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A COMPARISON OF THE EFFECTS OF CISPLATIN AND CARBOPLATIN UPON KIDNEY FUNCTION IN THE RAT

By

James M. Fadool

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ABSTRACT

A COMPARISON OF THE EFFECTS OF CISPLATIN AND CARBOPLATIN UPON KIDNEY FUNCTION IN THE RAT

By

James M. Fadool

Platinum coordination complexes have drawn considerable attention since their introduction as anticancer agents. To better understand the differences and similarities between the actions of cisplatin, the parent compound, and carboplatin, a second generation analogue, comparative investigation of their effects upon urinary water excretion, the neurohypophysis, calcium excretion and the parathyroid gland was undertaken. Wistar rats given a single intraperitoneal injection of cisplatin demonstrated a dose dependant decline in urine output and water consumption relative to controls. Carboplatin treatment elicited no significant change in either parameter relative to control treatment. Cytochemical and electron microscopic examination of the neurohypophyses from cisplatin-treated animals revealed a decrease in neurohormone, consistent with the change in urine output. Furthermore considerable rounding of the pituicytes after 9 mg/kg and large accumulations of glycogen were apparent. Urine calcium and phosphate excretion also changed dramatically in the cisplatin-treated animals while no significant effect was observed following carboplatin treatment. The variations in kidney handling of calcium and phosphate were associated with changes in the parathyroid gland

morphology consistent with increased synthesis and secretion of parathyroid hormone. Parathyroid gland activity of cisplatin-treated animals which received a single injection of calcium chloride the day of and each day following cisplatin administration, was reduced and the calcium and phosphate excretion returned to normal. These animals also demonstrated less severe gastrointestinal toxicity compared to cisplatin treatment alone while effects of the calcium supplementation upon nephrotoxicity varied. To examine the effect of cisplatin and carboplatin upon cytosolic calcium, Sarcoma-180 cells were loaded with the intracellular indicator indo-1 for the measurement of cytosolic calcium concentration using the ACAS interactive laser cytometer. Following treatment with either cisplatin or carboplatin no effect upon $[Ca^{2+}]_i$, actin distribution, membrane topography and cell viability were observed, thereby demonstrating no immediate alteration of $[Ca^{2+}]_i$ following platinum complex treatment. Both cisplatin and carboplatin treatment resulted in giant cell formation. The series of events responsible for giant cell formation was not clear.

ACKNOWLEDGMENTS

First and foremost, I wish to thank my mentor, Dr. Surinder K. Aggarwal who gave me the opportunity to pursue graduate work in his laboratory; offered encouragement and guidance but also the latitude to explore areas other than that originally intended; but most importantly, for those many insightful discussions encompassing every subject other than science. I would also like to thank the members of my graduate committee Dr. R. Neal Band, Dr. Stanley L. Flegler and Dr. Charles D. Tweedle.

Last but not least, I would also wish to acknowledge the other graduate students in Dr. Aggarwal's laboratory with whom I had the pleasure of working and past and present members of the office staff of the Department of Zoology who assisted with course paperwork and the usual graduate school paper chase.

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INTRODUCTION

INTRODUCTION

Cisplatin (cis-diamminedichloroplatinum II; CDDP) (Figure I) is a potent anticancer agent, used successfully in the management of several human malignancies (Bosl, et al., 1980; Rozencweigh, et al., 1977; Williams and Einhorn, 1982). CDDP was first synthesized in 1845, and had been used by chemists during the formation of the coordination theory. The biological activity of the compound was not reported until 1965 (Rosenberg, et al., 1965); while studying the effects of an electric field upon the division of E. coli, it was discovered that platinum compounds had an effect upon cell division. Subsequently, Rosenberg and colleagues tested the compounds for antitumor activity (Rosenberg, et al., 1969; Rosenberg, et al., 1970). Structural requirements for the compounds to possess antitumor activity were neutrality and a pair of labile chloride groups in the cis configuration while the trans isomers were much less potent.

The mode of action of CDDP in tumor regression is believed to be through interaction with DNA and the inhibition of DNA, RNA and protein synthesis (Harder and Rosenberg, 1970; Roberts and Pascoe, 1972). DNA synthesis has been proposed to be the primary target of platinum complexes while RNA and protein synthesis are effected secondarily. In both primary cultures of rabbit kidney proximal tubule cells and in cultures of tumor cell lines, the inhibition of DNA replication precedes inhibition of RNA transcription and protein synthesis (Harder and Rosenberg, 1970; Tay, et al., 1988).

The effect upon DNA by CDDP is most likely the result of both intrastand and interstrand crosslinking, representing greater than 90 % and less than 10 % of the total bound platinum respectively (Pascoe and Roberts, 1972; Roberts, et al., 1988). DNA interstrand cross-linking occurs predominantly between two N-7 atoms of guanine residues on opposite DNA strands particularly at 5'-GC-3' sequences (Lemaire, et al., 1991). The intrastrand crosslinking is primarily between GG or GA adducts (Roberts, et al., 1988). DNA crosslinking by CDDP blocks DNA replication and RNA transcription at the site of the adduct by interfering with the function of DNA or RNA polymerase, in an undetermined manner (see Lemaire, et al., 1991 and references therein).

In addition to interaction with nucleic acids, platinum coordination complexes affect a myriad of other cellular functions. CDDP inhibits ATPase activity (Guarino, et al., 1979), causes the loss of membrane associated transport enzymes and sulfhydryl groups (Aggarwal and Niroomand-Rad, 1983; Batzer and Aggarwal, 1986; Levi, et al., 1980), decreases mitochondrial accumulation of divalent cations and alters mitochondrial respiration (Aggarwal, et al., 1980; Binet and Volfin, 1977; Gordon and Gatton, 1986), and increases lipid peroxidation (Bombart, 1989; Sugihara and Gemba, 1986; Sugihara, et al., 1987). Though the effects of such changes have not been correlated to specific toxic side-effects, it is suspected that such interactions result in deregulation of intracellular ion homeostasis or cellular signalling mechanisms.

Additional mechanisms of action which may or may not be associated with the afore mentioned interactions have received considerable attention in recent years. Most notable is the inhibition of cell division after the replication of DNA, commonly referred

to as G_2-arrest. This was first described by Aggarwal (1974) and Aggarwal and Sodhi (1973), while studying the effect of short duration CDDP treatment on a number of tumorigenic cell lines in vivo and in vitro, when it was observed that CDDP treatment resulted in the formation of multinucleated giant cells. Synchronized cultures treated with CDDP, continued through nuclear division but cytokinesis was inhibited. Subsequently, several other investigators have documented G2 arrest with the formation of giant cells following CDDP treatment (Just and Holler, 1989; Vates, et al., 1985) however the exact mechanism governing the process has not been established. It has been hypothesized that G2 arrest results from either inhibition of transcription necessary for the initiation of mitosis or interruption of key signaling events (Sorenson and Eastman, 1988a & 1988b; Sorenson, et al, 1990). These experiments confirm that events other than inhibition of DNA synthesis are critical to CDDP-induced cytotoxicity.

Clinical trials were begun in the early 1970s and promising results were seen in the treatment of ovarian and testicular cancers, however, early success was plagued by serious toxicity, namely nephrotoxicity, ototoxicity, neurotoxicity and gastrointestinal toxicity in the form of nausea and vomiting. The nephrotoxicity was characterized by both renal functional impairment and morphological damage as proximal tubule necrosis common in most animals studied including humans (Daugaard, 1990; Nitsche, 1981; Rossof, et al., 1972; Rozencweigh, et al., 1972). Subsequently the nephrotoxicity could be managed through hydration and forced diuresis.

Diuresis therapy and saline infusions have been the most common methods employed to reduce the nephrotoxicity associated with CDDP treatment. In laboratory experiments, mannitol prevented the CDDP-induced azotemia and creatinine elevation

and has been successfully used to reduce the nephrotoxicity in clinical trials (Hayes, et al,. 1977; Pera, et al., 1979). Use of furosemide as a diuretic has encountered resistance due to conflicting results (Pera, et al., 1979; Ward, et al., 1977). There have been reports that furosemide protects kidney function (Pera, et al., 1979) even while aggravating the histopathological damage of the kidney, while others have suggested that furosemide actually enhances CDDP nephrotoxicity (Lehane, et al., 1979). High dose furosemide treatment alone has been attributed with proximal tubular necrosis (McMurty and Mitchell, 1977), therefore, use of furosemide has not been warranted due to these conflicting results.

Litterest (1981) first demonstrated that increased chloride ion concentration in the administration vehicle, significantly reduced the mortality rate of CDDP in rodents. It had been proposed that CDDP itself has relatively low toxicity compared to hydrolysis products formed by the substitution exchange of the labile chloride groups. Elevation of the chloride ion concentration would presumably decrease or inhibit this exchange of chlorides by -OH or H_2O. This mechanism has also been extended to explain the cytotoxicity of CDDP once it has entered the cell cytosol. The intracellular chloride concentration is much lower than that of the extracellular fluid, favoring hydrolysis of CDDP to a more reactive aquated species. However, recent data indicates that NaCl loading in rats leads to a significant reduction in the total uptake of platinum by the kidney (Mistry, et al., 1989) and a reduction in the various species of metabolites formed within the cytosol. This suggests that decreased platinum accumulation rather than an alteration in metabolite formation accounts for the reduction in the severity of the nephrotoxicity following saline or chloride ion treatment.

Alternative methods to diuresis therapy and saline infusions have been pursued, to better manage the nephrotoxicity and to diminish the incidence and severity of the neurotoxicity, gastro-intestinal toxicity and ototoxicity. A variety of chemical agents have been employed (for review see Gandara, et al., 1991); diethyldithiocarbamate (DDTC) (Gale, et al., 1982); WR-2721 (ethiofos) (Gandara, et al., 1991; Mollman, et al., 1988); ORG-2766, an adrenocorticotropic hormone analogue (Mollman, 1990; Terheggen, et al., 1989), as have agents which stimulate detoxification mechanisms (Naganuma, et al., 1987), use of antioxidants (Sugihara and Gemba, 1986; Sugihara, et al., 1987), treatment regimes which take advantage of physiological changes during the circadian rhythm (Boughattas, et al., 1988; Levi, et al., 1982).

DDTC has been used extensively in the treatment of nickel and cadmium poisoning (Gandara, et al., 1991). It forms complexes with a number of divalent cations including silver and ferric ions but does not bind calcium or magnesium. The mechanism of protection against CDDP toxicities is believed to be through the removal of tissue bound platinum without alteration of the platinum-DNA crosslinking (Gandara, et al., 1991).

The organothiosufate WR-2721 offers protection from CDDP-induced nephrotoxicity, ototoxicity and neurotoxicity (Gandara, et al., 1991; Mollman, et al., 1988). The mode of action is believed to be through chelation of platinum within the cell. WR-2721 is dephosphorylated by cellular phosphatases and enters the cell as ethiofos. Higher pH and alkaline phosphatase levels of normal tissues compared to malignant cells results in greater uptake and a greater degree of protection in these tissues.

ORG-2766 prevents the occurrence of CDDP-induced neurotoxicity in rats (Terheggen, et al., 1989). It has no known side effects and does not inhibit the antitumor activity of the platinum compound. ORG-2766, a corticotropic like peptide is believed to enhance peripheral nerve repair without the endocrine effects of the parent compound (Gandara, et al., 1991; Mollman, 1990; Terheggen, et al., 1989). However, like many of the other compounds tested, variations in treatment schedules, dosages and evaluation have made clinical studies difficult to compare.

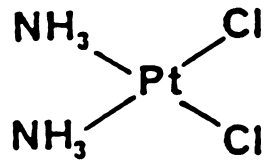
Analogues of CDDP have been synthesized in efforts to alleviate the toxic side effects while maintaining or improving the therapeutic index (Harrap, et al., 1980; Hydes, et al., 1984; Prestayko, et al., 1979). In contrast to CDDP, the second generation analogue carboplatin (cis-diammine 1,1-cyclobutane dicarboxylate platinum (II); CBDCA; JM-8) (Figure 1) has no nephrotoxicity, ototoxicity or peripheral neuropathy (Canetta, et al., 1985; Foster, et al., 1990) but exhibits similar antineoplastic activity. Myelosuppression appears to be a dose limiting factor in its use (Canetta, et al., 1985; Foster, et al., 1990).

As previously stated, the most widely accepted target of both CDDP and CBDCA is believed to be the cell nucleus through interaction with DNA (Roberts and Pascoe, 1972). The various platinum coordination complexes, however, have different rates of interaction with DNA assumingly dependent on their different rates of hydrolysis to more reactive species (Knox, et al., 1986). DeNeve et al. (1990), however, reported that only 3- to 4-fold more CBDCA had to be given compared to CDDP to obtain equal amounts of DNA crosslinking, while to elicit equitoxicity, a 13- to 16-fold increase in dose was needed. Furthermore, even high dose CBDCA treatment does not result in similar

pathological effects as CDDP. The discrepancies are more outstanding when one considers that following CDDP or CBDCA administration, platinum concentrations of the kidney are similar (Siddik, et al., 1988; Terheggen, et al., 1987). Therefore, inconsistencies between tissue concentrations, DNA crosslinking and toxicity suggest the toxic effects may in part be related to sites of action by CDDP other than the cell nucleus.

To better understand their similarities and differences, comparative studies of the effects of CDDP and CBDCA upon kidney function and hormonal changes related to kidney function were undertaken. Alteration of water metabolism in relation to neurohypophysis function and calcium excretion to parathyroid gland function were examined. From these observations, alteration of intracellular ion homeostasis seems to be the likely basis for some of the toxicities particular to CDDP treatment. Therefore, alteration in the intracellular calcium ion concentration was monitored in cells in vitro.

A. Cisplatin



B. Carboplatin

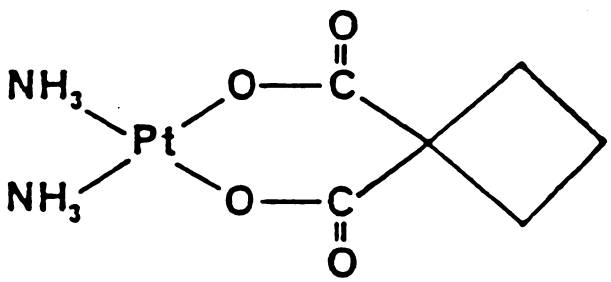


Figure 1. Platinum Coordination Complexes. Structure of Cisplatin (A) and Carboplatin (B).

I. Immunocytochemical Demonstration of Vasopressin Binding in Rat Kidney.[1]

[1], Reproduced, with permission, from authors: James M. Fadool and Surinder K. Aggarwal. Immunocytochemical demonstration of Vasopressin Binding in Rat Kidney. *Journal of Histochemistry and Cytochemistry* 38: 7-12 (1990).

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Original Article

Immunocytochemical Demonstration of Vasopressin Binding in Rat Kidney

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We investigated the immunoperoxidase demonstration of vasopressin (VSP) bound to paraffin-embedded sections of rat kidney and the effects of various fixatives. Slices of rat kidney from normal and 4-day water-deprived rats were incubated with 10^{-7} M VSP, fixed, and embedded in paraffin. Hydrated sections of these tissues were again incubated with 10^{-7} M VSP or 10^{-7} M VSP and 10^{-5} M oxytocin (OXY). VSP bound to the sections was demonstrated using rabbit anti-Arg^8 VSP antiserum and peroxidase-labeled second antibody. In sections of kidney from both normal and water-deprived rats, immunoperoxidase labeling was most intense in the renal papilla and was restricted to the cells of the ducts of Bellini and loops of Henle. In the medulla, the collecting ducts and medullary thick ascending limbs of Henle were moderately stained. In the normal kidney sections there was no staining of the proximal tubules, distal convoluted tubules (DCT), and only slight staining of the cortical collecting ducts (CCD). However, in the water-deprived rats there was a considerable increase in the staining of the DCT and CCD. Simultaneous incubation in OXY and VSP resulted in reduced immunoperoxidase labeling of the tubules. Omission of VSP incubation led to a similar decrease in stain intensity, indicating a specificity for the sites of VSP binding. This technique allows the identification of cells responsible for the binding of VSP in the kidney. (*J Histochem Cytochem 38:7–12, 1990*)

KEY WORDS: Vasopressin; Kidney; Rat; Immunocytochemistry.

Introduction

The standard methods for demonstration of hormone binding to target tissues include incubation of radiolabeled hormone with isolated membranes, tissue homogenate, or frozen sections (8,9, 31,35,36). However, in many cases the exact cell type responsible for the hormone binding cannot be determined. Homogenized tissues often contain cell components from a heterogeneous population of cells, and autoradiographic localization of binding is often limited by histological resolution (35,36). Recently immunohistochemical demonstration of proteins and steroids has been applied to the demonstration of hormones bound to target tissues (3,14,27,28,40), and has aided in the identification of the exact cells capable of binding the specific hormone.

