

SPIN TRAPPING STUDY OF SUPEROXIDE PRODUCTION IN FERROUS ION OXIDATION

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The oxidation of Fe^{2+} was investigated by electron paramagnetic resonance (EPR) spin trapping techniques with *N-t*-butyl- α -phenylnitrone (PBN) and dimethyl sulfoxide. Under pure oxygen, the spin adduct $\text{PBN} \cdot \text{OCH}_3$ was rapidly generated by the addition of Fe^{2+} (0.2–1.2 mM) into phosphate buffer containing ethylenediaminetetraacetate (EDTA), dimethyl sulfoxide and PBN at pH 7.4, but it decayed. The decay process of $\text{PBN} \cdot \text{OCH}_3$ consists of two components. The fast decay was dependent on Fe^{2+} concentration. Another was due to destruction of the spin adduct by superoxide anion ($\cdot\text{O}_2^-$), because superoxide dismutase (SOD) markedly prevented the decay. Catalase decreased the yield of $\text{PBN} \cdot \text{OCH}_3$. When Fe^{3+} -EDTA and ascorbate were used instead of Fe^{2+} -EDTA, similar phenomena were detected. These results demonstrate that Fe^{2+} reacts with O_2 to generate $\cdot\text{O}_2^-$, then H_2O_2 , which produces $\cdot\text{CH}_3$ by the reaction with Fe^{2+} and dimethyl sulfoxide. The $\cdot\text{OCH}_3$ radical results from the reaction between $\cdot\text{CH}_3$ and O_2 . The adduct $\text{PBN} \cdot \text{OCH}_3$ decays by the reaction with Fe^{2+} and $\cdot\text{O}_2^-$.

KEY WORDS: EPR, Spin trapping, Iron, Ferrous, Ferric, Superoxide, Oxidation, Ascorbate
Abbreviations EPR, electron paramagnetic resonance; EDTA, ethylenediaminetetraacetic acid; $(\text{CH}_3)_2\text{SO}$, dimethyl sulfoxide; PBN, *N-t*-butyl- α -phenylnitrone; SOD, superoxide dismutase; Tempol (2,2,6,6-tetramethyl-4-hydroxypiperidine-1-oxyl); AH^- , ascorbate monoanion; $\cdot\text{A}^-$, ascorbate radical.

INTRODUCTION

The reaction between Fe^{2+} and O_2 is a fundamental process in biological systems. The mechanism is still a subject of controversy¹⁻⁵. There have been no reports on spin trapping of $\cdot\text{O}_2^-$ produced in the reaction between Fe^{2+} and O_2 , as well as in the Udenfriend system (Fe^{3+} + ascorbate + O_2), because of the difficulty to detect $\cdot\text{O}_2^-$ directly in Fe^{2+} oxidation, i.e., most of $\cdot\text{O}_2^-$ may react with Fe^{3+} and Fe^{2+} .^{2,3}

For *N-t*-butyl- α -phenylnitrone (PBN), it was impossible to detect stable nitroxide species because of the very short half-life of $\cdot\text{OH}$ or $\cdot\text{O}_2^-$ spin adducts of PBN⁶. In a reaction system that contains $(\text{CH}_3)_2\text{SO}$ in excess of PBN, $(\text{CH}_3)_2\text{SO}$ reacts rapidly with $\cdot\text{OH}$, but not with $\cdot\text{O}_2^-$, to yield methyl radical ($\cdot\text{CH}_3$)⁷⁻⁹. Recently, Britigan *et al.*⁸ found that $\text{PBN} \cdot \text{OCH}_3$, which is fairly resistant to $\cdot\text{O}_2^-$, is formed by $\cdot\text{OH}$ in the xanthine oxidase system or in the Fenton reaction under air in the presence of $(\text{CH}_3)_2\text{SO}$. Thus, it seems worthy to try the spin trapping by PBN in the presence of $(\text{CH}_3)_2\text{SO}$ to investigate the reaction between Fe^{2+} and O_2 .

