ascorbate test is an excellent method to determine the status of the chelating resin); (4) glassware may recontaminate the solution[8]; and (5) dust, fingers, and phantom sources of contamination may seem to appear magically.

Using this simple, inexpensive method, it is easy to verify that the procedures used to demetal buffer and salt solutions are successful. The method provides a tool for troubleshooting when problems occur, and it is an easy and repeatable way to determine and report experimental conditions.

[8] Standard laboratory glassware is used for solution storage. After this glassware has been successfully cleaned, however, it is never returned to the usual pool of laboratory glassware for general use. Rather, when fresh solutions are required, the glassware is rinsed with high-purity water and refilled with the same solution.

[9] Spin-Trapping Methods for Detecting Superoxide and Hydroxyl Free Radicals *in Vitro* and *in Vivo*

By GARRY R. BUETTNER and RONALD P. MASON

Spin trapping has become a valuable tool in the study of transient free radicals as evidenced by the many investigations in which it has been employed.[1] Oxygen-centered radicals are of particular interest because they have been implicated in many adverse reactions *in vivo*. Their short lifetimes and broad linewidths make many of these radicals difficult, if not impossible, to detect by direct electron spin resonance (ESR) in room temperature aqueous solutions. Spin trapping provides a means, in principle, to overcome these problems, but it is not without its pitfalls and limitations. We discuss some of these problems in this chapter.

Choice of Spin Trap

Two types of spin traps have been developed, nitrone and nitroso compounds. In aqueous solutions, however, oxygen-centered spin adducts of nitroso spin traps such as MNP (2-methyl-2-nitrosopropane) are, in general, quite unstable. Thus, the nitrone spin traps are by far the most popular. The most useful radical trap for the study of oxygen-centered free radicals is 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), which has

[1] G. R. Buettner, *Free Radical Biol. Med.* **3**, 259 (1987).

been used extensively to study superoxide[1,2] and hydroxyl radicals[1,3] as well as peroxyl radical formation[1,4,5] in biochemical and biological systems.

Superoxide

The spin trapping of superoxide has been of much interest because of the involvement of superoxide in many physiological processes. DMPO–·OOH (the superoxide spin adduct of DMPO) has a distinctive spectrum (a^N = 14.2 G, a_β^H = 11.3 G, a_γ^H = 1.25 G)[1] that is easily recognizable. However, other peroxyl adducts of DMPO will have a similar appearance. Thus, the real proof that the spectrum observed is indeed due to DMPO–·OOH is gained by using SOD (superoxide dismutase) to inhibit the signal.[6]

Although the DMPO–·OOH spectrum is distinctive, the spin trapping of superoxide is not without its problems. The actual reaction of superoxide with DMPO is very slow (k_{obs} is 60 M^{-1} sec^{-1} at pH 7 and only 30 M^{-1} sec^{-1} at pH 7.4).[7] Thus, in most superoxide-generating systems the spin trap concentration must be quite high (~0.1 M) in order to outcompete the self-decay, namely, dismutation of superoxide. In addition, the DMPO–·OOH adduct is unstable, decaying by a first-order process with a half-life of about 60 sec at pH 7.[8] Therefore, one must always be prepared to deal with a relatively weak signal, that is, [DMPO–·OOH] will, under most circumstances, be less than approximately 10 μM.

Hydroxyl Radical

The DMPO–·OH adduct is the most often reported radical adduct of DMPO ($a^N = a_\beta^H$ = 14.9 G).[1] Much of the interest in the spin trapping of ·OH is due to its formation in the superoxide-dependent Fenton reaction:

$$O_2^{\cdot-} + HO_2\cdot \xrightarrow{H^+} H_2O_2 + O_2 \quad (1)$$

$$O_2^{\cdot-} + \text{Fe(III)} \longrightarrow \text{Fe(II)} + O_2 \quad (2)$$

$$\text{Fe(II)} + H_2O_2 \longrightarrow \cdot OH + OH^- + \text{Fe(III)} \quad (3)$$

[2] P. J. Thornalley and J. V. Bannister, *in* "Handbook of Methods for Oxygen Radical Research" (R. A. Greenwald, ed.), p. 133. CRC Press, Boca Raton, Florida, 1985.

