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Spin Trapping of Superoxide

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SUMMARY

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Due to conflicting reports in the literature, the spin trapping of superoxide by the nitrone, DMPO, has been re-investigated. We found that superoxide can indeed be trapped and that the DMPO-OOH and DMPO-OH adducts are distinguishable, in agreement with the original work of Harbour *et al.* (10). In contrast to their work, we found that DMPO-OOH was highly unstable and that it decomposed into DMPO-OH. We observed that TMPO, a spin trap structurally similar to DMPO, but lacking a β-hydrogen, formed a stable nitroxide upon reaction with superoxide. Rate constants for the reaction of superoxide with DMPO and TMPO were measured and indicated that spin trapping was an extremely inefficient method for the detection of superoxide. The new spin trap described by Janzen *et al.* (18), 4-POBN, was also capable of distinguishing the difference between superoxide and hydroxyl radical. The nitroxide formed upon the reaction of superoxide with 4-POBN (4-POBN-OOH) was found to be unstable. However, unlike DMPO-OOH, 4-POBN-OOH does not decompose into the hydroxynitroxide, 4-POBN-OH.

INTRODUCTION

Superoxide is a highly reactive product of univalent reduction of dioxygen (1–3). The existence of an enzyme developed specifically to remove superoxide from biological systems attests to the importance of superoxide as a potentially dangerous radical species (4–7). The identification and quantitation of superoxide in biological systems is often complicated by the presence of other factors. For example, the measurement of superoxide by reduction of cytochrome *c* cannot be carried out in the presence of other enzymes (e.g., cytochrome P-450 reductase) that readily reduce cytochrome *c* (8).

Spin trapping (9) was envisioned as a technique which could specifically identify and quantitate superoxide and hydroxyl radicals (1, 10, 11). In order to fulfill this goal, spin trapping must be able to distinguish the difference between hydroxyl rad-

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ical and superoxide. The resultant nitroxide should be relatively stable and the rate constant for the reaction of the radical with the spin trap should be high enough to allow for the efficient detection of short lived free radicals.

Nitrone spin traps such as DMPO[2] have been used in an attempt to meet the criteria stated above (12–17). It remains to be shown whether these criteria have ever been met. Evidence for the uniqueness of the hydroxyl and superoxide DMPO adducts rests upon the initial observations of Harbour *et al.* (10), using a system where the hydroxyl radical was produced by ultraviolet photolysis of hydrogen peroxide. At low hydrogen peroxide concentrations a four line epr spectrum was formed which was attributed to the DMPO-OH adduct, whereas at higher concentrations of hydrogen peroxide a different adduct was formed which was attributed to superoxide produced by the reaction of hydroxyl radical with hydrogen peroxide. Other than this concentration effect, no other evidence was presented to verify the assignment of the spectrum, as Janzen *et al.* (18) have already pointed out. Indeed the trapping of superoxide anion is controversial. One group claimed that superoxide cannot be trapped because it is charged (16), whereas another investigator stated that superoxide can be trapped but that DMPO-OOH is unstable (19, 20) and a third study suggests that DMPO-OOH is sufficiently stable such that this nitroxide can participate in a disproportionation reaction (12). However, to date, no study has unequivocally proved the existence of a spin trapped superoxide adduct.

In this report, the trapping of superoxide is re-investigated. We find that the original work of Harbour *et al.* (10) is essentially correct; however, DMPO-OOH is highly unstable under normal experimental conditions and decomposes into a false DMPO-OH adduct.

[2] The abbreviations used are: DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; DMPO-OH, 5,5-dimethyl-2-hydroxylpyrrolidinoxyl; DMPO-OOH, 5,5-dimethyl-2-hydroperoxypyrrolidinoxyl; TMPO, 2,5,5-trimethyl-1-pyrroline-N-oxide; TMPO-OOH, 2-hydroperoxy-2,5,5-trimethylpyrrolidinoxyl; 4-POBN, α-4-pyridyl 1-oxide N-tert-butyl nitrone; 4-POBN-OH, α-hydroxymethyl 4-pyridyl 1-oxide N-tert-butyl nitroxide; 4-POBN-OOH, α-hydroperoxylmethyl 4-pyridyl 1-oxide N-tert butyl; DMF, N,N-dimethylformamide.

