Environmental Health Perspectives
Vol. 64, pp. 321-330, 1985

Free-Radical Production and Oxidative Reactions of Hemoglobin

by Christine C. Winterbourn*

Mechanisms of autoxidation of hemoglobin, and its reactions with H_2O_2, O_2^-, and oxidizing or reducing xenobiotics are discussed. Reactive intermediates of such reactions can include drug free radicals, H_2O_2, and O_2^-, as well as peroxidatively active ferrylhemoglobin and methemoglobin–H_2O_2. The contributions of these species to hemoglobin denaturation and drug-induced hemolysis, and the actions of various protective agents, are considered.

The red blood cell has been extensively studied both as a source of free radicals and as a target for oxidative damage. This is largely because a wide variety of drugs and xenobiotics that can undergo oxidation-reduction reactions have been found to cause red cell destruction and hemolytic anemia. Interaction between the xenobiotic and hemoglobin is of prime importance in the process, which is usually characterized by hemoglobin oxidation to methemoglobin, and formation within the red cells of Heinz bodies, inclusions of denatured and precipitated hemoglobin. The incidence of this type of anemia first became apparent in dye or rubber industry workers exposed to compounds such as aniline and its derivatives (*1*). Subsequently, it attracted more attention when individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency were recognized to be susceptible to hemolysis induced by antimalarial drugs (*2*). Recent studies also suggest that environmental pollutants can give rise to intraerythrocytic hemoglobin oxidation (*3*).

There is an extensive literature on compounds capable of undergoing redox reactions with hemoglobin, both *in vitro* and *in vivo*, and for a comprehensive survey up to 1974, readers are referred to an excellent review by Kiese (*1*). In the past 10 years, developments in our understanding of the mechanisms of these reactions relate particularly to the use of superoxide dismutase as a probe for superoxide involvement and of electron spin resonance (ESR) techniques for identifying free-radical intermediates.

Before discussing various reaction mechanisms, I shall define the terms used for the various oxidized hemoglobin derivatives. Methemoglobin (Hb^{3+}) is the reversibly formed ferric derivative in which the six coordination position of the heme iron is occupied by OH^- or H_2O. If the globin structure is destabilized or a suitable ligand is available, methemoglobin can convert to hemichrome, in which either the distal histidine or an external ligand occupies the sixth coordination position.(*4*). Hemichromes can be formed reversibly or irreversibly. They tend to be unstable and to precipitate readily and are the main constituent of Heinz bodies (*5,6*). Choleglobin is a loose term used to describe denatured hemoglobin in which the porphyrin ring has been hydroxylated or broken open (*7*). This is thought to occur through internal peroxidation by $Hb^{3+}H_2O_2$. Ferrylhemoglobin, represented as Fe^{4+}, is equivalent to a $Hb^{2+}H_2O_2$ complex (peroxidase compound II), although it appears to be unable to produce H_2O_2 by dissociation (*8–10*). $Hb^{3+}H_2O_2$ refers to a short-lived ferryl radical intermediate produced in the reaction of methemoglobin with H_2O_2 (*9,10*). Absorption spectra of some of these species are shown in Figure 1.

Hemoglobin Autoxidation

Although predicted by Weiss (*11*), it was first shown by Wever et al. (*12*) and Misra and Fridovich (*13*) that the superoxide radical (O_2^-) is produced when oxyhemoglobin autoxidizes. It was subsequently shown that this O_2^-, and the H_2O_2 produced from it, in the absence of catalase or superoxide dismutase, cause oxidation of further hemoglobin (*14–16*). The mechanism can therefore be written

$$Hb^{2+}O_2 \rightarrow Hb^{3+} + O_2^- \quad (1)$$

$$2H^+ + O_2^- + Hb^{2+}O_2 \rightarrow Hb^{3+} + O_2 + H_2O_2 \quad (2)$$

$$H_2O_2 + 2Hb^{2+}O_2 \rightarrow 2Hb^{3+} + 2O_2 + 2OH^- \quad (3)$$

Because reaction (2) is slow, it occurs in competition with reaction (4)

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (4)$$

*Department of Pathology, Christchurch Clinical School of Medicine, Christchurch Hospital, Christchurch, New Zealand.

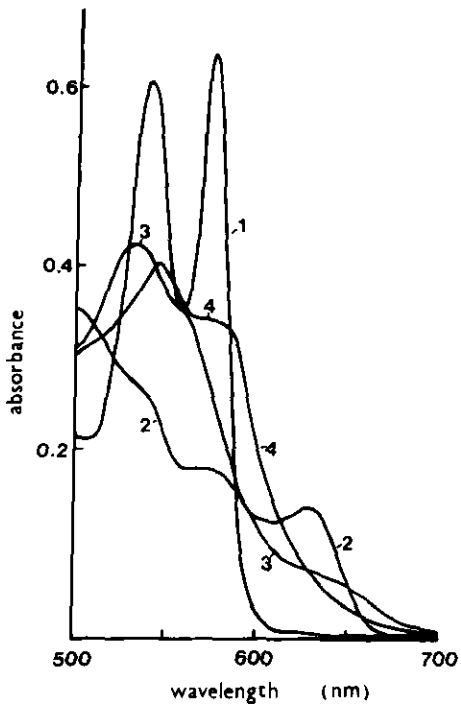


FIGURE 1. Absorption spectra of approximately 10 μM oxyhemoglobin (*1*), methemoglobin (*2*), hemichrome (*3*), and ferrylhemoglobin at pH 7.4.

without changing the overall stoichiometry. The rate of autoxidation increases as the pH is lowered, and also as P_{O_2} decreases, reaching a maximum at approx. half saturation (*17,18*). For this reason, there is some uncertainty whether reaction (1) occurs as shown, or is a reaction between deoxyhemoglobin and molecular oxygen (*19,20*). Regardless of the mechanism, however, autoxidation, and thus O_2^- production, are highest in partially deoxygenated blood. The mechanism of reaction (3) has not been studied during autoxidation. However, Whitburn (*21*) has shown that ferrylmyoglobin is produced as an intermediate in the equivalent reaction between oxymyoglobin and H_2O_2, suggesting that reaction (3) occurs by a similar mechanism:

$$Hb^{2+}O_2 + H_2O_2 \rightarrow Hb^{4+} + O_2 + 2OH^- \quad (5)$$

$$Hb^{4+} + Hb^{2+}O_2 \rightarrow 2Hb^{3+} + O_2 \quad (6)$$

If this is the case, virtually all the ferryl species must react with oxyhemoglobin to account for the observed stoichiometry of autoxidation (*18*):

$$2H^+ + 4Hb^{2+}O_2 \rightarrow 3O_2 + 2OH^- \quad (7)$$

Reactions of H_2O_2 with Hemoglobin

Some ferrous complexes react with H_2O_2 to give the hydroxyl radical ($OH^\cdot$) and this possibility could be considered for hemoglobin. However, if this were a major pathway, the stoichiometry of reaction (7) would require almost all the $OH^\cdot$ formed to cause heme oxidation. This would not be expected in view of the high reactivity of $OH^\cdot$, favoring reactions within the heme pocket before the radical could escape (*9*).Spectral changes with oxymyoglobin and H_2O_2 indicate formation of the ferryl species, which is reasonably stable with excess H_2O_2, but gradually converts excess oxymyoglobin to metmyoglobin (*20*). Ferrylhemoglobin and ferrylmyoglobin also oxidize a variety of electron donors including ascorbate, quinols, iodide and ferrocyanide (*8*).

