EPR Spin-Trapping Study on the Oxidizing Species Formed in the Reaction of the Ferrous Ion with Hydrogen Peroxide

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Abstract: Using 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) as a spin-trapping reagent for HO•, we compared the ratio of rate constants for the reaction of HO• with HO• scavengers (k) to those for the reaction with DMPO (k_{DMPO}) in a photolysis or a Fenton (Fe^{II}–H_2O_2) system. Assuming that the k/k_{DMPO} ratio measures the extent to which HO• is free in solution relative to 100% in a photolysis system, we concluded that HO• formed in the Fenton reaction is not totally free in solution. The extent to which it is not free, but bound in some kind of complex, depended upon the type of chelator used and increased in the order Fe^{II}ADP < Fe^{II}-phosphate = Fe^{II}EDTA < Fe^{II}DETAPAC. There was a remarkable difference in the mode of the Fenton reaction between Fe^{II}DETAPAC and Fe^{II}EDTA, particularly at high Fe^{II} concentrations (0.1 mM). An ethanol-oxidizing species other than HO•, presumably the ferryl ion, was detected in the Fe^{II}EDTA reaction but not in the Fe^{II}DETAPAC reaction. The major oxidizing species in the Fe^{II}EDTA–H_2O_2 reaction changed from the ferryl ion to HO• as the H_2O_2 concentration was increased, while it was invariably HO• alone in the Fe^{II}DETAPAC–H_2O_2 reaction. Benzoate and *tert*-butyl alcohol, known as typical HO• scavengers, were shown to react not only with HO• but also with the ferryl ion in the Fe^{II}EDTA reaction. Similar scavenging effects were observed with histidine, formate, and mannitol.

It was nearly a century ago when Fenton[1] reported that H_2O_2 acts as a strong oxidant in the presence of the ferrous ion. The combination of H_2O_2 and a ferrous salt is called Fenton's reagent. Forty years later, Haber and Weiss[2] proposed the formation of the hydroxyl radical (HO•) in the reaction of the Fenton reagent.

$$Fe^{II} + H_2O_2 \rightarrow Fe^{III} + HO^- + HO^\bullet \quad (1)$$

This highly reactive oxidizing species is now believed to be involved in oxygen toxicity in biology.[3] This oxygen toxicity mechanism has been suggested as the cause of many clinical conditions,[4] and a number of biochemical studies on Fenton's reagent have been reported.[5]

Although a considerable number of investigators, using the EPR spin-trapping technique, have found support for the formation of HO• from Fenton's reagent,[6] it has also been reported by others[7,8] that the oxidizing intermediate is not HO•, but some type of iron species such as the ferryl ion. We thought that the most pertinent approach to solving this contradiction might be to measure the stoichiometry of reaction 1 under various conditions by using the spin-trapping technique. The quantitative measurement of reaction 1 has shown that a stoichiometric amount of HO• is spin-trapped by 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) when the ferrous ion concentration is less than 1 μM.[9] It has also been shown that iron(II) diethylenetriaminepentaacetate (DETAPAC) is a very efficient Fenton reagent for producing HO•.[9] This observation apparently contradicts that of Rahhal and Richter,[8] who reported that the oxidizing species produced from the Fe^{II}DETAPAC–H_2O_2 reaction is not HO•, but an iron–oxo species such as the ferryl ion. The conclusion reported by Rush and Koppenol[7] regarding the iron(II) ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetate (EDTA) system is similar to that of Rahhal and Richter. Their conclusion is mostly derived from their kinetic analysis which suggests that benzoate and *tert*-butyl alcohol (*t*-BuOH), known as HO• scavengers, do not scavenge the oxidizing species produced in the Fenton reaction. Their analysis, based on kinetic and stoichiometric data for the overall Fenton reaction, assumes a variety of possible steps and therefore is inevitably complicated.

Using EPR spin trapping, we have determined that three types of oxidizing species are produced in the Fenton reaction and that all are scavenged by benzoate and *t*-BuOH.

Experimental Section

Reagents. DMPO was obtained from Sigma and used after redistilling. DETAPAC and ADP were obtained from Sigma, EDTA from MCB Manufacturing Chemicals, Inc., and 4-hydroxy-2,2,6,6-tetramethylpiperidinyl-1-oxy (Tempo–OH) from Aldrich. A 5 mM ferrous ion solution was prepared before every experiment by dissolving ferrous ammonium sulfate, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, in anaerobic water. All other compounds were reagent grade and were used as received.

EPR Spin Trapping. EPR assays were carried out in a flat cell by using a computer-controlled Varian E-9 EPR spectrometer. The modulation amplitude used was 1 G. All reactions were performed at room temperature (24 ± 1 °C) by using a flow apparatus (Model RX 1000, Applied Photophysics Ltd.). For standard experiments, reactions were initiated by mixing an equal volume of an aerobic solution (A) containing 150 mM KCl, 40 mM potassium phosphate buffer (pH 7.4), 40 mM DMPO, an iron chelator, and 0.4 mM H_2O_2 with an anaerobic solution (B) containing 150 mM KCl and 0.2 mM ferrous ion. The concentrations of iron chelators in A were 0.4 mM for DETAPAC and EDTA and 4 mM for ADP. The final concentrations of these compounds, therefore, became half of their starting concentrations except for that of KCl. Benzoate was added to solution A, and ethanol and *t*-BuOH were added to solution B. Premixing of the ferrous ion with iron chelators was avoided because the ferrous ion became more autoxidizable in the presence of chelators, particularly EDTA.[10] The pH of the stock solutions of HO• scavengers was adjusted to about 7.4 by HCl or NaOH.

