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Effect of 3-Amino-1,2,4-triazole on Catalase and Formation of Methemoglobin from Oxyhemoglobin in Erythrocyte by Superoxide Radicals[1)]

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The formation of methemoglobin (metHb) from oxyhemoglobin (oxyHb) in the erythrocyte by O_2^- was studied under the O_2^--generating system of photoactivated riboflavin with oxygen by using a catalase inhibitor, 3-amino-1,2,4-triazole (AT). In the AT-treated erythrocytes *in vitro* which have little catalase activity, the rate of metHb formation was increased remarkably compared with that of untreated normal erythrocyte. Furthermore, catalase added to 60 unit/ml to the suspending medium contained AT-treated erythrocytes caused a strong inhibition of formation of metHb, suggesting that H_2O_2 is generating in the external medium of erythrocytes by the reaction of photoactivated riboflavin or spontaneous dismutation of O_2^-. Addition of superoxide dismutase (5 μg/ml) caused an acceleration but not inhibition of the metHb formation. Hemolysis was scarcely observed in the time course of this experiment. These results may be interpreted that H_2O_2 generated in the outside of cells moves across the membrane and results in oxidative attack to oxyHb in the erythrocyte without the hemolytic effect. Another possible participation of O_2^- may be suggested to exert its enhanced oxidation of oxyHb, namely that O_2^- generated in the outside of cells may pass through the membrane directly.

Keywords——superoxide radical; hydrogen peroxide; erythrocyte; superoxide dismutase; catalase; 3-amino-1,2,4-triazole; oxyhemoglobin; methemoglobin

Superoxide radicals (O_2^-) are now known to be produced in a large number of biochemical reactions.[3)] On the other hand, superoxide dismutase has been proposed to protect cells against potential damaging effects of O_2^- by scavenging catalytically with a great efficiency.[4)] To research in the damaging effects of O_2^- within cells, erythrocytes having a definite life span are particularly suitable because of their high contents of superoxide dismutase, catalase and hemoglobin. As the first study attempting to elucidate the role of superoxide dismutase in erythrocytes, we have examined the possible participation of O_2^- in the formation of methemoglobin (metHb) from oxyhemoglobin (oxyHb) under the O_2^--generating system of photoactivated riboflavin through the use of an enzyme inhibitor, diethyldithiocarbamate (DDC) and suggested that catalase rather than superoxide dismutase is more responsible for the inhibition of metHb formation by O_2^-.[1)]

It is well known that 3-amino-1,2,4-triazole (AT) is a specific inhibitor of catalase and a potent cataractogenic agent.[5)] Heim, *et al.*[6)] have shown that catalase activity in the liver and kidney, but not blood cells, of rats and mice was inhibited markedly within a few hours

1) *a*) T. Miura, N. Ogawa and T. Ogiso, *Chem. Pharm. Bull.*, **26**, 1261 (1978); *b*) A part of this study was presented at the 98th Meeting of the Pharmaceutical Society of Japan, Okayama, 1978.
2) Location; *7-1, Katsuraoka-cho, Otaru, 047-02, Japan.*
3) *a*) H.P. Misra and I. Fridovich, *J. Biol. Chem.*, **247**, 6969 (1972); *b*) R. Wever, B. Oudega and B.F. Gelder, *Biochem. Biophys. Acta*, **302**, 475 (1973); *c*) J. Forman and J. Kennedy, *Arch. Biochem. Biophys.*, **173**, 219 (1976); *d*) M. Brunori, G. Falcoini, E. Floretti, B. Giardina and G. Rotillio, *Eur. J. Biochem.*, **53**, 99 (1975); *e*) C.C. Winterbourn, B.M. McGrath and R.W. Carall, *Biochem. J.*, **155**, 493 (1976); *f*) H.C. Sutton, P.B. Robert and C.C. Winterbourn, *ibid.*, **155**, 503 (1976).
4) M. McCord and I. Fridovich, *J. Biol. Chem.*, **244**, 6048 (1969).
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after the injection of AT, although the liver cytochrome c and hemoglobin level were not affected. The catalase activity in blood hemolysate has been shown to be inhibited irreversibly in the presence of hydrogen peroxide or ascorbic acid *in vitro* by AT.[7] The present paper is concerned with further investigation on hypothesis by using the catalase inhibitor, AT, that catalase but not superoxide dismutase in erythrocytes has protective ability for the conversion of oxyHb to metHb by O_2^-.

