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Biochemical studies of phenylmercuric acetate (PMA) and mercuric acetate toxicity in rats carried out during this investigation were (1) the metabolism of $^{203}Hg(Ac)_2$ and ^{203}Hg-PMA within kidney and liver tissues following oral ingestion, (2) the effect on some soluble enzyme activities and determinations of ^{203}Hg binding in the enzyme fractions, and (3) the metabolism of ^{14}C-labeled substrates by kidney slices from control animals and animals receiving PMA and $Hg(Ac)_2$.

In vitro ^{203}Hg binding of the kidney soluble proteins was determined after incubating the soluble fraction with ^{203}mercury labeled $Hg(Ac)_2$ and PMA. The proteins were separated by Sephadex G-100 gel filtration and the ^{203}Hg and O.D.$_{260m\mu}$ were determined in the eluate. Hg-binding depended on the type of mercurial and its concentration, and Hg did not seem to combine selectively with any

particular group of proteins.

Incubation of kidney slices in PMA solution resulted in mercury binding to groups of proteins of the soluble fraction in the 100,000 (peak I), 40,000 to 60,000 (peak II) and the 8,000 to 13,000 (peak III) molecular weight ranges, and incubation of $Hg(Ac)_2$ showed mercury binding to the peak I and III proteins. These binding patterns were different from those obtained with in vitro incubation of the soluble fraction.

In vivo metabolism of ^{203}Hg from a single dose of PMA or $Hg(Ac)_2$ resulted in a very large portion of ^{203}Hg being bound to the peak III proteins, especially after 48 hours. PMA and $Hg(Ac)_2$ treatment resulted in different ^{203}Hg-binding patterns the first 48 hours, especially in peaks I and II, and gradually became similar after that.

Rats receiving multiple doses of PMA or $Hg(Ac)_2$ also showed similar labeling patterns in the kidney soluble proteins. However, the O.D. profile was definitely altered, with an increase of O.D. in the area of peak III.

Single dose of PMA or $Hg(Ac)_2$ slightly increased the level of kidney soluble lactic acid dehydrogenase (LDH). Multiple doses of PMA or $Hg(Ac)_2$ decreased the LDH activity levels in the kidney soluble fraction. The maximum bindings were around 0.32 and 0.50 mole Hg/mole protein in the LDH fraction for $Hg(Ac)_2$ and PMA respectively. When the kidney soluble fraction was incubated in

1×10^{-4}M $Hg(Ac)_2$, about 2-4 moles of Hg were bound per mole of protein, and the LDH activity was not affected. LDH in the rat kidney did not return to a normal value with a significant decrease in mercury binding when a 2 week period of time was allowed to lapse following $Hg(Ac)_2$ dosage. It is highly possible that mercury binding at the active site of LDH enzyme is not the prime factor involved for the reduction of activity. It may be that the synthesis of enzyme is affected.

Single or multiple PMA dosage did not affect malic acid dehydrogenase (MDH) activity. A single dose of $Hg(Ac)_2$ showed no effect on MDH activity, but multiple doses showed a decrease. The Hg-binding was less than one mole of Hg/mole of protein in the MDH fraction. It seems doubtful that Hg binding at the active site of the MDH molecule is the main reason for the decrease of MDH activity following in vivo treatment.

When kidney slices were incubated with lactate-1-^{14}C, -2-^{14}C, or glutamate-3,4-^{14}C, a marked difference between those taken from control rats and from rats receiving oral doses of PMA (Hg accumulation; 198 μg ^{203}Hg/gr. fr. wt. kidney) or $Hg(Ac)_2$ (98 μg ^{203}Hg/gr. fr. wt. kidney) was observed. The effects were not due to an inhibition of lactate or glutamate uptake by the slices. Complete metabolism of labeled lactate or glutamate to $^{14}CO_2$ and lactate-1- or -2-^{14}C incorporation into glutamate were definitely affected more by

PMA, and to a lesser degree by $Hg(Ac)_2$. It seems possible that in vivo mercurial treatment induced an inhibition of decarboxylase activity in the kidney. Due to a reduction of lactate-^{14}C conversion through the decarboxylating pathway, a greater amount of lactate would become available for its metabolism through another pathway (a combination of assimilation followed by a degradation). A larger second peak which was found in all runs with kidney slices from mercurial treated rats illustrates this alteration. A delay of 2 or 3 hours for the appearance of the second peak may be explained by the overall depression of metabolic reactions. A decrease in respiration of isolated kidney tissues following toxic doses of both inorganic and organic mercurials has been reported.

Some Biochemical Studies of Phenylmercuric Acetate and Mercuric Acetate Toxicity in Rats

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INTRODUCTION

The use of organic mercurial diuretics has stimulated an interest in their metabolism by the animal. Extensive studies suggested that the diuretic action of these compounds is due to the slow release of mercuric ions. Organic mercury fungicides may be metabolized in a similar manner. Better understanding of mercurial metabolism will greatly assist in interpreting the toxicological observations.

Comparative studies of chronic toxicity of phenylmercuric acetate (PMA) and inorganic mercury (Hg^{2+}) by Fitzhugh et al. (15) showed that as little as 0.5 ppm. mercury given in the form of PMA will produce renal lesions, whereas from 10 to 20 times as much mercury is required to produce similar effects with mercuric acetate. The pharmacological properties of these two types of mercurials are not understood well enough to explain the observation.

Comparative studies on the accumulation, distribution, and rate of elimination of mercurials in experimental animals were reported (54, 55, 56, 45, 14, 60). Swensson et al. (54) reported that their study of the distribution and excretion of mercuric nitrate, PMA and methylmercuric hydroxide in rats, rabbits and dogs revealed considerable differences after intravenous supply. The two organic compounds are bound to the erythrocytes to a considerable extent,

while the inorganic mercury was bound to plasma. Mercuric nitrate and PMA were deposited chiefly in the kidneys, whereas the methylmercuric hydroxide was distributed more uniformly throughout the body. In their long term study in rats after subacute administration of mercurial through drinking water (55), they concluded that the organic compounds gave much higher mercury concentrations in the brain, liver, and kidney than the inorganic mercury. The ratio between the blood and the brain mercury contents seems to be constant for each substance and indicates a distribution equilibrium. Miller et al. (39) reported that PMA was absorbed unchanged when given intravenously, intramuscularly, or orally to chicks, rats, and dogs. Metabolism was fairly rapid and appeared to take place in both the liver and kidneys. Inorganic mercury was detected in both organs and accumulated mainly in the kidneys. Rothstein and Hayes (45) studied the metabolism of inorganic mercury following intravenous and intramuscular injection of $^{203}Hg(NO_3)_2$. They indicated that a large fraction was taken up by the liver and was cleared via fecal excretion in a few days. The kidney was the major site of mercury deposition, which was increased to about 85 to 90% of the total body burden in about 2 weeks. The clearance of mercury from the rats took place in three phases: a rapid phase involving 35% of the dose with a half-time of a few days; a second phase involving 50% of the dose with a half-time of 30 days; and finally a slow phase involving

the remaining dose with a half-time of approximately 100 days. Since most of the mercury is located in the kidney after the first few days, the two slow phases of excretion represented mainly the clearance from the kidney. Recently, Takeda et al. (56) reported that the alkylmercury compounds were excreted more slowly and were retained in higher concentration for a longer time in the body than inorganic mercury and phenylmercuric compounds after subcutaneous administration to rats. Distribution of mercury in the vital organs, particularly in the brain, was found to depend on the structure of the mercury compounds. Relatively high accumulation in the brain was observed for alkylmercury compounds, but not for inorganic and phenylmercury compounds. This difference in the distribution of mercury in the brain appears to be correlated with the specific neurotoxicity.

The subcellular distribution of mercury in several vital organs of laboratory animals has been reported (14, 20, 69). Greif et al. (20) reported a detailed study of the subcellular localization of radiomercury in the kidneys of rats and dogs after intramuscular or intravenous injection of labeled chlormedrin. The highest concentration of mercury was in the soluble fraction. The amount of mercury per milligram of nitrogen in all fractions increased with the increasing dosage, ranging between 0.1 to 10 mg Hg per kg. Ellis and Fang (14) reported on the subcellular distribution of mercury in

the rat liver and kidneys following an oral administration of PMA and Hg^{2+}. The percentage distribution of mercury varied slightly between the liver and kidney and showed no significant differences among those dosed with PMA or Hg^{2+}. Throughout a 72-hour post dosage period, the subcellular distribution of mercury remained quite constant even though the mercury binding in each fraction showed an increase during the early period, followed by a decrease at later ones. Yoshino et al. (69) reported on the subcellular distribution of mercury in the brain of animals after an intraperitoneal dose of methylmercurythioacetamide. The mercury content about 6 hours after administration was highest in the mitochondrial fraction, next highest in the microsomal fraction, and lowest in the supernatant. But after that time (from 1-16 days) the average radioactivities were 31, 26, and 24 percent of the total accumulation in the mitochondrial, microsomal, and supernatant fractions respectively. This is in contrast to those found in the kidney and liver (14, 20). Almost all radioactivity was bound to the protein, and there was little in either the lipid or the nucleic acid fractions. Since the turnover of mercury in the central nervous system (CNS) is rather slow, they theorized that the alkylmercury may have a greater affinity with those proteins in the CNS which have longer biological half lives.

As a first step in understanding the biochemical mechanisms

underlying the preferential accumulation of mercury by the kidney, Clarkson and Magos (7) carried out in vitro studies on the binding of Hg^{2+} in tissue homogenates. They indicated that there were two classes of mercury-binding sites in both kidney and liver homogenates which have binding capacities of 1.0×10^{-7} and 30×10^{-7} M Hg per gram fresh weight of tissue respectively. The results of the in vivo experiments did not differ significantly from those obtained when the mercury was added in vitro. Their findings, in addition to the results of several other investigators, ruled out four possible mechanisms of preferential kidney uptake: (1) kidney possessed binding sites of uniquely high affinity; (2) glomerular filtration and uptake from the filtrate; (3) mercury carried to the tissues as a soluble complex is precipitated in the tissue; and (4) kidney binding sites of uniquely high affinity exist in vivo but are constantly breaking down and are being regenerated.

By determining protein-bound mercury, Yagi and White (68) found that intravenous injections of $HgCl_2$ led to Hg binding in the soluble proteins which was similar to that in in vitro binding.

The metabolism of mercurials and their protein binding determine, to a great extent, the accumulation of mercury in the organ and their intracellular distribution, which may govern the sites of toxic action. Various mercurials have different properties of reactivity and toxicological behavior. Phenylmercuric acetate, PMA, and

mercuric acetate, $Hg(Ac)_2$, have selective action upon the kidney, due primarily to the mercurial accumulation.

Mercurials have also been observed to affect various enzymes, either through their reaction with sulfhydryl groups at or near the active site or with nonenzymic proteins which may modify complex systems unrelated to metabolism. A large number of enzymes in the kidney have sulfhydryl groups. Bickers et al. (2) studied the effect of meralluride on five renal tubular enzyme systems by histochemical methods and revealed that meralluride produced inhibition of the following enzyme activities: succinic dehydrogenase, diphosphopyridine nucleotide diaphorase, triphosphopyridine nucleotide diaphorase, and glucose-6-phosphatase. Inhibition of these enzymes was always accompanied by apparent necrobiosis, and was detected sooner with a higher dose (4 to 20 mg Hg/kg). Shore and Shore (49) reported a 40% decrease of alkaline phosphatase and no TCA cycle activity in the kidneys from rats 24 hours after receiving an intravenous injection of 3 mg/kg $HgCl_2$. The greatest inhibition of TCA activity was not observed until several hours after the maximal level of mercury in the kidney had been reached. The Hg level in the soluble fraction was 7.22 mμmole Hg/mg protein after three hours.

In vitro studies of intestinal mucosa alkaline phosphatase by Lazdunski and Ludovic (32) led them to conclude that a sulfhydryl group may be partially involved at the active center. Iodosobenzoate,

iodoacetamide, and p-chloromercuribenzoate (PCMB) did inhibit the enzyme but iodoacetic acid and N-ethylmalimide were not inhibitors. Since inhibition was observed, some thiols are probably bound but the ratio of inhibitor to enzyme concentration appears too great to be certain that a sulfhydryl group is required at the active center. If there is a thiol group near the active center it may not be readily available to certain mercurials. Heppel et al. (22), in working with Escherica Coli alkaline phosphatase, found that 1×10^{-3}M p-hydroxy mercuribenzoate, HMB, had no effect on the enzyme and 1.5×10^{-2}M HMB actually caused stimulation. Nimmo-Smith and Standen (41) observed that one-μmolar concentration of $Hg(Ac)_2$ caused 38% inhibition of an acid phosphatase from Schistosoma Mansoni.

Lectic acid dehydrogenase has been reported as extremely sensitive to mercurials (11) and was found to have 17-27 sulfhydryl groups per mole of enzyme. However, the reaction of PMB or $HgCl_2$ with LDH from beef heart, chicken heart and chicken muscle causes a loss of about 25% of the enzymatic activity for each mole of sulfhydryl reagent bound per mole of enzyme. It appears therefore that the "active" sulfhydryl groups are among the first four reacting rapidly and homogenously with the sulfhydryl reagents. Activity can be recovered by treatment with cysteine. The optical rotation characteristics, fluorescence, sedimentation coefficient, and immunological properties were not modified by mercurial binding.

The organic moiety attached to Hg does influence its binding properties. Theorell (58) observed that LDH was rapidly inhibited by Hg^{2+} but only slowly by PMB. Nielands (40) found the reversal of PMB inhibition of LDH was faster than inhibition.

In vitro studies of malic acid dehydrogenase, MDH, suggest that it is somewhat less sensitive to mercurials than LDH (59). Wolfe and Nielands (67) found values of about 7 moles of SH per mole of soluble MDH. Mercurials did not completely suppress the enzymatic activity until all seven SH's had been reacted. The inhibition could not be reversed with cysteine or glutathione. Seigel and Englard (51) found a MDH in beef heart mitochondria which differed from one found in the supernatent. The mitochondrial MDH had 12 SH's/mole and lost most activity after three equivalents of PMB had been added. The supernatant MDH had 6 SH's/mole and lost no activity after 3 SH's had been titrated.

The mercurials are extremely reactive with the functional groups of enzymes and other proteins. In determining the toxicological effects of mercurials, it is unfortunate that the sulfhydryl groups, which are the most reactive of the functional groups, seem to lack specificity toward some particular enzymes or classes of enzymes.

In addition to the studies of mercurial effects on enzyme activities, kidney metabolism studies may contribute to the elucida-

tion of the toxic action of PMA and $Hg(Ac)_2$ on kidneys. Changes in the metabolism of ^{14}C-labeled substrates by kidney slices can be a useful indicator of toxicity.

The fate of pyruvate-1-^{14}C and pyruvate-3-^{14}C incubated with rabbit kidney cortex slices was determined by Busella et al. (6). The radioactivity of carbon atoms 1 and 3 of pyruvate was incorporated into CO_2, lactate, alanine, glutamate, glucose, and glycogen (trace). Lactate is a direct precursor of pyruvate, which is involved in a central area of metabolism, providing a direct link between catabolic and anabolic pathways. It may be completely oxidized to CO_2 and H_2O in the mitochondria, or may also be converted to glucose via reactions occurring in the mitochondria and coupled with reactions in the cytoplasm. Pyruvate is metabolized by kidney slices via either one or both of those pathways, depending on the concentration (42). Any toxicity affecting pyruvate metabolism or lactate metabolism could reflect changes in mitochondrial activity, including the TCA cycle and mitochondrial systems involving the introduction of the substrate to the TCA cycle. This assumption is based on the fact that the bulk of the carbon atoms from lactate and pyruvate traverse reactions of the TCA cycle. Many investigations have indicated this. The distribution of labeled carbon in liver glycogen derived from labeled lactate was demonstrated by Lorber (34) to agree with predictions based on schemes of glycolysis and the TCA cycle. Carboxyl-

labeled pyruvate can give rise to glutamate labeled in carbon 1 (via oxaloacetate and fumarate) without prior conversion to bicarbonate (28).

Glutamate metabolism is directly concerned with TCA cycle activity (66). L-glutamate is converted to α-ketoglutarate which is further catabolized in rat kidney and liver slices. D-glutamic acid was enzymatically converted to D-pyrrolidone carboxylic acid.

The level of TCA cycle activity is much greater in the cortex of the kidney than in the medulla. James Lee and co-workers (33) incubated rabbit kidney cortex and medulla slices with various ^{14}C-labeled substrates. They found a highly aerobic metabolism in the cortex and a predominantly anaerobic glycolytic metabolism in the medulla. The oxygen consumption of kidney cortex is one of the highest in any tissue, while its respiratory quotient is one of the lowest, suggesting extensive oxidations of fatty acids. A high rate of anaerobic glycolysis occurs in the medulla associated with a respiratory quotient of about 0.99. The oxygen consumption of the medulla is about one-sixth that of the cortex, whereas anaerobic glycolysis in the inner medulla is about four times that of the cortex.

Oxygen consumption reflects the degree of biosynthesis occurring in addition to the amount of secretory work performed by the kidney. Much of the oxygen consumption of the intact organ is known to connect with the absorption of sodium (30).

Various compounds are actively transported and secreted by the kidneys. Para-aminohippuric acid (PAH) is actively accumulated by kidney slices. This process appears to be closely related to the tubular excretion of PAH in the intact animal. PAH accumulation in the kidney slices was measured following the addition of various metabolic intermediates to the suspending medium. Lactate and pyruvate consistently increased the uptake, although never to quite the same degree as acetate. Minimal and less reproducible positive effects were also observed with glucose, hexose diphosphate, propionate, butyrate, isobutyrate, and acetoacetate (17).

Substrate uptake is also dependent on the expenditure of energy. Rat kidney cortex slices accumulate concentrations of glutamate or succinate more than six times those of the medium. Anaerobically, the concentrations in medium and tissue rapidly become equal (21).

Changes occur in kidney metabolism and active transport capabilities after in vivo mercurial treatment. The nature and degree of the changes depend upon the dosage level, route of administration, and the type of mercurial. Mercurial diuretics such as meralluride increase the volume of urine at low doses. Toxic doses cause renal shut down (63, p. 927). Other mercurials such as p-chloromercuribenzoate (PCMB) have no effect on urine volume at lower doses, but will also bring about renal shut down at higher

doses (25).

Mercurial diuretics given to rats at doses of 2-5 mg Hg/kg produce marked diuresis. The compounds are not considered toxic at this dosage level. Mercury accumulates primarily in the area of the proximal tubules in the cortex and diuretic doses produce changes mainly in the proximal tubules. These changes vary with the dose and with the time after dosage. The most prominent effect seems to be inhibition of Na^+ transport, which is associated with a membrane-bound Na^+ and K^+ stimulated ATPase (25). This system is able to stimulate glycolysis. Also, the rate of passive outflow of K^+ from rat kidney slices is depressed (3). A non-diuretic, PCMB, does not achieve these effects at the same dosage level (25). However, the amount of Hg accumulated in the kidneys after diuretic treatment (170-190 μg Hg/gr. fr. wt.) is at least twice that following PCMB treatment (80 μg Hg/gr. fr. wt.). Electron microscopy revealed maximal changes in the proximal tubules 30 minutes after 1-2 mg Hg/kg injections of meralluride (46). Intracytoplasmic oedema occurs in the cells. Mitochondrial swelling occurs with vacuolation of the matrix and disappearance of the cristae. It was suggested that these effects are due to slower absorption of water and solutes in the proximal tubule, and temporary disturbances in the energy control mechanism which is responsible for reabsorption.

Toxic doses of diuretics and most other mercurials range

from 15 to 40 mg Hg/kg, depending on the compound. At these levels kidney function is impaired and metabolism is disturbed.

Mercaptomerin produced diuresis at 10.7 mg Hg/kg and at this level no change of O_2 uptake and P:O ratio in kidney slices were observed (63, p. 927). However, with a toxic dose of 26.7 mg Hg/kg, the O_2 uptake was depressed 35% and the P:O ratio dropped from 0.55 to 0.092.

Multiple high doses of mersalyl produced an increase in lactate and α-ketoglutarate levels in kidney tissue, but a decrease in lactate in the liver tissue (12). Carbohydrate metabolism in the whole animal was altered, even though the bulk of the Hg accumulates in the kidney.

Doses of mercuric chloride (3 mg Hg/kg I. V.) lower than the toxic range of most other mercurials gave Hg levels in the kidney about the same as that of diuretic dosages (180 μg Hg/gr. fr. wt.) (49). TCA cycle activity, using α-ketoglutarate as the substrate, was completely abolished within 24 hours of dosage. The rats died of renal failure within 5-7 days.

Some mercurials are not accumulated principally in the kidneys. Alkylmercury has a tendency to concentrate in the brain. Accumulations of around 50 μg Hg/gr. fr. wt. in the brain produce marked changes in CNS function and metabolism. A latent period exists between accumulation of Hg in the tissue and symptoms and

metabolic disturbances of CNS. There is a decrease in protein synthesis during the latent period which is thought to be responsible for the symptoms following this period (69).

Mercury levels which accumulate under in vivo conditions and produce metabolic inhibitions are most often less than levels required for in vitro inhibition. Webb (63, p. 928) makes the comment concerning in vitro studies of diuretics that "the lowest concentration of the mercurials which exerts an effect in vitro is much greater than the maximal tolerated plasma concentration in rats in vivo, so it is doubtful if these inhibitions of respiration are relevant to the diuretic action." Ethylmercury chloride inhibited the oxidation of glucose, pyruvate, and glutamate in rat brain slices and kidney slices (9). The mercury/tissue ratio required for 50% inhibition was 3600 μg Hg/gr. fr. wt. However, liver mitochondria are unable to take up inorganic phosphate in the presence of p-mercuribenzoate in the μmolar range (16). At high concentrations of ethylmercury chloride, α-keto-acid oxidases are inhibited (1). It is obvious that if the concentration of mercurials is great enough under in vitro conditions, metabolic processes such as substrate oxidation can be inhibited.

The amount of Hg causing inhibition of various enzyme activities and metabolic processes seems to vary greatly depending on the mercurial and the experimental conditions. Since many papers

do not include Hg-binding data, it is difficult to assess the real potency of the mercurial. Most investigators hold in common that the inhibition of respiration in kidney tissue does occur under high in vitro concentrations of mercurials and in vivo toxic doses of mercurials.

The present investigation has involved three general areas of study in comparing the toxicity of PMA and $Hg(Ac)_2$ in rats: (1) the metabolism of $^{203}Hg(Ac)_2$ and ^{203}Hg-PMA within kidney and liver tissues following oral ingestion; (2) the effect on some enzyme activities and determinations of ^{203}Hg binding in the enzyme fractions; and (3) the metabolism of ^{14}C labeled substrates by kidney slices from control animals and animals receiving PMA and $Hg(Ac)_2$.

MATERIALS AND METHODS

Administration of ^{203}Hg-$Hg(Ac)_2$ and ^{203}Hg-PMA to Rats

Adult male rats of Wistar strain, weighing 350-450 grams, were lightly anesthetized with ether prior to oral administration by intubation of solution containing $^{203}Hg(Ac)_2$ or ^{203}Hg-PMA. The mercuric acetate solution was prepared in water and the PMA was prepared in Wesson Oil. The concentration of both solutions was 1.3 mg Hg/ml. After dosing, the animals were housed individually in glass metabolism cages and were given food and water, ad libitum, during the entire experimental period.

Preparation of Kidney and Liver Soluble Fractions

The liver and kidneys were quickly removed and placed on ice. The tissue was first minced with scissors, then ground in cold 0.25M sucrose solution in a motor-driven homogenizer with a teflon pestle. The ratio of sucrose solution to the fresh weight of tissue was 5 to 1. Portions of these homogenates were subjected to centrifugation in a Sorvall refrigerated centrifuge at 35,000 x g for 90 minutes to sediment most of the particles. The supernatant, containing the soluble materials of cells and some unsedimented particles, was designated the soluble fraction.

Separation of Kidney and Liver Soluble Proteins by Sephadex Gel Filtration

A 2.5 x 60 cm. Sephadex G-100 column was prepared as follows. A piece of glass wool was placed in the bottom of the column with glass beads layered to a height of two centimeters. The column was then filled with Sephadex which had been soaked in 0.4 M NaCl and 0.1 M NaAc at pH 6.0 for two or three days. The entire operation was carried out in the cold room at 3° to 5°C. The buffer solution was fed to the column continually from a reservoir constructed to maintain a constant hydrostatic pressure to insure a constant flow rate.

Three substances of known molecular weights [(1) Gamma-globulin (Bovine) Cohn Fraction II purchased from Mann Research Laboratories, Inc., molecular weight 160,000, (2) twice recrystallized Bovine hemoglobin, molecular weight 68,000, and (3) Cytochrome-C from horse heart Type III purchased from Sigma, molecular weight 12,270] were each dissolved in a minimum volume of the same buffer and passed separately through the column in order to determine the elution volumes of these substances, as described by Whitaker (64).

About 5 to 10 ml of kidney or liver soluble fraction were placed on top of the column and the elution started when the soluble fraction was introduced into the Sephadex column. The eluate was

collected in test tubes mounted in a fraction collector. The volume, the optical density, and several enzymic activities were determined in each fraction. Aliquots of the fractions were also taken for the measurements of ^{203}Hg and total nitrogen content.

Detection of ^{203}Hg

Measurement of ^{203}Hg radioactivity in all samples was carried out with a Technical Associate well-scintillation spectrometer equipped with a 2 x 5/8 inch NaI(Tl) crystal. The background of this instrument was between 30 to 35 counts per minute and remained unchanged during these years. The initial counting rates were approximately 500,000 and 300,000 cpm per 1μ mole of mercuric acetate and PMA, respectively. In general, duplicate aliquots of 2 ml liquid samples were counted for 10 minutes to ensure accuracy. The radioactivities were corrected for background and decay.