Photolysis experiments were carried out by illuminating solutions in the EPR cavity with water-filtered UV light from a high-pressure mercury arc lamp. The UV intensity was controlled with an adjustable iris. The solutions contained 150 mM KCl, 20 mM phosphate (pH 7.4), 20 mM DMPO, 0.2 M H_2O_2, and varying amounts of ethanol (EtOH), benzoate, or *t*-BuOH. Spectra were taken immediately after 30-s illuminations.

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Table I. Effect of Benzoate and *t*-BuOH on Fe^{II}-H_2O_2-EtOH Reactions

chelator	EtOH (0.1 M)	scavenger	[spin adduct]$_{max}$, μM DMPO–OH	DMPO–Et	DMPO–tB	figure[a]
EDTA	–		11.0			3B
	+		2.8	30.6		6B, 7a
	+	0.1 M benzoate	0.5	3.0		6B
	+	1.2 M *t*-BuOH	1.0	3.6	10.5	7b
	–	1.2 M *t*-BuOH	1.0		11.4	7c
DETAPAC	–		45.0			1a, 3A
	+		11.7	25.2		3A
	+	0.1 M benzoate	0	0.4		6A
	–	0.2 M *t*-BuOH	3.5		3.7	1b

[a] The spin concentrations were measured from experiments shown in the figures listed in this column.

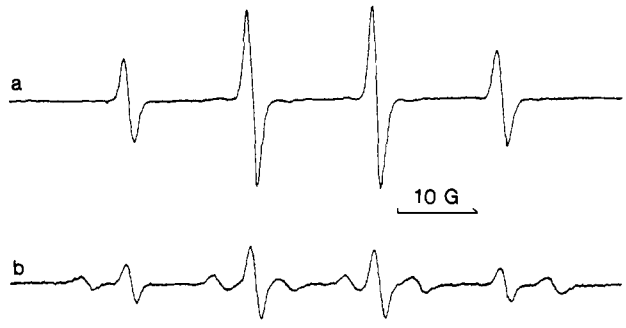
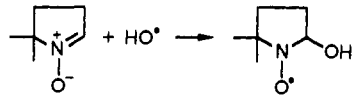


Figure 1. Effect of *t*-BuOH on the reaction of Fe^{II}DETAPAC with H_2O_2: (a) control; (b) 0.2 M *t*-BuOH. EPR spectra were taken 30 s after mixing Fe^{II} and H_2O_2. [Fe^{II}] = 0.1 mM and [H_2O_2] = 0.2 mM. The spin concentrations are shown in Table I. Spectrum b was taken at a gain 5 times higher than that of spectrum a.

Spin concentrations of the DMPO adducts of HO• (DMPO–OH), the EtOH radical (DMPO–Et), and the *t*-BuOH radical (DMPO–tB) were determined by double integration of their respective EPR signals, using a 39.0 μM Tempo–OH solution as an integration standard. The Tempo–OH concentration was determined by using an extinction coefficient at 240 nm of 1440 M^{-1} cm^{-1}.[11]

The reaction of DMPO with HO• is formulated as follows:[12]



Results

As reported previously,[9] DMPO–OH was formed from the reaction of H_2O_2 with Fe^{II}DETAPAC more effectively than from that with Fe^{II}EDTA. This difference became significant as the Fe^{II} concentration was increased. Table I shows that 45 μM DMPO–OH was produced at the expense of 100 μM Fe^{II}DETAPAC. This formation of DMPO–OH was inhibited by a HO• scavenger, *t*-BuOH. In this case, we observed a mixture of EPR signals for DMPO–OH and DMPO–tB, which was very similar to DMPO–Et, a DMPO spin adduct of the α-hydroxyethyl radical[6] (Figure 1b). The spin concentration was calculated to be 3.5 μM for DMPO–OH and 3.7 μM for DMPO–tB. The latter amount was only 9% of the expected spin concentration, assuming 100% efficiency in spin trapping of the scavenger radical. Because these signals were stable once formed, the formation of DMPO–tB from the reaction of *t*-BuOH with HO• was concluded not to be very efficient.

