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DIRECT ESR DETECTION OF A FREE RADICAL INTERMEDIATE DURING THE PEROXIDASE-CATALYZED OXIDATION OF THE ANTIMALARIAL DRUG PRIMAQUINE

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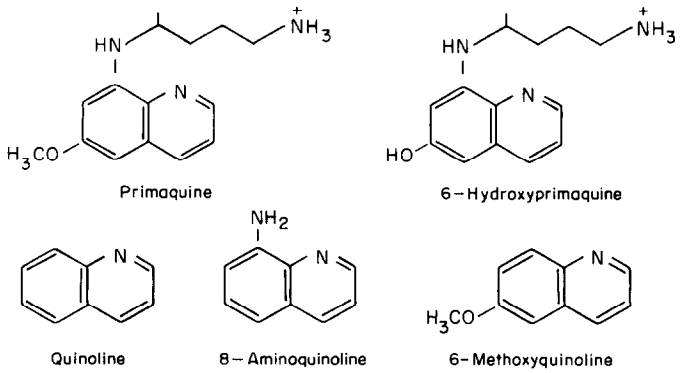
Abstract—Oxidation of the antimalarial primaquine by horseradish peroxidase and H_2O_2 was demonstrated by visible light absorption and ESR spectroscopy. Initial product analysis indicated a 15% yield of O-demethoxylation products, methanol and the quinone-imine derivative, and organic extractable polymeric material. Horseradish peroxidase was substituted by methemoglobin, and both enzymes showed greater activity at acidic pH values. During the enzymatic oxidation of primaquine, a drug-derived free radical was detected by direct ESR spectroscopy. A similar ESR spectrum was obtained during enzymatic oxidation of 6-hydroxyprimaquine at pH 9.0. Computer simulations of the ESR spectra obtained in normal and deuterated buffer indicated that the detectable free radical contains two primaquine moieties. This *in vitro* oxidation of primaquine to a free radical intermediate that is stable in the presence of oxygen might be considered a new mechanistic route for analyzing the pharmacological effects of primaquine.

Primaquine, an 8-aminoquinoline, is the only tissue schizontocide currently available for radical treatment of malarial infections. Its utility is compromised by its toxic effects on erythrocytes, and indeed primaquine was one of the first agents recognized to produce oxidative stress [1, 2]. Despite its importance, it is still not clear whether pharmacological effects of primaquine are due to the parent compound or to its metabolites [3–5]. Recently, oxidative metabolites such as 6-hydroxyprimaquine and 5,6-dihydroxyprimaquine [5–7] and the primaquine-dependent oxygen-derived free radicals superoxide anion and hydroxyl radical [8–10] have been identified. Historically, these metabolites have been connected by the hypothesis that primaquine would first be oxidized to a phenol and subsequently to a quinone-imine. This derivative, through redox-cycling, would generate active oxygen species [2]. However, it was reported recently that quinone-imine derived free radicals do not necessarily react with oxygen to generate oxygen-derived free radicals [11, 12]; also, there is no reported evidence for the generation of free radicals during primaquine oxidative metabolism.

The intermediacy and contribution of free radical species in the mechanism and cytotoxic action of many agents, including antiparasitic drugs, have been recognized recently [13–15]. Primaquine reductive metabolism has been shown to generate oxygen-derived radicals [8–10]; however, to get a better understanding of the routes by which the drug can be activated to free radicals, its oxidative metabolism should be considered [2–7]. In this context, we have examined the possibility of free



Scheme I

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radical generation by primaquine oxidation as catalyzed by horseradish peroxidase and H_2O_2. This enzyme system is known to oxidize a variety of compounds that bear structural features similar to the primaquine molecule (structures shown in Scheme I) such as aromatic amines [13–16], aminophenols [17–19] and methoxylated aromatic compounds [20–22]. In addition, hemoglobin, an important protein in the expression of primaquine pharmacological effects [5–9], has peroxidative activity [23–26]. Horseradish peroxidase is the best understood of the peroxidases. We report here that oxidation of primaquine catalyzed by horseradish peroxidase and H_2O_2 or methemoglobin and H_2O_2 involves oxidation of more than one reactive group in the molecule and results in generation of a free radical species that can be detected by direct ESR spectroscopy.

