The ubiquinone found in a respiratory-deficient mutant is therefore identical in structure and almost equal in amount to that of the corresponding wild-type yeast. Whatever the nature of the primary phenotypic difference between the two it is probably also not referrable to any gross differences in the synthesis or utilization of unsaponifiable lipids. These statements hold true so far only for the intact cell; whether or not they are applicable to respiratory particles and whether there are differences in the intracellular localization of these components is presently under study.

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ADDED IN PROOF

In a more recent publication T. Sugimura, K. Okabe and H. Rudney (1964, *Biochim. Biophys. Acta 82*, 350) report values of 315 and 411 μg Q/100 mg N for wild type and 24-85 μg Q/100 mg N for their mutants.

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Generation of Hydrogen Peroxide in Erythrocytes by Hemolytic Agents*

GERALD COHEN AND PAUL HOCHSTEIN†

From the Departments of Biochemistry and Psychiatry, Columbia University College of Physicians and Surgeons, and the New York State Psychiatric Institute, New York City

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The generation of H_2O_2 in intact erythrocytes was detected by demonstration of catalase-H_2O_2 complexes I and II. 3-Amino-1,2,4-triazole reacted with complex I to yield irreversibly inhibited catalase; inhibition was prevented but not reversed by employing ethanol to decompose complex I. Complex II was detected by its lack of catalatic activity which could be recovered by treatment with ethanol. The presence of H_2O_2 in intact cells was detected after the addition of several 8-aminoquinolines (e.g., primaquine) but not after the addition of the nonhemolytic 4-aminoquinoline, chloroquine; after the addition of the hemolytic agents phenylhydrazine and menadione; after the addition of the hydroquinone–*p*-quinone redox system, but not after the addition of the nonautoxidizable resorcinol; and, last, after the addition of exogenous H_2O_2. The generation of H_2O_2 from the 8-aminoquinolines required the presence of oxyhemoglobin and could be blocked by preliminary conversion of oxyhemoglobin to methemoglobin. The generation of H_2O_2 from phenylhydrazine was derived in part from a reaction with oxyhemoglobin and in part from autoxidation. The detection of H_2O_2 generated from hemolytic agents supports the concept that H_2O_2 toxicity plays a major role in drug-induced hemolysis of glucose-6-phosphate dehydrogenase–deficient erythrocytes.

Hemolysis in individuals with deficient erythrocyte levels of glucose-6-P dehydrogenase is known to occur upon exposure to various drugs (Beutler, 1960; Tarlov *et al.*, 1962). It has been suggested that the oxidative changes observed during hemolysis, e.g., loss of GSH and oxidation of hemoglobin, are manifestations of the presence of H_2O_2 (Mills and Randall, 1958; Cohen and Hochstein, 1961, 1963). Glucose-6-P dehydrogenase–deficient erythrocytes are sensitive to H_2O_2 by virtue of diminished generation of NADPH.

In this paper we present evidence that H_2O_2 is in fact generated in intact erythrocytes when hemolytic agents are added. Since H_2O_2 does not accumulate

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† Present address: Duke University Medical Center, Durham, North Carolina.

within erythrocytes to an extent to permit direct isolation and characterization, it was necessary to trap the evanescent peroxide by a chemical reaction of high specificity. For this purpose we utilized the reaction of 3-amino-1,2,4-triazole in inhibiting the enzyme catalase (Margoliash *et al.*, 1960; Tephley *et al.*, 1961; Nicholls, 1962).

Two different types of inhibition of catalase by aminotriazole have been distinguished (Margoliash and Novogrodsky, 1958; Margoliash *et al.*, 1960). The first is a simple reversible inhibition in which the enzymatic activity can be recovered by dilution (Fig. 1, reaction 1). In the second type of inhibition aminotriazole reacts with catalase-H_2O_2 complex I (Fig. 1, reaction 3), and as a result the aminotriazole is firmly affixed to the enzyme protein and can no longer be dissociated by dilution. This latter reaction serves as a detection system for H_2O_2: when irreversible inhibition of catalase by aminotriazole is observed, it may be inferred that H_2O_2 had been present in the test system. With this technique H_2O_2 generation has been detected in erythrocytes incubated with phenylhydrazine, menadione, and several 8-aminoquinoline antimalarials (e.g., primaquine, pamaquine).