Mercury Binding of Soluble Proteins from Kidney and Liver

One gram of tissue slices, which were cut with a Harvard tissue slicer to 0.5 mm thickness, were incubated in 15 ml of Krebs-Ringer phosphate buffer containing 1 x 10^{-5}M ^{203}Hg-labeled mercuric acetate or PMA at 25°C. After 2.5 hours of incubation with continuous shaking, the tissue was removed, rinsed with water and homogenized immediately in 0.25M sucrose solution. The soluble fraction was prepared and passed through a Sephadex G-100 column

as described in previous sections. The optical density at 260 mμ and the radioactivity of each effluent fraction were measured in order to estimate mercury bindings from Hg^{2+} and PMA.

Mercury bindings of soluble proteins from kidney were also measured after mixing an equal volume of soluble fraction and mercurial solution. The mixture was allowed to stand at 0° to 3°C for 30 minutes before separation by Sephadex G-100 gel filtration.

Assay Procedures for Acid and Alkaline Phosphatase

Alkaline phosphatase activity was determined according to the method described by Melani and Guerritore (37). The assay was carried out at pH 10.4 using p-nitrophenyl phosphate (PNPP) as the substrate. The reaction yielded p-nitrophenol (PNP), the concentration of which was determined with a Bausch and Lomb Spectronic 20 at 405 mμ. The change in optical density/minute at 405 mμ was converted to mμm PNPP/minute.

Measurement of acid phosphatase activity also utilized PNPP as the substrate. PNP is very sensitive to pH and has a lower extinction coefficient below pH 8.5, so the assay was run at pH 6.4. The enzyme from the kidney soluble fraction has a pH optimum of 4.9, however there was substantial activity at pH 6.4.

The following reaction mixtures were used in measuring alkaline and acid phosphatase activity.

Alkaline Phosphatase

0.2 M Glycine Buffer, pH 10.4	1.50 ml
1.5 mM $MgSO_4$	0.40
30.4 mM PNPP	0.50
Enzyme Solution	0.10
Distilled Water	0.50
Total Volume	3.00 ml

Acid Phosphatase

0.2 M Na Acetate Buffer, pH 6.4	1.50 ml
1.5 mM $MgSO_4$	0.40
30.4 mM PNPP	0.50
Enzyme Solution	0.10
Distilled Water	0.50
Total Volume	3.00 ml

Alkaline phosphatase activity was measured in both kidney soluble and microsomal fractions, from both treated and non-treated rats, while acid phosphatase activity was determined only in the kidney soluble fractions.

In Vitro Inhibition of Acid and Alkaline Phosphatase by PMA and $Hg(Ac)_2$

In vitro inhibition studies were made using Sephadex fractions containing phosphatase activity. The assay mixture, containing all constituents except PNPP, was adjusted with various concentrations of PMA and $Hg(Ac)_2$. The mixture was incubated at room temperature for 15 minutes before adding PNPP to initiate the reaction. The percent of inhibition of enzymic activity was then calculated. The

effects of the mercurials on a commercial enzyme preparation were also determined.

$^{203}Hg(Ac)_2$ Binding to Commercial Alkaline Phosphatase

To determine the relationship between the binding of mercurials and its effect on phosphatase activity, about 30 mg of calf mucosal alkaline phosphatase, purchased from Sigma, was incubated in 30 ml of 5×10^{-5} M $Hg(Ac)_2$ for 30 minutes. The enzymic activity was determined. This mixture was then separated on a 2.5 by 150 cm Sephadex G-100 column in the cold room. The alkaline phosphatase activity, ^{203}Hg concentration, and nitrogen concentration were determined in each fraction collected.

Assay Procedures for Lactic and Malic Acid Dehydrogenase

Lactate dehydrogenase activity was assayed by the method of Kornberg (29), according to the following reaction: Pyruvate + NADH $\rightleftarrows$ Lactate + NAD^+. The assay mixture contained the following reagents:

Na pyruvate (0.01 M)	0.10 ml
NADH (0.002 M) kept at a slightly alkaline pH	0.10
KH_2PO_4-K_2HPO_4 (0.1 M, pH 7.4)	1.00
NaCl(0.01 M)	
Enzyme preparation (make dilutions with NaCl solution)	0.10
Distilled Water	1.70
Total Volume	3.00 ml

After mixing, the change of O. D.$_{340}$ was followed in a Spectronic 30 spectrometer.

Malic dehydrogenase was assayed using the same mixture with the exception that a 0.0076 M oxaloacetate solution was used in place of the sodium pyruvate solution.

Lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) activities of both kidney and liver soluble fractions from control and mercurial treated rats were analyzed.

In Vitro Inhibition of Lactic and Malic Acid Dehydrogenase by PMA and $Hg(Ac)_2$

In vitro inhibition studies were made with Sephadex fractions containing LDH and MDH activity after removal of excess NaCl by dialyzing the fraction in cold 0.01 M tris buffer for 24 hours with several changes of buffer. The assay mixture, with everything present except pyruvate or oxaloacetate, was maintained at 37°C. The enzyme preparation was incubated for 15 minutes with varying amounts of mercurials, then the substrate was added to initiate the reaction.

In vitro inhibition studies of LDH were also carried out with the kidney soluble fraction. Equal volumes of kidney soluble fraction and $Hg(Ac)_2$ solution were incubated for 30 minutes at 3°C. After incubation, an aliquot of this mixture was then assayed for

LDH activity and another aliquot was passed through the Sephadex column for the determination of ^{203}Hg binding in the LDH fractions.

Radiorespirometry and Metabolite Detection

Preparation of Incubation Media

The media, for experiments with slices, were made up of 15.0 ml Krebs-Ringer phosphate buffer containing ^{14}C-labeled substrate. In the experiments with homogenates and soluble fractions the media were made up with 7.5 ml double strength Krebs-Ringer phosphate buffer, 5.5 ml distilled water, 2.0 ml homogenate or soluble fraction, and 50 μl of ^{14}C-labeled substrate solution.

The concentrations of ^{14}C-labeled substrates in the media were as follows:

DL-sodium lactate-1-^{14}C

Group I -- Two shipments of sodium DL-lactate-1-^{14}C were used. The specific activity of the first sample was 5.47 mc/mmole and the lactate concentration of the final media was 4.75×10^{-5} M. The specific activity of the second sample was 28 mc/mmole and the lactate concentration was 4.85×10^{-6} M.

Group II -- The specific activity of DL-sodium lactate was 28 mc/mmole. More lactate was used and the final concentration of lactate was 7.3×10^{-6} M.

The final concentration of DL-sodium lactate was 2.4×10^{-6} M

with a specific activity of 28 mc/mmole. All runs with kidney homogenates and soluble fractions were made with this media.

DL-sodium lactate-2-^{14}C. The specific activity of this sample was 36.5 mc/mmole and the final concentration was 4.87×10^{-6} M.

DL-sodium glutamate-3-4^{14}C. The specific activity of this sample was 5.5 mc/mmole and the final concentration was 2.0×10^{-5} M.

All countings for ^{14}C were carried out with a liquid scintillation spectrometer (Packard Tricarb Model 314ES). The counting solution of Bray which consists of an equal volume of (A) Toluene phosphor solution: 4 grams of 2, 5-diphenyloxazol, and 50 mg of 1, 4-bis-2-(5-phenyloxazol) benzene per liter of toluene, and (B) Methyl-cellosolve-napthalene solution: 50 grams of napthalene dissolved in a liter of methyl-cellosolve was used. The counting efficiency of this medium was 44%. All countings were standardized with benzoic acid as an internal standard.

Radiorespirometric System

The apparatus used in all in vitro experiments for continuous monitoring of respiratory $^{14}CO_2$ evolved by the kidney slices, utilizing ^{14}C-labeled substrates, is made up of the following components: (1) the reaction chamber, which is made from a 50 ml Erlenmeyer flask with two outlets to permit removal of respiratory $^{14}CO_2$ from

the chamber; the temperature of the chamber is regulated and maintained by a constant temperature bath, and the chamber can be shaken continuously; (2) a pump providing a stream of air to sweep through the respiration chamber at a prescribed rate directly into an ion chamber; (3) a flow ion chamber; 500 ml size chambers from Cary and Chicago Nuclear were used; (4) an electrometer: either electrometer, a Cary Model 38 or the Dynacon Model 6000, is capable of measuring current at the order of magnitude of 10^{-14}A with a sensitivity of 0.05 millivolt; (5) a recorder: a Morsely Model 681 strip chart recorder was connected to the Cary electrometer system and an Esterline Angus Graphic Ammeter recorder was connected to the Dynacon system for continuous recording of radioactivity. A schematic diagram of this is shown in Figure 1.

Analysis of Tissue and Media Samples

Samples of tissue slices (0.1 gm) and external media (0.5 ml) were collected at 4, 8, 12 and 24 hours during some of the experiments. Tissue slices were frozen quickly and then freeze-dried. Freeze-dried samples were ground with a mortar and pestle, weighed, and the radioactivity measured in a Geiger-Mueller counter. Counts were corrected for self-absorption and background. Aliquots (0.1 ml) of the media were counted in a liquid scintillation counter.

The freeze-dried samples were extracted twice with hexane,

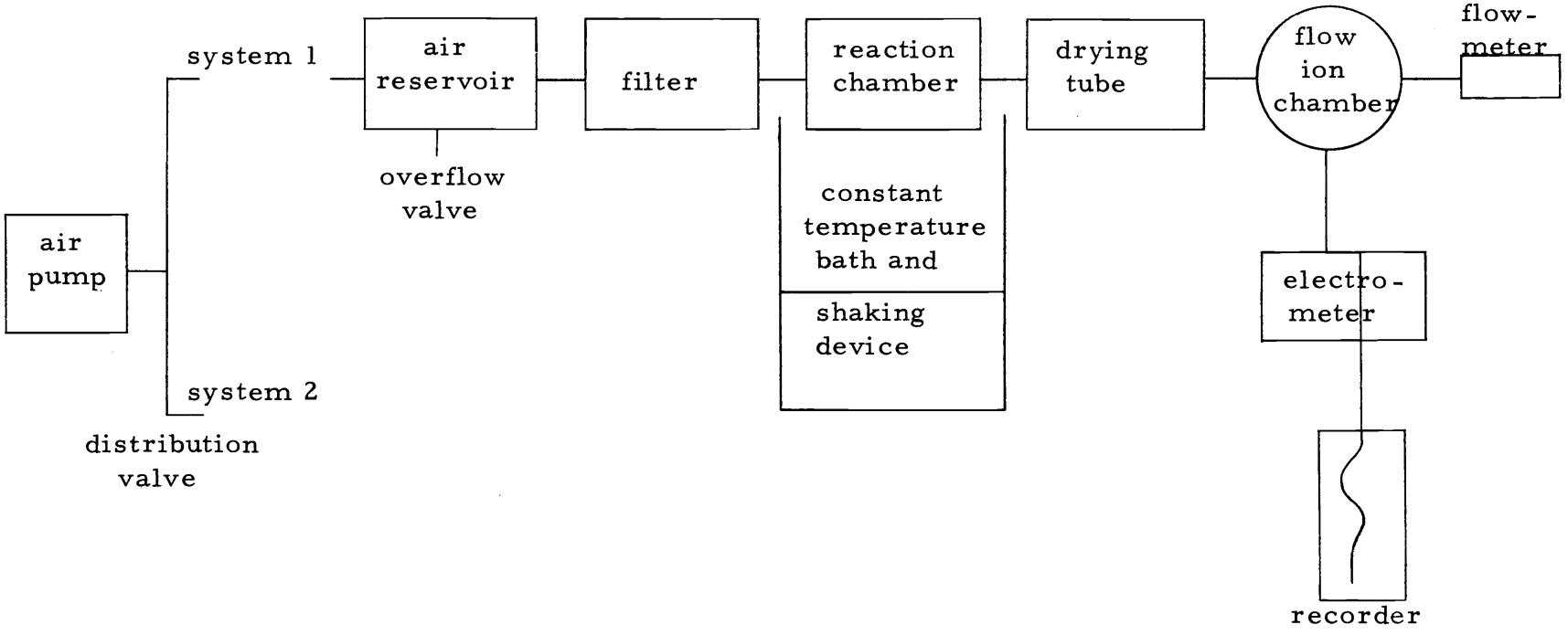


Figure 1. Radiorespirometric system.

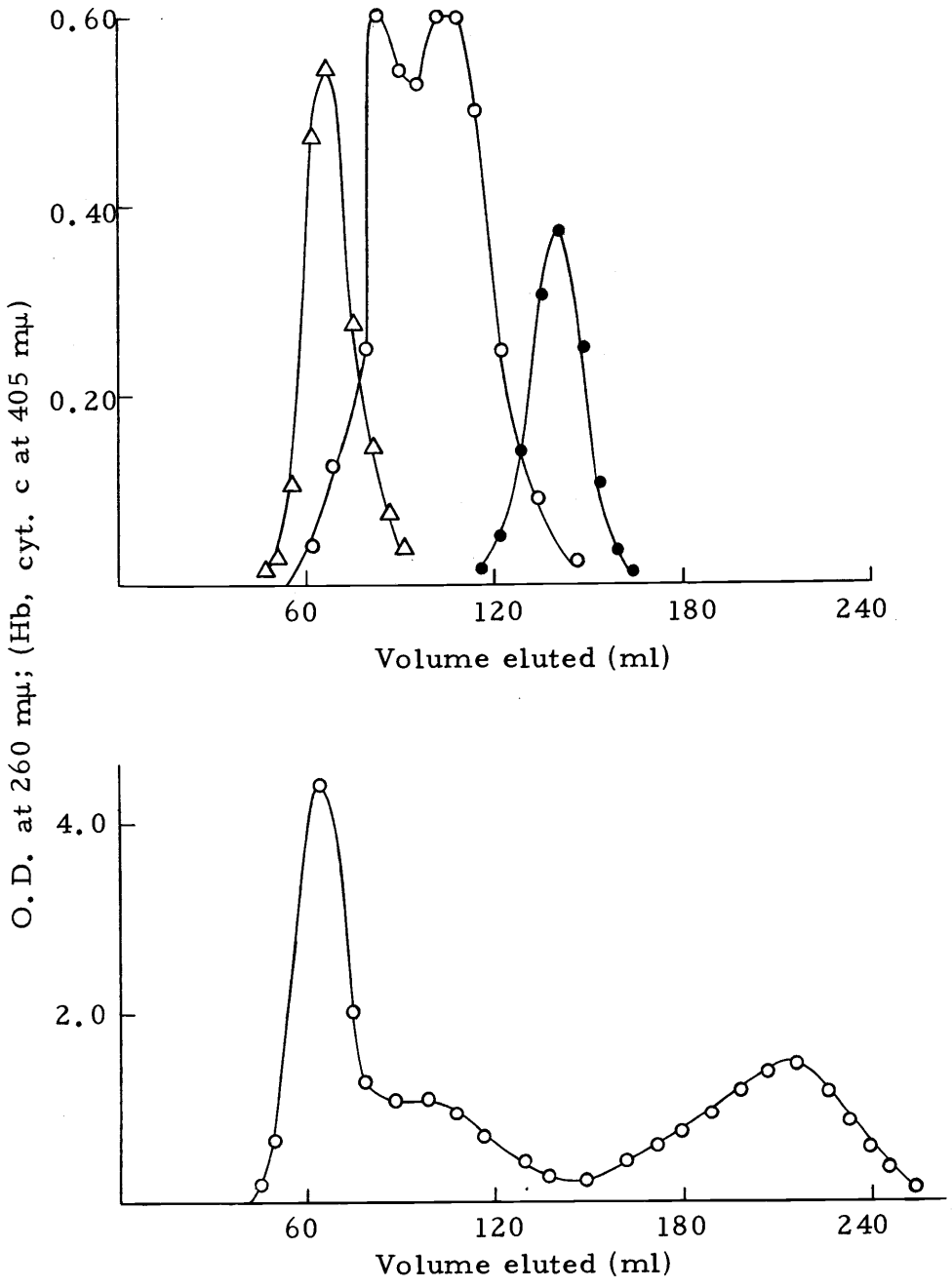
followed by a methanol extraction. The methanol extract and aliquots of media were applied to strips of Whatman #1 chromatography paper which were 1 x 18 1/2 inches. The strips were developed at room temperature in the solvent system of BAW (n-butanol: acetic acid : water, 12 : 3 : 5, V/V/V). The paper strips were allowed to equilibrate in the solvent chamber for two hours before adding the BAW solvent, which was freshly prepared each time. After being developed, the paper strips were air-dried in a hood for one hour and the radioactive spots were detected by the Radiochromatogram Scanner Packard Model 7201. The relative activity under each peak was determined.

RESULTS

O. D.$_{260\,m\mu}$ Profile and Molecular Weight Range of Kidney Soluble Proteins

The Sephadex gel filtration method described by Whitaker (64) was used to estimate the range of molecular weights and to separate the proteins in the kidney and liver soluble fractions. A linear correlation is reported to exist between the logarithm of the molecular weight of a protein and the ratio of its elution volume, Ve, to its void volume, Vo. Gamma-globulin, hemoglobin, and cytochrome-C were passed through the same column and the elution volume of each was determined, as seen in Figure 2.

The volume at which the O. D. peak appeared following passage of gamma-globulin through the column was 65 ml. Since gamma-globulin has a molecular weight much greater than 100,000, which is the exclusion limit of the gel, this volume is the void volume. Cytochrome-C from horse heart, reported to have a molecular weight of 12,270, resulted in an observed Ve/Vo ratio of 2.19. The elution of two x recrystallized Bovine hemoglobin resulted in two O. D. peaks. The first was used to obtain a Ve/Vo ratio of 1.26. It is probable that the second peak resulted from some dissociation. A plot of Ve/Vo values against the logarithm of their molecular weight is shown in Figure 3. The O. D.$_{260\,m\mu}$ profile of kidney



Top: Gamma globulin, hemoglobin, cytochrome-c respectively

Bottom: Control rat kidney soluble fraction.

Figure 2. Sephadex G-100 separation of some standard proteins and O. D. $_{260}$ mμ profile of rat kidney soluble proteins.

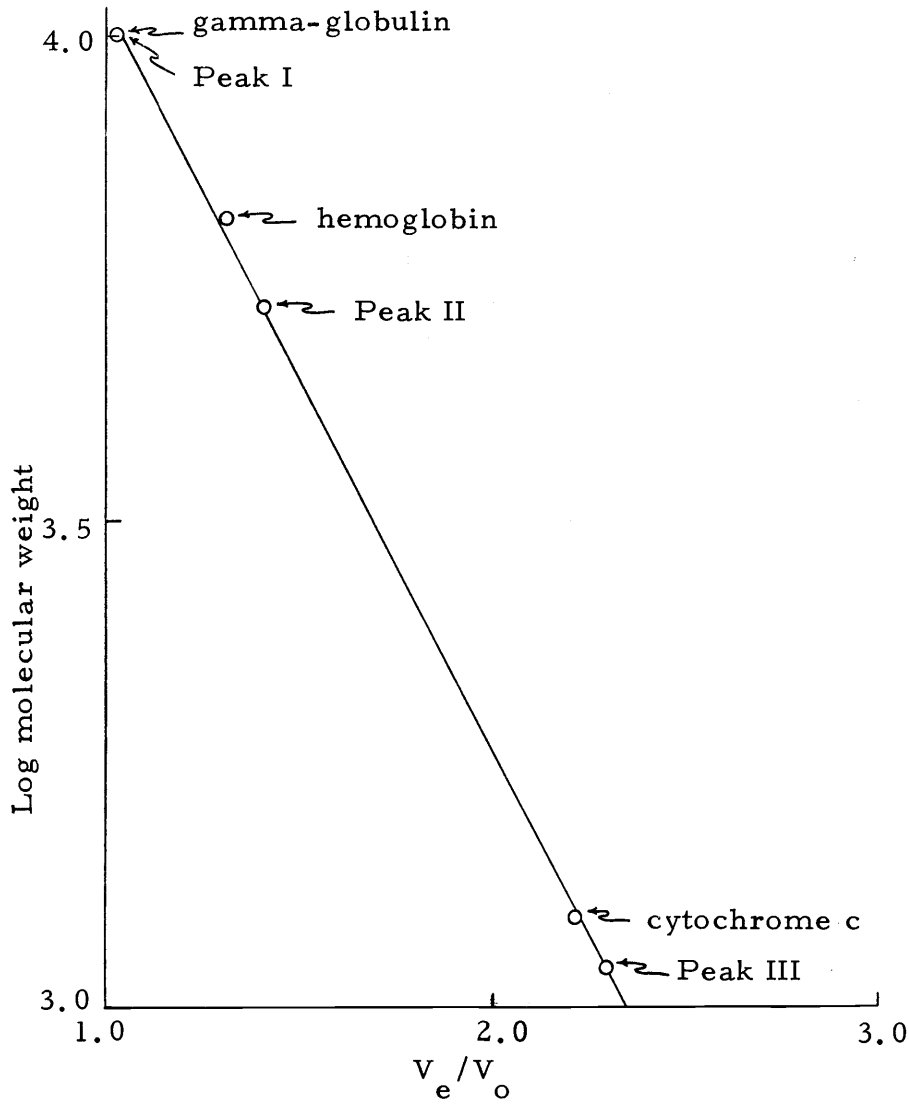
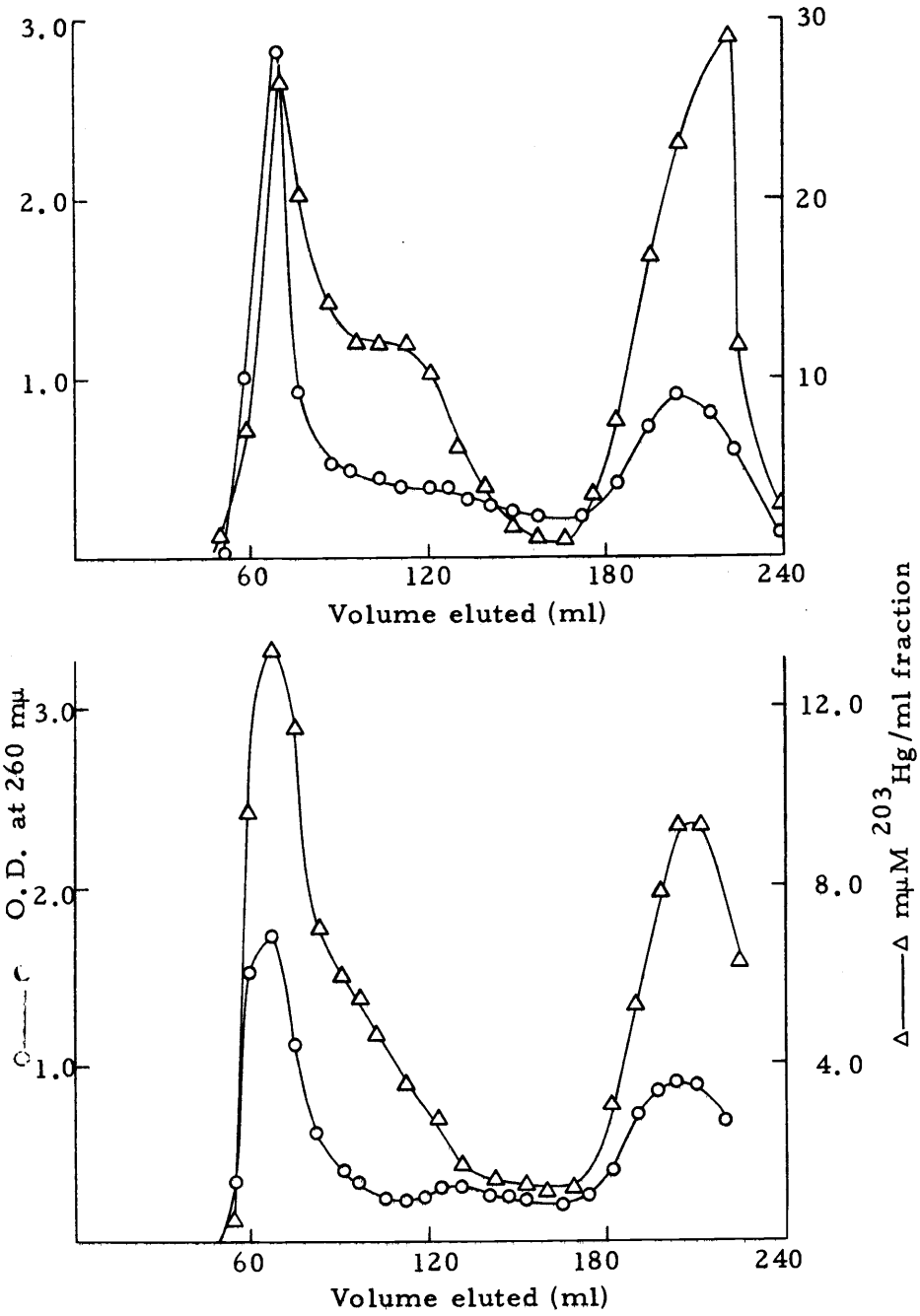


Figure 3. Relationship of log molecular weight and volume of elution for several proteins.

soluble fraction proteins is shown in Figure 2 and their ranges of molecular weight are plotted in Figure 3. Whitaker points out that in order to obtain a molecular weight which is significant to three figures without interpolation to the nearest 0.01 ml, one should use a column with Vo greater than 100 ml.

