Bovine erythrocyte green hemoprotein was shown to undergo rapid and complete bleaching during aerobic reduction by dithionite. In order to explain this phenomenon, we have studied the reactions of the ferrihemoprotein with dithionite and \( \text{H}_2\text{O}_2 \) and the reaction of the ferrihemoprotein with \( \text{H}_2\text{O}_2 \) and \( \text{O}_2 \). Bleaching caused by aerobic dithionite was shown not to involve either superoxide or bisulfitic anions. Inhibition by the ligands CO and pyridine suggested that a free heme site was necessary for bleaching and that the ferrous hemoprotein may be involved. Catalase strongly inhibits this bleaching, implying that \( \text{H}_2\text{O}_2 \) (produced in the aerobic dithionite solution) is involved. The ferrihemoprotein is bleached by \( \text{H}_2\text{O}_2 \) at pH 7.2, 25°C, in a reaction which involves a complex with \( \text{H}_2\text{O}_2 \) (\( K_2 = 1.3 \text{ mm} \)) that decays at a rate of 0.14 s\(^{-1}\). This reaction is inhibited by \( \text{CN}^- \) (\( K_1 = K_2 = 0.083 \text{ mm} \)). \( \text{H}_2\text{O}_2 \) reacts more rapidly with ferrohemoprotein; within 5 s, 35 to 50% of the hemoprotein is bleached and the remainder is oxidized to ferrihemoprotein. The anaerobic reduction of ferrihemoprotein by dithionite is very fast. These findings lead to the conclusion that in the aerobic bleaching reaction dithionite both generates \( \text{H}_2\text{O}_2 \) by reaction with \( \text{O}_2 \) and maintains the heme in the more reactive ferrous state. The ferrohemoprotein reacts rapidly with \( \text{O}_2 \) to form a transient complex \( (K_0 = 8.7 \times 10^{-5} \text{ M}) \) which decays to the ferrihemoprotein at a rate of 501 s\(^{-1}\) at 5°C. This oxidation is accompanied by a loss of 13 to 20% of the original absorbance of the ferrihemoprotein. Like green hemoprotein, cytochrome P-450 is bleached by \( \text{H}_2\text{O}_2 \), but at a much slower rate; ferrocytochrome P-450 reacts more rapidly than does ferricytochrome P-450.

The mechanism for the bleaching is envisioned to be similar to the peroxidatic cycle of Yokota and Yama- zaki (1965) *Biochim. Biophys. Acta* 105, 301) with the important difference that the addition of reducing equivalents to Compounds I and II of the peroxidatic cycle has been replaced by the loss of \( \text{HO}^+ \) radicals. We postulate that \( \text{HO}^+ \) generated by such a mechanism attacks, and thereby bleaches, the porphyrin ring.

Novel green hemoproteins have been isolated from human (1–3) and, more recently, from bovine (4, 5) erythrocytes. The bovine hemoproteins exist in two forms with electrophoretic, immunological, and gel exclusion properties indistinguishable from one another. Form I and Form II are separable upon chromatography on DEAE-Sephadex and, as a result of possessing different hemin prosthetic groups, are spectrally distinguishable. Each of the two forms exists as a monomer in solution, with one hemin per monomer. Each form undergoes ligand exchanges accompanied by spectral changes as would be expected for a hemoprotein possessing one strong-field ligand and an exchangeable weak-field ligand. From spectral, electrochemical, and ligand-binding experiments it has been concluded that the two forms apparently possess identical protein moieties but different hemin prosthetic groups which are unique to these hemoproteins.

Spectral characterization of the green hemoproteins has been hampered by their marked instability toward both oxidizing and reducing agents. Most notable was the inability to obtain a spectrum of the human ferrohemoprotein by the usual method of addition of sodium dithionite to an aerobic solution of the ferrihemoprotein (6). The same is true for the bovine hemoprotein. Inasmuch as the introduction of dithionite to an aerobic solution of the ferrihemoprotein resulted in an immediate disappearance of the Soret band of the spectrum, it would appear that the reagent destroyed the conjugation of the heme rather than just reducing the ferric iron.

