The Mechanism of Superoxide Anion Generation by the Interaction of Phenylhydrazine with Hemoglobin*

B. Golberg† and A. Stern§

From the Department of Pharmacology, Stella and Charles Guttman Laboratories for Human Pharmacology and Pharmacogenetics, New York University School of Medicine, New York, New York, 10016

J. Peisach¶

From the Departments of Pharmacology and Molecular Biology, Albert Einstein College of Medicine of Yeshiva University, Bronx, New York, 10461

The mechanism by which superoxide anion is generated by the interaction of phenylhydrazine with either oxy- or methemoglobin was investigated. Rather than superoxide anion generation resulting from an accelerated autooxidation of oxyhemoglobin, it was found that both oxy- and methemoglobin function as peroxidases toward phenylhydrazine with the resultant oxidation of this compound to phenyldiazine. Generation of phenyldiazine from the oxidation of phenylhydrazine by hemoglobin or by the hydrolysis and subsequent decarboxylation of methyl phenylazoformate \( \text{C}_6\text{H}_5\text{N}==\text{NCOOCH}_3 \) resulted in the production of superoxide anion. It is suggested that under certain conditions hemoglobin may function as a drug-metabolizing peroxidase.

The discovery of superoxide dismutase has led to much speculation about the function of this enzyme in protecting cells against possible damage by superoxide anion. One of the most promising systems for study of superoxide anion toxicity on the cellular level is the erythrocyte. The generation of superoxide anion during the autooxidation of the hemoglobin molecule has been well documented (1, 2). This process, under ordinary circumstances, is extremely slow and indeed, to detect superoxide anion release during hemoglobin autooxidation, the protein must be stressed by lowering the pH and raising the ionic strength beyond the physiological range (2).

Recently the hemolytic agent phenylhydrazine has been demonstrated to react with hemoglobin to cause the very rapid generation of superoxide anion (3). Phenylhydrazine-induced hemolysis may therefore prove to be an excellent experimental model for the study of the cellular toxicity of superoxide anion. In the present investigation, the nature of the interaction of phenylhydrazine with oxy- and methemoglobin is explored. As methemoglobin is ultimately formed by the interaction of phenylhydrazine with oxyhemoglobin, the relationship of this reaction to the autooxidation of oxyhemoglobin is of considerable interest. Moreover, a knowledge of the mechanism by which superoxide anion is chemically generated in the presence of hemoglobin may allow the prediction of potentially hemolytic agents.

MATERIALS AND METHODS

Oxyhemoglobin and methemoglobin were prepared as described previously (4). After the oxyhemoglobin was separated from osmotically shocked red cells, it was passed through a Sephadex G-25 column and the effluent was applied to a CM-cellulose-Sephadex column equilibrated with 50 mM phosphate buffer, pH 6.0. The column was washed with the same buffer containing 50 mM NaCl and the hemoglobin was removed using the same buffer containing 100 mM NaCl. With this procedure, superoxide dismutase as well as the reductase enzymes of the red cell were separated from the hemoglobin (5).

Superoxide dismutase was obtained from Truett Labs, Dallas, Texas, catalase (45,000 units/ml) from Biochemical Labs, Gardena, Cal. Phenyldiazine-HCl (recrystallized), a-chymotrypsin, nitroblue tetrazolium, and lactoperoxidase were purchased from Sigma Chemical Co., St. Louis, Mo. Methyl phenylazoformate was acquired from Calbiochem, San Diego, Cal. Epinephrine bitartrate was purchased from Schwarz/Mann, Orangeburg, N. Y. The 10% oxygen balance N-, argon, and carbon monoxide were obtained from Matheson Gas Products, East Rutherford, N. J.

Superoxide anion generation by the interaction of phenylhydrazine with hemoglobin was assayed as described previously (3).

For experiments under controlled atmospheres, a Thunberg cuvette having a side-arm equipped with a rubber septum stopper was utilized in place of the sample cuvette.
The benzene recovery using this procedure was 60% of standard benzene precipitation, cyclohexane extraction, and gas chromatographic assay. Benzene concentration was determined from the Varian Aerograph Series 1400 gas chromatograph with flame ionization detector. Standard curves were determined by adding known quantities of benzene to aqueous solutions of oxyhemoglobin followed by trichloroacetic acid precipitation, cyclohexane extraction, and gas chromatographic assay. The benzene recovery using this procedure was 60% of standard benzene concentrations.