The peroxidative activity of methemoglobin and metmyoglobin has been well studied (*8–10,21–24*). It was first suggested by George and Irvine (*8*) that the reaction between metmyoglobin and H_2O_2 might produce $OH^\cdot$. They observed ferrylmyoglobin as a product, a metmyoglobin:H_2O_2 stoichiometry of approximately 1:1, and evidence of a short-lived reactive intermediate. However, it was subsequently shown that the intermediate is equivalent to an $Hb^{3+}H_2O_2$ complex, with one oxidizing equivalent located on the heme (ferryl) and one on the globin, i.e., giving a ferrylheme–globin radical (*9,10*). The radical nature of this species has been observed by ESR spectroscopy, with both hemoglobin and myoglobin (*23–25*). The radical appears to be located initially on a histidine or phenylalanine residue, then shifts to a tyrosine (*25*). In theory, it could arise from $OH^\cdot$ produced in the heme pocket, but there is no proof of this. (This species is different from the $Fe^{3+}H_2O_2$ complex of horseradish peroxidase, in which both oxidizing equivalents are associated with the heme group.) This radical intermediate undergoes a number of reactions, including

$$Hb^{3+}H_2O_2 + Hb^{3+} \rightarrow 2Hb^{4+} + 2OH^- \quad (8)$$

and radical dismutation and other reactions that give rise to irreversibly oxidized heme derivatives, e.g., choleglobin or modified globins. Globin cross-linking results from one such reaction(*26*). With myoglobin, at least 10 steps in the overall reaction have been identified (*10*). This short-lived methemoglobin–peroxide radical should also be a strong oxidant of exogenous electron donors (*27*) and is a likely contributor to redox drug-hemoglobin reactions.

Thus any reaction involving oxyhemoglobin or methemoglobin and H_2O_2 can produce these oxidizing species which are capable of at least some of the reactions of $OH^\cdot$ radicals. For example, H_2O_2 and either hemoglobin species readily oxidize methional to ethylene (*28*). Before other reactions involving hemoglobin can be attributed to $OH^\cdot$, therefore, hemoglobin–peroxide complexes must be excluded. Furthermore, these species must rank highly in ability to cause cell damage, and whether $OH^\cdot$ is also formed may not be of crucial importance.

Reactions of O_2^- with Hemoglobin

O_2^-, in addition to oxidizing oxyhemoglobin [reaction (2)], can also reduce methemoglobin (*14*).

$$O_2^- + Hb^{3+} \rightarrow Hb^{2+}O_2 \qquad (9)$$

Both reactions are relatively slow (*29*); at pH 7.8, $k_2 = 6 \times 10^3$/M-sec and $k_9 = 4 \times 10^3$/M-sec. This means that in concentrated hemoglobin solutions (as in the red cell) O_2^- will react predominantly with hemoglobin, but in more dilute solutions, most will spontaneously dismutate. In the red cell cytoplasm, however, sufficient superoxide dismutase is present to outcompete hemoglobin for more than 99% of any O_2^- produced (*29*). Since O_2^- reacts with oxyhemoglobin and methemoglobin at almost the same rate, it cannot cause complete oxidation or reduction. Provided H_2O_2 is removed with catalase, continued exposure to O_2^- will produce a steady-state mixture containing approx. 40% methemoglobin.

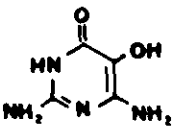
Redox Reactions of Hemoglobin with Xenobiotics

Hemoglobin reacts with a wide variety of redox active compounds. These compounds can be either reducing agents or oxidizing agents, and the reactions are predominantly of two types: either oxidation of the former by the heme-bound oxygen, or reduction of the latter by the heme iron. Other compounds may react only slowly with hemoglobin directly, but cause heme oxidation through generation of H_2O_2.

Some compounds such as phenylhydrazines, hydroxylamines and quinones, react directly with hemoglobin (*1,30*). Others, e.g., aniline and other substituted aromatics, are active *in vivo* but not *in vitro* because they first require metabolism. This commonly involves enzymatic deacylation and/or N- or ring-hydroxylation by the cytochrome P-450 system, predominantly in the liver. Into this category fall a number of drugs including primaquine, sulfanilamide, dapsone and phenacetin (*1,2*). Thus the ability of such drugs to cause methemoglobinemia or hemolysis will depend on the efficiency of these metabolic pathways as well as on red cell factors, both of which could be sources of individual variation.

Reactions Mediated Through H_2O_2

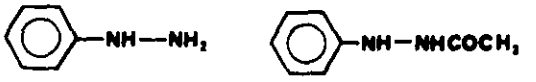
Reduced glutathione (GSH) can cause the oxidation and denaturation of hemoglobin (in addition to its ability to reduce methemoglobin). Almost all the heme oxidation is inhibited by catalase and, therefore, primarily due to H_2O_2, presumably formed on autoxidation of GSH (*31*). Another compound of toxicological interest, whose effects on hemoglobin are primarily mediated by H_2O_2, is divicine (I). Divicine, along with the structurally related isouramil, is responsible for red cell hemolysis in susceptible G6PD-deficient individuals who ingest fava beans (*32*). Divicine readily autoxidizes in solution. The initial step in this reaction is metal ion-catalyzed and proceeds by a chain reaction with O_2^- as an intermediate (*33*). H_2O_2 is produced in this reaction, and in hemoglobin solutions is responsible for the accelerated heme oxidation. Thus catalase inhibits this reaction, as does superoxide dismutase by inhibiting divicine autoxidation and thence H_2O_2 production.



I

Reaction of Oxyhemoglobin with Reducing Agents

Most drugs that cause Heinz body formation and hemolysis in G6PD deficiency, as a result of metabolic hydroxylation, react by this mechanism (*1,34*). It is typified by the reactions of phenylhydrazine (PH) (II) and acetylphenylhydrazine (APH) (III), which I shall discuss in some detail. The reactions of other compounds follow the same general pattern, although as a result of differences in reactivity or stability of intermediates, the end result can vary considerably. For example, the proportions of methemoglobin, hemichrome and choleglobin produced vary for different compounds, and radical scavengers in some cases accelerate, and in others protect against hemoglobin oxidation and denaturation.