Complications arising from the use of sodium dithionite as a reducing agent have long been observed. Sodium dithionite is unstable in an aerobic aqueous environment; hydrogen peroxide and bisulfitic are produced (7) which may then react with various hemoproteins to form unstable intermediates. Hemoglobin, for example, reacts with hydrogen peroxide to form choleglobin (7–10), a product in which the porphyrin ring has apparently been degraded by oxidative cleavage at the methyne bridge carbon(s). The mechanisms proposed to describe these oxidative attacks have generally been based (11–18) on the mechanism of Fenton’s reagent (19), a hydroxylating system which generates the hydroxyl radical, \( \text{HO}^- \), from the reaction of iron, a reducing agent such as ascorbate, and \( \text{H}_2\text{O}_2 \).

In this paper we describe reactions of Form I of the bovine erythrocyte green hemoprotein with oxygen, sodium dithionite, and hydrogen peroxide in order to ascertain the mechanism of the bleaching caused by dithionite. We find that the possible explanation for the bleaching of the green hemoprotein is to be found in the context of a peroxidatic cycle.1

**MATERIALS AND METHODS**

The green hemoprotein (Form I) and superoxide dismutase were isolated from bovine erythrocytes as described previously (4). Superoxide dismutase was assayed (20) by its ability to inhibit the catalysis of nitro blue tetrazolium reduction by xanthine oxidase. One-tenth

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1 Some of these findings have been presented previously in abstract form (L. J. DeFilippi, and D. P. Ballou (1976) *Fed. Proc. 35*, 1383).
microliter of superoxide dismutase stock solution (approximately 0.3 mg of protein/ml) in the 3-ml assay system was found to inhibit the reduction by 75%.

Catalase was obtained from the soluble fraction of washed human erythrocytes as follows. Cells were lysed by the addition of 3 volumes of cold water. The supernatant fraction (80 ml) was adjusted to pH 6.0 with 10% acetic acid. Ethanol (95%) was added slowly to a final concentration of 25%; the temperature was decreased simultaneously from 0°C to −15°C. The precipitate obtained by centrifugation at −30°C was washed with a small amount of 55% ethanol at −30°C and then dissolved at 0°C in 0.1 M potassium phosphate buffer, pH 8.1. The dissolved precipitate was concentrated to 3.6 ml by pressure dialysis on an Amicon PM-10 membrane and then applied to a column (3.5 x 115 cm) of Bio-Gel P-100 equilibrated with 0.1 M potassium phosphate buffer, pH 8.1. The catalase eluted ahead of contaminating hemoproteins as a grey-green band. Catalase activity was measured by monitoring the disappearance of H$_2$O$_2$ at 240 nm and H$_2$O$_2$ (Merck) was quantitated spectrophotometrically using a molar extinction coefficient of 40.6 cm$^{-1}$ at 240 nm (21).

A purified sample of the LM$_1$ form of rabbit liver cytochrome P-450 (22) was a gift of the laboratory of Dr. Minor J. Coon of this department. The sample contained 14 nmol of P-450/mg of protein.

RESULTS AND DISCUSSION

Reaction of the Green Hemoprotein with Dithionite

Bleaching by Dithionite in the Presence of Oxygen—The addition of solid dithionite (final concentration of approximately 0.3 mM) to a 0.76 μM solution of ferrihemoprotein resulted in an immediate disappearance of the spectrum in the Soret region (see Curves A and D of Fig. 1). At greater protein concentrations, complete destruction did not occur. This suggests that at the higher protein concentrations the reactive components are either consumed before destruction is complete or are present in limiting quantities. However, in the absence of O$_2$, Na$_2$S$_2$O$_4$ reacted rapidly with the protein to yield the stable ferrous form as reported previously (5). Aeration of such an anaerobic solution caused destruction of the hemoprotein as long as any Na$_2$S$_2$O$_4$ was present. These results suggest that some reactive product of Na$_2$S$_2$O$_4$ and O$_2$ was causing destruction.