Methods

Heparinized blood specimens obtained from normal adult subjects were centrifuged and the buffy coat and supernatant plasma were removed. The isolated cells were washed three times with 6–10 volumes of isotonic saline. Washed erythrocytes were suspended in 19 volumes of isotonic sodium phosphate buffer, *p*H 7.4 (Dacie, 1954), or were lysed in 19 volumes of 0.1% buffer.

For the preparation of methemoglobin-containing specimens, 2 volumes of 1% $NaNO_2$ were added to 1 volume of unwashed cells or its equivalent in whole blood. After 30 minutes' incubation at room temperature the cells were isolated by centrifugation and washed three times with isotonic saline to remove the excess nitrite. Standard suspensions or lysates were then prepared.

3-Amino-1,2,4-triazole (Mann Laboratories) was recrystallized from ethanol. The antimalarial agents primaquine phosphate, pamaquine citrate, pentaquine phosphate, and isopentaquine phosphate were obtained from the Sterling-Winthrop Research Inst. and were recrystallized from methanol–petroleum ether. Chloroquine phosphate was not recrystallized. Hydroquinone, resorcinol, and phenylhydrazine hydrochloride were recrystallized from ethanol. Menadione and *p*-quinone were purified by sublimation. Crystalline beef liver catalase was obtained as a suspension from the Worthington Biochemical Corp. All other chemicals were reagent grade.

Menadione was prepared as a suspension in 5% polyvinylpyrrolidone (PVP, Antara Chemicals). All other agents were dissolved in either 0.1% buffer or 0.9% buffer. The antimalarials and phenylhydrazine hydrochloride were neutralized by titration with NaOH. In experiments with commercial catalase, the enzyme crystals were isolated by centrifugation and then dissolved in 0.1% buffer.

Catalase analyses were performed with a permanganate titration method, essentially that as described by Euler and Josephson (1927), but altered according to the precautions indicated by Maehly and Chance (1954). Five ml of 0.006 M H_2O_2 in 0.01 M sodium phosphate buffer, *p*H 7.0 were incubated with 0.5 ml of a 1:100 dilution of the experimental samples for 3 minutes at 0°. The reaction was stopped by the addition of 1 ml 6 N H_2SO_4. Residual H_2O_2 was measured by titration with 0.01 N $KMnO_4$. The reaction appeared to follow first-order kinetics, i.e., the catalase activity was linearly related to the logarithm of the 3-minute titer. Catalase activity was determined graphically with the untreated control specimen defined as 100% activity. For the decomposition of catalase-H_2O_2 complex II (Chance, 1950), samples (4 ml) were treated with 2 ml buffered 0.45 M ethanol for 20 minutes at room temperature. Standard dilutions were then prepared for analyses.

In these experiments, 4-ml aliquots of cell suspensions or lysates were incubated with the indicated additions for 1 hour at 37° unless otherwise indicated.

Table I
Structure-Activity Relationships for Inhibition of Catalase by Aminotriazole (AT) in Erythrocyte Lysates[a]

Expt	Additions		Catalase Activity at 1 Hour (%)
1	None		100
	AT	(0.05 M)	100
	AT + hydroquinone	(0.7×10^{-3} M)	0
	AT + *p*-quinone	(0.7×10^{-3} M)	0
	AT + resorcinol	(1.4×10^{-2} M)	94
2	None		100
	AT	(0.05 M)	100
	AT + primaquine	(1.25×10^{-3} M)	0
	AT + pamaquine	(1.25×10^{-3} M)	0
	AT + pentaquine	(1.25×10^{-3} M)	10
	AT + isopentaquine	(1.25×10^{-3} M)	58
	AT + chloroquine	(2.5×10^{-3} M)	105

[a] Small inhibitory effects were noted for some of these agents in the absence of aminotriazole; these effects were similar to those illustrated in Table II.

