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Lipid Peroxidation of Erythrocyte Membrane induced by Xanthine Oxidase System: Modification of Superoxide Dismutase Effect by Hemoglobin[1)]

Toshiaki Miura* and Taketo Ogiso

Hokkaido Institute of Pharmaceutical Sciences, 7-1 Katsuraoka-cho, Otaru, 047-02, Japan

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Lipid peroxidation of erythrocyte ghosts was caused by incubation in a xanthine oxidase system. Addition of superoxide dismutase to this system strongly inhibited the lipid peroxidation, implying that O_2^- is an essential intermediate in the lipid peroxidation reaction. However, catalase did not inhibit but greatly promoted the lipid peroxidation, suggesting that catalase enhances net O_2^- production through the stabilization of xanthine oxidase. Chemical scavengers of singlet oxygen ($^1O_2$) inhibited the peroxidation reaction, suggesting that the extremely reactive radical of $^1O_2$ may be produced from O_2^- generated by the xanthine oxidase system. Hydroxyl radical scavengers were without effect. Furthermore, the lipid peroxidation was greatly accelerated with increasing concentration of oxyHb up to 2 μM. At concentrations above 2 μM, however, the lipid peroxidation reaction was inhibited. In the presence of 2 μM Hb, addition of superoxide dismutase or scavengers of $^1O_2$ inhibited the lipid peroxidation to the same extent as in white ghosts. In the presence of 10 μM Hb, however, catalase markedly prevented lipid peroxidation, whereas superoxide dismutase or chemical scavengers of $^1O_2$ had little effect. These results indicate that catalse was more effective than superoxide dismutase in providing protection against lipid peroxidation induced in the presence of a relatively high concentration of Hb and that the reaction mechanism of oxygen radicals with membrane lipids was modified in the presence of Hb.

Keywords——Erythrocyte membrane; lipid peroxidation; superoxide dismutase; catalase; hemoglobin

The erythrocyte is susceptible to damage by oxygen radicals and has a variety of biochemical mechanisms which serve to protect it. Superoxide dismutase (EC 1.15.1.1), catalase (EC 1.11.1.6) and the peroxidases are postulated to protect cells by scavenging reactive intermediates such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2).

Toxic effects of active oxygens on erythrocytes have recently been studied to elucidate the mechanisms of hemolysis and lipid peroxidation. Among the active oxygens, O_2^-, formed by one-electron reduction of molecular oxygen, has been reported to initiate the peroxidation and hence destruction of membrane lipids of erythrocytes, leading to liberation of hemoglobin (Hb).[2)] Therefore, high contents of superoxide dismutase in erythrocytes have been thought to protect the cells against such peroxidative damage by dismutation of O_2^- to H_2O_2 and oxygen.

However, several workers[3)] demonstrated that lipid peroxides could not be detected in erythrocytes exposed to O_2^- generated through the autoxidation of dihydroxyfumaric acid and by the xanthine oxidase system, while hemolysis was induced. We have reported previously that O_2^- did not appear to be a peroxidizing species for oxidative degradation of Hb and particularly for hemolysis, since superoxide dismutase had no inhibitory effect on these oxidative effects induced by the exposure of cells to the xanthine oxidase system.[4)]

This paper presents some evidence for the participation of O_2^- in the lipid peroxidation of erythrocyte membrane, and for protective effects of superoxide dismutase, catalase and some chemical scavengers of active oxygens against the lipid peroxidation induced by the xanthine oxidase system in the presence or absence of Hb.

Experimental

Materials——Xanthine oxidase (from butter milk), superoxide dismutase (from bovine blood), catalase (from bovine liver, thymol-free) and hemoglobin (human, type IV) were obtained from Sigma Chemical Co., St. Louis. Mo., U.S.A. Xanthine oxidase was passed through a Sephadex G-25 column equilibrated with water before use. Rat hemoglobin was prepared by gel filtration on a Sephadex G-100 column. Human Hb was reduced with sodium dithionite. Excess reducing agents were removed by gel filtration on Sephadex G-25 and then oxyHb was prepared by bubbling oxygen through the solution. Denatured superoxide dismutase was prepared by autoclaving for 30 min at 110°C and denatured catalase was obtained by heating an enzyme solution for 10 min at 100°C. Histidine, 1,4-diazabicyclo(2,2,2)octane (Dabco), mannitol and sodium benzoate were purchased from Wako Pure Chemical Industries Ltd. while thiobarbituric acid (TBA) was from Merck Japan Ltd. and was recrystallized three times from water before use. Malondialdehyde was prepared by the hydrolysis of 1,1,3,3-tetraethoxypropane, which was obtained from Tokyo Kasei Kogyo Co., Ltd.

