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THE SPIN TRAPPING OF SUPEROXIDE AND HYDROXYL FREE RADICALS WITH DMPO (5,5-DIMETHYLPYRROLINE-N-OXIDE): MORE ABOUT IRON

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The reaction of superoxide with the spin trap DMPO (5,5-dimethylpyrroline-N-oxide) is widely used to study superoxide production as well as issues associated with the superoxide-related formation of hydroxyl radical. However, the interpretation of observed intensities of DMPO/·OOH and DMPO/·OH signals in electron paramagnetic resonance spin trapping experiments is not without its difficulties. In this paper, I report experiments that demonstrate:

1. That the flux of superoxide formation in a DMPO spin trapping experiment can alter the apparent importance of weak DMPO/·OH signals;
2. That iron can influence the DMPO/·OOH spin trapping results;
3. That there is very little spontaneous breakdown of DMPO/·OOH to form DMPO/·OH.

KEY WORDS: EPR, Spin Trapping, Superoxide, Free Radical, DMPO (5,5-dimethylpyrroline-N-oxide), Iron.

Abbreviations DETAPAC: diethylenetriaminepentaacetic acid, DMPO: 5,5-dimethylpyrroline-N-oxide, EPR: electron paramagnetic resonance, HX: hypoxanthine, $O_2 \cdot^-$ superoxide; in this paper I use $O_2 \cdot^-$ to represent the equilibrium mixture of $O_2 \cdot^-$ and its protonated form $HO_2 \cdot$, $pK_a = 4.8 \pm 0.1$, X.O.: xanthine oxidase.

INTRODUCTION

The EPR spin trapping of superoxide with the spin trap DMPO is a widely used approach to study the production of superoxide in chemical, biochemical, and biological systems. Since the first report of the successful spin trapping of superoxide from the xanthine/xanthine oxidase system,[1] this system has become a widely used tool for the generation of superoxide as well as a standard for the comparison of other sources of superoxide in EPR spin trapping experiments. Unfortunately, the EPR detection of DMPO/·OOH is not without its shortcomings and pitfalls such as: Interference from iron,[1,2] the short lifetime of DMPO/·OOH,[3] the reaction of $O_2 \cdot^-$ with DMPO/·OOH and DMPO/·OH,[4,5] and the reported possibility that DMPO/·OOH decays to form DMPO/·OH.[6] In this paper I address additional aspects of the interference of iron and the formation of DMPO/·OH from DMPO/·OOH.

MATERIALS AND METHODS

Hypoxanthine, xanthine oxidase, riboflavin, chelating resin, and DMPO were from Sigma Chemical Co., St. Louis, MO. DMPO was purified with charcoal and stored

as a frozen 1.0 M aqueous solution before use.[3] Adventitious catalytic metals were removed from all buffers with chelating resin;[7] the absence of catalytic metals was verified with ascorbate.[7] Adventitious transition metals were removed from EDTA by repeated recrystallizations.[8]

When xanthine oxidase was used to generate superoxide, solutions contained: 50 mM DMPO, 50 μM DETAPAC or 250 μM EDTA, 1.0 mM hypoxanthine, and xanthine oxidase in 50 mM pH 7.8 phosphate buffer. When riboflavin was used, solutions contained; 50 mM DMPO, 50 μM riboflavin, and 1.0 mM DETAPAC in 50 mM pH 7.4 phosphate buffer. The riboflavin solutions in an EPR flat cell were illuminated with white light ($\approx$5–10 s) from a slide projector while in the EPR cavity,[3] or when appropriate, in a glass test tube outside the cavity. The intensities of DMPO/·OH and DMPO/·OOH were determined by double integration of the high field lines. These lines were chosen because they are the best separated, and therefore, have the least influence on each other. Quantitation of DMPO/·OOH and DMPO/·OH was accomplished using 3-carboxyproxyl as the standard.[4]

RESULTS

DMPO/·OOH to DMPO/·OH Conversion

Riboflavin is a photosensitizer that produces a strongly oxidizing triplet. This triplet is reduced by electron donors; both EDTA and DETAPAC can be oxidized by the triplet state of riboflavin. The reduced form of riboflavin will in turn convert dioxygen to superoxide. Thus, riboflavin can serve as a source of superoxide, but it will only generate superoxide in the presence of light. We have used riboflavin in this study as a "pulsed" source of superoxide to generate DMPO/·OOH.[3,9] After producing DMPO/·OOH with a 5–10 s pulse of light, we then scanned the high field DMPO/·OH and DMPO/·OOH lines repetitively to monitor the possible conversion of DMPO/·OOH to DMPO/·OH.