Fixing the magnetic field at a peak of the second line of the DMPO–OH signal, we could obtain its time-dependent formation curve (Figure 2a). Since the DMPO concentration was nearly saturated at 20 mM for trapping HO• generated from this reaction, the formation curve for DMPO–OH was regarded as that

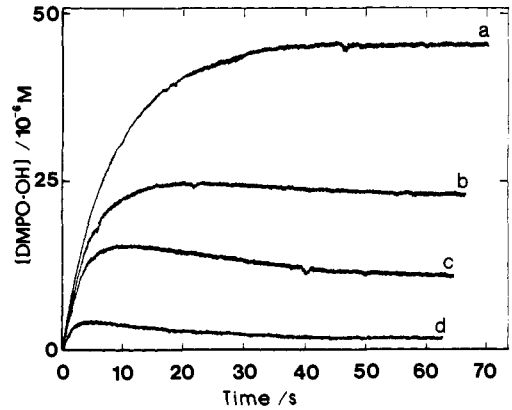


Figure 2. Effect of benzoate on DMPO–OH formation in a Fe^{II}DETAPAC-H_2O_2 reaction. [Fe^{II}] = 0.1 mM and [H_2O_2] = 0.2 mM. The magnetic field was set at a peak of the second line of the DMPO–OH EPR signal. [Benzoate] = 0 (a), 5 mM (b), 10 mM (c), 20 mM (d).

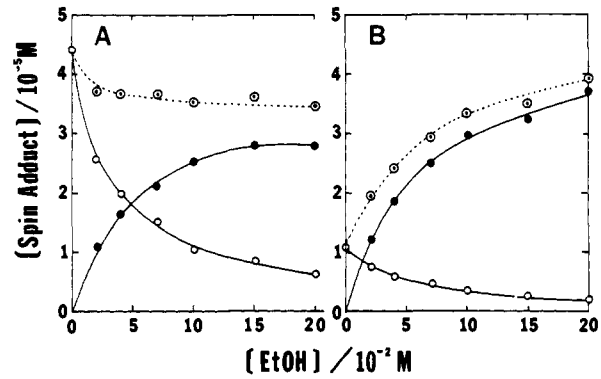


Figure 3. For (A) Fe^{II}DETAPAC and (B) Fe^{II}EDTA, effect of EtOH concentration on the maximum amounts of DMPO spin adducts accumulated in Fe^{II}-H_2O_2 reactions: (○) DMPO–OH; (●) DMPO–Et. [Fe^{II}] = 0.1 mM and [H_2O_2] = 0.2 mM. Dotted lines show the sum of the spin adducts.

for HO•. As seen in Figure 2, benzoate also inhibited the accumulation of DMPO–OH. In this case, however, we did not detect the formation of a new spin adduct which might be expected from benzoate. Probably, free radicals formed from the reaction of benzoate with HO• decayed without formation of the stable DMPO spin adducts. Figure 2 also shows that DMPO–OH gradually decomposed by some unknown mechanism when the reaction was started with high benzoate concentrations.

In spin-trapping experiments, EtOH has frequently been used for the confirmation of HO• formation, because the EPR signal of DMPO–OH is efficiently converted to that of DMPO–Et when EtOH is added to an HO•-generating system in the presence of DMPO.[12] Figure 3 shows the effect of EtOH concentration on the amounts of DMPO–OH and DMPO–Et accumulated during the reaction. Figure 3A shows that EtOH competed with DMPO for HO• and that the DMPO–OH lost in the presence of EtOH was recovered as DMPO–Et at a yield of about 75% in the reaction of Fe^{II}DETAPAC with H_2O_2. We assumed that DMPO–Et was

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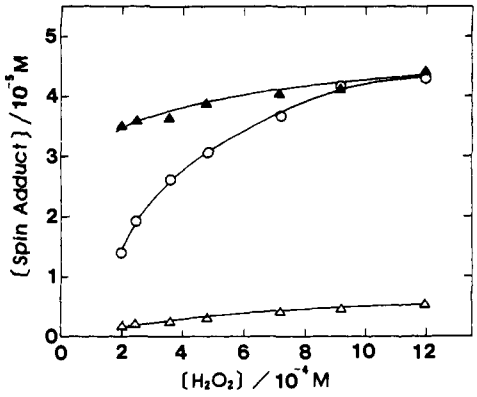

Figure 4. Effect of H_2O_2 concentration on the maximum amounts of DMPO spin adducts accumulated in a $Fe^{II}EDTA-H_2O_2$ reaction: (○) DMPO–OH at [EtOH] = 0; (△) DMPO–OH and (▲) DMPO–Et at [EtOH] = 0.2 M. [Fe^{II}] = 0.1 mM. In this experiment DMPO was premixed in solution B (see Experimental Section).

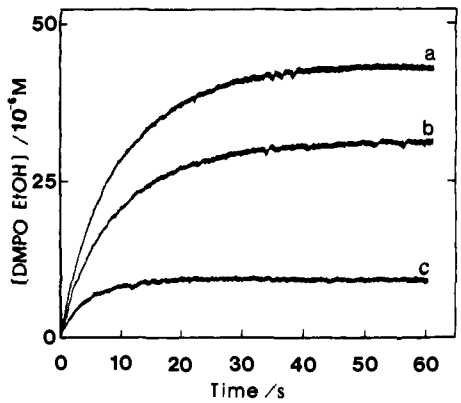

Figure 5. Effect of benzoate on DMPO–Et formation in a Fe^{II}DETAPAC–H_2O_2–EtOH reaction. [Fe^{II}] = 0.1 mM, [H_2O_2] = 0.2 mM, and [EtOH] = 0.4 M. The magnetic field was set at a peak of the second line of the DMPO–Et EPR signal. [Benzoate] = 0 (a), 20 mM (b), and 100 mM (c).