Experimental

Materials——Catalase (from beef liver, thymol free) and superoxide dismutase were obtained from Sigma Chemical Co., 3-amino-1,2,4-triazole (AT) from Wako Pure Chemical Industries, Ltd. Other agents used were of analytical grade.

Animals——Male Wistar strain rats (180 to 200 g) were used throughout this experiment. A solution of AT dissolved in 0.9% NaCl was injected intraperitoneally into rats (1 g/kg of body weight). Control rats received the same volume of saline vehicles. Rats were sacrificed 6 hr later and blood was taken from the common carotid artery into heparinized tubes. The liver and kidney were removed and then homogenized in 50 mM phosphate buffer, pH 7.0, followed by centrifugation at $10000 \times g$ for 10 min at 4°. The supernatant obtained was subjected to the subsequent enzyme assay as described below.

Preparation of AT-treated Erythrocyte and Lysate——Erythrocytes were sedimented by centrifugation at $1500 \times g$ for 10 min at 4°, washed with 0.9% NaCl three times and then incubated at 37° for various times in phosphate buffer, pH 7.4, containing 0.15 M NaCl, 2 mM ascorbic acid and 20 mM AT. AT-treated erythrocytes obtained were sedimented by centrifugation at $1500 \times g$ for 10 min at 4° and washed with 0.9% NaCl three times. Erythrocytes were lysed in 10 volume of distilled water. After 10 min, ghosts were sedimented by centrifugation at $10000 \times g$ for 10 min at 4°. The supernatant was removed and kept on ice until use.

Enzyme Assay——Assay for catalase is based on the decomposition of H_2O_2, which can be followed spectrophotometrically at 240 nm.[8] One unit of activity was defined as the amount of enzyme which decomposes 1 μl of H_2O_2/ml at 25°. Superoxide dismutase activity was determined by the method described previously,[1] which is based on its ability to inhibit the autoxidation of 6-hydroxydopamine. Catalase inhibitor, AT, had no effect on the activity of superoxide dismutase under the conditions used. Protein was quantified by the method of Lowry, *et al.*[9] using bovine serum albumin as a standard.

Gel Electrophoresis——Red cell proteins were separated by polyacrylamide disc gel electrophoresis according to the method of previous investigators.[10] Electrophoresis was performed at 2 mA per tube until the bromphenol blue marker dye reached the bottom of tubes. Gels were stained for superoxide dismutase by nitroblue tetrazorium[10] and catalase by iodine–starch procedure.[11]

Oxidation of Hemoglobin——O_2^- for reaction with oxyHb was generated by the reaction of photoactivated riboflavin with oxygen.[3b] Erythrocyte suspension of 0.5% hematocrit was incubated in O_2-saturated 10 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl, 1 mM EDTA and 5.3 μM riboflavin. The illumination was carried out in an alminium foil lined box equipped with two 15W fluorescent lamps as described previously.[1] The oxidation of oxyHb by O_2^- was measured by determining the rate of increase in absorbance at 630 nm or by recording the spectral changes between 520 and 660 nm on a spectrophotometer (Hitachi, Model, 200—20). The concentration of oxyHb in the lysate or reaction mixture was determined at 576 nm using the extinction coefficient given by Benesh, *et al.*[12] Amount of metHb was quantified by measuring the decrease in absorbance at 630 nm by the formation of cyanmethemoglobin in the reaction with KCN.[13]

Results

Inhibition of Catalase and Superoxide Dismutase in Erythrocyte by AT *in Vitro*

In order to obtain erythrocyte having no catalase activity, erythrocyte were pretreated with AT in the presence of ascorbic acid and a condition in which catalase is completely in-

