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LEAD MOBILIZING ACTIVITY OF DMPS, DMSA, AND DMPA FOLLOWING ORGANIC AND INORGANIC LEAD EXPOSURE

The University of Arizona M.S. 1986

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LEAD MOBILIZING ACTIVITY OF DMPS, DMSA, AND DMPA FOLLOWING ORGANIC AND INORGANIC LEAD EXPOSURE

by

Joan Mary Dooley

A Thesis submitted to the Faculty of the

DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

MASTER OF SCIENCE
WITH A MAJOR IN TOXICOLOGY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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H. V. Aposhian
H. V. Aposhian
Molecular and Cellular Biology

5/5/86
Date

To My Family

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ABSTRACT

The effectiveness of three water soluble metal binding agents, meso-dimercaptosuccinic acid (DMSA), 2,3-dimercapto-1-propane sulfonic acid, Na salt, (DMPS), and N-(2,3-dimercaptopropyl)phthalamidic acid (DMPA) were studied for their ability to prevent lead poisoning. Rats were given 2 mg Pb/kg/day i.p. for 7 days, followed by the metal binding agents, 0.2 mmol/kg/day, i.m., for 2 days. Urinary excretion of lead increased ($p<0.05$) with any of the compounds. Lead measured in brain tissue was decreased ($p<0.05$) by any of the compounds. DMSA lowered lead levels in bone tissue ($p<0.05$). DMSA and DMPS were given orally, for 8 days, at the same dose, to rats exposed to lead acetate. DMPS lowered lead levels in the kidney and the brain ($p<0.05$), while DMSA was only effective in the kidney ($p<0.05$). DMSA and DMPS were given i.m. to animals exposed to triethyllead, 1 mg Et_3Pb/kg, i.p. No changes in lead concentrations were found in brain, kidney or bone tissue as a result of these compounds.

CHAPTER 1

INTRODUCTION

History of Lead Poisoning

Lead is a metal that has been used since antiquity. Large quantities have been smelted in areas of southwest Asia and Europe since 2500 B.C. (Lin-Fu, 1980). The toxic effects of lead have also been recognized since antiquity. The Romans and Greeks, however, knowing the harmful effects of lead, still continued to expose themselves to it by cooking and storing food and wine in leaden vessels (Mack, 1973). Outbreaks of lead poisoning from contaminated foods continued to occur in many cultures until the 20th century.

Lead Poisoning in Children

The industrial revolution brought increased uses for lead and increased exposure of workers to lead. Occupational lead poisoning in the lead industries became a major problem. It was not until the 1920's, however, that childhood lead poisoning unrelated to parental exposure was also recognized as a serious problem. Lead paint on toys, furniture, and housing structures, coupled with a child's desire for pica (a craving for unnatural foods) was reported

as the major source of poisoning in children (Ruddock, 1924). Airborne lead is another source of poisoning. It can be inhaled or can settle into the dust where children play.

It was in the 1950's and 1960's that health officials became aware of the fact that lead poisoning occurred commonly in U.S. children. Epidemiological studies showed that lead poisoning occurred almost exclusively in children aged 1 - 6 who resided in old housing painted with lead-based paint, especially in large inner city slums. Children aged 1 - 3 were at the highest risk (Lin-Fu, 1972a).

With the recognition that lead poisoning was an important pediatric public health problem, the Lead-Based Paint Poisoning Prevention Act was passed by Congress in 1971. This act appropriated funds to detect and treat lead-based paint poisoning and eradicate its source. Mass screening of children for lead poisoning followed and with it came the discovery of thousands of "asymptomatic" children with elevated (greater than 30 µg/dl whole blood) blood lead levels. Health officials became concerned with possible subclinical toxic effects in young children (Lin-Fu, 1972b).

Today, lead-based paint found in the home (chips, dust, etc.) that children can ingest is the major source of

lead poisoning, but not the only one. Airborne lead from automobile exhaust that settles into dust is another major source, especially in well populated areas. Lead glazed pottery is still available, and children are exposed to lead dust brought home on the clothes of others working in the lead industries.

The Uses of Lead

In general, there are two main forms of lead in use today, commonly known as inorganic lead and organic lead. Inorganic lead, manufactured as lead salts and lead oxides, is used in paints, solders, batteries and cable sheathing. Lead oxide was used in interior house paint until 1945. The first organolead compound, hexaethyldilead, was synthesized in 1853. Now the most widely used organic lead compounds are tetraethyllead and tetramethyllead. General Motors discovered that tetraethyllead was an effective antiknock additive to gasoline. It controls the combustion of the air/fuel mixture, so the fuel burns smoothly and evenly when ignited. In 1923, the first gallon of gasoline with tetraethyllead was sold in Dayton, Ohio (Nickerson, 1984). Upon combustion in the engine the greater part of organic lead is converted to lead halogenides and lead oxide. These deposit in the engine or escape through the exhaust into the environment (Grandjean and Nielsen, 1978).

Inorganic Lead Toxicity

Absorption, Distribution and Excretion

In humans the route of absorption of inorganic lead is mainly through the respiratory tract and the gastro-intestinal tract. Only small amounts are absorbed through the skin. After absorption inorganic lead is distributed first to soft tissues such as kidney and liver. Lead is redistributed to bone, teeth, and hair, until 95% of the body burden of lead is found in these three tissues (Klaassen, 1980). Circulating lead is almost completely associated with erythrocytes. The half life of lead in blood is one month. Therefore, the concentration of lead in whole blood is the accepted indicator of recent intoxication (Hammond and Beliles, 1980). The target organs of lead, those organs where damage occurs, are the central nervous system, the peripheral nerves, the kidney, and the hemato-poietic system. Urine is the dominant excretion route of inorganic lead in humans. In experimental animals lead is excreted into the bile and more lead is excreted in the feces than in urine (Klaassen, 1980).

Central Nervous System

An often fatal condition is a central nervous system effect from chronic or subchronic exposures of high doses of inorganic lead that cause lead encephalopathy. The symptoms

are dullness, restlessness, muscle tremor, and ataxia, which lead to convulsions, coma and death. If the patient recovers, there is a high incidence of residual damage such as epilepsy, hydrocephalus, and idiocy. At low levels of toxicity, the central nervous system may produce subtle learning and behavioral changes, especially in children. In children the most sensitive target organ is the brain. The results of an investigation by Needleman (1979) that found even a low-dose absorption of lead caused irreversible intellectual damage in children, influenced the way many addressed the problem of low level toxicity. But there exists controversy about his findings, and his work has been criticized by the EPA (Marshall, 1983) as invalid because of sampling and statistical errors.

Renal Toxicity

In the kidney there are two types of renal damage from lead poisoning. First there is damage to the proximal tubules such that tubular reabsorption is impaired. Second, with prolonged high doses, kidney damage progresses to interstitial fibrosis and glomerular atrophy. Death from renal failure is possible. (Hammond and Beliles, 1980).

Blood Toxicity

The earliest effect that lead is known to have is on the hematopoietic system, Figure 1. Lead inhibits the

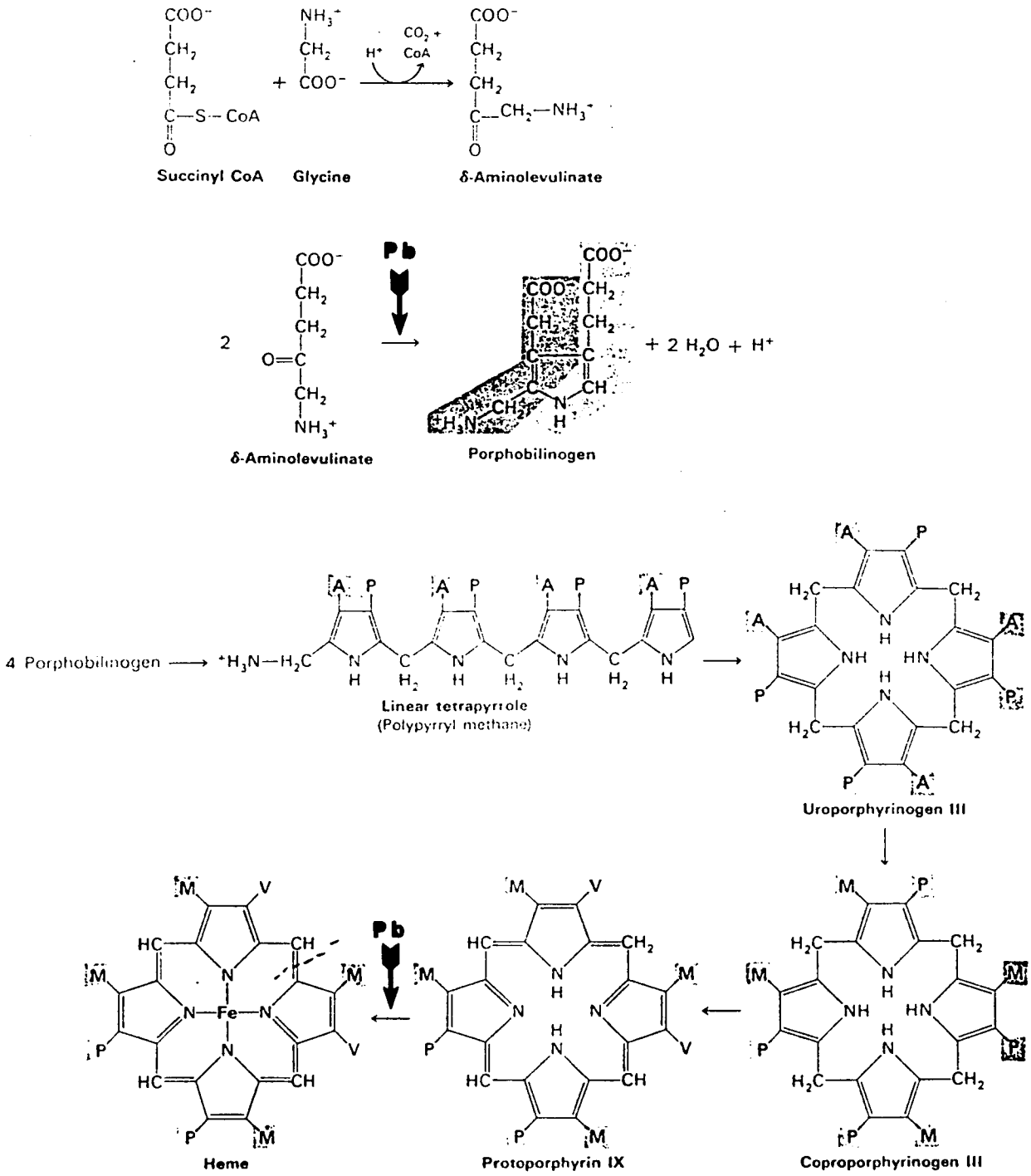


Figure 1. Heme Metabolism and Lead Inhibition

incorporation of iron into protoporphyrin IX either by direct inhibition of ferrochelatase or by interfering with iron's entry into the mitochondria (Hammond and Beliles, 1980). Hernberg and Nikkan (1970) demonstrated that delta-aminolevulinic acid dehydratase (d-ALAD) is inactivated by lead with no apparent threshold. d-ALAD catalyzes two molecules of aminolevulinic acid into one molecule of porphobilinogen. The accumulation of aminolevulinic acid, when d-ALAD is inhibited, occurs in the hypothalmus where it inhibits Na/K ATPase and affects neuromuscular function (Becker et al., 1973). Other toxic affects associated with lead poisoning include colic, anemia, and muscle weakness leading to palsy.

Organic Lead Poisoning

Of all the target organs, organic lead is most toxic to the central nervous system. Its effects are more mental or CNS oriented than those of inorganic lead. Hallucinations, delusions and excitement are the symptoms which can lead to delirium and death. Other central nervous system related symptoms include insomnia, nightmares and anorexia. Nausea, vomiting and diarrhea also appear, along with hypothermia, bradycardia and hypotension (Klaassen, 1980, Hammond and Beliles, 1980).

Absorption, Distribution and Excretion

Due to their lipophilicity, organic leads such as tetraethyllead are readily absorbed through the skin, GI tract and respiratory tract. Dealkylation of tetraethyllead takes place in the liver microsomes. This occurs quickly and it is the metabolite triethyllead, Et_3Pb^+, that exerts the toxic effects (Cremer, 1959). Triethyllead is found in highest concentration in liver, followed by kidney, brain and muscle (Bolanowska, 1968). The concentration of lead as triethyllead is not as high in blood as with inorganic lead so blood is not an indicator of organic lead poisoning. Triethyllead has no effect on the metabolism of porphyrins as inorganic lead does. Organic lead that is inhaled is excreted mainly in the feces. After oral intake, urine is the major excretory route. In triethyllead exposed rats excretion was mainly in the feces, but never exceeded one percent of the dose per day (Bolanowska, 1968).

Lead Determination

Until the turn of the century the analytical method for determining inorganic lead in blood or tissues was precipitation of lead with hydrogen sulfide and weighing the black precipitate, lead sulfide. This method was not specific for lead, but was the best available. From then until the late 1940's the method of choice was chelation with dithizone, then determination by polarography. Flame

emission photometry then came into practice, but it was the advent of atomic absorption spectroscopy in the 1960's that made accurate determination of even low levels of lead feasible (Grandjean and Olsen, 1984, Johnson and Stevenson, 1978). Atomic absorption is at present the most widely used method for the determination of lead in blood and other biological samples (Grandjean and Olsen, 1984). The use of a graphite furnace instead of a flame to atomize the sample has increased the sensitivity even further. The latest technology includes anodic stripping voltammetry and inductively-coupled plasma emission spectroscopy. The reference method is mass spectrometry.

Organolead compounds, like triethyllead, can be determined in blood, urine or tissue by GC/MS procedures or GC/AAS methods. Both of these methods can differentiate and quantitate the different alkyl groups on organolead compounds (Chau et al., 1984). Before these instruments were available the standard method of determining organic lead species was by complexing them with diphenylthiocarbazone (dithizone). This method is not as sensitive or reliable as the others.

Measuring Lead Exposure

Blood and Urine

Blood lead concentrations are the accepted indicator of inorganic lead exposure and intoxication. Twenty-four

hour urine specimens are also used to determine the presence and extent of lead poisoning. Since children are more sensitive to lead's adverse effects, they can develop encephalopathy at lower levels. Yet such levels are more difficult to measure accurately. It is also known that blood lead concentrations do not necessarily reflect the total body burden of lead, especially if it has already been sequestered into bone. So biochemical changes in the heme biosynthesis pathway which are altered by very low levels of lead can be measured to further assess damage due to lead exposure (Figure 1).

d-ALAD

When lead inactivates the enzyme d-ALAD there is an increase in aminolevulinic acid in the blood and urine, which can be measured by reacting with Ehrlich's reagent to form a colored complex. A more sensitive assay is to measure a change in the activity of d-ALAD itself. This is accomplished by incubating blood with aminolevulinic acid and measuring the amount of porphobilinogen formed. This assay correlates very well with blood lead levels and is especially relevant in the blood lead range of 10 µg/dl, where inhibition begins, to 60 µg/dl (Grandjean and Olsen, 1984).

ZPP

Lead also inhibits the incorporation of iron into the erythrocyte. This causes an accumulation of zinc-protoporphyrin (ZPP) in the red blood cell. ZPP is stable for the life of the red blood cell, making the ZPP level in blood an average measure of lead toxicity during the previous three to four months. ZPP is measured quickly by a hematofluorometer. ZPP levels correlate well with blood lead levels, especially above 30 µg/dl (Grandjean and Olsen, 1984). The major drawback of this assay is that ZPP is also increased in iron deficiency states.

Evaluation of Lead Levels

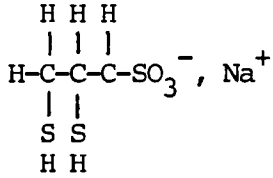
With lead so prevalent in our environment, natural lead poisoning occurs even in those who are not exposed to lead through an occupational setting. In industrialized countries the average blood lead values are between 10 and 20 µg/dl. 30 µg/dl has been designated as elevated. Because children are more susceptible, mean blood lead levels in children should be kept below 15 µg/dl (EPA, 1978). If symptoms are present, and blood lead levels are at least 40 µg/dl, treatment by chelation therapy is recommended (Chisholm, 1970).

Treatment of Lead Poisoning

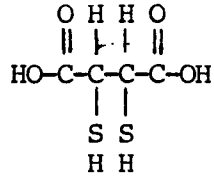
Before 1945 lead poisoning was treated with cod liver oil, calcium gluconate, parathyroid extract and sodium phosphate. These were given to enhance new bone formation and to increase the deposition of lead into the skeletal system (Byers et al., 1943). In the search to antagonize the effects of the arsenical war gas Lewisite, it was observed that arsenic had a high affinity for sulfhydryl containing compounds. Through experimentation it was found that sulfhydryl groups on adjacent carbon atoms competed successfully with binding sites responsible for arsenic's toxic effects. In 1945 British Anti-Lewisite (BAL), or dimercaptopropanol, was synthesized.

