Spin Trapping of Superoxide and Hydroxyl Radicals with Substituted Pyrroline 1-Oxides

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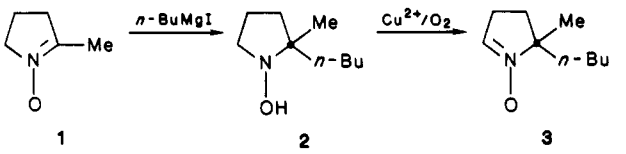
The synthesis of three nitrones, 5-butyl-5-methyl-1-pyrroline 1-oxide (BMPO), 5,5-dipropyl-1-pyrroline 1-oxide (DPPO), and 2-aza-2-cyclopentenespirocyclopentane 2-oxide (CPPO), was conducted with use of the zinc/ammonium chloride reduction of appropriately substituted γ-nitrocarbonyl compounds. The lipophilicity of these nitrones was estimated by determining their partition coefficients in a 1-octanol/water system. These nitrones were found to possess more lipophilic character than the most frequently used cyclic nitrone, 5,5-dimethyl-1-pyrroline 1-oxide (DMPO), which exhibits a partition coefficient of only 0.02. Hyperfine coupling constants for the spin trapping of superoxide and hydroxyl radical by the various nitrones were determined. The rate of spin trapping of superoxide with each nitrone was conducted by competitive kinetics with superoxide dismutase (SOD). In addition, the ability of DPPO and BMPO to spin trap free radicals generated during the metabolism of menadione by rat enterocyte cells was investigated. From these studies, DPPO and BMPO appear to be more suitable spin traps than DMPO when one is interested in monitoring free radicals generated intracellularly.

In recent years, a renewed interest in superoxide and hydroxyl radicals has taken place as these reactive intermediates have been proposed to mediate a variety of cellular responses including phagocytosis,[1] ischemic tissue injury,[2] aging,[3] and cancer.[4] Yet, despite enormous efforts to study the role of free radicals in cell injury, the most difficult obstacle remains the identification of these highly reactive species. One method that has been used successfully is spin trapping.[5] This technique consists of using a nitrone or nitroso compound to "trap" the initial unstable free radical as a "long-lived" nitroxide that can be observed at room temperature by using conventional electron spin resonance (ESR) spectrometric procedures.[6] Because the stable nitroxide accumulates, spin trapping is an integrative method of measurement and is inherently more sensitive than procedures that determine instantaneous or steady-state concentrations of free radicals.

Despite the above, spin trapping methods are not without their limitations and artifacts. For example, during the spin trapping of superoxide by 5,5-dimethyl-1-pyrroline 1-oxide (DMPO), it was determined that the half-life of the corresponding nitroxide, 2-hydroperoxy-5,5-dimethyl-1-pyrrolidinyloxyl (DMPO-OOH), was only 8 min.[7] However, the problem arises that during its decomposition a number of products are formed, including 2-hydroxy-5,5-dimethyl-1-pyrrolidinyloxyl (DMPO-OH) and hydroxyl radical, the latter of which can then be spin trapped by DMPO.[8] Because of these secondary reactions, it is not always possible to assign an ESR spectrum to a specific free radical trapped.

Despite these and other limitations, more investigators are turning to spin trapping methods as a means to study free-radical processes in biological milieu. Furthermore, with the observation that free radicals can be spin trapped in cell suspensions as a consequence of cellular drug metabolism,[9] the necessity to minimize artifacts has become even more crucial. Clearly, these observations point to the fact that new and more specific spin traps will be needed in order to address a number of important questions pertaining to how the cell deals with free radicals. A review of the literature indicates few studies that are devoted to the synthesis of 5,5-dialkyl-1-pyrroline 1-oxides. Two of the most important investigations, namely, Lee and Keana[10] and Haire and Janzen,[11] only synthesized several cyclic nitrones without further inquires into their biologic applicability.

Scheme I



In this paper, we report on the synthesis of a number of 5,5-dialkyl-1-pyrroline 1-oxides, the kinetics of their reaction with superoxide in aqueous medium, and their partition into a lipid environment. In addition, for several nitrones, we examine their ability to spin trap free radicals as the result of rat enterocyte metabolism.

Results and Discussion

The cyclic nitrone 5,5-dimethyl-1-pyrroline 1-oxide (DMPO) has been utilized as a spin trapping agent to detect biologically generated free radicals.[12] However, as discussed above, DMPO possesses some characteristics that makes it less attractive for many biologic applications. For example, this nitrone is extremely hygroscopic and in the presence of oxygen and trace metal ion impurities (e.g., 0.1 M potassium phosphate buffer contains 1 μM of iron as a contaminant), paramagnetic species will rapidly arise. In the case cited above, the resulting nitroxide has been reported to be 2-hydroxy-5,5-dimethyl-1-pyrrolidinyloxy (DMPO-OH).[8] As will be shown later in this report, DMPO-OH can also arise as a consequence of 2-hydroperoxy-5,5-dimethyl-1-pyrrolidinyloxy (DMPO-OOH) reduction by the enzyme glutathione peroxidase.