The neurohormone vasopressin (VSP) has an antidiuretic effect on the mammalian kidney (21,37), pressor action in the circulatory system (6,23,31), and has been implicated in the central nervous system functions of memory and behavior (9,11,13,15,36). Autoradiographic localization of VSP binding sites in the kidney has demonstrated the renal papilla and medulla to be the major regions of binding (9,35,36). Studies using isolated tubules of rat kidney have demonstrated the medullary ascending thick limb of Henle (MAL), the cortical collecting ducts (CCD), and the medullary collecting ducts (MCD) to be the major sites of antidiuretic action (4,16,21,30) of VSP. However, there is still a need for a reliable method to demonstrate the exact tubule segments in the intact kidney responsible for the action of VSP. In the present study we describe an accurate and reproducible method for immunocytochemical demonstration of VSP bound to specific regions of the nephron in paraffin-embedded sections of rat kidney.

Materials and Methods

Animals. Male Wistar rats [Crl: (WI) BR; Charles River, Wilmington, MA] weighing between 200–250 g were used throughout the study. The animals were housed individually in stainless steel metabolism cages for daily collection of urine. All animals had free access to laboratory animal feed. Half of the animals were provided with water and the other half were deprived of water for 4 days. The experiment was run on three separate occasions involving a total of 30 animals in all treatment groups.

Tissue Preparation. On Day 4, animals were killed by decapitation without anesthesia, with the pituitary and kidneys quickly dissected out and placed on an upturned petri dish over ice. Each pituitary was sliced longitudinally into two halves and was immersed in 4% formaldehyde in 0.05 M PBS, pH 7.2. A cross-section of the kidney (3 mm thick) containing the renal papilla was cut and immersed in PBS at 4°C. These slices of kidney were then incubated in 10^{-7} M VSP (Sigma Chemical; St Louis, MO) in PBS for 15 min before being fixed. Care was taken to wash these tissues thoroughly in two changes of PBS to remove any unbound hormone. The

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kidney slices were then fixed for 4 hr in either 4% formaldehyde in PBS or Bouin's fluid at 4°C or snap-frozen in liquid nitrogen, followed by freeze-substitution with 95% ethanol at −20°C. After fixation, all tissues except for those that were freeze-substituted were rinsed overnight in two changes of PBS, dehydrated in an ascending ethanol series, cleared with xylene, and embedded in paraffin. The freeze-substituted tissues were immediately moved to 100% ethanol, cleared, and embedded like the others. During infiltration and blocking, special care was taken not to exceed 58°C; temperatures greater than this were found to greatly reduce the subsequent immunohistochemical reaction. Thick sections (7 μm) adhered to glass slides were used in all immunohistochemical localizations.

VSP Binding. For demonstration of VSP, sections were deparaffinized and hydrated. Endogenous peroxidase activity was inhibited by incubation in 70% methanol containing 3% H_2O_2 for 40 min. Sections were rinsed in PBS, followed by incubation in 30% normal goat serum (NGS) in PBS to block nonspecific binding of the secondary antibody. The sections were subsequently treated in the following stepwise fashion in a humid environment at 20°C for 30 min each: (a) incubated in PBS containing 10^{-7} M VSP + 10% NGS; (b) rinsed with PBS + 10% NGS; (c) incubated in primary antisera, rabbit anti-Arg^8-VSP diluted 1:60 in PBS (polyclonal) (Biomedia; Foster City, CA); (d) rinsed with PBS + 10% NGS; and (e) incubated in peroxidase-labeled secondary antiserum, goat anti-rabbit IgG (Boehringer–Mannheim; Indianapolis, IN) diluted 1:50 with PBS. Peroxidase-labeled antibody was demonstrated by incubation in medium containing 1 mg 3,3-diamminobenzidine (Aldrich; Milwaukee, WI) per 1 ml 0.05 M Tris buffer (pH 7.6) and 0.05% H_2O_2. Sections were rinsed in 0.05 M Tris buffer, dehydrated, cleared, and mounted with permount.

According to the manufacturer (Biomedia), the specificity of the anti-VSP antiserum was judged by lack of crossreactivity with oxytocin (OXY), as tested using the method of Watkins and Choy (38). Hypothalami from Long–Evans rats and the homozygous Brattleboro strain of rat (a genetically defective strain which lacks production of VSP but not OXY) were incubated with the antiserum at 1:20–1:40 dilutions, followed by immunoperoxidase demonstration of bound antibody. Lack of staining in the Brattleboro rats of the magnocellular neurons of the supraoptic nuclei and paraventricular nuclei and staining of the same cell bodies in the Long–Evans rats was considered as specific binding of VSP with no crossreactivity for OXY.

To determine the specificity of the VSP binding, control sections were alternatively treated in the following manner: (a) PBS without added VSP; (b) PBS containing both VSP (10^{-7} M) and OXY (10^{-5} M; Sigma); (c) in medium lacking the primary antibody; or (d) in 10% NGS rather than secondary antiserum. In all experiments, paraffin sections of posterior pituitary from both normal and water-deprived animals were run as positive controls for VSP demonstration, whereas the intermediate and anterior pituitary served as negative controls.

Statistical Analysis. The data for urine volume were analyzed with the Student's *t*-test (34).

Results

The best immunohistochemical demonstration of VSP bound to kidney sections was obtained after formaldehyde fixation. Bouin's fixation provided excellent structural preservation but caused indiscriminate binding of VSP, as demonstrated by the immunoperoxidase-labeled antibody. The freeze-substituted tissues also had good structural preservation; however, staining intensity for immunoperoxidase-labeled antibody was not as intense when compared with formaldehyde-fixed tissues and could not be greatly enhanced with subsequent VSP incubations. Therefore, all subsequent descriptions are based on examination of immunohistochemical demonstrations of VSP binding from formaldehyde-fixed tissues.

Tissues not incubated in VSP (10^{-7} M) before fixation had little staining of any tubule segments; however, incubation in VSP (10^{-7} M) before fixation did demonstrate positive staining. Added incubation in VSP (10^{-7} M) of the sections from tissues previously incubated in VSP before fixation demonstrated a further increase in staining intensity.

The demonstration of the peroxidase-labeled antibody was observed as brownish staining of specific segments of the nephron, with no staining over the cell nuclei or the tubule lumen. In normal rats, the most intense staining was of the ducts of Bellini (DB) in the tip of the renal papilla (Figure 1A). The squamous epithelium of the thin loop of Henle (TLH) within the tip of the papilla also showed positive reaction for peroxidase activity. This staining decreased in intensity towards the inner papilla. In serial sections simultaneously incubated in VSP (10^{-7} M) and OXY (10^{-5} M), a reduction in staining intensiy of the above tubules of the papilla was observed (Figure 1B). Furthermore, adjacent sections lacking VSP incubation showed minimal staining reaction for peroxidase activity (Figure 1C).

In the medulla, moderate staining of the MCD and the MAL was observed, which could also be identified in the corticomedullary zone among the non-stained proximal straight tubules (PST). In the renal cortex there was no staining of glomeruli, proximal convoluted tubules (PCT), and distal convoluted tubules (DCT) (Figure 1D). However, the CCD showed slight positive reactivity, but only after sections from tissues pre-incubated in 10^{-7} M VSP were again incubated in 10^{-7} M VSP. In these regions there was no staining of the TLH, endothelial cells, or connective tissue.

The water-deprived rats had a statistically significant decrease in urine output relative to controls, 0.4 ± 0.1 ml/24 hr vs 20.16 ± 5.3 ml/24 hr, respectively ($p \leq 0.01$). The staining intensities for immunoperoxidase-labeled antibody in such animals were similar to the normal rat kidneys in the medullary region and the papilla. However, in the cortex a considerable increase in staining was observed (Figure 1E). In these sections the DCT and the CCD were positive, whereas there was no such staining in kidneys of normal animals. In water-deprived animals, the staining of the CCD was localized to the principal cells, and no staining of intercalated cells was observed (Figure 1F). Again, addition of OXY (10^{-5} M) to the VSP-containing incubation solution led to a decrease in the staining intensity.

Sections of pituitary from normal rats demonstrated intense staining of the posterior lobe, with no staining of the intermediate and anterior lobes (Figure 2A). Demonstration of VSP in sections of the pituitaries from water-deprived rats showed greatly reduced staining of the posterior lobe as compared with those from normal animals (Figure 2B). There was no staining of the tissues when either the primary or the secondary antiserum was omitted from the incubation medium (Figure 2C).

Discussion

Immunohistochemical demonstration of VSP was applied to the study of VSP bound to specific nephron segments of paraffin-embedded sections of rat kidney. The method of fixation had a considerable effect on the final demonstration of VSP in the kidney. Buffered formaldehyde, a routinely used fixative for immunocytochemistry (22,24,33,39), resulted in the best overall histo-

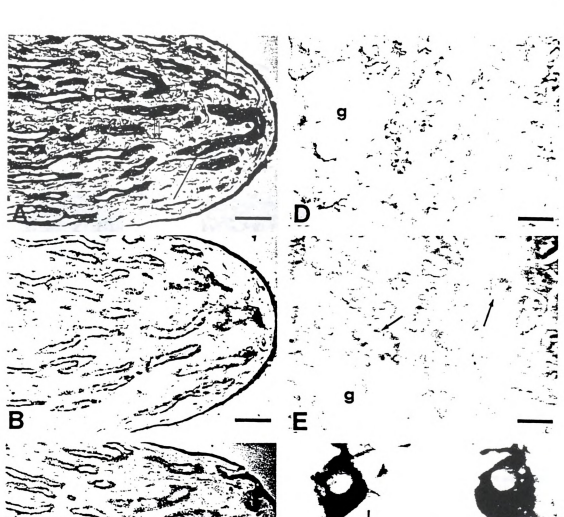

Figure 1. Immunoperoxidase demonstration of VSP bound to sections of paraffin-embedded rat kidney from normal and water-deprived rats. Tissues were incubated in VSP before fixation in 4% formaldehyde. Unless otherwise stated, sections (7 um) were further treated with VSP before its immunohistochemical demonstration. (A) Renal papilla from a normal rat, showing intense labeling of the ducts of Bellini (arrows) and the thin loops of Henle (double arrows). (B) An adjacent section to A simultaneously incubated in 10^{-7} M VSP and 10^{-5} M OPK. Note decreased intensity of staining of the ducts of Bellini and loops of Henle. (C) An adjacent section incubated in medium without VSP. Note the reduced intensity of staining. (D) Cortex of a kidney from a normal rat showing no staining of glomerulus (g), PCT, DCT, or CCD. (E) Renal cortex from a water-deprived rat counterstained with the PAS method. Note immunoperoxidase staining of the DCT and CCD (arrows), nephron segments lacking peroxidase labeling, the glomerulus (g), and the PCT with characteristic PAS-positive tubule lumens. (F) Higher-power view of a CCD from a water-deprived rat demonstrating the presence of principal cells (P) and intercalated cells (I) distinguished by their ability to bind VSP. Original magnifications: A, B, C × 50; D, E × 200; F × 1250. Bars: A–C = 240 um; D, E = 60 um; F = 12 um.

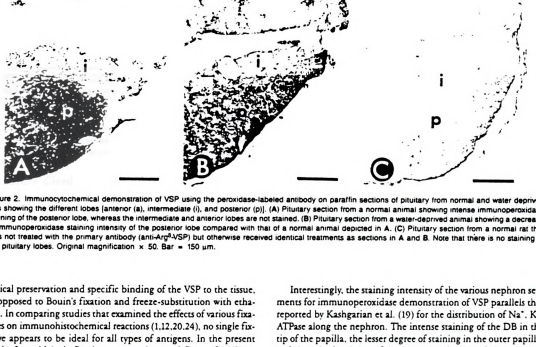

Figure 2. Immunocytochemical demonstration of VSP using the peroxidase-labeled antibody on paraffin sections of pituitary from normal and water deprived rats showing the different lobes [anterior (a), intermediate (i), and posterior (p)]. (A) Pituitary section from a normal animal showing intense immunoperoxidase staining of the posterior lobe, whereas the intermediate and anterior lobes are not stained. (B) Pituitary section from a water-deprived animal showing a decrease in immunoperoxidase staining intensity of the posterior lobe compared with that of a normal animal depicted in A. (C) Pituitary section from a normal rat that was not treated with the primary antibody (anti-Arg^8-VSP) but otherwise received identical treatments as sections in A and B. Note that there is no staining of the pituitary lobes. Original magnification × 50. Bar = 150 μm.

logical preservation and specific binding of the VSP to the tissue, as opposed to Bouin's fixation and freeze-substitution with ethanol. In comparing studies that examined the effects of various fixatives on immunohistochemical reactions (1,12,20,24), no single fixative appears to be ideal for all types of antigens. In the present study, formaldehyde fixation apparently served first to fix VSP to the binding molecules, as evidenced by the fact that there was slight staining of specific tubule segments in tissues incubated in VSP before fixation. Second, formaldehyde acted to preserve binding molecules in a receptive state, which were then able to bind VSP on subsequent incubations. This was apparent in the ability of a subsequent VSP incubation to amplify the staining intensity of specific nephron segments.

Autoradiographic demonstration of the bound VSP (7,9,35,36) and biochemical evidence for most of its activity (17,18,26) have been shown in the renal papilla and medulla, with slight activity in the cortex. The exact nephron segments responsible for the activity have been isolated only through microdissection of intact tubules, with the MAL and MCD accounting for the majority of the activity in the medulla (16,21,30) and the CCD in the cortex (21). The results obtained in this study by the immunohistochemical method conform with those documented after biochemical analysis. Furthermore, our technique offers improvements over other methods by providing increased resolution to permit identification of the exact tubule segments to which VSP is binding in the intact kidney. This method eliminates the biohazard associated with the use of radiolabeled probes and replaces the tedious manipulations of cell isolation techniques and microdissection with standard histological procedures.

Interestingly, the staining intensity of the various nephron segments for immunoperoxidase demonstration of VSP parallels that reported by Kashgarian et al. (19) for the distribution of Na^+, K^+-ATPase along the nephron. The intense staining of the DB in the tip of the papilla, the lesser degree of staining in the outer papilla, and the mixed staining of the intercalated cells and the principal cells of the CCD coincide exactly (19). It has been suggested that these nephron segments function to maintain the high interstitial tonicity of the respective kidney region through movement of sodium from the tubule lumens into the surrounding tissue spaces (19,30,37). This plays an important role in the reuptake of water by the nephron segments. The similarities in staining distribution and intensities suggest a close link between VSP stimulation and sodium resorption in specific cells of the nephron.

The increased immunoperoxidase staining of the DCT and CCD of the kidney sections from water-deprived rats compared with that of normal rats indicates a possible increase in VSP binding sites or higher affinity for VSP after exposure to elevated levels of endogenous hormone. Water deprivation stimulates the release of stored VSP from the neurohypophysis (32), resulting in increased levels of circulating VSP. The primary response of the kidney to the increase in circulating VSP is increased adenylate cyclase (AC) activity, leading to greater water permeability of the stimulated nephron segments (4,16,25,26,30) with a resultant decrease in urine output. Short-term exposure of cells in culture or of tissues in vivo to VSP results in a rapid decline in the number of receptors available to respond to a second round of exposure to the hormone until recovery has taken place (10,25). Conversely, it has been demonstrated in Wistar rats, Long-Evans rats, and the Brattleboro strain

that prolonged exposure to VSP, VSP analogues, or chronic saline infusions leads to increased VSP-stimulated AC activity in the kidneys (2,10,25). Our results demonstrate an increase in binding of VSP to the target tissues, i.e., the DCT and CCD, which parallels such an increase in AC activity (2,10,25).

The specificity of the VSP binding was evaluated by simultaneous incubation of the sections with OXY and VSP. These sections from both normal and water-deprived rats had a much lower staining intensity, as demonstrated by the immunoperoxidase method for VSP, than those tissues incubated in VSP alone. OXY also has known antidiuretic activity (5) and competes with VSP for binding sites on the tubule membrane (8,26,29). Therefore, competition for VSP binding sites by OXY would account for such a decrease in immunoperoxidase staining specific for VSP.

With this immunohistochemical technique, we are able to localize the exact cells responsible for VSP binding, an improvement over the other hormone binding techniques presently used. This may be helpful in routine examination of tissues for altered hormone binding due to physiologic or pathologic conditions.

