Liposome Oxidation and Erythrocyte Lysis by Enzymically Generated Superoxide and Hydrogen Peroxide*

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Xanthine oxidase, acting on acetaldehyde under aerobic conditions, produces a flux of \( \cdot O_2^- \) and \( H_2O_2 \) which attacks artificial liposomes and washed human erythrocytes. The liposomes were peroxidized and the erythrocytes suffered oxidation of hemoglobin followed by lysis. The oxidation of hemoglobin, within the exposed erythrocytes, could be largely prevented by prior conversion to carbon monoxide-hemoglobin, without preventing lysis. Hemolysis thus appeared to be a consequence of direct oxidative attack on the cell stroma. The enzyme-generated flux of \( \cdot O_2^- \) and of \( H_2O_2 \) also inactivated the xanthine oxidase. Superoxide dismutase or catalase, present in the suspending medium, protected the liposomes against peroxidation, the erythrocytes against lysis, and the xanthine oxidase against inactivation. Scavengers of \( \cdot O_2^- \) such as histidine or 2,5-dimethylfuran, which do not react with \( \cdot O_2^- \) or \( H_2O_2 \), also prevented peroxidation of liposomes and lysis of erythrocytes when present at low concentrations. In contrast, a scavenger of \( OH^- \), such as mannitol was ineffective at low concentrations and provided significant protection only at much higher concentrations. It is proposed that \( \cdot O_2^- \) and \( H_2O_2 \) cooperated in producing \( OH^- \) and \( \cdot O_2^- (\Delta g) \), which were the proximate causes of lipid peroxidation and of hemolysis.

The superoxide anion radical \( (\cdot O_2^-) \) is a common product of the reduction of molecular oxygen. Superoxide dismutases, which catalytically scavenge this radical (1), are essential components of the biological defense against oxygen toxicity (2-4). This implies that \( \cdot O_2^- \), or products derived from it, threaten the integrity of living cells. Yet our knowledge of the actions of \( \cdot O_2^- \) and its products upon cellular components is limited. In an attempt to gain such information, polyunsaturated fatty acids were previously exposed to an enzymic source of \( \cdot O_2^- \) and \( H_2O_2 \) which caused their peroxidation (5). This oxidative attack was completely prevented by superoxide dismutase, catalase, scavengers of \( \cdot O_2^- (\Delta g) \), and partially by scavengers of \( OH^- \). It was concluded that \( \cdot O_2^- \) and \( H_2O_2 \) had cooperated in generating \( OH^- \) and \( \cdot O_2^- (\Delta g) \), which then directly oxidized the unsaturated fatty acid. The abundance of polyunsaturated fatty acids in cell and organelle membranes and the rapidity of the reaction of \( \cdot O_2^- (\Delta g) \) with such fatty acids (6-9) lends biological significance to these results. A logical extension of the work with a single polyunsaturated fatty acid (5) would entail investigations of the effects of \( \cdot O_2^- \) and of \( H_2O_2 \) first on artificial liposomes and then on some relatively simple cell type, such as the erythrocyte, in which all of the lipid is located in the stroma (10, 11). The present report presents the results of such investigations.

MATERIALS AND METHODS

L-α-Phosphatidylcholine (type III E from egg yolk), cholesterol, dietyl phosphate, mannitol, hypoxanthine, xanthine, inosine, catalase, and bovine serum albumin were obtained from the Sigma Chemical Co. Sucrose, glucose, sodium chloride, chloroform, and ethylenediaminetetraacetic acid were from Matheson. Coleman and Bell. Starch (soluble) and potassium iodide were from Baker and Adamson, while uric acid and tertiary butanol were from the Fisher Chemical Co. Potassium phosphate and Silica Gel 1B thin layer sheets were from the J. T. Baker Chemical Co. 2,5-Dimethylfuran, obtained from the Aldrich Chemical Co., was purified by passage over a column of aluminum oxide (W200 basic, activity grade super 1) purchased from the International Chemical and Nuclear Corp. Xanthine oxidase from bovine cream (12) and superoxide dismutase from bovine erythrocytes (1) were isolated as previously described. Acetaldehyde from the Eastman Chemical Co. was distilled and then stored in the deep freeze until needed. Carbon monoxide was obtained from Matheson Gas Products, Inc., while \( H_2O_2 \) was from Mallinckrodt and histidine was from the Nutritional Biochemical Corporation.

