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EFFECTS OF NINE SYNTHETIC PUTATIVE METABOLITES OF PRIMAQUINE ON ACTIVITY OF THE HEXOSE MONOPHOSPHATE SHUNT IN INTACT HUMAN RED BLOOD CELLS *IN VITRO**

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Abstract—Suspensions of washed human red blood cells were treated with nine synthetic putative metabolic derivatives of primaquine (PQ'), and their individual effects on activity of the hexose monophosphate shunt (HMS) were quantitated by radiometric analysis of $^{14}CO_2$ from [^{14}C]glucose. The most potent HMS stimulant was 5-hydroxy-6-methoxy-8-aminoquinoline (5H6MQ), which caused 10-fold elevation of HMS activity at an estimated concentration of 0.004 mM. Ten millimolar primaquine (PQ) was required to achieve the same effect. Thus, 5H6MQ was approximately 2500-fold more reactive with the HMS than PQ. Other analogs achieved <0.4- to 154-fold increases in HMS reactivity. Patterns of effects on HMS activity indicated that 5-hydroxylation and/or N-dealkylation of PQ strongly enhanced HMS reactivity. In contrast, none of the putative metabolites of PQ activated the proteolytic system known to degrade oxidized protein in red cells, indicating that stimulation of the HMS by the PQ analogs was not related to an injurious oxidative stress. Red cells pretreated with 1.0 mM *N*-ethylmaleimide (NEM) or with 1.0% (w/v) sodium nitrite to cause glutathione sulfhydryl blockage and conversion of red cell hemoglobin to methemoglobin (metHb), respectively, also showed elevation of HMS activity when exposed to 5H6MQ. These observations suggested that 5H6MQ-induced elevation of HMS activity was at least partially independent of glutathione redox reactions, hydrogen peroxide accumulation and reaction with oxyhemoglobin. The relevance of these observations to proposed mechanisms of hemolytic toxicity of PQ is discussed.

Persons having an inherited deficiency of erythrocytic glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49) may suffer a mild to severe hemolytic anemia following administration of a therapeutic regimen of the schizonticidal antimalarial, primaquine [PQ; 6-methoxy-8-(4-amino-1-methylbutylamino)quinoline]. Various studies indicate that metabolic derivatives of PQ may cause the hemolytic toxicity in sensitive persons [1–5]. Identification of the responsible derivative(s) has proven to be a difficult problem because little is known of PQ metabolism in man and the elucidation of the mechanism of PQ-induced hemolysis has eluded intense investigation.

Since the identification of G6PD-deficiency as the probable underlying cause of PQ sensitivity [6], several hypotheses for the mechanism of this drug-induced hemolysis have been offered [7–11]. Limitation by G6PD of the overall activity of the hexose monophosphate shunt (HMS) has been cited most frequently as the basis of PQ hemolytic toxicity. This view is based largely on the fact that glutathione redox reactions which are coupled to the HMS afford the red cell primary protection against oxidative insult [12–14]. Persons deficient in G6PD thus would suffer a diminished ability to detoxify an oxidative challenge presented by certain drugs. Three observations seem to relate this relationship to the specific case of PQ-induced hemolysis: (a) PQ generates hydrogen peroxide in red cells *in vitro* [15], (b) G6PD-normal volunteers given a therapeutic dose of PQ appear to experience elevated levels of erythrocytic HMS activity [16], and (c) PQ-induced hemolysis *in vivo* is preceded by diminution of reduced glutathione levels in red cells [17]. These observations have been considered to be evidence that PQ initiates an oxidative attack which is of significance *in vivo*.

Recent studies in this laboratory, however, indicate that the hemolytic toxicity of PQ may not be attributable to generation of oxidizing radicals [18, 19]. It was reported that, in the case of two dihydroxylated PQ analogs, concentrations causing relatively potent elevation of HMS activity did not create a net oxidation of protein in G6PD-normal or deficient red cells *in vitro* [19]. An accumulation of oxidizing radicals would lead to oxidation of protein in red cells [12], observed as proteolytic degradation of oxidized protein attending elevation of HMS activity at near maximum rates [18, 20]. Data reported from this laboratory [19] and by Kelman *et al.* [21] indicated that PQ' and PQ-induced elevation of HMS activity was due to oxidation of NADPH independently of glutathione redox reactions. This

* The views of the authors do not purport to reflect those of the Department of the Army or the Deparment of Defense.

