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STUDIES ON THE MECHANISMS OF OXIDATION IN THE ERYTHROCYTE BY METABOLITES OF PRIMAQUINE

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Abstract—The interaction of certain metabolites of the 8-aminoquinoline antimalarial primaquine with both normal and glucose-6-phosphate dehydrogenase (G6PD)-deficient erythrocytes and with haemoglobin preparations was studied in an attempt to elucidate the mechanisms of methaemoglobin formation and haemolytic anaemia associated with the use of primaquine. Studies using erythrocytes revealed that oxidation of haemoglobin and reduced glutathione (GSH) was due to the metabolites rather than the parent drug. Incubation of free haemoglobin with 5-hydroxylated metabolites of primaquine also led to oxidation of oxyhaemoglobin and GSH. Oxidation of GSH also occurred in the absence of oxyhaemoglobin. The results suggest a dual mechanism for these oxidative effects, involving autoxidation of the 5-hydroxy-8-aminoquinolines and their coupled oxidation with oxyhaemoglobin. The initial products of these processes would be drug metabolite free radicals, superoxide radical anions, hydrogen peroxide and methaemoglobin. Further free radical reactions would lead to oxidation of GSH, more haemoglobin and probably other cellular constitutents. NADPH had no effect on the oxidative effects of the primaquine metabolites in these experiments. In the G6PD-deficient erythrocyte, the oxidation of haemoglobin and GSH leads to Heinz body formation and eventually to haemolysis, the mechanisms of which are as yet unclear. The possible role of oxygen free radicals in the mode of action of 8-aminoquinolines against the malaria parasite is also briefly discussed.

Since its introduction in 1952, the 8-aminoquinoline antimalarial primaquine [6-methoxy-8-(4-amino-1-methylbutylamino)quinoline, I, Fig. 1] has become the drug of choice for the radical cure of *Plasmodium vivax* infections. The usefulness of the drug is limited, however, by its toxic side effects, namely methaemoglobinaemia and, when the patient has a deficiency of erythrocyte glucose-6-phosphate dehydrogenase (G6PD), haemolytic anaemia. Whether it is the drug itself or one or more metabolites which is responsible for its toxicity and/or antimalarial activity is unclear. The present study was undertaken to determine which is responsible for methaemoglobin formation, and to attempt to elucidate the mechanisms which could lead to haemolysis in G6PD-deficient subjects.

Brodie and Udenfriend [1] isolated two active metabolites from the urine of dogs which had received an earlier-used 8-aminoquinoline antimalarial pamaquine. The metabolites were not identifed, but it was thought that the haemolytic and methaemoglobin-producing activities were due to the 5-hydroxy derivative, since 5-hydroxy-8-aminoquinoline caused similar effects *in vitro* whereas 8-aminoquinoline did not. Also, Greenberg *et al.* [2] observed that the 5-hydroxy derivative of pentaquine was much more active than the parent 8-aminoquinoline against *P. gallinaceum in vitro*. A metabolite of pamaquine was isolated from the droppings of chickens fed the drug and identified as the 5,6-quinolinequinone derivative of the drug, which was found to have superior antimalarial properties to the parent drug *in vitro* and to possess methaemoglobin-forming properties [3, 4].

Fraser and Vesell [5] working with 5,6-dihydroxy-8-aminoquinoline (DHAQ, II) and the 5,6-dihydroxy derivative of pentaquine found that these compounds decreased the GSH content and increased the methaemoglobin content and mechanical fragility of normal and more so of G6PD-deficient erythrocytes at concentrations where primaquine was inactive. These results were later confirmed by others using lower concentrations of DHAQ [6]. Fraser *et al.* [7], reported that the 5-hydroxy and 5-hydroxy-6-desmethyl derivatives of primaquine (III and V respectively) as well as DHAQ were much more active than primaquine or 6-desmethylprimaquine (IV) in generating hydrogen peroxide, forming methaemoglobin and depleting GSH in normal and G6PD-deficient erythrocytes at concentrations lower than those used in their earlier study [5]. In contrast to these findings, Tudhope and Leece [8] found that primaquine itself caused methaemoglobin and Heinz body formation when incubated with enzyme-normal erythrocytes.

In a study of the metabolism of tritium-labelled primaquine in the dog, Strother *et al.* [9] reported at least five metabolites and tentatively identified the main ones as 5-hydroxyprimaquine and 6-desmethylprimaquine. Recent studies in this laboratory using gas chromatography–mass spectrometry as well as TLC have identified 5-hydroxyprimaquine and 6-desmethylprimaquine as metabolites of the drug in studies in rats and rhesus monkeys. A tentative

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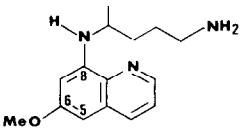
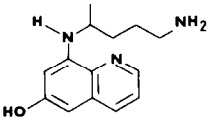
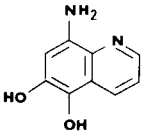


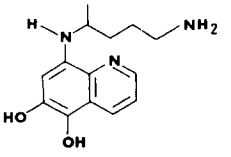
primaquine, I



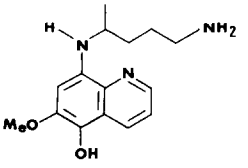
6 - desmethylprimaquine, IV



5,6 - DHAQ, II



5 - hydroxy - 6 - desmethyl primaquine, V



5 - hydroxyprimaquine (5 - HPQ), III

Fig. 1. Primaquine and its suspected metabolites.

identification of 5-hydroxy-6-desmethylprimaquine as a metabolite has also been obtained [10].

