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Methemoglobin formation by potassium ferrocyanide in the hemolysate of acatalasemic mice.

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Methemoglobin Formation by Potassium Ferrocyanide in the Hemolysate of Acatalasemic Mice

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Increased methemoglobin (metHb) formation is harmful to the normal oxygen transport. There are some mechanisms to reduce metHb formation in the erythrocyte. In the present study, effect of catalase (EC 1.11.1.6) on metHb formation by potassium ferrocyanide was examined spectrophotometrically in the hemolysates of acatalasemic, homozygous hypocatalasemic and normal mice. MetHb formation in the hemolysate of acatalasemic mice was significantly higher than that of normal mice. The addition of catalase reduced the metHb level, while superoxide dismutase (SOD) had little effect on it. A negative intimate correlation was observed between the logarithm of catalase activity (PU/gHb) and the metHb level (%) in the hemolysate. 3-Amino-1,2,4-triazole (AT), a specific catalase inhibitor, significantly increased the formation of metHb. These results indicate that hydrogen peroxide is generated in the process of metHb formation by potassium ferrocyanide and that catalase inhibits the metHb formation by destroying the H_2O_2 produced.

Key words : methemoglobin, potassium ferrocyanide, catalase, acatalasemia, biological monitoring

Inborn enzyme deficiency may affect the toxicokinetics and toxicodynamics of chemicals. It is suggested that patients of NADH diaphorase deficiency might suffer from serious methemoglobinemia due to hypersensitivity to methemoglobin (metHb) inducers (1). Aniline derivatives, nitrobenzene and nitrite are well-known metHb inducers (2).

MetHb reduction systems such as NADH diaphorase (EC 1.6.99.3), reduced glutathione and ascorbate are necessary for maintenance of the oxygen transport function of erythrocyte (3). It is indicated that some reductants, reacting with

HbO₂, produce H₂O₂ as well as metHb (4, 5, 6). Inhibitory effect of catalase (EC 1.11.1.6) on metHb formation is suggested by the same studies (4, 5, 6).

There are some reports on the reaction of oxyhemoglobin (HbO₂) with iron (II) compounds (4, 5). Potassium ferrocyanide [K₄Fe(CN)₆] is regarded to produce metHb by reacting with HbO₂ in the hemolysate (4, 5). Hydrogen peroxide is supposed to be another product of this reaction and it may promote metHb formation independently (4, 5).

Human acatalasemia was first recognized by Takahara in 1948 (7, 8). It is characterized by low or trace activity of catalase in the blood. A

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murine model of acatalasemia was developed by Feinstein *et al.* (9). Some studies show that catalase in the liver was irreversibly inhibited by 3-amino-1,2,4-triazole (AT)(10, 11).

The purpose of the present study was to investigate the effect of catalase on methHb formation and clarify the mechanism of catalase against methHb formation. We use the hemolysate of acatalasemic mice and examined spectrophotometrically the effect of catalase on methHb formation. Potassium ferrocyanide was used as the reagent for methHb induction and AT was used to certify H_2O_2 generation.

Materials and Methods

Mice. Male acatalasemic mice (C3H/AnL Cs^bCs^b), homozygous hypocatalasemic mice (C3H/AnL Cs^cCs^c) and normal mice (C3H/AnL Cs^aCs^a) were used in this study. The animals were 8 to 16 weeks old and their body weight was 31 to 45 g. Radiation induced acatalasemic and hypocatalasemic mice were generous donation from Dr. R. N. Feinstein (9).

Reagents. Potassium ferrocyanide [$K_4Fe(CN)_6$] was obtained from Wako Pure Chemical Ind., Osaka, Japan. Bovine liver catalase (42,000 Sigma Units/mg of protein) and superoxide dismutase (2,900 U/mg) were obtained from Sigma Chemical Co., St. Louis, MO, USA. 3-Amino-1,2,4-triazole was obtained from Tokyo Kasei Co., Tokyo, Japan. All the reagents were of analytical grade or equivalent.

Assay of catalase activity. Catalase activity was determined at 20°C, according to the perborate method of Feinstein (1949), which was expressed in perborate units per gram of hemoglobin (PU/gHb)(12).

Preparation of hemolysate. Blood samples were collected from orbital vein with heparinized capillaries. These samples were centrifuged and the sediments were washed three times with physiological saline. Three volumes of distilled water was added to the sediments to hemolyze erythrocytes. The hemolysates, thus obtained, were centrifuged at 10,000 rpm for 60 min at 0°C. The supernatant was diluted with 0.1 M Na, K-phosphate buffer (pH 6.8) to adjust the hemoglobin concentration to 0.6 mg/ml.

