

AN ABSTRACT OF THE THESIS OF

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Title: CALCIUM REGULATION AND PHYSIOLOGICAL FUNCTION
IN ANIMALS EXPOSED TO LEAD

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Tissue distribution studies of calcium and lead in brain tissue of rats were increased subsequent to a single intravenous injection of lead, 10 mg/kg, in the form of lead acetate. Time-course studies demonstrated that brain concentrations of calcium and lead (in terms of wet tissue weight) were found to be increased at days 1, 3 and 7 post treatment. At 24 hours brain levels of calcium and lead were elevated 70 and 350%, respectively. Over the subsequent 48 hours, brain levels of calcium reached maximal values and brain lead decreased slightly from day 1 values. At the end of 7 days, brain concentrations of calcium and lead were still elevated significantly above controls. The effect of lead on calcium in liver, heart, kidney, skeletal muscle, smooth muscle, hair, bone and serum of lead-exposed rats were not significantly altered.

Studies were undertaken to determine if the effect was mediated

by the thyroid-parathyroid complex. Calcium concentrations in the brain of lead-treated thyroparathyroidectomized (TX-PTX) rats were 30, 47 and 70% lower at days 1, 3 and 7, respectively, when compared to levels of intact lead-injected rats at 10 mg/kg. Brain levels of lead in lead-treated TX-PTX rats were comparable to levels in treated intact animals. This implies that the thyro-parathyroid system was necessary for complete expression of increased levels of brain calcium, but not essential for lead distribution in brain tissue of rats.

Concentrations of brain lead were dose related, but the effect of lead on brain calcium was not. At 5 mg/kg of lead, brain calcium was increased maximally after 24 hours when compared to the effects produced by other doses of lead, e.g., 10, 25 and 50 mg/kg. At 72 hours, brain calcium was elevated about two-fold in lead-treated rats regardless of the dose of lead administered; these levels of calcium were found unchanged or slightly lower at 7 days. Doses of lead at 25 and 50 mg/kg were not associated with altered calcium levels in the other tissues. In comparison, levels of brain lead associated with the various doses of lead changed little over the same time-course except for the 50 mg/kg dose of lead; at this dose brain lead continued to increase over 7 days.

Employment of dry tissue weight established that increased hydration of brain tissue was another effect of lead on brain tissue in

lead-treated rats. This additional effect of lead was associated only with the 25 and 50 mg/kg doses and was prominent at days 3 and 7. Elevated brain concentrations of calcium and lead with increased brain water suggested that lead's actions were on the permeability of brain tissue.

Alterations of biological parameters were determined in male rats chronically exposed to dietary lead, 300 parts per million for 8 weeks in the form of lead acetate. Equal concentrations of lead in water and in food differed in influencing various biological parameters. Whole-blood concentrations of lead were significantly elevated in all animals exposed to dietary lead. Lead-food and the combined effect of lead-water and lead-food significantly increased whole-blood levels of lead (10.5 and 17.9 $\mu\text{g}/100\text{ ml}$), respectively, above the levels in the lead-water group. Muscle concentrations of lead in the tibialis anterior of lead-food groups were increased significantly above the muscle levels in control, reconstituted food and lead-water groups. Absorption and distribution of lead depended on whether lead was administered in food or water.

Calcium concentrations in brain, blood and muscle (tibialis anterior) and lead levels in brain tissues of rats fed lead in their chow were increased, although not statistically significant. These elevated levels were associated with altered neuromuscular parameters that were determined in situ. The ratio of muscle twitch

amplitude/tetanus amplitude of the tibialis anterior was reduced significantly in the lead-food groups. Prolongation of the active state of contraction in tetanus due to increased intracellular calcium and/or lead's influence on the duration of the active state of muscle contraction mediated by the motor axons were suggested to account for the results. The tibialis anterior of lead-food groups tetanized at significantly lower frequencies than the other groups. Increased neuromuscular transmission and/or responsiveness at the junctional sites were suggested to explain these observations. It was not ascertained if elevated levels of calcium in blood, brain and muscle and/or the body burden of lead produced the alterations of the examined neuromuscular system.

Infusion of calcium (120 mg/kg/hr) to intact and conscious rabbits caused progressive increases in the serum concentration of thyrocalcitonin as determined by radioimmunologic methods. The hormone was detected in the blood of six normal rabbits at a mean concentration of 0.80 ng/ml \pm S.E. 0.25; an increased level of thyrocalcitonin was detected within 15 minutes after infusion of calcium, and peak concentrations were found during maximal hypercalcemia. Peak levels of thyrocalcitonin declined to pre-infusion levels within 30 minutes after cessation of calcium infusion in the normal rabbit. Also, the infusion of lead, 6 mg/kg/hr, tended to increase concomitantly serum calcium and thyrocalcitonin levels in rabbits, although the elevations were not statistically significant.

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in Animals Exposed to Lead

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TABLE OF CONTENTS

<u>Chapter</u>	<u>Page</u>
I. GENERAL INTRODUCTION	1
II. STUDIES ON THE INFLUENCE OF ACUTE LEAD INTOXICATION ON CALCIUM LEVELS IN VARIOUS TISSUES OF THE RAT	10
Introduction	10
Methods	11
Animals	11
Experimental	12
Administration of Metal Salts	14
Preparation of Tissue Samples for Analysis	14
Calcium Analysis	16
Lead Analysis	18
Results	19
Assessment of Analytical Procedures	19
Characteristics of Tissue Distribution of Calcium and Lead in Lead-Exposed Rats	32
Discussion	52
Methodology	52
Biological Effects	57
III. THE INFLUENCE OF CHRONIC LEAD EXPOSURE ON THE RESPONSE OF NEUROMUSCULAR PREPARATIONS <u>IN SITU</u>	64
Introduction	64
Methods	66
Animals	66
Dietary Treatment Regime	66
Procedure	67
Results	71
Influence of Dietary Lead on Various Biological Parameters	71
Influence of Dietary Lead on Neuromuscular Parameters	74
Discussion	76

<u>Chapter</u>	<u>Page</u>
IV. THE INFLUENCE OF INORGANIC LEAD ON SERUM LEVELS OF THYROCALCITONIN IN RABBITS	84
Introduction	84
Methods	85
Materials	85
Immunization Procedure	86
Radioiodination of Thyrocalcitonin or Para- thyroid Hormone	87
Separation of Iodinated Hormone From Remaining Free (^{125}I)-Iodide	87
Assay for Binding Capacity	88
Radioimmunoassay	91
Treatment of Rabbits	92
Results	93
Antibody Production During the Course of Immunization	93
Radioiodination of Parathyroid Hormone and Thyrocalcitonin	96
Binding Capacity of ^{125}I -Thyrocalcitonin	106
Radioimmunoassay of Thyrocalcitonin	114
Discussion	125
V. SUMMARY AND CONCLUSIONS	129
BIBLIOGRAPHY	133

LIST OF FIGURES

<u>Figure</u>		<u>Page</u>
1.	The effect of increasing concentrations of phosphoric acid on calcium absorption.	22
2.	Standard curve and recovery curve for blood lead.	24
3.	Temporal patterns of whole-brain levels of calcium among control (C), severed lateral recurrent-laryngeal nerves (LRLN), and thyroparathyroidectomized rats, following intravenous administration of lead, 10 mg/kg.	34
4.	Temporal patterns of whole-brain levels of lead among control (C), severed lateral recurrent-laryngeal nerves (LRLN), and thyroparathyroidectomized rats, following intravenous administration of lead, 10 mg/kg.	36
5.	Dose-response and temporal relationships of whole-brain levels of calcium in rats to various doses of lead.	41
6.	Dose-response and temporal relationships of whole-brain levels of lead in lead-exposed rats.	43
7.	Dose-response and temporal relationships of whole-brain levels of calcium in terms of dry brain weight (mg/100 g) in rats to various doses of lead acetate.	49
8.	Dose-response and temporal relationships of whole-brain levels of lead in terms of dry brain weight ($\mu\text{g/g}$) in rats to various doses of lead.	51
9.	The effects of variable delay between two stimulating pulses on the response of muscle twitch in the rat.	77
10.	Quantifications of antibody titers of sera from guinea pigs immunized with bovine thyrocalcitonin.	95

<u>Figure</u>	<u>Page</u>
11. Radioiodination of 5 μ g bovine parathyroid hormone.	98
12. Radioiodination of 5 μ g bovine thyrocalcitonin.	100
13. Radioiodination of 10 μ g bovine thyrocalcitonin.	102
14. Radioiodination of 20 μ g bovine thyrocalcitonin.	104
15. Binding capacity of ^{125}I -thyrocalcitonin by sera from guinea pigs immunized with bovine thyrocalcitonin. Fraction #3 from 5 μ g radioiodination.	109
16. Binding capacity of ^{125}I -thyrocalcitonin by sera from guinea pigs immunized with bovine thyrocalcitonin. Fractions #3, #4 and #5 from 10 μ g radioiodination.	111
17. Binding capacity of ^{125}I -thyrocalcitonin by sera from guinea pigs immunized with bovine thyrocalcitonin. Fractions #5, #6, and #7 from 20 μ g radioiodination.	113
18. Regression line of the standard curve for the radio-immunoassay system of bovine thyrocalcitonin.	116
19. The influence of serum calcium levels on serum thyrocalcitonin in the rabbit.	118
20. The effects of increasing levels of blood lead on serum calcium and thyrocalcitonin in the rabbit.	121
21. The effects of EDTA on serum calcium in the rabbit.	123

LIST OF TABLES

<u>Table</u>		<u>Page</u>
1.	Comparison of wet digestion methods of tissues for analysis of calcium and lead.	26
2.	Recovery of calcium.	30
3.	Recovery of lead.	31
4.	Time-course of blood, kidney and liver levels of lead in control, severed lateral recurrent-laryngeal nerves and thyroparathyroidectomized rats, following intravenous administration of lead, 10 mg/kg.	39
5.	Dose-response and temporal studies of blood, kidney and liver levels of lead in rats.	46
6.	Hydration of brain tissue in lead-exposed rats.	53
7.	Influence of dietary lead on various biological parameters.	72
8.	Influence of dietary lead on neuromuscular parameters.	75

CALCIUM REGULATION AND PHYSIOLOGICAL FUNCTION IN ANIMALS EXPOSED TO LEAD

I. GENERAL INTRODUCTION

This investigation was undertaken to determine the effects of lead on calcium metabolism. The influence of lead on tissue distribution and physiological function of calcium was examined in rats. In rabbits, an effort was made to determine the relationship of lead on serum levels of the calcium-regulatory hormones, namely, thyrocalcitonin and parathyroid hormone.

In view of its great toxicologic importance lead has been the subject of many pharmacologic studies. Some historical and comprehensive reviews of lead and its biologic effects are presented by Cantarow and Trumper (1944), Sobel and Burger (1955), Kehoe (1961a, 1961b), California Department of Public Health (1967), and in *Lead: Airborne Lead in Perspective* (1972). Lead intoxication can give rise to several well-known but nonspecific clinical syndromes of illness in man: anemia, acute colic, acute and chronic encephalopathy, peripheral neuropathy and/or myelopathy, and chronic or late lead nephropathy with or without secondary gout. Clinical descriptions of lead intoxication are reported by Tanquerel (1848), Aub et al. (1926), in modern texts by Chisolm (1968), Goodman and Gilman (1970), and in various reviews by Byers (1959), Emmerson

(1968), American Academy of Pediatrics (1969) and in the reviews cited above.

Relationships between calcium and lead metabolism have been recognized for many years. Lead, like calcium, is a bivalent cation under physiological conditions; however, unlike calcium, lead currently lacks a role as a necessary or essential metal in the functioning of biologic systems, but its distribution and association with topical and/or systemic calcification in exposed experimental animals suggest that calcium and lead may share the same regulatory system in vivo. The view that certain syndromes of lead intoxication are the result of impairment or dysfunction of calcium regulation has been proposed by Aub et al. (1926), Cantarow and Trumper (1944), Sobel and Burger (1955) and Kehoe (1961a and 1961b). The fact that lead tends to be deposited in the skeleton led to the assumption that calcific deposits attract lead salts (Aub et al., 1926; Sobel, Grawon and Kramer, 1938; Sobel et al., 1940; Kehoe, 1942; Cantarow and Trumper, 1944; Kehoe, 1961a, 1961d; Selye, 1962; Selye, Tuchweber and Gabbiani, 1962, 1963; Hass et al., 1967). Thus, the adage arose that the "lead stream follows the calcium stream." Lead that is retained within the skeleton may be mobilized and released to soft tissues via blood if demineralization of bone occurs. A high calcium intake in the absence of elevated phosphate intake mobilizes lead in bone and elevates lead's content in various

soft tissues, owing to the competition with lead for available phosphorus (Sobel, Grawon and Kramer, 1938; Lederer and Bing, 1940; Kehoe, 1942; Cantarow and Trumper, 1944; Schields and Mitchell, 1941; Kehoe, 1947, 1961a; Hass et al., 1967).

There is some similarity between the response of calcium and lead to parathyroid hormone administration. Parathyroid hormone administered to humans increases urinary excretion of lead as well as calcium (Hunter and Aub, 1926-27). Since parathyroid hormone is believed to affect deposition and reabsorption of the mineral component of bone, the mobilization of lead as well as calcium from bone suggests that lead must be deposited with bone calcium (Sawin, 1969).

The effect of vitamin D on lead metabolism appears similar to the vitamin's action on calcium. Addition of vitamin D to the diet increases the lead content of blood (Sobel et al., 1940) and of the femur (Sobel, Grawon and Kramer, 1938). Furthermore, lead suppresses enhanced formation of osseous and extraosseous intercellular bone matrix consequent to hypervitaminosis D (Hass et al., 1967).

Ironically, the attraction of calcium by lead was virtually unknown until recently. At the beginning of this century, Kumita (1909) reported that if large amounts of lead acetate were repeatedly injected subcutaneously or intramuscularly in rabbits, hard whitish

nodules were produced. This paper attracted little attention and stimulated no further research along these lines; however, in 1962 it was found that lead and certain other metals were capable of producing systemic or topical calcification, which normally is identifiable as such by histochemical techniques. This phenomenon occurred in various animal species, including the rat, in which connective tissue necrosis was rarely accompanied by calcification; furthermore, these compounds were effective even at dose levels which produced no necrosis (Selye, 1962). Selye and his coworkers were responsible for formulating the bases of pathological calcification of rat tissues, hypotheses of its mechanisms, and identifying its predisposing factors.

The manner in which the calcium-regulating hormones mediate the metabolism of inorganic lead is not understood. Selye (1958) demonstrated in rats that either thyroparathyroidectomy or parathyroidectomy was capable of preventing the development of osteitis fibrosa and nephrocalcinosis. Lehr et al. (1958) and Lehr (1959) reported that parathyroidectomy averted calcification of uremic myocardial foci produced by deprivation of the animal's kidneys. This evidence suggested that the parathyroids and/or thyroid-parathyroid complex were intimately associated with certain pathological processes which were characterized by soft tissue calcification. This view was modified sharply when Selye (1958) demonstrated

osteitis fibrosa in intact and parathyroidectomized rats with the antibiotic, stylomycin aminonucleoside, which induced severe renal lesions. In addition, Selye, Tuchweber and Gabbiani (1963) showed that osteitis fibrosa and cutaneous or local calcinosis induced by lead acetate could be successfully produced in parathyroidectomized rats. Consequently, the role of the parathyroids on soft tissue calcification induced by the action of lead is unclear.

Evidence that thyrocalcitonin influences lead metabolism is scarce and fragmentary. Gabbiani et al. (1968) and Hirsch and Munson (1969) demonstrated in rats that thyrocalcitonin inhibited experimental soft tissue calcification and hypercalcemia provoked by lead acetate. Thyrocalcitonin significantly reduced blood and skin lead levels. This action was attributed to the influence of the polypeptide on calcium and phosphorus blood levels. Gabbiani and Tuchweber (1970) showed that thyrocalcitonin counteracted the hepatotoxic manifestations of holmium and indium in rats. As a result these authors modified their earlier view and proposed that thyrocalcitonin may exert its inhibitory activity against soft tissue calcification, which lead provokes, through its influence on the distribution of lead rather than through an alteration of calcium and phosphorus metabolism.

On the other hand, there is a possibility that endogenous thyrocalcitonin might aggravate soft tissue calcification. Bajusz

et al. (1963) reported that in rats with experimental lesions of the coronary artery, myocardial calcification was increased following parathyroidectomy. Increased calcification of soft tissues was not observed in thyroparathyroidectomized rats whether they were untreated or given thyroxine. Bajusz et al. postulated the existence of a thyroid principle other than thyroxine that was responsible for the greater severity of the lesions in parathyroidectomized rats. Because this study was conducted before the discovery of thyrocalcitonin was published, the possible association of thyrocalcitonin with this effect was not discussed.

Rosen (1972) reported that porcine thyrocalcitonin significantly reduced blood lead levels in lead-intoxicated rats. Moreover, glucagon and imidazole (thyrocalcitonin-mimicking agents) are capable of depressing blood lead levels in rats chronically exposed to lead (Rosen, 1972). He suggested that the concentration of blood lead is determined, to an extent, by hormones and agents known to regulate bone resorption through participation of an adenylyl cyclase system.

One of the major reactants of the adenylyl cyclase system is adenosine triphosphate (ATP), whose physiologic role in muscle activity is becoming more clearly understood. The sequestering and release of calcium ions by the longitudinal cisterns and tubules of the sarcoplasmic reticulum apparently require ATP or an active

calcium pump. These events result in muscle relaxation and contraction, respectively (Davson, 1970). Moreover, it has been demonstrated that calcium mediates the release of the neurotransmitter at the neuromuscular junction (Thies, 1965). Thus, it is possible that calcium couples the events of excitation and contraction of muscle (Davson, 1970). Lead palsy is commonly associated with chronic plumbism and is attributed to the actions of lead on neuromuscular transmission and muscle activity that are intrinsically associated with calcium (Cantarow and Trumper, 1944; Kehoe, 1961c; Johnstone, 1964; Manalis and Cooper, 1973). Manalis and Cooper (1973) reported that lead influenced both pre and postsynaptic events of neuromuscular transmission in vitro in the isolated sciatic nerve-sartorius muscle preparation of the frog. Similar electrophysiologic studies on mammals are absent. Kostial and Vouk (1957) found that lead nitrate in concentrations as low as 12.1 μM blocked ganglionic transmission of the perfused superior cervical ganglion of the cat.

The apparent relationship between calcium and lead metabolism has been well documented. A fuller understanding of the biologic significance of this relationship is needed to establish a more comprehensive diagnostic and therapeutic approach to lead intoxication. For example, the obvious adverse effects of excessive lead on the nervous system, kidneys and hematopoiesis are well recognized at the clinical level, but are not adequately understood at the

subclinical level. Consequently, additional information on dose-response and time-course relations of lead to various tissue levels of calcium, prior to conspicuous soft tissue calcification, were conducted and are reported in this thesis, to determine the "threshold" of tissues for this effect. It is likely that altered tissue levels of calcium would result in pathological changes in the animal. The effects of thyroparathyroidectomy on tissue levels of calcium in animals exposed to lead were carried out to provide a better understanding of the possible role of the calcium-regulatory system in plumbism. Such studies may provide information as to whether hypothyroid and/or hypoparathyroid individuals are more susceptible to lead toxicity than euthyroid and/or euparathyroid subjects.

The importance of determining the influence of lead on the neuromuscular system in situ is apparent. Case studies suggest that neuropathy and myopathy may be significant but inadequately recognized aspects of lead toxicity both clinically and subclinically. Moreover, the scanty information of lead's effects on the neuromuscular system does not afford an adequate basis for the evaluation of the etiology of lead palsy. Research concerning the effects of chronic lead exposure on myologic parameters was conducted by this investigator to define more precisely the site of lesion and to assess the body burden of lead (muscle) in lead palsy.

Since radioimmunologic techniques are currently available for

monitoring many endocrine secretions, the apparent effects of lead on various endocrine products can be assessed. In this thesis, studies concerning the influence of lead on thyrocalcitonin and parathyroid hormone levels were directed to determine if plasma levels of these polypeptides were altered in mammalian species upon exposure to lead. With this information a more comprehensive picture could be constructed regarding the significance of the calcium-regulatory system to lead metabolism and the metal's clearance from the body.

The primary intent of the investigations reported herein was to develop a better understanding of the metabolic relations of lead and calcium in experimental animals. This information may serve as a basis for reference in the study of other less well understood adverse metabolic and functional effects that have been attributed to lead in man.

II. STUDIES ON THE INFLUENCE OF ACUTE LEAD INTOXICATION ON CALCIUM LEVELS IN VARIOUS TISSUES OF THE RAT

Introduction

Lead acetate belongs to the defined category of "direct calcifiers", i.e., substances that cause calcium deposition in connective tissue whenever they are injected (Selye, 1962). It has been shown that massive calcium deposits develop within 6 days in rats, following repeated intravenous administration of lead acetate, two 50 mg/kg doses 5 hours apart (Selye, Tuchweber and Gabbiani, 1962). The induced calcific deposits were conspicuously localized in the portal area, particularly around the portal vein and its branches, and in the splenic capsule and the surroundings of the ileocecal valve. This response rarely occurred when a single dose of lead was administered.

It is unclear what role the thyroid-parathyroid complex plays in this soft tissue calcification provoked by lead acetate. Selye (1958) and Lehr (1958 and 1959) noted that the thyroid-parathyroid system and/or the parathyroids were indispensable in the production of osteitis fibrosa and calcified uremic myocardial foci respectively, in bilaterally nephrectomized rats. Later it was revealed in rats that the parathyroids were not essential for the production of osteitis fibrosa and cutaneous calcinosis induced by lead acetate (Selye,

Tuchweber and Gabbiani, 1963). Analogously, Gabbiani et al. (1968) and Hirsch and Munson (1969) demonstrated that soft tissue calcification induced by lead salts was inhibited by thyrocalcitonin. Blood levels of lead were reduced significantly in rats by porcine thyrocalcitonin and other thyrocalcitonin-mimicking agents (Rosen, 1972). Hirsch, Gauthier and Munson (1963) reported that unavoidable trauma to the recurrent laryngeal nerves during thyroparathyroid-ectomy elevated serum calcium or retarded its fall. Knowledge of the influence of this effect on the calcium levels of other tissues is unknown.

In view of the action of lead on the calcification of soft tissue and the evidence of neuroendocrine effects on calcium regulation, elevated tissue levels of calcium induced by lead should be detectable prior to calcinosis. This dissertation reports dose-response and time-course studies which were conducted to determine if lead influences tissue levels of calcium in vivo. Also, the effects of surgical removal of the thyroid-parathyroid complex and severance of the recurrent laryngeal nerves on this response were studied.

Methods

Animals

Male Sprague-Dawley albino rats, with a mean body weight of

363 g (range 262-492 g) were used in these experiments. The animals were housed in suspended, stainless steel cages (five per cage) until the day of sacrifice. The rats were previously divided into treatment groups by formal randomization. Purina Rat Chow[®] and tap water were supplied ad libitum except to the thyroparathyroid-ectomized group; a 2% calcium lactate drinking solution was substituted for regular tap water in the postoperative care of this surgically-manipulated group (Zarrow, Yochum, McCarthy, and Sanborn, 1964). The room in which the rats were housed was maintained at $22^{\circ} \pm 1^{\circ}\text{C}$ with 17 changes of fresh air/hour and an automatically controlled 12-hour light-dark cycle. The animals were under veterinary surveillance and they were monitored daily by a trained animal caretaker, as well as periodically day and night by the investigating scientist.

Experimental

Four groups of animals were used with five animals assigned to each group: (a) control group (C); (b) sham-thyroparathyroid-ectomized group (STX-PTX); (c) lateral recurrent laryngeal nerve severance group (LRLN); and (d) thyroparathyroidectomized group (TX-PTX).

Thyroparathyroidectomy. The rats were anesthetized with sodium pentobarbital, 50 mg/kg, i. p. and secured on a small

animal board. A ventral midline incision was made from the cephalic border of the sternum and extending about 3 cm anteriorly. The incision was deepened by blunt dissection of the underlying subcutaneous connective tissue until the sternohyoid and sternomastoid muscles were visible. The trachea and thyroid gland were exposed by dividing the sternohyoid muscle longitudinally and by retracting the muscle with blunt wire retractors. The thyroid isthmus was divided at the midline and both thyroid lobes with embedded parathyroids were gently separated from the trachea by the use of iridectomy scissors. Despite the special care employed to avoid damaging the recurrent laryngeal nerves, it was not possible to remove the thyroid gland without stretching these nerves somewhat.

Following surgery, the retractors were removed allowing the muscles to return to their normal positions, and the incision was closed with silk sutures. A recovery period of 1 week was allowed to elapse before administration of lead acetate or physiological saline (0.9% sodium chloride in doubly distilled water).

Sham-Thyroparathyroidectomy. The general surgical procedure described above was utilized for this group except that the thyroid gland and associated structures were exposed but were not disturbed.

Lateral Recurrent Laryngeal Nerves Severance. Both recurrent laryngeal nerves posterior to the thyroid gland were located and

separated from the trachea and accompanying vasculature. The nerves were cut with iridectomy scissors. This surgery produced a characteristic wheeze in some animals upon their recovery.

Administration of Metal Salts

With the rat anesthetized with ether and secured on a surgical board, a superficial incision about 1 cm in length was made on the medial surface of the thigh, over the femoral artery and vein and along the boundary between adductor and extensor muscles. The incision was deepened by blunt dissection, thereby exposing the femoral vein and artery. With the vein occluded by thumb pressure proximal to the point of injection, physiological saline at 1 ml/kg or lead acetate (Mallinckrodt Chemical Works, St. Louis, Mo.), equivalent to 10 mg/kg of the lead ion (at the concentration, 10 mg/ml) was administered. A 27 gauge, stainless steel needle attached to a 1 ml glass syringe was utilized for the injections. The rate of injection was about 0.3 ml/min. The incision was closed with silk sutures.

Preparation of Tissue Samples for Analysis

Animals were sacrificed precisely 1 day, 3 days and 7 days subsequent to administration of metal salts. The following tissues were removed and subsequently analyzed for calcium and lead:

brain (whole), liver (left lobe), heart (whole), right kidney (whole), skeletal muscle (complete diaphragm), smooth muscle (entire small intestine), hair (removed from shoulder region and visibly free of debris), bone (diaphysial portion of femur), blood (whole and serum).

Sampling of Tissues. At the appropriate sacrifice time, the rat was anesthetized with ether. Five milliliters of blood were collected by cardiac puncture with a 22 gauge disposable needle attached to a 5 ml glass syringe. Immediately following collection, 3 ml of blood were dispensed into a tared, liquid scintillation counting vial for lead analysis; the remaining 2 ml were dispensed into a disposable culture tube, 12 x 75 mm, for serum calcium analysis.

After clot formation and followed by centrifugation to separate the cellular elements, the serum was removed and stored in the refrigerator at 4^oC until analyzed for calcium. The rat was sacrificed by cervical dislocation followed by exsanguination. The tissues were removed from the animal, rinsed briefly in physiological saline (except hair and bone), blotted with paper towels, placed into tared, liquid scintillation vials and weighed to the nearest 100 µg. The hair sample was removed from the carcass, placed into a tared vial and weighed. The small intestine was slit longitudinally and rinsed several times in physiological saline to remove its contents. Bones were freed from adhering soft tissue, split, and

the marrow removed before weighing. All tissues except the serum sample were dried in an oven at 90 - 92°C for 96 hours and reweighed. The tissues were ground to a fine powder with a round ended glass rod.

Digestion. Preliminary comparison of three methods of wet digestion were made prior to employing the mixed nitric and perchloric acid procedure. To each dried and ground tissue sample (except blood, bone and hair) the acid mixture (equal volumes of 12N HNO₃ and 35% HClO₄) was added, 0.4 ml for each 100 mg of wet tissue; to blood and hair samples, 4 ml of the mixed acid were added. The dried bone samples were digested with 4 ml of 33% formic acid to each 100 mg of wet bone. The tissue vials were sealed with Parafilm[®] and allowed to digest for a period of 3 weeks at room temperature. After centrifugation, aliquants of the supernatant were analyzed for calcium and lead as described below.

