0026-895X/93/010257-07$03.00/0

MOLECULAR PHARMACOLOGY, 43:257-263

Electron Spin Resonance Spin-Trapping Investigation into the Effects of Paraquat and Desferrioxamine on Hydroxyl Radical Generation during Acute Iron Poisoning

MARK J. BURKITT, MARIA B. KADIISKA,[1] PHILLIP M. HANNA, SANDRA J. JORDAN, and RONALD P. MASON

The Rowett Research Institute, Bucksburn, Aberdeen, AB2 9SB, Scotland, UK (M.J.B.), and Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709 (M.B.K., P.M.H., S.J.J., R.P.M.)

Received July 2, 1992; Accepted September 30, 1992

SUMMARY

We have previously described a secondary radical-trapping technique for the detection of *in vivo* hydroxyl radical generation during acute iron overload. With this technique, the hydroxyl radical (·OH) reacts with dimethylsulfoxide to form the methyl radical (·CH_3), which is then detected by ESR spectroscopy as its adduct with the spin trap phenyl-*N*-*tert*-butylnitrone in the bile of treated animals. In this study, we report both the individual and combined effects of the futile-cycling agent paraquat (PQ^{2+}) and the iron-chelating agent desferrioxamine (DFO) on iron-dependent ·OH generation. Interactions between iron and the partially reduced oxygen species superoxide and hydrogen peroxide, which are generated during the metabolism of PQ^{2+}, might be expected to stimulate ·OH generation to a level above that seen in the presence of the metal ion alone. Although PQ^{2+} was often found to promote further ·OH generation when administered to animals also given iron, the large variation observed between individual animals in response to the reagent meant that the effect was not statistically significant ($p < 0.05$). DFO was found to abolish iron-dependent ·OH generation, both in the presence and in the absence of PQ^{2+}. This is believed to result from the chelation of iron by DFO, to form an essentially redox-inert iron(III) complex that is unable to catalyze ·OH radical formation. In addition, it was found that the iron(II) complex of DFO can reduce PQ^{2+} to its radical cation *in vitro*, indicating, therefore, that the chelation of iron by DFO may not necessarily prevent its participation in free radical reactions.

The herbicidal activity of PQ^{2+}, the 1,1′-dimethyl-4,4′-bipyridylium dication, is attributed to its ability to catalyze the formation of cytotoxic oxygen-centered radicals within chloroplasts during photosynthesis (1, 2). The herbicide is believed to undergo an enzymatic single-electron reduction to form the paraquat radical cation, $PQ\cdot^{+}$ (reaction 1), which is then oxidized by molecular oxygen ($k = 7.7 \times 10^8$ M^{-1} sec^{-1}) to form the superoxide radical, $O_2\cdot^{-}$ (reaction 2) (3). Via its participation in repeated cycles of reduction and oxidation, PQ^{2+} is believed to generate a continuous flux of superoxide radicals. Similarly, the formation of superoxide radicals during the "futile cycling" of PQ^{2+} is believed to be responsible for its toxicity towards mammals (4).

$$PQ^{2+} \xrightarrow[\text{reduction}]{\text{enzymatic}} PQ\cdot^{+} \quad (1)$$

$$PQ\cdot^{+} + O_2 \rightarrow PQ^{2+} + O_2\cdot^{-} \quad (2)$$

Since 1968, PQ^{2+} has been known to undergo reduction to a radical in microsomal systems, as determined by its characteristic anerobic absorption spectrum (5). More recently, the radical has been detected using the more specific and informative technique of ESR spectroscopy in microsomal (6), hepatocyte (7), and lung epithelial cell incubations (8). The corresponding radicals from the related bipyridilium compounds diquat, benzyl viologen, and morfamquat have also been observed in hepatocyte incubations using ESR spectroscopy (7).

In their studies on the toxicity of PQ^{2+} towards the microorganism *Escherichia coli*, Hassan and Fridovich (9–12) have demonstrated the requirement for superoxide generation in the toxicity of the reagent. Indeed, it has been demonstrated that the exposure of *E. coli* to PQ^{2+} leads to the induction of superoxide dismutase, an enzyme of major importance in the protection of biological systems from oxygen radicals (9).

The reactivity of superoxide itself may be too low to account for the damage observed in biological systems exposed to the radical. It is often considered more likely that many of the biological effects of superoxide are indirect, resulting from its conversion to the more reactive hydroxyl radical (·OH) after its interaction with a redox-active metal ion (often believed to be iron or copper complexes) (see reactions 3–5 for iron, in

[1] Permanent address: Institute of Physiology, Bulgarian Academy of Sciences, "Academician Georgy Borchev" Street, Building 23, 1113 Sofia, Bulgaria.

ABBREVIATIONS: PQ, paraquat; DFO, desferrioxamine mesylate; DMSO, dimethylsulfoxide; 2,2′-DP, 2,2′-dipyridyl; PBN, phenyl *N*-*tert*-butylnitrone.

which the metal ion is assumed to exist as a low molecular weight chelate) (13, 14).

$$O_2\cdot^- + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad (3)$$

$$2O_2\cdot^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (4)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^- \quad (5)$$

Hydroxyl radical generation via reactions 1–5 has been demonstrated in model systems. For example, using ESR spectroscopy both the superoxide and hydroxyl radicals can be detected as their adducts with the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide when PQ^{2+} is incubated in the presence of NADPH-cytochrome P-450 reductase and NADPH (15). However, such direct evidence for the operation of reactions 1–5 *in vivo* is lacking.