On the basis of these findings, 5-hydroxyprimaquine (V) and DHAQ (II) a "model" metabolite for 5-hydroxy-6-desmethylprimaquine, were used in the present study.

MATERIALS AND METHODS

(i) *Chemicals*

Primaquine diphosphate was purchased from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). 5-6-Dihydroxy-8-aminoquinoline dihydrobromide (DHAQ) was a gift from Sterling-Winthrop Research Institute (New York, U.S.A.) and 5-hydroxyprimaquine dihydrobromide was donated by Walter Reed Army Institute for Research (Washington, DC). 5,5-Dithiobis-(2-nitrobenzoic acid), sodium diethyldithiocarbamate (DDC), catalase, glutathione peroxidase and superoxide dismutase (SOD) were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Desferrioxamine mesylate (Desferal) was supplied by CIBA Laboratories (Horsham, Sussex, U.K.) DE52 cellulose (diethylaminoethyl-cellulose) was obtained from Whatman Chemical Separation Ltd. (Maidstone, Kent, U.K.). All other chemicals were supplied by BDH Chemicals Ltd. (Liverpool, U.K.). Visking tubing (10 mm width) was obtained from J. W. Turner Ltd. (Liverpool, U.K.).

(ii) *Blood*

Blood was drawn from healthy volunteers (three G6PD-normal Caucasians and one G6PD-deficient Asian) into heparinized containers. The blood was centrifuged at 5000 *g* for 10 min and the plasma and buffy coat removed by aspiration. The erythrocytes were washed three times with 0.15 M phosphate-buffered saline (Dulbecco "A", pH 7.4, PBS), then resuspended in PBS to give a packed cell volume (PCV) of 30–40%. In some experiments, the haemoglobin in the cells was converted to methaemoglobin by incubating 5 ml of washed cells suspended in 10 ml PBS with 0.5 ml 0.18 M sodium nitrite at 37° for 20 min. The cells were then washed a further three times with PBS before being suspended in PBS to give a PCV of 30–40%. In some experiments erythrocytes, collected over heparin, were "aged" at 22° for 24–48 hr to deplete endogenous glucose. These cells were subsequently washed as described above. G6PD-normal cells when aged for 30–48 hr were found to simulate the behaviour of deficient cells in being more sensitive to oxidant effects.

(iii) *Free haemoglobin substrates*

Distilled, deionized water (60 ml) and DE52 cellulose (1.6 g) were added to 10 ml of packed red cells from G6PD-normal subjects which had previously been twice washed with PBS. The mixture was allowed to stand for 10 min with occasional mixing, then centrifuged at 5000 *g* for 5 min. The supernatant was decanted and treatment with fresh DE52 cellulose repeated twice more. After the final treatment with DE52 cellulose the haemoglobin concentration was adjusted to give a final haemoglobin concentration of about 30 g l^{-1}. This procedure was designed to provide a haemoglobin substrate free of NADH-methaemoglobin reductase [11]. Subsequent

assay showed that this had been achieved. G6PD activity was also absent. Methaemoglobin substrate was prepared by converting the haemoglobin of washed cells to methaemoglobin by incubation with 0.18 M sodium nitrate solution as described above followed by lysis and treatment with DE52 cellulose. After preparation of haemoglobin substrates, dialysis was carried out using Visking tubing against distilled, deionized water (500 ml) at 4° overnight.

(iv) *Estimations*

Methaemoglobin levels were estimated using a modification of the method of Evelyn and Malloy [12].

Haematocrit estimations were carried out in heparinised capillaries using a micro-haematocrit centrifuge.

Estimation of reduced glutathione was by the method of Beutler *et al.* [13].

Blood was screened for G6PD activity using the Bernstein dye decolourisation method (Sigma Chemical Co. Ltd., Poole, U.K.). Enzyme-deficiency was confirmed quantitatively by a UV spectrophotometric method [11].

In some experiments, cells stained with methyl violet were examined for the presence of Heinz bodies [12]. Results are presented as the number of erythrocytes containing one or more Heinz bodies.

Incubations using erythrocytes. Aliquots (2.4 ml) of red cell suspensions in PBS were placed in boiling tubes and primaquine, 5-hydroxyprimaquine (5-HPQ) or DHAQ were added in 0.3 ml PBS to give a final concentration of 3 mg l^{-1}. The total volume was made up to 3 ml with PBS, which sometimes contained other additions (see Results). The tubes were then stoppered and placed in a shaking water bath and incubated at 37° for 4 hr in the dark. Samples were taken before and during the incubation for estimation of methaemoglobin and GSH. The packed cell volume was measured before and at the end of the incubation period and an aliquot of red cell suspension was stained and examined for the presence of Heinz bodies.

