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OXIDATIVE ACTIVITY OF HYDROXYLATED PRIMAQUINE ANALOGS

NON-TOXICITY TO GLUCOSE-6-PHOSPHATE DEHYDROGENASE-DEFICIENT HUMAN RED BLOOD CELLS *IN VITRO**†

J. KEVIN BAIRD,‡§ DAVID E. DAVIDSON, JR. and JOAN E. DECKER-JACKSON

Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, DC 20307-5100, U.S.A.

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Abstract—The individual effects of two putative metabolites of primaquine (5,6-dihydroxyprimaquine and 5,6-dihydroxy-8-aminoquinoline) on the hexose monophosphate shunt (HMS) and on the ATP-dependent proteolytic system which rapidly degrades oxidized erythrocyte protein were measured in intact red blood cells *in vitro* from two blood donors. In red cells treated with nitrite (1–40 mM) or phenylhydrazine (0.01–10 mM), proteolytic activity was detected only with concentrations (7.5 mM $NaNO_2$ and 0.25 mM phenylhydrazine) causing >15-fold elevation of HMS activity, and glucose-6-phosphate dehydrogenase (G6PD)-deficient (25% of normal activity) red cell suspensions thus treated showed approximatly 30% greater proteolysis. G6PD-normal and deficient red cells treated with the primaquine analogs, however, did not experience proteolysis with concentrations (0.25 mM) in excess of those causing 17-fold elevation of HMS activity. Stimulation of the HMS by the primaquine analogs thus appears unrelated to an erythrotoxic oxidative stress. Methylene blue is known to cause an elevation of HMS activity through direct and diaphorase II-dependent oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) which is independent of injurious oxidative stress. It was found that the putative primaquine metabolites also caused direct and diaphorase II-dependent oxidation of NADPH in dilute hemolysate, thus suggesting that the putative primaquine metabolites have a methylene blue-like redox disposition in red blood cells. Results obtained in this study suggest that the hemolytic toxicity of primaquine may be unrelated to processes which lead to oxidative deterioration of red cell protein.

Erythrocytes deficient in glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49) may be susceptible to oxidative injury caused by certain therapeutic drugs. G6PD is rate limiting for the hexose monophosphate shunt (HMS), and glutathione redox reactions coupled to this pathway are believed to afford the erythrocyte primary protection against oxidative attack [1, 2]. A diminution of G6PD catalytic activity would impair this mechanism of defense against oxidative stress [3, 4]. The fact that G6PD-deficient erythrocytes more readily suffer depletion of reduced glutathione (GSH) following treatment with oxidizing substances is well established.

The antimalarial drug primaquine causes acute intravascular hemolysis in some G6PD-deficient individuals when administered in therapeutic doses [5, 6]. Cohen and Hochstein [7] demonstrated that primaquine may cause formation of hydrogen peroxide in intact erythrocytes *in vitro*, and Welt *et al.* [8] showed that *in vivo* levels of human metabolic derivatives of primaquine in serum induced elevation of HMS activity *in vitro*. These studies are frequently cited as indicative of the ability of primaquine to initiate an oxidative attack culminating in acute hemolysis. The pathological defect in G6PD-deficient erythrocytes which manifests primaquine hemolytic toxicity seems to be the inability of these cells to maintain protective levels of GSH during a potentially injurious oxidative stress.

The observation that primaquine causes hydrogen peroxide formation [7] and elevation of HMS under *in vivo* conditions [8] is consistent with this hypothesis. However, a recent study by Kelman *et al.* [9] demonstrated that stimulation of HMS induced by primaquine in intact red cells *in vitro* was not a process mediated by oxygen radical formation or glutathione redox reactions coupled to the HMS, but was similar to the NADPH-dependent stimulation induced by methylene blue. This observation raises doubts about the interpretation of elevation of the HMS by primaquine as an indication of the hemolytic potential of this drug. This problem has been discussed in a recent report from this laboratory in which it was shown that elevation of the HMS by methylene blue was not associated with an injurious oxidative stress [10]. If the oxidative activity of hemolytic metabolites of primaquine is methylene blue-like then it would appear unlikely that the hemolytic toxicity is due to the reactions described above (i.e.

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

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§ Address inquiries and requests for reprints to: J. K. Baird, Department of Infectious and Parasitic Disease Pathology, Armed Forces Institute of Pathology, Washington, DC 20306-6000.

depletion of GSH followed by oxidative deterioration of the red cell). It was our intent to explore this possibility in the present study.

The primary aim of this investigation was to determine whether the oxidative activities of hydroxylated derivatives of primaquine were associated with oxidative attack (i.e. similar to nitrite and phenylhydrazine) or not (i.e. similar to methylene blue). Levels of HMS activity, reactivity with NADPH, and activity of the ATP-dependent proteolytic system in erythrocytes which hydrolyzes oxidized protein [11] were measured to characterize the oxidative activity of test compounds in G6PD-normal and -deficient red cell suspensions.

MATERIALS AND METHODS

Buffers

PBSS, pH 7.4. A physiologically balanced salts solution (PBSS) was prepared as described elsewhere [12].