formed only through the oxidation of EtOH by HO• with a small loss of the spin:

$$CH_3CH_2OH + HO^\bullet \rightarrow CH_3\dot{C}HOH + H_2O \quad (2)$$

$$DMPO + CH_3\dot{C}HOH \rightarrow DMPO\text{–}Et \quad (3)$$

When DETAPAC was replaced by EDTA, the DMPO–OH formation decreased by about one-quarter in the absence of EtOH, but the addition of 0.1 M EtOH produced about 4 times more DMPO–Et than the amount of DMPO–OH lost (Figure 3B). With an increase in the DMPO concentration above 40 mM, we observed a contamination of the EPR signal with unidentified signals in our Fe^{II}EDTA reaction systems; therefore we carried out the experiments at [DMPO] = 20 mM, where about 90% of the HO• formed was trapped by DMPO. Despite this incomplete spin trapping, Figure 3B clearly indicates that EtOH was oxidized not only by HO• but also by another chemical species. It would be reasonable to assume the existence of a high-valence iron species such as the ferryl ion in the reaction of Fe^{II}EDTA with H_2O_2.

The formation of high-valence iron species in the $Fe^{II}EDTA-H_2O_2$ reaction greatly depended upon the H_2O_2 concentration. The amount of accumulated DMPO–OH increased markedly as the H_2O_2 concentration was increased (Figure 4). In the presence of 0.2 M EtOH, however, the increase of H_2O_2 concentration resulted in only a slight increase in the amount of DMPO–Et (Figure 4). Since this DMPO–Et increment was much less than the concomitant increase in the amount of DMPO–OH lost (○ minus △ in Figure 4) in the presence of EtOH, we concluded that the high-valence iron species concentration actually decreased with an increase in H_2O_2 concentration. This suggests that the oxidizing species in the $Fe^{II}EDTA-H_2O_2$ reaction changed from the high-

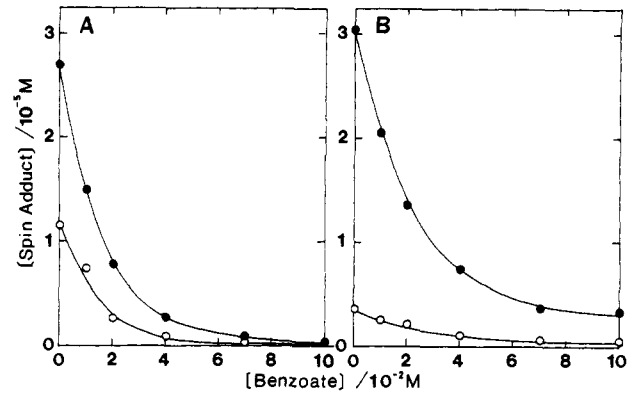

Figure 6. For (A) Fe^{II}DETAPAC and (B) Fe^{II}EDTA, effect of benzoate concentration on the maximum amounts of DMPO spin adducts accumulated in $Fe^{II}-H_2O_2$–EtOH reactions: (○) DMPO–OH; (●) DMPO–Et. [Fe^{II}] = 0.1 mM, [H_2O_2] = 0.2 mM, and [EtOH] = 0.1 M.

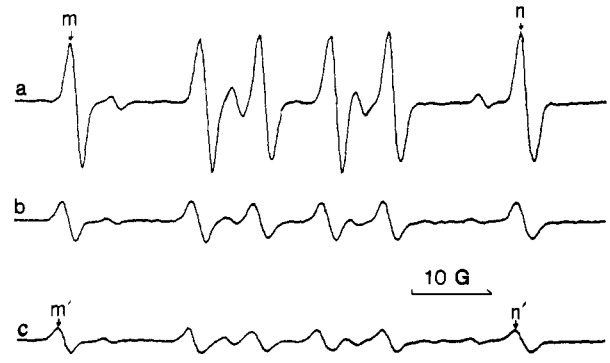

Figure 7. Effect of *t*-BuOH on the reaction of Fe^{II}EDTA with H_2O_2 in the presence of EtOH: (a) [EtOH] = 0.1 M; (b) [EtOH] = 0.1 M and [*t*-BuOH] = 1.2 M; (c) [*t*-BuOH] = 1.2 M. [Fe^{II}] = 0.1 mM and [H_2O_2] = 0.2 mM. The spin concentrations are shown in Table I. All spectra were taken at the same gain. Arrows (m, m′, n, and n′) indicate magnetic fields of the signal peaks.

valence iron species to HO• with the increase in H_2O_2 concentration. At these higher H_2O_2 concentrations, the reaction was over in about 1 s and the autoxidation of Fe^{II}EDTA could be neglected. In the $Fe^{II}DETAPAC-H_2O_2$ reaction, no significant increase in DMPO spin adducts was observed over the entire range of H_2O_2 concentrations, which is shown in Figure 4.

Benzoate was an effective HO• scavenger, as shown in Figure 2, but very high concentrations of benzoate were needed to inhibit the HO•-mediated formation of DMPO–Et in the reaction with Fe^{II}DETAPAC (Figure 5). Benzoate is competing only with DMPO for HO• in Figure 2, but in Figure 5 the competition is mainly with EtOH because the EtOH concentration was much higher than that of DMPO.