MATERIALS AND METHODS

General comments. Xanthine oxidase was obtained from Sigma Chemical Company, St. Louis, MO; Calbiochem-Behring, Corp., La Jolla, CA; and generously given to us by Dr. Irwin Fridovich, Department of Biochemistry, Duke University. Superoxide dismutase, cytochrome *c*, diethylenetriamine pentaacetic acid (DETAPAC), and xanthine were purchased from Sigma Chemical Company. Chelex 100 was purchased from Bio-Rad. Catalase (grade A) was obtained from Calbiochem-Behring Corp. Electron paramagnetic resonance spectra were recorded using a Varian Associates model E-9 spectrometer.

Tetramethylammonium superoxide was prepared according to the method of McElroy and Hashman (21). The spin traps, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) and 2,5,5-trimethyl-1-pyrroline-N-oxide (TMPO), were prepared according to the method of Bonnet *et al.* (22). The spin trap, α-4-pyridyl 1-oxide N-tert-butyl nitrone (4-POBN), was a gift from Dr. William Yamanashi, Department of Ophthalmology, Duke University.

Unless otherwise indicated, all phosphate buffers used were passed through a chelex-100 column according to the method of Poyer and McCay (23), in order to remove trace metal impurities such as iron. This minimizes the possibility of hydroxyl radical formation, since several investigators have shown that iron is required to catalyzed hydroxyl radical formation from superoxide and hydrogen peroxide (20, 24, 25). Unless indicated DETAPAC (1 mM) was also included in the buffers to prevent hydroxyl radical formation because studies have shown that iron-DETAPAC is unable to catalyze hydroxyl radical formation (20, 24).

In the tetramethylammonium superoxide (TMAS) system 50 λ of a 1 mg TMAS/ml DMF solution was added to 0.5 ml of 0.1 M chelexed potassium phosphate buffer pH 7.8 containing 1 mM DETAPAC and 0.18

M DMPO and the spectrum was recorded immediately.

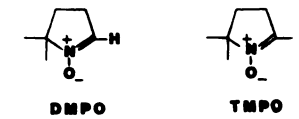
A typical xanthine/xanthine oxidase experiment contained 15 nM xanthine oxidase, 400 μM xanthine, 1 mM DETAPAC, 50 mM chelexed phosphate buffer pH 7.8. Concentrations of DMPO used were up to 0.18 M, TMPO up to 0.16 M and 4-POBN up to 0.18 M. By monitoring the conversion of xanthine to uric acid at 290 nm, it was found that no significant inhibition of xanthine oxidase by DMPO occurred under the conditions described above.

RESULTS AND DISCUSSION

Spin trapping of chemically pure superoxide. A freshly prepared solution of tetramethylammonium superoxide (TMAS) in DMF was used as the source of superoxide anion for these spin trapping experiments (Fig. 1). This compound is directly soluble in certain aprotic organic solvents such as DMF, acetonitrile and DMSO (1). Addition of an aliquot of TMAS/DMF to an aqueous solution of cytochrome *c* at pH 7.8 resulted in reduction of cytochrome *c*. This reduction was prevented by the prior addition of superoxide dismutase. It was also observed that cytochrome *c* reduction did not occur if the TMAS/DMF was added to an aqueous buffer just prior to cytochrome *c* addition. This demonstrates that the dismutation of superoxide is complete within the time required for these manipulations.

To determine if superoxide is trapped by DMPO, an aliquot of TMAS/DMF was added to a solution of DMPO in buffered phosphate solution at pH 7.8. The spectrum shown in Figure 2 was obtained. This spectrum can be interpreted as being a combination of two species whose hyperfine splitting constants are listed in Figure 2. The observed hyperfine splitting constants are consistent with those that have been attributed to the DMPO-OH and the DMPO-OOH adducts, respectively (10). Addition of superoxide dismutase prior to TMAS/DMF addition prevented the appearance of both spectra; however, addition of catalase prior to TMAS/DMF had no effect. These observations suggest that the formation of both species was dependent upon the trapping of superoxide and that hydrogen peroxide (formed via the dismutation of superoxide) is not involved. The ratio of these two species changed with time. During the 2.4 min required to scan the spectrum, a large decrease in the height of the DMPO-OOH spectrum was noted. Concomitant with this decrease, we observed a smaller increase in the height of the DMPO-OH spectrum. It was shown that this increase was not due to further trapping of radicals. Addition of excess superoxide dismutase and catalase immediately following the addition of TMAS/DMF did not prevent the increase in the DMPO-OH spectrum. We also observed an overall decrease in signal intensity as a function of time. An explanation for these phenomena is that DMPO-OOH is unstable and with time it decomposes partially into DMPO-OH and partially into a nonradical species, as shown in Figure 3. In contrast to this, the DMPO-OH spectrum is relatively stable, having a half life of 2.5 hours under these conditions. The conversion of one species into another is consistent with their structural assignments. These results also show that DMPO-OH can be produced by a mechanism which does not involve hydroxyl radical trapping. These points are further illustrated in the next section.

