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ONE-ELECTRON REDUCTION OF THE ANTIMALARIAL DRUG PRIMAQUINE, STUDIED BY PULSE RADIOLYSIS

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One-electron reduction of the antiparasitic drug primaquine has been studied by pulse radiolysis. Primaquine is reduced by the hydrated electron at neutral pH with a rate constant of $(2.47 \pm 0.1) \times 10^{10} dm^3 mol^{-1} s^{-1}$. Reduction by formate and isopropanol radicals is relatively slow ($\leqslant 10^7 dm^3 mol^{-1} s^{-1}$) at neutral pH, but increases in rate with decreasing pH on protonation of the quinoline moiety. The one-electron reduction product form reaction of the hydrated electron with primaquine at neutral pH reacts with O_2, benzyl viologen and NAD^+ with rates of $(1–2.3) \times 10^9 dm^3 mol^{-1} s^{-1}$. The relevance of these observations to the mechanisms proposed by Thornalley *et al.* (*Biochem. Pharmacol.* 32, 357, (1983)) for oxygen free radical generation in solutions of NADPH and primaquine and the antiparasitic action of the drug is discussed.

KEY WORDS: Primaquine, antiparasitic drug, one-electron reduction, pulse radiolysis.

INTRODUCTION

Many parasitic infections are susceptible to reactive oxygen species such as hydrogen peroxide, superoxide radicals and hydroxyl radicals.[1] The normal host response of phagocytes to these infections depends at least in part to the generation of such species[2,3] and many antiparasitic drugs appear to generate reduced forms of dioxygen as their mechanism of action.[4,5] Enhancement of oxidative stress, either through addition of hydroperoxides[6] or by inhibition of cellular antioxidant capacity,[1] is a new approach to the chemotherapy of parasitic infections.

Primaquine [8-(4-amino-1-methylbutylamino)-6-methoxyquinoline, PQ] is an antimalarial drug[7] which is also active against Leishmanial[8] and trypanosomal[5] infections. Within the red blood cell, primaquine stimulates the hexose monophosphate shunt as a result of oxidation of NADPH and leads to the generation of H_2O_2.[9,10] Subsequent reactions of hydrogen peroxide, possibly through generation of hydroxyl radicals in a Fenton-like reaction, have been proposed as the origin of the antimalarial effect of primaquine.[6] Although some authors have suggested that products of primaquine metabolism may also be responsible for the observed therapeutic effects,[11] there is now direct evidence from *in vitro* spin trapping experiments that aerobic incubation of primaquine and NAD(P)H in solution results in production of both superoxide and hydroxyl radicals.[12–14] Thornalley, Stern and Bannister[12] reported that NADPH and PQ form a charge transfer complex detected by quenching of NADPH fluorescence. This complex is proposed to be oxidised by molecular oxygen to give superoxide and a reduced primaquine radical ($PQH^{\cdot}$). The formation of a complex between NAD(P)H and PQ was confirmed using circular dichroism by Augusto *et al.*,[13] who

also showed that oxyhaemoglobin causes enhanced oxygen consumption and free radical generation in solutions containing NAD(P)H and PQ. Hydroxyl radical generation involving PQ has also been demonstrated in extracts of *Trypanosoma cruzi*.[14] These observations support the previous suggestion[6] that the hydroxyl radical is the ultimate toxic product of NAD(P)H oxidation mediated by PQ.

The overall scheme for free radical generation in solutions of PQ and NAD(P)H proposed by Thornalley *et al*,[12] and supported by Augusto *et al*.[13,14] is decribed by reactions (1) to (8):-

$$\text{NAD(P)H} + \text{PQ} \rightarrow (\text{NAD(P)}\ldots\text{H}\ldots\text{PQ}) \quad (1)$$

$$(\text{NAD(P)}\ldots\text{H}\ldots\text{PQ}) + \text{O}_2 \rightarrow \text{NAD(P)}^+ + \text{PQH}^{\cdot} + \text{O}_2^{\cdot-} \quad (2)$$

$$\text{PQH}^{\cdot} + \text{O}_2 \rightarrow \text{PQ} + \text{H}^+ + \text{O}_2^{\cdot-} \quad (3)$$

$$\text{O}_2^{\cdot-} + \text{H}_2\text{O}_2 \rightarrow \text{OH}^- + {}^{\cdot}\text{OH} + \text{O}_2 \quad (4)$$

$$\text{PQH}^{\cdot} + \text{H}_2\text{O}_2 \rightarrow \text{PQ} + {}^{\cdot}\text{OH} + \text{H}_2\text{O} \quad (5)$$

$$(\text{NAD(P)}\ldots\text{H}\ldots\text{PQ}) + \text{O}_2^{\cdot-} + 2\text{H}^+ \rightarrow \text{NAD(P)}^+ + \text{PQH}^{\cdot} + \text{H}_2\text{O}_2 \quad (6)$$

$$2\text{PQH}^{\cdot} \rightarrow \text{PQ} + \text{PQH}_2 \quad (7)$$

$$2\text{O}_2^{\cdot-} + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad (8)$$