Preparation of Erythrocyte Membrane——Blood was taken from the common carotid artery of male Wistar strain rats weighing about 200 g into heparinized test tubes and centrifuged at $1500 \times g$ for 10 min. The plasma and buffy coat were removed by aspiration. Erythrocytes were washed three times with 5 volumes of isotonic NaCl solution. Hemolysis was achieved by suspending the packed erythrocytes in 30 volumes of cold hypotonic 20 m Osm phosphate buffer, pH 7.4, containing 1 mM EDTA. The stroma was isolated by centrifugation at $10000 \times g$ for 20 min and washed 3 or 4 times with hypotonic phosphate buffer containing 1 mM ethylenediaminetetraacetic acid (EDTA) until the stroma was no longer colored. To remove EDTA, the white ghosts were further washed 3 times with hypotonic phosphate buffer without EDTA. No hemoglobin could be detected in the white ghosts preparation by the cyanmethemoglobin and pyridine hemochromogen method.

System for Generation of O_2^- and Reaction with Erythrocyte Ghosts——Generation of O_2^- in the xanthine oxidase system was assayed by the cytochrome c reduction procedure of McCord *et al.*[5] Xanthine oxidase activity was assayed at 25°C by measuring the absorption of uric acid at 293 nm. One unit of activity was defined as 1.0 μmol of xanthine converted to uric acid per min at pH 7.5. Protein concentrations were estimated by the method of Lowry *et al.* using bovine serum albumin as a standard.[6] Unless otherwise noted, all reaction procedures were as follows; erythrocyte ghosts (400 μg protein) were suspended in 3.0 ml of 10 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl, 5 mM hypoxanthine and 0.05 unit of xanthine oxidase. The reaction was carried out by incubation with shaking at 37°C.

Determination of Lipid Peroxide——Malondialdehyde (MDA) formation, a manifestation of lipid peroxidation in the white ghosts, was measured by the method of Uchiyama *et al.*;[7] 1.0 ml of 4% phosphoric acid and 1.0 ml of 0.6% TBA were added to the reaction mixture after a specified period of incubation and then heated for 45 min at 100°C. The mixture was cooled in water, then 4 ml of *n*-butanol was added and mixed vigorously. The butanol phase was separated by centrifugation and the optical density of the butanol layer was measured at 535 and 520 nm. The difference was taken as the TBA value. The determination of lipid peroxide formed in erythrocyes was carried out as described by Fee *et al.*[8]

Results

Lipid Peroxidation of Erythrocyte Membrane

As shown in Fig. 1, lipid peroxidation of erythrocyte ghosts as measured from the formation of MDA was caused by incubation in the xanthine oxidase system. Formation of MDA in the ghosts increased with time and the amount of MDA formed at 120 min after the incubation was about 20 nmol per protein (mg) of ghosts. No significant peroxidation reaction occurred on omission of xanthine oxidase and/or hypoxanthine from the complete system. Addition of a xanthine oxidase inhibitor, allopurinol, also resulted in no significant peroxidation reaction.

With intact cells incubated in the xanthine oxidase system, formation of MDA was not detected at any time of incubation. During the course of incubation, the cellular color changed to dark brown and the formation of cells resistant to hypotonic hemolysis was observed, as described previously.[4]

Effects of Superoxide Dismutase and Catalase

Since the xanthine oxidase system is known to generate H_2O_2 and O_2^-,[5] the effects of catalase and superoxide dismutase on the lipid peroxidation were examined. As shown in Table I, superoxide dismutase was a powerful inhibitor of the lipid peroxidation of erythrocyte

ghosts. This inhibitory effect of superoxide dismutase was concentration-dependent. Denatured superoxide dismutase which had been inactivated by autoclaving, retained some inhibitory activity on lipid peroxidation. This might be due to the liberation of copper from the denatured enzyme. Although the data are not shown, addition of copper to the complete system at the same concentration as in the enzyme sample inhibited the peroxidation reaction to the same extent as the denatured enzyme. Zinc, contained in superoxide dismutase together with copper, was without effect.