Liposomes...A solution containing 0.175 m phospatidylcholine, 0.050 m cholesterol, and 0.025 m dietyl phosphate was prepared in CHCl₃ and was stored in vacuo at \(-20^\circ\). Then 1 ml of this lipid solution was evaporated under a stream of \( N_2 \) at \( 0^\circ \) and the resultant lipid film was dispersed in the cold, by sonication under \( N_2 \) into 25 ml of 50 mM potassium phosphate and 0.1 mM EDTA at pH 7.8. Metal impurities had previously been removed from this buffer by passage over a column of Chelex 100. This dispersion of liposomes in phosphate buffer, which was 10 mM with respect to total lipids, was kept under \( N_2 \) until used. Reaction mixture contained liposomes at 3.3 mM total lipids, 50 mM potassium phosphate, 0.1 mM EDTA, 48 mM acetaldehyde, and 3.9 x 10⁻⁶ m xanthine oxidase, in a total volume of 2.0 ml, in 28 ml Erlemeyer flasks, were incubated at 37° for 60 min on a Gyrotory Water Bath model G-76 (New Brunswick Scientific Co.) at 116 rpm. Reactions were stopped by chilling in ice and products were extracted with 0.6 ml of CHCl₃:CH₃OH (1:1). Aliquots (20 μl) of these extracts were chromatographed on Silica Gel 1B thin layer sheets with CHCl₃:CH₃OH (1:2). Peroxides were visualized by a modification of the Stahl (13) starch-iodine procedure as previously described (5).
Oxidation of Liposomes and Erythrocytes by $O_2^{-}$ and $H_2O_2$

Erythrocytes—Blood was drawn each day by venipuncture into heparinized Vacutainers from a single normal 39-year-old male volunteer. Erythrocytes were washed at least four times with 10-volume portions of cold 0.9% NaCl. Reaction mixtures containing 16.7 mm potassium phosphate, 82.2 mm NaCl, 0.033 mm EDTA, 6 mm acetaldehyde, 0.13 $\mu$m xanthine oxidase, and erythrocytes, in a volume of 3.0 ml in 25-ml Erlenmeyer flasks, were incubated at 37$^\circ$C on a rotary shaker at 116 rpm. The concentration of erythrocytes was such (hematocrit = 0.15%) that $A_{414}$ was 3.0 after complete lysis. Reactions were stopped by chilling in ice and particulates were removed by centrifugation at 5000 $\times$ g for 5 min, yielding Supernatant 2 ($S_2$), which was assayed for catalase and for $A_{409}$ nm, a pellet, which was dispersed in 0 $\mu$l of H$_2$O, to achieve complete hemolysis. $S_2$ was then pooled with the resuspended and hemolyzed cells and protein precipitates were removed by centrifugation yielding Supernatant 3 ($S_3$). $S_3$ was assayed for catalase and for absorbances at 409 and 575 nm. Percentage of hemolysis was calculated on the basis of heme release or catalase release as $[S_3]/[S_1]$ (100), $[S_3]$ having been corrected for dilution. Since the absorption at 409 nm was equivalent for both oxyhemoglobin and the hemoglobin product, percentage of precipitation could be estimated by the relative decrease in $A_{409}$ after complete lysis of the treated cells (by dilution into H$_2$O) as compared to the untreated cell. This quantitative measurement agreed with the qualitative observation in the size of the pellet remaining after centrifuging $S_3$. Incubations under approximately 10% CO were performed by injecting 3.9 ml of CO into the gas space of the 25-ml flasks, through serum caps, which were then left in place until the reactions were terminated. All of the data presented were obtained with samples of blood drawn as needed from a single donor; however a great deal of preliminary work was done with pooled samples obtained from the Duke Hospital Blood Gases Laboratory. These mixed samples of unspecified origin gave results qualitatively similar in all respects to those later obtained with blood from a single donor; but they did exhibit a variable resistance to hemolysis which made quantitative comparisons difficult. It was apparent that different blood samples can exhibit marked variability in resistance both to mechanical and oxidant-induced stress. For example a 5-fold increase in xanthine oxidase concentration was necessary for good hemolysis of erythrocytes from our healthy volunteer, as compared to that required for equivalent lysis of most clinical laboratory samples.

RESULTS

Co-oxidation of Liposomes during Xanthine Oxidase Reaction

The oxidation of acetaldehyde by xanthine oxidase caused a co-oxidation of liposomes present in the reaction mixture. This co-oxidation was indicated by the appearance of a spot of the peroxide of phosphatidylcholine on developed thin layer chromatograms. Superoxide dismutase or catalase prevented this lipid peroxidation, whereas a control of bovine serum albumin had no effect. These results, which are shown in Fig. 1, indicated that both $O_2^-$ and $H_2O_2$ were essential intermediates in causing this peroxidation.

Since previous studies have suggested that $O_2^-$ and $H_2O_2$ can cooperate in the generation of OH$^-$ (14-23) and of $O_2'-(\Delta g)$ (5), these species were considered as the direct causes of the lipid peroxidation. Fig. 2 demonstrates that histidine or 2,5-dimethylfuran, which are effective scavengers of $O_2'-(\Delta g)$ (24, 25), prevented lipid peroxidation, when present at 2 and 3 $\mu$m, respectively; whereas mannitol, which should be able to react rapidly with OH$^-$ (26), did not have any clearly noticeable effects at these low levels. Mannitol, sucrose, or tertiary butanol, however, did diminish the yield of lipid peroxide by 20 to 50% when used at 100 $\mu$m.

