Reduction of δ -aminolevulinate dehydratase concentration by bromobenzene in rats

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ABSTRACT The effects of bromobenzene on δ -aminolevulinate (ALA) dehydratase (EC 4.2.1.24) were examined in rats. The enzyme in the bone marrow was irreversibly inhibited after 12 hours of treatment with bromobenzene (intraperitoneally) without any change in the enzyme concentration. When bromobenzene treatment was prolonged to 72 hours, the concentrations of the enzyme in the bone marrow and in the liver were reduced proportionally to the decrease in the enzyme activity. Neither the activity nor the concentration of ALA dehydratase in the peripheral erythrocytes were reduced even after 72 hours treatment with bromobenzene. These findings indicate that bromobenzene decreases ALA dehydratase activity in a biphasic manner; firstly, through an irreversible inhibition probably due to the formation of mercaptide with the essential SH groups and, secondly, through a reduced synthesis of the enzyme.

The activity of δ -aminolevulinate (ALA) dehydratase (EC 4.2.1.24) is well known to be inhibited by exposure to lead and the degree of the inhibition of the enzyme activity is recognised as an indicator of lead uptake.¹⁻³ The inhibition by lead is due to the formation of mercaptide between the essential SH groups of the enzyme molecule and lead.⁴ Therefore, substances able to bind to essential SH groups might also have an inhibitory effect on ALA dehydratase activity.

Recently, several organic solvents such as trichloroethylene,⁵ styrene,^{6,7} and bromobenzene⁸ have been reported significantly to reduce ALA dehydratase activity. These solvents are used widely in industry and studies of their biological effects should provide important information for evaluating the health status of workers exposed to them. Trichloroethylene is metabolised by cytochrome P-450 dependent mixed function oxidase to form reactive metabolite(s), which are considered to inhibit ALA dehydratase activity irreversibly by binding to the essential SH groups.⁵ Styrene, however, decreases the enzyme concentration by reducing enzyme synthesis at transcriptional and post-translational levels and this reduction in enzyme concentration results in reduced ALA dehydratase activity.^{6,7} These reports indicated at least two different ways to decrease ALA dehydratase activity, though both trichloroethylene

and styrene are oxidised by the cytochrome P-450 system.⁹⁻¹¹

Bromobenzene is also metabolised by the cytochrome P-450 system to form highly reactive intermediates (bromobenzene-3,4-oxide and -2,3-oxide),¹² which possibly decrease the dehydratase activity in two different ways. One is through the inhibition of the enzyme activity due to the formation of mercaptide with the essential SH groups as in the case of lead⁴ and trichloroethylene.⁵ The other is by the reduced synthesis of the enzyme protein as in the case of styrene.^{6,7} The first mechanism has been reported *in vitro*,⁸ but the second has not previously been examined.

Materials and methods

ANIMALS AND TREATMENT WITH BROMOBENZENE
Male Wistar rats, 7 weeks old and weighing 200-300 g, were divided into a control (n = 8) and a bromobenzene treated group (n = 8). Bromobenzene was administered intraperitoneally once a day in a dose of 4 mmol/kg body weight (dissolved in 0.5 ml of soybean oil)/kg body weight. Soybean oil (0.5 ml/kg body weight) was administered to the controls once a day. Four rats from each group were killed at 12 hours and 72 hours after the first administration of bromobenzene and blood, liver, and bone marrow from tibias and femurs were collected. Samples were assayed for packed cell volume (blood), protein concentration (liver and bone marrow), and activity and

concentration of ALA dehydratase (blood, liver, and bone marrow). Rats were fed with a certified chow (Funahashi Nojo, Hamamatsu, Japan) and distilled water ad libitum. They were housed four to a wire bottom cage in a room designed to control temperature (24–26°C), relative humidity (50–70%), and photocycle (12 hours). In all the experiments the rats were killed or treated between 0900 and 1000.