Figure 6 shows the effect of benzoate concentration on the amounts of DMPO–OH and DMPO–Et accumulated during the reactions of H_2O_2 with Fe^{II}DETAPAC and Fe^{II}EDTA in the presence of 0.1 M EtOH. The molar concentrations of spin adducts accumulated at [benzoate] = 0 were 2.8 μM DMPO–OH and 30.6 μM DMPO–Et for EDTA and 11.7 μM DMPO–OH and 25.2 μM DMPO–Et for DETAPAC. In the DETAPAC system (Figure 6A), both [DMPO–OH] and [DMPO–Et] decreased to about half at [benzoate] = 15 mM. The result could simply be explained in terms of the scavenging effect of benzoate on HO•. In the EDTA system (Figure 6B), the concentration of DMPO–Et formed from the reaction of EtOH with the high-valence iron species is roughly estimated to be as follows: total [DMPO–Et] minus [DMPO–Et] derived from HO• (30.6 μM–8.2 μM = 22.4 μM), which was reduced to 3.0 μM in the presence of 0.1 M benzoate (Table I). The important conclusion derived from this result is that benzoate scavenged not only HO• but the high-valence iron species as well in the $Fe^{II}EDTA-H_2O_2$ reaction. In the previous analyses of the overall kinetics of the Fenton

Table II. Effect of Scavenger Concentrations on k/k_{DMPO}[a]

[scavenger], mM	k/k_{DMPO} DETAPAC	k/k_{DMPO} EDTA	[scavenger], mM	k/k_{DMPO} DETAPAC	k/k_{DMPO} EDTA
		EtOH Scavenger			
20	0.71 (41)	0.38 (28)	100	0.67 (77)	0.34 (65)
40	0.59 (54)	0.34 (41)	150	0.56 (81)	0.38 (74)
70	0.56 (67)	0.36 (56)	200	0.59 (83)	0.38 (80)
		Benzoate Scavenger			
1	5.3 (21)		8	12.7 (84)	
2	5.3 (34)		10		4.0 (66)
4	7.7 (60)		15		4.0 (75)
5		3.4 (46)	20		4.3 (78)

[a] Percent inhibition for DMPO–OH accumulation is shown in parentheses.

reaction, it has been assumed that benzoate and *t*-BuOH scavenge HO•, but not the high-valence iron species.[7,8] Since Figure 6B shows that benzoate scavenged the high-valence iron species as well as HO•, we then tried to see whether or not *t*-BuOH also reacts with the high-valence iron species.

In Figure 7, we examined the effect of *t*-BuOH on the DMPO–Et accumulation during the reaction with Fe^{II}EDTA. In this case, however, the analysis was not simple because DMPO–Et and DMPO–tB gave similar EPR spectra. The splitting constants were measured to be A_N = 15.92 G and A_H = 23.10 G for DMPO–Et and A_N = 16.02 G and A_H = 23.58 G for DMPO–tB. These values produced a slight difference in the peak m to peak n separation shown in Figure 7. Computer simulation showed that the difference was proportional to the mixing ratio of the two signals, and spectrum b in Figure 7 was calculated to be for a mixture of 1.0 μM DMPO–OH, 3.6 μM DMPO–Et, and 10.5 μM DMPO–tB (Table I). Similarly as described above, the concentration of DMPO–Et formed via the high-valence iron species was decreased from 22.4 to 3.6 μM by the addition of 1.2 M *t*-BuOH. Therefore, we concluded that *t*-BuOH also scavenged the high-valence iron species and that a part of the lost DMPO–OH and DMPO–Et was recovered as DMPO–tB:

$$CH_3CH_2OH + Fe^{IV}{=}O \rightarrow CH_3\dot{C}HOH + Fe^{III} + HO^- \quad (4)$$

$$\text{benzoate} + Fe^{IV}{=}O \rightarrow \text{benzoate radical} + Fe^{III} \quad (5)$$

$$t\text{-BuOH} + Fe^{IV}{=}O \rightarrow \text{monodehydro } t\text{-BuOH} + Fe^{III} + HO^- \quad (6)$$

$$\text{DMPO} + \text{monodehydro } t\text{-BuOH} \rightarrow \text{DMPO–tB} \quad (7)$$

The possibility that benzoate and *t*-BuOH inhibited the accumulation of DMPO–Et through their oxidation by $CH_3\dot{C}HOH$ was excluded because $CH_3\dot{C}HOH$ is known to act as a reductant:[7b,13]

$$CH_3\dot{C}HOH + Fe^{III} \rightarrow CH_3CHO + Fe^{II} + H^+ \quad (8)$$

DMPO–tB once formed was stable as well as DMPO–OH and DMPO–Et under the present experimental conditions.

