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The Mechanism of Oxidative Hemolysis Produced by Phenylhydrazine

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SUMMARY

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Superoxide anion was found to be unimportant in phenylhydrazine-induced hemolysis and destruction of oxyhemoglobin. Phenyldiazene, presumably produced via the 2-electron oxidation of phenylhydrazine by oxyhemoglobin, was found to hemolyze red cells rapidly and convert oxyhemoglobin into methemoglobin, hemichromes, and other hemoglobin breakdown products. Phenyldiazene-induced hemolysis requires the presence of molecular oxygen, although superoxide anion, a known product of the reaction of phenyldiazene with oxygen, was demonstrated not to be the active hemolytic agent. The hemolysis produced in the presence of phenyldiazene and oxygen is related to lipid peroxidation in the red cell membrane. Benzenediazonium ion and benzene, oxidation products of phenylhydrazine, were not found to be hemolytic when compared with equimolar quantities of phenyldiazene. It is concluded that the active hemolytic agent produced from phenylhydrazine in red cells is a phenyldiazene-derived free radical produced by the reaction of phenyldiazene with oxygen, and is most probably the phenyl free radical.

INTRODUCTION

Use of phenylhydrazine has long been known to result in the oxidation of cellular components and lysis in red cells (1), and for this reason phenylhydrazine has been utilized clinically, to curb the increase in red cell mass occurring in polycythemia vera (2), and experimentally, to produce hemolytic anemias (3). The mode of action of phenylhydrazine, as well as the reason for its high degree of specificity toward the erythrocyte (4), has been a topic of interest for several years. The generation of "hydrogen peroxide-like" substances during the reaction of phenylhydrazine with hemoglobin (5) and of hydrogen peroxide itself in red cells exposed to phenylhydrazine (6) has been reported. Recently the highly reactive superoxide anion radical was demonstrated to be a product of the reaction of phenylhydrazine with hemoglobin (7). In this reaction phenyldiazine, an oxidation product of the interaction of phenylhydrazine and oxyhemoglobin, reacts with molecular oxygen (8) to form superoxide anion. It was postulated that phenylhydrazine-induced hemolysis results from the formation of a highly reactive oxidizing species, such as superoxide anion, which can then react with cellular constituents and cause lysis of the cell. As hemoglobin is apparently required for the production of these oxidizing species from phenylhydrazine, the high degree of specificity of phenylhydrazine in its toxic action toward the erythrocyte may be explained.

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The purpose of the present study was to elucidate the role of the oxidants generated by the reaction of phenylhydrazine and hemoglobin in the hemolysis produced by phenylhydrazine.

MATERIALS AND METHODS

Phenylhydrazine (twice recrystallized) and α-chymotrypsin were products of Sigma Chemical Company. Methyl phenylazoformate and catalase were obtained from Calbiochem; potassium ferricyanide and benzene, from Fisher Scientific Company; malonaldehyde bis(dimethylacetal) and benzenediazonium hexafluorophosphate, from Aldrich Chemical Company; carbon monoxide, from Matheson Gas Products; and 2-thiobarbituric acid, from J. T. Baker Chemical Company.

Human blood was drawn fresh daily in heparin and washed three times with 0.9% NaCl. Experiments concerning hemolysis induced by phenyldiazene were performed with an isotonic suspension of red cells at room temperature. Hemolysis was followed by the decrease in light scattering at 740 nm that occurs upon cell disruption (9). Aerated suspensions of carbonmonoxyhemoglobin-containing red cells were prepared by bubbling carbon monoxide through an isotonic red cell suspension for 10 min, followed by replacement of the buffer after centrifugation with fresh aerated buffer. Phenyldiazene was usually generated by the action of α-chymotrypsin on methyl phenylazoformate (see below) (9).