As shown in Fig. 1, the inhibitory effect of superoxide dismutase could be seen most clearly by adding 1.0 μg/ml of superoxide dismutase to the complete system 30 or 60 min after initi-

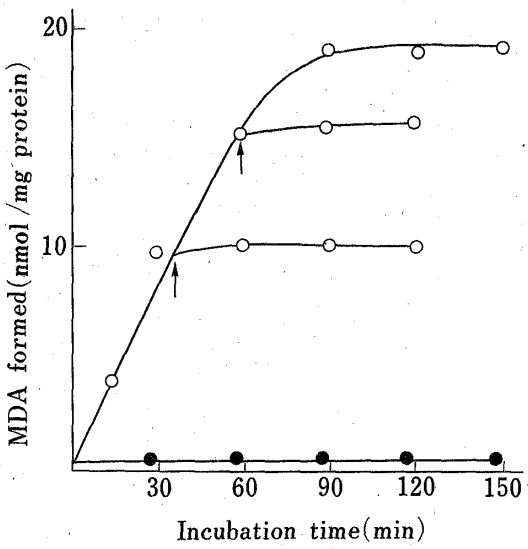

Fig. 1. Time Course of Lipid Peroxidation of Erythrocyte Ghosts in the Xanthine Oxidase System

The reaction mixture contained 5 mM hypoxanthine, 0.15 M NaCl and 400 μg of erythrocyte ghosts in 3.0 ml of 10 mM phosphate buffer, pH 7.4. Xanthine oxidase (0.05 unit) was added to start the reaction at 37°C. After incubation for various times, MDA formed was measured as described in this text. Superoxide dismutase was added to the reaction system at the points indicated by arrows. —○—, erythrocyte ghosts; —●—, erythrocytes (1 % hematocrit).

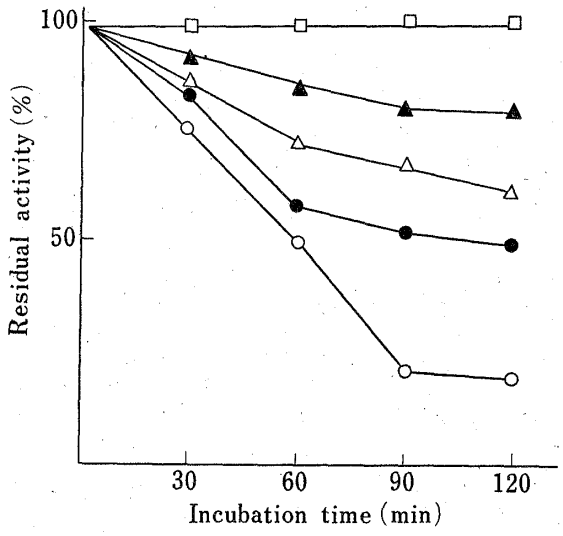

Fig. 2. Self-inactivation of Xanthine Oxidase

Reaction conditions were the same as described in the legend to Fig. 1. Catalase and/or superoxide dismutase were added to the xanthine oxidase system before initiation of the reaction. At intervals, an aliquot was removed from the reaction mixture and the xanthine oxidase activities were measured, —○—, complete system; —●—, + catalase (0.1 μg/ml); —△—, + catalase (0.5 μg/ml); —▲—, + catalase (1.0 μg/ml); —□—, + catalase (1.0 μg/ml) and superoxide dismutase (1.0 μg/ml).

