Mechanisms of ALA-D inhibition by lead and of its restoration by zinc and dithiothreitol

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ABSTRACT To induce the inhibition of ALA-D (delta-aminolaevulinic acid dehydratase) activity by lead in vitro, it is necessary to preincubate the enzyme fraction with lead ions and the Hb fraction (factors) together. The combination of two of the three (ALA-D fraction, lead acetate, and Hb fraction) in the preincubation has only a small effect on the activity. Lead preincubated with ALA-D and Hb fractions does not alter the affinity of the enzyme for the substrate, suggesting that the substrate can bind to the enzyme molecules (non-competitive inhibition). The restoration of activity by zinc with dithiothreitol is associated with the removal of lead from ALA-D fraction proteins to which it has bound in vivo and in vitro. The mode of the inhibitory action of tin on ALA-D is similar to that of lead because the inhibition is intensified by the addition of Hb fraction and is restored by heating. The inhibition concentration is, however, higher than that of lead. Of the three methods recovering decreased activity, heating is the most specific to detect inhibition by lead.

Erythrocyte ALA-D (δ -aminolaevulinic acid dehydratase) activity is widely used for the evaluation of lead exposure or for the rough estimation of lead in blood that is currently regarded as the most sensitive index of lead exposure. Sakai *et al*¹ have shown that the decrease in ALA-D activity is an indicator of the extent of lead saturation of ALA-D fraction proteins which have the highest affinity for lead among erythrocyte constituents. Nevertheless, the mechanisms of ALA-D inhibition by lead in the fraction have not been elucidated, although some erythrocyte factors are known to be involved in the inhibition.²

Depressed ALA-D activity in lead-exposed subjects is restored by heating the enzyme solution^{3,4} or by adding SH-compounds^{5,6} or zinc ions^{7,8} to the reaction mixture. Many workers^{5,9-16} have tried to evaluate lead exposure, using these agents or procedures recovering the activity. The extent of restoration in activity (activity ratio or VHE) is well correlated with blood lead concentrations, and the correlation is better than that between ALA-D activity itself and blood lead concentrations. This is probably because the normal range of activity ratio is much narrower than that of ALA-D activity itself,

and that the ratio is independent of some disorders that affect the activity.¹⁰ Thus the activity ratio has recently received considerable attention as an index of lead exposure. There has been, however, no standardised method of restoring the decreased activity to evaluate lead exposure nor have the mechanisms of the restoration been clarified.

Heavy metals other than lead can also affect ALA-D activity in vivo. Erythrocyte ALA-D activity in man exposed to either cadmium¹⁷ or methylmercury¹⁸ is reported to decrease as in exposure to lead. Chiba and Kikuchi¹⁹ reported that the activity is decreased in an animal experimentally exposed to tin. The activity is well known to be inhibited by many metals in vitro.^{14,20-22} By contrast, zinc ions injected subcutaneously into an animal temporarily reverses the lead-inhibition of ALA-D activity in vivo.^{23,24} Zinc exposure also affects the activity in man to a small extent.²⁵ As yet, however, there has been no evidence to show whether the lead effect on ALA-D activity differs from that of other inhibitory metals as regards the restoration of activity by the above-mentioned agents. If it were so, the mechanisms of the inhibition by lead should differ from those of other metals, and the restoration index (activity ratio) would be more specific and helpful than the activity determination itself in the evaluation of lead exposure.

The purpose of the present study was, therefore,

to disclose the mechanisms of ALA-D inhibition by lead and of its restoration by some agents, and to clarify the difference between the inhibition of ALA-D activity by lead and that of other heavy metals.

Materials and methods

Heparinised venous blood was obtained from workers occupationally exposed to lead and from normal subjects with no history of exposure to lead.

Preparation of the Hb and ALA-D fractions from normal blood was carried out by the method described previously.² Erythrocyte supernatant was gel-filtrated by passing through a Sephadex G-200 column (2.6 × 90 cm). Peak fractions with ALA-D activity and with absorption by Hb were pooled separately and used for the experiments. ALA-D activity was determined by the method of Sakai *et al.*² The methods of inducing ALA-D inhibition by metal ions and of restoring the inhibition were also carried out as described previously.²

In the experiment in which the effect of zinc and dithiothreitol (DTT) was examined on the distribution of lead and zinc in erythrocytes, 10 ml of erythrocyte lysate that had been adjusted to the original haematocrit value with 40 mM Tris-HCl buffer pH 7.0 were added to 1 ml of the solution containing 5 mM zinc acetate and 250 mM DTT. The lysate was incubated at 4°C for 40 hours and then centrifuged at 24 000 g for 30 minutes. Ten millilitres of the supernatant were passed through a Bio Gel A 5M column (2.6 × 90 cm) that had been equilibrated with 40 mM Tris-HCl buffer pH 7.0. Each fraction was collected in 13-ml aliquots and subjected to determinations of protein and lead concentrations and of ALA-D activity by the method previously reported.¹ Zinc concentrations were determined in the same MIBK (methyl-isobutylketone) extract as used to determine lead concentrations, using a flame atomic absorption spectrometer.

