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# Ligands and oxidants in ferrihemochrome formation and oxidative hemolysis*

(arylhydrazine/aryldiazene/hemoglobin/oxidative denaturation/Heinz body anemia)

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ABSTRACT **We have investigated the effect of size of a single neutral ring substituent on the induction of hemolytic anemia and the formation of a ferrihemochrome by substituted phenylhydrazines. The severity of induced anemia decreased with increase in size of a halogen atom or an alkyl group *ortho* to the hydrazino group, little anemia resulting from 2-iodophenylhydrazine and no anemia from 2-*tert*-butylphenylhydrazine. The size of a halogen atom or an alkyl group at the *meta* or *para* position had relatively little effect on the severity of induced anemia. The ability of an arylhydrazine to induce hemolytic anemia paralleled its ability to produce a ferrihemochrome with an exogenous ligand, probably the corresponding aryldiazene. In general, rapid and complete formation of ferrihemochrome occurred with arylhydrazines that induced severe anemia. The degree of hemolysis induced by an arylhydrazine was not related to its rate of autooxidation, i.e., the rate at which oxidants are formed by the reduction of oxygen. We propose a mechanism of arylhydrazine-induced oxidative denaturation based on the simultaneous formation of hydroxyl radical and aryldiazene ferrihemochrome in a reaction of oxyhemoglobin with arylhydrazine. We suggest that after oxidation of the porphyrin ring is initiated by a hydroxyl radical, oxidative cleavage of the ring is facilitated by the presence of a large ligand in the heme crevice. Thus, aryldiazene ferrihemochrome may contribute to instability in a hemoglobin molecule, whereas globin ferrihemochrome results from instability.**

Oxidative denaturation of hemoglobin is a process in which oxidative changes in the molecule cause its disruption and precipitation. This process takes place when the concentration of oxidants in a red cell is increased, when the ability of the red cell to dispose of oxidants is decreased, or when the resistance of hemoglobin to oxidation is impaired (1). The intracellular aggregates of denatured hemoglobin produced when oxidative denaturation occurs in the red cell are called Heinz bodies (2). Heinz bodies result from the action of a large number of drugs and other chemicals (3, 4) and from inherited amino acid substitutions that cause instability of the hemoglobin molecule and diminish its resistance to oxidative changes (5, 6). Inasmuch as the precipitation of hemoglobin within a red cell sets off a series of events that lead to a premature destruction of the cell (7–9), the formation of Heinz bodies is directly associated with the induction of hemolytic anemia.

Phenylhydrazine is the most effective chemical inducer of Heinz body hemolytic anemia, but ring-substituted phenylhydrazines differ in effectiveness according to the position, number, and charge of substituents (10, 11). The nature of its ring substitutions also determines the ability of an arylhydrazine to produce an exogenously liganded ferrihemochrome in the presence of an oxidant (12, 13). The present work is a systematic investigation of the effect of differences in size of a single uncharged substituent both on the induction of hemolytic anemia and on the formation of ferrihemochrome. The results are consistent with our suggestion that oxidative denaturation may be enhanced when an exogenous ligand produced by the oxidation of an arylhydrazine is bound to ferrihemoglobin (12).

A majority of the compounds referred to as oxidant compounds in oxidative hemolysis are reducing agents that do not react with normal hemoglobin in the absence of oxygen to produce Heinz bodies. Active oxidants capable of reacting rapidly with components of the hemoglobin molecule result from the reduction of oxygen by these compounds or their metabolic products (14). The rates of reaction of ring-substituted phenylhydrazines with oxygen (autooxidation) were shown by Stroh and Ebert (15) to depend on the nature of their ring substitutions. We have tested most of the compounds from their study for effectiveness in inducing hemolytic anemia and in producing a ferrihemochrome with an exogenous ligand.

## METHODS

Substituted phenylhydrazines were obtained from commercial sources or were synthesized as described (16) from the corresponding substituted anilines, except that the hydrochloride of ethyl 2-hydrazinobenzoate was synthesized from ethyl anthranilate (17). Each compound was purified by recrystallization from 2 M HCl or ethanol. Rabbits were injected with these compounds, and hematological data were obtained as described (11). Between two and six animals were studied with each compound. Arylhydrazines were added to ferrihemoglobin in the presence of potassium ferricyanide to obtain the absorption spectra of ferrihemochromes in the Cary model 17 recording spectrophotometer (12, 13).

