THE EFFECTS OF CIS-PLATINUM ON THE ISOLATED PERFUSED RABBIT KIDNEY AND ISOLATED KIDNEY TUBULES

by

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STATEMENT BY AUTHOR

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ABSTRACT

Cis-dichlorodiammineplatinum has been shown to be highly successful in arresting cell division, however, clinical Phase II trials have shown a development of tubular necrosis after repeated doses. Therefore, an investigation of the effects of this agent on isolated tubules and intact perfused kidneys of the rabbit were performed. A cellular hypertrophy with vacuolization preceded the loss of the luminal brush border in the proximal tubule. The results indicated a cytotoxic effect from platinum, rather than a specific enzymatic inhibition. Membrane transport and metabolism of the isolated proximal tubules paralleled the effects of the in situ perfused kidney while under the influence of cis-DDP. The kidney's ability to concentrate cis-DDP supported the findings that continuous exposure of the drug led to the elevated levels of platinum that could be responsible for the change in morphology and cell function seen in this investigation.

CHAPTER I

INTRODUCTION

Platinum coordination complexes form a new class of active antitumor agents in animals in man. cis-Dichlorodiammineplatinum II (DDP), a widely investigated drug, has completed Phase I clinical trials and is now in Phase II. It has provided objective remissions in 10-25% of terminally ill patients, encompassing 28 different tumor types (DeConti, 1973). DDP appears to be a broad spectrum antitumor drug. The mode of action is unknown, but probably involves binding to DNA and the enhancement of the patient's immune system. So far, only square planar and octahedral complexes of platinum, with a wide variety of inorganic and organic ligands, have shown marked antitumor activity (Baslo, Gray and Pearson, 1960).

Until 1970, the metal coordination complexes had been largely ignored in the search for new drugs for cancer chemotherapy. However, numerous laboratories and institutes are making extensive efforts to redress this issue. Some workers have shown a broader scope of biological activities for these complexes. These include bacteriocidal and viricidal, immunosuppressive, and antiarthritic activities. Thus, we have a new class of chemical structures with a wide variety of potential biological and medical applications. However, with most agents administered at chemotherapeutic doses, platinum complexes are quite toxic. Therefore, assessments of their biological and toxicological effects are important. This paper reviews our present knowledge of platinum compounds in humans

and experimental animals and reports results of original research on the toxicity and mechanism of action of cis-DDP on the isolated perfused kidney and isolated renal tubules.

Nature of Antitumor Platinum

The ability of certain platinum coordination compounds of tumor cells depends on the chemical structures of the complexes. The active complexes can be represented by Figure 1. "A" are the carrier ligand(s) (usually amines, either monodentate (NH_3) or bidentate (NH_2 CH_2 CH_2 NH_2)) and "X" represents the anionic leaving group(s) (either monodentate (Cl-) or bidentate (-OOCCH COO-)). Among these complexes, certain common features appear to be required for antitumor activity.

The central metal cation must be at a low oxidation state (Baslo and Pearson, 1967). In bivalent platinum compounds, the special inertness of the platinum-ligand bond may be the key to the unique activity of platinum (II) complexes as antitumor drugs.

Of the two possible isomers, only the cis configurations are the active complexes (Clease and Hoeschelle, 1973a). Among the "X" leaving groups, chloride, bromide, oxalate, or malonate ligands are the most active complexes. The antitumor activity can be established from the rates of substitution (hydrolysis?); if hydrolysis of the ligand occurs rapidly, then the complex is prevented from reaching the site. Ligands such as cyanide, on the other hand, form strong bonds with platinum and tend not to interact either rapidly enough or to a sufficient degree to elicit the antitumor response (Talley and O'Bryan, 1973).

The complex should contain relatively inert carrier ligands (A). In general, chemotherapeutic activities of N-donor atom ligands decrease

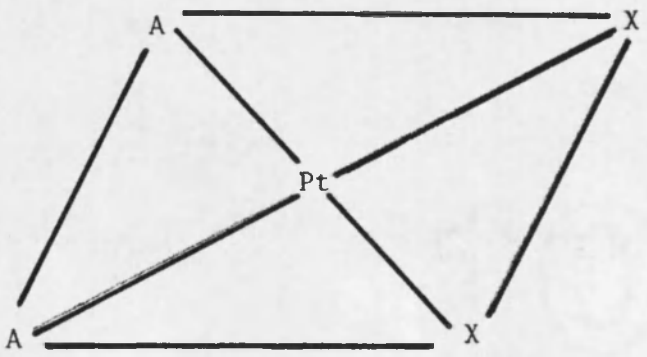

FIGURE 1 - Diagram of Active Coordination Complexes

along the series, NH_3 <RNH_2>R_2NH>R_3N. The increase in the size of R and the substitution of the hydrogen atom by alkyl or other functional groups similarly decreases activity. As R becomes larger and more hydrophobic, the complex may become less soluble, and therefore, less effective (Clease and Hoeschele, 1973b).

The hydrogen bonding interactions between amine ligands and biological receptors is believed to be important for the stabilization of the receptor-drug complex. A decrease of the strength in the hydrogen bonds could be related to decrease in the activity of alkyl substitution in ammonia and ethylenediamine complexes. It has also been shown that toxicity is unrelated to antitumor action; substitution of the hydrogen atom by an alkyl group decreases activity, but has virtually no effect in toxicity (Connars, 1972).

A known carrier ligand A capable of producing active complexes are ammonia, ethyleneimine, pyrolidine, alicyclic amines, cyclohexanine-trans 1, 2-diamine, and o-phenyl enediamine. A group of pyrimidines constitute another series of active complexes. They exhibit a deep blue color and are generically referred to as "platinum blues", but their structures are unknown (Davidson, 1975; Hoffmann and Bugge, 1908). Some of these active complexes can be seen in Figure 2.

The mechanism for transport of these platinum complexes across all membranes remains questionable; however, only the neutral platinum derivatives possess antitumor activity. Since high levels are required for platinum's efficacy, then the solubility must be high to meet these concentrations.

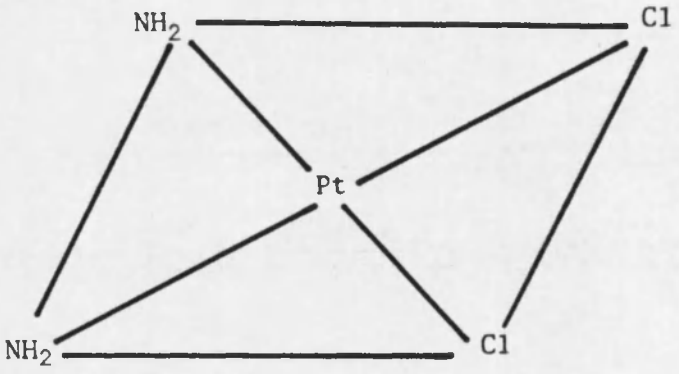

FIGURE 2 - Structure of the Active Complex, cis-DDP

Mechanism of Action

Rosenberg, VanCamp and Krigas (1965) first reported platinum's action on a biological system. He delivered a direct current between two platinum electrodes that had been submerged in a culture of E-coli. The disruptive effect was an inhibition of cell division with individual cells of E-coli attaining lengths that were 300 times those of normal cells (Rosenberg, VanCamp and Trasko, 1969). Radiolabelled complexes were prepared for the determination of the distribution of platinum in E-coli. Using ^{191}Pt, it was proposed that two different modes of action occurred contingent on the parent compound. Cis-DDP was associated with nucleic acids and cytoplasmic proteins, while cells which were inhibited by the tetrachloroplatinate ion localized the labelled platinum associated only with the cytoplasmic protein (Hawle and Gale, 1970). Rosenberg concluded that once the complex penetrated the cell wall of these bacteria, it was metabolized to various intermediates such as ($Pt(NH_3)_2 Cl^+$) and ($Pt(NH_3)_2^{++}$). The parent compound was considered to be devoid of activity per se. It was hypothesized that the monoaquated species inhibited synthesis of DNA, RNA, and protein and that biaquated species was selective toward the inhibition of only DNA synthesis (Hawle and Gale, 1970). The delayed onset of action seen for platinum could be explained by this mechanism. Furthermore, both RNA and protein synthesis are gradually restored to normal rates within 72-96 hours (Haracek and Drobnik, 1971), while DNA synthesis is inhibited for several days after drug application. Slow diffusional rates and membrane interaction of these lipid-insoluble compounds are also hypothesized to explain the delayed onset of inhibiting action (Gale, Manis and Atkins, 1973).

The interaction of ^{14}C-labelled dichloroethylenediamine with E-coli seems to be mediated via the guanine, cystosine, and adenine bases of DNA (Taylor, Tew and Jones, 1976). Hyperchromic changes due to a direct interaction of platinum with the bases has been confirmed by ultraviolet spectras copy (Taylor, Jones and Robins, 1973). The formation of platinum-nucleic acid bonds probably determines the inhibitory actions of platinum compounds on tumor growth and DNA synthesis. DNA interaction with platinum forms an acid resistant bond when incubated with intact cells. This interaction is inhibited by sodium chloride and nitrogen mustard compounds. The dissociation of both chlorine atoms from the central platinum atom may be a requirement for the antitumor and antimitotic action. The two neighboring chlorine groups in the cis formation could act as a bifunctional agent, whereas the trans configuration could only behave as a monofunctional agent. The subcellular distribution of ^{195m}Pt and ^{14}C-Ethylenediammine labelled cis-Dichloroethylenediammineplatinum is similar to cis-DDP for normal and tumor tissue. Localization of the radionucleides was mainly in the cytosol in the form of a low molecular weight complex. Over 50% of the injected dose localized in the kidney as a highly insoluble complex (Litterst, 1976; Harder and Rosenberg, 1971). Drobnik's work with E-coli concluded that cis-DDP caused a slight inhibition when present at 100 μM concentration during the induction of β-galactosidase and β-galactoside permease. The induction process remains unaffected by the short term presence of cis-DDP at concentrations far above the therapeutic dose, while some decrease occurred after a prolonged treatment (Harder and Rosenberg, 1971). These results inferred that either there was an alteration of the permease or there was a direct effect upon the cells, e.g., catabolic repression.

Structure activity studies have shown similarities in the spacing between two chlorine atoms of the platinum complex (3.3Å), and the spacing of the bases in the Watson-Aich model (3.4Å). The spatial structures of these compounds could account for this bifunctional alkylating agent which has been known to form inter- and intrastrand crosslinks (Haracek and Drobnik, 1972).

Another hypothesis relates the antitumor effect of platinum compounds with that of the stimulation of immune processes. Strong immunosuppressive effects with decreased antibody formation are observed after cells have been exposed to platinum. Inhibition of blastogenesis occurs 15 minutes following the administration of the drug in doses of 1-4 mg/kg. The relatively short immunosuppressive effects last for 18-72 hours. This could be advantageous because a rapid recovery of immune system may help in control of the malignant disease (Rosenberg, 1972). Rosenberg proposed that platinum drugs enhance the antigenicity of the tumor tissue so that even a weakened immune competency can still produce an adequate response to repress tumor growth.

Antitumor Activity

The platinum compounds have one of the broadest spectra of action of any class of antitumor agents yet discovered. They have exhibited complete inhibition of large growing tumors over a short period of time. Complete regression of dimithylbenzanthracene-induced mammary tumors and Rous sarcoma virus induced tumors was seen after cis-DDP treatment (Talley, 1970).

The effectiveness of cis-dichlorodiammineplatinum against human cancers has also been demonstrated (Harder and Rosenberg, 1971). Phase

II trials reported good results using platinum against testicular tumors, carcinomas of the bladder, breast, and thyroid. Taylor and associates (1976) observed remissions in neuroblastomas, malignant thymoma, and lymphoma. Current Phase II trials have utilized combination therapy with fluorouracil and adriamycin. Encouraging results are being found with combined therapy toward various carcinomas and lymphosarcomas.

