This is to certify that the

thesis entitled

Effect of Cisplatin on the Plasma Membrane Phosphatase Activities in Ascites Sarcoma-180 Cells: A Cytochemical Study

presented by

Iran Niroomand-Rad

has been accepted towards fulfillment of the requirements for

MASTER OF SCIENCE degree in Zoology

S. K. Agarwal

Major professor

Date 3/25/82

EFFECT OF CISPLATIN ON THE PLASMA MEMBRANE PHOSPHATASE ACTIVITIES IN ASCITES SARCOMA-180 CELLS: A CYTOCHEMICAL STUDY

By

Iran Niroomand-Rad

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Zoology

1982

ABSTRACT

EFFECT OF CISPLATIN ON THE PLASMA MEMBRANE PHOSPHATASE ACTIVITIES IN ASICTES SARCOMA-180 CELLS: A CYTOCHEMICAL STUDY

By

Iran Niroomand-Rad

In order to study the effects of cisplatin [cis-dichorodiammine-platinum (II)] on the tumor cells in presence or absence of the immune system, animals with ascites sarcoma-180 tumor burden were treated with therapeutic dose levels (9 mg/kg). Similarly, ascites sarcoma-180 cells were maintained in tissue culture media containing the same levels of the drug. Cell samples were taken from the animals at 12 hour intervals for 3 days where as samples were drawn from the tissue cultures at 15, 30, 45, 60 minutes, 2, 3, 4 and 5 hour intervals. Treated and untreated cells from *in vitro* and *in vivo* experiments when checked for alkaline-phosphatase, 5'-nucleotidase, Ca^{2+}-ATPase and Na^{+}-K^{+}-ATPase show a gradual decrease in activity on the plasma membrane. It takes about 60 minutes for inactivation of any enzyme *in vitro* while it takes 2 days *in vivo* experiments. Quantitative analysis show alkaline phosphatase activity drops from 9.7 to 4.9 nmoles in just 15 minutes further droping to 0.79 nmoles after 2 hours. Inactivation of various plasma membrane enzymes resulting in permeability changes are probably responsible for cell death.

Dedicated to my parents and Masood
to whom I owe everything

ACKNOWLEDGEMENTS

I wish to thank Dr. S. K. Aggarwal, my research advisor, for his constant support of this research with regard to the facilities and research materials provided. I would like to express my appreciation to him, not only for his expert technical advice and assistance, but also for the many things he taught me that were non-science related. His constant help and advice are greatly appreciated.

Appreciation is due also to Drs. J. L. Edwards and S. L. Flegler, who served as guidance committee members and assisted in the preparation of this thesis.

The author also expresses appreciation to Dr. K. M. Brown for his advice concerning experimental technique.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Amminoplatinum (II) complexes have attracted considerable attention as potential chemotherapeutic agents (2,3,46,48) against a broad spectrum of chemically-induced, virally-induced and transplantable tumors both in animals (49) and in humans (27). Of these, cis-dichlorodiammineplatinum (II) (cisplatin, DDP) is now available for the treatment of testicular (54) and ovarian cancers (28). However, the drug is not free from its toxic side effects on the kidney (proteinuria and shedding of the renal enzymes in the urine, together with changes in renal morphology), intestine (emesis, anorexia and diarrhea), and toxicity to the hematopoietic organs (bone marrow aplasia, splenic atrophy, atrophy of thymus and lymph nodes) (3). Renal toxicity remains the most limiting factor in its use. A second generation of platinum coordination complexes with little or no nephrotoxicity is currently being tested in clinical trials (16). Ways are still being sought to combat the toxic side effects of the available compound through the use of various diuretics and antagonists without rendering the drug ineffective against tumor systems (46).

Although various symptoms due to use of the drug have been well classified since its first discovery, the mechanisms of its action in tumor regression or its various toxicities have not yet been fully understood (42,47). Inhibitory effects of some platinum compounds on DNA, RNA and protein synthesis have been demonstrated in vitro using mammalian cells (41,25). The interaction of cisplatin with DNA seems to

be through the cross-linking of the complementary strands of DNA (42,60). If the DNA has already been replicated, cisplatin has been shown to inhibit the process of karyokinesis (1). Again, if the nucleus has already replicated the process of cytokinesis has been shown to be arrested, probably through the depolymerization of microfilaments (1). Further possible enhancement of the antigenicity of tumor cells (48) and regression of the tumor through the immune system have been proposed (3,7,45).

In order for cells to perform their essential metabolic functions, they must be able to transport ions and molecules against a concentration gradient. Present investigations were undertaken to study various plasma membrane enzymes such as Ca^{+2} ATPase, Na^{+}-K^{+}-ATPase, Alkaline phosphatase and 5'-nucleotidase that are involved with the transport of ions across the membrane, better to understand the mechanism of action of cisplatin in tumor regression and the cause of its various toxicities.

MATERIALS AND METHODS

In Vitro Studies

Highly inbred Swiss Webster female mice 4-5 weeks old (Spartan Research Animals, Haslett, Michigan) were implanted intraperitoneally with ascites sarcoma-180 according to protocols of the Cancer Chemotherapy National Service Center (50). The day of tumor implant was taken as day 0. The animals were sacrificed by cervical dislocation on day 8 of the tumor implant. Ascites fluid was removed with the help of a disposable pipette and transferred to culture flasks containing Eagle's Basal Medium (BME) with Eagle's Salt (Gibco, Grand Island, N.Y.) containing 2x amino acids with glutamine supplemented with 10% calf serum. Cultures were maintained at 37°C. Cell counts were made at the start of each experiment using a Coulter counter model ZB1. A concentration of 10^7 cells/ml was a standard for each experiment.

Cisplatin (Johnson Matthey Research Laboratories, Sonning Commons, Reading, U.K.) was added to the cultures as a freshly prepared solution (stock solution 9 mg/10 ml of 0.75% sterile saline) at a concentration of 0.9 mg/100 ml of the culture. Aliquotes of cells were withdrawn at 0, 15, 30, 45 and 60 minutes, 2, 3, 4 and 5 hours and were fixed in 1% buffered glutaraldehyde (0.05 M cacodylate buffer, pH 7.3) for 10 minutes at 4°C. After fixation, cells were washed twice in buffered sucrose (3.5% sucrose in 0.1 M cacodylate buffer, pH 7.3) and stored at 4°C until use.

Animal Studies

Swiss Webster female mice 4-5 weeks old with ascites sarcoma-180 were injected with sterile cisplatin solution (9 mg/kg) as intraperitoneal injection on day 8 of the tumor implant. The animals were sacrificed at 12 hour intervals (6 in each group) for a period of 8 days and the cells were collected using a disposable pipette and processed for Ca^{2+}-activated ATPase studies.

Cytochemical Studies

Ascites sarcoma-180 cells after various intervals of drug treatment and fixation were incubated in one of the several media as described below.