Indirect evidence for the role of iron in the toxicity of PQ^{2+} is provided by the findings from studies of the effects of DFO, an iron-chelating agent, on the toxicity of PQ^{2+} (16). DFO is often found to protect biological systems from oxygen radicals, presumably due to its stabilization of iron in the ferric oxidation state, which prevents its participation in reaction 3 and thereby prevents hydroxyl radical formation (17, 18). Whereas some workers do find that DFO protects animals from PQ^{2+} (16), others report that the reagent has, if anything, a slight accentuating effect on toxicity (19). Indeed, in the recent literature the "antioxidant" properties of DFO have been brought into question, and mechanisms have been proposed to support the alleged prooxidant actions of the reagent (20, 21).

In a recent paper we described an application of the spin-trapping technique for the *in vivo* detection of the hydroxyl radical using an animal model of acute iron poisoning (22). This technique utilizes a well known reaction in which the hydroxyl radical is converted to the methyl radical ($\cdot CH_3$) via its reaction with DMSO ($k = 7 \times 10^9$ M^{-1} sec^{-1}) (reaction 6) (23). The methyl radical is then detected as its adduct with the spin-trap PBN by using ESR spectroscopy (reaction 7).

$$(CH_3)_2SO + \cdot OH \rightarrow CH_3SO_2H + \cdot CH_3 \quad (6)$$

$$PBN + \cdot CH_3 \rightarrow PBN/\cdot CH_3$$

In the present study we have applied this model to investigate the effects of both PQ^{2+} and DFO on iron-dependent hydroxyl radical formation. When animals are intoxicated with iron alone, it is believed that the hydrogen peroxide required for the formation of the hydroxyl radical (reaction 5; the Fenton reaction) arises via the direct reduction of molecular oxygen by iron(II) (22). Therefore, it was expected that the exposure of animals to both PQ^{2+} and iron would promote $\cdot OH$ generation further by providing an additional source of H_2O_2 for this reaction. In addition, the administration of DFO to animals treated with either iron or iron plus PQ^{2+} was expected to provide insight into the mechanism of action of DFO at the level of the hydroxyl radical and, thereby, rationalize the pharmacological effects of the drug.

Materials and Methods

DMSO, ferrous sulfate, and PBN were from Aldrich Chemical Co. (Milwaukee, WI). DFO (deferoxamine mesylate), 2,2′-DP, and PQ were from Sigma Chemical Co. (St. Louis, MO), and [$^{13}C_2$]DMSO (minimum of 99% ^{13}C) was from Isotec (Miamisburg, OH). Male Sprague-Dawley rats (Charles River Laboratories, Raleigh, NC), fed a standard chow mix (NIH open formula; Zeigler Brothers Inc., Gardner, PA), were used in all experiments.

***In vivo* studies.** Nonfasted rats (330–400 g) were anesthetized (Nembutal) and their bile ducts were cannulated using ~7-cm PE10 tubing. Anesthesia was maintained throughout the experiments, which were initiated by the intraperitoneal injection of 1 ml/kg DMSO containing PBN (70 mg/ml), followed by the intragastric injection of 3 ml/kg ferrous sulfate solution (1 M, prepared immediately before use in N_2-purged water). Where indicated, DMSO was omitted and the PBN (70 mg/kg) was administered as an aqueous solution (26 mg/ml). In some experiments, additional reagents were administered via intraperitoneal injection before the injection of ferrous sulfate; PQ was given at a dose of 0.25 ml/kg from a 100 mg/ml aqueous stock solution, and DFO was administered at the indicated dose from a 1 M aqueous stock solution.

In all experiments, 20-min bile samples were collected (for 2 hr) into Eppendorf tubes containing 2,2′-DP solution (30 mM in water; 25 μl/100 g of rat weight). Samples were frozen immediately on dry ice and stored at −70° until ESR analysis was performed. All spectra shown are from samples collected between 100 and 120 min after the administration of ferrous sulfate.

***In vitro* reaction of iron(II) with PQ.** In order to prevent oxidation of the PQ radical, reactions were performed under anerobic conditions. A solution of PQ (20 mM), with or without DFO (5 mM), was purged with a gentle stream of nitrogen from a needle inserted through a rubber septum. After 5 min, a ferrous sulfate solution (prepared in N_2-purged water) was added to the mixture to a final concentration of 5 mM, and the sample was then aspirated into an ESR flat cell positioned in the cavity of the spectrometer for analysis (24).

ESR spectroscopy. Spectra were recorded on a Varian E-109 spectrometer with the following instrument conditions: 20-mW power, 80-G sweep width, 1-G modulation, 0.5-sec time constant, and 16-min sweep time. Hyperfine coupling constants and spectral simulations were obtained with a computer program, SIMEPR (written by D. R. Duling),[2] which sequentially varies all parameters of each radical species until a minimum in the error surface is located. Goodness-of-fit was judged by both a minimum in the sum of the squared residuals and visual comparisons.

Results

When rats were given an intraperitoneal injection of PQ^{2+} (25 mg/kg) followed by an intraperitoneal injection of PBN in DMSO (1 ml/kg of a 70 mg/ml solution) and an intragastric injection of ferrous sulfate (3 mmol/kg), a prominent six-line ESR signal was detected in bile samples collected into a 2,2′-DP solution (Fig. 1a). The hyperfine coupling constants for this signal (a^N = 16.3 G, a_β^H = 2.7 G) correspond to those determined previously for the PBN/$\cdot CH_3$ adduct detected in bile from rats treated with ferrous sulfate, DMSO, and PBN (22). Indeed, when rats were given ferrous sulfate, DMSO, and PBN with the omission of PQ^{2+}, the PBN/$\cdot CH_3$ adduct was again detected in the bile, but the signal was smaller than when rats were also given PQ^{2+} (Fig. 1b). The additional small doublet signal in this spectrum, often detected in bile samples, is from the ascorbate radical. A poorly resolved spectrum, believed to be from a mixture of radical adducts formed by the trapping of radicals centered on endogenous molecules (e.g., lipids), was obtained when animals were given ferrous sulfate, PQ^{2+}, and PBN without DMSO (Fig. 1c). When animals were given PQ^{2+}, DMSO, and PBN without iron, only a very weak signal was detected, thereby confirming the iron dependence of radical formation (Fig. 1d). Although the spectra shown in Fig. 1, a

[2] D. R. Duling. Computer analysis of multiple species EPR spectra. Manuscript in preparation.

