[Chem. Pharm. Bull. 26(4)1261—1266(1978)]

UDC 577.158.04 : 546.21-128.08.09

Involvement of Superoxide Radicals in the Formation of Methemoglobin from Oxyhemoglobin: Inhibition of Superoxide Dismutase by Diethyldithiocarbamate[1]

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(Received October 7, 1977)

Diethyldithiocarbamate (DDC), a copper chelate agent, was used to examine a possible participation of superoxide radicals (O_2^-) in the formation of methemoglobin (metHb) under the O_2^- generating system of photoactivated riboflavin. Superoxide dismutase in the lysate from DDC-injected rats was inhibited to 93% and catalase remained unchange, however, the formation of metHb was scarcely observed. In the experiments *in vitro*, superoxide dismutase was completely inhibited by the pretreatment with DDC above 3 mM whereas catalase was partially inhibited. In this case, the formation of metHb was accelerated with increase of DDC concentration. Addition of catalase to the dialyzed lysate resulted in the inhibition of formation of metHb to almost the same level as that in the normal lysate. Superoxide dismutase alone, however, caused a slight increase in the rate of formation of metHb, indicating that catalase but not superoxide dismutase has protective ability to the formation of metHb by O_2^-. From these results, O_2^- appears to be the source of hydrogen peroxide which accounts for most the observed metHb formation.

Keywords——superoxide dismutase; catalase; inhibition; erythrocyte; oxyhemoglobin; methemoglobin; superoxide radicals

Superoxide dismutase, which is copper-zinc protein, has been detected widely in living organisms and proposed to protect cell from a potential damaging effect of superoxide radicals (O_2^-) by dismutation to hydrogen peroxide and oxygen.[3] The O_2^- is now known to be produced in a large number of biological reactions, one of which is the autoxidation of oxyhemoglobin (oxyHb) to methemoglobin (metHb).[4] Hemolgtic agents, phenylhydrazine and menadione, have been demonstrated to react with oxyHb to cause a rapid generation of O_2^- overwhelmed superoxide dismutase, in erythrocyte.[5] Moreover, Lynch, *et al.*[6] have shown that the formation of metHb from oxyHb in the O_2^- generating system of photoactivated riboflavin was inhibited by superoxide dismutase, suggesting that this ability may represent one of its function in cells. The exact significances of this enzyme in erythrocyte, however, have not been clearly understood yet.

The recent demonstration by Heikkila, *et al.*[7a] that diethyldithiocarbamate (DDC), a copper chelating agent, acts as a potent *in vivo* inhibitor of superoxide dismutase provides

1) A part of this study was presented at the 66th Meeting of Hokkaido Branch of Pharmaceutical Society of Japan, Sapporo, 1977.

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3) *a*) I. Fridovich, *Ann. Rev. Biochem.*, **44**, 417 (1975); *b*) F.J. Yost and I. Fridovich, *Arch. Biochem. Biophys.*, **175**, 514 (1976); *c*) J.M. MacCord, B.B. Keele, and I. Fridovich, *Pro. Natl. Acad. Sci. U.S.*, **68**, 1024 (1971).

4) *a*) H.P. Misra and I. Fridovich, *J. Biol. Chem.*, **247**, 6969 (1972); *b*) R. Wever, B. Oudega, and B.F. Van Gelder, *Biochem. Biophys. Acta.*, **302**, 475 (1973); *c*) M. Brunori, G. Falcoini, E. Floretti, B. Giardina, and G. Rotillio, *Eur. J. Biochem.*, **53**, 99 (1975); *d*) C.C. Winterbourn, B.M. McGrath, and R.W. Carall, *Biochem. J.*, **155**, 493 (1976); *e*) H.C. Sutton, P.B. Roberts, and C.C. Winterbourn, *ibid*, **155**, 503 (1976).

5) *a*) B. Goldberg and A. Stern, *J. Biol. Chem.*, **250**, 2401 (1975); *b*) *idem*, *Biochem. Biophys. Acta.*, **437**, 628 (1976); *c*) *idem*, *Arch. Biochem. Biophys.* **178**, 218 (1977).