Although the primaquine radical, $\text{PQH}^{\cdot}$, plays an essential this proposed mechanism, it could not be detected using electron spin resonance spectroscopy by either Thornalley *et al*.[12] or Augusto *et al*.[13] due to the anticipated high reactivity of this radical and its resulting low steady state concentration in static incubations of PQ and NAD(P)H.

It is now reported that a one-electron reduced product of PQ may be observed in suitable pulse radiolysis experiments, and that this technique allows reactions of the radical species to be studied.

MATERIALS AND METHODS

Primaquine diphosphate (Sigma), isopropanol (Aldrich, Gold Label), benzyl viologen (BDH) and NAD^+ (Boehringer) were used as received. t-Butanol (Fluka, puriss.) was recrystallised before use. Other reagents were AnalaR grade where possible. Solutions were prepared in baked glassware, using water purified by a Milli Q system (Millipore Ltd.). Before irradiation solutions were bubbled with the appropriate gas for at least 20 minutes. Varying oxygen concentrations in solutions were obtained by purging with mixtures of N_2 and air, measured using flow meters (BOC).

Pulse radiolysis was undertaken with the facility at the University of Salford, employing a Febetron 705 which produced 20ns pulses of 1.5 MeV electrons, Solutions were pulse irradiated in a 2 cm pathlength silica cell thermostatted to 25C. Appropriate optical filters were inserted in the monitoring light beam to minimise photodecomposition of the sample. Dosimetry was performed with air saturated KSCN solutions ($10^{-2}\,\text{mol}\,\text{dm}^{-3}$) taking $\varepsilon\ [(\text{SCN})_2^{\cdot-}]_{480\,\text{nm}} = 7.6 \times 10^3\,\text{dm}^3\,\text{mol}^{-1}\,\text{cm}^{-1}$ and $G\ [(\text{SCN})_2^{\cdot-}] = 0.29\,\mu\text{mol}\,\text{dm}^{-3}\,\text{Gy}^{-1}$

One-electron reduction of primaquine was studied in three systems. Reaction of e_{aq}^- was studied in deaerated (N_2-purged) solutions containing t-butanol as a hydroxyl radical scavenger. The radical formed from t-butanol (reaction 10) was found to be

unreactive over the timescale of the experiments reported. Reduction by isopropanol radicals (IPA˙) was observed in N_2O-saturated solutions containing isopropanol (reactions (11) and (12)). The formate radical anion was similarly generated in N_2O-saturated solutions containing formate (reactions (10) and (13)):-

$$H_2O \rightsquigarrow {}^{\cdot}OH, e^-_{aq}, H^{\cdot} \quad (9)$$

$$^{\cdot}OH + (CH_3)_3COH \rightarrow {}^{\cdot}CH_2(CH_3)_2COH + H_2O \quad (10)$$

$$e^-_{aq} + N_2O \rightarrow {}^{\cdot}OH + OH^- + N_2 \quad (11)$$

$$^{\cdot}OH + (CH_3)_2CH\text{-}OH \rightarrow H_2O + (CH_3)_2{}^{\cdot}C\text{-}OH \ (IPA^{\cdot}) \quad (12)$$

$$^{\cdot}OH + HCO_2^- \rightarrow H_2O + CO_2^- \quad (13)$$

RESULTS AND DISCUSSION

Reactions of Reducing Radicals with Primaquine

The rates of reaction of three reducing radical species (e^-_{aq}, CO_2^- and IPA˙) with primaquine were studied by pulse radiolysis. The rate of reaction of e^-_{aq} was determined by measurement of the decay of the e^-_{aq} absorption at 700 nm over a range of primaquine concentrations (0–20 μmol dm^{-3}) as shown in the inset to Figure 1. At pH 7.3 a second order rate constant of (2.47 ± 0.1) × 10^{10}dm^{3}mol^{-1}s^{-1} was determined, whereas at pH 9.1 a value of (1.8 ± 0.1) × 10^{10}dm^{3}mol^{-1}s^{-1} was obtained.

