Role of Alloxan Radical in Generation of Hydroxyl Radical by Reaction of Alloxan with Glutathione in the Presence of Fe^{3+}-Ethylenediaminetetraacetic Acid

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The degradation of deoxyribose, an indicator of hydroxyl radical (HO·) generation, was observed in the reaction of alloxan with glutathione (GSH) in the presence of Fe^{3+}-ethylenediaminetetraacetic acid (EDTA). Catalase and HO· scavengers strongly inhibited this degradation, but superoxide dismutase (SOD) did not do so effectively, suggesting that the HO· was generated *via* the Fenton reaction but not the Haber–Weiss reaction. The reduction of Fe^{3+}-EDTA was rapid in the reaction of alloxan with GSH. The generation of alloxan radical in the reaction system was diminished by the addition of Fe^{3+}-EDTA, indicating that Fe^{3+}-EDTA was directly reduced by the alloxan radical. However, only a large amount of SOD inhibited both reduction of Fe^{3+}-EDTA and generation of alloxan radical, suggesting that superoxide radical (O_2^-) may participate indirectly in both reactions. The generation of O_2^- and H_2O_2 in the reaction was confirmed by the formation of compound III from lactoperoxidase and by partial regeneration of consumed oxygen upon addition of catalase, respectively, suggesting that O_2^- may be a source of H_2O_2. From these results, we concluded that HO· was generated by alloxan radical-dependent Fenton reaction.

Keywords alloxan; alloxan radical; oxygen radical; iron reduction; hydroxyl radical

Alloxan is widely used to produce experimental diabetes.[1–3] Although the mechanism of alloxan toxicity is not fully understood, the action is thought to be initiated by generation of oxygen radicals.[4,5] Hekkila *et al.*[3,6] found that the injection of hydroxyl radical (HO·) scavengers such as ethanol and thiourea into animals protected them against the diabetogenic action of alloxan. Several workers[7–11] have also demonstrated that the cytotoxic action of alloxan on isolated pancreatic islets of Langerhans was inhibited by superoxide dismutase (SOD), catalase and diethylenetriaminepentaacetic acid and a HO· scavenger, dimethylurea. Therefore, the action of alloxan is thought to be mediated by HO·.

Many workers[3,7,10,12,13] have provided evidence that HO· is generated from superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) *via* the iron-catalyzed Haber-Weiss reaction. As previously reported,[14,15] however, alloxan radical (HA·) generated from alloxan in the presence of reduced glutathione (GSH) could reductively release iron from ferritin. These findings strongly suggest that ferric iron is directly reduced by this radical rather than O_2^-. Therefore, we investigated the role of HA· in the generation of HO· by the reaction of alloxan with GSH in the presence of Fe^{3+}-ethylenediamine tetraacetic acid (EDTA).

Experimental

Materials Alloxan, GSH and 2-deoxy-D-ribose (DOR) were purchased from Wako Pure Chemical Industries Ltd., Japan. SOD (from bovine erythrocytes), catalase (from bovine liver, thymol-free), and lactoperoxidase (from bovine milk) were from Sigma Co., St. Louis, Mo. Other chemicals used in this experiment were of analytical grade from commercial suppliers.

Deoxyribose Degradation Thiobarbituric acid (TBA)-reactive oxidation products of DOR were determined according to the method of Halliwell and Gutteridge[16] with minor modifications. DOR (2.0 mM) was incubated in 3.0 ml of 10 mM phosphate buffer, pH 7.4, containing 1.0 mM alloxan, 0.3 mM GSH, 100 μM Fe^{3+}-EDTA and 0.15 M NaCl. The degradation of DOR was terminated after incubation for 10 min at 37 °C by mixing with 0.03 ml of 30% trichloroacetic acid, and then 0.5 ml of 0.6% TBA was added. The solution was heated for 10 min at 100 °C and cooled. The absorbance at 532 nm was measured with a spectrophotometer (Hitachi model 200-20).

