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Denaturation of Erythrocytes by Exposure to photochemically Produced Superoxide Radicals

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Rat erythrocytes were incubated in the photoactivated riboflavin system, in which the formation of erythrocytes resistant to hypotonic hemolysis occured. Cells illuminated for 60 min were as sensitive to hypotonic hemolysis as unilluminated cells. In contrast, cells illuminated for 120 min showed complete resistance to hypotonic hemolysis. Experiments with catalase, superoxide dismutase and other radical scavengers showed that the formation of protected cells appears to be due to H_2O_2 generated in the photoactivated riboflavin system. Microscopic examination revealed that cells exposed to O_2^- for 120 min were enlarged and contained more intensely intracellular materials around the interior periphery. These results suggest that rigid cells resistant to hypotonic hemolysis may be formed by H_2O_2 oxidation of hemoglobin and membrane elements of the cells.

Keywords——erythrocyte; photoactivated riboflavin; osmotic fragility; resistance to hypotonic hemolysis; hydrogen peroxide; catalase

Active oxygen species such as the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH^\cdot$) and singlet oxygen (O_2^*) have been shown to produce cell damage.[2)] Among these active oxygens, O_2^- is now known to be produced in a number of biological reactions as an intermediate in the reduction of molecular oxygen.[3)] Superoxide dismutase, which catalytically scavenges this radical, has been proposed to be an essential component of biological defence against oxygen toxicity.[4)]

The toxic effects of active oxygen species on erythrocytes have been extensively studied with respect to the mechanism of hemolysis and lipid peroxidation in the cell membrane.[5)] Several workers have demonstrated that cells are hemolyzed by exposure to O_2^- produced in many model systems.[5a-c,f)] In the previous papers,[6)] we attempted to elucidate the role of superoxide dismutase in erythrocytes, demonstrating that catalase but not superoxide dismutase has an inhibitory effect on the oxidative formation of methemoglobin (metHb) by an O_2^--generating system of photoactivated riboflavin. In the course of this study, we observed the formation of erythrocytes resistant to hypotonic hemolysis.

This paper deals with the results of an investigation using the photoactivated riboflavin system as a source O_2^- and describes the formation of cells resistant to hypotonic hemolysis as well as some of the effects of hemolysis. Furthermore, the effects of catalase and su-

1) Location: *Katsuraoka-cho, Otaru, 047-02, Japan.*

2) "Biochemical and Medical Aspects of Active Oxygens," ed. by O. Hayaishi and K. Asada, Japan Scientific Societies Press, Tokyo, 1977.

3) I. Fridovich, *Science*, **201**, 875 (1978).

4) *a*) M. MacCord and I. Fridovich, *J. Biol. Chem.*, **244**, 6048 (1969); *b*) E.M. Gregory and I. Fridovich, *J. Bacteriol.*, **114**, 1193 (1973); *c*) *Idem, ibid.*, **117**, 456 (1974); *d*) H.M. Hassan and I. Fridovich, *J. Bacteriol.*, **129**, 1574 (1977).

5) *a*) B. Goldberg and A. Stern, *J. Biol. Chem.*, **251**, 6468 (1976); *b*) *Idem, Arch. Biochem. Biophys.*, **178**, 218 (1977); *c*) A. Valenzueda, H. Rios, and G. Neiman, *Experientia*, **33**, 926 (1977); *d*) N.R. Brownlee, J.J. Huttner, R.V. Panaganamala, and D.G. Cornwell, *J. Lipid Res.*, **18**, 635 (1977); *e*) E.W. Kellog and I. Fridovich, *J. Biol. Chem.*, **252**, 671 (1977); *f*) A.M. Michelson and P. Durosay, *Photochem. Photobiol.*, **25**, 55 (1977); *g*) R.E. Lynch and I. Fridovich. *J. Biol. Chem.*, **253**, 1838 (1978).

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peroxide dismutase added externally to the medium were examined to clarify the possible participation of active oxygen species in the cellular damage.

Experimental

Materials——Catalase (from beef liver, thymol-free), superoxide dismutase (from bovine blood) and cytochrome c were obtained from Sigma Chemical Co., while riboflavin and nitro-blue tetrazolium were purchased from Wako Pure Chemical Industries, Ltd. and Japan Chemical Industries Co., respectively. All other chemicals were of the highest purity commercially available.

Preparation of Erythrocyte Suspension——Blood was taken from the common carotid artery of male Wistar strain rats (180 to 220 g) into heparinized tubes and centrifuged at $1500 \times g$ for 10 min at 4°. The plasma and buffy layer were carefully removed by aspiration. Erythrocytes were washed three times with 5 volumes of isotonic NaCl solution.

