DISTRIBUTION AND HISTOPATHOLOGICAL EFFECTS OF CIS-PLATINUM(II) DIAMMINODICHLORIDE ON NON-TUMORED AND TUMORED (SARCOMA 180) SWISS WHITE MICE

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This is to certify that the

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DISTRIBUTION AND HISTOPATHOLOGICAL EFFECTS OF CIS-PLATINUM(II)DIAMMINODICHLORIDE ON NON-TUMORED AND TUMORED (SARCOMA 180) SWISS WHITE MICE

presented by

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ABSTRACT

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Cis-platinum(II)diamminodichloride (cis-Pt(II)$(NH_3)_2Cl_2$) has been shown to be an effective cancer chemotherapeutic agent in laboratory animals. As a first step toward understanding the metabolism and mode of action of this compound, studies were undertaken to determine the relative lifetime of platinum in the Swiss white mouse (both non-tumored and tumored), as well as its distribution, with time, in the major organs. Neutron activation analysis---with gamma-ray spectrometry---was the analytical technique employed for this purpose. Three animals were sacrificed at 1, 6, 12 and 24 hours after injection of a therapeutic dose (8 mg/kg), and then every 12 hours for 144 hours. The major organs were removed and placed in pre-weighed polyethylene irradiation vials, which were then weighed and heat-sealed. (Blood samples were likewise collected, by heart puncture from 3 animals per time, at various intervals for 24 hours.) A fourth animal was simultaneously sacrificed at each time interval, for histopathological studies, and its major organs or representative sections thereof immediately placed in buffered formalin for fixation.

The samples for platinum determination were irradiated for 8 hours in a Triga Mark I reactor at a flux of approximately 2×10^{12} neutrons/cm^2/second. ^{199}Pt, the radioactive isotope most abundantly formed, has a relatively short half-life (31 minutes) and its characteristic γ-rays are low energy. In a biological matrix, even

qualitative detection of such an isotope is practically impossible. However, ^{199}Pt decays to another radionuclide, ^{199}Au, whose half-life is 3.15 days. Its characteristic γ-rays are, likewise, of low energy, and high concentrations of ^{24}Na tend to interfere with accurate detection. Thus, after a lapse of approximately 60 hours after irradiation---for efficient formation of ^{199}Au and radioactive decay of matrix elements---a radiochemical separation was undertaken. After complete digestion of the samples in a 1:1 solution of HNO_3 and H_2SO_4, the solutions were brought to volume with 6N HCl and passed over a Dowex 1-X8 anion-exchange column. The hexachloro salt of the gold remained bound to the surface of the resin, while most interfering ions passed through. After a suitable wash with 6N HCl, the column was placed in a 3" by 3" well-type NaI(Tl) crystal and the area under the 158keV photopeak of ^{199}Au determined and compared with that of a control. Results were expressed as μg Pt/gm tissue and μg Pt/cc blood.

The samples for histopathological study were dehydrated, cleared, infiltrated and embedded according to the paraffin method. 6μ sections were cut and a simple hematoxylin-eosin stain used for all sections. The slides were then studied for evidence of pathological alterations and their time course.

The distribution data revealed a low recovery of platinum at all times measured. (Although the entire animal was not assayed for platinum content, the level is still suspiciously low.) This and the relatively high amounts consistently present in filtering (liver and spleen) and excretory (kidney and intestines) organs indicates a possible rapid excretion of large quantities, primarily via the urine.

Although a therapeutic dose was employed, the amount of platinum present in the tumor is relatively low. This eliminates the possibility

that the potent anti-tumor properties result from specific uptake into neoplastic tissue. However, platinum is present as early as 1 hour following treatment, and a greater sensitivity of neoplastic, as compared to normal, tissue is a plausible alternative.

The total absence of platinum from brain samples suggests that the original neutral molecule is rapidly metabolized to form a charged molecule, incapable of passing the blood-brain barrier. The extreme reactivity of the compound, as a result of the lability of its chloride ions, supports such a suggestion.

The fall and subsequent rise in the amount of platinum present in the major organs, during the first 24 hours following treatment, suggests a temporary storage elsewhere in the body. Blood studies revealed a similar pattern, ruling out combination with and retention in the blood elements. However, peripheral shunting, as a temporary edema, or merely temporary distribution into muscle, etc., are possible, and present data are insufficient to resolve the problem.

Histopathological studies revealed little if any relationship between platinum concentration and amount of damage. Although large amounts of platinum are consistently found in liver and kidney tissue, minimal damage is evidenced. Most pathological alterations are seen in rapidly proliferating tissue---gastrointestinal mucosa and lymphoid elements of thymus and spleen. Thus, as with most cancer chemotherapeutic drugs and X-radiation, tissues with high mitotic activity are primary targets. However, the general damage resulting from platinum ---at least as evidenced in mice and rats---is considerably less than that found for other such agents.

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Jean Toth-Allen

A THESIS

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To my husband

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INTRODUCTION

Cancer may be defined as a "disease of multicellular organisms which is characterized by the seemingly uncontrolled multiplication and spread within the organism of apparently abnormal forms of the organism's own cells."[1] As such, it differs from most diseases afflicting man in that the obvious stages of the disease are represented by invasion by his own (albeit abnormally transformed) cells, rather than those of a foreign organism. It represents an infringement on the normal homeostasis of the body and may arise from the breakdown of a normal control mechanism or the loss of response to such control in a target tissue or cell. The causative agent---virus, chemical or physical carcinogen, or some as yet unknown agent---may produce an alteration in the affected cells which directly or indirectly results in a malignant transformation. Cytological studies of the chromosome constitution of various tumors revealed that abnormalities, both in number and structure, are a common occurrence However, no characteristic and exclusive chromosome pattern emerges (except for chronic myeloid leukemia) and, thus, it is proposed that such abnormalities, in general, represent a predisposition of the cell to malignancy, rather than a cause thereof.[2] Theories concerning deletion of an essential cellular activity, quantitative or qualitative alterations in DNA or RNA, variations in the nature or function of the electron transport system, changes in hormone levels or interrelationships, alteration of the normal electrolyte balance and/or of an ion

essential to this balance, or changes in membrane properties of the cells affected have been proposed to explain the cause---or at least the initial phase---of malignant growth. Any and all such alterations, however, may merely represent a symptom, or even a result, of the disease rather than its cause.

Present knowledge of the cause or causes of the diseases called cancer is, at best, incomplete. A preventative approach is therefore presently unfeasible. However, numerous approaches to cures are possible. Surgery and radiation, alone or in combination, have had good success in the early stages of a malignancy. However, the general characteristic of invasiveness---manifested as neoplastic metastasis---makes such treatment of little use in later phases of the disease. In this case, chemotherapy may play a vital role.

The concept of treating cancer by chemotherapy is by no means of recent vintage. Such endeavors began more than a thousand years ago, but efforts were sporadic and bore little fruit.[3] However, since World War II there has been a great upsurge in activity in the field of cancer chemotherapy. The first major breakthrough came with the discovery in 1946 of the chemotherapeutic activity of an alkylating agent---nitrogen mustard (HN-2)---against tumors in mice. This was followed in 1948 by a similar discovery concerning the action of an antimetabolite, amethopterin. In the decade that followed, the types of agents which powerfully alter experimental neoplasms were expanded to include narcotics (e.g. urethane), antibiotics (e.g. actinomycin D), mitotic poisons (e.g. colchicine), and several as yet unclassified agents.[4]

The success of cancer chemotherapy to date has been variable.

Numerous factors can influence the effectiveness of the individual drugs. Response can vary widely according to route of administration, as a result of activation or deactivation by various organs. Rate of excretion of a drug can likewise affect its activity, although in many cases the potency rather than the selectivity of the drug is most affected in this way. For certain compounds the dosage schedule employed is extremely critical in determining whether or not tumor regression will occur. Moreover, extra-cellular deactivation by compounds in the sera, and low permeability into neoplastic tissue can present formidable problems.[5] Even for those tumor systems which initially prove amenable to drug therapy, drug resistance can fairly readily develop. Despite such complications, various agents or combinations of such have produced successful remissions, or, at least, considerable extension of life.

Despite widely differing chemical structures, most cancer chemotherapeutic agents (with the possible exception of hormones) act by interfering with some stage of nucleic acid (mainly DNA) or protein synthesis.[6] Figure 1 shows schematically the stages where the various agents are believed to exert their effects. Of these agents, the various alkylating agents are perhaps the most widely known and used. These biological alkylating agents add a compound radical to the molecules with which they react, rather than merely an alkyl group as the name implies.[3] They are electrophilic reagents which react with nucleophilic centers of biological molecules, deactivating these molecules in most cases. If the target molecules are essential for cell division, mitosis is inhibited; if not the altered molecules are discarded after variable delays and cell division proceeds.[4] The effects on cells

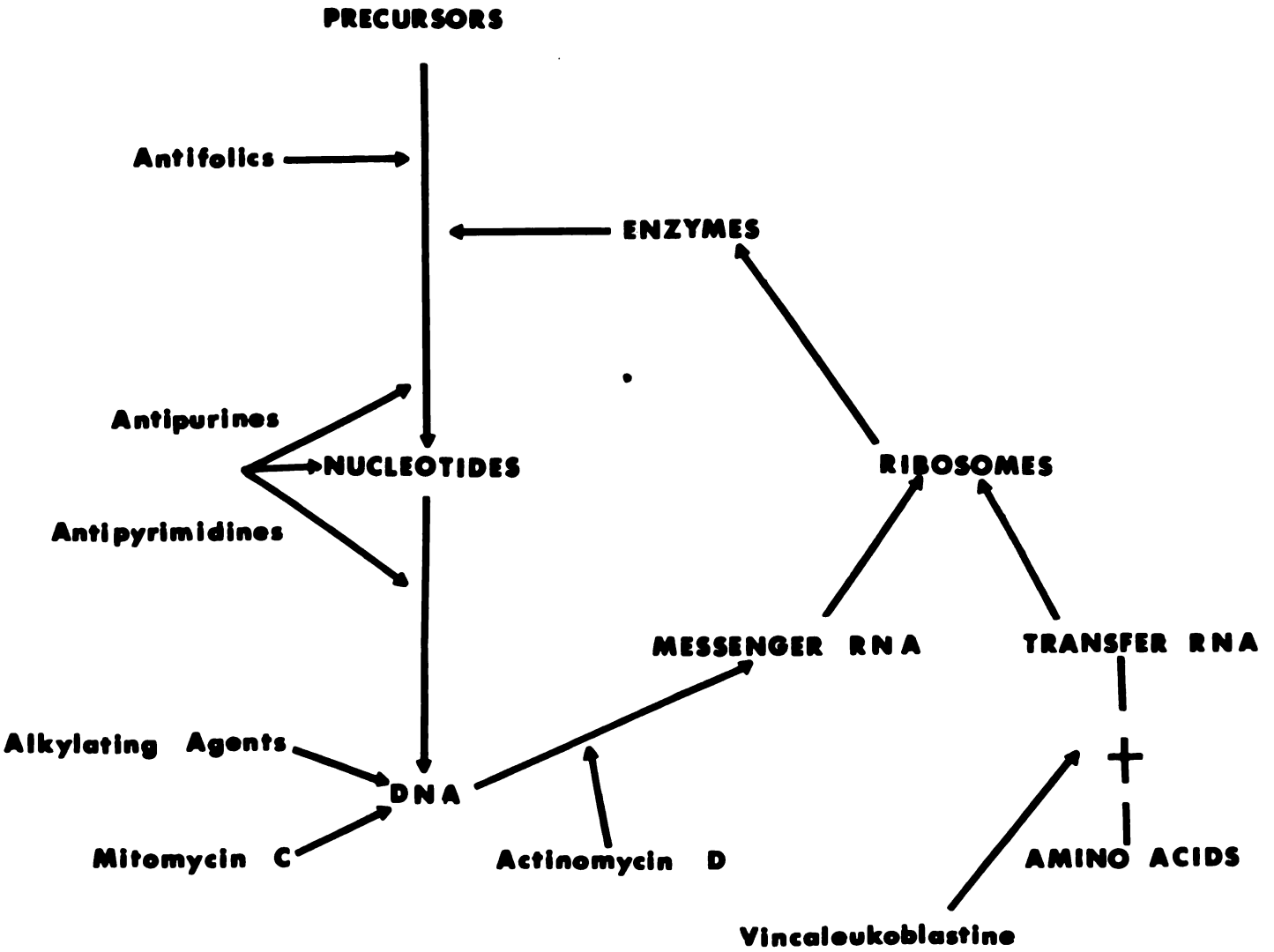

Figure 1: Schematic representation of the proposed stages of drug effectiveness. (After Connors[6])

range from temporary blockade of division to a cessation of division accompanied by continued growth (giant cell formation) to irreversible toxicity.

There are four main types of alkylating agents: nitrogen and sulfur mustards, ethylene imines, alkyl methane sulfonates and bis epoxides.[7] The most effective agents contain at least two reactive groups---are bifunctional---, and all such agents are relatively indiscriminate in their attack on cells, reacting with proteins, RNA and DNA alike. At dose levels just sufficient to cause cell death, evidence has accumulated that DNA is the most sensitive molecule to alkylation.[6] The exact nature of the interaction of these agents with DNA is still uncertain but several possibilities exist. Cross-linkage of the double helix by bifunctional agents, most probably involving guanine residues, is very possible. Such a linkage would be relatively stable and result in inhibition of cell division. Another possibility is a linkage formed between the DNA molecule and its associated protein, producing an abnormal connection between the two and thus preventing separation and duplication, or possibly interfering with mitotic separations of chromosomes.[4]

Bifunctional alkylating agents have a number of characteristics in common, many or all of which may be shared by other antitumor agents. they interfere with cell mitosis; produce fragmentation and clumping of chromosomes; are mutagenic and carcinogenic; usually have profound effects on blood and bone marrow; damage the intestinal mucosa; interfere with growth; have antifertility and teratogenic properties and suppress the immune response mechanism.[3,7,8] Central nervous system stimulation with convulsions is also common with most of these agents,

with the exception of the ethylenimmonium derivatives.[9] They show variable effects against numerous tumor systems, and resistance is fairly readily developed, with cross-resistance among the various agents in this class common.

Another major class of chemotherapeutic agents contains the anti-metabolites---basically folic acid antagonists, antipurines and anti-pyrimidines. These agents have molecular structures similar to those of the natural metabolites and interfere with the function of the latter. The folic acid antagonists---mainly aminopterin and amethopterin (methotrexate)---interfere with the reduction of folic acid to tetrahydrofolic acid, the active form of this coenzyme. This inhibition is mediated by the strong, specific binding of these agents with the active site of the reducing enzyme. In tumor cells, it is believed that these compounds mainly inhibit thymidine formation, resulting in inhibition of DNA synthesis.[6] Alone and in combination with other drugs these antagonists have been used against a variety of cancers. Unfortunately, as with the alkylating agents, resistance is fairly readily developed. Moreover, these agents result in bone marrow depression and anemia; ulcerations of oral and gastrointestinal mucosa; hepatic dysfunction; alopecia; skin rashes; abortion and teratogenic effects.[7]

6-Mercaptopurine is probably the most widely used purine antagonist and 5-fluorouracil (5-FU) or its deoxyriboside (5-FUDR) the most common antipyrimidine. The ribotide of 6-mercaptopurine is formed intra-cellularly and appears to act by inhibiting purine synthesis at an early stage in its biosynthetic pathway by so-called "pseudonegative feedback".[3] (Another well known purine analogue, 6-thioguanine is

believed to cause inhibition by direct incorporation into DNA, thus resulting in the formation of an abnormal molecule.[6]) Resistance to 6-mercaptopurine is readily developed and severe bone marrow depression, gastrointestinal disorders, hepatotoxicity and renal impairment are fairly common side reactions.[7,8] It has been successfully employed in various cases, however, particularly those involving acute childhood leukemia or chronic granulocytic leukemia.[7]

The catabolism and anabolism of uracil and its fluoro derivatives are extremely similar. Thus, 5-FU takes part in a multitude of biochemical reactions in the cell. It is believed to block the methylation of deoxyuridylic acid to form thymidylic acid, and it is incorporated as an odd base in RNA.[3] It has been found effective against various carcinomas but, unfortunately, resistance is developed once again. Among its toxic side-effects are gastrointestinal injury leading to dehydration and electrolyte imbalance; bone marrow toxicity; mild anemia; and alopecia and dermatitis.[8] Its riboside, 5-FUDR, is somewhat less toxic though equally as active and has thus replaced 5-FU, for the most part, in clinical practice.

In general, the antimetabolites have not proved as successful clinically as tests with laboratory animals seemed to predict. It appears that the pharmacology of these drugs in humans is sufficiently different from laboratory animals to prevent achievement of doses effective on tumor cells without producing excessive toxicity in the hosts. This appears to result from a greater instability of these agents in humans as compared to animals.[4]

Although not included in the scheme in Figure 1, various sulfhydryl inhibitors have been shown to have anti-tumor properties. Sulfhydryl

proteins are an essential portion of the nucleic protein, which is indispensable for the structural integrity of the chromosomes. Moreover, the mitotic apparatus---asters, centrioles, spindles, etc.--- is extremely rich in sulfhydryl proteins, and, in fact, such protein may account for a large mass of the dividing cells---perhaps up to 50%. Such agents are in some sense selective for cancer cells, possibly because the sulfhydryl-containing enzyme succinic dehydrogenase is necessary for maintenance of cancer cell membranes. Also cancer cells are streamlined for proliferation, implying that they may be mainly sulfhydryl-protein in structure.[7] However, like most anti-cancer agents they are not highly selective for tumor cells per se but tend to be selectively toxic to rapidly dividing cells.[6] Thus numerous toxic side-effects are likewise encountered with these agents.

Various antibiotics have been employed as cancer chemotherapeutic drugs. Actinomycin D, possibly the most commonly used of these drugs, is thought to combine physio-chemically with DNA. It does not interfere with DNA synthesis but RNA formation is inhibited. It is believed that the actinomycin molecule "fits" into the minor groove of the DNA helix, thus inhibiting formation of the RNA synthetase.[3] Mitomycin C, another frequently used antibiotic, is believed to attack essential sulfhydryl groups in vivo.[7] Varied success has been attributed to these agents. Toxic effects on bone marrow, liver, kidney and the gastrointestinal tract, as well as depression of the immune response, are common side-effects.

The Vinca alkaloids---extracts of the Vinca rosea plant, the periwinkle---have also found some success in cancer chemotherapy. The principle agents are vinblastine and vincristine. Their mechanism of

action is presently unknown, but they produce metaphase arrest and general mitotic inhibition.[10] They have produced some success against a variety of tumor systems and cross-resistance within the group is unknown. Their toxic side-effects are those common to most anti-cancer agents, with the addition of neurologic disorders, which may be severe.

Various other miscellaneous compounds, such as methylglyoxal bisguanylhydrazone (MeGAG), hydrazines and various hormones, have been used in cancer chemotherapy with varying degrees of success. Most compounds attack fast growing tissue rather than just cancerous tissue and, therefore, toxic side-effects are common and a balance between therapeutic efficiency and host toxicity must be carefully maintained. The number of agents of all classes both in clinical and preclinical testing, as well as in use, is legion. Workers in the field are optimistic that chemotherapy will supply a suitable control. In this regard, a new class of chemotherapeutic compounds---inorganic platinum compounds---has recently been discovered. Early animal studies indicate that these compounds may surpass those presently available.

These agents were first discovered while investigating the possible effects of an electric field on the growth processes of bacteria.[11] Under the experimental conditions E. coli formed long filaments, up to 300 times the length of a normal cell. Further studies revealed that a platinum salt, produced by electrode reactions, was the active agent for this phenomenon. The complex formed was tentatively identified as $(Pt(IV)\ Cl_6)^{-2}$. However, when bacterial chambers were inoculated with fresh solutions of $(NH_4)_2(Pt(IV)\ Cl_6)$, higher concentrations then originally estimated were necessary to cause filamentous growth. It was soon discovered that solutions of $(NH_4)_2(Pt(IV)Cl_6)$ exposed to light

for some time were more efficient in inhibiting cell division than freshly prepared solutions. UV-irradiated solutions were found to contain three species of platinum compounds---the doubly negative species which was found to be bacteriocidal; a singly negative species, initially found to cause neither inhibition of growth nor cell division; and a neutral species which inhibits cell division but not cell growth.[12,13] (Later studies showed that the singly negative species is extremely unstable, even in the dry, crystalline form, and fresh solutions of this compound were found to inhibit cell division.[14]) The active neutral species formed was eventually identified to be cis-$Pt(IV)(NH_3)_2Cl_2$.

With further testing it was found that various group VIIIB transition metal ions were capable of inhibiting cell division in E. coli, although not apparently interfering with growth. When various bacterial species were similarly tested gram-negative bacilli were found most sensitive, while gram-positive bacilli responded only at near toxic-levels. No coccus tested showed any effect. With the removal or decrease of platinum, the filamentous bacteria were seen to reinitiate cross-septation and, in time, only normal sized bacteria were again present.[13]

The distribution of an active neutral platinum species (cis-$Pt(IV)(NH_3)_2Cl_4$) in both E. coli and gram-positive cell lines (Bacillus cereus and Staphylococcus aureus), as well as the distribution of the growth inhibitor $Pt(IV)Cl_6^{-2}$ in E. coli, were investigated, using radioactive ^{191}Pt (half-life approximately 3 days). In the induced filamentous forms of E. coli, platinum was found associated with metabolic intermediates, nucleic acids and cytoplasmic proteins. For those E. coli inhibited by $Pt(IV)Cl_6^{-2}$, the platinum was found only in association with

cytoplasmic proteins. For the unaffected gram-positive cell line it was found that the platinum did indeed enter the cell, combining predominantly with metabolic intermediates.[15]

Various inorganic platinum compounds, with platinum in either the two or four valence state, were either synthesized or commercially obtained and purified. Tests for the effect of such compounds on experimental tumor systems were subsequently carried out. Significant regression of the mouse solid Crocker Sarcoma 180 was achieved with several of these compounds. Subsequent tests on the L1210 strain of mouse leukemia by Microbiological Associates, Inc., under contract to the Cancer Chemotherapy National Screening Service (CCNSC), showed several compounds to be fairly potent antileukemic agents, with significant increases in mean survival time attained.[16] In accordance with the Protocols of CCNSC for tests with the Sarcoma 180, tumors were implanted on day 0 and treatment started day 1. Table 1 gives the results for various platinum compounds on Sarcoma 180 in both ICR and random-bred Swiss mice.[17] T/C x 100 is a measure of compound efficacy, T/C being the ratio of treated tumor weight to control tumor weight at times of sacrifice. The host toxicity varied tremendously from compound to compound and such effects for a given compound were often inconsistent. It is of interest to note that the $(Pt(II)(NH_3)_4)^{++}$ salt, which had no evident tumor effect, was tolerated as high as 400 mg/kg on a daily dose schedule.