[3] G. R. Buettner, *in* "Handbook of Methods for Oxygen Radical Research" (R. A. Greenwald, ed.), p. 151. CRC Press, Boca Raton, Florida, 1985.

[4] M. J. Davies, *Biochim. Biophys. Acta* **964,** 28 (1988).

[5] M. J. Davies, *Chem. Phys. Lipids* **44,** 149 (1987).

[6] E. Finkelstein, G. M. Rosen, E. J. Rauckman, and J. Paxton, *Mol. Pharmacol.* **16,** 676 (1979).

[7] E. Finkelstein, G. M. Rosen, and E. J. Rauckman, *J. Am. Chem. Soc.* **102,** 4994 (1980).

[8] G. R. Buettner and L. W. Oberley, *Biochem. Biophys. Res. Commun.* **83,** 69 (1978).

Thus, SOD will inhibit DMPO–· OOH and/or DMPO–· OH formation if this reaction sequence is operative. However, catalase will inhibit the formation of · OH in reaction (3) above. A failure of catalase to inhibit the formation of DMPO–· OH when the superoxide-driven Fenton reaction is suspected indicates that something artifactual is occurring or that another mechanism must be sought.

Two additional SOD-inhibitable routes to DMPO–· OH from DMPO–· OOH itself should be considered: (1) the reduction of DMPO–· OOH[6] (a hydroperoxide) to the alcohol DMPO–· OH, for example, by glutathione peroxidase[9]; and (2) the possible homolytic cleavage of the oxygen–oxygen bond of DMPO–· OOH to produce free · OH, which is subsequently trapped by unreacted DMPO.[10,11] Thus, weak DMPO–· OH signals that are not catalase inhibitable should always be viewed cautiously because they quite often are artifactual rather than the result of the spin trapping of free · OH generated by the system under study.

To establish the existence of free hydroxyl radical in spin-trapping experiments, it is necessary to perform kinetic competition experiments with hydroxyl radical scavengers.[12,13] For example, ethanol, formate, and dimethyl sulfoxide can be used in these competition experiments, because upon hydroxyl radical attack they form carbon-centered radicals that can subsequently be trapped by DMPO:

$$\cdot OH + DMPO \xrightarrow{k_1} DMPO\text{–}\cdot OH \quad (4)$$

$$\cdot OH + HCO_2^- \xrightarrow{k_2} CO_2^{\cdot -} + H_2O \quad (5)$$

$$CO_2^{\cdot -} + DMPO \xrightarrow{k_3} DMPO\text{–}CO_2^{\cdot -} \quad (6)$$

$$\cdot OH + CH_3CH_2OH \xrightarrow{k_4} CH_3\dot{C}HOH + H_2O \quad (7)$$

$$CH_3\dot{C}HOH + DMPO \xrightarrow{k_5} DMPO\text{–}CH_3\dot{C}HOH \quad (8)$$

[9] G. M. Rosen and B. A. Freeman, *Proc. Natl. Acad. Sci. U.S.A.* **81,** 7269 (1984).

[10] E. Finkelstein, G. M. Rosen, and E. J. Rauckman, *Mol. Pharmacol.* **21,** 262 (1982).

[11] Finkelstein *et al.*[10] indicate that approximately 3% of DMPO–· OOH decomposes to produce · OH. Unfortunately, no experimental data or details are given to indicate how this estimate was made; thus, it is difficult to assess how this number should be used.

[12] A. L. Castelhano, M. J. Perkins, and D. Griller, *Can. J. Chem.* **61,** 298 (1983).