Alkaline Phosphatase (AP) activity was detected after incubation of cells at 37°C for 60 minutes in a medium containing 0.2 M Tris-maleate buffer (pH 8.2), 1.2% sodium-β-glycerophosphate, 0.2 mM magnesium chloride, 1% lead nitrate according to the method of Hugon and Borgers (29). The control incubation medium contained in addition 0.5 mM levamisole hydrochloride (13) or 50 mM L-phenylalanine (33) as the inhibitors of AP.

5'-Nucleotidase (5'-N). For the visualization of 5'-N, the cell suspension was incubated at 37°C for 60 minutes according to the technique of Uusitalo and Karnovsky (52). The medium consisted of 0.1 M Tris-maleate buffer (pH 7.3), 1.4 M adenosine-5'-monophosphate (AMP), 1% lead nitrate, 10 mM magnesium sulfate and 5% sucrose. The control medium contained in addition 50 μM of α,β-methylene adenosine diphosphate (ADP) (43) or else the substrate AMP was omitted.

Ca^{2+}-activated ATPase (Ca^{2+}-ATPase) was detected by incubating the cell suspensions in a medium containing 0.1 M Tris-maleate

(pH 7.3), 5% sucrose, 2 mM lead nitrate, 1 mM adenosine triphosphatase (ATP), 1 mM calcium chloride, 0.2 mM Magnesium chloride (17,43). The incubation lasted for 60 minutes at 37°C. The control incubation medium contained in addition 15 μg/ml quercetin (an inhibitor of Ca^{2+}-ATPase (19)) or the ATP was omitted from the incubations.

Na$^+$-K$^+$-activated ATPase (Na^+-K^+-ATPase) activity was determined by incubating the cell suspension in a medium consisting of 0.1 M Tris-maleate buffer (pH 7.3), 1 mM ATP, 1% lead nitrate, 10 mM magnesium sulfate, 5% sucrose and 100 mM sodium chloride (36). Controls were incubated in the same medium but without sodium chloride or ATP. Incubations were performed at 37°C for 60 minutes.

Thick (1μm) frozen sections of normal mouse liver and kidney were used to check for the reactivity of the various incubation media while also incubating various sarcoma-180 cell samples. All cell samples from various incubation media were washed in ice-cold corresponding buffer identical to the one used for incubation. Half of the cells from each sample were stained with 1% light ammonium sulfide and mounted in glycerine jelly after proper washings. Such slides were viewed using a Zeiss Photomicroscope II. Photomicrographs were prepared of random areas from the various samples. The other half of the cell samples were fixed in buffered 1% OsO_4 (pH 7.3) for 1 hr at 4°C and processed for routine electron microscopy. Ultrathin sections were cut on an LKB ultratome III ultramicrotome and were viewed in a Hitachi-HU11E electron microscope with or without uranyl acetate and lead citrate staining. The enzyme reaction product was visualized as an electron dense deposit on the surface of the cells. Records were made from the electron micrographs of

very high response (++++), moderate response (+++), low response (++), or a very poor response (+) and compared for the effects of the drug.

Biochemical assays

Detailed biochemical quantitations were carried out in the case of alkaline phosphatase only. Cells from various intervals of cisplatin treatment were homogenized in carbonate-bicarbonate buffer (pH 10.0) (1:5 w/v) using 16 full strokes of a Potter-Elvehyem homogenizer fitted with a teflon pestle rotating at 2,000 rpm (57). 0.3 ml of the homogenate was added to 0.6 ml of carbonate-bicarbonate buffer (pH 10.0) and mixed with 0.03 ml of 100 mM magnesium sulfate. The mixture was incubated at 30°C for 10 minutes before adding 0.07 ml of 87.6 mM disodium-ρ-nitrophenyl phosphate dissolved in 0.1 ml of the same buffer to start the reaction (32). An increase in the absorption at 400 mμ was recorded as a result of the release of inorganic phosphate (Pi) (34). The controls consisted of incubation medium without the cell sample or of medium and cells to which 0.5 mM levamisole had been added (13). Increase in absorption at 400 mμ was measured using a Beckman 25 spectrophotometer with recorder. The cuvettes used had a 1 cm path length and a 1 ml capacity. Enzyme activity was further calculated using Beer's law and expressed as nM nitrophenyl/min/mg protein (53).

Protein determination

Standard protein solutions containing 1.0-10.0 μg of bovine serum albumin (Sigma Chemical Co., St. Louis, MO) were prepared in 50 μl of Tris-buffered saline and 950 μl of the dye reagent (39). A standard curve was prepared from an absorption at 595 mμ to determine the protein concentrations in unknown samples.

It was found necessary to further dilute the above cell homogenates by a factor of 20x in order for the readings to fall within the standard curve. 50 µl of the diluted samples were added to 950 µl of the dye reagent and read for absorption at 595 mµ for 2 minutes.

RESULTS

Various effects of cisplatin on cellular morphology have already been reviewed (8) and therefore will not be repeated here. Since no attempt was made to synchronize the cells in a certain phase of the cell cycle, the following observations are based on random cells in various stages of the cell cycle.

Alkaline Phosphatase

Alkaline phosphatase activity as depicted by the reaction product (r.p.) after 1 hr of incubation appears as dense deposits only on plasma membranes (Fig. 1). It may be pointed out that not all the cells in a random section show positive r.p. As many as 10-20% of the cell total may be negative. The number of the dense deposits decreases as the length of exposure of the cells to cisplatin is increased (Fig 2). The activity totally disappears after 45 minutes of such an exposure (Fig. 3 and Table I). At the electron microscope level, the reaction product appears as a thin, continuous, electron dense layer interspersed with dense patches on the outer surface of the cells (Fig. 4). The undulating segments of the plasma membrane, together with the microvilli, show the highest amount of deposits. The number of electron dense patches gradually decreases after cisplatin treatment, until, after 2 hours of treatment one can hardly see any r.p. on the cell surface (Fig. 5). Similarly no r.p. could be observed in cells incubated in a medium containing either levamisole or L-phenylalanine and without cisplatin treatment.

Fig. 1 Localization of alkaline phosphatase around normal sarcoma-180 cells. Note the uniform distribution of reaction product (r.p.) on the plasma membrane (arrows). Original magnification x3600. Bar = 5 μm.

Fig. 2 Light mirograph taken with Nomarski optics showing the r.p. in patches (arrow) after 30 minutes of cisplatin. Original magnification x1600. Bar = 10 μm.

Fig. 3 No r.p. can be observed after 45 minutes of cisplatin treatment. Photomicrograph taken with Nomarski Optics. Original magnification x1600. Bar = 10 μm.

