Free Rad. Res. Comms., Vol. 6, No. 1, pp. 19–28
Reprints available directly from the publisher
Photocopying permitted by license only

AN ARTIFACT IN THE ESR SPECTRUM OBTAINED BY SPIN TRAPPING WITH DMPO

KEISUKE MAKINO*, HIROMASA IMAISHI, SHUUJI MORINISHI, TAKUYA HAGIWARA, TAMIO TAKEUCHI and AKIRA MURAKAMI

Department of Polymer Science and Engineering, Faculty of Textile Science, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606, Japan

and

MASATOSHI NISHI

Faculty of Pharmaceutical Science, Setsunan University, Nagaotouge-cho, Hirakata, Osaka 573-01, Japan

(Received May 10, 1988; in final form October 1, 1988)

In order to overcome a common problem in spin trapping with high concentrations of 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) where ESR spectra are obtained which include an unidentified set of lines composed of a triplet of doublets, commercial DMPO was analyzed for its impurities by high-performance liquid chromatography, mass spectrometry, and nuclear magnetic resonance spectroscopy. It has been determined that this undesirable ESR spectrum is due to an impurity included in the spin trap. This compound has been assigned to the hydroxylamine which is a DMPO-derivative having an epoxy ring located at the 2 and 3 positions.

KEY WORDS: Spin trapping, DMPO, DMPO impurity

INTRODUCTION

As the interest in free radical formation in living systems increases,[1,2] usage of spin trapping has increased.[3,4] In particular, spin trapping with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO)[5,6] has been used exclusively for this purpose because a large amount of the spin trap is readily dissolved in aqueous solutions to detect free radicals at low concentrations in such systems.

In such a case, ESR spectra obtained when commercial DMPO is used are always accompanied by an unidentified set of lines consisting of a triplet of doublets.[7] These undesirable ESR lines disturb accurate analysis of the spectra obtained by this method. In particular, quantitative measurements of free radicals by this method are unreliable because the intensities of this signal influence those of others appearing in the spectrum. The identity of the species producing these ESR lines has not been studied in detail before.

The present study was, therefore, undertaken to characterize the species leading to these ESR lines so that one can analyze the data obtained without the disturbance of these lines.

* To whom correspondence should be addressed.

EXPERIMENTAL SECTION

DMPO was purchased from Aldrich Chemical Co. (Wisconsin, USA) and purified DMPO was a gift from Mitsui-Toatsu Co. (Tokyo, Japan). Egg lecithin phosphatidylcholine (LPC) and other chemicals were purchased from Nakarai Chemicals (Kyoto, Japan). Serum was obtained from a New Zealand white rabbit weighing 2.5 kg.

ESR spectra were obtained by spin trapping in rabbit serum and in the system consisting of LPC (18 mg) dispersed in 60 μL of phosphate buffer (60 mM, pH 7.8) containing DMPO (15 μL) and N,N-bis(2-[bis(carboxymethyl)amino]ethyl)glycine (1 mM),[8] both of which were allowed to stand for 10 min at 37 °C.[7] The ESR spectrometer used was the JEOL Model PE-3X (X-band,, 100 kHz field modulation, JEOL, Tokyo, Japan). The hyperfine splitting (hfsc) constants were measured by Mn^{2+} in MgO as a reference.

A high-performance liquid chromatograph utilized was HLC-803D (Tosoh, Tokyo, Japan) equipped with a UV-8000 detector (Tosoh, tuned to 260 nm). Commercial DMPO was separated on an ODS column (TSKgel ODS-120T, 4 mm × 15 cm, Tosoh) using methanol/water (5/95) as an eluent. The flow rate was 0.5 mL/min.

A mass spectrometer used was JMS DX-300 (JEOL, Tokyo, Japan). For the measurement, electron impact ionization was utilized.

A nuclear magnetic resonance spectrometer utilized was JNM GX-400 (400 MHz, JEOL). The measurement was carried out in DMSO-d_6. The reference was tetramethylsilane.

Sonolysis of DMPO solutions was carried out in a Bransonic 52 ultrasonic bath (50 kHz, Branson Instruments Co., U.S.A.) with Ar-bubbling.[9]

All the experiments were carried out in the dark.

