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METABOLISM OF ETHANOL TO 1-HYDROXYETHYL RADICALS IN RAT LIVER MICROSOMES: COMPARATIVE STUDIES WITH THREE SPIN TRAPPING AGENTS

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Metabolism of ethanol to 1-hydroxyethyl radicals by rat liver microsomes was studied with three nitrone spin trapping agents (POBN, PBN, and DMPO) under essentially comparable conditions. The data indicate that POBN was the superior spin trapping agent for 1-hydroxyethyl radicals, and that DMPO was least efficient. Addition of deferoxamine completely prevented detection of 1-hydroxyethyl radicals with PBN or DMPO, but caused only 50% decrease in EPR signals when POBN was the spin trap. However, superoxide dismutase only decreased 1-hydroxyethyl radical formation when POBN was the spin trap. Other experiments demonstrated that POBN was the most effective of these nitrones for reduction of Fe(III) in aqueous solutions. Furthermore, 1-hydroxyethyl radical adducts were formed when POBN was added to mixtures of ethanol, phosphate buffer, POBN and $FeCl_3$, but this effect did not occur with either PBN or DMPO. Thus, these data indicate that undesirable effects of POBN on iron chemistry may influence results of spin trapping experiments, and complicate interpretation of the resulting data.

KEY WORDS: DMPO, PBN, ethanol radicals, POBN, iron chemistry, spin trapping.

INTRODUCTION

The spin trapping method is useful to study mechanisms of free radical formation and reaction in chemical and biological systems. In spin trapping, a highly reactive radical is allowed to react with a spin trapping agent to form a secondary, more stable radical which is often referred to as a "spin adduct". Electron paramagnetic resonance (EPR) studies may then be conducted with the spin adduct in order to characterize free radical reaction intermediates.

A number of spin trapping agents have been developed and are commercially

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available, but they may differ substantially in their ability to form stable adducts with radicals of interest, their water solubility, and their toxicity. Although some radicals form sufficiently stable spin adducts with a variety of spin trapping agents, there have been few comparative studies to determine whether the same conclusions about free radical mechanisms can be obtained when different traps are used.

Our studies into mechanisms of ethanol metabolism to the 1-hydroxyethyl radical have indicated that iron and hydrogen peroxide are important determinants of the rate of this reaction.[1,2] A recent report that 1-hydroxyethyl radical formation from ethanol was dependent on superoxide[3] seemed inconsistent with our results, and led us to perform direct comparative experiments with three spin trapping agents which have proven useful in studies with ethanol.

MATERIALS AND METHODS

The spin trapping agents used in these studies are PBN (α-phenyl-*N*-t-butylnitrone), POBN (α-[4-pyridyl 1-oxide]-*N*-t-butylnitrone), and DMPO (5,5-dimethylpyroline-*N*-oxide). All of the spin trapping agents were purchased from the Sigma Chemical Co., (St. Louis, MO), and were used at concentrations found in separate experiments to be optimal for microsomal studies. PBN and POBN were used as received, but DMPO was purified further by filtration through activated charcoal. Superoxide dismutase (SOD), deferoxamine (desferrioxamine, DFO), 2,2′-dipyridyl, and other biochemicals were also purchased from Sigma.

The spin trapping agents were added to liver microsomes from male, Sprague-Dawley rats that had been prepared with methods to minimize contamination with catalase and non-heme iron.[4] Other components of the microsomal incubations were ethanol (50–100 mM), phosphate buffer (40 mM, pH 7.4), and an NADPH-generating system composed of $NADP^+$ (0.3 mM), glucose-6-phosphate (50 mM) and glucose-6-phosphate dehydrogenase (0.5 U/ml). Where indicated, 0.1 mM azide was added to inhibit residual catalase activity which always contaminates the microsomes.[2,4] The final concentrations of reagents and other additions are shown in the figure legends. All solutions were thoroughly treated with Chelex-100 resin (Bio-Rad) to decrease concentrations of contaminating trace metals.

