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CATCHING FREE RADICALS: NEW TRICKS WITH OLD TRAPS

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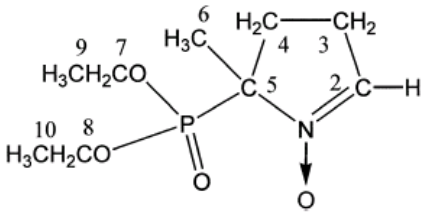
This paper describes some new and novel applications of spin trapping to biological problems. As a timely subject the use of NMR/MRI methods are updated. The enzyme, NAD(P)H:quinone oxidoreductase 1 or (NQO1) catalyzes the reduction of quinone substrates, generating stable hydroquinones. The studies outlined here were designed to determine if NQO1 can scavenge superoxide, taking on an additional function of a superoxide reductase. The second part of this review describes the technique of NMR spin trapping wtih nitrone spin traps that contain a stable isotope enabling the detection of diamagnetic products as a result of bioreduction or disproportionation reactions. Examples are shown for carbon- and oxygen based radicals with 31-P or 19-F containing nitrones. The real power of this method was demonstrated with $SO_3^{\bullet -}$ detection using nitrone spin traps that showed that the radical adduct occurred as a result of non-radical addition reactions followed by oxidation, rather than the scavenging of the reactive radical species.

INTRODUCTION

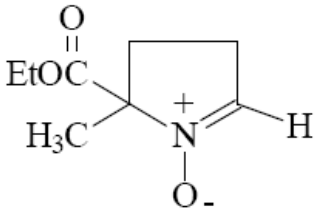
This article is an overview of methods of trapping and identifying free radical intermediates in biologically interesting reactions that exemplify some interesting property of the system that might not otherwise be found by alternative spectroscopic methods. In addition, the incorporation of NMR/MRI methods is discussed. Some of these were introduced in an earlier volume of this journal and will be discussed only briefly here (Berliner, Khramtsov, Clanton & Fujii, 2002).

SPIN TRAPPING

The principle of spin trapping is that one can chemically trap short-lived free radicals as longer-lived radical adducts. The most frequently used spin trapping agents are nitrone compounds, some of which are shown in Fig. 1. These compounds yield nitroxide adducts with fairly straightforward spectral characterization. There are nonetheless several problems: many nitrone adducts last only 5-25 minutes; the steady-state production rate of biological free radicals are frequently well below the detection limits of most EPR systems; radical capture rate constants are quite slow, requiring very high concentrations of spin trap for detectable levels. Hence the goal is always to synthesize newer, more reactive nitrones with longer lived adducts. Some of the nitrones discussed here, in fact, fall into that 'new generation' of spin traps yet, as we shall see, the problems of bioreduction and disproportionation are extremely difficult to overcome. Obviously, the ideal spin trap should yield some easily detectable signal that accumulates with time and is stable over long periods of time.



DEPMPO



EPMPO

Fig. 1 Structures of recently developed nitrone spin traps: DEPMPO (diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide); EMPO (5-Ethoxycarbonyl-5-methyl-1-pyrroline-N-oxide)