Noninvolvement of Bisulfite and Superoxide Anion—Bisulfite and superoxide are products of the reaction of dithionite and oxygen (7) and therefore were tested for their possible involvement in the bleaching reaction. Curve A of Fig. 1 depicts the Soret band of the ferric form of the hemoprotein. Curve B represents the Soret band immediately after adding the addition of 0.5 ml of 0.10 M NaHSO$_3$ and Curve C shows the spectrum 10 min later. Curves D and E show spectra of the bleached hemoprotein after the addition of a few grains of Na$_2$S$_2$O$_4$ (final concentration approximately 0.3 mM) to a solution identical with that shown in Curve A except that 10 μl and 100 μl of superoxide dismutase were included, respectively. A spectrum identical with that shown in Curve D was obtained upon addition of Na$_2$S$_2$O$_4$ in the absence of superoxide dismutase. Curves F and G show spectra of partially bleached hemoprotein after the addition of Na$_2$S$_2$O$_4$ to a solution identical with that shown in Curve A except that 400 units and 2000 units of catalase were included, respectively.

Involvement of Hydrogen Peroxide—The possible role of H$_2$O$_2$ (another product of the reaction of S$_2$O$_4^{2-}$ and O$_2$) was tested by including catalase in the reaction mixture. Curve A shows that 400 units of catalase/ml partially prevents bleaching (Curve F) and that 2000 units/ml results in 85 to 90% bleaching (Curve G). Thus, H$_2$O$_2$ produced by the reaction of S$_2$O$_4^{2-}$ with O$_2$ is an essential component of the bleaching phenomenon.

Requirements for Dithionite—A diluted (approximately 0.3 mM) solution of Na$_2$S$_2$O$_4$, which had been allowed to react completely with O$_2$ was added to the hemoprotein to test whether the products of such a reaction could cause bleaching. No rapid bleaching was observed. Thus, bleaching requires the presence of O$_2$.
combination of $S_2O_4^-$ and $H_2O_2$, or else a short lived intermediate formed upon reaction of $S_2O_4^-$ with $O_2$. The experiments described in the following section indicate that the first alternative is correct.

Protection from Dithionite Bleaching by Carbon Monoxide and Pyridine/Alkali—In order to ascertain that the $H_2O_2$ (or other reactive species generated in the presence of $O_2 + Na_2S_2O_4$) must bind to the heme iron prior to the bleaching of the heme, the protective effects of the iron ligands, CO and pyridine, were investigated. The protein was diluted into 1 part oxygen-saturated buffer plus 4 parts CO-saturated buffer (i.e. the same $O_2$ concentration in buffers saturated with air). Upon addition of $Na_2S_2O_4$ to this solution no bleaching occurred and the typical CO-reduced spectrum was obtained (Curve B, Fig. 2). Irradiation of this closed cuvette with visible light for 5 min resulted in a drop in absorbance to Curve C. When the cuvette was left in the dark for 10 min the spectrum did not change. An additional 15 min of irradiation yielded a further decrease in absorbance (Curve D). Aeration of the cuvette for 30 s gave an oxidized spectrum (Curve E) which exhibited approximately two-thirds of the absorbance at the Soret of the original oxidized hemoprotein (Curve A). Irradiation of the ferric protein (which had not been treated with $Na_2S_2O_4$) either in the presence or absence of CO did not result in any change in the spectrum.

