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ETHYL ACETATE EXTRACTION OF SPIN-TRAPPED FREE RADICALS: A REEVALUATION

JAMES R. TRUDELL
Department of Anesthesia, Stanford University School of Medicine, Stanford, CA 94305-5117, USA

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Abstract—We have suggested the use of ethyl acetate for extraction of hydroxyl or superoxide radical adducts of the spin trap phenyl *N*-tert-butyl nitrone (PBM). The technique produced EPR spectra with narrow line widths, the radical adducts were more stable, and there were sufficiently large differences between the isotropic nitrogen hyperfine coupling constant (a_N) and the beta hydrogen coupling constant ($a_{H\beta}$) for both the hydroxyl and superoxide radical adducts to allow their simultaneous quantitation in mixtures. However, Kalyanaraman, Mottley, and Mason have suggested that our assignments of a_N and $a_{H\beta}$ were incorrect and that extraction of spin-trapped adducts into ethyl acetate is not as useful as we had proposed. This paper demonstrates that their objections are unfounded and are based on a computational error that they made when they attempted to calculate the hyperfine splittings in their spectra.

Keywords—EPR, ESR, Free radicals, Hydroxyl radical, Superoxide, Spin trapping, Phenyl *N*-tert-butyl nitrone (PBN), 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO)

INTRODUCTION

In a previous publication, Bösterling and Trudell[1] suggested the use of ethyl acetate for extraction of hydroxyl or superoxide radical adducts of the spin trap phenyl *N*-tert-butyl nitrone (PBN). We proposed that the technique had the following advantages as compared with measuring the EPR spectra in the aqueous solutions in which they were produced: the line widths in degassed ethyl acetate are narrow, the radical adducts are more stable, and there are sufficiently large differences between the isotropic nitrogen hyperfine coupling constant (a_N) and the beta hydrogen coupling constant ($a_{H\beta}$) for both the hydroxyl and superoxide radical adducts to allow their simultaneous quantitation in mixtures. However, in two recent publications, Kalyanaraman et al.[2,3] have suggested that our assignments of a_N and $a_{H\beta}$ were incorrect and that extraction into ethyl acetate is not as useful as we had proposed. It is the purpose of the present paper to demonstrate that their objections are unfounded and are based on a computational error that they made when they attempted to calculate the hyperfine splittings in their spectra.

It is important to clarify the literature on this point because the potential usefulness for a reliable method to identify and quantitate hydroxyl radicals and superoxide radical anions has increased greatly in recent years. It has been shown that release of superoxide is part of the cytotoxic burst of macrophages.[4] In addition, superoxide formation by xanthine oxidase is an important factor in reperfusion damage following anoxia.[5] The use of various spin traps for detection of hydroxyl and superoxide radicals has been the subject of much research effort.[6–13] This paper will focus on a reevaluation of extraction of spin-trap adducts into ethyl acetate as a means to increase their stability and improve the resolution of their EPR spectra. This reevaluation is somewhat complicated because, in our original paper,[1] we chose to synthesize the PBN adducts that had been shown to be more stable than the corresponding 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) adducts.[11] However, when Kalyanaraman et al. reinvestigated the usefulness of ethyl acetate extraction, they used the less stable DMPO adducts. Therefore, a direct comparison of spectra and hyperfine splittings is not possible.

METHODS

The synthesis of the PBN adducts of hydroxyl and superoxide radicals as well as the conditions of the measurement of their spectra were described previously.[1] Briefly, the PBN-superoxide adduct (PBN—OOH) was prepared by addition of 20 mg of

powdered potassium superoxide (KO_2)/ml to 0.3 M $KHPO_4$ buffer containing 0.14 M PBN at 20°C. The adduct was extracted into ethyl acetate immediately to prevent hydrolysis. The hydroxyl radical adduct (PBN—OH) was produced by adding H_2O_2 to 0.3 M $KHPO_4$ buffer containing 20 μM Fe EDTA and 0.14 M PBN, as well as by alkaline hydrolysis of PBN—OOH.[11] The PBN—OH was extracted into ethyl acetate for measurement of the spectra. It should be noted that spin traps are reduced in biological systems. We found it necessary to saturate the ethyl acetate solutions with air to reoxidize the spin trap to the free radical state and then resaturate the solutions with nitrogen. EPR measurements were carried out on a Varian E-104A spectrometer that had been calibrated with tetracene radical. All spectra were measured in nitrogen-saturated ethyl acetate at 22°C with a modulation amplitude of 1 G and 10 mW of microwave power.

