QUANTITATIVE DETERMINATION OF THE SUPEROXIDE RADICALS IN THE XANTHINE OXIDASE REACTION BY MEASUREMENT OF THE ELECTRON SPIN RESONANCE SIGNAL OF THE SUPEROXIDE RADICAL SPIN ADDUCT OF 5,5-DIMETHYL-1-PYRROLINE-1-OXIDE

IKUKO UENO,* MASAHIRO KOHNO,** KUNITOSHI YOSHIIHIRA,*** AND IWAO HIRONO

Department of Carcinogenesis and Cancer Susceptibility, Institute of Medical Science, University of Tokyo,* Shirokanedai, Minato-ku, Tokyo, 108, Japan, ESR Application Laboratory, JEOL Ltd.,** Nakagami Akishima, Tokyo, 196, Japan and Division of Environmental Chemistry, National Institute of Hygienic Sciences,*** Yoga, Setagaya-ku, Tokyo, 158, Japan

(Received January 20, 1984)

Reactivities of biologically active compounds with superoxide radicals were examined by measurement of electron spin resonance (ESR) spectra of 5-hydroperoxy-2,2-dimethyl-1-pyrolidinylxoyl formed from 5,5-dimethyl-1-pyrroline-1-oxide and the superoxide radicals generated by xanthine-xanthine oxidase system.

Quantitative determination of the superoxide radicals was performed by comparing the intensity of the spin adducts with that of 4-hydroxy-2,2,6,6-tetramethyl-pipero-
dinoxyl whose spin concentration was determined.

Methyl viologen enhanced the formation of this spin adducts, while, rutin and luteoskyrin prevented it.

Keywords — superoxide radical; ESR spin-trapping; 5,5-dimethyl-1-pyrroline-1-oxide; antioxidant; methyl viologen; rutin; luteoskyrin

INTRODUCTION

Superoxide radicals are known to be produced in the respiratory cells and seem to have both useful and harmful effects.1) Reduction of cytochrome c2 or nitroblue tetrazolium3) is commonly used as an index of the formation of superoxide radicals. However, certain enzyme systems or reagents directly reduce these compounds. Therefore, the reduction of these compounds does not necessarily provide proof of the presence of superoxide radicals.

In this experiment we tried to detect the reactivities of chemical compounds with the superoxide radicals by measuring intensities of the first signal of the electron spin resonance (ESR) spectra of the spin adduct, 5-hydroperoxy-2,2-dimethyl-1-pyrrolidinylxoyl (DMPO-OOH) formed from superoxide radicals and 5,5-dimethyl-1-pyrroline-1-oxide (DMPO).

We used the xanthine-xanthine oxidase system as a superoxide radical generator. Quantitative determination of the superoxide radicals was performed by comparing the intensity of the DMPO-OOH adduct with that of 4-hydroxy-2,2,6,6-tetramethyl-pipero-dinoxyl.

MATERIALS AND METHODS

Materials — DMPO (Sigma), 4-hydroxy-2,2,6,6-tetramethyl-pipero-dinoxyl (Sigma), catalase (c-40, Sigma), xanthine oxidase (Boehringer, cow milk), butylated hydroxyanisole (BHA, Wako), butylated hydroxytoluene (BHT, Wako), benzo[b]pyrene (Wako), methyl viologen (Wako), α-naphthoflavone (Tokyo Kasei), rutin (Tokyo Kasei), β-naphthoflavone (Aldrich) and aflatoxin B1 (Makor) were used. Luteoskyrin and T-2 toxin were kindly supplied by Dr. Y. Ueno, Tokyo Uni-
versity of Science, Tokyo. Other chemicals used were of analytical grade.