Tris–HCl *EDTA, pH* 8.0. Tris–HCl EDTA buffer was prepared as described by Beutler [13].

Mercaptoethanol-EDTA. Mercaptoethanol-EDTA was prepared as described by Beutler [13].

Reagents

Saturated $Ba(OH)_2$. Saturated barium hydroxide solution was prepared as described in a previous report [12].

1% *(w/v) 1-Nitroso-2-naphthol.* Nitrosonaphthol reagent was prepared in 95% ethanol as described by Waalkes and Udenfriend [14].

20% (v/v) Nitric acid/nitrite. Nitric acid/nitrite reagent was prepared as described by Waalkes and Udenfriend [14].

0.2 *mM NADPH.* NADPH solution was prepared by addition of 1.36 ml PBSS to 0.3 mg $Na_4NADPH \cdot 2H_2O$ (Sigma Chemical Co., St. Louis, MO) within 30 min of use.

Isotopes

D[1-^{14}C]Glucose and D[2-^{14}C]glucose were prepared as described elsewhere [12].

Test compounds

Sodium nitrite. Reagent grade sodium nitrite was obtained from the Aldrich Chemical Co., Milwaukee, WI and stored in a desiccated container.

Phenylhydrazine. Reagent grade phenylhydrazine was obtained from the Aldrich Chemical Co. and stored at 4°.

Methylene blue chloride. Pharmaceutical grade methylene blue chloride was obtained from the Mallinckrodt Chemical Works, St. Louis, MO, and stored in a desiccated container.

Stock solutions (250 μM) of the above compounds were prepared in PBSS within 2 hr of use. Test solutions were prepared by dilution of stock with PBSS.

Primaquine. Primaquine [6-methoxy-8-(4-amino-1-methylbutylamino)quinoline] diphosphate was obtained from Ash Stevens Inc., Detroit, MI, and stored in a desiccated container protected from light. Stock solution (25 mM) was prepared in PBSS within 30 min of use. Primaquine test solutions were prepared by dilution with PBSS, and, when necessary, the pH of these solutions was adjusted to 7.4 using 1 M NaOH.

*WR*250016. 5,6-Dihydroxy-8-(4-amino-1-methylbutylamino)quinoline (WR250016) was obtained from the U.S. Army Drug Development Program inventory and stored in a desiccated jar under N_2 atmosphere at 4°. Stock solution (2.5 mM) was prepared in PBSS within 10 min of use. WR250016 test solutions were prepared by dilution with PBSS.

*WR*6865. 5,6-Dihydroxy-8-aminoquinoline hydrobromide (WR6865) was obtained from the U.S. Army Drug Development Program inventory and stored in a desiccated jar, under N_2 atmosphere at 4°. Stock solution (2.5 mM) was prepared in PBSS within 10 min of use. WR6865 test solutions were prepared by dilution with PBSS.

Primaquine, WR250016, and WR6865 solutions were protected from extended exposure to light.

Red blood cells

G6PD-normal. Whole blood (40 ml) was drawn by venipuncture from a 25-year-old Caucasian male with no family history of hemolytic sensitivity and washed as described elsewhere [12]. Red cell suspensions were kept at 0° and used within 1 hr.

G6PD-deficient. Whole blood (100 ml) was drawn by venipuncture from a 27-year-old Negroid male and washed as described previously [15]. The donor had previously been clinically diagnosed as G6PD-deficient following a hemolytic episode suffered during a viral infection for which chemotherapy had been administered. This individual was in a healthy condition and not under medication at the time of blood donation. To document the G6PD-deficiency condition of the donor, HMS activity measurements were taken from cell suspensions treated with 15 mM $NaNO_2$ or PBSS in parallel with G6PD-normal red cells thus treated. The following ratio (where the subscript denotes incubation medium and the "Test" or "Normal" prefix denotes cell type) was used to calculate %G6PD activity of normal in test red cell suspension:

$$\frac{\text{Test HMS}_{NaNO_2} \times \text{Normal HMS}_{PBSS}}{\text{Test HMS}_{PBSS} \times \text{Normal HMS}_{NaNO_2}} \times 100$$

$$= \begin{array}{l}\%\text{ of normal G6PD} \\ \text{activity in test} \\ \text{erythrocytes}\end{array}$$

Measurement of NADPH-dependent diaphorase reactivity

The ability of test compounds to serve as electron acceptors in the oxidation of NADPH via diaphorase II enzyme was measured using a modification of the method reported by Beutler [13]. Methylene blue readily accepts electrons in this reaction [13, 16] and served as a standard for positive diaphorase reactivity.

Red cell lysate. Red cell lysate served as the diaphorase source. Red cells kept in 10 mM glucose in PBSS at 4° for no longer than 5 days were centrifuged (500 × *g*, 10 min, 4°), resuspended in PBSS, and centrifuged again. Two millilitres of packed cells was transferred into 18.0 ml mercaptoethanol-EDTA

(25°), mixed well and centrifuged at 22,000 *g* for 10 min at 4°. An aliquot of supernatant fraction was transferred into a screw-cap vial, kept at 0° and used within 2 hr.