MATERIALS AND METHODS

The following reagents were obtained from commercial sources: primaquine diphosphate and quinoline (Aldrich Chemical Co., Milwaukee, WI); horseradish peroxidase (Type VI), methemoglobin (from sheep), 6-methoxyquinoline, 8-aminoquinoline, GSH,* DTPA, ^2H_2O and DMPO (Sigma Chemical Co., St. Louis, MO); and H_2O_2 (Mallinckrodt Inc., Paris, KY). Each was used without further purification. 6-Hydroxyprimaquine was synthesized according to Allahyari *et al.* [7].

Absorption spectra were recorded on an 8451 A Hewlett–Packard spectrophotometer at room temperature. ESR spectra were recorded with an ESR 200 D-SRC IBM spectrometer equipped with a TM_{110} cavity and a quartz flat cell for aqueous solutions. The reaction mixtures were transferred to the quartz flat cell by means of a rapid sampling device (Gilford Instruments) [27]. Simulations were carried out on a Varian E-935 data acquisition system. A computer-aided tuning procedure was applied to optimize the hyperfine coupling constants using the correlation between experimental and simulated spectra as the criterion for goodness of fit [28]. Oxygen consumption was measured polarographically with an oxygen monitor (Gilson 5/6 Oxygraphy).

The typical reaction mixture (3.0 ml final volume) contained primaquine (20 mM), H_2O_2 (10 mM), horseradish peroxidase (100 μg/ml) and DTPA (1 mM) in acetate buffer, pH 4.2. Additional components or changes in the incubation mixtures are given in the text and figure legends. The reactions were always started by enzyme addition. For the ESR experiments under anaerobic conditions the reaction mixtures, placed in rubber-stoppered serum bottles, were bubbled with nitrogen for 10 min before enzyme addition and then transferred to the quartz flat cell under nitrogen [27]. Deuterated acetate buffer was prepared by dissolving the conjugated acid-base pair in deuterium oxide; the pH was adjusted following the relation pH = pD − 0.4. Methanol was identified and quantitated by gas chromatography (Hewlett–Packard 5890 gas chromatography) by direct injection of the reaction mixtures (10-min incubation time) onto a Durabond 5 column (30 m) maintained at 30° for 6 min, followed by a gradient of 20°/min to 200°. Methanol was quantitated by comparison of the corresponding area in the reaction mixture with those of a standard curve prepared by additions of known quantities of methanol to the incubation buffer [25]. To estimate primaquine transformation, the reaction mixture was alkalinized with NaOH to pH 9.5 and extracted three

* Abbreviations: DMPO, 5,5-dimethylpyrroline *N*-oxide; DTPA, diethylenetriaminepentaacetic acid; and GSH, reduced glutathione.

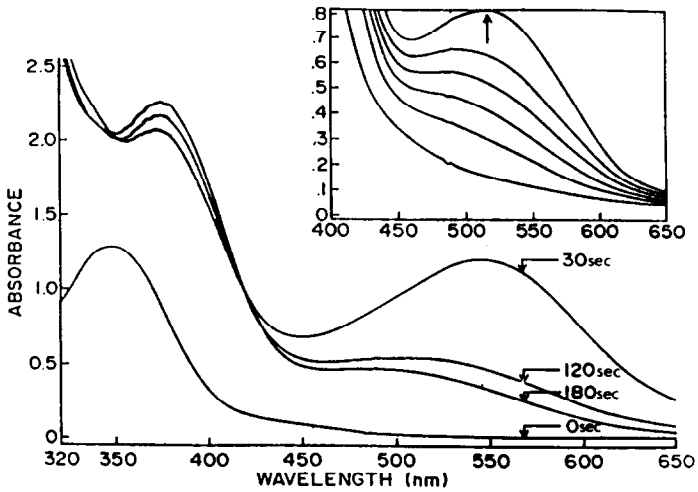


Fig. 1. Optical absorption spectra of primaquine (20 mM) oxidation by horseradish peroxidase (100 μg/ml) and H_2O_2 (20 mM) on 0.1 M acetate buffer, pH 4.2, containing 1 mM DTPA. Aliquots were withdrawn at the indicated times after initiation of the reaction and diluted thirty times. The inset shows successive scans of the same reaction with primaquine (1 mM), H_2O_2 (1 mM) and horseradish peroxidase (50 μg/ml); the first scan was 30 sec after initiation of the reaction, and there are 42-sec intervals between the scans except for the last which was run 500 sec after the reaction was initiated.