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mechanism of elevation of HMS activity accounts for the absence of oxidative injury associated with this effect [18]. These observations indicate that the stimulation of HMS activity by PQ and some of its putative metabolic derivatives was not associated with a toxic accumulation of oxidizing radicals. Thus, it appears that the estimated low levels of stimulation of the erythrocytic HMS (10–20% above baseline) *in vivo* following PQ administration [16] reflect an oxidative challenge which is insufficient to cause oxidative injury.

In the present study, the individual effects of several putative PQ metabolites on HMS activity were quantitated. The possibility that these PQ analogs might elevate the HMS as a consequence of oxidative attack (i.e. as a consequence of increased demand for reduced glutathione) was examined by testing for proteolysis at maximum levels of HMS activity and by measuring 5-hydroxy-6-methoxy-8-aminoquinoline (5H6MQ)-induced elevation of HMS activity in the presence of a sulfhydryl group blocking agent (*N*-ethylmalmeimide, NEM) or in the absence of appreciable amounts of oxyhemoglobin. The results of these experiments support the earlier contention that PQ-induced hemolysis may be a consequence of reactions not associated with an accumulation of oxidizing radicals (i.e. oxidative attack).

MATERIALS AND METHODS

Test compounds. All test compounds were obtained from the U.S. Army Drug Development Program inventory in powder form.* The following test compounds were employed: 4-hydroxy-6-methoxy-8-(4-amino-1-methylbutylamino)quinoline, $2HCl \cdot H_2O$ (4HPQ); 6-methoxy-8-(1-methyl-3-carboxypropylamino)quinoline (CPQ); 5-hydroxy-6-methoxy-8-(4-amino-1-methylbutylamino)quinoline, 2HBr (5HPQ); 5,6-dihydroxy-8-(4-amino-1-methylbutylamino)quinoline, 3HBr (5H6DPQ); 6-hydroxy-8-(4-amino-1-methylbutylamino)quinoline (6DPQ); 6-methoxy-8-aminoquinoline (MQ); 5-hydroxy-6-methoxy-8-aminoquinoline, 2HBr (5H6MQ); 5,6-dihydroxy-8-aminoquinoline, 2HBr (5,6DHQ); 6-hydroxy-8-aminoquinoline (6HQ); and 6-methoxy-8-(4-amino-1-methylbutylamino)-quinoline, $2H_3PO_4$ (PQ).

All test compounds were stored at 4° and protected from exposure to light. Some compounds (5HPQ, 5H6DPQ, and 5,6DHQ) were kept under N_2 atmosphere with desiccant, as recommended by the supplier.

Stock solutions (1.0 mM) of PQ analogs were prepared in phosphate-buffered physiological saline (PBSS, pH 7.4, prepared as described elsewhere [22]). Some test compounds (CPQ, 6HQ, 6DPQ, and MQ) were initially dissolved in dimethyl sulfoxide (DMSO). DMSO concentrations in incubation mixtures did not exceed 0.5% (v/v), a concentration which did not significantly affect baseline or elevated HMS activity levels ($P > 0.1$) (unpublished observation). Subsequent dilutions of test compounds were performed using PBSS as diluent. All test compound solutions were used within 15 min of stock solution preparation.

Red blood cell suspensions. Whole blood (10 ml) was drawn by venipuncture from one 25-year-old Caucasian male volunteer with normal levels of G6PD activity (data not shown) and immediately mixed with 40 ml PBSS prepared with deletion of $CaCl_2$. These cell suspensions were washed and white cells were removed as described elsewhere [22]. Red cell suspensions were kept at 0° and used within 2 hr.

In experiments to assess the possible dependence of 5H6MQ-induced elevation of HMS activity on glutathione redox reactions, hydrogen peroxide accumulation and/or oxyhemoglobin, red cell suspensions prepared as described above were further treated with 1.0 mM NEM or with 1% (w/v) $NaNO_2$ as described elsewhere [23].

HMS activity measure. HMS activity was measured by quantitation of $^{14}CO_2$ released from red cell suspensions containing D[1-^{14}C]glucose or D[2-^{14}C]glucose (both 0.35 μCi/μmole; 5.0 mM glucose) using a micro-volume procedure described in detail elsewhere [22]. The latter compound was employed to detect HMS activity due to first recycling of glucose through the HMS [24].

