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Kinetics of Ferrihemoglobin Formation by Some Reducing Agents, and the Role of Hydrogen Peroxide

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SUMMARY

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The rate of oxidation by hydrogen peroxide of human hemoglobin, virtually free from catalase, glutathione peroxidase, and superoxide dismutase, was found to be proportional to the concentrations of hemoglobin and hydrogen peroxide, the second-order rate constant at pH 7.4 and 37° being $k = 125\ M^{-1}\ sec^{-1}$. Formation of ferrihemoglobin by reduced glutathione in air was found to be slow, gaining its maximal velocity after a lag phase. Kinetic data and the effect of catalase or glutathione peroxidase demonstrated that hydrogen peroxide is an essential intermediate which produces ferrihemoglobin in solutions of hemoglobin and reduced glutathione. The much higher rate of ferrihemoglobin formation by phenylhydroxylamine than by hydrogen peroxide and the failure of catalase to inhibit the reaction showed that hydrogen peroxide is not an important intermediate in the formation of ferrihemoglobin by phenylhydroxylamine. The reaction rate was found to be proportional to the concentrations of phenylhydroxylamine and hemoglobin. The second-order rate constant was calculated to be $k = 2350\ M^{-1}\ sec^{-1}$. With the formation of ferrihemoglobin by 4-dimethylaminophenol also, reaction rates and the failure of catalase to inhibit the reaction demonstrated that hydrogen peroxide is of no importance. The lag phase of the reaction suggests that oxidation products of 4-dimethylaminophenol produced by the reaction between oxyhemoglobin and 4-dimethylaminophenol are essential intermediates.

INTRODUCTION

Most of the ferrihemoglobin-forming substances of toxicological importance are reducing agents. Many of them, such as arylhydroxylamines or aminophenols, may be produced biochemically *in vivo* from inert substances, e.g., arylamines. In addition to these foreign substances a biological reducing compound, glutathione, produces ferrihemoglobin (1). This is of particular interest, as about 2 mM reduced glutathione is present in human red cells (2).

The mechanisms by which reducing agents in the presence of oxygen oxidize ferrohemoglobin to ferrihemoglobin are not yet fully understood. Present knowledge has recently been reviewed by Kiese (3, 4). Since autoxidation of reducing agents can produce hydrogen peroxide, and hydrogen peroxide may oxidize hemoglobin to ferrihemoglobin (5, 6), hydrogen peroxide has been discussed as an active intermediate in the formation of ferrihemoglobin by reducing agents (7, 8). The appearance of hydrogen peroxide during the formation of ferrihemoglobin by phenylhydroxylamine has been demonstrated by Ellederová *et al.* (9).

We compared the kinetics of ferrihemo-

globin formation by hydrogen peroxide and some reducing agents and the effect of catalase on these reactions and found that there are reactions in which hydrogen peroxide is an essential intermediate and others in which it is not.

MATERIALS AND METHODS

Hemolysates were prepared by ultrasonic disintegration of human red cells washed five times with a 2-fold volume of 0.2 M phosphate. For ultrasonic treatment the packed cells were stirred for 30 min with 2 volumes of water. Then they were exposed in an ice bath for six 30-sec periods with 1-min intermissions to a field of 20 KHz with an amplitude of 6 μm (peak to peak). After addition of five grams of Celite per 100 ml of hemolysate and 10 min of stirring, the hemolysate was centrifuged for 20 min at 15,000 $\times$ g.

Purified hemoglobin was prepared by chromatography of the hemolysate on Sephadex G-100, on DE_{52}-cellulose (Whatman), and on Sephadex G-25. The hemolysate was diluted with 0.01 M Tris-HCl, pH 8.3, to 10 g of hemoglobin per 100 ml. Then 60 ml of this solution were applied to a 7 $\times$ 100 cm column of Sephadex G-100 (fine) and eluted with 0.01 M Tris-HCl, pH 8.3, from bottom to top.