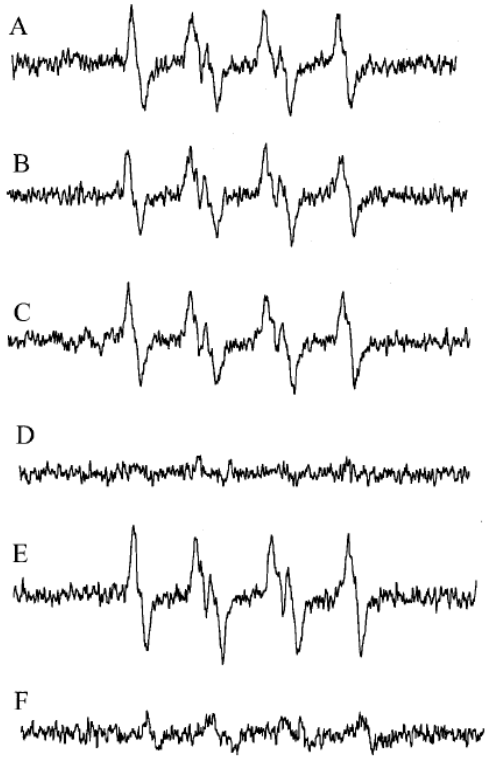


Fig. 2. The effect of NQO1 on EMPO: $O_2^{\bullet-}$ adduct formation. In addition to potassium superoxide and EMPO, the reactions contained the following: A, no additions; B, NADH (0.5 mM); C, NQO1 (25 µg/ml); D, NADH (0.5 mM), NQO1 (25µg/ml); E, NADH (0.5 mM), NQO1 (25 mg/ml), and ES936 (5 µM); and F, SOD (10 U/ml). Spectra were obtained 5 min after the addition of potassium superoxide. From Siegel et at. (2004) with permission.

NAD(P)H:QUINONE OXIDOREDUCTASE 1

NAD(P)H:quinone oxidoreductase 1 or DT-diaphorase (NQO1) is an obligate two-electron reductase that contains FAD and catalyzes the reduction of a broad range of quinone substrates. It generates stable hydroquinones, imparting good antioxidant properties. NQO1 is also reported to have good chemoprotective (cancer preventive) properties. It associates with p53 in human cancer cells, preventing the involvement of p53 in various tumor related processes. Of note is the fact that NQO1 is absent in human liver, yet moderately high expression is found in most other tissues, suggesting that its function may have evolved from quinone detoxification to a more broadened antioxidant role (Ross, Kepa, Winski, Beall, Anwar & Siegel, 2000). Of the novel chemoprotective mechanisms mediated by NQO1, we note that NQO1 can generate redox active hydroquinones which produce superoxide on autoxidation. There are also reports of marked induction of NQO1 under conditions of oxidative stress. Hence, without some mechanism of non-malevolent superoxide breakdown, the protective role would be in question. The purpose of the studies outlined here were to investigate if NQO1 can indeed scavenge superoxide.

There is a 1:1 correlation of O_2 consumption with NADH oxidation. Both processes are slowed by superoxide dismutase (SOD). In order to determine if H_2O_2 or $O_2^{\bullet-}$ were generated, the autoxidation was studied by luminol chemiluminescence using horseradish peroxidase and catalase, where it was shown that only H_2O_2 was generated. Furthermore, NADH oxidation was accelerated in the presence of $O_2^{\bullet-}$ yet no NADH oxidation with KO_2 occurred unless NQO1 was present.

NQO1 'Scavenging' of $O_2^{\bullet-}$

The xanthine/xanthine oxidase (X/XO) generation of $O_2^{\bullet-}$ was followed by DHE (dihydroethidium) oxidation, a common fluorescence method for $O_2^{\bullet-}$ detection. The reaction was suppressed by NQO1/NADH, however, both must be present in order to effect the inhibition. Only in the presence of both NQO1/NADH or SOD was $O_2^{\bullet-}$ generation suppressed. This was particularly evident when the enzyme was incubated with ES936, a mechanism-based inhibitor of NQO1. In addition, NQO1 is an effective superoxide scavenger in pyrogallol autooxidation. The addition of NQO1 in combination with NAD(P)H resulted in inhibition of pyrogallol autooxidation as shown. Oxygen consumption studies demonstrated that the combination of NQO1 and NADH had no effect of the rate of molecular oxygen consumption during the autooxidation. Lastly, Fig. 2 depicts EPR spectra using the spin-trap EMPO (5-Ethoxycarbonyl-5-methyl-1-pyrroline-N-oxide), which is very diagnostic for $O_2^{\bullet-}$ and OH radicals.

NQO1 as a superoxide reductase?

