Spin-Trapping Studies on the Reaction of Iron Complexes with Peroxides and the Effects of Water-Soluble Antioxidants

Masahiro Kohno,* Masako Yamada, Keiichi Mitsuta, Yokio Mizuta, and Toshikazu Yoshikawa†
ESR Application Laboratory, Analytical Instruments Division, JEOL Ltd., 1-2 Musashino 3-Chome, Akishima, Tokyo 196
† First Department of Medicine, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602
(Received October 29, 1990)

Using ESR spin-trapping techniques we measured the levels of free radical species generated from six different systems (hypoxanthine–xanthine oxidase, iron(II)–hydrogen peroxide, iron(III)–hydrogen peroxide, iron(II)–*t*-butyl hydroperoxide, iron(III)–*t*-butyl hydroperoxide, and catalase (CAT)–*t*-butyl hydroperoxide). Six types of radicals ($O_2^{-\cdot}$, $\cdot OH$, $\cdot H$, $\cdot CH_3$, $(CH_3)_3CO\cdot$, and $(CH_3)_3COO\cdot$) were detected as spin adducts of spin traps 5,5-dimethylpyrroline 1-oxide (DMPO) or 3,5-dibromo-4-nitrosobenzene sulfonate (DBNBS). Quantitative analysis of the levels of generated radicals by means of an ESR instrument also presents important information regarding the reduction of peroxides [hydrogen peroxide (H_2O_2) or *t*-butyl hydroperoxide (ROOH)] by iron(II) or iron(III) as well as catalase. In addition, the scavenging potencies of different water-soluble antioxidants such as L-ascorbic, D-isoascorbic, gallic, sorbic, and protocatechuic acids were evaluated in terms of their ability to reduce the peaks of spin adducts.

Active oxygens such as superoxide anion radical ($O_2^{-\cdot}$, $\cdot OOH$), hydroxyl radical ($\cdot OH$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) have been implicated as being major damaging species in pathology and have been widely investigated.[1] These compounds react with the lipids of membranes and through a series of reactions generate carbon-centered (R·), alkoxyl (RO·), and then peroxyl (ROO·) radicals, all of which are used as markers of lipid peroxidation and the disruption of cellular homeostasis.[2,3] Also widely explored are studies scavenging phenomena of these active oxygens and free radicals by such antioxidants as vitamins C and E, and glutathione, and by scavengers such as superoxide dismutase (SOD) as well as catalase (CAT).[4–7]

The ESR spin-trapping technique is very useful for stabilizing short-lived free radicals. This technique has therefore been a powerful tool for studying generation mechanisms of free radicals and active oxygens[8–10] as of free radicals.[11–23]

The present study was carried out in order to establish a detection method for such free radicals as $O_2^{-\cdot}$, $\cdot OH$, $\cdot H$, $\cdot CH_3$ (R·), $(CH_3)_3CO\cdot$ (RO·), and $(CH_3)_3COO\cdot$ (ROO·). The six radical-generating systems were: hypoxanthine–xanthine oxidase which generates $O_2^{-\cdot}$ and $\cdot OOH$; iron(II)– hydrogen peroxide which gives ·OH; iron(III)–hydrogen peroxide, ·OH; iron(II)-*t*-butyl hydroperoxide and iron(III)-*t*-butyl hydroperoxide, R·, RO·, ROO·, and CAT–*t*-butyl hydroperoxide, RO·. The mechanism in Fenton's or catalase reactions between iron(II), iron(III), or CAT and ROOH or H_2O_2, are also described based on the results obtained from experiments performed under several conditions. Finally, the potencies of water-soluble antioxidants (L-ascorbic acid, D-isoascorbic acid, gallic acid, sorbic acid, and protocatechuic acid) in scavenging $O_2^{-\cdot}$, ·OH, ROO·, and/or RO· are described.