Incubation. Reaction mixtures contained the following components:

Test	*Control*
20 μl Test compound (0.5 mM)	20 μl Test compound (0.5 mM)
20 μl RBC lysate	No lysate
40 μl NADPH solution (0.2 mM)	40 μl NADPH solution (0.2 mM)
100 μl Tris–HCl, EDTA	100 μl Tris–HCl EDTA
820 μl PBSS	840 μl PBSS

All components except NADPH were added to quartz cuvettes and mixed well. After approximately 1 min, NADPH was added, the mixture was shaken gently for about 5 sec, and relative fluorescence intensity (RFI) was measured immediately at room temperature in an Aminco Bowman spectrophotofluorometer. The wavelength of excitation was set at 340 nm and of emission at 456 nm. The cuvette was covered and maintained at 37° for 30 min, and a final RFI measurement was obtained subsequently.

Diaphorase reactivity determination. Because NADPH fluoresces, but $NADP^+$ does not, diminution of RFI is indicative of oxidation of NADPH to $NADP^+$. To obtain a relative measure of this reaction, the following ratio was employed:

$$\frac{RFI_i - RFI_f}{RFI_i} = \text{total relative NADPH oxidation}$$

where RFI_i and RFI_f were the initial and final RFI respectively. This ratio provided an indication of the extent of total NADPH oxidation which had occurred in the reaction mixture.

Subtraction of the control total relative NADPH oxidation ratio from that on test gives a measure of NADPH oxidation attributable to the presence of diaphorase; this value is referred to as "relative diaphorase reactivity". In experiments in which test compound was omitted from the reaction mixture but lysate was not, the resulting total relative NADPH oxidation ratios were essentially the same as those in reaction mixtures containing neither lysate or test compound (data not given). This indicates that diaphorase did not oxidize NADPH in the absence of added electron acceptor. The assay thus provides a relative measure of the ability of the test compound to serve as an electron acceptor for diaphorase-mediated NADPH oxidation.

A measure of the ability of the test compound to oxidize NADPH directly ("relative NADPH reactivity") was obtained by subtraction of the relative NADPH oxidation value of a blank sample (no test compound or lysate) from that of a control sample.

Measurement of HMS activity

A micro-volume (50 μl) procedure reported from this laboratory [12] was employed in the present study; incubation, HMS activity determination, and statistical analyses were conducted as described in that report. HMS activity was determined from $^{14}CO_2$ recovered from D[1-^{14}C]glucose and D[2-^{14}C] glucose incubations.

Measurement of proteolytic activity

A modification of the procedure described by Goldberg and Boches [11], as reported elsewhere [10], was employed in the present study. In this procedure free tyrosine was recovered from erythrocyte TCA extracts, tagged with naphthol, and quantitated using spectrofluorometry.

RESULTS

Diaphroase reactivity

The oxidation of NADPH in various reaction mixtures over a 30-min period is shown in Table 1. The data indicate that WR6865 and WR250016 were more effective than either phenylhydrazine or nitrite in acting as NADPH electron acceptors (enzymatic or non-enzymatic), but they were less effective than methylene blue.

Table 1. Oxidative reactivity of test compounds (10 μM) with NADPH (8 μM) as measured by the decrease in fluorescence emission intensity at 465 nm (340 nm excitation) from dilute hemolysate (1:1000) over a 30-min period (37°)

Compound	Relative diaphorase reactivity*	Relative NADPH reactivity†
Saline	0.00	0.00
Nitrite	0.01	0.01
Phenylhydrazine	0.06	0.00
WR250016	0.15	0.03
WR6865	0.17	0.09
Methylene blue	0.34	0.23

* NADPH oxidation attributable to diaphorase. Each value is the mean of three determinations, and tests conducted on separate occasions gave values in good agreement with those shown.

† NADPH oxidation attributable to test compound. Each value is the mean of three determinations, and tests conducted on separate occasions gave values in good agreement with those shown.

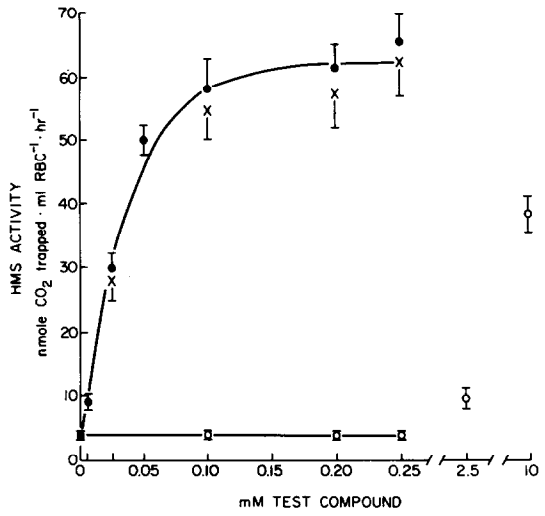

Fig. 1. Stimulation of the HMS by treatment of G6PD-normal red cells with primaquine (○), WR250016 (●), or WR6865 (×). Data points are the numerical mean from replicate groups of ten samples. Brackets indicate the limits for the 95% confidence interval. Each HMS stimulation profile was obtained from a separate experiment. Each experiment was repeated and gave values not significantly different ($P > 0.05$) from those shown.