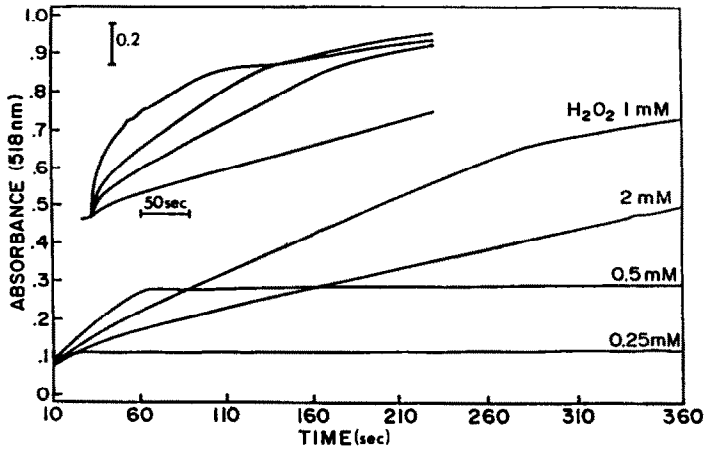


Fig. 2. Time-dependent increase in the absorbance at 518 nm during the reaction of primaquine (1 mM) and horseradish peroxidase (50 μg/ml) in the presence of various H_2O_2 concentrations: 0.25 mM, 0.5 mM, 1 mM, and 2 mM. The inset shows the time-dependent increase in the absorbance of 518 nm during the reaction of primaquine (1 mM) and H_2O_2 (1 mM) in the presence of various horseradish peroxidase concentrations; from the lower curve to the upper curve, the following enzyme concentrations were used: 25 μg/ml, 50 μg/ml, 100 μg/ml, and 250 μg/ml. The zero time absorbance was 0.05, and the readings started 10 sec after mixing.

times with an equal volume of dichloromethane. The organic layers were combined and dried with sodium sulfate, and the solvent was removed under vacuum. The residue was dissolved in methanol, and its UV-visible light absorption spectrum and TLC properties (in 2:1 benzene–methanol plus 0.5% of glacial acetic acid) were compared with those of primaquine and 6-hydroxyprimaquine [6].

RESULTS

Primaquine was readily oxidized by horseradish peroxidase and H_2O_2 at acidic pH, as shown by the changes in its visible light absorption spectrum (Fig. 1). At high substrate concentrations (10–20 mM), a transient species absorbing at 500 nm reached its maximum concentration at about 30 sec and subsequently decayed, leaving a reddish product with absorbance peaks at 375 and at 518 nm. At lower substrate concentration (0.25 to 2 mM), the peak at 550 nm was undetectable, but the increase of the absorbance at 518 can be followed (Fig. 1, inset). The time-dependent curve of the absorbance increases was proportional to the substrate, H_2O_2 and enzyme concentrations and showed a maximum when stoichiometric concentrations of primaquine and H_2O_2 were used (Fig. 2). The oxidation of primaquine by horseradish peroxidase and H_2O_2 was dependent on the pH and the nature of the buffer (Fig. 3). Oxidation was undetectable in phosphate

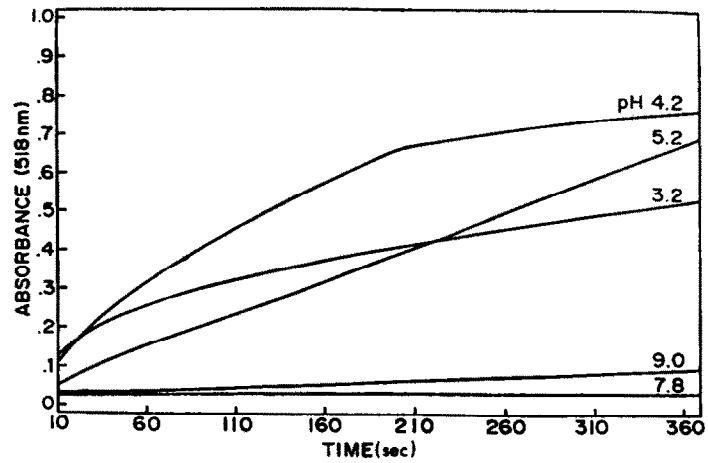


Fig. 3. Effect of pH upon the rate of the oxidation of primaquine (1 mM) by H_2O_2 (1 mM) and horseradish peroxidase (50 μg/ml); from the lower to the upper curve, the following buffers were used: phosphate, pH 7.8; borate, pH 9.0; acetate, pH 3.2; acetate, pH 5.2; and acetate, pH 4.2. Buffer concentrations were 0.1 M and all contained 1 mM DPTA. The zero time absorbance changed with pH but was close to 0.05; the readings started 10 sec after mixing.

buffer, pH 7.8, but was measurable, albeit slow, in borate buffer, pH 9.0 (Fig. 3).