Calcium Analysis

Apparatus. The Jarrell-Ash Dial-Atom, atomic absorption-flame emission spectrophotometer, Model Mark II was used in conjunction with a Hetco burner with a laminar-flow head. The light source was a Jarrell-Ash multi-element (Ca, Mg, Al, Fe) hollow-cathode lamp. The fuel was acetylene and the oxidant compressed air. The manufacturer's standard setting of 10 Ma was used for the

lamp current. Initial fuel and air pressure were 12 and 40 p. s. i. respectively. A slit of 1 mm was used, and calcium absorption at 422.7 m μ was recorded. The fuel-air mixture was adjusted to give a yellow reducing flame for calcium.

Reagents. A standard solution of calcium, 1 g/l was prepared by dissolving 36.680 g of dry analytical grade CaCl₂ in a slight excess of 1 N HNO₃ and diluting appropriately with doubly distilled water. A stock solution of 5% lanthanum nitrate was made by wetting 58.65 g of La₂O₃ (American Potash and Chemical Corp.) with doubly distilled water, then adding 75 ml of 70% HNO₃ and diluting to 1 liter with doubly distilled water. Additional calcium standards of 0.020, 0.10, 1, 5, 10, 15 and 20 mg per 100 ml were prepared by diluting the original standard solution appropriately with 5% lanthanum nitrate and 0.1 N HNO₃ to give a final concentration of 1% lanthanum. All other reagents were analytical grade and doubly distilled water was used throughout. The blank was 1% lanthanum in 0.1 N HNO₃. All glassware was soaked overnight in 35% HNO₃.

Analysis Procedure. After centrifugation of the digested samples, aliquants of the supernatant were diluted (1:5) by the stock lanthanum solution to give a final concentration of 1% lanthanum for calcium analysis. These solutions were compared in the atomic absorption spectrophotometer with the calcium standards.

Serum and bone samples were diluted directly 1/50 with 1%

lanthanum nitrate and 0.1 N HNO_3 prior to absorption flame photometry for calcium; the same standards were used as for the other tissues.

Lead Analysis

Apparatus. The same atomic absorption spectrophotometer was employed as described in Calcium Analysis. A lead hollow-cathode lamp (Jarrell-Ash, single element) was utilized. Acetylene was the fuel and compressed air the oxidant. A lamp current of 5 Ma was used as recommended by the manufacturer. A burner slit of 1 mm was utilized. The air and fuel pressure were 40 and 12 p. s. i. respectively. The primary wavelength of 217.0 $\text{m}\mu$ was employed for lead absorption. The air flow rate of 10 scfh (equivalent to 8 lbs/in^2 gauge pressure as empirically derived by the manufacturer) was employed, whereas the acetylene flow rate was set when aspiration of methyl isobutyl ketone (MIBK) was started by reducing the flow to the point at which yellow luminescence disappeared from the base of the flame. No significant differences in blank absorbance at this wavelength between pure MIBK and water-saturated MIBK were observed, although the latter is sometimes recommended.

Reagents. A commercially available standard of lead perchlorate (Orion Research Inc., 11 Blackstone St., Cambridge, Mass.) at 0.1 moles of lead per liter was employed for the original standard. Additional lead standards of 0.010, 0.015, 0.025, 0.050, 0.100, 0.300, 0.700, and 1.0 mg per 100 ml were prepared by diluting

the original standard appropriately with 0.1 N HNO_3 .

A 2% (weight/volume) of ammonium pyrrolidine dithiocarbonate (APDC) solution was utilized for complexing lead in the samples. Methyl isobutyl ketone (MIBK) was utilized as an extraction agent for lead. All solutions were stored in polyethylene bottles.

Analytical Procedure. An equal volume of water was added to an equal volume of tissue supernatant for dilution of the acidic contents. Usually 3 ml of supernatant were removed from blood, bone, brain, heart, kidney and liver samples, whereas 0.4 ml were used from hair, skeletal muscle and smooth muscle samples. To each vial containing 3 ml of tissue supernatant, 1 ml of 2% APDC was added, whereas 0.5 ml of APDC was added to each vial containing 0.4 ml of supernatant. The contents of the vials were mixed and 1.5 ml of MIBK was added, followed by 1 ml of water. The samples were centrifuged for 10 minutes. The MIBK layer was aspirated into the AAS and lead absorption recorded. These solutions were compared to the lead standards. A 0.2% APDC solution (weight/volume) in MIBK was used as the blank.

Results

Assessment of Analytical Procedures

Effect of Phosphate on Calcium Absorption. It has been

recognized that phosphate when present in solution with calcium, depressed calcium absorption measurements (Willis, 1961; Fuwa, 1971). Willis (1961) found that lanthanum overcame this depression by forming a complex with phosphate and consequently displaced calcium from its complex with phosphate. This analytical problem was assessed for this research in the following manner. Phosphoric acid was added to aliquots of a 0.0002% calcium solution to provide various concentrations of phosphate. These samples were analyzed for calcium in the absence and in the presence of a final sample concentration of 1% lanthanum. In the absence of lanthanum, a marked decrease in absorption by calcium was demonstrated by increasing the phosphate concentration from 0 to 5 mM (Figure 1). Thereafter, higher concentrations of phosphoric acid changed the absorption of calcium only slightly. In the presence of lanthanum, the absorption of calcium was not sharply affected until the phosphate concentration approached 10 mM.

Standard and Recovery Curve for Blood Lead. Lead standards were added to aliquots of the supernatant from a digested pooled-blood sample to assess the overall reliability and sensitivity of the analytical procedure for lead determinations (Figure 2). Recovery of lead added to the blood was determined by atomic absorption spectrophotometry (AAS) as described in Methods, over a range of 10 to 500 μg per 100 ml. The recovery curve obtained was linear and

Figure 1. The effect of increasing concentrations of phosphoric acid on the reading of a solution containing 0.2 mg per 100 ml calcium in the presence and absence of 1% lanthanum nitrate. The read-out units of the Jarrell-Ash Mark II represent the logarithmic absorption corrected to linearity. The symbol (I) represents the standard errors of the means of 10 determinations per point. In the absence of lanthanum, increasing the phosphate from 0 to 5 mM caused a marked decrease in absorption; thereafter, the change in absorption was less. In the presence of lanthanum, there was little effect on the reading until the phosphate concentration reached 10 mM.

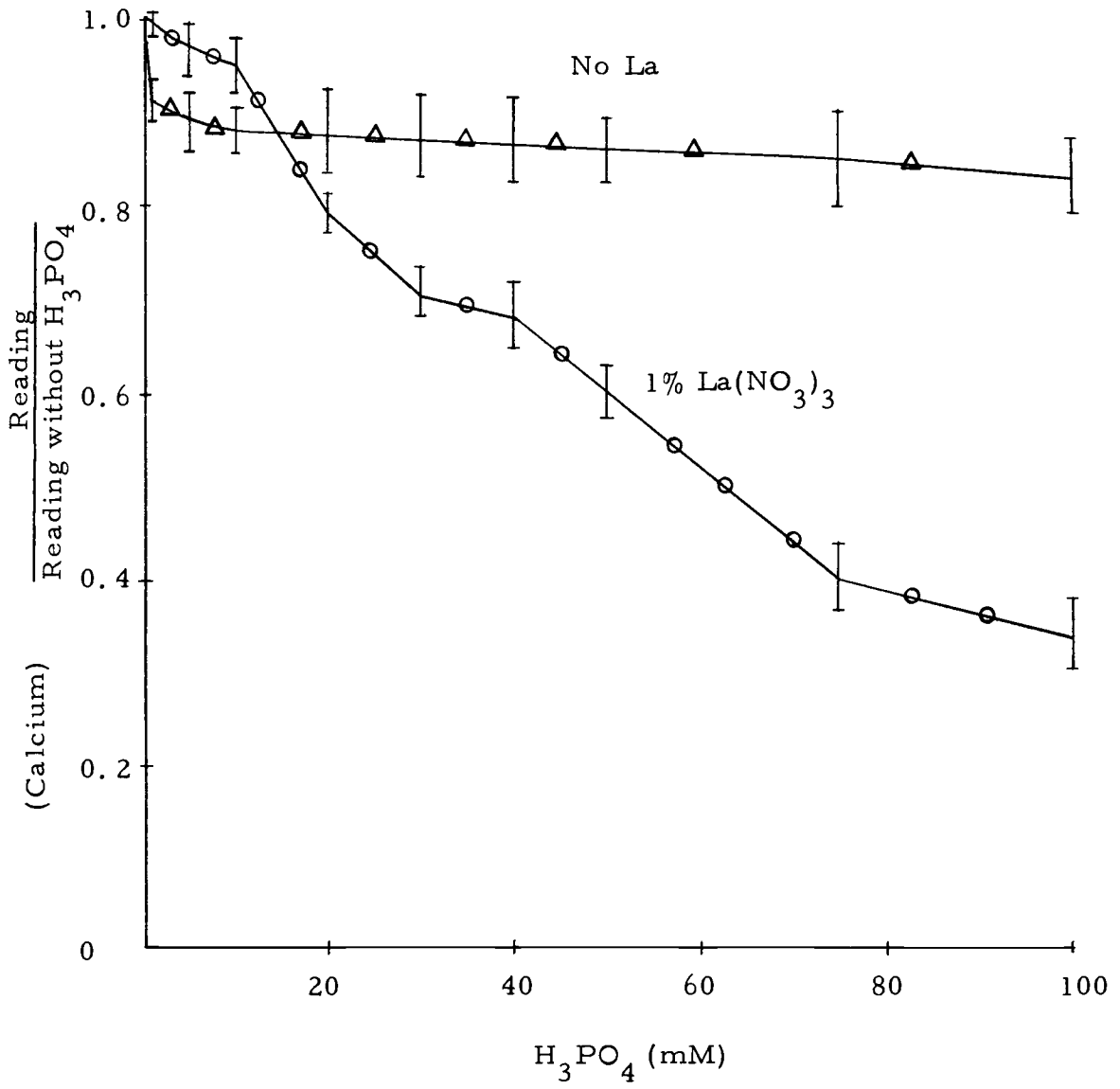
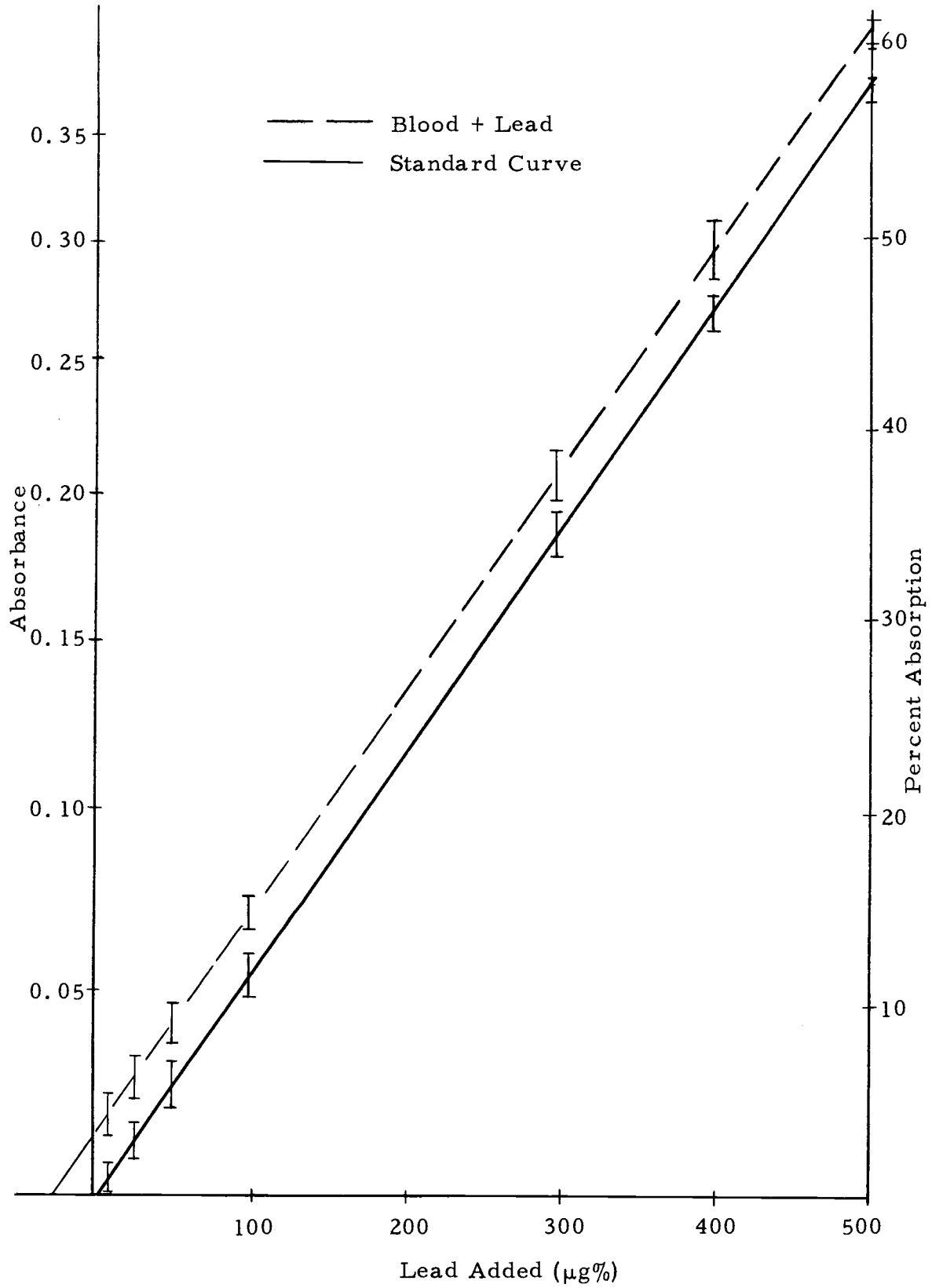


Figure 2. Standard curve and recovery curve for blood lead obtained by adding lead standards to aliquots of a pooled blood sample. Absorbance (A) and percent absorption ($\%A$) are on the ordinates. The symbol (I) represents the standard error of the mean for five determinations for each point. Note that the recovery curve remains parallel to the standard curve over a range of 0 to 500 μg per 100 ml of added lead. The intersection of the recovery curve with the abscissa represents the endogenous lead concentration (30 μg per 100 ml).



parallel to the curve obtained with the aqueous lead standard solution, up to a concentration of 500 μg per 100 ml (Figure 2). The sensitivity of the procedure as exhibited by the standard curve was 0.10 $\mu\text{g}/\text{ml}/1\%$ absorption. The endogenous lead concentration was 30 μg per 100 ml as illustrated by the intersection of the recovery curve with the Absorbance abscissa.

Comparison of Tissue Digestion Methods. Since calcium is present in considerably greater concentrations than lead in tissues, its analytical determination by AAS is not difficult. Tissue digestion by a conventional wet procedure was selected instead of complete ashing by incineration in a muffle furnace. The major reason for this selection was the lack of availability of equipment for dry tissue ashing; consequently, certain wet digestion methods were examined and compared to determine if they met certain established criteria for elementary tissue analyses of calcium and lead. These criteria were: (a) completeness of digestion; (b) an absence of significant interference with atomic absorption spectrophotometric analyses of calcium and lead; (c) little or no compromise with the practical detection limits (sensitivity) when small tissue samples are encountered. In Table 1 the data are recorded from a comparison study which examined three different methods of tissue digestion. The respective tissues were removed from three groups of rats, and each of the digestion methods was employed for the digestion of

Table 1. Comparison of wet digestion methods for analysis of calcium and lead of tissues.

Tissue	% Water	Element	(10) 12 N HNO ₃ digest	(10) 12 N HNO ₃ & 35% HClO ₄ digest	(10) 35% HClO ₄ digest
Blood	80.1±1.9	Ca	3.67± 1.29	4.24± 1.14	3.47± 1.23
		Pb	17.9 ± 2.0	30.0 ± 5.1	17.6 ± 3.3
Brain	78.6±1.1	Ca	3.32± 1.09	3.95± 0.60	3.08± 1.11
		Pb	0.20± 0.06	0.30± 0.02	0.20± 0.04
Hair	7.1±0.9	Ca	138.60±18.05	167.51±12.43	130.34±16.15
		Pb	35.76± 6.62	53.66± 4.38	33.91± 5.68
Heart	78.9±1.8	Ca	2.15± 0.26	2.59± 0.17	2.35± 0.73
		Pb	1.02± 0.17	1.27± 0.10	1.14± 0.15
Kidney	80.2±2.0	Ca	5.59± 1.11	6.56± 0.67	5.39± 1.29
		Pb	13.37± 3.56	15.87± 1.46	13.47± 2.49
Liver	70.7±0.09	Ca	2.20± 0.86	2.73± 0.30	1.97± 0.40
		Pb	13.62± 2.64	18.08± 1.47	14.81± 2.46
Skeletal Muscle	76.5±2.1	Ca	2.45± 0.70	2.78± 0.32	2.41± 0.86
		Pb	3.24± 0.93	3.65± 0.43	3.14± 1.05
Smooth Muscle	76.0±2.2	Ca	2.93± 0.82	3.85± 0.47	2.70± 0.60
		Pb	3.52± 0.54	4.74± 0.33	3.29± 0.68
Bone	30.1±1.9	Ca	8750 ±412.62 ^{a/}		
		Pb	35.5 ± 2.8 ^{b/}		

Table 1. Continued.

Tissue	% Water	Element	(10) 12 N HNO ₃ digest	(10) 12 N HNO ₃ & 35% HClO ₄ digest	(10) 35% HClO ₄ digest
Serum Calcium			5.10 ± 0.15 ^{c/}		

The mean calcium content (fluid, milliequivalent per liter; solid tissues, milliequivalent per kilogram of wet weight) ± standard error. The mean lead content (fluid, microgram per 100 milliliters; solid tissues, milligram per kilogram of wet weight) standard error. Figures in parentheses refer to number of individual analyses for each element per tissue. Large differences between the basal tissue values of calcium and lead, obtained by the mixed-acid versus either of the other two methods of digestion, were statistically significant ($P < 0.05$, Student's t-test) for all tissues analyzed.

^{a/} Aliquots were taken from 33% HCOOH digest and diluted 1:50 with 1% lanthanum solution.

^{b/} Aliquants were taken from 33% HCOOH digest and analyzed for lead as described in text.

^{c/} Aliquots were taken from serum sample and diluted 1:50 with 1% lanthanum solution.

tissues from 10 rats. The percent water values were reported as the mean \pm standard error of the mean for 30 samples per tissue. Upon drying and reweighing, the differences in the water content of the tissues among the three groups of rats were generally in the range of 0.5 to two percent, and in no case exceeded three percent. These differences were considered not to be of sufficient magnitude to necessitate reporting them in Table 1. The mixed-acid method yielded significantly higher basal levels of calcium and lead ($P < 0.05$) for all tissues analyzed when compared to the other two methods. Digestion of the tissues with 12 N HNO_3 generally produced greater levels of calcium and lead upon analysis than digestion with 35% HClO_4 .

Among the different tissues analyzed, bone and heart tissues contained the highest and the lowest levels of calcium respectively. Hair and brain or blood tissues were found to contain the highest and the lowest levels of lead respectively. Based upon the ratio of tissue calcium to tissue lead (in terms of parts per million of wet weight), the following (Ca/Pb) values for the various tissues were computed from the mixed-acid values of Table 1 to determine if a relationship existed between basal levels of calcium and lead: bone (4930), whole blood (282), brain (263), kidney (83), hair (62), heart (41), smooth muscle (16), skeletal muscle (15) and liver (3).

Recovery of Calcium and Lead. Table 1 clearly indicated that

recovery studies were required to determine which values represented reliable data in the study. In the recovery study (Tables 2 and 3) tissues from three groups of rats (10 rats per group) were removed and digested. Each of the digestion methods was employed for one group of animals. To the tissues from five of these rats and prior to digestion, additions of sufficient amounts of calcium and lead were made to approximately double the basal concentrations of these ions. The amounts of calcium and lead that were added to each tissue were based on mean values. These mean values were computed from the sum of individual values reported with each of the three digestion methods comprising Table 1. The tissues from the other five rats of each group were used for the basal analysis.

Upon comparison of the three digestion methods, a significant difference ($P < 0.05$) was found for all tissues in the recovery of added calcium and lead; the recovery values of the mixed-acid method were higher and evinced less variation (Table 2 and Table 3). The recovery of calcium and lead for tissues that were digested with mixed-acids was generally greater than 100%. In the case of whole-blood, the recovery of calcium of 98.5% was considered low. Serum gave more reliable recovery values for blood calcium than whole blood (Table 2). As a result, serum was used for monitoring blood calcium in subsequent studies.

Digestion of bone with 33% formic acid appeared to be

Table 2. Recovery of calcium.

Tissue	Calcium			
	Dilution in 1% La-0.1 N HNO ₃	12 N HNO ₃ digest	12 N HNO ₃ & 35% HClO ₄ digest	35% HClO ₄ digest
Serum	101.4±0.8			
Bone	101.1±0.6 ^{a/}			
Blood		95.3±1.5	98.5±1.1	95.1±1.8
Brain		97.0±1.0	99.6±0.7	96.4±1.3
Hair		97.6±1.2	101.9±0.9	97.0±1.4
Heart		98.1±1.1	101.1±0.8	98.4±1.3
Kidney		97.5±1.2	101.6±0.6	97.1±1.7
Liver		97.2±1.3	99.6±0.8	96.7±1.0
Skeletal Muscle		96.8±0.9	101.0±1.1	97.1±1.1
Smooth Muscle		96.3±1.4	102.1±0.9	95.9±1.5

Recovery (amount analyzed/amount added x 100, of added calcium). See text. Values in body of table are mean ± S. E. of five individual recoveries. A significant difference ($P < 0.05$, Student's t-test) in recovery of calcium from all tissues except serum and bone, was found when the mixed-acid digestion method was compared to the other two methods.

^{a/} Aliquots were taken from 33 percent formic acid digest and diluted 1:50 with one percent lanthanum nitrate solution.

Table 3. Recovery of lead.

Tissue	Lead		
	12 N HNO ₃ digest	12 N HNO ₃ & 35% HClO ₄ digest	35% HClO ₄ digest
Blood	97.5±1.2	101.3±1.0	98.1±1.3
Brain	97.4±1.0	99.5±0.9	96.9±1.1
Hair	98.8±0.8	102.6±1.3	98.1±1.4
Heart	97.1±1.0	100.6±0.7	98.0±0.7
Kidney	98.0±0.7	101.1±0.9	98.4±1.1
Liver	96.8±1.1	99.5±1.0	97.4±0.9
Skeletal Muscle	97.6±0.9	100.9±0.8	97.2±1.2
Smooth Muscle	98.6±0.8	101.6±0.7	98.0±1.0
Bone ^{a/}	101.4±0.9		

Recovery (amount analyzed/amount added x 100, of added lead). See text. Values in body of table are mean ± S. E. of five individual recoveries. The recovery of lead was found to be significantly lower ($P < 0.05$, Student's t-test) in the other two digestion methods when compared to mixed-acid method.

^{a/} Aliquots taken from formic acid digest.

adequate as based on recovery studies (see Table 2 and 3).

Characteristics of Tissue Distribution
of Calcium and Lead in Lead-
Exposed Rats

Temporal Patterns of Whole-Brain Levels of Calcium and Lead. Calcium levels in other previously-mentioned tissues except the brain of lead-exposed rats were not changed over the 7 day time-course. As illustrated in Figures 3 and 4, whole-brain levels of calcium and lead were significantly elevated ($P < 0.05$) in lead-treated rats (10 mg/kg), when compared to their non-treated counterparts. These findings were consistently observed in all lead-exposed groups, *i. e.*, control (C), animals with severed lateral recurrent laryngeal nerves (LRLN), and those which had been thyroparathyroidectomized (TX-PTX). Since the findings from the sham-thyroparathyroidectomized group and the control group were statistically similar, the individual values from each of these groups were combined and reported as control group values.

Brain levels of calcium and lead in all lead-exposed rats were found to be elevated about 70 and 350%, *i. e.*, 4 mEq/kg to 6.8 mEq/kg and 0.25 mg/kg to 1.12 mg/kg respectively within 24 hours after treatment. Over the next 6 days, brain levels of calcium in all lead-exposed rats, regardless of the group, continued to increase; however differences in the rates of increase were noticed. On days 3

Figure 3. Temporal patterns of whole-brain levels of calcium among control (C), severed lateral recurrent-laryngeal nerves (LRLN), and thyroparathyroidectomized rats, following intravenous administration of lead (10 mg/kg) of the ion in the form of lead acetate. Each bar with symbol (I) represents the mean \pm standard error of five animals, except for the control group which represents the mean \pm S.E. of 10 animals. Pb-0 = no lead; Pb-T = lead-treated. The brain levels of calcium in lead-exposed rats were significantly elevated ($P < 0.05$, Student's t-test) when compared to their counterparts which were not exposed to lead. The increase was evident within 24 hours and extended through 7 days.