HMS stimulation and proteolysis in G6PD-normal erythrocytes

HMS stimulation capabilities of the hydroxylated primaquine analogs, as well as that of primaquine, are presented in Fig. 1. It is evident that hydroxylation markedly increased the effect of primaquine on HMS activity. With respect to the amount of compound required to elevate the HMS 10-fold, the hydroxylated primaquine analogs were 200-fold more potent than primaquine itself (10-fold HMS elevation at 0.05 and 10 mM respectively).

Nitrite-induced HMS and proteolytic activities are shown in Fig. 2. Between 0 and 2.5 mM $NaNO_2$, HMS activity increased 3-fold above baseline, a significant elevation of activity ($P < 0.001$), but no proteolytic activity was detected (< 5 nmoles tyrosine released · (ml RBC)$^{-1}$ · hr^{-1}). In the presence of 7.5 mM $NaNo_2$, the HMS was elevated 15-fold above baseline and proteolytic activity was observed (10 nmoles tyrosine released · (ml RBC)$^{-1}$ · hr^{-1}). Nitrite concentrations greater than 10 mM caused as much as 20-fold elevation of HMS activity and a maximum proteolytic activity of 45 nmoles tyrosine released · (ml RBC)$^{-1}$ · hr^{-1}.

Phenylhydrazine-induced HMS and proteolytic activities are shown in Fig. 3. Elevation of HMS activity was significant (1.8-fold, $P < 0.001$) in the presence of as little as 0.015 mM phenylhydrazine (data not shown). In the presence of 0.025 mM phenylhydrazine, HMS activity was 5.5-fold above baseline and proteolytic activity was not observed. Phenylhydrazine concentrations greater than 0.10 mM caused as much as 30-fold elevation of HMS activity and maximum proteolytic activity (120 nmoles tyrosine released · (ml RBC)$^{-1}$ · hr^{-1}) (Fig. 3).

WR6865 and WR250016 produced results similar to those reported elsewhere for methylene blue [10], i.e. potent elevation of HMS activity (Fig. 1) in the absence of proteolytic degradation (Table 2).

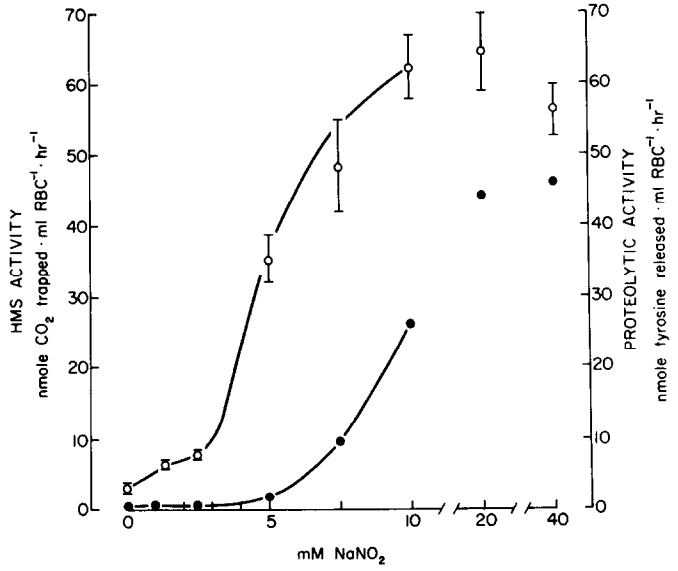

Fig. 2. Nitrite-induced elevation of HMS activity (○) and activation of a proteolytic system (●) in G6PD-normal red cells. HMS activity points are the numerical mean from replicate groups of ten samples. Brackets indicate the limits for the 95% confidence interval. This HMS stimulation profile was obtained from a single experiment which was repeated and gave uniformly similar ($P > 0.05$) values to those shown above. Proteolytic activity points are the numerical mean of values obtained from duplicate treatments in a single experiment. The proteolysis measurement experiment was repeated and gave values in good agreement with those shown.

