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ESR SPIN TRAPPING STUDIES INTO THE NATURE OF THE OXIDIZING SPECIES FORMED IN THE FENTON REACTION: PITFALLS ASSOCIATED WITH THE USE OF 5,5-DIMETHYL-1-PYRROLINE-*N*-OXIDE IN THE DETECTION OF THE HYDROXYL RADICAL

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Several investigators have challenged the widely held view that the hydroxyl radical is the primary oxidant formed in the reaction between the ferrous ion and hydrogen peroxide. In recent studies, using the ESR spin trapping technique, Yamazaki and Piette found that the stoichiometry of oxidant formation in the reaction between Fe^{2+} and H_2O_2 often shows a marked deviation from the expected value of 1:1 (I. Yamazaki and L. H. Piette (1990) *J. Am. Chem. Soc.* **113**, 7588–7593). In order to account for these observations, it was suggested that additional oxidizing species are formed, such as the ferryl ion (FeO^{2+}), particularly when iron is present at high concentration and chelated to EDTA.

In this paper it is shown that secondary reactions, involving the redox cycling of iron and the oxidation of the hydroxyl radical adduct of the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) by iron, operate under the reaction conditions employed by Yamazaki and Piette. Consequently, the stoichiometry of oxidant formation can be rationalized without the need to envisage the formation of oxidizing species other than the hydroxyl radical. It is also demonstrated that the iron(III) complex of DETAPAC can react directly with DMPO to form the DMPO hydroxyl radical adduct (DMPO/$^{\cdot}$OH) in the absence of hydrogen peroxide. Therefore, to avoid the formation of (DMPO/$^{\cdot}$OH) as an artefact, it is suggested that DETAPAC should not be used as a reagent to inactivate containating adventitious iron in experiments using DMPO.

KEY WORDS: Iron, hydroxyl radical, Fenton reaction, 5,5-dimethyl-1-pyrroline-*N*-oxide, ESR spin trapping.

ABBREVIATIONS: DETAPAC, diethylenetriaminepentaacetic acid; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; EDTA, ethylenediaminetetraacetic acid; HEDTA, N-(2hydroxethyl)ethylenediamine triacetic acid; TEMPO-OH, 4-hydroxy-2,2,6,6 tetramethypiperidinyl-1-oxyl.

INTRODUCTION

The controlled reduction of molecular oxygen to highly reactive and potentially cytotoxic species is believed to occur in all aerobic cells.[1,2] Under normal circumstances the formation of such species is confined to specific metal centers, such as those of the enzymes cytochrome P-450[3] and cytochrome c oxidase,[4] and the

exposure of the cell to non-specific oxidation by "free" reactive oxygen species (e.g., the superoxide radical, hydrogen peroxide and the hydroxyl radical) is believed to be minimal. However, during the last twenty five years or so it has become increasingly apparent that reactive oxygen species play an important role in the pathogenesis of many diseases, including cancer[5] and atherosclerosis.[6]

It is generally believed that the oxidative damage resulting from the exposure of cell components to the superoxide radical ($O_2^{\cdot -}$) and hydrogen peroxide is indirect and requires their conversion to the considerably more reactive hydroxyl radical ($^{\cdot}OH$) via interaction with a redox-active metal ion, often assumed to be iron.[7]

$$Fe^{3+} + O_2^{\cdot -} \rightarrow Fe^{2+} + O_2 \quad (1)$$

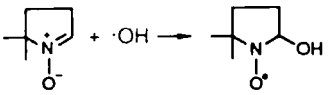
$$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (2)$$

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

Although there now exists a considerable body of evidence to suggest that the oxidant formed in the Fenton reaction (Reaction 3) is the hydroxyl radical,[7-10] many investigators have challenged this view and suggested that the oxidant may be a high valent iron-oxo species, such as the ferryl ion, FeO^{2+}.[11-17]

$$Fe^{2+} + H_2O_2 \rightarrow FeO^{2+} + H_2O \quad (4)$$

This controversy has arisen, in part, because of the difficulties associated with the detection of the $^{\cdot}OH$ radical, particularly in complex biological systems. Electron spin resonance (ESR) spectroscopy is the most direct method for the detection of free radicals. However, the hydroxyl radical has never been observed in free solution using ESR spectroscopy, probably due to the fact that, in the absence of any external pertubation, the unpaired electron is in an orbitally degenerate state.[18] Therefore, ESR evidence for the presence of the $^{\cdot}OH$ radical is usually obtained via the detection of secondary radicals formed following the reaction of $^{\cdot}OH$ with a suitable substrate. This may involve using continuous flow techniques, in which secondary radicals are observed directly as they are formed within the ESR cell.[9,10] Alternatively, by allowing the radical to react with a suitable spin trap compound, the relatively stable hydroxyl radical adduct to the spin trap can be observed using a static system.[19] The most frequently employed spin trap for the detection of the $^{\cdot}OH$ radical is 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO). Since the $^{\cdot}OH$ radical becomes, upon trapping, a structural component of the DMPO adduct that is observed (DMPO/$^{\cdot}OH$), it might be expected that the detection of this species provides unambiguous evidence for $^{\cdot}OH$ formation.