Another problem encountered with DMPO is its limited

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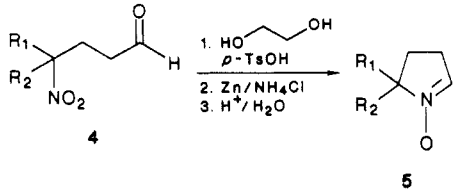
Scheme II[a]



[a] **a**, R_1 = Me, R_2 = Bu; **b**, R_1 = R_2 = Pr; **c**, R_1 and R_2 form $(CH_2)_4$.

diffusion into lipids. For example, we had earlier demonstrated[13] that the 1-octanol/phosphate buffer partition coefficient was only 0.09, which attests to its sparingly lipid soluble character. This is of particular importance since the second-order dismutation rate of superoxide at physiological pH is 6×10^5 M^{-1} s^{-1},[14] whereas the second-order rate constant of DMPO with superoxide is only 10 M^{-1} s^{-1}.[15] Thus, a high concentration of DMPO (usually around 0.1 M) in biological preparations is essential in order to allow for the detection of superoxide. From the standpoint of spin trapping free radicals in biological milieu, it becomes imperative that artifacts and limitations be minimized.

With the above discussion in mind, we designed and synthesized various nitrones that would exhibit increased lipophilicity. Preparation of these nitrones was undertaken by two different synthetic pathways. For example, the first involved the addition of *n*-butylmagnesium bromide to 2-methyl-1-pyrroline 1-oxide (**1**), giving the corresponding hydroxylamine, 2-butyl-2-methyl-1-hydroxypyrrolidine (**2**). Copper(II)-catalyzed aerial oxidation gave the corresponding nitrone 5-butyl-5-methyl-1-pyrroline 1-oxide (**3**), as shown in Scheme I. Although initial analysis of **3** by IR and NMR suggested that the conversion from **1** to **3** was complete, all attempts at purification of **3** via either chromatography or fractional distillation gave rise to a mixture of nitrones (**1** and **3**). Purification of other nitrones using the procedure described above was also unsuccessful. For this reason a second synthetic scheme was utilized. The key step involved cyclization of various appropriately substituted γ-(hydroxyamino)carbonyl compounds (Scheme II). For example, 5-butyl-5-methyl-1-pyrroline 1-oxide (**5a**) was prepared from 4-methyl-4-nitrooctanal ethylene acetal by using the zinc/ammonium chloride reduction procedure outlined by Bonnett et al.[16] Nitrones prepared by this synthetic route could then be purified to the degree necessary for spin trapping experiments. This purification was twofold: first, fractional elution was performed with silica gel (mesh 230–400) and a dichloromethane/methanol solvent system and second, the purified fraction containing the nitrone was Kugelrohr distilled. Nitrones at this point were essentially devoid of any paramagnetic impurities and were subsequently stored under argon at −70 °C until use.

The spin trapping of superoxide with nitrones depicted in Scheme II was conducted by using a superoxide-generating system consisting of xanthine in the presence of the enzyme xanthine oxidase at pH 7.8. The rate of superoxide formation, as measured by superoxide dismutase inhibitable reduction of cytochrome *c*, was 10 μM/min. The addition of superoxide dismutase (10 μg/mL) to the above reaction mixture confirmed that the ESR spectrum resulted from the reaction of superoxide with each nitrone. Hyperfine splitting constants for superoxide spin trapped adducts are summarized in Table I.

Table I. Summary of Experimental Data for Nitrones

	BMPO (**4a**)	DPPO (**4b**)	CPPO (**4c**)	DMPO
A_N ($O_2^{-\bullet}$), G	13.7	13.4	14.1	14.3[b]
A^{H_β} ($O_2^{-\bullet}$), G	11.8	11.2	11.5	11.7[b]
A^{H_γ} ($O_2^{-\bullet}$), G	2.0	1.4	*a*	1.25[b]
A_N ($^\bullet$OH), G	15.8	14.9	15.8	14.9[b]
A_H ($^\bullet$OH), G	15.8	14.9	22.3	14.9[b]
k M^{-1} s^{-1}	4.6	2.0	2.1	10[c]
K (octanol/water)	1.1	4.9	0.8	0.02 (0.09)[d]

[a] The spectrum was too broad to accurately determine this parameter. [b] Reference 7. [c] Reference 15. [d] The partition coefficient was obtained with use of 1-octanol/10 mM phosphate buffer at pH 7.4 (ref 13).

Spin trapping of hydroxyl radical was conducted by the addition of ferric ammonium sulfate (0.1 mM) to the above superoxide-generating system. The addition of catalase (300 units/mL) to the mixture confirmed that the ESR spectrum resulted from the reaction of hydroxyl radical with each nitrone. Hyperfine splitting constants for hydroxyl radical spin trapped adducts are also summarized in Table I.

To estimate the relative lipophilicity of the various nitrones synthesized in Scheme II, we determined the partition coefficient (K) of each nitrone in a water/1-octanol system. We found that each nitrone was more lipophilic than its analogue DMPO, which exhibited a partition coefficient of only 0.02. 5,5-Di-*n*-propyl-1-pyrroline 1-oxide (**5b**) was found to be more lipophilic than the other nitrones, making it particularly suitable for spin trapping experiments that involve intracellularly generated free radicals. The partition coefficients obtained are given in Table I.

We found that as the lipophilic character of the spin trap increased, aggregation in aqueous solution became more apparent. For example, anisotropic effects were observed in the ESR spectrum of 2-aza-3-hydroperoxycyclopentanespirocyclopentane-2-oxyl (**5c**). This phenomenon was also noted when **5c** was used to detect free radicals generated by rat enterocytes, suggesting the difficulty in the free rotation of the nitroxide in a lipid environment such as a cell preparation.