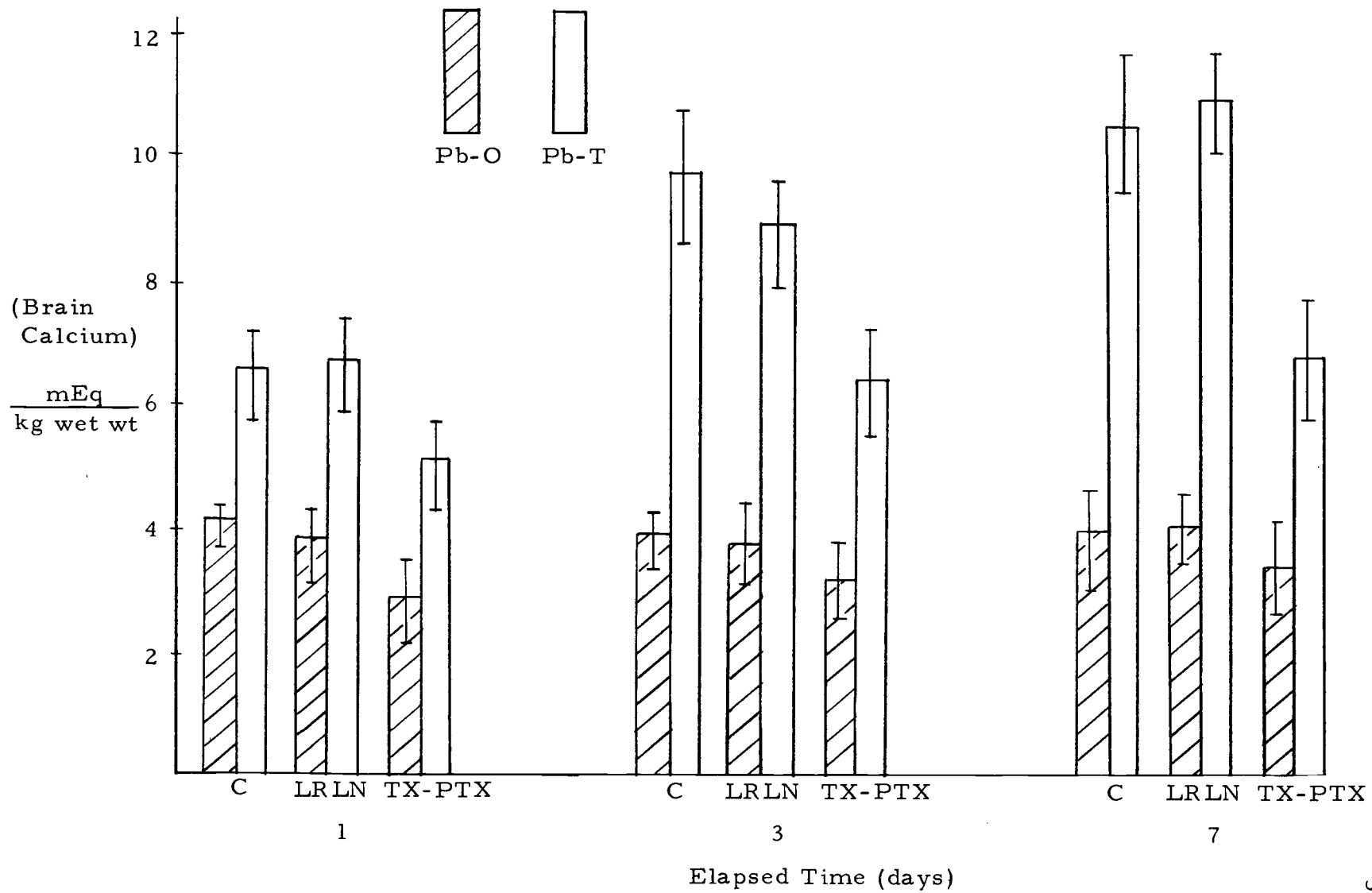
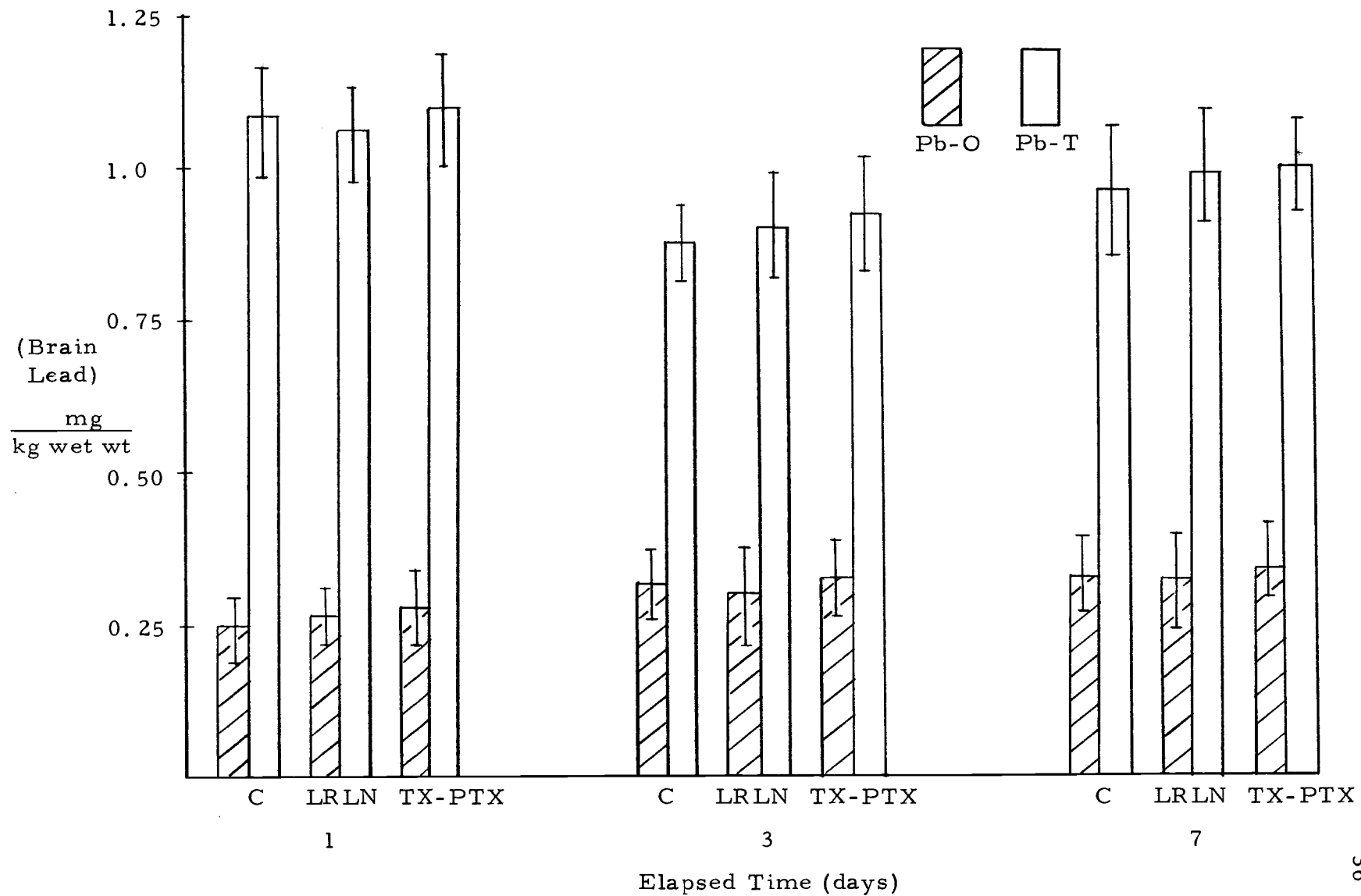


Figure 4. Temporal patterns of whole-brain levels of lead among control (C), severed lateral recurrent-laryngeal nerves (LRLN), and thyroparathyroidectomized rats, following intravenous administration of lead (10 mg/kg) of the ion in the form of lead acetate. Each bar with symbol (I) represents the mean \pm standard error of five animals, except for the control group which represents the mean \pm S. E. of 10 animals. Pb-O = no lead; Pb-T = lead-treated. The brain levels of lead were found to be elevated significantly ($P < 0.05$) in all lead-exposed animals on all sampling dates when compared to their control counterparts. This elevation of brain lead was prominent within 24 hours post-injection and declined slightly over the following 6 days.



and 7, the respective brain levels of calcium in lead-exposed rats of C and LRLN groups were found to be elevated about 135 and 175% (5.5 and 7.0 mEq/kg) more than C and LRLN controls. In comparison, calcium levels of brain tissue in lead-treated TX-PTX rats were increased approximately 90% (3.0 mEq/kg) when compared to their control counterparts on the same days. Moreover, prior surgical extirpation of the thyroid and parathyroid glands resulted in significant ($P < 0.05$) inter-group differences (Figure 3). Calcium levels of brain tissue in lead-treated TX-PTX rats were 30, 47 and 70% (1.6, 2.9 and 4.2 mEq/kg) lower on days 1, 3 and 7, respectively, when compared to levels found in treated rats of the C and LRLN group.

In contrast to behavior of calcium, lead levels of brain tissue in lead-exposed rats gradually declined after day 1 over the subsequent 6 days, *i. e.*, from the initially observed peak value of (1.12 mg/kg) 350% (greater than unexposed rats) to approximately 275% (0.85 mg/kg) on days 3 and 7. In addition, brain levels of lead in lead-exposed rats were not affected upon loss of thyroid-parathyroid functions (Figure 4).

These results strongly indicated that in lead-exposed rats, the thyro-parathyroid complex mediated the effect of lead in altering brain levels of calcium. Also, the brain distribution of lead was not directly dependent on thyro-parathyroid functions.

Calcium levels in the other previously-mentioned tissues of lead-exposed rats, regardless of the group, were not changed significantly ($P > 0.05$) over the same time-course.

Time-Course of Blood, Kidney and Liver Levels of Lead.

Blood, kidney and liver tissues of all groups of lead-exposed rats were found to contain significantly increased concentrations of lead ($P < 0.05$) at 24 and 72 hours (Table 4). By day 3, blood levels of lead in lead-treated animals were observed to decrease about 40% (40 $\mu\text{g}/100\text{ ml}$) from the average value 24 hours after administration of 100 μg per 100 milliliters. A further decrease of 48% (30 $\mu\text{g}/100\text{ ml}$) was noted over the next 4 days. Also, a similar decay rate was typified in kidney and liver tissues. In kidney, the levels of lead were reduced approximately 50% (39 mg/kg) over each time interval from day 1 to day 3 and day 3 to day 7. Liver levels of lead declined approximately 50 and 45% (34.5 and 17 mg/kg) respectively over the same time intervals. In all cases, the levels of lead in these particular tissues decreased to levels similar to those in untreated animals in 7 days.

Dose-Response and Temporal Relations of Brain Calcium and Lead.

Calcium and lead in brains of lead-exposed rats were significantly increased 1, 3 and 7 days after various doses of lead (5, 10, 25, and 50 mg/kg) (Figures 5 and 6). Brain concentrations of lead appeared to be dose related, as expected (Figure 6). In

Table 4. Time-course of blood, kidney and liver levels of lead in control (C), severed lateral recurrent-laryngeal nerves (LRLN) and thyroparathyroidectomized (TX-PTX) rats, following intravenous administration of lead, 10 mg/kg.

Tissue	Treatment ^{a/}	Elapsed time (days)	Groups		
			(10) C	(5) LRLN	(5) TX-PTX
Blood	Pb-O	1	25.2±3.3	27.1±4.2	23.6± 3.8
	Pb-T		105.4±7.8	94.3±8.5	108.6±10.2
	Pb-O	3	24.5±3.2	26.2±4.1	28.1± 3.5
	Pb-T		58.4±5.1	61.6±6.8	64.5± 8.2
	Pb-O	7	28.6±4.1	25.5±5.0	26.7± 4.6
	Pb-T		32.4±3.7	33.6±4.8	34.5± 6.1
Kidney	Pb-O	1	16.3±1.2	15.5±1.0	15.3± 1.5
	Pb-T		74.6±8.0	79.7±8.4	80.6± 9.2
	Pb-O	3	15.9±0.9	16.6±1.3	15.5± 0.7
	Pb-T		38.2±4.5	33.8±5.5	37.9± 6.2
	Pb-O	7	15.1±1.1	15.8±0.8	16.3± 0.8
	Pb-T		17.3±1.6	16.9±1.0	18.3± 1.5
Liver	Pb-O	1	17.5±1.0	18.1±0.7	17.8± 1.3
	Pb-T		66.5±6.4	71.6±5.7	68.3± 6.0
	Pb-O	3	17.9±0.7	17.4±0.5	17.4± 0.8
	Pb-T		31.8±3.5	34.2±4.4	33.5± 4.0
	Pb-O	7	17.3±0.8	17.6±0.7	18.0± 1.0
	Pb-T		19.1±1.1	18.3±0.9	18.7± 0.9

Mean lead content (fluid, microgram per 100 milliliters; solid tissues, milligram per kilogram of wet weight) ± standard error. Figures in parentheses refer to number of animals sampled at each sampling time for each treatment. The respective tissues of lead-treated animals, regardless of the group, exhibited significantly increased levels of lead ($P < 0.05$, Student's t-test) at sampling times of day 1 and day 3; however, elevated tissue levels of lead declined to control values by day 7.

^{a/} Pb-O (0.9% NaCl administered); Pb-T (10 mg/kg of lead administered).

Figure 5. Time-course of brain calcium concentrations in rats after various doses of lead. Each bar with accompanying symbol (I) represents the mean \pm standard error of 20 animals. The brain levels of calcium in lead-exposed rats were significantly elevated ($P < 0.05$, Student's t-test) when compared to untreated animals. A typical dose-response pattern was not evident 24 and 72 hours after administration of lead.

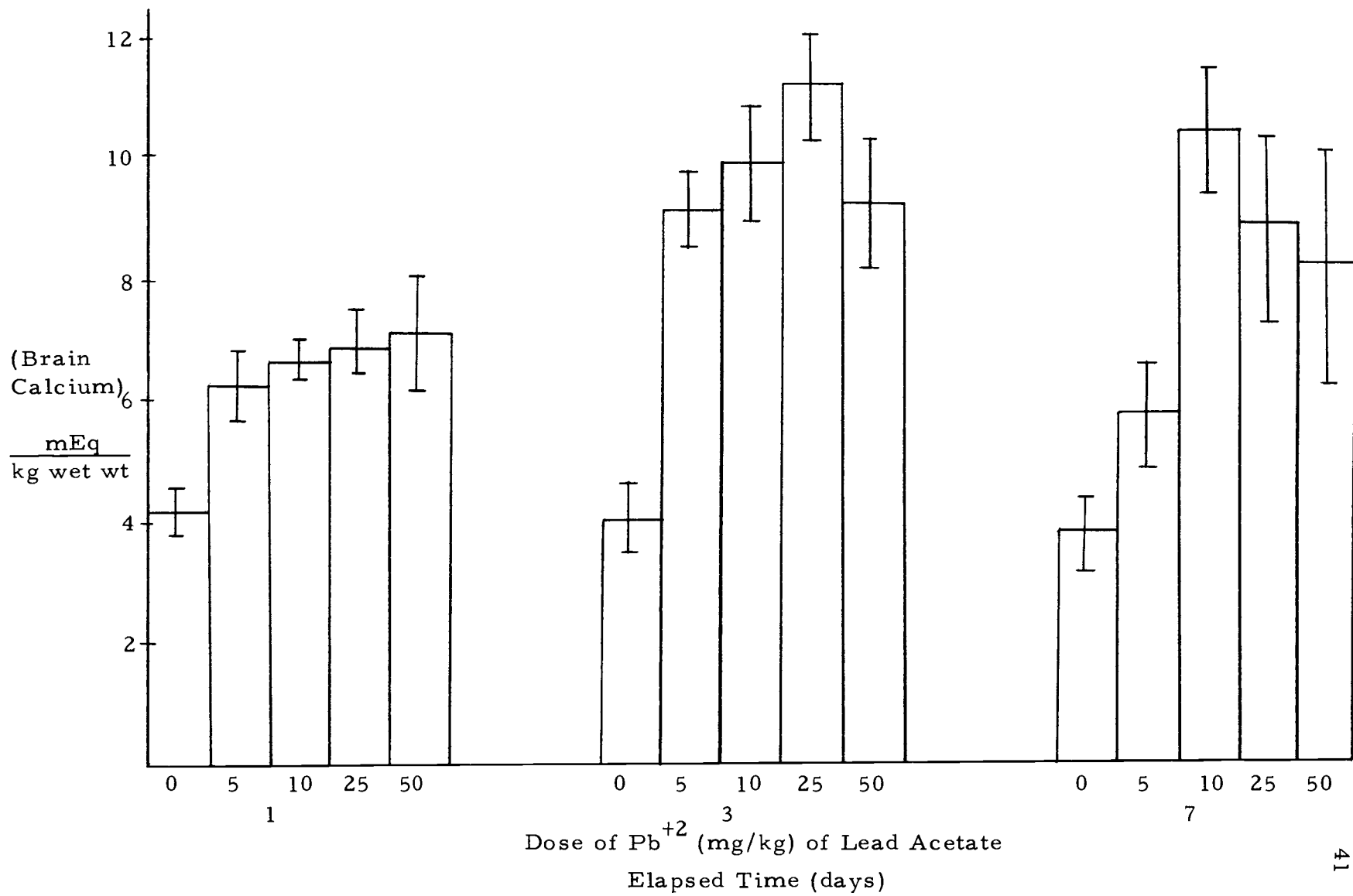
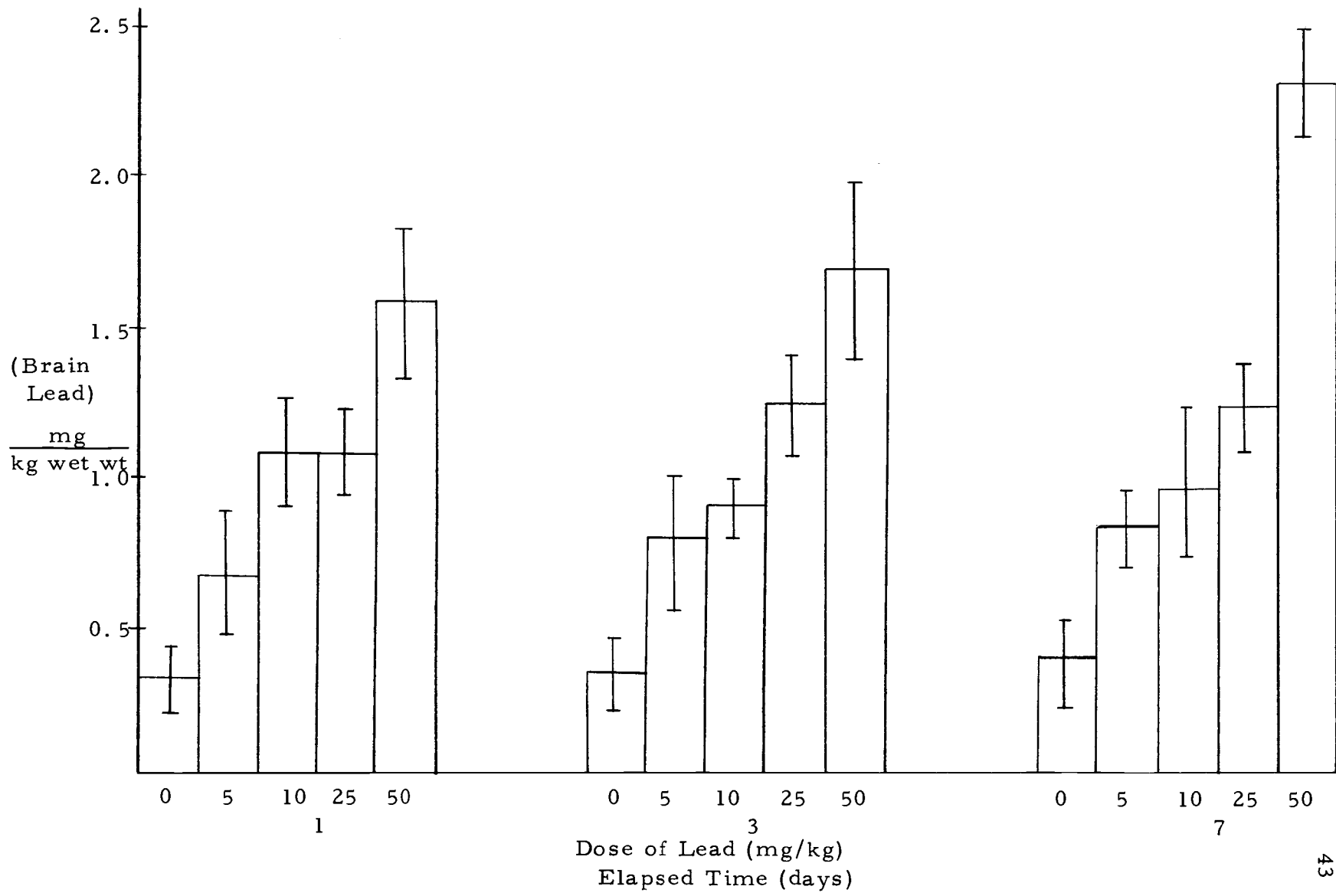


Figure 6. Dose-related and temporal patterns of whole-brain levels of lead in rats following intravenous administration of various doses of lead as the ion in the form of lead acetate. Each bar with symbol (I) represents the mean \pm standard error of 20 animals. Note that a dose relation was evident on each sampling date. Brain levels of lead continued to increase over 7 days for the 5 and 50 mg/kg doses; this effect was not observed for the 10 and 25 mg/kg doses of lead.



contrast, the effect of lead on brain calcium did not follow a typical dose-related pattern over the 7 days after treatment. Brain calcium was altered maximally 24 hours after administration of 5 mg/kg of lead; no significant additional increase of brain calcium was found at higher doses. Brain levels of calcium in lead-treated rats were observed to reach maximal levels within 72 hours. Brain levels of calcium were elevated about two-fold on day 3 by all doses of lead. At day 7, brain calcium in rats at 5 mg/kg of lead was elevated only about 50% (2 mEq/kg) above controls, whereas, the corresponding brain levels of calcium to the higher doses of lead were increased over 100% (4 mEq/kg) compared to controls.

Correlation between time-courses of brain lead and calcium levels was not evident. In comparison, the levels of brain lead associated with the various administered doses of lead appeared to change little over the same time-course. The only exception was observed at 50 mg/kg of lead; at this dose, brain levels of lead continued to increase over 7 days. None of the elevated levels of lead in brain tissue returned to control levels in 7 days.

The response of brain calcium to 50 mg/kg of lead after 3 and 7 days was lower than the response to 10 or 25 mg/kg doses of lead. This seeming inconsistency will be elucidated later.

Dose-Response and Time-Course Relations of Blood, Kidney and Liver Levels of Lead. Lead concentrations of blood in control

animals were approximately 27 $\mu\text{g}/100\text{ ml}$; within 24 hours after administration of 5, 10, 25 and 50 mg/kg of lead, blood lead levels in treated rats were elevated about three, four, five and six times respectively (Table 5). These elevated levels declined about 50% over the next 48 hours. Blood levels of lead decreased to control values by day 7 only in animals which received 5 and 10 mg/kg doses; in the other two groups blood lead was still significantly elevated at 7 days.

Kidney and liver tissues of controls were found to contain approximately 15.8 and 18.4 mg/kg of lead of wet weight, respectively. Within 24 hours relatively high concentrations of lead were demonstrated in kidney and liver tissues in lead-treated rats. Over a time-course of 7 days, these marked tissue levels were observed to decline at a rate comparable to that displayed by the blood. As in blood the levels associated with the 5 and 10 mg/kg doses reached control levels within 7 days and those associated with the 25 and 50 mg/kg doses of lead remained significantly elevated.

Hydration of Brain Tissue. At 3 and 7 days after administration of lead, the 50 mg/kg dose of lead appeared less effective than lower doses in altering brain calcium concentrations in rats (Figure 5). This result appeared inconsistent and required further study. The concentrations of brain lead were characteristic for the three lower doses and remained more or less constant (Figure 6). The

Table 5. Time-course of blood, kidney and liver lead concentrations in rats following various doses of lead acetate.

Tissue	Elapsed time (days)	Dose of Pb ⁺² (mg/kg)				
		0	5	10	25	50
Blood	1	26.8±3.7	78.4±6.3	101.3±7.7	122.9±9.1	153.3±10.4
	3	28.4±3.4	44.7±4.8	54.4±5.6	70.5±7.4	84.7± 8.1
	7	25.5±4.1	31.7±4.3	33.4±3.8	37.6±5.4	41.3± 4.7
Kidney	1	15.8±1.4	50.7±7.4	76.4±9.1	89.2±9.8	109.0±11.4
	3	16.3±1.0	28.1±3.6	35.9±6.2	44.7±6.5	58.4± 8.6
	7	15.3±1.2	18.6±1.6	20.4±3.3	23.1±3.0	26.7± 4.9
Liver	1	18.9±1.4	47.7±5.1	63.7±7.4	78.4±8.3	93.7± 9.1
	3	17.8±0.9	28.1±3.2	32.3±4.4	37.6±5.8	42.2± 5.4
	7	18.0±1.0	19.9±1.7	21.4±2.3	23.5±3.4	27.6± 4.1

Mean lead content (fluid, microgram per 100 milliliters; solid tissues, milligram per kilogram of wet weight) ± standard error of 20 animals sampled at each specific sampling time. All levels of lead exposure significantly elevated tissue levels of lead ($P < 0.05$, Student's t-test) in the respective tissues from day 1 through day 3; however, the increased tissue levels of lead declined to control levels by day 7 for the 5 and 10 mg/kg doses, but remained elevated for the 25 and 50 mg/kg doses.

50 mg/kg dose produced a progressive increase of brain lead over the time-course of 7 days. To determine if tissue hydration was responsible for such results, whole-brain levels of calcium and lead were determined in terms of dry weight (Figures 7 and 8). On this basis whole-brain levels of calcium in lead-exposed rats were less variable and typified a more definitive dose-response pattern (Figure 7). An average value of 37.5 $\mu\text{g/g}$ of dry weight was found for brain calcium levels in control rats. Peak levels of brain calcium were attained in lead-treated rats by day 3, regardless of the magnitude of lead exposure. The brain levels of calcium were found to decline over the subsequent 96 hours, but were still significantly elevated ($P < 0.05$) when compared to control levels. Interestingly, the effects of the three highest doses of lead on brain calcium were not significantly different ($P > 0.05$) on days 3 and 7. A three-fold elevation of brain calcium was demonstrated in rats that were exposed to any one of the three higher doses of lead. The brain lead concentration ($\mu\text{g/g}$ dry weight) was linearly related to the lead dose on all sampling dates subsequent to administration (Figure 8). Brain concentrations of lead were also less variable when presented in terms of dry weight. Control levels of brain lead were found to average about 1.40 $\mu\text{g/g}$. One day following administration, brain lead levels in treated rats were elevated significantly for all doses and continued to increase throughout the observation period. Between

Figure 7. Dose-response and temporal relationships of whole-brain levels of calcium (mg/100 g of dry weight) in rats to various doses of lead in the form of lead acetate. The same data were presented in Figure 5, but in terms of mEq/kg of wet weight. Each bar with symbol (I) represents the mean \pm S. E. of 20 animals. A significant increase ($P < 0.05$) of brain calcium was observed over the time-course of 7 days. The effect was similar in magnitude for the three higher doses of lead on each sampling date in contrast to Figure 5.

