AVAILABILITY OF ACTIVITY RATIO OBTAINED FROM ACTIVITY OF 5-AMINOLEVULINATE HYDRO-LYASE IN HUMAN BLOOD BEFORE AND AFTER HEAT TREATMENT TO EVALUATE LEAD EXPOSURE

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The activity of 5-aminolevulinate hydro-lyase (ALAD) in blood decreases in lead-exposed subjects. The activity of the hemolysed blood is restored by heat treatment (60°C, for 5 min) almost completely. A parameter of "activity ratio", dividing the enzyme activity before heat treatment by that after heat treatment, is proposed for evaluation of lead exposure, and this ratio was calculated in 167 human subjects, including those exposed to lead in work, lead concentration in their blood ranging 3.5 to 82 $\mu\text{g}/100\text{ g}$ blood. Correlation of the ratio against blood lead concentration was higher than that of the ALAD activity itself. As there are, in addition, subjects with lower-than-normal ALAD activity values regardless of lead exposure, the ratio is available as an index to evaluate the extent of lead exposure.

In 1963, Lichtman and Feldman¹⁾ reported that the activity of 5-aminolevulinate hydro-lyase (δ -aminolevulinic acid dehydrase, ALAD, EC 4.2.1.24) in blood was reduced by exposure of humans to lead. It is generally accepted that blood ALAD activity decreases even when the organisms are exposed a low level of lead that will not give any clinical symptoms or signs, and that determination of the activity has a good reproducibility and specificity. Therefore, it is used as a sensitive index in medical examination for evaluating lead exposure. Bonsignore *et al.*,²⁾ Vergnano *et al.*,³⁾ and Candura *et al.*⁴⁾ found and we also confirmed^{5,6)} that the activity lowered by lead can be restored by heating the hemolysate before the activity assay. We proposed, therefore, to use the activity ratio as a new parameter which was calculated from the ALAD activity of blood with and without heat treatment, for evaluating the effect of lead on organisms. This ratio shows a closer negative correlation with lead concentration in blood (Pb-B) rather than the activity of ALAD itself. This activity ratio was applied to 167 human subjects, ranging in lead concentration of 3.5 to 82 $\mu\text{g}/100\text{ g}$ of blood and its usefulness was confirmed.

MATERIALS AND METHODS

Subjects

Blood samples were obtained from the following three groups of subjects:

Group A (control). Thirty healthy persons (6 male and 5 female laboratory personnel, 15 male medical students, 2 male office workers, and 2 male factory workers). Their age ranged from 20 to 52 (mean 28) years. They were living in urban area, and had had no occupational or other contact with lead.

Group B (moderate lead-exposure). This group consisted of 87 (including 4 female) printing factory workers (15 type-pickers, 29 typesetters, 28 printing press workers, 11 type foundry and stereotypers, and 4 engineering workers and storekeepers). Their mean age was 40 (range 18 to 63) years and mean working period was 21 (range 2 to 42) years. The atmospheric lead content was not higher than 0.54 mg/m³.

Group C (high lead-exposure). Fifty male workers from a steel rope factory. They are engaged in annealing with molten lead or in bringing raw materials and unfinished products around the molten lead furnace. Their mean age was 36 (range 19 to 54) years. Their mean working period was 10 years (range 1 to 28). The maximum atmospheric lead content in their working area was 3.38 mg/m³. Two of these workers who had complained of arthralgia were moved from their working shop just after this examination. All the subjects participated voluntarily.

Sampling

All the blood samples were collected from the antecubital vein, put into heparinized tubes, and the tubes were placed in a jar with Dry Ice immediately. Blood was collected from the subjects in the laboratory for Group A and in the medical office of the factories for Groups B and C.