[13] Additional sources of artifactual DMPO–· OH signals are (1) hydrolysis of DMPO to produce DMPO–· OH as an impurity signal [R. A. Floyd and B. B. Wiseman, *Biochim. Biophys. Acta* **586,** 196 (1979)]; (2) the one-electron oxidation of DMPO followed by hydration of DMPO$^+$ [H. Chandra and M. C. R. Symons, *J. Chem. Soc., Chem. Commun.*, 1301 (1986)]; (3) the apparently concerted hydrolysis–oxidation reaction by photochemically excited molecules [V. S. F. Chew and J. R. Bolton, *J. Phys. Chem.* **84,** 1903 (1980)]; and (4) the presence of a strong oxidant such as hypochlorous acid [E. G. Janzen, L. T. Jandrisits, and D. L. Barber, *Free Radical Res. Commun.* **4,** 115 (1987)].

Most artifacts leading to DMPO–· OH radical adduct formation will be excluded by the use of hydroxyl radical scavengers if the scavenger-derived radical adduct is detected, if a corresponding decrease in the DMPO–· OH radical adduct concentration is found, and if quantitative kinetic criteria are used.[12]

Measurement of the initial rates of formation of the DMPO–· OH and DMPO–scavenger radical adducts removes the effects of the differential radical adduct decay rates.[12] Using this approach, the relative efficiency of two hydroxyl radical scavengers is quantitatively predictable from the known rate constants for the reactions of the hydroxyl radical with these scavengers. For example, using formate (k_2) and ethanol (k_4) we can calculate k_2/k_4 from the ratio of the rates of formation of these two radical adducts:

$$\frac{k_2}{k_4} = \frac{d[\text{DMPO–}\cdot\text{CO}_2^-]/dt}{d[\text{DMPO–CH}_3\dot{\text{C}}\text{HOH}]/dt} \times \frac{[\text{CH}_3\text{CH}_2\text{OH}]}{[\text{HCO}_2^-]} \tag{9}$$

In Eq. (9), the ratio k_2/k_4 from spin trapping should agree with the ratio of rate constants for the reaction of the hydroxyl radical with these scavengers as determined from pulse radiolysis. It should be kept in mind that to arrive at this expression, it is assumed that the predominant route of scavenger radical decay is via the trapping reaction. This kinetic approach has been successfully applied to an enzyme-dependent hydroxyl radical-generating system.[14]

A similar approach has been presented by Buettner *et al.*[15] In this approach a · OH scavenger is included in the spin-trapping mixture at a concentration calculated to reduce the intensity of the DMPO–· OH signal by 50%. In other words, the rate of the reaction of · OH with scavenger (Scav) is equal to its rate of reaction with DMPO:

$$k_{\text{scav}}[\text{Scav}][\cdot\text{OH}] = k_{\text{DMPO}}[\text{DMPO}][\cdot\text{OH}] \tag{10}$$

$$[\text{Scav}] = k_{\text{DMPO}}[\text{DMPO}]/k_{\text{Scav}} \tag{11}$$

Data must be obtained under circumstances where the rate of loss of DMPO–· OH is low compared to its rate of formation. In this approach possible side reactions of the scavenger radical are not a problem, unless of course they destroy DMPO–· OH, enabling a much wider range of experimental conditions to be used.

Recently, Samuni *et al.*[16] have demonstrated that $O_2^{\cdot-}$ reacts very

[14] K. M. Morehouse and R. P. Mason, *J. Biol. Chem.* **263,** 1204 (1988).

[15] G. R. Buettner, A. G. Motten, R. D. Hall, and C. F. Chignell, *Photochem. Photobiol.* **44,** 5 (1986).