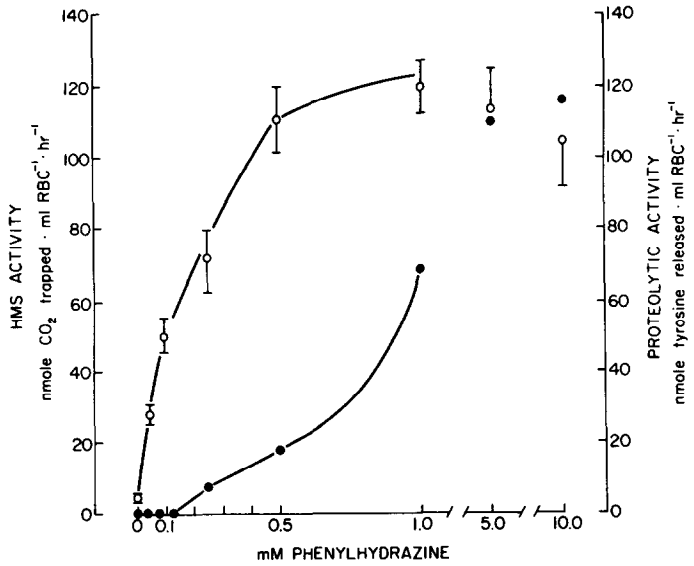

Fig. 3. Phenylhydrazine-induced elevation of HMS activity (○) and activation of a proteolytic system (●) in G6PD-normal red cells. HMS activity points are the numerical mean from replicate groups of ten samples. Brackets indicate the limits for the 95% confidence interval. This HMS stimulation profile was obtained from a single experiment which was repeated and gave uniformly similar ($P > 0.05$) values to those shown above. Proteolytic activity points are the numerical mean of values obtained from duplicate treatments in a single experiment. The proteolysis measurement experiment was repeated and gave values in good agreement with those shown.

Elevation of HMS activity by methylene blue was approximately 30-fold at maximum [10]. Thirty-five-fold HMS elevation by 0.25 mM of another hydroxylated primaquine analog (5-hydroxy-6-methoxy-8-aminoquinoline) was not accompanied with detectable proteolysis (unpublished observation).

Proteolysis in G6PD-deficient erythrocytes

Overall G6PD activity in red cell suspensions obtained from the G6PD-deficient donor was determined to be 25% of that in normal red cell suspensions. This value was calculated employing the HMS activity measurements listed in Table 3, as described in Materials and Methods.

Nitrite-induced proteolytic activity in G6PD-normal and G6PD-deficient red cells is shown in Fig. 4. In both cell types, proteolytic activity reached a plateau level; in G6PD-deficient erythrocytes this level was 70 nmoles tyrosine released · (ml RBC)$^{-1}$ · hr^{-1} (30% greater than that from G6PD-normal erythrocytes, 50 nmoles tyrosine released · (ml RBC)$^{-1}$ · hr^{-1}). Phenylhydrazine-induced proteolysis in G6PD-deficient erythrocytes was about 30% greater than that in normal erythrocytes (Fig. 5). In both G6PD-normal and -deficient erythrocytes, nitrite-induced proteolysis was about 50% of that induced by phenylhydrazine.

Methylene blue, WR6865 and WR250016 did not cause detectable proteolytic activity in G6PD-deficient red cells treated with concentrations in excess of those required for maximal elevation of HMS activity (Table 2).

Methylene blue does not interfere with proteolytic activity or the assay used for its measurement [10].

Table 2. Proteolysis in G6PD-normal and -deficient red cells in the presence of quantities of test compound sufficient for ≥20-fold elevation of HMS

	Proteolysis		
	G6PD-normal		
Compound	Saline	20 mM $NaNO_2$	G6PD-deficient
Saline	0	50	0
Nitrite (10 mM)	48		65
Phenylhydrazine (0.5 mM)	20		37
Methylene blue (0.25 mM)	0	58	0
WR250016 (0.25 mM)	0	27	0
WR6865 (0.25 mM)	0	38	0

All values are given in nmoles tyrosine release · (ml RBC)$^{-1}$ · hr^{-1} and are the mean of duplicate determinations conducted on at least two occasions.

Table 3. Measurement of HMS activity in G6PD-normal and -deficient red cell suspensions

Donor	Incubation	HMS activity*
G6PD-normal	PBSS	7.6 ± 0.4
	15 mM $NaNO_2$	71 ± 7
G6PD-deficient (Test)	PBSS	6.3 ± 0.4
	15 mM $NaNO_2$	14 ± 1.1

* Values are nmoles CO_2 trapped·(ml RBC)$^{-1}$·hr^{-1}. Each value is the mean ± the standard error of ten determinations. This experiment was repeated employing 0.25 mM WR250016 instead of 15 mM $NaNO_2$, and the difference in elevation of HMS activity between the two red cell suspensions was not significant ($P > 0.05$).

In the present study, WR250016 or WR6865 did not eliminate nitrite-induced proteolysis (Table 2) or interfere with the proteolytic activity assay (data not given).

DISCUSSION

Results obtained in this investigation indicate that at least two putative metabolites of primaquine exhibit methylene blue-like oxidative reactivity in intact red cells and that this pattern of activity does not appear to cause oxidative deterioration of G6PD-normal or -deficient red blood cells. Conversely, patterns of oxidative activity shown by nitrite or phenylhydrazine did cause generation of a toxic oxidizing potential which led to degradation of red cell protein. It is suggested that the hemolytic toxicity of primaquine may be unrelated to processes which cause oxidative deterioration of the red cell.