With or without zinc and DTT treatment, the erythrocyte supernatant from a lead worker was also gel-filtrated through a Sephadex G-75 column (1.6 × 90 cm) that had been equilibrated with 40 mM Tris-HCl buffer pH 7.0. ALA-D activity and protein concentrations in the fractions were determined in the manner described above, and lead concentrations in the fractions were directly determined by the method previously reported, using a flameless atomic absorption spectrometer.¹

Results

We have reported that to induce ALA-D inhibition by lead in vitro it is necessary for the enzyme solu-

Fig 1 Effect of preincubation of enzyme solution (E) or factors (F) with lead acetate (Pb, 50 nM) on inhibition of ALA-D activity. After first preincubation (4°C, 20 hours) of Pb + F (●) or of E + Pb (○), the third factor (E and F, respectively) was added and further preincubated at 4°C for described time (second preincubation). Control (Δ) was subjected only to second preincubation in combination of E, Pb, and F together. ◇: E + F (without Pb).

tion to be preincubated with lead acetate and the erythrocyte factors in the Hb fraction.² Figure 1 shows the effect of the preincubation of the enzyme solution (E, ALA-D fraction) or of the Hb fraction (F) with lead acetate (Pb) on the inhibition of ALA-D. After the first preincubation of lead with either E or F for 20 hours at 4°C, the third factor was added and the mixture further preincubated at 4°C for 0 to 23 hours (second preincubation). ALA-D activity was assayed in the final combination. None of the combinations first preincubated (4°C, 20 hours) induced the pronounced inhibition of ALA-D activity observed when the third factor was introduced. With increasing time of second preincubation, however, the extent of ALA-D inhibition is intensified significantly. At 23 hours of the second preincubation, the extent of ALA-D inhibition first preincubated with lead is almost identical to that observed when the Hb fraction is first preincubated with lead. These observations indicate that the second preincubation, where the three components are incubated together, is essential to induce the inhibition. Because a long preincubation time is necessary for the enzyme to be inhibited by lead in vitro, it seems that a ternary complex of E, Pb, and F is formed during the preincubation.

Table 1 indicates the extent of lead inhibition of ALA-D in the presence or absence of the Hb fraction. The Vmax value and the Km for substrate are determined from double reciprocal plots for the enzyme from Sephadex G-200 gel filtration of normal erythrocyte supernatant. In the absence of the Hb fraction, 50 nM lead acetate does not inhibit the activity of ALA-D, while 1 μM lead inhibits 30% of

Table 1 ALA-D inhibition by lead in the presence or absence of Hb fraction

	Added lead							
	None		50 nM		100 nM		1 μ M	
	Vmax	Km	Vmax	Km	Vmax	Km	Vmax	Km
Enzyme alone	5.71	0.057	5.70	0.057	5.56	0.057	4.00	0.057
Enzyme plus Hb fraction	3.64	0.154	1.27	0.154				
Heat	5.75	0.154	5.59	0.154				
Zinc	5.75	0.154	5.59	0.154				
DTT	10.87	0.154	9.09	0.154				

Vmax (μ moles/min/1), Km (mM), heat (60°C, 5 min), zinc (100 μ M), and DTT (10 mM).

Fig 2 Gel filtration chromatography of erythrocyte zinc and lead. (a): normal subject, (b) and (c): lead-exposed subjects. Before application on to column, erythrocyte lysate from a lead-exposed subject (c) was incubated with zinc and DTT to recover ALA-D activity as described in materials and methods. Blood lead concentrations and ALA-D activity of the three subjects are indicated in table 2.

the activity. The inhibition is non-competitive in type. Both Vmax and Km are altered by the addition of the Hb fraction to the mixture. The percentage of inhibition of the Hb fraction alone is 36%, and the type of inhibition is complex. In the presence of the Hb fraction, 50 nM lead acetate inhibits the activity significantly (65.1%). Thus the presence of the Hb fraction intensifies the inhibition of ALA-D by lead. The type of inhibition is non-competitive, indicating that the substrate can bind to the enzyme molecules. Heat treatment (60°C, 5 min), zinc (100 μ M), and DTT (10 mM) restore the non-competitive inhibition.

