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OXIDATIVE ACTIVITY OF PRIMAQUINE METABOLITES ON RAT ERYTHROCYTES *IN VITRO* AND *IN VIVO*

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Abstract—The oxidative activities of primaquine [6-methoxy-8-(4-amino-1-methylbutylamino)quinoline] and its metabolites, the quinone-imine derivatives of 5-hydroxyprimaquine [5-hydroxy-6-methoxy-8-(4-amino-1-methylbutylamino)quinoline] and 5-hydroxydemethylprimaquine [5-hydroxy-6-demethyl-8-(4-amino-1-methylbutylamino)quinoline], 6-methoxy-8-amino quinoline and hydrogen peroxide, were studied on rat erythrocytes *in vitro* and *in vivo*. In both cases, the most effective metabolites in oxidizing hemoglobin and depleting non-protein sulfhydryl groups from erythrocytes were the quinone-imine derivatives of the ring-hydroxylated metabolites, 5-hydroxyprimaquine and 5-hydroxydemethylprimaquine. The latter quinone-imines were shown by light absorption spectroscopy and oxygen consumption studies to be able to oxidize purified rat hemoglobin to methemoglobin but to be unable to react directly with reduced glutathione. In agreement with these results, no radical adduct was detected by electron paramagnetic resonance spectroscopy in incubations of rat erythrocytes with the quinone-imines and the spin-trap 5,5-dimethyl-1-pyrroline-*N*-oxide; metabolite-derived free radicals were detected instead. Taken together, the results suggest that 5-hydroxyprimaquine and 5-hydroxydemethylprimaquine are important metabolites in the expression of primaquine hemotoxicity, in contrast to 6-methoxy-8-aminoquinoline. Additionally, the results indicate that hydrogen peroxide is the ultimate oxidant formed from the ring-hydroxylated metabolites by redox-cycling of the corresponding quinone-imine derivatives both *in vitro* and *in vivo*.

Key words: primaquine, antimalarial drugs, hemotoxicity, primaquine metabolites, oxidative stress, methemoglobinemia

Malaria is one of the most common infectious diseases, affecting 100–300 million people per year. Primaquine [6-methoxy-8-(4-amino-1-methylbutylamino)quinoline] (PRQ)† plays a unique role in malaria treatment because, in addition to having a slight action on asexual blood stages of the parasite, it is the only drug capable of eliminating the persistent liver forms responsible for relapses in *Plasmodium vivax* and *Plasmodium ovale* infections [1]. The clinical usefulness of primaquine, however, is compromised by toxic effects such as hemolytic anemia and methemoglobinemia caused in patients, particularly in those with glucose-6-phosphate dehydrogenase deficiency [1–5]. The latter genetic disorder results from a number of different mutations, but the basic defect is a decreased or absent ability of the erythrocyte to generate NADPH [6] and a consequent susceptibility to drug-mediated oxidative stress [1–5]. These facts, considered together with the low hemotoxicity of primaquine *in vitro*, have led to widespread acceptance of the idea that the side-effects of primaquine are due to the oxidative activity of a metabolite; which metabolite is the ultimate oxidant, however, is still a matter of debate.

Primaquine has several biologically reactive groups in its structure, and its metabolism can render different compounds capable of oxido-reduction activity such as 5-hydroxyprimaquine [5-hydroxy-6-methoxy-8-(4-amino-1-methylbutylamino)quinoline] (5-HPQ) and 5-hydroxydemethylprimaquine (5-hydroxy-6-demethyl-8-(4-amino-1-methylbutylamino)quinoline] (5-H-DPQ), ring-hydroxylated metabolites identified in biological fluids of experimental animals [7–9] and 6-methoxy-8-aminoquinoline (MAQ), an identified human metabolite [10] (Fig. 1). MAQ has been implicated in primaquine hemotoxicity through conversion to an N-hydroxylated derivative with methemoglobin-forming activity (Fig. 1) by analogy with aniline metabolism and toxicity [11]. On the other hand, ring-hydroxylated metabolites such as 5-HPQ and 5-H-6-DPQ (Fig. 1) have been studied extensively *in vitro*, and have been shown to be more effective than primaquine in oxidizing hemoglobin and depleting reduced glutathione (GSH) from human erythrocytes [7, 8, 12–15]. The latter effects can probably be ascribed to the corresponding quinone-imine derivatives [13] which, together with hydrogen peroxide, are the main products of the fast auto-

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† Abbreviations: PRQ, 6-methoxy-8-(4-amino-1-methylbutylamino)quinoline); 5-HPQ, 5-hydroxy-6-methoxy-8-(4-amino-1-methylbutylamino)quinoline or 5-hydroxyprimaquine; 5-H-6-DPQ, 5-hydroxy-6-demethyl-8-(4-amino-1-methylbutylamino)quinoline or 5-hydroxydemethylprimaquine; MAQ, 6-methoxy-8-aminoquinoline; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); GSH, reduced glutathione; NPSH, non-protein sulfhydryl; PBS, phosphate-buffered saline; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; and mT, millitesla(s).