(5)

The DMPO/$^{\cdot}OH$ adduct can, however, also arise as an artefact; for example, via interaction of the corresponding superoxide adduct (DMPO/$^{\cdot}OOH$) with adventitious redox active metal ions.[20] Such reactions can usually be identified by performing suitable control experiments.[20,21] Another difficulty associated with the use of DMPO

to detect ·OH from the Fenton reaction lies in demonstrating that the DMPO/·OH adduct has not formed via the direct oxidation of the trap by, for example, FeO^{2+}.

$$FeO^{2+} + DMPO + H^+ \rightarrow Fe^{3+} + DMPO^{\cdot+} + {}^{-}OH \quad (6)$$

$$DMPO^{\cdot+} + H_2O \rightarrow DMPO/{\cdot}OH + H^+ \quad (7)$$

Indeed, DMPO/·OH formation via such a mechanism, involving the initial formation of an adduct between the oxidant and DMPO, has been demonstrated to occur during the reaction of a variety of reactive species with the spin trap.[22]

Yamazaki and Piette have recently described trapping experiments using DMPO that appear to challenge the view that the oxidant of the Fenton reaction is exclusively the hydroxyl radical.[23,24] They measured the stoichiometry of oxidant formation via the Fenton reaction and performed competition experiments to obtain the ratio of rate constants for the reaction of the oxidant with hydroxyl radical scavengers and DMPO. In order to rationalize their observations, it was proposed that at least three possible oxidants can be formed, namely free ·OH, bound (or confined) ·OH, and a high valent iron species, probably FeO^{2+}. It was suggested that the nature of the dominant oxidizing species formed depends very much on the nature of the iron chelator being used.[23,24]

The identity of the oxidizing species formed in the Fenton reaction is considered to be of crucial importance to the understanding of the role of iron in inducing biomolecular damage (see ref. 25 for a recent review). Indeed, differences in the selectivity with which the hydroxyl radical and iron oxo species are expected to attack substrate molecules may well have implications for the cellular sites at which oxidative damage occurs under pathological conditions. In view of the known difficulties associated with the quantification of oxygen radical formation using spin trapping,[19] it was decided to investigate further the application of the technique to the study of the Fenton reaction.

MATERIALS AND METHODS

Catalase (thymol-free), chelating resin (iminodiacetic acid), DMPO (5,5-dimethyl-1-pyrroline-*N*-oxide), DETAPAC (free acid), EDTA (disodium salt), and TEMPO-OH (4-hydroxy-2,2,6,6 tetramethy-piperidinyl-1-oxyl) were from Sigma. All other reagents were from BDH (Dorset, UK) and of analytical quality.

KCl-phosphate buffer (300 mM KCl-100 mM $KHPO_4$, pH 7) was treated with chelating resin using the batch method.[26] DMPO, dissolved in KCl-phosphate buffer to give a concentrated stock solution, was washed with activated charcoal prior to use. Ferrous sulfate stock solutions were always prepared using nitrogen-purged water.

For reactions involving the addition of iron(II), appropriate aliquots from 50 mM stock solutions of either EDTA or DETAPAC (in KCl-phosphate, pH 7) were added to incubations prior to the addition of iron. Premixing of the chelators with iron(II) was avoided because it would cause rapid autoxidation of the metal ion. For reactions involving the addition of iron(III), stock solutions of premixed iron complexes were used: an appropriate amount of $FeCl_3$ was added to the chelator (DETAPAC or EDTA) solution at low pH (around 3) to give final concentrations of iron(III) and

the chelator of 4 mM and 5 mM, respectively. No further pH adjustment was made: at the amounts used the addition of these stock solutions caused no significant changes in the pH of incubations.

Reactions between iron(II) and H_2O_2 were initiated by adding $FeSO_4$ to a tube containing appropriate quantities of KCl-phosphate buffer, water, DMPO, hydrogen peroxide and chelator stock solution (when used) to give the final reagent concentrations indicated in the legend to Figure 1. Similarly, reactions between iron(III) and H_2O_2 were initiated via the addition of an aliquot of the iron(III) complex stock solution to appropriate quantities of KCl-phosphate buffer, water, DMPO and H_2O_2 to give the final concentrations indicated in the legend to Figure 2 and Table 1.