6) R.E. Lynch, G.R. Lee, and G.E. Cartwright, *J. Biol. Chem.*, **251** 1015 (1976).

7) *a*) R.E. Heikkila, F.S. Cabbat, and G. Choen, *J. Biol. Chem.*, **251**, 2181 (1976); *b*) B. Goldberg and A. Stern, *J. Biol. Chem.*, **251**, 6468 (1976).

a useful tool for investigation of toxic action of O_2^- and a physiological role of this enzyme in cells.[7b] The purpose of the present study is to elucidate a role of this enzyme through the use of this inhibitor and to examine a possible participation of O_2^- in the formation of metHb in erythrocyte.

Experimental

Materials——Pure superoxide dismutase and hemoglobin (bovine erythrocyte) were obtained from Sigma Chemical Co. Catalase from Miles Lab. Lnc. was dialyzed against 0.02 M phosphate buffer, pH 7.4, prior to use. Hemoglobin was reduced with sodium dithionate. Excess reducing agents were removed by gel filtration on Sephadex G-100 and then oxyHb was prepared by bubbling oxygen through the solution. Sodium diethyldithiocarbamate and 6-hydroxydopamine HBr salt were purchased from Eastman Kodak and Aldrich Chemical Co. respectively. Other agents used were of analytical grade.

Animals——Male Wistar strain rats (180 to 200 g) were used for this experiment. Sodium diethyldithiocarbamate, 1.5 g/kg of body weight, dissolved in an isotonic saline was injected intraperitoneally into rats. Control rats received the same volume of the saline vehicle (1.0 ml). Rats were sacrificed 2 hr later and blood was taken from the common carotid artery in heparinized centrifuge tubes.

Preparation of Lysate——Erythrocyte was sedimented by centrifugation at 4°, washed with 0.9% NaCl three times, then lysed in 10 volumes of distilled water. After 10 min, ghosts were sedimented by centrifugation at 4° for 10 min at $10000 \times g$. The supernatant was removed and kept on ice untill use for subsequent experiments.

Enzyme Assay——Superoxide dismutase was measured by the method of Heikkila, *et al.*,[8] which is based on its ability to inhibit the autoxidation of 6-hydroxydopamine (6-HDA). The medium consisted of 50mM phosphate buffer, pH 7.4, containing 10^{-3}M EDTA. A solution of 6-HDA (0.2M) was prepared in distilled water sparged with nitrogen gas and added to the medium to be 2×10^{-2} M in a final volume of 10 ml. The increase in an absorbance at 490 nm was then measured for 30 sec on a spectrophotometer equipped with a flow cell attached to shipper unit (Hitachi, Model 200-20) and the increase at 15 sec was taken as the initial rate of autoxidation of 6-HDA. A standard curve relating the amount of superoxide dismutase to the percentage of inhibition of initial rate was constructed, which was virtually linear up to 100 μg/ml of superoxide dismutase. Assay methods based on the reduction of nitro blue tetrazorium and ferricytochrome c[9] were unfavorable to quantify superoxide dismutase in the presence of DDC, because the rate of reduction was so much affected by DDC. The assay for catalase was based on the decomposition of hydrogen peroxide, which can be followed spectrophotometrically at 240 nm.[10] One unit of activity was defined as the amount of enzyme which decomposes 1 μl of H_2O_2/ml at 25°.

Oxidation of Hemoglobin by O_2^-——Superoxide radicals were generated by the reaction of photoactivated riboflavin with oxygen.[4b] A reaction mixture containing 1 mM EDTA and 5.3 μM riboflavin in phosphate buffer, pH 7.4, was put in a quartz cuvette and bubbled with oxygen for 10 min. The illumination was carried out in an alminum foil lined box equipped with two 15 W fluorescent lamps. To provide homogeneous illumination, the cuvette was usually placed at a definite position in the box throughout experiments. To make sure that O_2^- is generating in the reaction mixture, the rate of reduction of nitro blue tetrazorium and ferricytochrome c was measured spectrophotometrically at 600 and 550 nm, respectively. In both cases, the rate of reduction was inhibited by superoxide dismutase (10 μg/ml) to 90—95% and only 10% by catalase (60 unit/ml), thereby indicating the generation of O_2^- in the reaction mixture. The time course of oxidation of oxyHb by O_2^- was measured at an initial concentration of 30 μM of oxyHb. At time intervals, the cuvette was transfered to a recording spectrophotometer (Shimazu, MPS-5000) and scanned between 500 and 680 nm. In some experimenrts, the decrease in absorbance at 576 nm was measured as an estimate of the rate of formation of metHb. The concentration of oxyHb in the lysate or reaction mixture was determined at 576 nm using the extinction coefficient given by Benesh, *et al.*[11]

