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CISPLATIN NEPHROTOXICITY:

IN VITRO STUDIES

by

Jennifer Suzanne Phelps

A Thesis Submitted to the Faculty of the
DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY
In Partial Fulfillment of the Requirements
For the Degree of
MASTER OF SCIENCE
WITH A MAJOR IN TOXICOLOGY

In the Graduate College
THE UNIVERSITY OF ARIZONA

1986
STATEMENT BY AUTHOR

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APPROVAL BY THESIS DIRECTORS

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Nov. 17, 86
Date

4/18/86
Date
Dedicated to my parents, Pharo and Lois
and to Pharo Thomas, Andrew and John
who have always supported me in my
educational endeavors
ACKNOWLEDGEMENTS

I would like to thank the members of my committee for their assistance and guidance in completing this project. Thanks to Dr. Jay Gandolfi for his financial support and enthusiasm and to Dr. Klaus Brendel for his enlightening discussions and love of learning. Also, thanks to Dr. Bob Dorr for providing the initial idea and unending encouragement.
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>List of Illustrations</th>
<th>vii</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Tables</td>
<td>ix</td>
</tr>
<tr>
<td>Abstract</td>
<td>x</td>
</tr>
</tbody>
</table>

1. Introduction

- Chemistry .......................... 2
- Pharmacokinetics .................. 4
- Chemotherapeutic Uses ............ 7
- Antineoplastic Action ............ 8
- Toxicities ........................ 9
- Renal Toxicity Effects .......... 10
- Mechanisms ......................... 12
- Protection of Cisplatin Nephrotoxicity .......................... 13
- Mannitol .......................... 14
- Sulphhydryl Agents ................ 15
- General Kidney Toxicity .......... 16
- In Vivo Systems .................... 17
- In Vitro Systems ................... 18
  - Isolated Perfused Kidney ....... 18
  - Proximal Tubule Systems ....... 19
  - Renal Cortical Slices .......... 21
  - Renal Membrane Vesicles ....... 23
- Purpose of Study .................. 24

2. Materials and Methods

- Animals ................................ 26
- Buffer and Incubation Medias ...... 26
- Chemicals ............................ 27
  - Platinate Compounds ............. 27
- Tissue Preparation ................ 30
  - Cortical Slices ................. 30
  - Proximal Tubules ............... 31
- Incubation Methods ........ ....... 32
  - Cortical Slices ................. 32
  - Proximal Tubules ............... 34
TABLE OF CONTENTS--Continued

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay Procedures</td>
<td>35</td>
</tr>
<tr>
<td>Cortical Slices</td>
<td>35</td>
</tr>
<tr>
<td>Histology</td>
<td>39</td>
</tr>
<tr>
<td>Proximal Tubules</td>
<td>40</td>
</tr>
<tr>
<td>Statistics</td>
<td>44</td>
</tr>
<tr>
<td>3. RESULTS</td>
<td>45</td>
</tr>
<tr>
<td>Cortical Slices</td>
<td>45</td>
</tr>
<tr>
<td>Cisplatin Effects on Cortical Slices</td>
<td>45</td>
</tr>
<tr>
<td>Proximal Tubules</td>
<td>57</td>
</tr>
<tr>
<td>Cisplatin Effects on Cortical Slices</td>
<td>57</td>
</tr>
<tr>
<td>Examination of Analogues in the Slices</td>
<td>60</td>
</tr>
<tr>
<td>Iproplatin</td>
<td>63</td>
</tr>
<tr>
<td>Carboplatin</td>
<td>63</td>
</tr>
<tr>
<td>Examination of Potential Protective Agents</td>
<td>68</td>
</tr>
<tr>
<td>Mannitol</td>
<td>68</td>
</tr>
<tr>
<td>Glutathione</td>
<td>68</td>
</tr>
<tr>
<td>4. DISCUSSION</td>
<td>72</td>
</tr>
<tr>
<td>Overview of Cisplatin Nephrotoxic Models</td>
<td>72</td>
</tr>
<tr>
<td>Precision Cut Slices</td>
<td>73</td>
</tr>
<tr>
<td>Methodology</td>
<td>73</td>
</tr>
<tr>
<td>Evaluation of Cisplatin Effects in Slices</td>
<td>75</td>
</tr>
<tr>
<td>Isolated Proximal Tubules</td>
<td>77</td>
</tr>
<tr>
<td>Comparison of Slices and Tubules</td>
<td>79</td>
</tr>
<tr>
<td>Analysis of Viability Parameters</td>
<td>80</td>
</tr>
<tr>
<td>Examination of Cisplatin Nephrotoxicity</td>
<td>82</td>
</tr>
<tr>
<td>Evaluation of Cisplatin Analogues</td>
<td>85</td>
</tr>
<tr>
<td>Evaluation of Potential Protective Measures</td>
<td>88</td>
</tr>
<tr>
<td>Summary</td>
<td>90</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>92</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>95</td>
</tr>
</tbody>
</table>
### List of Illustrations

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Chemical structure of cisplatin and transplatin</td>
<td>3</td>
</tr>
<tr>
<td>2.</td>
<td>Chemical reactions between cisplatin and water</td>
<td>5</td>
</tr>
<tr>
<td>3.</td>
<td>Chemical structure for iproplatin, an analogue of cisplatin</td>
<td>28</td>
</tr>
<tr>
<td>4.</td>
<td>Chemical structure for carboplatin, an analogue of cisplatin</td>
<td>29</td>
</tr>
<tr>
<td>5.</td>
<td>Uptake of platinum in cortical slices</td>
<td>46</td>
</tr>
<tr>
<td>6.</td>
<td>Intracellular LDH content of cisplatin treated slices</td>
<td>48</td>
</tr>
<tr>
<td>7.</td>
<td>Intracellular K⁺ content of cisplatin treated slices</td>
<td>49</td>
</tr>
<tr>
<td>8.</td>
<td>Intracellular ATP content of cisplatin treated slices</td>
<td>51</td>
</tr>
<tr>
<td>9.</td>
<td>Oxygen consumption of cisplatin treated slices</td>
<td>52</td>
</tr>
<tr>
<td>10.</td>
<td>Photomicrographs of a control slice after 18 hr in culture</td>
<td>53</td>
</tr>
<tr>
<td>11.</td>
<td>Photomicrographs of a cisplatin treated slice (10⁻⁵M) treated slice after 18 hr in culture</td>
<td>54</td>
</tr>
<tr>
<td>12.</td>
<td>Photomicrographs of a cisplatin treated slice (10⁻⁴M) treated slice after 18 hr in culture</td>
<td>55</td>
</tr>
<tr>
<td>13.</td>
<td>Photomicrographs of a cisplatin treated slice (10⁻³M) treated slice after 18 hr in culture</td>
<td>56</td>
</tr>
<tr>
<td>14.</td>
<td>Leakage of LDH of cisplatin treated proximal tubules</td>
<td>58</td>
</tr>
<tr>
<td>15.</td>
<td>Intracellular K⁺ content of cisplatin treated proximal tubules</td>
<td>59</td>
</tr>
<tr>
<td>16.</td>
<td>Intracellular ATP content of cisplatin treated proximal tubules</td>
<td>61</td>
</tr>
</tbody>
</table>
LIST OF ILLUSTRATIONS--Continued

17. Organic ion accumulation of cisplatin treated proximal tubules.......................... 62
18. Intracellular K⁺ content of iproplatin treated slices..... 64
19. Intracellular ATP content of iproplatin treated slices.... 65
20. Intracellular K⁺ content of carboplatin treated slices.... 66
21. Intracellular ATP content of carboplatin treated slices... 67
22. Intracellular K⁺ content of cisplatin-mannitol treated slices................................ 69
23. Intracellular K⁺ content of cisplatin-GSH treated slices.. 71
<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Comparison of c x t products for Pt analogues using intracellular $K^+$ and ATP endpoints</td>
<td>87</td>
</tr>
</tbody>
</table>
ABSTRACT

Cisplatin nephrotoxicity was examined in two in vitro systems, precision cut cortical slices and isolated proximal tubules. Ultra-thin, reproducible and uniform slices were mechanically cut and cultured for 18 hr at 37°C. Cisplatin treated cortical slices demonstrated a curvilinear uptake of platinum. Dose- and time-response for clinically relevant concentrations of cisplatin (10^{-3}M - 10^{-5}M) was apparent for four indices of viability including intracellular K^+, ATP, LDH and histopathology. Non-nephrotoxic transplatin was not absorbed by slices and caused only decreased intracellular ATP. Viability parameters were evaluated and intracellular K^+ was determined to be the optimal indicator due to reliability, sensitivity, and ability to predict cisplatin nephrotoxicity. Other platinum analogues were examined and also demonstrated a dose- and time-response for both intracellular K^+ and ATP. Non-enzymatically obtained, cisplatin treated, proximal tubules, the second in vitro system assessed, demonstrated no toxic change over a 6 hr incubation at 37 °C.
CHAPTER I

INTRODUCTION

The first inorganic anticancer drug introduced in the armamentarium of antineoplastic agents, was a platinum containing molecule, cis-diaminedichloro platinum or cisplatin (Zwelling and Kohn, 1982). Cisplatin has been a remarkably successful antineoplastic agent in a number of human neoplasms (Einhorn and Donahue, 1977; Prestayko et al, 1979; Zwelling and Kohn, 1982). Unfortunately, the drug does not provide solely beneficial effects; toxic effects have been severe and warrant attention, with the major toxic concern in the kidney (Harker, Stone and McCoy, 1974; Krakoff, 1979; Jacobs et al, 1980; Letterst, 1984; Bitran et al, 1982; Buamah et al, 1982). The scientific and medical communities have made great efforts to develop new dosing regimens and analogues of cisplatin in an attempt to arrive at a therapy with high antineoplastic activity and low toxicity; however cisplatin remains the drug of choice for many cancers. Experimental examination of cisplatin's nephrotoxicity has been primarily through clinical and in vivo studies. However, disadvantages exist for in vivo studies; large numbers of animals are required and experimental parameters are not easily manipulated. In vitro systems eliminate these problems and allow for screening of analogues prior to in vivo and clinical studies as well as enabling more intricate mechanistic
studies. To date, in vitro experimental kidney systems for the examination of cisplatin toxicity have been limited. Therefore, an in vitro model is needed to aid in the elucidation of the mechanism of cisplatin nephrotoxicity as well as aid in the search for new analogues of cisplatin. This study will examine the cytotoxicity of cisplatin in isolated renal proximal tubules and precision-cut renal cortical slices.

Chemistry

Cisplatin is a planar molecule comprised of a central platinum(II) atom with 2 chlorine atoms and 2 ammonia molecules in a cis configuration (Figure 1). Platinum is a transition metal containing eight electrons in the outer d shell. Nickel and palladium, also containing eight electrons in their outer d shell, show no antitumor activity. Due to the fact that Pt contains a larger number of electrons than Pd or Ni, Pt is able to form stronger covalent bonds which are purported to account for the antineoplastic activity of platinate compounds (Zwelling and Kohn, 1982). Some analogues of cisplatin exist as platinum(IV) and are thought to first degrade to a platinum(II) state before exerting their antineoplastic action (Cleare and Hoeschele, 1973).

Active platinum coordination compounds consist of 2 carrier ligands (NH₃ for cisplatin) and 2 leaving groups (Cl⁻ for cisplatin) (Cleare and Hoeschelle, 1973). The most important biological reaction involves displacement of the leaving groups. Stability of the leaving groups affects the antitumor activity; if substitution of a leaving
Figure 1. Chemical structure of a) cis-diaminedichloro platinum (cisplatin) and b) trans-diaminedichloro platinum (transplatin).
group occurs too rapidly, the compound may never reach the desired site. On the other hand, substitution may be nearly impossible if the "leaving group" is bound tightly, as with cyanide (Thomson, Williams, Reslova, 1972). Stability increases with decreasing electronegativity (I- > Br- > Cl-) with the exception of NH$_3$ and OH- which form rather strong bonds. Ligand replacement reactions also depend upon the identity of other ligands in the complex.

General consensus contends that the hydrated platinum species plays the major role reacting with important macromolecules within the cell (Litterst, 1984; Prestayko et al, 1979). The Pt-Cl bond is stronger than the Pt-H$_2$O bond, but the bond stability is greatly influenced by the concentrations of Cl or H$_2$O. Figure 2 shows the reactions of cisplatin and water. The chemistry within a biological system will be addressed later.

Pharmacokinetics

Cisplatin is administered most successfully by intravenous dosing in isotonic NaCl solution to avoid hydration of cisplatin. Care must be taken when administering cisplatin with divalent metals (ie. aluminum) because Pt(II) goes through oxidation displacement reactions with divalent metals (Prestayko, Cadiz, and Crooke, 1979).

Administration of radioactive cisplatin was performed to study the organ distribution of cisplatin in dogs (Litterst et al, 1976; LeRoy et al, 1979) and rats (Manaka and Wolf, 1977). The highest concentrations of cisplatin were found in the kidney, liver, skin, gonads, spleen and adrenals 1 to 2 hr after cisplatin injection. Six
Figure 2. Chemical reactions between cisplatin and water (Harrison and McAuliffe, 1978).
days post cisplatin treatment, the concentrations remained elevated only in kidney, liver, ovary and uterus.

In addition to animal studies, human studies have been performed with $^{195}$Pt-cisplatin using whole body scanning (Lange, Spencer and Harder, 1973; Smith and Taylor, 1974; Manaka and Wolf, 1980; Campbell, Kalman and Jacobs, 1983; Dumas et al, 1985). At 3 hr post administration, highest concentrations were in the kidneys and head regions, excluding the brain. At 40 hr, kidney, liver, and intestines contained the highest radioactivity.

