Activity of erythrocyte δ -aminolevulinic acid dehydrase and its change by heat treatment as indices of lead exposure

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Chiba, M. (1976). *British Journal of Industrial Medicine*, 33, 36-42. **Activity of erythrocyte δ -aminolevulinic acid dehydrase and its change by heat treatment as indices of lead exposure.** The effect of heat treatment on erythrocyte δ -aminolevulinic acid dehydrase (ALAD) activity in the blood of human subjects was examined in relation to their lead exposure. A marked difference in the response of ALAD activity to heat treatment was found between the lead-exposed and unexposed subjects; a distinct restoration of ALAD activity after the heat treatment was noted as being a feature of the lead-exposed subjects. On this basis, the activity ratio was calculated from values of the optical density (OD), that is, OD without heat treatment/OD with heat treatment at 60°C for 5 min, and the ratio was proposed as a new parameter for the evaluation of lead exposure. This activity ratio shows a close negative correlation with lead concentration in the blood (correlation coefficients between log ratio and blood lead concentration, $r = -0.92$, $n = 25$, in all subjects; $r = -0.75$, $n = 14$, in normal persons; $r = -0.71$, $n = 11$, in lead-exposed persons) and covers the deficiency in evaluating lead exposure by the ALAD activity alone. This method is of value for identifying unexposed subjects in whom ALAD values are lower than normal. Applicability of this ratio to frozen blood was also assured.

δ -aminolevulinic acid dehydrase (ALAD, δ -aminolevulinic acid dehydrase; EC 4.2.1.24) converts 2 mol δ -aminolevulinic acid (ALA) into 1 mol porphobilinogen (PBG) by dehydration and condensation in the process of porphyrin synthesis. Recently ALAD was isolated from various animals, plants, and bacteria. Its molecular weight and K_m value have been reported by many investigators (Gibson, Neuberger, and Scott, 1955; Granick and Mauzerall, 1958; Batlle, Ferramola, and Grinstein, 1967; Nandi, Baker-Cohen, and Shemin, 1968; Yamasaki and Moriyama, 1971; Candura, Franco, and Pozzi, 1971; Ho and Lascelles, 1971; Wilson, Burger, and Dowdle, 1972). Wilson *et al.* (1972) and Coleman (1966) have reported that ALAD is relatively heat stable.

It is known that the ALAD activity in erythrocytes decreases markedly in the early stage of lead absorp-

tion (de Bruin and Hoolboom, 1967; de Bruin, 1968; Hernberg *et al.*, 1970, 1972). The ALAD activity is measured by a relatively simple procedure with good reproducibility; it is, therefore, employed in the medical examination for lead absorption. Variation of ALAD activity among normal persons is, however, fairly large and in some cases, as will be shown later, it is difficult to distinguish the effect of lead only by the lowered activity of ALAD. On the other hand, a few groups of Italian investigators have reported that human ALAD activity is increased by heating haemolysate (Bonsignore *et al.*, 1968; Vergnano, Cartasegna, and Ardoino, 1969; Candura *et al.*, 1971; Franco, Pozzi, and Candura, 1971). In the present investigation, heat treatment of haemolysate preceding the measurement of ALAD activity was introduced, and the effect of the temperature and time of the heat treatment on the ALAD activity

was examined by a series of experiments on the human blood. As a result of these experiments, it was found that the response of ALAD activity to heat treatment was quite different for the normal and the lead-exposed blood. On this basis we propose the activity ratio as a new parameter for the evaluation of lead exposure in human beings.

Materials

Blood from the antecubital vein and random spot samples of urine were collected from the following five groups of subjects:

Group 1

Seven male workers engaged in annealing with molten lead in a steel rope factory. The maximum lead concentration in the air in their working area was 3.38 mg/m^3 .

Group 2

Four male workers engaged in soldering in a canning factory. The maximum lead concentration in the air in their working area was 0.13 mg/m^3 .

Group 3

Three male workers in the same canning factory not exposed to lead.

Group 4

Two male and three female laboratory personnel.

Group 5

Six male medical students.

After collection, the samples were analysed in the laboratory as quickly as possible. In addition, to examine the effect of storage on the ALAD activities, blood samples were collected from 28 lead workers belonging to Group 1 and 30 persons from the general urban population. The specimens were kept in a freezer at -20°C until analysis.