Results

Effect of DDC *in Vivo*

Table I shows a marked decrease in superoxide dismutase in the blood of rats 2 hr after injection of DDC. In this case, the loss of activity was about 93%. No enzymatic activity of superoxide dismutase was found in the serum from both groups, therefore, the change

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9) C. Beauchamp and I. Fridovich, *Anal. Biochem.*, **44**, 276 (1971).
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Table I. Superoxide Dismutase and Catalase Contents in the Blood from Rats Injected with DDC

	Superoxide dismutase μg/ml	Catalase unit ($\times 10^{-4}$)/ml
Normal	188±28	10.5±0.6
DDC-injected	14± 6[a)]	8.7±0.6[b)]

Each group of rats was killed 2 hr after *i.p.* injection of DDC (1.5 g/kg). The assay for each enzyme was carried out in blood by the method as described in the text. Values represent mean ± standard error of 5 rats.
a) Significantly different from normal, $p < 0.01$.
b) No significantly different from normal, $p > 0.1$.

of this enzyme is mainly attributed to that in erythrocyte. Catalase level in DDC-injected rats was significantly different from that of normal. Superoxide dismutase in the liver, kidney, and spleen was also strongly inhibited, while in brain and testis it diminished to a lesser extent (data not shown). The magnitudes of decrease in superoxide dismutase as described above were nearly consistent with the results reported by earlier workers on the brain, liver and blood of mice 3 hr after injection of DDC.[7a)]

Such an animals which has little superoxide dismutase was of particular interest to us to demonstrate whether or not superoxide dismutase plays a protective role on the conversion of oxyHb to metHb by O_2^-. Effects of O_2^- on the formation of metHb in the lysate from DDC-injected rats were examined and illustrated in Fig. 1. In the lysate from DDC-injected rats, absorption maximum at 576 nm characteristic of oxyHb showed a slow decrease with time and no increase in absorbance at 630 nm corresponding to the formation of metHb was observed. Although the data not shown, these changes were almost the same extent as that in the lysate from the normal blood. On the other hand, oxyHb prepared from pure hemoglobin was progressively oxidized as indicated by the decrease in absorbance at 576 nm and an absorption peak appeared at 630 nm. These results indicated that under the reaction conditions used, the oxyHb was not readily oxidized to metHb even if superoxide dismutase were inhibited up to about 93% by injection of DDC.

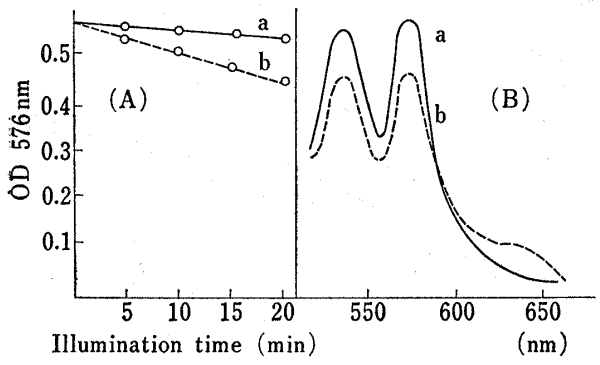

Fig. 1. The Rate of Oxidation (A) and Change in Spectra of OxyHb (B) after Exposure to O_2^-

Spectra (B) were recorded 20 min after illumination.
a) Lysate from DDC-injected rats.
b) Pure-oxyHb from bovine erythrocytes.

