of University of Missouri's 2nd Annual Conference on Trace Substances in Environmental Health. Sponsored by University of Missouri Environmental Health Center and Extension Division. Edited by Delbert D. Hemphill. Held at Memorial Union - University of Missouri - Columbia - Columbia, Missouri - July 16, 17, and 18, 1968.

Lead + Enzymes 1555

EFFECTS OF LEAD ON BIOCHEMICAL SYSTEMS

by

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INTRODUCTION

During the past few decades, effective health regulations and environmental control procedures have largely eliminated lead intoxication as a cause of industrial illness in the United States. In recent years, however, interest in the deleterious effects of lead has shifted from the industrial field to that of potential hazards to the community at large. In this regard, the possible implications to health of the lead concentrations present in the ambient environment, particularly in urban areas, have been summarized and discussed widely (U.S. Public Health Service Reports, 1965, 1966; Goldsmith and Hexter, 1967) although the data is scanty and their interpretation a matter of speculation.

Frank lead intoxication presents a well-characterized clinical syndrome manifested by colic, lead line, anemia, neuropathy, or encephalopathy, and is accompanied by the excretion of porphyrins and their precusors into the urine. The concentrations of lead required to produce this state are rather accurately known. In contrast, the biological consequences of exposure to lesser concentrations of lead, particularly the low amounts present in the average urban environment, are completely unknown.

Investigations designed to better delineate the biochemical effects of lead provide one approach toward better understanding the circumstances in which this element could come to constitute a health hazard. It is known, for example, that lead, like other heavy metals, may act as an enzyme

inhibitor. Several steps in heme synthesis are inhibited by lead concentrations of 10^{-4} to 10^{-6}M (Goldberg et al., 1956; Haeger-Aronsen, 1960; Rubino, 1962), and such inhibition likely contributes importantly to the anemia of overt lead intoxication. Similarly, certain ATPases are sensitive to small concentrations of lead (Hernberg et al., 1967; Sraczynski and Zajusz, 1966) but the physiological consequences of these observations, if any, are as yet uncertain. Many other enzymes, such as acetylcholinesterase, are also inhibited by lead but only at higher concentrations, 10^{-3}M (Chiesura et al., 1966), suggesting that such inhibition may not be biologically significant. There are suggestions that lead may disrupt biochemical functions such as glucose transport, detoxification mechanisms, and oxidative phosphorylation (Teras and Kahn, 1966), but these effects have not been studied in any detail. Nor is it clear that all the biological actions of lead are necessarily deleterious. Many elements, once judged to be only "poisonous contaminants," are now known to perform, in minute concentrations, essential biochemical functions, and such a role has not been excluded for lead.

The need for additional information concerning lead metabolism has prompted us to begin exploring the effects of this metal on diverse biological systems ranging in complexity from isolated enzymes to whole animals. Heavy metals are known to have a strong affinity for common biochemical ligands such as phosphates, cysteinyl and histidyl side chains of proteins, purines, pteridines, and porphyrins.

Therefore, it is not surprising that lead appears to act at a large number of biochemical sites. It seemed possible that this element could affect metabolism through several discrete mechanisms, e.g., by altering cellular oxidation, inhibiting protein synthesis, interfering with tetrapyrrole metabolism, and binding to nucleic acids. Evidence for such effects has, indeed, been demonstrated as will be exemplified in the discussion to follow, which constitutes a synopsis of several different approaches now employed in our laboratory.

A. Lead as an Enzyme Inhibitor: Lipoamide Dehydrogenase

Lead, despite its well-known affinity towards sulfur, is not a very effective inhibitor of the so-called sulfhydryl enzymes, i.e., those possessing a single thiol group at the active site. In fact, the affinity of lead toward monovalent sulfur ligands in proteins and enzymes is actually considerably smaller than that of many other elements, e.g., silver, mercury, or cadmium. Concentrations of 10^{-3} to 10^{-4}M lead are required to inhibit sulfhydryl enzymes in vitro and, since this value far exceeds concentrations present in tissues and body fluids in states of lead intoxication, it is evident that lead is not likely a very potent inhibitor of such enzymes in vivo.

