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ON THE GENERATION OF HYDROGEN PEROXIDE IN ERYTHROCYTES BY ACETYLPHENYLHYDRAZINE

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Abstract—The catalase inhibitor 3-amino-1,2,4-triazole was employed to detect hydrogen peroxide within erythrocytes; inhibition of catalase requires the presence of H_2O_2. H_2O_2 was detected during incubation of erythrocytes with the hemolytic drug acetylphenylhydrazine (APH). This result refutes a prior suggestion that APH does not generate H_2O_2, and supports the concept of H_2O_2 involvement during drug-induced hemolysis of glucose 6-phosphate dehydrogenase-deficient cells. The following agents were more active than APH in generating H_2O_2: phenylhydrazine (100-fold), ascorbate (10-fold), and hydroquinone (3-fold). The reasons why a system based on methemoglobin formation in catalase-deficient cells failed to detect the H_2O_2 generated by APH are discussed.

INTRODUCTION

IT HAS been proposed (1) that H_2O_2 generated in low concentrations within erythrocytes is detoxified mainly by glutathione peroxidase[1] (GSH : H_2O_2 oxidoreductase, EC 1.11.1.9) rather than by catalase (H_2O_2 : H_2O_2 oxidoreductase, EC 1.11.1.6); and (2) that decreased protection by GSH peroxidase underlies the sensitivity to drug-induced hemolysis exhibited by individuals who are deficient in the hexosephosphate shunt enzyme, glucose 6-phosphate dehydrogenase[2, 3] (D-glucose 6-phosphate : NADP oxidoreductase, EC 1.1.1.48). As indicated in the equations below, oxidized glutathione, which is formed as a product of GSH peroxidase activity (reaction 1), is normally reduced by GSSG reductase (reaction 2) with NADPH as the required cofactor; the NADPH is provided by glucose 6-phosphate dehydrogenase activity. Insufficient generation of NADPH results in a decrease in the concentration of GSH[4] and, hence, in exposure of the cells to oxidative damage by H_2O_2.

$$(1)\quad 2\,GSH + H_2O_2 \xrightarrow{\text{GSH peroxidase}} GSSG + 2\,H_2O$$

$$(2)\quad GSSG + NADPH + H^+ \xrightarrow{\text{GSSG reductase}} 2\,GSH + NADP^+$$

In a recent study of erythrocytes from subjects with hereditary acatalasia, Jacob *et al.*[5] adduced evidence in part supportive and in part contrary to the above concepts. They observed that actively metabolizing cells did not accumulate methemoglobin when exposed for several hours to H_2O_2 vapor or to two H_2O_2-generating agents, viz. ascorbate or hydroquinone. Concomitantly, a stimulation of hexosephosphate

shunt activity was noted. They concluded that the H_2O_2 in catalase-deficient cells was destroyed by GSH peroxidase activity which, in turn, was sustained by glucose-dependent hexosephosphate shunt activity. These results supported a major role for GSH peroxidase (proposal 1, above).

In correlative experiments[5] with metabolically inactive (glucose-deprived) cells, preferential accumulation of methemoglobin was observed in the catalase-deficient cells as compared to normal (catalase-rich) cells. The appearance of methemoglobin was preceded by a loss in GSH. The authors concluded that in the absence of GSH peroxidase activity, the catalase-deficient cells were more sensitive than normal cells to H_2O_2. Then, upon observing that the drug acetylphenylhydrazine (APH) did not cause preferential methemoglobin formation, they reasoned that, since catalase-deficient cells were necessarily more sensitive to H_2O_2, it could be concluded that H_2O_2 had not been formed. They further concluded that H_2O_2 was not involved in oxidative hemolysis induced by APH and its congeners. These conclusions were contrary to proposal 2 above, and conflicted with reports of H_2O_2 formation in erythrocytes treated *in vitro*[3, 6] or *in vivo*[7] with the APH congener phenylhydrazine.