Methods

Measurement of ALAD activity

ALAD activity was measured by the methods of Osanai (1970) and Wada *et al.* (1970) with partial modification. The 0.2 ml blood collected was placed in a heparinized tube, the tubes were placed in dry ice, and the blood was haemolysed. After 30 to 45 min, the blood was thawed by immersing the tubes in a 20°C water bath for about 30 s and kept in crushed ice. Then 0.5 mol/l phosphate buffer (pH 7.4) was added to each tube. The next step was heat treatment, the key point of this experiment, in which the sample tubes were shaken in a water bath and then kept in crushed ice. Various combinations of temperatures in the water bath and times of keeping the samples in the bath were tried to find the most suitable heat treatment. The control blood samples were taken directly to the next step without heat treatment. The substrate solution was added to the samples and the mixture was incubated for 1 h at 38°C .

The accuracy of thermoregulation in heat treatment and incubation was $\pm 0.08^\circ\text{C}$ and the temperature distribution in the bath was kept within $\pm 0.1^\circ\text{C}$. Temperature calibration of the water bath was made accurately, using a standard thermometer.

Each measurement was carried out in duplicate. The

coefficients of variation in this method were 2.8% ($n = 35$) for normal blood and 2.6% ($n = 31$) for the lead-exposed blood.

Measurement of ALA in the urine

The method described by Wada *et al.* (1969) was used.

Measurement of lead concentration in the blood

About 10 g whole blood was ashed by the wet technique. Lead was extracted with dithizone-methyl isobutyl ketone and its concentration was measured by atomic absorption spectrophotometry. The coefficient of variation of values obtained was 8.35% ($n = 24$).

Results

Effect of heat treatment temperature on the ALAD activity

For 5 min of heat treatment, the ALAD activity was measured at a temperature ranging from 35° to 70°C at 5°C intervals. In this experiment, three kinds of blood were used (Fig. 1); from three men of Group 1,

○—○ Normal person (Group 4)
□—□ Normal person (Group 5)
●—● Lead worker (Group 1)
* Subject 17 in Table
** Subject 20 in Table
▲ Before heat treatment
Each line represents an individual subject

FIG. 1. Effect of temperature of heat treatment for 5 min on ALAD activity.

two men and one woman of Group 4, and Subjects 17 and 20 in the Table. The last two subjects (marked with * and **, respectively, in Fig. 1) belonged to the normal groups but showed low ALAD activity. In all cases, the maximum activity appeared when their blood was treated at $55^\circ \sim 60^\circ\text{C}$. The activity curves in Fig. 1 varied between the normal and the lead-exposed subjects; a characteristic rise took place at $55^\circ \sim 60^\circ\text{C}$ in the exposed group. It is interesting that the pattern of the activity curves of Subjects 17 and 20 was similar to that of the other normal subjects. This fact will be referred to later.

FIG. 2. Effect of heat treatment and time on ALAD activity.

Effect of temperature and time of heat treatment on the ALAD activity

ALAD activity was determined at various temperatures and times of heat treatment on both normal and lead-exposed blood (Fig. 2). Blood samples were taken from 3 ~ 5 subjects in whom exposure to lead was relatively high among Group 1, and from 2 ~ 4 normal subjects belonging to Group 4. Each blood sample was assayed separately and the results were expressed as a mean value for the two groups. As the temperature of heat treatment rose the peak of the ALAD activity appeared earlier; the maximum activity from heat treatment of the blood appeared at 70°C for 30 s in normal group and at 80°C for 20 s in exposed group. Half-life of the ALAD activity of normal blood was 30 s at 80°C, 15 min at 60°C, and 50 min at 50° and 40°C. Whole blood samples could not be assayed because they had coagulated after heat treatment longer than 10 s at 98°C, 30 s at 80°C, 2.5 min at 70°C, and 20 min at 60°C. When the samples were treated at 60°C, the activity reached maximum after 1 min in the normal group and 2.5 min in the lead-exposed group. The levels of activity of these two groups after treatment at 60°C for 5 min did not agree with each other, which might be because the subjects of the exposed group were more heavily exposed than in the above results (Fig. 1). Although, the activity lowered by lead was restored almost completely by heat treatment at 60°C for 5 min, we found that the restoration of the activity

from the heat treatment was limited to the lower level in the case of heavy lead exposure beyond a certain level (Chiba and Kikuchi, 1974).