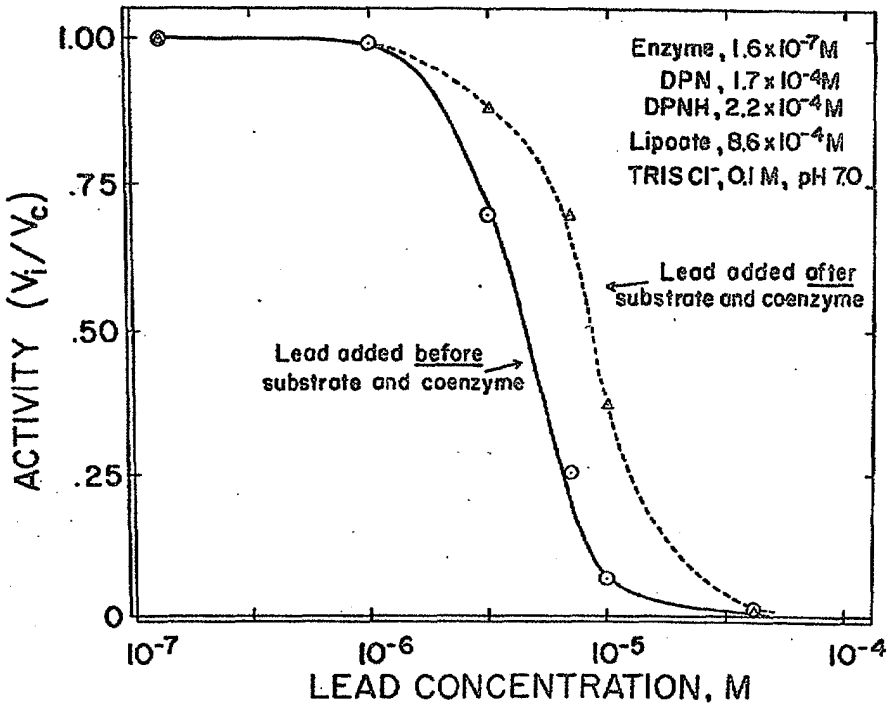
The principles of chelate chemistry indicate that the binding of lead should be markedly stronger when the metal interacts with more than one ligand group of the same molecule to form a chelate structure. The strong complexes of lead with 2,3-dimercaptopropanol (BAL), and the effectiveness of this agent in binding and eliminating lead from the body, are illustrations of this point. This suggests that enzymes possessing more than one thiol at their catalytic site should exhibit much greater sensitivity to lead inhibition than ordinary sulfhydryl enzymes.

Lipoamide dehydrogenase, an oxidative FAD-dependent enzyme from pig heart is an important component of the pyruvate and α-keto-glutarate complexes of mammalian mitochondria and, therefore, crucial to cellular oxidation. This enzyme has been reported to contain a dithiol configuration at the active site (Massey, 1963) and, on this basis, would be predicted to interact strongly with lead. As shown in Figure 1, lead markedly inhibits the enzymatic activity of lipoamide dehydrogenase: addition of 6.5×10^{-6}M lead to the assay causes a 50% inhibition of activity and, if lead is preincubated with the enzyme, even smaller amounts of the metal are inhibitory. Coenzymes and substrate provide partial protection against lead (Figure 1) and complete reversal of the inhibition occurs upon addition of equal concentrations of EDTA (Table I). It appears that lead binds to the

active center of lipoamide dehydrogenase, since coenzymes and substrate both afford protection against inactivation (Figure 1) and prevent restoration of activity by EDTA (Table I).

The interaction of lead with lipoamide dehydrogenase generates two new absorption bands at 310 and 260 mμ, identified by difference absorption spectrophotometry. The similarity of these absorption bands to those of lead-sulfur model complexes suggests that lead binds to sulfhydryl groups at the active center of this enzyme.

Figure 1



Effect of lead concentration on enzymic activity of pig heart lipoamide dehydrogenase. Lead is a strong inhibitor, only 50% activity remaining at a lead concentration of 6.5×10^{-6} M. Substrate and coenzymes partially protect against lead inhibition.

TABLE I Effect of EDTA on Lead Inhibition of Lipoamide Dehydrogenase

	Conditions†	% Activity
1.	Enzyme + Coenzymes-Substrate (control)	100
2.	Enzyme + Coenzymes-Substrate + EDTA* (control)	106
3.	[Enzyme + Coenzymes-Substrate] + Pb^{2+}	3
4.	[Enzyme + Coenzymes-Substrate] Pb^{2+} + EDTA	106
5.	[Enzyme + Pb^{2+}] + Coenzymes-Substrate + EDTA	2

†Assay Conditions: Enzyme or Enzyme-Pb complex, 1.65×10^{-7} M; DPN^{+}, 1.7×10^{-4} M; DPNH, 2.2×10^{-4} M; lipoate, 8.6×10^{-4} M; Tris-Cl, 0.1M, pH 7.0; ±Lead Acetate, 3.3×10^{-6} M.

In line 4, lead is added to the enzyme after addition of coenzymes and substrate whereas, in line 5, the order of addition is reversed.

*EDTA -- 3.4×10^{-4} M

The data indicate that lead is an extremely potent inhibitor of lipoamide dehydrogenase in vitro and suggests that lead might, through this mechanism, serve to disrupt cellular oxidation in vivo. Efforts to test this hypothesis are now in progress.

