Reactions of *N,N*-Dimethylaniline-*N*-oxide with Hemoglobin

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(Received July 12, 1966)

SUMMARY

N,N-Dimethylaniline-*N*-oxide reduces ferrihemoglobin (methemoglobin) under anaerobic conditions. In solutions of hemoglobin and *N,N*-dimethylaniline-*N*-oxide under air ferrihemoglobin is formed. Oxygen is necessary for this reaction. After a short lag phase the reaction rapidly increases in rate. Partial oxidation of the hemoglobin accelerates the reaction. Reagents which react with —SH groups also accelerate the reaction. However, the "autocatalytic" course is retained after the —SH groups are blocked.

INTRODUCTION

In a study of the role of *N,N*-dimethylaniline-*N*-oxide in the formation of ferrihemoglobin (methemoglobin) which is observed after the injection of *N,N*-dimethylaniline (1) it was observed that after the intravenous injection of the *N*-oxide the formation of ferrihemoglobin begins slowly and then increases in rate.

A similar course of reaction has been observed with ferrihemoglobin formation by nitrite (2–4), chlorate (5–7), and phenylenediamines (Kiese and Rauscher, in preparation). Since various mechanisms were found to cause this course of reaction, it appeared necessary to study the reaction between *N,N*-dimethylaniline-*N*-oxide and hemoglobin more closely.

METHODS

N,N-Dimethyl- and *N,N*-diethylaniline-*N*-oxide were prepared by oxidizing the amines with hydrogen peroxide according to Belov and Savich (8) and isolating the hydrochlorides of the *N*-oxides as described by Bamberger and Leyden (9). Ziegler and Pettit's (10) method was used for determining the *N*-oxides.

Beef hemoglobin was used in all experiments. The red cells were washed three times with several volumes of 0.9% sodium chloride solution. For experiments with red cells the washed cells were suspended in an equal volume of Krebs-Ringer phosphate solution. Hemoglobin solutions were prepared by hemolyzing washed red cells with water and separating the cell membranes in the centrifuge. Ferrihemoglobin solutions were prepared from red cells which had been incubated with nitrite and then had been washed four times with several volumes of 0.9% sodium chloride solution.

Ferrihemoglobin was determined by the increase in extinction at 550 mμ caused by adding cyanide to a suitably diluted sample of the reaction mixture.

In order to remove the oxygen from hemoglobin solutions or suspensions of red cells, nitrogen was passed over them for 1 hour at a rate of 4 liters per minute while they were spread as a thin fast-moving film on the wall of a rapidly rotated cylinder (11). In order to reduce hemolysis of red cell suspensions by this procedure, 2.5% polyvinylpyrrolidone (kollidon) was added to the suspensions. Furthermore, when the oxygen had been removed and the *N*-oxide had been added, the rapid rotation of the red cells was limited to periods of 1 min after samples had been taken from the suspensions. All experiments were carried out at 37°. When the effect of reagents which react with —SH groups was studied, hemoglobin solutions

were incubated, before the addition of *N,N*-dimethylaniline-*N*-oxide, for half an hour at 37° with 5×10^{-3} M sodium *p*-chloromercuribenzoate, 10^{-2} M *N*-ethylmaleimide, or 2.5×10^{-2} M iodoacetamide. These concentrations had been found most effective in experiments relating to ferrihemoglobin formation by durenediamine (Kiese and Rauscher, in preparation).

RESULTS

Reduction of Ferrihemoglobin by N,N-Dimethylaniline-N-oxide

N,N-Dimethylaniline-*N*-oxide reduces ferrihemoglobin. Figure 1 shows the results of experiments with solutions of about 3.5×10^{-3} equivalent ferrihemoglobin and 10^{-2} M *N,N*-dimethylaniline-*N*-oxide under carbon monoxide. After the *N*-oxide had been added to the ferrihemoglobin solution, the reaction did not begin at full speed but increased in rate. This course of reaction is even more evident with the much more slowly reacting *N,N*-diethylaniline-*N*-oxide. About 2 moles of *N,N*-dimethylaniline-*N*-oxide were found to disappear with each equivalent of ferrihemoglobin reduced.