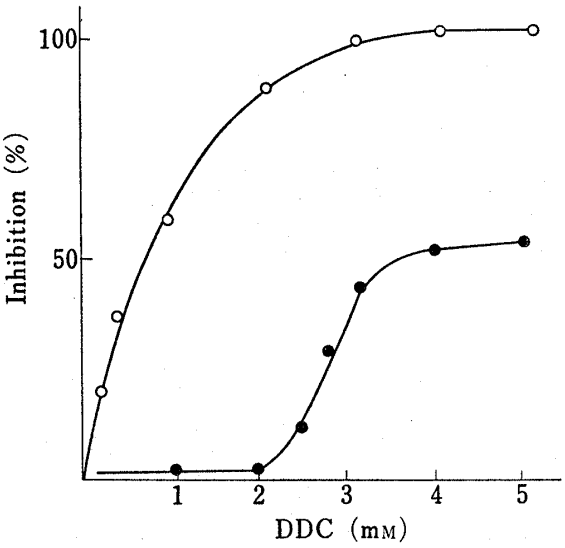

Fig. 2. Inhibitory Effect of DDC on Superoxide Dismutase and Catalase in the Lysate

Reaction mixture contains 1 mM EDTA and erythrocyte lysate (30 μM of oxyHb) in 3.0 ml of phosphate buffer, pH 7.4. Enzymes were estimated after the incubation with DDC for 60 min at 37°. Data are the mean of triplicate experiments.
—○—: superoxide dismutase, —●—: catalase.

Inhibition of Superoxide Dismutase and Catalase by DDC *in Vitro*

The normal lysate were pretreated with DDC *in vitro* and the conditions in which superoxide dismutase is completely inhibited by DDC were determined. Fig. 2 shows the relationship between DDC concentration and percent inhibition of both superoxide dismutase and catalase in the lysate. The inhibitory effect of DDC on superoxide dismutase increased with increasing concentration of DDC and total activity was completely lost at concentration of above 3 mM. Catalase, on the other hand, was much less sensitive to DDC and partially inhibited at level above 3 mM but not at below 2 mM.

As shown in Fig. 3, inhibitory effects of DDC on each enzyme were time-dependent. Preincubation together with 3 mM DDC for 60 min resulted in a complete inhibition of superoxide dismutase, while 30 min of preincubation caused about 60% inhibition. The rate of inhibition on catalase was fairly slow, reaching a steady level of around 50% after 90 min of the preincubation. In addition, there was little effect of DDC on each enzyme at zero time of the preincubation. The data presented here indicated that superoxide dismutase in the lysate is inactivated completely by the preincubation with DDC above 3 mM for 60 min associated with the partial inactivation of catalase.

Formation of MetHb from OxyHb in the Lysate Treated with DDC

The effect of O_2^- on the oxidation of oxyHb were examined in the lysate treated with various concentrations of DDC and illustrated in Fig. 4 by the decrease in absorbance at 576 nm. DDC accelerated the formation of metHb in concentration of DDC above 3 mM. In the normal lysate, the oxidation proceeded at a slow rate which was the same extent as that of concentration of DDC below 2 mM. Each enzymatic activity of these preparation was determined at the end of illumination. Consequently, the reaction mixture containing DDC above 3 mM did not have any enzymatic activity of superoxide dismutase but catalase of 25 to 30 unit/ml. In the normal lysate, superoxide dismutase and catalase were 5 μg/ml and

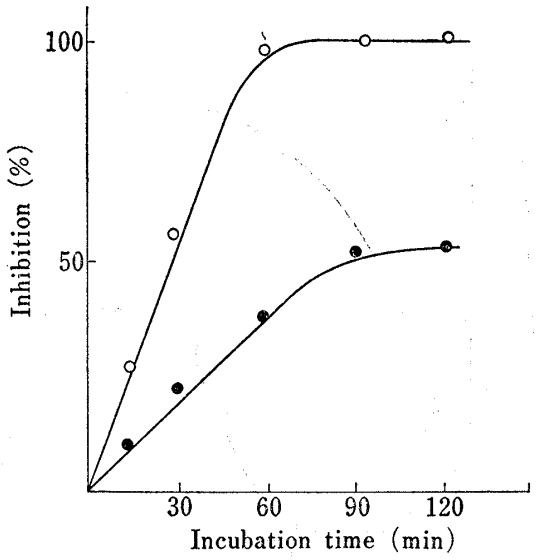

Fig. 3. Inhibition of Superoxide Dismutase and Catalase in the Lysate after Incubation with 3.0 mM DDC for Various Times at 37°

The condition are the same as those given in the legend to Fig. 1.
—○—: superoxide dismutase, —●—: catalase.