We may make several conclusions about NQO1:

a. During NQO1 auto-oxidation H_2O_2 is produced;
b. $O_2^{\bullet-}$ accelerates a NQO1 auto-oxidation in a fashion reminiscent of the auto-oxidation of flavins and flavoproteins;
c. The primary metabolic functions of NQO1 are:
    i. catalysis of quinone → hydroquinone

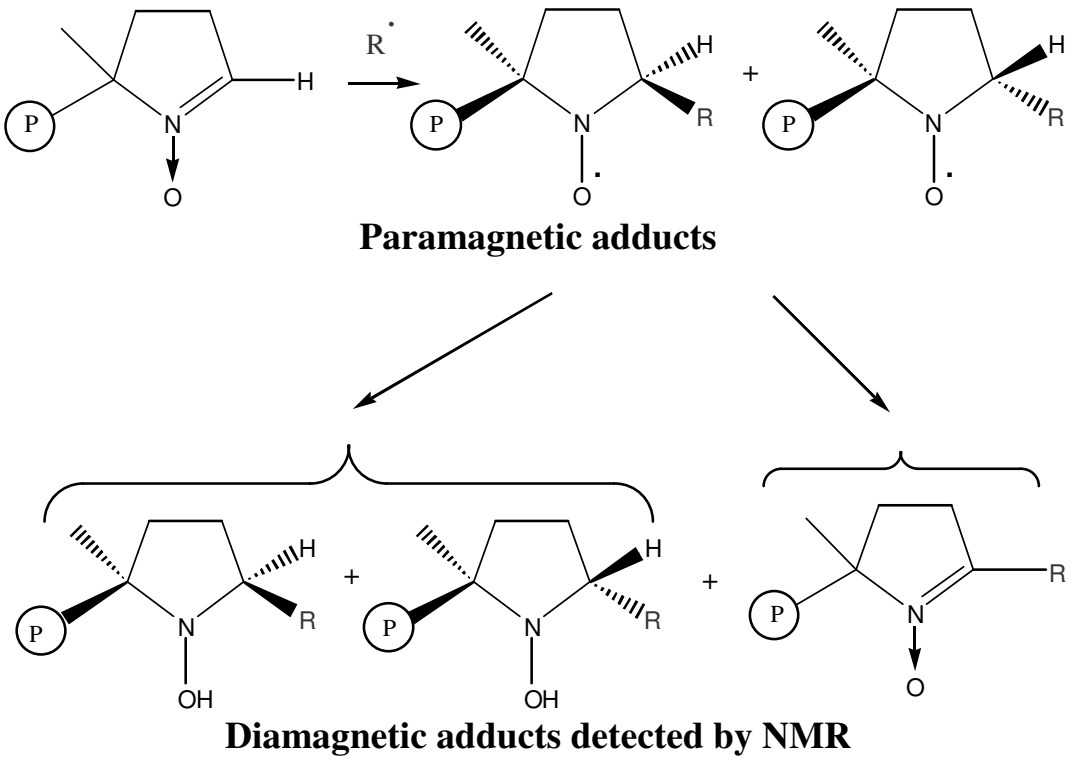


Fig. 3 Schematic of the reaction of DEPMPO with a free radical, R· and the diamagnetic hydroxylamine and nitrone disproportionation products. The hydroxylamine products will also result from biological reduction reactions.

ii. hydroquinone oxidation leading to $O_2^{\bullet-}$ generation where the $O_2^{\bullet-}$ is scavenged at the scavenged at the site of generation.

There is a very pertinent relevance of NQO1 $O_2^{\bullet-}$ scavenging at the cellular level as well. In two cancer cell lines: MDA468/NQ16 (human breast cancer) and CHO/812 (overexpressing NQO1):

a. there is a significant increase in $O_2^{\bullet-}$ scavenging activity;
b. the cell lines inhibit pyrogallol autoxidation in the presence of NAD(P)H;
c. there is more efficient $O_2^{\bullet-}$ removal than SOD due to high NQO1 levels in cells; it may function as an additional 'SOD enzyme in cellular systems;
d. note that in tissue with high natural exposure to $O_2^{\bullet-}$, such as the cornea and lens epithelium, there are high NQO1 levels.