## RESULTS

**Induction of Hemolytic Anemia.** Control animals injected with physiological saline did not develop anemia or reticulocytosis, while animals injected with unsubstituted phenylhydrazine developed severe hemolytic anemia. The effect on hemolytic activity of a halogen atom or an alkyl group at the *ortho* (2–), *meta* (3–), or *para* (4–) position of the benzene ring of phenylhydrazine was determined. The severity of hemolytic anemia induced by a halophenylhydrazine (Fig. 1) or an alkylphenylhydrazine (Fig. 2) was related to the size and position of its ring substituent. Compounds with small substituents induced severe hemolysis irrespective of the position of the sub-

Abbreviations: $HbO_2$, $Hb^+$, HbOH, and HbOOH are subunits of oxyhemoglobin, ferrihemoglobin, ferrihemoglobin hydroxide, and ferrihemoglobin peroxide, respectively; Ar, aryl; Ph, phenyl.

* Use of the term ferrihemochrome instead of hemichrome conforms to the recommentation (1964) of the International Union of Biochemistry (1965) in *Enzyme Nomenclature*, (Elsevier Publishing Company, Amsterdam), p. 19. The compound formed in the reaction of an aryldiazene with ferrihemoglobin is designated a ferrihemochrome because its visible absorption spectrum is typical of the latter type of compound.

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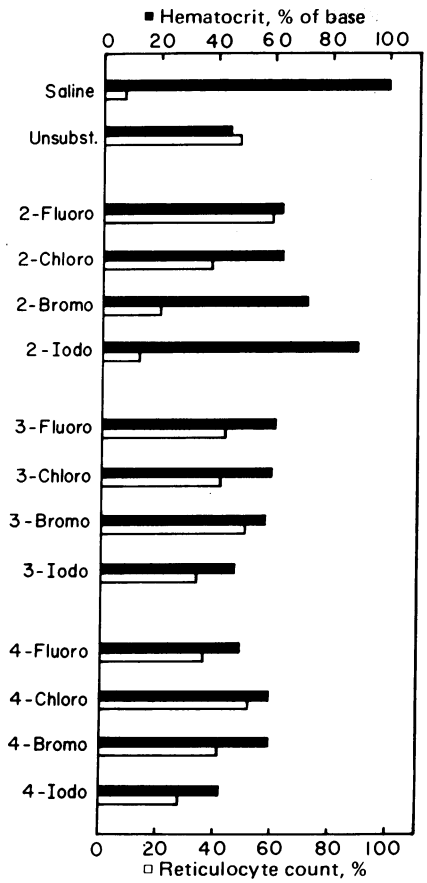


FIG. 1. Relative hematocrits and reticulocyte counts in rabbits injected with emulsions of halophenylhydrazines in sodium alginate. A baseline sample of blood was taken just before the first injection of 52 μmol of arylhydrazine per kg of body weight. The same dose was given on each of the three following days. The relative hematocrit is 100 times the ratio of the hematocrit on the day after the final injection to the hematocrit of the baseline sample. The reticulocyte count was taken on the day after the last injection. Mean values are shown.

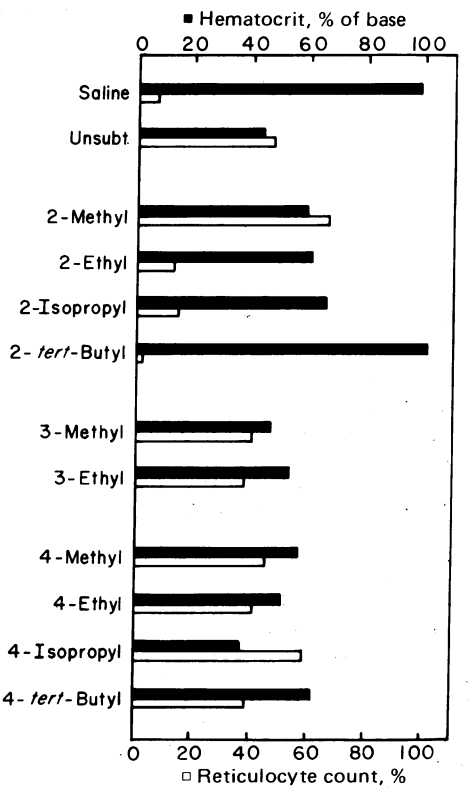


FIG. 2. Relative hematocrits and reticulocyte counts in rabbits injected with alkylphenylhydrazines as in Fig. 1.