Subsequently, tests were undertaken with mice in which the Sarcoma was allowed to attain a reasonably large size (8 day growths, typically averaging 0.5-1.5g in weight) before treatment was initiated. One or two injections, appropriately spaced, of either cis-$Pt(II)(NH_3)_2Cl_2$ or

Table 1: Results with various platinum compounds.

Compound	Dose Schedule	T/C x 100
$(Pt(II)Cl_4)^{-2}$	daily	60 or greater
$(Pt(IV)Cl_6)^{-2}$	daily	10-35
$(Pt(II)NH_3Cl_3)^{-}$	daily	16-20
$(Pt(IV)NH_3Cl_5)^{-}$	daily	50 or greater
cis-$Pt(II)(NH_3)_2Cl_2$	daily 1 shot, day 1	0.2 (at 2mg/kg) 6.0 (at 8mg/kg)
cis-$Pt(IV)(NH_3)_2Cl_4$	daily 1 shot, day 1	30-82 11 (at 12mg/kg)
cis-$Pt(II)(NH_2)_2(CH_2)_2Cl_2$	daily 1 shot, day 1	33 or greater 18 (at 16mg/kg)
cis-$Pt(IV)(NH_2)_2(CH_2)_2Cl_4$	daily 1 shot, day 1	not reproducible 48 (at 8mg/kg)
trans-$Pt(II)(NH_3)_2Cl_2$	daily	70 or greater
trans-$Pt(IV)(NH_3)_2Cl_4$	daily 1 shot, day 1	75 or greater 75-120
$(Pt(IV)(NH_3)_3Cl_3)^{+}$	daily	45-70
$(Pt(II)(NH_3)_4)^{++}$	daily	no tumor effect
$(Pt(IV)(NH_3)_4Cl_2)^{++}$	daily	70 or greater

Although most of the above data was collected using Swiss white mice, a few values were obtained using ICR mice.

cis-Pt(IV)$(NH_3)_2Cl_4$ were quite efficacious. A stasis of growth was initially evident and after several days the tumor became necrotic in appearance, began to be "pinched off" externally and "fell out" quite literally in many cases. For those animals in which a "fall out" was accomplished, the gaping hole closed and scarred and eventually no sign of the tumor's presence was apparent. This type of tumor "cure" is typical of that for a spontaneous regression, but the percentages of cures are far greater for platinum treatment than for spontaneous regression.[18]

Animals successfully cured of Sarcoma 180, after treatment on day 1 or day 8 after implant, were found to be immune to repeated implantation ---that is, a later implant either failed to grow or grew to a small extent and was then reabsorbed. As far as presently tested, such immunity appears to remain as long as one year after cure and quite possibly considerably longer.[19]

One male and two female random-bred Swiss mice, which had been cured by treatment with cis-Pt(II)$(NH_3)_2Cl_2$ by day 8 treatment, were mated to test for possible genetic mutations resulting from drug activity. Both females delivered normal sized litters (10 and 11 young) which appeared normal and were observed to remain so beyond weaning.[20]

As a test for possible problems in pregnancy as well as teratogenic effects, several pregnant female mice were obtained and administered a therapeutic dose (8mg/kg) of cis-Pt(II)$(NH_3)_2Cl_2$ at various stages of gestation---from the 14^{th} to the 20^{th} day. All offspring born appeared grossly normal. However, some animals had smaller than normal litters (possible reabsorption of abnormal young?) and several young died after birth, with no reason for death readily apparent.[21] It is postulated

that, since in most instances the mother mouse was herself ill from the effects of the platinum compound (treated animals commonly remain visibly ill 4 to 7 days after treatment), the death of her young was possibly a result of her inability to nurse efficiently. However, such tests were merely preliminary and further work is necessary along this line.

Tissue culture tests of the effects of various platinum compounds, using human amnionic AV_3 cells, indicate a decrease in uptake of tritiated thymidine, tritiated uridine and tritiated leucine after treatment with various compounds. The extent of inhibition is greatest with those compounds showing the greatest anti-tumor activity and at drug levels comparable to tumor therapeutic levels. Moreover, the effect is irreversible in this system, in the sense that inhibition is not removed with removal of the platinum compound.[22] Preliminary tests with a primary tissue culture line reveal less inhibition with comparable drug doses and apparent reversibility of effect.[23] This may result from a greater sensitivity of transformed or malignant cells, to the action of the platinum compounds, as compared to normal cells.

From the above and similar studies it became evident that various platinum compounds---in particular cis-platinum (II) diamminodichloride ($cis\text{-}Pt(II)(NH_3)_2Cl_2$), cis-platinum (IV) diamminotetrachloride ($cis\text{-}Pt(IV)(NH_3)_2Cl_4$), and cis-platinum (II) ethylenediaminedichloride ($cis\text{-}Pt(II)(NH_2)_2(CH_2)_2Cl_2$)---are potent anti-tumor agents in test animals. Moreover, it is quite evident that, as with all cancer chemotherapeutic agents, they are toxic to the host. However, at therapeutic doses, any injury to the host is evidently repairable, as cured animals continue to survive with no apparent ill effects.

In an effort to gain some insight into the mode of action of these compounds, the author undertook distribution studies of cis-Pt(II)$(NH_3)_2Cl_2$ in both tumored and non-tumored animals, as well as histopathological investigations. Cis-Pt(II)$(NH_3)_2Cl_2$ was chosen because of its tremendous and consistent success in treating solid Sarcoma 180 (both small and advanced tumors), as well as mouse L1210 leukemia and other mouse and rat tumor systems. The thesis material presented herein thus includes data concerning the distribution and histopathology of the drug in laboratory animals, as well as several minor experiments with the same system, all designed to provide some initial data for elucidation of a mode and site of action for this drug.

EXPERIMENTAL METHODS

General Procedures

For all experiments random-bred, specific pathogen free, female Swiss white mice were obtained from Spartan Research Laboratories (Williamston, Michigan). The mice weighed 18 to 20 grams on arrival. Translucent plastic, box-like mouse cages, with wire mesh lids, containing a food bucket and through which a typical laboratory water bottle could be inserted, served as animal housing. Corn husk litter, supplied by Spartan Research Laboratories, was used for bedding. Six to fourteen mice were housed per cage, depending upon the needs of the particular experiment. Both food (Purina laboratory chow) and water were continuously available, ad libitum, unless otherwise specified, and their supply was checked daily.

Usually animals to be used in tumor tests received their tumor on the day of arrival. The solid Sarcoma 180 used was originally obtained from Mr. S. Poilly of NIH and had been successfully passed through numerous generations of both ICR and random-bred Swiss white mice. A six to eight day (rapidly growing) tumor growth was obtained from one of the mice designated for transfer purposes. (Transfer of the tumor line, every six to ten days, into six to twelve animals, is necessary for maintenance of fresh, viable tissue for test purposes.) The animal was sacrificed by cervical dislocation and the tumor excised. (All instruments used for removal, preparation and implantation of the

tumor were sterilized before use.) The excised tumor was placed in a sterile petri dish containing a 0.1% solution of chloramphenicol, with 0.85% NaCl. Ten to twenty milligram pieces were cut from obviously "healthy" portions of the tumor mass and were loaded into 13g stilleto-type needles. Tumor implantation was into the left, forward axillary region, with initial entrance of the needle at the lower region of the animal's rib cage.

Cis-platinum(II) diamminodichloride (cis-Pt(II)$(NH_3)_2Cl_2$) used for all reported experiments was synthesized and purified by Thomas Krigas, according to procedures described elsewhere.[24] The compound was maintained in a dry, crystalline state and all solutions were prepared immediately before use, with sterile physiological saline as the solvent. A Mettler balance and volumetric flasks were used for the preparation of all solutions. The solutions were prepared in such a way that 0.025cc of the solution per gram of body weight was required to attain the desired dosage. All animals were weighed immediately before injection and received an injection amount, according to the individual weight to the nearest gram, by the intraperitoneal (IP) route. (Animals were individually coded and weighed in a 400ml beaker on a single pan, 16000 gram capacity, Dial-O-gram balance. The balance, with the beaker, was initially balanced at 100 grams and all initial weights were recorded.) A 1:200 solution of Amphyl was used as a disinfectant for all cages, equipment and work surfaces.

LD_{50} Studies

As a preliminary estimate of the toxicity of the cis-Pt(II)$(NH_3)Cl_2$

a single-dose, LD_{50} study was undertaken, according to the procedures outlined by the CCNSC.[25] Ten female animals were employed per dose level. All animals weighed 18 to 25 grams initially and were then fasted overnight, prior to injection. Initial weights were recorded and the mice were checked daily for deaths. Autopsies were performed as deaths occurred, none later than one hour after death. The animals were observed for 14 days after treatment. Final weights and total weight changes were recorded for all survivors and approximately ½ of the survivors were autopsied, following sacrifice by cervical dislocation.

Weight Studies

Early studies, in particular with tumored animals, indicated that the treated animals are grossly ill for 4 to 6 days following injection with a therapeutic dose (8mg/kg) of cis-Pt(II)$(NH_3)_2Cl_2$. In order to investigate more carefully the time course of the grossly-evident effects of this platinum compound, as monitored by weight changes, 4 sets of animals were prepared. Two cages containing 6 animals each were set aside for each set---one set to receive neither tumor nor platinum; the second to receive only platinum; the third to receive only a tumor; the fourth to receive both a tumor and platinum. The animals were weighed and tumors implanted the day after arrival. Individual weights were measured at the same time of day, each day. Injection of the platinum compound into the treated animals, according to individual weight, was accomplished on the 8^{th} day of tumor growth. Daily weight measurements were continued for 6 days after treatment. All animals were then sacrificed, by cervical dislocation, and autopsies performed on two, randomly chosen animals from each set.

Neutron Activation Analysis

The principle purpose of this thesis investigation was to discover the time dependent distribution of the platinum compound in the laboratory mouse, as well as any concommitant histopathological effect. In order to follow the distribution of the compound---or at least that portion with which the element platinum remains attached---a tracing method had to be established. The commonly used practice of employing radioactively labeled tracers did not prove feasible at the time. Platinum exists as but few convenient radioisotopes which are not readily available. Although the isotope ^{195m}Pt did become readily available, no personnel or facility to produce cis-$^{195m}Pt(II)(NH_3)_2Cl_2$ was available. Moreover, the half-life of approximately 4 days presented a problem, in that the time span of interest, after injection, was unknown and large amounts of material would need be handled within considerably short periods of time. To avoid these and other formidable problems, interest was turned to neutron activation analysis. Activation analysis, in general, involves the formation of a radioactive isotope by bombardment with such sub-atomic particles as neutrons, protons, alpha particles. (See the Appendix for details of the theory of neutron activation analysis and associated γ-ray spectroscopy.) Use of such a procedure provided many technical advantages. Since no radioactive isotope exists before activation, the samples of interest could be collected, and later activated, when convenient. However, problems still remained. Biological material contains high concentrations of such elements as Na, K and Cl. These elements are readily activated by neutron irradiation, interfering with the identification of elements, especially those present in only

trace quantities, whose characteristic γ-rays are low energy. (See Appendix for explanation of this phenomenon.) The radioactive platinum species most efficiently formed, ^{199}Pt, emits low energy γ-rays and, moreover, has a relatively short half-life---approximately 31 minutes. Thus, with a biological matrix, even qualitative analysis of ^{199}Pt becomes a formidable task.

Fortunately ^{199}Pt decays by β^--emission to the radioactive isotope ^{199}Au. Although its characteristic γ-rays are also of low energy (158 and 208 keV)[26], its half-life is approximately 3.15 days. The interference from high concentrations of ^{24}Na (half-life - 15 hours), however, still presented a problem. A radiochemical separation, for the removal of any and all interfering γ-ray emitters, was thus sought.

Initial tests were done with normal mouse liver samples, spiked with a cis-Pt(II)$(NH_3)_2Cl_2$ solution containing 6μg of platinum. (Eventually, tests with spiked samples of all tissues of interest were used to calibrate the entire system.) A control solution of the compound, containing the same amount of platinum in doubly distilled water, was used for comparison and calibration. These test samples, and all subsequent samples, were irradiated in the Michigan State University Triga Mark I research reactor (Gulf General Atomic, Inc.). Figures 2 and 3 show cut-away views of the reactor tank and its components and a close-up of the sample holders, respectively. As seen, the sample holders (40 in number) are equally spaced around a lazy-susan type apparatus, which is kept rotating constantly during irradiation to insure even flux distribution for all samples. For all tests described here, an 8 hour irradiation at the maximum allowable sustained power level (250kW and approximately 2×10^{12} neutrons/cm^2/sec.) was employed. The samples were

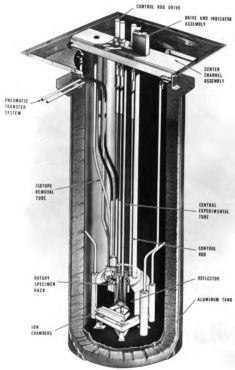

Figure 2: Cut-away view of the Triga Mark I reactor.

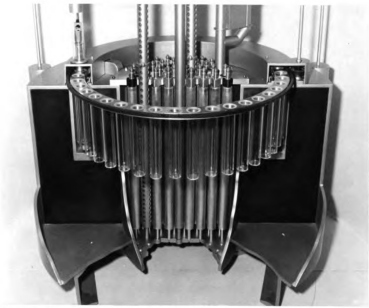

Figure 3: View of the lazy susan-type holder of the Triga Mark I.

heat-sealed in 2 dram polyethylene vials supplied by the reactor personnel and then placed within reactor sample containers and loaded, by means of an electrically operated fishrod assembly, into the reactor sample holders.

Approximately 60 hours were allowed to elapse between termination of irradiation and initiation of radiochemical separation procedures, both for personal safety (the samples were initially considerably radioactive as a result of the high concentration of ^{24}Na, ^{38}Cl, etc. formed) and for efficient conversion of ^{199}Pt to ^{199}Au (a lapse of at least 10 half-lives of the parent nuclide is recommended for efficient production of the daughter isotope[27]). The biological samples were then removed from the polyvials and digested in a minimum amount of a 1:1 solution of HNO_3 and H_2SO_4 acids, which was heated until digestion was complete and all HNO_3 fumes were driven off. (A small amount of H_2O_2 was also added to clear colored solutions of both liver and intestine samples.) The solution was then brought to volume (5 or 10 milliliters depending upon the amount of tissue initially present) with 6N HCl, to convert the heavy metal---the ^{199}Au---to its hexachloro salt. When cool the solution was passed over a Cl^- balanced anion-exchange column (Dowex 1-X8). (The hexachloro salt of the ^{199}Au remains tenaciously affixed to the top portions of the resin and the interfering ions, principally cations, pass through.) The column was then washed with 6N HCl to insure the complete removal of interferences. Initial studies were undertaken to ascertain the amount of wash for a given amount of a given tissue. The integrated area, under the 158keV photopeak of the ^{199}Au γ-ray spectrum, of both the washed column material and a simultaneously irradiated control solution were compared, as a measure of radiochemical separation

efficiency. When the agreement between the two measurements reached a reproducible 2-5%, the separation was considered suitable and the conditions for that tissue then set. Five inch lengths of 5/8" inner diameter polyvinylchloride tubing (Plastics Manufacturing and Supply Inc., Lansing, Michigan) were used as the ion-exchange columns and the top of the washed resin merely pushed flush with the upper end of the tube, the entire column suitably packaged in polyethylene bagging to prevent contamination and the entire column placed within the well of the detection crystal. This allowed for a consistent and readily disposable system. (The column was prepared using a one-hole rubber stopper, from which a short glass tube, equipped with a short length of tubing, extended. The flow rate of the column, which was supported in a burette holder, was controlled, with the use of a simple Hoffmann clamp, to approximately 3 or 4ml per minute.) Subsequent to irradiation, all work with samples was carried out in a vented hood, behind a wall of lead bricks, and rubber gloves and apron were constantly employed. A detection devise was placed close to the work area to constantly monitor the radiation levels and Michigan State University Public Safety officers performed weekly tests for radioactive contamination. All contaminated solid and liquid waste was suitably disposed of by the Department of Public Safety.

Each tissue of interest was individually calibrated with a control solution, irradiated simultaneous with tissue being tested. Thus, the conditions of the radiochemical separation were determined prior to actual data collection, and it was assumed that approximately 100% retention of the ^{199}Au salt was constantly achieved. (The calibration work, as well as other work with such an ion-exchange system,[28,29] indicate this to be an accurate assumption.)

The ^{199}Au activity of the anion-exchange columns was detected with the use of a 3" by 3" well type Packard Instruments NaI(Tl) scintillation crystal. (The well measured approximately 1 1/8" by 2".) This well-type crystal was in a lead housing, lined with cadmium and copper to suppress lead X-rays. The plastic tubing plus a "Lucite" disc served to suppress bremsstrahlung. The anion-exchange column was placed in the well such that the surface of the resin containing the ^{199}Au salt was flush with the bottom of the well. All samples and the individual control solutions (one 6 μg platinum control solution was included per reactor run, containing approximately 11 test samples) were analyzed for a 20 minute "live time". An EMI ten stage photomultiplier tube was directly connected to the scintillation crystal, located within the lead shielding apparatus. The output of the photomultiplier tube was fed directly into a Nuclear Data 512-channel analyzer-computer (Series One-thirty), which, for test purposes, was set to cover a 500keV range. The analyzer was connected to a Tetronix RM503 Oscilloscope, a Moseley Autograf x-y recorder and an IBM electric typewriter, model B, "input-output" type. The final γ-ray spectrum could thus be obtained by any or all of these methods---visually, graphically and/or digitally. The analyzer, moreover, was equipped with an integrator, with which the integrated area under the photopeak of interest could be automatically ascertained. This integration system was employed to provide the integrated area under the 158keV ^{199}Au photopeak. This integrated area, as well as the counts in the channels immediately abutting the photopeak of interest, were then typed out. The integrated area could then be corrected for background (by subtracting the average of the counts abutting the photopeak, times the number of channels in the peak) and compared with the corrected count so obtained for the control solution. A lapse of approximately four hours, or about 5% of the ^{199}Au lifetime, elapsed

between counting of the control and the last sample per run. This was considered negligible and not taken into consideration at any time.

A pilot test, undertaken to obtain some idea of the time course of the platinum within the body, was carried out as soon as a calibration for each tissue was successfully obtained. Four cages, containing four animals each, were prepared. Each animal received a tumor on day 0. On the 8^{th} day of tumor growth the animals received 8mg/kg of the cis-$Pt(II)(NH_3)_2Cl_2$. A 15 minute time interval was maintained between the injection of each of the 4 cages. At various intervals after injection ---1, 6, 12 and 24 hours---one animal from each cage was sacrificed by cervical dislocation. (The 15 minute spacing of the injections allowed for autopsy and maintenance of accurate timing of sacrifices.) The liver + gall bladder; the complete intestinal tract (from duodenum to rectum) with contents; the stomach, with contents, + the esophagus; the spleen; the lungs; the kidney + urinary bladder; endocrines (adrenal glands, pancreas and thymus); the heart; the brain; and the tumor were removed and placed into separate, pre-weighed polyethylene vials. Three tissues of each type were pooled per time (except for the intestinal tract, since only two complete tracts could be placed in a polyvial) for neutron activation analysis. The tissues from the animals in the fourth cage were placed in buffered formalin solutions for histopathological examination. The activation analysis vials were weighed to ascertain the wet tissue weight and then heat-sealed. They were kept refrigerated until irradiated.

The results of this pilot study indicated that longer, rather than shorter time intervals following injection were still necessary. It also indicated that measurement of samples of blood, obtained within the first 24 hours following treatment, would be of considerable interest. Conse-

quently, 0.6cc of blood, obtained by heart puncture, (0.2cc from each of 3 mice) was collected every hour for the first 6 hours following injection, then 8, 10, 12, 16, 20 and 24 hours respectively. Separate samples were simultaneously obtained from both tumored and non-tumored groups of animals. No anti-coagulent solutions of any sort were added. The poly-vials were heat-sealed and treated in the same manner as the other animal tissues. (Calibration of spiked (6μg platinum) samples of blood was carried out before the actual data was accumulated.)

For further tests on the other body tissues, 2 sets of animals, 4 cages apiece, were obtained, for the collection of all necessary samples. Ten animals per cage in the one set received tumors, while the 14 animals in each cage of the 2^{nd} set remained non-tumored. IP injection with the platinum compound was accomplished on the 8th day of tumor growth. Non-tumored animals were sacrificed, and tissues for both activation analysis and histopathology collected, at 1, 6, 12 and 24 hours after treatment. Both tumored and non-tumored animals were subsequently sacrificed and tissue so collected at 12 hour intervals, from 24 hours on, for 144 hours or 6 days. All samples were weighed, heat-sealed and kept refrigerated until irradiated.

The integrated areas obtained from these test samples were graphically converted into μg of platinum. A calibration curve was prepared for each separate irradiation run, by employing the value obtained for the 6μg platinum control for the given run. A plot of counts versus μg Pt was then developed. The corrected 6μg Pt value obtained was plotted and counts for multiples or fractions of this 6μg value calculated and plotted. (Preliminary tests had shown such a calibration curve gave results accurate to within 5% or less.)

Histopathology

The tissue samples for histological study were collected, as described above, at the same time as samples were collected for activation analysis studies. Thus, tissue samples were examined at times corresponding to those at which the platinum distribution within these same tissues was ascertained. The entire spleen, two sections of the main lobe of the liver, the section of the pancreas between the stomach and the duodenum (same as for activation studies), the esophagus at its gastric end, the glandular portion of the stomach, a section of duodenum and of either jejenum or ilium, the adrenals, the kidneys, the urinary bladder, the entire thymus, the heart, two sections of lung, and the brain (halved) were removed and immediately placed in a buffered formalin solution for fixation. (Tissues can remain indefinitely in buffered formalin and its action is progressive. This fixative consists of 1 liter 10% formalin solution containing 4.0gm $NaH_2PO_4 \cdot H_2O$ and 6.5gm Na_2HPO_4.[30]) The entire tumor was excised in all cases and sectioned according to the particular size. All sections cut included central (possibly necrotic) and outer tissue portions. The animal marked for sacrifice and histological use at 84 hours after injection lost the majority of its tumor, by "fall out," by the time of sacrifice. However, some tumor tissue did remain around the open wound and this was removed and placed into fixative. After at least a month in fixative the various sets of tissues were dehydrated, cleared, infiltrated and embedded according to the paraffin method.[30] (The brain sections were never embedded, as no platinum was found in brain tissue at any time, and other available data, with the same platinum compound, revealed no

change in brain tissue following treatment.[31])

The tissues were first washed overnight under running water to remove the formalin. They were then progressively dehydrated by a series of increasing percentages of ethyl alcohol in distilled water---50%, 70%, 95% and absolute alcohol. The time in these particular alcohols was 1 hour, 1 hour, two 3/4 hour washes and two 3/4 hour washes, respectively. The tissues were then cleared by two 3/4 hour washes in analytical reagent grade toluene. (The toluene was supplied by Mallinckrodt, and 95% and absolute alcohol, used for the preparation of all other dilutions, was purchased from Michigan State University Stores.) Two 3/4 hour washes in liquid tissue mat, melting point $55.0^{o}C \pm 0.5^{o}$, followed. (The tissue mat was supplied by Fisher Scientific Company and the temperature of the liquid paraffin was maintained just slightly above its melting point in a Stabil-therm Blue M Electric Oven, Blue Island, Illinois.) The tissues were now ready for embedding. Bond boxes, fashioned from 5" x 3" index cards, were used for the embedding. These were filled 3/4 full with liquid paraffin and the tissues transferred to them with the use of heated forceps, two or three tissues per box depending upon tissue size. Each box flap was marked with the identification of the tissues placed therein. The paraffin was cooled immediately in water, at a temperature of $10\text{-}15^{o}C$. When a solidified scum had formed on the surface, the potential block was dipped below the surface of the water. These were then stored in a cool placed until ready for trimming and slicing.

