Report to International Lead Zinc Research Organization on

Project No. IH-94

STUDY OF THE BIOLOGICAL BASIS FOR LEAD INTOXICATION

August 1, 1966 to July 1, 1967

Practice Project Level [illegible]

1. Introduction

[illegible] preliminary "screening" investigations undertaken in order to discern suitable systems for exploring in detail the [illegible]

from the kidneys of animals small enough in size to be convenient for experimental manipulation. Clearly, this would be an important consideration in view of anticipated investigations requiring dietary control, administration of precise quantities of various heavy metals, collection of excrement, etc. To this end, we first attempted to ascertain if it would be possible to isolate a protein having the characteristics of metallothionein from rabbit kidneys.

Homogenates of kidneys obtained from adult rabbits were chromatographed on columns of Sephadex G-50 (fine) in 0.02 M Tris-0.005 M Cl buffer, pH 8.6. The fractionation was followed by the absorbancy at both 254 and 280 mμ(metallothionein containing essentially no aromatic amino acid residues does not absorb, as do most proteins, at 280 mμ) and the cadmium content of each fraction was determined by means of atomic absorption spectroscopy. The results are shown in Figure 1. A shoulder of 260 mμ absorbing material is evident in the trailing edge of the major protein peak and coincides with a sharp cadmium peak. This pattern on Sephadex chromatography is precisely as would be anticipated for a small molecular weight protein such as metallothionein and coincides with the known behavior of this protein when isolated from equine and human sources.

For further purification, the cadmium containing fractions of the protein were lyophilized and chromatographed on columns of DEAE cellulose

column (Figure 2) with a gradient composed of 0.01 M Tris-[illegible] to 0.1 M Tris-0.03 M CS_2, pH 8.6. This procedure enabled us to profile the chromatographic sensitivity of this material with [illegible] of metallothionein from other sources. Preliminary experiments suggested that it might be feasible to similarly isolate metallothionein from [illegible] animals and ascertain if lead were bound to this protein in vivo.

[illegible] Metallothionein from [illegible] Rabbits

[illegible] ranging from [illegible] to [illegible]

while the liver tissue is currently being preserved for isolation of RNA in an effort to explore the possibility of interaction of lead with RNA in vivo(vide infra).

The results of the isolation of metallothionein from kidneys of control animals correspond to these shown in Figures 1 and 2, confirming the results of our preliminary experiments. The cadmium and zinc contents of the two principal peaks resolved by DEAE chromatography(Figure 2) are shown in Table 1. The chromatographic results are characteristic of metallothionein from other species as observed in our laboratory, but the limited amount of material available after completion of the fractionation rendered precise metal analysis difficult.

Kidneys obtained from the lead-intoxicated rabbits were [illegible]. The results of the Sephadex and DEAE chromatography are shown in Figures 3 and 4 respectively. Metal analyses of the fractions resolved on DEAE are listed in Table 2. Interestingly, lead is found in direct association with the cadmium and zinc containing peaks on Sephadex and DEAE chromatography. [illegible]

The distinct association of E_{250} absorbing material, cadmium, and lead strongly suggested(Figure 3) that lead, like cadmium, is bound to metallothionein in rabbit kidneys. The results from DEAE chromatography are in accord with this conclusion(Figure 4) since the major quantity of lead-containing material is distributed in the fractions through which the two metallothionein peaks emerge from the column. Unfortunately, the rather large salt concentration in this area, coupled with the small amounts of material available at this stage of the isolation procedure, precluded accurate quantitative determinations of the metal contents and, hence, the results in Table II cannot be considered to be final. Thus, it was not possible to establish, as hoped, the precise effect on the metal content of metallothionein of [illegible] in the rabbit, largely due to the small size of the kidneys yielding insufficient amounts of material for characterization. There can be little doubt, however, of the association of lead with metallothionein in this material, and the experiment suggests that the rabbit will be a highly suitable experimental animal for (in vivo) studies of displacement by [illegible] metals. We intend to further pursue such studies during the coming year.

IV. Lead Intoxication in the Equine(Pony)

In order to obtain the desired quantitative estimates of the interrelationship of the various heavy metals with metallothionein in states of lead intoxication, as well as to have suitable quantities of material available for amino acid analysis, peptide mapping, and further characterization of lead metallothionein, we have recently carried out a chronic poisoning experiment in a larger animal---a pony. We were able to purchase this animal approximately five months ago, and in the intervening period, with the aid of the Harvard Veterinary Service, have administered to the animal slowly increasing quantities of lead acetate, by the oral route, up to a final total dose of 12 grams daily. Intravenous or intramuscular administration was precluded owing to the ready formation of thromboses and abscesses. Hence, initially, this material was given by stomach tube. It was subsequently found that, if thirsty, the pony would drink the material mixed with a quart of ordinary water, the taste apparently not being too unpleasant. Since no specific information on lead intoxication in this species was available, the doses had to be gauged empirically. While the absorption of lead from the intestinal tract of this species is probably quite small, the large doses administered eventually enabled significant intoxication to be obtained. We had excellent success with our goal to poison the animal slowly, and induce anemia, but at no time to render him critically ill.

Poisoning was monitored by means of bimonthly measurements of the lead concentration in whole blood, which were maintained in the range of approximately 80 to 100 μg%, and hematocrits, which ultimately fell to nearly half the normal values. During the final month of the experiment, the animal exhibited signs of lethargy and significant weight loss, although paralysis, convulsions, or signs of gastrointestinal dysfunction were never observed.

The animal was sacrificed on July 11, and samples of all major organs and tissues obtained at postmortem examination were quickly frozen and are being preserved for future study. Presently, we are working on the isolation procedure for metallothionein in the kidney tissue and this should enable us to obtain quantitative information concerning the interaction of lead with this protein and the relative displacements of other metals upon binding here.

During the coming year we also plan to attempt a similar isolation for metallothionein from liver since the protein has been reported to be present in this tissue as well as kidney. [illegible] of the animal will also be used to isolate [illegible]

whether or not lead might replace the native metal of certain metalloenzymes known to be present in liver tissue, e.g., liver alcohol dehydrogenase, a zinc containing enzyme. Preliminary evidence suggests that it may be possible to carry out such a displacement of zinc by lead *in vitro* and the availability of suitable quantities of tissue from the lead poisoned animal permits us to search for the *in vivo* counterpart.

Prostatic tissue is known to contain very high concentrations of zinc although the precise functional role of the metal in this organ is uncertain. Prostatic tissue also has a high affinity for calcium and is a site of injury in calcium intoxication in [illegible]. We were able to secure the prostate from the lead intoxicated pony, and the concentrations of lead in this tissue will be ascertained and the possible displacement of normal zinc stores. Much of the analytical work of this nature to be undertaken during this next year will benefit from the availability of suitable spectrographic methods developed in this laboratory permitting the simultaneous determination in biologic materials of a large number of elements quantitatively. It is proposed to conduct further studies in this species if this pilot experiment will prove it to have the desired characteristics as is hoped.

V. Effect of lead on Growth and Metabolism of Rhodospseudomonas spheroides

We have indicated previously that the facultative anaerobe,

whether or not lead might replace the native metal of certain metalloenzymes known to be present in liver tissue, e.g., liver alcohol dehydrogenase, a zinc containing enzyme. Preliminary evidence suggests that it may be possible to carry out such a displacement of zinc by lead *in vitro* and the availability of suitable quantities of tissue from the lead poisoned animal permits us to search for the *in vivo* counterpart.

Prostatic tissue is known to contain very high concentrations of zinc although the precise functional role of the metal in this organ is uncertain. Prostatic tissue also has a high affinity for lead and is a site of injury in calcium intoxication in rabbits. We were able to secure the prostate from the lead intoxicated pigs, and the concentrations of lead in this tissue will be ascertained and the possible displacement of normal zinc stores. Much of the analytical work of this nature to be undertaken during this next year will benefit from the availability of suitable spectrographic methods developed in this laboratory permitting the simultaneous determination in biologic materials of a large number of elements quantitatively. It is proposed to conduct further studies in this species if this pilot experiment will prove it to have the desired characteristics as is hoped.

b. Effect of lead on growth and metabolism of *Rhodopseudomonas spheroides*

We have indicated previously that the facultative anaerobe,

Streptococcus sanguis, might prove attractive as a model system for studies of heavy metal metabolism and intoxication. Our last progress report noted preliminary studies employing this organism in attempts to identify a metabolic site sensitive to lead intoxication and our survey of the interactions of lead with a large number of soluble enzyme systems obtained from the bacterial cultures. These screening efforts have now shown additional approaches which have proven rewarding.

During the past year we have been able to establish reproducible conditions for the incubation [illegible] of the growth of Streptococcus sanguis and a considerable number of experimental [illegible] to evaluate the effect of various growth factors in the presence and absence of lead have [illegible] these studies we [illegible] a chemically [illegible] medium in which [illegible] metal sensitivities [illegible] effects of lead at the [illegible] The [illegible] [illegible] and we are [illegible] in the [illegible] and [illegible].

carbon sources and thiamin, nicotinic acid, and biotin provided as cofactors. Tungsten lamps were used as a source of both heat and light, and temperature during growth is maintained at approximately 30°. When several grams of organisms are required, growth is carried out in 2 liter Erlenmeyer flasks, while for screening procedures and many nutritional experiments it was found to be convenient to grow duplicate cultures of the organisms in 25 ml test tubes. Stocks were maintained from agar slants made up of the same media plus "Difco" proteose peptone media #3. Growth was monitored by measurement of the optical density at both 650 and 540 mμ, the former reflecting the turbidity while the latter served as a guide to pigment formation.

Typical growth curves obtained under these conditions are shown in Figure 2. The lag phase varies from 4 to 24 hours depending on the size of the inoculum, the rapid growth phase required 8 to 24 hours, and the cultures entered the stationary phase at 48 to 72 hours. Figure 3 also depicts the growth curves at two different concentrations of iron. It appears [illegible] the culture media, the organisms [illegible] found that iron was [illegible] the [illegible]

[illegible]

...ease inhibition by lead is observed at all concentrations of ...iron, inhibition is most pronounced when iron is absent or present in only small concentrations. Higher concentrations of iron both increase the total growth of the organisms and, in addition, partially overcome the inhibition imposed by lead. The effect of lead at varying concentrations on total growth determined at the end of 20 hours, by measurement of O.D. at both 540 and 420 mμ, is shown also in figures 5 and 9. These experiments confirm that the lead inhibition is more pronounced at lower iron concentrations; however, enough iron must be present to ... for the ... lead effect to ... [illegible]. Therefore, for this experiment, it would appear that ... iron is adequate for ... [illegible]

occurs in the presence of 1 mg per liter each of thiamine and nicotinic acid and 10 mg per liter of biotin. Under the conditions employed for the present experiments an absolute requirement for each of these cofactors was confirmed although we obtained somewhat differing results for the optimal concentration for each of the cofactors. The growth of the organisms as a function of variation in cofactor concentration is shown in Figure 2. These results were obtained at an iron concentration of 10 μM/liter. However, the optimal cofactor concentration is somewhat dependent upon available iron (Figure 3); notably, growth at the highest cofactor concentrations is greater when iron in the media is increased to 100 μM/liter. These experiments provide a basis for optimization of the [illegible]

[illegible]

[illegible]

h. Effect of Nicotinic Acid on Lead Inhibition of *Rhodopseudomonas spheroides*

In a fashion identical to that employed for thiamine, the effect of nicotinic acid on the lead-induced inhibition of *Rps. spheroides* was assessed as is shown in Fig. 14 and 15. No clear relationship is observed between the concentrations of nicotinic acid and the inhibition induced by lead. It would seem evident that lead also does not act by interfering directly by competing with this cofactor. The variable growth of the organisms as a function of concentration of both nicotinic acid and iron was given in figure 15.

i. Effect of Biotin on Lead Inhibition of *Rhodopseudomonas spheroides*

In a series of experiments similar to those described above, the possible relationships of biotin to the lead inhibition of *Rps. spheroides* was investigated. The results are presented in figures 16 and 17. Concentrations of biotin ranging from 20 micrograms per liter had no effect upon the lead tolerance of the organism. However, a concentration of 10 micrograms per liter was found to result in some increase in growth in the presence of lead but no significant potentiation of the lead inhibition of growth was observed. Appropriate explanation was given at the beginning of [illegible]

but was overcome when iron concentrations were optimal. It seems not unlikely that high concentrations of biotin act to effectively remove iron from availability for metabolism and, thereby, both limit growth and potentiate the lead-induced inhibition.

This relationship was also further examined at still higher biotin concentrations(1000 micrograms per liter) and at iron concentrations from 0 to 10^{-5} molecules per liter,figures 18 and 19). While there is some variation in the experimental data, it is clear again that the inhibition by biotin is more pronounced at lower iron concentrations. Shown on the same figures, for [illegible] and [illegible] lead inhibition at high iron concentrations. Notably, with these very large excesses of cofactors and iron, lead inhibition is almost completely reversed. [illegible] is not due to the high concentration of cofactors alone, as is shown in figures 20 and 21. Here, where no iron is added to the growth medium, increasing cofactor concentrations up to 500 times normal either had no effect on, further enhanced the inhibition induced by lead. While it appears that biotin and [illegible] can [illegible] the lead inhibition [illegible] these conditions make the significance of this observation somewhat doubtful.

[illegible], alkaline tris, and ferrous, were also tested at an intermediate iron

concentration, e.g., 10 micromoles/l (Figures 22 and 23). At this iron concentration, total growth is of course considerably improved over that at "no added iron"; while high concentrations of nicotinic acid are still inhibiting, they are less so than when iron is absent from the growth media.

Finally, the effect of cofactors on the lead-induced inhibition of *Rps. spheroides* was also studied in several multi-variable experiments, an example of which is shown in Figures 24 and 25. Again, the inhibition by lead was found to be relatively independent of cofactor concentration in the growth medium.

In summing up this series of experiments, it has been possible to show conclusively that the inhibition of growth [illegible] addition of lead does not [illegible] [illegible] with the known essential vitamin [illegible] [illegible]

[illegible], it was then determined to explore the metal ion requirements for growth of *Rps. spheroides* in order to ascertain if lead might act by interfering with an essential trace element. These experiments have led to observations of particular interest [illegible]

[illegible]

[illegible]

systems, this metal is known to activate a host of enzymatic reactions involving transferases, hydrolases, lyases, isomerases, and ligases, and manganese has already been shown to be an essential element in the biotin dependent pyruvate carboxylase of chicken and turkey and likely serves analogous roles in other metalloenzymes. Manganese has also been found to be firmly associated with ribonucleic acid isolated from diverse sources and, in RNA, the metal may serve as an important determinant of structure and it may also function in protein synthesis. Manganese has also been postulated to play a role in oxidative phosphorylation, metabolism of fatty acids, and in cholesterol synthesis. The effect of this metal on the growth of [illegible] was tested since it has been asserted that under certain conditions manganese might alter [illegible]

The effect of manganese on the growth and the lactobacillus [illegible] in the absence of iron, or in the presence of 10 milliliter iron, is shown in Figures 2a and 2b. In the control, without added manganese, final signal [illegible] decreases greatly either in the presence or absence of added iron, as noted by [illegible]

As indicated by the absorbancy at 650 nm(Figure 26), the total growth is sharply increased in the lead-exposed iron-sufficient cells upon addition of 0.1 mg/l of manganese, even beyond that observed in the controls. This effect is even more striking, however, when the absorbancy at 400 nm, reflecting pigment production, is determined(Fig. 27). Pigment is almost double in concentration in the lead plus manganese exposed cells. Figure 28 will show that high concentrations of iron [illegible] reverse the apparent stimulation of growth of the manganese cells, although this is not that relative [illegible] growth in the controls is actually [illegible] two [illegible] at these high concentrations of iron. However, the [illegible]

[illegible] were found at 450, 500, 540, and 560 mμ (Lowe and O'Brien, [illegible] 3, 79, 130, 1973).

[illegible] the experimental conditions employed for these [illegible] appear to induce a lesion in porphyrin synthesis resulting in the appearance of coproporphyrin III in the culture [illegible] In the absence of iron, lead does not appear to alter this phenomenon. However, in the presence of added iron, lead [illegible] the effect of exposure. The mechanism for [illegible] was then explored with the goal of identifying [illegible] of porphyrin synthesis at the [illegible]

[illegible]

[illegible] as shown in Figure [illegible]

[illegible]

could lead to accumulation of coproporphyrin III. On the other hand, manganese and lead might act to stimulate the production of precursors involved in steps prior to the synthesis of coproporphyrinogen and increase these precursors and coproporphyrin III beyond the capacity for further metabolism. A similar phenomenon could be observed if the metals acted to block the synthesis of a metabolite which ordinarily served as a repressor of early steps during the reaction. For example, it is known that heme acts as a feedback inhibitor for the first step in tetrapyrrole synthesis, the condensation of [illegible] to form [illegible]. We tested this hypothesis by determining the effect of manganese and lead on total growth and on the [illegible] of the synthesis scheme, [illegible] the [illegible] the existence of a [illegible] the presence of [illegible] [illegible]

[illegible]

when variable pigment production might be expected to influence the absorbancy measurements. Determination of the dry weight of cells per unit volume of cultures provides such an index of total growth but is quite time-consuming. It was found possible to obtain extremely convenient estimates, however, by measurement of the packed cell volume after centrifugation of the cultures in Wintrobe tubes, quite analogous to the procedure employed for determination of blood hematocrits. The correlation of packed cell volumes, determined by this means, with the weight of cells after heating to dryness at 104° is shown in Figure 31. On this basis, each millimeter of packed cell volume(PCV) [illegible] corresponds to 1.274 mgs of salt-free dried cells. Subsequent measurements were referred to this index of growth for purposes of normalizing the data.

C. Effect of Combinations of Manganese and Lead on Growth of Rhodopseudomonas spheroides

The effect of manganese concentrations on the growth of Rps. spheroides at three different concentrations of lead is shown in Figure 32. At all concentrations of lead, the addition of small amounts of manganese, less than [illegible] significant enhancement of growth. Higher concentrations of manganese, up to [illegible] had little effect on the growth of Rps. spheroides in the absence of lead but the presence of [illegible]

[illegible] enhancement of growth. These observations suggest, in fact, that it is manganese which is the growth-limiting factor for [illegible] peroxides in the presence of sufficient iron, while it is iron which becomes limiting in the presence of adequate manganese. Such a relationship has not, to our knowledge, been defined previously. While it remains to be determined if any other metal will substitute for manganese, to date we have found that copper(II), nickel, and zinc are not effective [illegible]. The inhibition of growth by lead could [illegible]

[illegible] effect of [illegible] on the [illegible] oxidation

[illegible] of copper(II) ion [illegible] the surrounding [illegible] It is known that under [illegible]

dependent site.

The effect of lead in these circumstances is shown in Figure 34. Conditions are as in Figure 33 other than the presence of 1×10^{-4} M Pb acetate. In general, it is apparent that lower concentrations of manganese are required to induce coproporphyrin III production if lead is present. Thus, in cultures grown in the presence of 10 µM/l of iron and 10 µg/l of manganese, coproporphyrin III production is minimal. However, if lead is present in this same culture, coproporphyrin production is increased nearly four times. Thus, lead and manganese exert [illegible] effect to induce the [illegible]

f. Effect of Manganese on Intracellular [illegible]

The effect of manganese on production [illegible] of low, intermediate and high concentrations [illegible] shown in Figure 35. Saccharomicoprophil was determined in the presence [illegible] extracts of centrifuged cells (Schneider, Fiterman, and Stanier, J. Cell. Comp. Physiol. 47, 23, 1957). Despite the marked stimulation of growth induced by manganese [illegible] this metal [illegible] to a striking decrease in [illegible] saccharomicoprophil [illegible] manganese [illegible] of saccharomicoprophil [illegible] manganese [illegible]

notably, the effect of iron in this regard appears rather minimal. Cultures grown in the presence of lead display an identical interference with bacteriochlorophyll production when manganese is added. Thus, it is suggested on the basis of these experiments that the increased excretion of coproporphyrin III into the media of Rps. spheroides, when grown in the presence of manganese, can be attributed to a block by this element in the synthetic pathways between protoporphyrinogen and bacteriochlorophyll.

F. Effect of Manganese on Heme Production

The effect of manganese on heme production was [illegible] under the same experimental conditions as those employed for [illegible] ntrations. Total heme was measured spectrophotometrically based upon the difference in absorption of the oxidized and reduced forms of the alkaline pyridine hemochromogens of whole cell extracts of the microorganisms (Porra and Lascelles, [illegible], 1965). It was found that accurate estimates of total heme were obtained [illegible] for the above experiments, due to [illegible]. Therefore, in order to [illegible] the interrelationships of the metals with total heme [illegible], both in the presence and absence [illegible]

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of manganese and lead, and at two different iron concentrations. The results are shown in Table IV.

Either in the presence or absence of added iron, or added iron plus lead, total heme in the cultures was about .8 to .95 [illegible] cell volume. In the cultures without added iron, however, addition of manganese decreased total heme present by nearly 50%. Lead did not further enhance this inhibition in heme synthesis. [illegible] [illegible] per liter of iron, [illegible] was without effect on heme synthesis. Now, however, addition of lead decreased total heme by more than 1/3. Thus, these results are in keeping with the observations on coproporphyrin production: when iron is present, lead and manganese act synergistically; in the absence of iron, manganese alone is sufficient to exert [illegible] effect.

[illegible] these [illegible] [illegible] and lead appear to interfere with tetrapyrrole synthesis [illegible] production of [illegible] heme [illegible] leads to accumulation in the growth [illegible] of coproporphyrin [illegible] to figure [illegible] the conversion of [illegible] the conversion of [illegible] inhibition [illegible] [illegible] with [illegible]

[illegible] Moreover, other sites of inhibition by [illegible] not yet be excluded. Having narrowed the field to these steps in tetrapyrrole synthesis, however, we are currently trying to identify unequivocally the precise reaction [illegible] blocked or inhibited, we shall then examine in detail the mechanism of that defect, hopefully with the isolated enzyme or enzymes which are responsible.