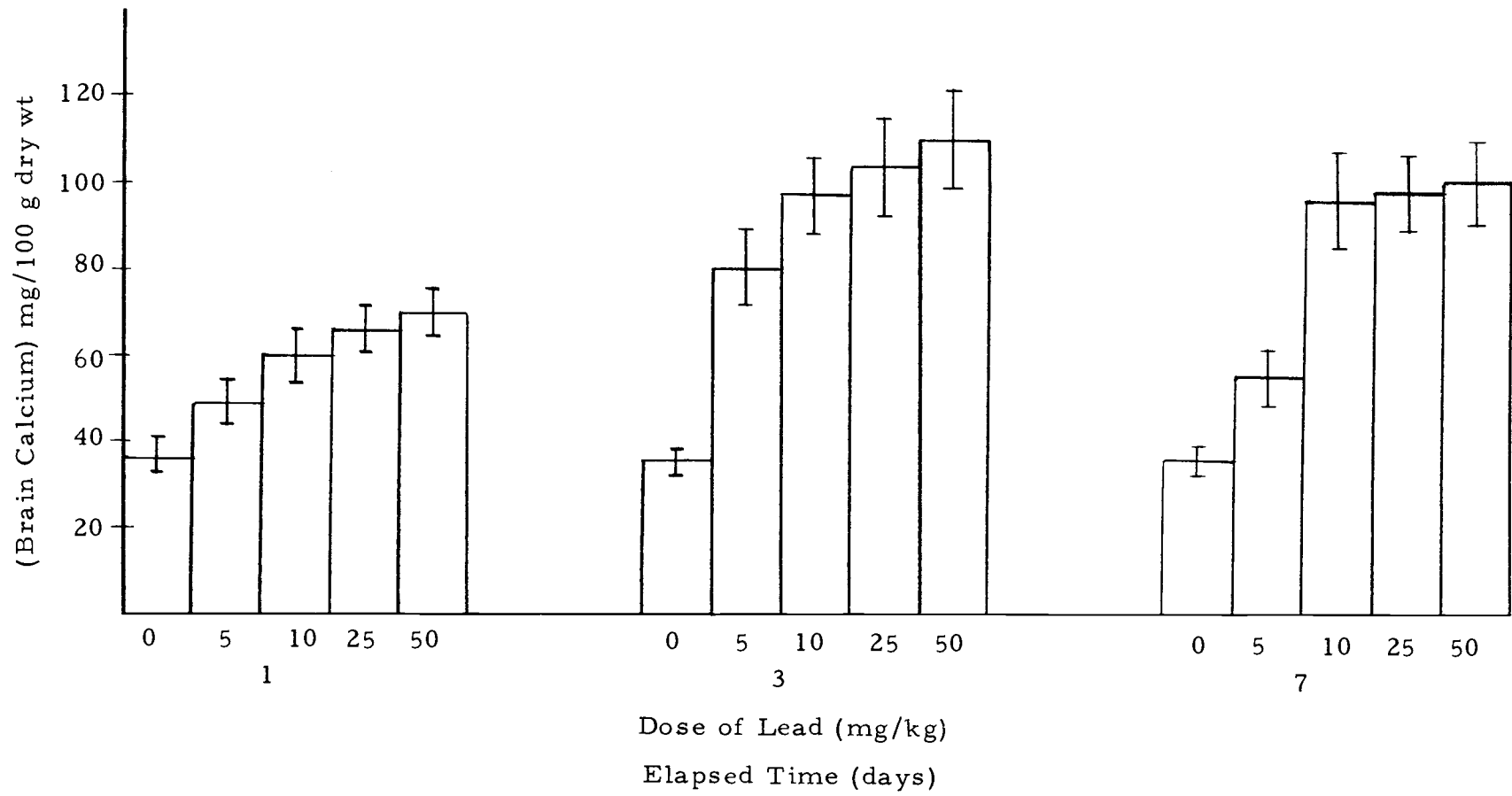
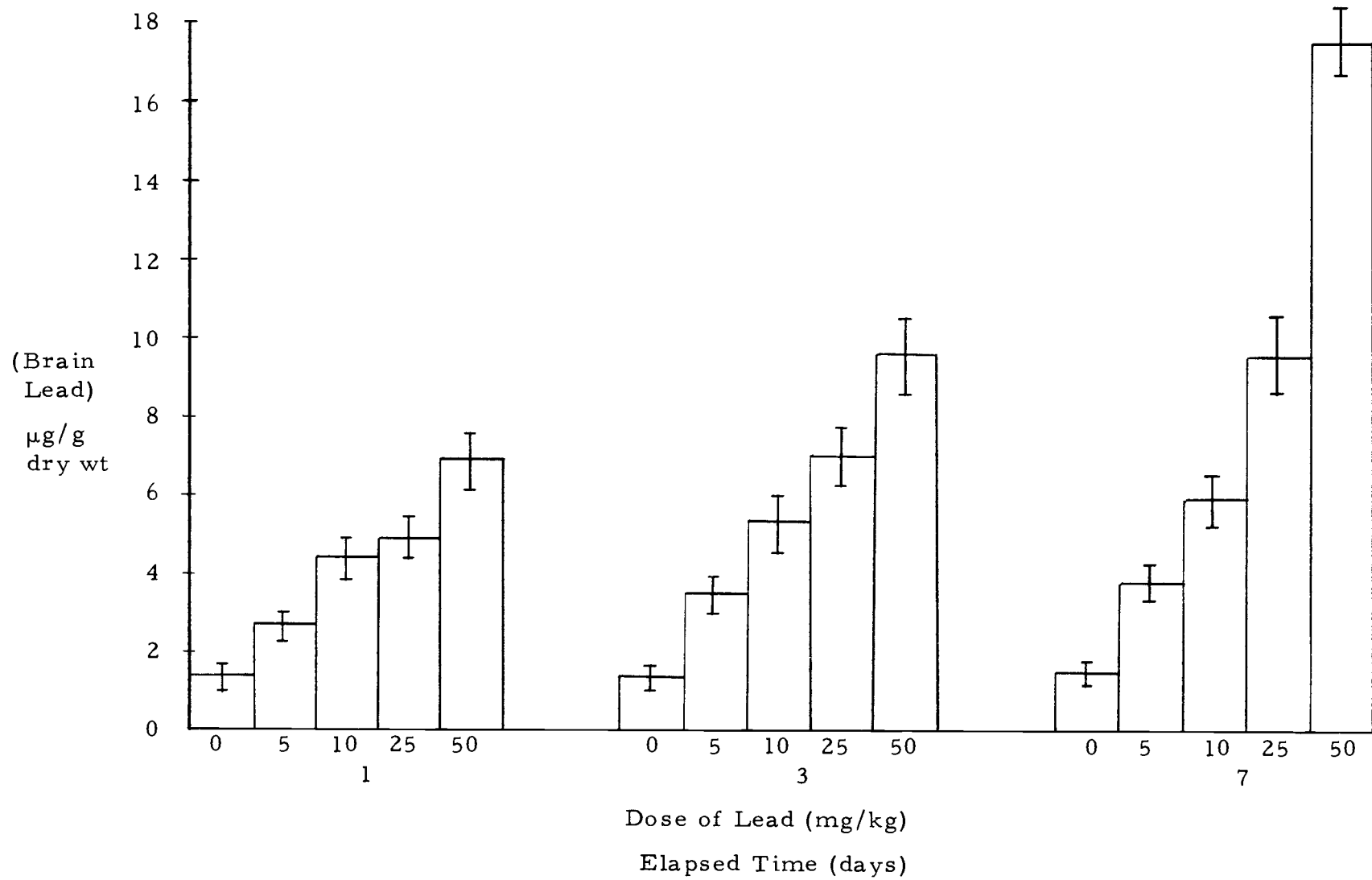


Figure 8. Dose-response and temporal relationships of whole-brain levels of lead, $\mu\text{g/g}$ of dry weight, in rats to various doses of lead in the form of lead acetate. The same data were presented in Figure 6, but in terms of mEq/kg of wet weight. Each bar with the symbol (I) represents the mean \pm S.E. of 20 animals. When presented in terms of dry weight, a typical dose-response pattern was clearly established on each sampling date.



days 3 and 7, brain lead concentrations increased 75% (10 $\mu\text{g/g}$ to 17.5 $\mu\text{g/g}$) in animals treated with 50 mg of lead/kg, and 35% (7.4 $\mu\text{g/g}$ to 10.0 $\mu\text{g/g}$) at the next lower dose. There was no increase in brain concentrations of lead after day 3 in response to the 5 and 10 mg/kg doses.

A subsequent study was made to confirm the results presented in Figures 7 and 8. The data reported in Table 6 corroborated these findings. Other soft tissues were examined to determine if increased tissue hydration was a universal feature among other organs of lead-exposed animals. While a trend for increased water content of kidney and liver tissues from lead-treated rats seemed evident, statistical analysis indicated that treatment caused no significant difference. Thus, brain tissue appeared uniquely predisposed to this action of lead.

Discussion

Methodology

The observed effects of phosphate interference with calcium measurement by atomic absorption spectroscopy are consistent with the findings of Willis (1961) and Fuwa (1971). When phosphate is present in the same chemical environment (aqueous or gaseous) as calcium, it produces a depression in calcium absorbance

Table 6. Hydration of brain tissue in lead-exposed rats.

Element	Wet weight ^{a/} and Dry weight ^{b/}	Elapsed time (days)	Dose of Lead (mg/kg)				
			0	5	10	25	50
Calcium	mEq/kg ^{a/} mg/100 g ^{b/}	1	3.88±0.88 34.15±5.2	6.04± 1.02 53.69± 8.3	6.44± 0.87 66.47±12.9	6.81± 0.42 71.47±13.2	7.15± 1.23 86.81±11.0
	mEq/kg mg/100 g	3	4.12±0.77 40.60±4.0	8.88± 1.14 89.19±14.5	9.42± 1.20 109.15±16.4	11.03± 1.59 120.71±17.0	8.97± 1.81 116.41±12.9
	mEq/kg mg/100 g	7	3.97±0.64 37.32±6.2	5.51± 1.09 59.46±10.3	10.5 ± 1.71 117.66±15.9	9.12± 2.74 119.12±16.5	7.33± 3.31 119.36±16.2
Lead	mg/kg ^{a/} µg/g ^{b/}	1	0.35±0.08 1.49±0.4	0.74± 0.23 2.61± 0.6	1.12± 0.30 4.53± 0.8	1.27± 0.29 5.07± 0.8	1.73± 0.42 7.31± 1.1
	mg/kg µg/g	3	0.24±0.06 1.47±0.3	0.87± 0.33 3.72± 0.8	1.11± 0.27 5.70± 1.2	1.39± 0.44 6.88± 1.2	1.96± 0.51 9.45± 1.6
	mg/kg µg/g	7	0.29±0.05 1.36±0.3	0.94± 0.39 4.11± 0.8	1.19± 0.24 5.92± 1.2	1.30± 0.34 10.01± 1.7	2.62± 0.65 16.81± 2.0

Mean brain concentrations of calcium and lead expressed in terms of wet and dry tissue weight ± standard error of 15 animals. When presented in terms of dry tissue weight (mg/100 g), whole-brain levels of calcium and lead in lead-exposed rats were less variable and typified a more definitive dose-response pattern than the wet weight expressions.

measurements. It is postulated that phosphate forms a complex with calcium, altering its specific light-absorbing nature (Fuwa, 1971).

The effect of phosphate on calcium absorbance, therefore, must be annulled before reliable calcium determinations can be made.

Lanthanum, in a concentration of 1%, appears adequate to suppress this interference in tissue digest, especially for tissues containing less than 100 millimoles per kilogram of phosphate. In the present study, all the tissues analyzed contained less than 100 millimoles per kilogram of phosphate according to values cited in the handbook by Spector (1956).

Tissue digestion by a mixture of equal volumes of 12 N HNO_3 and 35% HClO_4 appears to meet the following established criteria for elementary tissue analyses of calcium and lead: (1) completeness in digestion; (2) an absence of significant interference with atomic absorption spectrophotometric analyses of calcium and lead; (3) little or no compromise with the practical detection limits (sensitivity) when small tissue samples are encountered. Evidence that these criteria were met is represented by the results of the recovery studies. Upon evaluation of recovery data, each acid alone appears to be inadequate in digestion of tissues for subsequent analyses of calcium and lead. The significant improvement achieved with the two acid combination may be due to greater oxidizing action when the acids are combined.

During the last 7 years, atomic absorption spectrophotometry (AAS) has emerged in the United States as a very rapid, specific and sensitive method for the analysis of metallic elements, including lead, in any type of sample that can be put into solution. Using an air-acetylene flame to produce the "atomic cloud" of sample constituents, a sensitivity of 10 $\mu\text{g}/100$ g of whole-blood lead is now being achieved routinely with acid solutions of ashed and unashed blood samples. The accuracy and precision of this method are extremely high for lead, which makes it superior to other methods employed for lead analysis.

Most atomic absorption methods employ chelation by ammonium pyrrolidine dithiocarbamate (APDC) with extraction of the lead-APDC complex into methyl isobutyl ketone (MIBK) and subsequent measurement of the organic layer by AAS (Willis, 1962; Berman, 1964; Hessel, 1968; Farrelly and Pybus, 1969; Cernik, 1970), whereas methods not employing extraction have a very much lower sensitivity (Einarrson and Lindstedt, 1969). Extraction into MIBK yields higher sensitivity by concentration of the chelated metal and by more efficient atomization in the flame; extraction also minimizes the effect of viscosity variations and ionic interferences (Fuwa, 1971). The matrix interferences present in sampling boat techniques are also avoided (Fuwa, 1971).

It appears that the extraction of lead from acid-digested tissue

samples by MIBK is satisfactory for lead determinations. Lead is ultimately concentrated four times by this method when the ratio of 6 ml of diluted supernatant of the acid digest to 1.5 ml of MIBK is used. In the case of blood samples, this method yields a sensitivity of 0.10 μg lead per milliliter per one percent absorption. This indicates that smaller volumes of blood may be used if necessary.

The 217.0 $\text{m}\mu$ line was selected for lead determinations in preference to the one at 283.3 $\text{m}\mu$ because the latter is less sensitive and more subject to interference by flame and light scattering (Hessel, 1968; Cernik, 1970; Fuwa, 1971).

In this investigation, the values recorded for the calcium and water content of the various tissues of the rat agree with those reported in the handbook by Spector (1956). Bradbury et al. (1968), using flame photometry, reported values of 4.54 ± 0.01 and 5.78 ± 0.19 mEq/kg of basal calcium for brain and skeletal muscle of the rat respectively. While the value for brain calcium (Bradbury et al.) is in accordance with the recorded value in this study, the reported calcium value for skeletal muscle (Bradbury et al.) is considerably greater than the observed value in this investigation. This disparity may be attributed to the type of skeletal muscle sampled, for Bradbury et al. (1968) employed muscle from a hind limb and extracted the muscle calcium with 5 N HNO_3 .

Sobel et al. (1940) reported basal lead levels in blood of rats

of 20 $\mu\text{g}\%$ by colorimetric determinations. Other tissue values of lead in the rat determined in this study could not be compared, for an extensive survey of the literature failed to reveal basal tissue levels of lead for the species of Rattus. The results of the recovery studies, however, indicate that the procedures for tissue digestion and the determination of calcium and lead from the tissue digest by atomic absorption spectrophotometry are adequate and reliable.

Biological Effects

The elevation of brain levels of calcium and lead in lead-treated rats may be the consequence of an increase in permeability of the blood-brain barrier to these elements. Such an increased permeability of the blood-brain barrier could result from an indirect and/or direct action of lead on the barrier. One mechanism may center on the production of anoxia, which has been shown to increase the passage of certain substances, from the blood to the cerebrospinal fluid (Lending, Slobody and Mestern, 1961). Furthermore, anoxia may result from intravascular hemolysis, and Beutler (1966), Waldron (1966) and Berk et al. (1970) reported that intravascular hemolysis is a clinical feature associated with acute lead poisoning. This may be the mechanism by which lead increases brain calcium levels in rats; however, there is no evidence offered in the present

study indicating whether intravascular hemolysis occurs in lead-treated rats.

From a direct point of view, it has been shown that lead can produce a variety of effects on membrane functions. Griggs (1964), Hanson and Herberg (1966) and Waldron (1966) found that lead produced the following effects on the erythrocyte in vitro: (1) increased mechanical fragility; (2) increased glucose consumption; (3) increased loss of potassium; (4) decreased sodium and potassium ATPase activity. Selye, Tuchweber and Gabbiani (1962) hypothesized that lead-induced calcinosis is due to extravasation of blood and plasma, resulting from increased vascular permeability. They observed petechial hemorrhages in the affected regions in lead-treated rats prior to calcification and Chisolm (1962, 1968) proposed that vascular injury was the basic lesion responsible for diffuse, severe cerebral edema and elevated intracranial pressure of acute lead encephalopathy in children. The mechanism by which lead produces this direct breakdown of the capillary endothelium remains to be elucidated. Chemical and microscopic examinations of spinal fluid and blood may establish whether lead affects the blood-brain barrier. Recovery studies utilizing barrier indicators such as trypan blue and fluorescein may permit the identification of the sites of abnormal permeability (Davson, 1967). The presence of erythrocytes in cerebrospinal fluid would strongly suggest an increase in

permeability of the blood-brain barrier. Since elevated pressures and protein of the spinal fluid are found to be associated with lesions to the brain, spinal cord or meninges, these parameters should be evaluated in lead-treated rats. The occurrence of intravascular hemolysis can be ascertained by simple hematological determinations (gross microscopic examination of blood, hemoglobin concentration and erythrocyte cell counts). Furthermore, determinations of $p\text{CO}_2$ and pH of cerebrospinal fluid and plasma may establish if acid-base disturbance occurs following lead administration in rats.

From dry-weight determinations, an increase in water content of brain tissue was found to occur only in rats exposed to 25 and 50 mg/kg of lead. This increase in water content indicates that an osmotic imbalance has occurred. Increased hydration would suggest that the brain cells have taken up additional solute to re-establish a new Gibbs-Donnan equilibrium across their membranes; this would have been accompanied by water to maintain isosmolality. In consequence, the amount of calcium per unit weight of wet brain tissue would hardly have changed, because the water content would have increased. The increase of water content in brain tissue of lead-treated rats found in this study appears to be correlated with high blood lead levels. Furthermore, Chisolm and Harrison (1956) and Chisolm (1965, 1968) found a correlation between acute lead encephalopathy and body burden of lead in children; when acute this

condition was found to be associated with blood lead levels in excess of 80 $\mu\text{g}/100$ ml of whole blood. In the present study, comparable blood lead levels were maintained longer in rats that were injected with 25 and 50 mg/kg of lead than in rats treated with 5 and 10 mg/kg of lead. Consequently, the maintenance of high blood lead levels may explain the increased brain hydration which was found only in lead-exposed rats at 25 and 50 mg/kg of lead.

Loss of thyroid and parathyroid functions has no apparent effect on brain levels of lead in lead-treated and control rats; in contrast, thyroparathyroidectomy reduced significantly ($P < 0.05$) elevated brain levels of calcium in lead-exposed rats but not in control animals ($P > 0.05$). These results suggest that the thyro-parathyroid complex mediates only the brain response of elevated calcium levels to lead. This observation finds qualified support in the work reported by Bajusz et al. (1963); they observed that there was greater myocardial calcification produced by experimental lesions in parathyroid-ectomized animals than in rats with intact glands. This increased calcification of soft tissue was not observed in thyroparathyroidectomized rats, whether they were given thyroxine or not. Bajusz et al. postulated that a thyroid principle other than thyroxine was responsible for the greater severity of the lesions in parathyroidectomized rats. Because this study was conducted before the discovery of thyrocalcitonin was published, the possible association of this effect

with thyrocalcitonin was not discussed. In contrast to Bajusz et al. findings, but more pertinent to the present investigations, Gabbiani et al. (1968) and Hirsch and Munson (1969) demonstrated in rats that administered thyrocalcitonin inhibited experimental soft tissue calcification and hypercalcemia induced by lead acetate. This action was attributed to the influence of the polypeptide on calcium and phosphorus blood levels. Later, Gabbiani and Tuchweber (1970) proposed that thyrocalcitonin may exert its inhibitory action on soft tissue calcification, which lead induces, through the polypeptide's influence on the distribution of lead rather than through an alteration of calcium and phosphorus metabolism. Such studies implicate that thyrocalcitonin and not the parathyroid hormone is responsible for modifying soft tissue calcification. Further support for this implication has been offered by Selye, Tuchweber and Gabbiani (1963). They demonstrated that cutaneous calcinosis and osteitis fibrosa was produced by lead acetate in parathyroidectomized rats. Ironically, Selye (1962) found that parathyroid hormone acts as a "sensitizer" in eliciting the phenomenon of calciphylaxis in rats. The results from the present investigation can not champion or discredit any of these findings, for this investigator was unsuccessful in parathyroidectomizing rats, either by cautery or surgical extirpation. Comparative studies in parathyroidectomized, thyroparathyroidectomized and thyroidectomized rats may clarify these

apparently different findings. However, the results from radio-immunologic studies presented in Chapter IV of this thesis show that lead is associated with increased plasma levels of thyrocalcitonin in conscious rabbits following an intravenous infusion of lead.

Because the extrapolation of preliminary findings from one species to another species is scientifically difficult, similar studies as reported in this chapter should be conducted in the rabbit or other large mammalian species to determine if thyrocalcitonin and/or parathyroid hormone mediates the brain response to lead.

In the present investigation, hypercalcemia was not observed in lead-treated rats as mentioned by Gabbiani et al. (1968) and Hirsch and Munson (1969). The failure to observe elevated serum calcium levels could be because differences in treatment protocols exist between the two studies. They administered polymixin, an antibiotic (100 $\mu\text{g}/\text{kg}$, s. c.) with lead (30 mg/kg , i. v.) or lead, two doses at 50 mg/kg , i. v. 5 hours apart. The effects of polymixin on serum calcium regulation is unknown.

Elevated lead levels in brain, kidney and liver tissues on the sampling days of the present study correlate well with the associated blood lead levels. This relationship requires further study, for the amount of residual blood in each of these tissues, following sacrifice, would contribute only a small part to the observed elevated levels of lead.

The results indicate that the brain response of elevated calcium to lead is not altered by severance of the lateral recurrent laryngeal nerves. Severance of these nerves is postulated to release thyrocalcitonin, which produces a transitory decrease in serum calcium levels (Hirsch, Gauthier and Munson, 1963). Therefore, the elapse of time between the severance of the nerves and the determination of serum calcium could explain the failure to observe a brief hypocalcemia. Similarly, this explanation would seem to apply to brain calcium in response to lead; the effects of thyrocalcitonin may have already waned before lead produced its effect on brain calcium. Earlier sampling times of serum and brain tissue in lead-treated rats with severed nerves may reveal the specific effects of this type of surgery on these findings.

III. THE INFLUENCE OF CHRONIC LEAD EXPOSURE ON THE RESPONSE OF NEUROMUSCULAR PREPARATIONS IN SITU

Introduction

Lead palsy or peripheral neuropathy has been attributed to actions of lead on the neuromuscular junction (Cumings, 1959; Lead: Airborne Lead in Perspective, 1972) following chronic lead intoxication. Other reports, however, claim it does not require a prolonged exposure to lead, for subacute rates of lead exposure were found associated with lead palsy in man (Cantarow and Trumper, 1944; Catton et al., 1970).

Clinical signs and symptoms of lead palsy are characterized primarily as muscle weakness, fatigue and atrophy of the extensor muscles of hands and feet; an associated tenderness, severe pain, tremors and eventually paralysis of the involved muscles are found in more advanced cases (Catton et al., 1970). In some cases neuropathic and myopathic features that are associated with lead toxicity are essentially indistinguishable (Lead: Airborne Lead in Perspective, 1972).

The site of the lesion of lead palsy is controversial. Evidence has been offered that lead produces degenerative changes in the motorneurons and their axons (Cantarow and Trumper, 1944).

Impairment of high-energy phosphate metabolism in the muscle itself has been described by Kehoe (1961a). Johnstone (1964) reported that atrophy and contractures were signs sometimes observed in patients suffering from lead palsy. He interpreted these signs as being consistent with neural lesions rather than with interference of muscle phosphocreatine synthesis (Johnstone, 1964).

Prior to 1973, electrophysiologic studies of the effects of lead on neuromuscular systems were lacking. In 1957, Kostial and Vouk demonstrated that lead nitrate, in concentrations as low as 12.1 μM , blocked ganglionic transmission in the perfused superior cervical ganglion of the cat. Recently, Manalis and Cooper (1973) found that lead influenced both the pre and postsynaptic events in neuromuscular transmission, with the presynaptic ones being more sensitive.

Because so little is known about the effects of lead on neuromuscular function, it was considered necessary to obtain information about these effects. Neuromuscular parameters were assessed in situ on the tibialis anterior of chronically lead-exposed rats (300 parts per million in the diet for 8 weeks). The results indicated that lead, when present in the food, prolongs the "active state" of tetanus and increases neuromuscular transmission or responsiveness at the junctional sites in rats.

Methods

Animals

Male Sprague-Dawley albino rats were used exclusively in this study. At the initiation of the study, the subjects had a mean body weight of 172 g (range 130-200 g). The animals were housed in stainless steel cages (five per cage).

Dietary Treatment Regime

The rats were randomized into five separate groups consisting of the following: (1) a control group which received Purina Rat Chow[®] and tap water, (2) a lead-water group which received Purina Rat Chow[®] and doubly-distilled water containing lead at 300 parts per million (ppm) in the form of lead acetate (Mallinckrodt Chemical Works, St. Louis, Mo.), (3) a lead-food group which received Purina Rat Chow[®] containing 300 ppm of lead and tap water, (4) a lead-water lead-food group which were fed 300 ppm of lead in water and in Purina Rat Chow[®], (5) a reconstituted Purina Rat Chow[®] group which were fed Purina Rat Chow[®] that had been imbibed with doubly-distilled water and later dried to its former unaltered weight; they also received tap water for drinking purposes. All groups of animals received their respective dietary regime ad libitum for a period of

8 weeks, at the end of which the neuromuscular experiments were carried out. Dietary intake and body weight were monitored during the study.

Procedure

Cannulation. The rats were anesthetized with sodium pentobarbital 50 mg/kg, i. p. Subsequent to induction of anesthesia, the neck and right leg of the subject were shaved. The animal was secured to a surgical board and a ventral midline incision was made from the cephalic border of the sternum and extended about 3 cm anteriorly. The incision was deepened by blunt dissection of the underlying subcutaneous connective tissue until the sternohyoid and sternomastoid muscles were visible. The internal jugular vein was isolated and cannulated with PE-10 polyethylene tubing for drug administration. The trachea was exposed by dividing the sternohyoid muscle longitudinally; subsequently, the trachea was isolated and cannulated with PE-140 polyethylene tubing. The tracheal cannula was tied firmly in place with silk thread. On the same side as the cannulated internal jugular vein, the common carotid was isolated and cannulated with PE-50 polyethylene tubing for continuous blood pressure monitoring with a Dynograph[®] recorder.

Tibialis Anterior Preparation. The animal was placed in a recumbent position and a small patch of skin was excised from the

lateral portion of the shaved leg just above the knee and parallel to the femur. By blunt dissection, the common peroneal nerve was located and isolated. A ligature was placed on the common peroneal nerve; all exposed nerves closely associated with the common peroneal nerve were excised to prevent central influence on the preparation. The nerve stump and adjacent structures were covered with mineral oil. The skin over the top of the ankle was removed. The tendon from the tibialis anterior muscle was located and isolated. After a tie of silk thread was placed on the tendon, the tendon was severed at the distal end, folded-over and securely tied several times. The foot was ligated from the leg and amputated.

The lower ends of the tibia and fibula of the amputated foot were attached securely to a rigid metal framework by a clamp specifically designed for this purpose. The knee was flexed such that direction of muscle pull was about 5° from the horizontal plane. The thread tied to the tendon of insertion of the tibialis anterior was attached to an isometric strain gauge. The length of tendon between the muscle and tendon tied to the strain gauge was kept to a minimum to insure that the long anterior tibial tendon did not distort the tension output. A counterweight of 15 g was applied to the strain gauge. The same amount of tension was applied to all preparations by adjustment of the strain gauge.

All animals were administered 300 U/kg of sodium heparin

(Eli Lilly and Co., Indianapolis, Ind.) i. v. to maintain patency of the arterial cannula which was used in monitoring blood pressure. The heparin solution was preserved with 1% benzyl alcohol and contained NaCl and NaOH for isotonicity and pH adjustment, respectively. The animals temperature was maintained with a lamp or a heating pad.

Stimulation of Preparation. The tibialis anterior was stimulated through the peripheral nerves by platinum electrodes. Supramaximal, square wave pulses were generated by a stimulator. In the refractory period determinations, the pulses were generated by two stimulators that were connected in series. Stimulus-isolation units were employed to reduce the electrical stimulus artifacts.

Parameters and Protocol. The following neuromuscular parameters were studied to characterize the effects of lead on the neuromuscular system: (1) response characteristics of muscle twitch and tetanus; (2) response to increasing frequencies of stimulation (follow-through frequencies); (3) response to the delay between two pulses (refractory period determination); (4) response to prolonged tetanizing frequencies of stimulation (fatigue evaluation factor); (5) response to drugs.

The following protocol was applied to each preparation: (1) muscle twitches followed by tetanus (100 pulses/sec for 10 sec); (2) maximal duration of tetanus (100 pulses/sec); (3) follow-through frequency; (4) muscle twitches followed by tetanus; (5) follow-through

frequency; (6) delay between two pulses; (7) muscle twitches followed by tetanus; (8) follow-through frequency; (9) maximal duration of tetanus; (10) delay between two pulses; (11) muscle twitches followed by tetanus; (12) follow-through frequency; (13) response to the anticholinesterase drug, Eserine ^(R), 200 $\mu\text{g}/\text{kg}$, i. v.; (14) response to sodium pentobarbital, 50 mg/kg, i. v. (administered terminally). Before initiating each succeeding step of the protocol, the reference lines of the muscle twitch and/or the blood pressure were returned to the original recording level by readjusting the strain gauge.

Tissue Sampling and Analytical Determinations. Three milliliters of whole blood were removed (2 ml for blood lead and 1 ml for serum calcium determinations) via the carotid cannula. The blood was withdrawn at a rate of about 1 ml per minute, after completion of Step 10 in protocol. Immediately after blood withdrawal, an equal volume of 0.9% NaCl solution was administered through the venous cannula to replace the blood volume. Usually the original blood pressure was restored within 15 minutes following blood sampling.

Upon sacrifice of the animal with sodium pentobarbital, the tibialis anterior and the brain were removed and processed with the blood as described in Chapter II. The tissue levels of calcium and lead were determined as previously described.

Results

Influence of Dietary Lead on Various Biological Parameters

Effect of Dietary Lead on Weight Gain, Food and Water

Consumption. The animals readily consumed the experimental diets and showed weight gains over the 8 week period (Table 7). Rates of food and water consumption did not differ significantly ($P > 0.05$) between the various groups of rats. All groups ingested about the same volume of water daily except the reconstituted-food group. The dried food furnished to this group appeared more crumbly when compared to the rations of the other groups. It was assumed that this apparently different texture of the food was responsible for the increased water consumption recorded for this group. A small reduction in daily weight gains for groups supplied with lead-food was noted which appeared to be correlated with the food intake. The combined effect of lead-water and lead-food did not depress body weight gains significantly below that shown for other dietary regimes.

Effect of Dietary Lead on Whole-Blood Levels of Lead and

Serum Calcium. Whole-blood concentrations of lead were significantly elevated ($P < 0.05$) in all animals exposed to dietary lead (Table 7). Lead-food and the combined effect of lead-water and lead-food significantly increased ($P < 0.05$) whole-blood levels of

Table 7. Influence of dietary lead on various biological parameters.