The embedded blocks were trimmed into rectangles or squares, depending upon the shape of the tissues, attempting to keep opposite sides parallel. These trimmed blocks were mounted, using a hot knife,

onto wooden blocks cut to fit the vise attachment of the microtome used (Swift #650120 microtome). The paraffin blocks were sectioned until the tissues were exposed, to the level of interest, and the tissues then soaked in a Tide detergent solution to soften. The soften tissues were then cooled in the freezer to allow for easier sectioning of the paraffin and 6μ slices were cut. (A Lipshaw, stainless steel microtome blade was used for all sectioning.) Ribbons of tissue were removed to a Fisher Tissuemat water bath ($55^{o}C \pm 1.5^{o}C$) where the sections were flattened. Several consecutive sections of the given tissue were then mounted on pre-cleaned, non-corrosive microscope slides (Sargent) with the use of Mayer's Albumen fixative, which had been personally prepared, according to standard formulations.[30] The slides were then allowed to dry, until excess water had evaporated, and were then heated over an alcohol burner to just melt the paraffin and thus allow the tissue sections to flatten out on the slide surface. The slides were individually coded with a diamond-point marker and stored in wooden slide boxes until ready for staining.

A simple hematoxylin-eosin staining procedure was used for all sections. Harris' hematoxylin was the specific hematoxylin employed, and an adequate amount for all staining was graciously supplied by Mrs. Mae Sunderlin of the Michigan State University Department of Pathology. Eosin Y was obtained from the National Biological Stains Department of Allied Chemical and an alcohol solution prepared according to standard procedures.[30] The staining series employed is diagrammatically shown in Figure 4. The timing in all solutions was standard. After staining was complete, 2 to 3 drops of undiluted Permount (Fisher Scientific Company) were placed over the tissue sections and a #1 24" x 60" Sargent

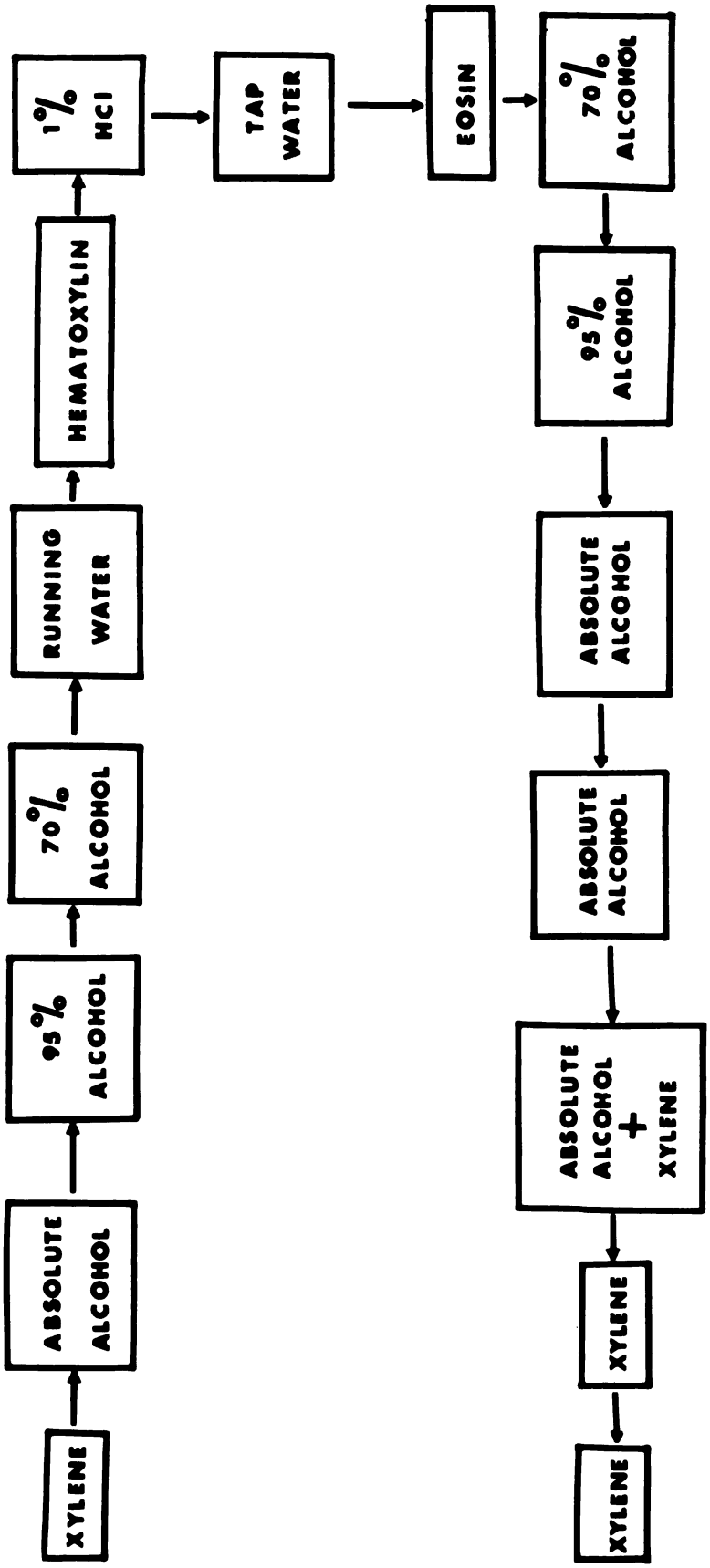

Figure 4: Staining series employed for all slide preparation.

cover glass affixed. After the mounting solution was sufficiently dry (several weeks) the slides were cleaned with xylene and labeled. Histopathological investigation of these tissues was then undertaken with the aid of Dr. Richard Kociba. All pathological changes, as well as their time course, were noted, and representative photomicrographs taken. (The photomicrographs were taken with the use of an American Optical Microstar 10 microscope and camera ensemble---the camera back being produced by Kodak. Kodak Ektachrome-X, EX 135-20 film was used for all photographs.)

RESULTS

LD_{50} Test

The results of the LD_{50} study are shown in Figure 5, as a plot of percent mortality versus the dose level (the dosages being plotted on a logarithmic scale). A straight line is tentatively drawn "through" the data points, as is typically done for such data.[32] It is obvious, however, that this data does not fit a straight line when so plotted. The data point for 16mg/kg deviates most drastically from such a simple curve, and appears to represent a dose level at which some change in physiological treatment of the compound occurs. This test was repeated by another member of the laboratory, and the results showed the same general trend.[33] From the present data, the LD_{50} appears to lie between 14 and 15mg/kg for a one injection dose schedule.

66.6% of the deaths occurring during this study occurred on the 4^{th} day after treatment, with 18% of the deaths on the 5^{th} day and only 3, 2 and 2 deaths respectively on the 3^{rd}, 6^{th} and 7^{th} days following treatment.

Gross Changes Resulting from Treatment

Figure 6 graphically presents the data obtained from the weight studies on the various sets of animals, ranging from complete controls to animals receiving both a tumor and platinum. Each data point

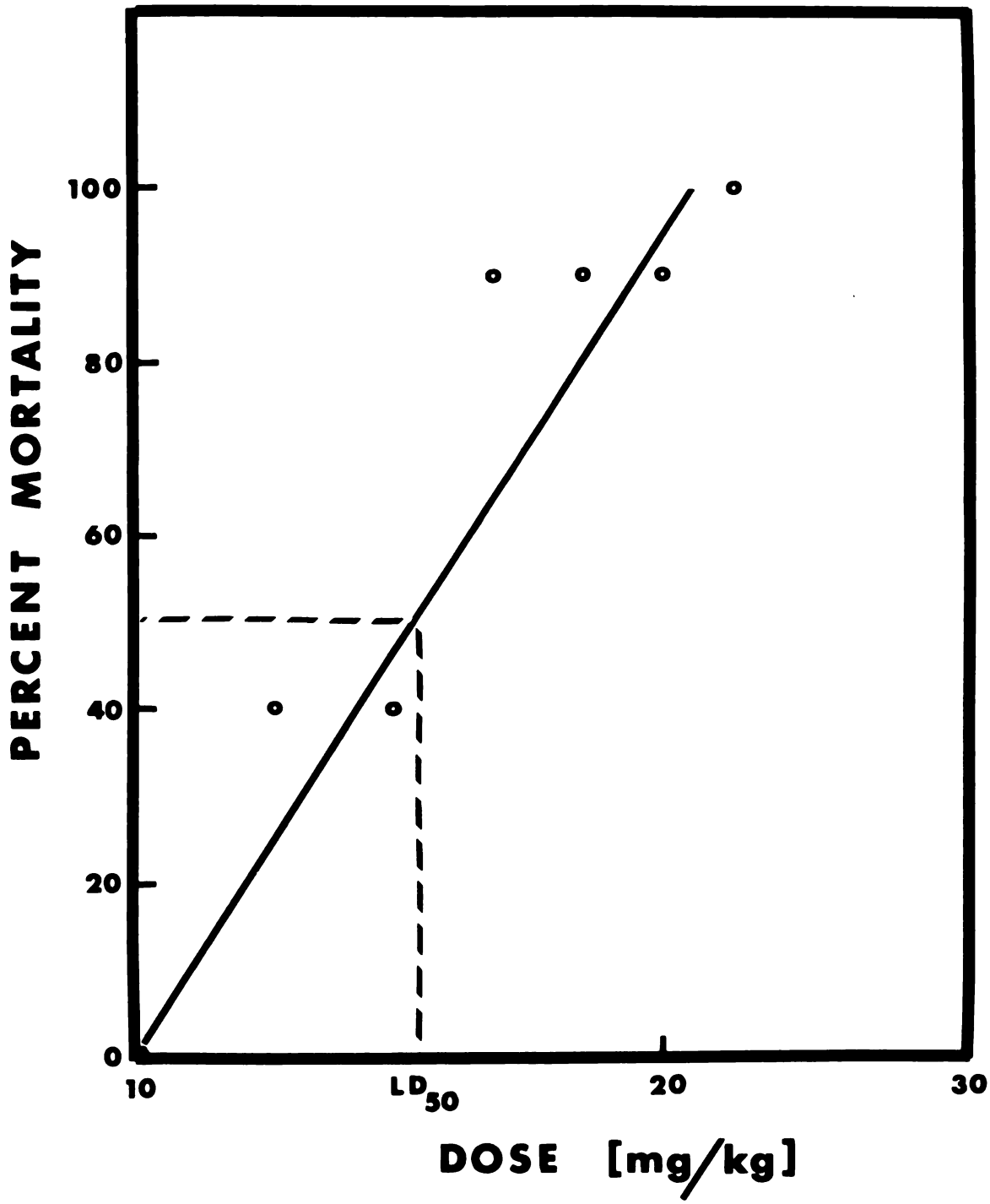

Figure 5: Results of LD_{50} study.

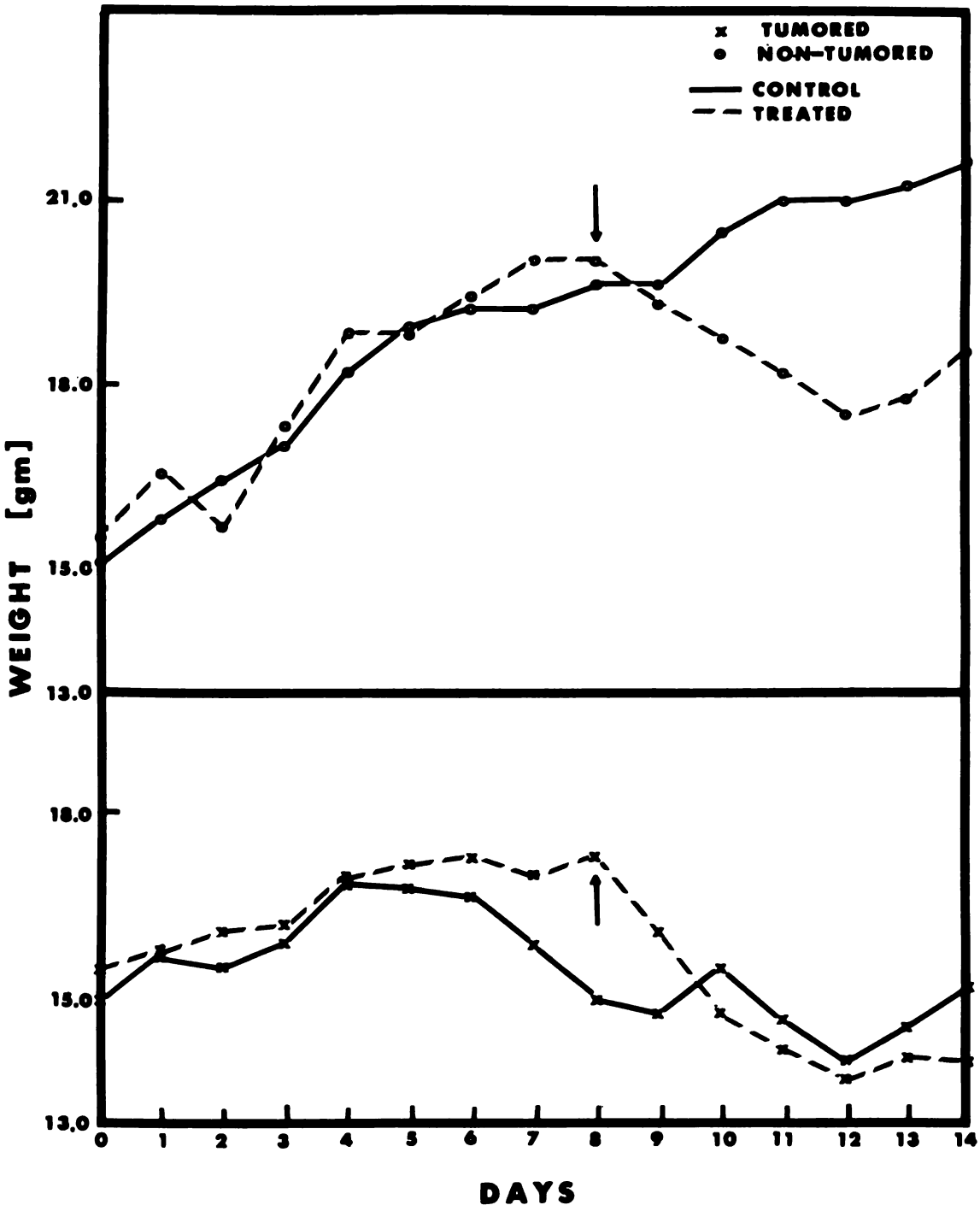

Figure 6: Average daily weights of various groups of mice.

represents the average of 12 animals, individually weighed (except the last points for both tumored animal plots, as one tumored control animal died on day 12 and one treated tumored animal died on day 13). The arrows indicate the respective average weights on the day on which 8mg/kg of the platinum compound was administered. It is obvious that the tumored animals are ill prior to platinum injection, as revealed by the stability or even loss in their average weight during the first 8 days of tumor growth, as compared to the obvious weight gain for non-tumored animals during this same time period. It is also obvious that the treated, tumored animals lose more weight following platinum injection than those animals receiving only platinum---an average weight loss of 2.5 grams for the non-tumored animals and 3.6 grams for the tumored animals. Both groups of treated animals, however, are capable of recovering from the drug effects, as evidenced by the reversal in their average weight plot at 12 days, or 4 days following treatment.

Autopsies were performed during the course of the LD_{50} studies and at the completion of the above-mentioned weight studies. Moreover, observations concerning organ differences were noted when samples were collected for both the activation analysis and histopathology studies. The spleen was an organ in which differences were consistently observed. Following tumor implantation, the spleen increased in over-all size and thickness as the tumor developed, so that for an animal with an 8 day tumor growth, the spleen weighed approximately twice as much as that of a non-tumored animal. Treatment with even a therapeutic dose of cis-$Pt(II)(NH_3)_2Cl_2$ caused a general shrinkage in spleen size, for both the tumored and non-tumored animals, with a gradual return to normal as the animal recovered from the platinum effects. (See Table 2.)

Table 2: Weights (in grams) of various tissues which show changes following injection with 8mg/kg cis-Pt(II)$(NH_3)_2Cl_2$. (Three tissues pooled per measurement.)

		Hours After Injection												
Tissue		1hr	6hr	12hr	24hr	36hr	48hr	60hr	72hr	84hr	96hr	108hr	120hr	132hr	144hr
liver	non-tumored	3.19	3.66	4.18	3.44	3.62	3.37	3.49	3.09	3.27	3.08	3.13	3.13	3.47	3.66
	tumored	3.59	3.25	3.51	3.51	2.53	2.38	2.82	2.19	2.22	1.90	2.34	2.98	2.84	2.79
spleen	non-tumored	0.23	0.23	0.22	0.18	0.18	0.19	0.17	0.18	0.16	0.15	0.15	0.18	0.18	0.19
	tumored	0.48	0.42	0.44	0.36	0.22	0.21	0.18	0.18	0.22	0.17	0.21	0.36	0.37	0.34
endocrines	non-tumored	0.69	0.69	0.79	0.81	0.67	0.71	0.64	0.66	0.62	0.58	0.67	0.68	0.62	0.66
	tumored	0.83	0.56	0.51	0.60	0.32	0.36	0.38	0.37	0.38	0.28	0.27	0.34	0.38	0.41

The thymus appeared similar in size in the non-treated tumored and non-tumored animals, and, in both sets of animals, it responded quite dramatically to platinum treatment, shrinking quite markedly, until it was almost non-existant. (The greatest amount of thymus atrophy was evident in tumored as compared to non-tumored animals.) As with the spleen, the thymus appeared to recover as the animal appeared to grossly rebound from the drug effects.

In animals which received toxic doses of platinum---the LD_{50} test ---post-mortem autopsies revealed distended and hemmorhaged upper intestinal tracts, with food accumulation in a grossly over-stretched stomach. Tumored animals, sacrificed at various times following a therapeutic dose, showed empty stomachs and intestinal tracts, evidencing at least an anorexia even at therapeutic doses.

At toxic doses, moreover, the adrenals appeared shrunken and darker in color than normal and the gall bladder was grossly enlarged. Adrenal effects were never evident at therapeutic doses and gall bladder enlargement rare.

Although not grossly evident, the tissue weights measured for activation analysis purposes revealed that the liver in the tumored animal shrinks somewhat following treatment at therapeutic levels. This can be seen from the values in Table 2. These values were measured for activation analysis studies and represent the weight of three pooled organs per time. The livers of animals receiving toxic doses of platinum were occasionally observed to be grossly shrunken, in post-mortem autopsies.

Most 8 day tumor growths have extensive blood supplies, evident as a complex system of capillaries. The inner portions of the tumor are bloody and necrotic in appearance; the outer portions appearing pink and

"healthy". Following treatment with 8mg/kg cis-Pt(II)$NH_3)_2Cl_2$, it can be observed that a large number of tumors progressively lose their extensive blood supply and the remaining tumor mass appears to be walled off from the surrounding, normal tissue. (A normally-growing tumor, in the forward axillary region, is quite firmly anchored to both the wall of the rib cage and to sub-cutaneous tissue.)

Neutron Activation Analysis

A typical γ-ray spectrum for ^{199}Au is shown in Figure 7. It was plotted after a 20 minute count of a typical 6µg platinum control sample. (Note the ordinate is logarithmic.)

The data obtained from both the tumored and non-tumored animal tissue samples can be expressed both numerically and graphically. Since each data point was obtained from a single pooled sample of tissue, the absolute values may not be statistically representative, although the general trend of values should be typical. Although, in general, all tissues were measured but once at a given time period, three sample values for tumored spleens, obtained 24 hours after injection (three spleens per sample), are available---1.58, 1.49 and 1.59 µg Pt/gm tissue. These values point out the reproducibility of the technique as well as the relative consistency of the platinum distribution.

Tables 3 and 4 present the data, as µg Pt/gm tissue for the tumored and non-tumored samples, respectively, as well as total percent recoveries for the various times. (Since only two complete intestinal tracts were measured at each time, the percent recovery from the three pooled animal tissues would be slightly, though not significantly higher,

if the third intestinal tract had been included at each time.) Figure 8 graphically presents this percent recovery data for both sets of animals. Figures 9 through 15 present the distribution data for the various body organs, directly comparing the values for tumored and non-tumored animals in all cases. Figure 16 presents the distribution data for the tumor.

The peculiar distribution pattern over the first 24 hours following injection---the fall and subsequent rise in the amount of platinum recoverable in the major body organs---suggested the possible retention of the platinum in the blood, combined with some blood element or chemical. Thus, the distribution of platinum in the circulating blood was examined and the results, for both sets of animals, are presented in Table 5 and Figure 17.