The synthesis of the hydroxyl radical adduct of DMPO (DMPO—OH) and measurement of the spectrum shown in Figure 1 was described by Kalyanaraman et al.[3] In order to calculate coupling constants a_N and $a_{H\beta}$ from their published spectra,[3] their spectra were photographically reproduced, as shown in Figure 1. All possible spectral splittings, as well as the 10-G reference bar, were measured with an accurate vernier caliper, and finally the measured splittings were converted into G. The integer spin of ^{14}N causes the nitrogen isotropic hyperfine coupling to form a triplet of lines of equal intensity. The beta hydrogen hyperfine coupling constant $a_{H\beta}$ splits each line of the triplet into a doublet. In the case of Figure 1A, which was measured in water, the a_N and $a_{H\beta}$ are almost exactly the same and the result is the observed 1:2:2:1 intensity pattern. In the case of Figure 1B, which was measured in ethyl acetate, the two coupling constants are different and a six-line spectrum of equal intensity is observed. When the spectrum in Figure 1B by Kalyanaraman et al. was analyzed in this laboratory, the only possible assignment of a pair of triplets was that indicated at the bottom of the figure.

RESULTS

The a_N and $a_{H\beta}$ values calculated as described here in methods from the spectra of the hydroxyl radical adduct DMPO—OH in ethyl acetate (Fig. 1B) published by Kalyanaraman et al. were 13.60 and 10.87

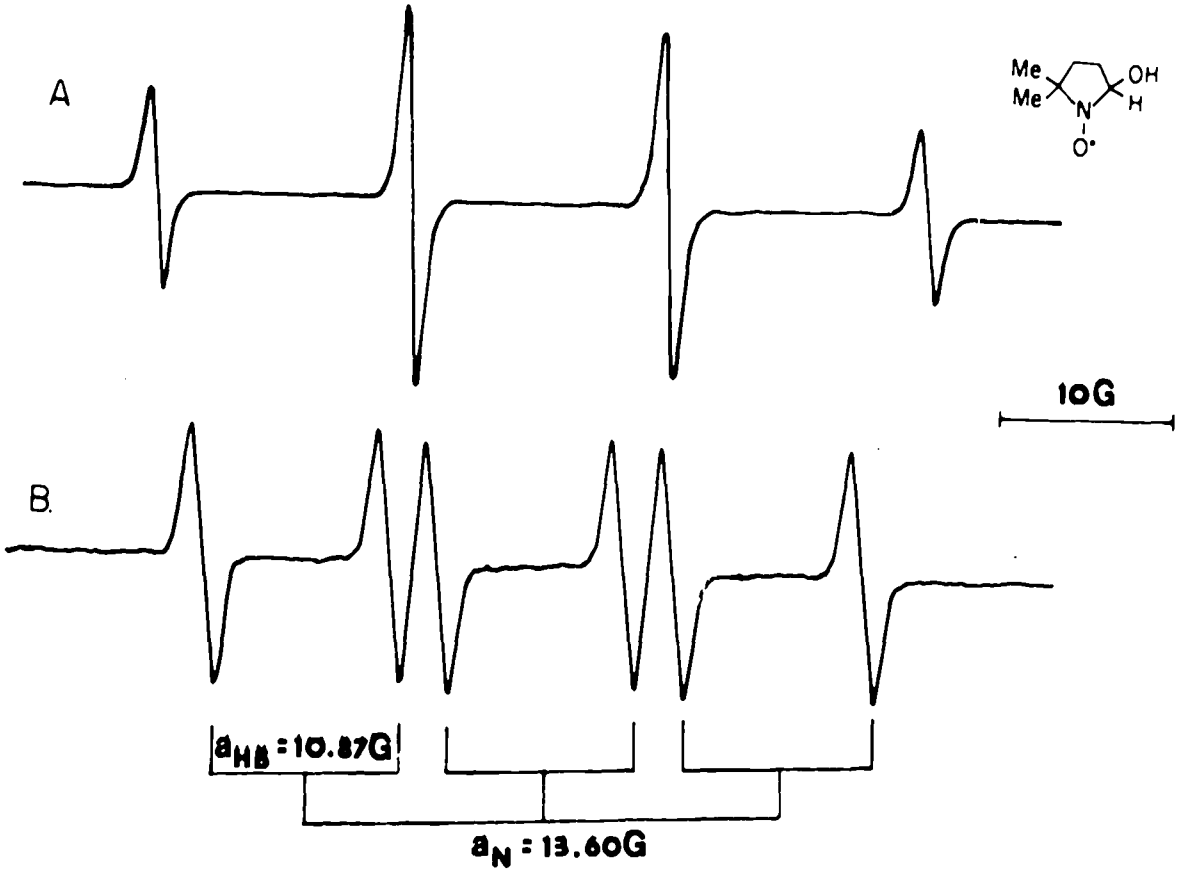

Fig. 1. Spectra A (DMPO—OH in water) and B of (DMPO—OH in ethyl acetate) of Figure 1 of Ref. 3 were reproduced from the original; the splittings were measured with a vernier caliper and converted to G by reference to the 10-G calibration bar. All possible assignments for a pair of triplets in spectrum B were considered and the unique possible correct assignment was made. This pair of triplets was drawn below spectrum B with the calculated splittings in G.