The incubation systems were allowed to preincubate at 37 °C for a few minutes, and the reactions were started with addition of the NADPH-generating system. After 10 min, the suspensions were siphoned into a flat EPR cell placed in the cavity of a Bruker EPR 300E spectrometer. Typical spectrometer operating conditions were: center field, 3480 G; sweep width, 100 G; sweep time, 84 sec; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; time constant, 328 ms; microwave frequency, 9.75 GHz; and microwave power, 19.9 mW. Other conditions are given in the figure legends.

RESULTS

When POBN (30 mM) was incubated with rat liver microsomes, ethanol, and an NADPH-generating system, a six-line EPR signal which was typical of the 1-hydroxyethyl radical adduct of POBN was observed (Figure 1). When this experiment was repeated with 1-^{13}C-ethanol, additional splitting of the EPR signals occurred (not shown), confirming the assignment of the spin adduct to the

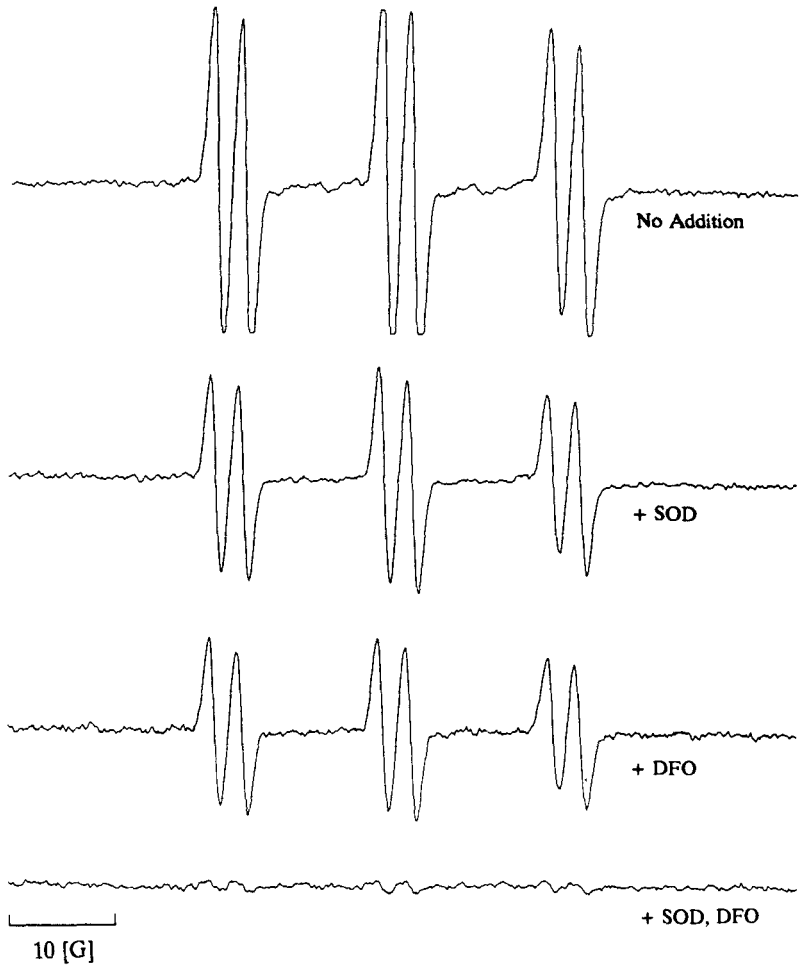

FIGURE 1 Spin trapping of 1-hydroxyethyl radicals formed by rat liver microsomes in the presence of POBN. Rat liver microsomes (approximately 2 mg/ml) were incubated for 10 min at 37 °C with ethanol (50 mM), POBN (30 mM) and an NADPH-generating system. At the end of the incubation period, the suspensions were siphoned into a flat EPR cell placed in the cavity of the EPR spectrometer. The spectra shown were obtained with 2 scans using a spectrometer gain of 5×10^5, and all spectra are shown on the same scale. Other conditions are as described in Materials and Methods. Where indicated, SOD (100 units/ml) and DFO (1 mM) were included in the incubation mixtures.