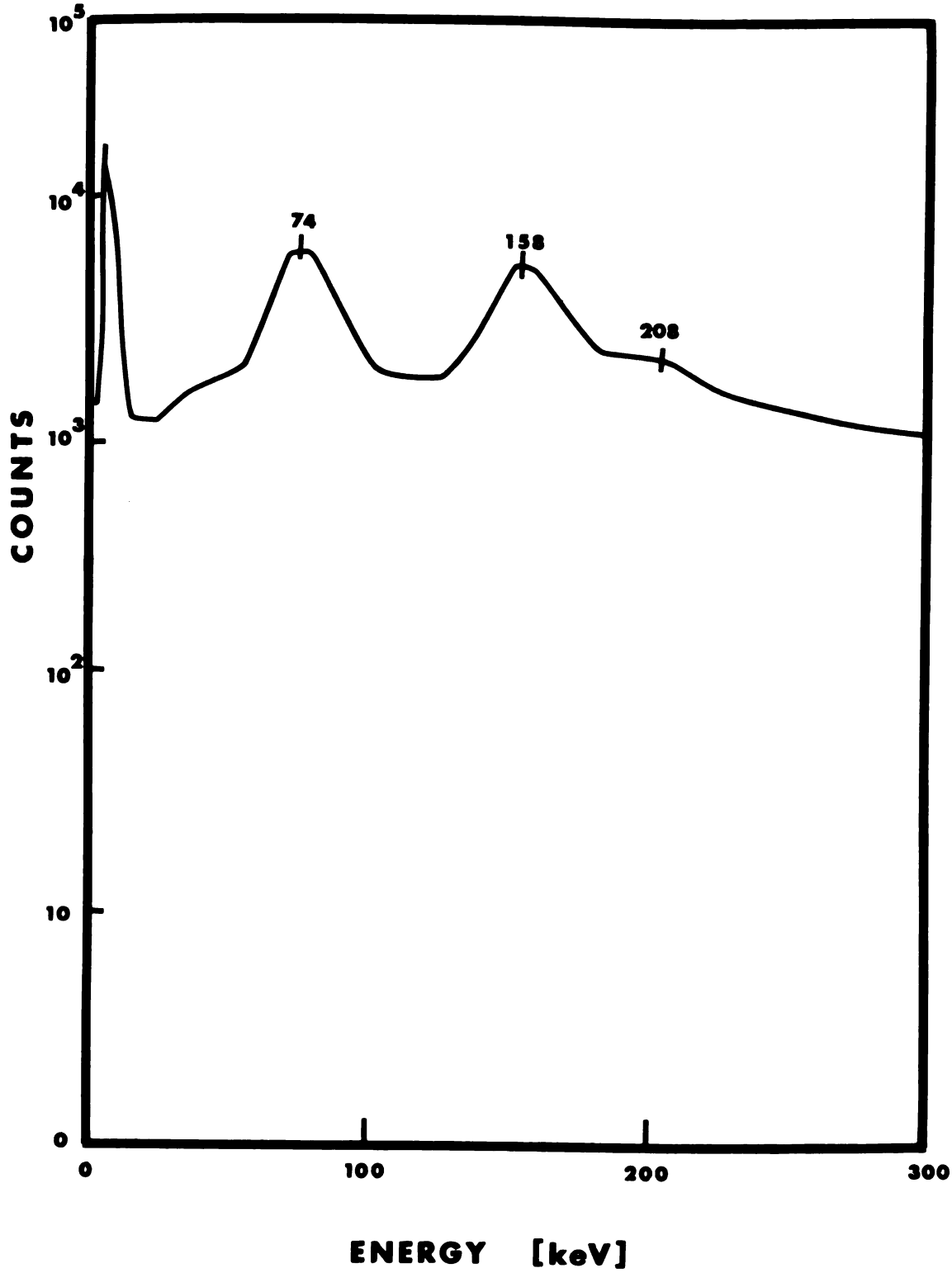

Figure 7: γ-ray spectrum of ^{199}Au.

Table 3: Platinum distribution (μg Pt/gm tissue) in the major organs of the tumored animals.

	Hours After Injection													
Tissue	1hr	6hr	12hr	24hr	36hr	48hr	60hr	72hr	84hr	96hr	108hr	102hr	132hr	144hr
liver + gall bladder	4.62	1.14	2.04	3.44	4.04	0.78	2.61	1.06	1.83	5.02	2.36	0.62	3.28	2.79
intestines + contents	2.21	1.71	1.88	1.78	1.57	1.94	0.60	0.62	0.63	0.63	0.56	----	----	N.D.
stomach + esophagus	3.36	0.98	2.17	0.79	0.65	----	----	----	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
spleen	1.41	0.91	1.17	1.58	1.72	1.69	2.06	1.75	1.29	2.15	1.59	0.22	1.49	1.25
lungs	1.58	1.18	1.29	1.00	0.59	0.42	0.47	0.57	0.96	0.40	0.37	0.19	0.60	0.57
kidneys + bladder	5.10	4.41	4.40	4.68	4.37	2.02	1.57	3.20	2.39	3.22	2.15	1.42	2.91	1.63
endocrines	1.34	2.35	0.54	1.04	1.24	1.96	1.46	0.49	1.50	1.71	1.41	0.63	1.37	1.14
heart	1.04	0.64	0.75	0.71	0.68	0.51	0.52	0.26	1.12	0.41	0.65	0.24	0.39	0.37
brain	----	----	----	----	----	----	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
tumor	1.48	1.15	0.85	1.31	1.15	0.35	0.42	0.55	0.80	0.74	0.64	0.15	0.77	0.70
% Pt recovered	15.2	8.29	7.52	9.03	8.62	4.12	4.44	2.96	3.53	6.10	3.69	1.32	4.85	3.52

N.D. = not done---sample not irradiated as platinum content assumed to have reached a consistent negligible level.

Table 4: Platinum distribution (μg Pt/gm tissue) in the major organs of non-tumored animals.

	Hours After Injection													
Tissue	1hr	6hr	12hr	24hr	36hr	48hr	60hr	72hr	84hr	96hr	108hr	120hr	132hr	144hr
liver + gall bladder	5.36	2.45	3.04	4.48	3.46	3.62	2.22	3.32	2.86	3.08	1.95	2.18	2.22	2.02
intestines + contents	1.94	1.05	0.86	1.00	0.43	0.38	----	----	----	N.D.	N.D.	N.D.	N.D.	N.D.
stomach + esophagus	1.39	0.28	----	----	----	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
spleen	1.65	0.51	1.75	1.29	1.53	1.59	1.19	1.35	1.22	1.29	1.45	1.14	1.24	1.34
lungs	1.70	0.66	1.25	1.19	0.83	0.91	0.64	0.71	0.69	0.58	0.50	0.48	0.37	0.42
kidneys + bladder	4.28	1.98	2.78	4.74	4.07	2.26	1.99	1.76	2.39	2.16	2.13	1.99	2.01	2.00
endocrines	1.45	0.58	0.53	1.29	1.22	0.73	0.75	0.52	1.00	0.66	0.57	0.67	0.62	0.94
heart	1.01	0.26	0.62	0.79	0.59	0.69	0.62	0.37	0.53	0.47	0.43	0.47	0.53	0.53
brain	----	----	----	----	----	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
% Pt recovered	10.4	5.44	6.78	8.24	6.12	5.12	3.28	3.58	3.68	3.66	2.68	2.88	3.06	2.98

N.D. = not done---sample not irradiated as platinum content assumed to have reached a consistent negligible level.

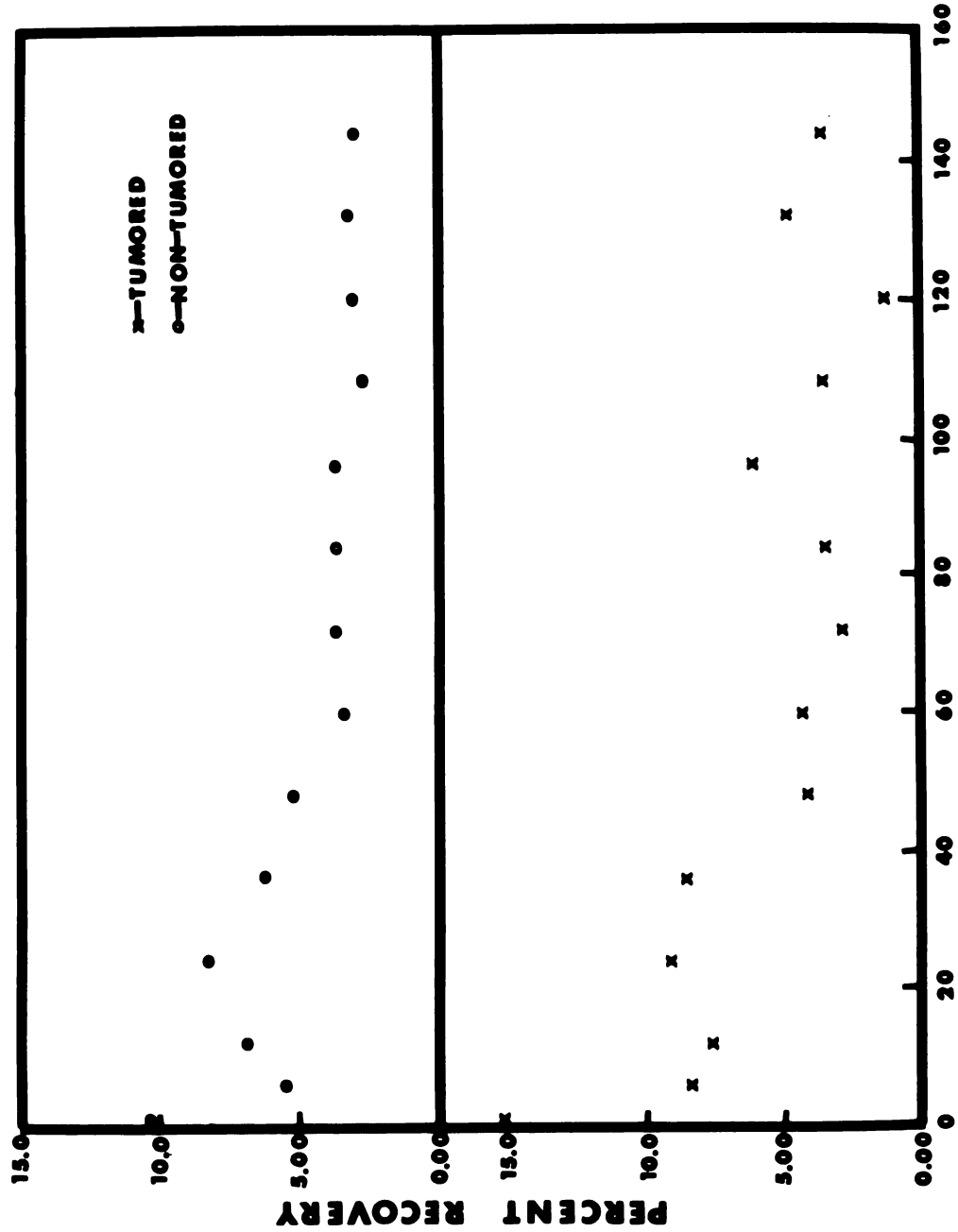

Figure 8: Platinum recovery in both non-tumored and tumored animal tissues.

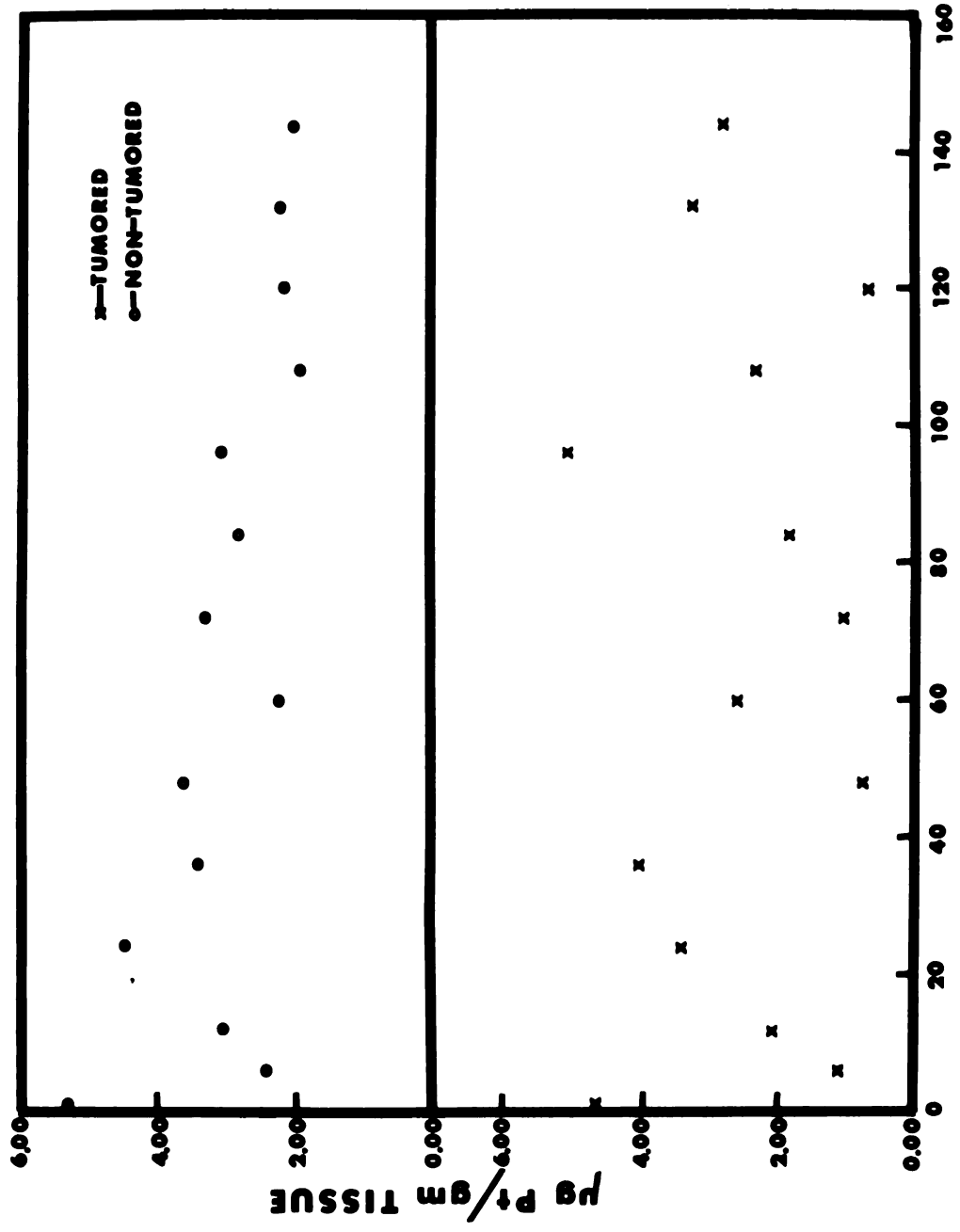

Figure 9: Platinum distribution data for liver + gall bladder samples.

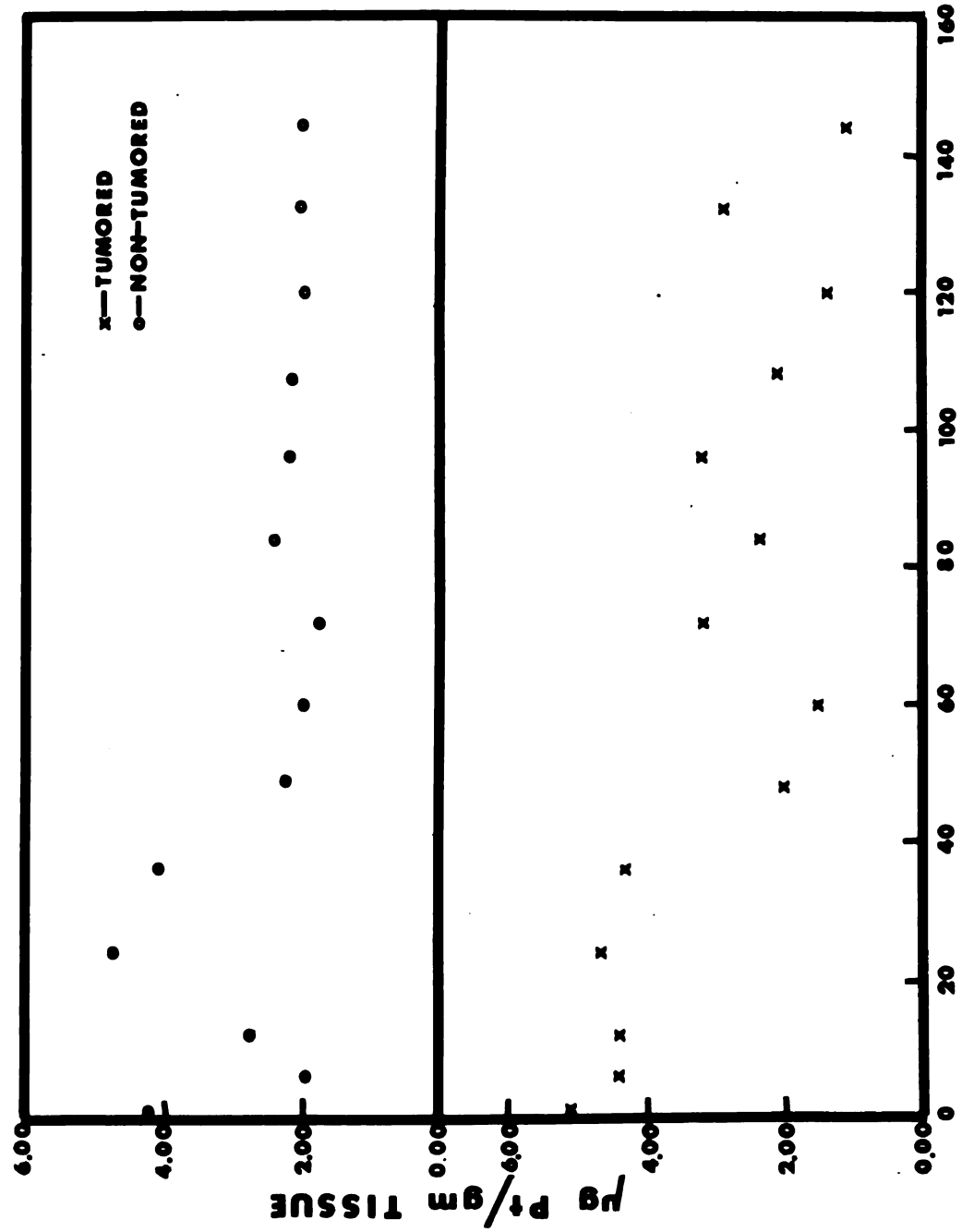

Figure 10: Platinum distribution data for kidney + bladder samples.

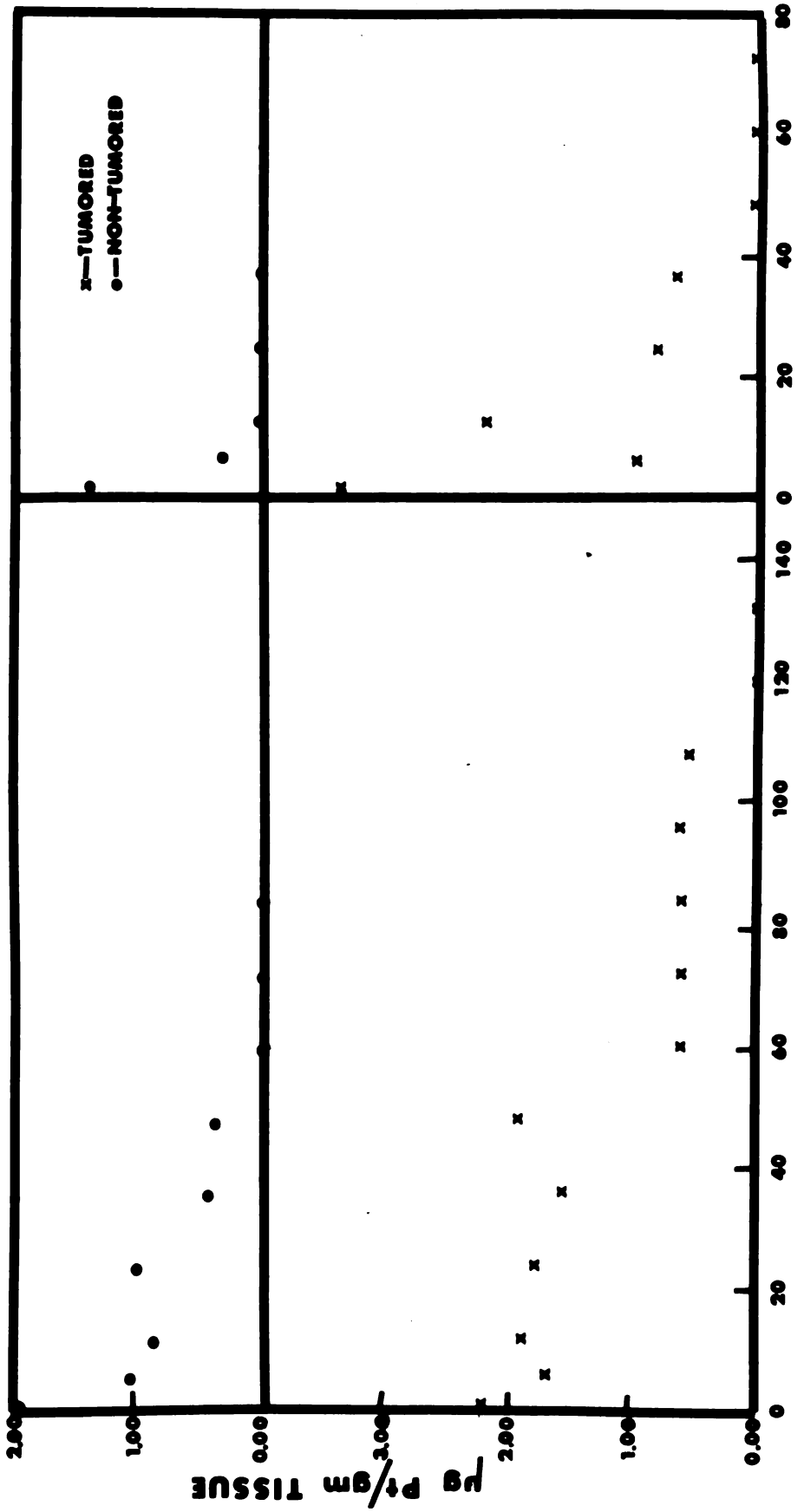

Figure 11: Platinum distribution data for intestines (left) and stomach + esophagus (right) samples.

