SPIN TRAPPING ARTIFACTS DUE TO THE REDUCTION OF NITROSO SPIN TRAPS

Carolyn MOTTLEY†, B. KALYANARAMAN and Ronald P. MASON

Laboratory of Environmental Biophysics, National Institute of Environmental Health Sciences, PO Box, 12333 Research Triangle Park, NC 27709, USA

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1. Introduction

The use of nitroso compounds as spin traps in chemical and biological systems has become widespread, and many interesting, unstable radicals have been trapped in this manner [1–4]. However, as we will show, extreme caution must be taken when trapping radicals in a reducing medium.

The technique of spin trapping involves producing the unstable free radical of interest and allowing it to react with a diamagnetic compound (the spin trap, usually a nitroso compound or a nitrone) to form a relatively stable free radical (the spin adduct) which can be observed by electron spin resonance (ESR). Observation of a stable free radical, however, is no guarantee that the radical of interest has been trapped. Spectral artifacts can arise due to nitroxide impurities or nucleophilic addition to nitroso compounds followed by oxidation to the nitroxide [5]. We report another way in which spin trapping artifacts may arise, that of direct reduction of a nitroso spin trap to a nitroxide free radical.

Though nitrose spin traps have been used extensively [1–4], apparently not much attention has been given to the possibility of this reduction, though reduction of the spin adduct has been proposed as a decay process [3,6]. The reduction of the spin trap itself is particularly important in biological systems because of the presence of endogenous reducing agents such as ascorbate.

Abbreviations: ESR, electron spin resonance; TBNB, 2,4,6-tri-*tert*-butylnitrosobenzene

† Permanent address: Department of Chemistry, Luther College, Decorah IA 52101, USA

2. Materials and methods

The spin traps nitrosobenzene and 2,4,6-tri-*tert*-butyl-nitrosobenzene were obtained from Aldrich Chemical Co. Ascorbic acid and epinephrine were obtained from Sigma Chemical Co. The drug dithranol was a gift from August C. Stiefel Research Institute (Oak Hill NY). Nitrosobenzene (5 mM) was prepared in Tris buffer (0.15 M, pH 7.4) by stirring overnight. ESR measurements were made on a Varian century series E-104A spectrometer equipped with a TM_{100} cavity. The g-values were determined using Fremy's salt as a secondary standard ($g = 2.0055$) in a capillary tube attached to the side of the flat cell containing the spin trap solution.

3. Results and discussion

It has been postulated that the active form of the drug dithranol (1,8,9-trihydroxyanthracene), used in the topical treatment of psoriasis, is free radical in nature. In an attempt to prove the existence of a free radical intermediate in the autoxidation of dithranol, the entrapment of the 1,8-dihydroxy-9-anthrone radical with the spin trap 2,4,6-tri-*tert*-butylnitrosobenzene (TBNB) was attempted [6]. A stable radical was observed [7] with the hyperfine coupling constants $a^N = 12.0$ G, $a_\beta^H = 13.6$ G and $a_m^H = 0.75$ G and a g-value of 2.0066. The observed radical was claimed to be the 1,8-dihydroxy-9-anthrone adduct with TBNB, although studies of radicals similar to the 1,8-dihydroxy-9-anthrone adduct have β-proton hyperfine coupling constants an order of magnitude smaller [8] than that reported in [6]. We propose that rather than adduct formation, the authors observed the reduction of the spin trap TBNB by the reducing agent dithranol,

and rather than a β-proton giving rise to the hyperfine splitting constant of 3.6 G, it is the proton attached to the nitrogen which causes this splitting.

Fig.1A shows the ESR spectrum seen by reducing TBNB with ascorbic acid in pyridine. This spectrum is essentially identical to the spectrum observed [6] with hyperfine splitting constants a^N = 12.3 G, a^H_{NH} = 13.4 G and a^H_m = 0.75 G and a g-value of 2.006. Using methanol as the solvent the same hydronitroxide radical was formed with the hyperfine splitting constants a^N = 12.7 G, a^H_{NH} = 15.0 G, and a^H_m = 0.90 G in excellent agreement with reported values for this radical [9]. The spectrum shown in fig.2A resulted from the addition of a small amount D_2O to the pyridine solution of TBNB and ascorbic acid. This spectrum clearly shows the exchange of a proton for a deuteron indicating an exchangeable hydrogen on the nitrogen. The hyperfine splitting constants obtained through computer simulation are a^N = 12.3 G, a^D_{ND} = 2.05 G