Rate constants for the reaction of various nitrones with superoxide are given in Table I. For these experiments we chose xanthine/xanthine oxidase as a source of superoxide because the rate of its production by this enzyme was found to be independent of nitrone concentration. For the studies described herein, the rate of spin trapping superoxide by various nitrones was conducted at pH 7.8. In an earlier paper,[15] we developed equations to determine the rate constant for the reaction of superoxide with DMPO. Because of this, we will now only summarize our approach. Rate constants for the reaction of superoxide with various nitrones were determined by competitive kinetics at pH 7.8. In this model, a rate constant is determined by using a substance with a known rate constant to inhibit the reaction of a nitrone with superoxide. For our experiments, superoxide dismutase (SOD) was chosen as the competitive inhibitor. In each case, the concentration of nitrone was held constant while the concentration of SOD was varied. SOD was standardized against cytochrome *c* according to the method of McCord and

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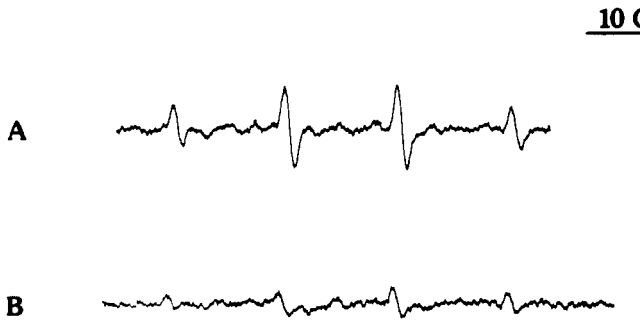

Figure 1. ESR spectra obained when enterocytes were incubated with DMPO in the presence (A) and absence (B) of menadione (0.1 mM in 0.14 M Me_2SO). The ESR spectrum in A is characteristic of DMPO-OH. Microwave power was 20 mW; the modulation frequency was 100 kHz with an amplitude of 1 G. Sweep time was 12.5 G/min and the receiver gain was 8×10^3 with a response time of 1 s; $A_H = A_N = 14.9$ G.

Fridovich.[17] Data were analyzed by using the following equation:

$$(V/v) - 1 = k_{SOD}[SOD]/k_{nitrone}[nitrone]$$

In this equation, V and v represent the rates of spin trapping of superoxide in the absence and presence of SOD, respectively. The second-order rate constants k_{SOD} and $k_{nitrone}$ are for the reaction of superoxide with SOD and nitrone, respectively. Control experiments indicated that there was no inhibition between SOD and the spin traps. As shown in Table I, rate constants for the various nitrones were found to fall into a narrow range of 2.0–4.6 M^{-1} s^{-1}, suggesting that the increased lipophilicity of the nitrone does little to enhance the reaction rate of superoxide in a physiologic buffer at pH 7.8.

Our initial studies were aimed at determining which cell model would allow us to address the issue as to the ability of various 5,5-dialkyl-1-pyrroline 1-oxides to spin trap superoxide as a consequence of cellular drug metabolism. From our earlier investigations using endothelial cells and the drug menadione,[9] it was clear that this model would not be adequate for these studies since most of the superoxide spin trapped, under the above described conditions, was extracellular. Freshly isolated rat hepatocytes presented another problem in that these cells contain high concentrations of several reductases that rapidly reduce the resulting nitroxide, obtained from the reaction of superoxide with the nitrone, to the corresponding hydroxylamine.[18] Thus, with hepatocytes, it is not possible to make a judgement as to the ability of various spin traps to diffuse across the plasma membrane of the cell and react with the superoxide generated, in situ. However, we did have preliminary data that suggested that rat intestinal cells, enterocytes, had low levels of nitroxyl reductases and as such might allow for the detection of nitroxides resulting from the reaction of nitrones with superoxide.

With this cellular preparation, we first sought to determine whether or not free radicals would be generated during the metabolism of a vitamin K derivative, menadione. It is now established that menadione when reduced by one-electron pathways can form a semiquinone free radical, which in the presence of oxygen generates superoxide.[19] Incubation of freshly isolated enterocytes with menadione (0.1 mM in 0.14 M Me_2SO) and DMPO resulted in the ESR spectrum shown in Figure 1A. In the absence of either menadione or menadione and cells, a very small signal was detected (Figure 1B). This spectrum apparently resulted from metal ion catalyzed aerial oxidation of DMPO to DMPO-OH. If the Swim's buffer, used in the above experiments, is changed to a simpler buffer containing only sodium phosphate (50 mM), NaCl (0.15 M), and glucose (5 mM), which had been passed through an ion-exchange column and then *N,N*-bis[2-[bis(carboxymethyl)amino]ethyl]glycine (DTPA, 1 mM) added, the ESR signal shown in Figure 1B was not present. Unfortunately, unlike our earlier studies with endothelial cells, rat enterocytes do not survive well in this simplified buffer.

The ESR spectrum depicted in Figure 1A is characteristic of DMPO-OH.[7] This observation, at first, suggests that, during enterocyte metabolism of menadione, hydroxyl radical was generated and spin trapped accordingly. However, we believe this to be unlikely. Evidence from previous investigations and current data point to the fact that superoxide is the only free radical spin trapped under the experimental conditions employed and that rapid decomposition of DMPO-OOH to DMPO-OH accounts for the observed ESR spectrum.