We report here marked formation of $\text{PBN} \cdot \text{OCH}_3$ in the autoxidation of Fe^{2+} -EDTA, during the reaction between Fe^{3+} -EDTA and ascorbate (the Udenfriend Reaction)¹⁰, showing the involvement of $\cdot\text{O}_2^-$ in these reactions.

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MATERIALS AND METHODS

Spin Trapping

EPR detection of spin adducts was performed with a Varian E12 EPR spectrometer (Varian Associates, Palo Alto, CA). Reactions were started by mixing a 900 μ l solution containing PBN, $(\text{CH}_3)_2\text{SO}$ and EDTA (final concentration; 10, 140 and 6.5 mM, respectively) in 50 mM phosphate buffer (pH 7.4) with a 100 μ l anaerobic solution containing ferrous ions at 20°C. Therefore, the final concentration of O_2 was about 0.25 mM (air-bubbled) or 1.2 mM (pure O_2 -bubbled). The anaerobiosis had been attained by bubbling with nitrogen gas. The solution of ferrous ion was prepared before every experiment by dissolving ferrous sulfate in anaerobic distilled water containing 0.0012 N HCl¹¹. The premixing of ferrous ion with EDTA was avoided because ferrous ions became more autooxidizable in the presence of EDTA^{12,13}. Boiled superoxide dismutase (SOD) was prepared by heating for 30 min in an autoclave. Desired reaction mixtures (1 ml) were prepared in microtubes and transferred to a quartz EPR flat cell, which was in turn placed in the cavity of the EPR spectrometer. Sequential EPR scans were then recorded at 20°C. The EPR spectrometer settings were incident microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 0.8 G; response time, 1 s; and sweep rate, 12.5 G/min. Spin concentration of $\text{PBN}/\cdot\text{OCH}_3$ was determined by double integration of the EPR signal using a Tempol (2,2,6,6-tetramethyl-4-hydroxypiperidine-1-oxyl) solution as an integration standard¹².

RESULTS

1) Reactions performed in air and O_2 with Fe^{2+} -EDTA

When 0.1 mM ferrous ion was added into phosphate buffer containing $(\text{CH}_3)_2\text{SO}$, PBN and EDTA under air, two spin adducts of PBN were detected. One nitroxide showed the hyperfine splitting constants $A_N = 16.56 \pm 0.39$ G ($n = 8$) and $A_H = 3.58 \pm 0.12$ G ($n = 6$) and was assigned as $\text{PBN}/\cdot\text{CH}_3$ ⁸. The other showed $A_N = 15.16 \pm 0.25$ G ($n = 8$) and $A_H = 3.45 \pm 0.10$ G ($n = 6$) and was $\text{PBN}/\cdot\text{OCH}_3$ ⁸. When ferrous ion was increased to 0.88 mM, reactions performed in air yielded mostly $\text{PBN}/\cdot\text{CH}_3$, which was stable over 30 min.

Under pure oxygen, initially $\text{PBN}/\cdot\text{OCH}_3$ appeared by 0.88 mM Fe^{2+} , but it decayed rapidly, and the presence of a small amount of $\text{PBN}/\cdot\text{CH}_3$ was recognized. Figure 1 shows the time course of $\text{PBN}/\cdot\text{OCH}_3$. SOD (500 units/ml), but not boiled SOD, prevented $\text{PBN}/\cdot\text{OCH}_3$ degradation and $\text{PBN}/\cdot\text{CH}_3$ generation. The addition of catalase (750 units/ml) diminished $\text{PBN}/\cdot\text{OCH}_3$ formation. When catalase concentration was increased to 6500 units/ml, $\text{PBN}/\cdot\text{OCH}_3$ were markedly decreased. Simultaneous addition of SOD and catalase protected $\text{PBN}/\cdot\text{OCH}_3$ from decomposition (Figure 1).