Biological Distribution

The distribution of cis-DDP labelled with ^{193m}Pt and ^{195m}Pt has been evaluated in mice, rabbits, and humans. There seems to be no selective uptake by tumors, but the rate of clearance in animals with tumors is significantly higher than in the control groups (Wolf, 1974). This has been seen only in the first few minutes after injection and the effect is dependent on the tumor size. Altered renal clearance in tumor animals could account for this increased rate of removal. Some investigators believe that there may be certain proteins in the blood which are affected; this could result in altered binding of platinum to serum proteins.

Figure 3 illustrates the temporal distribution of the radiolabelled Pt following parenteral administration. There is an apparent half life of less than one hour for the first four hours, followed by a considerably slower rate of decrease with significant amounts of platinum in the plasma 12 days after administration. The apparent $t_{\frac{1}{2}}$ for this slow phase is 4-5 days. Urinary levels of Pt reach 50-60% of the administered dose within the first four hours after treatment.

The organ distribution of platinum can be seen in Table 1. Highest levels are found in the kidney, which correlates with the high urinary

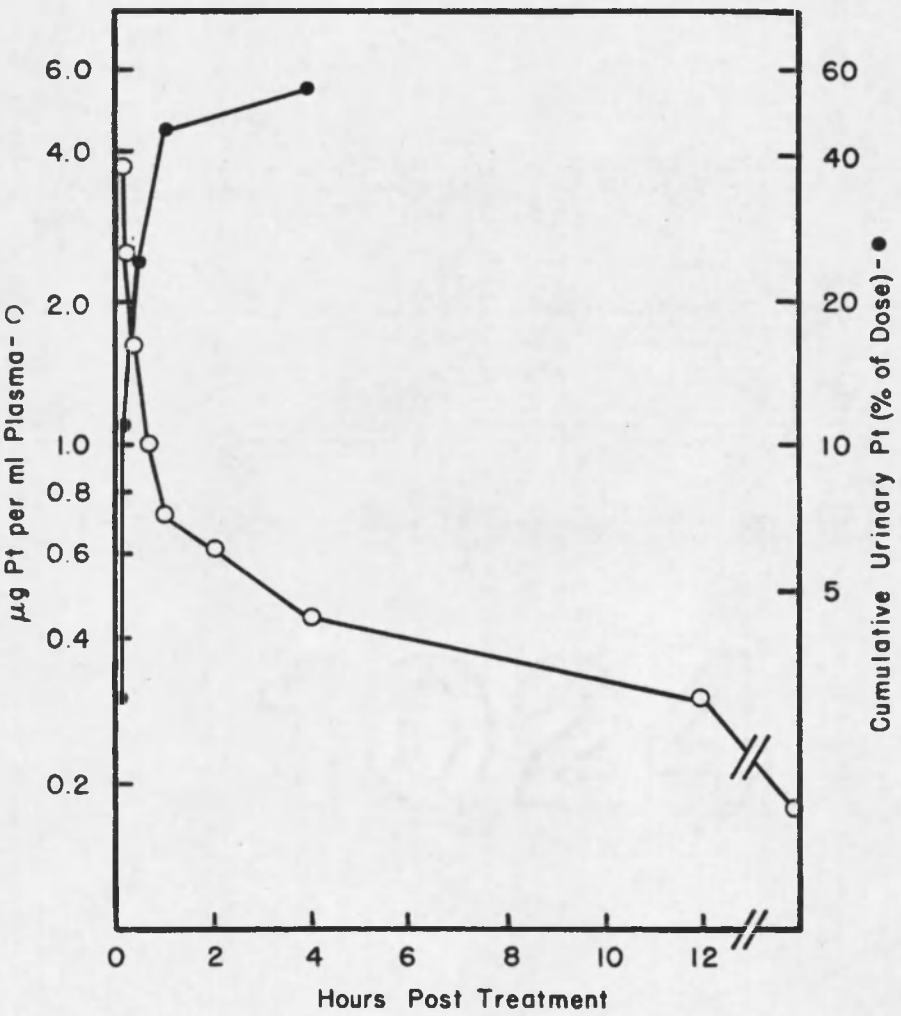

FIGURE 3 - The Temporal Distribution of Radiolabelled Platinum

Table 1: Organ Distribution of Platinum Following IV Administration of DDP, 1 mg/kg to Dogs.

	µg/g, wet weight					
	minutes		hours		days	
ORGAN	10	60	4	24	4	12
kidney	6.65	1.95	1.87	2.15	0.75	0.55
plasma	2.64	0.71	0.45	0.27	0.18	0.15
ovary	1.81	0.64	1.04	0.78	0.73	
liver	1.50	1.39	1.34	1.17	0.35	0.32
uterus	1.36	0.70	1.18	1.53	0.92	
skin	1.25	0.16			0.61	0.18
lung	1.21	0.80	0.78	0.80	0.66	0.14-0.24
stomach	1.20	0.68			0.31	
adrenal	1.06	0.34	0.93	1.08	0.26	
colon	0.70	0.40			0.23	
muscle	0.35	0.80	0.24	0.26	0.07	0.04
heart	0.32	0.11	0.58	0.36	0.03	
intestine	0.31	0.35			0.22	
pancreas	0.28	0.18	0.47	0.66	0.42	
spleen	0.26	0.15	0.52	0.43	0.33	
brain				0.06	0	
fat			0.23	0.04		

levels seen in the first phase of clearance. Within the first day after treatment, plasma values declined by approximately 90%, while many tissues maintained nearly constant levels of the element. Figure 4 shows the tissue:plasma ratio of platinum for several tissues during the first week past treatment. Although the absolute values are low, relative to those of the first day following administration, the concentration of platinum was significantly higher in several tissues than was found in plasma at corresponding times. When analysis of renal cortex and medulla were separated, it was found that the medulla had slightly higher concentrations of platinum. By the end of the first day, there were similar levels of the complex in both cortical and medullary portions, but as time progressed, the cortex had a higher tissue:plasma ratio than the medulla. This latter distribution was maintained as long as 12 days after administration (Taylor et al., 1976).

The organ distribution of platinum at various times after administration of the drug is in qualitative agreement with that shown for DDP and its analogs and in several species, including man. The localization of platinum in organs of excretion (kidney, liver, and lung) is a common finding reported for several species. The renal toxicity seen in man and animals is correlated with the high renal content of platinum in tissues following injection. Likewise, therapeutic efficacy also correlates with organ accumulation of Pt compounds. There are no tissues with higher platinum concentrations (except the kidney) after four days than the ovary and uterus; uterine cancer is one of the most responsive organs to platinum therapy (Ellerby, 1974).

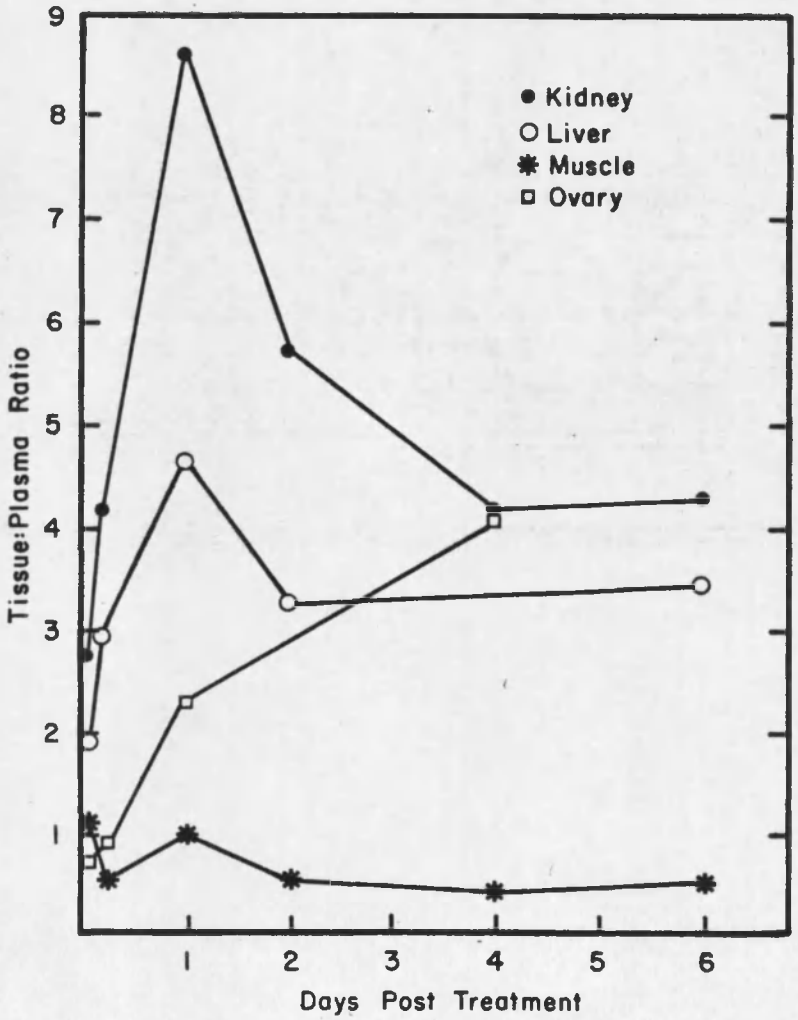

FIGURE 4 - Diagram of Platinum's Distribution in Tissue

Toxicity and Dosage

Animals receiving maximally tolerated doses recover from the toxic effects within 14-24 days. Renal lesions are the most severe toxic changes and are characterized by increasing excretion of urinary lactic dehydrogenase. A single I.P. injection of a toxic dose of 12.2 mg/kg in rats showed elevated levels of blood urea nitrogen, serum glucose, and serum albumin, whereas, total protein levels were depressed. Histological alterations were most pronounced in tissues having cellular constituents. Renal tubular sloughing occurred and rats that survived the toxic effects regenerated the cellular constituents in those tissue affected (Schaeppe, 1973).

In humans, a single dose of 1.95 mg/kg of cis-DDP was likely to produce renal impairment resulting in severe morbidity. The human tolerance has been shown to be similar to dogs and monkeys. The nephrotoxicity is manifested in creatinine and uric acid clearances and overall concentrating defects (Schaeppe, 1973).

Isolated Tubules

Kidney tubules have previously been isolated using techniques involving perfusion and digestion of the organ with collagenase, followed by centrifugation of the resulting suspension. The disadvantages of this technique involve prolonged incubation with an expensive, potentially damaging enzyme and contamination of the preparations with glomeruli. A method developed by Brendel and Meezan (1975) permits the isolation of a purified preparation of rabbit kidney tubules without collagenase digestion.

Rabbit kidneys are perfused with buffer, the cortex is homogenized by hand, and put through nylon sieves of varying mesh sizes. This separates large pieces of tissue from the glomeruli and tubules which are caught on the smaller mesh sieves. Tubules isolated in this way are morphologically intact, as shown by electron microscopy, and have intact basement membranes in contrast to those isolated with the use of collagenase as determined by measurements of oxidative metabolism and gluconeogensis and in transport functions such as PAH and TEA uptake (Brendel and Meezan, 1975).

The Intact Isolated Perfused Kidney

Studies with the isolated perfused rabbit kidney overcomes certain problems of *in vitro* and *in vivo* experiments. Correlation of renal function and energy state are not possible with cortical slices, homogenates, or isolated tubules. Sodium reabsorption is either absent or present at only a fraction of its maximum activity in most of the *in vitro* systems studied. The *in vivo* experiments that should be most contributory are largely not useful because flow through the kidney is so high as to obscure possible arteriovenous differences in the concentration of metabolites. Studies with the isolated perfused kidney have overcome many of these objections. A constant volume of defined and oxygenated medium is recirculated through the kidney via the renal artery. Urine is continuously formed and can be compared with the medium at sampling intervals. The reabsorption of glucose by the kidney tubules will be used to determine the renal damage that has occurred. *In vivo* studies have found depressed reabsorption of glucose within a few hours after exposure to *cis*-DDP.

