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ERYTHROCYTIC Δ -AMINOLAEVULINATE
DEHYDRATASE: A POTENTIAL BIOLOGIC
INDEX OF EXPOSURE TO TOXIC METALS

BY

JEAN-PIERRE FARANT, B. Sc.

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN
PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF
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OTTAWA, ONTARIO

The undersigned recommend to the Faculty
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"Erythrocytic δ -Aminolaevulinate Dehydratase:

A Potential Biologic Index of Exposure
To Toxic Metals"

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ABSTRACT

The reliability, sensitivity, specificity and applicability of a test based upon the measurement of the activity of the erythrocytic enzyme δ -aminolaevulinic acid dehydratase, in its present or altered form, as a viable alternative to other presently accepted biological indices of intoxications by lead, zinc, cadmium, copper and mercury present in the workplace and/or urban environment were thoroughly investigated.

The investigation of procedural factors culminated in the development of a novel micro-assay procedure which requires only 10 μ L of human blood and effectively avoids procedural pitfalls.

A study of the metals of interest, both "in vivo" and "in vitro", revealed that they can radically influence enzyme measurement at pH values in the range 5.8 to 7.4 and, thus, seriously prejudice the results of an assay performed at a single pH value. A search for an alternative, improved assay led to the development of a test based upon the ratio of enzyme activity measured at specific pH values before and/or after pre-incubation of the buffered haemolysate. Tests conducted with several lead-exposed populations clearly demonstrated that the activity-ratios could function as biological indicators of lead intoxication and replace the presently accepted index namely, blood-lead concentrations. The activity-ratios can also be used to monitor zinc or copper exposure although not with the same degree of sensitivity and specificity.

The study of the interaction of the metals of interest with the erythrocytic enzyme also shed new light upon its mechanism of action and several interpretations of these results are offered.

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INTRODUCTION

With the present trend towards an ever-increasing industrial use and production of metals, concern is growing about the rising levels of toxic metals which man introduces into his workplace and residential environment and the potential threat this may represent to his health and well-being. Such concern is justified when one considers that modern man has a lead body burden some 500 times greater than his early ancestors, and that relatively low-lead exposure can cause certain behavioral changes in children (1). Such findings attest to the special consideration which must be given to metal contaminants such as lead whose concentration in the environment may have already reached levels which could be harmful to man if combined with other factors that increase their absorption, retention, and toxicity.

Human exposure to metals occurs in several ways as the metals pass through the physical and biological components of the environment. During this passage, the physical state and chemical form of metals may change, their concentration ratios may be altered and some of them may accumulate selectively in food chains. The total exposure of the general population can include significant contribution from air, water, food, and other sources such as drugs, consumer products, tobacco smoke, and various beverages. Important elements of the general population are further exposed at work via inhalation of dusts, fumes, vapors, and occasional ingestion and skin absorption of toxic metals or their compounds.

The increasing environmental insult by toxic metals endured by man, both at work and at home, has resulted in a pressing need for specific, sensitive and accurate biological monitoring methods for assessing exposure to these metals. Such methods can play an important role by providing clinicians, epidemiologists, toxicologists, and researchers with means of detecting excessive exposures as early as possible to avert possible biological changes or by discovering biological disturbances while still reversible. Biological measurements offer important advantages over the usual air monitoring methods employed to assess exposure by considering absorption via all routes, not only through the lungs, and by considering individual differences in susceptibility to the toxic metal(s), metabolism or lifestyle. The exposed person effectively becomes his own integrator of the total exposure.

These biological monitoring methods would, of necessity, be based on a biological parameter evolving from basic toxicological investigations of the fate of the toxic metal in the human body and a careful consideration of the various factors which govern this fate. Furthermore, before being adopted for routine monitoring of exposed populations, the parameter must also meet the following prerequisites: It must reflect a frank biological change whose health significance is known, the intent being to propose a meaningful threshold value for the parameter selected; the relationship between the rate of variation of the biological

parameter and the rate of uptake or body burden ⁽¹⁾ of the metal over a wide range of exposure must be known if the parameter is to be used to evaluate total uptake of the metal via all portals of entry or its level of retention in the organism; the parameter must display a high degree of specificity for a particular metal and must also be sensitive to changes in the metal's concentration in the critical organ(s); the measurement of the parameter should be sufficiently simple to perform, free of interferences, non-time consuming and allow for safe storage of biological samples for extended periods of time; the precision and accuracy of the analysis should be satisfactory.

Biological monitoring of populations exposed to toxic metals invariably entails the collection of biological liquids such as, urine, saliva, placenta, blood, and sweat; of biological materials such as hair, nails, teeth, feces; or of biopsy or autopsy samples and their analysis for metal content. The usefulness of these biological materials as indices of exposures and retention of toxic metals has been discussed in several excellent articles (2, 3). Unfortunately, at present, there appears to be a general tendency towards anticipating too much from the results of such tests and their correlation with clinical observations as a means of obtaining meaningful conclusions concerning potential health effects of exposure. Such an approach is burdened with difficulties. In most instances, problems are encountered in establishing definitive

(1) Body burden = (uptake x time) - (elimination x time)

relationships between the level of metal measured in the indicator media and that found in the critical organ. Such problems are compounded when one considers that, for a particular biological material to be a good indicator, especially for metals having long halftimes, the relationship must be constant during both steady-state conditions and the period of active accumulation. For most biological media, including blood, there are few cases when this criterion applies (4). The level of toxic metals such as cadmium and lead in blood and urine, for instance, is only an index of current exposures and reflects a dynamic balance between exposure, amounts in tissue and, excretions. The concentration of cadmium and mercury in urine, on the other hand, correlates relatively well with kidney accumulation; but, substantial inter-individual variations make their use as estimates of body burden doubtful. Conversely, the levels of lead in bone, mercury in brain and cadmium in kidney are most accurate indices of body burden. However, samples can only be obtained via painful or improbable biopsies.

In addition to these fundamental shortcomings, the use of biological material as indicators of exposure is also subject to the wide array of difficulties which naturally beset the analysis of metals in such media (5) and to the problems of obtaining representative samples. There are serious contamination problems, inherent variability in results obtained, matrix effects and interferences, to name only a few of the analytical difficulties

which must be surmounted. Furthermore, the analyses are usually expensive to perform and require costly equipment and skilled personnel.

All the aforementioned tests have one additional common failing--they usually do not reflect a frank biological change. Correlations between measurable biochemical changes and metal content in biological media are often not obvious. Since biochemical approaches are indispensable to our understanding of the effects of toxic metals, it is clear that alternatives to the measurement of metals in biological media must be sought. Unfortunately, to date, the knowledge of biochemical changes resulting from toxic metal exposure, and the availability of tests based on biochemical effects are very limited. Such tests could be based on one or more of the following: The interaction of metals with ligands such as sulfhydryl groups at the active site of enzymes resulting in a measurable loss of activity; the interaction of metals with ligands on the surface of membranes causing changes in surface-active lipids and in membrane permeability; the displacement of essential metals from metalloenzymes by toxic metals; alteration of the rate of enzymic reaction by metal interaction with substrates and coenzymes; the interruption of essential metabolic pathways; structural changes in proteins resulting in their denaturation, alteration of bioelectric properties and loss of other vital functions; and effects on the energy metabolism system of non-enzymic molecules such as haemoglobin. Although the foregoing list of biochemical effects is by no means

exhaustive, it does give an idea of the scope of measurable changes available to assess toxic metal exposures. In the present study, the loss or enhancement of activity shown by an erythrocytic enzyme δ -aminolaevulinic acid dehydratase (δ -ALA-D, porphobilinogen synthase, 5-aminolaevulinate hydrolyase, EC 4.2.1.24) on interaction either "in vivo", "in vitro" or both with lead, cadmium, mercury, zinc and copper was selected as a likely basis for a biochemical test of intoxication by these common workplace or residential environmental contaminants. The test is by no means new. It has been used extensively as a most sensitive index of a subcritical effect of lead exposure for the past 17 years. Although one of the objectives of this investigation proposes a reappraisal of the methodology and usefulness of this test for lead exposure, the applicability of the test in its present or altered form to the assessment of intoxication by the other environmental metal contaminants mentioned above was also studied. Prior to providing details of the investigational strategy adopted, it would seem appropriate to give a brief review of this enzyme's properties, functions, and mechanism of action.

δ -ALA-D: Properties, Functions, and Mechanisms of Action

δ -ALA-D has been the subject of intensive investigations for the past twenty-five years or more and the findings have been reviewed and summarized in several excellent articles (6, 7, 8). The enzyme is an important link in the biosynthetic pathway shared by all tetra-pyrole-derived substances such as haeme, chlorophyll and vitamin B₁₂ (Figure 1). δ -ALA-D's existence was first

FIGURE 1: METABOLIC STEPS IN THE SYNTHESIS OF HAEME

detected by Dressel and Falk (9) when they found that porphobilinogen (PBG) was formed from aminolaevulinic acid (ALA) by haemolyzed chicken erythrocytes. It was subsequently demonstrated by Schmid and Shemin (10) that PBG synthesized from ^{14}C -labelled ALA contained the theoretical activity calculated on the basis of the condensation of two molecules of ALA. Based on this evidence, Nandi and Shemin proposed a basic mechanism (11), depicted in modified form in Figure 2A, for the reactions catalyzed by δ -ALA-D (explanations and evidence supporting this mechanism are given in Appendix "A"). This mechanism remained largely unchallenged and unproven through the years, apart from minor suggested modifications (12), until Jordan and Seehra (13) demonstrated the apparent order in which the two substrate molecules bind to the enzyme and condense to form PBG and proposed a mechanism (Figure 2B) which differed radically from what originally proposed. Consideration of the Nandi-Shemin mechanism and experimental observations have indicated the presence of an $-\text{NH}_2$ group belonging to lysine (11), which forms a Schiff base with the substrate; of a positive group tentatively assigned to a histidine residue which orientates the incoming substrate molecule (14); and of two separate essential thiol groups (6) at the active site. Barnard et al. (15) have proposed a modification of the Nandi-Shemin mechanism to incorporate the role of the thiol groups and histidinyl residue as participants in the acid catalysis dictated by the required protonation/deprotonation sequences.

A.

B.

FIGURE 2: BASIC MECHANISM FOR REACTIONS CATALYZED BY Δ -ALA-D

A. Nandi and Shemin Postulate (11).

B. Jordan and Seehra Postulate (13).

Evidence accumulated to date indicates that the enzyme is a homomultimer having a weight of 280,000-290,000 Daltons (16, 17) and a subunit weight of 34,500 Daltons (14, 16, 18). The molecule reportedly possesses 56 sulfhydryl groups which can be activated by thiol (17). Electron microscopy of the enzyme isolated from bovine liver (18) showed its quaternary structure as consisting of eight discrete lobes arrayed at the corners of a square having dihedral (D_4) symmetry. The diameter of each subunit is 44 Å. Shemin reported that only four of the eight subunits react with substrate to form a Schiff base linkage and concluded that the enzyme exhibited the phenomenon of half-site reactivity (19). Cheh and Neilands (7) have speculated that, based on its known quaternary structure, it is probable that through either asymmetric dimerization, negative cooperativity in binding to the two protomers of a dimer (20) or interprotomer construction of sites in the same binding domains, δ -ALA-D could, in fact, exhibit half-site reactivity.

The inhibition of an enzyme by a chelator is often the first indirect indication that the enzyme has a metal requirement. Removal of all the essential metal results in lowered activity which is directly proportional to the amount of metal still bound (21). Gibson (22) was the first to indicate that liver ALA-D might be a metalloenzyme when he demonstrated inhibition by ethylenediaminetetraacetic acid (EDTA), O-phenanthroline and 2, 2' dipyridyl. Komai and Neilands (23) reported inhibition by neocuproine and bathocuproinedisulfonate and found EDTA to be

the most effective inhibitor (10^{-6} M EDTA). Findings by Wilson (17) and Cheh (7) on the reversal of enzyme inhibition by 10^{-6} M EDTA through the addition of metals indicated that, although Zn^{+2} and Cd^{+2} were the only metals to restore full activity, substantial reactivation was observed with other metals. This ability to reverse inhibition, apparently occurs in the order $\text{Cd}^{+2} \gg \text{Zn}^{+2} > \text{Co}^{+2} > \text{Ni}^{+2} > \text{Mn}^{+2} > \text{Fe}^{+2} \gg \text{Fe}^{+3}, \text{Cr}^{+3}, \text{Mg}^{+2} > \text{Cu}^{+2}$. All these observations suggest that inhibition is due to metal chelation and that other metals besides Zn^{+2} may have an essential role to play.

There appears to be universal agreement that ALA-D might be a zinc metalloenzyme (7, 17, 24) although all attempts to date to determine the stoichiometry in the native enzyme have been unsuccessful. Results have ranged from 0.5 to 1.8 Zn^{+2} atoms per 280,000 Daltons (7, 24)--rather unseemly numbers for an octameric enzyme. The stoichiometry of 4-6 Zn^{2+} atoms per octameric enzyme was obtained by Cheh (25) and others (17) by titrating the apo-enzyme with Zn^{+2} and determining the extent of its reconstitution (it is of interest to note that this could only be accomplished after the enzyme was first reduced by dithiothreitol). Cheh also found that Cd^{+2} was equally effective in restoring activity, suggesting that the initial binding site could accommodate ions of different sizes. Reconstitution of the apoenzyme with cadmium also resulted in the appearance of an absorption band around 235 nm and a positive CD band below 260 nm indicating a possible asymmetry

about the metal binding site and a possible distortion in the metal's ligand geometry. Both Zn^{+2} and Cd^{+2} inhibited the enzyme at higher concentrations. Similar trials conducted with other divalent ions showed either minimal restoration of activity (Co^{+2} , Mn^{+2} , Ni^{+2}) or no restoration of activity (Cu^{+1} , Cu^{+2} , Fe^{+2} , Fe^{+3} , Cr^{+3} , Mg^{+2} , and Cd^{+2}). In fact, Cu^{+1} and Cu^{+2} , probably as a result of their binding to or oxidation of essential sulfhydryl groups, are inhibiting at the 10^{-7} to 10^{-8} M level and make reconstitution practically impossible.

Erythrocytic δ -ALA-D

The previous discussion has dealt almost exclusively with the properties of δ -ALA-D isolated from plant or animal sources and with experimental results obtained "In Vitro" with purified enzyme, with little reference to the erythrocytic enzyme. Since this form of the enzyme, which has been the subject of comparatively few investigations, is of great import to this study, the remainder of this review will be concerned with its known properties.

Erythrocytic δ -ALA-D displays most of the properties found for forms of the enzyme from other sources. At present, its function in red blood cells is unclear and believed to be limited. Its activity in red blood cells ranges widely, a fact variously attributed to genetic variation (26, 27), sex (28), age (28), and diet (29). ALA-D activity has also been shown to be significantly decreased in alcoholics (30), diabetics,

cancer patients and workers exposed to organic solvents, such as toluene, xylene, and benzene (31).

Because most researchers investigating erythrocytic δ -ALA-D were primarily interested in its potential as a biological monitor of metal intoxication, many of the studies of this form of the enzyme have dealt with the interactions of the enzyme "In Vitro" and "In Vivo" with zinc and/or lead, cadmium, mercury, copper, tin, manganese, and with factors which could influence these interactions. Thus, several "In Vitro" and "In Vivo" studies of the effects of Zn^{+2} on δ -ALA-D activity in red blood cells have been conducted. Abdulla and Häger-Aronsen (32) were the first to demonstrate that zinc, both "In Vitro" and "In Vivo", causes a marked increase in the enzyme's potency in human erythrocytes. They also observed that the metal has an inhibitory effect at relatively low concentrations. Finelli et al. (33) conducted studies with rats maintained on a low zinc diet and found that there was a significant decrease in the enzyme's activity both in erythrocytes and in the liver. Conversely, they reported no similar effects for copper. Border et al. (34) reported that the reactivation of the enzyme in blood with zinc after EDTA inhibition was pH-dependent. They concluded that this type of inhibition only involved peripherally bonded metal ions and ruled out a structural role for zinc in maintaining the enzyme's quaternary structure.

Much of the work performed to date on erythrocytic ALA-D has been concerned to a large extent with the enzyme's remarkable sensitivity to lead both "In Vivo" and "In Vitro". Reduced enzyme

activity associated with lead intoxication was first demonstrated by Lichtman and Feldman (35). Several authors have since confirmed this observation and studied various other aspects of the relationship between δ -ALA-D and blood lead level (36-42). All available evidence to date indicates that the level of activity of the enzyme in red blood cells is a specific and sensitive index of exposure to lead. Increases in lead level parallel commensurate decreases in ALA-D activity in the blood lead concentration range of 15 to 60 μg per deciliter. At higher blood lead concentrations, the activity of ALA-D plateaus at very low levels and this apparently limits the use of this test. All doubts concerning the possibility that the inhibition might be an "in vitro" artifact arising during the assay were allayed by Roëls et al. (43) who demonstrated that the observed phenomenon is a true reflection of enzyme activity which, while not indicative of a proven health hazard, is interpreted as a subclinical sign that exposure is taking place. As an index of intoxication, the enzyme displays a better correlation to body lead and to lead absorption than do other physiological parameters such as a rise in urinary ALA (43, 45) and coproporphyrin excretion (46), increase in erythrocyte protoporphyrin IX (47), inhibition of sodium-potassium adenosine triphosphatase (48) and a fall in haemoglobin level. In fact, Lauwerys et al. (49) have shown that urinary ALA only increases significantly when ALA-D activity is reduced to 30% of its initial value, corresponding to a blood lead level of 45 $\mu\text{g}/\text{dL}$.

Several studies have established the minimal inhibitory effect of lead on ALA-D at between 10 and 20 $\mu\text{g/dL}$ (44, 50-53). Inhibition is observed soon after initial exposure, long before any other biological effects are measureable (45, 53, 54) and is present throughout the period of exposure. After cessation of exposure to lead, ALA-D's activity recovers very slowly and may even precede a detectable drop in blood lead concentration (54-56). Regeneration is apparently faster in individuals who have endured a short exposure or who had a low initial lead concentration in their blood.

Cerklowski and Forbes (57) have demonstrated that excess dietary zinc alleviates many of the toxic effects of lead in rats intoxicated by this metal as shown by a decreased inhibition of kidney ALA-D and a concomitant decrease in blood and tissue lead content. Similarly, Finelli et al. (58) had earlier reported that enzymic activity returned to normal levels more quickly in lead-fed rats maintained on a high zinc diet and that "In Vitro" addition of zinc to the erythrocytes of these rats reactivated the enzyme to control values. They concluded that this suggested a competition between lead and zinc in binding to the enzyme with Pb^{+2} as the inhibitor and zinc as the activator. Meredith et al. (59) obtained similar results. This antagonistic effect of zinc on the inhibition of ALA-D by lead was investigated and confirmed "In Vitro" and "In Vivo" by several workers at concentrations of zinc and lead ranging from the physiological to that far in excess of that expected in

heavy industrial exposure (60-65). In all instances, zinc effectively masked the inhibitory effect of lead and placed the value of this test in jeopardy as an index of lead exposure. However, although a case study of a lead-intoxicated human subject appeared to support this contention (66), Meredith and Moore (67), in investigations conducted with workers concomitantly exposed to lead and zinc, concluded that the presence of zinc does not negate the value of ALA-D as a potent biological parameter of lead exposure. It is noteworthy that a decrease in urinary ALA excretion has been reported in workers exposed to both lead and zinc (68).

In two separate studies of Belgian workers exposed to both cadmium and lead, Roels et al. (69, 70) demonstrated conclusively that, unlike lead, cadmium has no significant effect on ALA-D activity. In the second study (70), they also found that the overall activity of the glutathione-oxidation-reduction pathway is not impaired by either lead or cadmium and ruled out a regulatory role for this enzyme in the activity of ALA-D in erythrocytes. It is of interest to note that Wada (71) had earlier reported a transient decrease in ALA-D activity in mice red blood cells in the early stages of their treatment with cadmium or mercury. In both instances this was followed by an increase in activity to a value in excess of the initial level.

As was the case for cadmium, there have been comparatively few studies performed on the effects of mercury or its organic compounds on erythrocytic ALA-D activity. Wada et al. (72) studied

a population of workers exposed to mercury in a tungsten rod factory and reported a definite inhibition of ALA-D by this metal. However, since exposure to lead in such a work environment is a possibility and no blood lead measurements were made, this result is in doubt. Two studies, conducted by Hernberg et al. (73) and Lauwerys et al. (74) respectively, involved populations exposed to low levels of mercury and consequently, an expected "no effect" verdict was arrived at. The only study of organic mercury compounds was carried out by Schutz and Skerfving (75) who reported a statistically significant decrease in ALA-D activity with increasing methyl mercury concentrations (range 15-340 ppb).

The effect on erythrocytic ALA-D of only a few other metals, besides lead, mercury, and cadmium, has been investigated. These included aluminum (76) which was found to behave as an activator, in a manner reminiscent of zinc and with which metal it produced a stoichiometrically additive activation. Recently, Chiba and Kikuchi (77) investigated the effect of tin on the enzyme and found that it inhibited the enzyme like lead. Finally, Melgaard et al. (78) reported a significant correlation between low blood manganese concentrations and decreasing ALA-D levels.

A large section of the general population is auto-exposed daily to two common toxicants, namely, carbon monoxide in tobacco smoke and ethanol in alcoholic beverages. Concern about the potential effect these two substances could have on ALA-D has led to their investigation. Thus, Moore et al. (30) demonstrated that

the enzyme's activity is significantly depressed in the blood of men in an advanced state of inebriation and in the blood, liver, and kidney of intoxicated rats. The inhibition is removed as blood ethanol levels drop. Conversely, it was found that the enzyme inhibited by lead was activated by high ethanol concentrations. Both effects were linked to increasing glutathione (GSH) levels with increasing ethanol concentrations, as high GSH levels lower normal ALA-D activity and increase that of the lead inhibited enzyme. Corroborative results were obtained by Abdulla et al. (79) and Prpic-Majic et al. (80). Moore and Meredith (81), on the other hand, did not find a significant depression of the enzyme at HbCO levels normally found in heavy smokers. Higher HbCO levels "in vitro" did show a slight depression of ALA-D activity.

Aside from the aforementioned efforts made to determine the extent to which erythrocytic ALA-D's activity is affected by its interaction with various metals, common toxicants and exogenous activators and inhibitors, a substantial part of the research on this enzyme has been devoted to the study of problems influencing its assay in whole blood. A review of the assay in its various forms leading to the present day's generally accepted "European Standardized Method" (82) follows.

Assay Methods

Two general methods have been used to assay δ -ALA-D activity. Both are based upon the addition of excess substrate ALA to buffered haemolysate, incubation at 37 °C for a specified period, reaction

of the product PBG with Erlich's reagent (p-dimethylaminobenzaldehyde) and measurement of the absorption of the colored product at 555 nm. One of these methods, the Gibson assay (22), utilizes phosphate buffer pH 6.8 (for either investigations involving the pure enzyme or ALA-D in whole blood or tissue), a thiol activator and substrate ALA in its reaction mixture; copper sulfate is used to stop the reaction; following centrifugation, the supernatant is mixed with an equal volume of regular Erlich reagent. The other method, the Granick-Mauzerall assay (83), also uses phosphate buffer ⁽¹⁾, pH 6.4, and substrate ALA but uses trichloroacetic acid-mercuric chloride to stop the reaction. The supernatant, after centrifugation, is mixed with an equal volume of modified perchlorate-Erlich reagent (84). An overall preference has been shown by many users for the Granick-Mauzerall method. This preference is due in large part to the better response linearity, color stability and greater effectiveness of Hg over Cu in blocking SH groups which interfere with color development. The latter assay procedure, in a modified form, has been adopted for this study.

Several investigations have been conducted over the years to optimize the assay's performance in conjunction with the enzyme's use as an index of lead exposure. The parameters investigated included collection, handling and storage of blood samples (85-93), the haemolysis procedure (90-95), the selection of appropriate buffer system and pH-optimum (86, 90-95), the colorimetric reaction (90),

- (1) Potassium salt not recommended because of formation of insoluble perchlorate salt.

the role of activators and inhibitors (86, 93, 95), the haemolysate and substrate concentration (86, 90, 92) and the incubation temperature and duration (86, 90, 92, 95). The last two factors have been well established and do not usually vary from assay to assay. These investigations have culminated with the proposal in 1974 of a standardized procedure by Berlin and Schaller, the "European Standardized Method" (82). Although a certain measure of inter-assay comparison of results is now attainable since the advent of this procedure, the assay nevertheless still displays certain unacceptable characteristics including: an inherent scatter in the results obtained; an analysis which must be performed within 24 hours of blood collection; and, in instances when the enzyme is used as an index of lead exposure, a curvilinear correlation with blood lead concentrations is obtained. The method's shortcomings have contributed to some extent to the general lack of acceptance of erythrocytic ALA-D as a viable index of exposure to metals such as lead, despite its reported simplicity, sensitivity, and specificity.

In recent years, several attempts have been made to adopt an alternative approach to the use of erythrocytic ALA-D activity as an index of metal (lead) exposure. Thus, Granick et al. (93) have proposed the use of a ratio of dithiothreitol (DTT)-activated to non-activated enzyme activity measured at pH 6.3 as an indicator of lead exposure (Granick test). Mitchell et al. (86) and Sakai et al. (97) have suggested improvements to this test such as the use of zinc, heat or both instead of DTT as a means of activating the enzyme. Having confirmed earlier observations by Nikkanen et al. (92)

that the pH of maximal activity for the enzyme, apparently was shifted to a lower pH (6.0) in the blood of lead-exposed workers than that found in non-exposed individuals (6.4 to 6.8) Tomokuni (95) demonstrated the use of the ratio of the enzyme's activity measured simultaneously at 6.8 and 6.0 as a more accurate test of lead exposure (Tomokuni test). This approach has recently been assessed by Chmielnicka et al. (98) and found to give a better correlation than the results of the "European Standardized Method" over a wider range of blood lead concentrations. Tomokuni (99) had also observed that the pH optimum of the enzyme in the blood of lead-exposed workers shifted back to pH 6.6 with a 3.6-fold concomitant increase in activity when the blood haemolysate was heated at 60 °C for 5 minutes prior to the actual enzyme activity measurement. He also noted that only a slight shift and 1.3-fold activation occurred in the blood of non-exposed persons. This method of enzyme activation was then proposed by Tomokuni (100), Chiba (101), and Ushio et al., (102) as an alternative to thiol activators in the Granick test (Tomokuni-Granick test). Finally, Beritic et al. (103) have recommended the use of the ratio of erythrocytic ALA-D activity to the value of free erythrocytic porphyrin (EP) as a possible measure of lead exposure (Beritic test) on the basis that a combination of the two most sensitive tests of lead exposure would yield a more viable alternative to their separate use.

Tomokuni (104) had also suggested that erythrocytic ALA-D activity could be determined more accurately by measuring the amount of substrate ALA consumed during the assay, thus taking into account the PBG converted into porphyrins. This procedure tended to yield results which correlated better with blood lead values (105). However, this method and the tests described above, have not enjoyed a significant acceptance amongst researchers as indices of lead exposure. Since none of the tests presented so far has successfully supplanted the measurement of metals in blood and other biological media as an index of exposure to toxic metals, a reassessment of the status of erythrocytic ALA-D as a potential biological parameter of metal intoxication appears appropriate.

Erythrocytic δ -ALA-D as a Biological Parameter of Metal Intoxication

Although erythrocytic δ -ALA-D has been used by many epidemiologists, toxicologists, clinicians and researchers as a sensitive index of subclinical lead intoxication and, in that capacity, has fulfilled many of the prerequisites for a viable biological parameter, several facts have contributed to its general lack of acceptance and its inability to rival the measurement of lead and other metals in blood in this role.

- 1) Haeme synthesis occurs in the bone marrow in nucleotide precursors of red blood cells, and it would appear that the haeme biosynthetic pathway enzymes, including erythrocytic δ -ALA-D, are devoid of any biological significance. However, commenting on the subject

of lead intoxication, Haeger-Aronsen (37) concluded that an abnormally low δ -ALA-D activity in erythrocytes is a clear sign of a biochemical disorder, even at lead concentrations considered to be of no clinical significance. This conclusion was based upon the fact that when ALA-D activity falls to one third its normal value, haeme synthesis is affected, as manifested by an increase in urinary ALA excretion. What is not yet known is whether such a manifest disorder can affect health.

2) δ -ALA-D activity in the blood of the general population ranges over a four-fold range and varies with several factors including genetic variation, sex, age, diet, diseases, and exposure to certain organic solvents. Such variability obviously makes the setting of a meaningful threshold limit impractical if not impossible.

3) Although the test's main use, to date, has been almost exclusively as an index of lead exposure, its specificity in this aspect is suspect viz. the reported antagonistic effect of zinc on lead "in vivo" (60-65). Its use as a specific test for other metals besides lead has yet to be fully exploited.

4) Inhibition of the enzyme's activity by lead occurs at a relatively low level, 15 $\mu\text{g/dL}$, and occurs shortly after initial exposure. Inhibition by this metal varies with blood lead concentrations in a curvilinear fashion to a level of some 60-80 $\mu\text{g/dL}$ where it plateaus at a low activity level. This marked sensitivity to lead has been cited as both an advantage and disadvantage.

5) As detailed earlier on, the assay of the enzyme itself is fraught with many difficulties. The method is in need of a better protocol and an extension of its capability to the analysis of small (10 μ L) blood samples, as required for the screening of large populations of children exposed to metals in the urban environment.

Until these many problems are effectively resolved, it is clear that the ALA-D test will remain an adjunct to other more acceptable tests. The strategy adopted during this study to investigate the test's many shortcomings follows.

Study Objective and General Strategy

The main objective of this study is to investigate thoroughly the reliability, sensitivity, specificity and applicability of the erythrocytic δ -ALA-D test, in its present or altered form, as a viable alternative to other presently accepted biological indices of intoxication by metals such as lead, mercury, cadmium, copper and zinc present in the workplace and/or urban environment, and to gain some understanding of the mechanism of action of the enzyme when affected by these toxicants. Ancillary objectives include the optimization of the protocol of the δ -ALA-D assay to accommodate small blood samples; and the development of reliable and accurate methods for the analysis of metals in blood.

At the very outset of this study, it seemed obvious that the reproducibility, precision and accuracy of the δ -ALA-D test were inextricably linked to procedural and exogeneous factors

capable of influencing the enzyme's pH-activity profile. Apparent shifts of this curve had been observed in the blood of lead-exposed individuals (92,95), after heating the blood heamolysate at 60 °C for 5 minutes (100-102) and after treatment of the blood with chelating agents such as EDTA (86, 99). In addition, different pH's of maximal activity have been reported by workers using essentially identical assay conditions. The investigation of these causative factors in turn necessitated the optimization of the widely accepted "European Standardized Method" to accomodate the 10 µL blood samples used to perform replicate analyses at several pH values with the same blood sample. In its final form, the protocol of the modified assay incorporated the appropriate safeguards against the adverse procedural and exogeneous factors found in the first instance. It was hoped that this approach would afford both reproducible and precise ALA-D results and thus provide a useful tool to investigate its potential as a viable biologic parameter.

Various alternatives to the existing test, based loosely on the Granick, Tomokuni, and Chiba tests presented earlier, were contemplated in an attempt to optimize the test's overall performance. Four alternate tests were tried using blood samples obtained from selected populations or rabbits exposed to either lead, cadmium, mercury, copper or zinc or combinations of these. These were:

- 1) Obtain the ratio of the activity of non-activated ALA-D to the activity of enzyme activated by dithiothreitol--a test

devised by Granick et al. (93) and highly recommended by other workers (86, 97);

- 2) obtain the ratio of activities measured at two different pH values to take advantage of the apparent shift in pH optimum with metals like lead--approach taken by Tomokuni (95);
- 3) obtain the ratio of activity measured at two pH values after incubation of the buffered haemolysate minus substrate (ALA) to take advantage of the optimum shift back towards its normal value and the increase in activity observed;
- 4) obtain a ratio of activity measured at a pH value for non-incubated and at the same or different pH value with incubated buffered haemolysates.

Ideally, one or more of the above tests would

- require small amounts of blood;
- simplify the present assay procedure;
- improve upon the present test's time of assay limitations (24 hours);
- show specificity for one or more metals, depending on assay conditions;
- be valid over a wide range of metal-in-blood concentrations;
- demonstrate a lack of sensitivity to common adventitious metals and toxicants such as CO and ethanol;
- relate to some biological change(s);

- provide a linear relationship between δ -ALA-D activity or activity-ratio values and blood metal concentrations;
- demonstrate an acceptable degree of precision and accuracy for both within-day and between-day assays;
- reduce the range of control values:

Once an acceptable test has been found, its applicability as a biological monitoring method for populations exposed to the metals cited could be conducted. Finally, it was also hoped that the results of the investigation would shed some light upon the mechanism of action of ALA-D.

RESULTS AND DISCUSSION

One of the aims of the study is the design of a biochemical test for metal intoxication based upon the measurement of δ -ALA-D activity at a single pH value, with and without activation, or at two separate pH values either with or without prior treatment of the enzyme with heat. Since several procedural, exogenous and endogenous factors can potentially affect the enzyme's pH-activity relationship and, most likely, would affect the results of the proposed tests, they were investigated first.

Factors Influencing the pH-Activity Relationship of δ -ALA-D

δ -ALA-D activity is strongly pH-dependent and the enzyme's assay requires a critical control of reaction mixture pH to ensure optimum reproducibility of results (91). Other factors such as adventitious metals or substances introduced at the time of blood collection, blood storage temperature and duration, preparation and treatment of the haemolysate and the presence of certain metals and toxicants in blood can modify the pH-activity profile. Such changes in the pH-activity profile can give rise to anomalous results obtained either with the usual assay or with a method based upon the ratio of enzyme activity measured at two different pH values. A general disregard by most researchers for potential analytical problems arising from changes in the pH-activity profile may, in large part, have contributed to the poor quality of the results obtained. Surprisingly, to date, only one known investigation has been devoted to the study of the pH-activity relationship of δ -ALA-D and then, only as a convenient means of comparing assays conducted

with whole blood versus washed erythrocytes (94). A thorough study of the aforementioned factors, under different sets of assay conditions, was undertaken to determine their nature, extent, and potential impact upon assay results.

Procedural Factors

Buffer systems

Two buffer systems, namely, dibasic sodium phosphate-citric acid (P-C) and the dibasic sodium phosphate-monobasic sodium phosphate (P-P) have been used extensively to date in the assay of δ -ALA-D with a decided preference being shown by many for the P-P buffer. Both have been investigated using blood samples collected from individuals who had no known unusual exposure to toxic metals and neither smoked cigarettes nor drank alcoholic beverages prior to testing. All pH-activity profiles obtained with both buffer systems at the outset of the investigation are typified by curves shown in Figure 3. Maximal activity in P-C buffer occurred at a different pH value (6.4 ± 0.1) than in P-P buffer (6.1 ± 0.1) and was consistently 15 to 20% lower in value.

The pH values obtained with both buffers were remarkably reproducible and were backed by comparison with NBS standards. However, they differed significantly from values obtained by others using similar assay conditions: 6.40 (93) or 6.80 (95) in P-P buffer and 6.65 (90) in P-C buffer. At the time, the discrepancy was partially ascribed to the use by these workers of insufficient buffer to overcome the buffering capacity of blood.

FIGURE 3: pH - ACTIVITY PROFILE OF HUMAN ERYTHROCYTIC Δ -ALA-D -
 Obtained in (A) 0.3 mol/liter P-P and (B) 0.3 mol/liter
 P-C buffer systems. Each point in this figure and in
 all subsequent figures is the mean of three separate
 assays with a mean deviation within $\pm 3\%$.

In view of the substantial buffering capacity of blood, measurement of pH-activity curves of blood haemolysates is more demanding than measuring those of pure enzymes since, at least at some pH values, the buffering capacities of both blood and buffer will be pitted one against the other. Clearly, the use of insufficient buffer may result in essentially no directed buffer action. Figure 4A illustrates the effect of four buffer concentrations of P-P buffer on the enzyme's profile. Similar results were obtained with the P-C buffer. In both instances, a buffer concentration of 0.3 mole/liter appears to be the minimum concentration beyond which no further significant change occurs. A 0.3 mole/liter P-P buffer system was adopted as part of the standard conditions for this study.

To facilitate comparison of results with those of others, the enzyme's activity was plotted versus the added buffer pH (Figure 4A). Although values of the actual pH of the incubated reaction mixture (pH_{inc}) are more meaningful than those of pH buffer, they are experimentally more involved to obtain, requiring an extra determination with a combination micro probe electrode. For comparison purposes, the set of $\text{pH}_{\text{buffer}}$ -activity curves shown in Figure 4A are given in Figure 4B as pH_{inc} -activity curves. It is interesting and noteworthy to see the difference between the sets of curves in Figures 4A and B based on the same data. In this study, all pH-activity curves will use buffer pH only.