Xanthine oxidase caused peroxidation of the liposomes when acting on acetaldehyde, but not when acting on xanthine or hypoxanthine. This was reminiscent of previous work which detected chemiluminescence during the oxidation of acetaldehyde, but not of xanthine, by xanthine oxidase (20, 27). This was explained on the basis of the scavenging of reactive intermediates by xanthine and urate but not by acetaldehyde (20). Fig. 3 demonstrates that the peroxidation of liposomes by xanthine oxidase acting upon 48 mm acetaldehyde was completely prevented by 0.5 mm hypoxanthine, xanthine, or urate. Certain of the purines, notably guanine and urate, have been implicated as singlet scavengers in photodynamic systems (28, 29). In our hands, xanthine, at 0.5 mm, were able to cause inhibition of lipid peroxidation in a rose bengal photosensitized $O_2'-(\Delta g)$-generating system. Since scavengers of $O_2'-(\Delta g)$ also prevented lipid peroxidation in the xanthine oxidase system, when present at 0.5 mm, whereas scavengers of OH$^-$ had no effect at this concentration, we tentatively conclude that xanthine and urate prevented lipid peroxidation by scavenging $O_2'-(\Delta g)$.

Oxidative Attack on Erythrocytes

Exposure of washed human erythrocytes to the xanthine oxidase-acetaldehyde reaction caused first a profound change in their color and then hemolysis. Curve 1 in Fig. 4 is the spectrum of a lysate of untreated erythrocytes; whereas Curves 2 and 3 were obtained from lysates of cells exposed to the xanthine oxidase reaction for 30 and 180 min, respectively. The shift in the position of the Soret maximum, from 414 to 405 nm, and the disappearance of the $\alpha$ and $\beta$ bands were complete within 30 min of exposure. There was no significant emergence of new peaks in the spectral region 600 to 800 nm. The absorbance at 409 nm was equivalent for both oxyhemoglobin and the hemoglobin oxidation product and this wave-length was used, therefore, for determination of hemolysis and of the precipitation of denatured hemoglobin derivatives. The decrease in total absorbance during more prolonged exposure was due to the formation of precipitates of denatured hemoproteins. These changes in hemoglobin were undoubtedly due to $H_2O_2$, since a gradual addition of $H_2O_2$ to suspensions of erythrocytes by the method of gaseous diffusion (30) produced changes identical with those shown in Fig. 4. Furthermore, catalase added to the xanthine oxidase system prevented these spectral changes, while superoxide dismutase, and scavengers of $O_2'-(\Delta g)$ and of OH$^-$ did not. $H_2O_2$, either added directly or generated by the autooxidation of ascorbate, is known to convert hemoglobin to methemoglobin, choleglobin, and other products (30-34).

Exposure of washed erythrocytes to the xanthine oxidase-acetaldehyde reaction caused hemolysis which became extensive after a lag of 2 h. As shown in Fig. 5, the extent of hemolysis could be assessed either in terms of the release of hemoprotein (Line 2) or of catalase (Line 1). Line 5 shows that precipitation of hemoglobin became extensive at 80 min and did not directly correlate with hemolysis. Omission of acetaldehyde or of xanthine oxidase from these reaction mixtures completely prevented hemolysis and oxidation of hemoprotein, and denatured xanthine oxidase was unable to replace the active enzyme. It is thus clear that neither xanthine oxidase nor acetaldehyde separately caused the hemolysis and oxidation, but that some product of their interaction was the active agent. The ineffectiveness of acetaldehyde by itself is an important control in view of the report that acetaldehyde can diminish the flexibility of erythrocytes (35). Such a direct effect of acetaldehyde upon the red cell stroma was evidently not a significant factor in the hemolysis caused by the xanthine oxidase-acetaldehyde reaction.

Mechanisms of Oxidative Hemolysis

Role of $O_2^-$ and $H_2O_2$—Superoxide dismutase, catalase,
Oxidation of Liposomes and Erythrocytes by \( O_2^- \) and \( H_2O_2 \)

**FIG. 1 (left).** Detection of lipid peroxides on thin layer chromatograms. Complete reaction mixtures contained 2.31 mM phosphatidylcholine, 0.66 mM cholesterol, and 0.33 mM dicetyl phosphate, as liposomes, in 50 mM potassium phosphate, 0.1 mM EDTA, 48 mM acetaldehyde, and 26.5 \( \mu \)g/ml of xanthine oxidase, in a total volume of 3.0 ml at pH 7.8 and 37°, under constant agitation on an oscillating platform. Lipids were extracted and chromatograms developed as described under "Materials and Methods." 1, acetaldehyde omitted; 2, complete reaction mixture; 3, complete mixture plus 10 \( \mu \)g/ml of superoxide dismutase; 4, complete mixture plus 10 \( \mu \)g/ml of catalase; 5, complete mixture plus 10 \( \mu \)g/ml of bovine serum albumin.

**FIG. 2 (center).** Effects of scavengers of \( O_2^- \) and \( \cdot \)OH on lipid peroxidation. Conditions as described in the legend of Fig. 1 except for a reduction in the concentration of total lipid to 2.5 mM. 1, zero time complete mixture (kept in ice and extracted with CHCl\(_3\)/MeOH immediately after addition of acetaldehyde); 2, complete mixture incubated as described under "Materials and Methods;" 3, complete mixture plus 2 mM histidine; 4, complete mixture plus 3 mM dimethylfuran; 5, complete mixture plus 3 mM mannitol.