Standard HMS stimulants (10 mM $NaNO_2$, Aldrich Chemical Co., Milwaukee, WI, 0.5 mM phenylhydrazine, Aldrich Chemical Co.; and 0.010 mM methylene blue chloride, Mallinckrodt Chemical Works, St. Louis, MO) were included in each experiment.

Proteolytic activity measure. Release of free tyrosine from oxidant-treated intact erythrocytes *in vitro* has been shown to reflect a proteolysis caused by activation of an ATP-dependent proteolytic system which specifically degrades oxidized protein [20]. In the present study, acid-soluble tyrosine was recovered from 30% trichloroacetic acid extracts of red cell suspensions and tagged with naphthol for quantitation by fluorescence spectroscopy. The procedure has been detailed elsewhere [18]. Proteolysis caused by standard oxidants (20 mM $NaNO_2$ or 1.0 mM phenylhydrazine) was measured in each experiment.

Statistical analyses. Tests of significance of difference between two means were conducted using an analysis of variance (ANOVA) F-test described by Sokal and Rolf [25]. Tests of significance of difference among means were conducted using the ANOVA Student–Newman–Keuls (SNK) test also described by Sokal and Rolf [25]. Statistical tables employed were those compiled by Rolf and Sokal [26].

RESULTS AND DISCUSSION

HMS activities. The increases in HMS activity following treatment with the standard HMS stimulants ($NaNO_2$, phenylhydrazine or methylene blue) were not appreciably different for each standard ($P > 0.05$). In each of ten experiments, ten samples

* The following individuals or organizations supplied these compounds to the U.S.A.D.D.P. inventory: Prof. L. H. Schmidt, University of Alabama; Dr. J. D. McChesney, University of Mississippi; Dr. A. Strother, Loma Linda University; Ash Stevens Inc., Detroit, MI; and Starks Associates, Buffalo, NY.

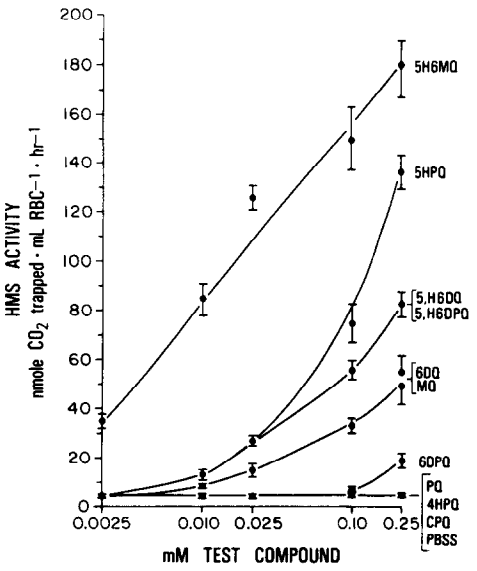

Fig. 1. Effects of test compounds on HMS activity. The identity of each plot is indicated in the right margin; two or more compounds given for a single plot indicate that the mean levels of HMS activity for these compounds were essentially the same. Each point represents the mean of a replicate set of ten samples. Brackets indicate the limits of the 95% confidence interval. The mean coefficients of variation for these data averaged 5.5% and ranged between 1.0 and 9.0%. Select concentrations of each test compound were retested and gave HMS activity values in good agreement with those shown.

of each HMS stimulant were tested, giving a total of one hundred individual HMS activity determinations per standard. The coefficient of variation within these groups (sample to sample) did not exceed 5% and the coefficient of variation among these groups (experiment to experiment) did not exceed 7%. These results indicate that the HMS activity values described below were obtained in a uniform series of HMS activity measurements.

Profiles of HMS activity in the presence of test compounds at 0.0025 to 0.25 mM are shown in Fig. 1. It is evident that 5H6MQ was the most potent HMS stimulant. Greater concentrations of PQ produced increased $^{14}CO_2$ production; at a PQ concentration of 10 mM a 10-fold elevation of HMS activity was observed [19]. The concentration of test compounds giving a similar 10-fold elevation of HMS activity was estimated from the plotted data, and the fold-increase in HMS reactivity was calculated. These values are listed in Table 1 and showed the following order of increase in HMS reactivity: 5H6MQ > 5HPQ > 5,6DHQ = 5H6DPQ > MQ = 6HQ > 6DPQ > PQ > CPQ > 4HPQ.

