

Relationships between distribution of lead in erythrocytes in vivo and in vitro and inhibition of ALA-D

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ABSTRACT Proteins in the ALA-D (δ -aminolaevulinic acid dehydratase) fraction from gel filtration of erythrocyte supernatant (ES) have the highest affinity for lead among erythrocyte constituents in vivo and in vitro. It takes 20-40 hours for erythrocyte components to be equilibrated with lead added in vitro. At low lead concentrations, under $60 \mu\text{g}/100 \text{ ml}$ ES, the extent of ALA-D inhibition correlates with that of lead concentrations in the ALA-D fraction, and the decrease in ALA-D activity indicates the extent of lead saturation of ALA-D fraction proteins. The saturation is attained at $80-110 \mu\text{g}/100 \text{ ml}$ ES. Although an appreciable amount of lead is also found in the haemoglobin fraction that contains certain factors concerned in ALA-D inhibition, lead responsible for inducing the inhibition is not bound to haemoglobin fraction proteins but to ALA-D fraction proteins. Of three treatments or agents recovering the enzyme from lead effects, zinc is the only one that can fully restore the inhibition.

Blood lead concentrations are currently regarded as the most reliable index of exposure to lead. Over 90% of the blood lead is bound to the erythrocytes and seems to be in dynamic equilibrium with plasma lead.^{1,2} Plasma lead is more diffusible than erythrocyte lead and more important for evaluating the toxic effect of lead because the diffusible form exerts an influence on lead concentrations of other compartments and produces critical effect in the various organs.¹⁻³ Erythrocytes rather behave as a repository for lead,⁴ which might produce some disturbances in erythrocyte metabolism such as Na, K-ATPase, ALA-D (δ -aminolaevulinic acid dehydratase) activity.^{5,6}

Recently, Ong and Lee⁶ have reported that more than 85% of the ²⁰³Pb incorporated into erythrocytes in vitro is associated with the cytoplasmic components and the rest is bound to the stromal membrane. They suggested that the membrane-bound lead might affect the activity of Na, K-ATPase. Several workers⁷⁻⁹ have indicated that most of the labelled lead incorporated into erythrocytes in vitro is found in haemoglobin fractions. By contrast, Sakai *et al*¹⁰ reported that half the lead in erythrocyte haemolysate from a lead-exposed worker was found in protein fractions with a high

molecular weight, including ALA-D. There is little information relating to lead-binding components in erythrocytes from a lead-exposed subject so far.

Erythrocyte ALA-D activity is the most sensitive indicator for blood lead concentrations, because the activity is specifically inhibited by lead at concentrations between 20 and $100 \mu\text{g}/100 \text{ g}$ blood.¹¹⁻¹⁴ The highly purified enzyme from human erythrocytes is a homo-octamer with a molecular weight of 252 000 and is inhibited by lead at 1 and $5 \mu\text{M}$ ¹⁵; the crude enzyme is also inhibited at a concentration exceeding $1 \mu\text{M}$.¹⁶ These concentrations of lead in the reaction mixture are tenfold to fiftyfold higher than those in the mixture containing haemolysate from a lead-exposed worker. Sakai *et al*¹⁰ suggested that certain factors detected in the haemoglobin fraction account for this discrepancy between in-vivo and in-vitro activity. As mentioned above, lead is found in the ALA-D fraction and also in the haemoglobin fraction that contains the factors. There are three possibilities in relation to lead inducing ALA-D inhibition; it may be due to lead in ALA-D fraction, lead in the haemoglobin fraction, or both.

To clarify further the nature and the mechanisms of ALA-D inhibition by lead we have investigated the relationships between ALA-D inhibition and the distribution of lead among erythrocyte components in vivo and in vitro.

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Materials and methods

LEAD BINDING IN VIVO

Ten millilitres of heparinised venous blood were obtained from workers occupationally exposed to lead and from normal subjects with no history of exposure to lead.

All procedures for fractionating erythrocyte constituents were carried out at 4°C as previously described.¹⁰ Packed erythrocytes from 10 ml of whole blood were washed three times in cooled saline (0.9% NaCl) and adjusted to the original haematocrit value with 25 mM phosphate buffer pH 7.0. Erythrocytes were completely lysed by sonicating. The lysate was centrifuged at 24 000 g for 30 minutes. The erythrocyte supernatant (ES) was applied to a Sephadex G-200 column (2.6 × 90 cm) that had been equilibrated with 25 mM phosphate buffer pH 7.0 and eluted with the same buffer. Each fraction was collected in 11 ml (fig 1(a), (b)) or 13 ml (fig 1(c), (d)) aliquots; protein and lead concentrations and ALA-D activity were determined on each aliquot.