In Vitro Mercury Binding of Kidney Soluble Proteins

When kidney soluble proteins were incubated with Hg^{2+} or PMA for 30 minutes at 3°C, mercury binding to proteins of large molecular weight (collected around void volume) and small molecular weight (O. D. peak, Ve about 200 ml) was relatively greater than Hg-binding to proteins of intermediate molecular weight (Figure 4). Using a higher concentration of PMA resulted in an overall greater mercury binding. The patterns of the two mercurials did not differ extensively. When the soluble fraction was obtained from tissue slices incubated at 25°C in 1.0×10^{-5}M $^{203}Hg(Ac)_2$ or ^{203}Hg-PMA for two and one half hours, different labeling patterns were observed as compared to those obtained with the soluble proteins (Figure 5). In addition to the Hg-binding peak at void volume, two peaks were observed from PMA at 40,000 to 60,000 and 8,000 to 13,000 molecular weight ranges, while only one peak was observed at 8,000 to 13,000 molecular weight range from Hg^{2+}. Also, the binding level of Hg from PMA or $Hg(Ac)_2$ was greatly decreased in the fractions

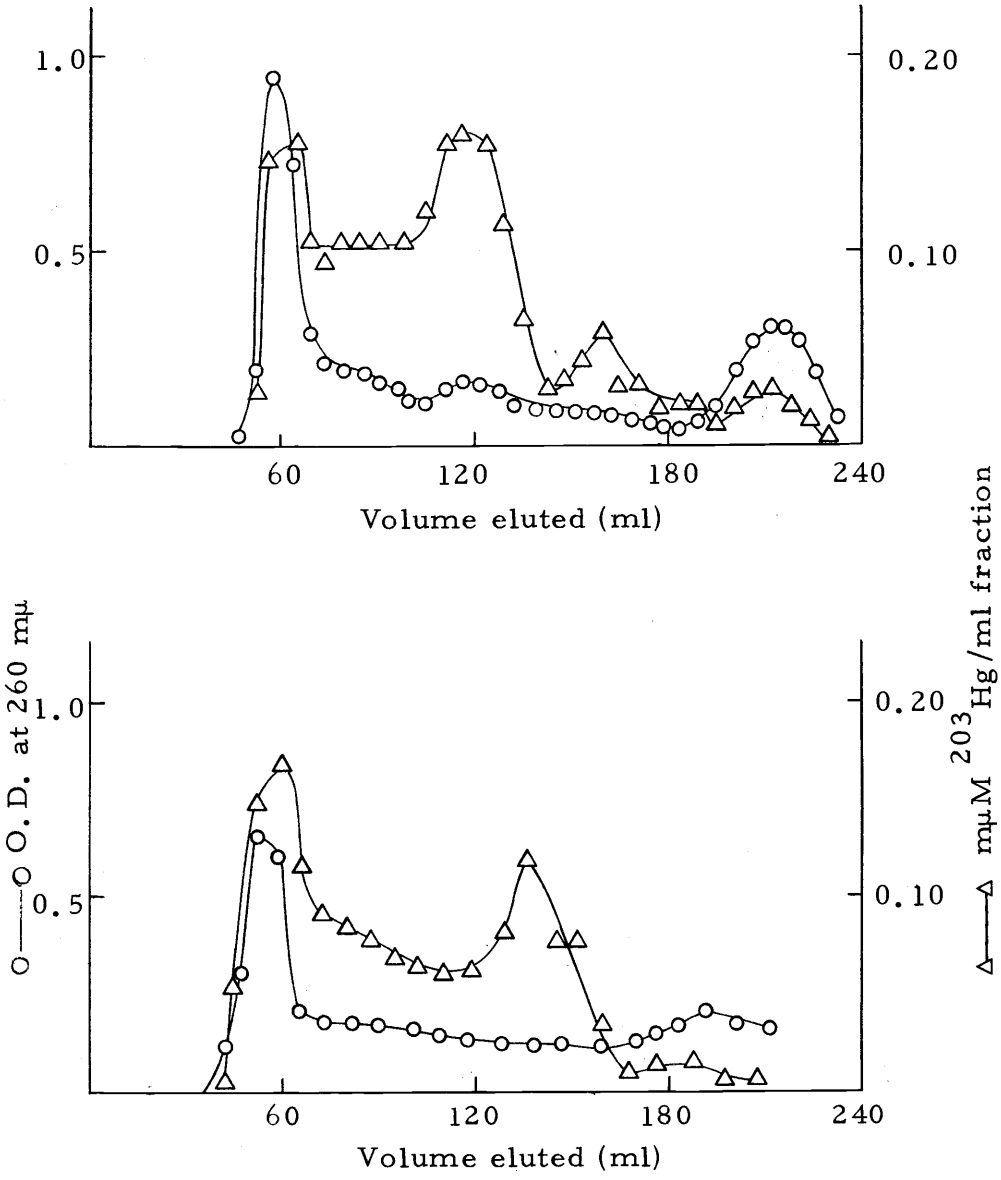


Top: Rat kidney soluble fraction; in vitro incubation 2.5×10^{-4} M PMA

Bottom: Rat kidney soluble fraction; in vitro incubation 1.0×10^{-4} M $Hg(Ac)_2$

Temperature 3^{o} Time 30 minutes

Figure 4. O. D.$_{260}$ mμ profile and ^{203}Hg-binding of soluble proteins after incubating kidney soluble fraction with $^{203}Hg(Ac)_2$ and ^{203}Hg-PMA.



Top: Rat kidney slices; in vitro incubation 1.0×10^{-5} M PMA

Bottom: Rat kidney slices; in vitro incubation 1.0×10^{-5} M $Hg(Ac)_2$

Temperature 25° C Time 2-1/2 hours

Figure 5. O.D.$_{260}$ mμ profile and ^{203}Hg-binding of soluble proteins after incubating kidney slices with $^{203}Hg(Ac)_2$ and ^{203}Hg PMA.

eluted which contain the last O. D. peak.

In Vivo Mercury Binding in Kidney Soluble Fraction

Figure 6 describes the accumulation of ^{203}Hg in the kidneys and liver with respect to time following single oral doses of 2 mg PMA or $Hg(Ac)_2$. The accumulation of ^{203}Hg in the kidneys, up to 48 hours after dosage, was about two-fold greater after PMA treatment. The level of ^{203}Hg in the kidneys was about the same 100 hours after dosage with either mercurial. The amount of ^{203}Hg from either mercurial retained in the kidneys was much greater than that retained in the liver. Up to 24 hours following dosage much greater amounts of ^{203}Hg from PMA than from $Hg(Ac)_2$ accumulated in the liver. The amount of ^{203}Hg from PMA decreased rapidly from 24 to 48 hours, and at 48 hours only low levels of ^{203}Hg from either mercurial were present in the liver.

The patterns of mercury binding in the kidney soluble proteins following oral mercurial administration differed a great deal from those obtained from direct incubation of the soluble proteins with mercurials. Figure 7 shows the patterns of mercury binding of kidney soluble proteins following oral PMA and $Hg(Ac)_2$ administration. They show three peak areas of mercury binding. The first area, designated peak 1, was collected between 60 and 80 ml and consists of proteins with molecular weight greater than 100,000. The fraction

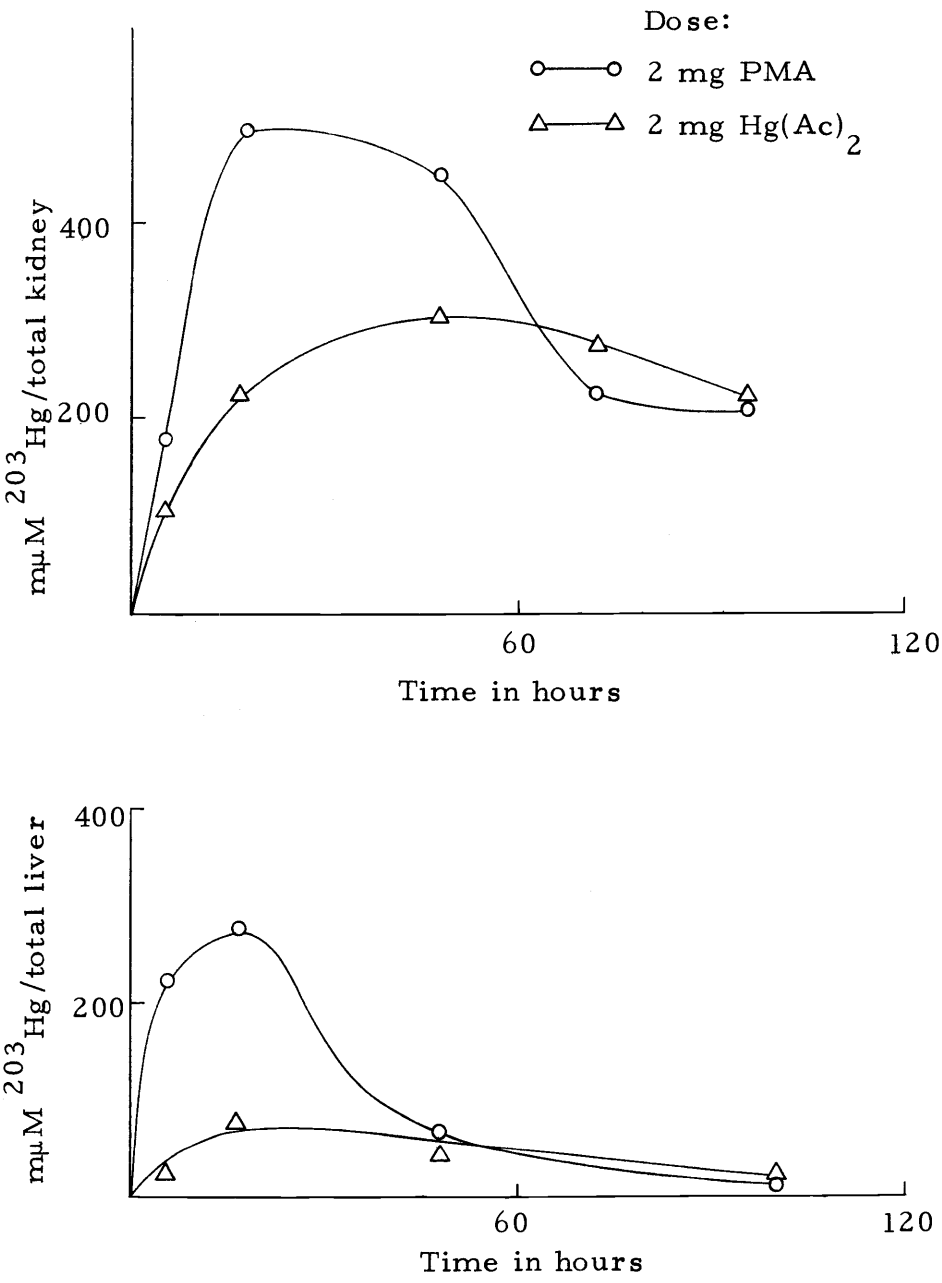
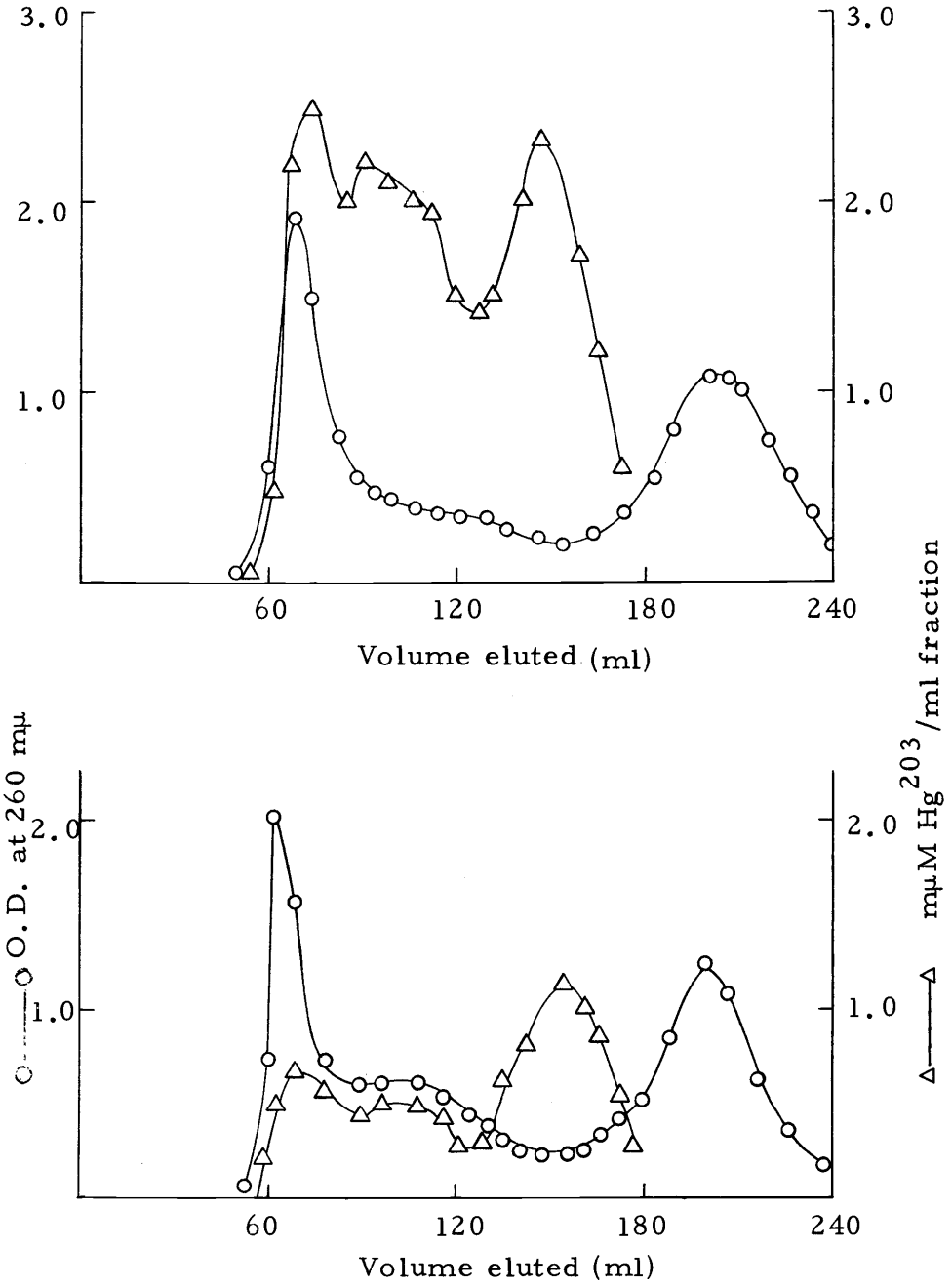


Figure 6. ^{203}Mercury accumulation in rat kidney and liver of animals receiving P^{203}MA and ^{203}Hg(Ac)$_2$, as post dosage time varies.



Top: Single dose; 2 mg PMA; 18 hours after dose

Bottom: Single dose; 2 mg $Hg(Ac)_2$; 13 hours after dose

Figure 7. $O.D._{260}$ mμ profile and ^{203}Hg-binding of rat kidney soluble proteins after single oral dosage of $^{203}Hg(Ac)_2$ and ^{203}Hg-PMA.

between 80 and 100 ml was referred to as peak II with a molecular weight range from 40,000 to 60,000. Peak III was eluted between 140 and 160 ml and the molecular weight range of the proteins was approximately 8,000 to 13,000. To obtain the comparable value of specific binding, the area for each peak, taken from the graph (Figure 7), under the mμm Hg/ml fraction curve, was calculated and was divided by the corresponding area under the O. D. curve. The Hg-binding patterns following oral PMA and $Hg(Ac)_2$ treatment differ greatly, which was not in agreement with those observed after direct incubation of soluble proteins. The results obtained in Figure 7 suggest that various dynamic metabolic factors may be involved in the distribution and binding of Hg from the two mercurials. The animals receiving Hg^{2+} and PMA treatment were not sacrificed at exactly the same time after dosage. This difference in time may influence the Hg distribution. The time course changes of mercury binding for each peak after a single oral dose are shown in Figures 8 and 9 for PMA and Hg^{2+} respectively. The mercury binding from PMA in all three peaks showed an increase during the first twenty hours. The rate of increase was greatest for peak III, next peak II, and the least, peak I. From 20-35 hours, peaks I and II showed a net reduction with the rate of reduction of peak II greater than that of peak I. In contrast, peak III was still rapidly accumulating Hg during this period. From around 45 to 168 hours, peaks I and II had similar

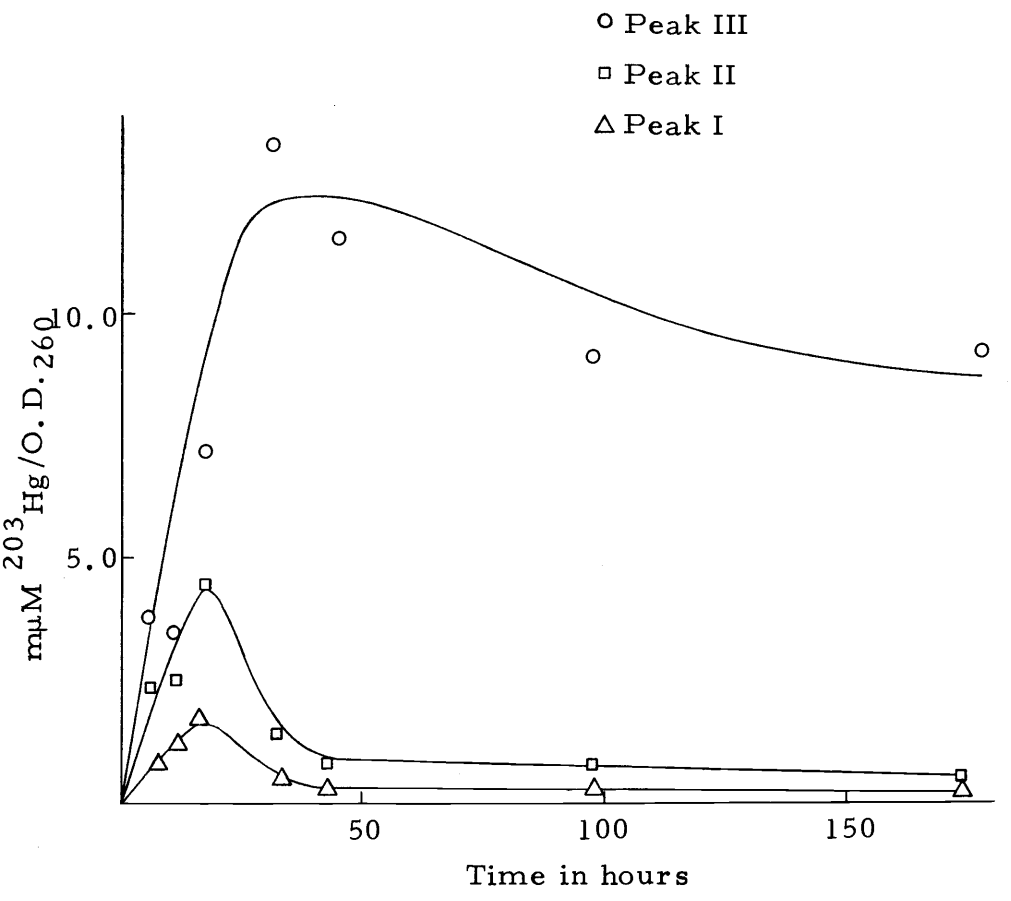


Figure 8. Time course study of ^{203}Hg-binding in kidney soluble fraction proteins following 2 mg ^{203}Hg-PMA dosage.

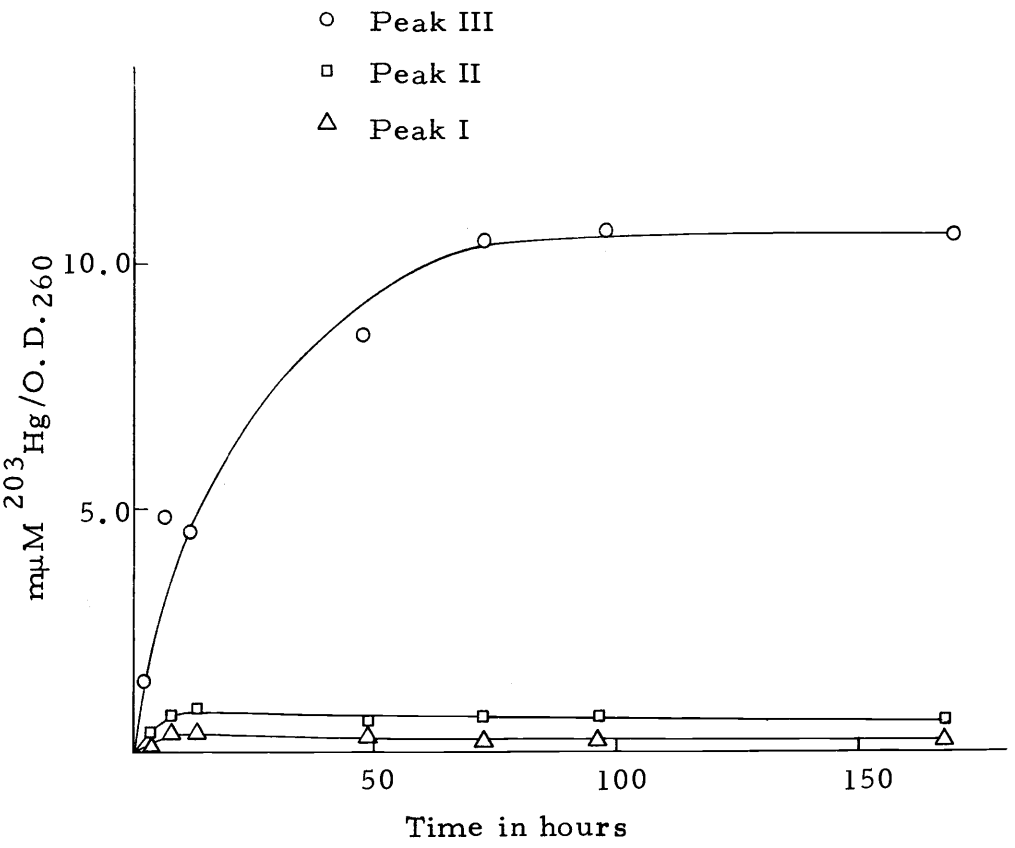


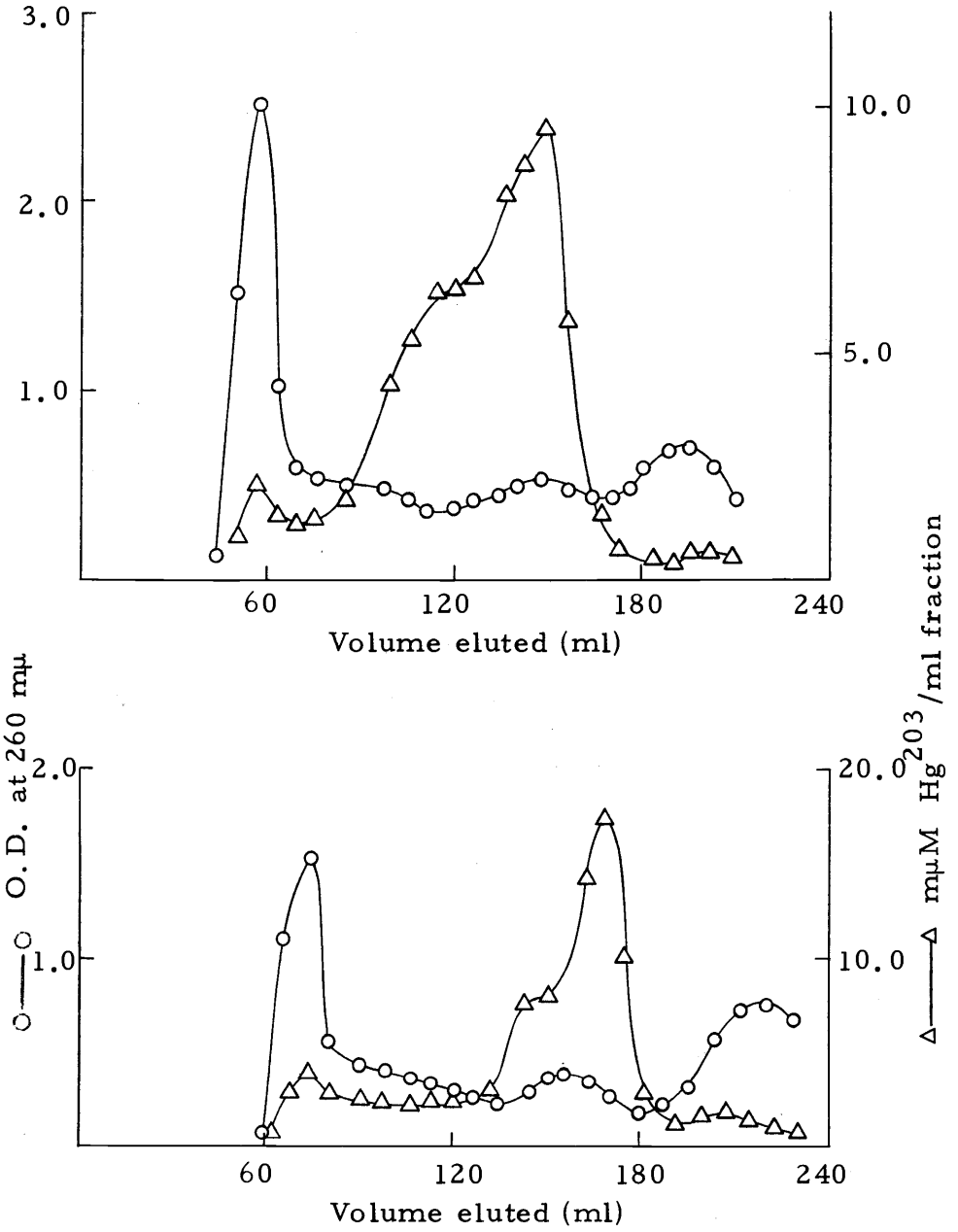
Figure 9. Time course study of ^{203}Hg-binding in kidney soluble fraction proteins following 2 mg $^{203}Hg(Ac)_2$ dosage.

gradual rates of Hg reduction. Peak III showed a somewhat rapid reduction rate from 45 to 100 hours and then remained fairly constant to 168 hours. The sample at 168 hours was the last recorded. The Hg-binding rates of peaks I and II from Hg^{2+} were quite different from those seen in Figure 8 following PMA dosage. The level of mercury bound is much smaller and the elimination rates are very gradual during the 170 hour period. At least ten-fold more mercury was bound to peak III, and the amount of binding increased continuously till 70 hours, and then remained constant till 168 hours or more.

