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PHENYL *N-TERT*-BUTYL NITRONE FORMS NITRIC OXIDE AS A RESULT OF ITS FE(III)-CATALYZED HYDROLYSIS OR HYDROXYL RADICAL ADDUCT FORMATION

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Phenyl *N-tert*-butyl nitrone (PBN) is commonly employed in spin-trapping studies. We report here evidence that PBN in aqueous solutions is decomposed by two pathways leading to the generation of nitric oxide ('NO). The first pathway is by hydrolysis of PBN, which is strongly catalyzed by ferric iron. The second pathway is via PBN-hydroxyl radical adduct formation. 'NO was trapped in the presence of cysteine and ferrous iron to form a $[(cys)_2Fe(NO)_2]^{-3}$ complex, which was measured by use of electron paramagnetic resonance (EPR) spectroscopy. A concomitant metabolite, benzaldehyde, was detected from both reaction mixtures. We propose that PBN is hydrolyzed by Fe^{3+} or attacked by hydroxyl radical, leading eventually to a common transient species, *tert*-butyl hydronitroxide [*t*-BuN(O˙)H], which is further oxidized to a 'NO source, *t*-BuNO. Our data imply that PBN may decompose to 'NO when used in biological models with oxidative stress conditions.

KEY WORDS: Spin traps, nitric oxide, EPR, nitrosyl-iron complexes, nitrones, hydroxylamines.

Abbreviations: PBN, phenyl *N-tert*-butyl nitrone; *t*-BuNHOH, *tert*-butyl hydroxylamine *t*-BuN(O·)H, *tert*-butyl hydronitroxide; *t*-BuNO, *tert*-nitrosobutane or 2-methyl-2-nitrosopropane; 4-POBN, α-(4-pyridyl-1-oxide) *N-tert*-butyl nitrone; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; GC/MS, gas chromatography/mass spectrometry.

INTRODUCTION

Nitrone spin traps are commonly used in free radical research.[1] Numerous reports have shown that nitrone spin traps exhibit protective effects against oxidative damage during various processes, *e.g.*, ischemia/reperfusion,[2,3] aging,[4] oxidation of low density lipoprotein,[5] CCl_4-induced rat liver injury,[6] and adriamycin-induced cardiotoxicity.[7] It is generally proposed that the scavenging of reactive radical species by nitrone spin traps is the mechanism responsible for this protection.[1–7]

We have recently reported that phenyl *N-tert*-butyl nitrone (PBN) is decomposed by light to a transient paramagnetic species, *tert*-butyl hydronitroxide [*t*-BuN(O·)H], which is further oxidized to *tert*-butyl nitrosobutane (*t*-BuNO), which in turn decomposes to 'NO.[8] In the previous spin trapping literature, *t*-BuN(O˙)H (a^N = 14.5 G

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and $a_{\alpha}^{H} = 14.0$ G) was detected in oxidizing systems when PBN or α-(4-pyridyl-1-oxide) *N-tert*-butyl nitrone (4-POBN) was used, *e.g.*, photolysis of hydrogen peroxide,[9–11] cyanide/horseradish peroxidase/hydrogen peroxide,[12] xanthine/xanthine oxidase/deferoxamine,[13] 22:6-enriched L1210 cells/$FeSO_4$,[14] and brain ischemia/reperfusion.[15] Therefore, the oxidative decomposition mechanisms of *N-tert*-butyl nitrones to *t*-BuN(O·)H[9] or *t*BuNO[8] are of potential importance because *t*-BuNO in aqueous solutions is thermally decomposed to ·*t*-Bu and ·NO[8,16] at 37°C. This is because ·NO is known to have important regulatory functions in various biological systems.[17–19]

It is known that *N*-alkyl nitroxides disproportionate to yield a hydroxylamine and a nitroso compound.[20] Therefore, the products, *tert*-butyl hydroxylamine (*t*-BuNHOH) and *t*-BuNO, are expected to be present in systems where *t*-BuN(O·)H is observed. Furthermore, it has been shown that the oxidation of *t*-BuNHOH forms *t*-BuN(O·)H and subsequently *t*-BuNO as initiated by either light,[8] ferric iron, or hydrogen peroxide (or the Fenton reagents).[21]

We previously reported that the hydroxyl radical adduct of PBN is a precursor of *t*-BuNO during light-induced hydrolysis of PBN.[8] In the present report we have further investigated such possibilities in reactions involving PBN and 1) ferric iron or 2) Fenton reagents. We have employed a technique to trap ·NO by binding it with cysteine and ferrous iron such that the resulting paramagnetic species, $[(cys)_2 Fe(NO)_2]^{-3}$, can be detected by EPR spectroscopy.[8,22] In addition, gas chromatography/mass spectrometry (GC/MS) and UV-spectrophotometry were performed for product identification.

MATERIALS AND METHODS

Materials L-cysteine (free base), PBN, sodium periodate, sulfanilamide, naphthylethylenediamine·dichloride, phosphoric acid, ascorbic acid, and *tert*-nitrosobutane (*t*-BuNO) were purchased from Sigma. Sodium nitrite was obtained from Fisher Scientific Co. Ferrous sulfate·heptahydrate was obtained from Alfa Products, MA. Ferric chloride was obtained from Fluka, NY. α(4-Pyridyl-1-oxide)-*N-tert*-butyl nitrone, benzaldehyde and *N*-(*tert*-butyl) hydroxylamine·hydrochloride (*t*-BuNHOH) were purchased from Aldrich. All experiments were done in glass, not plastic, containers.

