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# Postulated Mode of Action of Lead on Aminolevulinic Acid Dehydratase in Chronic Exposure

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**Summary.** The authors studied in vitro the variations of erythrocyte ALA D activity in subjects exposed and not exposed to lead risk before and after three tests: heat treatment at 60° C for five minutes, addition of GSH ($1 \times 10^{-2}$ mol/l) and of zinc ($1.5 \times 10^{-4}$ mol/l). A study of the ratios before and after treatments showed that GSH and zinc were better than heating in identifying false positives, and that zinc was preferable, owing to lesser dispersion of results around the mean. The ratio of ALA D activity before and after treatment is more closely correlated with PbB levels than ALA D activity without treatment. The addition of GSH restored the enzyme activity of the exposed subjects to values equal to those obtained with heating; this did not happen in the non-exposed subjects. It is postulated that, in lead exposure, the metal acts only on the thiol groups, which may then be reactivated by an optimal concentration of GSH.

**Key words:** Aminolevulinic acid dehydratase – Glutathione – Heating – Lead – Zinc

## Introduction

ALA D (E.C. 4.2.1.24) is an allosteric enzyme (Vergnano et al. 1968), and its activity is known to be inhibited by lead (Bonsignore et al. 1965; De Bruin and Hoolboom 1967).

There are still some uncertainties on the mechanism by which lead acts on this enzyme, in particular in subjects with past poisoning where a very low enzyme activity is found even with PbB levels within normal limits.

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*Abbreviations:* ALAD = Aminolevulinic acid dehydratase; ALA = Aminolevulinic acid; ALAU = Aminolevulinic acid in urine; FEP = Free erythrocyte protoporphyrin; PbB = Lead blood levels.

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The hypothesis has been put forward (Bonsignore et al. 1968) of the presence of a negative allosteric effector, thermolabile, of probable proteic nature, inactivated by heating at 60°C for five min according to the method of Gerhart and Pardee (1962), with consequent enzyme reactivation; this inhibition is also present in subjects not exposed to lead.

On the basis of these principles Chiba (1976) proposed a new parameter for the evaluation of lead exposure and for identifying unexposed subjects in whom ALA D values are lower than normal more often on genetic basis; this method is based on the determination of the ratio between enzyme activity before and after heating at 60°C for five min.

Two other important positive allosteric effectors, however, are present in the regulation of the enzyme: GSH and zinc. There are no in vivo or in vitro proofs of a close link between reduction of ALA D activity and reduction of GSH (Vergnano et al. 1969). Instead, as regards zinc, Cheh and Neilands (1973) demonstrated its presence in the enzyme molecule in a site different from the thiol groups, called the metallic site.

In the light of these three mechanism of enzyme activation, we believed it would be useful to study the behaviour of enzyme activity after three different methods of treatment, partially recreating Chiba's experiments (1976), with the aim of finding the most selective method of identifying the subjects in whom ALA D values were lower than normal without lead exposure and beginning a study on the inhibitory mechanism of lead during chronic exposure, when enzyme activity is greatly inhibited.

## Materials and Methods

PbB, FEP, ALA U were measured, and ALA D activity was studied in 20 male subjects: 10 were exposed to modest risk of lead poisoning in the decorating sector of a ceramic factory, and ten were non-exposed adult healthy males working in our laboratory. The blood was collected from subjects who had fasted overnight. Smoking habit of all subjects (exposed and not exposed) were insignificant small.

*PbB:* This was determined by direct reading after haemolysis using a Perkin-Elmer mod. 305 spectrophotometer, equipped with a graphite furnace HGA 76 and background corrector, following Fernandez's method (1975). The precision of the method shows a coefficient of variation (V.C.) of ±5%; accuracy is around 96–98%.

*FEP:* Assays were carried out with Piomelli's FEP test (1973), using Coproporphyrin I (Sigma Chemical Co., St. Louis, U.S.A.) as standard. For this method, V.C. is ±5% and accuracy 93–96%. The values obtained, corrected for the haematocrite, are expressed in µg/100 ml of red blood cells. The assays was carried out using a Perkin-Elmer mod. 204 spectrophotofluorimeter.

*ALA U:* This metabolite was determined using a commercial kit (Sibar, Perugia, Italy). The values obtained were corrected for a standard specific weight of 1024 and expressed in mg/l. The spectrophotometric readings was carried out using a Perkin-Elmer mod. 550 spectrophotometer. The method showed a V.C. of ±10% and an accuracy of 92–99%.

*ALA D:* The activity of this enzyme was determined using the method of Bonsignore et al. (1965), using as a substrate ALA HCl 0.01 mol/l (Sigma Chemical Co., St. Louis, U.S.A.) at a pH of 7.0. It is of the greatest importance that pH be 7.0 when GSH is added. At lower pH the activating action of GSH disappears until it becomes inhibitory (at pH 5.8). This event is related to the

shifting of the re-dox potential towards the reduced form of glutathione which thus leaves its optimal range of activity (Trevisan, unpublished data). 80 U/ml was considered the lower normal limit (Bonsignore et al. 1965). These are also the normal limit values found in our laboratory with this method.