II III

The hemoglobin spectral changes caused by APH (*35*) are shown in Figure 2. Similar changes are seen with PH but they occur about 40 times faster (*36*). They indicate the formation of predominantly hemichrome, with the shoulder at 630 nm indicating some methemoglobin (relatively more with APH than PH). The broad increase in absorbance evident above 650 nm represents modified porphyrin derivatives such as choleglobin. *N*-Phenylheme derivatives have also been detected with PH (*37*) and would contribute to this absorbance. Benzene and N_2 are also formed in the PH reaction (*1,34,38*), as are H_2O_2 and O_2^- (*39–41*) and the phenyl radical has been detected by ESR and spin trapping (*42*). Oxidation of PH follows the sequence

$$PhNHNH_2 \rightarrow PhN{=}NH \rightarrow PhH + N_2$$

Breakdown of the diazine (PhN=NH) is fast and O_2^--dependent, but it is stabilized in the presence of methemoglobin by formation of hemichrome (*43–46*). With APH the diazine also forms hemichrome, but does not readily break down to benzene (*44*). H_2O_2 and O_2^- have

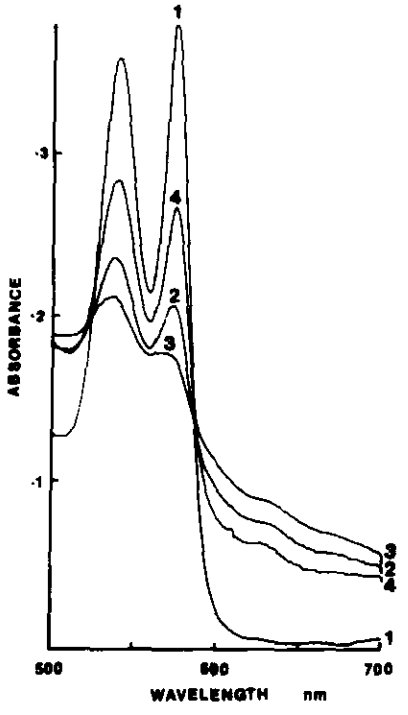


FIGURE 2. Spectral changes after reacting oxyhemoglobin (6 μM) with acetylphenylhydrazine (0.67 mM) for 30 min at 37°C (*35*): (1) oxyhemoglobin; (2) plus APH; (3) plus APH + superoxide dismutase; (4) plus APH + catalase.

also been detected with APH (*35,47*). Itano and Matteson (*36*) have interpreted the spectral changes differently, as indicating one reaction product that is none of those considered above, based on the maintenance of isosbestic points during the course of the reaction. However, this would also be the case if a complex mixture of products were formed in constant proportion. They also concluded that methemoglobin is not an important intermediate, since they could see little effect of CN^- (*36*). However, with PH or with APH and CN^- (*48* and C. Winterbourn, unpublished), cyanmethemoglobin can be a major reaction product, suggesting that methemoglobin is on the main reaction pathway.

Thus although some authors (*46,49*) have proposed that the initial reaction of PH with oxyhemoglobin is a $2e^-$ oxidation

$$PhNHNH_2 + Hb^{2+}O_2 \rightarrow Hb^{4+} + PhN{=}NH + H_2O_2 \quad (10)$$

the bulk of current evidence (*35,41,42,48,50*) with PH and other reducing agents favors $1e^-$ oxidation by the heme-bound oxygen, producing a free-radical intermediate, methemoglobin and H_2O_2 [reaction (11)]. Kawanishi and Caughey (*51*) have studied the kinetics of this reaction with $Fe(CN)_5H_2O^{3-}$. Its reaction with oxyhemoglobin (in the presence of catalase) is uncomplicated by formation of intermediate radicals or heme-binding products, and in this case the only products are methemoglobin, H_2O_2 and $Fe(CN)_5H_2O^{4-}$.

What is not clear is whether a methemoglobin-H_2O_2 complex is formed as shown in reaction (11) and then dissociates [reaction (12)], or the reverse. Reactions attributable to $Hb^{3+}H_2O_2$ include choleglobin formation due to internal peroxidation of the porphyrin. Such changes are slowed but not prevented by catalase or CN^-, suggesting at least some direct production of the complex. The initial products of the reaction are all capable of undergoing further reactions, with themselves, with the initial reactants, and with O_2, giving rise to other radicals such as the phenyl radical and O_2^- and reactive intermediates such as the diazine and hemoglobin–peroxide complexes. It is probably not possible to give a complete sequence of events occurring during the reaction, since these intermediates will react nonselectively with many components of the system. However, the reactions of the heme groups, and those resulting in free radical production, should be reasonably represented by the scheme summarized in Eqs. (11)–(21).

$$PhNHNH_2 + Hb^{2+}O_2 + H^+ \rightarrow PhNHNH^{\cdot} + Hb^{3+} + H_2O_2 \quad (11)$$

$$Hb^{3+}H_2O_2 \leftrightarrow Hb^{3+} + H_2O_2 \quad (12)$$

$$Hb^{2+}O_2 + H_2O_2 \rightarrow Hb^{4+} + O_2 + 2OH^- \quad (5)$$

$$PhNHNH^{\cdot} + Hb^{2+}O_2 + H^+ \rightarrow PhN{=}NH + Hb^{3+} + H_2O_2 \quad (13)$$

$$Hb^{3+} + PhN{=}NH \rightarrow Hb^{3+}PhN{=}NH \quad \text{(hemichrome)} \quad (14)$$

$$PhNHNH^{\cdot} + O_2 \rightarrow PhN{=}NH + O_2^- \quad (15)$$

$$PhN{=}NH + O_2 \rightarrow Ph^{\cdot} + O_2^- + N_2 + H^+ \quad (16)$$

$$Hb^{3+}H_2O_2 \rightarrow \text{choleglobin} \quad (17)$$

$$Hb^{3+}H_2O_2 + RH_2 \text{ or} \begin{cases} \nearrow Hb^{4+} + RH^{\cdot} + 2H_2O & (18a) \\ \searrow Hb^{3+} + R^{2+} + 2H_2O & (18b) \end{cases}$$

$$Hb^{4+} + RH_2 \text{ or} \begin{cases} \nearrow Hb^{3+} + RH^{\cdot} + H^+ & (19a) \\ \searrow Hb^{2+} + R + 2H^+ & (19b) \end{cases}$$

$$Ph^{\cdot} + \text{heme} \rightarrow \text{N-phenyl heme} \quad (20)$$

$$PhNHNH_2 + 2Hb^{3+} \rightarrow 2Hb^{2+} + PhN{=}NH \quad (21)$$

RH_2 in reactions (18) and (19) could be any oxidizable group, including PH itself or susceptible groups on the protein. Such groups are also targets for the free radicals produced in the reaction. With PH there is suggestive evidence for such modifications to the globin structure, resulting in decreased stability of hemichrome

(*35*). Hemichrome formed in the reaction of oxyhemoglobin with PH, although spectrally similar, is less stable and precipitates more readily than hemichrome formed directly from methemoglobin and phenyl diazine. The hemichrome formed from oxyHb and PH is considerably more stable, however, if the reaction is carried out in the presence of catalase or the free radical scavengers, ascorbate and GSH.

With APH, this overall sequence is applicable, except that reaction (15), rather than reactions (15) and (16), is likely to be the main source of O_2^-, and reaction (20) should have limited significance. It is possible, however, that other radicals apart from phenyl could also react directly with the porphyrin ring. In terms of the general applicability of this sequence, main sources of variation will be that some compounds form oxidation products which are either stable, or poor ligands for hemichrome formation.