Rationale and Objective

There are several renal secretory mechanisms which exhibit absolute limitation of transport capacity. We have decided to use the secretory mechanism for organic acids as the measure of proximal tubular activity and, more specifically, as the measure of proximal tubular activity in the assessment of renal function under the influence of cis-DDP. Examples of organic acids include phenol red, hippuric acid, p-aminohippuric acid (PAH), penicillin, chlorothiazide, and a variety of glucuronides as well as sulfuric acid esters. The Tm_{PAH} was chosen as the measurement of choice because of the ease and precision of analysis. Although numerically different, the functional significance of Tm_{PAH} is the same as that of $Tm_{GLUCOSE}$. In chronic renal disease, both have diminished in rough proportion to the tubular distribution and replacement of functional tubular tissue by fibrosis. In acute renal disease, the correlation is less evident; the Tm values are depressed not only through the loss of secretory and reabsorptive tissue, but also by a biochemical lesion of intact cells. Phase I trials of cis-DDP have shown depressed $Tm_{GLUCOSE}$ (Hill, 1975), along with lowered values in the secretory processes such as creatinine clearance.

Definition of Tubular Clearance

In order to correlate previous findings with our in vitro model of PAH uptake, an understanding of renal tubular cells is necessary. The ability of the kidneys to adapt to filtration rates and plasma concentrations are critical factors which regulate its clearance.

When the kidney removes a substance from the plasma by filtration only (e.g., insulin), the tubular clearance is zero. The amount

excreted will be solely proportional to the plasma concentration. If the clearance of a substance involves filtration and secretion, then the tubular clearance will depend on the plasma concentration and the tubular transport capacity for secretion. This rate of tubular transport, defined as T_X at a given plasma concentration, will depend on the kind of substance being transported. If the tubular cells have a "maximum tubular capacity" (Tm_X) for the particular substance, then as long as the plasma concentration remains below the threshold concentration that saturates the transport process, a maximal fraction of the substance that enters the peritubular capillaries which escapes filtration will be secreted. The following equation applies to para-aminohippuric acid which is filtered and secreted; a maximum transport capacity is maintained by the renal tubular cells.

$$V_{PAH} = GFR \times K_{PAH}/(PAH)p$$

Total Clearance = Glomerular Clearance + Tubular Clearance

The total renal clearance of a substance that is removed by filtration and secretion will decrease as the plasma concentration is increased. Since the amount of a substance transported maximally in a minute is constant (Tmp_{PAH}), then the varying quantity $[Tm/(PAH)_p]$ will be decreased as the plasma concentration is increased. Figure 5 illustrates how PAH approaches the Inulin clearance as the concentration of plasma PAH is increased.

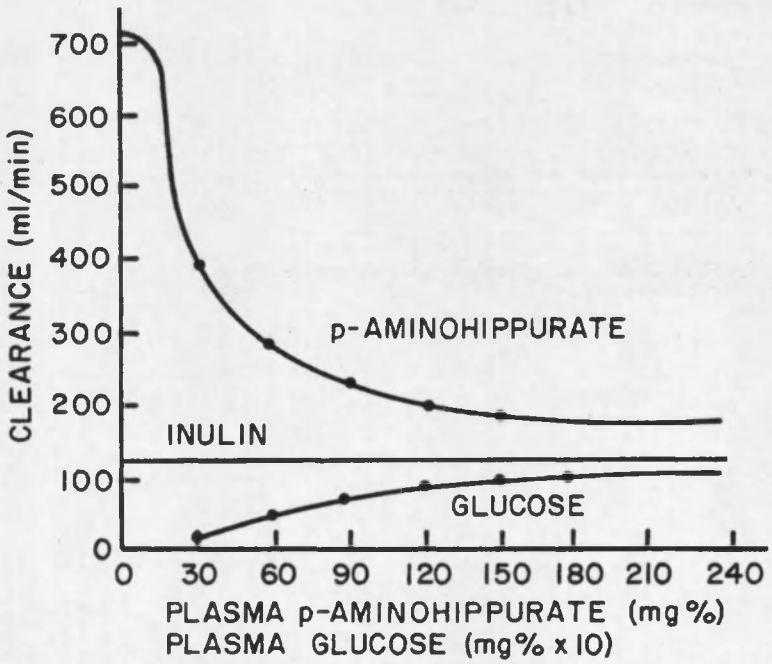

FIGURE 5 - Illustration of PAH Clearance in Relation to Insulin

Mechanisms for PAH Transport

Various investigators have shown a bidirectional transport for PAH in the kidneys (Rennick, 1977). A small quantity of PAH is reabsorbed, but does not seem to have an appreciable influence on the clearance rate. An active carrier-mediated process is believed to transport the substance. Only a single concentrating step is involved in the transport of PAH in the rabbit. A concentration gradient is established in which the compound is allowed to diffuse down from the blood capillaries into the interstitial fluid and enter the tubular cell through an energy dependent process. The high concentrations maintained in the intracellular compartment allow for another concentration gradient in which PAH can be diffused into the tubular lumen.

Previous studies have used renal slices in phosphate buffer containing PAH. Cross and Taggart (1950) used a slice:medium (S:M) ratio in which the amount of PAH in 1 gm of slice was divided by the corresponding amount in 1 ml of buffer. Values varied between 1.8-6.2 with an average of 4.0. The uptake of PAH has been characterized by the following observations: 1) The system is energy dependent and is supplied through aerobic metabolism which is dependent on oxidative phosphorylation; 2) When acetate was used as a substrate, an alkaline pH caused a depressed S:M ratio. This gave conclusive evidence that acetate entered its intracellular locus in a non-ionic form; 3) The uptake of PAH was shown to be calcium dependent; 4) When potassium levels were reduced, the S:M ratios declined rapidly. Optimum levels were shown to vary between 5-10 mM; 5) Glucose had no significant effect on PAH transport; 6) Intermediate chain length fatty acids seemed to have a competitive effect on PAH

transport; 7) Anoxia depressed PAH uptake, but increases in renal work did not require further increases in oxygen consumption.

Effect of cis-DDP on Renal Function

The use of DDP as a chemotherapeutic agent has been hampered by the high toxicity found in the kidney. There is a lack of information on how cis-DDP can elicit its toxic effects on growing cells. The question still remains as to whether an active metabolite must be formed in order to see a response. The delayed onset of toxicity gives support to this theory, but there is a need for an isolated intact cell to study. The proximal tubule is ideal, since a direct toxic effect has occurred due to the compound. Brendel and Meezan (1975) have developed a procedure for the isolation of a highly purified proximal tubule preparation in which the metabolic functions can be maintained to assess renal damage through renal function. A study of in vivo and in vitro kidneys will be made to determine the underlying causes of renal toxicity.

Kidney damage has been minimized with increased urinary flows so that patients can receive the therapeutic benefits of larger doses (Krakoff and Lippman, 1974). Thus, the second objective of this study will be to examine the effects of cis-DDP on the isolated intact kidney and demonstrate metabolic or morphologic changes that might accompany fluctuations in urine flow rates. Various parameters such as glucose reabsorption, phenol red secretion, and morphologic changes will be used to assess renal function and damage. The acute renal failure that accompanies platinum treatment will be monitored at various concentrations so that there can be a proper evaluation of the renal toxicity.

High concentrations of DDP in the kidney have paralleled the concentration seen in the liver. The lack of liver damage raises questions relating to the toxicity of an active metabolite formation. The rate of cell division cannot be the sole contributing factor to cell death. This study will investigate the results in *in vitro* experiments with platinum compound to those observed *in vivo*.

CHAPTER II

MATERIALS AND METHODS

Isolated Rabbit Tubule Preparations

Male New Zealand white rabbits weighing 2-3 kg were used in all experiments. The rabbits were maintained on Wayne Rabbit Blend and tap water ad libitum. Initial experiments employed sacrificing the rabbits with a 0.22 calibre high compression pellet directed to the cranium. A vertical midline incision was made, thereby fully exposing the visceral organs. Forceps were used to tease away fascia and fatty tissue covering the renal artery of the right kidney. Two sutures were loosely tied around the artery. One suture was .4 cm distal to the kidney and the other suture was 2 cm from the kidney. A 45° angulated incision was made halfway through the artery between the two sutures. A 21 gauge grooved and dulled butterfly needle was then used to cannulate the artery. Perfusion was started after the first suture secured the cannula at 20 mls/min. Immediately the renal vein was cut to allow for drainage. The kidney was then excised and freed of any abdominal tissue and perfused with Krebs-Ringer bicarbonate solution (115 mM NaCl, 5 mM KCl, 10 mM Na acetate, 1.2 mM $KH_2PO_4 \cdot H_2O$, 1.2 mM $MgSO_4 \cdot 7\ H_2O$, 1.0 mM $CaCl_2$, 25 mM Na HCO_3) for 20 minutes while gassed with 95% O_2/5% CO_2. The perfusion was non-recirculatory. The left kidney was then cannulated, excised, and perfused in a similar fashion. The blanched appearance of the kidneys gave evidence of the removal of any blood cells or other particles which might interfere with the ensuing perfusion. The perfusion apparatus consisted

of a variable speed peristaltic pump, connective tubing, and a reservoir container for the buffer solution.

After the 20 minute period, the kidneys were carefully removed and the renal capsule pulled off with forceps. The cortex was removed with the surgical forceps and suspended in buffer. These pieces were then homogenized by five strokes in a hand-held, loose fitting Teflon glass homogenizer which was especially designed for this purpose.

The homogenate was then transferred to a 210 μ nylon mesh sieve. Buffer solution was poured onto the sieve by a steady stream from a polyethylene wash bottle. The particles which came through were collected in a plastic liter beaker, while the remaining tissue was homogenized a second time as before. This tissue was again washed with buffer over the 210 μ sieve and collected in a 1 liter beaker. The material in the two 1 liter beakers was poured onto a 64 μ nylon sieve so that the kidney tubules could be recollected on the smaller size mesh. The final tubule concentration varied from 40-55 mg/ml of incubation buffer. Light microscopic observation confirmed that 95% tubules were isolated to 5% glomeruli.

The tubules were washed with a final buffer containing a 1% bovine serum albumin. This seemed to maintain the natural tubular form and prevent a clumping phenomenon at the outer ends. The washed tubules were then suspended to a final volume of 40 ml in albumin buffer where 35 μCi of tritiated water was immediately added. Five ml each of the tubule suspension in labelled incubation buffer was transferred to 25 ml siliconized Erlenmeyer side armed flasks. The flasks were designed with a conical impression placed in the bottom which prevented the tubules from

settling out of solution upon shaking. The flasks were placed in a gyro-rotary water bath shaker with a shaking speed of 90 rpm. Each flask was hooked up to a steady air supply of 95% O_2 and 5% CO_2 so that the suspension was aired, but not bubbled.

Assay Procedure for Transport and Incorporation of PAH and TEA

Transport studies involving the uptake of para-aminohippuric acid were conducted by the use of a radiolabel assay. ^{14}C-para-aminohippuric acid and tetraethylammonium bromide (TEA) were purchased from New England Nuclear. The labelled substrate was added simultaneously along with unlabelled PAH or TEA for the assessment of active transport and thus, tissue viability. The radiolabelled assay was similar to that reported by Brendel (personal communication, 1978). The tritiated water is used as an internal standard and represents the volume of tubules contained in the aliquot sampled. The labelled substrate represents the amount of substrate incorporated into that volume of tubules. The ratio of the labelled PAH (or TEA) to tritiated water gave a tissue:medium ratio which could be used to assess the active transport by the tubule cells. The radiolabel was added to the reaction flask in the following manner. Solutions of radioactive compound were prepared by adding 0.5 µCi of ^{14}C-PAH (specific activity .2 Ci/mM) to the swirling reaction flasks which contained 5 ml of incubation buffer, labelled water and cells. The concentration of the solution was then adjusted by the unlabelled PAH. Preliminary studies ranged from concentrations of .004 mM to 2 mM of PAH. During the course of the incubation (½, 1, 1½, 2, 4, 6, 8, 10, 12, and 15 min), .2 ml aliquots of swirling cells or tubules was removed by pipet,

The aliquots were placed on polycarbonate membrane filters which were assembled onto a vacuum chamber. The aliquots were filtered by suction and the filters placed on Kimax scintillation vials. Each vial contained 1 ml of 3% Triton solution. A final aliquot was taken after the 15 min sampling time and placed directly into the scintillation vial without filtering. The unfiltered aliquot was used to determine the $^{14}C/^{3}H$ ratio in the total volume of medium tissue.