For ion-exchange chromatography on DE_{52}-cellulose the pooled fractions containing hemoglobin were applied to a 3 $\times$ 8 cm column which had been equilibrated with 0.01 M Tris-HCl, pH 8.3. Hemoglobin was eluted with a linear gradient of 500 ml of 0.01 M Tris-HCl, pH 8.3, and 500 ml of 0.1 M Tris-HCl, pH 7.0.

Before chromatography on Sephadex G-25 the concentration of hemoglobin was increased by adsorption on another column of DE_{52}-cellulose as described above and elution with 0.5 M sodium chloride in 0.1 M Tris-HCl, pH 7.0. The fractions containing hemoglobin were pooled, adjusted to pH 7.4, and applied to a column of Sephadex G-25, 4 $\times$ 20 cm, equilibrated with 0.2 M phosphate, pH 7.4. Elution with 0.2 M phosphate, pH 7.4, yielded solutions of 4–8 g of hemoglobin per 100 ml, which contained about 70% of the hemoglobin applied to the Sephadex G-100 column. The proportion of ferrihemoglobin was below 1% of the total hemoglobin. The catalase content per gram of hemoglobin, expressed as a first-order rate constant, was $k < 1$. In the hemolysate the corresponding value was about 300. Superoxide dismutase and glutathione peroxidase were not detectable.

Superoxide dismutase (EC 1.15.1.1) was prepared from bovine red cells according to McCord and Fridovich (10). Gel filtration on Sephadex G-75 with 50 mM phosphate, pH 7.4, reduced glutathione peroxidase in the preparation to a negligible amount. Catalase was not detectable. The final superoxide dismutase preparation had a specific activity of 2470 units/mg.

Glutathione peroxidase (EC 1.11.1.9) with a specific activity of 225 units/mg and superoxide dismutase content of 400 units/mg were obtained by gel filtration of the superoxide dismutase preparation as described above.

Catalase (EC 1.11.1.6) from beef liver, analytical grade, with a specific activity of 115 k/mg, was purchased from Boehringer, Mannheim. Superoxide dismutase activity was 0.5 unit/mg. Glutathione peroxidase was not detectable.

Enzyme assays. Superoxide dismutase was measured using the method and units of McCord and Fridovich (10). For determination in hemolysates or solutions of purified hemoglobin, the hemoglobin was precipitated according to Tsuchihashi (11) and the activity of the supernatant was measured. Catalase was determined according to Aebi (12). As proposed by the author, the first-order rate constant in sec^{-1}, was used to express activity. Glutathione peroxidase was determined according to Paglia and Valentine (13). As proposed by Günzler *et al.* (14), *tert*-butyl peroxide was used as peroxide. It is not affected by catalase and shows a low rate of uncatalyzed reaction with glutathione. The assay mixture contained 0.2 M phosphate (pH 7.4), 2 mM EDTA, 10 mM glutathione, 0.15 mM NADPH, and 10 μg/ml of glutathione reductase. The reaction was started by addition of 0.5 mM *tert*-butyl peroxide. The decrease in absorbance at 340 nm was followed at 37°. One unit corresponds to

the formation of 1 μmole of oxidized glutathione per minute. When hemoglobin was present, the determination of enzyme activity was carried out under carbon monoxide.

Ferrihemoglobin was determined by the increase in absorbance at 550 nm caused by the addition of cyanide (15). Total hemoglobin was measured as ferrihemoglobin after ferrohemoglobin had been oxidized by ferricyanide.

For measuring the yield of ferrihemoglobin during infusion of hydrogen peroxide into hemolysate or solutions of purified hemoglobin, samples of 10 ml in 100-ml Erlenmeyer flasks were shaken in a gyrotory water bath. Nitrogen was passed over the solutions, beginning 30 min before the hydrogen peroxide solution was infused at a rate of 0.6 ml/hr.