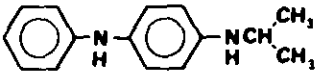
Reactions (16) and (20) or reaction (14) will not then be important. Other differences will relate to the rates of the different steps. The effects of catalase on the reaction illustrate this point. With APH, heme oxidation is slowed to less than half (*35*), while with PH its effect is slightly less (*36*). With phenylhydroxylamine (IV), however, catalase has no effect on the reaction rate (*31*).

IV

The effect of catalase will depend on the relative rates of reactions (11) and (5). With phenylhydroxylamine $k_{11} >> k_5$, with other compounds the rates are presumably more comparable. With nitrite (see below) reaction (11) appears to be much slower, and the effect of catalase related more to preventing reactions (18) and (19).

Superoxide dismutase has an acceleratory effect on the reaction of APH with oxyhemoglobin (Fig. 2), but with PH it inhibits (*36*). In this system O_2^- could either oxidize or reduce the heme groups (slow and probably not very significant) (*29*), act as a general radical scavenger (*52*), or directly oxidize PH (*41*). (It is not known whether it can also oxidize APH.) The acceleratory effect of the enzyme could be due to its preventing O_2^- acting as a radical scavenger, its inhibitory effects could relate to prevention of PH oxidation by O_2^-. The possibility of superoxide dismutase influencing reactions of the organic radicals with O_2 (as described below) is also possible but more information is required before its role can be fully understood.

GSH inhibits hemoglobin oxidation and denaturation by APH, and ascorbate can be almost completely inhibitory (*35*). Elevated ascorbate can also dramatically inhibit Heinz body formation in APH-treated red cells (*53*). This protection is over and above that of catalase, and is therefore not due to H_2O_2 removal. At least some of the effects appear to be due to radical scavenging, since the ascorbate radical has been detected, and ascorbate prevents spin-trapping of the phenyl radical (C. Winterbourn and B. Gilbert, unpublished data, but reduction of hemoglobin-peroxide complexes, via reactions (17) and (18), is also a strong possibility. The amount of protection given by these and other radical scavengers varies, depending on the nature of the particular drug, i.e., on the properties of the radical intermediates. Ascorbate and GSH scavenge only oxidizing radicals. GSH normally reacts by donating an electron, ascorbate by hydrogen donation (*54*), so their specificities will also be different. For example, *N*-isopropyl-*N'*-phenyl-*p*-phenylenediamine (IPPD) (V), which is used as a rubber antioxidant, rapidly oxidizes hemoglobin, and this reaction is partially inhibited by GSH but not ascorbate (*55*).



V

Thus xenobiotics such as PH react with oxyhemoglobin in a complex, multistep process. This produces many reactive intermediates, which are responsible for hemoglobin denaturation and potentially for other deleterious reactions in a red cell environment. The question has often been asked, what is the 'damaging species' responsible for drug-induced hemolytic anemia? H_2O_2 (*39,56*), O_2^- (*57–59*), and the drug radicals (*41,60*) have variously been proposed to fill this role. However, from the complexity of the reaction, it is clear that no one species can be held responsible, with H_2O_2, the drug free radicals, peroxidation by hemoglobin-H_2O_2 complexes, and hemichrome formation, all making a contribution. A major feature of drug induced hemolysis is the dramatic protection given by the hexose monophosphate shunt (absent in G6PD deficiency). The importance of this pathway for removing H_2O_2 via GSH and GSH peroxidase is clear (*39,61,62*), but free-radical scavenging and direct reduction of ferryl hemoglobin and methemoglobin–H_2O_2 could also be major factors.

Reaction of Oxyhemoglobin with Nitrite

This reaction shows the overall characteristics of the above mechanism, but with several distinctive features. Nitrite differs from the organic reductants in giving nearly quantitative conversion of oxyhemoglobin to methemoglobin, and is often used experimentally for this purpose (*64*). It can also cause methemoglobinemia in individuals ingesting foodstuffs or drinking water high in nitrite or nitrate (*1*). Nitrate in drinking water or vegetables usually originates from fertilizers, and high levels in well water can cause severe methemoglobinemia in infants. This cause of methemoglobinemia was recognized in 1945, and continues to be a problem in some areas (*1*). Only young infants are susceptible, because the pH of their upper intestinal tract is high

enough to harbor nitrate-reducing bacteria. After about 3 months of age, it appears that the pH drops below that required for bacterial growth, and nitrate is no longer such a problem. Even though nitrite can induce very high levels of methemoglobin, there is little evidence for hemoglobin denaturation and Heinz body formation. However, nitrite increases the proteolytic susceptibility of hemoglobin (*63*), suggesting that the globin may be somewhat modified.

The reaction of nitrite with deoxyhemoglobin is reasonably straight-forward, producing 1 equivalent of methemoglobin per NO_2^- and 1 equivalent of NO bound to ferrohemoglobin (*1*). In the presence of O_2, the stoichiometry can be described by (*64*)

$$4HbO_2 + 4NO_2^- + 4H^+ \rightarrow 4Hb^{3+} + 4NO_3^- + O_2 + 2H_2O \quad (22)$$

The kinetics of the reaction show a characteristic lag followed by a rapid autocatalytic phase (*65,66*). ESR studies by Kosaka et al. (*67*) and Jung and co-workers (*68*) have demonstrated the presence of a free radical intermediate with an ESR spectrum similar to the methemoglobin–H_2O_2 complex during the rapid phase of the reaction, and they propose that this is the autocatalytic agent. Kosaka et al. (*69*) propose the following mechanism:

Initiation (slow) step

$$Hb^{2+}O_2 + NO_2^- + 2H^+ \rightarrow Hb^{3+}H_2O_2 + NO_2^{\cdot} \quad (10)$$

Autocatalytic (fast) steps

$$Hb^{3+}H_2O_2 \leftrightarrow Hb^{3+} + H_2O_2 \quad (12)$$

$$Hb^{3+}H_2O_2 + NO_2^- \rightarrow Hb^{4+} + NO_2^{\cdot} + 2OH^- \quad (18a)$$

$$Hb^{4+} + NO_2^- \rightarrow Hb^{3+} + NO_2^{\cdot} \quad (19a)$$

$$Hb^{2+}O_2 + NO_2^{\cdot} \rightarrow Hb^{3+} + O_2 + NO_2^- \quad (13)$$

Chain termination

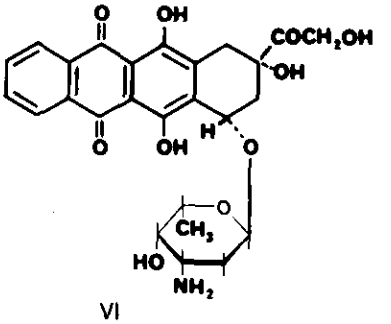
$$2NO_2^{\cdot} + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+ \quad (23)$$

In support of this mechanism, Kosaka et al. (*69*) observed that catalase or CN^- increased the lag period. Jung and Spolaczyk (*70*), on the other hand, found that although H_2O_2 shortened the lag phase, catalase had no effect.