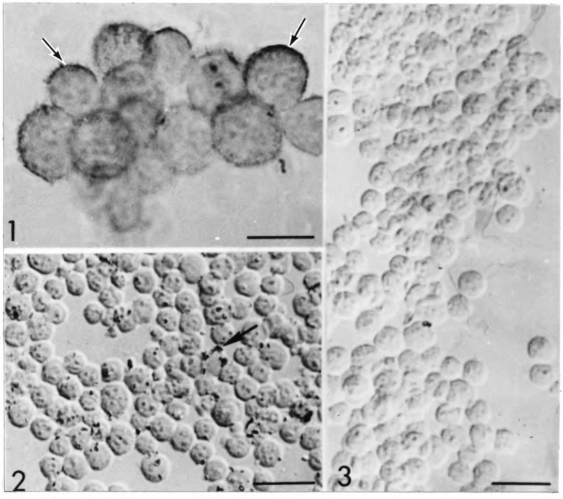

1
2
3

Fig. 4 Electron micrograph depicting alkaline phosphatase (arrows) on the surface of sarcoma-180 cells. N, nucleus. Original magnification x12000. Bar = 1 μm.

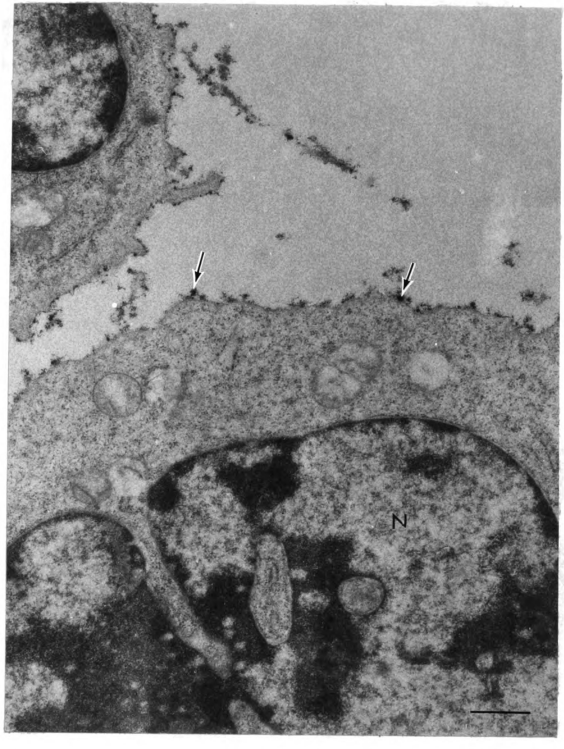
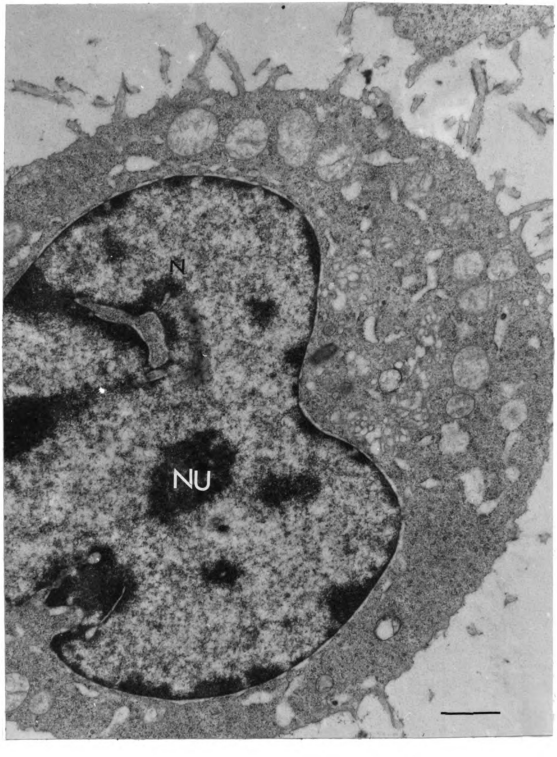

N

Fig. 5 No r.p. on the surface of cisplatin (2 hrs) treated cell. N, nucleus; Nu, nucleolus. Original magnification x8600. Bar = 2 μm.


N
NU

The visual cytological observations are further confirmed by biochemical determination of AP activity (Fig. 6). The treated cells show a change in alkaline phosphatase activity from 9.7 to 4.9 nmoles after just 15 minutes of exposure to cisplatin. These values drop to 0.79 nmoles after about 2 hours. Since observation ceased after 3 hours, it is not known if the value ever drops to zero.

5'N

As in the case of alkaline phosphatase, the cytochemical reaction for 5'-nucleotidase is mainly localized on the plasma membrane (Fig. 7). The reaction product is more abundant on the microvilli and on the undulations than on the nonundulated regions of the plasma membrane. Enzyme activity is vastly affected by cisplatin over a time period of 2 hrs (Table I). Again no r.p. is observed after incubation in a medium containing β-methyl ADP, or when the substrate is omitted.

Ca^{2+}-ATPase

Cells that have been incubated in a medium for Ca^{2+}-ATPase activity, when viewed under the light microscope, show r.p. on the cell surface as dark granules (Fig. 8). This granulation decreases when the cells are incubated in a culture medium containing cisplatin (Fig. 9). The r.p. completely disappears after 120 minutes of exposure to cisplatin (Fig 10). At the electron microscope level, the r.p. appears on the surface of almost all the cells as electron dense granules (in both the resting and the actively dividing cells) (Figs. 11-12). At times, r.p. can also be observed inside some vacuoles and around lipid globules. The r.p. decreases gradually from the surface as the time of treatment with cisplatin is increased from 0 to 120 minutes (Figs. 13-15 and Table I). Quercetin exposed cells show no reaction product.

Fig. 6 Phosphatase activity (n moles ρ-nitrophenol/min/mg protein) in ascites sarcoma-180 cell homogenates before and after cisplatin treatment (0.9 mg/100 ml).