Incubations using haemoglobin substrate. Aliquots (1.7 ml) of haemoglobin substrate (approximate haemoglobin concentration 30 g l^{-1}) were used with 1 ml of glutathione solution (3 g l^{-1} in PBS). 5-HPQ or DHAQ (in 5% acetone/95% PBS) was added in 0.1 ml to give a final concentration of 3 mg l^{-1}, and the volume made up to 3 ml with PBS solution which sometimes contained other additions (see Results). Samples were withdrawn as for whole cells.

RESULTS

Preliminary experiments involving the incubation of G6PD-normal erythrocytes with primaquine or the metabolites in PBS containing glucose (1 g l^{-1}) showed little effect on the levels of reduced glutathione and methaemoglobin levels because of active glycolysis and pentose phosphate pathway activity. In subsequent experiments glucose was omitted and the cells were aged overnight or longer to deplete intracellular glucose. The results from typical incubations of red blood cells from both a normal and a G6PD-deficient subject with primaquine, 5-HPQ and DHAQ are presented in Table 1.

The results show little effect on methaemoglobin levels induced by primaquine but a small decline in reduced glutathione levels compared to the control level was observed with G6PD-deficient erythrocytes. The control reduced glutathione level was also significantly reduced in these erythrocytes. Moderate methaemoglobin production was induced in both normal and G6PD-deficient cells by 5-HPQ. This compound also caused a considerable fall in GSH levels although the effect was more pronounced with the erythrocytes of the G6PD-deficient subject. DHAQ produced even greater increases in methaemoglobin levels and induced more precipitous falls in GSH levels. Again this effect was most pronounced with the G6PD-deficient cells.

The effects of 5-HPQ and DHAQ on GSH and methaemoglobin levels after prior conversion of the haemoglobin in the cells to methaemoglobin was also investigated (Table 2). Since primaquine showed

Table 1. Effects of primaquine and metabolites (3 mg l^{-1}) on methaemoglobin formation and reduced glutathione levels in erythrocytes from normal and G6PD-deficient subjects

	Normal				G6PD-deficient			
Hours	Control	PQ	DHAQ	5-PHQ	Control	PQ	DHAQ	5-HPQ
Methaemoglobin levels (percentage of total haemoglobin)								
0	0				0			
1	0	0	17.0	2.0	0.2	0	14.0	2.0
2	0	0.5	26.0	3.5	1.0	1.0	23.5	5.0
3	0	0.7	33.0	4.5	1.0	1.5	32.0	6.5
4	0	1.0	38.5	5.5	1.5	1.5	—	8.0
Reduced glutathione (percentage of initial levels)								
0	100				100			
1	80	77	69	64	92	88	52	58
2	76	75	40	43	84	67	19	29
3	77	80	18	34	73	65	17	32
4	85	85	10	42	66	54	0	22
Heinz bodies (%)	1	3	—	68	3	5	—	95

Incubations were at 37°; cells aged at 22° for 42 hr.
Mean values from 3 separate experiments.

Table 2. Effects of primaquine metabolites (3 mg l^{-1}) on levels of methaemoglobin and reduced glutathione when haemoglobin in cells is mainly in the oxidised form

Hours	Control	DHAQ	5-HPQ
Methaemoglobin levels (percentage of initial levels)			
0	90.0		
1	95.0	95.0	95.0
2	93.0	94.0	96.0
3	97.5	92.0	95.0
4	97.5	96.5	97.0
Reduced glutathione (percentage of initial levels)			
0	100		
1	115	114	88
2	102	97	98
3	98	82	97
4	98	78	72

Cells aged at 22° for 42 hr.
Mean values from 3 separate experiments.

little effect in the previous experiment, it was omitted from these incubations. Only aged G6PD-normal blood was used since enzyme-deficient blood was not available. The results show that neither DHAQ nor 5-HPQ much altered the methaemoglobin levels and although the levels of GSH were depressed to around 75% of the control levels, the effect was not noticeable until after three and four hours of incubation with DHAQ and 5-HPQ respectively.

In an attempt to elucidate the mechanism of oxidation of haemoglobin and GSH and to explain the lack of reaction when the haemoglobin was initially in the oxidised ferric state, the iron-chelating agents desferrioxamine [14] or EDTA were included in some incubation mixtures. These substances would reduce any autoxidation of GSH and determine if autoxidation of the metabolites was necessary. Little difference was observed in methaemoglobin or GSH levels of either normal or enzyme-deficient cells when these substances were added at concentrations of 0.67 g l^{-1} for desferrioxamine and 0.11 g l^{-1} for EDTA.

To determine if any of the oxidation observed with the metabolites was due to superoxide formation, sodium diethyldithiocarbamate (DDC) was included in some incubations (Table 3). This compound has been reported to be a good inhibitor *in vivo* of superoxide dismutase (SOD) [15, 16]. The experiments indicated that DDC, although an inhibitor of SOD was having a powerful oxidant effect *per se* on haemoglobin and GSH and was therefore not helpful in understanding the oxidant mechanisms of the primaquine metabolites. Similar conclusions were made from the findings of Strömme [17] and later by Sinet *et al.* [18].