First, it is known that quinones can be biologically reduced to semiquinones, which then autoxidize, yielding superoxide.[19] Although superoxide dismutes to afford hydrogen peroxide, subsequent formation of hydroxyl radical depends upon the ability of an intracellular metal ion (e.g., Fe^{2+}) to reduce hydrogen peroxide.[20] There is presently little data to support the existence of this reaction taking place within a cell. In fact, on the basis of a recent review article[21] that addressed the issue of hydroxyl radical formation in cell suspensions, one may surmise that hydroxyl radical is probably not being generated. However, even if hydroxyl radical were produced intracellularly, our methodology should have detected its presence. This is because the addition of a high concentration of Me_2SO (0.14 M, 40% greater than the spin trap DMPO) would have produced methyl radicals as a result of hydroxyl radical reaction with Me_2SO.[22-24] Spin trapping would then give 2,2,5-trimethyl-1-pyrrolidinyloxyl (DMPO-CH_3). However, as shown in Figure 1A, there is no evidence for the presence of this species (see Figure 2 for computer-simulated spectra of DMPO-OH and DMPO-CH_3). If the ESR spectrum shown in Figure 1A does not arise from the spin trapping of hydroxyl radical, then what is its source? Previous studies in our laboratory documented that DMPO-OOH decomposes into several species, one of which is DMPO-OH.[8] This decomposition could either be chemical or enzymatic. If chemical, the half-life of DMPO-OOH is approximately 8 min,[15] and DMPO-OOH should be observable. It was not, suggesting a more rapid elimination. If enzymatic, the reduction of DMPO-OOH to DMPO-OH suggests the presence in these cells of glutathione peroxidase whose physiological function is the reduction of organic hydroperoxides to alcohols.[25] For this reason, we determined the glutathione peroxidase activity of these cells and found it to be 20 ± 2 nmol min^{-1}

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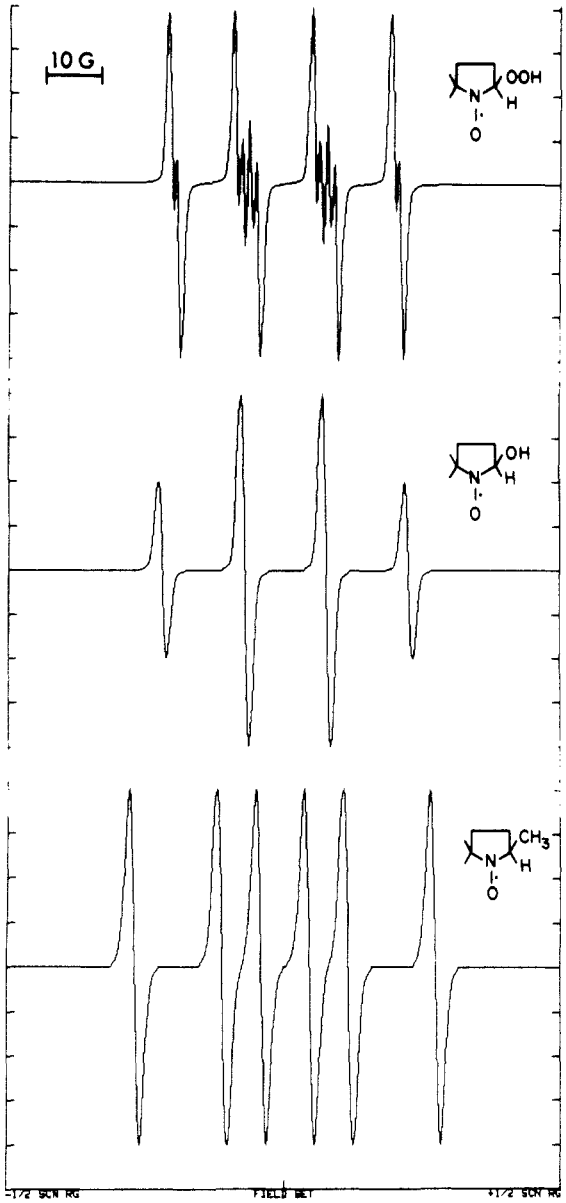

Figure 2. Computer simulation of ESR spectra for 2,2-dimethyl-5-hydroxy-1-pyrrolidinyloxyl (DMPO-OH) and 2,2,5-trimethyl-1-pyrrolidinyloxyl (DMPO-CH_3). The hyperfine splittings are as follows: for DMPO-OOH, A_N = 14.3 G, A^{H_β} = 11.7 G, and A^{H_γ} = 1.25 G; for DMPO-OH, $A_N = A_H$ = 14.87 G; for DMPO-CH_3, A_N = 15.31 and A_H = 22.00 G.

(mg of protein)$^{-1}$. We have previously demonstrated that this enzyme would reduce DMPO-OOH to DMPO-OH.[9] Thus, we suggest that only superoxide is spin trapped by DMPO during the metabolism of menadione and that the characteristic DMPO-OH ESR spectrum shown in Figure 1A results primarily from the enzymatic reduction of DMPO-OOH and not the direct spin trapping of hydroxyl radical.

Results reported above raise the question as to whether the ESR spectrum in Figure 1A was a composite of extra- and intracellular spin trapped superoxide. To answer this query, we incubated enterocytes with menadione and DMPO in the presence of superoxide dismutase (10 μg/mL). The resulting ESR spectrum was that of Figure 1A, demonstrating that no extracellular superoxide was spin trapped by DMPO.

With the knowledge that superoxide can be spin trapped intracellularly as a consequence of enterocyte metabolism, we felt that this model would be an appropriate test to

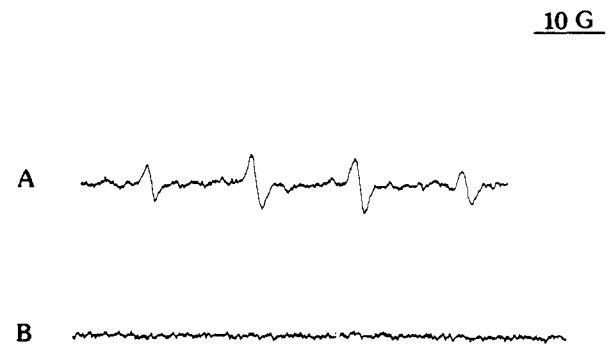

Figure 3. ESR spectra obtained when enterocytes were incubated with DPPO in the presence (A) and absence (B) of menadione (0.1 mM in 0.14 M DMSO). The ESR spectrum in A is characteristic of DPPO-OH. Microwave power was 20 mW; the modulation frequency was 100 kHz with an amplitude of 1 G. Sweep time was 12.5 G/min and the receiver gain was 8×10^3 with a response time of 1 s, $A_H = A_N$ = 14.9 G.