These data show that the bleaching effect due to $S_2O_4^-$ and $O_2$ is inhibited by binding of CO to the iron of the ferrohemoprotein. Light irradiation reverses this inhibition, presumably by photodissociating the CO from its site on the iron. CO is known to be easily photodissociated from hemoproteins such as hemoglobin (24). The above irradiation experiment also shows that the bleaching was not caused by reaction of the hemoprotein with a short lived intermediate of the $S_2O_4^- + O_2$ reaction, since removal of CO by photo-irradiation 15 min after mixing $Na_2S_2O_4$ and $O_2$ still resulted in hemoprotein bleaching.

Pyridine was also shown to inhibit the bleaching reaction. When pyridine (20%) plus aqueous NaOH (final concentration of 0.05 M) were added to a dilute, aerobic solution of the protein prior to the addition of sodium dithionite, the reduced pyridine hemochrome spectrum (25) was observed. However, if the sequence of additions was reversed, no hemochrome of any kind was obtained, demonstrating that the porphyrin ring had been destroyed. When the hemoprotein solution was made alkaline without pyridine, the addition of solid Na$_2$S$_2$O$_4$ resulted not in bleeding, but rather in approximately 30% conversion of the heme to a pentydopent-like compound as visualized by the subsequent addition of pyridine. Pentydonpent (so called because of its absorption band at 525 nm) is a degradation product formed by oxidation of two opposite bridge carbon atoms of hemin (26, 27).

These data show that when the heme of the denatured hemoprotein forms a complex with the strong field ligand, pyridine, bleaching is almost completely inhibited. Only partial degradation occurs in alkaline solution; under these conditions one would expect the heme to be bound to a hydroxide ion, which is a weak field, but tightly binding ligand. Extensive degradation in neutral aqueous solution indicates that $H_2O_2$ a loosely bound and weak field ligand, offers no protection.

The free heme (isolated by polyacrylamide gel electrophoresis in the presence of cyanide (28)) is also bleached by $Na_2S_2O_4$ under aerobic conditions when water is the only ligand present. As was found in the presence of the alkali-denatured protein, pyridine prevents the bleaching.

These observations support the contention that the protection offered by CO or pyridine is effected by liganding the heme iron rather than by changing the conformation of the protein. The data also allow us to conclude that $Na_2S_2O_4$ bleaches the heme by both generating $H_2O_2$ and maintaining the heme iron in the reduced state.

Reaction of Ferrohemoprotein with $H_2O_2$

The ferrohemoprotein was found to be rapidly bleached by $H_2O_2$. Under anaerobic conditions and in the presence of an excess of dithionite, a low concentration of $H_2O_2$ (86 $\mu$M) caused complete bleaching of the hemoprotein by the time a spectrum could be recorded (approximately 25 s). When this experiment was repeated under an anaerobic CO atmosphere, the ferrohemoprotein-CO complex was quite stable to the $H_2O_2$. After 30 min only 10% of the absorbance at the Soret peak had disappeared and there was a small red-shift (4 nm) of its maximum.

To examine whether $Na_2S_2O_4$ participated in this bleaching reaction, the reaction was carried out in the absence of dithionite with ferrohemoprotein prepared by photochemical reduction (Fig. 3). The ferrihemoprotein (Curve A) was photochemically reduced (29) with lumiflavin-3-acetate, EDTA, and light under anaerobic conditions (Curve B). Addition of $H_2O_2$ (final concentration of 86 $\mu$M) resulted in rapid bleaching of 35 to 50% of the hemoprotein and conversion of the remainder to the ferric form (Curve C). These reactions occurred within the 25-s period needed to monitor the Soret peak and were followed by a slower bleaching of the ferrihemoprotein (Curves D to J) at a rate identical with that obtained (vide infra) in the reaction of ferrihemoprotein with $H_2O_2$. Since 35 to 50% of the protein is bleached rapidly, it is apparent that
Ferrihemoprotein + H2O2 \rightarrow \text{intermediate} \rightarrow \text{product(s)} \tag{1}