Protein concentrations were determined by the absorbance at 280 nm; ALA-D activity was deter-

mined by the method of Ushio *et al*¹⁷ except that the assay mixture contained 0.5 ml of 0.2 M phosphate buffer pH 7.0, 0.5 ml of 20 mM ALA (δ -aminolaevulinic acid) solution, 0.5 ml of 50 mM DTT (dithiothreitol), 0.1 ml of the fraction, and 0.9 ml of distilled water; lead concentrations were also determined by the method by Ushio *et al*,¹⁷ using flame atomic absorption spectrophotometer.

LEAD-BINDING TO ERYTHROCYTE LYSATE

Normal erythrocyte lysate was prepared in the way described above, except that 40 mM Tris-HCl buffer pH 7.0 was used to adjust the volume back to the original haematocrit value. Twenty millilitres of the lysate were added to 2 ml of 25 μ M lead acetate and incubated at 37°C. Half the lysate was taken out 5 minutes after the addition of lead, and the rest was incubated for a further 55 minutes and then at 4°C for 40 hours. At the end of each incubation the lysate was centrifuged at 24 000 g for 30 minutes. ES thus obtained was applied to a Bio-gel A 1.5 m column (2.6 × 90 cm) that had been equilibrated with 40 mM Tris-HCl buffer pH 7.0 and eluted with the same buffer. ALA-D activity and concentrations of protein and lead in the fractions were determined

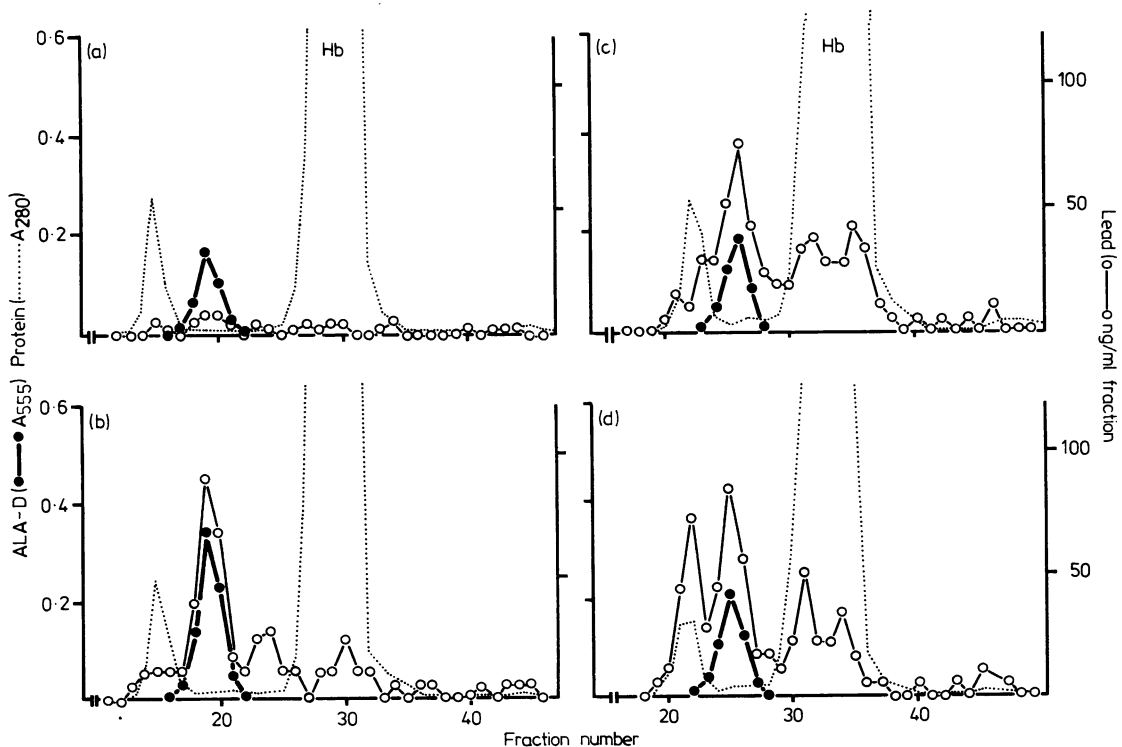


Fig 1 Distribution of *in-vivo* burdened lead among erythrocyte components. Blood lead concentrations of subjects (a)–(d) were 10.2, 48.6, 69.6, and 76.3 μ g/100 g respectively.