The mechanism of elevation of HMS activity by the hydroxylated primaquine analogs appears to be similar to that of methylene blue. Elevation of the HMS by methylene blue is independent of glutathione redox reactions [9, 16, 17], as is that by primaquine [9, 17]. This mechanism involves direct and diaphorase II-dependent oxidation of NADPH to $NADP^+$ [18] which directly mediates activation of G6PD enzyme and, consequently, the HMS [3, 19]. The NADPH-dependent and glutathione-independent mechanism of stimulation of the HMS by methylene blue was first reported Jacob and Jandl [16], and has been reiterated recently by Carson *et al.* [17]. Primaquine also directly oxidizes NADPH [20] with concomittant elevation of HMS [9]. The data given in Table 1 indicate that the two putative metabolites of primaquine were also capable of direct and diaphorase-dependent oxidation of NADPH. These findings suggest that the elevation of HMS activity by primaquine (or hydroxylated analogs) was a methylene blue-like process involving oxidation of NADPH.

Autoxidation of methylene blue in red cells is believed to account for its relatively potent effect on the HMS. Oxidized methylene blue in red cells is reduced rapidly by NADPH and reoxidized by molecular oxygen [21]. In this manner, NADPH may be continously oxidized by a limited quantity of methylene blue to effect a relatively potent HMS stimulation. By comparison, nitrite does not autoxidize [22] or react appreciably with NADPH (Table 1), and 200 moles are required for the oxidation of 1 mole glucose through the HMS, whereas 1 mole methylene blue may cause the oxidation of 160 moles glucose in the HMS [10]. This disparity is reflected

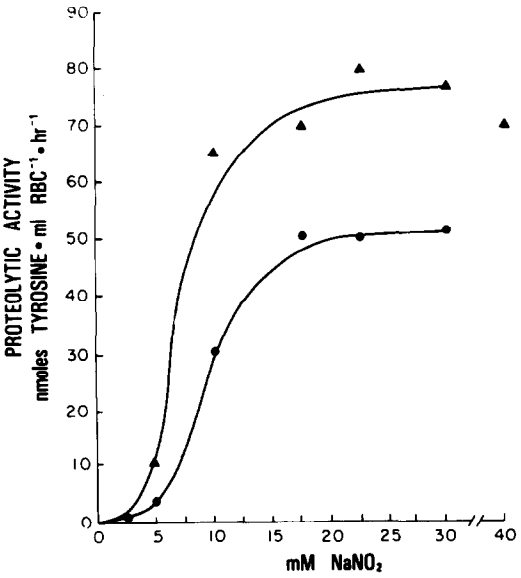

Fig. 4. Nitrite-induced proteolytic activity in G6PD-normal (●) or G6PD-deficient (▲) red cells. Data points are the numerical mean of values obtained from duplicate samples on a single occasion. These experiments were repeated and gave values in good agreement with those shown.

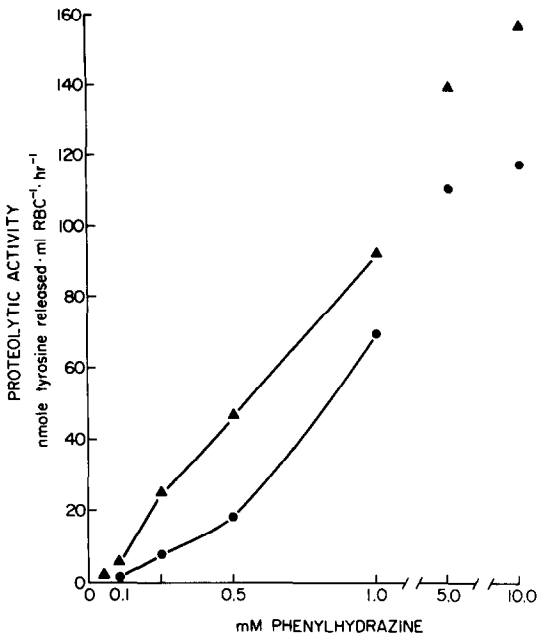

Fig. 5. Phenylhydrazine-induced proteolytic activity in G6PD-normal (●) or G6PD-deficient (▲) red cells. Data points are the numerical mean of values obtained from duplicate samples on a single occasion. The experiments were repeated and gave values in good agreement with those shown.

in the NADPH oxidation values given in Table 1. The hydroxylated primaquine analogs also effected relatively potent stimulation of HMS and reactivity with NADPH (Fig. 1 and Table 1). It thus appears that these compounds may also be autoxidized in the red cell. Autoxidation of primaquine has been demonstrated already [9, 20].

Autoxidative processes yield superoxide ion and thus create a potential for oxidative attack. Results obtained in a previous study [10] suggest that, even when autoxidation rates would be expected to be maximal, oxidative challenge by the oxygen reduction products thus derived did not present a toxic oxidizing potential to red cell protein. A maximum rate of methylene blue autoxidation would be reflected in a maximal elevation of HMS activity because the latter process is a direct consequence of the former [16]; diminution of the $NADPH/NADP^+$ ratio to very low levels (i.e. stimulation of HMS) reflects rapid reduction of methylene blue to leukomethylene blue and thus oxidation of this product by molecular oxygen. Proteolysis does not occur under these conditions [10]. This is consistent with the observation that no hydrogen peroxide accumulates with concentrations of methylene blue sufficient to cause maximum HMS activity [16]. Furthermore, methylene blue does not cause depletion of GSH *in vitro* [23] or *in vivo* [24]. These observations indicate that the autoxidation of methylene blue does not present a toxic oxidative challenge to the red cell. The data given in Table 2 indicate that no oxidative injury was incurred as a result of the suggested autoxidation of the hydroxylated primaquine analogues in G6PD-normal or -deficient red cells.