Haemolysate Preparation

It appears to be a widely accepted practice in the δ -ALA-D assay to incubate the haemolysate, buffered or non-buffered, at

FIGURE 4: Δ -ALA-D ACTIVITY AS A FUNCTION OF (A) P-P BUFFER pH AND (B) INCUBATION MIXTURE pH AT DIFFERENT P-P BUFFER CONCENTRATIONS: (●) 0.6 mole/liter; (▲) 0.3 mole/liter; (◻) 0.15 mole/liter; (○) 0.06 mole/liter.

room temperature or at 37 °C for periods up to 30 minutes to achieve complete lysis prior to the initiation of the actual assay (34, 59, 82, 92). This is a disastrous practice as far as enzyme assay is concerned. Figure 5 exemplifies the consequences of such a procedure for both buffered and non-buffered lysates. The enzyme is seriously de-activated and its pH-activity profile markedly affected by treating the lysates in this manner. Enzyme activities, obtained from an assay including heating of the haemolysates prior to the assay, will be lower than those in which this approach was not adopted. This de-activation phenomenon was also noted by Granick and his co-workers (52). Furthermore, while it is possible to obtain consistent results with procedures which include lysate incubation, this is only possible when the heating period is carefully timed and the temperature carefully controlled; in view of the rapid enzyme inactivation, a few minutes shorter or longer will destroy the consistency of results. This point is evident from the data of Figure 5.

The extensive fundamental work performed by Shemin and his co-workers on bacterial ALA-D, mentioned earlier on, has shown that preparations of the enzyme are extremely sensitive to air oxidation (15) and thus, the destruction of enzyme activity on heating the haemolysate is not entirely surprising.

A second crucial feature is illustrated in Figure 5. In addition to the loss of activity on heating the buffered haemolysate, the pH of maximum enzyme activity apparently undergoes a

FIGURE 5: Δ -ALA-D PH - ACTIVITY PROFILE AFTER INCUBATION OF P-F BUFFERED (A) AND NON-BUFFERED HAEMOLYSATE (B) (●) 0 min.; (○) 30 min. at 25 °C; (▲) 60 min. at 30 °C; (□) 30 min. at 30 °C;

dramatic shift to a higher pH. The shift of the pH optimum is of serious practical consequence regarding both the usual enzyme assay and, more so, the proposed activity ratio method. In the first instance, the value of the enzyme's activity, determined at the customary pH of 6.4 after incubating the buffered haemolysate at 37 °C, will be substantially lower than if the lysate had not been treated in this manner. The size of the decrease will depend upon both the temperature and duration of the incubation period. In the second instance, a shift in the pH-activity profile will adversely affect the ratio of activities measured at two pH values. In view of these effects, it is strongly recommended that enzyme assays commence as soon as possible (within a few minutes) of blood haemolysis.

Blood Collection and Storage

For convenience, the common practice of collecting blood samples by venipuncture with "low-lead" vacutainers (Becton Dickinson BD4410) was adopted without reservation at the beginning of the investigation. The results reported in the previous sections were obtained with blood samples collected in this fashion. One year, or so, into the investigation, several articles signaled the presence of metals and, especially zinc, in this type of blood collection device (106, 107). Kneip (85) and Mitchell (86) earlier on, had also suspected that an unknown substance with chelating properties, present in these tubes, was responsible for the poor quality of results obtained with the δ -ALA-D assay. Because of these adverse reports, Becton Dickinson discontinued the production

of BD4410 tubes and offered "trace metal-free" tubes (BD6527, blue top) and new "lead-free" tubes (BD6488, brown top) as replacements.

While making the transition from the older BD4410 to the newer BD6527 tubes, significantly different pH-activity profiles were observed for the enzyme in the blood collected in the newer devices (Figure 6). For one, erythrocytic ALA-D now had a pH of optimal activity at a higher pH value (6.4 ± 0.1), more in keeping with that reported elsewhere and distinctly different from that reported in the previous sections (6.1 ± 0.1). Furthermore, the activity for the same blood sample was markedly less in the new tubes. The interferring substance(s) not only caused a shift to lower pH values but also activated the enzyme. The activation phenomenon had also been reported by Kneip (85), Mitchell (86) and Burch (108).

The assumption by Kneip and Mitchell that the causative agent(s) had chelating properties is probably wrong, since the action of chelators on the enzyme most often results in de-activation through the removal of essential metals such as zinc (7, 17, 22, 23). Thus, no efforts were made to determine if such substances were present. On the other hand, metals such as Zn, Cu, Pb, Hg, and Cd which are known to affect the enzyme to some extent "in vitro" are most likely candidates. Based on this premise, the level of these metals in three types of vacutainers: BD6488, brown top; BD6527, blue top; and BD4716, green top were measured. The tubes were filled with 10 mL of 1% nitric acid solution, the tubes allowed to rest on their side at room temperature for one week and the metal content of the solution measured by atomic absorption. The results are shown in Table I.

FIGURE 6: EFFECT OF ADVENTITIOUS CONTAMINANTS IN BLOOD COLLECTION DEVICES ON Δ -ALA-D'S pH - ACTIVITY PROFILE

TABLE 1: METAL CONTAMINANT CONCENTRATION IN BLOOD SPECIMEN CONTAINERS

Container	Zinc (ppm)	Cadmium (ppm)	Lead (ppb)	Copper (ppb)	Mercury (ppb)
Vacutainer Dark Blue Stopper No. BD6527	0.14 ± 0.11* (0.01 - 0.62)	<0.006	1.72 ± 1.34 (N.D. - 8.00)**	6.81 ± 3.68 (2.30 - 17.8)	< 1.0
Vacutainer Green Stopper No. BD4716	76.2 ± 4.50 (57.4 - 82.6)	0.26 ± 0.11 (0.10 - 0.49)	13.4 ± 10.5 (3.00 - 40.0)	11.0 ± 7.82 (0.65 - 39.0)	< 1.0
Vacutainer Brown Stopper No. BD4488	62.4 ± 6.40 (53.1 - 72.7)	2.30 ± 1.50 (0.07 - 4.73)	4.96 ± 3.87 (1.50 - 36.3)	6.76 ± 4.22 (1.60 - 16.8)	< 1.0
Corning Polystyrene Tube No. 2570	0.12 ± 0.08 (0.01 - 0.18)	<0.007	N.D.	N.D.	< 1.0

* Mean ± standard deviation; Range of concentration is in parentheses.

** N.D. - Not detected

Zinc is ubiquitous in all tubes and especially prevalent in tubes BD6488 and BD4716. Since zinc is known to activate the enzyme "in vitro" (32, 34), its presence at least explains one of the phenomena observed in the earlier results. The shift to a lower pH value is as yet unexplained since lead, which is known to cause such a shift "in vivo" was present in relatively small amounts in all tubes. The effect(s) of the presence of cadmium or copper in the amounts shown on the pH-activity profile of ALA-D is, at present, unknown. The "in vitro" trials described in the next section were initiated in part as an effort to clarify this situation.

In view of the poor reliability of the commercially available vacutainer devices for blood collection, a better alternative was sought. Various types of plastic containers were tested in the manner detailed for the vacutainers. The results in Table I attest to the general absence of all metals of concern in these devices. Blood collection with plastic syringes (Becton Dickinson) and storage in plastic (Corning) tubes was selected for this study.

The proposed new δ -ALA-D assay would, hopefully, enable the analyst to perform the test much later than the twenty-four hours after blood collection required by the present protocol. Changes in the pH-activity profile of the enzyme in an aging blood sample are shown in Figure 7. It is encouraging to note that, aside from the drastic drop in activity during a period of two weeks (45%), there results no marked change in the overall shape of the curve and only a slight shift of the pH optimum from 6.5 to 6.3.

FIGURE 7: CHANGES IN THE Δ -ALA-D pH - ACTIVITY RELATIONSHIP
IN BLOOD STORED AT 4.°C FOR UP TO 17 DAYS.

Exogenous Factors

Either by design or by accident, certain substances which can change erythrocytic δ -ALA-D's pH-activity profile, may become part of the reaction mixture. Two such substances will be discussed here, namely, dithiothreitol (DTT, Cleland's reagent) a thiol group protective reagent, and trichloroacetic acid (TCA) which is used to stop the enzyme reaction.

Dithiothreitol, by virtue of its low redox potential, can maintain monothiols in the reduced state and reduce disulfides quantitatively (109). Granick and co-workers (52, 93) were amongst the first to demonstrate that incubation of δ -ALA-D with DTT prior to the addition of the substrate not only reactivates the enzyme but also removes the effects of lead poisoning. The action of this agent on the enzyme is exemplified in Figure 8A: The enzyme's activity is substantially increased (20%), a very slight shift of the pH optimum to a higher value occurs and, the profile is most often broader.

While developing a micro-method for the δ -ALA-D assay, an annoying non-reproducible shift in the pH-activity profile to higher values was often obtained. Careful elimination of all possible causative factors in the procedure revealed that the culprit was TCA, the substance used to stop the reaction. Apparently, through a change of procedure requiring the manual dispensing of this chemical, instead of the use of a semi-automatic reagent dispensing device, some TCA escaped into the laboratory and found its way into the reaction mixtures. Typical results, involving the deliberate TCA contamination of a blood haemolysate with varying amounts of this

FIGURE 8: Δ -ALA-D pH-ACTIVITY PROFILE BEFORE (■) AND AFTER (●) THE ADDITION OF DITHIOTHREITOL (A) AND TRICHLOROACETIC ACID (B) TO THE HAEMOLYSATE.

chemical, shown in Figure 8B and Table II, illustrate this point. Clearly, great care should be exercised in handling this material to prevent its escape into the laboratory. It is entirely possible that similar incidents in other laboratories have been one of the reasons for the poor interlaboratory comparison of results.

The δ -ALA-D pH-activity profile shown in Figure 8B is typical of such curves (pH optimum 6.4 ± 0.1) obtained for blood samples collected from individuals (with no known unusual exposures to metals or common toxicants) with an assay protocol based upon the findings detailed in this section.⁽¹⁾ This protocol, which was implicitly adopted for all δ -ALA-D determinations made during this study, was further modified to accommodate micro blood samples ($<10 \mu\text{L}$) to facilitate the investigation of the effects of metals both "in vivo" and "in vitro" on the enzyme's pH-activity relationship. Details of this final protocol are given in a later section.

(1)

The results reported earlier of the effects of buffers and haemolysate preparation on the δ -ALA-D pH-activity curve and obtained with contaminated blood are still nevertheless valid. In fact, the experiments were repeated in toto using the new protocol given above and the results obtained were essentially similar to those described earlier. Hence, there appeared to be no purpose in reiterating the results here.

TABLE II: ALA-D Activity as a Function of Trichloroacetic
Acid Concentration

TCA ¹ (ppm)	ALA-D Activity ² (μ M PBG/h/L _{RBC})
0.00	656 $\pm$ 34
0.10	649 $\pm$ 25
0.50	638 $\pm$ 33
1.00	571 $\pm$ 30
2.50	560 $\pm$ 6
5.00	560 $\pm$ 22

1 Final concentration in blood, after addition of TCA 0.02% in saline.

2 At pH 6.4.

Effects of Metals "In Vivo" and "In Vitro"

In 1972, Nikkanen and his co-workers (92) observed an apparent shift of erythrocytic δ -ALA-D's pH optimum from pH 6.4 to pH 5.8 concomitant with an increase in blood lead concentration in blood samples collected from workers occupationally exposed to the metal; and, they attributed this dependence of the pH optimum on blood lead levels to the allosteric nature of the enzyme (110). All attempts by Nikkanen and others (62, 92) to duplicate this pH optimum shift "in vitro" by the addition of lead salts have been unsuccessful to date, even though the reduction of the enzyme's activity was commensurate with the amount of lead added. This led to the conclusion that lead ions added "in vitro" to haemolysates fixed themselves to the active center in a manner different than "in vivo". No explanation has yet been offered for this ambivalent action of lead on δ -ALA-D.

These few reports constitute, in essence, the sum total of the efforts made to date to investigate the effects of lead, both "in vivo" and "in vitro", on the shape of erythrocytic δ -ALA-D's pH-activity profile. The possibility that metals other than lead could cause similar pH-optimum shifts and the implications such shifts would have upon the enzyme's assay results have been inexplicably ignored.

It is highly probable that the enzyme is subjected concurrently to the action of several metals both "in vivo", as a result of an individual's exposure to a mixture of metals viz.

welders, foundry workers, and "in vitro", via the possible contamination of the blood sample during collection (Table I) or of the assay mixture itself. It was, therefore, apparent that the effects of the metals of concern, namely, lead, copper, zinc, cadmium, and mercury upon the enzyme's pH-activity profile, both "in vivo" and "in vitro" and either singly or in combination, required investigation.

It is readily acknowledged that there are certain inherent difficulties in extrapolating the results of "in vivo" experiments performed with laboratory animals to man. However, the impossibility, in most instances, of conducting "in vivo" tests with human subjects necessitated the use of animals. Rabbits were selected for "in vivo" study because of the similarity of their erythrocytic δ -ALA-D profile to that of man. ⁽¹⁾ Wherever possible, attempts will be made to draw a comparison between the behaviour of the two species' erythrocytic δ -ALA-D towards "in vivo" and "in vitro" metals and to point out the similarities or disparities, if such exist.

All "in vitro" investigations were conducted with blood obtained from human subjects whose exposure to metals was well known.

(1) Both rhesus monkeys and rats were considered and found unsuitable for this study. The maximal blood δ -ALA-D activity for both species was less than 500 μ M PBG/h/L_{RBC} and their δ -ALA-D pH-activity profile differed markedly from that of man.

Lead

Lead is a toxic non-essential trace element inhibiting a large number of enzymes with functional sulfhydryl groups (111) such as δ -ALA-D.

Lead poisoning was already recognized by Hippocrates, four centuries B. C. The metal affects mainly three systems, namely, haematological, neurological and renal. Clinical manifestations of the disease (112) are characterized by impairment of the functional activity of various organs as a consequence of the accumulation of absorbed lead in the body. In addition to anemia, fatigue, headache, weight loss and chronic renal disease, many other factors are observed in lead poisoning, such as proteinuria, aminoaciduria and hyperuricemia, and neurological alterations manifested as intestinal colic, peripheral neuropathy and encephalopathy.

It is admitted that lead induces a certain number of biochemical injuries at the cellular or subcellular level that will explain the polymorphism of clinical manifestations as well as the variability of the toxicological aspects according to the degree and duration of exposure.

In asymptomatic lead-exposed people, other effects have also been demonstrated, namely, subclinical nervous damage, psychological disturbances and chromosomal aberrations. A summary of these effects and their relationship to blood lead levels is shown in Figure 9 (113). A study of one of these effects, that on δ -ALA-D in red blood cells is reported here.

Figure 9. Relationship between blood lead levels and the onset of a number of effects (113).

"In vivo". Rabbits were maintained on a lead diet by adding lead acetate to their drinking water. The δ -ALA-D pH-activity profiles for these rabbits changes dramatically as blood lead levels increases during lead feeding (Figure 10A). Thus, a pH-optimum shift from 6.60 to 6.00 and a concomitant decrease in the enzyme's activity with increasing blood lead concentrations are clearly observed.

The relationship between increasing blood lead concentrations and the enzyme's activity, measured at pH 6.0, 6.4, and 6.8 (arbitrarily selected) is best depicted in Figure 10B. At pH 6.4 and 6.8 a rather rapid decrease in enzyme activity ensues at blood lead concentrations $>0.4 \mu\text{M/L}$ ⁽¹⁾ followed by a tendency to plateau

(1) This value is difficult to ascertain and can only be considered as approximate.

FIGURE 10A: Changes in a Rabbit's Blood δ -ALA-D pH-Activity Profile with Increasing Blood Lead Levels
(o) 0.1 $\mu\text{M Pb}^{2+}/\text{L}$ (Δ) 1.5 $\mu\text{M Pb}^{2+}/\text{L}$
(∇) 2.1 $\mu\text{M Pb}^{2+}/\text{L}$

Figure 10B: Relationship Between Residual Rabbit Red Blood Cell δ -ALA-D Activity Measured at Different pH Values and Blood Lead Levels.

at blood lead levels $>2.0 \mu\text{M/L}$. A more gradual decrease in enzyme activity with increasing blood lead levels occurs at pH 6.0.

Although it was not possible to conduct a similar experiment with human subjects, a closely analogous test was achieved by collecting blood samples from firing range instructors before and after a shooting exercise.

The pH-activity profiles for the enzyme in the blood of a non-exposed individual, blood lead $0.43 \mu\text{M/L}$, and in the blood of two exposed instructors, blood lead $0.87 \mu\text{M/L}$ and $1.59 \mu\text{M/L}$ respectively, shown in Figure 11A, display a pH-optimum shift from 6.4 to 6.0.

On close examination, the three pH-activity profiles selected show an apparently anomalous relationship. Namely, the enzyme activity measured at certain pH values (pH 6.0, for instance) in blood containing relatively high lead concentrations (0.87 or $1.59 \mu\text{M/L}$) is significantly ($P < 0.01$) higher than that measured, at the same pH, in the control subject ($0.43 \mu\text{M/L}$). This frequent occurrence could be attributed to individual genetic variation in pH-activity profiles and is likely responsible for the usual intractable scattergrams obtained by researchers who relate blood leads to the enzyme's activity. It appears likely that, had the profiles been obtained for the blood of a single individual exposed to progressively higher lead concentrations, a set of curves similar to that of the rabbit (Figure 10A) would have been obtained.

FIGURE 11A: Erythrocytic δ -ALA-D pH-Activity Curves for Firing Range Instructors Exposed to Lead (●) 0.43 $\mu\text{M Pb/L}$ (○) 0.87 $\mu\text{M Pb/L}$ (▲) 1.59 $\mu\text{M Pb/L}$

FIGURE 11B: Relationship Between δ -ALA-D Activity in Human Blood Measured at Various pH Values and Blood Lead Levels.

The relationship between blood lead concentrations and residual enzyme activity measured at pH 6.0, 6.4, and 6.8 (Figure 11B) although admittedly obtained under less controlled circumstances than with rabbits, follows a pattern very similar to the latter. However, the pH-optimum shifts and the rapid decrease in enzyme activity at pH's 6.4 and 6.8 happen at higher blood lead levels, $>0.8 \mu\text{M/L}$, and the tendency to plateau occurs at $>3.0 \mu\text{M/L}$ blood lead.

This difference in sensitivity to lead between the two species could be attributed to an established adaptation of man to his lead-polluted environment as compared to rabbits who reside in a controlled relatively lead-free environment. This adaptation has been suggested by Hapke et al. (114) who regarded the enzyme patterns of all organisms to be under the influence of lead and the gradual changes in lead intake as being compensated by healthy organisms. Conversely, the observed difference could be due to a species-related difference in sensitivity to lead insult.

"In vitro". According to the results of Table I, blood specimens can be contaminated by lead present in devices used to collect and store blood samples. The question, therefore, arises as to whether this exogenous lead can influence the pH-activity profile of δ -ALA-D of blood stored for extended periods (>2 weeks) and at low temperature (4°C) as required in the proposed protocol. To date, few studies have looked at the effects of "in vitro" lead on the erythrocytic δ -ALA-D pH-activity profile, and none has been conducted

using the conditions mentioned. Most studies have involved the addition of various solutions of a lead salt (acetate, chloride, nitrate) ⁽¹⁾ to blood or its haemolysate and incubation of the mixture at 4, 25, or 37 °C for 0, 0.25, 0.50, 0.75, 1, 3, 6 or 14 hours before performing the enzyme assay at pH 6.4, 6.6, 6.7, 7.0 or over a pH range (43, 58-64, 76, 92, 114-116). Invariably, results have indicated a dose-related inhibition of the enzyme and a reduction in enzyme activity which was significantly less than that obtained "in vivo" for identical blood lead concentrations. However, no pH-optimum shift of the magnitude shown in Figure 11A was ever observed (62, 92).

An attempt was made to duplicate a gross contamination of a blood sample as follows: 30-mL blood specimens were collected from three control donors; the samples were combined, split into 10-mL aliquots and each aliquot was placed into polyethylene tubes containing saline lead acetate solutions of progressively increasing concentrations; the samples were mixed for 1 hour at 4 °C and subdivided into 10 equal portions which were placed in plastic micro tubes; all samples were stored undisturbed at 4 °C until assayed. A pH-activity curve was obtained for δ -ALA-D at each blood lead concentration after 16 hours, 2, 4, 7, 10, 14, and 20 days of storage. Two samples from each blood lead concentration set were fractioned into plasma and erythrocytic cytosol to determine the distribution of exogenous lead in whole blood. Plasma was removed

(1) The particular anion used apparently makes no difference to the action of lead (64).

from the remaining sample in each set, the red cells washed with cold saline solution and the pH-activity of the erythrocytic enzyme derived in the usual fashion to determine if plasma-borne lead played a role in the effects observed. The results of one of the several tests performed are shown in Figures 12A to 12D inclusively. Thus, after only 16 hours of contact, there occurs a progressive shift of the pH optimum from its initial position at 6.5 to 6.1 and a significant ($P < 0.01$) decrease in enzyme activity concomitant with increasing exogenous lead concentrations. As storage time lengthens, an optimum shift to 6.0 and a change in the overall shape of the profile analogous to that observed for "in vivo" lead take place at ever lower concentrations of adventitious metal. The optimum shift and overall profile shape, usually observed in the blood of lead intoxicated individuals, are approximated at all concentrations of exogenous lead after 20 days of contact at 4 °C (Figure 12D). Note the apparent activation of the enzyme at pH's 6.0 at all concentrations after 16 hours of contact. This activation is only observed at low concentrations as the time of contact increases.

The effects of "in vitro" lead concentrations on the enzyme's activity, measured at pH's 6.0, 6.4, and 6.8, after 1, 4, 10, and 20 days of contact at 4 °C, are illustrated in Figure 13. The relationship between residual enzyme activity, measured at pH 6.4 after 20 days, and exogeneous lead concentrations, correspond

FIGURE 12: Effects of Varying Concentrations of Lead on Normal Human Blood ALA-D "In Vitro", After 16 Hours (A), 7 Days (B), 14 Days (C), and 20 Days (D) of contact at 4 °C

(●) Control Blood 86 μM Zn, 14 μM Cu, 0.53 μM Pb/L; (▲) + 0.41 μM ; (Δ) + 0.81 μM ; (○) + 1.22 μM ; (◻) + 2.43 μM ; (◻) + 3.24 μM ; (x) + 4.05 μM ; and (T) + 8.10 μM Pb^{2+} /L Blood added.

FIGURE 12: Continued.

FIGURE 13: Effect of Increasing Concentrations of Pb^{2+} Added to Normal Human Blood on the Activity of its δ -ALA-D as Measured at Different pH Values After Varying Periods of Storage. Each Point Represents the Mean of Results Obtained From Three Separate Trials.

closely with that obtained by Davis and Avram⁽¹⁾ (115). As was discovered earlier by Mauras and Allain (64), the reduction in the enzyme's activity becomes more pronounced with time of contact. Eventually it approximates that obtained "in vivo" (Figure 11B). These workers and others (116) had also found that the inhibitory effect of "in vitro" lead could be enhanced by incubation at higher temperatures (37 °C).

It is interesting to observe that, although most of the "in vitro" Pb²⁺ investigations were performed under different sets of conditions (Table III), the results nevertheless compare favorably. The differences in the extent of enzyme inhibition obtained could be imputed to the selection of either whole blood or its lysate for the test, the pH of measurement, and the temperature and duration of the incubation period employed or combinations of some of these factors.

The present test results and those obtained by Roëls et al. (43) indicate that lead "in vivo" causes a significantly ($P < 0.01$) larger inhibition of the enzyme than lead "in vitro" at identical blood lead concentrations. Conversely, results obtained by Mauras and Allain (64) and others (63) appear to show that the exact opposite is true. It is quite likely that this apparent disagreement is the result of the selection of different test conditions.

(1) Davis and Avram measured enzyme activity at pH 6.7 in P-C buffer, a condition which corresponds approximately to measurement at pH 6.4 in P-P buffer.

TABLE III: Studies of the Interactions of Pb^{2+} "In Vitro" with Erythrocytic δ -ALA-D - Summary and Comparison of the Experimental Conditions and Results Obtained.

Reference		Pb^{2+} Addition		δ -ALA-D Assay Buffer	pH	δ -ALA-D Inhibition
		Media	Temp. Time			
Thompson (63)	(1)	RBC	4 °C 18 h	P-P	7.0	38% at 5.0 $\mu M/L Pb^{2+}$ 85% at 15 $\mu M/L Pb^{2+}$
Oavis (112)		Haemolysate	4 °C 18 h			
			---	P-C	6.7	50% at 1.9 $\mu M/L Pb^{2+}$ 90% at 9.3 $\mu M/L Pb^{2+}$
Davis (115)		Haemolysate	37 °C 1 h	P-C	6.7	50% at 0.62 $\mu M/L Pb^{2+}$
Mauras (64)	(1)	Blood	4, 25, 37 °C, 0, 1, 3, 4, 6, 14, 24 h	P-C	6.7	50% at 0.96 $\mu M/L Pb^{2+}$ (14 h at 25 °C)
Meredith (59)		Haemolysate (2)	---	P-P	6.6	60% at 1.95 $\mu M/L Pb^{2+}$
Roels (43)	(3)	Blood	37 °C 45 min. 20 °C 15 min. 4 °C 16 h	P-P	6.4	50% at 5.31 $\mu M/L Pb^{2+}$
Raphe (114)		Haemolysate	37 °C 15 min.	P-P	6.8	50% at 1.5 $\mu M/L Pb^{2+}$
Despaux (117)		Purified Enzyme	37 °C 10 min.	P-P	6.6	52% at 1.5 $\mu M/L Pb^{2+}$
Farant (3)		Blood	4 °C 20 days	P-P	6.4	50% at 2.25 $\mu M/L Pb^{2+}$

(1) δ -ALA-D Inhibition "In vitro" > "In vivo"

(2) Rat Blood

(3) δ -ALA-D Inhibition "In vivo" > "In vitro"

The progressive shift of the pH optimum with increasing exogenous Pb^{2+} levels and time of contact, observed in Figures 12A-D, is as yet unexplained. The shift is, without doubt, caused in large part by erythrocyte-borne lead, as the results shown in Figure 14 attest. One possible explanation for the delayed shift phenomenon would be that plasma-borne lead, bound to albumin and globulin (118) or as a peptized lead phosphate sol (119), encounters difficulties in penetrating the erythrocytic membrane and gaining access to the enzyme in the cytosol. However, the data shown in Table IV ⁽¹⁾ do not support this contention. The apportion of lead between the erythrocytic cytosol and the plasma does not change measurably after 7 days contact. An equilibrium in the distribution of lead in blood is apparently established very early on; and, this is in full agreement with the results reported by Barltrop and Smith (120) and Mortensen and Kellog (121) whereby equilibrium is achieved within 15 minutes of lead addition to blood. Thus it would appear that the delay in observing the pH-optimum shift is not related to the effective amount of lead within the erythrocytic membrane. Since over 90% of the lead in the erythrocytic cytosol

(1) These results are in apparent disagreement with those obtained by others such as Roëls et al. (43), and Ong and Lee (118), which indicate that over 80% of the lead is located within the erythrocyte regardless of the amount of lead added, and the results of this test and those of Mortensen and Kellog (124), which indicate that the incorporation of lead by erythrocyte appears dose-related i.e. proportionally decreases with increasing amounts of lead added. Mortensen and Kellog (121) also reported that the process of uptake by the erythrocytes was reversible and markedly temperature dependent. Since Roëls and Ong both incubated whole blood with the lead salt for 45 and 90 minutes respectively during the incorporation step and that, in an attempt to duplicate gross contamination, this procedure was not adopted here, it is highly probable that the difference in the results observed is attributable to a difference in experimental approach.

FIGURE 14: Effect of Varying Concentrations of Erythrocyte-Borne Lead on δ -ALA-D 10 days After Addition of Pb^{2+} to Blood.

(●) Control Blood 0.58 μM Pb/L
 Test Blood (Δ) 1.5 μM (△) 1.9 μM
 (○) 2.96 μM , (■) 5.99 μM Pb^{2+} added/L Blood.

TABLE IV: Effect of Storage at 4 °C Upon the Distribution of Varying Concentrations of Pb²⁺ Added to Human Blood.

Sample No.	Hct. %	Amount Added (μM/L)	Whole Blood Concentration (μM/L)	Plasma Concentration (μM/L)	Plasma/Whole Blood %	Erythrocyte Concentration (μM/L)	Erythrocyte/Whole Blood %
<u>Day 1</u>							
H-660	42	8.10	8.58 ± 0.5	7.74 ± 1.2	64.3	5.99 ± 0.5	29.3
		6.48	6.80 ± 0.6	6.94 ± 0.8	59.2	3.94 ± 0.2	24.3
		4.05	4.05 ± 0.6	3.75 ± 0.2	53.7	2.96 ± 0.2	30.7
		1.62	1.37 ± 0.2	1.48 ± 0.1	52.7	1.63 ± 0.1	49.9
		0.41	0.43 ± 0.2	0.45 ± 0.2	43.9	0.72 ± 0.1	70.3
		----	0.24 ± 0.1	N.D.	----	0.58 ± 0.1	----
<u>Day 7</u>							
H-660	42	8.10	9.01 ± 0.9	8.09 ± 0.1	54.7	5.37 ± 0.3	26.3
		6.48	6.53 ± 0.7	6.06 ± 0.2	51.7	4.05 ± 0.2	25.0
		4.05	3.95 ± 0.7	3.75 ± 0.2	53.7	3.18 ± 0.2	33.0
		1.62	1.32 ± 0.2	1.39 ± 0.2	58.8	1.45 ± 0.1	44.5
		0.41	0.51 ± 0.1	0.42 ± 0.1	47.8	0.72 ± 0.1	59.3

is reportedly attached to the haemoglobin molecule (118), one could postulate that lead is exchanged between this molecule and the less abundant δ -ALA-D molecule via a time-dependent (and possibly temperature-dependent) process. "In vivo", the situation is clearly different--over 98% of the affordable lead is located within the erythrocyte (122) and the temperature is maintained at a comparatively higher 37 °C. Thus, it would appear reasonable that a progressive shift in pH optimum is not observed in this case.

Possible reasons for the occurrence of the apparent pH optimum shift will be dealt with later.

Copper

The biochemical role of copper as an essential part of important metalloenzymes and other metalloproteins is well known. Furthermore, much is known of the manner of its absorption, distribution in the body, and excretion (123).

In blood, caeruloplasmin represents 95% of the plasma copper and its level reflects disorders of copper metabolism such as hepatolenticular disease (Wilson's disease). Significant increases in blood copper levels have been observed in such diseases as lymphoma, rheumatoid arthritis, myocardial infarction, cirrhosis of the liver, carcinoma of the prostate, in eclamptic patients and in women during pregnancy (124). Blood copper levels can also increase following occupational exposure to copper fumes during the refining, smelting and welding of the metal. Copper deficiency in humans has never been reported.

The effect of increased copper concentration on erythrocytic δ -ALA-D and its pH-activity profile has never been studied "in vivo". The results of such a study are reported in the following.

"In vivo". Human subjects occupationally exposed to copper were not available for this study, and rabbits injected subcutaneously with solutions of copper glycinate were used instead. The effects of a 2.5-fold rise in blood copper concentration on the enzyme's pH-activity curve are shown in Figure 15A. Two effects are observed: firstly, a shift in the pH optimum from 6.35 to 6.60 and secondly, a significant decrease in the enzyme's activity measured at pH 6.4. Higher concentrations of copper produce still larger shifts of the pH optimum and a more pronounced reduction of the enzyme's activity.

FIGURE 15A: Rabbit Erythrocytic δ -ALA-D pH-Activity Relationship, before (●, 12.6 $\mu\text{M Cu/L}$) and after (Δ, 31.5 $\mu\text{M Cu/L}$) Subcutaneous Injection with Copper Glycinate.

FIGURE 15B: Effect of "In Vivo" Cu on Rabbit Erythrocytic δ -ALA-D Activity Assayed at Varying pH Values.

The shift in the pH optimum and reduction of enzyme activity are not accompanied by an overall change in the shape of the profile as is observed with lead-poisoned δ -ALA-D.

Figure 15B shows changes in the residual enzyme activity, as measured at pH 6.0, and 6.8 (arbitrarily selected), with rising blood copper levels. Thus, contrary to lead, one observes a more gradual reduction in enzyme activity with increasing blood copper concentrations. Moreover, larger reductions are obtained at acid pH 6.0 than at the more basic pH 6.8.

The distribution of copper in rabbit whole blood with increasing copper concentrations is listed in Table V. The results clearly indicate that as copper levels rise from a normal $11.1 \pm 2.5 \mu\text{M/L}$ to $135 \mu\text{M/L}$, plasma copper concentrations rise from $12.9 \pm 4\%$ to 97% of whole blood total copper. Conversely, the amount of copper in the erythrocytic cytosol drops from $32.8 \pm 7.8\%$ of whole blood to 12.4% . Since most of the copper remains plasma-bound, probably as part of caeruloplasmin and other proteins, it seemed appropriate to determine whether this plasma-copper played an important role in the inactivation of the enzyme. The pH-activity profile of δ -ALA-D in saline-washed erythrocytes from blood containing $106 \mu\text{M Cu/L}$ is shown in Figure 16. Hence, it is readily seen that the effects of copper upon the enzyme are largely attributable to copper located in the plasma which gains access to the erythrocytic enzyme following haemolysis.

"In vitro". Despite the low level of copper contamination found in blood collection devices (Table I), the inadvertent introduction

TABLE V: Distribution of Endogenous Cu in Rabbit Blood

Sample No.	Hct. %	Amount Injected (mg/kg)	Whole Blood Concentration ($\mu\text{M/L}$)	Plasma Concentration ($\mu\text{M/L}$)	Plasma/Whole Blood $\bar{x}$	Erythrocyte Concentration ($\mu\text{M/L}$)	Erythrocyte/Whole Blood $\bar{x}$
R-280	45	---	6.30	7.60	66.3	6.02	42.9
R-242	45	---	8.22	9.41	63.0	7.83	42.8
R-230	39	---	8.80	8.22	56.8	9.51	42.1
R-221	39	---	9.44	10.1	63.5	8.71	36.1
R-240	42	---	9.41	10.1	62.3	8.80	39.3
R-227	42	---	10.1	10.1	58.0	8.82	36.6
R-234	42	---	10.1	10.7	61.4	10.8	44.9
R-232	33	---	11.3	12.6	74.7	11.3	33.0
R-256	33	---	11.3	11.3	67.0	7.40	21.6
R-266	41	1.0	12.0	14.4	70.1	6.80	23.2
R-218	45	---	12.6	14.5	63.3	8.10	28.9
R-237	45	---	12.6	17.6	76.8	6.82	24.3
R-267	42	---	12.6	14.5	67.2	10.2	34.0
R-278	43	---	13.2	19.5	63.5	8.10	26.4
R-260	40	1.0	13.8	17.6	76.5	9.82	28.4
R-243	45	1.0	14.5	16.7	63.3	8.40	26.1
R-219	39	---	14.5	19.5	82.0	8.72	23.4
R-265	41	1.0	14.5	14.5	59.0	10.8	30.5

TABLE V: Distribution of Endogenous Cu in Rabbit Blood (cont.)

Sample No.	Hct. %	Amount Injected (mg/kg)	Whole Blood Concentration ($\mu\text{M/L}$)	Plasma Concentration ($\mu\text{M/L}$)	Plasma/Whole Blood %	Erythrocyte Concentration ($\mu\text{M/L}$)	Erythrocyte/Whole Blood %
R-262	44	1.0	14.5	18.3	70.7	6.14	18.5
R-264	42	---	15.7	17.3	63.9	9.54	25.4
R-261	33	1.0	17.0	20.1	79.2	10.8	21.0
R-268	44	1.0	17.6	21.4	68.1	9.13	22.8
R-263	44	1.0	19.5	25.7	73.8	8.84	19.9
R-241	41	0.5	20.1	26.4	77.5	10.2	20.8
R-228	42	1.0	21.4	26.8	72.6	11.2	22.0
R-254	41	1.0	22.7	30.0	78.0	11.5	20.8
R-257	42	1.0	22.7	29.0	74.1	12.2	22.6
R-275	44	2.5	23.2	32.7	78.9	15.6	29.6
R-216	41	1.0	23.6	33.0	82.5	16.2	28.1
R-238	45	1.0	24.6	35.3	78.9	8.83	16.1
R-281	42	2.5	24.6	39.7	67.8	12.9	22.0
R-259	46	1.0	24.6	37.8	83.0	7.43	13.8
R-235	42	1.5	25.8	34.6	77.8	12.2	19.9
R-258	43	1.0	26.4	39.7	85.7	8.10	13.2
R-217	39	1.0	26.4	31.0	71.6	16.9	25.0
R-233	33	1.5	27.1	34.6	85.5	13.4	16.3

TABLE V: Distribution of Endogenous Cu in Rabbit Blood (cont.)