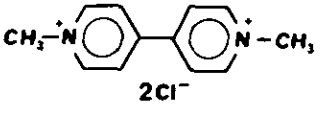
Of note is that in bistris buffer, the mechanism changes to include an O_2^--dependent step (*71*). This appears to be a result of the hemoglobin-peroxide complexes reacting with the buffer ions, presumably producing a bistris radical that reacts with O_2 to give O_2^-. This observation may have wider significance, since buffer radicals could similarly affect other oxidative reactions of hemoglobin.

Reaction of Hemoglobin with Oxidizing Compounds

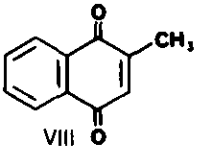
Compounds such as quinones, anthracyclines [Adriamycin, (VI)], methylene blue, paraquat (VII) and nitrofurantoin react with oxyhemoglobin by oxidizing the heme iron (*1,72-74*). Menadione (VIII), the vitamin K analog, is a significant member of this class of compound, since its administration to newborn infants can cause hemolysis.



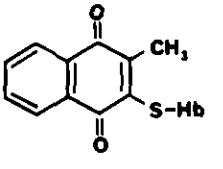
VI



VII



VIII



IX

The reaction produces a free radical intermediate (the semiquinone of naphthoquinone-6-sulfonate has been demonstrated directly by ESR (*75*) which is in most cases a reducing radical and reacts with O_2 to give O_2^- (*75,76*) as in reactions (24) and (25):

$$\text{quinone} + Hb^{2+}O_2 \leftrightarrow Hb^{3+} + O_2 + \text{semiquinone}^{\cdot} \quad (24)$$

$$\text{semiquinone}^{\cdot} + O_2 \leftrightarrow \text{quinone} + O_2^{\cdot} \quad (25)$$

H_2O_2 can then be produced by dismutation of O_2^- (*39*), a mixture of radicals, H_2O_2 and hemoglobin-peroxide complexes is again obtained, and hemoglobin denaturation and Heinz body formation ensues (*1*).

The spectral changes obtained with menadione (*75*) are shown in Figure 3. With most of these compounds, methemoglobin is the major product, with considerably less hemichrome and choleglobin formation than with phenylhydrazines. Oxidizing compounds cannot form

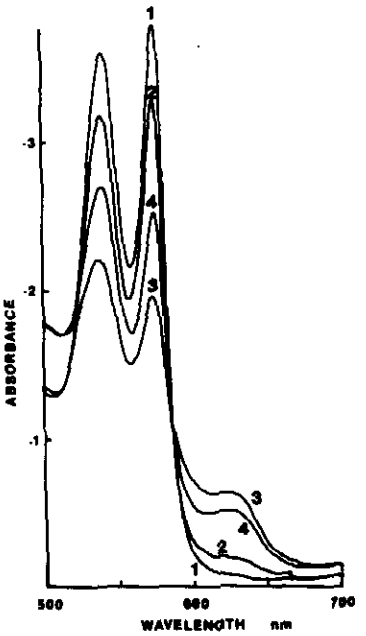


FIGURE 3. Spectral changes after reacting oxyhemoglobin (6 μM) with menadione (80 μM) for 40 min at 37°C (75): (1) oxyhemoglobin; (2) plus menadione; (3) plus menadione + superoxide dismutase; (4) plus menadione, superoxide dismutase + catalase.

phenylhydrazines. Oxidizing compounds cannot form $Hb^{3+}H_2O_2$ directly, but only by reaction (12), which may explain the lower choleglobin levels. Whether reaction (24) occurs as shown or involves deoxyhemoglobin appears to depend on the oxidant. We have found that menadione and Adriamycin do not react under N_2 (*72,75*), whereas benzoquinone and naphthoquinone-6-sulfonate readily react with deoxyhemoglobin (*75,77*). Other authors have reported a similar reaction for menadione (*76*). In general, reaction (24) is reversible. The reverse, reduction of methemoglobin, has been demonstrated with a variety of semiquinone radicals (*78*), and with paraquat (*73*) and Adriamycin (*72*). Furthermore, hemoglobin oxidation by menadione is accelerated by CN^-, which traps methemoglobin and prevents re-reduction (*75*).

Hemoglobin oxidation is generally inhibited by catalase [approximately 50% with menadione or Adriamycin (*72,75*)], demonstrating that further oxidation by H_2O_2 produced during the reaction occurs (Fig. 3). Superoxide dismutase, on the other hand, markedly accelerates oxidation, as in Figure 3 with menadione (*75*), and also with Adriamycin (*72*) and paraquat (*73*). This cannot be attributed to the enzyme preventing O_2^- from reacting with hemoglobin. As described above, with predominantly oxyhemoglobin present, O_2^- should cause slow oxidation and superoxide dismutase would then be slightly inhibitory.

The reason for this acceleration is clear when the effect of superoxide dismutase on the reactions of semiquinone radicals with methemoglobin is considered. A variety of semiquinones, produced from the parent compound either by ultraviolet or γ-irradiation, or with xanthine oxidase, reduce methemoglobin to (oxy)-hemoglobin in air or N_2 (*72,78–80*). In air, but not in N_2, these reactions are inhibited by superoxide dismutase. The semiquinones, rather than O_2^-, are responsible for the aerobic methemoglobin reduction since it is much faster than reduction by the same flux of O_2^- and almost complete reduction can be achieved, which is not possible with O_2^- (see the reactions of O_2^- above). The explanation is that reaction (25) is reversible, with the position of equilibrium depending on the redox potentials of the quinone and O_2. Superoxide dismutase, by removing O_2^-, can displace this equilibrium to the right, thus removing the semiquinone and inhibiting its reaction with methemoglobin (*78*). The superoxide dismutase concentrations required to prevent methemoglobin reduction vary for different quinones (*79*), and are in close agreement with those predicted for this mechanism from their redox potentials and the rate constants of the semiquinone/methemoglobin reactions (*80*). Superoxide dismutase also inhibits aerobic reduction of cytochrome c by semiquinone radicals (*81*), and again, the rate constant and redox potential data support a similar mechanism (*80*).

Returning to oxyhemoglobin oxidation, it is evident that by removing O_2^-, superoxide dismutase will prevent the reverse of reactions (24) and (25), and the overall effect will be a net increase in rate of conversion of oxyhemoglobin to methemoglobin, as observed.

