# Inhibitory Effect of Chloroquine on the Peroxidase Activity of Ferriprotoporphyrin IX

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The effect of the antimalarial chloroquine {7-chloro-4-[4-(diethylamino)-1-methylbutylamino]quinoline} on the oxidation of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (abts) promoted by ferriprotoporphyrin IX [(3,7,12,17-tetramethyl-8,13-divinylporphyrin-2,18-dipropanoato)iron(III)] and $H_2O_2$ was studied. Under the conditions [abts] ≫ [$H_2O_2$] ≫ [catalyst], the kinetic results showed a first-order dependence on both the peroxide (up to $8 \times 10^{-3}$ mol dm$^{-3}$) and the catalyst concentration (up to $4 \times 10^{-6}$ mol dm$^{-3}$), although a saturation effect was observed at higher concentrations. The values of the apparent second-order rate constants in the absence and presence of chloroquine were $(1.71 \pm 0.05) \times 10^5$ and $(1.23 \pm 0.04) \times 10^5$ dm$^6$ mol$^{-2}$ s$^{-1}$ at pH 6.86, 303.1 ± 0.2 K, and $I = 0.10$ mol dm$^{-3}$ NaCl. Therefore, the antimalarial inhibits the peroxidase activity by about 28% due the formation of a catalytically inactive complex with the haem. A first-order dependence on the concentration of abts was also verified, followed by a zero-order dependence at concentrations higher than $5 \times 10^{-3}$ mol dm$^{-3}$. Some EPR spin-trapping experiments, and comparative studies in the presence of bromide ions, provided evidence for the participation of analogues of the horseradish peroxidase compounds I and II rather than hydroxyl radicals, in the peroxidase activity of the haem. The results are discussed in the context of the antimalarial activity of chloroquine.

The catalytic activity of iron(III) porphyrins in reactions of hydrogen peroxide has been long studied as a model of haemoproteins, including cytochrome P-450, catalases and peroxidases.[1–3] All these proteins have ferriprotoporphyrin (haemin) IX {(3,7,12,17-tetramethyl-8,13-divinylporphyrin-2,18-dipropanoato)iron(III) [Fe(por)]$^+$} as their prosthetic group, although they participate in quite different biological processes such as drug metabolism, peroxide disproportionation, and electron transport. The intrinsic reactivity of the common haem group thus should reflect differences in the protein structure, controlling haem, protein and substrate interactions.[4]

Since structural features of the protein modulate haem reactivity, it is expected that complexing agents can also control the reactivity of haem in aqueous solution where it dimerizes, aggregates, and can suffer destructive oxidation.[5–7] When released from the globin moiety, haem can be hazardous to red-cell membranes, where it intercalates into the phospholipid bilayer and, after causing many structural changes,[8] finally leads to haemolysis.[9] By examining the haem–$H_2O_2$–dependent lipid peroxidation of rat-liver microsomes, it was verified[10] that haem-binding proteins may enhance, decrease, or completely inhibit haem-catalysed reactions. The extent of lipid peroxidation is dependent on the relative affinity of the protein and biological membranes for haem. Thus, human albumin and rat glutathione S-transferases, with moderate affinities for haem, decreased the haem-catalysed lipid peroxidation, whereas bovine albumin, with low affinity for haem, enhanced peroxidation, and haemopexin which strongly binds haem, completely inhibited the reaction.

Low-molecular-weight compounds can also effect haem reactivity. For instance, inhibitory effects by glutathione (γ-glutamylcysteinylglycine)[11] and desferrioxamine[12] in haem-induced red-cell lysis have been observed, and attributed to the chelation of these substrates to the central iron ion. By contrast, the antimalarial chloroquine (cq) {7-chloro-4-[4-(dimethylamino)-1-methylbutylamino]quinoline}, which appears to intercalate between dimers of haem forming a complex by π–π interactions and hydrophobic forces in a molar ratio of 2:1,[13,14] did not prevent haem-induced lysis of malarial parasites,[15] and it was able to increase haem-mediated lipid peroxidation in a model system.[16]

Although there is some controversy on the mode of action of 4-aminoquinoline antimalarials, it seems that their high affinity for haem may play a role in their activity.[17–19] The coordination of chloroquine to ferriprotoporphyrin IX was shown[20] to be essential for the drug to acquire a lipid peroxidative ability. More recently the antimalarial activity was attributed to the inhibition of a newly discovered haem polymerase enzyme by chloroquine,[21] which causes accumulation of a non-haemozoin ferrihaem, extremely toxic to the malaria parasites.[22] In both mechanisms, a toxic form of ferrihaem is implicated in the antimalarial action of chloroquine. Consequently, it is important to elucidate the mechanism by which the antimalarial drug interferes with the catalytic efficiency of the haem. Here we report kinetic studies of the influence of chloroquine on the oxidation of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (abts) promoted by haem–$H_2O_2$.

## Experimental

*Materials and General Methods.*—Ferriprotoporphyrin IX was obtained from Sigma (St. Louis, MO, USA) as 'haemin chloride' (Bovine, Type I). Stock solutions of it were prepared by dissolving accurately weighted amounts in a small volume of 0.10 mol dm$^{-3}$ NaOH solution, and diluting first to 0.87 mmol dm$^{-3}$ in deionized water, followed by further dilution in appropriate buffered-saline solutions.[23] The stock solutions of chloroquine (Sigma) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (Aldrich Chemical Co., Milwaukee, WI, USA) were prepared by dissolving appropriate amounts of the reagent

in deionized water, followed by dilution, respectively, to 1.02 and 23.0 mmol $dm^{-3}$. All stock solutions were prepared daily, immediately prior to use.

Hydrogen peroxide was obtained as a 35% by weight unstabilized aqueous solution from Peroxidos do Brasil Ltd (Sao Paulo, Brazil). More diluted solutions prepared from this material were assayed spectrophotometrically[24] ($\varepsilon_{452}$ = 360 $dm^3$ $mol^{-1}$ $cm^{-1}$) by using the vanadate method, based on the formation of a peroxo complex of vanadium(v).[25] 5,5-Dimethyl-1-pyrroline *N*-oxide (dmpo), from Sigma, was used as the spin trap in the EPR experiments, after purification by filtration through charcoal.[26] Phosphate buffer solutions (0.025 mol $dm^{-3}$, pH 6.86) were prepared from Merck (Darmstadt, Germany) $Na_2HPO_4$ and $KH_2PO_4$, and the ionic strength was adjusted to 0.10 mol $dm^{-3}$ with NaCl (Merck). Other chemicals of analytical grade were used without further purification.