Experimental

Materials. Spin trapping reagents 5,5-dimethylpyrroline 1-oxide (DMPO) and 3,5-dibromo-4-nitrosobenzene sulfonate (DBNBS), were supplied by Mitsui Toatu Chemicals and by Sigma Chemical Co., Ltd., respectively. Diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DETAPAC), used to chelate trace metal impurities, was obtained from Wako Pure Chemical. The following were the sources of different radicals and their corresponding suppliers: sources of superoxide radical [hypoxanthine (HPX) from Sigma Chemical, and xanthine oxidase (XOD) from Boehringer Mannheim, cow milk]; source of hydroxyl radical [iron(II) sulfate heptahydrate (ferrous iron) and iron(III) sulfate *n*-hydrate (ferric iron) from Wako Pure Chemical Ins., Ltd., and hydrogen peroxide (H_2O_2)]; source of methyl, *t*-butoxyl, *t*-butylperoxyl radicals [iron(II) or iron(III) and *t*-butyl hydroperoxide (ROOH) from Nakarai Kagaku Co.]. Antioxidant reagents L-ascorbic acid, D-isoascorbic acid (erythorbic acid), and gallic acid were purchased from Daiichi Pure Chemical, while sorbic acid and protocatechuic acids were obtained from Wako Pure Chemical Ins.

Instruments. ESR spectra were recorded on a JEOL JES-RE1X spectrometer using aqueous quartz flat cell (Inner size 60 mm×10 mm×0.31 mm) with an effective sample volume 160 μl.

Preparation of Samples. All measurements were carried out both in 0.1 M (M=mol dm^{-3}) of PBS (sodium phosphate buffer solution) (pH=7.8) and in pure water at room temperature. Both peroxides, H_2O_2 and ROOH, were used as aqueous solutions. The concentration of the antioxidants used in this experiment was 1.0 mM (1 mM=1.0×10^{-3} mol dm^{-3}). Superoxide radicals were generated from a hypoxanthine xanthine oxidase reaction system under the conditions reported previously.[15,17]

Results and Discussion

Detection and Identification of Radicals [$O_2^{-\cdot}$, ·OH, ·H, R·, RO·, and ROO·]. The ESR spectra of the spin