Methods — ESR Spectra consisting of DMPO-OOH, 5-hydroxy-2,2-dimethyl-1-pyrrolidinoyl (DMPO-OH) and DMPO spin adduct of unknown radical with six lines (DMPO-X)\(^4\) were recorded in a JEOL JES-FE2XG spectrometer equipped with 100 kHz field modulation (X-Band) as follows: (A) Twenty \(\mu\)l of DMPO were added to 195 \(\mu\)l of 100 mM sodium phosphate buffer (pH 7.4) containing 147 mM xanthine in a small test tube. After 5 \(\mu\)l of 1.6 \(\mu\)M xanthine oxidase were added to the solution, 200 \(\mu\)l of the reaction mixture were taken into an ESR cuvette. Recording of the ESR spectra was started 50 s after the xanthine oxidase addition, and repeated 10 times at 1 min intervals. Each scanning lasted 30 s. When effects of superoxide dismutase (SOD) and other chemical compounds on the DMPO-OOH formation were examined, test compounds dissolved in 10 \(\mu\)l of water or dimethylsulfoxide (DMSO) were added to the test tube prior to the addition of DMPO.

Signals of DMPO-OOH, DMPO-OH and DMPO-X were determined by analysis of their ESR parameters, as described by Finkelstein et al.\(^4\) In each recording, intensities of the first signal of all adducts (DMPO-OOH, DMPO-OH and DMPO-X) were calculated and represented as relative values of the standard signal of MnO. (B) For quantitative determination of the superoxide radical formation, ESR spectra in 60 \(\mu\)l of the solution containing xanthine, xanthine oxidase and DMSO in (A) were recorded 50 s after the addition of xanthine oxidase. Intensity of the first signal of DMPO-OOH adduct observed was multiplied by six because its spectrum consists of six lines, and the value was regarded as total intensity of \(5 \times 10^{-4} \text{ M} 4\)-hydroxyl-2,2,6,6-tetramethyl-pipperidinoxyol solution.

RESULTS

Experiment A

Fifty seconds after adding xanthine oxidase marked signals were observed in the ESR spectrum of the reaction mixture containing xanthine, DMPO, and xanthine oxidase, as shown in Fig.1 (A). By comparing G-values of the signals with those reported by Finkelstein et al.\(^4\) we

![FIG. 1. ESR Spectra of DMPO Spin Adducts Formed in the Solution Containing DMPO and the Xanthine—Xanthine Oxidase System with or without Addition of Superoxide Dismutase and Dimethyl Sulfoxide](image)

ESR spectra were recorded at room temperature under the following conditions: microwave power, 4.0 mW; modulation amplitude, 2G; scanning field, 3350±50G; amplitude, 1.25×1000; sweep time, 30 s. (A) ESR spectra were recorded in 200 \(\mu\)l of a solution consisting of 195 \(\mu\)l of 147 mM xanthine in 100 mM sodium phosphate buffer (pH 7.4), 20 \(\mu\)l of DMPO, and 5 \(\mu\)l of 1.6 \(\mu\)M xanthine oxidase. Spectra recorded 50 and 290 seconds respectively after the addition of xanthine oxidase were shown. (B) Ten \(\mu\)l of SOD (30 units) were added to the solution described in (A) prior to the addition of DMPO. (C) Ten \(\mu\)l of DMSO was added to the solution in (A) prior to DMPO. ESR parameter; DMPO-OOH \((A_N = 14.2, A_H = 11.6, A_H = 1.2G)\), DMPO-OH \((A_N = 14.8, A_H = 14.8G)\) (4).

Other experimental conditions are described in Methods.
identified the signals of DMPO-OOH, DMPO-OH and DMPO-X. Addition of SOD prior to
xanthine oxidase to the reaction mixture prevented the appearance of the signals correspond-
ing to the DMPO-OOH adduct. We suggest that these signals represent DMPO-OOH
adduct that originated from superoxide radicals.

Since DMSO, a hydroxyl radical scavenger, was used as a solvent in the experiments, the
effect of DMSO on the formation of DMPO spin adducts was examined. Addition of DMSO
(4.4%) prior to xanthine oxidase completely prevented the appearance of the DMPO-OH signals
(Fig. 1 (C)). To confirm the formation of the DMPO-OH signals from the hydroxyl radicals,
ethanol, another hydroxyl radical scavenger, was added to the reaction mixture. Ethanol (4.4%)
also prevented the appearance of the DMPO-OH signals (data not shown).