1-hydroxyethyl radical adduct of POBN, as previously reported.[3] When superoxide dismutase (100 U/ml) or deferoxamine (DFO, 1 mM) were added separately to the incubation systems, the EPR signal intensities were decreased by about 40% to 50%, respectively (Figure 1). When SOD and DFO were added together, no EPR signal was observed. These results are comparable to those of Knecht *et al.*,[3] who reported inhibition of 1-hydroxyethyl radical formation by SOD in the presence of DFO, using liver microsomes from ethanol-fed, alcohol dehydrogenase-deficient deermice.

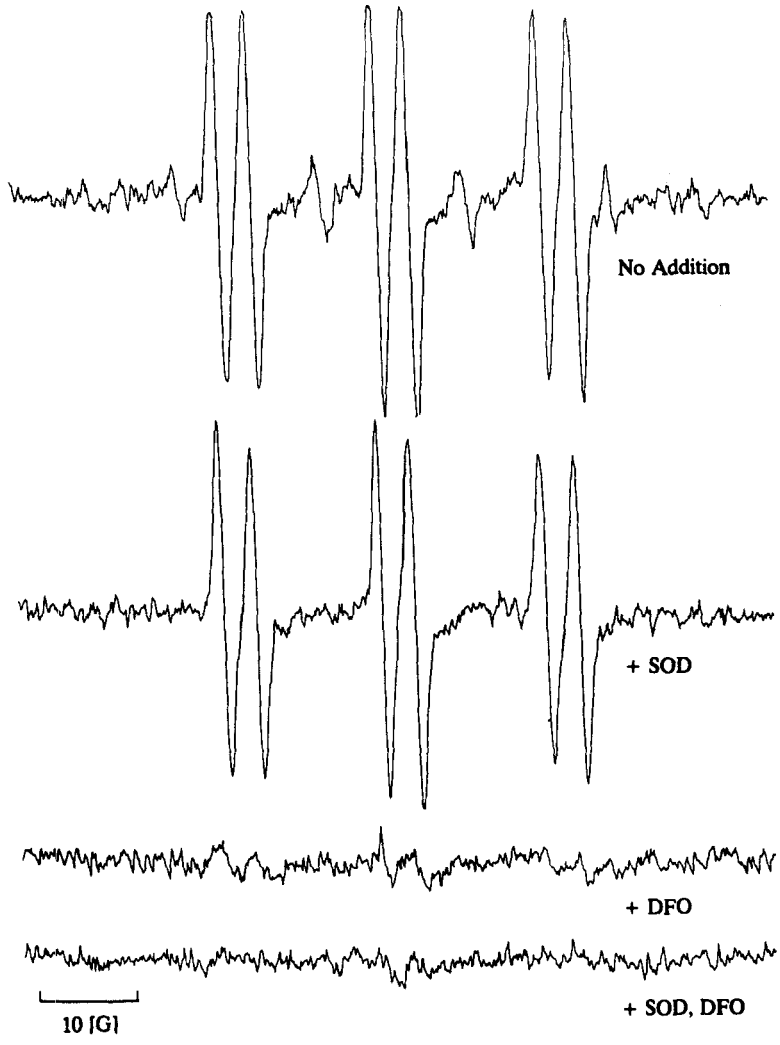

FIGURE 2 Spin trapping of 1-hydroxyethyl radicals formed by rat liver microsomes in the presence of PBN. Conditions are as indicated in the legend to Figure 1, with the exceptions that the PBN concentration was 20 mM, and the spectra were obtained with 10 scans using a gain of 1×10^6.

However, when the same experiments were conducted with PBN as a spin trap, different results were obtained. The EPR spectrum of the 1-hydroxyethyl radical adduct of PBN is the major signal shown in Figure 2, and its identity has been previously confirmed.[5] Weak signals of another unassigned spin adduct were also observed. Addition of SOD by itself eliminated the signal of the unassigned adduct, but had little effect on the signal of the ethanol radical adduct (Figure 2). When DFO was added to the incubation mixture, both EPR signals were almost entirely eliminated. As observed with POBN, there was no detectable EPR signal with the combination of DFO and SOD.