Rapid rates of oxidation of ferrohemoglobin were measured in a Gibson-Durrum stop-flow apparatus. The increase in absorbance at 630 nm was used as a measure of the increase in ferrihemoglobin. Since phenylhydroxylamine had to be dissolved under nitrogen, a small volume of its solution was mixed with a larger volume of hemoglobin solution in order to keep the changes in oxygen pressure and saturation of hemoglobin low. The ratio of the volumes of the syringes used for the injection of solutions into the mixing chamber was 1:7.22. For comparison the solutions of hydrogen peroxide were also prepared under nitrogen.

Slow rates of ferrihemoglobin formation, as with the reaction between hemoglobin and glutathione, were measured with recording spectrophotometers after the reactants had been mixed quickly in a cuvette.

In the experiments with various oxygen pressures all solutions were equilibrated at 37° with the respective gas in modified Thunberg tubes. The oxygen saturation of hemoglobin was determined by recording the absorbance at 577 nm. The gas mixtures were analyzed in a gas chromatograph.

Hydrogen peroxide was determined iodometrically. Phosphate solution, 0.2 M, pH 7.4, contained 0.172 M Na_2HPO_4 and 0.028 M KH_2PO_4.

4-Dimethylaminophenol was synthetized by Farbwerke Hoechst. Phenylhydroxylamine was prepared according to Utzinger (16) and recrystallized several times from benzene until the needles were colorless, m.p. 81°.

NADPH, cytochrome *c*, oxidized glutathione, glutathione reductase, xanthine oxidase, and catalase were purchased from Boehringer, Mannheim, and *tert*-butyl peroxide from Elektrochemische Werke, München; all other reagents were obtained from Merck, Darmstadt.

RESULTS

Formation of ferrihemoglobin by hydrogen peroxide. The protection afforded to hemoglobin by catalase, glutathione peroxidase, and other factors in red cells against attack by hydrogen peroxide is shown by Table 1. Slow infusion of hydrogen peroxide into solutions of purified hemoglobin virtually free from catalase, glutathione peroxidase, and superoxide dismutase resulted in a more than 500-fold yield of ferrihemoglobin relative to infusion of hydrogen peroxide into hemolysate.

In kinetic studies of the reaction between hydrogen peroxide and hemoglobin, the initial rates of ferrihemoglobin formation were found to be proportional to the hemoglobin and hydrogen peroxide concentrations. Thus a second-order reaction may be assumed:

$$-\frac{d\,[HbFe^{2+}]}{dt} = k \times [HbFe^{2+}] \times [H_2O_2]$$

k being 125 M^{-1} sec^{-1} in 0.2 M phosphate, pH 7.4, at 37° in air.

Determinations of rates of ferrihemoglobin formation by hydrogen peroxide under various oxygen pressures showed that the rate was highest under nitrogen and that it decreased with increasing oxygen pressure. As may be seen in Fig. 1, the rate under nitrogen was nearly 30 times higher than the rate under 680 mm Hg of oxygen.

Formation of ferrihemoglobin by reduced glutathione. Addition of 10 mM reduced glutathione to a solution of purified human hemoglobin (1 mM iron) in 0.2 M phosphate and 2 mM EDTA, pH 7.4, at 37°

TABLE 1

Yield of ferrihemoglobin after infusion of hydrogen peroxide

Hydrogen peroxide was infused for 1 hr into a hemolysate of human red cells or a solution of purified human hemoglobin in 0.2 M phosphate, pH 7.4, under nitrogen at 37°. The concentration of Fe^{2+} in hemoglobin was 3 mM.

Preparation	H_2O_2 infused in 1 hr	Heme oxidized after 1 hr	Ratio of heme oxidized to H_2O_2 infused
	mole	*mole*	*moles/mole*
Hemolysate	9×10^{-2}	2×10^{-4}	0.0022
Purified hemoglobin	1.5×10^{-3}	1.8×10^{-3}	1.2

caused a marked increase in absorbance at 630 nm. After a lag phase of about 3 min, the rate of increase in absorbance corresponded to ferrihemoglobin formation of about 1 μM Fe^{3+} per second. Spectral analysis revealed that this increase was due not only to ferrihemoglobin but, to a lesser extent, to the formation of green hemoglobin derivatives.