Sample No.	Hct. %	Amount Injected (mg/kg)	Whole Blood Concentration (μM/L)	Plasma Concentration (μM/L)	Plasma/Whole Blood $\bar{x}$	Erythrocyte Concentration (μM/L)	Erythrocyte/Whole Blood $\bar{x}$
R-255	38	1.0	32.7	45.3	85.9	9.55	11.0
R-277	43	1.5	46.0	77.4	95.9	12.9	12.1
R-231	39	1.0	47.2	71.0	91.8	13.5	11.2
R-276	44	2.5	95.7	148.	86.6	40.6	18.7
R-282	41	2.5	106.	178.	99.1	26.4	10.2
R-279	45	2.5	135.	238.	97.0	37.2	12.4

Control Rabbits

N = 15 -- --- 11.1 ± 2.5 12.9 ± 4.0 66.1 ± 6.9 8.75 ± 1.3 32.8 ± 7.8

FIGURE 16: pH-Activity Profile of Rabbit Erythrocytic δ -ALA-D of Blood Containing $106 \mu\text{M Cu}^{2+}/\text{L}$ Before (Δ) and After ($\square$) the Removal of Plasma; ($\bullet$) Control Blood $55 \mu\text{M Zn}^{2+}$, $7.6 \mu\text{M Cu}^{2+}$ and $0.7 \mu\text{M Pb}^{2+}/\text{L}$ Blood.

of exogenous copper into a blood specimen or the reaction mixture itself is always a possibility. Therefore, tests involving gross contamination of a blood sample were conducted in the manner described for lead to determine the effects of prolonged contact with the metal at low temperature. The results for 16 hours and 13 days of storage are shown in Figure 17 A and B. A gradual shift of the pH optimum from 6.25 to 6.6 and a progressive reduction in enzyme activity is observed with increasing adventitious copper concentration after 16 hours of contact. Only slight changes in pH-optimum and profile shape occur with time of contact. Unlike lead "in vitro", the pH-activity profiles for "in vitro" copper closely resemble those obtained "in vivo" at the very onset of contamination.

A plot of residual enzyme activity versus blood copper concentrations, shown in Figure 18, indicates that, although enzyme reduction increases with time of contact at pH 6.8, it reverts to original values at pH 6.0 after lengthy storage. The slight shift of the profile to the left may be responsible for this result.

It is worth noting that Thompson et al. (63) have obtained a similar non-linear inhibition of blood δ -ALA-D by copper and observed a 90% inhibition of the enzyme at a concentration of 94 $\mu\text{M/L}$, using the experimental conditions listed in Table IV. Although Despaux et al. (117) have reported a no-effect level of 0.3 $\mu\text{M Cu}^{2+}/\text{L}$, Davis and Avram (116) have found significant

FIGURE 17: Addition of Varying Concentrations of Copper to Human Blood and its Effects on Erythrocytic δ -ALA-D after 16 Hours (A) and 13 Days (B) of Storage at 4 °C.

(●) Control Blood - 79.8 μM Zn, 0.48 μM Pb, 11.3 μM Cu/L
 (▲) 0.54 μM , (▲) 1.08 μM , (○) 3.10 μM , (■) 13.2 μM , and 33.0 μM Cu^{2+} /L blood added.

FIGURE 18: Effect of Copper "In Vitro" on the Activity of Human Erythrocytic δ -ALA-D Measured at Specific pH Values After Varying Periods of Storage at 4 °C. Every Point is the Mean of Two Separate Trials.

inhibition at 0.93 $\mu\text{M/L}$ as well as a 50% inhibition of the enzyme at 2.5 $\mu\text{M Cu}^{2+}/\text{L}$. The reasons for the disparities between these few studies of the action of Cu^{2+} on $\delta\text{-ALA-D}$ could very well be the same as those discussed earlier on for Pb^{2+} "in vitro" that is, differences in experiment conditions.

Comparison of Figures 15A and B and 17A and 18, reveals that copper "in vitro" apparently can effect a larger reduction in the enzyme's activity than "in vivo" at similar copper concentrations. For instance, at pH 6.0, the enzyme is 50% inhibited by 8 μM exogeneous Cu^{2+}/L after 13 days of contact, while at least 20 $\mu\text{M/L}$ of the metal "in vivo" is required to achieve the same inhibition. Notwithstanding the fact that human and rabbit blood are being compared ⁽¹⁾, a close examination of the distribution of copper in rabbit whole blood Table V and in human blood after addition of copper (Table VI), indicates that copper is apportioned between plasma and the erythrocytic cytosol in approximately the same amounts in both cases. Hence, the reason(s) for the more potent "in vitro" Cu^{2+} effect on $\delta\text{-ALA-D}$ must lie elsewhere.

(1) Copper is similarly distributed between plasma and erythrocytes in the normal blood of both species; and, in fact, a few preliminary "in vitro" copper trials performed with rabbit blood yielded results similar to that obtained with human blood.

TABLE VI: Distribution of "In Vitro" Cu^{2+} in Human Blood After Varying Periods of Storage at 4 °C.

Sample No.	Hct. %	Amount Added ($\mu\text{M/L}$)	Whole Blood Concentration ($\mu\text{M/L}$)	Plasma Concentration ($\mu\text{M/L}$)	Plasma/Whole Blood %	Erythrocyte Concentration ($\mu\text{M/L}$)	Erythrocyte/Whole Blood %
<u>Day 1</u>							
H-660	42	33.1	41.2 ± 1.2	58.1 ± 3.0	81.7	10.4 ± 0.7	10.7
		6.60	17.7 ± 0.8	22.7 ± 0.7	74.4	8.52 ± 0.3	20.2
		1.61	13.5 ± 0.7	14.5 ± 1.2	64.2	8.50 ± 0.4	26.4
		0.53	13.0 ± 0.7	15.7 ± 0.8	70.1	8.03 ± 0.3	25.9
		0.27	12.2 ± 0.9	13.2 ± 0.4	62.8	8.50 ± 0.4	29.3
		----	11.3 ± 0.9	12.4 ± 1.05	63.6	7.93 ± 0.86	29.5
<u>Day 7</u>							
H-660	42	33.1	42.1 ± 0.6	52.9 ± 2.1	72.8	17.9 ± 0.7	17.9
		6.60	18.2 ± 0.6	22.0 ± 1.5	70.1	11.8 ± 0.6	27.2
		1.61	17.1 ± 0.2	19.8 ± 1.9	67.2	11.3 ± 0.6	27.8
		0.53	13.2 ± 0.3	13.2 ± 1.1	58.0	10.4 ± 0.3	33.1
		0.27	12.5 ± 0.7	13.8 ± 1.5	64.0	7.90 ± 0.8	26.5
		----	11.5 ± 0.9	11.7 ± 1.31	59.0	8.80 ± 1.1	32.1

Zinc

Like several other trace elements, zinc functions as both a structural component of many metalloenzymes (125) and as a co-factor of several enzymes. Zinc ions can either activate or inhibit enzyme processes and are also required in the synthesis of nucleic acids.

Zinc's metabolic role and metabolism have been the subject of several reviews (126, 127) and, its uptake, distribution and excretion have been studied extensively (128).

The primary disorder of zinc metabolism is not known in man. Rare cases of zinc deficiency diseases do occur, however, and these include pernicious anemia, hypogonadism, dwarfism, geophagia and alcoholic cirrhosis of the liver. Abnormal levels have also been related to carcinoma of the prostate, wound healing, atherosclerosis, acute and chronic infections and, in women taking oral contraceptives (124).

Study of the effect of zinc on erythrocytic δ -ALA-D, either "in vivo" or "in vitro", has almost invariably been conducted at a select pH value; and, as yet, no thorough investigation has been made of the metal's influence on the shape of the enzyme's pH-activity curve. Such an investigation is reported in this section.

"In Vivo". Blood samples collected from workers exposed to zinc were available for this study. Unfortunately, these workers were also concurrently exposed to either lead, cadmium, methyl mercury or a combination of these. As was the case for the lead and copper "in vivo" studies, the investigation was conducted with rabbits

injected subcutaneously with solutions of zinc lactate. (1)

Two significant changes are evident in the pH-activity profile of erythrocytic δ -ALA-D in the blood of a rabbit showing a 1.5-fold increase in its whole blood zinc level (Figure 19A): a marked increase in enzyme activity at all pH values and an apparent shift of the pH optimum from pH 6.5 to pH 6.0. It would appear then that both lead and zinc have the ability to shift the pH maximum to pH 6.0; however, the effect of the two divalent metal ions is divergent namely, lead strongly inhibits whereas zinc strongly activates the enzyme at pH 6.0.

The greatest increase in δ -ALA-D activity and blood zinc level usually occurred within the first hour after injection. The level of zinc in blood and erythrocytic δ -ALA-D gradually diminished thereafter such that both had almost returned to pre-injection level 24 hours later.

For reasons stated earlier, fewer results than anticipated were obtained from the rabbit zinc "in vivo" study. Nevertheless, it was possible to establish a relationship between net "in vivo" zinc blood concentration-increases and residual enzyme activity at arbitrarily selected pH values, 5.9, 6.4, and 6.8. The relationships obtained and illustrated in Figure 19B, indicate the following:

(1) Repeated subcutaneous injection of this solution at various sites on the back of several rabbits used specifically for the "in vivo" zinc study, resulted in the appearance of large bleeding sores which took months to heal. This effect, also observed by Haeger-Aronsen (65), greatly reduced the number of results which could be obtained from this group of rabbits.

FIGURE 19A: Elevated Whole Blood Zinc and its Effects on Rabbit Erythrocytic δ -ALA-D Before (▲) and After (■) Washing Erythrocytes with 0.9% Saline. (●) Control Blood 57 μ M Zn, 6.8 μ M Cu, 0.43 μ M Pb/L (▲) 88 μ M Zn/L (■) Saline Washed Erythrocytes.

FIGURE 19B: Changes in Residual δ -ALA-D Activity with Net Increases in Endogenous Whole Blood Zinc.

- 1) at pH 5.9 - an initial marginal decrease in enzyme activity with small blood zinc level increases; a rapid rise in δ -ALA-D activity as blood zinc levels increase by 40 μ M/L; and a tendency for the enzyme's activity to plateau at further increases in blood zinc >40 μ M/L. 2) At pH 6.4 - a smaller drop in enzyme activity is observed as blood zinc levels increase by 10 μ M/L, followed by a more modest rise in activity until blood zinc concentrations increase 30 μ M/L and a tendency for the activity to plateau thereafter. 3) At pH 6.8 - no decrease in δ -ALA-D and a tendency for the activity to plateau after a very moderate rise in enzyme activity as blood zinc levels attain a concentration >10 μ M/L above normal.

Whenever increases in a divalent ion's concentration cause changes in δ -ALA-D's pH-activity profile, the question may be asked as to whether the changes noted can be imputed to either the metal in plasma, which interacts with the erythrocytic enzyme following lysis or, the metal already in the cytosol or both. In Figure 19A, one observes that the profile obtained from erythrocytes washed free of plasma demonstrates most of the effects noted for whole blood. Therefore, it would appear that zinc in erythrocytes, like lead, is, in large part, responsible for the pH-optimum shift and enzyme activation observed. Plasmic zinc, nonetheless, also contributes significantly following haemolysis. These results are supported by the data for the distribution of zinc in rabbit whole blood listed in Table VII. A cursory examination of the table reveals that, as whole blood zinc values increase more than three fold, plasmic zinc likewise increases markedly and progressively

TABLE VII: Distribution of Varying Concentrations of Zinc in Rabbit Whole Blood

Sample No.	Hct. %	Amount Injected (mg/kg)	Whole Blood Concentration (µM/L)	Plasma Concentration (µM/L)	Plasma/Whole Blood $\bar{x}$	Erythrocyte Concentration (µM/L)	Erythrocyte/Whole Blood $\bar{x}$
R-230	39	---	45.9	22.3	29.6	89.5	76.0
R-241	41	---	47.4	31.8	39.6	84.2	72.8
R-231	39	---	49.0	24.8	30.8	91.4	72.7
R-237	45	---	49.0	28.1	31.5	86.2	79.2
R-240	42	---	50.5	36.7	42.2	82.6	68.7
R-228	42	---	50.5	23.3	26.7	77.0	64.0
R-255	38	---	51.0	24.2	29.4	83.9	62.5
R-265	41	---	52.0	27.8	31.5	89.3	70.4
R-242	45	---	52.0	32.1	33.9	94.0	81.0
R-181	37	0.2	52.0	23.8	28.8	103.	73.3
R-227	42	---	52.0	27.5	30.7	77.6	62.7
R-232	33	---	52.0	26.0	33.5	122.	77.4
R-281	42	3.0	52.0	24.0	26.8	89.0	71.9
R-178	37	0.2	53.0	26.7	31.7	103.	71.9
R-257	42	---	53.2	31.2	34.0	80.6	63.6
R-264	42	---	54.5	28.8	30.6	79.5	61.3
R-234	42	---	55.1	24.5	25.8	113.	86.4
R-235	42	---	55.1	26.0	27.4	90.4	68.9

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TABLE VII: Distribution of Varying Concentrations of Zinc in Rabbit Whole Blood (cont)

Sample No.	Hct. %	Amount Injected (mg/kg)	Whole Blood Concentration ($\mu\text{M/L}$)	Plasma Concentration ($\mu\text{M/L}$)	Plasma/Whole Blood %	Erythrocyte Concentration ($\mu\text{M/L}$)	Erythrocyte/Whole Blood %
R-217	39	---	56.0	30.6	33.3	93.5	65.1
R-282	41	3.0	56.0	27.0	28.4	89.0	65.2
R-243	45	---	56.6	34.3	33.3	94.0	74.7
R-216	41	---	57.0	35.1	36.3	87.0	62.6
R-263	44	---	57.8	32.1	31.7	84.2	62.6
R-238	45	---	58.1	45.0	42.6	92.1	71.3
R-254	41	---	58.1	27.8	28.2	86.8	61.3
R-256	33	2.0	62.0	43.4	46.9	96.0	51.0
R-258	43	2.0	63.6	40.4	36.2	89.8	60.7
R-267	42	2.0	64.2	45.6	41.2	87.2	57.0
R-266	41	2.0	64.5	41.6	38.1	65.8	41.8
R-262	44	2.0	67.3	47.1	39.2	70.1	45.8
R-259	46	2.0	68.8	50.2	39.7	86.8	58.0
R-261	33	2.0	69.8	54.5	52.3	104.	49.2
R-233	33	1.5	70.4	47.4	45.0	111.	52.0
R-275	44	3.0	75.0	61.0	45.5	101.	59.3
R-268	44	2.0	75.0	43.4	32.4	82.3	48.3
R-260	40	2.0	75.0	61.2	49.0	94.1	50.2

TABLE VII: Distribution of Varying Concentrations of Zinc in Rabbit Whole Blood (cont.)

Sample No.	Hct. %	Amount Injected (mg/kg)	Whole Blood Concentration (μM/L)	Plasma Concentration (μM/L)	Plasma/Whole Blood $\bar{x}$	Erythrocyte Concentration (μM/L)	Erythrocyte/Whole Blood $\bar{x}$
R-221	39	1.0	88.0	85.7	59.4	84.1	37.3
R-276	44	3.0	88.0	75.0	47.7	108.	54.0
R-280	45	4.0	88.0	80.0	50.0	102.	52.2
R-278	43	3.0	91.0	85.0	53.2	102.	48.2
R-279	45	4.0	94.0	95.0	55.6	109.	52.2
R-277	43	3.0	100.	97.0	55.3	109.	46.9
R-218	45	2.0	106.	125.	64.9	92.5	39.3
R-219	39	3.0	148.	174.	71.7	110.	28.9

Control Rabbits

N = 21 -- --- 52.9 ± 3.6 29.5 ± 5.3 32.5 ± 4.6 89.5 ± 10. 69.8 ± 7.2

(from 29.6 to 71.7% of total value). Conversely, erythrocytic zinc, although showing a definite, but modest and uneven, increase in concentration, eventually contributes a lower proportion of total blood zinc content (from 81% to 28.9% of total value). The present findings are consistent with the animal studies of Haeger-Aronsen et al. (65) who noted a positive correlation between erythrocytic δ -ALA-D activity and plasmic zinc but were unable to demonstrate any correlation between enzyme activity and erythrocytic zinc concentrations.

"In vitro". In view of the gross zinc contamination of blood collected in brown- and green-top Vacutainers at the beginning of this study (Table I), it was imperative that the effect of "in vitro" zinc on erythrocytic δ -ALA-D be investigated. The levels of zinc selected for the study were chosen to duplicate conditions to be expected "in vivo" with individuals such as smelter workers (1) exposed to zinc fumes. Thus, various amounts of zinc in saline solution were added to aliquots of a common human blood sample as described earlier for lead and copper and the pH-activity profile of δ -ALA-D in the zinc-poisoned aliquots determined after 16 hours and 14 days of storage at 4 °C. The results of one of the tests are shown in Figures 20A and B respectively. After 16 hours of contact, zinc activated the enzyme at pH values <6.4 at the various levels tested, the degree of activation being proportional to the

(1) "In vivo" levels encountered often exceed double normal levels of blood zinc i.e. approximately $160 \mu\text{M/L Zn}^{2+}$ in whole blood ().

FIGURE 20: Addition of Varying Concentrations of Zn^{2+} to Human Whole Blood and its Effects on δ -ALA-D After 16 Hours (A) and 14 Days (B) of Storage at 4°C

(●) Control Blood: 77 μM Zn, 11.8 μM Cu, 0.58 μM Pb/L
 (▲) 15.3 μM , (▲) 30.5 μM , (○) 76.5 μM , (●) 153 μM and
 (□) 765 μM Added Zn^{2+} /L Blood

exogenous zinc concentration and being greatest at the lower end of the pH range. The changes wrought on the enzyme's profile by zinc are readily apparent and closely mimic the changes observed with "in vivo" zinc in rabbit blood (Figure 19A) --a gradual shift of the optimum to a more acid pH value, from 6.3 to 5.9 and, a progressive and rapid increase in activity at pH 5.9. Interestingly, Cantrell et al. (62) reported that the enzyme's activity was significantly enhanced without a shift in the pH optimum following the addition of 530 $\mu\text{M/L}$ of zinc to blood haemolysate.

It is noteworthy that, after 16 hours of contact, a drop in enzyme activity takes place at relatively low concentrations of exogenous zinc for pH values >6.4 . While Abdulla et al. (32) have reported a similar slight inhibitory effect at a zinc concentration of 40 $\mu\text{M/L}$, Border et al. (61) failed to observe any inhibition by zinc at a level of 5.4 $\mu\text{M/L}$. It is quite likely that these contradictory results could be explained in terms of the different experimental conditions employed. The inhibitory effect of low level zinc disappears after 14 days of contact and the enzyme's activity is increased at all pH values much as is observed "in vivo". Also, after 14 days of storage, the pH optimum has shifted to pH 5.9 for all concentrations of exogenous zinc.

The relationship between added Zn^{2+} concentrations and residual δ -ALA-D activity, measured at pH's 5.9, 6.4, and 6.8 after 16 hours and 14 days of contact, is illustrated in Figure 21. Keeping in mind that normal physiological zinc levels in human blood, 80 to 120 $\mu\text{M/L}$ (129), are significantly higher than that found in rabbit blood, 40 to 80 $\mu\text{M/L}$, and that the metal ion's

FIGURE 21: Relationship Between Human Erythrocytic δ -ALA-D Activity and the Concentration of Added Zn^{2+} After Varying Periods of Storage at 4°C .

distribution between plasma and erythrocytes is also dissimilar, 1/2 for rabbits as compared to about 1/10 for man, a tentative comparison can be made with results presented earlier for "in vivo" zinc in rabbit blood (Figure 19B). Thus, with "in vitro" Zn^{2+} there occurs a similar but more rapid rise in enzyme activity at pH 5.9 with no initial drop in activity at low zinc levels, and a very similar pattern of activation and inhibition at pH 6.4 and 6.8 (especially for the 16-hours curves). All in all, the curves obtained in both instances are remarkably alike considering that the effects of zinc "in vivo" and "in vitro" on the enzyme are being contrasted using the blood of two species. In the "in vitro" case the marked enhancement of enzyme activation after 14 days of contact could be indicative of a protective-like role for zinc ions against enzyme losses during storage as observed by Haamp and Kriebitzsch (130) and Davis and Avram (116).

A summary of the investigations performed, to the present, on the effects of "in vitro" zinc on erythrocytic δ -ALA-D (Table VIII) exemplifies the many experimental conditions used. Such a diversity of approaches usually leads to equally diverse results and makes a meaningful comparison of data difficult. Nevertheless, the results obtained for this "in vitro" study do conform to some degree with the results listed in Table VIII.

The distribution of "in vitro" zinc in human blood is shown in Table IX. Notwithstanding the inherent difference in distribution of this trace metal in rabbit and human blood mentioned earlier, a similar apportioning of zinc between plasma and erythrocytes occurs with rising zinc levels in the blood of the two species:

TABLE VIII: Summary of the Conditions and Results of Investigations of the Effects of "In Vitro" Zn^{2+} on Erythrocytic δ -ALA-D Activity.

Reference	Zn^{2+} Addition			δ -ALA-D Assay		Effects
	Media	Temp.	Time	Buffer	pH	
Abdulla (32)	Blood	37 °C	4 h	P-P	6.4	(1) 46 μ M/L - slight inhibition 764 μ M/L - 72% (2) 1530 μ M/L - 101%
Border (61)	Haemolysate	37 °C	30 min.	P-P	6.5 7.5	Activation varies with pH Greater at lower pH range; 5.4 μ M/L - minimal activation.
Border (34)	Haemolysate	37 °C	30 min.	P-P	7.0	3.5 μ M/L - 89.8% 350 μ M/L - 137.7% 35 μ M/L - 114.8%
Davis (115)	Haemolysate			P-C	6.7	370 μ M/L - 157% >370 μ M/L - inhibition
Davis (116)	Haemolysate	37 °C	1-4 h	P-C	6.7	Prevented loss of activity

TABLE VIII: Summary of the Conditions and Results of Investigations of the Effects of "In Vitro" Zn^{2+} on Erythrocytic δ -ALA-D Activity (cont.)

Reference	Zn ²⁺ Addition			δ -ALA-D Assay		Effects
	Media	Temp.	Time	Buffer	pH	
Trevfssan (131)	Haemolysate	RT (?)	10 min.	NaHCO ₃	7.0	150 μ M/L - 133% 15 μ M/L - 127%
Thompson (63)	RBC	4 °C	18 h	P-P	7.0	No inhibition noted
	Haemolysate	4 °C	18 h			652 μ M/L - Maximal activation 955 μ M/L - Inhibition begins
Cantrell (62)	Haemolysate	37 °C	10 min.	P-P	6.0-	530 μ M/L - Activation
	(Baboon Blood)				7.5 (incubation pH)	without shift optimum
Meredith (59)	Haemolysate (2)	--	--	P-P	6.6	180 μ M/L - Optimal activation- 240%

TABLE VIII: Summary of the Conditions and Results of Investigations of the Effects of "In Vitro" Zn^{2+} on Erythrocytic δ -ALA-D Activity(cont.)

Reference	Zn^{2+} Addition			δ -ALA-D Assay		Effects
	Media	Temp	Time	Buffer	pH	
Meredith (76)	Blood	37 °C	1 h	P-P	6.6	Inhibition at 74 mM
	Haemolysate	--	--	P-P		200 $\mu M/L$ - 259 %
Farant	Blood	4 °C	16 h	P-P	6.4	80 $\mu M/L$ - 136%
				P-P	6.8	2 $\mu M/L$ - 89%

(1) Concentration of added zinc.

(2) % of control blood δ -ALA-D activity.

TABLE IX: Distribution of Exogenous Zn^{2+} in Human Whole Blood After Varying Periods of Storage at 4 °C

Sample No.	Hct. %	Amount Added ($\mu M/L$)	Whole Blood Concentration ($\mu M/L$)	Plasma Concentration ($\mu M/L$)	Plasma/Whole Blood %	Erythrocyte Concentration ($\mu M/L$)	Erythrocyte/Whole Blood %
<u>Day 1</u>							
H-660	42	153.	240. $\pm$ 8.2	249. $\pm$ 22.	60.2	181. $\pm$ 10.	31.7
		76.5	162. $\pm$ 10.	138. $\pm$ 13.	49.4	167. $\pm$ 15.	43.3
		38.3	110. $\pm$ 12.	58.9 $\pm$ 8.2	31.1	169. $\pm$ 12.	64.5
		15.3	101. $\pm$ 5.6	47.0 $\pm$ 5.6	27.0	175. $\pm$ 15.	72.8
		9.6	94.2 $\pm$ 8.2	36.0 $\pm$ 3.2	22.2	169. $\pm$ 12.	75.4
		----	86.0 $\pm$ 7.8	15.4 $\pm$ 1.7	10.4	165. $\pm$ 4.5	80.6
<u>Day 7</u>							
H-660	42	153.	250. $\pm$ 17.	266. $\pm$ 30.	61.7	229. $\pm$ 9.1	38.5
		76.5	172. $\pm$ 20.	146. $\pm$ 3.6	49.2	207. $\pm$ 2.1	50.5
		38.3	122. $\pm$ 8.3	63. $\pm$ 1.8	30.0	203. $\pm$ 10.	69.9
		15.3	114. $\pm$ 8.4	54.2 $\pm$ 2.1	27.5	197. $\pm$ 12.	72.3
		9.6	115. $\pm$ 9.2	54.1 $\pm$ 5.1	27.2	198. $\pm$ 11.	72.3
		----	95.7 $\pm$ 6.5	21.3 $\pm$ 3.7	12.9	199. $\pm$ 23.	87.8

the portion of Zn^{2+} in plasma rises rapidly with increasing blood zinc levels while erythrocytic zinc, although showing a modest increase, eventually accounts for a markedly lesser proportion of total zinc at high concentrations. Thus, it would appear that as zinc levels rise in blood, only a small fraction of the zinc penetrates the erythrocytic membrane and gains access to the cytosol and eventually δ -ALA-D. This distribution does not change measurably after 7 days of storage at 4 °C indicating that equilibrium is achieved early.

From the results of this "in vitro" study it can be concluded that every effort must be made to exclude access by adventitious zinc to either the blood sample during its collection, the haemolysate during its preparation or the assay reaction itself, since this trace element can severely affect the pH-activity profile (and hence the assay results obtained at a single pH value) at relatively low concentrations, $<10 \mu\text{M/L}$, and after a very short period of contact.

Mercury

The short-term toxic effects of mercury on man are well known and its potential harm has generated justified concern, now reinforced by public cognizance of these effects. However, more worrisome still are the prospects of long range consequences, about which little is yet known.

The toxicity, metabolism, distribution, absorption and excretion of metallic mercury, its inorganic salts and various organic compounds have been examined extensively in recent years (13, 132-138). ⁽¹⁾ At present, the selective toxicity of metallic mercury can be explained only on the basis of its distribution (139).

Exposure to mercury vapour at a concentration of 0.1 to 0.2 mg/m³ may result in intention tremor and erethism mercurialis (135). Although salivation and gingivitis are also possible signs of intoxication they are not as frequent as is tremor. Idiosyncrasy of mercury may also lead to proteinuria and nephrotic syndrome or sometimes to pink disease (140).

Hg²⁺ interacts with -SH and -S-S- groups of proteins in a multitude of systems and the consequences for structure and function of proteins have been reviewed thoroughly (141, 142). The biochemical basis of toxicological effects of mercury and its derivatives are generally thought to be through such mercury-sulfur interactions (136). However, even though the formation of mercury mercaptide is highly specific, mercury is nevertheless highly unspecific in terms of the proteins with which it reacts (143).

(1) Sample of a large body of literature on the subject.

TABLE XIII: Changes in Blood Lead Levels Following the Subcutaneous Administration of Zinc Lactate to a Lead-Intoxicated Rabbit. (1)

Subcutaneous Injection		Date	Lead ($\mu\text{g/dL}$)	Zinc ($\mu\text{g/dL}$)
Metal	Amount (mg)			
Lead (2)	25	21/8/80	49.0	327
--	--	28/8/80	48.1	370
--	--	4/9/80	41.7	340
--	--	11/9/80	36.0	348
Zinc	1.0	20/9/80	40.0	400
Zinc	5.0	23/9/80	38.4	470
Zinc	5.0	29/9/80	36.7	570
Zinc	10	10/10/80	59.5	762
Zinc	10	17/10/80	68.5	852
--	--	24/10/80	23.4	400

(1) Rabbit Weight $\approx$ 5 Kgm during test duration.

(2) Rabbit developed bleeding sore following injection.

FIGURE 22: Rabbit Erythrocytic δ -ALA-D pH-Activity Relationship Before (●) $<0.01 \mu\text{M Hg/L}$ and After (○) $2.5 \mu\text{M Hg/L}$ Subcutaneous Injection with Mercuric Acetate.

TABLE X: Distribution of Varying Concentrations of Mercury in Rabbit Whole Blood

Sample No.	Hct. %	Amount (1) Injected (mg/kg)	Whole Blood Concentration ($\mu\text{M/L}$)	Plasma Concentration ($\mu\text{M/L}$)	Plasma/Whole Blood %	Erythrocyte Concentration ($\mu\text{M/L}$)	Erythrocyte/Whole Blood %
R-281	41	--	0.025	0.036	84.0	0.010	16.0
R-285	42	--	0.020	0.024	63.0	0.016	29.0
R-289	40	--	0.020	0.030	80.0	0.010	16.0
R-283	41	--	0.032	0.034	62.0	0.026	33.0
R-287	42	--	0.032	0.037	66.0	0.023	30.0
R-291	40	--	0.045	0.052	72.0	0.030	27.0
R-286	42	0.1	0.122	0.186	90.0	0.030	10.0
R-282	41	0.3	0.234	0.378	95.0	0.023	4.0
R-290	40	0.5	0.374	0.568	91.0	0.040	4.0
R-284	41	0.5	0.508	0.742	86.0	0.147	12.0
R-292	40	1.0	0.798	1.171	88.0	0.140	7.0

(1) Hg^{2+} mg/Kg - injected subcutaneously as mercuric acetate.

of contamination found in the laboratory.

The interaction of mercury "in vitro" with δ -ALA-D has been examined to a greater extent than "in vivo". Thus, in 1955, Gibson et al. (22) showed that mercury was strongly inhibitive to δ -ALA-D preparations obtained from ox liver. Calissano et al. (144) and Hernberg et al. (73) arrived at similar conclusions using δ -ALA-D either in whole blood or isolated from erythrocytes. Avram and Davis (116) also reported a 50% inhibition of the enzyme at a concentration of $4 \times 10^{-6} M$, an inhibition which increased markedly after incubation of the metal with the haemolysate for 1 hour at $37^{\circ}C$ (50% inhibition at $1.9 \times 10^{-6} M$). Conversely, both Coleman (145) and Mitchell et al. (86) observed that mercury activated the enzyme in mouse liver and human erythrocytes (at $10^{-5} M$) respectively. Despaux et al. (117) on the other hand, reported both a marginal activation at low mercury concentrations and inhibition at levels of mercury $6 \times 10^{-6} M$. The results of this mercury "in vitro" investigation, shown in Figure 23A and obtained in the manner described for lead, zinc and copper, may resolve this apparent contradiction. It is clear that three possible conclusions can be arrived at as to the effects of exogenous mercury on erythrocytic δ -ALA-D, depending upon which buffer pH is selected by the investigator;

- at a pH < 6.60 - inhibition at all Hg^{2+} concentrations $> 5 \mu M Hg^{2+}/L$
- at a pH of 6.60 - no effects at concentrations $> 5 \mu M Hg^{2+}/L$
- at a pH > 6.60 - marginal inhibition at low Hg^{2+} levels and activation thereafter.

FIGURE 23A: Addition of Hg^{2+} to Human Whole Blood and its effects on δ -ALA-D After 3 Days of Storage at 4 °C.
 (●) Control Blood 86 μM Zn, 11.8 μM Cu, 0.24 μM Pb, <0.01 μM Hg/L (▲) 5 μM (△) 40 μM , (○) 60 μM , (■) 80 μM , and (□) 100 μM added Hg^{2+}/L Blood

FIGURE 23B: δ -ALA-D Residual Activity at Selected pH values as a Function of Added Hg^{2+} Concentrations.

The relationship between the enzyme's activity, the concentration of adventitious mercury and the buffer pH value used is illustrated in Figure 23B. Although it is impossible to ascertain exactly which assay pH was used for the studies described above as compared to those used in this investigation, it is highly likely that the diverse conclusions reported could be traced to a difference in assay pH. These results, as well as those obtained for lead, copper, and zinc, clearly indicate that, to perform "in vitro" or "in vivo" studies at a single pH value rather than over a range of pH values with an enzyme as pH-dependent as δ -ALA-D, can only lead to conjectures as to the effects of these metals on the enzyme.

The effect of mercury on erythrocytic δ -ALA-D after an extended period of contact with the metal is shown in Figure 24. Thus, at low mercury concentrations, a slight shift of the pH-activity profile to a higher pH value occurs and, similarly to zinc, mercury apparently preserves the enzyme's activity at high pH values at the metal concentrations used.

The results of the distribution of exogenous mercury in blood, listed in Table XI, clearly demonstrate that the metal is overwhelmingly found in plasma; a much smaller proportion ingresses the cytosol where it probably combines with haemoglobin. The data is not very unlike that obtained for "in vivo" mercury distribution (Table X).

In summary, mercury is unlikely to affect erythrocytic δ -ALA-D at the concentrations normally found "in vivo" after even

FIGURE 24: Preservation of δ -ALA-D Activity by Added Hg^{2+} After (A) 3 days and (B) 15 days of storage at 4 °C. (●) Control Blood: 77 μ M Zn^{2+} , 11.8 μ M Cu^{2+} , 0.08 μ M Pb^{2+} , <0.01 μ M Hg^{2+} (Δ) 5 μ M and (■) 100 μ M added Hg^{2+} /L Blood.

TABLE XI: Distribution of Exogenous Hg^{2+} in Humna Whole Blood After 7 Days of Storage At 4 °C

Sample No.	Hct. %	Amount Added ($\mu M/L$)	Whole Blood Concentration ($\mu M/L$)	Plasma Concentration ($\mu M/L$)	Plasma/Whole Blood %	Erythrocyte Concentration ($\mu M/L$)	Erythrocyte/Whole Blood %
H-690	42	90	84.8	142.7	98.0	1.14	0.6
		70	70.9	120.5	97.8	1.10	0.8
		40	40.4	68.9	98.6	1.02	1.0
		30	28.9	49.2	99.0	0.40	0.9
		-2	0.023	0.03	78.0	0.012	23.0

an acute intoxication. Moreover, unless a gross contamination of the blood sample, haemolysate or assay mixture occurs, there is little chance that adventitious mercury will be of any serious consequence to the assay results.

Cadmium

Cadmium is one of the most toxic metals known to man and there has been increased concern about its presence in the environment and human exposure through food, water, and air. Although the metal has only recently been fingered as an environmental hazard which contributes to human illness (146-149), it has long been recognized as a health hazard to miners and exposed industrial workers, most notably producers of alkaline batteries.

The toxicity, metabolism, distribution, absorption and excretion of Cd in many species, including man, has been examined extensively in recent years (3, 111, 148, 150). Like mercury and lead, no biological functions as yet have been defined for cadmium. This non-essential element tends to accumulate in an individual throughout his lifetime since the body does not have a biological mechanism to rid itself of this toxicant. Chronic poisoning in

man causes kidney damage with proteinuria (151, 152). Osteomalacia is also thought to result, in part, from chronic cadmium poisoning (ouch-ouch disease) (153).

Interaction of Cd with polythiols is particularly strong and likely of considerable biological significance. Indeed, this element, like lead, can inhibit numerous enzymes with functional sulfhydryl groups (111). The metal often competes with zinc for the same sulfhydryl or imidazole groups (154, 155) and often substitutes for Zn in metalloenzymes e.g. carboxypeptidase (111). It also interacts with phosphatidylethanolamine and phosphatidylserine monolayers and nucleic acids (111). It should also be mentioned that cadmium activates a large number of enzymes "in vitro" (111).

"In vivo". The only two "in vivo" investigations of cadmium conducted to date have been those of Lauwerys et al. (69) and Roels et al. (70) with cadmium-exposed workers. ⁽¹⁾ Both indicated that the metal has no significant effect on erythrocytic δ -ALA-D at concentrations of the metal in blood found in cases of chronic cadmium exposure. The results of this investigation with rabbits, fed cadmium acetate in their drinking water ad libitum, reaffirm this conclusion; no significant changes in the enzyme's pH-activity profile were detected even at relatively high levels of cadmium in blood, 0.2 μ M/L.

The distribution of the endogeneous metal in blood has been

(1) range of whole blood cadmium concentrations observed in exposed workers is 0.9 to 8.9×10^{-8} M/L (70).

the subject of several investigations (70, 156, 157) and there appeared to be no point in repeating the determination. Invariably, results indicated that cadmium is found in the erythrocytes bound either to metallothionin (60%) (157) or haemoglobin (156, 157) and hardly detectable in plasma.

"In vitro". As was the case for mercury, conflicting data and hypotheses are found in the literature as to the "in vitro" action of cadmium on δ -ALA-D. Abdulla et al. (158) and Wada et al. (71) both reported an inhibitive action of the metal on the enzyme at 4.4 μ M/L and 20 μ M/L respectively. Conversely, Wilson et al. (17) observed that the effect, whether activation or inhibition, was wholly dependent upon the concentration of the substrate. Despaux et al. (117) and Davis and Avram (115, 116) on the other hand, both were able to demonstrate that cadmium stimulated the enzyme's activity at low concentrations ($< 4 \times 10^{-6}$ M/L) followed by a progressive inhibition at higher levels of the metal (50% inhibition at 8×10^{-5} M). A one-hour incubation of the haemolysate with cadmium apparently enhanced the inhibition by cadmium (115).