PURIFICATION OF ALA DEHYDRATASE

ALA dehydratase was purified from rat erythrocytes as described previously.¹³ The purified enzyme preparation was homogenous on gel filtration chromatography and in analytic polyacrylamide electrophoresis both in the absence and presence of sodium dodecyl sulphate. The enzyme was labelled with 125-I (Amersham International Ltd, Buckinghamshire, UK) as reported previously.¹³ The specific radio activity of 125-I ALA dehydratase was 6.0 μ Ci/ μ g of enzyme protein.

PREPARATION OF ANTIBODY AGAINST ALA DEHYDRATASE

The antibody was prepared by the method described in an earlier report⁶ using the ALA dehydratase purified from rat erythrocytes as described above. At a dilution of 1:15 000 the IgG preparation could bind approximately 50% of the labelled antigen (= labelled ALA dehydratase).

PREPARATION OF MICROSOMES

Liver microsomes from rats treated with phenobarbital were prepared as reported previously.⁸

ASSAY OF ALA DEHYDRATASE ACTIVITY

ALA dehydratase activity was assayed both in the absence and presence of 10 mM dithiothreitol and 0.1 mM zinc acetate.¹³ Unless otherwise stated, the

enzyme activity in the text represents the activity after the reactivation with dithiothreitol and zinc. One unit of the dehydratase activity was defined as 1 μ mole of porphobilinogen formed at 37°C per hour. Radioimmunoassay of the enzyme was carried out as described previously.¹³ The effect of the mixed function oxidase system on the enzyme after the administration of bromobenzene was investigated in vitro according to the previous method.⁸ When the incubation was ended, ALA dehydratase was reactivated with dithiothreitol and zinc and the enzyme concentration was determined.

REAGENTS

ALA hydrochloride was purchased from Sigma Chemicals Co (St Louis). Other chemicals used were all of analytical grade.

STATISTICS

Student's *t* test (two tailed) was applied for the examination of significant differences between the control groups and groups treated with bromobenzene.

Results

The activity of ALA dehydratase decreased significantly after 12 hours of bromobenzene administration only in the bone marrow (table 1). It was not accompanied with a decrease in the enzyme concentration. Treatment with dithiothreitol and zinc could not restore the decreased activity of ALA dehydratase, indicating an irreversible inhibition of ALA dehydratase by bromobenzene. By contrast, ALA dehydratase activity in the erythrocytes and in the liver was unaltered after 12 hours of treatment with bromobenzene. Table 1 indicates the significant decrease of ALA dehydratase concentration and of

Table 1 Effect of treatment with bromobenzene on ALA dehydratase

ALA dehydratase (No of rats)		Duration of bromobenzene treatment	
		12 hours	72 hours
Bone marrow (n = 4)	Activity	79 (8.6)*	45 (13.8)**
	Concentration	102 (5.0)	47 (9.6)**
Erythrocytes (n = 4)	Activity	105 (17.7)	90 (22.1)
	Concentration	101 (16.9)	97 (8.2)
Liver (n = 4)	Activity	92 (7.3)	59 (18.8)**
	Concentration	95 (4.9)	62 (11.8)**

To compare the enzyme activity with its concentration, both values were described as relative values (%) in comparison with the means of concurrent controls.

Means and standard deviations (in parentheses) of ALA dehydratase activities and concentrations in the pooled control—that is, the control at 12 hours and 72 hours, eight animals in total—were $17.9 (2.15) \times 10^{-3}$ unit/mg protein and 854 (108.0) ng/mg protein in the bone marrow, $194 (27.6) \times 10^{-3}$ unit/ml of packed cells and $7.73 (2.43) \mu$ g/ml of packed cells in erythrocytes, and $6.19 (1.38) \times 10^{-3}$ unit/mg protein and 615 (99.8) ng/mg protein in liver.

*Difference from the control is significant at $p < 0.01(**)$ or $< 0.05(*)$.