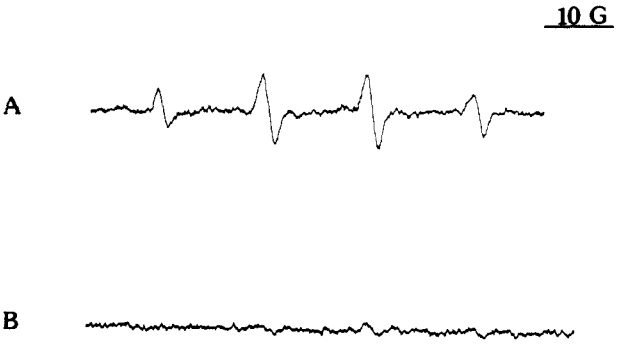

Figure 4. ESR spectra obtained when enterocytes were incubated with BMPO in the presence (A) and absence (B) of menadione (0.1 mM in 0.14 M DMSO). The ESR spectrum in A is characteristic of BMPO-OH. Microwave power was 20 mW; the modulation frequency was 100 kHz with an amplitude of 1 G. Sweep time was 12.5 G/min and the receiver gain was 8×10^3 with a response time of 1 s, $A_H = A_N$ = 15.8 G.

evaluate the ability of the nitrones in Scheme II to diffuse into cells and, in so doing, spin trap superoxide. When either 5,5-dipropyl-1-pyrroline 1-oxide (DPPO, 0.1 M) or 5-butyl-5-methyl-1-pyrroline 1-oxide (BMPO, 0.1 M) was incubated with enterocytes in the presence of menadione (0.1 mM in 0.14 M Me_2SO), ESR spectra shown in Figures 3 and 4 were obtained. Of interest is the finding that although the rate of spin trapping of superoxide by DMPO in aqueous solution was two- or fivefold greater than that of BMPO and DPPO, respectively, in freshly isolated enterocytes, however, there was little difference in the concentration of the corresponding superoxide spin trapped adduct among the various nitrones examined. These results point to the fact that even though the rate constants for the spin trapping of superoxide by either BMPO or DPPO is considerably less than for DMPO, in cell suspension studies, the difference can be overcome by increasing the lipophilicity of the spin trap. One final observation is worth noting. We have found that for BMPO and DPPO the metal ion catalyzed aerial oxidation of the nitrone to 1-hydroxy-5,5-dipropylpyrrolidine (DPPO-OH) and 1-hydroxy-5-methyl-5-propylpyrrolidine (BMPO-OH) is less prevalent than for DMPO. This conclusion was reached by comparing ESR spectra obtained from a 0.1 M solution of each trap after the samples were stored under argon at −70 °C for a period of 1 month. Paramagnetic impurities were present in the sample of DMPO. However, none were detected in the BMPO and DPPO samples. We conclude, on the basis of results presented

herein, that either BMPO or DPPO are better spin traps than DMPO when one is interested in the detection of free radicals generated intracellularly as a consequence of metabolism.

Experimental Section

General Comments. Xanthine oxidase, bovine erythrocyte superoxide dismutase (SOD), diethylenetriaminepentaacetic acid (DTPA), and hypoxantine were purchased from Sigma Chemical Co. Chelex 100 was purchased from Bio-Rad. All buffers were passed through a Chelex-100 column according to the method of Poyer and McCay[26] to remove trace metal impurities. All other reagents were used as obtained from commercial suppliers unless otherwise noted. Acrolein was freshly distilled prior to use. Tetrahydrofuran was distilled from sodium benzophenone ketyl during passage of an inert gas (nitrogen or argon).

Infrared spectra were recorded on a Perkin-Elmer Model 1330 infrared spectrophotometer. Proton NMR spectra were obtained on a Perkin-Elmer R-32 90-mHZ spectrometer utilizing tetramethylsilane (Me_4Si) as an internal standard. In many cases only the characteristic peaks were recorded in the NMR spectrum due to overlap of signals in the high-field region. Electron spin resonance spectra were recorded on a Varian Associates Model E-9 spectrometer. Elemental analyses were performed by Atlantic Microlab, Inc., Atlanta, GA. Where analyses are indicated only by the symbols of the elements, the analytical results were within the acceptable range of ±0.4% of the theoretical values for those elements.

Column chromatographic separations were performed with silica gel (230–400 mesh) obtained from American Scientific Co. Elution of the components was monitored by analytical thin-layer chromatography utilizing Eastman Chromagram silica gel sheets without fluorescent indicator. All solvents were used as obtained commercially.

5,5-Dimethyl-1-pyrroline 1-oxide (DMPO) was prepared according to the procedure of Bonnett et al.[17] The nitrone was Kugelrohr distilled at 23–25 °C (0.05 mm) prior to use to remove any paramagnetic impurities.

Syntheses. 2-Nitrohexane. A solution containing 2-bromohexane (16.51 g, 0.1 mol), sodium nitrite (12.0 g), phloroglucinol dihydrate (17.5 g), and urea (13.5 g) in 300 mL of *N,N*-dimethylformamide was stirred in the dark at ambient temperatures for 4 days. The mixture was then poured into 300 mL of ice water layered over with 200 mL of hexane. The hexane layer was removed and the aqueous DMF layer extracted with three 200-mL portions of hexane. The hexane extracts were combined, washed with 200 mL of saturated saline solution, and dried over calcium chloride. The solvent was removed and the residue fractionally distilled to afford 6.55 g (50%) of the product as a colorless liquid, bp 70–72 °C (10 mm) [lit.[27] 95 °C (50 mm)]: IR (neat film) 1550 (N–O) cm^{-1}; ^1H NMR (CCl_4) δ 4.48 (sextet, 1 H, $CHNO_2$).