MetHb induction. Hemolysate, which was prepared as explained above, was placed in a glass cuvet with a

light path of 1cm and then incubated in the air at 37°C without shaking. The reaction was initiated by addition of 2, 4, 8 and 16 mM final concentration of potassium ferrocyanide. MetHb formed was measured at various time intervals (5, 10, 15, 20 and 30 min), spectrophotometrically with a Hitachi model 100-50 spectrophotometer at 541 and 630 nm, according to the modified Van Kampen method (13). MetHb level was expressed as percentage of methHb to total hemoglobin [(methHb/total Hb) × 100].

To examine the effect of catalase and superoxide dismutase (SOD) on methHb formation, these substances were added before initiating the reaction of ferrocyanide with HbO_2 . AT was added in the same way to certify that H_2O_2 was produced in the reaction. Final concentrations of the reagents were as follows : catalase, 1.6 PU/ml, SOD : 80 U/ml and AT : 40 mM.

Statistical study. The difference between the means of two independent groups was tested for statistical significance. Student's *t*-test was applied when variances of two populations were assumed to be equal, while the Welch test was used in cases where they were different.

Results

Catalase activity. Catalase activities (mean ± SD) in the hemolysates of normal, homozygous hypocatalasemic and acatalasemic mice were 849.24 ± 77.18 , 227.77 ± 25.21 (26.8 % of normal) and 57.64 ± 10.48 (6.8 % of normal) PU/gHb, respectively.

MetHb formation by potassium ferrocyanide. Effect of potassium ferrocyanide on methHb formation in the hemolysates of normal and acatalasemic mice are shown in Table 1. At and after 10 min percentage of methHb level formed in acatalasemic hemolysates are significantly higher than normal in all concentrations ($p < 0.05$, *t*-test). As shown in Fig. 1, an intimate correlation is observed between the reciprocal of potassium ferrocyanide concentration and the reciprocal of methHb level. The regression equations are determined as follows :

$$\begin{aligned} \text{Acatalasemic : } Y &= 2.99 + 5.78 X \quad (r=0.989) \\ \text{Normal : } Y &= 4.06 + 7.26 X \quad (r=0.994), \end{aligned}$$

Table 1 Methemoglobin formation in the hemolysate of normal and acatalasemic mice at various time intervals in the presence of various concentrations of potassium ferrocyanide at 37°C.

Conc. of $K_4Fe(CN)_6$ (mM)	Mice ^a	Methemoglobin level (%) ^b				
		5 min	10 min	15 min	20 min	30 min
None	N	0.65 ± 0.19	0.83 ± 0.14	1.19 ± 0.23	1.34 ± 0.38	1.62 ± 0.43
	A	0.78 ± 0.10	1.04 ± 0.27	1.21 ± 0.34	1.42 ± 0.26	1.73 ± 0.49
2	N	4.92 ± 0.56	7.88 ± 0.76	10.82 ± 0.83	13.16 ± 1.47	18.10 ± 0.91
	A	5.52 ± 0.81	9.46 ± 1.30*	13.88 ± 1.39**	17.24 ± 1.01**	23.60 ± 1.54**
4	N	5.86 ± 0.68	9.42 ± 1.06	12.98 ± 1.68	16.54 ± 1.60	22.84 ± 1.77
	A	6.42 ± 0.98	12.26 ± 1.48**	16.80 ± 1.08**	21.78 ± 2.37**	28.64 ± 1.90**
8	N	6.30 ± 0.73	11.12 ± 0.40	15.72 ± 1.27	19.88 ± 1.59	27.06 ± 1.11
	A	8.84 ± 0.80**	15.74 ± 1.16**	21.34 ± 1.39**	26.04 ± 1.54**	33.60 ± 1.39**
16	N	7.62 ± 1.22	13.44 ± 0.74	18.28 ± 0.62	22.98 ± 1.90	30.58 ± 1.99
	A	12.80 ± 0.97**	21.88 ± 1.51**	27.72 ± 1.09**	31.66 ± 1.56**	38.58 ± 1.74**

a : N means normal and A means acatalasemic mice.
b : Values are expressed as mean ± SD of five mice.
The difference of means between normal and acatalasemic mice is certified by Student's *t*-test. *, *p* < 0.05 ; **, *p* < 0.01.

where Y represents the reciprocal of metHb level (total Hb/metHb) and X expresses the reciprocal

of potassium ferrocyanide concentration (1/mM). A dose effect relationship was found in both groups. Michaelis constants (*K*_m) obtained from these two equations are 1.93 mM (acatalasemic) and 1.79 mM (normal).