RESULTS AND DISCUSSION

When DMPO (20 μL) was added to rabbit serum (200 μL) and the system was incubated for 10 min at 37 °C, the ESR spectrum represented in Figure 1a was obtained. With the hyperfine splitting constants listed in Table 1, as depicted in Figure 1b, computer simulation reproduced this spectrum. This spectrum is composed of three different sets of lines, as indicated by the stick diagrams in Figure 1c. Although as represented in Figure 1c, two of the ESR sets of lines in Figure 1a are attributed to the spin adducts of hydrogen atoms (DMPO-H) and hydroxyl radicals (DMPO-OH), the third component having six ESR lines has remained unidentified.[7] We have already found that the intensities of DMPO-H and DMPO-OH are influenced by the production of "Six-lines" (Details of this result will be reported elsewhere).

In order to determine whether Six-lines is due to the impurity included in commercial DMPO, the following experiments were carried out. First, sonolysis of various concentrations of aqueous DMPO solutions was performed with Ar-bubbling.[9] It is known that by sonication, water molecules are cleaved homolytically to generate hydrogen atoms and hydroxyl radicals.[10–12] The results obtained are summarized in Figure 2, where the ESR intensities of the three ESR spectra are plotted against the DMPO concentrations of the solutions. As is seen in the figure, only the intensity of Six-lines increased as a function of the DMPO concentration while those of the other two did not. Since it has been reported that even at concentrations as low as 10 mM,

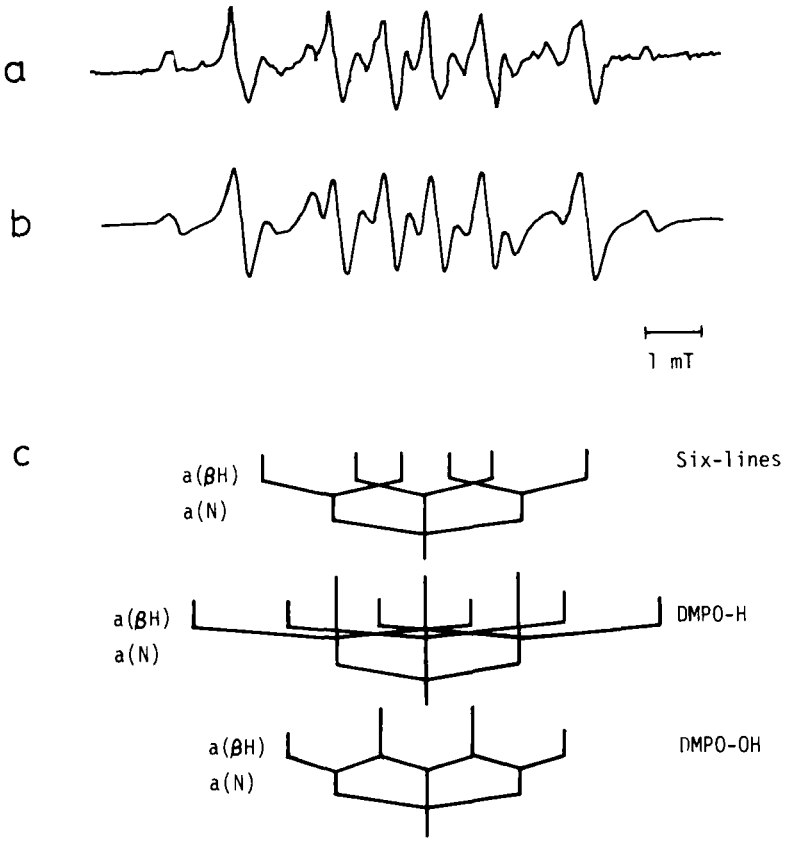


FIGURE 1 ESR spectra obtained (a) from rabbit serum (200 μL) incubated in the presence of DMPO (20 μL) for 10 min at 37 °C and (b) by computer-simulation using the hfsc values listed in Table 1. The spectrum in (a) is composed of the three different ESR patterns, as indicated by the stick diagrams in (c).

the trapping efficiency of DMPO is already high in aqueous solutions,[11] this increase in the intensity of the Six-lines spectrum is believed to be unusual. Since the intensity of Six-lines was low at low DMPO concentrations where the concentration of the impurity is also low while it was high at the high concentrations where that of the impurity is high, it is implied that Six-lines arises from an impurity in DMPO. Secondly, the change in the ESR intensities of the three components was investigated