Optical absorption measurements were performed on a Beckman DU-70 spectrophotometer, at 303.1 ± 0.2 K. The EPR spectra were recorded at room temperature on a Bruker ER 200-SRC spectrometer, using flat-faced quartz cells. Samples of reaction solutions, in the presence of an excess of dmpo (0.1 mol $dm^{-3}$), were transferred to EPR containers, immediately after hydrogen peroxide addition and the spectra scanned.

*Kinetic Measurements.*—Kinetic experiments were usually performed by adding sample solutions of the iron porphyrin or of its 2:1 complex with cq (final concentration 0.5–4.0 μmol $dm^{-3}$) to abts solution (8.4 mmol $dm^{-3}$) held in a cuvette in the thermostatted spectrophotometer cell compartment. The 2:1 complex was prepared by adding stoichiometric amounts of the chloroquine solution to buffered-saline solutions of the haem. The reaction was then initiated by addition, with mixing, of hydrogen peroxide solution (final concentration 0.20–0.90 mmol $dm^{-3}$). The increases in the absorption at 660 nm, corresponding to a maximum in the spectra of the radical cation $abts^{\bullet+}$ formed ($\varepsilon$ = 1.05 × $10^4$ $dm^3$ $mol^{-1}$ $cm^{-1}$) were then monitored.[27,28] Typical curves of absorbance *versus* time, up to 90% completion of reaction, obeyed first-order kinetics for all the concentration ranges studied. Initial rates, $v_i = (dA/dt)_{t=0}$, where $A$ = absorbance at 660 nm, were obtained by computing a theoretical fit to the experimental curves, during the first 5% of the reaction.[29] Deviations in the values of the rate constants were ≤3%, as indicated, estimated from repeated experiments.

## Results

*Kinetic Studies.*—At constant pH (phosphate buffer 0.025 mol $dm^{-3}$, pH 6.86) and [abts] ≫ $[H_2O_2]$ ≫ [catalyst], the oxidation of abts obeyed first-order kinetics in relation to both the concentrations of haem and hydrogen peroxide. Plots of initial rate ($v_i$) *versus* [Fe(por)] are shown in Fig. 1(*a*), for different $H_2O_2$ concentrations, at constant [abts]. By using the 2:1 $Fe(por)^+$–cq complex[13,14] as the catalyst, an inhibitory effect in the presence of the antimalarial was evident [Fig. 1(*b*)]. The apparent second-order rate constants were calculated from the variation of the slope of these curves ($k_{obs}$) with the $H_2O_2$ concentration. A linear dependence was observed (Fig. 2), with intercept $k_0$ = 29.8 ± 0.2 $dm^3$ $mol^{-1}$ $s^{-1}$, and values of $k$ = (1.71 ± 0.05) × $10^5$ $dm^6$ $mol^{-2}$ $s^{-1}$ and $k'$ = (1.23 ± 0.04) × $10^5$ $dm^6$ $mol^{-2}$ $s^{-1}$ for the reactions catalysed by $[Fe(por)]^+$ and by the $Fe(por)^+$–cq adduct, respectively, according to equations (1)–(3), where $v_i$ = initial rate = $(dA/dt)_{t=0} = \varepsilon_{abts^{\bullet+}}\, b\{d[abts^{\bullet+}]/dt\}$ and $b$ = 1.000 cm.

$$v_i = k_{obs}[\text{catalyst}] \quad (1)$$

$$v_i = (k_0 + k[H_2O_2])[\text{Fe(por)}] \quad (2)$$

$$\text{or} \quad v_i = (k_0 + k'[H_2O_2])[\text{Fe(por)–cq}] \quad (3)$$

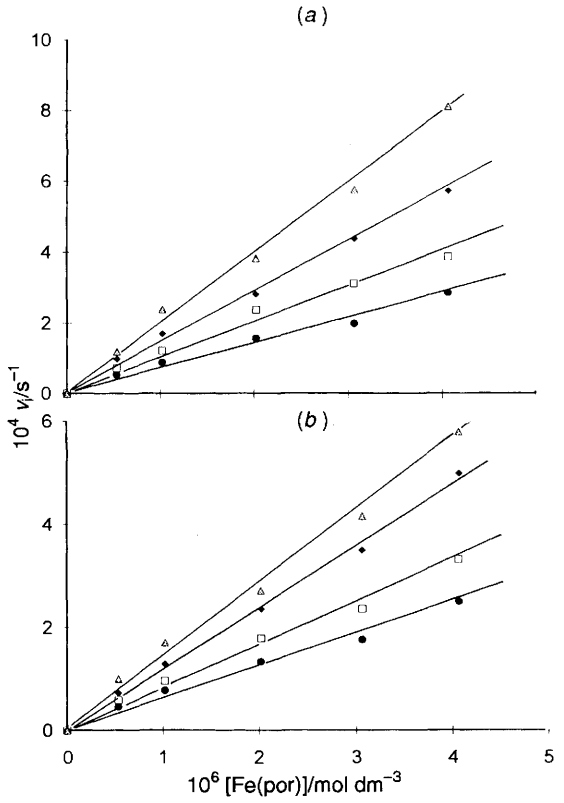



**Fig. 1** Dependence of the initial rate ($v_i$) on [catalyst] at different $[H_2O_2]$ values: [abts] = 8.40 × $10^{-3}$ mol $dm^{-3}$, at pH 6.86 (0.025 mol $dm^{-3}$ phosphate), 303.1 K and $I$ = 0.10 mol $dm^{-3}$ in NaCl. (*a*) Reaction catalysed by iron protoporphyrin IX, Fe(por). (*b*) Reaction catalysed by $Fe(por)^+$–cq $v_i = (dA/dt)_{t=0} = \varepsilon_{abts^{\bullet+}}\, b\{d[abts^{\bullet+}]/dt\}$, where $b$ = 1.000 cm and $\varepsilon_{abts^{\bullet+}}$ = 1.05 × $10^4$ $dm^3$ $mol^{-1}$ $cm^{-1}$. $10^4[H_2O_2]$ = 2.44(●), 4.33 (□), 6.52 (◆) or 9.31 mol $dm^{-3}$ (△)

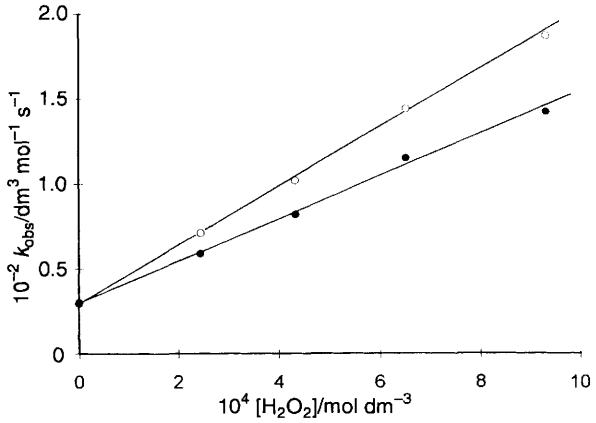



**Fig. 2** Influence of $[H_2O_2]$ on the first-order rate constant in the absence (○) and in the presence (●) of chloroquine. Experimental conditions as in Fig. 1