EPR Spectra of Nitrosyl-iron Complex We have employed the protocol previously reported by Woolum *et al.*[22] to form nitrosyl-iron complexes. This protocol (or "An EPR assay for ·NO") involves the trapping of ·NO by cysteine and ferrous iron to form the $[(cys)_2Fe(NO)_2]^{-3}$ complex detectable at g_{iso} ~2.03. Typically, cysteine (30 mg/ml), $FeSO_4$ (1 mM) and ascorbic acid (20 mM) were added into incubation mixtures. Ascorbic acid performs the following functions: 1) it reduces nitrite to ·NO, and 2) it maintains iron in the lower oxidation state. The EPR spectra were recorded at room temperature using a Bruker ESP300 EPR spectrometer. The spectrometer was operated at 9.77 GHz with a 100 kHz modulation frequency. The incubation mixtures were pipetted into a quartz flat cell centered in a TM_{110} cavity, and EPR spectra were recorded. The *g*-values were calibrated by using potassium peroxylamine disulfonate ($g = 2.0055$).[23]

Product Determination by GC/MS Hexane extracts of reaction mixtures (with 2 vol of hexane) were analyzed by GC/MS. The column used in the Hewlett-Packard

GC 5880A gas chromatograph was a Perkin Elmer methyl 5% phenyl 0.25 mm × 3 m, 0.25 μm film with helium pressure of 5 psi. The program rate was 40°C (1 min hold), increased to 250°C at 10°C per min. The mass spectrometer was a Finnigan 700 Ion Trap Detector, and was scanned over the mass range 40–650 dalton at a scan rate of 1 s/scan. An injection volume of 5 μl was used. Mass spectrometric data are presented as reconstructed ion chromatograms. A reconstructed ion chromatogram is a plot of the relative abundance of a selected ion *vs* time.

UV-spectrophotometry The formation of *t*-BuNO was monitored by measuring the absorbance at 662 nm[16] with a Hewlett-Packard 8415A diode array spectrophotometer.

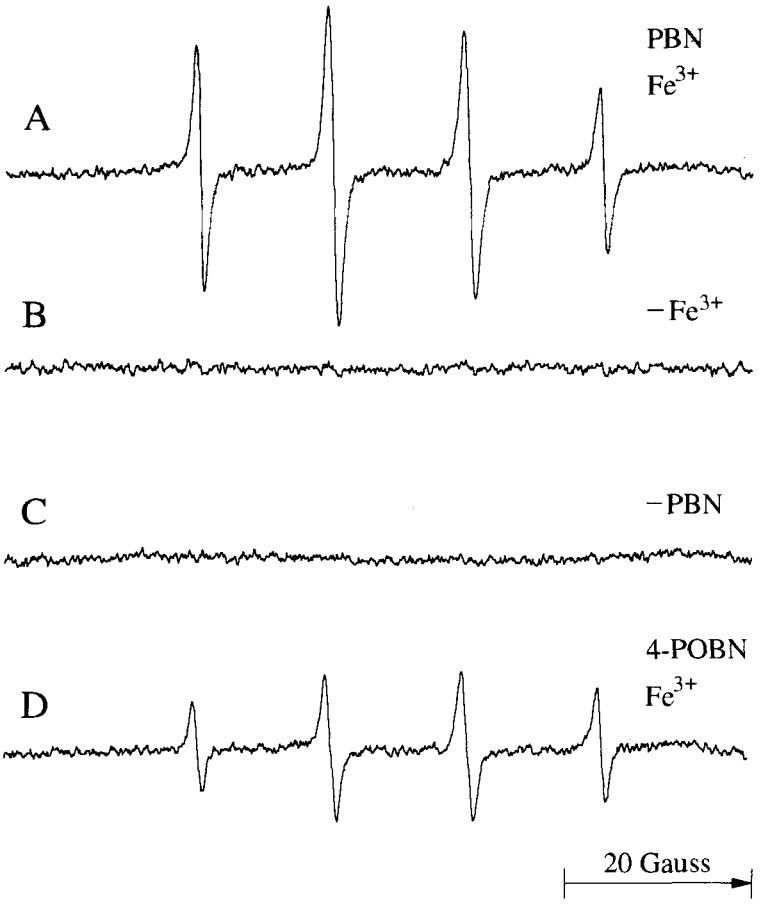


FIGURE 1 Room temperature EPR spectra of *t*-BuN(O˙)H from the oxidation of PBN by ferric iron. *A*, 50 mM PBN and 1.25 mM $FeCl_3$ in 50 mM Tris·HCl, pH 7.0. *B*, Same as in *A* except that $FeCl_3$ was omitted. *C*, Same as in *A* except that PBN was omitted. *D*, 50 mM 4-POBN and 1.25 mM $FeCl_3$ in 50 mM Tris·HCl, pH 7.0. Spectrometer conditions were: modulation amplitude, 0.7 G; microwave power, 20 milliwatts; time constant, 0.66 s; scan rate, 14 G/min.

RESULTS

·NO from the Hydrolysis of PBN Catalyzed by Ferric Ions

When PBN reacted with 1.25 mM ferric iron in either deionized water, Tris·HCl, pH 7.0, or HEPES, pH 7.0, a four-line EPR spectrum attributable to *t*-BuN(O·)H was detected (Figure 1*A* and Table I). The assignment for *t*-BuN(O·)H is based on the hyperfine coupling constants, which are in agreement with the literature values.[24] The omission of ferric iron or PBN resulted in no detectable EPR signals (Figures 1, *B–C*). When ferric iron concentrations greater than 5 mM were used, no detectable signals were obtained (data not shown), suggesting that ferric iron was also involved in the oxidation of *t*-BuN(O·)H to a non-radical species. When 4-POBN replaced PBN in Figure 1*A*, a lower concentration of *t*-BuN(O·)H was detected (Figure 1*D*).