Polymorphism: NQO1*2 alleles (diminished NQO1 activity) show increased rates of leukemia, lung, colon, skin, bladder cancer and Parkinson's disease (Harada et al., 2001).

Overall, the results suggest that NQO1 serves as a superoxide reductase:

$$O_2^{\bullet-} + e^- + 2H^+ \rightarrow H_2O_2$$

which is to be compared with superoxide dismutase (SOD)

$$2O_2^{\bullet-} \rightarrow H_2O_2 + O_2$$

NMR SPIN TRAPPING: ST-NMR

As discussed earlier, it would be desirable to have a more persistent, sensitive spectroscopic method to observe free radical reactions. For example, it might be possible to "visualize" regional inflammation and oxidant stress using a combination of redox sensitive chemical probes (e.g. spin traps) and nuclear magnetic resonance. Of course, we must consider the shortcomings as well:

ADVANTAGES	DISADVANTAGES
(1) Depth of penetration	(1) Low sensitivity
(2) Maintains signal specificity	
(3) Measures stable diamagnetic reduction products	

To date the field of NMR spin trapping has shown some promise with nitrone spin traps containing stable isotopes (e.g., 31-P, 19-F) whereby one can examine the product of a radical trapping reaction in the diamagnetic products. For example, Fig. 3 depicts both the paramagnetic and diamagnetic products obtained with the 31-P containing spin trap, DEPMPO. The spectra are rich in information and the diamagnetic products show up as unique peaks in the 31-P NMR spectrum.

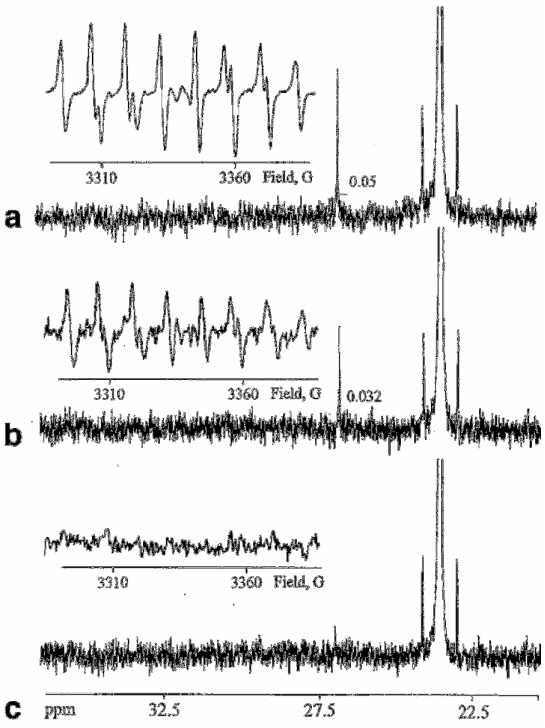
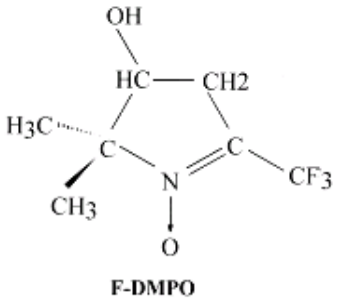


Fig. 4. 31 P-NMR spectra of DEPMPO: $O_2^{\bullet-}$ and neutrophil-derived reaction products. a: 31 P-NMR spectra of 50 mM DEPMPO in 0.1 M potassium phosphate buffer, pH 7.4, 2 mM DTPA after 1 hr incubation in the presence of 0.4 unit/ml xanthine oxidase and 0.4 mM hypoxanthine. b: 1 hr incubation in the presence of 2×10^6 neutrophils, stimulated by formyl-methionyl-leucyl-phenylalanine (fMLP). c: 1 hr incubation in the presence of 2×10^6 neutrophils without stimulation with fMLP. Spectrometer settings were: frequency 121.5 MHz ; number of scans 512; acquisition time 1.69 sec. Insets: ESR spectra obtained simultaneously. Spectrometer settings were as follows: microwave power, 20 mW; modulation amplitude, 0.8 G; receiver gain (a, 6.3×10^2; b, 5×10^3; c, 1×10^4). From Khramtsov *et al.* (1999) with permission.