	Animal groups				
	Control	Regular food and lead-water	Lead-food and regular water	Lead-food and lead-water	Reconstituted food and regular water
Number of animals	18	18	18	10	18
Daily food intake ^{a/}	24.4 ±3	25.2 ±4	21.1 ±4	22.6 ±3	25.5 ±4
Daily water intake	32.5 ±6	30.3 ±7	31.9 ±6	30.4 ±5	35.6 ±7
Daily weight gains/group	4.8 ±2.4	4.9 ±2.7	4.5 ±2.2	4.2 ±2.4	4.6 ±2.8
Brain calcium	36.22±4.7	39.81±3.8	41.35±3.2	41.69±4.8	38.44±3.3
Brain lead	1.42±0.2	1.50±0.3	1.58±0.5	1.66±0.6	1.45±0.5
Muscle calcium	23.91±3.4	24.73±4.1	26.19±3.8	28.09±4.3	24.11±3.6
Muscle lead	13.70±1.6	16.12±1.8	22.4 ±3.3*	25.87±3.6*	14.2 ±2.2
Serum calcium	4.94±0.2	5.05±0.1	5.12±0.09	5.24±0.22	4.97±0.18
Whole-blood lead	28.3 ±4.8	37.2 ±5.2*	47.7 ±5.5*	55.1 ±6.6*	26.7 ±5.0

Mean calcium content (serum, milliequivalents per liter; solid tissues, milligrams per 100 grams of dry weight) ± standard error. Mean lead content (whole-blood, micrograms per 100 milliliters; solid tissues, microgram per gram of dry weight) ± standard error. Mean daily food intake (grams/animal ± S.E.). Daily water intake (milliliters/animal ± S.E.). Mean daily weight gains per group (grams/day ± S.E.).

^{a/} For 20 monitoring periods (food and water intake, weight change per group).

* (P < 0.05) when compared to controls and reconstituted groups.

lead (10.5 and 17.9 $\mu\text{g}/100\text{ ml}$, respectively) above the levels in the lead-water group. No apparent difference in the fragility of the erythrocytes was observed in the blood among the different groups of rats, for blood samples of treated rats did not show a greater degree of hemolysis.

Serum calcium levels of the lead-exposed groups of animals were slightly higher than levels of untreated animals; however, the true significance of this observation is unknown, since the serum proteins were not determined.

Effect of Lead on Brain and Muscle Concentrations of Calcium and Lead. Interestingly, while a trend for increased brain levels of calcium and lead in chronically lead-exposed rats was evident, statistical analysis indicated that treatment caused no significant difference. This was in contrast to the increased brain levels of calcium observed with an acute dose of lead (Chapter II). Muscle levels of calcium followed a similar trend; however, lead-levels in tibialis anterior of lead-food groups were increased significantly ($P < 0.05$) above the muscle levels in the other three groups. This difference was not anticipated for the lead-water group, for the rate of lead exposure was greater in this group of rats than in the lead-food groups.

No difference in behavior was noted among the various groups of rats during the time-course of the study, even though significant

differences in the body burden of lead were found among the groups of rats.

Influence of Dietary Lead on Neuromuscular Parameters

Muscle Twitch/Tetanus. The ratio of maximal isometric twitch and tetanus amplitudes has been reported to be about 0.2 - 0.3 in most limb muscles of adult animals at 35 - 37°C (Close, 1972). In the present study similar values were found in control, lead-water and reconstituted-food groups (Table 8). The ratio of muscle twitch to tetanus was significantly ($P < 0.05$) less in the lead-food groups when compared to the other groups. Interestingly, the exposure rate was greater in the lead-water group than in the lead-food group (Table 7), because the rats ingested more water than food daily (in terms of grams).

Follow-Through Frequency. Follow-through frequency was defined by the present investigator as the minimal stimulating frequency necessary to convert the muscle twitch to a tetanic response. More specifically, it was the frequency which did not produce a standardized displacement greater than 2 mm at the peak amplitude attained by the recorder stylus during the stimulating frequency.

As illustrated in Table 8, the tibialis anterior of the lead-food groups tetanized at frequencies significantly lower than the

Table 8. Influence of dietary lead on neuromuscular parameters (300 ppm x 8 weeks).

Group	Number of observations	Muscle twitch tetanus	Follow-through frequency (pulses/second)	Absolute refractory period (milliseconds)	Relative refractory period (milliseconds)
Control	72	0.20±0.07	32±2	45±4	90±5
Lead-water	72	0.18±0.08	30±3	48±5	90±5
Lead-food	72	0.15±0.07*	25±2*	52±5	100±5
Lead-water and lead-food	40	0.15±0.06*	24±2*	52±5	100±5
Reconstituted food	72	0.21±0.08	31±3	48±4	90±5

Mean values ± standard error. Absolute values are given for number of observations.

* (P < 0.05) when compared to control and reconstituted groups.

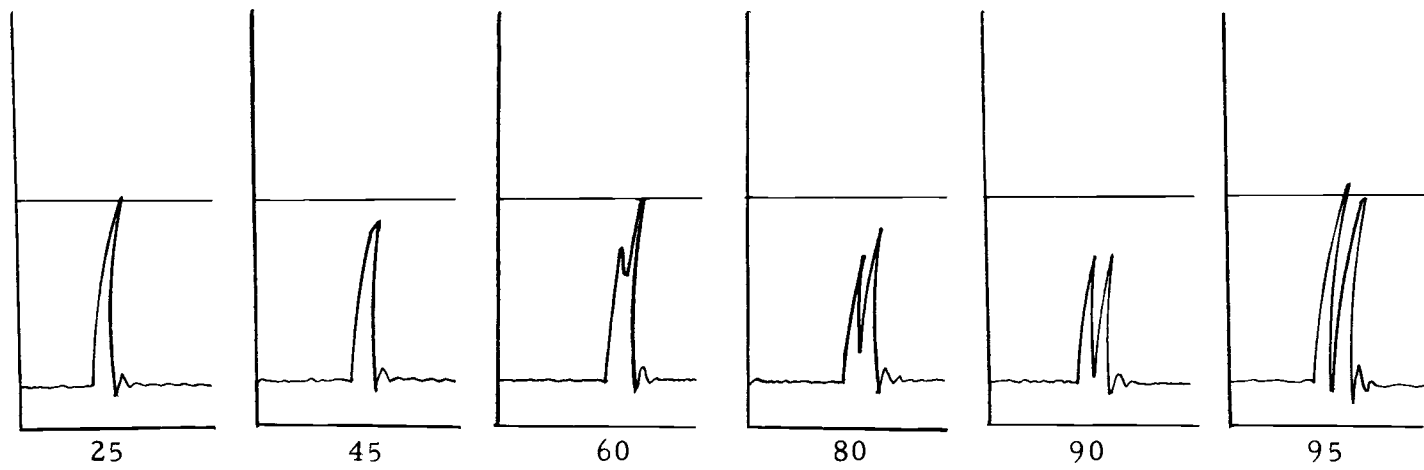
preparation of the other groups. Only small differences in the magnitude of this parameter were found in preparations from control, lead-water and reconstituted-food animals. In general, the variations associated with the determinations of this parameter were small.

Refractory Period. In the present study, the absolute and the relative refractory periods of the tibialis anterior were determined by applying two similar pulses with variable delay between them. If the two pulses were separated by a delay of 45 to 50 milliseconds, the relative refractory period was elicited (Figure 9). A longer duration between the two pulses allowed full expression of a second contraction. Lead did not change these parameters significantly ($P > 0.05$) (Table 8), when compared to the other experimental diets; however, a trend was shown that lead-food diets had a greater effect on both the absolute and relative refractory periods than lead-water in chronically lead-exposed rats.

Discussion

It is recognized that chronic lead exposure affects various biological parameters, e. g., hematopoiesis, myologic, renal and neurologic (Lead:Airborne Lead in Perspective, 1972). According to Six and Goyer (1970), 200 $\mu\text{g}/\text{ml}$ (ppm) of lead in the drinking water is the maximum dose of lead that the rat can tolerate for a

Figure 9. The effects of variable delay between two stimulating pulses on the response of muscle twitch in the rat. The relative refractory period became evident as the delay between two similar stimulating pulses was 45 milliseconds in duration.



Delay (milliseconds)

period of 10 weeks without producing significant alterations in hemato-
topoiesis or renal size, histology and function when accompanied by
0.9% dietary calcium. The rats at a lead-exposure rate of 300 parts
per million for 8 weeks in the diet of the present study did not exhibit
any overt signs of lead toxicity. Furthermore, the results of the
present study show disparity to the findings of Six and Goyer (1970);
they reported a mean blood lead concentration of 10 $\mu\text{g}\%$ in control
rats and a significantly elevated serum calcium in lead-treated rats.
This could be due to differences in analytical methods in estimating
these two parameters. "An automated technique" was employed for
the analysis of calcium, while whole-blood lead was determined in
non-extracted samples by atomic absorption spectrophotometry.

Results of the present study indicate that equal concentrations of
lead in water and food differ widely in influencing various biological
parameters. This would suggest that lead in the drinking water is
not absorbed to the same extent as lead in food. Some unknown
influence on the availability of lead for intestinal absorption when
present in the water may account for this interesting observation.
Solubility and precipitation of lead acetate in the drinking water do not
appear to be factors influencing this phenomenon, for lead acetate
is readily soluble in water after the water is slightly acidified with
acetic acid. Kehoe (1961c) reported that the motor activity of the
bowel is the major determinant for the absorption of inorganic lead

from the G.I. tract; for lead is mainly absorbed from the small intestine, to a lesser extent from the colon, and not at all from the stomach. But the difference of motor activity of the bowel between the lead-water group as compared to the lead-food groups was not noteworthy. Dietary balance studies of lead in rats may offer some explanation for the phenomenon; however, such results may be difficult to interpret if lead undergoes enterohepatic circulation.

The elevated brain and muscle levels of calcium and lead in the lead-food groups may result from the action of thyrocalcitonin in body redistribution of lead and calcium. The elevated serum calcium and/or increased blood levels of lead may stimulate secretion of thyrocalcitonin. Thus, both blood lead and calcium could be redistributed and deposited to soft tissues such as brain and muscle.

The reduced muscle twitch/tetanus ratio in rats of the lead-food groups correlates well with the elevated muscle and blood levels of lead and calcium. An enhancement in the prolongation of the active state of contraction in tetanus would account for this reduced ratio. The relationship of the amplitude of muscle twitch and tetanus has been recognized as an index of the active state of contraction (Davson and Eggleton, 1962). Hill (1949) defined the active state of contraction as a sudden change in the mechanical properties of the muscle. The active state of twitch is acknowledged to decay sooner than that of tetanus. It is well known that calcium ions within the contractile

cell is related to the activation of the contractile process, i.e., the process of excitation-contraction coupling. Increased calcium influx into the sarcoplasmic reticulum would allow an increase in the active state of contraction and result in a reduced twitch/tetanus ratio. Alternately, a reduced ratio would occur if in a muscle fiber, the extent of activation was less than the average or in which calcium ion was removed more rapidly; the twitch of this fiber would be faster than average if other factors were equal; however, this mechanism seems unlikely because muscle and blood concentrations of calcium were elevated. This mechanism could be revealed by determination of the relationship between twitch time to peak and twitch/tetanus ratio. According to Bagust et al. (1973) one of the possible mechanisms controlled by the motor axons is the duration of the active state. It may be that lead affects only the motor nerves but not the contractile machinery, and consequently influences the activation mechanisms and thus produces differences in twitch/tetanus ratios.

The follow-through frequency data indicate that the tibialis anterior in rats of the lead-food groups tetanized at frequencies significantly lower when compared to similar data of the other groups of rats in the study. These results would suggest an increase in neuromuscular transmission or responsiveness at the junctional sites. Again, increased calcium contents of the muscle and/or blood

in these rats may account for this finding, since calcium ions are associated with the liberation of the transmitter (Davson, 1970). Also, Davson reported that an increase in calcium ions increases the amount of acetylcholine release per impulse. The results of the present study are not in accordance with Kostial and Vouk (1957), for they reported that lead nitrate as low as 12.1 μM reduced significantly the amount of acetylcholine released from the stimulated preganglionic fiber of the superior cervical ganglion of the cat. It should be pointed out that lead nitrate may differ from lead acetate in its actions in vivo, and that a cat ganglion is dissimilar to the neuromuscular system of the rat in many respects. Thus, a valid comparison between the two studies may not be tenable.

The investigations of Manalis and Cooper (1973) suggest that lead chloride increases the frequency of the miniature endplate potential but depresses the phasic release of acetylcholine on the in vitro isolated sciatic nerve-sartorius muscle preparation of the frog. An increase in the frequency of the miniature endplate potential by lead would facilitate the responsiveness of the junctional membrane to the phasic release of acetylcholine by a nerve impulse or lead to the growth of muscle action potentials. These mechanisms would increase the contraction of the muscle, i. e., the time from the beginning of the action potential to the point of maximal tension by decreasing the latent period of the muscle action potential. Many

differences between the present study and Manalis and Cooper's are recognized: (1) in vitro vs in vivo; (2) non-mammalian vs mammalian species; (3) lead chloride vs lead acetate; (4) lead-exposure, in a bath vs dietary. Therefore, the relevancy of the Manalis and Cooper (1973) study to the mammalian neuromuscular system awaits further clarification.

IV. THE INFLUENCE OF INORGANIC LEAD ON SERUM LEVELS OF THYROCALCITONIN IN RABBITS

Introduction

The manner in which the calcium-regulatory hormones mediate the metabolism of inorganic lead has received attention but is poorly understood. Selye, Tuchweber and Gabbiani (1963) demonstrated that cutaneous calcinosis and osteitis fibrosa induced by lead acetate could be produced successfully in parathyroidectomized rats. Evidence that thyrocalcitonin influences lead metabolism was presented by Gabbiani et al. (1968) and Hirsch and Munson (1969). Both groups of investigators showed that experimental soft tissue calcification provoked by lead acetate in rats was inhibited by thyrocalcitonin. In addition, Rosen (1972) found that blood levels of lead in lead-intoxicated rats were reduced significantly by exogenous thyrocalcitonin. The hypotheses advanced by these investigators to explain their findings did not share a common mechanism.

The effects of inorganic lead on serum levels of thyrocalcitonin and parathyroid hormone are unknown. Establishing the nature of lead's influence at this level of calcium-regulation is essential for understanding the apparent relations that exist between lead and calcium metabolism. Further elucidation of the clinical significance and mechanism of these effects are needed for the management of

clinical lead intoxication and for comprehensive medical and environmental control programs.

Immunological methods have been used previously to assay various polypeptides (Unger et al., 1961; Utiger, 1965), hormones (Utiger, 1965; Berson and Yalow, 1959; Yalow et al., 1964; Utiger et al., 1962) and drugs (Smith et al., 1969; Spector and Parker, 1970; Spector and Flynn, 1971). These radioimmunoassays can be performed with a small sample of biological fluid and without the need of extraction. The present investigation was undertaken to develop such immunoassays for thyrocalcitonin and parathyroid hormone. Antibodies specific for thyrocalcitonin were elicited by immunizing guinea pigs with bovine thyrocalcitonin; these antibodies were capable of detecting the presence of nanogram quantities of thyrocalcitonin. With this sensitive and specific radioimmunoassay, the circulatory levels of thyrocalcitonin in the sera of rabbits were measured, before and after infusion of lead acetate.

Methods

Materials

The following materials (with their suppliers) were used in the study: crystalline bovine thyrocalcitonin (100 mu/mg), bovine parathyroid hormone (832 u/mg), Freund's complete

adjuvant-Perrin's modification (Calbiochem, San Diego, Cal.); rabbit anti-guinea pig gamma globulin (Nutritional Biochemical Corp., Cleveland, Ohio); polyacrylamide gel, P-4 and P-10 (Bio-Rad Lab., Richmond, Cal.); crystalline bovine albumin (Fraction V) and sodium metabisulfite (J. T. Baker Chem. Co., Phillipsburg, N. J.); chloramine-T (Sargent-Welch Scientific Co., Anaheim, Cal.); sodium ethylmercurithiosalicylate (Aldrich Chemical Co. Inc., Milwaukee, Wis.); ^{125}I -sodium iodide (New England Nuclear, Boston, Mass.). The phosphate-buffered saline (PBS) solution used was 0.01M phosphate, pH 7.5, and 0.15M NaCl with 0.01% sodium ethylmercurithiosalicylate.

Immunization Procedure

Each guinea pig was injected with 750 μg of thyrocalcitonin or 500 μg of parathyroid hormone. The immunogen (750 μg or 500 μg) in 0.01M phosphate-buffered saline, pH 7.5 was emulsified with an equal volume of complete Freund's adjuvant. A 1.5 ml dose was injected subcutaneously into multiple sites on the shaved dorsum of the animal. The injections were made four times a week for 4 weeks and then once a week for 24 weeks. The animals injected with parathyroid hormone were treated for a total of 12 weeks. Blood was collected from the animals by cardiac puncture 5 to 7 days after immunization beginning with the sixth week, and was tested for

existing antibody titers of the injected hormones.

Radioiodination of Thyrocalcitonin
or Parathyroid Hormone

Thyrocalcitonin and parathyroid hormone were radioiodinated by a modification of the method of Hunter and Greenwood (1962), *i. e.* ^{125}I was used instead of ^{131}I . Usually, 5 μg samples of the respective hormones were iodinated by 0.5 mCi of ^{125}I . Oxidation of (^{125}I)-sodium iodide for the labelling of the hormone was accomplished by reacting 40 μg of chloramine-T with the hormone and radionuclide mixture for exactly 2 minutes. The oxidation reaction was arrested upon the addition of excess sodium metabisulfite (125 μg).

Separation of Iodinated Hormone From
Remaining Free (^{125}I)-Iodide

Immediately following the last iodination step, the reaction mixture was applied to a polyacrylamide gel column (P-4 for thyrocalcitonin and P-10 for parathyroid hormone) which had been hydrated with 0.05M sodium phosphate buffer, pH 7.5 for 24 hours. The dimensions of the column were: 15 cm long and 0.9 cm inside diameter. Elution of the iodinated hormone mixture from the column was accomplished by 0.05 M phosphate buffer. One milliliter fraction of eluant was collected in 1 ml of 5% bovine serum albumin solution. The flow rate of the column was approximately 200 $\mu\text{l}/\text{min}$.

After 18 of the 1 ml fractions were collected, the albumin-containing fractions were mixed with a vortex mixer and 5 μ l aliquots from each fraction were dispensed into clean, dry culture tubes for counting purposes. A Packard Auto-Gamma[®] spectrometer was used to determine the amount of radioactivity of each fraction. By plotting the activity of each fraction, the degree of labelling of the hormone was ascertained. The fractions which contained high amounts of labelled hormone were stored at 4^oC until use.

Assay for Binding Capacity

Initially, a crude estimation of the antibody titer for anti-thyrocalcitonin or anti-parathyroid hormone was made to screen the antisera. This was done by incubating various concentrations of antisera with a 0.001% non-labelled hormone solution at 37^oC for 2 hours and evaluating macroscopically the amount of precipitation of each dilution. Antisera which demonstrated precipitation at high dilutions were selected for the binding capacity assay.

The main objective of the binding capacity assay was to ascertain the binding capacity of the labelled hormone from the various gel filtration fractions that had been collected in the radioiodination experiments. Consequently, this assay determines if the labelled hormone in a given fraction is satisfactory for the conduction of a radioimmunoassay. In addition, the binding capacity assay enables

determination of the optimal dilutions of antiserum and second antibody that should be used in the radioimmunoassay.

A typical incubation mixture for the assay contained the following: 100 μ l of a diluted ^{125}I -thyrocalcitonin fraction sufficient to provide 20,000 counts per minute; 200 μ l of guinea pig antisera; 500 μ l of 1% bovine serum albumin-phosphate buffered saline (BSA-PBS). The antisera were first diluted 1:400 with 0.05M ethylenediaminetetraacetic acid - phosphate buffered saline, pH 7.5 (EDTA-PBS); further dilutions were made with 1:400 normal guinea pig serum (NGPS). The reaction mixture was agitated by a vortex mixer and subsequently incubated at 4°C for 24 hours.

A double antibody precipitation method, similar to that of Morgan and Lazarow (1963), was used to separate the antibody-bound hormone from unbound hormone. Gamma globulins are the major precipitating antibodies in serum; in radioimmunoassays, the labelled hormone is found associated with this fraction of serum. Thus, an anti-gamma globulin can be used to precipitate the labelled hormone-gamma globulin complex, and consequently separate the labelled-bound hormone from the labelled-unbound hormone.

After the 24 hours incubation period, 200 μ l of rabbit anti-guinea pig gamma globulin (RAGPGG) at various dilutions (1:60, 1:80, 1:100) were added to the reaction tubes. By using various dilutions of the RAGPGG, the optimal concentration of the RAGPGG

was determined for precipitation of ^{125}I -thyrocalcitonin-gamma globulin complex. Again, the contents of the tubes were mixed and incubated for 72 hours at 4°C . Then, 3 ml of phosphate buffered saline (PBS) were added to all tubes except the total count tubes (only labelled hormone was added to total count tubes) to wash the precipitate and further dilute the unbound ^{125}I -thyrocalcitonin in the reaction tubes. The supernatant was decanted following centrifugation, and the precipitate at the bottom was counted for radioactivity.

Background radioactivity and radioactivity due to nonspecific protein binding were determined by omitting the 200 μl samples of various dilutions of antiserum from the assay tubes. If the omitted antiserum was replaced by 200 μl of 1:400 NGPS, the nonspecific protein binding was determined. The amount of radioactivity from background and nonspecific protein binding (in counts per minute) was subtracted from all the tubes which contained antiserum. Each determination (background, nonspecific protein binding and degree of binding for each antiserum dilution) was done in duplicate. The fraction of labelled thyrocalcitonin and the optimal dilutions of antiserum and RAGPGG that were associated with the highest degree of specific binding of labelled thyrocalcitonin were used in the radioimmunoassay. A 25% binding of the labelled hormone is considered adequate for most radioimmunoassays (Dr. L. Swanson, personal communication).

Radioimmunoassay

The radioimmunoassay for thyrocalcitonin depends upon a competition between unlabelled thyrocalcitonin and a standard of labelled thyrocalcitonin for combination with specific antibody binding sites. A standard curve was established by adding known concentrations of unlabelled thyrocalcitonin to a constant incubation mixture containing ^{125}I -thyrocalcitonin and antiserum. Unknown quantities were determined by comparison with the standard curve.

The incubation mixture for the radioimmunoassay of the endogenous thyrocalcitonin of rabbits contained the following: guinea pig antiserum to bovine thyrocalcitonin (200 ml, 1:800 dilution) was added to all tubes except background and nonspecific protein binding determination tubes; ^{125}I -thyrocalcitonin, (100 μl with approximately 20,000 counts per minute) was added to all tubes; sera from rabbits that had been infused with lead (2.5 and 10 mg/ml) or CaCl_2 (50 mg/ml) or EDTA (50 mg/ml) and which contained unknown amounts of thyrocalcitonin were diluted 1:2 or 1:5 with 1% PBS, and 500 μl of each mixture was added to different tubes.

To prepare a standard curve, 500 μl of unlabelled thyrocalcitonin in 1% PBS and in a concentration range of 0.75 to 600 ng/ml, was assayed in place of unknown serum.

The background and nonspecific protein binding were

determined as described in Binding Capacity Assay. This amount of radioactivity was subtracted from all other tubes to establish net counts per minute for each sample. To determine the maximum binding of ^{125}I -thyrocalcitonin for antibody binding sites, the above assay was carried out with the unlabelled thyrocalcitonin omitted. The contents of the tubes were mixed and subsequently incubated at 4°C for 24 hours. Following the elapsed time of 72 hours, the double antibody method was used as described in Binding Capacity Assay to separate the labelled-bound thyrocalcitonin from unbound ^{125}I -thyrocalcitonin. A dilution of 1:60 was used for the RAGPGG. The washing of the precipitate and the counting were done as described above.

Treatment of Rabbits

Rabbits were chosen as an experimental animal in which to study the effects of lead on endogenous thyrocalcitonin levels because of the ease in obtaining repetitive arterial blood samples and in maintaining an intravenous infusion in the conscious animal for prolonged periods. New Zealand white rabbits were restrained in a box designed for this purpose; a 23-gauge needle was placed in a marginal ear vein and a polyethylene cannula (inside diameter 0.023 inches) was threaded into the central ear artery. Heparin (Sodium heparin, 300 U/kg i. v.) was administered to maintain patency

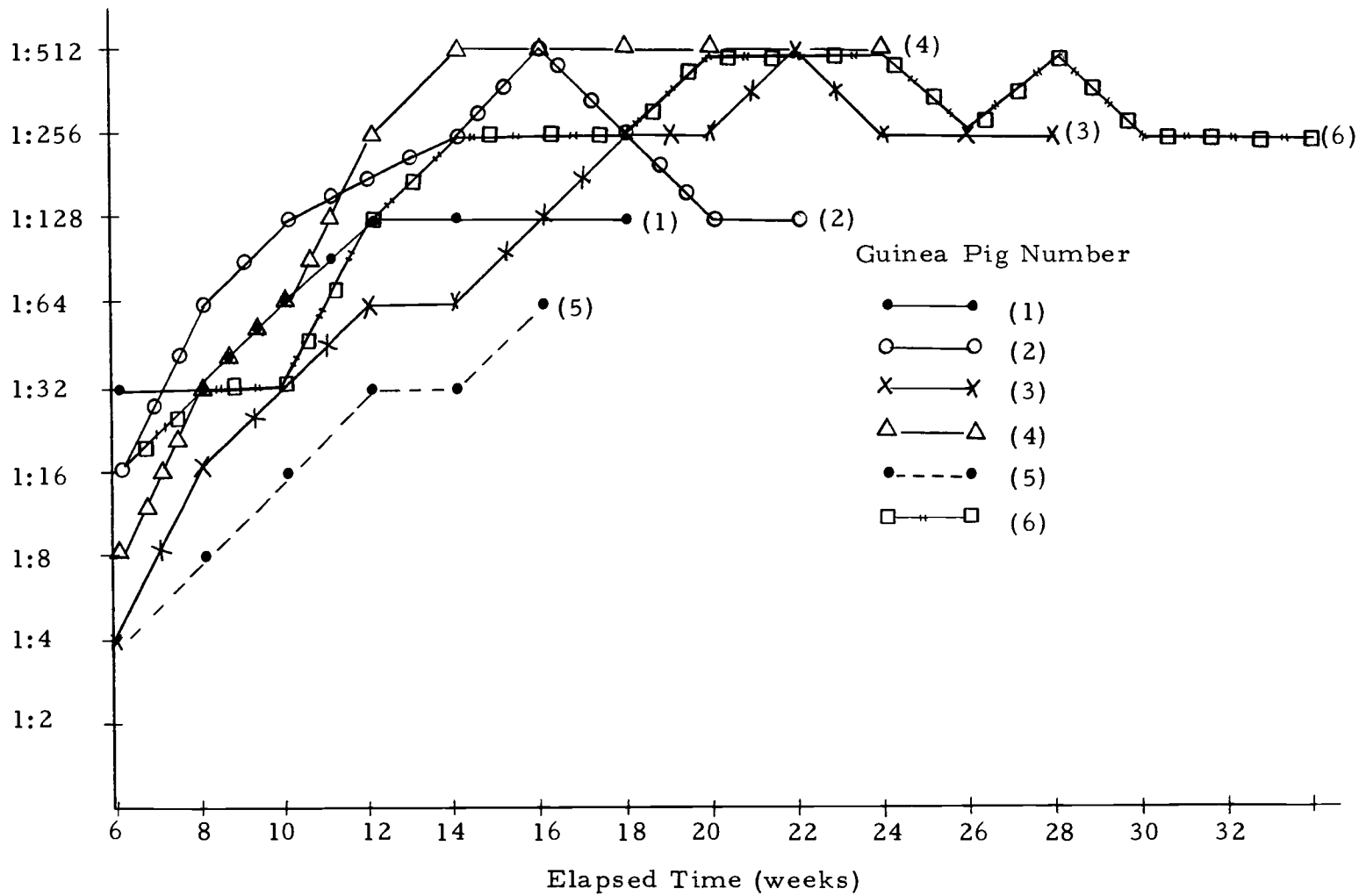
of the cannula and needle. Blood samples were withdrawn through the arterial cannula; infusions of dextrose, calcium, EDTA and lead were performed through the marginal ear vein at a rate of 6 ml per hour by an infusion pump. Each experiment consisted of a 30-minute control period (infusion of 5% dextrose in water) followed by a 60 minute infusion period of the following solutions: CaCl_2 , 50 mg/ml (120 mg/kg/hr); lead in the form of lead acetate, 2.5 and 10 mg/ml (6 and 24 mg/kg/hr); EDTA, 50 mg/ml (120 mg/kg/hr). Each experiment was completed by a 30 minute infusion period of 5% dextrose in water. Seven normal and two thyroidectomized rabbits were studied. Calcium and lead were determined in serum and whole blood, respectively, as described in Chapter II.