The production of ·NO from *t*-BuNO during the oxidation of *t*-BuNHOH by UV-photolysis has been demonstrated.[8] In the event that *t*-BuNHOH is oxidized to *t*-BuNO by catalytic ferric iron, hydrogen peroxide, or the Fenton reagents,[21] ·NO is expected to form. We therefore employed an EPR assay for ·NO to determine whether ·NO was formed in these systems. When *N*-(*tert*-butyl) hydroxylamine (*t*-BuNHOH) was mixed with Fe^{3+} in 25 mM phosphate buffer, pH 7.0, an EPR spectrum of *t*-BuN(O·)H was detected (Figure 2*A*). This signal was about two-fold more intense than that of air-oxidized *t*-BuNHOH in the absence of added iron (data not shown). When an EPR assay for ·NO was performed in a 50-min pre-incubated mixture of *t*-BuNHOH and Fe^{3+}, a signal at g_{iso} ~2.03 indicative of $[(cys)_2Fe(NO)_2]^{-3}$ was obtained (Figure 2*B*). The same $[(cys)_2Fe(NO)_2]^{-3}$ complex was obtained in a 1.5 h pre-incubated reaction mixture of *t*-BuNHOH and Fenton reagents (Figure 2*C*). There was no detectable EPR signal in the controls when either hydrogen peroxide or *t*-BuNHOH was omitted (Figure 2, *D–E*). There was only a weak signal when hydrogen peroxide and *t*-BuNHOH were present in the incubation mixture (Figure 2*F*). When an equivalent amount of Fe^{3+} was mixed with *t*-BuNHOH, the $[(cys)_2Fe(NO)_2]^{-3}$ complex was not produced under the same experimental conditions (Figure 2*G*).

The detection of *t*-BuN(O·)H during the oxidation of *t*-BuNHOH correlates with

TABLE I

Hyperfine coupling constants from radical adducts obtained during the oxidation of *t*-BuNHOH and PBN

System	Radical	Hyperfine Coupling Constants (Gauss)			Source
		a^N	a^H_α	a^H_β	
PBN/Fe^{3+}	*t*-BuNO/·H	14.5	14.0	–	Figure 1*A*
t-BuNHOH/Fenton	*t*-BuNO/·H	14.5	14.0	–	Figure 2*A*
PBN/Fenton	PBN/·OH	15.5	–	2.7	*a*
	unknown	15.3	–	–	
t-BuNHOH/Fenton	*t*-BuNO/·H	14.5	14.0	–	*b*
	t-BuNO/·*t*-Bu	17.1	–	–	
	unknown	15.2	–	–	

[a] The complete reaction mixture consisted of 25 mM PBN, 2 mM $FeSO_4$ and 100 mM H_2O_2 in deionized water and was measured 30 min after mixing.

[b] The complete reaction mixture consisted of 25 mM *t*-BuNHOH, 1 mM $FeSO_4$ and 5 mM H_2O_2 in 25 mM Tris·HCl, pH 7.1, and was measured 10 min after mixing.