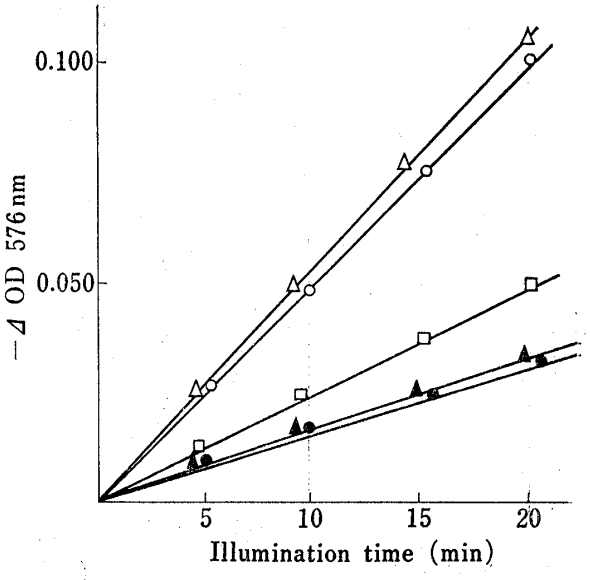

Fig. 4. Effect of Superoxide Dismutase and Catalase on the Rate of Oxidation of OxyHb in the DDC-treated Lysate

Erythrocyte lysate (30 μM of oxyHb) treated with DDC as described in the legend to Fig. 3, was dialyzed against 50 mM phosphate buffer, pH 7.4, for 24 hr and then the oxidation reaction was initiated by illumination. —○—: no addition, —△—: + superoxide dismutase (5 μg/ml), —□—: + catalase (18 unit/ml), —▲—: + catalase (48 unit/ml), —●—: normal lysate.
Data are the mean of triplicate samples in representative experiment.

60 unit/ml respectively. These results were nearly consistent with the data as shown in Fig. 2 and indicating that each enzyme was not inactivated by O_2^- during the course of illumination for 20 min. No precipitation was apparent during the time of observation. On the contrary, the lysate treated with DDC above 5 mM for 60 min began to exhibit increase in light scattering at 576 nm due to the precipitation of hemoglobin. These results can be interpreted that the enhanced formation of metHb caused by added DDC may be attributed to the total loss of enzymatic activity of superoxide dismutase or partial inhibition of catalase in the lysate.

Formation of MetHb from OxyHb in the Dialyzed Lysate

To avoid complications arising from the presence of DDC, the lysate whose superoxide dismutase and catalase have been inhibited by DDC were dialyzed against 50 mM phosphate buffer. Typical results on the lysate treated with 3 mM DDC were shown in Fig. 4. Neither enzymatic activity was restored during the course of this procedure and catalase furthermore decrease to 12 unit/ml. Catalase added at 48 unit/ml to the reaction mixture to give the original level, clearly inhibited the formation of metHb to almost the same extent as that in the normal lysate, whereas catalase at 18 unit/ml inhibited partially. This result implies an active role for hydrogen peroxide in the formation of metHb. Addition of 5 μg/ml of superoxide dismutase, however, did not caused the inhibition but a slight acceleration of the formation of metHb, suggesting that hydrogen peroxide is formed from O_2^- by the enzymatic activity of superoxide dismutase. From these results, the acceleration of the formation of metHb at the concentration of DDC above 3 mM as shown Fig. 4, seems likely to be due to the depression of catalase activity rather than that of superoxide dismutase.

