BIOPHYSICS RESEARCH LABORATORY
Department of Biological Chemistry
Harvard Medical School
at
Peter Bent Brigham Hospital

August 2, 1965

Mr. Don C. Fowler, Project Manager
International Lead Zinc Research Organization
292 Madison Avenue
New York 17, New York

Dear Mr. Fowler:

I am enclosing a copy of our report on the project entitled "Studies on the Biological Basis for Lead Intoxication" covering the first four months of its operation, starting from April 1, 1965. As you can see we have gone quite a way in that short time and I am quite pleased with the progress we have made.

In accord with your suggestion I am hereby requesting a renewal of the grant as of January 1, 1966 to December 31, 1966. We intend to continue the work under the same headings and in the same direction indicated in our application last year. Since the project has been in operation for such a brief period we have, of course, not had an opportunity to extend these investigations beyond the plans and hypotheses previously advanced under the headings of I. Methodology, II. Lead and Heme Synthesis and III. Metallothionein and the Urine Excretion of Lead, noted both in our initial application and progress report. It is our intent to do so and to intensify the work that has been launched so successfully. I do hope that you and your Committee will share our enthusiasm about the progress of the work and we are looking forward to a continuation of our pleasant association. This application has the approval of Dr. Elkan R. Blout, Professor and Chairman of the Department of Biological Chemistry. The budget for next year is attached.

Sincerely yours,

Bert L. Vallee
Professor of Biological
Chemistry

BLV:dem

Approved:

Elkan R. Blout, Chairman
Department of Biological Chemistry

Report to International Lead Zinc Research Organization on

Project No. LII-94

STUDY OF THE BIOLOGICAL BASIS FOR LEAD INTOXICATION

April 1, 1965 to August 1, 1965

Submitted by:

Bert L. Vallee
Professor of Biological Chemistry
Biophysics Research Laboratory
Department of Biological Chemistry
Harvard Medical School
at Peter Bent Brigham Hospital
Boston, Massachusetts

August 2, 1965

Progress Report--Lead Study

I. Introduction

During the first four months of this project our efforts have been concentrated along two specific lines. First, we have explored the analytical chemistry of lead with the goal of improving methods for the determination of this metal in biological materials. The availability of suitable analytical procedures is a clear prerequisite of any significant advance in this field, basic or applied. Second, we have conducted preliminary investigations of the interaction of lead with several biological systems of varying complexity. These were chosen with the intent of discerning suitable experimental systems for detailed investigation of the biochemical effects of lead along the lines indicated in our draft proposal. The pertinent studies and results which contribute to both of these goals will be described briefly. They should be considered largely exploratory at this juncture much as some of the leads already detected may eventually serve as the basis for extended investigations.

II. Analytical Chemistry of Lead

The availability of a simple, rapid, precise, and accurate method for the determination of lead in biological materials is essential for our contemplated investigations into the biochemical effects of lead. Most methods for the determination of traces

of lead in urine, for example, require the prior removal of organic matter and concentration of lead by ashing. The undesirable features of these methods are obvious: the time required for ashing is considerable; some of the ashing procedures engender losses during this preparatory step; other metals potentially interfere; reagents need to be purified to keep contamination at a low level.

To obviate these difficulties we have investigated the suitability of atomic absorption spectroscopy for the determination of lead. This procedure, based on physical phenomena known for more than 100 years, became practical but a brief 7-8 years ago through the design of appropriate hollow cathode sources by Walsh in Australia. One of his colleagues, Willis, has applied it to the analysis of lead in urine(Nature 191, 381, 1961). In this procedure lead is extracted from urine using ammonium pyrrolidine dithiocarbamate and methyl n-amyl ketone at acid pH, and the element determined by atomic absorption using the lead line at 2833 Å. In this manner lead may be concentrated by a factor of 100 or more enabling the determination of concentrations of 0.4 ppm using 50 ml samples of urine or even concentrations as low as 0.2 ppm when 100 or 200 ml samples of urine are used.

The principles uncovered in our laboratory through the introduction of long absorption cells, taking advantage of the

Beer-Lambert law,(Fuwa and Vallee, Analytical Chemistry 35, 942, 1963) first made possible the lowering of the limits of detection for many metals by orders of magnitudes. As might have been predicted on the basis of the underlying theory, these fundamentals apply also to the specific problem of lead determination and we have now succeeded, indeed, in improving the sensitivity obtained by atomic absorption spectroscopy for this element. Our preliminary efforts have been directed toward obviating the extraction procedure while achieving the sensitivity which is required for routine analytical work.

The establishment of optimal spectral conditions is basic to this procedure and, hence, we investigated the lead spectrum to uncover the ideal spectral line to employ for the measurement of lead by means of atomic absorption. Up to the present most workers have used the lead line at 2833 Å for this purpose. Investigation of accessible lines revealed, however, that the line at 2169.99 Å is inherently more than twice as sensitive. This discovery is itself based upon our general investigation of the spectral range from 1820-2300 Å, a region not widely considered practical by many analysts, but which sometimes has special advantages for certain elements. During the examination of other heavy metals we have found in some instances unlisted, very sensitive spectral lines at such relatively short wavelengths. For this reason we have screened the entire spectral range from

2170 Å to 1870 Å(while flushing with hydrogen gas) for other possible sensitive lines. However, the absorption at 2169.99 Å has, thus far, proved superior to all others. The relative absorption of the various ultraviolet spectral lines examined is shown in Table 1.

In investigating the most suitable pathlength for the determination of lead we have found, thus far, that with a standard Beckman burner and hydrogen-oxygen gas a 09 centimeter pathlength Alundum cell is optimal. Employing these conditions a calibration curve for lead in aqueous solution appears as shown in Fig. 1. A similar curve is obtained in the presence of 0.1 M phosphate buffer so that under these conditions there is no marked suppression of absorption due to the presence of phosphate anion--an important consideration for the analysis of urine.

In an attempt to increase sensitivity, calibration curves were prepared in the presence of varying concentrations of perchloric acid, an adjuvant we have found very helpful for the analysis of other metals. While at concentrations of 0.001 to 0.1 M, perchloric acid increases sensitivity slightly, concentrations above 0.1 M perchlorate give no significant enhancement of sensitivity. The lead calibration curve in the presence of 0.05 M perchlorate is compared with that in water in Fig. 2.

