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THE REDUCTION OF NITROSO-SPIN TRAPS IN CHEMICAL AND BIOLOGICAL SYSTEMS. A CAUTIONARY NOTE

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Abstract: The $NaBH_4$ or enzymatic reduction of 2-methyl-2-nitrosopropane results in a four-line ESR spectrum due to t-butyl hydronitroxide. This spectrum is identical to a previously reported ESR spectrum (C.S.Lai and L.H. Piette, Tet. Letters, 9, 775 (1979)) obtained during a Fenton reaction using 2-methyl-2-nitrosopropane, which had been assigned to the hydroxyl radical adduct. This note presents evidence that this four line spectrum can arise from the chemical reduction of the spin trap.

The use of nitrones and nitroso compounds as spin-traps[1] in biological systems[2] has become widely applied. Two commonly used nitroso spin-traps are 2-methyl-2-nitrosopropane and nitrosobenzene, while phenyl-t-butyl nitrone and 5,5-dimethyl-1-pyrroline-1-oxide are among the most widely used nitrone spin-traps. Rat liver microsomes catalyze many electron transfer reactions in NADPH-dependent oxidation and reduction reactions. Several investigators have applied spin-trapping for the detection of superoxide and hydroxyl radicals in microsomal systems[2]. Recently, Lai and Piette claimed that the hydroxyl radical produced by the Fenton reaction was spin-trapped using 2-methyl-2-nitrosopropane ($a^N=a^H=14.4G$).[3] They explained the unexpectedly large hyperfine splitting constant of the OH hydrogen as due to hydrogen bonding between the hydrogen atom of the hydroxyl group and the oxygen atom of the nitroxide. In this note, we present evidence that the ESR spectrum obtained by Lai and Piette[3] could arise from the chemical reduction of the spin trap by ferrous iron resulting in t-butyl hydronitroxide. We would like to emphasize that the reduction of 2-methyl-2-nitrosopropane also occurs in microsomal incubations containing NADPH.

The chemical reduction in air of 2-methyl-2-nitrosopropane was carried out by the addition of one equivalent of $NaBH_4$ to two equivalents of 2-methyl-2-nitrosopropane in a Tris buffer (0.15M) pH 7.5, (equation 1).

$$H_3C - \underset{CH_3}{\overset{CH_3}{C}} - N=O \xrightarrow[H_2O]{NaBH_4} H_3C - \underset{CH_3}{\overset{CH_3}{C}} - \underset{H}{N} \dot{-} O \qquad (1)$$

t-butyl hydronitroxide

The ESR spectrum of t-butyl hydronitroxide is shown in Fig. 1A ($a^N=a^H=14.4G$). The first product of $NaBH_4$ reduction is presumably the corresponding hydroxylamine. The t-butyl hydronitroxide

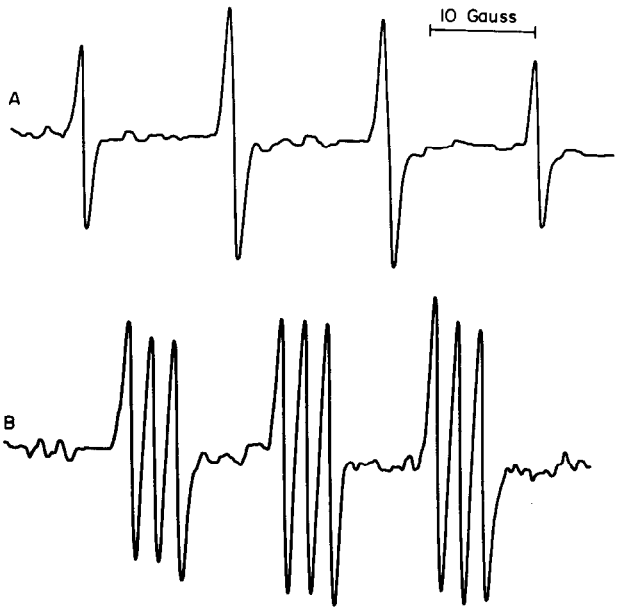

Fig. 1A. The first derivative ESR spectrum of t-butyl hydronitroxide obtained upon reduction of MNP with $NaBH_4$ in H_2O.

Fig. 1B. The first derivative ESR spectrum of t-butyl deuteronitroxide obtained upon reduction of MNP with $NaBH_4$ in D_2O.