The repetitive EPR scans of the high field lines of DMPO/·OOH and DMPO/·OH show that each species decays after cessation of illumination, albeit at quite different rates (Figures 1 and 2). Within the limits of the noise in the data, the decay of DMPO/·OH is linear in the time of the experiment while the DMPO/·OOH signal follows the predicted first-order decay rate[3] with $t_{1/2} \approx 65$ s. (At higher concentrations, DMPO/·OH decays by a first-order process, $t_{1/2} = 23$ mins., but at lower concentrations such as observed here, the decay appears much more complex and slower.[10])

In examining Figures 1 and 2, there is no evidence that DMPO/·OOH decays to form DMPO/·OH in this system, because there is no detectable change in the slope of the DMPO/·OH decay curve after the depletion of DMPO/·OOH. Thus, I conclude that in this system there is no significant decay of DMPO/·OOH to DMPO/·OH. This is in contrast to results reported using tetramethylammonium superoxide to generate DMPO/·OOH.[6] This apparent discrepancy is unexplained but may well be a result of the source of superoxide. However, Finkelstein *et al.*[6] estimate that only a very small portion of DMPO/·OOH is converted to DMPO/·OH, <3%. This low estimate along with data presented here indicates that this source of DMPO/·OH is indeed minor and is vastly overused by researchers to rationalize experimental observations.

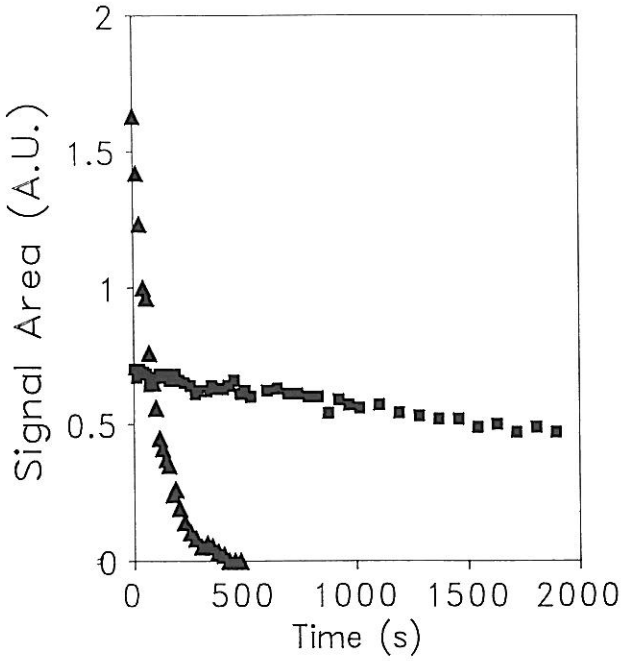

FIGURE 1 Decay of DMPO/·OOH(▲) and DMPO/·OH (■) generated by the riboflavin/DETAPAC and light system described in Materials and Methods. All time points represent time after the cessation of illumination.