Chelating Agents

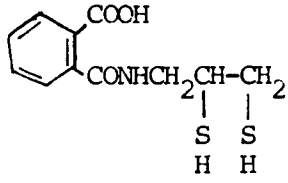
BAL. Chelating agents form stable coordinate-covalent bonds with cationic metal atoms. BAL is a chelating agent because it contains two potential donor atoms that can bind with a metal to form a heterocyclic ring (Figure 2). The donor atoms that are present in electronegative functional groups (OH, SH, NH etc.) donate electrons for coordination with the metal atom. The optimum stability of a chelate appears to be the formation of a five membered ring. Chelating agents are generally non-specific for metals. So after World War II, BAL was used for the



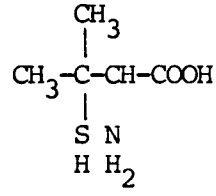
DMPS
(2,3-Dimercapto-1-propane-sulfonic acid, Na salt)
Unithiol



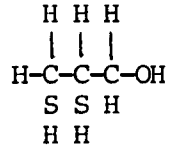
DMSA
(Meso-dimercapto-succinic acid)
Succimer



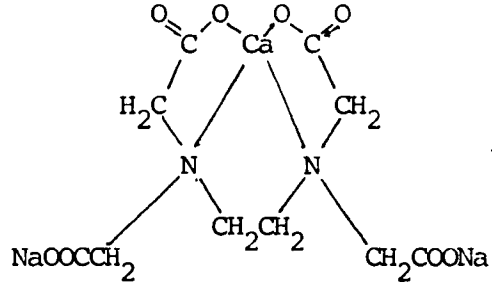
DMPA
(N-(2,3-Dimercaptopropyl)-phthalamidic acid)



D-Penicillamine



BAL
(British Antilewisite)
(2,3-Dimercapto-1-propanol)



EDTA
(Ethylenediaminetetraacetic acid, $CaNa_2$ salt)

Figure 2. Structures of Metal Binding Agents

treatment of poisoning by other heavy metals, including lead, mercury, copper and arsenic (Klaassen, 1980). Lead exerts its toxic effect by inhibiting certain enzyme systems that depend on free sulfhydryl groups. BAL is effective as a chelating agent because lead has more affinity for it than for an endogenous ligand. Because of its lipophilicity, BAL may be thought of as an intracellular chelator. BAL has its limitations, however, because of its side effects. These include lacrimation, blepharospasm, nausea, tachycardia and increased systolic and diastolic blood pressure (Chisholm, 1970). Because of its limited water solubility BAL is available for i.m. injection only, in a peanut oil base, and causes pain and local tissue necrosis at the injection site.

EDTA. Another chelating agent that was developed in the early 1940's was EDTA, ethylenediaminetetraacetic acid, used as the disodium salt of the calcium chelate. EDTA binds lead via nitrogen and oxygen atoms (Figure 2). It removes lead from bone cells but not erythrocytes. EDTA penetrates cell membranes poorly, and can be considered an extracellular chelator (Becker and Correia, 1982). BAL is more effective than EDTA in reversing the toxic effects of lead, including lowering amino-levulinic acid levels. EDTA is given i.v. or i.m. only, because it enhances lead absorption from the gut. It too has toxic side effects, the

most important being massive renal tubular necrosis (Chisholm, 1970). If fever, rashes, hypercalcemia or renal damage occur, EDTA therapy is stopped. In severely lead poisoned patients, especially children, BAL and EDTA are given together to increase the chelant to lead ratio without using toxic doses of either chelate. $CaNa_2EDTA$ is less effective in children than in adults (Chisholm, 1970).

D-Pen. A third chelating agent in use today is D-Penicillamine (D-Pen). D-Pen is not as effective as BAL or EDTA in lowering lead levels, but it can be given orally, which the others can not. With chronic use D-Pen can also increase lead absorption from the gut. As with any chelation therapy, it is always important to remove the patient from the source of the lead poisoning. Excessive doses of D-Penicillamine can cause nephrotic syndrome, bleeding phenomena, leucopenia and neutropenia (Chisholm, 1970).

Water Soluble Analogs of BAL

DMPS and DMSA

Chelation therapy is necessary to prevent lead encephalopathy. It is also needed to prevent neurological impairment when lead is present in low levels, especially in children. The use of BAL, EDTA and D-Pen have decreased mortality and morbidity of lead poisoning greatly. However,

the toxicity associated with these compounds has made it feasible to look for other alternatives, specifically, less toxic water soluble compounds that could be administered orally. This has been done in the Soviet Union and China since the 1950's, but not in the western world until recently. Two water soluble analogs of BAL, 2,3-dimercapto-1-propanesulfonic acid, Na salt (DMPS), and meso-dimercaptosuccinic acid (DMSA), were developed. These compounds retain the adjacent sulfhydryl groups necessary for stable ring formation with a metal, but each contain an ionizable functional group which make them more water soluble than BAL. DMPS has been the drug of choice for treating lead poisoning in the Soviet Union since 1958, where it is called Unithiol. There it is the drug of choice for treating lead, mercury or arsenic poisoning, and is given prophylactically to prevent occupational heavy metal poisoning (Aposhian, 1984).

The effectiveness of DMSA against heavy metal poisoning was first reported by Liang et al., (1957) in Shanghai, but it was first used to increase the uptake of antimony for schistosomiasis therapy (Friedheim et al., 1954). DMPA (N-(2,3-dimercaptopropyl)-phthalamidic acid), is another water soluble analog of BAL. It was first reported synthesized by Portnyagina and Morgun in 1966 from Kiev, Soviet Union. It was found to be more potent than BAL

for mercury mobilization and excretion (Yonaga and Morita, 1981). Studied in our lab, DMPA was found to be as toxic as BAL in mice, and prevented the lethal effects of sodium arsenite (Stine et al., 1984). The structures of these compounds are given in Figure 2. These compounds are being studied in the U.S. now for their ability to bind lead and increase its excretion. The oral bioavailability of some of these compounds in experimental animals has been studied. The bioavailability of DMPS when given p.o. to rats was found to be 30-40% (Gabard, 1978). The G.I. absorption of DMSA may be species dependent, ranging from 20-30% in the monkey to more than 90% in the hamster (McGown et al., 1984) DMPS is in the clinical stage of testing at John Hopkins University (Chisholm and Thomas, 1985). DMSA is being given in clinical trials at Columbia University (Graziano et al., 1985). Nowhere are they being studied together, however, under the same conditions. Nor are they compared to D-Penicillamine, the only other orally effective metal binding agent.

Thesis Objectives

When the enormity of the problem of lead poisoning in America was realized, the need arose for less toxic, more palatable compounds that successfully removed lead from target organs, and prevented harmful effects, especially in

children. Those that were developed, using the dithiol arrangement that worked so well with BAL, are being tested now, against each other and against the established methods of treatment. It is the objective of this research to compare three water soluble analogues of BAL, DMPS, and DMPA, for their effectiveness, by mouth and i.m., in preventing the effects of lead poisoning in minimally exposed rats. These will be compared to control animals and to D-Pen, which is also water soluble and effective orally. The comparisons will be made by examining urinary lead excretion resulting from the administration of these compounds, and by measuring tissue levels of lead in target organs following a course of therapy. Animals will be exposed to inorganic lead as lead acetate, and organic lead as triethyllead. At present there is no treatment available for organolead poisoning.

In testing these compounds, a number of different parameters will be used. The focus of this research is not to study in depth the activity of these metal binding agents, but to explore different doses and different routes of administration for an initial insight into how well the compounds work to treat lead poisoning in rats.

In hypothesizing the possible outcomes of metal binding therapy following triethyllead administration, the need for a method of determining levels of inorganic lead

and organic lead in the same animal tissue arose. If any metal binding agent lowered lead levels in target organs, it would be advantageous to know which lead species was being removed. A review of methods for this kind of determination revealed no procedure that was appropriate, taking into consideration the available instrumentation. It became the second objective of this research to explore various methods of determining organic and inorganic lead compounds in a biological matrix, and to develop a working method for this kind of determination.

CHAPTER 2

MATERIALS AND METHODS

Animals

The animals used in the lead mobilization studies were male Sprague-Dawley rats, from the CD colonies of Charles River Breeding Labs, Inc., Wilmington, MA. All animals were shipped from the Portage, MI facility. When shipped, the animals weighed between 145 and 160 grams. The animals were housed at the animal handling facility in the Bio Sciences West building, initially in tiered metal cages, 3 or 4 to a cage. They were fed Wayne Lab Blox and tap water ad libitum and maintained on a 12 hour light/dark cycle, at a temperature of 22 degrees C. There was a minimum of 5 days for acclimation before experiments were begun. By then, the rats weighed 190 - 210 grams.

During the lead-loading portion of the dosing procedure, the animals remained in the metal cages. If urines were not to be collected, the rats remained there during metal binding therapy also. Their drinking water was changed from tap to distilled during this time. When urines were collected, the animals were housed individually in

plastic metabolic cages. They also received distilled water at this time. The metabolic cages were washed daily in soap and water, acid cleaned with 20% nitric acid, and rinsed with distilled water.

Triethyllead and Metal Binding Agents

Triethyllead Chloride was purchased from Alfa Products, Morton Thiokol, Inc., Danvers, MA. Because the purity was unknown, it was purified by washing first with ether, then with hexane, in a darkened room. The purified product was stored in the dark, at -25 degrees C. D-Penicillamine and DMPS as the sodium salt were obtained from the Heyl Co., Berlin, West Germany. DMPA was obtained from Dr. T. Yonaga, Tokyo, Japan. DMSA was obtained from Johnson and Johnson, Skillman, NJ. Sodium bicarbonate was purchased from Mallinckrodt Chemical Works, St. Louis, MO.

Dosing solutions of lead were made in water immediately before use. Each metal binding agent was dissolved in a saline solution of 5% $NaHCO_3$. Aliquots were frozen, and then thawed immediately prior to use. A new aliquot was used for each dosing.

Atomic Absorption

Lead levels in tissues and urines were determined by Atomic Absorption spectroscopy. The flame atomic absorption

spectrometer (AAS) used was a Varian 475. The AAS used an air acetylene flame, a lead hollow cathode lamp, and the wavelength measured was 283.3 nm. It had a lower detection limit for lead of 200 ppb. Brain lead levels in rats were lower than 200 ppb, so brain tissues, and all tissues from animals dosed with triethyllead, were analyzed on an IL Video 12 graphite furnace atomic absorption spectrometer (GFAAS). The GFAAS used a lead hollow cathode lamp, nitrogen or argon was the carrier gas, and the wavelength measured was 283.3 nm. The furnace temperature rose from 60 to 2000 degrees C in 10 seconds.

When using GFAAS, it was imperative to have matrix matched samples and standard curves. A small difference in a sample's viscosity will greatly affect the aspiration rate of the sample into the cuvette, and false readings will result. In all experiments using GFAAS, the standard curve had the same matrix as the samples. This was accomplished by simultaneously digesting the same tissue from an untreated animal and adding the same volume of digest as in the sample dilutions to the standards, or by treating a blank tissue sample to the same chromatography steps used to separate triethyllead from inorganic lead, and adding this to the standards made.

When triethyllead was used in an experimental procedure, it was also used to make the standard curves, and the same for lead nitrate. After tissue samples were digested in nitric acid, they contain only inorganic lead, so lead nitrate was used for those standard curves.

Glassware

All glassware, nonmetallic portions of plastic metabolic cages, econocolumns and Tygon tubing were soaked in 20% v/v solution of nitric acid, and rinsed in double distilled, deionized water before use. DI water was obtained from the Veterinary Diagnostic Lab.

Mobilization of Inorganic Lead

IM administration of Metal Binding Agents

In this experiment, there were six dosing groups, with 5 animals per group. Due to the limited number of metabolic cages, 6 animals were treated at one time, one from each group, and the experiment was repeated 5 times.

The six groups consisted of a control group; these animals received only the vehicle during lead loading and metal binding therapy, a lead control group; where no metal binding agents were given but lead was, and four groups receiving lead and one metal binding agent each; DMPS, DMSA, DMPA or D-Pen (Figure 3).

Day	Procedure
1	
2	
3	
4	2 mg Pb/kg/day, i.p., daily, as lead acetate
5	
6	
7	
8	24 hour urine collection
9	
10	0.2 mmol/kg DMSA, DMPS, DMPA or D-Pen, i.m.,
11	3 times/day. 24 hour urine collection
12	sacrifice

Figure 3. Dosing Procedure for the i.m. Route of Administration of DMSA, DMPS, DMPA and D-Pen Following Lead Loading With Lead Acetate.

Lead acetate was administered, i.p., in a dose of 2 mg Pb/kg/day, in a dosing volume of 2 ml/kg, for 7 days. This dose was chosen from other research (Twarog and Cherian, 1983) which showed this dose did not cause overt lead toxicity but provided adequate lead stores for the metal binding agents. For 6 of these 7 days, the animals were kept in tiered metal cages, on the 7th day they were moved to plastic metabolic cages for a one day acclimation period before urine collections began. The animals then remained in the metabolic cages for the rest of the experiment. There were 2 days following lead loading when no injections were made, only 24 hour urines were collected. The day following this 48 hour delay, the metal binding agents were given, at a dose of 0.2 mmol/kg, 3 times a day for 2 days. The dosing volume was 1 ml/kg. These were administered i.m., in the gastrocnemius muscle, in alternating legs for each injection. Urines were collected concurrently each 24 hour period.

The last day of the experiment was the day of sacrifice, immediately following the two days of metal binding therapy. The animals were anesthetized with nembutal, and their diaphragms were cut. Their kidneys, brains with cerebellum, and femurs were removed. Although

lead is found in the highest concentration in liver, these organs were chosen because the brain is the critical organ of lead toxicity, the kidney receives the most overt damage in lead poisoning, and bone is the storage depot for lead.

Nitric Acid digestion

The capsules on the kidneys as well as much of the tissue around the femurs as possible were removed. These organs were weighed whole, then portions approximately 0.5 g for kidneys, 1.0 g for brains, and one whole femur were weighed out into acid cleaned test tubes which are the digestion vessels. Three ml of concentrated nitric acid was added to each test tube, and they were heated in an oil bath at 60 - 85 degrees, until the tissues had dissolved. Then the temperature was raised to greater than 120 degrees, and the nitric acid was evaporated to dryness. The brains only were digested and analyzed in duplicate. Similarly, a 2.5 ml aliquot from each day of each animal's 24 hour urine collections was added to 5 ml of concentrated nitric acid. The urines were digested by heating at 60 - 85 degrees for one hour, then evaporated to dryness. All samples were redissolved in 5 ml of 50% nitric acid.

This procedure was suitable for running on the flame AAS, but 1:10 to 1:50 dilutions were necessary for samples analyzed by GFAAS. Each lead value reported for kidney,

femur and urine by flame AAS was the mean of 2 samplings. Brain tissue was sampled 3 times, 2 samples from each brain, by the GFAAS, so 2 means of 3 were recorded. Lead levels were reported as µg Pb/g tissue or µg Pb/24 hour urine excretion.

Oral Administration Of Metal Binding Agents

In this experiment, 4 groups of 5 rats were used to determine the p.o. efficacy of the metal binding agents DMPS and DMSA following lead loading with lead acetate (Figure 4). The route of administration was p.o. instead of i.m., to determine their efficacy by that route. No urines were collected in this experiment, so the animals were kept in tiered metal cages throughout the study, and all 20 animals were experimented upon at one time. For 7 days, 3 of the 4 groups received daily i.p. injections of lead acetate, at a dose of 2 mg Pb/kg/day, and a dosing volume of 2 ml/kg. The 4th group was the control group, and they were injected with the vehicle only. After a 2 day equilibration period, one lead loaded group received DMPS, one lead loaded group received DMSA, and the other two groups received the vehicle $NaHCO_3$ in saline. The dose of DMSA and DMPS was 0.2 mmol/kg, given 3 times a day, for 8 days, by gastric lavage. The dosing volume was 1 ml/kg. The animals were fasted during the dosing of the metal binding agents until one hour

Day	Procedure
1	
2	
3	2 mg Pb/kg/day, i.p., daily, as lead acetate
4	
5	
6	
7	
8	
9	
10	
11	
12	
13	
14	0.2 mmol/kg DMSA or DMPS, p.o., 3 times/day
15	
16	
17	
18	sacrifice

Figure 4. Dosing Procedure for the p.o. Route of Administration of DMSA or DMPS Following Lead Loading With Lead Acetate.

following the last dose for that day. Food was then available for a 5 hour period. This was to prevent the metal binding agents from interacting with any food.

The day following the 8th day of orally administered metal binding agents, the animals were sacrificed as previously described. The same organs were removed, digested and analyzed for their lead content.

Mobilization Of Triethyllead

IM Administration Of Metal Binding Agents

The effectiveness of the metal binding agents DMPS or DMSA was tested on the organic form of lead, triethyllead, in rats (Figure 5). Three groups of 5 rats were given triethyllead chloride, i.p., at at dose of 1.0 mg Et_3Pb/kg/day, for 7 days. This dose was chosen after a preliminary study was done to determine an adequate dose that did not produce overt signs of toxicity (see results). The dosing volume was 2 ml/kg. No urines were collected, so the animals remained in tiered metal cages throughout the experiment. After 48 hours without treatment, DMPS was given, i.m., to one group of rats, and DMSA was given to another group, while the third group became the lead control group and received only the vehicle. The dose of the metal binding agents was 0.2 mmol/kg, the dosing volume was 1 ml/kg. These were administered 3 times/day, for 2 days, in

Day	Procedure
1	
2	
3	
4	1.0 mg Et_3Pb/kg/day, i.p., daily, as Et_3PbCl
5	
6	
7	
8	
9	
10	0.2 mmol/kg DMSA or DMPS i.m., 3 times/day
11	
12	sacrifice

Figure 5. Dosing Procedure for the i.m. Route of Administration of DMSA and DMPS Following Lead Loading With Triethyllead.

the gastrocnemius muscle, alternating legs with each dose. The following day the animals were sacrificed, and the brain with cerebellum, kidneys and femurs with tibias were removed and frozen in acid cleaned containers until digested.