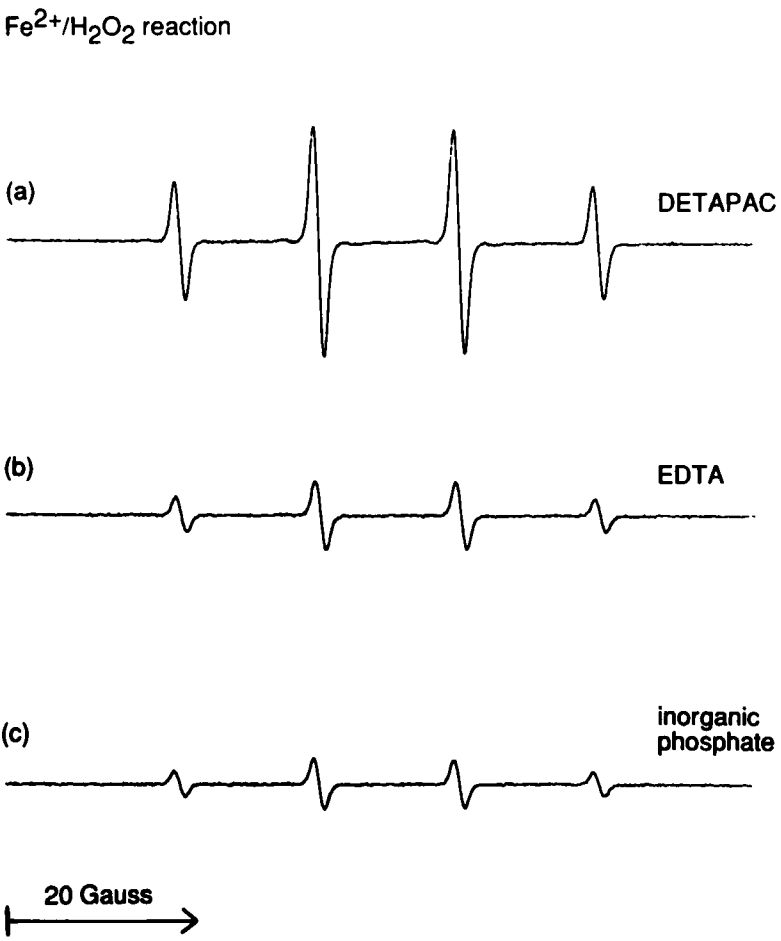


Figure 1 Effect of the chelating agent used on the concentration of the DMPO hydroxyl radical adduct detected following the reaction of 25 μM Fe^{2+} with 100 μM hydrogen peroxide in 150 mM KCl, 50 mM $KHPO_4$, pH 7.0, containing 100 mM DMPO. (a) 200 μM DETAPAC. (b) 200 μM EDTA. (c) No chelator was added (the metal ion is chelated by the buffer).