TABLE I
The hfsc values (mT) obtained from the ESR spectrum of rabbit serum depicted in Figure 1a.

	a(N)	a(μH)
DMPO-H	1.55	2.34
DMPO-OH	1.43	1.43
Six-lines	1.61	2.45

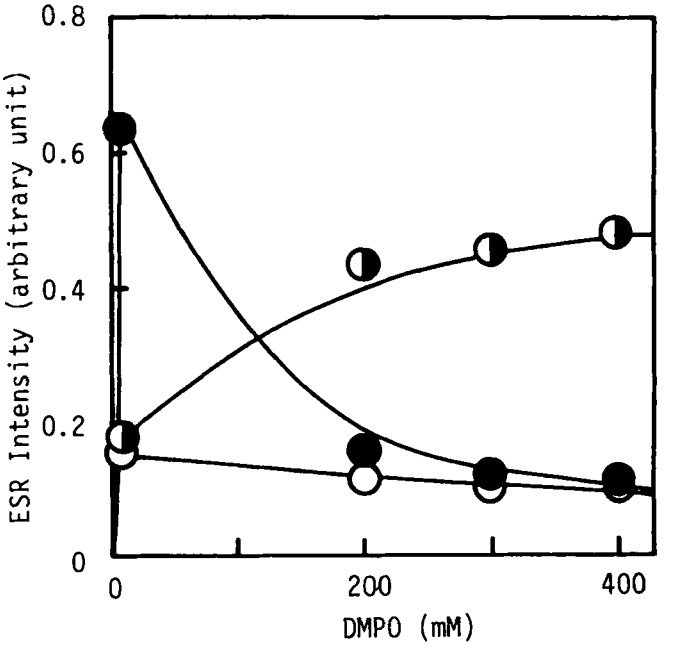


FIGURE 2 Effect of the DMPO concentration on the ESR intensities of aminoxyl radicals produced by sonolysis in aqueous DMPO solutions. ○; DMPO-H, ●; DMPO-OH, ◑; Six-lines.

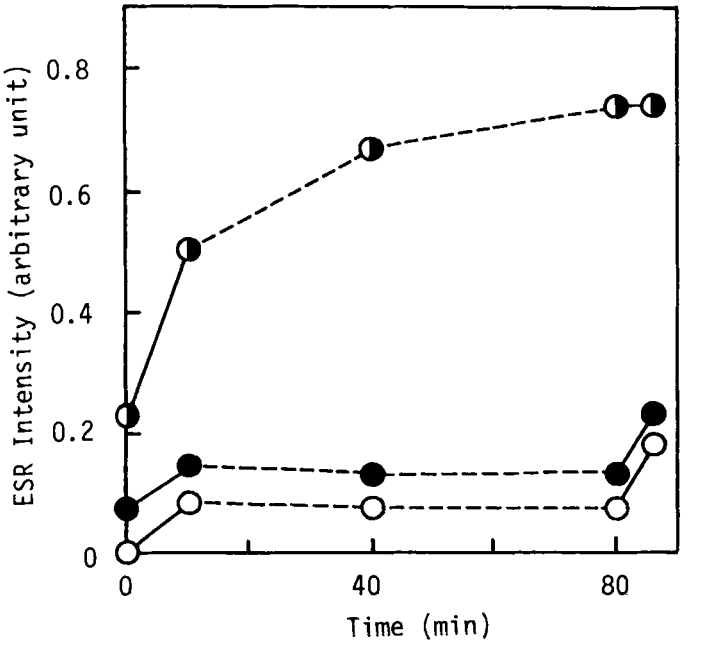


FIGURE 3 Change in the ESR intensities of aminoxyl radicals produced in the LPC system incubated for 10 min at 37 °C under (——) aerobic and (- - - -) unaerobic conditions. ○; DMPO-H, ●; DMPO-OH, ◑; Six-lines.