by the methods described above. ALA-D activity of the two types of ES sample was also determined with or without heating.¹⁸

LEAD-BINDING TO WHOLE BLOOD

Heparinised blood was obtained from normal donors with no history of exposure to lead. Four millilitres of blood were added to 1 ml of lead acetate solutions (0–7 $\mu\text{g/ml}$ of 0.9% NaCl) and incubated at 37°C for 60 minutes and then at 4°C for 20 hours. All procedures for the preparation of each fraction were carried out at 4°C. After centrifugation of lead-loaded blood, the packed erythrocytes were washed five times with cooled saline (0.9% NaCl); the original haematocrit value was restored with 40 mM Tris-HCl buffer pH 7.0. Erythrocytes were completely lysed by sonicating, and ES was obtained by centrifugation in the manner described above. Altogether 2.5 ml of ES was fractionated by passing through a Bio-gel A 1.5 m column (1.6 \times 90 cm), which had been equilibrated with 40 mM Tris-HCl buffer pH 7.0 and eluted with the same buffer. Each fraction was collected in 4.7 ml aliquots on which ALA-D activity and protein concentrations were determined. After the addition of 1.5 N HClO₄ to each fraction (final 0.15 N), the lead concentrations were directly determined using flameless atomic absorption spectrophotometer. Measurement of ALA-D activity of ES and its restoration by heat, zinc, or dithiothreitol were carried out by the method previously described.¹⁸

Results

Figure 1 shows the Sephadex G-200 gel filtration chromatography of ES from a normal subject and

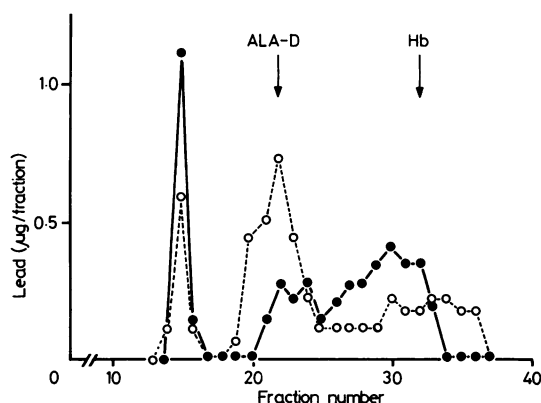


Fig 2 Distribution of lead loaded to erythrocyte lysate in vitro. Erythrocyte lysate was incubated with lead acetate (50 $\mu\text{g}/100\text{ ml}$) for 5 minutes (—●—) or 40 hours (---○---) as described in materials and methods.

from the lead workers. In fig 1(a) which shows the chromatography of ES from a normal subject there is no significant lead peak. By contrast, in figs 1(b)–(d) there are three or four lead peaks, the largest of which corresponds with that of ALA-D activity. About half the lead in the ES from a worker (b) is found in a peak that exactly corresponds to the ALA-D activity, suggesting that proteins in ALA-D fraction have the highest affinity for lead. With the rise in blood lead concentrations (figs 1(c) and (d)), no more increase of lead can be seen in the ALA-D fraction proteins, while the lead in other fractions is increasing. These observations suggest that lead-binding to ALA-D fraction proteins attains a plateau at high concentrations of blood lead (over 70 $\mu\text{g}/100\text{ g}$).

Figure 2 shows the gel filtration chromatography of lead bound to erythrocyte lysate. After 5 minutes' incubation of the lysate with lead acetate, lead found in ALA-D and the haemoglobin fractions are 21% and 41% respectively. After 40 hours' incubation, 43% and 27% of ES lead are found in the ALA-D and haemoglobin fractions respectively. After 40 hours' incubation, lead in the ALA-D fraction increased and that in the haemoglobin fraction decreased. The distribution pattern of lead after 40 hours' incubation was similar to that in fig 1, indicating that it takes a long time for erythrocyte constituents to attain an equilibrium with added lead. Table 1 shows the ALA-D activity of ES; the lead concentrations of the different fractions are shown in fig 2. An increase in the amount of lead in the ALA-D fraction after 40 hours' incubation is associated with an increased inhibition of ALA-D activity. Based on these findings it appears that the lead responsible for inducing the inhibition of ALA-D is not bound to haemoglobin fraction proteins but to the ALA-D fraction proteins.