Relationship between ALAD activity, activity ratio, lead concentration in the blood, and ALA concentration in the urine

Figs 1 and 2 show that, if the heat treatment condition were selected appropriately, for example at 60°C for 5 min, the ALAD activities of the lead-exposed subjects would show a more marked increase than those of the normal subjects.

Since we have found that in the case of the ALAD activities of $0.2 \sim 1.6 \mu\text{mol PBG} \cdot \text{h}^{-1} \cdot (\text{ml RBC})^{-1}$ by our method, the blood lead concentrations against ALAD value were widely scattered and the ALAD activity was not always a clear-cut index of lead exposure, we considered that the effect of lead on ALAD might be parallel with the degree of the change in the activity induced by the heat treatment rather than to the ALAD value itself. Hence the ratio of the activities with and without heat treatment was introduced in the following experiment and its usefulness for the evaluation of lead exposure was examined.

In 25 subjects, the ALAD activity (A), the activity after heat treatment at 60°C for 5 min (B), lead concentration in the blood (Pb-B), and ALA concentration in the urine (ALA-U), were measured and the activity ratio (A/B) was calculated from the optical density (OD) directly. The results are shown in the Table. The values of the activities were low in all lead workers and normal subjects Nos. 17, 20, and 21. The value of the ratio was lower than 0.40 in the lead workers, whereas it was higher than 0.61 in the others. Although Subjects 17, 20, and 21 had low ALAD activities, suggesting a possible lead absorption, they should be considered normal, as mentioned later. It is noteworthy that their activity ratios were in the same range as the other normal subjects.

Figs 3 and 4 show the correlation of ALAD activity v. Pb-B, and activity ratio v. Pb-B, respectively. It is evident from these graphs that the correlation of the activity ratio against Pb-B is higher than that to ALAD activity itself. In general, the relationship between these parameters is weak in the unexposed group and the interrelation becomes stronger after a person has become burdened with lead (Tsuchiya, 1974). In the present work, there was no correlation between ALAD activity or log ALAD activity and Pb-B in the unexposed group, but the correlation between the log activity ratio and Pb-B was statistically significant ($r = -0.75$, $n = 14$).

ALAD activity and activity ratio of the stored blood
Generally, activity of most enzymes decreases with

TABLE
ALAD Activity, Activity Ratio, Pb-B, and ALA-U IN LEAD-EXPOSED AND UNEXPOSED SUBJECTS

Case	Group no. (*1)	ALAD activity (*2)	Activity ratio (*3)	Pb-B(*4)	ALA-U(*5)
1	1	0.37	0.37	1.16	19.44
2	1	0.12	0.13	1.93	32.18
3	1	0.06	0.06	2.55	56.96
4	1	0.23	0.24	1.84	12.66
5	1	0.13	0.13	2.30	54.45
6	1	0.46	0.39	1.16	17.31
7	1	0.26	0.27	2.08	29.59
8	2	0.56	0.30	2.40	9.23
9	2	0.43	0.40	1.79	11.67
10	2	0.28	0.29	1.62	13.73
11	2	0.31	0.36	1.52	14.95
Exposed group mean $\pm$ SD		0.29 ± 0.15	0.27 ± 0.11	1.85 ± 0.44	24.74 ± 16.12
12	3	1.07	0.70	0.98	15.25
13	3	0.95	0.79	0.66	9.84
14	3	0.80	0.67	0.90	13.27
15	4	0.79	1.00	0.51	9.53
16	4	0.92	0.88	0.52	12.66
17	4	0.46	0.72	0.37	12.81
18	4	1.57	1.35	0.17	13.12
19	4	0.91	1.02	0.35	10.83
20	5	0.25	0.96	0.35	16.55
21	5	0.42	0.61	0.51	14.49
22	5	1.24	1.04	0.41	16.47
23	5	0.79	0.99	0.39	14.26
24	5	0.84	1.18	0.23	13.12
25	5	0.85	1.00	0.37	15.78
Unexposed group mean $\pm$ SD		0.85 ± 0.32	0.92 ± 0.20	0.48 ± 0.22	13.43 ± 2.17

Conversion factors to: $\mu\text{g}/100\text{ ml Pb-B}=20.7$; $\text{mg}/100\text{ ml ALA}=0.0131$

*1 Groups 1 and 2, Lead workers; Groups 3, 4, and 5, unexposed persons.