Assay of Fe^{3+}-EDTA Reduction Fe^{3+}-EDTA (100 μM) was incubated in 10 mM phosphate buffer, pH 7.4, containing 1.0 mM alloxan, 0.3 mM GSH, 0.15 M NaCl and 1.0 mM bathophenanthroline sulfate. The reaction was initiated by addition of alloxan at 37 °C and continuously monitored at 534 nm. The amount of reduced iron was calculated from the increase in the absorption, based on a coefficient of 22140 $M^{-1} \cdot cm^{-1}$.[17] For anaerobic experiments, all solutions containing 5.0 mM glucose and 10 U/ml of glucose oxidase were purged with N_2 gas to remove any remaining oxygen.[18] To prevent ferrous oxidation by H_2O_2, 420 U/ml of catalase was also included.

Electron Spin Resonance (ESR) Measurements ESR measurements were made at room temperature with a JEOL model JES-RE1X. Spectrometer settings for HA· were 3365 G magnetic field, 5.0 mW microwave power, 9.450 GHz modulation frequency, 0.20 G field modulation width, 0.3 s time constant and 2.5 G/min scan rate. Samples were mixed outside of the cavity, then rapidly aspirated into the aqueous flat cell for ESR measurements. The g-value was measured using Mn^{2+} as a marker. The relative intensity of HA· was determined by measuring the peak height at the center hyperfine line of the spectrum.

Detection of O_2^- Production O_2^- was detected by the production of compound III from lactoperoxidase.[19] Lactoperoxidase (10 μM) was incubated in 10 mM phosphate buffer, pH 7.4, containing 1.0 mM alloxan, 0.3 mM GSH and 0.15 M NaCl. To avoid any effect of H_2O_2, 420 U/ml catalase was added to all the reaction mixtures.[20] The reaction was initiated by addition of alloxan at 37 °C and continously monitored at 588 nm.

Oxygen Consumption Oxygen consumption was measured polarographically at 37 °C with a Clark-type oxygen electrode (Yanaco model PO-100A). The reaction mixture consisted of 1.0 mM alloxan and 0.3 mM GSH in 0.6 ml of 10 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl. The amount of produced H_2O_2 was calculated from the amount of oxygen release upon addition of catalase to the reaction mixture, using the following relationship: $H_2O_2 \rightarrow 1/2\,O_2 + H_2O$.[21]

Results

Generation of Hydroxyl Radical As summarized in Table I, degradation of DOR, an indicator of HO· generation, occurred in the reaction system of alloxan with GSH in the presence of Fe^{3+}-EDTA. The DOR degradation was scarcely observed in the reaction system in the absence of Fe^{3+}-EDTA. When alloxan or GSH was omitted from the complete system, the degradation of DOR was not observed. Addition of 50 U/ml of SOD to the complete system had no significant effect on the degradation of DOR, but a large amount of SOD (500 or 1500 U/ml) was slightly inhibitory. Addition of catalase (140 U/ml) caused 80% inhibition. The scavengers of HO·, mannitol and benzoate, at a concentration of 100 mM also inhibited the degradation

TABLE I. Inhibition of Deoxyribose Degradation by SOD, Catalase and Hydroxyl Radical Scavengers

Conditions	OD 532 nm	% inhibition
Complete system	0.342	—
+SOD (50 U/ml)	0.355	−4
+SOD (500 U/ml)	0.262	23
+SOD (1500 U/ml)	0.228	33
+catalase (140 U/ml)	0.069	80
+mannitol (10 mM)	0.126	63
+mannitol (100 mM)	0.006	98
+benzoate (10 mM)	0.120	65
+benzoate (100 mM)	0.050	85

The complete system consisted of 2.0 mM deoxyribose, 1.0 mM alloxan, 0.3 mM GSH, 0.15 M NaCl and 100 μM Fe^{3+}-EDTA in 10 mM phosphate buffer, pH 7.4. Other conditions were described in Experimental. Each value represents the mean of triplicate experiments.