An initial analysis of the reaction products was attempted based on a possible horseradish peroxidase-catalyzed O-demethoxylation [20]. Both the 1:1 primaquine–H_2O_2 stoichiometry of product formation (Fig. 2) and its reddish color with an absorbance peak at 518 nm are consistent with quinone-imine formation [19, 20, 22], which would also yield methanol as a product [20]. However, determination of methanol by gas chromatography accounted for only 15% of the initial substrate concentration (Table 1), suggesting that the quinone-imine formation is not quantitative. Indeed, a gross estimation of the final concentration of the quinone-imine can be made by assuming a value of 8000 $M^{-1} cm^{-1}$ for ε at 518 nm [19, 20, 22]. With either 20 mM primaquine or 1 mM primaquine (Fig. 1) the estimated quinone-imine yield (10%) was comparable to that of methanol (Table 1). This indicates that, by contrast with the horseradish peroxidase-catalyzed oxidation of 9-methoxyellipticine [20], O-demethoxylation is not the main path of primaquine oxidation. At alkaline pH, a yellow product was obtained by organic extraction, while the reddish product remained in the aqueous phase. In methanol, the yellow extract had a UV-visible light absorption spectrum (max = 213, 265, 382 nm) similar to that of primaquine (max = 207, 265, 359 nm) with the peaks shifted to higher wavelengths. The TLC properties of the yellow extract were also different from those of primaquine and 6-hydroxy-primaquine; its lower mobility and tendency to aggregate in methanol suggest polymeric products.

Primaquine oxidation was accompanied by the generation of a free radical species that was detected by direct ESR spectroscopy (Fig. 4). This radical was detected with concentrations of primaquine above 5 mM and in excess of H_2O_2; in other experimental conditions, only a broad one-line ESR signal was observed (not shown). Radical generation was dependent on the complete system (Fig. 4, A–C), and it was also observed when methemoglobin was substituted for horseradish peroxidase (Fig. 4D). Radical stability was not affected by oxygen as demonstrated by incubation under anaerobic conditions; the same ESR spectra (Fig. 4, A–D) were obtained under air or nitrogen. Also, parallel experiments showed that the standard incubation mixture did not consume oxygen. Consequently, all the ESR spectra shown here (Figs. 4–7) were recorded in air-saturated buffer. The fact that the primaquine-derived free radical did not react with oxygen was confirmed by the experiments showing that primaquine oxidation required stoichiometric concentrations of H_2O_2 (Fig. 2). By contrast, the stability of the primaquine-derived free radical was decreased at higher pH values (Fig. 5) or in the presence of GSH (Fig. 5, inset). The effect of GSH did not seem to be due to competitive inhibition of primaquine oxidation by GSH [29] because spin-trapping experiments in the presence of DMPO have shown that no DMPO–GS adduct is detected in the experimental conditions of the inset of Fig. 5.

To minimize changes in the ESR spectrum peak heights due to radical decay during the scanning time, the reaction was run at pH 3.2 in normal and in deuterated buffer (Fig. 6); the calculated hyperfine coupling constants from spectrum simulations indi-

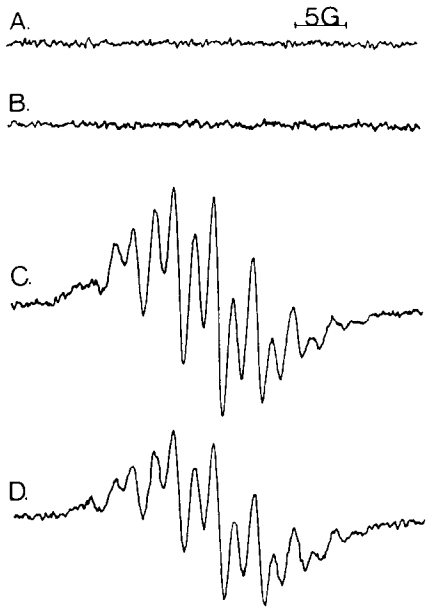


Fig. 4. ESR spectra obtained during the oxidation of primaquine (20 mM) by horseradish peroxidase (100 μg/ml) and H_2O_2 (10 mM): (A) in the absence of enzyme, (B) in the absence of H_2O_2, (C) complete system, and (D) 20 μM methemoglobin substituted for horseradish peroxidase. Instrumental conditions: microwave power, 20 mW; modulation amplitude, 0.5 G; time constant, 0.5 sec; scan rate, 0.25 G/sec.

