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Enhanced Lipid Peroxidation of Erythrocyte Membranes and Phosphatidylcholine Liposomes Induced by a Xanthine Oxidase System in the Presence of Catalase[1)]

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When rat erythrocyte membranes or phosphatidylcholine (PC) liposomes were incubated in a xanthine oxidase system, lipid peroxidation was stimulated by the addition of catalase. Addition of a large amount of xanthine oxidase caused no significant increase in the lipid peroxidation of PC liposomes unless catalase was present in the reaction system. Enhanced peroxidations of both membranes and liposomes observed in the presence of catalase were strongly inhibited by superoxide dismutase (SOD), suggesting that O_2^- is an essential species in these reactions. Several iron-chelators strongly inhibited the lipid peroxidations, suggesting an involvement of iron in the peroxidation reaction. Unless catalase was present, the rate of liposome peroxidation was stimulated only slightly by addition of Fe^{3+}, but in the presence of catalase, the addition of Fe^{3+} markedly accelerated the peroxidation reaction, which was inhibited by SOD or desferrioxamine. Furthermore, the addition of Fe^{3+} to the xanthine oxidase system in the presence of catalase caused a significant decrease in the rate of cytochrome c reduction, suggesting that Fe^{3+} may be reduced to Fe^{2+} by O_2^-. The peroxidations enhanced by catalase may be induced by supply of sufficient O_2^- and Fe^{2+} for lipid peroxidation. Histidine and 1,4-diazabicyclo-(2,2,2)-octane, quenchers of $^1O_2$, inhibited the peroxidation of membranes and liposomes. Mannitol and benzoate, scavengers of $OH^\cdot$, showed no significant effect on the peroxidation of membranes or liposomes. These results indicate possible participation of $^1O_2$ species in the O_2^--dependent lipid peroxidation induced by the xanthine oxidase system in the presence of catalase.

Keywords——lipid peroxidation; erythrocyte membrane; phosphatidylcholine liposome; xanthine oxidase; catalase; superoxide dismutase; singlet oxygen

It is well recognized that superoxide (O_2^-), formed by one-electron reduction of molecular oxygen, is produced in many biological reactions, namely, autoxidation of oxyhemoglobin,[2)] certain enzymatic reactions[3)] and mammalian phagocytosis.[4)] The enzyme superoxide dismutase (SOD, EC 1.15.1.1) dismutates O_2^- to hydrogen peroxide (H_2O_2) and oxygen.[5)] H_2O_2, in turn, is metabolized by catalase or glutathione peroxidase.[6)]

The toxicity of O_2^- radicals has been examined extensively to clarify the role of SOD in the lipid peroxidation of biological membranes, in which active oxygen causes many pathologic conditions. A number of experiments indicate that neither O_2^- nor H_2O_2 is the species directly causing lipid peroxidation.[7)] Several investigators pointed out that highly reactive oxygen species such as hydroxyl radical ($OH^\cdot$) could be generated by iron-catalyzed interaction of O_2^- with H_2O_2 (Haber–Weiss reaction).[8)] Other workers concluded that both $OH^\cdot$ and singlet oxygen ($^1O_2$) were responsible for initiating lipid peroxidation, since SOD and catalase inhibited the peroxidation of lenolenate.[9)]

Previously, we demonstrated that lipid peroxidation of erythrocyte membranes in a xanthine oxidase system was enhanced by the addition of catalase, presumably due to the enhancement of net O_2^- production through protection of xanthine oxidase against self-inactivation by H_2O_2. In addition, initiation of this peroxidation was inhibited by SOD or chemical scavengers of $^1O_2$ but not by $OH^\cdot$ scavengers, suggesting that $^1O_2$ may play a critical

role in the peroxidation reaction.[10] However, the mechanism of enhanced lipid peroxidation induced by the xanthine oxidase system in the presence of catalase is not yet fully understood.

In this paper, we describe in detail our results on the possible participation of $^1O_2$ and traces of iron in the $\cdot O_2^-$-dependent lipid peroxidation of liposomes and erythrocyte membranes enhanced by catalase.