A likely resolution to the divergent results lay in the possibility that the methemoglobin detection system was not a reliable indicator of H_2O_2 formation. Accordingly, the present experiments were undertaken to compare in a more direct manner the amounts of H_2O_2 generated by APH, ascorbate, and hydroquinone. The catalase inhibitor 3-amino-1,2,4-triazole (AT) and endogenous intraerythrocytic catalase were employed as the detection system: AT inhibits catalase by reacting with catalase-H_2O_2 complex I[8–10] (reaction 3) and, therefore, the rate of inhibition provides a

$$\text{(3)}\quad \text{catalase} + H_2O_2 \rightleftharpoons \text{complex I} \xrightarrow{\text{AT}} \text{inhibited catalase}$$

measure of the steady-state concentration of free H_2O_2 in equilibrium with complex I. In these experiments, the generation of H_2O_2 from APH was clearly evident. Hence, the premise that glucose-deprived, catalase-deficient cells are necessarily sensitive to H_2O_2-generating drugs was not substantiated. The formation of H_2O_2 from APH (and from phenylhydrazine) is consistent with the hypothesis that H_2O_2 *is* a toxic intermediate during oxidative hemolysis in glucose 6-phosphate dehydrogenase-deficient cells.

EXPERIMENTAL

Blood specimens were drawn from normal donors into heparinized Vacutainers (Becton-Dickinson), stored at 5°, and used within 24 hr. Specimens were centrifuged, and the buffy coat and supernatant plasma were removed. For some experiments, the cells were washed three times with 6–10 volumes of isotonic saline and were then suspended in isotonic buffer,[11] pH 7·4. In other experiments, the cells were treated with N-ethylmaleimide to remove intracellular GSH,[12] for reasons presented in the Results section. In the latter experiments, the cells were washed once, suspended in buffer at a 50 per cent hematocrit, and the intracellular GSH was measured with the DTNB [5,5′-dithiobis-(2-nitrobenzoic acid), Aldrich Chemical Co.] procedure described by Beutler *et al.*;[13] an equimolar amount of N-ethylmaleimide in 0·1 volume isotonic buffer, was then added. After 10-min incubation at room temperature, the samples were centrifuged, the remaining two washes completed, and the cells resuspended in buffer. This treatment resulted in losses of 90–95 per cent of the GSH.

Four-ml aliquots of cell suspension were equilibrated at 37°, with shaking, in the presence of 0·05 M AT (3-amino-1,2,4-triazole, c.p., Mann Res. Labs.). Then concentrated solutions of the drugs in volumes of less than 0·2 ml were added. APH (acetylphenylhydrazine, i.e. acetophenylhydrazide, Eastman Organic Chemicals) and phenylhydrazine hydrochloride (c.p., Fisher) were recrystallized from ethanol. Hydroquinone (c.p.) and L-ascorbic acid (reagent grade) were obtained from Fisher. Samples were removed at various time periods and analyzed for catalase activity. The rate of inhibition of catalase provided a measure of the rate of generation of H_2O_2 by the various agents (cf. Introduction). Catalase assays were performed with a permanganate titration procedure.[3] Aliquots of cell suspension were first treated with 5 volumes of buffered 0·45 M ethanol for 20 min at room temperature as a precaution to decompose any inactive catalase-H_2O_2 Complex II. Catalase activity was expressed in terms of a zero-time control whose activity was defined as 100 per cent.

RESULTS

In preliminary experiments, the erythrocytes were supplemented with glucose in order to support reduction of GSSG (reaction 2), thereby to maintain a constant GSH level during the incubation with the various drugs. This was desirable since the reaction of H_2O_2 with catalase, leading to enzyme inhibition (reaction 3), was in competition with GSH peroxidase activity (reaction 1); it had been shown previously[3] that elimination of competitive reactions—oxidation of GSH or hemoglobin—sensitized erythrocytes to inhibition of catalase. However, despite the presence of glucose, one agent, hydroquinone, caused the GSH to disappear (most probably as the result of formation of the *p*-quinone–GSH adduct). For example, in an experiment with a 33 per cent suspension of cells supplemented with glucose (11 mM), 2 mM hydroquinone caused a decline of GSH from 2·1 mM to 1·0 mM in half an hour and to 0·1 mM by the second hour, while with 5mM ascorbate or 5 mM APH the GSH was virtually unchanged. The loss of GSH with concomitant elimination of the GSH peroxidase activity resulted in accelerated inhibition of catalase (Fig. 1). When