Previous incubation of the ferrihemoglobin solution with 5×10^{-3} M *p*-chloromercuribenzoate did not change the initial rate of decrease in *N*-oxide concentration. But the rate of ferrihemoglobin reduction increased to more than twice the velocity observed in the absence of *p*-chloromercuribenzoate. After all the ferrihemoglobin was reduced the concentration of the *N*-oxide remained constant. Only about 1 mole of *N,N*-dimethylaniline-*N*-oxide was found to disappear with the reduction of one equivalent of ferrihemoglobin in the presence of *p*-chloromercuribenzoate.

With constant initial concentrations of

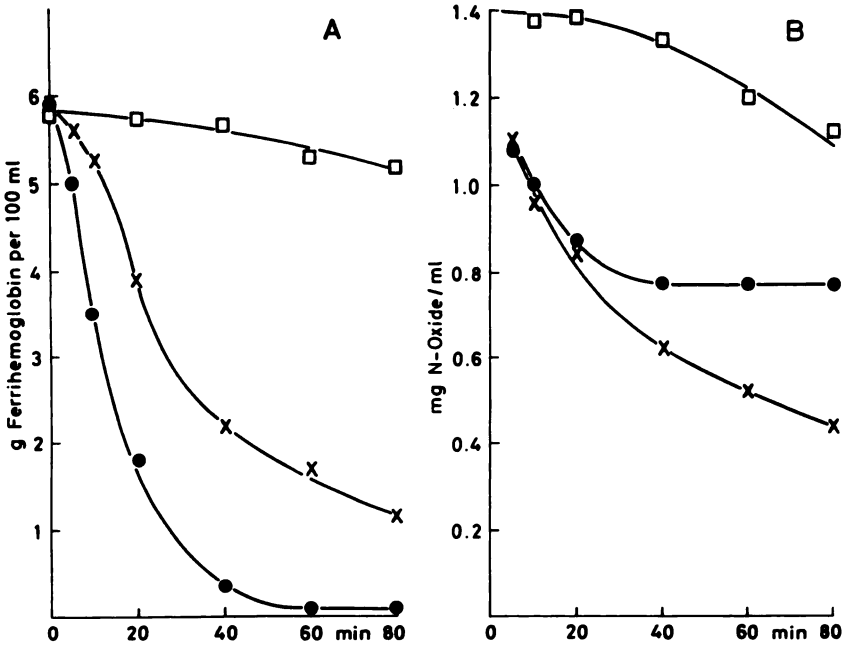

FIG. 1. *Reduction of ferrihemoglobin (A) and disappearance of N,N-dimethylaniline-N-oxide and N,N-diethylaniline-N-oxide (B) under carbon monoxide at 37°*

The ferrihemoglobin concentration was about 6 g/100 ml (3.5×10^{-3} equivalent per liter); the concentration of *N*-oxides was 10^{-3} M. ×, Solutions of ferrihemoglobin and *N,N*-dimethylaniline-*N*-oxide; ●, solutions of ferrihemoglobin which had been incubated with 5×10^{-3} M *p*-chloromercuribenzoate before adding *N,N*-dimethylaniline-*N*-oxide; □, solutions of ferrihemoglobin and *N,N*-diethylaniline-*N*-oxide. The symbols indicate the averages of three experiments.

N,N-dimethylaniline-*N*-oxide the velocity of ferrihemoglobin reduction was observed to increase with the ferrihemoglobin concentration. The increase in reaction rate exceeds the increase in ferrihemoglobin concentration. In view of the complicated kinetics it is hard to define the relationship between the ferrihemoglobin concentration and the rate of its reduction more closely.

If *N,N*-diethylaniline-*N*-oxide and ferrihemoglobin were allowed to react under air the ferrihemoglobin concentration decreased only a little. The *N*-oxide, however, disappeared more rapidly than in the absence of oxygen. After 80 min it was no longer detected in the reaction mixtures.