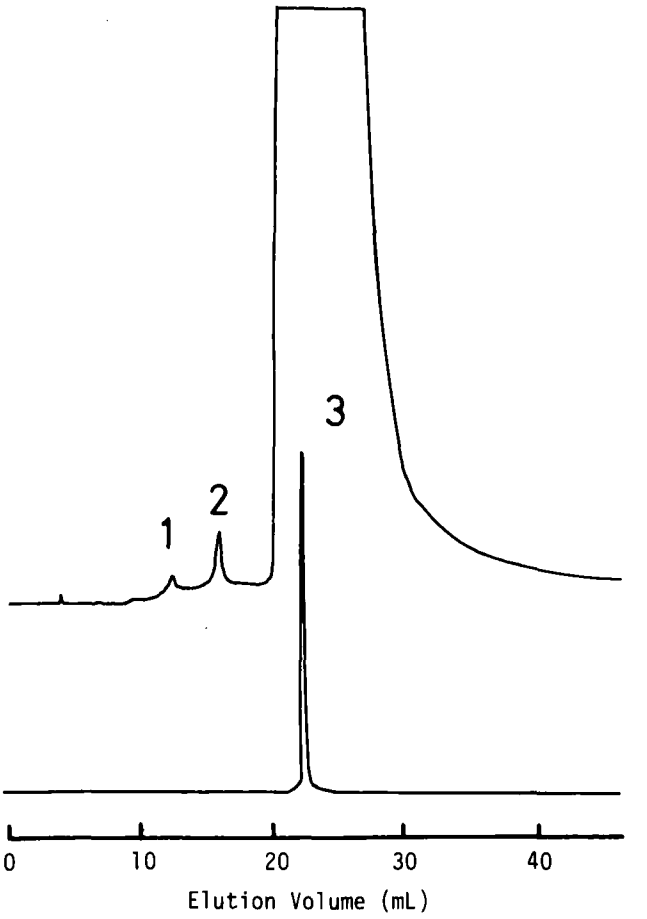


FIGURE 4 Separation of commercial DMPO by high-performance liquid chromatography. Conditions: column, TSKgel ODS-120T; eluent, methanol/water (5/95); flow rate, 0.5 mL/min; detection, 260 nm.

in the LPC dispersion system (see Experimental Section). As represented in Figure 3, under aerobic conditions, the intensities of DMPO-H and DMPO-OH increased while under unaerobic conditions, they did not. On the other hand, Six-lines increased in intensity even under unaerobic conditions. Consequently, assuming that Six-lines is due to an impurity in DMPO, it seems to be reasonable that this impurity is a compound oxidized readily to give an aminoxyl radical (nitroxide radical) producing Six-lines. A hydroxylamine is a candidate for this impurity compound because it has been known that hydroxylamines are formed from nitrone compounds by known path ways and that they are readily converted to aminioxyl radicals by oxidation.[13]

In order to identify the impurity compound, chromatographic separation of commercial DMPO was carried out. Although it was found that in gas chromatographic columns, even in inert columns, conversion of the impurities and even DMPO to other molecules occurred, by high-performance liquid chromatography peaks due to the

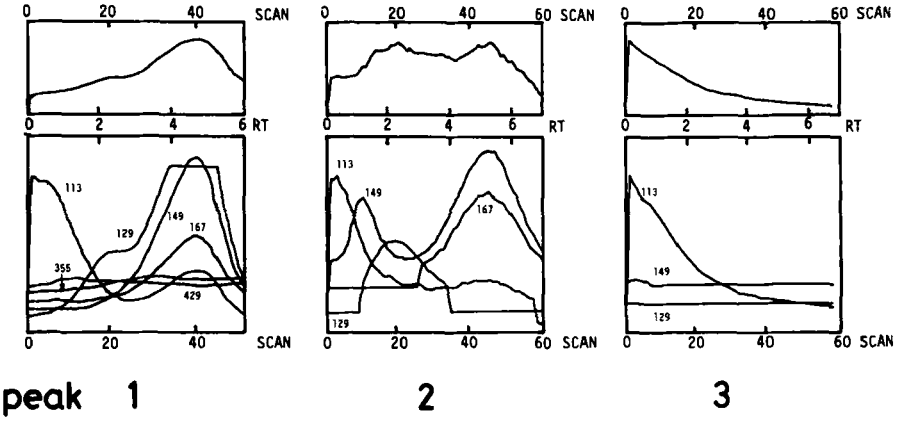
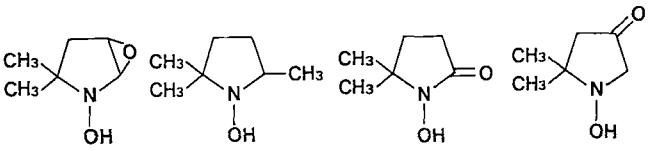