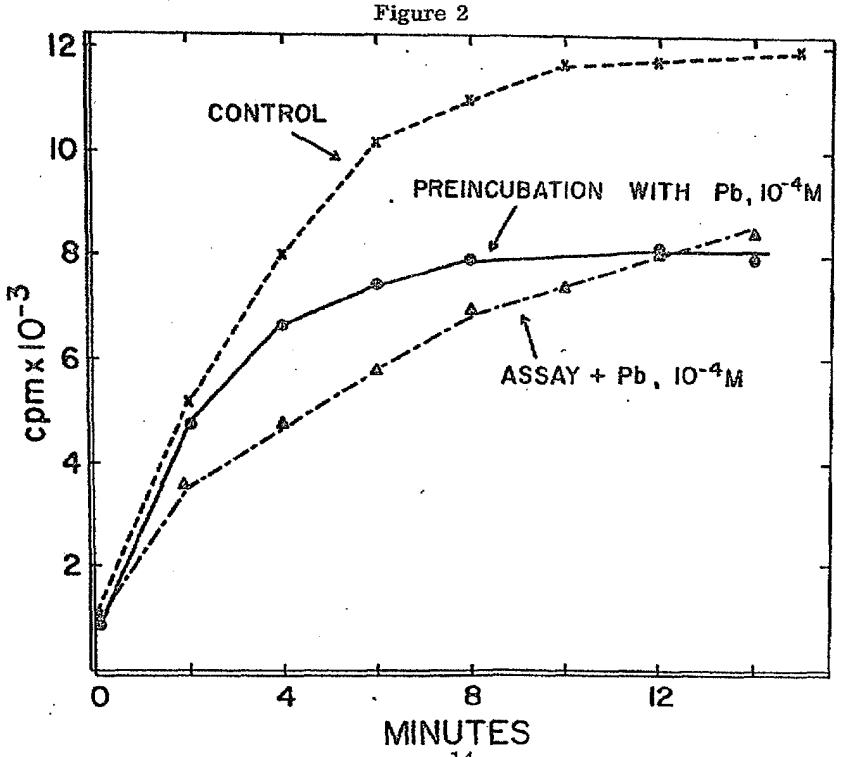
B. Effect of Lead on Protein Synthesis

At a higher level of biological organization, the effects of lead become more complex. This is evident upon investigation of the influence of lead on an isolated step in the protein synthesizing apparatus--the incorporation of a single amino acid into transfer RNA. We have employed, for this study, the incorporation of ^{14}C-leucine into t-RNA, utilizing the amino acid activating system obtained from E. coli, strain B (Figure 2).

The addition of 10^{-4} M lead acetate results in nearly 50% inhibition of enzymatic activity over the first few minutes of the reaction, likely due to the interaction of lead with the aminoacyl synthetase, believed to be a sulfhydryl enzyme. While this inhibition does not appear unusual in its

kinetics, a quite different form of inhibitory effect is observed when lead is preincubated with t-RNA, at 37° (Figure 2). In this instance, the reaction is measured by diluting the "preincubated" lead--t-RNA solution ten times into the assay mixture; thus, the lead concentration in the final assay is only 10^{-5}M. Notably, during the early part of the reaction, the lead-containing sample exhibits activity identical to that of the control suggesting that 10^{-5}M lead is not sufficient to inhibit the aminoacyl synthetase. However, the reaction plateaus early and only 60% as much ^{14}C-leucine is

Figure 2



Effect of lead on incorporation of ^{14}C-leucine into t-RNA from E. coli. Addition of lead to the assay mixture decreases the rate of ^{14}C-leucine incorporation, most likely due to inhibition of the aminoacyl synthetase. Preincubation of t-RNA with lead at 37° decreases the extent of amino acid incorporation, most likely due to hydrolysis of the nucleic acid.

incorporated over the total time course (Figure 2). This form of inhibition likely indicates that part of the leucyl t-RNA was hydrolyzed during the preincubation in keeping with the known effects of metal ions, particularly lead, in bringing about hydrolysis of RNA (Matsushita and Ibuki, 1960; Britten, 1962; Huff et al., 1964; Farkas, 1968).

These experiments suggest that lead might affect protein synthesis by two quite different mechanisms: first, by a direct effect, through inhibition of the activating enzyme and, secondly, by bringing about hydrolysis of t-RNA and, presumably, similar RNA species.

C. Effect of Lead in Metal-Ion Antagonism

At the cellular level, lead appears to affect biological functions by still additional mechanisms. Studies of the photosynthetic microorganism, Rhodopseudomonas spheroides, serve as an example of lead toxicity on the basis of "metalion antagonism," a phenomenon wherein one metal induces a biological effect by altering the requirement for a different element (Martin, 1951; Ulmer, 1966).