Analyses

Measurement of ALAD activity in blood. Determination of ALAD activity in blood was started within 4 hr after sampling, because the activity fell slightly even on Dry Ice if blood was kept till the next day. (a) Reagents: Buffer—0.5 mol/l phosphate buffer (pH 7.4) was made from monopotassium and disodium phosphate. Substrate solution—100 mg of 5-aminolevulinic acid (ALA) was dissolved in 3 ml of the buffer and 4 ml of 0.1 mol/l potassium hydroxide (86 mmol/l ALA). This solution was prepared freshly just before using. Modified Ehrlich reagent—1 g of p-dimethylaminobenzaldehyde was dissolved in the mixture of 42.8 ml of glacial acetic acid, 7.7 ml of 70% perchloric acid, 2.5 ml of 0.2 mol/l mercury dichloride, and 2.5 ml of conc-hydrochloride. TCA—5% trichloroacetic acid (b) Procedure: 0.1 ml of buffer and 0.1 ml of substrate solution were added to 0.2 ml of blood hemolyzed on Dry Ice, and the mixture was incubated at 38°C for 1 hr.

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To stop the reaction, 2 ml of TCA was added to the samples. After centrifugation (3000 rpm, 10 min), 3 ml of Ehrlich reagent was added to 1 ml of supernatant. After 15 min accurately, optical density (OD) at 556 nm was read. Each measurement, both with and without heat treatment, was carried out in duplicates.

Heat treatment. This step was a key point of this procedure. Hemolysate containing the buffer solution was shaken for 5 min in a 60°C water bath.

Measurement of Pb-B. After ashing about 10 g of blood by the wet technique and dithizone-methyl isobutyl ketone extraction, Pb-B was determined by atomic absorption spectrophotometry.

Calculations

ALAD activity [$\mu\text{mol PBG} \cdot \text{h}^{-1} \cdot (\text{ml RBC})^{-1}$] = $\frac{\text{OD (sample)} - \text{OD (blank)}}{6.2 \times 10^4} \times 10^6 \times 48 \times \frac{100}{\text{Ht}} \times 10^{-3} = \frac{\text{JOD}}{\text{Ht}} \times 77$ where Ht is the hematocrit value, 6.2×10^4 is molar absorbance of porphobilinogen (PBG) ($\text{liter} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$), 48 is the dilution factor of the sample blood. OD (blank) may be negligible because the value is below 0.005 in most cases.

$$\text{Activity ratio} = \frac{\text{OD of the sample without heat treatment}}{\text{OD of the sample activated with heat treatment}}$$

RESULTS

Fig. 1 shows the mean value and standard deviation (SD) of blood ALAD activity, activity ratio, and Pb-B for each group. In three parameters, the differences were statis-

Fig. 1. Mean values of three parameters for each group.

tically significant between Group A (control) and the others ($p < 0.001$), and between Group B and Group C. Table 1 shows that all subjects are rearranged according to Pb-B values independent of their groups. Fig. 2 shows the relationship between blood ALAD activity and Pb-B. The points from Group C scattered horizontally at right side of the graph. Fig. 3 shows the relationship between the activity ratio and Pb-B. The ratio in Group A is above 0.6 and most of Group C is under 0.4. It is evident from Figs. 2 and 3 that a certain value of ALAD activity corresponds to a relatively wide Pb-B levels rather than to the activity ratio. Furthermore, correlation coefficient between Pb-B and the activity ratio ($r_1 = -0.874$) is higher than that between Pb-B and the ALAD activity ($r_2 = -0.768$).

Fig. 2. Relationship between blood ALAD activity and Pb-B.

Table 1. Blood ALAD activities before and after heat treatment and activity ratios in different Pb-B groups in all subjects.

(A) Grouping at Pb-B levels of 20 $\mu\text{g}/100\text{ g}$ blood.

Pb-B	No. of Subjects	ALAD activity before heating		ALAD activity after heating		Activity ratio		Correlation coefficient	
		mean	SD	mean	SD	mean	SD	vs. Activity	vs. Ratio
<20	93	0.81	0.23	0.97	0.21	0.83	0.16	-0.226†	-0.680††
20 ≤ <40	48	0.48	0.21	0.97	0.20	0.48	0.16	-0.571††	-0.749††
40 ≤ <60	16	0.16	0.06	0.88	0.17	0.18	0.05	-0.404	-0.478
60 ≤ ≤82	10	0.12	0.03	0.92	0.13	0.13	0.03	-0.394	+0.148

(B) Grouping into two groups of below 20 $\mu\text{g}/100\text{ g}$ of Pb-B.