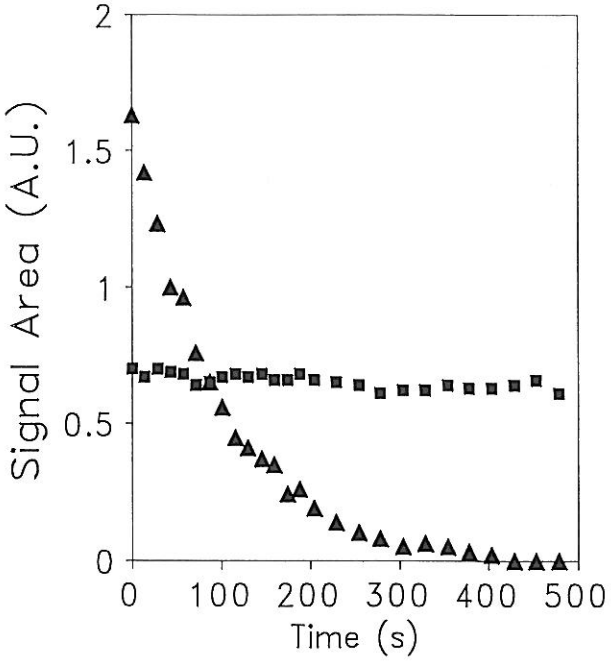

FIGURE 2 Initial 500 s of Figure 1. Decay of DMPO/·OH (▲) and DMPO/·OOH (■).

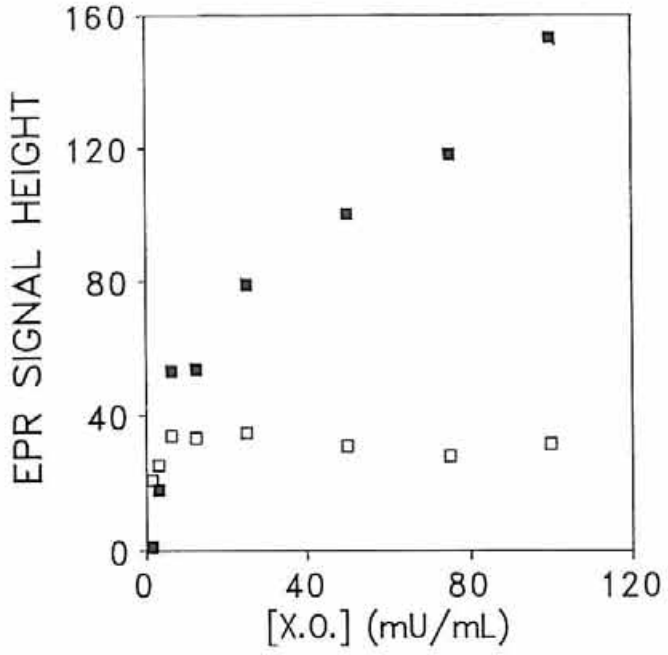

FIGURE 3 Relative EPR signal intensity of DMPO/·OOH (■) and DMPO/·OH (□) as the concentration of xanthine oxidase is varied in a HX/X.O. superoxide-generating system.

Superoxide Flux and DMPO/·OOH

Xanthine oxidase will oxidize hypoxanthine to uric acid; the electrons from this oxidation are passed to dioxygen to produce both H_2O_2 and $O_2\cdot^-$.[11] The flux of superoxide is a function of many variables, but keeping all variables constant, the flux of $O_2\cdot^-$ produced can be varied by changing the concentration of X.O. In Figure 3, we see that the relative intensity of DMPO/·OOH increases as a function of the X.O. concentration; however, the intensity of the weak DMPO/·OH background signal remains fairly constant. Thus, when viewing a spectrum generated by a low flux of $O_2\cdot^-$, the importance of DMPO/·OH may be misjudged when compared to a spectrum generated with a higher flux of $O_2\cdot^-$, Figure 4.

DMPO/·OOH and Fe

It is now well recognized that iron greatly influences the course of free radical reactions in superoxide-generating systems. Spin trapping results observed in iron-containing systems are no exception. The principal change is that superoxide in the presence of redox active iron, such as FeEDTA, will result in the formation of HO·; in DMPO spin trapping systems, one sees increased amounts of DMPO/·OH.[1]

However, a consideration that is not typically taken into account is the competition for $O_2\cdot^-$ between the spin trap and the iron-chelate. For example, at pH 7.4 with FE(III)EDTA the rate of disappearance of superoxide will be