Effect of catalase on metHb formation. As indicated in Table 2, addition of catalase to the hemolysates of acatalasemic and normal mice reduced the metHb formation by potassium ferrocyanide. Thus catalase prevented the formation of metHb from HbO₂. The degree of inhibition depends on the dose of catalase added to the hemolysate. There was a decreasing order of metHb formation in the hemolysates of acatalasemic, hypocatalasemic and normal mice. Fig. 2 shows that a negative intimate correlation is recognized between the logarithm of catalase activity and the metHb level from the above results. The regression equation is determined as follows:

$$Y = 45.03 - 6.43 X \quad (r = 0.968),$$

where Y represents the metHb level (%) and X expresses the logarithm of catalase activity (PU/gHb).

Effect of AT. As indicated in Fig. 3, signif-

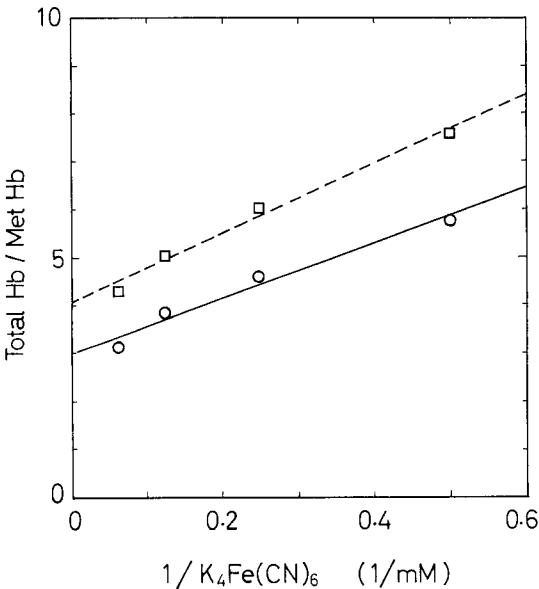


Fig. 1 Relationship between the reciprocal of potassium ferrocyanide concentration (1/mM) and the reciprocal of methemoglobin (metHb) level (total Hb/metHb) at 20 min after the addition of potassium ferrocyanide.
○, acatalasemic ; □, normal hemolysate. Each point expresses the mean of five mice.

icantly increased levels of metHb were induced by potassium ferrocyanide in normal and acatalasemic hemolysates in the presence of AT ($p < 0.01$, t -test). Catalase activities (mean $\pm$ SD, PU/gHb) in the hemolysates of normal and acatalasemic mice decreased from 849.24 ± 77.18 , 57.64 ± 10.48 to 39.18 ± 7.07 (4.6% of

the control), 4.42 ± 2.50 (7.7% of the control) at 30 min after addition of AT. The inhibitory effect of AT on catalase seems to indicate that hydrogen peroxide is produced in the reaction of HbO_2 with potassium ferrocyanide.

Effect of SOD. The results of experiments are shown in Table 3, in which SOD alone, or

Table 2 Effect of catalase on methemoglobin (metHb) formation in the hemolysate of acatalasemic and normal mice by potassium ferrocyanide^a at 37°C.

Additions ^b	Mice ^c	Catalase activity (PU/gHb)	MetHb level (%) ^d		
			10 min	20 min	30 min
None	A	57.6	15.74 ± 1.16	26.04 ± 1.54	33.60 ± 1.39
	N	849.2	11.12 ± 0.40	19.88 ± 1.59	27.06 ± 1.11
Catalase (50 μl)	A	1,383.3	12.23 ± 1.17	19.48 ± 1.52	26.81 ± 0.87
Catalase (100 μl)	A	2,633.3	10.25 ± 0.35	16.30 ± 0.73	23.43 ± 0.79
	N	3,474.2	10.08 ± 0.54	15.43 ± 0.54	21.25 ± 1.23
Catalase (200 μl)	A	5,316.7	9.15 ± 0.95	14.50 ± 0.64	20.04 ± 1.11

a : Final concentration of $\text{K}_3\text{Fe}(\text{CN})_6$ was 8 mM.

b : 50 PU/ml of catalase was added.

c : A means acatalasemic and N means normal mice.

d : Values are expressed as mean $\pm$ SD of five mice.