Lipid peroxidation in phenyldiazene-treated red cells was assessed by the thiobarbituric acid assay for the lipid breakdown product malonaldehyde (10). Phenyldiazene was generated for the lipid peroxidation experiments by hydrolyzing methyl phenylazoformate with alkaline buffer (50 mM sodium carbonate, pH 11.8) to phenyldiazenecarboxylate, which is moderately stable at alkaline pH (11). Addition of a small volume of the concentrated alkaline carboxylate dispersion to a red cell suspension buffered at pH 7.4 results in very rapid decarboxylation of the phenyldiazenecarboxylic acid to phenyldiazene (8, 11). The pH of the red cell suspension after the addition of the carboxylate was found to remain at 7.4. This method of phenyldiazene generation was preferred to that of treating methyl phenylazoformate with α-chymotrypsin, as the presence of the enzyme in the reaction medium tended to decrease the color development in the assay. Lipid peroxidation was expressed as nanomoles of malonaldehyde by comparing experimental assay results with a standard curve prepared by assaying known quantities of malonaldehyde as the bis(dimethylacetal).

Hemoglobin was prepared by methods previously described (8). All optical measurements were performed on a Cary 14 recording spectrophotometer.

RESULTS

Oxidation of phenylhydrazine and hemolysis. Addition of phenylhydrazine to an erythrocyte suspension containing potassium ferricyanide, a compound which oxidizes phenylhydrazine extremely rapidly (12) but does not penetrate the red cell (13), resulted in relatively rapid hemolysis of the cells (Fig. 1). The hemolysis was preceded by a lag phase and was unaffected by the addition of superoxide dismutase (EC 1.15.1.1, superoxide:superoxide oxidoreductase) or catalase (EC 1.11.1.6, H_2O_2:H_2O_2 oxidoreductase) to the red cell suspension. Conversion of the cellular oxyhemoglobin to carbonmonoxyhemoglobin, followed by suspension of these cells in an aerated environment, resulted in marked enhancement of lysis when compared with oxyhemoglobin-containing red cells (Fig. 1). Again, superoxide dismutase and catalase added to the external medium did not inhibit hemolysis (Fig. 2). Complete exclusion of oxygen from the potassium ferricyanide-containing red cell suspension resulted in no hemolysis when phenylhydrazine was added (Fig. 2).

The role of an oxdized form of phenylhydrazine as the active hemolyzing agent was further investigated by exposing an aerated suspension of carbonmonoxyhemoglobin-containing erythrocytes to various intermediates of phenylhydrazine oxidation. Only phenyldiazene, in the presence of molecular oxygen, produced hemolysis

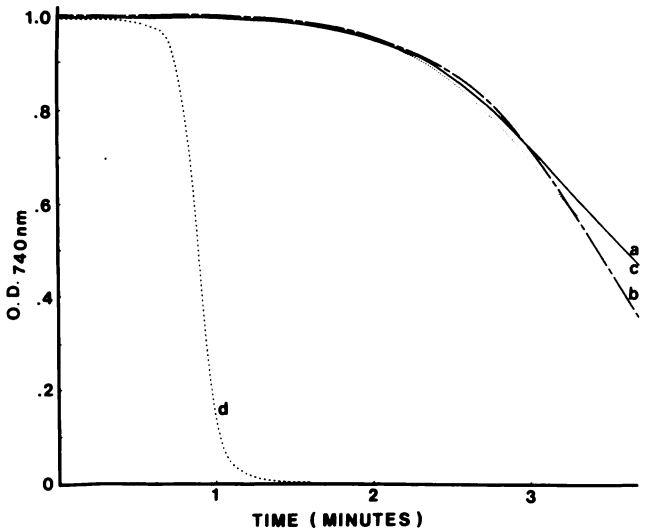


FIG. 1. *Lysis of red cells exposed to phenylhydrazine and potassium ferricyanide followed as decrease in light scattering at 740 nm*