[illegible] investigations of the general [illegible] system [illegible] Here, [illegible] of interest is [illegible] with other metals, to influence a metabolic system.

proteins and enzymes is considerably smaller than for many other metals, e.g., Ag^{+}, Hg^{++}, or Cu^{++}. Thus, lead is a relatively weak inhibitor of enzymes possessing a single [illegible] at the active site(sulfhydryl enzymes). Concentrations of lead required to inhibit such enzymes *in vitro* are usually of the order of 10^{-4}-10^{-3}M and, therefore, far exceed the concentrations present in tissues and body fluids of lead-intoxicated animals. On this basis, it might be thought [illegible] that lead would inhibit such enzymes *in vivo*.

Significantly, the ability of lead to bind to such systems is drastically altered when the metal forms a chelate structure [illegible] of interacting with more than one ligand group of the [illegible] molecule. [illegible] well-established relationships of chelate [illegible] of [illegible] metal complexes with an [illegible]dentate chelating agent [illegible] [illegible] the binding constant of the [illegible] [illegible] the number of ligand atoms [illegible] [illegible] with a relatively high [illegible] [illegible] increase by 100,000-fold [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] related directly to [illegible]

[illegible] with these enzyme groups in this protein, [illegible] their proximity.

[illegible] such circumstances it would be predicted that [illegible] containing both the thiol group at their catalytic [illegible], in proper spacing, should also be much more sensitive to [illegible] lead than enzymes still enzymes.

[illegible] interference in the oxidative ATP dependent [illegible] lead with [illegible] of the pyruvate decarboxylase [illegible] a heterogeneous [illegible]

[illegible]

[illegible] to be oxidized to lipoic acid in the oxidized form and, [illegible] subsequently, reduced by NADH. Hence, interaction of the dithiol [illegible] with a metal, such as lead, should interfere with the [illegible] cycle and abolish enzymatic activity. To test this [illegible] we have examined the interaction of lead with this [illegible] and the effect of lead on the reduction of lipoic acid [illegible].

[illegible]

observed, indicated by the loss of 50% of the antiarrhythmic activity in the presence of 6.1 x 10^{-6} M Pb^{++} (solid line).* The oxidation appears to be time-dependent, increasing more rapidly as the course of the assay. Thus when the rates of oxidation of PPNH are measured 4 minutes after initiation of the reaction(broken line) the point of 50% of inhibition is shifted towards a Pb^{++} concentration of 5 x 10^{-6}M. From this and related observations, it appears that [illegible] equilibrium within the period of these [illegible]

is added after DPN(NPN$^+$) and lipoate, only 20% inhibition was observed at the same concentration of lead producing 50% inhibition when the order of addition of reactants was reversed. Consequently, the inhibition curve is shifted toward higher lead concentrations, denoting an apparent "lower affinity" of lead for the enzyme. Thus, these results demonstrate partial protection by substrates and [illegible] against inactivation of the enzyme by lead.

The effect of coenzymes and substrates on the reversibility of the lead inhibition by EDTA provides additional evidence that, indeed, lead binds at the active site of [illegible] As noted above, the [illegible]

[illegible]

[illegible]

Significantly, a very analogous inhibition is also observed with cadmium, long known as a potent inhibitor of lipoamide dehydrogenase. Owing to its greater affinity for thiol ligands cadmium is an even stronger inhibitor than lead. As in the case of lead, inhibition by cadmium is not reversed when NaEDTA is added *after* the inactive Cd-lipoamide dehydrogenase has formed a complex with coenzyme and substrate(Table 1).

[illegible] of lead and cadmium [illegible] apparent dissociation [illegible] which are consistent with [illegible] the lead-enzyme [illegible] and the two [illegible] to those observed [illegible] of lead [illegible] with the [illegible] ligands. These results indicate [illegible] the two [illegible]

renders the absorption spectrum of bound FAD more similar to that of free FAD, suggesting that it interferes with the strong association of FAD with the protein. This is consistent with the suggestion that FAD interacts with the dithiol group during certain steps of catalysis (Massey, The Enzymes, Vol. 7, p. 271).

The physico-chemical properties of lipoamide dehydrogenase and its interaction with lead will be further explored during the coming year, and we intend to investigate the possibility that this enzyme also is sensitive to mutation by lead in vivo. These studies serve as a model for the examination of other analogous enzymatic systems which are activated or inhibited by metal interactions.

Table I

METAL CONTENT OF METALLOTHIONEIN FROM NORMAL RABBIT KIDNEYS

DEAE Chromatography	Cadmium µg/gm protein	Zinc µg/gm protein
Peak I	200	560
Peak II	590	1360

Reference BRL 223-36

Table II

METAL CONTENT OF METALLOTHIONEIN FROM LEAD-POISONED RABBIT KIDNEYS

DEAE Chromatography	Cadmium μg/gm protein	Zinc μg/gm protein	Lead μg/gm protein
Peak I	88	206	1065
Peak II	375	1303	151

Reference S.L.-P3-26

Table III

MEDIUM FOR CULTURE OF *RHODOPSEUDOMONAS SPHEROIDES*

Sodium-L-glutamate monohydrate	3.8 g
DL-malic acid	2.7 g
NH_2PO_4	600.0 mg
K_2HPO_4	500.0 mg
$(NH_4)_2HPO_4$	800.0 mg
$MgSO_4 \cdot 7H_2O$	200.0 mg
$CaCl_2$	40.0 mg
Nicotinic acid	1.0 mg
Thiamine-HCl	1.0 mg
Biotin	10.0 µg

Distilled water to 1 liter

pH is adjusted to 6.8 with 1 *N* NaOH before autoclaving for 10 minutes at 10 lbs/sq in.

Reference: Lascelles, Biochem. J. 82, 78, 1956.

Table IV

EFFECT OF MICRONUTRIENTS ON CLONE CONTAINING [illegible] SYNTHETASE

Iron (μg/liter)	Manganese (μg/liter)	Lysine (g/liter)	Total Hemoglobin (g/dl PCV)
0	0	3	0.77
0	0	4	0.94
3	3	3	0.48
0	3	4	0.46
1.5	3	0	0.54
3	0	4	0.85
3	3	0	0.93
	3	4	0.63

[illegible]

Table V

REACTIVATION OF LEAD AND CADMIUM COMPLEXES OF LIPOAMIDE DEHYDROGENASE (LAD) WITH EDTA

Reference: SRL (269-263; 269-275)

EDTA, M	Order of Addition of EDTA	ACTIVITY %[1] LAD(control)	Pb-LAD[2]	Cd-LAD[3]
--	--	93	3	3
3.4×10^{-5}	after coenzymes and substrate	100	2	3
5.4×10^{-3}	before coenzymes and substrate	130	106	94

1 Assay: LAD or LAD-metal complex, 1.65×10^{-9} M; Ca^{++}, 1.2×10^{-4} M; EDTA, 1.2×10^{-4} M; Lipoate, 3.5×10^{-4} M; TRIS Cl^-, 0.1M, pH 7; Na_2EDTA as indicated.

2 Pb-LAD prepared by incubating 10^{-5} M LAD with 2×10^{-4} M Pb^{++} in 0.5 M Tris-chloride, pH 7.

3 Cd-LAD prepared by incubating 10^{-5} M LAD with 2×10^{-4} M Cd^{++} in 0.5M Tris-chloride, pH 7.

Sephadex Chromatography - Normal Rabbit Kidney:

reference BRL 323-4

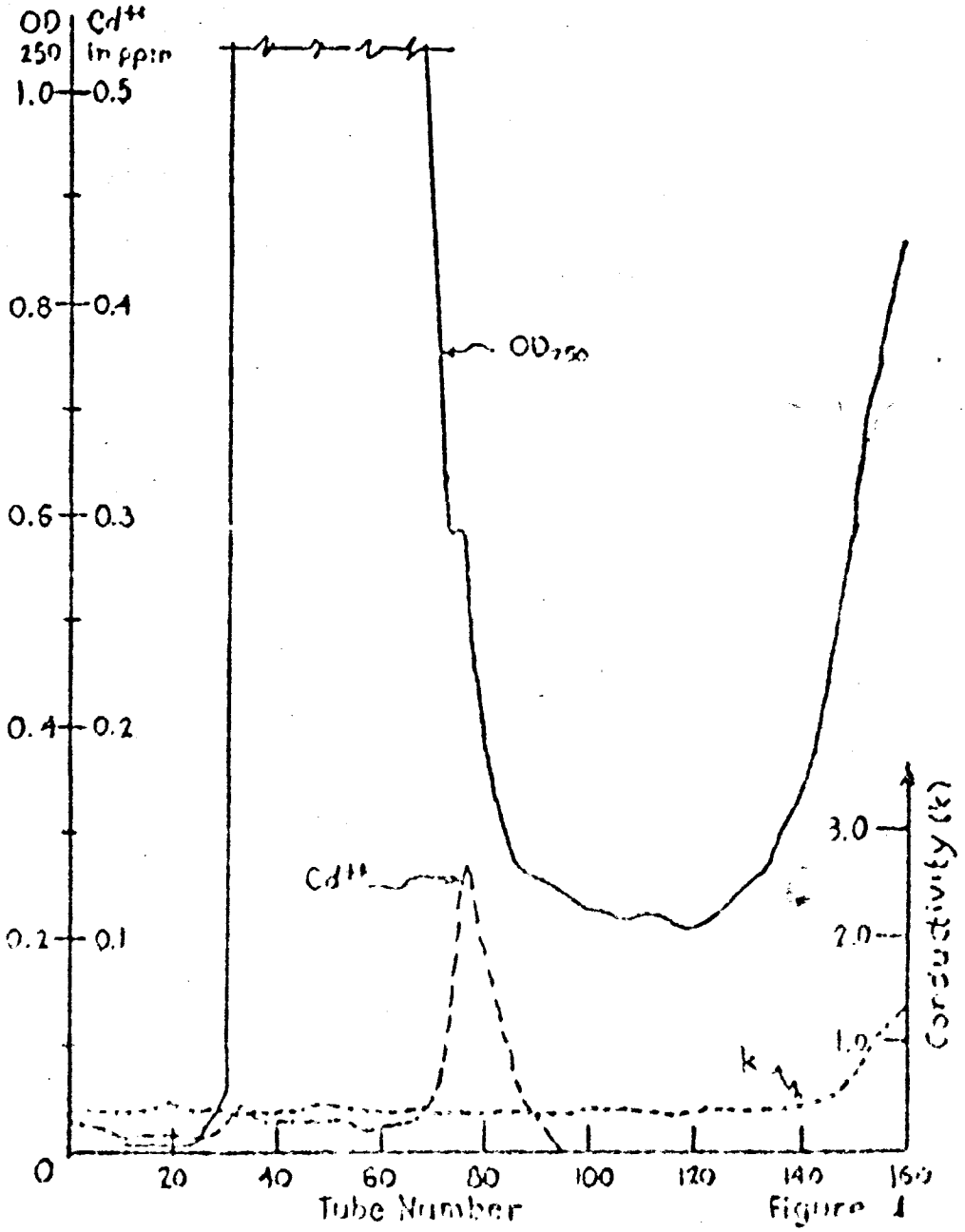


Figure 1

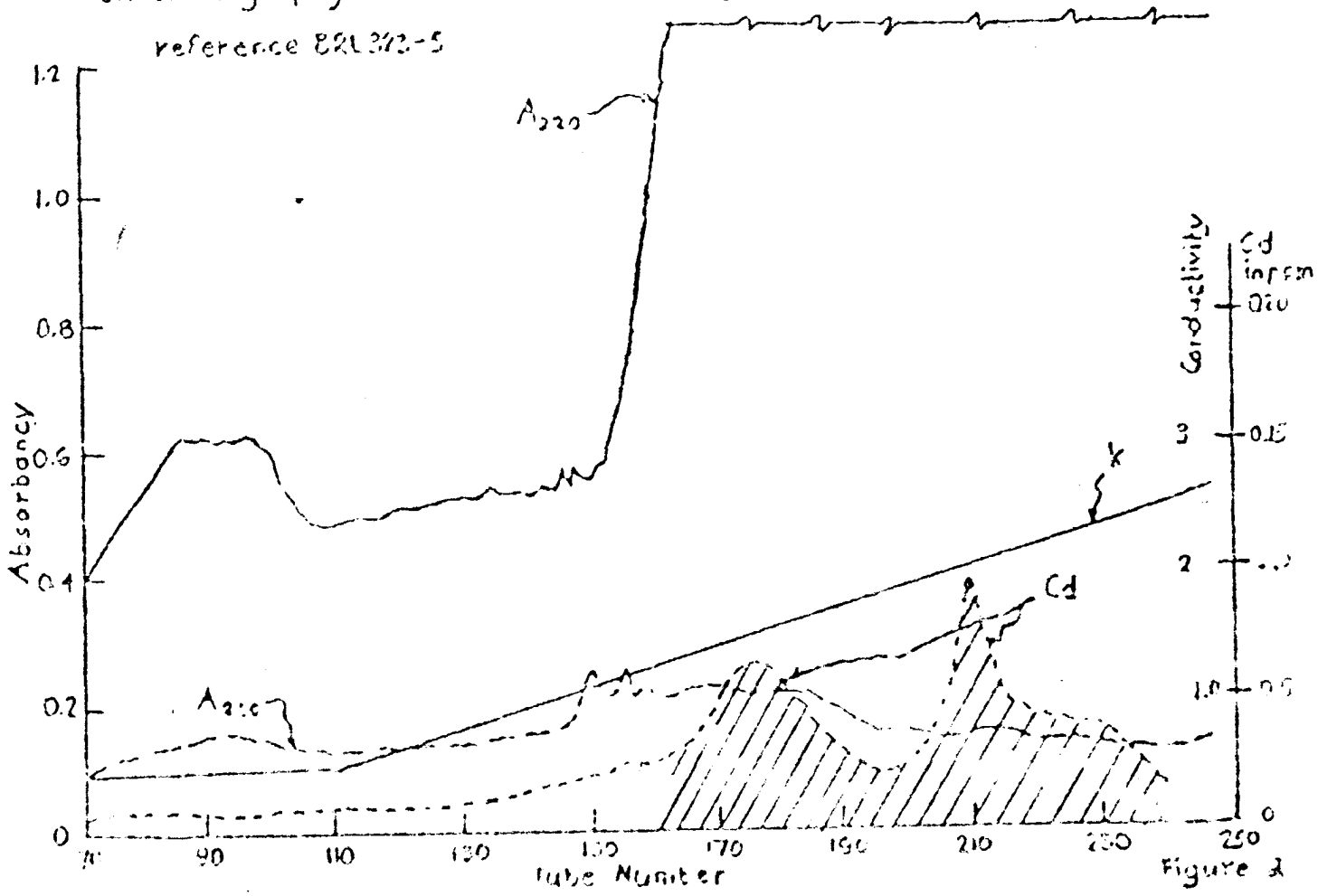

Chromatography of Normal Rabbit Kidney DEAE Column
reference BRL 342-5
A_{220}
A_{280}
Absorbancy
1.2
1.0
0.8
0.6
0.4
0.2
0
Conductivity
Cd
3
2
1.0
0
90
110
130
150
170
190
210
230
250
Tube Number