FIGURE 5 Mass chromatograms obtained from the fractions of Peak 1, 2 and 3 in Figure 4. Conditions: ionization, electron impact; ionizing energy, 20 eV; temperature of sample probe, raised from 20 °C at the rate of 16 °C/min.

impurities could be obtained. With an ODS column and under isocratic conditions with the solvent of methanol/water (5/95), a chromatogram shown in Figure 4 was obtained, where three peaks appear. Peak 3 was readily determined by co-chromatography to be due to DMPO. Peak 1 and 2 appearing in an enlarged chromatogram are due to the impurities. All the peaks were fractionated and subsequently concentrated by vacuum-evaporation at room temperature.

The samples obtained from these three chromatographic peaks were analyzed by mass spectrometry where electron impact ionization was applied and the temperature of the sample probe was increased gradually from 20 °C at the rate of 16 °C/min. The ionizing energy applied was 20 eV. The resultant mass-chromatograms are shown in Figure 5. In the chromatogram obtained from Peak 1, useful information could not be obtained because all the peaks except that of m/z 113 (due to DMPO) eluted at the same position (ca. Scan No. 40) indicating that all the peaks may be due to the cleavage of large molecules, presumably that of m/z 429. The fraction of Peak 3 gave only one peak due to DMPO (m/z 113). From the fraction of Peak 2, informative results were obtained. As is seen in the figure, the independent peak of m/z 129 is observed while other peaks eluted at the same position (ca. Scan No. 50).

DMPO derivatives, which have the molecular weight of 129, must have one additional oxygen atom and hydrogen. Since it has been known that hydroxylamines are readily converted to aminoxyls by oxidation,[13] a possible compound is the hydroxylamine, which is a DMPO analogue and has molecular weight of 129. A few likely possibilities are listed below.