stituent on the benzene ring. Compounds with larger substituents also induced severe hemolysis when the *meta* or *para* position was occupied; however, progressive decrease in hemolysis was associated with increase in size of a substituent at the *ortho* position; 2-iodophenylhydrazine induced the least hemolysis among the halogen compounds, and 2-*tert*-butylphenylhydrazine induced no hemolysis. The latter compound is the first substituted phenylhydrazine with a single neutral substituent found not to have hemolytic activity.

Induction of anemia and reticulocytosis by various substituted phenylhydrazines for which rates of autooxidation were determined by Stroh and Ebert (15) is shown in Table 1. Results from a previous report (11) and from Figs. 1 and 2 have been included for the purpose of direct comparison with autooxidation rates. No correlation between rate of autooxidation and severity of induced anemia is evident. Among the bromophenylhydrazines, the *meta* isomer was autooxidized at the lowest rate, but the *ortho* isomer induced the least hemolytic anemia. Disubstituted chloro and bromo compounds were autooxidized less rapidly and also resulted in less anemia than the respective monosubstituted compounds; on the other hand, 2,4-dimethylphenylhydrazine was autooxidized more rapidly but induced less anemia than any of the monomethyl compounds. The ethyl ester of 4-hydrazinobenzoic acid induced moderate hemolytic anemia; the anion of this acid, which was autooxidized three times as rapidly, induced no anemia. The anemia induced by the rapidly autooxidized 2-methoxy and 4-methoxy compounds was moderate.

**Formation of Ferrihemochrome.** The reaction of a ferricyanide-oxidized halophenylhydrazine or alkylphenylhydrazine with ferrihemoglobin to produce a compound with the absorption spectrum of a ferrihemochrome depended upon its ring substitution. Most of these compounds produced stable ferrihemochrome spectra rapidly, but a few yielded unstable spectra that began to revert to that of ferrihemoglobin within 5 min. The latter behavior was shown by 2-iodophenylhydrazine, ethyl 2-hydrazinobenzoate, and all of the 2-alkylphenylhydrazines except 2-*tert*-butylphenylhydrazine, which showed slow and partial formation of ferrihemochrome. Fig. 3 shows results from rapid, complete reactions in which the recorded spectra showed no significant change between 5 and 10 min after each of the three isomers of chlorophenylhydrazine was added to ferrihemoglobin in the presence of ferricyanide. The appearance of a different spectrum with each isomer is consistent with the binding of exogenous ligands that include the respective chlorophenyl groups. Fig. 4 shows the effect of charge on ferrihemochrome formation—slow and incomplete with the anion of 4-hydrazinobenzoic acid but moderately rapid and nearly complete after 10 min with the ethyl ester.

The formation of ferrihemochromes by ferricyanide-oxidized substituted phenylhydrazines for which rates of autooxidation were known have been summarized in Table 1. Compounds that produced stable ferrihemochromes rapidly and completely induced moderate to severe hemolytic anemia. With the exception of 2-methylphenylhydrazine, which induced a severe anemia, compounds with unstable ferrihemochromes or incompletely formed ferrihemochromes induced mild anemia or no anemia.