RESULTS

Role of $H_2O_2$ in Oxidation of Epinephrine and Phenylhydrazine by Methemoglobin—Previous studies have demonstrated that when phenylhydrazine is added to a solution of methemoglobin and epinephrine, a rapid oxidation of the epinephrine takes place which is completely inhibited by superoxide dismutase and almost completely inhibited by catalase (3). This phenomenon suggests that the oxidation of epinephrine from the reaction of phenylhydrazine with methemoglobin is due to superoxide anion and that most of this superoxide anion is derived from a peroxide intermediate. Therefore, it was decided to study the role of $H_2O_2$ in the formation of superoxide anion by the reaction of phenylhydrazine with methemoglobin.

When $H_2O_2$ is added to a solution of epinephrine, no significant oxidation of the epinephrine occurs. If $H_2O_2$ is added to a solution of methemoglobin and epinephrine, a rapid oxidation of the epinephrine takes place (Fig. 1B, curve c). This oxidation of epinephrine is not inhibited by superoxide dismutase (Fig. 1A, curve a). This experiment illustrates the role of methemoglobin in serving as a peroxidase toward phenylhydrazine oxidation. If phenylhydrazine is added at the same time as $H_2O_2$ to a solution of methemoglobin and epinephrine, inhibition of the peroxidation of epinephrine is obtained in the presence of superoxide dismutase (Fig. 1A, curve b), but not in the absence of the enzyme (Fig. 1B, curve d). These results indicate that phenylhydrazine can inhibit the peroxidation of epinephrine catalyzed by methemoglobin and that this inhibition of epinephrine peroxidation is related to superoxide anion production.

The role of methemoglobin as a peroxidase was further investigated by studying the aerobic oxidation of phenylhydrazine to benzene in the presence of methemoglobin (Fig. 2). This oxidation is inhibited either by catalase or by an excess of thiocyanate, a known substrate for the methemoglobin-peroxidase reaction (6). Thiocyanate also causes almost total inhibition of epinephrine oxidation by phenylhydrazine and methemoglobin. Superoxide dismutase has no inhibitory effect on the aerobic oxidation of phenylhydrazine catalyzed by methemoglobin.

The importance of peroxidase activity of hemoglobin for superoxide anion generation by phenylhydrazine was investigated by substituting ferrilactoperoxidase for methemoglobin in reaction mixtures containing phenylhydrazine and epinephrine. Although a slow oxidation of epinephrine occurs in solutions containing lactoperoxidase, this oxidation is markedly enhanced when phenylhydrazine is added (Fig. 3a). Oxidation of epinephrine by the reaction of phenylhydrazine and lactoperoxidase is completely inhibited by superoxide dismutase (Fig. 3c) and partially inhibited by catalase (Fig. 3b). The incomplete inhibition by catalase is probably due to the inability of catalase to adequately compete with the peroxidase for $H_2O_2$. It should be noted that the concentration of lactoperoxidase is 1/100 the concentration of methemoglobin used in demonstrating superoxide anion production with phenylhydrazine and methemoglobin. The much greater ability of ferrilactoperoxidase compared to methemoglobin to cause the generation of superoxide anion with phenylhydrazine was also demonstrated by using equimolar concentrations of both proteins in the reactions (Fig. 3, curve d).

Relationship of Superoxide Anion Production by Reaction of Phenylhydrazine and Oxyhemoglobin to Autooxidation of Oxyhemoglobin—An interesting question regarding the generation of superoxide anion by the reaction of phenylhydrazine with oxyhemoglobin...
Drazine is added to oxyhemoglobin solutions may be explained by the oxidation of epinephrine during the reaction of phenylhydrazine and oxyhemoglobin (1, 2). This was investigated by studying the effects of varying oxygen tension on superoxide anion generation by the reaction of phenylhydrazine and fully saturated oxyhemoglobin. If the superoxide anion generated during the reaction of phenylhydrazine with oxyhemoglobin is derived from the heme, its production should remain constant as the oxygen tension of oxyhemoglobin solutions is lowered as long as the heme, its production should remain constant as the oxygen saturation of hemoglobin remains the same. When phenylhydrazine is added to an oxyhemoglobin solution equilibrated with 20% oxygen at pH 6.8, the oxidation of epinephrine is observed (Fig. 4c). This oxidation is partially inhibited either by superoxide dismutase or catalase and totally inhibited by both enzymes together (3). When an oxyhemoglobin solution at pH 6.8 is equilibrated with 10% oxygen in a Thunberg cuvette, a procedure which does not significantly reduce the oxygen saturation of hemoglobin as verified spectrophotometrically, a decrease in the oxidation of epinephrine is seen when phenylhydrazine is added (Fig. 4b). As Misra and Fridovich have demonstrated, the stoichiometry at pH 6.8 for the reaction of superoxide anion with epinephrine remains nearly the same with either 10% or 50% oxygen (7). Therefore, the decrease in epinephrine oxidation observed under 10% oxygen when phenylhydrazine is added to oxyhemoglobin solutions may be explained by a decreased production of superoxide anion.