Hemoglobin also reacts with menadione and other quinones with free 2 and 3 positions to form a thioether (IX) with the β-93 cysteines (*75*). A similar reaction is known to occur with GSH and other thiols (*1,82–84*). When menadione reacts with methemoglobin, formation of the thioether is accompanied by heme reduction to oxyhemoglobin (*75*). Blocking the β-93 cysteines with iodoacetamide prevents both reactions, but with GSH added to substitute for the blocked thiols, heme reduction occurs. Superoxide dismutase does not affect thioether formation but does inhibit heme reduction. This reduction is apparently caused by the semiquinone, thus implicating the semiquinone as an intermediate in thioether formation. Unless the β-93 thiol groups are blocked, this reaction decreases the rate of oxyhemoglobin oxidation by menadione. Thioether formation, therefore, provides two mechanisms whereby compounds such as menadione could be detrimental to the cell; firstly, by blocking key thiol groups, and secondly, as a means of semiquinone radical generation. The reaction of oxyhemoglobin with *p*-aminophenols has also been shown to produce a thioether, presumably by the reaction of the β-93 cysteines with the oxidized quinoneimine form of the drug (*85*).

The reactions of some compounds with oxyhemoglobin involve both oxidative and reductive mechanisms. Hydroquinones, which form quinones on oxidation, are one example. Another is the rubber antioxidant IPPD (V).Both it and its oxidation product react extremely rapidly, and the overall effect is the catalytic oxidation of hemoglobin by very low concentrations of IPPD (*55*).

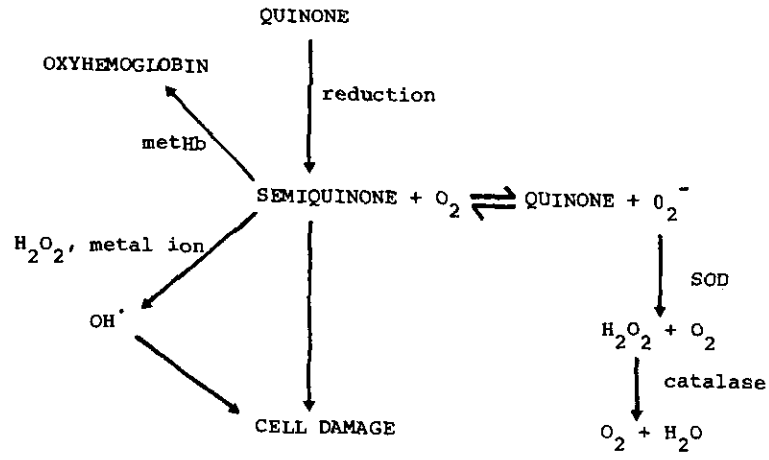


FIGURE 4. Reaction sequence showing how superoxide dismutase (SOD) could inhibit cell damage by semiquinone (or other reducing) radicals.

Protective Function of Superoxide Dismutase

Superoxide dismutase is thought to be required to protect cells against damage by O_2^-. It is usual to consider this in terms of reactions of O_2^- itself or of more reactive products such as $OH^\cdot$ formed directly from O_2^-. However, the ability of superoxide dismutase to inhibit reactions of radicals such as semiquinones with methemoglobin (and cytochrome c) suggests that it could have a more general protective function against other radical precursors or products of O_2^-. Radical reactions can proceed through a series of equilibria, and superoxide dismutase, like other enzymes, can displace such equilibria and exert control over more than its immediate substrate. In this scheme, O_2^- can be considered (as illustrated in Fig. 4) as an intermediate in a radical detoxification pathway involving O_2, O_2^- and superoxide dismutase (*86*). Although methemoglobin reduction by semiquinones is not disadvantageous, this mechanism provides protection against other less desirable reactions of such radicals.

The assistance of Mrs. S. Scandrett in preparing the manuscript is gratefully acknowledged.

REFERENCES

1. Kiese, M. Methemoglobinemia: A Comprehensive Treatise. CRC Press, Cleveland, 1974.
2. Beutler, E. Glucose-6-phosphate dehydrogenase deficiency. In: The Metabolic Basis of Inherited Disease (J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, Eds.), McGraw-Hill, New York, 1972, pp. 1358–1388.
3. Medeiros, M. H. G., Bechara, E. J. H., Naoum, P. C., and Mourao, C. A. Oxygen toxicity and hemoglobinemia in subjects from a highly polluted town. Arch. Environ. Health 38: 11–16 (1983).
4. Rachmilewitz, E. A., Peisach, J., and Blumberg, W. E. Studies on the stability of oxyhemoglobin A and its constituent chains and their derivatives. J. Biol. Chem. 246: 3356–3366 (1971).
5. Peisach, J., Blumberg, W. E., and Rachmilewitz, E. A. The demonstration of ferrihemochrome intermediates in Heinz body formation following the reduction of oxyhemoglobin A by acetylphenylhydrazine. Biochim. Biophys. Acta 393: 404–418 (1975).
6. Winterbourn, C. C., and Carrell, R. W. Characterization of Heinz bodies in unstable haemoglobin haemolytic anaemias. Nature 240: 150–152 (1972).
7. Lemberg, R., Legge, J. W., and Lockwood, W. H. Coupled oxidation of ascorbic acid and haemoglobin. 2. Formation and properties of choleglobin. Biochem. J. 35: 339–350 (1941).
8. George, P., and Irvine, D. H. The reaction between metmyoglobin and hydrogen peroxide. Biochem. J. 52: 511–517 (1952).
9. King, N. K., and Winfield, M. E. The mechanism of metmyoglobin oxidation. J. Biol. Chem. 238: 1520–1528 (1962).
10. Fox, J. B., Nicholas, R. A., Ackerman, S. A., and Swift, C. E. A multiple wavelength analysis of the reaction between hydrogen peroxide and metmyoglobin. Biochemistry 13: 5178–5186 (1974).
11. Weiss, J. J. Nature of the iron-oxygen bond in oxyhaemoglobin. Nature 202: 83–84 (1964).
12. Wever, R., Oudega, B., and Van Gelder, B. F. Generation of superoxide radicals during the autoxidation of mammalian oxyhemoglobin. Biochim. Biophys. Acta 302: 475–478 (1973).
13. Misra, H. P., and Fridovich, I. The generation of superoxide radical during the autoxidation of hemoglobin. J. Biol. Chem. 247: 6960–6962 (1972).
14. Winterbourn, C. C., McGrath, B. M., and Carrell, R. W. Reactions involving superoxide and normal and unstable haemoglobins. Biochem. J. 155: 493–502 (1976).
15. Lynch, R. E., Lee, G. R., and Cartwright, G. E. Inhibition by superoxide dismutase of methemoglobin formation from oxyhemoglobin. J. Biol. Chem. 251: 1015–1019 (1976).
16. Demma, L. S., and Salhany, J. M. Direct generation of superoxide anions by flash photolysis of human oxyhemoglobin. J. Biol. Chem. 252: 1226–1230 (1977).
17. George, P., and Stratmann, C. J. Oxidation of myoglobin by oxygen. Biochem. J. 51: 418–425 (1952).
18. Brown, W. D., and Mebine, L. B. Autoxidation of oxymyoglobins. J. Biol. Chem. 244: 6696–6701 (1969).
19. Banerjee, R., and Stetzkowski, F. Heme transfer from hemoglobin and ferrihemoglobin to some new ligands and its implication in the mechanism of oxidation of ferrohemoglobin by air. Biochim. Biophys. Acta 221: 636–639 (1970).
20. Wallace, W. J., Houtchens, R. A., Maxwell, J. C. and Caughey, W. S. Mechanism of autooxidation for hemoglobins and myoglobins. Promotion of superoxide production by protons and anions. J. Biol. Chem. 257: 4966–4977 (1982).
21. Whitburn, K. D. The interaction of hydrogen peroxide with oxymyoglobin. In: Oxygen Radicals in Chemistry and Biology (W. Bors, M. Saran and D. Tait, Eds.), Walter de Gruyter, Ber-