The above equations fitted well the experimental data, showing an inhibitory effect of the order of 28% when chloroquine is present. The value $k_0$ is independent of the presence of chloroquine, and probably includes a dependence on [abts], the oxidation of which in the absence of hydrogen peroxide was verified to be marginal. In the case of higher concentrations of [Fe(por)] (>5 μmol $dm^{-3}$), significant deviation from direct proportionality was observed, even in the presence of chloroquine (data not shown). Such behaviour has been observed before, during oxidation of abts by a water-soluble haem derived from cytochrome c, the octapeptide

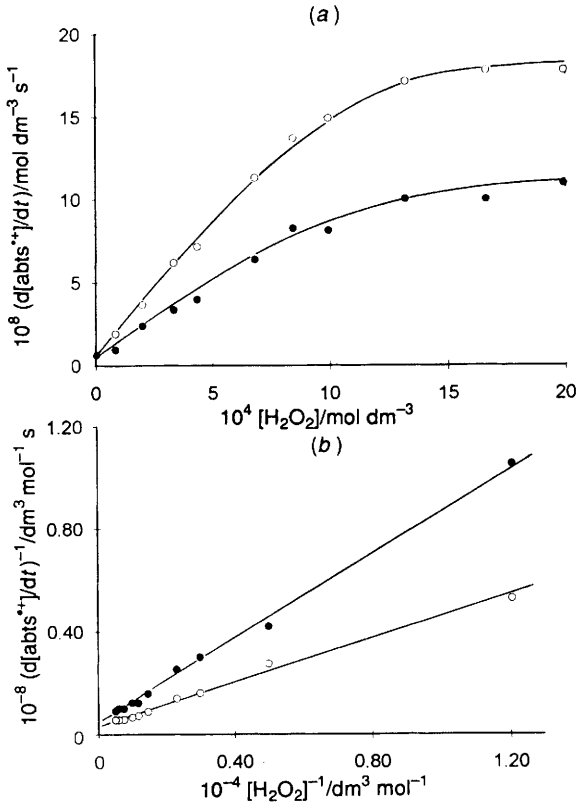



**Fig. 3** Saturation effect of the reaction rate with increasing peroxide concentration: [Fe(por)] = $3.15 \times 10^{-6}$ mol dm$^{-3}$, [cq] = $1.61 \times 10^{-6}$ mol dm$^{-3}$, [abts] = $8.41 \times 10^{-3}$ mol dm$^{-3}$, at pH 6.86 (0.025 mol dm$^{-3}$ phosphate), 303.1 K and $I$ = 0.10 mol dm$^{-3}$ in NaCl. (*a*) Dependence of the initial rate (d[abts$^{\bullet+}$]/d$t$) on [$H_2O_2$]. (*b*) Lineweaver–Burk plot of 1/(d[abts$^{\bullet+}$]/d$t$) *versus* 1/[$H_2O_2$]. (○) Without cq, (●) with cq

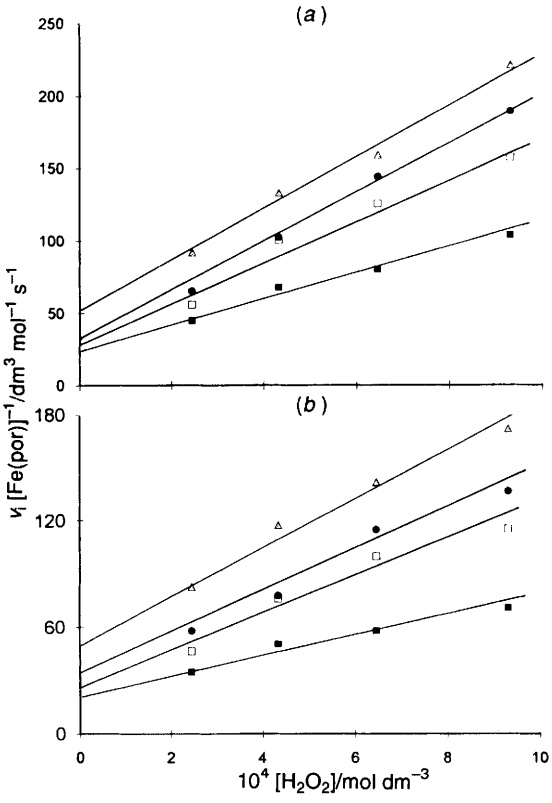



**Fig. 4** Influence of [abts] on the initial rate of the oxidation of abts by $H_2O_2$ catalysed by Fe(por): (*a*) in the absence and (*b*) in the presence of chloroquine. [Fe(por)] = $3.05 \times 10^{-6}$ mol dm$^{-3}$, [cq] = $1.57 \times 10^{-6}$ mol dm$^{-3}$; at pH 6.86 (0.025 mol dm$^{-3}$ phosphate), 303.1 K and $I$ = 0.10 mol dm$^{-3}$. $10^3$[abts] = 3.25 (■), 6.40 (□), 8.40 (●) or 13.00 mol dm$^{-3}$ (△)

microperoxidase-8 (MP-8).[30] In addition, when hydrogen peroxide concentrations $> 9 \times 10^{-4}$ mol dm$^{-3}$ were used a similar saturation effect was observed, and equation (4) applies.

$$\frac{d[abts^{\bullet+}]}{dt} = \frac{k_3[H_2O_2][catalyst]}{K_M + [H_2O_2]} \quad (4)$$

The kinetic parameters $k_3 = 14.8 \times 10^{-2}$ s$^{-1}$, $K_M = 2.31 \times 10^{-3}$ mol dm$^{-3}$, and $k'_3 = 6.51 \times 10^{-3}$ s$^{-1}$, $K'_M = 1.59 \times 10^{-3}$ mol dm$^{-3}$ were determined, respectively in the absence and in the presence of chloroquine (Fig. 3). Therefore, a first-order dependence on both peroxide and catalyst concentrations is only observed when more diluted solutions are used.

On studying the influence of the [abts] on the initial rate of reaction a more complicated dependence was verified. For different concentrations of the substrate abts a linear dependence on [$H_2O_2$] was obtained (Fig. 4), either in the absence or in the presence of chloroquine. However, the dependence of the slope of these curves with [abts] showed a saturation effect (Fig. 5). These results are fitted by the equation: $v_i = (k_0 + k[H_2O_2])$[catalyst], where $k_0 = 22.5 + (1.29 \times 10^3)$ [abts] and $1/k = 2.43 \times 10^{-6} + 3.08 \times 10^{-8}$ (1/[abts]) if the catalyst is Fe(por), or $k_0 = 20.5 + (1.12 \times 10^3)$ [abts] and $1/k = 2.47 \times 10^{-6} + 5.45 \times 10^{-8}$ (1/[abts]) if the catalyst is the Fe(por)$^+$–cq adduct.