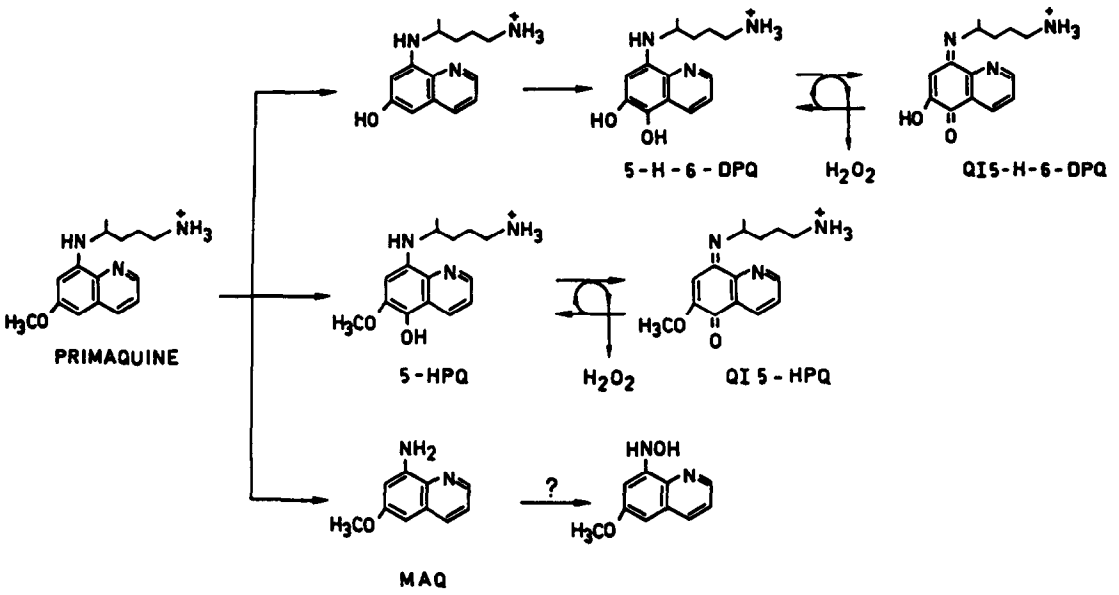

Fig. 1. Schematic representation of primaquine biotransformation to metabolites with oxidative activity.

oxidation undergone by 5-HPQ and 5-H-6-DPQ at neutral pH (Fig. 1) [16]. Although MAQ, 5-HPQ and 5-H-6-DPQ have been identified as primaquine metabolites *in vivo*, their oxidative properties have been either inferred or examined in *in vitro* studies. Consequently, an important step for the understanding of primaquine hemotoxicity is to examine the oxidative activity of the identified metabolites *in vivo*.

In this report, we compare the oxidative activities of primaquine and its metabolites, the quinone-imine derivatives of 5-H-PQ and 5-H-6-DPQ, MAQ and hydrogen peroxide (Fig. 1) on rat erythrocytes *in vitro* and *in vivo*. The results suggest that 5-H-PQ and 5-H-6-DPQ are important metabolites in the expression of primaquine hemotoxicity, whereas MAQ appears not to be relevant. Additionally, the results support the view that hydrogen peroxide is the ultimate oxidant formed from 5-H-PQ and 5-H-6-DPQ by redox-cycling of the corresponding quinone-imine derivatives [16].