Results

A. Detection of H_2O_2 in Erythrocyte Lysates.—In *in vivo* studies of inhibition of catalase in various rat tissues after administration of aminotriazole, it was noted that erythrocyte catalase was unaffected (Heim *et al.*, 1956; Feinstein *et al.*, 1958). Failure to inhibit erythrocyte catalase *in vivo* appeared to be due to insufficient generation of H_2O_2 since erythrocyte catalase could be inhibited *in vitro* by the addition of H_2O_2, or agents which generated H_2O_2 (Margoliash and Novogrodsky, 1958; Tephley *et al.*, 1961). These observations with H_2O_2-generating agents have been extended by the results presented in this paper to include many of the hemolytic drugs. For instance, Table I contains representative data for several 8-aminoquinoline drugs and for some model compounds. In these particular experiments, lysates were employed rather than intact cells in order to study structure-activity relationships in the absence of complicating permeability phenomena. However, all the active compounds were effective with intact cells also (cf. Table II).

p-Quinone and hydroquinone represent examples of a redox pair in which the oxidized form can be converted into the phenolic form by reducing agents in tissues, while the reduced form can react directly with molecular oxygen to produce H_2O_2. As a negative control, the structurally similar dihydric phenol, resorcinol (1,3-dihydroxybenzene), which cannot be oxidized to a corresponding quinone and therefore cannot generate H_2O_2, was shown to be inactive as a catalase inhibitor. In the illustrative example in Table I, resorcinol was

TABLE II
DETECTION OF HYDROGEN PEROXIDE IN INTACT ERYTHROCYTES[a]

	Catalase Activity at 1 Hour (%)					
					After Treatment with Ethanol	
	(A)	+ AT (B)	+ AT + Ethanol (C)	+ Ethanol (D)	(A′)	(B′)
Controls	100	100	124	126	118	118
H_2O_2	87	0	124	124	114	0
Hydroquinone	58	0	118	114	114	0
Primaquine	72	0	124	124	111	0
Pamaquine	68	0	111	114	104	0
Menadione	65	0	118	124	100	0
Phenylhydrazine	79	0	124	124	111	0

[a] The final concentrations of the added substances were: aminotriazole, 0.05 M; hydroquinone, menadione, or phenylhydrazine, 1.0×10^{-3} M; primaquine or pamaquine, 2.5×10^{-3} M; and ethanol (columns C and D) 0.45 M. Hydrogen peroxide (4.5 μmoles) was added by gaseous diffusion during the 1-hour incubation period. Column A: Complex I→Complex II (reaction 6, Fig. 1). Column B: Complex I + AT→(Catalase-AT) (reaction 3, Fig. 1). Column A′: Complex II + ethanol→Acetaldehyde + 2 H_2O + free catalase (reaction 7, Fig. 1). Column B′: (Catalase-AT) + ethanol→No reaction. Column C or D: Complex I + ethanol→Acetaldehyde + 2 H_2O + free catalase (reaction 5 Fig. 1).

inactive even at a 20-fold increase in concentration over hydroquinone.

In experiment 2 of Table I, results are presented for a series of 8-aminoquinoline antimalarials. The 4-aminoquinoline antimalarial chloroquine, which is nonhemolytic, was included as a negative control. In this experiment and in others with either lysates or intact cells, pamaquine was the most effective H_2O_2-generating agent in the 8-aminoquinoline group, while primaquine was more effective than either pentaquine or isopentaquine.

B. Detection of H_2O_2 in Intact Erythrocytes.—The presence of H_2O_2 in intact erythrocytes treated with various hemolytic agents and model compounds is illustrated in Table II by demonstration of irreversible inhibition of catalase with aminotriazole (cf. columns A and B). Included in Table II are data obtained with the hemolytic agents phenylhydrazine and menadione, as well as with H_2O_2 itself added to the cells by means of a gaseous-diffusion technique (Cohen and Hochstein, 1961).

In these experiments with intact cells, as well as in the experiments with lysates, it was noted that some loss in catalatic activity occurred even in the absence of aminotriazole (column A). In other experiments it was possible to eliminate this effect by employing lower concentrations of drugs. However, the loss in catalatic activity observed in the absence of aminotriazole possessed properties associated with the formation of catalase-H_2O_2 complex II, and hence was actually confirmatory evidence for the generation of H_2O_2 from the various hemolytic agents. As indicated in Figure 1, complex II is formed from catalase-H_2O_2 complex I (Chance, 1950; Keilin and Nicholls, 1958). The reaction is relatively slow and proceeds only if complex I is not decomposed by a suitable hydrogen donor such as ethanol (reaction 5) or another molecule of H_2O_2 (reaction 4). Complex II does not exhibit catalatic activity, but it can be decomposed to free enzyme by ethanol.[1] As indicated in column A′ of Table II, the loss in catalatic activity observed in the absence of aminotriazole was recovered by treatment with ethanol and therefore could be attributed to the presence of complex II. On the other hand, the inhibition of catalatic activity observed with aminotriazole was not reversed by ethanol (column B′) and hence possessed

[1] Chance (1950) and Keilin and Nicholls (1958) suggest that ethanol does not react directly with complex II, but rather with complex I with which it is in equilibrium. Other donors such as phenol, formate, or nitrite, react directly with complex II.