Wever et al. (2), have demonstrated that the spontaneous generation of superoxide anion from bovine oxyhemoglobin was barely detectable at pH 7.0, but was increased greatly when the pH was lowered to 6.0 and the ionic strength of the hemoglobin solution was increased. As this increase in superoxide anion production by oxyhemoglobin at lower pH parallels an increase in the spontaneous formation of methemoglobin (8), it became essential to explore the effect of pH on superoxide anion generation by the reaction of phenylhydrazine and oxyhemoglobin. Raising the pH from 6.0 to 7.5 results in an increase in the oxidation of epinephrine (Fig. 5). The percentage of inhibition of this oxidation of epinephrine by superoxide dismutase and catalase throughout the pH range of 6.0 to 8.0 remains constant at 80%; but inhibition by superoxide dismutase increases from 0 at pH 6.0 to a maximum of approximately 50% at pH 7.0 (Fig. 6). The inhibition by catalase, on the other hand, decreases from a maximum of 80% at pH 6.0 to a minimum of about 50% at pH 7.0 (Fig. 6). As the pH profile of these enzymes indicates that their activity remains constant within the pH range studied (9, 10), this suggests that an increase in the ratio of univalent to divalent electron flux to molecular oxygen (11) is occurring as the pH of solutions of oxyhemoglobin containing phenylhydrazine and epinephrine is increased from pH 6.0 to 7.0. This increase in the ratio of univalent to divalent electron flux to molecular oxygen with increasing pH has been demonstrated for xanthine oxidase (11) and ferredoxin (7). As with oxyhemoglobin, increasing the pH from 6.0 to 7.5 in solutions of methemoglobin containing phenylhydrazine and epineph-
Phenylhydrazine, Hemoglobin, and Superoxide Generation

Initial rates are linear measured after a 40-s dead time. The initial short lag observed in the presence of superoxide dismutase was disregarded and the linear rate after the lag was used. The inhibitory effect of thiocyanate on the oxidation of epinephrine by catalase appear not to exert a significant inhibitory effect on phenylhydrazine oxidation by oxyhemoglobin. Concentrations and conditions are the same as in Fig. 5. Percentage of inhibition is defined as:

\[
100 - 100 \times \frac{\text{rate of epinephrine oxidation in the presence of enzyme(s)}}{\text{rate of epinephrine oxidation in the absence of enzyme(s)}}
\]

Percentage of inhibition is defined as:

\[
\frac{\text{rate of epinephrine oxidation in the presence of enzyme(s)}}{\text{rate of epinephrine oxidation in the absence of enzyme(s)}} \times 100
\]

Initial rates are linear measured after a 40-s dead time. The initial short lag observed in the presence of superoxide dismutase was disregarded and the linear rate after the lag was used. The inhibitory effect of thiocyanate on the oxidation of epinephrine by catalase appear not to exert a significant inhibitory effect on phenylhydrazine oxidation by oxyhemoglobin. Concentrations and conditions are the same as in Fig. 5. Percentage of inhibition is defined as:

\[
100 - 100 \times \frac{\text{rate of epinephrine oxidation in the presence of enzyme(s)}}{\text{rate of epinephrine oxidation in the absence of enzyme(s)}}
\]

Initial rates are linear measured after a 40-s dead time. The initial short lag observed in the presence of superoxide dismutase was disregarded and the linear rate after the lag was used. The inhibitory effect of thiocyanate on the oxidation of epinephrine by catalase appear not to exert a significant inhibitory effect on phenylhydrazine oxidation by oxyhemoglobin. Concentrations and conditions are the same as in Fig. 5. Percentage of inhibition is defined as:

\[
100 - 100 \times \frac{\text{rate of epinephrine oxidation in the presence of enzyme(s)}}{\text{rate of epinephrine oxidation in the absence of enzyme(s)}}
\]

