjected to two-dimensional gel electrophoresis.

*Two-Dimensional Gel Electrophoresis.* Lyophilized nuclear proteins were dissolved in 20–40 µl sample buffer, 9 *M* urea/1.0 m*M* dithiothreitol/2% ampholines (pH 3.5–10) (LKB, Inc. Rockville, MD).

The first-dimensional separation was by non-equilibrium isoelectric focusing with the samples loaded on the acid side. Gels (6.3 × 0.2 cm) were poured into one-half of a 200-µl disposable glass micropipet. The gel solution contained 9 *M* urea/4% acrylamide/0.2% *N,N'*-methylenebisacrylamide/2% ampholines, pH 3.5–10, 0.02% ammonium persulfate/0.1% *N,N,N',N'*-tetramethylethylenediamine. The upper reservoir was filled with 0.01 *M* $H_3PO_4$ (anode) and the lower was filled with 0.02 *M* NaOH (cathode). The samples were electrophoresed (a) for 30 min at 50 V; (b) for 30 min at 100 V; (c) at 200 V until the crystal violet tracking dye (5 µg) ran off the alkaline end (approximately 1.5 h); and (d) for 20 min at 400 V [14]. The gels were removed and adapted for 3 min in 0.05 *M* Tris/HCl, pH 7.5/1% SDS/5.0% 2-mercaptoethanol. The gels were then placed on top of the second-dimension slab gel.

The second-dimension slab SDS gel electrophoresis was carried out in a glass cell (102 mm × 83 mm × 1.2 mm). The slab gels consisted of a separation gel (80 mm) and a stacking gel (15 min). The separation gel was polymerized from a solution containing 9.6% acrylamide, 0.3% *N,N'*-methylenebisacrylamide, 0.1% SDS, 0.4 *M* Tris/HCl, pH 8.8, 0.03% ammonium persulfate, and 0.05% *N,N,N',N'*-tetramethylethylenediamine. The stacking gel contained 4.5% acrylamide, 0.1% *N,N'*-methylenebisacrylamide 0.125 *M* Tris/HCl, pH 6.8, 0,1% SD5, 0.03% ammonium persulfate and 0.1% *N,N,N',N'*-tetramethylethylenediamine. The running buffer contained 25 m*M* Tris base, 192 m*M* glycine and 0.1% SDS. The slab gels were electrophoresed at 25 mA/slab gel for 2 h or until the bromphenol blue tracking dye reached the anodic end. The slab gels to be autoradiographed only were removed and fixed in 10% trichloroacetic acid, 10% acetic acid, and 20% methanol for at least 1 h. They were then impregnated with EnHance for 1 h, followed by rinsing in $H_2O$ for 1 h. Slab gels to be stained to detect the standard proteins were removed and placed in 0.25% Coomassie Brilliant Blue K in methanol/acetic acid/$H_2O$ (5/1/5).

The gels were destained in the same solution, then treated exactly as described above prior to drying. Gels were dried on filter paper in a gel dryer (Bio-Rad Laboratories, Richmond, CA) for 2 h with heating. Autoradiograms were prepared using Kodak X-omat X-ray film with exposure at −70° C.

*Determination of Isoelectric pH(pI) and Molecular Weights.* The pI values and molecular weights of the separated polypeptides were estimated by the inclusion of internal standards, which were detected by Coomassie staining prior to autoradiography (see above). The standards were phosphorylase b (mol. wt 94,000, pI 8.1), bovine serum albumin (mol. wt) 67,000, pI 6.7), and carbonic anhydrase (mol. wt 29,000, pI 7.4). The position of these proteins on the gel was used to construct a mol. wt-pI grid.

*Quantification of Spot Intensity.* To quantify the intensities of protein spots, autoradiograms were scanned with a Transidyne Scanning Densitometer (Model 2955, Transidyne General Corp., Ann Arbor, MI). Slit width and height were adjusted to encompass the largest spot in a series to be measured. Individual spots were scanned at least six times, and in both the horizontal and vertical directions when possible. The integrated intensities obtained were averaged.

*Reproducibility of the Gels.* The pattern of protein spots within a given gel series from a single experiment was highly reproducible (cf. figures). Between experiments or gel runs some expansion and/or contraction of the pattern was often found. All observations reported here have been found in at least two independent experiments.