Experimental

Materials——Catalase (from bovine liver, thymol-free), SOD (from bovine blood), xanthine oxidase (from buttermilk), iron-free transferrin (from humans) and iron-free lactoferrin (from human colostrum) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A., while iron-saturated transferrin was from AB KABI Stockholm, Sweden. Chelex-100 resin was purchased from Japan Bio-Rad Laboratories. Lyophilized powder of catalase was dissolved in 10 mM phosphate buffer, pH 7.4. After centrifugation for 30 min at 20000 × *g* at 4 °C, the enzyme solution was dialyzed against the same buffer in the cold and then passed through a Sephadex G-100 column (2.8 × 80 cm) equilibrated with 10 mM phosphate buffer, pH 7.4 containing 0.15 M NaCl. This preparation of catalase was found to be pure by polyacrylamide gel electrophoresis. Xanthine oxidase was passed through a Sephadex G-25 column equilibrated with water before use. Phosphatidylcholine (PC, from egg), histidine, 1,4-diazabicyclo-(2,2,2)-octane (DABCO), diethylenetriaminepenta-acetic acid (DETAPAC), mannitol and sodium benzoate were obtained from Wako Pure Chemical Industry Co., Ltd., while thiobarbituric acid (TBA) was from Merck Japan Ltd. TBA was recrystallized three times from water before use. Malondialdehyde (MDA) was prepared by the hydrolysis of 1,1,3,3-tetraethoxypropane, which was obtained from Tokyo Kasei Kogyo Co., Ltd. Desferrioxamine was purchased from CIBA Laboratories. All other chemicals used were of the highest purity commercially available.

Preparation of Erythrocyte Membranes and Liposomes——Erythrocyte membranes were prepared as described previously.[10] PC liposomes were prepared by the method of Kashnitz *et al.*[11] with a minor modification. PC dissolved in chloroform was evaporated to dryness and dispersed in 10 mM phosphate buffer containing 0.15 M NaCl with a vortex mixer. Lipid phosphate determination was performed by the method of Bartlett.[12] Amounts of iron in erythrocyte membranes and PC liposomes were determined by atomic absorption measurement.

Enzyme Assay——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 hypoxanthine converted to uric acid per min at pH 7.5. SOD activity was measured by the method of McCord *et al.*[5] The assay for catalase activity was based on the decomposition of H_2O_2, which was followed spectrophotometrically at 240 nm; one unit of activity was defined as 1.0 μmol of H_2O_2 decomposed per min.[13] Determination of H_2O_2 was followed by the method of Hildebrandt and Roots.[14] Protein concentration was estimated by the method of Lowry *et al.*[15] Specific activities of SOD and catalase used in this study were 2.9×10^3 and 2.2×10^4 U/mg, respectively.

Reaction System——Erythrocyte membranes (400 μg protein) or PC liposomes (10 μmol) were suspended in 3.0 ml of 10 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl, 5 mM hypoxanthine and 0.025 unit of xanthine oxidase. Other additions or deletions were as specified in the figures and tables. To remove trace metals, all the buffer solutions used were treated with Chelex-100 resin. The reaction was initiated by addition of xanthine oxidase with shaking at 37 °C.

Lipid Peroxidation——Malondialdehyde (MDA) formation, a measure of lipid peroxidation, was determined as described previously.[10] To prevent further peroxidation of lipids during the assay procedure, 0.1 ml of 1% butylated hydroxytoluene in dimethylsulfoxide was added to the TBA reagent. A large amount of H_2O_2 has been reported to prevent the efficient detection of MDA by the TBA colorimetric method,[16] but the amount of H_2O_2 generated in the reaction system used in this study scarcely affected the detection of MDA.