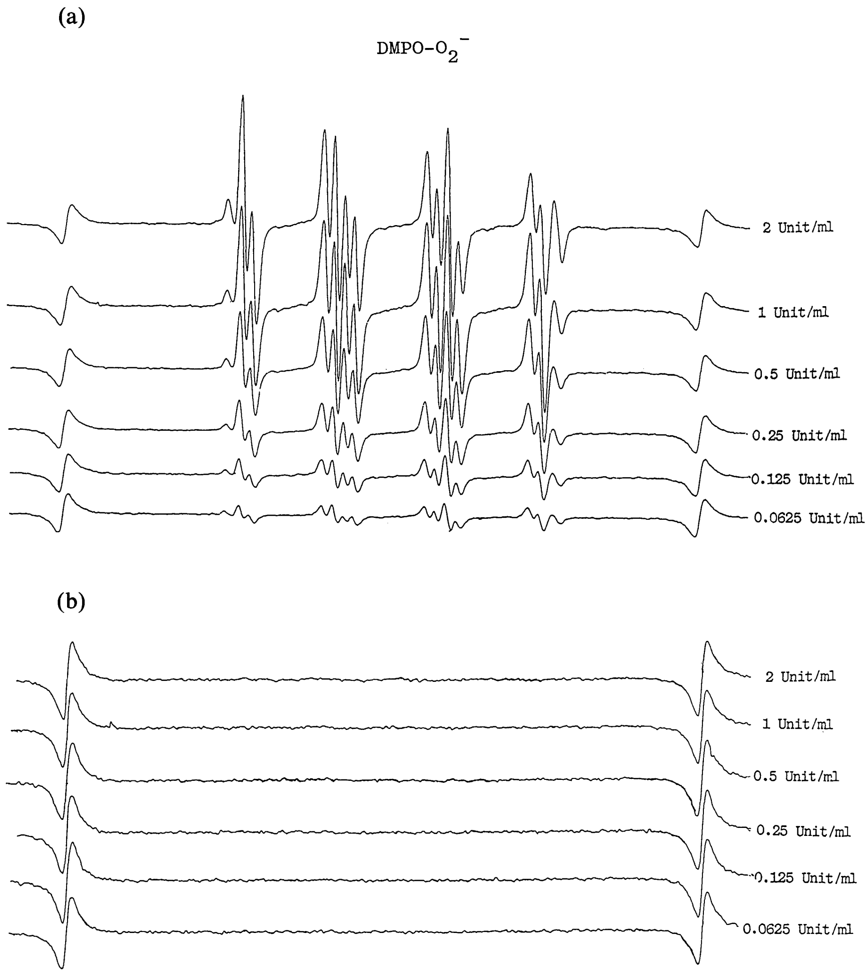

Fig. 1. ESR spectra of spin adducts of $O_2^{-\cdot}$, observed by the use of spin traps DMPO (a) and DBNBS (b). Spectrum (a) recording started 30 s after mixing 50 µl 2 mM HPX, 35 µl 5.5 mM DETAPAC, 15 µl 9.2 M DMPO (a), 50 µl 0.1—0.5 U ml^{-1} XOD, and 50 µl PBS. Spectrum (b) was observed under same conditions but without 15 µl 60 mM DBNBS.

adducts obtained in the hypoxanthine–xanthine oxidase reaction by using two kinds of spin traps, DMPO and DBNBS, are shown in Figs. 1a and 1b. In these figures, the peaks at either ends are of Mn^{2+} in MgO, which is used as an internal standard. The *g* values of both peaks are 2.0334 and 1.9810 at the resonance frequency of 9450.0 MHz, respectively. The method of measurement and the reaction conditions used in the experiments have been described previously.[10,17] As shown in Fig. 1a, a spin trapped by DMPO increases with the XOD concentration, and the hyperfine coupling constants (hfcc) obtained from the spectra coincide with the values of DMPO–O_2^- reported previously.[8] Using the spin trap DBNBS instead of DMPO, no signals were observed under the same conditions (Fig. 1b). However, the addition of DBNBS (60 mM) reduced the signal intensity of DMPO–O_2^-. From the experimental results for changing the concentration of DBNBS, the value of 50% inhibition(ID_{50}) of DMPO–O_2^- was measured to be 0.3 mM. The reaction rate constant (k_2) of DBNBS with $O_2^{-\cdot}$ in a hypoxanthine–xanthine odixase reaction were determined to be 3.9×10^4 M^{-1} s^{-1} at pH=7.8 from ID_{50} by treating for competitive reaction.[14] This value is 5.9-times larger than the previous reported value, 6.6×10^3 M^{-1} s^{-1} at pH=7.0, obtained by a pulse radiolysis method.[26] The difference may be due to differences in the pH and generation system of $O_2^{-\cdot}$. The results clearly indicate that both DMPO and DBNBS react with $O_2^{-\cdot}$. However, only DMPO gives a spectrum of the $O_2^{-\cdot}$ spin adduct. No $O_2^{-\cdot}$ spin adduct signals can be detected in the case of DBNBS. It is speculated that nitrones are better than nitroso compounds in detecting $O_2^{-\cdot}$ as a spin adduct.

Figures 2a and 2b show the spectra of the spin adducts obtained using DMPO or DBNBS, respectively, in reactions between 0.1 mM iron(II) chelated by DETAPAC (0.1 mM) and H_2O_2 (1 mM) in pure water.