We next attempted to determine the effect of urine upon the atomic absorption of lead. Urine was extracted with dithizone (diphenylthiocarbazone) and carbon tetrachloride at pH 2, 4, 6, and 8. A calibration curve of lead was then carried out in the presence of this extracted urine and compared to that of water (Fig. 3). When metal-extracted urine is used as a blank, as in this instance, the calibration curve for lead is very similar to that in water. However, compared to a water blank the absorption of urine is significant. It is not immediately apparent if this absorption is due to lead contamination of the urine sample used or to other effects (e.g. presence of anions) but the intrinsic urine absorption was not removed by extraction with dithizone.

A number of experiments were carried out to evaluate the extractability of lead from urine by dithizone since to determine the concentration of the metal by this convenient means offered many analytical advantages.

Lead was added to urine and extractions were carried out over a wide range of dithizone concentrations and with varying pH. Using recovery experiments it was determined that lead was not extractable at acid pH and only partially extracted in the pH range 4-7. Complete extraction could not be obtained in any of these preliminary experiments and thus the possibility of increasing sensitivity by concentrating lead in the urine with

dithizone extraction does not appear practical on the basis of present observations. We shall next carry out the same series of experiments employing the ammonium pyrrolidine dithiocarbamate reagent.

Since the mixture of gases and volatilization of the sample by the Beckman burner may not be ideal in the case of lead we have also investigated the suitability of utilizing specially designed burners to achieve more optimal conditions. A ring microburner built in our shop, for example, permits fine flow regulation and mixture of three gases, adding considerable flexability to the shape and temperature control of the flame and to aspiration of the sample. The increased sensitivity for lead employing such a burner is shown in Fig. 4. In this instance hydrogen was employed as the gas feeding the ring, while air and nitrogen are supplied to the microburner. Using this new burner, sensitivity is further increased about two times suggesting that further progress along this line can be achieved.

The present limit of detection of our atomic absorption method is within the range required for lead in urine by direct analysis and but a small lowering of this limit will make the procedure suitable for wide, routine application.

In brief, these preliminary efforts to obtain adequate sensitivity for the measurement of lead have proven very

encouraging and extension of these efforts should enable us to design a suitable, sensitive, rapid and precise method for the measurement of lead in biological materials.

III. Transferrin and conalbumin

We have pointed out in our initial proposal that lead is known to interfere with heme synthesis at a number of differing enzymatic steps. It has also been thought possible, however, that lead might influence heme synthesis by interfering with iron transport directly and thus preventing the arrival of iron at synthetic sites. In view of the known metal binding propensities of serum transferrin it was postulated that lead might bind to this protein and thus interfere with the transport of iron.

We have attempted to test this hypothesis by ascertaining if lead does indeed bind to serum transferrin in a manner which interferes with the binding of iron. We have also carried out similar studies with the closely related metal-binding protein, conalbumin.

In order to increase the solubility of lead the binding studies were carried out in 1 M Tris buffer. Under these conditions, about 1.3 μgms of iron are required to saturate the iron binding sites of 1 mg of conalbumin and about 1 μgm of iron to saturate the binding sites of each mg of transferrin. The results of the competition experiments between lead and iron

for binding to conalbumin and transferrin are shown in Fig. 5 and 6. Lesser concentrations of lead were also tested with similar results. From the figures it may be seen that up to a 20-fold excess of lead does not interfere significantly with the binding of iron to either conalbumin or transferrin. On the basis of these studies it does not appear likely that lead binds very readily to the iron binding site of either transferrin or conalbumin and, thus, it seems unlikely that the competition of lead for iron-binding in the serum plays a significant role in lead intoxication.

IV. Metallothionein

Metallothionein is a small molecular weight protein of unique chemical composition first isolated in this laboratory from horse kidney cortex(Margoshes and Vallee, J. Am. Chem. Soc. 79, 4813, 1957). Electrophoretically and ultracentrifugically homogenous preparations contain as much as 5.9% cadmium, 2.2% zinc, 0.2% iron, 0.1% copper and 9.3% sulfur(Kägi and Vallee, J. Biol. Chem. 235, 3460, 1960; 236, 2435, 1961). Cadmium and zinc appear to compete for binding at the same site and may be isomorphic in metallothionein. Most of the sulfur is accounted for by 27 cysteine residues and there is some evidence that each atom of cadmium or zinc is bound to three sulfhydryl groups. The molecular weight of metallothionein is 10,000.

Even though metallothionein represents 1 to 2% of the

total weight of soluble protein in horse kidney cortex, efforts to identify its biological function have not been successful thus far. Its physical characteristics are consistent with a role in a wide range of potential homeostatic mechanisms, either in detoxication, catalysis, storage or immune phenomena. The possibility that thionein, the metal-free protein might bind other heavy metals, such as lead has not hitherto been investigated. The potential significance of such binding, if demonstrated, in terms of regulation or detoxification of lead within the body is obvious. Therefore, an investigation of the interaction of lead with metallothionein was undertaken.

Metallothionein was prepared according to the method of Kägi and Vallee(J. Biol. Chem. 235, 3460, 1960). The metal-free protein, thionein, was prepared by acidification and gel filtration(Sephadex G-25). Immediately after separation, lead was added to an aliquot of the metal-free protein and the pH restored to neutrality by addition of 0.1 N sodium hydroxide. The final concentrations employed were: protein = 5×10^{-6}M and lead = 1×10^{-4}M. The absorption spectra and optical rotatory dispersion of the lead metallothionein were then determined. The results are shown in Fig. 7 and 8.