## Results

CDDP inhibits the incorporation of $^3$H-thymidine, $^3$H-uridine and $^3$H-leucine into cellular macromolecules [11]. The incorporation of $^3$H-thymidine into DNA was inhibited at concentrations 2- and 5-fold lower than those needed for comparable decreases in RNA or protein synthesis in HeLa cells. However, no appreciable decrease in synthesis of these macromolecules was detected at concentrations between 0 and 10 µg/ml CDDP [13]. In addition, cell viability is unaffected at these concentrations [10]. We have found no significant decrease in $^{35}$S-methionine incorporation in HeLa cells after a 3.5-h CDDP treatment at concentrations of 0–10 µg CDDP/ml. The distribution of recovered L-$^{35}$S-methionine in the sequential nuclear washes was as follows: the 0.025 *M* NaCl–0.075 *M* EDTA extract 24%; the 0.01 *M* Tris-HCl extract, 19%; and the 0.6 *M* NaCl extract, 30% of the total recovered radioactivity. This is similar to the protein distribution found by others using this extraction technique [12, 14]. No difference in the distribution was found after CDDP treatment of cells.

### *Proteins of the Saline-EDTA Extract*

Figure 1 shows the two-dimensional gel electrophoretic analysis of the proteins of the 0.025 *M* NaCl–0.075 *M* EDTA nuclear extract. Most of the cytonuclear proteins are released by this extraction, as has been previously reported [12]. These autoradiograms were overexposed to reveal the minor spots, and the pattern of polypeptides was found to be highly

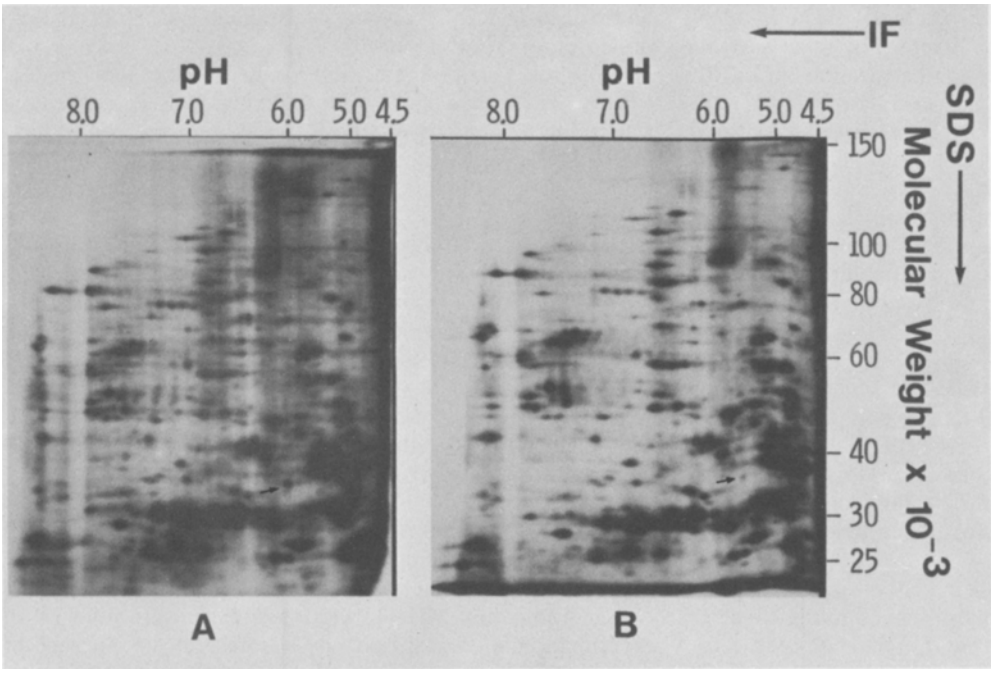


**Fig. 1A** and **B.** Two-dimensional gel electrophoretic pattern of $0.9 \times 10^5$ cpm of L-$^{35}$S-methionine-labeled protein of the 0.025 *M* NaCl–0.075 *M* EDTA extracts of HeLa cell nuclei purified as in *Materials and Methods.* The proteins of the sample were separated according to isoelectric point by non-equilibrium isoelectric focusing in the first dimension and according to molecular weight by SDS electrophoresis in the second dimension. *Abscissa,* pH range; *ordinate,* molecular weight ($\times 10^{-3}$) **A** non-drug treated control; **B** treatment at 10 μg CDDP/ml. *Arrow,* spot which decreases in intensity after CDDP treatment

