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THE EFFECT OF LOW LEAD DOSES IN VIVO ON LEAD
AND δ -AMINOLEVULINIC ACID DEHYDRATASE ACTIVITY LEVELS
IN BLOOD, LIVER, KIDNEY AND BRAIN OF THE RAT

by

© Sandra C. Wright, B.Sc.

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of
the requirements for the degree of
Master of Science
Department of Chemistry

Carleton University

Ottawa, Ontario

10 September, 1984

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**"THE EFFECT OF LOW LEAD DOSES IN VIVO ON LEAD
AND δ -AMINOLEVULINIC ACID DEHYDRATASE ACTIVITY LEVELS
IN BLOOD, LIVER, KIDNEY AND BRAIN OF THE RAT"**

submitted by Sandra C. Wright, B.Sc.
in partial fulfilment of the requirements for
the degree of Master of Science

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ABSTRACT

Rats were exposed to low lead levels of 0, 50, 100 and 1000 ppm in their drinking water for durations of 3, 7, 21 and 35 days. Blood, liver, kidney and brain samples were analyzed for lead content and d-Aminolevulinic Acid Dehydratase (ALA-D) activity. Both restored (by incubation with dithiothreitol and Zn^{+2}) and nonrestored activities were measured.

Blood lead and ALA-D activity levels reflected the amount of the lead dose but not the duration of the exposure to lead. The ratio of restored to nonrestored erythrocyte ALA-D activities was found to correlate reasonably well with blood, liver and kidney lead levels.

Exposure of blood and kidney ALA-D to lead caused inhibition of the enzyme which appeared to trigger a compensatory mechanism in both tissues which resulted in an increased amount of the enzyme. Liver and brain samples showed little effect of low lead exposure on ALA-D activity, possibly due to a low lead accumulation in these tissues.

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INTRODUCTION

Lead has been widely used since ancient times in lead-glazed pottery, water pipes and roofing and was until recently, widely used in paints. It is now used in batteries, solders, ammunition and various alloys. A very important use of lead is in gasoline as an anti-knock additive. It is a prevalent toxin with cases of chronic lead poisoning also dating back to ancient times. Lead poisoning is revealed through a wide variety of symptoms such as fatigue, constipation, abdominal pain, colic, anorexia, altered sleep, irritability, anemia, pallor, neuropathy and encephalopathy. Symptoms in children can include vomiting, behavioral changes, speech disturbances, seizures, fever and dehydration. Encephalopathy is more common in children and is one of the most serious consequences since irreversible neurological and psychological effects can result.(1) An individual can have any combination of symptoms and to varying degrees of severity. Clinical signs may not be obvious at all unless blood lead levels exceed 80 $\mu\text{g}/100\text{ mL}$.(2) Detection of the symptoms can be very subjective so that diagnosis is also based on a history and evidence of lead exposure. Whole blood lead concentrations are the most widely used monitor of lead exposure.

Lower lead levels, where clinical signs are absent or very nebulous, are known to affect the hematopoietic system.(1) With the elucidation of the structure and the synthesis of the porphyrins, the identification of the urinary pigments and red cell porphyrin changes accompanying lead exposure were characterized and applied to the

diagnosis of lead poisoning. Free erythrocyte protoporphyrin (FEP) levels were known to increase in lead exposed individuals and were shown to be essentially zinc protoporphyrin (ZPP).(3) ZPP levels are measured directly and simply (via fluorescence measurements) in whole blood which has made it a good indicator of lead poisoning.(4) ZPP levels can be directly related to high lead concentrations in the blood of both children and adults but its appearance is delayed and therefore increases some time after blood lead levels have increased. The delay is due to the lag time required for developing erythrocytes from the bone marrow, where lead is having an effect on heme synthesis, to appear in the peripheral blood. Thus, ZPP levels are considered a measure of lead hazard and not just of lead exposure. Problems with using the ZPP assay include the requirement for a mathematical correction for hematocrit or hemoglobin levels (significant in cases of more than moderate anemia) and the fact that zinc protoporphyrin levels are also increased in cases of iron deficiency anemia.

The characterization of the heme biosynthetic pathway (see Figure 1) illustrated the effects of lead (in vitro) on the enzymes of the pathway. Lead acts to depress the activity of delta-Aminolevulinic Acid Dehydratase (ALA-D), Coproporphyrinogen Oxidase (CPG-O) and Ferrochetalase. Delta-Aminolevulinic Acid Synthetase (ALA-S) activity is increased.(5,6) Increased ZPP levels found in lead exposure are due to decreased Ferrochetalase activity. Chronic lead poisoning leads to an increased Aminolevulinic Acid (ALA) excretion in the urine due to increased ALA-S and decreased ALA-D activities. The level of ALA in urine has been investigated as a biological monitor of lead poisoning.(1) It correlates with body lead burden well in children but

FIGURE 1 - Pathway of synthesis of heme.

Figure 1 (cont)

not in adults where normal levels are often found in known cases of lead poisoning.(6) Also, the 24-hour urine samples required can be difficult to obtain from children.

ALA-D catalyzes the condensation of two ALA molecules to form porphobilinogen.(7) The enzyme consists of eight subunits in a cubic arrangement and is an example of half-site reactivity in that it appears that only four of the eight subunits react with the substrate.(8) Seven to eight sulfhydryl groups are present in each subunit and are required for activity as are four to eight atoms of divalent zinc.(7-9) The enzyme reaction proceeds via the formation of a Schiff base with the substrate (10), with the involvement the amino acid lysine at the active site (see Figure 2). Two histidine residues also function at the active site along with one zinc atom and two cysteine residues.(9) Two subunits are required for activity with one involved in the formation of the Schiff base with one molecule of ALA while the other is involved in a non-covalent bonding with the second molecule.(11) Routine clinical assays have been developed for the enzyme.(12) They consist of the reaction of blood hemolysate or tissue homogenate with the substrate, ALA, combining the product, PBG, with a modified Erlich's reagent and reading the absorbance of the resulting compound at 550 nm. The assay is sensitive to many interferences (13) such as from extraneous lead, cadmium and zinc from the containers used and from airborne TCA (used in the assay to stop the reaction). Storage of the enzyme is also a problem because of the susceptibility of the sulfhydryl groups to oxidation.(7,14,15)

ALA-D is susceptible to very low lead levels (down to 10 $\mu\text{g}/100\text{ mL}$ of blood and less).(1) The enzyme inhibition caused by lead is through

FIGURE 2 - Mechanism of condensation of two molecules of δ-Aminolevulinic Acid to form Porphobilinogen.(49)

its high affinity for sulfhydryl groups. Of about 56 to 64 thiol groups found per enzyme molecule, 20 to 26 are free and it is these that are thought to be susceptible to binding by lead (and other divalent metals).(5) The degree of inhibition of erythrocytic ALA-D activity has been found to correlate with blood lead concentration and there is no delay in its response to lead exposure as is found in ZPP levels. It has therefore been extensively studied as a biological indicator of lead exposure.(16-27) Problems arise in its use as a diagnostic tool due to the shifts in the pH optimum of ALA-D activity on exposure to metal (21,28), individual-to-individual scatter in levels of ALA-D activity (20) and the non-specificity of the assay for lead (due to possible exposure to other metals, especially zinc, at the same time as the individual's exposure to lead).(29) Possible alternatives have been the use of ratios of ALA-D activity at different pHs (21,24,26,27) and/or of activity before and after restoration of lead inhibited activity (20,23,26,27,30), which have been found to correlate better with blood lead levels. Restoration is achieved through the use of heat, Zn^{+2} , and sulfhydryl compounds such as glutathione and dithiothreitol (DTT), alone or in combinations before the enzyme assay.(15) Zinc and DTT act to restore lead-inhibited activity by displacing lead from the sulfhydryl groups. Heat (60°C, 5 min.) is thought to activate the enzyme by causing conformational changes in the enzyme which displaces either lead itself or a factor which is concerned in some way in the inhibition by lead.(31) These three methods of activation have been found to be additive in their effect indicating that they act in different ways to restore activity. Lead is thought to bind to at least two different types of site on the

enzyme.(32) Zinc can effectively compete with lead at some of these sites but not others and DTT also cannot completely reverse inhibition, but activity can be almost completely restored if the two are used in combination.(33)

Blood sampling is the method of choice for monitoring exposure to lead due to the relative ease of taking samples on a routine basis but blood lead, and biological parameters correlated with blood lead concentrations, really only reflect the blood lead burden and not necessarily the body burden of lead. Lead is incorporated into other tissues such as bone marrow (where it competes with divalent calcium and is therefore relatively tightly bound), kidney, liver and brain. Lead can move in and out of the erythrocytes and therefore blood lead may reflect only very recent exposure to lead and not the organ levels.