A spin trapping experiment using TMPO[3] provided some insight into the na-



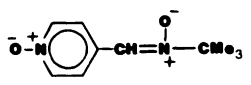


FIG. 1. *Structures of spin traps*

[3] TMPO has been used to trap other radicals. Note: Janzen, E. G., C. A. Evans and J. I. P. Liu. Factors Influencing Hyperfine Splitting in the ESR Spectra of Five-Membrane Ring Nitroxides. J. Mag. Res. **9**: 513–516, 1973 and Janzen, E. G. and C. A. Evans. Rate Constants for Spin Trapping tert-Butoxy Radicals as Studied by Electron Spin Resonance. J. Amer. Chem. Soc. **95**: 8205–8206, 1973.

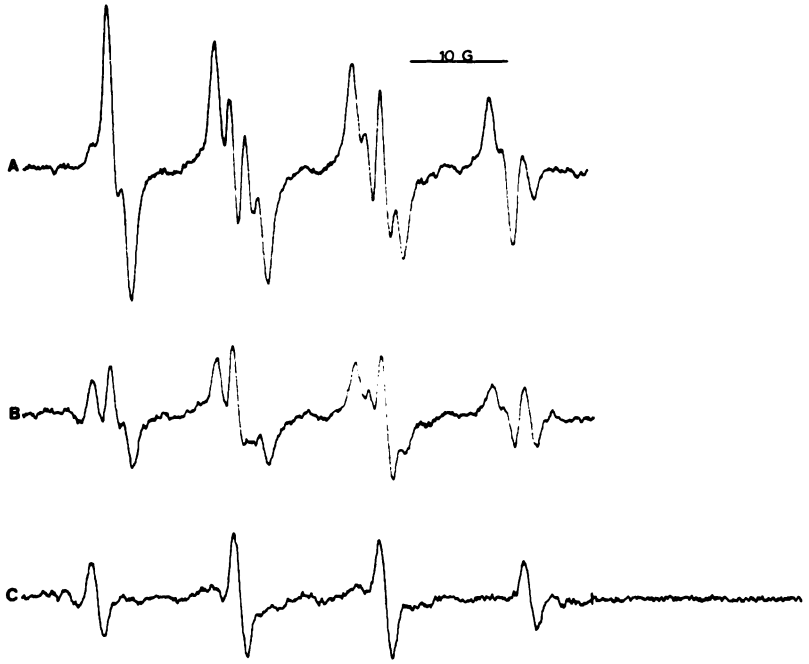


FIG. 2. *Electron paramagnetic resonance spectra obtained 30 seconds, 3 min, and 12 min after the addition of tetramethylammonium superoxide/DMF solution to 50 mM pH 7.8 chelexed phosphate buffer containing 0.18 M DMPO and 1 mM DETAPAC*

The spectra can be attributed to two species, DMPO-OH (A_N = 14.8, A_H = 14.8), and DMPO-OOH (A_N = 14.2, A_H^β = 11.6, A_H^γ = 1.2-gauss). Panel A is predominately DMPO-OOH, B is a combination of DMPO-OOH and DMPO-OH and C is predominately DMPO-OH. Modulation complitude was 1 gauss, microwave power 10 mW, time constant 0.3 sec., scan time 4 min., scan range 100 gauss.

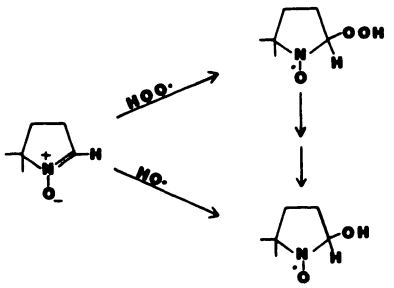


FIG. 3. *Alternate mechanisms by which DMPO-OH can be formed*

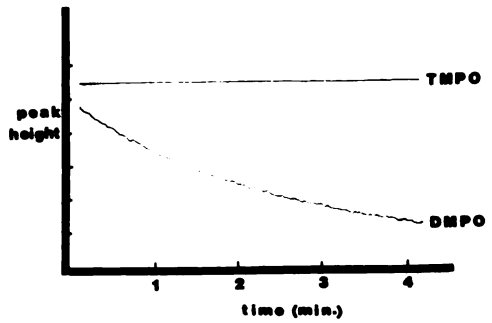


FIG. 4. *Comparative stability of the adducts formed by reaction of tetramethylammonium superoxide with DMPO (0.18 M) and TMPO (0.16 M)*