TABLE I. Effects of Superoxide Dismutase and Catalase on Lipid Peroxidation

Experimental conditions	MDA formed nmol/mg of ghosts protein	% inhibition of peroxidation	% stimulation of peroxidation
Complete system	19.3±2.4	—	—
+Superoxide dismutase (0.1 μg/ml)	9.8±1.0	49.3	—
+Superoxide dismutase (0.5 μg/ml)	4.7±0.5	75.7	—
+Superoxide dismutase (1.0 μg/ml)	1.7±0.1	91.2	—
+Denatured superoxide dismutase (1.0 μg/ml)	17.3±0.9	10.4	—
+Catalase (0.1 μg/ml)	28.0±0.3	—	45.1
+Catalase (0.5 μg/ml)	32.8±0.2	—	69.9
+Catalase (1.0 μg/ml)	38.9±0.3	—	101.0
+Denatured catalase (1.0 μg/ml)	20.1±2.4	—	4.1
+Superoxide dismutase (1.0 μg/ml) and catalase (1.0 μg//ml)	1.5±0.1	92.3	—

The complete system consisted of 5 mM hypoxanthine, 0.15 M NaCl, erythrocyte ghosts (400 μg protein) and 0.05 unit of xanthine oxidase in 3.0 ml of 10 mM phosphate buffer, pH 7.4. Enzymes were added to the complete system and then incubated for 120 min at 37°C. Each value represents the mean ± S.E. of triplicate experiments.

ation of the lipid peroxidation reaction. The addition of allopurinol during the incubation caused an immediate inhibition of MDA production. These results indicate that a continuous generation of O_2^- is required for the initiation of lipid peroxidation of ghost membrane.

On the other hand, catalase did not inhibit but greatly promoted the lipid peroxidation of ghosts at any concentration of catalase employed. Heat-inactivated catalase showed essentially no promotive effect. Addition of both catalase and superoxide dismutase to the complete system gave an effect similar to that obtained with superoxide dismutase alone.

Figure 2 shows that xanthine oxidase activity was markedly inactivated during the course of incubation, and that this inactivation was completely prevented by addition of both catalase and superoxide dismutase at concentrations of 1.0 μg/ml each to the reaction medium. On the other hand, catalase alone prevented the inactivation of xanthine oxidase depending on the concentration of catalase. These results seem to be in agreement with those of other workers,[2c] who found that H_2O_2 arising from the enzymatic reaction is involved in the inactivation of xanthine oxidase. Thus, the promotion of lipid peroxidation resulting from addition of catalase as shown in Table I is probably due to the enhancement of net O_2^- production induced through the stabilization of xanthine oxidase.

Effect of Some Radical Scavengers

The mechanism by which MDA is formed in the cell membrane may involve other radicals such as singlet oxygen ($^1O_2$) and hydroxyl radical (OH·). To examine this possibility, compounds which are known to scavenge these radicals were tested. As can be seen in Table II, histidine and Dabco employed as scavengers of $^1O_2$ strongly inhibited the peroxidation reaction. Carotene and 2,5-dimethylfuran could not be used in this experiment, because they interfered with the colorimetric determination of MDA. Sodium benzoate and mannitol, which are known to react with OH·, had no significant effect. These results suggest that $^1O_2$ possibly produced from O_2^- is an essential intermediate for the lipid peroxidation of erythrocyte ghosts in the xanthine oxidase system.

Table II. Effects of Various Compounds on Lipid Peroxidation

Experimental conditions	MDA formed nmol/mg of ghosts protein	% inhidition of peroxidation
Complete system	19.3±2.4	—
+Histidine	3.7±0.4	80.9
+Dabco	6.8±0.5	64.8
+Sodium benzoate	18.5±1.8	4.2
+Mannitol	20.0±3.4	3.6[a]

All chemicals were added to the complete system at 10 mM concentration. The conditions were the same as given in Table I. Each value represents the mean ± S.E. of triplicate experiments.

a) % stimulation of peroxidation.

Lipid Peroxidation of Ghosts in the Presence of Hemoglobin

Lipid peroxidation was scarcely observed in the intact cells as shown in Fig. 1. Furthermore, during the course of the experiment, we have observed that the exposure of ghosts contaminated with a trace of Hb to the xanthine oxidase system caused only a slight increase in the formation of MDA compared with that of white ghosts. Therefore, to examine the possible partipation of Hb in the lipid peroxidation, the white ghosts were exposed to the xanthine oxidase system in the presence of human oxyHb.