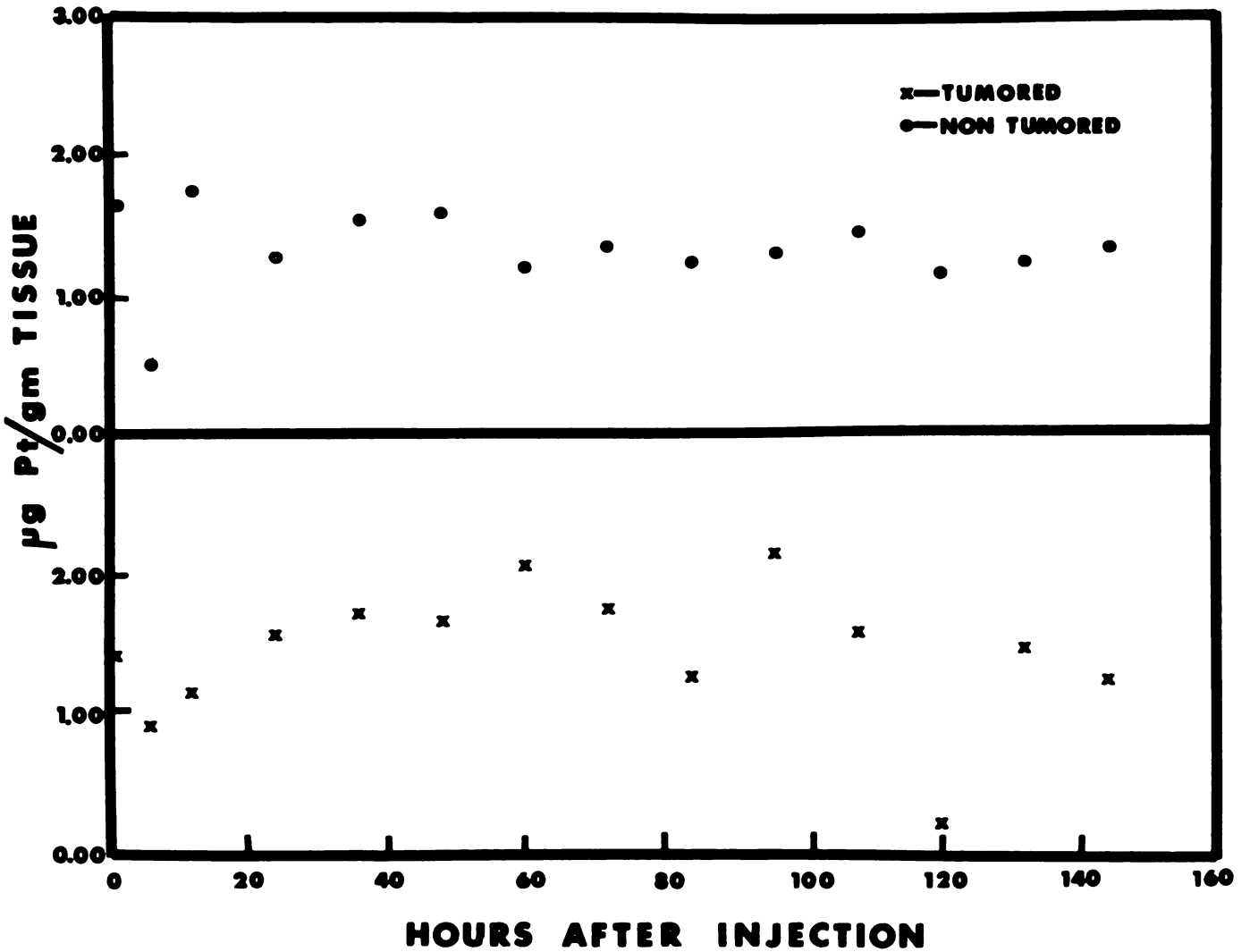

Figure 12: Platinum distribution data for spleen samples.

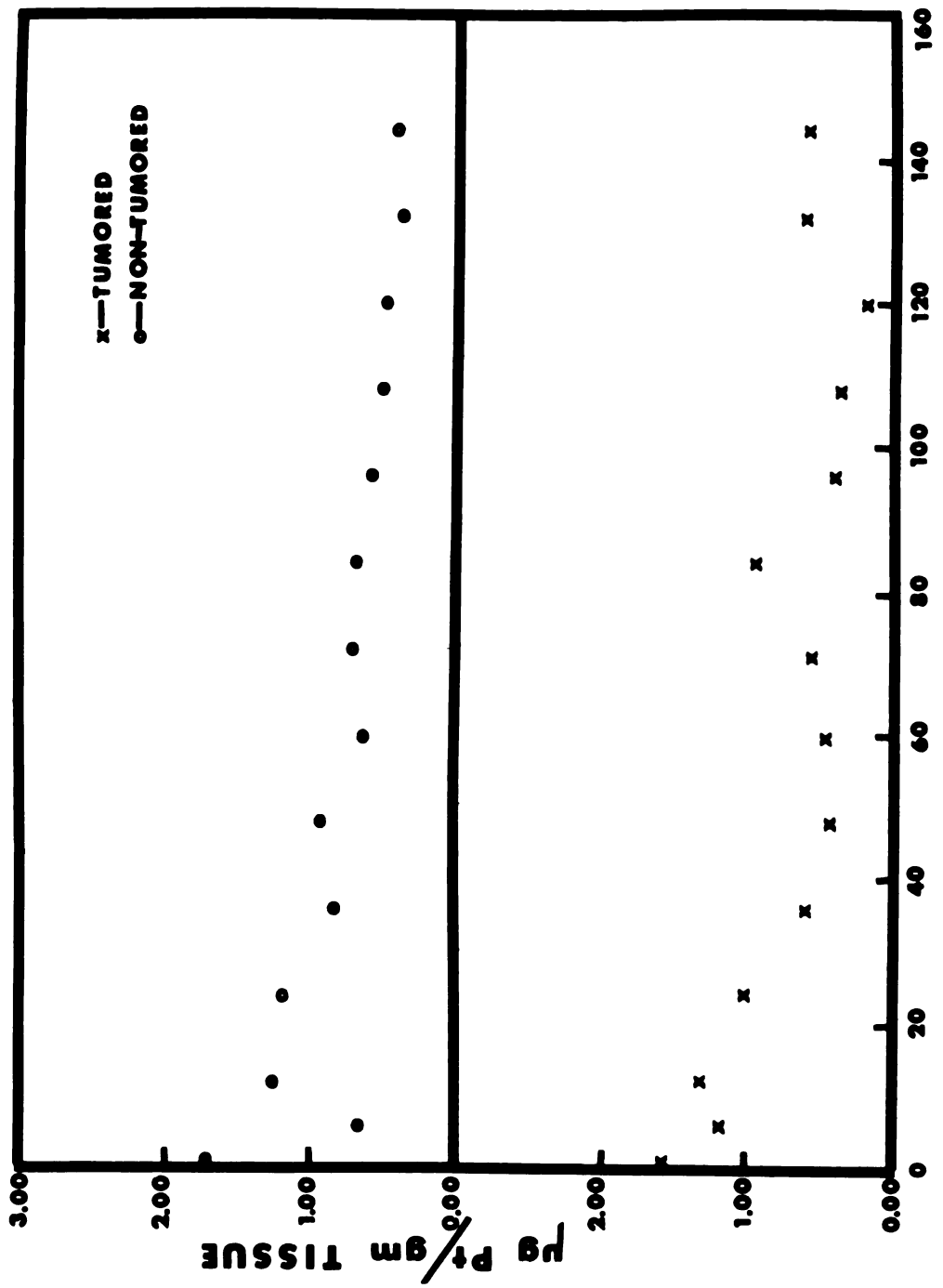

Figure 13: Platinum distribution data for lung samples.

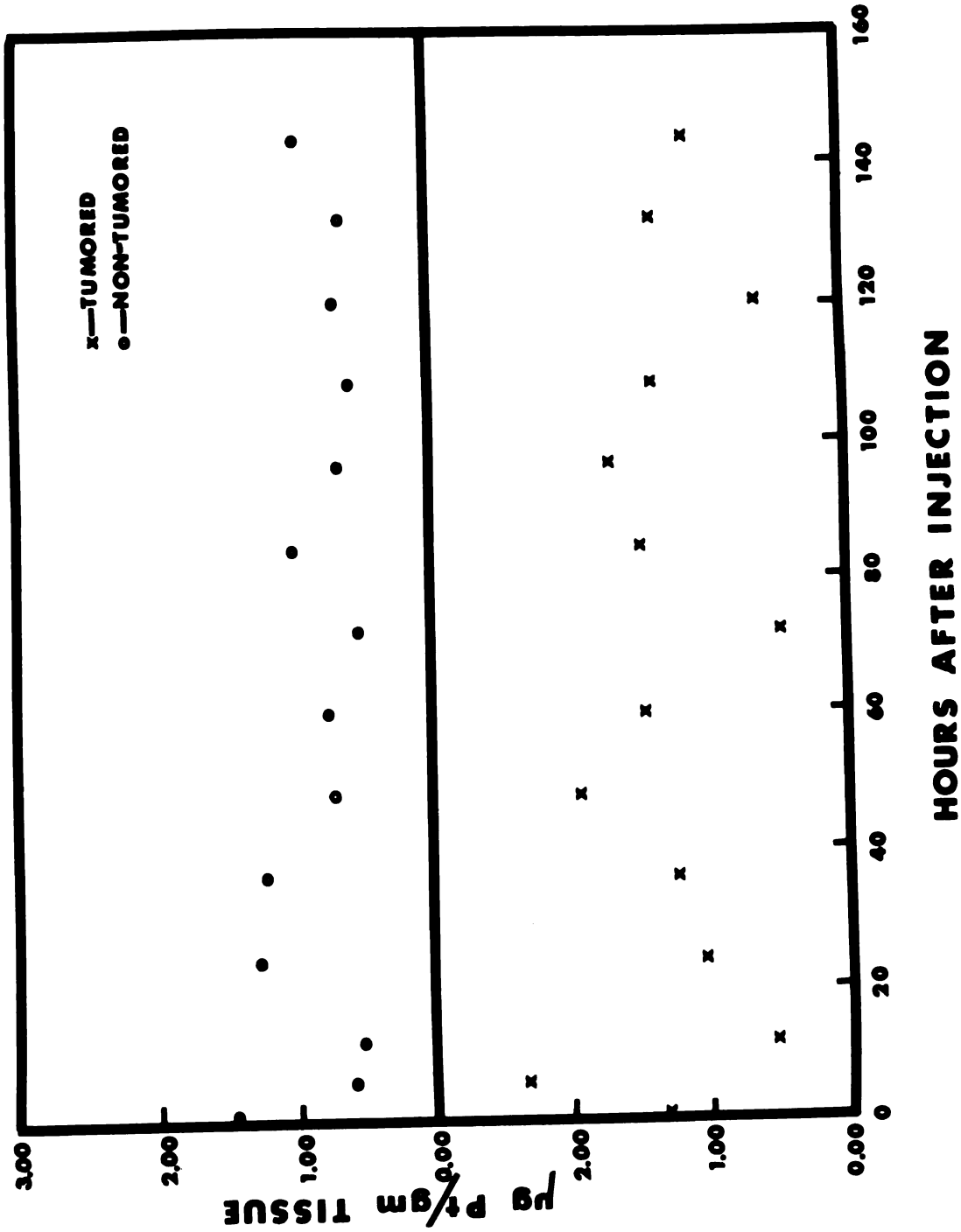

Figure 14: Platinum distribution data for endocrine samples.

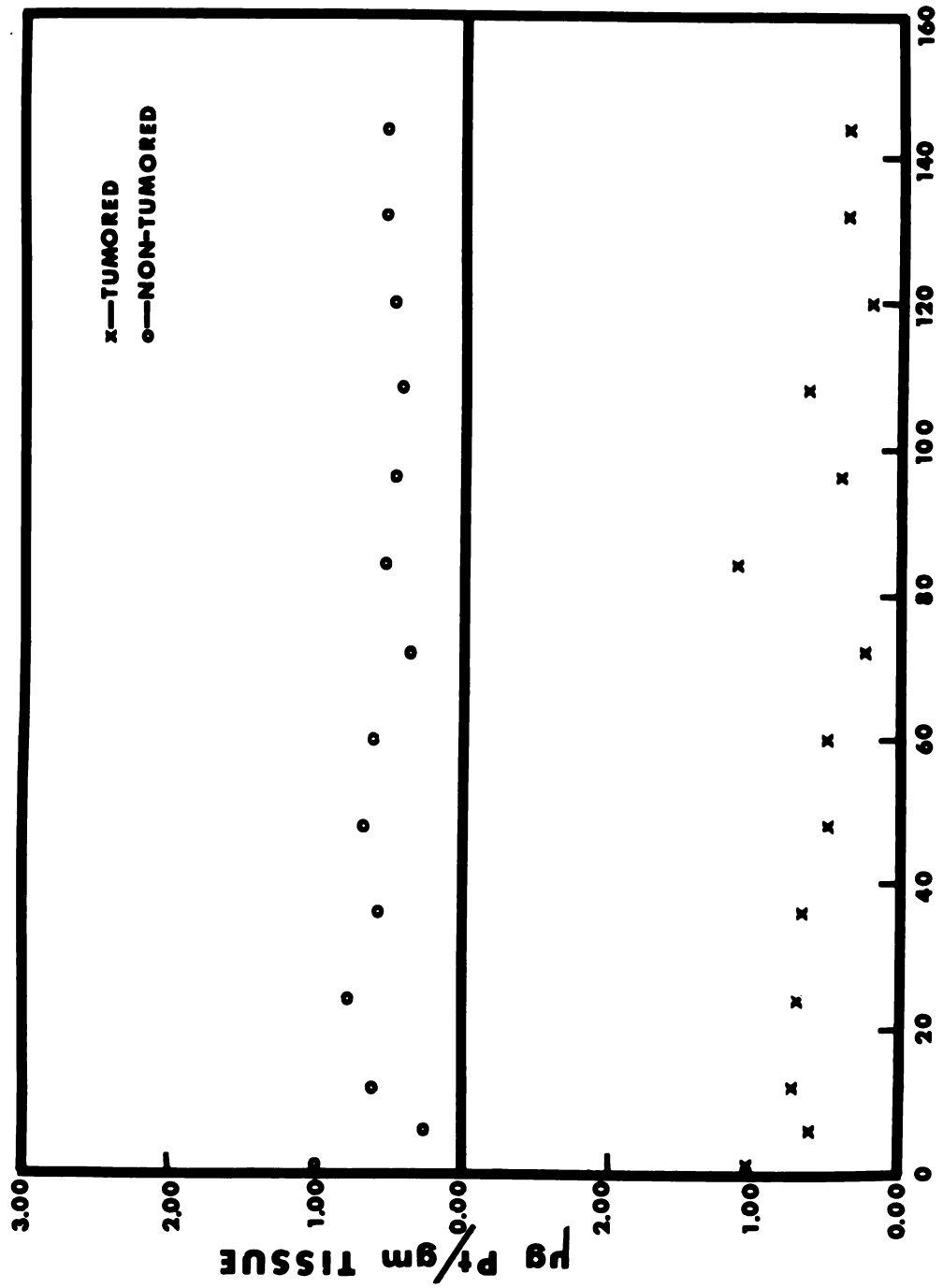

Figure 15: Platinum distribution data for heart samples.

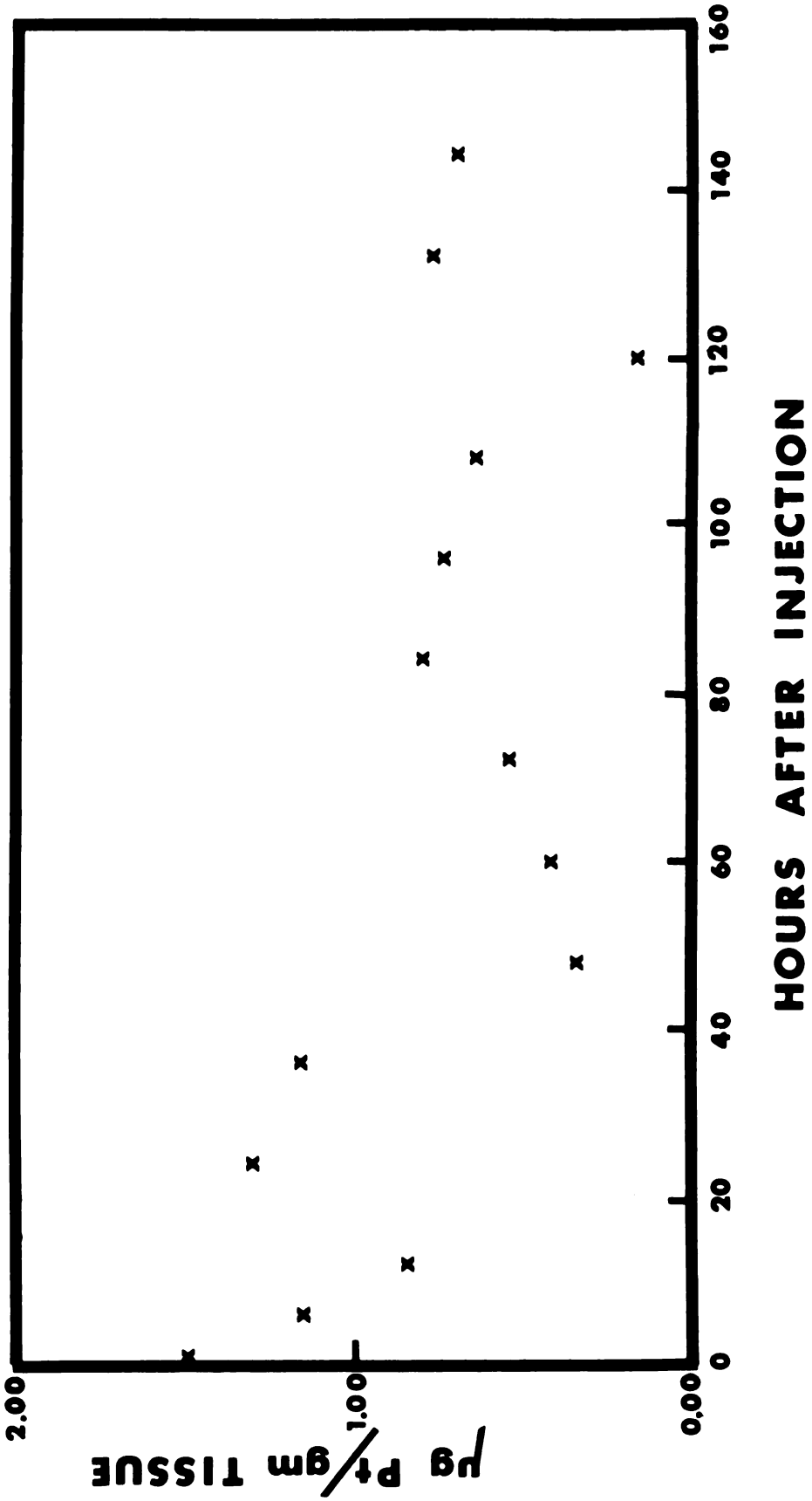

Figure 16: Platinum distribution data for tumor samples.

Table 5: Amount of platinum (μg Pt/cc blood) measured in the blood samples from both tumored and non-tumored animals

Hours After Injection	Tumored	Non-Tumored
1	2.50	3.23
2	1.30	0.60
3	0.83	1.37
4	1.53	2.47
5	1.22	1.67
6	0.90	1.20
8	0.57	0.63
10	0.82	0.57
12	1.00	1.47
16	0.50	0.17
20	1.67	3.53
24	1.63	2.67

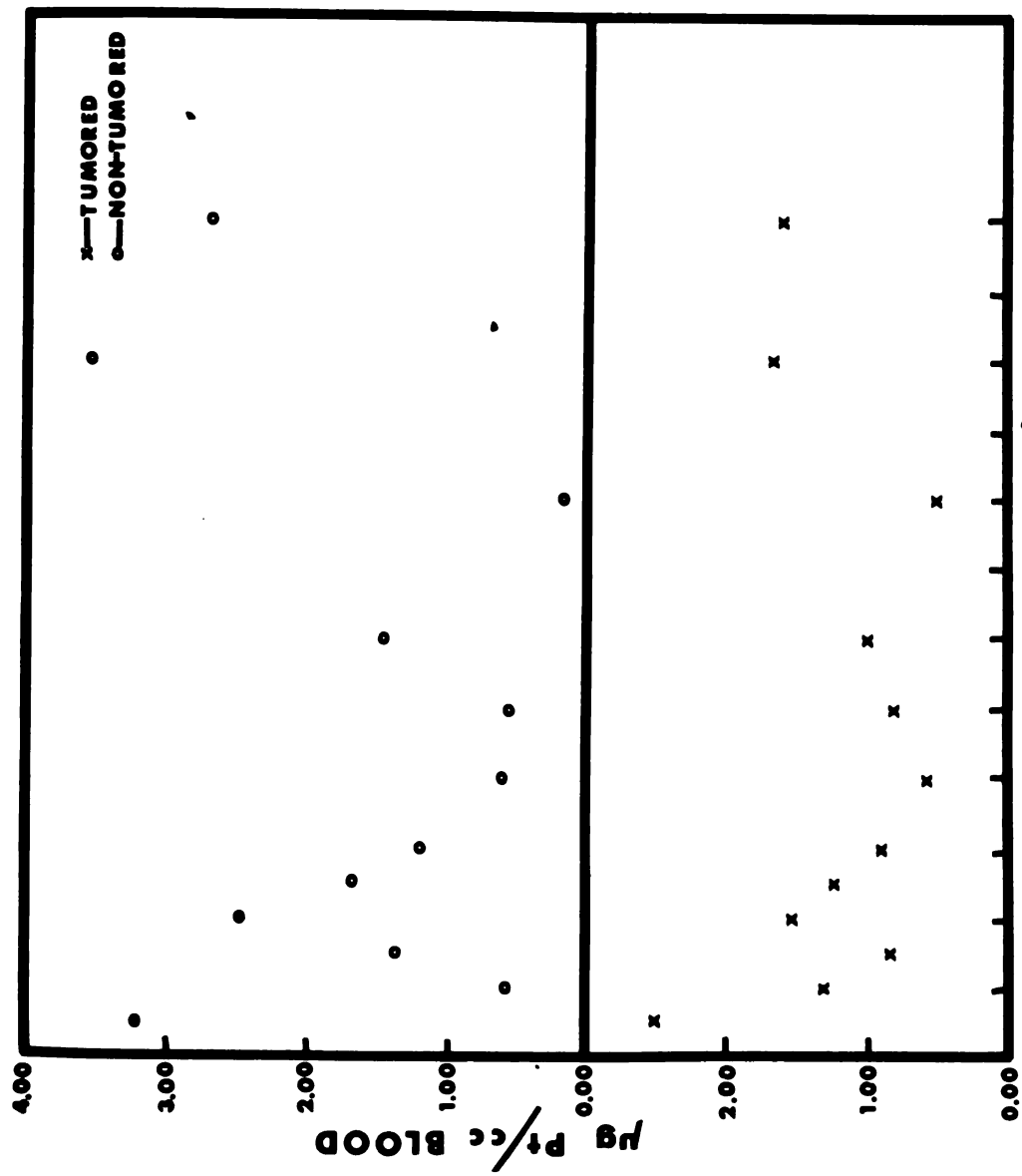

Figure 17: Platinum distribution in the blood.

Histopathology

As with most anti-cancer drugs, $(cis\text{-}Pt(II)NH_3)_2Cl_2$ affects tissues with relatively high turn-over rates most dramatically. In the present study, normal body tissues in this class include intestines, stomach, and the lymphoid elements of spleen and thymus. Tissue samples, from both tumored and non-tumored animals, of pancreas, lung, heart, and urinary bladder showed no histological alterations of any type. Esophagus samples from tumored animals revealed more extensive keratinization than normally appears, at least for the first few days following treatment. However, as mentioned above, anorexia was common in tumored animals following treatment and lack of food passage through the esophageal lumen would easily explain such keratinization.