In order to eliminate the participation of the enzymes and cofactors of the pathways controlling the reduction of methaemoglobin and oxidised glutathione, which may to varying extents mask the effects of the primaquine metabolites, subsequent experiments were performed using free haemoglobin preparations. The mean results from three separate experiments are presented in Table 4. Similar experiments were performed using free methaemoglobin preparations (Table 5). The results with free oxyhaemoglobin preparations showed significant increases in methaemoglobin formation induced by 5,6-DHAQ and 5-HPQ. Oxidation of GSH in these experiments was also appreciable with both metabolites. When most of the haemoglobin was initially present as methaemoglobin some reduction of the methaemoglobin was observed. GSH levels also rapidly declined with 5-HPQ but only slowly with the lower concentration of DHAQ.

The effect of EDTA in incubations containing haemoglobin and 5-HPQ is shown in Table 6. The effects of EDTA on the oxidation of GSH, both autoxidation and that induced by 5-HPQ, in the absence of haemoglobin was also investigated (Table 7). The results show that EDTA protects GSH from oxidation by 5-HPQ both in the presence of haemo-

Table 3. Effects of diethyldithiocarbamate (DDC) on methaemoglobin formation and glutathione oxidation in cells in the presence and absence of primaquine metabolites

	Normal erythrocytes				G6PD-deficient erythrocytes		
Hours	Control	DDC	DHAQ	DHAQ + DDC	Control	5-HPQ	5-HPQ + DDC
Methaemoglobin levels (percentage of total haemoglobin)							
0	0				0.5		
1	0.5	7.0	19.0	25.0	1.0	11.0	18.0
2	0.5	16.0	27.5	49.0	1.5	20.0	31.0
3	1.0	17.0	35.0	69.0	2.5	26.0	39.0
4	1.0	21.5	42.5	78.0	3.0	30.0	44.0
Reduced glutathione (percentage of initial values)							
0	100				100		
1	99	85	46	15	72	60	34
2	112	78	42	9	73	47	12
3	115	54	25	0	61	21	10
4	110	7	19	0	56	7	7

DDC 4 mg l^{-1}, DHAQ 10 mg^{-1}, 5-PHQ 5 mg l^{-1}.
Erythrocytes aged at 22° for 18 hr.

Table 4. Effects of primaquine metabolites on free oxyhaemoglobin solutions containing reduced glutathione

Hours	Control	DHAQ	5-HPQ
Methaemoglobin levels (percentage of total haemoglobin)			
0	0		
1	0	15.5	21.5
2	0	23.0	28.0
3	2.0	31.5	37.0
4	7.5	36.8	55.0
Reduced glutathione levels (percentage of initial values)			
0	100		
1	95	93	70
2	95	93	68
3	91	83	56
4	89	76	49

Oxyhaemoglobin 16.5 g l^{-1}, GSH 1.0 g l^{-1}, DHAQ 2 mg l^{-1}, 5-HPQ 10 mg l^{-1}.
Haemoglobin prepared from normal erythrocytes.

Table 5. Effects of primaquine metabolites on free methaemoglobin solutions containing reduced glutathione

Methaemoglobin levels (percentage of total haemoglobin) Hours	Control	DHAQ	5-HPQ
0	87.5		
1	95.0	95.0	85.7
2	93.0	93.5	81.0
3	94.0	97.0	78.0
4	94.0	88.0	80.0
Reduced glutathione levels (percentage of initial values) Hours	Control	5,6-DHAQ	5-HPQ
0	100		
1	92	92	79
2	93	90	61
3	88	88	55
4	91	86	41

Methaemoglobin 16.5 g l^{-1}, GSH 1.0 g l^{-1}, DHAQ 2 mg l^{-1}, 5-HPQ 10 mg l^{-1}.
Haemoglobin prepared from normal erythrocytes.

globin (Table 6) and in its absence (Table 7) and also from autoxidation (Table 7). The presence of EDTA had only a slight effect, if any, on methaemoglobin formation however.

The effects of added NADPH on the oxidation of haemoglobin and GSH by DHAQ and 5-HPQ were also investigated. While it was not possible to see if NADPH offered any protection to reduced glutathione against autoxidation, results showed that NADPH did not protect against oxidation of haemoglobin or GSH by DHAQ or 5-HPQ.

Table 6. Effects of EDTA on the oxidation of free haemoglobin solutions containing reduced glutathione by 5-hydroxyprimaquine

Hours	Control	5-HPQ	5-HPQ + EDTA
Methaemoglobin levels (percentage of total haemoglobin)			
0	0	0	0
1	0	16.5	19.0
2	2.0	39.0	31.5
3	4.5	47.5	41.0
4	8.0	58.0	55.0
Reduced glutathione (percentage of initial values)			
0	100		
1	96	82	87
2	92	74	85
3	88	62	77
4	81	54	73

Haemoglobin concentration 16.5 g l^{-1}, reduced glutathione concentration 1.0 g l^{-1}, 5-HPQ 10 mg l^{-1}, EDTA 78 mg l^{-1} (0.3 mM).