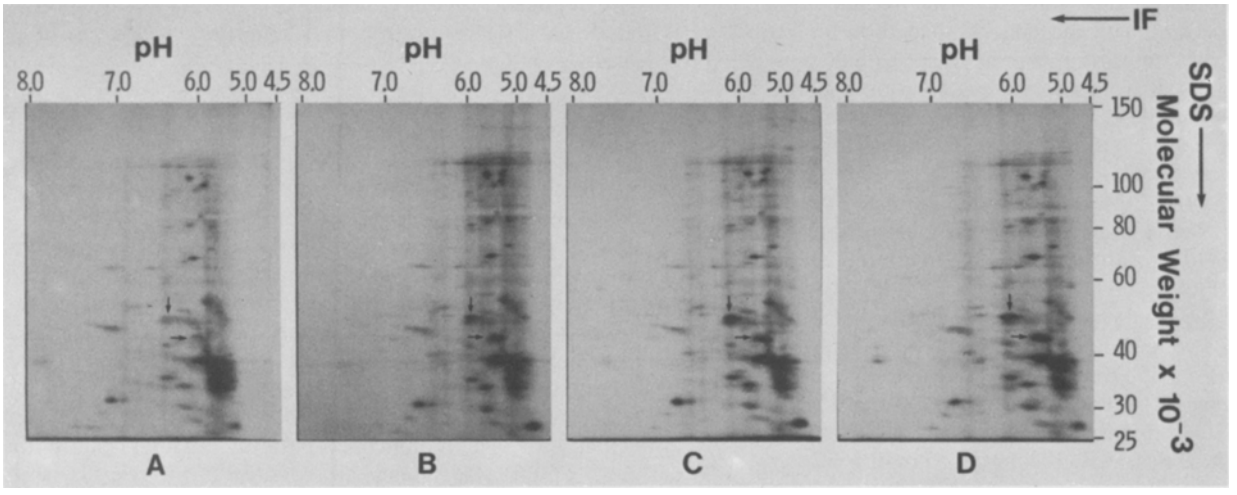


**Fig. 2A–D.** Two-dimensional gel electrophoretic pattern of $1.0 \times 10^5$ cpm of labeled protein of the HeLa nuclear Tris extract. **A** Non-drug treated control; **B** 0.2 μg CDDP/ml; **C** 1.0 μg CDDP/ml; **D** 5.0 μg CDDP/ml. See legend to Fig. 1 for details. *Arrows,* spots which increase in intensity after CDDP treatment

reproducible. Overall, the extractable protein patterns were remarkably similar in control cells and those treated with CDDP. A few minor spots decreased in intensity at the highest CDDP concentrations tested (10 μg CDDP/ml). The most notable alteration was the spot at 36K, 5.8 (see Fig. 1, arrows). Spots at 75K, 7.1 and 38K, 7.5 also appear to decrease in intensity at 10 μg CDDP/ml.

*Proteins of the Tris Extract*

Tris-extractable proteins are generally more acidic than those extracted with saline-EDTA. The Tris extract has been reported to contain several proteins of the nuclear matrix. These polypeptides, which are in the region with molecular weights between 40,000 and 60,000 and with pI in the range of 6.0–6.5 [12], were not affected by CDDP treatment in our experiments. The most striking changes seen after CDDP treatment was an increase in intensity of two spots at a molecular weight of 50,000 and pI of 6.0 and a molecular weight of 45,000 and pI of 5.3 (Fig. 2, arrows). There was also an apparent decrease in intensity in the major group of Tris proteins at molecular weights of 32,000–40,000 and pI of 4.8–5.5.