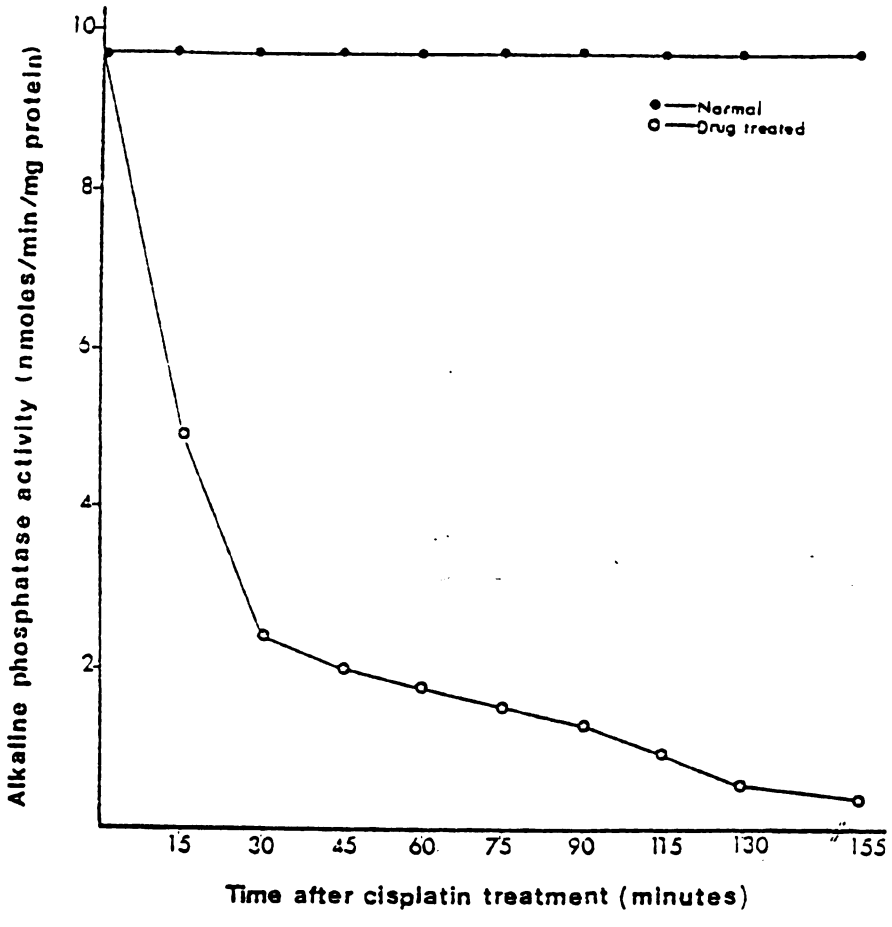

Alkaline phosphatase activity (nmoles/min/mg protein)
10
8
6
4
2
Normal
Drug treated
15
30
45
60
75
90
115
130
155
Time after cisplatin treatment (minutes)

Fig. 7 5'-N r.p. (arrows) distributed on the plasma membrane. N, nucleus; Nu, nucleolus. Original magnification x20,000. Bar = 1 μm.

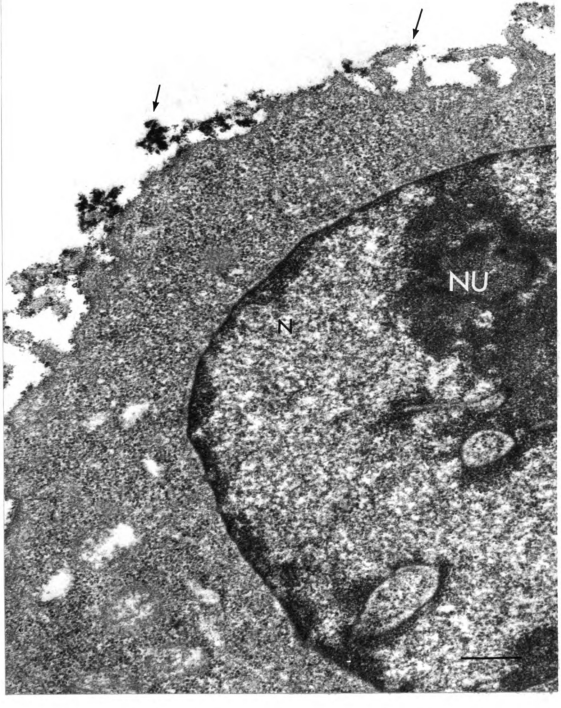

NU
N

Table I. Plasma membrane phosphatase activities before and after cisplatin treatment in Ascites sarcoma-180 cells *in vitro*

Time of cisplatin treatment (minutes)	Alkaline Phosphatase	5'- Nucleotidase	Ca^{2+} -ATPase	Na^{+} K^{+} -ATPase
0	++++	++++	++++	++++
15	++	++	+++	+++
30	++	±	++	++
45	±	-	+	+
60	-	-	±	±
120	-	-	-	-
180	-	-	-	-
240	-	-	-	-
300	-	-	-	-

++++ a very strong reaction
+++ moderate reaction
++ not a very dense reaction
+ low activity
± Less than 10% reaction limited to few spots
- negative reaction

Fig. 8 Reaction product (arrows) depicting Ca^{2+}-ATPase on the surface of untreated sarcoma-180 cells. Original magnification x3000. Bar = 5 μm.

Fig. 9 Ascites sarcoma-180 cells after 30 minutes of cisplatin treatment showing only patches of reaction product indicative of Ca^{2+}-ATPase activity. Original magnification x3000. Bar = 5 μm.

Fig. 10 Ascites sarcoma-180 cells after 2 hrs of cisplatin treatment showing no r.p. for Ca^{2+}-ATPase. Original magnification x3600. Bar = 5 μm.

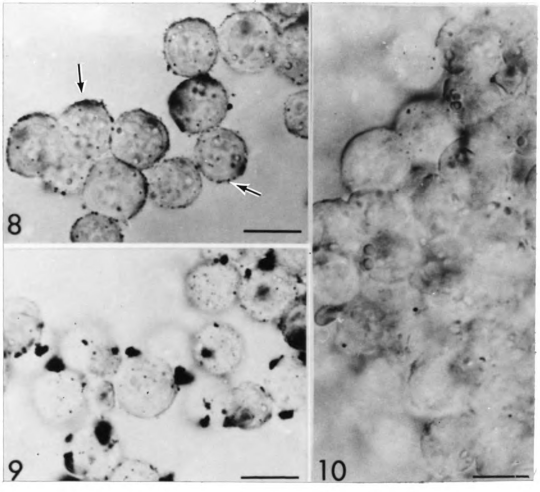

8
9
10

Fig. 11 Electron micrograph show Ca^{2+}-activated ATPase r.p. (arrows) on the surface of a sarcoma-180 cell during interphase. M, mitochondria; N, Nucleus. Original magnification x25000. Bar = 0.5 μm.

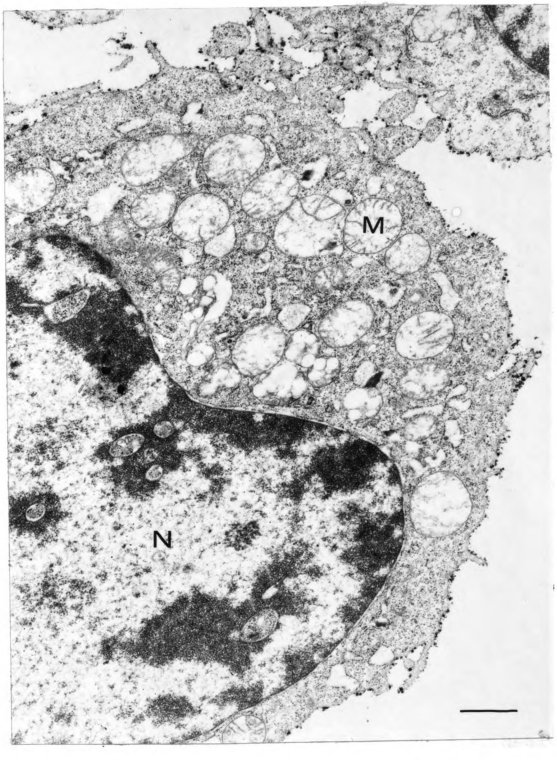

M
N

Fig. 12 Ascites sarcoma-180 cell in metaphase showing Ca^{2+}-ATPase r.p. (arrows) on the surface. CH, chromosomes. Original magnification x22500. Bar = 1 μm.