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II. The Effects of Cisplatin and Carboplatin Upon the Rat Neurohypophysis and Water Balance

ABSTRACT

The effects of cisplatin (CDDP) and carboplatin (CBDCA) upon the neurohypophysis were analyzed in an effort to correlate vasopressin (VSP) release to any changes in urine output using Wistar rats (Crl:(WI)BR). CDDP (2.5 - 9 mg/kg) resulted in morphological and cytochemical changes consistent with a release of VSP from the neurohypophysis and a paralleled decrease in urine output in a dose-dependant fashion. Cytochemical and ultrastructural analyses show a complete loss of neurosecretory granules from the axonal endings, large glycogen accumulations within the pituicytes, and pycnosis especially after high dose treatments. No mitotic activity was observed in the surviving cells. In comparison, 50 mg/kg CBDCA produced a slight increase of neurosecretory material 3 days post-treatment followed by a modest decrease 5 days post-treatment. However only slight changes in urine output were observed. The observed changes in neurohypophyses and the associated urine output following CDDP treatment would be counter-productive to measures taken to alleviate the toxicity.

Index terms: CDDP; cisplatin; cis-dichlorodiammineplatinum (II); CBDCA; carboplatin; cis-diammine 1,1-cyclobutane dicarboxylate platinum II; neurohypophysis; vasopressin; kidney; immunohistochemistry.

INTRODUCTION

CDDP (cis-dichlorodiammineplatinum (II); cisplatin)is a valuable antineoplastic agent primarily used in the treatment of ovarian, testicular and head and neck tumors (Bosl, et al., 1980; Loehrer and Einhorn, 1984; Williams and Einhorn, 1982). The clinical use of CDDP however, is not without severe toxic side effects of which nephrotoxicity is the major dose limiting factor (Foster, et al., 1990). The nephrotoxicity is manifest both as structural and functional impairment of the kidney (Nitschke, 1981; Daugaard and Abildgaard, 1989). Intensive hydration and forced diuresis have been found to be effective in controlling the severity of CDDP nephrotoxicity (Hayes, et al., 1977; Litterest, 1981; Walker and Gale, 1981), however, other side effects such as gastro-intestinal toxicity, myleosuppression, ototoxicity and peripheral neuropathy remain problematic (Foster, et al., 1990; Roelefs, 1984; Wright and Schaefer, 1982).

Analogues of CDDP have been synthesized in efforts to alleviate the toxic side effects while maintaining or improving the therapeutic index (Harrap, et al., 1980; Hydes, et al., 1984; Prestayko, et al., 1979). Of these second generation complexes, CBDCA (cis-diammine 1,1-cyclobutane dicarboxylate platinum II; carboplatin; JM-8) has been the focus of much attention and is now available for clinical use. CBDCA has not been plagued by severe nephrotoxicity, ototoxicity or peripheral neuropathy, but rather myleosuppression appears to be the dose limiting factor in its use.

The most widely accepted target of both CDDP and CBDCA is believed to be the cell nucleus through interaction with DNA (Knox, et al., 1986; Roberts and Pascoe, 1972) and inhibition of macromolecule synthesis (Harder and Rosenberg, 1970). Additional factors such as inhibition of ATPase activity (Guarino, et al., 1979; Batzer and Aggarwal, 1986) and altered mitochondrial function (Aggarwal, et al., 1980; Gordon and Gatton, 1986) may play an important role in the initiation or potentiation of the toxicities associated with CDDP treatment. Molecular studies involving the interaction with DNA alone (Deneve, et al., 1990), can not explain the discrepancies between the toxicities associated with CDDP and CBDCA treatment. Therefore, studies which compare dissimilarities of physiological effects of the two complexes may aid in elucidating the basis of these differences in toxicities.

In laboratory investigations, changes in urine output have been described following CDDP treatment of Sprague-Dawley rats (Gordon, et al., 1982; Clifton, et al. 1982; Safristein, et al., 1981), Fisher rats (Goldstein, et al., 1981), Wistar rats (Appenroth and Braunlich, 1984) and Long-Evans strain (Batzer and Aggarwal, 1986) while a comparative study using Sprague-Dawley rats is available following both CDDP and CBDCA treatments (Batzer and Aggarwal, 1986). CDDP has been found to be diuretic in the Long-Evans rat, while CBDCA is antidiuretic in the same strain. Previous work has linked the diuresis observed in Spague-Dawley rats following CDDP treatment to an altered plasma vasopressin (VSP) levels one day post treatment and lack of kidney response to increased circulating VSP levels 8 days post-treatment (Gordon, et al., 1982).

The changes in the neurohypophysis which resulted in the observed increase in circulating VSP and any comparative studies to access the differences between CDDP

and CBDCA treatment have not been explored. The present investigation was undertaken to compare cytochemical and ultrastructural changes in the neurohypophysis following CDDP or CBDCA treatment using Wistar rats.

MATERIALS AND METHODS

Animals. Male Wistar rats (Crl:(WI)BR) (Charles River Laboratory, Portage MI) weighing between 200 - 300 gm were used through out the study. Animal care was provided through the University Laboratory Animal Care facilities at Michigan State University. Animals were housed in metabolic cages with free access to food and deionized water for up to 8 days post treatment. Urine output, water consumption and body weight were monitored daily.

Drugs. CDDP and CBDCA were the generous gift of the National Cancer Institute and Johnson-Matthey, Inc. Animals were divided into groups of six and were given a single bolus i.p. injection of 2.5, 5, 7, or 9 mg/kg CDDP dissolved in 0.85% NaCl or 50 mg/kg CBDCA dissolved in 5% glucose while controls received the injection vehicle only. Animals were also alternatively treated with 3 weekly injections of 2.5 mg/kg CDDP or 25 mg/kg CBDCA for total doses of 7.5 mg/kg CDDP or 75 mg/kg CBDCA.

In preliminary studies, CDDP treatment of Wistar rats resulted in decreased water consumption, therefore, to access the effects of low water consumption upon the neurohypophysis, rats were pair-watered for 5 days.

Animals were killed on days 1, 3, 5, 8, and 45 following drug treatment and representative tissues from animals in each treatment group were removed for histological

analysis. Pituitary and kidney were removed and fixed in Bouin's solution, dehydrated, cleared and embedded in paraffin. Sections (7μm thick), adhered to glass slides, were stained with either hematoxylin and eosin (H&E) for structural evaluation or by periodic acid/Schiff's method for demonstration of carbohydrates (Humason, 1979). Sections of pituitary were alternatively stained with chromealum hematoxylin for the demonstration of neurosecretory material.

For electron microscopy, pituitaries were fixed in a solution of 1 % glutaraldehyde and 1 % osmium tetroxide in 0.05 M cacodylate buffer (pH 7.2) for 4 hrs at 4 °C. Tissues were dehydrated in acetone and embedded in araldite. Thick sections (1 μm) were stained with methylene blue for structural observations. Ultrathin sections (70 nm) were picked up on formvar coated copper grids, contrasted with lead citrate and uranyl acetate and viewed under a Hitachi HU-11E electron microscope operated at 75 kV.

For immunocytochemical demonstration of VSP pituitaries from various treatment groups were fixed in 4 % formaldehyde in 0.05 M phosphate buffered saline (PBS; pH 7.2) for 4 hrs at 4 °C, dehydrated, cleared with xylene and embedded in paraffin. Immunocytochemical demonstration of VSP was performed as previously described (Fadool and Aggarwal, 1990). The primary antiserum was rabbit anti-Arg8-VSP (Biomedia; Foster City, CA) and the secondary antibody was peroxidase labeled goat anti-rabbit IgG (Boehringer-Mannheim; Indianapolis, IN). Peroxidase activity was demonstrated by the diaminobenzidine reaction.

Immunocytochemical demonstration of VSP binding in kidney sections of CDDP-treated and control rats was performed by the modified method (Fadool and Aggarwal,

1990) of Ravid, et al. (1985). Hydrated sections of kidney were incubated in medium containing 10^{-6} M VSP and 1 % bovine serum albumin, washed with the hormone diluent and VSP was demonstrated as in case of pituitary sections.

Statistical analysis was performed using a one-way analysis of variance with Student-Newman-Keals follow-up test (Steele and Torrie, 1980).

RESULTS

All CDDP-treated animals registered significant weight loss with those in the highest dose groups registering the greatest losses. The CBDCA-treated and control animals gained weight throughout the study.

A striking feature of CDDP treatment in Wistar rats was a marked dose-related decrease in urine output by day 3 post treatment parallelled by a similar decrease in water consumption (Figure 1). This trend reversed by day 6 post treatment with urine output higher in the CDDP-treated than control animals (Figure 2). The increase in urine output one day post treatment was only observed in 5 mg/kg treatment group; 2.5 mg/kg had no significant effect and 7 and 9 mg/kg treatment resulted in decreased urine output for the first 24 hr period. CBDCA-treated rats exhibited only a slight changes in urine output and water consumption, however, neither was significantly different from controls.

The CDDP-treated rats, consistently demonstrated an increased hematocrit value relative to controls through 5 days post treatment. These data are consistent with the considerable dehydrated state following CDDP treatment.

The neurohypophysis is comprised of axons, which have their origins mainly in the supraoptic nucleus and paraventricular nucleus of the hypothalamus, and glial cells (pituicytes) which are distinguishable by their prominent nuclei. The gland is profusely

supplied by capillaries which appear to encircle the pituicytes and axonal endings.

Morphological and cytochemical studies of the neurohypophysis demonstrated characteristic changes corresponding to the fluctuations in the urine output. One day post treatment with 7 or 9 mg/kg CDDP or 3 days post treatment with 5 mg/kg CDDP, there was observed considerably less PAS/chromealum hematoxylin positive staining compared to control (Figure 3A & B), representative of loss of carbohydrate-bearing VSP-associated neurophysin (Ivell, et al., 1983) and neurosecretory material, respectively. In saline-treated animals, the neurosecretory material within the axons could be resolved as distinct granules in 1 μm thick sections of the neurohypophysis (Figure 3C). Three days post 9 mg/kg CDDP treatment there was a considerable decrease in the number of neurosecretory granules contained within the axons (Figure 3D) and loss of cytoplasmic extensions of the pituicytes. Three days post 50 mg/kg CBDCA resulted in a slight increase in neurosecretory granules within the axons (Figure 3E). This was reversed by 5 days post treatment (Figure 3F). Immunocytochemical studies further demonstrated a decrease in VSP specific staining in the neurohypophyses of the CDDP-treated rats (Figure 4).

Concurrent to the loss of neurosecretory material following CDDP treatment were observed characteristic changes in pituicyte morphology. Large accumulations of PAS-positive material which tested positive for glycogen were observed in the pituicytes (Figure 3B). These accumulations were most pronounced 5 days post 7mg/kg treatment. The glycogen accumulations were also observed in the 5 mg/kg treatment group but only 10 days post treatment and only in the periphery of the gland. Pair-watered animals showed a considerably less amount of glycogen accumulations when compared to drug-

treated animals. No accumulations were seen in the control or the CBDCA-treated animals.

CDDP treatment induced rounding of the pituicytes which was most pronounced in the 9 mg/kg treatment group (Figure 5). Such pituicytes no longer maintained cytoplasmic extensions, had increased numbers of lipid droplet accumulations, and demonstrated clumping of nuclear material within the nucleus and degeneration of their cytoplasmic organelles. Rounding of pituicytes was observed by day 1 post-treatment and persisted through the duration of the study. Forty-five days post CDDP treatment, acellular vacuoles remained in place of the degenerated pituicytes.

Nephrotoxicity was accessed by morphological alterations in the kidney. Five days post CDDP treatment (5,7, or 9 mg / kg), lesions of the P_3-segments of the proximal convoluted tubules were evident. The PAS-positive glycocalyx had been lost and carbohydrate containing casts were prevalent in the lumens of collecting tubules. At 45 days post-treatment, the kidney was highly vacuolated; dilated cysts being lined by squamous epithelium were prevalent. No morphological damage was apparent following single dose CBDCA (50 mg / kg) treatment.

VSP binding to kidney sections of control animals as revealed by immunocytochemical staining of exogenous VSP was highest in the papilla with labeling of the epithelial lining of the collecting ducts and the thin loops of Henle (Figure 6). There was moderate staining of the thick ascending limb and little staining of any other tubule segment in the cortex. Following CDDP treatment, there was considerably less staining of the thin loop of Henle in the renal papilla 8 days post treatment while staining of other tubule segments remained unchanged. In kidneys of pair-water rats, staining

similar to that in control animals was observed in the papilla and the medulla, while increased staining for bound VSP was observed in the cortical collecting tubules (Fadool and Aggarwal, 1990).

Chronic damage was observed following 3 weekly injections of 2.5 mg/kg CDDP (Figure 7). Unlike acute CDDP toxicity, these changes were not limited to the cortico-medullary region of the kidney, but were also present in the cortex. Tubule segments were lined by an highly irregular epithelium characterized by cells of grossly enlarged size, with enlarged hyperchromatic-nuclei and thickening of the basement membrane. On the other hand, there was little evidence of kidney damage following 3 weekly injections of 25 mg/kg CBDCA for a cummulative dose of 75 mg/kg (Figure 7). There was observed an occasional focal necrosis of tubule cells but no dramatic alteration comparable to CDDP treatment.

DISCUSSION

The primary mode of action of CDDP in tumor regression is believed to be through its interaction with DNA. Nephrotoxicity has prevented the administration of doses greater than 200 mg/m^2 per administration cycle and has limited the use of CDDP in some cases. The kidney's vulnerability to CDDP may stem from its ability to accumulate and retain CDDP to a greater degree than other organs (Choie, et al., 1980; Terheggen, et al., 1987). In an effort to decrease the accumulation of CDDP in the tubule cells, diuresis therapy has been used in clinical treatments (Hayes, et al., 1977; Walker and Gale, 1981). In an effort to improve the therapeutic index for CDDP, analogues have been synthesized with changes made at the chloride labile groups (Harrap, et al., 1980) of which CBDCA has received attention (Foster, et al., 1990).

Immunocytochemical and morphological evidence establishes that the changes in urine output, following platinum complex treatment of the Wistar rats, coincided with the release of VSP from the posterior pituitary. There was little storage of neurohormone in dense core vesicles and increased incidence of smaller translucent vesicles at the release zones in the axon terminals, consistent with previous ultrastructural observations describing increased release of hormone from the neurohypophysis (Krisch, 1974; Nordmann, 1985). These changes in the neurohypophysis correspond to decreased urine output in a dose dependent manner similar to that previously reported for Wistar

rats (Appenroth and Braunlich, 1984).

The exact mechanism governing the release of neurohormone from the neurohypophysis is not known, although interaction between the pituicytes and the axonal endings is believed to play an important role in the process (Tweedle, 1983; Tweedle and Hatton, 1980a & 1980b; Wittkowski and Brinkmann, 1974). In the normal gland, pituicyte cytoplasmic processes encircle nearby axonal endings, limiting axonal contact with the basement membranes of the capillaries, thereby preventing hormone release. Stimuli which result in the release of VSP or oxytocin (OXY), trigger the retraction of the pituicyte cytoplasmic processes allowing greater axonal/capillary basement membrane contacts thereby facilitating hormone release (Tweedle, 1983; Tweedle and Hatton, 1980a & 1980b; Wittkowski and Brinkmann, 1974). With prolonged stimuli, pituicytes go through a series of morphological changes characterized by increased lipid inclusions, increased glycogen accumulation and lastly, rounding up with apparent swelling of cytoplasmic organelles and pycnosis (Krsulovic and Bruckner, 1969; Olivieri-Sangiacomo, 1972; Rechardt and Hervonen, 1975; Tweedle and Hatton, 1980a & 1980b). The pituicytes, however, retain the plasticity to reverse the process and again encircle the axonal endings following physiological stress (Tweedle and Hatton, 1980a & 1980b). CDDP treatment, however, accelerated the processes leading to pituicyte rounding and initiated irreversible changes in the pituicyte morphology. The glycogen accumulations observed 3 days post 7 mg/kg treatment and the excessive rounding of pituicytes after 9 mg/kg treatment were to a much greater extent than that elicited in pair watered-controls, a potent stimulator of these alterations. Moreover, vacuolization of the gland was observed 45 days after treatment indicating that the degenerated pituicytes

were not replaced by the mitosis of existing glia (DuBois, et al., 1985; Krsulovic and Bruckner, 1969). Therefore, it is suggested that CDDP has dramatic effects upon the functional relationship between pituicytes and axonal endings leading to increased hormone release.