lin, 1984, pp. 447–451.
22. Keilin, D., and Hartree, E. F. Reaction of methaemoglobin with hydrogen peroxide. Nature 166: 513–514 (1950).
23. Gibson, J. F., and Ingram, D. J. E. Location of free electrons in porphyrin ring complexes. Nature 178: 871–872 (1956).
24. Gibson, J. F., Ingram, D. J. E., and Nicholls, P. Free radical produced in the reaction of metmyoglobin with hydrogen peroxide. Nature 181: 1398–1399 (1958).
25. King, N. K., Looney, F. D., and Winfield, M. E. Amino acid free radicals in oxidized metmyoglobin. Biochim. Biophys. Acta 113: 65–82 (1976).
26. Rice, R. H., Young, M. L., and Brown, W. D. Interactions of heme proteins with hydrogen peroxide: protein crosslinking and covalent binding of benzo[a]pyrene and 17β-estradiol. Arch. Biochem. Biophys. 221: 417–427 (1983).
27. Keilin, D., and Hartree, E. F. Catalase, peroxidase and metmyoglobin as catalysts of coupled peroxidatic reactions. Biochem. J. 60: 310–325 (1955).
28. Winterbourn, C. C. Hydroxyl radical production in body fluids: Roles of metal ions, ascorbate and superoxide. Biochem. J. 198: 125–131 (1981).
29. Sutton, H. C., Roberts, P. B., and Winterbourn, C. C. The rate of reaction of the superoxide radical ion with oxy- and met-haemoglobin. Biochem. J. 155: 503–510 (1976).
30. Mason, R. P. Free radical intermediates in the metabolism of toxic chemicals. In: Free Radicals in Biology, Vol. 5 (W.A. Pryor, Ed.), Academic Press, New York, 1982, pp. 161–222.
31. Eyer, P., Hertle, H., Kiese, M., and Klein, G. Kinetics of ferrihemoglobin formation by some reducing agents and the role of hydrogen peroxide. Molec. Pharmacol. 11: 326–334 (1975).
32. Mager, J., Chevion, M., and Glaser, G. Favism. In: Toxic Constituents of Plant Foodstuffs, 2nd ed. (L. I. Liener Ed.), Academic Press, New York, 1980, Chapt. 9, pp. 265-294.
33. Winterbourn, C. C., Benatti, U., and de Flora, A. (manuscript in preparation).
34. Beavan, G. W., and White, J. C. Oxidation of phenylhydrazines in the presence of oxyhaemoglobin and the origin of Heinz bodies. Nature 173: 389–391 (1954).
35. French, J. K., Winterbourn, C. C., and Carrell, R. W. Mechanism of oxyhaemoglobin breakdown on reaction with acetylphenylhydrazine. Biochem. J. 173: 19–26 (1978).
36. Itano, H. A., and Matteson, J. L. Mechanism of initial reaction of phenylhydrazine with oxyhemoglobin and effect of ring substitutions on the bimolecular rate constant of this reaction. Biochemistry 21: 2421–2426 (1982).
37. Ortiz de Montellano, P. R., and Kunze, K. L. Formation of N-phenylheme in the hemolytic reaction of phenylhydrazine with hemoglobin. J. Am. Chem. Soc. 103: 6534–6536 (1981).
38. Rostorfer, H. H., and Totter, J. R. The reduction of methemoglobin by phenylhydrazine under anaerobic conditions. J. Biol. Chem. 221: 1047–1055 (1956).
39. Cohen, G., and Hochstein, P. Generation of H_2O_2 by hemolytic agents. Biochemistry 3: 895–900 (1964).
40. Goldberg, B., and Stern, A. The generation of O_2^- by the interaction of the hemolytic agent, phenylhydrazine, with human hemoglobin. J. Biol. Chem. 250: 2401–2403 (1975).
41. Misra, H. P., and Fridovich, I. The oxidation of phenylhydrazine: Superoxide and mechanism. Biochemistry 15: 681–687 (1976).
42. Hill, H. A. O., and Thornalley, P. J. Phenyl radical production during the oxidation of phenylhydrazine and in phenylhydrazine-induced haemolysis. FEBS Letters 125: 235–238 (1981).
43. Itano, H. A. Phenyldiimide, hemoglobin, and Heinz bodies. Proc. Natl. Acad. Sci. (U.S.) 67: 485–492 (1970).
44. Mannen, S., Itano, H. A. Stoichiometry of the oxidation of arylhydrazines with ferricyanide. Quantitative measurements of absorption spectra of aryldiazenes. Tetrahedron 29: 3497–3502 (1973).
45. Itano, H. A., Hirota, K., and Hosokawa, K. Mechanism of induction of haemolytic anaemia by phenylhydrazine. Nature 256: 665–667 (1975).
46. Itano, H. A., Hirota, K., and Vedvick, T. S. Ligands and oxidants in ferrihemochrome formation and oxidative hemolysis. Proc. Natl. Acad. Sci. (U.S.) 74: 2556–2560 (1977).
47. Cohen, G. On the generation of hydrogen peroxide in erythrocytes by acetylphenylhydrazine. Biochem. Pharmacol. 15: 1775–1781 (1966).
48. Castro, C. E., Wade, R. S., and Belser, N. O. Conversion of oxyhemoglobin to methemoglobin by organic and inorganic reductants. Biochemistry 17: 225–231 (1978).
49. Goldberg, B., Stern, A., and Peisach, J. The mechanism of superoxide anion generation by the interaction of phenylhydrazine with hemoglobin. J. Biol. Chem. 251: 3045–3051 (1976).
50. Caughey, W. S., and Kawanishi, S. Mechanisms for superoxide and peroxide production from dioxygen and hemoglobins. In: Oxy Radicals and Their Scavenger Systems, Vol. I. Molecular Aspects (G. Cohen, and R. A. Greenwald, Eds.), Elsevier Science, New York, 1983, pp. 105–110.
51. Kawanishi, S., and Caughey, W. S. Aquopentacyanoferrate(II): An effective probing electron donor in the conversion of oxyhemoglobin to methemoglobin and peroxide. Biochem. Biophys. Res. Commun. 88: 1203–1208 (1979).
52. Lee-Ruff, E. The organic chemistry of superoxide. Chem. Soc. Rev. 6: 195–214 (1977).
53. Winterbourn, C. C. Protection by ascorbate against acetylphenylhydrazine-induced Heinz body formation in glucose-6-phosphate dehydrogenase deficient erythrocytes. Brit. J. Haematol. 41: 245–252 (1979).
54. Forni, L. G., Mönig, J., Mora-Arellano, V. O., and Willson, R. L. Thiyl free radicals: direct observations of electron transfer reactions with phenothiazines and ascorbate. J. Chem. Soc. Perkin Trans. II: 961–965 (1983).
55. Williamson, D., Winterbourn, C. C., Swallow, W. H., and Missen, A. W. Oxidative hemoglobin breakdown induced by a rubber additive. Hemoglobin 5: 73–84 (1981).
56. Jain, S. K., and Hochstein, P. Generation of superoxide radicals by hydrazine. Its role in phenylhydrazine-induced hemolytic anemia. Biochim. Biophys. Acta 586: 128–136 (1979).
57. Goldberg, B., and Stern, A. Superoxide anion as a mediator of drug-induced oxidative hemolysis. J. Biol. Chem. 251: 6468–6470 (1976).
58. Goldberg, B., and Stern, A. The role of the superoxide anion as a toxic species in the erythrocyte. Arch. Biochem. Biophys. 178: 218–225 (1977).
59. Valenzuela, A., Rios, H., and Neiman, G. Evidence that superoxide radicals are involved in the hemolytic mechanism of phenylhydrazine. Experientia 33: 962–964 (1977).
60. Goldberg, B., and Stern, A. The mechanism of oxidative hemolysis produced by phenylhydrazine. Molec. Pharmacol. 13: 832–839 (1977).
61. Mills, G. C. Hemoglobin catabolism. I. Glutathione peroxidase, an erythrocyte enzyme which protects hemoglobin from oxidative breakdown. J. Biol. Chem. 229: 189–197 (1957).
62. Nicholls, P. Contributions of catalase and glutathione peroxidase to red cell peroxide removal. Biochim. Biophys. Acta 279: 306–309 (1972).
63. Goldberg, A. L., and Boches, F. S. Oxidized proteins in erythrocytes are rapidly degraded by the adenosine triphosphate-dependent proteolytic system. Science 215: 1107–1109 (1982).
64. Kosaka, H., Imaizumi, K., Imai, K., and Tyuma, I. Stoichiometry of the reaction of oxyhemoglobin with nitrite. Biochim. Biophys. Acta 581: 184–188 (1979).
65. Jung, F., and Remmer, H. Über die Umsetzung zwischen Nitrit und Hämoglobin. Arch. Exp. Pathol. Pharmakol. 206: 459–274 (1949).
66. Jung, F., and Kahl, R. Über die Reaktion zwischen Hämoglobin und Natriumnitrit. Acta Biol. Med. Ger. 19: 853–868 (1967).
67. Kosaka, H., Imaizumi, K., and Tyuma, I. A mechanism of autocatalytic oxidation of oxyhemoglobin by nitrite: an intermediate detected by electron spin resonance. Biochim. Biophys. Acta 702: 237–241 (1982).
68. Ebert, B., Lassman, G., and Jung, F. Analysis of paramagnetic intermediates at the reaction of hemoglobin with nitrite using EPR. Biomed. Biochim. Acta 42: S154–S158 (1983).
69. Kosaka, H., Tyuma, I., and Imaizumi, K. Mechanism of autocatalytic oxidation of oxyhemoglobin by nitrite. Biomed. Biochim. Acta 42: S144–S148 (1983).