<10	24	0.89	0.24	0.89	0.21	1.00	0.13	-0.050	-0.506††
10 ≤ <20	69	0.78	0.22	1.00	0.20	0.78	0.13	-0.205	-0.427††

† $p < 0.05$

†† $p < 0.01$

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Fig. 3. Relationship between activity ratio and Pb-B.

Fig. 4. Frequency distribution of ALAD activity before and after heat treatment and activity ratio for each group.

Assuming simple Michaelis-Menten kinetics of the non-competitive inhibition type for lead *in vivo*, inhibitor constant (K_i) may be calculated from the following formula:

$$\frac{V}{V_i} = 1 + \frac{1}{K_i} [Pb]$$

where V is the maximum velocity of this enzyme without an inhibitor, which represents the enzyme activity after heat treatment, and V_i , the velocity in the presence of an inhibitor, which represents the activity before heat treatment. If reciprocal of the ratio is plotted on the ordinate against Pb-B on the abscissa, as in Fig. 3, the apparent K_i is expressed as $1/\text{slope}$ of a regression line. Calculated value of K_i was $0.4 \mu\text{mol/liter}$.

Fig. 4 is a histogram of frequency distribution of blood ALAD activities, activities after heat treatment, and the calculated ratios. As is generally known, blood ALAD activity fell markedly as the Pb-B level increased (upper column). On the other hand, the activities after heat treatment lie in a relatively same range independent of lead exposure. While the range of ALAD activities of control group was relatively wide,

Table 2. Relationship among Pb-B, blood ALAD activity, and activity ratio.

Group	Pb-B ($\mu\text{g}/100 \text{ g blood}$)	ALAD activity	Activity ratio
A	6	0.68	0.99
A	8	0.63	0.85
B	10	0.58	0.78
B	12	0.59	0.76
B	14	0.69	0.67
B	14	0.67	0.58
B	15	0.64	0.65
B	15	0.59	0.63
B	17	0.68	0.71
B	17	0.65	0.70
B	19	0.65	0.67
B	20	0.68	0.57
B	20	0.58	0.61
C	20	0.69	0.59
B	21	0.61	0.57
B	22	0.69	0.70
B	23	0.64	0.66
B	23	0.60	0.57
B	25	0.60	0.59
B	34	0.65	0.54

The subjects with 0.58 to $0.69 \mu\text{mol PBG} \cdot \text{h}^{-1} \cdot (\text{ml RBC})^{-1}$ of blood ALAD activity are listed in the order of Pb-B levels

r_{s1} (between Pb-B and ALAD activity) = -0.042 (NS)

r_{s2} (between Pb-B and activity ratio) = -0.689 ($p < 0.01$)

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that of the ratio was considerably narrower. A few persons are sometimes found in non-exposed group whose ALAD activity is so low as to suggest lead exposure. These can be distinguished from lead-exposed group by using their activity ratio (upper left and lower left). The availability of the activity ratio will be mentioned in detail later (Table 2).

There was no correlation between the blood ALAD activity or the activity ratio and age or duration of lead exposure of subjects in both Groups B and C.