Initial rates are linear measured after a 40-s dead time. The initial short lag observed in the presence of superoxide dismutase was disregarded and the linear rate after the lag was used. The inhibitory effect of thiocyanate on the oxidation of epinephrine by catalase appear not to exert a significant inhibitory effect on phenylhydrazine oxidation by oxyhemoglobin. Concentrations and conditions are the same as in Fig. 5. Percentage of inhibition is defined as:

\[
100 - 100 \times \frac{\text{rate of epinephrine oxidation in the presence of enzyme(s)}}{\text{rate of epinephrine oxidation in the absence of enzyme(s)}}
\]

Initial rates are linear measured after a 40-s dead time. The initial short lag observed in the presence of superoxide dismutase was disregarded and the linear rate after the lag was used. The inhibitory effect of thiocyanate on the oxidation of epinephrine by catalase appear not to exert a significant inhibitory effect on phenylhydrazine oxidation by oxyhemoglobin. Concentrations and conditions are the same as in Fig. 5. Percentage of inhibition is defined as:

\[
100 - 100 \times \frac{\text{rate of epinephrine oxidation in the presence of enzyme(s)}}{\text{rate of epinephrine oxidation in the absence of enzyme(s)}}
\]

Initial rates are linear measured after a 40-s dead time. The initial short lag observed in the presence of superoxide dismutase was disregarded and the linear rate after the lag was used. The inhibitory effect of thiocyanate on the oxidation of epinephrine by catalase appear not to exert a significant inhibitory effect on phenylhydrazine oxidation by oxyhemoglobin. Concentrations and conditions are the same as in Fig. 5. Percentage of inhibition is defined as:

\[
100 - 100 \times \frac{\text{rate of epinephrine oxidation in the presence of enzyme(s)}}{\text{rate of epinephrine oxidation in the absence of enzyme(s)}}
\]

Initial rates are linear measured after a 40-s dead time. The initial short lag observed in the presence of superoxide dismutase was disregarded and the linear rate after the lag was used. The inhibitory effect of thiocyanate on the oxidation of epinephrine by catalase appear not to exert a significant inhibitory effect on phenylhydrazine oxidation by oxyhemoglobin. Concentrations and conditions are the same as in Fig. 5. Percentage of inhibition is defined as:

\[
100 - 100 \times \frac{\text{rate of epinephrine oxidation in the presence of enzyme(s)}}{\text{rate of epinephrine oxidation in the absence of enzyme(s)}}
\]

Initial rates are linear measured after a 40-s dead time. The initial short lag observed in the presence of superoxide dismutase was disregarded and the linear rate after the lag was used. The inhibitory effect of thiocyanate on the oxidation of epinephrine by catalase appear not to exert a significant inhibitory effect on phenylhydrazine oxidation by oxyhemoglobin. Concentrations and conditions are the same as in Fig. 5. Percentage of inhibition is defined as:

\[
100 - 100 \times \frac{\text{rate of epinephrine oxidation in the presence of enzyme(s)}}{\text{rate of epinephrine oxidation in the absence of enzyme(s)}}
\]

Initial rates are linear measured after a 40-s dead time. The initial short lag observed in the presence of superoxide dismutase was disregarded and the linear rate after the lag was used. The inhibitory effect of thiocyanate on the oxidation of epinephrine by catalase appear not to exert a significant inhibitory effect on phenylhydrazine oxidation by oxyhemoglobin. Concentrations and conditions are the same as in Fig. 5. Percentage of inhibition is defined as:

\[
100 - 100 \times \frac{\text{rate of epinephrine oxidation in the presence of enzyme(s)}}{\text{rate of epinephrine oxidation in the absence of enzyme(s)}}
\]

Initial rates are linear measured after a 40-s dead time. The initial short lag observed in the presence of superoxide dismutase was disregarded and the linear rate after the lag was used. The inhibitory effect of thiocyanate on the oxidation of epinephrine by catalase appear not to exert a significant inhibitory effect on phenylhydrazine oxidation by oxyhemoglobin. Concentrations and conditions are the same as in Fig. 5. Percentage of inhibition is defined as:

\[
100 - 100 \times \frac{\text{rate of epinephrine oxidation in the presence of enzyme(s)}}{\text{rate of epinephrine oxidation in the absence of enzyme(s)}}
\]