Experiments with various concentrations of GSH at constant hemoglobin concentration showed that the rate of ferrihemoglobin formation is not proportional to the concentration of GSH but to its square (Fig. 2).

When the hemolysate was used in place of purified hemoglobin, addition of GSH did not cause formation of ferrihemoglobin. This result and the production of green hemoglobin derivatives in mixtures of purified hemoglobin and GSH pointed to hydrogen peroxide as a possible active intermediate. This role of hydrogen peroxide was confirmed by results of experiments in which catalase or glutathione peroxidase was added to mixtures of purified hemoglobin and GSH. Figures 3 and 4 show that catalase and glutathione peroxidase inhibited the formation of ferrihemoglobin by GSH. In each case the rate of ferrihemo-

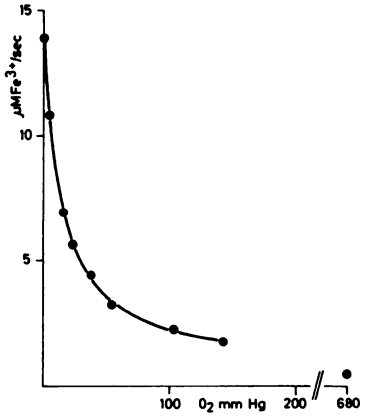

FIG. 1. *Effect of oxygen pressure on rate of oxidation of purified human hemoglobin (0.12 mM iron) by 0.12 mM hydrogen peroxide in 0.2 M phosphate, pH 7.4, at 37°*

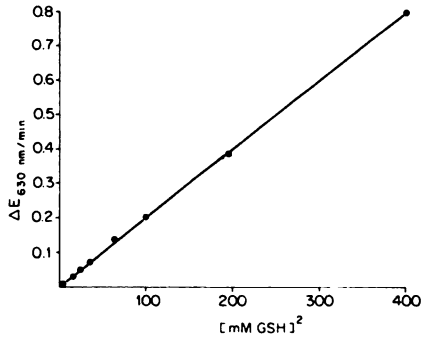

FIG. 2. *Dependence of rate of ferrihemoglobin formation on concentration of reduced glutathione*

Purified human hemoglobin (1 mM iron) in 0.2 M phosphate and 2 mM EDTA, pH 7.4, at 37° under air. The ordinate shows the maximal rate of increase in absorbance at various GSH concentrations.

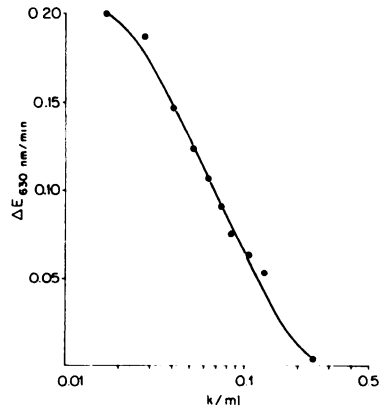

FIG. 3. *Effect of catalase on formation of ferrihemoglobin by reduced glutathione*

Purified human hemoglobin (1 mM iron), 10 mM GSH, in 0.2 M phosphate and 2 mM EDTA, pH 7.4, at 37° under air. The abscissa shows catalase concentration in *k* units per milliliter, and the ordinate, the maximal rate of increase in absorbance.

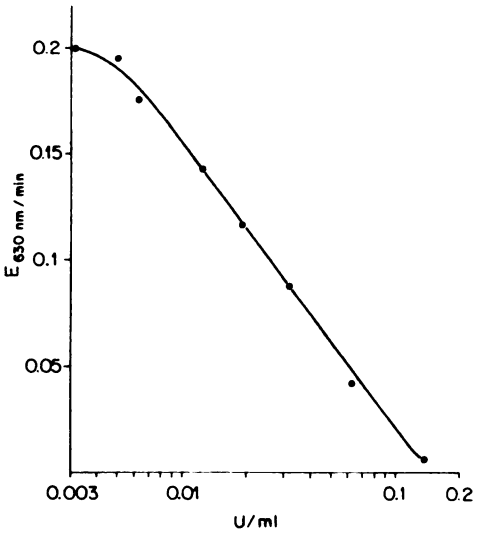

FIG. 4. *Effect of glutathione peroxidase on formation of ferrihemoglobin by reduced glutathione*
For experimental conditions, see Fig. 3.

globin formation decreased nearly proportionally to the logarithm of the enzyme concentration. Addition of superoxide dismutase, 600 units/ml of reaction mixture, did not affect the rate of ferrihemoglobin formation by GSH.