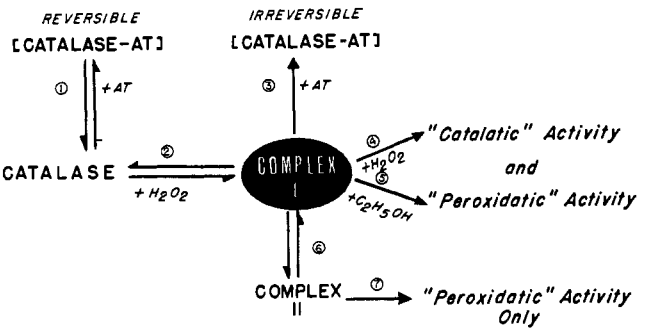

FIG. 1.—Reactions of catalase with aminotriazole and with hydrogen peroxide. The presence of H_2O_2 can be detected by observing irreversible inhibition of catalatic activity by aminotriazole (AT) (reaction 3) and/or by demonstrating formation of complex II (reaction 6). The intermediate formation of complex I can be confirmed by employing ethanol (reaction 5) to compete with and prevent reaction 3. Complex II is defined operationally as a loss in catalatic activity which can be recovered by incubation with ethanol (reaction 7).

the characteristics of the catalase-aminotriazole complex formed from complex I (reaction 3).

Still further evidence for the presence of H_2O_2 was obtained in experiments in which ethanol was added at zero time. Ethanol and aminotriazole are competitive with each other for reaction with complex I (reactions 3 and 5). It has been shown that oxidation of ethanol is the favored reaction (Margoliash *et al.*, 1960; Tephley *et al.*, 1961); i.e., by decomposing complex I, ethanol prevents inhibition of catalase by aminotriazole. Since ethanol protected erythrocyte catalase in our experiments (column C), reaction 3 was confirmed as the mechanism of inhibition. Ethanol also prevented accumulation of complex II when aminotriazole was omitted (column D).

Erythrocyte catalase could be sensitized to irreversible inhibition by aminotriazole when the elimination of H_2O_2 via alternate pathways was prevented. Two such pathways are oxidation of GSH and hemoglobin (Cohen and Hochstein, 1963); in particular, oxidation of GSH catalyzed by GSH peroxidase represents the major pathway of reaction of H_2O_2 in erythrocytes. In the experiment illustrated in Figure 2, cells were sensitized to the H_2O_2 generated from hydroquinone by pretreatment to oxidize GSH or hemoglobin. It is probable that GSH is the dialyzable sulfhydryl substance which has been reported by Margoliash and Schejter (1961) to protect erythrocyte catalase.

TABLE III
COMPARISON OF THE EFFECTS OF VARIOUS DRUGS ON CRYSTALLINE CATALASE AND ON ERYTHROCYTE CATALASE, IN THE PRESENCE OF AMINOTRIAZOLE (AT)[a,b]

Expt	Additions		Catalase Activity at 1 Hour (%)		
			Lysate	Crystalline Catalase	Crystalline Catalase + Lysate
1	AT	(0.06 M)	100	100	100
	AT + hydroquinone	(2.3×10^{-4} M)	58	0	30
	AT + hydroquinone	(6.8×10^{-4} M)	0	0	0
	AT + primaquine	(7.0×10^{-4} M)	31	102	38
	AT + primaquine	(4.2×10^{-3} M)	0	98	0
2	AT	(0.06 M)	100	100	100
	AT + hydroquinone	(7.0×10^{-4} M)	0	0	
	AT + *p*-quinone	(3.5×10^{-4} M)	0	0	
	AT + menadione	(7.0×10^{-4} M)	0	21	
	AT + pamaquine	(5.0×10^{-4} M)	4	92	10
3	AT	(0.06 M)		100	
	AT + phenylhydrazine	(8.6×10^{-6} M)		9	