After administration, it is known that cisplatin is converted to other products in the body although the details of these reactions and their products have not been well elucidated (Daley-Yates and McBrien, 1984). Previous studies in man concluded that a biphasic (De Conti, Toftness and Lange, 1974) action occurs. The first phase consisted of a short half life of approximately 25-49 min and a second, slower phase with a half life of 58 to 73 hr. By 3 hr, 90% of the cisplatin has been reported to be protein bound (DeConti et al, 1973).

The kinetics of cisplatin were more accurately described utilizing radiopharmacokinetics. From this, a seven-state system was presented (Manaka and Wolf, 1980) to predict platinum levels in the kidney, urine, blood, and body, in both bound and unbound (mobile) forms. This model suggests that cisplatin is rapidly absorbed by the tissues and binds to macromolecules (DNA and proteins) and that free drug returns from organs to blood. These binding reactions involve the hydrolysis products of cisplatin. The high concentration of chlorine
in the plasma or in the extracellular fluid prevents hydrolysis from occurring outside the cell (Harrison and McAuliffe, 1978).

Based on urinary platinum analysis, excretion of cisplatin is primarily through the kidney in 2 phases (LeRoy et al, 1979). The first phase is rapid (50 ml/min) and the second phase much slower (0.6 ml/min).

**Chemotherapeutic Uses**

In 1965, Rosenberg, VanCamp and Krigas made a serendipitous discovery. In an experiment performed to analyze bacterial growth in an electric current, it was discovered that the electrolysis products from a platinum electrode could cause inhibition of cell division in E. coli while not interfering with growth. Platinum compounds were then tested for their antitumor activity and Rosenberg et al (1969) reported inhibition of sarcoma 180 and leukemia L1210 in mice. The most efficacious compounds tested at this time were cis-Pt(IV)(NH₃)₂Cl₄, cis-Pt(II)(NH₃)(O₂)Cl₂, Pt-(II)NH₂CH₂CH₂NH₂Cl₂, and Pt(IV)(NH₂CH₂CH₂NH₂)Cl₄. The cis compounds are more active than the trans compounds (Cleare and Hoeschele, 1973). Transplatin is neither active antineoplastically nor nephrotoxically.

Cisplatin is effective in many experimental tumors (Rosenberg et al, 1969; Prestayko et al, 1979). In 1972, the National Cancer Institute introduced cisplatin to clinical trials. Cisplatin has since become widely accepted and is used as a primary antineoplastic agent in a number of human neoplasms.
Experimental systems such as sarcoma 180, leukemia L1210 (Rosenberg et al., 1969), B16 melanoma and Walker 256 carcinoma (Kociba and Sleight, 1971) have shown antineoplastic activity with cisplatin. Mice with L1210 leukemia achieved remission for 10 mo post cisplatin administration (Rosenberg et al., 1969). Regression of tumors occurred in 63-100% of animals with sarcoma 180 (Rosenberg et al., 1969). In DMBA induced rat mammary tumors, cisplatin also demonstrated antineoplastic action (Welsch, 1971).

Human neoplasms demonstrating beneficial effects from cisplatin therapy are numerous (Higby, Wallace, and Albert, 1974). The highest antineoplastic activity is seen in testicular cancers (Einhorn and Donahue, 1977). Though non-seminomatous germinal neoplasms account for only 1% of malignant tumors in men, it is the neoplasm with the highest cancer death rate for men age 25-34 (Zwelling and Kohn, 1982). This tumor does not respond to radiotherapy but in combination therapy with cisplatin, vinblastine and bleomycin, 100% response rate with 74% complete remission is reported (Einhorn and Donahue, 1977).

The second most effective use of cisplatin is in ovarian cancers where response rates range from about 30-80% with various single agent and combination therapy regimens (Prestayko et al., 1979). Other tumors responding to cisplatin therapy include bladder, head and neck, and lung, all to a lesser extent than gonadal cancers.

**Antineoplastic Action**

Antineoplastic action is believed to occur through DNA binding. As mentioned before, the hydrolysis reactions are crucial to the anti-
neoplastic action of cisplatin. Stereochemically, transplatin cannot link two adjacent guanine nucleosides in DNA like cisplatin can. Studies have shown that a greater variety of intrastrand crosslinks between bases with one or more intervening nucleotides are formed by transplatin (Pinto and Lippard, 1985). Cisplatin forms the adduct, cis-[Pt(NH3)2 (d(pGpG))], which is accommodated in duplex DNA with a very small and localized disruption of the Watson-Crick base pairing (Kozelka et al, 1985; Hartog et al, 1985; Sherman et al, 1985). It has been proposed by Sherman et al (1985) that these differences in adduct formations result in transplatin adducts being more profoundly disruptive to the duplex DNA, and hence, more easily recognized and repaired by the cell. The cisplatin adducts, however, are so small that the repair mechanisms fail to recognize the error.

Toxicities

Toxicities of cisplatin are wide and varied; nausea, vomiting, renal damage, ototoxicity, peripheral neuropathy, myelosuppression, tetany and allergic reactions have all been reported. All demonstrate a dose-response while nephrotoxicity and ototoxicity also demonstrate a cumulative dose response (Prestayko et al, 1979). In fact, the nephrotoxicity has been the major dose limiting factor of cisplatin therapy.

Of the other reported toxicities, nausea and vomiting are the most severe. In fact, cisplatin has been referred to as the most "barfogenic" drug ever used clinically (Dorr, 1986). Antiemetics, nabilone and prochlorperazine, have been effective in most incidences to control emesis.
Myelosuppression appears to be dose related to cisplatin. Leukopenia, thrombocytopenia and anemia have been seen in 27, 16, and 11%, respectively, of nearly 300 patients (DeConti et al, 1974; Higby et al, 1974). Incidence and severity of myelosuppression are not altered by hydration or diuresis.

Tinnitus and high frequency hearing loss are representative of the aforementioned ototoxic effects of cisplatin. Approximately 11% of the patients treated with cisplatin manifest ototoxicity (Lippman et al, 1973; Prestayko et al, 1979). This toxicity is both dose related and cumulative but some reversibility is possible upon cessation of cisplatin chemotherapy.

Another toxic effect is neurotoxicity, manifested by peripheral neuropathies including paresthesia in upper and lower extremities, tremor, weakness, loss of taste and light headedness (Prestayko et al, 1979). More recently, this has become an area of concern as it appears to be related to cumulative cisplatin doses.

Finally, anaphylactic reactions have been reported but are quite rare (Rozenweig et al, 1977). The reactions seen include facial edema, wheezing, tachycardia and hypotension which occur within minutes after IV infusion.

Renal Toxicity

Effects

Phase I trials reported nephrotoxicity to be the most severe side effect. Both animal studies and clinical studies have demon-
strated this nephrotoxicity which is the major limitation of cisplatin use in chemotherapy.

Cisplatin appears to be directly toxic to renal tubules affecting the proximal tubules most severely, though distal tubule and collecting ducts also appear damaged (Walker and Gale, 1981). Hydropic degeneration in the S3 segment of the proximal tubules presents the earliest tubular change (Dobyan et al, 1980). Following hydropic degeneration, various histopathological changes are seen. These changes include loss of brush border microvilli, mineralization of tubular epithelial cells, granular casts within lumina, disorganization and necrosis (Dobyan et al, 1980; Ward and Faurie, 1976). The most feared side effect of cisplatin chemotherapy is renal failure resulting in death. Upon cessation of drug therapy, regeneration has been seen to occur (Prestayko et al, 1979). No glomerular damage has been seen in either animal or human studies (Madias and Harrington, 1978).

Dogs and monkeys showed renal lesions, azotemia, hypochloremia, proteinuria, increased LDH excretion in urine (Schaeppi et al, 1973). Toxic effects are dose-related and are visible between 55 and 124 d after treatment (Madias and Harrington, 1978).

In testicular cancer treatment, patients normally receive a total dose of 100 mg/m\(^2\) cisplatin per 5 wk which is administered 20 mg for 5 d; this 5 d dosing pattern is repeated every 5 wk. Patients receiving long term cisplatin therapy exhibit regular and persistent decreases in glomerular filtration rate. This is usually visible after
the second 5 wk cycle (Dentino et al, 1978; Prestayko et al, 1979).
Serum creatinine levels also are seen to increase indicating renal impairment.

Mechanisms

Many theories have been proposed to explain the nephrotoxic mechanism of cisplatin. After cisplatin administration, the nuclei, microsomes and cytosol of kidney cells are seen to concentrate platinum (Choie, del Campo and Guarino, 1980). When, cisplatin is added to kidney homogenates in vitro platinum is not shown to concentrate in the nuclear or microsomal fractions (Leh and Wolf, 1976). It is thought that aquation is necessary for cisplatin to become reactive and this may not occur in cell free conditions (Choie et al, 1980). Cisplatin has also been purported to bind to metallothione (Naganuma et al, 1984; Mason and Edwards, 1985; Bakka et al, 1985). The interaction with sulphydryl containing compounds has been the focus of much attention. For example, Litterst and associates (1982) demonstrated only 30% binding of cisplatin to glutathione which suggests that other binding sites may be important in the mechanism of cisplatin nephrotoxicity.

Other binding sites have been suggested by Mason, Hogg, and Edwards (1986). Within the blood of cisplatin treated rats, Mason and associates observed the formation of several low molecular weight Pt-containing fractions. These fractions are rapidly removed by a) urinary excretion b) protein binding and c) kidney uptake. Low molecular weight Pt-containing fractions have also been isolated from the urine;
these fractions have also been observed in the kidney cells as indicated by similar chromatographic properties.

In addition, many enzymes have been examined as possible sites of cisplatin interaction. Enzyme activity has been shown to be inhibited by cisplatin only in prolonged in vitro exposures (Aull et al, 1979). ATPase has been proposed as a possible critical cisplatin interaction site but the studies utilize an unrealistically high dose of cisplatin (Guarino et al 1979; Daley-Yates and McBrien, 1982a). Other enzymes showing inhibition in vitro were again plagued by unrealistic doses (Aull et al, 1979).

Although the earliest histopathologic lesion observed is a swelling of the mitochondria, cisplatin did not effect calcium transport nor mitochondrial oxygen consumption in vitro (Aggarwal et al, 1980).

Many mechanisms have been proposed; however, there is no singular explanation for the renal toxicity of cisplatin. Further elucidation of cisplatin's nephrotoxic mechanism is currently a priority in cisplatin research.

**Protection of Cisplatin Nephrotoxicity**

The first measures to eliminate nephrotoxicity consisted of changing the dosing schedule. Dose fractionation was employed in clinical studies. Einhorn and Donahue (1977) split the typical dose of approximately 100mg/m² into 20mg/m² for 5 days. Toxicity was still observed though it was noticed later than single dose administration. Vigorous hydration was then induced with the cisplatin but toxicity was
only seen to decrease slightly (Einhorn and Donahue, 1977). Vigorous hydration was also examined in the dog (Cvitkovic et al, 1977) when vigorous hydration was used before, during and after dosing in dogs, protection was apparent but again slight. A number of other protection agents were examined including diuretics (Cvitkovic et al, 1977; Hayes et al, 1977; Per and Harder, 1979; Frick et al, 1979; Daley-Yates and McBrien, 1982b; Finley, Fortner and Grove, 1985), probenecid (Ross and Gale, 1979; Jacobs et al, 1984), chloruresis (Earhart et al, 1983; Litterst, 1981), superoxide dismutase (McGinness et al, 1977; Walker and Gale, 1981) and sulphhydril compounds (Borch and Pleasants, 1979; Walker and Gale, 1981; Filipske et al, 1979; Burchenal et al, 1978; Howell and Taetle, 1980; Evans et al 1984; Gale and Atkins, 1981; Howell et al, 1982; Levi et al, 1980; Uozumi et al, 1984). Two of the most promising were mannitol and the sulphhydril compounds, both of which were examined in this nephrotoxic in vitro study.

Mannitol

Animal models demonstrated that prehydration and concomitant osmotic diuresis can prevent serious nephrotoxicity (Cvitkovic, 1977; Holdener et al, 1978; Rainey and Alberts, 1978). Clinical studies were undertaken by Hayes et al (1977) who demonstrated a higher therapeutic index than previously reported. The higher therapeutic index was seen when mannitol (12.5 g in 50 ml water) was administered immediately prior to rapid infusion of cisplatin and followed by 50 ml of 20% mannitol per hour for 6 hr.
Mannitol unexpectedly favors the retention of platinum rather than aiding in excretion (Muggia et al, 1984). The nephrotoxicity of high dose cisplatin is lessened but not eliminated by use of prehydration and mannitol induced diuresis (Hayes et al, 1977).

**Sulphhydryl Reagents**

**Diethylidithiocarbamate (DDTC).** Disulfiram converts to DDTC in lab animals and man thus providing DDTC to the kidney (Borch, Markovitz and Pleasants, 1979). DDTC administered to male F344 rats after LD50 doses of cisplatin reduced nephrotoxic effects (Borch et al, 1979). The antitumor action of cisplatin in rats (Borch et al, 1979) and mice (Gale, Atkins, and Walker, 1982) is not affected.

DDTC can be effective when given after cisplatin rather than before like most other chelating agents indicating that perhaps DDTC is acting as a competitive chelator and removes cisplatin from protein-bound sulphhydryl groups in the kidney (Borch et al, 1979).