In all instances where two compounds differed only in the presence or absence of a 5-hydroxy substitution, the 5-hydroxy analog produced a greater increase in HMS reactivity (5HPQ > PQ; 5H6MQ > 6HQ; 5,6DHQ > 6DPQ). N-Dealkylation also caused increases in PQ HMS reactivity (MQ > PQ; 5H6MQ > 5HPQ; 6HQ > 6DPQ), except in the case of the dihydroxy PQ analogs (5,6DHQ = 5H6DPQ). 6-Demethylation of PQ and PQ analogs had two distinct effects: (a) 6-demethylation of PQ and PQ analogs lacking a 5-hydroxy substituent caused increases in HMS reactivity (6DPQ > PQ; 6HQ > MQ), and (b) 6-demethylation of PQ analogs having a 5-hydroxy substitution diminished the increase in HMS reactivity afforded by the latter alteration alone (5H6MQ > 5,6DHQ; 5HPQ > 5H6DPQ). Hydroxylation of the 4-position of PQ diminished HMS reactivity (PQ > 4HPQ), as did carboxylation of the N-alkyl chain (PQ > CPQ). These observations indicate that 5-hydroxylation and/or N-dealkylation of PQ result in the greatest increases in HMS reactivity.

5H6MQ-induced elevation of HMS with NEM or methemoglobin. The effects of 1.0 mM NEM or of conversion of red cell hemoglobin to metHb on 5H6MQ-induced elevation of HMS activity are shown in Table 2.

Treatment of red cells with NEM caused decreases in HMS activity of all experimental groups. Jacob

Table 1. Effect of substitutions to PQ structure on reactivity with red cell HMS

Compound	Substitution	Fold-increase in HMS reactivity*
PQ	None	1.0
CPQ	8-NHCHCH$_3$(CH$_2$)$_2$COOH	0.4†
4HPQ	4-OH	<0.4†
5HPQ	5-OH	154
5H6DPQ	5-OH, 6-OH	117
6DPQ	6-OH	14
MQ	8-NH$_2$	40
5H6MQ	5-OH, 8-NH$_2$	2500
5,6DHQ	5-OH, 6-OH, 8-NH$_2$	117
6HQ	6-OH, 8-NH$_2$	47

* Millimolar amount of PQ required to cause 10-fold elevation of HMS activity (10 mM) divided by that of test compound. Values were obtained from the data presented in Fig. 1.

† Values were obtained from HMS activity measurements taken using ≤10 mM test compound because there was no effect at ≤0.25 mM.

Table 2. Effect of 1.0 mM NEM or metHb on elevation of HMS activity by 5H6MQ and standard HMS stimulants

Test compound	HMS activity*		
	Control	NEM	MetHb
PBSS	4.9 ± 0.34	3.1 ± 0.15	40 ± 2.2
Nitrite (10 mM)	48 ± 2.4	5.4 ± 0.40	39 ± 2.0
Phenylhydrazine (0.5 mM)	37 ± 2.2	11.4 ± 0.5	43 ± 1.4
Methylene blue (0.010 mM)	57 ± 3.8	27 ± 1.5	60 ± 4.0
5H6MQ (0.010 mM)	60 ± 1.8	23 ± 1.4	74 ± 4.8

* Values indicate nmoles CO_2 trapped · (ml RBC)$^{-1}$ · hr^{-1} ± standard error of ten determinations.

and Jandl [27] observed that this effect accompanied complete deactivation of glutathione in saline-treated (i.e. control) red cells, whereas NEM concentrations insufficient to cause complete shutdown of glutathione redox reactions were accompanied by elevated or baseline levels of HMS activity in these cells. In the present study, the observed diminution of HMS activity in saline-treated red cells exposed to 1.0 mM NEM (Table 2) was considered indicative of complete or nearly complete blockage of glutathione sulfhydryl by NEM.

Nitrite-treated red cells did not show appreciably elevated HMS activity levels following NEM treatment. The stimulation of HMS by nitrite thus appears to be entirely dependent upon glutathione redox reactions. Phenylhydrazine maintained HMS stimulation (approximately one-third of that in control red cells) in the presence of NEM, indicating at least partial independence of its effect from glutathione redox reactions. This finding has been reported elsewhere [5, 19] and may be attributed to the ability of phenylhydrazine to oxidize NADPH [19].