System for the Production of O_2^- and Reaction with Erythrocytes——O_2^- was generated by the reaction of photoactivated riboflavin with oxygen as described previously.[6] Packed cells were suspended in 3 ml of O_2-saturated 10 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl, 1 mM EDTA and 20 μM riboflavin to give a final hematocrit of 0.1%. This reaction mixture was approximately iso-osmolar with 0.9% NaCl. A quartz cuvette containing the above solution was thermostated at 37°, and then illuminated in an aluminium foil-lined box equipped with two 15 W fluorescent lamps. The rates of reduction of nitro-blue tetrazolium and ferricytochrome c were measured spectrophotometrically at 600 and 550 nm to confirm that O_2^- was generated in the reaction mixture. In the cases of both nitro-blue tetrazolium and ferricytochrome c, the rate of reduction was inhibited to 95% by superoxide dismutase (30 μg/ml) and to only 10% by catalase (10 μg/ml), as described previously.[6] The anaerobic reaction with cells was carried out in a modified Thunberg tube with a quartz cell under an atmosphere of nitrogen gas.

Hemolysis and Osmotic Fragility——Hemolysis was followed in terms of decrease in turbidity of the cell suspension at 740 nm, as described by earlier workers.[5b,7] Complete hemolysis was carried out by adding cells to distilled water. Tests of osmotic fragility were carried out as follows. Cells exposed to O_2^- for various times were washed three times with isotonic solution, followed by centrifugation at $1500 \times g$ for 10 min. The packed cells were resuspended in test tubes containing 3 ml of NaCl at various concentrations. All NaCl solutions used were prepared by dilution of a stock solution equivalent to 10% as described by Parpart *et al.*[8] After standing for 10 min at room temperature, the percentage hemolysis was determined in terms of the decrease in turbidity of cells by the method already described. In some experiments, the cells exposed to O_2^- were subjected to hypotonic hemolysis in 0.2% NaCl. Optical measurements were made using a Hitachi 200-20 recording spectrophotometer. The osmolarities of the reaction mixtures containing the O_2^--generating system were determined with osmometer (OS osmometer, Fiske Associates). All the scavengers to be added in these experiments were prepared as isotonic solutions or were made isotonic by addition of NaCl.

Microscopic Examination——Cell suspensions exposed to O_2^- were examined microscopically for inclusion bodies after superstaining with an isotonic aqueous solution of crystal violet and brilliant cresyl blue.[9] Cell suspensions were also treated with phenylhydrazine for the preparation of Heinz bodies, and examined in the same way.

Results and Discussion

Hemolysis During Incubation with the O_2^--generating System

The incubation of erythrocytes in the isotonic O_2^--generating photoactivated riboflavin system caused a profound change in their color to dark brown, but no hemolysis was detectable at any time of incubation, as shown in Fig. 1. Even prolonged incubation (over 120 min) did not lead to hemolysis. Oxidative hemolysis is known to be dependent on the cell concentration.[5] However, hemolysis was scarcely observed at any concentration under the experimental conditions used. In the course of this study, the cells were found to undergo hemolysis when serum was added to the O_2^--generating system. A typical result is shown in Fig. 1.

Effect of O_2^- Exposure on the Fragility of Erythrocytes

The cells incubated for 60 min and 120 min in the O_2^--generating system, in which no hemolysis could be detected as shown in Fig. 1, were subjected to a general test for osmotic

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8) A.K. Parpart, P.B. Lorenz, E.P. Parpart, J.R. Gregg, and A.M. Chase, *J. Clin. Invest.*, **26**, 636 (1947).
9) "Modern Medical Technology," Vol. 3, ed. by S. Miwa, Igaku Shoin Ltd., Japan, 1972, p. 243.

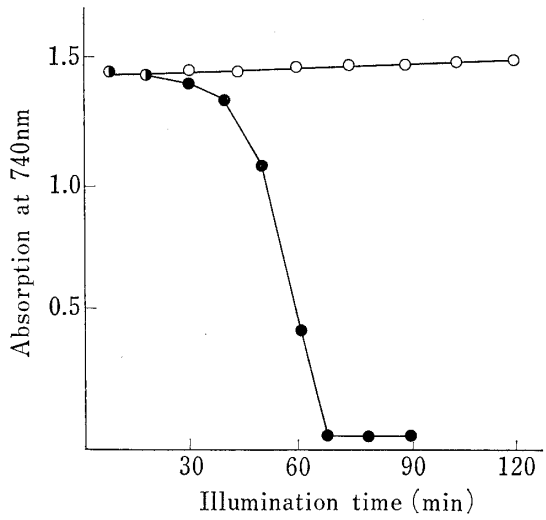

Fig. 1. Time Course of Hemolysis of Erythrocytes on O_2^- Exposure

The cells were suspended in 10 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl, 1 mM EDTA and 20 μM riboflavin to give 0.1% hematocrit. After illumination for various times under fluorescent lamps, the degree of hemolysis was determined at 740 nm. Each point represents the mean of triplicate experiments. ——○——, complete system; ——●——, addition of 30% (v/v) serum to the complete system.