The adrenal showed no pathological changes for either set of animals. However, a hyperplasia of medulla and/or cortex was evident at various times. This may merely reflect the "stress" conditions platinum treatment produces within the animal. Liver samples, likewise, revealed no damage as a result of treatment. A non-consistent picture of fat accumulation, known as fatty metamorphosis, was seen in various sections of both tumored and non-tumored animal tissue. Its significance is unknown and it is not considered a pathological alteration.[34]

The pathological alterations of the intestinal mucosa were quite marked. Although no changes were evident 1 hour following injection, by 6 hours inhibition of division in the crypt cell regions was complete. Both pyknosis and karyorrhexis of the crypt cell nuclei were evident and the villi were edematous in appearance. By 12 hours after treatment, vacuolization of the crypt region was evident. At this time, the villi

of non-tumored tissue sections were squared in appearance, most probably as a result of a general shrinkage of the villi, resulting from lack of generation of replacement cells in the inhibited crypt regions. In tumored tissue sections, the villi tips appeared to be sloughing into the lumen and their lamina propria areas were edematous. Although the tissue appearance was similar at 24 hours, by 36 hours several mitotic figures were evident in sections of non-tumored tissue. The onset of regeneration in the intestines of the tumored animals was not apparent until 48 hours, however. Regeneration progressed rapidly in both sets of tissue, with a regenerative increase evident. The intestines of the non-tumored animals appeared completely normal, with no remnants of inhibition remaining, by 72 hours after treatment. Debris resulting from earlier inhibition remained for a longer period of time in tumored animals, however, normal appearing sections not found until 108 hours after treatment. Figures 18 through 21 show photomicrographs of normal crypt regions and villi as well as the appearance of these regions following platinum treatment.

Inhibition in the stomach appeared quite similar to that explained above for the intestines. All mitotic activity was inhibited by 6 hours and the rugae and gastric pits became shrunken in appearance. However, by 24 hours for the non-tumored animal and 36 hours for the tumored animal, regeneration was evident and a normal appearing section was again evident by 48 and 60 hours, respectively. (See Figure 22 for a comparison of a normal and inhibited sections of stomach.)

Figure 23 shows a normal section of thymus tissue. Note the thick outer section (cortex) which appears quite heavily stained. This region contains large numbers of closely packed lymphoid elements. Following

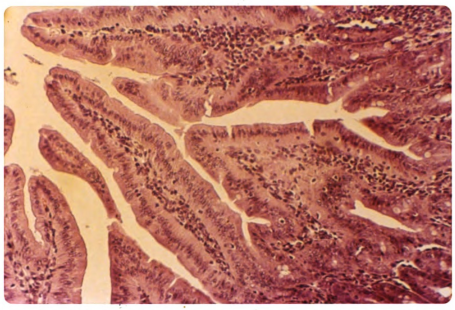

Figure 18a: Villi of control small intestine section. Magnification = 60x.

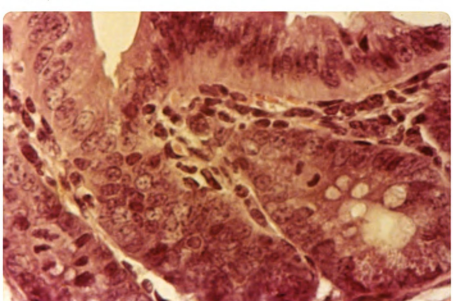

Figure 18b: Crypt region of control small intestine section. Magnification = 240x.

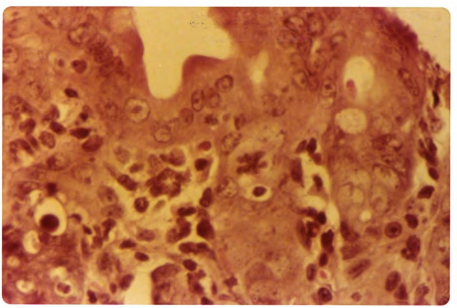

Figure 19: Crypt region of treated small intestines. Tumored animal, 48 hours after treatment. Magnification = 240x.

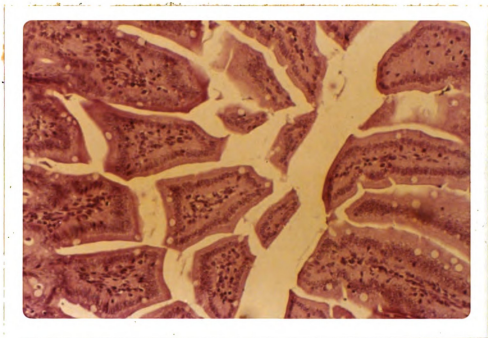

Figure 20: Squared villi of treated small intestines. Non-tumored animal, 12 hours after treatment. Magnification = 60x.

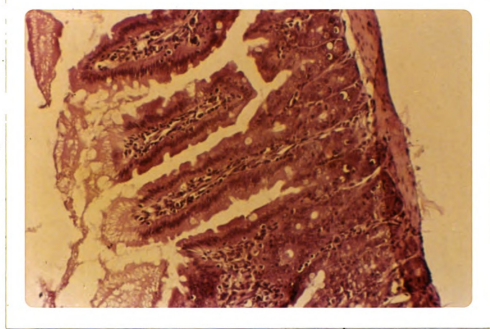

Figure 21: Sloughing villi of treated small intestines. Tumored animal, 12 hours after treatment. Magnification = 60x.

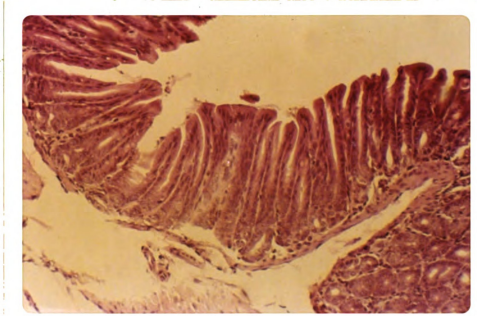

Figure 22a: Control section of stomach. Magnification = 60x.

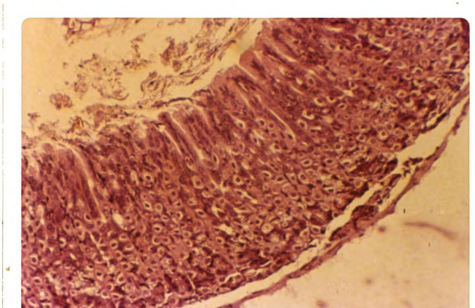

Figure 22b: Section of treated stomach. Non-tumored animal, 12 hours after treatment. Magnification = 60x.

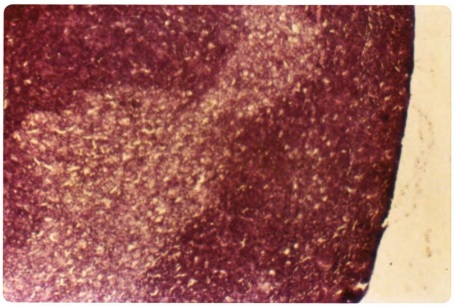

Figure 23: Control section of thymus. Magnification = 24x.

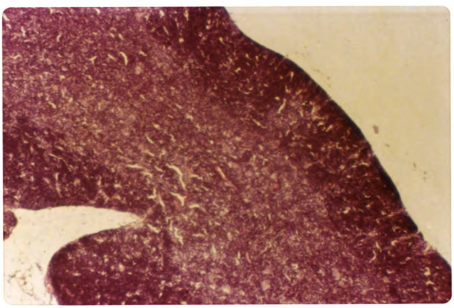

Figure 24: Section of treated thymus. Non-tumored animal, 36 hours after treatment. Magnification = 24x.

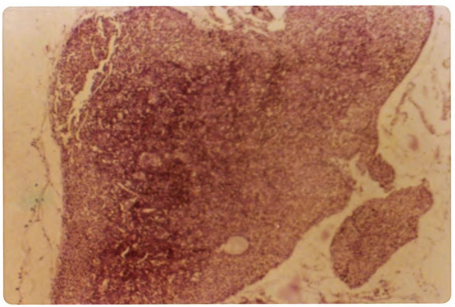

Figure 25: Apparent reversal of thymus regions. Tumored animal, 108 hours after treatment. Magnification = 24x.

treatment with platinum, these elements are greatly depleted. In the non-tumored animal this depletion never progresses beyond that illustrated in Figure 24 (maximum at 36 and 48 hours), and regeneration is evident by 60 hours, with the normal appearance again obvious at 108 hours. In the tumored animal, however, depletion progresses considerably beyond this point, until the section shows an apparent reversal of regions. This apparent reversal results from such a dramatic depletion of lymphoid elements in the cortical region, that the scantily populated medullary region becomes the area of greatest cellularity. (See Figure 25.) This apparent reversal was first evident 36 hours following treatment. Regeneration appeared obvious from 48 to 72 hours, but an apparent reversal was again evident from 84 to 120 hours. Regeneration did again occur, following this second depletion, but no normal appearing sections were yet obvious at the longest time interval examined (144 hours).

The white pulp of the spleen, as the cortical aspect of the thymus, is largely lymphoid in composition. It exists as relatively distinct regions, separated by the splenic red pulp---the region of extramedullary hematopoiesis, particularly in young animals. (Figure 26 is a photomicrograph of a section of normal spleen.) Although descriptions of the progressive development of Sarcoma 180 in mice, indicate no metastatic invasion and only rare local invasion into the lungs[35,36], sections of spleen from animals with 8 day or older tumor growths revealed the presence of neoplastic invasion. In one animal, the metastatic tissue had completely obliterated the normal splenic architecture. However, most spleens merely contained metastatic nodules admixed with normal splenic tissue---as shown in Figure 27.

Treatment with a therapeutic dose of cis-Pt(II)$(NH_3)_2Cl_2$ resulted

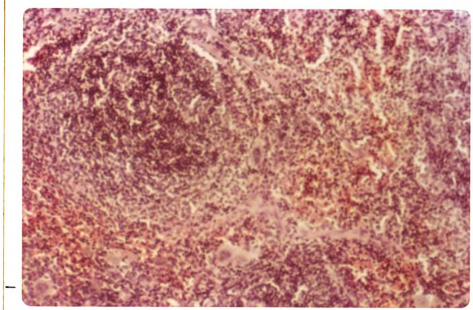

Figure 26: Control spleen. Magnification = 60x.

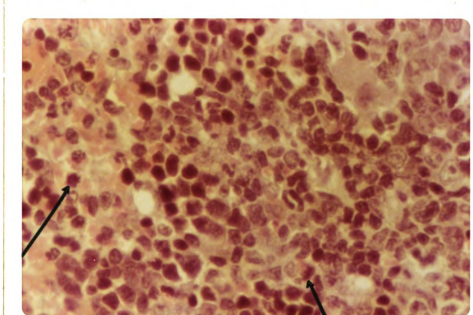

Figure 27: Control spleen of tumored animal. Magnification = 240x. (Arrows indicate neoplastic cells.)

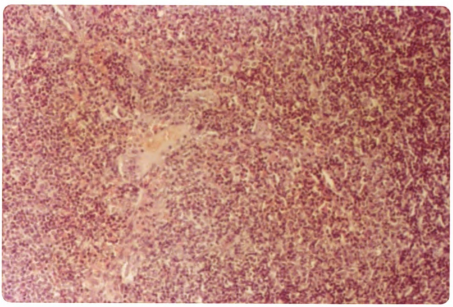

Figure 28a: Section of treated spleen. Non-tumored animal, 12 hours after treatment. Magnification = 60x.

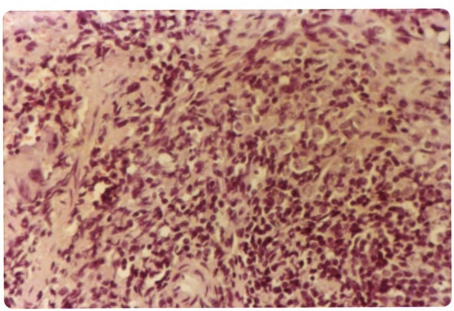

Figure 28b: Section of treated spleen. Tumored animal, 84 hours after treatment. Magnification = 120x.

in a depletion of lymphoid elements and a destruction and inhibition of the hematopoietic elements, in both tumored and non-tumored animals. In the tumored animal tissue, any and all neoplastic tissue was likewise inhibited. As with all other inhibited tissue, splenic regeneration rapidly occurred (evident by 24 hours in non-tumored samples and 36 hours in tumored samples). However, unlike the other tissues, the normal appearance of the spleen was not recovered in the time interval examined. Instead, a hyperplasia existed, of both the lymphoid elements and those of the extramedullary hematopoietic system, as long as 144 hours following treatment, with no leveling off of these elements evident.

Although not an organ whose tissue normally shows rapid turn-over, the kidney also showed some indications of pathological damage. Unlike the above-mentioned tissues, however, no damage was evident until 24 hours following treatment. A capillary congestion, in both cortical and medullary regions (mainly medullary), became obvious at that time. This persisted and several instances of pyknosis and vacuolization of the cells of the tubular epithelium were observed. Tubular epithelium, so damaged, eventually sloughed into the tubular lumen and was replaced. Such tubular damage, however, was minimal. See Figures 29 through 31 for control and treated sections of kidney.

The changes in the tumor tissue, subsequent to platinum treatment, were similar to those obvious for all tissues with rapid turn-over rates. Mitotic activity was inhibited and nuclear degeneration was evidenced by pyknosis and karyhorrexis. Unlike the other tissues, however, abnormal mitotic figures could be seen in treated tumor sections (Figures 33, 34 and 35b). Moreover, the general appearance and staining properties of the entire cell changed, as evident from a comparison of the control

and treated sections. (Figure 35a shows a capsule forming around the outer aspects of the tumor mass.)

Tissue sections, prepared from tumors excised 132 and 144 hours following treatment, appeared almost identical to control tumor sections. Thus, as with all other affected tissue, the tumor is capable of regeneration, under the proper conditions.

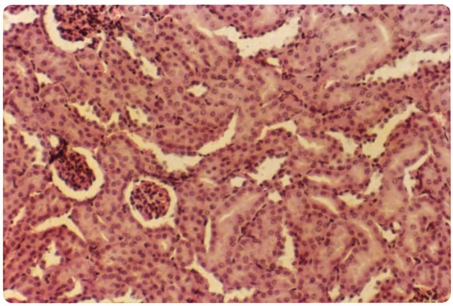

Figure 29: Control section of kidney. Magnification = 60x.

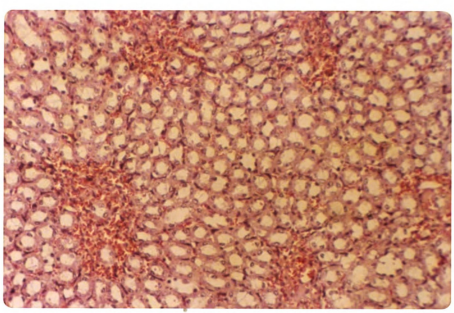

Figure 30: Capillary congestion in medullary region of kidney. Non-tumored animal, 24 hours after treatment. Magnification = 60x.

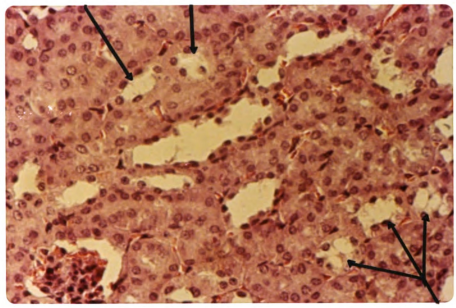

Figure 31: Tubular sloughing in treated section of kidney. Non-tumored animal 48 hours after treatment. Magnification = 120x. (Arrows indicate sloughing tubular epithelium.)

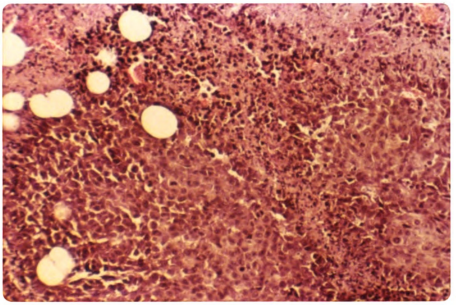

Figure 32a: Control section of tumor. Magnification = 60x.

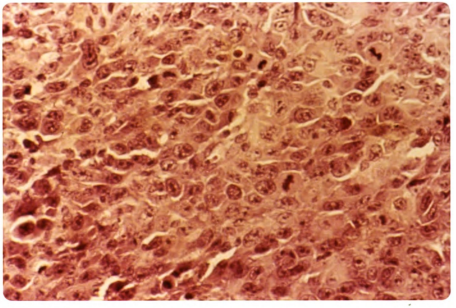

Figure 32b: Control section of tumor. Magnification = 120x.

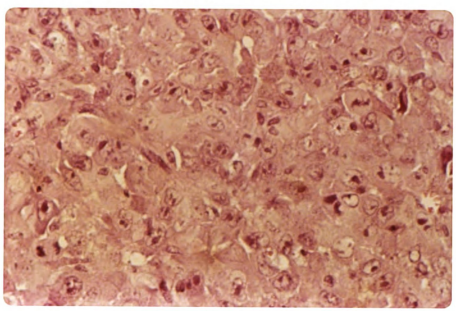

Figure 33: Section of tumor, 48 hours after treatment. Magnification = 120x.

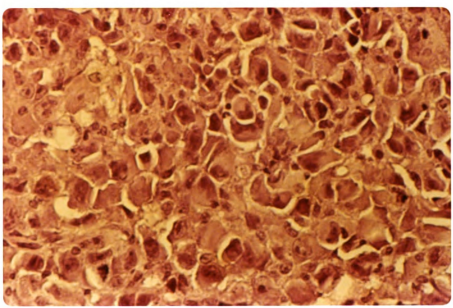

Figure 34: Section of tumor, 72 hours after treatment. Magnification = 120x.

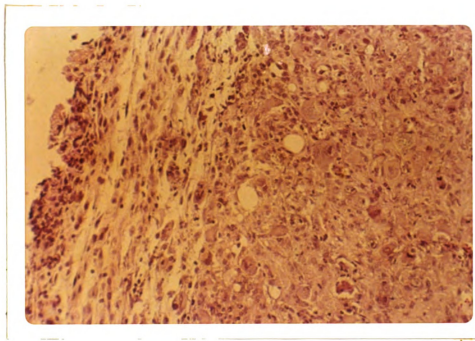

Figure 35a: Section of tumor, 120 hours after treatment, showing formation of a capsule. Magnification = 60x.

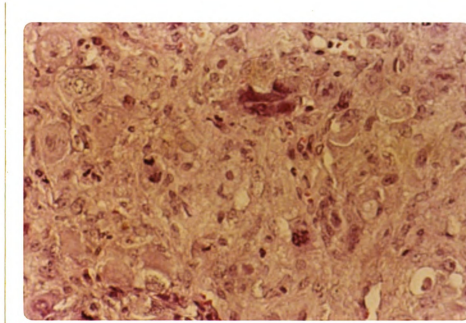

Figure 35b: Section of tumor, 120 hours after treatment. Magnification = 120x.

DISCUSSION

Platinum Distribution

It is readily obvious from the distribution data that there is no specific uptake into tumor tissue. In fact the greatest portion of the platinum was found in the filtering (liver and spleen) and excretory (kidney mainly) organs. This raises a problem as to how the platinum compound, generally inhibitory to all rapidly proliferating tissue, can successfully regress both small and large established tumor growths without permanent damage to such vital tissues as bone marrow, intestinal and gastric mucosa and lymphoid tissue. A readily obvious solution to this dilemma is the suggestion that, for some as yet unknown reason, tumor tissue is more sensitive to the action of the platinum compound than is normal body tissue. Although no conclusive evidence for this postulate exists, the finding that primary tissue culture lines appear less sensitive to platinum inhibition than transformed lines, and are apparently reversibly inhibited while transformed lines appear to respond irreversibly,[23] gives such a suggestion at least some probability.

The data also reveal a low total percent recovery of platinum. (This figure was calculated using the total amount of platinum injected into the three animals, sacrificed at the given time interval, as the base value.) As mentioned above, if the platinum content of the third intestinal tract, for the given set of tissues measured, was included, the percent recovery would be somewhat, though not significantly, higher.

Unfortunately, not all parts of the test animals were assayed for platinum content. Data with rats indicate a marked affect of the platinum on bone marrow.[37] Thus, some platinum would most probably be found in the bone marrow, at least for the first day or so following treatment. The amount, however, may not be significant, as present data shows little if any relationship between tissue damage and platinum content. Moreover, the animals' skeletal muscle; bone, nails and teeth; and skin and fur may also obtain various quantities of platinum. Despite such unmeasured possibilities, even the highest percent recovery actually attained appears considerably low. This and the high, early levels of platinum in the liver and kidneys suggest a high, rapid excretion rate via the urine. Such a suggestion would also satisfactorily explain the delayed minimal renal damage, which is more suggestive of a constant level of irritation than of a direct renal toxicity. Whole body tests with a radioactive platinum isotope, to measure the total amount of platinum remaining within the animal at any given time following injection, are soon to be accomplished and should provide valuable information along this line.

The distribution pattern for the first 24 hours after treatment---the fall and subsequent rise in total platinum recovered---presents some interesting possibilities. Earlier suggestions of retention of the platinum in the circulating blood can be ruled out from the distribution pattern measured from the blood samples. It is possible, however, that the platinum combines with some element of the serum and is shunted to the periphery as a result of an initial, temporary edema. Most of the animals are somewhat sluggish following injection, so that a temporary, mild edema cannot be completely ruled out from presently available data.

It is also possible that there is a temporary peripheral shunt of the platinum---to skeletal muscle, skin, etc.---and that redistribution throughout the major body organs is rapidly accomplished. Any and all such suggestions are, however, tentative and further information concerning the in vivo reactivity of the platinum compound and its total metabolism scheme is necessary before anything further can be ascertained in this regard.

Complete absence of platinum in brain tissue suggests that, by as early as 1 hour after injection, the initially neutral platinum compound has been converted into a charged molecule, incapable of passing the blood-brain barrier. (Early entrance into the brain tissue, with subsequent exist, of minute quantities of neutral compound, cannot be completely ruled out, however.) The ease of reaction of cis-$Pt(II)(NH_3)_2Cl_2$, as a result of the extreme lability of its chloride ions, argues for rapid and complete reactivity of the initial compound with reactive biological molecules present in the blood or even the intraperitoneal fluid. The actual rate of reactivity and the compound or compounds formed in vivo are presently unknown, however, although various workers are presently investigating such problems.