70. Jung, F., and Spolaczyk, M. The reaction between nitrite and hemoglobin. Biomed. Biochim. Acta 42: S149–S153 (1983).
71. Kosaka, H., and Tyuma, I. Production of superoxide anion by N,N-bis(2hydroxyethyl)-iminotris(hydroxymethyl)methane buffer during oxidation of oxyhemoglobin by nitrite and effect of inositol hexaphosphate on the oxidation. Biochim. Biophys. Acta 709: 187–193 (1982).
72. Bates, D. A., and Winterbourn, C. C. Reactions of Adriamycin with haemoglobin. Biochem. J. 203: 155–160 (1982).
73. Winterbourn, C. C. The reaction of hemoglobin with paraquat radicals in the presence and absence of O_2. Biochem. Internatl. 7: 1–8 (1983).
74. Dershwitz, M., and Novak, R. F. Generation of superoxide via the interaction of nitrofurantoin with oxyhemoglobin. J. Biol. Chem. 257: 75–79 (1982).
75. Winterbourn, C. C., French, J. K., and Claridge, R. F. C. The reaction of menadione with haemoglobin. Mechanism and effect of superoxide dismutase. Biochem. J. 179: 665–673 (1979).
76. Goldberg, B., and Stern, A. Production of superoxide anion during the oxidation of hemoglobin by menadione. Biochim. Biophys. Acta 437: 628–632 (1976).
77. Kakizaki, T., Sato, M., Tsuruta, H., and Hasegawa, H. Oxidation of haemoglobin by *p*-quinone. Ind. Health. 7: 13–21 (1969).
78. Winterbourn, C. C., French, J. K., and Claridge, R. F. C. Superoxide dismutase as an inhibitor of reactions of semiquinone radicals. FEBS Letters 94: 269–272 (1978).
79. Winterbourn, C. C. Superoxide dismutase: Potential for a wider role as an inhibitor of free radical reactions. In: Chemical and Biochemical Aspects of Superoxide and Superoxide Dismutase (J.V. Bannister and H. A. O. Hill, Eds.), Elsevier/North-Holland, Amsterdam, 1980, pp. 372–379.
80. Sutton, H. C., and Sangster, D. The reactivity of semiquinone radicals and its relationship to the biochemical role of superoxide. J. Chem. Soc. Faraday Trans. I 78: 695–711 (1982).
81. Winterbourn, C. C. Cytochrome c reduction by semiquinone radicals can be indirectly inhibited by superoxide dismutase. Arch. Biochem. Biophys. 209: 159–167 (1981).
82. Fieser, L. F., and Fieser, M. Organic Chemistry, G. G. Harrap, London, 2nd. ed., 1953, p. 763.
83. Nakai, N., and Hase, J. The reaction of 2-methyl-1,4-naphthoquinone with bovine serum albumin and papain. Chem. Pharm. Bull. Tokyo 16: 2339–2342 (1968).
84. Mezick, J. A., Settlemire, C. T., Brierley, G. P., Barefield, K. P., Jensen, W. N., and Cornwell, D. G. Erythrocyte membrane interactions with menadione and the mechanism of menadione-induced hemolysis. Biochim. Biophys. Acta 219: 361–371 (1970.
85. Eyer, P., Lierheimer, E., and Strosar, M. Site and mechanism of covalent binding of 4-dimethylaminophenol to human hemoglobin, and its implication to the functional properties. Mol. Pharmacol. 24: 282–290 (1983).
86. Sullivan, S. G., Winterbourn, C. C., and Stern, A. Hypothesis: Oxygen, superoxide dismutase and catalase form a metabolic pathway that protects against oxidative damage in the red cell. In: Oxy Radicals and Their Scavenger Systems, Vol. 11 (R. Greenwald and G. Cohen Eds.), Elsevier/North Holland, New York, 1983, pp. 105–112.