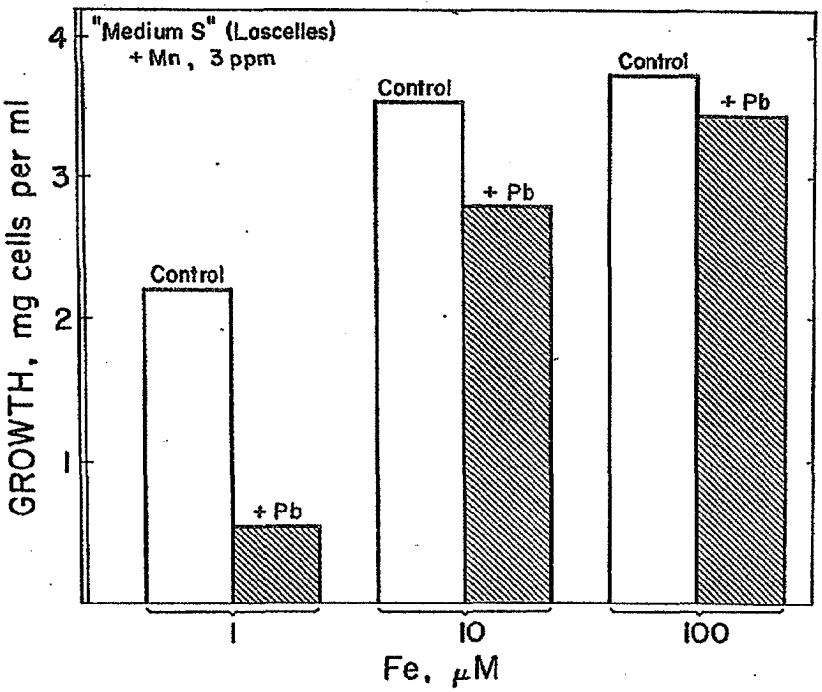
Rps. spheroides can grow aerobically, synthesize heme and employ oxidative pathways. However, dependent upon the conditions of culture, particularly light and oxygen tension, the organism may also grow as a facultative anaerobe, manufacture bacteriochlorophy 11, and carry out photosynthesis (Van Niel, 1963). This dual capability has made Rhodopseudomonas a favorite system for studies of the pathways of tetraphyrrole synthesis (Lascelles, 1964). For the same reason, we thought it an interesting organism on which to study the effects of lead toxicity in whole cells.

When Rps. spheroides is cultured on basic media (Lascelles Medium S), without added iron, lead causes a pronounced inhibition of growth (Figure 3). This inhibition is much less pronounced, although still evident, in iron-supplemented cultures suggesting that lead may affect growth by competing with iron at an essential metabolic step. Additional evidence

for such a competition comes from studies of porphyrin metabolism in this microorganism.

Lascelles has shown that iron-deficiency induces a marked increase in coproporphyrin excretion by Rps. spheroides (Lascelles, 1965). We have found that coproporphyrin excretion is also critically influenced by other metals, particularly lead, manganese, and cobalt.

Figure 3

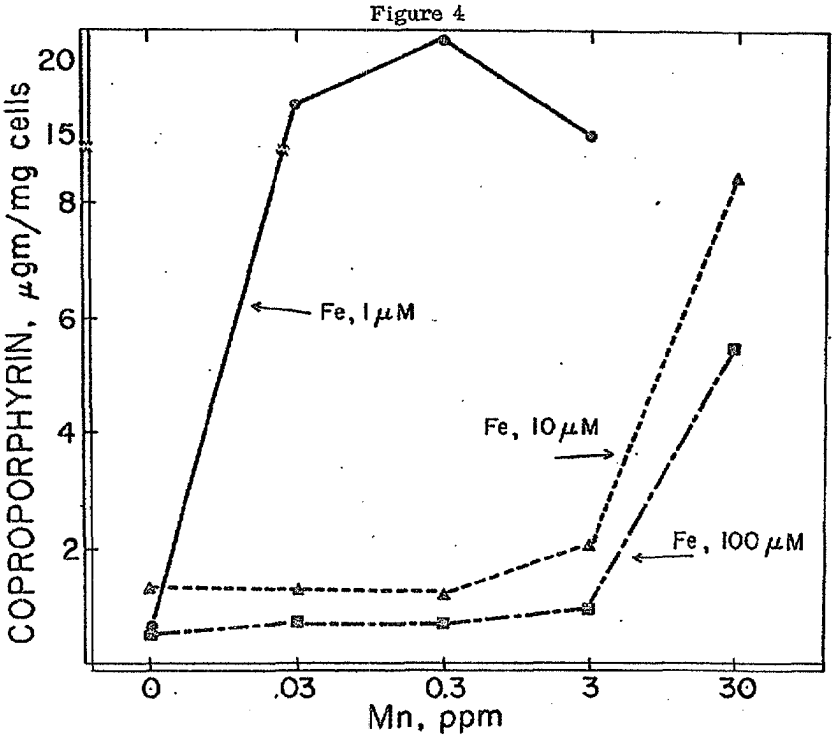


Effect of lead on growth of Rhodopseudomonas spheroides. At an iron concentration of 1 μM, lead markedly inhibits semi-anaerobic, light-grown cultures. This inhibition is largely overcome by higher iron concentrations.