It was thought that the addition of catalase or SOD to haemoglobin incubations could reduce the degree of GSH oxidation, but these enzymes were found to be without effect when tested at concentrations of 10, 20 or 30 mg l^{-1}. Glutathione peroxidase similarly did not appreciably enhance the degree of glutathione oxidation by DHAQ when present at a concentration of 200 units l^{-1}.

No changes in haematocrit from initial values were seen in any experiment when estimations were carried out after incubation.

DISCUSSION

The first biochemical abnormality shown to characterise drug-sensitive erythrocytes, was a low level of GSH [19]. Most drugs that produced haemolytic anaemia in G6PD-deficient cells also induce some degree of intraerythrocytic methaemoglobin formation, but a relationship between methaemoglobin production and haemolysis has not been firmly established. Deficiency of G6PD makes the erythrocyte incapable of sufficiently rapid regeneration of GSH because of insufficient turnover of NADPH. Hence the already low level of GSH can become rapidly depleted leaving the erythrocyte exposed to oxidant damage.

Table 7. Effect of EDTA on glutathione oxidation in the absence of haemoglobin

Reduced glutathione (percentage of initial values) Hours	GSH alone	GSH + EDTA	GSH + 5-HPQ	GSH + 5-HPQ + EDTA
0	100			
1	98	104	98	96
2	93	100	90	100
3	85	100	83	98
4	85	101	74	102

Reduced glutathione 1.0 g l^{-1}, 5-HPQ 10 mg l^{-1}, EDTA 78 mg l^{-1}.

These studies show that primaquine has no significant oxidant effect on normal and G6PD-deficient erythrocytes even in the absence of glucose. In contrast the metabolites 5-HPQ and DHAQ caused an appreciable increase in methaemoglobin levels and a sharp decrease in GSH levels. These effects were accompanied by Heinz body formation. The effects were most marked with G6PD-deficient erythrocytes. These results are in agreement with the findings of Fraser and Vesell [5] and Fletcher *et al.* [6] using DHAQ and with those of Fraser *et al.* [7] using 5-HPQ. The results described here conflict with the findings of Cohen and Hochstein [20], Tudhope and Leece [8] and Kelman *et al.* [21] that primaquine itself is responsible for the oxidant effects. However, these workers used much higher concentrations of the drug than those used in the present study.

The results with aged normal erythrocytes, in which most of the haemoglobin was first oxidised to methaemoglobin prior to incubation with DHAQ or 5-HPQ, suggest that the metabolites are much more reactive towards oxyhaemoglobin than methaemoglobin and that the glutathione oxidation observed towards the end of the experiments may be due to reaction with a species produced by reaction of the metabolites with molecular oxygen. The iron-chelating agents desferrioxamine and EDTA did not protect GSH in cells against oxidation unlike when using free haemoglobin. This suggests the failure of the chelating agents to penetrate the red cell membrane.

The experiments with free oxyhaemoglobin (Tables 3 and 4) produced results which agree generally with those obtained with erythrocytes (Tables 1 and 2). In experiments with free haemoglobin the concentration of 5-HPQ was increased from 3 mg l^{-1} to 10 mg l^{-1} to observe the effects shown in Table 3. This is an acceptable adjustment since the drug concentration is higher at times within the erythrocyte than in plasma. This has been shown in studies in this laboratory on the uptake of [^{14}C]-primaquine and its metabolites in primates. Some 40% of the total radioactive dose is located in the erythrocytes at 4 hr following an oral dose. Determination of primaquine levels shows that after 2–3 hr the radioactivity is largely due to metabolites [22].

NADPH was observed to have little effect on the oxidation of oxyhaemoglobin or GSH by DHAQ or by 5-hydroxyprimaquine in a cell-free system. Kelman *et al.* [21] showed that primaquine itself interacts with NADPH but not GSH in a non-cellular system and therefore stimulates pentose phosphate pathway activity in erythrocytes by a mechanism similar to that with methylene blue. However, these workers used a much higher concentration of primaquine (260 mg l^{-1}) than those of the primaquine metabolites or parent drug used in the experiments reported here (2–10 mg l^{-1}). Similar findings with primaquine metabolites have also been reported [23]. A lack of redox cycling of oxidised forms of metabolites with NADPH in G6PD-deficient erythrocytes would tend to increase oxidant effects in such cells.

Overall, the results reported here suggest a dual mechanism for the oxidative effects on haemoglobin and GSH by the primaquine metabolites DHAQ and 5-HPQ. This is a coupled, one-electron oxidation of both drug metabolite and oxyhaemoglobin, which would result in the formation of hydrogen peroxide by a mechanism similar to that following reaction of oxyhaemoglobin with phenols [24], and autoxidation of the drug metabolite by molecular oxygen, possibly catalysed by metal ions, producing superoxide radicals. The coupled oxidation of drug metabolite and oxyhaemoglobin would give rise to the corresponding semiquinomine radical, methaemoglobin and hydrogen peroxide (Fig. 2). Seminquinonimine radicals are also likely to arise from the autoxidation of 5-hydroxylated metabolites of primaquine. Semiquinonimine radicals may themselves undergo autoxidation to 8-imino-5(8H) quinolinones (Fig. 2) forming more superoxide in the process. Oxidation of semiquinonimines to 8-imino-5(8H)quinolinones may also occur by reaction with methaemoglobin, the latter being reduced to haemoglobin. Superoxide, produced by autoxidation of 5-hydroxylated metabolites and semiquinonimine radicals, may bring about the oxidation of more metabolite or semiquinonimine radicals, being reduced to peroxide in the process.