[16] A. Samuni, C. D. V. Black, C. M. Krishna, H. L. Malech, E. F. Bernstein, and A. Russo, *J. Biol. Chem.* **263,** 13797 (1988).

efficiently with DMPO–· OH and DMPO–· CH_3 adducts, destroying the nitroxide and thus producing an ESR-silent species. If the flux of superoxide is high enough, the DMPO–· OH adduct may not even be observed because of its rapid removal. Thus, a high flux of superoxide would not be desirable if additional free radical reactions are expected in a superoxide spin-trapping system.

Peroxyl Radical Trapping

Peroxyl radicals have been successfully spin trapped with DMPO and PBN (α-phenyl-*N*-*tert*-butyl nitrone).[1,4,5] The appearance of the DMPO–· OOR spectrum is similar to that of DMPO–· OOH, albeit the splitting constants are somewhat different. As expected, DMPO–· OOR formation cannot be inhibited by superoxide dismutase.[17] The major experimental problem encountered is that DMPO–· OOR decays very quickly in aqueous solutions[4]; thus, time is a major consideration in any spin-trapping protocol.

In Vivo and In Vitro Superoxide

Many studies are pursuing the possible production of superoxide or hydroxyl radicals by cell organelles, intact cells, and organs. The detection of superoxide by spin trapping with DMPO has been achieved in all of the above. For success, however, experimental protocols must allow for the relatively short lifetime of DMPO–· OOH[8] and the possible interference by metal catalysts such as iron.[18] For example, in studying free radicals produced in myocardial ischemia/reperfusion, Arroyo *et al.*[19] immediately froze the coronary effluents in liquid nitrogen to prevent spin adduct decay. By monitoring the ESR spectra of the effluents immediately after thawing, they were successful in observing DMPO–· OOH.

DTPA, EDTA, and Desferal

The presence of transition metals (particularly iron) and various chelating agents can significantly alter the results of spin-trapping experiments.[18] Although contaminating catalytic metals can be removed from buffer and biochemical systems,[20] this would be a difficult and uncertain (perhaps impossible) process for cells and organs. Thus, chelating agents

[17] B. Kalyanaraman, C. Mottley, and R. P. Mason, *J. Biol. Chem.* **258,** 3855 (1983).

[18] G. R. Buettner, L. W. Oberley, and S. W. H. C. Leuthauser, *Photochem. Photobiol.* **28,** 693 (1978).

[19] C. M. Arroyo, J. H. Kramer, B. F. Dickens, and W. B. Weglicki, *FEBS Lett.* **221,** 101 (1987).

[20] G. R. Buettner, *J. Biochem. Biophys. Methods* **16,** 27 (1988).

are much needed tools. When studying a superoxide-generating system, EDTA will, in general, enhance the catalytic activity of iron in the reaction sequence,[1-3,18,21] thereby increasing the yield of DMPO–· OH while decreasing or eliminating the appearance of DMPO–· OOH. DTPA (diethylenetriaminepentaacetic acid) reduces or eliminates many of the problems generated by catalytic iron in superoxide-generating systems,[18] but under circumstances where a reducing agent stronger than superoxide is responsible for iron reduction, DTPA can increase DMPO–· OH formation.[14] In studying stimulated neutrophils, Britigan *et al.*[22,23] found DTPA (1–100 μM) to be a very useful tool; it had no effect on neutrophil superoxide production or oxygen consumption, whereas it enhanced the detection of superoxide by DMPO in their cellular experiments.

The iron chelator Desferal (deferrioxamine mesylate) renders iron essentially catalytically inactive in reactions (1)–(3) above.[21] Unfortunately the hydroxamic acid moieties of Desferal can undergo one-electron oxidation by superoxide (most likely · OOH), hydroxyl radical, and horseradish peroxidase.[24-26] The nitroxide radical so formed is stable for a free radical, but nevertheless it reacts rapidly with cysteine, methionine, glutathione, ascorbate, and a water-soluble form of vitamin E.[25] This radical may also deactivate enzymes, as demonstrated for alcohol dehydrogenase.[25] If Desferal is present at a relatively high concentration (compared to spin trap), it can effectively compete for superoxide and hydroxyl radical.[26] Since adventitious transition metals are present at only micromolar concentrations and spin traps are used at millimolar concentrations, scavenging by Desferal is perhaps less of a problem than the interference caused by the detection of the Desferal nitroxide radical itself. In any case, the Desferal concentration should be kept as low as possible to minimize scavenging.