**FIG. 3 (right).** Effect of purines on lipid peroxidation. Conditions as described in Fig. 1 except for a reduction in the concentration of total lipid to 2.5 mM. 1, complete mixture, but xanthine oxidase omitted; 2, complete reaction mixture, but with acetaldehyde omitted; 3, complete mixture plus 0.5 mM xanthine as substrate; 4, complete mixture plus 0.5 mM hypoxanthine; 5, complete mixture plus 0.5 mM xanthine; 6, complete mixture plus 0.5 mM urate.

**FIG. 4 (left).** Changes in the optical spectrum of hemoglobin during exposure of erythrocytes to the xanthine oxidase-acetaldehyde reaction. Reaction mixtures contained 16.7 mM potassium phosphate, 82 mM NaCl, 0.033 mM EDTA, 6 mM acetaldehyde, and 120 \( \mu \)g of xanthine oxidase in 3.0 ml at pH 7.8 at 37°. Erythrocytes were present in an amount which gave an absorbance of 3.0 at 409 nm upon complete lysis in 3.0 ml of \( H_2O \). Samples were incubated in an oscillating water bath and after lysis were diluted 4-fold prior to recording of their spectra. 1, 30-min incubation with acetaldehyde omitted; 2, 30-min incubation in complete mixtures; 3, 180-min incubation in complete mixture.

**FIG. 5 (right).** Hemolysis and precipitation of hemoglobin due to the xanthine oxidase-acetaldehyde reaction. Conditions were as described in the legend of Fig. 4. Hemolysis was estimated as described under "Materials and Methods." 1, complete reaction mixture with hemolysis based upon release of catalase; 2, complete reaction mixture with hemolysis based upon release of heme protein; 3, same as 1, but acetaldehyde omitted; 4, same as 2, but acetaldehyde omitted; 5, precipitation of hemoglobin in complete reaction mixture assessed by decrease in \( A_{590} \) after thorough osmotic lysis as described under "Materials and Methods."
Oxidation of Liposomes and Erythrocytes by $O_2^-$ and $H_2O_2$

and mannitol were tested for their ability to blunt the attack by the xanthine oxidase-acetaldehyde reaction upon erythrocytes. The results, summarized in Table I, demonstrate that superoxide dismutase protected against hemolysis, but actually augmented the extent of precipitation of hemoglobin, while catalase prevented both hemolysis and precipitation. Control proteins, such as bovine serum albumin, or heat-inactivated superoxide dismutase were without effect. Both superoxide dismutase and catalase, which diminished hemolysis when present from the inception of the reaction, were without effect when added after 1 h of incubation. The data in Fig. 5 show that very little hemolysis had occurred during 1 h of the reaction. It is thus apparent that latent damage to the erythrocyte, mediated by $O_2^-$ and $H_2O_2$, was complete within the 1st h and would then lead to lysis by a process which could not be interrupted by superoxide dismutase or catalase. In fact, erythrocytes, which had been washed after only 30-min exposure to the xanthine oxidase reaction and which were then resuspended in the buffer/saline solution, still showed complete lysis after an additional 210 min of incubation at 37°C. Mannitol (10 mM) prevented hemolysis, but had no effect on precipitation of hemoglobin. The degree of hemolysis assessed in terms of the release of catalase (Experiment 2, Table I) appeared to be greater than that based upon the release of hemoprotein. This difference was actually due to a gradual inactivation of catalase after exposure to and latent damage by the xanthine oxidase-acetaldehyde reaction. Since $S_0$ was unavoidably assayed up to several hours later than $S_t$, its content of catalase had diminished to a greater extent, thus giving rise to an artificial increase in percentage of lysis, based upon catalase release. Superoxide dismutase eliminated this discrepancy between extent of lysis based upon release of catalase and that based upon release of hemoprotein (Experiment 3), presumably because superoxide dismutase protected the catalase in solution against the oxidative attack of the xanthine oxidase-acetaldehyde reaction and therefore eliminated the instability caused by that attack.