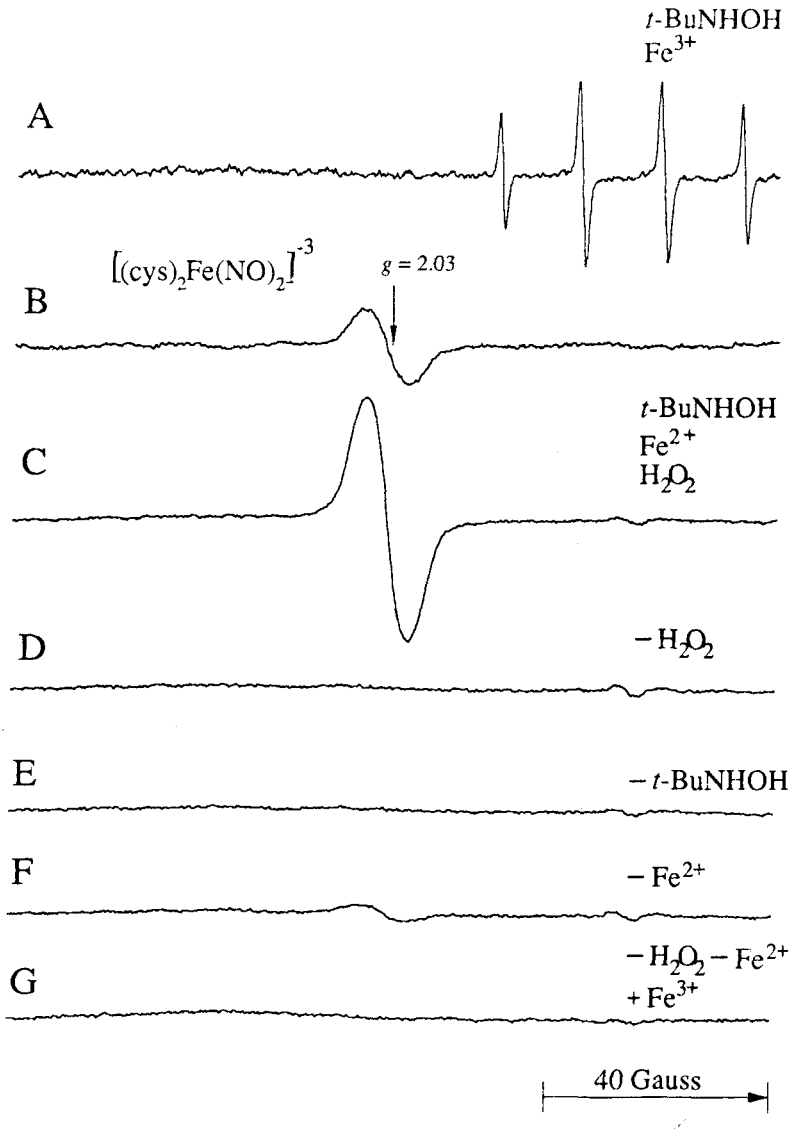


FIGURE 2 Room temperature EPR spectra of the $[(cys)_2Fe(NO)_2]^{-3}$ complex where $^{\cdot}NO$ was generated from the oxidation of *t*-BuNHOH at 37°C in 25 mM phosphate buffer, pH 7.0. Complexing agents consisting of cysteine (30 mg/ml), Fe^{2+} (1 mM) and ascorbic acid (20 mM) were added to the incubation mixtures of *B–G*. *A*, An incubation consisting of 25 mM *t*-BuNHOH and 1.25 mM Fe^{3+}. *B*, Complexing agents were added to the mixture described in *A* after a pre-incubation at 37°C for 50 min. *C*, Complexing agents were added to a mixture of 25 mM *t*-BuNHOH, 125 μM Fe^{2+} and 10 mM H_2O_2 after a pre-incubation at 37°C for 1.5 h. *D*, Same as in *C* except that H_2O_2 was omitted. *E*, Same as in *C* except that *t*-BuNHOH was omitted. *F*, Same as in *C* except that Fe^{2+} was omitted. *G*, Same as in *C* except that H_2O_2 and Fe^{2+} were omitted and 125 μM Fe^{3+} was added. Spectrometer conditions were: modulation amplitude, 3.0 G; microwave power, 20 milliwatts; time constant, 0.66 s; scan rate, 27 G/min.

the formation of ·NO (Figure 2). Since *t*-BuN(O·)H was observed in the PBN/ferric iron mixtures (Figure 1), an EPR assay for ·NO was performed as well. The PBN/ferric iron reaction and the controls were allowed to react for 50 min. An EPR signal indicative of the $[(cys)_2Fe(NO)_2]^{-3}$ complex was detected from the sample (Figure 3*A*). The omission of either PBN or ferric iron resulted in no detectable EPR signals (Figure 3, *B–C*).

The reaction of PBN with ferric iron appears to have resulted in the production of *t*-BuN(O·)H (Figure 1), but this species could be due to the oxidation of a significant contaminant, *t*-BuNHOH, in the PBN. To exclude such a possibility, we oxidized *t*-BuNHOH with a mild oxidant, sodium periodate. As a result, the signal of *t*-BuN(O·)H present in buffer alone was increased fourfold (Figure 4*A*) when compared with *t*-BuNHOH in buffer alone. In contrast, when PBN was oxidized by sodium periodate, there was no detectable signal (Figure 4*B*). It is concluded that *t*-BuN(O·)H detected in Figure 1*A* was indeed a product of the reaction of PBN with ferric iron.

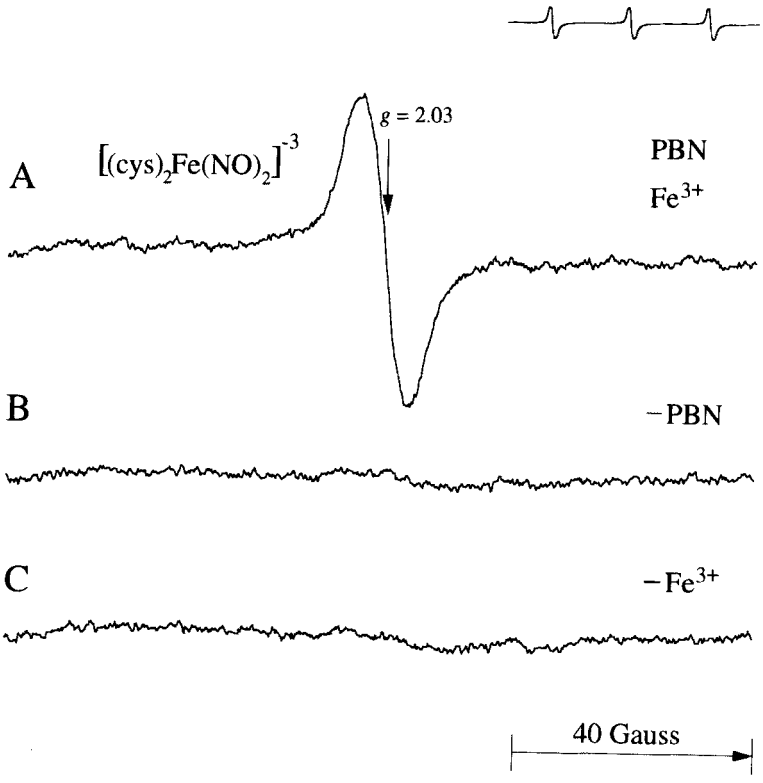


FIGURE 3 Room temperature EPR spectra of the $[(cys)_2Fe(NO)_2]^{-3}$ complex where ·NO was generated from a reaction between PBN and $FeCl_3$ after 50-min incubation time at room temperature. Complexing agents consisting of cysteine (30 mg/ml) and ascorbic acid (20 mM) were added to the incubation mixtures. *A*, 50 mM PBN and 3 mM $FeCl_3$. *B*, Same as in *A* except that PBN was omitted. *C*, Same as in *A* except that $FeCl_3$ was omitted. Spectrometer conditions were: modulation amplitude, 3.0 G; microwave power, 20 milliwatts; time constant, 0.66 s; scan rate, 27 G/min. The three-line signal of peroxylamine disulfonate was used as a *g*-marker.