To examine further the relationship between ALA-D inhibition by lead and lead-binding to ALA-D fraction proteins, we carried out in-vitro experiments, in which blood from a normal subject was loaded with varying amounts of lead. In these experiments we incubated blood with lead at 37°C for 60 minutes and then at 4°C for 20 hours, where ALA-D inhibition attained a plateau (data not shown). Table 2 indicates the relation between the amount of loaded lead and lead absorption by erythrocytes in vitro. The percentage of lead absorbed by the ES decreases with increasing amounts of loaded lead, suggesting that the rate of lead incorporation into erythrocytes is decreased at a high dose level in vitro.

Figure 3 shows the distribution of varying amounts of lead that are loaded to erythrocytes in vitro (table 2). As the amount of lead is increasingly

Table 1 ALA-D inhibition by lead acetate incubated for 5 minutes and 40 hours

| | ALA-D activity (A_{555}) | | | VHE (%) |
|------------|------------------------------|--------|-------|---------|
| | NH | (%) | H | |
| Control | 0.280 | (100) | 0.335 | 83.6 |
| Pb (5 min) | 0.208 | (74.3) | 0.201 | 104.0 |
| Pb (40 h) | 0.031 | (11.5) | 0.200 | 15.5 |

ES added distilled water instead of lead acetate was used as control. Pb (5 min) and Pb (40 h) were same as shown in fig 2. NH and H were non-heated and heated (60°C, 5 min) activity, respectively. VHE: value for heat effect (NH/H × 100).¹⁷

Table 2 Absorption of increasing amounts of lead acetate by erythrocytes *in vitro*

| | Loaded lead acetate ($\mu\text{g}/100\text{ ml}$) | Lead concentrations in ES ($\mu\text{g}/100\text{ ml}$) | Absorbed lead (%) | |
|-----|---|---|-------------------|-------|
| I | 0 | 9.1 | 0 | — |
| II | 21 | 32.4 | 23.3 | 110.9 |
| III | 42 | 55.1 | 46.0 | 109.5 |
| IV | 63 | 64.9 | 55.8 | 88.6 |
| V | 105 | 81.9 | 72.8 | 69.3 |
| VI | 140 | 111.2 | 102.1 | 72.9 |

loaded, four lead peaks appear in the gel filtration chromatography of ES—void volume, ALA-D, haemoglobin and low molecular weight fractions. These distribution patterns are similar to those seen in fig 1. At comparatively low concentrations of lead (under 60 $\mu\text{g}/100\text{ ml}$ ES), the largest lead peak is found in the ALA-D fraction. At high levels (greater than 80 $\mu\text{g}/100\text{ ml}$) the lead peak corresponding to ALA-D activity is not raised further, although the amounts of lead in the other fractions are still increasing. These results are summarised in fig 4. Apparently lead-binding to ALA-D fraction proteins reaches saturation level at lead concentrations exceeding 80 $\mu\text{g}/100\text{ ml}$ ES.

Figure 5 shows the ALA-D activity of ES (table 2, figs 3, 4). The activity declines in a linear fashion as the ES lead concentration increases up to 60 $\mu\text{g}/100\text{ ml}$ but no further decline is found at lead concentrations exceeding 60 $\mu\text{g}/100\text{ ml}$. This is 20 $\mu\text{g}/100\text{ ml}$ lower than those resulting in saturation of ALA-D fraction proteins with lead (figs 3, 4). These observations further confirm that most of the lead found in the ALA-D fraction is responsible for inducing ALA-D inhibition. The inhibited activity is