The oxidizing activities of nitrite and phenylhydrazine were different from those of the methylene blue-like compounds. In Figs. 2 and 3 it is evident that nitrite- or phenylhydrazine-induced stimulation of the HMS was coupled to oxidation of red cell protein. Proteolysis was detected after elevation of HMS to near-maximal activity (>15-fold). This was attributed to stimulation of the HMS in a glutathione-dependent manner, accompanied by depletion of GSH [4, 16], leading to irreversible oxidation of red cell protein [1, 2]. That proteolysis was more pronounced in G6PD-deficient red cells (Figs. 4 and 5) indicates that maintenance of GSH was G6PD-dependent and essential for protection of red cell protein against oxidative attack.

These relationships seem well-established and are the basis of the hypothesis which attributes the hemolytic toxicity of primaquine in G6PD-deficient persons to the ability of primaquine and/or its metabolites to generate oxygen radicals [1, 4, 7, 8, 17, 25]. It is difficult to reconcile the results obtained in this study with this hypothesis on two accounts: (1) generation of oxygen radicals through autoxidative processes does not appear to have been oxidatively toxic to the red cell, even with diminished ability to maintain protective levels of GSH; and (2) the absence of a net oxidation of red cell protein following treatment with the putative primaquine metabolites indicates that these compounds do not generate a toxic oxidizing stress, regardless of its origin (i.e. neither through oxygen nor drug radicals).

The marked differences between the nitrite-like and methylene blue-like oxidants may be attributable to the formation of nitrite [26] and phenylhydrazine radicals [27, 28] which may directly oxidize red cell protein and thus create a glutathione-dependent stimulation of the HMS. Such radicals would be longer-lived than those of oxygen [28, 29], and the red cell probably would not possess efficient systems for their detoxification, as it does for the oxygen radicals [1]. That the stimulation of HMS by the methylene blue-like compounds is independent of GSH ([9, 16, 17] and Table 3) and occurs without proteolysis suggests that the oxygen radicals produced in their autoxidation were sufficiently detoxified by glutathione-independent enzymes (e.g. superoxide dismutase and catalase).

G6PD-deficient red cells also appear to have been capable of full protection against oxygen radicals, but suffered diminished protection against oxidative challenge to red cell protein presented by nitrite or phenylhydrazine oxidizing radical moieties. That G6PD-deficient red cells may possess normal levels of catalase activity [30, 31]* would support the notion that self-protection against oxygen radical toxicity may be complete in these cells. Alternatively, the absence of oxidative injury to G6PD-deficient cells treated with methylene blue-like compounds may be due to the limiting amount of NADPH in these cells [15, 19], i.e. the extent of autoxidation of the methylene blue-like compounds and the comcomitant generation of oxygen radicals may be limited.

Formation of a toxic oxidizing radical similar to those formed by nitrite or phenylhydrazine of some metabolic derivative of primaquine would seem to account for the primaquine sensitivity of G6PD-deficient individuals; however, the available experimental evidence does not support this explanation. Estimates of elevation of HMS in human red cells by human metabolic derivatives of primaquine ranged between 10 and 20% above baseline activity [8]. This activity reflects an oxidizing potential which appears trivial in comparison to those found necessary to cause detectable levels of nitrite or phenylhydrazine-induced proteolysis *in vivo* (>15-fold elevation of HMS). Thus, there is no indication that an appreciable oxidative challenge is incurred in *in vivo* whether derived from oxygen radicals or a phenylhydrazine-like primaquine radical.

In summary, this study has shown that two hydroxylated primaquine analogs exhibit methylene blue-like oxidative activity in red blood cells. The most striking feature of this pattern of activity was the inability of the putative primaquine metabolites tested to generate a net oxidation of G6PD-deficient red cell protein. This activity was contrasted with that shown by nitrite and phenylhydrazine. These latter compounds caused stimulation of the HMS which was coupled to oxidation of red cell protein,

* Tarlov and Kellermeyer [32] measured 40% diminution of catalase activity in G6PD-deficient individuals and suggested that the finding of normal catalase activity by Beutler *et al.* [30] and Szeinberg *et al.* [31] was due to experimental error.

an effect which was more pronounced in G6PD-deficient red cells. All of the test compounds employed in this study are known or believed to generate oxygen radicals. This process alone, however, did not appear to be sufficient to cause a net oxidation of red cell protein. The oxidative damage in nitrite- and phenylhydrazine-treated red cells may be attributed to formation of peroxynitrite [26] or phenyl radicals [27, 28] which may be longer-lifed [28, 29] and less efficiently detoxified by the red cell. This interpretation provides a possible explanation for the absence of proteolysis in red cells treated with compounds which autoxidize and thus produce oxygen radicals. The formation of an oxidatively toxic primaquine radical *in vivo* or as explanation for primaquine sensitivity of G6PD-deficient individuals is not supported by estimates of primaquine-induced elevation of HMS *in vivo*. Those estimates [8] reflect an oxidative stress which is shown in the present study to be insufficient to cause a net oxidation of red cell protein (regardless of G6PD activity) *in vitro*. The observations reported herein, based upon two blood donors, support the possibility that the hemolytic toxicity of primaquine may be unrelated to processes which lead to the oxidative deterioration of sensitive red cells.