Rats receiving multiple doses of PMA or $Hg(Ac)_2$ also showed similar labeling patterns in the kidney soluble proteins. However, the O. D. profile was definitely altered, with an increase of O. D. in the area of peak III (Figure 10).

In Vivo Mercury Binding of Liver Soluble Fraction

The livers, following single 2 mg PMA dosages, were removed at various post-dosage times and treated the same as the kidneys. The O. D. and mercury binding profiles, shown in Figure 11, are quite similar to those of the kidney soluble fraction. The kinetics of Hg-binding (mμm Hg/O. D.) to proteins were calculated and presented in Table 1. The binding of mercury in Peak I was low and did not change greatly with time. Within the Peak II area, the most Hg was bound during the first 9 hours, then the level quickly decreased.



Top: Multiple dose; 9 x 3 mg $Hg(Ac)_2$ at daily intervals; 24 hours after last dose.

Bottom: Multiple dose; 5 x 3 mg PMA at daily intervals; 24 hours after last dose.

Figure 10. $O.D._{260}$ mμ profile and ^{203}Hg-binding of rat kidney soluble proteins after multiple oral dosage of $^{203}Hg(Ac)_2$ and ^{203}Hg-PMA.

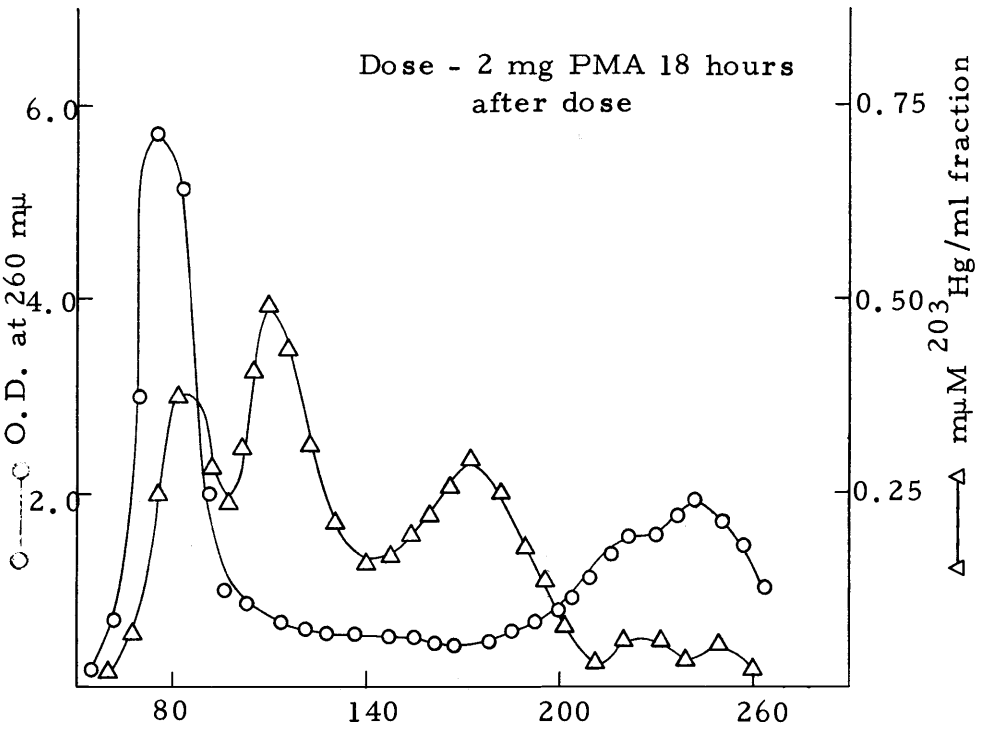


Figure 11. O.D.$_{260}$ mμ profile and ^{203}Hg-binding of rat liver soluble proteins after single oral dose of ^{203}-Hg-PMA.

Table 1. In vivo kinetics of mercury binding into rat liver soluble proteins after an oral dose of ^{203}Hg-PMA at 3.6 mg/kg body weight.

Sephedex-G-100 Column 2.2 x 60 cm

Time after dosage in hours	Mercury Binding, mμm/O. D.		
	Peak I mol wt. ≥ 100,000	Peak II mol wt. 36,000-50,000	Peak III mol wt. 11,000-14,000
4.5	0.074	0.502	0.254
9	0.145	0.762	0.380
12	0.074	0.535	0.315
18	0.058	0.468	0.482
34	0.070	0.190	1.08
41	0.064	0.172	0.320

Peak III showed a continuing increase in mercury binding until 34 hours, after which the Hg was rapidly eliminated from that peak. Peak III of the liver soluble fraction did not accumulate or retain Hg to the extent observed in the kidney soluble fraction.

Soluble Enzymes of Kidney and Liver

Alkaline and Acid Phosphatases

After Sephadex G-100 gel filtration of the kidney soluble fraction, the acid and alkaline phosphatase activities, ^{203}Hg concentration, and optical density of the eluate were determined (Figures 12 and 13). The mercury binding data are expressed by dividing the mg protein of the fraction into the ^{203}Hg concentration (Figure 13).

The mercurials did not significantly affect the acid and alkaline phosphatase levels in the kidney soluble fractions of both male and female rats following various dosages of $Hg(Ac)_2$ or PMA (Figure 13). The Hg-binding data showed that more ^{203}Hg from PMA than from Hg^{2+} was present in the fractions containing enzymatic activity. The mole ^{203}Hg per mole of protein ratio was much less than one. Following Sephadex G-100 separation, the alkaline phosphatase fraction had about 0.2 Mole Hg bound per mole protein in the alkaline phosphatase fraction.

The in vitro studies made with Sephadex fractions containing enzymatic activity showed that both acid and alkaline phosphatase

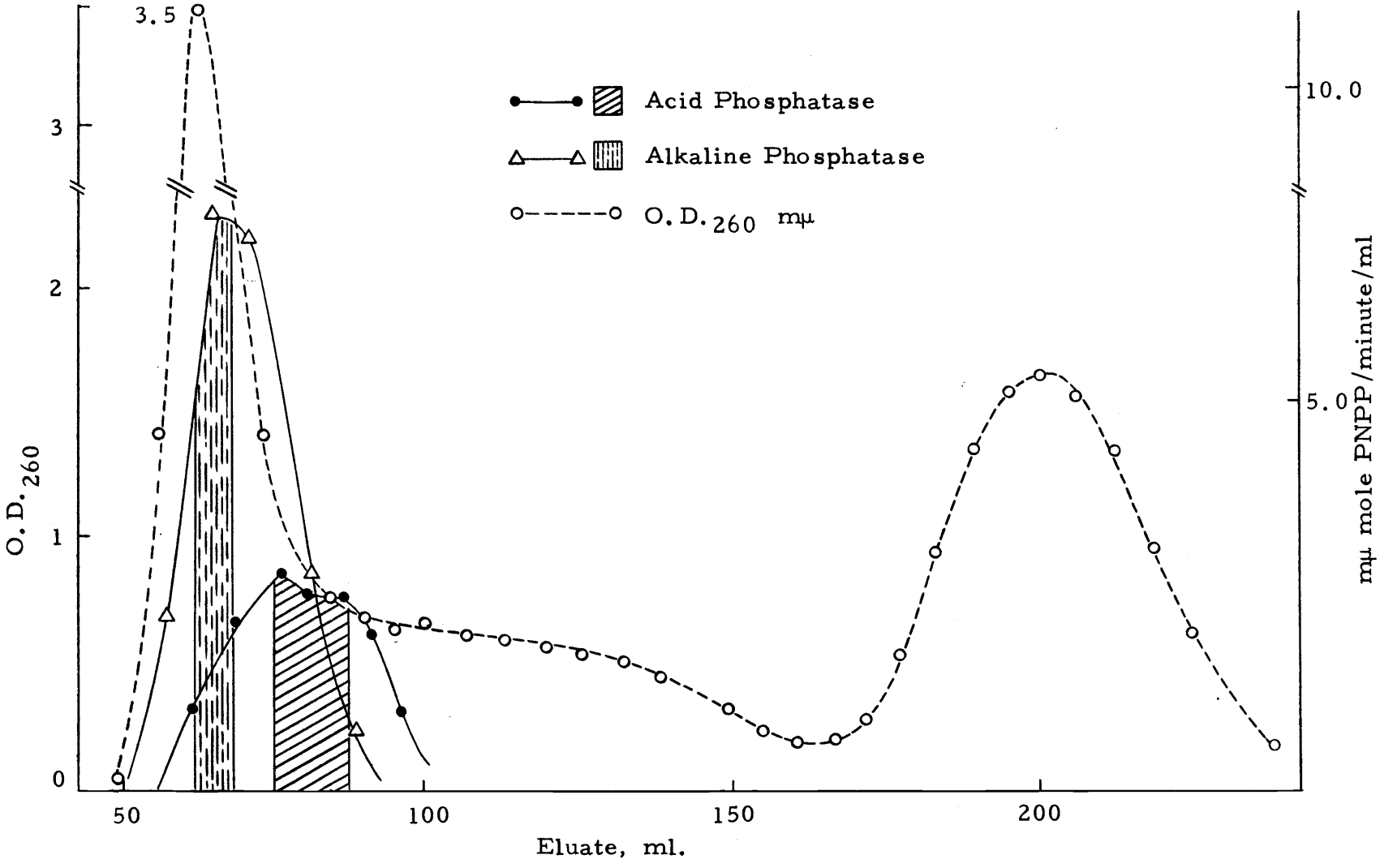
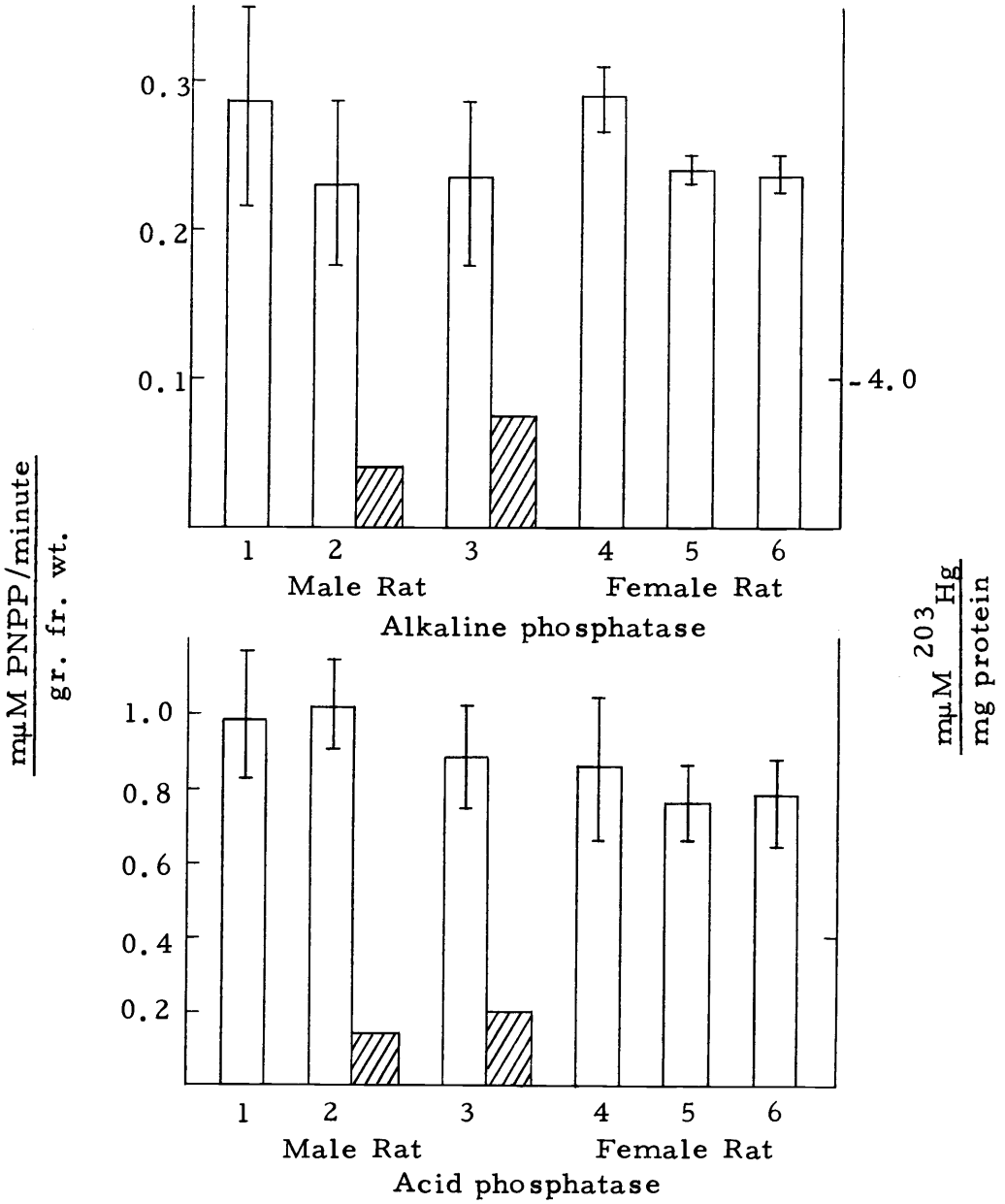


Figure 12. Sephadex G-100 separation of alkaline and acid phosphatase in rat kidney soluble fraction.



		dose	time of sacrifice
1.	control		
2.	$Hg(Ac)_2$	single; 2-5 mg	6-48 hours
3.	PMA	single; 1-3 mg	6-48 hours
4.	control		
5.	$Hg(Ac)_2$	single; 2-5 mg	12-24 hours
6.	PMA	single; 1-3 mg	12-24 hours

Figure 13. Alkaline and acid phosphatase activity in kidney soluble fraction of rats receiving PMA and $Hg(Ac)_2$. Open bar, enzymatic activity; lined bar, Hg-binding.

were much more sensitive to $Hg(Ac)_2$ than to PMA (Figure 14). Similar results were obtained with calf mucosal alkaline phosphatase from Sigma. A 1 mg/ml enzyme solution of the commercial alkaline phosphatase preparation incubated in 5.0×10^{-5} M $Hg(Ac)_2$ showed 20% inhibition of enzymatic activity. After sephadex separation, the alkaline phosphatase fraction had about 2.5 Moles Hg/Mole protein in the alkaline phosphatase fraction.

Lactic and Malic Acid Dehydrogenases

The elution pattern of lactic and malic dehydrogenase from the sephadex column is shown in Figure 15. Lactic and malic dehydrogenase activities and optical density in the eluate were determined. The Hg-binding data are given by ^{203}Hg concentration/O. D. in Figure 16.

There was a slight stimulation of LDH in the kidney soluble fraction but no change in the MDH level following single doses of PMA and $Hg(Ac)_2$, even though the binding of PMA was about twice that of $Hg(Ac)_2$. In the case of multiple doses of $Hg(Ac)_2$, the levels of both LDH and MDH decreased. Mercury-binding was about 3-fold that resulting from the single doses. If the animals were allowed to recover for two weeks following the last dose of the multiple dose series, LDH levels in the kidney soluble fraction still did not seem to return to the control level. However, MDH levels recovered to

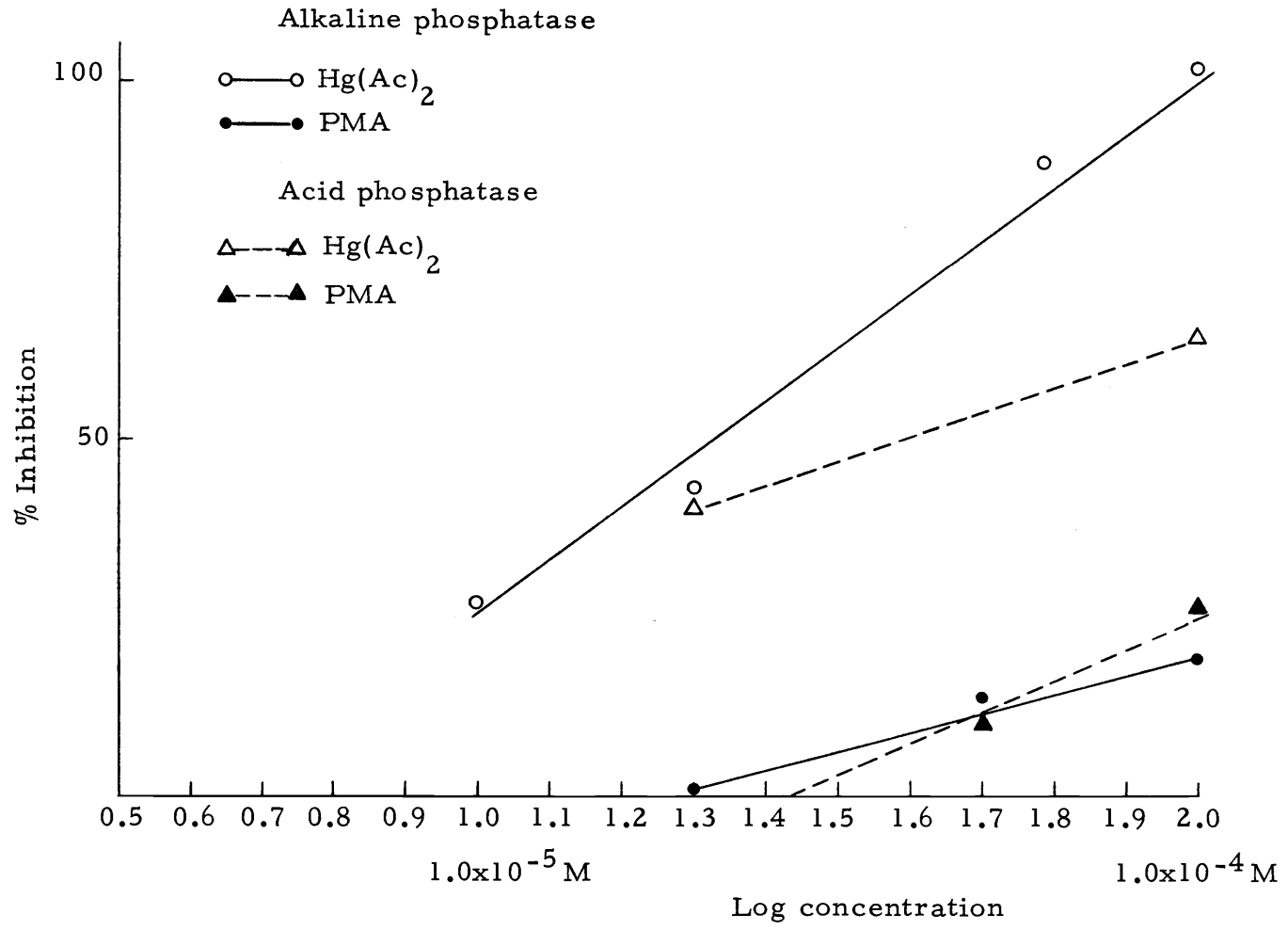


Figure 14. In vitro effect of PMA and $Hg(Ac)_2$ on acid and alkaline phosphatase of rat kidney soluble fraction.

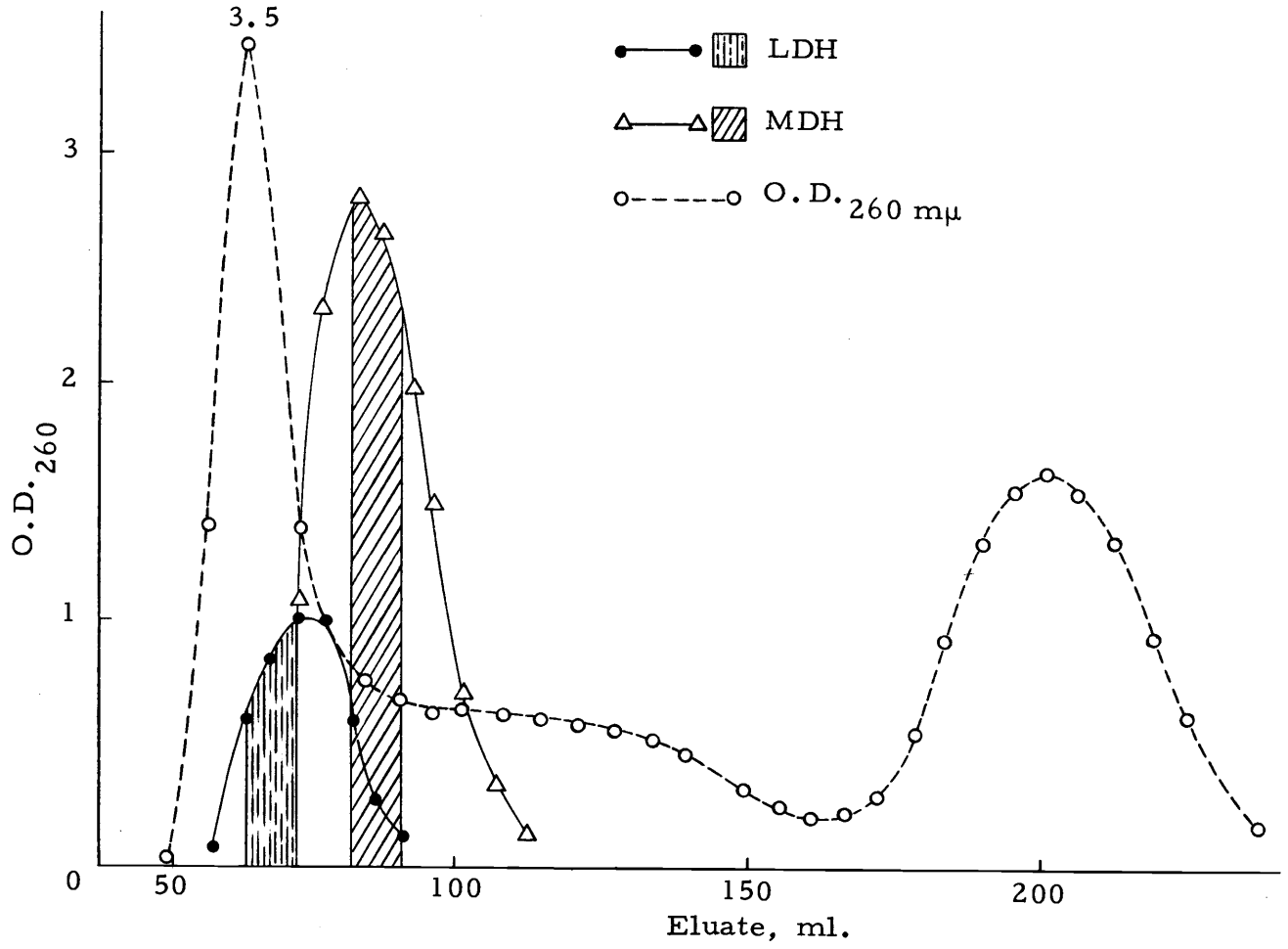
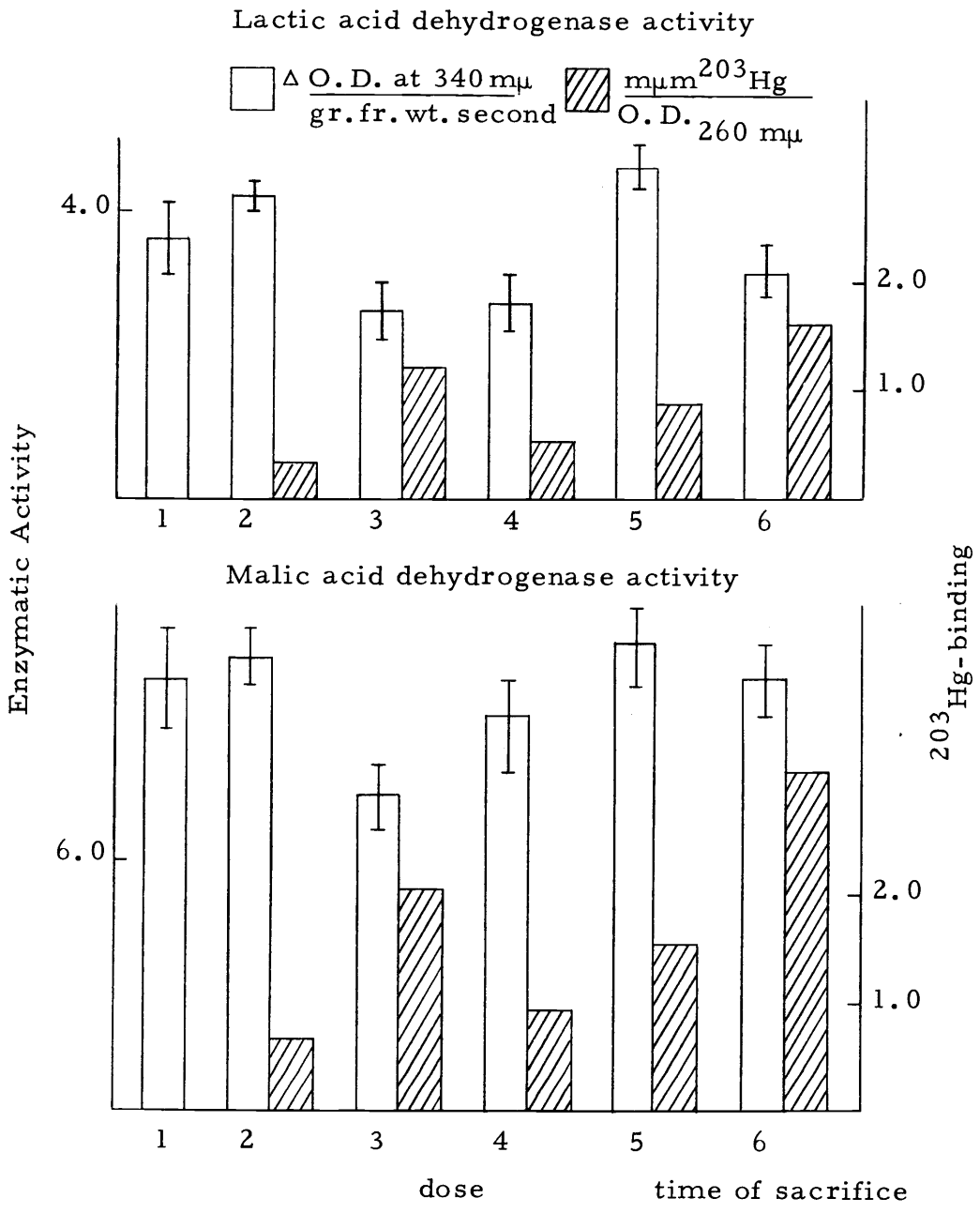


Figure 15. Sephadex G-100 separation of lactic and malic acid dehydrogenase in rat kidney soluble fraction.