Table 1. Formation of methanol during the oxidation of primaquine catalyzed by horseradish peroxidase*

Incubation mixture	Methanol formation Concentration† (mM)	Yield (%)
20 mM Primaquine + 20 mM H_2O_2	3.05	15
10 mM Primaquine + 10 mM H_2O_2	1.45	14.5

* The reaction was started by addition of horseradish peroxidase (100 μg/ml) in acetate buffer, pH 4.2, containing 1 mM DTPA, and was incubated for 10 min at room temperature.

† The results are the average of two independent determinations.

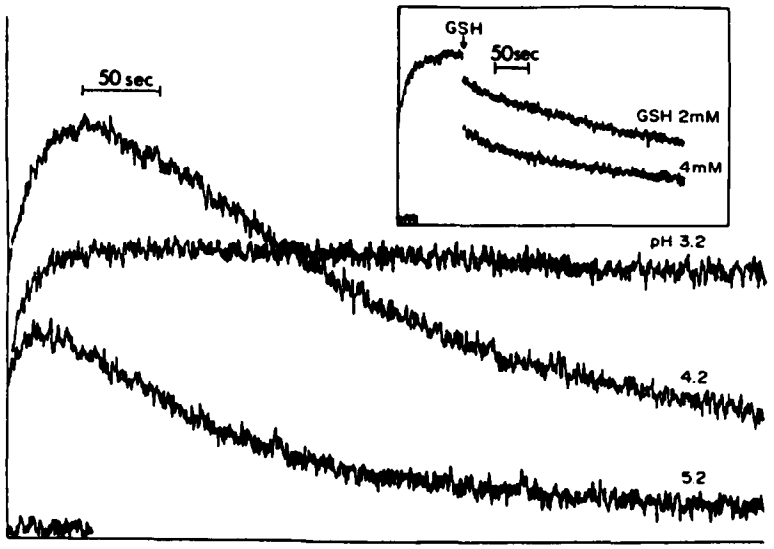


Fig. 5. Time course of the amplitude of the ESR signal obtained during oxidation of primaquine (20 mM) by horseradish peroxidase (100 μg/ml) and H_2O_2 (10 mM) in acetate buffer at the indicated pH values. The magnetic field was centered at the central peak of the primaquine radicals. The inset shows the time course of the primaquine radical at pH 3.2 upon addition of 2 and 4 mM GSH.

cated two equivalent nitrogens with $a^N = 3.646$, two equivalent protons with $a^H = 4.095$, and two equivalent protons with $a^H = 1.505$ (Table 2). The spectrum obtained in deuterated buffer (Fig. 6C) was simulated when the four protons were substituted with deuterium [spin 1/2; $a^{^2H} = 0.154 \times a^H$].

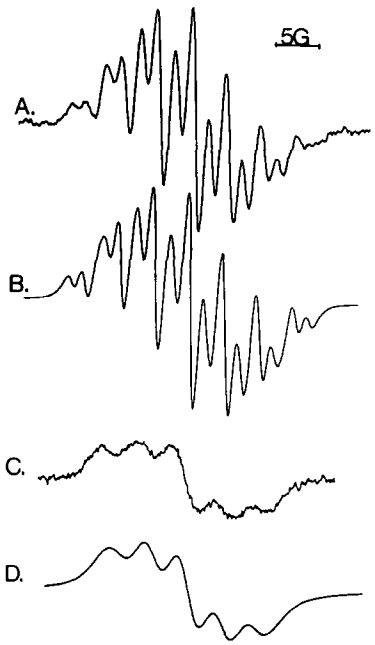


Fig. 6. ESR spectra obtained during the oxidation of primaquine (20 mM) by horseradish peroxidase (100 μg/ml) and H_2O_2 (10 mM) at pH 3.2 in (A) acetate buffer, (B) computer simulation of (A), (C) deuterated acetate buffer and (D) computer simulation of (C). Instrumental conditions: Microwave power, 20 mW; modulation amplitude, 0.5 G for (A) and 1.0 G for (C), time constant, 2 sec; scan rate, 0.05 G/sec.