Erythrocytic ALA-D is not considered to be biologically significant since heme synthesis does not occur in mature red blood cells. It is possible that it may reflect the effect of lead in bone marrow though, as indicated by the observations of increased erythrocytic ALA-D activity in lead-exposed animals and humans.(34-38) In young animals, the increased activity was due to an increased reticulocyte level in the blood. Reticulocytes are immature red blood cells and have a higher ALA-D activity than mature cells.(37) In adult animals and humans, the increased activity was due to an increase in the level of ALA-D itself in the red blood cells with no concomitant change in the blood profile.(35,36,38) This type of an effect has not been found in the ALA-D levels in the organs.

Discussions continue in the literature as to whether blood lead levels, blood ALA-D activity or FEP levels are the most suitable

measure of the total body lead burden and the biological effects of lead.(6,25) It is also of interest to define a method which would determine the mode of lead exposure, whether chronic (long-term) or acute (one-time exposure), at high or low lead doses. This may be important where an individual's history of lead exposure is not known.

In this study, rats are exposed to relatively low levels of lead (0, 50, 100 and 1000 ppm) in their drinking water. At these levels, no clinical signs of lead poisoning are expected. A profile of the response in blood, liver, kidney and brain to different levels of lead (due to both varying concentrations of lead given and the duration of exposure) is to be obtained by measuring lead concentrations and ALA-D activity. Both restored, using DTT and Zn^{+2} , and nonrestored ALA-D activity are measured. FEP values are not included in this report. Blood samples are taken in order to characterize the relationship between erythrocyte lead content, ALA-D activity and lead exposure. Liver, kidney and brain samples are taken in order to measure lead accumulation in these tissues and thereby attempt to estimate body lead burden and to relate blood data to tissue data. ALA-D activity is measured in the tissues to evaluate possible biochemical effects of lead. Heme synthesis does occur in these tissues as it is required for incorporation into various hemoproteins. Hemoproteins include the cytochromes which are required for electron transfer and catalase which acts to detoxify hydrogen peroxide in the body.

The animals are exposed to lead for up to five weeks which would represent "sub-acute" or chronic exposure. Acute lead poisoning is not considered in this study since its occurrence is relatively rare with only occasional cases found in young children.

Low lead doses, those that might be found in environmental or moderate occupational exposure, are used. At low lead exposure the effect of lead on the level of ALA-D activity is more precise than its effect on FEP levels where there is a greater incidence of false positives and negatives. Buchet et al (40) found no significant FEP response in the animals given the similar lead doses as those in this study. Although low lead levels are known to effect ALA-D activity, their effects on the synthesis of heme, and therefore their biological significance, are not known.

RESULTS

In this study, rats were exposed to lead (as lead acetate) via their drinking water at 0, 50, 100 and 1000 ppm dose levels. The rats were approximately 10 weeks old at the beginning of the study and can therefore be considered as "adult" animals. Liver, kidney, brain and blood samples were taken at 3, 7, 21 and 35 day time periods with at least four samples for each tissue taken at each time point and dose level.

Tissue samples were homogenized in Tris buffer in an attempt to reduce the possibility of lead loss due to its chelation in phosphate buffer. No difference in the level of enzyme activity was found with the use of Tris buffer as compared to phosphate buffer. The samples were analyzed for lead content and d-Aminolevulinic Acid Dehydratase (ALA-D) activity. Blood samples were analyzed fresh. Lead analysis was performed on freeze-dried tissue homogenates. For ALA-D activity assay, tissue homogenates were stored frozen and measured within two weeks. Activation of ALA-D in lead-exposed kidney samples appeared to occur with prolonged storage presumably due to lead loss. ALA-D activity was assayed in buffer solutions ranging in pH from 5.8 to 7.2. The value of the buffer pH is used as the actual pH of the reaction mixture since it is similar to the actual pH of the reaction mixture and is less difficult to measure. A range of pH values is used instead of the single pH used by most other authors (12) since lead has been found to cause a shift in the pH optimum for ALA-D activity as well as the inhibition of enzyme activity.

"Restored" ALA-D activity (ALA-D(+DTT)) was also measured at the same time as the nonrestored activity. Restoration was achieved by incubating the enzyme with dithiothreitol (DTT) and Zn^{+2} (at concentrations of 10 mM and 40 μ M respectively in the reaction mixture) before reaction with the substrate. The inhibition of the enzyme is removed by displacement of lead from the enzyme and the reversal of any inactivation caused by air oxidation. Inactivation is known to occur with incubation of the enzyme without substrate.(13) This occurred with liver samples assayed for ALA-D(+DTT) activity at the beginning of the study when a lower DTT concentration was used. With these low DTT levels, the restored ALA-D activity in control liver samples was actually lower than the nonrestored activity due to inactivation during the preincubation with DTT and Zn^{+2} . The DTT concentration was not high enough to even counteract air oxidation during this extra incubation time and was therefore probably not high enough to remove lead inhibition. The DTT concentration was therefore increased from the 4 mM final concentration originally used to a 10 mM final concentration in the reaction mixture. The change in DTT concentration did not appear to change the degree to which blood and kidney samples were restored therefore the earlier data obtained with 4 mM DTT is included (and meaned) with the later data obtained using 10 mM DTT. The earlier liver samples were repeated using the 10 mM DTT since frozen liver samples were available.

The levels of lead administered to the rats had no obvious clinical effects. From Table 1 it can be seen that there was no significant difference in hematocrit, blood or organ protein levels, organ weights or body weights between lead-exposed and control animals.

TABLE 1 - Body weights, organ weights, protein levels and blood hematocrit levels in rats exposed to 0, 50, 100 and 1000 ppm lead acetate in their drinking water for 3, 7, 21 and 35 days.

DURATION OF DOSE (days)	DOSE (ppm)	FINAL BODY WEIGHT (g)	BLOOD HEMATOCRIT (%)	PROTEIN (mg/mL)	LIVER ORGAN WEIGHT (g)	PROTEIN (mg/mL)
3	0 (5) ^a	268 ± 13 ^c	41 ± 2	284 ± 45	14.1 ± 1.3	45.4 ± 5.9
	50 (5)	265 ± 18	40 ± 3	271 ± 50	13.4 ± 1.2	47.4 ± 4.8
	100 (4)	271 ± 22	42 ± 2	293 ± 21	13.4 ± 1.4	49.5 ± 4.9
	1000 (5)	273 ± 13	41 ± 1 (4) ^b	277 ± 20	13.7 ± 1.0	49.6 ± 4.6
7	0 (5)	299 ± 20	41 ± 2	304 ± 49	14.6 ± 1.6	53.2 ± 10
	50 (5)	289 ± 18	42 ± 1	310 ± 53	14.0 ± 1.4	48.0 ± 4.3
	100 (5)	302 ± 9	41 ± 2	304 ± 51	14.8 ± 1.4	51.0 ± 8.3
	1000 (5)	290 ± 12	41 ± 1	298 ± 49	13.9 ± 1.1	50.7 ± 4.2
21	0 (4)	358 ± 16	43 ± 2	333 ± 56	16.5 ± 1.7	51.4 ± 9.3
	50 (4)	358 ± 24	42 ± 4	313 ± 36	15.5 ± 1.6	50.8 ± 8.6
	100 (4)	355 ± 6	45 ± 1	335 ± 38	15.7 ± 0.6	52.2 ± 6.9
	1000 (4)	340 ± 9	43 ± 2	319 ± 25	14.9 ± 1.3	56.5 ± 8.0
35	0 (4)	389 ± 47	46 ± 1	330 ± 40	16.1 ± 4.2	47.0 ± 3.9
	50 (4)	406 ± 18	45 ± 2	339 ± 53	16.5 ± 1.5	51.1 ± 0.6
	100 (4)	426 ± 27	45 ± 2	331 ± 19	17.9 ± 0.4	52.0 ± 4.2
	1000 (4)	397 ± 24	43 ± 2	317 ± 26	16.6 ± 1.5	51.2 ± 8.4

a : number of animals sacrificed at each point
b : number of samples (if different from "a")
c : standard deviation of mean value

TABLE 1 (cont)