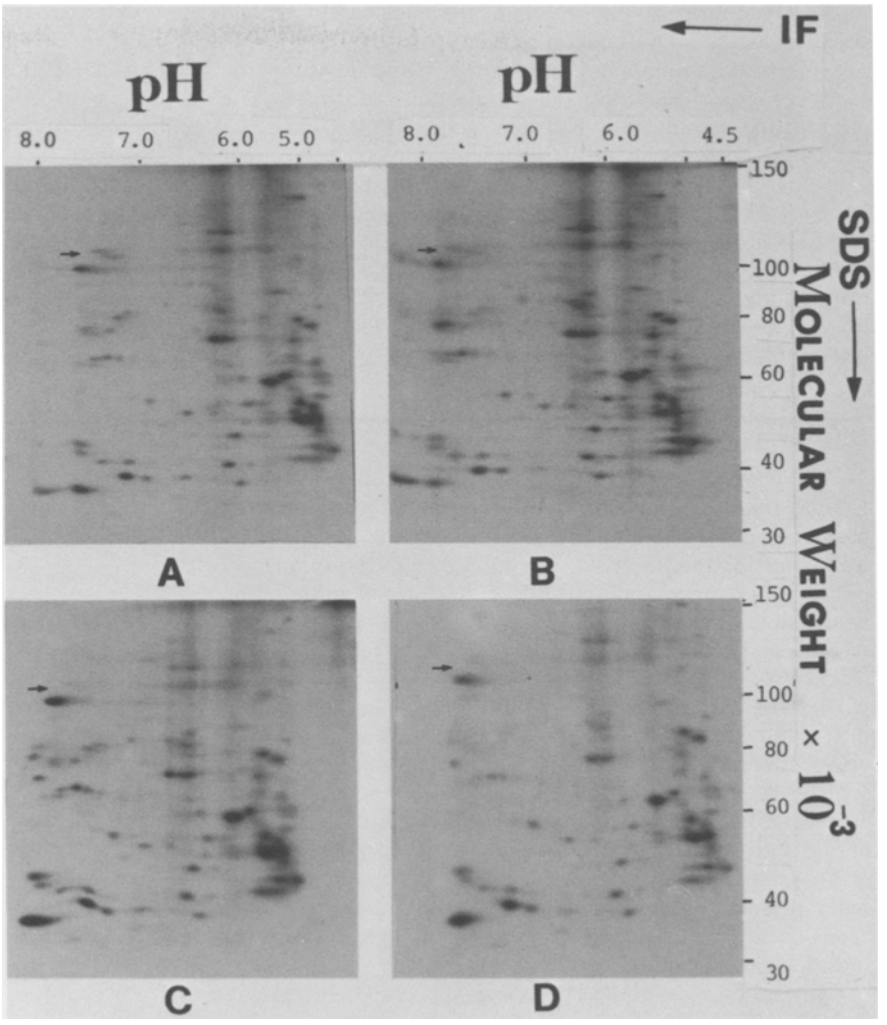


**Fig. 3A–D.** Two-dimensional gel electrophoretic pattern of $1.0 \times 10^5$ cpm of labeled protein of the HeLa nuclear 0.6 *M* NaCl extract. **A** non-drug treated control; **B** 0.2 μg CDDP/ml; **C** 1.0 μg CDDP/ml; **D** 5.0 μg CDDP/ml. *Arrow*, spot at (110K, 7.7)

**Table 1.** Quantitation of the 0.6 *M* NaCl-extracted nuclear proteins of HeLa cells

| CDDP concentration (μg/ml) | Integrated spot intensities (area in arbitrary units) Peptide: (mol. wt × $10^{-3}$, pI) | | | | |
|---|---|---|---|---|---|
| | 40, 6.2 | 50, 6.1 | 55, 6.8 | 39, 7.3 | 110, 7.7 |
| 0 | 1.00 ± 0.03 | 0.83 ± 0.04 | 0.48 ± 0.02 | 2.08 ± 0.06 | 1.05 ± 0.08 |
| 0.2 | 1.00 ± 0.05 | 0.87 ± 0.06 | 0.40 ± 0.02 | 2.00 ± 0.03 | 0.43 ± 0.16 |
| 1.0 | 1.00 ± 0.11 | 0.74 ± 0.01 | 0.34 ± 0.05 | 2.47 ± 0.51 | 0.23 ± 0.12 |
| 5.0 | 1.00 ± 0.08 | 0.86 ± 0.14 | 0.32 ± 0.06 | 2.26 ± 0.02 | 0.02 ± 0.01 |

The integrated intensities of the nuclear protein spots of the 0.6 *M* NaCl extract were measured by scanning densitometry. The measured areas for each spot were arbitrarily normalized to the intensity of the spot at 40K, 6.2. Values given are averages ± standard deviations

*Proteins of the 0.6 M NaCl Extract.* Most of these more tightly bound nuclear proteins are present in control and drug-treated samples (Fig. 3). The pattern of peptides at molecular weights of 30,000–60,000 and pI 7.5–8.0 represents peptides derived from hnRNP [12]. No apparent changes in these proteins were induced by increasing concentrations of CDDP.