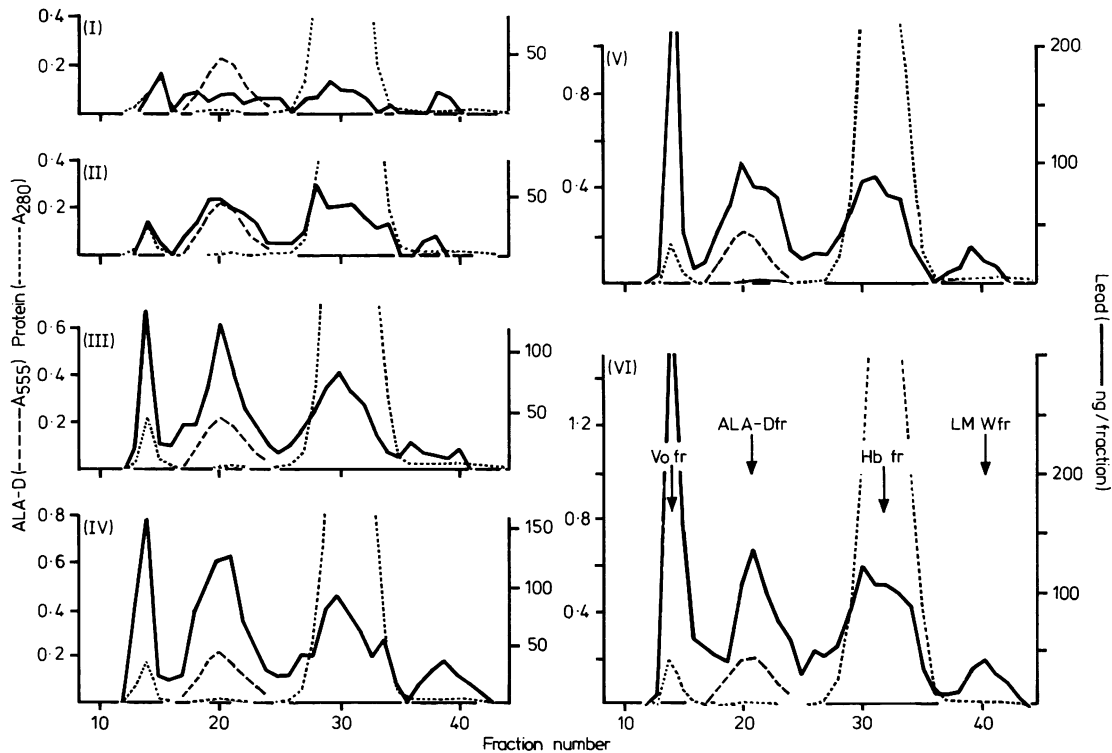


Fig 3 Distribution of increasing amounts of lead loaded to erythrocytes *in vitro*. Lead concentrations in ES of (I)–(VI) are indicated in table 2. Vo fr: void volume fraction, ALA-D fr: ALA-D fraction, Hb fr: haemoglobin fraction, LMW fr: low molecular weight fraction.

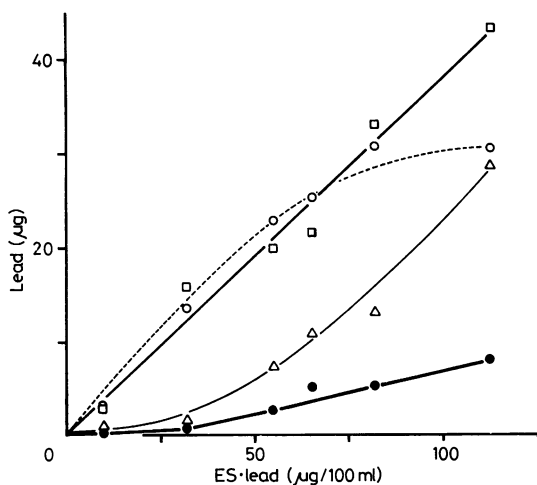


Fig 4 Relationship between ES lead concentration and lead in four fractions as shown in fig 3. Amounts of lead in four fractions were expressed as lead (μg) originated from 100 ml of ES. \circ : ALA-D fraction, \square : Hb fraction, \triangle : void volume fraction, \bullet : low molecular weight fraction.

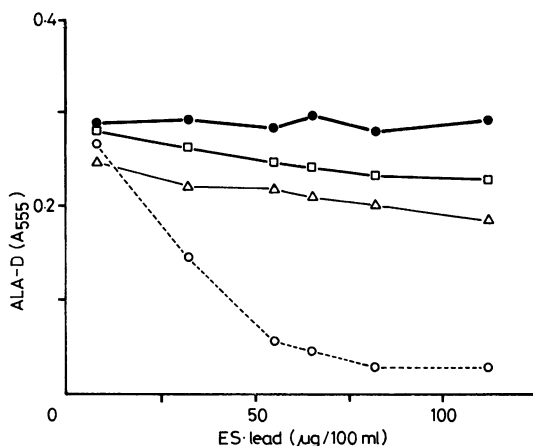


Fig 5 ALA-D inhibition by ES lead and its restoration by three treatments. \circ : no treatment, \bullet : zinc ($100 \mu\text{M}$), \square : dithiothreitol (10 mM), \triangle : heat (60°C , 5 min).

totally restored to the control level by zinc, while neither dithiothreitol nor heat is sufficient to restore the inhibition, especially at high concentrations of lead.