MATERIALS AND METHODS

Primaquine diphosphate, GSH, rat hemoglobin, DTNB, aniline and DMPO were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Catalase, H_2O_2:H_2O_2 oxidoreductase (EC 1.11.1.6) was from Boehringer Mannheim (Mannheim, Germany). The metabolites 5-HPQ, 5-H-6-DPQ, and MAQ were obtained from the World Health Organization, and were synthesized by Prof. A. Strother, University of Loma Linda (CA, U.S.A.) as part of the SWG-CHEMAL, UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. DMPO was purified by charcoal filtration [17]. Rat oxyhemoglobin was obtained from the commercial product as previously described [18]. Aniline hydrochloride was obtained by titration of the free base with concentrated HCl followed by product recrystallization from ethanol. Stock solutions of MAQ (5 mM) were prepared in PBS containing ethanol (2%, v/v) for the *in vitro* experiments. Stock solutions of 5-HPQ and 5-H-6-DPQ (5–10 mM) were prepared in aerated PBS, and the final pH was adjusted to 7.4 by the addition of concentrated NaOH. These stock solutions contain equimolar concentrations of hydrogen peroxide and the corresponding quinone-imines of the ring-hydroxylated metabolites (Fig. 1) [16], and were used in most *in vitro* experiments. The only exceptions were the experiments with purified rat oxyhemoglobin and GSH in which the quinone-imines were purified from hydrogen peroxide, as previously described [16].

Male Wistar rats (200–250 g) were killed, and red blood cells were obtained as previously described [13] and resuspended in PBS; a final packed cell volume of 30% was used in most experiments. All *in vivo* studies employed male Wistar rats (200–250 g) fasted for 18 hr before the experiments. Basal levels were determined in blood collected from the tail vein before any treatment. EDTA was used as an anticoagulant, and the samples were kept on ice and examined within 2 hr of collection. The animals were anesthetized with ether and injected intravenously with the test compounds. Primaquine, the quinone-imines, aniline and hydogen peroxide were administered in 0.85% NaCl with the final pH adjusted to 7.4. MAQ was prepared in ethanol:0.85% NaCl (1:1, v/v) and dimethyl sulfoxide:0.9% NaCl (1:1, v/v) for intravenous and oral administration, respectively.

Methemoglobin levels were determined as previously described [11]. Non-protein sulfhydryl groups were determined in erythrocytes and in whole blood

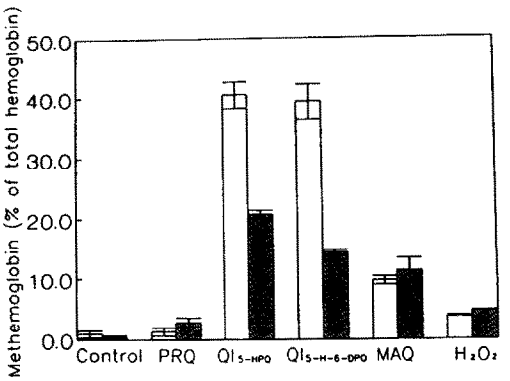

Fig. 2. Methemoglobin formation in rat erythrocytes incubated with either primaquine or its metabolites. Methemoglobin levels were measured after a 2-hr incubation at 37° of rat erythrocytes (30%) with the compounds (0.5 mM) in PBS, pH 7.4, in the absence (open bars) or in the presence (striped bars) of glucose (0.5 mM). The results are the means ± SD of three experiments.

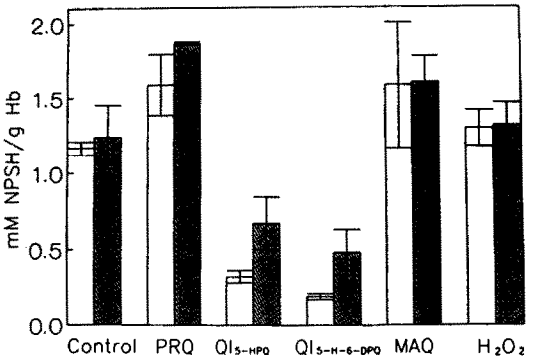

Fig. 3. NPSH levels in rat erythrocytes incubated with either primaquine or its metabolites. NPSH levels were measured after a 2-hr incubation at 37° of rat erythrocytes (30%) with the compounds (0.5 mM) in PBS, pH 7.4, in the absence (open bars) or in the presence (striped bars) of glucose (0.5 mM). The results are the means ± SD of three experiments.

as described by Maples *et al.* [19], and the values obtained were normalized in relation to the total hemoglobin concentration of each sample [20]. Erythrocyte osmotic fragility was determined by measuring hemoglobin release after erythrocyte incubation with 0.55% NaCl for 30 min at room temperature. The values obtained were expressed in comparison with the hemoglobin released after incubation of each erythrocyte sample with 0.85% NaCl (0% hemolysis) and 0.1% NaCl (100% hemolysis) [21].

Absorption spectra were recorded on an Aminco DW-200 spectrophotometer, oxygen uptake was followed in an oxygen monitor (Gilson 5/6 Oxygraph), and EPR spectra were recorded on a Bruker ER 200 D-SRC spectrometer. All of these measurements were performed at room temperature unless otherwise specified.