G, respectively. However, the values of a_N and $a_{H\beta}$ calculated by computer synthesis by Kalyanaraman et al. were 10.95 and 13.75 G, respectively. (In their paper, they referred to these values as "the correct spectral parameters," but, needless to say, this nomenclature will not be adhered to here.) Comparison of the values calculated here with those in Table 1 of their publication revealed that they had apparently misassigned the nitrogen hyperfine splitting as the beta hydrogen splitting.

DISCUSSION

It is surprising that Kalyanaraman and Mason would publish an a_N value as low as 10.95 G for a DMPO adduct, especially when they use it as a basis to take issue with the work of others. In an extensive review of the literature of nitroxides, Janzen[10] found that a_N of DMPO adducts was lowest in dimethyl formamide (12.8 G) or hexane (12.9 G) and highest in water (14.1 G). Intuition would suggest that the a_N of DMPO adducts in a solvent with a moderately high dielectric constant and a polar carbonyl group, such as ethyl acetate, would be between 12.8 and 14.1 G.

The factors that cause the shift in a_N of nitroxides in polar solvents are well understood. Interactions of the polar solvents with the lone pair electrons of the nitroxide oxygen atom increase its electron affinity, resulting in a redistribution of the π-electrons such that there is maximum unpaired electron density on the nitrogen atom.[14,15] This theoretical prediction is borne out experimentally. For example, Janzen[10] supported the finding of Wajer that a plot of the isotropic nitrogen hyperfine coupling constant was linear with respect to Reichard's solvent polarity parameter. Thus, the a_N of a nitroxide radical can be used to give information about the polarity of the environment in which the probe is found. Trudell et al.[16] measured the decrease in a_N of nitroxide spin labels attached to fatty acids at increasing depths toward the center of a phospholipid bilayer. They interpreted this change as reflecting a decrease in dielectric constant and polarity as the probes were moved from near the phospholipid headgroups into the hydrocarbon interior of the bilayer. A similar study by Griffith et al.[17] used the change in a_N as a function of depth of spin probes in the bilayer as evidence that water penetrated into phospholipid bilayers.

The a_N and $a_{H\beta}$ values calculated from spectra of PBN adducts measured in ethyl acetate by Bösterling and Trudell[1] were: 14.90 and 4.28 G for PBN—OOH and 13.71 and 2.1 G for PBN—OH, respectively. The point made in that paper was that in ethyl acetate, the large differences between the four coupling constants allowed simultaneous quantitation of the two radical adducts without using computer subtraction. In a review of hydroxyl and superoxide adducts of PBN in different solvents, Janzen[10] found that a_N ranged from 13.4 in benzene to 15.98 G in water. The a_N calculated here from the spectrum of PBN—OOH in ethyl acetate by Bösterling and Trudell[1] was 14.90 G, a value intermediate between the extremes of solvent polarity.

Janzen[10] has pointed out that the a_N and $a_{H\beta}$ for a given spin-trap adduct in different solvents should be related by a linear equation of the form $a_{H\beta} = a(a_n) + b$. Kalyanaraman et al.[3] suggested that a_N and $a_{H\beta}$ for DMPO—OH listed in their Table 1 could be related by the equation $a_{H\beta} = 0.2832\ a_N + 10.9$. However, when their computer calculated values for DMPO—OH in ethyl acetate are inserted into this equation, the resulting fit is the poorest among all the solvents tabulated in their Table 1. Even when the values for their spectra as calculated in this paper from Figure 1B are inserted in the same equation, a poor fit is obtained. It is likely that incorrect a_N and $a_{H\beta}$ assignments have led to an incorrect fitting equation.

Similar equations were derived by Janzen et al.[9] for the hydroxyl and superoxide radical adducts of PBN in various solvents (but not including ethyl acetate). Insertion of the $a_{H\beta}$ (2.1 G) and a_N (13.71 G) values calculated from PBN—OH[1] into the appropriate equation $a_{H\beta} = 0.60a_N - 6.5$ gives a result of 1.73 G, a 0.37-G error between observed and predicted. Insertion of the corresponding values of $a_{H\beta}$ (4.28 G) and a_N (14.90 G) calculated for PBN—OOH[1] into the appropriate equation from Janzen, $a_{H\beta} = 1.26a_N - 15.7$, gives a result of 3.07 G, a 1.2-G error between observed and predicted.

