Generation of Hydrogen Peroxide During the Reaction of Nitrite with Oxyhemoglobin*

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3-Amino-1,2,4-triazole was employed to detect H_2O_2 generated during the reaction of nitrite with oxyhemoglobin. Aminotriazole inhibited erythrocyte catalase by reacting with catalase-H_2O_2 complex I. Catalase was protected against inactivation by higher concentrations of nitrite which decomposed complex I in a peroxidatic reaction. The failure of nitrite to act as a hemolytic agent in glucose-6-phosphate dehydrogenase–deficient individuals, despite its ability to generate H_2O_2 and to oxidize both hemoglobin and reduced glutathione, is attributed to: (1) the limited generation of H_2O_2 and (2) the ability of nitrite to act as a substrate for catalase.

Individuals with deficient levels of glucose-6-P dehydrogenase are sensitive to a variety of hemolytic drugs (Beutler, 1960; Tarlov *et al.*, 1962). Oxidation of hemoglobin is an often-noted accompaniment to erythrocyte destruction in these individuals (Brewer *et al.*, 1962).

Recently, Harley and Robin (1962) demonstrated that the methemoglobin-forming agent sodium nitrite, added to glucose-6-P dehydrogenase–deficient erythrocytes *in vitro*, promoted a loss of reduced glutathione. The lability of GSH in glucose-6-P dehydrogenase–deficient erythrocytes after administration of hemolytic drugs both *in vivo* and *in vitro* has been well documented (Beutler, 1957, 1959); oxidation of GSH appears to be a necessary prerequisite to erythrocyte destruction. However, it is known that nitrite does not produce hemolysis (Brewer *et al.*, 1962).

It has been proposed that the instability of GSH in glucose-6-P dehydrogenase–deficient cells is brought about via oxidation by H_2O_2 catalyzed by GSH peroxidase (Mills and Randall, 1958; Cohen and Hochstein, 1961, 1964). Hence the observations of Harley and Robin represented tentative indication for the generation of H_2O_2 during the oxidation of oxyhemoglobin by nitrite. In the experiments described in this current report, 3-amino-1,2,4-triazole was employed as a tracer to detect H_2O_2 generation in erythrocyte lysates incubated with sodium nitrite. Aminotriazole inhibits catalase irreversibly by reacting with catalase-H_2O_2 complex I (Margoliash *et al.*, 1960); that is, the reaction requires the presence of H_2O_2 (Fig. 1). The aminotriazole technique has been employed to detect H_2O_2 in intact erythrocytes incubated *in vitro* with hemolytic agents (Cohen and Hochstein, 1964). The failure of $NaNO_2$ to act as a hemolytic agent can be explained, and is discussed.

METHODS

The methodology was as described in the preceding publication (Cohen and Hochstein, 1964), with the following exceptions: Heparinized blood specimens, either untreated or first incubated with excess $NaNO_2$ (25 mg/ml packed cells) at room temperature for 30 minutes to oxidize hemoglobin, were washed five times with 5 volumes 0.9% saline. The cells were then mixed with 19 volumes 0.1% sodium phosphate buffer *p*H 7.4 for the preparation of lysates, or with 0.9% buffer for the preparation of cell suspensions. The 0.1% buffer was employed for experiments with crystalline catalase.

Experimental samples (4 ml) were incubated for 1 hour at 37° in the presence or absence of 3-amino-1,2,4-triazole (0.18 M). At the end of the incubation period the samples were treated with 2 ml buffered 0.45 M ethanol for 20 minutes at room temperature as a precaution to decompose any catalase-H_2O_2 complex II (Chance, 1950a). Although nitrite also decomposes complex II (Keilin and Nicholls, 1958) it appeared advisable to employ ethanol routinely in order to provide similar treatment for the control specimens.

RESULTS

Figure 2 illustrates that the addition of sodium nitrite to erythrocyte lysates results in inhibition of catalase by aminotriazole (curve A). The initial increase in inhibition with increasing dose of nitrite appeared to follow the extent of hemoglobin oxidation (see legend, Fig. 2). At higher nitrite concentrations, less inhibition of catalase was observed; this result is in accord with the ability of nitrite to serve as a substrate for catalase-H_2O_2 complex I (cf. Discussion).

Curve B shows that no inhibition of catalase was observed in lysates prepared from methemoglobin-containing cells. In addition no inhibition was observed when the addition of aminotriazole was delayed until after the nitrite and oxyhemoglobin had reacted (curve C). These data illustrate a requirement for the presence of oxyhemoglobin.