It will be noted in Fig. 7 that thionein, the metal-free protein, curve 1, does not absorb light at wavelengths longer than 240 mμ. This is consistent with the amino acid analyses

for thionein which demonstrates virtually complete absence of any aromatic amino acids which give rise to the usual protein absorption near 280 mμ. In contrast the absorption spectrum of metallothionein, curve 2, shows a shoulder between 240 and 270 mμ. Difference spectra(metallothionein minus thionein) demonstrate this shoulder to arise from an absorption band centered at 250 mμ which compares favorably with the known absorption bands of cadmium mercaptides. Lead thionein, curve 3, shows still greater absorption, with the presence of a shoulder at wavelengths longer than 300 mμ and a diffuse increase in absorption at wavelengths shorter than 300 mμ. This suggests that lead binds to thionein and generates new chromophoric metal-protein ligand sites.

As shown in Fig. 7, the absorption bands which appear upon binding of lead to thionein are optically active as has been shown previously for the chromophore characteristic of cadmium binding to this protein(Ulmer and Vallee, Biochem. Biophys. Res. Commun. 8, 327, 1962). Thus, the optical rotatory dispersion is anomalous at wavelengths shorter than 350 mμ due to the presence of multiple Cotton effects. The optically active lead-thionein chromophore indicates that the metal-binding site of the protein is asymmetric and, thereby, provides a method for further investigation of the physical chemical properties of this system(Ulmer and Vallee, Advances in Enzymology 27, 37, 1965).

It would be suspected that sulfur is the ligand which binds lead in thionein, and some indication that this may be the case has been found by studying the interaction of lead, as well as other metals, with model systems. For example our studies of the metal complexes of mercaptoethanol are shown in Table 2. It may be seen that at pH 8, with a 30:1 ratio of ligand to metal, the interaction of lead with mercaptoethanol produces absorption bands at 250 and 310 mμ, with molar extinction coefficients at 14,000 and 3600 respectively. The maxima of the absorption bands of lead mercaptoethanol coincides quite well with the midpoint of the extrinsic Cotton effects shown for lead metallothionein in Fig. 8. These investigations will form the basis of a continued study of the binding of Pb^{2+} by various macromolecular species involved either in storage, transport or disposal of the element.

V. Rhodopseudomonas spheroides

Our plans for the investigation of the biochemical effects of lead include studies of its action upon heme synthesis. As a likely model system for such work, due to its easy accessibility and known characteristics, we have examined the suitability of Rhodopseudomonas spheroides, a photosynthetic micro-organism which has been successfully employed by others in the study of porphyrin synthesis (e.g. Gibson et al., Biochem. J. 83, 539, 1962).

In preliminary experiments we have grown Rhodopseudomonas spheroides, in the light, in the presence and absence of 10^{-4}M lead. Both the growth rate and the extent of growth are diminished by about 50% in the presence of lead. Moreover, measurement of the chlorophyll content of the cells grown in the presence of lead revealed a 50% decrease in chlorophyll compared to the control, using protein as a baseline. At higher concentrations of lead, porphyrins appear to be excreted into the culture medium.

These preliminary experiments demonstrate striking effects of lead upon the total growth and chlorophyll synthesis by these microorganisms. Rhodopseudomonas would appear to offer a unique opportunity to study the effects of lead toxicity in a simple system and we next intend in this manner to examine the effect of lead on the activity of various enzymes known to be required for porphyrin synthesis.

VI. Conclusions

During the brief duration of this project we have undertaken investigations of the methodology for determination of lead in biological systems and have investigated the interactions of lead with several proteins. We have also shown that lead affects the growth and metabolic characteristics of a simple micro-organism, Rhodopseudomonas spheroides, which may serve as a model system for studies of porphyrin synthesis. Significant progress in these areas is already evident and based upon this work further efforts will proceed along the lines indicated in the initial proposal.

TABLE I

Examination of Ultraviolet Spectrum for Lead Lines By Atomic Absorption Spectroscopy (Hydrogen-Air Flame)

Lines (Å)	Io(μA)	I(μA)
2833	80	54
2169.99	"	33
2164	"	(less Absorption than at 2833 Å)
2152	"	"
2130	"	"
2093	"	"
2081	"	"
2065	"	"
2026	"	"
2005	"	"
1976	"	"
1962	"	"
1941	"	"
1916	"	"
1908	"	"
1900	"	"
1888	"	"
1880	"	"
1878	"	"
1872	"	"
1870	"	"

Reference BRL 175-61

TABLE II

METAL COMPLEXES OF MERCAPTOETHANOL

All at pH 8, with a 50:1 ratio of ligand:metal

GROUP	METAL ION	WAVELENGTH, MAX. OR SHOULDER	MOLAR EXTINCTION
IIA	calcium(II)	266	1800
	strontium(II)	--	--
	barium(II)	--	--
VIII	iron(II)	236	3000
	cobalt(II)	280	20000
	nickel(II)	255	3400
		330	3000
IB	copper(I)	264	11600
	copper(II)	264	9600
	silver(I)	275	6000
		375	5200
IIB	zinc(II)	226	5600
	cadmium(II)	248	17400
	mercury(II)	<225	>2400
IVA	lead(II)	250	14000
		310	3600

CALIBRATION CURVE FOR LEAD (aqueous soln.)