[a] The concentration of crystalline catalase was 0.25 mg/ml; catalatic activity was equivalent to that in the lysates. Negligible inhibition of catalase (less than 10%) was noted in control samples incubated without aminotriazole but with each of the other agents at the concentrations indicated in the table. [b] The concentration of phenylhydrazine (8.6×10^{-6} M) employed in the experiment illustrated in Table III was very much lower than that required to demonstrate an aminotriazole effect with the other agents. At this concentration, phenylhydrazine alone had no effect on catalase activity. However, at higher phenylhydrazine concentrations, crystalline catalase was strongly inhibited even in the absence of aminotriazole and the inhibition could not be reversed by treatment with ethnol. This phenomenon appeared to be dependent upon the presence of H_2O_2 since it could be blocked by incorporating ethanol into the reaction mixture to decompose complex I. For example, in an experiment with 1.7×10^{-4} M phenylhydrazine in the absence of aminotriazole, the catalase activity after 1 hour was 0% compared to the control, and the activity could not be recovered by subsequent treatment with ethanol. A second sample prepared with ethanol (0.4 M) added at zero time exhibited full enzymatic activity. In experiments with lysed or intact erythrocytes, strong inhibition of catalase by phenylhydrazine alone, irreversible by ethanol, was *not* observed (cf. Table IV).

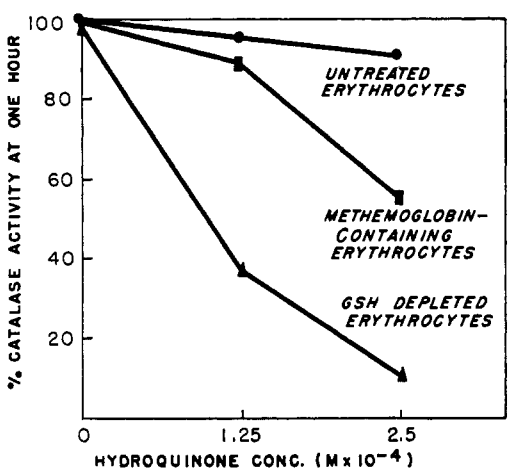

FIG. 2.—Sensitization of erythrocytes to aminotriazole. Erythrocytes were depleted of GSH by subjecting washed cells at 37° to H_2O_2 vapor for 3 hours (Cohen and Hochstein, 1963). The control specimen was also incubated for 3 hours at 37°, but without H_2O_2 vapor. The methemoglobin-containing specimens were prepared separately as described in the experimental section. At the start of the aminotriazole experiment the GSH levels were: control, 60 mg %; GSH-depleted specimen, 15 mg %; and methemoglobin-containing specimen, 70 mg %. GSH was measured with the nitroprusside method of Grunert and Phillips (1951). The methemoglobin values as judged by the absorption at 625 mμ at the start of the experiment were: control and GSH-depleted specimen, less than 5%; nitrite-treated specimen, over 95%. Aminotriazole was added to a final concentration of 0.05 M to initiate the experiment.

C. The Role of Oxyhemoglobin.—Inhibition of catalase by aminotriazole in the presence of autoxidizable substances such as hydroquinone or phenylhydrazine was clearly anticipated from the previous results of Margoliash and Novogrodsky (1958) and Tephley *et al.* (1961). Likewise, H_2O_2 formation from quinones such as menadione was anticipated on the grounds that cellular reducing systems would promote formation of the corresponding autoxidizable dihydric phenols. However, the activity of the 8-aminoquinoline drugs could not be predicted solely on the basis of structure: these agents are not readily autoxidizable nor can they be reduced to such derivatives.

The data of Table III indicate that the H_2O_2-generating activity of primaquine and pamaquine required the presence of a catalyst present in erythrocytes: these agents were ineffective against crystalline catalase, unless the crystalline enzyme was first added to the lysate. Readily autoxidizable compounds, on the other hand, were active with both crystalline catalase and erythrocyte catalase. The activity of quinones with crystalline catalase was no doubt derived from preliminary reduction to dihydric phenols by reducing agents present in the enzyme preparation.

The possibility that oxyhemoglobin was the catalyst required for generation of H_2O_2 from primaquine was tested by the experiment illustrated in Figure 3. Preliminary conversion of oxyhemoglobin to methemoglobin blocked the generation of H_2O_2 when primaquine was subsequently added to the cells. Similar results were obtained with pamaquine. On the other hand, the activity of the autoxidizable phenol, hydroquinone, was not eliminated by removal of oxyhemoglobin. It should be noted that the activity of primaquine and pamaquine were blocked despite the fact that methemoglobin-containing cells were more sensitive to H_2O_2-generating agents (Fig. 2).