1.	control		
2.	$Hg(Ac)_2$	single; 2-15 mg	24 hours
3.	$Hg(Ac)_2$	multiple; 25-40 mg	5-24 hours
4.	$Hg(Ac)_2$	multiple; 25-35 mg	5-14 days
5.	PMA	single; 2 mg	24 hours
6.	PMA	multiple; 4-24 mg	24 hours

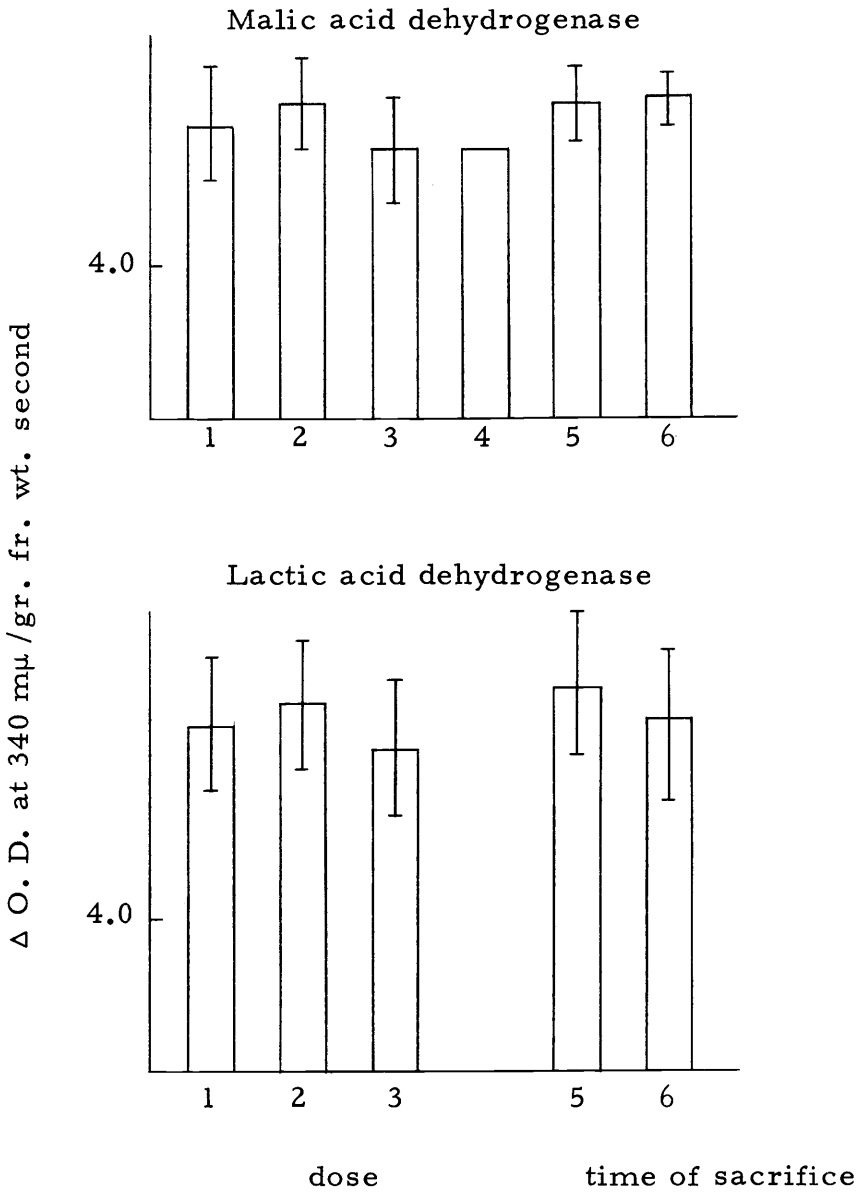
Figure 16. Lactic and malic acid dehydrogenase activity in kidney soluble fraction of rats receiving PMA and $Hg(Ac)_2$.

some extent, even though Hg-binding decreased by 1/2 or 1/3 that of the maximum binding observed (which was about 0.3 Mole of Hg bound to one mole of protein).

The liver soluble fractions from the rats treated as described showed no significant alteration of LDH or MDH activity levels (Figure 17).

When the kidney soluble fraction was incubated in vitro with $Hg(Ac)_2$, concentrations as high as 1.0×10^{-4} M did not affect the LDH activity. Even when the concentrations were increased to 3.0×10^{-4} M, only a slight inhibition of LDH activity was observed. The soluble fraction incubated in 1.0×10^{-4} M $^{203}Hg(Ac)_2$ was separated by Sephadex G-100 column to determine Hg-binding of the LDH fraction. The results revealed that about 3.2 M Hg/M protein was bound in the LDH fraction, or about ten times the amount observed from in vivo experiments. This suggests that inhibition of LDH activity is not directly related to the Hg-binding in vivo.

When LDH and MDH of kidney soluble fraction were partially purified through Sephadex G-100 gel filtration, the enzyme preparations were found to be very sensitive to $Hg(Ac)_2$ and PMA, as shown in Figures 18 and 19. To achieve the same degree of inhibition, two fold or more PMA than $Hg(Ac)_2$ was required. When the enzyme preparation was prepared from an animal which had received an oral dose of 6 mg $Hg(Ac)_2$, these enzymes were found to be more sensitive



1.	control		
2.	$Hg(Ac)_2$	single; 2-15 mg	24 hours
3.	$Hg(Ac)_2$	multiple; 25-40 mg	5-24 hours
4.	$Hg(Ac)_2$	multiple; 25-35 mg	5-14 days
5.	PMA	single; 2 mg	24 hours
6.	PMA	multiple; 4-24 mg	24 hours

Figure 17. Lactic and malic acid dehydrogenase activity in liver soluble fraction of rats receiving PMA and $Hg(Ac)_2$.

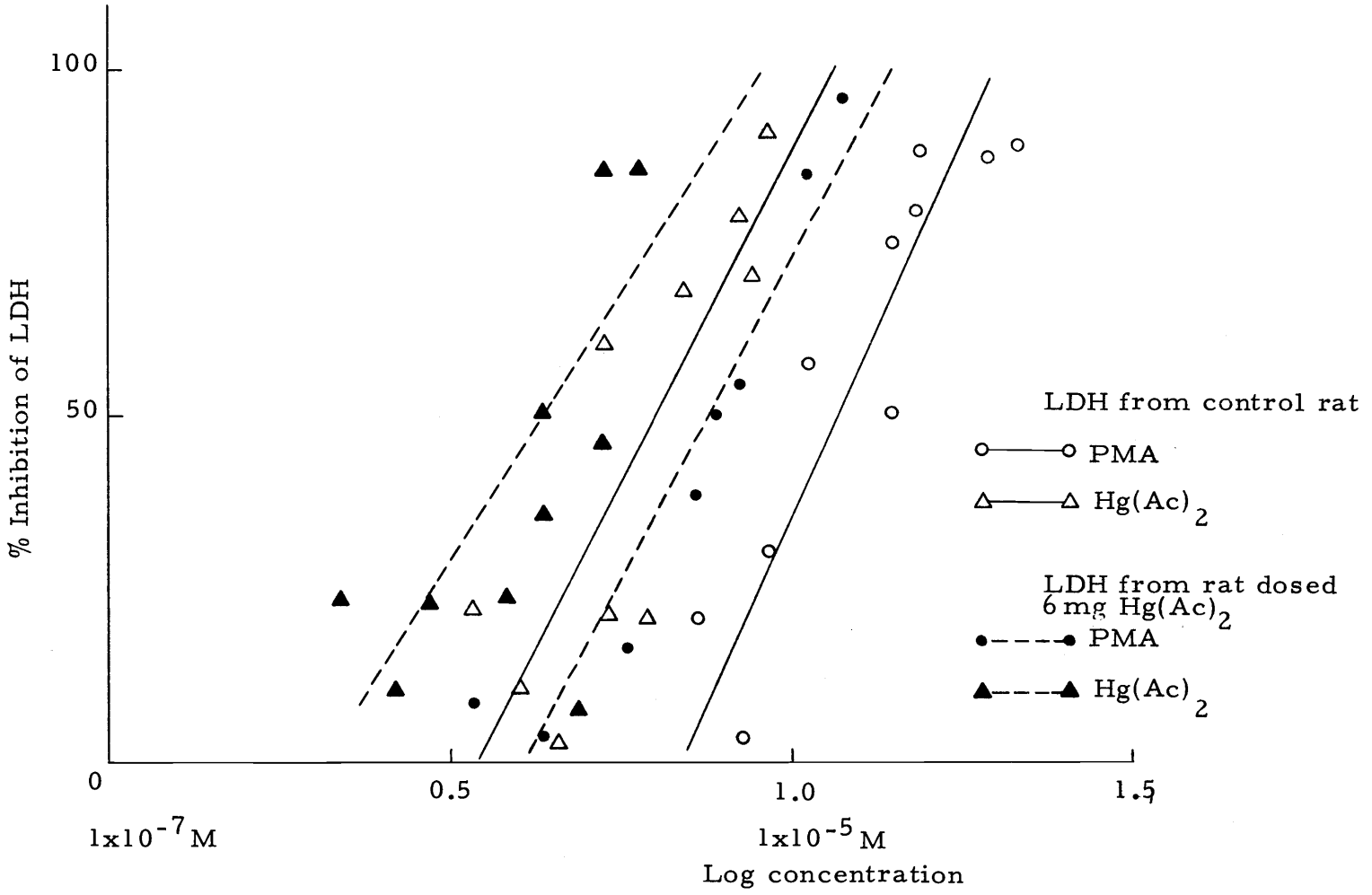


Figure 18. In vitro effect of PMA and $Hg(Ac)_2$ on kidney soluble lactic acid dehydrogenase from $Hg(Ac)_2$ treated and control rats.

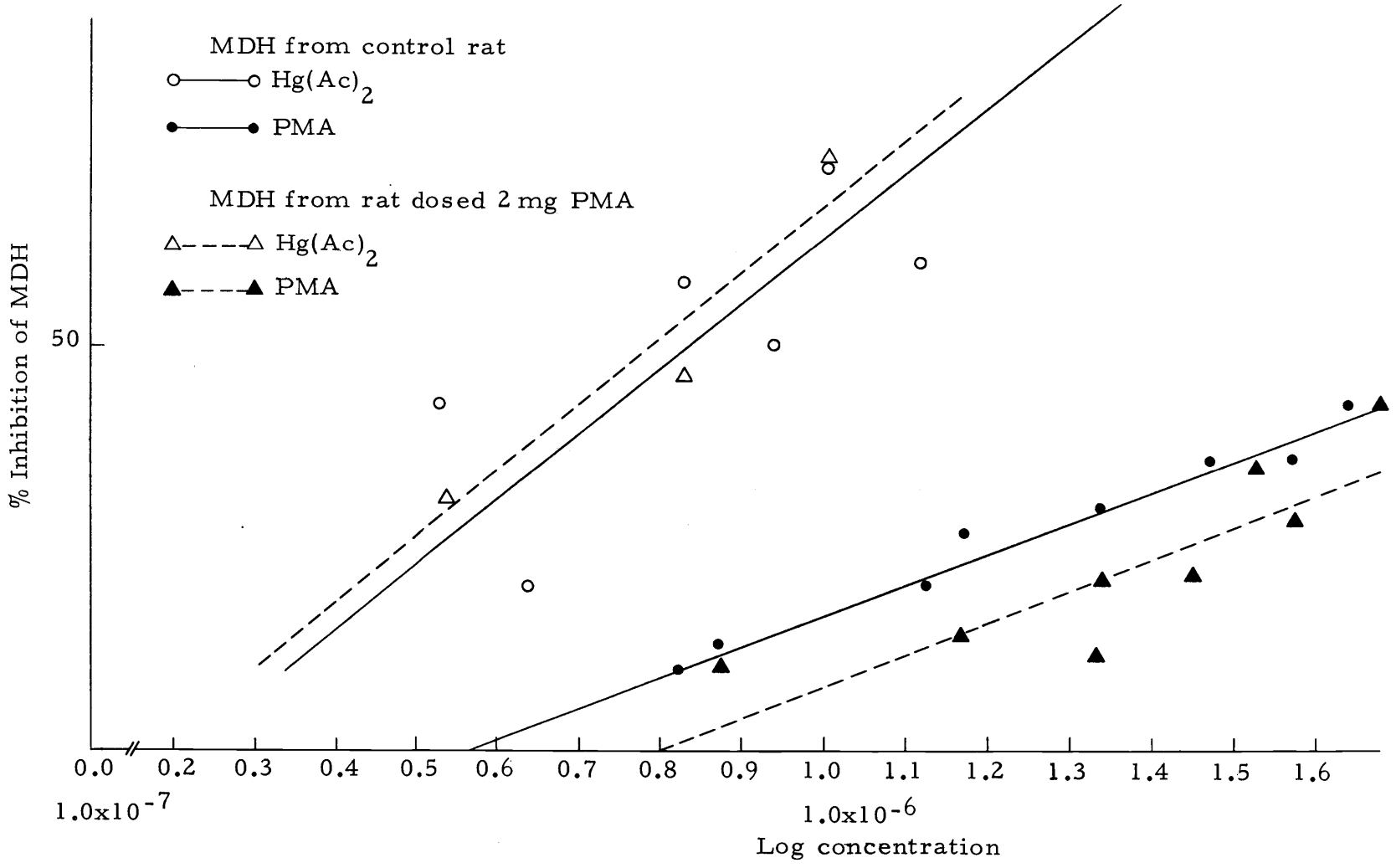


Figure 19. In vitro effect of PMA and $Hg(Ac)_2$ on kidney soluble malic acid dehydrogenase from PMA treated and control rats.

to $Hg(Ac)_2$ or PMA inhibition than those prepared from an untreated animal. As shown in Figure 18, the slope of LDH inhibition was similar for $Hg(Ac)_2$ and PMA, regardless of whether the enzymes were prepared from either treated or untreated animals. The slope of inhibition of MDH, as shown in Figure 19, indicated that MDH activity was not affected as markedly as LDH activity when the enzyme was incubated with either of the two mercurials. Similarly, as in the LDH studies, PMA showed less inhibition than $Hg(Ac)_2$ in untreated animals. When the enzyme preparation was prepared from an animal which had received an oral dose of 2 mg PMA, these enzymes showed inhibition levels similar to the control animals. The curves of Figures 18 and 19 were plotted using the least squares method of data analysis.

Radiorespirometry and Metabolite Detection

The total dosage level of PMA and $Hg(Ac)_2$ required to decrease the activity of some enzymes in the kidney soluble fraction ranged from 15 to 30 mg administered over a period of several days. It was decided to examine the metabolism of several ^{14}C-substrates by kidney slices from animals receiving 15 to 30 mg of PMA or $Hg(Ac)_2$ and to determine whether or not changes in metabolism could be related to the changes in enzyme activity levels or the levels of ^{203}Hg accumulation in the kidney tissue. These determina-

tions were carried out 24 hours and 2 weeks following the last dose.

The animals were orally dosed with PMA and $Hg(Ac)_2$ and sacrificed as follows:

a) PMA (24 hours) - rats were dosed with 3 mg PMA each day for 5 successive days and killed 24 hours after the last dose

b) PMA (2 weeks) - rats were dosed with 3 mg PMA each day for 5 successive days and killed 2 weeks after the last dose

c) Hg (24 hours) - rats were dosed with 3 mg $Hg(Ac)_2$ each day for 10 successive days and killed 24 hours after the last dose

d) Hg (2 weeks) - rats were dosed with 3 mg $Hg(Ac)_2$ each day for 10 successive days and killed 2 weeks after the last dose.

The above treatment designations are used in the following results section.

The ^{203}Hg concentrations that were present in the kidneys at the time of sacrifice were:

PMA (24 hours) - 990 mμm or 198 μg ^{203}Hg/gr. fr. wt.

PMA (2 weeks) - 430 mμm or 86 μg ^{203}Hg/gr. fr. wt.

Hg^{2+} (24 hours) - 465 mμm or 93 μg ^{203}Hg/gr. fr. wt.

Hg^{2+} (2 weeks) - 190 mμm or 38 μg ^{203}Hg/gr. fr. wt.

The kidneys were puffy and lighter in color following multiple doses of the mercurials. They were increased in size and weights as indicated in the following: a) controls, 2.4 ± 0.2 gm (16 animals sacrificed), b) PMA (24 hours), 3.3 ± 0.3 gm (10 animals sacrificed),

c) PMA (2 weeks), 2.8 ± 0.2 gm (6 animals sacrificed), d) Hg^{2+} (24 hours), 2.9 ± 0.2 gm (11 animals sacrificed), and e) Hg^{2+} (2 weeks), 2.7 ± 0.2 gm (6 animals sacrificed). The kidney weights after single dose exposures of PMA and $Hg(Ac)_2$ were the same as in controls.

In Vitro Metabolism of ^{14}C-Lactate

Lactate-1-^{14}C Metabolism by Kidney Slices

Group I (using kidney slices from rats receiving multiple doses)

The metabolic rates of lactate-1-^{14}C conversion by kidney slices from control and mercurial treated rats as shown in Figure 20 indicated that oral administration of toxic dosages of mercurials bring about some changes in the capacity of the kidney to metabolize lactate-1-^{14}C. Kidney slices from treated rats exhibited an inhibition of $^{14}CO_2$ production during the initial period and a stimulation during later hours. The overall $^{14}CO_2$ production from lactate-1-^{14}C was not changed. This experiment and several following experiments with kidney slices indicated that the pathway leading from lactate-1-^{14}C to $^{14}CO_2$ operates at a lower rate following mercurial treatment, while the pathway leading to ^{14}C-alanine formation functions at the same rate. PMA treatment resulted in a greater disturbance than a greater dose of $Hg(Ac)_2$. A post-dosage time of two weeks allowed the kidneys to recover a significant portion of their initial capacity

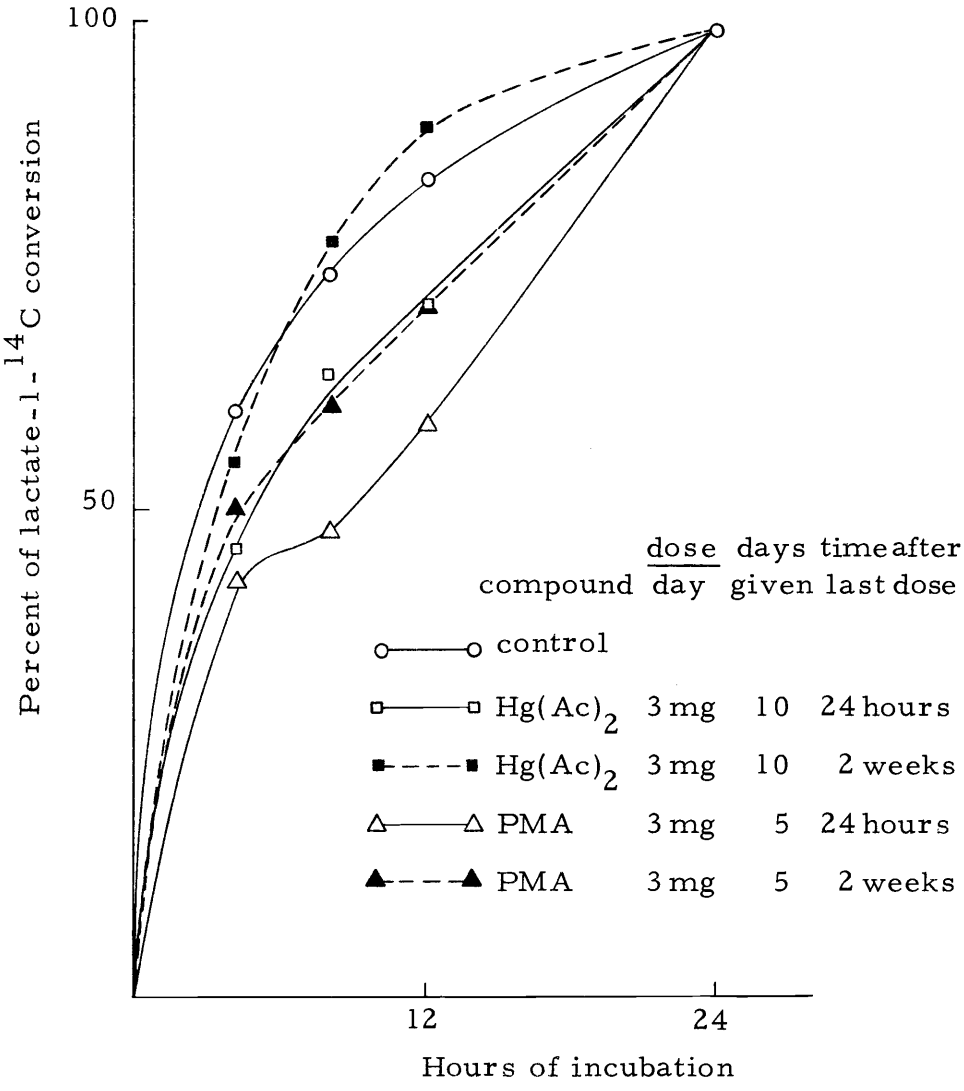


Figure 20. Rate of lactate-1-^{14}C conversion by kidney slices, Group I Series.

for metabolic activity. The extent of metabolic disturbance correlates, in most cases, with the ^{203}Hg content present in the kidney tissue. Kidney slices from control and treated animals converted about the same amount of lactate-1-^{14}C to $^{14}CO_2$ within the 24-hour period. Control slices converted about 75% of the lactate-1-^{14}C to $^{14}CO_2$ within the first 12 hours and 15% during the second 12 hours, with the major peak of $^{14}CO_2$ appearing between the first and third hours and a second peak at 14 to 16 hours. Slices from PMA and $Hg(Ac)_2$ treated animals sacrificed after 24 hours (Figures 21a and b), showed a decreased amount of $^{14}CO_2$ output the first twelve hours and an increased quantity respired the second twelve hours, with the appearance of the second peak at 17 to 18 hours. The slices from PMA-treated animals exhibited this effect to a greater degree than the slices from the Hg^{2+} treated animals. $^{14}CO_2$ production patterns from kidney slices from treated animals 2 weeks after the final $Hg(Ac)_2$ dosage returned to normal.

$^{14}CO_2$ output from 12-24 hours may be affected by microbial contamination since antiseptic conditions were not used on any except two control runs, which were carried out after steam sterilizing the media, flasks, and syringes. The media were clouded by this treatment, indicating some occurrence of precipitation. $^{14}CO_2$ electrometer graphs, using steam sterilized materials, were the same as with the non-sterilized materials for the first twelve hours, but

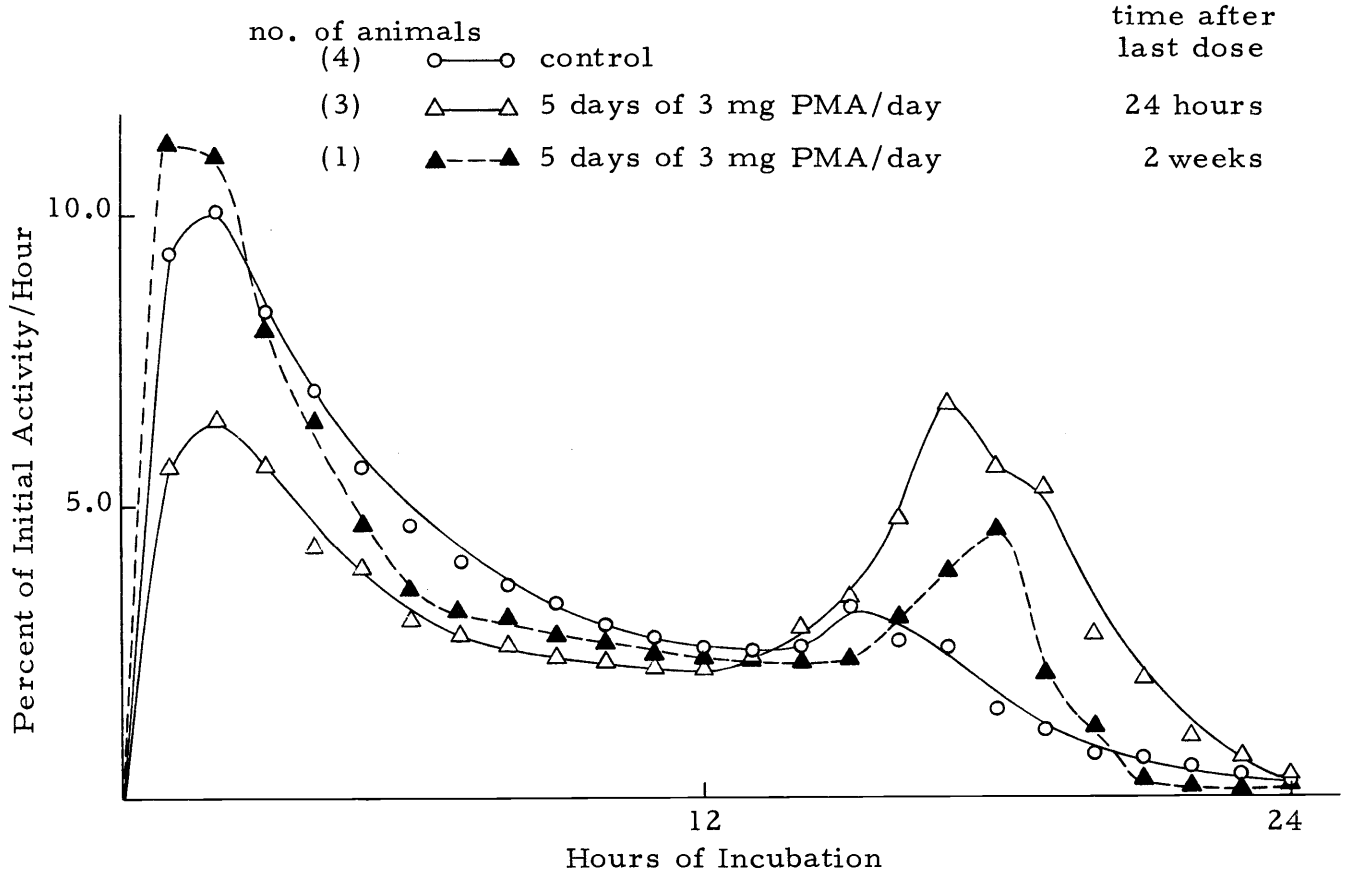


Figure 21 a). Electrometer recordings of $^{14}CO_2$ respired by kidney slices incubated with lactate-1-^{14}C, Group I Series.