CDDP-induced neurotoxicity now has been frequently observed following cumulative doses greater than 300 mg/m^2 (Roelofs, et al., 1984). The peripheral neuropathy was characterized by axonal degeneration and demyelination (Thompson, et al., 1984). Terheggen, et al., (1989) have recently reported relatively high levels of platinum-DNA binding in the satellite cells in rat dorsal root ganglion compared to neuron nuclei of the ganglion, the brain and the spinal cord. Therefore, effects upon glia may be an important component of CDDP-induced neurotoxicity and may be the basis for the dramatic changes observed in the pituitary. Although, CDDP does not penetrate the brain after systemic administration, presumable due to its inability to cross blood tissue barriers (Douple, et al., 1979), CDDP may have greater access to the pituitary because it is profusely supplied with fenestrated capillaries which lack such barriers.

The elevation of urine output during the first 24 hrs post 5 mg/kg CDDP treatment and the morphologic data consistent with decreased release of neurohormone coincided with previously reported data of urine output and circulating VSP levels following CDDP treatment of Sprague-Dawley rats (Clifton, et al., 1982; Gordon, et al., 1982). The increased urine output was negated with VSP injections demonstrating that the diuresis was not of nephrogenic origin but rather of neuronal origin. It is unlikely, though, that these alterations in urine output resulted from direct inhibition of VSP release by platinum coordination complexes (Clifton, et al., 1982) as suspected. In the

present study, higher dosages of CDDP (7 and 9 mg/kg) elicited changes in pituicyte morphology and urine output during the initial 24 hrs post treatment consistent with VSP release . Furthermore, increased levels of circulating VSP have been reported within 3 hrs of CDDP treatment in dogs (Cubeddu, et al., 1990). Therefore, the inhibitory effects of CDDP upon VSP release in vitro may differ from those effects observed in vivo.

The diuresis observed 6 days post CDDP treatment was similar to that observed for Sprague-Dawley rats (Gordon, et al., 1982; Safirstein, et al., 1981), and the diuresis was unresponsive to the administration of exogenous VSP (Gordon, et al., 1982). The concentration defect was attributed to decreased interstitial tonicity (Gordon, et a., 1982; Safirstein, et al., 1981) as a result of a defect in urea cycling following CDDP administration (Safirstein, et al., 1981). In the present study, decreased binding of VSP to nephron segments, particularly the thin loop of Henle, was observed following CDDP treatment. The role of VSP binding to the thin loop of Henle (TLH) in the antidiuretic response is unknown. However, in the rat the TLH contains a VSP sensitive adenylate cyclase (Morel, 1981). Therefore, lack of hormone response in conjunction with the changes in interstitial tonicity are the likely basis of the increased urine output several days after CDDP administration.

The kidney is the major excretory organ for both CDDP and CBDCA (Saddik, et al., 1988) and diuresis therapy and saline infusion are the most accepted practices for the prevention of CDDP nephrotoxicity (Mistry, et al., 1989). The data presented here as well as previously published results demonstrate increased VSP release from the neurohypophysis following CDDP treatment and dramatic alterations in neurohypophysis morphology. It is suggested that stimulation of VSP release following CDDP treatment

may potentiate the toxic side effects associated with CDDP treatment and impede those steps taken to reduce CDDP-induced nephrotocixity.

Figure 1. Dose dependent changes in water metabolism. Graph illustrating the dose dependency of the changes in water consumption and urine output following CDDP treatment (2.5, 5, 7, and 9 mg/kg) on day 3 (A) and day 5 (B) post treatment (each point represents mean $\pm$ SD; n = 6). (* = $P \leq 0.05$).

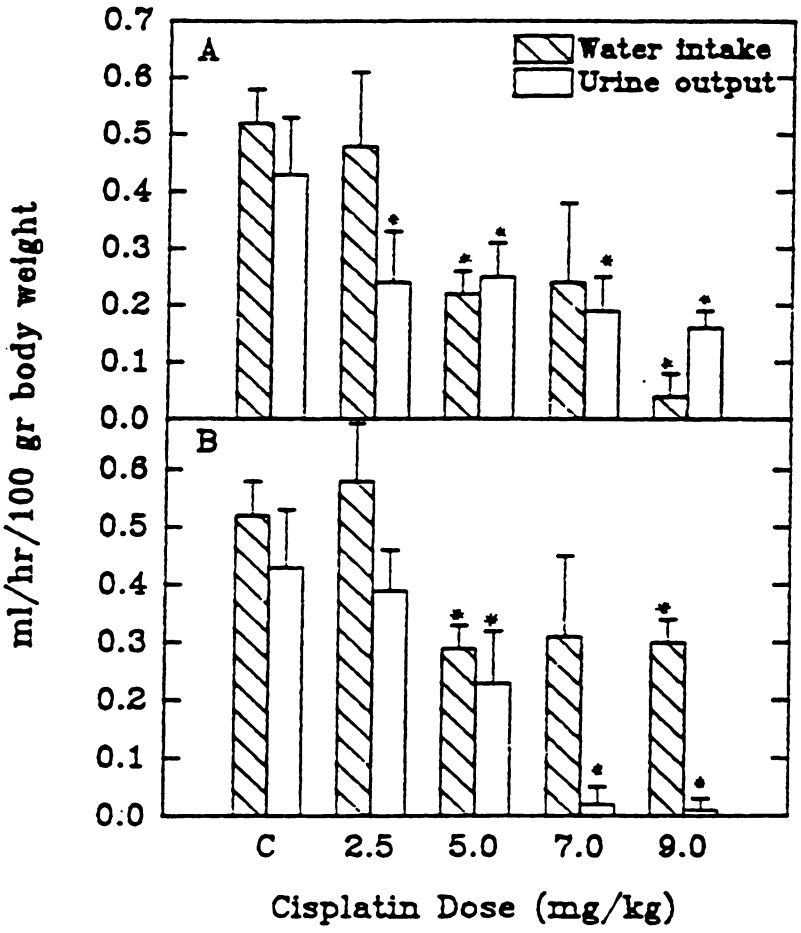
A
B
Water intake
Urine output
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
C
2.5
5.0
7.0
9.0
ml/hr/100 gr body weight
Cisplatin Dose (mg/kg)

Figure 1.

Figure 2. Daily changes in water metabolism. Graph illustrating the daily water intake (A) and urine output (B) following saline, CDDP (5 mg/kg) or CBDCA (50 mg/kg) treatment. Note the decrease in both parameters following CDDP treatment while no change was observed following CBDCA (each point represents mean $\pm$ SD; n = 6).(* = $P \leq 0.05$).

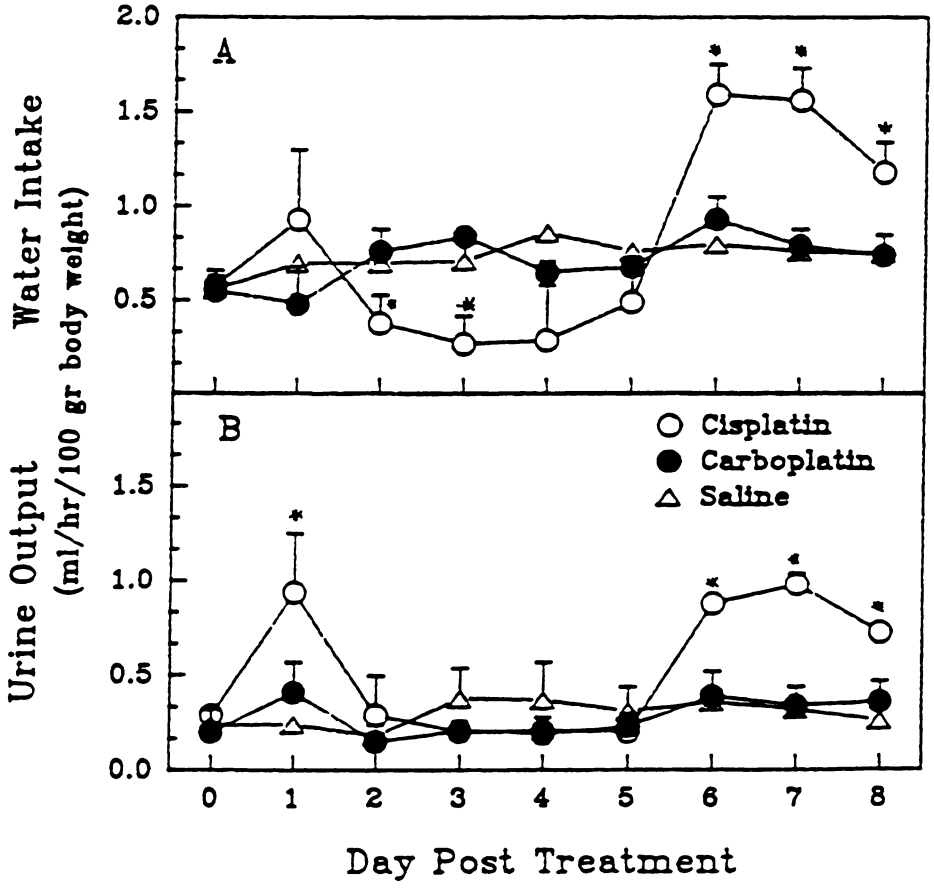
A
B
Cisplatin
Carboplatin
Saline
Urine Output Water Intake
(ml/hr/100 gr body weight)
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
5
6
7
8
Day Post Treatment

Figure 2.

Figure 3. Cytochemical changes in the neurohypophysis. A. Light micrograph of a PAS/Orange-G stained section of a neurohypophysis from a saline-treated rat showing the carbohydrate nature of the neurosecretory material within the axons and the large axonal endings (arrows). B. Light micrograph of a PAS/Orange G stained section 3 days post-CDDP treatment (7 mg/kg). Note the many glycogen accumulations within the pituicytes (arrows) and absence of PAS positive material from the axonal endings. C-F. Light micrographs of a 1 μm thick sections of plastic embedded tissue stained with methylene blue. C. Section showing the neurosecretory granules within the axonal endings (arrows). D. Section 3 days post treatment with 9 mg/kg CDDP showing the loss of neurosecretory granules from the axonal endings (arrows) and rounding of the pituicytes (arrowheads). E. Section 3 days post 50 mg/kg CBDCA showing the increase in neurosecretory material with in the axonal endings(arrows). F. 5 days post 50 mg/kg CBDCA the in considerably less neurosecretory material within the axonal endings (arrows). (Magnification = 1000X); (Bar = 10 μm).

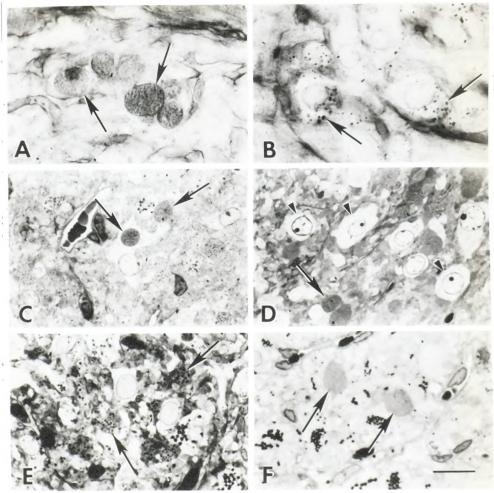

Figure 3.

Figure 4. Immunoperoxidase demonstration for VSP. Immunohistochemical demonstration for VSP in pituitaries of control (A), 5 mg/kg CDDP-treated 5 d (B), and water deprived rats (C) showing the anterior (a), intermediate (i) and neural (n) lobes. Note the intense staining of the posterior lobe of the gland from the control rat, while the posterior lobe of the CDDP-treated and water deprived rats have greatly reduced staining intensity. (A, B & C, x 50); (Bar = 150 μm).

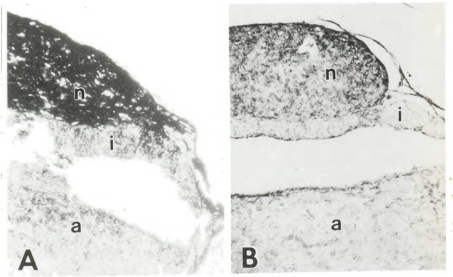

Figure 4.

Figure 5. Ultrastructural changes of pituicytes. A. In the control animal, the pituicytes have long cytoplasmic processes that encircle the axonal endings, limiting axon/capillary basement membrane contact. B. Following 9 mg/kg CDDP treatment for 5 days, the pituicytes retract their cytoplasmic extensions, have swollen and disrupted mitochondria and endoplasmic reticulum, and show condensation of the nuclear material. C. 3 days post 7 mg/kg CDDP treatment, pituicytes show large glycogen accumulations and many cytoplasmic lipid inclusions however the cytoplasmic degeneration and rounding-up is not as striking as in the 9 mg/kg treatment group. (n = nucleus; a = axons; l = lipid inclusions; g = glycogen)(A & B, x 8000); (C x 12,000).

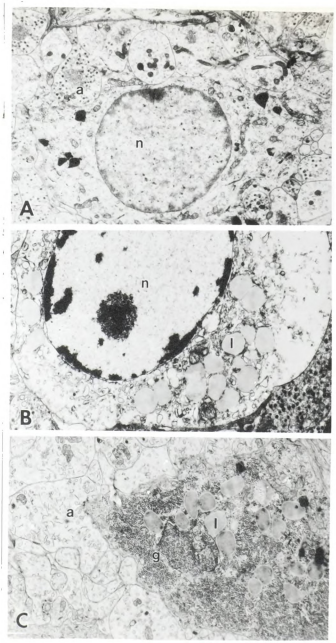

Figure 5.

Figure 6. Demonstration of VSP binding to kidney. Immunohistochemical demonstration of VSP binding to paraffin-embedded sections of kidney from control (A & B) and CDDP-treated (C & D) rats. A. Renal papilla from a control rat showing intense labelling of the ducts of Bellini (arrows) and the thin loops of Henle (arrowheads). B. At a higher magnification, the staining of the loops of Henle in more evident (arrowheads). C. Renal papilla from a rat 8 days-post 5 mg/kg treatment. Note the general decrease in staining intensity and the lack of staining of the thin loops of Henle (arrowheads). D. These alterations are prominent at a higher magnification (arrowheads). (A & C, x 50); (B & D, x 200). (A & C, Bar = 240 μm); (B & D, Bar = 60 μm).

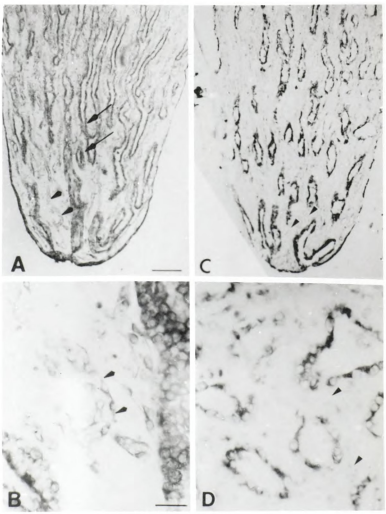

Figure 6.

Figure 7. Chronic damage to kidney. PAS/hematoxylin stained paraffin sections of kidney following chronic CDDP treatment (3 X 2.5 mg/kg/week) (A) or CBDCA treatment (3 X 25 mg/kg/week) (B). A. Gross tubular necrosis, enlarged cells (arrows) and hyperchromatic nuclei were evident following CDDP treatment. B. Little damage was observed following repeated CBDCA administration. (A & B, x 40); (Bar = 50 μm).

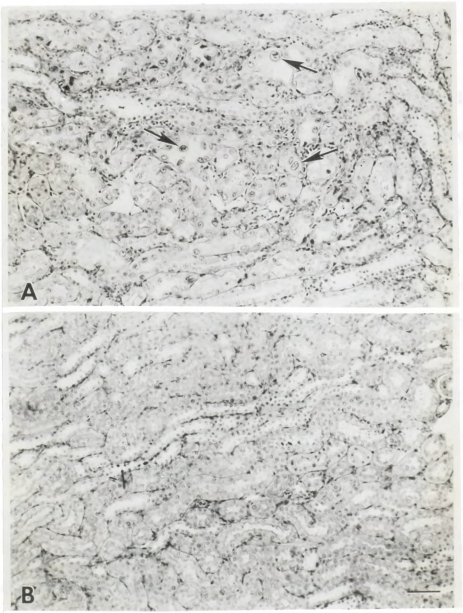

Figure 7.

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III: Role of Calcium in cis-Diamminedichloroplatinum(II) Toxicities and Their Prevention.

ABSTRACT

The role of calcium in CDDP-induced toxicities was examined. A single intraperitoneal injection of CDDP (7 mg/kg) resulted in a significant decrease in urinary calcium excretion with a significant elevation in urinary phosphate excretion while no changes were observed following a single dose of CBDCA (50 mg/kg). The alterations of urinary calcium and phosphate excretion were prevented by injections of 5 mg of calcium as 1.1 ml of 1.3 % $CaCl_2$ immediately proceeding and each day following CDDP treatment. Analysis of parathyroid gland morphology revealed increased activity following CDDP treatment and a reversal of this trend in the calcium plus CDDP-treated rats. Calcium supplementation reduced the severity of the CDDP-induced weight loss and decreased the severity of gastric distension frequently observed following CDDP treatment. In the kidney, histological alterations of the P_3-segment of the nephron and carbohydrate containing casts within tubule lumens were evident following CDDP and calcium plus CDDP treatments, although, there appeared to be some protective effect upon the loss of membrane associated transport enzymes and decreased lipid peroxidation. Calcium supplements seem to reduce the CDDP-induced toxicities.