Results

Enhanced Effect of Catalase on Lipid Peroxidation

When rat erythrocyte membranes were incubated in the xanthine oxidase system, lipid peroxidation was observed as indicated by an increase in the formation of MDA as shown in Fig. 1. The addition of catalase to the reaction system (1.0 μg/ml) caused a significant increase of MDA formation, indicating that lipid peroxidation is stimulated by catalase together with xanthine oxidase as described previously.[10] When liposomes were incubated in the reaction system in the presence of catalase, MDA formation was greatly enhanced and the amount of MDA formed was about 6.8 nmol in 120 min. In contrast, the incubation in the absence of catalase resulted in no significant peroxidation of lipids. When catalase (1.0 μg/ml) was added

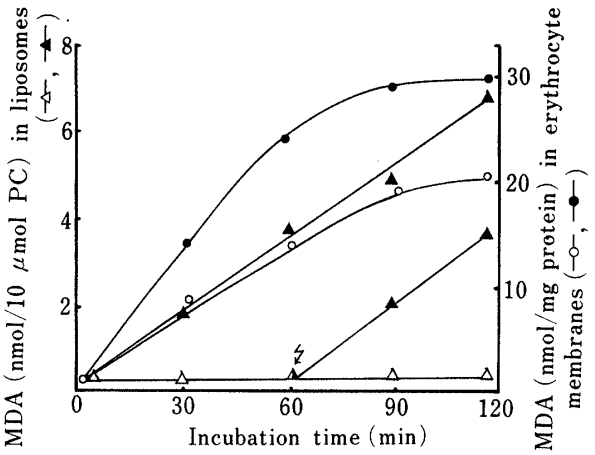

Fig. 1. Time Course of Lipid Peroxidations of Erythrocyte Membranes and PC Liposomes Induced by the Xanthine Oxidase System in the Presence of Catalase

The xanthine oxidase system contained 0.15 M NaCl, 5 mM hypoxanthine and 0.025 unit of xanthine oxidase in 3.0 ml of 10 mM phosphate buffer, pH 7.4. Erythrocyte membranes (400 μg protein) and PC liposomes (10 μmol) were added to the complete system and incubated for various periods of time at 37 °C. —○—, erythrocyte membranes; —●—, erythrocyte membranes + catalase (1.0 μg/ml); —△—, PC liposomes; —▲—, PC liposomes + catalase (1.0 μg/ml). Catalase (1.0 μg/ml) was added to the reaction system at the point indicated by the arrow. Each point represents the mean of triplicate experiments.

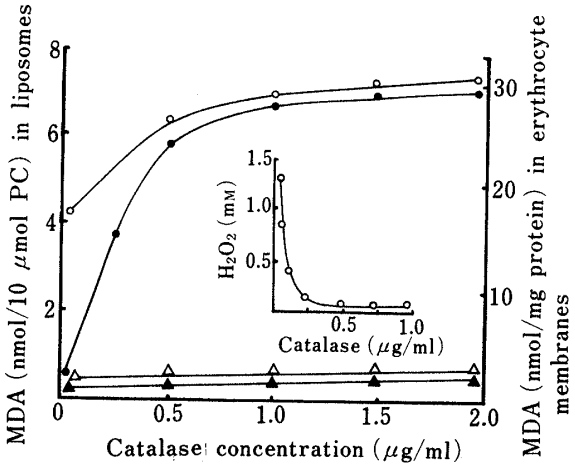

Fig. 2. Effect of Catalase Concentration on Lipid Peroxidation of Erythrocyte Membranes and PC Liposomes Induced by the Xanthine Oxidase System

Erythrocyte membranes and PC liposomes were added to the reaction system in the presence of various concentrations of catalase and incubated for 120 min at 37 °C. The insert shows the concentration of H_2O_2 generated in the reaction system at 120 min after the start of incubation. Each point represents the mean of triplicate experiments. —○—, erythrocyte membranes; —●—, PC liposomes; —△—, erythrocyte membranes + SOD (1.0 μg/ml); —▲—, PC liposomes + SOD (1.0 μg/ml).

60 min after the start of incubation, the peroxidation reaction proceeded linearly at a rate similar to that seen when catalase was present from the start. Further addition of a large amount of xanthine oxidase (over 0.025 unit) at 60 min caused no significant increase in MDA formation unless catalase was present in the reaction system.