Table 1. Induction of hemolytic anemia, reaction with oxygen, and formation of ferrihemochrome by ring-substituted phenylhydrazines

| Phenylhydrazine | Anemia* Rel. hct. | Anemia* Retic. ct. | $v_0 \times 10^6$ † | Ferrihemochrome spectrum‡ |
|---|---|---|---|---|
| Unsubstituted | 47 | 48.7 | 66 | Stable |
| 2-Methyl | 60 | 67.2 | 83 | Unstable |
| 3-Methyl | 47 | 41.3 | 85 | Stable |
| 4-Methyl | 62 | 45.2 | 93 | Stable |
| 2,4-Dimethyl | 70 | 29.1 | 140 | Unstable |
| 2-Chloro | 62 | 38.4 | 31 | Stable |
| 3-Chloro | 58 | 41.2 | 19 | Stable |
| 4-Chloro | 58 | 52.7 | 29 | Stable |
| 2,3-Dichloro | 77 | 43.6 | 4.45 | Stable |
| 2,4-Dichloro | 71 | 34.5 | 4.2 | Stable |
| 2-Bromo | 69 | 20.3 | 25 | Stable |
| 3-Bromo | 55 | 50.7 | 17 | Stable |
| 4-Bromo | 58 | 40.7 | 26 | Stable |
| 2,4-Dibromo | 90 | 3.3 | 1.4 | Unstable |
| 2-Methoxy | 82 | 31.2 | 118 | Stable |
| 3-Methoxy | 83 | 32.7 | 50 | Stable |
| 4-Methoxy | 78 | 22.1 | 135 | Stable |
| 2-Carboxy§ | 95 | 4.0 | ~250¶ | Partial (3%) |
| 4-Carboxy§ | 92 | 4.4 | 36 | Partial (17%) |
| 2-Ethyl ester‖ | 89 | 3.9 | ~250¶ | Partial (51%) unstable |
| 4-Ethyl ester‖ | 80 | 12.0 | 11 | Stable |

* Determined as in Figs. 1 and 2. Data from ref. 11 and Figs. 1 and 2 are included. Tabulated values are the mean of eight experiments for phenylhydrazine and the mean of between two and six experiments for substituted phenylhydrazines. Rel. hct., relative hematocrit; Retic. ct., reticulocyte count.

† In mol × liter$^{-1}$ × min$^{-1}$. Initial rate of autooxidation of arylhydrazine by oxygen at a constant pressure of oxygen as determined by Stroh and Ebert (15). $v_0 = -dC_{OHa}/dt$, where $C_{OHa}$ is the initial concentration of arylhydrazine.

‡ Spectra obtained by the addition of ferricyanide and arylhydrazine to ferrohemoglobin as in Figs. 3 and 4. Unless otherwise noted, unstable spectra showed essentially complete conversion to ferrihemochrome followed by reversion toward ferrihemoglobin.

§ Hydrazinobenzoic acid.

¶ These anomalously high values may have resulted from a failure of the analytical method for phenylhydrazines with *ortho*-carboxy substituents.

‖ Ethyl hydrazinobenzoate.

## DISCUSSION

The present results confirm previous findings (11, 12) of the parallel relationship between the induction of anemia by an arylhydrazine on the one hand and the formation of ferrihemochrome by an oxidation product of the arylhydrazine on the other. The ligand, which can be produced by the oxidation of an arylhydrazine with two equivalents of ferricyanide, probably is the corresponding aryldiazene (ArN=NH) or aryldiazenyl anion (ArN=N$^-$) (13, 18–20). Although aryldiazenes are unstable and undergo spontaneous bimolecular decomposition (21, 22), complexes of these compounds are stable (23, 24). A pair of chlorine atoms or methyl groups at the *ortho* positions or a carboxy group at either the *ortho* or the *para* position inhibits both the formation of ferrihemochrome and the induction of hemolytic anemia by an arylhydrazine (10–13). In the present study we found that a single *tert*-butyl group at the *ortho* position suffices to inhibit both effects.