Modified Nitric Acid Digestion

The digestion procedure used for tissues from animals dosed with triethyllead was a modified version of the nitric acid procedure developed in the U of A Veterinary Diagnostic laboratory. The thawed tissues were weighed whole, and then one whole kidney without its capsule, both halves of the brain, and one femur/tibia were weighed separately and placed in acid cleaned 250 ml beakers. Ten ml HNO_3 was added along with 3-4 acid washed boiling stones. To each beaker a 65 mm acid cleaned watch glass was placed snugly over the top. The beakers were heated at 70 degrees C on a hot plate until the tissue had dissolved completely. The watch glass was then tipped to vertical and the solution was evaporated at 70 degrees until approximately 2 ml remain. Five ml of 30% H_2O_2 was added slowly to the beaker. The H_2O_2 foamed and boiled vigorously when in contact with the acid solution. The solution was re-evaporated to approximately 3 ml. It was then quantitatively transferred to a 25 ml erlenmeyer flask, and diluted to volume with water. The H_2O_2 helped oxidize fat globules in the tissue

sample, but did not remove all of them. These were transferred into the erlenmeyer flasks also, but were not in the dilutions made for atomic absorption. All samples were read by GFAAS.

Statistics

To determine significant changes in lead levels in tissues due to the administration of metal binding agents, the non-parametric Wilcoxon Rank Sum W test was performed, and p values from this test that are lower than 0.05 were taken as significant (Siegal, 1956). The Q test described by Dean and Dixon (1951) was used to test for extraneous outliers. If the Q value was found to be larger than would occur in a normal population 90% of the time, the value was rejected. Means and standard errors reported in Figures 13 through 23, and Table I, are determined by 2-way Analysis of Variance. All statistical analysis was done by members of the U of A Cancer Center, using the Statistical package for the Social Sciences (SPSS) (Nie et al., 1975).

Quantitative Determinations Of Organic and Inorganic Lead

Spectrophotometry

Work done by Cremer in the late 1950's established a spectrophotometric assay for the determination of triethyllead and diethyllead using diphenylthiocarbazone

(dithizone). At best this method could only be used to estimate organic lead levels in tissues (Cremer, 1959).

In 1961, a method was developed by Henderson and Snyder to simultaneously determine triethyllead, diethyllead and inorganic lead. Dithizone was used because each of these species forms a different chloroform-soluble dithizonate that can be measured spectrophotometrically. Triethyllead forms a yellow complex, diethyllead forms an orange complex, and inorganic lead forms a red complex. The absorption maxima are separate enough that the individual absorbances can be measured following Beer's law.

This method was tried for the purpose of simultaneously determining triethyllead and inorganic lead in rat tissue samples. The presence of diethyllead as a contaminant was not tested. The spectrophotometer was calibrated with aqueous samples of 20 μg Pb per ml as lead nitrate or triethyllead chloride. These were reacted separately with dithizone as per the procedure, and scanned on the spectrophotometer to find the maximum wavelength. The maximum wavelength for inorganic lead is 515 nm, the maximum wavelength for triethyllead is 435 nm. With the known amount of lead in the dithizone complex, calibration constants were obtained, which was the absorbance at each wavelength, 435 and 515, per μg Pb, for each dithizone complex. These

values were necessary to determine the amounts of inorganic lead and triethyllead in unknown samples.

The Derivation of the Calibration Constants

let a and b equal the absorbance measurement of the sample at 435 and 515 nm respectively,

let x and y equal the µg of lead as triethyllead and the µg of inorganic lead present respectively,

let t_1 and t_2 equal the absorbance per µg of triethyllead at 435 and 515 nm respectively,

let i_1 and i_2 equal the absorbance per µg of inorganic lead at 435 and 515 nm, respectively,

therefore, $a = xt_1 + yi_1$

$b = xt_2 + yt_2$

solve for x and y in terms of a and b, using t_1, t_2, i_1 and i_2 calibration constant values.

The Procedure for the Dithizone Reaction.

Reagents: Ammoniacal buffer solution

2.5 g ammonium citrate

1.0 g potassium cyanide

6.0 g sodium sulfite

dissolve in 40 ml H_2O. Dilute to 250 ml with concentrated ammonium hydroxide. The citrate solubilizes cations, the cyanide complexes interfering cations, the sulfite maintains

a reducing system, because dithizone is readily destroyed by oxidants. Also, triethyllead will not react with dithizone under acidic conditions.

Dithizone Solution

Fifteen mg diphenylthiocarbazone dissolved in 250 ml chloroform. Store in an opaque bottle. Triorganolead dithizonates are converted to inorganic lead dithizonates rapidly if exposed in light. A Varian 2290 spectrophotometer was used for all experiments.

Procedure. In a 125 ml stoppered erlenmeyer flask, 10 ml distilled water, 4 ml ammoniacal buffer solution and 5 ml dithizone solution were added. The flask was wrapped completely in foil and shaken vigorously by hand for one minute. The organic and aqueous phases separate, and an aliquot of the organic phase was pipetted into two identical glass cuvettes. Each cuvette was read twice at both 515 and 435 nm, so there were 4 measurements at each wavelength. These were averaged and that value was the blank absorbance for that sample. The readings were taken as rapidly as possible to avoid light degradation of the dithizone. The placement of the 2 cuvettes into the spectrophotometer was alternated for the four readings, first one cuvette, then the other, then the first, etc., so each cuvette was equally exposed to light.

The dithizone solution was returned to the foil wrapped erlenmeyer and the cuvettes were washed with alcohol. One ml of a lead sample was added to the flask along with 0.08 ml of 50% v/v HNO_3. The erlenmeyer was again shaken for one minute. An aliquot of the organic phase was placed into the two cuvettes, and again both cuvettes were read twice at 515 and 435 nm, with the placement of the cuvettes alternated between pairs of absorbance measurements. The average of these four values minus the blank values became the a and b values in the derived equations. The equations were then be solved for x and y.

This method was found to be not sensitive enough at the low levels of lead believed to be in the tissues of animals given triethyllead. Tissues from animals dosed with triethyllead should not be digested in nitric acid, because triethyllead decomposes to inorganic lead at high temperatures, so the tissues were prepared another way. Tissue was homogenized in a hand-held Kontes glass tissue grinder, with water. The homogenate was spiked with lead, then centrifuged. The supernatant was analyzed for lead by the dithizone method. Proteins were precipitated with 1 ml of 30% perchloric acid in one-half of the samples to see if that had any effect. The addition of tissue homogenates to the dithizone reaction caused interferences that gave false readings by the spectrophotometer. A new method was needed.

Chelating Ion Exchange Chromatography

A chelating carboxylic acid cation exchange resin was developed that has a selectivity for divalent ions over monovalent ions by a factor of approximately 5000 to 1. It is made of a styrene divinylbenzene copolymer with pairs of iminodiacetate ions that act as chelating groups. It has a very strong attraction for transition metals. It is used to remove metal contaminants from buffers, and to concentrate trace metals from seawater samples which are then eluted and analyzed.

Kingston et al. (1978) developed a method for determining the concentration of several divalent metal cations in seawater in trace amounts, by separating them from alkali and alkali earth metals using Chelex 100 chelating resin, then analyzing by GFAAS. The interfering monovalent cations such as Na and K were eluted from the resin first with ammonium acetate, then the divalent cations were eluted with nitric acid. The principle of this method was used to try to separate triethyllead, a monovalent cation, from inorganic lead, a divalent cation, in a biological matrix. In order to optimize conditions of this method, standards in nitric acid were used first.

Procedure. A slurry of Chelex 100 was poured into an 11 ml acid cleaned polypropylene econocolumn to the 2 ml

mark. One ml of water was already in the column to facilitate the packing of the resin. The bottom of the column was attached to a Buchler peristaltic pump with Tygon tubing. The peristaltic pump provided a flow rate in the column of 1.0 ml/min. The resin was washed of contaminants with two 5 ml aliquots of 2.5 M HNO_3, which caused the resin to shrink to approximately half the original volume. Excess acid was removed with a 5 ml aliquot of water pulled through the column. The resin was transformed into the ammonium form with 10 ml of 2.0 M NH_4OH. The pH of the effluent was checked for basicity. The column was rinsed of excess NH_4OH with 5 ml H_2O. If there were any bubbles in the resin at this point, water was pumped backwards through the column from below, which, with tapping the side of the column, dislodged any air bubbles and brought them to the surface of the resin. During column packing and throughout the separation procedure the column was never allowed to run dry.

The fluid level in the column was brought to just above the surface of the resin, and the Tygon tubing was emptied. A 5 ml lead sample pH adjusted to the range 5.0 to 5.5, was added to the top of the resin, pulled through the resin by the peristaltic pump, and deposited into a 5 ml polypropylene tube. Fifteen ml of 2.0 M ammonium acetate (pH 5.0 - 5.5) was added to 5 ml aliquots, followed by 5 ml H_2O, then 15 ml 2.5 M HNO_3. Each 5 ml aliquot was collected

in a 5 ml polypropylene tube. Lead levels in the effluents were measured by GFAAS. The resin and column were discarded.

Ammonium acetate suitable for trace metal analysis was not available, and there was an appreciable amount of lead found in the crystals that were available. Prior to use, a 2.0 M solution of ammonium acetate was made and the pH was adjusted to the range of 5.0 - 5.5 with concentrated HNO_3. Then this solution was run through another econo-column filled with Chelex 100 resin that had been converted to the NH_4^+ form. The collected effluent was analyzed for lead, and found to be lead free. There was no detectable pH change in a 100 ml volume after passing through the resin. This lead free ammonium acetate was used throughout.

This method was first tried with water spiked with lead, until conditions were optimized. The parameters changed from the published procedure included increasing the ammonium acetate concentration from 1.0 M to 2.0 M and increasing the amount of 2.5 M HNO_3 from 7 ml to 15 ml. Figures 6 and 7 show the elution profiles of triethyllead and lead nitrate in the 5 ml volumes of the effluents. When the best achievable separation of lead nitrate and triethyl-lead was obtained, this method was tried using a biological matrix.

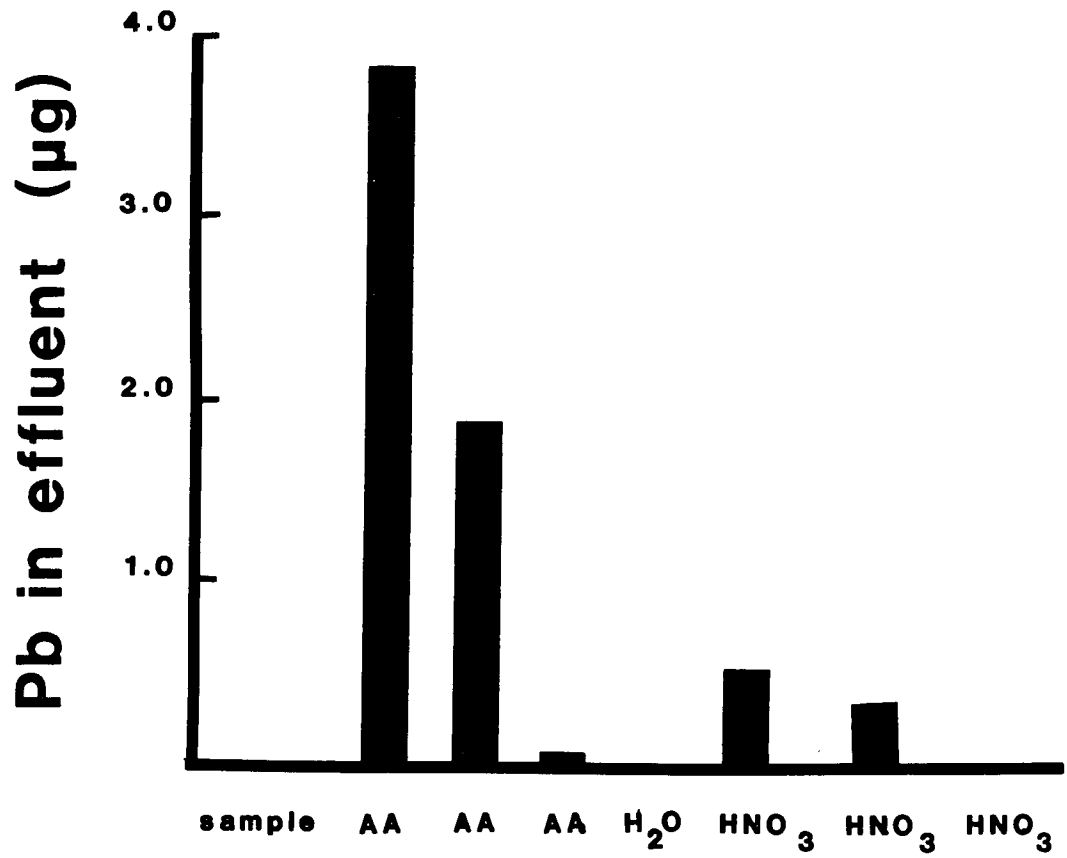


Figure 6. Elution Profile of an Aqueous Solution of Triethyllead Chloride on Chelex 100 Cation Exchange Resin.

5 ml of a 1 µg/ml Pb solution was applied to a prepared Chelex 100 column. 15 ml of the eluents AA and HNO_3 were added to the column in 5 ml aliquots, and collected for Pb determination by GFAAS. 5 ml H_2O was added as a rinse preceding the HNO_3.
AA = ammonium acetate

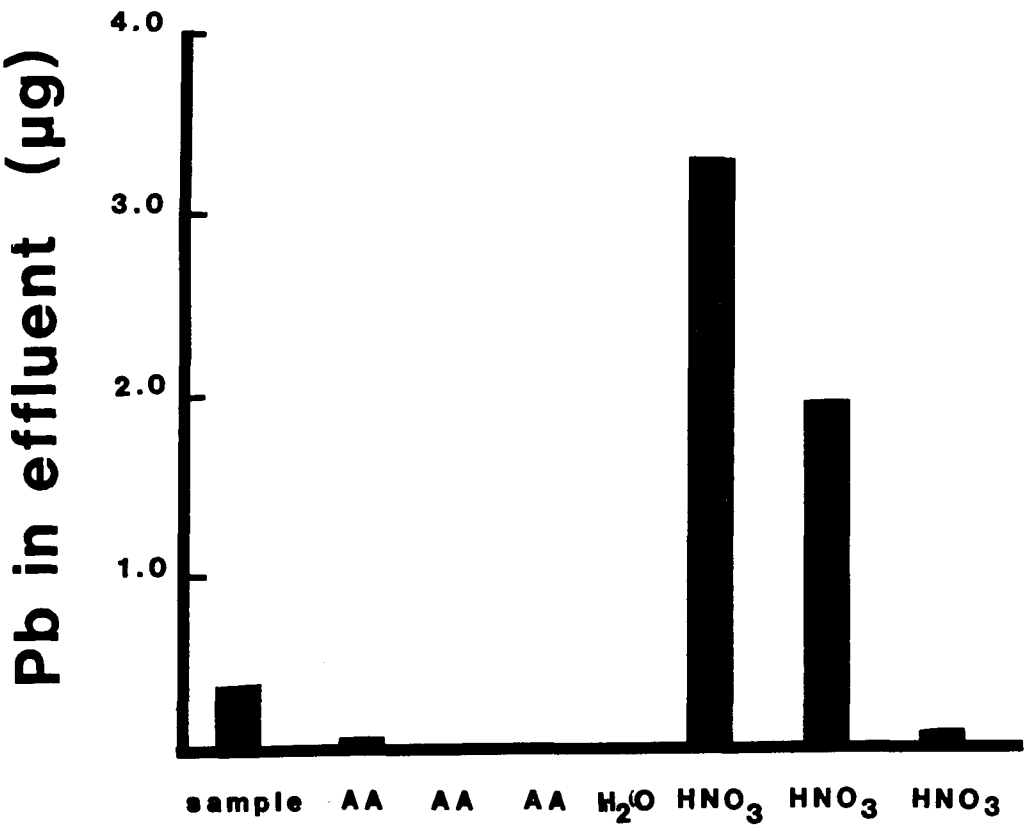


Figure 7. Elution Profile of an Aqueous Solution of Lead Nitrate on Chelex 100 Cation Exchange Resin.

5 ml of a 1 µg/ml Pb solution was applied to a prepared Chelex 100 column. 15 ml of the eluents AA and HNO_3 were added to the column in 5 ml aliquots, and collected for Pb determination by GFAAS. 5 ml H_2O was added as a rinse preceding the HNO_3.
AA = ammonium acetate

Tissue Solubilization

In order to preserve the triethyllead ion if present in animal tissue, a digestion procedure other than hot nitric acid, which decomposes triethyllead to inorganic lead ions, was needed. A method developed by Chau et al. (1984) that solubilizes tissue without altering the chemical form of any alkyllead species was used.

Procedure. Approximately 1.0 g of tissue was weighed and cut into small pieces and placed into an acid washed test tube. Desired amounts of lead from stock solutions were added to the test tube. Two and one half ml of 25% tetramethylammonium hydroxide (TMAH) was added. The test tube was heated in a water bath at 70 degrees C until all the tissue was in solution, approximately 40 minutes. The digest was diluted to 10 ml with H_2O, and was then ready to be applied to the Chelex column.