RESULTS

In vitro *oxidative activity*. Formation of methemoglobin in rat erythrocytes incubated for 2 hr at 37° with primaquine or its metabolites is shown in Fig. 2. Primaquine (0.5 mM) had a marginal effect on hemoglobin oxidation, whereas the quinone-imine derivatives of either 5-HPQ or 5-H-6-DPQ were potent methemoglobin inducers at the same concentration (Fig. 2). Qualitatively, these results are in agreement with those previously obtained with canine hemolysates [12] and human erythrocytes [13–15]. Methemoglobin formation observed in the presence of the quinone-imines was not due to the hydrogen peroxide present in the stock solutions of these compounds [16] since 0.5 mM hydrogen peroxide was much less effective in promoting hemoglobin oxidation (Fig. 1). MAQ was also ineffective since the low methemoglobin level observed in its presence (about 10%) (Fig. 2) was due to the solvent ethanol as verified in control experiments.

Physiological concentrations of glucose (5 mM) significantly inhibited the accumulation of methemoglobin caused by primaquine metabolites (Fig. 2). In the case of the quinone-imine derivatives of 5-HPQ and 5-H-6-DPQ, glucose inhibited hemoglobin oxidation by 49 and 63%, respectively, but methemoglobin levels were still significantly higher than in control erythrocytes (Fig. 2).

The oxidative activity of the metabolites was also examined by measuring the levels of non-protein sulfhydryl groups (NPSH) in the incubated rat erythrocytes (Fig. 3). Again the more effective metabolites were the quinone-imine derivatives of 5-HPQ and 5-H-6-DPQ (Fig. 3). As observed in the case of hemoglobin oxidation (Fig. 2), glucose was partially effective in inhibiting the NPSH depeletion caused by the quinone-imine derivatives (Fig. 3).

Oxidation of hemoglobin and NPSH was not accompanied by rat erythrocyte hemolysis. Indeed, under the experimental conditions shown in Fig. 2, in the absence or in the presence of glucose, none of the metabolites induced hemolysis as verified by measurements of hemoglobin release in the red blood cell supernatant (not shown).

Quinone-imine reactivity. The most effective oxidizing metabolites in erythrocytes, the quinone-imines derived from 5-HPQ and 5-H-6-DPQ (Figs 2 and 3), were purified (see Materials and Methods) and examined for their abilities to interact with hemoglobin and GSH. The quinone-imines oxidize purified rat hemoglobin, predominantly producing methemoglobin as demonstrated by the spectral changes observed during the reaction (Fig. 4A). Catalase (100 μg/mL) inhibited the rate of hemoglobin oxidation by 60% under the experimental conditions shown in Fig. 4A, indicating participation of hydrogen peroxide in the process. Hemoglobin oxidation by the quinone-imine metabolites or primaquine was accompanied by oxygen liberation (not shown) as observed before for other quinones [18]. On the other hand, the quinone-imines did not

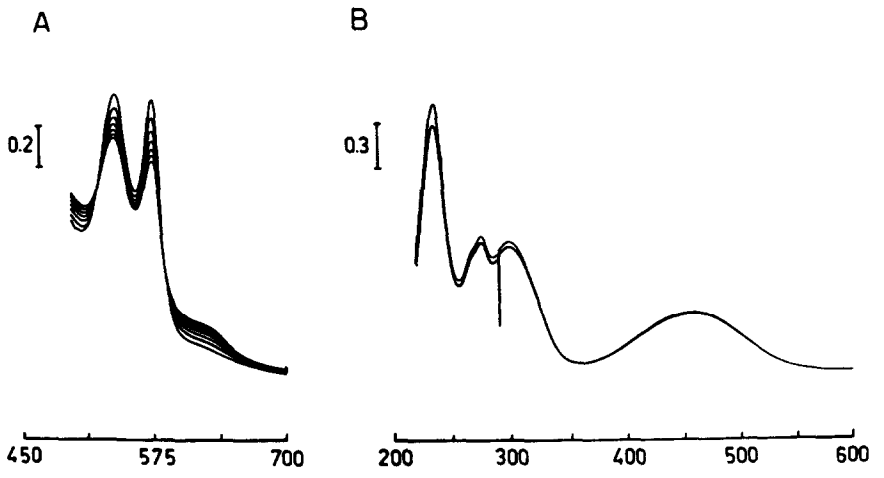

Fig. 4. Spectral changes observed during the interaction of the quinone-imine derivative of 5-H-6-DPQ with hemoglobin (A) and GSH (B). (A) Oxyhemoglobin (95 μM) was incubated with the quinone-imine (50 μM) at 37° in PBS, pH 7.4, and the spectra were scanned at 5-min intervals. (B) The spectrum of the 5-H-6-DPQ quinone-imine (150 μM) was scanned at room temperature before (upper spectrum) and 20 min after the addition of GSH (1 mM) (lower spectrum).