Results

Antibody Production During the Course of Immunization

As illustrated in Figure 10, guinea pigs immunized with bovine thyrocalcitonin at repeated intervals produced measurable levels of anti-thyrocalcitonin when tested by a precipitation method. All animals responded to the antigenic hormone but differences were noted regarding the rate and intensity of antibody production. Titers of the antibody rose sharply during the first 4 months and plateaued shortly thereafter; subsequent administrations of the antigen did not

Figure 10. Quantification by a precipitation method of antibody titers of sera from six guinea pigs immunized with bovine thyrocalcitonin over the course of several months. A dose of 750 μg /animal in complete Freund's adjuvant was administered at each injection time as described in the text. Sera were collected 5 to 7 days subsequent to antigen administration. Sera from animals coded #3, #4 and #6 were used for specific binding studies.



increase the antibody titers, and the titers of antibody actually decreased in guinea pigs numbered 2, 3 and 6. Animals identified as #1 and #5 died relatively early in the course of immunization. The sera from guinea pigs coded #3, #4 and #6 were selected and subsequently stored at -20°C for specific binding studies of radioactively labelled thyrocalcitonin.

A low antibody titer against bovine parathyroid hormone was demonstrated in the sera of treated guinea pigs; the animals received repeated injections of the hormone (500 μg /injection) in complete Freund's adjuvant for 12 weeks; however, results from specific binding experiments of radioactively labelled parathyroid hormone were negative.

Abscesses developed on some animals treated with thyrocalcitonin and parathyroid hormone during the course of immunization; the lesions did not respond to topically administered antibiotics. Cultures from the abscesses were negative upon bacteriologic examination. Furthermore, moderate to severe nephrocalcinosis was revealed by necropsy in animals treated with parathyroid hormone.

Radioiodination of Parathyroid Hormone and Thyrocalcitonin

The results of radioiodination of bovine parathyroid hormone and thyrocalcitonin are shown in Figures 11, 12, 13 and 14. The smaller peak in each of these Figures represents the radioactivity

Figure 11. Radioiodination of bovine parathyroid hormone. Five micrograms of the hormone were reacted with 0.5 mCi ^{125}I and 40 μg of chloramine-T for 2 minutes. The labelled hormone was separated from free ^{125}I on a P-10 polyacrylamide gel column. The iodination efficiency was 40%.

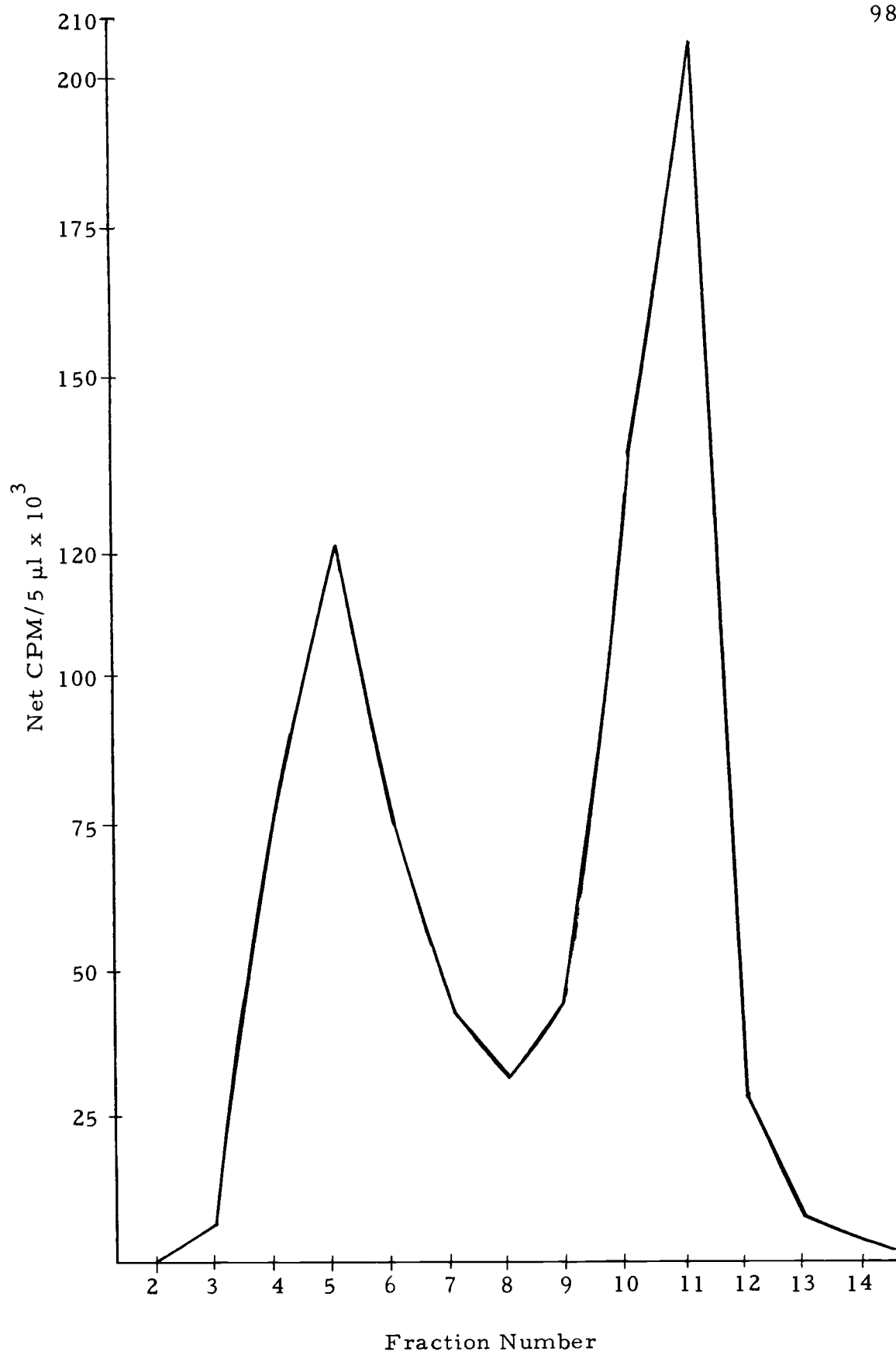


Figure 12. Radioiodination of bovine thyrocalcitonin. Five micrograms of the hormone were reacted with 0.5 mCi ^{125}I and 40 μg of chloramine-T for 2 minutes. The labelled hormone was separated from free ^{125}I on a P-4 polyacrylamide gel column. The iodination efficiency was 16%.

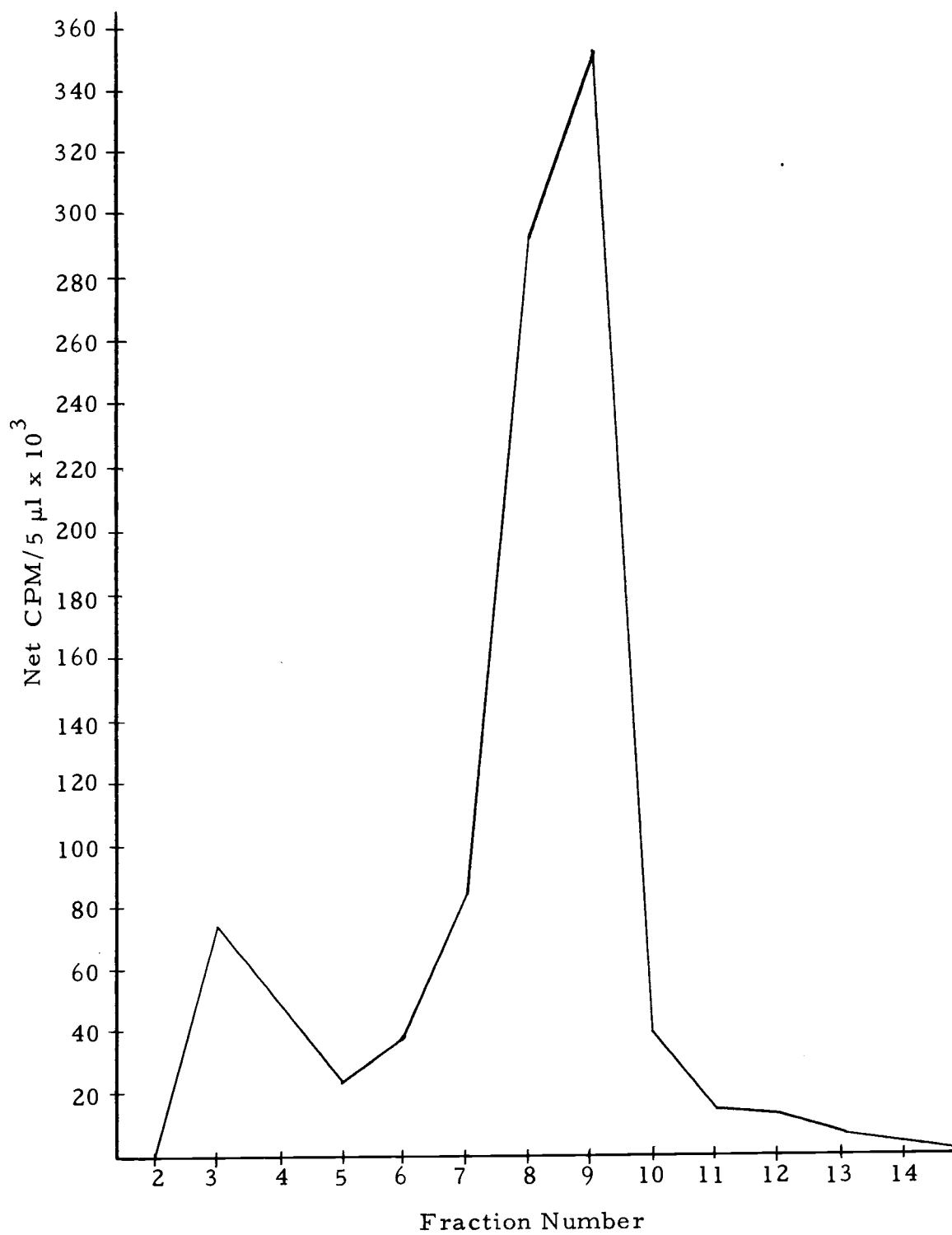


Figure 13. Radioiodination of bovine thyrocalcitonin. Ten micrograms of the hormone were reacted with 0.5 mCi ^{125}I and 40 μg of chloramine-T for 2 minutes. The labelled hormone was separated from free ^{125}I on a P-4 polyacrylamide gel column. Iodination efficiency was more than 40%.

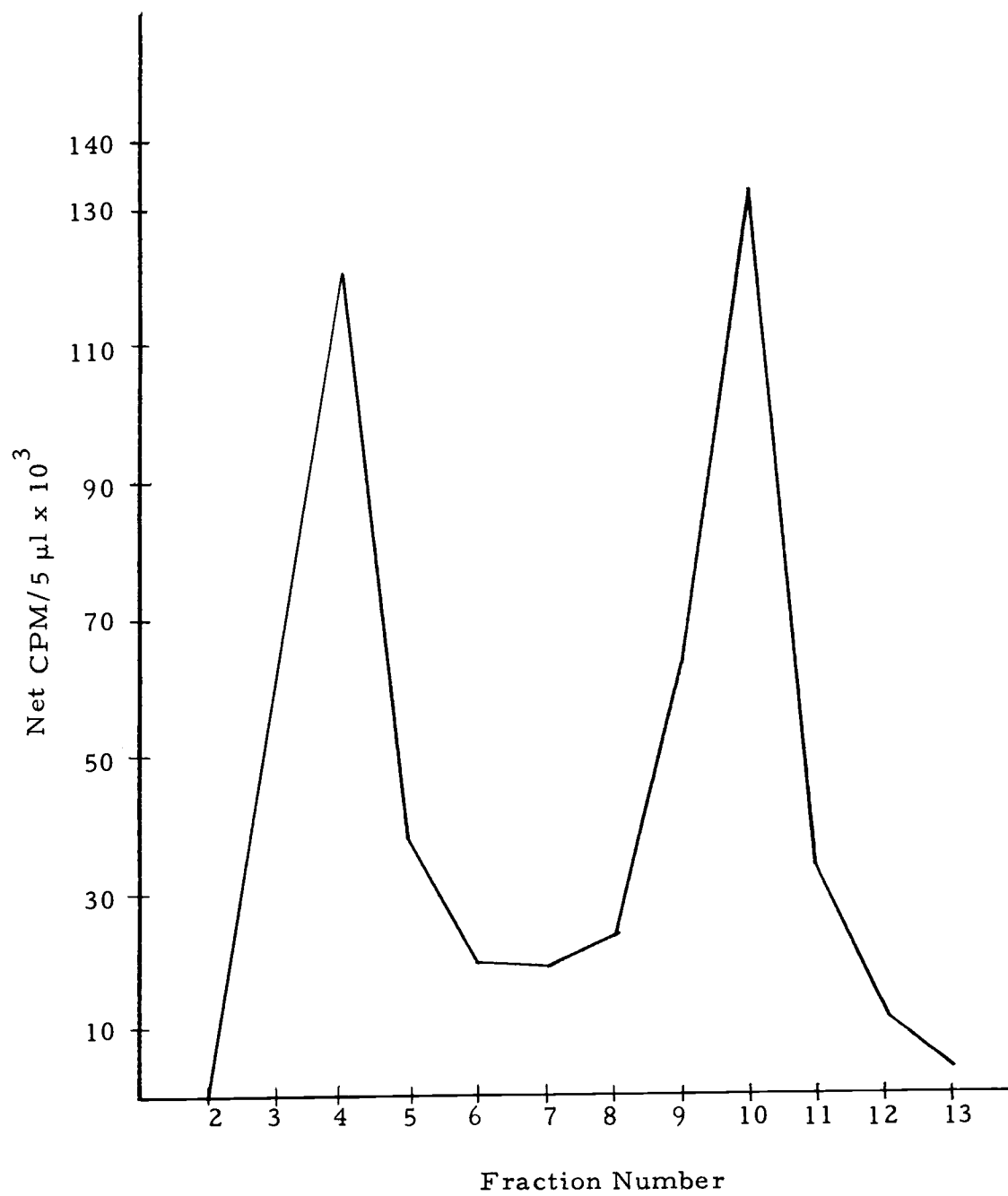
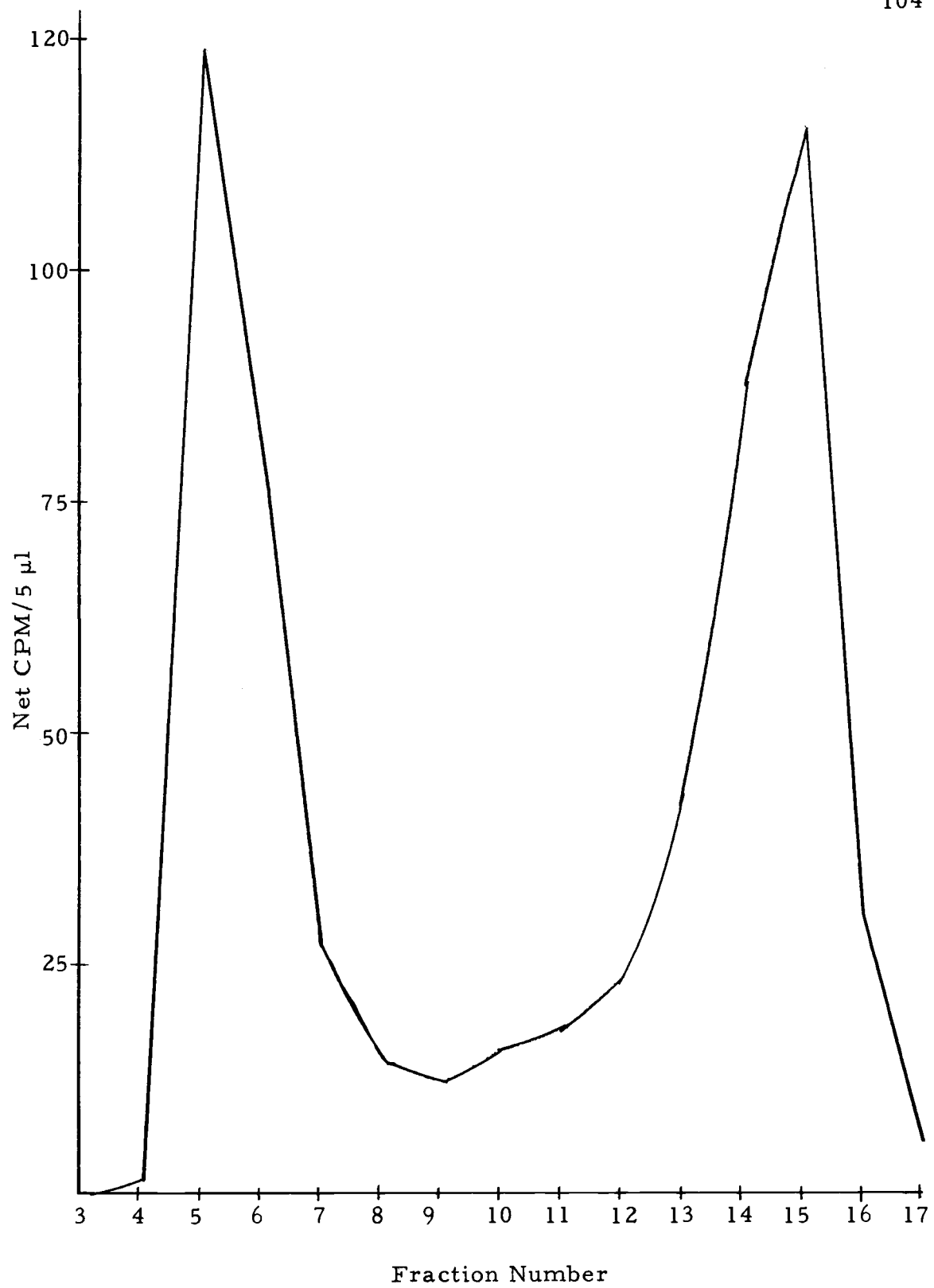


Figure 14. Radioiodination of bovine thyrocalcitonin. Twenty micrograms of the hormone were reacted with 0.5 mCi of ^{125}I and 40 μg of chloramine-T for 2 minutes. The labelled hormone was separated from free ^{125}I on a P-4 polyacrylamide gel column. An iodination efficiency of 40% was achieved.



of the labelled hormone, whereas free ^{125}I is associated with the latter peak.

In Figure 11, the results of iodination of bovine parathyroid hormone are shown; the reaction mixture consisted of 5 μg of hormone and 0.5 mCi of ^{125}I in the presence of 40 μg of chloramine-T. The reaction was allowed to proceed for exactly 2 minutes before excess sodium metabisulfite was added. Approximately 40% of the added radioactivity was found in the smaller peak. This was considered a satisfactory iodination, from the standpoint of specific activity; however, the results of binding capacity assays of the labelled hormone on sera from animals injected repeatedly with parathyroid hormone, over 12 weeks, indicated inadequate binding with fractions #4, #5 and #6 of this iodination. Since subsequent experiments produced similar results, the decision was made to discontinue further radioimmunologic studies on the parathyroid hormone and to proceed with experiments using thyrocalcitonin.

Under similar conditions as described for the radioiodination of parathyroid hormone, only 16% of the total activity of the iodination was found in the labelled hormone peak for thyrocalcitonin (Figure 12). Fraction #3 was saved and tested later in a binding assay. The labelled hormone was relatively stable when stored at 4°C for periods up to a month; however, because of the possible radiation damage to the hormone, the labelled hormone was not

used for radioimmunoassay purposes more than a month after iodination.

Experimental conditions were varied in subsequent radio-iodinations of thyrocalcitonin to increase the amount of labelled hormone. In Figures 13 and 14 are shown the results when 10 and 20 μg , respectively, of bovine thyrocalcitonin were reacted with 0.5 mCi of ^{125}I and 40 μg of chloramine-T for 2 minutes. In each radio-iodination, an efficiency of 40% or more was accomplished. This was a marked improvement in iodination efficiency when compared to the previous iodination of 5 μg of thyrocalcitonin. The fractions that were associated with high radioactivity of the ^{125}I -thyrocalcitonin were subsequently tested for binding by immunized sera.

Binding Capacity of ^{125}I -Thyrocalcitonin

Fractions of the labelled hormone peak from various radio-iodinations of thyrocalcitonin were tested for binding capacity by sera from guinea pigs immunized with bovine thyrocalcitonin. Sera from guinea pigs coded #4 and #6 were used exclusively, since preliminary binding experiments showed that the binding by serum from guinea #3 was less satisfactory than sera from animals coded #4 and #6.

The results of the binding capacity assay on fraction #3 from

the initial radioiodination of thyrocalcitonin are presented in Figure 15. Three dilutions of the sera and second antibody were tested to determine the optimal dilutions for subsequent binding assays.

The corresponding dilutions of 1:8000 and 1:60 of the sera and second antibody were found optimal for the binding of ^{125}I -thyrocalcitonin in this experiment. The findings strongly indicated that antibody in the sera was "recognized" by the labelled hormone and lower dilutions of the sera were needed to increase the binding of ^{125}I -thyrocalcitonin.

Subsequent binding capacity assays confirmed this need, demonstrating the binding of ^{125}I -thyrocalcitonin by the sera was enhanced when lower dilutions of the sera were used in conjunction with higher specific activity of the labelled hormone fractions from other radioiodinations (Figure 16 and 17). The dilution of 1:800 of the sera was found to be optimal for the binding of labelled thyrocalcitonin when testing the various fractions. In addition, the fraction of the labelled hormone associated with the zenith of the graph gave the highest binding of ^{125}I -thyrocalcitonin when compared with the ascending and descending limb fractions. The serum from guinea pig #4 was selected for radioimmunoassay experiments, since it was associated with the highest binding of ^{125}I -thyrocalcitonin. The results from these two binding assays clearly indicated that the

Figure 15. Binding capacity of ^{125}I -thyrocalcitonin by sera from guinea pigs immunized with bovine thyrocalcitonin. Fraction #3 of the initial radioiodination of thyrocalcitonin was used to bind the antibody from sera of guinea pigs coded #4 and #6.

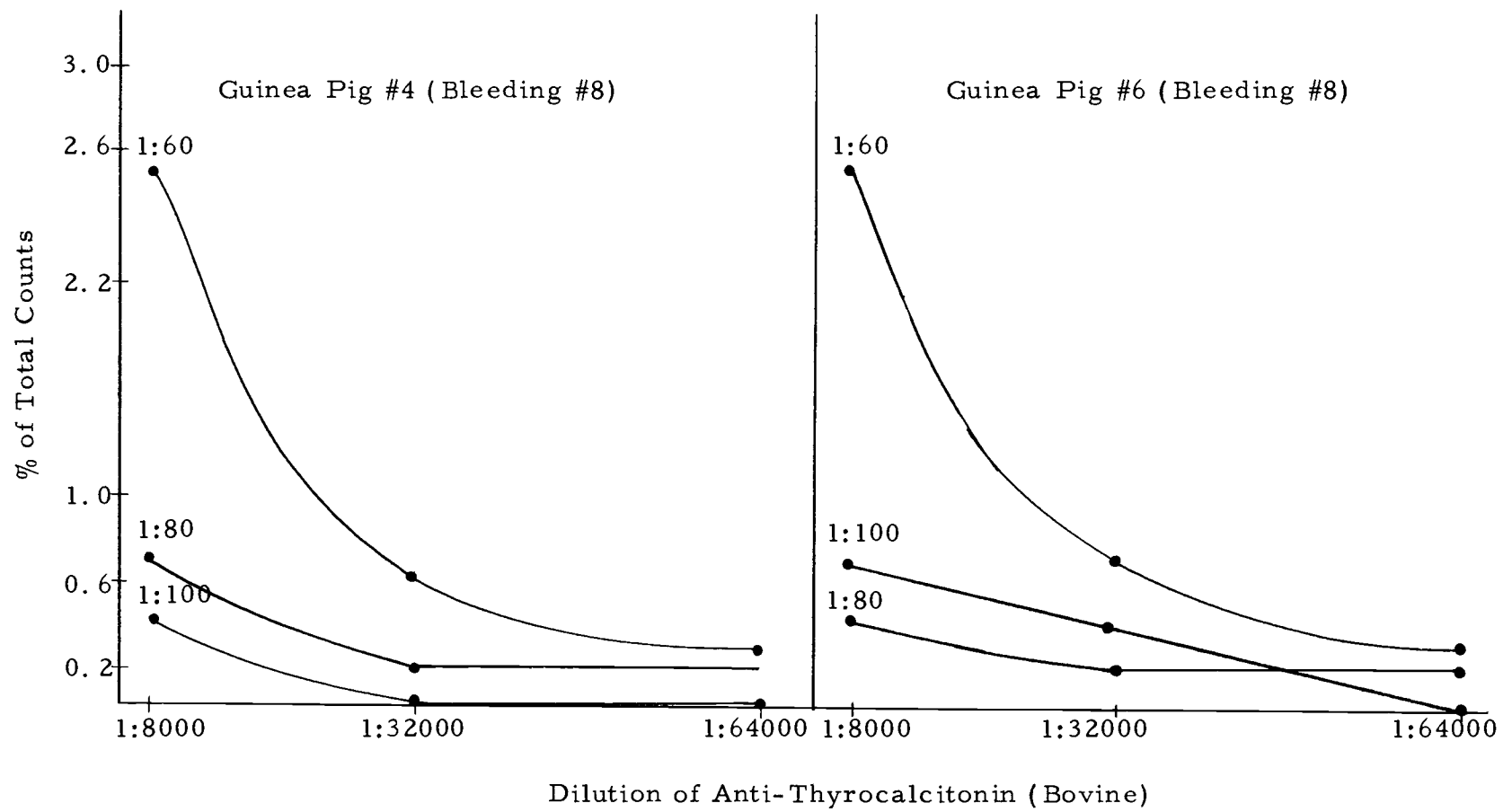


Figure 16. Binding capacity of ^{125}I -thyrocalcitonin by sera from immunized guinea pigs coded #4 and #6. Fractions #3, #4 and #5 correspond to the fractions on the ascending limb at the zenith, and on the descending limb of the labelled hormone peak, respectively, in Figure 13.