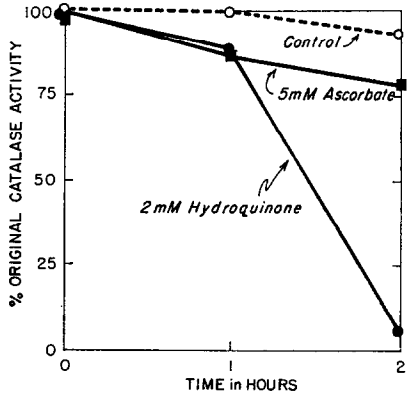

FIG. 1. Discontinuity in the catalase inhibition curve with hydroquinone (no pretreatment with N-ethylmaleimide). Erythrocytes were suspended at a 25 per cent hematocrit in the presence of 11 mM glucose.

glucose was omitted from the medium, GSH losses were observed for ascorbate and APH as well, but at slower rates than for hydroquinone (Fig. 2). In order to bypass problems of interpretation arising from initial lag periods followed by varying degrees and rates of sensitization, the cells were presensitized by removal of GSH as the

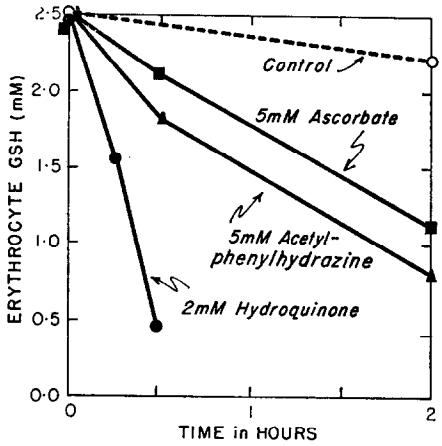

FIG. 2. GSH loss in the absence of glucose. Erythrocytes were suspended at a 33 per cent hematocrit; they were not treated with N-ethylmaleimide. Samples were washed three times with 20 volumes of isotonic saline in order to remove the interfering drugs before proceeding with GSH analyses.

N-ethylmaleimide adduct.[12] As a result, the lag periods for catalase inhibition were eliminated, and this facilitated direct comparison of the various drugs (Fig. 3).

An underlying assumption in this study was that the rate of inhibition of catalase was proportional to the rate of generation of H_2O_2; however, this had not been explicitly

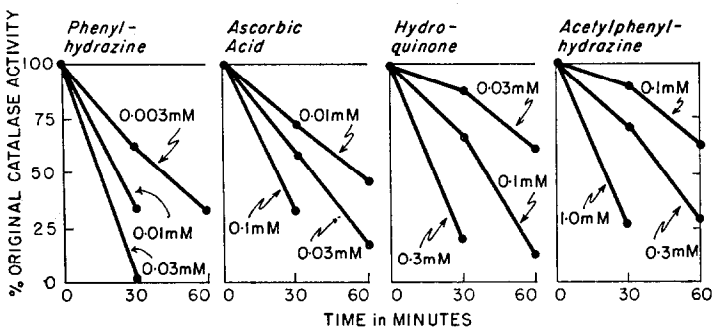

FIG. 3. Comparison of H_2O_2 generation rates for various drugs. Erythrocytes were suspended at a 5 per cent hematocrit in buffer without glucose. Intracellular GSH after N-ethylmaleimide treatment was 0·12 mM. A control sample incubated with AT, but without drug, exhibited a 16 per cent decline in catalase activity after 1 hr. Samples incubated with drug, but without AT, were unchanged. The relative rates of inhibition of catalase provide a measure of the relative rates of H_2O_2 generation.

demonstrated in prior studies[8–10] where the reaction mechanism proceeding through catalase-H_2O_2 complex I (reaction 3) had been established. Therefore, N-ethylmaleimide-treated cells were exposed to varying amounts of reagent H_2O_2, added continuously by means of a vapor-state diffusion method:[4] the extent of inhibition

increased with increasing rates of addition of H_2O_2 (see Table 1). This result verified the applicability of the test system for studying varying rates of H_2O_2 generation by the drugs. Other, more detailed, studies performed with crystalline catalase indicated a direct linear relationship between the rate of H_2O_2 generation by the

TABLE 1. INHIBITION OF CATALASE WITHIN ERYTHROCYTES AS A FUNCTION OF THE RATE OF ADDITION OF H_2O_2

Estimated rate of addition of H_2O_2 (μmoles/hr)	% Inhibition of catalase by aminotriazole (mean ± average deviation for duplicate vessels)*	
	After 1 hr	After 2 hr
0·000		6
0·015	11 ± 0	26 ± 1
0·030	17 ± 0	55 ± 8
0·060	65 ± 19	98 ± 2