As shown in Fig. 3, the formation of MDA was greatly accelerated with increasing concentration of Hb up to 2 μM. At concentrations above 2 μM of Hb, however, the lipid peroxida tion reaction was partially inhibited. Rat Hb behaved in essentially the same way as human Hb.

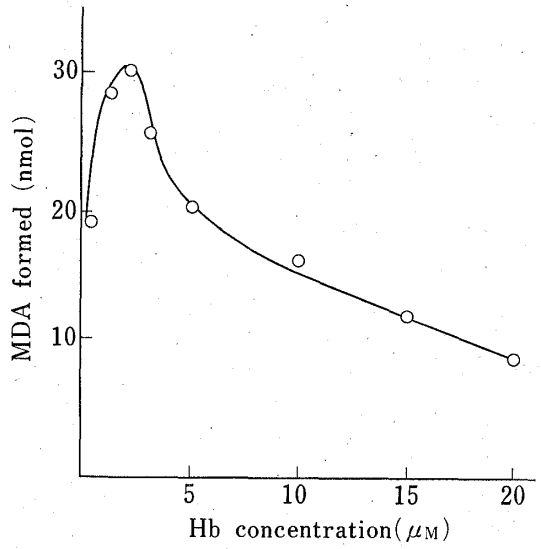

Fig. 3. Lipid Peroxidation of Erythrocyte Ghosts in the Presence of Hemoglobin

Various amounts of Hb were added to the complete system. After incubation for 120 min, MDA formed was measured as described in the text.

Subsequently, the effects of superoxide dismutase, catalase and some chemical scavengers on the lipid peroxidation were examined in the presence of 2 or 10 μM Hb. Typical results are summarized in Table III. In the presence of 2 μM Hb, the action of enzymes and scavengers on the lipid peroxidation was similar in magnitude to that on white ghosts, as mentioned above. In the presence of 10 μM Hb, however, catalase added at 1.0 μg/ml markedly reduced the lipid peroxidation, whereas superoxide dismutase had little effect. Heat-denatured catalase was without effect. Addition of both enzymes inhibited the lipid peroxidation to nearly the same extent as that of catalase alone, suggesting a possible involvement of H_2O_2 in this lipid peroxidation. Chemical scavengers of $^1O_2$ or of OH· (Table II) did not necessarily inhibit the lipid peroxidation reaction. These results indicate that catalase was a more effective enzyme than superoxide dismutase for providing protection against the lipid peroxidation induced in the presence of a relatively high concentration of Hb, and that the reaction mechanism of oxygen radicals with membrane lipids was modified in the presence of Hb.

Table III. Effects of Superoxide Dismutase, Catalase and Chemical Scavengers on Lipid Peroxidation in the Presence of Hemoglobin

Experimental condtions	MDA formed nmol/mg of ghosts protein	% inhibition of peroxidation
Hb (2 μM)	30.8±0.9	—
+Superoxide dismutase (1.0 μg/ml)	4.9±0.3	84.1
+Catalase (1.0 μg/ml)	58.2±3.6	89.0[a)]
+Both enzymes	2.9±0.1	90.6
+Histidine (10 mM)	7.6±0.2	75.4
+Dabco (10 mM)	7.9±0.5	74.4
Hb (10 μM)	16.6±0.4	—
+Superoxide dismutase (1.0 μg/ml)	11.4±0.5	31.3
+Catalase (1.0 μg/ml)	3.0±0.4	81.9
+Both enzymes	1.5±0.1	91.0
+Histidine (10 mM)	12.6±0.3	24.1
+Dabco (10 mM)	16.4±0.3	1.2

Hb was added to the complete system before the initiation of reaction. The conditions were the same given in of Table I. Each value represents the mean ± S.E. of triplicate experiments.
a) % stimulation of peroxidation.

Discussion

The peroxidation of membrane lipids occurred on exposure of white ghosts to the O_2^--generating system of xanthine oxidase, whereas lipid peroxidation was scarcely observed in intact erythrocytes. Addition of superoxide dismutase to this system strongly inhibited the lipid peroxidation of white ghosts, implying that O_2^- is an essential intermediate in the lipid peroxidation reaction. The inability of either catalase or OH· scavengers to inhibit this

peroxidation reaction ruled out the involvement of H_2O_2 or OH·. Furthermore, the chemical scavengers of $^1O_2$ employed in this experiment inhibited the peroxidation reaction, suggesting that the extremely reactive $^1O_2$ radical may be produced from O_2^- generated in the xanthine oxidase system.