Figure 4 shows the effect on coproporphyrin excretion of iron as opposed to manganese. Little coproporphyrin is produced in the absence of manganese, even at the lowest iron concentration (1μM); however, marked coproporphyrin excretion appears upon addition of only 0.03 ppm manganese

to the culture. Cobalt has an effect similar to manganese. In iron-supplemented cultures, e.g., 10 to 100 μM, much higher quantities of manganese (or cobalt) are tolerated before the appearance of excess coproporphyrin.

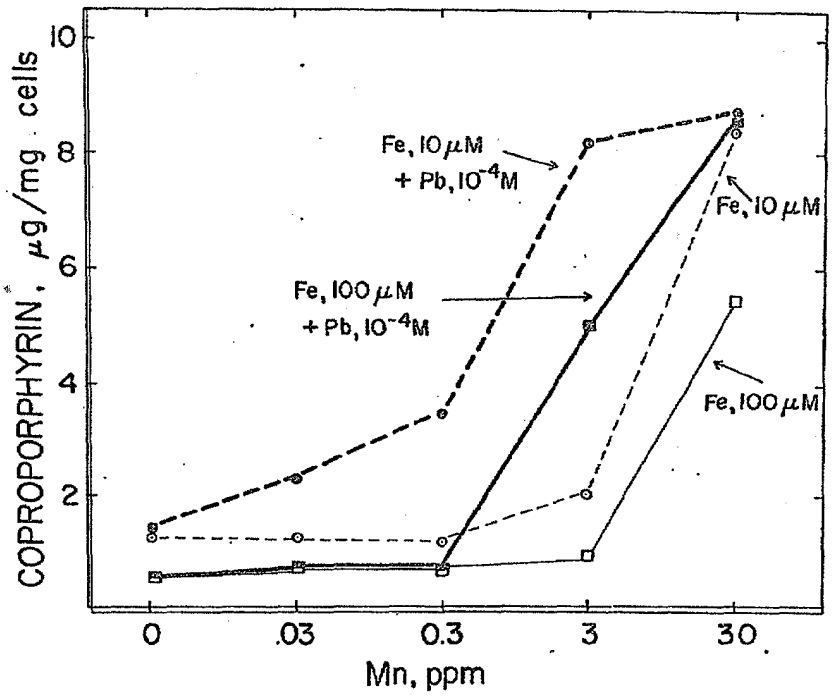
Figure 4



Metal-ion antagonism in Rhodopseudomonas spheroides. At low Fe concentrations (1 μM/liter) Mn, 0.03 ppm, induces marked coproporphyrin excretion. In the presence of 10 or 100 μM/liter Fe, increased coproporphyrin appears only at much greater Mn concentrations.

Lead potentiates the effect of manganese in causing increased coproporphyrin excretion by this system. Thus, in the iron-supplemented cultures, little coproporphyrin is found at a manganese concentration of 3ppm; however, the addition of lead results in substantial coproporphyrin excretion (Figure 5). The insolubility of lead at this pH precludes achieving concentrations sufficient to increase coproporphyrin excretion in the absence of some other metal, such as manganese or cobalt.

Figure 5



Synergistic antagonism of Fe, by Mn and Pb, in Rhodopseudomonas spheroides. In the presence of Pb, lesser concentrations of Mn induce coproporphyrin excretion.

It appears that manganese, cobalt, and lead act singly, and in concert, to antagonize iron and, thereby, alter tetrapyrrole synthesis in Rps. spheroides. Such competitive effects of other metals for iron are indicative of metalion antagonism, an observation long recognized, both in plants and animals (Martin, 1951; Ulmer, 1966), and constituting an important mechanism whereby trace element imbalance may result in disease states in all forms of life.[1] The present observations would also appear to constitute an example of a "conditioned iron deficiency" wherein lead, manganese, and cobalt act as conditioning factors causing an increased

[1] Antagonism of manganese and iron has been recognized previously, for example, in rice (Tanaka and Navasero, 1966) and in poultry (Woerpel and Balloun, 1964).

requirement for iron in Rhodopseudomonas (Ershoff, 1948).

The biochemical locus of this metal-ion antagonism has been further investigated by determining the concentration of the various intermediates and end-products of tetrapyrrole synthesis, the pathways of which are shown in abbreviated form in Figure 6. Table II shows the results of such an investigation. Addition of manganese to cultures limited in iron, or addition of both manganese and lead to iron-supplemented cultures, consistently induces production of large quantities of coproporphyrin III. This alteration is accompanied by a decrease in both of the principal end-products of tetrapyrrole synthesis, bacteriochlorophyll (Table II-B) and heme (Table II-C). Moreover, the metal-induced increase in coproporphyrin is not accompanied by a corresponding increase in protoporphyrin (Table II-D). Reference to Figure 6 suggests that the competition of manganese and lead with iron most likely affects the conversion of coproporphyrinogen to protoporphyrinogen. This conclusion is consistent with previous suggestions that the enzyme involved, coproporphyrinogen oxidase, or "coprogenase," may be iron-dependent (Lascelles, 1964). Preliminary experiments suggest that manganese and lead may also affect ferrochelatase, the enzyme involved in the conversion of protoporphyrin to heme, albeit to a lesser extent (Figure 6).