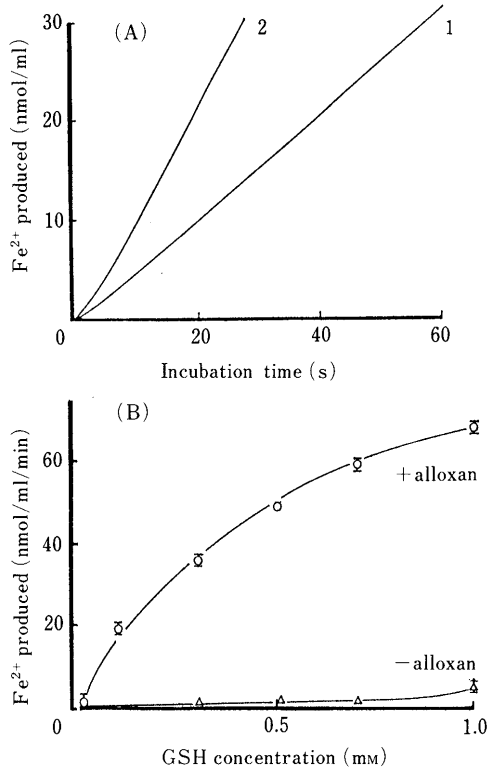

Fig. 1. Reduction of Fe^{3+}-EDTA by Alloxan with GSH

(A) The reaction mixture contained 1.0 mM alloxan, 0.3 mM GSH, 100 μM Fe^{3+}-EDTA and 0.15 M NaCl in 10 mM phosphate buffer, pH 7.4. The reaction was started by addition of alloxan under aerobic (1) and anaerobic conditions (2). Other conditions were as noted in Experimental. (B) The experimental conditions were the same as in (A) under aerobic conditions, except that the concentration of GSH was varied. Each point indicates the mean ± S.E. of triplicate experiments. If no S.E. is given, the S.E. was smaller than the symbol used.

by 98 and 85%, respectively. These results indicate that HO· is generated in the reaction of alloxan with GSH in the presence of Fe^{3+}-EDTA.

Reduction of Fe^{3+}-EDTA Figure 1 (A) shows the time course of the reduction of Fe^{3+}-EDTA in the reaction system of alloxan with GSH. Fe^{3+}-EDTA was rapidly reduced and the amount of Fe^{2+} increased linearly with time for at least 60 s under aerobic conditions. Neither alloxan nor GSH alone reduced Fe^{3+}-EDTA. Under anaerobic conditions, the rate of Fe^{3+}-EDTA reduction was about twice that under aerobic conditions. When EDTA was displaced by other iron-chelators such as adenosine diphosphate (ADP) or citrate, almost the same results were

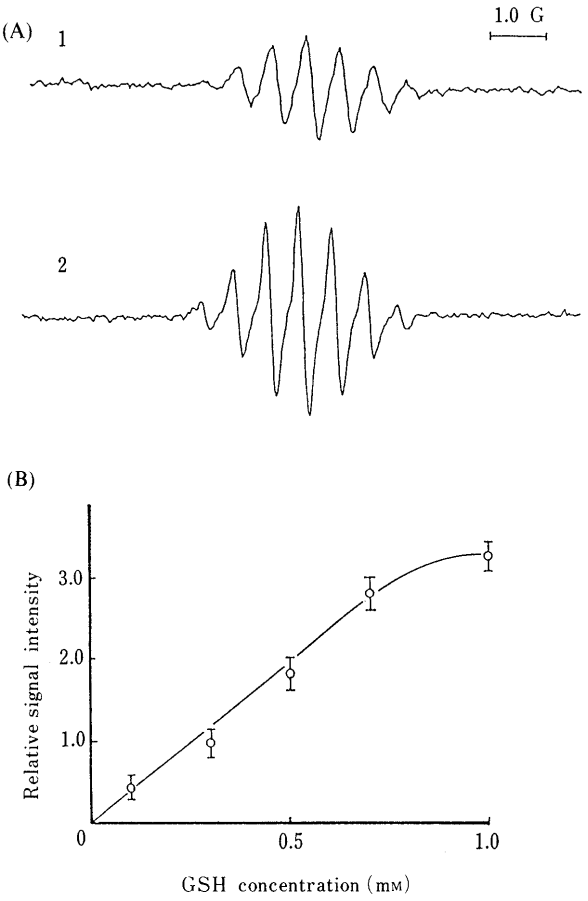

Fig. 2. ESR Spectrum of the Alloxan Radical

(A) Alloxan was incubated with GSH (0.3 mM) in 10 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl. The reaction of alloxan with GSH was carried out under aerobic (1), or anaerobic conditions (2). Other conditions were as noted in Experimental. (B) The experimental conditions were the same as in (A) under aerobic conditions, except that the concentration of GSH was varied. Each point indicates the mean ± S.E. of triplicate experiments.

obtained (data not shown).