The results of the "in vitro" cadmium study, conducted as previously described, are shown in Figure 25A and B. One can observe (Figure 25A) that minimal changes in the profile occur at approximately pH 6.45 at all the Cd^{2+} concentrations used; below this pH value there is significant ($P < 0.005$) activation at low cadmium blood concentrations followed by progressive inhibition with increasing levels of the metal; above pH 6.45, the reverse

FIGURE 25A: Addition of Cadmium to Human Whole Blood - Its Effects on δ -ALA-D pH-Activity Profile After 3 Days of Storage at 4 °C.
 (●) Control Blood: 86 $\mu\text{M Zn}^{2+}$, 11.8 $\mu\text{M Cu}^{2+}$, 0.24 $\mu\text{M Pb}^{2+}$, <0.01 $\mu\text{M Cd}^{2+}$ /L
 (Δ) 8.8 $\mu\text{M Cd}^{2+}$ (▲) 35 $\mu\text{M Cd}^{2+}$ (○) 53 $\mu\text{M Cd}^{2+}$ (■) 70 $\mu\text{M Cd}^{2+}$ (□) 88 $\mu\text{M Cd}^{2+}$ /L Blood.

FIGURE 25B: Relationship Between the Concentration of Added Cd^{2+} and Residual δ -ALA-D Activity in Human Blood.

is observed, that is, inhibition at low cadmium levels and a progressive activation with increasing concentrations of the metal in blood. Notably, there occurs no significant ($P > 0.01$) changes in the enzyme's profile over the whole range of pH values when a Cd^{2+} concentration of $53 \mu\text{M/L}$ is added to whole blood. Once again, it is readily observable that the effects "in vitro" of a metal such as cadmium, determined at a single pH value above or below pH 6.45, can lead to opposing conclusions. This may explain the contradictory reports found in the literature as to the effects of cadmium upon δ -ALA-D.

Long-term contact of the metal and enzyme at 4°C (Figure 26) does not produce a detectable shift in the enzyme's profile. However, after 15 days of incubation at this low temperature, there is activation of the enzyme over the whole pH range at a low Cd^{2+} concentration ($22 \mu\text{M/L}$) indicating that the metal may be protective against enzyme activity loss during storage as observed by Hampp and Kriebitzsch (130).

The distribution of cadmium in whole blood, shown in Table XII is much the same as given elsewhere (70), that is; approximately 75% of the amount of metal added is found in the plasma and only 10 to 20% is taken up by the erythrocytes. This radically contrasts the situation observed "in vivo" and given earlier.

From the above results, one may conclude that cadmium exposure, whether acute or chronic, will not likely introduce

FIGURE 26: δ -ALA-D pH-Activity Profile 3 Days (A) and 15 Days (B) After the Addition of $22 \mu\text{M Cd}^{2+}/\text{L}$ (Δ) to Control Human Blood ($\bullet$) $77 \mu\text{M Zn}^{2+}$, $11 \mu\text{M Cu}^{2+}$, $0.58 \mu\text{M Pb}^{2+}$ and $<0.0 \mu\text{M Cd}^{2+}$.

TABLE XII: Distribution of Exogenous Cd^{2+} in Human Blood After 7 Days of Storage At 4 °C

Sample No.	Hct. %	Amount Added ($\mu\text{M/L}$)	Whole Blood Concentration ($\mu\text{M/L}$)	Plasma Concentration ($\mu\text{M/L}$)	Plasma/Whole Blood $\bar{x}$	Erythrocyte Concentration ($\mu\text{M/L}$)	Erythrocyte/Whole Blood $\bar{x}$
H-691	40	8.80	8.41	19.7	93.0	0.71	5.0
		4.40	4.25	9.16	86.0	0.60	8.0
		1.88	1.93	4.09	85.0	0.48	15.0
		1.25	1.44	3.02	84.0	0.39	19.0
		—	<0.04	—	—	—	—

enough metal in the blood to produce either a shift in the pH optimum or activation or inhibition. "In vitro", this is not the case. As the data in Table I indicates, cadmium can be a serious contaminant of blood collected with devices such as Vacutainers. Thus, the possible contamination of blood collected in brown-stoppered Vacutainers (mean $20.5 \mu\text{M Cd}^{2+}/\text{L}$) is indeed sufficient to cause a mild shift to a lower pH value and a concomitant increase in activity, somewhat similar effects to that observed in Figure 6 for blood collected in these devices. However, the concurrent effects of the presence of large amounts of zinc in these Vacutainers cannot be discounted and will be examined shortly.

Plausible Combinations of Toxic Metals

An individual's exposure to toxic metals at work or at home, more than likely involves concurrent exposure to a combination of several metals at various individual levels of concentration. A welder, for instance, is subjected to fumes containing iron, lead, copper, zinc, and a veritable host of other metals; a lead-zinc miner endures iterative exposure to clouds of mineral dust containing lead, zinc and cadmium; a copper smelter worker inhales copper and zinc fumes daily; and the same holds true for several other occupations involving workday exposure to metal fumes and dusts. It therefore seems appropriate, at this point, to examine the impact of some of the most plausible metal combinations of the five toxic metals under consideration on erythrocytic δ -ALA-D's pH-activity profile to determine what effects, if any, such

combinations would have on the enzyme's proposed assays.

The results obtained to date for individual metals have indicated that they can exert a measurable effect only if their concentration in blood exceeds a specific value proper to that metal. Hence, in what will follow, the combination of metals used will include a concentration of individual metals equal to or in excess of, that known to cause an effect.

Obviously, not all 26 possible combinations of the five toxic metals ⁽¹⁾ in question are of equal importance vis-à-vis human exposure to environmental contaminants. Only those considered to be commonplace will be examined here. These include: lead/zinc, lead/copper, copper/zinc, lead/copper/zinc, lead/mercury and lead/cadmium. The basis for this selection is the availability of test populations exposed to such combinations at the time of this study. All other possible combinations will be presented briefly later.

Lead/zinc

Of all the pairs of metals investigated, the combination of lead and zinc has received the most attention from researchers. The main objective of the studies was to determine if zinc could stimulate the enzyme and reverse its lead-induced inhibition "in vitro" as well as "in vivo", and also to test the specificity of the δ -ALA-D assay for lead intoxication.

(1) 10 pairs and triplets; 5 quartets; 1 set of 5 metals.

"In vivo". The study of the antagonistic effect of zinc on the inhibition of δ -ALA-D by lead has been the subject of relatively few "in vivo" investigations as compared to those performed "in vitro". In one such study, Meredith and Moore (159), using 22 subjects concurrently exposed to lead and zinc, were able to demonstrate that, although elevated blood zinc levels has a significant effect on inhibited δ -ALA-D, the effect was small at zinc concentrations between 80 and 120 μ M/L (within the normal physiological range) relative to the effects of lead on the enzyme's activity. A case study of a lead-poisoned human subject reported by Thomasino et al. (66) has suggested that relatively high zinc concentrations may have a protective role in the effect of lead toxicity. This finding gained support from observations made by Haeger-Aronsen et al. (65) that zinc almost completely eliminates the inhibitory effect of lead in rabbits injected with both lead and zinc. Experiments conducted by Finelli et al. (58) with rats maintained on a lead diet further indicated that there seems to be a competition between Pb^{2+} and Zn^{2+} in binding to the enzyme with lead as the inhibitor and zinc as the activator; and, that the enzyme has a greater affinity for lead than zinc since relatively high concentrations of zinc are required to reactivate lead-inhibited ALA-D. Cantrell et al. (62) and Schlipkötter et al. (60) were likewise able to demonstrate a zinc antagonism towards lead inhibition of δ -ALA-D in baboons and mice respectively. It is noteworthy that only in one of the aforementioned studies were the combined effects of

lead and zinc on the pH-activity profile of δ -ALA-D investigated (62).

In the present study, rabbits, fed lead acetate in their drinking water over a long period of time, were injected subcutaneously with zinc lactate. The dramatic results of increasing blood zinc levels on the erythrocytic δ -ALA-D pH-activity profile of a single rabbit intoxicated with lead are shown in Figure 27. The profile does not shift to lower pH values and the main stimulation of activity (609%) occurs at pH 5.9 with lesser activation at higher pH values (for instance, 280% at pH 6.4). The pH-activity profile does not return to its pre-lead inhibited state even when whole blood zinc levels attain a concentration some 2.5 times higher than normal value. It must be understood here that, as indicated earlier for normal blood, an important portion of the activation of δ -ALA-D by endogenous zinc results from the action of plasma-bound zinc on the enzyme after haemolysis. An observation worth noting at this point relates to the significant increases ($P < 0.01$) in blood lead levels in rabbits treated with zinc (Table XIII); and, the fact that their blood lead concentrations returned to significantly ($P < 0.008$) lower levels several days after zinc treatment, an indication that zinc may have substituted for lead at certain binding sites in blood and tissue. These variations in blood lead levels made it difficult to draw meaningful relationships between increased blood zinc levels and residual enzyme activity.

FIGURE 27: Subcutaneous Injection of Lead-Intoxicated Rabbit with Zn^{2+} --- Effects on δ -ALA-D pH-Activity Profile.

(●)	Control	50 μM Zn^{2+}	10.0 μM Cu^{2+}	0.01 μM Pb^{2+}/L
(Δ)		53 μM Zn^{2+}	10.3 μM Cu^{2+}	2.06 μM Pb^{2+}/L
(▲)		54 μM Zn^{2+}	9.9 μM Cu^{2+}	1.74 μM Pb^{2+}/L
(○)		72 μM Zn^{2+}	9.1 μM Cu^{2+}	1.85 μM Pb^{2+}/L
(■)		130 μM Zn^{2+}	11.3 μM Cu^{2+}	3.31 μM Pb^{2+}/L

TABLE XIII: Changes in Blood Lead Levels Following the Subcutaneous Administration of Zinc Lactate to a Lead-Intoxicated Rabbit. (1)

Subcutaneous Injection		Date	Lead ($\mu\text{g/dL}$)	Zinc ($\mu\text{g/dL}$)
Metal	Amount (mg)			
Lead (2)	25	21/8/80	49.0	327
--	--	28/8/80	48.1	370
--	--	4/9/80	41.7	340
--	--	11/9/80	36.0	348
Zinc	1.0	20/9/80	40.0	400
Zinc	5.0	23/9/80	38.4	470
Zinc	5.0	29/9/80	36.7	570
Zinc	10	10/10/80	59.5	762
Zinc	10	17/10/80	68.5	852
--	--	24/10/80	23.4	400

(1) Rabbit Weight $\approx$ 5 Kgm during test duration.

(2) Rabbit developed bleeding sore following injection.

"In vitro". By far the most universal application of δ -ALA-D assay to date, has been for the assessment of human lead exposure. Therefore, it was felt that, more useful information would be gained from metal combination studies, if a common blood sample containing a high level of "in vivo" lead was mixed with increasing concentrations of a single exogenous metal--the purpose here being to determine the effects of adventitious metal contaminants on the proposed assays when used for the assessment of lead exposures.

Zinc was added to lead-poisoned blood. The results as determined after 3 and 16 days of storage at 4 °C are shown in Figure 28A and B. Although the effects on the pH-activity profile are similar to those obtained "in vivo", the stimulation of activity at pH 5.9 appears to be much less pronounced. No significant difference is noted after storage of blood samples at 4 °C for 16 days, in direct contrast with the enhancement of δ -ALA-D activity observed in stored normal blood doped with zinc (Figure 20).

Very different results are obtained when less than 10 μ M zinc is added to blood (Figure 29). At these low concentrations zinc appears to inhibit δ -ALA-D activity at most pH values--much as was obtained with normal blood at pH values <6.4. The relationships between the concentration of zinc added and residual δ -ALA-D activity are illustrated in Figures 30A and B, and are similar in many aspects to those obtained for normal blood (Figure 21) albeit not as pronounced in lead-poisoned blood at pH 5.9. The inhibitory

FIGURE 28: Addition of Zn^{2+} to Lead-Poisoned Human Blood - Its Effects on δ -ALA-D pH-Activity Profile After 3 Days (A) and 16 Days (B) of Storage at 4 °C.
 (●) Control Blood: 100 μM Zn^{2+} , 13.5 μM Cu^{2+} , 2.91 μM Pb^{2+} /L.
 (▲) 30.6 μM (▲) 76.5 μM , (○) 153 μM and (■) 765 μM Added Zn^{2+} /L.

FIGURE 29: Addition of Small Concentrations of Zn^{2+} to Lead-Poisoned Human Blood - Effects on δ -ALA-D pH-Activity Profile After 7 Days of Storage at 4 °C. (●) Control Blood - 78 μM Zn^{2+} , 13.4 μM Cu^{2+} , 0.87 μM Pb^{2+} /L. (Δ) 4 μM (▲) 8 μM and (○) 12 μM added Zn^{2+} /L Blood.

FIGURE 30: Residual δ -ALA-D Activity in Lead-Poisoned Human Blood (A) $2.91 \mu M$ (B) $1.45 \mu M Pb^{2+}$ /L Measured at Specific pH Values as a Function of Added Zn^{2+} Concentrations After 3 Days of Storage at $4^{\circ}C$.

effect of zinc at low concentrations depicted in Figure 30B, is noteworthy.

The antagonism of exogenous zinc towards "in vivo" lead and its inhibition of erythrocytic δ -ALA-D conform to reported observations (Table XIV). Border et al (61), were also able to demonstrate a pH dependency in zinc's ability to reverse lead-caused inhibition of the enzyme; however, the pH-activity curve they obtained was very different from that shown here. The pH-activity curve for lead-poisoned δ -ALA-D in baboon blood after treatment with zinc, both "in vivo" and "in vitro", obtained by Cantrell et al. (62) was more different still--they reported a pH-activity curve having two optima, one at 6.8 and the other at 6.2. They attributed this latter optimum to "de novo" production of an enzyme with δ -ALA-D activity. Note the similarities of the results presented here with those obtained by others for the action of exogenous lead and zinc together on δ -ALA-D in normal blood (Table XIV).

According to Hill and Matrone (161), elements which have similar physical and chemical properties are expected to be biologically antagonistic. Zn^{2+} and Pb^{2+} approximate these conditions (both are borderline Lewis acids and have a sp^3 - tetrahedral and $sp^3 - \pi$ - tetrahedral electronic configuration respectively); and, therefore, it is with no surprise that one finds that these ions are in fact antagonistic in their behaviour towards δ -ALA-D.

A more detailed hypothesis, attempting to explain the diverse behaviour of the elements towards δ -ALA-D, will be presented later. Suffice, at this point, to briefly summarize the salient

TABLE XIV: Summary and Comparison of Investigations of the Combined Effects of Lead and Zinc on Erythrocytic δ -ALA-D Activity.

Reference	Zn ²⁺ Addition			δ-ALA-D Assay		Effects
	Media	Temp.	Time	Buffer	pH	
A. Pb "In Vivo"						
Pinelli (58)	Blood	--	--	P-P	6.8	Maximal reactivation at 10 ⁻⁴ M Zn ²⁺
Bonner (34)	Haemolysate	37 °C	30 min.	P-P	6.9	370% activation DF 4.3 μM/L Pb ²⁺ poisoned δ-ALA-D by 1.5 x 10 ⁻⁴ M/L Zn ²⁺
Mauras (64)	Haemolysate	37 °C	1 h.	P-C	6.7	Maximal activation at 10 ⁻² M Zn ²⁺
Cantrell (62)	Haemolysate	37 °C	10 min.	P-P	6.0-7.5	Two optima observed at pH 6.2 and 6.8
Sakai (97)	Buffered Haemolysate	60 °C	5 min.	P-P	7.0	Maximal reaction at 10 ⁻⁴ M/L Zn ²⁺ Highest at higher pH values
B. Pb "In Vitro"						
Border (34)	Haemolysate	37 °C	30 min.	P-P	6.9	5.3 x 10 ⁻⁴ M/L Zn ²⁺ reactivated δ-ALA-D inhibited by 5.3 μM/L Pb ²⁺
Trevisan (126)	Haemolysate	R.T.	10 min.	NaHCO ₃	7.0	50% reactivation of 5 x 10 ⁻⁶ M/L Pb ²⁺ poisoned blood by 1.5 x 10 ⁻⁴ M/L Zn ²⁺
Meredith (59)	Haemolysate	--	--	P-P	6.6	19% activation on addition of 2 x 10 ⁻⁴ M/L Zn ²⁺ and 1.86 μM/L Pb ²⁺
Davis (112)	Haemolysate	--	--	P-C	6.7	15% reactivation of blood containing 1.86 μM/L Pb ²⁺ by 3.7 x 10 ⁻⁴ M/L Zn ²⁺
Davis (113)	Haemolysate	37 °C	1 h.	P-C	6.7	140% reactivation of blood containing 1.8 μM/L Pb ²⁺ by 3.7 x 10 ⁻⁴ M/L Zn ²⁺

observations made, namely:

- the action of zinc on δ -ALA-D in lead-poisoned blood is concentration-dependent, being inhibitive at low metal concentrations ($<10 \mu\text{M}$) and stimulatory thereafter;
- the extent of the activation or inhibition is strongly pH-dependent;
- maximal effect on the enzyme occurs, whether activation or inhibition, at pH 5.9 in common with the respective individual effect of lead and zinc on the enzyme.

Comment has been made earlier about the ubiquitous contaminant nature of zinc as exemplified by the metal's presence in all blood collection devices (Table I). Thus, if a blood sample were collected from a severely lead-exposed person, in a zinc contaminated vacutainer (green or brown top, as is customary today), it is highly likely that, regardless of the blood lead level, a false negative result will be obtained from an assay conducted at a single pH value. This action of zinc upon lead-poisoned δ -ALA-D does appear to place the reliability and usefulness of such an assay in question. It remains to be seen, if an assay, based upon the ratio of measurements of enzyme activity at two separate pH values, will suffer a similar fate.

Lead/copper

Plumbers, welders and smelter workers are but a few examples of tradesmen subject to copper fume fever which results

from an overexposure to copper fumes. Since lead is also one of the metals employed by these workers, it is highly probable that these individuals are concurrently exposed to lead and copper fumes during their workday. In view of this, it is rather surprising that the combined impact of "in vivo" copper and lead on erythrocytic δ -ALA-D has not been studied.

"In vivo". To duplicate this type of exposure rabbits were either given lead acetate in their drinking water "ad libitum" and injected with a copper glycinate solution or injected with this solution then force fed a solution of lead acetate. Identical results were obtained for both approaches that is, additive inhibition at pH 6.0. An example is shown in Figure 31. Thus, a worker concomitantly exposed to both lead and copper would have a lower δ -ALA-D value than that expected for his blood lead concentration i.e. a false positive result would be obtained.

"In vitro". To simulate, as for zinc above, the contamination of a lead-poisoned blood by copper, various concentrations of the metal were added to blood samples collected from lead exposed individuals. The results shown in Figures 32A and B are complementary in the sense that they represent the effect of high and low concentrations of the adventitious metal respectively. These pH-activity curves are singularly similar to those obtained for the "in vivo" experiments, that is, a progressive additive reduction of the enzyme activity at pH 6.0 is noted. This relationship between added metal concentrations and residual enzyme activity is illustrated in Figure 33; and although the pattern obtained is

FIGURE 31: Subcutaneous Injection of a Lead-Poisoned Rabbit ((●) $52 \mu\text{M Zn}^{2+}$, $8.2 \mu\text{M Cu}^{2+}$, $1.17 \mu\text{M Pb}^{2+}/\text{L}$) with Cu^{2+} ((Δ) $52 \mu\text{M Zn}^{2+}$, $14.5 \mu\text{M Cu}^{2+}$, $1.06 \mu\text{M Pb}^{2+}/\text{L}$) - Effects on pH-Activity Profile.

FIGURE 32: Addition of Cu^{2+} to Lead-Poisoned Human Blood - Effects on δ -ALA-D pH-Activity Profile After 3 Days of Storage at 4°C .

- (A) (●) Control $102 \mu\text{M Zn}^{2+}$, $13.2 \mu\text{M Cu}^{2+}$, $1.46 \mu\text{M Pb}^{2+}/\text{L}$. (Δ) $6 \mu\text{M}$, ($\blacktriangle$) $15 \mu\text{M}$, ($\circ$) $30 \mu\text{M}$ and ($\blacksquare$) $160 \mu\text{M}$ Added Cu^{2+}/L .
- (B) (●) Control $78 \mu\text{M Zn}^{2+}$, $9.6 \mu\text{M Cu}^{2+}$, $0.87 \mu\text{M Pb}^{2+}/\text{L}$. (Δ) $0.2 \mu\text{M}$, ($\blacktriangle$) $0.5 \mu\text{M}$, ($\circ$) $1 \mu\text{M}$ and ($\blacksquare$) $3 \mu\text{M}$ Added Cu^{2+}/L .

FIGURE 33: Relationship Between Residual δ -ALA-D Activity in Lead-Poisoned Human Blood at Specific pH Values and Added Cu^{2+} Concentrations After 3 Days of Storage at 4°C .

similar to that observed for the addition of the metal to normal blood (Figure 18), it differs markedly in other aspects. Firstly, there appears to be a uniform reduction of activity at all pH values and secondly, the reduction is significantly larger in the case of lead-poisoned blood. Thus, contamination of a blood sample, haemolysate or assay mixture with copper would likely result in a false positive result.

Parenthetically, all investigations conducted to date on the combined effects of lead and copper on erythrocytic δ -ALA-D involved the addition of both metals to normal blood or its haemolysate (63, 131). Nevertheless, the results obtained were identical to those shown above namely, the inhibition of δ -ALA-D, was greater than that observed with either of the two metals alone. This additive inhibition has been attributed to the simultaneous action of the two metals at different sites on the enzyme (131).

Cu^{2+} has a dsp^2 square planar coordination geometry whilst Pb^{2+} has a sp^3 - π -tetrahedral configuration. Although both metals are borderline Lewis acids, they have distinctively different chemical and physical properties. Hence, assuming that the Hill and Matrone hypothesis (160) is correct, the metals are not expected to be biologically antagonistic. This is in fact observed here with δ -ALA-D--the metals apparently behave in an additive manner towards this enzyme.

Copper/zinc and copper/zinc/lead

Brass foundry workers are subject to metal fume fever, actually due to an exposure to the fumes of several metals including zinc, and copper or combinations thereof. The effects of the copper/zinc pair, on δ -ALA-D, either "in vivo" or "in vitro" have not been examined extensively to date.

"In vivo". Copper and zinc solutions were administered subcutaneously to rabbits either simultaneously (at different sites on the back) or in sequence (2 hours apart). An example of the results obtained is shown in Figures 34A and B respectively. In Figure 34A, note the activation of the enzyme which results as the proportion of copper to zinc drops (5 hours after injection). Thus, "in vivo" zinc can reverse the Cu induced inhibition of δ -ALA-D (Figure 34A and B) as it does the inhibition caused by lead. Therefore, zinc would appear to be similarly biologically antagonistic to copper. This antagonism is well documented (161, 162). Since copper and zinc have similar chemical and physical properties, the Hill-Matrone hypothesis (160) apparently applies here also.

Copper, lead and zinc were administered in this order at 2-hour intervals to rabbits as described earlier. The results, Figure 35, illustrate the dramatic sequential changes in the enzyme's pH-activity curve which occurred during this test. These results confirm zinc's antagonism towards both lead and copper. The results also reiterate the difficulties inherent in interpreting δ -ALA-D assay results obtained at a single pH value for an individual exposed to a mixture of metals such as lead, copper and zinc.

FIGURE 34A: Simultaneous injection of a Rabbit with Cu^{2+} and Zn^{2+} - Effects on δ -ALA-D pH-Activity Profile; Blood Samples Collected 2 Hours Apart.
 (●) Control: 54 μM Zn^{2+} , 10.2 μM Cu^{2+} , 0.98 μM Pb^{2+} /L
 (□) 68.8 μM Zn^{2+} , 24.6 μM Cu^{2+} , 0.75 μM Pb^{2+} /L
 (Δ) 67.3 μM Zn^{2+} , 14.5 μM Cu^{2+} , 0.86 μM Pb^{2+} /L

FIGURE 34B: Sequential (2 Hours Apart) Injection of a Rabbit with Cu^{2+} and Zn^{2+}
 (●) Control: 55 μM Zn^{2+} , 7.6 μM Cu^{2+} , 0.70 μM Pb^{2+} /L
 (Δ) 56 μM Zn^{2+} , 10.6 μM Cu^{2+} , 0.70 μM Pb^{2+} /L
 (□) 88 μM Zn^{2+} , 9.6 μM Cu^{2+} , 0.70 μM Pb^{2+} /L

FIGURE 35: Sequential (2 hours apart) Administration of Cu^{2+} , Pb^{2+} , and Zn^{2+} to a Rabbit - Effects on the δ -ALA-D pH-Activity Profile.

(●) Control	- 64 $\mu\text{M Zn}^{2+}$	10.1 $\mu\text{M Cu}^{2+}$	0.34 $\mu\text{M Pb}^{2+}/\text{L}$
(Δ)	64 $\mu\text{M Zn}^{2+}$	26.4 $\mu\text{M Cu}^{2+}$	0.66 $\mu\text{M Pb}^{2+}/\text{L}$
(▲)	58 $\mu\text{M Zn}^{2+}$	20.0 $\mu\text{M Cu}^{2+}$	3.20 $\mu\text{M Pb}^{2+}/\text{L}$
(□)	75 $\mu\text{M Zn}^{2+}$	18.0 $\mu\text{M Cu}^{2+}$	3.10 $\mu\text{M Pb}^{2+}/\text{L}$

Lead/mercury

A simultaneous exposure to lead and mercury is a fairly rare event especially at the levels of mercury required to induce changes in δ -ALA-D's pH-activity curve. For this reason, this combination of metals was examined only "in vitro" (in an attempt to mock a possible but highly unlikely contamination of lead-poisoned blood by exogenous mercury) via the addition of increasing concentrations of Hg^{2+} to blood having a high endogenous lead level. The results of one of these experiments are shown in Figure 36A and the relationship between added mercury concentrations and residual enzyme activity is seen in Figure 36B. According to these results, mercury gradually reduces the enzyme's activity at pH 6.0 as its concentration increases in a manner somewhat reminiscent of copper (Figure 32A and B). However, at relatively elevated metal concentrations a second optimum begins to appear at a more basic pH value (≈ 7.1) and the enzyme's activity at pH values >6.4 increases rapidly. Notwithstanding the singular occurrence of two optima, the results are not entirely unexpected as they follow a pattern already observed for the addition of Hg^{2+} to normal blood (Figure 27). At this point, it is tempting to conjecture that the two optima indicate the presence of two different species of the enzyme at high mercury and lead concentrations.

Only Davis and Avram (116) have also studied the combined effects of lead and mercury "in vitro" on the enzyme; and, they reported observing a slow decline in activity with increasing metal concentrations (measured in P-C buffer at pH 6.7)

FIGURE 36A: Addition of Hg^{2+} to Lead-Poisoned Human Blood and its Effects on δ -ALA-D's pH-Activity Profile.
 (●) Control Blood 94 μM Zn 13 μM Ca, 1.54 μM Pb and 0.02 μM Hg.
 (Δ) 1.25 μM , (▲) 2.5 μM , (○) 5.0 μM (■) 20 μM Added Hg^{2+} /L Blood.*

FIGURE 36B: Residual δ -ALA-D Activity of Human Lead-Poisoned Blood as a Function of the Concentration of Added Hg^{2+} , After 3 Days of Storage at 4 °C.

In view of the two effects demonstrated by Hg^{2+} on the enzyme, that is, additive inhibition at low pH values (<6.4) similar to copper and activation at high pH values (>6.4) and high metal concentrations, it is somewhat difficult to classify this metal ion as being either antagonistic to or additive with lead.

Lead/cadmium

Although a combined lead/cadmium occupational or environmental exposure is a most definite possibility, exposure to cadmium levels required to produce a discernable change in the enzyme's pH-activity relationship is highly unlikely. Therefore, as for the Hg/Pb^{2+} pair, the combination will be considered "in vitro" only because of its potential as a contaminant in blood collection devices (Table I).

Cadmium at low concentrations activates lead inhibited δ -ALA-D at pH 5.9 (Figure 37). As the metal's concentration increases to relatively large values, there occurs an apparent shift of the pH optimum to pH 6.2 and a marked increase in the enzyme's activity ($P < 0.005$) at pH values >6.0. Thus, cadmium appears to be able to overcome all inhibition of the enzyme by lead and to restore the pH-activity curve to almost its original state. The results again, are in keeping with those obtained for the addition of cadmium to normal blood (Figure 25A).

Davis and Avram (115, 116), who also studied the joint action of lead and cadmium on the enzyme, demonstrated that following an initial inhibition, the metal enhanced the enzyme's

FIGURE 37: Addition of Cd^{2+} to Lead-Poisoned Human Blood - Effects on δ -ALA-D pH-Activity Profile.
 (●) Control Blood 86 μM Zn^{2+} , 15 μM Cu^{2+} , 2.08 μM Pb^{2+} and $<0.01 \mu\text{M}$ Cd^{2+} /L.
 (▲) 8.8 μM and (Δ) 44 μM added Cd^{2+} /L Blood.

activity at levels of $\text{Cd}^{2+} > 10^{-6}\text{M}$, peaked at 10^{-5}M (111%) and decreased thereafter. The investigators also observed that cadmium was a far more potent activator of the enzyme than zinc. This is indeed true if the activity is measured at high pH values (as they did) but not necessarily so at low pH values.

It is noteworthy that both mercury and cadmium are strong Lewis acids, unlike the three other metals copper, zinc, and lead which are borderline Lewis acids. Cd^{2+} is very like Zn^{2+} in chemical and physical properties (it also has a sp^3 tetrahedral electronic configuration) and in fact can replace zinc in certain proteins (111). Hence, it is not surprising to find this metal behaving like zinc and being antagonistic to lead.

The effect of pairs of metals each including lead and one of the other metals as components, each at equimolar concentration (60 $\mu\text{M/L}$) added simultaneously to control blood (having a higher than "normal" lead level) is illustrated in Figure 38. All results are as expected from the foregoing discussion except that obtained for Hg/Pb which shows a pH-activity curve with an optimum at pH 6.5 (lower than that shown in Figure 36A). The reason for this difference in pH optimum is not entirely clear at present. However, it could be speculated that such a change occurs at very high Hg^{2+} concentrations.

FIGURE 38: Addition of $60 \mu\text{M/L Pb}^{2+}$ $\pm$ other Metal Ions to Human Blood - Effects on δ -ALA-D pH-Activity Profile After 3 Days of Storage at 4°C .
 (●) Control Blood: $87 \mu\text{M Zn}^{2+}$, $12.5 \mu\text{M Cu}^{2+}$, $0.96 \mu\text{M Pb}^{2+}$, $0.01 \mu\text{M Hg}^{2+}$, $<0.01 \mu\text{M Cd}^{2+}$ /L Blood.

Other Possible Combinations of Toxic Metals

It could easily be argued that some of the other possible combinations of the five toxic metals should have also been given a more thorough treatment either because of the potential exposures they represent, Cd/Cu for instance, or, because the combination is a likely contaminant of blood samples as, for example, the zinc/cadmium pair. However, the prime aim of the present investigation was to determine which of the five metals and their most likely combinations, "in vivo" and/or "in vitro", would lead to changes in the enzyme's pH-activity profile and, consequently, to erroneous assay results. This objective has been met in the foregoing sections. The remaining possible combinations of pairs of toxic metals are briefly presented here mainly, in the hope that some light may be shed on the mode of action of these metals on the enzyme.

Equimolar amounts (60 μ M/L) of all possible pairs of the five toxic metals being investigated were added to an aliquot of a common control blood sample (endogenous lead level slightly higher than normal value), mixed for 1 hour at 4 °C and allowed to stand 16 hours at 4 °C before assay. The results are shown in Figures 38 and 39 A-D inclusively.

Note that several of the combinations already described are also included and that several curves are repeated. This has been done to afford an easier comparison of the possible combinations. Thus, each figure represents the effects on erythrocytic δ -ALA-D of one metal paired in turn with one of the other four elements.

FIGURE 39: Addition of Pairs of Metal Ions (60 μM/L) to Human Blood - Effects on δ-ALA-D pH-Activity Profile After 3 Days of Storage at 4 °C.

(●) Control Blood: 87 μM Zn²⁺, 12.5 μM Cu²⁺, 0.96 μM Pb²⁺, 0.01 μM Hg²⁺, <0.01 μM Cd²⁺/L Blood.

(A) Zinc

(B) Copper

(C) Mercury

(D) Cadmium ± Metal Pair.

FIGURE 39 Continued

At first glance, these figures present a rather bewildering array of curves which clearly exemplify the enzyme's numerous and diverse responses to the action of individual metals or their pair combinations. To simplify the interpretation of these results, they are summarized in Table XV as % of control blood δ -ALA-D activity at the pH optimum of each metal or its pair combinations (*) and, for each pair, as residual enzyme activity measured at the optimum pH value of each member of a pair group. Each value is further classified as to the pH range in which it was obtained and listed in order of increasing ability to activate the enzyme. Furthermore, if one assumes that potency in effecting a change, either enzyme activation or inhibition, can be equated to a metal ion's ability to cause a greater change in enzyme activity at the pH optimum of its rival ion than that ion can effect at the metal ion's own pH optimum value, the following facts emerge from these values.

- Cu^{2+} and Pb^{2+} (1) are enzyme inhibitors
- Cd^{2+} , Hg^{2+} (1) and Zn^{2+} (1) are enzyme activators
- The overall most potent enzyme effector is Cu^{2+}
- The order of metal ions as to potency to effect a change, either activation or inhibition, on the enzyme appears to be as follows:

(1) This statement is true at a concentration of 60 $\mu\text{M/L}$; at relatively lower concentrations it has been shown that all three metals reverse their effect i.e. inhibitors become activators and vice-versa.

TABLE XV: Summary of the Net Effects of Metal Ions and Paired Metal Ions on the Activity of Erythrocytic δ -ALA-D.

Metal Ion	Ion Pair	% of Control Blood δ -ALA-D Activity				Net Effect
		5.9 $\pm$ 0.2**	6.34 $\pm$ 0.2	6.7 $\pm$ 0.2	7.1 $\pm$ 0.2	
Pb ²⁺	Cu ²⁺	6	14*	16	--	Additive Inhibition
Pb ²⁺	--	32*	--	--	--	Inhibition
Cu ²⁺	--	--	--	32*	--	Inhibition
Cu ²⁺	Zn ²⁺	16	--	40*	--	Inhibition
Pb ²⁺	Zn ²⁺	57*	--	--	--	Inhibition
Cu ²⁺	Cd ²⁺	--	43	80*	--	Inhibition
Cu ²⁺	Hg ²⁺	--	--	41	80*	Inhibition
Cd ²⁺	Pb ²⁺	75	110*	--	--	Activation
Cd ²⁺	--	--	119*	--	--	Activation
Zn ²⁺	--	125*	--	--	--	Activation
Hg ²⁺	Pb ²⁺	88	138*	--	160	Activation
Cd ²⁺	Zn ²⁺	96	143*	--	--	Activation
Hg ²⁺	Zn ²⁺	53	--	153*	178	Activation
Hg ²⁺	Cd ²⁺	--	116	163*	184	Activation
Hg ²⁺	--	--	--	--	167*	Activation

* pH optimum

** pH range

that is, under the experimental conditions annotated above.

- The pH optimum of the profile that results from the action of equimolar amounts of two metal ions on δ -ALA-D is usually within 0.2 pH unit of that of the most potent ion. If the two ions are matched or, nearly so, the pH optimum usually appears midway between that of the two respective ions.

These conclusions pertain to results obtained under "in vitro" conditions and may not extrapolate to an "in vivo" situation. Unfortunately, it is not possible to verify "in vivo" if all ion pairs including either Hg^{2+} or Cd^{2+} agree with these conclusions since these metals are much too toxic to mammals at the levels required to produce changes in the enzyme. It is nevertheless possible to examine the results described earlier for zinc, lead and copper "in vivo" to determine if these statements apply. Thus, from the results in Figure 27 it is clear that lead is a more potent effector than zinc and can cause enzyme inhibition at lesser concentrations than that required by zinc to activate the enzyme. From the results in Figure 31 it is obvious that lead and copper additively inhibit the enzyme; and, from consideration of several tests involving the administration of both copper and lead to rabbits, it would appear that lead and not copper is the more potent inhibitor of δ -ALA-D "in vivo". This fact could be attributed to lead's greater ability to gain immediate access to the erythrocytes's cytosol and δ -ALA-D "in vivo" than copper. A

similar consideration of the results for "in vivo" zinc and copper in Figures 34 A and B clearly indicate that copper is decidedly a more potent inhibitor of the enzyme than zinc is an activator. Close scrutiny of the results of a sequential administration of all three metals to a rabbit (Figure 35) confirm the conclusions that "in vivo" $Pb > Cu > Zn$ as to potency as effectors of changes in erythrocytic δ -ALA-D.