As shown in Fig. 2, added catalase caused an increase in the lipid peroxidation of both erythrocyte membranes and liposomes in a concentration-dependent manner up to 1.0 μg/ml, while heat-inactivated catalase had no effect. The enhanced lipid peroxidations observed in the presence of catalase were strongly inhibited by SOD (1.0 μg/ml) but were unaffected by heat-denatured SOD. The insert of Fig. 2 shows the effect of catalase on the concentration of H_2O_2 formed in the reaction system during 120 min of incubation. In the absence of catalase, H_2O_2 accumulated was 1.4 mM in 120 min. The addition of catalase up to 0.5 μg/ml resulted in a progressive decrease of H_2O_2 with an increase of MDA formation. H_2O_2 was completely decomposed by catalase above the concentration range of 0.5 to 1.0 μg/ml. No lipid peroxidation of erythrocyte membranes or liposomes was induced during the decomposition of 1.5 mM H_2O_2 added to the reaction medium by catalase. These results suggest that the enhanced lipid peroxidation of erythrocyte membranes and liposomes observed in the presence of catalase is entirely dependent on O_2^- but not H_2O_2.

Involvement of a Trace of Iron

The process of lipid peroxidation has been suggested to be mediated by trace amounts of iron salts,[8b] so the possible participation of iron in the enhanced lipid peroxidation by catalase was examined by using several iron-chelators. As shown in Table I, the addition of diethylenetriaminepenta-acetic acid (DETAPAC) or a specific iron chelator, desferrio-

TABLE I. Effect of Iron-Chelators and Iron-Binding Proteins on Lipid Peroxidations in the Presence of Catalase

Experimental conditions	Concentration	Erythrocyte membranes		PC liposomes	
		MDA (nmol)	Inhibition (%)	MDA (nmol)	Inhibition (%)
None	—	28.8±0.7	—	6.8±0.9	—
DETAPAC	10 (mM)	1.1±0.1	96.2	0.8±0.1	88.2
Desferrioxamine	10 (mM)	1.2±0.1	95.8	0.7±0.2	89.7
Apotransferrin	30 (μg/ml)	3.2±0.1	88.9	0.3±0.1	95.6
Transferrin	30 (μg/ml)	30.3±1.0	5.2[a)]	7.3±0.2	7.4[a)]
Denatured apotransferrin	30 (μg/ml)	29.5±0.3	2.4[a)]	5.4±0.3	20.6
Apolactoferrin	30 (μg/ml)	2.8±0.1	90.3	1.8±0.1	74.6
Denatured apolactoferrin	30 (μg/ml)	29.0±0.3	0.7[a)]	5.8±0.2	14.7

Erythrocyte membranes and PC liposomes were added to the xanthine oxidase system in the presence of catalase (1.0 μg/ml). Iron-chelators and iron-binding proteins were added to the reaction system before initiation of the reaction. Each value represents the mean ± S.E. of triplicate experiments. *a*) Stimulation.

xamine, at a final concentration of 10 μM, caused about 96% inhibition of the peroxidation in the erythrocyte membranes and about 88% inhibition in the liposome system. Apotransferrin and apolactoferrin were potent inhibitors of both lipid peroxidations. Their inhibitory effects can be attributed to their high iron-binding capacity, since neither iron-saturated nor denatured protein has any significant effect on the peroxidation reaction. Iron-chelators themselves had little effect on O_2^- generation in the xanthine oxidase system. These results suggest that a trace of iron may be required for the O_2^--dependent lipid peroxidation in the presence of catalase. The reaction mixtures used were treated with Chelex-100 resin to remove contaminating metals as described in Experimental. The trace of iron involved in the peroxidation reaction, therefore, appears to arise from endogeneous iron in the erythrocyte membranes and liposomes. Actually, the erythrocyte membranes and liposomes used in this study contained about 140 ng Fe/mg of protein and 50 ng/mg of phospholipid, respectively.