As shown in Fig. 1 (B), the initial rates of Fe^{3+}-EDTA reduction increased with increasing concentration of GSH up to 1.0 mM under aerobic conditions. In these cases, GSH alone at below 1.0 mM slightly reduced Fe^{3+}-EDTA under the experimental conditions used. These results indicate that Fe^{3+}-EDTA is easily reduced depending on the concentration of GSH in the reaction system, even under aerobic conditions.

Generation of Alloxan Radical ESR spectroscopy was used to detect HA· in the reaction system in the absence of Fe^{3+}-EDTA. As shown in Fig. 2 (A), the ESR spectrum of HA· was obtained when alloxan was incubated with GSH under aerobic conditions. The spectrum obtained here agreed with that of the HA· reported by other workers.[22,23] When GSH was omitted from the reaction system, no generation of HA· was observed. Under anaerobic conditions, a high signal intensity of HA· was observed, indicating that the generation of HA· is enhanced under anaerobic as compared to aerobic conditions.

As shown in Fig. 2 (B), the signal intensity of HA· was increased with increasing concentration of GSH under aerobic conditions.

Effect of Fe^{3+}-EDTA on Alloxan Radical As shown in Fig. 3, addition of Fe^{3+}-EDTA to the reaction system of alloxan with GSH resulted in a decrease in the signal

intensity of HA· under aerobic conditions, reaching about 85% diminution at 100 μM Fe^{3+}-EDTA. Similarly, under anaerobic conditions, the addition of Fe^{3+}-EDTA caused a decrease in the signal intensity of HA·. These results indicate that the HA· directly reacts with Fe^{3+}-EDTA under both aerobic and anaerobic conditions.

Effect of SOD As shown in Table II, a small amount of SOD (50 U/ml) had no significant effect on the reduction of Fe^{3+}-EDTA and the generation of HA·, while a large amount of SOD (500 or 1500 U/ml) was slightly inhibitory. No inactivation of SOD was observed through the course of incubation. Under anaerobic conditions, the effects of SOD were scarcely observed. These results indicate a requirement of a high concentration of SOD to inhibit the reduction of Fe^{3+}-EDTA and generation of AH·.

Generation of O_2^- We next attempted to detect O_2^- in the reaction of alloxan with GSH in the absence of Fe^{3+}-EDTA. The lactoperoxidase reacts with O_2^- to produce compound III, which has a characteristic absorption at 546 and 588 nm.[19] As shown in Fig. 4 (A), the characteristic absorption of compound III was obtained in the reaction system of alloxan with GSH. When GSH was omitted from the reaction mixture, the production of compound III was scarcely observed. As shown in Fig. 4 (B), the absorbance at 588 nm increased depending on the concentration of GSH. In addition, the increase of absorption at 588 nm was completely inhibited by addition of SOD (50 U/ml) (data not shown). These results indicate that O_2^- is generated depending on the concentration of GSH in the reaction of alloxan with GSH.

Oxygen Consumption and Generation of H_2O_2 As shown in Fig. 5, oxygen consumption was rapidly initiated by the addition of alloxan to the reaction medium containing 0.3 mM GSH. When the reaction had ceased, the addition of catalase (420 U/ml) caused return to the solution of about 42% of the consumed oxygen, and the amount of H_2O_2 produced was about 45 nmol/ml. Neither alloxan nor GSH alone caused any oxygen consumption. Increasing the concentration of GSH resulted in an increase