DISCUSSION

There are several reports that blood ALAD activity which was reduced by lead *in vivo* can be increased by some *in vitro* treatments. For this purpose, Bonsignore *et al.*,²⁾ Candura *et al.*,⁴⁾ Vergnano *et al.*,³⁾ and Chiba⁶⁾ adopted heat treatment of hemolyzed blood samples, Nikkanen *et al.*,⁷⁾ and Tomokuni⁸⁾ made use of assays at various pH, and Lichtman and Feldman,¹⁾ Hopke and Prigge,⁹⁾ and Granick *et al.*¹⁰⁾ added sulfhydryl reagent such as glutathione or dithiothreitol to hemolyzed samples. Tomokuni⁸⁾ and Granick *et al.*¹⁰⁾ proposed an activity ratio calculated from values obtained by two different assays as one of the indices for lead exposure. According to Tomokuni,⁸⁾ blood ALAD activity itself is better than the activity ratio (pH) in relation to correlation coefficient against Pb-B, and restoration of the activity by pH shift is incomplete. In the ratio (SH) by Granick *et al.*,¹⁰⁾ some points agree with our results, although the way of activation employed is different. Points of agreement are (1) restoration of the activity with the SH reagent treatment is complete, and the blood ALAD activities after activation are about $1 \mu\text{mol PBG} \cdot \text{h}^{-1} \cdot (\text{ml RBC})^{-1}$, (2) distribution of each subject's ratio against Pb-B value is narrower than that of the activity itself under $50 \mu\text{g/dl}$, (3) the borderline of the ratio between lead-exposed and non-exposed subjects is about 0.5, or reciprocal 200%, and (4) the calculated inhibitor constant (Ki) is $0.4 \mu\text{mol/liter}$.

In Table 1, we consider that the so-called normal value of the blood ALAD activity is above $0.58 \mu\text{mol PBG} \cdot \text{h}^{-1} \cdot (\text{ml RBC})^{-1}$ by our method. This is based on the mean value minus SD of ALAD activity of blood samples with Pb-B below $20 \mu\text{g}/100 \text{ g}$ which is the Pb-B range of non-exposed subjects. Since the mean value plus SD of the activity, for the subjects with Pb-B of 20 to $40 \mu\text{g}/100 \text{ g}$, in which range the activity decreases lineally, is $0.69 \mu\text{mol PBG} \cdot \text{h}^{-1} \cdot (\text{ml RBC})^{-1}$, the subjects whose Pb-B values are beyond those of non-exposed ones may be included in the group of 0.58 to $0.69 \mu\text{mol PBG} \cdot \text{h}^{-1} \cdot (\text{ml RBC})^{-1}$ of the ALAD activities. Our activity ratio is useful in distinguishing them as shown in Table 2, in which cases with 0.58 to $0.69 \mu\text{mol PBG} \cdot \text{h}^{-1} \cdot (\text{ml RBC})^{-1}$ of blood ALAD activity are listed in the order of their Pb-B values. The rank correlation coefficients were calculated between Pb-B and the activity itself [$r_{s1} = -0.042$ (NS)], and between Pb-B and activity ratio, $r_{s2} = 0.689$ ($p < 0.01$). This reveals that the activity ratio reflects Pb-B level better than the activity itself. Other advantages of using our ratio are (1) while the activity cannot be compared unless the assay methods are

same, the ratios are comparable without such consideration, and (2) the ratio shows a close negative correlation against Pb-B rather than the activity itself as mentioned above. We considered that the reason for the latter is because individual variation may be eliminated by division of the activities by the values with and without heat treatment, by which the effect of lead on the organisms remains. The disadvantage of this ratio is that when Pb-B is over 40 $\mu\text{g}/100\text{ g}$ blood the correlation between the ratio and Pb-B becomes weaker as the activity itself. Perhaps, the blood ALAD activity is extremely sensitive to lead and its fall reaches the lowest at about 40 $\mu\text{g}/100\text{ g}$ Pb-B.

We made a hypothesis for the explanation of the activity change after heat treatment. ALAD in blood includes at least two components⁶⁾; the one is inherently active and is inhibited by heat treatment and lead *in vivo*, and the other is inherently inactive at a lower temperature and is activated by heat treatment and stable to lead *in vivo*. On the other hand, Dineen and Rullet¹¹⁾ has considered that two enzyme fractions were present in blood, which had pH optimum of 6.8 and 6.1. This interesting problem deserves further elucidation.

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