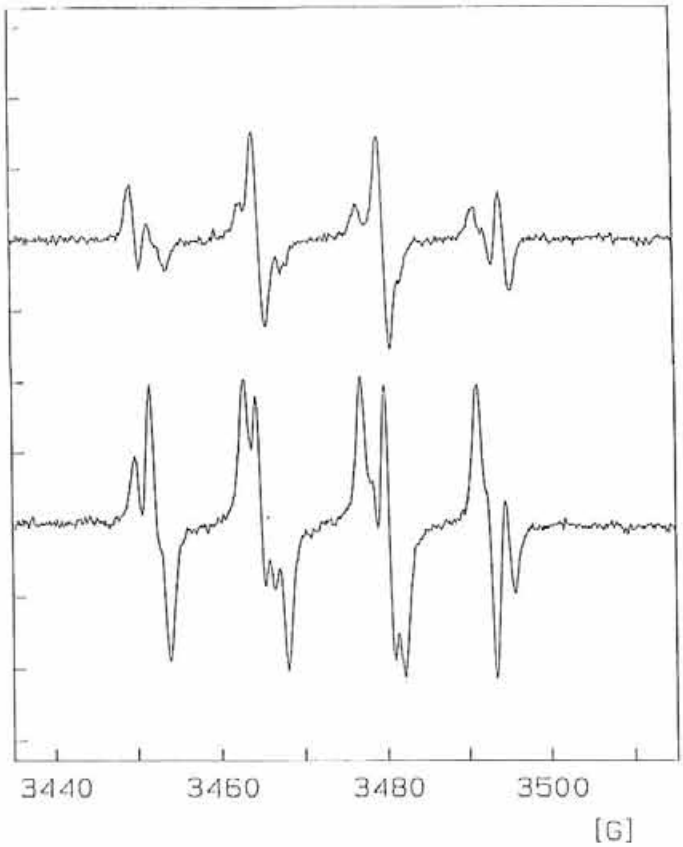

FIGURE 4 EPR spectra of DMPO/·OOH generated by the HX/X.O. system. *Upper spectrum*: The system contained: 50 mM DMPO; 0.5 mM hypoxanthine; 50 μM DETAPAC; and xanthine oxidase, ≈3 mU/mL in 50 mM pH 7.8 phosphate buffer. *Bottom spectrum*: All ingredients were the same except that xanthine oxidase was present at ≈25 mU/mL. Instrument settings were: 40 mW microwave power; 0.96 gauss modulation amplitude; 0.33 s time constant; 80 gauss/168 s scan rate; receiver gain, 1×10^6 top, 5×10^5 bottom. The EPR scan was initiated ≈3.5 minutes after the introduction of X.O.

$$\frac{-d[O_2\cdot^-]}{dt} = k_1^{obs}[Fe(III)EDTA]\,[O_2\cdot^-] + k_3^{obs}[O_2\cdot^-]^2 \qquad (1)$$

where $k_1^{obs} = 1 \times 10^6\,M^{-1}\,s^{-1}$ [12] and $k_3^{obs} = 2.4 \times 10^5\,M^{-1}\,s^{-1}$ [13] while in the DMPO spin trapping experiment,

$$\frac{-d[O_2\cdot^-]}{dt} = k_2^{obs}[DMPO]\,[O_2\cdot^-] + k_3^{obs}[O_2\cdot^-]^2 \qquad (2)$$

where $k_2^{obs} = 30\,M^{-1}\,s^{-1}$ at pH 7.4 [14,15]

If we consider experiments where sufficient Fe(III)EDTA and/or DMPO is/are present so that $k_3^{obs}[O_2\cdot^-]^2$ is negligible, then when comparing the rates of these two competing reactions, we have:

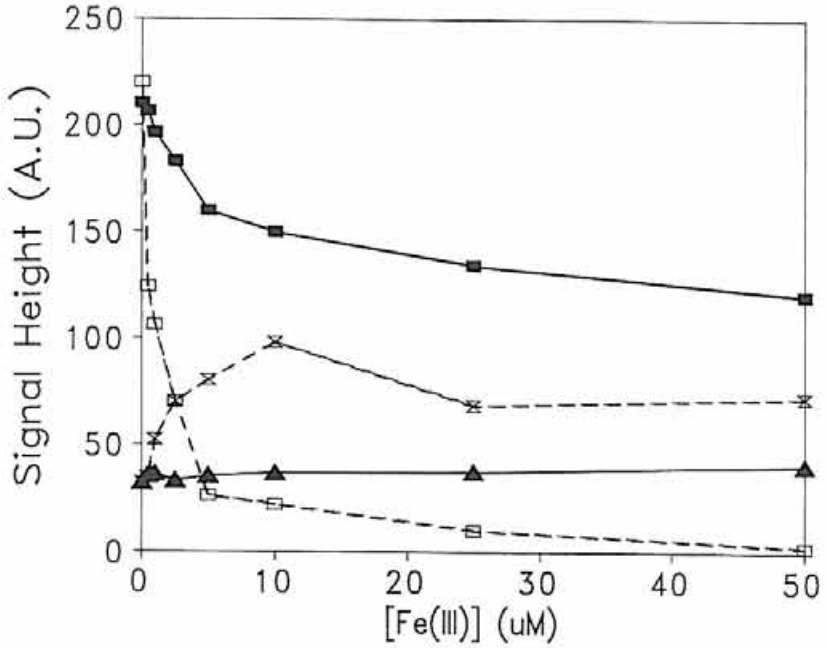

FIGURE 5 Relative intensity of DMPO/·OOH (■ and □) and DMPO/·OH (▲ and X) in a pH 7.4 phosphate buffer solution that contains 1 mM HX, 25 mU/mL X.O., 50 mM DMPO, 250 μM EDTA (dashed lines) or 250 μM DETAPAC (solid lines), and varying concentrations of Fe(III).

$$\frac{\text{rate with Fe(III)EDTA}}{\text{rate with DMPO}} = \frac{1 \times 10^6\,M^{-1}s^{-1}\,[\text{Fe(III)EDTA}]\,[O_2\cdot^-]}{30\,M^{-1}s^{-1}\,[\text{DMPO}]\,[O_2\cdot^-]}$$

or,

$$\text{ratio} = 3.3 \times \frac{10^4[\text{Fe(III)EDTA}]}{[\text{DMPO}]}$$

If in a spin trapping experiment [Fe(III)EDTA] = 5 μM and [DMPO] = 50 mM, then this ratio of rates = 3.3. Thus, the intensity of the DMPO/·OOH signal will decrease substantially just from the competition with Fe(III)EDTA.

Indeed, examination of Figure 5 shows that the intensity of the DMPO/·OOH signal is greatly diminished by the addition of Fe(III) to the EDTA chelate present in the HX/X.O. solution. As predicted, the intensity of the DMPO/·OH signal increases with an increase in added iron. However, when DETAPAC is substituted for EDTA, the addition of Fe(III) results in only a small loss of DMPO/·OOH signal intensity, Figure 5. This is consistent with a much lower rate constant for the reaction of $O_2\cdot^-/HO_2\cdot$ with Fe(III)DETAPAC as compared to Fe(III)EDTA. Pulse radiolysis experiments have put an upper limit of $1 \times 10^4\,M^{-1}s^{-1}$ for the reaction of $O_2\cdot^-/HO_2\cdot$ with Fe(III)DETAPAC.[12] These spin trapping results are consistent with this rate constant.