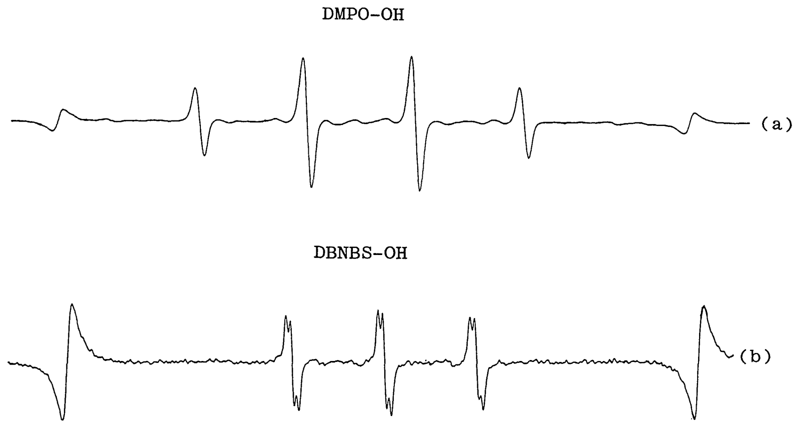

Fig. 2. ESR spectra of spin adducts of ·OH observed by the use of spin traps DMPO (a) and DBNBS (b). Spectra recording started 30 s after mixing 75 µl 0.1 mM iron(II) chelated by 0.1 mM DETAPAC before use [iron(II)-DETAPAC], 75 µl 1 mM H_2O_2, 50 µl 60 mM DMPO and 60 mM DBNBS, in 50 µl pure water.

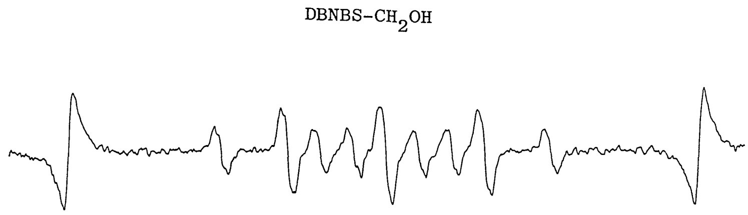

Fig. 3. ESR spectra of spin adducts of ·CH_2OH after mixing 75 µl 0.1 mM iron(II)-DETAPAC, 75 µl 1 mM H_2O_2, 15 µl 60 mM DBNBS and 50 µl 1.6 mM methanol.

In the case of DMPO (Fig. 2a), a typical hfcc was obtained as a_N=1.48 and $a_{H\beta}$=1.48 mT, which is the same as the typical value of DMPO–OH.[12] Using iron(III) (0.1 mM) instead of iron(II), less DMPO–OH is generated (Fig. 2b).

The results of the above-mentioned experiments demonstrate that iron(II) is more active than iron(III) in the reduction reaction of H_2O_2. With DBNBS, the hfcc of the signal is analyzed as a_N=1.24 mT and a_H=0.063 mT (Fig. 2b), which coincide with those reported previously.[26,27] However, the previous studies have many uncertainties,[27] since this adduct was assigned to DBNBS–O_2^-. In order to confirm that this radical species was DBNBS–OH, the methanol, which is a specific scavenger of ·OH, was added to the reaction system. Then, the signal decayed with increasing methanol concentration. When 1.6 mM methanol was added, another spin adduct, which is assigned as methanol radical (DBNBS–CH_2OH; a_N= 1.36 mT, a_H=0.91 mT and a_H=0.06 mT),[20,27] is detected as is shown in Fig. 3. In addition, a concomitant increase in the intensity of the DBNBS–OH signal is observed with increasing amount of added H_2O_2 (Fig. 4). Thus,·OH can be trapped not only by DMPO, but also by DBNBS. The rate constant (k_2) for the reaction of DBNBS with ·OH is determined to be 2.2×10^{10} M^{-1} s^{-1}, as measured from its competitive reaction with DMPO in trapping ·OH radicals. In this treatment, $k_2=3.4\times10^9$ M^{-1} s^{-1} [19] was used as the rate constant for the reaction of DMPO to trap ·OH. From these values, DBNBS is more sensitive than DMPO for detecting ·OH.

In the reaction between iron(II) of 0.1 mM chelated by DETAPAC of 0.1 mM, and H_2O_2 (0.01 mM), two kinds of spin adducts, DMPO–OH and DMPO–H, are observed (Fig. 5). Thus, from these spectra, it is confirmed that at least two types of radicals (·H and ·OH) are generated in the reaction. The concentration of the generated DMPO–OH agrees with that of DMPO–H under this reaction.