In similar experiments with phenylhydrazine (Table IV), only a portion of the catalase-inhibiting activity could be blocked by preliminary conversion of oxyhemoglobin to methemoglobin. However, it should be noted that phenylhydrazine can reduce methemoglobin (Rostorfer and Totter, 1956) and then generate a small amount of H_2O_2 by subsequent reaction with oxy-

TABLE IV
GENERATION OF HYDROGEN PEROXIDE FROM PHENYLHYDRAZINE IN OXYHEMOGLOBIN-CONTAINING AND METHEMOGLOBIN-CONTAINING ERYTHROCYTES

Additions	Catalase Activity at 1 Hour (%)[a]	
	Oxyhemoglobin	Methemoglobin
None	100	100
AT (0.06 M)	96	100
AT + phenylhydrazine (3.5×10^{-5} M)	62	85
AT + phenylhydrazine (8.5×10^{-5} M)	31	58
AT + phenylhydrazine (3.5×10^{-4} M)	0	4
Controls:		
Phenylhydrazine alone[b] (3.5×10^{-5} M)	96	94
Phenylhydrazine alone[b] (8.5×10^{-5} M)	92	94
Phenylhydrazine alone[b] (3.5×10^{-4} M)	92	96

[a] Samples were routinely treated with ethanol at the end of the incubation period in order to decompose complex II.
[b] Although the addition of phenylhydrazine by itself resulted in the formation of complex II (cf. Table II), which in this experiment was subsequently decomposed by treatment with ethanol, there was on the other hand *no* pronounced irreversible inhibition of catalase in the absence of aminotriazole, such as that observed when these concentrations of phenylhydrazine were added to crystalline catalase (discussed in footnote *b*, Table III).

hemoglobin. In addition, phenylhydrazine exhibited activity against crystalline catalase in the absence of hemoglobin (Table III). These results are consistent with a dual activity of phenylhydrazines, namely, generating H_2O_2 via autoxidation and also in a coupled reaction with oxyhemoglobin.

DISCUSSION

Beutler (1957, 1960) reported that the hemolytic agent, primaquine, when added to glucose-6-P dehydrogenase-deficient erythrocytes *in vitro*, caused a decline in intracellular GSH; similar results were obtained with phenylhydrazine and hydroxylamine. One mechanism to explain this effect is via intermediate generation of H_2O_2 with subsequent oxidation of GSH to GSSG, catalyzed by GSH peroxidase (Mills and Randall, 1958; Cohen and Hochstein, 1961, 1963). Hence, Beutler's observations might be interpreted to indicate that primaquine can bring about the generation of H_2O_2 in erythrocytes *in vitro*. Evidence for H_2O_2 generation during the reaction of phenylhydrazine or hydroxylamine with oxyhemoglobin was reported by Rostorfer and Cormier (1957); "H_2O_2-like" substances were detected with a luminescence technique. The formation of H_2O_2 during coupled oxidations of hemoglobin and various hydrogen-donor molecules was suggested previously by Lemberg and Legge (1949). In this current report, the presence of H_2O_2 in intact erythrocytes treated with phenylhydrazine or primaquine (as well as with menadione, pamaquine, pentaquine, or isopentaquine) was detected by demonstrating the formation of catalase-H_2O_2 complexes I and II.

In the experiments of Beutler *et al.* (1957) with acetylphenylhydrazine an altered form of hemoglobin, which had the property of oxidizing GSH, was isolated. The active derivative may have been methemoglobin-H_2O_2 (Keilin and Hartree, 1951; Bodansky, 1951) or some other hematin-peroxide complex. Beutler *et al.* (1957) concluded that the hemoglobin derivative should be included in the mechanism postulated to explain the loss of GSH in the presence of acetylphenylhydrazine.