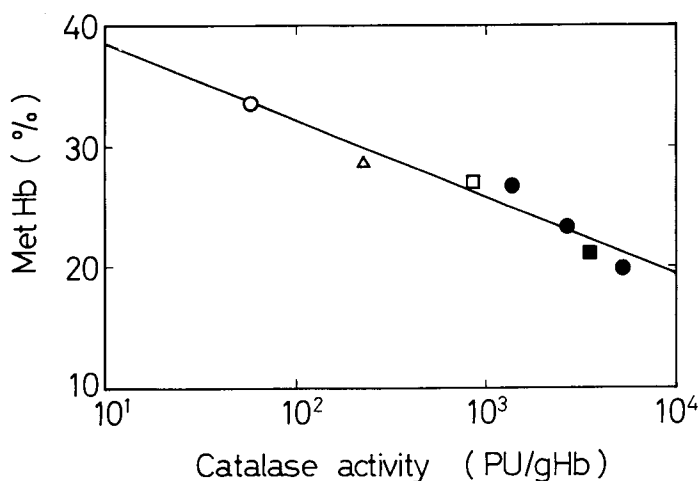


Fig. 2 Relationship between the catalase activity (PU/gHb) and the methemoglobin (metHb) formation (%) at 30 min after the addition of potassium ferrocyanide at a final concentration of 8 mM.

○, acatalasemic ; ●, acatalasemic with catalase (50 PU/ml), △, hypocatalasemic ; □, normal ; ■, normal hemolysate with catalase. Each point expresses the mean of five mice.

Table 3 Effect of superoxide dismutase (SOD) on methemoglobin (metHb) formation in the hemolysate of acatalasemic mice by potassium ferrocyanide^a at 37°C.

Additions	MetHb level (%) ^d		
	10 min	20 min	30 min
None	15.74 ± 1.16	26.04 ± 1.54	33.60 ± 1.39
SOD ^b	16.08 ± 1.56	25.64 ± 1.35	31.30 ± 0.62
Catalase ^c	11.24 ± 1.06	16.04 ± 0.45	19.94 ± 1.23
SOD ^b + catalase ^c	10.48 ± 1.40	15.14 ± 0.86	19.32 ± 1.25

a : Final concentration of K₄Fe(CN)₆ was 8 mM.
b : 80 U/ml final concentration of SOD was added.
c : 1.6 PU/ml final concentration of catalase was added.
d : Values were obtained from five mice each and expressed as mean ± SD.

SOD and catalase were added. Nearly the same levels of metHb as those without SOD were observed in each group. Thus SOD had little

effect on metHb formation by potassium ferrocyanide.

Discussion

For the maintenance of the oxygen transport function of erythrocytes, it is necessary that metHb reduction systems such as NADH diaphorase, reduced glutathione and ascorbate are able to reduce metHb to deoxyHb. It is also suggested by some studies that catalase, glutathione peroxidase (EC 1.11.1.9) and superoxide dismutase (EC 1.15.1.1) are related to the control of metHb formation (6, 14, 15).

Scott *et al.*(3) attributed the metHb reduction mainly to NADH diaphorase (73%), and to a lesser extent, to ascorbate (12%), reduced glutathione (9%) and other reductant systems (6 %). In contrast, according to the studies carried out on acatalasemic Japanese humans, and mice, slightly higher activity of glutathione peroxidase, higher to normal activity of SOD (14, 15, 16) and almost normal activity of NADH diaphorase (15, 16) were reported. It was also reported that a negative correlation ($r=-0.88$) was found between the catalase activity and the metHb concentration in the blood of mice (15). In addition, increased metHb formation was observed in the hemolysates and erythrocytes of acatalasemic Japanese humans and mice exposed

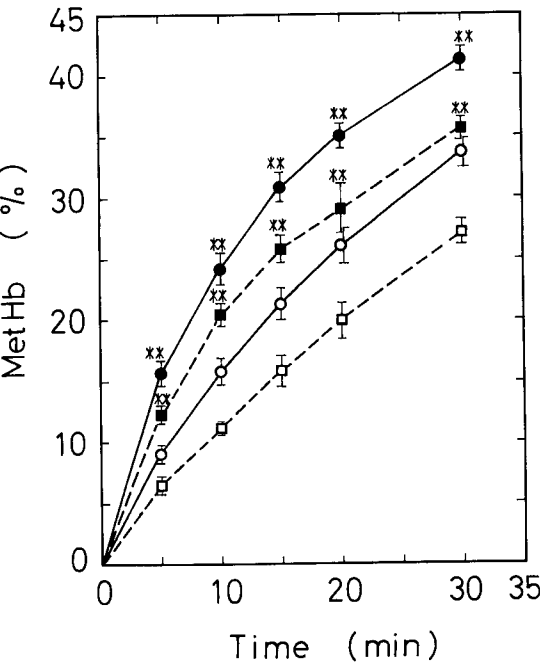
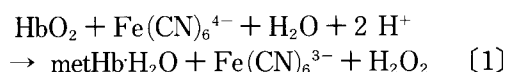