CONCLUSIONS

The calculations presented in this paper suggest that both the DMP—OH radical adduct prepared by Kalyanaraman et al. and the PBN—OH radical adduct prepared by Bösterling and Trudell were made by correct synthetic techniques. However, it would appear that the objections of Kalyanaraman et al. to the ethyl acetate extraction technique were based on their unfortunate transposition of a_N and $a_{H\beta}$ in their calculations and their failing to realize that the resulting a_N was not in the range of possiblities. It is hoped that this paper confirms the value of extraction of spin-trap adducts before measurement of their EPR spectra. The stability of the adducts and the ability to observe clear differences between hydroxyl and superoxide radical adducts, without a requirement for computer synthesis and subtraction, would seem to be of considerable value.

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REFERENCES

1. Bösterling B, Trudell JR. Spin trap evidence for production of superoxide radical anions by purified NADPH-cytochrome P450 reductase. Biochem Biophys Res. Commun. 1981; 98:569–575.
2. Kalyanaraman B. Detection of toxic free radicals in biology and medicine. In: Hodgson E, Bend JR, Philpot RM, eds. Reviews in biochemical toxicology 4. New York: Elsevier Biomedical; 1982: 73–139.
3. Kalyanaraman B, Mottley C, Mason RP. On the use of organic extraction in the spin-trapping technique as applied to biological systems. J. Biochem Biophys Methods. 1984; 9:27–31.
4. Korchak HM, Eisenstat BA, Hoffstein ST, Dunham PB, Weissmann G. Anion channel blockers inhibit lysosomal enzyme secretion from human neutrophils without affecting generation of superoxide anion. Proc Natl Acad Sci USA. 1980; 77:2721–2725.
5. McCord JM. Oxygen-derived free radicals in postischemic tissue injury. N Eng J Med. 1985; 321:159–163.
6. Harbour JR, Chow V, Bolton JR. An electron spin resonance study of the spin adducts of OH and HO2 radicals with nitrones in the ultraviolet photolysis of aqueous hydrogen peroxide solutions. Can J Chem. 1974; 52:3549–3553.
7. Janzen EG, Wang YY, Shetty RV. Spin trapping with alpha-pyridyl 1-oxide N-tert-butyl nitrones in aqueous solutions: a unique electron spin resonance spectrum for the hydroxyl radical adduct. J Am Chem Soc. 1978; 100:2923–2935.
8. Sugiura Y, Kikuchi T. Formation of superoxide and hydroxyl radicals in iron(II)-bleomycin-oxygen system: electron spin resonance detection by spin trapping. J Antibiot (Tokyo). 1978; 31:1310–1312.
9. Janzen EG, Nutter DE Jr, Davis ER, Blackburn BJ, Poyer JL, McCay PB. On spin trapping hydroxyl and hydroperoxyl radicals. Can J Chem. 1978; 56:2237–2242.
10. Janzen EG. A critical review of spin trapping in biological systems. In: Pryor, WA, Ed. Free radicals in biology, New York: Academic Press; 1980: 115–186.
11. Finkelstein E, Rosen GM, Rauckman EJ. Production of hydroxyl radical by decomposition of superoxide spin-trapped adducts. Mol Pharmacol. 1982; 21:262–265.
12. Zs.-Nagy I, Floyd RA. Hydroxyl free radical reactions with amino acids and proteins studied by electron spin resonance spectroscopy and spin-trapping. Biochim Biophys Acta. 1984; 790:238–250.
13. Floyd RA, Henderson R, Watson JJ, Wong PK. Use of salicylate with high pressure liquid chromatography and electrochemical detection (LCED) as a sensitive measure of hydroxyl free radicals in adriamycin treated rats. J Free Rad Biol Med. 1986; 2:13–18.
14. Nordio PL. General magnetic resonance theory. In: Berliner LJ, ed. Spin labeling: theory and practice. New York: Academic Press; 1976: 5–52.
15. Carrington A, McLachlan AD. Introduction to magnetic resonance. New York: Harper and Row; 1967: 72–85.
16. Trudell JR, Hubbell WL, Cohen EN. The effect of two inhalation anesthetics on the order of spin-labeled phospholipid vesicles. Biochim Biophys Acta. 1973; 291:321–327.
17. Griffith OH, Dehlinger PJ, Van SP. Shape of the hydrophobic barrier of phospholipid bilayers: evidence for water penetration in biological membranes. J Mem Biol. 1974; 15:159–192.