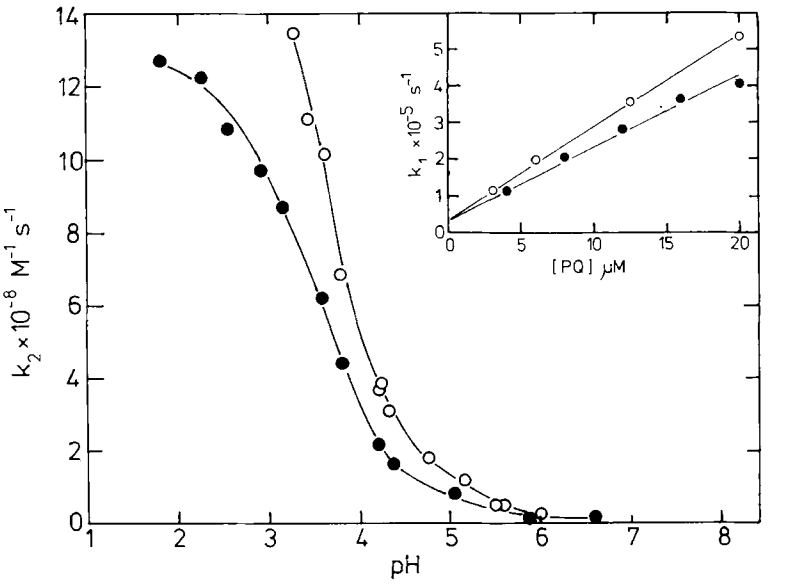

FIGURE 1 Effect of pH on the second order rate constants for reaction of CO_2^- (○) and IPA˙ radicals (●) with primaquine. *Inset*:- First order rate constants versus primaquine concentration for the decay of the hydrated electron in solutions at pH 7.3 ± 0.1 (○) and pH 9.1 ± 0.1 (●).

Spectrophotometric titration of primaquine in solution gave a value of 3.4 ± 0.1 for the pK_a for protonation of the nitrogen atom in the quinoline ring, close to pK_a's reported for similar 8-aminoquinoline analogues.[15] On the basis of published rate constants for e_{aq}^- reactions,[16] the aliphatic side chain of primaquine is expected to have a low ($< 10^8 dm^3 mol^{-1} s^{-1}$) reactivity with e_{aq}^-. These results therefore indicate that the unprotonated 8-aminoquinoline ring of primaquine is the site for reduction by e_{aq}^- at a diffusion-controlled rate.

Rate constants for reactions of CO_2^- and IPA$^\cdot$ with primaquine were obtained by measurement of the rate of formation of the transient product absorption at 480–500 nm. At neutral pH the reactions of both of these radicals are relatively slow ($k \leqslant 10^7 dm^3 mol^{-1} s^{-1}$). The rate constants for reactions of IPA$^\cdot$ and CO_2^- with primaquine increase with decreasing pH, as shown in Figure 1. Measurements of the rate of reaction of CO_2^- with PQ are limited to pH $\geqslant$ 3.3 due to competition for reaction of e_{aq}^- with H^+ and N_2O in acidic solutions, and the low rate constant for scavenging of $^\cdot OH$ by formic acid.[17,18] The second order rate constant for reaction of IPA$^\cdot$ with primaquine increases to $1.3 \times 10^9 dm^3 mol^{-1} s^{-1}$ at pH 1.8. The pH-dependence of the rate constant for reaction of IPA$^\cdot$ with PQ follows a curve with $pK_a = 3.5 \pm 0.1$, corresponding to protonation of quinoline ring. These results are similar to those obtained for reactions of CO_2^- and IPA$^\cdot$ with substituted pyridines,[18] although e_{aq}^- is