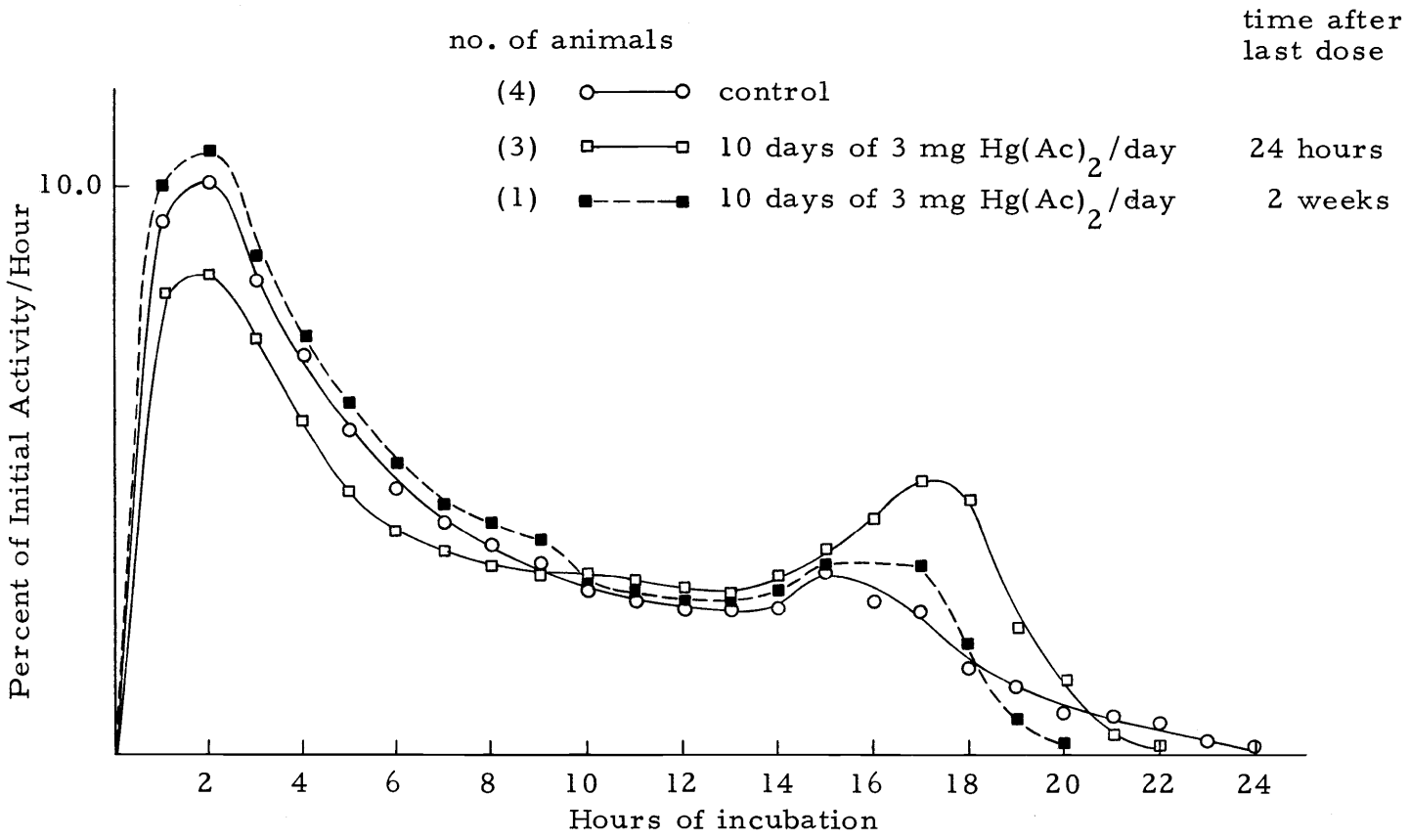
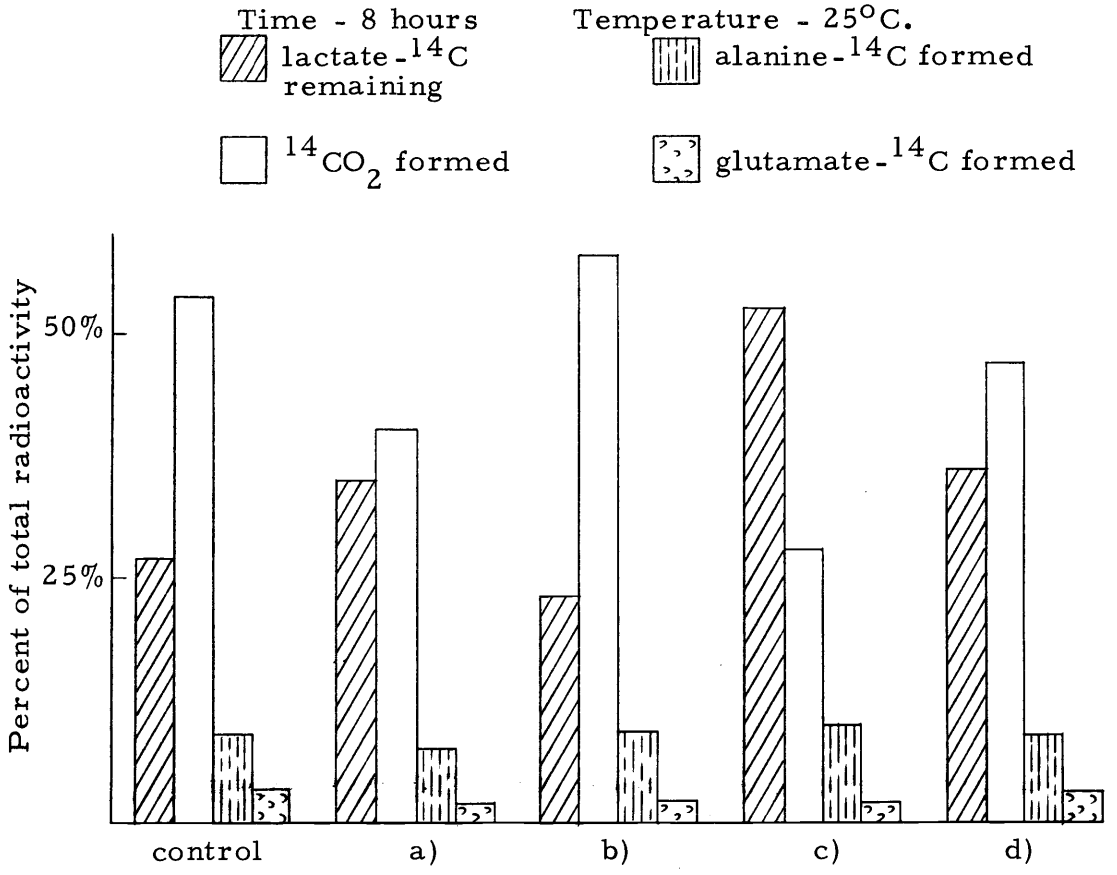


Figure 21 b). Electrometer recordings of $^{14}CO_2$ respired by kidney slices incubated with lactate-1-^{14}C, Group I Series.

showed some differences from twelve to twenty-four hours. The principle difference was a 2 or 3 hour delay in the appearance of the second peak.

The time course conversions of lactate-1-^{14}C by kidney slices from control and treated animals are shown in Figure 20. Significant reduction was observed with treated slices, particularly during the first 12-hour period. PMA induced a greater effect than a higher dosage of Hg^{2+}. Also, a quicker recovery from Hg^{2+} treatment was evident. This was probably not due to an inhibition of lactate uptake by the slices, since slices of control and treated animals accumulated equal amounts of lactate-1-^{14}C within the time period designated in the graph. Metabolite formation from lactate-1-^{14}C other than CO_2 was determined by paper chromatography of the media and tissue slice extracts. Three labeled metabolites, in significant quantities, were found after development of the strips in a BAW solvent system. Two major peaks were lactate and alanine, which have R_F's of about 0.70 and 0.30 respectively. Another smaller peak, or R_F between 0.20 and 0.23, was identified as glutamate. Several small peaks of $<$1 to 2% of total activity on the strip were detected, however it is difficult to precisely determine the area of such small peaks. For this reason, they are not presented in the isotopic balance sheet.

An isotopic balance sheet accounting for the distribution of radioactive products is presented in Figure 22. Slices from treated



	compound administered	dose/day	days given	time after last dose
a)	$Hg(Ac)_2$	3 mg	10	24 hours
b)	$Hg(Ac)_2$	3 mg	10	2 weeks
c)	PMA	3 mg	5	24 hours
d)	PMA	3 mg	5	2 weeks

Figure 22. Metabolites formed during 8 hours incubation of kidney slices with lactate-1-^{14}C, Group I Series.

animals formed about the same amount of ^{14}C-alanine within 8 hours as controls. It was difficult to precisely evaluate the effect of mercurial treatment on ^{14}C-glutamate formation since the amount formed by control slices was quite small. However, ^{14}C-glutamate formation seemed to be decreased to some degree by mercurial treatment. The principle effect reflected in the isotope distribution graph is a reduction in the rate of lactate-1-^{14}C to CO_2 by slices from the mercurial treated animals sacrificed after 24 hours.

Group II (using kidney slices from rats receiving single doses)

Kidney slices from rats given a single dose of mercurial showed less effect on lactate-1-^{14}C metabolism than the multiple doses described previously. Control slices converted 70.5% of initial lactate-1-^{14}C to $^{14}CO_2$ during the first 8 hours, and the slices from $Hg(Ac)_2$ and PMA-treated animals converted 64.0% and 56.5% respectively to $^{14}CO_2$ (Figure 23). Paper chromatographic results of tissue slice extracts and external media indicated the presence of 3 major metabolites with the R_F values as in the previous lactate-1-^{14}C experiments. The percentages of lactate-1-^{14}C remaining and metabolite formation (based on initial activity) after 8 hours are presented in Figure 24. The reduction of the conversion of lactate-1-^{14}C to $^{14}CO_2$ was about twice that with PMA than that with $Hg(Ac)_2$ treatment. Conversion of lactate to alanine and glutamate did not seem to be affected by either mercurial at this dosage level.

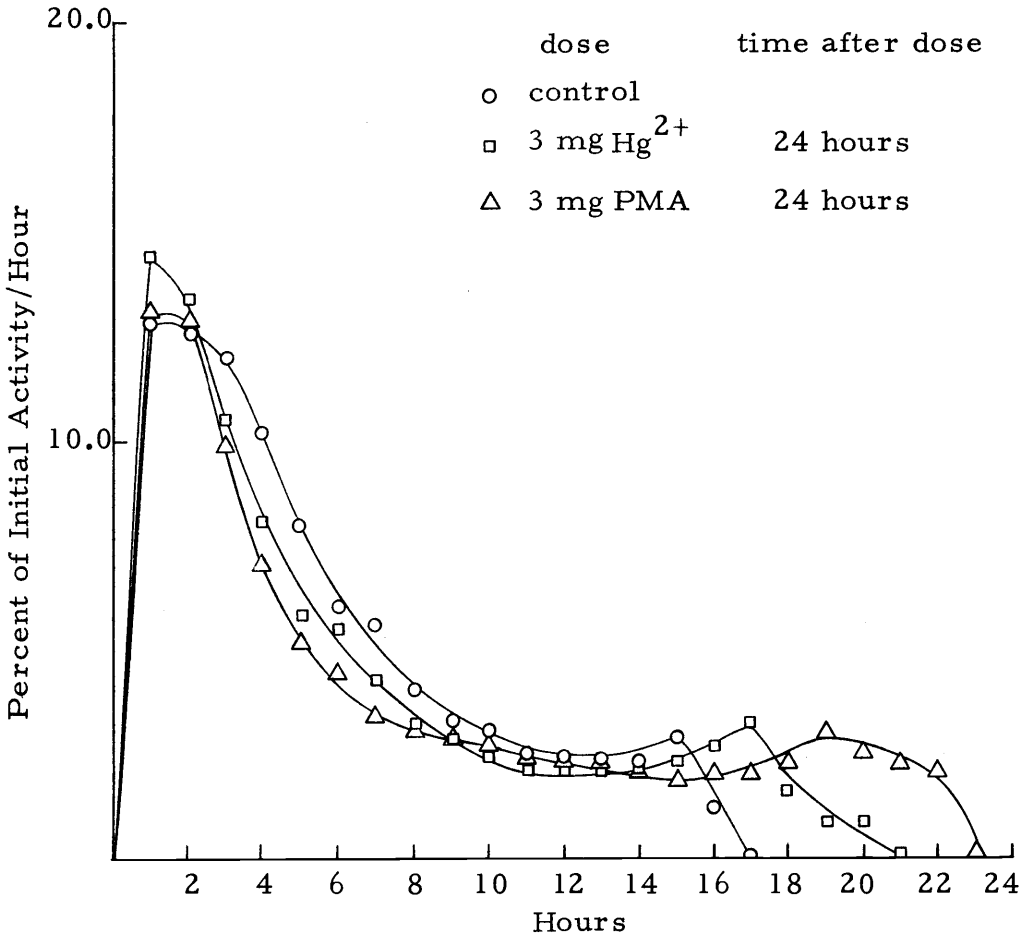
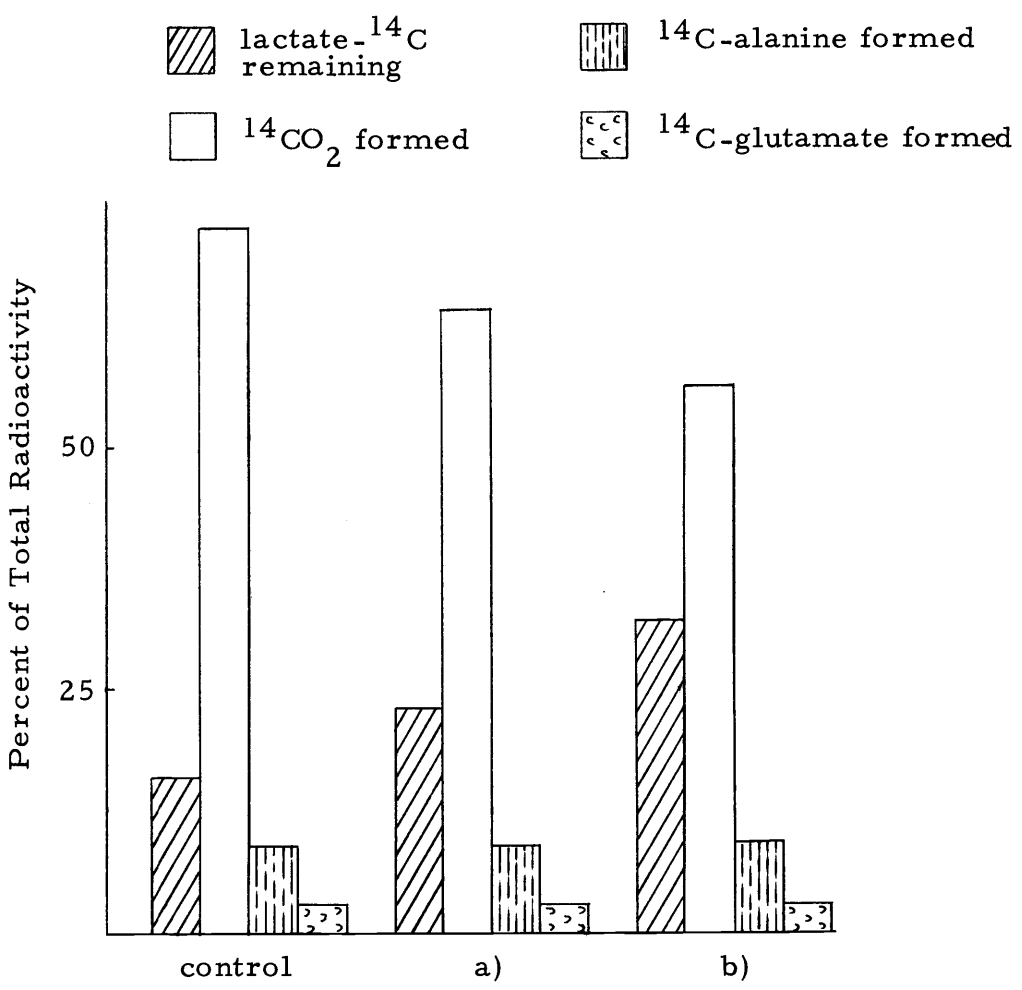


Figure 23. Electrometer recordings of $^{14}CO_2$ respired by kidney slices incubated with lactate-1-^{14}C, Group II Series.



	dose	time after dose
a)	3 mg $Hg(Ac)_2$	24 hours
b)	3 mg PMA	24 hours

Figure 24. Metabolites formed during 8 hours incubation of kidney slices with lactate-1-^{14}C, Group II Series.

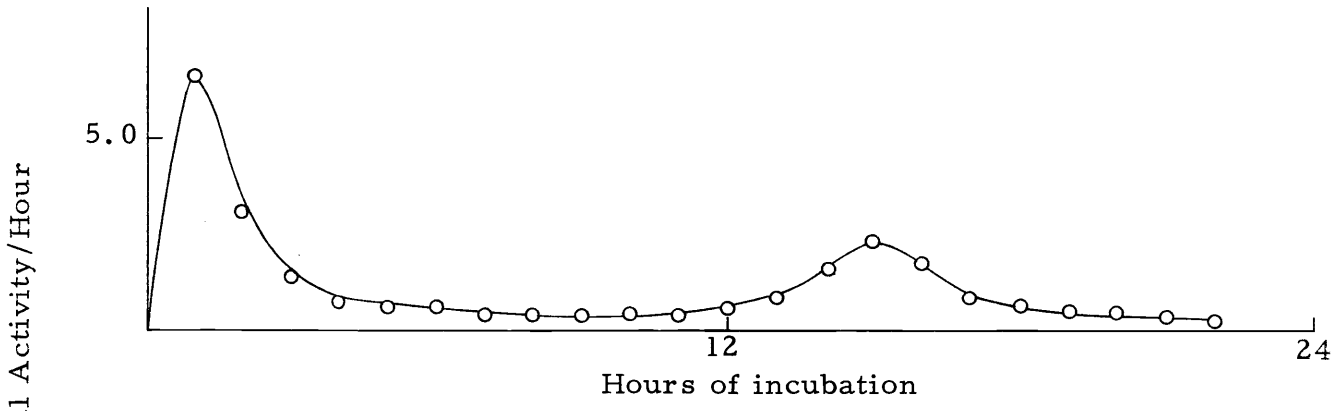
Lactate-1-^{14}C Metabolism by Kidney Homogenates and Soluble Fraction

Since it has been shown in previous sections that soluble LDH activity in the kidney was reduced by in vivo mercurial treatment, it would be interesting to check whether or not this cell-free preparation and the homogenate will convert labeled lactate to CO_2 and other metabolites as we observed with kidney slices.

Equivalent amounts of kidney soluble fractions or homogenates were incubated with lactate-1-^{14}C. $^{14}CO_2$ production and the formation of labeled metabolites were determined. $^{14}CO_2$ electrometer recordings from duplicate experiments using kidney homogenates from (a) controls, (b) 10 x 3 mg $Hg(Ac)_2$, and (c) 5 x 3 mg PMA, each sacrificed 24 hours later, showed very little difference (Figure 25).

Metabolite formation by homogenates after 8 hours is shown in Figure 26. The values from PMA-treated animals indicated a decrease in $^{14}CO_2$ and ^{14}C-alanine, while those from $Hg(Ac)_2$-treated animals indicated a slight increase in $^{14}CO_2$ production and no effect on labeled alanine formation. Labeled glutamate formation by homogenates from control and treated animals was extremely small.

An isotopic distribution of metabolite formation following 8 hours of incubation with kidney soluble fractions is shown in Figure 26. The results from the $Hg(Ac)_2$-treated animal showed similar



Electrometer recording of $^{14}CO_2$ respired by kidney homogenate incubated with lactate-1-^{14}C.

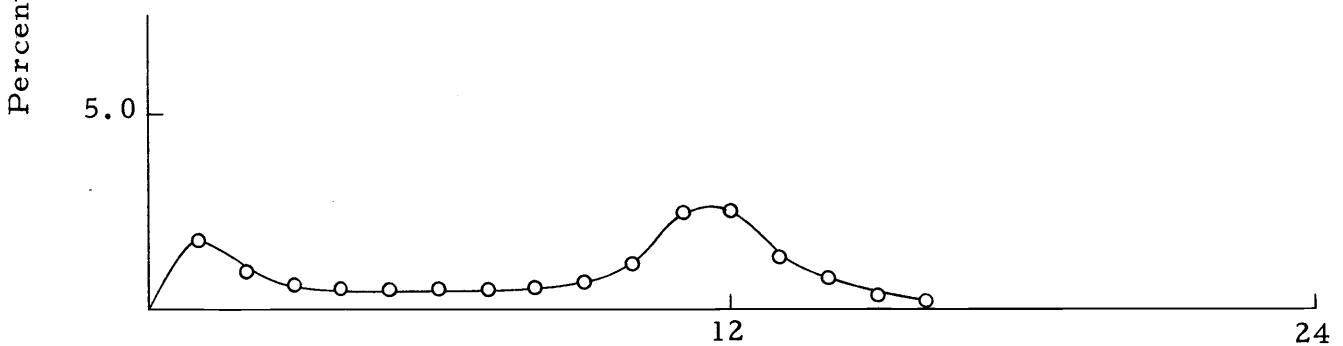


Figure 25. Electrometer recordings of $^{14}CO_2$ respired by kidney homogenates and soluble fractions incubated with lactate-1-^{14}C.

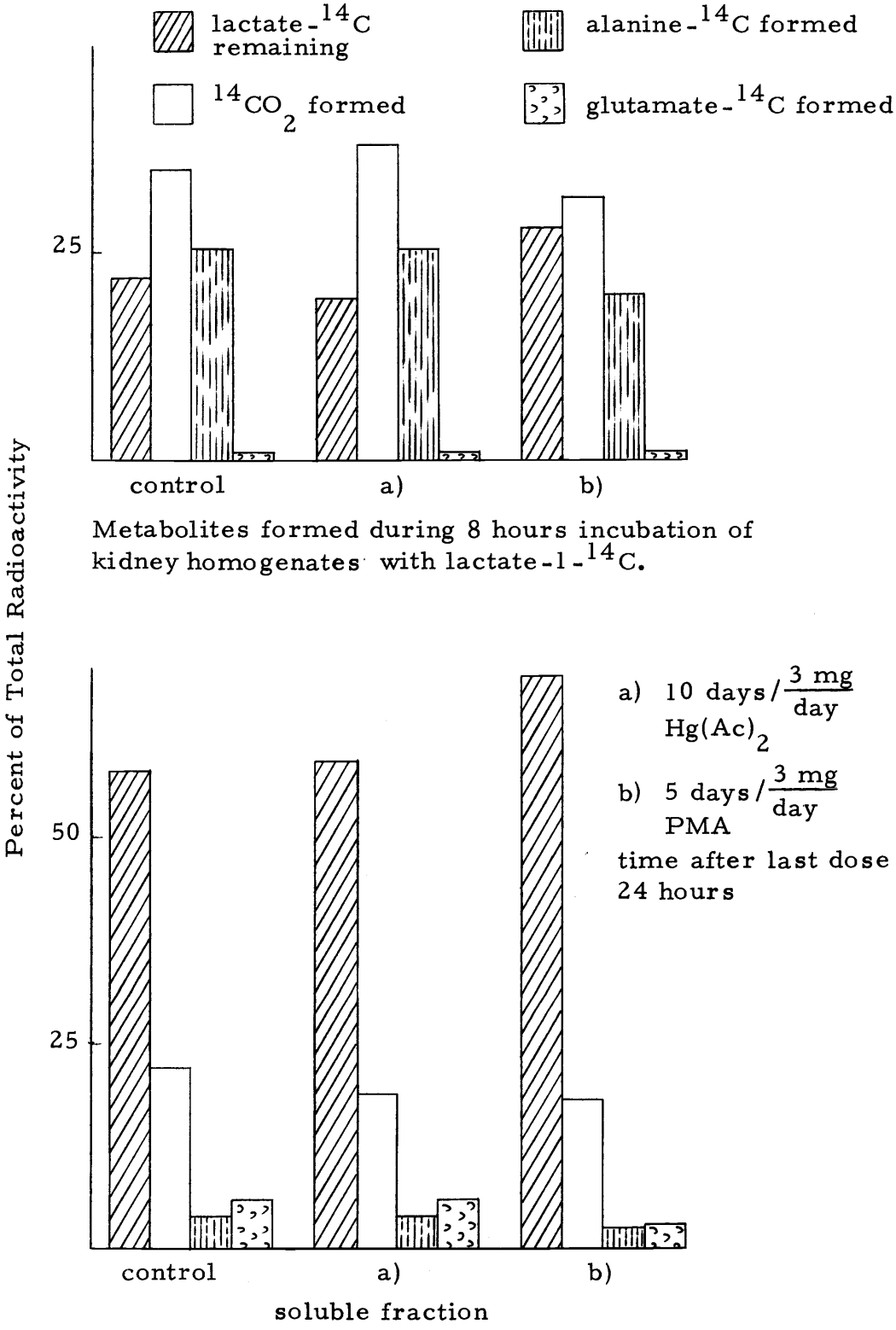


Figure 26. Metabolites formed during 8 hours incubation of kidney homogenates and soluble fractions with lactate-1-^{14}C.

amounts of labeled glutamate and alanine formed and slightly less $^{14}CO_2$ given off. Less labeled glutamate, alanine, and CO_2 were formed by kidney soluble fractions from PMA-treated animals. The extent of the decrease of metabolite formation with kidney soluble fractions from mercurial-treated animals was not dramatic, however. The alteration of lactate-1-^{14}C metabolism following mercurial treatment was greater with slices than with homogenates or soluble fractions.