KIDNEY ORGAN WEIGHT (g)	PROTEIN (mg/mL)	BRAIN ORGAN WEIGHT (g)	PROTEIN (mg/mL)
2.8 ± 0.3	17.0 ± 2.3	1.6 ± 0.2	14.1 ± 1.5
2.7 ± 0.3	17.5 ± 2.4	1.7 ± 0.2	15.0 ± 1.9
2.5 ± 0.2	20.7 ± 4.0	1.7 ± 0.3	14.9 ± 0.4
2.6 ± 0.3	17.9 ± 2.0	1.9 ± 0.1	14.8 ± 1.2
2.8 ± 0.5	18.1 ± 2.9	1.8 ± 0.3	14.0 ± 1.8
2.7 ± 0.2	17.2 ± 2.1	1.7 ± 0.2	14.7 ± 0.8
2.9 ± 0.3	17.9 ± 1.2	1.9 ± 0.1	14.2 ± 1.0
2.8 ± 0.2	20.1 ± 2.4	1.9 ± 0.1	14.8 ± 0.8
3.1 ± 0.3	18.8 ± 3.6	1.8 ± 0.2	14.8 ± 0.4
3.2 ± 0.1	19.7 ± 3.1	1.7 ± 0.3	14.7 ± 0.4
3.2 ± 0.1	20.5 ± 1.4	1.8 ± 0.1	15.9 ± 0.3
3.1 ± 0.3	20.3 ± 2.5	2.0 ± 0.4	13.9 ± 2.9
3.2 ± 0.6	18.2 ± 2.2	1.8 ± 0.1	14.8 ± 0.7
3.3 ± 0.2	18.5 ± 1.6	1.5 ± 0.5	12.4 ± 3.6
3.4 ± 0.4	18.3 ± 2.0	2.0 ± 0.1	14.2 ± 2.1
3.4 ± 0.2	20.0 ± 1.7	1.8 ± 0.2	16.4 ± 1.4

Table 2 gives the lead concentrations found in the tissues and blood at each dose level and time point. The values represent the mean of at least four samples. Figures 3 to 5 illustrate the lead content of liver, kidney and brain (relative to tissue protein) respectively as a function of the duration of each lead dose. Lead concentrations appear to have reached a steady state in liver and kidney samples by the third day of exposure to lead. The lead concentration in brain samples continued to rise until the 21 day time point. The concentration of lead in each tissue corresponded to lead dose but there was no change over the duration of the experiment. The accumulation in the tissues was greatest in kidney (relative to protein level), which had an approximately ten fold greater concentration of lead than liver. Lead concentration in liver was about 2.5 times that in brain. Blood lead concentrations are expressed per 100 mL of blood and are given as a function of time for each lead dose in Figure 6. Lead concentrations were around the 70 $\mu\text{g}/100\text{ mL}$ level in the animals given 1000 ppm lead in water. The samples from the lower dosed animals were far below this level. The 100 and 50 ppm lead exposed animals showed blood lead concentrations of about 25 and 15 $\mu\text{g}/100\text{ mL}$ respectively. The control animals had lead concentrations of less than 10 $\mu\text{g}/100\text{ mL}$ of blood.

Figures 7, 8, 9 and 10 illustrate ALA-D activity as a function of the buffer pH for blood, liver, kidney and brain respectively for all lead doses at each time point. Each curve is an average of at least four samples. The ALA-D activity was taken to be the activity at the optimum pH. Blood was found to contain the lowest amount of ALA-D activity, that is the lowest amount of PBG formed in one hour per

TABLE 2 - Lead Levels in blood and tissue samples of rats exposed to 0, 50, 100 and 1000 ppm lead in their drinking water for 3, 7, 21 and 35 days.

DURATION OF DOSE (DAYS)	LEAD DOSE (ppm)	LEAD CONCENTRATION			
		BLOOD ($\mu\text{g}/100 \text{ mL}$)	LIVER ($\mu\text{g}/\text{g prot}$)	KIDNEY ($\mu\text{g}/\text{g prot}$)	BRAIN ($\mu\text{g}/\text{g prot}$)
3	0	5.9 $\pm$ 2.7	0.14 $\pm$ 0.06	3.1 $\pm$ 5.5	0.41 $\pm$ 0.16
	50	14 $\pm$ 7	0.31 $\pm$ 0.08	5.2 $\pm$ 2.6 *	0.37 $\pm$ 0.20 *
	100	23 $\pm$ 13	1.0 $\pm$ 0.7	20 $\pm$ 14	0.60 $\pm$ 0.16
	1000	69 $\pm$ 17	3.8 $\pm$ 1.9	58 $\pm$ 20	0.87 $\pm$ 0.33
7	0	7.5 $\pm$ 4.3	0.18 $\pm$ 0.04	0.70 $\pm$ 0.2	0.57 $\pm$ 0.49
	50	19 $\pm$ 5	0.49 $\pm$ 0.22	9.4 $\pm$ 5 *	0.53 $\pm$ 0.33 *
	100	25 $\pm$ 7	0.97 $\pm$ 0.35	15 $\pm$ 11	0.65 $\pm$ 0.49 *
	1000	68 $\pm$ 26	4.0 $\pm$ 2.0	52 $\pm$ 17	1.2 $\pm$ 0.7
21	0	6.8 $\pm$ 3.1	0.088 $\pm$ 0.028	0.76 $\pm$ 0.59	0.39 $\pm$ 0.14
	50	18 $\pm$ 8	0.69 $\pm$ 0.44	10 $\pm$ 6	0.57 $\pm$ 0.11
	100	28 $\pm$ 6	1.8 $\pm$ 0.7	23 $\pm$ 14	0.92 $\pm$ 0.60
	1000	56 $\pm$ 20	4.8 $\pm$ 1.8	51 $\pm$ 14	2.0 $\pm$ 1.1
35	0	11 $\pm$ 2	0.16 $\pm$ 0.11	0.42 $\pm$ 0.23	0.35 $\pm$ 0.16
	50	24 $\pm$ 9	0.51 $\pm$ 0.21	12 $\pm$ 4	0.59 $\pm$ 0.28
	100	31 $\pm$ 9	0.93 $\pm$ 0.56	20 $\pm$ 11	0.66 $\pm$ 0.41
	1000	76 $\pm$ 14	4.0 $\pm$ 0.7	60 $\pm$ 25	1.8 $\pm$ 0.5

* : no significant difference from control value

milligram of total protein. Liver contained the highest amount of enzyme activity in terms of tissue protein. The specific activity of ALA-D in kidney samples was approximately one half that found in liver. Brain samples contained an extremely low ALA-D activity per amount of protein, about one-tenth of the amount found in liver. ALA-D in kidney and brain samples was allowed to react for two hours to produce a larger amount of PBG to allow a more accurate measure. The activities are expressed as the amount of PBG produced in two hours per milligram protein.

Table 3 gives the erythrocyte ALA-D activity and the optimum pH values for the samples from control and lead-dosed animals. Figure 11 gives the activity as a function of time for each dose. It can be seen that erythrocyte ALA-D activity was inhibited by exposure to lead with the degree of inhibition increasing with the lead dose. Table 3a gives the ALA-D activity in blood samples at pH 6.4, the pH at which most authors measure ALA-D activity. The ALA-D activity measured at 6.4 can be seen to be slightly different than that measured at the pH optimum. The degree of inhibition remained relatively constant in the samples from animals given 50 and 100 ppm lead over the duration of the exposure to lead. Erythrocyte ALA-D from the 50 and 100 ppm lead-exposed samples were inhibited to approximately 74% (± 5) and 64% (± 4) of control values respectively. The degree of inhibition at the 1000 ppm dose level was constant at about 35% (± 1) over the 3 and 7 day time points but then appeared decreased (ie was less inhibited compared to controls) at the 21 day point. At 35 days, the inhibition is significantly less than it was at 3 days. This relative increase in the ALA-D activity at 21 days was concomitant with a very large

TABLE 3 - Erythrocyte ALA-D Activity (restored and nonrestored) in rats exposed to 0, 50, 100 and 1000 ppm lead in their drinking water for 3, 7, 21 and 35 days. Activity measured at peak pH.