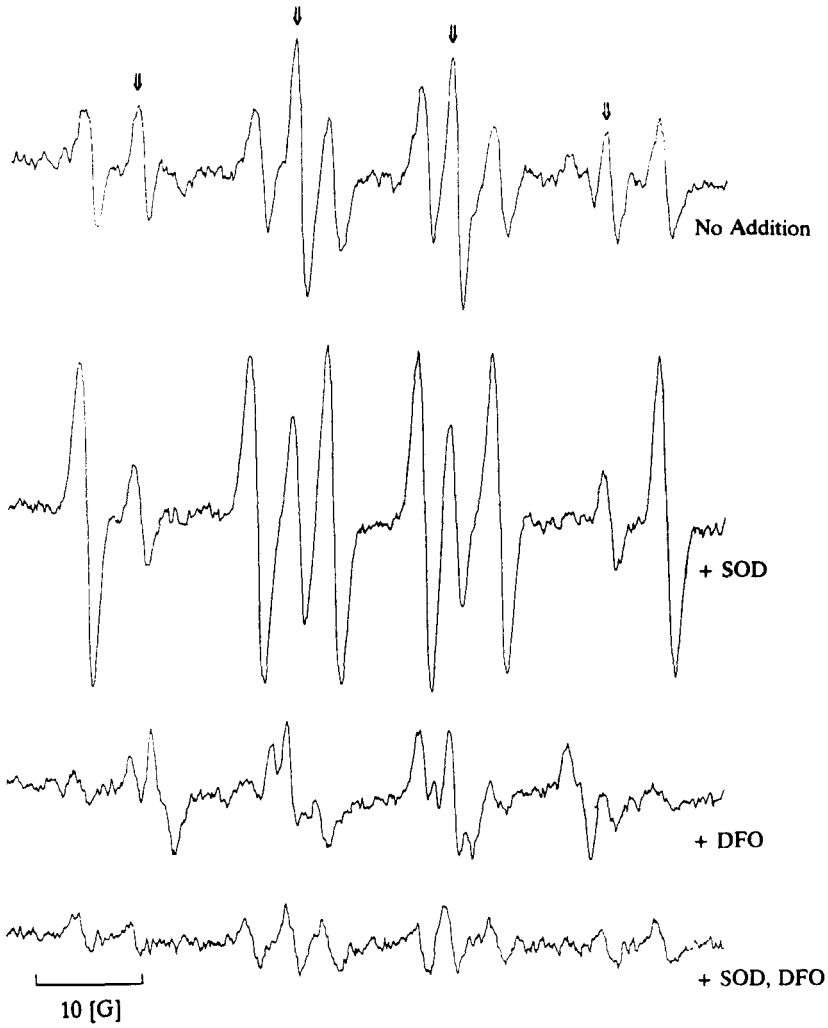

FIGURE 3 Spin trapping of 1-hydroxyethyl radicals formed by rat liver microsomes in the presence of DMPO. Conditions are as indicated in the legend to Figure 1, but with higher concentrations of both DMPO (40 mM) and ethanol (100 mM) and accumulating 5 scans using a spectrometer gain of 1×10^6. The catalase inhibitor azide (0.1 mM) was also added to increase the signal intensity,[2] but azide did not alter the effects of SOD and DFO indicated in this figure. The arrows (⇓) designate the signals of the ·OH adduct of DMPO.

In view of the different results obtained with POBN and PBN, the experiments were extended by using DMPO as a spin trapping agent. Both hydroxyl radicals (·OH) and superoxide radicals (·OOH) form relatively stable spin adducts with DMPO, and we have previously reported detection of both spin adducts in rat liver microsomal suspensions.[2,4] When DMPO was incubated with liver microsomes, ethanol, and 0.1 mM azide to inhibit catalase activity,[2,4] the EPR signals observed were typical of mixtures of ·OH and ethanol radical spin adducts (Figure 3), as reported elsewhere.[2] In contrast to effects observed with the other spin trapping

agents, addition of SOD increased the signal intensity of both spin adducts of DMPO (Figure 3), but this effect was most noticeable with the 1-hydroxyethyl radical adduct. This effect of SOD might be explained by either increased rates of hydrogen peroxide formation from dismutation of superoxide, or protection of the adducts from superoxide-mediated destruction.[6] Preliminary studies with photolytically generated hydroxyl and 1-hydroxyethyl radical adducts of DMPO have confirmed some superoxide-dependent loss of EPR signals (data not shown). When DFO was added to the incubation system, the EPR signal from the 1-hydroxyethyl radical adduct was absent from the spectrum, and the remaining signal was typical of the ·OOH adduct of DMPO. When SOD was present along with DFO, only very weak and poorly defined EPR signals were observed (Figure 3).