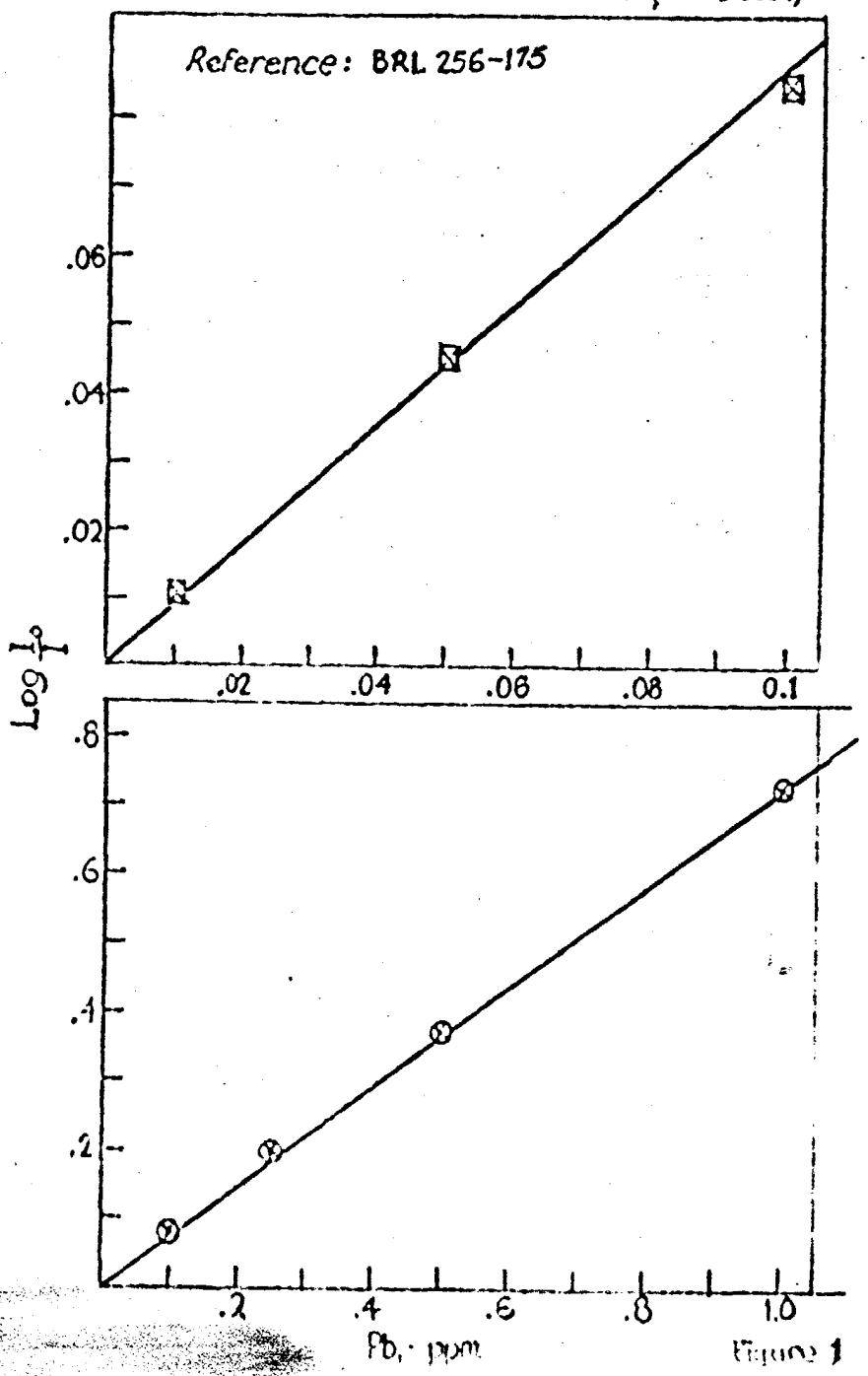


Figure 1

EFFECT OF PERCHLORATE ON ATOMIC ABSORPTION OF LEAD