A final word of caution is required at this point concerning a certain temptation to extrapolate these conclusions to a situation where these metals are added to an isolated and purified preparation of erythrocytic δ -ALA-D. With such a preparation, it is more than likely that different conclusions would be arrived at since the exogenous metals do not have to contend with the complex matrix that is blood and, more specifically, its haemolysate and the numerous alternative binding sites offered to these metals in such a milieu as compared to those available on δ -ALA-D. The conclusions above, therefore, apply to the action of metals on δ -ALA-D present in the complex matrix that is blood and its haemolysate.

This section effectively concludes the investigation of the potential effects of common toxic metals "in vivo" and "in vitro" on the pH-activity profile of erythrocytic δ -ALA-D and the possible consequences these effects may have on the proposed enzyme assays. Admittedly, it was not an exhaustive investigation of all metals which have been reported to cause changes in the enzyme's activity such as silver (22, 116, 163), manganese (96, 116), aluminium (57, 116), nickel (163), tin (77, 116) and others. However, as mentioned

on several occasions before, the main objective of this present study was to restrict it to a consideration of the most prevalent toxic metals in man's work and home environment. There remains one final causative factor which should be examined to complete this investigation of effectors which can impinge on the δ -ALA-D assay that is, the common toxicants carbon monoxide (in tobacco smoke) and ethanol (in alcoholic beverages). These are studied in what follows.

Common Toxicants as Effectors

Man, today knowingly subjects himself at home and at play to a wide variety of chemicals either by the ingestion of drugs, food additives and alcoholic beverages, by inhalation, mainly through the smoking of tobacco products and, by skin absorption via the topical application of commercial health and beauty products. Any one of these chemicals, their by-products and metabolites could conceivably act on erythrocytic δ -ALA-D, cause significant changes in its pH-activity profile and consequently in its assay results. It is obviously beyond the scope of this study to undertake a thorough investigation of all possible effectors from this type of auto-exposure; and, only the two most common and prevalent toxicants which have been reported by certain investigators to cause changes in enzyme activity, namely, ethanol in alcoholic beverages and carbon monoxide from the smoking of tobacco products will be investigated here.

Ethanol

Ethanol can affect haeme and porphyrin metabolism in many ways. For instance, it can inhibit ALA synthetase (164) the initial and rate limiting enzyme of haeme biosynthesis. The discovery that it can also interfere with δ -ALA-D was made by M. R. Moore et al. (30). These investigators are, in the main, responsible for most of the work published on this subject. They reported that erythrocytic δ -ALA-D was significantly depressed in the blood of men intoxicated with alcohol and that the enzyme regained its normal level *pari passu* with the decrease in ethanol level. It was also noted that this return to normalcy required one week or more for chronic alcoholics (165). They also claimed to have observed marked increases in the activity of lead inhibited erythrocytic δ -ALA-D in rats intoxicated with both alcohol and lead. (166).

Moore et al. (30) have postulated that these effects can be linked to the oxidation of ethanol. According to this hypothesis, the oxidation of ethanol in the cell produces reduced nicotinamide adenine dinucleotide (NADH) which in turn reduces glutathione (GSH) and alters the GSH/GSSG ratio of the cell. Since glutathione is known to minimize the effects of δ -ALA-D lead inhibition (111), it is expected that the activity of the lead affected enzyme would rise. However, these same investigators were also able to demonstrate that this rise in GSH adversely affects normal δ -ALA-D whose activity falls with increasing GSH concentration..

These results have been verified by several other workers (79, 167, 168) although some contradictions have emerged (168). It is the purpose of this investigation to verify if these reported changes in δ -ALA-D activity translate into meaningful alterations in the enzyme's pH-activity profile.

For this investigation, a blood sample was collected from control and lead intoxicated rabbits, 50% aqueous solutions of ethanol were administered orally to each and, a second blood sample ⁽¹⁾ collected approximately two hours ⁽²⁾ after alcohol loading. The alcohol and δ -ALA-D level in both samples from each rabbit was determined and compared. The effects of the highest levels of ethanol found in the blood of a control and lead-intoxicated rabbit on the pH-activity profile of erythrocytic δ -ALA-D is shown in Figure 40. The results are as anticipated-- a small but significant ($P < 0.01$) change in activity for the control (inhibition) and lead-poisoned animal (activation). Note that the largest changes appear to occur in the region of the pH optimum in both cases.

Interestingly, a plot of the % residual enzyme activity versus blood alcohol content, Figure 41, reveals some new information concerning the indirect action of this chemical on the enzyme.

(1) As shown earlier, blood letting in rabbits (<3 mL) two hours apart does not appear to stimulate erythropoiesis and cause an increase in δ -ALA-D activity due to a greater number of circulating reticulocytes (169).

(2) According to Moore et al. (30) maximum blood alcohol level is reached within two hours of alcohol intake.

FIGURE 40: Effect of Ethanol (EtOH) on Erythrocytic δ -ALA-D pH-Activity Curve
 (A) Control Rabbit (●) no EtOH (○) 0.122 g EtOH/dL Blood.
 (B) Lead-Poisoned Rabbit 2.01 $\mu\text{M Pb}^{2+}/\text{L}$ (●) no EtOH (○) 0.130 g EtOH/dL Blood.

FIGURE 41: Relationship Between Residual Erythrocytic δ -ALA-D Activity Measured at pH 6.4 and Blood Ethanol Level.

- (●) "Normal" human subjects : $<12 \mu\text{g Pb}^{2+}/\text{dL blood}$
- (▲) Normal rabbits : $<2 \mu\text{g Pb}^{2+}/\text{dL blood}$
- (□) Lead intoxicated rabbits: $>30 \mu\text{g Pb}^{2+}/\text{dL blood}$

Thus, at relatively low ethanol concentrations' (<0.9 g/dL) there is a definite activation of the enzyme in the blood from the control rabbit. Above this level of ethanol, the enzyme's activity is inhibited by ethanol. A similar phenomenon is observed with lead-treated rabbits except that the % increase in enzyme activity reaches higher levels and inhibition does not set in until comparatively higher ethanol levels are attained (>0.15 g/dL).

A somewhat similar experiment was conducted with human volunteers who were asked to imbibe varying quantities of Rhum. The results of this test are also depicted in Figure 41 and show a like pattern. However, increases in human erythrocytic δ -ALA-D activity occur at levels of blood ethanol <0.05 g/dL and do not exceed 12%. These results are not that dissimilar from those of Moore et al. (30) who reported a 30% decrease in enzyme activity for a mean blood alcohol concentration of 0.225 g/dL (extrapolation of the graph in Figure 41 to this value of blood alcohol indicates a 22% decrease in enzyme activity).

The data above lends support to the Moore postulate for the indirect action of ethanol on δ -ALA-D in blood (30). Thus, at low ethanol concentrations only a small amount of GSH would be produced, just enough to activate both normal and lead inhibited enzymes. As the concentration of alcohol rises so does that of GSH until it becomes inhibitive to the normal enzyme (possibly by removing the essential element zinc) but not to the lead affected enzyme which it still has the ability to activate by removing lead. However, at high enough levels of ethanol, GSH

would be expected to become inhibitory to the lead-poisoned enzyme also.

In a normal test population, it would be quite unlikely for a researcher to come across a subject with >0.1 g/dL alcohol blood content (more than legally drunk!). Hence, the results of main interest above are those in the range of ethanol concentration <0.1 g/dL. And, as Figure 41 illustrates, the % increase or decrease of δ -ALA-D activity is 10% or close enough to the value of the coefficient of variance usually obtained with δ -ALA-D assay (6%) not to be a problem. Furthermore, the enzyme's pH-activity profile does not appear to be unduly affected by ethanol and this forbodes well for an assay protocol based on measurement of enzyme activity at two pH values.

Carbon monoxide

Carbon monoxide (CO) is produced by the incomplete combustion of carbonaceous material. Although any given person is exposed to this gas from a multitude of sources in his environment, either at home or at work, by far the most important source for some individuals is cigarette smoke which may contain upwards of 4-5% CO. In fact, with every puff from a cigarette, a smoker literally exposes himself to the equivalent of a 400 ppm CO atmosphere.

Carbon monoxide is not a cumulative poison, but is excreted or absorbed in the lungs at a rate dependent upon its partial pressure in ambient air, the amount of carboxyhaemoglobin (COHb),

in the blood, the duration of exposure, the rate of ventilation of the lungs and the health and metabolism of the exposed individual. The gas is absorbed through the lungs into capillary blood where it reacts with the iron in porphyrin. Thus, it affects haemoglobin, myoglobin, cytochrome oxidase, cytochrome P-450 (170) and according to Azar et al. (171) and others (81, 172) it also can affect δ -ALA-D in blood.

Symptoms of CO intoxication in man are directly related to blood COHb levels. In a non-smoker, the normal background level of COHb ranges between 0.4 and 0.8%, levels which are attributed to endogenous sources, such as haeme catabolism (173). In smokers, however, these values can attain a median value of 5% and a maximum near 15%.

Since smoking has been claimed by some to be associated causally with higher blood lead levels (174, 175) it was decided not to use a protocol involving smokers versus non-smokers but to conduct all experimental work with rabbits.

Blood was collected from each test rabbit which was then placed in a large Saran plastic bag; 100% CO gas was admitted into the bag till the atmosphere inside the bag reached $\approx$ 500 ppm. The animals were kept inside the exposure chamber for periods ranging from 5-30 minutes. A second blood sample was collected immediately after the exposure period.

The effect of a level of 54.9 and 18.4% COHb in blood on δ -ALA-D is shown in Figure 42. It can readily be seen that even these high levels of COHb have little or no effect on δ -ALA-D

FIGURE 42: Effect of Carbon Monoxide (CO) on the pH-Activity Profile of δ -ALA-D

(A) (●) no CO

(□) 54.9 % COHb

(B) (●) no CO

(□) 18.4 % COHb

activity and its pH-activity profile. In essence, these results agree with those of Moore and Meredith (81) who reported reductions of activity of 2.1% or less in smokers as compared to non-smokers and concluded that it is unlikely that CO levels found in heavy smokers will be of any consequence in the use of δ -ALA-D as a biological monitor of toxic metals, and this, despite anecdotal evidence suggesting that such levels have a profound effect on the enzyme (81).

Micro Scale δ -ALA-D Assay

Several methods have been proposed for measuring δ -ALA-D activity in erythrocytes based upon the enzyme's conversion of two molecules of δ -ALA to porphobilinogen (PBG) which is then measured spectrophotometrically by reaction with Erlich's reagent (82, 86, 90, 92, 95). In general, these involved modifications of the original Granick-Mauzerall method (83) and usually included a suggested change in the buffer system utilized to achieve better reaction pH control. Assay methods varied from study to study and this made intercomparison of results almost impossible. In an effort to achieve some uniformity in the assay of the enzyme, Berlin and Schaller (82) in 1974, proposed the "European Standardized Method" which has since become widely accepted.

The methodology as it stands, however, has a number of drawbacks, one of which is the requirement for venipuncture in order to obtain a sufficient quantity of blood. In some instances, it may be significantly preferable to collect blood for ALA-D assay by skin puncture rather than by the customary venipuncture. These may include: a need for frequent blood sampling to monitor occupational exposure to heavy metals; reticence on a donor's part to submit to venipuncture; veins difficult to tap owing to their size and location. With children or infants, venipuncture can be distressful and excessively hazardous and skin puncture is definitely preferable. There may be other reasons to opt for finger prick collection such as difficulties encountered in collecting, transporting, storing and shipping large numbers of blood samples from remote and relatively inaccessible sites.

Such an approach would also greatly facilitate the collection of blood samples from the test populations which form an integral part of this study. Another conceivable benefit would be an improved control of trace contamination of collected blood samples as Vacutainers would no longer be needed.

The collection of blood samples via finger pricks entails a drastic reduction of the size of sample required to conduct an assay since only 200-500 μL of blood are usually collected by this procedure. Most δ -ALA-D assay methods presently require 200 μL or more of blood per test (or 800 μL or more for triplicate test and control samples). This large sample requirement also makes the conduct of several concurrent and repetitive tests on the same blood sample, as described in the previous section, very difficult unless a researcher has ready access to large quantities of blood. The assay, therefore, should have a requirement for a relatively small blood sample.

In view of the numerous exogenous and procedural factors which can drastically affect assay results (Table XVI), the adopted procedure must, of necessity, incorporate rigid safeguards against these effectors.

Assay Protocol

From the foregoing it is obvious that the objectives which must be considered in the development of a micro-assay for δ -ALA-D include:

- reduction of the quantity of blood required to the minimum volume which will afford optimal reproducibility;
- incorporation of safeguards to avoid all procedural pitfalls and interferences which have been

TABLE XVI: Summary of Effectors and the Change(s) each Causes in the pH-Activity Profile of Normal Erythrocytic α -ALA-D

Effector	Effects		
	pH-optimum shift	Activation	Inhibition
Buffer type and concentration	Slight ⁽¹⁾	--	--
Buffered haemolysate incubated at 37 °C, 30 min.	Large ⁽²⁾ $\longrightarrow$	--	Strong ⁽³⁾
Haemolysate incubated at 37 °C, 30 min.	Slight $\longrightarrow$	--	Strong
Storage at 4 °C >2 weeks	$\longleftarrow$ Slight	--	Strong
Dithiothreitol (0.02M)	Slight $\longrightarrow$	Strong	--
Trichloroacetic acid (>1 ppm)	Slight $\longrightarrow$		Mild ⁽⁴⁾
Endo ⁽⁵⁾ -lead (>15 μ g/dL)	$\longleftarrow$ Large	--	Strong
Exo ⁽⁵⁾ -lead (>10 μ g/dL)	$\longleftarrow$ Large	--	Strong
Endo-copper (>30 μ g/dL)	Large $\longrightarrow$	--	Strong
Exo-copper (>3 μ g/dL)	Large $\longrightarrow$	--	Strong
Endo-zinc (> μ g/dL)	$\longleftarrow$ Large	Strong	--
Exo-zinc (>10 μ g/dL)	$\longleftarrow$ Large	Strong	--
Endo-mercury (<50 μ g/dL)	No effects		
Exo-Mercury (>100 μ g/dL)	Large $\longrightarrow$	Strong	--
Endo-cadmium (<30 μ g/dL)	No effects		
Exo-cadmium (>90 μ g/dL)	$\longleftarrow$ Slight $\longrightarrow$	Mild	--
Ethanol	No effects		
Carbon Monoxide	No effects		

(1) Slight - ≤ 0.2 pH unit shift(2) Large - > 0.2 pH unit shift; arrow indicates predominant direction of shift(3) Strong - $> 10\%$ change in activity(4) Mild - $< 10\%$ change in activity at any point on curve

(5) Endo - endogenous metal } amount of metal in parentheses represents an estimate of minimum level required to produce effect(s).
 Exo - exogenous metal }

identified to date in the study;

- favorable comparison of results obtained with those of a corresponding universally accepted macromethodology, the "European Standardized Method" (82).

Each of these factors are considered and discussed in turn in what follows.

Blood sample

Collection. 250-500 μ L finger prick samples can best be obtained with polyethylene collection tubes. These tubes have been checked and found to be relatively free of metal contaminants. The tubes are heparinized by wetting with a solution of 5% (w/v) sodium heparin. However, care should be taken to verify that the sodium heparin solutions used are free of metals, as some commercially available products contain surprisingly large quantities of zinc. If larger blood samples are needed, these can be collected with a plastic syringe and transferred to an heparinized plastic tube, as noted earlier.

Size. At the onset of this study, attempts were made to use 5 μ L of whole blood. Difficulties were encountered in obtaining reproducible results and this was attributed to errors inherent in pipetting such a small volume of blood. Good reproducibility was achieved by pipetting 10- μ L blood samples with a positive displacement micro pipette and this approach was adopted for the assay.

Haemolysate preparation

Several methods were explored to achieve complete lysis of the blood samples. Although the freeze-thaw method tended to yield marginally higher activity values, the non-ionic detergent Triton X-100

was selected for the preparation of haemolysate because it afforded greater ease in the preparation of the lysates for large numbers of blood samples. Lysis was assumed virtually complete within minutes of adding the reagent. For the reasons discussed at length elsewhere, the practice of pre-incubating either the buffered or non-buffered lysates at 37 °C for varying periods of time, as recommended by others (82, 92, 178) was not even considered.

Buffer system

As noted earlier, 0.3 M/L sodium phosphate buffer appears to be the best choice for the assay. Since the δ -ALA-D curves for individuals having 5-10 $\mu\text{g/dL Pb}^{2+}$ in their blood consistently peaked at a pH of 6.4 ± 0.1 , this pH value was selected for the assay. This value also agreed with that selected by the majority of researchers.

Reaction conditions

The optimum δ -ALA-D substrate concentration, 0.125 M/L, incubation temperature, 37 °C, and duration of incubation, 60 minutes, have been fully established in several previous studies and no attempts were made to modify these conditions.

Color Formation

For a protocol requiring the use of 10 μL of blood, the reaction of PBG in the supernatant with modified Erlich reagent is best performed in the optical micro cell itself. Sample throughput can be increased if a more rapid development of the Erlich's colored salt than the 15 minutes normally required is achieved. This is

effectively accomplished simply by using double the aliquot of the reagent added to the supernatant. Color development is essentially complete between 2 to 5 minutes and tends to diminish slightly thereafter. The resulting medium is devoid of precipitant and no filtration is required as suggested by others (82).

Precision and reproducibility

The reproducibility of the method either day-to-day or within-run was very good as the following shows:

Mean ALA-D concentration μ mole PBG/h/L _{RBC}	Within-run (n = 20)		Day-to-day (n = 14)	
	SD	CV%	SD	CV%
1588			78	4.9
1065	54	5.1		
557	22	4.0		
308	11	3.6		

The relative variability about the mean of the control samples was within acceptable limits.

Comparison with the "European Standardized Method" (82)

The level of δ -ALA-D in the blood of 24 workers exposed to heavy metals was determined simultaneously with the proposed micro-method and the "European Standardized Method". The results obtained were evaluated by the least-squares linear regression method. The scattergram shown in Figure 43 demonstrates a good correlation ($r = 0.95$),

FIGURE 43: Correlation Between Proposed Micro-Method and "European Standardized Method" for Assay of δ -Aminolaevulinic Acid Dehydratase.

a negligible Y-intercept, and a proportionality error (slope = 0.95) in favor of the micro-method. This disparity between the two sets of values was expected since pre-incubation of the haemolysate as required by the protocol of the "European Standardized Method" will reduce δ -ALA-D activity significantly. To negate all possible bias, the δ -ALA-D activity in split blood samples collected from 49 children living in the vicinity of a lead smelter was determined by the "European Standardized Method" at the Centre de Toxicologie de Laval, Quebec, laboratory, and by the micro-method in our laboratory. The results, shown in Figure 44, also demonstrate that relatively higher results are obtained with our technique.

The ALA-D activity loss through pre-incubation of the haemolysate can be restored by treatment with a chemical agent, such as dithiothreitol, which provides exogenous thiol groups. If pre-incubation is the main cause of the disparity between the two methods, determination of the activated enzyme should remove this difference. The results of 33 samples, obtained from persons with no known exposure to heavy metals, shown in Figure 45 indicate that this was indeed the case. However, although a good correlation was again obtained ($r = 0.97$), the regression line, slope of 1.04, now indicated a positive bias in favor of the "European Standardized Method". Interlaboratory comparison of the two methods for the assay of the activated enzyme confirmed these results. ($r = 0.90$, $Y = 1.01 x - 179$, $S_{y/x} = 131$). Therefore, it would appear that treatment of blood with Triton X-100 as suggested by the protocol does not lead to total lysis. Nevertheless, when faced with the possible alternative procedures for performing lysis such as preheating the haemolysate or the more cumbersome freeze-thawing technique, the use

FIGURE 44: Interlaboratory Comparison of δ -ALA-D Assay Results Obtained with Micro-Method (Ottawa) and "European Standardized Method" (Quebec) on Same Set of Blood Samples.

FIGURE 45: Correlation Between Proposed Micro-Method and "European Standardized Method" for Assay of δ -Aminolaevulinic Acid Dehydratase After Enzyme Activation with Dithiothreitol.

of Triton X-100 to achieve lysis was decidedly preferable for the determination of inactivated δ -ALA-D with the micro-method.

Advantages and disadvantages

The statistically significant correlation of results obtained from inter and intra laboratory comparison of analysis performed on aliquots of the same samples indicates that it can function as a sensitive and precise alternative to the widely accepted "European Standardized Method" when only small quantities of blood are available for δ -ALA-D assay. The procedure was adopted for all investigations reported herein and has yielded very consistent results during the past three years.

For the clinician, the method presents irrefutable advantages. These include the requirement for relatively inexpensive equipment, small quantities of reagents and, consequently, reduced cost per assay, and a relatively short period of training. An inexperienced person usually can perform 100 to 150 assays per day after two or three days of training. The method also incorporates important safeguards against the factors (Table XVI) which can drastically affect assay results.

Unfortunately, the micro-assay method described above, although not subject to errors caused by either procedural or exogenous factors which plague other available methods, nevertheless still possesses many inherent characteristics which decidedly diminish its ready acceptance as a biologic monitor of exposure to toxic metals by clinicians and researchers alike. These systemic drawbacks include:

- requirement to perform the assay within 24 hours of blood sample collection;

- the need to determine the blood hematocrit;
- the need to perform fairly involved calculations;
- lack of agreement in the units to use in reporting the results;
- the large range of normal δ -ALA-D values obtained ;
500 - 2500 $\mu\text{M PBG/h/L}_{\text{RBC}}$;
- the curvilinear correlation obtained between δ -ALA-D levels and toxic metal concentrations (more specifically lead);
- the apparent lack of specificity of the test for a metal such as lead in the presence of important concentrations of either zinc or copper in blood.

It is hoped that a protocol making use of a ratio of δ -ALA-D activities, measured at different pH values, as a biologic index will eliminate some, if not all, of these drawbacks while still incorporating some of the micro-assay's obvious advantages. Such a study is reported in the next section.

Ratios of δ -ALA-D Activity Measured at Specific pH Values:

Trials as Biologic Indices of Metal Intoxication

From the preceding discussion, it is clear that an alternative to the customary measurement of enzyme activity at a set pH value must be sought if erythrocytic δ -ALA-D is to achieve wide acceptance as a useful biologic monitor of metal intoxication. Consideration of the δ -ALA-D pH-activity profiles of normal and lead-poisoned blood (Figure 11A) reveals such a possibility--that is, measurement of the enzyme's activity at two judiciously selected pH values on the profile, division of one value by the other, and correlation of the ratio so obtained with the blood's metal content. This approach is promising if one considers the results in Figure 7, which indicate that the overall shape of the profile, and consequently any ratios derived thereof, is not expected to change significantly during the storage of the blood at 4 °C for periods of 2-3 weeks. Thus, one of the micro-assay's shortcomings, that is, the need to perform the test within a specified time after blood collection, would be overcome.

Figure 46 exemplifies yet another alternative. The pH-activity profiles of δ -ALA-D for the buffered haemolysate of normal and lead-poisoned human blood, before and after pre-incubation at 37 °C for 30 minutes are shown. Accordingly, advantage can be taken of the difference in pH optimum shift and loss of activity shown by the lead-poisoned blood δ -ALA-D as compared to that of normal blood. A ratio derived from the activity of the enzyme measured at carefully selected pH values before and after pre-incubation

FIGURE 46: pH-Activity Relationship of δ -ALA-D in Lead-Poisoned Human Bloods Before (●) 51 $\mu\text{g/dL}$, (▲) 13 $\mu\text{g/dL}$ Lead) and After (□) 51 $\mu\text{g/dL}$, (○) 13 $\mu\text{g/dL}$ Lead) Incubation of the P-P Buffered Haemolysate at 37 °C for 30 Minutes.

could effectively extend the useful range of blood lead concentrations over which the test could be used. It also should be noted that such approaches negate the need to perform blood hematocrits and involved δ -ALA-D activity calculations.

The use of ratios of δ -ALA-D activities to assess exposure to metals, and more specifically lead, is not without precedent. In 1973, Granick et al. (52) proposed the use of a ratio of dithiothreitol-activated enzyme to non-activated enzyme as a test for lead intoxication. Since then, other means of activating the enzyme either by heating (100), with zinc (97) or zinc and dithiothreitol (97) or reduced glutathione (86), have been suggested. In general, the test has met with limited acceptance. The following year, in 1974, Tomokuni (95) confirmed the occurrence of the pH-optimum shift in lead-poisoned blood earlier observed by Nikkanen et al. (92); and attempted to make use of this phenomenon by suggesting a test based on the ratio of the enzyme's activity measured at pH 6.8 (pH optimum for normal blood according to Tomokuni?) and that measured at pH 6.0 (pH optimum for lead-poisoned blood). However, he reported a poor correlation ($r = 0.54$) between this ratio and the concomitant blood lead levels. Recently, Chmielnicka et al. (98) compared results obtained with Tomokuni's method with those obtained with the "European Standardized Method" over a wide range of blood lead concentrations and reported that Tomokuni's method showed superior correlation at high blood lead levels. A report of investigations of similar methods based on the ratio of δ -ALA-D activity at specific pH values as potential biologic monitors of exposure to metals follows.

Selection of Appropriate δ -ALA-D Activity Ratio(s)

Obviously, a very large number of possible combinations of pairs of pH values exist at which the enzyme activity could be measured to derive a ratio. Instead of making an arbitrary choice based upon uncertain criteria, the most plausible activity ratios were determined from rabbit metal intoxication trials.

Lead

The changes in δ -ALA-D pH-activity profiles caused by exogenous and endogenous lead and other factors, presented earlier, form the basis of the initial pH value selection. Thus, in Figure 47, consideration of the profiles of δ -ALA-D in normal and lead-poisoned rabbit blood, with and without prior incubation of the buffered lysate, indicate that such a preliminary selection ought to include the pH optimum of each profile that is, pH 6.0, 6.4, 6.6 ^{Δ} (1) and 7.2 ^{Δ} . However, pH 6.0, chosen by Tomokuni, was eliminated at the very outset as previous observations of the effects of low-level cadmium or zinc contamination and zinc "in vivo" (Figures 19, 20, 21, 25) indicated that ratios including measurements at this pH value were susceptible to drastic changes. As will be shown later, this was a fortuitous decision.

Thirty possible ratios of enzyme activity are obtained by pairing measurements made at the three remaining pH values with or without pre-incubation of the lysate. Four plausible ratios namely;

(1) ^{Δ} Indicates prior incubation of the buffered lysate at 37 °C for 30 minutes.

FIGURE 47: Activity Ratio $6.6^{\Delta}/6.4$ Derived from the pH-Activity Profile of δ -ALA-D Before and After Incubation of the P-P Buffered Haemolysate at 37°C for 30 Minutes: Normal Rabbit Blood Before (●) and After (○) Incubation; Lead-Poisoned Rabbit Blood Before (□) and After (■) Incubation.

6.4/7.2, $6.6^{\Delta}/7.2^{\Delta}$, $6.6^{\Delta}/6.4$ and $7.2^{\Delta}/7.2$ were selected on the basis that they would give a positive linear correlation between each ratio and blood lead levels, sizable change in ratio value with increasing blood lead concentration and would reflect some meaningful changes in the enzyme's pH-activity profile. One of these ratios, $6.6^{\Delta}/6.4$, is depicted in Figure 47 for both normal and lead-poisoned rabbits.

Rabbits were maintained on a lead diet and their blood collected weekly as described earlier. The enzymic determination was conducted, as detailed in the micro-assay procedure, simultaneously at pH 6.4, 7.2, 6.6^{Δ} and 7.2^{Δ} . The correlation between each ratio and the blood lead levels was excellent ($r \geq 0.93$) in each instance (Figures 48A-D). As mentioned earlier, rabbit erythrocytic δ -ALA-D appears more sensitive to lead insult than man's and few ratios were obtained above 50 $\mu\text{g}/\text{dL}$ blood lead for this reason. This was especially true for ratios which included pH measurement of the enzyme's activity at pH 7.2. At this pH, the Erlich colored salt tended to have an absorbance less than 0.1 unit and standard deviations for triplicate readings were consistently large.

Assuming that rabbits with less than 2 μg lead/dL blood are normal, the following facts arise from the determination of the four ratios, as described above, in the blood of thirty-six normal rabbits.

FIGURE 48: Correlation of the 6.4/7.2 (A), 6.6 Δ /7.2 Δ (B), 6.6 Δ /6.4 (C) and 7.2 Δ /7.2 (D) 6-ALA-D Activity Ratios with Rabbit Blood Lead Concentrations. Deviation of Each Ratio From the Regression Line in the Presence of Varying Net Increase in the Concentration ($\mu\text{g/dL}$) of Zinc (Δ) or Copper ($\square$) is also Shown.

FIGURE 48 (cont.)

FIGURE 48 (cont.)

FIGURE 48 (cont.)

<u>Ratio</u>	<u>Mean $\pm$ S.D.</u>	<u>C.V. (%)</u>	<u>Range</u>
6.4/7.2	1.17 $\pm$ 0.10	8.6	0.90 - 1.43
7.2 ^{Δ} /7.2	0.91 $\pm$ 0.08	9.0	0.37 - 1.00
6.6 ^{Δ} /6.4	0.46 $\pm$ 0.18	39	0.17 - 1.00
(6.6/7.2) ^{Δ}	0.58 $\pm$ 0.23	40	0.23 - 1.40

 δ -ALA-D(μM PBG/h/L_{RBC})

1454 $\pm$ 317	22	674 - 2128
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Comparison of these results with δ -ALA-D activity measurements for the same rabbits reveals a marked reduction in the range of normal values for ratios 7.2 ^{Δ} /7.2 and 6.4/7.2 as compared to that obtained from enzymic activity.

As shown in Figure 49, the between-day reproducibility of the activity ratio method, over a period of 34 days of storage at 4 °C, was excellent for all ratios at both high and low concentrations of blood lead. The following results further attest to the remarkable stability of each ratio over an extended period of storage at 4 °C, as compared to that of the enzyme's intrinsic activity during the storage period.

<u>Blood lead</u>	<u>Ratio</u>	<u>Mean $\pm$ S. D.</u>	<u>C. V. (%)</u>
4 μg/dL	6.4/7.2	1.39 $\pm$ 0.11	8.0
	7.2 ^{Δ} /7.2	0.98 $\pm$ 0.06	6.0
	6.6 ^{Δ} /6.4	0.64 $\pm$ 0.04	6.0
	6.6 ^{Δ} /7.2 ^{Δ}	0.88 $\pm$ 0.06	7.0

FIGURE 49: Comparison of the Between-Day Reproducibility of the Activity-Ratios 6.4/7.2 (O), 6.6/7.2 (■), 6.6/6.4 (□), 7.2/7.2 (Δ) and δ -ALA-D Activity Values (●) for Normal (solid lines) and Lead-Poisoned (broken lines) Rabbit Blood.

<u>Blood lead</u>	<u>Ratio</u>	<u>Mean</u> <u>S. D.</u>	<u>C. V. (%)</u>
59 μ g/dL	6.4/7.2	not measurable	—
	7.2 ^{Δ} /7.2	not measurable	—
	6.6 ^{Δ} /6.4	2.91 $\pm$ 0.45	15
	6.6 ^{Δ} /7.2 ^{Δ}	5.25 $\pm$ 0.52	10

Within-day variation of each ratio was similarly excellent (C. V. < 6%) at high and low levels of blood lead.

According to the results presented above, the four activity ratios selected would appear to be superior to the measurement of δ -ALA-D as a biologic measure of lead intoxication. Thus, the determination can be made days or weeks after blood collection, correlation with blood lead is linear, a narrower range of normal values is possible and the test can operate over a wider range of blood lead values. It is also procedurally simpler to perform as it does not require the measurement of hematocrits and the performance of involved calculations. It remains to be seen if the test is specific for lead or if it can be used to assess intoxication by other metals.

Zinc

Rabbits (blood lead < 2 μ g/dL) were injected subcutaneously with solutions of zinc lactate and their blood collected within four hours as described earlier. The four activity-ratios selected to assess lead intoxication were tested for zinc poisoning. Examples of the δ -ALA-D activity ratios obtained from the blood of zinc-intoxicated rabbits follow:

3

<u>Rabbit No. 1</u>				
<u>Zinc $\mu\text{g/dL}$</u>	<u>6.4/7.2</u>	<u>7.2$^{\Delta}$/7.2</u>	<u>6.6$^{\Delta}$/6.4</u>	<u>6.6$^{\Delta}$/7.2$^{\Delta}$</u>
350 (Normal)	1.03 $\pm$ 0.02	0.93 $\pm$ 0.12	0.62 $\pm$ 0.04	0.92 $\pm$ 0.02
410	1.16 $\pm$ 0.02	0.99 $\pm$ 0.02	0.65 $\pm$ 0.02	0.99 $\pm$ 0.04
528	1.15 $\pm$ 0.05	0.92 $\pm$ 0.04	0.64 $\pm$ 0.03	0.80 $\pm$ 0.04
964	1.15 $\pm$ 0.07	0.92 $\pm$ 0.04	1.09 $\pm$ 0.02	1.15 $\pm$ 0.02

<u>Rabbit No. 2</u>				
400	1.29 $\pm$ 0.03	1.00 $\pm$ 0.03	0.70 $\pm$ 0.01	0.91 $\pm$ 0.02
598	1.36 $\pm$ 0.07	1.03 $\pm$ 0.05	0.75 $\pm$ 0.05	0.99 $\pm$ 0.07
692	1.27 $\pm$ 0.03	1.10 $\pm$ 0.03	0.98 $\pm$ 0.02	1.13 $\pm$ 0.02

Other results, not given here, indicate a similar trend that is, ratios 6.4/7.2 and 7.2 $^{\Delta}$ /7.2 are not significantly ($P > 0.01$) affected by increases in blood zinc close to three times normal levels, whereas ratio 6.6 $^{\Delta}$ /6.4 and, to a lesser extent, 6.6 $^{\Delta}$ /7.2 $^{\Delta}$, increase with rising zinc levels. Examination of Figures 19A and B reveals why the 6.4/7.2 ratio does not change measurably: commensurate changes occur at pH 6.4 and 6.8 (also 7.2, not shown) over a wide range of blood zinc increases and, hence, the ratio 6.4/7.2 is not expected to and does not show a significant change in value. Figure 50 exemplifies the effects of a large concentration of endogenous zinc upon the enzyme's pH-activity profile in zinc-poisoned blood with and without prior incubation of the buffered haemolysate at 37 °C for 30 minutes. From a comparison with Figure 47, it is clearly observed that zinc markedly enhances the activity of the enzyme at pH 6.6 after pre-incubation; and,

FIGURE 50: δ -ALA-D's pH-Activity Profile of Zinc and Copper-Poisoned Rabbit Blood Before and After Incubation of the P-P Buffered Haemolysate at 37 °C for 30 Minutes:
 Zinc-Poisoned (964 $\mu\text{g/dL}$) Blood Before (○) and After (●) Incubation.
 Copper-Poisoned (257 $\mu\text{g/dL}$) Blood Before (▲) and After (△) Incubation.

consequently, increases the value of the ratios $6.6^{\Delta}/6.4$ and $6.6^{\Delta}/7.2^{\Delta}$. The ratio $7.2^{\Delta}/7.2$ is not affected.

The dramatic increase in enzyme activity at pH 6.0, shown in Figure 19B, clearly vindicates an earlier decision not to use this pH value for ratios to measure lead poisoning since a ratio such as $6.0/7.2$ would have given false positive results for lead. Nevertheless, on the basis of a few results (examples shown), it could be concluded that this ratio, and possibly the ratio $6.6^{\Delta}/6.4$, could be used to assess exposure to zinc, albeit in the absence of changes in blood lead levels and for sizable increases in zinc levels.

<u>Ratio</u>	<u>Zinc $\mu\text{g/dL}$</u>	<u>Mean $\pm$ S. D.</u>
6.0/7.2	350	0.56 ± 0.04
	408	0.65 ± 0.02
	416	0.59 ± 0.03
	548	0.82 ± 0.06
	964	1.29 ± 0.02

Copper

Rabbits (blood lead $< 2 \mu\text{g/dL}$) were injected subcutaneously with solutions of copper glycinate and their blood collected within four hours as described earlier. Again, the same four activity-ratios were tested as biologic indices of copper intoxication. Examples of the values obtained for the blood of a single rabbit whose blood copper attained nearly ten times normal level follow:

<u>Copper $\mu\text{g/dL}$</u>	<u>6.4/7.2</u>	<u>7.2$^{\Delta}$/7.2</u>	<u>6.6$^{\Delta}$/6.4</u>	<u>6.6$^{\Delta}$/7.2$^{\Delta}$</u>
72	1.24 $\pm$ 0.02	0.82 $\pm$ 0.03	0.41 $\pm$ 0.01	0.62 $\pm$ 0.02
148	1.17 $\pm$ 0.04	0.78 $\pm$ 0.03	0.18 $\pm$ 0.03	0.25 $\pm$ 0.04
168	1.11 $\pm$ 0.03	0.64 $\pm$ 0.06	0.14 $\pm$ 0.03	0.24 $\pm$ 0.03
200	1.09 $\pm$ 0.02	0.58 $\pm$ 0.02	0.10 $\pm$ 0.02	0.20 $\pm$ 0.03
220	1.09 $\pm$ 0.05	0.53 $\pm$ 0.01	0.03 $\pm$ 0.01	0.70 $\pm$ 0.01
368	0.91 $\pm$ 0.03	0.45 $\pm$ 0.04	0.04 $\pm$ 0.01	0.07 $\pm$ 0.01
672	0.87 $\pm$ 0.05	----- not measurable -----		

According to these results, the ratio 6.4/7.2 is not significantly ($P > 0.01$) affected by rising blood copper levels until these reach approximately five times normal levels. Conversely, the ratio 7.2 $^{\Delta}$ /7.2, and to a greater extent the ratios 6.6 $^{\Delta}$ /6.4 and 6.6 $^{\Delta}$ /7.2 $^{\Delta}$, are dramatically reduced as blood copper doubles its value. Figure 15A and B and 50 show why. Activity values at pH 6.4, 6.8 (and 7.2 not shown) (Figure 15A, B) are not inordinately reduced as blood copper levels rise; and, the ratio 6.4/7.2 is likewise not expected to be greatly reduced. As described earlier, most of the copper "in vivo" is located in the plasma. Haemolysis of copper-poisoned blood followed by incubation lengthens the time of contact between the metal and the enzyme, and strongly favors their interaction. As a result (Figure 50), activity at all pH values is greatly reduced, the pH-activity profile is shifted to more basic pH (pH optimum > 7.4) and the activity-ratios which include pre-incubation of the haemolysate are drastically affected.