DURATION OF DOSE (DAYS)	LEAD DOSE (ppm)	ALA-D ACTIVITY ($\times 10^4$ μmol PBG/hr/mg prot)	pH OPTIMUM	PERCENT OF CONTROL ACTIVITY	ALA-D(+DTT) ACTIVITY ($\times 10^4$ μmol PBG/hr/mg prot)
3	0	6.8 $\pm$ 1.0	6.5 $\pm$ 0.1	100	8.5 $\pm$ 1.4
	50	5.2 $\pm$ 1.5	6.4 $\pm$ 0.1	77	9.3 $\pm$ 2.3 *
	100	4.4 $\pm$ 1.1	6.1 $\pm$ 0.1	65	10.6 $\pm$ 1.2
	1000	2.3 $\pm$ 0.3	6.1 $\pm$ 0.1	34	9.9 $\pm$ 1.1
7	0	5.0 $\pm$ 1.2	6.6 $\pm$ 0.1	100	6.4 $\pm$ 1.6
	50	3.9 $\pm$ 0.8	6.2 $\pm$ 0.1	75	7.9 $\pm$ 1.8
	100	3.4 $\pm$ 0.9	6.2 $\pm$ 0.1	66	8.0 $\pm$ 1.6
	1000	1.9 $\pm$ 0.6	6.1 $\pm$ 0.1	36	8.0 $\pm$ 1.7
21	0	3.7 $\pm$ 0.4	6.4 $\pm$ 0.1	100	4.0 $\pm$ 0.9
	50	2.9 $\pm$ 0.4	6.2 $\pm$ 0.1	78	5.1 $\pm$ 0.9
	100	2.1 $\pm$ 0.2	6.2 $\pm$ 0.1	58	5.1 $\pm$ 0.1
	1000	1.7 $\pm$ 0.3	6.2 $\pm$ 0.1	46	6.4 $\pm$ 0.4
35	0	3.2 $\pm$ 1.1	6.5 $\pm$ 0.2	100	4.4 $\pm$ 2.4
	50	2.1 $\pm$ 0.2	6.2 $\pm$ 0.2	67	4.1 $\pm$ 0.8 *
	100	2.1 $\pm$ 0.4	6.1 $\pm$ 0.1	65	5.0 $\pm$ 1.2 *
	1000	1.6 $\pm$ 0.4	6.1 $\pm$ 0.1	49	5.5 $\pm$ 0.7 *

* : no significant difference from control value

TABLE 3a - Erythrocyte ALA-D Activity in rats exposed to 0, 50, 100 and 1000 ppm lead in their drinking water for 3, 7, 21 and 35 days. Activity measured at pH 6.4.

DURATION OF DOSE (DAYS)	LEAD DOSE (ppm)	ALA-D ACTIVITY ($\times 10^4$ μ mol PBG/ hr/mg prot)	PERCENT OF CONTROL ACTIVITY
3	0	6.6 $\pm$ 1.1	100
	50	5.1 $\pm$ 1.5	78
	100	4.0 $\pm$ 1.0	60
	1000	1.8 $\pm$ 0.3	27
7	0	4.7 $\pm$ 1.1	100
	50	3.7 $\pm$ 0.7	79
	100	3.0 $\pm$ 0.6	64
	1000	1.5 $\pm$ 0.6	32
21	0	3.5 $\pm$ 0.3	100
	50	2.6 $\pm$ 0.3	76
	100	1.8 $\pm$ 0.1	51
	1000	1.3 $\pm$ 0.1	37
35	0	2.8 $\pm$ 0.7	100
	50	2.0 $\pm$ 0.2	71
	100	1.9 $\pm$ 0.4	69
	1000	1.2 $\pm$ 0.2	43

increase in the restored level of ALA-D, over and above the generally higher ALA-D(+DTT) levels in lead exposed samples as compared to controls. Figure 12 gives ALA-D(+DTT) activity with duration of each lead dose. The restored activity is taken at the pH maximum which generally occurred at pH 6.3. ALA-D(+DTT) activity was increased in the 50 ppm lead exposed samples over controls at the 7 day time point. It remained higher until the 35 day time point where there was no difference between lead-dosed and control blood samples. The 100 and 1000 ppm lead exposed samples were similar, except that they showed an ALA-D(+DTT) activity greater than ALA-D activity occurring earlier, at the 3 day time point. As seen in Figure 7, the pH optimum for erythrocyte ALA-D generally occurred in control samples at 6.5. The 50 ppm lead-exposed samples showed a slight shift in this optimum to lower values at 3 days on lead. This shift gradually increased with the duration of lead exposure to give a final pH shift of 0.3 units at 35 days. The 100 and 1000 ppm dosed samples showed an immediate downward shift of 0.3 to 0.4 pH units which was constant for the duration of exposure.

ALA-D activity, both restored and nonrestored, in kidney samples is quoted at the pH optimum for the enzyme and given in Table 4. ALA-D activity in the kidney samples was also inhibited by lead exposure and showed a 0.2 to 0.3 downward shift in the pH optimum in lead-exposed samples from the control value of 6.5. Figure 9 illustrates this change in pH optimum with lead dose and duration of exposure. The ALA-D activity as a function of time and lead dose is given in Figure 13. The levels of ALA-D activity at the three day time point were 81 (± 8), 54 (± 7) and 44 (± 4)% of controls for the 50, 100 and 1000 ppm

TABLE 4 - Kidney ALA-D Activity (restored and nonrestored) in rats exposed to 0, 50, 100 and 1000 ppm lead in their drinking water for 3, 7, 21 and 35 days..

DURATION OF DOSE (DAYS)	LEAD DOSE (ppm)	ALA-D ACTIVITY (x10 ³ umol PBG/ 2hr/mg prot)	pH OPTIMUM	PERCENT OF CONTROL ACTIVITY	ALA-D(+DTI) ACTIVITY (x10 ³ umol PBG/ 2hr/mg prot)	
3	0	5.4 ± 0.5	6.5 ± 0.1	100	6.9 ± 0.9	*
	50	4.4 ± 0.8	6.2 ± 0.1	81	6.7 ± 1.3	*
	100	2.9 ± 0.3	6.2 ± 0.1	54	5.8 ± 1.2	*
	1000	2.4 ± 0.6	6.2 ± 0.1	44	6.8 ± 1.3	*
7	0	5.2 ± 1.0	6.7 ± 0.3	100	6.8 ± 2.4	*
	50	3.1 ± 0.8	6.2 ± 0.1	66	6.3 ± 2.0	*
	100	2.8 ± 0.9	6.2 ± 0.1	60	6.0 ± 2.0	*
	1000	2.2 ± 0.7	6.2 ± 0.1	46	6.0 ± 2.0	
21	0	4.5 ± 0.3	6.4 ± 0.2	100	5.6 ± 0.3	*
	50	3.6 ± 0.5	6.3 ± 0.1	80	6.2 ± 0.9	*
	100	3.1 ± 0.2	6.2 ± 0.1	68	5.9 ± 0.9	*
	1000	2.4 ± 0.3	6.2 ± 0.1	52	5.6 ± 1.2	*
35	0	4.9 ± 0.4	6.4 ± 0.1	100	6.3 ± 0.4	
	50	4.5 ± 0.5	6.2 ± 0.1	91	7.3 ± 0.7	
	100	4.4 ± 1.1	6.2 ± 0.1	90	7.7 ± 1.8	
	1000	3.0 ± 0.4	6.2 ± 0.1	61	7.0 ± 0.8	

* : no significant difference from control values

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dose levels respectively. The degree of inhibition was relatively constant until 35 days. At this point the 50 and 100 ppm dosed samples showed an increase in ALA-D activity so that they showed no difference from control values. The activity for the 1000 ppm exposed samples also increased at this point though not back to control levels. These increases in activity, with no change in the lead concentration in the organ, correspond to an increased ALA-D(+DTT) activity (as compared to controls) in all three dose levels. Unlike the blood samples, the ALA-D(+DTT) activity in lead-exposed kidney samples was generally below or indistinguishable from the controls until the 35 day time point. Restored ALA-D activity with time and lead dose is given in Figure 14.

Table 5 gives the ALA-D activity at maximum pH values for liver samples from control and lead-exposed rats. Figure 15, giving ALA-D activity with time at each lead dose, indicates that lead has had little effect on hepatic ALA-D activity. Only the 3 day, 1000 ppm dosed sample had a statistically significant decrease from control. Small shifts of about 0.2 to 0.3 units in the pH optimum occurred in the ALA-D activity of samples given 100 ppm lead for 3 and 35 days and 1000 ppm lead for 3, 21 and 35 days. The control values had a pH optimum of 6.5. Figure 8 illustrates the effect of lead on the maximum pH of hepatic ALA-D activity.

Brain ALA-D activity values are given in Table 6. They also show little difference between lead-exposed and control samples as indicated by Figure 16. The pH optimum of ALA-D of brains from control samples occurs at about 6.5 to 6.7 but is difficult to estimate because of the flat pH profiles (shown in Figure 10). Some small differences from control values for pH optimum and ALA-D activity were seen but these appear to be random occurrences and not dose-related.

TABLE 5 - Liver ALA-D Activity in rats exposed to 0, 50, 100 and 1000 ppm lead in their drinking water for 3, 7, 21 and 35 days.