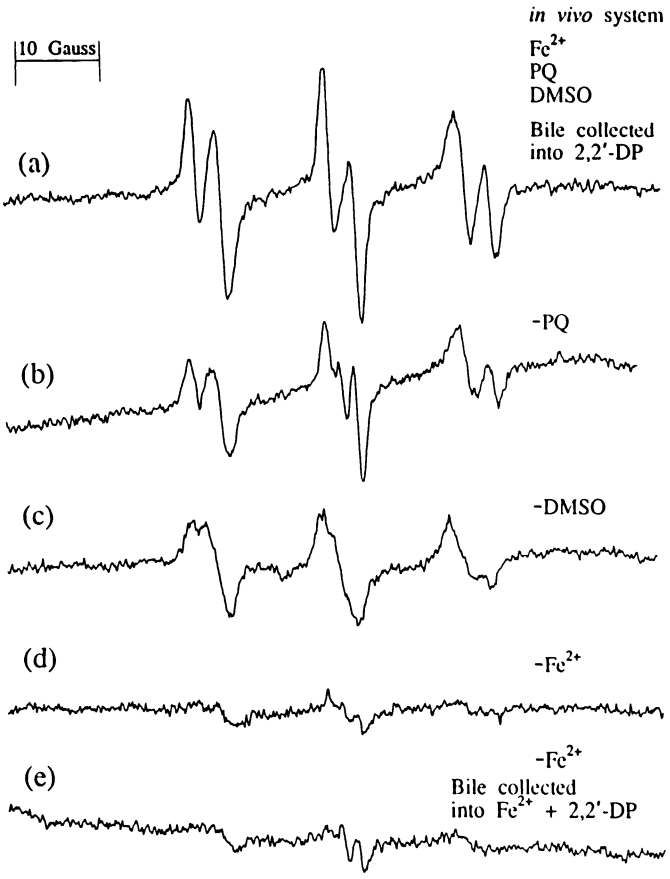

Fig. 1. Spectra of radical adducts detected in bile from rats given $FeSO_4$ (3 mmol/kg, intragastrically), PQ^{2+} (25 mg/kg, intraperitoneally), and DMSO (1 ml/kg, intraperitoneally) containing PBN (70 mg/ml). Bile samples were collected into a 30 mM 2,2′-DP solution (25 μl/100 g of rat weight). a, Complete system; b, complete system minus PQ^{2+}; c, complete system minus DMSO; d, complete system minus $FeSO_4$; e, complete system minus $FeSO_4$, with bile collected into a 30 mM 2,2′-DP/2 mM $FeSO_4$ solution (25 μl/100 g of rat weight).

and b, demonstrate a typical response to PQ^{2+} treatment, the difference was not statistically significant ($p < 0.05$), as a result of the variability between animals in the extent of stimulation of radical generation by PQ^{2+}.

We have demonstrated previously that, in order to prevent the occurrence of reactions between iron, oxygen, DMSO, and PBN in bile samples during their collection from animals treated with these reagents, samples must be collected directly into solutions of the iron(II)-stabilizing agent 2,2′-DP (22). If this precaution is not taken, signals from radical adducts generated *ex vivo* may be mistaken for those from adducts generated *in vivo* and excreted in the bile. In order to verify that the collection of bile samples into 2,2′-DP solution can prevent reactions occurring *ex vivo* when animals are also treated with PQ^{2+}, further control experiments were performed. Bile samples from animals treated with PQ^{2+}, DMSO, and PBN, but not ferrous sulfate, were collected directly into aqueous solutions of 30 mM 2,2′-DP (25 μl/100 g of rat weight) containing ferrous sulfate to give a final iron concentration of ≈0.4 mM. Even at this concentration of ferrous sulfate, which is comparable to that measured in the bile of rats given a 3 mmol/kg intragastric injection of ferrous sulfate (22), no detectable radical adduct formation occurred in the sample (Fig. 1e). This finding confirms, therefore, that the ESR signals shown in Fig. 1, a-c, are from radical adducts formed *in vivo*.

Via the substitution of $[^{13}C_2]$DMSO for DMSO ($^{12}C_2$), it has been confirmed previously that the signal detected in the bile of rats treated with ferrous sulfate, DMSO, and PBN is that of the PBN/$\cdot CH_3$ adduct. The aforementioned isotope substitution leads to the formation of PBN/$\cdot {}^{13}CH_3$, which can be readily identified from its characteristic ESR signal showing coupling to carbon-13 (22). In order to confirm that the signal detected when animals are also treated with PQ^{2+} (Fig. 1a) is that of the PBN/$\cdot CH_3$ adduct (as evidence for $\cdot$OH generation), similar experiments were carried out using $[^{13}C_2]$DMSO. It is apparent that, in addition to the characteristic 12-line signal from the PBN/$\cdot {}^{13}CH_3$ adduct (of which only nine lines are resolved in bile), the spectrum shown in Fig. 2a also contains signals from other radicals. Using a computer program developed at NIEHS, it was possible to simulate this spectrum when,