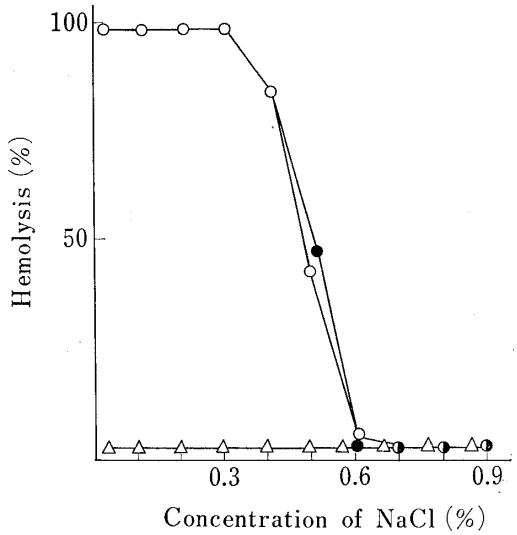

Fig. 2. Effect of Exposure to O_2^- on Hypotonic Hemolysis of Erythrocytes

The cells were incubated in the photoactivated riboflavin O_2^--generating system for 0 (——○——), 1 (——●——) and 2 (——△——) hr under the conditions given in Fig. 1. After centrifugation for 10 min at 1500 × *g*, the packed cells were suspended in test tubes containing various concentrations of NaCl. After standing for 10 min at room temperature, hemolysis was measured by the method described in "Experimental". Each point represents the mean of duplicate experiment.

fragility. As shown in Fig. 2, cells illuminated for 60 min were as sensitive to hypotonic hemolysis as unilluminated cells. In contrast, cells illuminated for 120 min were no longer sensitive to hypotonic hemolysis. With cells incubated under anaerobic conditions for 120 min, almost no such resistance to hypotonic hemolysis was observed. These results indicate possible involvement of oxygen species in the formation of these resistant cells. Cells incubated for 120 min with the hypotonic O_2^--generating system at an osmotic pressure of 180 m osmoles/l (equivalent to that of 0.6% NaCl) also acquired resistance to hypotonic hemolysis (data not shown). Furthermore, to determine whether the oxidative reaction to form resistant cells is reversible or not, resistant cells washed three times with isotonic NaCl were tested for osmotic fragility after standing overnight at room temperature. The results of this experiment showed that osmotic fragility was little affected by this treatment, and that the effect was irreversible. The resistant cells obtained by O_2^- exposure could not be solubilized with 1% Triton X-100 and were not disrupted by sonication at 20 kHz for 5 min. The formation of resistant cells by O_2^- exposure was also observed in both human and sheep erythrocytes under experimental conditions employed.

The protection and stabilization of erythrocytes against hypotonic hemolysis have been reported to be induced by many kinds of drugs, such as tranquilizers, anesthetics and antihistamines, at very low concentrations.[10] These phenomena have been reported to be reversible and were thought to result from changes in the conformational state of membrane components induced by these drugs.[10] On the other hand, several workers, have shown that chemical modification of erythrocyte membranes with acetic anhydride at high concen-

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trations or with glutaraldehyde "fixes" the cells and make them resistant to degradation.[11] Based on these results, the formation of cells resistant to hypotonic hemolysis by O_2^- exposure may be due to denaturation of the erythrocyte membranes by direct oxidative attack.

Microscopic Observation

The resistant cells obtained by O_2^- exposure were found to contain large amounts of dark brown pigments. Therefore, morphological changes of the cells were compared with those of phenylhydrazine-treated cells, which are known to contain inclusion bodies (Heinz bodies) composed of precipitates of denatured hemoglobin (Hb). As shown in Fig. 3, the phenylhydrazine-treated cells were somewhat crenated and contained granules or membrane-associated particles within the cells. In contrast, the resistant cells were enlarged, and showed a mottled appearance, probably due to precipitates of denatured Hb or breakdown products of Hb. In addition, the cells appeared to contain more intensely stained intracellular materials around the interior periphery. Denatured Hb and subsequent Heinz body formation are known to be intermediate events in the process of oxidative hemolysis.[12] On the basis of these results, we suggest that rigid cells protected against hypotonic hemolysis as described above may be formed through the irreversible modification of membrane components by oxidation due to O_2^- exposure.

