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[^{17}O]OXYGEN HYPERFINE STRUCTURE FOR THE HYDROXYL AND SUPEROXIDE RADICAL ADDUCTS OF THE SPIN TRAPS DMPO, PBN AND 4-POBN

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Summary. [^{17}O]oxygen hyperfine coupling constants are reported for the superoxide and hydroxyl radical adducts with the spin traps 5,5-dimethyl-1-pyrroline N-oxide, N-t-butyl-α-phenylnitrone and α-(4-pyridyl 1-oxide)-N-t-butylnitrone. These couplings provide spectroscopic evidence that the spin adducts have been correctly identified. © 1986 Academic Press, Inc.

Introduction. Superoxide and hydroxyl radicals have been postulated as oxygen-derived radicals in many biochemical reactions. The spin trapping of these radicals to form more stable nitroxide spin adducts observable by electron spin resonance (ESR) has proven useful in the study of these species, especially in biological systems (1-6).

The DMPO -hydroxyl and DMPO-superoxide radical adducts and the PBN-hydroxyl and PBN-superoxide radical adducts were first assigned in 1974 (7). In those experiments H_2O_2 was photolyzed with UV light to produce hydroxyl and superoxide

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Abbreviations used: DMPO, 5,5-dimethyl-1-pyrroline N-oxide; PBN, N-t-butyl-α-phenylnitrone; 4-POBN, α-(4-pyridyl 1-oxide)-N-t-butylnitrone; DTPA, diethylenetriaminepentaacetic acid.

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radicals which were subsequently trapped by DMPO or PBN. In 1978 the first report of the 4-POBN-hydroxyl and superoxide radical adducts produced by the UV photolysis of H_2O_2 solutions appeared (8). In addition, the 4-POBN-hydroxyl radical adduct was produced with chelated Fe^{3+} and H_2O_2, with sodium persulfate at pH 2-6, and by acid-catalyzed addition of water to the nitrone double bond followed by oxidation by H_2O_2. The proof of the structure of the 4-POBN-hydroxyl radical adduct was based on the detection of a splitting of about 0.3 G, which was attributed to the γ-hydrogen of the hydroxy group because it was exchanged in D_2O (8).

There is no definitive spectroscopic evidence that the assignments of the DMPO-hydroxyl and -superoxide radical adducts (7), the PBN-hydroxyl and -superoxide radical adducts (9), or the 4-POBN-hydroxyl and -superoxide radical adducts (8) are indeed oxygen-centered radical adducts. In this paper we confirm the assignments of these radical adducts through the formation of [^{17}O]oxygen-centered radical adducts which were monitored with ESR spectrometry.

Materials and Methods. Sodium persulfate, silver nitrate, NADPH, xanthine, xanthine oxidase (Grade III from buttermilk), diethylenetriaminepentaacetic acid (DTPA), 5,5-dimethyl-1-pyrroline N-oxide (DMPO), N-t-butyl-α-phenylnitrone (PBN) and α-(4-pyridyl 1-oxide)-N-t-butylnitrone (4-POBN) were all obtained from Sigma Chemical Company. Paraquat (methyl viologen) was obtained from Aldrich Chemical Company. [^{17}O]oxygen gas (64 atom %) and [^{17}O]water (60 atom %) were obtained from MSD Isotopes. The DMPO was distilled under vacuum and stored under nitrogen below 0°C. All other chemicals were used without further purification. Rat liver microsomes were prepared (10) and stored below -70°C before use.

Superoxide anion radicals were generated by adding NADPH and rat liver microsomes to a solution of paraquat in pH 7.4 phosphate buffer containing spin trap and 1 mM DTPA (final concentrations were 1 mM paraquat, 1 mM NADPH, 100 mM spin trap and 2.7 mg microsomal protein/ml). [^{17}O]oxygen experiments were carried out by initially bubbling nitrogen through the spin trap solution in a small vial with a rubber septum. A gas tight syringe was used to remove 10 ml of nitrogen gas from the vial and replace it with 10 ml of [^{17}O]oxygen. The vial was shaken well and paraquat, NADPH and microsomes were added using syringes. The sample was aspirated into the flat cell using a rapid sampler device (11).