The rate of GSH oxidation in solution of GSH and hemoglobin was not diminished by the addition of catalase. When catalase, 0.2 *k*/ml, was added after the formation of ferrihemoglobin by GSH had gained full speed, the reaction stopped immediately.

If hydrogen peroxide were the active intermediate in the formation of ferrihemoglobin by GSH, the lag phase would reflect the buildup of a steady-state concentration of hydrogen peroxide. Then the lag phase should be eliminated by the addition of hydrogen peroxide, as demonstrated by Fig. 5. Addition of the same concentration of hydrogen peroxide to hemoglobin without GSH produced only little ferrihemoglobin.

In order to test whether hydrogen peroxide reacted directly with ferrohemoglobin or whether $OH^{\cdot}$ radicals were involved (17, 18), 2 mM ethanol was added as a $OH^{\cdot}$ radical scavenger (19) to mixtures of ferrohemoglobin and GSH, as described above. No effect on the kinetics of ferrihemoglobin formation was observed. The effect of oxygen pressure on the rate of ferrihemoglobin formation by GSH is shown in Fig. 6. The rate attained a maximum around the oxygen pressure of air and was about half as high under 1 atm of oxygen.

Formation of ferrihemoglobin by phenylhydroxylamine. The reaction of hemoglobin with phenylhydroxylamine produced ferrihemoglobin much more rapidly than the reaction with hydrogen peroxide. The

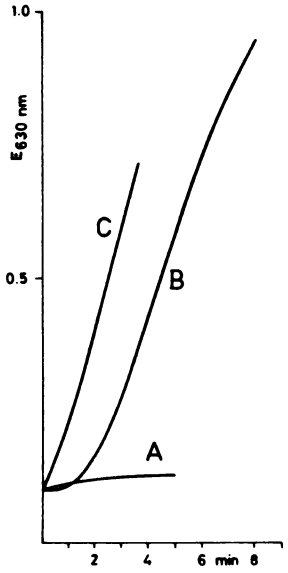

FIG. 5. *Formation of ferrihemoglobin by 10 μM hydrogen peroxide (A), 10 mM reduced glutathione (B), and 10 μM hydrogen peroxide plus 10 mM GSH (C)*
Purified human hemoglobin (1 mM iron) in 0.2 M phosphate and 2 mM EDTA, pH 7.4, at 37° under air

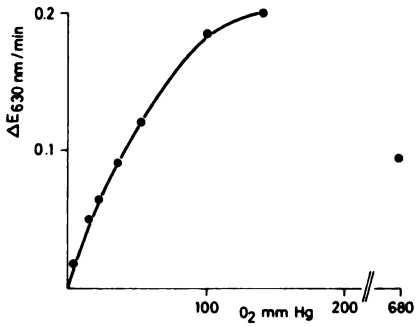

FIG. 6. *Dependence on oxygen pressure of rate of ferrihemoglobin formation by reduced glutathione*
Purified human hemoglobin (1 mM iron), 2 mM EDTA, and 10 mM GSH in 0.2 M phosphate, pH 7.4, at 37°.

high rate of reaction is illustrated in Fig. 7. This also shows that at constant hemoglobin concentration the reaction rate was proportional to the concentration of phenylhydroxylamine. Since at constant phenylhydroxylamine concentration the rate was also proportional to the hemoglobin concentration, a second-order reaction may be assumed:

$$-\frac{d\,[HbFe^{2+}]}{dt} = k \times [HbFe^{2+}] \times [\text{phenylhydroxylamine}]$$

k was calculated to be 2350 $M^{-1}\,sec^{-1}$ in 0.2 M phosphate, pH 7.4, at 37°.