INTRODUCTION

CDDP (cis-diamminedichloroplatinum II; cisplatin), a potent anticancer agent, has been incorporated into the regimes for the treatment of ovarian, testicular and head and neck tumors (Loerhrer, 1984). Nephrotoxicity, a major limiting side-effect in its use, has been controlled through intensive hydration and forced diuresis (Hayes, et al., 1977; Walker and Gale, 1981). However, many other toxicities such as neurotoxicity, gastro-intestinal toxicity, ototoxicity, and renal ion wasting still remain problematic (Thompson, et al.,1984; Roelofs, et al., 1984; Schilsky and Anderson, 1979).

The severe hypomagnesemia has been accompanied by increased urinary magnesium wasting which indicates altered handling of magnesium by the kidney (Schilsky and Anderson, 1979). Hypocalcemia has become more prevalent with high dose CDDP treatment and has been correlated with the incidence of hypomagnesemia (Danngaurd, 1990). In rabbit, chronic administration of CDDP, resulted in a rise in fractional excretion of magnesium; attributed to a significant reduction in both magnesium and calcium transport by proximal straight tubule segments, however, hypocalcemia has not been reported in these studies (Wong, et al., 1988).

In clinical treatment, the gastro-intestinal toxicity is characterized by nausea and vomiting within hours of treatment and the occurrence of delayed emesis being pronounced 48 to 72 h post-treatment in 60 % to 80 % of the patients (Richtsmeier, 1986; Kris, et al., 1985). In laboratory investigations involving rodents, CDDP-induced

gastro-intestinal toxicity is characterized by stomach bloating, diarrhea, weight loss, and paralysis of intestinal peristalsis (Aggarwal, et al., 1980). The incidence of stomach bloating has been found to parallel the emesis associated with clinical use of CDDP and various chemotherapeutic agents (Roos, et al., 1981; Roos and Oliver, 1987).

The clinical use of the second generation analogue, cis-diammine 1,1-cyclobutane dicarboxylate platinum (II) (CBDCA, JM-8, carboplatin) (Harrap, et al., 1980), on the other hand, has not been associated with gastro-intestinal toxicity or peripheral neuropathy commonly observed following CDDP treatment, nor have there been reports of altered renal handling of ions following CBDCA treatment (Foster, et al., 1990). Altered ion homeostasis and differences in toxic side effects of the two drugs has led us to examine the relationship between calcium homeostasis and various toxicities associated with CDDP treatment but not routinely observed subsequent to CBDCA treatment.

Present is an effort to survey the effects of CDDP and CBDCA treatment upon the mechanisms responsible for calcium homeostasis and to examine the chemotherapeutic potential of calcium supplementation prior to and during CDDP treatment.

MATERIALS AND METHODS

Animals. Male Wistar rats (Crl:(WI)BR) (Charles River Laboratory, Portage MI) weighing between 200 - 300 g were used through out the study. Animal care was provided through the University Laboratory Animals Care Facilities at Michigan State University. Animals were housed in metabolic cages with free access to food and deionized water. Urine output, water consumption and body weight were monitored daily.

Chemicals. CDDP and CBDCA were the generous gift of the National Cancer Institute and Johnson-Matthey, Inc. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals were divided into groups of six or eight and were given a single bolus i.p. injection of 7 mg/kg CDDP dissolved in 0.85% NaCl or 50 mg/kg CBDCA dissolved in 5% glucose while controls received the injection vehicle only. In a separate set of experiments, CDDP-treated rats (7 mg/kg) and control rats were given a daily injection of 5 mg of soluble calcium as 1.1 ml of 1.3 % CaCl2 (w/v) immediately preceding CDDP treatment and each day thereafter while controls received saline only. Calcium supplementation experiments were repeated twice.

Blood samples were collected daily via the orbital sinus or tail vein under light ether anesthesia into heparinized tubes and the plasma separated and frozen for later calcium analysis.

Rats were sacrificed 1, 4 or 8 days after the last treatment. Parathyroid glands (PTG) were dissected free of thyroid tissue and fixed in a solution of 1 % glutaraldehyde and 1 % osmium tetroxide in 0.05 M cacodylate (pH 7.2) for 1 hr at 4°C. Glands were dehydrated in acetone series and embedded in araldite. Sections, 1 μm in thickness, were stained with methylene blue and used for morphometric analysis. Ultrathin sections were picked up on formvar coated copper grids, stained with lead citrate and uranyl acetate and viewed under an Hitachi HU-11E electron microscope operated at 75 kV.

For histological analysis, slices of kidney and liver were removed and fixed in Bouin's solution, dehydrated, cleared and embedded in paraffin. Sections (7 μm thick), adhered to glass slides, were stained by the periodic acid/Schiff's method for demonstration of carbohydrates. For biochemical procedures, the left kidney was excised, the capsule and adjacent adipose tissue were removed and the kidney sliced in half longitudinally. The cortex was dissected free of medullary tissue with a scalpel. The cortex was then weighed and homogenized in ten volumes of deionized water with a Teflon Potter-Elvehjem tissue homogenizer. Samples of liver were removed and homogenized in an identical manner. The homogenates were centrifuged in a clinical centrifuge at low speed to remove large tissue pieces and the supernatant was frozen at -20 °C until used for calcium and lipid peroxidase determinations.

From animals sacrificed 4 days post-treatment, the stomachs were removed; the weight of the stomachs and contents were recorded.

Lipid peroxidation in kidney and liver homogenates was determined by the thiobarbituric acid reaction (Ohkawa, et al., 1979). Briefly, 0.2 ml of the homogenized sample were boiled for 60 minutes in 4.0 ml of solution containing 2% sodium dodecyl

sulfate, 7.5 % acetic acid (pH 3.5), 0.3% thiobarbituric acid. Samples were cooled on ice and spun at 1000 g for 10 min. The absorbance of the supernatant was read at 532 nm and the concentration of malondialdehyde (MDA) was calculated using 1,1,3,3,-tetramethoxy-propane (TMA) as a standard.

The secretory activity of the PTG was measured by computer-assisted image analysis using a General Imaging Inc. (Gainesville, FL) analysis system. Secretory activity was assessed by comparing the area of the gland (in pixels) demonstrating high secretory activity as indicated by dark staining with methylene blue versus the total gland area. Specifically, 1 μm thick sections of plastic embedded glands were stained with a 1% solution of methylene blue in 1% borax and viewed under a Leitz Dialux 20 microscope. Images of the entire gland were taken with a Dage MTI 67M video camera and digitized with an FG100 frame grabber (Imaging Technology Inc., Woburn, MA). For area determination, a preset range of grey tones indicative of intense staining with methylene blue were automatically highlighted and the number of pixels in the highlighted area was automatically counted. The total area of the section (in pixels) was then determined and the percentage of highlighted area versus total area was calculated. Images were processed and analyzed using software from Micro Science Inc. (Federal Way, WA). Three sections from each gland separated by a distance of no less than 50 μm were used in this analysis.

Urine, serum, and tissue calcium analyses were performed by colorometric assay using the indicator phthalein purple and measuring absorbance at 565 nm (Ray Sarkar and Chauhan, 1967). Protein concentration was determined using the Biorad (Richmond, CA) reagent. Inorganic phosphate concentration of urine samples and serum was

determined using Sigma Chemical Co (St. Louis, MO) diagnostic kit 670.

RESULTS

Following CDDP treatment, as a single bolus injections of 7 mg/kg, there was observed considerable weight (Figure 1) loss compared to controls and CBDCA-treated rats (50 mg/kg). However, calcium supplementation during CDDP treatment, had a significant effect upon the severity of weight loss compared to CDDP treatment alone (Figure 1).

CDDP treatment caused a significant decline in urinary calcium excretion and a paralleled increase in urinary phosphate excretion (Figure 2). Calcium supplementation during CDDP treatment (7 mg/kg) reversed this trend resulting in a normal pattern of calcium and phosphate excretion. No changes in calcium and phosphate excretion were observed following CBDCA treatment (Figure 2).

Plasma calcium concentration and kidney calcium content in CDDP and CBDCA-treated rats are listed in Table 1. No alteration of plasma calcium levels and liver calcium content (data not shown) were observed. However, there was a significant elevation of kidney calcium content 24 hours after CDDP and CBDCA treatment. By day 4 post treatment, this trend was reversed in the CBDCA-treated rats, but remained elevated in the CDDP treatment group.

CDDP treatment markedly altered PTG morphology. At the light microscope level, CDDP treatment resulted in a dramatic increase in the number of dark chief cells (Figure 3) compared to PTG from control animals. Fine structural analysis of the PTG 4 days post CDDP treatment revealed many dark chief cells with highly involuted plasma

membranes, irregular nuclear membranes and swollen endoplasmic reticulum and Golgi (Figure 4) as compared to the glands of control animals. Calcium-supplementation before and after CDDP treatment reversed this trend (Figures 3 and 4). These PTG were composed of predominantly light chief cells with no involutions of their plasma and nuclear membranes. These cells also demonstrated little ER and Golgi in the cytoplasm.

Image analysis of 1 μm thick section of parathyroid glands (Table 2) revealed a significant increase in the activity of the gland from CDDP-treated animals (75.8 ± 10.6) when compared to controls (59.8 ± 5.8). Calcium supplementation greatly reduced the hyperactivity of the gland (15.9 ± 12).

Four days post-treatment with CDDP (7 mg/kg), the stomachs and their contents exhibited a significant increase in mass compared to control animals (Figure 5). Calcium supplementation did significantly affect the mass of the stomachs compared to CDDP alone although the gastric distension was not completely eliminated in all animals tested (Table 2).

Although calcium supplementation had a notable effect upon gastro-intestinal toxicity, only a slight protective effect from nephrotoxicity was appearent. Acute CDDP treatment (7 mg /kg) resulted in gross tubular lesions predominantly in the cortical-medullary region of the kidney. This was first evident one day post treatment. By 4 days, there was an obvious loss of the PAS positive brush border of the cells of the P_3-segment of the proximal tubule and accumulation of PAS positive material in the lumen of distal tubules of the cortex and the loops of Henle and collecting ducts of the renal medulla (Figure 6). Kidney morphology in the CDDP plus-calcium treatment group was

effected to a lesser degree. CDDP treatment was also characterized by a loss of phosphatase activity from kidney tubule segments as previously reported (Batzer and Aggarwal, 1986), however, calcium supplementation appeared to offer some protective effect, with a significant reduction in the severity of the CDDP-induced decrease in membrane transport enzymes activity. Calcium supplementation was also associated with decreased lipid peroxidation in the kidney cortex compared to CDDP treatment alone (Table 2). Again, no morphological or cytochemical damage was evident following CBDCA treatment (50 mg/kg).

DISCUSSION

Clinical use of CDDP is plagued by a number of toxicities of which nausea and vomiting remain problematic and are often responsible for the interruption of treatment. In laboratory investigations, the stomach bloating in the rat has paralleled the emesis associated with CDDP treatment (Roos, et al., 1981) and has been used as a model to study the incidence, severity and prevention of gastro-intestinal toxicity (Roos and Oliver, 1987).

In the present study, we report that the CDDP-induced gastric distension could be lessened by prior treatment with calcium chloride and the calcium-supplementation protected some animals from the decline in body weight frequently observed following CDDP treatment. The effects may be related to the prevention of altered calcium homeostasis following CDDP treatment.

Hypocalcemia has been observed following CDDP treatment (Blachley and Hill, 1981; Hayes, et al., 1979; Schilsky and Anderson, 1979), and it has become more

prevalent with high dose CDDP treatment with a significant correlation with the decreased plasma magnesium levels (Daugaard, 1990). It has been suggested that the CDDP induced hypocalcemia was related to the lack of end organ response (bone) to circulating parathyroid hormone as a result of magnesium deficiency (Blachley and Hill, 1981) or to decreased production and release of PTH (Hayes, et al., 1981). It has also been demonstrated that CDDP inhibits PTH-stimulated bone-resorption in vitro, using an embryonic mouse bone assay (Abramson, et al., 1988). Therefore it would be anticipated that magnesium supplementation would correct these alterations. However, Blom, et al., (1985) have reported decreased serum calcium levels in spite of magnesium supplementation and normal serum magnesium levels. PTH maintains serum calcium levels through increased organic matrix breakdown and calcium and phosphate release (Wong, 1982). In the present study CDDP treatment resulted in decreased renal calcium excretion, increased phosphate excretion and morphological changes in PTG activity corresponding to hyperactivity (Swivastav and Swarup, 1982; Wild, et al., 1982) while no alterations of plasma calcium concentration were observed. These data are consistent with parathyroid hormone stimulated changes of renal excretion of calcium and phosphate (Bourdeau, et al., 1988; Armbrecht, et al., 1981; Burnatowski, et al, 1977). Furthermore, Mavichak, et al. (1984;1985) reported decreased bone phosphate and magnesium content following chronic administration of CDDP in rats which, in the context of hyperparathyroidism, could have also resulted from PTH-induced bone resorption. Therefore, hyperparathyroid activity was associated with CDDP treatment in the rat in an attempt to maintain normal serum calcium levels.

Although, CDDP treatment of bone tissue in vitro reportedly decreased PTH

stimulated calcium release (Abramson, et al., 1988), this may have been due to cytotoxic effects upon the bone cells. CDDP decreased the number of osteoclasts in this system. Alternatively, CDDP at the dosage used in the culture system could be altering intracellular signalling mechanism responsible for the PTH-stimulated osteoclast proliferation and bone resorption, however, further studies are needed to support this conclusion.

Calcium supplementation had a significant effect upon CDDP-induced gastro-intestinal toxicity. San Antonio and Aggarwal (1984) demonstrated that fundal strips from CDDP-treated animals and control animals contract in a similar manner while maintained in calcium containing medium but CDDP treatment resulted in hypercontractility to acetylcholine and serotonin in a dose dependant fashion provided they were placed in a calcium free medium. These data demonstrate that the stomach bloating was likely to be due to altered neurotransmitter release (San Antonio and Aggarwal, 1984) with the nonselective increase in sensitivity of the muscle to neurotransmitter. Such an action by CDDP has been supported by the demonstration that CDDP effusion decreased calcium dependant action potentials in electrically excitable cells (Oakes, et al., 1987). Inhibition of calcium-dependent action potentials would conceivably inhibit calcium evoked neurotransmitter release (Zucker and Haydon, 1988) and influence stomach bloating. Further support is taken from experiments which demonstrated ruthenium compounds, which alter calcium channel activity, alter smooth muscle contractility in vivo, and treatment results in symptoms characteristic of CDDP-induced gastro-intestinal toxicity (Kruszyna, et al., 1980). Therefore, alteration of calcium homeostasis which produced the hyperparathyroid activity may underlie the

gastric distension observed following CDDP treatment.

Although calcium supplementation effectively decreased the gastrointestinal toxicity associated with CDDP treatment, only a slight protective effect upon kidney toxicity was observed. Parathyroid hormone stimulates an increase in intracellular calcium in renal tubular epithelium (Goligorsky and Hruska, 1988) and Nagata and Rasmussen (1970) have also demonstrated that a rise in calcium levels causes increased gluconeogenesis in isolated kidney tubules which is also observed following CDDP treatment. Changes in intracellular calcium concentration have been linked to the pathogenesis of cellular toxicity (Trump and Berezesky, 1984). Therefore, it is speculated that parathyroid hormone may be potentiating the nephrotoxic effects of CDDP through an alteration of intracellular calcium concentration.

Capasso and associates (1989; 1990) have also examined the role of PTH in CDDP-induced nephrotoxicity and reportedly have achieved some success in decreasing the severity of the toxicity through parathyroidectomy. Furthermore, injections of PTH increase blood urea nitrogen (BUN) and serum creatinine (SCR) levels over CDDP treatment alone, strengthening the argument that hormonal influence upon kidney function play a role in potenciation of the nephrotoxicity. However, as previously stated, PTH may only be a contributing factor in the severity of the nephrotoxicity but not the cause or initiator of the damage, since severe nephrotoxicity was still prevalent in the parathyroidectomized animals (Capasso, et al., 1989; Capasso, et al., 1990).