The effect of added $FeCl_3$ (10 μM) on the lipid peroxidation of liposomes is shown in Fig. 3. In the absence of catalase, the rate of peroxidation was stimulated only slightly by addition of Fe^{3+}. In the presence of catalase, however, added Fe^{3+} markedly accelerated the peroxidation reaction. The addition of desferrioxamine (10 μM) 60 min after initiation of the reaction caused a marked depression of MDA formation. When SOD (1.0 μg/ml) was added to the liposome system in the presence of both catalase and Fe^{3+}, the lipid peroxidation was strongly inhibited to the extent of 87%.

Effect of Added Fe^{3+} on Ferricytochrome c Reduction in the Xanthine Oxidase System

As shown in Fig. 4, the rate of cytochrome c reduction in the absence of catalase rapidly decreased about 3 min after the start of incubation. In the presence of catalase, however, the xanthine oxidase reaction caused a time-dependent reduction of cytochrome c, which was almost completely inhibited by SOD (1.0 μg/ml). These results indicate that the net decrease in the reduction of cytochrome c in the absence of catalase resulted from reoxidation of ferrocytochrome c by H_2O_2 accumulated during the incubation. The addition of Fe^{3+} to the xanthine oxidase system in the presence of catalase caused a slight but significant decrease in the rate of cytochrome c reduction, suggesting that Fe^{3+} may be reduced to Fe^{2+} by O_2^-. On the other hand, when Fe^{3+} was added to the xanthine oxidase system in the absence of catalase, no significant effect was observed. This result suggests that Fe^{3+} reduced by O_2^- is

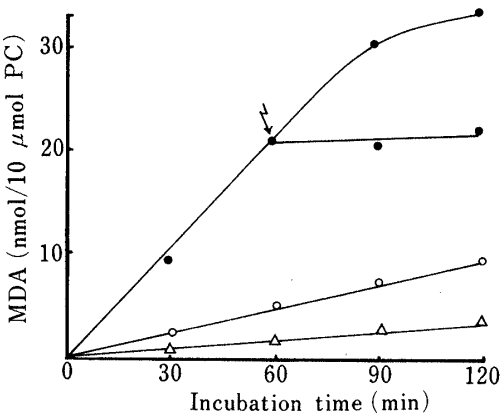

Fig. 3. Effect of Added Fe^{3+} on Lipid Peroxidation of PC Liposomes Induced by the Xanthine Oxidase System

Ferric chloride (10 μM) was added to the reaction system containing PC liposomes and the whole was incubated for various periods of time at 37 °C. Desferrioxamine, at a final concentration of 10 μM, was added at the point indicated by the arrow. Each point represents the mean of triplicate experiments. —●—, + catalase (1.0 μg/ml); —○—, − catalase; —△—, + catalase (1.0 μg/ml) and SOD (1.0 μg/ml).

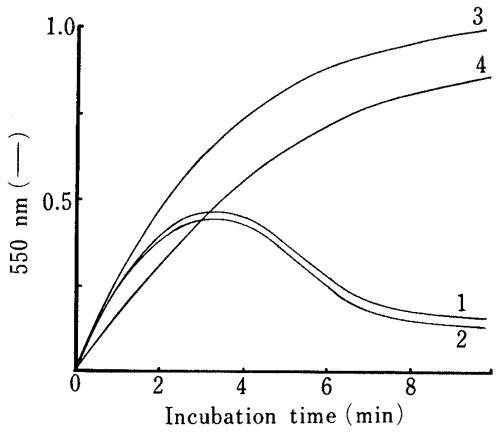

Fig. 4. Effect of Catalase and Fe^{3+} on the Rate of O_2^- Generation in the Xanthine Oxidase System

The reduction of ferricytochrome c (0.1 mM) in the complete system as described in Fig. 1 was monitored by following the change in absorbance at 550 nm. Components of the reaction system were as follows; 1, complete system; 2, + 10 μM Fe^{3+}; 3, + catalase (1.0 μg/ml); 4, + catalase (1.0 μg/ml) and 10 μM Fe^{3+}.