Similar experiments were carried out with histidine, formate, mannitol, and acetate in order to investigate the reactivity of the high-valence iron species. Histidine, mannitol, and formate were found to react with the high-valence iron species. The EPR signals of DMPO–Et and DMPO–OH shown in Figure 7a were replaced by that of the DMPO adduct of $CO_2^{\bullet-}$[6] in the presence of 0.2 M formate (spectra not shown). No DMPO spin adduct was derived from histidine and mannitol. The effects of acetate were so weak that we could not conclude whether or not acetate reacted with the iron species at an acetate concentration of 0.3 M.

The rates of the Fenton reactions are relatively slow, being 10^2–10^4 M^{-1} s^{-1}, and vary with the nature of the iron chelators.[9] Therefore, rate constants for the reactions of HO• with EtOH, benzoate, and *t*-BuOH cannot be measured directly in these Fenton reactions. However, from competitive kinetics of the reactions of DMPO and of scavengers for HO•, it was possible to measure relative rate constants, as reported by Finkelstein et al.[12a] Since the DMPO–OH concentration could be measured directly from a peak of the last line of its EPR signal even in the presence of DMPO–Et and DMPO–tB, and since DMPO–OH once formed is stable except in the presence of high benzoate concentrations, the ratio of rate constants was obtained according to the following equation:

$$\frac{k}{k_{DMPO}} = \frac{[\text{DMPO}]([\text{DMPO–OH}]_0 - [\text{DMPO–OH}])}{[\text{scavenger}][\text{DMPO–OH}]} \quad (9)$$

where k_{DMPO} and k are rate constants for the reactions of HO• with DMPO and a scavenger, respectively, and [DMPO–OH] and $[\text{DMPO–OH}]_0$ are concentrations of DMPO–OH accumulated during the reaction in the presence and absence of a scavenger, respectively. In most cases, the k/k_{DMPO} ratio thus obtained at different scavenger concentrations was constant within experimental error (Table II). In the benzoate–Fe^{II}DETAPAC–H_2O_2 reaction, however, the ratio increased as the benzoate concentration was increased because the DMPO–OH formation was slow, being accompanied by the decay of DMPO–OH especially when the reaction was started at high benzoate concentrations (Figure 2). In this case, therefore, the ratio was estimated from data obtained at low benzoate concentrations. The results are summarized in Table III, which also shows the k/k_{DMPO} ratios measured for free HO• produced by photolysis in comparison with those reported previously.[7a,12a]

Table III. k/k_{DMPO} for HO• Formed in Photolysis and the Fenton Systems

	k/k_{DMPO} *t*-BuOH	EtOH	benzoate
photolysis	0.15[a]	0.53[a]	1.6[a]
	0.20	0.38	1.5
Fenton reaction[b]			
DETAPAC	*0.56*[c]	0.59	*5.3*[c]
EDTA	0.17	0.37	*3.4*[c]
ADP	0.15	0.36	2.2
phosphate	0.18	*0.17*[c]	1.9

[a] These ratios were calculated from rate constants (10^9 M^{-1} s^{-1}) for free HO•, being 0.52 for *t*-BuOH, 1.8 for EtOH, 3.4 for DMPO, and 5.5 for benzoate.[7] [b] Rate constants (10^4 M^{-1} s^{-1}) for the Fenton reaction are 0.041 for DETAPAC, 1.4 for EDTA, 0.82 for ADP, and 2.0 for phosphate.[9] [c] The italic ratios differ significantly from those measured for free HO•.

Discussion

The oxidizing species generated in the Fenton reaction have been discussed by many investigators but are still controversial.[14] There may be at least three oxidizing species, namely free HO•, bound (or confined) HO•, and high-valence iron. How can these species be discriminated from each other experimentally? Despite many experimental efforts, a unique solution is not yet known. Since the EPR spin-trapping technique appears to be the most

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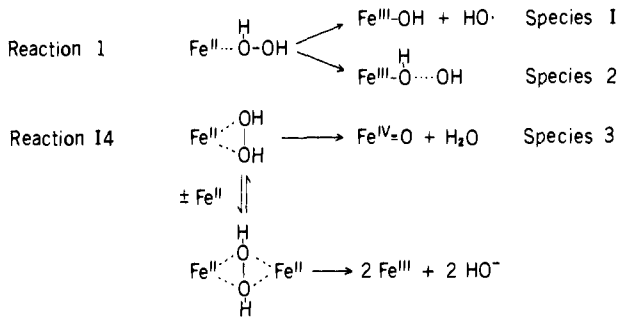
Scheme I



direct method to detect HO•, we will discuss the nature of these oxidizing species on the basis of EPR spin-trapping data. According to our experimental data, the oxidizing species will be classified as follows: (1) free HO• (species 1), which is trapped by DMPO as DMPO–OH and gives the same k/k_{DMPO} ratio as that measured in a photolysis or a pulsed-radiolysis system; (2) bound HO• (species 2), which is also trapped by DMPO as DMPO–OH but gives a k/k_{DMPO} ratio that differs significantly from that measured in a photolysis or a pulsed-radiolysis system, probably because the scavenger reactions with HO• occur through steric or electrostatic interactions with the iron chelators; (3) a high-valence iron species (species 3), which is probably a ferryl ion and is not trapped by DMPO as DMPO–OH but gives DMPO–Et when EtOH is present.