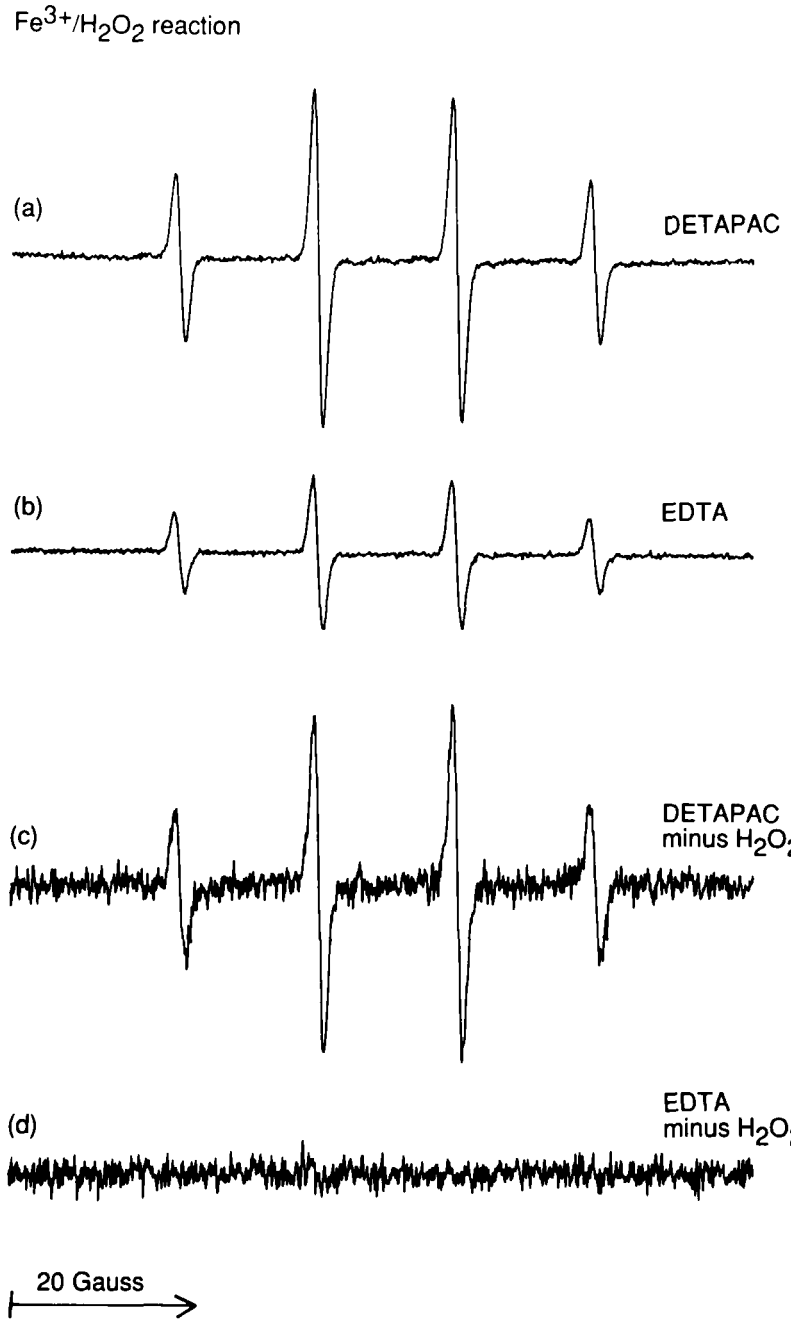


Figure 2 Effect of the chelating agent used on the concentration of the DMPO hydroxyl radical adduct detected following the reaction of 150 μM Fe^{3+} with 150 μM hydrogen peroxide in 150 mM KCl, 50 mM $KHPO_4$, pH 7.0, containing 100 mM DMPO. (a) 187.5 μM DETAPAC. (b) 187.5 μM EDTA. (c) 600μM Fe^{3+} – 750μM DETAPAC with the omission of hydrogen peroxide. (d) as (c), but using EDTA.

Table 1 Effects of Fe^{3+} and H_2O_2 relative concentrations on the amount of the DMPO hydroxyl radical adduct detected in the presence of either DETAPAC or EDTA (in 150 mM KCl, 50 mM $KHPO_4$ buffer, pH 7)

$[Fe^{3+}]$ (mM)	$[H_2O_2]$ (mM)	[DMPO/·OH] (μM)[1]		
		DETAPAC	EDTA	DETAPAC/EDTA
0.15[2]	1.00	3.8	2.3	1.6
1.00[3]	0.15	22.7	5.4	4.2

[1]Concentrations of DMPO/·OH reported are representative of at least three experiments, showing a variation of less than 5%.
[2]0.187 mM chelator.
[3]1.25 mM chelator.

In order to investigate any reaction between iron(II) and DMPO/·OH, the radical adduct was first prepared by adding 75 μl $TiCl_3$ (4 mM, prepared freshly in N_2-purged water) to a tube containing 820 μl water, 690 μl KCl-phosphate buffer, 300 μl 100 mM DMPO and 90 μl 4 mM H_2O_2. After 30 s, 5 μl catalase (1400 units) was added to remove the remaining H_2O_2. After 1 min 10 μl 50 mM EDTA or 50 mM DETAPAC was added, followed by 10 μl 4 mM $FeSO_4$. In control experiments, 20 μl water was added instead of the chelator and $FeSO_4$. Similarly, in order to investigate any reaction between iron(III) and DMPO/·OH, the adduct was first prepared as above, but using 790 μl water and 700 μl KCl-phosphate buffer. After incubation with catalase the iron(III) complex was added: 40 μl either 4 mM $FeCl_3$-5 mM DETAPAC or 4 mM $FeCl_3$-5 mM EDTA (40 μl water was added to control incubations). In order to detect any reaction between either the iron(II) or iron (III) complexes with the DMPO/·EtOH adduct, 1.57 M ethanol was included in the incubations described above for the preparation of DMPO/·OH.

After initiation via the addition of iron, incubations were transferred to an ESR flat cell positioned and tuned within the cavity of the ESR spectrometer using a rapid sampling device[27] and recording commenced immediately. Subsequent recordings indicated that the spectra shown here are of stable signals that do not undergo any significant changes within several minutes of recording. Spectra shown in a given figure, and described in a given table, were recorded under identical conditions, without removing the ESR cell from the cavity or retuning. Consequently, the radical concentrations presented were reproducible with less than 5% variation.

Radical concentrations were determined by double integration of spectra, using TEMPO-OH as a standard. The concentration of TEMPO-OH was deterined using the extinction coefficient at 240 nm of 1440 M^{-1} cm^{-1}.[24] Spectra were recorded using a Bruker E 106 spectrometer with the following instrument settings: modulation frequency, 100 kHz; sweep width, 80 G; modulation amplitude, 0.8 G; time constant, 41 ms; sweep time, 168 s; power, 20 mW.

RESULTS

Because the hydroxyl radical reacts with DMPO at a very high rate ($k = 3.4 \times 10^9$ M^{-1} s^{-1}) to form the hydroxyl radical adduct, DMPO/·OH,[28] the formation of