In these experiments, the samples were diluted 1000-fold in order to analyze for catalatic activity. When the residual nitrite in the assay medium exceeded 2×10^{-6} M, an inhibition of catalatic activity was observed both in the presence or absence of aminotriazole. This effect did not require incubation of experimental samples with concentrated nitrite, but could be reproduced by incorporating the calculated dilution of nitrite into the catalase assay medium. The effect is illustrated in Table I with crystalline catalase: The entire "apparent" aminotriazole inhibitory action (column A) could be attributed solely to the presence of nitrite (column B). In contrast, the "true" aminotriazole inhibitory action illustrated in Figure 2 required the presence of oxyhemoglobin, and could not be reproduced simply by incorporating the appropriate dilution of nitrite into the assay medium.

In experiments with intact erythrocytes it was demonstrated that this effect with nitrite alone was

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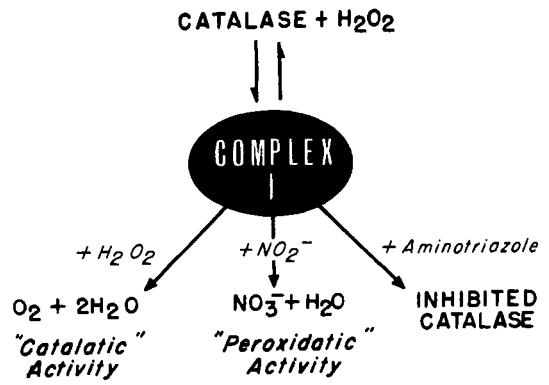

FIG. 1.—Reactions of catalase-H_2O_2 complex I.

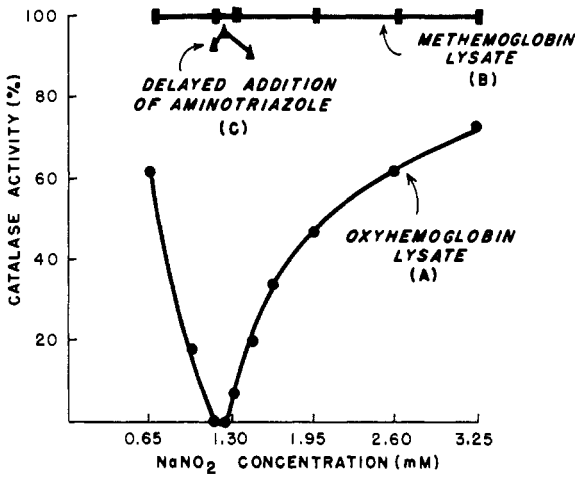

FIG. 2.—Inhibition of catalase by aminotriazole in lysates treated with sodium nitrite. For curve C, samples were incubated with $NaNO_2$ for 1 hour, then aminotriazole was added and the samples were reincubated for another hour. For curve A, the extent of oxidation of hemoglobin at the end of the incubation period was estimated by measurements at the 630 mμ absorption peak of methemoglobin. The following values were observed: at 0.65 mM $NaNO_2$, 68%; at 0.98 mM $NaNO_2$, 87%; at 1.13 mM $NaNO_2$, 100%; all others, 100%.

reversible: It could be eliminated by washing the cells several times with saline to remove the excess nitrite prior to preparing lysates for analyses. For example, in an experiment with erythrocytes at a 10% hematocrit incubated with 0.14 M nitrite for 1 hour, the following catalase activities were observed: control, 100%; nitrite, unwashed, 15%; nitrite, washed, 96%. Reversible inhibition of catalatic activity can be explained in terms of the ability of nitrite to act as a substrate for catalase-H_2O_2 complex I (cf. Discussion).

In Figure 2, the amounts of nitrite added to the lysates were small enough so that no inhibition by nitrite alone was observed after dilution of samples containing up to 2 mM nitrite. For higher concentrations of nitrite, the data obtained with aminotriazole (curve A) were corrected for small inhibitions of less than 10% observed with nitrite alone.

DISCUSSION

During the oxidation of oxyhemoglobin by nitrite very little oxygen is evolved (Marshall and Marshall, 1945). Other oxidants, such as ferricyanide, liberate stoichiometric quantities of oxygen upon conversion of ferrous heme to the ferric form (Marshall and Marshall, 1945; Peters and Van Slyke, 1932). The H_2O_2 detected in our experiments with sodium nitrite may have been derived from the oxygen which was originally bound to ferrous hemoglobin. Nitrite may act in part to destabilize the oxygen-hemoglobin complex and to promote oxidation of hemoglobin by oxygen; the H_2O_2 would then represent the reduced form of the oxidizing agent.