The effects of NEM on methylene blue and 5H6MQ-induced elevation of HMS activity were similar. Both compounds maintained an appreciable elevation of HMS activity in NEM-treated red cells (approximately one-half of that in control red cells). The latter effect, partial inhibition of HMS, may be due to one of two activities: (a) the partial elevation of HMS activity by these compounds in the presence of NEM may reflect a proportionate degree of dependence on glutathione redox reactions for this activity; or (b) the non-specificity of sulfhydryl blockage by NEM [28] may have caused an overall inhibition of red cell glucose catabolism through the HMS (e.g. in the presence of 2.0 mM NEM there was no elevation of HMS activity induced by methylene blue, phenylhydrazine, or 5H6MQ; data not shown). The former explanation seems unlikely because Jacob and Jandl [27] observed that methylene blue induced stimulation of HMS activity to control levels in NEM-treated red cells (this was not reproduced in the present study). Thus, the latter explanation may account for the lower levels of methylene blue- or 5H6MQ-induced elevation of HMS in the presence of NEM.

Conversion of hemoglobin to metHb would prevent possible peroxigenic reactions between test compounds and oxyhemoglobin and would not permit accumulation of hydrogen peroxide [15, 21]. The latter activity has been attributed to the action of metHb as a scavenger of one-electron donors [21] or to peroxidative removal of H_2O_2 by metHb itself [29]. Thus, elevation of HMS activity in red cells containing metHb appears to result from a mechanism which is independent of oxidant reaction with oxyhemoglobin and/or of accumulation of H_2O_2.

In the present study, saline-treated red cells containing primarily metHb had levels of HMS activity which were approximately 10-fold greater than that in control red cells (Table 2), as has been reported elsewhere [19, 23, 29]. It was suggested that this

Table 3. Proteolysis in red cell suspensions treated with 0.25 mM test compound and/or standard oxidants

Test compound	Proteolytic activity*			
	Test compound	Test compound + 20 mM $NaNO_2$	20 mM $NaNO_2$ standard†	1.0 mM Phenylhydrazine standard†
PQ	0	22	25	18
CPQ	0	17	17	16
4HPQ	0	23	25	17
5HPQ	0	12	33	33
5H6DPQ	0	17	27	31
6DPQ	0	13	27	25
MQ	0	19	30	21
5H6MQ	0	16	24	23
5,6DHQ	0	23	21	19
6HQ	0	22	33	28

* All values are as nmoles tyrosine released · (ml RBC)$^{-1}$ · hr^{-1}. Proteolytic activity values <5 nmoles tyrosine released · (ml RBC)$^{-1}$ · hr^{-1} were considered to be zero.

† Listed corresponding to each experiment during which results were obtained.

effect may be due to the high number of centrifugations (five) required to remove added nitrite after the conversion of hemoglobin to metHb [29], but Sullivan and Stern [23] found that repeated centrifugation of red cells did not affect HMS activity. An alternative explanation may be that the nitrite treatment initially oxidized red cell components which were not restored to their reduced state until an exposure to glucose-provided HMS reducing equivalents. Since in this study metHb red cells were not exposed to glucose until the determination of HMS activity, it seems likely that the high level of HMS activity in saline-treated metHb red cells was the result of an oxidative stress created during the initial nitrite treatment. The presence of metHb *per se* would not account for elevated levels of HMS activity [27].

Stimulation of the HMS above the baseline levels in metHb red cells, therefore, reflects activity due to mechanisms which are independent of reaction with oxyhemoglobin or accumulation of hydrogen peroxide. The absence of such elevation in nitrite-treated metHb red cells (Table 2) indicates dependence on these reactions for the nitrite-induced elevation of HMS. This interpretation is consistent with the reports that generation of oxygen and peroxynitrate radicals by nitrite requires reaction with oxyhemoglobin [30–32]. Phenylhydrazine also failed to cause elevation of HMS activity in metHb red cells (Table 2). This suggests that the glutathione-independent stimulation of HMS by phenylhydrazine in NEM-pretreated red cells required reaction with oxyhemoglobin to yield an NADPH-oxidizing form of this compound which would elevate the HMS independently from glutathione redox reactions. This interpretation is supported by the observation that phenylhydrazine in cell-free physiological saline oxidized NADPH only in the presence of added red cell lysate [19].

Methylene blue and 5H6MQ caused appreciable elevation of HMS activity in metHb red cells (Table 2), indicating that the mechanism of elevation of HMS activity was at least partially independent of reaction with oxyhemoglobin and accumulation of H_2O_2. Furthermore, this implies that the explanation offered above for the partial inhibition of methylene blue-induced elevation of HMS activity in NEM-treated red cells may also apply to that observed with 5H6MQ. On the basis of these results it is suggested that the mechanism of 5H6MQ-induced elevation of HMS is a methylene blue-like process (i.e. direct oxidation of NADPH in the absence of an appreciable accumulation of oxidizing radicals).