Ten ml of scintillation cocktail[1] was added to each vial. The vials were capped, shaken, and transferred to a Searle Mark VI liquid scintillation counter and counted for two minutes for double label counting. Quench curves were programmed and efficiency was corrected by the external standard ratio method. The counting conditions employed normally resulted in 35% counting efficiency for the full ^{3}H window and 64% counting efficiency for the narrow ^{14}C window. A ratio of 1:10 was maintained for ^{14}C:^{3}H so that spill down into the lower energy isotope window would be negligible to total number of counts.

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1. Scintillation cocktail consisted of two parts of a solution of 22.8 g omnifluor (New England Nuclear) per gallon of spectral grade toluene and one part Triton X-100.

Assay Procedure for Competitive Inhibitors of Uptake and Transport

Isolation procedures for kidney tubules were carried out as in previous experiments. Known competitive antagonists were assayed against PAH (or TEA). The antagonists employed were phenol red (Eastman Kodak Laboratories) and probenecid (Merck Sharp and Dohme). Equimolar concentrations ranged from .01 mM to 1 mM against the labelled substrates. Probenecid was extracted from Benimid with alcohol, dried with a rotary evaporator, and solubilized with incubation buffer. The addition of the antagonists preceded the labelled substrate just prior to the sampling. Sampling time was 15 minutes using .2 ml aliquots for filtration.

Dichlorodiammineplatinum (II) was donated by the Southwestern Oncology Group in the form of NSC-119875[1]. The same protocol was used for the preliminary experiments as in previous uptake studies. Subsequent experiments employed a two hour incubation of cis-DDP with the tubules and cells. Concentrations of platinum were varied over the range of .003 mM to 3 mM. All samples were solubilized with Triton X-100 (3%) and received 10 ml of omnifluor for liquid scintillation counting.

Assay Procedure for Double Reciprocal Plot

The kinetic studies were carried out by isolating kidney tubules by the procedure of Brendel and Meezan (1975). The washed tubules were suspended to a final volume of 60 ml in buffer where 50 μCi of tritiated water was added. Eleven ml each of tubule suspension was transferred to five separate 25 ml Erlenmeyer side armed flasks. Two flasks received final concentrations of .02 mM and .2 mM cis-DDP, while the remaining

flasks were given equal volumes of buffer. A two hour incubation period preceded the sampling time. Two ml each of the incubated tubule suspension was transferred to five separate 10 ml flasks that were aired with 95% O_2 and 5% CO_2 on the shaker. The concentrations of PAH used for each flask were .62 mM, 1.2 mM, 2.5 mM, 5 mM, and 10 mM respectively. Solutions of the radioactive compound were prepared by adding .3 μCi of ^{14}C-PAH (S.A. = .2 Ci/mM) to the swirling flasks. A five minute sampling period (1, 2, 3, 4, 5) was employed where .2 ml aliquots were removed and placed on the polycarbonate membrane filters for filtration and counting.

The Isolated Perfused Kidney

New Zealand white rabbits received .6 ml/kg sodium nembutal IV for a surgical plane of anesthesia. The rabbits were given an injection of 3 mg Papaverine (Lilly) and 3 mg Vasodilan (Mead Johnson). A 10% solution of mannitol and 500 units of heparin were infused. This established a substantial diuresis and prevented renal thrombosis. All glassware was clean and dust-free while solutions were filtered through a millipore filter (.5 micron pore size). A midline incision was made and the abdominal musculature retracted. The mesenteric artery was ligated and loose ligatures were placed on the descending aorta above and below the branches to the renal arteries. The renal artery was cannulated without renal ischemia by introducing the cannula into the descending aorta via the superior mesenteric or celiac arteries and then into the renal artery. The ascending vena cava was then severed to permit drainage of blood. Approximately 50 ml of medium that had been gassed with 95% O_2 and 5% CO_2 was perfused through the renal artery by a hand syringe. The washed out kidneys were then placed on the perfusion apparatus.

The preparation of the medium was modified with considerable improvement in renal function. Minimal Essential Medium 199 (GIBCO) contained all the nutrients with the following electrolyte balance: (Na^+ 154 mEq/L, K^+ 5.4 mEq/L, Ca^+ 2.8 mEq/L, and Cl^- 125 mEq/L). Twenty-two hundred mg of sodium bicarbonate was added for a concentration of 25 mM. The medium received ampicillin (100 mg), papaverine (50 mg), vasodilan (10 mg), and insulin (200 units) to give a final volume of one liter. Before each experiment, the perfusate was prepared as follows: 3 parts of medium to 1 part bovine serum was millipored, gassed, and warmed in the perfusion apparatus for 30 minutes. The serum had previously been heated at 56°C for 30 minutes in order to remove complement. In addition to prefiltration of the perfusate, filters (14 micron pore size) were included in the circuit to filter the medium during the perfusion. The pH was maintained at 7.4 throughout the experiment with phenol red as an indicator.

Figure 6 shows the apparatus hooked up to a pulsatile pump in series. Gassed water is warmed and circulated so that oxygen and carbon dioxide are allowed to permeate the silastic tubing in the gas exchange chamber. Approximately 70 ml of perfusate was necessary for proper circulation in the system. The pulsatile pressure was recorded and kept constant during a single perfusion. The resistance-pressure had to be adequate to produce a copious urine flow; this was monitored with a manometer attached to each kidney chamber. Readings were taken before the kidneys were hooked up. This allowed for the actual internal resistance due to the kidney. Physiologic pressures were maintained with the pump in this way.

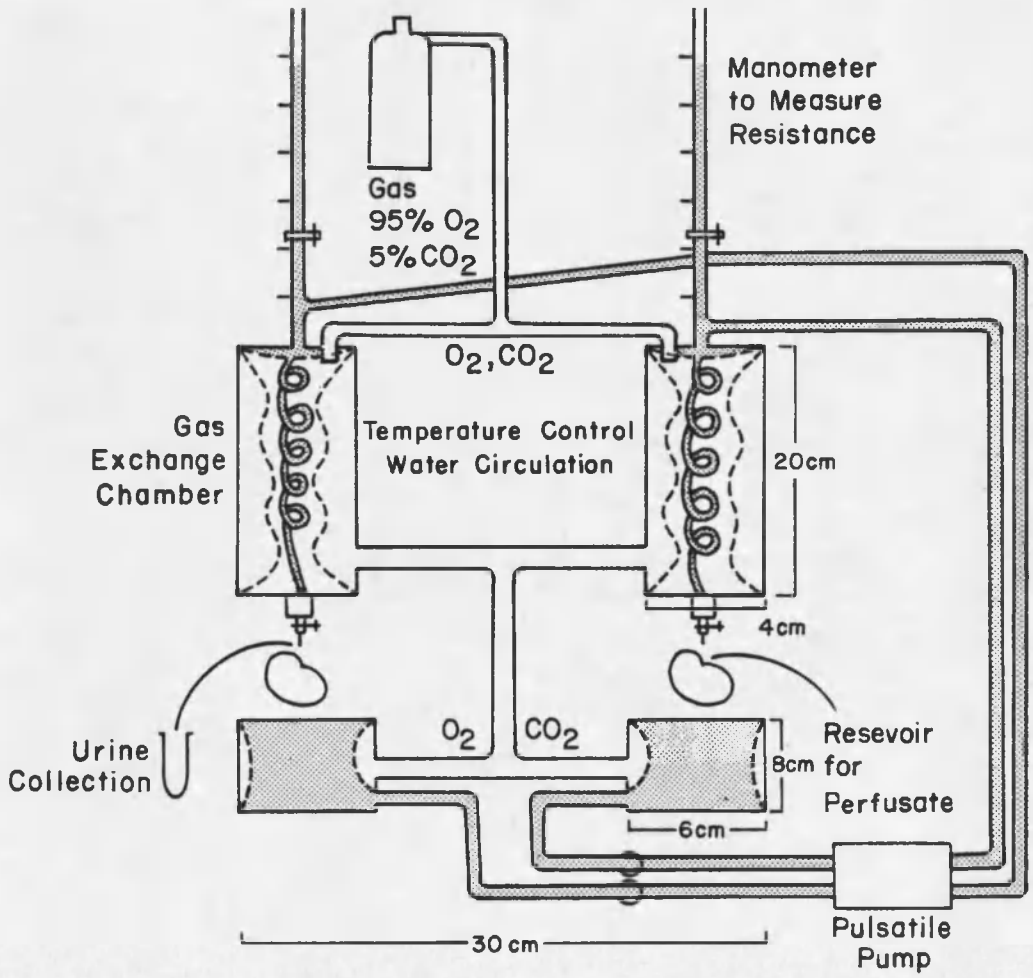

FIGURE 6 - Diagram of the Apparatus for Kidney Perfusion

Urine was collected over a four hour perfusion. Glucose levels were measured with a glucose analyzer (Beckman) and phenol red was measured spectrophotometrically. The urine was alkalinized in the colorimetric assay for phenol red. Urine protein levels were quantified by COMBISTIX (Ames) reagent strips.

Tissue slices were prepared for control and treated kidneys. A 3% glutaraldehyde solution (Karnovsky's) was used for light microscope and electron microscope studies. Slices were sectioned for the outer medullary zone in which the straight portion of the proximal tubules was believed to undergo necrosis.

CHAPTER III

RESULTS AND DISCUSSION

Proximal Tubule Isolation

Tubules obtained by hand homogenization with subsequent washings through sieves were shown to be 95% tubules with only 5% glomeruli when viewed at the light microscope level. Figure 7 illustrates the intact tubule with its surrounding basement membrane smooth and flawless. The overall outside diameter of the tubule is approximately 65 microns. Electron microscopy revealed an abundance of microvilli along with lateral interdigitations with adjacent cells. This can be seen in Figure 8 along with the basilar "infoldings" in the mid and basal regions of the cell. It was concluded that the isolation procedure did not result in damage to the gross or fine structure of the isolated rabbit tubules (Hjelle, 1975).

The loose fitting pestle was necessary to minimize disruption of tubular cells. The second hand homogenization along with vigorous washing of the tubules improved the yield of tubules by as much as 20%.

Metabolism

Transport and Incorporation

Optimal concentrations for PAH uptake by the rabbit renal proximal tubules was found at .1 mM. Tissue:medium ratios attained 25 during a time course of uptake of 15 minutes as in Figure 9. Farah, Rennick