A) A second sample was heated at 60°C for 5 min according to the method of Gerhart and Pardee (1962) in order to denature the allosteric sites, in a thermostatic bath; the haemolized blood was cooled in ice.

B) GSH (Merck, Darmstadt, Western Germany) was added to a third sample at the optimal concentration of $1\times10^{-2}$ mol/l (final concentration) and incubated at room temperature for 10 min.

C) Lastly, a fourth sample received zinc in a final concentration of $1.5\times10^{-4}$ mol/l and was incubated at room temperature for 10 min.

Following the indications of Chiba (1976), we calculated the ratios between the optical density (O.D.) of ALA D activity and the same activity after treatment with heat, with GSH and with zinc.

The spectrophotometer assay using a Perkind-Elmer mod. 550 shows a V.C. of ±5%.

Lastly, we calcualted the correlation *r* coefficient and the regression function between the logarithm of ALA D and that of treated ALA D with PbB and other indexes of biological effect (FEP and ALA U).

Student's *t*-test was applied for a comparison among the means.

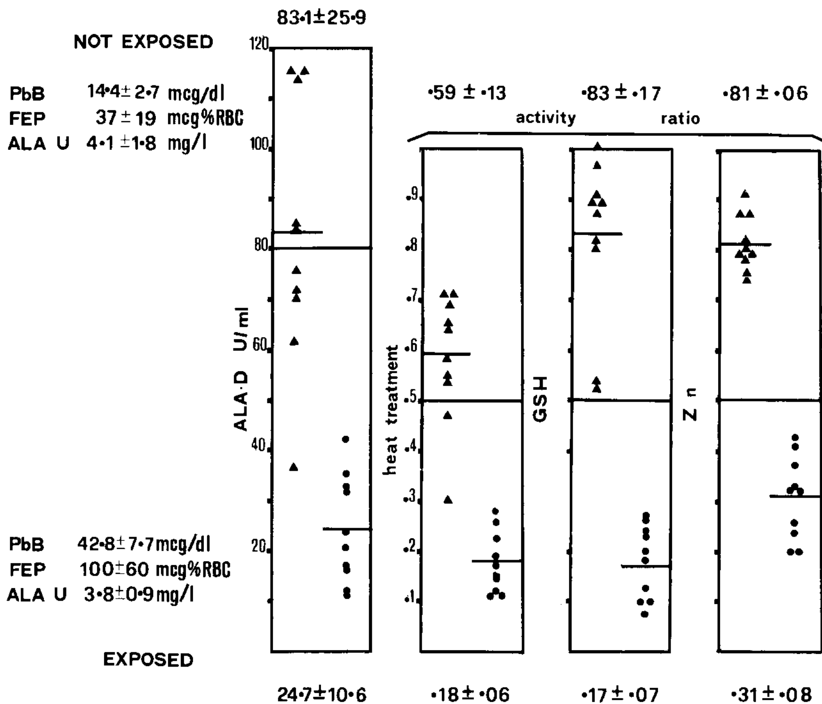


**Fig. 1.** Histogram of the distribution of the values of ALA D and ALA D activity ratio after treatment. The difference between the mean values of exposed (●) and non-exposed (▲) is significant for $P<0.001$. The line that divides the histogram into two parts shows the lower normal limit values. The half line shows the mean of the values for exposed *(right)* and non-exposed *(left)*

**Results**

Figure 1 shows graphically the values of ALA D activity found in exposed and non-exposed subjects, and the ratios between the O.D. of ALA D and the same density of ALA D after treatment with heat, GSH and zinc. It should be noted that five non-exposed subjects had values of enzyme activity below normal (80 U/ml of red blood cells): they may be considered false positives. A further study of the ratios showed that only two of these five showed a ratio lower than 0.5 (the limit indicated by Chiba 1976) in samples treated with heat, whereas none of the samples treated with GSH and zinc fell under that value among normal subjects. This limit separated the two populations in vitro treated with GSH and zinc.

None of the exposed subjects showed ALA D activity higher than 50 U/ml of red blood cells and a ratio higher than 0.5.