hydroxyl radicals via the Fenton reaction can be followed by measuring DMPO/·OH formation. By locking the field of the spectrometer at one of the signal peaks of the DMPO/·OH adduct, Yamazaki and Piette were able to measure *initial* rates of adduct formation and thereby obtain rate constants for the reaction of various iron(II) complexes with H_2O_2.[23] The values they obtained are in reasonable agreement with those obtained using other methods.[13,29,30] However, when attempts were made to measure the stoichiometry of the Fenton reaction (i.e., $[Fe^{2+}]$:[oxidant]), it was found that the *maximum levels of DMPO/·OH accumulated* (typically after 25 to 100 s) do not reflect the rate constant of the Fenton reaction for a given iron(II) complex. In similar experiments reported here it was also found that, although iron(II)EDTA is known to react faster than iron(II)DETAPAC with H_2O_2 ($k = ca.\ 7 \times 10^3$ and $8 \times 10^2\ M^{-1}\ s^{-1}$, respectively),[23] the concentration of DMPO/·OH detected from the Fenton reaction using EDTA was much lower than when using DETAPAC. For example, when Fe^{2+} at a concentration of 25 μM was reacted with excess H_2O_2 in the presence of DETAPAC, the concentration of DMPO/·OH detected was 17 μM (Figure 1A). In contract, when EDTA was used as the iron chelator, the concentration of DMPO/·OH detected was only 5 μM (Figure 1B). Similarly, in the absence of an added chelator, when it is believed that the metal ion is chelated to the phosphate buffer, the concentration of the adduct detected was 4 μM (Figure 1C).

Although such observations have led Yamazaki and Piette to question the mechanism of the Fenton reaction,[23] it would seem more likely that the concentration of DMPO/·OH detected reflects not only the stoichiometry (and rate) of the Fenton reaction, but also the occurrence of additional, secondary reactions.

Since hydrogen peroxide can reduce Fe^{3+},[31-33] it seemed possible that the Fe^{3+} formed in the Fenton reaction might undergo reduction to Fe^{2+} by excess H_2O_2 and initiate a second cycle of reaction. Indeed, when Fe(III)DETAPAC was added to an equimolar amount of H_2O_2 in the presence of DMPO, the signal from the DMPO/·OH adduct was detected (Figure 2A). Similarly, when the same experiment was performed using EDTA, the DMPO/·OH adduct was again detected (Figure 2B), but at a lower concentration than when using DETAPAC. Interestingly, when a higher concentration of Fe(III)DETAPAC was employed, DMPO/·OH was also detected in incubations from which H_2O_2 was omitted (Figure 2C), but at much a lower concentration. No signal was detected when H_2O_2 was omitted from incubations using Fe(III)EDTA (Figure 2D). These findings demonstrate that under conditions of excess H_2O_2, redox cycling of iron, with the formation of additional DMPO/·OH, must occur. One possible explanation for the detection of DMPO/·OH following the addition of Fe(III)DETAPAC to DMPO alone is that DMPO can undergo direct oxidation by the metal complex to form a radical cation, which then hydrates to form DMPO/·OH.

In order to investigate further the ability of Fe^{3+} to undergo redox cycling in the presence of H_2O_2, additional experiments were carried out at both low and high $[Fe^{3+}]$ to $[H_2O_2]$ ratios. As shown in Table 1, the difference in the concentration of the DMPO/·OH adduct detected between experiments performed using either DETAPAC or EDTA is most pronounced when Fe^{3+} is present in excess: using 1 mM Fe^{3+} and 150 μM H_2O_2, over four times as much DMPO/·OH is detected when using DETAPAC when compared with EDTA. In contrast, when using 150 μM Fe^{3+} and 1 mM H_2O_2, just over one and a half times as much adduct is detected in the presence of DETAPAC when compared with EDTA (Table 1).

These findings, plus the observation that the ESR signals detected under such

involving Fe^{3+}, experiments were carried out in which the iron(III) complexes were added to the adduct. When iron(III)DETAPAC (final Fe^{3+} concentration, 80 μM) was added to DMPO/·OH, formed as described above using 150 μM Ti^{3+}, after catalase addition, the concentration of the adduct fell from 8.3 μM to 5.7 μM (Table 2), indicating that Fe(III)DETAPAC can oxidize DMPO/·OH. When similar experiments were carried out using iron(III)EDTA, the concentration of the adduct fell to 2.1 μM (Table 2), indicating that iron(III)EDTA is the more effective oxidant for DMPO/·OH. It is believed, therefore, that the concentration of DMPO/·OH detected in the experiments described in Figure 1 reflects not only the relative rate constants of the Fenton reaction for the respective iron(II) complexes (EDTA > DETAPAC), but also the relative rates of reduction of the iron(III) complexes by H_2O_2 and the relative rates of oxidation of the adduct by the iron(III) complexes.

Much of the data that would appear to question the assertion that the hydroxyl radical is the oxidant formed in the Fenton reaction is derived from competition experiments in which the rate of attack of the oxidant upon DMPO is compared with its rate of attack upon a scavenger molecule, such as ethanol.[23,24] When ethanol is oxidized by ·OH, the major radical formed is $\cdot CHOHCH_3$,[11,29] the α-hydroxyethanol radical, which reacts with DMPO to form an adduct, DMPO/·EtOH. Yamazaki and Piette noted that when ethanol was included in incubations using Fe(II)DETAPAC, the amount of DMPO/·OH lost was recovered as DMPO/·EtOH at a yield of about 75 per cent. In contrast, when EDTA was used instead of DETAPAC, far more DMPO/·EtOH was produced than needed to account for the amount of DMPO/·OH lost. This observation was taken to indicate that, when using EDTA in the Fenton system, ethanol is oxidized not only by ·OH but also by an additional chemical species.[23,24] Therefore, it was also decided to investigate further the mechanisms of DMPO/·EtOH formation and removal.