4-Nitroheptane. A solution of 4-bromoheptane (17.0 g, 95 mmol), sodium nitrite (11.4 g), phloroglucinol dihydrate (16.6 g), and urea (12.8 g) dissolved in 300 mL of *N,N*-dimethylformamide was treated as above to give 6.92 g (50%) of the product as a colorless liquid, bp 44–46 °C (0.25 mm) [lit.[28] 58–60 °C (3.0 mm)]: IR (neat film) 1547 (N–O) cm^{-1}; ^1H NMR (CCl_4) 4.39 (m, 1 H, $CHNO_2$).

4-Methyl-4-nitrooctanal (4a). A solution of 2-nitrohexane (6.0 g, 46 mmol) and Triton B (0.31 mL) in 50 mL of dry tetrahydrofuran was allowed to cool in an ice bath. Freshly distilled acrolein (3.1 mL, 46 mmol) in 10 mL of THF was added dropwise over a period of 20 min to the ice-cooled, stirring solution. After complete addition the solution was allowed to stir in the ice bath for an additional 5 h. The reaction was quenched with 3 mL of 1 N hydrochloric acid solution, the mixture diluted with 200 mL of dichloromethane and washed once with 100 mL of saturated saline solution, and the dichloromethane layer dried over anhydrous sodium sulfate. The solvent was removed and the residue vacuum distilled to afford 3.0 g (35%) of **4a** as a pale yellow liquid, bp 112–114 °C (1.0 mm): IR (neat film) 2738 ((C=O)–H), 1727 (C=O), 1535 (N–O) cm^{-1}; ^1H NMR (CCl_4) δ 9.73 (s, 1 H, (C=O)–H). Anal. ($C_9H_{17}NO_3$) C, H, N.

4-Nitro-4-propylheptanal (4b). The conjugate addition of 4-nitroheptane (12.0 g, 83 mmol) and acrolein (2.8 mL, 41 mmol) was performed as indicated above to give 4.07 g (49%) of **4b** as a light yellow oil, bp 100–102 °C (0.25 mm): IR (neat film) 2730 ((C=O)–H), 1723 (C=O), 1533 (N–O) cm^{-1}; ^1H NMR (CCl_4) δ 9.97 (s, 1 H, (C=O)H), 2.52 (m, 4 H), 2.12 (m, 4 H), 1.10–1.70 (m, 10 H). Anal. ($C_{10}H_{19}NO_3$) C, H, N.

3-(1-Nitrocyclopentyl)propanal (4c). Nitrocyclopentane (9.51 g, 83 mmol) was added to a solution of sodium methoxide (prepared by dissolving 0.2 g of sodium metal in 50 mL of absolute methanol). The solution was heated to reflux, the external heat removed, and freshly distilled acrolein (5.52 mL, 83 mmol) added dropwise at a rate sufficient to maintain controlled refluxing. The solution was then refluxed for an additional hour. After cooling, the solution was acidified with glacial acetic acid, methanol was evaporated, and the residue was shaken with 50 mL of saturated saline solution. The resulting mixture was extracted with three 50-mL portions of diethyl ether, and the combined extracts were dried over anhydrous sodium sulfate. Removal of the solvent and fractional distillation afforded 4.18 g (30%) of **4c** as a pale-yellow oil, bp 94–97 °C (0.05 mm): IR (neat film) 2745 ((C=O)–H), 1725 (C=O), 1535 (N–O), 1360 (N–O) cm^{-1}; ^1H NMR (CCl_4) 9.68 (s, 1 H, ((C=O)H), 2.40 (m, 6 H), 1.74 (br s, 6 H). Anal. ($C_8H_{13}NO_3$) C, H, N.

4-Methyl-4-nitrooctanal Ethylene Acetal. A solution of 4-nitro-4-nitrooctanal (2.45 g, 13 mmol), ethylene glycol (0.89 g, 14 mmol), and *p*-toluenesulfonic acid (0.04 g) in 50 mL of benzene was refluxed for 2 h, while any water produced was collected in a Dean–Stark trap. Once the reaction was complete, the solution was cooled and then washed once with 50 mL of distilled water, 50 mL of saturated aqueous sodium bicarbonate, and then 50 mL of saturated saline solution. The benzene layer was dried over anhydrous magnesium sulfate, the solvent removed, and the residue vacuum distilled to afford 2.31 g (76%) of the product as a colorless oil, bp 115–118 °C (0.6 mm): IR (neat film) 1535 (N–O) cm^{-1}; ^1H NMR (CCl_4) δ 4.78 (m, 1 H), 3.84 (d, 4 H), 1.88 (m, 5 H), 0.8–1.70 (complex m, 11 H). Anal. ($C_{11}H_{21}NO_4$) C, H, N.

4-Nitro-4-propylheptanal Ethylene Acetal. A solution of 4-nitro-4-propylheptanal (3.50 g, 17 mmol), ethylene glycol (1.19 g, 19 mmol), and *p*-toluenesulfonic acid (0.05 g) was treated as above to afford 3.21 (75%) of the product as a colorless oil, bp 108–110 °C (0.1 mm): IR (neat film) 1530 (N–O), 1352 (N–O) cm^{-1}; ^1H NMR (CCl_4) δ 4.78 (t, 1 H, CHO_2), 3.85 (m, 4 H, OCH_2CH_2O), 1.85 (m, 6 H), 0.85–1.55 (complex m, 12 H). Anal. ($C_{12}H_{23}NO_4$) C, H, N.