Discussion

Previous investigators[12] have shown that superoxide dismutase was inhibited *in vitro* by a number of metal chelate agents, cyanide, azide, *o*-phenanthroline and DDC, which form complexes with the copper as a active site of this enzyme. In present study, administration of DDC to rats caused the pronounced lowering of superoxide dismutase in erythrocyte without the effect on catalase activity (Table I). The experiments *in vitro* showed that superoxide dismutase in the lysate was inhibited completely but catalase was partially inhibited in a time-dependent fashion (Fig. 2 and 3). DDC is known to be without effect of catalase activity,[10] therefore, the inactivation of catalase presented here may be regarded as reflecting a direct reaction of DDC with this enzyme molecular except with an active site, possibly by the prolonged preincubation employed in this experiment. Similar results were obtained with pure superoxide dismutase and catalase.

Superoxide dismutase has been believed to play a role in scavenging O_2^- as a potentially toxic species within cells.[3] Therefore, the erythrocyte with lowered this enzymatic activity can be assumed to be readily injured by O_2^-. In fact, Stern *et al.*[7b] reported that inhibition of this enzyme lead to a shortening of the life span of cells in hypotonic media. Moreover, copper deficient swine have been shown that superoxide dismutase in erythrocyte decreased to 15% of control value and that the survival time of such cell is shortened, the osmotic fragility is increased and the membrane is excessively permeable to sodium ions.[13] However whether these changes are a consequence of membrane damage due to O_2^- brought about by superoxide dismutase deficiency is speculative. If superoxide dismutase functions to prevent metHb

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formation in erythrocyte as discussed by previous workers,[6] cells with lowered content of this enzyme should exhibit an enhanced rate of metHb formation when exposed to O_2^- *in vitro*. In the present study, superoxide dismutase in erythrocite was inhibited to 93% and catalase remained unchange by injection of DDC but the conversion of oxyHb to metHb was scarcely observed. On the other hand, in the reaction system containing oxyHb prepared from purified hemoglobin which contains neither superoxide dismutase nor catalase, the rate of formation of metHb was significantly increased compared with that of the lysate from DDC-injected rat (Fig. 1). These differences between the results with lysate and pure hemoglobin can be interpreted that the enzymatic activity of superoxide dismutase in the lysate remains enough to prevent the formation of metHb even if the DDC inhibits this enzyme to 93%. Lynch, *et al.*[6] have pointed out that the content of superoxide dismutase in normal erythrocyte is more than ten times that required to inhibit completely the formation of metHb by O_2^-. An attempt to inhibit completely superoxide dismutase in erythrocyte *in vivo* was actually impossible because the dose of DDC used in this experiment is nearly the upper limit being capable of administration. The total loss of enzymatic activity of superoxide dismutase in the lysate was achieved by the pretreatment with DDC. In this case, the formation of metHb proceeded under the O_2^- generating system (Fig. 4). Futhermore, this conversion of oxyHb to metHb was almost completely inhibited by the addition of catalase and somewhat accelerated by superoxide dismutase in the dialyzed lysate (Fig. 4). These results offer an evidence that hydrogen peroxide rather than O_2^- plays a role for the formation of metHb under the conditions used. The acceleration for the formation of metHb by superoxide dismutase, on the other hand, suggests that O_2^- is appears to be one of the source of hydrogen peroxide involved in the formation of metHb, as superoxide dismutase catalyses the formation of hydrogen peroxide from O_2^-. Another possible source of hydrogen peroxide may come into play, namely that O_2^- formed under the illumination undergoes spontaneous dismutation to hydrogen peroxide in the presence of oxyHb. Indeed, the evidence for this possibility has been discussed by Demma, *et al.*[14] Eyer, *et al.* have noted that when hemoglobin is treated with hydrogen peroxide, a greater than 500 fold yield of metHb per hydrogen peroxide is obtained.[15] This would explain the great inhibition of the formation of metHb by catalase in this experiment. On the basis of these results, it seems most likely that the lack of O_2^- effect on the formation of metHb in the lysate from DDC-injected rats (Fig. 1) may be due to their full complement of catalase activity. In the normal erythrocyte, O_2^- either by superoxide dismutase or by acting as an oxidizing agent toward some cellular constituents is converted to hydrogen peroxide which is decomposed by catalase. This could be considered as one important role for these enzymes. However, the exact significance of these enzyme in the physiological conditions must be cleared by further studies.

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