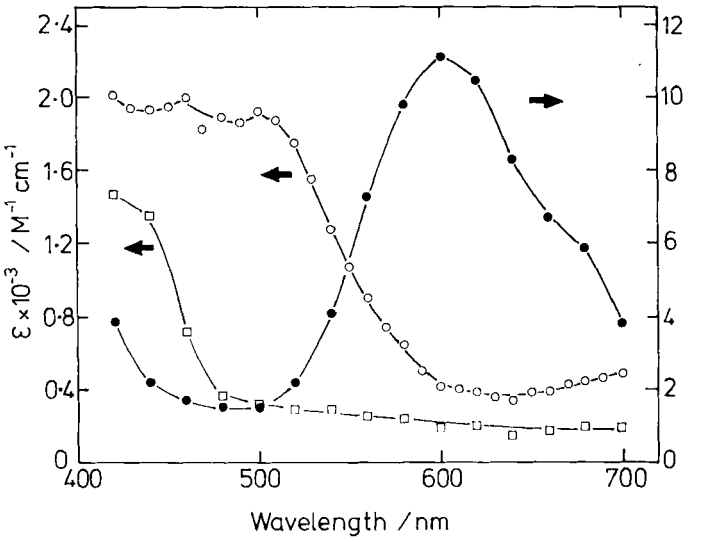

FIGURE 2 Transient absorption spectra from pulse radiolysis of primaquine solutions. Extinction coefficents were calculated assuming the yield of e_{aq}^- to be 0.3 μmol dm^{-3}Gy^{-1}.[17] All solutions were deaerated by bubbling with N_2. ○:- Transient spectrum from reaction of e_{aq}^- with PQ measured 5 μs after the pulse in solutions containing PQ (0.5 mmol dm^{-3}), t-butanol (0.425 mol dm^{-3}) at pH 6.9. ●:- Transient spectrum measured following reaction of the PQ radical with BV^{2+}. Measured 30 μs after the pulse in solutions containing PQ (2 mmol dm^{-3}), BV^{2+} (55 μmol dm^{-3}), t-butanol (0.425 mol dm^{-3}) and phosphate (20 mmol dm^{-3}) at pH 6.8. □:- Transient spectrum measured following reaction of the PQ radical with NAD^+. Measured 20 μs after the pulse in a solution containing PQ (2 mmol dm^{-3}), NAD^+ (200 μmol dm^{-3}), t-butanol (0.425 mol dm^{-3}) and phosphate (20 mmol dm^{-3}) at pH 7.0.

significantly more reactive with PQ than with pyridine ($k = 10^9 dm^3 mol^{-1} s^{-1}$) at neutral pH.

Due to the low reactivity of $IPA^{\cdot}$ and $CO_2^{\cdot-}$ with PQ at neutral pH, subsequent experiments to study the one-electron reduced product from PQ were undertaken following reaction of PQ with e_{aq}^-. The transient absorption spectrum, uncorrected for the bleaching of the primaquine ground state absorption ($\varepsilon < 100 M^{-1} cm^{-1}$ for $\lambda > 420$ nm at pH 7), formed on reaction of e_{aq}^- in deaerated solutions containing PQ (0.5 mmol dm^{-3}) and t-butanol (0.425 mol dm^{-3}) at pH 6.9 is shown in Figure 2. The spectrum contains a broad maximum between 440 nm and 520 nm with $\varepsilon_{max} \sim 2 \times 10^3 dm^3 mol^{-1} cm^{-1}$, together with an together with an indication of a weaker absorption maximum at $\lambda > 700$ nm. Measurements at wavelengths shorter than 400 nm were precluded by the strong absorbance of PQ solutions at those wavelengths.

Reactions of the Reduced Primaquine Radical

The reaction between benzyl viologen (BV^{2+}) and the radical species produced by reduction of PQ with e_{aq}^- was studied by pulse radiolysis. The rate of this reaction at pH 6.8 was measured by following the formation of the $BV^{+\cdot}$ radical cation at 600 nm in deaerated solutions containing PQ (2 mmol dm^{-3}), BV^{2+} (0–0.274 mmol dm^{-3}) and t-butanol (0.425 mol dm^{-3}). The results shown in Figure 3A give a second order rate constant for this reaction of $(2.0 \pm 0.1) \times 10^9 dm^3 mol^{-1} s^{-1}$ at pH 6.8. The identity of $BV^{+\cdot}$ as a product of this reaction is confirmed by the spectrum in Figure 2, measured 20 μs after pulse radiolysis of a deaerated solution containing PQ (2 mmol dm^{-3}), BV^{2+} (55 μmol dm^{-3}) and t-butanol (0.425 mol dm^{-3}), which is identical to that for $BV^{+\cdot}$ reported previously.[19] In a similar solution containing PQ and BV^{2+} (274 μmol dm^{-3}), the extinction coefficient calculated for $BV^{+\cdot}$ is $\sim 1.4 \times 10^4 dm^3 mol^{-1} cm^{-1}$, which is similar to that quoted by Wardman and Clarke,[19] indicating essentially quantitative electron transfer from the reduced PQ radical to BV^{2+}.