Conditions are the same as listed in Figure 2. The TMPO-O_2^- adduct spectrum was a nitroxide triplet, A_N = 15.6 gauss.

ture of the decomposition of DMPO-OOH. In contrast to DMPO, the nitroxide formed by the reaction of superoxide with TMPO was rather stable (see Figure 4). Prior addition of superoxide dismutase could prevent the formation of this nitroxide, whereas catalase had no effect. The stability of the ensuing nitroxide can be attributed to the fact that it lacks a β-hydrogen and therefore, compared to DMPO, it is less susceptible to H·abstraction followed by rearrangement leading to an epr invisible species.

Spin trapping of superoxide in a biologic system. The xanthine/xanthine oxidase system was used to generate superoxide for our spin trapping experiments. As with the chemical system discussed previously, the spectra observed were found to vary with time, as shown in Figure 5. The initial spectrum consists mainly of the DMPO-OOH adduct. (A more detailed analysis is provided by the computer simulation in figures 6 and 7.) After five minutes, most of the DMPO-OOH spectrum was gone, and the dominant species present was the DMPO-OH adduct. With increasing time, a six-line spectrum appeared, $A_N = 15.31$, $A_H = 22.0$, and eventually predominated. Superoxide dismutase prevented the formation of both DMPO-OOH and DMPO-OH. Catalase could not prevent the formation of DMPO-OH. Thus, neither a Fenton reaction (1) nor a Haber-Weiss reaction (1, 25, 26) was involved in the formation of DMPO-OH. Therefore, the generation of DMPO-OH was due mainly to decomposition of DMPO-OOH, as already described for the chemical system. Addition of peroxidase (lactoperoxidase or horseradish peroxidase) increased the ratio of DMPO-OH to

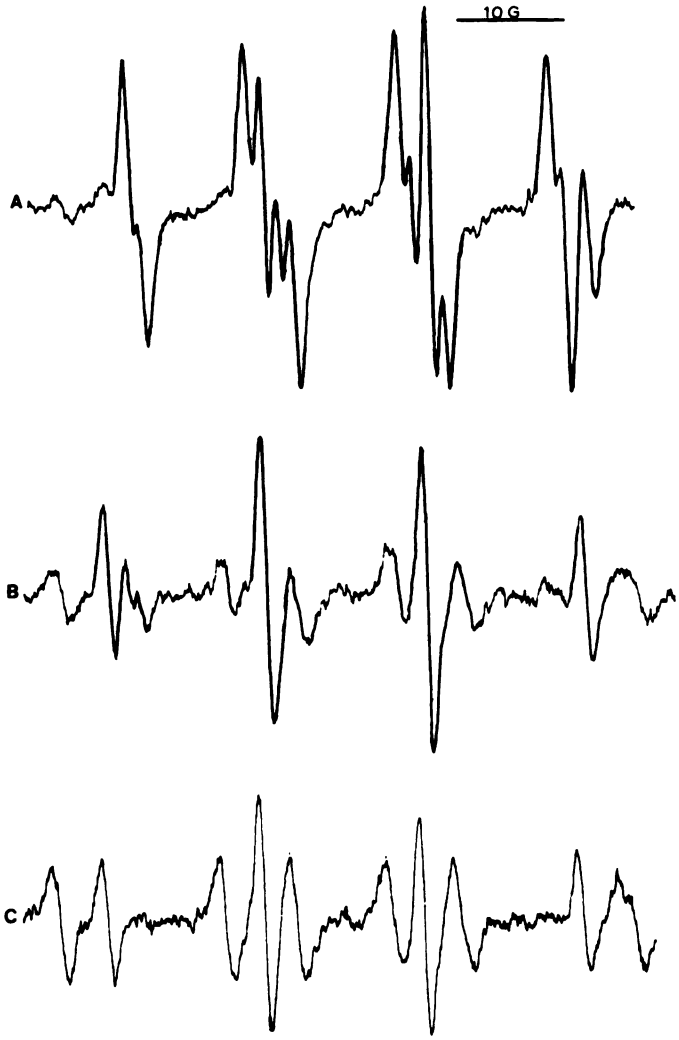


FIG. 5. *Spectra generated by DMPO spin trapping 24 sec, 5 min, and 10 min after addition of xanthine oxidase (75 nM final concentration) to 50 mM chelexed pH 7.8 phosphate buffer containing 4×10^{-4} M xanthine and 0.18 M DMPO*