The ESR literature on quinoline derivatives is restricted to quinoline anion radicals obtained by the reduction of these compounds [36]. In order to generate comparative data for the enzymatically generated primaquine-derived radical (Figs. 4 and 6), we examined the ability of the horseradish peroxidase–H_2O_2 system to oxidize to free-radical intermediates four compounds that resemble the primaquine molecule, namely quinoline, 8-aminoquinoline, 6-methoxyquinoline and 6-hydroxy-

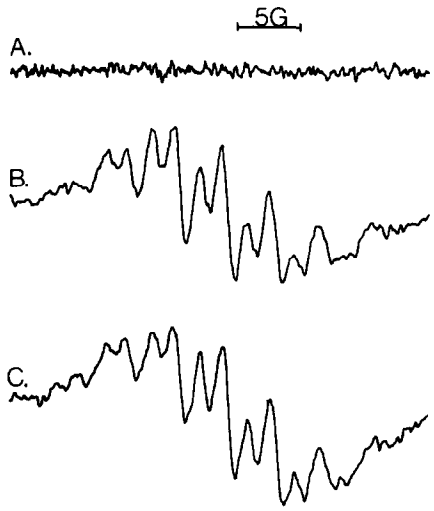


Fig. 7. ESR spectra obtained during horseradish peroxidase (100 μg/ml) and H_2O_2 (10 mM) oxidation of (A) 100 mM 6-hydroxyprimaquine in acetate buffer, pH 3.2; (B) 200 mM 6-hydroxyprimaquine in borate buffer, pH 9.0; and (C) 100 mM primaquine in borate buffer, pH 9.0, containing 20% (v/v) ethanol. Instrumental conditions: microwave power, 20 mW; modulation amplitude, 1 G; time constant, 0.5 sec; scan rate, 0.5 G/sec for (A), and 0.25 G/sec for (B) and (C).

Table 2. Hyperfine splitting constants of some nitrogen cation radicals.

	Primaquine-derived free radical*	Benzidine-like free radicals†	Phenazine-like free radicals‡
a^N	3.646	3.1–4.8	6.0–6.6
a^H_{NH} or $a^H_{NCH_2R}$	4.095	3.3–5.0	6.0–6.5
a^H ring	1.505	0.8–1.6	0.6–3.2

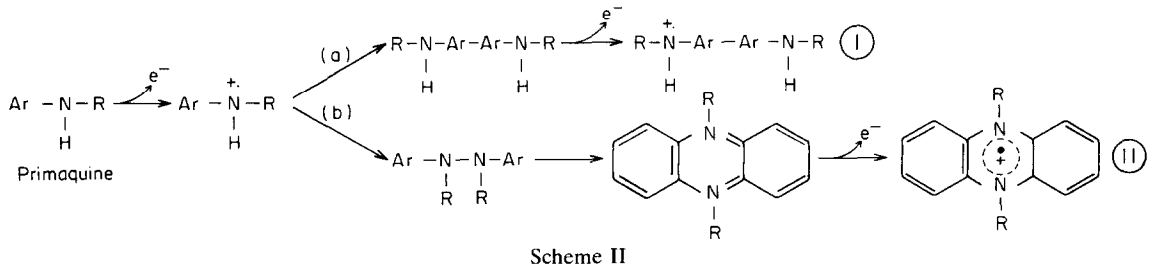
* This work.
† Refs. 30–32.
‡ Refs. 32–35.

primaquine (Scheme I). In all conditions used, substrate concentration varied from 10 to 400 mM and pH varied from 3.2 to 9.0; only 6-hydroxyprimaquine oxidation at pH 9.0 gave rise to an ESR spectrum similar to that obtained by primaquine oxidation (Fig. 7). Primaquine was a better substrate for horseradish peroxidase–H_2O_2, including at pH 9.0, as ascertained by the time-dependent changes in the absorbance at 518 nm or by the substrate concentration-dependent radical yield (Fig. 7). A detailed comparison of the primaquine-generated spectrum with the 6-hydroxyprimaquine-generated spectrum was precluded by the fast radical decay at pH 9.0.