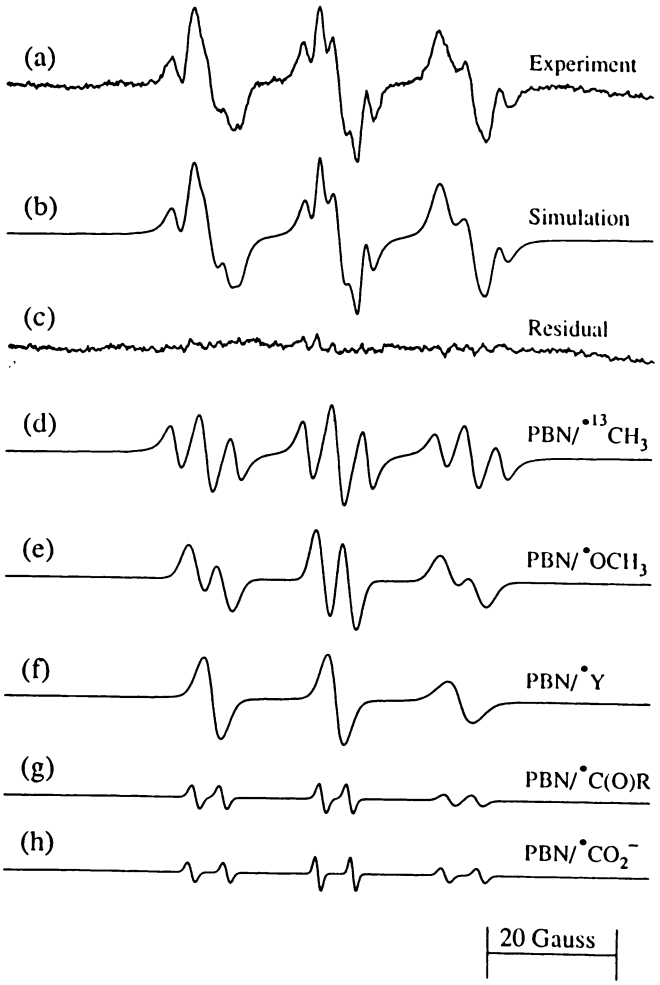

Fig. 2. Experimental and simulated spectra of radical adducts detected in bile from rats given $FeSO_4$ (3 mmol/kg, intragastrically), PQ^{2+} (25 mg/kg, intraperitoneally), and $[^{13}C_2]$DMSO (1 ml/kg, intraperitoneally) containing PBN (70 mg/ml). Bile was collected into a 30 mM 2,2′-DP solution (25 μl/100 g of rat weight). a, Experimental spectrum; b, complete simulation; c, residual simulated spectrum after subtraction of b from a; d, simulated spectrum of PBN/$\cdot {}^{13}CH_3$ (mole ratio, 0.50); e, simulated spectrum of PBN/$\cdot O^{13}CH_3$ (mole ratio, 0.26); f, simulated spectrum of unknown PBN/$\cdot$Y (mole ratio, 0.21); g, simulated spectrum of PBN/$\cdot$C(O)R (mole ratio, 0.02); h, simulated spectrum of PBN/$\cdot CO_2^-$ (mole ratio, 0.01).

TABLE 1

Summary of hyperfine coupling constants for PBN radical adducts detected in bile from rats treated with iron, PQ; and [$^{13}C_2$]DMSO

Values were determined from five separate computer simulations of Fig. 2a using different starting values.

Parent radical	a^N	a_β^H	a_β^{13C}	a^H (unknown)
	G	G	G	G
$\cdot{}^{13}CH_3$	16.31 ± 0.10	3.36 ± 0.13	4.00 ± 0.15	
$\cdot O^{13}CH_3$	16.07 ± 0.25	3.06 ± 0.39		
·Y	15.20 ± 0.07			1.20 ± 0.06
$\cdot CO_2^-$	15.69 ± 0.08	4.36 ± 0.06		
·C(O)R	15.61 ± 0.10	3.36 ± 0.09		

[3] M. J. Burkitt, M. B. Kadiiska, P. M. Hanna, and R. P. Mason, unpublished observations.

in addition to the signal from the PBN/$\cdot{}^{13}CH_3$ adduct, signals from four other radical adducts were included (see Fig. 2, b-h, and Table 1). Additional experiments (data not shown) indicated that at least three of these radicals (Fig. 2, f-h) are DMSO independent and, therefore, result from the trapping of radicals centered, presumably, on endogenous molecules.

From the computer simulation, species f is found to show a very small or no hydrogen coupling, which, if present, is not resolved at the line width adopted by the simulation program (PBN/·Y) (Table 1). This coupling appears to be too small to be from a β-hydrogen and may be from a more distant γ-hydrogen, the β-hydrogen being absent. This hydrogen splitting is better resolved in simulations of spectra of radicals detected in the bile of rats treated with the deuterated spin trap d_{14}-PBN.[3] Janzen (25) has suggested that nitroxides lacking a β-hydrogen may be formed via a mechanism involving the abstraction of the β-hydrogen of a radical adduct by another (nontrapped) radical. The hyperfine coupling constants determined for species g do not correspond well to those published for other PBN adducts in bile; one possible identity is PBN/·C(O)R (Table 1). After a comparison of the hyperfine coupling constants determined for radical h (see Table 1) with those published previously for radical adducts in bile, we found that the values for this species corresponded well to those of the $\cdot CO_2^-$ adduct of PBN (26). This radical adduct has been detected previously in the bile of carbon tetrachloride-intoxicated rats, where it was derived from CCl_4, as shown by ^{13}C-labeling (26). The origin of the PBN/$\cdot CO_2^-$ adduct in the experiments described here is not so clear; it may be formed by oxidative degradation of an endogenous molecule such as formate. The hyperfine coupling constants for species e provide, again, only limited information on its identity. This species may be the methoxyl adduct of PBN (PBN/$\cdot O^{13}CH_3$), but because of the presence of similar six-line signals from other species in the spectra it was not possible to establish unambiguously that the formation of this species is DMSO dependent. Our earlier *in vitro* experiments provide evidence for the formation of the methoxyl radical during the oxidation of DMSO by ·OH (22), but the absence of any reference hyperfine coupling constant values for this adduct in bile means that our assignment here can be only speculative.