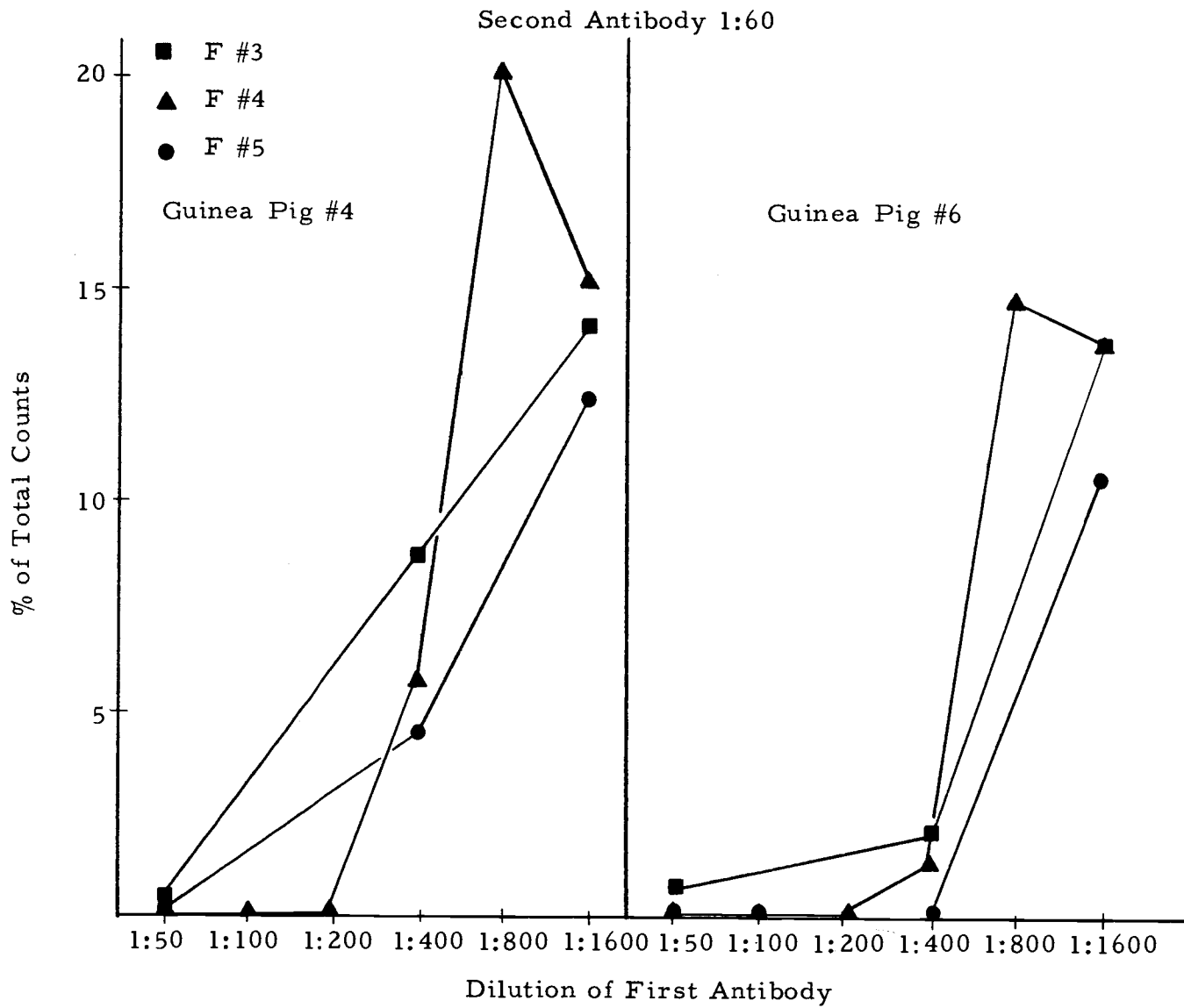
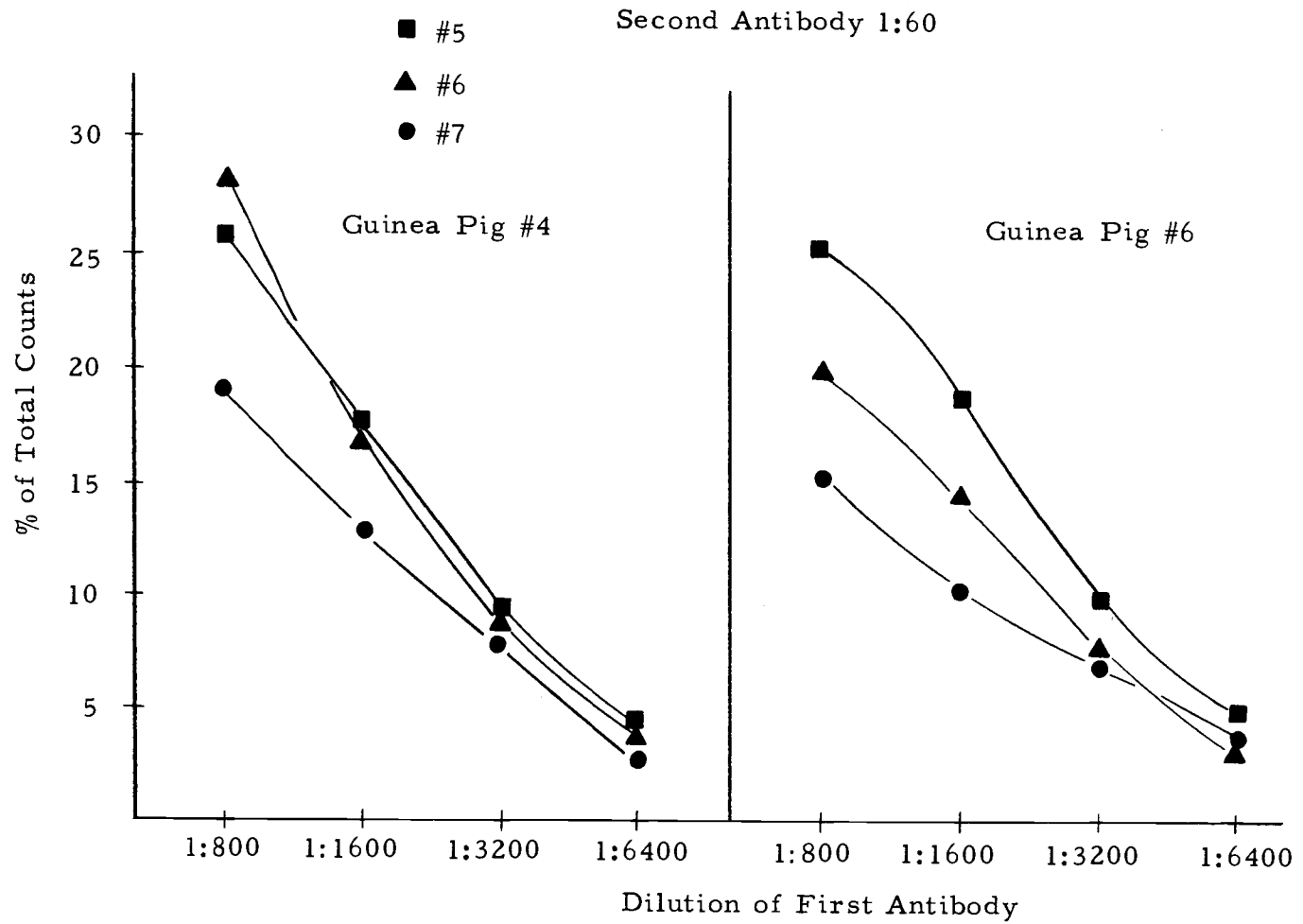


Figure 17. Binding capacity of ^{125}I -thyrocalcitonin by sera from immunized guinea pigs coded #4 and #6. Fractions #5, #6 and #7 correspond to the fractions on the ascending limb, at the zenith, and on the descending limb of the labelled hormone peak, respectively, in Figure 14.



antibody titer and the labelled hormone were adequate to generate a standard curve.

Radioimmunoassay of Thyrocalcitonin

As illustrated in Figure 18, a linear regression line with 95% confidence limits was constructed by the computer using logarithmic transformation of the dose to represent the standard curve of this assay system. All points were "weighed" equally by the computer in establishing the regression line. The concentration range of the line ranged from 0 to 600 ng/ml of bovine thyrocalcitonin. The assay was capable of consistently detecting as little as 0.80 ng/ml of rabbit thyrocalcitonin in assays when guinea pig #4 serum, at a final dilution of 1:800, was incubated for 72 hours. The specificity and cross reactivity of this assay system were not ascertained.

Experimental Studies. A direct relationship between serum levels of calcium and serum levels of thyrocalcitonin was found under experimental conditions in rabbits. The hormone was detected in the blood of six normal rabbits. The mean concentration in these animals was 0.80 ng/ml \pm S.E. 0.25; in contrast, thyrocalcitonin was not detectable in the sera of one normal and three thyroidec-tomized animals. The responses obtained in the assay with 500 μ l of serum from a normal and a thyroidectomized rabbit are shown in Figure 19. Five hundred microliters of serum from a

Figure 18. Regression line representing the standard curve of the radioimmunoassay system with 95% confidence limits. As little as 0.80 ng/ml of rabbit thyrocalcitonin could be measured consistently in the assay system.

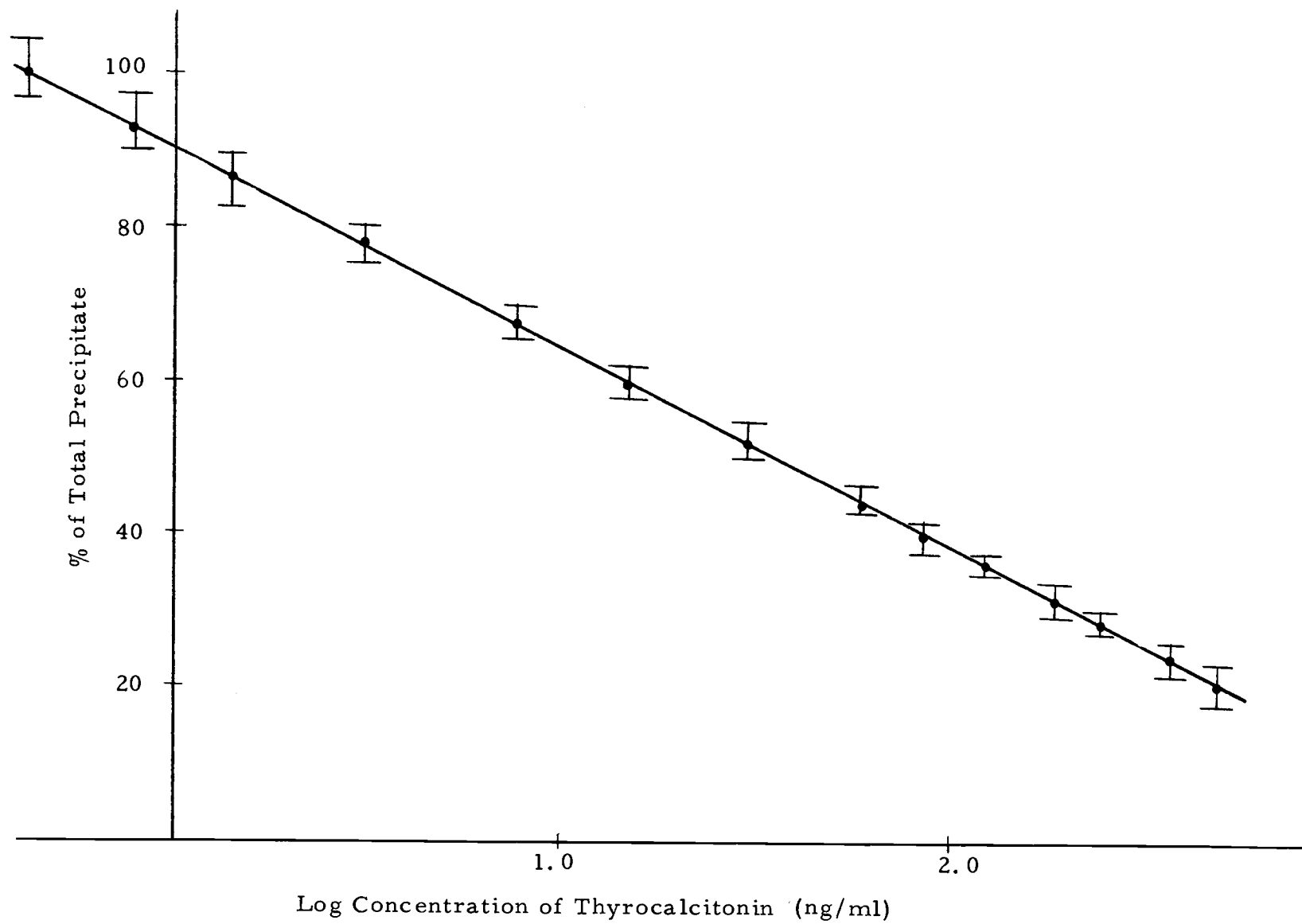
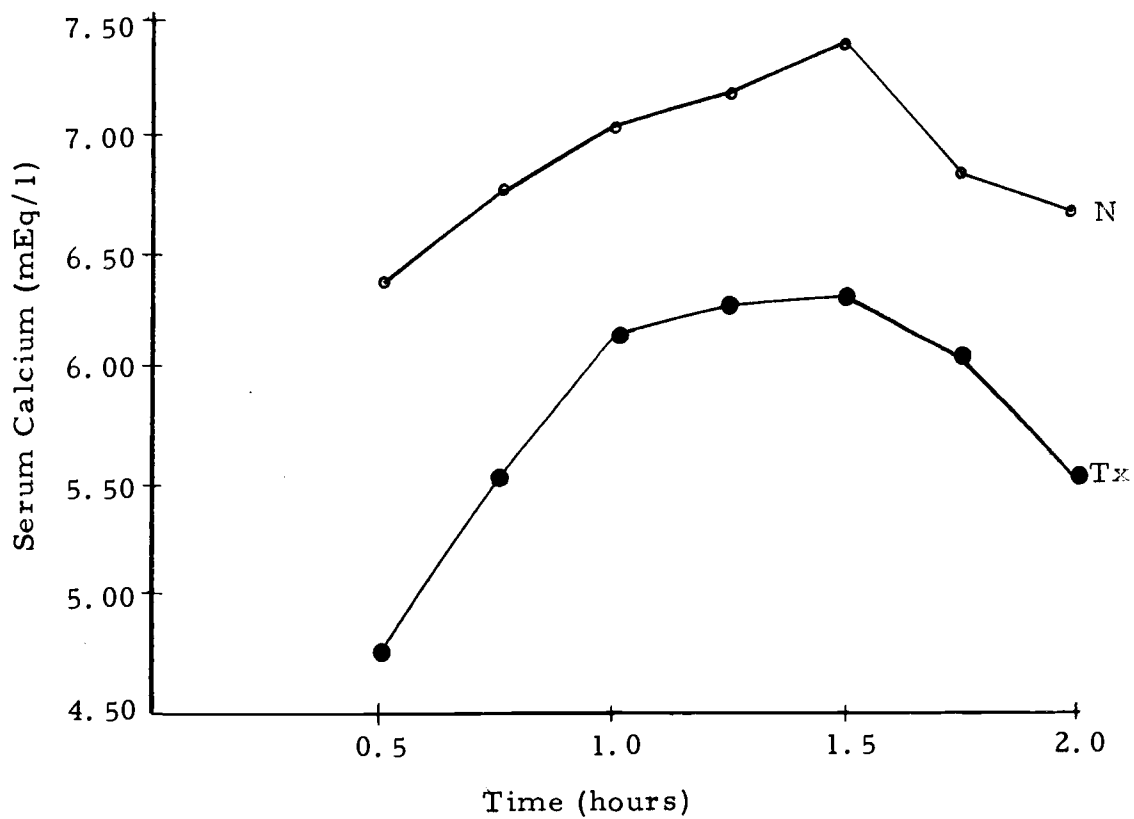
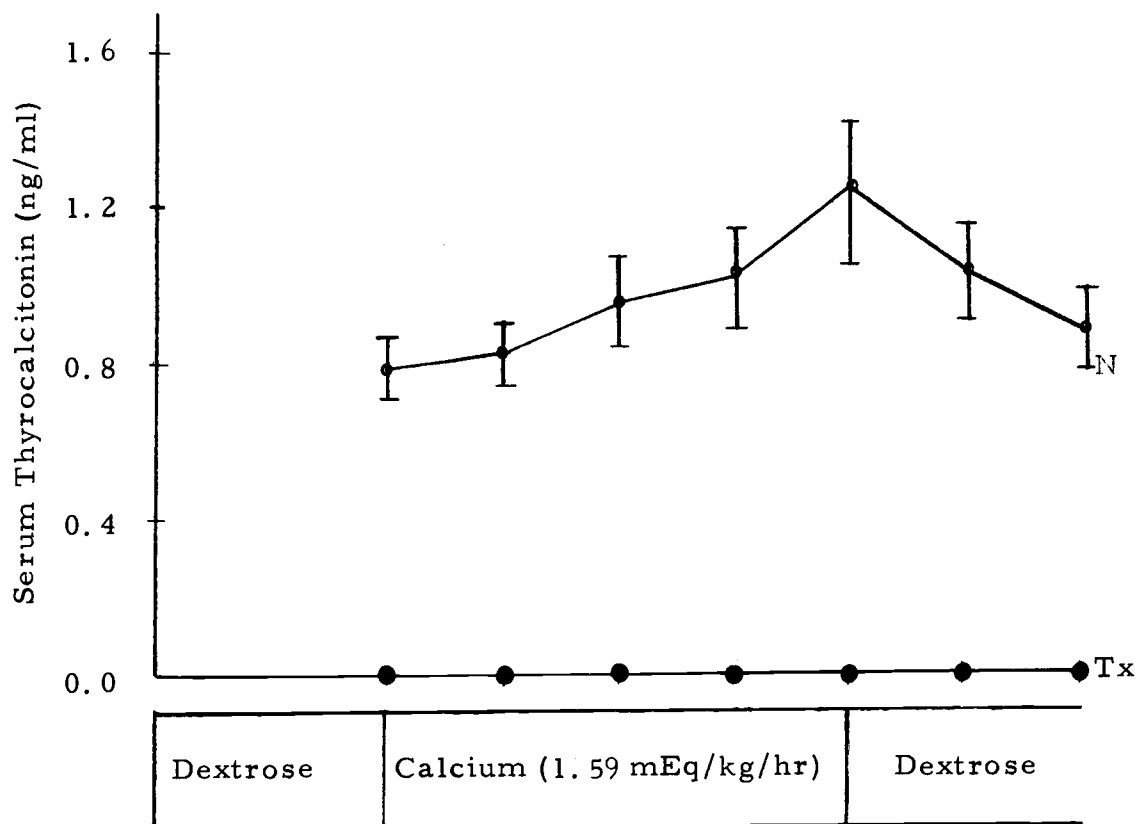


Figure 19. The influence of serum calcium concentration on serum thyrocalcitonin in rabbits. The vertical bars represent the standard errors of the means for three determinations. The pre-infusion level of thyrocalcitonin in the serum of a normal (N) rabbit was $0.80 \text{ ng/ml} \pm 0.08$; in contrast, similar aliquots of serum from a thyroidectomized rabbit (Tx) did not displace ^{125}I -thyrocalcitonin from antibody.



thyroidectomized rabbit infused with calcium did not displace ^{125}I -thyrocalcitonin from antibody, in contrast to the displacement produced by similar aliquots of serum from a normal animal under similar experimental conditions. Infusion of calcium to intact rabbits caused progressive increases in the concentration of thyrocalcitonin. The increased concentration of hormone was detected within 15 minutes of initiation of the infusion of calcium, and peak concentration was achieved during the period of maximal hypercalcemia. The hormone concentration rapidly returned to normal when the infusion of calcium was stopped.

The relationships of blood lead concentrations on serum calcium and thyrocalcitonin levels were investigated in the rabbit by infusion of lead, 6 mg/kg/hr, in the form of lead acetate (Figure 20). At a blood lead level of 0.25 $\mu\text{g}/\text{ml}$, a serum calcium concentration of 6.16 mEq/l was determined, but the hormone was not detected by the assay system. When the blood level of lead reached 15 $\mu\text{g}/\text{ml}$, after 15 minutes of infusion of lead, the serum calcium concentration had increased to 6.33 mEq/l and ^{125}I -thyrocalcitonin was displaced from the antibody. A progressive displacement of the labelled hormone with a concomitant increase in serum calcium was found with increases in blood levels of lead. The rate of rise in concentrations of thyrocalcitonin followed the slope of serum calcium more closely than the increasing slope of lead. Peak levels of serum

Figure 20. The effects of increasing concentrations of blood lead on serum calcium and thyrocalcitonin in the rabbit. The vertical bars represent the standard errors of the means of three determinations. Following a marked increase in concentrations of blood lead by infusion of lead, serum calcium and thyrocalcitonin levels rose gradually in the rabbit, but thyrocalcitonin levels were not statistically significant ($P > 0.05$).

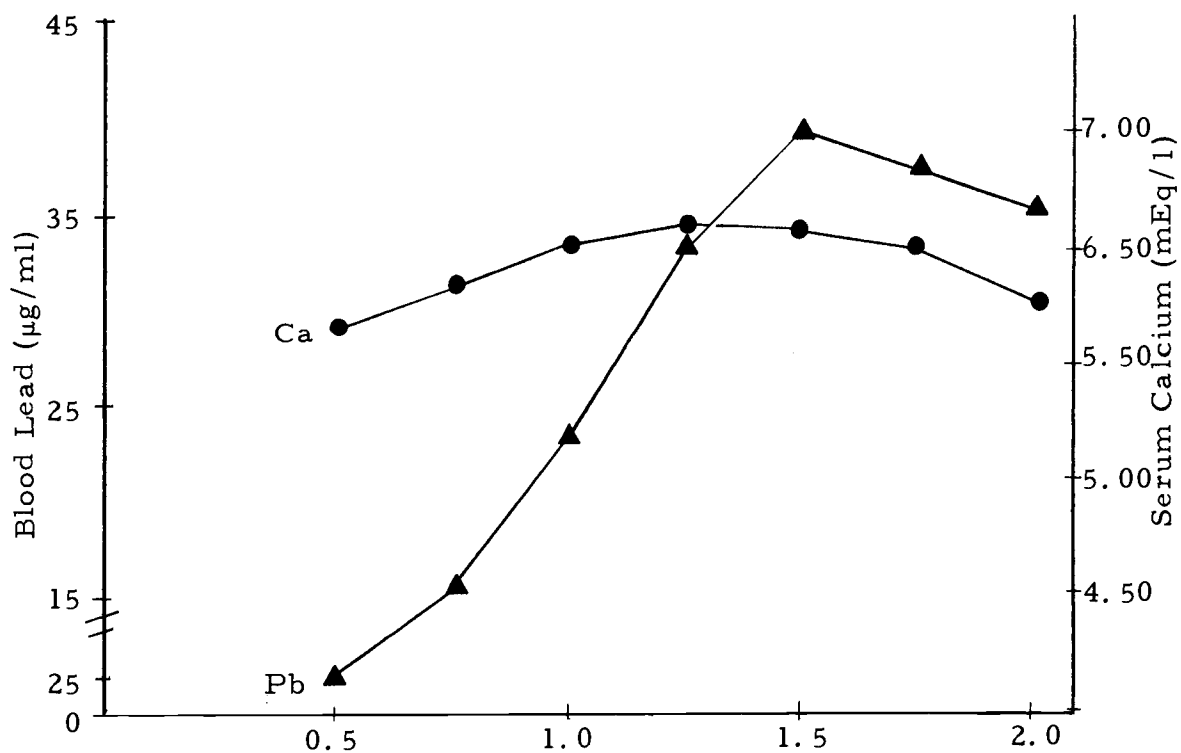
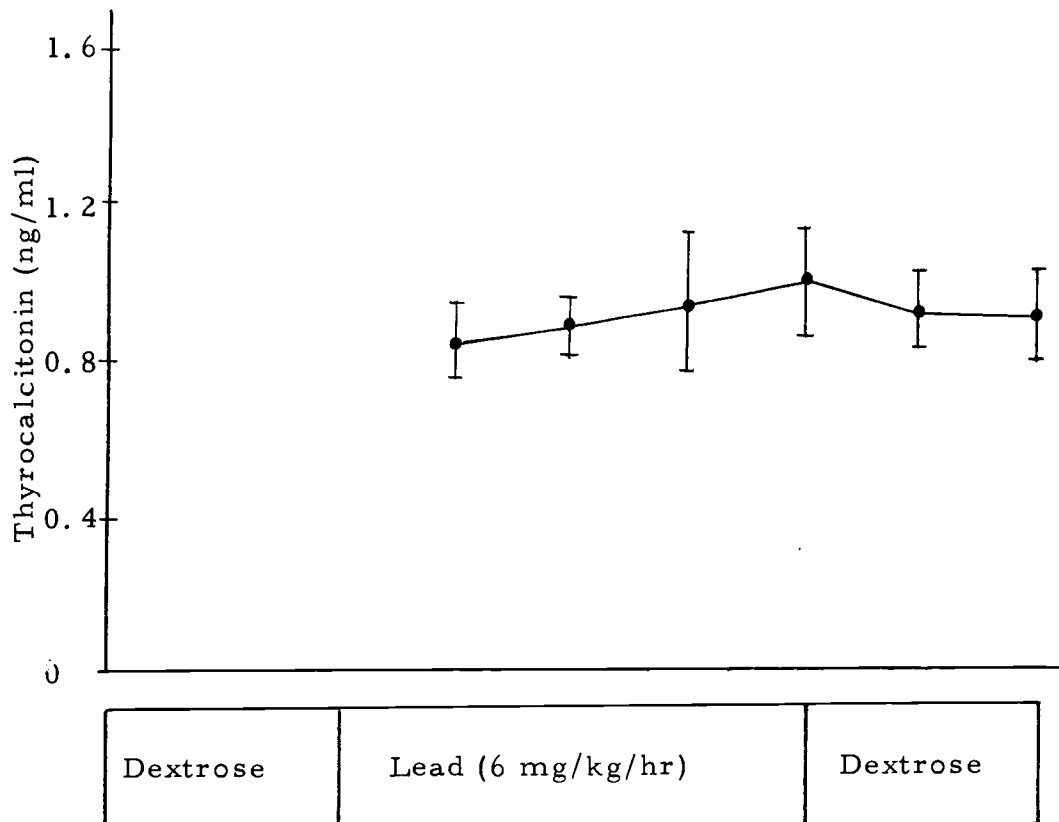
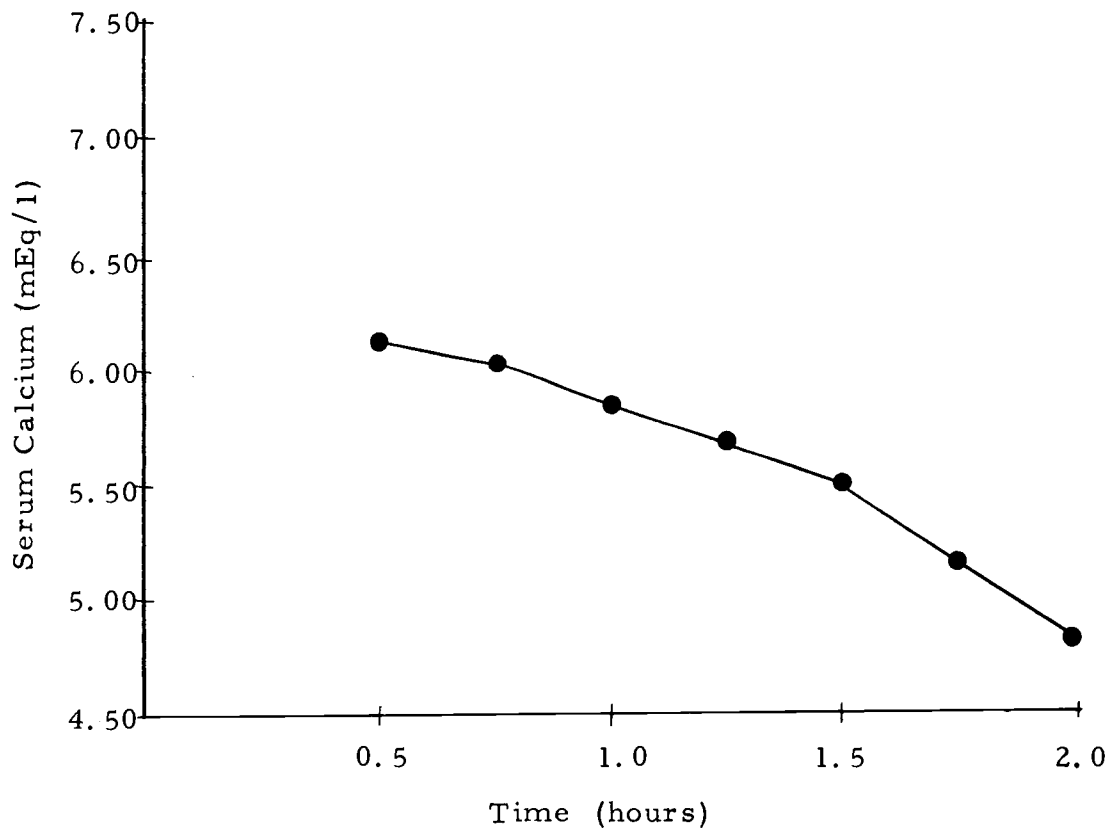
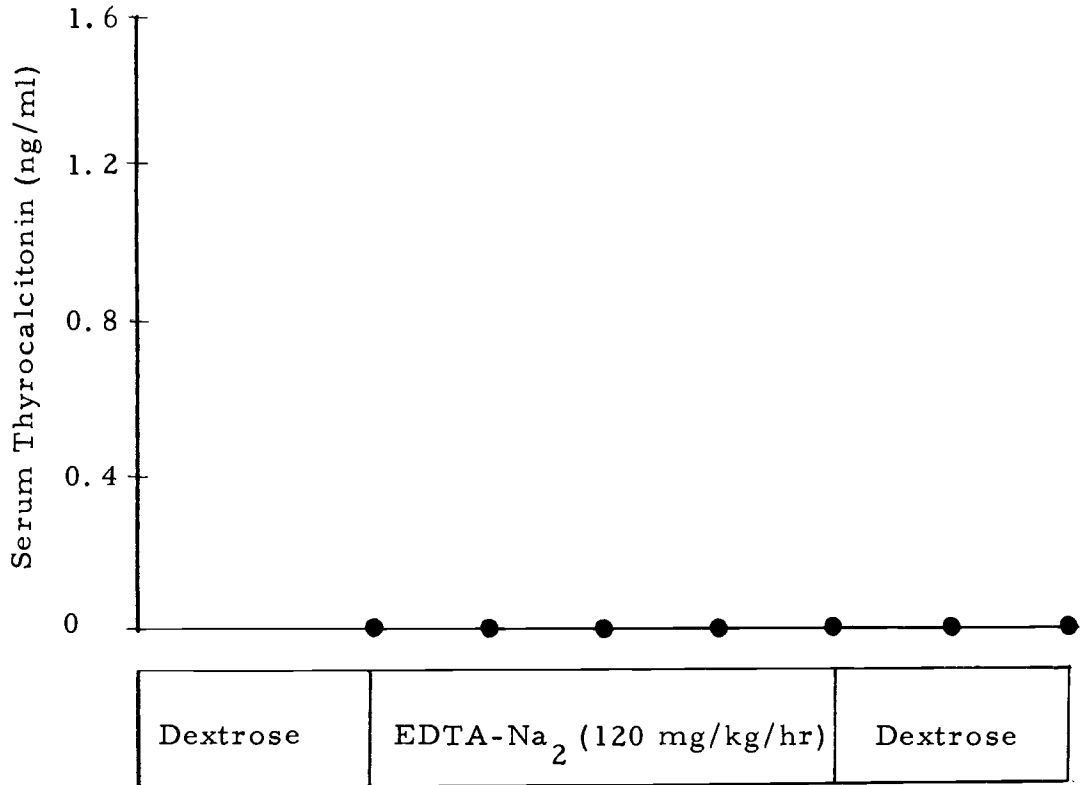


Figure 21. The effects of EDTA on serum calcium in the rabbit. Endogenous thyrocalcitonin was not detected at any time during the experiment by the assay system. A marked decline of serum calcium was evident within 45 minutes after infusion of EDTA (120 mg/kg/hr); hypocalcemic levels were found at the termination of the experiment.



calcium and thyrocalcitonin were associated with the maximal concentration of blood lead. The peak levels of calcium and the hormone declined to pre-infusion levels after termination of lead infusion, but blood lead levels did not return to normal values within the time-course of the experiment.