Figure 2 shows the chromatographic pattern of gel filtration of erythrocyte lead and zinc. There is little difference in the distribution pattern of erythrocyte zinc between normal and lead-exposed subjects,

while lead peaks in the latter are higher than those in the former.¹ In the erythrocyte lysate incubated with zinc and DTT before the application on a column (fig 2(c)), a large quantity of lead appears in the peak with low molecular weight—that is, not found in non-treated erythrocytes (fig 2(b))—whereas the amount of lead in the other peaks decreases. Most of zinc added to lysate with DTT is found in the Hb fractions after gel filtration. Normal erythrocyte lysate that had been incubated with lead acetate to induce the inhibition of ALA-D was also chromatographed with or without the addition of zinc and DTT (fig 3). When zinc and DTT are present, lead bound to the proteins with high molecular weight, including ALA-D, is removed and appears in the low molecular weight fractions. The removal of lead from ALA-D fraction proteins to which it

Fig 3 Gel filtration chromatography of erythrocyte lead loaded *in vitro*, with or without treatment with zinc and DTT. Erythrocyte lysate from a normal subject (blood lead concentration 10 µg/100 g) was incubated with lead acetate (60 µg/100 ml) to induce ALA-D inhibition by method previously reported.¹ Half the lysate lead-loaded was treated with zinc and DTT as described in materials and methods (-----), to the other half distilled water was added instead of zinc and DTT (—————).

Table 2 ALA-D activity and blood lead concentrations of subjects examined in fig 2

Subjects	Blood lead concentrations (µg/100 g)	ALA-D activity	
		Unit*	A ₅₅₅
(a)	10	49.9	
(b)	53	15.3	
(c)	57	15.4	
(c') without			0.022
with			0.218

*µmoles delta-aminolaevulinic acid/min/ml RBC. (a), (b), and (c) are the subjects examined in fig 2. (c') was erythrocyte supernatant from the subject (c), and ALA-D activity was determined with or without treatment of zinc and DTT.

Table 3 ALA-D activity of erythrocyte supernatant used for gel filtrations in fig 3

	ALA-D activity (A ₅₅₅)
No addition of lead	0.210
Plus lead	0.015
Plus lead plus zinc and DTT	0.145

has been bound *in vivo* (fig 2) and *in vitro* (fig 3) accompanies the restoration of ALA-D inhibition (tables 2 and 3).

A Sephadex G-75 column was used to make a rough estimation of the molecular weight of the lead peak that appeared after the addition of zinc and DTT (fig 4). Lead removed from the proteins is eluted together with DTT and is found in an elution volume encompassed by a V_e/V_0 of 3.0. The lead eluted together with DTT seems not to be protein bound.

From these experiments we have been able to show the nature of ALA-D inhibition by lead at low

concentrations and its restoration by heating, zinc, or DTT or both. We have also examined whether the nature of ALA-D inhibition is specific to lead. In the presence or absence of the Hb fraction, the ALA-D fraction was preincubated at 4°C for 20 hours with the chlorides of various heavy metals (table 4). In the absence of the Hb fraction, Cu^{++} , Hg^{++} , methylmercury, and Cd^{++} powerfully inhibit the activity of ALA-D whereas the presence of the Hb fraction decreases the extent of inhibition by these metals. The inhibition is not restored by heat treatment and this differs from the inhibition produced by lead. The inhibition by Sn^{++} is similar to that of lead because it increases in the presence of

Fig 4 Form of lead removed from high molecular weight proteins by zinc and DTT. Erythrocyte lysate from a lead worker was gel filtrated by Sephadex G-75 column with (b) or without (a) the treatment of zinc and DTT. Concentrations of lead in blood, zinc protoporphyrin in RBC, and δ -aminolaevulinic acid and coproporphyrin in urine of the subject were 48 µg/100 g, 233 µg/100 ml, 11.8 mg/100 ml, and 149 µg/l respectively. Proteins with known-molecular weight, DTT, and amino-acid were also gel filtrated by the same column: I ovalbumin (45 000), II chymotrypsinogen (25 000), III cytochrome C (12 500), IV DTT, and V tryptophane.