2) Reactions following O_2 purging with high Fe^{2+}

Fe^{2+} in EDTA was increased from 0.88 mM to 1.2 mM, nearly equal to the concentration of dissolved O_2 . The decay of $\text{PBN}/\cdot\text{OCH}_3$ was markedly enhanced and the signal of $\text{PBN}/\cdot\text{CH}_3$ was intensified. SOD (500 units/ml) did not fully suppress $\text{PBN}/\cdot\text{OCH}_3$ degradation while $\text{PBN}/\cdot\text{CH}_3$ was hardly detected. Catalase (750

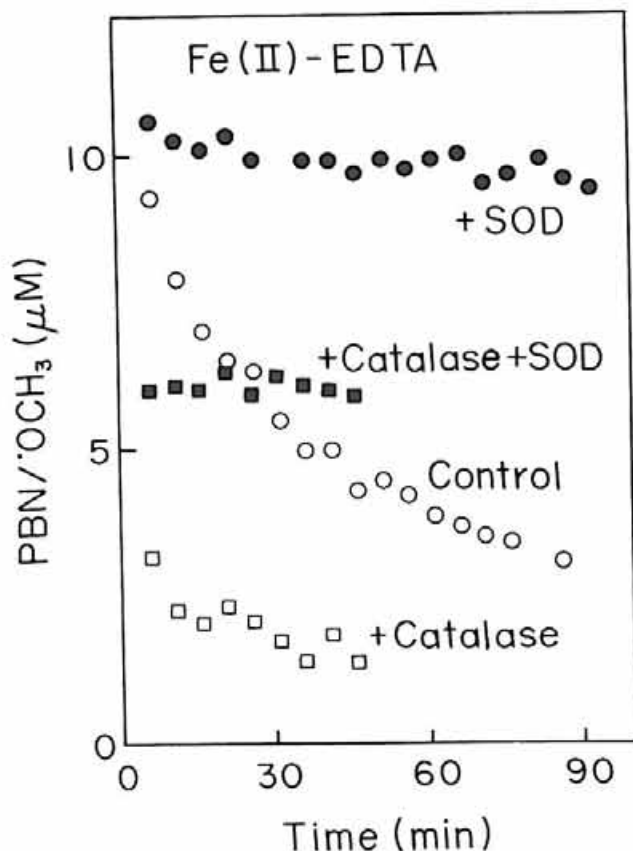


FIGURE 1 Formation and decay of PBN/ \cdot OCH₃ by Fe²⁺-EDTA. Spin concentration was calculated, comparing the upper portion of the first derivative spectra of the rightest of the six peaks of PBN/ \cdot OCH₃ with the respective upper height of PBN/ \cdot OCH₃ of known amounts. Reaction mixture was: Fe²⁺ (0.88 mM) to a mixture of phosphate buffer (50 mM, pH 7.4), EDTA (6.5 mM), PBN (10 mM), and (CH₃)₂SO (140 mM) under bubbling with O₂. ○, no addition; ●, SOD (500 units/ml); □, catalase (750 units/ml); ■, SOD (500 units/ml) + catalase (750 units/ml).

units/ml) inhibited generation of PBN/ \cdot OCH₃ to a similar extent as in 0.88 mM Fe²⁺. When 1.2 mM Fe²⁺ was added in the absence of chelators or in the presence of 24 mM ADP, the signal of PBN/ \cdot OCH₃ decreased to 1/6-fold or 1/10-fold, respectively. These results are in accord with the *in vivo* experiment⁵.

3) Reactions performed in O₂ with low Fe²⁺

In these conditions where O₂ (1.2 mN) is in excess over the concentrations of Fe²⁺ in EDTA, only PBN/ \cdot OCH₃ was detected. At 0.4 mM Fe²⁺, the decomposition of PBN/ \cdot OCH₃ became slow. SOD prevented the decay of PBN/ \cdot OCH₃. Catalase decreased the intensity of PBN/ \cdot OCH₃. At 0.2 mM Fe²⁺, PBN/ \cdot OCH₃ decayed slowly after an apparent lag. The effect of SOD and/or catalase was essentially the same. At 0.1 mM Fe²⁺, PBN/ \cdot OCH₃ was apparently stable.

A semilogarithmic plot of PBN/ $\cdot\text{OCH}_3$ destruction showed that the decay process consists of two components: i.e., the rate of fast decay increased with the concentration of ferrous ion. The rate of slow decay was independent of Fe^{2+} concentration. There was no spin adduct formation by the addition of ferrous ion (0.88 mM) to PBN following N_2 purging. However, if the anaerobic EPR-silent sample (containing Fe^{2+} -EDTA, $(\text{CH}_3)_2\text{SO}$ and PBN) was exposed to air, PBN/ $\cdot\text{CH}_3$ was rapidly formed.