Lead intoxication in both animals and humans results in the urinary excretion of large quantities of δ-aminolevulinic acid and accumulation of protoporphyrin in erythrocytes; these alterations are consistent with the demonstrated inhibition by lead of both δ-aminolevulinic acid denydrase and ferrochelatase in bone marrow and red blood cells (Goldberg et al., 1965; Haeger-Aronsen, 1960). However, plumbism is also accompanied by a marked increase in urinary coproporphyrin III and the mechanism of this abnormality has not been defined. The present experiments, with Rps. spheroides, suggest that through antagonism of essential iron, lead inhibition of coprogenase is a most likely explanation for the coproporphyrinuria of lead intoxication.

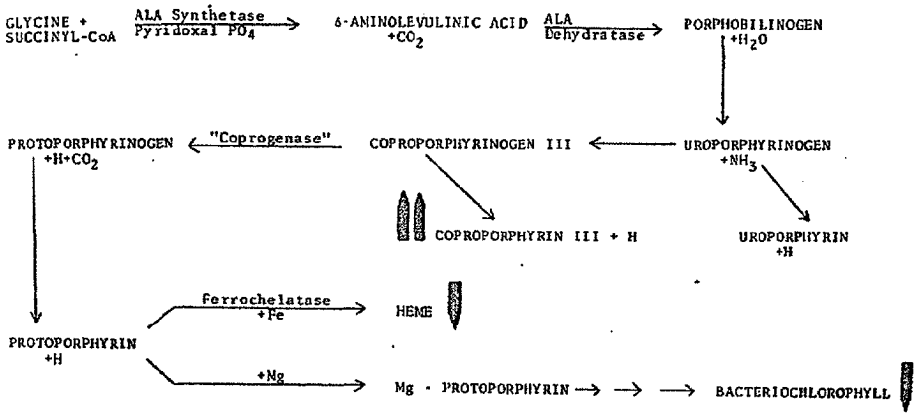
TABLE II Effect of Pb and Mn on Tetrapyrrole Synthesis in Rhodopseudomonas spheroides

Metals Added†			A	B	C	D
Fe_{-6} $-x10^{-6}M$	Mn_{-5} $2x10^{-5}M$	Pb_{-4} $1x10^{-4}M$	Coproporphyrin $\mu g/mg$*	Bacteriochlorophyll $\mu g/mg$*	Heme $m\mu M/mg$*	Protoporphyrin $\mu g/mg$*
1	-	-	1.2	5.8	0.8	0.8
1	-	+	1.7	3.4	1.0	1.1
1	+	-	32.4	1.4	0.5	< 1.0
1	+	+	36.8	1.4	0.5	< 1.0
10	-	-	1.3	7.6	1.0	1.6
10	-	+	1.5	10.8	0.9	1.4
10	+	-	0.6	3.3	1.0	0.3
10	+	+	7.3	2.2	0.7	0.1

†Lascelles Medium S with metals as indicated

*Tetrapyrrole concentration per mg cell protein

Figure 6



Pathway of tetrapyrrole synthesis in Rhodopseudomonas spheroides. In the presence of Mn and Pb, coproporphyrin III is markedly increased while both principal end-products, heme and bacteriochlorophyll, are decreased (arrows).

D. Lead Distribution in Higher Animals

At the highest level of biological complexity we have poisoned experimental animals with lead with the aim of discerning the general distribution of this element and its specific interaction with critical subcellular constituents.

Chronic lead intoxication of the pony was induced by oral administration of lead acetate over a four-month period; the degree of poisoning was monitored by assessing the development of anemia and determination of lead concentrations in the blood. The animal was sacrificed and the organs and tissues were analyzed for their metal concentrations. The organ distribution of lead in this species is shown in Table III. Lead was localized primarily to liver, bone, kidney, spleen, lung and hair, similar to the distribution observed in lower species.

E. Interaction of Lead with Metallothionein

It was of interest to determine if lead, like zinc, copper, iron, cobalt, manganese, and cadmium might associate specifically with known

TABLE III Equine Lead Intoxication: Distribution of Lead in Tissues

Organ or Tissue	Lead Concentration* µg/g
Liver	31.7
Bone (rib)	12.7
Kidney	9.8
Spleen	5.0
Lung	4.7
Pancreas	3.2
Urethra	0.3
Brain	Trace†
Heart	Not Detected
Aorta	Trace
Muscle	Trace
G-I Tract	
Esophagus	0.6
Stomach	Trace
Duodenum	Trace
Ileum	0.7
Endocrine Glands	
Thyroid	Trace
Adrenal	Trace
Pituitary	Trace
Skin	Trace
Hair (tail)	13 to 100

*Lead determined by quantitative spark emission spectrography

†Trace is equivalent to 0.2 ppm or less

metal-binding proteins in vivo. This possibility has led us to study the interaction of lead with one such protein, equine renal metallothionein.