Proteolysis activities. None of the putative PQ metabolites caused oxidative degradation of red cell protein at 0.25 mM or eliminated proteolysis caused by 20 mM $NaNO_2$ (Table 3). These compounds did not affect the tyrosine standard curve used to estimate proteolytic activity (data not shown). Standard oxidants tested in each proteolysis experiment gave proteolytic activity values which were in relatively good agreement (Table 3). These results demonstrate that the absence of proteolysis following treatment with the PQ analogs was not a result of PQ analog-induced proteolytic system shut-down, interference with the measurement of the tyrosine–naphthol complex, or chance failure of the assay system.

Thus, elevation of HMS activity by the putative PQ metabolites was not associated with oxidative attack of labile red cell protein components. This is additional evidence that elevation of HMS by these compounds was achieved through a methylene blue-like mechanism—that involving oxidation of NADPH independent of glutathione redox reactions.

Relevance to proposed mechanisms of primaquine hemolytic toxicity. Data given in this report demonstrate that certain alterations in structure of PQ resulted in marked increases in HMS reactivity. Despite these effects, none of the PQ analogs activated a proteolytic system known to degrade oxidized red cell protein. Potent stimulation of the HMS in the absence of proteolysis suggests methylene blue-like redox activity in the red cell [18]. That the stimulation of the HMS by 5H6MQ was at least partially independent of glutathione redox reactions and of reaction with oxyhemoglobin supported this interpretation. These observations are relevant to proposed mechanisms of PQ hemolytic toxicity.

Methylene blue is known to cause elevation of HMS activity as a consequence of direct NADPH oxidation; NADPH reduces methylene blue which may then reduce hemoglobin or molecular oxygen [33, 34], thus becoming oxidized and again available to accept NADPH electrons. This redox recycling activity is believed to account for the ability of this compound to effect relatively potent elevation of HMS activity. That is, continuous oxidation of NADPH by methylene blue causes the NADPH/$NADP^+$ ratio to decrease so that, in the competition between these allosteric effectors of G6PD (negative and positive, respectively) for binding sites on G6PD, $NADP^+$ is favored and elevation of HMS activity results [27, 35, 36]. This mechanism accounts for the glutathione independence of methylene blue-induced stimulation of HMS [27, 36, 37] and the absence of oxidative injury associated with this effect [18].

Several similarities between this intracellular redox activity and that shown by PQ and some of its putative metabolites have been reported. PQ [21], 5H6DPQ and 5,6DHQ [19] oxidize NADPH and elevate the HMS without dependence upon glutathione redox reactions or oxyhemoglobin. Reduced PQ is oxidized by molecular oxygen [38] or hemoglobin [21], thus permitting redox recycling activity. The dependence of the reduction of metHb by PQ upon glucose suggests a direct dependence on HMS reducing equivalents (i.e. NADPH) for reduction of PQ. Several putative metabolites of PQ cause potent elevation of HMS activity without causing proteolysis ([19] and Table 3). Furthermore, the elevation of the HMS by the most potent stimulant among the PQ analogs, 5H6MQ, appears to be independent of glutathione redox reactions, hydrogen peroxide accumulation, and reaction with oxyhemoglobin. All of the intracellular redox activities described above are exhibited by methylene blue. These observations suggest that, like methylene blue, PQ and some of its putative metabolic derivatives are in an intraerythrocytic redox equilibrium which is mediated by NADPH.

This activity probably accounts at least in part for the ability of PQ to generate oxygen radicals in red cells [15]; reduced PQ, like methylene blue [39], reacts with O_2 to produce superoxide radical [38]. Copious production of superoxide leads to formation of hydrogen peroxide and hydroxyl radical through the univalent reduction of superoxide to water [12, 40].