The common finding with all three spin trapping agents was that DFO addition decreased the EPR signal intensity of the 1-hydroxyethyl radical spin adducts,

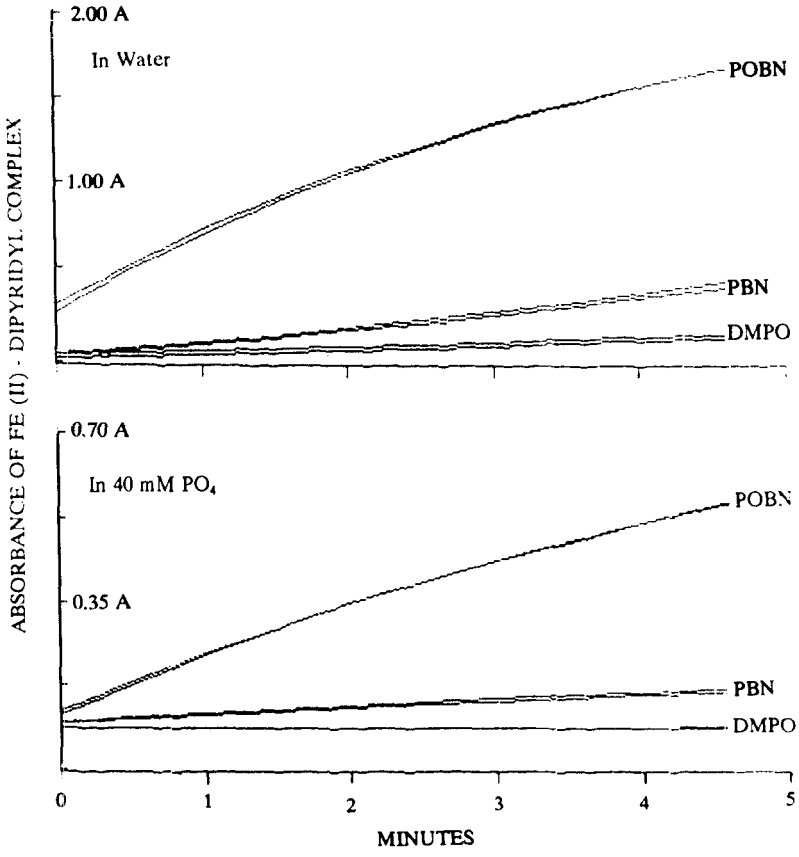

FIGURE 4 Reduction of Fe(III) by nitrone spin traps. $FeCl_3$ (1 mM) was incubated at room temperature with the indicated spin trapping agents (40 mM) and 2,2'-dipyridyl (0.05%). The absorbance of the Fe(II)-dipyridyl complex at 522 nm was monitored continuously using a Perkin-Elmer Lambda 4B UV/VIS Spectrophotometer. In the top panel, the reactions were run in water, using a vertical scale of 2.0 absorbance units. In the lower panel, the reactions were run in 40 mM phosphate buffer (pH 7.4), using a vertical scale of 0.7 absorbance units. Each experimental condition was run in duplicate. The experimentally determined extinction coefficient for the ferrous-dipyridyl complex is 8.3 cm^{-1} mM^{-1}.

indicating an important role of iron in its formation. A residual 1-hydroxyethyl radical signal was observed only when POBN was the spin trapping agent (Figure 1). Similarly, SOD by itself decreased the signal intensity only when POBN was used as the spin trapping agent. Taken together, these data suggested that POBN might uniquely influence some aspect of iron-dependent 1-hydroxyethyl radical formation.