With this classification, a question might be raised as to the possibility of the ferryl ion reacting with DMPO to form DMPO–OH. There may be two possible reaction paths. One of them is a direct reaction of the ferryl ion with DMPO:

$$Fe^{IV}{=}O + DMPO + H^+ \rightarrow Fe^{III} + DMPO\text{–}OH \quad (10)$$

For the second possible reaction path, HO• is suggested to be formed directly from the ferryl ion. The reaction has been formulated by Walling and Amarnath[15] as

$$Fe^{II} + H_2O_2 \rightarrow Fe^{IV}\text{–}OH \rightleftharpoons Fe^{III} + HO^\bullet \quad (11)$$

or by Sugimoto and Sawyer[16] as

$$Fe^{IV}{=}O + H_2O \rightarrow Fe^{III}\text{–}OH + HO^\bullet \quad (12)$$

Reactions 11 and 12 were formulated under conditions of very high Fe^{II} concentrations in a strongly acidic solution and in a dry acetonitrile medium, respectively.

We have no direct evidence that would permit us to exclude these possibilities, but it seems reasonable to conclude that the high-valence iron species does not form DMPO–OH in our aqueous system. If it does, we have to assume the existence of another oxidizing species that does not form DMPO–OH but oxidizes EtOH to the free radical. The oxygen atom transfer via the ferryl ion shown in reaction 10 appears to occur only in organic media.[16,17] Reaction 11 might occur at extremely low pH[15] but not at neutral pH, where the ferryl ion is deprotonated. Although the ferryl ion is a strong oxidant, its reduction potential is considerably lower than that of the $HO^\bullet/H_2O$ couple, as described by Koppenol and Liebman,[18] and reaction 12 can be excluded.

Rahhal and Richter concluded that the reaction of Fe^{II}DETAPAC with H_2O_2 produces a high-valence iron species but not HO•.[8] This conclusion apparently contradicts that derived from the EPR spin-trapping data reported here. Their conclusion is based upon the kinetic and stoichiometric analysis of the overall Fe^{II}DETAPAC–H_2O_2 reaction, the procedure being essentially the same as that described by Walling[14a] and by Rush and Koppenol.[7] They compared the rates of Fe^{III} production and the $\Delta[Fe^{III}]/\Delta[H_2O_2]$ ratios measured in the presence and absence of HO• scavengers. The rate and the stoichiometry are assumed to be modified by specific HO• scavengers if the product in the Fenton reaction is HO• (reaction 1) but not if it is the ferryl ion (reaction 14).

$$Fe^{II} + H_2O_2 \rightarrow Fe^{III} + HO^- + HO^\bullet \quad (1)$$

$$Fe^{II} + HO^\bullet \rightarrow Fe^{III} + HO^- \quad (13)$$

$$Fe^{II} + H_2O_2 \rightarrow Fe^{IV}{=}O + H_2O \quad (14)$$

$$Fe^{II} + Fe^{IV}{=}O + H_2O \rightarrow 2Fe^{III} + 2HO^- \quad (15)[19]$$

The conclusion that HO• is not produced in the Fenton reaction is derived from the result that well-known HO• scavengers such as benzoate and *t*-BuOH do not affect the rate and the stoichiometry of Fe^{III} formation. Therefore, this result has also led to the conclusion that benzoate and *t*-BuOH are significantly less reactive toward the high-valence iron species. Our spin-trapping data, however, clearly show that benzoate and *t*-BuOH inhibit the Fenton reaction in both the Fe^{II}EDTA and the Fe^{II}DETAPAC systems. Furthermore, Figure 1 indicates that *t*-BuOH reacts with an oxidizing species formed in the Fe^{II}DETAPAC–H_2O_2 reaction and a part of the oxidation product of *t*-BuOH is trapped as DMPO–tB. Why Rahhal and Richter did not detect the effect of *t*-BuOH on the Fe^{II}DETAPAC reaction is not clear, but there is the possibility that the reaction of HO• with *t*-BuOH does not change the rate of Fe^{III}DETAPAC formation and the Δ-$[Fe^{III}DETAPAC]/\Delta[H_2O_2]$ ratio when the free radical of *t*-BuOH acts as an oxidant.

$$t\text{-BuOH} + HO^\bullet \rightarrow \text{monodehydro } t\text{-BuOH} + H_2O \quad (16)$$

$$Fe^{II} + \text{monodehydro } t\text{-BuOH} + H^+ \rightarrow Fe^{III} + t\text{-BuOH} \quad (17)$$

Although *t*-BuOH[7,8,20] and benzoate[7,20c,21] have been used as typical HO• scavengers, it should be emphasized here that these molecules scavenge the high-valence iron species as well as HO•.