The sizable impact of copper on enzyme activities determined at pH 6.0 (Figures 15A and B), further reinforces the decision not to use this pH value to assess lead intoxication. As the following results show, copper poisoning, similar to zinc intoxication, could be assessed by determining the activity ratio 6.0/7.2 that is, in the absence of changes in zinc and lead levels and at fairly high copper levels. The activity ratios $6.6^{\Delta}/6.4$ and $6.6^{\Delta}/7.2^{\Delta}$ could likewise be used.

<u>Ratio</u>	<u>Copper ($\mu\text{g}/\text{dL}$)</u>	<u>Mean $\pm$ S. D.</u>
6.0/7.2	54	1.24 ± 0.08
	73	1.18 ± 0.10
	144	0.63 ± 0.04
	220	0.50 ± 0.02

Mercury

The results of mercury intoxication experiments, conducted as earlier described, and listed in Table XVII, clearly indicate that the metal has only minimal effects on the activity ratios being tested at levels that can be found in mercury-poisoned humans. These results are not surprising. A cursory examination of Figure 22 readily shows that little or no meaningful changes in the enzyme's pH-activity profile and, consequently, in the ratios, are expected at concentrations of mercury $< 5 \mu\text{M Hg} / \text{L}$ (or $100 \mu\text{g}/\text{dL}$). (1)

(1) Although not shown here, similar minimal changes in pH-activity profile are observed for the enzyme in incubated buffered lysate for mercury-poisoned blood.

TABLE XVII : EFFECTS OF METAL INTOXICATION - RABBIT EXPERIMENTS

Blood Sample Number	α -ALA-O Activity (μ M PBGM/100C)		Activity Ratios at Specific pH Values						Metal Concentrations (μ g/dl)			
	-DTT (a)	+DTT/-DTT	(6.4/7.2) (b) -DTT	(6.4/7.2) +DTT	(6.4/7.2) (b) -DTT	(6.4/7.2) +DTT	(6.5/7.2) (c) -DTT	(6.5/7.2) +DTT	Lead	Zinc	Copper	Mercury
R-117	2015 $\pm$ 36	---	1.09 $\pm$ 0.06	---	0.63 $\pm$ 0.01	0.51 $\pm$ 0.01	0.88 $\pm$ 0.05	0.88 $\pm$ 0.05	3.7	373	83	0.42
R-121	1832 $\pm$ 35	---	1.03 $\pm$ 0.02	---	0.43 $\pm$ 0.03	0.21 $\pm$ 0.01	0.86 $\pm$ 0.01	0.86 $\pm$ 0.01	< 2.0	399	72	0.57
R-133	1756 $\pm$ 57	---	1.06 $\pm$ 0.03	---	0.41 $\pm$ 0.06	0.36 $\pm$ 0.05	0.92 $\pm$ 0.02	0.92 $\pm$ 0.02	< 2.0	373	74	0.53
R-147	1810 $\pm$ 48	---	1.07 $\pm$ 0.03	---	0.49 $\pm$ 0.04	0.39 $\pm$ 0.03	0.86 $\pm$ 0.01	0.86 $\pm$ 0.01	< 2.0	346	66	1.69
R-163	1639 $\pm$ 60	---	1.06 $\pm$ 0.02	---	0.53 $\pm$ 0.01	0.47 $\pm$ 0.02	0.95 $\pm$ 0.02	0.95 $\pm$ 0.02	< 2.0	352	70	3.30
R-173	1746 $\pm$ 29	---	1.18 $\pm$ 0.05	---	0.89 $\pm$ 0.02	0.78 $\pm$ 0.02	1.04 $\pm$ 0.04	1.04 $\pm$ 0.04	< 2.0	430	78	2.14
R-183	1593 $\pm$ 75	---	1.07 $\pm$ 0.05	---	0.85 $\pm$ 0.02	0.82 $\pm$ 0.04	1.03 $\pm$ 0.02	1.03 $\pm$ 0.02	< 2.0	380	81	2.04
R-193	1509 $\pm$ 18	---	1.28 $\pm$ 0.03	---	0.74 $\pm$ 0.02	0.59 $\pm$ 0.02	1.02 $\pm$ 0.02	1.02 $\pm$ 0.02	< 2.0	344	88	3.83
R-211	1626 $\pm$ 24	---	1.15 $\pm$ 0.03	---	0.73 $\pm$ 0.02	0.70 $\pm$ 0.03	1.10 $\pm$ 0.03	1.10 $\pm$ 0.03	< 2.0	360	88	19.1
R-225	1509 $\pm$ 0	---	1.26 $\pm$ 0.06	---	0.96 $\pm$ 0.04	0.80 $\pm$ 0.04	1.05 $\pm$ 0.02	1.05 $\pm$ 0.02	< 2.0	340	92	19.5
R-239	1119 $\pm$ 3	---	1.32 $\pm$ 0.00	---	0.79 $\pm$ 0.02	0.64 $\pm$ 0.00	1.08 $\pm$ 0.03	1.08 $\pm$ 0.03	< 2.0	290	83	12.4
R-249	1530 $\pm$ 64	---	1.29 $\pm$ 0.05	---	0.96 $\pm$ 0.02	0.72 $\pm$ 0.02	0.96 $\pm$ 0.03	0.96 $\pm$ 0.03	3.6	350	78	27.8
R-252	267 $\pm$ 12	---	6.82 $\pm$ 2.00	---	3.94 $\pm$ 0.25	3.25 $\pm$ 0.95	5.64 $\pm$ 0.56	5.64 $\pm$ 0.56	46.5	366	104	12.5

Cadmium

From previous observations, it was found that no detectable changes in the enzyme's pH-activity profile occur at blood concentrations of $0.2 \mu\text{M/L}$ (or $27.4 \mu\text{g/dL}$) that is, at levels of the metal found in intoxicated humans. Therefore, it was not unexpected to find that the activity-ratios were likewise not significantly ($P > 0.1$) affected at these levels of blood cadmium.

Specificity of the Activity-Ratio Method

On the basis of the preceding results, one could conclude that the activity-ratios $6.4/7.2$ and $7.2^{\Delta}/7.2$ both demonstrate acceptable specificity for lead whereas the other two ratios, both affected by copper and zinc, do not. However, it must not be forgotten that the trials were conducted with normal rabbits, blood lead $< 2 \mu\text{g/dL}$. In view of previous results on the effects of pair mixtures of metals, which included lead, upon the enzyme's pH-activity profile, this conclusion may indeed be premature. In what follows, the possible concurrent effect(s) of lead and either zinc, copper, mercury or cadmium, "in vivo" and "in vitro", on the activity-ratios will be examined.

Lead/zinc

"In vivo" lead/"in vitro" zinc. The effects of increasing endogenous zinc levels upon the pH-activity profile of the enzyme in the blood of a lead-poisoned rabbit were described earlier (Figure 27).

Accordingly, zinc activates the enzyme proportionally more as the pH becomes more acid and exerts its greatest effect at pH 5.9. As

a result, activation at pH 6.4 is measurably larger than at pH 7.2 and, it is expected that the ratio 6.4/7.2 will be greater in the presence of zinc than in its absence. This effect is demonstrated in Figure 48A. In general, a combined intoxication with lead and zinc will produce false positive results, and this, even at relatively low levels of both metals. This occurrence effectively demonstrates that although the ratio 6.4/7.2 is specific for lead, the presence of endogenous zinc constitutes a serious interference.

As shown in Figure 51, zinc severely limits the extent to which the pH-activity profile of lead-poisoned blood shifts to more basic values during incubation of the buffered haemolysate (pH optimum at 6.2 instead of at 6.6). Consequently, the other three activity-ratios are also larger to varying degrees in the presence of zinc (Figures 48B-48D).

"In vivo" lead/"in vitro" zinc. The action of adventitious zinc upon the pH-activity profile of lead-poisoned blood has been reported to be inhibitory at low concentrations of added zinc (<50 $\mu\text{M/L}$) (Figure 29) and to be activating at high levels of the metal (Figure 28). However, it should be noted that zinc, as in the "in vivo" situation, exerts its effect mainly at acid pH values until relatively large zinc levels are reached, after which, it activates at all pH values equally well. The effect on the ratio 6.4/7.2 is predictable (Figure 52A): an initial increase in ratio value at low zinc levels followed by a gradual decline with rising levels until the ratio almost attains a normal value. Thus, if a blood sample were to be collected from a lead-poisoned

FIGURE 51: pH-Activity Profile of δ -ALA-D in Rabbit Blood Containing Endogenous Lead, in the Absence or Presence of Zinc or Copper, Before and After Incubation of the Buffered Lysate at 37 °C for 30 Minutes.

Lead-Poisoned Blood: 35.7 $\mu\text{g/dL}$ Lead, 64 $\mu\text{g/dL}$ Copper, 346 $\mu\text{g/dL}$ Zinc; Before ($\blacktriangle$) and After ($\triangle$) Incubation.

Lead/Zinc-Poisoned Blood: 68.5 $\mu\text{g/dL}$ Lead, 72 $\mu\text{g/dL}$ Copper, 852 $\mu\text{g/dL}$ Zinc; Before ($\bullet$) and After ($\circ$) Incubation.

Lead/Copper-Poisoned Blood: 37.3 $\mu\text{g/dL}$ Lead, 164 $\mu\text{g/dL}$ Copper, 420 $\mu\text{g/dL}$ Zinc; Before ($\blacksquare$) and After ($\square$) Incubation.

FIGURE 52A: The Effect of Varying Concentrations of Adventitious Zinc Upon the Activity-Ratios $6.4/7.2$ (● Normal, ○ Lead-Poisoned) and $7.2^{\Delta}/7.2$ (▲ Normal, △ Lead-Poisoned) Obtained from Normal (9 $\mu\text{g/dL}$ Lead) and Lead-Poisoned (55 $\mu\text{g/dL}$ Lead) Human Blood.

individual in a grossly zinc contaminated device (green or brown-stoppered vacutainer for instance, Table I) the ratio would indicate that the person is normal, that is, zinc would effectively mask lead intoxication. This situation was in fact encountered during the early stage of this study. Blood samples from 100 lead-exposed workers (blood lead 23-84 $\mu\text{g}/\text{dL}$) were received in green-stoppered vacutainers. Without exception, the 6.4/7.2 ratio test indicated that these workers had had no unusual lead exposure. Similar results were obtained for the other three ratio tests. This occurrence again demonstrates the great care that must be exercised by the researcher or clinician in collecting in contaminant-free devices blood samples destined for δ -ALA-D tests.

Lead "in vitro"/zinc "in vitro". Lead added to normal human blood causes a progressive shift of the pH optimum with increasing levels of added metal and time of contact (Figures 12, 13). After a week of storage at 4 °C, the pH-activity profile closely resembles that observed with endogenous lead. It is not surprising therefore to witness a similar progression in the value of the activity ratios from normal to that expected for the blood of lead-poisoned individuals (Figure 52B). The most striking effects of adventitious lead are observed for the ratios 6.4/7.2 and 7.2^A/7.2. However, the values attained by these two ratios, even after 20 days of contact, are considerably less than those observed at comparable blood metal levels in human blood.

A combined contamination of a blood sample by zinc and

FIGURE 52B: Activity Ratios 6.4/7.2 (O), 7.2/7.2 (Δ), 6.6/7.2 (x) and 6.6/6.4 ($\square$) of Normal Human Blood 16 Hours (solid lines) and 20 Days (broken lines) at 4 °C after the Addition of Lead.

lead is a definite possibility, as the results of tests on blood collection devices attest (Table I). Such a contamination would follow a pattern of effects on the ratios reminiscent of that found for lead "in vivo" and zinc "in vitro" (Figure 52A).

Lead/copper

Lead "in vivo"/copper "in vivo". The pH activity profile of δ -ALA-D in the blood of a rabbit concurrently intoxicated with copper and lead displays an additive reduction of the enzyme's activity, which is more pronounced at acid pH values and lessens to near normal value at basic pH (Figures 31 and 51). As expected, the 6.4/7.2 ratio diminishes gradually with increasing copper levels at a set blood lead concentration. Relatively large copper levels are required to produce this effect at low blood concentrations and vice versa at high lead levels (Figure 48A).

Although similarly reduced in value by the presence of "in vivo" copper, the 7.2^A/7.2 ratio reduction is considerably less than that shown by the other two ratios (Figures 48B and C). Figure 51 demonstrates the effects of "in vivo" copper on the enzyme profile of lead-poisoned blood after incubation of its buffered lysate; and, the reasons for the aforementioned observations are self-evident.

Lead "in vivo" or "in vitro"/copper "in vitro". The contamination of lead-poisoned blood by copper or, the joint contamination of a normal blood sample by both metals, affects the activity-ratios as

in the "in vivo" situation (Figures 32, 33). The 6.4/7.2 and 7.2^Δ/7.2 activity-ratios are both significantly ($P < 0.01$) reduced; but, to a much lesser extent than the other two ratios for any given concentrations of lead and copper.

Other mixtures

The combinations of trace metals described above are the most likely to be encountered during the everyday use of the activity-ratio method. Other possibilities, presented earlier, are less likely to occur and the effects of only the most important of these on the activity ratios will be briefly considered here.

Lead/copper/zinc (Figures 34, 35). Examples of several "in vivo" tests conducted with rabbits using this mixture of metals follow:

Lead ($\mu\text{g/dL}$)	Copper ($\mu\text{g/dL}$)	Zinc ($\mu\text{g/dL}$)	6.4/7.2	7.2 ^Δ /7.2	6.6 ^Δ /6.4	6.6 ^Δ /7.2 ^Δ
9.0	70	390	1.19	0.84	0.42	0.30
9.0	148	492	1.21	0.69	0.34	0.18
14.6	48	358	1.54	0.97	0.62	0.39
15.0	608	578	1.13	--- not measurable ---		
10.6	43	374	1.18	0.78	0.28	0.19
11.0	860	612	0.56	--- not measurable ---		
6.5	74	397	1.28	1.00	0.68	0.53
63.2	112	490	18.6	9.80	3.18	1.73

The results are paired in sets to give before and after intoxication test data. Although the results do not lend themselves to an easy interpretation, some trends are clear. The 6.4/7.2 activity-ratio is by far the least affected by the presence of zinc and copper; and the effects of copper are diminished by zinc as long as copper

levels in blood remain less than those of zinc. This is to be expected in view of zinc's antagonism towards both lead and copper.

Lead/mercury. A combined occupational overexposure to both lead and mercury is most likely a rare event. Nevertheless, assuming it did occur, the presence of mercury in blood, at levels found in mercury intoxicated persons (20-50 $\mu\text{g/dL}$), would not affect the activity-ratios being considered.

Lead ($\mu\text{g/dL}$)	Mercury ($\mu\text{g/dL}$)	6.4/7.2	7.2 Δ /7.2 Δ	6.6 Δ /6.4	6.6 Δ /7.2 Δ
43.7	0.1	5.24	3.62	3.19	3.17
45.0	40.0	5.12	3.81	2.40	3.16
27.4	0.45	2.73	1.28	1.16	1.98
25.2	31.1	2.26	1.29	1.31	1.75

A gross mercury contamination of a human blood sample containing a relatively high level of endogenous lead, if it were to occur, would have the net effect of decreasing three of the activity ratios as shown. Only the 6.6 Δ /6.4 ratio is not unduly affected. Pre-incubation of the buffered haemolysate of a mercury and lead-poisoned blood shifts the pH optimum of the profile (not shown) to more basic pH values (>7.2). This fact and a close examination of Figures 36A and B readily explain the results observed.

Lead ($\mu\text{g/dL}$)	Mercury ($\mu\text{g/dL}$)	6.4/7.2	7.2 Δ /7.2 Δ	6.6 Δ /6.4	6.6 Δ /7.2 Δ
60	0.1	7.58	4.31	2.05	3.60
60	50	5.86	3.76	2.29	3.58
60	100	3.69	2.63	2.12	2.98
60	500	0.78	2.24	2.09	0.91

Lead/cadmium. An occupational exposure to cadmium that would result in blood cadmium levels required to produce discernable changes in δ -ALA-D's pH-activity profile and hence, in the value of the activity-ratios, is highly unlikely. This is borne out by the following data:

Lead ($\mu\text{g/dL}$)	Cadmium ($\mu\text{g/dL}$)	$6.4/7.2$	$7.2^{\Delta}/7.2$	$6.6^{\Delta}/6.4$	$6.6^{\Delta}/7.2^{\Delta}$
2.0	0.1	1.26	0.89	0.50	0.71
2.0	2.01	1.24	0.91	0.56	0.77

Even at high concentrations (986 $\mu\text{g/dL}$), adventitious cadmium has only minimal effect on the activity ratios (Figure 25). However, this is not the case when cadmium is introduced into lead-poisoned blood (Figure 37) as the following data indicates.

Lead ($\mu\text{g/dL}$)	Cadmium ($\mu\text{g/dL}$)	$6.4/7.2$	$7.2^{\Delta}/7.2$	$6.6^{\Delta}/6.4$	$6.6^{\Delta}/7.2^{\Delta}$
55	0.1	6.19	3.57	2.30	3.84
55	99	4.69	3.36	1.99	2.79
55	493	2.13	1.99	1.24	1.32

All ratios are reduced. These results are not surprising since cadmium, like zinc, is antagonistic to lead and tends to restore the pH-activity profile and the activity-ratios to normalcy (Figure 37).

Common toxicants - Ethanol and Carbon Monoxide

Figures 40 and 42 both indicate that ethanol or carbon monoxide intoxication have no significant effect on the erythrocytic

δ -ALA-D's pH-activity profile and, therefore, are likely not to cause changes in the activity-ratios considered. The results of the trials of ethanol in humans and rabbits (Table XVIII A and B) and of carbon monoxide in rabbits (Table XIX) (Appendix B) indicate that this is so. A paired-t test of this data reveals that, without exception, the changes in all four ratios are not significant ($P > 0.01$).

These experiments effectively conclude the investigation of the specificity of the four selected activity-ratios for the toxic metals being studied. Before making final conclusions as to the usefulness of these ratios as biological indices of toxic metal exposure, it would seem appropriate to test them in actual human population studies. Such investigations are reported in the next section.

Human Population Studies

The populations chosen for this study include: children of the Rouen-Noranda, Quebec area who are exposed daily to toxic metal contaminants emanating from nearby smelters; United Technologies Corporation (U. T. C.), U. S. A. workers subjected to a variety of metals during welding and other metal processing operations; firing range instructors and armourers exposed to high levels of lead during pistol firing exercises; and miners and millworkers working in the Nanisivik lead/zinc mine, Arctic Bay, Northwest Territories who are subjected to lead, zinc, cadmium and other metals during their workday. Moreover, the Inuits employed at the mine are also exposed to a dietary source of mercury.

The control population was composed of laboratory workers of the Occupational Health Unit, Medical Services Branch, Health and Welfare Canada and other volunteers whose exposure to toxic metals was not significant during the study.

The test populations have one common characteristic. All were exposed to lead to varying degrees. This happenstance dovetails remarkably well with the results detailed in the previous section, which indicated that two of the chosen activity ratios namely, $6.4/7.2$ and $7.2^{\Delta}/7.2$, show promise as specific indicators of lead intoxication. Furthermore, each of the test populations can be regarded as presenting a unique test for the ratios. The Rouen-Noranda children will determine the applicability of the ratios for the assessment of low-lead exposures; the U.T.C. workers will test the relevance of the ratios to moderate lead exposure in an adult population; the firing range instructors should test the ratios over a wide range of blood lead levels; and, the Nanisivik miners and millworkers should determine the specificity of the ratios to lead in the presence of their main interferences namely, zinc and cadmium. The Inuits in this latter population will further test the ratios in the presence of methyl mercury.

Unfortunately, there was no population exposed to either zinc or copper available during the study and, therefore, there was no opportunity to test the applicability of the $6.6^{\Delta}/6.4$, $6.6^{\Delta}/7.2^{\Delta}$ or $6.0/7.2$ ratios in assessing exposure to these metals.

For each of the populations studied, painstaking precautions were taken to circumvent all the procedural pitfalls

identified in the previous sections. Blood samples were collected with plastic syringes, (1) the blood stored in plastic containers at 4 °C and, the micro-assay method was used to measure their δ -ALA-D activity.

Control population

The baseline data obtained from the control population study were as follows:

Activity-ratios/ δ -ALA-D activity

	6.4/7.2	7.2 ^{Δ} /7.2	6.6 ^{Δ} /6.4	6.6 ^{Δ} /7.2 ^{Δ}	δ -ALA-D ⁽²⁾	(δ -ALA-D) ^{+DTT}	+DTT/-DTT ⁽²⁾
<u>N</u>	109	116	108	100	107	80	66
<u>$\bar{X}$</u>	1.61	1.14	0.90	1.30	1231	1706	1.38
<u>S. D.</u>	0.14	0.17	0.21	0.31	423	506	0.55
<u>C. V. (%)</u>	9.0	15.0	23.0	24.0	34.0	30.0	34.0
<u>S. E.</u>	0.02	0.02	0.02	0.03	41.0	56.0	0.07
<u>Range</u>	1.25- 2.12	0.77- 1.51	0.48- 1.38	0.63- 2.09	510- 2175	663- 3104	1.00- 3.33

(1). There are exceptions, and these will be identified in the text.

(2) Determined within 24 hours of collection.

Metal concentration

	Lead ($\mu\text{g/dL}$)	* Zinc ($\mu\text{g/dL}$)	Copper ($\mu\text{g/dL}$)	Mercury ($\mu\text{g/dL}$)	Cadmium ($\mu\text{g/dL}$)
N	110	107	109	50	30
$\bar{X}$	11.4	577	93.6	0.1	0.1
S.D.	2.7	93	18.5	--	--
C. V. %	24.0	16.0	20.0	--	--
S. E.	0.25	9.0	1.8	--	--
Range	4.0- 15.6	354- 778	64- 159		

According to this data, the range of normal values for the activity ratios $6.4/7.2$ and $7.2^{\Delta}/7.2$ obtained from males and females of a wide age range and of different ethnic background, was considerably less than that given by the usual δ -ALA-D test. The inclusion in the normal bloods of several samples inadvertently collected in brown-stoppered vacutainers of unknown contamination may, in fact, have contributed to producing a wider range of activity ratios. Some 52 samples collected as specified in the protocol gave the following ratios:

$$\underline{6.4/7.2} \quad 1.55 \pm 0.10 \text{ (C. V. 6.5\%)} \text{ and}$$

$$\underline{7.2^{\Delta}/7.2} \quad 1.09 \pm 0.08 \text{ (C. V. 7.3\%)}$$

that is, nearly within the experimental error for the micro-assay method.

Another point worth mentioning is the remarkable stability of the test over a prolonged period, for any given individual, barring any unusual exposure to toxic metals. For example, the $6.4/7.2$ and $7.2^{\Delta}/7.2$ ratios for one such subject, measured at

regular intervals (12 times) over a period of three years afforded the following results:

6.4/7.2	1.36 ± 0.06	range 1.26 - 1.42
$7.2^{\Delta}/7.2$	1.06 ± 0.04	range 0.99 - 1.11

This is not an isolated example. Several other subjects tested during the same period, albeit not as often, gave similar results. The other two ratios were likewise stable over the test period.

Test Populations

The results for the test populations are summarized in Table XX. It should be noted that samples suspected of having been grossly contaminated during collection were arbitrarily eliminated.

Children, Rouen-Noranda, Quebec. Forty-nine children of the Rouen-Noranda area of Quebec, exposed to smelter-generated environmental contaminants, formed this population. Their blood lead levels ranged from 5.5 to 20.2 $\mu\text{g}/\text{dL}$. Notwithstanding the narrowness of this range of blood lead concentrations and the fact that this range has been established by several studies (44, 50-53) as that in which lead exhibits its minimal inhibitory effect on δ -ALA-D in humans, a respectable correlation (considering the narrow range) was obtained between blood lead levels and the ratios $6.6^{\Delta}/7.2^{\Delta}$ ($r = 0.62$) $6.6^{\Delta}/6.4$ ($r = 0.52$) and $7.2^{\Delta}/7.2$ ($r = 0.49$). The results for the other ratio, 6.4/7.2, and tests including δ -ALA-D, $\text{FEP}^{\dagger}$ and Granick's (+DTT/-DTT) were comparably less favorable.

The linear correlation between blood lead levels and the activity-ratio $7.2^{\Delta}/7.2$ is shown in Figure 53. The scatter observed

TABLE XX: SUMMARY OF STATISTICAL DATA FOR ACTIVITY RATIO TESTS RESULTS

Test Population	Activity Ratio Test	Statistical Data						
		N(1)	Y(2)	Sy/x (3)	r (4)	F(5)	t (6)	Others
Rabbit	-- (7)	147	-0.07x+ 3.42	0.15	0.48	--	--	--
	6.4/7.2	143	0.13x+ 0.79	0.53	0.95	1340	--	--
	(6.6) ^A /6.4	133	0.05x+ 0.39	0.19	0.96	1380	--	--
	(6.6/7.2) ^A	129	0.07x+ 0.40	0.26	0.93	708	--	--
	(7.2) ^A /7.2	147	0.07x+ 0.73	0.25	0.97	2720	--	--
Rouen-Noranda Children	-- (7)	45	-0.01x+ 3.28	0.09	0.22	2.01	--	--
	+DTT/-DTT	42	0.02x+ 1.16	0.30	0.14	0.78	--	--
	6.4/7.2	45	0.05x+ 1.31	0.29	0.37	6.31	--	--
	(6.6) ^A /6.4	45	0.03x+ 0.67	0.155	0.52	16.3	--	--
	(6.6/7.2) ^A	45	0.06x+ 0.79	0.19	0.62	24.3	--	--
U. T. Corporation	(7.2) ^A /7.2	45	0.04x+ 0.83	0.168	0.49	13.5	--	0.33 (6)
	-- (9)	45	-0.05x+13.0	2.50	0.10	0.30	--	--
	-- (7)	38	-0.02x+ 3.26	0.11	0.49	12.9	--	--
	6.4/7.2	36	0.04x+ 1.10	0.08	0.79	54.5	--	--
	(6.6) ^A /6.4	38	0.03x+ 0.69	0.14	0.56	14.3	--	--
	(6.6/7.2) ^A	36	0.05x+ 0.80	0.13	0.74	41.3	--	--

TABLE XX: SUMMARY OF STATISTICAL DATA FOR ACTIVITY RATIO TEST RESULTS.

Test Population	Activity Ratio Test	Statistical Data					
		N(1)	Y(2)	Sy/x(3)	r(4)	p(5)	t(6)
Firing Range Instructors	(7.2) ^A /7.2	38	0.06x+0.43	0.12	0.81	66.1	--
	-- (9)	51	0.15x+13.0	5.70	0.12	0.74	--
	-- (7)	36	0.06x+3.19	0.11	0.60	19.2	--
	+DTT/-DTT	28	0.02x+1.7	0.47	0.50	8.8	--
	6.4/7.2	38	0.13x-0.06	0.88	0.91	166	--
	(6.6) ^A /6.4	39	0.02x+0.65	0.19	0.84	92.1	--
	(6.6/7.2) ^A	36	0.03x+0.88	0.27	0.87	106	--
	(7.2) ^A /7.2	39	0.07x+0.08	0.46	0.92	--	--
	6.4/7.2+DTT	35	0.06x+0.39	0.50	0.87	103	--
	-- (7)	95	-0.09x+2.96	0.15	0.28	4.03	--
Nanisivik Mines	6.4/7.2	95	0.09x+0.05	0.48	0.92	487.9	8.56
	(6.6) ^A /6.4	95	0.02x+0.54	0.19	0.81	152.8	--
	(6.6/7.2) ^A	95	0.02x+0.89	0.35	0.52	30.5	--
	(7.2) ^A /7.2	95	0.09x+0.09	0.32	0.96	1,026.79	2.0486
	+DTT/-DTT	81	0.08x+1.86	3.05	0.30	7.51	--
	-- (7)	95	-0.09x+2.96	0.15	0.28	4.03	--

TABLE XX: SUMMARY OF STATISTICAL DATA FOR ACTIVITY RATIO TEST RESULTS

Test Population	Activity Ratio Test	Statistical Data					
		N (1)	$\bar{Y}$ (2)	$S_{Y/X}$ (3)	r (4)	F (5)	t (6)
Human Test Population	6.4/7.2	191	$0.09x + 0.53$	0.40	0.93	1244	11.9
	$(6.6)^A/6.4$	200	$0.02x + 0.72$	0.20	0.73	233	--
	$(6.6/7.2)^A/6.4$	214	$0.02x + 1.00$	0.35	0.57	103	--
	$(7.2)^A/7.2$	230	$0.08x + 0.27$	0.35	0.93	1440	--
(1) Test Population Number			(4) Correlation Coefficient			(7) log ALA-D	
(2) Linear Regression Equation			(5) F Test Statistic			(8) Spearman's Rank Correlation Coefficient	
(3) Estimated Error of Y on X			(6) t Test Statistic			(9) Free Erythrocytic Porphyrin

FIGURE 53: Correlation of the 7.2 Δ /7.2 δ -ALA-D Activity Ratio Values and Blood Lead Concentrations. Children in the Rouen-Noranda Area of Quebec.

cannot be imputed solely to the activity ratio test. Part of the blame for the scatter of results must be attributed to blood lead determinations. This is particularly true in this case. Not enough blood was collected (<1.5 mL) to perform blood lead measurements by the more precise and accurate ($\pm 5\%$) coprecipitation method; and, the less reliable and less precise ($\pm 2 \mu\text{g/dL}$) anodic stripping voltammetry technique was used instead. It can readily be seen that such an inherent error would introduce serious scatter in so narrow a range of blood lead values. It is highly probable that a much better correlation would have been obtained for all ratios if the alternate more precise blood lead analysis had been performed. This, in fact, will be demonstrated in the next section.

Nevertheless, a definable trend in the results is noticed in Figure 53 and the range of activity ratio values obtained, 0.74 ± 0.15 to 1.75 ± 0.05 , is usable. Thus, it can be concluded that the activity-ratios can be used to assess low level lead exposure in populations such as children with a precision which may rival blood lead determination.

Workers - United Technologies Corporation (U. T. C.) U. S. A.

Blood samples were collected from 42 workers prior to their full employment with U. T. C. The pre-employment blood lead values, determined by the coprecipitation method, ranged from 8.2 to 23.0 $\mu\text{g/dL}$. A remarkably good linear correlation was obtained between the ratios $7.2^\Delta/7.2$ ($r = 0.81$); $6.4/7.2$ ($r = 0.79$); $6.6^\Delta/7.2$ ($r = 0.74$) and blood lead levels. The results for the ratio $6.6^\Delta/6.4$ and the.

δ -ALA-D and ZPP tests were definitely inferior.

The linear correlation between the activity-ratio $7.2^{\Delta}/7.2$ and blood lead concentrations, Figure 54, clearly exemplifies the reduced scatter and even distribution of results about the prediction line.

These tests lend strong support to the conclusion made in the preceding section that some of the ratio tests, namely $6.4/7.2$, $7.2^{\Delta}/7.2$ and $6.6^{\Delta}/7.2^{\Delta}$, could be used to assess low lead exposure in humans; and, in view of the present notoriety enjoyed by blood lead values as biologic indicators for lead, these ratios could conceivably be more suitable indices.

Firing range instructors and armourers. Blood samples were collected from 39 firing range instructors and armourers over a period of three years. (1) The blood lead concentrations determined with both the anodic stripping voltammetry and coprecipitation methods ranged from 11 to 66 $\mu\text{g}/\text{dL}$. Unfortunate circumstances did not allow these bloods to be collected in the recommended fashion. They were collected instead with brown-stoppered vacutainers (Table I) although precautions were taken to isolate the stopper, suspected of being a principal source of contamination, from the blood. However, blood zinc levels ranged from 484 to 791 $\mu\text{g}/\text{dL}$ (mean 620 ± 82) and this indicated a distinct possibility of low level zinc contamination.

(1) Collected December 13, 1977, November 28, 1978, June 3 and July 15, 1980.

FIGURE 54: Correlation Between the δ-ALA-D Activity Ratio 7.2/7.2 Values and Blood Lead Concentrations. United Technologies Corporation Workers.

Taking into consideration that the population under test is a composite of four chronologically separate entities, all four activity-ratio tests listed in Table XX demonstrate an excellent linear correlation ($r \geq 0.84$) with blood lead levels. The linear correlation of the activity ratio $7.2^{\Delta}/7.2$ ($r = 0.92$) with blood lead concentrations, shown in Figure 55, attests to this excellent agreement.

The results obtained for the δ -ALA-D and Granick tests clearly perform poorly in comparison. Hence it would appear that the activity ratios can also be used successfully as biologic indices over a wider range of lead intoxication.

Miners, millworkers and attendant surface workers, Nanisivik Mines, Arctic Bay, Northwest Territories. Ninety-five samples were collected as described in the protocol from workers at the Nanisivik Mines over a period of two years. (1) The population included 43 Inuits. Their collective exposure to various metals during this period was <0.01 to 0.92 mg/m^3 lead; <0.01 to 40.36 mg/m^3 zinc; <0.01 to 0.12 mg/m^3 cadmium; and <0.01 to 0.05 mg/m^3 copper. Blood lead concentrations for this population ranged from 4 to $85 \text{ } \mu\text{g/dL}$, zinc levels from 380 to $739 \text{ } \mu\text{g/dL}$ (mean 569 ± 73) and cadmium blood concentrations at $<0.16 \text{ } \mu\text{g/dL}$. Total mercury (inorganic + organic) in the blood of Inuits ranged from 0.3 to $9.8 \text{ } \mu\text{g/dL}$ and $<0.6 \text{ } \mu\text{g/dL}$ in Caucasians. The total mercury measured was in large part methyl mercury derived from eating fish and/or meat contaminated by this environmental pollutant.

(1) Blood collections were made September 19, 1979, March 28 and April 12, 1980

FIGURE 55: Correlation Between δ -ALA-D Activity-Ratio 7:2 Δ /7.2 Values and Blood Lead Concentrations. Firing Range Instructors and Armourers.

The linear correlation between the activity ratios $6.4/7.2$ ($r = 0.92$) and $7.2^{\Delta}/7.2$ ($r = 0.96$) and blood lead concentrations is excellent (Table XX). The poorer results for the ratios $6.6^{\Delta}/6.4$ and $6.6^{\Delta}/7.2$ could be attributed to their greater sensitivity to endogenous zinc as demonstrated in the earlier section. The results for the Granick test and δ -ALA-D test are distinctly inferior.

The linear correlation between blood lead levels and the activity ratio $7.2^{\Delta}/7.2$, shown in Figure 56, clearly demonstrate the excellent agreement between these two parameters. A close examination of this correlation (slope 0.09) reveals a possible one to ten relationship between each ratio value and its corresponding concentration. That is, a blood lead value of 10 $\mu\text{g/dL}$ has a ratio of 0.99, 20 $\mu\text{g/dL}$, 1.89 and 100 $\mu\text{g/dL}$, 9.09. In fact, if one was to multiply each $7.2^{\Delta}/7.2$ ratio value by a factor of ten and perform a paired-t test between these values and their corresponding blood lead values, a respectable $t = 2.05$ is obtained for a $p = 0.02$, indicating that there is not a very significant difference between the two paired sets of values. A similar treatment of the $6.4/7.2$ ratio data gives a $t = 8.56$ indicating a more significant (but not overwhelming) difference between this ratio (that is multiplied by ten) and its corresponding blood lead value.

It is now clear that the activity ratios tested and, more specifically the $6.4/7.2$ and $7.2^{\Delta}/7.2$ ratios, can function as bone fide biologic indices of lead poisoning over a range of lead

FIGURE 56: Correlation Between Blood Lead Concentration and the δ -ALA-D Activity Ratio Value 7.2/7.2. Miners, Mill-workers and Attendant Surface Workers, Nanisivik Lead/Zinc Mine, Arctic Bay, Northwest Territories.

concentrations found in normal to acutely intoxicated humans.

The zinc exposure endured by this population apparently did not raise blood zinc levels sufficiently to interfere with these ratios.