The digested lead sample in TMAH had a pH of greater than 12. If this was lowered to the recommended pH of at least 5, which was the optimum pH for the chelating efficiency of Chelex 100, proteins precipitate. It was found that there was a 20 - 30% loss of lead due to acid precipitation in digested samples, so the tissue digest was applied to the column at the basic pH.

The elution pattern of triethyllead and lead nitrate changed with the presence of tissue and TMAH, figures 8 and 9. The amount of lead that passed through the resin without adhering increased, so not only was there a problem with some overlap in the elution of the lead species, but the tissue matrix interfered with the resin's ability to retain lead ions. Consequently, a new resin was tried.

Adsorption Chromatography

Diatomaceous earth, (Celite 545). This is a sedimentary composite of the fossil shells of microscopic unicellular algae. The principle compound in diatomaceous earth is silica. It is water insoluble, and is capable of retaining four times its weight of water. In chromatography it is used to retain lipophilic substances from aqueous media that can subsequently be eluted by organic solvents. Diatomaceous earth is also known as kieselguhr.

Da Silva and Diehl (1983) used Celite 545 to separate tetraethyllead and liver microsomes from biological samples to isolate triethyllead. This method was modified to separate and differentially elute triethyllead and inorganic lead. It was hypothesized that the more lipophilic triethyllead would be retained on a column of Celite 545, whereas inorganic lead would not.

Procedure. A slurry of Celite 545 was poured into an econocolumn to the 5 ml mark. The column was attached to

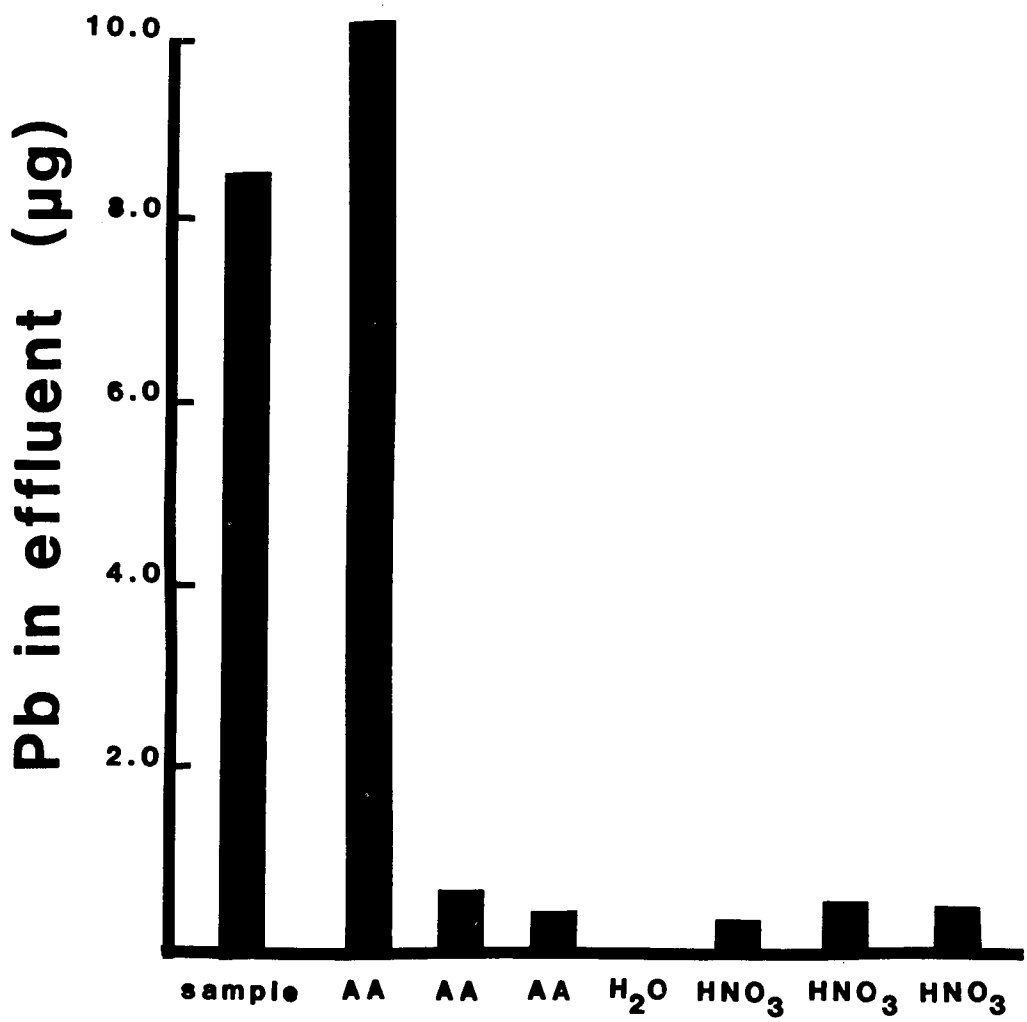


Figure 8. Elution Profile of Triethyllead in Tissue Digested with TMAH from a Chelex 100 Cation Exchange Column.

200 µl of a 100 µg Pb/ml stock solution of Et_3PbCl was added to 1.0 g liver tissue, and digested in TMAH until dissolved. The sample was diluted to 5 ml and applied to a prepared Chelex 100 column. 15 ml of the eluents AA and HNO_3 were added in 5 ml aliquots and collected for Pb determination by GFAAS. A 5 ml H_2O rinse was added preceding the HNO_3.
AA = ammonium acetate

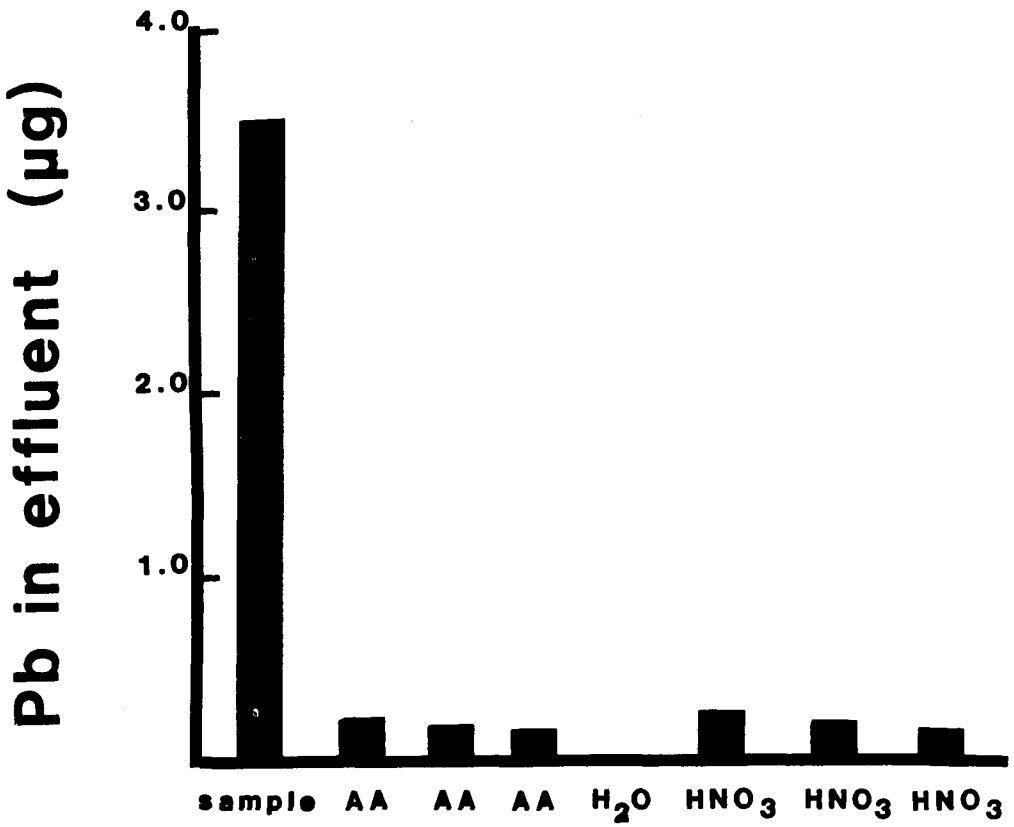


Figure 9. Elution Profile of Lead Nitrate in Tissue Digested with TMAH from a Chelex 100 Cation Exchange Column.

100 µl of a 100 µg Pb/ml stock solution of $Pb(NO_3)_2$ was added to 1.0 g liver tissue, and digested in TMAH until dissolved. The sample was diluted to 12 ml, and 5 ml was placed on a prepared Chelex 100 column. 15 ml of the eluents AA and HNO_3 were added in 5 ml aliquots and collected for Pb determination by GFAAS. A 5 ml H_2O rinse was added preceding the HNO_3.
AA = ammonium acetate

a peristaltic pump with Tygon tubing. The resin was washed with 10 ml of 2.0 M HNO_3, then rinsed with 10 ml H_2O. Lead samples were spiked into a stock solution of liver tissue that was digested in 5 ml TMAH and diluted to 50 ml. Triethyllead was applied to one column, and remained in contact with the resin for 5 minutes before being eluted and replaced with 5 ml H_2O. To remove the triethyllead, 15 ml of 2.0 M HNO_3 was added in 5 ml aliquots. All effluents were collected in 5 ml polypropylene tubes. This was repeated with a new column and new resin and lead nitrate. If lead nitrate was not retained by the lipophilic resin, it would remain in the sample volume or elute with the 5 ml H_2O. Only the sample containing lead was allowed to remain in the resin for the 5 minute period. Elution profiles of the lead species are shown in figures 10 and 11.

Amberlite XAD. This resin is made of agglomerated microspheres, that provide a large surface area for interaction with mobile phases. Adsorption by XAD resins is governed by the hydrophobicity of the solute. The more hydrophobic, the more firmly the solute is bound to the solid beads. Non polar solvents are used to elute compounds adsorbed to the resin. Since triethyllead is much more lipophilic than inorganic lead, it was postulated that it would be retained on an XAD resin, whereas inorganic lead would not. A proposed method for separating triethyllead

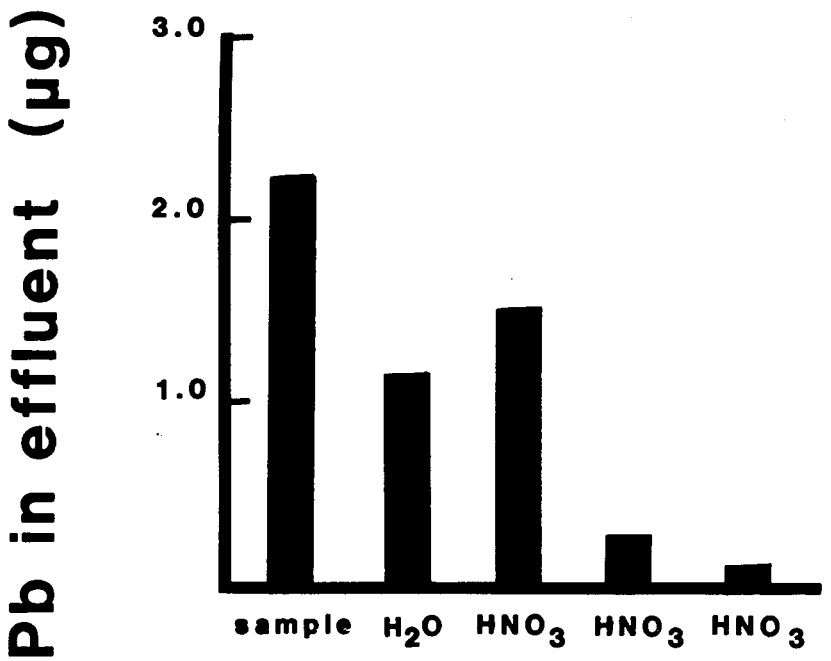


Figure 10. Elution Profile of Triethyllead in Tissue Digested with TMAH on Celite 545.

50 µl of a 100 µg Pb/ml stock solution of Et_3PbCl was added to 5 ml of a stock solution of TMAH digested liver tissue. The sample was shaken and applied to a prepared Celite column. Eluents of 5 ml H_2O then 15 ml HNO_3 in 5 ml aliquots were added and collected for Pb determination by GFAAS.

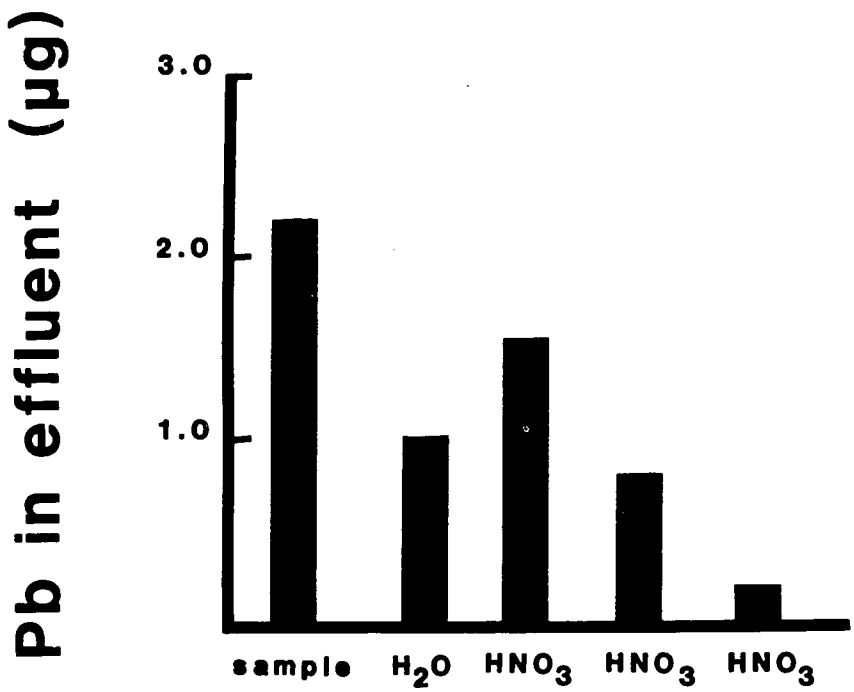


Figure 11. Elution Profile of Lead Nitrate in Tissue Digested with TMAH on Celite 545.

50 µl of a 100 µg Pb/ml stock solution of $Pb(NO_3)_2$ was added to 5 ml of a stock solution of TMAH digested liver tissue. The sample was shaken and applied to a prepared Celite column. Eluents of 5 ml H_2O then 15 ml HNO_3 in 5 ml aliquots were added and collected for Pb determination by GFAAS.

from inorganic lead using water and isopropanol as eluents was developed.

Procedure. One g XAD resin was transferred to an econocolumn. This amount of resin corresponds to a 4 ml volume in the column. A piece of glass wool was placed over the resin because the XAD beads float. The column was attached to the peristaltic pump with Tygon tubing. The resin was washed with 10 ml methanol, then 20 ml H_2O. The column was then made basic with 10 ml of 1.25% TMAH. The resin was now coated with the same matrix as the lead sample. Fifty µl of a 100 µg Pb/ml stock solution of lead nitrate or triethyllead was spiked into 5 ml of a stock solution of TMAH digested liver tissue. This was applied to the column, followed by 10 ml H_2O in 5 ml aliquots, then 5 ml isopropanol. Effluents were collected in 5 ml polypropylene tubes and analyzed for Pb by GFAA.

The XAD resin was not retaining all the triethyllead ions, for later elution by isopropanol. A large percentage was coming out in the pass through of the same application. An ion pairing agent was added, hexane sulfonate, to increase the lipophilicity of the stationary phase.

Procedure. Four ml XAD resin was poured into an econocolumn. Ten ml methanol was eluted through the column, followed by 20 ml H_2O. Then 10 ml 0.1% hexane sulfonate was

added, instead of TMAH. The lead sample was prepared by adding 1 ml 0.01% hexane sulfonate to 4 ml digested liver tissue, and spiking with 50 µl of a 100 µg Pb/ml stock solution of either triethyllead or lead nitrate. The lead sample, 10 ml H_2O and 5 or 10 ml isopropanol were eluted through the column and collected for Pb analysis. If lead nitrate was used, 5 ml isopropanol was applied to the column. If triethyllead was used, 10 ml isopropanol was applied instead for more complete elution of any organic lead. Figure 12 shows the elution profile of triethyllead on an XAD column with the addition of hexane sulfonate.

Cation Exchange Chromatography

A strongly acidic cation exchange resin such as Bio Rad's AG 50W-X8 can be used to separate amino acids, remove cations from buffers and separate metals. The functional anion group is sulfonic acid, attached to a styrene divinylbenzene copolymer matrix. The ionic form of the resin is hydrogen. The relative selectivities of a few cations are listed: H^+ - 1.0

NH_4^+ - 1.95

Ca^{++} - 3.9

Pb^{++} - 7.5.

Lead is one of the most strongly bound ions. To elute it from a column, either an ion with a higher affinity is added to the column, or a large excess of another cation is used.