The above reactions all involve the oxidation of 5-hydroxylated metabolites of primaquine or their first oxidation products, the corresponding semiquinonimine radicals. The latter species may undergo reduction by reaction with GSH (Fig. 2) or possibly by reaction with the free sulphydryl groups of haemoglobin and other proteins and enzymes in the red cell giving rise to oxidised glutathione or mixed disulphides. Semiquinonimine radicals may also be neutralised by disproportionation. The superoxide produced by autoxidation of 5-hydroxylated metabolites and semiquinonimines may be neutralised by superoxide dismutase, by spontaneous disproportionation, or may be scavenged by methaemoglobin [25] or oxidise oxyhaemoglobin to methaemoglobin with the formation of more hydrogen peroxide.

Radicals produced from 5-hydroxylated metabolites of primaquine are more resonance stabilised than any corresponding radicals produced from primaquine which are less likely to be formed. Hence primaquine itself was observed to have little effect in either aged normal or G6PD-deficient erythrocytes (Table 1). The slightly increased GSH oxidation over that observed in the control G6PD-deficient erythrocytes could represent the effects of some primaquine autoxidation. The oxidation of GSH in the presence of 5-HPQ and DHAQ could result from the reduction of hydrogen peroxide and drug metabolite free radicals. The greater effect observed with DHAQ may be because the presence of the 8-amino aliphatic side-chain of 5-HPQ does not permit such a close interaction with the haemoglobin as can be attained by DHAQ, i.e. because of steric hindrance.

The protecting effect of EDTA against glutathione oxidation and to a very small extent against methaemoglobin formation (Table 6) suggests that autoxidation of the metabolites of primaquine is responsible in part for the oxidative effects of the 8-aminoquinolines in the erythrocyte. This protecting effect was also evident in the absence of haemoglobin (Table 7). Although autoxidation of GSH occurred,

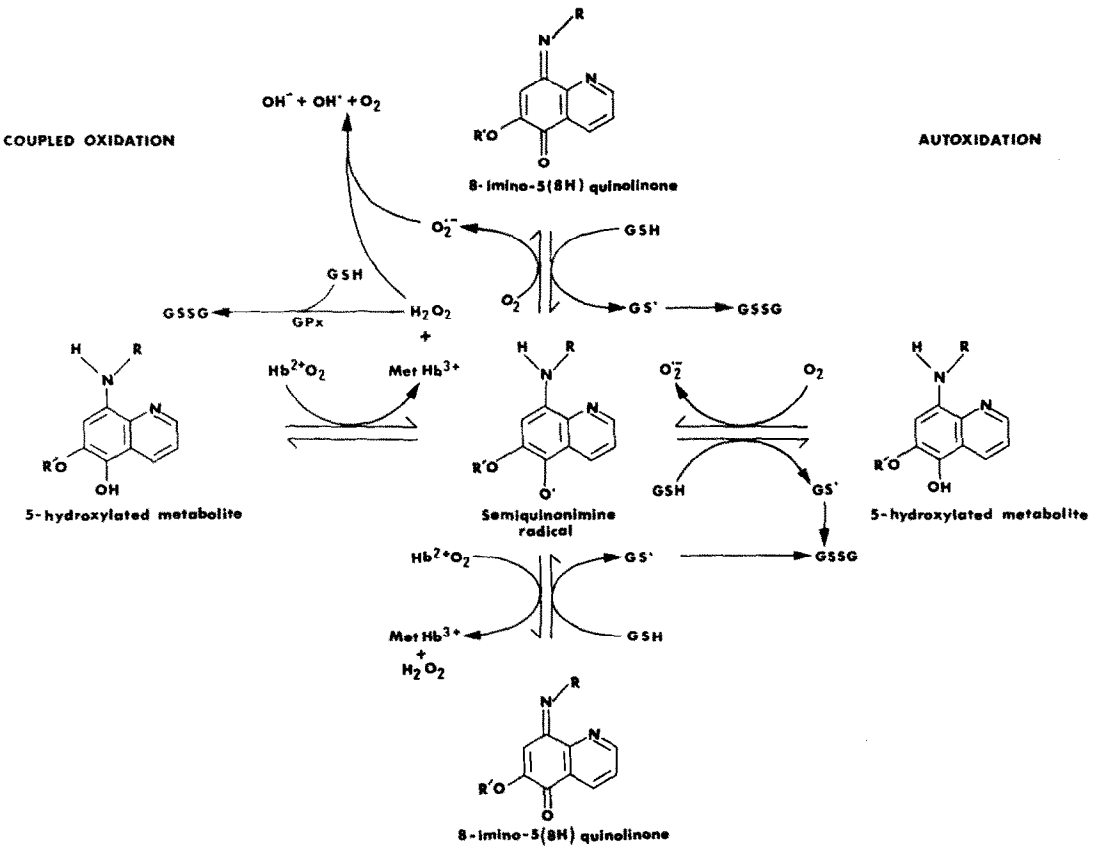


Fig. 2. Proposed dual mechanism for the oxidative effects of primaquine metabolites in the erythrocyte. (GPx, glutathione peroxidase.)

its oxidation was enhanced by 5-HPQ, possibly because of semiquinonimine radicals formed by autoxidation of the metabolite.