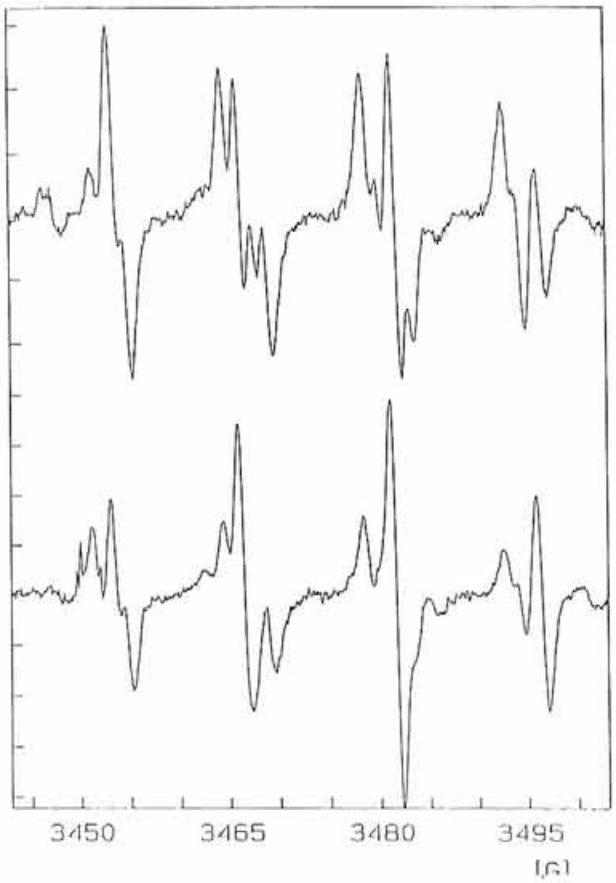

FIGURE 6 EPR spectra of DMPO/·OOH and DMPO/·OH. These samples were generated outside the cavity with the riboflavin/DETAPAC and light system. Within 5 s after cessation of illumination, the sample was drawn into a flat cell in the cavity and spectral scans were initiated. *Top*: No added iron. *Bottom*: 20 μM Fe(II) added immediately after cessation of illumination. Instrument settings: 0.95 G mod. amp.; 5×10^5 receiver gain; 40 mW power; time constant 0.17 s; scan 60 G/84 s. With this scan rate, there is 55 s between the high and low field lines of DMPO/·OOH.

Another facet of the influence of iron that is generally overlooked is the possible reaction of DMPO/·OOH with Fe(II). To examine this possibility, I generated DMPO/·OOH with the riboflavin/DETAPAC and light system outside the cavity. Immediately after the cessation of illumination, 20 μM Fe(II) was added to the solution and then drawn by vacuum into the prepositioned and tuned flat cell in the cavity. EPR scans were initiated within 5 s after illumination ceased. As seen in Figure 6. the addition of Fe(II), *after* the generation of $O_2 \cdot^-$ had ceased, caused the rate of decay of DMPO/·OOH to increase. Comparing the low-field and high-field lines of DMPO/·OOH, we see a 35 ± 1% decrease in signal height in the absence of Fe(II), Figure 6. However, in the presence of 20 μM Fe(II), this decay is accelerated. In comparing these same lines, there is a 54 ± 1% decrease in signal

height with Fe(II) present. This observation is consistant with the reaction of Fe(II) with DMPO/·OOH.

DISCUSSION

The influence of iron on spin trapping experiments is well known,[1,2] but the focus is typically on the formation of ·OH via the Fenton reaction. However, the presence of iron can provide the spin trap with significant competition for $O_2\cdot^-/HO_2\cdot$, thereby bringing about a decrease in the DMPO/·OOH signal intensity. The actual effect depends on the chelate associated with the iron; DETAPAC results in a relatively redox-inactive iron that will provide little interference for the detection of superoxide.

Thus, the typical "superoxide and iron" spin trapping result should be interpreted with at least four points in mind: (1) the competition between the iron-complex and the spin trap for superoxide; (2) the reaction of Fe(II) with DMPO/·OOH; (3) the formation of ·OH from the Fe(II) complex and H_2O_2;[1,12] and (4) the possible lack of H_2O_2 formation from the dismutation of superoxide because of competition from the spin trap[16] and/or from the iron chelate present.

In addition to the influence of iron, the flux of superoxide must be considered when interpreting spin trapping results in superoxide-generating systems. Levels of DMPO/·OH can easily be given improper emphasis due to the superoxide flux in the experiment.

Even though there are many things to consider in the interpretation of EPR spin trapping results, they provide a wealth of unique information to aid researchers in their study of free radical processes.

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