Although the relative contributions of the radical adducts in Fig. 2, g and h, appear to be small, satisfactory simulations of the experimental spectrum in Fig. 2a were not obtained when either or both were omitted. Nevertheless, only the methyl radical adduct in Fig. 2d can be considered to be a unique assignment, based on the ^{13}C-labeling experiment. A simulation of Fig. 1a was obtained with all five radical adducts when the values determined in Fig. 2b were used with the omission of the ^{13}C-splitting, where the six-line signal of the [^{12}C]methyl adduct of PBN predominates (data not shown).

In order to investigate further the role of iron in the generation of the hydroxyl radical during PQ^{2+} intoxication, additional experiments were carried out in which animals were also treated with DFO. Before investigation of the effect of DFO on ·OH generation induced by simultaneous PQ^{2+} and iron intoxication, the effect of the reagent on ·OH generation induced by iron alone was examined. Because the intragastric injection of DFO, along with iron, would be expected to modify the metabolism of iron by simply inhibiting its absorption from the gastrointestinal tract, experiments were performed in which the chelating agent was administered via intraperitoneal injection. After the treatment of rats with ferrous sulfate (3 mmol/kg), DFO (3 mmol/kg), DMSO, and PBN, the six-line signal from the PBN/$\cdot CH_3$ adduct, otherwise detected in the absence of DFO (Fig. 3a), was almost abolished (Fig. 3b), suggesting that DFO can inhibit hydroxyl radical generation during iron overload. Similarly, the treatment of animals with DFO caused a complete inhibition of the signal from the PBN/$\cdot CH_3$ adduct when animals were given PQ^{2+} in addition to the aforementioned reagents (Fig. 4), suggesting, again, that ·OH generation is inhibited.

In order to identify possible reactions that might lead to the increase in the toxicity of PQ^{2+} in the presence of DFO reported by Borg and co-workers (19), *in vitro* experiments were performed in which the reactivity of the iron-DFO complex towards PQ^{2+} was investigated. Borg and Schaich (20) have demonstrated that the PQ radical can reduce iron(III)-DFO to

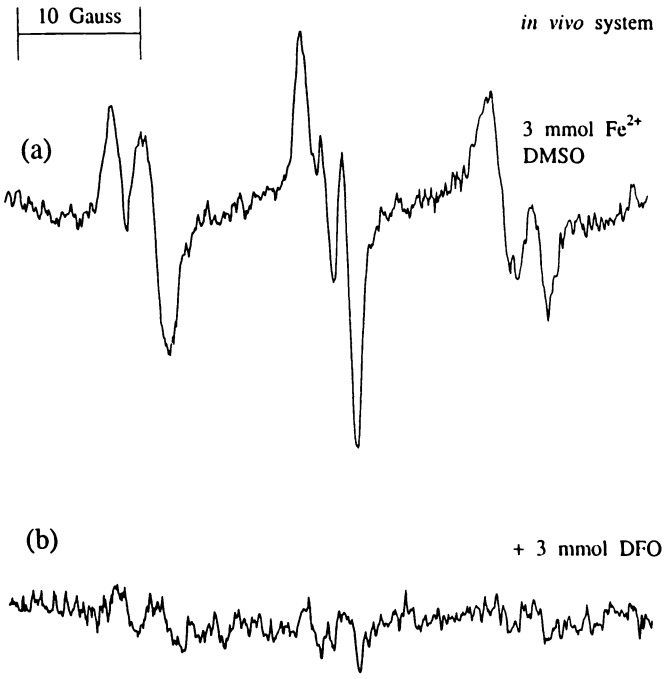

Fig. 3. Effect of DFO treatment on spectra detected in bile from rats given iron, DMSO, and PBN. Rats were given $FeSO_4$ (3 mmol/kg, intragastrically) plus DMSO (1 ml/kg, intraperitoneally) containing PBN (70 mg/ml). Bile samples were collected into a 30 mM 2,2′-DP solution (25 μl/100 g of rat weight). a, Complete system; b, complete system plus DFO (3 mmol/kg, intraperitoneally).

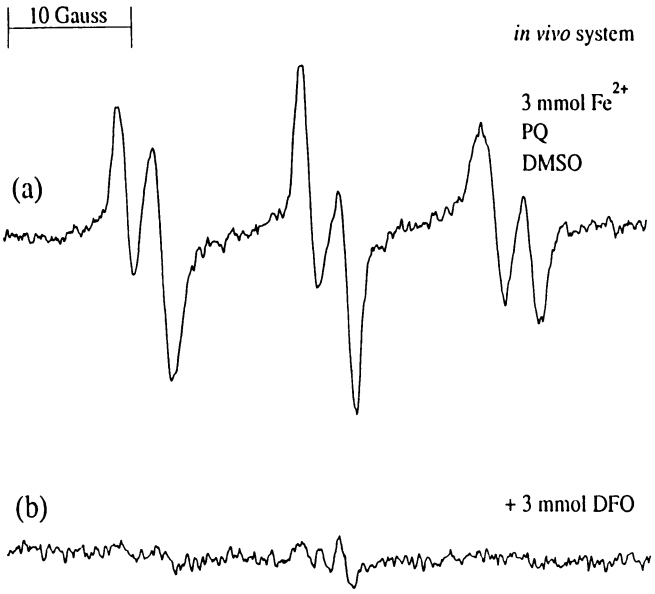

Fig. 4. Effect of DFO treatment on spectra detected in bile from rats given iron, PQ^{2+}, DMSO, and PBN. Rats were given $FeSO_4$ (3 mmol/kg, intragastrically), PQ^{2+} (25 mg/kg, intraperitoneally), and DMSO (1 ml/kg, intraperitoneally) containing PBN (70 mg/ml). Bile samples were collected into a 30 mM 2,2′-DP solution (25 μl/100 g of rat weight). a, Complete system; b, complete system plus DFO (3 mmol/kg, intraperitoneally).