react directly with GSH since incubation of excess thiol reagent with the quinone-imines under anaerobic conditions did not change the light absorption spectra of the metabolites (see, for instance, Fig. 4B) [22, 23]. In agreement, the addition of GSH also did not induce oxygen consumption by the purified quinone-imines under aerobic conditions (not shown).

To obtain further information about the interaction of the quinone-imines and erythrocytes, we performed EPR–spin-trapping experiments in the presence of DMPO. This compound has been shown previously to trap both the hemoglobin-thiyl radical and the glutathionyl radical formed by oxidation of hemoglobin or GSH in rat erythrocytes incubated *in vitro* with hemotoxic drugs [19, 24, 25]. For instance, the DMPO-hemoglobin thiyl free radical adduct has been trapped in the case of phenylhydrazine [24] and *tert*-butylhydroperoxide [19], whereas both the DMPO-glutathionyl and the DMPO-hemoglobin thiyl free radical adducts have been trapped in the case of rat erythrocytes incubated with phenylhydroxylamine [25]. In the case of primaquine-derived quinone-imines, however, no DMPO free radical adducts were detected in incubations of rat erythrocytes (8 or 100%), DMPO (100 mM) and the metabolites (2–20 mM); drug-derived free radicals were detected instead (see, for instance, Fig. 5). The radical observed in the case of 5-HPQ was the corresponding semiquinone-imine detected before in auto-oxidation and redox-cycling experiments (Fig. 5A) [16]. In the case of 5-H-6-DPQ, the detected radical changed depending on the experimental conditions employed. In the presence of high concentrations of the quinone-imine (>10 mM), erythrocytes (100%) and Mg^{2+} (0.5 M), the corresponding *o*-semiquinone-imine radical was detected (Fig. 5B) [16]. The intrinsic

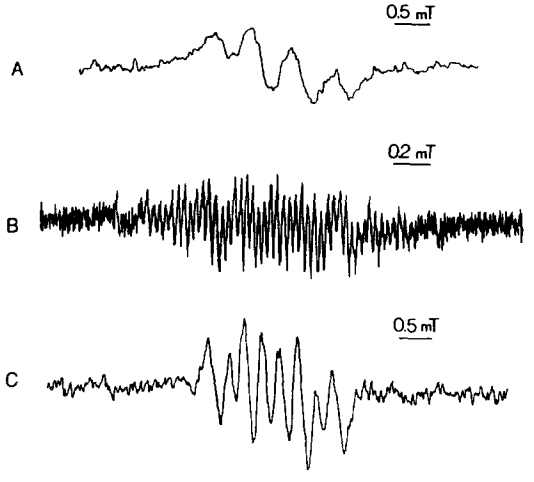

Fig. 5. EPR spectra obtained from incubations of rat erythrocytes with the quinone-imine derivatives of 5-HPQ and 5-H-6-DPQ in the presence of DMPO. The incubation mixtures in PBS, pH 7.4, at room temperature contained DMPO (100 mM) and: (A) rat erythrocytes (8%) and 5-HPQ-derived quinine-imine (2.0 mM); (B) rat erythrocytes (100%), Mg^{2+} (0.5 M) and 5-H-6-DPQ-derived quinine-imine (20 mM); and (C) rat erythrocytes (8%), Mg^{2+} (0.5 M) and 5-H-6-DPQ-derived quinone-imine (20 mM). Instrumental conditions: microwave power, 31 mW for (A) and (C) and 2.5 mW for (B); modulation amplitude, 0.2 mT for (A) and (C) and 0.01 mT for (B); time constant, 1 sec; scan rate, 0.05 mT/sec for (A), 0.00125 mT/sec for (B) and 0.02 mT/sec for (C).