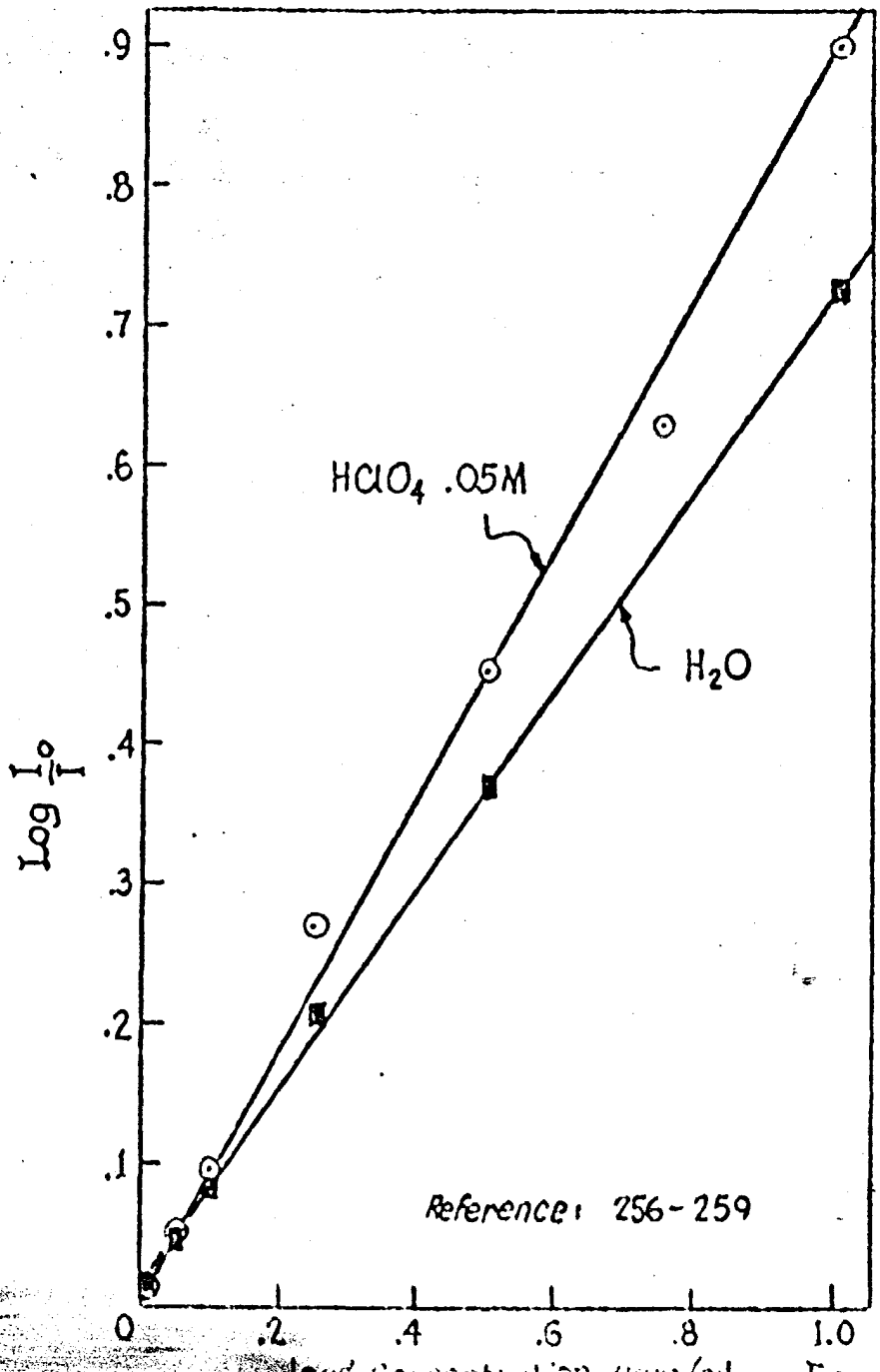
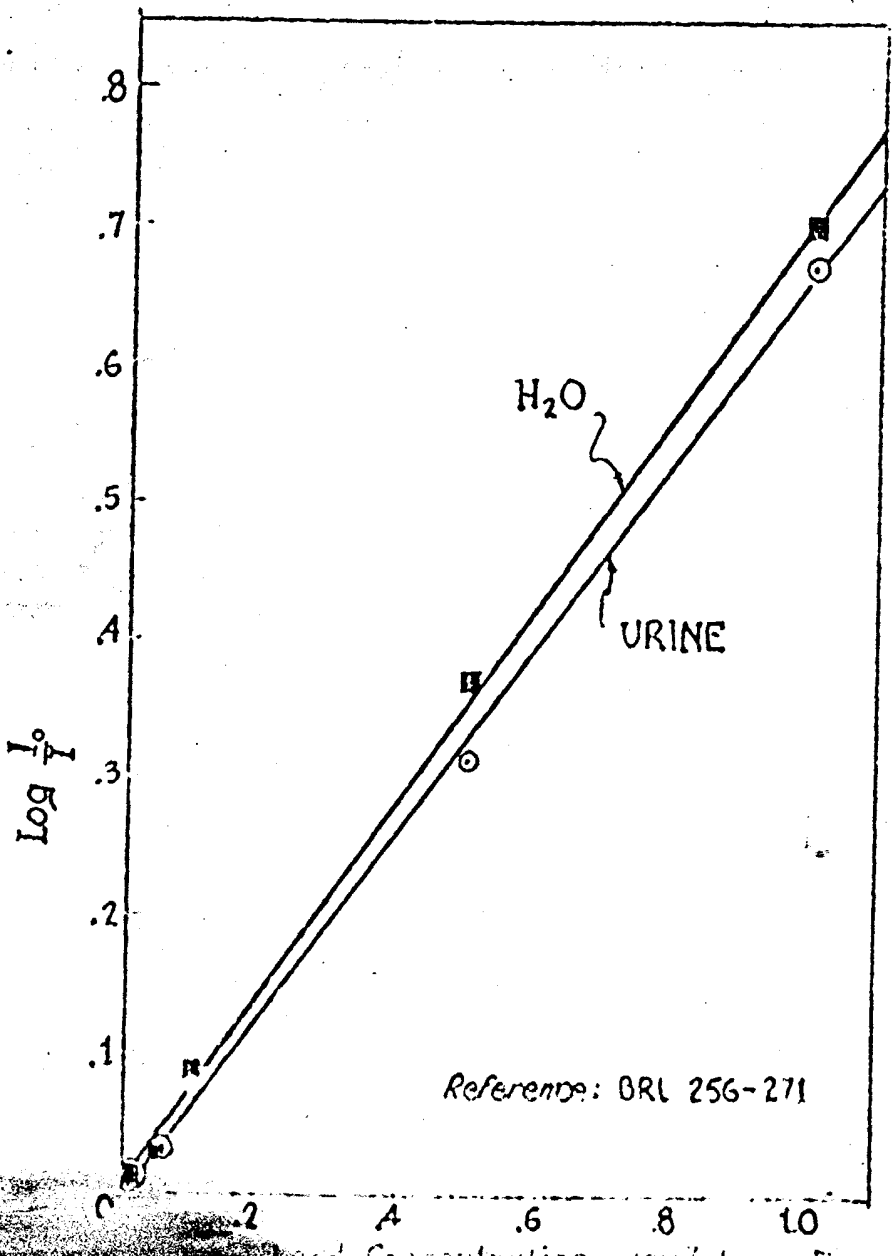