In similar experiments, when ethanol was added to incubations using Fe(II)EDTA, there was a marked increase in the concentration of total sin adduct detected (results not shown). In contrast, when ethanol was added to incubations using Fe(II)DETAPAC, there was a decrease in the concentration of total spin adduct detected (not shown). Further experiments were performed using iron(III) complexes to initiate oxidant formation. When Fe(III)DETAPAC (final Fe^{3+} concentration, 150 μM) was incubated with equimolar H_2O_2, the DMPO/·OH adduct was detected at a concentration of 5.3 μM (Figure 3A). When ethanol was included in the incubation (1.57 M), the total spin adduct concentration (DMPO/·OH plus DMPO/·EtOH) detected was lower, at 1.1 μM (Figure 3B). This poor recovery of spin concentration is to be expected when it is remembered that *two* radical scavenging steps are involved in the formation of DMPO/·EtOH: the ·OH radical must first react with ethanol and then the $\cdot CHOHCH_3$ radical must react with the trap. The $\cdot CHOHCH_3$ radical may undergo oxidation by Fe^{3+} before trapping,[30] and it is known that the ·OH radical can also attack ethanol at the β-hydrogen to form $\cdot CH_2CH_2OH$,[11,30] which is not detected as an adduct to DMPO. Despite these considerations, when ethanol was added to incubations using Fe(III)EDTA, the total spin adduct concentration detected was found to increase, from 1.9 μM to 3.0 μM (Figure 3C and D).

The observations above demonstrate that DMPO/·EtOH can be formed in reactions involving the reduction of iron(III) by H_2O_2 and that the rate constant of the Fenton reaction *is* reflected in the yield of DMPO/·EtOH detected (i.e., EDTA > DETAPAC). In order to explore the possibility that this is because, when compared with DMPO/·OH, the DMPO/·EtOH adduct is relatively resistant to destruction by iron