Table 2 Time course of the decrease of ALA dehydratase in the bone marrow. (Figures are means and standard deviations in parentheses)

Rats	Activity ($\times 10^{-3}$ unit/mg protein)	Concentration (ng/mg protein)	Activity/mass ratio (units/mg enzyme)
Control	17.9 (2.15)	854 (108)	21.2 (1.40)
12 hours treated	14.1 (1.54)*	869 (43)	16.8 (1.34)**
72 hours treated	8.1 (2.47)**	403 (92)**	20.1 (4.76)

Activity/mass ratio was calculated as activity of ALA dehydratase/concentration of ALA dehydratase.

Other conditions as in legend to table 1 are in the text.

*Difference from the control is significant at $p < 0.01$ (**) or $p < 0.05$ (*).

ALA dehydratase activity in the bone marrow and liver but not in erythrocytes 72 hours after the first administration of bromobenzene. Results of radioimmunoassay show that the reduced enzyme concentration was proportional to the reduced enzyme activity in the bone marrow and in the liver (to 47% and 67% of the control, respectively).

Difference was observed between rats treated with bromobenzene for 12 hours and those treated for 72 hours (table 1) when the activity/mass ratio (= activity of ALA dehydratase/concentration of ALA dehydratase) was examined in the bone marrow (table 2). The activity/mass ratio of the control rats was 21.2 units/mg of enzyme, which is almost compatible with the specific activity of purified ALA dehydratase (rat, 23.8–27.0 units/mg of enzyme⁶). This observation shows the accuracy of our purification and of the determination of the enzyme. As shown in table 2, a significant decrease of the activity/mass ratio in the bone marrow was observed after only 12 hours of the first treatment with bromobenzene. This shows that the reduced enzyme activity in the early stage of intoxication should be an inhibition of enzyme activity. The activity/mass ratio in the rats treated for 72 hours, however, was almost identical with that in the control rats (20.1 v 21.2) indicating that the reduced enzyme activity in rats treated for 72 hours is due to a reduced enzyme concentration.

To elucidate the site of the reduced enzyme concentration by bromobenzene, the following experiments were carried out. ALA dehydratase activity was decreased to 61% of the control when the enzyme was

incubated in vitro with the mixed function oxidase system and bromobenzene (table 3). The enzyme concentration determined by radioimmunoassay, however, did not decrease after in vitro incubation. It is, therefore, unlikely that the enzyme concentration was depressed by post-translational degradation.

Discussion

Lead inhibits the activity of ALA dehydratase by the formation of mercaptide with the essential SH groups of the enzyme molecule⁴ and the inhibition of ALA is one of the most sensitive indicators of lead exposure.¹⁻³ ALA dehydratase activity in the peripheral blood is routinely determined in the medical examination of lead workers.¹ Recently, clear evidence of the increased synthesis of ALA dehydratase by lead was reported in rats¹³ and in man.³ Therefore, attention should be given to enzyme synthesis as well as to enzyme activity in the study of effects of chemicals on ALA dehydratase.

Several organic solvents such as trichloroethylene,⁵ styrene,^{6,7} and bromobenzene⁸ have been reported to decrease ALA dehydratase activity in vivo. In general, two mechanisms should be considered when we examine mechanisms by which enzyme activity is reduced in vivo. One is the inhibition of the enzyme activity due to an interaction between the enzyme molecules and the chemicals or the metabolite(s), or both. In the case of trichloroethylene, ALA dehydratase is inhibited by the chemical after bioactivation with mixed function oxidases and the

Table 3 Effects of bromobenzene on ALA dehydratase in the presence of the mixed function oxidase system in vitro. Figures in the table are means ($n \times 3$) and standard deviations (in parentheses)

Incubation conditions			ALA dehydratase	
Microsomes	NADPH	Bromobenzene	Activity (%)	Concentration (%)
–	–	–	100 (5)†	100 (7)†
+	+	–	102 (3)	103 (8)
+	+	+	61 (4)*	98 (6)

Purified ALA dehydratase (7 μ g protein/ml) was incubated with the mixed function oxidase system and bromobenzene as described previously.⁸

*Significantly decreased when compared with the control ($p < 0.01$).

