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Considerations in the Spin Trapping of Superoxide and Hydroxyl Radical in Aqueous Systems Using 5,5-Dimethyl-1-pyrroline-1-oxide

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SUMMARY: The superoxide radical spin adduct of the spin trap 5,5-dimethyl-1-pyrroline-1-oxide was found to be relatively unstable in aqueous solution. The half-life of the electron spin resonance signal is approximately 80 sec at pH 6 and only about 35 sec at pH 8. These observations as well as the possible reaction products of $O_2^{\cdot}$ that may develop in the time course of an experiment, must be considered when planning or interpreting data from a spin trapping experiment.

INTRODUCTION: The technique of spin trapping (1) has recently been applied to studies of free radicals in biological systems (2-6). Much of this increased interest results from studies on the role of the oxygen centered radicals $O_2^{\cdot}$ and ·OH in biological systems. Our studies using the spin trap 5,5-dimethyl-1-pyrroline-1-oxide have revealed some potential limitations in the technical application of this technique. It is the purpose of this communication to relate these observations as they can significantly affect the interpretation of spin trapping data.

MATERIALS AND METHODS: The spin trap 5,5-dimethyl-1-pyrroline-1-oxide was purchased from Aldrich Chemical Co., Inc., Milwaukee, Wisconsin. This DMPO typically contains a colored impurity. An aqueous solution of this DMPO will result in an ESR signal typical of what is expected from the spin trapping of carbon-centered radicals (7) (Fig. 2) and of a simple nitroxide i.e., no β-hydrogen splitting. (This example also has a signal from the ·OH spin adduct.) The intensity of these signals will change slowly with time. If the colored impurity is removed, no signal is observed. This indicates that the impurity in the DMPO rather than the DMPO itself is labile in aqueous solution, contrary to what has been reported by Lai and Piette (4). The colored impurity present in this commercial DMPO was removed by filtration with neutral decolorizing charcoal using approximately 10 parts water to one part DMPO. The resulting aqueous solution was divided into small aliquots and frozen until used to slow the process of thermal signal growth. Xanthine, xanthine oxidase, riboflavin, and

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Abbreviations: DMPO, 5,5-dimethyl-1-pyrroline-1-oxide; DETAPAC, diethylene-triaminepentaacetic acid; ESR, electron spin resonance.

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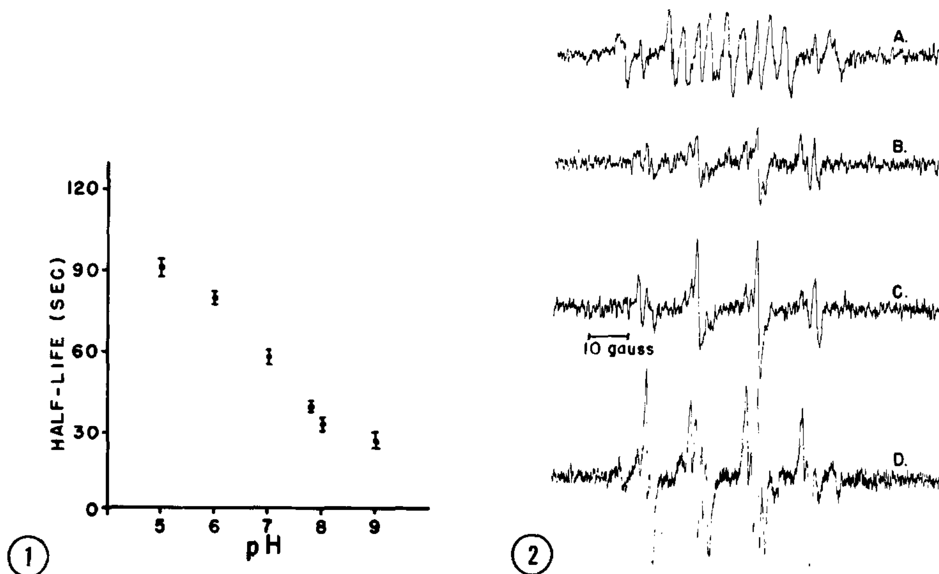


Figure 1: The half-life of the $O_2^{\cdot}$ spin adduct signal of DMPO in aqueous solution as a function of pH.