The reactivity of $^1O_2$ with unsaturated fatty acids and biological membrane has been demonstrated directly by photo-oxygenation using several sensitizers to generate $^1O_2$.[9] The autoxidation of fatty acids is also believed to be initiated by $^1O_2$.[10] Kellogg *et al.*[11] have noted that xanthine oxidase, while acting aerobically upon acetaldehyde in the absence of iron salts, could cause the peroxidation of linolenate. Since both superoxide dismutase and catalase inhibited this lipid peroxidation, they proposed that the combination of O_2^- and H_2O can directly give rise to $^1O_2$ as follows: $O_2^- + H_2O_2 \rightarrow OH^- + OH\cdot + {}^1O_2$. The present study, however, demonstrated that catalase did not inhibit lipid peroxidation, but rather increased the rate of MDA formation, indicating that the $^1O_2$ which caused lipid peroxidation of white ghosts is probably not generated secondarily from a reaction of H_2O_2 with O_2^-. The promotive effect of catalase on the lipid peroxidation presented here may occur through the protection of xanthine oxidase against self-inactivation by H_2O_2 (Fig. 2).

Pederson *et al.*,[12] on the other hand, have shown that peroxidation of microsomal lipid was promoted by incubation in the xanthine oxidase system in the presence of some iron salts and chelating agents such as adenosine diphosphate (ADP) or EDTA. They also showed that the microsomal lipid peroxidation was inhibited by either superoxide dismutase or 1,3-diphenylisobenzofuran, suggesting that $^1O_2$ is generated by the non-enzymatic dismutation of O_2^- without involvement of H_2O_2 or OH· and that lipid peroxidation is initiated by the reduction of Fe^{3+} followed by the decomposition of hydroperoxides to generate alkoxyl radicals. A recent report has shown that lipids of microsomes undergo peroxidation with production of MDA in the presence of H_2O_2 and hematin, suggesting that the formation of hydroperoxides is due to small amounts of $^1O_2$ produced by slow non-enzymatic disproportionation of H_2O_2, and that hematin promotes the radical chain reaction leading to decomposition of hydroperoxides and ultimately to the formation of MDA.[13]

The results described in this paper also demonstrate that $^1O_2$ appears to be involved in the lipid peroxidation of white ghost membrane. It should be pointed out, however, that the lipid peroxidation was accelerated by exposure of the ghost membrane to the xanthine oxidase system in the presence of 2 μM oxyHb (Fig. 3) and that this peroxidation reaction was inhibited by either superoxide dismutase or scavengers of $^1O_2$. Although the data are not shown, the membrane of white ghosts contained about 90 ng of ferric iron per mg of ghost protein despite repeated washing as described in the text. Moreover, addition of 10 μM EDTA to the complete system resulted in nearly complete inhibition of MDA formation, in agreement with the results of Pederson *et al.* that EDTA at concentrations in excess of that of iron salt effectively inhibits microsomal lipid peroxidation.[12] On the basis of these results, it seems likely that the lipid peroxidation is initiated by $^1O_2$ generated from O_2^- through some interaction with iron salt as a trace contaminant in the ghost membrane. However, the lipid peroxidation of white ghosts in the xanthine oxidase system did not proceed as a chain reaction but depended on the continuous generation of O_2^-, in contrast to that in the autocatalytic radical chain reaction.[14]

In contrast to the enhanced formation of MDA in the presence of 2 μM Hb, a high concentration of Hb partially inhibited the lipid peroxidation reactions of white ghosts (Fig. 3). Moreover, the lipid peroxidation reactions of white ghosts in the presence of 10 μM Hb were strongly inhibited by catalase but not by superoxide dismutase or scavengers of $^1O_2$, suggesting a possible involvement of H_2O_2 rather than O_2^- or $^1O_2$ (Table III). These results indicate that catalase is a more effective protector against the lipid peroxidation of erythrocytes in the presence of a high level of Hb. Thus, a high concentration of Hb apparently modifies the reaction mechanism of oxygen radicals with membrane lipid in the xanthine oxidase system. As reported previously, catalase prevents the degradation of oxyHb in the xanthine oxidase

system.[4b] The inhibitory effect of catalase on lipid peroxidation seems to involve protection of Hb from oxidative attack of H_2O_2 generated in the xanthine oxidase system. However, we are unable to explain the modifying effect of Hb as yet.