Modulation amplitude was 1 gauss, time constant 1 second, scan time 8 minutes, scan range 100 gauss.

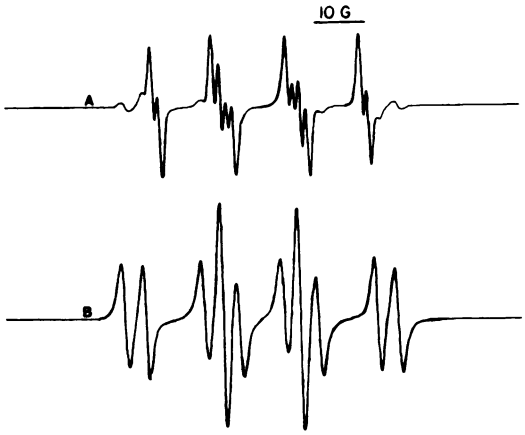


FIG. 6. *Spectra A and B are computer simulations of the spectra in figures 4A and C respectively*

A is composed of 80% DMPO-OOH, 15.4% DMPO-OH, 4.6% "six line spectrum". B is composed of 54% DMPO-OH, and 46% "six line spectrum".

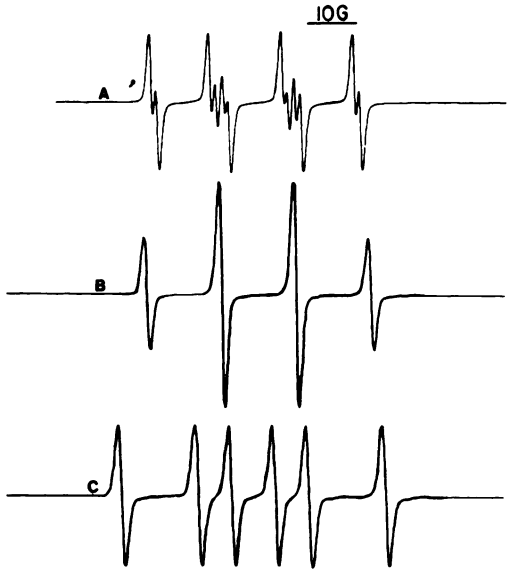


FIG. 7. *Computer simulations*

A. Computer simulation of DMPO-OOH, A_N = 14.3g, A_H^β = 11.7g and A_H^γ = 1.25g.

B. Simulation of DMPO-OH A_N = 14.87g, A_H = 14.81g.

C. Simulation of "six line spectrum" A_N = 15.31, A_H = 22.

DMPO-OOH, which is consistent with the structures assigned to these species. At low ratios of DMPO to xanthine oxidase, a three-line nitroxide spectrum appeared, A_N = 14.7, which was not present at higher ratios. We attribute this phenomenon to a multiple attack on DMPO by superoxide, where DMPO-OOH is initially formed, then is further attacked by superoxide resulting in the loss of the β-hydrogen and its distinctive spectral characteristics.

Rate constant for superoxide trapping. Determination of the rate constant for superoxide trapping by epr techniques requires the resultant nitroxide to be sufficiently stable so that kinetic measurements may be followed over several minutes. For this reason, TMPO was chosen over DMPO.

A solution of superoxide dismutase, standardized against cytochrome *c* according to the method of McCord *et al.* (4), was used to inhibit the formation of TMPO-OOH by xanthine/xanthine oxidase. The method of kinetic competition was then used to determine the rate constant for the trapping of superoxide by TMPO. Let us initially consider the fate of superoxide produced in a system consisting of xanthine/xanthine oxidase, TMPO and superoxide dismutase. As shown in equation 1, superoxide can react with itself (spontaneous dismutation), TMPO or superoxide dismutase.