As mentioned previously, it is not possible to measure directly rate constants for the reactions of HO• formed in the Fenton reaction, because the HO• formation is slow when compared with its decay. By the EPR spin-trapping technique, only the k/k_{DMPO} ratio can be measured; see Table III. If this ratio differs significantly from that measured in a photolysis or a pulsed-radiolysis system, HO• formed in the Fenton reaction cannot be free and must be considered either bound, complexed,[20a] caged,[8] or crypto[22] HO•. If the ratio is the same as that measured in the photolysis system within experimental error, then HO• is very likely to be free. Since the ratio is significantly different in the case of Fe^{II}DETAPAC (Table III), HO• generated from this reaction is concluded to be not free but bound in some type of complex (species 2 in Scheme I). According to our previous paper,[9] characteristic features of the Fe^{II}DETAPAC–H_2O_2 reaction are (1) it forms DMPO–OH most efficiently, (2) it produces no appreciable amount of high-valence iron species, and (3) the rate of DMPO–OH formation is more than 10 times slower than the rates of the other Fe^{II}–H_2O_2 reactions (see footnote *b* in Table III). Probably, the binding of H_2O_2 to Fe^{II}DETAPAC occurs via a constrained conformation, which makes the reaction slower but more efficient for DMPO–OH formation. Because of these and other features, DETAPAC is sometimes reported to act as

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an inhibitor of the Fenton reaction, as discussed in our previous paper.[9]

If we assume that the reaction of species 2 with a scavenger cannot be faster than that of free HO•, the fact that the k/k_{DMPO} ratio is 1.5 for free HO• and 5.0 for HO• in the DETAPAC system has led us to conclude that the reaction of DMPO with HO• formed from Fe^{II}DETAPAC is at least 3 times slower than that with free HO•. HO• formed from the Fenton reaction in the presence of EDTA and phosphate is similarly grouped as species 2. k/k_{DMPO} for the HO• generated from Fe^{II}ADP suggests that it is free HO•. Although the number of scavengers tested here is limited, the results are summarized in such a way that the extent to which the HO• radical is not free but bound in some type of complex increases in the order Fe^{II}ADP < Fe^{II}-phosphate = Fe^{II}EDTA < Fe^{II}DETAPAC. It should also be noted that the Fe^{II}ADP–H_2O_2 system also produces the high-valence iron species even at relatively low Fe^{II} concentrations.[9] This Fe^{II}ADP system has been used as a typical Fenton reagent to induce oxidative damage in tissues.[23]

Our results for the DETAPAC system (Table III), however, contrast with those of Tanigawa,[24] who reported a linear relationship of the rate constants for various scavengers between the free and the Fe^{II}DETAPAC-generated HO• radicals.

Halliwell et al.[25] have reported a similar comparison of rate constants for HO•-scavenger reactions in pulse radiolysis and Fe^{II}EDTA systems. They have determined the rate constants from competitive kinetics in the deoxyribose degradation. In this case, the interpretation of their results is complicated by possible chain reactions involving intermediate radical species and also by the possibility that the reaction is initiated not only by an HO• species but also by the high-valence iron species. The advantage of the spin-trapping method is that it measures only the reaction of the HO• species and thus minimizes the effects of the propagation of chain reactions.

The characteristics of the Fenton reaction can also be analyzed either from the nature of either the free-radical intermediates formed[26] or the final products.[3,15,27] Walling and Amarnath[15] have concluded that a cage reaction of HO• is involved in the reaction of H_2O_2 with Fe^{II} which is complexed with an easily oxidizable ligand. Analyzing EPR spectra of free radicals formed in the Fenton reaction, Shiga has concluded that HO• is involved in the oxidation of benzoic acids[26b] but not in the oxidation of alcohols.[26a] In these reactions, the Fe^{II} concentration used is more than 10 times higher than that used in our results presented here, and one can expect a considerable amount of high-valence iron species to be produced as the oxidizing intermediates.

The analysis of DMPO spin adducts at varying concentrations of EtOH (Figure 3) and benzoate (Figure 6) does not show any difference in reactivity between HO• and the ferryl radical. In order to explain that the mechanism of Fenton reactions depends on the nature and the concentration of the Fe^{II} complexes and that the extent to which HO• is free depends on the nature of the Fe^{II} complexes, we present a tentative scheme for the Fenton reaction. This scheme may also explain the switchover from reaction 14 to reaction 1 by the increase in H_2O_2 concentration in the Fe^{II}EDTA reaction (Figure 4).

An Fe^{II} concentration of about 0.1 mM has frequently been used in the analysis of the Fenton reaction, but oxygen toxicity in biology is thought to be mediated by a few micromolar or less iron concentration:[3a,19c] at such concentrations the Fe^{II}–H_2O_2 reaction generates nearly a stoichiometric amount of DMPO–OH, irrespective of the nature of the Fe^{II} complex.[9] In order to measure the k/k_{DMPO} ratio at these physiological Fe^{II} concentrations, one must use reaction systems that involve the recycling of Fe^{III} in the presence of a suitable reducing system. This study is under investigation in our laboratory.

Registry No. DMPO, 3317-61-1; DETAPAC, 67-43-6; EDTA, 60-00-4; ADP, 58-64-0; DMPO–OH, 55482-03-6; DMPO–Et, 40936-09-2; DMPO–tB, 116186-10-8; H_2O_2, 7722-84-1; HO•, 3352-57-6; *t*-BuOH, 75-65-0; ferrous ion, 15438-31-0; benzoate, 65-85-0; histidine, 71-00-1; mannitol, 87-78-5; formate, 64-18-6; ferryl radical, 73133-33-2.

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