Metallothionein is an unusual protein of small molecular size which represents 1-2% of the total weight of soluble protein in equine kidney cortex (Margoshes and Vallee, 1957). In purified form it contains as much as 6% cadmium, more than 2% zinc, 8 ½ % sulfur and small amounts of other metals such as copper and iron (Kägi and Vallee, 1960; 1961). While first isolated from the horse, we have recently purified metallothionein from the kidneys of other species, including man, and have found an identical or very similar protein to be present in liver. The physical characteristics of metallothionein are well established (Kägi and Vallee, 1960;1961), but efforts to identify its biological function have, thus far, been unsuccessful. Its properties are consistent with a role in a wide range of potential homeostatic mechanisms, either in catalysis, storage, immune phenomena, or perhaps in detoxification of heavy metals. The latter possibility was tested by ascertaining if metallothionein might interact with lead.

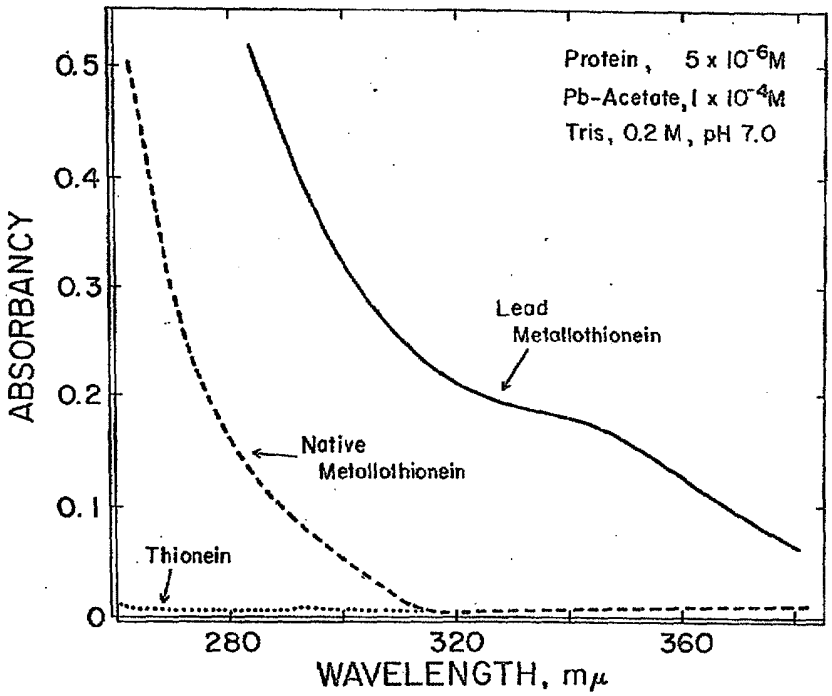
The binding of lead to metallothionein in vitro can be shown readily by the appearance of a specific absorption spectrum (Figure 7). Lead metallothionein exhibits a broad absorption band with a maximum between 320 and 330 mμ, characteristic of lead-sulfur interactions. The association constant for lead to thionein, the metal-free protein, appears to be approximately 10^{15}, midway between that of cadmium and zinc, the metals most abundantly present in this protein in normal animals. Since lead binds more firmly than zinc in thionein, it readily displaces the zinc of the native protein in vitro.

The possible in vivo interaction of lead with metallothionein was investigated by isolation of this protein from the kidneys of the lead-poisoned pony. The last step in the purification procedure, chromatography on DEAE cellulose is shown in Figure 8. Metallothionein appears as a discrete symmetrical peak. Significantly, the principal lead peak present in the

chromatographic material is coincident with that of metallothionein indicating that this protein does, indeed, bind lead in vivo as well as in vitro.