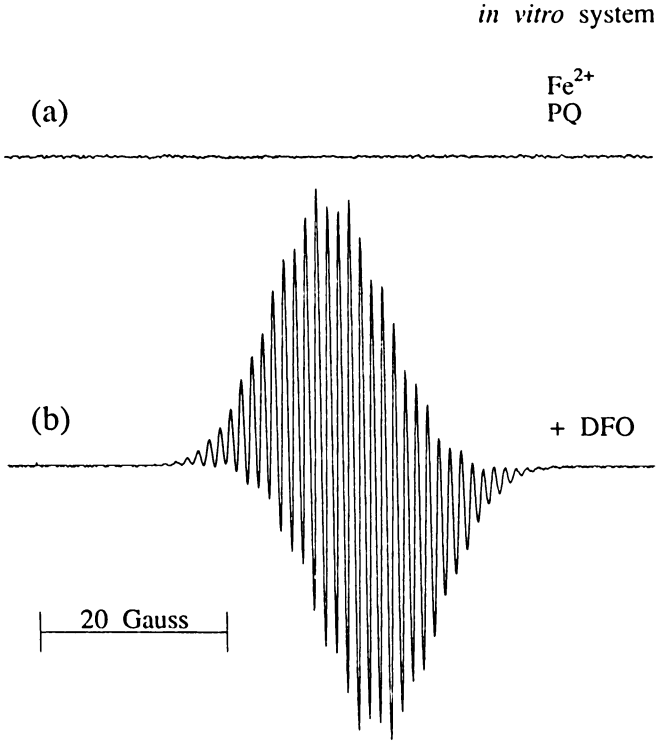

Fig. 5. *In vitro* reduction of PQ^{2+} to the $PQ\cdot^{+}$ radical cation by iron(II) in the absence (a) and presence (b) of DFO, demonstrated via direct ESR spectroscopy. A deoxygenated $FeSO_4$ solution (5 mM final concentration) was added to a deoxygenated solution of PQ^{2+} (20 mM final concentration) and transferred to an ESR flat cell mounted within the spectrometer cavity, using an anaerobic aspiration system (a). When indicated, DFO was included in the PQ^{2+} solution (at 5 mM final concentration) to which the $FeSO_4$ was added (b).

iron(II)-DFO. In view of the similarity in the redox potentials of the $PQ^{2+}/PQ\cdot^{+}$ and Fe(III)-DFO/Fe(II)-DFO redox couples, both approximately −0.45 V (27, 28), we considered it highly likely that the reverse reaction would also occur, that is, the reduction of PQ^{2+} to its radical by iron(II)-DFO (reaction 8).

$$\text{Fe(II)-DFO} + PQ^{2+} \rightarrow \text{Fe(III)-DFO} + PQ\cdot^{+} \qquad (8)$$

When ferrous sulfate was added to a deoxygenated solution of PQ^{2+}, no ESR signal could be detected (Fig. 5a). However, when DFO was included in the system, the characteristic ESR signal (6) from the $PQ\cdot^{+}$ radical was detected (Fig. 5b). This finding demonstrates the reduction of PQ^{2+} to its radical cation by an iron complex and suggests that, in the presence of iron(II), DFO may provide an additional route to the formation of $PQ\cdot^{+}$.

Discussion

The experiments described above provide evidence to suggest that the hydroxyl radical is generated during PQ^{2+} intoxication. In order to achieve a detectable level of ·OH generation using our technique, it was necessary to simultaneously load animals with iron. However, it is expected that generation of the radical also occurs when animals are given PQ^{2+} alone, but at a level not detectable by the ESR spin-trapping technique. In support of this, Thomas and Aust (29) have reported that the $PQ\cdot^{+}$ radical can mobilize iron from ferritin and thereby provide a source of iron.

It is expected that only a minuscule proportion of the hydroxyl radicals generated in an iron-intoxicated animal react with DMSO to form the methyl radical. Most of the ·OH radicals are expected to react with endogenous biomolecules, which far exceed the concentration of DMSO attainable in an animal. The same argument can be applied to the trapping of the methyl radical with PBN; all but a minute proportion of the methyl radicals formed are expected to be lost via other reactions. Furthermore, any PBN/$\cdot CH_3$ adduct that is formed must also escape reduction to an ESR-silent hydroxylamine before reaching the ESR sample cell (30). Despite these considerations, it has been claimed that PBN can protect animals from PQ toxicity by acting as a free radical scavenger (31).

Because Kohen and Chevion (16) have demonstrated that DFO can protect rats from PQ^{2+} toxicity, our demonstration that DFO suppresses hydroxyl radical generation in rats intoxicated with PQ^{2+} suggests that the ·OH radical is, indeed, responsible for the toxic effects of the toxicant and is not simply an innocuous side product of its metabolism.

DFO is known to inhibit hydroxyl radical formation via mechanisms that are dependent on iron(III) reduction but not iron(II) oxidation; iron(II) complexed with DFO undergoes only one oxidation reaction (18, 22). Therefore, our finding that the drug abolishes detectable hydroxyl radical formation in animals challenged with iron indicates that the metal ion undergoes redox cycling *in vivo*. Glutathione and ascorbate may act as endogenous reductants necessary for the redox cycling of iron. An alternative, and perhaps less likely, explanation for the effect of DFO is that, after binding of iron(II), the drug scavenges hydroxyl radicals at their site of formation and thereby prevents their reaction with the DMSO detector molecule.

