Oxidative Degradation of Haemoglobin by Nitrosobenzene in the Erythrocyte

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Substitutions on the benzene ring of nitrosobenzene did not have the same effect on oxidative haemolysis as substitutions on phenylhydrazine. We previously found that the haemolytic effect of arylhydrazines paralleled their oxidative conversion into ligands of ferrihaemoglobin. In contrast, although most substituted nitrosobenzenes that are ligands of ferrohaemoglobin caused haemolysis and most that are not ligands failed to cause haemolysis, several exceptions to these relations were observed. The haemolytic effect of nitrosoarenes appeared to be related more closely to the ease of their reduction to arylhydroxylamines than to their properties as ligands. We propose a mechanism of oxidative degradation whereby the cyclic formation of phenylhydroxylamine from nitrosobenzene within an erythrocyte leads to the accumulation of H_2O_2 , which then reacts with ferrohaemoglobin to initiate the oxidative cleavage of haem. The postulated active intermediate in this reaction is the same as that previously proposed in the oxidative degradation of haemoglobin by phenylhydrazine and in the coupled oxidation of ascorbic acid and haemoglobin.

Haemolytic anaemia associated with the formation within erythrocytes of aggregates of denatured haemoglobin known as Heinz bodies results from the action of phenylhydrazine. The effectiveness of a ring-substituted phenylhydrazine in the induction of Heinz-body anaemia depends on the nature of its substitutions (Itano *et al.*, 1976). The binding of phenyldiazene, a product of oxidation of phenylhydrazine, to ferrihaemoglobin is also affected by ring substitutions, and the effectiveness of a substituted phenylhydrazine as a haemolytic agent parallels the ability of this product to bind to ferrihaemoglobin (Itano *et al.*, 1975, 1977). Nitrosobenzene, a ligand of ferrohaemoglobin, is iso-electronic with phenyldiazene. The induction of ferrihaemoglobinaemia by nitrosobenzene has been reported (Kiese, 1966), but the induction of anaemia has not. The induction of Heinz-body anaemia by nitrosobenzene and the effect on this process of substituents on the benzene ring of nitrosobenzene are reported here, and a mechanism by which nitrosobenzene results in the oxidative degradation of haemoglobin in erythrocytes is proposed.

Materials and Methods

Nitrosobenzene, 2-methylnitrosobenzene and ethyl 4-nitrosobenzoate (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) were recrystallized from ethanol. 3-Methylnitrosobenzene (m.p. 53–53.5°C) and 3-ethylnitrosobenzene (m.p. 49–50°C) were synthesized

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from the corresponding anilines (Bamberger, 1895; Lutz & Lytton, 1937). The preparation and purification of the other ring-substituted nitrosobenzenes of the present study have been described (Hirota & Itano, 1978). A fresh emulsion of each compound in aqueous 0.5% sodium alginate (Sigma Chemical Co., St. Louis, MO, U.S.A.) containing 20% (v/v) ethanol was prepared each day and stored at 4°C until injected. Daily subcutaneous injections of 150 μmol of test compound/kg body weight contained in 1.0 ml of emulsion were made into female domestic rabbits (*Oryctolagus cuniculus*) of 2.2 ± 0.25 kg body weight, and haematological measurements were performed as described previously (Itano *et al.*, 1976). Each packed cell volume was the mean of three determinations, and each reticulocyte count was the mean of three values based on a count of 500–1000 erythrocytes by each of three individuals. Of the indicators of degree of anaemia, packed cell volume is the one that can be determined with the greatest accuracy (Biggs & MacMillan, 1948). Reticulocytosis occurs in response to the loss or destruction of erythrocytes and is a reliable indicator of haemolysis in the absence of loss of blood from other causes.

Results

The dosage of nitrosobenzene required to produce an anaemia of severity comparable with that previously obtained with phenylhydrazine (Itano *et al.*, 1976) was determined. The daily dosage was 3 times

as great and had to be administered for 6 days instead of 4. The experimental protocol based on these determinations and the results obtained with several compounds are shown in Fig. 1.

Fig. 1. Severity of anaemia and reticulocytosis induced in rabbits with selected nitrosobenzene derivatives

For each kg of body weight, 150 μ mol of compound was injected daily for 6 days (arrows). The relative packed cell volume is 100 times the packed volume on a given day divided by the packed cell volume before the first injection. The values shown for relative packed cell volume (—) and reticulocyte counts (----) are the means of four or five determinations. Δ , Sodium alginate (control); $\blacktriangle$, nitrosobenzene; $\circ$, 2-chloronitrosobenzene; $\bullet$, 2-iodonitrosobenzene; $\square$, 2,6-di-isopropylnitrosobenzene.