Hydrogen peroxide, produced in reactions resulting from the autoxidation of the 5-hydroxylated metabolites or formed by their coupled oxidation with oxyhaemoglobin may be detoxified in the erythrocyte by catalase or by glutathione peroxidase. Alternatively hydrogen peroxide, in the presence of superoxide and metal ions, may undergo the Haber–Weiss reaction, forming highly reactive hydroxyl free radicals (Fig. 2).

In the enzyme-normal red cell, oxidised glutathione produced by the effects of 5-hydroxylated metabolites of primaquine can be reduced back to its functional state. G6PD-generated NADPH may also augment the reduction of methaemoglobin to haemoglobin by NADH-methaemoglobin reductase via the NADPH-dependent reductase when the red cell is challenged by excessive oxidative stress. In the G6PD-deficient subject the erythrocyte is incapable of increasing the turnover of NADPH. Hence, once formed, oxidised glutathione cannot be reduced again and methaemoglobin reduction may also be impaired, although methaemoglobin reduction can still occur via NADH-methaemoglobin reductase. Faced with the oxidant stress caused by the active metabolites of primaquine, the already low level of GSH in the enzyme-deficient erythrocyte becomes rapidly depleted. The free radicals produced may then react with the sulphydryl groups of haemoglobin (and those of thiol-containing enzymes and proteins) caused mixed disulphide formation and the precipitation of Heinz bodies. Haemolysis may then occur by two processes. Firstly, Heinz body formation could lead to increased red-cell membrane fragility and permeability with a loss of red cell potassium ions, which has been implicated as a factor leading to lysis with oxidant drugs [26]. These processes may lead to intravascular haemolysis. Secondly, damaged erythrocytes may be sequestered and destroyed in the spleen, and possibly other sites of the reticulo-endothelial system.

A similar mechanism for the haemolytic anaemia of favism has been proposed, involving generation of hydrogen peroxide from molecular oxygen brought about by the redox cycling of two pyrimidine aglycones, isouramil and divicine, from the bean *Vicia fava* [27].

The likelihood of the production of "activated oxygen" and free radicals from the reactions of 5-hydroxylated metabolites of 8-aminoquinoline antimalarials is interesting in view of the more recent findings linking oxygen free radical species and the death of malaria parasites. The intravenous administration of alloxan to mice infected with *P. vinckei* was shown to produce a rapid reduction in parasitaemia and transient haemolysis [28]. These effects were attributed to the generation of hydroxyl radicals by the alloxan-dialuric acid system, and were blocked

by desferrioxamine and DDC and partially blocked by propanol. Similar antimalarial activity has also been observed upon treatment of infected mice with t-butyl hydroperoxide [29]. Also, some degree of haemolysis and suppression of parasitaemia has been shown to occur in infected mice treated with hydrogen peroxide or phenylhydrazine, a compound which is well-known to induce haemolysis by a mechanism involving hydrogen peroxide and superoxide [28]. The hypothesis presented here that the oxidant effects of primaquine may be due to the generation of drug metabolite free radicals, superoxide and hydrogen peroxide, when considered with these recent observations linking parasite death with oxygen free radicals, lends further support to the hypothesis of Alving *et al.* [29] that the toxic properties of primaquine and its antimalarial activity may be due to the same metabolite(s).

