

THE ORIGIN OF THE HYDROXYL RADICAL OXYGEN IN THE FENTON REACTION

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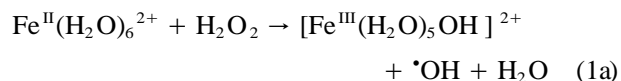
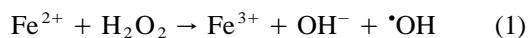
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Abstract—There is an ongoing discussion in the chemical literature regarding the nature of the highly reactive hydroxyl radical formed from the reaction between ferrous iron and hydrogen peroxide (the Fenton reaction). However, the fundamental experiment of directly determining the source of the hydroxyl radicals formed in the reaction has not yet been carried out. In this study, we have used both hydrogen peroxide and water labeled with ^{17}O , together with ESR spin trapping, to detect the hydroxyl radicals formed in the reaction. ESR experiments were run in phosphate buffer with 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) as a spin trap, and either H_2O_2 or H_2O labeled with ^{17}O . The hydroxyl radical was generated by addition of Fe^{2+} ion to H_2O_2 , or as a control, by photolysis of H_2O_2 in the ESR cavity. Observed ESR spectra were the sum of DMPO/ ^{16}OH and DMPO/ ^{17}OH radical adduct spectra. Within experimental accuracy, the percentage of ^{17}O -labeled hydroxyl radical trapped by the DMPO was the same as in the original hydrogen peroxide, for either method of hydroxyl radical generation, indicating that the trapped hydroxyl radical was derived exclusively from hydrogen peroxide and that there was no exchange of oxygen atoms between H_2O_2 and solvent water. Likewise, the complementary reaction with ordinary H_2O_2 and ^{17}O -labeled water also showed that none of the hydroxyl radical was derived from water. Our results do not preclude the ferryl intermediate, $[\text{Fe}=\text{O}]^{2+}$ reacting with DMPO to form DMPO/ $^{\bullet}\text{OH}$ if the ferryl oxygen is derived from H_2O_2 rather than from a water ligand. Copyright © 1997 Elsevier Science Inc.

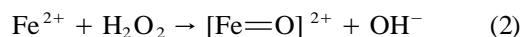
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INTRODUCTION

There is an ongoing discussion in the chemical literature regarding the nature of the highly-reactive hydroxyl radical formed from the reaction between ferrous iron and hydrogen peroxide (the Fenton reaction).¹ The classical Fenton mechanism (Eq. 1, or with ligands included, Eq. 1a) predicts that hydrogen peroxide is reduced at the iron center with generation of free hydroxyl radical.



It has been argued, however, that some, if not all of the hydroxyl radical produced in the Fenton reaction may remain bound at the iron center, either as the $[\text{Fe} \cdots \cdot\text{OH}]^{3+}$ or the $[\text{Fe}=\text{O}]^{2+}$ intermediate (Eq. 2).



These intermediates are proposed to have oxidizing properties similar to, but distinguishable from, free hydroxyl radical, and have been promoted based on comparison of Fenton reaction kinetics with that of the hydroxyl radical generated independently of iron.

Recently Sawyer et al.² studied ‘‘Fenton reagents’’ with hydrocarbons as radical scavengers. From product analysis, they concluded that free $\cdot\text{OH}$ is not the dominant reactant at all, and that with chelated iron, a nucleophilic adduct reacts directly with substrates. Wink et al.³ used stopped-flow kinetics and competition stud-

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ies to probe the reaction of a Fenton intermediate with *N*-nitrosodimethylamine. They proposed a reversible reaction between Fe^{2+} and H_2O_2 to an intermediate X, whose reactivity patterns were consistent with an iron complex and not the $\cdot\text{OH}$ radical. However, Walling and Amarnath,⁴ in a study of the oxidation of a mandelic acid–iron complex by H_2O_2 , had previously concluded that both an Fe^{IV} species and the hydroxyl radical were involved.

For reactions involving free radical intermediates, ESR spectroscopy would appear to be the method of choice, but the $\cdot\text{OH}$ radical in solution cannot be directly detected by ESR. Thus, indirect techniques must be used, such as spin trapping with 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO). Based on relative yields in competitive trapping experiments, Yamazaki and Piette^{5,6} presented evidence for the presence of additional oxidizing species other than hydroxyl radical and stated that, although the $\cdot\text{OH}$ radical is present, it is not all free in solution. However, it has been shown that, under reaction conditions commonly employed, the DMPO/ $\cdot\text{OH}$ adduct can arise as an artifact, such as by oxidation of the DMPO itself followed by reaction with water, or by reaction of a precursor DMPO/superoxide adduct.^{7,8}

Although the reactivity of iron complexes has been recognized,² the existence of “secondary” reactions having different reaction rates for different complexes has not always been considered. Burkitt⁷ has shown that such reactions can involve complexed iron with spin trap-hydroxyl radical adducts under commonly employed reaction conditions. For example, the Fe^{III} DTPA (diethylenetriaminepentaacetic acid) complex, in particular, can be reduced by oxidation of the DMPO/ $\cdot\text{OH}$ spin adduct. If not recognized, these reactions can confuse kinetic comparisons and stoichiometric calculations in the Fenton reaction.