Data presented in Fig. 8 show that the rate of ferrihemoglobin formation by phenylhydroxylamine with the hemolysate was virtually the same as with purified hemoglobin. Catalase added to purified hemoglobin in concentrations 10 times higher than found in the hemolysate did not diminish the reaction rate. The lack of a difference between hemolysate and purified hemoglobin in the rate of ferrihemoglobin formation by phenylhydroxylamine indicated that superoxide dismutase did not affect the reaction, since purified hemoglobin was also free from red cell

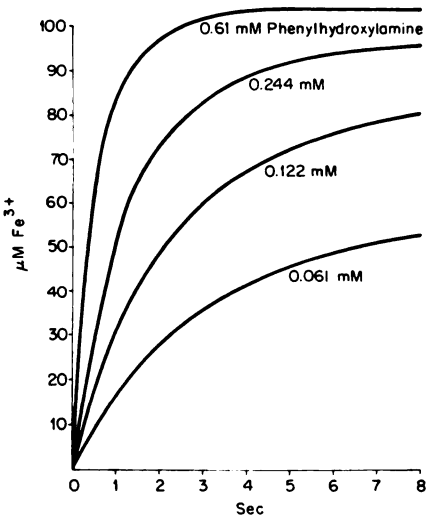

FIG. 7. *Formation of ferrihemoglobin by reaction of 0.061–0.61 mM phenylhydroxylamine and purified human hemoglobin (0.115 mM iron) in 0.2 M phosphate, pH 7.4, at 37°*

Catalase content was 0.45 *k*/g of hemoglobin; oxygen pressure, 550 mm Hg.

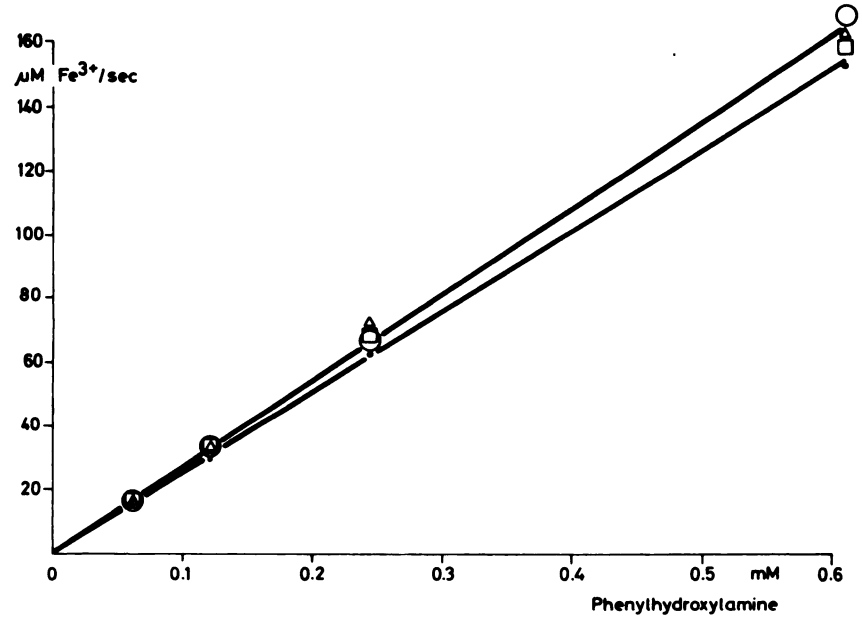

FIG. 8. *Formation of ferrihemoglobin by phenylhydroxylamine in the absence and presence of catalase*

Hemoglobin (0.116 mM iron) in 0.2 M phosphate, pH 7.4, at 37°. ●, hemolysate, catalase content about 300 *k*/g of hemoglobin; △, purified hemoglobin, catalase content 0.6 *k*/g; □, purified hemoglobin, catalase content more than 5.5 *k*/g; ○, purified hemoglobin plus catalase, final catalase content 3000 *k*/g.

superoxide dismutase. An extremely high concentration of the enzyme, 11,000 units/ml, changed the rate of ferrihemoglobin formation in a solution of human hemoglobin (0.112 mM Fe^{2+}) and 0.122 mM phenylhydroxylamine only from 33 to 29 μM Fe^{3+} per second.