The production of oxygen radicals by PQ has been considered to be the basis of its hemolytic toxicity [9, 12, 15, 28, 37, 41]. It has been reasoned that superoxide production creates a non-specific oxidative stress (i.e. production of hydrogen peroxide and, particularly, hydroxyl radical) in which red cell components most susceptible to oxidative attack (e.g. protein sulfhydryls [12]) become oxidized. In normal red cells, glutathione redox reactions would dissipate an oxidizing potential and restore electrons to oxidized groups employing reducing equivalents generated in the HMS. In G6PD-deficient red cells, however, such detoxification would not occur because replenishment of reduced glutathione is directly dependent on reduction of $NADP^+$ by G6PD activity. The marked decreases in reduced glutathione levels which precede PQ-induced hemolysis in some G6PD-deficient individuals [17] have thus been considered to show a cause-and-effect relationship. On the basis of these and similar observations, it is widely held that oxygen radicals formed through drug oxidation (of hemoglobin) or reduction (of O_2) reactions in the compromised red cell cause hemolysis through a general oxidative attack. The mechanism through which such an oxidative attack may lead to hemolysis is not as yet understood.

This hypothesis is not supported by the observed absence of oxidative damage to red cell protein in the presence of PQ analogs which cause very high levels of elevation of HMS activity ([19] and Tables 1 and 3). This has also been observed from G6PD-deficient red cell suspensions [19]. These observations indicate that a toxic accumulation of oxidizing radicals may not occur as a consequence of the intracellular redox activity of at least the nine putative metabolites of PQ employed in this study. Furthermore, because the actual elevation of HMS activity *in vivo* by true metabolic derivatives of PQ (of undetermined structure) has been estimated to be only 10–20% above baseline [16], it seems unlikely that the PQ-induced hemolysis is achieved through a non-specific oxidative attack of sensitive red cell components; compounds which did exhibit a capability for causing such an oxidative stress (nitrite and phenylhydrazine) did not cause detectable levels of proteolytic activity at HMS activity levels <1500% above baseline *in vitro* [19]. Finally, the relative scarcity of NADPH in G6PD-deficient red cells [42, 43] would appear to limit production of superoxide through reaction of drug reduced by NADPH [21] with molecular oxygen [38]. These observations from a single blood donor suggest that an oxidative attack is not the underlying cause of PQ-induced hemolysis in persons deficient in erythrocytic G6PD.

It is difficult to attribute the severe hemolytic anemia which PQ induces in G6PD-deficient Caucasians to oxidative attack when *in vivo* reduced glutathione levels in red cells decrease by only 15% prior to hemolysis [44]. It would be expected that an 85% complement of reduced glutathione would be sufficient to prevent irreversible oxidative deterioration of the red cell, e.g. Jacob and Jandl [27] noted that reduced glutathione must be completely exhausted before oxidative damage to hemoglobin is incurred. Furthermore, persons having an inherent deficiency of reduced glutathione suffer only mild hemolytic anemias or none at all [45, 46], which is not consistent with an oxygen radical-mediated hemolysis. It may be that even extreme diminution of reduced glutathione prior to hemolysis in G6PD-deficient individuals [17] is a result of drug-mediated exhaustion of NADPH and is not directly related to reactions which culminate in hemolysis.

Reactivity of PQ derivatives with NADPH may explain the PQ-sensitivity of G6PD-deficient persons. Red cells with normal levels of G6PD activity possess NADPH/$NADP^+$ ratios which are very high in comparison to those of G6PD-deficient red cells [42, 43]. Thus, upon exposure to NADPH-reactive compound (e.g. methylene blue or PQ′) the normal red cell would maintain the drug in reduced form, whereas in the G6PD-deficient red cell the oxidized form of drug would predominate. It is possible that, in the presence of an excess of oxidized drug in the red cell, oxidations may occur which would not occur in equilibrium conditions which strongly favored the reduced form (i.e. an intact HMS). This interpretation is supported by the observation that a metabolite of the hemolytic drug pamaquine, 5-hydroxy-6-methoxy-8-(4-diethyl-1-methylbutylamino) quinoline, oxidized hemoglobin when in its oxidized form (presumably the quinonimine) but was unable to do so when reduced [1]. Strother *et al.* [47] reported similar observations from PQ metabolites isolated from mouse liver microsomes; it was found that, while 5H6DPQ caused greater increases in metHb content in G6PD-deficient red cells than in normal, a quinonimine form of that compound caused increases in the metHb content of G6PD-deficient red cells only. Blanchard and Schmidt [48] hypothesized that the methemoglobinemia resulting from administration of many 8-aminoquinoline derivatives was a consequence of *in vivo* conversion of drug to a quinonimine form. These observations are consistent with the proposed relationship between an NADPH-mediated drug redox equilibrium and oxidation of hemoglobin by what is probably a quinonimine oxidation product of PQ′.