The effects of size, position and number of halogen atoms are shown in Fig. 2. Each compound with one halogen atom induced haemolysis, and those with the smaller atoms at the *ortho*- (2-) position were the most effective. Compounds with a pair of halogen atoms at both *ortho*- (2- and 6-) positions induced little or no anaemia, and compounds with a pair of atoms elsewhere on the benzene ring induced intermediate degrees of anaemia. Similar results were obtained with alkyl substituents, as shown in Fig. 3. Compounds with a pair of *ortho*-groups were ineffective, and the 2,4-dimethyl and 3,5-dimethyl compounds were but slightly more effective. Fig. 4 shows that a carboxy group at either the *ortho*- or the *para*-position prevented the induction of anaemia, but that the ester (ethoxy-carbonyl) group did not. Examination of stained blood films revealed that all compounds that induced anaemia also resulted in Heinz bodies. Usually an erythrocyte contained only one inclusion, and this inclusion was located at the periphery of the cell.

For the most part, substitutions that blocked the haemolytic effect of phenylhydrazine did likewise with nitrosobenzene. These included paired *ortho*-substitutions and the carboxy group. Differences in the relative effects of the same substitutions on nitrosobenzene and phenylhydrazine were, however, found among compounds that induced anaemia. Nitrosobenzenes with an alkyl group at the *ortho*-position induced more anaemia than their respective *para*-isomers. Among the *ortho*-substituted nitrosobenzenes, the ethyl and isopropyl derivatives were more effective than the methyl. These effects of

Fig. 2. Relative packed cell volume and reticulocyte counts determined on blood samples from rabbits injected with halonitrosobenzenes

Relative packed cell volume (solid blocks) and reticulocyte counts (open blocks) were measured as in Fig. 1 and are the values obtained at day 7.

Fig. 3. Relative packed cell volume and reticulocyte counts determined on blood samples from rabbits injected with alkyl-nitrosobenzenes

Relative packed cell volume (solid blocks) and reticulocyte counts (open blocks) were measured as in Fig. 1 and are the values obtained at day 7.

Fig. 4. Relative packed cell volume and reticulocyte counts determined on blood samples from rabbits injected with nitrosobenzoic acids and their esters

Relative packed cell volume (solid blocks) and reticulocyte counts (open blocks) were measured as in Fig. 1 and are the values obtained at day 7.

position and size of alkyl substituents are the reverse of those previously obtained with correspondingly substituted phenylhydrazines. An ethyl ester group

at the *ortho*-position resulted in more anaemia than the same group at the *para*-position in nitrosobenzene, but the same substitutions resulted in the opposite relative effect in phenylhydrazine (Itano *et al.*, 1977).

Discussion

Substituents on the benzene ring of nitrosobenzene affect the equilibrium of its binding by ferrohaemoglobin and in some cases prevent binding altogether (Hirota & Itano, 1978). Most of the compounds that induced anaemia in the present work are ligands of ferrohaemoglobin, and most of the compounds that did not induce anaemia are not ligands. However, the 4-*t*-butyl and pentafluoro compounds, which are ligands, resulted in little or no haemolysis, and the 2-*t*-butyl and 2-ethoxycarbonyl compounds, which are not ligands, resulted in haemolysis. Thus binding of a nitrosoarene to ferrohaemoglobin appears not to be a necessary condition for nitrosoarene-induced haemolysis.

Inactive as a direct source of oxidant, nitrosobenzene is reduced enzymically in the erythrocyte to phenylhydroxylamine (Dannenberg & Kiese, 1950), which reacts with oxyhaemoglobin to produce H_2O_2 (Ellederová *et al.*, 1968). The participation of arylhydroxylamines in oxidative haemolysis is suggested by a comparison of the haemolytic efficacy of a substituted nitrosobenzene with the electrode potential of its reduction to the corresponding hydroxylamine. Lutz & Lytton (1937) found that the potential of the reversible reaction:

was greater for *ortho*-substituted nitrosobenzenes than for their respective *para*-isomers. In other words, an *ortho*-substituted compound was more readily reduced than its *para*-isomer. The only exception was *o*-iodonitrosobenzene, which was more difficult to reduce than its *para*-isomer. Each *ortho*-substituted halo, alkyl or ethoxycarbonyl compound in the present study resulted in more haemolysis than its *para*-isomer, again with the exception of *o*-iodonitrosobenzene. Therefore, among the isomeric pairs for which both haemolytic efficacy was determined in the present study and electrode potentials were determined by Lutz & Lytton (1937), the relative severity of the haemolysis induced by a nitrosoarene corresponds completely with the relative ease of reduction of the nitrosoarene to arylhydroxylamine.