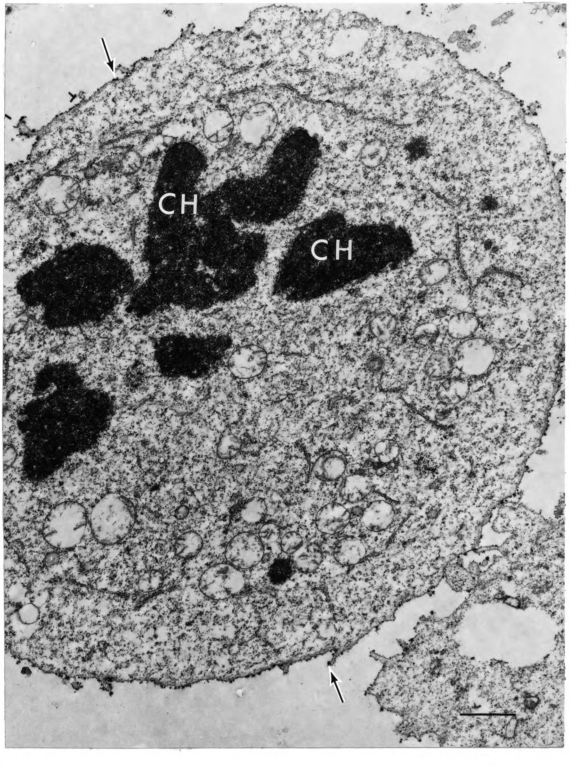

CH
CH

Fig. 13 Interface between two ascites sarcoma-180 cells showing Ca^{2+}-ATPase r.p. (arrows) after exposure to cisplatin for 30 minutes. N, nucleus. Original magnification x25,000. Bar = 1 μm.

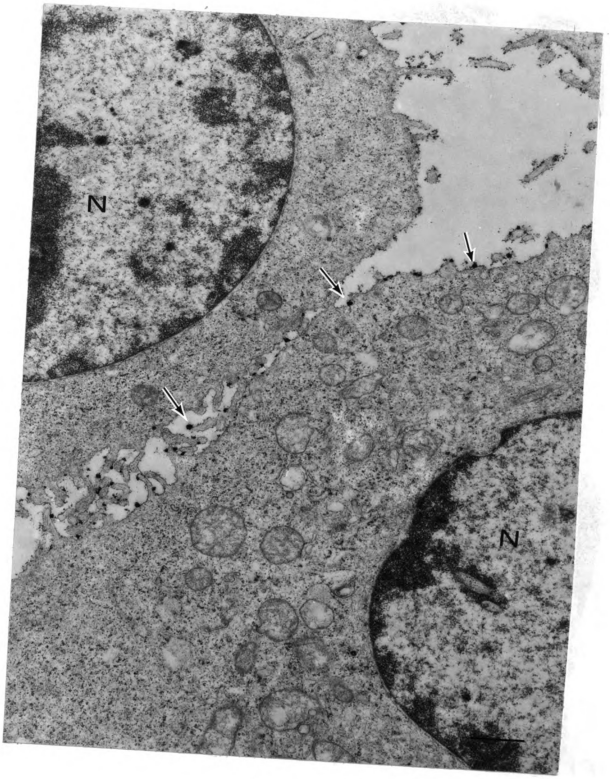

N
N

Fig. 14 Interface between two ascites sarcoma cells showing Ca^{2+}-ATPase r.p. after 45 minutes of cisplatin treatment (arrow). Note the sparse distribution of reaction product. N, nucleus. Original magnification x18000. Bar = 1 µm.

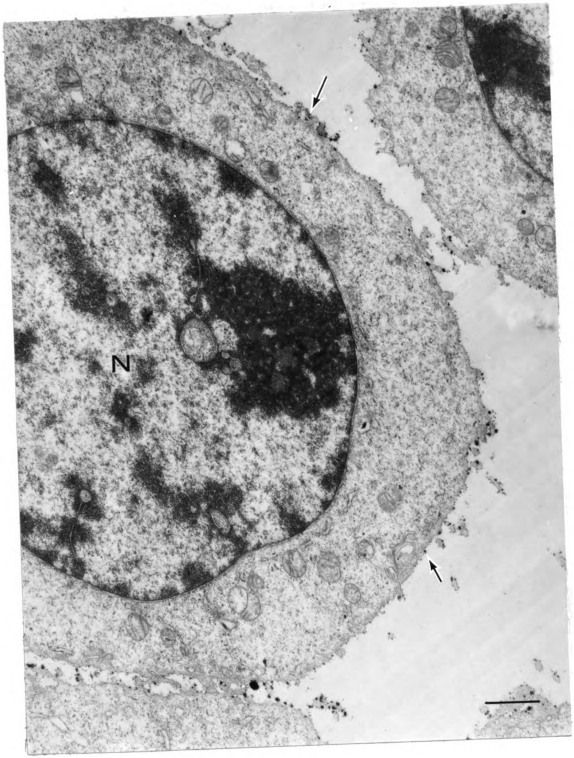

N

Fig. 15 Ascites sarcoma-180 cell in metaphase exposed to cisplatin for 2 hrs showing no r.p. for Ca^{2+}-ATPase. Compare with Fig. 12 for normal reaction. CH, chromosomes. Original magnification x25,000. Bar = 1 μm.

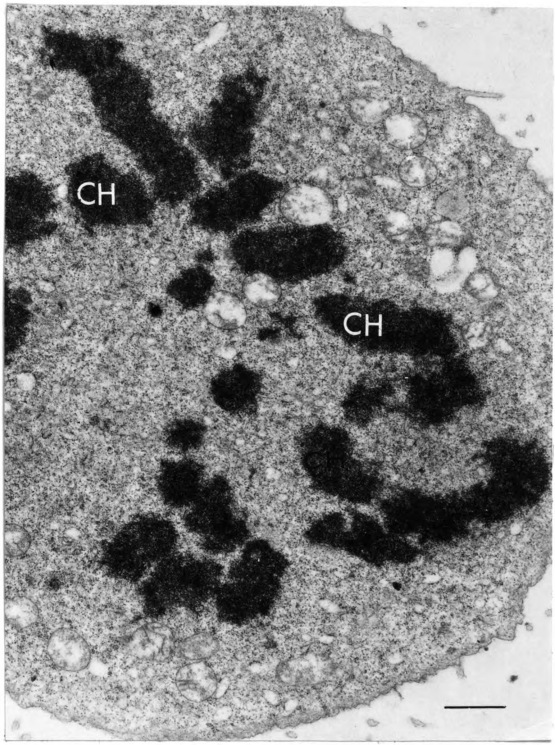

CH
CH

Fig. 16. Graph showing changes in the Ca^{2+}-activated ATPase as depicted by the concentration of reaction product of various incubations in ascites sarcoma-180 *in vitro* and *in vivo* after cisplatin treatment.