Challenges with O-centered radicals

The efficacy of this method in the detection of reactive oxygen species (ROS), particularly superoxide and hydroxyl radical, are depicted in Fig. 4, where superoxide radical was generated from either the X/XO system or stimulated neutrophils. The NMR spectra of the superoxide adduct reaction products was essentially indistinguishable from the corresponding ·OH adduct reaction products. Unstimulated neutrophils showed no ESR signal (Fig. 4c) and this signal was completely inhibited by co-incubation with 85 units/ml SOD (data not shown). In all cases, the NMR signals for superoxide formation both from the enzymatic (Fig. 4a), the NMR signals for neutrophil stimulation (Fig. 4b), were relatively small and essentially indistinguishable from the spectra for hy-



F-DMPO

Fig. 5. 4-Hydroxy-5,5-dimethyl-2-trifluoromethyl-pyrroline-1-oxide (FDMPO).

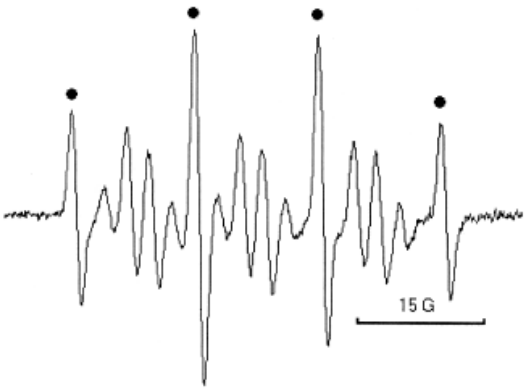


Fig. 6. X-band EPR spectra showing the competition of FDMPO and DMPO (•) for • OH-radical. ESR spectra were obtained in 0.1 M potassium phosphate buffer, pH 7.0, 2 mM, DTPA, 10 mM H_2O_2, 100 mM FDMPO, 100 mM DMPO, after addition of 10 mM $FeSO_4$ solution. From Khramtsov *et al.* (2001b) with permission.

droxyl radical (27.05 ppm). In every case with superoxide and hydroxyl radical trapping, the EPR and NMR signals slowly disappear with time. This appears to be due to the elimination of either H_20 or H_20_2, respectively (Khramtsov, Berliner & Clanton, 1999). Despite both this ambiguity and the observation that even the NMR signals disappear with time, the possibility that NMR may be potentially useful for ''detection'' of free radicals in some biological settings is enticing.

^{19}F labeled spin traps

Khramtsov, Reznikov, Berliner, Litkin, Grigor'ev & Clanton (2001b) have reported the synthesis and characterization of 19-F containing nitrones, such as FDMPO shown in Fig. 5. This spin trap forms relative stable adducts which usually require ascorbate reduction before the 19-F NMR spectrum is visible. The reactivity of FDMPO vs. DMPO is equivalent as shown in Fig. 6.

MECHANISMS THAT DO NOT INVOLVE DIRECT RADICAL TRAPPING

Liu et al (Liu, Miyake, Panz & Swartz, 1999a; Liu, Bechara, Kotake & Swartz, 1999b) have shown previously the efficacy of nitrone spin traps, such as DEPMPO, for the detection of biological radicals. Sulfite anion radical can be generated, in principle, by at least two pathways shown below:

$$SO_3^{2-} + O_2SO_3^{\bullet-} \longleftrightarrow SO_3^{\bullet-} + O_2^{\bullet-}$$

$$SO_3^{2-} + Cr(VI) \longleftrightarrow SO_3^{\bullet-} + Cr(V)$$

In a study designed to trap the sulfite in vivo they examined the dichromate induced production of $SO_3^{\bullet-}$ in rodents where a distinct radical adduct signal was found (Liu *et al*., 1999b). In a followup ST-NMR study by Khramtsov, Potapenko, Bagrayanskaya and Clanton (2003) they found evidence for the hydroxylamine sulfite adduct to DEPMPO without the requirement for dichromate oxidation, as depicted in Fig. 7. That is, a high quantity of