REFERENCES

1. S. K. Srivastava, A. K. Lal and N. H. Ansari, *Red Blood Cell and Lens Metabolism* (Ed. S. K. Srivastava), p. 123. Elsevier North Holland, New York (1980).
2. E. R. Jaffé, *Red Blood Cell and Lens Metabolism* (Ed. S. K. Srivastava), p. 107. Elsevier North Holland, New York (1980).
3. H. N. Kirkman, W. G. Wilson and E. H. Clemons, *J. Lab. clin. Med.* **95**, 877 (1980).
4. E. Beutler, *Pharmac. Rev.* **21**, 73 (1969).
5. E. Beutler, *The Metabolic Basis of Inherited Disease* (Eds. J. B. Stanbury, J. B. Wyngaarden and D. S. Fedrickson), p. 1430. McGraw-Hill, New York (1978).
6. R. J. Dern, F. M. Weinstein, G. V. LeRoy, D. W. Talmage and A. S. Alving, *J. Lab. clin. Med.* **43**, 303 (1954).
7. G. Cohen and P. Hochstein, *Biochemistry* **3**, 895 (1964).
8. S. I. Welt, E. H. Jackson, H. N. Kirkman and J. C. Parker, *Ann. N.Y. Acad. Sci.* **179**, 625 (1971).
9. S. N. Kelman, S. G. Sullivan and A. Stein, *Biochem. Pharmac.* **31**, 2409 (1982).
10. J. K. Baird, *Int. J. Biochem.* **16**, 1053 (1984).
11. A. L. Goldberg and F. S. Boches, *Science* **215**, 1107 (1982).
12. J. K. Baird, J. E. Decker-Jackson and D. E. Davidson, Jr., *Int. J. Biochem.* **16**, 1049 (1984).
13. E. Beutler, *Red Cell Metabolism: A Manual of Biochemical Methods*. Grune & Stratton, New York (1975).
14. T. P. Waalkes and S. Udenfriend, *J. Lab. clin. Med* **50**, 733 (1957).
15. H. N. Kirkman, G. D. Gaetani, E. H. Clemons and C. Mareni, *J. clin. Invest.* **55**, 875 (1975).
16. H. S. Jacob and J. H. Jandl, *J. biol. Chem.* **241**, 4243 (1966).
17. P. E. Carson, R. Hohl, M. V. Nora, G. W. Parkhurst, T. Ahmad, S. Scanlan and H. Frischer, *Bull. Wld Hlth Org.* **59**, 427 (1981).
18. M. D. Sass, C. J. Caruso and D. R. Axelrod, *Clinica chim. Acta* **24**, 77 (1969).
19. G. D. Gaetani, J. D. Parker and H. N. Kirkman, *Proc. natn. Acad. Sci. U.S.A.* **71**, 3584 (1974).
20. P. J. Thornalley, A. Stern and J. V. Bannister, *Biochem. Pharmac.* **32**, 3571 (1983).
21. E. N. Metz, P. Balcerzak and A. L. Sagone, Jr., *J. clin. Invest.* **58**, 797 (1976).
22. A. Tomoda, A. Tsuji and Y. Yoneyama, *Biochem. J.* **193**, 169 (1981).
23. J. E. Smith, E. Mahaffey and M. Lee, *Biochem. J.* **168**, 587 (1977).
24. L. Bockris and R. H. Smith, *Nature, Lond.* **196**, 278 (1962).
25. A. M. Magon, R. M. Leipzig, V. G. Zahnoni and G. J. Brewer, *J. Lab. clin. Med.* **97**, 764 (1981).
26. M. P. Doyle, R. A. Pickering, R. L. Dykstra, C. L. Nelson and R. F. Boyer, *Biochem. biophys. Res. Commun.* **105**, 127 (1982).
27. H. A. Itano and S. A. Mannen, *Biochim. biophys. Acta* **421**, 87 (1976).
28. H. A. O. Hill and P. J. Thornalley, *Fedn Eur. Biochem. Soc.* **125**, 235 (1981).
29. J. V. Bannister and H. A. O. Hill, *Biochem. Soc. Trans.* **10**, 68 (1982).
30. E. Beutler, R. J. Dern, C. L. Flanagan and A. S. Alving, *J. Lab. clin. Med.* **45**, 286 (1955).
31. A. Szeinberg, C. Sheba, N. Hirshorn and E. Bodonyi, *Blood* **12**, 603 (1957).
32. A. R. Tarlov and R. W. Kellermeyer, *J. Lab. clin. Med.* **58**, 204 (1961).