Experiment 3 in Table I demonstrates that superoxide dismutase, while diminishing hemolysis, actually increased precipitation of the hemoglobin. Since the conversion of hemoglobin to methemoglobin and choleglobin, and then to the insoluble cholehemochromogen (36) is an oxidative process (30-36), this effect of superoxide dismutase seems paradoxical and demands explanation. $H_2O_2$ played a dominant role in this hemoprotein oxidation which then led to precipitation. This was demonstrated by the protective effect of catalase (Experiment 4). Two reasons can be advanced for this effect of superoxide dismutase. Firstly, $O_2^-$ is not only able to cause the oxidation of oxyhemoglobin to methemoglobin (37), but is also capable of the conversion of methemoglobin to oxyhemoglobin (38). In a reaction mixture generating both $O_2^-$ and $H_2O_2$, the net effect of the $O_2^-$ could be the reductive oxygenation of methemoglobin and the consequent protection of the hemoglobin against further oxidative degradation. In such circumstances, superoxide dismutase would eliminate the reductive action of $O_2^-$ and leave the oxidative action of $H_2O_2$ intact. A reason of secondary importance for the enhanced hemoglobin oxidation in the presence of superoxide dismutase relates to the stability of xanthine oxidase. This enzyme, in generating $O_2^-$ and $H_2O_2$, could cause its own inactivation. If superoxide dismutase prevented this inactivation it would allow a greater production of both $O_2^-$ and $H_2O_2$ by xanthine oxidase. Fig. 6 demonstrates that xanthine oxidase was extensively inactivated during its action on acetaldehyde and that superoxide dismutase, catalase, and histidine provided partial protection, while superoxide dismutase plus catalase protected markedly. The data in Fig. 6 indicate that $O_2^-$, $H_2O_2$, and possibly $O_2\,('Ag)$ were involved in the inactivation of xanthine oxidase. It is now clear that the direct antimetabolic action of superoxide dismutase was actually much greater than indicated by the data in Table I. For, while scavenging $O_2^-$ and thus protecting the stroma against this source of oxidative attack, it was preventing the reductive oxygenation of methemoglobin by $O_2^-$ and also enhancing the net production of both $O_2^-$ and $H_2O_2$ by extending the catalytic lifetime of xanthine oxidase in the reaction mixtures. These actions would tend to cause greater oxidative degradation of hemoglobin and more extensive oxidative attack on the cell stroma than otherwise might be expected.

**Role of $O_2\,('Ag)$, $O_2^-$, and $H_2O_2$**

$O_2\,('Ag)$ and $H_2O_2$ have repeatedly been shown to cooperate in the generation of very reactive products which exhibited the properties of $OH_•$ and of $O_2\,('Ag)$ (5, 14-23). The oxidative hemolysis caused by the xanthine oxidase-acetaldehyde reaction might be due to these derived products, rather than to $O_2^-$ and $H_2O_2$ directly. Compounds which scavenge $OH_•$ or $O_2\,('Ag)$ (or both), but which do not react with $O_2^-$ or $H_2O_2$, were used to test this proposal. Table II indicates that histidine and 2,5-dimethylfuran, which are known to react rapidly with $O_2\,('Ag)$, and xanthine and urate, which would be expected to do so, provided marked inhibition.

**Table I**

Hemolysis caused by xanthine oxidase-acetaldehyde reaction

<table>
<thead>
<tr>
<th>Experiment and condition</th>
<th>Precipitation</th>
<th>Lysis by catalase release</th>
<th>Lysis by hemol release</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>1. Xanthine oxidase omitted</td>
<td>0</td>
<td>1.4 ± 0.3</td>
<td>2.1 ± 0.7</td>
</tr>
<tr>
<td>2. Complete mixture</td>
<td>18.5 ± 1.5</td>
<td>108.0 ± 7.0</td>
<td>74.6 ± 0.7</td>
</tr>
<tr>
<td>3. Complete mixture + 10 μg/ml of superoxide dismutase</td>
<td>51.0 ± 1.0</td>
<td>52.8 ± 0.4</td>
<td>51.7 ± 0.8</td>
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<tr>
<td>4. Complete mixture + 10 μg/ml of catalase</td>
<td>1.9 ± 0.4</td>
<td>8.6 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>5. Complete mixture + 20 μg/ml of bovine serum albumin</td>
<td>26.6 ± 0.9</td>
<td>111.0 ± 1.0</td>
<td>81.7 ± 1.5</td>
</tr>
<tr>
<td>6. Complete mixture + 10 mM mannitol</td>
<td>18.9 ± 1.6</td>
<td>14.9 ± 1.2</td>
<td>14.9 ± 1.6</td>
</tr>
<tr>
<td>7. Complete mixture + 10 μg/ml of superoxide dismutase + 10 μg/ml of catalase</td>
<td>1.9 ± 1.0</td>
<td>17.1 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>8. Complete mixture + 10 μg/ml of superoxide dismutase added after 60 min of incubation</td>
<td>30.7 ± 0.6</td>
<td>94.9 ± 1.2</td>
<td>78.0 ± 0.7</td>
</tr>
<tr>
<td>9. Complete mixture + 10 μg/ml of catalase added after 60 min of incubation</td>
<td>17.6 ± 0.8</td>
<td>72.2 ± 1.5</td>
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</tr>
</tbody>
</table>
Effects of various compounds on hemolysis