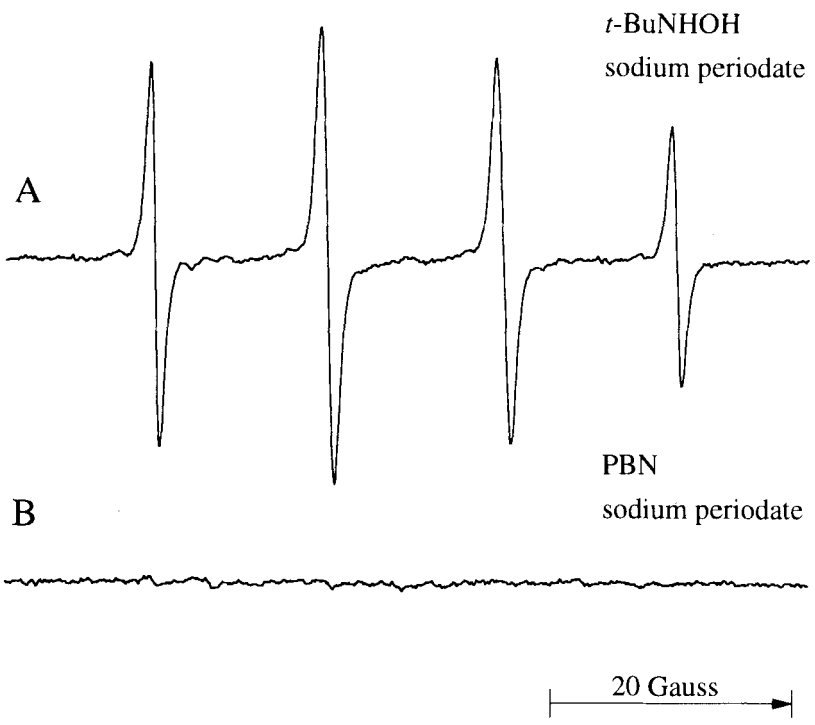


FIGURE 4 *A*, The oxidation of 50 mM *N-tert*-butyl hydroxylamine (*t*-BuNHOH) with 1.25 mM sodium periodate in 50 mM Tris·HCl, pH 7.0. *B*, 50 mM PBN and 1.25 mM sodium periodate in 50 mM Tris·HCl, pH 7.0. Spectrometer conditions were: modulation amplitude, 0.7 G; microwave power, 20 milliwatts; time constant, 0.66 s; scan rate, 14 G/min.

The reaction between PBN and ferric iron produced the odor of benzaldehyde. A PBN/ferric iron reaction mixture was allowed to proceed for 17 min and was extracted with hexane. The hexane extract was analyzed by GC/MS, and a product with a mass spectrum and retention time characteristic of benzaldehyde was detected (Figure 5). No benzaldehyde was detected when ferric iron was omitted from the reaction mixture.

The nitroso compound *t*-BuNO is expected to form under oxidizing conditions when *t*-BuN(O˙)H is observed.[8,20-21] Since *t*-BuN(O˙)H was formed during oxidation of PBN by ferric iron (Figure 1), we used a spectrophotometric technique to measure *t*-BuNO[16] at $\lambda_{max} = 662$ nm and $\epsilon_{662} \approx 20$ L mole^{-1} cm^{-1}. Figure 6 shows that 50 mM PBN incubated with 3 mM ferric iron for 15 min resulted in a strong absorbance at 662 nm. The maximum absorbance from this reaction corresponded to 2.3 mM *t*-BuNO. Control experiments with the omission of either ferric iron or PBN did not result in detectable absorbance (Figure 6). As expected, an absorbance at 662 nm was immediately detected in an incubation containing *t*-BuNHOH and ferric iron (data not shown).

The relative intensity of an EPR signal of *t*-BuN(O˙)H and the absorbance at 662 nm of *t*-BuNO were monitored from an incubation containing PBN and $FeCl_3$