Similar results were observed when the dependence of the initial rate on the [catalyst] was studied at different [abts], as shown in Figs. 6 and 7. In this case the determined values for the rate constants were consistent with equations (5) and (6), respectively, in the absence and in the presence of the antimalarial.

$$v_i = \frac{2.92 \times 10^2[abts]}{7.94 \times 10^{-3} + [abts]}[Fe(por)] \quad (5)$$

$$v_i = \frac{2.88 \times 10^2[abts]}{13.0 \times 10^{-3} + [abts]}[Fe(por)\text{–}cq] \quad (6)$$

On verifying the effect of the [abts] in the determined rate law, a pseudo-first-order dependence at concentrations up to $8 \times 10^{-3}$ mol dm$^{-3}$ was also observed. For higher concentrations an appreciable saturation effect was evident, which may be a consequence of the back reduction of the substrate[31] by the same haem–peroxo complex as was initially formed [equations (7) and (8)]. Indeed, the subsequent decrease in the

$$Fe(por)\text{–}H_2O_2 + abts^{\bullet+} \longrightarrow Fe(por) + O_2^{\bullet-} + 2H^+ + abts \quad (7)$$

$$O_2^{\bullet-} + abts^{\bullet+} \longrightarrow abts + O_2 \quad (8)$$

abts$^{\bullet+}$ concentration was evident in the spectrophotometric experiments, with increasing haem concentration, as shown in Fig. 8. This accounts for a consecutive reaction of the radical cation abts$^{\bullet+}$, in a reductive step [equation (7)]. In the presence of chloroquine this decrease in the abts$^{\bullet+}$ concentration is not observed, in the same range of catalyst concentration, until 240 min of reaction.

By considering all the determined kinetic parameters, the final kinetic expressions (9) and (10) were obtained for the reaction catalysed by [Fe(por)]$^+$ and by the complex Fe(por)$^+$–cq, respectively. In both cases there is a term

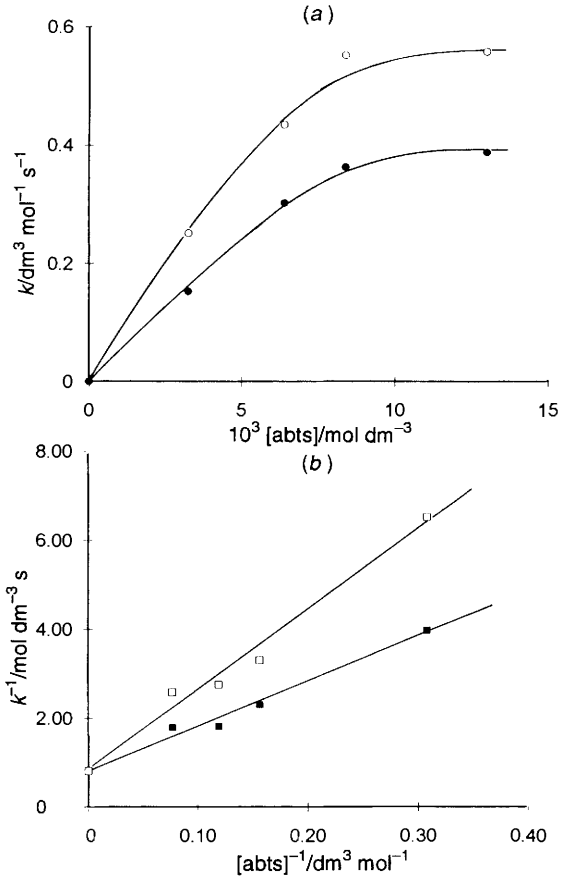



**Fig. 5** Dependence of the slopes in Fig. 4 on [abts]: (*a*) $k$ (second-order rate constant) *versus* [abts]; (*b*) $1/k$ *versus* 1/[abts] with (□) and without cq (■). Experimental conditions as in Fig. 4 with (●) and without cq (○)

$$\frac{v_i}{[\text{Fe(por)}]} = 22.4 + (1.29 \times 10^3)[\text{abts}] + \left(\frac{4.12 \times 10^5[\text{abts}]}{1.27 \times 10^{-2} + [\text{abts}]}\right)[H_2O_2] \quad (9)$$

$$\frac{v_i}{[\text{Fe(por)–cq}]} = 20.5 + (1.12 \times 10^3)[\text{abts}] + \left(\frac{4.05 \times 10^5[\text{abts}]}{2.21 \times 10^{-2} + [\text{abts}]}\right)[H_2O_2] \quad (10)$$

independent on either hydrogen peroxide or abts, and a second one dependent only on [abts].

*Reaction Intermediates.*—Two alternative mechanisms are possible for the reduction of a peroxide bond by iron(III) porphyrins.[32] Heterolytic cleavage of the O–O bond seems to be favoured in hydroxylic solvents with all classes of oxidants (peracids, hydroperoxides, and iodosylbenzenes),[33] while homolytic cleavage is predominant when a haem–$O_2R$ complex is produced at low temperature in aprotic solvents or in very basic solutions[34] (Scheme 1). Therefore, the formation of hydroxyl radicals in the studied system can be a major discriminant between these two types of mechanisms. To investigate the formation of free hydroxyl radicals as intermediates under our experimental conditions, EPR spin-trapping studies were performed in the presence of dmpo. The observed spectra in the presence and in the absence of the antimalarial drug are shown in Fig. 9 and can be ascribed to 5,5′-dimethyl-2-pyrrolidinone *N*-oxide, with $a_N$ = 0.72 mT