*2 Unit: $\mu\text{mol PBG} \cdot \text{h}^{-1} \cdot (\text{ml RBC})^{-1}$

*3 ALAD activity without heat treatment/ALAD activity with heat treatment; calculated directly from OD values

*4 Unit: $\mu\text{mol/l blood}$

*5 Unit: $\mu\text{mol/l urine}$ corrected for specific gravity

the lapse of time. The ALAD activity of the stored blood samples, both in the normal and exposed cases, is known to decrease with considerable individual variation; its reliability as an index of lead exposure has generally been considered to deteriorate (Nikkanen, Hernberg, and Tola, 1972). Measurement of the activity, therefore, should be made as soon as possible after the collection of samples but in the case of mass screening it would be convenient if the activity could be assessed correctly even after a few days. In this respect, an experiment was carried out with blood from 30 unexposed and 28 lead-exposed, the latter being divided into two groups according to the Pb-B level. Mean values of Pb-B were $1.74\text{ }\mu\text{mol/l blood}$ (range $1.06 \sim 2.17$, $n = 14$) for lower Pb-B blood and 2.66 ($2.27 \sim 3.38$, $n = 14$) for higher Pb-B blood. That of normal blood was about $0.43\text{ }\mu\text{mol/l blood}$. All of the blood was kept

in a freezer at -20°C after haemolysis by dry ice immediately after collection. Then, the ALAD activities with and without heat treatment at 60°C for 5 min were determined after one, two, and five days, and the activity ratios were calculated. This result is shown in Fig. 5. It was evident that the activity of normal blood decreased markedly after storage. After five days, normal and exposed blood could not be distinguished from the ALAD activity level. On the other hand, this result shows that the difference in the activity ratio between the normal and lead-exposed subjects persists even after storage. The activity ratio is expected to give a good index of lead exposure even in stored blood. The same experiment as described in Fig. 1 was performed on the stored blood. Although the absolute value of the activity was low, its characteristic behaviour to heat treatment was similar to that of the fresh blood.

○ Normal person (Groups 3, 4 and 5)
 ● Lead worker (Groups 1 and 2)
 I $\log \text{ALAD} = 0.039 - 0.341 \text{ Pb-B}$
 II $\text{Pb-B} = 0.530 - 1.677 \log \text{ALAD}$
 $r = -0.76$ in all subjects ($n = 25$), $r_N = -0.07$ in normal subjects ($n = 14$), $r_{\text{Pb}} = -0.55$ in Pb-workers ($n = 11$)

FIG. 3. Correlation between ALAD activity and Pb-B.

○ Normal person (Groups 3, 4, and 5)
 ● Lead worker (Groups 1 and 2)
 I $\log \text{activity ratio} = 0.142 - 0.413 \text{ Pb-B}$
 II $\text{Pb-B} = 0.461 - 2.061 \log \text{activity ratio}$
 $r = -0.92$ in all subjects ($n = 25$), $r_N = -0.75$ in normal subjects ($n = 14$), $r_{\text{Pb}} = -0.71$ in Pb-workers ($n = 11$)

FIG. 4. Correlation between activity ratio and Pb-B.

Discussion

The concentration of lead in the blood, Pb-B, is important for the evaluation of lead absorption by an organism. For this purpose, atomic absorption spectrophotometry is being used, but it requires a large sample volume and delicate care throughout the whole process because the measurement deals

○—○ unexposed group ($n = 30$) mean Pb-B: $0.43 \mu\text{mol/l}$ blood
 △—△ low exposed group ($n = 14$) mean Pb-B: $1.74 \mu\text{mol/l}$ blood
 ▲—▲ high exposed group ($n = 14$) mean Pb-B: $2.66 \mu\text{mol/l}$ blood
 I SD

FIG. 5. Effect of storage of blood samples on ALAD activity and activity ratio.

with a relatively low level; it is therefore troublesome for the mass screening of humans exposed to lead. Recently, a rapid micromethod has been developed (Delves, 1970; Joselow and Singh, 1974). It should be pointed out that there is a large coefficient of variation in the low Pb-B range in Delves's report. Joselow and Singh stated that this method was appropriate for high Pb-B, and could not avoid contamination in field work. Flameless atomic absorption spectrometry is also used for micro-analysis of Pb-B (Hwang *et al.*, 1971; Hwang, Ullucci, and Mokeler, 1973). When the sample volume is reduced, greater sensitivity of the analytical method becomes inevitable. Therefore, contamination from glassware, the effect of background, and errors of pipetting become serious problems. It is also desirable that the instrument and its accessories should not be expensive.