$$O_2\cdot^- + O_2\cdot^- + 2H^+ \rightarrow O_2 + H_2O_2$$

$$O_2\cdot^- + \text{TMPO} \rightarrow \text{TMPO-OOH} \qquad \text{Eq. 1}$$

$$O_2\cdot^- + O_2\cdot^- + 2H^+ \xrightarrow{\text{SOD}} O_2 + H_2O_2$$

At a level of TMPO high enough to trap out most of the superoxide, the spontaneous dismutation is insignificant, and TMPO and SOD are competing for the available superoxide. Under these conditions the following relationship applies where V equals the rate of enzymatic generation of superoxide and k_t and k_s are the second order rate constants for reaction of superoxide with TMPO and SOD, respectively.

$$V = \frac{-d(O_2\cdot^-)}{dT} \qquad \text{Eq. 2}$$

$$= k_t(\text{TMPO})(O_2\cdot^-) + k_s(\text{SOD})(O_2\cdot^-)$$

Let the rate of EPR adduct formation equal v, then

$$v = k_t(\text{TMPO})(O_2\cdot^-) \qquad \text{Eq. 3}$$

Dividing Equation 3 into Equation 2 and rearranging gives Equation 4

$$v = \frac{Vk_t(\text{TMPO})}{k_s(\text{SOD}) + k_t(\text{TMPO})} \qquad \text{Eq. 4}$$

or

$$\frac{1}{V - v} = \frac{1}{V} + \frac{k_t(\text{TMPO})}{k_s V(\text{SOD})} \qquad \text{Eq. 5}$$

The results are shown in Figure 8 and are consistent with the above equation. Since the SOD solution was standardized against cytochrome *c*, the rate constant for TMPO trapping of superoxide is 1.16×10^{-5} times the second order rate constant for the reaction of superoxide with cytochrome *c*. Assuming that this rate constant is 6×10^5 M^{-1} sec^{-1} (27), k_t becomes 7 M^{-1} sec^{-1}.

Since the DMPO-OOH adduct is unstable, its rate constant could not be determined by monitoring the formation of this product by epr. However, its rate constant was determined by its ability to inhibit the formation of TMPO-OOH. Its rate constant was found to be 1.5 times that of TMPO or 10 M^{-1} sec^{-1}.

Effect of chelating agents and metal ion impurities on superoxide trapping. The effect of metal ion impurities present in phosphate buffer and the effects of chelating agents on superoxide trapping were systematically investigated using the xanthine/xanthine oxidase superoxide generating system. Both spin trapping and optical techniques (cytochrome *c* reduction) were employed. These factors affect the amount of DMPO-OOH formed and their effects can be summarized as follows: DETAPAC + chelexed buffer = DETAPAC + non-chelexed buffer > EDTA + chelexed buffer > chelexed buffer > non-chelexed buffer. These observations can be explained on the basis that metal ions have been shown to possess superoxide dismutase activity (1). In our system, we found the EDTA inhibited most of this activity and that DE-