Discussion

This is the first report on the distribution of erythrocyte lead from lead workers in relation to ALA-D inhibition. We have shown that proteins in ALA-D

fraction have the highest affinity for lead among erythrocyte components *in vivo* and *in vitro*. The only investigation that has dealt with erythrocyte lead-binding proteins from lead-exposed workers is that by Raghavan *et al.*,⁵ who determined the lead content in four fractions of red blood cell haemolysate (haemoglobin lead, 10 000 molecular weight fraction lead, free lead, and residual lead). In their experiments, the haemoglobin fraction contained about half the erythrocyte lead. Several workers⁷⁻⁹ using radioactive lead have noted that haemoglobin has a high affinity for lead *in vitro*. Ong and Lee⁹ concluded that haemoglobin is the principal molecule interacting with lead in erythrocytes. In their experiments the duration of incubation of erythrocytes with lead was two hours. Their results are actually coincident with ours as seen in fig 2 (5-minute incubation of lysate with lead). We have, however, confirmed that erythrocyte components are not equilibrated with lead added *in vitro* within two hours of incubation at 37°C , because ALA-D inhibition by added lead does not attain the plateau level.¹⁹ After 40 hours' incubation of lysate with lead, ALA-D was almost completely inhibited (table 1), and the lead peak in ALA-D fraction was the most prominent (fig 2). This pattern of distribution of lead was similar to that found in ES from lead workers (fig 1). It takes 20-40 hours^{10,19} for the erythrocyte constituents to be equilibrated with lead added *in vitro*, as seen in blood from lead-exposed workers.

In a previous paper¹⁰ we found that to induce or intensify the inhibition of ALA-D *in vitro*, it is necessary for the enzyme fraction to be preincubated with lead acetate and the erythrocyte factors in the haemoglobin fraction. The enzyme activity can be restored by gel filtration and re-inhibited by preincubation with the haemoglobin fraction. The haemoglobin fraction containing bound lead also causes ALA-D inhibition if preincubated with a normal ALA-D fraction. The problem of whether the lead that causes ALA-D inhibition originates from the ALA-D fraction or the haemoglobin fraction has not been solved. The present study has shown that the lead which induces ALA-D inhibition is not bound to the factors in the haemoglobin fraction but to the ALA-D fraction proteins. Lead bound to the ALA-D fraction protein, however, cannot induce the inhibition unless it has been incubated with the factors.¹⁰

The inhibition of ALA-D by lead reaches a plateau at lead concentrations exceeding $60 \mu\text{g}/100 \text{ ml}$ ES, while the ALA-D fraction becomes saturated with lead at $80-110 \mu\text{g}/100 \text{ ml}$. The difference in these concentrations suggests that a small amount of lead ($20-40 \mu\text{g}/100 \text{ ml}$) might bind to the

proteins other than ALA-D. At concentrations under 60 $\mu\text{g}/100\text{ ml}$, however, most of the lead found in the ALA-D fraction seems to be bound to ALA-D protein and is responsible for the inhibition in the presence of the factors. Thus the decrease in ALA-D activity is an indicator of lead saturation of ALA-D fraction proteins which have the highest affinity for lead, because the extent of ALA-D inhibition correlates with that of lead saturation of the fraction at low lead concentrations (figs 4, 5). These results are consistent with those obtained from the determination of ALA-D activity in lead-exposed workers.¹¹⁻¹⁴

Data of the restoration experiments (fig 5) are also compatible with those previously reported.¹⁰ Zinc completely restored the inhibition of ALA-D activity to the control level at wide range of lead concentrations; dithiothreitol and heat did not completely restore activity, especially at high concentrations of lead. The blood used for the present study was fresh, and the enzyme was thought not to be oxidised because the activity was slightly increased by dithiothreitol at a lead concentration of 9 $\mu\text{g}/100\text{ ml}$ (fig 5). In the previous experiment¹⁰ the extent of restoration by dithiothreitol was larger than that of zinc, because the enzyme was partly oxidised during purification. Zinc does not bind to the enzyme unless the essential cysteinyl residues are reduced.²⁰ Although zinc cannot restore the inactivation of ALA-D caused by oxidation, it is the only agent capable of fully restoring the inhibition due to lead.

Haemoglobin fraction proteins also have a high affinity for lead.^{9,21} We do not know whether or not all the lead in the haemoglobin fraction is bound to the haemoglobin molecules. The effect of lead on the haemoglobin fraction and on fractions other than ALA-D has not been clarified.

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