Assuming that $k_{-1} \gg k_2$, the dependence of the pseudo-first order rate constant ($k_{obs}$) on bleaching on the concentration of H2O2 should be given by Equation 2 (30, 31) where $K_{D,H_2O_2}$ is the dissociation constant of the hemoprotein-H2O2 intermediate:

$$
\frac{1}{k_{obs}} = \frac{K_{D,H_2O_2} + 1}{k_2}
$$

A plot of $1/k_{obs}$ versus $1/[\text{H}_2\text{O}_2]$ is shown in Fig. 4 (solid circles). Through regression analysis, a value of $0.14 \pm 0.03$ s$^{-1}$ for $k_2$ and a value of $1.32 \pm 0.15$ mM for $K_{D,H_2O_2}$ were obtained. The $y$ intercept was shown to be nonzero by the Student $t$ test which yielded a confidence limit of $>0.9995$.

The assumption that $k_{-1} \gg k_2$ can be tested. Preliminary studies indicate that $k_1$ is approximately 400 m$^{-1}$s$^{-1}$ at 25°C and pH 7. From the value of 1.3 mM for $K_D$, $k_1$ is calculated to be 0.52 s$^{-1}$. Thus, $k_{-1}$ is not much larger than $k_2$. Simulations of the reaction described by Equation 1 using $k_1 = 400$ m$^{-1}$s$^{-1}$ and $k_{-1} = 0.52$ s$^{-1}$ yield log plots which are linear in the time range observed in the above experiments. The open squares in Fig. 4 are derived from these simulations. It is clear that when $k_{-1}$ is not much greater than $k_2$, the estimate for $K_D$ obtained from such reciprocal plots will be larger than the true $K_D$. The open circles are the results of simulations with $k_1 = 400$ m$^{-1}$s$^{-1}$ and $k_{-1} = 0.4$ s$^{-1}$. The excellent agreement between the simulations and the data suggests that $K_D = 1$ mM.

**Inhibition by Cyanide**—To determine whether the bleaching is preceded by binding of hydrogen peroxide to the ferric heme iron, the ability of cyanide, a known heme-ligand (5), to compete with the H2O2 for the binding site was tested. The reversible reaction of the hemoprotein with cyanide is described by Equation 3:

$$
\text{Ferrihemoprotein} + \text{CN}^- \rightarrow \text{ferrihemoprotein} - \text{CN}^- \tag{3}
$$

where $k_{-2}/k_3 = K_{D,\text{H},\text{CN}}$ is the dissociation constant of the hemoprotein-cyanide complex. Assuming that CN$^-$ binds rigidly to the same site of the ferrihemoprotein as does H2O2 and that the hemoprotein-cyanide complex is not bleached by H2O2, then Equation 4 describes the kinetics of the reaction:
Plots of $1/k_{obs}$ versus $[CN^-]$ are shown in Fig. 5 for three concentrations of H$_2$O$_2$. These plots yield similar $K_{D,CN}$ ($82 \pm 14 \mu M$ at 0.93 mM H$_2$O$_2$; 102 $\pm$ 29 $\mu M$ at 1.84 mM H$_2$O$_2$; and 65 $\pm$ 9 $\mu M$ at 2.86 mM H$_2$O$_2$). The average of the $K_{D,CN}$ values, 83 $\pm$ 13 $\mu M$, is very similar to the value of 84 $\pm$ 3 $\mu M$ that was obtained for the dissociation constant of green hemoprotein Form I from a static titration with cyanide (5). Thus it would appear that CN$^-$ and H$_2$O$_2$ compete for the same ligand site on the ferrihemoprotein and that this specific binding of H$_2$O$_2$ is required for the bleaching reaction which occurs when H$_2$O$_2$ is added to ferrihemoprotein.