Mercury and, more precisely, methyl mercury, has no effects on the ratios. The mixing of genetically different populations, Caucasians and Inuits, similarly did not affect the results obtained here.

Heterogeneous populations. To test the activity-ratios with a population including individuals of different age, sex, genetic background and occupation, the data collected for the various populations studied were grouped and statistically tested. The results, shown in Table XX, again indicate that the activity-ratios $6.4/7.2$ ($r = 0.93$) and $7.2^{\Delta}/7.2$ ($r = 0.93$) are markedly superior to the other two ratios.

The excellent correlation between the ratio $6.4/7.2$ and the blood lead levels for this heterogeneous population appears in Figure 57. Again, note the nearly complete absence of scatter of results over this wide range of blood lead values. A paired-t test performed between blood lead levels and their corresponding $6.4/7.2$ ratio value multiplied by a factor of ten yields a $t = 11.9$ indicating an acceptable agreement between the two values. Since the same equation was obtained for the prediction line for the correlation between this ratio and blood lead levels for both the heterogeneous and Nanisivik populations, it could be conjectured that this equation $Y = 0.09x + 0.05$ may apply to any human

FIGURE 57: Correlation Between Blood Lead Concentrations and the Values of the δ -ALA-D Activity-Ratio 6.4/7.2 for an Heterogeneous Population.

population tested, albeit assuming that the protocol detailed earlier for the test is strictly adhered to. Note for instance, that the results obtained for the control population 6.4/7.2 ratio (1.14 ± 0.17 for a mean blood lead level of $11.4 \pm 2.7 \mu\text{g/dL}$) agrees very well with this equation. However, it is more likely that extensive tests using larger and more diversified populations will be required to prove this postulate true. Similar comment can be made for the $7.2^\Delta/7.2$ activity-ratio.

Therefore, it would appear that the activity ratios 6.4/7.2 and $7.2^\Delta/7.2$ are biologic monitors of lead intoxication that can be used to assess lead exposure in any heterogeneous population. The ratios clearly rival presently universally accepted blood lead concentration values in this aspect.

Advantages and Disadvantages of the Activity-Ratio Method ⁽¹⁾

Advantages

The activity-ratio method introduced in the preceding sections essentially incorporates the micro-assay technique detailed earlier and, as such, possesses that procedure's inherent advantages including: a requirement for small blood samples, small quantities of reagents, inexpensive equipment and a relatively short period of training; a large sample throughput; good accuracy, precision, reproducibility and reliability; and a relative freedom from the effects of procedural pitfalls. However, in addition to these,

(1) Refers to the 6.4/7.2 and $7.2^\Delta/7.2$ δ -ALA-D activity ratio tests unless otherwise stated.

the method overcomes all of the shortcomings that have heretofore plagued procedures such as the micro-assay technique, and offers advantages of its own. Thus,

- the method can be used for the study of heterogeneous populations since it is not affected by age, sex, diet and genetic background;
- the method can be used for the study of populations exposed to low lead levels such as children;
- the ratios appear stable, that is, the ratio for a given individual remains constant over a long period, barring exposure to lead; this may make the test appropriate for monitoring exposed populations over a period of years;
- the test can be performed as much as a month after blood collection assuming that the sample is obtained and stored as detailed in the protocol;
- the test does not require ancillary measurements such as hematocrits and involved calculations, that is, it has inherent simplicity;
- a linear correlation is obtained between the ratio values and blood lead levels over a wide range of blood lead concentrations;
- the ratios can give a fair approximation of blood lead concentrations;
- the test is demonstrably specific for lead exposure;
- the test appears more reliable than the presently widely accepted index of lead exposure namely, blood lead concentrations;
- the method gives results vastly superior to those obtained with tests such as Granick's (Δ DTT/-DTT), FEP, ZPP and δ -ALA-D activity under similar test conditions.

- the test has no notable sensitivity to relatively high concentrations of endogenous cadmium, mercury (and methyl mercury);
- the test is not affected by the presence of common toxicants such as carbon monoxide and ethanol;
- other ratios such as 6.0/7.2 could be used to monitor other metals such as zinc and copper individually and in the absence of each other and lead;
- the method can be used equally well for animal intoxication studies.

Disadvantages

Although the activity-ratio method does meet and actually surpasses all of the objectives listed at the very outset of the study, it is by no means without flaw. The disadvantages of the method, along with suggestions for overcoming them, follow:

- the method is severely affected by the presence of relatively large concentrations of endogenous and exogenous zinc and/or copper and by large concentrations of exogenous cadmium and mercury. All effects due to the presence of exogenous metals can be easily overcome by simply adhering to the conditions detailed in the protocol for the collection, storage and treatment of blood samples. Effects due to the presence of endogenous zinc and/or copper, which may occur as the result of a concurrent exposure to lead, copper and zinc, could be eliminated by delaying the collection of blood samples from exposed subjects for a predetermined period after exposure to allow zinc and/or copper concentrations to regain

normal levels; (1)

- accuracy and precision is somewhat lost at high blood lead levels (>60 $\mu\text{g/dL}$) since the absorption of the Erlich colored salt becomes relatively low (<0.1 absorption unit). This handicap can be overcome by using an alternative more sensitive procedure for the determination of PBG such as fluorescence (176);
- the activity-ratio method results may not reflect a frank biological change; but, nor does blood lead. However, it should be noted that preliminary results have indicated that observed changes in the erythrocytic δ -ALA-D pH-activity relationship are paralleled in certain organs such as the liver (177). Thus, it may be that activity-ratio results will reflect a biological change occurring in a critical organ. Further tests will be required to prove this to be true.

(1) As the rabbit metal intoxication studies have indicated, endogenous zinc and copper have a considerably shorter biological half-time than lead.

Mechanism of Action of δ -ALA-D

Present State of Knowledge

The mechanism of action of δ -ALA-D in vogue today is essentially that postulated by Nandi and Shemin (11) and modified by Jordan and Seehra (13) (Figure 2A and B) (Appendix "A"). According to this mechanism, four groups are implicated at the active site as essential for enzyme activity; the -NH_2 group belonging to a lysine residue which forms a Schiff base with the incoming ALA molecule; a positive group belonging to histidine which orientates the incoming substrate molecule by forming an ion bond with the carboxylate ion in β position to the carbonyl of ALA; and two separate essential cysteinyl residues. The exquisite sensitivity of δ -ALA-D to air oxidation and observations that PBG protects the active site from the action of alkylating agents (15) have suggested that the two cysteinyl groups are sufficiently close ($\approx 5 \text{ \AA}$) for facile disulfide bond formation.

The ready interconversion of -S-S- to SH groups in δ -ALA-D is considered by some as a means of physiological control of enzymic activity mediated by the concentration of naturally occurring thiols (178). Several enzymes are known which also contain critical disulfide(s) (179-181). For instance, the presence of two similarly chemically non-equivalent cysteinyl residues has also been detected in enzymes such as yeast alcohol dehydrogenase (182) and glyceraldehyde-3-phosphate-dehydrogenase (183).

On the basis of the known facts concerning the active site, Barnard et al. (15) have proposed a further modification of the Nandi-Shemin mechanism to incorporate the role of the sulfhydryl groups as participants in the acid/base catalysis dictated by the required protenation/deprotenation sequences of the mechanism (Figure 58). This is similar to the acid/base role proposed for the cysteinyl residue in proline racemase (184) and histidine decarboxylase (185). An important feature of the proposed mechanism is the interposition of an imidazole bridge provided by the histidiny residue or a water molecule to alternate the acid/base status of the thiol groups.

Recently Bevan et al. (186) have investigated the role of metal ions and, more specifically Zn^{2+} , in the mechanism of action of bovine liver δ -ALA-D. From the results obtained, they concluded that Zn^{2+} was clearly essential to enzyme activity and that the octameric enzyme required 4 gram atoms of Zn^{2+} /mole to achieve maximal activity. However, the binding of Zn^{2+} to the enzyme and more specifically to its essential sulfhydryl groups only occurred after treatment of the enzyme with sulfhydryl reagents. The sensitivity of δ -ALA-D to sulfhydryl oxidation and the requirement for a sulfhydryl reagent prior to metal binding are not unique to this enzyme. Horse liver dehydrogenase (187) and leucine aminopeptidase (188) are two such enzymes.

Bevan et al. also demonstrated that alkylation of the enzyme with iodoacetamide decreased the binding of Zn^{2+} consistent with the requirement of reactive sulfhydryl groups for binding.

FIGURE 58: PROPOSED MODIFICATION OF THE NANDI-SHEMIN MECHANISM FOR G-ALA-B (15)

A. ACETIC ACID SIDE CHAIN

P. PROPIONIC ACID SIDE CHAIN

They also proved that Zn^{2+} was not required for substrate binding nor maintenance of quaternary structure; but, they did not rule out that the enzyme may play a role in the catalytic steps of the enzyme.

Cd^{2+} was the only other metal tested by Bevan which likewise activated the enzyme. The apoenzyme required 4 g atom of Cd^{2+} /mole for a maximal activity which was 10-15% higher than that obtained with Zn^{2+} . The binding of Cd^{2+} to the enzyme was also dependent upon the presence of reactive sulfhydryl groups.

Nature of Information Provided by Study

The results of the study of the effects of zinc, lead, copper, cadmium and mercury "in vivo" and "in vitro" upon the pH-activity relationship of erythrocytic δ -ALA-D have essentially formed the basis for the development of a novel biological index for assessment of exposure to these metals (effects summarized in Table XVI). The study of such pH effects can also give information about certain aspects of the reaction mechanism and particularly about the structure of the so-called active centre of the enzyme. (1) Thus, changes in the reaction mechanism of an enzyme with pH value might yield valuable information on the role of ionizing groups in the reaction. However, too much emphasis should not be placed on the use of such studies to identify specific ionizing groups. This is particularly true in this instance since the enzyme preparation is fairly complex;

(1) The pH curve most likely reflects the ionic forms of the active centre.

and there is the distinct possibility that the effect(s) observed may be due to the interaction of the metal with a substance other than the enzyme. Nonetheless, careful interpretation of the effects of metals upon δ -ALA-D's pH-activity profile may yield useful information about the enzyme's mechanism of action.

Plausible Interpretation of Results

Modes of metal-protein interactions which can influence enzymic activity may be grouped into three categories on the basis of the major effect(s) observed (189). Each category will be examined in the light of results obtained.

Effect on Metal (189)

Examples of this effect are found preponderantly among iron and copper oxidation-reduction enzymes. An obvious function for these enzymes is to keep the metals in solution. The protein may also contribute to the stability of the lower valence state and thereby facilitate the shuttling of electrons in the oxidation-reduction process. This effect is not likely responsible for the changes in pH-activity profile observed.

Effect on Protein

Thiol groups occupy a special place in their reactions with metallic ions. The association constant $k_{RSM} = [RSM] / [RS][M]$ for the various cations of interest are $>10^{20}$ for Hg^{2+} , $\approx 10^{19}$ for Cu^{1+} , 10^{11} for Pb^{2+} , 10^7 for Zn^{2+} and Cd^{2+} and considerably less for nearly all other ions (190). Thus all metals of interest can form mercaptides with the essential thiol groups of δ -ALA-D (190). The divalent ions are also known to bind to both the imidazole and carboxylate ions at the active site (191). Any such combination can result in the inhibition of the enzyme and, in fact, such an inhibition is observed with lead and copper. Lead most probably induces inhibition by reacting with the thiol groups (52, 92) and this contention is supported by the reversibility of this effect by treatment of the enzyme with sulfhydryl reagents (93). Although copper can similarly cause inhibition by reacting with the essential cysteinyl residues it could also produce this effect by inducing oxidation

of the vicinal thiols (192). Note the striking resemblance in pH-activity profiles obtained from the incubation of the enzyme in P-P buffer at 37 °C for 30 minutes (Figure 5) and from the action of Cu^{2+} on the enzyme "in vitro" (Figure 17).

It would be of interest at this point to consider the affinity of metal ions for hydroxyl ions ⁽¹⁾ as this may have a bearing on the extent to which each ion binds with the active site sulfhydryl groups at different pH values. At pH's between 6 and 7, which were used during this study, the following equilibrium is shifted strongly to the right for Cu^{2+} and Hg^{2+} but not for Zn^{2+} , Cd^{2+} and Pb^{2+} (189).

This is evident from the basic dissociation constants (K_b) for lead hydroxide 9.6×10^{-4} , cadmium hydroxide 1×10^{-3} , zinc hydroxide 9.6×10^{-4} and mercuric hydroxide 1.8×10^{-22} (193).

Thus, cadmium, zinc and lead would tend to form more of the hydroxide progressively as the pH increases from 6 to 7 whereas both $\text{Cu}(\text{OH})_2$ and $\text{Hg}(\text{OH})_2$ would already have formed. This occurrence would progressively remove both zinc and cadmium from participation in the enzymic reaction whilst mercury, which also has the ability to activate the enzyme, would not be available at low pH values. This would explain the pH optimum for lead, cadmium and zinc, which appears at a pH <6.2 and the lack

(1) This suggestion from Dr. Brian Hollebone is gratefully acknowledged.

of activation observed for Hg^{2+} at these low pH values. The maximal activation by Hg^{2+} and Cu^{2+} at pH values ≥ 6.8 is as yet unexplained.

Alternative reasons for the phenomena observed can be sought from the effects of the interaction of the metals with the protein. When such an interaction occurs, it is obvious that the net charge of the complex will change and this in turn may directly change the enzyme's affinity for the substrate (189). As a result of combination, the groups involved in catalytic activity may have their dissociation curves shifted markedly along the pH axis. In essence, the pH-activity curve of the enzyme may be shifted considerably.

The electrostatic effect on bound metal can also influence the association-dissociation of an octameric enzyme like δ -ALA-D. Combination with a cationic metal ion may substantially favor the existence of a specific polymer with either enhanced or reduced activity and which has a distinctly different pH-activity profile.

Combination with a metal may also result in a change of enzyme conformation (194). If, for instance, some of the 56 thiol groups which are located on the octameric enzyme, play an essential role in maintaining a particular conformation, the combination with a metal can change this configuration. This site of combination may not be the active site itself but a so-called allosteric site far removed from it (195). Since δ -ALA-D has long been considered as an allosteric enzyme (110), this is a distinct possibility. It is also highly probable that such a change in conformation would lead to a noticeable change in the enzyme's pH-activity curve.

A metal can also activate an enzyme via the removal of an inhibitor. Such appears to be the case with the activation of lead-inhibited enzyme by either zinc, cadmium or mercury. In all instances, a marked change in the pH-activity profile is noted.

If used with caution and in conjunction with other evidence, the dependence of binding upon pH may give good indication of the nature of the groups involved. However, for the reasons stated earlier, this was not done here, as it was felt that the evidence at hand was too limited to allow such determinations. It is distinctly possible, for instance, that some of the metals are binding with more than one group at the active site (196).

Cooperative Effects (189)

Metals can also serve as bridges in the formation of a complex between the enzyme and its substrate and this mechanism has been postulated for some time for metal-activated protein. A chelate can actually form between metal ion and substrate as a requirement for enzymic activity. The formation of a chelate could thus stabilize the ground state of a substrate-metal-enzyme complex.

This brief foregoing discussion does not exhaust all possible interpretations of the results obtained during this study. For instance, the metals could alter the rate of enzymic reaction by combining with the substrate (197) or with a co-enzyme. However, it is felt that the information provided by

the results of the study, by itself, is not sufficient to enable one to arrive at any firm conclusions concerning the mechanism of action of δ -ALA-D. Further research, based upon these results, could yield important findings about this mechanism.

SUMMARY AND CONCLUSIONS

According to the results of this study, the activity of the erythrocytic enzyme δ -ALA-D is particularly prone to the influence of many procedural, exogenous and endogenous effectors at pH values in the range 5.8 to 7.4. This inherent susceptibility to a host of factors in turn can seriously affect the enzyme's assay when performed at a specific pH value within this range.

Procedural factors, which include blood collection and storage, selection of an appropriate buffer system and its concentration and, haemolysate preparation and treatment, all have a significant impact upon the assay outcome. These effects are readily circumvented by adopting an optimal assay protocol and adhering closely to this procedure, once selected, for all future determinations. For this study, the haemolysate was prepared with Triton X-100 and the determination of enzyme activity initiated soon thereafter using a 0.3M/L P-P buffer system pH 6.4. Other procedural factors namely, incubation temperature and duration, substrate concentration, and color reaction have been the subject of several studies and were not examined here. Results obtained with this protocol have proven consistent over the three-year duration of the study.

Endogenous metals such as lead, copper, zinc and possibly others not investigated during the study, and present in blood at concentrations in excess of that normally found, cause changes in enzyme activity whose extent is related to the nature

of the metal itself, its concentration, its admixture with other metals and the pH of assay.

Of the metals investigated, lead, at blood concentrations $>0.8 \mu\text{M/L}$, apparently is the most potent effector. The metal exerts an overall inhibitive effect on the enzyme which is more pronounced at the basic end of the pH scale. Copper, at concentrations in excess of normal values, likewise causes inhibition of the enzyme although its strongest effects are exerted in the relatively acid region of the pH range. Together these two metals additively inhibit the enzyme over the whole range of pH values. Conversely, zinc activates the enzyme, its maximum activation occurring at pH 5.9. Being antagonistic to both lead and copper, this metal has the ability to reverse a lead or copper-induced inhibition, albeit at relatively high blood zinc concentrations. The effect of lead and zinc and/or copper can be distinguished by collecting blood samples for assay a predetermined number of days following exposure, to allow zinc and copper levels to regain normal levels. Mercury and cadmium were also studied and found not to cause a marked effect on assay results at levels usually found in cases of acute human intoxication.

Two other potential endogenous effectors were investigated, namely, carbon monoxide with principal exposure via tobacco smoking and ethanol which results from the drinking of alcoholic beverages. Whilst CO has no noticeable effect on the δ -ALA-D assay, ethanol has a significant activation effect on normal δ -ALA-D at low concentrations ($<0.05 \text{ g EtOH/L}$) and is inhibitive thereafter; and it has a similar but more pronounced effect on lead-poisoned

δ -ALA-D. These effects have been linked to the oxidation of ethanol and the concomitant reduction of glutathione (30). This potential problem can be avoided by selecting blood donors who have not imbibed alcoholic beverages for at least twenty-four hours prior to the test.

Exogenous effectors include a wide variety of substances or metals which, either by design or accidentally, find their way into the blood sample or reaction mixture where they affect the activity of the enzyme at the assay pH. The consequences of the introduction of an intentional, viz. dithiothreitol, and accidental, viz. trichloroacetic acid, exogenous substance upon enzyme activity were examined. The former simply activates the enzyme uniformly at most pH values whereas the latter reduces its activity and that, more notably at acid pH values. It is obvious that all effects due to accidentally incorporated substances can be avoided by strict adherence to the assay protocol which was designed to guard against such eventuality.

The introduction of lead, zinc, copper or their mixtures adventitiously into blood samples, haemolysate or reaction mixture yields results that are almost similar in nature, scope and intensity to those obtained "in vivo". However, for lead, comparable results are only obtained after a considerable period of contact at 4 °C (>6 days). This occurrence may explain why the so called "lead-induced" shift of the pH optimum noted "in vivo" has not, until now, been observed "in vitro". It is also noteworthy that, zinc in the presence of lead and in small concentrations (<10 μ M/L), does not activate but rather additively

inhibits the enzyme.

Although mercury and cadmium have no discernable effect "in vivo", at concentrations observed in cases of acute intoxication, these metals "in vitro" have a significant impact upon enzymic activity at the higher levels found in instances of contamination. Thus, depending upon concentration and pH of measurement, both metals can either inhibit, produce no effect or greatly enhance the enzyme's activity. Notably, cadmium can reverse lead-induced inhibition of δ -ALA-D at comparatively lower concentrations than zinc and, as such, is a more potent activator.

Mixtures of the five metals of interest produce a bewildering array of effects too numerous to discuss here. Suffice to say that the net effect will depend upon the metals involved, their concentrations and the assay pH. The metal ions, at equimolar concentration, can effect a change in enzymic activity "in vitro" according to the order $\text{Cu} \approx \text{Cd} > \text{Hg} > \text{Pb} > \text{Zn}$. All effects caused by exogenous metals can be circumvented by taking all precautions to avoid their introduction into blood samples during collection and storage, haemolysates during preparation and the reaction during its performance.

It must be noted that most investigations of the effects of metals on δ -ALA-D activity conducted to date, either "in vivo" or "in vitro", were performed at a single pH value. This approach is valid if the prime intent is to determine the impact of these metals upon the results of an assay conducted at that pH value. However, if the objective is to study the interactions of these metals with

a pH-dependent enzyme like δ -ALA-D, such an approach will lead to contradictory results. Such was the case for the investigations of mercury and cadmium interaction with the enzyme (115, 116). For an enzyme like δ -ALA-D, which has a marked propensity to display a strong pH-dependency in such tests, it would be strongly recommended that metal-enzyme interaction experiments be performed over a judiciously selected range of pH values to obtain meaningful results.

A thorough evaluation of the presently widely accepted "European Standardized Method" (82) of measuring δ -ALA-D activity at a set pH value, 6.4, yielded an improved micro-assay which, unlike all previous methods, is not subject to procedural pitfalls and affords several distinct advantages. Nonetheless, this novel assay, like all previous procedures, is plagued by several objectionable features. Furthermore, although the method could conceivably be used to assess exposure to lead, copper or zinc, it would be difficult to distinguish which metal was causing the observed effects based on a determination performed at a single pH value.

To overcome the present assay's many drawbacks, advantage was taken of the difference in response of the enzyme to the metals of interest before and after incubation of the P-P buffered haemolysate at 37 °C for 30 minutes (Δ) and a ratio of enzymic activity measured at two judiciously selected pH values was derived. After a careful process of elimination and extensive tests conducted with rabbits exposed to the metals, two likely activity ratios were found namely, activity at 6.4/ activity at 7.2 and

activity at 7.2^{Δ} / activity at 7.2. Extensive tests using these ratios with several metal-exposed populations (1) confirmed the choice of the ratios. The activity ratios not only surmount all the previous assay failings and offer unique advantages, but they also can successfully replace the presently accepted lead-in-blood test. Although less sensitive and less specific, an activity-ratio such as 6.0/7.2 could be used to determine exposure to zinc or copper in each other's absence and that of lead.

Notwithstanding the potential use of activity-ratios as biological indicators of exposure to copper or zinc, it is readily apparent that their foremost application as specific and sensitive indices is for lead intoxication. According to tests conducted with these ratios and the five most common environmental metal contaminants, the specificity of the activity ratios for lead is only questionable in the presence of endogenous zinc and to a lesser extent copper. (2) However, as explained earlier, this occurrence does not constitute a major obstacle if blood collection is delayed sufficiently long after joint exposure to these metals to allow zinc and/or copper to attain normal concentrations. Ethanol and carbon monoxide in blood do not affect the ratios.

(1) All populations studied were predominantly exposed to lead and to a lesser extent to the other metals of interest.

(2) Assuming that all necessary precautions have been taken to eliminate the presence of adventitious metals including copper, zinc, lead, cadmium and mercury.

Although it is eminently clear that the activity-ratios $6.4/7.2$ and $7.2^A/7.2$ fulfill most of the prerequisites for biologic indices as detailed at the very beginning of this report and, could beneficially replace or confirm blood lead levels as biologic indicators of lead exposure, it is also readily acknowledged that some resistance is anticipated from the medical, clinical and scientific community in accepting these ratios for this purpose. Clinical enzymology, for one, has relied throughout its history on the accurate and sensitive measurement of enzyme activity at a single pH value. Furthermore, method development in this field has, unfortunately, mostly limited itself to the progressive identification and control of all variables which impinge on enzyme activity, as was done here for δ -ALA-D and, in general, has rarely sought new insight into clinical enzymology. Methods used today are most often only improvements of older established techniques whose imperical principles were set out decades ago and which still guide the clinical interpretation of the enzymic activities obtained. However, the classical view of the progress of science is one of a steady accumulation of new facts which, as they are confirmed, are added to the growing body of knowledge and form the foundation of further advances. It is earnestly hoped that, in view of the many advantages offered, the use of activity-ratios as biologic indicators of metal exposure will be regarded as such an advance, and they will be accepted as bone fide alternatives to the more familiar measurement of δ -ALA-D at a single pH value as well as blood metal concentrations for the assessment of metal exposures.

The study has provided new insight into the mechanism of action of δ -ALA-D. Thus, it was found that lead was not the only metal which can cause a marked shift in the enzyme's pH optimum. Apparent shifts were also observed with copper, cadmium, mercury and zinc and, furthermore, the shift obtained was specific to the metal effecting it. Notably, copper, zinc, and lead caused identical shifts "in vivo" and "in vitro" indicating that a similar process was operative in both instances. Although attempts were made to interpret the occurrence of these shifts on the basis of well-known metal-protein interactions, it is felt that considerably more research is required before firm conclusions can be drawn about their significance. It is hoped that the results of this study will provide the basis for further exciting discoveries about the mechanism of action of this enzyme.

MATERIALS AND METHODS

Blood Sample Collection

Human blood samples were obtained by venipuncture from volunteer non-smokers with no known exposure to metals and from workers or from the general population with known exposure to one or more of the following: lead, cadmium, mercury, methyl mercury, zinc or copper. At the outset of this study, blood samples were drawn by venipuncture into heparinized Vacutainers (Becton Dickinson and Company, Rutherford, NJ, low lead BD4610) held in such a manner as to minimize contact with the tube's rubber stopper. Following collection, the stopper was removed, replaced with a piece of Parafilm (American Can Company, Dixie/Marathon, Greenwich, CT), and the tube inverted several times to heparinize the blood. The blood was transferred to polystyrene tubes (Falcon 2057, Falcon Company, Oxnard, CA) and stored at 4 °C.

Later, as it became apparent that there was a possibility of contamination by adventitious metals or substances in Vacutainers, blood samples were collected using a disposable 10 ml plastic syringe (Becton Dickinson and Company, Rutherford, NJ) and immediately transferred to polystyrene culture tubes (No. 2570, Corning Glass works, Corning, NY 14835) containing enough sodium heparinate (Abbott Laboratories Ltd., Montreal, Quebec, Canada) to yield a final concentration of 500 USP units per milliliter of blood.

Micro blood samples were obtained from volunteers via finger pricks, the blood being collected with heparinized micro

hematocrit tubes (Dade Division, American Hospital Supply Corporation, Miami, FLA 33152). The blood samples were transferred to disposable 400 μ L polypropylene tubes (Simport Plastics Ltd., Beloeil, Quebec, Canada). All blood samples were kept at 4 °C until used.

Rabbit blood samples were obtained from male white albino New Zealand rabbits. Blood samples were drawn from an ear artery by venipuncture using a 3 cc plastic disposable syringe (Becton Dickinson, Rutherford, NJ) and transferred to polypropylene tubes (Falcon 2057, Falcon Company, Oxnard, CA) containing sodium heparinate. (1)

For all blood samples, δ -ALA-D analyses and hematocrit (Hct) determinations were performed within one day of collection. The hematocrit of each blood sample was determined with a microcapillary centrifuge (IEC Model MB, Danson/IEC Division, Needham Heights, MA).

"In Vivo" Experiments

All "in vivo" experiments were conducted with white albino New Zealand rabbits, six months to two years old, weighing two to five kilograms, housed in individual stainless steel cages and fed standard rabbit pellets and water ad libitum.

(1) It has been observed by others (169) that frequent bloodletting of small animals can stimulate erythropoiesis and cause subsequent increases in the activity of δ -ALA-D due to a greater number of circulating reticulocytes. This possible occurrence was verified by comparing the activity of δ -ALA-D in two successive 3 cc blood samples collected one or two hours apart. The results indicated that there was no significant ($p < 0.010$) difference.

"In vivo" metal tests

Copper. Copper was administered as a sterile 1 to 5 mg Cu^{2+} /mL solution of copper glycinate ⁽¹⁾ carefully injected subcutaneously (0.5 to 1.5 mL) on the back of the test rabbit. Blood was collected within two hours of the injection, as it was noted that the level of copper in blood rapidly returned to its normal level.

Zinc. Zinc was similarly administered by injecting the rabbits subcutaneously on the back (0.5 to 1.5 mL) with a sterile 1, 5 or 10 mg Zn^{2+} /mL solution of zinc lactate. Blood was collected within four hours of the injection.

Unlike copper glycinate, zinc lactate injections produced large bleeding sores on the backs of certain (but not all) of the test rabbits. This occurrence seriously curtailed these tests as the sores required more than one month to heal.

It should be mentioned that all attempts at feeding copper or zinc to rabbits via the oral route, either by using feeding tubes or by placing salts of the metals in the animals' drinking water, met with dismal failure. This procedure did not raise the test animals' zinc or copper blood levels measurably even when relatively large amounts of the metals were administered.

Lead. Attempts to inject lead subcutaneously as the lead acetate, even at small concentrations, produced the above mentioned sores and this route had to be abandoned. The administration of lead to

(1) Prepared from cupric carbonate and glycine.

rabbits was successfully accomplished by placing the lead acetate,⁽¹⁾ in the animals' drinking water. This procedure gradually raised the rabbits' blood lead level over a period of one week to concentrations >50 µg/dL. Blood samples were collected on a weekly basis.

Mercury. Test rabbit blood mercury levels were successfully increased to levels exceeding 300 ppb (or 30 µg/dL) by feeding the animals mercury acetate in their drinking water ad libitum.⁽²⁾ The animals did exhibit some reticence in drinking water so treated over prolonged periods.

Cadmium. Cadmium was likewise administered to rabbits via their drinking water.⁽³⁾ In both instances, there appeared to be no outward signs of mercury or cadmium intoxication in the test animals. Blood samples were collected weekly.

Ethanol trials

5 to 10 mL solutions of 50% (V/V) ethanol in water were administered to test rabbits orally using 15"-long feeding tubes. Blood samples were collected prior to and one to two hours following feeding.

- (1) 5-20 mL of a 11.4 g Pb Ac₂/250 mL solution/liter of drinking water.
- (2) 10-20 mL of a solution of 0.9 g mercuric acetate/100 mL water placed in 1 L of drinking water.
- (3) 10-20 mL of a solution of 0.6 g cadmium acetate/100 mL in 1 L of drinking water.

In the only trials involving humans during this study, volunteers were asked to give a 10 cc blood sample and then to consume varying quantities of rum (20 to 240 mL each) within one hour. Another blood sample was collected within the next two hours from each volunteer.

Carbon monoxide exposures

A make-shift exposure chamber was fabricated by joining two 75 L Saran (ANSPEC Company, Inc., Ann Arbor, Michigan 48107) gas bags at their bottom ends. Blood samples were collected from the rabbits prior to placing them in the chamber. A small amount of 100% carbon monoxide was introduced into the chamber until its atmosphere attained a level in excess of 500 ppm CO. Exposure periods ranged from 5 to 30 minutes. The animals were again bled immediately after removal from the chamber.

Toxicant "In Vitro" Experiments

All "in vitro" trials were conducted with blood samples collected from three or more human volunteers either with no known unusual exposures to toxic metals or with known exposures to specific metals. The samples were carefully mixed and an aliquot of the pool added to 5 mL Falcon polypropylene tubes containing varying concentrations of the toxicant of interest in 0.9% (w/v) saline solutions. The "contaminated" blood samples were gently mixed at 4 °C on a Multipurpose rotator (Scientific Industries, Inc.) for 3/4 to one hour and then allowed to stand at 4 °C for periods of hours or days before performing the enzyme assay.

Trichloroacetic acid (TCA)

3 milliliters of blood is added to five Falcon polypropylene tubes containing 75, 37.5, 15, 15, 7.5 and 1.5 μL of 0.02% (w/v) TCA solution in 0.9% (w/v) saline. The samples are well mixed and allowed to stand one day at 4 $^{\circ}\text{C}$ before assay.

Metals

Stock and working solutions. 134.56 mg of lead (5N9 Ventron, ALFA Division, 152 Andover St., Danvers, MA 01923) is placed in a 100 mL volumetric and 0.5 mL concentrated nitric acid (British Drug House) is added. Following dissolution, 5 mL of deionized water is added and the solution evaporated almost to dryness at low heat to drive off all remaining nitric acid fumes. The solution is then made to volume with 0.9% (w/v) saline. 2.5 mL of this solution is then added to a second 100 μL volumetric flask and is diluted to the mark with 0.9% (w/v) saline giving a 33.59 $\mu\text{g Pb}^{2+}/\text{mL}$ saline working solution.

Similarly, the following stock (s) and working (w) solutions are prepared from 5N9-6N9 Ventron, ALFA Division metals.

Zinc. 1.039 mg Zn^{2+}/mL , (s) 0.208 mg Zn^{2+}/mL (w);

Copper: 0.210 mg Cu^{2+}/mL (s), 0.04 mg Cu^{2+}/mL (w);

Mercury: 0.101 mg Hg^{2+}/mL (s), 0.02 $\mu\text{g Hg}^{2+}/\text{mL}$ (w);

Cadmium: 0.101 mg Cd^{2+}/mL (s), 0.02 $\mu\text{g Cd}^{2+}/\text{mL}$ (w).

"In vitro trials." (1) 100 μ L and 50 μ L of stock, 100 μ L, 50 μ L, and 10 μ L of working solutions of each metal are placed into five sets of five 5-mL Falcon polypropylene tubes and two milliliter of pooled well mixed blood is added to each tube and the contaminated bloods treated as described above.

δ -ALA-D assays were conducted as prescribed by the protocol after a period of storage at 4 °C ranging from three hours to 20 days or more.

Determination of ALA-D Activity

Reagents

$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, citric acid, mercuric chloride and *p*-dimethylaminobenzaldehyde Analar grade chemicals were purchased from British Drug House Chemicals Ltd., Toronto, Ontario, Canada. Triton X-100, δ -aminolaevulinic acid and dithiothreitol were obtained from Sigma Chemical Company, St. Louis, MO. Trichloroacetic acid was purchased from Fisher Scientific Company, Ottawa, Ontario, Canada.

Buffer systems

All glassware and plastic containers are cleaned of possible heavy metal and organic contamination by soaking in a solution of 10% nitric - 5% perchloric acid for two days and rinsed five times with deionized water (Milli-Q System, Millipore Corporation, Toronto, Ontario, Canada). Dibasic sodium phosphate - citric acid and dibasic sodium phosphate - monobasic sodium phosphate buffers

(1) Conducted as described unless otherwise specified in the text.

are prepared from 0.3M solutions of the components at 0.1 pH units intervals between pH 5.8 to 7.4. The pH of each buffer is determined with an Accumet, Model 520 digital pH/ion meter (Fisher Scientific Company), calibrated with a 0.25M sodium phosphate buffer reference standard pH 6.878 ± 0.005 (20°C) (National Bureau of Standards, U.S.A. reference material 186 I & II-C). Buffer solutions are stored at 4°C and prepared monthly. Analysis by atomic absorption spectrometry indicates levels of lead, cadmium and mercury less than 10 ppb and of zinc and copper less than 10 ppb.

Preparation of reagents

Sodium phosphate buffer, pH 6.4. Dissolve 53.72 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ in 500 mL of deionized water (solution A) and 23.40 g of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ in 500 mL of deionized water (solution B). Mix 100 mL of solution A with 168 mL of solution B. Verify pH periodically, and prepare fresh monthly.

0.1% Triton X-100. Dissolve 0.5 mL Triton X-100 in 500 mL deionized water.

(0.125M) δ -aminolaevulinic acid. Dissolve 209.5 mg of this reagent in 100 mL deionized water, store at 4°C and prepare weekly.

(0.02M) dithiothreitol. Place 154.7 mg in 50 mL of deionized water, store at 4°C and prepare fresh weekly.

6% trichloroacetic acid/0.06M mercuric chloride. Dissolve 15 g trichloroacetic acid and 4 g mercuric chloride in 250 mL deionized water. Prepare fresh monthly.

Modified Erlich. Place 2 g p -dimethylaminobenzaldehyde in 60 mL glacial acetic acid, add 32 mL of 70% perchloric acid and make up to 100 mL with glacial acetic acid. Prepare fresh daily.

Assay procedures

Inactivated enzyme. The δ -ALA-D activity of blood is measured by determining the amount of porphobilinogen formed under specific conditions

A. Semi-micro method. Gently mix the blood samples for at least 5 minutes on a Multipurpose rotator (Scientific Industries, Inc.), remove the blood sample and allow it to stand in an ice bath for 2 minutes. Pipet 50 μ L of the sample with a Scientific Manufacturing Industries micro pipet (SMI-D) into each of four 1.5-mL disposable polypropylene micro test tubes (Brinkmann Instruments, Inc.) cap the tube, mix well for 15 seconds on a Vortex Genie (Scientific Industries, Inc., Springfield, Mass.) and place in an ice bath for 3 minutes to complete lysis. To the three test samples add a mixture made up of equal parts of the substrate (0.0125 mole/liter ALA) and the buffer (0.3 mole/liter sodium phosphate) pre-incubated at 37 $^{\circ}$ C for 30 minutes, cap, mix well and place in a water bath at 37 $^{\circ}$ C for 60 minutes. To the control sample, immediately add 500 μ L of a solution of 6% trichloroacetic acid - 0.06M mercuric chloride with a dispensette (Brinkmann Instruments) and treat as for the test samples. At the end of the incubation period, add the trichloroacetic acid solution, mix well on a Vortex Genie and centrifuge at 11,500 X g for 5 minutes in an Eppendorf microcentrifuge, Model 5413 (Brinkmann Instruments). Transfer 500 μ L of the supernatant to 100 mm cuvettes add one mL of modified Erlich reagent with a dispensette, mix well. Measure the absorbance within 5 minutes at 555 nm vs. a reagent blank

with a Beckman, Model 25 spectrophotometer calibrated with liquid absorbance standards (National Bureau of Standards, U.S.A. standard reference material 931b).