TABLE I
THE EFFECT OF NITRITE ON CATALASE ANALYSES[a]

	Final $NaNO_2$ Concentration in Assay Medium (M)	Catalatic Activity %: Preincubation with Nitrite and Aminotriazole (A)	Catalatic Activity %: No Aminotriazole. No Preincubation with Nitrite (B)
1.	0.0	100	100
2.	1.1×10^{-6}	96	104
3.	1.9×10^{-6}	96	90
4.	3.2×10^{-6}	92	94
5.	1.3×10^{-5}	80	72
6.	1.3×10^{-4}	29	20

[a] The samples contained crystalline catalase at a concentration of 0.125 mg/ml; the catalase activity was equivalent to that in the lysates in Fig. 2. The samples of column A were incubated with $10^3 \times$ the nitrite concentrations listed in the table; samples were subsequently diluted 1000-fold during analyses. The initial concentrations of nitrite in samples 2, 3, and 4 were identical to the initial concentrations for the specimens representing the third, eighth, and last points, respectively, in Fig. 2, curve A. The samples in column B were incubated in the absence of aminotriazole or nitrite; the nitrite was subsequently incorporated into the assay medium for the diluted samples.

The ability of nitrite to act as a substrate for catalase-H_2O_2 complex I has been studied by Chance (1950b) and by Keilin and Nicholls (1958). Nitrite, by competing with H_2O_2 for reaction with complex I (cf. Fig. 1), can inhibit the rate of decomposition of H_2O_2 (Table I). Decomposition of complex I explains the lack of marked inhibition of catalase by aminotriazole at high nitrite concentrations. In Figure 2, the strongest inhibition of catalase was observed when the amount of added nitrite corresponded closely to that required for total oxidation of hemoglobin. At higher nitrite concentrations the excess nitrite was competitive with aminotriazole for reaction with complex I, and thereby was increasingly effective in preventing irreversible inhibition of catalase. For example, when nitrite was added to a final concentration of 1.95×10^{-3} M (curve A) the excess nitrite was present at a concentration of roughly 8×10^{-4} M (cf. legend, Fig. 1), which is sufficient for rapid decomposition of complex I as indicated by marked interference with catalatic activity (Table I). The ability of higher concentrations of nitrite (viz., 10^{-1} M) to protect crystalline catalase from inactivation by aminotriazole in the presence of H_2O_2-generating agents had been noted previously by Margoliash *et al.* (1960). In the experiment illustrated in Figure 2, nitrite was acting both to generate H_2O_2 and to protect catalase. Hence inhibition was observed best in the narrow range where the balance between the two effects was favorable.

In experiments with methemoglobin-containing lysates, the conversion of oxyhemoglobin to methemoglobin was carried out by treatment of intact cells with excess nitrite. The nitrite was removed by multiple washings with saline, and the cells were then lysed for the experiment. Hence, in curve B of Figure 2, it was the absence of oxyhemoglobin and not the presence of residual nitrite which protected catalase against inhibition when the experimental dose of nitrite was

added. This point was confirmed in curve C where no excess nitrite was present.

The generation of H_2O_2 during the oxidation of hemoglobin by nitrite explains the loss in GSH noted by Harley and Robin (1962) for glucose-6-P dehydrogenase-deficient cells incubated with sodium nitrite. The erythrocyte enzyme GSH peroxidase catalyzes the oxidation of GSH by H_2O_2 (Mills, 1959); the loss in GSH is not readily reversed in glucose-6-P dehydrogenase-deficient cells (Cohen and Hochstein, 1961) due to insufficient generation of NADPH required for GSSG reductase activity. The increased sensitivity of glucose-6-P dehydrogenase-deficient individuals to methemoglobin formation during administration of sodium nitrite (Brewer *et al.*, 1962) may be related to the instability of GSH: After the GSH level has declined, the H_2O_2 can contribute to the oxidation of hemoglobin (Cohen and Hochstein, 1963).