Reaction of Ferrohemoprotein with O$_2$

Since one of the reactants in the aerobic reduction of the hemoprotein by Na$_2$S$_2$O$_4$ was O$_2$, we studied the reaction between ferrohemoprotein and O$_2$. The protein was reduced quantitatively with Na$_2$S$_2$O$_4$ under anaerobic N$_2$ and then was rapidly mixed with a solution of air-saturated buffer. The reaction, as monitored by stopped flow at 25°C, was complete in the dead time of the instrument (approximately 3 ms). However, upon lowering the temperature to 5°C, the reaction could be observed and was found to obey pseudo-first order kinetics with respect to the ferrihemoprotein.

An inverse plot of $k_{obs}$ versus [O$_2$]$^{-1}$ (see Fig. 6) yielded a non-zero $y$ intercept, evidence for the formation of an intermediate complex (C) between the ferrohemoprotein and O$_2$; the complex breaks down to form ferrihemoprotein and, presumably, superoxide anion (Equation 5) in a reaction analogous to the autoxidations of P-450 (32) and hemoglobin (33).

\[
\frac{1}{k_{obs}} = \frac{K_{D,CN}[CN^-]}{k_1[H_2O_2]} + \frac{1}{k_2[H_2O_2]} \tag{4}
\]

From the inverse plot the following parameters were calculated: $k_0 = 501 \pm 45$ s$^{-1}$ and $K_{D,0_2} = 8.7 \pm 1.3 \times 10^{-8}$ M where $K_{D,0_2}$ the dissociation constant for the postulated ferrohemoprotein-O$_2$ complex, is equal to $k_4/k_5$.

After autoxidation of the quantitatively reduced hemoprotein only 80 to 87% of the original ferrihemoprotein was recovered as judged by the absorbance of the Soret band. A second cycle of reduction and autoxidation gave an additional loss of 13 to 20% of absorbance. Similar data were obtained when the autoxidation was performed with ferrohemoprotein which had been prepared by photochemical reduction or when 1300 units of catalase/ml were included to remove any H$_2$O$_2$ which would form from the spontaneous dismutation of O$_2$.

The bleaching which occurs during autoxidation may account for the inability of catalase to inhibit completely the bleaching observed when dithionite is added to an aerobic solution of the hemoprotein (see Fig. 1).

Reactivity of Cytochrome P-450 toward H$_2$O$_2$

Since cytochrome P-450 has been found to degrade in rat liver microsomes whenever lipid peroxidation is proceeding (34, 35) or during substrate hydroxylation by peroxides (36, 38), it appears that the active site of cytochrome P-450 is labile toward peroxides. It has also been noted that P-420, an inactive form of P-450, is labile in aerobic solutions of dithionite and that the lability is arrested by the inclusion of CO (39, 40).

To test the possibility that ferric P-450 was more labile than ferric P-450 toward H$_2$O$_2$, purified rabbit liver cytochrome P-450 (22) was treated with combinations of O$_2$, Na$_2$S$_2$O$_4$, and H$_2$O$_2$ under the conditions employed with the green hemoprotein. Ferrocytochrome P-450 (that had been prepared using an excess of Na$_2$S$_2$O$_4$) reacted immediately with 0.58 mM H$_2$O$_2$ in an anaerobic N$_2$ atmosphere to form the ferric state; this was followed by a slow bleaching reaction which followed first order kinetics, had a half-life of approximately 4 min, and resulted in an overall 40% decrease in absorbance at the Soret band. When the experiment was repeated under an anaerobic CO atmosphere, there was no apparent destruction of the heme. The only observed reaction was a slow spectral change which was accounted for by a conversion of P-450 to P-420. When the same amount of H$_2$O$_2$ was added to an aerobic solution of ferricytochrome P-450 there was a very slow drop in absorbance with a half-time of approximately 3 h.