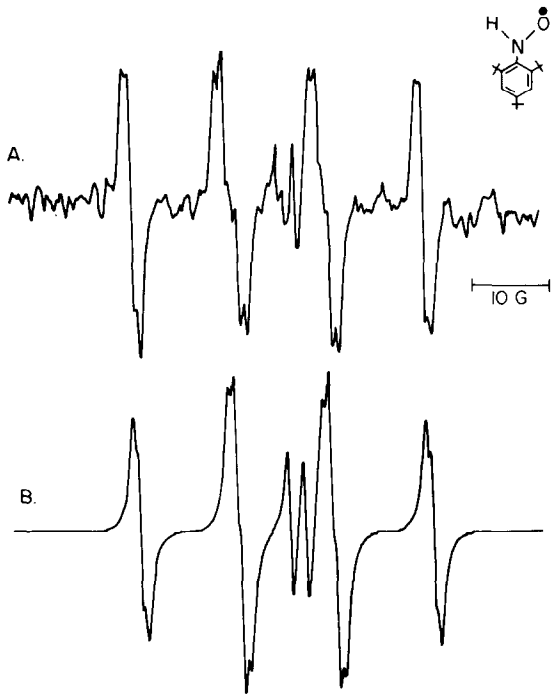

Fig.1. (A) ESR spectrum obtained from 20 mM TBNB and 10 mM ascorbic acid in pyridine. The doublet in the center of the spectrum is due to the ascorbate semidione radical. Microwave power was 20 mW; modulation amplitude was 0.41 G. (B) Computer simulation of the above spectrum with a Lorentzian line shape with a peak-to-peak width of 0.8 G. Hyperfine values for the nitroxide radical are given in the text. The g-value shift for the ascorbate semidione is 1.45 G down field from the nitroxide.

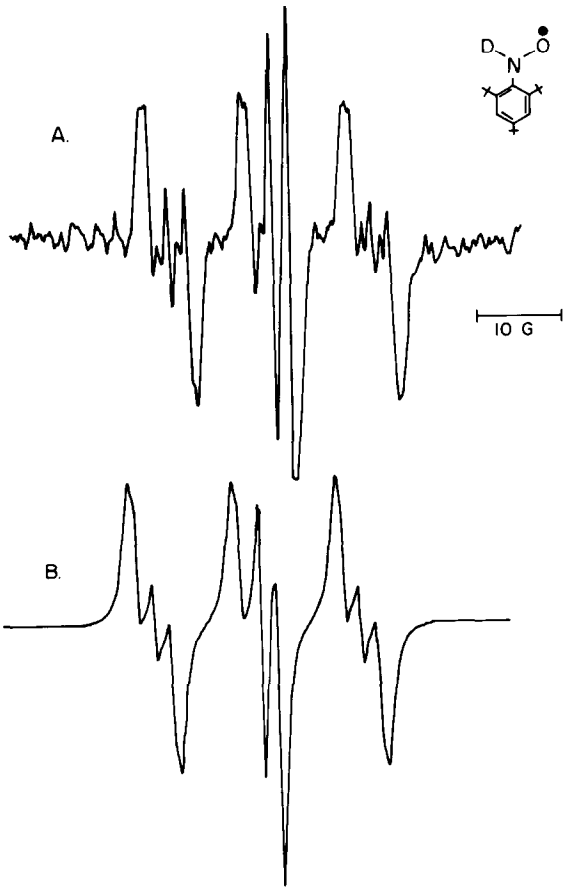

Fig.2. (A) ESR spectrum obtained from 20 mM TBNB, 10 mM ascorbic acid, amd 3 M D_2O in pyridine. The doublet in the center is due to the ascorbate radical. Microwave power was 20 mW; modulation amplitude was 0.41 G. (B) Computer simulation of above spectrum with a Lorentzian line shape with a peak-to-peak width of 0.8 G. Hyperfine values are given in the text.

(in accordance with the gyromagnetic ratios $a^D = (\gamma_D/\gamma_H)a^H$) and a^H_m = 0.75 G. Apparently dithranol merely reduced TBNB to its hydronitroxide and no adduct formation occurred, contrary to [6].

Similarly, reduction of the spin trap 2-methyl-2-nitrosopropane was incorrectly claimed to be the adduct of the spin trap and the hydroxyl radical produced by the Fenton reaction [10]. It has been shown that the ESR spectrum observed in this case could arise from the reduction of the spin trap by the ferrous ion resulting in *tert*-butyl hydronitroxide [11]. The same hydronitroxide is formed by reduction of 2-methyl-2-nitrosopropane with sodium borohydride or with microsomal incubations containing NADPH [6, 11].

A third example of spin trap reduction is the reduction of nitrosobenzene to the corresponding hydronitroxide by a variety of reducing agents. The spectrum shown in fig.3 (a^N = 10.63 G, a^H_{NH} = 13.15 G, $a^H_o = a^H_p$ = 3.26 G, a^H_o = 3.61 G, a^H_m = 1.10 G) was a result of reduction by ascorbate. The same spectrum is formed by reduction with catechols (such as epinephrine), zinc [12], or titanous ion [13]. The reduction of nitrosobenzene to a free radical by ascorbate [14] or epinephrine [15] has been reported. The ESR spectrum obtained with these biological reducing agents had not been analyzed, but was presumed to be of the phenyl hydroxylamine free radical with the structure PhNOH [14, 15].