DISCUSSION

Our results show that oxidation of primaquine catalyzed by horseradish peroxidase–H_2O_2 or methemoglobin–H_2O_2 is a complex process involving oxidation of more than one reactive group in the molecule. By contrast with the horseradish peroxidase-catalyzed oxidation of 9-methoxyellipticine [20], O-demethoxylation products accounted for only about 15% of the initial substrate concentration (Fig. 1, Table 1). This points to the aromatic amino group as an important target in primaquine oxidation. This possibility has been considered recently, by analogy with aniline oxidation [37]. In this case, generation of a phenylhydroxylamine derivative has been inferred, but this suggestion is not supported by our ESR studies. A phenylhydroxylamine derivative in oxidizing conditions would lead to a nitroxide radical [38, 39]. A free radical intermediate was generated during primaquine oxidation catalyzed by horseradish peroxidase–H_2O_2 or methemoglobin–H_2O_2; however, the nitrogen and hydrogen hyperfine constants calculated from spectrum simulations (Fig. 6, Table 2) indicate a nitrogen cation radical [16, 30–35] and not an aromatic nitroxide radical [39, 40]. Also, there is only one example reporting horseradish peroxidase as a N-hydroxylation catalyst [40]. Unambiguous identification of the primaquine-derived radical, however, is difficult due to low resolution of the ESR spectra (Figs. 4–7) and incomplete analysis of the reaction products. A complex product distribution can be expected during aromatic amine oxidations, as the initial products are more easily oxidized than the parent compound, undergoing rearrangements and addition reactions [33]. Scheme II summarizes reactions and structures that can be drawn for oxidation of the primaquine aromatic amino group [33, 41]. As shown in the scheme, path (a) could lead to a benzidine-like cation radical (structure I, Scheme II) and path (b) could lead to a phenazine-like cation radical (structure II, Scheme II). Both structures can be drawn for primaquine and 6-hydroxyprimaquine, in agreement with the similar ESR spectra displayed in Fig. 7. The structures can also account for the four exchangeable hydrogens demonstrated by the experiments with deuterated buffer (Fig. 6). In addition to the heteroatomic hydrogens, primaquine has two hydrogens in the ring at positions 5 and 7 that are exchangeable by deuterium [42]. Paths (a) and (b) are expected to be pH dependent, with low pH values favoring path (a) and high pH values favoring path (b) [32–34]. In the case of primaquine oxidation, basically the same ESR spectra were obtained in acidic and basic conditions (Figs. 6 and 7), although a higher radical yield was detectable at low pH values. At this point it is difficult to suggest a structure for the primaquine-derived free radical, but comparison of our calculated ESR parameters with those reported in the literature for structures I and II (Table 2; Scheme II) indicates that a benzidine-like radical (Structure I, Scheme II) is more plausible. Generation of a benzidine-like radical in the presence of nucleophilic groups can lead to further condensation reactions [43, 44], accounting for the polymeric nature of the reaction products.

Independently of the complete product identification and exact radical structure determination, the results reported here are the first demonstrations



Scheme II

that the antimalarial primaquine can be oxidized by horseradish peroxidase, generating a free radical intermediate. We have also demonstrated that (a) methemoglobin can catalyze radical formation; (b) the radical is not a nitroxyl [36–38]; (c) the radical is oxygen insensitive and consequently cannot be an efficient generator of oxygen radicals [11]; and (d) primaquine oxidation products are prone to condensation reactions and could react with several biomolecules [41, 42]. These results offer a new mechanistic route for understanding the pharmacological effects of primaquine [3–10] and should be explored further.