Figure 1

Sephadex Chromatography

Lead Poisoned Rabbit Kidneys

reference BRL-323-1

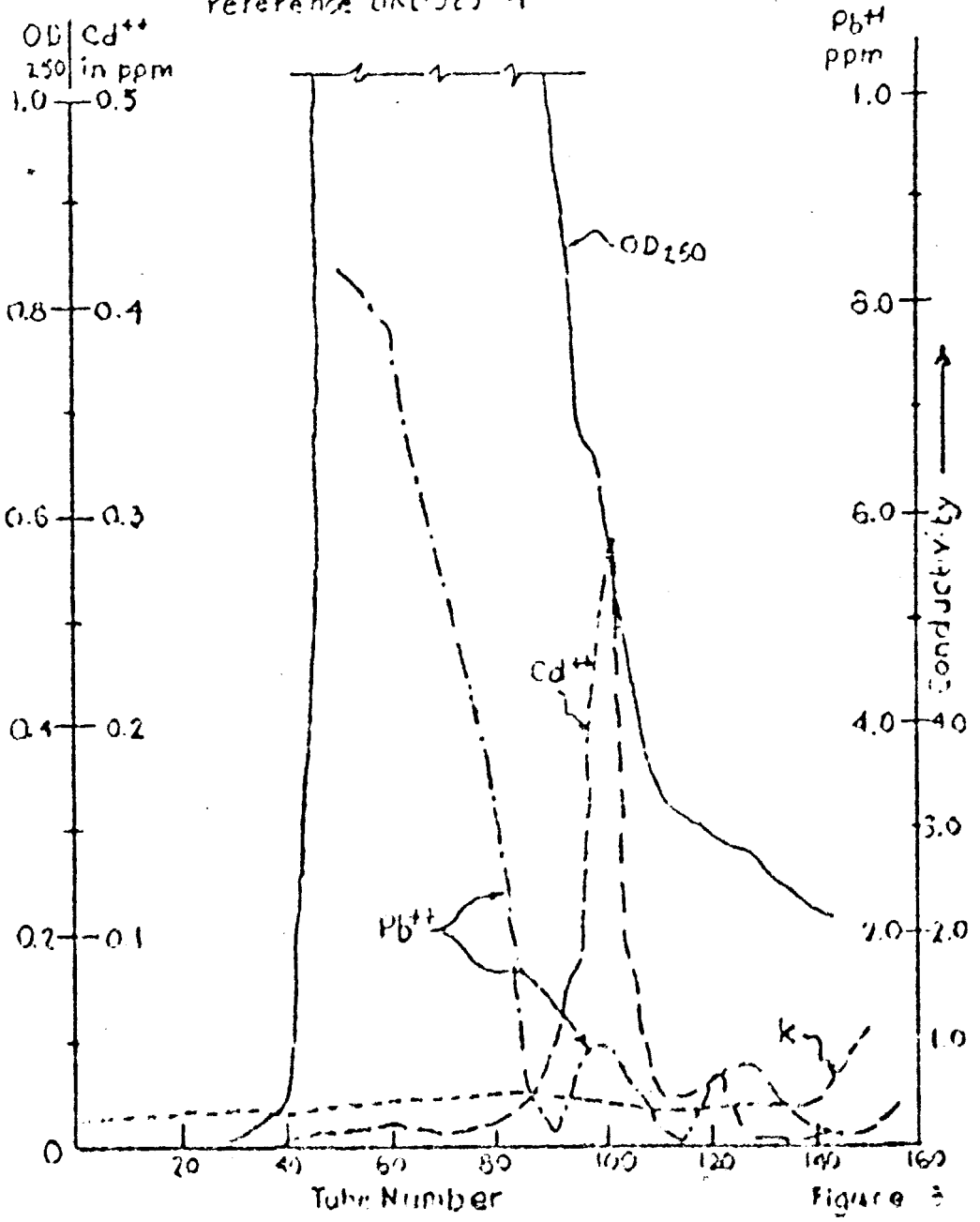


Figure 3

Chromatography of Kidneys from Lead Poisoned Rabbits - DEAE Column

reference BRL 328-10

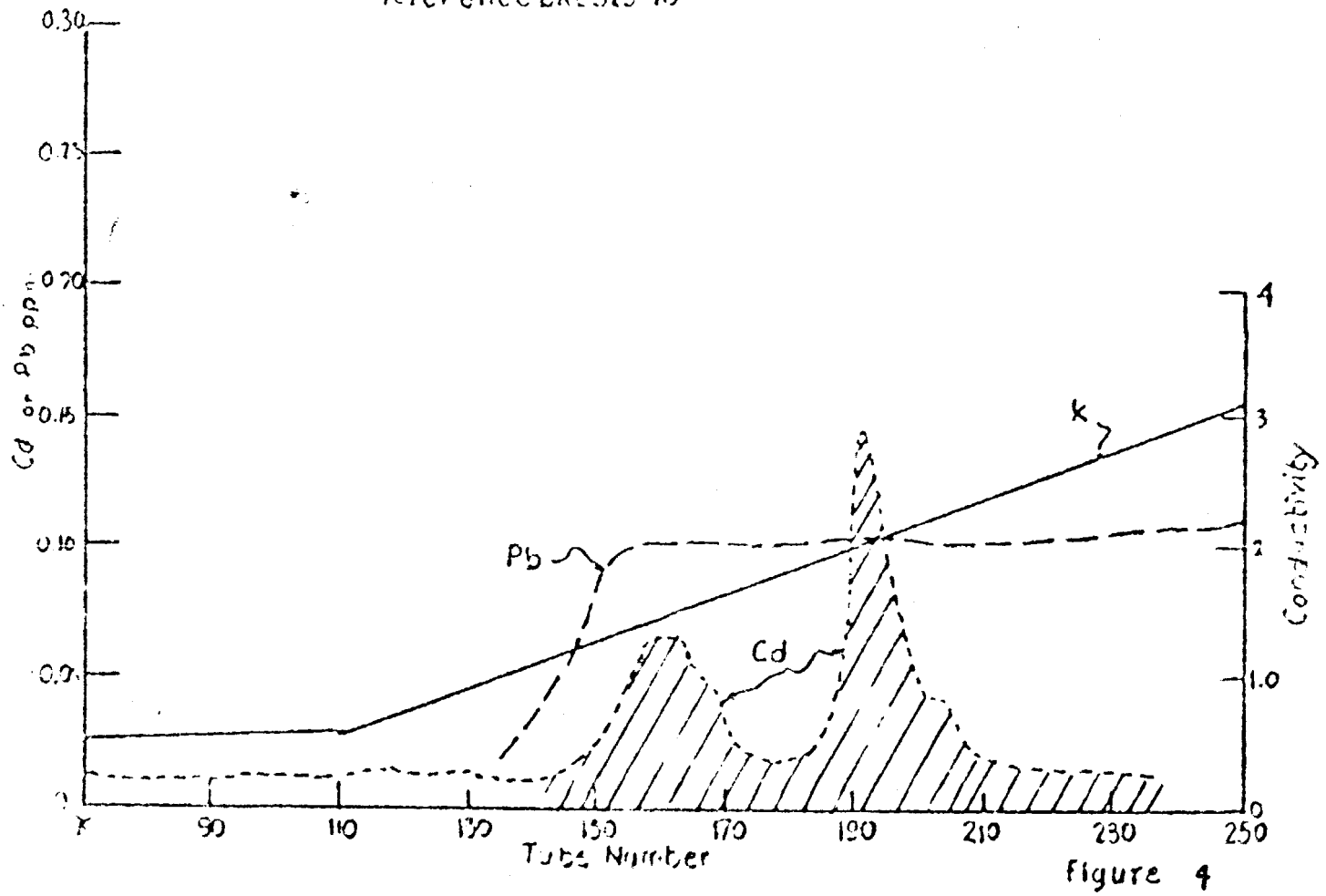


Figure 4

GROWTH CURVES FOR Rps sphaeroides (standard conditions)
reference BRL-323-24

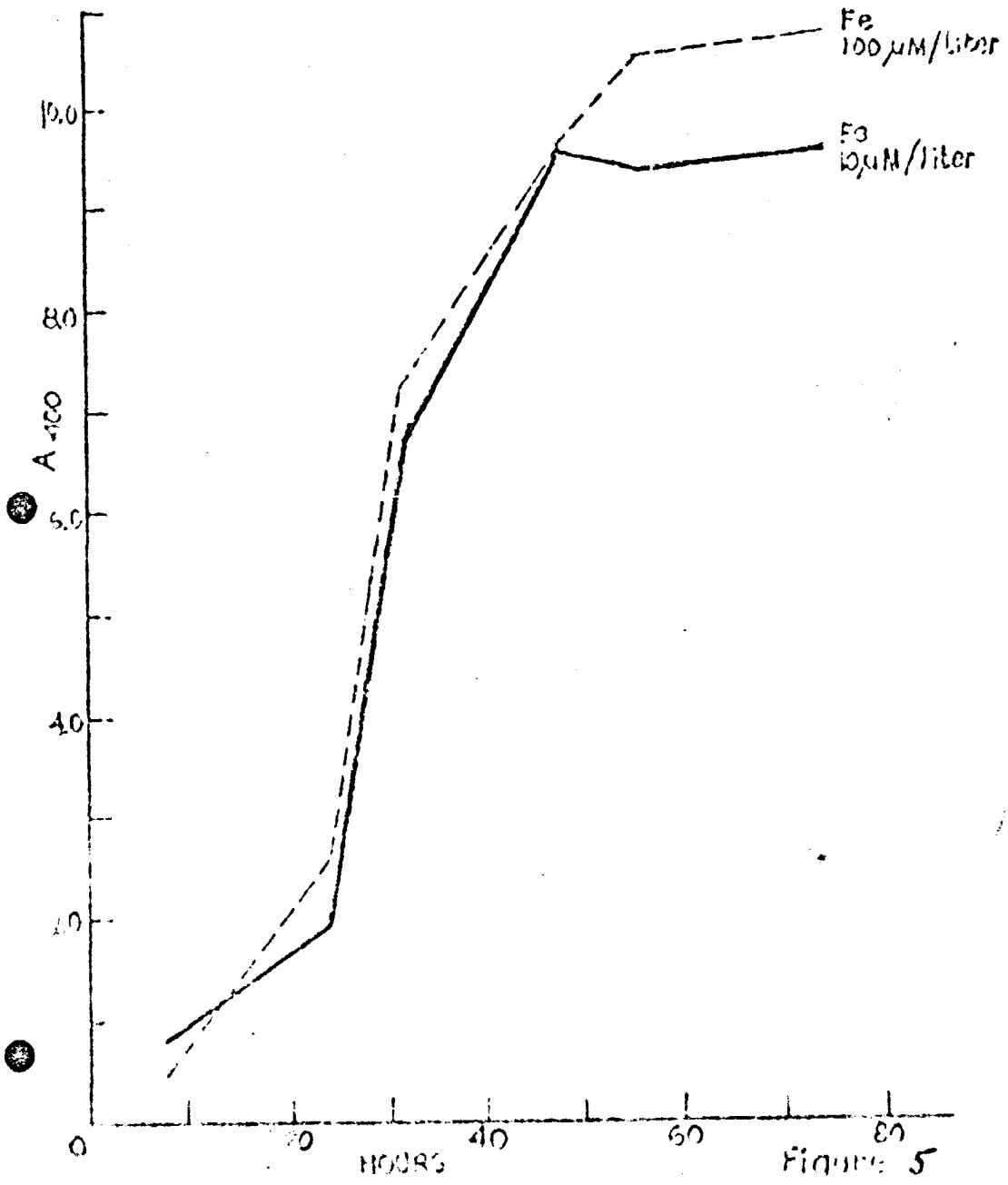


Figure 5

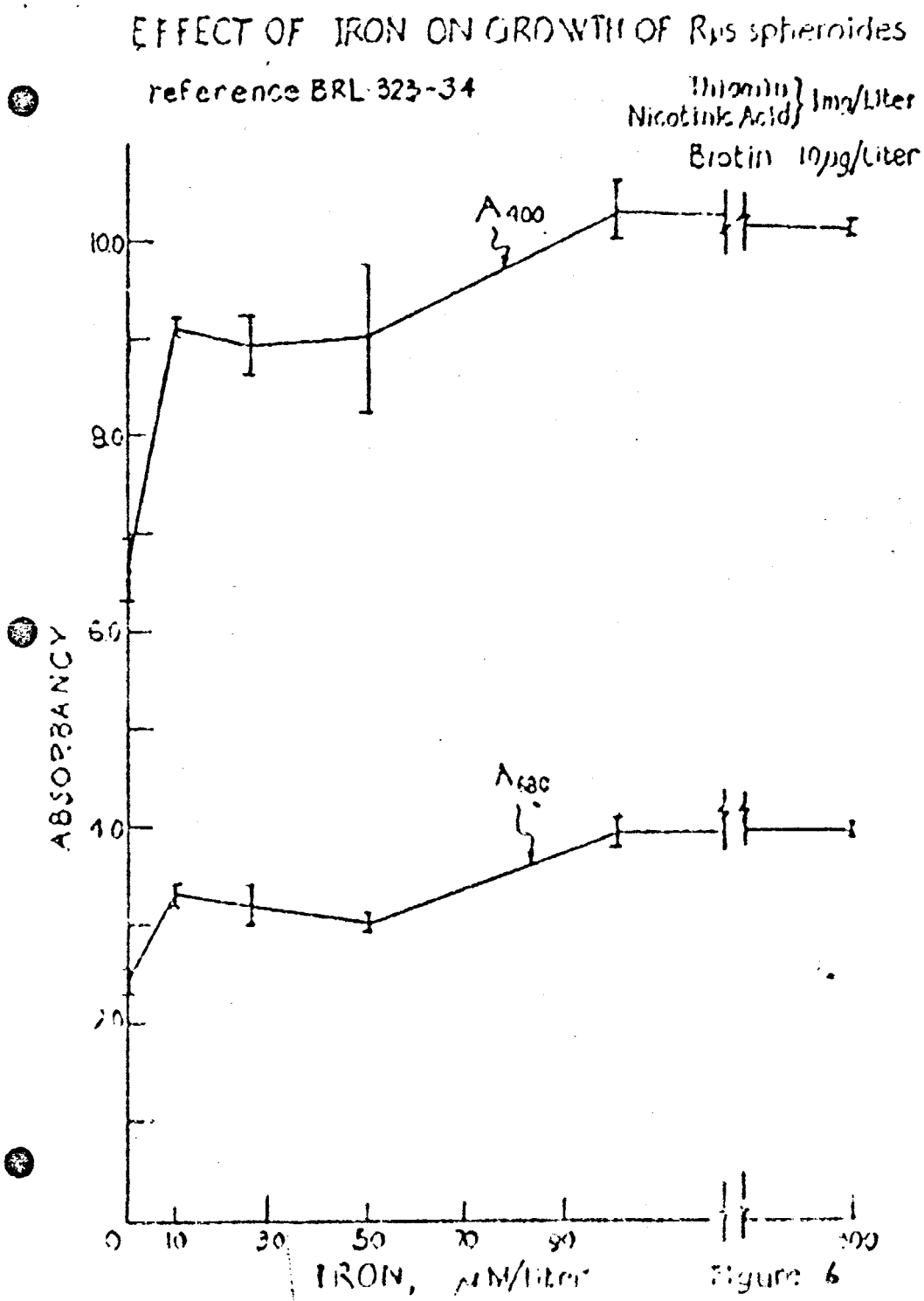

EFFECT OF IRON ON GROWTH OF Rps spheroides
reference BRL 323-34
Thiamin } 1mg/Liter
Nicotinic Acid }
Biotin 10µg/Liter
A400
A680
ABSORBANCY
10.0
8.0
6.0
1.0
0
10
30
50
70
90
100
IRON, µM/liter

Figure 6

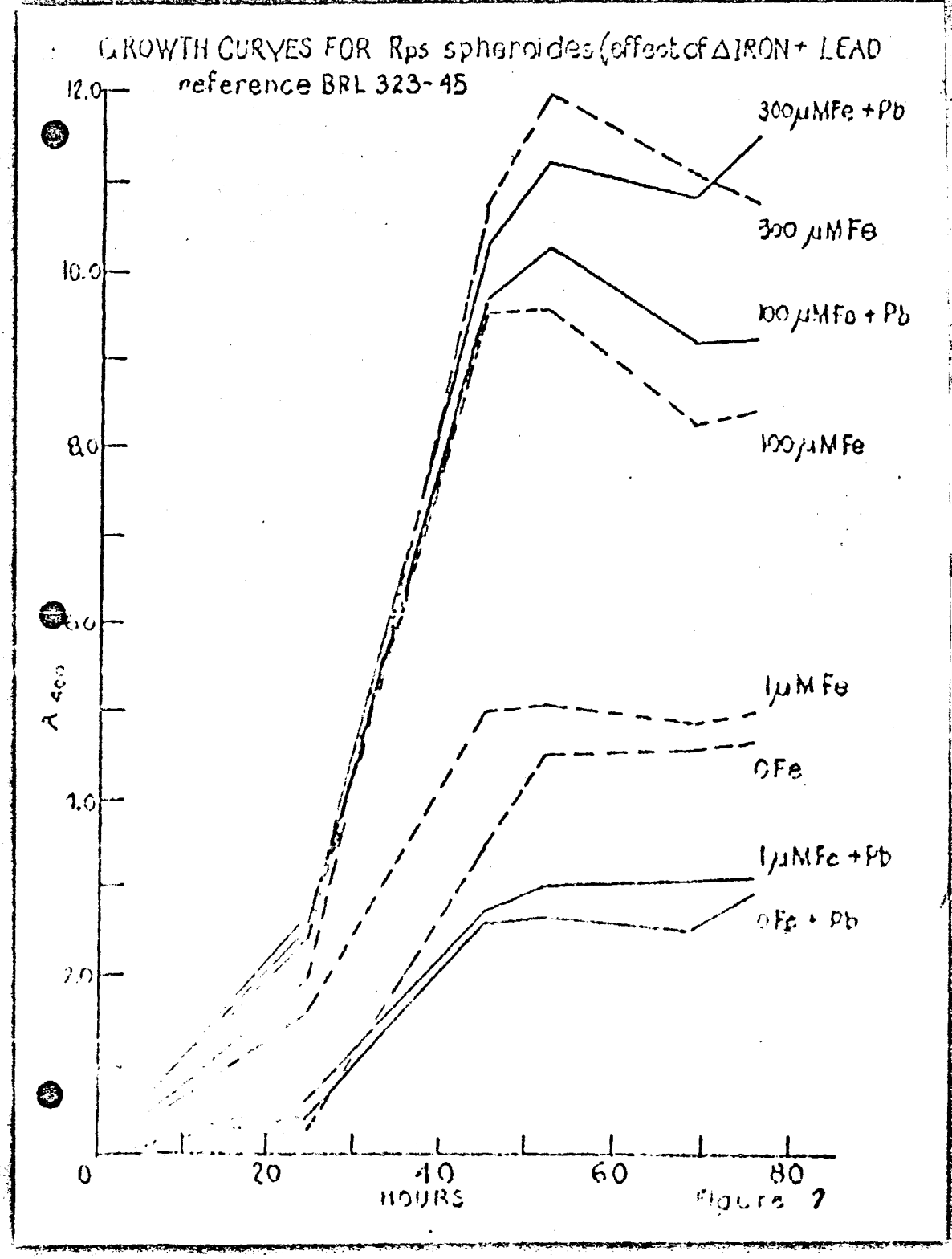

GROWTH CURVES FOR Rps spheroides (effect of ΔIRON + LEAD
reference BRL 323-45
12.0
10.0
8.0
2.0
0
20
40
60
80
HOURS
A 600
300 μM Fe + Pb
300 μM Fe
100 μM Fe + Pb
100 μM Fe
1 μM Fe
0 Fe
1 μM Fe + Pb
0 Fe + Pb

Figure 7

GROWTH OF Rps SPHEROIDES

Effect of Iron Concentration in Presence and Absence of 1×10^{-4} M Lead Acetate

reference BRL-342-41B

Biotin, 5µg/Liter
Thiamin, 1mg/liter
Nicotinic Acid, 1mg/Liter

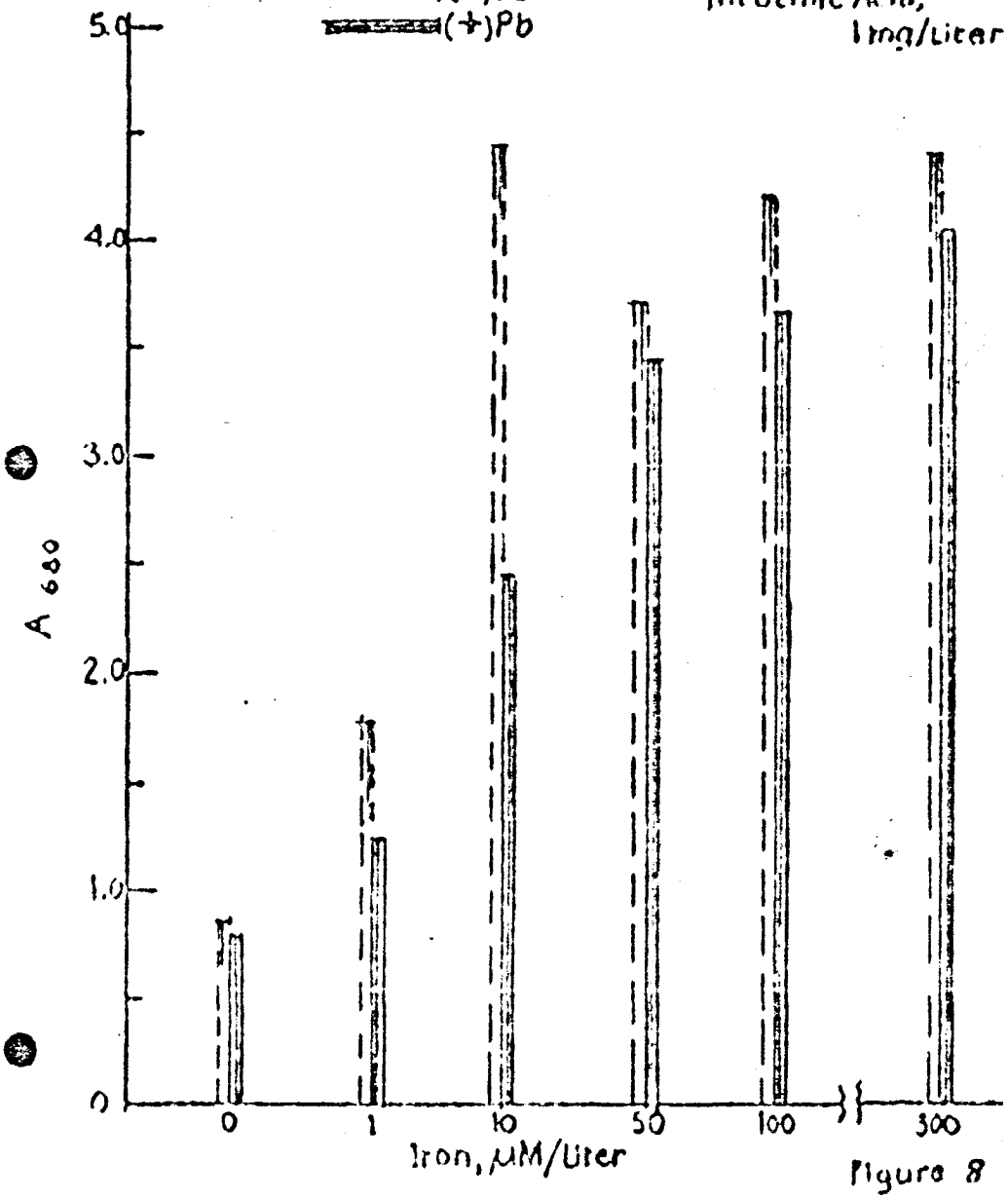


Figure 8

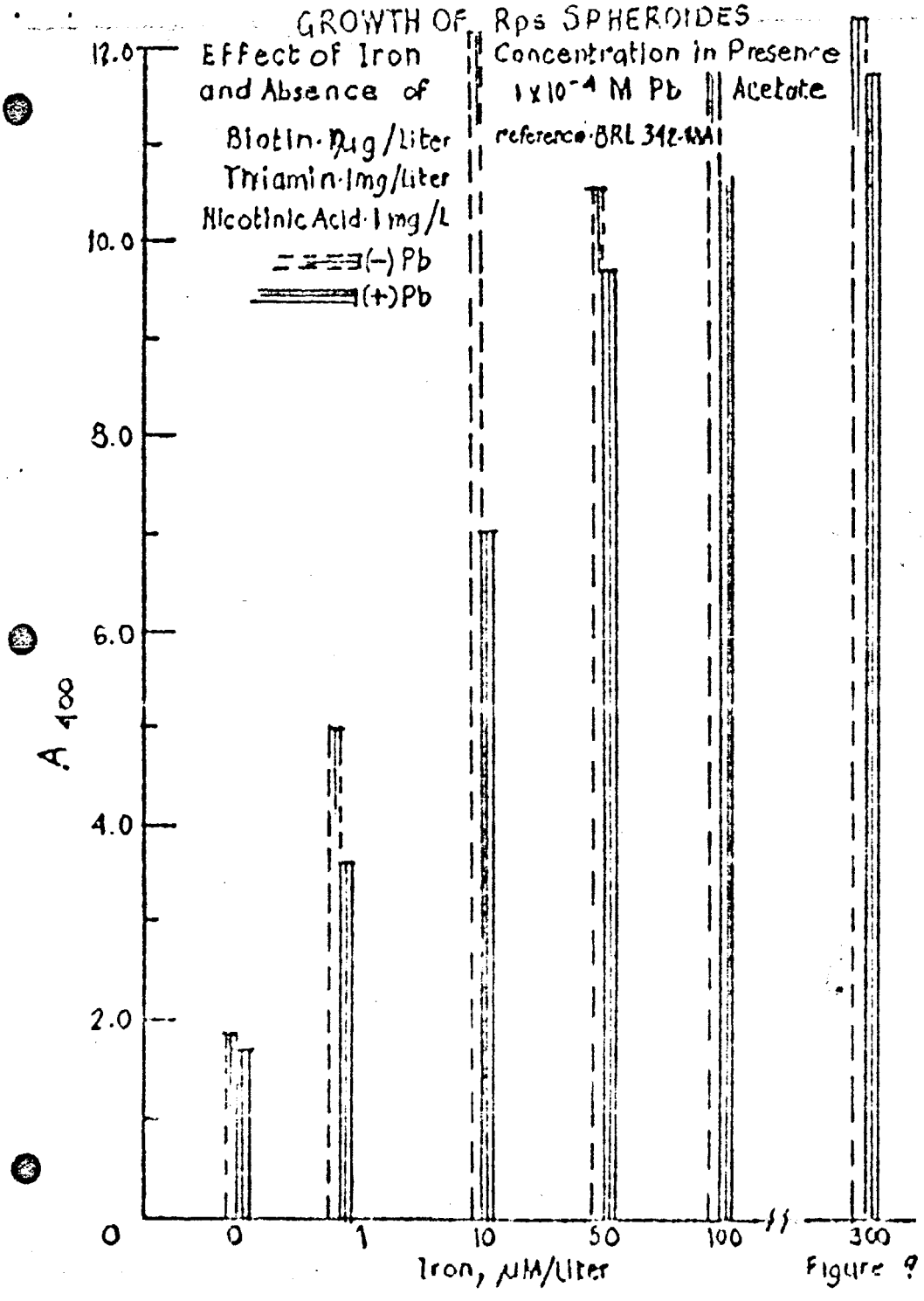

GROWTH OF Rps SPHEROIDES
Effect of Iron Concentration in Presence and Absence of
1 x 10⁻⁴ M Pb Acetate
Biotin · 10µg/Liter
Thiamin · 1mg/liter
Nicotinic Acid · 1 mg/L
(-) Pb
(+) Pb
reference · BRL 342-MI
A 480
12.0
10.0
8.0
6.0
4.0
2.0
0
0
1
10
50
100
300
Iron, µM/Liter