Na^+-K^+-ATPase

The plasma membrane distribution patterns for Na^+-K^+-ATPase are exactly similar to those for Ca^{2+}-ATPase, both before and after cisplatin treatment, and are summarized in Table 1.

In in vitro studies most of the above enzymes on the cell surfaces are totally inactivated within 2 hours of their exposure to cisplatin. However, in the in vivo studies it takes about 2 days to achieve the same results (Fig. 16).

DISCUSSION

In the present study we have investigated the effects of cisplatin on plasma membrane enzymes because of their importance in the homeostasis of the cell. Control of plasma membrane functions such as transport of metabolites (23), cell growth (25), synthesis and breakdown of cyclic nucleotides (24) and the process of mitosis (6) are known to be affected by cisplatin.

Alkaline phosphatase has been demonstrated to be mostly associated with the plasma membrane (40). In the liver, the enzyme is present predominantly in the plasma membranes of the bile canaliculi and does not extend appreciably to the basal or the lateral membranes beyond the tight juctions (20).

The discovery of alkaline phosphastase in the proximal convoluted tubules of the kidney, the intestinal microvilli, and the follicular epithelium of the ovary suggests some role for this enzyme in active transport of substances across the membrane (18). It is known that this enzyme is needed for the hydrolysis of extracellular phosphate esters, so that the reaction products such as orthophosphate and organic residues can then be transported into the cell (56). Further evidence for a transport function of alkaline phosphatase comes from the finding that some mutant strains of E. coli which lack alkaline phosphatase are unable to grow in phosphate-containing media (22). Since alkaline phosphatase is able to transfer phosphate groups from one alcohol to another, it is

possible that its physiological function depends on its transferase activity, rather than its hydrolase activity (55). Thus, membrane-associated alkaline phosphatase activities are closely related to, if not identical with, Ca^{2+}-dependent ATPase (11).

A parallel can be found between the activity of alkaline phosphatase and DNA synthesis in synchronized HeLa cells, in that high specific activity of AP has been used as a marker for the mitotic plasma membranes (35). However, just the contrary has been demonstrated using certain aliphatic monocarboxylates that inhibit cell division in HeLa cells, yet alkaline phosphatase activity is increased on the plasma membrane (53). Similar conclusions have been drawn concerning cultured human fibroblasts, which, when treated with DNA inhibitors like hydroxyurea and methothrexate, show elevated levels of alkaline phosphatase (58). However, using cisplatin, which is a known inhibitor of DNA synthesis and a mitotic inhibitor, we have observed a distinct drop in the enzyme activity in both *in vitro* and *in vivo* studies. In the *in vitro* system it takes about 60 minutes for an inactivation of the enzyme, whereas in the *in vivo* system it takes about 2 days using the same ascites sarcoma-180 cells. This is probably due to the fact that the levels of cisplatin in the *in vitro* system are much higher than in the animal. In the animal system, 70-90% of the injected drug is usually excreted in the initial minutes of its injection (45) and thus the amount available to the cells for interaction is very small. Inhibition of DNA by cisplatin has been determined to occur through an intrastrand and interstrand crosslinking with nuclear DNA (59,30). In either case, the prime interaction is with the purine and pyrimidine bases. Inhibition of DNA synthesis and increase or decrease of alkaline phosphatase activity therefore, do not seem to be interrelated.

A direct inhibition of various enzymes after cisplatin treatment has been reported (21), however, the drug concentrations used in such assay systems are much higher than those normally found in cells. Alkaline phosphatase activity in the urine of cisplatin-treated rats shows a 50-70% increase with a corresponding decrease in the kidney homogenates, indicating a stripping of the enzyme from the brush borders (5). A similar stripping effect of cisplatin has been demonstrated on sialic acid in normal splenocytes and transformed lymphocytes in culture (38).

Patients suffering from carcinoma of the testis when treated with cisplatin show a 75% decrease in plasma sialyltransferase that corresponds with a relative decrease in the size of the tumor (31). However, the enzyme by itself is again unaffected by cisplatin in the assay system.

Our observations show 5'-nucleotidase activity mostly on the plasma membrane of the ascites sarcoma-180 cells. 5'-Nucleotidase has been extensively studied as an ectoenzyme and is often bound to the brush borders of various cells (43). 5'-Nucleotidase is one of the enzymes directly involved in the metabolism of adenosine and indirectly in the synthesis of ATP and cyclic AMP and also in the breakdown of the latter (53). Plasma membranes are permeable to nucleosides whereas nucleotides are unable to traverse the membrane intact. Hence 5'-nucleotidase is involved in the dephosphorylation of nucleoside monophosphate and the nucleoside product may then be internalized (44,40). Thus inhibition of this enzyme would inhibit further DNA synthesis because of unavailability of various nucleotides needed. Cisplatin indeed does affect the enzyme activity at all stages of the cell cycle, therefore interfering with cell metabolism and replication.

Ca^{2+}-ATPase is involved in the transport of Ca^{2+} ions across the plasma membrane and in regulating the level of these ions inside the cell (44). Ca^{2+} plays a key role in regulating cell metabolic processes by stimulating or inhibiting key enzymes (14). In general, cells must keep the intracellular Ca^{2+} concentration at a very low level in order to maintain Na^{+}-K^{+}-ATPase in an active state. Even a small increase in internal Ca^{2+} would therefore act as a powerful messenger of extracellular membrane stimuli (44).

A correlation has been proposed between Ca^{2+}-ATPase activity and the chromosomal cycle to a point that the enzyme activity has been shown to be increased as the cell prepares for mitosis (37). However, we have not observed any significant increase or decrease in Ca^{2+}-ATPase activity on the plasma membrane of the interphase or actively dividing sarcoma-180 cells. Cisplatin knocks out all ATPase activity from the plasma membrane irrespective of the stage of cell cycle. Cisplatin has also been shown to cause an inactivation of Ca^{2+}-ATPase activity from the brush border of the proximal tubule cells (4). A parallel has further been established in the inactivation of Ca^{2+}- ATPase activity and Ca^{2+} ions within mitochondria (8,12). If the internal generation of Pi is prevented, Ca^{2+} is spontaneously released from the mitochondria (15). Lowering the internal concentration of Pi with the use of appropriate inhibitors leads to the release of accumulated Ca^{2+}, which is related to the permeability of the membrane.