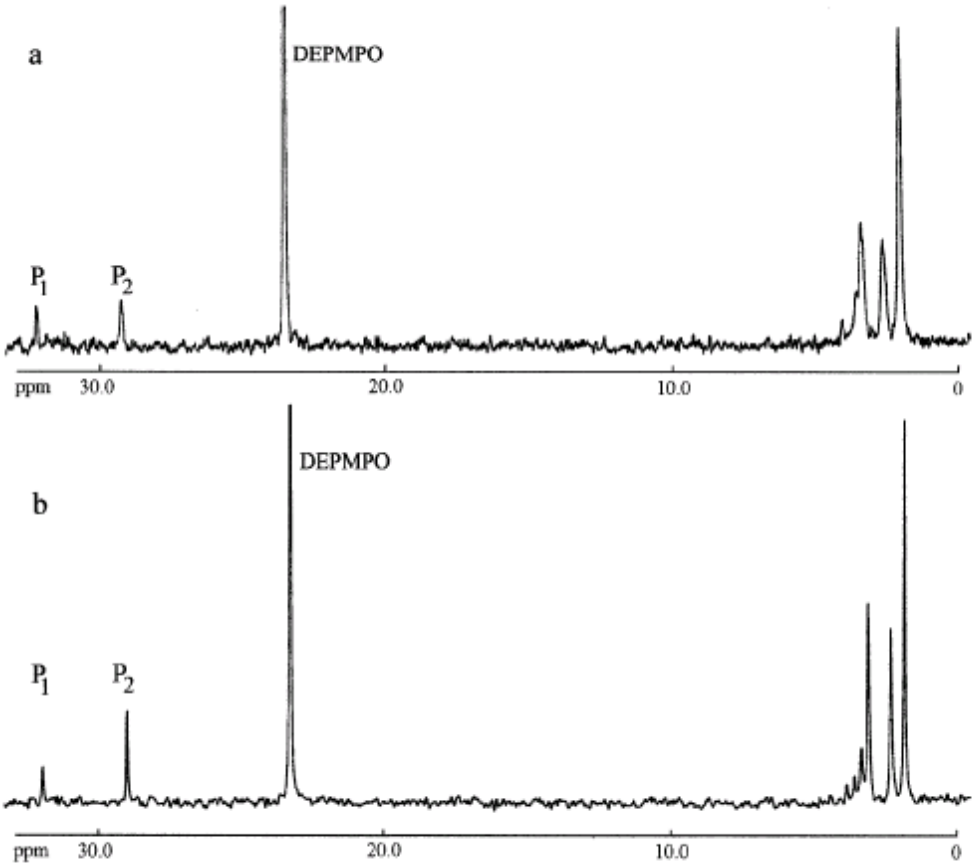


Fig. 7. 31 P-NMR spectra observed in blood samples obtained from animals treated i.p. with sulfite and DEPMPO with and without dichromate i.v. (a) dichromate addition; (b) without dichromate injection. Spectrometer settings were as: frequency 202.5 MHz, number of scans 640, acquisition time 0.54 s. From Khramtsov *et al.* (2003) with permission.

reduced sulfite adduct was obtained that did not depend on oxidizing agents. The results suggests a possible nonradical reaction between (bi)sulfite and DEPMPO, ie., nonradical nucleophillic addition to nitrones. This then leads to generation of nitroxyl radicals through oxidation of the resulting hydroxylamine giving EPR detectable species Hence, the results demonstrate that SO_3 detection using nitrone spin traps such as DEPMPO or DMPO may involve nonradical addition reactions. This was also evident from extended EPR studies where the slow formation of the other enantiomeric adduct of DEPMPO: $SO_3^{\bullet-}$ was formed, presumably via nonradical nucleophillic addition followed by oxidation of the hydroxylamine. Such examples for nonradical addition reactions have been found in the past, for example with the cardiac drug nifedipine (Fujii & Berliner, 1999).

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