Figure 9

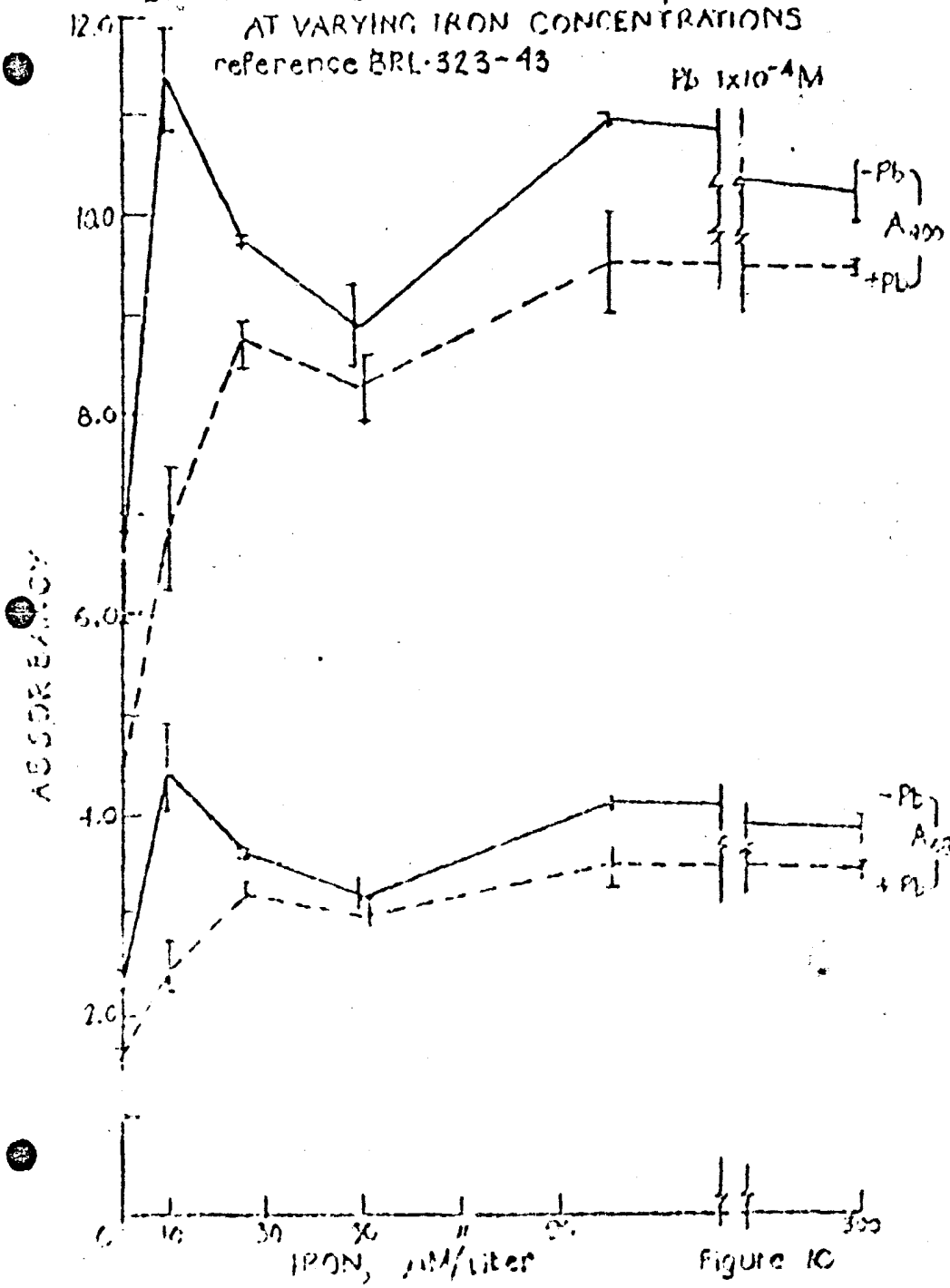

EFFECT OF LEAD ON GROWTH Rps spheroides
AT VARYING IRON CONCENTRATIONS
reference BRL-323-43
Pb 1x10⁻⁴M
-Pb
+Pb
12.0
10.0
8.0
6.0
4.0
2.0
0
10
30
IRON, µM/liter

Figure 10

GROWTH OF Rps SPHEROIDES
Effect of Cofactors on Growth
reference · BRL-323-34 COE

Fe = 10 μM/Liter

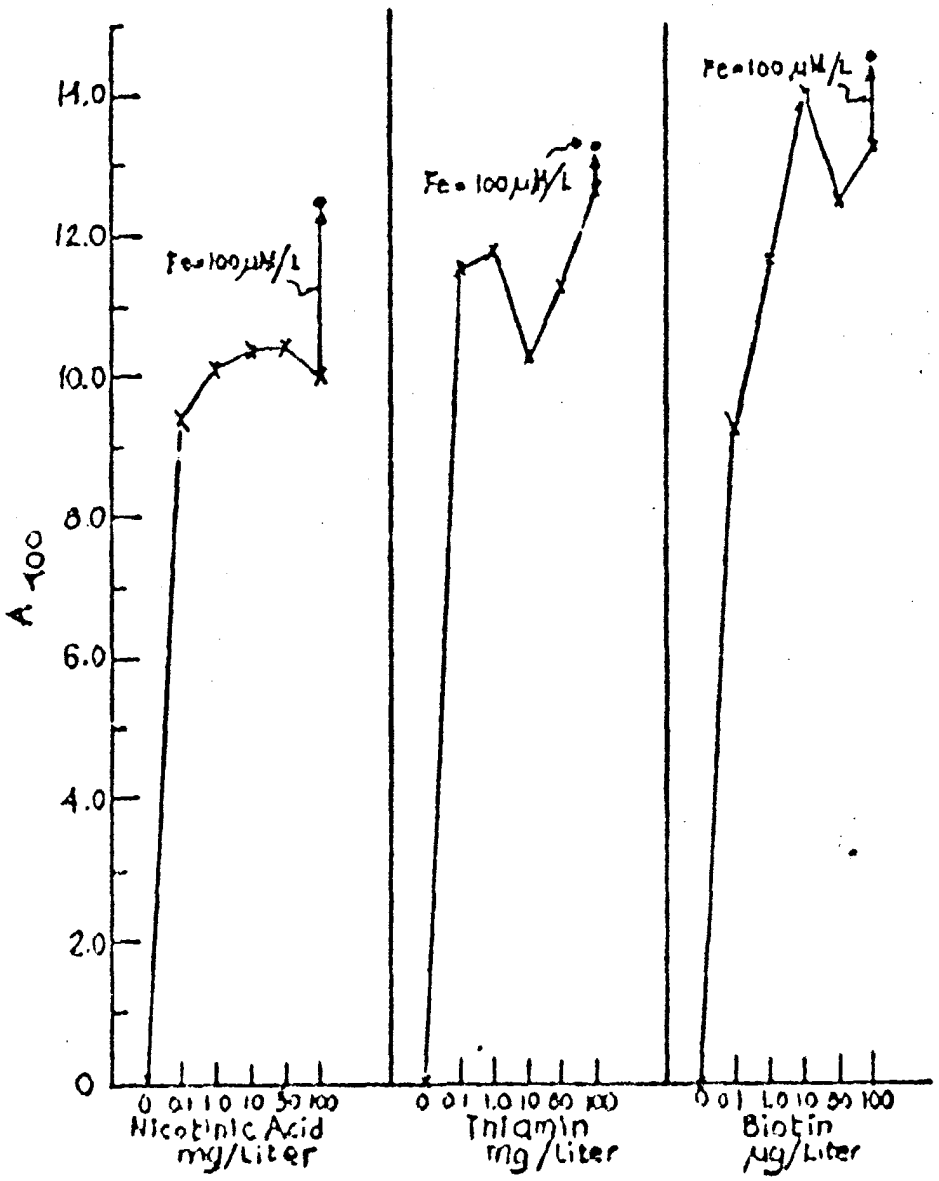


Figure 11

GROWTH OF Rps SPHEROIDES

Effect of Δ Iron vs. Δ Thiamin in Presence and Absence of Lead

reference BRL-323-40D.F.

Nicotinic Acid 1mg/Liter
Lead Acetate 1×10^{-4} M
Biotin 10μg/Liter

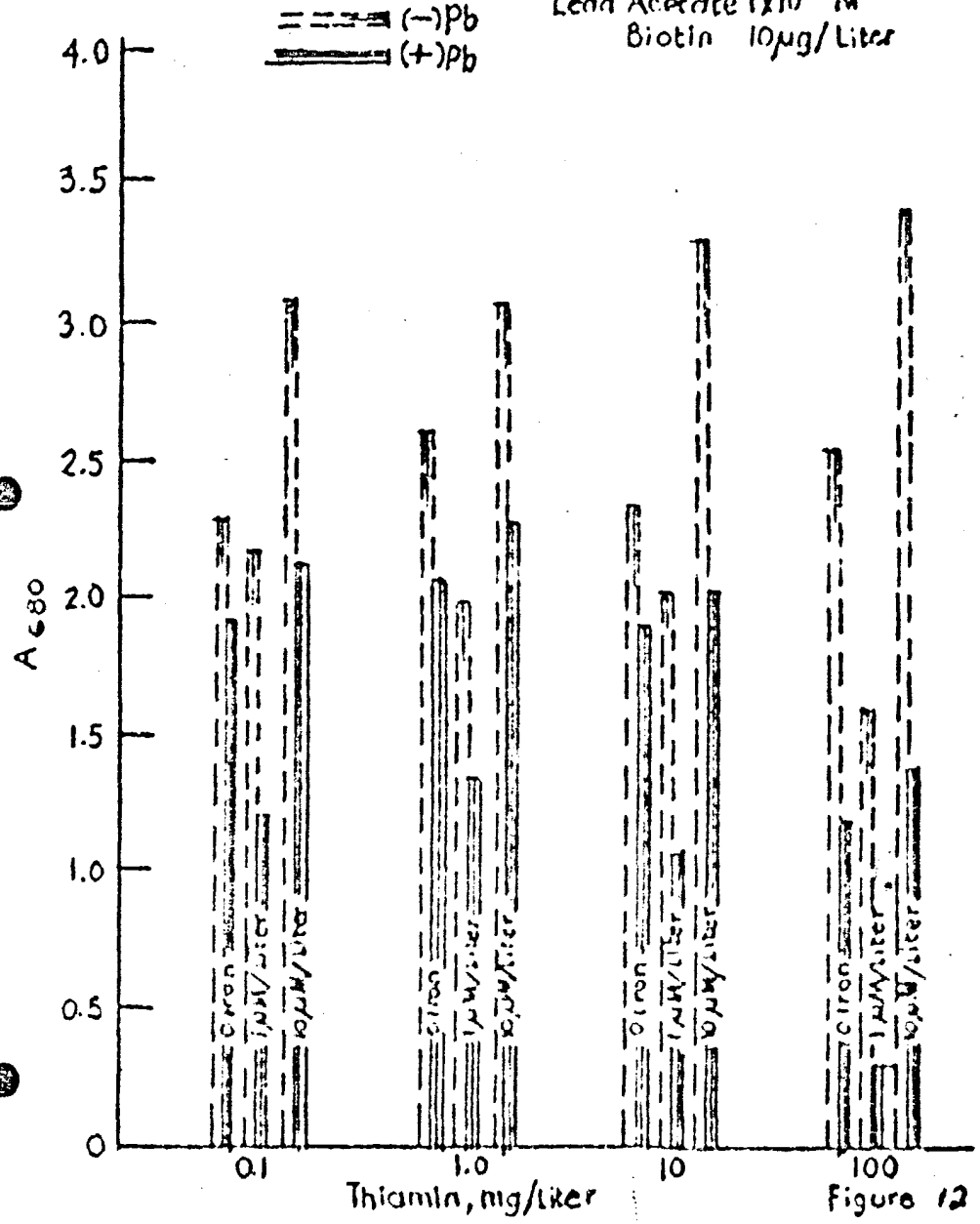


Figure 12

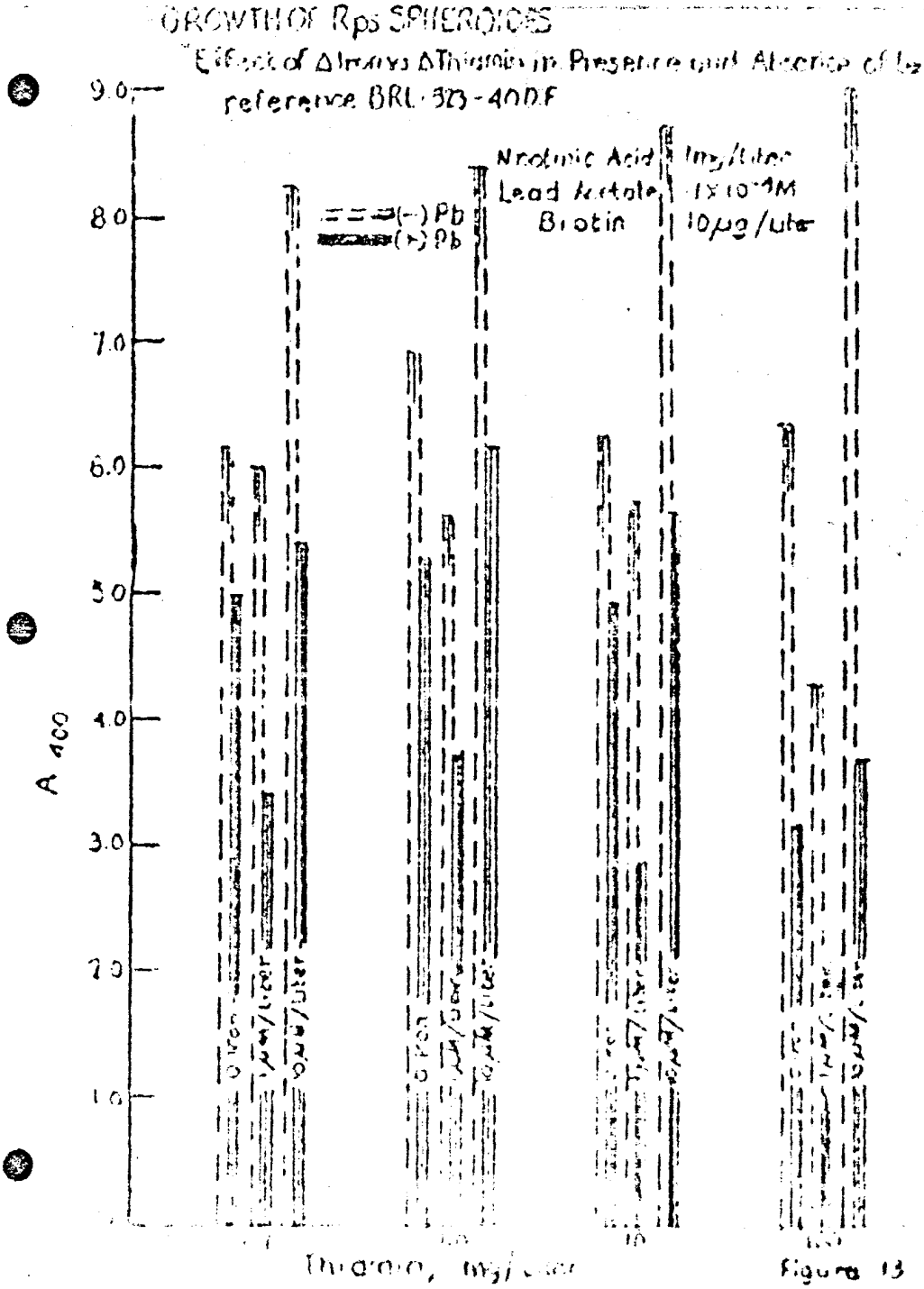

GROWTH OF Rps SPHEROIDES
Effect of Δ Imaxs ΔThiamin in Presence and Absence of Lead
reference BRL-323-400F
Nicotinic Acid 1mg/liter
Lead Acetate 1x10⁻⁴M
Biotin 10μg/liter
(-) Pb
(+) Pb
A 400
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
Thiamin, mg/liter

Figure 13

GROWTH OF Rps SPHEROIDES

Effect of Δ Iron vs. Δ Nicotinic Acid in presence and absence of Lead

Thiamin = 1 mg/liter
Pb = 1×10^{-4} M
Biotin = 10 µg/Liter

reference - BRL-323-90 A C
(-)Pb
(+)Pb

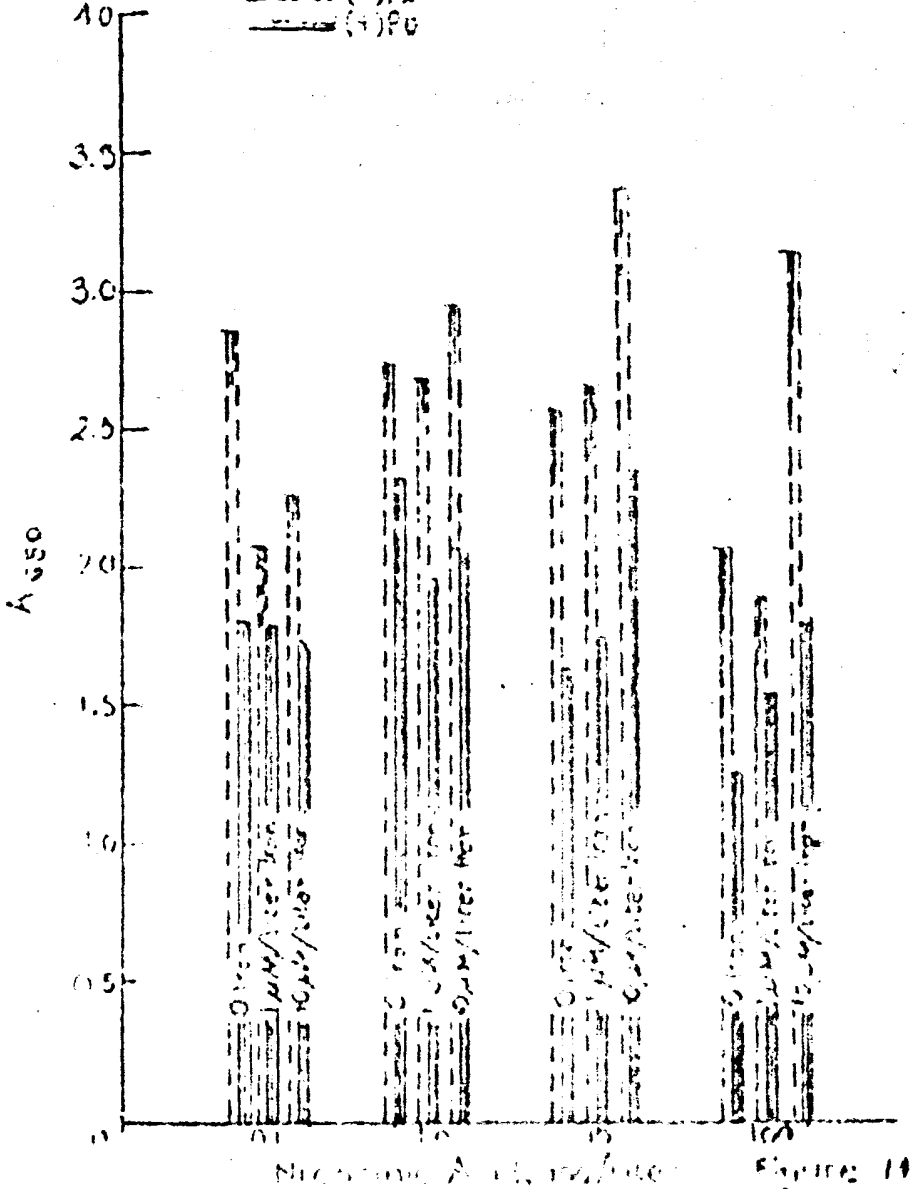
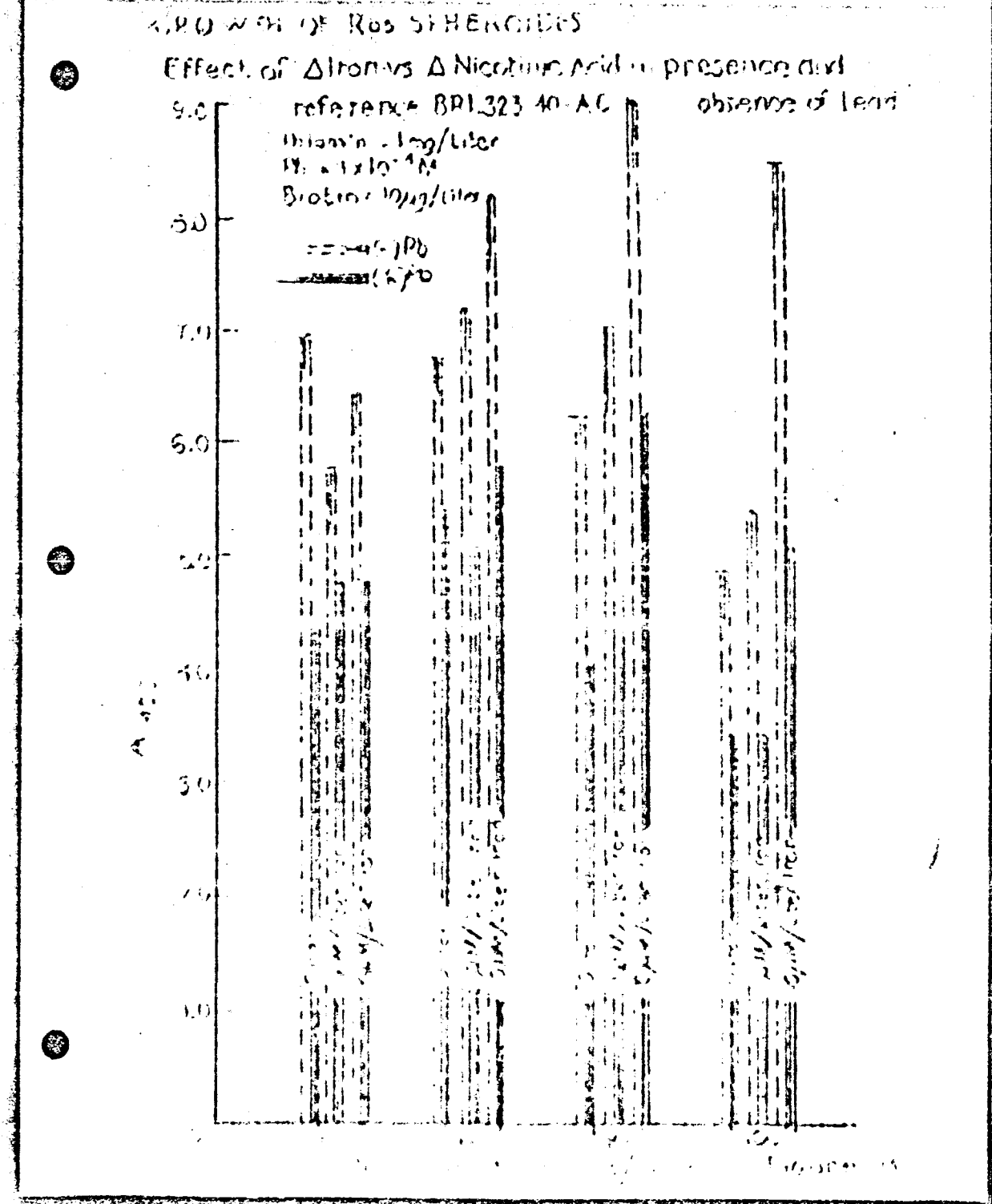