Hydroxyl radicals were produced in two different ways. The DMPO-hydroxyl radical adduct was formed by mixing xanthine and xanthine oxidase together in the presence of DMPO and Fe^{2+} (final concentrations were 0.1 mM xanthine, 0.14 units xanthine oxidase/3 ml, 100 mM DMPO and 0.5 mM Fe^{2+}) (12). A clean spectrum of DMPO-hydroxyl radical adduct was obtained after 8 minutes. The DMPO-[^{17}O]hydroxyl radical adduct was formed by introducing [^{17}O]oxygen gas into the system as described above. The PBN- and 4-POBN-hydroxyl radical adducts were formed by the reaction of 50 mM sodium persulfate and 100 mM spin trap catalyzed by 10^{-4} M $AgNO_3$ (8). In this case the sulfate anion radical from the persulfate does not form hydroxyl radicals but instead oxidizes the PBN or 4-POBN to a radical cation which is then hydrolyzed to form the hydroxy radical adduct (13).

The [^{17}O]oxygen adduct was generated using [^{17}O]water as the solvent.

The ESR spectra of the superoxide radical adducts and the DMPO-hydroxyl radical adduct were obtained using an IBM ER-200 ESR spectrometer operating at 9.7 GHz with 100 kHz modulation frequency and equipped with an ER-4103 TM cavity. The PBN- and 4-POBN-hydroxyl radical adduct ESR spectra were obtained using a Varian E-109 spectrometer operating at 9.5 GHz with 100 kHz modulation frequency and with an E-238 TM_{110} cavity. Simulations of spectra were carried out on an HP 9236 computer system with Varian software.

Results and Discussion. The experimental and simulated spectra for the DMPO-superoxide and hydroxyl radical adducts, the PBN-superoxide and -hydroxyl radical adducts, and the 4-POBN-superoxide and -hydroxyl radical adducts are shown in Figures 1, 2, and 3, respectively. In each case, arrows mark the lines from the [^{16}O]oxygen adducts. The splitting constants, based on the computer simulations, are summarized in Table 1. The spectra for the DMPO and 4-POBN adducts and the PBN-superoxide radical adduct are quite clean, i.e., there are no nitroxide impurities or unassigned adducts. In the case of the PBN-hydroxyl radical adduct, the experimental spectrum also includes the spectrum of tert-butyl hydronitroxide (lines marked by asterisks), a decomposition product of PBN often seen when persulfate is used (9).

In the initial studies (7), the structures of the DMPO-hydroxyl and superoxide radical adducts and the PBN-hydroxyl and superoxide radical adducts were based on well-characterized photochemical processes. Irradiation of 1% H_2O_2 is known to give hydroxyl radicals, while irradiation of 30% H_2O_2 gives rise to the secondary superoxide radical. Further chemical evidence that the DMPO-hydroxyl and PBN-hydroxyl radical adducts were correctly assigned was provided when Sargent and Gardy obtained the same spectra as Harbour, Chow and Bolton by the irradiation of N_2O-saturated aqueous solutions of DMPO or PBN with 4 MeV electrons (14). Since N_2O is known to react rapidly with electrons to form the hydroxyl radical, the spectrum obtained was attributed to the DMPO- or PBN-hydroxyl radical adduct. The DMPO-superoxide radical adduct assignment offered by Harbour, Chow, and Bolton was based on the sensitivity to superoxide dismutase of the signal produced by adding tetramethyl ammonium superoxide to an aqueous solution of DMPO (15). Superoxide dismutase, a specific enzyme which disproportionates superoxide, provides a unique method for determining the involvement of superoxide in the production of spin adducts. Finkelstein et