A peptide at a molecular weight of 110,000 and a pI of 7.7 (arrows, Fig. 3) appeared to decrease in intensity as the CDDP concentration was increased. This spot was revealed as a series of closely spaced polypeptides in the isoelectric focusing direction upon further exposure of the gel. This type of pattern is often associated with protein modification [1, 5].

*Quantitation of the 0.6 M NaCl-Extracted Proteins.* Since variations in the background level of the film may affect visual estimations of protein spot, intensity scanning densitometry was used to quantitate the decrease in the intensity of the protein complex at 110K, 7.7. This procedure also corrects for small variations in the amount of radioactive protein loaded onto the first-dimension gels. The integrated intensity of the 110K, 8.4 complex was compared with that of other protein spots in the same gel (Table 1). Table 1 lists averages and standard deviations for four spots in various areas of the gels and for the 110K, 7.7 complex. Results are shown for cells treated with 0, 0.2, 1.6, and 5 μg CDDP/ml. The intensities of

most of the spots in the autoradiogram appear to remain constant as the CDDP concentration was increased.

The visual estimation is confirmed by the densitometric scans for four of these spots in various areas of the gel. The intensity of the complex at molecular weight 110,000 and pI 7.7 decreased dramatically with increasing drug concentration. The other protein spots scanned displayed a relatively constant intensity as the CDDP concentration was increased. The decrease in intensity of the protein complex with molecular weight 110,000 and pI 7.7 was found over a CDDP concentration range that had previously been shown to be cytotoxic for HeLa cells [11] and had no significant effect on overall protein synthesis.

## Discussion

We have examined the proteins of HeLa cell nuclei after CDDP treatment by high-resolution two-dimensional gel electrophoresis. These studies demonstrate the utility of this technique for the study of drug-induced effects on specific polypeptides in several fractions of HeLa cell nuclei. The results obtained demonstrate highly reproducible separations of the non-histone nuclear proteins of HeLa cells after drug treatment.

Several differences between the proteins extracted from drug-treated and control cells were observed. Very few effects were noted on the saline-EDTA-extracted proteins, even after prolonged exposure of the gels to allow detection of minor proteins. The major change found was a decrease in intensity of one polypeptide with a molecular weight of 36,000 and a pI of 5.8 (see Fig. 1). However, decreases in the intensities of other protein spots with approximate molecular weights of 75,000 and pI values of 7.1–7.5, although difficult to substantiate, cannot be excluded.

Several proteins of the Tris extract appeared to increase in intensity after CDDP treatment of the cells. The most notable changes in these are spots at molecular weight 50,000 and pI 6.0 and molecular weight 45,000 and pI 5.3.

The 0.6 *M* NaCl extract is distinguished by the presence of a polypeptide complex at a molecular weight of 110,000 and a pI of 7.7, which decreased to almost undetectable levels after treatment with a CPPD concentration in excess of 1 μg CDDP/ml. The complex appears as a series of spots when the autoradiograms are exposed for a longer time. This may indicate that this polypeptide occurs in a post-translationally modified form, or there may be several similar proteins in the complex.

Changes in polypeptide intensities in these experiments may indicate either an effect on the synthesis or extractability from the nucleus of the proteins. DNA-protein crosslinks induced by CDDP may underlie the differences in extractability of certain of the nuclear proteins. The possibility that gel mobility changes for proteins were induced by direct modification of the $^{35}$S-methionine-labeled proteins by CDDP was investigated. Treatment of protein extracts with cynanide ion to remove CDDP prior to electrophoresis did not alter the $^{35}$S autoradiographic pattern of spots. Whether these changes play a role in the cytotoxic activity of CDDP or whether they are a consequence of its interaction with DNA is presently unclear.

These results suggest that the system can be employed to study specific drug-protein interactions. They demonstrate that CDDP does not induce a similar effect on all nuclear proteins of HeLa cells. Specific changes have been observed for only a few of the separated proteins, suggesting the possibility that there are specific DNA-protein interactions after CDDP treatment of HeLa cells, or that the synthesis of a few proteins may be altered in a highly specific manner by CDDP. Further studies employing a variety of analogs are required before we can understand the significance of these observations, and additional studies are in progress to determine whether the changes observed are due to variations in the extractability of the proteins or alterations in synthetic rate.

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