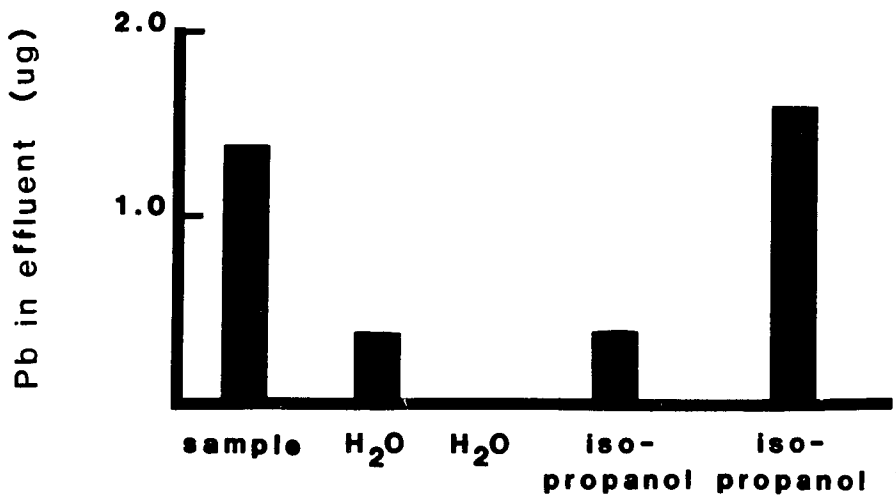


Figure 12. Elution Profile of Triethyllead from XAD2 Resin with The Addition of Hexanesulfonic Acid.

50 µl of a 100 µg Pb/ml stock solution of Et_3PbCl was added to 4 ml digested tissue and 1 ml 0.01% hexanesulfonic acid. This was eluted through a column of XAD2 resin that had previously been coated with 10 ml 0.1% hexanesulfonic acid. 10 ml H_2O followed by 10 ml isopropanol in 5 ml aliquots were eluted through the column and collected for Pb analysis by GFAAS.

For this procedure, Ca was chosen as the competing cation. In a 1 µg Pb/ml solution of lead nitrate or triethyllead, the lead concentration is 4.8×10^{-3} M. In this procedure, a 0.1 M concentration of Ca was used to elute inorganic lead ions, while a 1.0 M NH_4OH solution was used to elute the less tightly held organic lead ions.

Procedure. A 4 ml slurry of AG 50W-X8 resin was poured into an econocolumn, attached to a peristaltic pump. The resin was washed of contaminants with 15 ml 1.0 M HNO_3, then rinsed with 45 ml H_2O. The pH of the resin was then between 6 and 7, which was the same pH as the lead solutions. Fifty µl of a 100 µg Pb/ml stock solution of lead nitrate or triethyllead was diluted to 5 ml with H_2O. This was applied to the column, followed by 10 ml 1.0 M NH_4OH, 5 ml H_2O, and 10 ml 0.1 M $Ca(NO_3)_2$. $CaCl_2$ can not be used because $PbCl_2$ is insoluble. All effluents were collected in 5 ml polypropylene tubes and analyzed for Pb by GFAAS.

Reagents and Equipment

Mobilization of Inorganic Lead

Lead Acetate. J.T. Baker Chemical Co., Phillipsburg, NJ

Nitric Acid Digestion

Nitric Acid, Baker instra-analyzed for trace metal analysis. J.T. Baker Chemical Co.

Lead Nitrate. Baker analyzed, J.T. Baker Co.

Modified Nitric Acid digestion

Boiling Stones, 4 - 12 mesh size. Cargille Scientific Inc., Cedar Grove, NJ

Hydrogen Peroxide, AR, 30% solution. Mallinckrodt Inc., Paris, KY

Watch Glass, Pyrex, 65mm. Corning glass Works, Corning, NY.

Spectrophotometry

Ammonium Citrate, dibasic crystal. J.T. Baker Co.

Chloroform, G.R., ACS reagent. E.M. Industries, Gibbstown, NJ

Diphenylthiocarbazone, ACS reagent. Matheson, Coleman and Bell.

Potassium Cyanide. Fisher Scientific, Fair Lawn, NJ

Perchloric Acid, 70-72%. J.T. Baker Co.

Sodium Sulfite, anhydrous. Polaroid.

Chelex 100 Ion exchange Chromatography

Ammonium Acetate, crystals. J.T. Baker Co.

Ammonium Hydroxide, Baker instra-analyzed for trace metal analysis. J.T. Baker Co.

Chelex 100, 200 - 400 mesh, Na form. Bio Rad laboratories, Richmond, CA

Econocolumn, polypropylene. Bio Rad Laboratories.

Peristaltic pump. Buchler, Fort Lee, NJ

Polypropylene tubes with polythene caps. Sarstedt, Princeton, NJ

Tygon tubing, 1/16" OD, 1/8" ID. VWR Scientific

Tissue Solubilization

Tetramethylammonium Hydroxide (TMAH), 25% in water. Eastman Kodak Co., Rochester, NY

Adsorption Chromatography

Amberlite XAD 2 polystyrene resin, mesh size 20/60. Applied Science Division, State College, PA

Celite 545. Fisher Scientific Co.

Glass wool, Pyrex. Corning Glass Works.

1-Hexanesulfonic Acid, Na Salt. Eastman Kodak Co.

Isopropanol, certified ACS. Fisher Scientific Co.

Methanol, certified ACS. Fisher Scientific Co.

Cation Exchange Chromatography

AG 50W-X8 cation exchange resin, 100-200 mesh, hydrogen form. Bio Rad Laboratories.

CHAPTER 3

RESULTS

Animal Studies

Three different animal studies were performed to study the effectiveness of several water soluble metal binding agents in removing lead from the tissues of rats. Two studies used lead acetate for inorganic lead exposure. Changes in the dosing parameters of route and length of metal binding therapy were made. The third study used the same protocol of dosing the metal binding agents as the first experiment, but an organic form of lead, triethyllead, was used instead. This was done to test the ability of these agents to mobilize this form of lead. Since parameters were changed with each experiment, comparisons of effectiveness between groups should not be made. Only comparisons with the control groups within each experiment will be made.

Mobilization of Inorganic Lead

IM Administration Of Metal Binding Agents. In this experiment, following metal binding therapy, urines were collected, and three tissues were removed for lead analysis.

Urines were collected for four days, starting two days before the metal binding agents were given, and continuing throughout metal binding therapy. Therefore, dosing began at the 48 hour time point. The lead levels for each 24 hour collection are given in Table I, and are portrayed graphically in Figure 13. The total urinary excretion of lead during the 48 hours that the metal binding agents were given is shown in Figure 14. Three of the four agents significantly increased urinary lead excretion when compared to the lead control group. DMSA had the greatest effect, $p < 0.01$, while D-Pen had no effect. DMPS and DMPA also were significantly different from the lead control group, $p < 0.02$ and 0.01 respectively.

The metal binding agents did not influence the lead levels in the kidney after two days of treatment (Figure 15). Although levels in animals treated with DMSA, DMPS and DMPA are lower than those in the Pb control group, they are not low enough to be significantly different. One kidney value in the DMSA group was rejected by the Q test as an outlier and removed from statistical analysis. Not only are there no values that are significantly lower than the Pb control group, the inter-group comparisons show no significant differences either.

In Figure 16, the lead levels found in brain tissue of rats treated with DMSA, DMPS, and DMPA were all

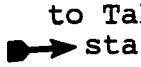
Table I. Urinary Lead Excretion Before and During Metal Binding Therapy.

Dosing group	0 - 24 hr	24 - 48 hr	↓ 48 - 72 hr	72 - 96 hr
control	9.2 ± 1.6	8.6 ± 1.6	15.2 ± 5.6	9.3 ± 1.6
Pb control	15.6 ± 3.6	19.1 ± 3.5	28.4 ± 10.0	18.0 ± 2.9
DMSA	24.3 ± 4.8	23.3 ± 3.9	*121.9 ± 14.9	**75.5 ± 5.0
DMPS	23.3 ± 2.7	21.0 ± 2.5	63.6 ± 16.0	**43.6 ± 3.6
DMPA	21.6 ± 3.2	21.5 ± 3.3	52.0 ± 5.1	*46.0 ± 6.3
D-Pen	23.8 ± 5.1	18.5 ± 1.7	26.6 ± 6.6	*34.8 ± 5.5

units are µg Pb/24 hours.
mean ± s.e.m. as determined by AAS (n=5).
→ start of metal binding administration.
* denotes significant difference from Pb control, $p<0.05$.
** denotes significant difference from Pb control, $p<0.01$.

Figure 13. Daily Urinary Excretion of Lead the 2 Days Prior and the 2 Days During Which Metal binding Agents Were Administered, i.m.

Lead acetate was given, i.p., daily for 7 days at a dose of 2 mg Pb/kg/day. Urine collection began the day following the last lead dose. During the 48-72 hr dosing collection time period, each metal binding agent was given, i.m., at a dose of 0.2 mmol/kg, 3 times a day, for 2 days. Values are means from 5 animals. Refer to Table I for standard errors.


start of metal binding agents.

control animals

Pb control animals

DMSA treated animals

DMPS treated animals

DMPA treated animals

D-Pen treated animals

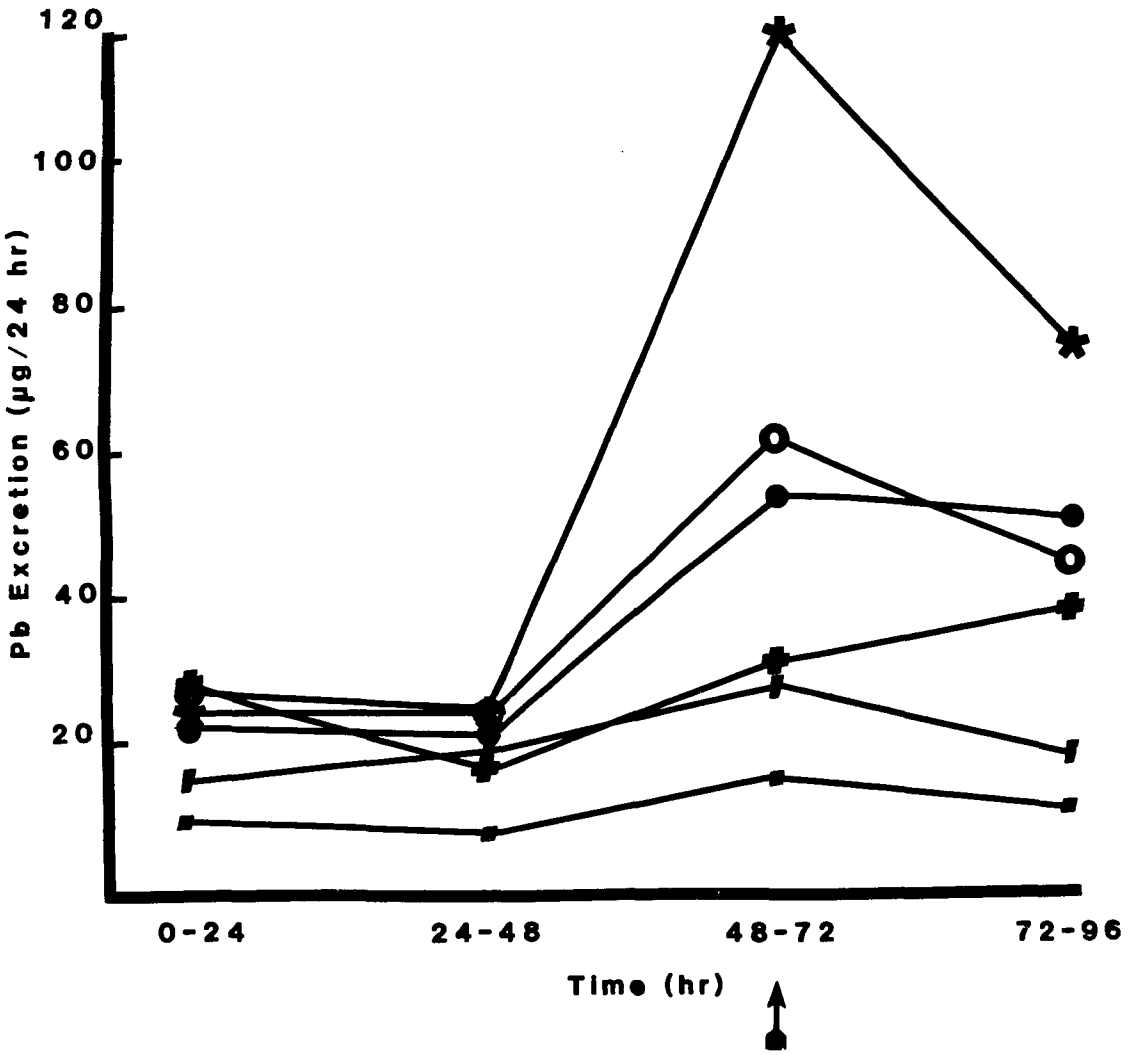


Figure 13. Daily Urinary Excretion of Lead the 2 Days Prior and the 2 Days During Which Metal binding Agents Were Administered, i.m.

Figure 14. Cumulative Urinary Excretion of Lead During the 2 Day Period That Metal Binding Agents Were Administered, i.m.

Lead acetate was given i.p., daily, for 7 days at a dose of 2 mg Pb/kg/day. 72 hours following the last lead injection, each metal binding agent was administered, i.m., at a dose of 0.2 mmol/kg, 3 times/day, for 2 days. Values are mean ± s.e.m. (n = 5) as determined by AAS.
*denotes significant difference from Pb control, $p < 0.05$
** denotes significant difference from Pb control, $p < 0.02$.
*** denotes significant difference from Pb control, $p < 0.01$.

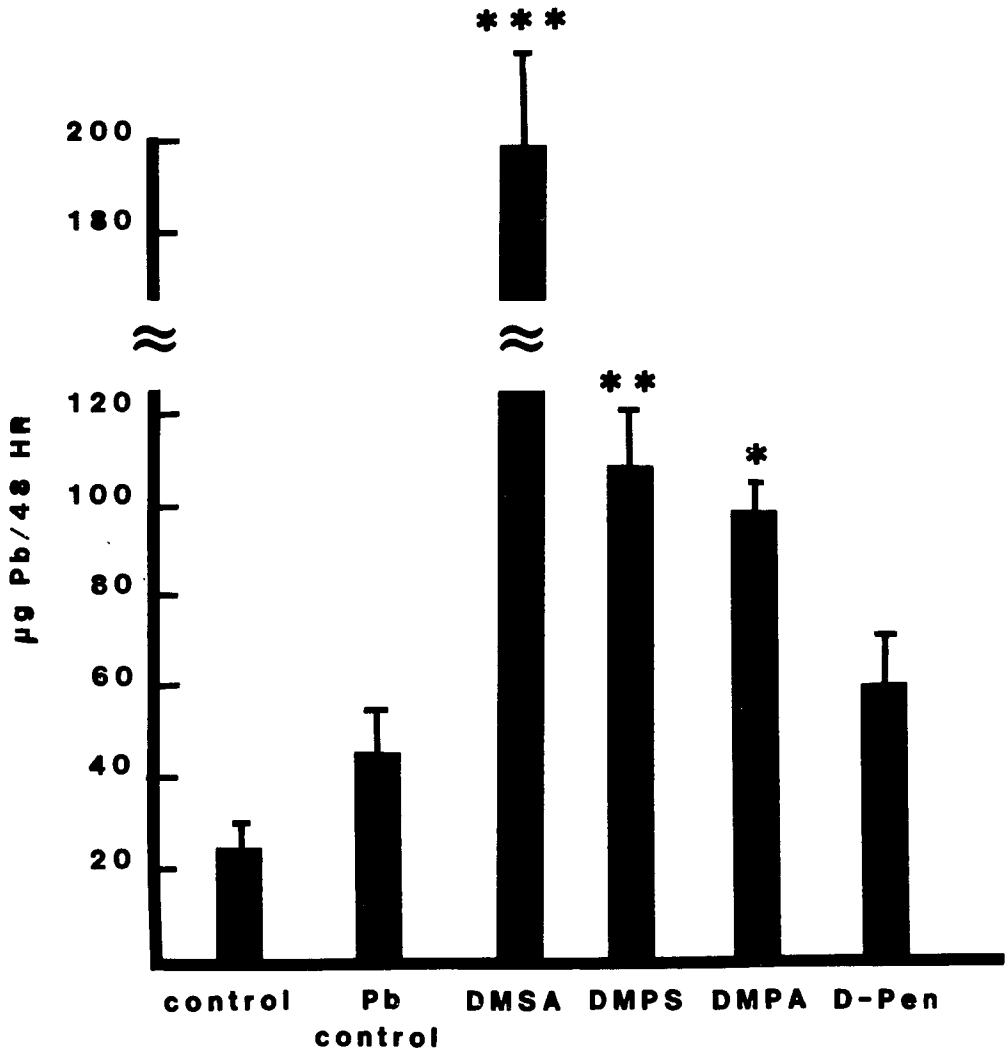


Figure 14. Cumulative Urinary Excretion of Lead During the 2 Day Period That Metal Binding Agents Were Administered, i.m.