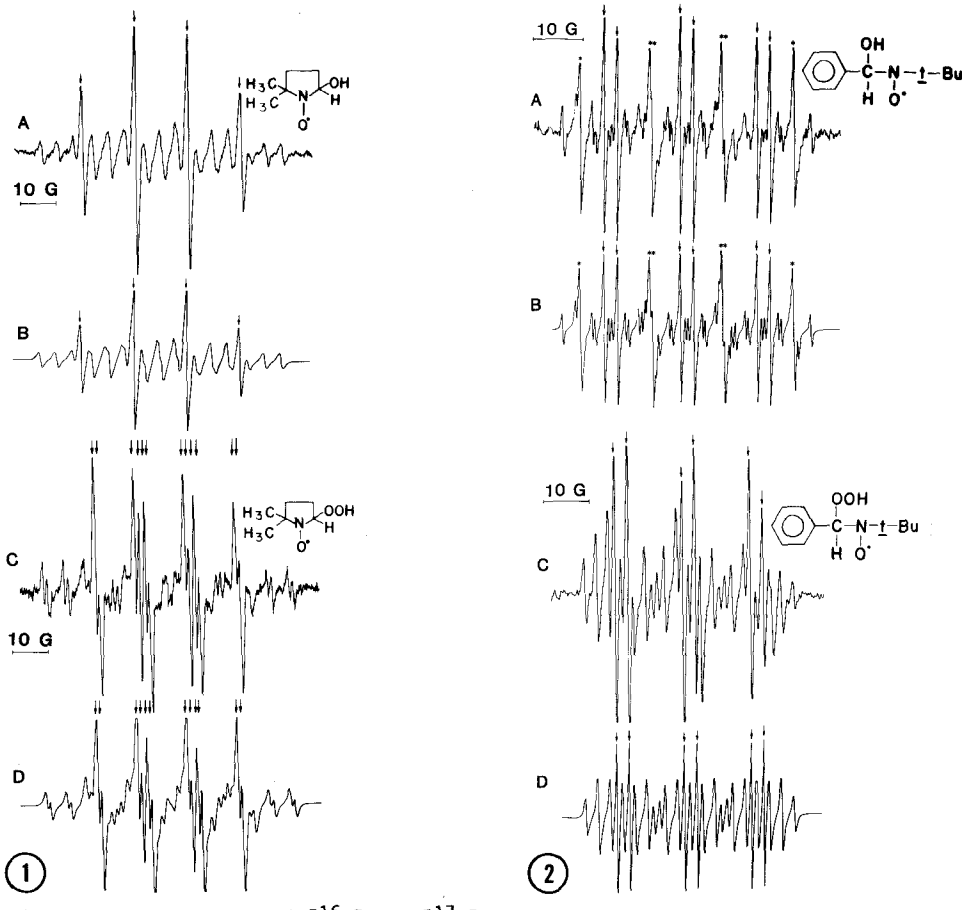

Figure 1. ESR spectra of [^{16}O] and [^{17}O] containing DMPO-hydroxyl and -superoxide radical adducts. Arrows indicate lines from the [^{16}O]oxygen adduct spectra. A. DMPO-hydroxyl radical adduct spectrum observed in an incubation containing 0.1 mM xanthine, 0.14 units xanthine oxidase/3 ml, 100 mM DMPO, 0.5 mM Fe^{2+} and [^{17}O]oxygen in pH 7.4 phosphate buffer. Instrumental conditions: microwave power, 20 mW; sweep rate, 12 G/min; modulation amplitude, 1 G; time constant, 1 s. B. Computer simulation of the DMPO-hydroxyl radical adduct spectrum. $a^N = a^H_\beta = 15.01$ G and $a^{17O} = 4.66$ G. C. DMPO-superoxide radical adduct spectrum observed in an incubation containing 1 mM paraquat, 1 mM NADPH, 100 mM DMPO, 1 mM DTPA, 2.7 mg microsomal protein/ml and [^{17}O]oxygen in pH 7.4 phosphate buffer. Instrumental conditions: microwave power, 20 mW; sweep rate, 30 G/min; modulation amplitude, 0.5 G; time constant, 0.2 s. D. Computer simulation of the DMPO-superoxide radical adduct spectrum. $a^N = 14.2$ G, $a^H_\beta = 11.34$ G, $a^H_\gamma = 1.25$ G, and $a^{17O} = 5.9$ G.