REFERENCES

1. G. Cohen and P. Hochstein, *Biochemistry* **3**, 895 (1964).
2. J. L. Holtzmam, *Life Sci.* **30**, 1 (1981).
3. A. Strother, I. M. Fraser, R. Allahyari and B. E. Tilton, *Bull. Wld Hlth Org.* **59**, 413 (1981).
4. P. E. Carson, R. Hohl, M. V. Nora, G. W. Parkhurst, T. Ahmand, S. Scalan and H. Frischer, *Bull. Wld Hlth Org.* **59**, 427 (1981).
5. TDR. Seventh Programme Report, January 83–December 94, World Health Organ., p. 2/13.
6. A. Strother, R. Allahyari, J. Buchholz, I. M. Fraser and B. E. Tilton, *Drug Metab. Dispos.* **10**, 35 (1984).
7. R. Allahyari, A. Strother, I. M. Fraser and A. J. Verbiscar, *J. med. Chem.* **27**, 407 (1984).
8. P. J. Thornalley, A. Stern and J. V. Bannister, *Biochem. Pharmac.* **32**, 3571 (1983).
9. O. Augusto, C. L. V. Weingrill, S. Schreier and H. Amemiya, *Archs Biochem. Biophys.* **244**, 147 (1986).
10. O. Augusto, M. J. M. Alves, W. Colli, L. S. Filardi and Z. Brener, *Biochem. biophys. Res. Commun.* **135**, 1029 (1986).
11. V. Fischer, P. R. West, S. D. Nelson, P. J. Harvinson and R. P. Mason, *J. biol. Chem.* **260**, 11446 (1985).
12. G. Powis, E. M. Hodnett, K. S. Santone, K. L. See and D. C. Melder, *Cancer Res.* **47**, 2363 (1987).
13. R. P. Mason and C. F. Chignell, *Pharmac. Rev.* **33**, 189 (1981).
14. R. Docampo and S. N. J. Moreno, in *Free Radicals in Biology* (Ed. W. A. Pryor), Vol. 6, p. 243. Academic Press, New York (1984).
15. R. Docampo and S. N. J. Moreno, *Rev. infect. Dis.* **6**, 223 (1984).
16. B. W. Griffin, D. K. Davis and G. V. Bruno, *Bioorg. Chem.* **10**, 342 (1981).
17. E. Perez-Reyes and R. P. Mason, *J. biol. Chem.* **256**, 2427 (1981).
18. P. R. West, L. S. Harman, P. D. Josephy and R. P. Mason, *Biochem. Pharmac.* **33**, 2933 (1984).
19. C. Auclair and C. Paoletti, *J. med. Chem.* **24**, 289 (1981).
20. C. Meunier and B. Meunier, *J. biol. Chem.* **260**, 10576 (1985).
21. N. Haim, J. Roman, J. Nemec and B. K. Sinha, *Biochem. biophys. Res. Commun.* **135**, 215 (1986).
22. J. Bernadou, G. Meunier, C. Paoletti and B. Meunier, *J. med. Chem.* **26**, 574 (1983).
23. P. George and D. H. Irvine, *Biochem. J.* **52**, 511 (1952).
24. P. George and D. H. Irvine, *J. Colloid Sci.* **11**, 327 (1956).
25. O. Augusto, K. L. Kunze and P. R. Ortiz de Montellano, *J. biol. Chem.* **257**, 6231 (1982).
26. P. R. Ortiz de Montellano and C. E. Catalano, *J. biol. Chem.* **260**, 9265 (1985).
27. P. R. Mason, *Meth. Enzym.* **105**, 416 (1984).
28. A. Motten and J. Schreiber, *J. magn. Resonance* **67**, 42 (1985).
29. L. S. Harman, D. K. Carver, J. Schreiber and P. R. Mason, *J. biol. Chem.* **261**, 7811 (1986).
30. P. D. Josephy, T. E. Eling and R. P. Mason, *J. biol. Chem.* **257**, 3669 (1982).
31. P. D. Josephy, T. E. Eling and R. P. Mason, *J. biol. Chem.* **258**, 5561 (1983).
32. G. Cauquis, J. Gognard and D. Serve, *Electrochim. Acta* **20**, 1011 (1975).
33. G. Cauquis, M. Genies and D. Serve, *Tetrahedron Lett.* 5009 (1972).
34. G. Cauquis, H. Delhomme and D. Serve, *Electrochim. Acta* **20**, 1019 (1975).
35. J-D. Cheng and H. J. Shine, *J. org. Chem.* **40**, 703 (1975).
36. J. Chaudhuri, S. Kume, J. Jagur-Grodzinski and M. Szwarc, *J. Am. chem. Soc.* **90**, 6421 (1968).
37. J. H. Harrison, Jr. and D. J. Jollow, *J. Pharmac. exp. Ther.* **238**, 1045 (1986).
38. A. Stier, R. Class, A. Lucke and I. Reitz, *Xenobiotica* **10**, 661 (1980).
39. I. Golly and P. Hlavica, *Biochem. J.* **226**, 803 (1985).
40. B. K. Sinha, *Biochem. Pharmac.* **32**, 2604 (1983).
41. F. T. Naylor and B. C. Sauders, *J. chem. Soc.* 3519 (1950).
42. C. D. Hufford, J. D. McChesney and J. K. Baker, *J. heterocyclic Chem.* **20**, 273 (1983).
43. P. D. Josephy, T. E. Eling and R. P. Mason, *Molec. Pharmac.* **23**, 766 (1983).
44. P. D. Josephy, *Fedn Proc.* **45**, 2443 (1986).