Figure 11


Effect of Δ Ironvs Δ Nicotinic Acid in presence and
reference BRL323 40 AC
absence of lead
9.0
5.0
7.0
6.0
3.0
1.0

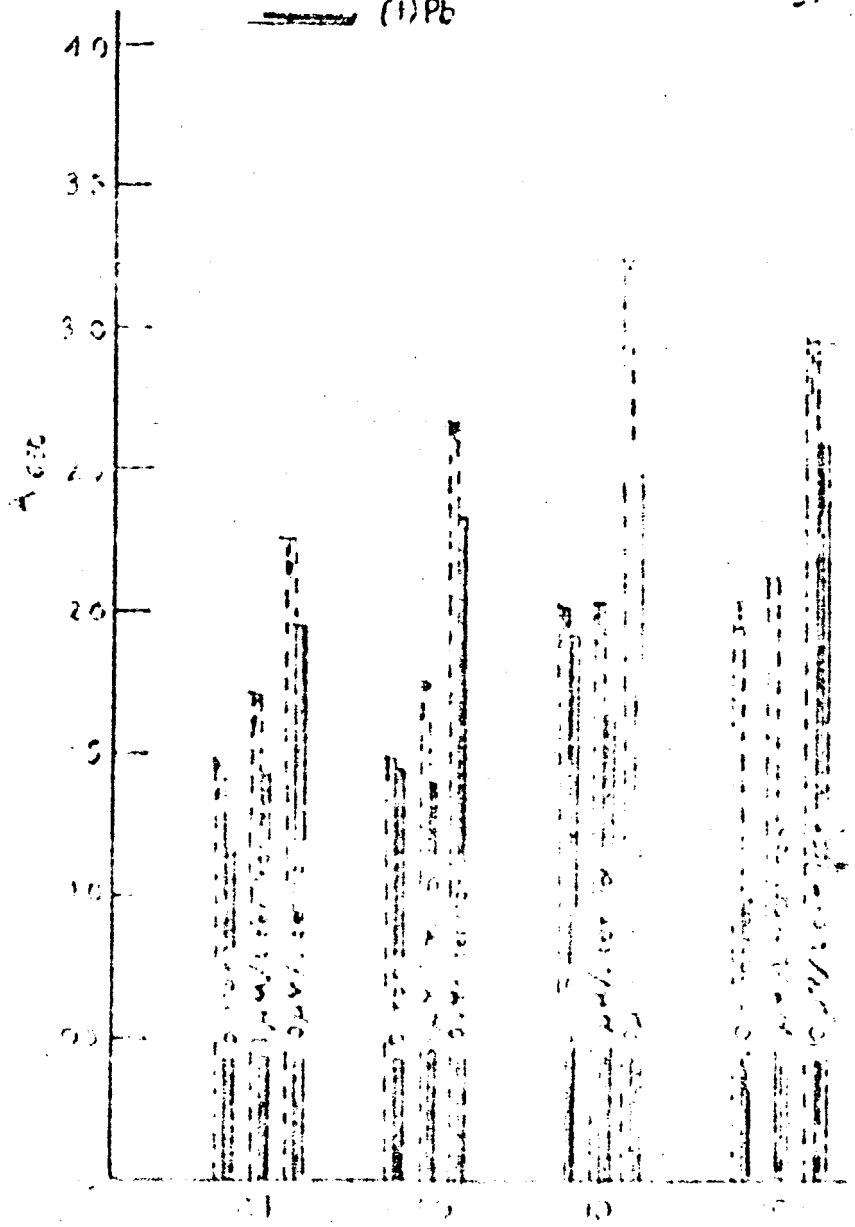
GROWTH OF Rps SPHEROIDES

Effect of Δ Nicotinic Δ Biotin in presence and absence of Lead

reference BRL-323-[illegible] G1

Lead Acetate [illegible] 10^{-4} M
Nicotinic Acid 1mg/liter
Thiamin 1mg/liter

(-) Pb
(+) Pb



GROWTH OF Rps SPHEROIDES

Effect of Δ Imp vs Δ Biotin in presence and absence of Lead

reference BRL 323-40-G2

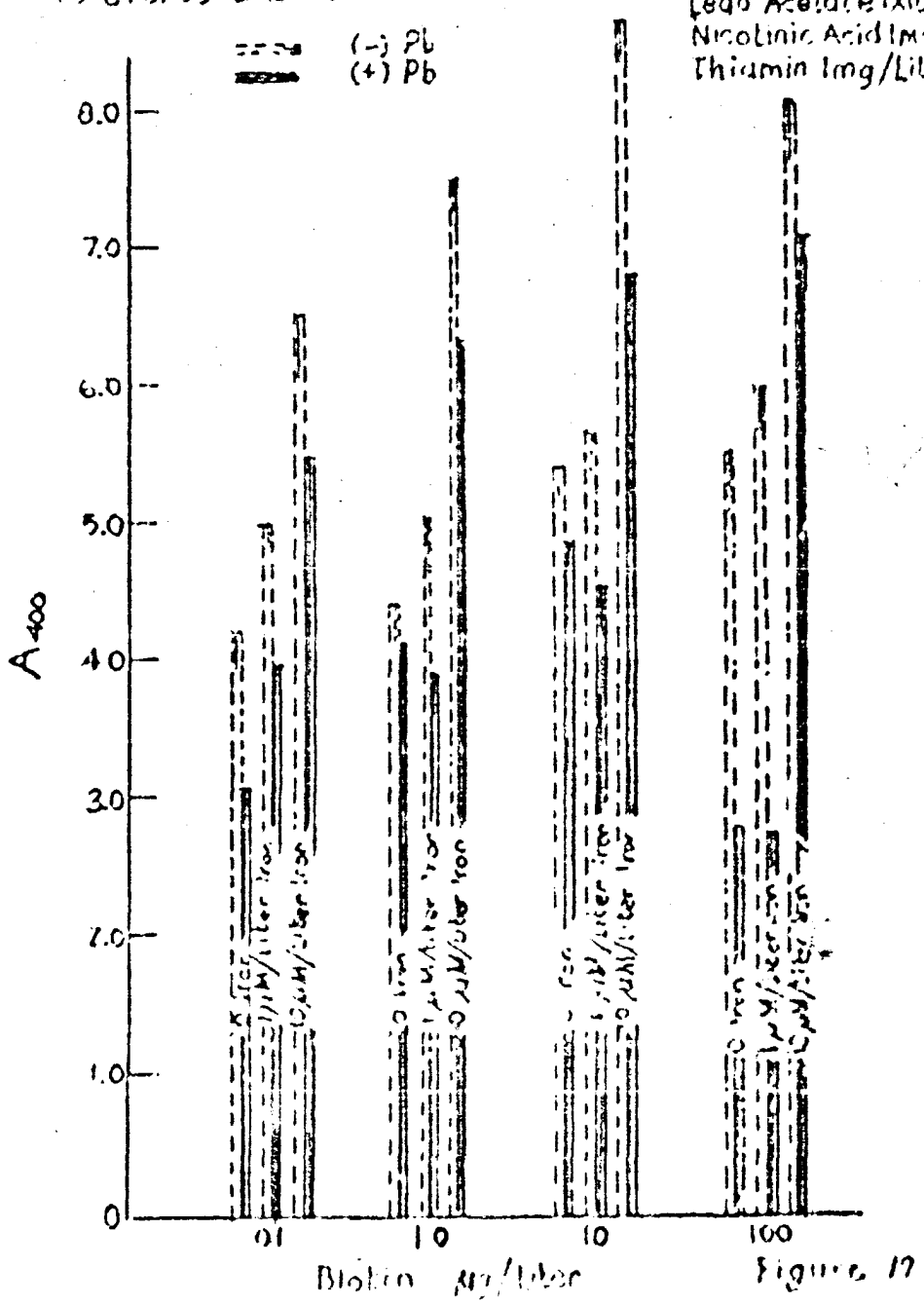
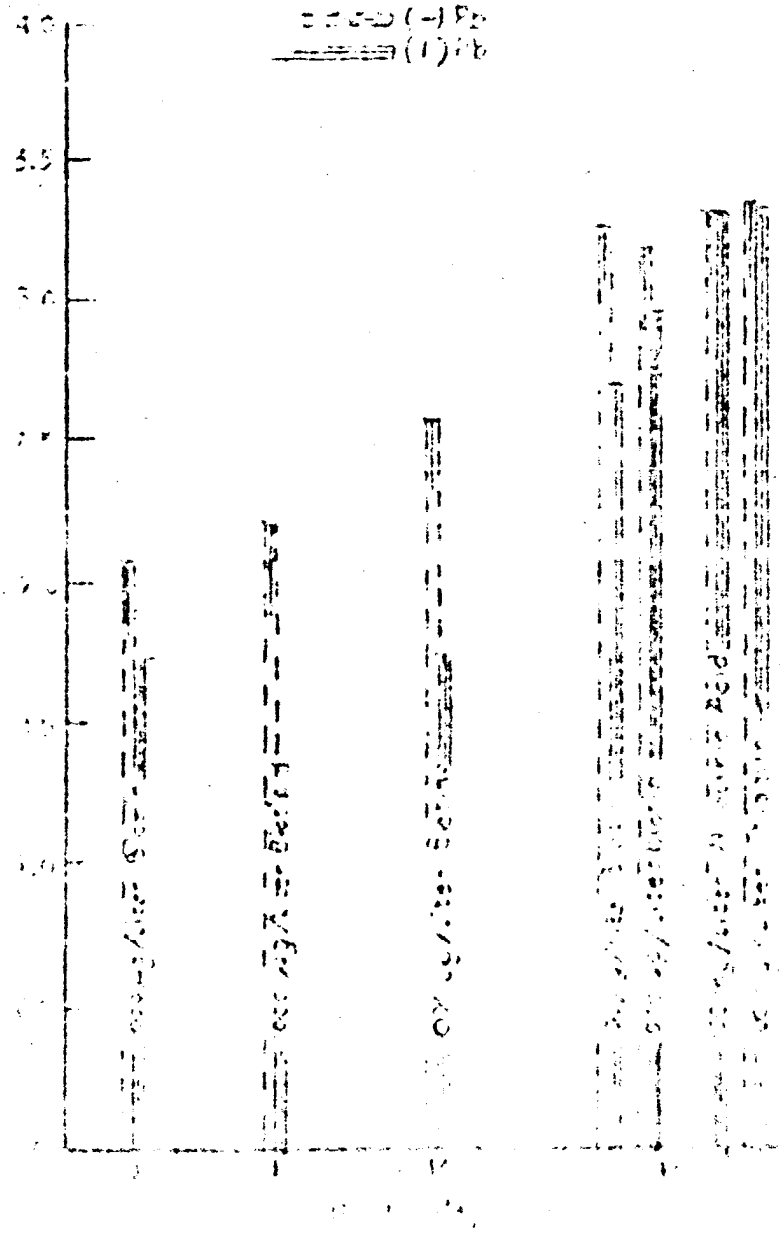


Figure 17

GROWTH OF Rp SPHEROIDES

Effect of Δ1800 vs Cofactors in presence of different level of lead reference PRL-373-44

Lead Acetate = 1×10^{-4} M



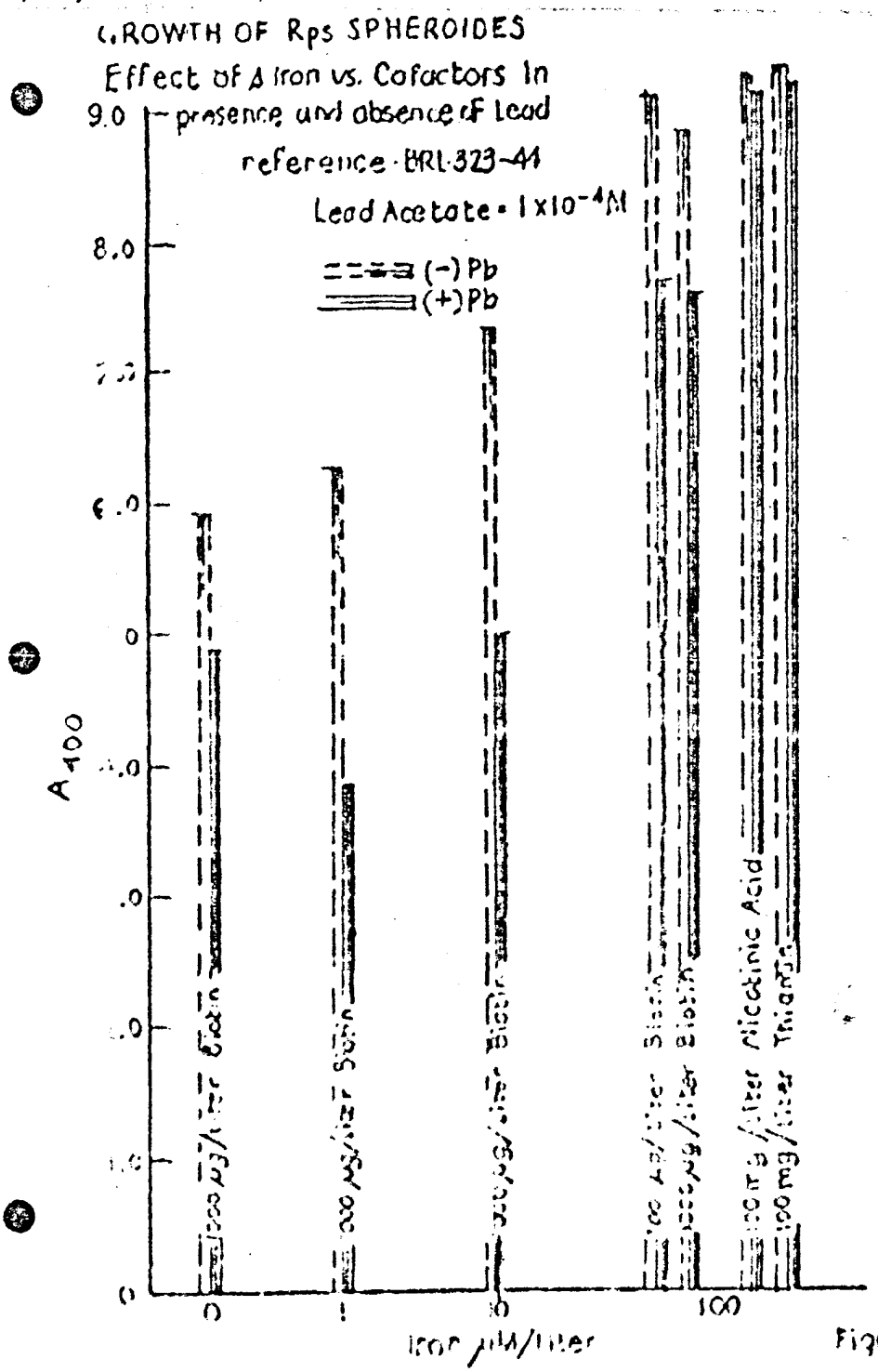

GROWTH OF Rps SPHEROIDES
Effect of Δ Iron vs. Cofactors in
presence and absence of Lead
reference · BRL-323-41
Lead Acetate = 1x10⁻⁴M
(-) Pb
(+) Pb
9.0
8.0
A 400
0
100
Iron μM/liter
Nicotinic Acid
Thiamine