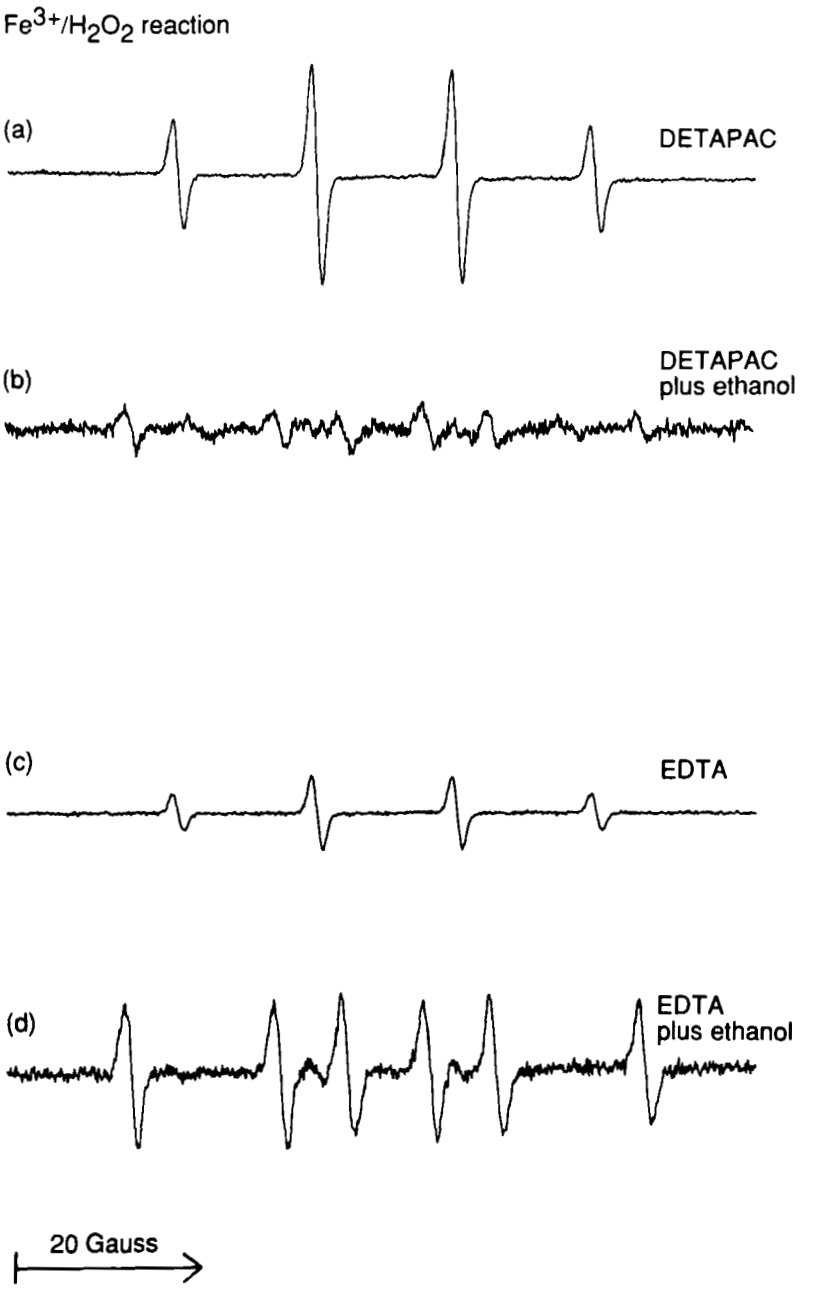


Figure 3 Effect of ethanol addition on the amount of spin adduct detected following the reaction of 150 μM Fe^{3+} with 150 μM hydrogen peroxide in 50 mM KCl, 50 mM $KHPO_4$, pH 7.0, containing 100 mM DMPO. (a) 187.5 μM DETAPAC. (b) 187.5 μM DETAPAC plus 1.57 M ethanol. (c) 187.5 μM EDTA. (d) 187.5 μM EDTA plus 1.57 M ethanol.

(particularly in the presence of EDTA), experiments were performed in which it was aimed to assess the ability of iron complexes to reduce and oxidize DMPO/·EtOH to ESR silent species. The adduct was prepared using Ti^{3+} as described for DMPO/·OH, but with the inclusion of ethanol in incubations. When using 150 μM Ti^{3+}, the concentration DMPO/·EtOH detected was 1.7 μM (not shown). When iron(II)DETAPAC (final Fe^{2+} concentration, 20 μM) was added to DMPO/·EtOH, after catalase addition, to give the same final volume, no change in the concentration of the adduct was observed (not shown), indicating that Fe(II)DETAPAC is a poor reductant of DMPO/·EtOH. When experiments were carried out using iron(II)EDTA, the concentration of the adduct fell by about 50% (not shown), indicating that Fe(II)EDTA can reduce DMPO/·EtOH. When either iron(III)DETAPAC or iron(III)EDTA was added to incubations up to a concentration of 80 μM, only a negligible loss of signal was detected (not shown), confirming that DMPO/·EtOH is resistant to oxidation by Fe^{3+}.

The above findings demonstrate that the increase in total spin adduct concentration detected when ethanol is added to incubations employing EDTA, and not DETAPAC, is a reflection of the relative stabilities of the DMPO/·OH and DMPO/·EtOH adducts in the presence of Fe(III)DETAPAC and Fe(III)EDTA, and cannot be taken to prove the presence of oxidants other than ·OH. As with DMPO/·OH, reduction of DMPO/·EtOH by iron(II) is not expected to occur to any significant extent in the presence of excess peroxide.

DISCUSSION

The identity of the oxidizing species formed in the Fenton reaction has been discussed widely and remains a controversial, yet central aspect of oxygen radical chemistry.[25] Several investigators have challenged the view that the hydroxyl radical is the primary oxidant formed. For example, using spectrophotometric techniques based on the measurement of iron(III) formation and cytochrome c oxidation, Rush and Koppenol have shown that the oxidizing species formed in the rate limiting step of the reaction between Fe(II)EDTA and hydrogen peroxide is not the hydroxyl radical, and fails to react with *tert*-butyl alcohol. This oxidant, which may be the ferryl-EDTA complex, is believed to react with hydrogen peroxide to form another transient which is scavenged by *tert*-butyl alcohol. It was suggested that this second oxidant may be the hydroxyl radical.[12] Similarly, Rahhal and Richter report that Fe(II)DETAPAC reacts with hydrogen peroxide to yield an oxidizing species which is not scavenged by *tert*-butyl alcohol under conditions where more than 90 per cent of any hydroxyl radicals present would be expected to be scavenged, and suggest, therefore, that the oxidizing species formed is an iron-oxo species, such as the ferryl ion.[16] Rush and Koppenol have also presented data which suggests that a highly oxidizing iron intermediate is formed in the reaction of Fe(II)DETAPAC, and particularly Fe(II)HEDTA, with hydrogen peroxide.[13] More recently, Koppenol and coworkers have described experiments in which the mechanisms of salicylate hydroxylation by radiolytically generated hydroxyl radicals and a Fenton system were compared. Their findings suggested that the primary hydroxylating species formed in the reaction of Fe(II)EDTA with hydrogen peroxide is the hydroxyl radical.[36]

A major problem encountered when using scavenger molecules to determine oxidant formation via the Fenton reaction is interference from secondary reactions in which

radicals generated on the scavenger molecule either reduce iron(III) or oxidize iron(II). Croft *et al.* have recently demonstrated that, if these secondary reactions are taken into account, the oxidation of a variety of organic substrates via the Fenton system (using EDTA and several other chelators) can be rationalized in terms of the free hydroxyl radical as the attacking species.[30]

Yamazaki and Piette employed the ESR spin trapping technique because it provides a direct method of detection and identification of the hydroxyl radical.[23,24] However, great care is needed in the interpretation of spin trapping experiments involving iron. Although it is expected that under the conditions of excess H_2O_2 employed here reduction of DMPO/·OH and DMPO/·EtOH by iron(II) is negligible, the finding that these adducts are reduced by iron(II) more effectively in the presence of EDTA than DETAPAC should prove useful in the interpretation of experiments carried out under other conditions.