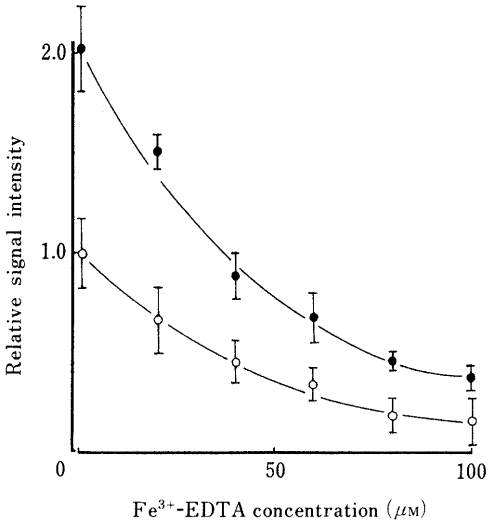

Fig. 3. Effect of Fe^{3+}-EDTA on the Generation of Alloxan Radical

The reaction mixture contained 1.0 mM alloxan, 0.3 mM GSH, 0.15 M NaCl and various concentrations of Fe^{3+}-EDTA in 10 mM phosphate buffer, pH 7.4. The reaction was performed under aerobic (○) or anaerobic conditions (●). Other conditions were as noted in Experimental. Each point indicates the mean ± S.E. of triplicate experiments.

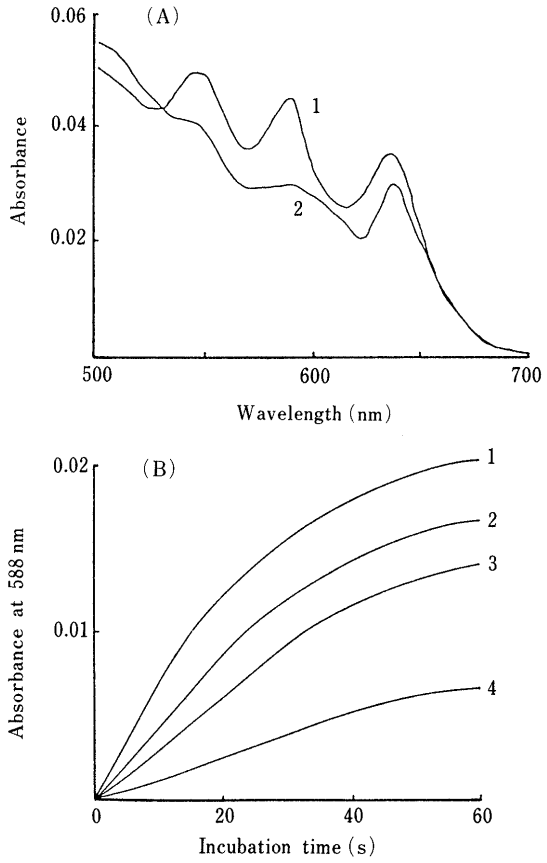

Fig. 4. Production of Compound III from Lactoperoxidase in the Reaction of Alloxan with GSH

(A) The reaction mixture contained 1.0 mM alloxan, 0.3 mM GSH and 0.15 M NaCl in 10 mM phosphate buffer, pH 7.4. Absorption spectra were recorded 5 min after the addition of alloxan to the complete system (1) and without GSH (2). Other conditions were as noted in Experimental. (B) The experimental conditions were the same as given in (A), except that the concentration of GSH was 1.0 mM (1), 0.5 mM (2), 0.3 mM (3), or 0.1 mM (4). The reaction was initiated by the addition of alloxan and continuously monitored at 588 nm.

TABLE II. Effect of SOD on Reduction of Fe^{3+}-EDTA and Generation of Alloxan Radical

Conditions	Fe^{3+}-EDTA reduction (nmol/min/ml)	Inhibition (%)	Alloxan radical intensity	Inhibition (%)
Reaction system	35.1 ± 0.6 (3)	—	1.33 ± 0.02 (8)	—
+SOD (50 U/ml)	32.5 ± 1.0 (3)	7.5	1.24 ± 0.03 (3)	6.8
+SOD (500 U/ml)	19.9 ± 0.6 (3)	43.3	0.92 ± 0.16 (3)	30.8
+SOD (1500 U/ml)	14.8 ± 0.5 (3)	57.8	0.68 ± 0.17 (6)	48.9
+denatured SOD (1500 U/ml)	34.7 ± 0.3 (3)	1.1	1.41 ± 0.04 (3)	−6.0

Reduction of Fe^{3+}-EDTA was carried out in the reaction system consisted of 1.0 mM alloxan, 0.3 mM GSH, 0.15 M NaCl, 1.0 mM bathophenanthroline sulfonate and 100 μM Fe^{3+}-EDTA in 10 mM phosphate buffer, pH 7.4. Measurement of alloxan radical intensity was performed in the reaction system without bathophenanthroline sulfonate and Fe^{3+}-EDTA. Other conditions were as described in Experimental. Each value represents the mean ± S.E. Numbers of experiments are given in parentheses.