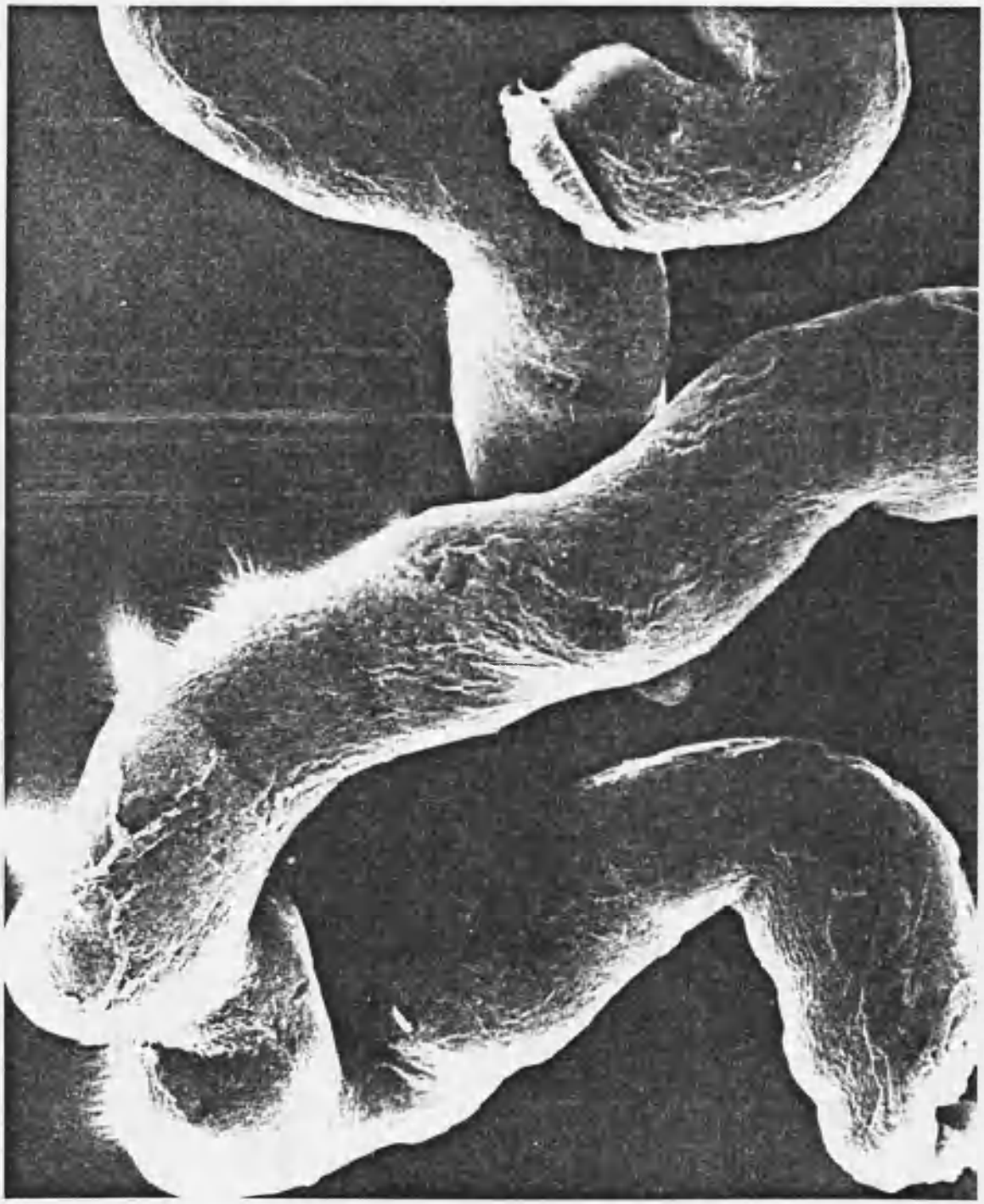

FIGURE 7 - Rabbit Renal Proximal Tubules Obtained by Hand Homogenization and Sieving

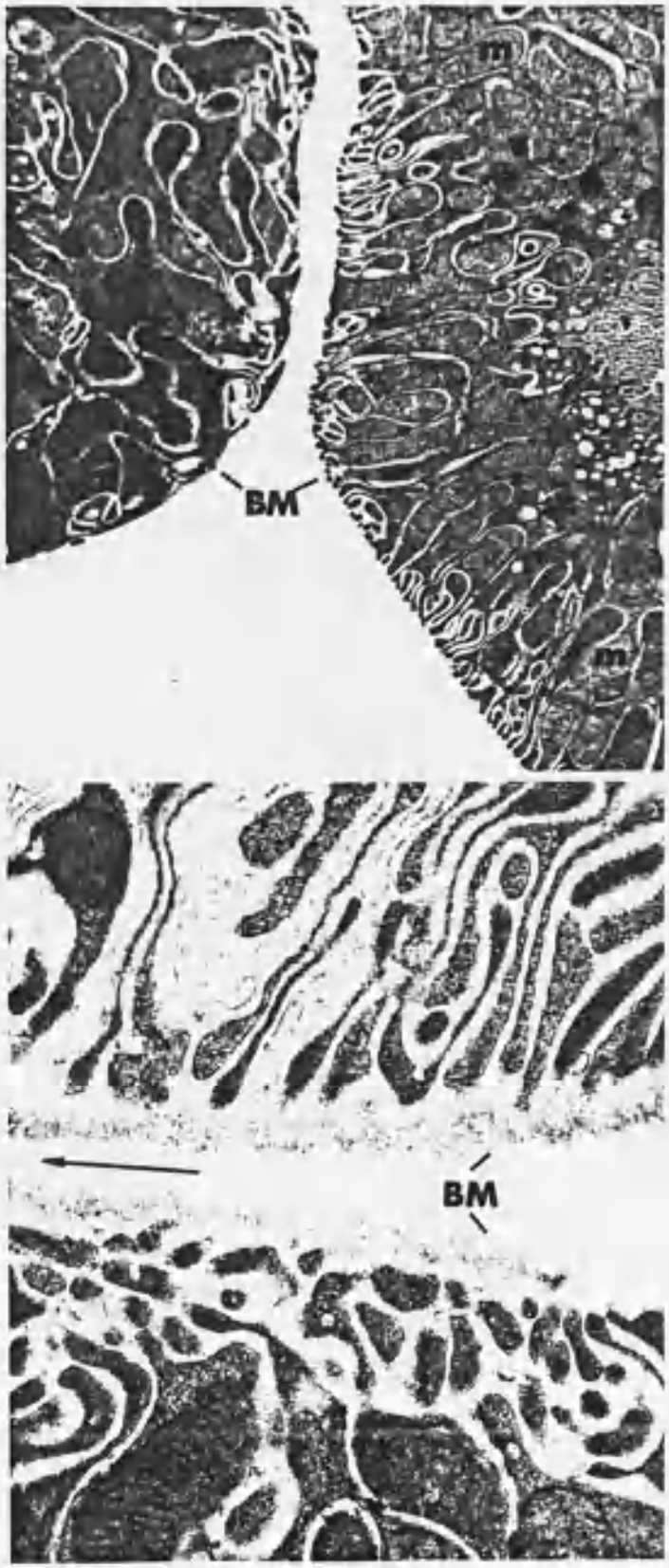

FIGURE 8 - An Electron Micrograph of Two Adjacent Isolated Tubules

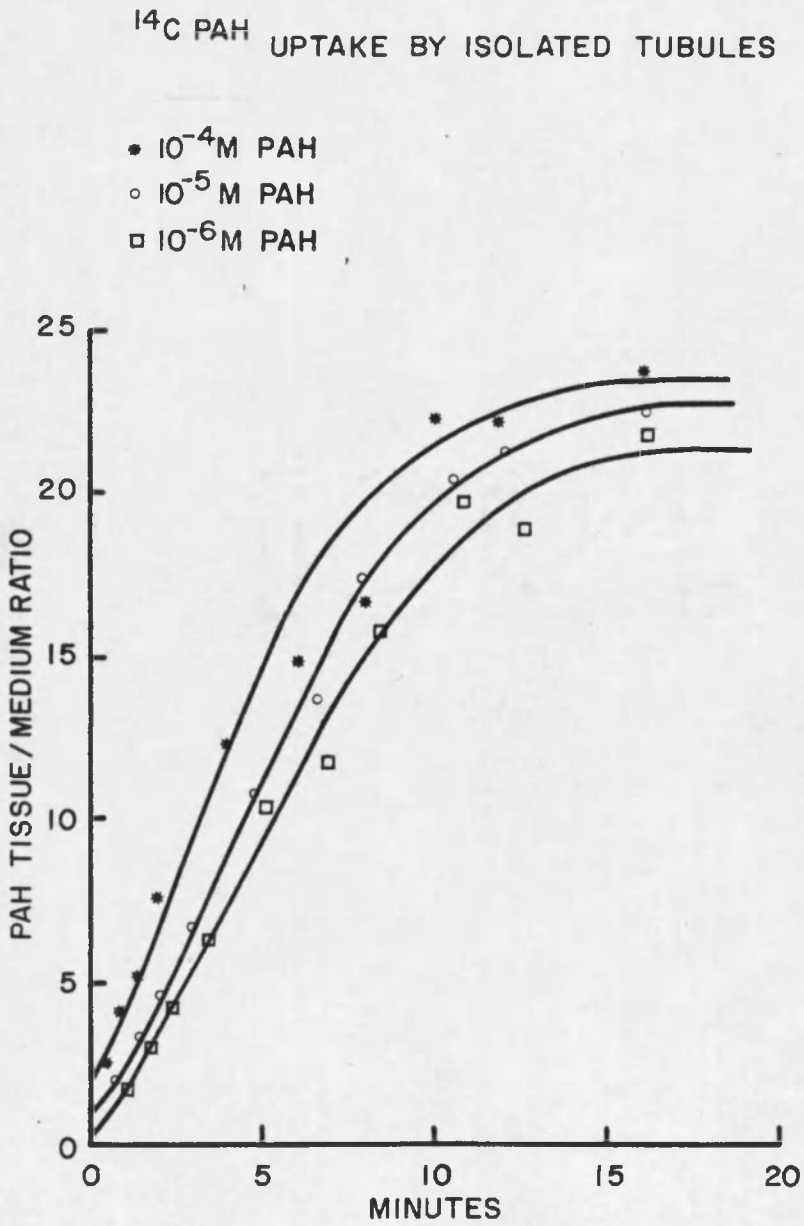

FIGURE 9 - The Incorporation of ^{14}C-PAH By Isolated Rabbit Tubules

and Frazer (1954) used a PAH concentration of .14 mM in the slide; medium procedure for rabbit tissue slices. The .1 mM range was used throughout the experiments conducted for PAH transport.

Known specific inhibitors of the organic acid transport system were used to provide reference values with which to compare the effects of platinum compounds. At comparable concentrations, phenol red depressed T/M values over 90% (Fig. 10). When probenecid was used in equimolar concentrations against PAH, a depression of 90% was exhibited by the competitive antagonist. Figure 11 illustrates the substantial inhibition of PAH uptake even at low concentrations of probenecid. The dose dependent inhibition by probenecid was used as a control competitive antagonist for PAH transport.

Probenecid did not exhibit inhibition of uptake when tetraethylammonium ion was used as a labelled substrate. Although T/M values were not as elevated as for PAH, there was no depression during the 15 minute sampling when probenecid was incubated with the tubules (Fig. 12). The presence of two separate transport systems in the isolated tubule preparation gave support to previous findings on renal tubules and provided an ideal one-cell model as an index of tissue viability. PAH uptake and transport was chosen as an index for renal function due to the higher T/M ratios seen in preliminary tests.

cis-Dichlorodiammineplatinum's Effect on Transport and Incorporation

cis-DDP was added to the incubated cells along with the labelled PAH. No significant depression of T/M was seen at equimolar concentrations. Only at 1 mM did DDP cause a significant inhibition of PAH uptake

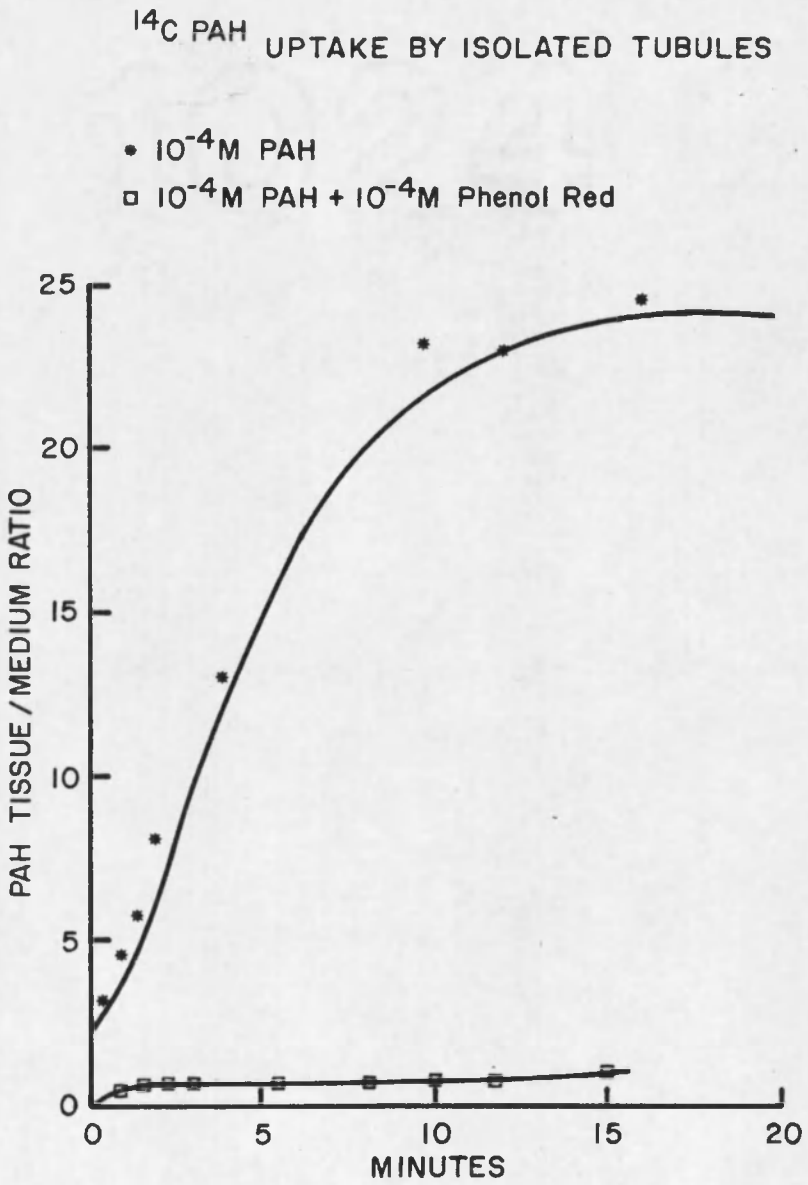

FIGURE 10 - The Incorporation of ^{14}C-PAH By Isolated Rabbit Tubules Under the Influence of Phenol Red