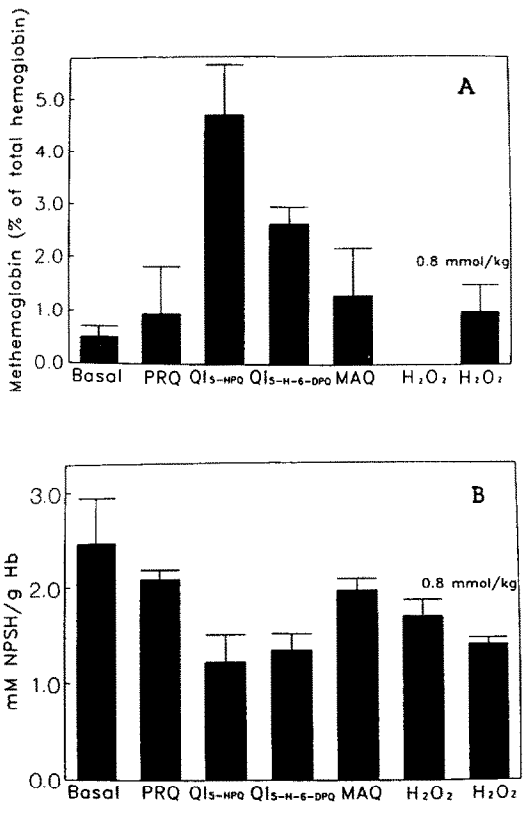

Fig. 6. Methemoglobin (A) and NPSH (B) levels in rats after intravenous administration of either primaquine or its metabolites. The compounds were administered as described in Materials and Methods at the following doses: primquine (0.1 mmol/kg), the quinone-imine derivatives of 5-HPQ and 5-H-6-DPQ (0.08 mmol/kg), MAQ (0.08 mmol/kg) and hydrogen peroxide (0.08 and 0.8 mmol/kg, as specified in the figure). The results are the means ± SD of a minimum of three experiments.

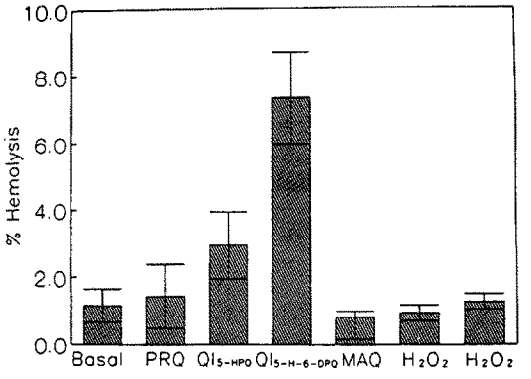

Fig. 7. Osmotic fragility of rat erythrocytes after intravenous administration of either primaquine or its metabolites. The compounds were administered as described in Materials and Methods at the following doses: primaquine (0.1 mmol/kg), the quinone-imine derivatives of 5-HPQ and 5-H-6-DPQ (0.08 mmol/kg), MAQ (0.08 mmol/kg) and hydrogen peroxide (0.08 and 0.8 mmol/kg, respectively, as shown in Fig. 6). The results are the means ± SD of a minimum of three experiments.

properties of this radical, such as the number of EPR active centers and saturation properties [16], are consistent with the requirements of high steady-state concentrations to allow EPR detection (Fig. 5B). Accordingly, lower concentrations of erythrocytes (8%) and quinone-imine, in the presence or in the absence of Mg^{2+}, led to detection of a different free radical intermediate (Fig. 5C). Formation of this radical probably depends on peroxidative reactions since its EPR spectrum is similar to that previously detected in incubations of primaquine with methemoglobin and hydrogen peroxide [26]. These EPR studies prove that erythrocytes are able to promote redox-cycling of the quinone-imine metabolites as has been suggested before [13]. This process, however, rendered undetectable levels of both DMPO-hydroxyl and DMPO-thiyl radical adducts (Fig. 5).

In vivo *oxidative activity*. Figure 6 presents the methemoglobin (Fig. 6A) and NPSH (Fig. 6B) levels determined 1 hr after intravenous administration to rats of primaquine (0.1 mmol/kg) or its putative metabolites, the quinone-imines of 5-HPQ (0.08 mmol/kg) and 5-H-6-DPQ (0.08 mmol/kg), MAQ (0.08 mmol/kg), and hydrogen peroxide (0.08 and 0.8 mmol/kg). Preliminary experiments led us to select the above exposure time and metabolite doses, since higher intravenous doses (≥2 mmol/kg) of the quinone-imine derivatives were toxic, causing animal death. Methemoglobin levels in rats were low but significantly higher than the basal level only in the case of the quinone-imine derivatives of 5-HPQ (4.7%) and 5-H-6-DPQ (2.6%) (Fig. 6A). These compounds were also more effective in depleting NPSH from erythrocytes; its levels dropped to about 50% of the basal value upon administration of the quinone-imines (Fig. 6B). Concentrations of hydrogen peroxide similar to those present in the stock solutions of the metabolites (0.08 mmol/kg) [16] marginally affected NPSH levels, whereas higher concentrations (0.8 mmol/kg) decreased NPSH levels to 62% of the basal level (Fig. 6B).