The incubation mixture contained red cells (0.2% in 10 mM sodium phosphate buffer, pH 7.4, containing 135 mM NaCl and 10 mM glucose), phenylhydrazine (0.104 mM), $K_3Fe(CN)_6$ (0.208 mM), and superoxide dismutase (23.1 μg/ml or catalase (2695 units/ml). Curve *a*, hemolysis in the presence of phenylhydrazine and $K_3Fe(CN)_6$; *b*, effect of superoxide dismutase on hemolysis; *c*, effect of catalase; *d*, hemolysis of an aerated suspension of carbonmonoxyhemoglobin-containing erythrocytes by phenylhydrazine and $K_3Fe(CN)_6$. Neither phenylhydrazine nor $K_3Fe(CN)_6$ caused lysis during the time course of this experiment.

(Table 1). This result confirms the earlier work of Kosower *et al.* (14), who demonstrated an oxygen-dependent hemolysis of carbonmonoxyhemoglobin-containing erythrocytes exposed to methyl phenylazoformate.

Lipid peroxidation and hemolysis produced by phenyldiazene and oxygen. Carbonmonoxyhemoglobin-containing red cells, when exposed to a source of phenyldiazene and molecular oxygen, undergo peroxidation of the lipids in their cell membranes, as demonstrated by the detection of the lipid oxidation product, malonaldehyde, in the cellular medium. Neither superoxide dismutase nor catalase inhibited lipid peroxidation (Table 2). A temporal correlation between lipid peroxidation and hemolysis indicates that the bulk of lipid breakdown occurred during the lag phase of hemolysis (Fig. 3).

Oxyhemoglobin destruction by phenylhydrazine and phenyldiazene. Treatment of purified oxyhemoglobin with phenylhydrazine results in the apparent conversion of oxyhemoglobin to a green pigment. Spectral examination of the products demonstrates the presence of hemichrome (15, 16). As addition of potassium cyanide causes a decrease in absorbance at 630 nm and an increase in absorbance between 580 and 540 nm, the presence of methemoglobin is also implied (17). The residual absorbance from 600 to 700 nm in the presence of cyanide indicates the formation of choleglobin and like compounds (15) (Fig. 4).

Treatment of an oxyhemoglobin solution with phenyldiazene generated by the hydrolysis and decarboxylation of methyl phenylazoformate results in the breakdown of the oxyhemoglobin into methemoglobin, choleglobin, hemichrome, and related compounds, as with phenylhydrazine. Analogous to the situation with phenylhydrazine, the breakdown of oxyhemoglobin by phenyldiazene is not inhibited by superoxide dismutase or catalase, but is inhibited by conversion of the oxyhemoglobin to carbonmonoxyhemoglobin.

Neither superoxide dismutase nor cata-

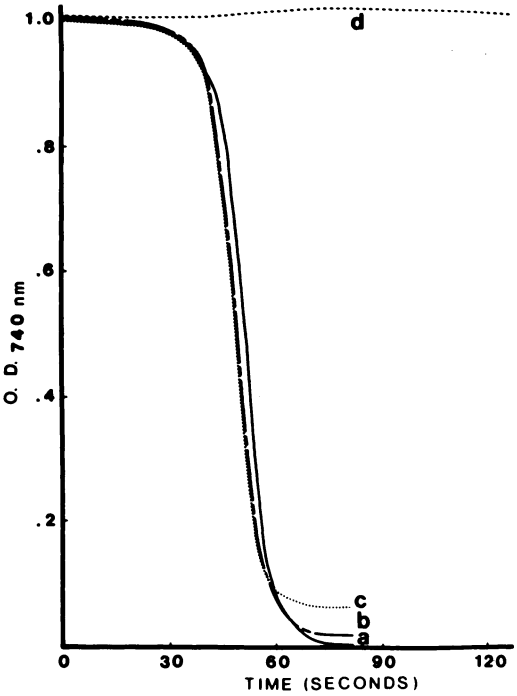


FIG. 2. *Lysis of carbonmonoxyhemoglobin-containing red cells by phenylhydrazine and $K_3Fe(CN)_6$*