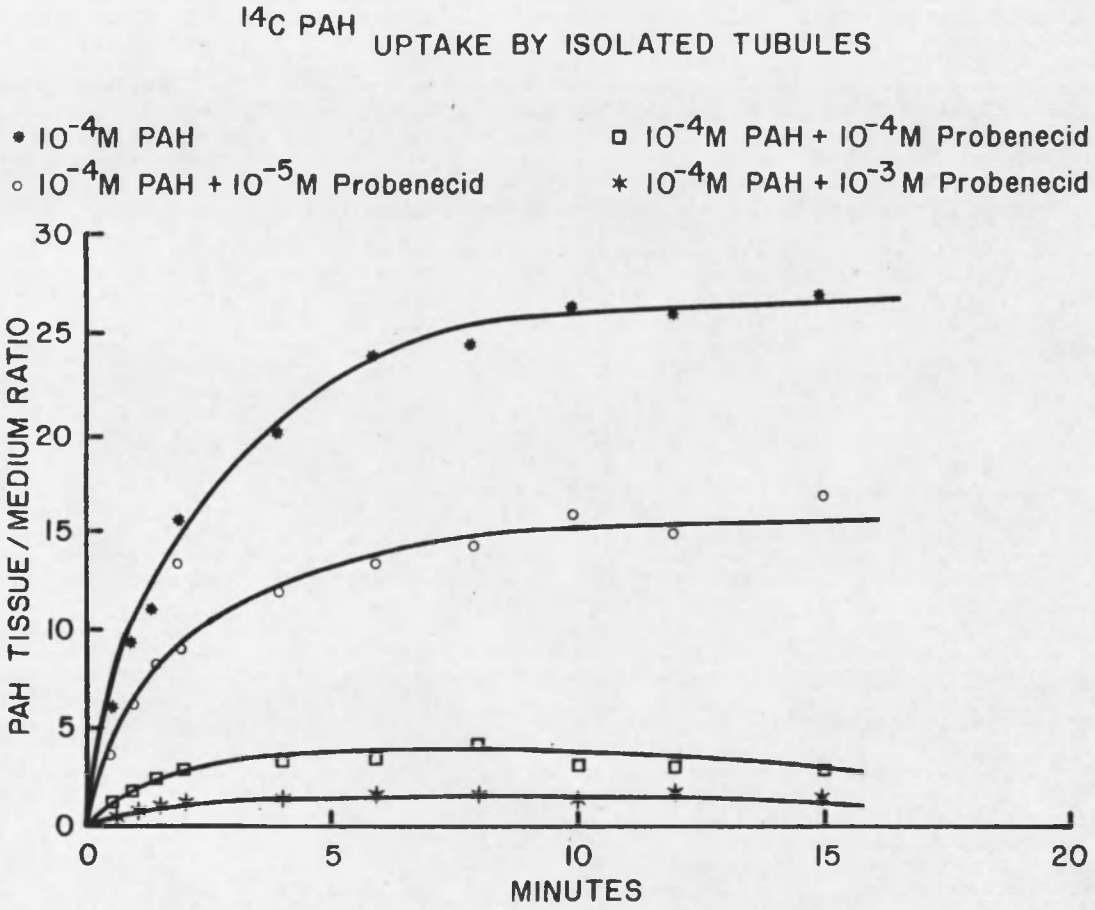

FIGURE 11 - The Incorporation of ^{14}C-PAH By Isolated Rabbit Tubules Under the Influence of Probenecid

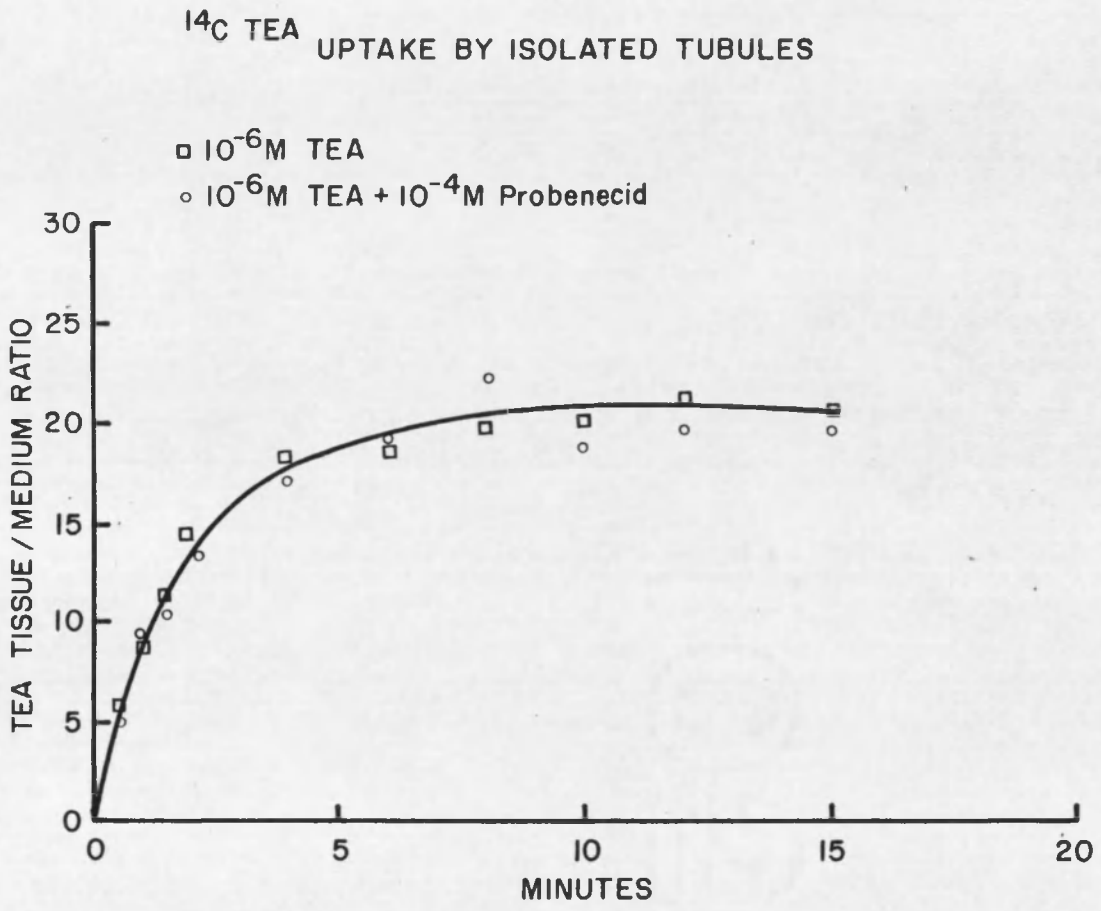

FIGURE 12 - The Incorporation of ^{14}C-TEA By Isolated Rabbit Tubules Under the Influence of Probenecid

by the tubules. The inhibition seen in Figure 13 was a general cytoinhibitory effect of platinum seen at the high concentrations. One, two, and three hour incubation periods were used to investigate the delayed response seen in vivo. Exposure of platinum complex to light for 24 hours was also used to investigate the mono and dihydroxy species thought responsible for platinum's activity (Moore and Hysell, 1975). Figure 14 was the result of two hour incubations. No significant inhibition was seen at equimolar concentrations when compared to probenecid. The substantial inhibition of PAH by cis-DDP at 1 mM was examined by using the kinetic protocol described in Materials and Methods.

Figure 15 is the Lineweaver Burke plot of PAH transport kinetics in the presence of cis-DDP. The V_{max} for PAH transport was determined to be .53 mM/µl/min. By extrapolation, the Km value was found to be approximately .37 mM. These values were similar to the results obtained by Cross and Taggart (1950). The graph demonstrates a lowering of both values in the presence of inhibitor suggesting that there was mixed inhibition by cis-DDP at high concentrations, rather than pure competitive or non-competitive inhibition. The results indicate a very slow rate of interaction of the enzyme with the organometallic complex.

This study emphasized the Pt-protein (enzyme) interaction. The delayed response was due to a necessary activation of the platinum complex within the tubule cells or a general cytoinhibition as in the case of a metabolic inhibitor. Intermediate metabolites of cis-DDP have been isolated (Taylor, Tew and Jones, 1976), but the toxic effects due to prolonged exposures have not revealed a direct cause and effect of these metabolites.

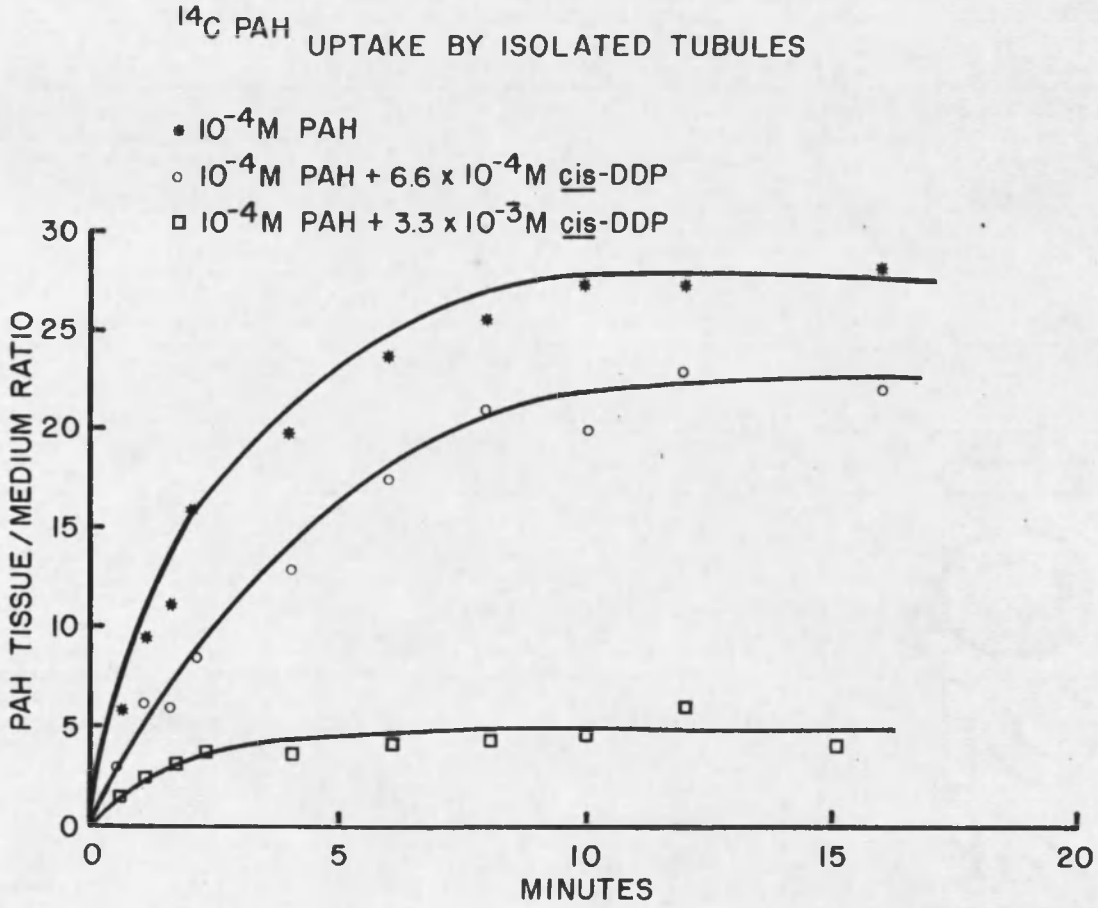

FIGURE 13 - The Incorporation of ^{14}C-PAH By Isolated Rabbit Tubules Under the Influence of cis-Platinum