N,N-Diethylaniline-*N*-oxide was found to react much more slowly with ferrihemoglobin than the dimethyl homolog. In a solution of about 3.5×10^{-3} equivalent of ferrihemoglobin per liter and 10^{-2} M *N,N*-diethylaniline-*N*-oxide, during 80 min incubation under carbon monoxide, 0.5×10^{-3} equivalent of ferrihemoglobin and 10^{-3} M *N*-oxide disappeared (see Fig. 1).

Formation of Ferrihemoglobin by N,N-Dimethylaniline-N-oxide

The course of the reaction and the role of oxygen; effect of —SH reagents. The characteristic course of the increase in ferrihemoglobin concentration in red cells caused by 10^{-2} M *N,N*-dimethylaniline-*N*-oxide under air is shown in Fig. 2. This course of the reaction is observed not only with hemoglobin within red cells, but also with hemoglobin in solution. The effect of hemoglobin concentration in solutions of hemoglobin on the reaction rate has been studied with solutions containing 4–12 g of hemoglobin per 100 ml. As calculated from the phase of most rapid increase in ferrihemoglobin concentration in three experiments, the velocity of ferrihemoglobin formation was found to be proportional to the hemoglobin concentration.

If suspensions of red cells were freed from oxygen by passing nitrogen over them, ferrihemoglobin formation by *N,N*-dimethylaniline-*N*-oxide was strongly inhibited, as may be seen in Fig. 2. The oxygen content of the red cell suspensions kept under nitrogen was not determined. Therefore it cannot be decided whether the very slow formation of ferrihemoglobin was due to oxidation of ferrohemoglobin by the *N*-oxide or whether traces of oxygen were involved in the reaction. In any case the data shown in Fig. 2 clearly illustrate the importance of oxygen for ferrihemoglobin formation by *N,N*-dimethylaniline-*N*-oxide.

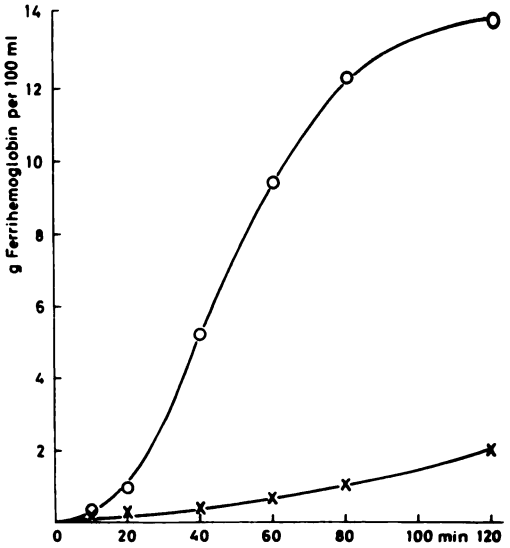

FIG. 2. *Formation of ferrihemoglobin by 10^{-2} M N,N-dimethylaniline-N-oxide in suspensions of red cells containing 16–20 g hemoglobin per 100 ml under air and under nitrogen at 37°*

In the experiments under nitrogen, before addition of the *N*-oxide oxygen was removed by passing nitrogen over suspensions rotated at 1400 rpm for 1 hour. The cell suspensions kept under air underwent the same treatment in order to avoid any effect of the technique of removing oxygen from red cell suspensions. The symbols indicate the averages of three experiments. ○, Suspensions of red cells under air; ×, suspension of red cells under nitrogen.

Pretreatment of the red cell suspensions with reagents which react with —SH groups increased the velocity of the reaction of *N,N*-dimethylaniline-*N*-oxide with oxyhemoglobin. However, it did not change the course of the reaction, i.e., the increase in rate after some ferrihemoglobin had been formed (Fig. 3).