Unlike phenylhydrazine, which causes the oxidative degradation of oxyhaemoglobin in aqueous solution as well as in the erythrocyte, phenylhydroxylamine produces ferrihaemoglobin, but little or no verdoglobin, in its reaction with oxyhaemoglobin in solution (Rostorfer & Cormier, 1957; Eyer *et al.*, 1975). The formation of ferrihaemoglobin and H_2O_2 from oxyhaemoglobin and a reductant such as phenylhydroxylamine results from the transfer of one electron each from ferrous haem and reductant to the liganded oxygen molecule:

where Hb is haemoglobin (Wallace & Caughey, 1975). However, Heinz bodies are not formed when reductants such as NaNO_2 and *p*-aminophenol react with oxyhaemoglobin to produce ferrihaemoglobin (Rentsch, 1968; Miller & Smith, 1970). Apparently the single of mol of H_2O_2 formed per mol of reductant consumed by this reaction does not suffice by itself to lead to the oxidative degradation of haemoglobin.

The oxidative degradation of haemoglobin by nitrosobenzene and phenylhydroxylamine must therefore be promoted by conditions within the erythrocyte. Nitrosobenzene is reduced enzymically in the erythrocyte to phenylhydroxylamine and is regenerated when the latter reacts with oxyhaemoglobin. This cycle can be repeated as many as 50 times with each molecule of phenylhydroxylamine (von Issekutz, 1939), and each cycle of reactions produces a molecule of H_2O_2 . Low concentrations of H_2O_2 in the erythrocyte are eliminated by glutathione peroxidase and higher concentrations by catalase; however, glutathione peroxidase and the enzymes that take part in the regeneration of glutathione are inactivated by sustained exposure to H_2O_2 in increased concentration (Cohen & Hochstein, 1963), and catalase is inhibited by phenylhydroxylamine (Seide, 1941). The introduction of nitrosobenzene into an erythrocyte accordingly leads not only to amplification of the

production of H_2O_2 , but also to impairment of the capacity of the cell to dispose of this oxidant.

Lemberg *et al.* (1939) proposed that the coupled oxidation of ascorbic acid and haemoglobin produces choleglobin via the formation of a complex, $\text{Hb(II)H}_2\text{O}_2$, of H_2O_2 with the ferrous haem of ferrohaemoglobin. Bonnett & Dimsdale (1972) suggested that hydroxylation of a *meso*-bridge carbon atom on the porphyrin ring of haem is accomplished by a hydroxyl radical released from a complex of H_2O_2 with pyridine ferrohaemochrome:

where Py is pyridine. In the presence of O_2 , oxidative cleavage of the porphyrin ring of haem follows the hydroxylation of a *meso*-bridge (Schmid & McDonagh, 1975). The requirement for ferrous haem as the reactive site of H_2O_2 may explain the virtual absence of oxidative degradation in the reaction of phenylhydroxylamine with oxyhaemoglobin in aqueous solution. H_2O_2 must transfer from its site of formation, which has become a ferric haem, to a ferrous haem at a different site. Each molecule of phenylhydroxylamine, in the absence of a mechanism for its regeneration from nitrosobenzene, can yield only one molecule of H_2O_2 , which consequently may not attain a concentration sufficient to compete successfully with O_2 for ferrohaemoglobin. Low partial pressure of O_2 was found by Lemberg *et al.* (1941) to favour the formation of choleglobin. The formation of $\text{Hb(II)H}_2\text{O}_2$ would therefore be especially favoured in venous blood that contains erythrocytes in which the concentration of H_2O_2 has been increased by repeated cycles of reduction of nitrosobenzene and oxidation of phenylhydroxylamine.

In the mechanism that we proposed for the oxidative action of phenylhydrazine (Itano *et al.*, 1977), $\text{Hb(II)H}_2\text{O}_2$ is formed in a single two-electron step by the direct transfer of two hydrogen atoms from phenylhydrazine to the liganded O_2 molecule of oxyhaemoglobin:

where Ph is phenyl. This reaction is consistent with the rapidity with which phenylhydrazine degrades haemoglobin in dilute solution as well as in the erythrocyte, because it requires neither accumulation of H_2O_2 by enzymic activity nor transfer of H_2O_2 from site of formation to site of oxidative reaction. Thus, although the pathway to its formation is different, the active intermediate that we postulated above in the oxidative action of nitrosobenzene and phenylhydroxylamine is the one that we previously postulated in the oxidative degradation of haemoglobin by phenylhydrazine; and this intermediate is the transient complex of H_2O_2 and ferrohaemoglobin proposed many years ago by Lemberg *et al.*

(1939) in the coupled oxidation of ascorbic acid and haemoglobin. The same intermediate would account for the formation of Heinz bodies by aniline and nitrobenzene inasmuch as both of these compounds are converted in the erythrocyte into phenylhydroxylamine and nitrosobenzene (Kiese, 1966). Indeed, a common basis for the oxidative activity of structurally dissimilar compounds may be their conversion into metabolites capable of repeated cycles of oxidation and reduction that lead to the accumulation of H_2O_2 , which can attack both haemoglobin and cell membrane. Whether oxidative haemolysis results from oxidative degradation of the haemoglobin molecule or oxidation of the membrane of the erythrocyte, or is the combined effect of both processes, remains to be determined.

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