The increase in inhibitory effect with increase in size of neutral *ortho* substituents, but not of *meta* or *para* substituents,

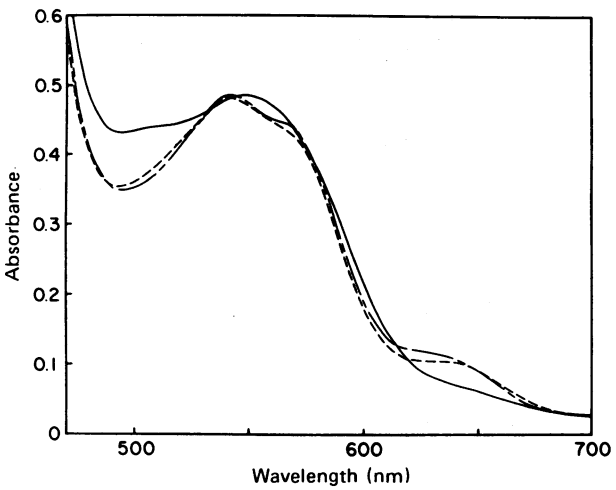


FIG. 3. Spectra of ferrihemochromes produced by reactions of ferrihemoglobin with the oxidized isomers of chlorophenylhydrazine in the presence of excess ferricyanide. To 4.00 ml of 50 μM (in Fe) ferrohemoglobin in 0.05 M sodium phosphate (1:1) buffer (pH 6.84) under nitrogen, 26 μl of 0.1 M potassium ferricyanide and 12 μl of 0.05 M chlorophenylhydrazine were added in succession. Spectra were recorded, 1, 5, and 10 min after the addition of arylhydrazine. Reactions were nearly complete after 1 min; the 5-min spectra shown here were nearly the same as the respective 10-min spectra. 2-Chlorophenylhydrazine (—); 3-chlorophenylhydrazine (- - - -); 4-chlorophenylhydrazine (- — -).

suggests a steric influence on reactions of the hydrazino or diazenyl group. The effect of a carboxy group must reside in the negative charge of its anion because the larger ester group is only partially inhibitory (11, 12). The same effects of size, position, and charge of ring substituents occur in the binding of substituted nitrosobenzenes by ferrohemoglobin (25).

Hydrogen peroxide and superoxide radical ion, suggested as oxidants in the denaturation of hemoglobin (26–28), are produced in the autooxidation of phenylhydrazine (15, 26, 29). It is evident from Table 1, however, that the severity of arylhydrazine-induced oxidative hemolysis is not closely related to the rate at which an arylhydrazine reacts with molecular oxygen. The same reduction products of oxygen are also formed in the reaction of phenylhydrazine with oxyhemoglobin (26–28, 30). Phenylhydrazine is oxidized in one-electron steps by in-

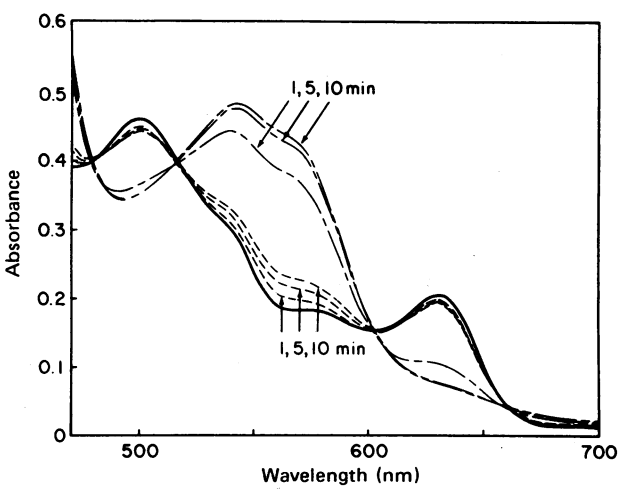


FIG. 4. Spectra of reactions of mixtures of ferrihemoglobin (—) with oxidized 4-hydrazinobenzoic acid (- - - -) and its ethyl ester (- — -), respectively, in excess ferricyanide. The conditions of the reactions were the same as in Fig. 3.

organic oxidants (31, 32). The reaction of a one-electron reductant with oxyhemoglobin can be represented as the formation of ferrihemoglobin peroxide (33, 34) by the transfer of a hydrogen atom to dioxygen ligand.

$$HbO_2 + H\cdot \rightarrow HbOOH \quad [1]$$

If no further reaction takes place, the final products are ferrihemoglobin and hydrogen peroxide (35).