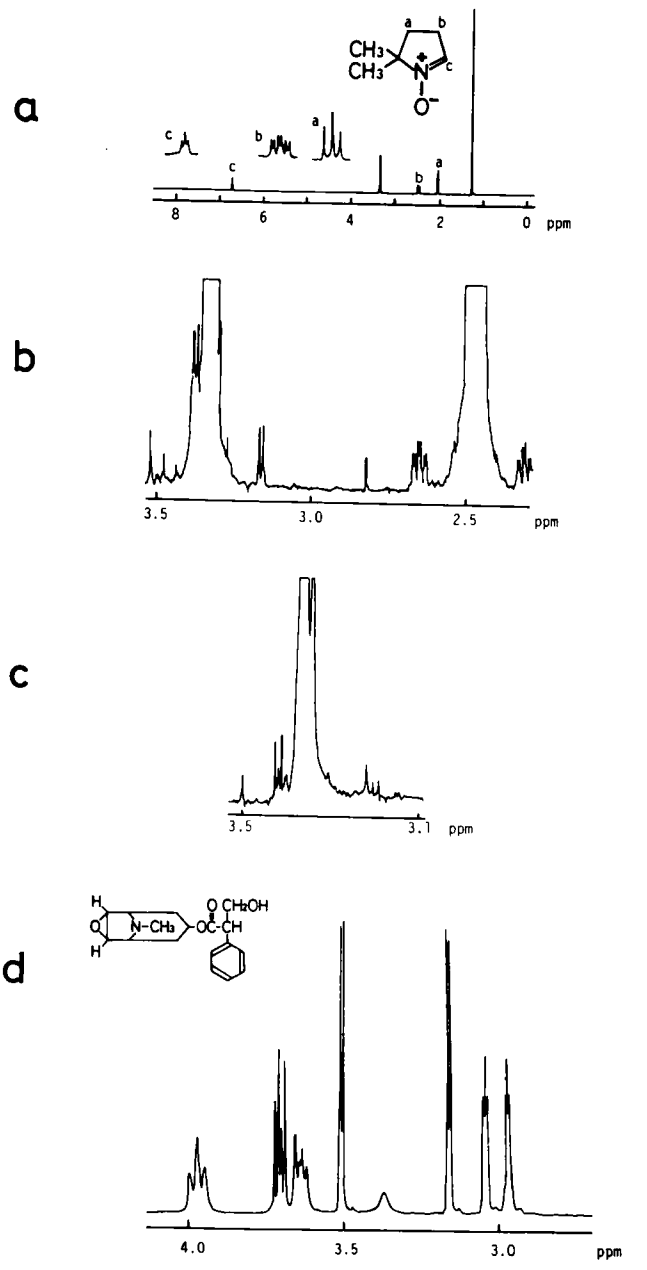


FIGURE 6 400 MHz ^{1}NMR spectra obtained from (a) and (b) commercial DMPO, (c) purified DMPO, and (d) scopolamine. Solvent, DMSO-d_6. Reference, tetramethylamine.

The compound III is readily ruled out since the hfsc values of this spin adduct have been reported to be a(N) = 0.71 mT and a(βH) = 0.42 mT.[14] Also, the compound IV is ruled out because the aminoxyl radical converted from this hydroxylamine must produce a triplet of triplets.

In order to obtain more informations for the assignment, ^{1}H-NMR spectroscopy (400 MHz) was applied to commercial DMPO in DMSO-d_6. The resulting spectrum is represented in Figure 6. The NMR spectrum depicted in Figure 6a shows the proton signals of DMPO. When the free induction decay was accumulated 10,000 times and subsequently Fourier-transformed, at the base line, the signals due to the impurities appeared, as shown in Figure 6b. Although the full assignment for the signals of the

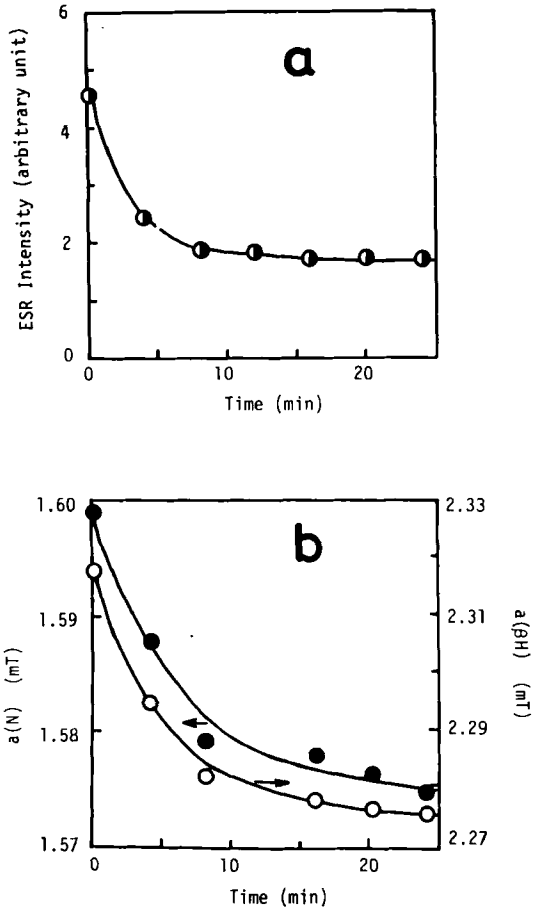


FIGURE 7 Progressive change (a) in the ESR intensity (-◑-) and (b) in the apparent hfsc values (a(N), -●- and a(βH), -○-) as a function of storage period, obtained for a doublet of triplets appearing in the ESR spectrum, which was obtained by the illumination (260 nm) of an aqueous DMPO solution (10 mM, 28 μL) containing DMSO (0.5 μL) and H_2O_2 (1%, 10 μL) which was followed by immediate addition of DMPO (15 μL) and LPC (18 mg) and by subsequent vigorous agitation. This triplet of doublets was produced by superposition of the ESR spectrum of a DMPO adduct of methyl radical and Six-lines. As shown in (a), the former disappeared in ca. 10 min when the hfsc values came down to those of Six-lines (see (b)).