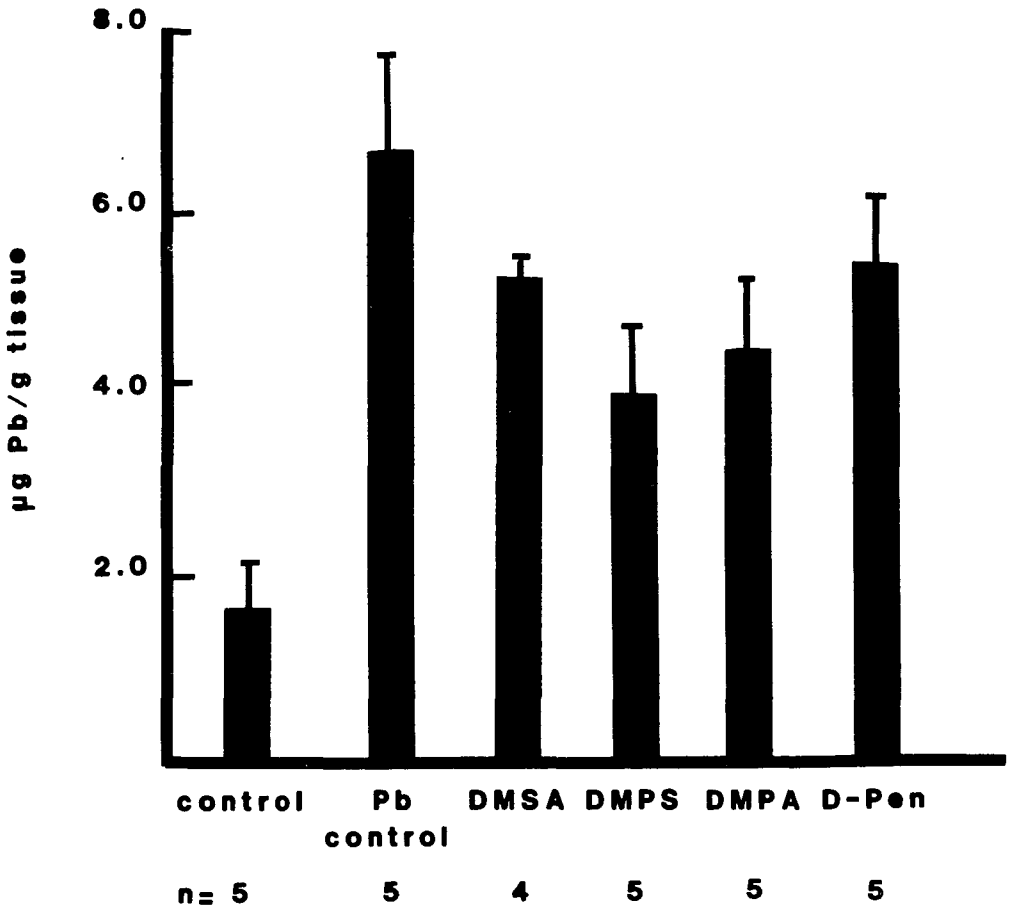


Figure 15. Kidney Lead Levels Do Not Differ Significantly Following Two Days of Metal Binding Agents, Administered i.m.

Lead acetate was given, i.p., daily, for 7 days at a dose of 2 mg Pb/kg/day. 72 hours following the last lead injection, each metal binding agent was administered, i.m., at a dose of 0.2 mmol/kg, 3 times/day, for 2 days. Values are mean ± s.e.m., as determined by AAS.

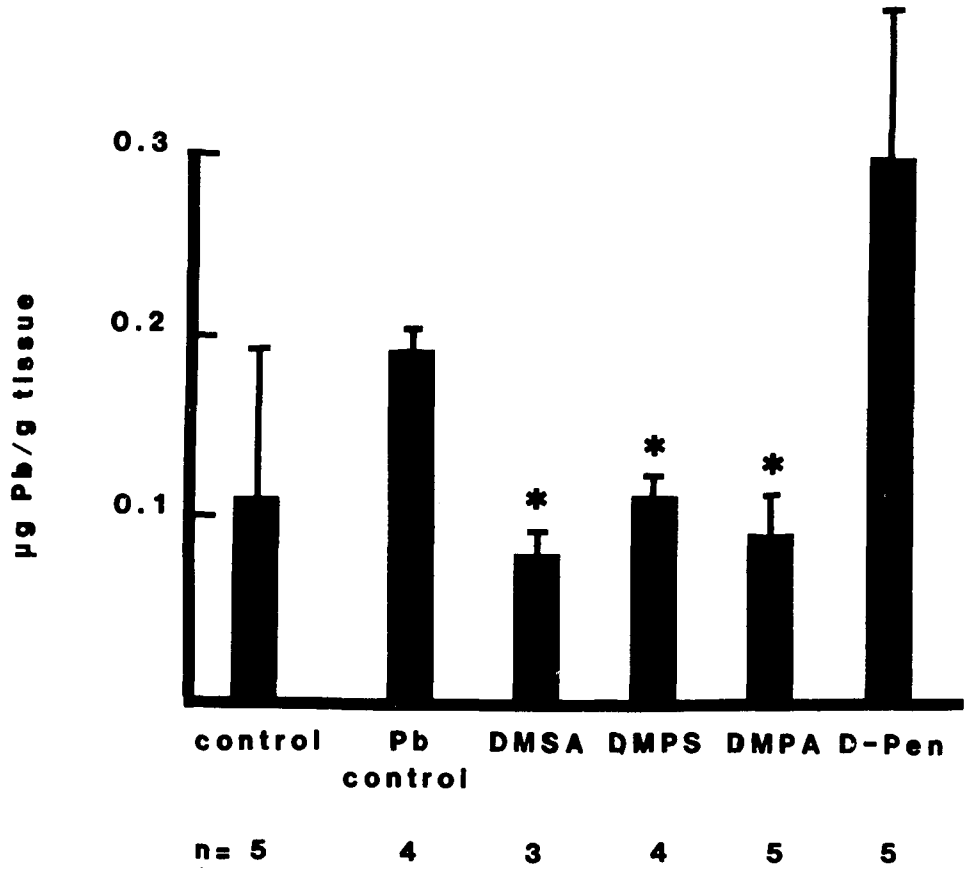


Figure 16. Brain Lead Levels Are Significantly Decreased After the i.m. Administration Of DMSA, DMPS, or DMPA.

Lead Acetate was given, i.p., daily, for 7 days at a dose of 2 mg Pb/kg/day. 72 hours following the last lead injection, each metal binding agent was administered, i.m., at a dose of 0.2 mmol/kg, 3 times/day, for 2 days. Values are mean ± s.e.m., as determined by GFAAS.
*denotes significant difference from Pb control, $p < 0.05$.

significantly lower than the lead control animals, $p < 0.05$. Only the animals receiving D-Pen did not have lowered brain levels. Some of the standard errors amongst the brain tissues are very large, especially in the control group and the D-Pen group. Also, three values were found to be outliers by the Q test, one in the Pb control group, 2 in the DMSA group, and 1 in the DMPS group. Of all tissues tested, brain tissue presents the most variability in measured lead levels.

In femoral tissue, only the animals treated with DMSA showed a significant lowering of lead levels as compared to the Pb control animals (figure 17). But that value did not approach the untreated control group level. It should be noted that this is only after 2 days of metal binding therapy. The Q test rejected one outlier in the control group and one in the Pb control group.

In sum, in animals loaded with lead acetate for 7 days, then given the metal binding agents by the i.m. route, the effects of the different metal binding agents on different tissues were varied. On urinary lead excretion, DMSA is clearly the most effective, but DMPS and DMPA also are active in removing lead from the body. There was no significant change from Pb control values by any of the four compounds in lowering lead levels in kidney tissue. DMSA, DMPS and DMPA each lowered the levels of lead in the brain

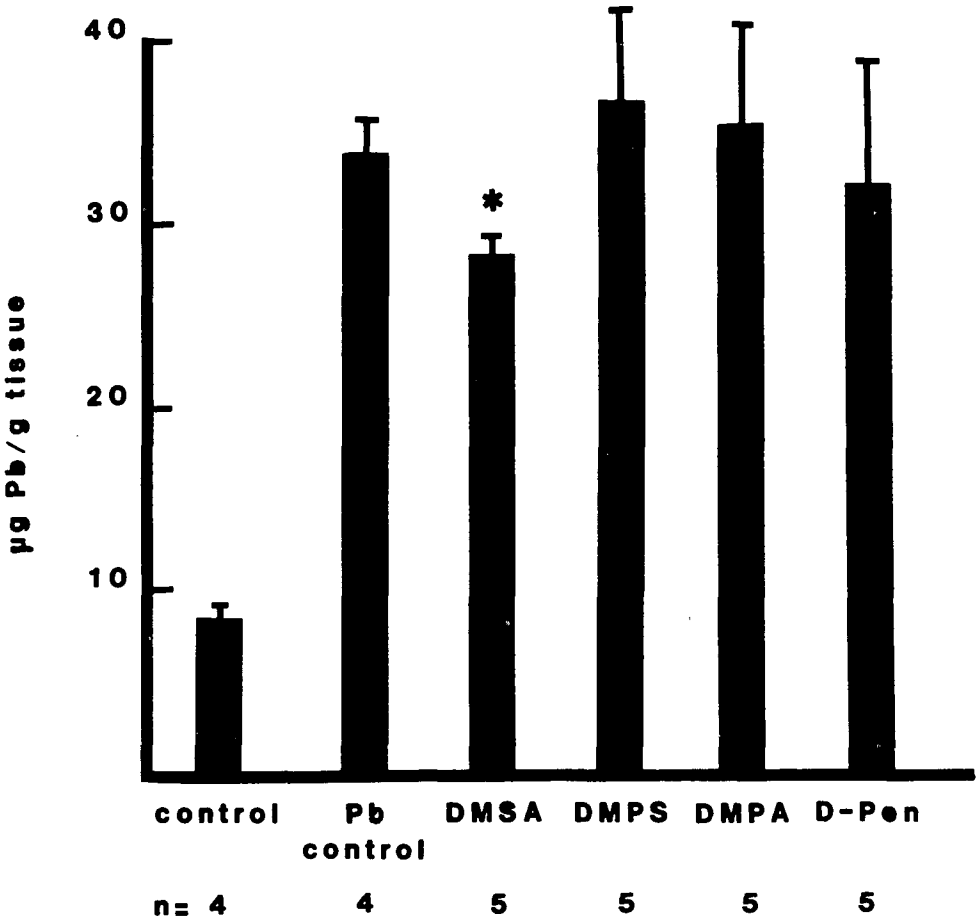


Figure 17. DMSA Lowered Lead Levels In Bone Tissue When Administered i.m.

Lead acetate was given, i.p., daily, for 7 days at a dose of 2 mg Pb/kg/day. 72 hours following the last lead dose, each metal binding agent was administered, i.m., at a dose of 0.2 mmol/kg, 3 times/day for 2 days. Values are mean ± s.e.m. as determined by AAS. * denotes significant difference from Pb control, $p < 0.05$.

by the same factor, causing a significant decrease compared to Pb controls. Only DMSA lowered bone lead levels significantly. After two days of parental administration, DMSA was the most effective in removing inorganic lead from 2 of 3 organs tested, and in the most critical organ, the brain, DMPA and DMPS were also effective. D-Pen was not effective in removing lead from any of the organs tested nor demonstrated any ability to excrete lead in the urine.

Graphically, only compounds from groups that differed significantly from the lead control groups were designated by asterisks. But some intergroup differences occurred; specifically in brain tissue, DMSA values were significantly lower than DMPS values ($p < 0.05$). Also in brain tissue, the control values were so varied that they were not different from Pb controls.

Oral Administration of Metal Binding Agents. Since DMSA decreased bone and brain Pb levels, it seemed worthwhile to see if it would do so as or more effectively if given for longer periods of time, and given p.o. DMPS was also included for comparison. D-Pen was not chosen for this experiment because of the poor response seen when it was administered i.m. The dosing schedule was increased from two days to eight to enhance any positive effects by DMPS and DMSA. Gastric lavage, however, as a dosing procedure carries with it the risk of perforating the esophagus or

stomach each time a dose is given. An eight day dosing schedule therefore was chosen as a reasonable length of time for this study. No problems with this dosing schedule occurred.

Brain, kidney and bone tissues were removed from the experimental animals to study the effects of DMSA and DMPS. Figure 18 shows the effects DMSA and DMPS have on removing lead from the kidney. Both compounds lowered lead levels significantly compared to lead control animals. The DMPS value approaches the control group value.

Those animals treated with DMPS had brain lead levels that were significantly lower than the lead control animals. The DMSA values were so varied that no change from Pb control is seen (Figure 19). This was not due to one animal, the values from all five animals were widely scattered. The levels of lead found in the femurs of rats were not changed with DMSA or DMPS treatment. Neither compound significantly removed lead from bone tissue as compared to the lead control animals (Figure 20).

In summary, after an 8 day regimen of 3 oral doses per day, both DMSA and DMPS were effective in lowering lead levels in kidney tissue, neither produced a change in bone tissue, and only DMPS was effective in removing lead from the brain. In a comparison between DMPS and DMSA values,

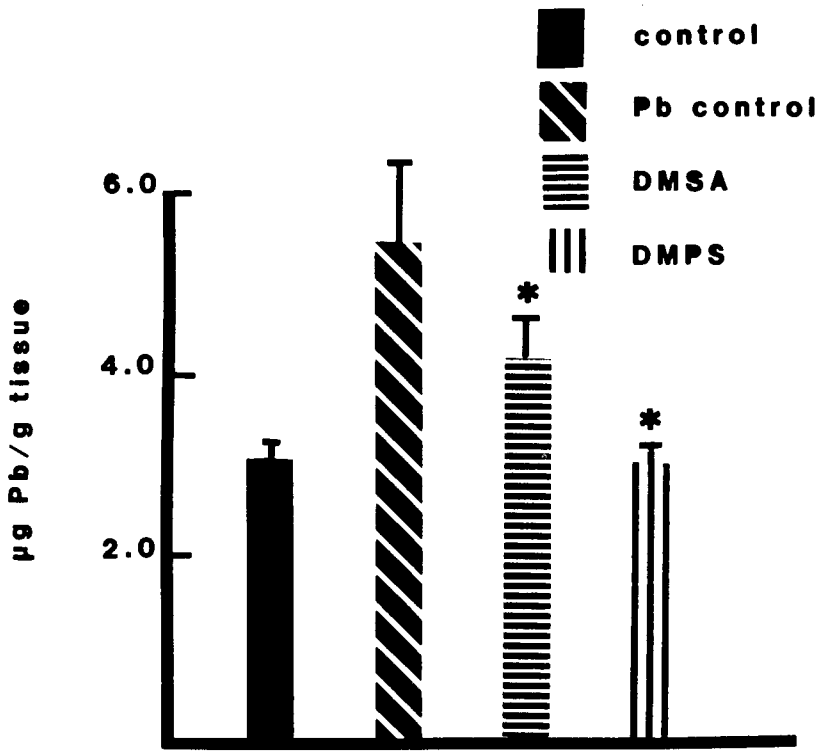


Figure 18. DMSA and DMPS Lowered Kidney Levels of Lead When Given p.o.

Lead acetate was given, i.p. daily, for 7 days at a dose of 2 mg Pb/kg/day. 72 hours following the last lead dose, each metal binding agent was administered, p.o. at a dose of 0.2 mmol/kg, 3 times/day, for 8 days. Values are mean ± s.e.m., as determined by AAS (n = 5).
* denotes significant difference from Pb control, $p < 0.05$.

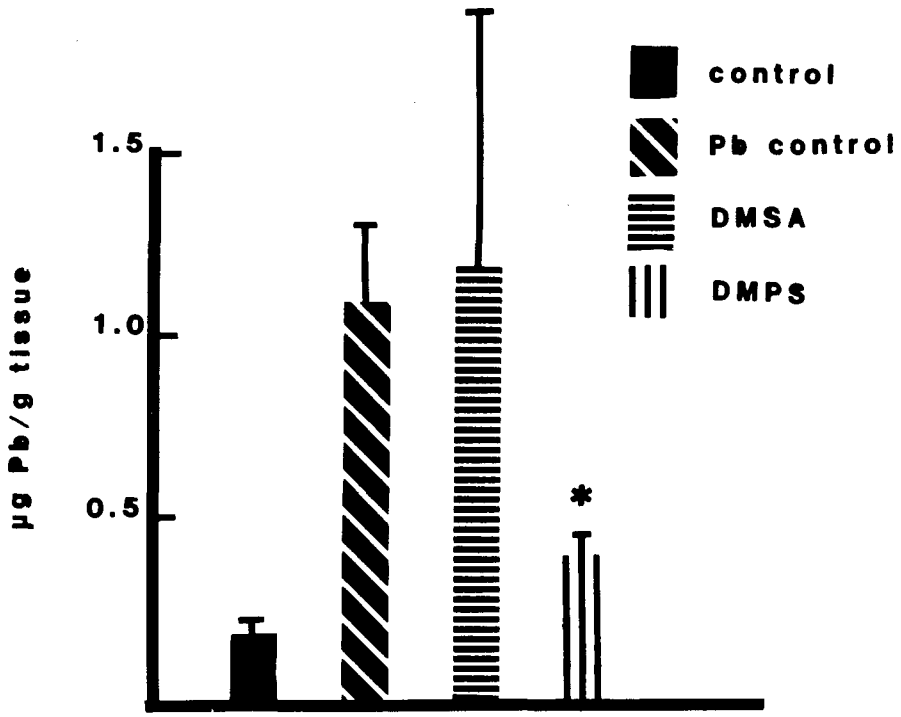


Figure 19. DMPS Was Effective in Lowering Lead Levels in Brain Tissue When Administered p.o.

Lead acetate was given, i.p. daily, for 7 days at a dose of 2 mg Pb/kg/day. 72 hours following the last lead dose, each metal binding agent was administered, p.o., at a dose of 0.2 mmol/kg, 3 times/day, for 8 days. Values are mean ± s.e.m, as determined by GFAAS (n = 5).
*denotes significant difference from Pb control, $p < 0.05$.