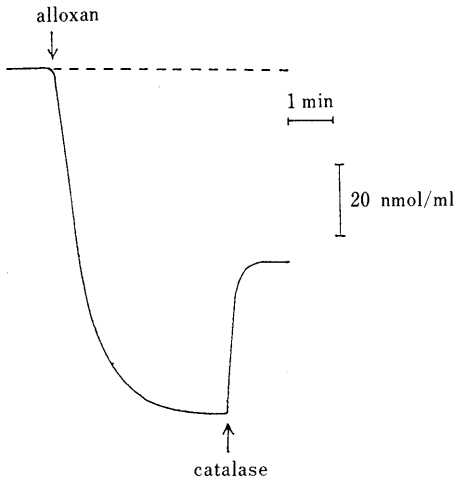

Fig. 5. Oxygen Consumption in the Reaction of Alloxan with GSH

The reaction mixture contained 1.0 mM alloxan, 0.3 mM GSH, 0.15 M NaCl in 10 mM phosphate buffer, pH 7.4. Oxygen consumption was measured with an oxygen electrode as described in Experimental. Addition of catalase (30 μg/ml) is indicated by arrows. The dotted line shows the oxygen consumption in the reaction system without GSH.

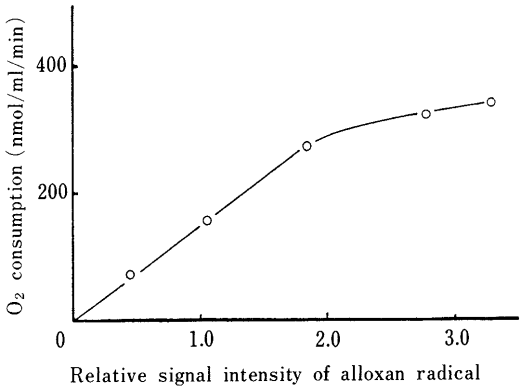

Fig. 6. The Rate of Oxygen Consumption Plotted against the Signal Intensity of Alloxan Radical

The rate of oxygen consumption was measured at various concentrations of GSH with a Clark-type oxygen electrode as described in Experimental. The signal intensity of alloxan radical was determined at various concentrations of GSH by ESR under the experimental conditions described in Fig. 2 (A).

of the rate of oxygen consumption (data not shown).

A relationship between the rate of oxygen consumption and the signal intensity of HA· was demonstrated by experiments performed with different concentrations of GSH. As summarized in Fig. 6, the rates of oxygen consumption increased with increasing generation of AH·. These results suggest a possible interaction of HA· with oxygen and a consequential generation of O_2^-.

Discussion

Hydroxyl radical, the most reactive species among active oxygens, has been implicated in the diabetogenic effect of alloxan.[3,6,7] The present study demonstrated that HO· was generated by the reaction of alloxan with GSH in the presence of Fe^{3+}-EDTA. HO· is known to be generated *via* the iron-catalyzed Haber-Weiss reaction[24] as follows:

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

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

This reaction is essentially O_2^--dependent, so SOD should strongly inhibit the generation of HO·. However, the generation of HO· in the reaction system of alloxan with GSH was strongly inhibited by catalase, but not effectively by SOD (Table I). This suggested that HO· was generated *via* the reaction between Fe^{2+} and H_2O_2 (reaction 2), namely, the Fenton reaction[25] but not *via* the Haber-Weiss reaction.

GSH has been reported to reduce alloxan by one electron to HA·.[22,23] On the other hand, HA· can be produced by reaction of alloxan with O_2^-.[26] The findings that SOD (50 U/ml) did not inhibit the generation of HA· (Table II) and that increasing yield of HA· was obtained with increasing concentration of GSH (Fig. 2B) indicate that alloxan is directly reduced by GSH rather than O_2^-, yielding HA·.