3-(1-Nitrocyclopentyl)propanal Ethylene Acetal. A solution of 3-(1-nitrocyclopentyl)propanal, ethylene glycol (1.20 g, 19 mmol), and *p*-toluenesulfonic acid (0.05 g) in 50 mL of benzene was treated as above to afford 3.08 g (82%) of the product as a colorless oil, bp 120–122 °C: IR (neat film) 1530 (N–O), 1355 (N–O) cm^{-1}; ^1H NMR (CCl_4) δ 4.75 (t, 1 H, $CH(CH_2O_2)$), 3.77 (m, 4 H, OCH_2CH_2O), 2.50 (m, 2 H), 2.10–1.30 (complex m, 10 H). Anal. ($C_{10}H_{17}NO_4$) C, H, N.

5-Butyl-5-methyl-1-pyrroline 1-Oxide (5a). Zinc dust (2.3 g) was added portionwise to a solution of 4-methyl-4-nitrooctanal ethylene acetal (2.00 g, 8.7 mmol) and ammonium chloride (0.49 g) in 100 mL of 50% aqueous ethanol. The resulting suspension was stirred vigorously for 12 h at ambient temperatures, the suspension was filtered, the filter cake was washed with four 25-mL portions of warm water, and the combined filtrate and washings were acidified with 20 mL of concentrated hydrochloric acid. The acidic solution was heated at approximately 70 °C for 1 h. The solution was then cooled in an ice bath and was basified with potassium carbonate. The mixture was concentrated to about 50 mL on a rotavap and the residue extracted with four 50-mL portions of dichloromethane. The combined extracts were dried over anhydrous sodium sulfate. The solvent was removed and the residue first purified by chromatographic separation on a silica gel column. Elution of the column with 5% methanol/dichloromethane afforded 1.33 g of **5a** (R_f 0.67) as a light yellow oil, which gave an EPR signal. The resulting fraction was Ku-

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gelrohr distilled at 30–35 °C (0.05 mm) to afford 1.09 g (81%) of **5a** as a colorless oil: IR (neat film) 1583 (C=N), 1245 (N–O) cm^{-1}; ^1H NMR (CCl_4) δ 6.58 (m, 1 H), 2.55 (m, 2 H), 2.12 (m, 2 H), 1.90–0.85 (complex m, 12 H). Anal. ($C_9H_{17}NO$) C, H, N.

5,5-Dipropyl-1-pyrroline 1-Oxide (5b). A solution of 4-propyl-4-methylheptanal ethylene acetal (2.6 g, 11 mmol) and ammonium chloride (0.63 g) in 150 mL of 60% aqueous ethanol was treated with zinc dust (3.0 g) as given above. The crude product was initially purified by column chromatography as described above (R_f 0.82) and then Kugelrohr distilled at 30–50 °C (0.1 mm) to give **5b** as a colorless oil: IR (neat film) 1580 (C=N), 1205 (N–O) cm^{-1}; ^1H NMR (CCl_4) δ 6.63 (m, 1 H, HC=N), 2.50 (m, 2 H, CH_2C=N), 2.07 (d of t, 2 H, CH_2CH_2C=N). Anal. ($C_{10}H_{19}NO$) C, H, N.

2-Aza-2-cyclopentenespirocyclopentane 2-Oxide (5c). A solution of 3-(1-nitrocyclopentyl)propanal ethylene acetal (2.80 g, 13 mmol) and ammonium chloride (0.74 g) in 150 mL of 50% aqueous ethanol was treated with zinc dust (3.5 g) as given above. The crude product was initially purified by column chromatography as described above (R_f 0.51) and then Kugelrohr distilled at 35–40 °C (0.02 mm) to give **5c** as a white solid. IR (neat film) 1580 (C=N), 1220 (N–O) cm^{-1}; ^1H NMR (CCl_4) δ 6.57 (m, 1 H, CH=N), 2.55 (m, 2 H), 2.12 (m, 2 H), 2.00–1.29 (m, 8 H). Anal. ($C_8H_{13}NO \cdot {}^1/_2H_2O$) C, H, N.

Trapping of Free Radicals. Spin Trapping of Superoxide. The superoxide generating system contained 400 μM hypoxanthine, 1 mM diethylenetriaminepentaacetic acid (DTPA), 50 mM sodium phosphate buffer (pH 7.8), and xanthine oxidase such that the rate of superoxide production was 10 μM/min at 25 °C. Measurement of superoxide was determined optically by the reduction of cytochrome *c* at 550 nm with use of an extinction coefficient of 20 mM^{-1} cm^{-1}. The concentration of the various spin traps ranged from 10 mM to 0.1 M. The reaction was initiated by the addition of xanthine oxidase. By monitoring the conversion of xanthine to uric acid at 292 nm, it was determined that the various nitrones did not inhibit the enzyme under the above experimental conditions. The formation of superoxide spin trapped adducts was monitored by ESR as the low-field peak and was observed to increase linearly for several minutes. The production of this nitroxide was completely inhibited by superoxide dismutase (10 μg/mL). No free radical could be trapped if any component of the above reaction mixture was not present.

Spin Trapping of Hydroxyl Radical. The spin trapping of hydroxyl radical was undertaken by the addition of ferric ammonium sulfate (0.1 mM) to the above described superoxide generating system. No free radical was spin trapped in the presence of catalase (300 units/mL).