7) *a*) E. Margoliash and A. Novogrodsky, *Biochem. J.*, **68**, 469 (1958); *b*) E. Margoliash, A. Novogrodsky and A. Shejter, *ibid.*, **74**, 339 (1959).
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hibited was determined. Figure 1 shows the relationship between AT concentration and per cent inhibition of both enzyme activities by the incubation for 2.5 hr at 37°. The inhibitory effect of AT on catalase increased with increasing concentrations of AT and the activity was lost about 90% at the concentration of 20 mM AT. At the concentration of AT above 30 mM, total activity of catalase in erythrocyte was almost completely lost and a spontaneous oxidation of oxyHb was observed. Superoxide dismutase, on the other hand, was not sensitive to AT and hemolysis was scarcely observed during the course of incubation. In the system without ascorbic acid, AT did not cause any inhibition of catalase activity, indicating that the inhibition by AT requires the presence of H_2O_2 or its sources such as ascorbic acid as reported by previous workers.[7]

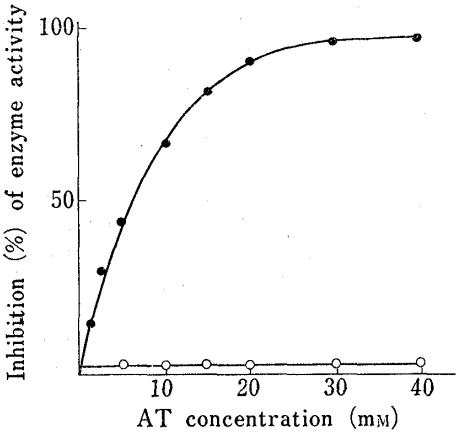

Fig. 1. Effect of AT on Catalase and Superoxide Dismutase in Erythrocyte *in Vitro*

Erythrocyte suspension of 5% hematocrit was incubated in phosphate buffer, pH 7.4, containing 0.15 M NaCl, 2 mM ascorbic acid and various concentrations of AT. Enzyme activity was estimated after the incubation with AT for 2.5 hr at 37°. —●—; catalase, —○—; superoxide dismutase.

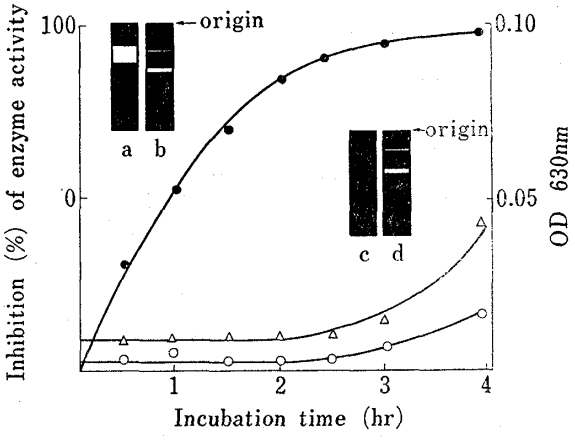

Fig. 2. Inhibition of Catalase and Superoxide Dismutase Activity and Changes in Absorbance of Hemoglobin in Erythrocyte after Incubation with 20 mM AT for Various Times at 37°

The conditions are the same as those given in the Fig. 1.
—●—; catalase, —○—; superoxide dismutase, —△—; change in absorbance.
Stained patterns on polyacrylamide gel of both enzymes in erythrocytes were as follows; (a); catalase and (b); superoxide dismutase before the incubation with AT, (c); catalase and (d); superoxide dismutase 2.5 hr after the incubation with AT.

Figure 2 shows that inhibitory effect of AT on each enzyme was time dependent in fashion. Per cent inhibition of catalase reached about 90% but superoxide dismutase did not change 2.5 hr after the incubation with 20 mM AT. In the time course of the incubation over 3 hr, erythrocytes began to exhibit the spontaneous oxidation of oxyHb and an increase in absorbance at 630 nm. It is possible that this increase in absorbance is not all due to the formation of metHb because the absorption peak did not disappear completely by the addition of excess KCN. Rate of the inhibition of superoxide dismutase by AT was fairly slow, remaining 85% of total activity, even 4 hr after the incubation. In this case also, hemolysis was not observed during the course of incubation. The data presented here demonstrated that catalase-inhibited erythrocytes which have less than 10% of the original activity, can be obtained by the incubation procedure with AT in the presence of 2 mM ascorbic acid for 2.5 hr at 37°. These results were confirmed by polyacrylamide gel electrophoresis as illustrated in Fig. 2. The enzyme activity of catalase in erythrocytes incubated with AT for 2.5 hr was not detected on gel by the staining procedure. However, superoxide dismutase remained unchange in stained bands giving two major bands in which the slower one was coincident with hemoglobin. This band is probably due to interaction of hemoglobin with O_2^- as discussed by previous workers.[10a]