<table>
<thead>
<tr>
<th>Experiment and condition</th>
<th>Precipitation</th>
<th>Lysis by catalase release</th>
<th>Lysis by heme release</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Xanthine oxidase omitted</td>
<td>0</td>
<td>2.7 ± 0.7</td>
<td>4.2 ± 1.2</td>
</tr>
<tr>
<td>2. Complete</td>
<td>23.6 ± 0.6</td>
<td>117.0 ± 1.0</td>
<td>79.7 ± 0.3</td>
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<tr>
<td>3. Complete + 0.5 mM histidine</td>
<td>16.6 ± 3.0</td>
<td>8.9 ± 0.3</td>
<td>8.9 ± 0.6</td>
</tr>
<tr>
<td>4. Complete + 0.5 mM dimethyluracil</td>
<td>35.0 ± 1.8</td>
<td>7.5 ± 2.0</td>
<td>5.6 ± 1.0</td>
</tr>
<tr>
<td>5. Complete + 0.5 mM mannitol</td>
<td>21.6 ± 0</td>
<td>115.0 ± 12.0</td>
<td>78.5 ± 0.2</td>
</tr>
<tr>
<td>6. Complete + 0.5 mM xanthine</td>
<td>37.6 ± 1.0</td>
<td>8.8 ± 0.7</td>
<td>8.6 ± 0.3</td>
</tr>
<tr>
<td>7. Complete + 0.5 mM urate</td>
<td>27.4 ± 1.0</td>
<td>11.6 ± 6.5</td>
<td>9.7 ± 5.5</td>
</tr>
<tr>
<td>8. Complete + 10 mM NaCl</td>
<td>28.5 ± 0.7</td>
<td>118.0 ± 2.0</td>
<td>83.6 ± 1.4</td>
</tr>
<tr>
<td>9. Complete + 0.5 mM inosine</td>
<td>17.9 ± 1.8</td>
<td>73.9 ± 5.4</td>
<td>65.1 ± 2.2</td>
</tr>
<tr>
<td>10. Complete + 0.5 mM inosine + 0.5 mM glucose</td>
<td>17.4 ± 0.4</td>
<td>68.2 ± 5.1</td>
<td>57.7 ± 2.0</td>
</tr>
</tbody>
</table>

Effects of Carbon Monoxide

The oxidative attack on hemoglobin with sequential formation of methemoglobin, choleglobin, and insoluble products, as seen in these studies, has its counterpart in a wide variety of hemolytic anemias (39). The precipitates of oxidized hemoglobin which can form within erythrocytes are called Heinz bodics and there is the possibility that they contributed to the hemolysis observed in the xanthine oxidase-acetaldehyde system. Thus Heinz bodies are known to become attached to the stroma (40), to produce dome-like outpouching of the cell membrane, and to cause a clustering of membrane-associated proteins in the area overlying the Heinz body (41). Heinz bodies also diminish the flexibility of the erythrocyte (42, 43). In view of these known interactions of Heinz bodies with the stroma, it was important to decide whether the hemolysis observed was due primarily to direct attack on the stroma, by products of the xanthine oxidase-acetaldehyde reaction, or to a secondary effect of the denatured hemoglobin precipitates. Carbon monoxide was therefore used to convert the hemoglobin to a stable derivative prior to exposure of the erythrocytes to oxidative attack. It must be noted that carbon monoxide does not inhibit xanthine oxidase or superoxide dismutase. The results summarized in Table III demonstrate that CO prevented precipitation of the hemeprotein but did not prevent protection against hemolysis. This protective effect was seen if these compounds were present from the inception of the xanthine oxidase-acetaldehyde reaction, but not if they were added after 60 min of exposure of the erythrocytes to that reaction. In contrast, compounds which were expected to react rapidly with \( \cdot \text{OH} \), but not with \( \cdot \text{O}_2^- \), such as mannitol, glucose, or inosine, had much less effect. NaCl was tested at a concentration 20-fold higher than that of any of the other compounds to eliminate the possibility that osmotic effects were important in the protections observed. NaCl had no effect. It appears likely that \( \cdot \text{O}_2^- \), or something with similar reactivity, was responsible for much of the hemolytic action of the xanthine oxidase-acetaldehyde reaction.

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hemolysis. It is thus clear that hemolysis was primarily due to direct attack on the stroma, rather than to interactions of oxidized hemoglobin with the stroma. Superoxide dismutase or catalase protected against hemolysis in the presence of CO, thus again exposing the importance of O$_2^-$ and of H$_2$O$_2$. The protection provided by 2 mM histidine or by 10 mM mannitol, which do not scavenge O$_2^-$ or H$_2$O$_2$, again indicates that O$_2^-$ and H$_2$O$_2$ were important as the precursors of more reactive species, possibly O$_2$(Dg) and OH-. It should be noted that in this experiment, where the denaturation of hemoglobin plays no part in hemolysis, superoxide dismutase protected as markedly as catalase, since the paradoxical effects of superoxide dismutase on the oxidation of hemoglobin had been eliminated by the presence of CO.