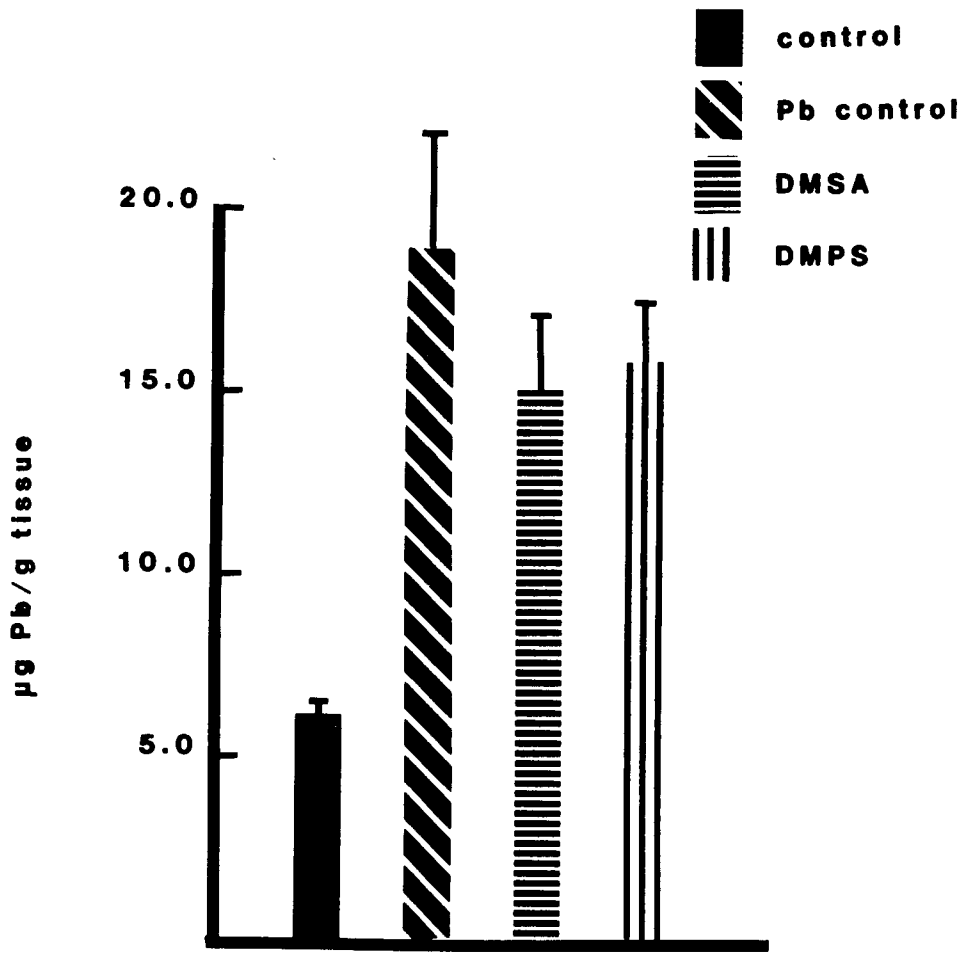


Figure 20. Bone Lead Levels Were Not Lowered by the p.o. Administration of DMSA or DMPS.

Lead acetate was given, i.p., daily, for 7 days at a dose of 2 mg Pb/kg/day. 72 hours following the last lead dose, each metal binding agent was administered, p.o., at a dose of 0.2 mmol/kg, 3 times/day, for 8 days. Values are mean ± s.e.m., as determined by AAS (n = 5).

there was no significant difference in any of the tissues analyzed.

Mobilization of Triethyllead

IM Administration of Metal Binding Agents. The activity of DMSA and DMPS to remove organic lead from rat tissues was studied. The lead levels of three different tissues, kidney, brain, and bone, were compared in animals that received triethyllead, and triethyllead followed by DMSA or DMPS. No control animals were used. The dose of 1 mg Et_3Pb/kg/day was chosen from a preliminary study that was done with 6 rats. Two received, 3 mg Et_3Pb/kg/day, i.p., for 7 days, 2 received 2 mg Et_3Pb/kg/day, and 2 received 1 mg Et_3Pb/kg/day. Animals in the highest dosing group died by the 6th and 7th day. The two animals receiving the 2 mg Et_3Pb/kg dose showed classic signs of organic lead toxicity; they were very jumpy, excitable and easily startled. Those animals receiving a dose of 1 mg Et_3Pb/kg had no overt signs of organic lead poisoning, so this dose was chosen for the experiment.

DMSA and DMPS did not have an effect on brain tissue after triethyllead was administered (Figure 21). Although both groups that received metal binding agents appeared to have higher lead levels than that of the lead control group, the differences were not significant.

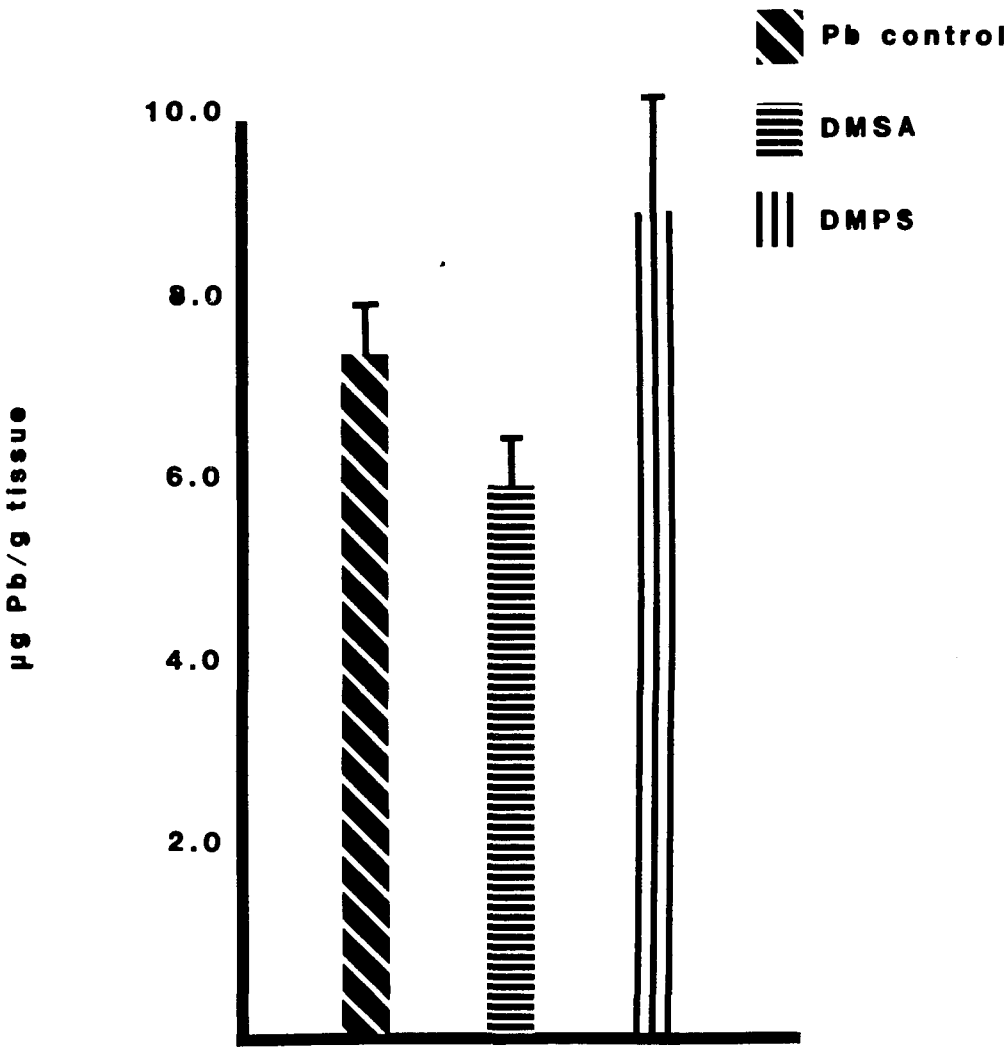


Figure 21. Brain Lead Levels in Rats Given Triethyllead Chloride Were Not Lowered by The i.m. Administration of DMSA or DMPS.

Triethyllead Chloride was given, i.p. daily, for 7 days at a dose of 1 mg Et_3Pb/kg/day. 72 hours following the last lead injection, each metal binding agent was administered, i.m., at a dose of 0.2 mmol/kg, 3 times/day, for 2 days. Values are mean ± s.e.m., as determined by GFAAS (n = 5).

There is no significant difference found in kidney tissue between the groups receiving DMSA or DMPS and the group that received only triethyllead (Figure 22).

Levels of lead in bone tissue were compared (Figure 23). Again, no differences occur between the animals receiving triethyllead and those receiving triethyllead and DMSA or DMPS.

To summarize, DMSA and DMPS were not effective under these conditions in lowering levels of lead administered as triethyllead in any of the tissues examined.

Quantitative Determination of Organic and Inorganic Lead

After a search for a method of determining levels of both organic lead and inorganic lead that did not require a major investment in analytical equipment (ie., GC/MS apparatus), it became apparent that a sensitive, practical method had yet to be developed. Several methods were explored and modified for the possibility of allowing accurate measurement of both lead species in biological samples.

Spectrophotometry

The method of Henderson and Snyder published in 1961 was accurate in detecting a wide range of triethyl, diethyl and inorganic lead levels in the same sample by measuring the absorbance of each dithizonate complex, when in an

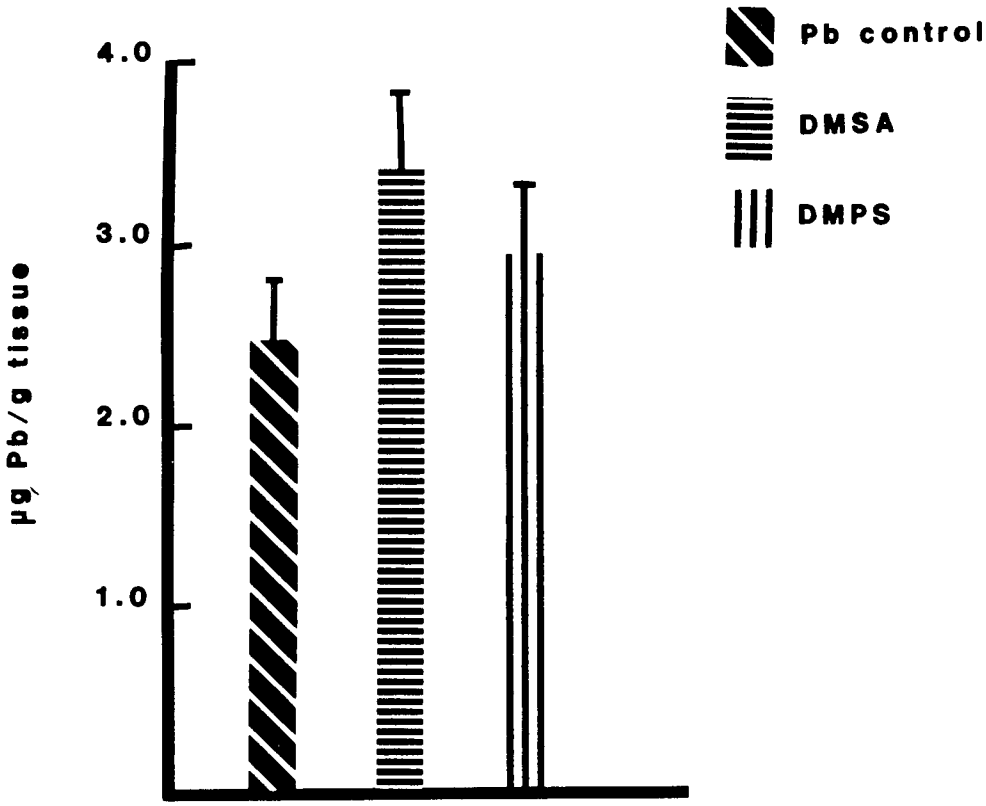


Figure 22. Kidney Lead Levels in Rats Given Triethyllead Chloride Were Not Lowered by The i.m. Administration of DMSA or DMPS.

Triethyllead Chloride was given, i.p. daily, for 7 days at a dose of 1 mg Et_3Pb/kg/day. 72 hours following the last lead injection, each metal binding agent was administered, i.m., at a dose of 0.2 mmol/kg, 3 times/day, for 2 days. Values are mean ± s.e.m., as determined by GFAAS (n = 5).

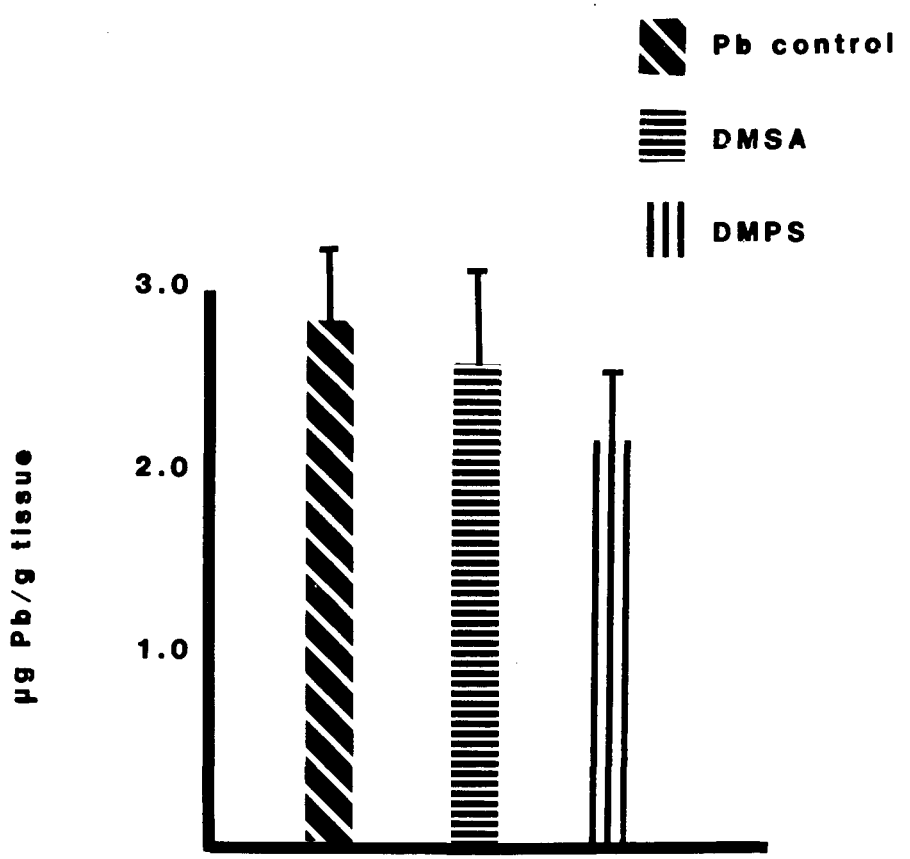


Figure 23. Bone Lead Levels in Rats Given Triethyllead Chloride Were Not Lowered by The i.m. Administration of DMSA or DMPS.

Triethyllead Chloride was given, i.p. daily, for 7 days at a dose of 1 mg Et_3Pb/kg/day. 72 hours following the last lead injection, each metal binding agent was administered, i.m., at a dose of 0.2 mmol/kg, 3 times/day, for 2 days. Values are mean ± s.e.m., as determined by GFAAS (n = 5).

aqueous medium. When the authors used this method to determine lead species in leaded fuel, interferences prevented a practical application of this method. The same results were found when this method was tried with triethyllead and lead nitrate in water and in a digested tissue matrix.

Table II shows the results of the recovery study of different amounts of lead in water as determined by the dithizone reaction. The interferences caused by the digested tissue matrix are shown in Table III. The results of this assay are not reproducible with the addition of acid digested tissue. In homogenized tissue, with or without acid precipitation of the proteins, there was so much interference that the organic and aqueous phases in the dithizone reaction mixture only partially separated, and a large emulsion formed at the interface. Spectrophotometric measurements were not reproducible and meaningless compared to the levels of lead added, in either form, and with or without acid precipitation. This method was not accurate at the low levels of lead believed to be in tissues of animals dosed with triethyllead. If there was not a method for determining lead species simultaneously, then a method for separating them was needed.

Table II. Recovery of Aqueous Solutions of Triethyllead and Lead Nitrate by the Dithizone Spectrophotometric Assay.

Pb added (μg)		Pb found (μg)		error (μg)	
Pb^{++}	Et_3Pb^+	Pb^{++}	Et_3Pb^+	Pb^{++}	Et_3Pb^+
10.0	10.0	10.6	11.0	+0.6	+1.0
20.0	20.0	17.6	17.8	-2.4	-2.2
10.0	5.0	12.6	6.9	+2.6	+1.9
5.0	5.0	7.0	7.0	+2.0	+2.0
2.0	2.0	1.9	3.1	-0.1	+1.1
1.0	1.0	0.8	3.0	-0.2	+2.0
0.0	2.0	-1.1	2.4	-1.1	+0.4
2.0	0.0	2.4	0.6	+0.4	+0.6
				$\bar{x}$ = 0.9	0.3
				SD = 1.4	1.6

Table III. Recovery of Lead in Acid Digested Tissue by the Dithizone Spectrophotometric Assay.

Pb added (µg)		Pb found (µg)		error (µg)	
Pb^{++}	Et_3Pb^+	Pb^{++}	Et_3Pb^+	Pb^{++}	Et_3Pb^+
0.0	0.0	-0.9	4.8	-0.9	+4.8
0.0	0.0	-0.4	5.1	-0.4	+5.1
3.6	0.0	7.0	5.0	+3.4	+5.0
3.6	0.0	6.6	5.8	+3.0	+5.8
0.0	8.9*	4.7	15.7	4.7	+6.8
			$\bar{x}$ =	2.0	5.5
			SD =	2.5	0.8

*spiked to aliquot of tissue digest.