Initial rates are linear measured after a 40-s dead time. The initial short lag observed in the presence of superoxide dismutase was disregarded and the linear rate after the lag was used. The inhibitory effect of thiocyanate on the oxidation of epinephrine by catalase appear not to exert a significant inhibitory effect on phenylhydrazine oxidation by oxyhemoglobin. Concentrations and conditions are the same as in Fig. 5. Percentage of inhibition is defined as:

\[
100 - 100 \times \frac{\text{rate of epinephrine oxidation in the presence of enzyme(s)}}{\text{rate of epinephrine oxidation in the absence of enzyme(s)}}
\]

Initial rates are linear measured after a 40-s dead time. The initial short lag observed in the presence of superoxide dismutase was disregarded and the linear rate after the lag was used. The inhibitory effect of thiocyanate on the oxidation of epinephrine by catalase appear not to exert a significant inhibitory effect on phenylhydrazine oxidation by oxyhemoglobin. Concentrations and conditions are the same as in Fig. 5. Percentage of inhibition is defined as:

\[
100 - 100 \times \frac{\text{rate of epinephrine oxidation in the presence of enzyme(s)}}{\text{rate of epinephrine oxidation in the absence of enzyme(s)}}
\]
globin and superoxide anion production. A logical conclusion from these studies is that the oxidation of phenylhydrazine produces some intermediate which can cause the univalent reduction of molecular oxygen with a subsequent conversion to benzene and N₂.

The oxidation of phenylhydrazine to benzene and N₂ by simple oxidants such as ferricyanide and quinones is known to proceed via a 2-electron process yielding phenylazidone (C₆H₅N=NH) as an intermediate (13). The extreme reactivity of phenylazidone with molecular oxygen, as well as evidence that this reaction produces free radicals (14), prompted an investigation of the ability of phenylazidone to generate superoxide anion.

Phenyldiazine may be generated by the hydrolysis and subsequent decarboxylation of methyl phenylazidone. As the azoformate is rather stable to hydrolysis in the neutral pH range the enzyme α-chymotrypsin may be added to catalyze the hydrolytic reaction and thus increase its rate to a level suitable for study (15). Nitroblue tetrazolium (16) was utilized to detect superoxide anion principally because of the powerful oxidizing action of methyl phenylazidone toward electron donors such as epinephrine. In Fig. 9A, the disappearance of methyl phenylazidone at pH 7.0 in the presence of α-chymotrypsin is detected by monitoring the decrease in optical density at 502.5 nm. The simultaneous increase in optical density at 560 nm due to the reduction of nitroblue tetrazolium occurring in the presence of azoester and α-chymotrypsin is also illustrated. In Fig. 9B the effect of superoxide dismutase on inhibiting the reduction of nitroblue tetrazolium during hydrolysis and subsequent decarboxylation of methyl phenylazidone is shown. It can be seen that approximately 50% of the reduction of nitroblue tetrazolium in the aerobic decomposition of phenylazidone is due to superoxide anion.

If the oxygen in a reaction mixture containing methyl phenylazidone, α-chymotrypsin, and nitroblue tetrazolium is replaced by argon, the reduction of the tetrazolium occurs at approximately the same rate as in the presence of oxygen except that now superoxide dismutase does not exert a significant inhibitory effect on the reduction. This illustrates the powerful reducing action of phenylazidone toward both nitroblue tetrazolium and molecular oxygen.

**DISCUSSION**

As we have demonstrated, the generation of superoxide anion and the oxidation of phenylazidone to benzene with methemoglobin is dependent upon H₂O₂ production since catalase will inhibit both of these phenomena. It is suggested that methemoglobin serves as a peroxidase catalyzing the oxidation of phenylazidone by H₂O₂ to an intermediate capable of the univalent reduction of molecular oxygen. Thus the aerobic oxidation of phenylazidone by methemoglobin, HbO₂(?) may be written as follows:

\[
\text{Hb}^{1+1} + \text{H}_2\text{O}_2 \rightarrow \text{[HbO]}^{2+} + \text{H}^+ + \frac{1}{2} \text{H}_2\text{O}_2 \text{(fate unknown)} \quad (1)
\]

\[
2\text{[HbO]}^{2+} + 4\text{NH}-\text{NH}_2 \rightarrow 2\text{Hb}^{1+} + 4\text{N}=\text{NH} + 2\text{H}^+ \quad (2)
\]

\[
\phi\rightarrow \text{N}=\text{NH} + \text{O}_2 \rightarrow \phi^+ + \text{O}_2^- + \text{H}^+ \quad (3)
\]