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REFERENCES

1. Brodie BB and Udenfriend S, Metabolites of pamaquine in urine. *Proc Soc Exp Biol Med* **74**: 845–848, 1950.
2. Greenberg J, Taylor DJ and Josephson ES, Studies of *Plasmodium gallinaceum in vitro* II. The effects of some 8-aminoquinolines against erythrocytic parasites. *J Infect Dis* **88**: 163–167, 1951.
3. Josephson ES, Taylor DJ, Greenberg J and Ray AP, A metabolic intermediate of pamaquine from chickens. *Proc Soc Exp Biol Med* **76**: 700–703, 1951.
4. Josephson ES, Greenberg J, Taylor DJ and Bami HL, A metabolite of pamaquine from chickens. *J Pharmacol Exp Ther* **103**: 7–9, 1951.
5. Fraser IM and Vesell ES, Effects of drugs and drug metabolites on erythrocytes from normal and glucose-6-phosphate dehydrogenase-deficient individuals. *Ann NY Acad Sci* **151**: 777–794, 1968.
6. Fletcher KA, Canning MV and Gilles HM, Metabolites of primaquine and their effects on erythrocytes. *Trans Roy Soc Trop Med Hyg* **71**: 111–112, 1977.
7. Fraser IM, Tilton BE and Strother A, The relationships of hydrogen peroxide production to glutathione depletion and methaemoglobin formation by model metabolites of primaquine and phenacetin in normal and glucose-6-phosphate dehydrogenase deficient erythrocytes. *Pharmacologist* **17**: 250, 1975.
8. Tudhope GR and Leece SP, Red-cell catalase and the production of methaemoglobin, Heinz bodies and changes in osmotic fragility due to drugs. *Acta Haematol* **45**: 290–302, 1971.
9. Strother A, Fraser IM, Allahyari R and Tilton BE, Metabolism of 8-aminoquinoline antimalarial agents. *Bull Wld Hlth Org* **59**: 413–425, 1981.
10. Price AH and Fletcher KA, The metabolism and toxicity of primaquine. In: *Ethnic Differences in Reactions to Drugs and Xenobiotics*, pp. 261–278. Alan R. Liss, New York, 1986.
11. Beutler E, *Red Cell Metabolism, a Manual of Biochemical Methods*. Grune & Stratton, London, 1971.
12. Dacie JV and Lewis SM, *Practical Haematology*, 5th Ed. Churchill Livingstone, London, 1975.
13. Beutler E, Duron O and Kelly BM, Improved method for the determination of blood glutathione. *J Lab Clin Med* **61**: 882–890, 1963.
14. Gutteridge JMC, Richmond R and Halliwell B, Inhibition of the iron-catalysed formation of hydroxyl radicals from superoxide and of lipid peroxidation by desferrioxamine. *Biochem J* **184**: 469–472, 1979.
15. Heikkila RE, Cabbat FS and Cohen G, *In vivo* inhibition of superoxide dismutase in mice by diethyldithiocarbamate. *J Biol Chem.* **251**: 2812–2185, 1976.
16. Goldberg B and Stern A, Superoxide anion as a mediator of drug-induced oxidative haemolysis. *J Biol Chem* **251**: 6468–6470, 1976.
17. Strömme JH, Methaemoglobin formation induced by thiols. *Biochem Pharmacol* **12**: 937–948, 1963.
18. Sinet P-M, Garber P and Jerome H, H_2O_2 production, modification of the glutathione status and methaemoglobin formation in red blood cells exposed to diethyldithiocarbamate *in vitro*. *Biochem Pharmacol* **31**: 521–525, 1982.
19. Beutler E, Dern RJ, Flanagan CL and Alving AS, The haemolytic effect of primaquine. VII. Biochemical studies of drug-sensitive erythrocytes. *J Lab Clin Med* **45**: 286–295, 1955.
20. Cohen G and Hochstein P, Generation of hydrogen peroxide in erythrocytes by hemolytic agents. *Biochemistry* **3**: 895–900, 1964.
21. Kelman SN, Sullivan SG and Stern A, Primaquine-mediated oxidative metabolism in the human red cell. Lack of dependence on oxyhaemoglobin, H_2O_2 formation or glutathione turnover. *Biochem Pharmacol* **31**: 2409–2414, 1982.
22. Fletcher KA, Price AH and Barton PF, The pharmacokinetics and biochemical pharmacology of primaquine in rhesus monkeys and rats. In: *Primaquine: Pharmacokinetics, Metabolism, Toxicity and Activity, Proceedings of a meeting of the Scientific Working Group on the Chemotherapy of Malaria, UNDP/World Bank/WHO Special Programme, Geneva, Switzerland, 27–28 February* 1984 (Eds. Wernsdorfer WH and Trigg PI), pp. 49–63. John Wiley, Chichester, 1987.
23. Baird KJ, McCormick GJ and Canfield CJ, Effects of nine synthetic putative metabolites of primaquine on activity of the hexose monophosphate shunt in intact human red blood cells *in vitro*. *Biochem Pharmacol* **35**: 1099–1106, 1986.
24. Wallace WJ and Caughey WS, Mechanism for the autoxidation of hemoglobin by phenols, nitrite and 'oxidant' drugs. Peroxide formation by one electron donation to bound dioxygen. *Biochem Biophys Res Commun* **62**: 561–567, 1975.
25. Winterbourn CC, McGrath BM and Carrell RW, Reactions involving superoxide and normal and unstable haemoglobins. *Biochem J* **155**: 493–502, 1976.
26. Miller A and Smith HC, The intracellular and membrane effects of oxidant drugs on normal red cells. *Br J Haematol* **19**: 417–428, 1970.
27. Chevion M, Navok T, Glaser G and Mager J, The chemistry of favism-inducing compounds. The properties of isouramil and divicine and their reaction with glutathione. *Eur J Biochem* **127**: 405–409, 1982.
28. Clark IA and Hunt NH, Evidence for reactive oxygen intermediates causing haemolysis and parasite death in malaria. *Infect Immun* **39**: 1–6, 1983.
29. Clark IA, Hunt NH, Cowden WB, Maxwell LE and Mackie EJ, Radical-mediated damage to parasites and erythrocytes in *Plasmodium vinckei* infected mice after injected of t-butyl hyperoxide. *Clin Exp Immunol* **56**: 524–530, 1984.
30. Alving AS, Powell RD, Brewer GJ and Arnold JD, Malaria, 8-aminoquinolines and haemolysis. In: *Drugs, Parasites and Hosts* (Eds. Goodwin LG and Nimmo-Smith RH), pp. 83–97. Churchill, London, 1960.