Fig. 3 Methemoglobin (metHb) formation by potassium ferrocyanide (8 mM) at various time intervals and effect of 3-amino-1,2,4-triazole (AT) (40 mM) on it.
○, acatalasemic ; ●, acatalasemic with AT ; □, normal ; ■, normal hemolysate with AT. The results are presented as mean ± SD of five mice. The differences of means (●—○ and ■—□) are certified by Student's *t*-test. * *, *p* < 0.01.

to NO and NO₂ gasses (6). Hydrogen peroxide was supposed to be another product of the reaction (6). According to these results, we can infer that catalase plays an important role in the protection against metHb formation.

In the present study, increased metHb formation was induced by potassium ferrocyanide in the homolysate of acatalasemic mice. Addition of catalase reduced the production of metHb. A negative intimate correlation ($r = -0.968$) was observed between the logarithm of catalase activity and the metHb level. These results also suggest the significance of catalase on the inhibition of metHb formation. Kawanishi and Caughey (4) proposed that the reaction of HbO₂ with ferrocyanide proceeds as follows :



Catalase is known to catalyze the reaction of H₂O₂ to H₂O and O₂. The effect of catalase in our study suggests that hydrogen peroxide is actually produced by Reaction 1. The H₂O₂ thus formed is involved in Reaction 2 and promotes the conversion of HbO₂ to metHb•H₂O. The production of H₂O₂ is supported by the decrease in the metHb level in proportion to the catalase activity. AT was used to confirm this. AT is known as an irreversible inhibitor of catalase, which reacts with catalase-H₂O₂ compound I (17). After the addition of AT, significantly increased levels of metHb formation were observed, and catalase activity was markedly reduced. In contrast, addition of SOD had little effect on metHb formation by ferrocyanide. This confirms that catalase participates in the protection against metHb formation.

The reaction system wherein HbO₂ is converted to metHb usually involves many different reactions, and therefore is complex. Kawanishi and Caughey reported a comparative study of iron

(II) compounds reacting with HbO₂ as electron donors (4, 5). They indicated that ferrocyanide as well as aquopentacyanide reacts with HbO₂ to produce metHb and H₂O₂. The production of H₂O₂ was demonstrated by the decrease in rate after the addition of catalase. They also suggested that the effect of catalase was pronounced for ferrocyanide, because the reaction of HbO₂ with aquopentacyanide was much faster, and that reaction which involved the generated H₂O₂ was less noticeable. We demonstrated the generation of H₂O₂ in the reaction from the effect of AT and catalase. The results of our experiments are consistent with the above reports. Our findings also support a mechanism, in which H₂O₂ is produced by one electron donation to heme-bound O₂ from both ferrocyanide iron (II) and heme iron (II). When H₂O₂ is produced in the reaction of HbO₂ with metHb inducers such as potassium ferrocyanide, the H₂O₂ may promote the metHb formation and catalase prevents it through destruction of the H₂O₂.

Many industrial chemicals are assumed to produce metHb and are called metHb inducers. Workers exposed to such chemicals may suffer from serious methemoglobinemia. People with hereditary enzyme deficiencies such as acatalasemia are considered to be hypersusceptible or unusually responsive to the inducers. Acatalasemia is a rare congenital anomaly. The number of acatalasemia in Japanese was estimated at about 423 by Ogata *et al.* (18), and only 90 cases among Japanese were reported to date (16, 19). Frequency of hypocatalasemia, catalase activity of which is about 50% of normal, among Japanese is calculated as 0.23% by Takahara (16). The frequency is relatively high. Monitoring of the metHb level in blood is useful to avoid the adverse health effects. In the field of biological monitoring, ACGIH (American Conference of Governmental Industrial Hygienists) listed metHb inducers, and recommended 1.5% of metHb in the blood as BEI (Biological Exposure Index) for them in 1989 (20). In performing biological monitoring, we should consider genetic factors

such as acatalasemia, hypocatalasemia, when data are interpreted. Such workers are regarded as high risk groups. Occupational physicians, therefore, should evaluate the extent, to which they require additional protection.

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