The effect of partial oxidation of hemo-

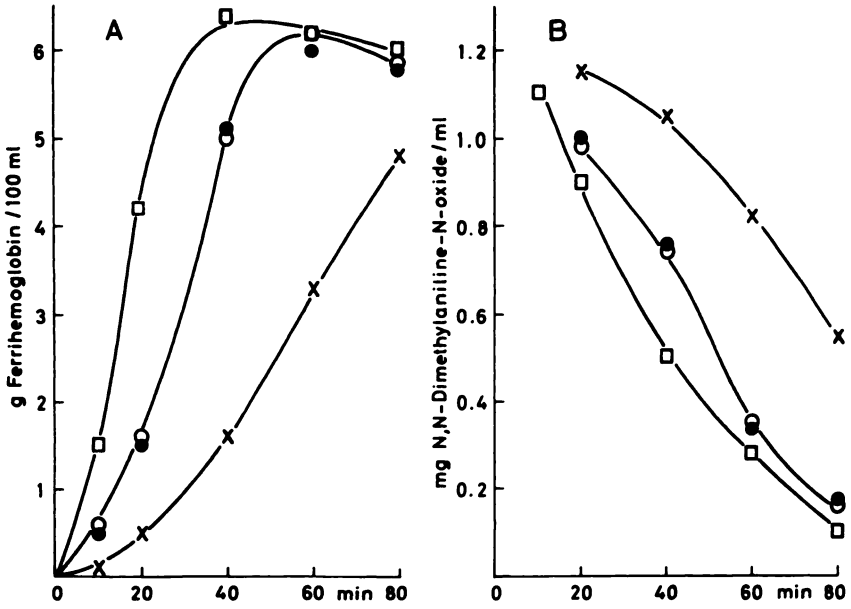

FIG. 3. *The effect of —SH reagents on the rate of ferrihemoglobin formation (A) and disappearance of 10^{-2} M N,N-dimethylaniline-N-oxide (B) in solutions of about 7 g hemoglobin per 100 ml under air at 37°*

The symbols indicate the means of three experiments. ×, No —SH reagent added; □, 10^{-2} M *N*-ethylmaleimide added before the *N*-oxide; ○, 2.5×10^{-2} M iodoacetamide added before the *N*-oxide; ●, 5×10^{-3} M *p*-chloromercuribenzoate added before the *N*-oxide.

globin on the formation of ferrihemoglobin by N,N-dimethylaniline-N-oxide and oxygen. The course of ferrihemoglobin formation by *N,N*-dimethylaniline-*N*-oxide in the presence of oxygen suggests that a reaction product enhances the reaction. In order to test whether the partial oxidation of hemoglobin enhances the reaction, the ferrihemoglobin formation in solutions of hemoglobin of which about 30% had been oxidized to ferrihemoglobin was compared with the ferrihemoglobin formation in solutions with the same content of ferrohemoglobin equivalents.

For partial oxidation of the hemoglobin, washed red cells were suspended in an equal volume of 0.9% sodium chloride solution. To 100 ml of red cell suspension, 16 mg sodium nitrite was added. After being kept for 30 min at room temperature, the cells were washed four times with several volumes of isotonic sodium chloride solution. Then about 30% of the hemoglobin was found to be oxidized to ferrihemoglobin.

Figure 4 shows the formation of ferrihemoglobin in solutions of hemoglobin of which about 30% had been previously oxidized and in solutions with the same ferrohemoglobin content and a negligible percentage of ferrihemoglobin. A similar accelerating effect was observed if a mixture of ferrohemoglobin and ferrihemoglobin was used in place of partially oxidized hemoglobin. The concentration of *N,N*-dimethylaniline-*N*-oxide was found also to decrease more rapidly in the solutions which contained ferrihemoglobin at the onset of the reaction.

Formation of Ferrihemoglobin by N,N-Diethylaniline-N-oxide

N,N-Diethylaniline-*N*-oxide was found to reduce ferrihemoglobin much more slowly than *N,N*-dimethylaniline-*N*-oxide (Fig. 1). The results presented in Fig. 5 show that the reaction with oxyhemoglobin, which produces ferrihemoglobin, also proceeds more slowly than the reaction between *N,N*-dimethylaniline-*N*-oxide and

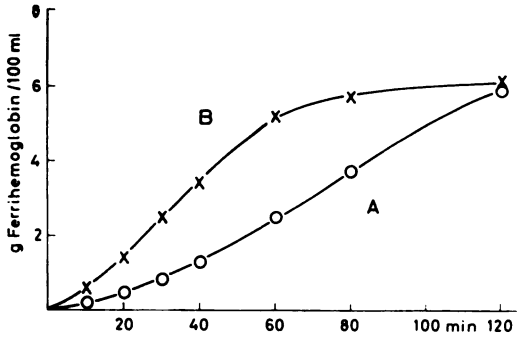

FIG. 4. *The influence of partial oxidation of hemoglobin on the formation of ferrihemoglobin by N,N-dimethylaniline-N-oxide under air*