Figure 19

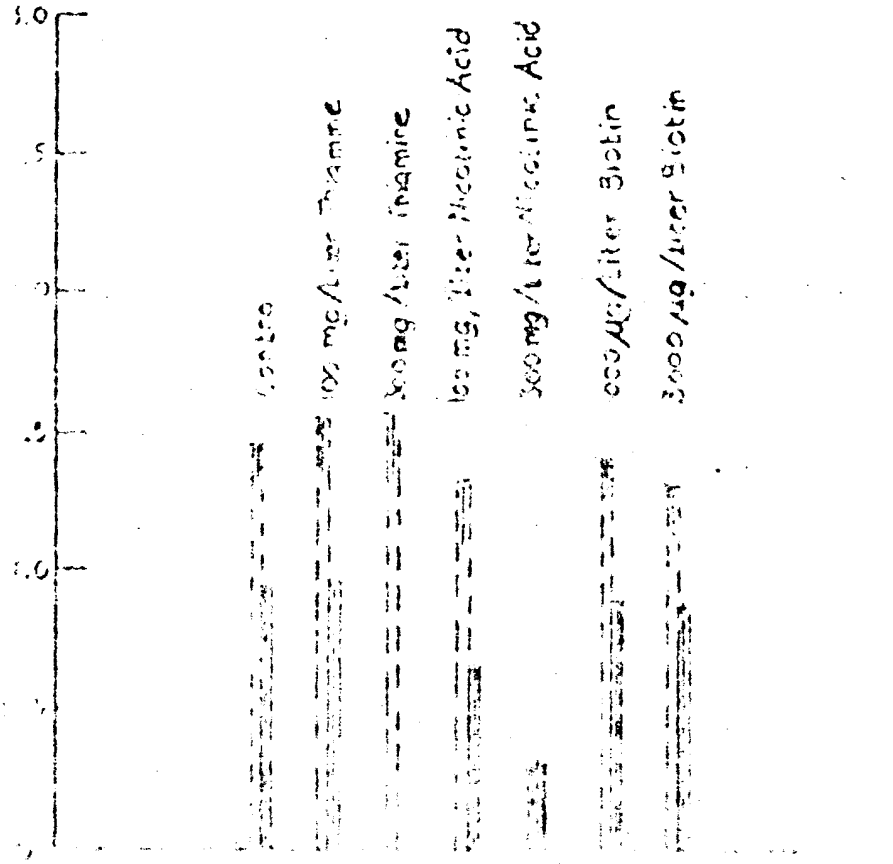
GROWTH OF Rps SPHEROIDES

Effect of High Concentrations of Cofactors - No Iron

reference BKL 342-51

(-) Pb

(+) Pb



20

GROWTH of Rps SPHEROIDES

Effect of High Concentrations of Cofactors - No Iron

reference BNL 34750

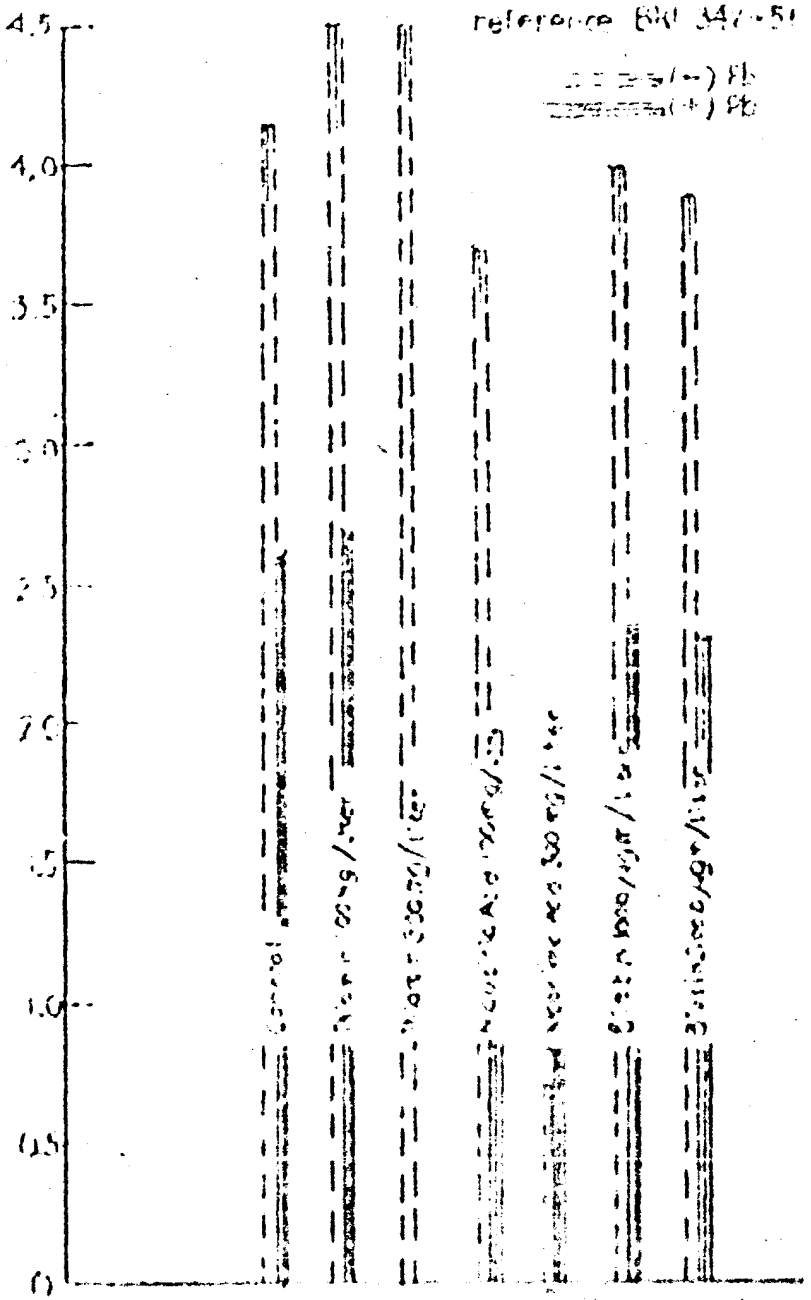


Figure 2

GROWTH OF RpsSPHEROIDES

Effect of High Concentration Cofactors + Iron

reference BRL-342-54

Iron, 10 μM/Liter

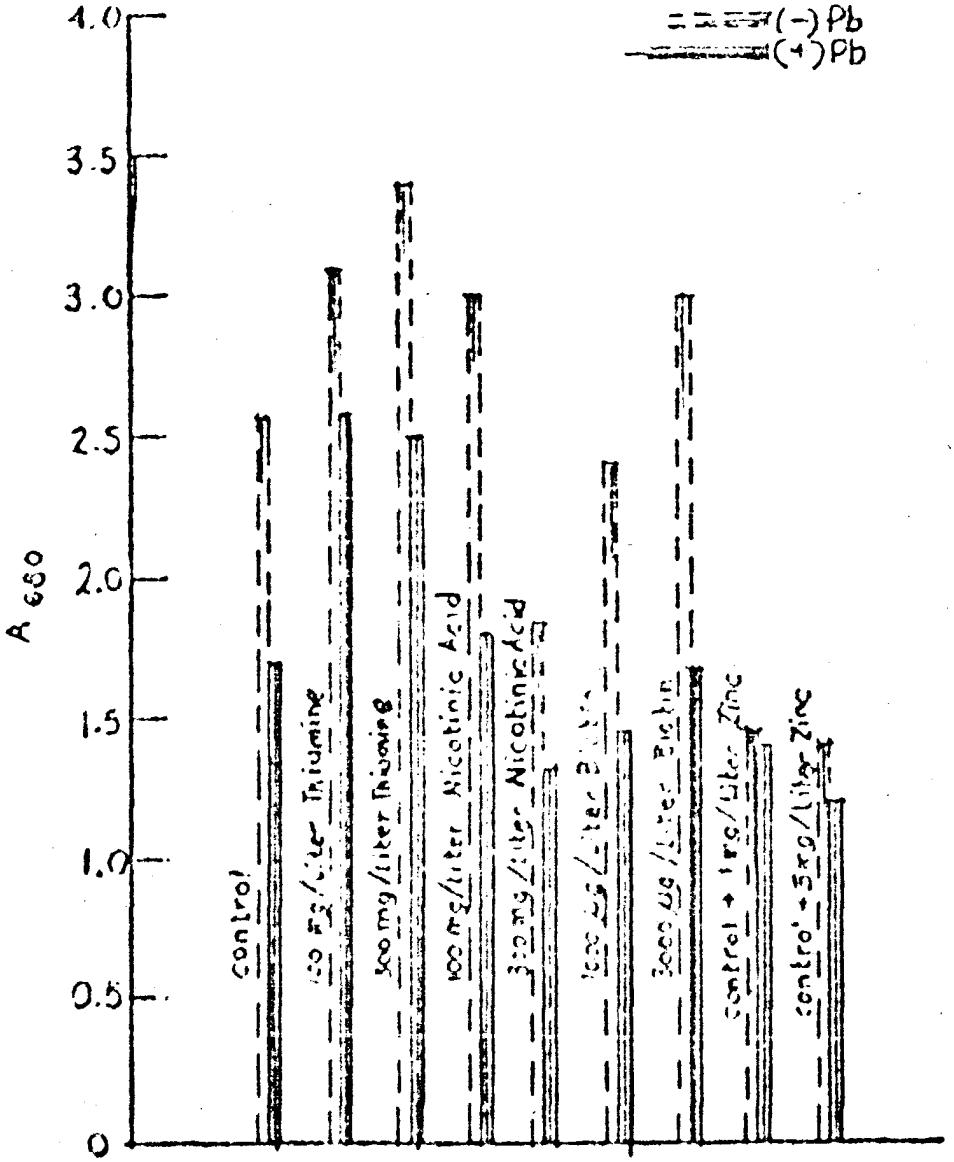


Figure 22

GROWTH OF Rps. SPHEROIDES

Effect of High Concentration Cofactors + Iron

reference BRL 342-54

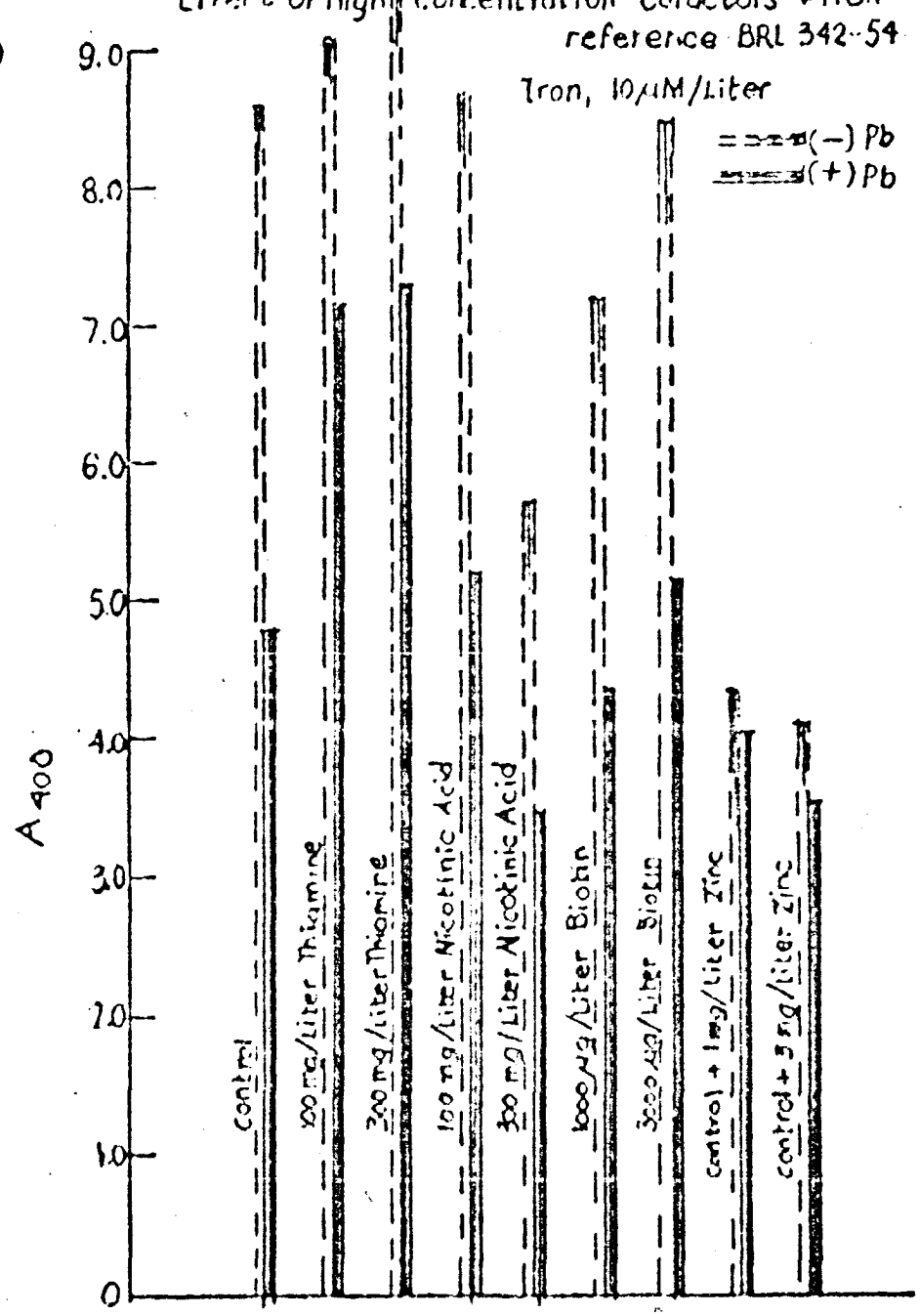


Figure 23

GROWTH OF Rps SPHEROIDES

Effect of Thiamin on Lead Inhibition. Growth 43-47 hours reference BRL 323-47

Pb Acetate = 1×10^{-4} M

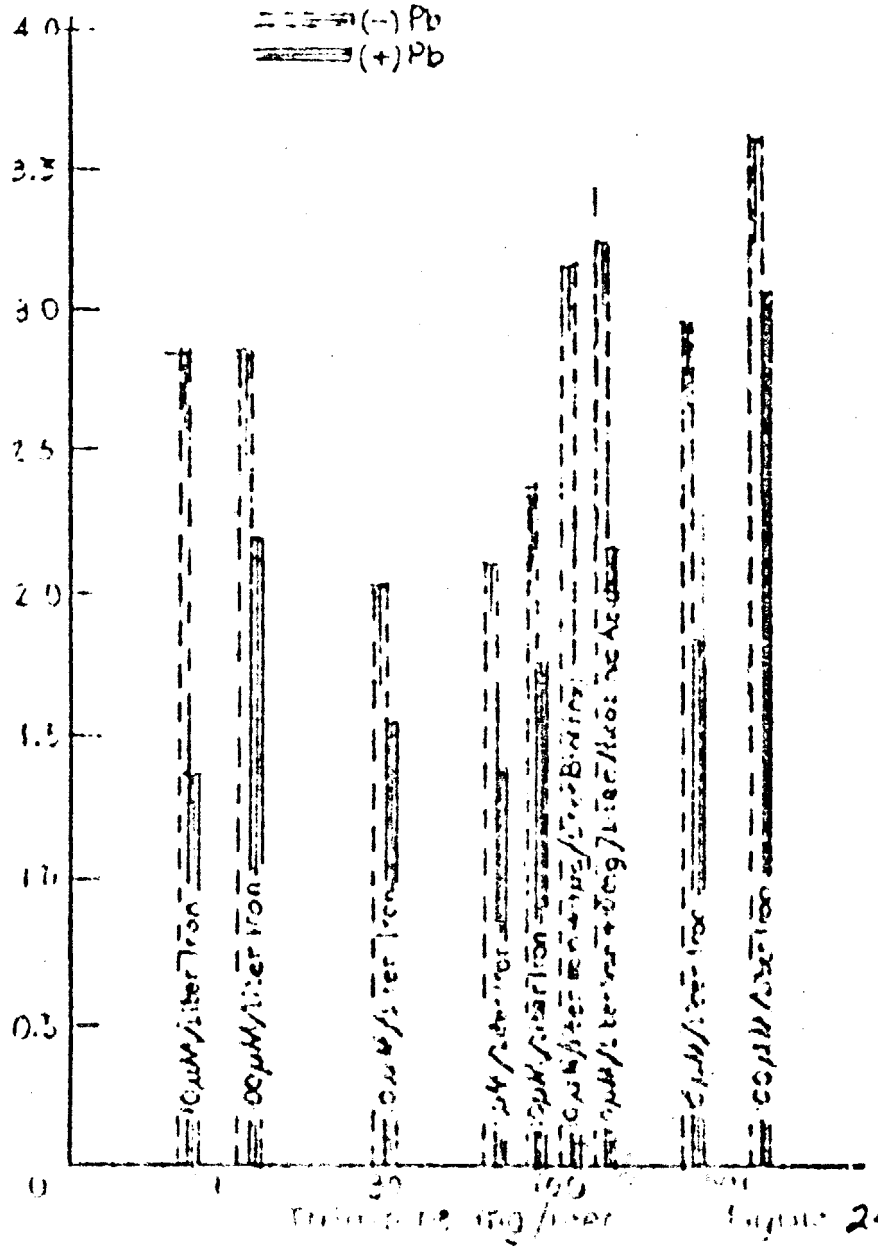


Figure 24

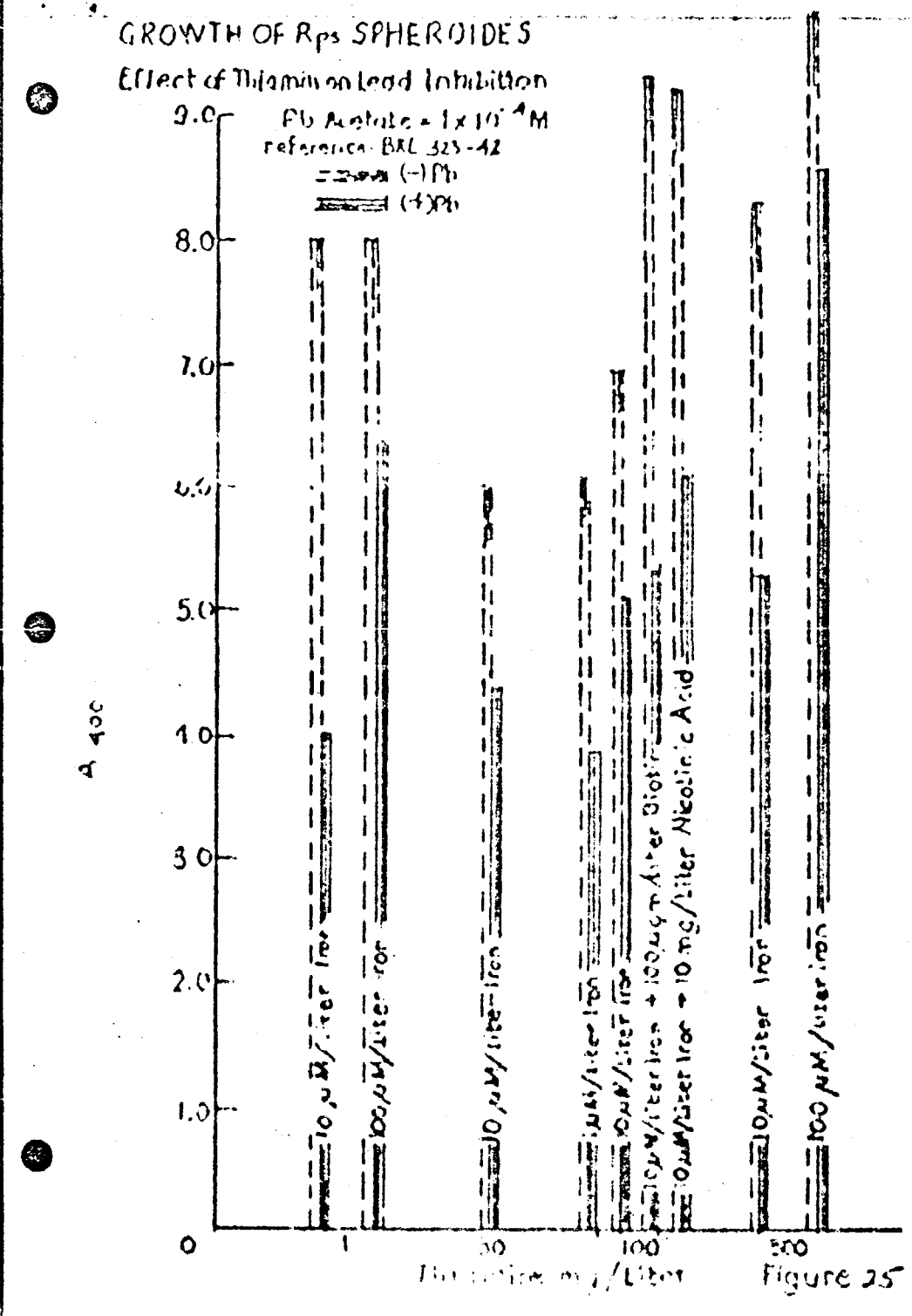

GROWTH OF Rps SPHEROIDES
Effect of Thiamin on Lead Inhibition
Pb Acetate = 1 x 10⁻⁴ M
reference BRL 325-42
(-) Pb
(+) Pb
A 400
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
1
30
100
300
10 μM/liter Iron
100 μM/liter Iron
10 μM/liter Iron
10 μM/liter Iron + 100 μg/liter Biotin
10 μM/liter Iron + 10 mg/liter Nicotinic Acid
10 μM/liter Iron
100 μM/liter Iron