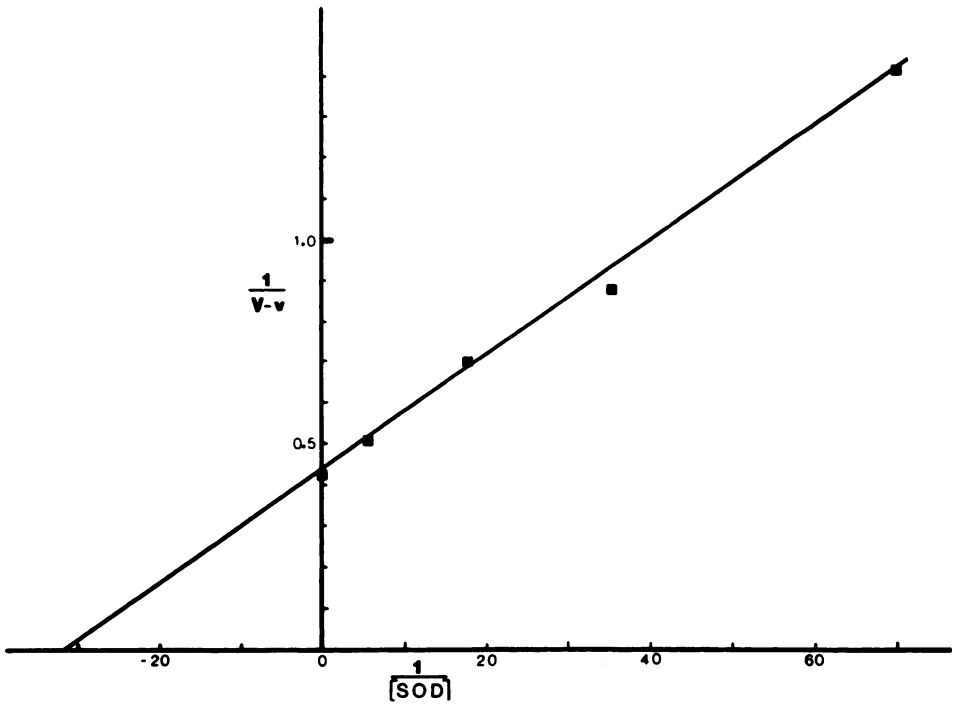


FIG. 8. *Competition between SOD and TMPO for O_2^- produced by the xanthine oxidase system*

The system contained 0.16 M TMPO, 1 mM DETAPAC, 15 nM xanthine oxidase, 400 μM xanthine, 50 mM pH 7.8 chelexed phosphate buffer, in a total volume of 0.5 ml. Units of SOD activity are defined by the assay of McCord and Fridovich (4). The ordinate refers to the rate of epr adduct formation, expressed in arbitrary units. Results are shown as the mean of duplicates.

TAPAC is even more effective. Buettner *et al.* (20) have found that DETAPAC-iron is inactive as a superoxide dismutase, in contrast to EDTA-iron.

In most cases, the sequence seen in Figure 5 was followed, i.e., the DMPO-OOH appeared initially, followed by DMPO-OH, and then with time a new six line spectrum was formed. With DETAPAC present little or none of the six line spectrum was observed. The effects of various factors were either to retard or accelerate this time sequence. Those factors that were most effective in increasing the amount of DMPO-OOH formed were also most effective in retarding the time sequence of DMPO-OOH decomposition. The reasons for these effects were not determined; however, it is well known that various metals, especially iron, can accelerate the decomposition of hydroperoxides (28).

In a control experiment, it was found that the addition of EDTA-iron (either Fe^{+2} or Fe^{+3}) to DMPO in pH 7.8 phosphate buffer resulted in a six-line spectrum, A_H = 22.0 and A_N = 15.3 gauss, which is identical to that observed in the xanthine/xanthine oxidase system. The presence of both iron and EDTA is essential to the generation of this spectrum. In contrast, DETAPAC-iron did not form this species. In addition, DETAPAC could prevent the EDTA-iron generated six line spectrum. Preliminary results suggest that this spectrum is due to an EDTA-iron catalyzed oxidation of DMPO. The mechanism of this phenomenon is under further investigation.

Trapping of Superoxide by 4-POBN

Recently, Jansen *et al.* (18) have reported the use of a nitrone, α-4-pyridyl 1-oxide N-tert-butyl nitrone (4-POBN), to investigate the trapping of hydroxyl radicals. These authors declare that 4-POBN is "unique in its suitability for the detection of hydroxyl radicals" at biologically acceptable pH ranges (18). In order to see whether 4-POBN could trap superoxide, it was incubated with xanthine/xanthine oxidase under conditions identical to those employed to produce the DMPO-OOH spectrum. As shown in Figure 9, the ensuing nitroxide gives a spectrum with an A_N = 14.16 and

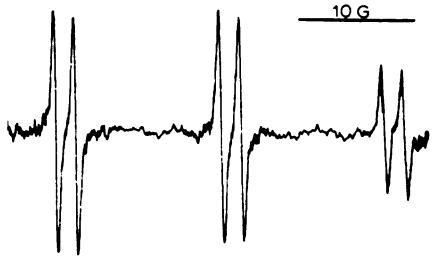


FIG. 9. *Epr spectrum formed by reaction of O_2^- with 4-POBN in a system consisting of 75 nM xanthine oxidase, 400 μM xanthine, 1 mM DETAPAC, 20 mg/ml 4-POBN, in 50 mM pH 7.8 chelexed phosphate buffer*

A_N = 14.16 g A_H = 1.75. Modulation amplitude was 0.5 gauss, time constant 1.0 sec.

A_H = 1.75 gauss. It rapidly decomposed, leaving no epr signal. Superoxide dismutase completely inhibited the formation of this species, but catalase did not. Based on this evidence, we suggest that this species is 4-POBN-OOH. As with DMPO, the inclusion of DETAPAC in the buffered solution greatly increased the intensity of the initially observed epr signal.