Figure 2. ESR spectra of [^{16}O] and [^{17}O] containing PBN-hydroxyl and -superoxide radical adducts. Arrows indicate lines from the [^{16}O]oxygen-adduct spectra. A. PBN-hydroxyl radical adduct spectrum observed in an incubation containing 50 mM sodium persulfate, 10^{-4} M $AgNO_3$, 100 mM PBN and [^{17}O]water. Asterisks indicate the lines of t-butyl hydronitroxide. Instrumental conditions: microwave power, 20 mW; sweep, 10 G/min; modulation amplitude, 0.33 G; time constant, 0.5 s. B. Computer simulation of the PBN-hydroxyl radical adduct spectrum. $a^N = 15.5$ G, $a^H_\beta = 2.72$ G, and $a^{17O} = 3.36$ G. Asterisks indicate the t-butyl hydronitroxide lines: $a^N = 14.6$ G and $a^H = 14.0$ G. C. PBN-superoxide radical adduct spectrum observed in an incubation containing 1 mM paraquat, 1 mM NADPH, 100 mM PBN, 2.7 mg/ml microsomal protein and [^{17}O]oxygen in pH 7.4 phosphate buffer. Instrumental conditions: microwave power, 20 mW; sweep rate, 9.6 G/min; modulation amplitude, 0.5 G; time constant, 2 s. D. Computer simulation of the PBN-superoxide radical adduct spectrum. $a^N = 14.81$ G, $a^H_\beta = 2.7$ G, and $a^{17O} = 2.7$ G.

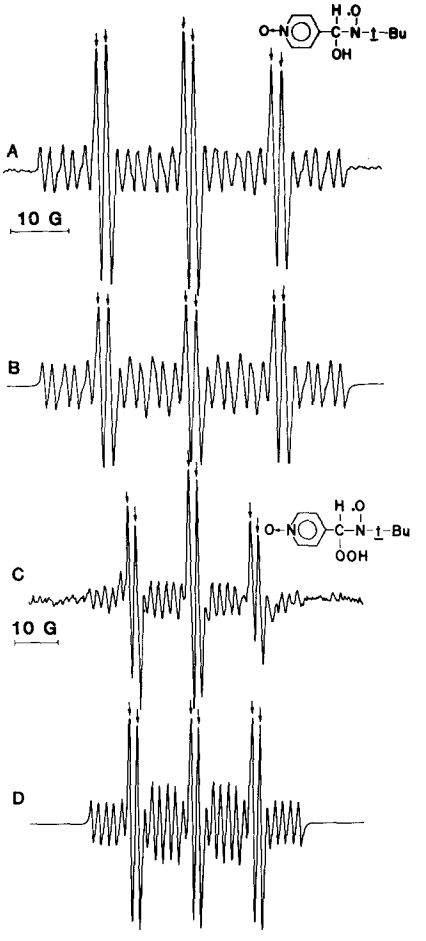

Figure 3. ESR spectra of [^{16}O] and [^{17}O] containing 4-POBN-hydroxyl and -superoxide radical adducts. Arrows indicate lines from the [^{16}O]oxygen-adduct spectra. A. 4-POBN-hydroxyl radical adduct spectrum observed in an incubation containing 50 mM sodium persulfate, 10^{-4} M $AgNO_3$, 100 mM 4-POBN and [^{17}O]water at pH 2.3. Instrumental conditions: microwave power, 20 mW; sweep rate, 10 G/min; modulation amplitude, 0.4 G; time constant, 1 s. B. Computer simulation of the 4-POBN-hydroxyl radical adduct spectrum. a^N = 15.1 G, a^H_β =1.66 G, a^H_γ = 0.3 G, and a^{17O} = 3.9 G. C. 4-POBN-superoxide radical adduct spectrum observed in an incubation containing 1 mM paraquat, 1 mM NADPH, 100 mM 4-POBN, 2.7 mg/ml microsomal protein, 1 mM DTPA and [^{17}O]oxygen in pH 7.4 phosphate buffer. Instrumental conditions: microwave power, 20 mW; sweep rate, 30 G/min; modulation amplitude, 1 G; time constant 0.5 s. D. Computer simulation of the 4-POBN-superoxide radical adduct spectrum. a^N = 14.18 G, a^H_β = 1.72 G, and a^{17O} = 3.6 G.

al. produced a spectrum of the 4-POBN-superoxide radical adduct using xanthine/xanthine oxidase (15). They demonstrated superoxide dismutase inhibition under conditions which gave rise to DMPO-superoxide radical adduct formation. Similarly, Kalyanaraman et al. showed that redox cycling of mitomycin C gave a superoxide dismutase-sensitive PBN-superoxide radical adduct under con-

Table 1. Hyperfine Coupling Constants of Superoxide and Hydroxyl Radical Adducts (in Gauss)