**Thiourea, Thiosulfate.** Upon coincubation of thiourea and cisplatin in vitro, DNA crosslinks and lethal lesions have been shown to reverse without the breakdown of DNA (Filipske et al, 1979). This suggested the potential of thiol protection for cisplatin toxicity. Burchenal (1978) demonstrated protection of toxic effects of cisplatin by thiol containing compounds showing dose related decreases of cisplatin induced nephrotoxicity when 800 mg/kg sodium thiosulfate was given immediately after cisplatin administration. However, antitumor activity was also greatly reduced in L1210 leukemia (Howell and Taetle, 1980; Uozumi et al 1984). Sodium thiosulfate possibly reduces toxicity
by direct interaction with cisplatin since the mixing of the two compounds prior to administration renders cisplatin devoid of antitumor activity (Sunderman, 1971).

**General Kidney Toxicity**

Due to the kidney receiving 25% of the cardiac output, large fractions of compounds can cause severe damage to the kidney (Foulkes and Hammond, 1975). The kidney also has the ability to concentrate agents and can be affected by the direct interaction of the agent and the kidney tissue. In addition to direct effects, toxins can also cause indirect alterations in renal function by affecting the renal blood flow.

Processes within the kidney may allow for very high localized concentration of toxins in the kidney. The high rate of metabolic activity of tubular cells and biotransformation leading to toxic metabolites both make the kidney vulnerable to damage. Many sites and functions in the kidney may be affected by a toxin. Changes in the blood flow, glomerular changes and tubular obstruction and proximal tubule destruction may all occur.

The high energy requirement and extensive exposure to toxins in the proximal tubule make it extremely susceptible to chemical insult. Many nephrotoxins are actively transported by proximal tubular cells resulting in high intra-luminal concentrations or high intracellular concentrations. Cell injuries occur through uncoupling of mitochondrial respiratory chain, enzyme activity modification, plasma membrane
interaction and other mechanisms (Porter and Bennett, 1981). Reabsorptive activities and energy dependent secretive activites can also be depressed in the proximal tubule.

**In Vivo Systems**

Nephrotoxicity has traditionally been evaluated by in vivo studies using physiological parameters such as blood urea nitrogen, glomerular filtration rate, urine analysis, and histopathology.

**Cisplatin Studies** Generally, cisplatin nephrotoxicity has been investigated with the use of in vivo rat studies. A typical experiment includes dosing animals just below the LD$_{50}$ dose sacrificing animals at various timepoints ranging from 12 hr - 5 d and examining histopathology. During the experiment, classical in vivo markers were monitored, ie. blood urea nitrogen, serum creatinine, urinary proteins, urinary glucose (Goldstein et al, 1981; Kawamura, Soeda and Yoshida, 1981; Safirstein et al, 1981; Doby any et al, 1980; Leonard et al, 1971; Taylor, Tew and Jones, 1976; Daley-Yates and McBrien, 1976; Braunlich et al, 1984; Jongejan et al, 1986). Platinum levels were sometimes measured in the blood or in the urine (Daley-Yates and McBrien, 1982a; Kawamura et al, 1981; Goldstein et al, 1981). The complexity of the kidney as well as extra-renal factors both serve to mask site specific damage. The development of in vitro systems provide methods for examination of early nephrotoxic events.
In Vitro Systems

Recently, there has been a strong emphasis on developing in vitro systems because of their great advantages over in vivo in greatly reducing the number of animals utilized and extensive costs. In vitro systems are designed to characterize compounds prior to in vivo testing (Kacew and Hirsch, 1981).

There are many sound reasons supporting the development of in vitro systems. Manipulation of system conditions allows for exact potency quantification, thorough mechanistic studies, and specific subtissue response of a compound.

Available in vitro systems include the isolated perfused kidney, isolation of various nephron parts, tissue pieces or slices, cell cultures and purified enzyme systems.

Isolated Perfused Kidney

The most basic in vitro system is the isolated perfused kidney which allows each animal to act as his own control. High dose toxicity problems of whole animals are eliminated. Perfusion pressure and nutrient delivery are readily manipulated providing more intricate experimentation. Disadvantages, however, are also present in the perfused kidney. Retention of viability is only approximately 4 hr, site specific damage is also difficult to pinpoint, and oxygen delivery is poor to the straight proximal tubule. Thus, an appropriate system that can address the study of proximal tubule toxins is needed.
Proximal Tubule Systems

Isolation of the nephron parts for experimental study has been mainly by the efforts of physiologists. The most extensively studied nephron segment has been the proximal tubule. A large portion of the kidney's functions are the responsibility of the proximal tubule (Kluwe, Harrington and Cooper, 1982; Hassall et al, 1983c; Rylander et al, 1986) which serves to reabsorb 60-80% of the glomerular filtrate, 65% of the sodium and chloride, all of the glucose, amino acids, proteins, and bicarbonate (Leaf and Coltran, 1985). The proximal tubule is the site of active hydrogen ion secretion and thus regulates the acid/base balance; it also has carrier sites for active secretion of organic ions in the basolateral and luminal membranes. Gluconeogenesis, ammoniagenesis and cytochrome P450 activity (Rush et al, 1984) are all at their highest point in the proximal tubule.

Proximal tubule fragments from rabbit kidney cortex were first isolated by Burg and Orloff (1962) for the study of transport. The preparation required a collagenase digestion and examined the uptake of para-aminohippuric acid and o-methyl-CH₃-D-glucoside. Various nephrotoxins were examined in this system; neomycin, kanamycin, viomycin, and mercuric chloride all demonstrated renal compromise of PAH transport mimicking in vivo results. Disadvantages to this collagenase preparation include the lack of a basement membrane (Balaban et al, 1980) which potentially modified the exposure of nephrotoxins as well as transport substrates to the plasma membrane. Another disadvantage is
possible contamination of plasma membrane proteins (Hjelle et al, 1981) which were inadvertently digested with the extracellular matrix.

Brendel and Meezan (1975) developed another technique to prepare proximal tubule suspensions that served to overcome some of the previous problems. Proximal tubules were isolated from the cortex of rabbit kidneys by gentle disruption of the tissue followed by differential sieving of the tissue. These suspensions contained approximately 95% animal tubules (Rylander et al, 1986) which still had an intact basement membrane which serves as a support to the tubule as well a permeability barrier. The presence of the basement membrane may play an important role in toxicologic responses and may provide better comparison to in vivo systems. Preparation time is only 45 min compared to collagenase preparation time of 2 hr; viability is extended to 4 hr instead of 60 min for the enzyme derived tubules. Other important features of the proximal tubule preserved in this preparation include epithelial junctions between cells, an intact brush border membrane, gluconeogenesis, PAH and 2-deoxyglucose (a glucose analogue) transport (Brendel and Meezan, 1975).

A variety of nephrotoxins including metals and bioactivated halogenated hydrocarbons have been examined in this non-enzymatically prepared proximal tubule suspension. S-(trans-1,2-dichlorovinyl)-L-cysteine (DCVC), a halogenated cysteine conjugate has shown inhibition of PAH and TEA transport and changes in intracellular potassium and glutathione levels (Hassall et al, 1983a,b; Rylander et al, 1986). Cadmium chloride has also been extensively characterized in this system.
demonstrating alterations from control values in organic ion transport, intracellular potassium, oxygen consumption and glutathione levels (Rylander et al, 1986). The non-enzymatically prepared proximal tubules have retained the essential features of proximal tubules, demonstrate metabolic activity and exhibit toxicity by known xenobiotics. Thus, this proximal tubule suspension system proves itself a viable in vitro experimental system available for the examination of other nephrotoxins.

**Cisplatin Studies.** Guarino (1979) used a modified system of proximal tubules (Forester et al, 1948) to examine the effects of cisplatin and analogues. The tubules were isolated from flounders and were dependent on seasonal availability. These studies examined the transport of PAH and TEA, oxygen consumption, sodium and potassium levels as affected by cisplatin. Cisplatin was seen to affect all of these parameters but the concentrations used were all 1mM and greater. The in vitro systems in this study use concentrations of 1mM and less. Maintenance of these flounder tubules necessitated a 20°C bath, far below normal mammalian body temperature.

**Renal Cortical Slices**

Tissue slices have been employed for a vast number of experimental studies; renal cortical slices have aided in the evaluation of a number of xenobiotics' nephrotoxic action to normal renal function (Bailie Smith and Hook, 1984; Bennett et al, 1978; Berndt and Mehendale, 1979; Berndt, 1976; Kacew and Hirsch, 1981; Kluwe et al, 1982). Most commonly, these studies have employed the use of animals
pretreated with the agent of interest and toxicity evaluated by organic ion accumulation, gluconeogenesis, and oxygen consumption. These parameters have demonstrated sensitivity equivalent to in vivo parameters such as blood urea nitrogen, proteinuria, enzymuria, glucosuria, and glomerular filtration rate (Berndt and Mehendale, 1979; Kacew and Hirsch, 1981). Bach and Lock (1983) demonstrated similar effects between in vivo and in vitro drug exposure. The methods of slicing have limited viability and are not always consistent in sub-tissue content.

A novel method of tissue slicing was developed with the invention of a new mechanical slicer (Krumdieck et al, 1980). This method allowed for numerous slices to be rapidly produced and with great consistency. These precision cut slices were a great improvement over the crude method of hand sliced preparations. The precision cut slices were first successfully employed for the liver (Smith et al, 1984) and then for the kidney (Ruegg et al, 1986). Ruegg et al (1986) demonstrated renal damage due to mercuric chloride, potassium dichromate, and ischemic conditions. Intracellular potassium and histopathology were used to evaluate the results of these in vitro exposures. After 12 hr of incubation in a circulating, aerating support vessel, the slices exposed to mercuric chloride demonstrated injury to the straight portions of the proximal tubules and left the convoluted regions unaffected. Both ischemia and potassium dichromate caused damage to the convoluted portions and not the straight portions of the proximal tubule although the time of damage differed for between the compounds.
These results correlate well with the specific damage seen in the histopathology of \textit{in vivo} studies for these conditions. Thus, this system allows for site-specific damage evaluation. In summary, the precision cut cortical slice provides an excellent reproducible, rapid and inexpensive method to examine the mechanisms of nephrotoxins.

\textbf{Cisplatin Studies.} Renal cortical slices made with a hand held microtome have been examined with cisplatin (Safirstein, Miller and Guttenplan, 1984). Slices were shaken on a water bath and incubated for a maximum of 4 hr. Organic base transport and probenicid both were inhibited by 60 μM cisplatin demonstrating feasibility of this system. However, these are both functional aspects of the slice rather than toxicity aspects. The slices obtained in this study were somewhat crude; the system does not allow for many slices to be obtained rapidly. Four hr may not be long enough to demonstrate toxicity of cisplatin \textit{in vitro}.

\textbf{Renal Membrane Vesicles}

\textbf{Cisplatin Studies.} Purified brush border and basolateral membrane vesicles were used by Williams and Hottendorf (1985) to examine cisplatin effects on transport. Cisplatin was found to inhibit TEA transport in this preparation indicating a functional disability. This preparation does not allow for site specific studies, nor does it allow for prolonged incubation periods.
Purpose of Study

Despite numerous studies using different experimental models, no single mammalian in vitro system has demonstrated the potential for intensive mechanistic studies, rapid analogue screening, and characterization of cisplatin nephrotoxicity.

The purpose of this thesis was to evaluate the potential of precision cut cortical slices and proximal tubule suspensions for increasing the understanding of cisplatin nephrotoxicity and predicting platinum analogue nephrotoxicity. Specific objectives set to fulfill this purpose included the following:

--Evaluation of the cytotoxicity of cisplatin in precision cut renal cortical slices with the use of the following viability parameters:
  a. Intracellular LDH
  b. Intracellular K^+  
  c. Intracellular ATP
  d. Histopathology

--Evaluation of the cytotoxicity of cisplatin in renal proximal tubule suspensions with the use of pre-established viability parameters:
  a. LDH leakage
  b. Intracellular ATP
  c. Intracellular K^+ 
  d. Organic ion transport
Upon evaluation of the above stated objectives, the cortical slices were determined to be an excellent cisplatin nephrotoxicity model. It became necessary to further test the system with potential protective agents and analogues. New objectives that developed included the following:

--Examination of cisplatin analogues
   a. Iproplatinum
   b. Carboplatinum

--Examination of protective agents of cisplatin nephrotoxicity
   a. Mannitol
   b. Glutathione
CHAPTER II

MATERIALS AND METHODS

Animals

Male New Zealand white rabbits were obtained, 800-1000g for tubule preparation and 1200-1500g for slice preparation, from Blue Ribbon Ranch in Tucson, AZ. Animals were housed individually or in double cages with 3-5 animals at 12h/12h light/dark cycles, food (Purina Rabbit Chow) and water were available ad libitum. Rabbits were killed by a shot at the base of the skull from an air pellet gun. This was found to be the most expedient way of preparing renal tubules or slices without contaminating drugs or additional trauma to the animal.

Buffers and Incubation Medias

For preparation of both proximal tubules and cortical slices a buffer containing 120 mM NaCl, 10 mM CH\textsubscript{3}COONa, 5 mM KCl, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, 1.2 mM MgSO\textsubscript{4}•7H\textsubscript{2}O, and 27 mM NaHCO\textsubscript{3} was used. This buffer and all other solutions were made with the use of deionized, distilled water. The pH was adjusted prior to use by bubbling with 95% O\textsubscript{2}/5% CO\textsubscript{2} for 30 min and titrating with 0.1 N NaOH to pH 7.4.