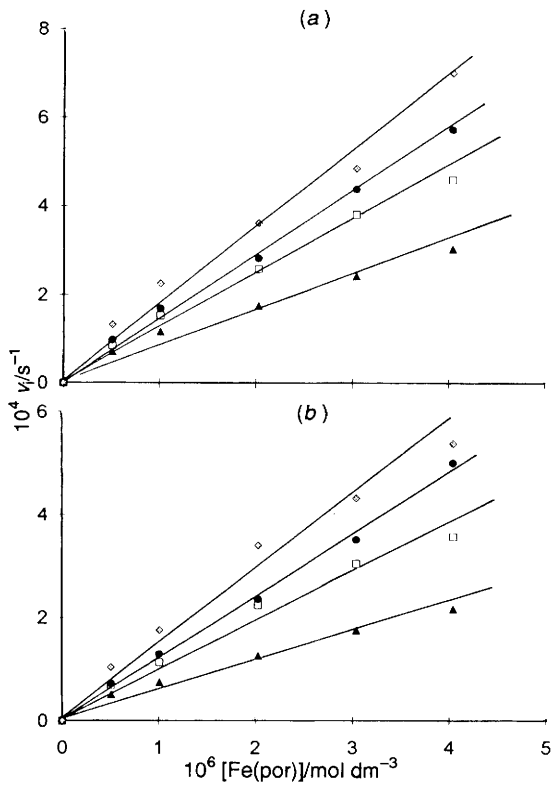
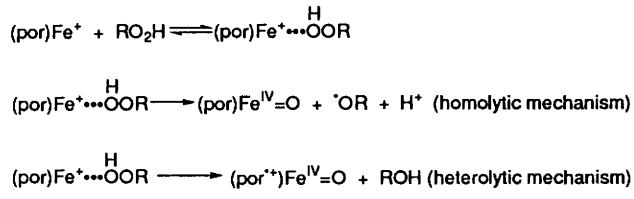



**Fig. 6** Dependence of the initial rate on [catalyst] at different [abts] values (*a*) in the absence and (*b*) in the presence of chloroquine ([Fe(por)]:[cq] = 2:1). $[H_2O_2]$ = 6.30 × 10$^{-4}$ mol dm$^{-3}$; pH 6.86 (0.025 mol dm$^{-3}$ phosphate), 303.1 K and $I$ = 0.10 mol dm$^{-3}$ 10$^3$[abts] = 3.25 (▲), 6.40 (□), 8.40 (●) or 13.0 mol dm$^{-3}$ (◇)




**Scheme 1** por represents the porphyrin group

and $a_H$ = 0.41 mT (2 H), and not to the dmpo•OH radical adduct ($a_N$ = 1.49 mT and $a_H$ = 1.49 mT).[35] Consequently, our spin-trap data do not support the homolytic scission mechanism. The species dmpox is usually formed in strongly oxidizing systems, such as horseradish peroxidase–$H_2O_2$ and haematin–1-methyl-1-phenylethylhydroperoxide.[36]

In the presence of chloroquine the adduct was formed in lower yields. From the corresponding areas it was possible to estimate the concentrations of dmpox as 7.10 × 10$^{-7}$ and 1.28 × 10$^{-7}$ mol dm$^{-3}$, respectively in the absence and in the presence of chloroquine, indicating an inhibitory effect of the order of 80%.

The reaction of abts with hydroxyl radicals occurs with 58% efficiency,[37] with a rate constant of $k$ = 1.2 × 10$^{10}$ dm$^3$ mol$^{-1}$ s$^{-1}$. However, ≈100% efficiency was observed in reactions with inorganic oxidizing radicals, such as $Br_2^{\bullet -}$, generated from bromide ions ($k$ = 2 × 10$^9$ dm$^3$ mol$^{-1}$ s$^{-1}$). Therefore, increases in the abts$^{\bullet +}$ formation in the presence of bromide can be indicative of the scavenging of hydroxyl radicals by those ions, according to equations (11) and (12). This effect has been

$$^\bullet OH + 2Br^- \longrightarrow Br_2^{\bullet -} + OH^- \quad (11)$$

$$Br_2^{\bullet -} + \text{abts} \longrightarrow \text{abts}^{\bullet +} + 2Br^- \quad (12)$$

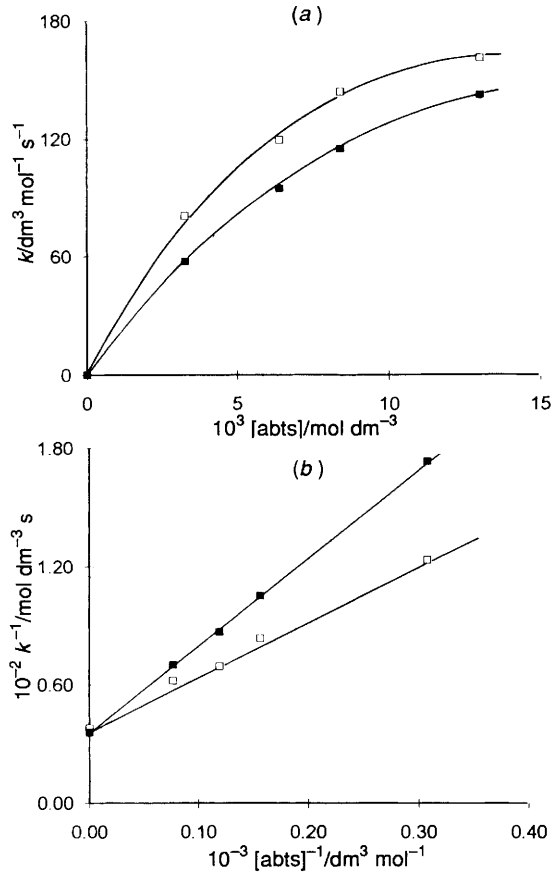



**Fig. 7** Dependence of the slopes in Fig. 6 on [abts] in the presence (■) and absence of cq (□): (*a*) $k$ (second-order rate constant) *versus* [abts]; (*b*) $1/k$ *versus* 1/[abts]. Experimental conditions as in Fig. 6

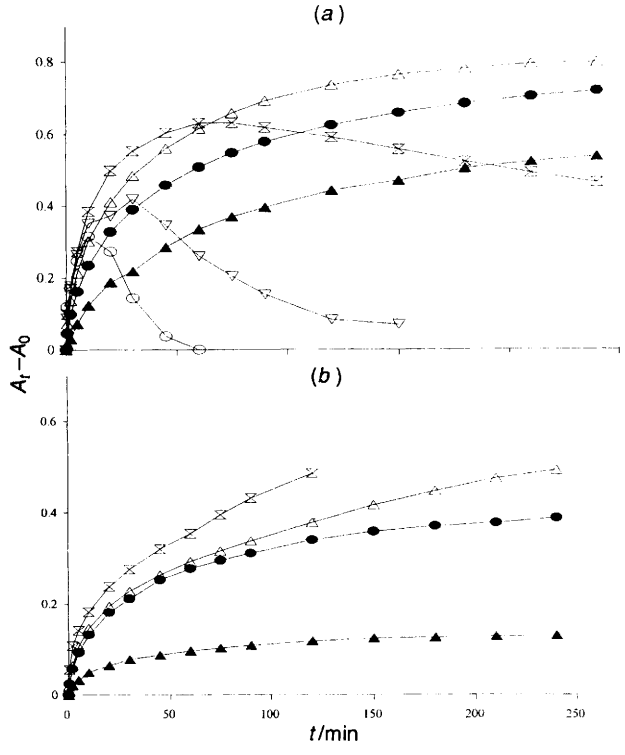



**Fig. 8** Formation and decay of abts$^{\bullet+}$ at different haem concentrations, monitored at 660 nm, at pH 6.86 (0.025 mol dm$^{-3}$ phosphate), 303.1 K and $I$ = 0.10 mol dm$^{-3}$ in NaCl, (*a*) in the absence and (*b*) in the presence of cq. [$H_2O_2$] = (8.20 ± 0.20) × $10^{-4}$ mol dm$^{-3}$, [abts] = 2.63 × $10^{-3}$ mol dm$^{-3}$ and [cq] = ½[Fe(por)]. $10^6$-[Fe(por)] = (*a*) 0.32 (▲), 2.23 (●), 9.40 (△), 31.5 (⊠), 94.7 (▽) or 160 (○); (*b*) 0.32 (▲), 2.23 (●), 19.0 (△) or 160 mol dm$^{-3}$ (⊠)