4) Reactions performed in O_2 with Fe^{3+} and ascorbate

When ascorbate was added into the reaction mixture containing Fe^{3+} , PBN/ $\cdot\text{OCH}_3$ was formed with a small amount of PBN/ $\cdot\text{CH}_3$. The adduct PBN/ $\cdot\text{CH}_3$ decayed rapidly, but SOD prevented the decay process (Figure 2). The spin adduct was hardly detected if EDTA was replaced with DTPA.

DISCUSSION

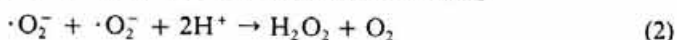
The present study first demonstrated the involvement of $\cdot\text{O}_2^-$ during the oxidation of Fe^{2+} , and during the reaction between Fe^{3+} and ascorbate (the Udenfriend Reaction)¹⁰.

(i) Superoxide production and dismutation

Ferrous-chelates react with dissolved O_2 to produce $\cdot\text{O}_2^-$,

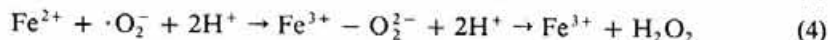
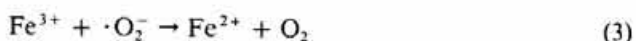


$\cdot\text{O}_2^-$ catalyzed by iron spontaneously dismutates to produce H_2O_2 .²



(ii) The reaction of superoxide with $\text{Fe}^{2+}/\text{Fe}^{3+}$

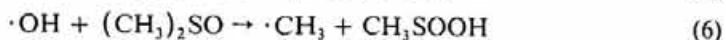
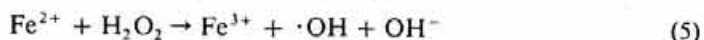
$\cdot\text{O}_2^-$ reacts with Fe^{3+} -EDTA and Fe^{2+} -EDTA with similar rate constants of about $2.0 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$ at pH 7.4^{2,3,14,15}.



Most of superoxide anion disappears; i.e., via Reactions 3 and 4, therefore it is difficult to detect $\cdot\text{O}_2^-$ directly by the spin trapping method under these conditions. Another reason is the very slow rate constant of spin trapping $\cdot\text{O}_2^-$ as compared to $\cdot\text{OCH}_3$ (fast) or $\cdot\text{CH}_3$ (fast).

(iii) PBN/ $\cdot\text{OCH}_3$ formation

H_2O_2 reacts with ferrous ion to form hydroxyl radical, or $\cdot\text{OH}$ -like species¹⁶, which reacts with $(\text{CH}_3)_2\text{SO}$ to produce $\cdot\text{CH}_3$. For simplicity, we write:



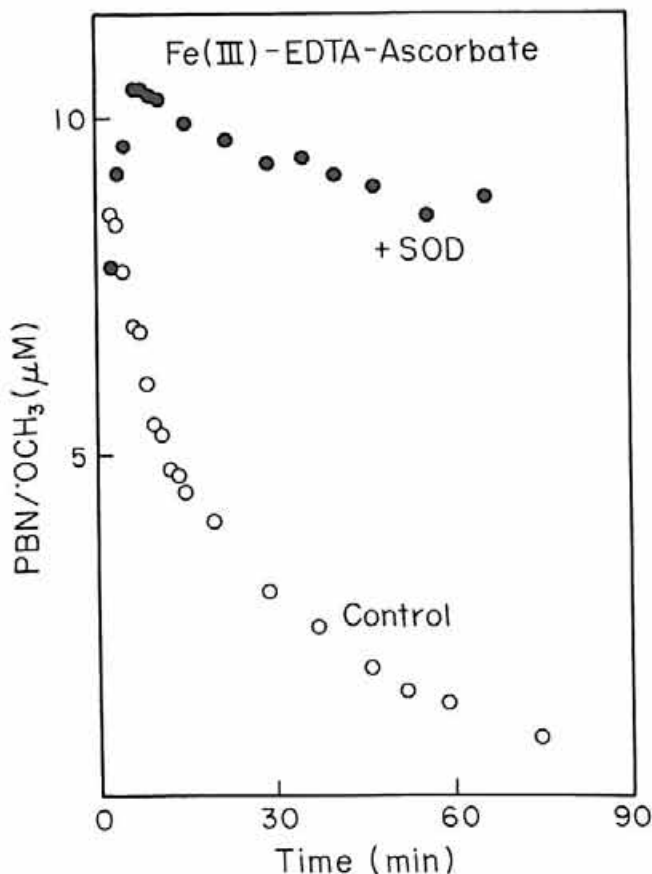
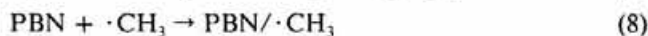
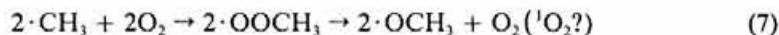