Figure 2

COMPARISON OF ATOMIC ABSORPTION OF LEAD IN URINE vs. WATER



Reference: BRL 256-271

Figure 3

ATOMIC ABSORPTION SPECTROSCOPY OF LEAD

Ring micro-burner vs Beckman burner

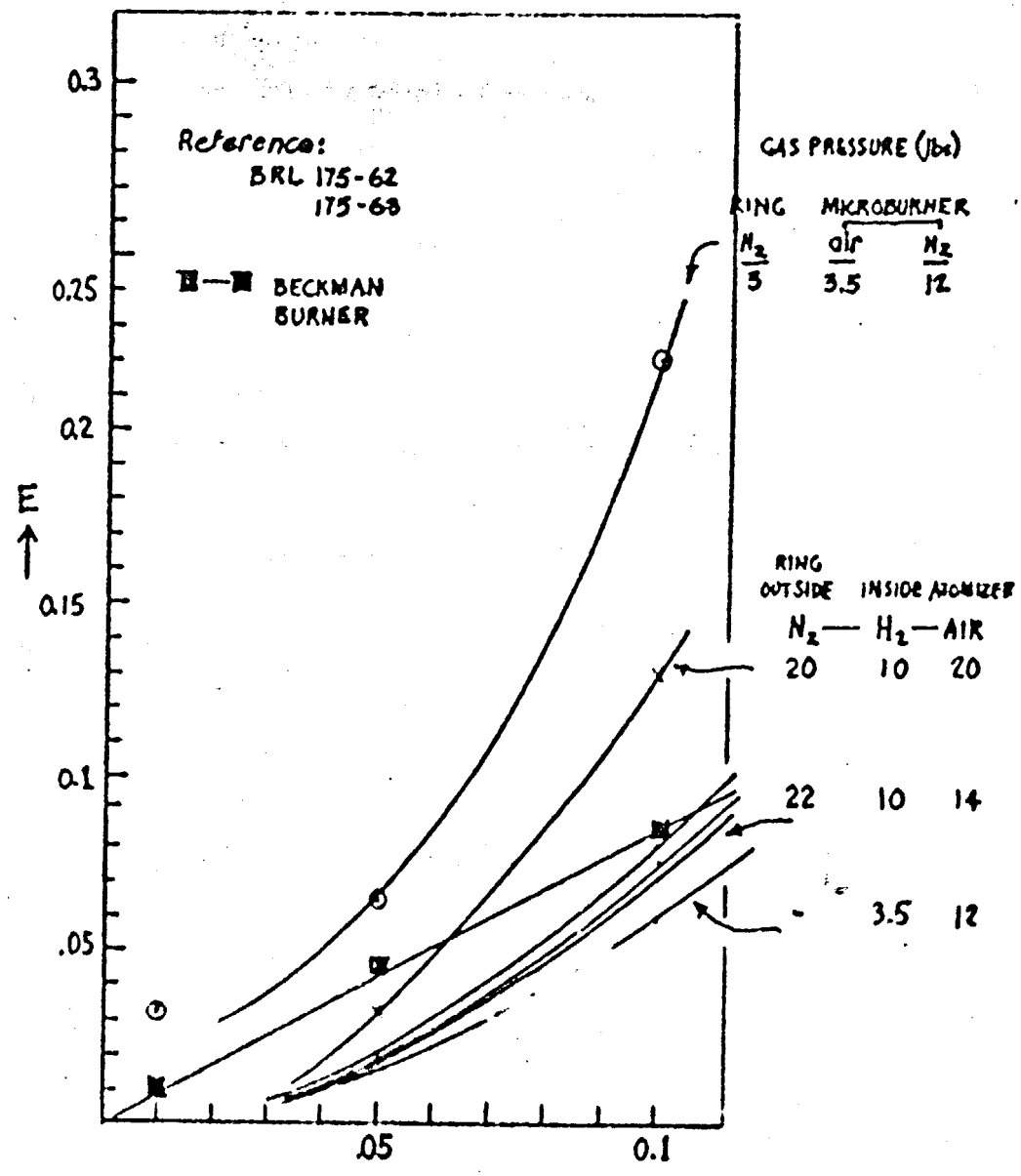


Figure 4

EFFECT OF LEAD UPON BINDING OF IRON TO CONALBUMIN

Protein = 13.6 mg
Lead = 207 γ

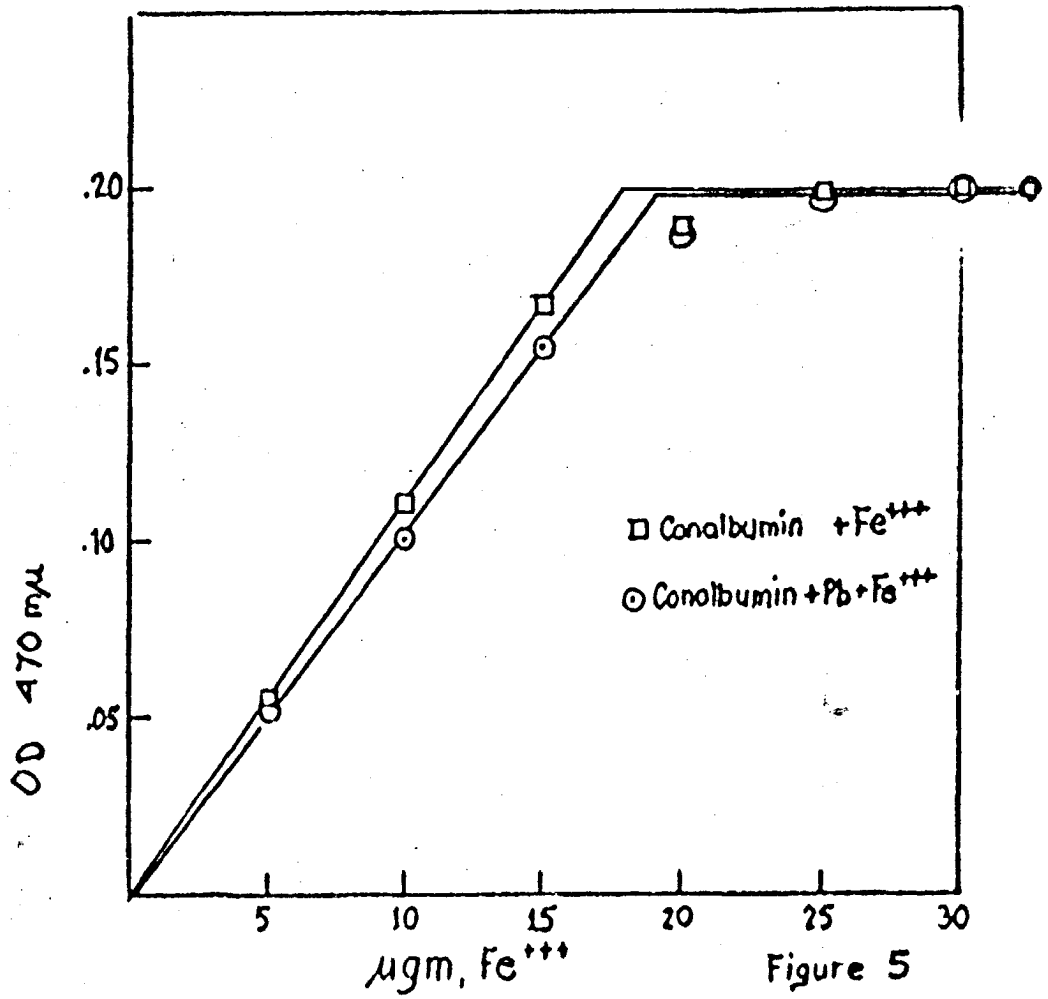


Figure 5

Reference: BRL 256-146
256-144

EFFECT OF LEAD UPON BINDING OF IRON TO TRANSFERRIN

Protein = 10.95 mg/ml (slightly turbid)
lead = 207 γ

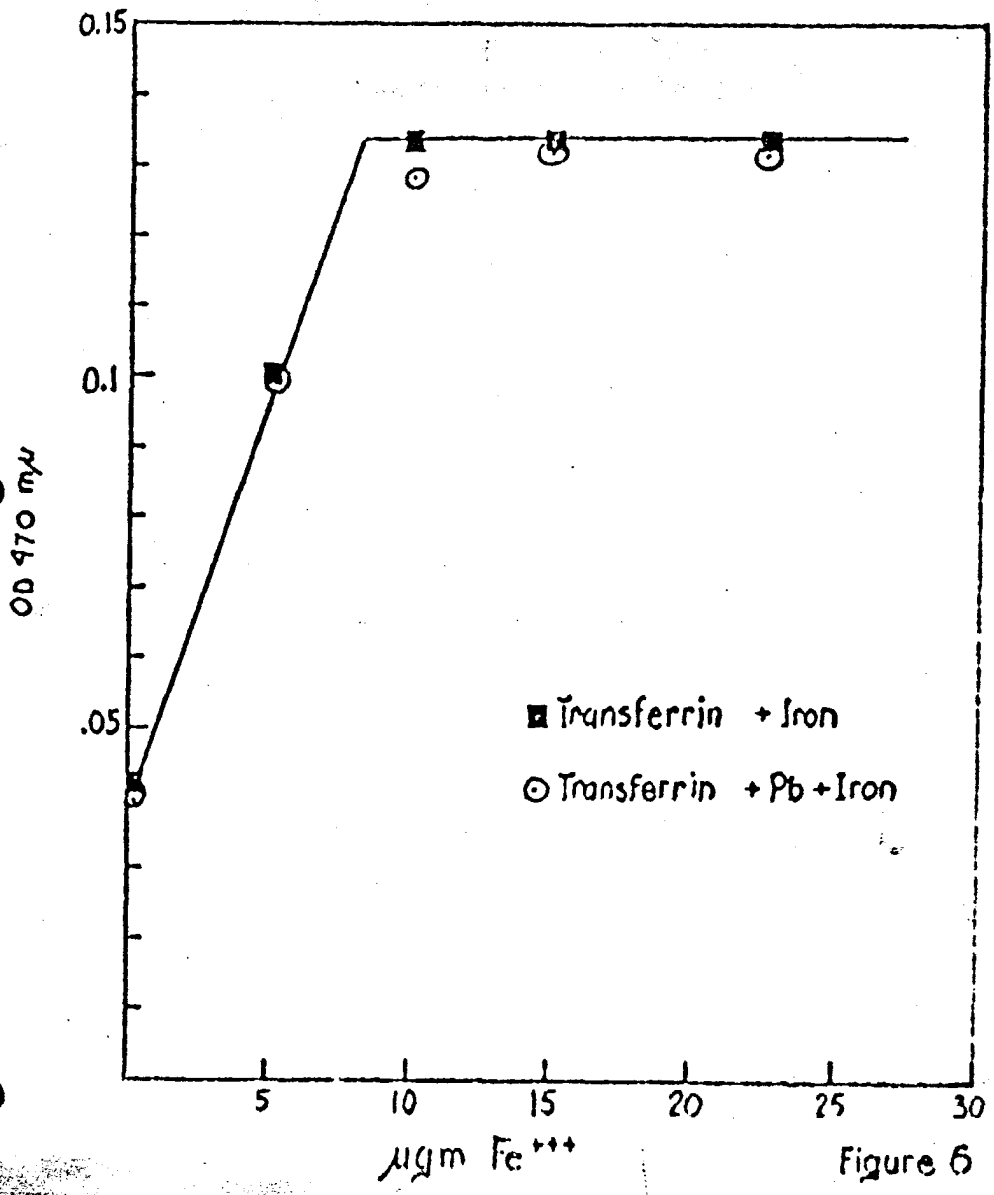


Figure 6

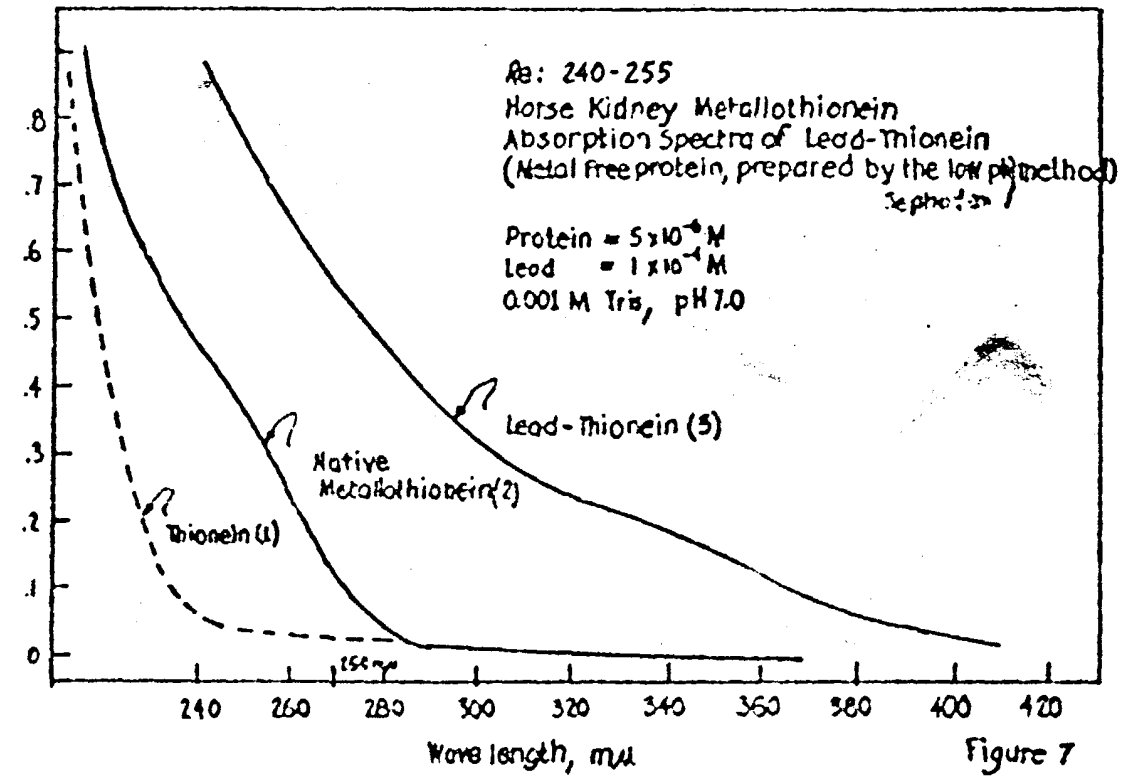

Rs: 240-255