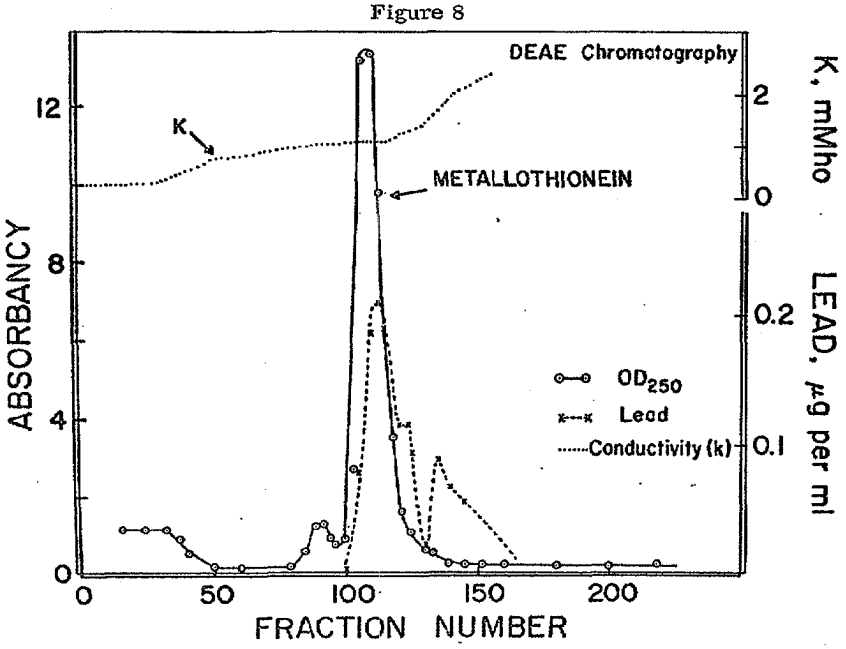
Only a fraction of the total lead present in kidney appears to bind to metallothionein and lead does not significantly displace cadmium and zinc from this protein in vivo. Thus, although it is possible to demonstrate an interaction of lead with metallothionein, the experiment does not provide evidence that this unusual protein serves a significant function in the detoxification of lead. Moreover, these observations indicate the necessity of testing, in whole animals, hypothesis generated by in vitro experimentation.

Figure 7



Absorption spectrum of equine renal metallothionein. The apoprotein, thionein, is devoid of aromatic amino acids and does not absorb at wavelengths longer than 240 mμ. Native metallothionein has an absorption band at 250 mμ, arising from cadmium-sulfur interaction. Lead metallothionein absorbs at still longer wavelengths, 320 to 340 mμ, indicative of a lead-sulfur chromophore.

Figure 8



DEAE chromatography of metallothionein from kidney of the lead-intoxicated pony. The principal protein and lead-containing fractions emerge together from the column.

F. Interaction of Lead with Robonucleic Acid

As indicated previously, lead appears to interact with ribonucleic acid in vitro and potentially, may interfere with protein synthesis on this basis (vide supra). It seemed important, therefore, to determine if lead also interacts with RNA in vivo. For this purpose, we have isolated and purified RNA from the liver of the lead-poisoned pony and analyzed this material for metals by emission spectrography (Wacker and Vallee, 1959). The results are shown in Table IV. It is evident that RNA binds considerable lead, as well as other metals noted previously. The significance of this observation, with its important implications in terms of transmission of genetic information, and fundamental cellular synthetic processes, will require a good deal of further work, but it may well prove of great interest in evaluating the consequences of long-term exposure to lead.

TABLE IV Equine Lead Intoxication: Metal Analysis of Liver RNA

METAL	CONCENTRATION μg/gm*
Zinc	135
Strontium	80
Barium	92
Iron	395
Calcium	1670
LEAD	103
Manganese	13
Aluminum	27
Nickel	55
Molybdenum	Trace
Magnesium	315
Chromium	9

*Emission of spectrographic analysis in duplicate of samples ashed at 450° C.

SUMMARY

Lead appears to act at a large number of biochemical sites and might contribute to metabolic alterations through several different mechanisms. Lead strongly inhibits lipoamide dehydrogenase, a purified enzyme crucial to cellular oxidation, probably through binding to the dithiol configuration at the active catalytic center. Protection is afforded by coenzymes and substrates and the inhibition is reversed by EDTA.

Lead inhibits the incorporation of ^{14}C-leucine into E. coli t-RNA; the data suggests that the metal may affect protein synthesis both by attacking the synthesizing enzymes and, perhaps, through binding and hydrolyzing t-RNA.

At the cellular level, lead inhibits the growth of the microorganism, Rhodopseudomonas spheroides, and appears to alter tetrapyrrole synthesis in this species through a complex metal-ion antagonism involving coproporphyrinogen oxidase (coprogenase). This action of lead may well be the basis for the increased urinary coproporphyrin observed in plumbism.

These and still other effects of lead are likely pertinent to evaluating the significance of exposure to this metal in higher forms of life. The distribution of lead and its binding to specific subcellular constituents, metallothionein and liver RNA, in the pony are reported as early efforts to delineate loci of interaction of the metal in intact animals.

These experiments of the biological effects of lead on systems of widely varying complexity were undertaken to provide a firmer basis for estimating potential hazards to health from this element in the ambient environment and to localize potential sites of its action in metabolism.

Acknowledgement: The authors gratefully acknowledge the contributions to this work by Drs. J.H.R. Kägi and W.E.C. Wacker.

This work was supported by a Grant-in-Aid from the International Lead Zinc Research Organization Incorporated, New York City, New York.

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