In Vivo and In Vitro Hydroxyl Radical

The hydroxyl radical adduct of DMPO has also been observed in cell organelles, intact cells, and organs. However, the actual determination

[21] G. R. Buettner, *Bioelectrochem. Bioenerg.* **18,** 29 (1987).

[22] B. E. Britigan, M. S. Cohen, and G. M. Rosen, *J. Leuk. Biol.* **41,** 349 (1987).

[23] Britigan *et al.*[22] have observed a concentration-dependent inhibition of neutrophil oxygen consumption by PBN (<10 mM). DMPO was without effect at similar concentrations. However, an apparent impurity in DMPO did produce a marked inhibition of stimulated neutrophil superoxide formation in one set of experiments, which emphasizes the necessity of always performing routine control experiments.

[24] K. M. Morehouse, W. D. Flitter, and R. P. Mason, *FEBS Lett.* **222,** 246 (1987).

[25] M. J. Davies, R. Donkor, C. A. Dunster, C. A. Gee, S. Jonas, and R. L. Willson, *Biochem. J.* **246,** 725 (1987).

[26] O. Hinojosa and T. J. Jacks, *Anal. Lett.* **19,** 725 (1986).

that free · OH has been trapped is somewhat problematic because the presence of classic · OH scavengers such as ethanol, dimethyl sulfoxide, or formate can have a severely perturbing influence on the system, especially at the high concentrations that are required to outcompete the reaction of any · OH formed with the numerous biochemicals present at millimolar concentrations. Thus, we believe that the unambiguous determination that free · OH has been spin trapped requires very careful experimental design and interpretation, especially when the goal is to examine free radical production in cells, organs, or whole animals.

[10] Detection of Singlet Molecular Oxygen during Chloride Peroxidase-Catalyzed Decomposition of Ethyl Hydroperoxide

By ROBERT D. HALL

Introduction

During the 1970s and 1980s, a number of researchers proposed that the production of $^1O_2$, the lowest excited state of molecular oxygen, occurs during certain biochemical reactions. However, most of the experimental evidence for these proposals has been indirect, usually relying on physical and chemical quenchers of $^1O_2$, deuterium oxide enhancement of postulated effects of $^1O_2$, or the red chemiluminescence associated with the simultaneous electronic transition of two $^1O_2$ molecules. Not surprisingly, objections have often been raised regarding the value of these indirect methods.

More recently, several groups have begun to utilize the unique luminescence of $^1O_2$ at 1268 nm to measure the production of $^1O_2$ by chemical,[1] photochemical,[2,3] or biochemical[4–7] reactions. This infrared luminescence can also be combined with the traditional methods of detection to further support or question hypotheses concerning $^1O_2$.

[1] J. R. Kanofsky, *J. Org. Chem.* **51,** 3386 (1986).
[2] A. U. Khan, *J. Am. Chem. Soc.* **103,** 6516 (1981).
[3] R. D. Hall and C. F. Chignell, *Photochem. Photobiol.* **45,** 459 (1987).
[4] J. R. Kanofsky, *J. Biol. Chem.* **258,** 5991 (1983).
[5] A. U. Khan, P. Gebauer, and L. P. Hager, *Proc. Natl. Acad. Sci. U.S.A.* **80,** 5195 (1983).
[6] J. R. Kanofsky and B. Axelrod, *J. Biol. Chem.* **261,** 1099 (1986).
[7] R. D. Hall, W. Chamulitrat, N. Takahashi, C. F. Chignell, and R. P. Mason, *J. Biol. Chem.* **264,** 7900 (1989).