It is considered more likely that the differences observed in the stoichiometry of DMPO/·OH and DMPO/·EtOH formation via the Fenton reaction using either iron(II)EDTA or Fe(II)DETAPAC result, in part, from differences in the abilities of the respective iron(III) complexes to participate in secondary reactions, namely redox cycling by H_2O_2 and reduction by DMPO/·OH. Consequently, although the initiating Fenton reaction is relatively slow when using Fe(II)DETAPAC, the iron(III)DETAPAC formed in the reaction is reduced back to iron(II) by H_2O_2 to generate further DMPO/·OH which is relatively resistant towards oxidation by Fe(III)DETAPAC. In contrast, although the initiating Fenton reaction is relatively fast for EDTA, additional DMPO/·OH generation via redox cycling of the metal ion is relatively inefficient, at least, in part, because the adduct is removed via oxidation. It should be stressed that it is not known whether iron(III) reduction by the peroxide is faster for DETAPAC than for EDTA; the finding that more DMPO/·OH is detected when using DETAPAC, compared with EDTA, may be a reflection of the relative stability of the adduct in the presence of the two iron chelates. The difference in the rate of DMPO/·OH oxidation by the DETAPAC and EDTA complexes of iron(III) becomes less significant at higher $[H_2O_2]$ to [iron] ratios: under such conditions the peroxide is able to compete more effectively with DMPO/·OH for oxidation by iron(III)EDTA. This may be why Yamazaki and Piette found that the difference in the yield of DMPO/·OH between experiments using Fe(II)DETAPAC and Fe(II)EDTA was less pronounced at very low iron(II) concentrations (see also Table 1), when the stoichiometry of DMPO/·OH formation approaches 1:1 for both iron complexes.[23]

Another difference in the behaviour between the two iron complexes is that Fe(III)DETAPAC, and not Fe(III)EDTA, reacts with DMPO to form DMPO/·OH in the absence of added H_2O_2. Although the contribution of this pathway to the overall levels of DMPO/·OH detected here is probably negligible, this finding indicates nevertheless that DETAPAC should not be used as a reagent to inactivate contaminating adventitious iron in experiments using DMPO.

The increase in total spin adduct concentration observed when ethanol is included in incubations employing Fe(II)EDTA, but not Fe(II)DETAPAC, is believed therefore to reflect differences in the ease of oxidation of DMPO/·OH and DMPO/·EtOH by Fe(III)EDTA: in the absence of the scavenger the ·OH radical is trapped as DMPO/·OH, which undergoes oxidation; whereas in the presence of ethanol the hydroxyl radical is converted to the more stable DMPO/·EtOH adduct, thus conserving the spin. This increase in total spin adduct concentration seen upon ethanol

addition is not observed when using DETAPAC because Fe(III)DETAPAC is a relatively poor oxidant of DMPO/·OH. The finding that the iron(II) and iron(III) complexes of DETAPAC are not as effective as the corresponding EDTA complexes in bringing about the reduction and oxidation of DMPO/·OH, respectively, may reflect the absence of a free coordination site on the iron-DETAPAC complex,[37] indicating that the reactions probably proceed via inner sphere mechanisms.

Perhaps the most direct evidence to support the assertion that the DMPO/·OH adduct is formed in a Fenton system by the addition of the free hydroxyl radical to DMPO, rather than via a mechanism involving DMPO oxidation by FeO^{2+} followed by hydrolysis, is provided by the findings of Mottley *et al.*[38] These workers used xanthine oxidase to incorporate [^{17}O]oxygen into hydrogen peroxide in the presence of Fe^{2+} and DMPO. The DMPO hydroxyl radical adduct they detected displayed hyperfine coupling to [^{17}O]oxygen. Interestingly, although Mottley *et al.* performed this experiment to provide evidence for the correct identification of the DMPO/·OH spin adduct, in doing so, they also provided evidence that the oxygen atom of the hydroxyl group in the adduct is derived from hydrogen peroxide and not the solvent.[38]

It appears, therefore, that although complicated by the operation of secondary reactions, the findings from spin trapping investigations into the identity of the oxidizing species formed in the Fenton reaction can be rationalized in terms of the free hydroxyl radical, without the need to involve the participation of other chemical species. Further experimentation is required to provide a full kinetic analysis of the reactions that occur during the spin trapping experiment; it is nevertheless clear at this stage that additional reactions, involving the redox cycling of iron and the oxidation of radical adducts by iron, must be considered along with the existing list of artefacts and pitfalls encountered when using the technique.[20,39]

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