B. Micro-method. For the assay, pipet 10 μL of each blood sample into four 400- μL microtubes with a Scientific Manufacturing Industries micro pipet (SMI-C), add 50 μL of Triton X-100, mix for 15 seconds and place in an ice bath for 3 minutes to complete lysis. To the haemolysate add 100 μL of a freshly prepared mixture containing equal parts of the sodium phosphate buffer pH 6.4, and the substrate, δ -aminolaevulinic acid, pre-incubated at 37 $^{\circ}\text{C}$ for 30 minutes. To the control sample, immediately add 100 μL of trichloroacetic acid-mercuric chloride mixture and mix well. Incubate all samples at 37 $^{\circ}\text{C}$ for 60 minutes. At the end of the incubation period, stop the reaction by adding 100 μL of the trichloroacetic acid/mercuric chloride solution with a dispensette (Brinkmann Instruments, Rexdale, Ontario, Canada). Mix vigorously and centrifuge at 11,500 X g for 5 minutes in an Eppendorf microcentrifuge, Model 5413 (Brinkmann Instruments). Transfer 200 μL of the supernatant to a 1-cm light path, 2 mm wide micro cell, add 400 μL of modified Erlich reagent, cap, mix well and read the absorbance within 5 minutes at 555 nm versus a blank containing 400 μL of modified Erlich reagent and 200 μL of water as described above.

Activated enzyme. For enzyme activation add 125 μL and 25 μL of the dithiothreitol solution to the haemolysate of the semi and micro-method respectively and incubate at 37 $^{\circ}\text{C}$ for 30 minutes prior to the addition of the buffer-substrate solution. The remainder of the assay is conducted as detailed above.

Calculations. The enzyme is expressed as micro moles of porphobilinogen (PBG) generated per hour, per liter of erythrocytes.

Units - (μ mole PBG/h/L_{RBC}) 37 °C

Sample calculation - microassay

Dilution factor	X	100	X	1
	<hr/>		<hr/>	
	% hematocrit		Extinction coefficient	
	X absorbance (A_{555})			
	= 78	X 100	X	1 X A_{555}
	<hr/>		<hr/>	
		% hematocrit		6.2×10^{-2}
	= 125.8	$\times 10^3$	X A_{555}	
			<hr/>	
			% hematocrit	

δ -ALA-D pH Activity Curves

Depending on whether the semi micro or micro-method is employed, place 1 mL or 200 μ L of well mixed blood into three 5 mL Falcon polypropylene tubes and add 7 or 1.4 mL of Triton X-100 respectively. Vortex the haemolysate for 30 sec. and allow it to stand in an ice bath for 3 minutes to complete lysis.

Add 500 or 100 μ L of the haemolysate to each one of a series of ten 1.5 mL or 400 μ L disposable polypropylene tubes containing 500 μ L or 100 μ L mixture of equal parts of the substrate ALA and the phosphate buffers pH 5.8 to 7.4 pre-incubated at 37 °C for 30 minutes. Cap, vortex and place the micro tubes in a water bath at 37 °C for 60 minutes. Perform the remainder of the assay as described above.

δ -ALA-D Activity Ratio Test

Place 200 μ L of well mixed blood in three 5-mL Falcon tubes with an SMI-I pipet, add 800 μ L of Triton X-100 with a dispensette, vortex and procede as described earlier. Add 100 μ L of each haemolysate to seven 400- μ L microtubes with an Eppendorf 100 μ L pipet--four of these tubes contain an ALA and phosphate buffer mixture, pH 6.4, 6.6, 6.8 and 7.2 mixture (first set), the remaining three tubes (second set) contain only phosphate buffer, pH 6.6, 6.8 and 7.2. Incubate the first set at 37 °C for 60 minutes. Incubate the second set at 37 °C for 30 minutes, add 50 μ L of the substrate solution and incubate at 37 °C for 60 minutes. In both instances, complete the assay essentially as described earlier. Various ratios are obtained by dividing the absorbance value for a particular sample by that of another. For instance, by dividing the absorbance value for the sample at buffer pH 6.4 by that of the sample at buffer pH 7.2 to give a 6.4/7.2 pH ratio test.

Determination of Toxicant Concentration in Blood, Plasma or Erythrocytes

Toxic metal analysis

Treatment of blood samples. All blood samples collected for blood metal determination, except those obtained for mercury analysis, are frozen until analysed. After thawing and prior to analysis, all samples are sonified (Sonicator cell disruptor, Heat Systems Ultrasonics, Inc., Plainsview, N.Y. 11803) to rid them of blood clots.

1.5-mL blood samples collected for the determination of metals in plasma and erythrocytes are centrifuged at 11,500 X g for 5 minutes in 1.5-mL micro tubes within three hours of collection. The plasma is carefully removed with an SMI-I 100 μ L pipet, placed in a 1.5-mL micro tube and frozen until analysed (however, not plasma samples destined for mercury analysis). The erythrocytes are washed three times with an ice-cold 0.9% (w/v) saline solution, the buffy layer removed with an SMI-I pipet and, 300 μ L transferred to a 1.5-mL micro tube with an SMI-I pipet. 1 mL of Triton X-100 is added, the samples vigorously vortexed for 1 minute and allowed to stand at 4 °C overnight. The samples are centrifuged for 30 minutes at 11,500 X g to remove the stromal membrane. The supernatant is decanted and frozen (except for mercury analysis) until analysed.

Zinc analysis (198). Place 500 μ L of well mixed blood, haemolysate or plasma in a 15-mL polystyrene culture tube (Corning) dilute twenty-fold with deionized water and make 1% nitric acid by the addition of 100 μ L of concentrated nitric acid (Aristar British Drug House). Aspirate the mixture into the flame of a Perkin-Elmer 603, atomic absorption spectrophotometer. Calibrate the instrument with prepared standards after each five determinations. Determine all samples in duplicate. The precision of the determination is estimated to be $\pm 10 \mu\text{g/dL Zn}^{2+}$ and a detectability of 10 $\mu\text{g Zn}^{2+}$ /dL blood can be attained.

Copper analysis (199). Determine in the samples prepared for zinc assay. The precision of the determination is estimated at

$\pm 5 \mu\text{g/dL}$ blood and the detectability at $5 \mu\text{g Cu}^{2+}/\text{dL}$ of blood.

Mercury analysis. Measure (total and inorganic) mercury concentrations in blood according to the method of Magos as modified by Farant et al. (200) to accomodate micro blood samples. The precision of the determination is estimated at $0.2 \mu\text{g/dL}$ and $0.05 \mu\text{g/dL}$ mercury can be detected. Interlaboratory tests of blood samples conducted periodically during this study indicated that the determinations were satisfactorily accurate.

Blood lead assays. Blood lead determinations presented certain difficulties at the very outset of the study. Thus, there were problems in obtaining reproducible results and participation in interlaboratory tests indicated that the results had a tendency to be too low by some 20% or more. It is noteworthy that our problems with blood lead determinations were not unique--the results obtained by the participants of the tests most often showed a 2-fold variation in value. The reproducibility problem was successfully circumvented by determining the blood lead concentration of every sample by two alternative techniques and averaging the results obtained.

Method 1. The lead level in blood samples is determined by anodic stripping voltammetry with an Environmental Sciences Associates voltammeter, Model 3010 using a modification of the procedure suggested by the manufacturer.

Adjust the instrument's setting as recommended by the manufacturer. Add $100 \mu\text{L}$ of well mixed blood to 2.9 mL of Metexchange reagent whose blank value has been instrumentally set

at zero. Perform two successive determinations of the blood's lead content. Repeat the determinations after the addition of 50 μL of a 0.5 $\mu\text{g/mL}$ Pb^{2+} standard solution. The lead concentration in $\mu\text{g/dL}$ is calculated according to the following formula:

$$\text{Metal Concentration } (\mu\text{g/dL}) = \frac{A_1 v C_s}{A_2 V - (A_2 - A_1) V}$$

where

A_1 = average peak area before standard addition, cm^2

A_2 = average peak area after standard addition, cm^2

v = standard solution volume, mL

V = solution volume before standard solution addition, mL

C_s = concentration of standard solution, $\mu\text{g/mL}$.

The precision of the determinations averages 2 $\mu\text{g/dL}$ Pb^{2+} over the range of concentrations of metal found. The detectability attainable is 2 $\mu\text{g/dL}$ lead.

Method 2.3 The lead is isolated from digested whole blood by coprecipitation with strontium sulfate, the sulfate precipitate converted to carbonate salt which is dissolved in nitric acid, and the metal analysed by atomic absorption spectrometry.

Preparation of reagents

0.75% strontium solution: Dissolve 11.25 g $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$ (Fisher Scientific Company) in 500 mL of deionized water.

5% nitric acid. Add 128 mL of concentrated nitric acid (Aristar, British Drug House) to 2 liters of deionized water.

20% ammonium carbonate solution. Dissolve 400 g of ammonium carbonate (Fisher Scientific Company) in 2 liters of deionized water.

0.05N sulfuric acid. Add 3 mL of concentrated sulfuric acid (Aristar, British Drug House) to two liters of deionized water.

Separation of lead

Add 1.0 mL of the strontium chloride solution to 50-mL Erlenmeyer flasks (10% HNO_3 acid washed) and dry in an oven for 1 1/2 hours at approximately 95 °C. Accurately weigh 3.0 g of well mixed blood into the flasks. Four blank samples (3 mL of water) and lead-spiked samples are prepared simultaneously. Allow samples to stand overnight at 4 °C.

Add 4 mL of concentrated nitric acid (Aristar, British Drug House) to each flask and heat cautiously till frothing subsides. A further 4 mL of nitric acid is added and heating is resumed until a clear yellow-brown solution is obtained. Cool the flasks, add 4 mL of concentrated sulfuric acid (Aristar, British Drug House) and heat the mixtures again till frothing ceases. Remove from heat, add 2 mL of nitric acid and 1 mL of 70% perchloric acid (Aristar, British Drug House). Heat mixture until dense white fumes cease to be evolved from the solution. Remove from the heat, cool and add 6 mL of deionized water. This causes the sulfates (lead and strontium as well as others) to precipitate.

Transfer the precipitate to 15 mL polystyrene centrifuge tubes with 2 mL of 0.05N sulfuric acid, centrifuge for 20 minutes at 2,000 rpm. Decant the supernatant and wash the precipitate with 1-2 mL warm 0.05N sulfuric acid. Add 10 mL of 20% ammonium carbonate solution and vortex mixture. Centrifuge, decant supernatant and discard it, and repeat treatment. Dry the carbonate salts, still in the centrifuge tubes, at 60 °C overnight.

Dissolve the dried precipitate in 2.5 mL of 5% nitric acid and determine the concentration of lead in the solution by atomic absorption spectrometry (flame) at 283.3 nm. For this study a Perkin-Elmer Model 603 atomic absorption spectrophotometer was used. The precision of the assay is $\pm 5\%$ (coefficient of variation) and 2-4 $\mu\text{g/dL Pb}^{2+}$ can be detected.

Cadmium assay. Because of its relatively low concentration in blood, ($<1.0 \mu\text{g/dL}$ in normal individuals), cadmium presents certain analytical difficulties. After attempting its determination by several different techniques, its measurement in blood by anodic stripping voltammetry was selected on the basis of its sensitivity and excellent reproducibility.

Nitric acid is used to precipitate the protein in a sonified sample. After centrifugation, the supernatant is submitted to a mild digestion with perchloric acid. The white precipitate from this digestion is dissolved in sodium acetate--acetic acid buffer, pH 6.5, and polarography performed on an aliquot of this sample. Quantification of cadmium and also lead is achieved by the method of standard addition.

Preparation of reagents. (All glassware and plasticware must be thoroughly washed with 10% HNO_3 solution).

1.0 mole/liter sodium acetate. Weigh 8.203 ± 0.0003 g of sodium acetate (Aristar, British Drug House) and transfer to a 100-mL volumetric flask. Dilute to volume with deionized water.

0.1 mole/liter sodium acetate-acetic acid buffer. (pH 6.5)

Pipet 20 mL of 1.0 mole/liter sodium acetate into a 200-mL volumetric flask and dilute to near volume (10 mL) with deionized water. Add glacial acetic acid (Aristar, British Drug House) until $\text{pH } 6.5 \pm 0.5$ is reached. Dilute to volume.

Standards. Prepared from standards used in "in vitro" experiments.

Determination of cadmium. Mix blood sample on Multipurpose rotator and sonify. Pipet 500 μL deionized water and 100 μL of concentrated nitric acid (Aristar, British Drug House) into 1.5-mL micro tubes. To each micro test tube add 500 μL of sonified blood as quickly as possible with an SMI-F pipet. Cap micro tubes and vortex to break clumps. Centrifuge in Eppendorf micro centrifuge Model 5413 at 11,500 X g for 2 minutes. Carefully decant the supernatant into 18-mL pre-cleaned glass scintillation vials (O. H. Johns Scientific Ltd.) Cover vials with 10-mL disposable micro beakers. Add 500 μL of deionized water to each micro tube, resuspend the precipitate and centrifuge. Combine supernatants in vials. Add 500 μL 70% perchloric acid (Aristar, British Drug House) to each vial and heat for 1-2 hours at $100-110^\circ\text{C}$. If the digest is still colored, add a further 50 μL 70% perchloric acid. Take to dryness on hot plate.

Remove vials, cool and dissolve crystalline white precipitate in 4 mL of Na Ac-Hac buffer. Transfer 2 mL of the sample digest and 1 mL of buffer to the polarographic cell.

The PAR 174A (Princeton Applied Research, Princeton, NJ) is set up for differential pulse anodic stripping voltammetry (DPASV) as recommended by the manufacturer. The following conditions are used:

- 1) initial potential - 1.0 V vs. SCE;
- 2) modulation amplitude 25 mV;
- 3) potential scan rate 5 mV/second;
- 4) potential scan range 1.5 V;
- 5) display direction - negative;
- 6) clock - 0.5 second;
- 7) output offset - 0;
- 8) low pass filter off;
- 9) chart speed - 1 inch/minute;
- 10) deposition time - 3 minutes;
- 11) rest period - 30 seconds;
- 12) current range 10 μ AMP.

DPASV was conducted as detailed in the operation manual.

Quantification of the cadmium in the sample was achieved by the method of standard addition.

Calculations. The concentration of cadmium in blood is determined by using the formula given in method 1. (lead). 0.2 μ g/dL cadmium can be detected in blood samples using this technique with a reproducibility of $\pm 10\%$ (coefficient of variation).

Carboxy haemoglobin (COHb) (201)

Estimation of molar absorbance values

Add 200 μ L of blood to 30 mL of deionized water, mix and centrifuge at 2,000 rpm for 15 minutes. Place 10 mL of this lysate in a 100-mL volumetric flask and dilute to the mark with 10 mM/L tris (hydroxymethyl) aminoethane (THAM) (Fisher Scientific Company). Transfer 30 mL to a 150-mL Erlenmeyer flask and bubble carbon monoxide gas (Matheson of Canada Ltd.) through the solution for 15 minutes followed by nitrogen gas for a further 15 minutes. Place three 4-mL aliquots in 15-mL test tubes and add 40 mg sodium hydrosulfite (Fisher Scientific Company). Stopper the tubes, mix and allow to stand for 15 minutes. Read their absorbance at 420 and 432 nm versus a blank made up of 2 mg/mL sodium hydrosulfite in 10 mM/L THAM. Calculate the molar absorptivity for COHb from the formula

$$\epsilon_{420}^{\text{COHb}} = A / (l \times c)$$

or
432

where A is the measured absorbance, c is the molar concentration of haemoglobin and l is the light path length.

To determine the molar absorptivity of haemoglobin (ϵ^{Hb}) place three 4-mL samples of solution (2) in 15-mL test tubes, add 40 mg sodium hydrosulfite to each and procede as described above. Calculate the molar absorptivity of haemoglobin from the formula

$$\epsilon_{420}^{\text{Hb}} = [A - (f \epsilon_{420}^{\text{COHb}} \cdot l \cdot c)] / [(1 - f) \cdot l \cdot c]$$

or
432

where A is the absorbance, f the total haemoglobin assumed to be present, c is the total haemoglobin concentration in moles of haemoglobin Fe /liter and l is the length of the light path.

COHb determinations

Draw a 2 mg/mL sodium hydrosulfite solution in 10 mM/L THAM into a 5-mL glass syringe fitted with an 18-gauge needle extended with a piece of polyethylene tubing. Place the tip of the polyethylene tubing at the bottom of a 10 mm cell and fill it until a meniscus forms above the top of the cell. Fill 4 cells (one blank and three test) ⁽¹⁾ in this manner. With a 10-μL Hamilton syringe fitted with a 7 cm long piece of polyethylene tubing, transfer two μL of well mixed blood. Shake the cells vigorously and read the absorbance at 420 and 432 nm 10 to 15 minutes after mixing. The fraction of the total haemoglobin present as COHb is determined by using the formula:

$$x = \frac{(A_{432} \cdot \epsilon_{420}^{Hb}) - (A_{420} \cdot \epsilon_{432}^{Hb})}{(A_{420} \cdot (\epsilon_{432}^{COHb} - \epsilon_{432}^{Hb}) - [A_{432} \cdot (\epsilon_{420}^{COHb} - \epsilon_{420}^{Hb})]}$$

(1) Blank and sample solutions must have identical amount of sodium hydrosulfite in 10 mM/L THAM

Blood ethanol

Standards. 0.35 g/dL, 0.18 g/dL and 0.05 g/dL ethanol standards are prepared by pipetting 4.43 mL, 2.28 and 0.63 mL of absolute ethanol (Consolidated Alcohols Ltd., Toronto, Ontario, Canada) respectively into three tared 100-mL volumetric flasks partially filled with deionized water. The volumetric flasks are then reweighed and brought to volume. The exact ethanol concentrations are determined using these weights and a working solution of each is obtained by diluting 1:10.

Ethanol determination. (202) The ethanol content of blood was determined by gas chromatography using an internal standard solution of acetonitrile (44%) prepared by diluting 0.22 g of the chemical (Aldrich Chemical Company, Inc., Milwaukee, WIS 53233) with deionized water and bringing up to volume of 500 mL.

200 μ L of either the standard ethanol solutions or blood samples are diluted with 1.8 mL of the internal standard solutions. 3 μ L of each solution is injected into a Gow-Mac series 750 chromatograph equipped with a flame ionization detector, a stainless steel 6' x 1/8" i.d. column packed with Porapak Q 80/100 med/2% Carbowax, a Fisher Recordall series 5000 recorder and a Spectra Physics (Minigrator) electronic integrator. The operating conditions used are: helium carrier gas, 20 mL/min, hydrogen gas, 50 mL/min, and air 450 mL/min; temperatures of injection port and column and the oven are 118 °C and 175 °C respectively. The electronic integrator is started after each injection and the areas corresponding to ethanol and acetonitrile recorded.

The ethanol concentration of the unknown sample is calculated in the following manner:

$$C_u = C_k \times (A_{ik} / A_{ek}) / (A_{iu} / A_{eu})$$

where

- C_u = concentration of ethanol in unknown sample (Zw/v)
- C_k = concentration of ethanol in ethanol standard (Zw/v)
- A_{ik} = area of internal standard peak in known standard
- A_{ek} = area of ethanol peak in known standard
- A_{iu} = area of internal standard in unknown sample
- A_{eu} = area of ethanol peak in unknown sample.

Experimental Data Assessment

Evaluation of assays

δ -ALA-D assays. The intra-day assay precision is determined by analysing blood samples from normal and toxic metal-exposed individuals. Since δ -ALA-D activity falls off with time after collection, inter-day assay precision is determined by measuring the enzyme's activity periodically in the blood of control subjects over a six-month period.

pH-ratio tests. Intra-day assay precision is determined by measuring the pH-ratios of twenty replicate samples from both normal and metal-intoxicated persons. Inter-day assay assessment is accomplished by measuring the pH-ratios on twenty consecutive days.

Metal analysis. The precision of each analysis is estimated by replicate analysis of blood samples on the same day and by performing daily analyses of the same samples on twenty consecutive days. Whenever possible, precision is expressed as the coefficient of variation. The minimum amount of metal detectable in blood is also estimated.

Method comparison

Methods developed during the study viz. the δ -ALA-D micro-assay procedure, are compared to existing methods such as the "European Standardized Method" (82) by conducting simultaneous analyses of common samples with both methods.

Statistical methods

Standard statistical methods are used throughout. Wherever possible all data in the study are expressed as the mean $\pm$ standard deviation. One notable exception is the metal analyses where a coefficient of variation is given.

The correlation between assays is tested by the method of linear regression. The standard error of estimate is also given, and is determined by the equation $S_{y/x} = S_y \sqrt{1 - r^2}$ where S_y = standard deviation of y and r = correlation coefficient.

An estimate of the significance between two means such as, the mean δ -ALA-D activity of a blood haemolysate to which no metal is added compared with that to which various concentrations of metals are added, is obtained using the paired-t test. A difference is considered significant when the corresponding P value is between 0.01 to 0.001.

In some special instances, the F ratio test is also used.

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APPENDIX "A"

Mechanism of Action for δ -ALA-D
Postulated by Napdi and Shemin (11)

APPENDIX "A"

The basic mechanism for the reactions catalyzed by δ -ALA-D as proposed by Nandi and Shemin (11) is depicted in a slightly modified form in Figure 2A. The condensation occurs in three distinct steps following the formation of an enzyme-substrate imine: 1) an Aldol condensation; 2) dehydration of the Aldol intermediate by loss of water from the C-C intrasubstrate bond; and 3) ring closure and displacement of the enzyme in a manner reminiscent of a transimination reaction; formation of a new intramolecular cationic Schiff base which provides the electrophilic center required for labilization of a hydrogen atom and formation of PHG. The Schiff base mechanism is supported by the observation that the enzyme is inactivated in the presence of the substrate by reduction with sodium borohydride with concomitant fixation of the label to the enzyme.

Apart from a recent modification suggested by Aboud and Atkar (12), concerning the stereospecific retention of the G-5 pro-S hydrogen atom of ALA in the aromatization process, the mechanism proposed by Nandi and Shemin for the enzyme isolated from *R. spheroides* has remained largely unchallenged and unproven. The mechanism is consistent with that of other aldolases (203) and amine catalyses (204) in that it postulates the formation of a Schiff base between the substrate and the enzyme stabilizing a carbanion (205) which then makes a nucleophilic attack on the carbonyl atom of a second ALA molecule and thus forms an intrasubstrate C-C bond. According to this mechanism, the ALA mole-

cule attached to the enzyme through a Schiff base linkage would be expected to give rise to the side of the pyrrole ring bearing the acetic acid side chain whereas the propionic acid side chain would originate from the δ -ALA in solution or attached at another site. The synthesis of heterologous pyrroles by Nandi and Shemin (11) from laevulinic acid and δ -ALA appeared to lend support to this contention. Laevulinic acid must be the molecule in Schiff base linkage with the enzyme, otherwise, if it were δ -ALA, the pyrrole would not be formed. However, as it will be shown, this is not conclusive proof--a different mechanism may be operative. Jordan and Seehra (13) using the ^{14}C -single turnover technique succeeded in shedding some light on the order in which the two substrates bind to the enzyme and condense to form PBG. They mixed an equal or lesser amount of $[5 - ^{14}\text{C}]$ -5-aminolaevulinic acid with δ -ALA-D isolated from bovine liver and followed this by an excess of the non-labelled substrate. Degradation of the PBG to 2-4 dinitrophenyl glycine demonstrated that the label was located preponderantly in the C-11 position of PBG demonstrating that, conversely to the mechanism proposed by Nandi and Shemin, the δ -ALA bound to the enzyme gives rise to the side of the pyrrole ring bearing the propionic acid side-chain (Figure 2b). It should be noted that attempts by Jordan and Seehra to repeat the preparation of an heterologous PBG with bovine δ -ALA-D and laevulinic acid were unsuccessful. At this point, one could conjecture that δ -ALA-D functions via different mechanisms in eucaryotic and procaryotic cells and this would lend support to the speculation by Cheh and

Neillands (7) that there exists two types of enzyme based on source. The situation could be clarified by repeating the Jordan-Seehra experiment with R. spheroides δ -ALA-D.

APPENDIX "B"

Effects of Common Toxicants Upon
The Activity-Ratio Test Results

TABLE XVIII: EFFECTS OF ETHANOL INTOXICATION - A. RABBIT EXPERIMENTS

Blood Sample Number	δ -ALA-D Activity μ M PBC/h/L RBC	Activity Ratios at Specific pH Values				Metal Concentrations (μ g/dL)			EtOH (g/dL)
		(6.6/7.2) Δ				Lead	Zinc	Copper	
		6.4/7.2	(6.6/7.2) Δ	(6.6) Δ /6.4	(7.2) Δ /7.2				
R-26	148 $\pm$ 5	5.19 $\pm$ 0.37	5.42 $\pm$ 0.01	2.48 $\pm$ 0.08	2.64 $\pm$ 0.00	34	340	51	0.0130
R-35	147 $\pm$ 19	4.37 $\pm$ 1.14	4.24 $\pm$ 0.07	2.74 $\pm$ 0.36	3.60 $\pm$ 0.96	---	---	---	---
R-27	2488 $\pm$ 17	1.08 $\pm$ 0.03	0.47 $\pm$ 0.01	0.44 $\pm$ 0.01	1.01 $\pm$ 0.02	4	445	83	---
R-37	2833 $\pm$ 49	1.10 $\pm$ 0.03	0.79 $\pm$ 0.02	0.69 $\pm$ 0.03	0.96 $\pm$ 0.04	3	431	89	0.0617
R-28	162 $\pm$ 19	5.59 $\pm$ 0.60	8.43 $\pm$ 0.73	2.79 $\pm$ 0.15	1.86 $\pm$ 0.19	22	418	57	---
R-30	141 $\pm$ 13	4.06 $\pm$ 0.01	6.58 $\pm$ 0.07	3.10 $\pm$ 0.28	1.40 $\pm$ 0.20	---	386	64	0.0347
R-33	1734 $\pm$ 57	1.08 $\pm$ 0.05	0.78 $\pm$ 0.07	0.74 $\pm$ 0.01	1.02 $\pm$ 0.03	< 2	399	64	---
R-36	2024 $\pm$ 49	1.08 $\pm$ 0.01	0.71 $\pm$ 0.02	0.64 $\pm$ 0.02	0.98 $\pm$ 0.06	< 2	379	57	0.0117
R-38	1708 $\pm$ 27	1.29 $\pm$ 0.21	0.76 $\pm$ 0.06	0.67 $\pm$ 0.11	1.15 $\pm$ 0.12	4	454	83	---
R-39	1463 $\pm$ 11	1.18 $\pm$ 0.04	0.77 $\pm$ 0.03	0.80 $\pm$ 0.04	1.22 $\pm$ 0.02	6	452	83	0.1216
R-41	2340 $\pm$ 24	1.18 $\pm$ 0.04	0.70 $\pm$ 0.04	0.61 $\pm$ 0.01	1.04 $\pm$ 0.05	12	---	---	---
R-42	1869 $\pm$ 88	1.26 $\pm$ 0.09	0.92 $\pm$ 0.06	0.76 $\pm$ 0.05	1.04 $\pm$ 0.06	---	---	---	0.0158

0.0130

0.0617

0.0347

0.0117

0.1216

0.0158

TABLE XVII: EFFECTS OF ETHANOL INTOXICATION -B. HUMAN TEST SUBJECTS

Blood Sample Number	6-ALA-D Activity mM PBG/h/L RBC	Activity Ratios at Specific pH Values			Metal Concentrations (µg/dL)			EtOH (g/dL)
		6.4/7.2	(6.6/7.2) ^Δ	(6.6) ^Δ /6.4	(7.2) ^Δ /7.2	Lead	Zinc	
H-437 A	1249±28	1.61±0.08	0.97±0.08	0.81±0.02	1.29±0.02	6	660	0.160±0.016
H-437 B	1327±72	1.46±0.04	1.05±0.00	0.85±0.04	1.18±0.09	-	---	0.173±0.018
H-438 A	1050±68	1.39±0.10	0.98±0.14	0.72±0.08	1.02±0.08	12	490	0.044±0.004
H-438 B	1166±40	1.39±0.07	1.11±0.06	0.74±0.03	0.92±0.04	-	---	0.070±0.007
H-439 A	1602±46	1.44±0.12	1.10±0.07	0.73±0.05	0.96±0.09	10	647	0.121±0.012
H-439 B	1637±46	1.37±0.06	1.12±0.01	0.72±0.02	0.89±0.01	-	---	0.114±0.012
H-440 A	1641±19	1.35±0.02	0.99±0.04	0.65±0.02	0.88±0.05	7	608	0.082±0.008
H-440 B	1579±40	1.32±0.03	1.01±0.06	0.72±0.01	0.94±0.04	-	---	0.074±0.008
H-441 A	1829±33	1.34±0.07	1.06±0.08	0.69±0.03	0.87±0.04	11	543	0.128±0.013
H-441 B	1892±59	1.34±0.05	1.07±0.11	0.70±0.05	0.88±0.03	-	---	0.111±0.011

TABLE XVIII: EFFECTS OF ETHANOL INTOXICATION - B. HUMAN TEST SUBJECTS

Blood Sample Number	δ -ALA-D Activity μ M PBG/h/L RBC	Activity Ratios at Specific pH Values			Metal Concentrations (μ g/dL)			EtOH (g/dL)
		6.4/7.2	(6.6/7.2) Δ	(6.6) Δ /6.4	(7.2) Δ /7.2	Lead	Zinc	
H-633	185 $\pm$ 4	1.25 $\pm$ 0.03	0.90 $\pm$ 0.03	0.60 $\pm$ 0.00	0.84 $\pm$ 0.03	10	549	0.009 $\pm$ 0.0001
H-634	1771 $\pm$ 21	1.27 $\pm$ 0.02	0.91 $\pm$ 0.05	0.67 $\pm$ 0.02	0.93 $\pm$ 0.05	--	571	---
H-635	2091 $\pm$ 48	1.36 $\pm$ 0.08	1.02 $\pm$ 0.04	0.79 $\pm$ 0.02	1.06 $\pm$ 0.06	9	601	0.0257 $\pm$ 0.0002
H-636	2166 $\pm$ 80	1.35 $\pm$ 0.08	1.20 $\pm$ 0.05	0.87 $\pm$ 0.03	0.98 $\pm$ 0.06	--	621	---
H-637	1213 $\pm$ 24	1.49 $\pm$ 0.08	1.20 $\pm$ 0.05	0.91 $\pm$ 0.05	1.13 $\pm$ 0.06	14	471	0.0165 $\pm$ 0.0002
H-638	1349 $\pm$ 81	1.70 $\pm$ 0.10	1.38 $\pm$ 0.10	0.91 $\pm$ 0.05	1.12 $\pm$ 0.08	--	486	---
H-639	1763 $\pm$ 99	1.36 $\pm$ 0.10	1.00 $\pm$ 0.03	0.78 $\pm$ 0.05	1.06 $\pm$ 0.05	9	631	0.0062 $\pm$ 0.0004
H-640	1840 $\pm$ 32	1.42 $\pm$ 0.09	1.18 $\pm$ 0.05	0.91 $\pm$ 0.04	1.09 $\pm$ 0.07	--	628	---
H-641	1568 $\pm$ 37	1.40 $\pm$ 0.04	1.02 $\pm$ 0.02	0.78 $\pm$ 0.02	1.06 $\pm$ 0.03	9	667	0.0316 $\pm$ 0.0002
H-642	1525 $\pm$ 41	1.44 $\pm$ 0.04	1.00 $\pm$ 0.02	0.76 $\pm$ 0.02	1.10 $\pm$ 0.02	--	669	---
H-643	1206 $\pm$ 53	1.53 $\pm$ 0.07	1.19 $\pm$ 0.09	0.81 $\pm$ 0.03	1.05 $\pm$ 0.08	10	594	0.0303 $\pm$ 0.0002
H-644	1254 $\pm$ 64	1.53 $\pm$ 0.08	1.40 $\pm$ 0.07	0.95 $\pm$ 0.05	1.04 $\pm$ 0.05	--	631	---

TABLE XIX: EFFECTS OF CARBON MONOXIDE EXPOSURE/RABBIT EXPERIMENTS

Blood Sample Number	δ -ALA-D Activity $\mu\text{M PBG/h/L RBC}$	Activity Ratios at Specific pH Values			Metal Concentrations ($\mu\text{g/dL}$)			COHB %
		6.4/7.2	(6.6/7.2) Δ	(6.6) Δ /6.4	(7.2) Δ /7.2	Lead	Zinc	Copper
R-82	1134 $\pm$ 66	1.39 $\pm$ 0.02	0.92 $\pm$ 0.04	0.70 $\pm$ 0.06	1.06 $\pm$ 0.06	< 2.0	353	74
R-91	1110 $\pm$ 36	1.41 $\pm$ 0.06	0.96 $\pm$ 0.03	0.71 $\pm$ 0.03	1.05 $\pm$ 0.06	< 2.0	353	62
R-86	1470 $\pm$ 78	1.34 $\pm$ 0.05	0.86 $\pm$ 0.03	0.65 $\pm$ 0.05	1.01 $\pm$ 0.03	3.0	360	65
R-92	1418 $\pm$ 68	1.34 $\pm$ 0.05	0.84 $\pm$ 0.02	0.63 $\pm$ 0.05	1.01 $\pm$ 0.02	4.0	---	---
R-87	2777 $\pm$ 100	1.20 $\pm$ 0.04	0.55 $\pm$ 0.04	0.43 $\pm$ 0.04	0.94 $\pm$ 0.04	2.0	464	64
R-93	2757 $\pm$ 26	1.19 $\pm$ 0.01	0.66 $\pm$ 0.07	0.53 $\pm$ 0.07	0.95 $\pm$ 0.03	2.1	438	63
R-88	3417 $\pm$ 79	1.23 $\pm$ 0.02	0.71 $\pm$ 0.01	0.55 $\pm$ 0.02	0.95 $\pm$ 0.02	< 2.0	418	87
R-94	3296 $\pm$ 110	1.20 $\pm$ 0.04	0.62 $\pm$ 0.05	0.47 $\pm$ 0.05	0.91 $\pm$ 0.04	< 2.0	438	88
R-90	2539 $\pm$ 17	1.20 $\pm$ 0.01	0.48 $\pm$ 0.03	0.37 $\pm$ 0.03	0.92 $\pm$ 0.03	< 2.0	399	93
R-95	2757 $\pm$ 36	1.24 $\pm$ 0.02	0.74 $\pm$ 0.02	0.54 $\pm$ 0.02	0.90 $\pm$ 0.02	< 2.0	431	100
R-89	1961 $\pm$ 58	1.09 $\pm$ 0.03	0.54 $\pm$ 0.05	0.48 $\pm$ 0.02	0.97 $\pm$ 0.05	< 2.0	399	95
R-96	2074 $\pm$ 35	1.13 $\pm$ 0.03	0.62 $\pm$ 0.02	0.52 $\pm$ 0.02	0.96 $\pm$ 0.03	< 2.0	431	101
R-102	1860 $\pm$ 36	1.01 $\pm$ 0.04	0.41 $\pm$ 0.02	0.36 $\pm$ 0.01	0.90 $\pm$ 0.04	< 2.0	399	65
R-106	2165 $\pm$ 25	1.13 $\pm$ 0.04	0.72 $\pm$ 0.02	0.59 $\pm$ 0.02	0.93 $\pm$ 0.04	< 2.0	353	72
R-103	2119 $\pm$ 20	1.04 $\pm$ 0.02	0.60 $\pm$ 0.05	0.51 $\pm$ 0.04	0.90 $\pm$ 0.03	< 2.0	346	74
R-107	2159 $\pm$ 30	1.09 $\pm$ 0.03	0.58 $\pm$ 0.02	0.48 $\pm$ 0.02	0.90 $\pm$ 0.03	5.5	346	65
R-104	1890 $\pm$ 154	1.19 $\pm$ 0.10	0.42 $\pm$ 0.03	0.37 $\pm$ 0.03	1.05 $\pm$ 0.07	< 2.0	346	64
R-108	1881 $\pm$ 58	1.12 $\pm$ 0.04	0.45 $\pm$ 0.01	0.36 $\pm$ 0.01	0.87 $\pm$ 0.03	< 2.0	340	70
R-105	2119 $\pm$ 70	1.04 $\pm$ 0.04	0.46 $\pm$ 0.07	0.40 $\pm$ 0.06	0.89 $\pm$ 0.04	< 2.0	379	82
R-109	2246 $\pm$ 20	1.15 $\pm$ 0.02	0.67 $\pm$ 0.03	0.52 $\pm$ 0.02	0.90 $\pm$ 0.02	< 2.0	353	89

END

3 1 H 0 8 H 8 2

FIN