The incubation media for the cortical slices was a serum free mixture of Delbecco's modified eagles (DME) and nutrient mix F-12 (1:1). Phenol red is omitted from the media as it is proposed to
interfere with transport (Berndt, 1976). The media is gassed for 15 min with 95% O₂/5% CO₂ and pH adjusted to 7.4 with NaOH or HCl.

The incubation medium used for the proximal tubules consisted of the above buffer supplemented with 14 mM glucose, MEM essential amino acids (50X; Gibco Laboratories, Grand Island, NY; 10 ml per 500 ml media), MEM vitamins mix (50X; Gibco Laboratories, Grand Island, NY; 10 ml per 500 ml media), and MEM non-essential amino acids (200X; Gibco Laboratories, Grand Island, NY; 2.5 ml per 500 ml media). This medium was used because of its similarities to other kidney cell culture media (Detrisac et al, 1984; Chernian, 1982). The medium was filter sterilized with a Nalgene Filter (Type LS, 0.45; Nalge Co., Rochester, NY.) and stored at 4°C. For each experiment 100 ml was warmed to 25°C, further supplemented with fetal bovine serum (5% volume/volume, Gibco Laboratories, Grand Island, NY), bubbled with 95% O₂/5% CO₂, and adjusted to pH 7.4 with NaOH.

Chemicals

Platinate compounds

Cis-platinum(II) diaminedichloro was obtained from Sigma Chemical Co. (St. Louis, MO) and dissolved in buffer described below. Trans-platinum(II) diaminedichloro (transplatin) was also obtained from Sigma Chemical Co. (St. Louis, MO). Since the transplatin is not as water soluble as cisplatin, it was dissolved in 25% DMSO. Iproplatinum
Figure 3. Chemical structure of iproplatin (CHIP), an analogue of cisplatin.
Figure 21. Chemical structure of carboplatin (CARBO), an analogue of cisplatin.
(CHIP; Bristol-Meyers Company; Syracuse, New York) and carboplatinum (CARBO; Ben Venue Laboratories; Bedford, Ohio) were both dissolved in buffer (Figures 3 and 4).

Glutathione was obtained from Sigma (St. Louis, MO) and dissolved directly in DME/F12 media. DME/F12 media was also used to dissolve mannitol obtained from Mallinckrodt (St. Louis, MO).

Tissue Preparation

Cortical Slices

Cortical slices were prepared using a method developed by Ruegg and associates (1985). A midline incision on the abdomen of a rabbit allowed exposure of the kidneys. Both kidneys were removed from the animal and the capsules removed. The kidneys were then placed into a beaker of slicing buffer. A drill (Model 8050 ValueCraft, Enfield, CT; 300 RPM) with a specially adapted circular bit (6mm inside diameter) was used to bore five cores from each kidney. The cores were oriented cortex to papilla allowing for possible positional slicing (Ruegg et al, 1986). Each core was immediately placed into a fresh beaker of buffer. The cores were processed into slices using a modification of a tissue slicer initially prepared by Krumdieck and associates (1980). This version of the slicer uses a motor-driven vibrating blade and has been designed to rapidly produce slices of similar thickness. Individual tissue cores were placed into a plastic cylinder such that the cortical section would be cut first. A small weighted piston was placed on top of the kidney core. This immobilized cylindrical tissue
holder is then pulled across the vibrating blade (Gillette "Super Stainless", Boston, MA) cutting the tissue perpendicular to the tubular axis in the kidney thus producing slices with short tubular segments. The freshly cut slices are then carried in the circulating buffer to a removable collecting sieve and the first two slices from each core are discarded as they have been subjected much handling. Approximately 10 cortical slices can then be obtained; 5-7 slices are kept and stored in ice-cold oxygenated (95% O₂/5% CO₂) buffer until ready for incubation.

Proximal Tubules

Proximal tubules were isolated from the kidney cortex of rabbits by a non-collagenase digestion (Brendel and Meezan, 1975) as modified by Hassall and associates (1983a). The abdomen was opened with a midline incision and the right kidney was exposed, cannulated and removed. The kidney was then perfused by gravity flow of the perfusion buffer from a height of 2 m. The left kidney was then similarly exposed, cannulated and removed while the right kidney was being perfused. The right kidney was then removed and placed in a beaker of fresh perfusion buffer while the left kidney was gravity perfused for 5 min. The capsules of both kidneys were removed prior to the gravity perfusion. Next, the kidneys were attached to a non-recirculating perfusion apparatus and perfused at a pressure of 120 mm Hg for 10 min with oxygenated perfusion buffer at 37°C. The cortex was gently plucked off after perfusion and placed into a hand homogenizer containing 10 ml of buffer. Ten strokes of a loosely-fitting tapered teflon pestle was used to homogenize the cortex. A 210 micron sieve
was used to filter the homogenate; 700 ml of buffer was sprayed to wash the homogenized cortex tissue. The filtrate was poured onto a 64 micron sieve and washed with 300 ml of buffer. Using a spray of incubation media, the tubules were collected into a corner of the sieve and then washed into a beaker containing the incubation media and the volume was brought up to 100 ml with incubation media.

**Incubation Methods**

Cortical Slices

Slices were prepared as mentioned previously, and placed in ice-cold oxygenated (95% O<sub>2</sub>/5% CO<sub>2</sub>) buffer in between preparation and incubation. Individual slices were removed with the bent end of a spatula and placed onto stainless steel mesh cylinders which had two stainless steel wheels (1.6 cm O.D., 0.16 cm wall; The Micro Group, Medway, MA). The stainless steel cylinders were made from a 250 micron pore size screen (Tetco Inc., Monterey Park, CA) cut into 3 cm x 2 cm rectangular pieces which were placed into the wheels; two millimeters of screen protruded off either end of the cylinder and was bent out in order to hold the wheels in place. After a slice was placed in the cylinder, the cylinder was removed from the buffer, blotted on tissue paper and loaded into a glass scintillation vial with 1.7 ml of the incubation media containing vehicle or platinum analogues.

**Platinate treated slices.** Each vial contained 1.5 ml of media (gassed for 15 min with 95%O<sub>2</sub>/5%CO<sub>2</sub> and titrated with NaOH to pH 7.4; 0.1% penicillin) and 0.2 ml of platinate compound or vehicle. Cispla-
tin was serially diluted to deliver 0.2 ml of compound for a final concentration range of $10^{-2} \text{M}$-$10^{-6} \text{M}$. transplatin (0.2 ml) was added for a final concentration of $10^{-3} \text{M}$. Analogues, iproplatin (CHIP) and carboplatin (CARBO), were serially diluted to deliver 0.2 ml of compound for a final concentration range of $10^{-2} \text{M}$-$10^{-5} \text{M}$). Mannitol and glutathione treated slices will be detailed below. Vials were gassed by placing the uncapped vial into a plastic box saturated with 95% O$_2$/5% CO$_2$ for 15 sec. Immediately, the vials were capped and horizontally placed onto a vial rotater (1.4 rpm) at 37°C, housed in a black acrylic box. The slices were removed for regassing every 2 hr during the first 6 hr and every 4 hr thereafter. Slices were removed for analysis after 6, 12, 18, and 24 hr exposure to test compound or vehicle.

**Mannitol Effects.** Mannitol (4 mM final concentration) and penicillin (0.1%) were added directly to the DME/F12 media; the media was bubbled with 95%O$_2$/5%CO$_2$ for 15 min and titrated to pH 7.4 with NaOH. Aliquots (1.5 ml) of the mannitol treated media were added to scintillation vials and 0.2 ml of cisplatin (final concentration of $10^{-3} \text{M}$) or vehicle added for a final volume of 1.7 ml. Slices were placed on metal screen inserts inside the vials, regassed as above and removed at 6, 12, 18, and 24 hr for intracellular potassium analysis.

**Glutathione Effects.** Glutathione and penicillin were added directly to the media which was then gassed as above and brought to pH 7.4. The glutathione ($10^{-2} \text{M}$) and penicillin (0.1%) containing media (1.7 ml) was added to vials; slices were then placed in the metal
screen inserts inside the vial. After 1 hr, the media was removed and fresh media (1.5 ml) and cisplatin (0.2 ml; final concentration of $10^{-3}$ M) or vehicle (0.2 ml) added. Slices were regassed as described above and removed for intracellular potassium analysis after 6, 12, 18, and 24 hr of exposure to cisplatin.

Proximal Tubules

Glass scintillation vials were silanized (Prosil-28, SCM Chemicals, Gainesville, FL) and two ml aliquots of proximal tubules suspensions were placed into the vials. The pipette tip used for this distribution was beveled with a razor blade and then the cut edges were polished with a quick wave through a flame; this minimized traumatization of the tubules. The vials receiving tubules earliest were used for the longest incubation times. All tubules were distributed immediately prior to incubation. After addition of vehicle, cisplatin (final concentration: $10^{-6}$ M-$10^{-3}$ M) or transplatin (final concentration: $10^{-3}$ M), the vials were swirled and capped with teflon-silicon capliners (Tuf-Bond Discs, Pierce Chemical Co., Rockford, IL) and vial caps which had a single hole drilled were used to hold the capliners in place. A 25 gauge needle was inserted through the capliner to oxygenate the tubules with 95% O$_2$/5% CO$_2$ and a second 25 gauge needle was used to prevent pressure build-up. As the oxygen line had six outlets, six vials were oxygenated at a time. After 3 min of oxygenation, the vials were loaded horizontally onto a heated 37°C vial rotater set at 1.4 rpm. In order to minimize temperature fluctuations, the vial rotater was housed in an acrylic plastic box. The vials were re-
oxygenated with 95% O₂/5% CO₂ for 3 min every 2 hr. Samples were removed for biochemical analysis after 1, 2, 4, and 6 hr of incubation. Methods for organic ion transport study are described below.

Assay Procedures

Cortical Slices

**Platinum Content of Slices.** Platinum content of slices was measured over the incubation time by flameless atomic absorption spectrometry (Litterst et al, 1984). Slices were treated as described in the platinate treated section above and removed at 6, 12, 18, and 24 hr, rinsed twice with buffer, then blotted with tissue paper and weighed. Only cisplatin 10⁻³M and transplatin 10⁻³M were examined. The slices were then digested in aqua regia (concentrated HCl and concentrated HNO₃ 1:1) overnight, diluted and measured by a spectrometer (Instrumentation Laboratories Video 12 Atomic Absorption Spectrophotometer equipped with a model 655 graphite furnace and Fastac II Sampling Accessory; Allied Analytical Systems, Waltham, MA) equipped with a platinum hollow cathode lamp (Starna Cells Inc., Atascadero, CA). Quantification of the samples was by the use of linear regression performed on a standard curve (10 - 100 ppb) made from a stock solution of PtCl (Alfa Products, Danvers, MA). Values were reported as ppm Pt/mg slice.

**DNA Content.** The slice was removed from the vial, blotted on tissue paper, and placed in a microfuge tube containing 0.5 ml of water. The slice was sonicated with ten pulses or the necessary number
to completely disrupt the tissue. Aliquots (400 µl) were removed for K⁺ and DNA analysis; and, the remaining 100 µl were used for protein and LDH analysis. The pellet remaining after the supernatent fraction for potassium analysis was used for the analysis of DNA. DNA was used to normalize the biochemical analyses. Ice cold acid alcohol (3.6 ml of 100% ethanol and 0.1 ml of concentrated HCl) was added to the pellet and the tubes were placed on a horizontal shaker for 30 min. The tubes were next placed upright in 4°C for 2 hr and centrifuged for 10 min at 3000 rpm. The supernatent fraction was discarded and the pellets were dried overnight in a hood. Next, diaminobenzoic acid (0.1 ml; 30% w/v) was added to the samples, the tubes were capped and placed in a water bath (70-80°C) for 35 min. The caps tended to "pop off" but were replaced immediately. The tubes were removed and allowed to cool to room temperature. HCl (1.0 ml; 1.0 N) was added to the tubes and the DNA content measured by fluorescence (Aminco-Bowman Spectrophotofluorometer, Silver Spring, MD), excitation at 410 nm; emission at 500 nm. A standard curve (0.5-20 µg DNA) was made from a 0.1 mg/ml stock solution of DNA from calf thymus (Sigma, St. Louis, MO) in ammonium hydroxide (1.0 N). DNA values were calculated from this standard curve and expressed as µg DNA.

Protein Content. The protein content was measured using the BioRad method described in the proximal tubule methods section. An aliquot (10 µl) of sample was added to 2.0 ml of BioRad and vortexed. After 15 minutes the absorbance at 595 nm was read. A standard curve (0-1 mg/ml) was made from a stock of bovine serum albumin (Fraction V,
Sigma Chemical Co., St Louis, MO; 0.5 mg/ml) and was used to quantify the protein content in the samples.

**K⁺ Content.** Bovine serum albumin (50 µl; 5 mg/ml solution) was added to the aliquot for K⁺ and DNA analysis. Perchloric acid (20 µl; 70% solution) was then added to precipitate all samples. The samples were then centrifuged at 3000 rpm for 10 min. The supernatent fractions were removed for K⁺ analysis. Flame photometry was used to determine the amount of potassium present. The same procedure was used as described in the methods for proximal tubules.