DURATION OF DOSE (DAYS)	LEAD DOSE (ppm)	ALA-D ACTIVITY ($\times 10^3$ μ mol PBG/hr/mg prot)	pH OPTIMUM	PERCENT OF CONTROL ACTIVITY
3	0	6.0 $\pm$ 0.8	6.5 $\pm$ 0.1	100
	50	6.2 $\pm$ 1.2	6.4 $\pm$ 0.2	103
	100	5.8 $\pm$ 0.8	6.3 $\pm$ 0.1 *	96
	1000	5.0 $\pm$ 0.4 *	6.2 $\pm$ 0.1 *	83
7	0	5.1 $\pm$ 1.1	6.4 $\pm$ 0.2	100
	50	5.7 $\pm$ 1.0	6.5 $\pm$ 0.2	113
	100	5.2 $\pm$ 0.5	6.3 $\pm$ 0.2	98
	1000	5.1 $\pm$ 0.7	6.3 $\pm$ 0.1	101
21	0	5.6 $\pm$ 1.2	6.5 $\pm$ 0.1	100
	50	5.9 $\pm$ 1.2	6.4 $\pm$ 0.1	105
	100	5.5 $\pm$ 1.3	6.4 $\pm$ 0.2	98
	1000	5.2 $\pm$ 0.8	6.2 $\pm$ 0.1 *	92
35	0	5.5 $\pm$ 1.6	6.5 $\pm$ 0.2	100
	50	4.8 $\pm$ 0.9	6.4 $\pm$ 0.2	87
	100	4.6 $\pm$ 1.2	6.3 $\pm$ 0.1 *	85
	1000	4.7 $\pm$ 1.5	6.2 $\pm$ 0.1 *	86

* : significant difference from control values

TABLE 6 - Brain ALA-D Activity in rats exposed to 0, 50, 100 and 1000 ppm lead in their drinking water for 3, 7, 21 and 35 days.

DURATION OF DOSE (DAYS)	LEAD DOSE (ppm)	ALA-D ACTIVITY (x103 μ mol PBG/ 2hr/mg prot)	pH OPTIMUM	PERCENT OF CONTROL ACTIVITY
3	0	1.1 $\pm$ 0.1	6.6 $\pm$ 0.3	100
	50	1.0 $\pm$ 0.2	6.4 $\pm$ 0.1	89
	100	0.9 $\pm$ 0.2 *	6.4 $\pm$ 0.1 *	81
	1000	1.0 $\pm$ 0.1 *	6.4 $\pm$ 0.1	89
7	0	1.1 $\pm$ 0.1	6.7 $\pm$ 0.4	100
	50	1.0 $\pm$ 0.1	6.6 $\pm$ 0.3	93
	100	1.0 $\pm$ 0.1 *	6.5 $\pm$ 0.2	91
	1000	1.0 $\pm$ 0.2	6.3 $\pm$ 0.1 *	91
21	0	1.0 $\pm$ 0.1	6.5 $\pm$ 0.2	100
	50	0.9 $\pm$ 0.2	6.5 $\pm$ 0.1	95
	100	1.0 $\pm$ 0.2	6.5 $\pm$ 0.1	95
	1000	0.9 $\pm$ 0.2	6.7 $\pm$ 0.1 *	90
35	0	1.3 $\pm$ 0.3	6.7 $\pm$ 0.2	100
	50	1.2 $\pm$ 0.3	6.4 $\pm$ 0.1 *	92
	100	1.2 $\pm$ 0.1	6.6 $\pm$ 0.1	92
	1000	1.0 $\pm$ 0.2	6.4 $\pm$ 0.1 *	77

* : significant difference from control values

FIGURE 3 - Lead concentration in liver samples from rats given lead acetate in their drinking water at 0, 50, 100 and 1000 ppm for 5 weeks.

FIGURE 4 - Lead concentration in kidney samples from rats given lead acetate in their drinking water at 0, 50, 100 and 1000 ppm for 5 weeks.

FIGURE 5 - Lead concentration in brain samples from rats given lead acetate in their drinking water at 0, 50, 100 and 1000 ppm for 5 weeks.

FIGURE 6 - Lead concentration in blood samples from rats given lead acetate in their drinking water at 0, 50, 100 and 1000 ppm for 5 weeks.

FIGURE 7a - ALA-D activity vs reaction buffer pH for blood samples from rats dosed at 0, 50, 100 and 1000 ppm lead for 3 days.

FIGURE 7b - ALA-D activity vs reaction buffer pH for blood samples from rats dosed at 0, 50, 100 and 1000 ppm lead for 7 days.

FIGURE 7c - ALA-D activity vs reaction buffer pH for blood samples from rats dosed at 0, 50, 100 and 1000 ppm lead for 21 days.

FIGURE 7d - ALA-D activity vs reaction buffer pH for blood samples from rats dosed at 0, 50, 100 and 1000 ppm lead for 35 days.

FIGURE 8a - ALA-D activity vs reaction buffer pH for liver samples from rats dosed at 0, 50, 100 and 1000 ppm lead for 3 days.

FIGURE 8b - ALA-D activity vs reaction buffer pH for liver samples from rats dosed at 0, 50, 100 and 1000 ppm lead for 7 days.

FIGURE 8c - ALA-D activity vs reaction buffer pH for liver samples from rats dosed at 0, 50, 100 and 1000 ppm lead for 21 days.

FIGURE 8d - ALA-D activity vs reaction buffer pH for liver samples from rats dosed at 0, 50, 100 and 1000 ppm lead for 35 days.

FIGURE 9a - ALA-D activity vs reaction buffer pH for kidney samples from rats dosed at 0, 50, 100 and 1000 ppm lead for 3 days.

FIGURE 9b - ALA-D activity vs reaction buffer pH for kidney samples from rats dosed at 0, 50, 100 and 1000 ppm lead for 7 days.

FIGURE 9c - ALA-D activity vs reaction buffer pH for kidney samples from rats dosed at 0, 50, 100 and 1000 ppm lead for 21 days.

FIGURE 9d - ALA-D activity vs reaction buffer pH for kidney samples from rats dosed at 0, 50, 100 and 1000 ppm lead for 35 days.

FIGURE 10a - ALA-D activity vs reaction buffer pH for brain samples from rats dosed at 0, 50, 100 and 1000 ppm lead for 3 days.

FIGURE 10b - ALA-D activity vs reaction buffer pH for brain samples from rats dosed at 0, 50, 100 and 1000 ppm lead for 7 days.

FIGURE 10c - ALA-D activity vs reaction buffer pH for brain samples from rats dosed at 0, 50, 100 and 1000 ppm lead for 21 days.

FIGURE 10d - ALA-D activity vs reaction buffer pH for brain samples from rats dosed at 0, 50, 100 and 1000 ppm lead for 35 days.

FIGURE 11 - Erythrocyte ALA-D activity with time of exposure (3, 7, 21 and 35 days) at 0, 50, 100 and 1000 ppm lead dose levels.

FIGURE 12 - Restored erythrocyte ALA-D activity with time of lead exposure (3, 7, 21 and 35 days) at 0, 50, 100 and 1000 lead dose levels.

FIGURE 13 - Kidney ALA-D activity with time of lead exposure (3, 7, 21 and 35 days) at 0, 50, 100 and 1000 ppm Lead dose levels.

FIGURE 14 - Restored kidney ALA-D activity with time of lead exposure (3, 7, 21 and 35 days) at 0, 50, 100 and 1000 ppm lead dose levels.

FIGURE 15 - Liver ALA-D activity with time of lead exposure (3, 7, 21 and 35 days) at 0, 50, 100 and 1000 ppm lead dose levels.

FIGURE 16 - Brain ALA-D activity with time of lead exposure (3, 7, 21 and 35 days) at 0, 50, 100 and 1000 ppm lead dose levels.

DISCUSSION

In this study, rats were exposed to low levels of lead (up to 1000 ppm) in their drinking water for up to five weeks. Lead concentrations and d-Aminolevulinic Acid Dehydratase (ALA-D) activity were measured in the blood, liver, kidney and brain from animals exposed to lead at 0, 50, 100 and 1000 ppm dose levels.

The kidney was found to accumulate the highest concentration of lead (per gram protein) as noted by other authors.(39-41) The kidney plays a major role in the in vivo metabolism of lead and is considered a "target tissue" for lead in the body.(5,40,42) Long-term exposure of the kidney to lead can result in the development of tubular nephropathy.(5,42) Liver and brain were found to accumulate lead by as early as the third day of lead exposure for most tissue samples and by the 21st day for all samples at all levels of lead exposure. This contradicts the findings of Buchet et al (39) who found no significant increase in lead concentrations in brain or liver samples from rats given up to 1000 ppm lead for three weeks. Only after 6 weeks was there an increase in lead concentration in the 1000 ppm dosed liver samples. Lead levels in kidney samples from 100 ppm lead-exposed animals in Buchet's study were increased at three and six weeks to a level equal to those found in this study. The levels of lead found in the 1000 ppm exposed kidney samples at three weeks though, were higher than those found in this study and by six weeks had increased even further to give a lead concentration of approximately three times that found in this study. One possible reason for differences in lead data

between the two studies could be due to the different techniques used in lead analysis. In this study, a solid sampling technique was used for the determination of lead concentrations in freeze-dried liver, kidney and brain samples as opposed to the digestion technique used in Buchet's study. The solid sampling should give less interference from extraneous lead and therefore both lower values for lead concentration and more precise values. Blood lead levels, measured using essentially the same analytical technique, were similar in the two studies. Miller (19) and Mykkanen (41), in experiments with infant animals given lead since birth, found a higher concentration of lead in brain samples than those found here. The concentrations of lead in the kidney were slightly lower and in liver were approximately the same as those found in this study. Their results suggest a difference in the distribution of lead in young animals as compared to adults. It is possible the brain accumulates a higher percentage of the lead absorbed by infant animals. This might be one reason (along with the fact that systems are still in the developmental stage) why the effects of lead on the brain are more pronounced in the young.