Parathyroidectomy had little effect upon the weight loss associated with CDDP treatment (Capasso, et al., 1990) while calcium supplementation had a considerable effect. These data suggest that the mechanism attributing the gastric toxicity in rodents

may be independent of those factors contributing to the nephrotoxicity.

In summary, alterations in urinary calcium and phosphate excretion following CDDP treatment could be explained by increase PTG activity however, no alteration in plasma calcium was observed. These changes could be reversed with calcium supplements, which also had a significant effect upon the gastrointestinal toxicity and a modest effect upon the renal toxicity associated with CDDP treatment. These data suggest that alteration of calcium homeostasis leading to stimulation of PTG activity may aggrevate CDDP-induced toxicities.

Figure 1. Body weight changes. A. Body weight changes for Wistar rats following ip injection of CDDP (7 mg / kg), CBDCA (50 mg / kg) or saline on day 0. (Mean $\pm$ SD; n = 6 for each treatment group). B. Body weight changes for Wistar rats following a single ip injection of 7 mg/kg CDDP, CDDP + daily injection of 5 mg ionized calcium, or saline. (Mean $\pm$ SD: n=8 for each treatment group).

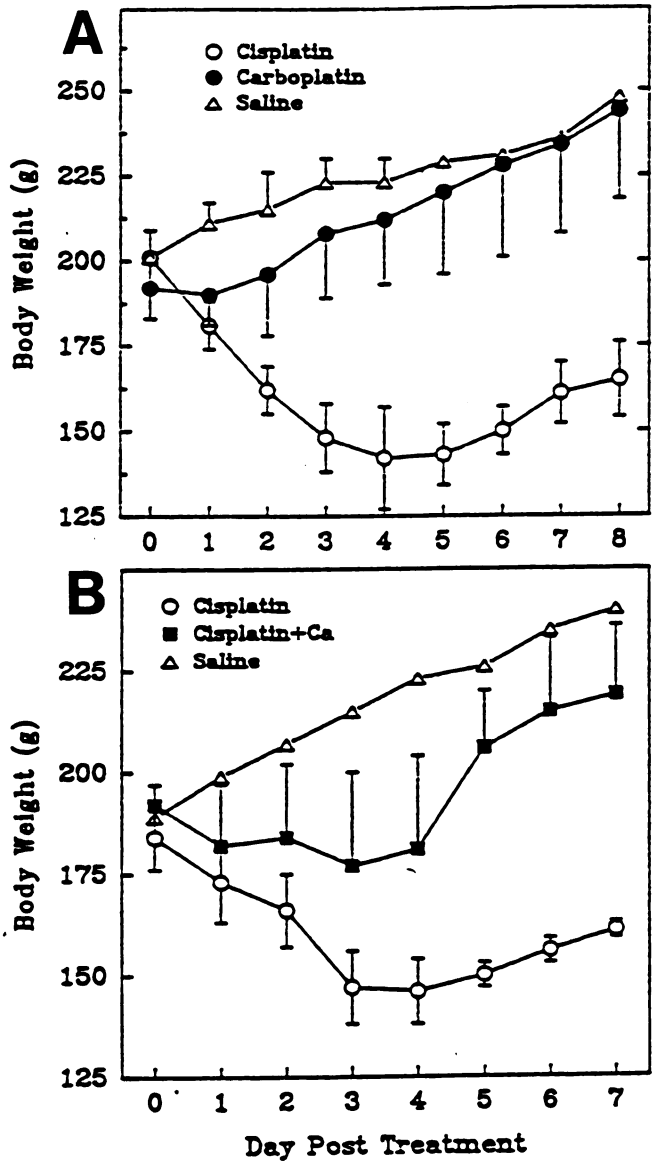
A
Cisplatin
Carboplatin
Saline
Body Weight (g)
250
225
200
175
150
125
0 1 2 3 4 5 6 7 8
B
Cisplatin
Cisplatin+Ca
Saline
Body Weight (g)
225
200
175
150
125
0 1 2 3 4 5 6 7
Day Post Treatment

Figure 1.

Figure 2. Divalent ion excretion. Urinary calcium (A) and phosphate excretion (B) following CDDP treatment or saline alone. (Standard error of the means was $\leq$ 10 % mean; n=8 for each data point).

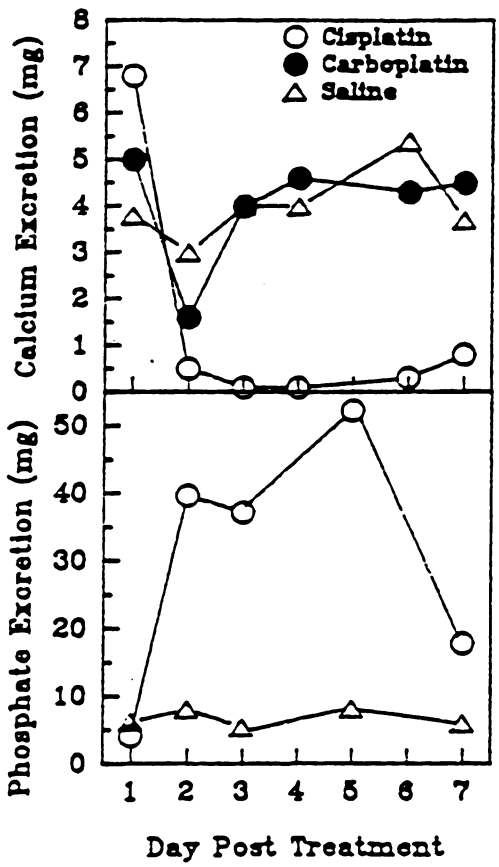
Cisplatin
Carboplatin
Saline
Calcium Excretion (mg)
Phosphate Excretion (mg)
Day Post Treatment
0
1
2
3
4
5
6
7
8
10
20
30
40
50

Figure 2.

Figure 3. PTG morphology. Light microscopic evaluation of PTG morphology of saline-treated (A), CDDP-treated (B), and calcium-supplemented CDDP-treated (C) rats. Note the preponderance of dark chief cells in the gland following CDDP treatment (B) compared to saline treatment (A). Calcium-supplementation (C) reversed this trend with light cells comprising the majority of this gland. (A & C, Dark chief cells are highlighted). (Bar = 100μm).

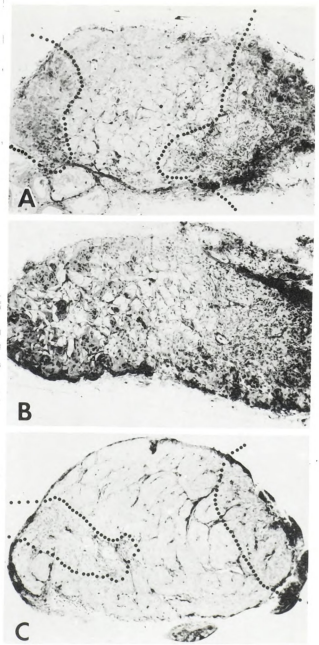

Figure 3.

Figure 4. PTG ultrastructure. Fine structural analysis of PTG 5 days post treatment. A. Note dark chief cells with highly irregular nuclei (N) and extensive endoplasmic reticulum (ER)(arrow). B. Light (L) and Dark (D) chief cells demonstrating the normal appearence of the gland. Golgi, G; mitochondria, M; nucleus, N. C. Light chief cells predominate in the glands from CDDP-treated plus calcium-injected animals. Note the relatively smooth appearence of the nucleus (N), regular plasma membranes, and inactive ER (arrows). (Bar = 1 μm).

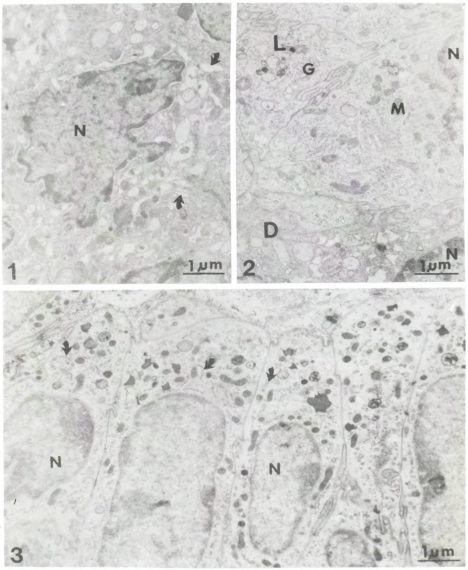

Figure 4.

Figure 5. Gastric distension. Comparative appearence of stomachs following CDDP treatments. Photomicrograph comparing the sizes of the stomachs from saline-treated (A), CDDP (7 mg/kg) plus daily calcium-treated (B) an CDDP-treated (7 mg/kg)(C) rats four days after initiation of treatment.

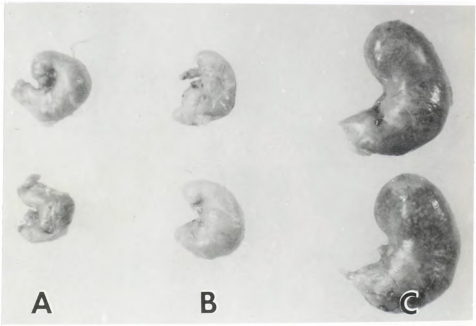

Figure 5.

Figure 6. Comparative nephrotoxicity. PAS stained kidney sections from saline-treated (A), CDDP-treated (B), and CDDP plus calcium-treated (C) rats. (A) Saline-treated rats demonstrate PAS positive glycocalyx in the lumens of the proximal tubules (arrows). (B) Staining of the glycocalyx was greatly reduced (arrows) and there were PAS positive casts in the lumens of the collecting ducts (arrowheads) following CDDP treatment. (C) CDDP plus calcium treatment also resulted in kidney damage. However there was not the complete loss of the PAS positive glycocalyx from the proximal tubule cells (arrows) and fewer casts in the collecting duct lumens (arrowheads). (Bar = 40 μm)

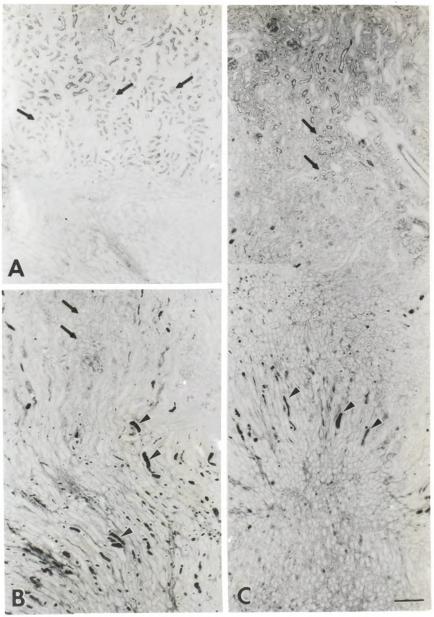

Figure 6.

TABLE 1. EFFECT OF CDDP AND CBDCA ON PLASMA AND TISSUE CALCIUM CONCENTRATIONS

	Pl_{Ca2+}[a]	K_{Ca2+}[b] Day 1	K_{Ca2+}[b] Day 4
Saline	9.2 ± 0.4	0.39 ± 0.01	0.40 ± 0.01
CDDP	9.1 ± 0.2	0.43 ± 0.01*	0.51 ± 0.02*
CBDCA	9.4 ± 0.3	0.43 ± 0.01*	0.42 ± 0.02

[a], Plasma calcium concentration, mg/dl, 4 day post treatment;
[b], Kidney cortical calcium concentration, μg/mg protein;
*, significantly different from saline-treated ($P < 0.05$)

TABLE 2. EFFECT OF CALCIUM UPON CDDP-INDUCED TOXICITIES

	PTG Morphology % Active	Mass of Stomachs[a] Plus Contents	Lipid[b] Peroxidation
Saline	59.8 ± 5.8	3.2 ± 1.2	72 ± 6
CDDP	75.8 ± 10.6*	13.2 ± 2.7*	96 ± 7*
CDDP + Calcium	15.9 ± 12**	7.4 ± 6.1***	72 ± 12

[a], mass of stomachs and their contents in grams.
[b], peroxidation values are nmol malondialdehyde per mg protein.
* , **, Significantly different from saline treatment ($P < 0.05$ and 0.01 respectively).
***, Significantly different from CDDP treatment ($P < 0.05$).

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IV: Effect of CDDP and CBDCA on Sarcoma-180 Cells In Vitro: Role of Intracellular Calcium in Toxic Assault.

ABSTRACT

The effect of CDDP and CBDCA upon intracellular calcium ion concentration ($[Ca^{2+}]_i$) was investigated. Monolayer cultures of sarcoma-180 cells were loaded with the intracellular fluorescent indicator indo-1, and $[Ca^{2+}]_i$ of single cells was monitored before and after platinum complex treatment. In all concentrations tested, neither CDDP nor CBDCA had a significant effect upon resting $[Ca^{2+}]_i$, mitochondrial sequestration of calcium, or mechanism responsible for the general maintenance of $[Ca^{2+}]_i$. Furthermore, during two hours of treatment no change in membrane topography, actin filament distribution and cell viability was observed. Following platinum complex treatment, cultures returned to normal medium and allowed to grow for 3 days illustrated a dosage dependant decline in cell density relative to controls. There was, however, a concurrent increase in cell diameter and the formation of multinucleated giant cells. The mechanisms resulting in the giant cell formation were not identified, however, this may result from an alteration in the localized signalling processes.

[1]Abbreviations: intracellular calcium ion concentration, $[Ca^{2+}]_i$; CDDP, cis-diamminedichloroplatinum (II); CBDCA, JM-8, cis-diammine 1,1-cyclobutane dicarboxylate platinum (II); adenisine triphosphate, ATP; sarcoma-180, S-180; CDDP hydrolysis products, DDP-OH; mercuric chloride, $HgCl_2$; Minimal Essential Medium, MEM; supplemented calf serum, CS; fluorescein-labeled phalloidin, Fl-Ph; N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, HEPES; indo-1 acetylmethoxyester, indo-1/AM; Erhlich tumor, ET; Hank's balanced salt solution buffered with 10 mM HEPES, HBSS; extracellular calcium-ion concentration, $[Ca^{2+}]_e$; phosphate buffered saline, PBS; intraperitoneal, i.p.

INTRODUCTION

For many years, dystrophic calcification of cells and tissues has been recognized as an indication of tissue necrosis. Recently, Several groups of investigators (Smith, et al., 1987; Trump and Berezesky, 1984; Trump, et al., 1989) have put forth a dynamic model characterizing the role of altered intracellular ion homeostasis in the initiation of cytotoxicity and tumorigenesis. The pattern of acute cytotoxicity can be described by an alteration of membrane function resulting in the elevation of intracellular ion concentrations with ensuing biochemical and morphological changes. Loss of membrane integrity may include the direct inhibition of ATPase activity, modification of ion channels, or the general increase of membrane permeability through activation of compliment, lipid peroxidation or alteration of sulfhydryl groups (Smith, et al., 1987; Trump and Berezesky, 1984; Trump, et al., 1989). Injury to intracellular processes or compartments would include inhibition of mitochondrial respiration, depletion of energy production and arrest of organelle function associated with ion homeostasis (Smith, et al., 1987; Trump and Berezesky, 1984; Trump, et al., 1989). In the proposed model, the elevation of intracellular calcium ion concentration ($[Ca^{2+}]_i$)[1] would initiate depolymerization of microtubules and microfilaments, bleb formation of the plasma membrane, and altered macromolecule synthesis.

It has been postulated that the anticancer agent CDDP (cis-

diamminedichloroplatinum II; cisplatin), may have an effect upon $[Ca^{2+}]_i$, and that the increase in $[Ca^{2+}]_i$ could be an underlining cause of the severe nephrotoxicity, ototoxicity and neurotoxicity associated with CDDP treatment (Aggarwal and Niroomand-Rad, 1983; Comis, et al., 1986; DeWitt, et al., 1988; Oakes, et al., 1987). CDDP has been demonstrated to inhibit adenisine triphosphatase (ATPase) activity (Daley-Yates and McBrien, 1982; Guarino, et al., 1979; Uozumi and Litterest, 1985), cause sloughing of membrane transport enzymes (Aggarwal and Niroomand-Rad, 1983; Batzer and Aggarwal, 1986), inhibit mitochondrial respiration (Aggarwal, et al., 1980; Gordon and Gatton, 1986), decrease mitochondrial accumulation of divalent cations (Aggarwal, et al., 1980; Gemba, et al., 1987), and decrease plasma membrane sulfhydryl groups (Batzer and Aggarwal, 1986; Levi, et al., 1980). Changes in membrane function, such as these, could conceivably result in alterations of intracellular ion homeostasis.