Nitrone spin trapping agents such as DMPO may be capable of reducing Fe(III) to produce artifactual signals.[7] For this reason, the ability of these three nitrone spin trapping agents to reduce Fe(III) was compared in simple chemical systems. $FeCl_3$ (1.0 mM) was added to an aqueous solution of dipyridyl (0.05%), and the absorbance of the ferrous-dipyridyl complex at 522 nm was monitored continuously. Fe(III) was reduced by all three nitrones, but the rate of reduction was greatest with POBN (Figure 4, top panel). When the same experiment was repeated in phosphate buffer as used in spin trapping experiments (40 mM, pH 7.4), Fe(III) reduction was essentially eliminated in the presence of DMPO or PBN, but continued at an appreciable rate (11.2 nmoles/ml/min) in the presence of POBN (Figure 4, lower panel).

These chemical reactions were also tested for their ability to form 1-hydroxyethyl radicals. When ethanol, phosphate buffer (40 mM, pH 7.4) and spin trap (40 mM) were mixed together and subjected to EPR spectroscopy, a readily detectable signal for the 1-hydroxyethyl radical was observed with POBN, but not with PBN or DMPO (Figure 5). If the phosphate concentration was increased to 100 mM, the signal intensity of the 1-hydroxyethyl radical adduct of POBN was approximately twice that observed at the lower concentration of phosphate (Figure 5), but signals were still undetectable with PBN or DMPO (data not shown).

DISCUSSION

POBN is a water-soluble spin trapping agent that forms relatively stable complexes with the 1-hydroxyethyl radical, and appears to be superior to either PBN or DMPO in trapping of this radical. This conclusion is based on the relative EPR signal intensities obtained with the three spin trapping agents under essentially comparable conditions. For example, the EPR signals obtained with POBN (Figure 1) were approximately ten times stronger than those obtained with PBN (Figure 2). DMPO was even less effective under these conditions, and addition of azide was required to intensify the EPR signals (Figure 3). Because of its favorable properties, POBN has become widely utilized in studies of 1-hydroxyethyl radical formation.[3,8-11] The excellent spin trapping properties of POBN may explain its ability to detect 1-hydroxyethyl radicals in the presence of DFO, which eliminated these signals in experiments with PBN and DMPO (Figures 1–3).

In studies of this type, it is obviously essential that the spin trapping agents themselves do not enter into the reaction mechanisms. A caution has already been raised in the case of DMPO, which can interact with Fe(III) to produce an EPR signal which is indistinguishable from that of the ·OH radical adduct.[7] However, this artifact is thought to be inconsequential in biological systems, because iron chelating agents such as phosphate prevent this problem.[12]

However, the data of this report clearly show that POBN is capable of directly reducing Fe(III) in aqueous solutions, even in the presence of phosphate buffer (Figure 4). Because Fe(II) catalyzes formation of other oxidants (e.g., ·OH, ferryl