Formation of MetHb from OxyHb in Erythrocyte Treated with AT

Effects of O_2^- on the formation of metHb in the erythrocytes treated with AT were examined and typical results were shown in Fig. 3. In the untreated-normal erythrocytes which have both enzymatic activities, the formation of metHb was scarcely observed during the course of illumination for 60 min. On the other hand, in the AT-treated erythrocytes which have little catalase activity, the formation of metHb was progressively proceeded with time of illumination. When the excess KCN was added to the reaction mixture, the absorption peak at 630 nm decreased completely, indicating that metHb is the major product in this reaction. Hemolysis was not observed in both AT treated- and untreated-erythrocyte during the course of illumination. These results indicated that the enhanced formation of metHb is attributed to the loss of enzymatic activity of catalase in the AT-treated erythrocytes and suggested that H_2O_2 rather than O_2^- may play a role for the oxidation of oxyHb as reported previously.[1)]

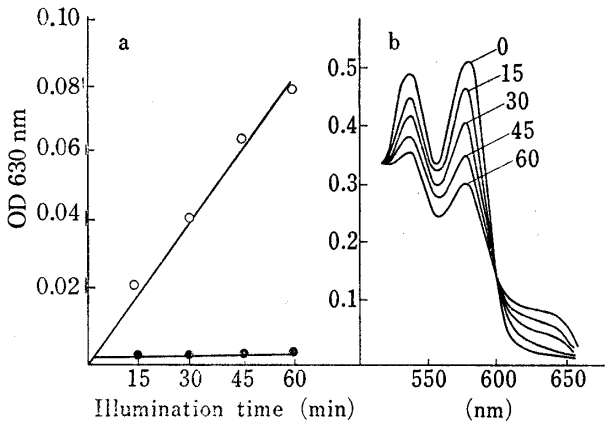

Fig. 3. Effect of Superoxide Radicals on the Rate of Oxidation (a) and Changes in Spectrum of OxyHb (b) in Erythrocyte Pretreated with AT

Catalase inhibited-erythrocytes (30 μM oxyHb) were incubated in 10 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl, 5.3 μM riboflavin and 1 mM EDTA at 37° and the oxidation reaction was initiated by illumination (a). Erythrocytes were lysed after various time of illumination and then changes in absorption spectrum were recorded (b). Numbered curves refer to illumination time.
—○—; AT-treated erythrocyte,
—●—; untreated-normal erythrocyte.

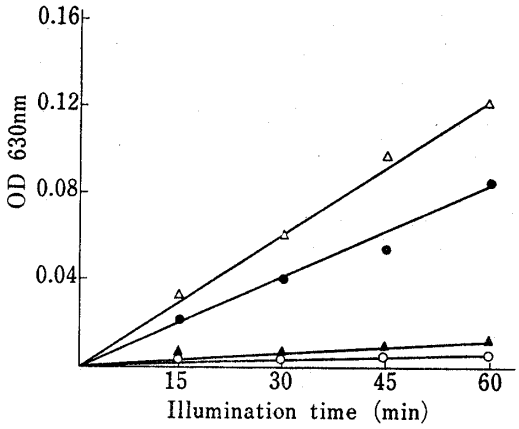

Fig. 4. Effect of Superoxide Radicals in External Fluid of Erythrocyte on the Rate of Oxidation of OxyHb

AT treated- or untreated-erythrocytes were incubated in 10 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl, 5.3 μM riboflavin and 1 mM EDTA at 37° and the oxidation reaction was initiated by illumination. —△—; +superoxide dismutase (5 μg/ml), —●—; no addition, —▲—; +catalase (60 unit/ml), —○—; untreated-normal erythrocyte.