Thus, cytochrome P-450 appears to undergo reactions parallel to those observed for the green hemoprotein, although at much slower rates. As described above for the green hemoprotein, the increased lability in the presence of Na$_2$S$_2$O$_4$
appears to result from repeated cycles of reduction by dithionite and a combination of bleaching and oxidation by H$_2$O$_2$. A difference between the reactivities of the two proteins may be the much slower reduction by dithionite of P-450 compared to green hemoprotein. Consequently, under these conditions P-450 remains principally in the less reactive ferric form.

Further Discussion—The studies presented here show that bleaching of green hemoprotein proceeds during the reactions of ferrihemoprotein with H$_2$O$_2$, ferrohemoprotein with O$_2$, and ferrohemoprotein with H$_2$O. The kinetic studies provide evidence that an intermediate complex is formed both in the reaction of ferrihemoprotein with H$_2$O$_2$ and in the reaction of ferrohemoprotein with O$_2$. Moreover, data to be presented in a subsequent paper provide evidence that ferrohemoprotein forms a spectrally detectable complex with H$_2$O$_2$.

We have constructed a speculative scheme, parallel to that envisioned for peroxidatic reactions (41–43), which can explain the oxidation-reduction reactions of green hemoprotein. Fig. 7 shows the peroxidatic scheme proposed by Yokota and Yamazaki (44, 45) and a similar scheme to describe the reactions of green hemoprotein. In the peroxidatic scheme (Plate a) the peroxidase first undergoes a two-step oxidation by H$_2$O$_2$ (a$_1$). This heterolytic cleavage of H$_2$O$_2$ yields H$_2$O and Compound I, which has a formal oxidation state of +3. To return the peroxidase to the +3 oxidation state it undergoes two 1-electron reductions, first (a$_2$) to Compound II (oxidation state of +4) and then (a$_3$) to the ferric peroxidase. Ferroperoxidase can react with O$_2$ (via a$_4$) to form Compound III (46, 47) which loses O$_2^-$ to form the ferric peroxidase (a$_5$). Ferroperoxidase can also react with H$_2$O$_2$ to form Compound II (47).

The green hemoprotein can be envisioned to interact with oxygen and peroxides in a parallel fashion (Plate b). The ferric form of the hemoprotein can be aerobically reduced (b$_1$) to the ferrohemoprotein and subsequently reacted with oxygen (b$_2$) to form a kinetically detectable intermediate (Compound III) which then rapidly re-forms (b$_3$) the ferrihemoprotein, presumably with formation of O$_2^-$, A small amount (approximately 15%) of the hemoprotein is bleached during this process. The bleaching is not due to reaction of free H$_2$O$_2$ since catalase has no effect on the bleaching of ferrohemoprotein with O$_2$. The bleaching may occur through loss of H$_2$O$_2$ from Compound III (via b$_4$) to form Compound II which can self-destruct as will be described later.

The observation that reaction of ferrohemoprotein with H$_2$O$_2$ yields ferrihemoprotein and colorless degradation products is compatible with the reaction sequence (b$_5$) and (b$_6$): the ferrohemoprotein would first react with H$_2$O$_2$ to form a Compound II-like intermediate which would then break down to the ferrihemoprotein. We have preliminary stopped flow evidence for saturation of this reaction with higher H$_2$O$_2$ concentrations, suggesting that an intermediate has been formed. Assuming that the ferrihemoprotein is derived from the ferrohemoprotein in a single turnover reaction, the approximately 50% destruction of the hem suggests that the actual agent of destruction is highly reactive.

The direct reaction of ferrohemoprotein with H$_2$O$_2$ (b$_7$) has been shown to yield an intermediate (Complex I). This intermediate is postulated to have the same oxidation state as peroxidase Compound I but, in contrast to Complex I, it would contain both of the oxygen atoms derived from H$_2$O$_2$. Complex I degradation to ferrihemoprotein is envisioned as proceeding through the intermediate formation of Compound II (Reactions b$_7$ and b$_8$). The observed inhibition of this pathway by cyanide would occur by direct binding of the cyanide to the ferrihemoprotein (b$_9$). Likewise, the observed CO-inhibition of bleaching of ferrohemoprotein with H$_2$O$_2$ would occur by direct binding of CO to the ferrohemoprotein (b$_{10}$).