The erythrocytes of rats receiving intravenous administration of the metabolites were also examined for osmotic fragility (Fig. 7). The effects were negligible except in the case of rats treated with the 5-H-6-DPQ-derived quinone-imine (Fig. 7). This increase in osmotic fragility did not correlate with the oxidative activity of the metabolites since both quinone-imines were equally efficient in depleting NPSH (Fig. 6B), whereas the 5-HPQ-derived quinone-imine was more effective in oxidizing hemoglobin (Fig. 6B). Previous *in vitro* studies with glucose-6-phosphate dehydrogenase-deficient red blood cells have also failed to correlate hemoglobin release with hemoglobin oxidation and GSH depletion promoted by 5-HPQ and 5-H-6-DPQ [14, 15].

Administration of higher doses of primaquine or MAQ (2 mmol/kg) intragastrically to rats was also ineffective in promoting methemoglobin formation

(not shown). By contrast, the same dose of aniline greatly increased formation of methemoglobin, the levels of which attained a maximum of about 40% of the total hemoglobin 2 hr after administration (not shown), as previously reported [11].

DISCUSSION

The oxidative activity of identified primaquine metabolites (Fig. 1) on rat erythrocytes was studied *in vitro* (Figs. 2 and 3) and *in vivo* (Figs. 6 and 7). In both cases, the most effective metabolites in oxidizing hemoglobin and depleting NPSH from erythrocytes were the quinone-imine derivatives of 5-HPQ and 5-H-6-DPQ. Consequently, our results do not support the proposition of a similar hemotoxic mechanism for primaquine and aniline [11]. Indeed, primaquine was not effective in producing methemoglobin *in vivo* in the rat (Fig. 6A; see also Results), in contrast to aniline [11]. Also, in the latter case, metabolic *N*-hydroxylation reactions producing phenylhydroxylamine have been established to be more important in leading to methemoglobinemia than ring-hydroxylation reactions producing aminophenols [11]. By contrast, in the case of primaquine the ring-hydroxylated metabolites (Fig. 6) were more effective *in vivo* than MAQ (Fig. 6), the immediate precursor of the putative primaquine-derived arylhydroxylamine (Fig. 1).

Taken together, our results also provide a mechanistic view for the oxidative activity of the quinone-imines derived from 5-HPQ and 5-H-6-DPQ. The toxicity of quinone-like compounds is usually ascribed either to formation of adducts with cellular constituents, such as GSH and protein-thiols, or to redox-cycling leading to the formation of active oxygen species and ulterior GSH oxidation [23, 27, 28]. The first mechanism appears unlikely in the case of 5-HPQ and 5-H-6-DPQ quinone-imines since the positions susceptible to 1,4-reductive addition [26] are blocked (Fig. 1). In agreement, a direct reaction between GSH and the quinone-imines was excluded by light absorption spectroscopy (Fig. 4B), oxygen consumption (see Results) and spin-trapping experiments in the presence of DMPO (Fig. 5). On the other hand, redox-cycling of the 5-HPQ and 5-H-6-DPQ quinone-imines has been demonstrated before in the presence of NADPH/ferredoxin:$NADP^+$ oxidoreductase [16] and here, in the presence of erythrocytes (Fig. 5, A and B). In this case, redox cycling can be accomplished by either hemoglobin (Fig. 8A) [13] or cellular reductases (Fig. 8B). A direct reaction between the quinone-imines and hemoglobin was demonstrated (Fig. 4A). In erythrocytes incubated *in vitro* without glucose, the latter reaction should be directly responsible for both semiquinone-imine (Fig. 5) and methemoglobin (Fig. 2) formation and indirectly, through the formed hydrogen peroxide, for NPSH depletion (Fig. 3) as schematically shown in Fig. 8A. In the presence of glucose, the NADH and NADPH contents of erythrocytes are replenished continuously and oxidative reactions are inhibited (Figs. 2 and 3) due to the NAD(P)H-dependent antioxidant processes such as glutathione reduction [29, 30], maintenance of catalase activity [30, 31], and methemoglobin reduction [32, 33]. However, the oxidative activity is not inhibited completely since there is a competition for NAD(P)H between the antioxidant and the oxidative processes, the latter producing hydrogen peroxide through redox-cycling of the quinone-imines by reductases (Fig. 8B). This mechanism is in agreement with the findings of Baird *et al.* [34] who demonstrated that both 5-HPQ and 5-H-6-DPQ oxidize NADPH through a diaphorase-like activity leading to a marked increase in the hexose monophosphate shunt but without any stimulation of red blood cell protein degradation. Since hydrogen peroxide does not stimulate proteolysis [35] but oxidizes hemoglobin [33, 36, 37] and depletes NPSH [36, 37], it is likely to be the