The reaction of the oxygen with the radical formed on one-electron reduction of PQ by e_{aq}^- was also studied by pulse radiolysis. The rate of decay of the transient absorption due to the PQ radical at 500 nm was measured in solutions containing PQ (0.5 mmol dm^{-3}) and oxygen (0–108 μmol dm^{-3}) at pH 6.9. The variation in first order rate for the decay of the PQ radical absorption with oxygen concentration, shown in Figure 3B, gives a second order rate constant of $(2.31 \pm 0.25) \times 10^9 dm^3 mol^{-1} s^{-1}$, assuming air saturated water contains 270 μmol dm^{-3} O_2. A similar experiment, except at pH 4.0 and a PQ concentration of 2 mmol dm^{-3}, gave the same rate constant $(2.18 \pm 0.28) \times 10^9 dm^3 mol^{-1} s^{-1}$).

The rate of reaction between NAD^+ and the radical formed on reduction of PQ by e_{aq}^- was measured by pulse radiolysis of deaerated solutions containing PQ (2 mmol dm^{-3}), NAD^+ (0–250 μmol dm^{-3}) and t-butanol (0.425 mol dm^{-3}) at pH 7.0. The reaction was observed from the decay of the PQ radical absorption at 500 nm. The results shown in Figure 3A give a second order rate constant of $(1.01 \pm 0.04) \times 10^9 dm^3 mol^{-1} s^{-1}$. The transient absorption measured at 20 μs after the decay of the PQ radical absorption in deaerated solutions containing both PQ and NAD^+ is shown in Figure 2. Whilst the strong absorbance of the PQ below 420 nm prevented measurement of the complete spectrum of this product, the spectrum is sufficiently similar to that of $NAD^{\cdot}$[20] within the spectral region examined to suggest that a one electron transfer occurs between the PQ radical and NAD^+, leading to $NAD^{\cdot}$.