Horse Kidney Metallothionein
Absorption Spectra of Lead-Thionein
(Metal free protein, prepared by the low pH method)
Protein = 5 x 10⁻⁶ M
Lead = 1 x 10⁻⁴ M
0.001 M Tris, pH 7.0
Lead-Thionein (5)
Native Metallothionein (2)
Thionein (1)
Absorbance
Wave length, mμ
240
260
280
300
320
340
360
380
400
420
0
.1
.2
.3
.4
.5
.6
.7
.8

Figure 7

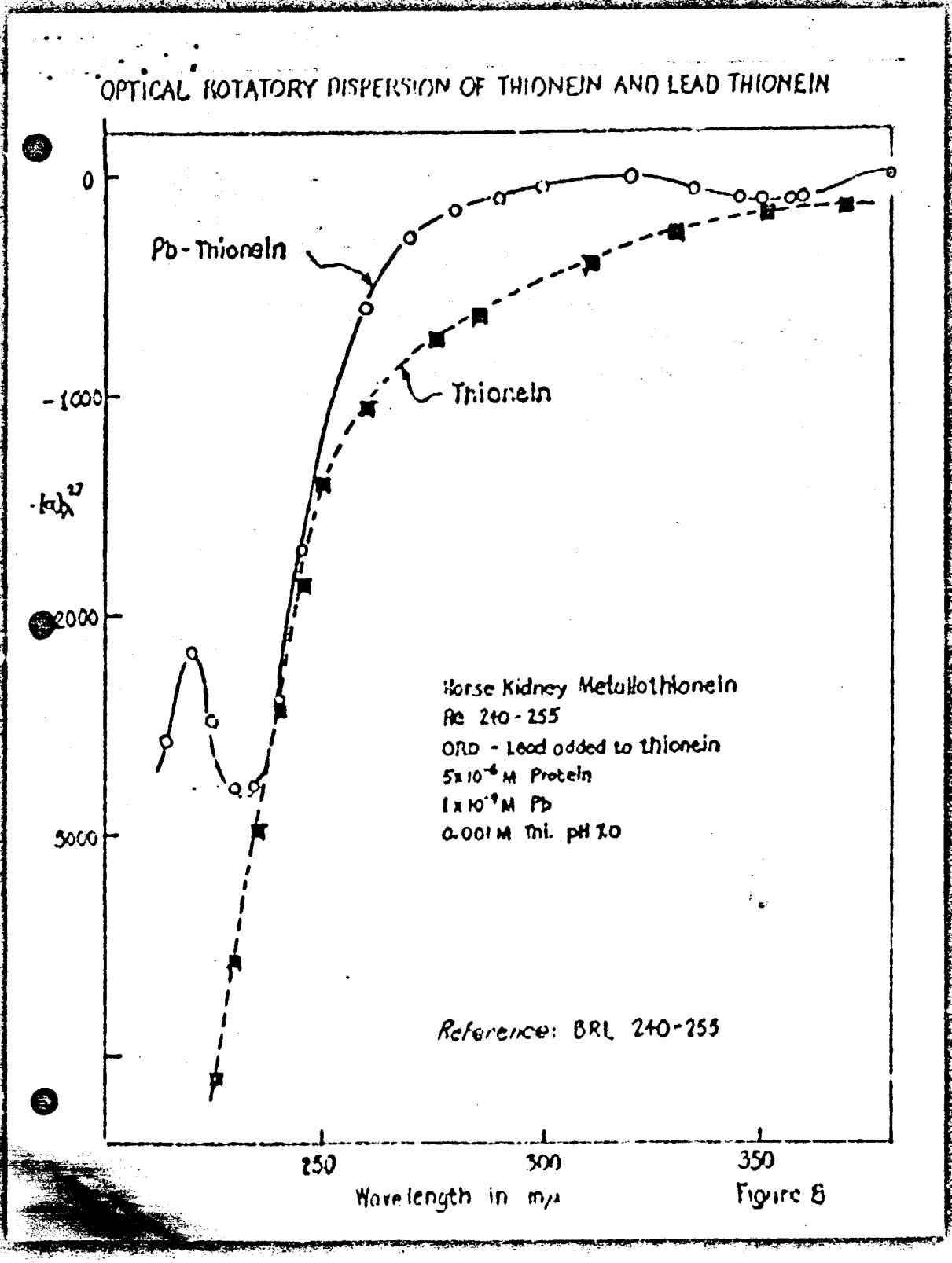

OPTICAL ROTATORY DISPERSION OF THIONEIN AND LEAD THIONEIN
0
-1000
-2000
-3000
-[a]λ²
Pb-Thionein
Thionein
Horse Kidney Metallothionein
Ac 240-255
ORD - Lead added to thionein
5x10⁻⁶ M Protein
1x10⁻⁴ M Pb
0.001 M Tri. pH 7.0
Reference: BRL 240-255
250
300
350
Wavelength in mμ

Figure 8

PROPOSED BUDGET FOR STUDY ON BIOLOGICAL BASIS OF LEAD INTOXICATION

Personnel:	
Research Associate, full time	$13,000.00
Retirement, Social Security, etc.--Harvard Composite Rate, 15%	1,950.00
Technician, full time	5,250.00
Harvard Composite Rate, 9 3/4%	511.88
Chemicals and Glassware	2,500.00
Instrumentation and Modifications	
Vanguard Model 1056A Automatic Ultraviolet Analyzer	3,180.00
GMK Model VI Fractionator with funnels and Volumetric Cylinders	1,200.00
Shop Time	725.00
Travel	250.00
Total	$28,566.88
Overhead, Harvard Rate 20%	5,713.38
Grand Total	$34,280.26