The concentration of lead in the blood and tissues of lead-exposed animals remained relatively constant over the five week study. Mykkanen et al (41) observed that lead concentrations in liver, kidney and brain samples from rats given lead since birth were maintained relatively unchanged throughout adult life. The lead concentration in blood samples was found by Buchet to plateau after four days of exposure to lead concentrations of 1000 ppm and below as observed in this study after three days of lead exposure. Fujita et al (35) also observed that blood lead levels in rats given lead at greater

concentrations given here, remained unchanged from that observed at four days of lead exposure.

Lead is assumed to be absorbed continuously from the gastrointestinal tract throughout the day which is superimposed on its constant excretion. This would prevent the appearance of peak values in the blood lead levels. The intake and output of lead is very balanced in normal individuals with excretion being very close to the amount absorbed.(43) In industrial societies, in subjects without occupational exposure, the total body burden of lead increases with age from about 2 mg in children to 200 mg in the elderly.(44) These small quantities of lead are stored in relatively inert form in bone. Schlick et al (45) postulate that the soft tissues (kidney, liver, etc) serve as a temporary store for lead in order to allow its quick removal from the blood. Lead is then later redistributed, with one part being directly excreted and the other being stored in the bones as a long term site of deposit. In studies where acute lead doses were given intravenously (45,46), the blood lead concentrations fell rapidly to normal whereas liver and kidney lead levels were slower to return to normal. Elimination of lead from brain samples was found to be slower than that found in liver and kidney samples. Bone lead remained raised for a longer period. If blood lead was being decreased by distribution to both soft tissues and bone for long-term storage, one would expect an increasing accumulation in the soft tissues with chronic exposure to lead. The tissues as well as the blood only appear to indicate on any given day, the level of lead exposure that day and therefore only show recent exposure to lead. In order to determine the total body burden of lead, it appears that one would have to measure lead levels in the

skeleton.

Blood sampling is used as a means of monitoring an individual's exposure to lead with blood lead levels and ALA-D activity being relatively easy to measure. There is considerable interest in determining which measure (if either), is a good method of determining the degree of lead exposure (both dose and duration) and the effects of lead exposure. Erythrocyte lead levels and ALA-D activities from samples of all doses and time periods covered in the study were plotted against each other and against the same parameters in tissue samples. The correlation coefficients are given in Table 7. As can be seen in Figure 17, ALA-D activity in blood decreases with increased blood lead concentration. As observed by other authors (39) there is a negative correlation between ALA-D activity and the logarithmic of blood lead, although the degree of correlation is not much better than found for activity vs blood lead. ALA-D activity is plotted against the logarithm of blood lead in Figure 18. A better correlation, as noted earlier in the Introduction section, is obtained by relating the ratio of restored to nonrestored activity (seen in Figure 19). Only a slightly better correlation than that obtained for ALA-D activity alone is obtained for the ratio of nonrestored activity at different pH values (pH 7.2/pH 6.4, seen in Figure 20). Blood lead levels relate well to liver and kidney lead concentrations but slightly less well to brain lead levels. These relationships are plotted in Figures 21, 22 and 23. The ratio of restored to nonrestored ALA-D activity in blood correlates to lead concentrations in tissues to approximately the same degree as the blood lead levels did (seen in Figures 24, 25 and 26), while erythrocyte ALA-D activity alone does not (seen in Figures 27, 28

TABLE 7 - Relationship of blood lead and ALA-D activity levels with each other and with tissue levels.

Factors Correlated	Correlation Coefficient
Erythrocyte ALA-D, Blood Lead	0.62
Erythrocyte ALA-D, Log Blood Lead	0.68
Restored:Nonrestored Activity, Blood Lead	0.87
Activities at pH 6.4:pH 7.2, Blood Lead	0.70
Blood Lead, Liver Lead	0.87
" , Kidney Lead	0.89
" , Brain Lead	0.63
Erythrocyte ALA-D, Liver Lead	0.61
" , Kidney Lead	0.59
" , Brain Lead	0.43
Restored:Nonrestored Activity, Liver Lead	0.83
" , Kidney Lead	0.86
" , Brain Lead	0.60
Erythrocyte ALA-D, Lead Dose (at 3 days)	0.76
Blood Lead, Lead Dose (at 3 days)	0.92

FIGURE 17 - Erythrocyte ALA-D activity vs lead concentration in the blood.

FIGURE 18 - Erythrocyte ALA-D activity vs the logarithm of the blood lead concentration.

FIGURE 19 - Ratio of restored ALA-D activity to nonrestored activity in blood vs blood lead concentration.

FIGURE 20 - Ratio of ALA-D activity at pH 6.4 to activity at pH 7.2 in blood vs blood lead concentration.

FIGURE 21 - Blood lead concentration vs liver lead concentration.

FIGURE 22 - Blood lead concentration vs kidney lead concentration.

FIGURE 23 - Blood lead concentration vs brain lead concentration.

FIGURE 24 - Ratio of restored to nonrestored ALA-D activity in blood vs liver lead concentration.

FIGURE 25 - Ratio of restored to nonrestored ALA-D activity in blood vs kidney lead concentration.

FIGURE 26 - Ratio of restored to nonrestored ALA-D activity in blood vs brain lead concentration.

FIGURE 27 --Erythrocyte ALA-D activity vs liver lead concentration.

FIGURE 28 - Erythrocyte ALA-D activity vs kidney lead concentration.

and 29).

As was found for the lead concentrations in blood and tissues, the degree of inhibition of erythrocyte ALA-D activity remained relatively constant over the five week study. It therefore also appears not to reflect the duration of an individual's exposure to lead, but rather the level of the lead dose. Both erythrocyte ALA-D activity and lead levels relate to the size of the lead dose with blood lead concentration giving a much better correlation. The effects of lead on blood and kidney ALA-D appear to change with time due to compensatory mechanisms present in these tissues (to be discussed later in this section). This will probably have a limiting effect on the correlations of blood ALA-D activity with other parameters. The degree of correlation will decrease as the duration of exposure increases.

The optimum pH of the erythrocyte ALA-D activity was found to shift to a more acid pH. It is possible that the binding of lead to an allosteric site on ALA-D causes conformational changes which lower the pH optimum for enzyme activity. Almost all authors have measured ALA-D activity at a single pH, generally pH 6.4.(12) By doing so, changes in the activity level may seem more or less pronounced than actual because a pH shift has caused the pH maximum to shift to a more acid pH. The activity is therefore being measured at a different point on the activity vs pH curve (ie not necessarily at the maximum). In this study, when the pH optimum shifted only slightly (for the samples exposed to 50 ppm lead for three days, for example) measuring at pH 6.4 gives a slightly less inhibited activity because the maximum of the activity vs pH curve has moved to pH 6.4. Where the curve shifted further (for higher dose levels), the enzyme seems slightly more

inhibited because the maximum has shifted past pH 6.4 and one would be measuring the activity at a low point on the activity vs pH curve. These errors would probably increase with higher lead levels where greater inhibition of the enzyme is occurring. In human samples where the pH curves are generally steeper, the error caused may be higher than those found here. Another advantage of measuring the activity over a pH range is that the shift in pH may occur more gradually than the inhibition of enzyme activity as was observed in the blood samples from animals exposed to 50 ppm lead. This might give more information as to the duration of the exposure to lead where the degree of inhibition does not.