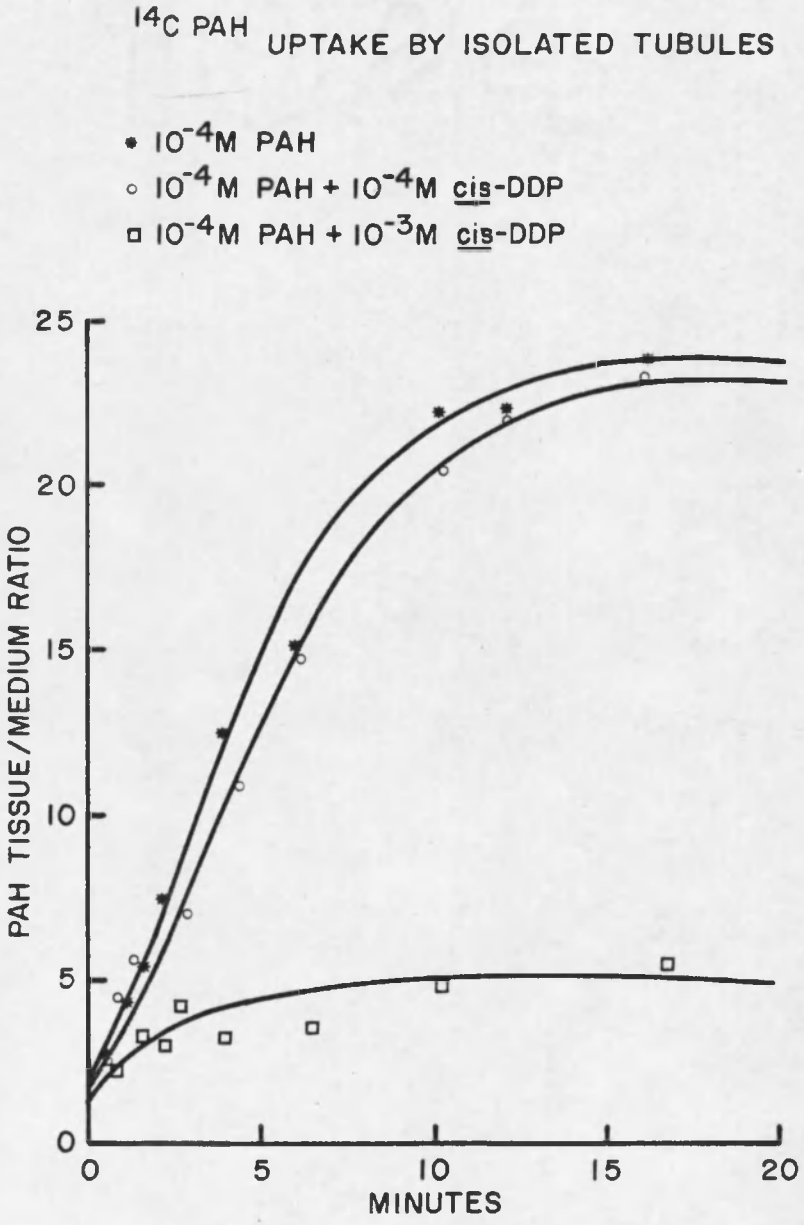

FIGURE 14 - The Incorporation of ^{14}C-PAH By Isolated Rabbit Tubules Under the Influence of Incubated cis-DDP

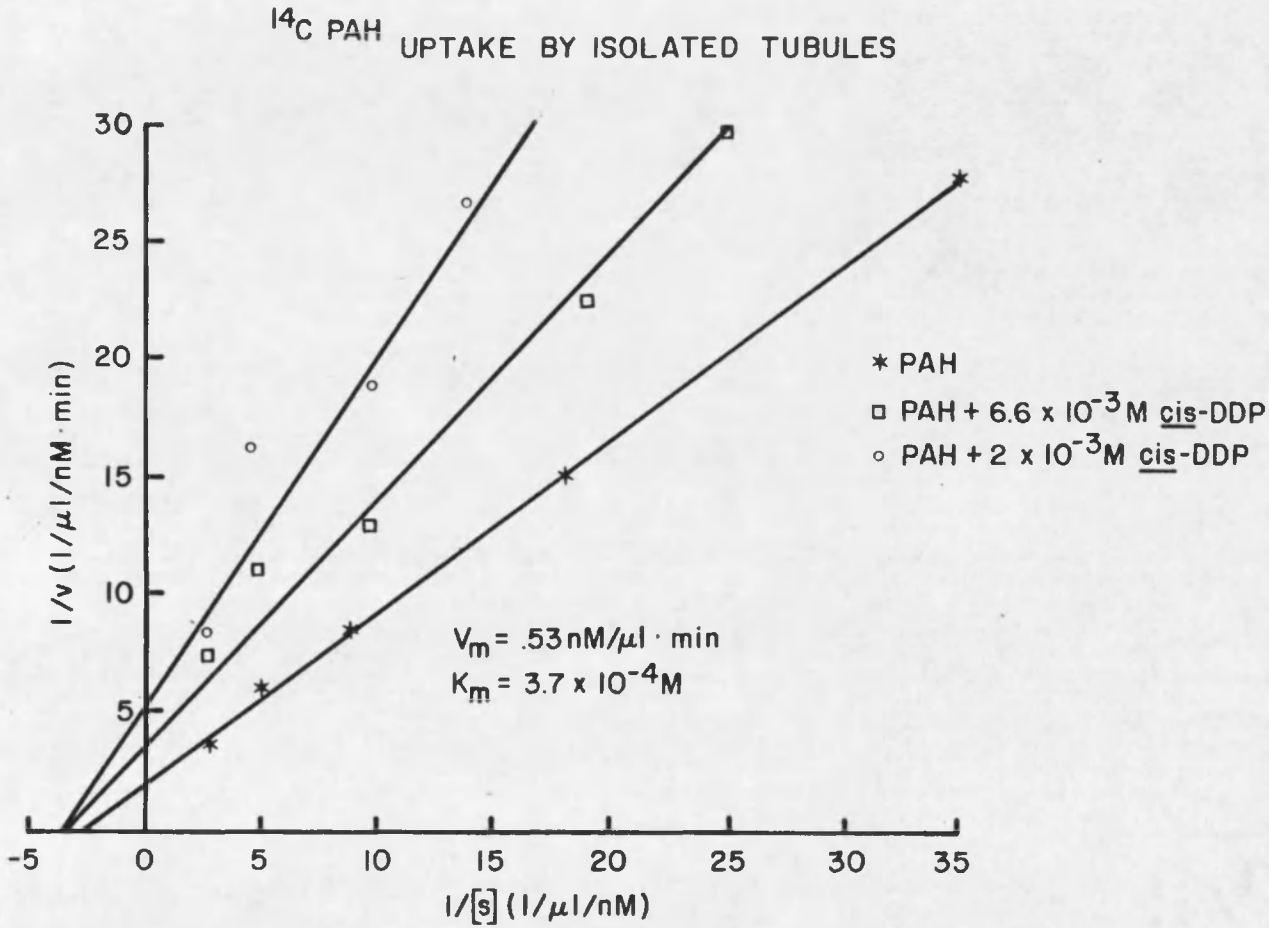

FIGURE 15 - Double Reciprocal Plot of ^{14}C-PAH Under the Influence of cis-DDP by Isolated Tubules of Rabbit Kidneys

The Isolated Perfused Kidney

Isolated kidneys were perfused for various time intervals ranging from one to four hours. As morphologic changes sometimes occurred in perfusions exceeding four hours in the normal kidney, we limited our drug studies to shorter perfusions. A light micrograph of the normal perfused kidney can be seen in Figure 16. The excised outer medullary zone demonstrates the "normal" morphology of the pars recta in the proximal tubule.

cis-DDP was added to the circulating perfusate of one of the kidneys during the three hour perfusion. Platinum concentrations were maintained at .1 mM by changing the perfusate every 30 minutes. No significant abnormalities were seen at the electron microscope level. The tubular epithelium appeared intact as well as the appearance of the mitochondria which were normal. Increased urinary flow rates occurred in both kidneys after two hours and remained constant (2 ml/min) thereafter. The renal function closely resembled the normal kidney over the three hour perfusion. Glucose level was maintained in the "normal" range and phenol red variations in the urine were insignificant.

Since the metal complex had little effect at the physiologic range, we decided that an induced renal lesion might be seen at higher concentrations of the drug. Platinum levels were maintained at 1 mM over the three hour time period. Histologic examination revealed a severe necrosis of the epithelium. Figures 17a and 17b are electron micrographs of the straight portion of the proximal tubules. The marked vacuolation of mitochondria with brush border loss is characteristic of tubular necrosis due to heavy metals. Similar results had been reported for the mercurial