Concentrations and conditions were the same as in Fig. 1. Curve *a*, hemolysis in the presence of phenylhydrazine and $K_3Fe(CN)_6$; *b*, effect of superoxide dismutase on hemolysis; *c*, effect of catalase; *d*, hemolysis of an oxygen-free suspension of carbonmonoxyhemoglobin-containing erythrocytes in the presence of phenylhydrazine and $K_3Fe(CN)_6$.

lase altered the rate of decrease of the absorbance at 576 nm of an oxyhemoglobin solution when phenylhydrazine was added (Fig. 5). Addition of the enzymes did not affect the over-all spectrum at the end of the reaction. Exposure of an aerated solution of carbonmonoxyhemoglobin to phenylhydrazine resulted in no conversion of the carbonmonoxyhemoglobin into other pigments (Fig. 5).

DISCUSSION

It is known from the experiments of Nizet (18) and of Beaven and White (19) that addition of phenylhydrazine to oxyhemoglobin results in the oxidation of the phenylhydrazine to benzene and molecular nitrogen. The oxidation of phenylhydrazine to benzene and nitrogen is a complex process and can proceed through several inter-

TABLE 1

Hemolysis of carbonmonoxyhemoglobin-containing red cells by phenyldiazene

The incubation mixture contained red cells (0.2% in 10 mM sodium phosphate buffer, pH 7.4, containing 135 mM NaCl and 10 mM glucose), phenylhydrazine intermediate (0.104 mM), superoxide dismutase (23.1 μg/ml) or catalase (2695 units/ml), α-chymotrypsin (0.36 unit/ml), and $K_3Fe(CN)_6$ (0.208 mM). Hemolysis was measured at room temperature as a decrease in light scattering at 740 nm. Experiments were performed under aerobic conditions unless otherwise noted. Refer to Scheme 1 for a description of the various intermediates of phenylhydrazine oxidation. Phenyldiazene was generated by the reaction of phenylhydrazine and $K_3Fe(CN)_6$ or methyl phenylazoformate and α-chymotrypsin. Neither $K_3Fe(CN)_6$ nor α-chymotrypsin caused lysis. Exposure time was 3 hr.

Intermediate	Lysis
Phenylhydrazine	–
Phenyldiazene	+
Phenyldiazene and superoxide dismutase	+
Phenyldiazene and catalase	+
Phenyldiazene (anaerobic)	–
Benzenediazonium (as the hexafluorophosphate)	–
Benzene	–

TABLE 2

Lipid peroxidation in carbonmonoxyhemoglobin-containing red cells exposed to phenyldiazene and oxygen

The incubation mixture contained red cells (7% in 10 mM sodium phosphate buffer, pH 7.4, containing 135 mM NaCl and 10 mM glucose), methyl phenylazoformate (0.919 mM), and superoxide dismutase (112 μg/ml) or catalase 1348 units/ml). Red cell samples were incubated with phenyldiazene for 2 hr at room temperature. Phenyldiazene was generated by hydrolyzing methyl phenylazoformate with alkaline buffer (50 mM sodium carbonate, pH 11.8) to phenyldiazenecarboxylate, which is moderately stable at alkaline pH (11). Addition of a small volume of the concentrated alkaline carboxylate dispersion to a red cell suspension buffered at pH 7.4 results in very rapid decarboxylation of the phenyldiazenecarboxylate to phenyldiazene (8, 11).