used [31] as an argument to distinguish whether the intermediate formed in reactions of hydrogen peroxide is the hydroxyl radical or an iron(IV) species. Under our experimental conditions 0.10 mol dm$^{-3}$ $Br^-$ did not give any significant effect on the determined rate constant for abts$^{\bullet+}$ formation nor on the efficiency of the reaction (Table 1). These results taken together with the described EPR studies provide strong evidence for heterolytic cleavage of the O–O bond.

Peroxide cleavage by haem and haem proteins can result in haem oxidation and destruction, particularly in the absence of oxidizing substrates. Chloroquine prevented the destruction of the haem by hydrogen peroxide, as shown in Table 2, where the initial absorbance of the solutions and the corresponding absorbance after 75 min of reaction are compared, for both the [Fe(por)]$^+$ and Fe(por)$^+$–cq catalysts. In the absence of chloroquine a more pronounced degradation of the haem ring was observed, as monitored by the Soret band at 364 nm. By contrast, in the presence of the antimalarial drug, the corresponding band at 390 nm was preserved. In the range of haem concentrations used [(10–35) × $10^{-6}$ mol dm$^{-3}$], the average decrease in the Soret band observed in the absence of cq was *ca.* 2.5 times larger than that observed in its presence. In other words, the inhibition by the antimalarial in the degradative oxidation of the catalyst, as calculated in Table 2, is *ca.* 60%.

## Discussion

It is well known the tendency of protein-free haems to undergo dimerization in aqueous solution. As the dimerization constant for ferriprotoporphyrin IX is large [5] ($K_d$ = 4.50, at 25 °C and $I$ = 0.1 mol dm$^{-3}$) and dependent on the pH [equation (13)] it

$$2[\text{Fe(por)}]^+ + H_2O \overset{K_d}{\rightleftharpoons} [\text{(por)Fe(OH)Fe(por)}]^+ + H^+ \quad (13)$$

is reasonable to assume that under our experimental conditions most of the catalyst is in the form of a dimer, probably bridged by an oxo or hydroxo group.[38] The method used in its preparation, by previous dissolution on NaOH, also favours the dimerization.[23] Earlier studies estimated the proportion of monomer in solutions of protoferrihaem as about 0.1–0.01%.[39] Therefore, the predominant species in the studied system is probably a dinuclear bridged species.

A dinuclear intermediate $Fe^{IV}_2O$ has recently been assigned as a functional analogue of enzyme compound I in the case of deuteroferrihaem [(3,7,12,17-tetramethylporphyrin-2,18-dipropanoato)iron(III)].[40] This species subsequently undergoes one-electron redox processes, regenerating the initial catalyst.

The kinetic results described here, however, indicate that the main catalytic active species in the oxidation of abts is the monomeric haem (see Scheme 2), as suggested before by Jones *et al.*[5] in the oxidation of hydroquinone by both haem and modified haems. In the presence of the antimalarial chloroquine, a second equilibrium (14) should be considered,

$$[\text{(por)Fe(OH)Fe(por)}]^+ + \text{cq} \overset{K_f}{\rightleftharpoons} \{[\text{(por)Fe(OH)Fe(por)}]\cdot\text{cq}\}^+ \quad (14)$$

leading to a decrease in the monomer concentration in the dimerization equilibrium (13) and forming a 2:1 adduct (or highly polymeric species of dimeric haem with intercalated chloroquine). The equilibrium constant $K_f$ = 2.86 × $10^8$ dm$^3$ mol$^{-1}$ was determined by Chou *et al.*[13] based on equilibrium dialysis studies. Consequently, the inhibitory effect of