**ATP Content.** ATP quantification was determined by the luciferase assay (Chappelle, Pecciolo, and Atland, 1967; Gorus and Schram, 1979). Slices were removed at the designated time points and were blotted on tissue paper. The slice was weighed and placed immediately thereafter into 1.0 ml of boiling water in a glass conical tube. The tube was then placed into a boiling water bath and concurrently sonicated until the slice was completely disrupted. The samples were left in the boiling water bath for a total of 10 min at which time they were removed, allowed to come to room temperature and restored to 5 ml volume with water. Aliquots (1.0 ml) were placed into microfuge tubes and the microfuge tubes were stored at -20°C for later measurement. The ATP levels were measured on a Turner Industries Model 25 Photometer (Turner Instruments, Mountain View, CA). Test samples (100 µl) were mixed with 25 µl of luciferase (Turner Industries, Mountain View, CA) and the light output recorded at 550 nm. Light output was compared between a standard solution of ATP and the test sample to determine the
amount of ATP in the sample. The ATP standard was made from mixing a known amount of ATP with 10.0 ml of water.

LDH Content. The LDH commercial kit was revised from the kit used for proximal tubules (previously Sigma No. 266-UV, currently LD-L 10). The new procedure employed the same assay as previously noted; it was less time consuming and required 500 μl of LD-L reagent and only 10 μl of sample which were added into a microcuvette and then inverted before reading. The change in absorbance was then read on a Beckman DU-7 Spectrometer (Beckman Instruments, Fullerton, CA) for 3 min. The temperature of the cell was noted to include a temperature correction factor.

Oxygen Consumption. Slice oxygen consumption was measured with a Clark-type electrode (Yellow Springs Instrument Co., Yellow Springs, OH) attached to a chart recorder (Omniscribe recorder, Houston Instruments, Austin, TX; Brendel and Meezan, 1975). The apparatus was calibrated using buffer bubbled with nitrogen (15 min) and oxygenated buffer (95% O₂/5% CO₂); the calibration was periodically checked.

At the desired incubation endpoints, slices were removed and blotted on tissue paper. Two slices were measured together in a specially adapted basket made of stainless steel screen. This basket was placed into the top of the incubation chamber and had adequate exposure to the buffer as demonstrated by watching the circulation of a drop of dye. The incubation well was heated to 37°C and contained the oxygen electrode. A small stir bar was positioned at the bottom of the well to ensure even distribution of oxygen.
Oxygen consumption was monitored for approximately 5 min. The slices were then removed and blotted and weighed to allow for normalization of values. Oxygen consumption of sample slices was reported as nanoatoms of oxygen per 10 mg (2 slices) of tissue.

Histology

**Embedding.** Slices were removed at the appropriate time point and placed into vials containing 3 ml of a fixative made from 6.5 g sodium phosphate, monobasic, 1.35 g sodium hydroxide, 430 ml water, 20 ml glutaraldehyde 25%, practical grade; J.T. Baker Chemical Co., Phillipsburg, N.J.), and 50 ml formaldehyde (37%, A.R. grade, Mallinckrodt, Inc., St. Louis, MO). The slices were left in fixative (4F-1G) for at least 24 hr. A JB-4 embedding kit (Polysciences, Inc., Worthington, PA) was used for glycol methacrylate embedding. Prior to embedding, tissues were dehydrated in 1 ml glass vials with increasing ethanol concentrations (30%-95%), 15 min at each concentration. Slices were then infiltrated for 4 hr (changing the infiltrate at 2 hr) with infiltrate solution which was a non-catalyzed embedding medium. The infiltrate was then replaced with 1 ml of catalyzed embedding medium and samples were poured into a plastic mold (Polysciences, Inc.) with the slices laying flat on the bottom of the mold. Aluminum chucks (Fisher Scientific, Phoenix, AZ) were placed over the molds to which embedding medium was then added to fill the mold. The samples were placed in the cold room (4°C) and allowed to polymerize overnight.

**Sectioning.** Methacrylate blocks were removed from the mold and rinsed in water. Blocks were then cut into thin sections (2 microns)
with a glass knife (Fisher Scientific, Phoenix, AZ) fitted on a Sorvall JB-4 microtome (Sorvall Instruments, Newtown, CT). The sections were placed on microscope slides and gently heated to fix the methacrylate to the glass slide.

**Staining.** Tissues were stained with a toluidine blue stain. Microscope slides were placed on a slightly hot heating plate and a drop of Toluidine Blue added to cover each tissue. As the edges of the dye turned a metallic color, the slides were removed from the heat and placed under distilled water for approximately 10 min. Slides were allowed to air dry before coverslipping.

**Proximal Tubules**

At the end incubation, a one ml aliquot of tubules was removed from the scintillation vial and placed into a microfuge tube. The microfuge tubes were centrifuged for 5 seconds at 12,500 rpm, and the supernatent fraction removed for LDH analysis. The pellet was retained for $K^+$, ATP, and protein content. One ml of trichloroacetic acid (TCA; 6% in water) was added to the pellet to precipitate the proteins; the samples were vortexed and placed on ice. The samples were sonicated with 10 pulses of a Sonifer cell disrupter adapted with a microtip (Branson Sonic Power Co., Danbury, CT) as soon as possible. Next, the samples were centrifuged for three minutes at 12,500 rpm (Beckman Microfuge B; Beckman Instruments, Palo Alto, CA) at 4°C. The supernatent fraction was removed and stored at 4°C in microfuge tubes for ATP and $K^+$ analysis. The pellets were used for analysis of protein content, and stored at 4°C until the assay was performed.
**Protein Content.** Protein quantification was used to normalize the K⁺, ATP, and LDH values obtained from slices. A commercial kit (BioRad Protein Assay; BioRad Laboratories, Richman, CA) was employed to analyze the total protein content. In this assay, Comassie Blue reacts with proteins in solution creating a quantitative change in color. Samples were dissolved in 500 µl of 0.2 N NaOH (Balalan et al, 1980) and sonicated. A ten-fold dilution was necessary with water. Tris-HCl (0.4 ml of 1.0 M; pH 7.2) was used to neutralize aliquots (100 µl) of the diluted protein samples, BioRad reagent (2.5 ml, diluted 1:4 with water) was added and the samples were vortexed. A standard curve (0-1 mg/ml) made from bovine serum albumin (Fraction V, Sigma Chemical Co., St Louis, MO) was carried through the above steps and used for quantification of the samples. A spectrometer (Gilford Model Staser II; Gilford Instrument Laboratories, Co., Oberlin, OH) was used to measure the absorbance at 595 nm, 20 min after the reagent was added. Linear regression was used to obtain a standard curve and the amount of protein in each sample was extrapolated from this line.

**K⁺ Content.** Flame photometry was employed to examine the K⁺ content. A standard curve was run (0-2 mM KCl in water) before the analysis of the samples to calibrate the instrument as well as enable quantification of the samples. The flame photometer (Model CA-51, Perkin Elmer, Danbury, CT) was set for urine K⁺ analysis. The sample K⁺ concentrations were calculated from the standard curve using linear regression and data was expressed in nmoles K⁺/mg protein.
ATP Content. ATP values were measured using an enzyme-linked assay adapted into a kit (Sigma procedure No. 366--UV; St. Louis, MO). A spectrometer (Acta II, Beckman Instruments, Fullerton, CA) was used to measure the conversion of NADH to NAD (a decrease in absorbance at 340 nm is seen). The amount of ATP present is proportional to the amount of NADH converted to NAD and can be determined with a standard equation. End point analysis was used to maximize the detection of the small amount of ATP present. The assay used microcuvettes which allowed a 10X reduction in reagents. The ATP values were normalized by tubule protein content and data were expressed as nmoles ATP/mg protein.

LDH Content. A colorimetric assay (Sigma kit No. 226-UV; Sigma Chemical Co., St. Louis, MO) was used to determine the Lactate dehydrogenase (LDH) content of the medium. The assay involves the conversion of lactate to pyruvate; the formation of NADH can be used as a proportional value of LDH activity. An absorbance increase can be monitored at 340 nm (ACTA V spectrometer) for the NADH. A standard equation is used to convert the NADH value to LDH activity and the data is reported as International Units per liter.

Organic Ion Transport. Active transport was measured using para-aminohippuric acid (PAH) and tetraethylammonium bromide (TEA) for the quantification of acid transport and base transport, respectively. Fifty µl of substrate mixtures, PAH or TEA were added with a Hamilton syringe (Hamilton Co., Reno, NV) through the septum of the vial containing tubules. The substrate mixtures were made from 0.01 M PAH
(para-[1-14C]-aminohippuric acid, 40.7 mCi/mmol, New England Nuclear, Boston, MA; diluted 1:25 with water; supplemented with non-radioactive PAH) or 0.01 M TEA([1-14C]-tetraethylammonium bromide (4.7 mCi/mmol, New England Nuclear, Boston, MA; diluted with 1:50 with water; supplemented with non-radioactive TEA) and 3H2O (100 mCi/ml; ICN Radiochemicals, Irvine, CA; diluted 1:25 or 1:50 with water; included as a volume marker). Substrates were added 20 min prior to completion of the desired incubation period to allow the organic ion accumulation to reach steady-state (Hassall et al., 1983a). A one ml aliquot of tubules from each sample was removed and placed in a microfuge tube containing 300 µl of silicone oil which allowed for the separation of tubules and media. These microfuge tubes were subsequently centrifuged at 12,500 rpm (Beckman Spinco Model 152 microfuge; Beckman Instruments, Palo Alto, CA) for 5 sec. The tubules formed a pellet on the bottom of the tube while the media remained on top, separated by the silicone oil.

Tissue to media ratios of PAH or TEA (as 14C) were then calculated. The tubule pellet from each sample was placed in a plastic scintillation vial containing 1 ml of 3% Triton X-100 overnight. Aliquots (200 µl) of the media from each sample were placed in another scintillation vial containing 1 ml of 3% Triton X-100. Scintillation cocktail (8 ml) was added the next morning and the vials were analyzed for 14C content and H3 content by a Searle Analytical 81 liquid scintillation counter (Searle Analytic, Des Plaines, IL). The 3H content which was used to correct for the volume differences between media and tubule pellet.
Statistics

Experiments for both tubules and slices were carried out with triplicate samples in all instances. Except where stated, three animals were used to compile values. Data are expressed as mean ± SEM except where stated otherwise. Ambiguous points between control and drug treated samples were compared by the use of a One-way Anova test. When the F-ratio in the Anova test was significant, Fisher's Least Significant Difference Test was applied and points with an F-ratio < .05 were considered to be significantly different.
CHAPTER III

RESULTS

Precision cut slices have demonstrated toxic effects due to ischemic conditions and mercuric chloride (Ruegg et al., 1986); viability was assessed with histopathology and intracellular $K^+$ levels. Isolated proximal tubules have also previously been used to demonstrate toxicity with halogenated hydrocarbons and heavy metals (Hassall et al., 1983; Rylander et al., 1986). Viability markers used to determine damage included organic ion transport, intracellular $K^+$, intracellular ATP, LDH leakage, and oxygen consumption. These two in vitro systems, precision cut cortical slices and isolated proximal tubules, were assessed for their potential as models of cisplatin nephrotoxicity.

Cortical Slices

Cisplatin Effects on Cortical Slices

Platinum slice content was first evaluated for both cisplatin and transplatin. The biochemical integrity of cortical slices treated with cisplatin ($10^{-3}$M - $10^{-6}$M) was examined by looking for changes from control values in oxygen consumption, intracellular potassium, intracellular lactate dehydrogenase, and intracellular ATP. Slices were dosed at time zero and removed for analysis after 6, 12, and 18 hr of incubation.
Figure 5. Uptake of platinum in cortical slices.

Uptake of platinum in cortical slices treated with $10^{-3}$M cisplatin and $10^{-3}$M transplatin. Data are expressed as means ± SEM (n = 3 slices for cisplatin and n = 2 slices for transplatin).
**Platinum Content.** Precision cut slices incubated with high dose (10^{-3}M) cisplatin were examined for their platinum uptake for 18 hr incubation period (Figure 5). Cisplatin appeared to have a possible biphasic uptake with approximately 80 ppm Pt absorbed in the first 12 hr and then by 18 hr approximately 180 ppm Pt absorption by the slice. Thus, a doubling of uptake rate occurs from 12 to 18 hr compared to the first 12 hr of incubation. Transplatin treated slices did not demonstrate any significant uptake of platinum.

**Intracellular LDH.** Decreases in intracellular LDH levels, indicating disruption of the membrane, were seen in cisplatin treated slices (Figure 6). Intracellular LDH levels were normalized to μg DNA present in each slice. Both a time and dose response were seen for a range of cisplatin doses (10^{-3}M - 10^{-5}M). No significant changes were seen at 6 hr of incubation and only the high dose demonstrated a marked change from control after 12 hr. By 18 hr, all doses demonstrated a significant change from control values. Non-nephrotoxic transplatin (10^{-3}M) was added to cortical slices and had no effect on LDH levels over an 18 hr incubation period.

**Intracellular K^+.** Intracellular K^+ levels, normalized to μg DNA, of cisplatin treated slices (10^{-3}M - 10^{-6}M) were measured and again, both dose and time responses were apparent (Figure 7). A low dose of 10^{-6}M showed no deviation from control values after 18 hr. After 12 hr of culture, only the high dose of 10^{-3}M demonstrated a significant change from control. After 18 hr of culture, cisplatin doses (10^{-3}M - 10^{-5}M) all were significantly decreased from control.
Figure 6. Intracellular LDH content of cisplatin treated slices.

Effect of cisplatin (10^{-3}M - 10^{-6}M) and transplatin (10^{-3}M) on intracellular LDH content of cortical slices. Data are expressed as means ± SEM (n = 6 slices; P < .05). Where error bars are not seen, they are contained within the symbol.
Figure 7. Intracellular K⁺ content of cisplatin treated slices.