Since the fact that HA· mediates the release of ferritin iron has been reported,[14,15] HA· would have a potent capacity to reduce chelated iron with a low redox potential such as Fe^{3+}-EDTA.[27] Data presented here showed that Fe^{3+}-EDTA was rapidly reduced by the reaction system depending on the concentration of GSH (Fig. 1B). Furthermore, HA· was diminished by the addition of Fe^{3+}-EDTA under anaerobic or aerobic conditions (Fig. 3), indicating that Fe^{3+}-EDTA can be reduced directly by HA·. Additional evidence that SOD (50 U/ml) did not inhibit the reduction of Fe^{3+}-EDTA (Table II) suggests that Fe^{3+}-EDTA can be reduced mainly by HA· rather than O_2^-. Another potential iron reductant may be glutathione thiyl radical. Harman *et al.*[28] have detected a thiyl free radical in the oxidation of GSH by horseradish peroxidase. However, we could not detect this radical in the reaction of alloxan with GSH.

The results of the present study demonstrated that oxygen consumption was rapidly induced by addition of alloxan in the presence of GSH (Fig. 5). Additional evidence that HA· was more effectively diminished under aerobic than anaerobic conditions (Fig. 2) and that the rate of oxygen consumption increased with increasing intensity of HA· (Fig. 6) suggests a possible interaction of HA· with oxygen, by which O_2^- is generated.

Since O_2^- is proposed to react with alloxan to produce HA·,[26] an equilibrium would be present between O_2^- and HA· as follows:

$$\text{alloxan} + O_2^- + H^+ \leftrightarrow HA\cdot + O_2 \quad (3)$$

On the other hand, both HA· and O_2^-[13] are able to reduce Fe^{3+}-EDTA as follows:

$$HA\cdot + Fe^{3+}\text{-EDTA} \rightarrow \text{alloxan} + H^+ + Fe^{2+}\text{-EDTA} \quad (4)$$

$$O_2^- + Fe^{3+}\text{-EDTA} \rightarrow O_2 + Fe^{2+}\text{-EDTA} \quad (5)$$

Data presented here showed that much higher SOD concentrations were required to inhibit both reduction of Fe^{3+}-EDTA and generation of HA· under aerobic conditions (Table II). A possible reason why a large amount of SOD is required to inhibit these reactions is as follows; SOD can remove O_2^- and displace equilibrium 3 to the left as postulated by Winterbourn.[26] Therefore, the concentration of HA· is decreased and consequently the reduction of Fe^{3+}-EDTA (reaction 4) may be inhibited by SOD. Alternatively, HA· may be a more potent reductant than O_2^-, so that reaction 4 will always predominate over reaction 5.

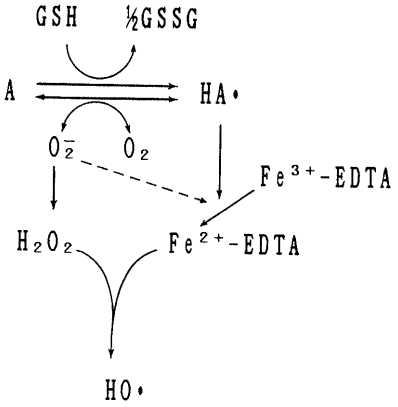

Chart 1. Proposed Mechanism for Generation of Hydroxyl Radical in the Reaction of Alloxan with GSH in the Presence of Fe^{3+}-EDTA

A, alloxan; HA·, alloxan radical.

Based on the above discussion, the generation of HO·, involving HA·, is proposed to occur as illustrated in Chart 1.

Based on the present results, it is strongly suggested that HO· is generated *via* HA· through a Fenton-type reaction, since a large amount of SOD is required to protect against the diabetogenic action of alloxan. Indeed, the protective effect of SOD has been confirmed by employing large amounts of the enzyme.[7,9,11] It seems, therefore, that scavengers of HA· would be more effective to protect against the diabetogenic action of alloxan.

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