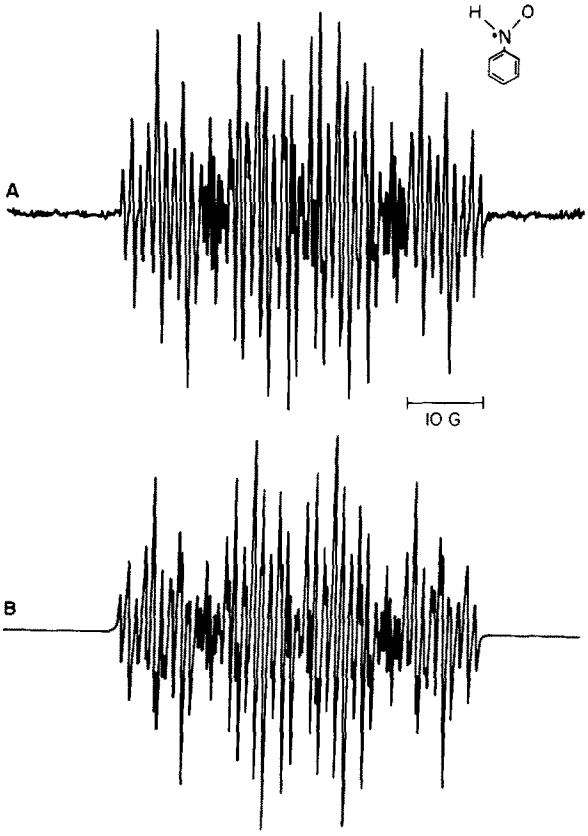

Fig.3. (A) ESR spectrum of 5 mM nitrosobenzene and 2.5 mM ascorbic acid in Tris buffer at pH 7.4. Hyperfine values are a^N = 10.63 G, a^H_{NH} = 13.15 G, $a^H_o = a^H_p$ = 3.26 G, a^H_o = 3.61 G, a^H_m = 1.10 G [16]. Microwave power was 20 mW; modulation amplitude was 0.33 G. The same ESR spectrum is obtained when ascorbate is replaced by 2.5 mM epinephrine. (B) Computer simulation of above spectrum using a Lorentzian line shape with peak-to-peak width of 0.3 G.

We show that 3 widely used nitroso spin traps, 2-methyl-2-nitrosopropane, 2,4,6-tri-*tert*-butylnitrosobenzene and nitrosobenzene, undergo reduction to the corresponding nitroxide under conditions that are often used in biological spin-trapping studies. Since nitroso compounds are easily reduced, it is imperative that caution be exercised when using nitroso spin traps particularly in biological or chemical redox systems. One must take a great deal of care in showing that spin adduct formation, rather than simple reduction of the nitroso spin trap, is responsible for the observed ESR spectrum. To our knowledge nitrone spin traps are not as prone to reduction as nitroso spin traps, as would be expected from their generally higher reduction potentials.

References

[1] Perkins, M. J. (1970) in: Essays on Free-Radical Chemistry, spec. publ. no. 24, 97. Chem. Soc., London.
[2] Lagercrantz, C. (1971) J. Phys. Chem. 75, 3466–3475.
[3] Evans, C. A. (1979) Aldrichim. Acta. 12, 23–29.
[4] Janzen, Edward G. (1980) in: Free Radicals in Biology (Pryor, W. A. ed) vol. 4, pp. 115–154, Academic Press, New York.
[5] Forrester, A. R. and Hepburn, S. P. (1971) J. Chem. Soc. 701–703.
[6] Kalyanaraman, B., Mason, R. P., Perez-Reyes, E., Chignell, C. F., Wolf, C. R. and Philpot, R. M. (1979) Biochem. Biophys. Res. Commun. 89, 1065–1072.
[7] Martinmaa, J., Vanhala, L. and Mustakallio, K. K. (1978) Experientia 34, 872–873.
[8] Terabe, S. and Konaka, R. (1972) J. Chem. Soc. Perkin II, 2163–2172.
[9] Shiotani, M., Murabayashi, S. and Sohma, J. (1976) Int. J. Radiat. Phys. Chem. 8, 483–495.
[10] Lai, C. A. and Piette, L. H. (1979) Tetrahedron Lett. 775–778.
[11] Kalyanaraman, B., Perez-Reyes, E. and Mason, R. P. (1979) Tetrahedron Lett. 4809–4812.
[12] Ayscough, P. B., Sargent, F. P. and Wilson, R. (1966) J. Chem. Soc. (B), 903–906.
[13] Gutch, C. J. W. and Waters, W. A. (1964) Proc. Chem. Soc. 230.
[14] Bigalow, J. E., Jacobson, B., Varnes, M. and Koch, C. (1978) Photochem. Photobiol. 28, 869–876.
[15] Sridhar, R. (1980) Radiat. Res. 83, 466 abst.
[16] Forrester, A. R. and Neugebauer, F. A. (1979) in: Landolt-Börnstein Zahlewerte und Funktionen aus Naturwissenshaften und Technik (Fischer, H. and Hellwege, K.-H. ed) Gruppe II: Atom- und Molekularphysik, vol. 9/pt c 1 Organische Radikals mit N als Zentralatom und Nitroxid- Radikale p. 539, Springer-Verlag, Berlin, New York.