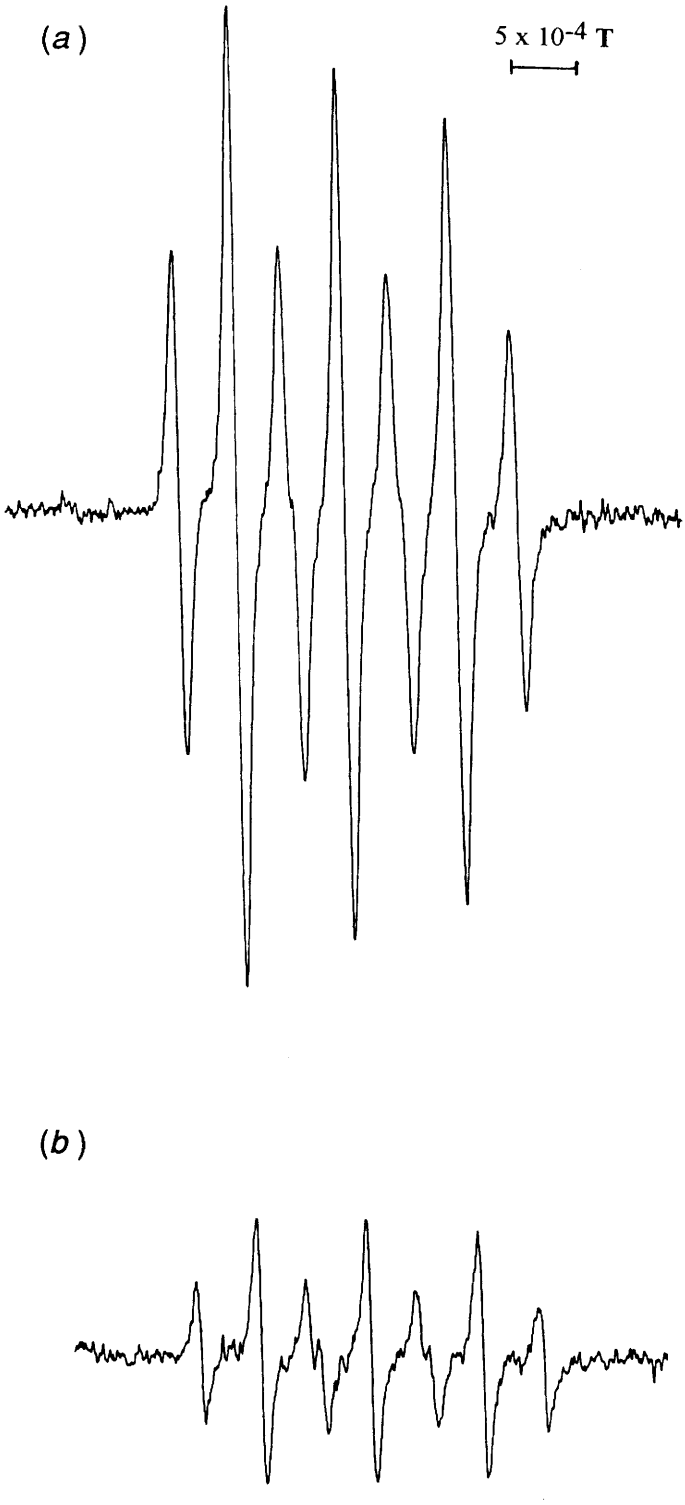



**Fig. 9** The EPR spectra obtained, by using dmpo as spin trap, in the reaction of hydrogen peroxide with ferriprotoporphyrin IX, in the absence (*a*) and in the presence (*b*) of chloroquine. $[H_2O_2]$ = 11.30 × $10^{-3}$ mol $dm^{-3}$, [Fe(por)] = 4.24 × $10^{-5}$ mol $dm^{-3}$, [cq] = 2.10 × $10^{-5}$ mol $dm^{-3}$, [dmpo] = 0.14 mol $dm^{-3}$, at pH 6.86 (0.025 mol $dm^{-3}$ phosphate), 303.1 K and $I$ = 0.10 mol $dm^{-3}$ in NaCl

chloroquine on the oxidation of abts can be explained by the corresponding decrease in the concentration of the monomeric haem in the presence of the antimalarial. Under stoichiometric conditions, and admitting that at least 99% of the haem is present as the complex haem–chloroquine, an inhibition of 34% can be calculated (Table 3), a value which is very close to the 28% verified experimentally. It is important to notice that under the experimental conditions used in the EPR measurements, with [catalyst] of the order $10^{-5}$ mol $dm^{-3}$, an inhibitory effect of *ca.* 80% was verified (Fig. 9), in accordance with the calculated value of 84% (Table 3).

Based on the experimental data (Fig. 9, Table 3), a heterolytic mechanism for the peroxidase activity of the $[Fe(por)]^+$, in the presence of chloroquine, was proposed. To explain the dependence of the rate constants on [abts] (Figs. 5 and 7), the formation of a complex between abts and the haem is assumed, which also presents catalytic activity with respect to hydrogen peroxide (Scheme 2), as has been described in peroxidase systems.[30,41]

By steady-state treatment of the mechanism shown in Scheme 2, assuming [abts] > $[H_2O_2]$ ≫ [catalyst], and that the reactions between abts and species **I** and **II** are very fast compared with the other steps, the rate law (15) can be derived.

$$v_i = \frac{\{A + B[\text{abts}]\}[H_2O_2][\text{Fe(por)}]_T}{1 + K[\text{abts}] + (A + B[\text{abts}])[H_2O_2]} \quad (15)$$

This equation is in agreement with the verified first-order dependence on [catalyst], and observed saturation effects with increasing $[H_2O_2]$ and [abts]. In this expression, according to Scheme 2, $A = k_1k_2/(k_{-1} + k_2 + k_3)$, $B = k_4k_5K/(k_{-4} + k_5)$, $K$ = equilibrium constant for the formation of the abts–haem complex and $[Fe(por)]_T$ is the total concentration of the haem catalyst.

An analogous catalytic cycle, involving complexation of substrates to the haem octapeptide MP-8 and resulting in inhibition of its peroxidatic activity, was recently described. In this case, a kinetic expression similar to (15) was verified, for the oxidation of phenols and aniline.[42]

As demonstrated by the kinetic data obtained (Fig. 2), basically the same rate law applies either in the absence or in the presence of chloroquine. The main effect of the antimalarial appears to be displacement of the dimerization equilibrium of the haem, through formation of a very stable complex. This complex efficiently protects the porphyrin ring from attack by peroxide, decreasing the rate constants, and precluding the degradative oxidation of the more active monomeric catalyst (Table 2).

The experimental rate law, (16) and (17) showed a first term

$$v_i = (k_0 + k[H_2O_2])[\text{catalyst}] \quad (16)$$

$$v_i = \left\{(a + b[\text{abts}]) + \left(\frac{c[\text{abts}]}{d + [\text{abts}]}\right)[H_2O_2]\right\}[\text{catalyst}] \quad (17)$$

independent of both $[H_2O_2]$ and [abts], which can be attributed to the reaction under non-saturation pseudo-first-order conditions. In this case the reaction of abts with oxidized haem species must be much faster than any other in the catalytic cycle. A limiting value of $a = 21 \pm 3$ $s^{-1}$ was estimated from the results, independent of the presence of chloroquine. This term may also reflect the catalytic activity of the haem dimer which has been shown to be much lower than that of the monomer[6,43] in the absence of chloroquine.

A second term, dependent only on [abts], could be interpreted as a result of the direct autooxidation to $abts^{\bullet+}$, catalysed by both the haem and the haem-chloroquine complex, although the abts–$abts^{\bullet+}$ system has been described as insensitive to oxygen.[32] Experimental data for this very slow reaction, verified without the addition of hydrogen peroxide, for [abts] = 2.60 × $10^{-3}$ mol $dm^{-3}$, gave a value of $b$[abts] = 3.6 $s^{-1}$. Calculated by expression (17), the corresponding value was 4.4 $s^{-1}$.