Generation of 4-POBN-OH by either acid-catalyzed addition of water to the double bond followed by hydrogen peroxide oxidation, or ultraviolet photolysis of hydrogen peroxide in 0.1 M phosphate buffer at pH 7.8 gave a species with a spectrum having A_N = 14.93 and A_H = 1.69 gauss, note Figure 10. Thus, 4-POBN-OOH and 4-POBN-OH give different splitting constants and are thus distinguishable.

In contrast to DMPO-OOH, the epr spectrum of 4-POBN-OOH did not decompose into the epr spectrum of 4-POBN-OH. Additionally, 4-POBN is found to be a relatively poor spin trap for superoxide. This precluded the possibility of determining an accurate rate constant; however, using kinetic competition experiments, as outlined previously, it was estimated that 4-POBN is approximately 30 times slower than DMPO at trapping superoxide.

In conclusion, we have demonstrated that superoxide may be trapped and identified using DMPO. The conversion of DMPO-OOH into DMPO-OH and the effect of peroxidase enzymes upon the ratio of these species is consistent with their

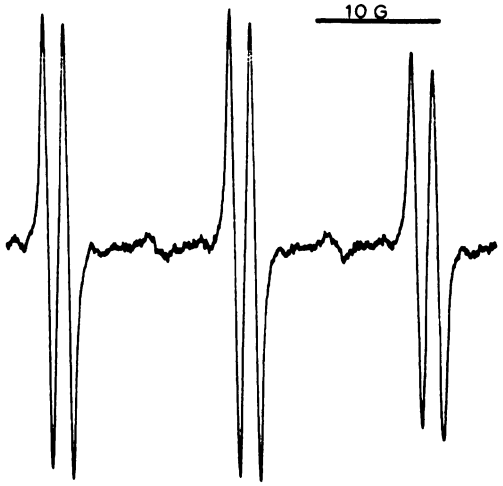


FIG. 10. *4-POBN-OH formed in a system containing 10 mg 4-POBN, 1% H_2O_2, and a catalytic amount of CH_3CO_2H, in a total volume of 0.5 ml*

A_N = 14.93, A_H = 1.69 gauss. Modulation amplitude was 0.5 gauss, microwave power 10 mW, time constant 1.0 sec, scan time 0.5 hr.

original structural assignments. These experiments also show that spin trapping with DMPO is highly prone to artifact unless careful attention is paid to the purity of the buffers, enzyme preparations, chelating agents and spin traps. The instability of those spin trap adducts possessing a β-hydrogen makes them only suitable for the qualitative detection of superoxide. The low rate constant for the reaction of superoxide with the spin traps tested, also makes them inefficient detectors of superoxide. TMPO, which forms a more stable nitroxide with superoxide, may be useful in quantitating this free radical under conditions where more conventional methods, such as cytochrome *c* reduction, cannot be used. However, superoxide dismutase must be employed as a control in this instance, since the TMPO-OOH spectrum (a nitroxide triplet) is not detailed enough to distinguish it from other radical species. The production of a six-line spectrum by iron-EDTA and DMPO is of interest since other investigators have used similar conditions; yet, to our knowledge, no group has reported this phenomenon. Despite this fact, a spectrum similar to that which we have obtained appears in published spectra in systems where iron-EDTA is used (16, 17). In addition, the iron-EDTA complex can act as a superoxide dismutase (20). Thus the use of EDTA in spin trapping experiments is contraindicated. The use of DETAPAC as a chelating agent decreases many of the problems associated with EDTA.

Another possible drawback to the use of spin trapping in biological systems, is that the products of spin trapping, nitroxides, are redox active. Nitroxides can be reduced to hydroxylamines (29), which are not detectable by epr. The hydroxylamines in turn can be reoxidized into nitroxides by superoxide or superoxide producing systems (30). Thus, redox cycling of the spin trapped products is possible. We did not find this to be a problem in the xanthine/xanthine oxidase system.

The use of DMPO to detect hydroxyl radical has the additional caveat in that DMPO-OH can arise via a mechanism that does not involve the trapping of hydroxyl radicals, e.g., from DMPO-OOH. Janzen (18) has already shown, in the case of 4-POBN, that a false 4-POBN-OH adduct can be formed by the addition of water across the double bond followed by oxidation of the resultant hydroxylamine. Therefore, the investigator should be aware that spin traps are reactive compounds that can form radical products by mechanisms not involving radical trapping.

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