TABLE II. Effect of Various Scavengers on the Lipid Peroxidations of Erythrocyte Membranes and PC Liposomes

Scavenger	Concentration (mM)	Erythrocyte membranes		PC liposomes	
		MDA (nmol)	Inhibition (%)	MDA (nmol)	Inhibition (%)
None	—	28.8±0.7	—	6.8±0.9	—
Histidine	10	4.8±0.4	83.3	1.1±0.3	83.8
DABCO	10	12.0±1.0	58.3	0.5±0.2	92.6
Mannitol	10	27.7±0.4	3.2[a)]	6.4±1.0	5.9[a)]
Benzoate	10	29.4±0.7	2.1[a)]	7.0±1.1	2.9[a)]

Erythrocyte membranes and PC liposomes were added to the xanthine oxidase system with catalase (1.0 μg/ml) and incubated for 120 min at 37 °C. Various scavengers were added to the reaction system before the initiation of the reaction. Each value represents the mean ± S.E. of triplicate experiments. *a*) Without significance.

rapidly reoxidized by H_2O_2 generated in the xanthine oxidase system.

Effect of Chemical Scavengers

Table II shows the effects of several radical scavengers on the lipid peroxidation of erythrocyte membranes and liposomes induced by the xanthine oxidase system in the presence of catalase. Histidine and DABCO, which are used as quenchers for $^1O_2$, inhibited the peroxidation of erythrocyte membranes to extents of about 83 and 60%, respectively. In the liposome system, both quenchers were also powerful inhibitors. Mannitol and benzoate, well-known scavengers of $OH^{\cdot}$, showed no significant effect on the lipid peroxidation of membranes or liposomes, indicating that $OH^{\cdot}$ was not involved in these peroxidation reactions. Although the data are not shown, in the presence of catalase, DABCO (10 mM) inhibited the enhanced lipid peroxidation induced by addition of Fe^{3+} (10 μM) by about 50%. These results indicate a possible participation of $^1O_2$ species in the O_2^--dependent peroxidation induced by the xanthine oxidase system in the presence of catalase.

Discussion

Xanthine oxidase is known to suffer from auto-inactivation because of its high reactivities with self-generated H_2O_2 and O_2^-.[17] Since catalase prevents this inactivation, the increase in net production of O_2^- was considered as one of the direct causes of enhanced lipid peroxidation.[10] However, even addition of a large amount of xanthine oxidase to the reaction system 60 min after incubation caused no lipid peroxidation of liposomes (Fig. 1), suggesting that the promotive effect of catalase on the lipid peroxidation could be due to destruction of H_2O_2 generated in the xanthine oxidase system rather than stabilization of xanthine oxidase. Aust *et al.* have demonstrated that direct addition of H_2O_2 (below 0.9 mM) during the xanthine oxidase-dependent peroxidation of liposomes in the presence of iron chelates causes a sharp decrease in the rate of MDA formation.[18] Unlike the case with the liposome system, lipid peroxidation of erythrocyte membranes actually occurred in the absence of catalase at rates significantly slower than those observed in the presence of catalase (Fig. 1). This may be a reflection of the different configuration of the model membranes or the presence of a small amount of catalase in the erythrocyte membranes as reported by Snyder *et al.*[19]

SOD and several iron chelators inhibited the enhanced lipid peroxidation of both erythrocyte membranes and liposomes in the presence of catalase (Fig. 1 and Table I). Furthermore, our results with liposomes show that the lipid peroxidation is greatly enhanced by addition of Fe^{3+} in the presence of catalase (Fig. 3). These results suggest that enhanced lipid peroxidation is initiated *via* an oxidation reaction of O_2^- by Fe^{3+}. The reduction of Fe^{3+} by O_2^- proceeds as follows;[20]

$$O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad (1)$$

The occurrence of this reaction was supported by the finding that added Fe^{3+} inhibits cytochrome c reduction by O_2^- (Fig. 4), in agreement with previous results.[21] The reaction of Fe^{2+} with H_2O_2, which is frequently referred to as the Fenton reaction, is known to produce the very reactive species $OH^{\cdot}$ as follows;