By considering the kinetic parameters determined, good agreement was observed when comparing the intercept and the slope in Fig. 2 with the corresponding values calculated by

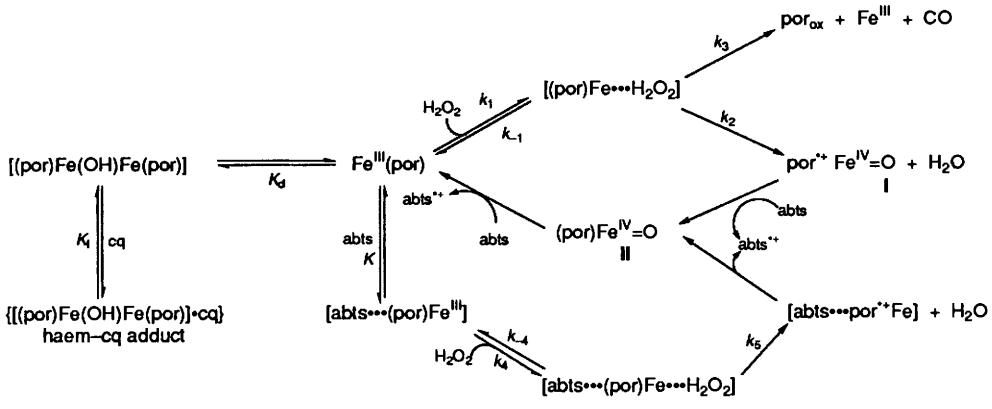
**Table 1** Effect of bromide ions on the haem-catalysed oxidation of abts by hydrogen peroxide, in the presence and absence of chloroquine: [Fe(por)] = $3.17 \times 10^{-6}$ mol dm$^{-3}$, [cq] = $1.62 \times 10^{-6}$ mol dm$^{-3}$, [$H_2O_2$] = $(8.40 \pm 0.20) \times 10^{-4}$ mol dm$^{-3}$, [abts] = $2.63 \times 10^{-3}$ mol dm$^{-3}$, 303.1 K and $I$ = 0.10 mol dm$^{-3}$ NaCl

| | $10^4 k_{obs}/s^{-1}$ ($\pm 0.08$) | | % Efficiency | |
|---|---|---|---|---|
| Catalyst | Without bromide | [$Br^-$] = 0.10 mol dm$^{-3}$ | Without bromide | [$Br^-$] = 0.10 mol dm$^{-3}$ |
| [Fe(por)]$^+$ | 2.75 | 2.55 | 8.97 | 9.38 |
| Fe(por)$^+$–cq | 2.37 | 2.25 | 6.25 | 6.46 |

**Table 2** Degradative oxidation of the haem ring by hydrogen peroxide in the absence and presence of chloroquine at 303.1 K, in 0.10 mol dm$^{-3}$ NaCl, pH 6.86 and [$H_2O_2$] = $2.04 \times 10^{-3}$ mol dm$^{-3}$. $\Delta A = A_0 - A_{75\ min}$

| | Absence of chloroquine | | [cq] = [Fe(por)]/2 | | Degradative oxidation of catalyst | |
|---|---|---|---|---|---|---|
| $10^5$[Fe(por)]/ mol dm$^{-3}$ | $\Delta A$ after 75 min | % Decrease in $A$ | $\Delta A$ after 75 min | % Decrease in $A$ | Relative oxidation[a] | % Inhibition[b] |
| 1.05 | 0.288 | 65.9 | 0.145 | 38.5 | 0.503 | 49.7 |
| 1.43 | 0.361 | 60.1 | 0.157 | 30.8 | 0.435 | 56.5 |
| 1.91 | 0.448 | 53.7 | 0.191 | 27.5 | 0.426 | 57.4 |
| 2.54 | 0.587 | 51.6 | 0.225 | 23.9 | 0.383 | 61.7 |
| 3.18 | 0.637 | 45.3 | 0.218 | 18.8 | 0.342 | 65.8 |
| 3.50 | 0.678 | 44.8 | 0.230 | 18.2 | 0.339 | 66.1 |

[a] Calculated as $(\Delta A)_{cq}/(\Delta A)_{cq=0}$. [b] Inhibition in the degradative oxidation of the catalyst, calculated as the percentage of unaltered catalyst, in the presence of cq: $1 - [(\Delta A)_{cq}/(\Delta A)_{cq=0}]$.




**Scheme 2** Overall charges removed for clarity

**Table 3** Calculated values of the monomeric haem concentration and the corresponding decrease in the catalytic activity expected for different total concentrations of ferriprotoporphyrin IX and chloroquine

| $10^6$[Fe(por)]/ mol dm$^{-3}$ | $10^6$[cq]/ mol dm$^{-3}$ | $10^8$[Monomeric haem][a]/ mol dm$^{-3}$ | % Inhibition expected[b] |
|---|---|---|---|
| 1.00 | 0.50 | 7.61 | 34.4 |
| 2.00 | 1.00 | 8.71 | 48.2 |
| 3.00 | 1.50 | 9.17 | 55.7 |
| 4.00 | 2.00 | 9.45 | 60.2 |
| 5.00 | 2.50 | 9.61 | 64.4 |
| 10.0 | 5.00 | 9.97 | 74.1 |
| 20.0 | 10.0 | 10.1 | 81.6 |
| 30.0 | 15.0 | 10.2 | 84.8 |

[a] Calculated by considering the dimerization equilibrium of haem ($K_d$ = 4.50), and the association equilibrium of haem and chloroquine ($K_f = 2.86 \times 10^8$ dm$^3$ mol$^{-1}$), as specified in the text, equations (13) and (14). [b] Estimated by comparison of the calculated concentrations of the monomeric species in the presence and absence of chloroquine: $1 - ([\text{monomeric haem}]_{cq}/[\text{monomeric haem}]_{cq=0})$.

equation (17): 36.6 s$^{-1}$ and $1.64 \times 10^5$ dm$^3$ mol$^{-1}$ s$^{-1}$, respectively. Analogous data in the literature, based also on $(dA/dt)_{t=0}$, for the oxidation of abts catalysed by MP-8 indicated[30] values of the intercept $k_0 = 2.963 \times 10^3$ [MP-8] and a slope = $1.188 \times 10^8$ [MP-8], emphasizing that the protein moiety modulates but does not determine the haem peroxidase activity. The rate constants are $\approx 10^3$ times higher when the catalyst is MP-8 instead of [Fe(por)]$^+$.

From the present data it is concluded that the formation of the 2:1 adduct Fe(por)$^+$–cq is responsible for the observed inhibitory effect of the antimalarial on the peroxidatic activity of Fe(por)$^+$ in aqueous solution. The adduct may also account for the observed protection of the haem ring from degradative oxidation since in the presence of chloroquine the destruction of the catalyst is minimized. Therefore, although chloroquine inhibits the peroxidative activity of haem in aqueous solution, it can protect the catalyst from degradation mediated by water-soluble peroxides. In this case, higher concentrations of haem will be available to interact with critical cellular targets such as membranes[20] and chromatin[44] which are sensitive to haem-mediated oxidative reactions. The fact that chloroquine can increase the concentration of toxic haem either by liberating haem to critical cellular targets or by inhibiting haem polymerase[21,45] further supports the hypothesis that this antimalarial drug may act by increasing haem-mediated oxidative stress of the malarial parasites.

**Acknowledgements**

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Pesquisa e Desenvolvimento Tecnològico (CNPq). We are grateful to Mr. Silvio S. Zavan for assistance in obtaining the EPR spectra.

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*Received* 13*th March* 1995; *Paper* 5/01568E