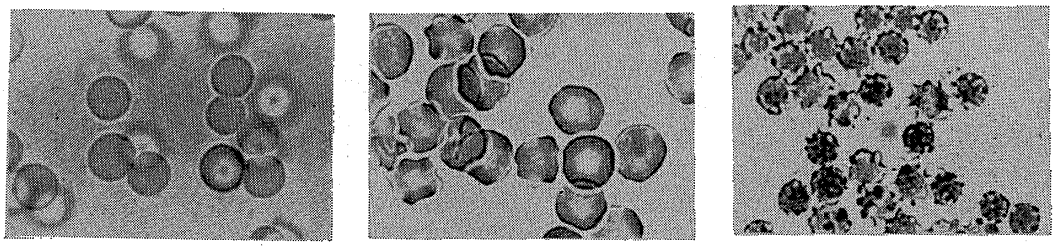

Fig. 3. Photomicrograph of Brilliant Cresyl Blue-Stained Normal Erythrocytes (left) Resistant Erythrocytes (middle) and Phenylhydrazine-treated Erythrocytes (right)

Resistant erythrocytes were obtained by incubation in the O_2^--generating system for 120 min. Phenylhydrazine-treated erythrocytes were prepared as described in "Experimental." (Magnification $\times 1000 \times 1/2$).

Effects of Catalase and Superoxide Dismutase

The results summarized in Table I demonstrate that the addition of catalase to the O_2^--generating system caused hypotonic hemolysis to various extents depending on the

Table I. Effect of Catalase and Superoxide Dismutase on the Formation of Erythrocytes Resistant to Hypotonic Hemolysis

Experimental conditions	Hypotonic hemolysis (%)	
1. Complete system	0 (no lysis)	(5)
2. Complete system+10 μg/ml of catalase	100	(3)
3. Complete system+5 μg/ml of catalase	94.0±0.6	(3)
4. Complete system+1 μg/ml of catalase	23.0±2.5[a)]	(3)
5. Complete system+30 μg/ml of superoxide dismutase	0	(3)
6. Complete system+1 μg/ml of catalase and 30 μg/ml of superoxide dismutase	49.3±8.3	(3)

The cell suspensions (0.1% hematocrit) were incubated in the O_2^--generating system with 20 μM riboflavin in the absence or presence of catalase and superoxide dismutase for 120 min and then tested for osmotic fragility in 0.2% NaCl. Values represent means ± standard error. Numbers of experiments are given in parentheses.
a) no significant difference between the results of experiments 4 and 6, $p>0.1$.

11) *a*) K.L. Carraway, D. Kobylka, J. Summers, and C.A. Carraway, *Chem. Phys. Lipids*, **8**, 65 (1972); *b*) R.A. Capaldi, *Biochem. Biophys. Res. Commun.*, **50**, 656 (1973).
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amounts of catalase added. The addition of catalase (10 μg/ml) to the reaction medium resulted in complete hypotonic hemolysis. These results indicate that catalase prevents the formation of resistant cells. This effect of catalase is apparently related to its enzyme activity, because heat-inactivated catalase showed almost no ability to inhibit the formation of resistant cells. Superoxide dismutase had no effect. Experiment 6 in Table I, involving the addition of both catalase and superoxide dismutase, shows that superoxide dismutase had no significant effect in comparison with experiment 4, in which catalase (1 μg/ml) alone produced partial inhibition of the formation of resistant cells. These results suggest that H_2O_2 rather than O_2^- may be involved in the formation of resistant cells.

The mechanism of formation of resistant cells may be associated with other radicals, *i.e.*, $OH^\cdot$ or O_2^*. To examine this possibility, several chemical scavengers were used (benzoate, formate, mannitol and tris(hydroxymethyl)aminomethane as $OH^\cdot$ scavengers, histidine, tryptophan, xanthine and urate as O_2^* scavengers). None of the hydroxyl radical scavengers used inhibited the formation of resistant cells. On the other hand, histidine and tryptophan had no effect, but xanthine and urate slightly delayed the time of formation of resistant cells (data not shown). The reason for the discrepancy between the effects of O_2^* scavengers is not clear, but O_2^* may play some role in the process of oxidative attack on the cell membrane.

In the previous papers,[6] we reported that H_2O_2 generated in the photoactivated riboflavin system by spontaneous dismutation of O_2^- participated in the oxidative conversion of oxyHb to metHb. The resistant cells obtained by O_2^- exposure changed in color to dark brown as mentioned above, whereas the cells illuminated in the presence of catalase remained unchanged. These results suggest that the oxidative attack of H_2O_2 on both cell membrane components and Hb molecules plays a key role in the events initiating the formation of resistant cells. Further experiments, using the xanthine oxidase O_2^--generating system, are required to elucidate the mechanism involved in the denaturation of erythrocytes.