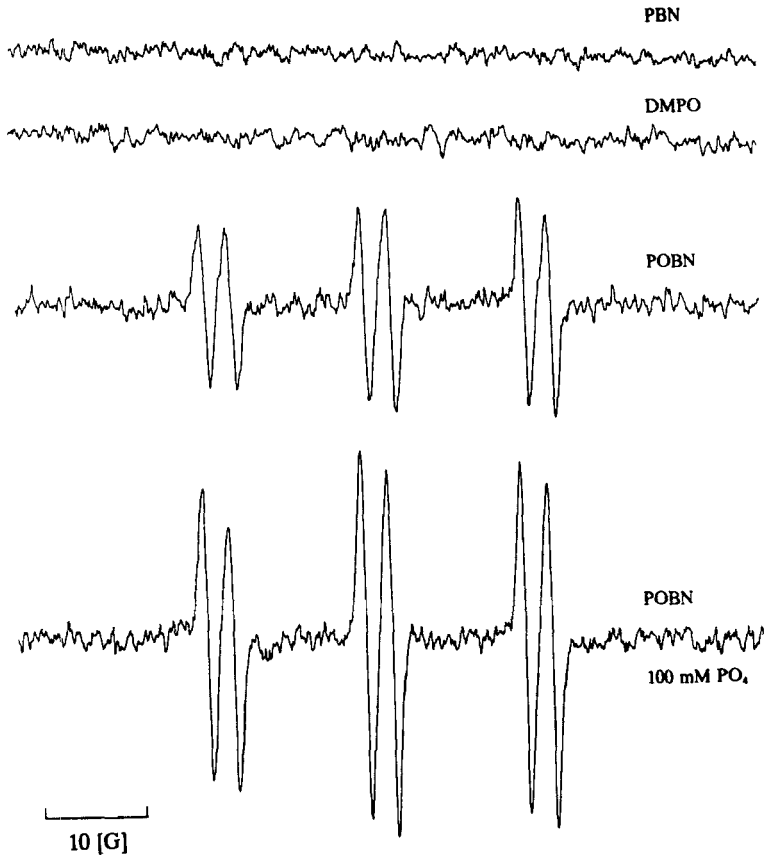

FIGURE 5 1-Hydroxyethyl radical formation in the presence of $FeCl_3$ and phosphate buffer. $FeCl_3$ (1 mM), ethanol (100 mM), and the indicated spin trapping agents (40 mM) were mixed in phosphate buffer (pH 7.4) and were transferred to a flat cell in the cavity of the EPR spectrometer. The phosphate concentration was 40 mM in the top three spectra, and 100 mM in the bottom spectrum. The spectra were obtained with an accumulation of 10 scans, using a spectrometer gain of 1×10^6, and other conditions as indicated in Materials and Methods.

ions), the presence of an agent that reduces Fe(III) may affect both the rate, and the mechanism, of iron-dependent oxidations.

Phosphate buffer is known to speed the autoxidation of Fe(II), and the presumed products of this reaction are Fe(III) and superoxide.[13] Many laboratories have shown that Fe(II) autoxidation in the presence of phosphate buffer forms one or more reactive intermediates which are capable of degrading deoxyribose and benzoate,[14] forming methyl and methoxy radicals from dimethylsulfoxide,[15] and 1-hydroxyethyl radicals from ethanol.[16] It is therefore reasonable to assume that Fe(II) formed by the POBN-dependent reduction of Fe(III), in the presence of phosphate buffer, would rapidly re-oxidize, thereby forming superoxide radicals and/or other oxidants. 1-Hydroxyethyl radicals detected in mixtures of POBN, ethanol, $FeCl_3$ and phosphate buffer (Figure 5) were most likely formed through such reactions.

The results also demonstrate that the concentration of phosphate in systems containing POBN, $FeCl_3$, and ethanol influences the reaction significantly. A higher concentration of phosphate enhanced POBN-dependent 1-hydroxyethyl radical generation, as shown in Figure 5. This effect may be due to higher rates of Fe(III) reduction and reoxidation, and consequently a higher rate of 1-hydroxyethyl radical production from ethanol. Because PBN and DMPO reduce Fe(III) very slowly in the presence of phosphate (Figure 4), it is not surprising that 1-hydroxyethyl radical signals were not detected in those systems.

Products formed in these types of chemical oxidation-reduction reactions may not necessarily be part of the biological system under study. There is no reason to doubt that POBN detects microsomal 1-hydroxyethyl radical formation. Nevertheless, it is bothersome that SOD inhibited 1-hydroxyethyl radical formation only when POBN was the spin trapping agent (Figure 1). Because POBN appears to have greatest sensitivity for detection of 1-hydroxyethyl radicals, as discussed above, it seems that even greater inhibitory effects of SOD should be observed with PBN or DMPO. However, there was no apparent effect of SOD when PBN was used as a spin trapping agent (Figure 2), and a paradoxical increase in signal intensity was observed in experiments with DMPO (Figure 3).

These conflicting results are difficult to explain. It is possible that POBN could enter a microenvironment which is not readily accessible to PBN or DMPO, but this seems unlikely because both POBN and DMPO are highly water soluble. Based on data which show reduction of Fe(III) by POBN (Figure 4) and subsequent formation of 1-hydroxyethyl radicals (Figure 5), it appears that undesirable effects of POBN on iron chemistry may have influenced reactions that would normally be attributed to microsomal enzyme systems.

The extent to which POBN may cause similar problems in other free radical reactions is still unknown. However, investigators who wish to employ POBN as a spin trapping agent in any reaction where iron may have a catalytic role should be aware that the potential for POBN-influenced radical formation exists. It may be appropriate to compare results obtained with other spin trapping agents in order to test for possible direct effects of the spin trapping agent itself.

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