A thyroidectomized and an euthyroid animal died within 15 to 30 minutes after the infusion of lead at rates of 6 mg/kg/hr and 24 mg/kg/hr, respectively. Immediately prior to death, the animals exhibited a marked increase in respiratory rate and cutaneous vasoconstriction. Blood samples taken during the infusion of lead showed a moderate degree of hemolysis.

The results of the effects of EDTA (calcium and lead chelating agent) on serum calcium in the rabbit are illustrated in Figure 21. At no time during the experiment was thyrocalcitonin detected in the serum by the assay system. Furthermore, blood lead levels were reduced to non-detectable levels after 30 minutes of EDTA infusion. A decrease in serum calcium concentration was quite evident within 30 minutes after the infusion of EDTA Na₂ at 120 mg/kg/hr. This was followed by a hypocalcemia at the termination of the experiment; however, the animals did not exhibit signs of tetany at any time during the study. Under similar experimental conditions, a thyroidectomized rabbit died after 20 minutes of EDTA infusion with the absence of any signs of tetany.

Discussion

Bovine parathyroid hormone could be iodinated with ^{125}I with an efficiency of about 40%. However, binding experiments indicated that antibodies to this antigen were not elicited in guinea pigs. The failure to demonstrate a binding titer of antibody in the sera from guinea pigs injected with parathyroid hormone could result from: (1) poor recognition or affinity of the antibody for the hormonal antigenic sites because of damage to the sites during the radioiodination or storage of the hormone; (2) interference of the binding of antibody to the labelled hormone by subunits of parathyroid hormone in the sera of immunized animals. Rasmussen, Sze and Young (1964) and Wong and Lindall (1971) reported that several fractions of the parathyroid hormone exist and may be responsible for the failure of some radioimmunoassay systems to detect circulating levels of parathyroid hormone in the plasma or serum of some animals; (3) antigenicity of injected hormone was inadequate to elicit antibody formation. The conjugation of parathyroid hormone to a larger carrier protein (e. g., albumin) for greater antigenicity may overcome this problem and allow sufficient specificity and affinity to permit quantification of nanogram or micromicrogram amounts of the hormone per milliliter of plasma or serum.

The present study indicates that 10 μg or more of

thyrocalcitonin produced a labelling efficiency 40% or greater. By doubling the amount of thyrocalcitonin, the radioactivity associated with the ^{125}I -thyrocalcitonin also doubled. The amount of ^{125}I -label that can be attached to a molecule is a function of the number of tyrosine residues present in the molecule. Since thyrocalcitonin contains only one tyrosine residue per molecule (Potts et al., 1968; and Neher et al., 1968), this limits the amount of ^{125}I -label that can be attached to the molecule. This factor should be considered when iodinating small peptides.

From the binding experiments of thyrocalcitonin, it is evident that the antibody in the sera of immunized guinea pigs binds the ^{125}I -thyrocalcitonin; however, the final dilution of 1:800 of the first antibody limits the detection or sensitivity of the radioimmunoassay system. Others have reported the development of antisera to unconjugated thyrocalcitonin under similar immunizing conditions for radioimmunoassay studies (Deftos, Lee and Potts, 1968; Tashjian, 1969). Deftos, Lee and Potts (1968) used a 1:50,000 dilution of antiserum from a guinea pig immunized with porcine thyrocalcitonin to develop the first radioimmunoassay for thyrocalcitonin which detected as little as 15-20 $\mu\mu\text{g/ml}$ of porcine thyrocalcitonin. Later, Lee, Deftos and Potts, (1969) reported that the mean normal level of ~~plasma~~ thyrocalcitonin in the rabbit was 0.14 ng/ml. This is considerably lower than 0.80 ng/ml found in the present study. Alternately, the

differences may be due to the well-known unique specificities of individual antisera or in thyrocalcitonin standards. Other factors that should be considered to explain the discrepancy in the normal circulating level of thyrocalcitonin in rabbits are: (1) the proximity of the sampling cannula in relation to thyroid gland and (2) subspecies variation. It is also possible that metabolites of thyrocalcitonin would be recognized by the antibody, but this is unknown and should be investigated.

Logarithmic transformation of the amounts of thyrocalcitonin added to the system produces a linear standard curve. This type of construction permits a more accurate estimate of the slope and intercept by graphical methods. As little as 0.75 ng/ml of bovine thyrocalcitonin is capable of displacing 7% of the ^{125}I -thyrocalcitonin from the antibody binding sites; whereas 1 ng/ml displaces 10% of the labelled hormone. The concentration of 0.75 ng/ml of bovine thyrocalcitonin is the valid detection limit of the assay system.

From the experimental studies in rabbits, it is evident that the radioimmunoassay developed in the present study is adequate to determine circulating levels of serum thyrocalcitonin in the normal rabbit. The failure to detect circulating levels of thyrocalcitonin in the rabbit (perfused with EDTA) was apparently related to the serum concentration of calcium.

The observation that the peak level of thyrocalcitonin had

declined to pre-infusion levels within 30 minutes after cessation of calcium infusion in the normal rabbit suggests a rapid turnover of thyrocalcitonin. Similarly, after lead infusion, thyrocalcitonin levels fell rapidly to pre-infusion levels. This would appear to confirm that thyrocalcitonin has a very rapid turnover.

From the lead infusion experiment, it is not clear if lead is directly responsible for the release of thyrocalcitonin or if lead indirectly mediates the release of thyrocalcitonin by increasing serum calcium levels; however, the slope of serum calcium level change corresponds more closely to the slope of the serum thyrocalcitonin plot. Further studies are required to clarify the effects of lead on circulating levels of thyrocalcitonin.

Since EDTA has a greater affinity for lead than for calcium, blood lead levels could not be determined accurately in this experiment. This particular analytical problem should be examined more intensively.

These observations in the rabbit show the usefulness of this animal for further studies on the regulation of thyrocalcitonin secretions.

V. SUMMARY AND CONCLUSIONS

Results of this investigation strongly indicated that lead affects calcium metabolism and function. Elevation of brain calcium levels to an intravenously administered dose of lead was observed to be unique among the various tissues of the rat. This response of the brain tissue was influenced by the thyro-parathyroid system, since the response was fully expressed in the intact rat, but attenuated in the thyroparathyroidectomized rat. These results suggested that the effects of lead on calcium levels in the brain of the rat are regulated to some extent by the hormones of the calcium-regulatory system; however, altered serum calcium levels were not detected in lead-treated rats. Possibly, lead may produce subtle changes in the circulating levels of thyrocalcitonin and/or parathyroid hormone which would intensify concentrations of brain calcium, but may not be reflected by measurable changes in serum calcium concentration. A related study on the infusion of lead in the rabbit indicated that increased blood concentrations of lead in the rabbit are associated with elevated thyrocalcitonin levels; however, the increases of circulating levels of the hormone were not statistically significant, and furthermore, were concomitantly accompanied with increases in serum calcium. It is recognized that the experimental conditions of the two studies were different in many aspects, but the results

of both studies lend evidence that lead and calcium metabolism share some common features.

Results of the study of chronic lead exposure in the diet of rats indicated that equal concentrations of lead in water and in food differ markedly in influencing various biological parameters. This would suggest that lead in the drinking water is not absorbed to the same extent as lead in food. Insolubility of the lead acetate or precipitation of the metal from the drinking water do not appear to be contributing factors to this observation. Also, no significant difference in bowel activity was noted between the two groups of lead-treated rats. Balance dietary studies may help interpret this observation.

Neuromuscular parameters of the tibialis anterior muscle, i. e., twitch/tetanus ratio and follow-through frequency, correlated well with concomitantly elevated levels of lead and calcium in muscle and blood of rats. Increased intracellular calcium levels in the sarcoplasmic reticulum would provide an explanation for a prolongation of the active state of contraction and reduce the ratio of twitch/tetanus. Lead may affect the motor axons and consequently alter the active state of muscle contraction. Elevated levels of calcium and/or lead may alter the miniature endplate potentials and thus increase the responsiveness of the junctional sites. An increase in the amount of acetylcholine released per impulse may result from elevated levels of calcium and lead; other considerations

are lead's influence on: (1) the cholinesterase system at the junctional sites, (2) axonal conduction of the involved motor fibers, and (3) diffusional characteristics of acetylcholine at the neuromuscular junction.

In conclusion, the influence of lead on calcium regulation and physiological function can be summarized as follows: (1) a single intravenous dose of lead (10 mg/kg) increased significantly both calcium and lead levels in brain tissue of treated rats; (2) the thyro-parathyroid complex was necessary for complete expression of lead's effect on elevated brain calcium levels, but was not essential for distribution of lead in brain tissue of injected rats; (3) at 5, 10, 25 and 50 mg/kg of lead, brain concentrations of lead were found to be dose-related; however, brain levels of calcium at these doses of lead did not follow a similar pattern; (4) at 24 hours, any dose of lead used produced about a 70% increase in brain calcium; at 72 hours, brain levels of calcium were elevated about two-fold and remained elevated significantly above control levels at day 7; (5) calcium levels in other sampled tissues were not elevated significantly over the same time-course; (6) increased brain hydration was demonstrated for 25 and 50 mg/kg doses of lead in rats at days 3 and 7; (7) equal concentrations of lead in water and in food (300 ppm for 8 weeks) differed widely in influencing various biological parameters in rats (blood, brain and muscle levels of calcium and

lead); (8) reduced twitch/tetanus ratios and decreased tetanizing frequencies of the tibialis anterior (found only among the lead-food groups of rats) correlated well with concomitantly elevated levels of lead and calcium in muscle and blood; (9) infusion of lead in the rabbit (6 mg/kg/hr) elevated serum levels of calcium and thyrocalcitonin, concomitantly; however, the peak levels of the hormone were not statistically different from levels of the polypeptide measured at the initiation of the infusion of lead.

Obviously, more studies are needed to bridge the gap between our current knowledge of lead and calcium metabolism, and our conspicuous ignorance of it.

BIBLIOGRAPHY

- American Academy of Pediatrics. 1969. Subcommittee on accidental poisoning. Prevention, diagnosis and treatment of lead poisoning in children. *Pediatrics*. 44:291-298.
- Aub, J. C., L. T. Fairhall, A. S. Minot, and P. Reznikoff. 1926. Lead poisoning. Vol. 7. In: *Medicine Monographs*. Baltimore, Williams and Wilkins Company.
- Bagust, J., S. Knott, D. M. Lewis, J. C. Luck and R. A. Westerman. 1973. Isometric contractions of motor units in a fast twitch muscle of the cat. *Journal of Physiology*. 231:87-104.
- Bajusz, E., G. Jasmin, and A. Mongeau. 1963. Thyroid-parathyroid interrelationship in processes governing myocardial calcification. *Endocrinology*. 73:92-96.
- Berk, P. D., D. P. Tschudy, L. A. Shepley, J. G. Waggoner, and N. I. Berlin. 1970. Hematologic and biochemical studies in a case of lead poisoning. *American Journal of Medicine*. 48:137-144.
- Berman, E. 1964. The determination of lead in blood and urine by atomic absorption spectrophotometry. *Atomic Absorption Newsletter*. 3:111-116.
- Berson, S. A. and R. S. Yalow. 1959. Quantitative aspects of the reaction between insulin and insulin-binding antibody. *Journal of Clinical Investigation*. 38:1996-2016.
- Beutler, E. 1966. Glucose 6-phosphate dehydrogenase deficiency. In: *The Metabolic Basis of Inherited Disease*. 2nd ed. (eds.) J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson. New York, McGraw-Hill. p. 1060-1089.
- Bradbury, M., C. R. Kleeman, H. Bagdoyan, and A. Berberian. 1968. The calcium and magnesium content of skeletal muscle, brain and cerebral spinal fluid as determined by atomic absorption flame photometry. *Journal of Laboratory and Clinical Medicine*. 74:884-892.

- Byers, R. K. 1959. Lead poisoning. Review of the literature and report on 45 cases. *Pediatrics*. 23:585-609.
- California Department of Public Health. 1967. Lead in the environment and its effects on humans. Berkeley, California Department of Public Health.
- Cantarow, A. and M. Trumper. 1944. Lead poisoning. Baltimore, The Williams and Wilkins Company.
- Catton, M., M. Harrison, P. Fullerton, and G. Kazantzis. 1970. Subclinical neuropathy in lead workers. *British Medical Journal*. 2:80-82.
- Cernik, A. A. 1970. Determination of lead chelated with ethylenediaminetetraacetic acid in blood after precipitation of protein with perchloric acid. *British Journal Industrial Medicine*. 27:40-42.
- Chisolm, J. J., Jr. 1962. Aminoaciduria as a manifestation of renal tubular injury in lead intoxication and a comparison with patterns of aminoaciduria seen in other diseases. *Journal of Pediatrics*. 60:1-17.
- Chisolm, J. J., Jr. 1965. Chronic lead intoxication in children. *Developmental Medicine Child Neurology*. 7:529-536.
- Chisolm, J. J., Jr. 1968. Lead poisoning (Plumbism). In: *Pediatrics*. 14th ed. (ed.) H. L. Barnett. New York, Appleton-Century - Crofts. p. 313-319.
- Chisolm, J. J., Jr. and H. E. Harrison. 1956. The exposure of children to lead. *Pediatrics*. 18:943-957.
- Close, R. 1972. Dynamic properties of mammalian skeletal muscles. *Physiological Reviews*. 52:129-197.
- Cummings, J. N. 1959. *Heavy Metals and the Brain*. Thomas, Springfield. p. 95.
- Davson, H. 1967. Breakdown of the blood-brain barrier. In: *Physiology of the Cerebrospinal Fluid*. Boston, Little, Brown and Company. p. 300-301.

- Davson, H. 1970. Aspects of muscular contraction. In: A Textbook of General Physiology. 4th ed. Vol. 2. Baltimore, The Williams and Wilkins Company. p. 1387-1455.
- Davson, H. and M. Eggleton. 1962. The contractile system. In: Starling's Human Physiology. 13th ed. Philadelphia, Lea and Febiger. p. 832-833.
- Deftos, L., M. Lee and J. Potts, Jr. 1968. A radioimmunoassay for thyrocalcitonin. Proceedings of National Academy of Sciences. 60:293-299.
- Einarrson, O. and G. Lindstedt. 1969. A non-extraction atomic absorption method for the determination of lead in blood. Scandinavian Journal of Clinical and Laboratory Investigation. 23:367-371.
- Emmerson, B. T. 1968. The clinical differentiation of lead gout from primary gout. Arthritis Rheumatism. 11:623-634.
- Farrelly, R. O. and V. Pybus. 1969. Measurement of lead in blood and urine by atomic absorption spectrophotometry. Clinical Chemistry. 15:566-572.
- Fuwa, K. 1971. Flame absorption spectrometry. Spectrochemical Methods of Analysis. 9:189-234.
- Gabbiani, G. and B. Tuchweber. 1970. Prevention by calcitonin of metallic salt intoxications. Calcified Tissue Research 4 (Supplement):144-145.
- Gabbiani, G., B. Tuchweber, G. Cote', and P. Lefort. 1968. Action of thyroxine and calcitonin on experimental soft-tissue calcification. Calcified Tissue Research. 2:30-37.
- Goodman, L. S. and A. Gilman. 1970. Heavy metals. In: The Pharmacological Basis of Therapeutics. 4th ed. London, Toronto, The Macmillan Company. p. 977-986.
- Griggs, R. C. 1964. Lead poisoning: Hematologic aspects. In: Progress in Haematology. Vol. IV. (eds.) C. V. Moore and E. B. Brown. New York, Grune and Stratton, Inc. p. 117-137.

- Hasan, J. and S. Hernberg. 1966. Interactions of inorganic lead with human red blood cells. *Work-Environment-Health*. 2:26-44.
- Hass, G. M., J. H. McDonald, R. Oyasu, H. A. Battifora, and J. T. Paloucek. 1967. Renal neoplasia induced by combinations of dietary lead subacetate and N-2-fluorenylacetamide. In: *Renal Neoplasia*. (ed.) J. S. King, Jr. Boston, Little, Brown and Company, Inc. p. 377-412.
- Hessel, D. W. 1968. A simple and quantitative determination of lead in blood. *Atomic Absorption Newsletter*. 7:55-58.
- Hill, A. V. 1949. The onset of contraction. *Proceedings of Royal Society, B*. 136:242-255.
- Hirsch, F., G. F. Gauthier, and P. L. Munson. 1963. Thyroid hypocalcemic principle and recurrent laryngeal nerve injury as factors affecting the response to parathyroidectomy in rats. *Endocrinology*. 73:244-252.
- Hirsch, P. F. and P. L. Munson. 1969. Thyrocalcitonin. *Physiological Reviews*. 49:549-622.
- Hunter, D. and J. C. Aub. 1926-27. Lead studies. IV. The effect of the parathyroid hormone on the excretion of lead and of calcium in patients suffering from lead poisoning. *Quarterly Journal of Medicine*. 20:123-129.
- Hunter, W. M. and Greenwood, F. C. 1962. Preparation of iodine-131 labeled human growth hormone of high specific activity. *Nature (London)*. 194:495-501.
- Johnstone, R. T. 1964. Clinical inorganic lead intoxication. *Archives of Environmental Health*. 8:250-255.
- Kehoe, R. A. 1942. Lead absorption and lead poisoning. *Medical Clinics of North America*. 26:1261-1279.
- Kehoe, R. A. 1947. Exposure to lead. *Occupational Medicine*. 3:156-171.
- Kehoe, R. A. 1961a. The metabolism of lead in man in health and disease. *Archives of Environmental Health*. 2:418-422.
- Kehoe, R. A. 1961b. The Harben Lectures. The metabolism of lead in man in health and disease. Lecture I. Research Institute of Public Health. 24:81-97.

- Kehoe, R. A. 1961c. The Harben Lectures. The metabolism of lead in man in health and disease. Lecture II. Research Institute of Public Health. 24:101-120.
- Kehoe, R. A. 1961d. The Harben Lectures. The metabolism of lead in man in health and disease. Lecture III. Research Institute of Public Health. 24:177-203.
- Kostial, K. and V. B. Vouk. 1957. Lead ions and synaptic transmission in the superior cervical ganglion of the cat. British Journal of Pharmacology and Chemotherapy. 12:219-222.
- Kumita. 1909. Virchows Archives. 198:401-405.
- Lead: Airborne Lead in Perspective. 1972. Washington, D. C., National Academy of Sciences.
- Lederer, L. B. and F. C. Bing. 1940. Effect of calcium and phosphorus on retention of lead by growing organisms. Journal of the American Medical Association. 114:2457-2461.
- Lee, M., L. Deftos and J. Potts. 1969. Control of secretion of thyrocalcitonin in the rabbit as evaluated by radioimmunoassay. Endocrinology. 84:36-40.
- Lehr, D. 1959. Causative relationships of parathyroid hormone to renogenic and reniprival cardiovascular disease. Annals of New York Academy of Sciences. 72:901-969.
- Lehr, D., I. Wajda, and M. Krukowski. 1958. Importance of adrenal cortex-parathyroid "axis" in the mechanism of experimental cardiovascular necrosis. Journal of Pharmacology and Experimental Therapeutics. 122:42A-43A.
- Lending, M., L. B. Slobody, and J. Mestern. 1961. Effect of hyperoxia, hypercapnia and hypoxia on blood-cerebrospinal fluid barrier. American Journal of Physiology. 200:959-962.
- Manalis, R. S. and G. P. Cooper. 1973. Presynaptic and postsynaptic effects of lead at the frog neuromuscular junction. Nature. 243:354-355.
- Morgan, C. R. and A. Lazarow. 1963. Immunoassay of insulin: Two antibody system. Diabetes. 12:115-126.
- Neher, R., B. Riniker, R. Maier, P. G. H. Byfield, T. V. Gudmundsson, and I. Macintyre. 1968. Human Calcitonin. Nature. 220:984-996.

- Potts, J. T., Jr., H. D. Niall, H. T. Keutman, H. B. Brewer, Jr., and L. J. Deftos. 1968. The amino acid sequence of porcine thyrocalcitonin. *Proceedings of the National Academy of Sciences, U.S.* 59:1321-1328.
- Rasmussen, H., Y. L. Sze, and R. Young. 1964. Further studies on the isolation and characterization of parathyroid polypeptides. *Journal of Biological Chemistry.* 239:2852-2857.
- Rosen, J. F. 1972. Actions of calcitonin-mimicking agents and calcitonin antagonists in lead intoxicated rats. *Pediatric Research.* 6:401.
- Sawin, C. R. 1969. *The hormones endocrine physiology.* London, Little, Brown and Company. p. 135.
- Selye, H. 1958. Prevention of experimental nephrocalcinosis with thyroxine. *Endocrinology.* 62:227-229.
- Selye, H. 1958. Production of osteitis fibrosa by stylomycin aminonucleoside in the parathyroidectomized rat. *Endocrinology.* 62:287-291.
- Selye, H. 1962. *Systemic calciphylaxis. Calciphylaxis.* The University of Chicago Press. p. 83-249.
- Selye, H., B. Tuchweber, and G. Gabbiani. 1962. Calcinosis induced by lead acetate. *Journal of Pharmacology and Experimental Therapeutics.* 138:131-138.
- Selye, H., B. Tuchweber, and G. Gabbiani. 1963. Role of the parathyroids in the production of osteitis fibrosa and soft tissue calcification by lead acetate. *Endocrinology.* 72:782-786.
- Shields, J. B. and H. H. Mitchell. 1941. The effect of calcium and phosphorus on the metabolism of lead. *Journal of Nutrition.* 21:541-552.
- Six, K. and R. Goyer. 1970. Experimental enhancement of lead toxicity by low dietary calcium. *Journal of Laboratory and Clinical Medicine.* 76:933-942.

- Smith, T., V. Butler, and E. Haber. 1969. Determination of therapeutic and toxic serum digoxin concentrations by radioimmunoassay. *New England Journal of Medicine*. 281:1212-1220.
- Sobel, A. E. and M. Burger. 1955. Calcification XIII. The influence of calcium, phosphorus and vitamin D on the removal of lead from blood and bone. *Journal of Biological Chemistry*. 212:105-110.
- Sobel, A. E., O. Grawon, and B. Kramer. 1938. Influence of vitamin D in experimental lead poisoning. *Proceedings of the Society for Experimental Biology and Medicine*. 38:433-437.
- Sobel, A. E., H. Yuska, D. D. Peters, and B. Kramer. 1940. The biochemical behavior of lead. I. Influence of calcium, phosphorus and vitamin D on lead in blood and bone. *Journal of Biological Chemistry*. 132:239-265.
- Spector, S. and E. J. Flynn. 1971. Barbiturates:Radioimmunoassay. *Science*. 174:1036-1038.
- Spector, S. and C. Parker. 1970. Morphine:Radioimmunoassay. *Science*. 168:1347-1348.
- Spector, W. S. 1956. *Handbook of Biological Data*. (ed.) W. S. Spector. Philadelphia and London, W. B. Saunders Co.
- Swanson, L. 1973. Associate Professor, Oregon State University, Department of Animal Science. Personal Communications. Corvallis, Oregon.
- Tanquerel Des Planches, L. 1848. *Lead Diseases:A Treatise*. With notes and additions on the use of lead pipes and its substitutes. (Translated from the French by S. L. Dana), Lowell, Mass., Daniel Bixby and Company, 1948.
- Tashjian, A. H., Jr. 1969. Immunoassay of thyrocalcitonin. I. The method and its serological specificity. *Endocrinology*. 84:140-148.
- Thies, R. E. 1965. Neuromuscular depression and the apparent depletion of transmitter in mammalian muscle. *Journal of Neurophysiology*. 28:427-442.

- Unger, R. H., A. M. Eisentraut, M. S. McCall, and L. L. Madison. 1961. Glucagon antibodies and an immunoassay for glucagon. *Journal of Clinical Investigation*. 40:1280-1289.
- Utiger, R. 1965. Radioimmunoassay of human plasma thyrotropin. *Journal of Clinical Investigation*. 44:1277-1286.
- Utiger, R. D., M. L. Parker, and W. H. Daughaday. 1962. Studies on human growth hormone. A radioimmunoassay for human growth hormone. *Journal of Clinical Investigation*. 41:254-261.
- Waldron, H. 1966. The anaemia of lead poisoning: A review. *British Journal of Industrial Medicine*. 23:83-100.
- Willis, J. B. 1961. Determination of calcium and magnesium in urine by atomic absorption spectroscopy. *Analytical Chemistry*. 33:556-559.
- Willis, J. B. 1962. Determination of lead and other heavy metals in urine by atomic absorption spectroscopy. *Analytical Chemistry*. 34:614-617.
- Wong, E. and A. Lindall. 1971. Human parathyroid gland immunoreactive peptides. Evidence for parathormone. *Journal of Laboratory Clinical Medicine*. 78:825-826.
- Yalow, R., S. Glick, J. Roth, and S. Berson. 1964. Radioimmunoassay of human plasma ACTH. *Journal of Clinical Endocrinology Metabolism*. 24:1219-1225.
- Zarrow, M. X., J. M. Yochum, J. L. McCarthy, and R. C. Sanborn. 1964. Parathormone. In: *Experimental Endocrinology*. New York and London, Academic Press. p. 365-382.