In order to study the appearance and decay of
the spin adducts of DMPO-OOH, DMPO-OH, and DMPO-X separately, relative intensities of
the first signals of each of the three adducts were plotted against the reaction time (Fig. 2 (A)).
The curve of the DMPO-OOH showed rapid formation of the superoxide radicals and a short-
lived adduct, while the curves of the DMPO-OH and DMPO-X showed slow formations. Inhibi-
tory effects of SOD, and DMSO and ethanol, on the formation of adducts of DMPO-OOH and
DMPO-OH respectively, are clearly demonstrated by the curves shown in Figs. 2 and 3. Interes-
tingly, in the presence of DMSO and ethanol, intensities of DMPO-OOH and DMPO-X were
greatly increased (Fig. 3). The effect of catalase on the formation of DMPO-OH adduct
was examined in the presence and absence of SOD. In both cases, catalase moderately sup-
pressed the formation of the adduct (Fig. 4).

To demonstrate that our method may be ap-

![Graph](A) ![Graph](B)

**FIG. 2.** *Time Courses of the Formations of the Adducts of DMPO-OOH, DMPO-OH and Unknown Radical with Six Lines in the Solution Containing the Xanthine–Xanthine Oxidase System and DMPO with and without Addition of Superoxide Dismutase*

(A) Intensities of the first ESR signals of DMPO-OOH, DMPO-OH and DMPO-X, which are shown in Fig. 1 (A) are plotted against time. (B) Intensities of the signals of DMPO-OOH, DMPO-OH, and DMPO-X observed in the solution with SOD in Fig. 1 (B) are plotted.
FIG. 3. Effects of Dimethyl sulfoxide and Ethanol on the Formation of the ESR Spin Adducts in the Solution Containing the Xanthine–Xanthine Oxidase System and DMPO

(A) Intensities of the first ESR signals of DMPO-OOH, DMPO-OH and DMPO-X, which were shown in Fig. 1 (C) are plotted against time. (B) Intensities of the signals of DMPO-OOH, DMPO-OH and DMPO-X, which were observed in the solution containing xanthine, xanthine oxidase, DMPO and ethanol, are plotted against time.

FIG. 4. Effect of Catalase on the Formation of the ESR Spin Adducts in the Solution Containing the Xanthine–Xanthine Oxidase System and DMPO with and without Addition of Superoxide Dismutase

(A) Intensities of the first signals of ESR spectra of DMPO-OOH, DMPO-OH and DMPO-X, which were recorded in 200 µl of a solution consisting of 195 µl of 100 mM sodium phosphate buffer (pH 7.4) and 147 mM xanthine, 10 µl of catalase (3 units), 20 µl of DMPO and 5 µl of 1.6 µM xanthine oxidase, are plotted against time. (B) Ten µl of SOD (30 units) were added to the solution described in (A) prior to the addition of DMPO.
plied to detecting reactivity of any chemical compound with superoxide radicals, we examined the effect of methyl viologen (a well-known superoxide radical amplifier) on the formation of DMPO-OOH adduct. Methyl viologen markedly enhanced the formation of DMPO-OOH adduct (Fig. 5 (A)). We then applied this method to certain biologically active compounds to determine their reactivities with superoxide radicals. Rutin (Fig. 5 (B)) and luteoskyrin (Fig. 5 (C)) showed inhibitory effects on the formation of DMPO-OOH adduct. In the presence of BHA, BHT, α- and β-naphthoflavone, and benzo[a]pyrene, the formation of the DMPO-OOH adduct was slightly enhanced (Table I). Aflatoxin B1 and T-2 toxin showed no effect (data not shown).

Experiment B

Initial rates of formation of superoxide radicals in solutions containing different concentra-
tions of xanthine oxidase were calculated from the amounts of DMPO-OOH adduct formed during the first 50 s after addition of xanthine oxidase (see Fig. 6). The adding of up to 0.22 μM of xanthine oxidase proved formation of DMPO-OOH adduct that was proportional to the enzyme added.

DISCUSSION

It has been reported that the superoxide radicals can be detected by ESR spin trapping technique using DMPO and other spin trapping reagents. However, life span of the DMPO spin adduct of superoxide radicals (DMPO-OOH) apparently is extremely short.