The failure of nitrite to produce hemolysis *in vivo* despite its ability to generate H_2O_2 and to oxidize hemoglobin might appear to be contradictory to the concept that H_2O_2 generation is an important factor in the drug-induced hemolytic anemias. However, it must be emphasized that nitrite possesses only a limited capacity to generate H_2O_2 since generation must cease after the hemoglobin has been oxidized. Furthermore, nitrite, by functioning as a substrate for complex I, can act *in vivo* to detoxify intracellular H_2O_2. In this manner, nitrite may compensate for the diminished GSH peroxidase pathway of glucose-6-P dehydrogenase-deficient individuals. It should be noted that decomposition of H_2O_2 by catalatic activity does not appear to be an important pathway in erythrocytes (Cohen and Hochstein, 1963). The failure of nitrite to act as an hemolytic agent is therefore not at all in conflict with the concept that hemolytic agents act by generating H_2O_2. Nitrite merely represents an example of an agent whose toxicity is self-limited because the necessary cofactor (oxyhemoglobin) is removed during the process of H_2O_2 generation, and because nitrite can act to detoxify H_2O_2 as well as to generate it.

References

Beutler, E. (1957), *J. Lab. Clin. Med. 49*, 84.

Beutler, E. (1959), *Blood 14*, 103.

Beutler, E. (1960), *in* The Metabolic Basis of Inherited Disease, Stanbury, J. B., Wyngaarden, J. B., and Fredrickson, D. S., eds., New York, McGraw-Hill, p. 1031.

Brewer, G. J., Tarlov, A. R., Kellermeyer, R. W., and Alving, A. S. (1962), *J. Lab. Clin. Med. 59*, 905.

Chance, B. (1950a), *Biochem. J. 46*, 387.

Chance, B. (1950b), *J. Biol. Chem. 182*, 649.

Cohen, G., and Hochstein, P. (1961), *Science 134*, 1756.

Cohen, G., and Hochstein, P. (1963), *Biochemistry 2*, 1420.

Cohen, G., and Hochstein, P. (1964), *Biochemistry 3*, 895 (accompanying paper).

Harley, J. D., and Robin, H. (1962), *Blood 20*, 710.

Keilin, D., and Nicholls, P. (1958), *Biochim. Biophys. Acta 29*, 302.

Margoliash, E., Novogrodsky, A., and Schejter, A. (1960), *Biochem. J. 74*, 339.

Marshall, W., and Marshall, C. R. (1945), *J. Biol. Chem. 158*, 187.

Mills, G. C. (1959), *J. Biol. Chem. 234*, 502.

Mills, G. C., and Randall, H. P. (1958), *J. Biol. Chem. 232*, 589.

Peters, J. P., and Van Slyke, D. D. (1932), Quantitative Clinical Chemistry, Vol. II, Methods, Baltimore, Williams and Walker, p. 229.

Tarlov, A. R., Brewer, G. J., Carson, P. E., and Alving, A. S. (1962), *Arch. Internal Med. 109*, 209.

Effect of Ligands and Oxidation State upon the Reaction of Myoglobin and Hemoglobin with Zinc*

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Additional supporting evidence is presented for the previous proposal that the *rate-controlling* step in the Zn-mediated suppression of the Soret band of myoglobin and hemoglobin involves macromolecular conformational changes concomitant with rupture of the otherwise inaccessible iron-imidazole linkage and occupancy of the imidazole group by Zn. Thus, carbonmonoxymyoglobin and carbonmonoxyhemoglobin are very much less reactive toward Zn than their corresponding ferriproteins, and the Zn-reacted ferroproteins are very sensitive to oxidation to the ferri state. Also, ferricytochrome c with its covalent iron-ligand bonds is spectrally refractory to Zn. The greater reactivity of ferrimyoglobin and ferrihemoglobin as compared with their corresponding ferro- forms is conceivably related to charge transfer from the ligands to the iron in the former.

Recently (Cann, 1963, 1964) it was shown that reaction of Zn ions with sperm whale ferrimyoglobin at *p*H 6.4 causes major changes in the ultraviolet and visible absorption spectra of the protein as well as loss of solubility at salt concentrations as low as 0.25 M and increased sensitivity to tryptic digestion. It seems clear, therefore, that Zn-reacted myoglobin is conformationally quite different from the unreacted protein. Reaction of the protein with Zn can be reversed to yield renatured ferrimyoglobin by one of the following three methods: (a) lowering the Zn concentration by dilution; (b) sequestering the Zn ions at *p*H 6.4 with ethylenediaminetetraacetate (EDTA) or citrate; or (c) lowering the *p*H of the reaction mixture to a value of 5.2. The renatured protein can be readily crystallized in the same crystal habit as ferrimyoglobin never exposed to Zn.

The most characteristic spectral change is a marked reduction in the Soret-band intensity accompanied by

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