Partition Coefficient Studies. A 1×10^{-4} M stock solution of each nitrone in deionized water was prepared. The stock solution was diluted to prepare solutions of varying concentrations and a calibration curve obtained from the absorbance measurements at 250 nm. Then 3 mL of each stock solution was combined with an equal volume of 1-octanol (Aldrich, gold label) and the biphasic mixture was shaken for at least 30 min. After separation, the lower aqueous layer was drained and the concentration of the nitrone determined from the calibration curve. The concentration of each nitrone in the 1-octanol layer was determined by difference and the partition coefficient calculated.

Isolation of Rat Enterocytes. Rats (male Sprague–Dawley from Charles River, Boston, MA) were anesthesized with pentobarbital and fixed on a small animal operative board. The portal vein was exposed through a wide abdominal incision which allowed the retraction cephalad of the entire abdominal wall. The vein was retrogradely cannulated by an 18-gauge Quick-Cath catheter (Travenol Laboratories, Inc., Deerfield, IL) and infused with buffer A (119.8 mM NaCl; 23.8 mM $NaHCO_3$; 2.4 mM KCl; 0.1 mM KH_2PO_4; 0.1 mM L-lysine; 0.1 mM EGTA, 20 mM dextrose, pH 7.4; and 200 U/L heparin) at a flow rate of 5 mL/min (Harvard pump, Model 1210, Harvard Instrument Co., Millis, MA) until all visible blood was removed from the intestinal vascular arcade. Immediately after the portal vein was cannulated, the superior mesenteric artery was cut near its origin to provide egress for the vascular infusate. The infusion was then changed to buffer B [buffer A plus 10.5 g/L bovine serum albumin and 438 mg/L collagenase (Worthington, type I)], which was infused at a rate of 5 mL/min with use of a nonrecirculating system. After infusion of 120 mL, the vascular perfusion was stopped. Both buffers A and B were warmed to 37 °C in a large capacity Haake water bath and oxygenated with use of a gas-permeable coil prior to their infusions. While the vascular perfusion was in progress, the intestinal lumen was also being perfused. The intestine was cannulated just distal to the ligament of Treitz with use of Tygon tubing and fixed by a silk tie (size 000). Another cannula was placed in the distal intestine, approximately 10 cm proximal to the cecum. Buffer A was infused through the proximal cannula at a flow rate of 11 mL/min (by means of a Harvard pump, Model 1210). After the perfusate became clear of residual intestinal contents, the remaining perfusate was collected in a 250-mL beaker at room temperature. Every 30 min the released cells were harvested and pelleted by centrifugation (500*g* for 5 min in a Beckman centrifuge Model TJ/6 at 40 °C, Beckman Instruments, Palo Alto, CA). The supernatant was removed, and the cells were gently suspended with use of a wide bore glass pipet. This process was repeated until the desired number of cells had been collected, usually 100 million. During the intestinal perfusion, which lasted up to 90 min, the intestine was gently kneaded by hand. Cell viability was tested by using the Trypan blue exclusion method, which indicated at least 85% viability.

Spin Trapping of Free Radicals Generated by Enterocyte Metabolism. Spin trapping experiments were designed to detect superoxide generated as a consequence of enterocyte metabolism of menadione. A typical reaction mixture contained 1 million cells, menadione (0.1 mM in 0.14 M Me_2SO, final concentration) or Me_2SO (0.14 M, final concentration), the spin trap (0.1 M), and sufficient Swim's buffer to bring the final volume to 0.5 mL. Reaction mixtures were then transferred to a flat quartz ESR cell and fitted into the cavity of the ESR spectrometer. The ESR spectrum was obtained within 1 min after initial mixing.

Glutathione Peroxidase Activity of Isolated Enterocytes. This enzyme activity was determined by the procedure of Flohe and Gunzler.[29] Cells were homogenized in a sucrose/phosphate buffer (0.25 M sucrose containing 1 mM EDTA and 50 mM sodium phosphate at pH 7.4) by a polytron homogenizer at speed 5 for 3 s (Model PT 10-35 with a PT-20-FT generator, Brinkman Instruments Co., Westburg, NY). These homogenates were centrifuged at 9000*g* for 20 min. The 9000*g* supernatant was then centrifuged at 100000*g* for 60 min. The supernatant was used to determine glutathione peroxidase activity as outlined by Flohe and Gunzler.[29] Protein was measured by the method of Lowry et al.[30]

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Registry No. **4a**, 104463-44-7; **4a** (ethylene oxide), 104463-47-0; **4b**, 104463-45-8; **4b** (ethylene oxide), 104463-48-1; **4c**, 104463-46-9; **4c** (ethylene oxide), 104463-49-2; **5a**, 104463-50-5; **5a** (peroxy radical), 104463-38-9; **5a** (hydroxy radical), 104463-41-4; **5b**, 104463-51-6; **5b** (peroxy radical), 104463-39-0; **5b** (hydroxy radical), 104463-42-5; **5c**, 104322-61-4; **5c** (peroxy radical), 104463-40-3; **5c** (hydroxy radical), 104463-43-6; $H_3CCH(NO_2)(CH_2)_3CH_3$, 14255-44-8; $H_3CCHBr(CH_2)_3CH_3$, 3377-86-4; $H_3C(CH_2)_2CH(NO_2)(CH_2)_2CH_3$, 2625-37-8; $H_3C(CH_2)_2CHBr(CH_2)_2CH_3$, 998-93-6; $OHCCH{=}CH_2$, 107-02-8; menadione, 58-27-5; superoxide, 11062-77-4; hydroxyl radical, 3352-57-6; nitrocyclopentane, 2562-38-1.

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