Effect of cisplatin (10⁻³M - 10⁻⁶M) and transplatin (10⁻³M) on intracellular K⁺ content of cortical slices. Data are expressed as means ± SEM (n = 9 slices; P < .05). Where error bars are not seen, they are contained within the symbol.
Both intracellular LDH and K\textsuperscript{+} levels dropped in a very similar pattern. Transplatin (10\textsuperscript{-3}M) treated slices showed no change from control values over 18 hr.

**Intracellular ATP.** ATP levels of cisplatin treated slices, normalized by the wet weight of each slice, also demonstrated a dose-response and time-response to cisplatin when compared to control levels. The time and dose response to cisplatin for intracellular ATP levels were more dramatic than intracellular K\textsuperscript{+} and LDH (Figure 8). Intracellular ATP evaluation was a more sensitive indicator than intracellular LDH or intracellular K\textsuperscript{+} as both 10\textsuperscript{-3}M and 10\textsuperscript{-4}M demonstrated a significant change from control by 12 hr.

**Oxygen Consumption.** A change in the ability of a cell to consume oxygen might indicate a poisoning of the electron transport chain. Oxygen consumption was examined at the high dose of cisplatin (10\textsuperscript{-3}M) to observe whether oxygen consumption was dropping before the intracellular ATP levels. The high dose of 10\textsuperscript{-3}M cisplatin shows no decrease from control values at 6 hr incubation and then drops rapidly to undetectable levels by 18 hr of culture (Figure 9). Thus, oxygen consumption does not drop before intracellular ATP.

**Histopathology.** The histology of cisplatin treated slices confirmed the results seen in the biochemical parameters. After 18 hours of incubation, a dose-response was apparent for cisplatin treated slices (10\textsuperscript{-3}M - 10\textsuperscript{-5}M; Figures 10, 11, 12, and 13). The patterned response demonstrated cisplatin to first affect the S\textsubscript{1} and S\textsubscript{2} segments of the proximal tubule, and then affecting the S\textsubscript{3} segments. The high
Figure 8. Intracellular ATP content of cisplatin treated slices.

Effect of cisplatin (10^{-3}M - 10^{-5}M) and transplatin (10^{-3}M) on intracellular ATP content of cortical slices. Data are expressed as means ± SEM (n = 3 slices; P < .05). Where error bars are not seen, they are contained within the symbol.
Figure 9. Oxygen consumption of cisplatin treated slices.

Effect of cisplatin (10^{-3}M) on oxygen consumption of cortical slices. Data are expressed as mean ± SEM (n = 3 samples; P < .05).
Figure 10. Although most proximal tubular cells contain increased vacuolization, the nuclei appear healthy with prominent nucleoli. There is good differential staining. Upper photomicrograph is 250X and lower is 100X.
Figure 10. Photomicrographs of a control slice after 18 hr in culture.
Figure 11. Nuclei appear healthy, with prominent nucleoli although most proximal tubules contain increased vacuolization. A few convoluta have pyknotic nuclei. Upper photomicrograph is 250X and lower is 100X.
Figure 11. Photomicrographs of a cisplatin ($10^{-5}$M) treated slice after 18 hr in culture.
Figure 12. Well patterned necrosis affecting the pars convoluta leaving the straight proximal tubules, ascending thicks and collecting ducts within the medullary rays intact. Medullary rays contain good differential staining and healthy nuclei with prominent nucleoli. The convoluted regions show nuclear pyknosis, loss of brush border and loss of cytoplasmic staining. The glomeruli appear relatively intact. Upper photomicrograph is 250X and lower is 100X.
Figure 12. Photomicrographs of a cisplatin (10^{-4}M) treated slice after 18 hr in culture.
Figure 13. All tubular cell types are damaged showing signs of nuclear pyknosis and sloughing of cells from brush border; however basement membranes appear intact. There is also lack of differential staining. The glomeruli appear relatively intact. Upper photomicrograph is 250X and lower is 100X.
Figure 13. Photomicrographs of a cisplatin ($10^{-3}$ M) treated slice after 18 hr in culture.
dose cisplatin demonstrated noticeable vacuolization in the $S_1$ and $S_2$ segments of the proximal tubules after 6 hr of incubation. By 12 hr this dose had destroyed most of the cells in the cortex with the exception of the glomeruli. The middle dose showed some damage to the $S_1$ and $S_2$ proximal tubules by 12 hr but was much more pronounced at 18 hr of incubation. The low dose damage was again in $S_1$ and $S_2$ tubules but slight even after 18 hr.

**Proximal Tubules**

Cisplatin Effects on the Tubules

High dose cisplatin ($10^{-3}$M) effects on proximal tubules were evaluated using the pre-established viability parameters, intracellular $K^+$, intracellular ATP, LDH leakage and organic ion transport.

**LDH Leakage.** LDH, a cytosolic enzyme, is a classic indicator of membrane damage and generally indicates severe cell damage (Goethals et al, 1984). Cisplatin treated tubule suspensions did not show any change in LDH leakage as compared to controls over a 6 hr incubation period. (Figure 14).

**Intracellular $K^+$.** Intracellular $K^+$ can indicate more subtle cell damage than LDH leakage. Changes in intracellular $K^+$ imply a disruption of ionic gradient control (Williams et al, 1981) and has been repeatedly used as an *in vitro* toxicity indicator (Smith et al, 1984; Rylander et al, 1986; Ruegg et al, 1986). Cisplatin ($10^{-3}$M)
Figure 14. Leakage of LDH of cisplatin treated proximal tubules.

Effect of cisplatin ($10^{-3}$M) on LDH release from proximal tubules. Values are means ± SD (n = 9 sample preparations).
Figure 15. Intracellular K⁺ content of cisplatin treated proximal tubules.

Effect of cisplatin (10⁻³ M) on intracellular K⁺ content of proximal tubules. Data are expressed as mean ± SD (n = 9 sample preparations).
added to proximal tubule suspensions did not induce any change in intracellular $K^+\,$ levels as compared to control values (Figure 15).

**Intracellular ATP.** Similar to intracellular $K^+\,$, intracellular ATP changes can also indicate more subtle cell damage than LDH leakage. When tubules suspensions were treated with $10^{-3}\text{M}$ cisplatin, there was no marked change in intracellular ATP levels as compared to controls (Figure 16).

**Organic Ion Transport.** Organic ion transport had proved to be a fairly reliable viability indicator for various known nephrotoxins. Cisplatin effects on transport have previously been examined in other systems (Williams and Hottendorf, 1985; Bird et al, 1980). Thus, organic ion transport was potentially useful for examining the proximal tubule system in cisplatin nephrotoxicity. The high dose cisplatin ($10^{-3}\text{M}$) was examined in the proximal tubule system using PAH and TEA as the organic ion prototypes (Figure 17). Both TEA and PAH accumulation were significantly decreased by 4 and 6 hr of incubation, respectively, in the presence of $10^{-3}\text{M}$ cisplatin. TEA transport was decreased 70% of controls by 4 hr and PAH transport was decreased 89% of controls by 6 hr. However, when non-nephrotoxic transplatin ($10^{-3}\text{M}$) was examined, it was found to inhibit organic ion accumulation in a similar manner to cisplatin. TEA transport was decreased 80% of controls and PAH was decreased 72% of controls by 6 hr.

**Examination of Analogues in the Slice System**

Two analogues of cisplatin were examined in the cortical slices; slice viability was measured by looking for changes in the
Figure 16. Intracellular ATP content of cisplatin treated proximal tubules.

Effect of cisplatin ($10^{-3}M$) on intracellular ATP content of proximal tubules. Data are expressed as mean ± SD ($n = 9$ sample preparations).
Figure 17. Organic ion accumulation of cisplatin treated proximal tubules.

Effect of cisplatin ($10^{-3}$M) on organic ion accumulation in proximal tubules. Data are expressed as means ± SD ($n = 9$ sample preparations).
intracellular potassium and intracellular ATP levels as compared to controls. Intracellular ATP was included as a toxicity indicator because of its potential to detect very subtle changes in cell viability. The analogues, iproplatinum (CHIP) and carboplatinum (CARBO) were added to the media at the start of incubation in the same manner that cisplatin was added and slices were incubated for to 24 hr.

Iproplatin

Iproplatin was added at $10^{-2}$M, $10^{-3}$M, and $10^{-4}$M and demonstrated marked changes in intracellular ATP and intracellular K$^+$ from control values. Intracellular K$^+$ levels of CHIP treated slices ($10^{-2}$M and $10^{-3}$M) both showed decreases from control levels to 72% and 69%, respectively (Figure 18). After 6 hr of incubation, the high dose demonstrated a decrease of 6% from control intracellular ATP levels, while the middle dose demonstrated a decrease of 39% from control ATP levels (Figure 19). The ATP assay appeared to be a more sensitive indicator of toxicity than the K$^+$ assay.

Carboplatin

Carboplatin was also examined and intracellular K$^+$ and ATP levels were compared to control, untreated values over a 24 hour incubation period. Similar to CHIP, CARBO demonstrated a dose-response and time-response in treated slices for both intracellular K$^+$ and intracellular ATP (Figures 20 and 21). The ATP assay was a more sensitive indicator showing a wider spread of the dose response than K$^+$ levels.
**Figure 18.** Intracellular K\textsuperscript{+} content of iroplatin treated slices.

Effect of iroplatin (CHIP; 10\textsuperscript{-2}M - 10\textsuperscript{-4}M) on intracellular K\textsuperscript{+} content in cortical slices. Data are expressed as means ± SEM (n = 3 slices; P < .05). Where error bars are not seen, they are contained within the symbol.
Figure 19. Intracellular ATP content of iproplatin treated slices.

Effect of iproplatin (CHIP; $10^{-2}$M - $10^{-4}$M) on intracellular ATP content of cortical slices. Data are expressed as means ± SEM (n = 3 slices; P < .05). Where error bars are not seen, they are contained within the symbol.
Figure 20. Intracellular $K^+$ content of carboplatin treated slices.

Effect of carboplatin (CARBO; $10^{-2}$M - $10^{-4}$M) on intracellular $K^+$ content of cortical slices. Data are expressed as means ± SEM (n = 3 slices; P < .05). Where error bars are not seen, they are contained within the symbol.
Figure 21. Intracellular ATP content of carboplatin treated slices.

Effect of carboplatin (CARBO; $10^{-2}$M - $10^{-4}$M) on intracellular ATP content of cortical slices. Data are expressed as means ± SEM (n = 3 slices; P < .05).
Examination of potential protective agents

There have been various efforts to manipulate the dosing regimen of cisplatin in order to avoid nephrotoxicity including a change of dose schedule as well as coadministration of other substance. Some of the more successful agents previously examined include mannitol and sulfur containing compounds. Two compounds, mannitol and glutathione were added to the incubation media and examined for their effects on the intracellular K⁺ levels of precision cut slices. Slices were pretreated with glutathione for 1 hr, and mannitol was left in the incubation media for the entire incubation period. Intracellular K⁺ was chosen as an indicator due to its relative sensitivity and convenience of technique.

Mannitol

Mannitol present in the media of control slices demonstrated no visible change from untreated, control slices throughout the entire period of incubation (18 hr). Mannitol (4 mM) was administered to 10⁻³ cisplatin treated slices and was seen to decrease K⁺ 71% more than slices treated with only 10⁻³M CDDP at 6 hours. As seen in Figure 22, throughout 18 hours of incubation, mannitol/10⁻³M cisplatin K⁺ values were lower than the 10⁻³M cisplatin slices.

Glutathione

Glutathione was allowed to preincubate with the slices for 1 hr before administration of the cisplatin, the media was changed at this time and cisplatin was added in a concentration of 10⁻³M; no
Figure 22. Intracellular K⁺ content of cisplatin-mannitol treated slices.

Effect of 4mM mannitol on intracellular K⁺ content in cisplatin (10⁻⁴M) treated cortical slices. Data are expressed as means ± SEM (n = 3 slices; P < .05). Where error bars are not seen, they are contained within the symbol.
significant change was seen from the slices that were not incubated with the glutathione. Slices incubated with glutathione demonstrated no change from control, untreated slices (Figure 23).
Figure 23. Intracellular $K^+$ content of cisplatin-GSH treated slices.

Effect of 10mM GSH on intracellular $K^+$ content in cisplatin ($10^{-3}$M) treated cortical slices. Data are expressed as means ± SEM ($n = 3$ slices; $P < .05$). Where error bars are not seen, they are contained within the symbol.
Overview of Cisplatin Nephrotoxic Models

Very few in vitro investigations of the renal toxicity of cisplatin have been made. Of those that have been developed, flounder proximal tubules, cortical membrane vesicles, and hand cut kidney slices, none have been without major drawbacks.

Cisplatin nephrotoxicity has been examined in a flounder proximal tubule model (Guarino et al, 1979). Flounder tubules necessitate incubation at 20°C and are also not representative of a mammalian system. The system is dependent upon the availability of flounder and appropriate holding facilities.

Another in vitro system used to study cisplatin effects in the kidney is a membrane system (Williams et al, 1985). Brush border and basolateral membrane vesicles were purified, and cisplatin transport was investigated. Cortical membrane vesicles do not encompass the whole intact proximal tubule, the site of cisplatin damage, nor do these vesicles retain the architecture of the kidney cortex. Vesicles may offer clues to transport mechanisms, but are limited when mechanism of toxicity and analogue evaluation are attempted.

Slices obtained from a hand held microtome (Safirstein et al, 1981) and free hand cut slices (Goldstein et al, 1981) have also been
examined, but result in nonhomogeneous slice populations which are susceptible to damage during the cutting process. In addition, these slices are not usually oriented in a way that allows for easy examination of site specific damage. Most commonly slices are taken from animals dosed in vivo disregarding the advantages of true in vitro toxicology. With these problems in mind, further investigation of in vitro systems for cisplatin nephrotoxicity is warranted.