In comparison to CDDP, the second generation analogue CBDCA (JM-8, cis-diammine 1,1-cyclobutane dicarboxylate platinum (II); carboplatin), has not been found to dramatically alter membrane function in vivo or in vitro (Batzer and Aggarwal, 1986). Additionally, clinical use of CBDCA has not been associated with nephrotoxicity, ototoxicity or peripheral neuropathy frequently seen following CDDP treatment (Canetta, et al., 1985; Foster, et al., 1990). The differences in nephrotoxicity are more outstanding when one considers that following CDDP or CBDCA administration, platinum concentrations in the kidney are similar (Siddik, et al., 1988; Terheggen, et al., 1987). Furthermore, DeNeve et al. (1990) have reported that 3- to 4-fold more CBDCA had to be given compared to CDDP to obtain equal amounts of DNA crosslinking, on the other hand to elicit equitoxicity a 13- to 16-fold increase in dose was needed. The

discrepancies between tissue concentrations, DNA interstrand crosslinking and toxicity suggest the toxic effects may in part be related to sites of action by CDDP other than the cell nucleus.

The advent of fluorescent, ion-specific intracellular probes has enabled investigators to monitor $[Ca^{2+}]_i$ fluctuations of cultured cells in response to various stimuli (Goligorsky and Hruska, 1988; Rink, 1988). Using the intracellular probe indo-1, we have investigated the response of sarcoma-180 (S-180) cells, to toxic assault with CDDP, CBDCA, CDDP hydrolysis products (DDP-OH) and mercuric chloride ($HgCl_2$).

MATERIALS AND METHODS

Chemicals and Reagents. CDDP and CBDCA were the generous gift of Johnson Matthey Research Laboratories and the National Cancer Institute. Minimal Essential Medium (MEM), Hank's balanced salt solution without phenol red and sodium bicarbonate, and supplemented calf serum (CS) were obtained from GIBCO (Grand Island, NY). Indo-1 was purchased from Molecular Probes (Eugene, OR). ATP, fluorescein-labeled phalloidin (Fl-Ph), B-glycerophosphate, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), indo-1 acetylmethoxyester (indo-1/AM), $HgCl_2$, levamisole, ouabain, and trypsin-EDTA solution were obtained through Sigma Chemical Co., (St. Loius, MO). All other components were of the finest grade available. Water used throughout the study was first deionized and then glass distilled.

DDP-OH was prepared by dissolving CDDP into distilled water and allowing the solution to age for a period of 7 days at 37 °C. Previous chromatographic studies (Mistry, et al., 1989) have demonstrated the formation of a single neutrally charged specie with identical chromatographic properties as DDP-OH following this procedure. The formation of DDP-OH was confirmed by thin-layer chromatography (Hall, et al., 1976), using silica gel plates and isopropanol/ formic acid/ water (20:1:5) as the mobile phase. Platinum containing species were detected with $SnCl_2$. Rf values were compared to known standards of CDDP dissolved in 100 mM NaCl.

Male Wistar rats (Crl:(WI)BR) (Charles River Breeding Laboratories) weighing between 200 and 250 grams were housed and maintained in facilities provided by the University and managed by Laboratory Animal Care Service. All experiments involving the use of vertebrate animals were preapproved by the University Animal Use Committee.

S-180 cells, obtained from American Type Culture Collection (Rockville Pike, MD), were received at passage 92. All subsequent experiments were preformed on passage numbers 95-102. Erhlich tumor (ET) cells were generously provided by Irwin J. Goldstein of the University of Michigan. All cells were maintained in MEM buffered with 10 mM HEPES and supplemented with 10% CS.

In vitro studies.

Intracellular Calcium Measurement. $[Ca^{2+}]_i$ was measured using the fluorescent Ca^{2+} indicator indo-1 (Grynkiewicz, et al., 1985; Tsien, et al., 1985). The membrane permeable form of the dye, indo-1/AM, was stored at -20 °C as a 1 mM stock solution in dimethyl sulfoxide. The working solution was prepared by diluting the stock solution to a final concentration of 3 μM in Hank's balanced salt solution buffered with 10 mM HEPES, pH 7.4 (HBSS).

S-180 cells were plated at a density of 2.5×10^{-5} cells/ml in 35 mm culture dishes which had a portion of the bottom removed and an acid/alcohol cleaned coverglass affixed in its place. This step was necessary to accommodate the oil immersion objective of the Olympus IMT-2 inverted microscope. Twenty-four to 24 hrs after plating, the cultures were rinsed twice with HBSS and incubated in 1 ml of the working solution of

indo-1/AM for 45 min at 37 °C. Once within the cytosol, intracellular esterases cleave the membrane permeant form of the dye to the membrane impermeable form indo-1, thus trapping the dye within the cytosol (Tsien, 1981). After loading, cultures were rinsed with 2 changes of HBSS and stored in HBSS until use.

Fluorescent intensities were monitored using the ACAS 470 Interactive Laser Cytometer (Meridian Instruments, Inc.; Okemos, MI) outfitted with an Olympus inverted microscope and oil immersion objectives. Ultraviolet excitation was with the 351-363 laser lines with simultaneous emission recordings at 405 nm and 485 nm for the calcium-bound and calcium-free species of indo-1 respectively. Actual $[Ca^{2+}]_i$ was calculated by comparing the ratio of emission intensities at 405 nm/485 nm with those of a standard curve. The standard curve was generated by taking the ratio of the emission intensities recorded for 3 μM indo-1 in a solution containing 10 mM HEPES, 115 mM KCl, 20 mM NaCl, 1 mM $MgSO_4$, 1 uM EGTA, 10% ethanol and concentrations of $CaCO_3$ ranging from 0 to 1 mM. All experiments were run in the line scan mode of operation to enable a 1 sec delay between the initiation of scans and to minimize the photobleaching of the dye. All experiments were repeated on at least three separate occasions.

CDDP, CBDCA, $HgCl_2$, and KCN were dissolved in HBSS immediately prior to use. Groups of cells, which had characteristic fibroblast morphology, were selected for analysis. Scans were initiated at a 1 to 5 sec delay interval and repeated 300 to 500 times per trial. Test compounds were added after the initial 30 to 50 scans to allow adequate time for base line $[Ca^{2+}]_i$ determination.

In a second set of experiments, the effect of the various compounds upon $[Ca^{2+}]_i$ was monitored in calcium free HBSS and in the presence of high extracellular calcium-

ion concentration ($[Ca^{2+}]_e$). Cultures were loaded with indo-1 as described above. Immediately prior to calcium analysis, the HBSS was removed and replaced with calcium-free HBSS containing 10 mM EGTA. Cells were selected and treated with test compounds as previously stated. At various time points following the addition of the drugs to calcium free cultures, $CaCl_2$ dissolved in HBSS was added to the cultures to achieve a 1.37 mM $[Ca^{2+}]_e$ and fluorescence intensities were monitored for an additional 5 min.

Controls included monitoring untreated cultures for the time points indicated and monitoring cultures treated with the vehicle solution alone.

In all experiments, alterations of $[Ca^{2+}]_i$ were compared by peak height and the rate of change in $[Ca^{2+}]_i$. The rate of $[Ca^{2+}]_i$ elevation was calculated as the slope of the line from the onset of the change in $[Ca^{2+}]_i$ to the highest recorded concentration. The rate of decline in $[Ca^{2+}]_i$ was taken as the slope of the line from the highest measured value for $[Ca^{2+}]_i$ to the first plateau that occurred in the trace.

Cell viability: Cells were plated at a density of 2.5 x 10^5 cells/ml in 25 cm^2 closed culture flasks. After 24 hr, the medium was replaced with HBSS. CDDP, CBDCA, or $HgCl_2$ were added to the cultures and cell viability was monitored in treated and control cultures for 2 h by trypan blue exclusion. Cultures were rinsed twice with MEM + 10 % CS and returned to normal medium. Cell morphology and cell density were observed daily for several weeks using a Nikon Diaphot inverted microscope outfitted with a 20 x objective. On day 3 post-treatment, cell were removed from the culture vessels by trypsin digestion and cell volumes were calculated from the cell diameter as measured

with an ocular micrometer.

Actin visualization: S-180 cells and ET cells were plated at a density of 1 x 10^5 cells/ml on acid/alcohol cleaned glass coverslips. Twenty-four hours later, cultures were rinsed twice with HBSS and various amounts of CDDP, CBDCA, DDP-OH or $HgCl_2$ dissolved in HBSS were added to the cultures. At set time intervals ranging from 1 min to 120 min, samples were removed from the treatment solution and fixed for five minutes at room temperature in 3.7 % formaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4. Cells were rinsed in PBS, dehydrated in acetone at -20 °C and air dried. F-actin was visualized by inverting each coverslip on 150 μl of 2 μM Fl-Ph in PBS for 40 min at 37 °C in a humid chamber. Coverslips were then thoroughly rinsed with PBS and mounted on glass slides with a 1:1 mixture of glycerol and PBS. Photobleaching of the fluorescein was inhibited by the addition of p-phenylenediamine to the mounting medium. Stained cells were photographed under a Nikon Labophot epifluorescent microscope outfitted with 40 X neofluor objective lens.

Phosphatase Demonstrations. Two Wistar rats were killed by decapitation and their kidneys removed, mounted in OCT mounting medium and frozen in liquid nitrogen. Frozen sections 10 μm in thickness were cut using an IEC cryostat. Sections were picked up on glass coverslips, allowed to air dry and were briefly fixed at 4 °C in a solution of 1% glutaraldehyde and 7 % sucrose in 0.05 M tris-maleate buffer (pH 7.4). After fixations, sections were washed twice in the buffered sucrose solution.

To study the effects of drug treatment on phosphatase activity, kidney sections

were treated with CDDP dissolved in 0.05 M tris-HCl (pH 7.4) for up to 120 min. After drug treatment, sections were rinsed in buffered sucrose and processed for the cytochemical demonstration of alkaline phosphatase, Na^+/K^+-ATPase, and Ca^{2+}/Mg^{2+}-ATPase as previously described (Batzer and Aggarwal, 1986).

In vivo studies. Wistar rats were divided into treatment groups of six animals. Animals received a single intraperitoneal (i.p.) injection of CDDP (5 mg/ml) dissolved in saline or CBDCA (50 mg/ml) dissolved in 5% glucose solution while controls received the injection vehicle only. Animals were killed for analysis 1 day, 3 days and 5 days post injection. Kidneys were removed and frozen sections were obtained as previously described. After fixation, sections were stored in buffered sucrose solution for phosphatase demonstrations.

Statistical analysis was performed using Student's t-test for one way comparisons or a one way analysis of variance and Student-Newman-Keals follow-up test for multiple comparisons (Steel and Torrie, 1980).

RESULTS

Using the intracellular fluorescent probe indo-1, $[Ca^{2+}]_i$ was measured in S-180 cells grown in subconfluent cultures. The resting $[Ca^{2+}]_i$ was found to be 95 $\pm$ 6.5 nm (n=12). Addition of 7.5 to 75 μg/ml CDDP, 50 to 500 μg/ml CBDCA or 7.5 ug/ml DDP-OH had no immediate effect upon resting $[Ca^{2+}]_i$ (Figure 1). At the end of 120 min of treatment with 10 μg / ml CDDP the resting $[Ca^{2+}]_i$ was 96.3 $\pm$ 13.9 nm (n=12) which was not significantly different from pre-treatment levels. Addition of either 7.5 μg/ml $HgCl_2$ (Fgiure 1B) or 5 mM KCN (Figure 1C) to the platinum-treated cultures, resulted in rapid and sustained increases in $[Ca^{2+}]_i$ to 409 $\pm$ 35 nm and 231.5 $\pm$ 17 nm respectively.

To examine the possibility that extracellular calcium protects cells from an alterations of $[Ca^{2+}]_i$ during drug treatment, HBSS was replaced with Ca-free HBSS and the cells were treated with various concentrations of the drugs. Again, no change in $[Ca^{2+}]_i$ was observed following addition to the culture of CDDP or CBDCA. Interestingly, no elevation of $[Ca^{2+}]_i$ was observed following $HgCl_2$ treatment in the calcium free medium.

Following drug treatment for 4 minutes of the cells in Ca free HBSS, CaCl2 solution was added to the cultures to achieve a final $[Ca^{2+}]_e$ of 1.37 mM. In control cultures (Figure 2), a rapid increase in $[Ca^{2+}]_i$ followed by a gradual decline at a rate of -

7 nM Ca^{2+}/sec to near pre-treatment levels. As previously stated, treatment of cells in Ca-free HBSS with CDDP produced no change in $[Ca^{2+}]_i$. Elevation of the $[Ca^{2+}]_e$ in CDDP-treated cultures resulted in a rise in $[Ca^{2+}]_i$ and rate of decline similar to that observed with normal cells (Figure 2b). This was also the case following CBDCA treatment. However, raising the $[Ca^{2+}]_e$ of $HgCl_2$-treated cultures resulted in a rapid and sustained increase in $[Ca^{2+}]_i$ (Figure. 2C) indicating the alteration in $[Ca^{2+}]_i$ in response to $HgCl_2$ resulted from the loss of plasma membrane integrity and passage of extracellular calcium into the cytosol.

Treatment of cultures of S-180 cells with CDDP or CBDCA had no effect upon cell viability during the 2 hr of exposure. Treated cultures returned to normal media did however, illustrated a dosage dependant decline in cell density relative to controls with no surviving cells 24 hrs after treatment with either 75 mg/ml CDDP or 500 mg/ml CBDCA. At the lower concentrations of CDDP and CBDCA there was evidence of continued macromolecule synthesis with no cell division, resulting in the formation of giant cells. The giant cells retained the characteristic fibroblast morphology of the original cell line with apparent increases in cell size (Figure 3). Morphometric analysis of untreated S-180 cells and giant cells 3 days post-treatment with 50 ug/ml CBDCA revealed a 3 fold increase in cell volume relative to untreated cells [$314 \pm 10.6\ \mu m^3$ and $113 \pm 2.8\ \mu m^3$ ($p < 0.05$)]. No difference in cell volumes were found between platinum treatment groups. By the fourth day post-treatment, many of the giant cells appeared multinucleated or had micronuclear formations however no mitotic spindles were observed. The giant cells reached confluence in the 7.5 μg/ml CDDP treatment group and the 50 μg / ml CBDCA treatment group with no parallel increase in cell number.

Viability of the giant cells began to decrease by the tenth day post-treatment. None of the giant cells could be maintained in culture indefinitely.

Staining of S-180 cells with Fl-Ph revealed many stress fibers and web like structures of actin in the cell bodies and filopodia. Neither CDDP nor CBDCA had any effect upon the distribution of the actin filaments during 2 hr of treatment and no membrane blebbing was observed (Figure 4). In comparison, $HgCl_2$ treatment resulted in a large decrease in the number and distribution of actin filaments in both the cell bodies and filopodia (Figure 4) and there was considerable membrane blebbing after extended treatment (data not shown). Cell viability began to decrease within 2 hr of $HgCl_2$ treatment.

In cytochemical studies, no appreciable loss of phosphatase activity was observed following incubation of kidney sections from untreated rats in CDDP containing medium. However, a much different pattern was seen in kidney sections from drug-treated animals. As previously described (Batzer and Aggarwal, 1986), CDDP treatment resulted in a significant decrease in enzyme activity as compared to control animals. These changes were first evident 3 days post-treatment and were much more pronounced by the fifth day. Following CBDCA treatment, no alteration in the distribution or intensity of reaction product for any of the three enzymes was observed when compared to sections from control animals.

DISCUSSION

Using the intracellular fluorescent indicator, indo-1, it was demonstrated that neither CDDP nor the second generation analogue CBDCA have an effect upon $[Ca^{2+}]_i$, calcium buffering mechanisms, and mitochondrial sequestration of calcium in intact S-180 cells. Additionally, no alterations in the distribution or number of actin filaments and no membrane blebbing were observed for the duration of CDDP or CBDCA. These data demonstrated the lack of events associated with alteration of intracellular ion homeostasis following CDDP and CBDCA treatment.

Decreased plasma membrane enzyme activity had been considered a likely target of CDDP treatment. Guarino, et al. (1979) has demonstrated that CDDP was able to significantly inhibit ATPase activity in vitro, although, it was latter suggested by Uozumi and Litterest (1985) that it was unlikely that inhibition of ATPase is the cause of the nephrotoxicity. Although CDDP was able to inhibit ATPase activity in vitro (Daley-Yates and McBrien, 1982; Guarino, et al., 1979), excessively high concentrations or prolonged times of incubation are required. CDDP has been found to have no direct effect upon calcium and magnesium transport in perfused kidney tubules (Wong, et al., 1988). The present study as well as previous histochemical (Batzer and Aggarwal, 1986) and biochemical (Uozumi and Litterest, 1985) analyses of the changes observed in vivo, have demonstrated that the decrease in membrane associated phosphatase activity does

not occur for several days following CDDP treatment when other indicators of nephrotocixity, (e.g. elevated blood urea nitrogen) are already evident (Uozumi and Litterest, 1985). Furthermore, decreased alkaline phosphatase activity in tumor cells and in the kidney has been attributed to sloughing of the plasma membrane components as opposed to direct inhibition of enzyme activity, which suggests that other changes in cell structure must take place before the loss of enzyme activity becomes evident.