Conditions	Malonaldehyde formed
	nmoles/ml red cells
Control	0
+Phenyldiazene	81.6
+Phenyldiazene and superoxide dismutase	97.2
+Phenyldiazene and catalase	93.3

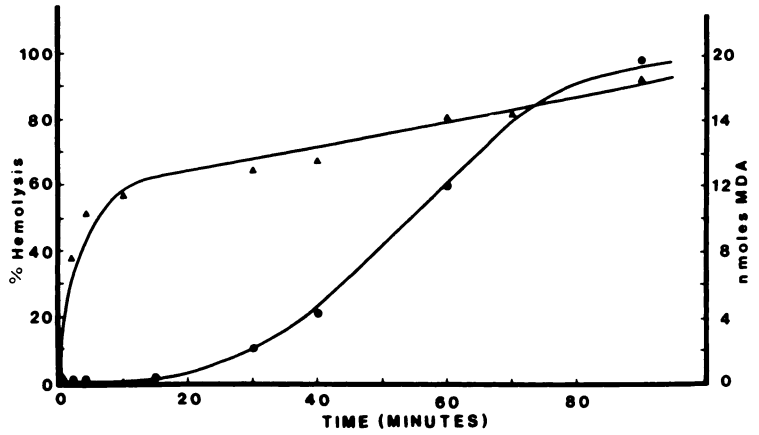


FIG. 3. *Temporal relationship between lipid peroxidation and hemolysis of an aerated suspension of carbonmonoxyhemoglobin-containing erythrocytes exposed to phenyldiazene*

The incubation mixture contained red cells (7% in 10 mM sodium phosphate buffer, pH 7.4, containing 135 mM NaCl and 10 mM glucose) and methyl phenylazoformate (0.919 mM). Phenyldiazene was generated by hydrolyzing methyl phenylazoformate with alkaline buffer (50 mM sodium carbonate, pH 11.8) to phenyldiazenecarboxylate, which is moderately stable at alkaline pH (11). Addition of a small volume of the concentrated alkaline carboxylate dispersion to a red cell suspension buffered at pH 7.4 results in very rapid decarboxylation of the phenyldiazenecarboxylate to phenyldiazene (8, 11). Hemolysis was followed at room temperature by removing a small aliquot of the phenyldiazene-treated red cell suspension, adding it to a cuvette of 1-cm path length, and following the decrease in light scattering at 740 nm due to cell disruption. Samples of the remaining reaction mixture standing at room temperature was analyzed at various time intervals for the lipid oxidation product malonaldehyde (MDA). ●, hemolysis; ▲, malonaldehyde formation.

mediates. A scheme for phenylhydrazine oxidation, in which it is evident that several highly reactive species are produced, is presented in Scheme 1.

Exposure of a purified oxyhemoglobin solution to phenylhydrazine or phenyldiazene results in degradation of the oxyhemoglobin to several products (15). This degradation is not inhibited by superoxide dismutase or by catalase, illustrating the lack of importance of O_2^- or H_2O_2 in the process. Carbonmonoxyhemoglobin present in aerated buffer to which phenylhydrazine has been added does not undergo oxidation or degradation. Interestingly, neither will phenyldiazene, the 2-electron oxidation product of phenylhydrazine, degrade carbonmonoxyhemoglobin in the presence of oxygen. This demonstrates that oxyhemoglobin not only oxidizes phenylhydrazine to phenyldiazene but also serves as a catalyst for its own destruction by phenyldiazene and oxygen.

Exposure of erythrocytes to an external source of phenyldiazene, generated either by the oxidation of phenylhydrazine with potassium ferricyanide or by using methyl phenylazoformate as a precursor, results in massive oxyhemoglobin breakdown in the cells, as expected. Although in previous work no hemolysis of ordinary red cells exposed to methyl phenylazoformate had been detected (14), we have observed hemolysis with phenylhydrazine and potassium ferricyanide. Conversion of the oxyhemoglobin in red cells to carbonmonoxyhemoglobin enables one to study hemolysis induced by phenyldiazene in the absence of hemoglobin breakdown. Carbonmonoxyhemoglobin-containing erythrocytes, when compared with oxyhemoglobin-containing erythrocytes, exhibited a marked increase in their rate of hemolysis when exposed to either of the phenyldiazene-generating systems and molecular oxygen. The reason for this enhanced sensitivity of carbonomonoxyhemoglobin-containing erythrocytes to phenyldiazene has been explained by Kosower *et al.* (14). They postulated that with oxyhemoglobin-containing erythrocytes, the reaction of phenyldiazene with oxygen occurs primar-