Scavenging experiments can also be difficult to interpret if the radicals formed from the scavenger molecules are capable of further reaction. Rush and Kopenol^{9,10} studied the Fenton reaction of Fe^{II} EDTA, Fe^{II} DTPA, and Fe^{II} HEDTA with alcohol scavengers and recognized that the nature of the chelator was an important factor. Results with Fe^{II} HEDTA suggested an intermediate other than hydroxyl radical, but, in the other two cases, the properties of the intermediate were reported to be similar to the hydroxyl radical. Rahhal and Richter¹¹ reported that Fe^{II} DTPA reacted with H_2O_2 to yield an oxidizing species whose properties in scavenging experiments with *tert*-butyl alcohol were not consistent with the $\cdot\text{OH}$ radical (Eq. 3).



Croft *et al.*¹² have shown, however, that the radicals formed from alcohols in the Fenton reaction can react with both iron(II) and iron(III) to yield kinetic results that can appear to differ from the hydroxyl radical.

According to existing mechanisms, the oxygen atom in both the ferryl intermediate and the hydroxyl radical originate from hydrogen peroxide, but this has not been tested. Furthermore, if free hydroxyl radical reacts with water, an exchange of oxygen atoms would occur. A more likely possibility is that iron-bound hydroxyl radical species would undergo exchange of oxygen with water. In any case, the fundamental experiment of directly determining the source of the hydroxyl radicals formed has not yet been carried out. In this study, we have used both hydrogen peroxide and water labeled with ^{17}O , together with ESR spin trapping, to detect the hydroxyl radicals formed in the reaction. We show that, within experimental accuracy, in the Fenton reaction there is no exchange of oxygen atoms between ^{17}O -labeled H_2O_2 and solvent water or vice-versa.

MATERIALS AND METHODS

Experiments were run in 100 mM phosphate buffer (pH 7.4) with 100 mM 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO, Sigma Chemical Co., St. Louis, MO) added as a spin trap. The DMPO was purified twice by vacuum distillation at room temperature and stored at -80°C before use, and the buffer was treated with Chelex® 100 ion-exchange resin (Bio-Rad Laboratories, Hercules, CA) to remove trace heavy metal contaminants. The ^{17}O -labeled H_2O_2 was from Isotec, Inc. (Miami, OH), and was received as a 2% aqueous solution, 82 atom % ^{17}O . For each experiment it was diluted fourfold to a final concentration of 0.5%. In the complementary experiment, the Fenton reaction was run with normal H_2O_2 in ^{17}O -enriched water (52.7 atom %, Isotec, Inc.). ESR spectra were obtained on a Varian E-109 ESR spectrometer with a TM_{110} cavity and a quartz aqueous flat cell. Spectra were stored on a computer for later analysis. The hydroxyl radical was generated by addition of 1 mM Fe^{2+} ion (as FeSO_4) to the reaction mixture. As a control, the hydroxyl radical was also generated directly by photolysis in the ESR cavity with a 1-kW Hanovia Xe/Hg compact-arc lamp in a Schoeffel housing.¹³ The solution for photolysis experiments was identical to the Fenton solution except without added iron. Experiments were run at least in triplicate. ESR hyperfine splittings and relative intensities were obtained by means of the program WINSIM.¹⁴

RESULTS AND DISCUSSION

The observed spectra were the sum of the DMPO/ $\cdot^{16}\text{OH}$ and DMPO/ $\cdot^{17}\text{OH}$ radical adduct spectra. The