$$HbOOH + H^+ \rightarrow Hb^+ + H_2O_2 \quad [2]$$

The ineffectiveness of a number of ferrihemoglobin-producing one-electron reductants as oxidative denaturants indicates that an equivalent amount of hydrogen peroxide does not cause significant damage to ferrihemoglobin and that reactions 1 and 2 do not account for the effectiveness of phenylhydrazine. The addition of a hydrogen atom in a second one-electron step to the proximal oxygen of the peroxide ligand would produce a more active oxidant.

$$HbOOH + H\cdot \rightarrow \begin{bmatrix} HOOH \\ | \\ Hb \end{bmatrix} \rightarrow HbOH + OH\cdot \quad [3]$$

Liganded hydroxide ion, which for the most part dissociates at physiological pH, results from the proximal oxygen; and free hydroxyl radical results from the distal oxygen. The postulated intermediate product of reaction 3 has the same structure as the complex suggested by Bonnett and Dimsdale (36) to be the source of hydroxyl radical in the hydroxylation of a *meso* (methene) bridge in pyridine ferrohemochrome by hydrogen peroxide. The validity of this mechanism in the *meso*-hydroxylation of heme in oxyhemoproteins is supported by the hydroxylation of specific *meso* positions in myoglobin and hemoglobin. Ligands are directed toward the $\alpha$ methene bridge in myoglobin and toward pyrrole II (situated between the $\alpha$ and $\beta$ bridges) in hemoglobin (37–39). The $\alpha$ methene bridge in myoglobin and the $\alpha$ and $\beta$ bridges in hemoglobin are the only bridges hydroxylated when these proteins undergo coupled oxidation with ascorbate (40). Thus, the bridges closest to the oxygen atom from which hydroxyl radical is formed, namely, the distal atom of dioxygen ligand, are also the bridges specifically hydroxylated.

Ascorbic acid produces Heinz bodies *in vitro* but not *in vivo*, except in the presence of alloxan or dialuric acid (3). We have ascertained that the reaction of ascorbate with oxyhemoglobin is very slow in comparison to the reaction of phenylhydrazine. The two-step mechanism of reactions 1 and 3, while consistent with the oxidative activity of ascorbic acid, fails to account for the exceptional rapidity and severity both *in vitro* and *in vivo* of phenylhydrazine-induced oxidative denaturation. We suggest that activation of molecular oxygen when bound to hemoglobin (41) and of hydrazine by the phenyl group (28), together with the similarity in interatomic distance of dinitrogen and dioxygen (42), promotes simultaneous or rapid consecutive transfer of a hydrogen atom to each oxygen atom of dioxygen ligand from the same molecule of phenylhydrazine. The initial products are phenyldiazene and the intermediate complex of reaction 3, which dissociates to ferrihemoglobin hydroxide and hydroxyl radical (36). Hydroxyl radical is released in the vicinity of pyrrole II, and phenyldiazene replaces hydroxide as the ligand of ferrihemoglobin.

$$HbO_2 + PhNHNH_2 \rightarrow \begin{bmatrix} HOOH \\ | \\ Hb \end{bmatrix} + PhN{=}NH \quad [4]$$

$$\rightarrow HbOH + OH\cdot + PhN{=}NH \rightarrow HbPhN{=}NH^+ + OH\cdot + OH^-$$

The entire sequence of reactions takes place within the hydrophobic heme crevice (43), which protects the unstable products from the aqueous medium surrounding the molecule and permits the efficient hydroxylation of a methene bridge.

2,6-Dichlorophenylhydrazine reacts slowly with oxyhemoglobin, and 2,6-dichlorophenyldiazene binds slowly to ferrihemoglobin (12). Thus, the same ring substitutions may inhibit both oxidant and ferrihemochrome formation. Does the ability of an arylhydrazine to produce a ligand of ferrihemoglobin merely parallel its ability to react with oxyhemoglobin to produce an oxidant, or does the formation of an exogenously liganded ferrihemochrome actually enhance the severity of oxidative denaturation? We suggest that the powerful oxidative activity of phenylhydrazine results from the combined action of oxidant and ligand. Hydroxyl radical hydroxylates either the $\alpha$ or the $\beta$ *meso* bridge (36, 40), and phenyldiazene ligand interferes with normal van der Waals contacts between heme and its crevice (43), thereby facilitating the entry of an oxygen molecule. Cleavage of the porphyrin ring at the hydroxylated *meso* bridge by oxygen (40, 44) leads to the loss of heme and the decomposition of phenyldiazene to benzene and nitrogen (45). A ring substitution can attenuate the activity of an arylhydrazine by inhibiting the formation of hydroxyl radical, aryldiazene ferrihemochrome, or both.