In this reaction scheme H₂O₂ oxidizes the methemoglobin to ferrylhemoglobin [HbO]⁺⁺ (17), 2 molecules of which then oxidize phenylhydrazine to phenylazidone while being converted back to methemoglobin. A similar reaction scheme was first suggested by Yamazaki and Piette (18) for the “peroxidase-oxidase” activity of horseradish peroxidase toward dihydroxyfumaric acid. Their scheme showed that H₂O₂ decomposition could be coupled to the formation of superoxide anion through the intermediacy of a partially oxidized form of dihydroxyfumarate. From our experiments related to superoxide anion generation by phenylazidone and the observation that phenylhydrazine appears to act as a 2-electron reductant (13), it is concluded that methemoglobin catalyzes the oxidation of phenylhydrazine to phenylazidone by H₂O₂ (Equations 1 and 2) and the phenylazidone then reacts with molecular oxygen to yield superoxide anion (Equation 3). The initial source of H₂O₂ required in Equation 1 is probably derived from a slow autooxidation of phenylhydrazine. Indeed H₂O₂ has been detected during this process (19). Once the reaction has been initiated, superoxide anion derived from the reaction of phenylazidone with molecular oxygen can be converted into H₂O₂ to perpetuate the process by a chain reaction. The conversion of superoxide anion into H₂O₂ can occur via spontaneous dismutation or by reduction with an exogenous electron donor such as epinephrine.

The generation of superoxide anion by the reaction of phenylhydrazine with oxyhemoglobin proved to be somewhat more complicated. Decreasing oxygen tension in oxyhemoglobin solutions while not significantly lowering the oxygen
saturation of the hemoglobin (Fig. 4), as well as lowering pH (Fig. 5), produces a decrease in the generation of superoxide anion when phenylhydrazine is added. These findings rule out a simple displacement of superoxide anion from ferriheme as supposedly occurs during the autooxidation of oxyhemoglobin (1, 2) as this displacement would not be expected to be dependent upon the oxygen tension as long as the oxygen saturation of hemoglobin remains the same. Also, spontaneous decomposition of oxyhemoglobin to yield superoxide anion and methemoglobin is reported to increase at lower pH (2) while we find that the formation of superoxide anion is increased at higher pH (Figs. 5 and 6). It is therefore suggested that phenylhydrazine reacts with oxyhemoglobin to form a new species which subsequently reacts with free molecular oxygen in solution to produce superoxide anion. As phenylhydrazine is known to be oxidized to benzene in the presence of oxyhemoglobin (12) one can again envision an oxidized intermediate of phenylhydrazine such as phenyldiazine as the superoxide anion source.

The viability of the mechanism outlined for the reaction of phenylhydrazine with methemoglobin in the reaction of phenylhydrazine with oxyhemoglobin was investigated through the use of thiocyanate. Thiocyanate is oxidized by \( \text{H}_2\text{O}_2 \) in the presence of methemoglobin to \( \text{SO}_2^- \) and \( \text{CN}^- \) (6) and thus can compete with phenylhydrazine for oxidation, and therefore, inhibits superoxide anion generation. Thiocyanate was shown to be ineffective in inhibiting phenylhydrazine oxidation and superoxide anion production by oxyhemoglobin (Figs. 7 and 8). Catalase had the same behavior as the thiocyanate indicating that the oxidation of phenylhydrazine and thus the generation of superoxide anion by oxyhemoglobin is independent of the methemoglobin-peroxidase activity. The observation that aerobic solutions of carbon monoxidehemoglobin failed to oxidize phenylhydrazine or produce superoxide anion indicate that the primary oxidant of phenylhydrazine must be oxyhemoglobin itself. Oxyhemoglobin may act in a manner similar to the structurally analogous oxyperoxidase (20) toward certain reducing agents (21). Thus, the reaction of phenylhydrazine with oxyhemoglobin or peroxidase may be written as follows:

\[
[HbO_2]^{+\text{II}} + \text{NH}_2\text{NH}_2 + [HbO]^{-\text{II}} + \text{H}_2\text{N}=\text{NH} + 2\text{H}^+ \quad (4)
\]