Various platinum coordination complexes including cisplatin have been shown to stimulate state 4 respiration which is similar to uncoupling of oxidative phosphorylation (8,12), again causing effux of Ca^{2+} from the mitochondria. The membrane permeability changes have

been implicated in the release of various hydrolytic enzymes from the lysosomes, thus inducing damage to the cell. Various compounds that are competitors or inhibitors of Ca^{2+} sites (26) and block Ca^{2+} transport mechanisms have also been proven to be potent antitumor agents (9).

Using frog skin as a model, it has further been demonstrated that cisplatin increases the permeability of the cellular pathway (10), an observation common to other antineoplastic agents such as adriamycin (51). Increased levels of intracellular Ca^{2+} are known to shut down Na^{+}-K^{+}-ATPase which is an integral glycoprotein of the plasma membrane. Guarino et al. (23) have demonstrated an inactivation of Na^{+}-K^{+}-ATPase in isolated kidney tubules under the influence of cisplatin. They have implicated this inactivation of ATPase to be responsible for the kidney toxicity so very prominent after cisplatin treatment.

From the above considerations, we conclude that cisplatin is responsible for the inactivation of various plasma membrane associated enzymes that are very important in the normal metabolic processes of the cell, such as transport, synthesis, breakdown of cyclic nucleotides and mitosis. The drug does not seem to be selective in its action as it affects normal cells and tumor cells alike. However, the tumor cells are known to be metabolically more active, and are probably destroyed faster than they have time to recover the various membrane enzymes. Thus, in addition to the inhibition of DNA synthesis by crosslinking the complementary strands, cisplatin also functions to alter the surface properties of cells to cause antitumor or cytotoxic effects.

REFERENCES

REFERENCES

1. Aggarwal SK: Inhibition of cytokinesis in mammalian cells by cis-dichlorodiammine-platinum (II). Cytobiologie 8:395 (1974).

2. Aggarwal SK: Fine structural studies using platinum coordination complexes. J Clin Hematol Oncol 7:760 (1977).

3. Aggarwal SK, Broomhead JA, Fairlie DP, Whitehouse MW: Platinum drug Combined anti-lymphoproliferative and nephrotoxicity assay in rats. Cancer Chemother Pharmacol 4:249 (1980).

4. Aggarwal SK, Hammouda EM: Fine structural changes in the Ca^{2+}, Ca^{2+} and Mg^{2+}-activated ATPase, and alkaline phosphatases in renal tubules after cisplatin treatment. Ed. GW Bailey, 38th Ann Proc Electron Microscopy Soc Amer, Reno, Nevada, p 444 (1980).

5. Aggarwal SK, Hammouda EM: Changes in calcium and various enzymes due to cisplatin treatment in rats: A biochemical and histochemical approach to nephrotoxicity. (In preparation).

6. Aggarwal SK, Sodhi A: Cytotoxic effects of cis-dichlorodiammine-platinum (II) on the mammalian cells in vitro: A fine stuctural study. Cytobiologie 7:366 (1973).

7. Aggarwal SK, Wagner RW, McAllister PK, Rosenberg B: Cell-surface-associated nucleic acid in tumorigenic cells made visible with platinum-primidine complexes by electron microscopy. Proc Natl Acad Sci USA 72:928 (1975).

8. Aggarwal SK, Whitehouse MW, Ramachandran C: Ultrastructural effectsof cisplatin. In: Cisplatin: Current Status and New Developments. Eds. AW Prestayko, S Crooke, and SK Carter. Academic Press, New York p 79 (1980).

9. Anghileri LJ: Effects of gallium and lanthanum on experimental tumor growth. Europ J Cancer 15:1459 (1979).

10. Berg Van den EG, Dennis VW, Lucas VS, Huang AT: Direct effects of cisplatin on membrane permeability of the frog skin (abstract). Proc Am Assoc Cancer Res 21:277 (1980).

11. Birge SJ, Gilbert HR: Indentification of an intestinal sodium and calcium-dependent phosphatase stimulated by parathyroid hormone. J Clin Invest 54:710 (1974).

12. Binet A, Volfin P: Effect of an anti-tumor platinum complex, Pt (II) Diamino-toluene on mitchondria. Biochimica et Biophysica Acta461:182 (1977).

13. Borgers M: The cytochemical application of new potent inhibitors of alkaline phosphatases. J Histochem cytochem 21:812 (1973).

14. Bygrave FL: The ionic environmental and metabolic control. Nature 214:667 (1967).

15. Bygrave FL, Ramachandran C, Smith RL: On the mechanism by which inorganic phosphate stimulates mitochondrial transport. FEBS Letters 83:155 (1977).

16. Cleare MJ, Hyde PC, Hepburn DR, Malerbi BW: Antitumor platinum complexes: Structure-activity relationships In: Cisplatin, Current Statusand New Developments Eds. AW Presteyko, St Crooke and SK Carter. Academic Press, New York p 149 (1980).

17. Cutler L, Rodan G, Fenstein MB: Cytochemical localization of adenylate cyclase and of calcium ion, magnesium ion-activated ATPases in the dense tubular system of human blood platelets. Biochim Biophys Acta 469:52 (1977).

18. Danielli JF: Structural factors in cell permeability and secretion. Symp Soc Exp Biol 6:1 (1952).

19. Fewtrell CMS, Comperts BD: Quercetin: A novel inhibitor of Ca^{2+} influx and exocytosis in rat peritoneal mast cells. Biochim Biophys Acta 469:52 (1977).

20. Fishman WH: Perspective on alkaline phosphatase isoenzymes. Am J Med 56:617 (1974).

21. Friedman ME, Musgrove B, Lee K, Teggins JE: Inhibition of malate dehydrogenase by platinum (II) complexes. Biochimica et Biophysica Acta 250:286 (1971).

22. Garen A: Genetic control of the bacterial enzyme, alkaline phosphatase. In: Microbial Genetics. Ed W Hayes and RC Clowes, Cambridge Univ Press, Cambridge p 239 (1960).

23. Guarino AM, Miller DS, Arnold ST, Pritchard JB, Davis RD, Urbanek MA, Litterst CL: Platinate toxicity: past, present, and prospects. Cancer Treat Rept 63:1475 (1979).

24. Harder HC: Renaturation of DNA, Poly DAT and double stranded homopolymeric nucleotides treated with cis-pt (II) $(NH_3)_2$ Cl_4. Biophy Soc Abs. 12:174A (1972).

25. Harder HC, Rosenberg B: Inhibitory effects of anti-tumor platinum of DNA,RNA and protein synthesis in mammalian cells in vitro. Int J Cancer 6:207 (1970).

26. Hart MM, Adamson RH: Antitumor activity and toxicity of salts of inorganic group 3a metals: aluminum, gallium, indium and thallium. Proc Nat Acad Sci (Wash) 68:1623 (1971).