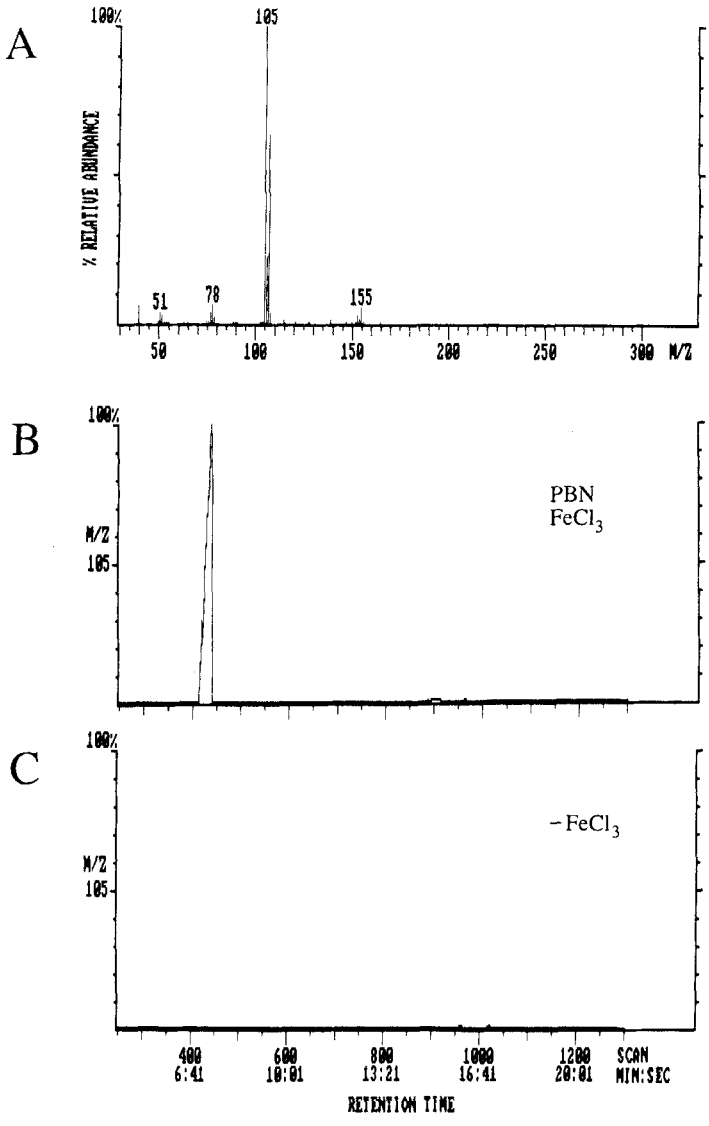


FIGURE 5 GC/MS analysis of the hexane extracts from a reaction mixture of 50 mM PBN and 3 mM $FeCl_3$ in water. *A*, Mass spectrum of benzaldehyde peak from the PBN/$FeCl_3$ reaction mixture; m/z = 105 is assigned to $[C_6H_5CO]^+$; m/z = 107 due to $(M + H)^+$ arising from self chemical ionization. *B*, A reconstructed ion chromatogram for m/z 105 from *A*. *C*, A reconstructed ion chromatogram for m/z 105 from the reaction mixture without $FeCl_3$.

at room temperature (Figure 7). It was found that the *t*-BuN(O˙)H signal reached a maximum intensity at ~12 min after mixing and then started to decay. As the *t*-BuN(O˙)H decayed to zero, the absorbance of *t*-BuNO at 662 nm reached a maximum. At incubation times longer than 30 min, the 662 nm absorbance of *t*-BuNO decreased in intensity (Figure 7). The time course of the 662 nm absorbance of the commercially available *t*-BuNO dissolved in water containing 3% dimethyl sulfoxide

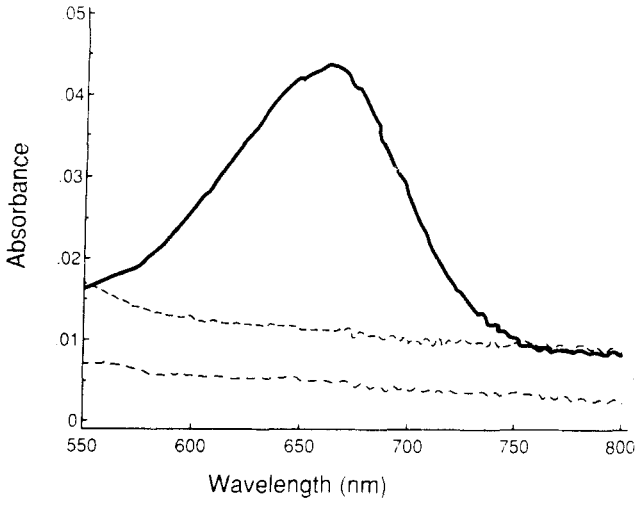


FIGURE 6 A UV-spectral profile of *t*-BuNO from the PBN/ferric iron reaction mixture in water, measured 15 min after mixing. *A*, 50 mM PBN and 3 mM $FeCl_3$ (————). *B*, Without $FeCl_3$ (– – – – –, lower). *C*, Without PBN (– – – – –, upper).

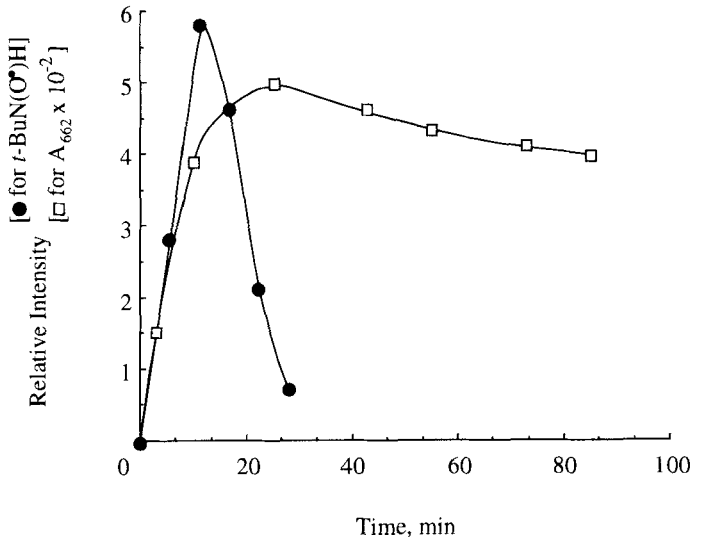


FIGURE 7 Time course of *t*-BuN(O˙)H EPR intensity (●), and absorbance at 662 nm of *t*-BuNO (□). The incubation consisted of 50 mM PBN and 3 mM $FeCl_3$ in 25 mM Tris·HCl, pH 7.0. Each point is an average of two separate experiments.

in a capped cuvette also exhibited a decrease in intensity (data not shown). Presumbably, *t*-BuNO decomposed to ˙NO and *t*-Bu˙. For the 4-POBN/ferric iron reaction, the rate of formation of *t*-BuN(O˙)H was about threefold slower than that obtained from the PBN/ferric iron reaction (data not shown). This time course study suggests that PBN decomposes to *t*-BuN(O˙)H, which is subsequently oxidized to *t*-BuNO.