impurities was not successful, a set of doublets characteristic of the protons of an epoxy ring located on a skewed ring could be observed; chemical shifts were 3.16 ppm and 3.37 ppm (J-coupling was 4 Hz). Protons on the epoxy ring located on a skewed ring frequently have non-equivalent chemical shifts. When DMPO purified by Mitsui-Toatsu was measured in DMSO-d_6, this doublet disappeared in the spectrum (see Figure 6c): the spectrum was also obtained by accumulating the free induction decay 10,000 times. For comparison, scopolamine (the structure being indicated in Figure 6d), which has an epoxy ring on a five membered ring including a nitrogen atom, was measured and the spectrum obtained is shown in Figure 6d. In the spectrum, a set of doublets characteristic of the epoxy protons appeared at 3.16 ppm and 3.51 ppm (J-coupling was 3 Hz). The above observation indicates that commercial DMPO includes a hydroxylamine which has the molecular weight of 129 and has an epoxy ring probably located at C2 and C3 positions of DMPO. This compound (structure I) can be converted by oxidation to an aminoxyl radical whose ESR spectrum consists of a triplet of doublets (Six-lines). It is probable that this compound is formed by the hydrogen abstraction at C3 of DMPO by oxidation, subsequent O_2 addition at C3, and the attack of thus produced peroxyl radical at C3 to C2 in the double bond of the molecule.

Since it has been reported in a previous paper that Six-lines may be assigned to a DMPO adduct of methyl radical,[3] the stability and the hfsc values of this adduct were compared to the values of the compound giving rise to Six-lines. DMPO-methyl adduct was produced by illuminating at 260 nm an aqueous DMPO solution (28 μL, 10 mM) containing DMSO (0.5 μL) and H_2O_2 (1%, 10 μL). Immediately after the illumination, 15 μL of DMPO and 18 mg of LPC was added to the system so that the hfsc values of the ESR signals could be compared to those obtained in the LPC dispersion system. The change in the ESR intensity and in the hfsc's are represented in Figure 7a and b, respectively. It has been clarified that the DMPO methyl adduct, the hfsc values (a(N) = 1.60 mt, a(βH) = 2.32 mT) of which are slightly different from those of Six-lines, decays much faster than Six-lines with the half-life time of ca. 2 min: the half-life time of Six-lines is of the order of hour.

In conclusion, the species producing Six-lines in the ESR spectra obtained upon usage of high concentration of DMPO is assigned to the aminoxyl radical formed from the epoxy hydroxylamine (structure I) by oxidation.

References

1. Pryor, W.A. "Free Radicals in Biology," Vol. 1, Chap 1, 1–68, Academic Press, New York, (1976).
2. Porter, N.A. "Free Radicals in Biology," Vol. 4, Chap 8, 51–68, Academic Press, New York, (1980).
3. Britigan, B.E., Rosen, G.M., Chai, Y. and Cohen, M.S. *J. Biol. Chem.*, **261,** 4426–4431, (1986).
4. Docampo, R., Casellas, A.M., Madeira, E.D., Cardoni, R.L., Moreno, S.N.J. and Mason, R.P. *FEBS Lett.*, **155,** 25–30 (19XX).
5. Finkelstein, E., Rosen, G.M. and Rauckman, E.J. *J. Mol. Pharmacol.*, **16,** 676–685, (1979).
6. Finkelstein, E., Rosen, G.M. and Rauckman, E.J. *Arch. Biochem. Biophys.*, **200,** 1–16, (1980).
7. Makino, K., Imaishi, H., Morinishi, S., Takeuchi, T. and Fujita, Y. *Biochem. Biophys. Res. Comm.*, **141,** 381–386, (1986).
8. Finkelstein, E., Rosen, G.M. and Rackman, E.J. *J. Am. Chem. Soc.*, **102,** 4994–4999, (1980).
9. McKee, J.R., Christman, C.L., O'Brien, W.D., Jr. and Wang, S.Y. *Biochemistry*, **16,** 4651–4654, (1977).
10. Makino, K., Mossoba, M.M. and Riesz, P. *J. Am. Chem. Soc.*, **104,** 3537–3539, (1982).
11. Makino, K., Mossoba, M.M. and Riesz, P. *J. Phys. Chem.*, **87,** 1369–1377, (1983).
12. Makino, K., Mossoba, M.M. and Riesz, P. *Radiat. Res.*, **96,** 416–421, (1983).

13. Bonett, R., Clark, V.M. and Todd, A. *J. Chem. Soc.*, 2102–2104, (1959).
14. Floyd, R.A. and Soong, L.M. *Biochem. Biophys. Res. Comm.*, **261,** 79–84, (1977).

Accepted by Prof. E.G. Janzen