The peroxidatic-type mechanism provides an explanation for the bleaching phenomenon within the framework of a relatively well understood series of reactions if we modify the scheme with the following postulation: since no reducing equivalents are added, the addition of reducing equivalents to Compounds I and II of peroxidase is replaced by the loss of oxidizing equivalents. These oxidizing equivalents would be formally identical with hydroxyl radicals. The peroxidatic scheme results in the heterolytic cleavage of H$_2$O$_2$ whereas the green hemoprotein is postulated to cleave H$_2$O$_2$ homolytically.

The bleaching of the ferrous and ferric forms of the hemoprotein upon interaction with H$_2$O$_2$ and of the ferrous hemo-

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*Fig. 7. Plate a, Yokota-Yamazaki scheme (44) for peroxidatic cycle. Plate b, parallel mechanism proposed for interaction of the green hemoproteins with H$_2$O$_2$. The Yokota-Yamazaki scheme shows the relationship between horseradish peroxidase derivatives which appear during the peroxidase-oxidase reaction. Numbers in parentheses show the effective oxidation level. The symbol "e" represents an electron obtained from a reducing agent such as ascorbate or dihydroxyfumarate. The second scheme tentatively explains the reactions that the green hemoprotein undergoes in the presence of H$_2$O$_2$, CN, CO, O$_2$, and reducing agents. H$^+$ is included simply to express the pH dependence of the reaction (the rate of bleaching decreased to 20% as the pH was decreased from 8.9 to 3.65). The formation of Compound I in Reaction b$_1$, can be considered to be the same as described by Equation 1 in the text, Reaction b$_{10}$ by Equation 3, and Reactions b$_2$, b$_3$, and b$_5$ by Equation 5. The HO$^-$ radicals postulated to be formed in Reactions b$_2$ and b$_5$ are thought to be the species which bleaches the hemoprotein. This bleaching is not shown here. It is not assumed that the HO$^-$ is actually free of the hemoprotein.

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1) P. Rallou, J. J. DeFilippi, and D. E. Hultquist, unpublished results.
incorporation of this concept into the framework of the generally accepted mechanism of peroxidase action generalizes radicals in similar reactions is not new (14-18). However, reaction proceeds in the absence of protein. The possibility that the apoprotein serves as an electron donor the other when Compound II is converted to ferrihemoprotein. H2O2 is postulated to produce two hydroxyl radicals; one is would be expected to be converted to ferrihemoprotein principally by direct loss of O2-. Reaction of ferrihemoprotein with H2O2 is postulated to produce two hydroxyl radicals; one is produced when Compound I is converted to Compound II and the other when Compound II is converted to ferrihemoprotein. The possibility that the apoprotein serves as an electron donor substrate could result in accelerated degradation of the enzyme prosthetic group in a self-destructing fashion.

A mechanism which proposes the formation of hydroxyl radicals in similar reactions is not new (14-18). However, incorporation of this concept into the framework of the generally accepted mechanism of peroxidase action generalizes the peroxidase scheme. This mechanism is consistent with proposals (see, for example, O’Carra (48)) that “activated oxygen” associated with various hemoproteins periodically and accidentally hydroxylates its own heme group. One could conceivably extend this to include control mechanisms for inactivation of enzymes whose substrates are not present. In this case, the absence of a suitable electron donor substrate could result in accelerated degradation of the enzyme prosthetic group in a self-destructing fashion.

Acknowledgements—We wish to thank Dr. Minor J. Coon for the use of various facilities in his laboratory and Dr. Vincent Massey for helpful discussions concerning this work and for the use of his laboratory. We thank Mr. Narlin Beatty for assistance in the design and implementation of the computer programming of the simulations.

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