Figure 2: A. Example of the signals seen from an aqueous solution of DMPO with the colored impurity present. The relative intensities of the carbon-like spin adduct and the simple nitroxide (no β-hydrogen splitting) changes slowly with time, the simple nitroxide signal gains intensity while the carbon-like spin adduct signal loses intensity. The OH spin adduct was introduced by a weak Fenton reagent, 0.01% H_2O_2 and 10^{-5} M Fe^{+2}. B. Time course of the DMPO spin adduct signal seen from the reaction mixture of 1 x 10^{-4} M xanthine, 1 x 10^{-3} M EDTA, 1 x 10^{-6} M Fe^{+2}, 0.05 M DMPO and approximately 6 x 10^{-8} M xanthine oxidase. The scan rate was 25 gauss/min. The scan was in the region of the low field ·OH and $O_2^{\cdot}$ spin adduct lines approximately 2 minutes after the introduction of xanthine oxidase into the reaction mixture. C. Same as B except the scan was in the region of the low field ·OH and $O_2^{\cdot}$ spin adduct lines approximately 6 1/2 minutes after the introduction of xanthine oxidase into the reaction mixture. D. Example of the $O_2^{\cdot}$ spin adduct spectrum of DMPO generated by the illumination (500 watt slide projector approximately 50 cm from sample in ESR cavity) of a solution containing 4 x 10^{-5} M riboflavin, 1 x 10^{-3}M DETAPAC and 0.05 M DMPO in 0.05 M phosphate buffer at pH 7.8.

diethylenetriaminepentaacetic acid were obtained from Sigma Chemical Co., St. Louis, Missouri.

To determine the lifetime of the $O_2^{\cdot}$ spin adduct of DMPO, the reaction mixture consisted of 2 x 10^{-4} M riboflavin, 1 x 10^{-3} M DETAPAC, and 0.05 M DMPO in buffered solution. Oxygen was bubbled through the mixture for approximately five minutes. The mixture was placed in an ESR quartz flat cell. With the cell in the cavity the mixture was irradiated for 10-30 sec using a 500 watt slide projector placed approximately 25 cm from the cavity. The height of the low field line in the $O_2^{\cdot}$ spin adduct signal of DMPO was monitored after termination of illumination.

In the spin trapping experiments using xanthine oxidase as an $O_2^{\overline{\cdot}}$ generator, typical reaction mixtures consisted of 1×10^{-4} M xanthine, 0.05 M DMPO, 1×10^{-3} EDTA or DETAPAC and 1×10^{-6}M iron as ferrous sulfate. Xanthine oxidase was present at approximately 6×10^{-8} M.

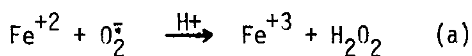
RESULTS AND DISCUSSION: In our studies of $O_2^{\overline{\cdot}}$ and •OH radical formation in biological systems using DMPO as the spin trapping agent, we have observed that an important consideration is the time course of the ESR signal detected. One of the reasons for employing the spin trapping technique is to convert a short-lived free radical by an addition reaction with the spin trap into a relatively long-lived free radical product which can easily be studied by ESR. The •OH radical, and usually the carbon-centered radicals, form stable spin adducts with DMPO in aqueous solution; the resultant signal often lasts for hours or even days depending on temperature. However, the $O_2^{\overline{\cdot}}$ spin adduct of DMPO is relatively unstable. This signal lasts for only a few minutes. A printing error in the paper by Harbour et al. (8) indicates that the $O_2^{\overline{\cdot}}$ spin adduct is more stable than the •OH spin adduct of DMPO. This may have caused some confusion since in fact we have observed the exact opposite.

The fact that the $O_2^{\overline{\cdot}}$ spin adduct of DMPO is so short lived is an important consideration when designing or interpreting data from a spin trapping experiment. Thus, if one waits too long before examining the signal, all the $O_2^{\overline{\cdot}}$ spin adduct signal may have decayed. We have examined some of the decay kinetics of the $O_2^{\overline{\cdot}}$ spin adduct of DMPO. The illumination of riboflavin will result in the production of $O_2^{\overline{\cdot}}$ in an aerobic solution. This reaction has even been used as the basis for an assay for the enzyme superoxide dismutase (9). The appearance of $O_2^{\overline{\cdot}}$ from photochemically reduced tetra-acetyl riboflavin occurs within 200-300 msec after its introduction into the reaction system (10). Thus, on the time scale of our experiment, production of $O_2^{\overline{\cdot}}$ and the subsequent spin trapping will essentially cease when illumination of the reaction mixture is terminated. To measure the lifetime of the $O_2^{\overline{\cdot}}$ spin adduct of DMPO, we monitored the height of the low field peak after the cessation of illumination of the riboflavin mixture. [To a first approximation the rate of change of concentration is propor-