The H_2O_2 was added by a vapor-state diffusion method.[4] The diffusion rate was varied by employing different concentrations of H_2O_2 (viz. 0·067 M, 0·133 M, and 0·267 M) as a source of the vapor. The rate of diffusion of H_2O_2, which was proportional to the H_2O_2 concentration, was calculated from prior calibrations[1, 4] with 10 M H_2O_2 (= approximately 2·25 μmoles/hr for these particular vessels). Four-ml aliquots of a 5 per cent suspension of erythrocytes in buffer, without glucose, were employed. The cells had been pretreated with N-ethylmaleimide; this caused a decline in intracellular GSH from 2·0 to 0·2 mM.

* Duplicate catalase assays on the same sample agreed to within 5 per cent; the greater scatter observed for duplicate vessels was due to flask-to-flask variations in geometry and/or other factors which affected the transfer of H_2O_2.

glucose oxidase reaction and the rate of catalase inhibition by AT; this latter work was performed during an evaluation of the rate of secretion of H_2O_2 by *Mycoplasma pneumonia* and will be reported elsewhere (G. Cohen and N. L. Somerson, in preparation).

Figure 3 compares the relative effectiveness of hydroquinone, ascorbate, phenylhydrazine, and APH as H_2O_2-generating agents (measured as inhibition of catalase by AT). In a prior study[3] with phenylhydrazine and hydroquinone, the intermediary formation of catalase-H_2O_2 complex I was confirmed by employing ethanol to block inhibition by AT. Visual inspection of the rate curves indicates roughly equivalent H_2O_2-generation rates for 0·01 mM phenylhydrazine, 0·1 mM ascorbate, 0·3 mM hydroquinone, and 1·0 mM APH. Thus, on a molar basis, phenylhydrazine, ascorbate, and hydroquinone were respectively 100-fold, 10-fold, and 3-fold more effective than APH. Similar relationships hold for the lower drug concentrations in Fig. 3.

Estimates of absolute H_2O_2-generation rates can be obtained from the catalase-inhibition data. For example, 0·003 mM phenylhydrazine and 0·3 mM APH each caused roughly 70 per cent inhibition of catalase in 1 hr (Fig. 3); the same effect was obtained by the addition, over the course of 1 hr, of 0·06 μmole H_2O_2 (Table 1) to the 4-ml reaction volume (or roughly 0·015 μmole/ml per hr). These calculations establish only an order of magnitude, since the vapor-state diffusion system employed for calibration, although providing a means for continuous addition, does not provide completely uniform addition of H_2O_2 to the cells: the steady-state conditions are

punctuated by the transfer of more concentrated H_2O_2 either as microdroplets of vapor condensate or by streaming of condensate from the side walls of the vessels.[3]

In the experiment of Fig. 3, the hematocrit (5 per cent) was less than that (33 per cent) employed by Jacob *et al.*,[5] and the drug concentrations were more dilute. However, H_2O_2 generation was evident under the more concentrated conditions as well. For example, in an experiment with N-ethylmaleimide-treated cells suspended at a 25 per cent hematocrit, the catalase activities (compared to a zero-time control) were as follows: AT only, 100 per cent at 60 min; AT plus 0·5 mM phenylhydrazine, 22 per cent at 30 min; AT plus 5 mM ascorbate, 21 per cent at 30 min; and AT plus 5 mM APH, 79 per cent at 30 min and 11 per cent at 60 min.

DISCUSSION

Relative rates of formation of H_2O_2 in erythrocytes incubated with acetylphenylhydrazine, phenylhydrazine, hydroquinone, or ascorbate were readily measured with aminotriazole plus intracellular catalase as the detection system. The ability of APH to generate H_2O_2 has not been demonstrated previously. The formation of H_2O_2 from hydroquinone,[3] phenylhydrazine,[3, 6] and ascorbate[8, 14] has been reported; the H_2O_2 is generated by the reaction of the drug with oxygen (autoxidation) and by a coupled oxidation of drug and oxyhemoglobin.