Lactate-2-^{14}C Metabolism by Kidney Slices

Kidney slice incubations with lactate-2-^{14}C produce $^{14}CO_2$ evolution patterns and metabolite distributions varied from those observed using lactate-1-^{14}C. $^{14}CO_2$ from lactate-2-^{14}C appeared less rapidly than from lactate-1-^{14}C, as would be expected. The C-2 of lactate has to traverse through the TCA cycle before being given off as CO_2, while the bulk of C-1 may be decarboxylated upon the entrance of pyruvate into the TCA cycle. An increase of ^{14}C incorporation into glutamate from lactate-2-^{14}C reflected the more extensive distribution of ^{14}C within TCA cycle intermediates, while the formation of equal amounts of labeled alanine indicated its pathway was independent of the TCA cycle.

Multiple doses of PMA seem to affect the metabolism of lactate-2-^{14}C by kidney slices more than $Hg(Ac)_2$. A two week period

following the last dosage of PMA or Hg^{2+} appears to be adequate for the rats to recover significantly.

$^{14}CO_2$ electrometer recordings indicated slices from all the above animals respired about the same amount of $^{14}CO_2$ within the first 12 hours, with the exception of rats dosed with 5 x 3 mg PMA and sacrificed 24 hours later, in which case less $^{14}CO_2$ was given off within this time period (Figure 28). Time course conversion of lactate-2-^{14}C as shown in Figure 27 revealed the greatest effect during the early experimental period. The relative distribution of metabolites is shown in Figure 29. Greater differences were found with kidney slices from rats receiving 5 x 3 mg PMA than from those receiving 10 x 3 mg $Hg(Ac)_2$. Also, a quicker recovery was evident with animals treated with $Hg(Ac)_2$.

In Vitro Metabolism of Glutamate-3,4-^{14}C by Kidney Slices

Conversion of C-3 and C-4 of glutamate to CO_2 required its entrance into the TCA cycle after conversion to α-ketoglutarate. The CO_2 production pattern by kidney slices from control rats showed low levels of $^{14}CO_2$ until about 14 hours, as seen in Figure 31a, and a large peak from 14 to 18 hours. In the experiment using slices from mercurial treated animals sacrificed after 24 hours, $^{14}CO_2$ production was slightly less during the low level output period, which was extended to 16 hours, and then followed with a major $^{14}CO_2$ peak.

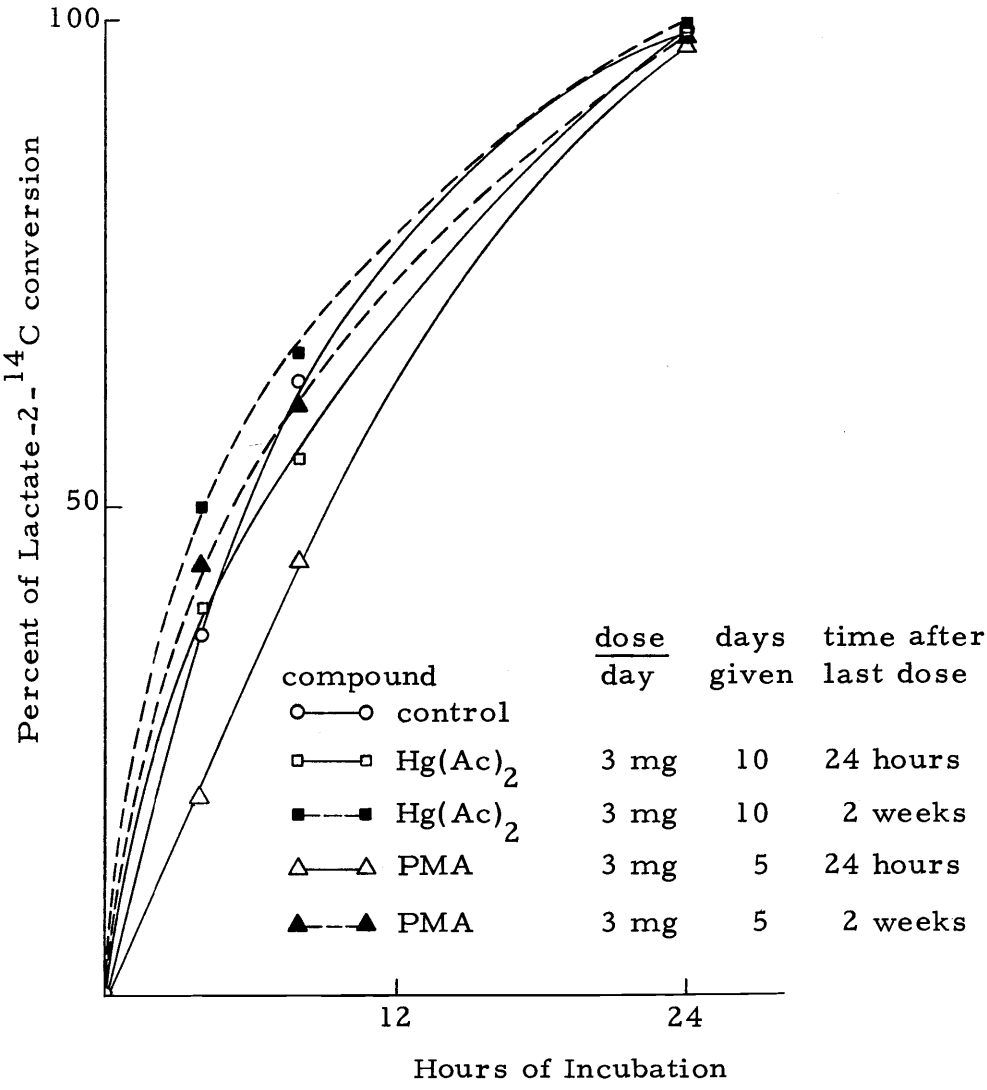


Figure 27. Rate of lactate-2-^{14}C conversion by kidney slices.

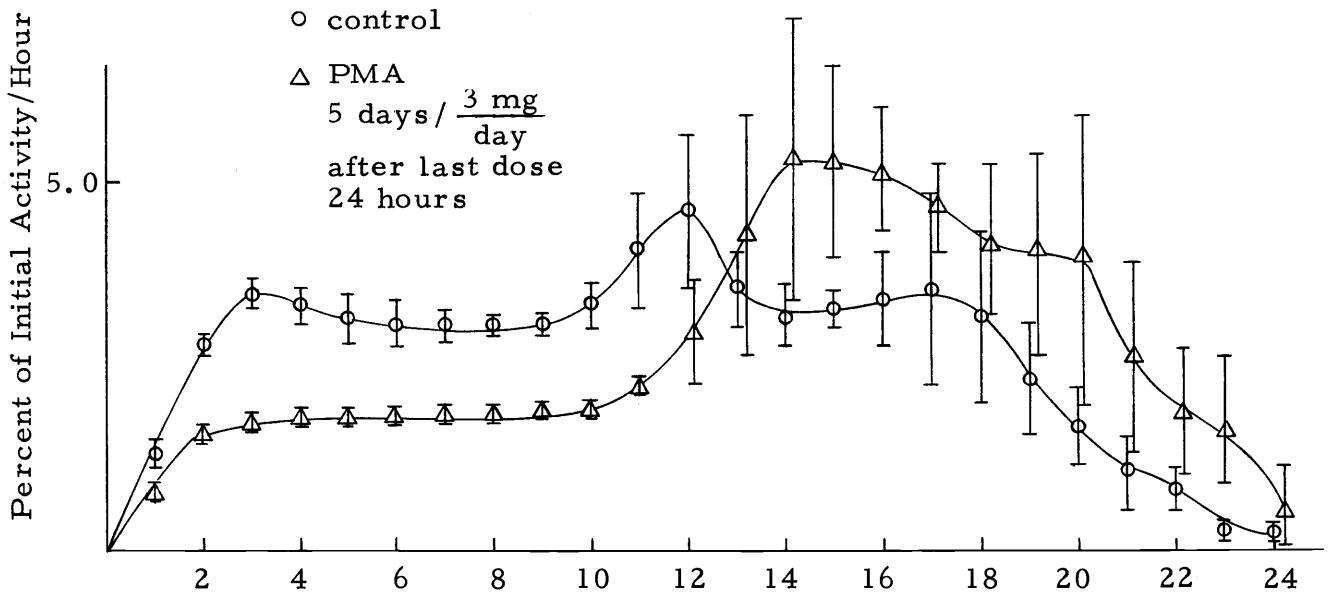


Figure 28 a). Electrometer recordings of $^{14}CO_2$ respired by kidney slices incubated with lactate-2-^{14}C.

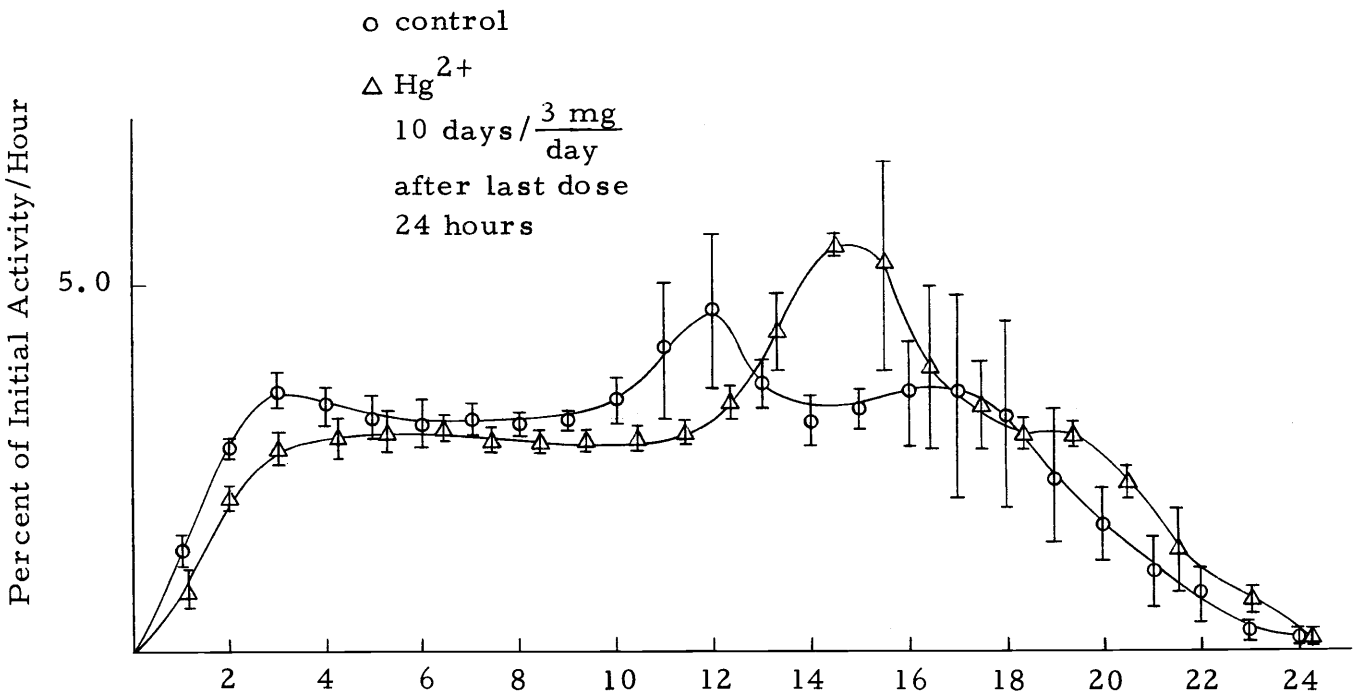
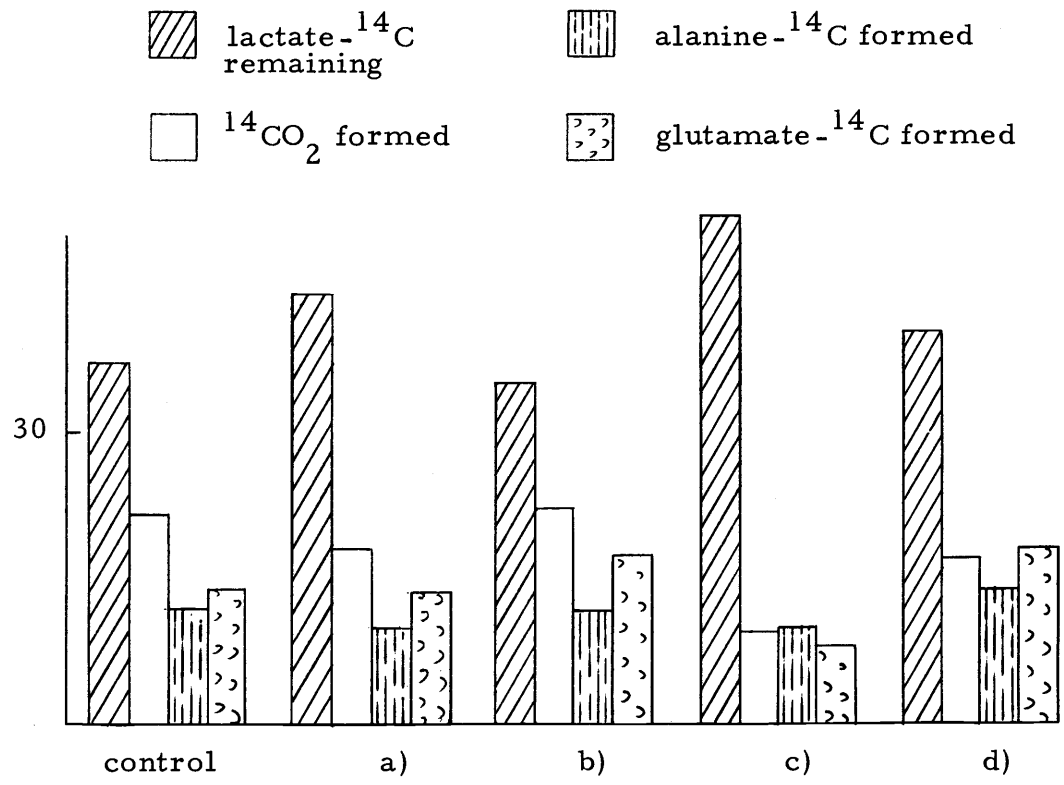


Figure 28 b). Electrometer recordings of $^{14}CO_2$ respired by kidney slices incubated with lactate-2-^{14}C.



	compound administered	dose/day	days given	time after last dose
a)	$Hg(Ac)_2$	3 mg	10	24 hours
b)	$Hg(Ac)_2$	3 mg	10	2 weeks
c)	PMA	3 mg	5	24 hours
d)	PMA	3 mg	5	2 weeks

Figure 29. Metabolites formed during 8 hours incubation of kidney slices with lactate-2-^{14}C.

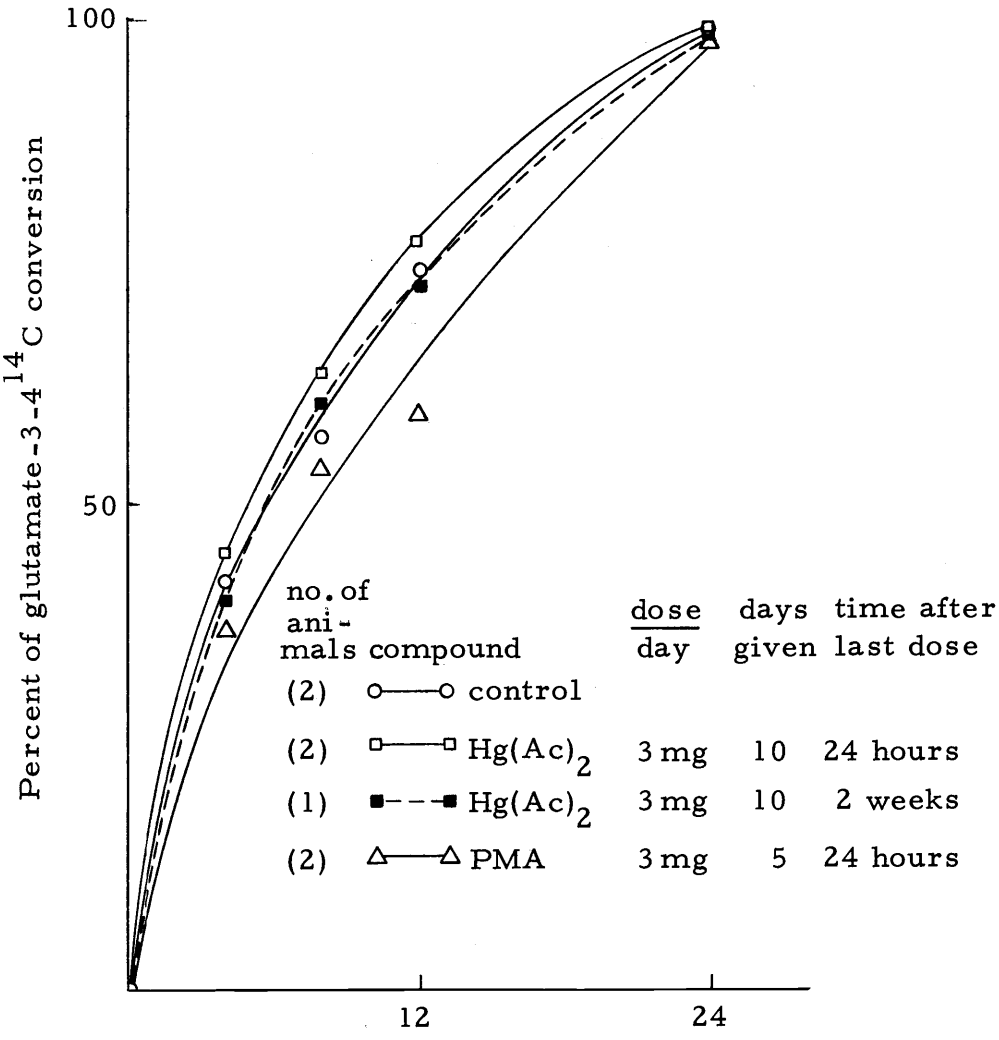


Figure 30. Rate of glutamate-3-4 ^{14}C conversion by kidney slices.

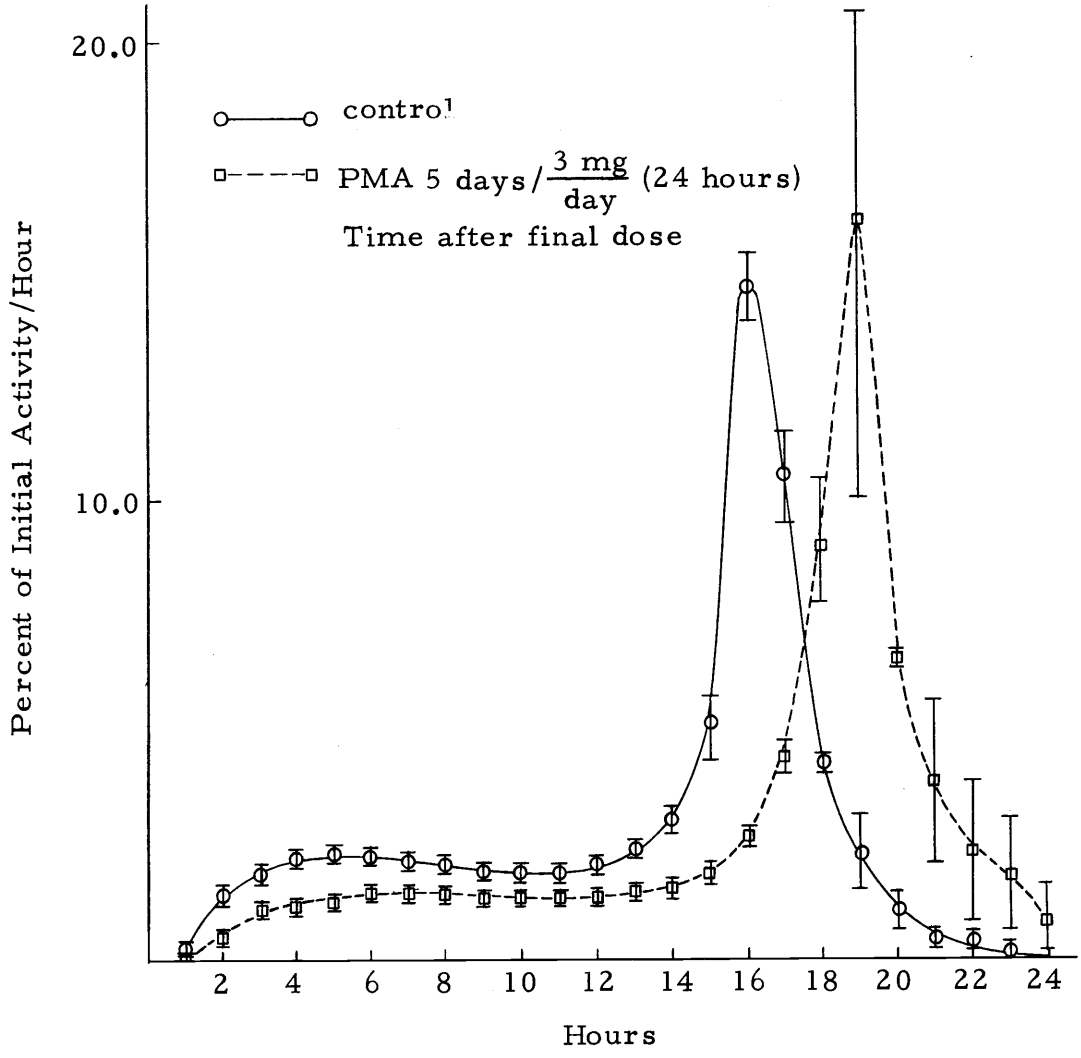


Figure 31 a). Electrometer recordings of $^{14}CO_2$ respired by kidney slices incubated with glutamate-3-4^{14}C.