Mitochondria have also been suspected as a target of CDDP treatment. Swelling of the cristae, decreased divalent cation accumulation and an altered state of respiration have been associated with CDDP treatment in vivo (Aggarwal, et al., 1980; Gordon and Gatton, 1986). Addition of CDDP o CBDCA to preparations of isolated mitochondrial produce alterations in mitochondrial function similar to those observed in vivo, however, these changes occur on the order of seconds to minutes (Aggarwal, et al., 1980; Binet and Volfin, 1977; Gemba, et al., 1987) rather than days. This may suggest that mitochondria are a possible target of platinum coordination complex treatment, however, other data appear contrary to this conclusion. The effects of the drug upon mitochndria in vivo are not apparent until several days after CDDP treatment, and as stated previously. As such, these alterations must be secondary to the initial mode of action of the drug. In the present study KCN was still able to elicit a release of mitochondrial stores of calcium even after 2 h of CDDP treatment of S-180 cells demonstrating no change in mitochondrial sequenctration of this divalent ion. Furthermore, Choie et al. (1980) have demonstrated that following CDDP treatment, the highest concentrations of platinum are found in the cell cytosol, followed by the nuclei and microsome fractions and lastly the mitochondria. Consequently, buffering by other cellular components

potentially dampened the effect of CDDP upon the mitochondria and therefore, the former experiments using isolated mitochondria may not properly depict the actual setting of the mitochondria within the intracellular milieu.

Although these studies ebb the possibility of global alterations of $[Ca^{2+}]_i$ by CDDP and CBDCA, this does not preclude the possibility of subtle effects upon mechanisms of calcium signaling within the cell. From a broad range of studies a trend has been uncovered describing the effects of CDDP treatment upon calcium-dependant signalling mechanisms. Oakes, et al. (1987) demonstrated that the extracellular application of CDDP to electrically excitable cells, decreased calcium-dependent action potentials although no concurrent effect upon the resting membrane potential was observed. CDDP has inhibitory effects upon the release of VSP from isolated pituitaries (Clifton, et al., 1982) and decreased parathyroid hormone-induced bone resorption in vitro (Abramson, et al., 1988); both calcium dependent processes (Robinson, et al., 1976; Wong, 1982). De Witt, et al. (1988) have reported that administration of nephrotoxic doses of CDDP resulted in stimulation of a renal endoplasmic reticulum calcium pump prior to the initiation of other indicators of nephrotoxicity. Additionally, differences in the biophysical properties of intracellular calcium channels prepared from CDDP-sensitive and CDDP-resistant cell lines have been reported (Vassilev, et al., 1987). Calcium channels from the CDDP-resistant cell line have been shown to have a larger mean open time and a higher probability of an open state compared to those of the sensitive cell line. The authors interpret these differences as related to a higher rate of CDDP efflux, but alternatively the channels from the resistant cell line may balance any detrimental effect CDDP may have upon intracellular calcium signaling mechanisms.

Aggarwal and Sodhi (1973) and Aggarwal (1974) first observed giant cells following CDDP treatment and suggested that inhibition of cytokinesis with continued macromolecule synthesis was central to their formation. Subsequently, several other investigators have linked the formation of giant cells following CDDP treatment to G2 arrest after replication of DNA (Just and Holler, 1989; Vates, et al., 1985) which suggests mechanisms of action other than the inhibition of DNA synthesis. In the present study we report the formation of giant cells in response to both CDDP and CBDCA treatment, which suggests that both complexes have a similar mode of action in this test system. Nonetheless, the exact mechanism governing the formation of giant cells has not yet been established.

Recent evidence suggests that localized changes in $[Ca^{2+}]_i$, trigger mitotic events such as nuclear envelope breakdown (Kao, et al., 1990) and movement of chromosomes during anaphase (Zhang, et al., 1990). The polymerization and depolymerization of actin filaments is also tightly coupled to changes in the $[Ca^{2+}]_i$ (Downey, et al., 1990). Taking these factors into account, as well as CDDP's possible inhibition of calcium coupled events, it is suggested that giant cell formation may result from an alteration of the intracellular signalling mechanisms responsible for the initiation and progression of mitotic events. Therefore, the absence of overall changes in $[Ca^{2+}]_i$ may imply that the changes elicited by platinum complexes upon signalling mechanism are too local to be detected.

In comparison to changes observed following CDDP or CBDCA treatment, were changes observed after $HgCl_2$ treatment. $HgCl_2$ induced a rapid rise in $[Ca^{2+}]_i$ in S-180 cells in the presence of extracellular calcium but no change was observed in its absence.

Subsequent to the rise in $[Ca^{2+}]_i$, there was plasma membrane blebbing and alteration in the distribution of actin filaments.

The sequence of events following mercury treatment was in good agreement with that proposed for the pathogenesis of cellular toxicity through the alteration of intracellular ion homeostasis (Smith, et al, 1987; Trump and Berezesky, 1984; Trump et al., 1989). Similar findings were observed in treatment of primary cultures of kidney tubule cells with mercury compounds, KCN, ouabain, and the calcium ionophore A23187 (Smith, et al., 1987; Trump, et al., 1989). The only subtle difference between our experiments and those previously reported for $HgCl_2$ was the requirement of extracellular calcium for initiation of the toxic effects. However, in review of several reports it is evident that the dependance upon extracellular calcium varies with the cell type used and the type of chemical assault.

In summary, no global change in $[Ca^{2+}]_i$ was observed following CDDP or CBDCA treatment of S-180 cells in vitro, although giant cell formation was observed following treatment with either platinum coordination compound. The exact mechanism of giant cell formation was not clear, though it may represent a phenomenon associated with interruption of localized signals responsible for triggering cell division.

Figure 1. $[Ca^{2+}]_i$ following platinum treatment. Alteration of $[Ca^{2+}]_i$ in response to CDDP, $HgCl_2$ and KCN treatments. Drugs were added at the indicated point (arrow) to cultures of S-180 cells maintained in HBSS. A. Treatment with CDDP (7.5 μg/ml) had no immediate effect upon $[Ca^{2+}]_i$. B. Addition of $HgCl_2$ (5 μg/ml) to CDDP-treated cultures elicited an immediate and sustained increase in $[Ca^{2+}]_i$. C. Addition of KCN (10 μg/ml) to CDDP-treated cultures resulted in a sustained increased in $[Ca^{2+}]_i$. The delay in response observed with KCN varied between cells presumably due to variation in rate of accumulation within the cells.

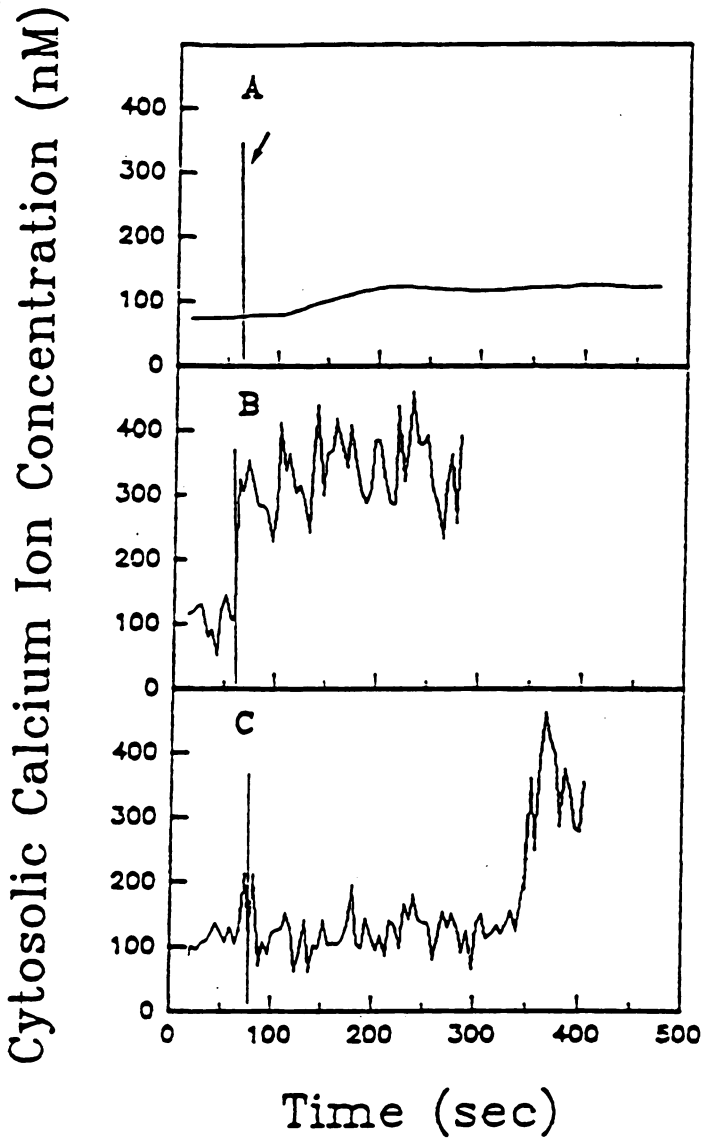
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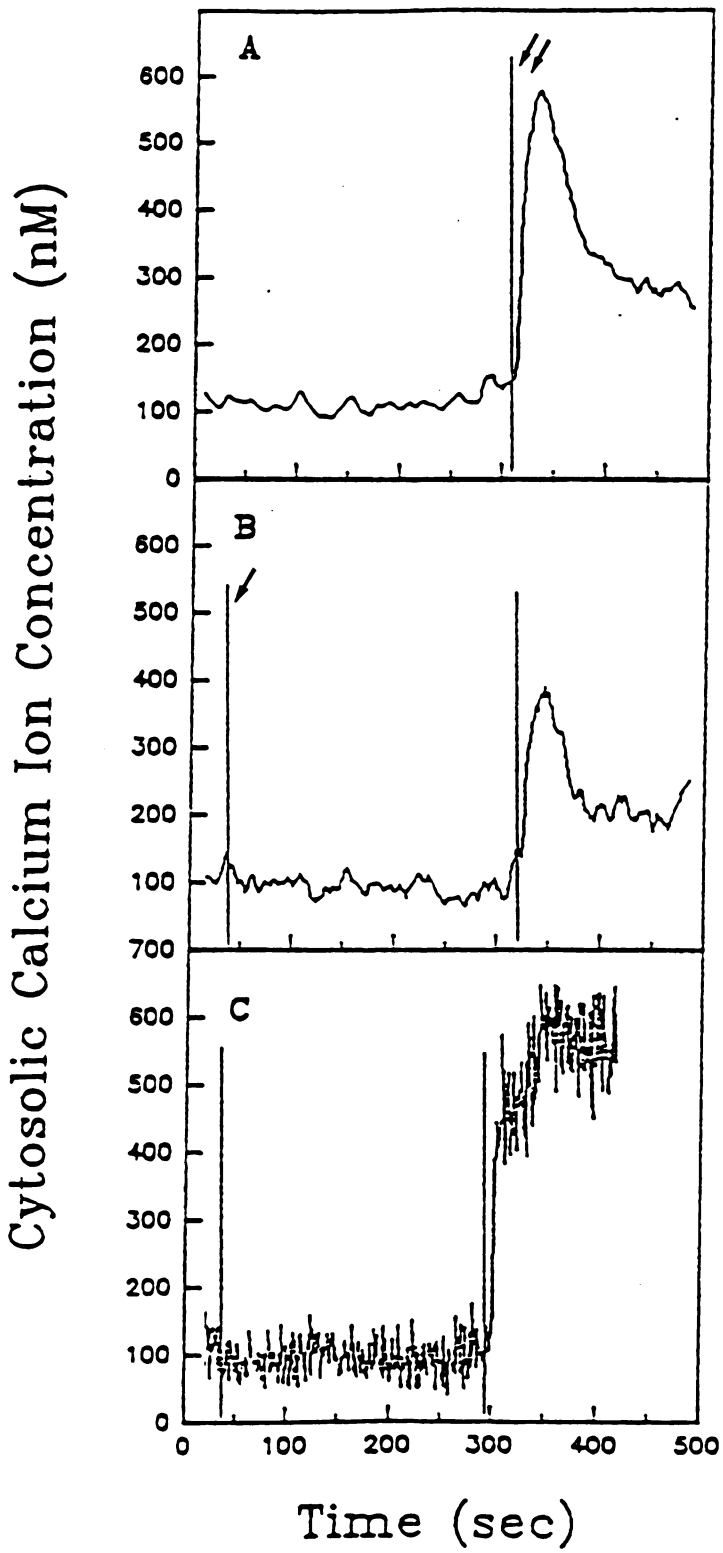
Figure 1.

Figure 2. $[Ca^{2+}]_i$ in Ca^{2+} free medium. Changes in $[Ca^{2+}]_i$ of control, CDDP-treated and $HgCl_2$-treated cells in calcium free-HBSS and after the addition of $CaCl_2$. HBSS was replaced with calcium free-HBSS and drugs were added at the point indicated (arrow). CaCl2 was then added to the cultures (double arrow) to increase the $[Ca^{2+}]_e$ to 1.37 mM for the final minute of observation. A. Representative trace of the effects of increasing the $[Ca^{2+}]_e$ to 1.37 mM on $[Ca^{2+}]_i$ of a control cell. The $[Ca^{2+}]_i$ initially rose at a rate of 7 μM/sec, reached a peak of 620 $\pm$ 120 nM and fell to near pretreatment levels. B. Trace demonstrating no effect by treatment with CDDP upon changes of $[Ca^{2+}]_i$ induced by an elevation of $[Ca^{2+}]_e$. C. Elevation of $[Ca^{2+}]_i$ in $HgCl_2$-treated cultures was observed only after the addition of calcium to calcium-free cultures.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Cytosolic Calcium Ion Concentration (nM)
Time (sec)

Figure 2.

Figure 3. Giant cell formation. A. Untreated culture demonstrating normal appearance of S-180 cells in monolayer culture. B. The appearance of giant cells following 7 days of recovery from a 2 hr exposure to 7 μg/ml CDDP. Note the grossly enlarged size and presence of multinucleated cells. C. Giant cells formed after 7 days recovery from 2 hr exposure to 30 μg/ml CDDP. Note the similar appearance as the lower dose treatment but the presence of fewer cells. Bar = 50 μm.

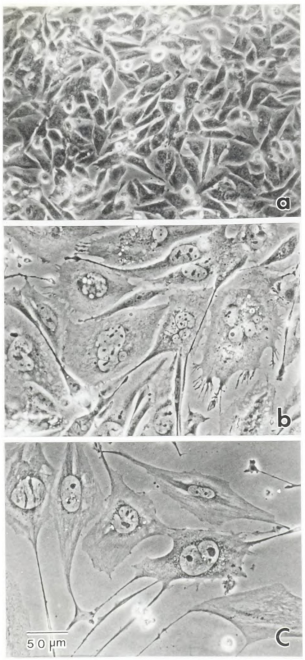

Figure 3.

Figure 4. Actin distribution in S-180 cells. Cultures of S-180 cells were treated with CDDP and $HgCl_2$ and actin filaments were demonstrated using FL-PH. a. Untreated cells demonstrated many stress fibers and fine web like filaments near the cell nucleus. b. No change of actin filament distribution was observed in CDDP-treated cultures (7.5 μg/ml for 2 hrs). c. Treatment with 5 μg/ml $HgCl_2$ for 15 min resulted in decreased staining of stress fibers and actin filaments associated with the peripheral areas of the cells and formation of large cluster of actin about the cell nucleus. Bar = 10 μm.

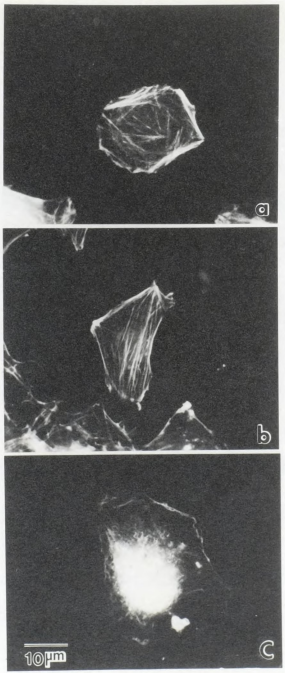

Figure 4.

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The material presented within the manuscript was undertaken in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Zoology at Michigan State University.

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