Formation of ferrihemoglobin by 4-dimethylaminophenol. The formation of ferrihemoglobin by 4-dimethylaminophenol showed complicated kinetics which did not permit characterization of the reaction rate by a simple equation. As shown in Fig. 9, the reaction attained maximal velocity after a lag phase. Data in Fig. 9 also show that the maximal reaction rate was about half as high as the rate of ferrihemoglobin formation by an equimolar concentration of hydrogen peroxide. Addition of catalase, 3000 *k*/g of hemoglobin, or superoxide dismutase, 11,000 units/ml, did not affect the rate of ferrihemoglobin formation by 4-dimethylaminophenol.

The dependence on oxygen pressure of the rate of ferrihemoglobin formation by 4-dimethylaminophenol is shown in Fig. 10. The reaction rate was found to rise quickly with oxygen pressure to a maximum around 40 mm Hg. Further increase in oxygen pressure diminished the rate. Under an oxygen pressure of 1 atm the rate still amounted to 60% of the maximum.

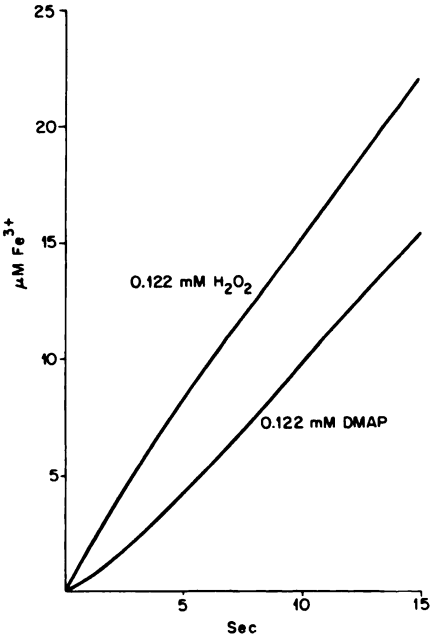

FIG. 9. *Formation of ferrihemoglobin by reaction of 4-dimethylaminophenol (DMAP) or H_2O_2 with purified human hemoglobin (0.115 mM iron) in 0.2 M phosphate, pH 7.4, at 37°*

The catalase content corresponded to 0.45 *k*/g of hemoglobin.

DISCUSSION

The results of our experiments demonstrate that the formation of ferrihemoglobin by GSH is due to hydrogen peroxide produced by autoxidation of GSH. Hydrogen peroxide produced ferrihemoglobin much more rapidly than GSH in equimolar concentration. The rate of ferrihemoglobin formation by GSH was proportional to the square of GSH concentration. The lag phase was abolished by addition of a suitable concentration of hydrogen peroxide. Catalase or glutathione peroxidase inhibited the formation of ferrihemoglobin by GSH. Thus the following sequence of reactions may be assumed:

$$2\ GSH + O_2 \rightarrow GSSG + H_2O_2$$

$$H_2O_2 + HbFe^{2+} \rightarrow HbFe^{3+}$$

Formation of hydrogen peroxide by autoxidation of GSH was demonstrated earlier (20, 21).

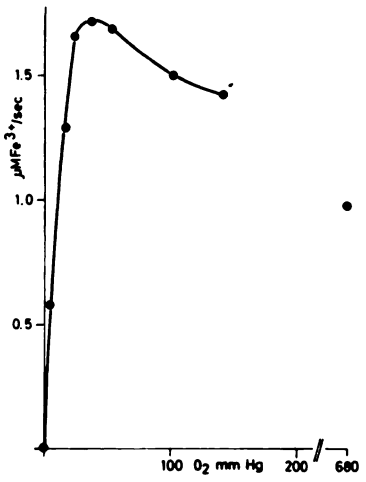

FIG. 10. *Dependence of rate of ferrihemoglobin formation by 4-dimethylaminophenol on oxygen pressure*

Purified human hemoglobin (0.1 mM iron) and 0.1 mM 4-dimethylaminophenol in 0.2 M phosphate, pH 7.4, at 37°.