Figure 25

GROWTH OF Rp. SPHEROIDES
Effect of Manganese
reference BRL-342-6.0

( ) Pb
(+) Pb

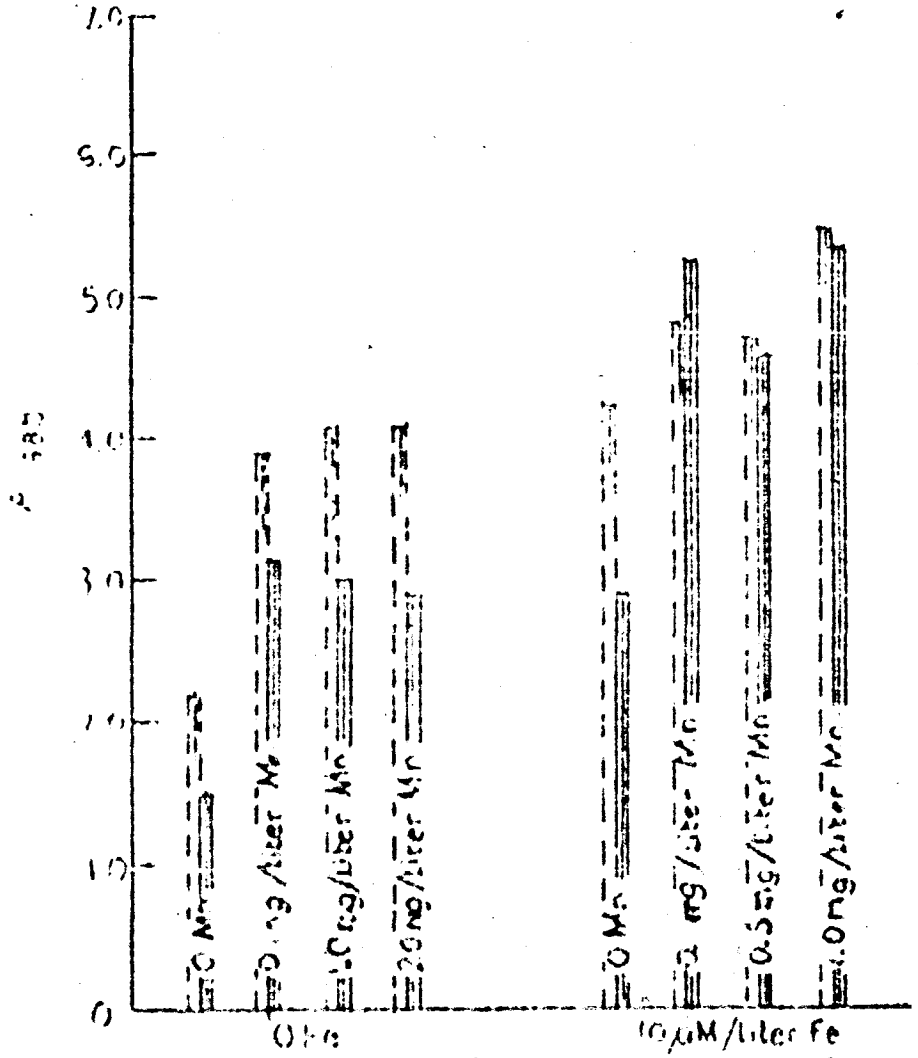


Figure 26

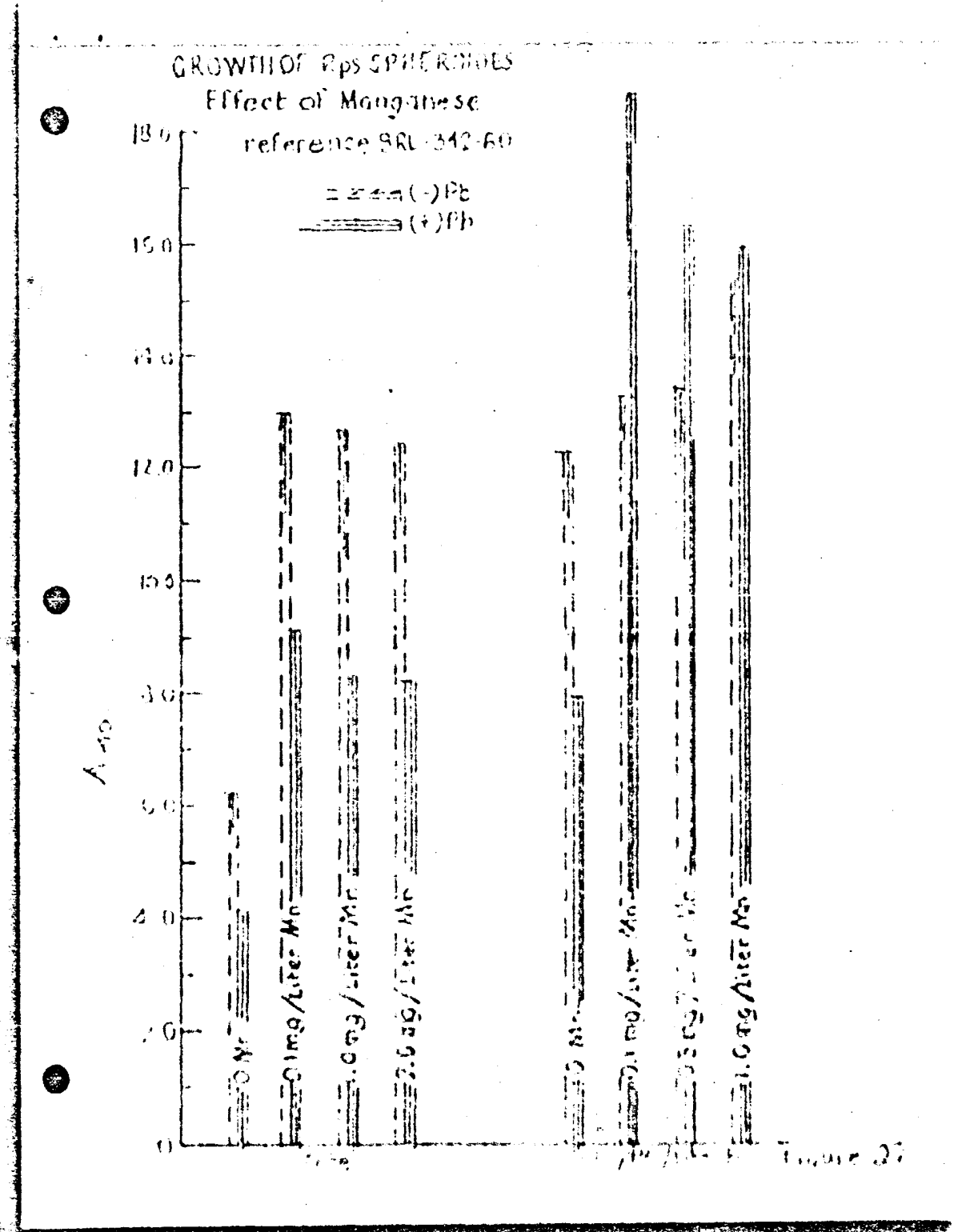


Figure 31

GROWTH OF Rhs SPHEROIDS
Effect of Mn^{++}
reference BRL 942-53

(-)Pb
(+)Pb

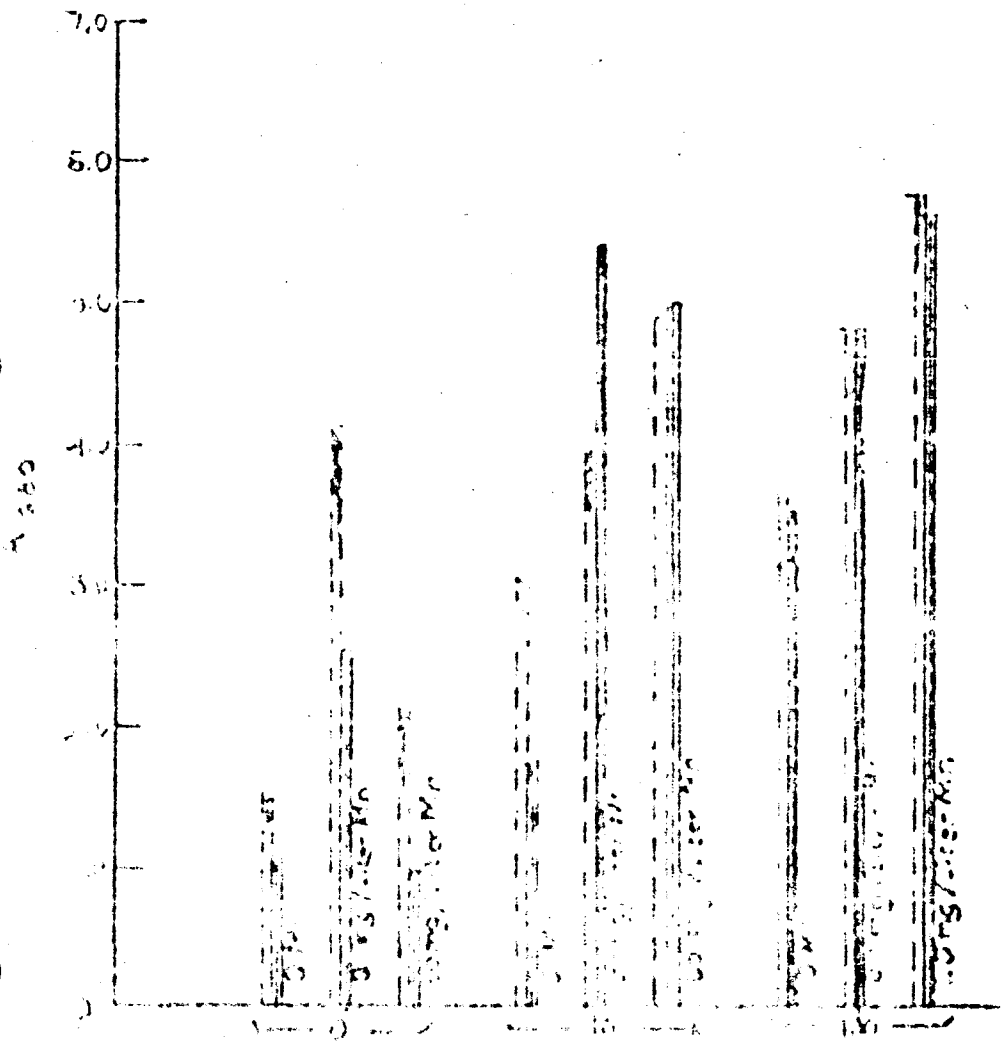
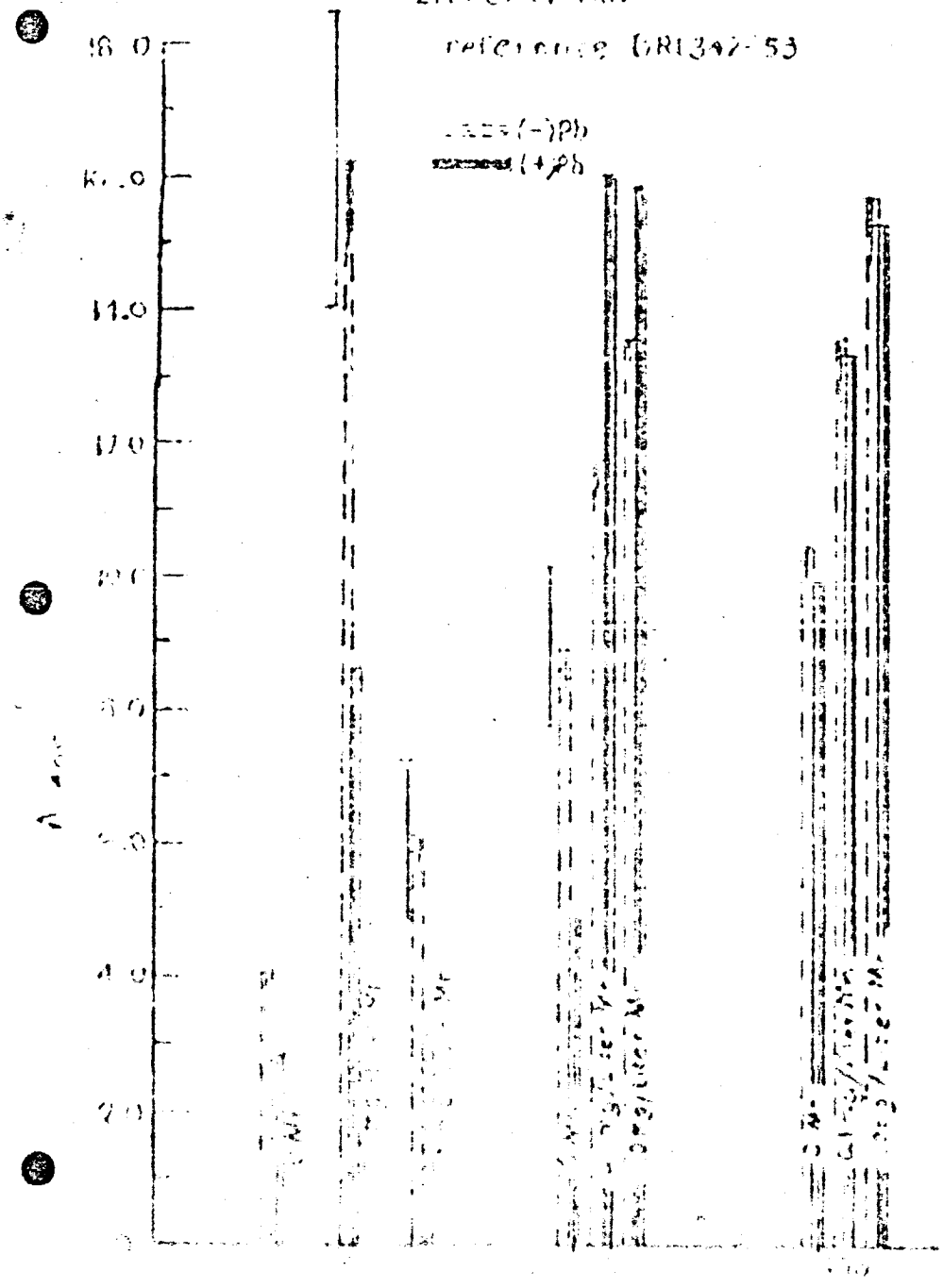


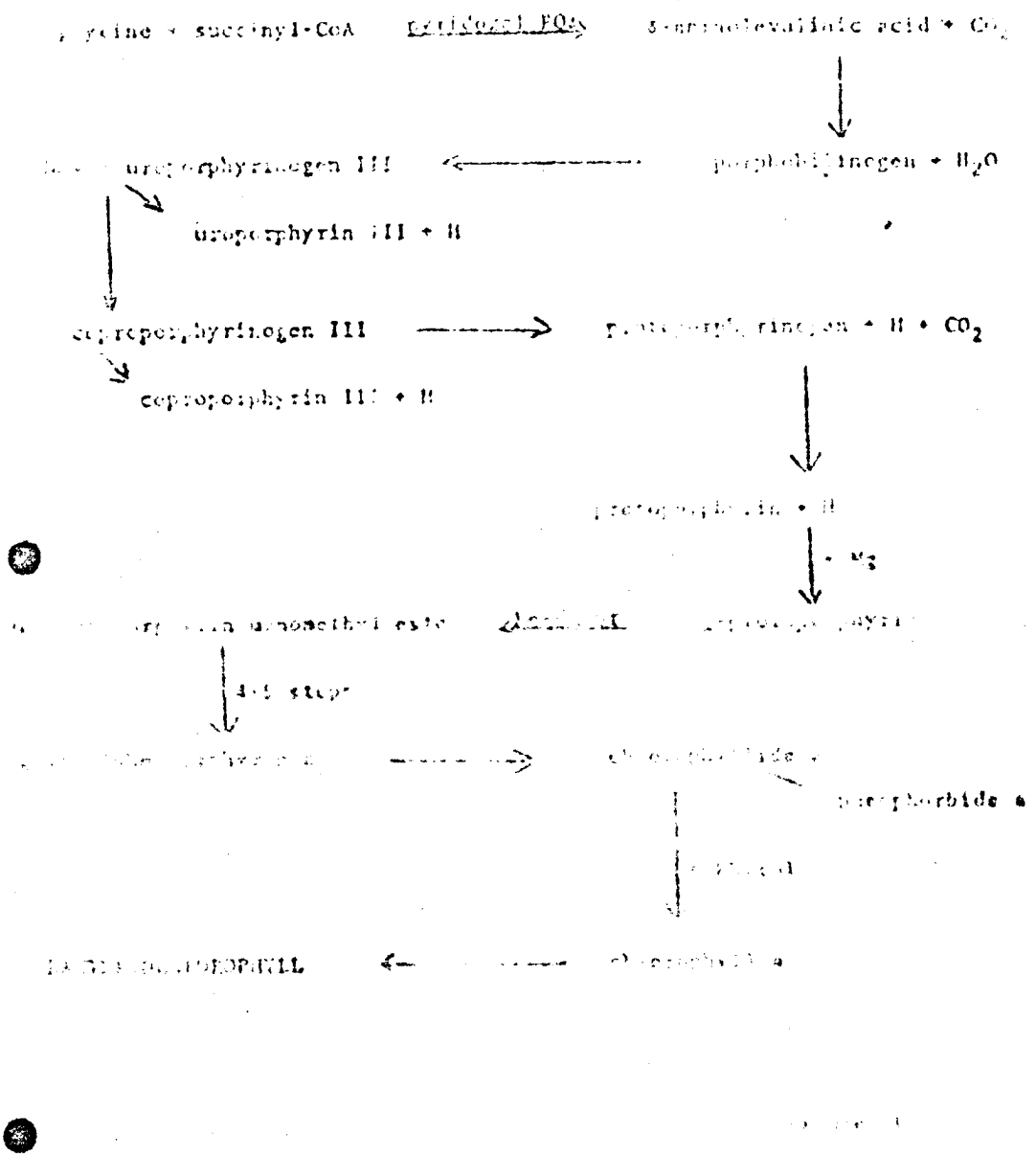
Figure 28

Effect of Mn^{++}

reference GR1342-53



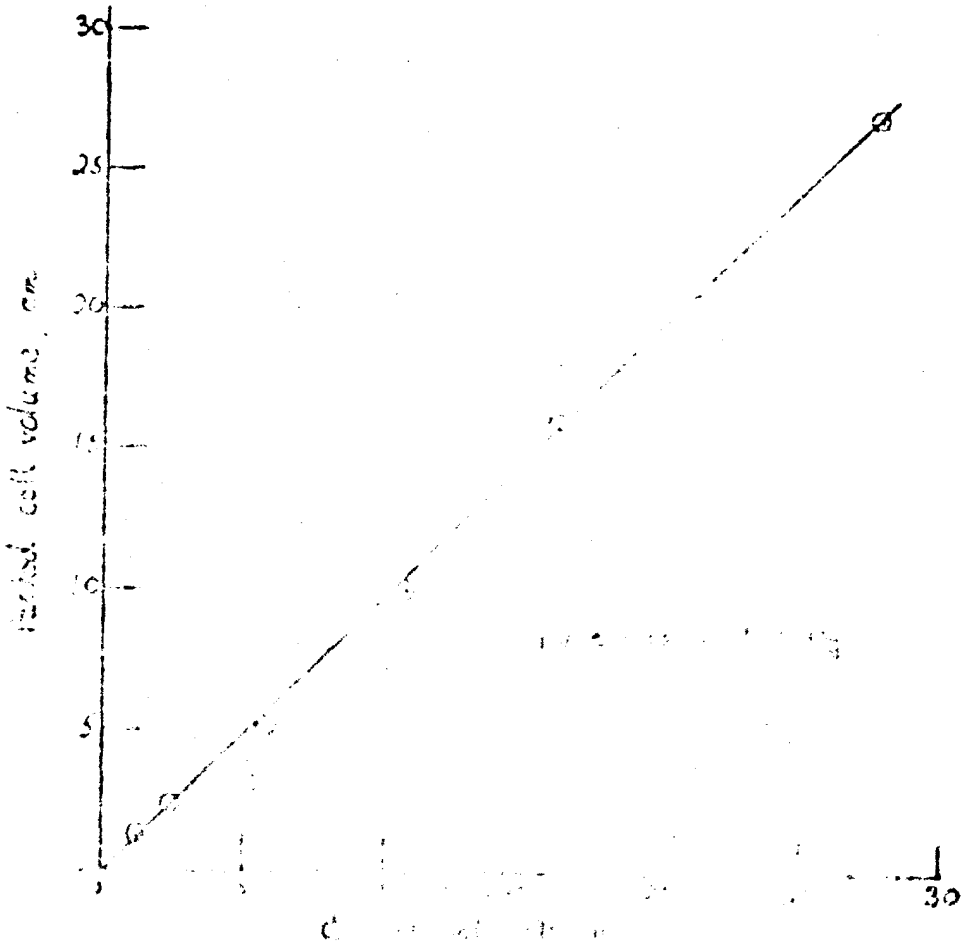
Tetrapyrrole synthesis - [illegible] porphyrides



GROWTH of Rps. SPHEROIDES

correlation of packed cell volume and dry weight of cells (10^9)

Reference: BRL 342-81



EFFECT OF Mn^{++} ON GROWTH OF Rps. SPHEROIDES

Reference: BRL 342-75-76

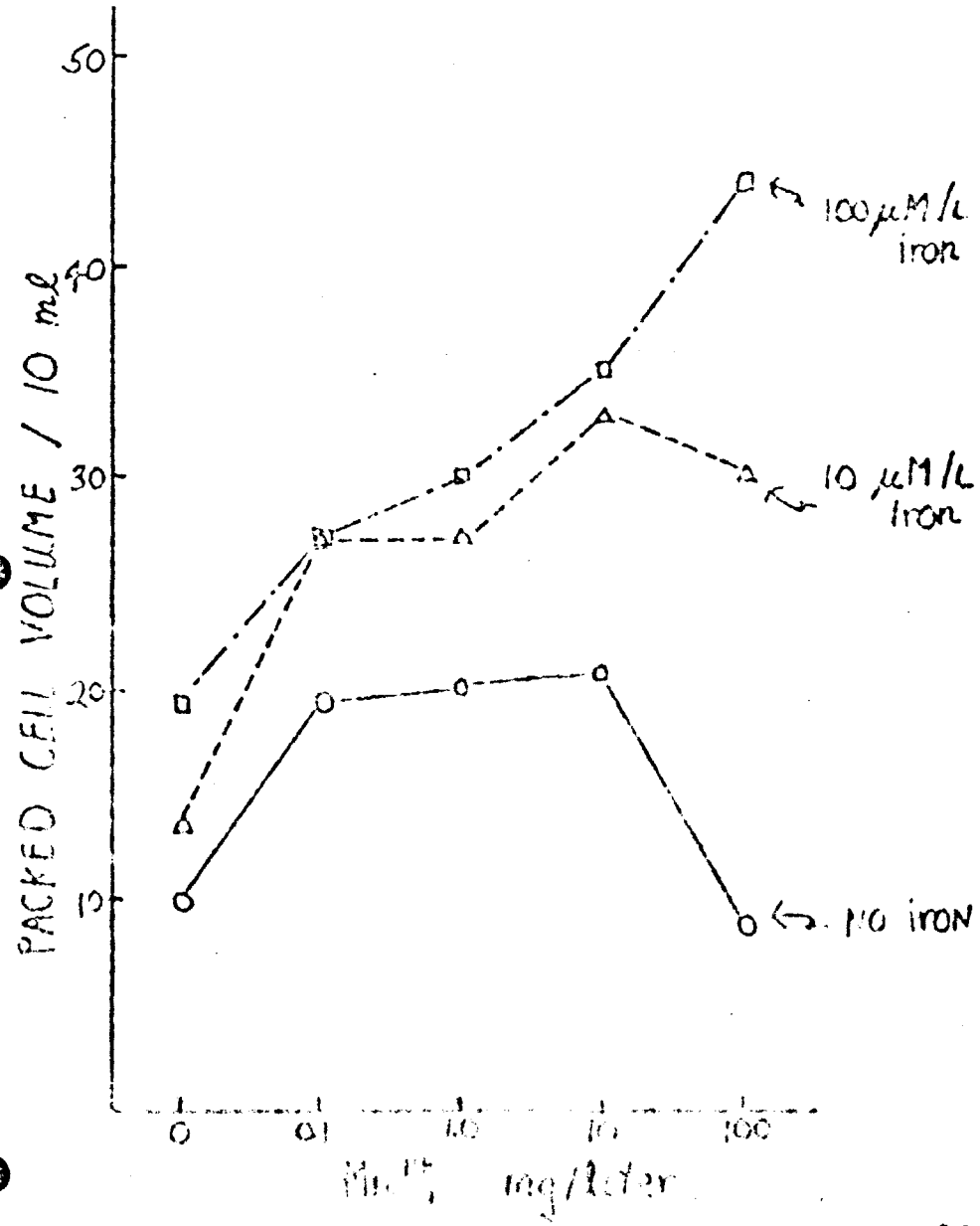
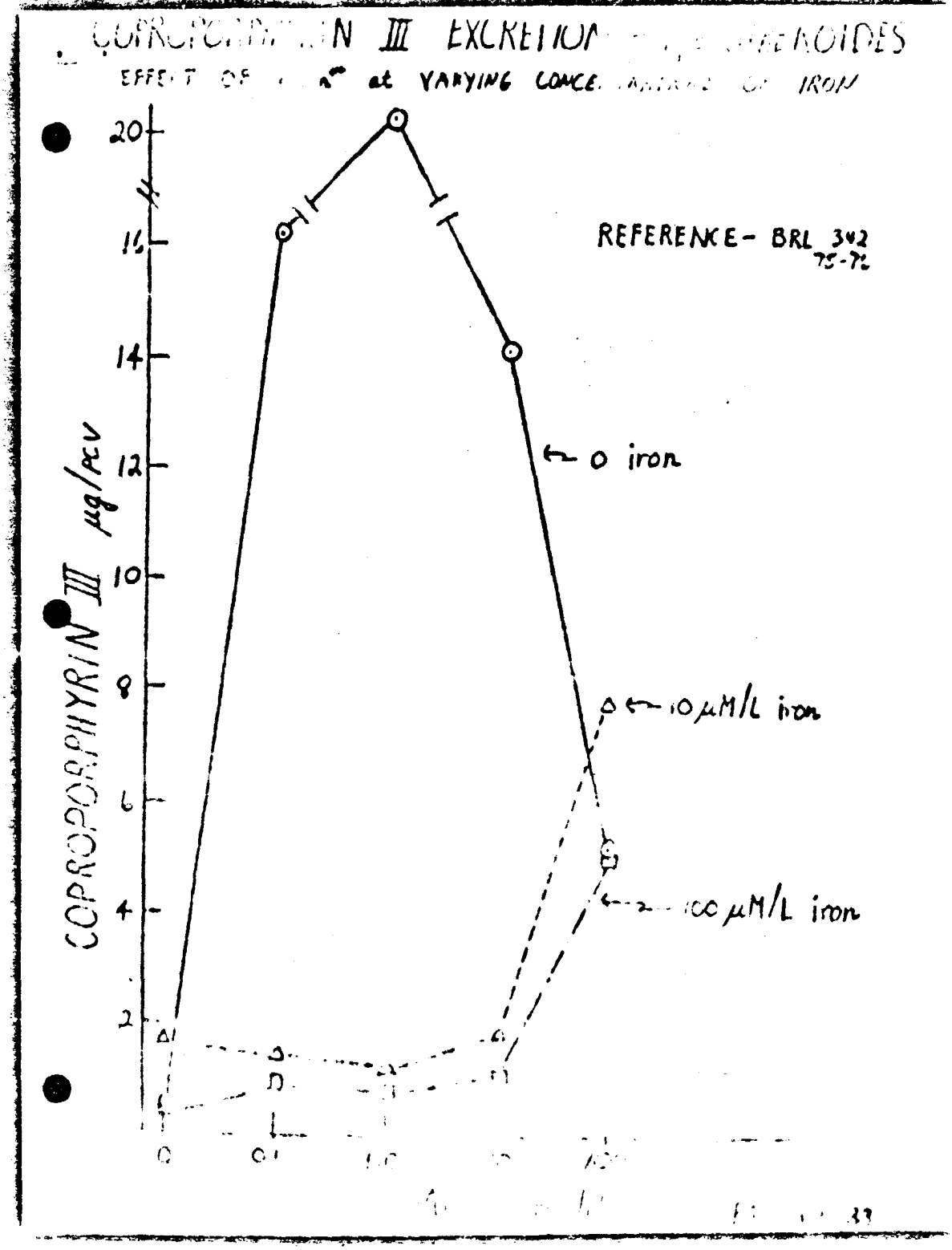
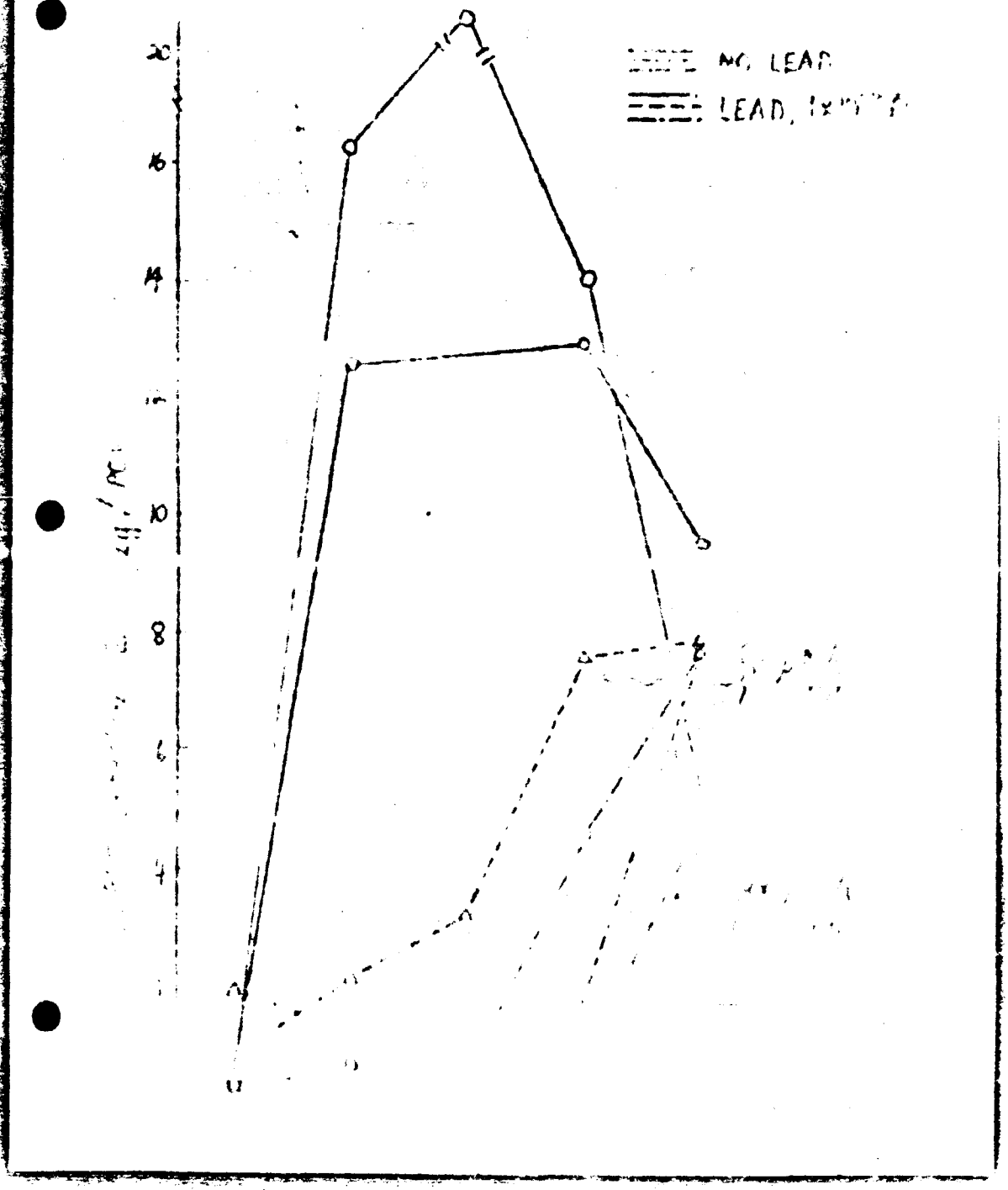