Table 4 ALA-D inhibition by various metals and its restoration by heat, DTT, or zinc

Metal ions	ALA-D activity (A_{555})				
	Enzyme alone	Enzyme plus Hb fraction			
		No further treatment	Heat	DTT	Zinc
Control	0.315	0.249	0.281	0.333	0.257
Cu ⁺⁺ (10^{-7} M)	0.087	0.255	0.148	0.332	0.252
(10^{-6} M)	0.012	0.194	0.092	0.310	0.226
Pb ⁺⁺ (10^{-7} M)	0.300	0.050	0.180	0.260	0.253
Sn ⁺⁺ (10^{-7} M)	0.303	0.256	0.283	0.322	0.260
(10^{-6} M)	0.320	0.165	0.275	0.332	0.260
Hg ⁺⁺ (5×10^{-5} M)	0.010	0.020	0.012	0.344	0.035
Methylmercury (10^{-5} M)	0.010	0.180	0.233	0.348	0.270
Cd ⁺⁺ (10^{-6} M)	0.095	0.140	0.035	0.273	0.107

Heat (60°C, 5 min), DTT (10 mM), and zinc (100 μ M).

Hb fraction and is partly restored by heating. The inhibition concentration of tin, however, is over 10-fold higher than that of lead. The inhibition by methylmercury is partly restored by zinc, but the Hb fraction does not intensify the inhibition. The addition of DTT to the reaction mixture non-specifically restores the inhibition by all the metals examined.

Discussion

The results presented here support and confirm our working hypothesis on the mechanisms of the ALA-D inhibition by lead at low concentrations.² The extent of ALA-D inhibition with the Hb fraction (factors) first-preincubated with lead was almost identical to that of the inhibition of ALA-D first-preincubated with lead (fig 1). This observation is consistent with that previously reported.¹ Lead added to the erythrocyte lysate from normal subjects is found mainly in the Hb fraction and ALA-D activity is not inhibited. When the lysate is further incubated the lead is transferred to the ALA-D fraction proteins and the activity is significantly inhibited. In the isolated ALA-D fraction where lead is bound to proteins, however, ALA-D inhibition is not found. If the fraction is incubated with the Hb fraction (factors) for 20–40 hours, there is a pronounced inhibition of the activity of ALA-D. Based on the present data, and the previous findings,¹ ternary complex of ALA-D (E), lead (Pb) and factors (F) is probably formed during the preincubation. The complex might be the non-active form of the enzyme. There was no change in the K_m values of the enzyme incubated with the Hb fraction in the presence or absence of lead acetate (table 1), indicating that lead incorporated into the complex does not affect the affinity of ALA-D for substrate. Thus the ternary complex can bind the substrate, but cannot dissociate the product (prophobilinogen).

Whether lead in the complex interferes with the process of the condensation of two moles of δ -aminolevulinic acid remains to be investigated. The restoration of inhibited activity by gel filtration³ is probably due to the dissociation of the complex.

In another paper² we have discussed the mechanisms of restoring ALA-D inhibition by heating, and the differences between the action of zinc or DTT, or both, and of heating. The present data from the restoration experiments (figs 2–4) indicate that the recovery of activity correlates well with the removal of lead from the ALA-D fraction proteins. The removal of lead would result in the dissociation of the ternary complex, restoring the activity. From the present findings alone it is not clear whether the zinc, added with DTT, is incorporated into the high affinity sites on the ALA-D molecules instead of lead.² Zinc, however, is required for the enzyme to become active, and 4-g atoms of zinc are bound to the octameric enzyme to produce maximum activity when the essential sulphhydryl groups are in the reduced state.²⁵

The lead removed from the high molecular weight proteins was eluted with DTT and seemed not to bind proteins (fig 4). In the classification of erythrocyte lead by Raghavan *et al*²⁶ the removed lead is free because the elution volume by Sephadex G-75 extends to a V_e/V_o of 3.0. The lead is either in an inorganic form or bound to amino-acids or small peptides; this point requires further investigation. Raghavan *et al*²⁶ also reported the induction of a 10 000 molecular weight protein in lead-exposed workers that has the function of detoxifying erythrocyte lead. As shown in fig 4, we failed to detect this protein-bound lead. This failure might be due to the appearance of toxicity in the subject who showed moderately high levels of blood zinc protoporphyrin, urinary δ -aminolaevulinic acid, and coproporphyrin.

The inhibitory action of tin on ALA-D activity was similar to that by lead and the inhibition by tin is restored by heat; the inhibitory concentrations of tin were 10-fold higher than those of lead, however (table 4). The blood tin concentration (6 μ moles in total blood) is of the same order as the blood lead concentration (7 μ moles in total blood) in man,²⁷ but no relationship between blood tin concentrations and ALA-D activity has so far been shown.

From our results we conclude that the recovery of the activity of ALA-D by heat is the most specific measure by which to detect exposure to lead.

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