Our results for ferrihemoglobin formation in the presence of GSH cannot explain the mechanism of hydrogen peroxide formation. The equations given above present over-all reactions. The occurrence of O_2^- radicals during autoxidation of GSH, which Misra (22) has demonstrated at alkaline pH and in the absence of EDTA, cannot be excluded. This is of no importance, however, for the mechanism of ferrihemoglobin formation, as Misra (23) and our own unpublished experiments have shown that O_2^- does not form ferrihemoglobin. The participation of OH radicals in the formation of ferrihemoglobin by GSH has been ruled out by the failure of the OH radical scavenger ethanol to affect the kinetics of ferrihemoglobin formation.

The kinetics of ferrihemoglobin formation by GSH differs from the reactions of the other reducing agents studied with respect to the optimal oxygen pressure. The reason why high oxygen pressure is needed for a maximal rate of ferrihemoglobin formation by GSH is not yet known.

The kinetics of the oxidation of hemoglobin by hydrogen peroxide points to complicated reaction mechanisms. Our results and data published by Aebi and Suter (24) show that hydrogen peroxide may oxidize more than 1 Eq of ferrohemoglobin. Since the rate of oxidation of ferrohemoglobin by hydrogen peroxide follows second-order kinetics a 1-electron transfer reaction is rate-limiting. Furthermore, there are side reactions, as shown by the formation of green precipitates during infusion of hydrogen peroxide into hemoglobin solutions.

The formation of ferrihemoglobin by GSH via hydrogen peroxide and not by a mechanism which is observed with other reducing agents is of biological importance. Thus catalase and glutathione peroxidase can prevent the formation of ferrihemoglobin by GSH in red cells. Hydrogen peroxide is not an active intermediate of any significance in the formation of ferrihemoglobin by phenylhydroxylamine. This reaction is much faster than the formation of ferrihemoglobin by hydrogen peroxide, and it is not inhibited by catalase. The second-order kinetics of the reaction points to the reaction between oxyhemoglobin and phenylhydroxylamine as the rate-limiting step. It is uncertain whether the impact with phenylhydroxylamine leaves the oxyhemoglobin behind as ferrihemoglobin. But this impact must produce some reactive oxidant (radical) which quickly reacts with deoxyferrohemoglobin. Such a reaction has to be assumed, because the formation of ferrihemoglobin by phenylhydroxylamine shows its maximal rate when the hemoglobin is not yet fully saturated with oxygen (25). The failure of superoxide dismutase to inhibit ferrihemoglobin formation by phenylhydroxylamine indicates that the reactive intermediate is not the superoxide ion.

The mechanism of ferrihemoglobin formation by 4-dimethylaminophenol does not involve hydrogen peroxide either. The rate of the reaction, although it is only about half as rapid as ferrihemoglobin formation by hydrogen peroxide, excludes hydrogen peroxide as a major active intermediate. The failure of catalase to inhibit the reaction confirms this conclusion. Experiments with superoxide dismutase showed that superoxide ion is not involved in the formation of ferrihemoglobin either. A scheme of the reactions that may occur in solutions of hemoglobin and 4-dimethylaminophenol in the presence of oxygen, published earlier (26), has to be corrected correspondingly. The lag phase in the formation of ferrihemoglobin and the maximal rate at low oxygen pressure indicate that an active intermediate(s) other than hydrogen peroxide is produced by the reaction of 4-dimethylaminophenol with oxyhemoglobin and reacts with deoxyferrohemoglobin. This confirms conclusions from results of earlier experiments (26), that oxidation products of 4-dimethylaminophenol which oxidize deoxyferrohemoglobin play an important role in the formation of ferrihemoglobin by 4-dimethylaminophenol.

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