ily inside the cell, where the oxygen concentration is highest. The reactive radical generated by the phenyldiazene-oxygen interaction would then preferentially degrade the oxyhemoglobin. Replacement of the oxyhemoglobin in erythrocytes by carbonmonoxyhemoglobin not only would limit the reaction of phenyldiazene with oxygen to the outside of the cell, but also would block the breakdown of hemoglobin (14). Under these circumstances, any reactive species produced would attack princi-

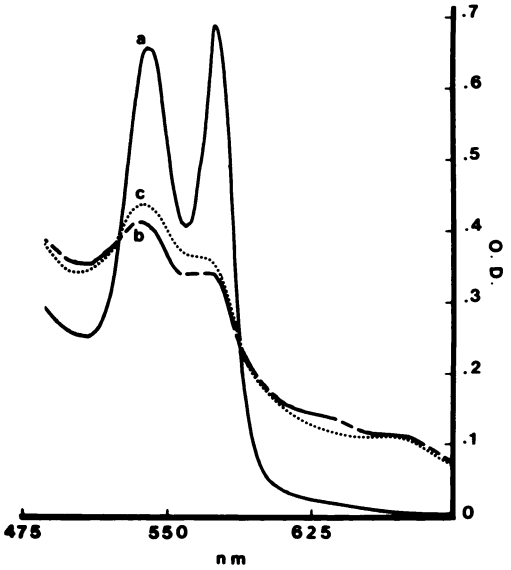


FIG. 4. *Effect of phenylhydrazine on optical spectrum of oxyhemoglobin*

The incubation mixture contained oxyhemoglobin (0.039 mM) and phenylhydrazine (0.104 mM) in 50 mM sodium phosphate buffer containing EDTA (100 μM) at pH 7 and 27°. Curve *a*, control, no phenylhydrazine added; *b*, phenylhydrazine added to oxyhemoglobin solution, spectrum recorded 5 min later; *c*, 20 μl of an aqueous solution of 0.1 M KCN, pH 7.4, added to 2.6 ml of reaction mixture *b*.

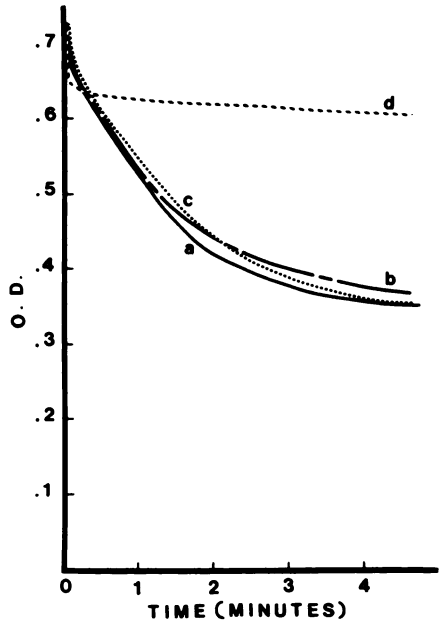
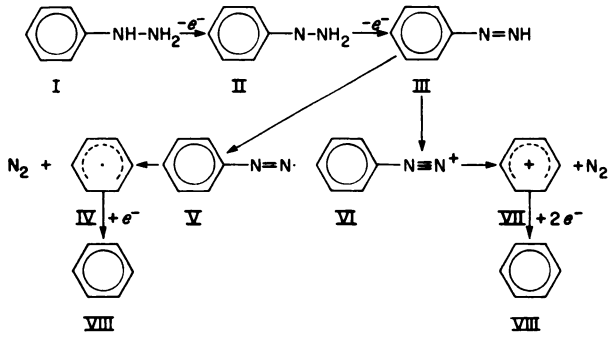


FIG. 5. *Kinetics of oxyhemoglobin destruction by phenylhydrazine*

Concentrations and conditions were the same as in Fig. 4. Curve *a*, addition of phenylhydrazine to an oxyhemoglobin solution; *b*, effect of superoxide dismutase on reaction *a*; *c*, effect of catalase on reaction *a*; with oxyhemoglobin destruction monitored as decrease in absorbance at 576 nm; *d*, addition of phenylhydrazine to an aerated solution of carbonmonoxyhemoglobin, with the reaction monitored at 569 nm.