DFO is also believed to have pro-oxidant properties; indeed, Borg and co-workers (19) report that the drug causes a small enhancement in the toxicity of PQ^{2+} to mice. Although the antioxidant properties of DFO are believed to result, in part,

from its ability to stabilize iron in the ferric form, its pro-oxidant properties are less well understood. It is often overlooked that DFO has ferroxidase activity. The binding of iron(II) by DFO results in a rapid oxidation of the metal ion, with the concurrent generation of oxygen radicals, including ·OH (18, 32). Furthermore, the very high stability constant of the Fe(III)-DFO complex has led Borg and Schaich (20) to suggest that DFO, particularly when present at high concentrations, may be able to accumulate iron from endogenous pools, including ferritin. Although iron(III)-DFO is generally resistant to reduction (e.g., by superoxide and ascorbate), it has been demonstrated by these workers that the complex can be reduced by the paraquat radical to give the iron(II) complex (20). Iron(II)-DFO may generate ·OH via either autoxidation or direct reaction with hydrogen peroxide (18).

Borg and Schaich (20) have argued further that the apparent biphasic antioxidant versus pro-oxidant behavior of DFO is dose dependent, with pro-oxidant behavior becoming increasingly important at higher doses. This may to some extent explain the discrepancy between their observations and those of Kohen and Chevion (16), who used a lower dose of DFO.

Additional insight into the mechanism of action of DFO in PQ-intoxicated animals is provided by the recent findings of Van der Wal and co-workers (33, 34). These workers report that DFO reduces the mortality rate in vitamin E-deficent rats challenged with PQ^{2+} (33), but not in their nondeficient counterparts (34). They suggest that in the deficient animals higher levels of membrane lipid peroxidation may promote the entry of DFO into cells, thereby allowing it better access to the subcellular iron pools believed to be responsible for catalyzing ·OH radical production (34). In support of the proposal that the rate of cellular uptake of DFO may be a factor that limits its ability to act therapeutically, Van der Wal and co-workers (34) also found that the more lipophilic chelator CP51 of the hydroxypyridione series protects nondeficient animals from PQ^{2+}. However, it is also expected that the cellular uptake of DFO is necessary for it to act as a pro-oxidant by the mechanisms described above.

Because the animals used in our experiments are given iron, mobilization of the endogenous metal ion is not required for ·OH generation. Therefore, although the dose of DFO administered to our animals is far greater than that used by Borg and co-workers (19), only antioxidant behavior (inhibition of ·OH formation) is expected.

Our analysis of radical adducts excreted via the biliary route may not reflect the effects of the toxicant at other target organs such as the lung (8). However, the liver is the major site of PQ^{2+} metabolism (35), and our findings provide the first ESR evidence for iron-dependent hydroxyl radical generation during PQ intoxication. Our findings also provide direct evidence to support the notion that the protective action of DFO towards animals intoxicated with either iron or PQ^{2+} can indeed be attributed to its ability to suppress iron-dependent hydroxyl radical formation.

Acknowledgments

The authors wish to thank Mr. D. Duling for his assistance with the computer simulation of spectra.