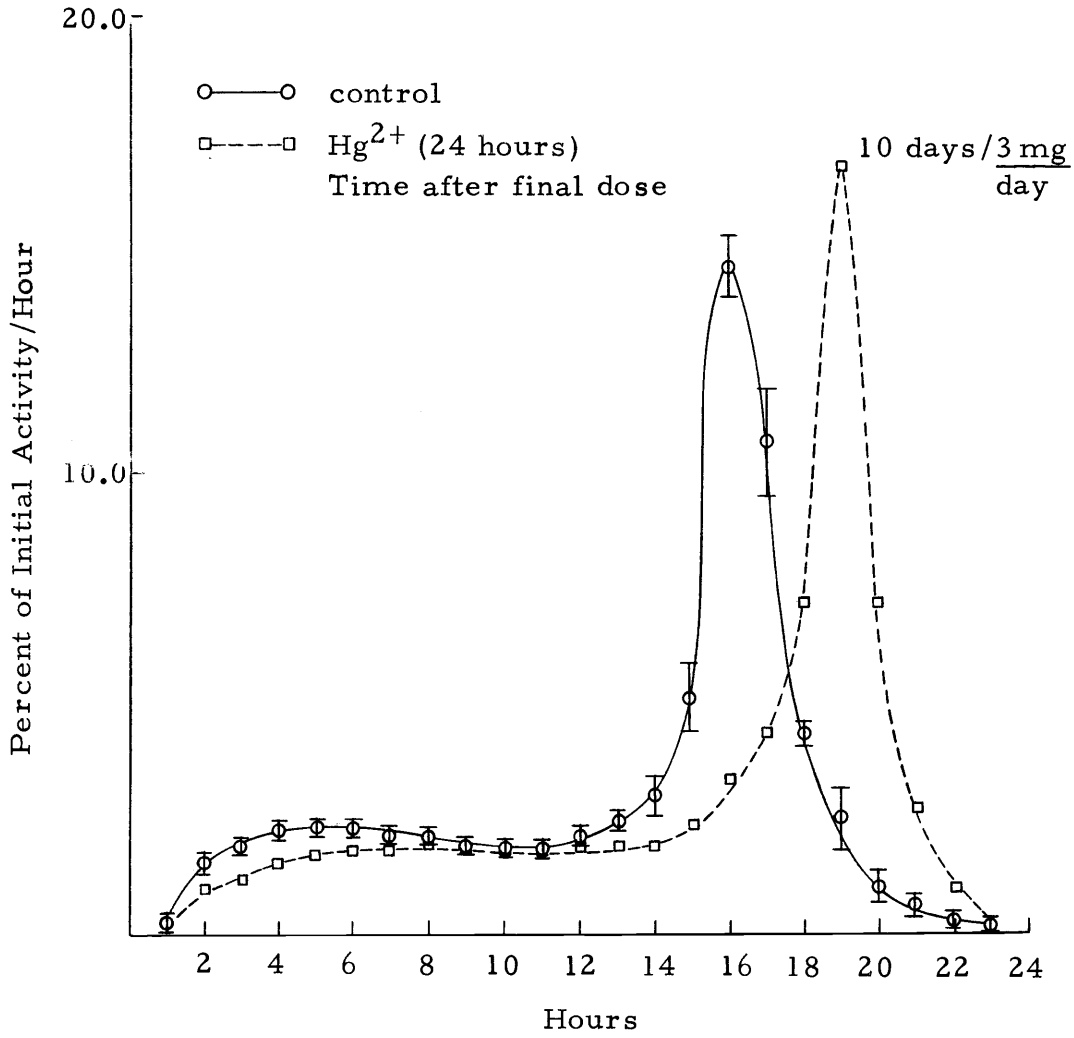
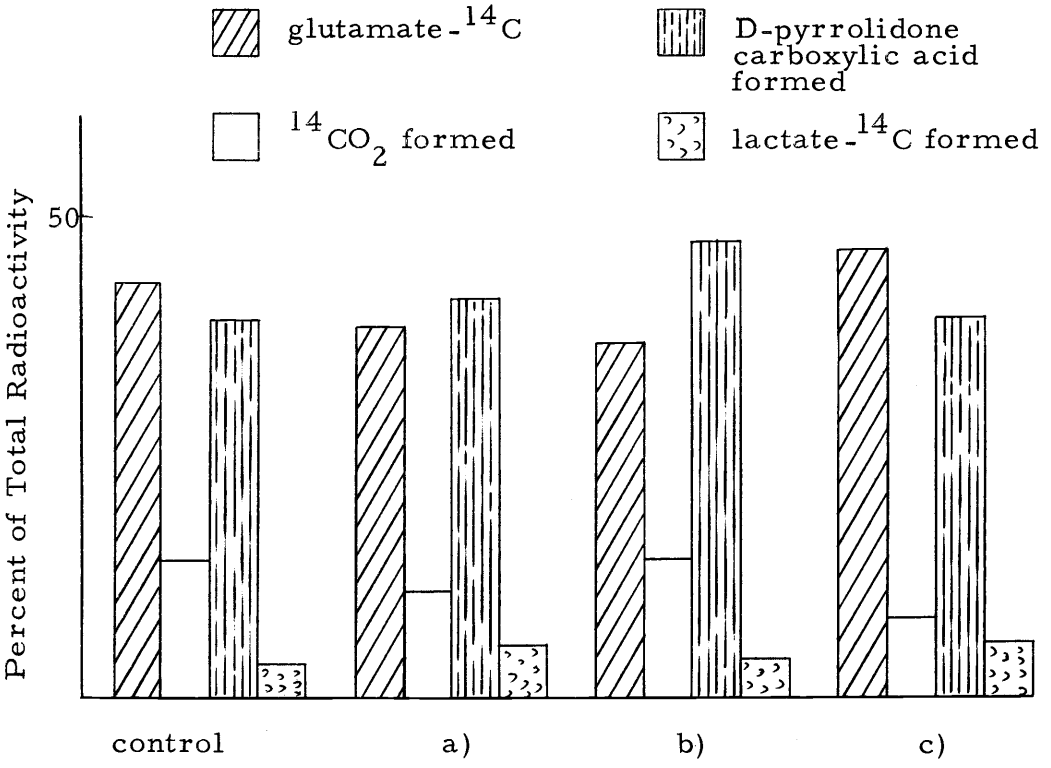


Figure 31 b). Electrometer recordings of $^{14}CO_2$ respired by kidney slices incubated with glutamate-3, $4\,^{14}C$.

This peak, which appeared two hours later, was of slightly larger magnitude than the control. The $^{14}CO_2$ production pattern with slices from the animal receiving 10 x 3 mg $Hg(Ac)_2$ and sacrificed after 2 weeks was the same as control patterns.

Time course comparison on the rate of glutamate conversion between control and mercurial-treated kidney slices showed only a slight difference during the first 8 hours (Figure 30). At twelve hours, slices from PMA-treated animals showed about 13% less glutamate conversion than with controls and this difference disappeared at 24 hours.

Paper chromatographic results of the media and tissue extracts of the 4, 8, and 12 hour samples indicated 3 major radioactive peaks of R_F values 0.24, 0.55, and 0.72. They were identified as glutamate, D-pyrrolidone-carboxylic acid, and lactate, respectively. Overall glutamate-3,4-^{14}C conversion by slices from controls and treated animals did not differ dramatically within the first 9 hours (Figure 32). $^{14}CO_2$ patterns and glutamate-^{14}C conversion rate curves indicated the greatest effects were manifest 24 hours after 5 x 3 mg PMA treatment, in which a reduction of $^{14}CO_2$ and an increase of lactate formation were observed.



	compound administered	dose/day	days given	time after last dose
a)	$Hg(Ac)_2$	3 mg	10	24 hours
b)	$Hg(Ac)_2$	3 mg	10	2 weeks
c)	PMA	3 mg	5	24 hours

Figure 32. Metabolites formed during 8 hours incubation of kidney slices with glutamate-3, 4^{14}C.

DISCUSSION

The incubation of kidney soluble fraction with either PMA or $Hg(Ac)_2$ resulted in mercury binding of proteins in all molecular weight ranges. The concentration of ^{203}Hg present in each fraction was dependent on the type of mercurial, its concentration, and the nature of protein in each fraction. Mercury did not seem to combine selectively with any particular group of proteins under these conditions.

Incubation of PMA with kidney slices resulted in mercury binding to groups of proteins of the soluble fraction in the 100,000, the 40,000 to 60,000 and the 8,000 to 13,000 molecular weight ranges, and incubation of $Hg(Ac)_2$ showed mercury binding to proteins in the 100,000 and the 8,000 to 13,000 molecular weight range. These binding patterns were different from those obtained with in vitro incubation of the soluble fraction and were undoubtedly influenced by mercurials under intracellular conditions.

In vivo metabolism of ^{203}Hg from PMA or $Hg(Ac)_2$ resulted in a very large portion of ^{203}Hg being bound to the peak III proteins, especially after 48 hours. PMA and $Hg(Ac)_2$ treatments resulted in different ^{203}Hg binding patterns during the first 48 hours and gradually became similar after that. In the early period after PMA administration, mercury may be in the organic form. However, by

48 hours after PMA treatment it is probable that the ^{203}Hg is in the inorganic form (39). Takeda (56) stated that in the early period after administration of mercurials, the distribution patterns of phenylmercury compound in the several organs and in the blood were rather more similar to those of alkylmercury compounds than to those of mercuric chloride, but in the later period they became quite similar to that of mercuric chloride.

It might be necessary for the animal to convert PMA in order to excrete the mercury from it. Miller (39) stated that PMA cannot be excreted rapidly, unchanged. He observed that less than 10% of PMA is excreted unchanged in the urine of rats in 48 hours. Substantial amounts of ^{203}Hg from PMA were eliminated in the urine within 48 hours, while very small amounts were excreted following $Hg(Ac)_2$ dosage (14). In our lab, chromatographic analysis of the urine from PMA -treated animals collected for 24 hours showed that less than 5% of the ^{203}Hg in the urine was present as free PMA. It would be interesting to determine if the appearance of ^{203}Hg in the urine from PMA was the result of metabolism of PMA associated with its binding to proteins of larger molecular weight in peaks I and II, for the following reasons: (a) peaks I and II bind mercury from PMA extensively during the initial 48 hour period which corresponds to the time during which the greatest amount of ^{203}Hg is excreted via the urine, (b) peaks I and II bind much less $^{203}Hg(Ac)_2$ at any

time, and (c) it has been determined that PMA is metabolized in the kidney (39).

The Hg-binding of peaks I and II had an increasing trend during the first 24 hours, followed immediately by a decreasing trend. The mercury binding of peak III showed a longer increasing trend and remained at a high level for a longer time. This observation would suggest that the peak III proteins may have a longer biological half-life than the peak I or II proteins. Yoshino et al. (69) explained the long retention period of alkylmercury in the brain as being due to a greater affinity of alkylmercury for those proteins in the CNS which have a rather longer biological half-life.

Following multiple doses of the mercurials the O. D. profile of kidney soluble fraction changed, with a definite increase at the peak III region. The kidney may increase the synthesis of peak III proteins which have a high mercury binding capacity. This may be the consequence of a toxic effect. It has been observed that an increased binding of Hg in the soluble fraction, following $HgCl_2$ injection in rats on a low protein, high sucrose diet, is associated with the ability to tolerate greater doses of $HgCl_2$ (53). Clarkson and Magos (7) found the same classes of binding sites in both liver and kidney homogenates. Two classes of mercury binding sites were observed, one class having a chemical affinity for mercury 30 fold greater than the other class. They speculated that the metabolism

of mercury might be responsible for the preferential uptake by the kidney. Our experiment revealed that the Hg-binding patterns of the kidney and liver soluble proteins were somewhat proportionately similar 18 hours after PMA dosage. However, the patterns obtained at 48 hours or later were quite different. Most of the Hg at this time had been eliminated from the liver while the kidney had accumulated substantial amounts in the peak III fraction. The metabolism of mercury seems to vary between species and between strains within a species. Miller et al. (38) reported a difference in the retention of mercury following injection of several mercurials into two strains of mice. This seems to strengthen the supposition that the preferential uptake of mercury by the kidney is coupled to metabolism.

Mercury may affect acid and alkaline phosphatase activity levels by forming a Hg-enzyme complex with decreased activity. From our studies, the acid and alkaline phosphatase levels in the kidney soluble fraction were not decreased by a single dose of PMA or $Hg(Ac)_2$ and less than one mole of ^{203}Hg was bound to each mole of these enzymes. From in vitro study with commercial alkaline phosphatase of calf mucosa, 20% inhibition was observed when almost 2.5 moles of ^{203}Hg of $Hg(Ac)_2$ were bound to one mole of enzyme. In order to attain in vivo inhibition of these enzyme activities via a Hg-enzyme complex, oral doses of either mercurial much greater than were given would be required.

$Hg(Ac)_2$ was a much more potent in vitro inhibitor of both alkaline and acid phosphatase than was PMA. Webb (63, p. 861) found in a survey of 160 reports on enzyme inhibition using Hg^{2+} and PMB (a compound differing from PMA in that there is a negatively charged group present) that 25% of them did not allow comparison, but of the remaining reports, Hg^{2+} was more potent in 65% and PMB in 29%, and in 6% they were of equal potency. On account of the similarity of PMA and PMB, the following ideas may be applicable for PMA to some extent. Hg^{2+} might be expected to be more potent than PMB because 1) it is smaller and might be able to penetrate and react with sulfhydryl groups inaccessible to the larger molecule, and 2) since it is bifunctional, it could, in some instances, induce dimerization of the enzyme (or even polymerization).

Single doses of PMA and $Hg(Ac)_2$ seemed to slightly increase the level of kidney soluble LDH. The increased level of activity could be due to either an increased number of LDH molecules or a modification of the enzymes. Soluble LDH prepared from the kidney of a rat which had been administered an oral dose of $Hg(Ac)_2$ was more sensitive to in vitro treatment by $Hg(Ac)_2$ or PMA. This could involve a modification of LDH due to the binding of the sulfhydryl groups or other reactive sites, and be due to a decrease in sulfhydryl concentration of other proteins in the LDH preparation. Shore and Shore (50) found that the sulfhydryl concentration in the kidney soluble

fraction decreased while the disulfide concentration increased after I. V. $HgCl_2$ treatment.

Following multiple doses of PMA or $Hg(Ac)_2$, the LDH activity levels in the kidney soluble fraction decreased. The decrease in LDH activity correlated with increased ^{203}Hg binding to proteins in the LDH fraction (eluate from Sephadex column). The maximum bindings were around 0.32 and 0.50 mole Hg/mole protein in the LDH fraction for $Hg(Ac)_2$ and PMA respectively. From the observations of Di Sabato (11), one expected at least one mole Hg/mole enzyme to be required to decrease the activity 25%. When the kidney soluble fraction was incubated in 1×10^{-4} M $Hg(Ac)_2$, about 2-4 moles of Hg were bound per mole of protein, and the LDH activity was not affected. Thus, it is highly possible that other reasons besides direct Hg-LDH active site binding exist for the effect of orally administered mercurials on LDH. It is possible that LDH may have a greater affinity for Hg than other proteins in the fraction. Therefore the mercury binding value would be less than the observation made by Di Sabato (11) using pure LDH. However, the activity of soluble LDH in rat kidney did not return to normal value with a significant decrease of mercury binding when a 2 week period of time was allowed to lapse following $Hg(Ac)_2$ dosage. This result seems to suggest that mercury binding at the active site of LDH enzyme is not the prime factor involved in the reduction of activity. It is possible

that the synthesis of enzyme is affected.

Single or multiple PMA dosage did not affect malic acid dehydrogenase activity. A single dose of $Hg(Ac)_2$ showed no effect on MDH activities, but multiple doses showed a decrease. In vitro studies indicated that $Hg(Ac)_2$ was a much more potent inhibitor than PMA. The Hg-binding, after in vivo treatments, was less than one mole of Hg/mole of protein in the MDH fraction. Siegel and Englard (51) indicated that around 6 moles of sulfhydryl must be bound from one mole of enzyme to suppress activity. It seems doubtful that Hg binding at the active site of the MDH molecule is the main reason for the decrease in MDH activity following in vivo treatment. Quicker recovery of kidney soluble MDH than LDH suggests a differential effect of mercurials on the biosynthesis of different types of proteins. More experiments would be needed to verify this speculation.

Radiorespirometry and Metabolite Detection

When kidney slices were incubated with lactate-1-^{14}C or -2-^{14}C, a marked difference between those taken from control rats and those from rats receiving oral doses of PMA or $Hg(Ac)_2$ was observed. The effects were not due to an inhibition of lactate uptake by the slices, since equal amounts of labeled lactate were accumulated by the slices from both control and treated animals. Complete metabolism of labeled lactate to $^{14}CO_2$ and its incorporation into

glutamate were definitely affected more by PMA, and to a lesser degree by $Hg(Ac)_2$. As the pattern of $^{14}CO_2$ production from lactate-1-^{14}C indicated, the catabolic oxidation of lactate-1-^{14}C by kidney slices may traverse through two distinctive pathways. The appearance of $^{14}CO_2$ at the first peak could be derived from a decarboxylation reaction of lactate or its metabolites (pyruvate, alanine) while the second peak might result from a combination of assimilation, followed by a degradation. A depression or a lack of $^{14}CO_2$ production from a given substrate in radiorespirometric experiments has been interpreted to be due to a lower or lack of enzyme system for such conversion (62). The inhibitory effects of mercurial on pyruvate decarboxylase in Acetobacter suboxydans (26), lysine decarboxylase in E. coli (57), and α-ketoisocaproate decarboxylase in Proteus valgaris (47) have been reported. It is reasonable to conclude that in vivo mercurial treatment also induced an inhibition of decarboxylase activity in the kidney.

Due to a reduction of lactate-1-^{14}C conversion through the decarboxylating pathway, a greater amount of lactate would become available for its metabolism through the other path. A larger second peak which was found in all runs with kidney slices from mercurial-treated rats illustrates this alteration. The delay of 2 to 3 hours for the appearance of the second peak may be explained by the overall depression of metabolic reactions. A decrease in respiration of

isolated kidney tissues following toxic doses of both inorganic and organic mercurials has been reported (12; 49; 63, p. 927). The dosage required for the depression seems to depend on the mercurial. Oxidation of α-ketoglutarate was inhibited 90% in kidney tissues from rats 24 hours after I. V. injection of 3 mg/kg $HgCl_2$ (49). The maximum mercury accumulation in the kidney was 180 μg Hg/gr. fresh tissue. In vitro experiments with chlormedrin carried out by Grief and Jacobs (19) showed 20% inhibition of mitochondrial respiration using a mercury level quite similar to that found in our experiment, 24 hours after 5 x 3 mg doses of PMA.

Inhibition of metabolic activity as revealed by radiorespirometric experiments could be due to a number of reasons, such as inhibition of enzyme systems, ion transport, energy transfer, and others. Isolated cortical tubules from rat kidneys showed tubular structural and mitochondrial alterations when exposed to minimal doses of mercuric chloride (31). A direct cytopathic mechanism for mercury toxicity was implicated. The electron transport system may not be involved as the site of toxic action (35). Study of the uptake of Mg^{2+} by heart mitochondria in the presence of mercurials led Brierly et al. (4) to conclude that hydroxymercuric benzoate and p-chlorophenylmercuric sulfonate should be classified as inhibitors of mitochondrial energy transfer. They speculated that a thiol group, sensitive to these mercurials, was involved between the dinitrophenol

and oligomycin sensitive points in the energy transfer system associated with oxidative phosphorylation. In vivo mercurial treatment also depressed Na^+ activated ATPase (3, 25) and Pi uptake by kidney slices (8) and by mitochondria (16). ATP turnover rate and ADP and Pi concentrations all have a great influence on respiration rate (23). It is difficult at this stage to speculate which factor(s) may contribute to the alteration of $^{14}CO_2$ output.

The rate of DL-glutamate-3,4-^{14}C metabolized to $^{14}CO_2$ by slices from PMA and $Hg(Ac)_2$-treated animals was depressed, while the uptake rate into the slices and conversion to D-pyrrolidone-carboxylic acid was not affected. Labeled L-glutamate is converted to $^{14}CO_2$ by the kidney slices after being transformed to α-ketoglutarate and subsequently metabolized in the TCA cycle (66). A large peak of $^{14}CO_2$ output was seen in the electrometer recordings from glutamate-1-^{14}C and -3,4-^{14}C incubations after 12 and 16 hours respectively. $^{14}CO_2$ from glutamate-1-^{14}C appeared in the first cycle of the TCA reaction sequence while -3,4-^{14}C had to cycle several times before $^{14}CO_2$ appeared. Lesser amounts of $^{14}CO_2$ were given off continuously before the $^{14}CO_2$ concentration peak appeared. The rate of $^{14}CO_2$ evolution from glutamate-3,4-^{14}C prior to the peak at 16 hours was depressed more with slices from PMA-treated animals than from $Hg(Ac)_2$-treated ones. The $^{14}CO_2$ peak was delayed about 2 hours by either mercurial. Glutamate-1-

^{14}C metabolism to $^{14}CO_2$ was affected in a similar manner by $Hg(Ac)_2$ treatment. As with labeled lactate metabolism, it seems that the main effect of mercurial treatment on ^{14}C-glutamate metabolism by kidney slices is the rate depression of one or more reactions associated with respiration.

Future investigations with acetate and succinate, which are key metabolic intermediates, will contribute to elucidating more specific information as to the mechanism of toxicity of various mercurials. A study of the effects of mercurials on their metabolism and associated ability to facilitate work functions performed by the kidneys (e.g. PAH uptake into slices) would be interesting. More specific information as to the intracellular location and quantities of Hg accumulated must be reported in conjunction with studies of the effects of mercurials on cellular activity. The measurement of general parameters of biological activity such as protein synthesis, O_2 uptake, etc. would provide a more complete picture of the overall effect. Finally electron micrographs of membrane structure and cellular appearance following mercurial treatment would be helpful. The action of mercurials appears to be a composite interaction and many parameters of biological and cellular activity seem to be affected.

SUMMARY

Biochemical studies of phenylmercuric acetate (PMA) and mercuric acetate toxicity in rats carried out during this investigation were (1) the metabolism of $^{203}Hg(Ac)_2$ and ^{203}Hg-PMA within kidney and liver tissues following oral ingestion; (2) the effect on some soluble enzyme activities and determinations of ^{203}Hg binding in the enzyme fractions; and (3) the metabolism of ^{14}C-labeled substrates by kidney slices from control animals and animals receiving PMA and $Hg(Ac)_2$.

In vitro ^{203}Hg-binding of the kidney soluble proteins was determined after incubating the soluble fraction with ^{203}mercury labeled $Hg(Ac)_2$ and PMA. The proteins were separated by Sephadex G-100 gel filtration and the ^{203}Hg and O. D.$_{260\ m\mu}$ were determined in the eluate. Hg-binding to proteins of large molecular weight (collected around void volume) and small molecular weight (O. D. peak, Ve about 200 ml) was relatively greater than Hg-binding to proteins of intermediate molecular weight. Hg-binding depended on the type of mercurial and its concentration, and Hg did not seem to combine selectively with any particular group of proteins.

Incubation of PMA with kidney slices resulted in mercury binding to groups of proteins of the soluble fraction in the 100,000 (peak I), 40,000 to 60,000 (peak II) and the 8,000 to 13,000 (peak III)

molecular weight ranges, and incubation of $Hg(Ac)_2$ showed mercury binding to the peak I and III proteins. These binding patterns were different from those obtained with in vitro incubation of the soluble fraction.

In vivo metabolism of ^{203}Hg from a single dose of PMA or $Hg(Ac)_2$ resulted in a very large portion of ^{203}Hg being bound to the peak III proteins, especially after 48 hours. PMA and $Hg(Ac)_2$ treatment resulted in different ^{203}Hg binding patterns the first 48 hours, especially in peaks I and II, and gradually became similar after that.

Rats receiving multiple doses of PMA or $Hg(Ac)_2$ also showed similar labeling patterns in the kidney soluble proteins. However, the O. D. profile was definitely altered, with an increase of O. D. in the area of peak III.

The $O.D._{260}$ and mercury binding profiles of the liver soluble proteins were quite similar to those of the kidney soluble proteins 18 hours after a single dose of PMA. After 48 hours, the Hg level in the liver was very low. Peak III did not accumulate or retain Hg to the extent observed in the kidney soluble fraction.

In vitro studies of inhibition of soluble acid and alkaline phosphatase, and lactic and malic acid dehydrogenase activities indicated that at least twice as much PMA as $Hg(Ac)_2$ was required to give the same degree of inhibition. Hg-binding studies indicated that more

than one mole of Hg/mole protein (protein in the enzyme fraction) was required to inhibit these enzymes.

Acid and alkaline phosphatase levels in the kidney soluble fraction were not decreased by a single dose of PMA or $Hg(Ac)_2$ and less than one mole of ^{203}Hg was bound to each mole of these enzymes.

Single doses of PMA and $Hg(Ac)_2$ slightly increased the level of kidney soluble lactic acid dehydrogenase (LDH). Multiple doses of PMA or $Hg(Ac)_2$ decreased the LDH activity levels in the kidney soluble fraction. The maximum bindings were around 0.32 and 0.50 mole Hg/mole protein in the LDH fraction for $Hg(Ac)_2$ and PMA respectively. When the kidney soluble fraction was incubated in 1×10^{-4} M $Hg(Ac)_2$, about 2-4 moles of Hg were bound per mole of protein, and the LDH activity was not affected. LDH in rat kidney did not return to a normal value with a significant decrease in mercury binding when a 2 week period of time was allowed to lapse following the final $Hg(Ac)_2$ dosage. It is highly possible that mercury binding at the active site of LDH enzyme is not the prime factor involved for the reduction of activity. It may be that the synthesis of enzyme is affected.

Single or multiple PMA dosage did not affect malic acid dehydrogenase (MDH) activity. A single dose of $Hg(Ac)_2$ showed no effect on MDH activity, but multiple doses showed a decrease. The Hg-binding was less than one mole of Hg/mole of protein in the MDH

fraction. It seems doubtful that Hg binding at the active site of the MDH molecule is the main reason for the decrease of MDH activity following in vivo treatment.

When kidney slices were incubated with lactate-1-^{14}C or -2-^{14}C, a marked difference between those taken from control rats and from rats receiving oral doses of PMA (Hg accumulation; 198 μg ^{203}Hg/gr. fr. wt. kidney) or $Hg(Ac)_2$ (98 μg ^{203}Hg/gr. fr. wt. kidney) was observed. The effects were not due to an inhibition of lactate uptake by the slices. Complete metabolism of labeled lactate to $^{14}CO_2$ or its incorporation into glutamate were definitely affected more by PMA, and to a lesser degree by $Hg(Ac)_2$. It seems possible that in vivo mercurial treatment induced an inhibition of decarboxylase activity in the kidney. Due to a reduction of lactate-^{14}C conversion through the decarboxylating pathway, a greater amount of lactate would become available for its metabolism through another pathway (a combination of assimilation followed by a degradation). A larger second peak which was found in all runs with kidney slices from mercurial-treated rats illustrate this alteration. A delay of 2 or 3 hours for the appearance of the second peak may be explained by the overall depression of metabolic reactions. A decrease in respiration of isolated kidney tissues following toxic doses of both inorganic and organic mercurials has been reported.

The rate of DL-glutamate-3, 4-^{14}C metabolized to $^{14}CO_2$ by

kidney slices from PMA or $Hg(Ac)_2$-treated rats was depressed, while the uptake rate into the slices and conversion to D-pyrrolidone-carboxylic acid was not affected. The rate of $^{14}CO_2$ evolution from glutamate-3, 4-^{14}C prior to a peak at 16 hours was depressed more from slices of PMA-treated animals than from $Hg(Ac)_2$-treated ones. The $^{14}CO_2$ peak was delayed about 2 hours by either mercurial.

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