The central theme of this thesis was based on the investigation of new in vitro systems for the analysis of cisplatin nephrotoxicity and for the development of a cisplatin analogue screen. Cisplatin's nephrotoxicity was examined by using a variety of viability indices in both precision cut cortical slices and isolated proximal tubules.

**Precision Cut Slices**

Methodology

A number of indicators in the precision cut slice indicated toxicity due to cisplatin. To date, this is the first in vitro system to demonstrate a dose-response and time-response toxic action due to cisplatin.

Methodology for precision cut slices was developed and these new slices demonstrated superior viabilities than those reported for hand cut slices. In this system mercuric chloride regional damage and ischemia induced selective damage was shown (Ruegg et al, 1986). Initially these slices were incubated in a glass support vessel. Due to the limited availability of the glass support vessels, a roller culture
incubation system which had been successful for liver slice culture (Smith et al., 1985) was used.

This system is advantageous because it allows for both sides of the slice to be exposed to a gas phase during the incubation period. This is achieved by placing slices on the inside wall of a stainless steel mesh cylinder that rotates inside a glass scintillation vial. The glass scintillation vials are maintained at a constant temperature and slowly rotate on a 37°C vial rotator.

The instrument used in the preparation of the kidney slices was demonstrated to produce highly uniform and viable liver slices (Smith et al., 1985) and was adapted to be used for kidney slices. The instrument allows for approximately 100 slices to be cut within 15 min. The reproducibility and uniformity of the slices was previously examined by Ruegg and associates (1986) and found to be approximately 300 μm ± 10%. The mechanical slicer is a convenient method of slicing that results in minimal damage to the slices. The tissue is submerged in the appropriate medium for the entire cutting time and a suitable internal current rapidly removes the slice from the blade to a holding vessel.

No single test can supply the full information concerning the effects of a test compound in this system. Therefore a variety of parameters were examined for assessing the potential of the system for characterizing cisplatin effects in the kidney. Platinum levels were measured in the slices for both cisplatin and transplatin treated slices. Intracellular LDH, K⁺, ATP and oxygen consumption were all monitored. Intracellular lactate dehydrogenase is a general viability
marker, indicating a disruption of the cell membrane, and was chosen due to it being a classic marker used in toxicological investigations. The next two parameters that were examined, intracellular potassium and intracellular ATP levels, are linked to ionic gradients and energy status in the cell and possibly detect more subtle damage to the cell. Oxygen consumption has often been used as a biochemical tool; the majority of the oxygen consumed in the cortex is used for ATP production to support the \( \text{Na}^+/\text{K}^+ \)-ATPase (Balaban et al, 1980; Gonick et al, 1980). Histopathology of cortical slices can indicate site specific toxicity and enable visualization of toxicity patterns (Ruegg et al, 1986).

Evaluation of Cisplatin Effects in Slices

In past in vitro studies, relevance to clinically observed concentrations has been severely neglected; previous in vitro systems have been limited to high concentrations (greater than \( 10^{-3}\text{M} \) of cisplatin). The system employed here demonstrates responses to cisplatin concentrations ranging from \( 10^{-3}\text{M} - 10^{-5}\text{M} \). The highest concentration is equivalent to that used in other in vitro studies (Guarino et al, 1980; Williams et al, 1985, Goldstein et al, 1981) and is approximately ten times the peak blood levels of cisplatin reported during therapeutic use. The lowest concentration (\( 10^{-5}\text{M} \)) used in the precision cut cortical slice has been determined to be approximately ten times less than peak blood levels of cisplatin in clinical studies.

The platinum uptake (approximately \( 10^{-3}\text{M} \) or 100 ppm at 12 hr) of cisplatin treated precision cut slices approximates reported in vivo
kidney platinum levels (Mason et al, 1986; Choie et al, 1980; Weiner and Jacobs, 1983). The platinum uptake for non-nephrotoxic transplatin treated slices stayed under 10 ppm. The low uptake of platinum in transplatin treated slices suggests that the stereochemistry of the platinum molecule is extremely important for the entry of the molecule into the cell.

Of the viability parameters examined in precision cut cortical slices, intracellular ATP appears to have been the most sensitive. Intracellular ATP demonstrated a significant decrease from control for the high dose (10^-3M) at 6 hr and a significant decrease for the middle dose (10^-4M) at 12 hr. Intracellular K^+ levels didn't show a significant change for the middle dose until 18 hr. Intracellular K^+, however, was also very a sensitive indicator and presented slightly less variability than intracellular ATP. Intracellular LDH and oxygen consumption were the last parameters to fall in response to cisplatin treatment; LDH also proved to be more variable than intracellular K^+. Non-nephrotoxic transplatin demonstrated no effect on intracellular K^+ but did produce a decrease in intracellular ATP levels. It is possible that an artificially low ATP level is not necessarily an irreversible early step leading to more severe nephrotoxicity as this has been observed in liver slices (Smith, 1986). As demonstrated by the drop in intracellular ATP, perhaps a tiny amount of transplatin is toxic to the cell and causes a decrease in the ATP.
Isolated Proximal Tubules

Proximal tubules isolated without the use of proteolytic enzymes have been well described as a model system for nephrotoxicity (Hassall et al, 1983a,b,c; Rylander et al, 1986). Since renal toxicity of cisplatin has been localized in the proximal tubule (Krakoff, 1979; Choie et al, 1980; Prestayko et al, 1979; Litterest, 1984; Weiner and Jacobs, 1983), this in vitro system held promise as a model for cisplatin nephrotoxicity. A battery of viability parameters have been investigated for this system (Rylander et al, 1986) four of which were chosen to aid in determining the effect of cisplatin on tubules. The effect of cisplatin on organic ion transport has previously been examined in vitro and was included in the study.

Cisplatin, at a dose of $10^{-3}M$, demonstrated no effect on either intracellular $K^+$, intracellular ATP, or LDH leakage of proximal tubules in this system. However, organic ion transport of the tubules was notably affected. Decreases in both TEA and PAH accumulation within cisplatin treated tubules were seen. If this inhibition of accumulation is part of the toxic effect, transplatin, a non-nephrotoxic isomer of cisplatin, should not produce a decline in accumulation. However, transplatin treated slices demonstrated a similar effect on these ion accumulations. Thus, organic ion accumulation probably does not play an important role as an indicator for the nephrotoxicity of cisplatin. Once a cell is irreversibly damaged, the energy production necessary for the various transport processes will diminish and the activity of many active transport systems will decline. Since TEA and PAH are
handled by independent transport systems, it is unlikely that the inhibition from cisplatin or transplatin is due to competitive inhibition at the substrate site, however it is possible that inhibition of both transport systems is effected via the common ATP requirement.

Goldstein et al (1981) also found both TEA and PAH transport to be inhibited in free hand cut slices. However, the effects of cisplatin on organic ion transport systems are still not well understood. To date, the research on cisplatin transport has resulted in conflicting reports. Safirstein et al (1982) found inhibition of accumulation of platinum in hand held microtome cut slices and free hand cut slices treated with probenecid. Isolated perfused rat kidney studies showed an increase in excretion of cisplatin with probenecid (Daley-Yates and McBrien, 1982). Cortical membrane vesicles demonstrated a competitive inhibition of TEA in the brush border of proximal tubules, however, inhibition of TEA in the basolateral membrane could not be distinguished as competitive. PAH transport was not affected by cisplatin in this membrane vesicle system (Williams et al, 1985). In vivo studies of transport have also produced conflicting results. An in vivo chicken study showed cisplatin to inhibit both PAH and TEA transport. In this study, administration of organic cation transport inhibitors, quinine and cyanine, demonstrated protection from cisplatin nephrotoxicity (Bird et al, 1984). Jacobs and associates (1984) showed that patients pretreated with probenecid, an anion transport inhibitor, demonstrated a significantly lower clearance of platinum compared to controls suggesting that secretion of cisplatin or one of its reaction
products was blocked by probenecid. Differences in experimental designs and animal species make it difficult to reconcile the differences in results; thus, further investigation of cisplatin and its effects on transport functions is needed.

Although the proximal tubule suspension system produced changes in organic ion accumulation due to cisplatin, virtually no indication of cisplatin toxicity was seen. Perhaps, tubules were not viable for long enough to express the toxicity of cisplatin. After 6 hr incubations, the tubule control parameters themselves declined and it was doubtful that longer incubations would be feasible.

Comparison of Slices and Tubules

Comparison of results from proximal tubule experiments with those from cortical slices demonstrate the potential of precision cut slices in investigations into the mechanism of cisplatin nephrotoxicity. Differences in the two systems might be attributed to two major factors, extent of damage introduced to the tissue in the isolation procedure and the morphology of the tissue. Cortical slices retain the differentiated, junctional architecture of the kidney as well as cell to cell interactions. Secondly, slices produced in the mechanical slicer are viable much longer than the proximal tubule suspensions produced by a homogenization procedure. Many of the parameters measured decline after 6 hr of incubation; this is especially true for lower concentrations of cisplatin. At 6 hr of incubation with \(10^{-3}\)M cisplatin, the intracellular content of \(\text{K}^+\) and ATP in the renal proximal tubules was identical to that in controls, while at 6 hr of
incubation with the renal slices there was a 27% and 38% loss in intracellular $K^+$ and ATP, respectively at the same concentration of cisplatin.

A longer period of incubation is desirable in the expression of cisplatin damage, especially at lower concentrations (ie. significant changes in viability for $10^{-5}$M cisplatin was not observed until 18 hr of incubation). Goldstein et al (1981) incubated hand cut renal slices for only 90 min, an obviously inadequate length of incubation to demonstrate cisplatin toxicity at lower doses. Safirstein et al (1984) increased this incubation period to 4 hr. Which, according to the results of the precision cut slices, is still not long enough to demonstrate low dose toxicity. Membrane vesicles were incubated with cisplatin for less than a minute (Williams et al, 1985). Guarino et al (1980) carried out experiments for only 6 hr in the flounder proximal tubules. In addition to time of exposure consideration, it is possible that the retention of the organ architecture in the slices allows for the expression of cisplatin nephrotoxicity.

**Analysis of Viability Parameters**

Two different approaches can be taken when examining viability parameters. First, when examining mechanisms of action, a variety of viability parameters should be studied that highlight specific cell characteristics as well as integrated cell functions. And secondly, when a system for rapid screening is desired only a few, sensitive, and easily measured indices of toxicity would be desirable.
A parameter specific to the renal proximal tubules is organic ion transport. Since the proximal tubule is intimately involved in reabsorption and secretion of organic ions, these functions have commonly been used as markers of renal cell compromise (Kluwe et al, 1981; Hassall et al, 1983a; Rylander et al, 1986).

As discussed above, organic ion transport has been examined in a number of cisplatin models (Kluwe et al, 1981; Williams et al, 1985; Hassall et al, 1983a; Rylander et al, 1986). Since it is possible that cisplatin acts as a substrate for the organic base transport carrier or the organic acid transport carrier, a decrease in PAH or TEA transport could indicate either a functional change or toxic damage. Transport was the principle parameter used for screening analogues in flounder proximal tubules (Guarino et al, 1980). Because of our results which showed that transport is equally effected by both nephrotoxic and non-nephrotoxic platinum complexes, there is a need for the development of additional sensitive indicators of nephrotoxicity.

Although a battery of parameters have been characterized for cisplatin, the optimal choice of indicator in terms of sensitivity, reliability and simplicity would be measurement of intracellular K⁺. Even though intracellular ATP is slightly more sensitive, it would be the second choice due to its increased variability. Also, intracellular ATP demonstrated a response to non-nephrotoxic transplatin at 12 hr of incubation. To gain more information about the lesion induced by cisplatin, a combination of intracellular K⁺, intracellular ATP, oxygen consumption and histopathology could be very informative.
Examination of Cisplatin Nephrotoxicity

The longer incubation periods possible with the slices allow the time course and dose-response of cisplatin's nephrotoxicity to be expressed. The toxic response of cisplatin in the slices can be analyzed by examining closely the time and dose effects on each individual parameter which reflect the sequence of events leading to cell death.

A current theory for cisplatin nephrotoxicity has been concerned with the interaction of cisplatin and Na⁺/K⁺ ATP-ase activity. (Guarino et al, 1980; Daley-Yates and McBrien, 1980). The rapid decline of both ATP and K⁺ in the precision cut cortical slices support this hypothesis. Several studies have demonstrated inhibition of the ATPase by cisplatin. Guarino and associates (1980) found cisplatin in the isolated flounder kidney tubules to inhibit Na⁺/K⁺-ATPase but only after exposure to high concentrations. In vitro mitochondrial ATPase inhibition by cisplatin and its diaquo has also been investigated (Aggarwal et al, 1980) however, only the diaquo analogue was found to cause inhibition. Kidney homogenate mixed with high dose cisplatin also demonstrated inhibition of ATPase. Furthermore, aged solutions of cisplatin were found to be 1,000 times more inhibitory to ATPase than to freshly made cisplatin (Daley-Yates and McBrien, 1980). These data suggest that the hydrated cisplatin is the toxic agent to Na⁺/K⁺-ATPase is known to be much higher in the pars convoluta rather than the pars recta (Ashton and Koepsell, 1976). Perhaps, this explains why the S₁ and S₂ tubules were more susceptible than S₃ to cisplatin in the histopathology of the cortical slices.
Another theory of cisplatin toxicity is through mitochondrial uncoupling. The results in the precision cut slices fit this hypothesis nicely. The initial response to mitochondrial uncoupling would be a decrease in ATP production as is seen in my system. Since ATP is responsible for the maintenance of the Na⁺/K⁺-ATPase, the balance of these ions would be disrupted, explaining the decline of intracellular K⁺. The decrease of oxygen consumption in cisplatin treated slices occurs at a later time compared to ATP and K⁺ decreases. In fact, oxygen consumption can increase in response to mitochondrial uncoupling. When the cell has reached a state of more extreme intoxication, the oxygen consumption will decrease. A late event of toxicity occurs at a time when cell membranes dissolve and cytosolic enzymes, such as lactate dehydrogenase are released. Aggarwal and associates (1980) demonstrate that the hydrolysis product of cisplatin can induce uncoupling of oxidative phosphorylation, swelling of the mitochondrial inner membrane and a spontaneous release of Ca²⁺ from the mitochondria. However, cisplatin itself does not demonstrate any effect on oxidative phosphorylation.