Lead, while inhibiting ALA-D enzyme activity, has been found to increase the synthesis of the enzyme in bone marrow resulting in its increased activity in the peripheral blood. (34-38) In all previous studies, animals were given very high doses of lead relative to those given in this study. Maes et al (34) noted an increased ALA-D activity and spleen weight in young rats given lead since birth. Lead treatment shortened the survival of erythrocytes, possibly due to membrane damage, and their increased destruction resulted in the increased organ size. More reticulocytes (immature erythrocytes) are therefore present in the blood circulation. ALA-D activity diminishes with the age of the erythrocyte and therefore increased reticulocyte levels give an increased ALA-D activity. Later studies (35,37,38) done on older adult rats found no such increase in reticulocyte level with lead poisoning. Using both enzyme activity (nonrestored and restored) and radioimmunoassay technique, an increase in the activity of restored ALA-D in lead-exposed animals over controls was found to be due to an

increased rate of synthesis of the enzyme in the bone marrow with no increase in numbers of blood cells or changes in the blood profile. It has been generally believed that ALA-D has a large capacity to counter change in its activity level since heme synthesis itself is not affected by low lead levels which do inhibit ALA-D. Prolonged lead exposure must trigger some mechanism which results in the increased synthesis of ALA-D in bone marrow by at least the fourth day of exposure. The amount of erythrocyte ALA-D begins to increase on the ninth day of lead exposure with the lag time being explained by the time required (4-7 days) for the appearance of mature erythrocytes in the peripheral blood.(35)

In this present study low lead doses, very much lower than those used in the previously mentioned studies, were also found to increase the ALA-D activity in blood samples from lead-exposed rats compared to those from control animals. This was evidenced by the higher level of restored ALA-D activity in blood samples from lead-exposed rats as compared to the restored levels in controls. The restored activity is the level of activity that would be present if there was no lead inhibition of ALA-D occurring and is usually be taken as representing the actual amount of enzyme present assuming the same specific activity in lead-exposed and control blood samples.

The restored activity in blood samples from lead-dosed animals was generally higher than the restored activity in control samples until the 35th day of exposure except in the 50 ppm dosed samples which did not show an increase in restored ALA-D activity until the 7 day time point. By the 35th day, there was no difference in the level of restored ALA-D activity between lead-exposed and control blood samples.

The increase in ALA-D appears to occur earlier than one would expect, that is, before the usual time required for mature erythrocytes to appear in the peripheral blood from the bone marrow. One possible explanation for this is that lead is having an effect on cells in the bone marrow during the late stages of synthesis where they are soon to be released into the blood. At this stage they still have a nucleus and may therefore be capable of enzyme synthesis. This could cause the small increase in restored ALA-D activity observed. Fujita et al (35) observed a 1.5 to 2 fold increase in erythrocyte ALA-D. Except for the 1000 ppm samples exposed to lead for 21 days, that magnitude of increase was not reached in the samples in this study. At this time point, cells that were affected by lead from the beginning of their synthesis would be present in the peripheral blood. At least two mechanisms of increased ALA-D in the blood must be present. One mechanism which causes a very small increase in the amount of ALA-D, while the other causes a relatively large increase due to the presence of cells which contain a higher amount of ALA-D due to its increased synthesis in the bone marrow. The latter appears to occur only with the highest lead level used in this study and must therefore be triggered when inhibition of the enzyme reaches a certain level. The fact that the restored activity decreases at 35 days appears to indicate that any form of increase in the amount of ALA-D is limited. This has been noted by Fujita et al (35) where a maximal increase in ALA-D in rat erythrocytes of about 2 fold was observed.

The increased synthesis of ALA-D partially compensates for the inhibition of ALA-D by lead. This is evident in the 1000 ppm samples exposed to lead for 35 days. The ALA-D activity in these samples

were actually less inhibited than was evident at the earlier 3 day time point. The small increase in ALA-D did not appear to significantly effect the amount of inhibition present in the 50 and 100 ppm lead-exposed blood samples. ALA-D remains inhibited to a relatively constant degree with exposure to constant lead levels in the five week study.

An increased amount of ALA-D due to lead exposure has also been observed in humans.(36) The increase in the enzyme was presumably also due to increased synthesis of the enzyme by blood cells in the bone marrow and not due to increases in the reticulocyte count since there was no observable difference in hematocrit or hemoglobin levels between non- and lead-exposed individuals. These increases were observed for blood lead levels as low as 10 and 20 $\mu\text{g}/100\text{ mL}$ and are therefore possible the most sensitive indicator of lead exposure. Up to blood lead levels of 40 $\mu\text{g}/100\text{ mL}$, increased ALA-D synthesis partially compensated for the inhibition of the enzyme such that it appeared to be at 40% of control levels when in fact 85% of the ALA-D content of the blood samples was inhibited. Above 40 $\mu\text{g}/100\text{ mL}$ blood lead concentrations, the increase in the amount of ALA-D reached a plateau. At the same time as ALA-D inhibition could no longer be compensated for urinary excretion of ALA increased. It would appear then that the apparent capacity for ALA-D to absorb changes in its activity and therefore the capacity of the heme biosynthesis pathway to be unaffected by changes in the activity of ALA-D is due to the cells limited ability to increase the synthesis of the enzyme. Erythrocyte ALA-D may therefore be of some use as a biological monitor of the effects of lead.

Liver ALA-D activity, as can be seen in Figure 30 which represents the activity vs liver lead concentration, appeared to show no significant effects of lead exposure. The pH optimums of the various samples though, were found to have shifted to more acid values. These results may occur due to the high amount of variation in ALA-D activity between liver samples. This variation makes it difficult to show clearly an effect of lead on activity above the background. The shifts in the pH though, would indicate that lead is having an effect on liver ALA-D. Buchet et al (39) also found no effect of lead on ALA-D activity in liver samples from animals given the same dose levels as those given in this study. Schlick et al (45) found only a slight inhibition of liver ALA-D compared to that found in other tissues in mice given low lead doses in their drinking water. A possible explanation given for the lower inhibition of liver ALA-D is the presence of a specific lead binding protein which inactivates the lead biologically. This explanation is not consistent with the fact that there is no increasing accumulation of lead in the liver unless the protein-lead complex is very short-lived. Kao et al (47) found ALA-D to be inhibited in studies where rats were given lead doses (one dose only) higher than those given in this study. The magnitude of inhibition was much less than that found in blood and kidney samples. The decrease in the activity was found to occur very quickly (within six hours) as occurred in the other tissues, but returned to control values within two days of dosing. It is possible then that the ALA-D activity had returned to normal by the 3 day time point in this study. This may be suggested by the fact that the 1000 ppm liver samples, dosed for 3 days, were slightly inhibited from controls. Earlier

Figure 30 - Liver ALA-D activity vs lead concentration in liver.

sampling may have showed a trend of increasing activity from some point before 3 days until controls levels are reached by the 7th day. With very high doses, the level of liver ALA-D actually "overshot" the control values to give an increased activity in lead exposed samples 2 days after the lead dose.(47) The levels of lead found in the liver samples from the 35 ppm lead-exposed animals only approached the levels found in the kidney samples from control rats. It is possible that the livers simply did not accumulate enough lead to cause a significant inhibition of ALA-D in that tissue. Millar et al (19) found a large decrease in ALA-D activity (approximately 30% of control levels) in suckling rats exposed to lead. The levels of lead found in the liver, as stated earlier, were similar to those found in this study. This might indicate an increased sensitivity to lead in young animals as compared to adults.

The effect of lead on ALA-D activity in brain was also minimal. Figure 31 representing activity vs lead concentration, there appears to be no change in ALA-D activity with increased lead levels in the brain. The levels of ALA-D in brain are extremely low which makes it difficult to observe small changes in its activity. The effect of lead on the pH optimum of the ALA-D activity is also difficult to assess due to the "flatness" of the activity vs pH curves. There is a large amount of error in attempting to assign a maximum pH value to the ALA-D activity in brain samples. Buchet et al (39) found no effect of lead on ALA-D in brain samples from animals given similar lead doses. The low levels of lead accumulated in the brain, even at the highest doses, may not have been sufficient to cause a decrease in ALA-D activity. Millar et al (19) observed a large inhibition, about 90%, in lead exposed samples

FIGURE 31 - Brain ALA-D activity vs lead concentration in brain.

from control values in suckling rats. As stated earlier, a larger proportion of lead was found to accumulate in the brains of infant rats. The high inhibition of brain ALA-D may be due to the higher concentration of lead present and/or an increased sensitivity of the enzyme in younger animals.

Figure 32 illustrates the relationship between the ALA-D activity in kidney samples to the concentration of lead in that tissue. The enzyme in kidney, like that in blood, is inhibited by lead. The degree of inhibition was related to the size of the lead dose and was relatively constant until the 35th day. An increase in the restored level of ALA-D activity over control values occurred at the 35 day time point for all dose levels indicating an increase in the level in the enzyme. This increased amount of enzyme caused a decrease in the observed amount of inhibition and this compensation resulted in the ALA-D activity in the 15 and 100 ppm lead-dosed samples to return to control levels. The increase in enzyme did not completely compensate for lead inhibition in the 1000 ppm samples. This transitory inhibition of kidney ALA-D by lead was also observed by Buchet et al (39) where inhibition present in samples exposed in samples to low lead levels was reduced by the sixth week of lead exposure. Fowler et al (40) found no effect of lead on ALA-D in kidney samples from rats exposed to low lead levels for nine months.

FIGURE 32 - Kidney ALA-D activity vs lead concentration in kidney.

CONCLUSIONS

The effects of low levels of lead on ALA-D activity and lead levels in the blood, liver, kidney and brain samples over a chronic (5 week) dosing period have been documented in this study.