In an earlier report, Strother *et al.* [5] measured metHb in G6PD-deficient red cells treated *in vitro* with several of the putative metabolites employed in the present study. A correlation between hemoglobin oxidation and stimulation of the HMS (i.e. oxidation of NADPH) should exist if intracellular predominance of oxidized drug causes the observed alteration to hemoglobin in G6PD-deficient (i.e. NADPH-deficient) red cells. The ranked order of PQ analogs causing metHb formation was essentially the same as that for HMS reactivity (see [5] and Table 1). This correlation suggests that elevation of HMS activity in normal red cells (through a methylene blue-like process) reflects the extent to which hemoglobin may be modified as a probable conse-

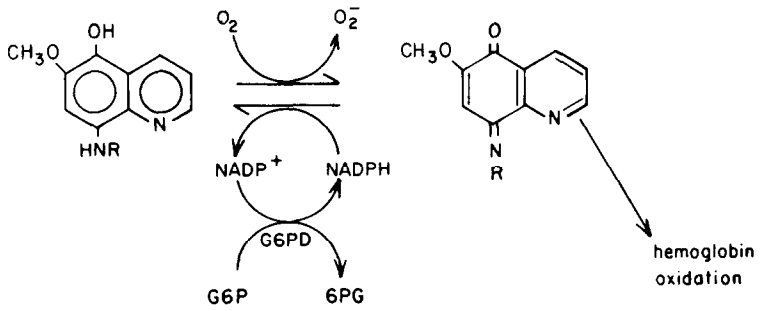

Fig. 2. Proposed mechanism of elevation of HMS activity and relationship between NADPH-mediated drug redox equilibrium and modification of hemoglobin as may occur in red cells lacking sufficient NADPH to maintain drug in reduced form (i.e. G6PD-deficient).

quence of failure to maintain drug in reduced form in G6PD-deficient red cells. These suggested relationships are illustrated in Fig. 2.

Oxidation of hemoglobin by PQ′ to form metHb *per se* probably does not account for the hemolytic toxicity of PQ. The most compelling evidence in this regard is the observation that even potentially fatal doses of nitrite in humans are not accompanied with hemolysis despite overwhelming production of metHb [49]. Also, G6PD-deficient persons exposed to nitrite do not suffer hemolysis despite production of metHb [50]. Similarly, persons deficient in metHb-reductase typically do not suffer hemolytic sensitivity to chemotherapeutic agents [51, 52]. Paradoxically, PQ tends to cause methemoglobinemia in G6PD-normal rather than deficient individuals [37]. This observation may be explained on the basis of the proposed drug redox equilibrium illustrated in Fig. 2. The scarcity of NADPH in G6PD-deficient red cells would limit superoxide production through reaction of reduced PQ′ with O_2 and thus prevent formation of methemoglobin in this manner. It seems likely that some other product of drug–hemoglobin reaction may be responsible for the PQ-induced hemolysis.

In this regard, Itano and colleagues [53–55] have demonstrated that the phenylhydrazine-induced Heinz body hemolytic anemia in rodents seems to be caused by formation of a phenyl-heme covalent complex rather than by oxidative deterioration of the red cell. The hemolytic effect of an analog of the antimalarial drug menadione (1,4-naphthoquinone-2-sulfonate) observed *in vitro* [56] may be attributed to a similar effect. The possibility that some metabolic derivatives of PQ may form lytic complexes with hemoglobin heme is being investigated presently in this laboratory.

In summary, this study has shown that 5-hydroxylation and/or *N*-dealkylation of PQ caused pronounced increases in HMS reactivity. The results of experiments employing measurements of 5H6MQ-induced elevation of HMS activity in the presence of glutathione redox blockade or metHb demonstrated that this effect was independent of glutathione redox activity or of reaction with oxyhemoglobin and hydrogen peroxide. The failure to cause net oxidation of red cell protein in blood from the single donor tested indicated that elevation of HMS by these compounds was not associated with an erythrotoxic accumulation of oxidizing radicals. It was suggested that PQ′ reactivity with NADPH may explain the PQ-sensitivity of G6PD-deficient persons; in these red cells the NADPH/$NADP^+$ equilibrium may allow maintenance of drug in oxidized form and thus allow reactions which may culminate in hemolysis. Formation of a covalent complex between PQ′ and hemoglobin heme to yield a lytic product is considered a possibility.

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