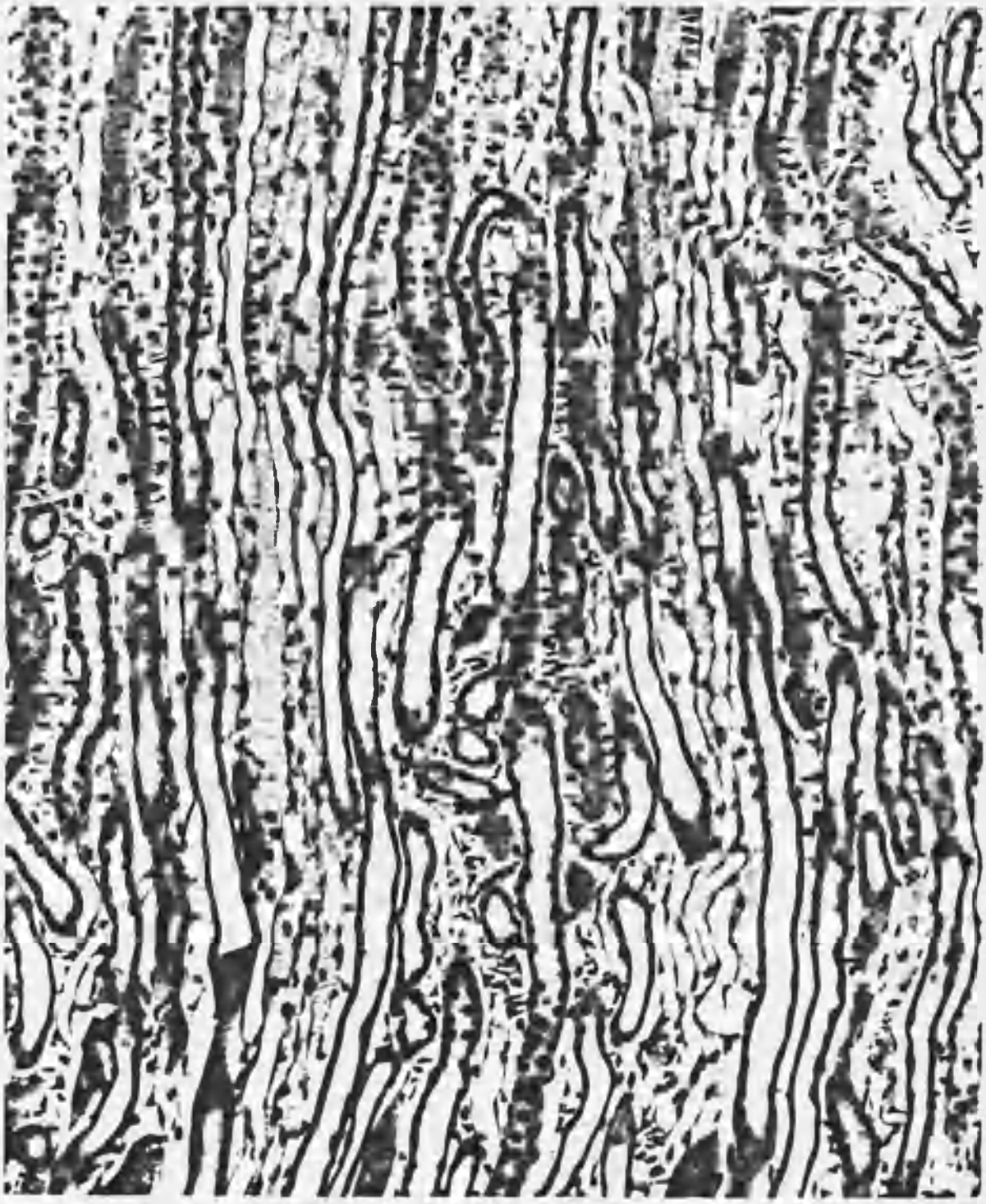

FIGURE 16 - A Light Micrograph of the Normal Isolated Perfused Kidney

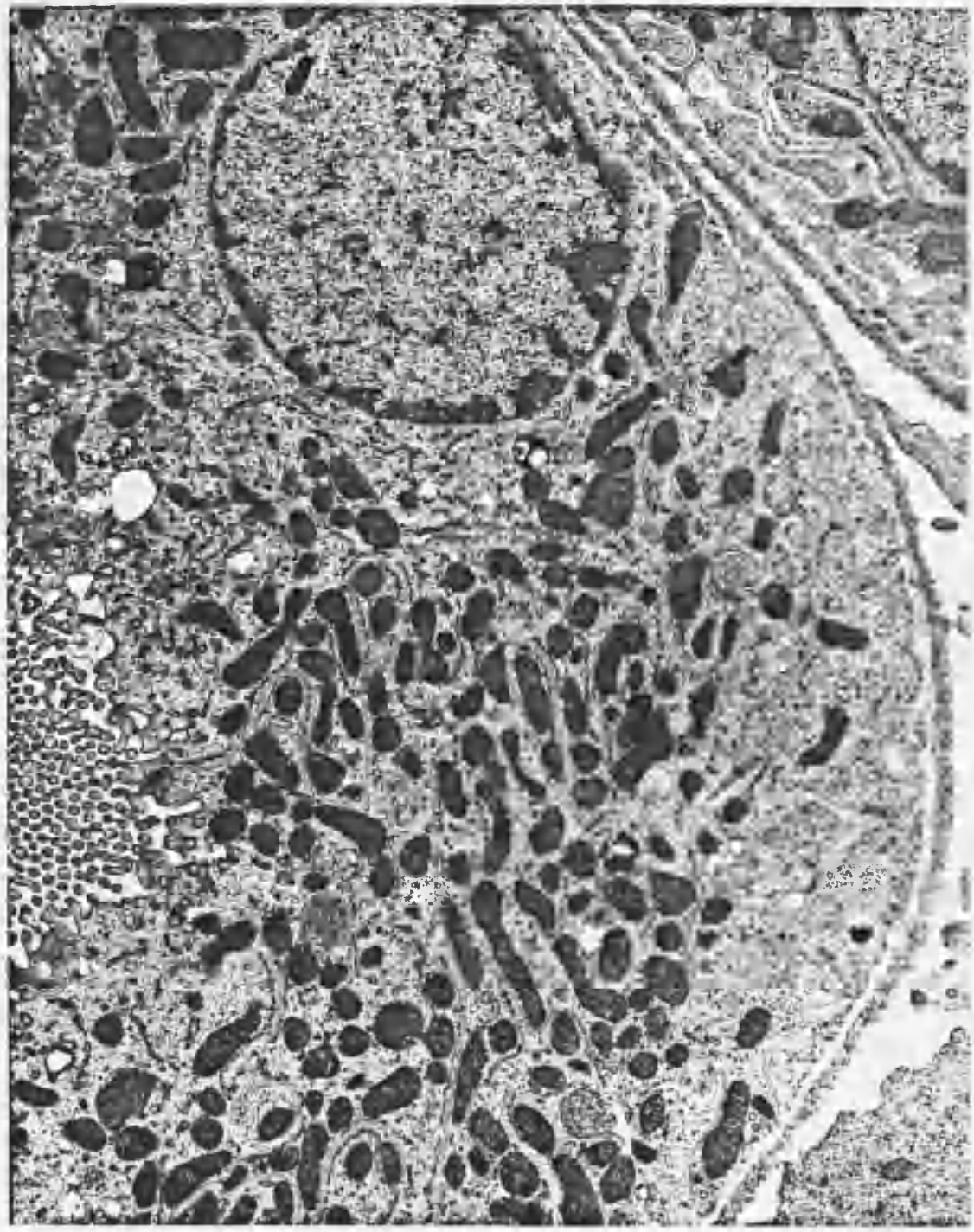

FIGURE 17a- Electron Micrograph of the Normal Isolated Perfused Kidney

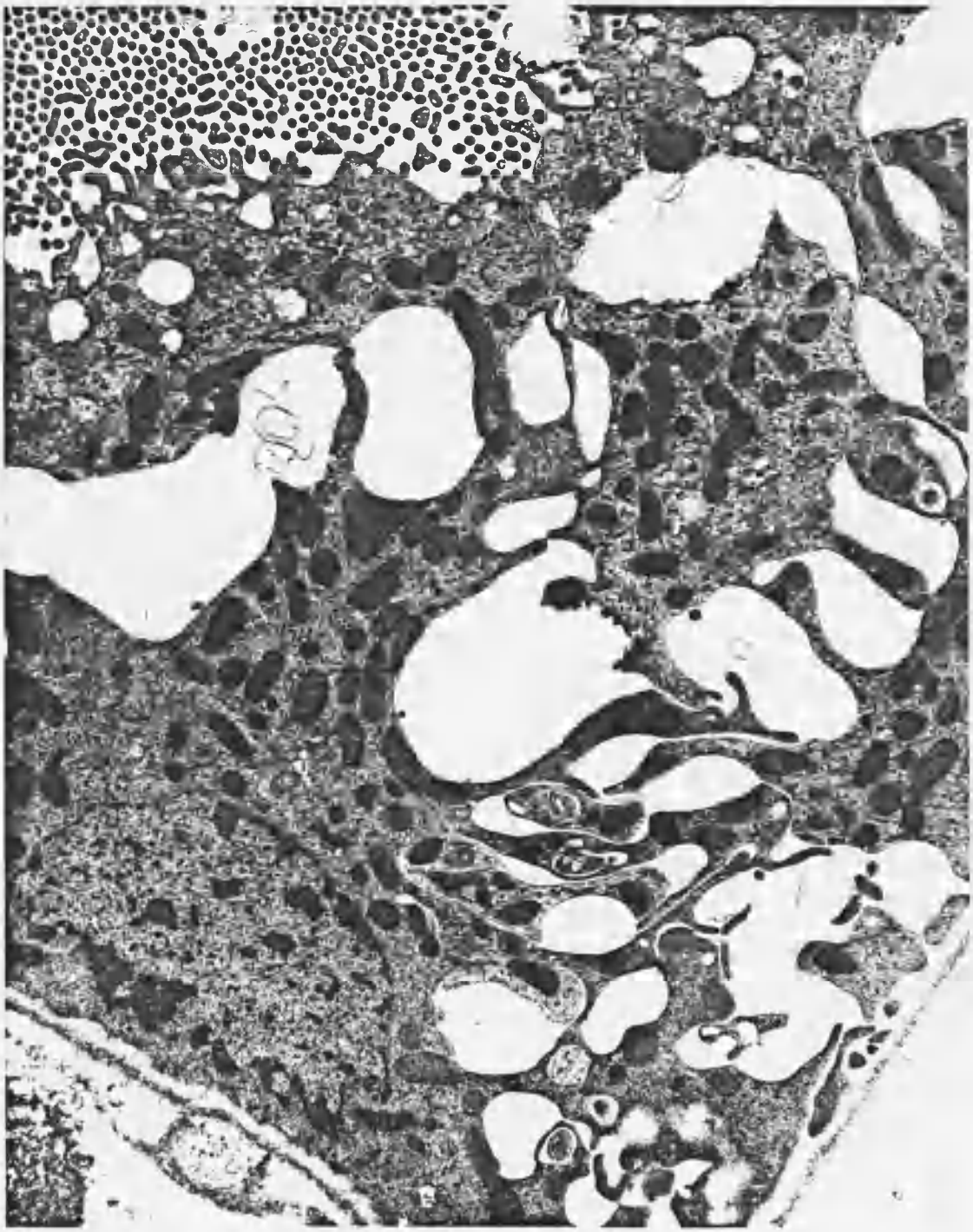

FIGURE 17b - Electron Micrograph of the Platinum Treated Isolated Perfused Kidney

compounds, except that no electron dense deposition was seen in our preparations. Results of the analyses of the collected urine are presented in Table 2. The perfusate contained concentrations of 1 gm/L of glucose and 2 mg/100 ml of phenol red. Values are expressed in percent reabsorption and percent secretion respectively. The slight improvement in renal function seen after two hours was attributed to the release of inhibition via autoregulation in the intact kidney. The administration of papaverine and vasodilan gave the same results. Increase of vascular flow rates presumably improved the passage of solutes from the extracellular compartments.

Structural alterations of kidney tissue as a result of lethal doses of cis-DDP poisoning were found to be in agreement with chronic studies done at sublethal doses. The juxtamedullary zone of the medulla, which is the general site of major portions of the proximal convoluted tubules, was injured preferentially by the drug. The reversibility in tubular damage seen in compensatory growth indicates a poisoning by sublethal doses of platinum. This is considered a chemical method for damaging kidney tissue while leaving a portion of the tissue capable of functioning. The observation of both severely damage and apparently uninjured secretory epithelium in kidneys during lethal doses of cis-platinum further supports this concept.

Renal function following smaller doses of cis-DDP as monitored by the reabsorption of glucose and extraction of phenol red show no significant impairment. Morphologic damage to the epithelium appeared minimal. Although these epithelial cells may not have been rendered incapable of function by the metal complex, studies performed with the isolated tubules

Table 2: Relative Reabsorption of Glucose & Removal of Phenol Red by the Perfused Rabbit Kidney

Treatment[a]	Percentage of reabsorbed glucose in 5 min collection	Percentage of normal removal of phenol red in 5 min collection
Control, 1 hr	68	100
Control, 2 hr	72	100
Control, 3 hr	79	100
cis-DDP (1 mM) 1 hr	60	100
cis-DDP (1 mM) 2 hr	48	71
cis-DDP (1 mM) 3 hr	45	15

[a]Kidneys were obtained by the same rabbit and run simultaneously. The circulatory perfusate was changed every 30 minutes in both reservoirs; the treated kidney received 1 mM of cis diaminedichloroplatinum in the perfusate.

support the formation of an active metabolite. The limited time of drug exposure accounted for the lack of toxicity and cytologic changes.

The functional studies in the course of events following platinum's administration were examined. Renal tubular necrosis or acute renal failure was the result of several mechanisms that apparently came into play and are the cause of these. The dosage of platinum seemed to be an important factor in determining these factors.

CHAPTER IV

CONCLUSIONS

The interaction of cis-DDP with DNA has accounted for the anti-tumor action seen in Phase II clinical trials, while the mechanism by which cis-DDP elicits tubular necrosis still remain to be elucidated. It is likely that the concentrating process of the kidney tubules play a large role in determining the sites of toxicity. The formation of an active metabolite with elevated levels of platinum permit its sequestering by the kidney, eliciting the response of hypertrophy, brush border loss, and the eventual irreversible necrosis.

The amount of cis-DDP needed to arrest cell division is incompatible with tubular function. Derivatives such as the uracils and pyrimidines need further investigation, since tubular function can withstand higher doses of these compounds. Combination therapy with diuretics has prolonged platinum's toxic effect and this has encouraged clinical trials. The success of platinum in cancer therapy will rely heavily on the prevention of the cytotoxic effects seen in the kidney.

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