27. Hill JM, Speer RJ, Loeb E, Maclellan A, Hill NG, Khan A: Clinical experience with cis-platinous diammine dichloride (PDD). In: Advances in Antimicrobial and Antineoplastic Chemotherapy. Ed. M. Hejzlar. Urban & Schwarzenberg Baltimore p 225 (1971).

28. Holland JF, Bruckner HW, Cohen CJ, Wallech RC, Crusberh SB, Greenspan EM, Goldberg J: Cisplatin therapy of ovarian cancer. In: Cisplatin, Current Status and New Developments. Eds. AW Presteyko, ST Crooke and SK Carter. Academic Press, New York p 383 (1980).

29. Hugon J, Borgers M: A direct lead method for the electron microscope visualization of alkaline phosphatase activity. J Histochem Cytochem 14:429 (1966).

30. Johnson NP, Hoeschele JD, Rahn RO: Kinetic analysis of the in vitrobinding of radioactive cis- and trans-dichlorodiammine-platinum (II) to DNA. Chem Biol Interact 30:151 (1980).

31. Kessel D, Samson MK, Shah P, Allen J, Baker LH: Alterations in plasma sialyltransferae associated with successful chemotherapy of a disseminated tumor (case report). Cancer 38:3132 (1976).

32. Leathwood PD, Plummer DT: Enzymes in rat urine: I. A metabolism cage for the complete separation of urine and faeces. Enzymologia 37:240 (1969).

33. Lin CW, Sasaki M, Orcutt ML, Miyayama H, Singer RM: Plasma membrane localization of alkaline phasphatase in HeLa Cells. J Histochem Cytochem 24:659 (1976).

34. Lojda Z, Gossrau R, Schiebler TH: Detection Methods: Alkaline phosphatase. In: Enzyme Histochemistry. Springer-Verlag, New York p 56 (1979).

35. Lucid SW, Griffin MJ: Cell cycle variations in HeLa 65 plasma membrane alkaline phosphatase. Biochem Biophys Res Comm 74:113 (1977).

36. Marchest VT, Palade GE: The localization of Mg-Na-K-activated adenosine triphosphatase on red cell ghost membranes. J Cell Biol 35:385(1967).

37. Petzelt C: Ca^{2+}-activated ATPase during the cell cycle of the sea urchin Strongylocentratus Purpuratus. Exp Cell Res 70:333 (1972).

38. Prasad SB, Sodhi A: Effect of cis-dichlorodiammine platinum (II) on the agglutin ability of tumor and normal cells with concanavalin A and wheat germ agglutinin. Chem Biol Interactions 36:355 (1981).

39. Read SM, Northcote DH: Minimization of variation in the responseto different proteins of the Coomassie Blue G dye-binding assay for protein. Analytical Biochem 116:53 (1981).

40. Riordan JR, Forstner CR: Glycoprotein membrane enzymes. In: Current Topics in Membrane and Transport. Vol II Ed. F Bronner, A Kleinzeller, RL Juliano and A Rothstein, Academic Press, New York p 146 (1978).

41. Roberts JJ, Pascoe JM: Cross-linking of complementary strands of DNA in mammalian cells by antitumor platinum compounds. Nature 235:28

42. Roberts JJ, Thomson AJ: The mechanism of action of antitumor platinum compounds. Prog Nucl Acid Res Mole Biol 22:71 (1979).

43. Rodan GA, BourretLA, Cutler LS: Increase in 5'-Nucleotidase and decrease in adenosine inhibitor of adenylate cyclase. J Cell Biol 72:493 (1977).

44. Ronquist G: Structural bound adenosine-triphosphate phosphohydrolase of the animal cell. In: Biological Transport. Ed. HN Christensen, WA Benjamin Inc. London, p 27 (1975).

45. Rosenberg B: Possible mechanisms for the antitumor activity of platinum coordination complexes. Cancer Chemother. Rep 59:589 (1975).

46. Rosenberg B: Nobel metal complexes in cancer chemotherapy. In: Inorganic and Nutritional Aspects of Cancer. Ed GN Schrauzer Plenum Press, New York p 129 (1977).

47. Rosenberg B: Cisplatin: Its history and possible mechanisms of action. In: Cisplatin, Current Status and New Developmens Eds. AW Presteyko, ST Crooke, and SK Carter. Academic Press, New York p 9 (1980).

48. Rosenberg B: Clincial aspects of platinum anticancer drugs. In: Metal Ions in Biological Systems. Ed. H Sigel. Marcel Dekker Inc. New York p. 128 (1980).

49. Rosenberg B, VanCamp L: The successful regression of large solid sarcoma-180 tumors by platinum compounds. Cancer Res 30:1799 (1970).

50. Sodhi A, Aggarwal SK: Effect of cis-dichlorodiammine-platinum (II) in the regression of Sarcoma 180: A fine structural study. J Natl Cancer Inst 53:85 (1974).

51. Solie TN, Yuncker C: Adriamycin induced changes in translocation of sodium ions in transporting epithelial cells. Life Sciences 22:1907 (1978).

52. Uusitalo RJ, Karnovsky MJ: Surface localization of 5'-nucleotidase on the mouse lymphocyte. J Histochem Cytochem 25:87 (1977).

53. Vorbrodt A, Borun TW: Ultrastructural cytochemical studies of plasma membrane phosphatase activities during the HeLa S3 cell cycle. J Histochem Cytochem 27:1596 (1979).

54. Williams SD, Einhorn LH: Cisplatin chemotherapy of testicular status and new developments. In: Cisplatin, Current Status and New Development. Ed AW Prestayko, ST Crooke and SK Carter. Acedemic Press, New York p 323 (1980).

55. Wilson IB, Dayan J, Cyr K: Some properties of alkaline phosphatase from Escherichia coli. J Biol Chem 239:4182 (1964).

56. Wilson TH, Wilson DH: Studies in vitro of digestion and absorption of pryimidine nucleotides by the intestine. J Biol Chem 233:1544 (1958).

57. Wright PJ, Leathwood PD, Plummer DT: Enzymes in rat urine: Acid phosphatase. Enzymologia 42:459 (1971).

58. Wjcik L, Buchwald M, Riordam JR: Induction of alkaline phosphatase in cultured human fibroblasts. Biochimica et Biophysica Acta 585:374 (1979).

59. Zwelling LA, Kohn KW: Effects of cisplatin on DNA and the possible relationships to cytotoxicity and mutagenicity in mammalian cells. In: Cisplatin, Current Status and New Development. Ed. AW Presteyko, ST Crooke and SK Carter. Academic Press, New York, p 21 (1980).

60. Zwelling LA, Kohn KW, Ross WE, Ewing RA, Anderson T: Kinetics of formation and disappearance of DNA cross-linking effect in mouse leukemia L1210 cells treated with cis- and trans-diammine-dichloroplatinum (II). Cancer Res 38:1762 (1978).