Using DMPO, both the reactions between iron(II) or iron(III) and ROOH give three kinds of spin adducts of methyl (R·), *t*-butoxyl (RO·), and *t*-butylperoxyl (ROO·) radicals (Fig. 6). However, when DBNBS was used, only the spin adduct of methyl radical (DBNBS–CH_3: a_N=1.37 mT and a_{CH_3}=1.35 mT and a_H=0.07 mT) was observed (Fig. 7). The reaction between the iron(III) and ROOH give a relatively weak signal of methyl

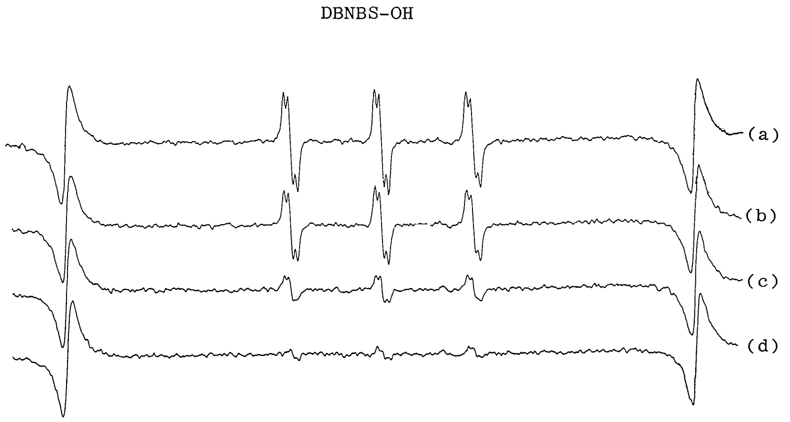

Fig. 4. ESR spectra of spin adducts of ·OH observed by the use of spin trap DBNBS. 75 µl 0.1 mM iron(II)-DETAPAC, 75 µl of (a) 1 mM, (b) 0.5 mM, (c) 0.25 mM and (d) 0.125 mM H_2O_2 concentration, 15 µl 60 mM DBNBS, in 50 µl pure water.

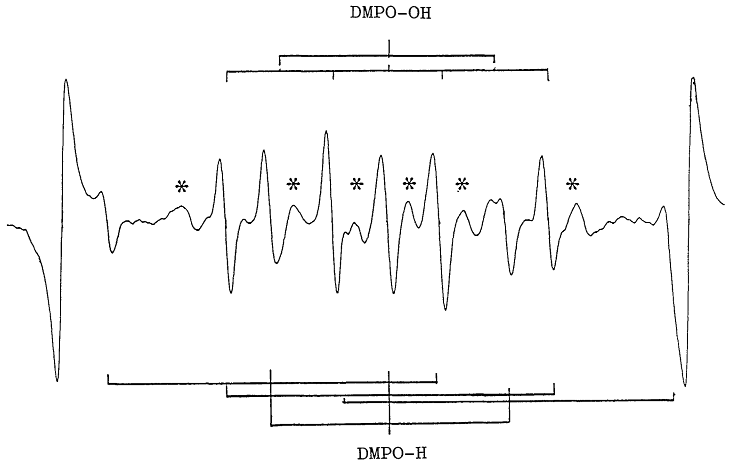

Fig. 5. ESR spectra of spin adducts of ·OH and ·H after mixing 75 µl 0.1 mM iron(II)-DETAPAC, 75 µl 0.01 mM H_2O_2, 15 µl, 0.92 M DMPO, in 50 µl pure water. The signal with "*" is the adduct of carbon center radical.