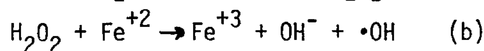
tional to the rate of change of peak height (11).] A plot of log (peak height) versus time resulted in a straight line indicating an apparent first order decomposition process. As a useful measure of this decay rate, the half-life of the ESR signal was determined. The decay rate was also found to be pH dependent (Fig. 1). There is an approximate three-fold increase in the half-life of the signal when going from pH 9 ($t_{1/2}$ = 27 sec) to pH 5 ($t_{1/2}$ = 91 sec). This is reasonable when it is realized that peroxides are, in general, more stable in acidic solution. However, at pH 3 and pH 4, no $O_2^{\bar{\cdot}}$ signal was observed, only the •OH spin adduct was detected.

Other obviously important considerations are the reactions that $O_2^{\bar{\cdot}}$ and subsequent products can undergo. In this case, we are looking at a relatively long rather than short time scale. We have previously shown that the amount of iron and the particular metal chelator present in a reaction mixture containing xanthine and xanthine oxidase (an $O_2^{\bar{\cdot}}$ generating system) can profoundly influence which spin adduct will be observed (6). The initial signal observed in the presence of 10^{-4} M (or less) iron (with or without EDTA) was that due to the $O_2^{\bar{\cdot}}$ spin adduct. However, at 10^{-3} M iron we were only able to observe the •OH spin adduct. With all iron concentrations tested (even at 10^{-6} M iron), the intensity of the $O_2^{\bar{\cdot}}$ spin adduct decreased with time, while that of the •OH spin adduct increased: the greater the iron concentration, the sooner the •OH signal appeared. For example, in a solution of 1 x 10^{-4} M xanthine, approximately 6 x 10^{-8} M xanthine oxidase, 1 x 10^{-3} M EDTA, 1 x 10^{-6} M iron (as ferrous sulfate) and 0.05 M DMPO, the initial signal observed is that of the $O_2^{\bar{\cdot}}$ spin adduct (Fig. 2). However, 3-4 minutes after the introduction of the xanthine oxidase, the •OH spin adduct signal is of greater intensity than the $O_2^{\bar{\cdot}}$ spin adduct, and after 8-10 minutes the signal seen is dominated by the •OH spin adduct. The presence of a signal from an •OH spin adduct of DMPO even with only 10^{-6} M iron present is a significant observation. However, when considering the relative intensities of the •OH and $O_2^{\bar{\cdot}}$ spin adducts, the relative lifetimes of the two signals must be taken into account.

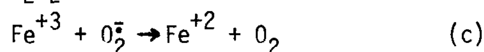
These observations as well as those of Ilan and Czapski (12), McClune et al. (13), Halliwell (14) and Buettner et al. (6) are consistent with the following mechanism:



$$Fe^{+2} + O_2^{\overline{\cdot}} \xrightarrow{H+} Fe^{+3} + H_2O_2 \quad (a)$$



$$H_2O_2 + Fe^{+2} \rightarrow Fe^{+3} + OH^- + \cdot OH \quad (b)$$



$$Fe^{+3} + O_2^{\overline{\cdot}} \rightarrow Fe^{+2} + O_2 \quad (c)$$

In the above reactions, iron may also be chelated with EDTA. If DETAPAC is used as a metal chelator in the reaction above, the only signal observed is that due to the $O_2^{\overline{\cdot}}$ spin adduct. This indicates that the iron is sequestered in such a form as not to be active in this sequence of reactions.

CONCLUSIONS: The $O_2^{\overline{\cdot}}$ spin adduct of DMPO in aqueous solution is relatively unstable: the half-life of the ESR signal varies as a function of pH. The half-life is approximately 80 sec at pH 6 and decreases to approximately 35 sec at pH 8. These observations, as well as the possible reaction products of $O_2^{\overline{\cdot}}$, must be considered when planning or interpreting data from spin trapping experiments.

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