Both blood lead levels and ALA-D activity have been examined as methods of monitoring exposure to lead. The concentration of lead in the blood has been shown to represent recent exposure to lead and to be dependent on the size of the lead dose. Blood lead levels cannot accurately reflect the total body burden of lead since they do not relate well to the duration of exposure. Blood ALA-D activity has been examined as a biological monitor of lead exposure. The degree of inhibition of ALA-D activity does relate to blood lead levels, with activity decreasing with increased lead levels. The ratio of restored ALA-D activity (via incubation of the enzyme with DTT and Zn^{+2}) to nonrestored activity correlates reasonably well with blood lead levels. A compensatory mechanism whereby ALA-D levels are increased, occurs over the time period examined. The correlation of ALA-D activity to blood levels therefore becomes increasingly poorer as the length of chronic exposure increase and is therefore of limited use. The changes in the synthesis of ALA-D, an enzyme required in heme biosynthesis, may be of use as an indicator of the biological effects of low lead levels. Blood lead concentrations more accurately reflect the size of the lead dose and the levels of lead in the liver and kidney. The lead levels however do not necessarily correlate to the biological effects of lead in these tissues. This study cannot fully address the question of

which form of monitoring is more significant.

The significance of exposure to low levels of lead, which generally result in blood lead levels below that at which clinical effects are observed, is unknown. The finding of constant lead levels in the blood and soft organs over the course of this study combined with the results of other workers, indicate that these tissues in adult animals can rapidly eliminate lead from themselves. This elimination of lead, coupled with the compensatory mechanisms present in both blood and kidney, indicates that the body deals well with low levels of lead, and raises questions concerning the biological effects (in adults) of low lead doses. A study concerned with the levels of lead and ALA-D activity during longer periods of lead exposure and following the discontinuation of lead feeding is needed. This type of study would aid in determining the duration of exposure, if any, required for low lead doses to begin to have detrimental biological effects and whether removal of an individual from a lead environment will return tissue lead and ALA-D activity to normal levels and decrease the total lead burden.

MATERIALS AND METHODS

All solutions were made with deionized water (Milli-Q System, Millipore Corp., Toronto, Ont.). All glassware used was soaked in 30 % nitric acid (Canlab, Ottawa, Ont.) for two days and rinsed five times with deionized water.

In Vivo Lead Dosing and Sample Preparation

Male Sprague Dawley rats (Canadian Breeding Farm and Sprague Dawley Laboratories Ltd., St. Constant, Que.), of initial body weight between 200 and 270 grams were used in the experiment. In order to allow time for the sacrifice of animals, processing of tissues and ALA-D assay on fresh blood samples, the experiment was done in parts. At each time point at least one animal given no lead was processed along with the lead dosed rats to serve as a control. Lead was administered in the drinking water, which, along with laboratory chow (Purina Rat Chow, Ritchie Feed and Seed, Ottawa, Ont.), was available to the animals ad libitum. The lead, as lead acetate (AnalaR, British Drug House Chemicals Ltd., Toronto, Ont.), was dissolved to the appropriate concentration in one litre of deionized water with the aid of three to four drops of glacial acetic acid (Baker Analyzed Reagent, J.T. Baker Chemical Co., Phillipsburg, N.J.). The control animals received acidified water only. The drinking water was changed every two days.

The rats were anaesthetised with ether and a blood sample taken by

cardiac puncture using a 10 mL syringe (Becton Dickenson and Co., Rutherford, N.J.) that had previously been rinsed with a heparin/saline solution (heparin - Sigma Chemical Co., St. Louis, MO; NaCl - AnalaR, BDH). The blood was put into a polystyrene tube (2057 Falcon Company, Oxnard CA) containing three to four milligrams of sodium heparin (168 units/mg) and stored on ice. One millilitre of the heparin/saline solution was injected into the heart, the thorax opened and the animal perfused with 150 mL of ice cold isotonic saline via the left ventricle (drainage via the right atrium). The liver, kidney and brain were removed and kept in saline on ice until processing.

The tissues were weighed and homogenized in 0.05 M Tris(hydroxymethyl)aminomethane (AnalaR, BDH) buffer (pH 7.2) using a Brinkmann polytron homogenizer (Brinkmann Instruments) for 30° to 40, 20 to 30 and 10 to 20 seconds on liver, kidney and brain samples respectively, followed by ten strokes using a glass mortar and pestle (Polter Elvehjeim, Fisher Scientific Ltd, Ottawa, Ont.). Buffer was added to give proportions of buffer to tissue of 5 w/w for liver and 10 w/w for kidney and brain. The homogenate and blood samples were divided for lead analysis and ALA-D activity assay. Homogenates for lead analysis were freeze dried, while those for enzyme assay were frozen in 1.5 mL tubes (Simport Plastics, Que.) in liquid nitrogen and stored at -80°C. Blood samples were assayed fresh.

Lead Analysis

Lead analysis was performed, using graphite furnace atomic absorption spectroscopy (Perkin-Elmer Model 603, heated graphite

atomiser HGA 76B and Deuterium Arc Background Corrector), by Dr. C.L. Chakrabarti's research group (Chemistry Dept., Carleton University). Blood samples were stored at 4°C until analysis which was generally carried out within 12 hours of blood collection. The samples were diluted with a matrix modifier consisting of 0.2 % w/v $\text{Na}_4\text{H}_2\text{P}_2\text{O}_7$ (BDH analyzed) and 0.5 % v/v of Triton X-100 (Eastman Organic Chemicals, Rochester, N.Y.). Lead levels were determined in the blood samples using a calibration curve obtained from acidified aqueous lead standards. Solid, freeze-dried tissue samples were analyzed using a platform technique where the sample is deposited directly onto a platform inside the graphite tube. Lead levels were determined by standard addition method.

ALA-D Assay

Each sample was assayed at ten pH values and in triplicate. 0.3 M phosphate ($\text{H}_2\text{NaPO}_4 \cdot 12\text{H}_2\text{O}$ and $\text{HNa}_2\text{PO}_4 \cdot \text{H}_2\text{O}$; AnalaR, BDH) buffers were prepared ranging in pH from 5.8 to 7.2. 50 μL of 0.125 M ALA (ALA-HCL; Sigma Chemical) and 50 μL of appropriate phosphate buffer were pre-incubated in a 400 μL microcentrifuge tube (Hughes and Hughes, Romford, Essex) at 37°C. An appropriate amount of blood or homogenate (200, 100, 300 and 300 μL of blood, liver, kidney and brain homogenates respectively) was mixed with 1 mL of 0.1% Triton X-100 (Sigma Chemical) solution for 1.5 minutes in a 1.5 mL microcentrifuge tube, added to the ALA - buffer solution and mixed on a vortex. The reaction was incubated for one hour in the case of blood and liver samples and two hours for kidney and brain. 100 μL of a 6% trichloroacetic acid

(Fisher Scientific)/1.6% mercuric chloride (Anachemia, Montreal, Que.) solution was added and the tube mixed on a vortex to stop the reaction. The mixture was centrifuged for 5 minutes at 15,000 rpm (Eppendorf Microcentrifuge Model 5413). 200 μ L of the supernatant was mixed with 400 μ L of modified Erlich's solution. The modified Erlich's solution consisted of 2 g of p-dimethylaminobenzaldehyde (Sigma Chemical) dissolved in 60 mL of glacial acetic acid plus 32 mL of 70% perchloric acid (Baker Analyzed Reagent) made up to 100 mL with glacial acetic acid. The absorbance of the product was measured at 555 nm on a Beckman Model 25 Spectrophotometer against a blank of 400 μ L of modified Erlich's solution and 200 μ L of water.

Restored ALA-D activity was determined by incubating 50 μ L of phosphate buffer, 100 μ L of the sample/Triton X-100 solution and 50 μ L of a 20 mM DTT (Sigma Chemical)/200 μ M zinc acetate (Nichols Chemical Co. Ltd., Montreal, Que.) solution for one half hour. The reaction was initiated by adding 50 μ L of ALA solution and proceeding as detailed above for the nonrestored activity.

The enzyme assay is expressed as micromoles of PBG generated per hour (or two hours) per milligram protein, which is obtained by the equation:

$$\frac{\text{dilution factor} \times \text{absorbance at 555 nm}}{\frac{\text{mg protein}}{\text{mL sample}} \times 1000 \times (6.2 \times 10^{-2})}$$

where 6.2×10^{-2} is the extinction coefficient of the reaction product.

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Protein concentrations were determined by the method of Lowry et al (48).

Blood hematocrit was determined using a microcapillary tube of blood (Dade Division, American Hospital Supply Co., Miami, FA) centrifuged for thirty minutes in an IEC Model MB Centrifuge (Danson/IEC Division, Needham Heights, MA).

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