To determine whether H_2O_2 is generated from O_2^- in the external medium of erythrocyte and whether H_2O_2 by itself could be diffused through the membrane of erythrocyte, pure catalase or superoxide dismutase was added to the suspending medium. As can be seen in Fig. 4, catalase added to 60 unit/ml to the suspending medium contained AT-treated erythrocytes caused a inhibition of formation of metHb to almost the same level as that in the normal erythrocytes, indicating that H_2O_2 is generating in the external medium of erythrocyte by the reaction of photoactivated riboflavin or spontaneous dismutation of O_2^-. Addition of superoxide dismutase at 5 μg/ml, however, resulted in an acceleration of the formation of metHb in the AT-treated erythrocytes, suggesting that H_2O_2 generated from O_2^- by enzymatic activity of superoxide dismutase may be possibly diffused into cells.

Effect of O_2^- on the Rate of Oxidation of OxyHb in the Lysate from Erythrocyte Treated with AT

Previous observations[1)] that H_2O_2 rather than O_2^- plays a role for the formation of metHb were confirmed by using the lysate obtained from catalase-inhibited erythrocyte treated with AT. The formation of metHb from oxyHb was proceeded during the course of illumination

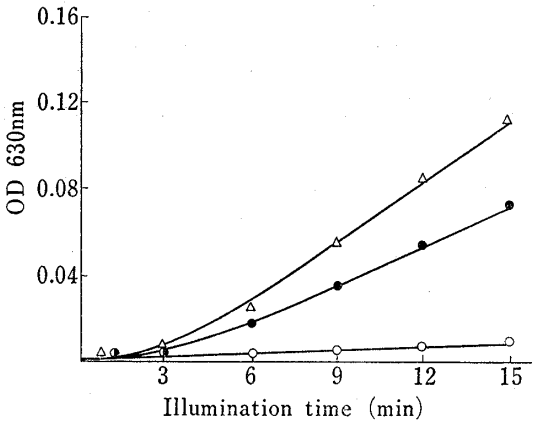

Fig. 5. Effect of Catalase and Superoxide Dismutase on the Rate of Oxidation of OxyHb in the Lysate from AT-treated Erythrocyte

Pure catalase or superoxide dismutase was added to the reaction mixture contained 1 mM EDTA, 5.3 μM riboflavin and erythrocyte lysate (30 μM oxyHb) in 3.0 ml of phosphate buffer, pH 7.4. The oxidation reaction was initiated by illumination.
—△—; +superoxide dismutase (5 μg/ml),
—●—; no addition,
—○—; +catalase (60 unit/ml).

as shown in Fig. 5. On the other hand, catalase added at 60 unit/ml to the reaction mixture caused a strong inhibition of metHb formation, whereas addition of 5 μg of superoxide dismutase caused an acceleration but not inhibition of the formation of metHb. These results suggested that H_2O_2 is formed from O_2^- by an enzymatic activity of superoxide dismutase. These results were nearly consistent with the previous observations.[1] Each enzyme was not inactivated by O_2^- during the course of illumination for 15 min as described previously.[1] Furthermore, no precipitation was apparent during time of this observation and the formation of metHb from oxyHb by O_2^- in the lysate occurred more rapidly compared with that in the AT-treated erythrocytes (Fig. 3).

The possible mechanism for the formation of metHb may be associated with other radicals, *i.e.* hydroxyl radicals or singlet oxygens. To testified this possibility, several scavengers were used as follow; benzoate and *tert*-butyl alcohol as hydroxyl radical scavengers, diphenylisobenzofuran and bilirubin as singlet oxygen scavengers. Although data not shown in Fig. 4, their all failure to protect the oxidation of oxyHb excluded the possibility of participation of these excited forms of oxygen under the conditions used.

Discussion

Heim, *et al.*[6] have shown that the injection of AT into rats causes a rapid decrease in the catalase activity of the liver and kidney, but has no effect on the catalase activity of blood, suggesting that this lack of AT effect on blood *in vivo* may be due to an inability of AT to penetrate the cell membranes. Whereas, Margoliash, *et al.*[7] have demonstrated that the presence of H_2O_2 is required for the irreversible inhibition of catalase, involving the reaction between the catalase-H_2O_2 complex I and AT, suggesting that the lack of AT effect on catalase of blood may possibly be ascribed to the lack of the available amount of H_2O_2 in blood.