In order to measure ESR signals with high accuracy and sensitivity, we used a specifically ordered, large-volume, ESR “flat cell” fixed in a cell holder. ESR spectra were recorded immediately after the beginning of the reaction of the

![Figure 5](image-url)

**FIG. 5. Effects of Methyl Viologen, Rutin and Luteoskyrin on the Formation of the ESR Spin Adducts in the Solution Containing the Xanthine–Xanthine Oxidase System and DMPO**

Intensities of the first signal of ESR spectrum of DMPO-OOH, which were recorded in 200 μl of a solution consisting of 195 μl of 100 mM sodium phosphate buffer (pH 7.4) and 147 mM xanthine, 10 μl of methyl viologen (A), rutin (B) and luteoskyrin (C) in DMSO, 20 μl of DMPO and 5 μl of 1.6 μM xanthine oxidase, are plotted against time.
xanthine oxidase system with a high concentration of DMPO. Intensities of the first signal of the DMPO-OOH adduct were measured. They are easily distinguished from other signals during the early stages of reaction. We then calculated the quantities of the superoxide radicals by comparing the intensities of the DMPO-OOH adduct with the intensity of the ESR spectrum of a known amount of 4-hydroxy-2,2,6,6-tetramethyl-piperidinoxy.

This method allows easy detection of reactivities of the chemical compounds with the superoxide radicals. Since during previous research, utilizing the spin-trapping method, superoxide radicals were found in biological systems, *i.e.* spinach chloroplasts and rat liver microsomes, we rationalized that our method would detect radicals produced in the biological systems. Recently, Hiramatsu *et al.* used a similar method to detect superoxide radicals in brain tissue with good results (personal communication).

Finkelstein *et al.* reported that the DMPO-OOH adduct is formed from the adduct of DMPO-OOH, and not from hydroxyl radicals, in a reaction system, containing diethylenetriamine pentaacetic acid and a phosphate buffer, which was first purified by passing it through a Chelex 100 column. However, our experiments demonstrated that hydroxyl radical scavengers, DMSO and ethanol, completely prevented formation of the DMPO-OOH adduct. Further more, catalase, both in the presence and absence of SOD, suppressed formation of the DMPO-

**FIG. 6. Quantitative Determination of the Superoxide Radicals formed in the Solution Containing the Xanthine–Xanthine Oxidase System and DMPO adduct and calculation of the concentrations of the adduct were performed as described in Methods.**

<table>
<thead>
<tr>
<th>Test compounds added</th>
<th>M</th>
<th>DMPO-OOH formed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Intensity</td>
</tr>
<tr>
<td>BHA</td>
<td>$1 \times 10^{-3}$</td>
<td>0.97</td>
</tr>
<tr>
<td>BHT</td>
<td>$1 \times 10^{-3}$</td>
<td>1.13</td>
</tr>
<tr>
<td>α-Naphthoflavone</td>
<td>$1 \times 10^{-3}$</td>
<td>1.33</td>
</tr>
<tr>
<td>β-Naphthoflavone</td>
<td>$1 \times 10^{-3}$</td>
<td>1.20</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>$1 \times 10^{-3}$</td>
<td>1.48</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>$1 \times 10^{-4}$</td>
<td>1.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.23</td>
</tr>
</tbody>
</table>

Intensities of the first ESR signal of DMPO-OOH were recorded 50 s after addition of a test compound in the solution described in Fig. 4(A), except a test compound dissolved in DMSO was added instead of methyl viologen. Other experimental conditions are described in Methods.
OH adduct. These observations suggest that, under our experimental conditions, DMPO-OH adduct was formed mainly from hydroxyl radicals, which probably are produced in a reaction between superoxide radicals and hydroperoxide, such as in the Harber-Weiss or Fenton reactions.\(^{10}\) The reason for the intensified DMPO-OOH adduct concentration by DMSO and ethanol is not clear from this experiment. Further experiments are now under way.

REFERENCES
1) I. Fridovich: The biology of oxygen radicals. Superoxide radical is an agent of oxygen toxicity; superoxide dismutases provide an important defense, *Science*, 201, 875—880 (1978).