Table 1. ESR Parameters for the DMPO/ $\cdot\text{OH}$ Radical Adduct

Method	Atom % ^{17}O	Radical	Hyperfine Coupling Constants, Gauss			% DMPO/ $\cdot^{17}\text{OH}$ Radical ^a
			N	H	$^{17}\text{O}^b$	
$h\nu$, $\text{H}_2^{17}\text{O}_2$	82	DMPO/ ^{17}OH	15.04	14.80	4.68	76.9 ± 1.7^c
$h\nu$, $\text{H}_2^{17}\text{O}_2$	82	DMPO/ ^{16}OH	15.00	14.88	—	—
Fe^{2+} , $\text{H}_2^{17}\text{O}_2$	82	DMPO/ ^{17}OH	15.04	14.80	4.68	80.4 ± 1.7
Fe^{2+} , $\text{H}_2^{17}\text{O}_2$	82	DMPO/ ^{16}OH	15.00	14.84	—	—
Fe^{2+} , H_2^{17}O	52.7	DMPO/ ^{17}OH	15.20	14.45	—	<1

^a The sum of the ^{17}OH and ^{16}OH radical intensities was normalized to 100%.

^b Literature value for $^{17}\text{O} = 4.66 \text{ G}$.¹⁵

^c Average $\pm \frac{1}{2}$ range.

results of the experiments are given in Table 1. Typical experimental spectra with simulations based on the parameters in Table 1 are shown in Figure 1.

Within the limits of experimental uncertainty, the percentage of ^{17}O -labeled hydroxyl radical trapped by the DMPO was the same as in the original hydrogen peroxide for either method of hydroxyl radical generation, indicating that the trapped hydroxyl radical was derived exclusively from hydrogen peroxide and not from water. Likewise, the Fenton reaction with ordi-

nary H_2O_2 and ^{17}O -labeled water also showed that, in the complementary reaction, none of the hydroxyl radical was derived from the water solvent. In either case, trapping with DMPO was sufficiently rapid to prevent any significant scrambling of the hydroxyl radical with the solvent water.

Because photolysis of hydrogen peroxide produces hydroxyl radicals by homolytic cleavage, these experiments indicated an insignificant exchange of ^{17}O before trapping by DMPO (Table 1). Comparison of the photolysis results with those for hydroxyl radical generated via the Fenton reaction, shows that, in the latter reaction, the labeled radical was also trapped before any isotopic exchange could occur. Equations 2 and 3 above imply that a significant fraction of the hydroxyl radicals formed are bound in an iron complex. If this is the case, dissociation of the radical as the original ^{17}O -labeled species must not be in competition with intramolecular electron transfer involving bound water molecules, resulting in isotope scrambling. In addition, our results do not preclude the ferryl intermediate $[\text{Fe}=\text{O}]^{2+}$ reacting directly with DMPO to form DMPO/ $\cdot\text{OH}$ if the ferryl oxygen is derived from H_2O_2 and does not exchange with a water ligand, but do exclude an exchangeable oxygen in any iron complex with hydroxyl radical-like reactivity or that such a species dissociates to hydroxyl radical. Finally, the possibility of a DMPO cation radical intermediate that reacts with hydroxide anion to form DMPO/ $\cdot\text{OH}$ ^{8,16} is excluded in the generation of DMPO/ $\cdot\text{OH}$ by the Fenton reaction or by the photolysis of H_2O_2 .

REFERENCES

- Sutton, H. C.; Winterbourn, C. C. On the participation of higher oxidation states of iron and copper in Fenton reactions. *Free Rad. Biol. Med.* **6**:53–60; 1989.
- Sawyer, D. T.; Kang, C.; Llobet, A.; Redman, C. Fenton reagents (1:1 $\text{Fe}^{\text{II}}/\text{HO}_2\text{H}$) react via $[\text{L}_x\text{Fe}^{\text{II}}\text{OOH}(\text{BH}^+)]$ (1) as hydroxylases ($\text{RH} \rightarrow \text{ROH}$), not as generators of free hydroxyl radicals (HO^\cdot). *J. Am. Chem. Soc.* **115**:5817–5818; 1993.