Although data not shown in this paper, *i.p.* injection of AT to rats caused a marked decrease in catalase activity of liver and kidney except blood. The catalase activity of lung and testis diminished to a lesser extent, while the spleen and heart were not affected by the injection of AT. On the other hand, superoxide dismutase in these tissues was not inhibited by this treatment. These results were consistent with the result reported by Heim, *et al.*[6]

The experiments *in vitro*, however, showed that catalase in the erythrocyte was inhibited strongly, but superoxide dismutase was little affected by the incubation with 20 mM AT in the presence of ascorbic acid as the source of H_2O_2 (Fig. 1,2). These results indicated the possible permeability of AT through the cell membranes at 20 mM concentration of AT and suggested that the lack of AT effect in blood *in vivo* may be regarded as being too low concentration of AT in blood rather than inability of AT to penetrate the cell membrane. Moreover, AT did not show any inhibition of catalase activity in the cells by the incubation in the absence of ascorbic acid as described in Fig. 1. These results seem to suggest that the intact cells have no sufficient H_2O_2 to induce the inhibitory effect of AT on catalase activity as discussed

by Morgoliash, *et al.*[7] Indeed, catalase and glutathione peroxidase (EC.1.11.1.7) are known to be present in the erythrocyte to diminish the toxic effects caused by H_2O_2.[14]

The authors have shown previously by using a potent inhibitor of superoxide dismutase, DDC, that catalase but not superoxide dismutase in the erythrocyte has a protective ability for oxidative effect of O_2^- on the conversion of oxyHb to metHb.[1] If catalase functions to prevent metHb formation, the cells with lowered content of this enzyme should exhibit an enhanced rate of metHb formation induced by the exposure to O_2^- *in vitro*. In the erythrocytes pretreated with AT *in vitro* which contain little catalase activity, the rate of metHb formation induced by the exposure to O_2^- was significantly increased compared with that of the normal erythrocytes (Fig. 3). Furthermore, this conversion of oxyHb to metHb was almost completely depressed to the normal level by the addition of catalase into the suspending medium and somewhat accelerated by superoxide dismutase (Fig. 4). These results offer evidences that H_2O_2 generated in the outside of cells moves across the membrane and results in oxidative attack to oxyHb in the erythrocyte. Another possible participation of O_2^- may be supposed to exert its enhanced oxidation of oxyHb, namely that O_2^- generated in the outside of cells may pass through the membrane directly. Lynch, *et al.*[15] have recently reported that O_2^- generated enzymatically in the internal vecicles prepared from erythrocyte stroma could move easily across the membrane and causes lysis of the stromal membrane. Whether O_2^- by itself could pass through the outer membrane of erythrocyte or not remains to be elucidated in further experiments.

There are some informations relating to role of O_2^- as a mediator of oxidative hemolysis.[16] The hemolysis of cells by O_2^- generated in the xanthine oxidase reaction is known to exhibit a marked induction period with a very slow rate and to depend on cell concentration, that is, no detectable hemolysis could be obtained at high concentration of cells.[15,16c] Furthermore, H_2O_2 is suggested to be not an important agent in the attack on membrane. In the present study, hemolysis was scarcely observed in both erythrocytes treated and untreated with AT through the course of illumination for 60 min, probably due to the shorter period of incubation and the higher concentration of cells employed in this experiment. During this incubation, oxyHb in the erythrocytes treated with AT was found to be converted to metHb, indicating probably that H_2O_2 is not correlated with hemolysis but the oxidative conversion of oxyHb to metHb.

The relative rate of metHb formation in the lysate from AT-treated erythrocyte was about 4 times greater than that in the AT-treated erythrocyte under the O_2^- generating system. This conversion of oxyHb to metHb was almost completely depressed by the addition of catalase and somewhat accelerated by superoxide dismutase (Fig. 5). These results also suggested that O_2^- is appeared to be one of the source of H_2O_2 by which the formation of metHb is accelerated and are in agreement with our finding that O_2^- by itself is not responsible for the oxidation of oxyHb in the O_2^- generating system.

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