With intact cells incubated in the xanthine oxidase system, no MDA was detected (Fig. 1), suggesting that catalase in the intact cells takes part in protection against membrane lipid peroxidation. In fact, when intact erythrocytes treated with 3-amino-1,2,4-triazole, which has been reported to inhibit catalase activity in cells,[15] were incubated in the xanthine oxidase system, the peroxidation reaction readily proceeded and the formation of MDA increased with time. On the other hand, the formation of MDA in intact cells treated with diethyldithiocarbamate, which was previously reported to inhibit superoxide dismutase activity in the cells almost completely,[16] was not detected at any time of incubation in the xanthine oxidase system (data not shown). Further studies are required to clarify the possible participation of Hb and the physiological role of superoxide dismutase in the lipid peroxidation of erythrocyte membrane.

References and Notes

1) A part of this study was presented at the 101st Meeting of the Pharmaceutical Society of Japan, Kumamoto, 1981.
2) *a*) J.A. Fee and H.D. Teiterbaum, *Biochem. Biophys. Res. Commun.*, **49**, 150 (1972); *b*) A. Valenzueda, H. Rios and G. Neiman, *Experientia*, **15**, 962 (1977); *c*) E.W. Kellogg and I. Fridovich, *J. Biol. Chem.*, **252**, 6721 (1977).
3) *a*) B. Goldberg and A. Stern, *Arch. Biochem. Biophys.*, **179**, 218 (1977); *b*) M. Taniguchi, M. Aikawa and T. Sakagami, *J. Biochem.* (Tokyo), **89**, 795 (1981).
4) *a*) T. Miura and T. Ogiso, *Chem. Pharm. Bull.*, **27**, 2815 (1979); *b*) *Idem*, *ibid.*, **29**, 1730 (1981).
5) M. McCord and I. Fridovich, *J. Biol. Chem.*, **244**, 6049 (1969).
6) O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
7) M. Uchiyama and M. Mihara, *Anal. Biochem.*, **86**, 271 (1978).
8) J.A. Fee, R. Bergamini and R.G. Briggs, *Arch. Biochem. Biophys.*, **169**, 160 (1975).
9) *a*) "Free Radicals in Biology," Vol. II, ed by Pryor, Academic Press, p. 120; *b*) M.M. King, E.K. Lai and P.B. McCay, *J. Biol. Chem.*, **250**, 6496 (1975).
10) *a*) H.R. Rawls and J. Van Santen, *J. Am. Oil. Chem. Soc.*, **47**, 121 (1970); *b*) S.M. Anderson, N.I. Krinsky and D.C. Clagett, *Photochem. Photobiol.*, **20**, 65 (1974).
11) E.W. Kellogg and I. Fridovich, *J. Biol. Chem.*, **250**, 6496 (1975).
12) *a*) T.C. Pederson and S.D. Aust, *Biochem. Biophys. Res. Commun.*, **52**, 1071 (1973); *b*) *Idem*, *Biochim. Biophys. Acta*, **385**, 232 (1975).
13) F. Ursini, M. Maiorino, L. Ferri, M. Valente and C. Gregolin, *J. Inorg. Biochem.*, **15**, 163 (1981).
14) *a*) S.E. Lewis and E.D. Wills, *Biochim. Biophys. Acta*, **70**, 336 (1963); *b*) Y. Nakamura and T. Nishida, *J. Lipid Res.*, **12**, 149 (1971).
15) T. Miura and T. Ogiso, *Chem. Pharm. Bull.*, **26**, 3540 (1978).
16) *a*) R.E. Heikkila, F.S. Cabbat and G. Cohen, *J. Biol. Chem.*, **251**, 1015 (1976); *b*) T. Miura and T. Ogiso, *Chem. Pharm. Bull.*, **26**, 1261 (1977).