**Table 1.** Regression functions between logarithm of ALA D and ALA D activity ratio, and PbB

| | Regression function | $r$ |
|---|---|---|
| ALA D | log ALA D = 2.16 − 0.019 PbB | −0.8999 |
| Activity ratio after heating | log activity ratio = 2.0044 − 0.018 PbB | −0.9470 |
| Activity ratio after GSH | log activity ratio = 2.2185 − 0.023 PbB | −0.9304 |
| Activity ratio after zinc | log activity ratio = 2.0997 − 0.014 PbB | −0.9350 |

**Table 2.** Regression functions between logarithm of ALA D and ALA D activity ratio, and FEP

| | Regression function | $r$ |
|---|---|---|
| ALA D | log ALA D = 1.97 − 0.005 FEP | −0.8270 |
| Activity ratio after heating | log activity ratio = 1.793 − 0.0044 FEP | −0.7900 |
| Activity ratio after GSH | log activity ratio = 1.924 − 0.0054 FEP | −0.7460 |
| Activity ratio after zinc | log activity ratio = 1.937 − 0.0036 FEP | −0.8084 |

**Table 3.** ALA D activity in non-exposed and exposed subjects before and after treatment with heat (60°C for 5 min), GSH ($1 \times 10^{-2}$ mol/l) and zinc ($1.5 \times 10^{-4}$ mol/l)

| | ALA D (U/ml) | ALA D after | | |
|---|---|---|---|---|
| | | Heating (U/ml) | GSH (U/ml) | Zinc (U/ml) |
| Not exposed | 83.1 ± 25.9 | 144.8 ± 48.3 | 107.1 ± 49.4 | 103.1 ± 34.6 |
| Exposed | 24.7 ± 10.6* | 134.1 ± 24.6 | 145.8 ± 29.9** | 81.0 ± 22.0 |

* Significant difference between exposed and non-exposed for $P < 0.001$, $t = 7.4752$
** Significant difference between exposed and non-exposed for $P < 0.05$, $t = 2.2800$

Highly significant was the difference between exposed and non-exposed subjects both on ALA D activity ($P < 0.001$), FEP ($P < 0.005$), and PbB ($P < 0.001$). No difference was found for ALA U between exposed and non-exposed subjects.

Table 1 shows the regression function between ALA D activity and the ratios between O.D. of such activity after the various treatments on one hand, and PbB on the other. Table 2 shows the regression function between ALA D and ALA D ratios, and FEP.

Table 3 shows the average values ± standard deviation of the activity of the enzyme in basic situations, after heat treatment (60° C for 5 min), and after addition of GSH ($1 \times 10^{-2}$ mol/l) and of zinc ($1.5 \times 10^{-4}$ mol/l).

## Discussion

Our results indicate that the three ratios: ALA D/ALA D after treatment are correlated with PbB levels more closely than ALA D alone, according to Chiba (1976) for heat treatment and to Lauwerys et al. (1978) for thiol group donors. Among the three procedures, reactivation by zinc appears technically simpler, and gives the smallest dispersion of the results around the means in the two groups studied (Fig. 1).

The correlation with FEP seems on the contrary to be practically identical both for ALA D activity and for the ratios ALA D/ALA D after treatment. This is presumably related to the greater variability of protoporphyrin IX, and to its lesser sensitivity as an index of biological effect to lead exposure with respect to ALA D.

Lastly, no correlation was found with ALA U. This is ascribable to the low exposure of the workers, since it is known that ALA U is modified only after inhibition of ALA D activity at the level or more of 70% (Haeger-Aronsen et al. 1971).

The comparison of the effects on ALA D of different reactivating treatments (Table 3) offers elements for interesting speculation. After heat treatment, ALA D activity reaches, in accordance with previous research (Bonsignore et al. 1968), a maximum value that is the same in normal and in lead-exposed subjects; an identical complete reactivation of enzyme activity in lead exposed subjects is obtained after in vitro treatment with GSH, but only a partial restoration is obtained by in vitro treatment with zinc. These results can be explained with the hypothesis that lead, in chronic exposure, is only bound to the thiol groups from which, in optimal conditions, GSH is able to displace it, restoring complete activity to the enzyme; on the contrary, the restoration by zinc must be ascribed to a positive allosteric effect, without deplacement of lead from the metallic site; at that site it binds only in case of acute or in vitro exposure, as we have recently demonstrated (Trevisan et al. 1980).

The effect of GSH treatment in normal subjects, causes only a partial restoration of the enzyme whose activity remains significantly lower than that obtained after treatment in lead exposed subjects; this is difficult to explain. We can make the hypothesis that this correlates with the existence of two forms of the allosteric enzymes (Monod et al. 1965) with different attack of metals on enzyme sites.

The results obtained are only indicative. Experimental research currently under way may give more decisive results regarding the different action on the enzyme by lead in acute, chronic, and past exposure.

We can conclude that the ratios between ALAD activity before and after treatment with heating, GSH and zinc allow identification of the false positives, i.e. those subjects who have low enzyme activity and normal PbB levels in according to Mauras and Allain (1979). We also observed that the use of the ratio between O.D. of enzyme activity and that of ALA D activity treated with zinc is particularly useful because it shows a smaller dispersion of the results around the mean.

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