Another interesting feature of the distribution data is the obvious cyclic nature of the platinum distribution in the livers of both tumored and non-tumored animals. The fact that a rhythmic response should be revealed for data concerning rodent liver, in general, is not unusual or surprising. Various mouse liver activities and properties show characteristic circadian rhythms---phospholipid, RNA and DNA metabolism; mitotic activity; glycogen content.[38] The stimuli which govern the expression of such rhythms arise within the animal rather than from any

cyclic aspect of the environment---daily cycles of light and dark of high and low temperatures merely regulating these rhythms so that they coincide with the 24-hour cycle of the natural environment. (For instance, a nocturnal animal subjected to constant light does not alter the periodicity of its daily activity period but merely progressively shifts its phase---i.e. shows a daily delay in onset of activity of about 30 minutes. Such a phase shift is continued only as long as the animal is maintained on a constant light regimen.)[39]

The real point of interest concerning the cyclic nature of the hepatic data is the fact that while the established rhythm for the non-tumored animals appears to have a 24-hour cycle, that of the tumored animals appears to have a 36-hour cycle. Is the shift in periodicity a result of the tumor or a combination of the platinum and the tumor? In this regard it is interesting to note that various workers in the field suggest that maintenance of circadian behavior in mammals may depend upon feedback to the central nervous system from periodic sequences of peripheral metabolic events, that are loosely locked in phase with each other via specialized hormonal (and autonomic nervous) controls. Thus, a given rhythm in an animal results from mixing and weighing of psychogenic, neurogenic, hormonal and metabolic factors as well as physicochemical and socioecological signals from the environment.[40] Thus, it seems possible that the 12-hour periodicity shift observed in liver samples from tumored animals could well reflect various metabolic, hormonal, neural changes resulting from the growth of the tumor and/or in combination with the platinum treatment. Since the cycle obvious in the livers of non-tumored animals is the common circadian rhythm, it would appear that tumor effects, or alterations in such effects by platinum treatment,

are most likely responsible for this shift in periodicity.

With regard to drug administration in general, it is interesting to note that the phase of the 24-hour changes in physiologic state can critically determine survival from noxious agents---i.e. periodic changes in susceptibility to a given agent occur. Moreover, such periodic changes in susceptibility are not the same from one agent to another.[40] It would be pharmacologically important if certain therapeutic-toxic ratios also change periodically.

Returning to the distribution data, it is obvious that measurable amounts of platinum are still present 144 hours or 6 days following treatment. That the animal has recovered from the initial inhibitory effects of cis-Pt(II)$(NH_3)_2Cl_2$ by this time, suggests that either the remaining platinum is no longer in a biologically active form or that its intracellular level is below threshold value for effectiveness. If the platinum is biologically inactive and cannot cumulatively act as a typical heavy-metal poison, no major problem exists. If, however, the level is merely sub-threshold or if the inactive platinum species can cumulatively act as a heavy-metal poison, dose schedules requiring several injections of the platinum compound may result in enhanced physiological damage. Further studies in this regard, are, therefore, necessary.

It would seem to be of major interest to compare the present distribution data with that for various other potent anti-cancer agents, as well as metallic elements normally present in the body. Such comparison is, however, difficult since the individual workers do not follow any general procedure for the presentation of their distribution data. Therefore, in order to be able to present a few pertinent comparisons,

the author has taken the liberty of converting all data presented to a consistent numerical system. For tumored animal samples, the value obtained for the tumor sample was arbitrarily given the value 1.00 and the values for all other tissues presented relative to the tumor value. For non-tumored animal systems, the value for the liver, the largest of the body organs, was arbitrarily given the value 1.00. Table 6 shows the pertinent portion of the platinum data evaluated according to such a system. It is readily obvious that the filtering and excretory organs contain the greatest percentage of platinum measured. Although the values for the tumored data are considerably different from the non-tumored, since the comparatively low tumor value was here used for a basis, the ratio of liver values to the other tissue values can be easily seen to lie within the same area for both sets, for most of the tissues.

Table 7 presents similar data, 24 hours after injection, for triethylenephosphoramide-^{32}P (TEPA), a potent alkylating agent. (All injections were given intraperitoneally.) The values for non-tumored animals show a more general distribution of this drug as compared to the platinum compound. Although the filtering and excretory organs do contain a considerable portion of the drug still present in the body, other organs measured contain relatively high portions also. The distribution for the solid tumors---the Sarcoma 37 and Lymphosarcoma 1---, however, are quite similar to the values for the platinum compound in Sarcoma 180-bearing animals. The different pattern for the leukemic animals---animals with L-1210-—may merely result from the more diffuse nature of the neoplasm. For all these systems, 60 to 80% of the ^{32}P was excreted within 24 hours. The major pathway for excretion was via the kidneys, with very little radioactivity found in the feces.[41] (Thus, a

Table 6: Comparative representation of pertinent cis-Pt(II)$(NH_3)_2Cl_2$ distribution data.

	Hours After Injection											
	Non-tumored						Tumored					
Tissue	1hr	6hr	12hr	24hr	36hr	48hr	1hr	6hr	12hr	24hr	36hr	48hr
liver + gall bladder	1.00	1.00	1.00	1.00	1.00	1.00	3.12	0.99	2.40	2.62	3.50	2.22
intestines + contents	0.36	0.43	0.28	0.22	0.12	0.10	1.49	1.49	2.21	1.36	1.19	5.55
stomach + esophagus	0.26	0.11	----	----	----	----	2.27	0.85	2.58	0.60	0.57	----
spleen	0.31	0.21	0.58	0.29	0.44	0.44	0.95	0.79	1.38	1.20	1.49	4.84
lungs	0.32	0.27	0.41	0.26	0.24	0.25	1.07	1.02	1.52	0.76	0.51	1.20
kidney + bladder	0.80	0.81	0.92	1.06	1.18	0.63	3.44	3.84	5.18	3.58	3.80	5.77
endocrines	0.27	0.24	0.17	0.29	0.35	0.20	0.91	2.04	0.64	0.79	1.08	5.62
heart	0.19	0.11	0.20	0.18	0.17	0.19	0.70	0.56	0.88	0.54	0.59	1.46
brain	----	----	----	----	----	----	----	----	----	----	----	----
tumor							1.00	1.00	1.00	1.00	1.00	1.00

Table 7: Distribution of triethylenephosphoramide-^{32}P (TEPA), a potent alkylating agent, in non-tumored and several tumored animal systems.

	24 Hours After Injection			
Tissue	Non-tumored	L-1210	Sarcoma-37	Lymphosarcoma 1
liver	1.00	1.67	1.31	1.05
G.I. tract	0.75	4.22	0.63	0.81
spleen	1.29	1.33	1.74	1.00
lungs	1.17	1.33	1.05	1.24
kidneys	0.88	1.11	0.89	0.86
brain	0.17	0.22	0.11	0.19
tumor		1.00	1.00	1.00
muscle	0.21	0.55	0.37	0.29
skin	0.33	0.78	0.58	0.67
bone	0.79	1.45	0.74	0.43

relatively high portion of drug in the digestive system need not indicate that excretion is considerable via this route.)

5-Fluorouracil-2-^{14}C, an antimetabolite, was administered, via the intraperitoneal route, to mice containing 10 day growths of Sarcoma 180. The distribution data collected is presented in Table 8. An obvious difference exists for this system---a specific uptake of the drug into tumor tissue, even as early as 1 hour after injection. The bone marrow contained a large portion of the drug, with filtering and excretory organs containing less but yet significant amounts. 88% of the administered dose was excreted within the first 24 hours. The majority of the ^{14}C was found in the urine, with small quantities of $^{14}CO_2$ collected in the early hours.[42]

Unfortunately, available distribution data for the potent folic acid inhibitor---amethopterin---are relatively sparse. The studies presented in Table 9 were performed following I.V. injection of the drug into Akm mice. A disc method of assay (analogous to antibiotic disc tests) was performed on various tissue homogenates, at various times after injection. Serum levels were initially high (on the order of 300-400 mγ/ml) but the concentration either reached or approximated zero within 4 hours after treatment. Amethopterin, not unexpectedly, was found to accumulate in tissues containing high concentrations of folic acid (particularly liver and kidney). In mice with advanced leukemia, results were suggestive of a more widespread distribution, with higher concentrations found within the tissues. This may be an indication of the affinity of leukemic cells for amethopterin. High serum levels over a considerable time period, however, suggest that a possible renal dysfunction, a secondary leukemic effect, may be responsible for the

Table 8: Distribution data following an intraperitoneal injection of 5-fluorouracil-2-^{14}C into mice containing 10 day growths of Sarcoma 180.

	Hours After Injection				
Tissue	1 hour	4 hours	12 hours	24 hours	48 hours
liver	0.40	0.21		0.13	0.16
intestine	0.54	0.30		0.38	0.03
spleen	0.41	0.27	0.21	0.23	0.11
lungs	0.11	0.07		0.05	0.07
kidney	0.55	0.15	0.13	0.11	0.22
heart	0.07	0.01		0.01	0.05
brain	0.10	0.03		0.03	0.05
tumor	1.00	1.00	1.00	1.00	1.00
bone marrow	0.86	0.55		0.33	0.73
muscle	0.05	0.01		0.01	0.00
ovaries	0.06	0.05		0.02	0.04
fat	0.18	0.09		0.03	0.08

Table 9: Distribution of I.V.-injected amethopterin in non-tumored Akm mice.

	Hours After Injection			
Tissue	1 hour	2 hours	4 hours	24 hours
liver	1.00	1.00	1.00	1.00
spleen	0.19	0.37	0.34	0.51
lung	0.08	0.10	0.14	----
kidneys	0.59	0.77	0.69	0.94
muscle	0.02	----	0.06	----

higher drug levels in this instance.[43] Since the data are so sparse, it is difficult to make any significant comparison between cis-Pt(II)$(NH_3)_2Cl_2$ and amethopterin. It is interesting to note, however, the difference in the time course of serum levels between the two drugs.

Table 10 presents distribution data for the tritiated methyl ester of streptonigrin, an antibiotic isolated from the broth of Streptomyces flocculus and found to show anti-tumor activity. This drug was administered I.P. to mice containing 5 to 10 day growths of Sarcoma 180. Although for this drug the greater portion can be found in the filtering and excretory organs, the kidney appears to be less important than the intestines. However, excretory measurements---indicating approximately 65% excretion by 12 hours and 90% by 24 hours---could not be easily partitioned into urinary and fecal portions, as such results varied widely from animal to animal and fecal samples were contaminated with urine. (Metabolic cages were used for excretion collection.)[44]

Radiomanganese, injected I.V. into the tail vein, was followed with time in the major mouse organs as well as in the excreta. Within the first 2 hours, approximately 83% of the administered dose was excreted, mainly in the urine. Small amounts of the radioactive manganese were then found in the fecal matter for several days, until approximately 88% was eliminated after 7 days. Table 11 shows the distribution of the manganese, 24 hours after injection. As with cis-Pt(II)$(NH_3)_2Cl_2$ the greater portion of the element at this time was found in liver and kidney. (The pancreas also contained a considerable portion, and such is not true for the platinum compound at this time.) However, the blood level is relatively insignificant in the manganese data, while 24 hours represents a time just past the second major blood level for the platinum

Table 10: Distribution of the tritiated methyl ester of streptonigrin, an antibiotic, in mice bearing Sarcoma 180.

	Hours After Injection	
Tissue	24 hours	48 hours
liver	1.39	0.98
small intestine	3.56	1.11
large intestine	1.10	0.72
stomach	0.34	1.35
spleen	1.39	1.98
lung	0.58	0.59
kidney	0.48	1.43
heart	0.24	0.47
brain	0.08	0.05
tumor	1.00	1.00
muscle	0.54	0.51
skin	0.47	0.20
plasma	0.27	0.73
blood cells	0.56	1.34
bile (1 mouse)	0.31	

Table 11: Distribution of radiomanganese in normal mouse tissue 24 hours after I.V. injection.

Tissue	24 Hours After Injection
Liver	1.00
Intestine	0.41
Spleen	0.33
Pancreas	1.90
Kidney	2.60
Brain	0.10
Blood	0.01
Muscle	0.11
Skin	0.15
Bone	0.22
Testis	0.22
Eye	0.10

compound. Similar to the platinum, however, manganese can still be measured as long as 7 days after injection, mainly in kidneys, liver and muscle.[45]

With the exception of 5-fluorouracil, probably the only anti-cancer drug currently known which shows specific tumor uptake, the general distribution pattern for such drugs as here included is similar. The filtering and excretory organs contain the greatest percentage of the compound remaining in the body. Moreover, excretion begins almost immediately, at a considerably high rate. Unlike most of these drugs, however, cis-Pt(II)$(NH_3)_2Cl_2$ does not appear in brain tissue at the times studied. This may be more fortunate than unfortunate, as toxic central nervous system effects can, thus, be largely avoided. Moreover, for treatment of cancers of the brain, direct introduction of the drug is always possible.

It is also interesting to note that, other than the relatively high pancreatic content, manganese distribution and excretion properties appear quite similar to those for platinum. The significance of such a comparison is presently unknown, however.

Histopathology

The data shows little, if any, relationship between the platinum content or its time course for a given tissue and the histological picture of that tissue. The liver and kidney, which consistently show the highest platinum levels, show little or no damage, while the stomach and intestines, which show considerably lower levels of platinum over a relatively short period of time, show considerable pathological altera-

tions. It would appear, therefore, that the platinum compound is selectively attacking tissues which show considerable mitotic activity---have short turn-over times. Table 12 gives the relative mitotic activity of the major organs. Even such a crude division of tissue type readily explains why agents toxic to tissue with high mitotic activity would affect such tissues as gastric and intestinal mucosa and lymphoid tissues more dramatically than liver, kidney, pancreas, adrenal or brain. It also predicts that the bone marrow---a tissue not investigated in the present study---would likewise be inhibited by platinum, as was found following platinum treatment of rats.[37] Such is, in fact, indicated by the hyperplastic nature of both lymphoid and hematopoietic elements of the recovered spleen. If the bone marrow was indeed inhibited, extramedullary hematopoietic sources would attempt to compensate whenever possible. (Preliminary blood smears from treated mice do indicate a general leukopenia, typical of bone marrow depression.[47])

Moreover, the recovery rates of the inhibited tissue are representative of the turn-over abilities of the tissues. Although the generation time for mouse intestinal crypt cells is only 11 hours, it takes from 1.5 to 2 days for total transit time---i.e. for transit of new cells, produced in the crypt regions, up to the tips of the villi.[46] Thus, from onset of regeneration, a lapse of approximately 2 days would not be unreasonable before the mucosal regions appear normal and totally free of the remnants of inhibition. This is, in fact, the approximate time period observed.

Lymphocyte depletion, of both the spleen and thymus, is likewise understandable in light of turn-over times. Moreover, a thymic recovery time---from onset of regeneration to apparent normality---of approximately

Table 12: Mitotic activity of body tissue (After Bond, et al.[46])

No mitosis and no cell renewal	Low mitotic index and little or no cell renewal	Frequent mitosis with cell renewal
CNS	liver	epidermis and appendages
sense organs	glands:	respiratory system
	exocrine	
adrenal medulla	pancreas	digestive system
	salivary	
	prostate	urinary system
	endocrine	genital system
	thyroid	
	anterior pituitary	blood-forming organs
	kidney	pulmonary alveolar cells (macropahages)
	adrenal cortex	
		mesothelia
	differentiated connective tissue (derma, tendon, cartilage, bone)	
	vascular endothelia	

2 days, for the non-tumored mouse, is within turn-over times for this tissue. The replacement time for thymus small lymphocytes in the mouse is 3 to 4 days, with mitotic indices 5 to 10 times higher for thymic lymphoid cells than for those of lymph nodes or spleen.[48] The greater and more prolonged atrophy of the thymus in tumored animals is not necessarily unusual either. It has been known for many years, for instance, that thymic atrophy is common with progressive growth of the Sarcoma 180 tumor itself.[49] Moreover, it is known that the mouse thymus may undergo involution following illness or severe stress, as well as following food restriction, irradiation and the action of certain "mitotic poisons" (such as nitrogen mustard and podophyllin).[50] All such factors, and many more common ones, are now believed to produce thymic atrophy by stimulating an increased production of lymphocytolytic adrenal corticosteriods. (Atrophy may likewise be induced via the action of sex steroids, malnutrition and vitamin B deficiencies.)[48] The hyperplastic nature of the adrenal cortex as well as the general "stress" conditions of the treated mouse---complicated by the presence of an advanced growth of Sarcoma 180---can thus fairly well explain the prolonged and varying involution picture of the thymus.

In general, the histopathological response of mouse tissue to cis-$Pt(II)(NH_3)_2Cl_2$ is similar to that found for the rat.[37] For the rat studies, however, LD_{50} (12.2 mg/kg for the rat) rather than therapeutic doses were administered to non-tumored animals only. Gastric and intestinal mucosa inhibition and degeneration was similar, with greater damage evident in the rat. (Regeneration of these elements was complete 9 days after treatment, in those animals which survived.) Thymic atrophy, with severe lymphocytic depletion and apparent reversal, was evident, regen-

eration again complete by the 9th day after treatment. The spleen also showed obvious lymphoid depletion with a hyperplastic regeneration, which persisted as long as 20 days following injection (The maximum pathology for all the above tissues was evident on the 4th day after treatment.) The kidney showed tubular epithelial necrosis, with hyalin and granular casts present in the lumen of sloughing tubules. Regeneration occurred, without evident persistent damage.

Unlike the mouse studies, rat experiments included tests of bone marrow and various blood-serum levels. As with other tissues evidencing high mitotic activity, bone marrow was greatly inhibited by platinum treatment. The marrow elements appeared to be attacked according to their level of maturity---immature precursors were relatively absent within 2 days after treatment. Typical nuclear degeneration was present with pyknosis, karyorrhexis and vacuolization evident. However, regeneration was evident, in those animals which survived, from 9 to 20 days following treatment.

Of the circulating blood elements, basically the more immature forms and those with short life spans were affected. Leukocytes, in general, were depressed to a low at day 3, with a subsequent, fluctuating return to normal. Platelets and reticulocytes (immature red blood cells) were likewise depressed, with the same time course. Mature red blood cells---with a life span of 60 to 80 days---were untouched, with no evidence of hemolysis. The various enzymes, and other protein constituents, of the blood were either relatively untouched or slightly depressed, indicating no damage to major organs and possibly a general enzyme-depressive effect of the platinum compound.

It is of interest to compare the pathology of this system with that

of some of the other anti-cancer agents as well. The general toxic effect to tissues of high mitotic activity is peculiar to cancer chemotherapeutic drugs as well as to X-rays. Although this is true, each particular drug has some more or less characteristic effect which is not necessarily shared by all.

The alkylating agents, in general, produce their most serious effect on the bone marrow. However, as with the other drugs, they attack rapidly dividing cells in general, causing marked lymphoid depletion and atrophy of the thymus and spleen as well as inhibition and degeneration of the intestinal mucosa. Nuclear swelling and loss of polarity occur in the crypt cells of the intestine and such "giant-appearing cells" can be seen along the villi on subsequent days. The same type of mucosal inhibition is evident in the stomach. Most drugs in this class---notably excluding the cyclic ethylenimmonium derivatives ---also cause central nervous system stimulation with convulsions.[9] Alkylating agents in general are also mutagenic and teratogenic, and suppress the immune mechanism.[7,8] Moreover, two side effects are seen with one agent each---alopecia with cyclophosphamide and skin pigmentation with busulfan.[51]

Various anti-metabolites show peculiar combinations of effects. As with most alkylating agents, the overt manifestations of 6-mercaptopurine intoxication are delayed. The pathological effects in mice and rats include hepatic necrosis, lesions of the intestinal epithelium and depletion of bone marrow. The changes encountered in the small and large intestine include epithelial atypia and edema, congestion and leukocytic infiltration of the mucosa.[52] Renal impairment may also occur.

The greater utilization of uracil by rat hepatomas, as compared to

normal rat liver, led to the design and synthesis of fluorouracil and its deoxyribose derivative. The two major toxic effects of these fluorinated pyrimidines, in man, are gastrointestinal damage and bone marrow suppression. Diarrhea, stomatitis, nausea, vomiting, alopecia, dermatitis, pharyngitis, esophagitis, epistaxis and leukopenia are also common side effects.[51]

Use of the main folic acid antagonist, methotrexate (amethopterin), results in damage primarily to normal rapidly proliferating tissues---bone marrow, gastrointestinal mucosa, hair follicles, gonads and fetal tissue. With bone marrow damage, anemia, granulocytopenia and thrombocytopenia are the chief factors limiting the dosage of these agents. Increased susceptibility to infection, bleeding, diarrhea, moderate to complete alopecia, and ulceration of oral and intestinal tract mucosa are also prominent. Depression of spermatogenesis and a variety of skin rashes, usually in the form of a perifolliculitis, vesiculation, and desquamation, are seen. Moreover, abortion or teratogenic effects may prevail during the early stages of pregnancy.[51]

Although present toxicologic data is limited to mice and rats, it appears that cis-Pt(II)$(NH_3)_2Cl_2$, while attacking rapidly proliferating tissue as most such drugs, is not as generally toxic as most cancer chemotherapeutic agents. Diarrhea does not occur until lethal doses are reached and therapeutic doses do not appear to cause any mutagenic or teratogenic effects. Moreover, no central nervous system involvement has been witnessed, even at LD_{50} doses or higher in mice and rats.

The general pathological effects of the platinum compound are somewhat similar to those resulting from acute radiation illness in mice. Table 13 summarizes the early lesions found in various mouse organs

Table 13: Summary of the early lesions of acute radiation illness in various organs of mice in order of increasing radioresistance.

Organ	Major Changes	Minor Changes	Regeneration	Resistant Elements
Lymph nodes	Almost complete destruction of lymphocytes	Edema, ecchymosis	Moderate by 75 hr.	Reticuloendothelial cells, plasmacytes
Spleen	Destruction of lymphocytes of white pulp and erythroblasts and myeloblasts of red pulp	Erythrocytophagia, increase of blood pigment	Lymphocytopoiesis returns, slight return of myelopoiesis, by 78 hr.	Reticuloendothelial cells, plasmacytes
Bone marrow	Almost complete destruction of erythroblastic and myeloblastic tissue	Dilated sinusoids "ground glass" matrix, erythrocytophagia	Slight return of myelopoiesis, by 78 hr.	Reticuloendothelial cells, fat, endothelial cells
Testis	Complete destruction of spermatogonia	Moderate destruction of spermatocytes, spermatids, spermia	None seen	Sertoli cells, interstitial cells, prespermatogonia
Small intestine	Extensive necrosis of crypt epithelium	Edema of lamina propria	Rapid, almost complete in 80 hr.	Surface epithelium, muscle
Large intestine	Moderate necrosis of epithelium at bases of glands	Edema of lamina propria	Rapid, complete by 80 hr.	Mucous cells, surface epithelium, muscle
Lung	None	Destruction of lymphoid aggregates about bronchi	----------	Total lung structure
Liver	None	None	----------	Total liver structure

following radiation.[53] Although lymph nodes were not specifically investigated for the mouse in the present studies, several lymph nodes were included in treated sections of intestine, and a depletion of lymphocytes, similar to that for spleen and thymus tissue at the same time interval, was evident. Although the gonads were not directly investigated, tests with mating of "cured" animals[20] tend to deny at least permanent destructive effects of the platinum compound on spermatogenesis.