Globin ferrihemochrome, in which the distal ligand of ferriheme is a group in the protein component of hemoglobin, has been identified as an intermediate in oxidative denaturation (46–48). Although the rate of spontaneous formation of this compound from normal, native ferrihemoglobin is extremely slow, its rate of formation from inherited unstable hemoglobins, hemoglobin H ($\beta$ chain tetramer), and free $\alpha$ and $\beta$ chains is rapid (46, 48, 49). Globin ferrihemochrome is also formed when oxyhemoglobin or ferrihemoglobin is added to a denaturing medium (46, 50). These reactions indicate that instability of a subunit of hemoglobin enables a binding site in the protein component of the subunit to become a ligand of its heme. In contrast, an aryldiazene ferrihemochrome is produced readily from native, stable ferrihemoglobin by the addition of an exogenous ligand under nondenaturing conditions (13).

Wallace *et al.* (51) have suggested that autooxidation of hemoglobin results from the displacement of superoxide from oxyhemoglobin by a nucleophilic ligand of ferrihemoglobin. Superoxide is produced in the autooxidation of isolated $\alpha$ and $\beta$ chains of oxyhemoglobin, but little or no ferrihemoglobin appears as an intermediate when the same subunits undergo conversion to globin ferrihemochrome (48, 49). These observations suggest that ferrihemoglobin is not an obligatory precursor of globin ferrihemochrome inasmuch as the latter can result directly from an unstable subunit of oxyhemoglobin through the displacement of superoxide by a globin ligand.

$$HbO_2 + \text{globin ligand} \rightarrow \text{globin ferrihemochrome} + O_2\cdot^- \quad [5]$$

Because of the extreme sensitivity of the hemoglobin molecule to small alterations of internal nonpolar contacts near the heme, an inherited amino acid substitution in each of an identical pair of subunits can result in instability of the entire molecule (52). It is accordingly likely that oxidative cleavage of the porphyrin ring in one or at most two of the subunits of a molecule of hemoglobin would result in instability not only of the oxidized subunits, but also of the remaining chemically unaltered subunits. The latter would then undergo spontaneous conversion to globin ferrihemochrome, and the intact heme groups in these otherwise normal subunits would be included in Heinz bodies. Differences between the catabolic products

of heme in Heinz bodies and the products of physiological breakdown (53) may stem from a lack of specificity in the cleavage of methene bridges in the coupled oxidation of denatured hemoglobin (54).

The formation of ferrihemoglobin has been regarded as an essential event in chemically induced oxidative denaturation by some workers (47, 55, 56) but not by others (57, 58). Ferrihemoglobin is inactive as a substrate in the oxidative cleavage of porphyrin and must be reduced to ferrohemoglobin before *meso*-hydroxylation, the initial step in the oxidative degradation of heme, can occur (40, 44). If, as proposed above, destabilization of the hemoglobin molecule in chemically induced oxidative denaturation depends on cleavage of the porphyrin ring, the formation of unliganded ferrihemoglobin would hinder rather than further the degradative process.

Finch (59) has pointed out that oxidant compounds range in effect from those that result in ferrihemoglobinemia without hemolysis to those that cause only red cell destruction. One-electron reductants, such as nitrite (35), that produce ferrihemoglobin without causing oxidative denaturation apparently do not proceed beyond reaction 1 to reaction 3. The formation of phenyldiazene and its binding by ferrihemoglobin (reaction 4) accounts for the relatively low concentration of the latter in phenylhydrazine-induced oxidative hemolysis. A reductant that produces ferrihemoglobin and hydroxyl radical from oxyhemoglobin (reactions 1 and 3) but does not produce a ligand may cause oxidative denaturation and at the same time yield ferrihemoglobin as a side product.

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