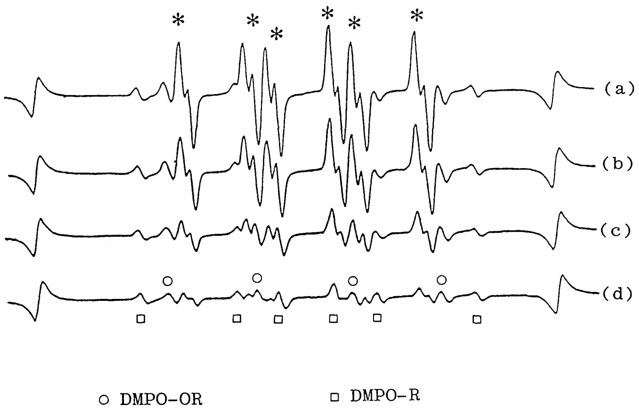

Fig. 6. ESR spectra observed in the reaction between 0.1 mM iron(II)-DETAPAC and ROOH of (a) 10 mM, (b) 5 mM, (c) 2.5 mM, (d) 1.25 mM ROOH concentration. The signal with "*" is the adduct of ROO·.

Table 1. Hyperfine Coupling Constants of Spin Adduct of Radicals, $O_2^{-\cdot}$, ·OH, ·H, R·, RO·, and ROO·[a)]

Spin adduct.	a_N	a_H^β	a_H^γ
DMPO-O_2^-	1.41	1.14	0.13
DMPO-OH	1.48	1.48	
DMPO-H	1.64	2.25	
DMPO-OOR	1.45 (1.45)	1.05 (1.05)	0.14 (0.15)
DMPO-OR	1.49 (1.48)	1.57 (1.60)	
DMPO-R	1.64	2.24	

a) ( ) is reported values.

adducts (Fig. 7). In the case of the reaction with CAT, only RO· is observed (Fig. 8). The spin adducts of R·, RO·, and ROO· were identified using the hfcc values listed in Table 1.[29,30] As shown in Figs. 7 and 8, the spin adducts of ROO· and RO· changed, depending on each concentration of iron(II), CAT and ROOH.

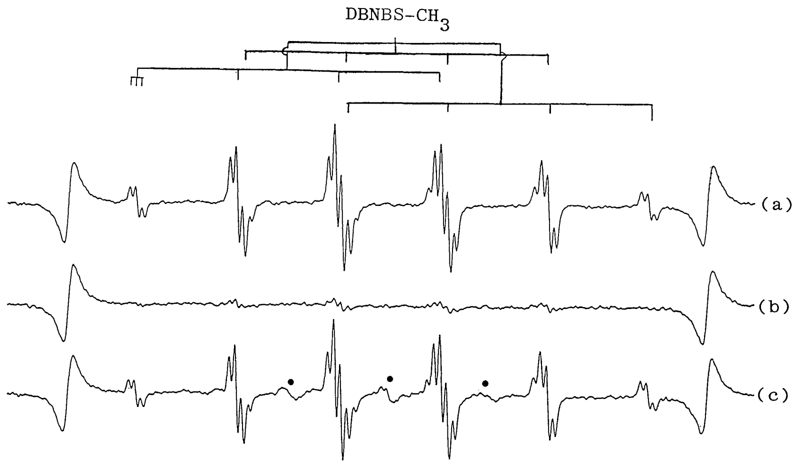

Fig. 7. ESR spectrum of $\cdot CH_3$ was observed by the use of DBNBS in the reaction between 1.0 mM iron(II) (a), iron(III) (b), iron(II)–DETAPAC (c) and 10 mM ROOH. The signal with "*" is the adduct of carbon center radical.