Chelating Ion Exchange Chromatography

The method of Kingston et al., (1978) effectively separated the interfering alkali and alkaline earth metals such as Na, K, Ca and Mg from transition metals such as Pb that occur in large concentrations in seawater. The presence of these other metals caused loss of analyte and dampening of the absorbance signal when analyzed by GFAA. The use of Chelex 100 resin was optimized for the best separation of the various metals which included choosing ammonium acetate over ammonium nitrate for a sharper elution peak, and finding the best pH range of 5.0 to 5.5 for eluting metals from the resin.

The principles of this method were used for the separation of triethyllead from inorganic lead in digested tissue. Aqueous solutions of each lead species were eluted through a Chelex 100 column. It was found that three 5 ml aliquots of 2.0 M ammonium acetate and three 5 ml aliquots of 2.5 M HNO_3, with 5 ml H_2O in between, eluted all the lead completely. In fact, the 5 µg Pb as Et_3PbCl theoretically placed on the column in Figure 6, eluted as 6.6 µg when analyzed by GFAA, a 130% recovery.

Six µg Pb as $Pb(NO_3)_2$ eluted from the column used for Figure 7, a 120% recovery. As shown graphically in Figure 6, most of the triethyllead elutes with the ammonium

acetate fraction, but 12% of the total lead elutes with HNO_3. This happened consistently when triethyllead was used. In Figure 7, 9% of the total lead eluted was found in the original sample volume that passed through the column and the first ammonium acetate fraction. This also happened consistently with $Pb(NO_3)_2$.

Though not perfect, this method was then tried with tissue digested in TMAH, and spiked with triethyllead or lead nitrate. The elution pattern changed in the presence of TMAH, as shown in Figures 8 and 9. In Figure 8, 20 µg Pb as Et_3PbCl was applied to the column, and 20.88 µg eluted, but 41% of the lead was not retained in the resin, instead it remained in the sample volume. Fifty-two percent was in the first ammonium acetate fraction, and the rest eluted in small portions with the other eluents. $Pb(NO_3)_2$ had similar results. Five and two-tenths µg Pb was applied to the column (this value was obtained with a 50 µl volume of sample removed for analysis by GFAAS immediately preceding the application of the sample). Four and two-thirds µg Pb eluted off the column, but 76% of that never was retained by the chelating resin. The other 24% did not selectively elute in the HNO_3 fractions. The interferences due to the addition of TMAH masked the chelating ability of the iminodiacetate ions to retain lead.

Adsorption Chromatography

Celite 545. The use of Celite 545 was based on the principle that triethyllead, with its lipophilic ethyl groups, would be retained on this matrix, whereas lead nitrate would not. The lead nitrate would then appear in the first fractions from the column, and the triethyllead would elute later; the reverse of the elution pattern used with Chelex 100 chelating resin. Triethyllead was eluted with HNO_3.

The purpose of holding the lead sample in the resin for a five minute period is to allow maximum adsorbance of the organic lead species onto adsorbing sites. Figures 10 and 11 show the elution of both lead species from Celite 545. They elute similarly, with good recovery of the lead species. There was 114% recovery of lead as triethyllead, and a 90% recovery of lead as lead nitrate. The Celite did not retain triethyllead, but allowed 44% to remain in the sample volume. Forty percent of the total amount of lead nitrate that eluted was in the sample volume. The similarity of elution between the two lead species made this resin impractical for the purpose of separation.

XAD Resin. The same principle of adsorption due to the lipophilicity of the resin matrix applies to XAD2 resin as it did to Celite 545. Triethyllead being more lipophilic

than lead nitrate would be retained, for later elution with an organic solvent. Isopropanol was chosen for its lipophilicity and its water solubility.

The XAD resin was not strong enough to retain the triethyllead ions from the sample volume. The addition of an ion-pairing agent, hexane sulfonate, was added, to increase the lipophilicity of triethyllead. The column was coated with 0.1% hexane sulfonate, and 0.01% was added to the sample. The SO_3^- group could ion-pair with the monovalent triethyllead, and could also ion-pair with Pb^{++}. The long hexyl group will adsorb well onto the solid phase XAD resin. A 0.01% hexane sulfonate solution contains 0.53 mM hexane sulfonate, and a 1 µg Pb/ml lead solution contains 4 mM Pb. A more concentrated solution of hexane sulfonate could not be used, due to interferences with the GFAAS. The addition of this concentration of the ion-pairing reagent caused a 10 fold depression of the Pb signal seen by the GFAA detector. The aspiration time of the standards and the samples was increased from 2 seconds to 10, and the absorbance values were still only one-half the normal values of a water matrix.

The elution pattern of triethyllead with these conditions is shown in Figure 12. Even with the addition of hexane sulfonate, 37% of the triethyllead remained in the sample volume. The total lead recovery was 76%, as 5.0 µg

Pb was applied to the column, and 3.8 µg eluted. Thirty-seven percent of the total organic lead eluting with the sample volume is too much to be able to differentiate it from inorganic lead.

Cation Exchange Chromatography

The use of AG 50W-X8 to bind cations such as Pb^{++} and Et_3Pb^+ was tested. Because lead is so strongly bound, a large excess of the competing compound was needed. It is known that calcium dampens the lead signal seen by the GFAA detector (Kingston et al., 1978). A preliminary study measuring lead nitrate spiked to aqueous solutions of various concentrations of $Ca(NO_3)_2$ proved this. $Pb(NO_3)_2$ was added to solutions of 1.0 M Ca, 0.1 M Ca and 0.01 M Ca. The lead levels measured were consistently 40% depressed at each calcium concentration. Knowing that a large excess of Ca was needed to elute the more tightly bound Pb^{++}, 10 ml of 0.1 M $Ca(NO_3)_2$ was chosen. Ten ml of 1.0 M NH_4OH is used to elute Et_3Pb^+ ions, which, being monovalent, would be less tightly held by the resin.

Unfortunately, 10 ml 0.1 M Ca did not successfully replace Pb^{++} ions from the cation exchange resin, nor did it cause the elution of Et_3Pb. Effluents collected after 5 µg Pb as $Pb(NO_3)_2$ was applied to the column contained no lead, and effluents collected after 5 µg Pb as Et_3PbCl was applied

contained only 1.7 µg Pb, a 34% recovery. It appears that Pb ions are bound too tightly to be eluted with calcium. Barium has a higher affinity for the resin than lead (8.7). Future studies with this resin should investigate this as a counter ion.

CHAPTER 4

DISCUSSION

The goals of this project were twofold, to study the ability of several water soluble metal binding agents to remove lead from specific tissues in the rat, and to find a viable method for determining levels of triethyllead in the tissues of animals exposed to it.

Animal Studies

When lead is introduced into the bloodstream, it can bind to many components, including sulfhydryl containing enzymes and proteins, heme and other porphyrins. Lead equilibrates with tissues such as kidney and liver, but is sequestered into bone. The equilibrium is directed towards moving lead into bone stores. The purpose of chelation therapy is to provide a compound that has more affinity for metal ions than any endogenous substance. Even if the chelating agent can not penetrate the sites of lead accumulation, such as crossing cell membranes, it can still have an effect by shifting the equilibrium from out of the affected cells into the extracellular space, as the chelator removes lead from this space. Enough chelating agent must

be available to remove the existing lead stores. These factors were in mind when the protocol for the animal experiments was developed.

The effectiveness of these metal binding agents were studied in three different ways. They were examined, after i.m. administration, and therefore completely absorbed by the body, for their ability to remove inorganic lead from specific organs. They were examined when given orally, which is the aim in the development of these compounds. And they were also studied for their effectiveness in removing lead from the body when the exposure was due to organic lead, and the agents were given i.m. Organic lead has different manifestations of toxicity and causes damage to different organ systems.

Mobilization of Inorganic Lead

IM Administration of Metal Binding Agents. The results of an i.m. administration of DMSA, DMPS, DMPA and D-Pen to rats previously injected i.p. with lead acetate revealed that DMSA increased urinary lead output fivefold, it decreased the level of lead in brain tissue significantly, and was the only compound to decrease bone stores of lead. In this study, DMSA was the most effective agent. DMPS and DMPA worked equally as well as DMSA in the brain, which is the most important of all tissues on which to have

an effect. DMPS and DMPA significantly increased urinary lead excretion also, but not to the extent DMSA did.

Only D-Pen failed to lower lead levels in any of the tissues measured, or increase urinary lead excretion. It is known that D-Pen is less effective than BAL or EDTA in mobilizing lead stores (Hammond, 1971). It is given when lead levels in patients are not so elevated that the extensive BAL/EDTA regimen is required. It is not surprising that the analogues of BAL, which retain the vicinyl dithiol groups, are more effective in binding lead and removing it from tissues and out of the body.

Kidney tissue was the only tissue that showed no significant changes in lead levels as a result of any metal binding therapy. This could be due to the fact that kidney tissue is the last place lead ions are before leaving the body, as evidenced by increased urinary excretion. The mechanism of urinary lead excretion is not completely known. The prevailing hypothesis is that lead is filtered by the glomerulus, with variable amounts of reabsorption (Vander et al., 1977). Lead toxicity in the kidney occurs in the proximal tubules, with depression of glucose, amino acids and phosphate. Lead may have accumulated in the kidney in stores unavailable to the metal binding agents. Or the majority of the compounds may have been metabolized before

reaching the kidney, therefore unable to bind any available lead. Also, knowing that the kidney does excrete lead when possible, the lead stores may have still been in a state of flux when levels were measured. If a longer period of metal binding therapy was allowed, or more time following therapy occurred, significant changes in lead levels might be seen.

The fact that these agents remove lead from the brain is important, especially in the case of children. The brain is the most sensitive target organ. There is no therapeutic value in a compound that increases urinary output of a metal if there is no corresponding decrease in concentration at the target organ (Aaseth, 1978).

The levels of lead measured in each tissue reflects the activity of each to absorb, retain and release lead. The bone tissues, as the storage place for lead, has ten times the levels seen in the kidney. As the excretory route, kidney has ten times higher levels than the brain. In these animals, the partitioning of lead between the three organs studied show that the organs that contain the most lead are not the ones most easily damaged.

It is noted that measurable amounts of lead were found in the control animals. An especially variable amount was seen in the brain. The animals, though only 2 - 3 months old, could have been exposed to lead via their food,

the drinking water, and the metal cages where they live and on which they chew.

Oral Administration of Metal Binding Agents. Due to the nature of the BAL analogues DMSA and DMPS, the next logical step in the study of lead mobilization is to administer them orally. These compounds have hydrophilic functional groups making them more water soluble, and absorbable by the GI tract. BAL is not useful orally due to its lipophilicity. It is administered only by a painful i.m. injection.

D-Pen was excluded from this oral dosing study because it was ineffective compared to the other compound when given i.m. D-Pen's usefulness is its activity when given orally. The oral effectiveness depends on the GI absorption. The absorption of some of these compounds has been studied. D-Pen is well absorbed from the GI tract. It is postulated that DMSA is well absorbed (Graziano et al., 1978), but others found it species dependent (McGown et al., 1984) and only 30-40% of DMPS is absorbed (Gabard, 1978).

Only DMSA and DMPS were used for the oral administration study. The dosing schedule was increased from two days to eight to allow for incomplete absorption of the compounds and to extend the deleading time.

Bone, brain and kidney lead levels were examined from control animals and treated animals for the effectiveness of these compounds via this route. This time, DMPS treated animals showed significant changes in lead levels of two of the three tissues tested. DMSA was effective in only one.

Both DMSA and DMPS lowered levels of lead in the kidney. Eight days of therapy was effective, when two days was not. This shows the need for providing sufficient dosages of the compounds to deplete all available lead stores, both in the kidney and in other organs that are later excreted through the kidney.

Only DMPS treated animals had lower levels of lead in the brain, DMSA values were too variable to be significantly different. The large standard error for this one treatment group is not due to just one animal, or the Q test would have rejected it. The possibility of lead partitioning into different areas of the brain, or DMSA removing lead from various portions of the brain was considered. Brain tissues were analyzed in duplicate, sectioned into two pieces sagitally when the whole brain was intact and could be oriented. When this could not be done, the brain was halved in a random manner. Further study needs to be done to evaluate this wide response in the brain lead levels.

There was no change seen from any treatment group in measured levels of lead in bone tissue. After eight days of treatment, there must be redistribution of the lead from hard tissues to soft, as the metal binding agents clear lead from extracellular spaces. Kidney levels were lower, and DMSA lowered bone levels after two days of administration, so either lead had resequestered back into bone during this extended period of testing, or perhaps there are two stores of lead in bone, one accessible to binding agents, and one that is not. The longer time allowed in this experiment before lead levels were measured may have allowed the partitioning of lead into inaccessible areas.

Lead levels in the control animals were examined for consistencies. Since these animals never received any lead loading or treatment, levels are due to environmental circumstances. Values of lead in kidney tissue ranged from 2 μg Pb/g in the first experiment, to 3 μg Pb/g in the second experiment. Brain levels were approximately 0.1 μg Pb/g in both experiments. Bone levels were 8 μg Pb/g in the first experiment, and 7 μg Pb/g in the second. These values show the universal availability of lead in the environment.

Similar research done with lead loaded rats given DMPS by i.p. injection 48 hours following the last lead dose instead of 72, at a lower dose than in this study, showed

increases in urinary lead excretion, and a decrease in levels of lead in the kidney, but no changes were seen in brain tissue or bone tissue (Twarog and Cherian, 1983). Other authors have suggested that DMPS acts specifically to remove lead from the kidney via a high renal clearance of the compound in the plasma ultrafiltrate, which passes through the proximal tubules, there binding to lead and removing it (Gabard 1978). Very little DMPS is reabsorbed by the tubules.

It has also been postulated that there exists in the kidney two depots of lead stores, those easily accessible to water soluble agents, located in extracellular spaces, and a pool that is intracellular and not attainable by hydrophilic compounds (Hammond, 1971). The results of this study to determine the ability of DMPS to remove lead from the kidney found lead levels reaching those of control animals when DMPS was given orally. DMPS values did not approach control values when given i.m. If this is due to the length of time and route of administration of DMPS or its ability to reach different storage spaces of lead is unknown.

As for other studies done with DMSA, the rate of excretion of lead in rats given DMSA i.p. increased and DMSA was found to be effective compared to lead controls and i.p.

D-Pen (Graziano et al., 1978). Also DMSA was 80% as effective when given p.o. as when given i.p. for urinary lead excretion, implying that DMSA is well absorbed from the GI tract. Although this study did not include urinary excretion levels of DMSA given p.o., it did find DMSA to be effective i.m., compared to both D-Pen and lead control animals.

Mobilization of Triethyllead

As stated in the results, DMSA and DMPS were ineffective in removing lead administered as triethyllead from any tissue tested. These results confirm other studies done with the established chelating agents BAL, EDTA and D-Pen, which had no effect when administered to animals (Cremer, 1959), or humans (Kitzmiller et al., 1954).

Brain, kidney and bone tissues were measured for total lead levels. Organic lead accumulates mostly in the liver, kidney and brain, and with time redistributes to bone tissue. In rats, the half-life of triethyllead in the kidney is 15 days, and in the brain the half-live is 7-8 days (Hayakawa, 1972).

Lead levels were measured in this study from animals sacrificed five days after the last triethyllead dose was given, before the measured half lives in brain or kidney tissue. It is noted that the levels of lead in the brain

are in the same range as those in bone tissue, between 2 and 3 µg Pb/g tissue. With the administration of lead acetate, these tissue values differed by a factor of 100. The concentration of lead administered to animals receiving lead acetate was 4.8 mM, daily for 7 days. The concentration of lead given as triethyllead was 1.7 mM, daily for 7 days. Yet ten times more lead was found in the brains of animals receiving triethyllead as lead acetate, demonstrating the greater ability of organic lead to deposit in the brain. The lack of an antidote for organic lead poisoning seems more disturbing in light of these findings. Only supportive and symptomatic care is recommended for organolead poisoned patients, including barbiturates to reduce delirium and insomnia (Grandjean, 1984).

Quantitative Determination of Organic and Inorganic Lead

Though many methods were tried and modified in the effort to find a viable method for determining different lead species in the same biological sample, none were found. Further investigation will probably uncover a working method, but in the span of this research this goal was not accomplished.

Of the methods tried, the typical problem encountered in chromatography was incomplete separation of the lead species. There was always some percentage of

triethyllead that overlapped with the elution of lead nitrate, so that accurate amounts of organic lead in unknown samples were unattainable.

This problem brought up the question of the purity of the triethyllead, obtainable only from Alfa Products. Reports gave the purity as greater than 98%. If a consistent percentage of the triethyllead appeared as inorganic lead, no matter the chromatography assay, it would imply a percentage of the triethyllead was contaminated or had degraded. Such was not the case. However, the different chromatography techniques are not all equal in their ability to separate the lead species, so conclusions regarding the purity of triethyllead can not be made from these findings. A qualitative measure of the purity by TLC was investigated but never actually done. It would be prudent to do this before any further work in this area is started.

Each of the methods had difficulties with the addition of the tissue matrix. TMAH may solubilize tissue without altering the chemical forms of organolead compounds, but it also provides interfering cations that mask a resin's ability to retain lead ions.

The cation exchange procedure seemed promising if a suitable replacement cation could be found that had a greater selectivity than Pb, such as Ba. Further

investigation with this ion is necessary. Knowing that Ca had the ability to suppress the lead signal by 40%, meant that at least 60% of any lead that eluted would be detected. That was enough to know that when no lead was detected in the effluents that should contain lead nitrate, no lead was there.

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