SCHEME 1.

I, phenylhydrazine; II, phenylhydrazyl radical; III, phenyldiazene; IV, phenyl radical; V, phenyldiazenyl radical; VI, benzenediazonium ion; VII, phenyl cation; VIII, benzene.

pally the cell membrane, resulting in the accelerated hemolysis observed. Thus the destruction of oxyhemoglobin by phenylhydrazine and phenyldiazene may serve the useful purpose of scavenging reactive species in the red cell which otherwise would attack the cell membrane and lead to lysis.

Detection of the lipid breakdown product malonaldehyde in the extracellular medium of red cells containing carbonmonoxyhemoglobin and exposed to phenyldiazene and oxygen indicates that the lipids in the red cell membrane have undergone peroxidation. Lipid peroxidation has been discussed as a triggering mechanism for cell lysis (20). The present data for phenyldiazene-induced hemolysis show a relationship between lipid peroxidation and hemolysis, with the bulk of lipid peroxidation occurring prior to the onset of lysis. The recent demonstration of lipid peroxidation in the red cells of rabbits exposed to phenylhydrazine (21) is further evidence in favor of a phenyldiazene-oxygen interaction mediating hemolysis by phenylhydrazine. The lipid peroxidation and hemolysis experiments done with carbonmonoxyhemoglobin-containing red cells exposed to phenyldiazene illustrate that the cell can undergo membrane changes when hemoglobin breakdown does not occur. This contrasts with similar experiments with carbonmonoxyhemoglobin-containing red cells exposed to dihydroxyfumaric acid. In that case, membrane changes were not observed (22).

Experiments in which red cells were exposed to extracellular and intracellular sources of O_2^- (22, 23) illustrated that O_2^- does not produce lipid peroxidation in erythrocytes. The reaction of phenyldiazene with oxygen must therefore produce, in addition to O_2^-, an oxidant more powerful than O_2^-. This latter conclusion was confirmed by adding superoxide dismutase or catalase to the red cell medium containing phenyldiazene and oxygen, in order to intercept any reactive oxygen intermediates as they were produced. As these enzymes failed to protect either oxy- or carbonmonoxyhemoglobin-containing red cells from lipid peroxidation and hemolysis by phenyldiazene in the presence of oxygen, it is apparent that a species other than O_2^-, H_2O_2, or any product of their reaction is involved in the hemolytic effect of phenyldiazene.

What is the species generated by the reaction of phenyldiazene with oxygen that is responsible for phenyldiazene-induced hemolysis? As the reaction of phenyldiazene with oxygen yields O_2^-, the phenyldiazenyl free radical must initially be formed. The ultimate production of benzene and nitrogen by the autoxidation of phenyldiazene (24) indicates that molecular nitrogen is lost from the phenyldiazenyl radical. This would result in the formation of the phenyl free radical. The detection of biphenyl, as a secondary product of the univalent oxidation of phenyldiazene in the presence of benzene, has been cited as evidence for the formation of phenyl radical in the reaction (25). The phenyl radical would behave as a potent oxidant, which could easily abstract hydrogen atoms from such red cell constituents as the porphyrin ring of hemoglobin and the lipids of the cell membrane, thereby initiating free radical chain reactions resulting in the destruction of the cell. As the reactivity of the phenyl radical would be expected to far exceed that of O_2^-, the relative unimportance of the latter species in the red cell damage produced by a drug which generates both species simultaneously is not surprising.

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