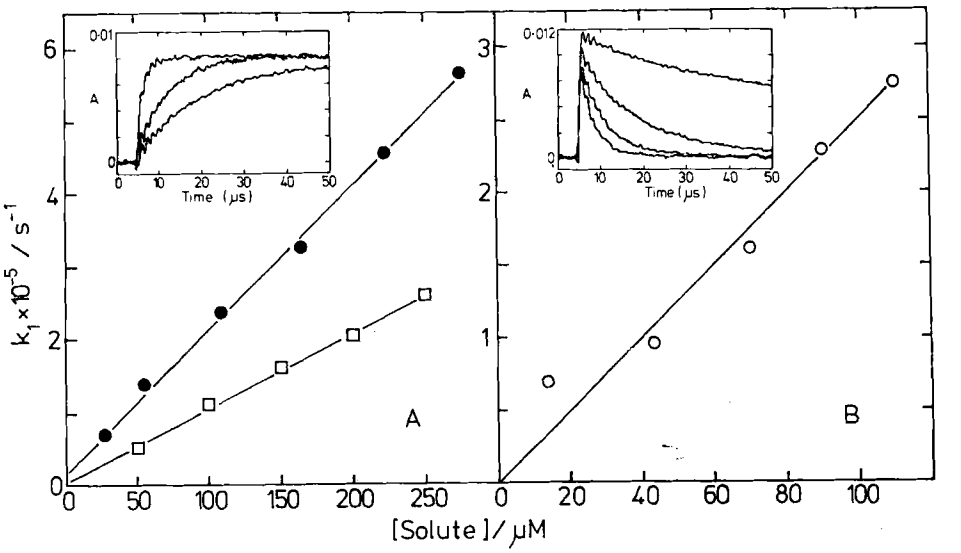

FIGURE 3 First order rate constants versus solute concentrations for reactions fo the PQ radical with BV^{2+}, NAD^+ and O_2. A; ●:- Reaction of the PQ radical with BV^{2+} measured in N_2-purged solutions containing PQ (2 mmol dm^{-3}), BV^{2+} (27.4–274 μmol dm^{-3}), t-butanol (0.425 mol dm^{-3}) and phosphate (20 mmol dm^{-3}) at pH 6.8 ± 0.1. The reaction was monitored by formation of the BV^{+} absorption at 600 nm, as shown in the inset for solutions containing (top to bottom) 274, 55 and 27.4 μmol dm^{-3} BV^{2+} (Dose = 1.02 Gy/pulse). □:- Reaction of the PQ radical with NAD^+ measured in N_2-purged solutions containing PQ (2 mmol dm^{-3}), NAD^+ (50–250 μmol dm^{-3}), t-butanol (0.425 mol dm^{-3}) and phosphate (20 mmol dm^{-3}) at pH 7.0 ± 0.1. The reaction was monitored by decay of the PQ radical absorption at 500 nm. B; Reaction of the PQ radical with O_2 in solutions containing PQ (0.5 mmol dm^{-3}), O_2 (17–108 μmol dm^{-3}), t-butanol (0.425 mol dm^{-3}) and phosphate (10 mmol dm^{-3}) at pH 6.9 ± 0.1. The reaction was monitored by decay of the PQ radical absorption at 500 nm as shown in the inset for solutions containing (top to bottom) 0, 17, 70 and 108 μmol dm^{-3} O_2 (Dose = 9.6 Gy/pulse).

The observation of electron transfer from the PQ radical to both NAD^+ and BV^{2+} with rates of (1–2) × 10^9 dm^3 mol^{-1} s^{-1}, strongly suggests that the reaction of O_2 with this radical is also a one-electron transfer, rather than one of addition to give a peroxy radical. This provides strong support, together with an evaluation of the rate constant, for reaction (3) above in the overall mechanism of PQ-mediated NAD(P)H oxidation proposed by Thornalley *et al.*[12] The present results do not indicate whether the one-electron reduced radical of PQ exists as $PQ^{\cdot-}$ or $PQH^{\cdot}$. Previous results for the related pyridinyl radical[21] show it to have $pK_a > 14$, corresponding to formulation of the primaquine radical as $PQH^{\cdot}$ by Thornalley *et al.*[12] However, the near diffusion-controlled rates of electron transfer from the PQ radical to NAD^+, BV^{2+} and O_2 suggest it to be the radical anion ($PQ^{\cdot-}$), since reactions involving radical deprotonation/protonation coupled to electron transfer tend to be significantly reduced in rate.[22] Further experiments are in progress to resolve this question. The observation that the primaquine radical undergoes electron-transfer reactions with rate constants close to the diffusion-controlled limit, and the lack of any evidence of transient equilibria being established in the electron-transfer reactions studied, implies that the PQ radical is very strongly reducing, with a one-electron reduction potential at least 100 mV more

negative than that of NAD^+ which has the lowest one-electron reduction potential ($E_7^1(NAD^+/NAD^\cdot) = -922\,mV$[24]) of the compounds studied, compared with the values for O_2 ($E_7^1(O_2/O2^{\cdot-}) = -160\,mV$[23]) and BV^{2+} ($E_7^1(BV^{2+}/BV^{\cdot+}) = -354\,mV$[19]).

In terms of the overall mechanism for free radical production by aerobic solutions of PQ and NAP(P)H (reactions (1)–(8) above) as formulated by Thornalley *et al.*, the most significant result of the present study is related to the dissociation of the [NAD(P)...H...PQ] charge transfer complex after one-electron oxidation by molecular oxygen (reaction (2)). Since it is shown that NAD^+ may oxidise the PQ radical very rapidly, it is most likely that this complex dissociates into $NAD(P)^\cdot$ and PQ, rather than $PQH^\cdot$ and $NAD(P)^+$ as originally proposed. Superoxide radical production by subsequent steps would not be inhibited by preferential formation of $NAD(P)^\cdot$ rather than $PQH^\cdot$, since it is known that $NAD^\cdot$ also rapidly ($k = 2 \times 10^9\,dm^3\,mol^{-1}s^{-1}$) reduces oxygen to the superoxide radical.[20] The possibility of free radical generation in oxygenated solutions of primaquine and NAD(P)H, despite the very negative one-electron reduction potentials of both compounds, must be due to the nature of the charge transfer complex (NAD(P)...H...PQ), allowing it to be oxidised by molecular oxygen, and to the subsequent oxidation of $NAD(P)^\cdot$ or $PQH^\cdot$ by oxygen. The charge transfer complex formed between NAD(P)H and primaquine therefore deserves further study. However, the results suggest that the primaquine radical ($PQH^\cdot$ or $PQ^{\cdot-}$) may play a less significant role in the aerobic oxidation of NAD(P)H, oxygen radical production and consequent antiparasitic effects of primaquine than has been previously supposed.

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