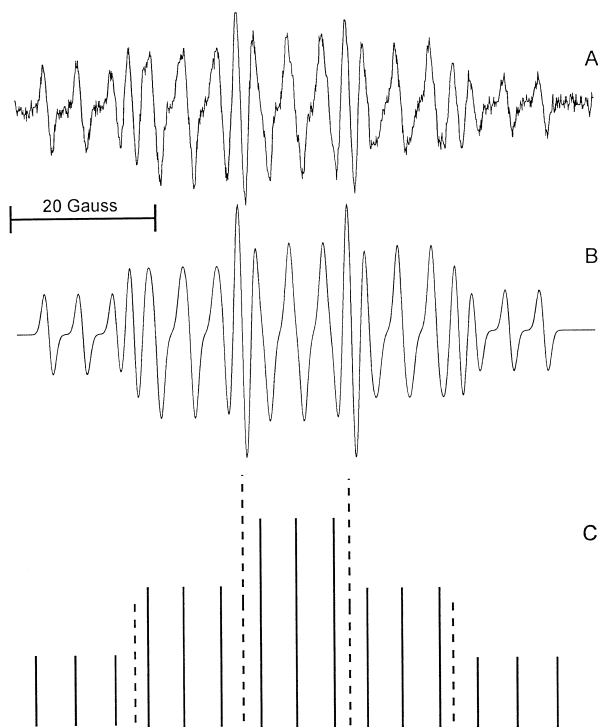


Fig. 1. A: First-derivative ESR spectrum of the DMPO/ $\cdot\text{OH}$ adduct; 0.5% $\text{H}_2^{17}\text{O}_2$, 1 mM FeSO_4 , 100 mM DMPO, 100 mM phosphate buffer (Chelex treated, pH 7.4). B: Computer simulation of spectrum A, based on the parameters in Table 1. C: Stick diagram. (---), Spectrum of the DMPO/ ^{16}OH radical. (—), Spectrum of the DMPO/ ^{17}OH radical.

3. Wink, D. A.; Nims, R. W.; Saavedra, J. E.; Utermahlen, W. E., Jr.; Ford, P. C. The Fenton oxidation mechanism: Reactivities of biologically relevant substrates with two oxidizing intermediates differ from those predicted for the hydroxyl radical. *Proc. Natl. Acad. Sci. USA* **91**:6604–6608; 1994.
4. Walling, C.; Amarnath, K. Oxidation of mandelic acid by Fenton's reagent. *J. Am. Chem. Soc.* **104**:1185–1189; 1982.
5. Yamazaki, I.; Piette, L. H. EPR spin-trapping study on the oxidizing species formed in the reaction of the ferrous ion with hydrogen peroxide. *J. Am. Chem. Soc.* **113**:7588–7593; 1991.
6. Yamazaki, I.; Piette, L. H. ESR Spin-trapping studies on the reaction of Fe²⁺ ions with H₂O₂-reactive species in oxygen toxicity in biology. *J. Biol. Chem.* **265**:13589–13594; 1990.
7. Burkitt, M. J. 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. *Free Rad. Res. Comms.* **18**:43–57; 1993.
8. Chandra, H.; Symons, M. C. R. Hydration of spin-trap cations as a source of hydroxyl adducts. *J. Chem. Soc., Chem. Commun.* 1301–1302; 1986.
9. Rush, J. D.; Koppenol, W. H. Oxidizing intermediates in the reaction of ferrous EDTA with hydrogen peroxide. *J. Biol. Chem.* **261**:6730–6733; 1986.
10. Rush, J. D.; Koppenol, W. H. The reaction between ferrous polyaminecarboxylate complexes and hydrogen peroxide: An investigation of the reaction intermediates by stopped flow spectrophotometry. *J. Inorg. Biochem.* **29**:199–215; 1987.
11. Rahhal, S.; Richter, H. W. Reduction of hydrogen peroxide by the ferrous iron chelate of diethylenetriamine-*N,N,N',N',N''*-pentaacetate. *J. Am. Chem. Soc.* **110**:3126–3133; 1988.
12. Croft, S.; Gilbert, B. C.; Lindsay Smith, J. R.; Whitwood, A. C. An E.S.R. investigation of the reactive intermediate generated in the reaction between Fe^{II} and H₂O₂ in aqueous solution. Direct evidence for the formation of the hydroxyl radical. *Free Rad. Res. Comms.* **17**:21–39; 1992.
13. Harbour, J.; Chow, V.; Bolton, J. R. An electron spin resonance study of the spin adducts of OH and HO₂ radicals with nitrones in the ultraviolet photolysis of aqueous hydrogen peroxide solutions. *Can. J. Chem.* **52**:3549–3553; 1974.
14. Duling, D. R. Simulation of multiple isotropic spin-trap EPR spectra. *J. Magn. Res., Ser. B* **104**:105–110; 1994.
15. Kondo, T.; Krishna, C. M.; Riesz, P. Effect of non-volatile scavengers of hydroxyl radicals on thymine radical formation induced by gamma rays and ultrasound. *Int. J. Radiat. Biol.* **53**:891–899; 1988.
16. Zubarev, V. E.; Brede, O. Generation of α -aminoxylcarbenium ions by electron-transfer oxidation of *N*-*tert*-butyl-3-phenyloxazirane and their role in nitron spin trapping chemistry. *J. Chem. Soc., Perkin Trans. 2.* 2183–2187; 1995.