References

1. Epel, B. L., J. Neumann, and R. Fishman. The mechanism of the oxidation of ascorbate and Mn^{2+} by chloroplasts: the role of the radical superoxide. *Biochim. Biophys. Acta* **325**:520–529 (1973).
2. Harbour, J. R., and J. R. Bolton. Superoxide formation in spinach chloroplasts: electron spin resonance detection by spin trapping. *Biochem. Biophys. Res. Commun.* **64**:803–807 (1975).
3. Farrington, J. A., M. Ebert, E. J. Land, and K. Fletcher. Bipyridylium quaternary salts and related compounds. V. Pulse radiolysis studies of the reaction of paraquat radical with oxygen: implications for the mode of action of bipyridyl herbicides. *Biochim. Biophys. Acta* **314**:372–381 (1973).
4. Bus, J. S., S. D. Aust, and J. E. Gibson. Superoxide and singlet oxygen catalyzed lipid peroxidation as a possible mechanism for paraquat (methyl viologen) toxicity. *Biochem. Biophys. Res. Commun.* **58**:749–755 (1974).
5. Gage, J. C. The action of paraquat and diquat on the respiration of liver cell fractions. *Biochem. J.* **109**:757–761 (1968).
6. Mason, R. P., and J. L. Holtzman. The role of catalytic superoxide formation in the O_2 inhibition of nitroreductase. *Biochem. Biophys. Res. Commun.* **67**:1267–1274 (1975).
7. DeGray, J. A., D. N. R. Rao, and R. P. Mason. Reduction of paraquat and related compounds to free radical metabolites by rat hepatocytes. *Arch. Biochem. Biophys.* **289**:145–152 (1991).
8. Horton, J. K., R. Brigelius, R. P. Mason, and J. R. Bend. Paraquat uptake into freshly isolated rabbit lung epithelial cells and its reduction to the paraquat radical under anaerobic condions. *Mol. Pharmacol.* **29**:484–488 (1986).
9. Hassan, H. M., and I. Fridovich. Regulation of the synthesis of superoxide dismutase in *Escherischia coli*: induction by methyl viologen. *J. Biol. Chem.* **252**:7667–7672 (1977).
10. Hassan, H. M., and I. Fridovich. Superoxide radical and the oxygen enhancement of the toxicity from paraquat in *Escherichia coli*. *J. Biol. Chem.* **253**:8143–8148 (1978).
11. Hassan, H. M., and I. Fridovich. Intracellular production of superoxide radical and of hydrogen peroxide by redox active compounds. *Arch. Biochem. Biophys.* **196**:385–395 (1979).
12. Hassan, H. M., and I. Fridovich. Paraquat and *Escherichia coli*: mechanism of production of extracellular superoxide radical. *J. Biol. Chem.* **254**:10846–10852 (1979).
13. Gilbert, B. C., and M. Jeff. Oxidative damage and repair: one-electron transfer reactions involving metal ions, peroxides and free radicals, in *Free Radicals: Chemistry, Pathology, and Medicine* (C. Rice-Evans and T. Dormandy, eds.). Richelieu Press, London, 25–49 (1988).
14. Halliwell, B. Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts: its role in the degradation of hyaluronic acid by a superoxide-generating system. *FEBS Lett.* **6**:238–242 (1978).
15. Yamazaki, I., L. H. Piette, and T. A. Grover. Kinetic studies on spin trapping of superoxide and hydroxyl radicals generated in NADPH-cytochrome P-450 reductase-paraquat systems: effect of iron chelates. *J. Biol. Chem.* **265**:652–659 (1990).
16. Kohen, R., and M. Chevion. Paraquat toxicity is enhanced by iron and reduced by desferrioxamine in laboratory mice. *Biochem. Pharmacol.* **34**:1841–1843 (1985).
17. Burkitt, M. J., and B. C. Gilbert. Model studies of the iron-catalysed Haber-Weiss cycle and the ascorbate-driven Fenton reaction. *Free Radical Res. Commun.* **10**:265–280 (1990).
18. Burkitt, M. J., and B. C. Gilbert. The autoxidation of iron(II) in aqueous systems: the effects of iron chelation by physiological, non-physiological and therapeutic chelators on the generation of reactive oxygen species and the inducement of biomolecular damage. *Free Radical Res. Commun.* **14**:107–123 (1991).
19. Osheroff, M. R., K. M. Schaich, R. T. Drew, and D. C. Borg. Failure of desferrioxamine to modify the toxicity of paraquat in rats. *J. Free Radicals Biol. Med.* **1**:71–82 (1985).
20. Borg, D. C., and K. M. Schaich. Prooxidant action of desferrioxamine: Fenton-like production of hydroxyl radicals by reduced ferrioxamine. *J. Free Radicals Biol. Med.* **2**:237–243 (1986).
21. Halliwell, B. Protection against tissue damage *in vivo* by desferrioxamine: what is its mechanism of action? *Free Radicals Biol. Med.* **7**:645–651 (1989).
22. Burkitt, M. J., and R. P. Mason. Direct evidence for *in vivo* hydroxyl-radical generation in experimental iron overload: an ESR spin-trapping investigation. *Proc. Natl. Acad. Sci USA* **88**:8440–8444 (1991).
23. Veltwisch, D., A. Janata, and K.-D. Asmus. Primary processes in the reactions of OH· radicals with sulphoxides. *J. Chem. Soc. Perkin Trans. II* 146–143 (1980).
24. Mason, R. P. Assay of *in situ* radicals by electron spin resonance. *Methods Enzymol.* **105**:416–422 (1984).
25. Janzen, E. G. Spin trapping and associated vocabulary. *Free Radicals Res. Commun.* **9**:163–167 (1990).
26. Knecht, K. T., and R. P. Mason. *In vivo* radical trapping and biliary secretion of radical adducts of carbon tetrachloride-derived free radicals. *Drug Metab. Dispos.* **16**:813–817 (1988).
27. Farrinton, J. A., M. Ebert, and E. J. Land. Bipyridilium quaternary salts and related compounds. 6. Pulse radiolysis studies of the reaction of paraquat radical analogues with oxygen. *J. Chem. Soc. Faraday Trans. I* **74**:665–675 (1978).
28. Cooper, S. R., J. V. McArdle, and K. M. Raymond. Siderophore electrochemistry: relation to intracellular iron release mechanism. *Proc. Natl. Acad. Sci. USA* **75**:3551–3554 (1978).
29. Thomas, C. E., and S. D. Aust. Reductive release of iron from ferritin by

cation free radicals of paraquat and other bipyridyls. *J. Biol. Chem.* **261:**13064–13070 (1986).
30. Sentjurc, M., and R. P. Mason. Inhibition of radical adduct reduction and reoxidation of the corresponding hydroxylamines in *in vivo* spin trapping of carbon tetrachloride-derived radicals. *Free Radicals Biol. Med.* **13:**151–160 (1992).
31. Sata, T., E. Kubota, H. A. Misra, M. Mojarad, H. Pakbaz, and S. I. Said. Paraquat-induced lung injury: prevention by *N-tert*-butyl-α-phenylnitrone, a free-radical spin-trapping agent. *Am. J. Physiol.* **262:**L147-L152 (1992).
32. Klebanoff, S. J., A. M. Waltersdorph, B. R. Michel, and H. Rosen. Oxygen-based free radical generation by ferrous ions and deferoxamine. *J. Biol. Chem.* **264:**19765–19771 (1989).
33. Van Asbeck, B. S., F. C. Hillen, H. C. M. Boonen, Y. de Yong, J. A. Dormans, N. A. Van der Wal, J. J. Marx, and B. Sangster. Continuous intravenous infusion of deferoxamine reduces mortality by paraquat in vitamin-E-deficient rats. *Am. Rev. Respir. Dis.* **139:**769–773 (1989).
34. Van der Wal, N. N. A., L. L. Smith, J. F. L. M. Van Oirschot, and B. S. Van Asbeck. Effect of iron chelators on paraquat toxicity in rats and alveolar type II cells. *Am. Rev. Respir. Dis.* **145:**180–186 (1992).
35. Baldwin, R. C., A. Pasi, J. T. MacGregor, and C. H. Hine. The rates of radical formation from the dipyridylium herbicides paraquat, diquat, and morfamquat in homogenates of rat lung, kidney, and liver: an inhibitory effect of carbon monoxide. *Toxicol. Appl. Pharmacol.* **32:**298–304 (1975).

Send reprint requests to: Mark J. Burkitt, The Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen, AB2 9SB, Scotland, UK.