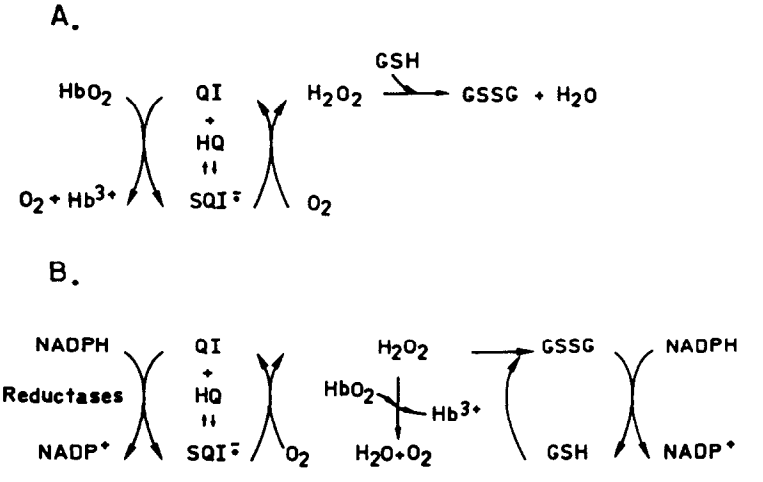

Fig. 8. Schematic representation of the suggested pathways for quinone-imine redox-cycling in erythrocytes. (A) Predominant pathway in the absence of glucose. (B) Predominant pathway in the presence of glucose.

ultimate oxidant formed from the quinone-imines in the presence of a continuous flux of NADPH (Fig. 8B).

Formation of hydrogen peroxide through redox-cycling of the 5-HPQ and 5-H-6-DPQ quinone-imines by red blood cell reductases can also explain the results obtained *in vivo* (Figs. 6 and 7). In this case, NPSH depletion was the most pronounced effect of the metabolites and a similar consumption of sulfhydryl groups was observed upon administration of hydrogen peroxide in 10-fold molar excess to the administered quinone-imines (Fig. 6B). The requirement of such a large excess of hydrogen peroxide to promote similar toxic effects appears to be reasonable since the quinone-imines produce hydrogen peroxide *in situ* through cellular reductases (Fig. 8B), whereas a bolus of hydrogen peroxide is distributed and detoxified in the whole animal.

Overall, the results reported here are important in emphasizing that oxidative stress can be imposed on erythrocytes by several mechanisms depending on the oxidant. Although glucose-6-phosphate dehydrogenase deficiency is characterized by an enhanced erythrocyte sensitivity to several drugs including primaquine, aniline and phenylhydrazine, the three compounds are likely to act by different mechanisms. Particularly important in this respect was our demonstration that the methemoglobin-forming activity of primaquine and its metabolites *in vivo* in the rat (Fig. 6) was much lower than the activity of aniline [11] and phenylhydrazine [38, 39]. The latter compound directly oxidizes hemoglobin *in vitro* and *in vivo* [23, 38, 39], whereas phenylhydroxylamine is the methemoglobin-forming metabolite of aniline [11, 24]. By contrast, the ultimate oxidant formed from primaquine is not effective in oxidizing hemoglobin under the continuous supply of NAD(P)H sustained *in vivo*. Such a property is to be expected for hydrogen peroxide [33].

The view that hydrogen peroxide could be the ultimate oxidant formed from primaquine metabolism has been supported in the literature for many years, although different metabolic routes have been proposed for hydrogen peroxide formation [5, 13, 16, 40–44]. Consequently, the merit of our results is to provide a chemically feasible route for hydrogen peroxide generation from primaquine metabolism through the redox-cycling of quinone-imine derivatives of 5-HPQ and 5-H-6-DPQ. The latter compounds, however, have not yet been identified as human metabolites [45, 46]. This fact, considered together with the low oxidative activity of primaquine *in vivo* in a convenient experimental animal such as the rat (Fig. 6; see also Results), indicates that metabolic studies in humans are important for further progress in the understanding of the hemolytic properties of this antimalarial drug.

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