·NO from Hydroxyl Radical Adduct Formation of PBN

We also investigated the decomposition of PBN under Fenton conditions. When measured immediately after the mixing of PBN, Fe^{2+} and H_2O_2 in nitrogen-purged deionized water, an EPR spectrum exhibiting a typical six-line signal of the hydroxyl radical adduct of PBN (PBN/·OH) ($a^N = 15.5$ G and $a_\beta^H = 2.7$ G) was obtained (Table I). With subsequent scans, a three-line signal of $a^N = 15.3$ G overlapped with PBN/·OH and continually increased in intensity with time (Table I). This three-line signal is an unknown species although this same species was also obtained when *t*-BuNHOH was mixed with either H_2O_2 alone or the Fenton reagents (Table I). A similar spectrum of $a^N = 15.3$ G has been reported from the Fenton reaction with a nitrone, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO). This species was characterized as a hydroxyl radical adduct of 2-hydroxy-5,5-dimethyl-1-pyrroline *N*-oxide (HDMPO/·OH).[25]

In order for the decomposition of PBN/·OH radical adduct to yield a detectable concentration of the $[(cys)_2Fe(NO)_2]^{-3}$ complex, a long reaction time was necessary. When the PBN/Fenton mixture was incubated at 37°C for 20 h, a multi-component EPR spectrum was obtained (Figure 8*A*). When the EPR assays for ·NO were performed in these incubations, a signal of the $[(cys)_2Fe(NO)_2]^{-3}$ complex was detected (Figure 8*B*). The $[(cys)_2Fe(NO)_2]^{-3}$ complex formation occurred only when the reaction was done in deionized water. Attempts to perform these experiments in Tris·HCl and phosphate buffers failed (data not shown). The omission of either H_2O_2 or PBN resulted in no (or little) detectable complex formation (Figures 8, *C–D*). A control incubation without ferrous iron resulted in a very weak $[(cys)_2Fe(NO)_2]^{-3}$ signal (Figure 8*E*) and the nitroxide spectrum with $a^N = 15.2$ G as observed previously (Table I). When an EPR assay for ·NO was performed in an incubation containing only PBN, a weak signal of $[(cys)_2Fe(NO)_2]^{-3}$ was obtained, indicating that PBN at 37°C underwent hydrolysis to form a ·NO source (Figure 8*F*).

A hexane extract from the PBN/Fenton reaction was also analyzed by GC/MS. As with the PBN/ferric iron reaction, benzaldehyde was detected (data not shown).

DISCUSSION

Nitric oxide has been found to play important regulatory roles in cellular function and communication.[17–19] Lack of nitric oxide may be an early event for development of diseases; thus, recent research has been focused on drugs which can release nitric oxide.[26,27] In this study, we have demonstrated through chemical experiments that when a nitrone, either PBN or 4-POBN, reacts with ferric iron, *t*-BuN(O·)H is formed as a transient species and is further oxidized to *t*-BuNO, a ·NO source. In addition, evidence for the decomposition of PBN by hydroxyl radical to ·NO is demonstrated as well.

It is known that *t*-BuN(O·)H disproportionates[20] or is oxidized by metals[21] to form *t*-BuNO, a ·NO source. Accordingly, we have demonstrated that the oxidation of *t*-BuNHOH by ferric iron and hydroxyl radical forms *t*-BuN(O·)H and, subsequently, ·NO from *t*-BuNO (Figure 2). *t*-BuNO at 662 nm absorbance was consistently measured from these reactions (data not shown). Thus, ·NO is expected to be a by-product in some *in vitro* spin-trapping experiments where PBN or 4-POBN is used, and whenever *t*-BuN(O·)H is detected.[9–15]

It is known that the hydroxyl radical adduct of PBN[9,10] and 4-POBN[11] are not

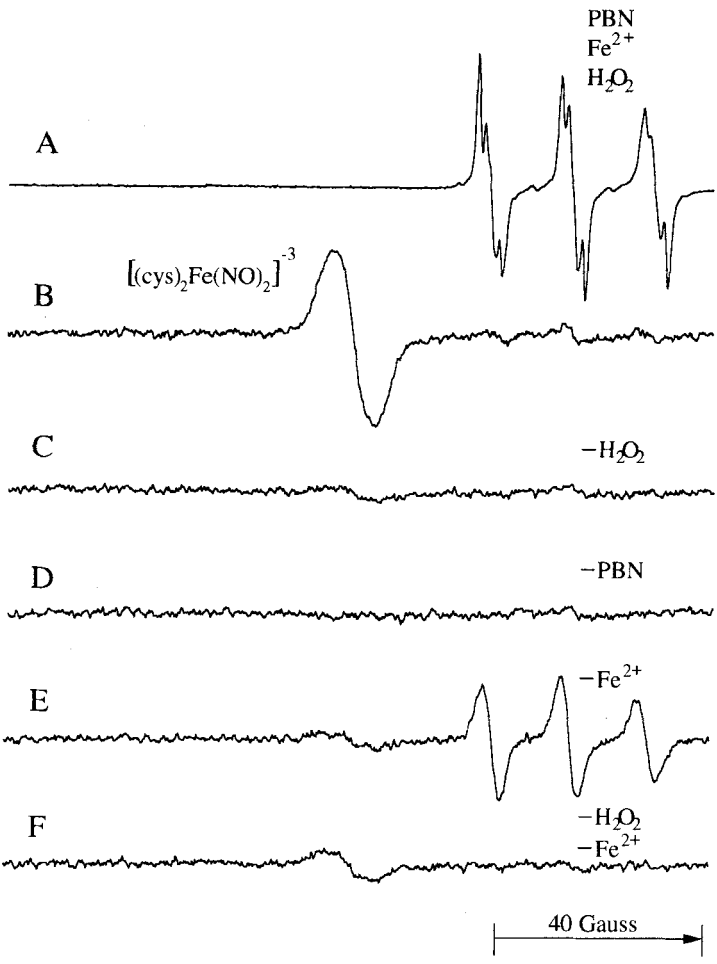
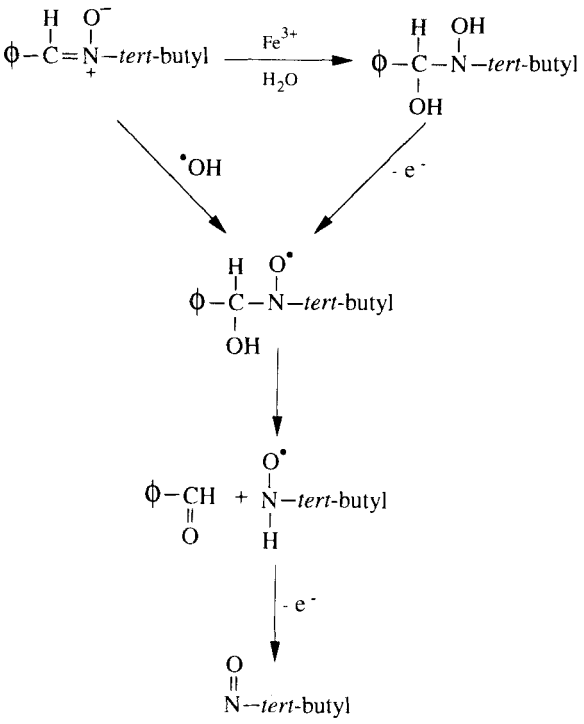