This reaction depicts a 2-electron reduction of oxygenated heme \([HbO_2]^{+\text{II}}\) to the ferryl state. The formation of the ferryl oxidation state during reaction with aromatic reductants was demonstrated by Peisach et al. (22) who trapped this intermediate in the presence of hemoglobin as sulfhemoglobin. The ferryl state could then be discharged as in Equation 2 with the utilization of additional phenylhydrazine and the formation of methemoglobin. Note that the formation of ferrylheme during the reaction of phenylhydrazine with methemoglobin involves an oxidative process and is inhibited by catalase while the formation of ferrylhemoglobin during the reaction of phenylhydrazine with oxyhemoglobin is a reductive process and is not inhibited by catalase. The phenyldiazine produced in Equation 4 and by the subsequent discharge of the resultant ferryl state (Equation 2) can then react as in Equation 3 with molecular oxygen to produce superoxide anion. The oxidation of epinephrine that is inhibited by catalase (3) and thiocyanate (Fig. 7) is no doubt due to the direct peroxidation of the epinephrine catalyzed by the methemoglobin formed during the reaction. The consumption of phenylhydrazine as in Equation 4 and the subsequent reduction of the resultant ferryl state to methemoglobin would leave the epinephrine free from competition for oxidation by methemoglobin and \( \text{H}_2\text{O}_2 \) formed from the superoxide anion generated by the phenyldiazine.

Phenylhydroxylamine structurally resembles phenylhydrazine. Phenylhydroxylamine reacts with oxyhemoglobin resulting in the formation of methemoglobin and nitrosobenzene via a 2-electron process (23). Eyer et al. (24) have studied the reaction and have concluded that free \( \text{H}_2\text{O}_2 \) is not involved in the conversion of oxyhemoglobin to methemoglobin. This would appear to be evidence in favor of a direct reduction of oxyhemoglobin to methemoglobin as proposed for the phenylhydrazine reaction. Nitrosobenzene unlike phenyldiazine is relatively stable and thus does not react with molecular oxygen to produce superoxide anion. Therefore, our observation of the absence of superoxide anion generation by the reaction of phenylhydroxylamine with oxyhemoglobin would be expected.

In our study it has been shown that both ferric and oxyhemoproteins may oxidize aromatic reducing agents to species which may be capable of causing the univalent reduction of molecular oxygen to superoxide anion. The calculated values of the oxidation-reduction potential of the \( O_2/\text{O}_2^- \) couple in water is reported as \(-0.27\) to \(-0.33\) V. (25). Thus, if any oxidation state of hemoglobin can oxidize a reduc tant to form an intermediate having an oxidation-reduction potential lower than this range of values, superoxide anion can be produced. This fact may prove useful in screening potentially hemolytic agents, once a firm connection between the generation of superoxide anion during the interaction of phenylhydroxylamine and hemoglobin and the hemolytic effect of this drug is established.

The oxidative activation of phenylhydrazine by oxyhemoglobin to a chemical species potentially toxic to the red cell demonstrates the possible role of the red cell as the site of metabolism of certain drugs. Since drugs are in the circulation before being taken up by the liver, one must consider the metabolites of the hemoglobin drug-metabolizing systems as potential precursors to the hepatic oxidative pathways.

In attempting to explain the hemolytic action of phenylhydrazine, the extremely high concentration of hemoglobin present in the red cell must be taken into account. If superoxide dismutase in the erythrocyte has evolved specifically to protect the red blood cell against lysis by the superoxide anion generated during the slow autoxidation of hemoglobin, the levels of this enzyme may be insufficient to protect the cell against the relatively large superoxide anion release caused by the interaction of hemolytic agents such as phenylhydrazine with hemoglobin. This hypothesis is currently being explored with the hope that the molecular basis of the hemolytic action of phenylhydrazine and related compounds may at last be explained.

Acknowledgments—We are much indebted to Drs. N. Eric Naftchi and M. Demeny for the use of the gas chromatography equipment.

REFERENCES

*B. Goldberg and A. Stern, unpublished observation.
The mechanism of superoxide anion generation by the interaction of phenylhydrazine with hemoglobin.
B Goldberg, A Stern and J Peisach


Access the most updated version of this article at http://www.jbc.org/content/251/10/3045

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/251/10/3045.full.html#ref-list-1