†Taken as control.

dehydratase concentration is not decreased.⁵ The other mechanism is the inhibition of enzyme synthesis. Styrene is metabolised by a cytochrome P-450 dependent system through a reactive intermediate, styrene-7,8-oxide,⁹⁻¹¹ which inhibits ALA dehydratase activity in vitro.⁷ Further investigation, however has, shown that styrene suppresses dehydratase synthesis at the transcriptional level in animals, resulting in a decrease in enzyme concentration, with no inhibition of the enzyme.⁶ Bromobenzene is also oxidised by the cytochrome P-450 system^{8,12} and the reactive intermediate(s) inhibit ALA dehydratase in vitro⁸ but a reduction in ALA dehydratase concentration has not been claimed.

The present study indicates that bromobenzene reduces ALA dehydratase activity by these two mechanisms. As shown in tables 1 and 2, in the bone marrow of rats treated for 12 hours the activity of ALA dehydratase decreased significantly with bromobenzene treatment with no reduction in enzyme concentration. Accordingly, the activity/mass ratio (= activity/enzyme concentration) in the bone marrow decreased to 79% of the control in the rats treated for 12 hours.

In the rats treated for 72 hours the concentration and the activity of ALA dehydratase were decreased significantly in the bone marrow and the liver. Both the concentration and the activity were decreased proportionally; therefore, the activity/mass ratio of rats treated for 72 hours (20.1 units/mg protein) was compatible with the control rats (21.2 units/mg protein) notwithstanding the reduced enzyme activity (table 2). These results indicate that the reduced activity of ALA dehydratase observed in rats treated for 72 hours is due not to the activity inhibition but to a reduced enzyme concentration. There are two possible ways to decrease the concentration of an enzyme; either there is a decrease in its synthesis or an acceleration in its degradation. Our in vitro study failed to confirm the latter possibility, since with the mixed function oxidase system, bromobenzene decreased only the ALA dehydratase activity but not the concentration of the enzyme (table 3). The reduction in ALA dehydratase is likely, therefore, to be due to an inhibition of its synthesis.¹⁴⁻¹⁶

The ALA dehydratase concentration in the peripheral erythrocytes was not decreased significantly by treatment with bromobenzene, notwithstanding the clear decrease of the enzyme concentration in the bone marrow (table 1). The maturation of erythrocytes from proerythroblast in the bone marrow usually requires 4-7 days in mammals.^{13,17,18} As erythrocyte ALA dehydratase is synthesised in the bone marrow, the absence of decrease in the dehydratase concentration in the peripheral erythrocytes of rats treated for 72 hours supports the hypothesis of reduced synthesis of

the enzyme in animals treated with bromobenzene.

The idea that bromobenzene reduces ALA dehydratase synthesis may explain the time lag between the decrease of non-protein SH (reduced form) content in the liver and the reduction of ALA dehydratase activity observed in the liver.⁸ The amount of non-protein SH in the liver reached a minimum after 24 hours of bromobenzene administration and, thereafter, the concentration was gradually restored.⁸ Non-protein SH group is considered to play a part in the protection against the toxicity of the reactive intermediates of bromobenzene¹² and trichloroethylene⁵ on macromolecules of animals. The addition of dithiothreitol prevents the inhibition of ALA dehydratase by intermediates of trichloroethylene in vitro.⁵ Thus the time course of the decrease in ALA dehydratase activity should be synchronised with the fluctuations in non-protein SH contents, since the interaction between intermediates and the enzyme should correlate inversely with the concentration of non-protein SH. In fact, this was not the case. Our results show that the activity of ALA dehydratase decreased progressively in the liver even after 72 hours, notwithstanding the partial recovery of non-protein SH content (48 hours in (8)). This may be due to the prolonged reduction in the synthesis of ALA dehydratase by the interaction between nucleic acids and the reactive intermediate(s) of bromobenzene.

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