Figure 32


COPROPORPHYRIN III EXCRETION
EFFECT OF
at VARYING CONCE
IRON
REFERENCE- BRL 342
75-7
COPROPORPHYRIN III µg/ml
20
16
14
12
10
8
6
4
2
0
0 iron
10 µM/L iron
100 µM/L iron


NO. LEAD
LEAD,
20
16
14
10
8
6
4

Bacteriochlorophyll Content of Rps. Spheroides

Effect of ΔMn^{++} at varying concentrations of Iron

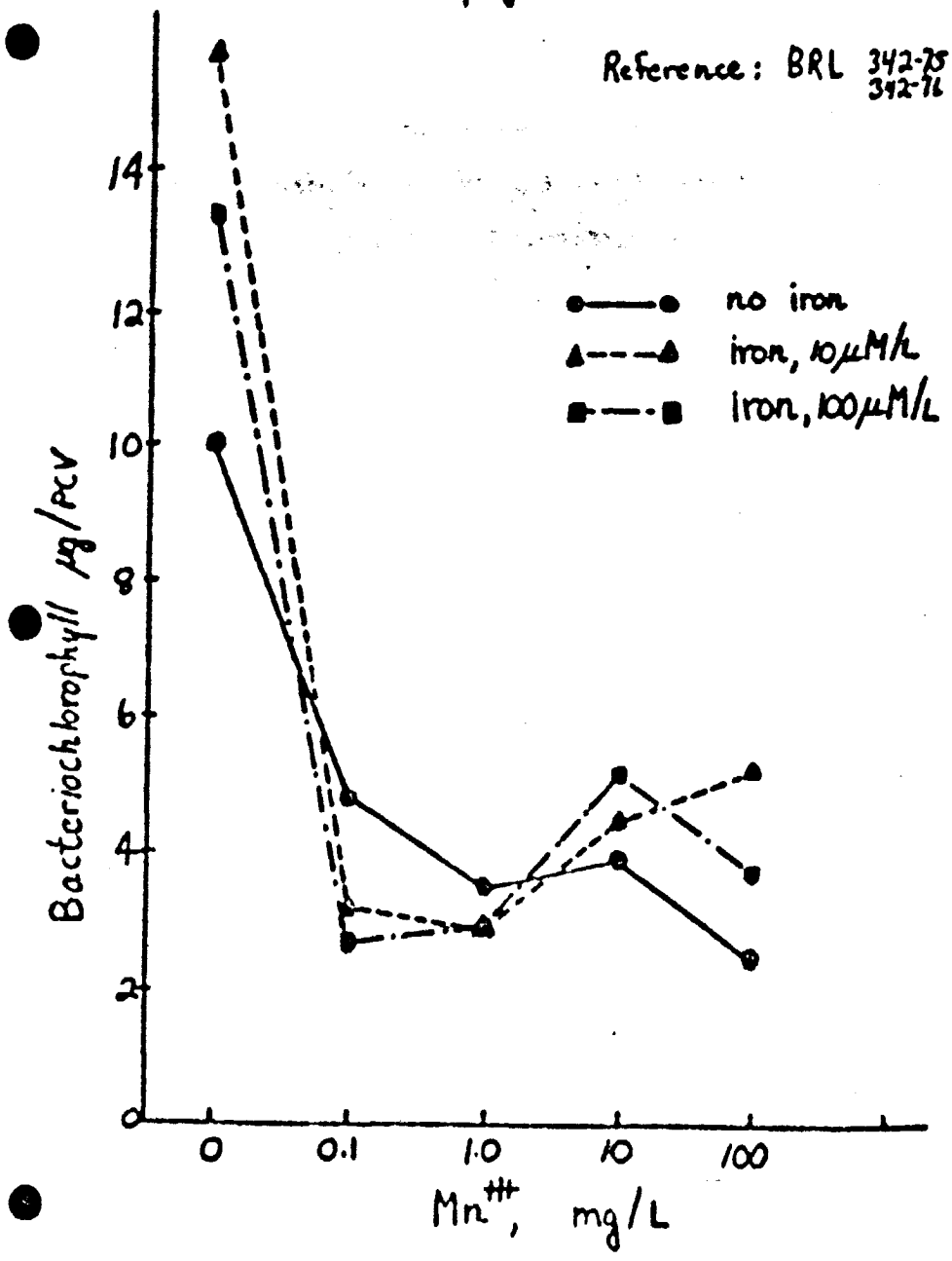


Figure 35

LIA12302

INHIBITION OF LIPOAMIDE DEHYDROGENASE BY LEAD
(269 – 275)

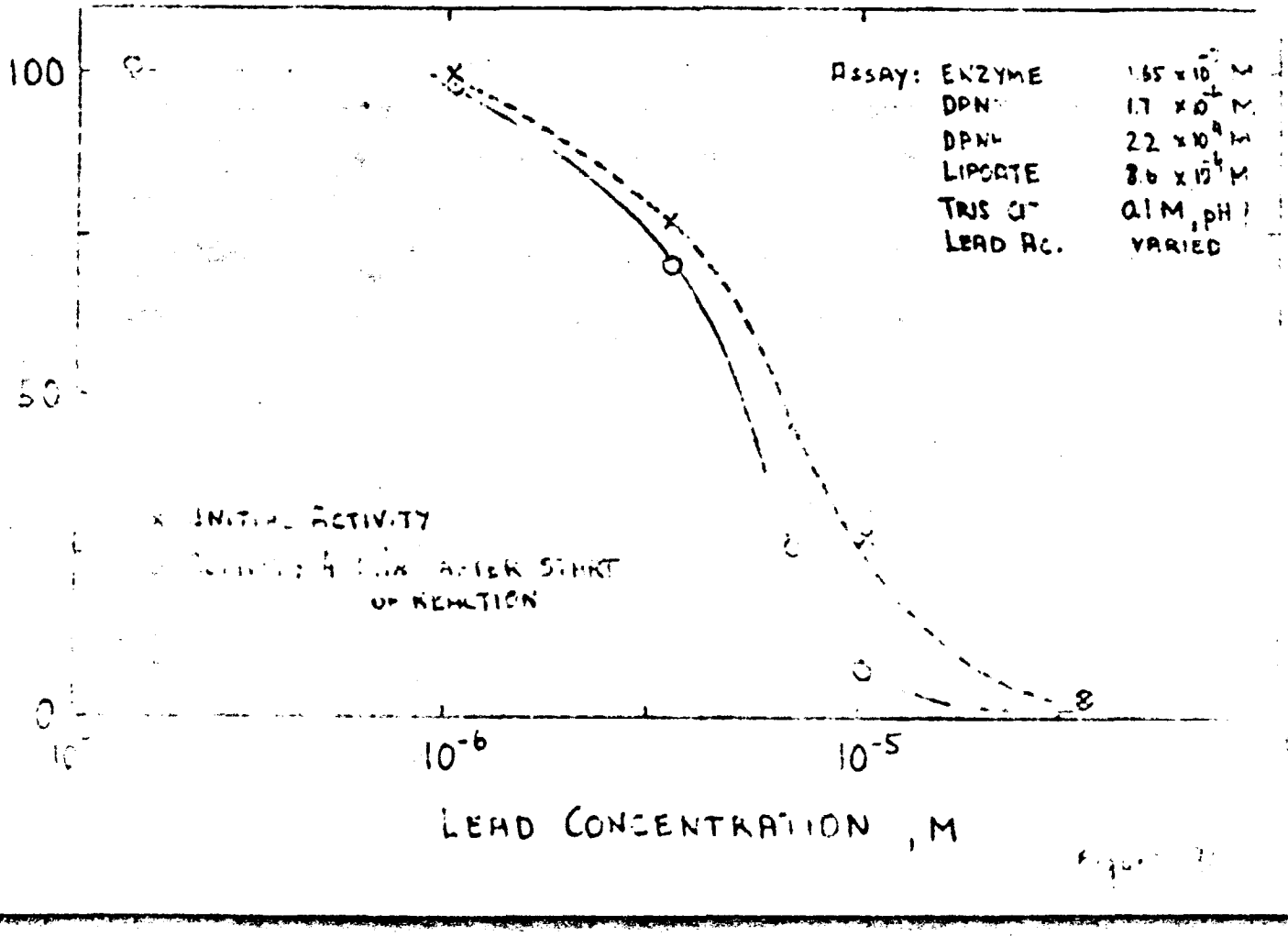


Figure 2

PARTIAL PROTECTION OF LIPOAMIDE DEHYDROGENASE BY SUBSTRATE AND COENZYMES AGAINST INHIBITION BY LEAD (269 - 275)

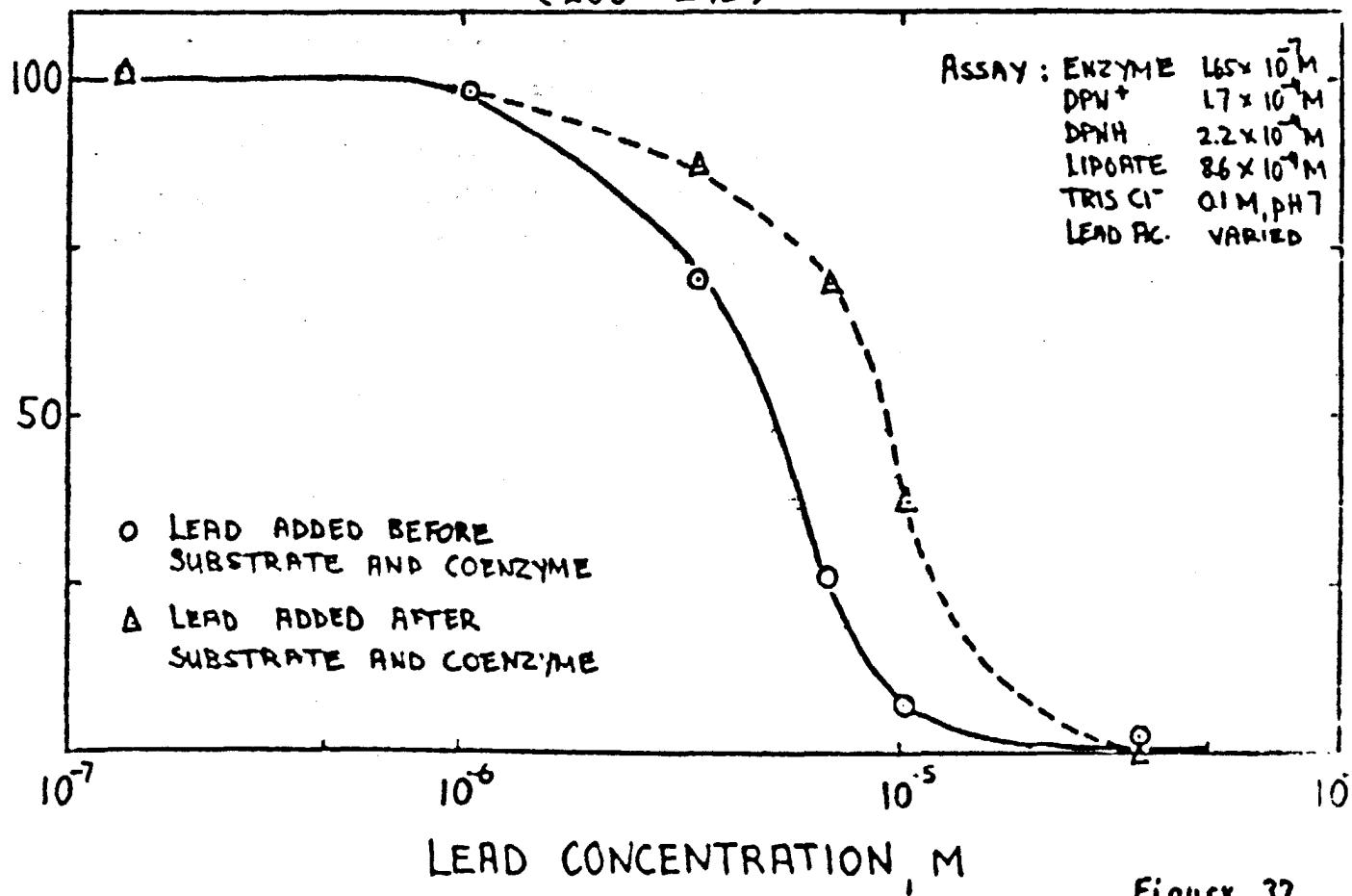


Figure 37

DIFFERENCE SPECTRA OF COMPLEXES OF LIPOAMIDE DEHYDROGENASE WITH LEAD AND CADMIUM (263 - 254)

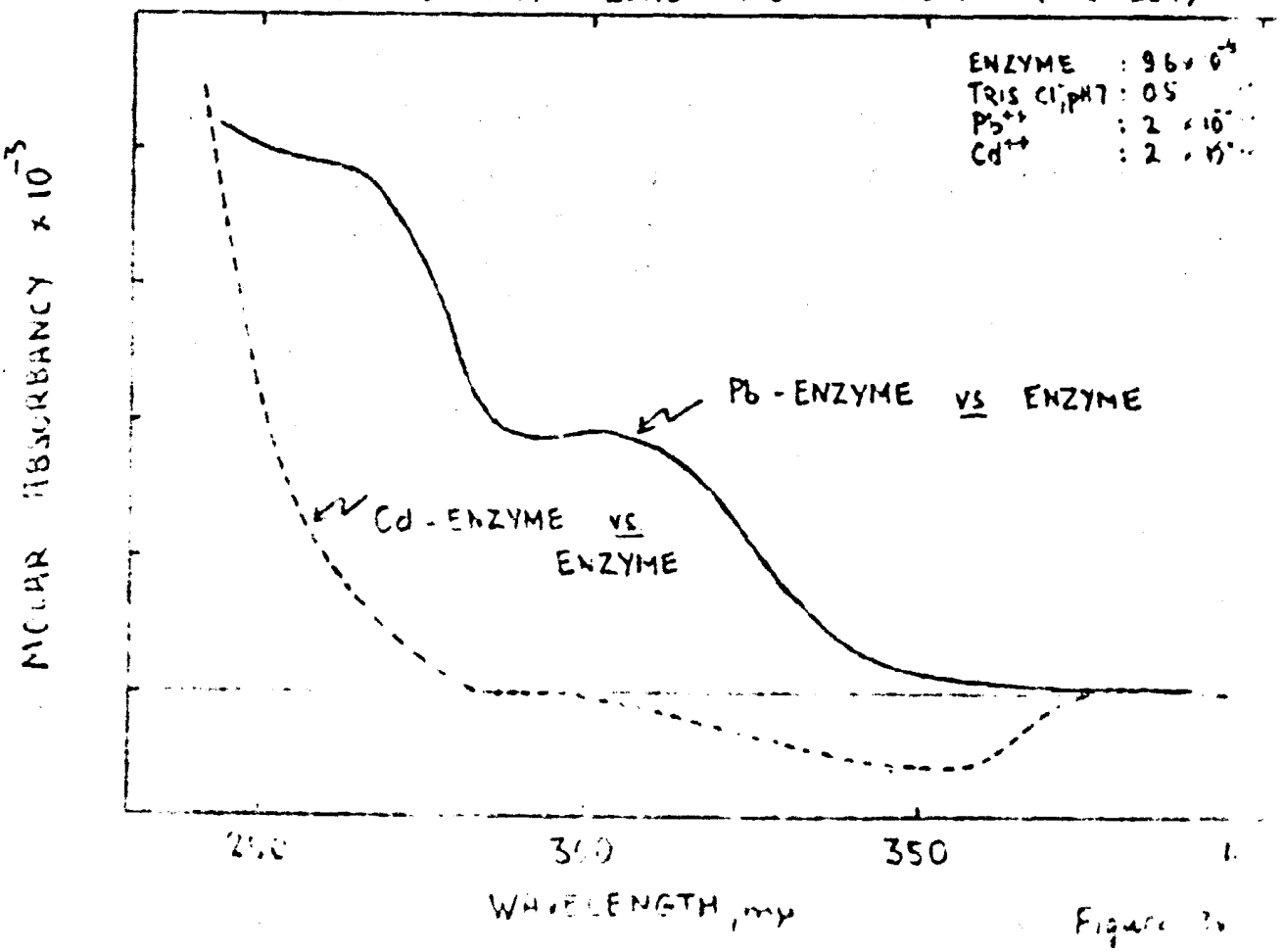


Figure 2