$$Fe^{2+} + H_2O_2 \rightarrow OH^{\cdot} + OH^- + Fe^{3+} \quad (2)$$

The OH radical generated by reaction (2) is unlikely to be a species responsible for the enhanced lipid peroxidation, however, because reaction (2) must be inhibited by catalase. Therefore, the enhanced lipid peroxidation described here may occur through the facilitation of reaction (1) by rapid decomposition of H_2O_2. The iron-catalyzed reactions (1) and (2) may be represented by the overall equation, namely, the Haber–Weiss reaction, as follows;[8b]

$$O_2^- + H_2O_2 \rightarrow OH^{\cdot} + OH^- + O_2 \quad (3)$$

Therefore, an alternative explanation for the marked stimulation of peroxidation by catalase is that the removal of H_2O_2 blocks the consumption of O_2^- by reaction (3), as postulated by Tyler.[21b] The enhanced lipid peroxidation observed upon addition of catalase may be induced by a sufficient supply of O_2^- and facilitation of Fe^{2+} formation for the peroxidation reaction.

Gutteridge *et al.* have demonstrated that iron chelators, desferrioxamine and DETAPAC, inhibit the peroxidation of membrane lipids induced by adding Fe^{2+} and also inhibit the iron-catalyzed formation of $OH^{\cdot}$ from O_2^- (Equation 3).[8b] Cohen *et al.*[22] postulated that the ability of DETAPAC to block oxidative phenomena in biological systems is probably due to suppression of Fe^{3+} reduction by O_2^-. Both lactoferrin and transferrin at physiological extents of iron-saturation have been reported to be powerful inhibitors of iron-dependent lipid peroxidation.[23] Addition of Fe^{3+} or Fe^{2+} to the xanthine oxidase system in the presence of catalase caused a rapid peroxidation which was strongly inhibited by SOD

(data not shown). These results and the observation that the peroxidation reaction was inhibited by several iron-chelators (Table I) indicate that Fe^{2+} plays an important role in the initiation or propagation of lipid peroxidation. The form of ferrous ion which promotes lipid peroxidation has been proposed to be some type of perferryl ion ($Fe^{2+}O_2$) or ferryl ion (FeO^{2+}), but the precise mechanism is not yet clear.[24]

In the present experiments, mannitol and benzoate, which are $OH^{\cdot}$ scavengers, did not significantly affect the lipid peroxidation (Table II), suggesting again that $OH^{\cdot}$ plays no part in the observed peroxidation reaction. Moreover, chemical scavengers of $^1O_2$ strongly inhibited the peroxidation reactions, indicating a possible participation of $^1O_2$. Singlet oxygen, which is a highly excited, energetic species of molecular oxygen, may be generated by spontaneous dismutation of O_2^-. Khan has demonstrated the production of $^1O_2$ *via* the spontaneous dismutation of O_2^- by using anthraquinone as a fluorphor and by direct detection of the emission spectrum at 1268 nm.[25] Other workers have also presented evidence for the production of $^1O_2$ by the xanthine oxidase system.[26] In contrast to these results, King *et al.* have found no evidence for production of $^1O_2$ by the xanthine oxidase system, suggesting that $^1O_2$ formed from the breakdown of lipid peroxides contributes to further peroxidative attack on the membrane lipids.[27] Furthermore, Aust *et al.* have proposed that $^1O_2$ generated by the breakdown of initially formed lipid hydroperoxides is not involved in the initiation but rather in the propagation step of lipid peroxidation.[24a] Our results together with these findings leads us to speculate that interaction of $^1O_2$ and Fe^{2+} with membrane lipids is important as a causative factor in the O_2^--dependent lipid peroxidation process.

Other workers have pointed out that SOD can function as a scavenger of $^1O_2$,[28] so the inhibitory effect of SOD on the lipid peroxidation induced by O_2^- might be explainable on the basis of its ability to remove O_2^- and $^1O_2$. Although $^1O_2$ has been suggested to occur in many biological reactions,[29] the mechanism by which $^1O_2$ is formed from O_2^- is not yet known. A more sensitive technique for identification of $^1O_2$ in biological systems is required to clarify the participation of $^1O_2$ in lipid peroxidation and the physiological significance of SOD in relation to biological damage caused by oxygen radicals.

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