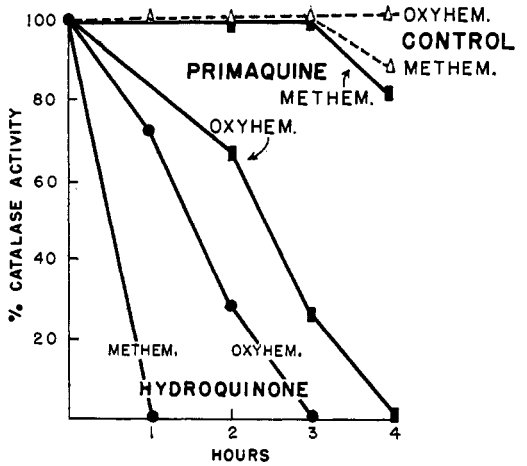

FIG. 3.—Blockade of primaquine-induced generation of hydrogen peroxide. The aminotriazole concentration in this experiment was 0.06 M. Intact erythrocytes were employed. Primaquine was added, where indicated, to a final concentration of 2.0×10^{-3} M, and hydroquinone to a final concentration of 2.5×10^{-4} M.

However, we distinguish at least two ways in which phenylhydrazine may act: first by direct reaction with molecular oxygen to generate H_2O_2, and second by reaction with oxyhemoglobin to form free H_2O_2 and/or a hematin-H_2O_2 complex. Hence it is not necessary to include the modified form of hemoglobin in the reaction sequence to explain the loss in GSH. The formation of H_2O_2 alone is a sufficient condition to bring about oxidation of GSH in the presence of GSH peroxidase (Cohen and Hochstein, 1963).

The source of the H_2O_2 in experiments with 8-aminoquinolines is unclear, as is the requirement for oxyhemoglobin. One possible explanation is that the 8-aminoquinolines promoted oxidation of ferroheme to ferriheme by oxygen; the H_2O_2 would then represent the reduced form of the oxidizing agent. In these experiments the erythrocytes were noted to darken, indicating that oxidation of hemoglobin was indeed taking place.

A second possible explanation is that the 8-aminoquinolines were converted into derivatives which were capable of undergoing autoxidation. It is known that 8-aminoquinolines are converted *in vivo* into hydroxylated derivatives and corresponding quinones or quinoneimines (Williams, 1959). These metabolites can form a reversible redox system analogous to the hydroquinone *p*-quinone system and therefore capable of generating H_2O_2. A likely site for hydroxylation of aromatic substances *in vivo* is in liver where a microsomal hydroxylating system is present (Mitoma *et al.*, 1956). If hydroxylated metabolites were formed in our experiments *in vitro* then oxyhemoglobin would appear to have been the catalyst (cf. Results, section C).

It is probable that the metabolites of the 8-aminoquinolines play a dominant role in the hemolytic process *in vivo*. These metabolites have been shown to bring about oxidation of hemoglobin and lysis of erythrocytes *in vitro* (Brodie and Udenfriend, 1950). Similar effects have been observed *in vitro* for low concentrations of H_2O_2 (Cohen and Hochstein, 1963) and for agents which can generate H_2O_2, e.g., menadione (Rose and Gyorgy, 1960), hallochrome (Hochstein and Cohen, 1963), and hydroquinone and other reversible redox compounds (Emerson *et al.*, 1949). It should be noted that many other hemolytic drugs (viz., aspirin, analine, acetanalide, phenacetin, sulfanilamide, etc.) are converted *in vivo* into autoxidizable derivatives (cf. Williams, 1959).

The generation of H_2O_2 from hemolytic agents provides a rational link between the molecular structure of the active drugs, the erythrocyte GSH levels, and the enzyme glucose-6-P dehydrogenase. Thus the hemolytic agents are substances which are autoxidizable (e.g., reduced menadione, phenylhydrazine) or which can be transformed into autoxidizable derivatives (e.g., the 8-aminoquinolines). H_2O_2 generated from these agents or their metabolites via autoxidation or via a coupled oxidation with oxyhemoglobin brings about oxidation of GSH, which in glucose-6-P dehydrogenase–deficient cells cannot be reversed due to insufficient generation of NADPH (Cohen and Hochstein, 1961). Since the rate of detoxication of H_2O_2 by GSH peroxidase declines with the intracellular GSH level and since catalase cannot protect erythrocytes (Cohen and Hochstein, 1963), the glucose-6-P dehydrogenase–deficient cells are exposed to further oxidative damage such as oxidation of hemoglobin and inhibition of glycolysis (P. Hochstein and G. Cohen, data to be published). The damaged cells may then be selectively eliminated by two mechanisms; intravascular hemolysis or removal by the reticuloendothelial system.

The data presented in this current report indicate that H_2O_2 is a toxic intermediate common to many of the hemolytic agents. We suggest that drug-induced hemolysis represents a manifestation of H_2O_2 intoxication *in vivo*.

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