On the other hand, ALAD activity is sensitive to lead and is measured by a relatively simple process requiring only 0.2 ml blood. Close negative correlation between ALAD activity and Pb-B has been reported by several authors (Nakao, Wada, and Yano, 1968; Hernberg *et al.*, 1970, 1972; Haeger-Aronsen, Abdulla, and Fristedt, 1971; Tola, 1973), and it seems that there is no ambiguity with the evaluation of lead exposure by ALAD determination.

There are many medical indices of lead exposure other than Pb-B and ALAD activity, such as concentration of lead, ALA, PBG, and coproporphyrin in the urine, and haemoglobin and punctate basophil cell count. The correlation between these parameters has been reported by many investigators (Cramér and Selander, 1965; Selander, Cramér, and

Hallberg, 1966; Gibson, Mackenzie, and Goldberg, 1968; Williams, King, and Walford, 1969; Selander and Cramér, 1970; Haeger-Aronsen, 1971; Waldron, 1971). Their results, however, do not always agree with each other, and the discrepancy is considered to be due to the degree of lead exposure and individual variations in sensitivity to lead. When attention is paid to the normal groups in these reports, there is no statistically significant correlation, except in the report by Hernberg and Nikkanen (1970), whose specimens included one particular person whose value was markedly biased. It seems that this affected the whole finding significantly.

We found a different behaviour of ALAD activity between exposed and unexposed subjects as described above. The levels of activity after heat treatment of the blood from exposed subjects agreed with that from unexposed ones, except for the especially low activity among individuals in the unexposed and heavily exposed group. It was assumed that raised ALAD activities of exposed subjects by heat treatment of their blood were correct normal values of their own.

The fact that the activity increases by heat treatment seems to be incompatible with a simple interaction: that the inhibition or restoration of enzyme activity is caused by capture or release of lead by the essential SH groups of the enzyme, because the affinity of lead to the SH groups is too strong to be affected by gentle heating. The heat treatment used in this experiment induces a substantial change in the enzyme conformation; hence the explanation based on allosteric site inhibition is plausible as pointed out by Nikkanen *et al.* (1972). Another possible hypothesis is that the ALAD in erythrocytes includes at least two components. One of them, type 1, is inherently active without heat treatment, inhibited by lead *in vivo*, and cannot be restored by heat treatment. The other, type 2, is inherently inactive, activated by heat treatment, and insensitive to lead *in vivo*, but type 2 may also be inactivated by lead levels above 1.93 $\mu\text{mol/l}$ (40 $\mu\text{g}/100\text{ g}$) blood. Both types of ALAD are subjected to reduction by overheating. Consequently, the activity of type 1 decreases steadily as a function of the period of heat treatment while that of type 2 increases initially, reaches a peak, and decreases.

The activity ratio introduced here takes advantage of these characteristics. The ratio makes it possible to discriminate between the low activity caused by lead from that of other origins; and this makes the evaluation of lead exposure by ALAD activity more reliable (Table). Subjects 17, 20, and 21 in this experiment are examples of such a case. They are actually normal in Pb-B and, from their personal history, have had no occupational or other contact with lead. The aetiology of this low activity is unknown.

In the normal group especially, the activity ratio reflects the effect of lead on the organism although the range of Pb-B is limited to only about 0.14 ~ 0.97 $\mu\text{mol/l}$ blood (log ratio v. Pb-B: $r = -0.75$, $n = 14$). The lower limit of the activity ratio for the normal subjects is considered to be about 0.5 (Table).

If Pb-B is beyond 1.93 $\mu\text{mol/l}$ blood, however, the variation of the ratio increases (Fig. 4). This is presumably due to the fact that two components of ALAD may be inactivated by lead with individual variations. This concentration of Pb-B, 1.93 $\mu\text{mol/l}$ (40 $\mu\text{g}/100\text{ g}$) agrees with the normal upper limit reported by Lane *et al.* (1968).

It is useful in practice that this ratio can be applied to frozen stored blood (Fig. 5). For instance, in mass screening ALAD activity is measured in the usual way at first, and then it is possible to re-examine the activity ratio of the stored blood for the subjects showing a low activity of questionable range.

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