FIGURE 8 Room temperature EPR spectra of the $[(cys)_2Fe(NO)_2]^{-3}$ complex where ·NO was generated from a reaction between 50 mM PBN, 3.0 mM Fe^{2+} and 75 mM H_2O_2 after a 20-h incubation time at 37°C in deionized water. Complexing agents consisting of cysteine (30 mg/ml), Fe^{2+} (1 mM) and ascorbic acid (20 mM) were added into the incubation mixtures (Figures *B–F*). *A*, Complete incubation consisting of PBN, Fe^{2+} and H_2O_2. *B*, Complexing agents were added in the complete incubation in *A*. *C*, Same as in *B* except that H_2O_2 was omitted. *D*, Same as in *B* except that PBN was omitted. *E*, Same as in *B* except that Fe^{2+} was omitted. *F*, Same as in *B* except that H_2O_2 and Fe^{2+} were omitted. Spectrometer conditions were: modulation amplitude, 0.7 G for *A* and 2.87 G for *B–F*; microwave power, 20 milliwatts; time constant, 0.66 s; scan rate, 27 G/min. Each spectrum was added from 2 scans.

persistent; they decompose to *t*-BuN(O·)H. PBN is also decomposed by ferric iron to the transient species, *t*-BuN(O·)H (Figure 1*A*), and, as consistent with results shown in Figure 2, the further oxidation of *t*-BuN(O·)H forms *t*-BuNO (Figures 6 and 7), followed by the release of ·NO (Figure 3). Benzaldehyde was also detected as one of the products (Figure 5). The PBN decomposition mechanism is likely to be a reverse of PBN synthesis where a condensation of benzaldehyde and *t*-BuNHOH[28,29] is performed. A proposed mechanism which accounts for our observations is shown in



Scheme 1

Scheme 1. PBN undergoes hydrolysis, which is catalyzed by ferric iron to form the hydroxylamine of PBN/·OH. This adduct may undergo cleavage to produce *t*-BuN(O·)H as proposed by others.[9-11] *t*-BuN(O·)H is oxidized to *t*-BuNO which ultimately undergoes further decomposition to ·NO (Figure 3).

In the reaction of PBN with hydroxyl radical, the PBN/·OH adduct is obtained and spontaneously may cleave to form benzaldehyde and *t*-BuN(O·)H (Scheme 1). Again, the further oxidation of *t*-BuN(O·)H forms *t*-BuNO. This decomposition pathway is in agreement with the previous report where PBN/·OH spontaneously decomposes to *t*-BuN(O·)H.[9-11] Although only a weak signal of *t*-BuN(O·)H was detected in the 20 h-incubation mixture, we were able to detect ·NO by EPR assay (Figure 8). We were able to separate and identify benzaldehyde in the hexane extracts from this reaction, which is consistent with Scheme 1.

The decomposition of PBN by ferric iron appears rather simple since only the transient *t*-BuN(O·)H was observed (Figure 1). On the other hand, the decomposition of PBN/·OH is clearly quite complex, since not only PBN/·OH radical adduct was observed, but a broad three-line species (a^N = 15.16 G) of unknown structure also appeared 30 min after mixing (Table I). This species, exhibiting only a nitrogen hyperfine coupling, may be a *t*-BuNO radical adduct of an unknown species, because the same spectrum was also obtained when *t*-BuNHOH was mixed with either H_2O_2 alone or with Fenton reagents (Table I). The presence of this unknown species may be of importance since a number of *in vivo* papers have reported PBN-dependent

species of similar hyperfine coupling constants. For example, a spectrum with $a^N = 15.2$ G (or as assigned PBN/$^{\cdot}$Y) was observed in bile from rats treated with iron or copper.[30,31] PBN/$^{\cdot}$Y was not present in any of the control experiments, suggesting that metals were necessary for its formation.[30,31] Thus, PBN/$^{\cdot}$Y was likely a decomposition product of PBN radical adducts under Fenton conditions.

Our data indicate that PBN can undergo oxidative degradation to benzaldehyde and *t*-BuN(O$^{\cdot}$)H which subsequently decomposes to $^{\cdot}$NO. That this oxidative decomposition of PBN occurs *in vivo* is supported by the detection of the three-line PBN-dependent nitroxide in models of iron and copper poisoning.[30,31]

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