**DISCUSSION**

The aerobic xanthine oxidase-acetaldehyde reaction mounts an oxidative attack on artificial liposomes and on washed human erythrocytes. In the former case, the production of lipid hydroperoxides was demonstrated by thin layer chromatography. Since all of the lipid in erythrocytes is in the stroma (11), these cells are analogous to large liposomes and it seems probable that lipid peroxidation was important in the observed lysis. Lipid peroxidation has been reported to precede lysis of tocopherol-deficient erythrocytes by H$_2$O$_2$, infused by gaseous diffusion or generated during the autooxidation of dialuric acid (31). Since superoxide dismutase or catalase prevented both peroxidation of the liposomes and lysis of the erythrocytes, we conclude that both O$_2^-$ and H$_2$O$_2$ were the active products of the xanthine oxidase reaction. Since peroxidation and hemolysis were also prevented by compounds which do not scavenge O$_2^-$ or H$_2$O$_2$, we must conclude that O$_2^-$ and H$_2$O$_2$ gave rise to more reactive species which then directly attacked the lipids in the liposomes and in the erythrocyte stroma. Previous studies with solutions of linolenate yielded similar results and it was then concluded that O$_2$(Dg) was produced by the interaction of O$_2^-$ and H$_2$O$_2$ and was mostly responsible for the peroxidation observed (5).

In the present studies, the same conclusion applies. Histidine, 2,5-dimethylfurane, xanthine, and urate prevented lipid peroxidation and hemolysis. Presumably this is the result of their O$_2$(Dg) scavenging capabilities. Mannitol, which scavenges OH-, but which does not scavenge O$_2$(Dg), did not protect at 0.5 mM, but was effective at 10 mM. O$_2$(Dg) appears to have been most important in directly causing lipid peroxidation with OH- possibly being a contributing factor. Mannitol (10 mM) might have protected by acting as an antioxidant or membrane stabilizer in the process of lipid peroxidation. Thus mannitol at 10 mM did protect against lysis when added 60 min after the inception of the xanthine oxidase-acetaldehyde reaction, at which time, superoxide dismutase, catalase, 2,5-dimethylfurane, or histidine were no longer effective. Lipid peroxidation can proceed by a free radical chain reaction (44). Once the antioxidants in an erythrocyte have been depleted, one can imagine such chain peroxidations proceeding for rather long periods of time. This might account for the observation that superoxide dismutase or catalase protected hemolysis, when present from the outset of the incubation, but had no effect when added 1 h later, even though very little hemolysis was evident at 1 h. Alternately, the delay in hemolysis could have been due to gradual swelling of damaged cells, until they reached the point of rupture. Thus increased permeability of the stroma, due to oxidative damage imposed by the xanthine oxidase reaction, would lead to gradual osmotic swelling and ultimately to lysis. Some workers have failed to see hemolysis when tocopherol-deficient rat erythrocytes were exposed to the xanthine oxidase reaction and concluded that O$_2^-$ and H$_2$O$_2$ are not hemolytic (45). These authors did not know that xanthine and urate prevent hemolysis in the xanthine oxidase system and so they used xanthine as the substrate rather than acetaldheyde. Other workers have succeeded in hemolyzing erythrocytes by exposure to the xanthine oxidase-xanthine reaction (46). However, they noted that xanthine was required and that superoxide dismutase did not protect and concluded that H$_2$O$_2$ and possibly OH- were the attacking species.

We have used the xanthine oxidase-acetaldehyde reaction as a convenient source of O$_2^-$ and of H$_2$O$_2$. This model system does have physiological correlates. Hemoglobin slowly autooxidizes and it was proposed in 1955 that this process would generate O$_2^-$, H$_2$O$_2$, and OH- (47). More recently, O$_2^-$ has definitely been shown to be a product of the autooxidation of hemoglobins (48-50) and of isolated hemoglobin chains (51). Naturally occurring substances, such as the vicine and convicine of fava beans and drugs such as the aminoquinolines, could augment this intraerythrocyte production of O$_2^-$, If O$_2^-$ is generated, then its dismutation would soon provide H$_2$O$_2$ and we have seen that O$_2^-$ and H$_2$O$_2$ interact to yield products which behave like OH- and O$_2$(Dg) and which cause lipid peroxidation and hemolysis.

To explain these results, we suggested the following mechanism, based on the Haber-Weiss reaction: H$_2$O$_2$ + O$_2^-$ + OH- → OH- + OH- + O$_2$(Dg) (6). Recently Noguchi et al. have proposed that O$_2$(Dg) was produced by the decomposition of paraeric acid which in turn was generated as a consequence of the oxidation of acetaldheyde, initiated by OH-, which was produced from O$_2^-$ and H$_2$O$_2$ by a Haber-Weiss reaction. This alternative proposal could also account for our results although no definitive evidence for either mechanism has yet been obtained.

Defenses against O$_2^-$ and H$_2$O$_2$ are clearly mandatory and the erythrocyte has them. Superoxide dismutase scavenges O$_2^-$ and keeps its concentration vanishingly low and catalase and glutathione peroxidase serve the same function vs a vs H$_2$O$_2$. Their combined effects would minimize the likelihood of interaction of O$_2^-$ and H$_2$O$_2$ and hence minimize the production of OH- and of O$_2$(Dg). Photochemically generated O$_2$(Dg) has been shown to be hemolytic (52). Erythrocytes can be depleted of superoxide dismutase by treatment with dietylthiocarbamate and this has been shown to sensitize these cells to hemolysis by O$_2^-$ generated internally by the action of naphthoquinone sulfonate on oxyhemoglobin (53).