Histopathological damage in the precision cut cortical slices is located mainly in the convoluted proximal tubules. Toxicity in in vivo rat kidneys has consistently demonstrated straight proximal tubule degeneration to be the location of damage (Dobyan et al, 1979; Safirstein et al, 1981; Kawamura et al, 1980; Weiner and Jacobs, 1983; Goldstein and Mayor, 1983). The damage in humans in postmortem studies
has been seen to be located mainly in the convoluted distal tubules and secondarily in the convoluted proximal tubules.

Several speculations can be made to account for the discrepancy in the location of cisplatin damage. There could, of course, be species differences and secondly, mechanistic differences may also explain the differences. As previously mentioned, the convoluted portion of the proximal tubule contains more Na\(^+\)/K\(^+\)-ATPase activity than the straight portion of the tubule. Considering that cisplatin or its hydrolysis products inhibits Na\(^+\)/K\(^+\)-ATPase, these cells may be more susceptible to damage by cisplatin. Also, the convoluted tubule has recently been shown to be the segment of the nephron which is most sensitive to inhibition of mitochondrial respiration and injury to this segment may be a reflection of its special dependence on oxidative metabolism (Shanley, Brezis and Spokes et al, 1986).

An important and related issue is the lesion seen in kidney slices in response to ischemia. Parallel work using the same precision cut rabbit kidney slices demonstrated ischemic damage to occur in the convoluted proximal tubules (Ruegg et al, 1986). However, ischemic damage is generally reported to occur in the straight portion of the proximal tubule \textit{in vivo}. It has been speculated that the early lesion of ischemic damage occurs in the convoluted portion of the tubule and that these cells are repaired \textit{in vivo} and ultimately, straight portions of the proximal tubules are damaged. Perhaps, the initial damage of cisplatin is similarly in convoluted cells of the tubule which are able to regenerate \textit{in vivo} but damage is ultimately expressed in the
straight proximal tubules. Cisplatin toxicity may be initiated in a
different manner in the cell than ischemia but then results in a sim­
ilar expression of the lesion. Oxidative phosphorylation and concom­
mitant inhibition of Na⁺/K⁺-ATPase is only one of many possible mech­
anisms of cisplatin toxicity. It appears that because of the potential
for cisplatin or its analogues to bind to macromolecules, the nephro­
toxic mechanisms of cisplatin might be multifaceted and complex and
warrant further investigation.

**Evaluation of Cisplatin Analogues**

Several platinum analogues have demonstrated equivalent antitumor activity to cisplatin and are currently under examination for
potential toxicities (Canetta, Rozenweig and Carter, 1985; Rose and
Schurig, 1985; Harrap, 1985). Platinum analogues, iproplatin and car­
boplatin, were examined in the precision cut slice system. Important­
ly, clinically relevant drug concentrations were chosen for both
analogues (10⁻²M – 10⁻⁴M). Iproplatin and carboplatin presented fan
shaped dose-response and time-response depressions of both intracel­
lular K⁺ and intracellular ATP. A comparison of cisplatin, trans­
platin, iproplatin and carboplatin c x t (concentration x time) to
reach 50% of control values is shown in Table 1. From this table, a
ranking of nephrotoxicity for equivalent dose levels can be determined:
cisplatin = iproplatin >> carboplatin >> transplatin. This ranking is
comparable to other reports for maximum tolerated doses (Canetta et al,
1985). As previously mentioned, transplatin has not shown nephrotox­
icity. Carboplatin has demonstrated less nephrotoxic than cisplatin by
Harland et al (1984) in patients. Comparison of blood urea nitrogen, serum creatinine and $^{51}$Cr-EDTA clearance has demonstrated carboplatin to be 59%, 67% and 80% less nephrotoxic, respectively (Canetta et al, 1985). In a similar comparison between carboplatin and iproplatin, carboplatin shows 56% and 3% less nephrotoxicity than iproplatin for blood urea nitrogen and proteinuria and iproplatin show 33% less than carboplatin for serum creatinine. The reasons for the difference has been suggested to be possibly related to differences in stability or mechanism of renal clearance. Regarding half-lives, carboplatin has the longest half-life, cisplatin has the shortest, and iproplatin is intermediate between these two. The relative concentration of reactive platinum species in the kidney tubule would be reduced because of the inherent stability of carboplatin or iproplatin. Thus, only a small fraction of reactive platinum species would be present in the nephron despite the high platinum levels reported. The renal clearance of carboplatin apparently does not involve a concentrative mechanism like cisplatin (Harland et al, 1984) and this may account for the lack of nephrotoxicity by carboplatin. The different ligands present on the analogues change the disposition of the agents and thus also the renal handling of these compounds.
Table 1

Comparison of c x t Products\textsuperscript{a} for Pt Analouges Using Intracellular $K^+$ and ATP Endpoints

<table>
<thead>
<tr>
<th>Compound</th>
<th>Intracellular $K^+$ 10\textsuperscript{-3M}</th>
<th>Intracellular $K^+$ 10\textsuperscript{-4M}</th>
<th>Intracellular ATP 10\textsuperscript{-3M}</th>
<th>Intracellular ATP 10\textsuperscript{-4M}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin</td>
<td>9.1 x 10\textsuperscript{-3}</td>
<td>14.1 x 10\textsuperscript{-4}</td>
<td>7.0 x 10\textsuperscript{-3}</td>
<td>13.1 x 10\textsuperscript{-4}</td>
</tr>
<tr>
<td>Transplatin</td>
<td>not achieved</td>
<td>----</td>
<td>11.1 x 10\textsuperscript{-3}</td>
<td>---</td>
</tr>
<tr>
<td>Iproplatin</td>
<td>4.1 x 10\textsuperscript{-3}</td>
<td>14.9 x 10\textsuperscript{-4}</td>
<td>6.9 x 10\textsuperscript{-3}</td>
<td>8.6 x 10\textsuperscript{-4}</td>
</tr>
<tr>
<td>Carboplatin</td>
<td>11.1 x 10\textsuperscript{-3}</td>
<td>not achieved</td>
<td>8.4 x 10\textsuperscript{-3}</td>
<td>33.8 x 10\textsuperscript{-4}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Concentration x time in M•hr to achieve 50% of control parameters
Evaluation of Potential Protective Measures

Many agents have been examined for their possible protective action against cisplatin nephrotoxicity. Two of the more widely recognized methods for partial amelioration of cisplatin renal toxicity have been with mannitol and sulphydryl compounds.

Precision cut renal slices demonstrated no improvement but an aggravation of cisplatin toxicity upon addition of mannitol. It is possible that the mannitol and cisplatin are able to complex with each other and that this modifies the renal effects of cisplatin. The precision cut slice model may not allow enough time for mannitol complexes to form before cisplatin damage is initiated.

The mechanism of protection from cisplatin nephrotoxicity by mannitol is not well understood. At first, it was thought that mannitol acted by increasing the rate of excretion but then it was discovered that plasma levels of platinum actually increased and elimination half times likewise increased indicating that diuresis was not responsible for the protection observed (Belt et al., 1983; Gormley et al., 1979). An in vitro study of D-mannitol platinum complexes has been performed and after approximately 24 hr a complex containing 2 molecules of cisplatin and 1 molecule of mannitol seems to have been formed (Eshaque, McKay and Theophanides, 1976). This complex formation may render the cisplatin less able to enter the renal cell. Clearly, this interaction requires more study including more chemical analysis as well as observation of cisplatin-mannitol effects in defined cellular systems. In conclusion, it is not possible to repeat the in vivo
response of mannitol protection in vitro; it appears that mannitol protection is due to extra renal effects, possibly a multi-organ factor is responsible.

A second potential protective measure for cisplatin nephrotoxicity is suggested with administration of sulfur containing compounds. It has been proposed that the electrophilic aquation site of the cisplatin molecule should interact avidly with various nucleophiles, most specifically sulfur or nitrogen containing molecules (Litterst et al, 1982; Zwelling and Kohn, 1982). Pre-incubation of the cortical slices with glutathione, a nucleophilic scavenger, should decrease toxicity of cisplatin. There appears to be an amelioration effect with the addition of glutathione for the first 6 hr of incubation. It has been demonstrated that renal glutathione is essentially depleted by 6 hr. Thus, after 6 hr, any protective effect from glutathione would not be seen from pretreatment with glutathione. If the glutathione were present in the incubation media for the entire experiment, it is likely more protection would have been observed as compared the pretreatment procedure. However, there is the danger that chemical interaction between the two agents would occur extracellularly and be responsible for possible renal toxicity changes that would then be observed.

Litterest et al (1982) reports only 30% binding to -SH groups by cisplatin in the kidney. Since the -SH binding is not overwhelming, it follows that a large improvement of toxicity should not be seen. Other reports in this area have commonly allowed coincubation of cis-
platin and a sulfur containing compound. For example, Uozumi et al (1984) and Howell and Taetle (1980) both demonstrate inactivation of cisplatin when sodium thiosulfate was administered in combination due to formation of the Pt-thiosulfate complex in the extracellular fluid. For reasons mentioned above, this approach is possibly inconclusive. In addition, a concurrent decrease in antitumor action is seen with coadministration of sodium thiosulfate and cisplatin (Howell and Taetle, 1980). The mechanism of protection by sulfur containing compounds from cisplatin nephrotoxicity is still investigated.

Summary

This study has shown that precision cut cortical slices represent a feasible model for the examination of the nephrotoxicity of platinate compounds. Toxicity of a model platinate, cisplatin, was characterized for a variety of viability parameters. Intracellular ATP was shown to be the earliest indicator, followed by intracellular K⁺, oxygen consumption, and intracellular LDH decreases. Intracellular K⁺ quantification when normalized to μg DNA present in the slices was the most reliable, reproducible marker of nephrotoxicity. The system allowed for extensive manipulation which can aid in the elucidation of toxic and protective effects.

The precision cut slice system demonstrated a) that biochemical parameters examined revealed toxicity of cisplatin b) that histopathological studies demonstrated site specific damage c) that cisplatin toxicity required nearly 6 hr for significant depression of some of the
viability indicators d) dose-and time-response damage for cisplatin nephrotoxicity were observed.

Future studies should include an examination of the effects of ischemia in the system and their possible interference with histopathological interpretations. Other potentially protective agents might be examined with the use of preincubations with amines, such as the basic amino acids, lysine, ornithine, and arginine as well as the polyamines, putriscine and spermidine. In vitro comparisons are warranted and would serve to verify the in vitro effects seen. Another area deserving attention includes more extensive study of in vitro enzyme interactions with cisplatin and metabolites, as well as cisplatin and mannitol in vitro interactions. Preincubations of mannitol and cisplatin before addition to slices would be informative as would measurement of platinum uptake in the presence of mannitol. Finally, this system has potential for more extensive mechanistic studies of cisplatin and analogues.
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>°C</td>
<td>degrees centigrade</td>
</tr>
<tr>
<td>CCl₂</td>
<td>cadmium chloride</td>
</tr>
<tr>
<td>Conc.</td>
<td>concentration</td>
</tr>
<tr>
<td>DCVC</td>
<td>S-(trans-1,2-dichlorovinyl)-L-cysteine</td>
</tr>
<tr>
<td>DME</td>
<td>Delbecco's modified eagles</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>mercuric chloride</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>in</td>
<td>inch</td>
</tr>
<tr>
<td>K⁺</td>
<td>potassium ion</td>
</tr>
<tr>
<td>KCl</td>
<td>potassium chloride</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>potassium phosphate, dibasic</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>μ</td>
<td>micrometer</td>
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</tbody>
</table>
LIST OF ABBREVIATIONS — Continued

M  molar
mCi  milliCurie
MEM  minimum essential media
mg  milligram
MgSO_4\cdot7H_2O  magnesium sulfate, heptahydrate
min  minute
ml  milliliter
mM  millimolar
mmole  millimole
μmoles  micromole
Na^+  sodium ion
NaCl  sodium chloride
NAD  nicotinamide adenine dinucleotide
NADH  reduces nicotinamide adenine dinucleotide
NaHCO_3  sodium bicarbonate
Na^+/K^+-ATPase  sodium- and potassium- dependent adenosine triphosphatase
NaOH  sodium hydroxide
nm  nanometer
O_2  oxygen
PAH  para-aminohippuric acid
rpm  revolutions per minute
SD  standard deviation from the mean
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>SEM</td>
<td>standard error from the mean</td>
</tr>
<tr>
<td>sec</td>
<td>second</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TEA</td>
<td>tetraethylammonium bromide</td>
</tr>
<tr>
<td>U/L</td>
<td>international units per liter</td>
</tr>
</tbody>
</table>
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