Spin Adduct	a^N	a^{H}_{β}	a^{H}_{γ}	$a^{17}O$	Reference
DMPO/$^{\bullet}$OOH	14.2	11.34	1.25[a]	5.9	this work
	14.3	11.7	1.25	-	7
DMPO/$^{\bullet}$OH	15.01	15.01	-	4.66	this work
	15.0	15.0	-	-	14
PBN/$^{\bullet}$OOH	14.81	2.7	-	2.7	this work
	14.8	2.75	-	-	7
PBN/$^{\bullet}$OH	15.5	2.72	-	3.36	this work
	15.3	2.75	-	-	7
POBN/$^{\bullet}$OOH	14.18	1.72	-	3.6	this work
	14.16	1.75	-	-	15
POBN/$^{\bullet}$OH	15.1	1.66	0.3[b]	3.9	this work
	14.97	1.68	0.34	-	8

[a]Splitting due to γ hydrogen

[b]Splitting due to hydroxyl hydrogen (8)

ditions where the DMPO-superoxide radical adduct was detected (16). In 1978, Janzen et al. further verified the PBN-hydroxyl adduct assignment by producing the adduct in a variety of different ways (9). Only in the use of the 4-POBN-hydroxyl radical adduct was GC/MS used to separate and positively identify the radical adduct (17).

Until now evidence of the correct assignments of the hydroxyl radical adducts of DMPO and 4-POBN, and the superoxide radical adducts of DMPO, PBN and 4-POBN has been primarily circumstantial as outlined above. Our observations of [^{17}O]oxygen hyperfine structure for each of the radical adducts firmly establish, for the first time, that they are oxygen-centered radical adducts and add spectroscopic evidence to the chemical evidence for their structures.

References

1. Evans, C.A. (1979) Aldrichim. Acta 12, 23-29.
2. Perkins, M.J. (1980) Adv. Phys. Org. Chem. 17, 1-64.
3. Finkelstein, E., Rosen, G.M., and Rauckman, E.J. (1980) Arch. Biochem. Biophys. 200, 1-16.
4. Janzen, E.G. (1980) In "Free Radicals in Biology" (W.A. Pryor, ed.), Vol. 4, pp. 115-154, Academic Press, New York.
5. Buettner, G.R. (1982) In "Superoxide Dismutase" (L.W. Oberley, ed.), Vol. II, pp. 63-81, CRC Press, Boca Raton, Florida.
6. Kalyanaraman, B. (1982) In "Rev. Biochem. Toxicol." (E. Hodgson, J.R. Bend, and R.M. Philpot, eds.) Vol. 4, pp. 73-139, Elsevier Biomedical, New York.

7. Harbour, J.R., Chow, V., and Bolton, J.R. (1974) Can. J. Chem. 52, 3549-3553.
8. Janzen, E.G., Wang, Y.Y., and Shetty, R.V. (1978) J. Amer. Chem. Soc. 100, 2923-2925.
9. Janzen, E.G., Nutter, D.E., Jr. Davis, E.R., Blackburn, B.J., Poyer, J.L. and McCay, P.B. (1978) Can. J. Chem. 56, 2237-2242.
10. Moreno, S.N.J., Docampo, R., Mason, R.P., Leon, W., and Stoppani, A.O.M. (1982) Arch. Biochem. Biophys. 218, 585-591.
11. Mason, R.P. (1984) in Packer, L. (ed.) Oxygen Radicals in Biological Systems, Methods in Enzymology, Vol. 105, 416-422.
12. Buettner, G.R., Oberley, L.W., and Chan Leuthauser, S.W.H. (1978) Photochem. Photobio. 28, 693-695.
13. Janzen, E.G., and Coulter, G.A. (1984) J. Amer. Chem. Soc. 106, 1962-1968.
14. Sargent, F.P., and Gardy, E.M. (1976) Can. J. Chem. 54, 275-279.
15. Finkelstein, E., Rosen, G.M., Rauckman, E.J., and Paxton, J. (1979) Mol. Pharmacol. 16, 676-685.
16. Kalyanaraman, B., Perez-Reyes, E., and Mason, R.P. (1980) Biochim. Biophys. Acta 630, 119-130.
17. Abe, K., Suezawa, H., Hirota, M., and Ishii, T. (1984) J. Chem. Soc. Perkin Trans. II, 29-34.