Oxyhemoglobin can be oxidized to methemoglobin by O$_2^-$ (37, 38) and methemoglobin can also be converted to oxyhemoglobin by this radical (36). The reactions involved and their rate constants are:

(A) HbFe$^{3+}$ + O$_2^-$ + 2H+ → HbFe$^{++}$ + H$_2$O$_2$ + O$_2$

$$k = 4 \times 10^{7} \text{ M}^{-1} \text{s}^{-1}$$

(B) HbFe$^{3+}$ + O$_2^-$ → HbFe$^{3+}$ + O$_2$(Dg)

$$k = 5.7 \times 10^{6} \text{ M}^{-1} \text{s}^{-1}$$

This suggests that hemoglobin could itself act as a catalytic scavenger of O$_2^-$ and raises the question of the essentiality of superoxide dismutase in erythrocytes. Indeed when a hemolyzate is electrophoresed on polyacrylamide gels and the activity

stain for superoxide dismutase (14a) is applied, a weak activity coincident with the band of hemoglobin is seen. A liter of packed erythrocytes contains 300 g or 4.7 mM hemoglobin and 0.150 g or 4.7 \( \mu \text{M} \) superoxide dismutase. The rate constant for the reaction of superoxide dismutase with \( \text{O}_2^- \) is \( 2 \times 10^9 \text{M}^{-1} \text{s}^{-1} \) (54, 55) and this is nearly 10-fold greater than the corresponding rate for hemoglobin. It follows that superoxide dismutase at the concentration present in erythrocytes is 1000-fold more effective in scavenging \( \text{O}_2^- \) than the hemoglobin could possibly be. Furthermore consider that the catalytic scavenging of \( \text{O}_2^- \) by hemoglobin requires that in the steady state, a substantial fraction of it be in the form of methemoglobin and this would diminish the oxygen-carrying capacity of the blood and raise the danger of the formation of Heinz bodies. There can be no doubt that superoxide dismutase in erythrocytes is a vastly more efficient scavenger of \( \text{O}_2^- \) than the hemoglobin.

In a recent publication, Goldberg and Stern (56) studied hemolysis using dihydroxyfumarate as a source of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \). They reported observations seemingly at odds with those described above. They found that superoxide dismutase or catalase diminished both the oxidative degradation of hemoglobin and the hypotonic hemolysis caused by dihydroxyfumarate. Since carbon monoxide, which stabilized the hemoglobin against oxidative attack, also prevented hemolysis, they concluded that lysis was a secondary consequence of hemoglobin oxidation and did not involve peroxidation of stromal lipids. To explain these results, they proposed the peroxide-dependent formation of \( \text{O}_2^- \) by dihydroxyfumarate, where \( \text{O}_2^- \) is the attacking species causing hemoglobin denaturation and hypotonic lysis. These workers failed to consider the possibility that superoxide dismutase or catalase might inhibit the autooxidation of dihydroxyfumarate and thus might prevent changes in the erythrocyte by decreasing the rate of production of both \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \). This seemed likely since autooxidations are frequently chain reactions and since \( \text{O}_2^- \) often functions as a chain carrier. Thus, the autooxidation of epinephrine (57), tetrahydropterins (58), 6-hydroxydopamine (59), and phenylhydrazine (60) are inhibited by superoxide dismutase.

We have investigated this possibility by following the autooxidation of dihydroxyfumarate at 300 nm (61) and observing the effects of superoxide dismutase and of catalase. Dihydroxyfumarate (0.3 mM) autooxidized rapidly at 25°C in 10 mM sodium phosphate buffer at pH 6.4 and exhibited a decrease in absorbance at 300 nm of 0.148/min. Under these conditions, which simulate those used by Goldberg and Stern (66), superoxide dismutase at 10 \( \mu \text{g} \)ml inhibited 72%, catalase at 10 \( \mu \text{g} \)ml inhibited 50%, and both enzymes present simultaneously inhibited 90%. The rate of autooxidation of dihydroxyfumarate was more rapid when the pH was raised to 7.4, but the inhibitory effects of superoxide dismutase and of catalase were virtually unchanged.

We would propose that the reported (56) effects of autooxidizing dihydroxyfumarate upon erythrocytes were primarily due to \( \text{H}_2\text{O}_2 \)-dependent denaturation of hemoglobin during autooxidation. Superoxide dismutase protected the red cells because it inhibited autooxidation and thus diminished the production of \( \text{H}_2\text{O}_2 \), while catalase both decreased production of \( \text{H}_2\text{O}_2 \) and scavenged that \( \text{H}_2\text{O}_2 \) which was produced. As reported above, oxidative attack on the stroma can lead to lysis after a lag of an hour or more. It seems likely that Goldberg and Stern might have observed hemolysis due to lipid peroxidation, in the case of erythrocytes exposed to carbon monoxide during dihydroxyfumarate autooxidation, if their observations had been made on a time scale of hours, rather than tens of seconds.
Oxidation of Liposomes and Erythrocytes by $O_2^-$ and $H_2O_2$

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Liposome oxidation and erythrocyte lysis by enzymically generated superoxide and hydrogen peroxide.
E W Kellogg, 3rd and I Fridovich


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