Jacob *et al.*[5] did not detect H_2O_2 generated by APH when methemoglobin accumulation in catalase-deficient cells was used as an indicator. Likewise, the methemoglobin test system failed to detect H_2O_2 generated from "fresh" (i.e. not exposed to u.v. irradiation) primaquine, although H_2O_2 generated by recrystallized primaquine has been observed[3] with the AT system. The reasons why some H_2O_2-generating agents such as hydroquinone or ascorbate caused preferential methemoglobin formation in catalase-deficient cells, while others such as primaquine or APH did not, may involve a variety of parameters.

(1) The relative abilities of the drugs to decrease the intracellular GSH concentration were important. In the absence of GSH peroxidase activity (reaction 1), the catalase of normal cells protected hemoglobin from oxidation, while catalase-deficient cells were fully exposed to the oxidant effects of H_2O_2. Since hydroquinone caused a rapid decline in GSH (Fig. 2), it was more effective than APH in causing differential methemoglobin formation. (2) A greater differential response would be expected from drugs producing greater amounts of H_2O_2. The response to ascorbate was in keeping with its greater effectiveness than APH (roughly 10-fold, cf. Fig. 3) in generating H_2O_2. (3) H_2O_2 was formed both by autoxidation of the drug and by the coupled oxidation of drug and oxyhemoglobin;[3, 14] in the latter reaction, methemoglobin is a product. A differential response requires that the amount of methemoglobin formed in response to H_2O_2 be detected as an increment over the methemoglobin formed during the coupled oxidation. Ascorbate and hydroquinone were better suited for the test system since they were roughly fifty times more active than APH in generating H_2O_2 via autoxidation.* (4) Ascorbate and hydroquinone were more active than APH in effecting some reduction of methemoglobin to oxyhemoglobin, thereby permitting

* In experiments with crystalline catalase and AT, performed as described elsewhere,[3] the rate of inhibition of catalase in the presence of 3 mM APH (i.e. the rate of generation of H_2O_2 via autoxidation) was comparable to that produced by 0·06 mM hydroquinone or 0·06 mM ascorbate.

broadening of any differential response.* (5) Protection by catalase against methemoglobin formation need not be derived from the peroxidation of a second molecule of H_2O_2 (catalatic activity) but rather from the peroxidation of the H_2O_2-generating drug itself (the peroxidatic activity of catalase). Hydroquinone can act as a substrate for catalase[15] and may create an artifact by destroying H_2O_2, thereby protecting hemoglobin, only in catalase-rich cells.

The demonstration that APH is indeed a potent H_2O_2-generating agent is in keeping with the concept that oxidant hemolysis in glucose 6-phosphate dehydrogenase-deficient individuals is a response to increased intracellular levels of H_2O_2. The relative insensitivity of human catalase-deficient cells (glucose-supplemented) to oxidant damage by H_2O_2 vapor or H_2O_2-generating drugs added *in vitro* has been documented by Aebi and co-workers[16] and Jacob *et al.*[5] The experiments *in vivo* of Jacob and co-workers[5] offer particularly compelling evidence for the unessential role of erythrocyte catalase under physiologic conditions: they found that ^{51}Cr-labeled catalase-deficient cells injected into normal recipients did not show decreased survival when challenged with primaquine, an H_2O_2-generating drug that causes profound oxidative hemolysis in glucose 6-phosphate dehydrogenase-deficient subjects. In addition, administration of primaquine directly to two acatalasic subjects failed to alter hematocrit, methemoglobin concentration, or reticulocyte count. These results *in vivo* correlate well with the observations *in vitro* and support the view that GSH peroxidase, sustained by glucose-consuming hexosephosphate shunt activity, provides the major protection against H_2O_2 in erythrocytes.

ADDENDUM

Further evidence substantiating the importance of GSH peroxidase in protecting cells from oxidant hemolysis can be found in the recent report by Prins and co-workers.[17] The report describes subjects with a genetic block in erythrocyte GSH synthesis. The subjects exhibited a sensitivity to hemolysis during administration of fava bean and/or primaquine, and their cells were sensitive *in vitro* (Heinz bodies) to APH. Erythrocyte glucose 6-phosphate dehydrogenase levels were normal, but GSH peroxidase activity was clearly lacking in the absence of GSH.

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* In an experiment with cells (33 per cent suspension) prepared at 95 per cent methemoglobin by treatment with $NaNO_2$, 2 mM hydroquinone caused a 34 per cent reduction in methemoglobin at 1 hr, 5 mM ascorbate a 29 per cent reduction in 3 hr, and 5 mM APH an 11 per cent reduction in 3 hr.

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