FIGURE 2 Formation and decay of PBN/·OCH₃ by Fe³⁺-EDTA and ascorbate. Reaction mixture was: anaerobically prepared ascorbate (0.6 mM) to a mixture of phosphate buffer (50 mM, pH 7.4), Fe³⁺ (0.9 mM), EDTA (6.5 mM), PBN (10 mM), and (CH₃)₂SO (140 mM) under bubbling with O₂. ○, no addition; ●, SOD (500 units/ml).

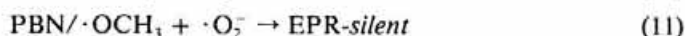
·CH₃ reacts either with O₂ to generate ·OOCH₃ or with PBN to yield PBN/·CH₃.



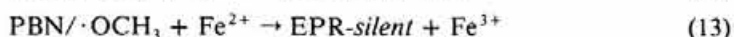
The self-reaction of methylperoxyl radicals (·OOCH₃) (Reaction 7) suggests one source of ·OCH₃.¹⁷ *tert*-Butylperoxyl radical adducts of PBN was detected only at low temperature¹⁷ or in the dark¹⁸. These PBN/·OOCH₃, if present, may undergo a change to PBN/·OCH₃.

(iv) decay of PBN/·OCH₃

Although PBN/·OCH₃ is far more resistant to ·O₂⁻ than PBN/·CH₃,⁸ ·O₂⁻ mediates destruction of PBN/·OCH₃ to EPR-silent species, because SOD prevented the decay of PBN/·OCH₃ (Figure 1).



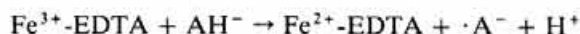
Fe²⁺ certainly reacts with PBN/·CH₃ and PBN/·OCH₃, because the fast part of the decay was dependent on Fe²⁺ concentration.



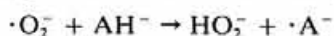
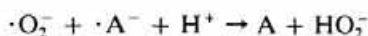
Some of EPR-silent species may be hydroxylamines, which are reoxidized to nitroxides¹⁹.

(v) the Udenfiend system

When Fe³⁺-EDTA and ascorbate were used instead of Fe²⁺-EDTA, the reduction of Fe³⁺-EDTA to Fe²⁺-EDTA by ascorbate occurs²⁰.



Then, above Reactions 1–13 occur. The formation rate of PBN/·OCH₃ depends on the reduction rate of Fe³⁺-EDTA by ascorbate. The yield of the adduct depends on the concentration of ascorbate where Fe²⁺-EDTA acts as a catalyst. The following reactions also occur^{21–23}.



Further, PBN/·OCH₃ and PBN/·CH₃ reduction by ascorbate may occur.

In short, the present study has demonstrated the involvement of ·O₂⁻ during the autoxidation of Fe²⁺-EDTA and also during the reaction between Fe³⁺-EDTA and ascorbate (the Udenfriend Reaction¹⁰). Two decay processes of generated PBN/·OCH₃ by Fe²⁺ and by ·O₂⁻ were recognized in these Reactions.

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