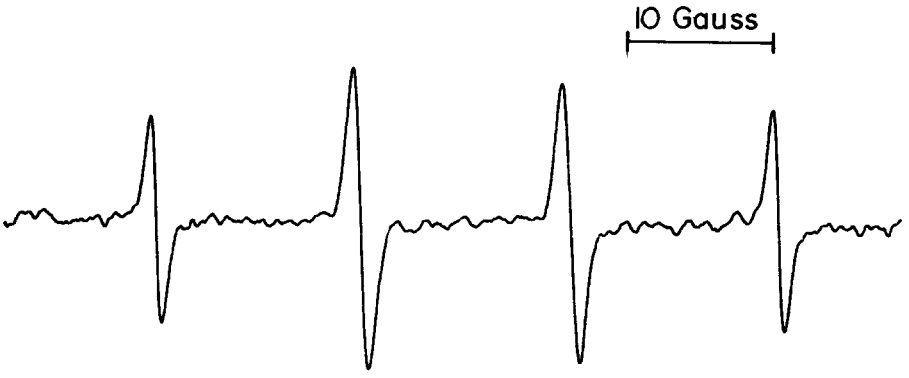

Fig. 2. The first derivative ESR spectrum of t-butyl hydronitroxide obtained upon reduction of MNP with microsomes and NADPH-generating system. The incubation mixture consisted of 0.02 M MNP in a Tris buffer (0.15 M) pH 7.5, 4 mg/ml microsomal protein 3.6 mg/ml glucose-6-phosphate, 0.66 mg/ml $NADP^+$ and 1.32 units/ml of glucose-6-phosphate dehydrogenase.

could form by air oxidation of the t-butyl hydroxylamine.[4] Alternatively, t-butyl hydronitroxide may form by the comproportionation of t-butyl hydroxylamine and 2-methyl-2-nitrosopropane. Note that the intensity of the four lines is not equal to 1:2:2:1, due to the fact that the proton and nitrogen hyperfine couplings are not exactly equal. Fig. 1B shows the ESR spectrum of t-butyl deuteronitroxide (a^N=14.0 G, a^D=2.2 G). From the experimentally observed a^H, an expected value of a^D=2.2 G can be calculated using the ratio of the nuclear moments and the assumption that the spin density is unchanged at the nucleus. It should be mentioned that the previously reported ESR parameters for t-butyl hydronitroxide in isopropanol are different from the present work (a^N=13.2 a^H=11.8).[5] The addition of isopropanol to the reaction mixture (MNP + $NaBH_4$ solution in Tris buffer) decreased the nitrogen and hydrogen coupling and hence the observed difference could be ascribed to a solvent effect. Irradiation of an argon-saturated aqueous solution of 2-methyl-2-nitrosopropane also gives t-butyl hydronitroxide ($a^N=a^H$=14.4G).[6]

We carried out the reduction of 2-methyl-2-nitrosopropane with rat liver microsomes and NADPH in air, and observed the four line spectrum due to t-butyl hydronitroxide (Fig. 2). This product of spin trap reduction should not be confused with a radical adduct. The concentration of this free radical increased for over 30 min. This free radical accumulated in the presence of catalase (30,000 units/ml), but not in presence of superoxide dismutase (30 μg/ml). Catalase should prevent the formation of any hydroxyl radical adduct formed from hydrogen peroxide. Inhibition by superoxide dismutase is consistent with superoxide oxidation of the t-butyl hydroxylamine reduction product[4] or reduction of the 2-methyl-2-nitrosopropane by superoxide. In situ irradiation of an aqueous solution of 2-methyl-2-nitrosopropane gave a nitroxide (a^N=28.0G) suggested to be the hydroxyl radical adduct.[6] All these results taken together make the assignment of the nitroxide observed from a Fenton reaction to a hydroxyl radical adduct[3] appear to be in error.

Experimental:

2-methyl-2-nitrosopropane (1 mg/ml) was prepared in Tris buffer (0.15 M, pH 7.5) in the dark by stirring overnight in a cold box. ESR measurements were made at room temperature with a Varian century series E-109 spectrometer equipped with a TM_{110} cavity. The microwave power was 20 mW and the magnetic field modulation was 1.0 G.

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