Although the effect and time course of irradiation on intestinal mucosa is similar to that for platinum, several specifics vary. Large nuclei, with prominent, rounded nucleoli and abundant nuclear sap were formed among the crypt cells which survived immediate cellular destruction by irradiation. These cells are commonly referred to as possessing "owl's eye" nuclei and are present in the deeper portions of the mucous glands of the large intestines and in the crypts of the jejenum and ileum, beginning as early as 12 hours after irradiation. Cells so affected did not appear to die but gradually moved up the sides of the glands and villi to the tips, whence, presumably, they were sloughed in the usual process of attrition.[53] Such cells were not evident in either mouse or rat intestinal tissues following platinum treatment.

Although it appears that soluble platinum compounds do not, as a rule, act as the soluble salts of heavy metals usually do (e.g. the extremely soluble $(Pt(II)(NH_3)_4)^{++}$ salts are tolerated as high as 400 mg/kg on a daily schedule), it is still of interest to compare toxic effects of platinum compounds with those of soluble heavy metal salts. Some heavy metals are known to exert their biological effects through combination with sulfhydryl groups. However, they are all known to

react with other essential biological ligands as well (-OH, $-COO^-$, $PO_4H_2^-$, NH_2 and imidazole).[54] As with cis-Pt(II)$(NH_3)_2Cl_2$, there is often no correlation between the organs and tissues affected by a metal and the tissues of greatest concentration.[55]

Soluble arsenic salts lead to mild vasodilation or even injury to capillary beds; edema resulting from transudation of plasma; disruption and inhibition of gastrointestinal epithelia; renal capillary, tubular and glomerular damage; bone marrow depression and anemia; and hepatic toxicity evidenced as fatty infiltration, central necrosis and cirrhosis. Such salts are slowly excreted in both urine and feces, starting 2 to 8 hours after administration. Their slow excretion is the basis for their cumulative toxic action. Chemically and biologically, antimony salts resemble those of arsenic, but are more caustic locally.[55]

Most silver salts are insoluble but the few that are can cause corrosion of the mucosa of the digestive tract, resulting in local trauma and hemmorhagic gastroenteritis, which often proceeds to shock and death. They have an initial stimulatory effect on the brain stem, followed by depressive effects, with respiratory depression leading to death. Such salts are cumulative in the body tissues.[55]

The most common toxic effects of soluble gold compounds involve skin and mucous membranes, usually of the mouth. Lesions of the mucous membranes, including stomatitis, pharyngitis, tracheitis, glossitis, gastritis, colitic and vaginitis, are common. Gold salts are also usually toxic to the hematopoietic organs, with thrombocytopenia, leukopenia, agranulocytosis and aplastic anemia not uncommon. Toxicity to the kidneys is usual, with renal insufficiency often resulting in death. Encephalitis, peripheral neuritis, hepatitis and nitroid crisis are also possible toxic effects.[55]

Soluble mercury compounds cause disruption and inhibition of gastric and intestinal mucosa; kidney damage; central nervous system involvement; and electrolyte imbalance. Like other heavy metal salts they are cumulative, as a result of their slow excretion rate. Lead salts also result in severe gastrointestinal distress; neuromuscular problems and paralysis; and severe central nervous system disorders.[55]

It is obvious that even the fairly toxic platinum compounds, such as the cis-Pt(II)$(NH_3)_2Cl_2$ used for the present studies, do not cause as general damage to major body organs as do typical soluble heavy metal compounds. The actual differences between platinum compounds and those of most heavy metals is presently unknown, however.

General Considerations

Available data concerning cis-Pt(II)$(NH_3)_2Cl_2$ suggest that this drug is a potent cancer chemotherapeutic agent. It successfully regresses and even cures a wide variety of transplantable tumor systems commonly employed for the screening of potential anti-cancer drugs. Although it shows effects in common with known classes of anti-tumor agents, it also evidences significant differences. Its distribution pattern, as presented here, is similar, in general, to that for various other agents, as well as for the naturally occurring metal element, manganese. However, the various differences obvious in this data---the peculiar distribution pattern for the first 24 hours; the return of measurable amounts of platinum into the circulation after as long as 20 hours; the long term retention of measurable quantities---suggest a possible basic difference in physiological treatment of this drug.

Moreover, the pathological effects induced appear minimal when compared to the numerous toxic side effects of the various other drugs. It appears to attack rapidly proliferating tissue (with the possible exception of the reproductive organs) almost exclusively, with little if any direct effect on other major body organs.

The present studies, by themselves, establish little, if anything, positive concerning the biological activity of the compound. What is, however, presented is evidence contrary to several previous suggestions ---such as the belief that the potent tumor activity resulted from specific uptake into neoplastic tissue---as well as guidelines, or even limits, to further experimentation and speculation. For instance, it suggests that a biologically active form of the compound (or a threshold level of the same) is relatively short-lived---only 1 or 2 days--- although measurable amounts of platinum are present for considerably longer periods of time. This, combined with the probability of rapid reactivity and possible temporary peripheral shunting, provides a starting point for investigation of the active chemical compound. Understanding of the fate and mode of action of cis-Pt(II)$(NH_3)_2Cl_2$ is obviously in its infancy, and no present theory can be satisfactorily supported or denied.

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APPENDIX

APPENDIX

Neutron Activation Analysis

Activation analysis is a method of chemical analysis primarily based on the principle that a stable isotope can be induced, by irradiation with nuclear particles or photons of sufficient energy, to undergo a nuclear transformation to a radioisotope, which can then be detected and analyzed---both qualitatively and quantitatively. Although activation with charged particles and high-energy photons is technically possible, these types of activation analysis have not been widely developed. For charged particles there is always a threshold energy which must be exceeded before activation can be achieved. Moreover, although light elements have reasonable capture cross-sections for charged particles, these particles have very little penetration power in solid materials and exposed targets are thus raised to high temperatures. For photon activation the threshold energies are even higher and the cross-sections of most nuclides much lower.[56]

Activation with neutrons and, in particular with thermal neutrons, has received the most attention and greatest degree of development. Neutrons as neutral particles face no energy threshold for nuclear interaction and most nuclei have high capture cross-sections for thermal neutrons. Moreover, high neutron fluxes are readily available from modern nuclear reactors.

Various reactions are possible following bombardment with neutrons, depending on the kinetic energy of the incident particles. For thermal neutrons, (kinetic energy at 20°C approximately 0.025eV) the predominant reaction is the (n,γ) process. With the capture of a neutron, the nuclide is raised in atomic weight but not atomic number, thus retaining its chemical identity. The new-formed nuclide is in an excited state and promptly drops to its ground state with the release of characteristic γ-ray(s) called prompt γ's.[27] (Although possible, it is not ordinarily feasible to perform analyses by detection of these prompt γ-rays.) If the new-formed nuclide is radioactive, as is the usual case, it will decay with time as any radioisotope. For thermal neutron reactions, the usual forms of decay are internal conversion or β^--particle emission, or a combination of the two. These reactions are usually accompanied by the release of one or more characteristic γ-rays.

Most neutron-rich nuclides decay by β^--emission, a transformation which tends toward nuclear stability by conversion of a nuclear neutron to a nuclear proton, with the emission of a β^--particle and a neutrino. The new, usually stable nuclide is one atomic number higher than the original nuclide (e.g. $^{24}Na \xrightarrow{-\beta^-} {}^{24}Mg$). Most artifically produced radionuclides are only slightly away from the stability diagonal and thus decay to stable nuclides in a single decay event as opposed to the long chains of consecutive decays found for the naturally radioactive species.

Bombardment with fast neutrons (kinetic energies in the MeV range) can produce several types of reactions---(n,n'), (n,p), (n,α) and (n,2n). Such processes seldom have large capture cross-sections. Moreover, the (n,p) and (n,α) reactions face Coulombic barriers against the escape of

the proton and alpha particle respectively. In the case of a few of the heaviest elements, bombardment with fast neutrons with kinetic energies in the 100MeV range, causes spallation or breakdown of the target nuclei into a mixture of lighter nuclides.

The activities produced by irradiation with neutrons can be expressed as follows:

$$A = N\sigma\emptyset\ (1 - e^{-\lambda t_i}); \quad \lambda = 0.693/T_{\frac{1}{2}}$$

where A = activity induced, disintegration/second
N = number of target atoms
σ = capture cross-section, cm^2
$\emptyset$ = neutron flux, neutrons/cm^2/second
t_i = irradiation time
$T_{\frac{1}{2}}$ = half-life of product isotope

The term $(1 - e^{-\lambda t_i})$ is called the saturation factor. As t_i becomes large compared to $T_{\frac{1}{2}}$ this factor approaches unity or $A = N\sigma\emptyset$.

As with any radioactive sample, the radioactivity induced decreases with time according to

$$A_t = A_o\ (e^{-0.693/T_{\frac{1}{2}}})$$

where A_o = initial activity
A_t = activity after time t
t = elapsed time

Therefore, at a given time the activity of a given radionuclide is dependent on several factors---some intrinsic to the nuclide; others variables of the irradiation process. In cases of long irradiation, radionuclides with relatively short half-lives may decay considerably before irradiation is complete. Therefore, irradiation variables must be suitably adjusted for the radionuclide(s) of interest. (For qualitative analysis of all elements present in a given sample, therefore,

several irradiations of different lengths may be required.)

Various instruments (Geiger-Mueller counters, ion chambers, scintillation counters, gas counters, spectrometers, and semiconductor detectors) are available for detection of the radioactive emissions induced---charged particles and/or γ-rays. In termal neutron activation analysis, β^--particles and γ-rays are the types of emission usually investigated. Beta particles radiated from a given nuclide are not monoenergetic but exhibit a continuous energy distribution from zero to some maximum energy characteristic of the emitting nuclide.[37] Since most irradiated samples will contain more than one β^--emitter, each with continuous energy spectra, a chemical separation of the nuclides is necessary before measurements can be made. After isolation of the various radionuclides, a simple counter is adequate for quantitative measurement of the individual nuclides---once the absence of extraneous γ-ray producers has been ascertained and the β^--spectrum has proved that of the separated β^--emitter only.

Since nearly all activated nuclides emit γ-rays of characteristic energy, the kind and amounts of elements present in an activated sample can be ascertained from the total γ-ray spectrum. When γ-rays pass through matter they can ionize atoms and molecules, losing all or a fraction of their kinetic energy in so doing. There are three basic modes of interaction: photoelectric interaction, Compton interaction and pair production. For the photoelectric effect the incoming γ-ray imparts all of its energy to an orbital electron, which is thus ejected with a kinetic energy equal to that of the original γ-ray minus the binding energy of the electron. It is a major mode of interaction for low-energy γ-rays (0.5 MeV or less) in elements of high atomic number.

The Compton effect (or Compton scattering) occurs when the incoming γ-ray loses only a fraction of its energy in an interaction with an orbital electron. Provided the γ-energy is appreciably greater than the electron binding energy, the γ-photon is deflected as though an elastic collison had taken place.[56] The photon moves off at some angle, dependent on the particular collision event, with less energy than the incident γ-ray. Likewise the Compton electron is scattered at an angle, with a given kinetic energy. This type of interaction is important at γ-energies above 1.0 MeV.

In pair production the incident γ-ray reacts with the nucleus of an atom and is completely converted into an electron-positron pair, with kinetic energy equal to that of the original photon less the energy of creation. Since the rest mass energy of each particle is 0.51MeV, the threshold for this event is 1.02 MeV.[57] When the positron slows down it reacts with any free electron present, annihilating both particles and producing two 0.51 MeV photons. Pair production predominates in the high photon-energy region, particularly in media of high atomic number.

If the above reactions occur in a phosphor (highly fluorescent solid or liquid substance such as doped NaI or anthracene) the de-excitation and recombination processes convert the absorbed energy into light pulses or scintillations, the brightness of which is proportional to the total energy absorbed.[56] Such is the basis of detection with scintillation detectors. The most commonly used detector of this type which is employed in activation analysis is the single-crystal NaI(Tl) scintillation counter---employed either as a flat or well-type crystal. The short-range photo-electrons generated by the photoelectric reactions

are stopped in the crystal, imparting all their energy to the crystal. This results in a total absorption or photoelectric peak with energy characteristic of the originally emitting substance. The original interaction takes place on an iodide atom leaving a vacancy in the atom. As electrons from higher orbitals "drop" to fill the vacancy created, a characteristic iodine X-ray of 28 keV is emitted. If this reaction occurs close to the crystal surface, the X-ray can escape the crystal without detection. Thus the photoelectric event would not possess the full energy of the original photon and a peak will instead appear at an energy corresponding to the energy of the photoelectric peak minus 28 keV. This peak is called an "escape peak".

Compton electrons formed have energy values from essentially zero to a maximum cutoff point called the Compton edge, which represents the maximum energy transfer between the photon and electron. Large angle Compton processes (180^{o}) cause a characteristic spectral feature called the "back scatter peak" which looks quite like a photopeak at an energy of about 200 keV. The ratio of photopeak height to Compton distribution increases with the size of the detection crystal. (The actual sizes, of course, are limited by numerous factors---a 3" by 3" NaI(Tl) crystal being a commonly used size.)

When the emitting substances produce γ-rays of sufficient energy to cause pair-production in the scintillation crystal, this is evidenced in the γ-spectrum by the appearance of an "annihilation peak" at 0.511 MeV. The annihilation of a positron and electron produces two 0.511 MeV photons, emitted in opposite directions. One or both of these photons may escape the crystal and thus detection, but those that are detected contribute to the height of the 0.511 MeV peak.

The "light flashes" or scintillations resulting from the above-mentioned interactions in the crystal are converted to voltage pulses and amplified by a multistage photomultiplier tube. For NaI(Tl) spectrometry, the crystal-photomultiplier ensemble is quite often connected directly to an analyzer---single-channel or multi-channel depending upon the requirements of the particular experiment. For most experiments a multi-channel analyzer is most practical, usually with 512 to 1024 channels. When the sample has been counted for a preset "live time" and the counts per channel suitably programmed in the memory, the resulting γ-ray spectrum can be read out in various ways, depending on the particular requirements and the equipment available. A visual display of the spectrum can be produced by channeling the output onto an oscilloscope screen or to a chart recorder. It can also be dumped out digitally as actual counts per channel or punched onto tapes. By suitable calibration the energy per channel, usually measured as keV, can be ascertained and energies associated with the various photopeaks assigned.

A "live time" reading was stressed above as all analyzers have a dead time which limits the counting rate which the equipment can handle. (For a multi-channel analyzer the dead time is in the order of 80 μsec.)[58] Therefore, for samples with high count rates---greater than the capability of the analyzer---counting times are in reality longer than the preset "live time".

(Although the NaI(Tl) scintillation system has been used effectively for γ-ray analysis, it lacks the peak resolution often required for analysis of multi-nuclide samples. To achieve greater resolution, semiconductor detectors can be employed---e.g. the lithium-drifted germanium detector, Ge(Li). Gamma-spectra measured with the use of such detectors

evidence excellent peak resolution but low counting efficiency.)

The information obtained either as a counts versus channel (or energy) plot or as actual digital counts per channel must now be analyzed according to the information desired. For a purely qualitative analysis of the elements present this can, in many cases, by a straight-forward identification of the nuclides present by the characteristic γ-ray energies found. From this information, identification of the elements present in the original sample can be determined. In many instances this analysis is complicated by various factors---several possible precursors forming the same radionuclide; γ-energies of nuclides being too close for complete resolution (at least with NaI(Tl) detectors); or a general matrix interference in which the chief constituents of the samples are activated very efficiently, causing high backgrounds and poor counting efficiencies for detection of trace element γ-rays. Except for the first complication, radiochemical separation following irradiation of the sample would be of benefit. The radionuclides formed are separated into small groups or individually and then counted. In this way interfering matrix constituents can be significantly removed and nuclides producing γ-rays of like energies can be separated from each other.

Quantitative measurements of the trace or matrix are possible. Although absolute measurements are possible, they are seldom employed as the variables of the detection system (counting efficiency, amplification factors, etc.) as well as those peculiar to the nuclide itself and to the irradiation process must be known with good accuracy. Comparative methods are instead used in which the area under the photopeak characteristic of the radionuclide of interest (suitably corrected for background) is compared with the corrected area resulting from a standard, containing

a known amount of the nuclide and irradiated and counted under conditions identical to the sample being analyzed. For exact comparison, flux variation at the various sample positions in the reactor or the other neutron source employed must be controlled or known accurately and then taken into consideration in the final calculations. Moreover, the standard must have the same type of matrix as the samples being measured, as far as feasible and necessary for the given system, as self-shielding effects can differ significantly from medium to medium. Also, the counting geometry of standard and sample should likewise be controlled.

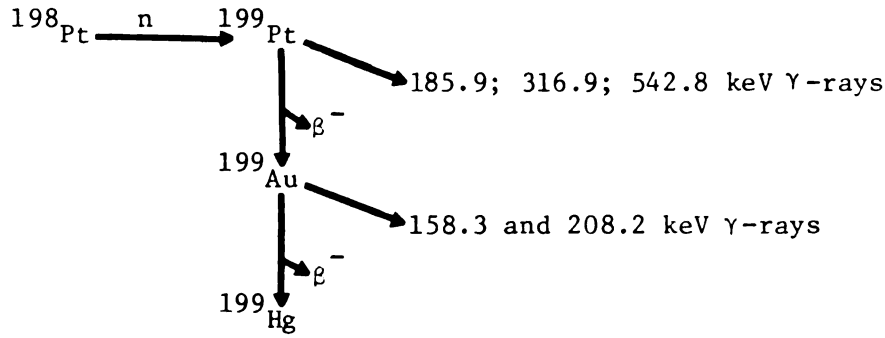
For accurate and reproducible quantitative measurement, adequate counting statistics must exist for the photopeak of interest. Suitable adjustment of the "live time" for counting, considering sample activity and the half-life of the nuclide, can produce satisfactory count statistics. (The radioactive decay process itself is statistical in nature---all randomly occurring processes being subject to fluctuations around a mean. Thus, even if all other variables are carefully controlled, the counting rate for a given sample will vary from measurement to measurement.) Numerous statistical techniques, usually computerized for convenience in handling large quantities of data, have been devised for determining quantitatively amounts of trace elements and major matrix constituents. Although such non-destructive methods of detection are preferred, interferences often make it difficult if not impossible to undertake accurate, reproducible quantitative measurements, even with the best statistical analyses of the data. (For example the large amount of ^{23}Na, which is readily converted to ^{24}Na, $T_{\frac{1}{2}}$ = 15 hours, by thermal neutron irradiation, makes it extremely difficult to measure short-lived isotopes of trace elements generated in biological matrices---

particularly for those nuclides whose characteristic -rays have low energies which would be hidden beneath the extremely high Compton scatter produced by large quantities of ^{24}Na.) In such cases radiochemical separation of the radionuclide(s) of interest must precede analysis. Separation of nuclides is usually performed after rather than before irradiation to avoid possible contamination which might be introduced in the separation procedure---either from the reagents used and/or from mechanical manipulation of the sample.

Since activation analysis is often used for the determination of the levels of trace elements present, a radiochemical separation can entail recovery of microgram amounts or less of the desired nuclide. Unless it has been previously ascertained that the particular procedure of separation consistently yields close to 100% recovery, carriers are usually added. A measured amount---usually several milligrams---of a compounds containing a known amount of the element to be separated, in the same valence state, is added to the sample before any separation steps are undertaken. Measurement of the amount of carrier present at the end of the separation procedure, by ordinary analytical techniques (e.g. spectral analysis), will give the percent recovery for the procedure in that particular instance. (No carrier was added in the present experiment, as the hexachloro salts of heavy metals are known to be, for all practical purposes, 100% retained on the resin.[28,29])

If all sources of error are considered and suitably controlled and/or included in the calculations, neutron activation analysis, with γ-ray analysis, can be a powerful qualitative and quantitative analytical tool. Since each field of interest presents different matrices and conditions, the applicability of this type of analysis will vary not only from field to field but among various problems of a given field.

For the present investigations---detection of microgram amounts of platinum in a biological matrix---a relatively long irradiation time, followed by a suitable radiochemical separation proved to be an amenable system. Table 14 lists various elements, found in relatively high concentrations in biological media, which form radionuclides with neutron bombardment. Many elements produce isotopes with half-lives comparable to or longer than that of ^{199}Pt (30.0 minutes) and with γ-ray photopeaks of considerably higher energy. However, ^{199}Au has a considerably longer half-life---3.15 days[26] and is formed as follows:



The extremely high concentration of ^{24}Na and ^{42}K in all tissue and ^{59}Fe in blood---each with reasonably long half-lives---still represent interferences to accurate γ-ray analysis. Use of the anion-exchange resin successfully eliminates these interferences.

Although actual limits of detectability were never accurately determined for this system, a sample containing 6μg Pt consistently gave approximately 3-4 x 10^3 counts above background at maximum peak height, for the 158 keV photopeak, for a 20 minute "live time" count (a total peak area of approximately 200,000, with corrected area approximately 90,000). Thus, for most samples (with the possible exception of the intestines, as interferences from fecal debris contamination were present here) 0.1μg Pt was easily detectable and smaller amounts were actually

detected, for several spleen samples, with reasonable counting statistics.

Table 14: Elements of biological media activated by neutron bombardment.[26]

Element	Radionuclide formed	$T_{1/2}$	Main γ-ray photopeak (keV)
Na	^{24}Na	15 h.	1368.4; 2753.6
K	^{42}K	12.52 h.	1524.7
Ca	^{47}Ca	4.7 d.	160.0; 1296.9
	^{49}Ca	8.8 min.	3083; 4071
Mg	^{27}Mg	9.45 min.	844.0; 1014.1
Cl	^{38}Cl	37.29 min.	1642.0; 2166.8
Fe	^{59}Fe	45.1 d.	192.5; 1098.6; 1291.5
Zn	^{65}Zn	245 d.	1115.4
	^{69m}Zn	13.8 h.	438.7
	^{71}Zn	2.2 min.	121.8; 511.6; 910.1
I	^{128}I	25.4 min.	442.7; 526.3
Cu	^{64}Cu	12.8 h.	1345.5
	^{66}Cu	5.1 min.	1039.0
Br	^{80}Br	4.5 h.	640.4; 617.0; 665.7
	^{82}Br	35.87 h.	554.3; 619.0; 776.6
Rb	^{86}Rb	18.66 d.	1076.6
	^{86m}Rb	1.02 min.	555.8
	^{88}Rb	17.8 min.	898.0; 1836.1; 2677.6