Curve *A*. Initial concentration of hemoglobin 7.5 g per 100 ml with a negligible amount of ferrihemoglobin. Curve *B*. Initial concentration of hemoglobin 10.7 g per 100 ml of which 3.1 g/100 ml was oxidized to ferrihemoglobin. The curves show the increase in ferrihemoglobin concentration beyond the ferrihemoglobin present at the onset of the reaction. *N,N*-dimethylaniline-*N*-oxide 10^{-2} M, temperature 37°. The symbols indicate the means of three experiments.

oxyhemoglobin. The decrease in *N*-oxide concentration corresponds to the rate of ferrihemoglobin formation. In 80 min the concentration of *N,N*-diethylaniline-*N*-oxide decreased by only about 10%, whereas about 75% of the *N,N*-dimethylaniline-*N*-oxide had disappeared in the same time.

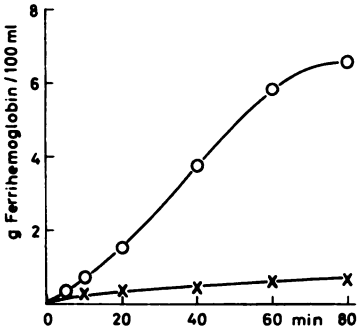

FIG. 5. *Formation of ferrihemoglobin by 10^{-2} M N,N-dimethylaniline-N-oxide and N,N-diethylaniline-N-oxide in solutions containing about 8.5 g hemoglobin per 100 ml under air at 37°*

The symbols indicate the averages of three experiments. ○, *N,N*-dimethylaniline-*N*-oxide; ×, *N,N*-diethylaniline-*N*-oxide.

DISCUSSION

The formation of ferrihemoglobin by *N,N*-dimethylaniline-*N*-oxide is not due to a direct oxidation of the ferrohemoglobin by the *N*-oxide. Oxygen is needed for the reaction. Since the *N*-oxide is rather stable in aqueous solution under air, oxyhemoglobin must be involved in the reaction. The reaction of oxyhemoglobin with *N,N*-dimethylaniline-*N*-oxide either yields ferrihemoglobin directly or produces a substance which on its part oxidizes ferrohemoglobin. A mechanism of the latter type seems to act when other reducing substances like arylhydroxylamines form ferrihemoglobin (12).

Apart from the reduction of ferrihemoglobin by *N,N*-dimethylaniline-*N*-oxide, other reactions complicate the course of ferrihemoglobin formation by the *N*-oxide. The reaction increases in rate after part of the hemoglobin is oxidized. Partial oxidation of the hemoglobin by another agent also accelerates the reaction. The mechanism by which the "autocatalytic" effect of ferrihemoglobin works is not yet understood. Oxidation of an iron atom in the hemoglobin molecule increases the affinity of the other ones for oxygen (13, 14). Heme-heme interaction may facilitate the reaction of oxygenated hemes with the *N*-oxide once one heme is oxidized. Another mechanism could be an activation of the *N*-oxide by combining with the ferriheme.

A third possibility involving reactions of —SH groups seems to be ruled out by the results presented. Pretreatment of the hemoglobin with —SH reagents accelerates its reaction with *N,N*-dimethylaniline-*N*-oxide. In a study of the reaction of phenylenediamines with hemoglobin, it has been observed that derivatives which oxidize the iron in hemoglobin also react with —SH groups of the protein. The data presented in Fig. 1 show that *N,N*-dimethylaniline-*N*-oxide or a derivative also react with —SH groups. However, the progressive blocking of —SH groups cannot be the full explanation for the autocatalytic type of reaction, because it is also observed after the —SH groups have

reacted with *p*-chloromercuribenzoate, *N*-ethylmaleimide, or *p*-iodoacetamide.

ACKNOWLEDGMENT

The author is grateful to Miss Maria Arnold and Miss Luise Richter for competent technical assistance.

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