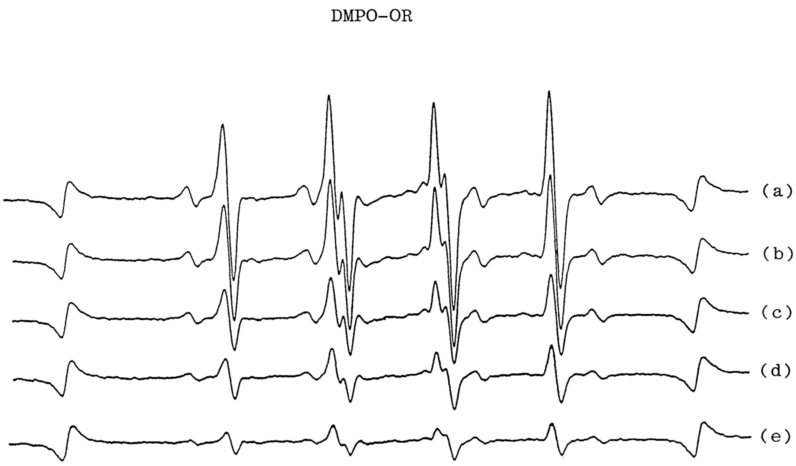

Fig. 8. ESR spectra observed in the reaction between ROOH and catalase of (a) 0.086 mM, (b) 0.043 mM, (c) 0.022 mM, (d) 0.011, (e) 0.006 mM catalase concentration.

In spin-trapping experiments, it can be confirmed that the results are very much affected by the concentration of DMPO used. For instance, if the DMPO concentration changed from 23 mM to 690 mM, the main observed spin adduct changed from RO· to ROO·, as well as the experiments for detecting ·OH and ·H. Thus, varying the DMPO concentration allows one to analyze specific radicals.

Reactivity of Water Soluble Antioxidants with $O_2^{-\cdot}$, ·OH, ROO·, and RO·. When DMPO was added to a solution of a radical-generating system, several spin adducts such as DMPO–O_2^-, DMPO–OH, DMPO–OOR, and DMPO–OR were detected. Various antioxidants such as L-ascorbic acid, D-isoascorbic acid, gallic acid, sorbic acid, and protocatechuic acid were diluted to 1 mM in pure water and added to a solution of the radical-generating system. Then, the amount of each spin adduct was found to decrease. These changes in the intensity of the ESR spectra were evaluated for the scavenging potencies of various antioxidants on $O_2^{-\cdot}$, ·OH, ROO·, and RO·. Table 2 shows the percent intensities of the spin adducts after the addition of antioxidants under the measurement conditions, as shown in the table, all of which were of equal concentration (1 mM). Based on Table 2, the scavenging action on $O_2^{-\cdot}$ of various antioxidants investigated decrease in the order D-isoascorbic acid > gallic acid > L-ascorbic acid > protocatechuic acid ≫ sorbic acid. As for ·OH, the potencies of antioxidants decrease in the order D-isoascorbic acid > L-ascorbic acid ≫ gallic acid = protocatechuic acid = sorbic acid=0%. For RO·, that order is gallic acid > protocatechuic acid ≫ sorbic

Table 2. Scavenging Activities(%) of Water Soluble Antioxidants L-Ascorbic Acid, D-Isoascorbic Acid, Sorbic Acid Protocatechuic Acid, and Gallic Acid against $O_2^{-\cdot}$, ·OH, ROO·, and RO·

Compound	$O_2^{-\cdot}$ [a]	·OH [b]	ROO· [c]	RO· [d]
L-Ascorbic acid	93.3	70.3	0	0
D-Isoascorbic acid	100.0	100.0	0	0
Sorbic acid	0	0	0	5.8
Protocatechuic acid	63.6	0	10.3	3.0
Gallic acid	96.5	0	0	28.5

Measurement conditions for generating $O_2^{-\cdot}$, ·OH, RO·, ROO· are shown as follows. a) $O_2^{-\cdot}$, 2 mM HPX+5.5 mM DETAPAC+0.4 U ml^{-1}+0.7 M DMPO. b) ·OH, 0.1 mM iron(II)–DETAPAC+1 mM H_2O_2+0.92 M DMPO. c) ROO·, 1.0 mM iron(II)–DETAPAC+5 mM ROOH+0.07 M DMPO. d) RO·, 1.0 mM iron(II)–DETAPAC+5 mM ROOH+0.07 M DMPO.

acid ≫ L-ascorbic acid = D-isoascorbic acid= 0%. In the case of ROO·, only protocatechuic acid show the function.

Reaction Mechanisms

The Fenton's Reaction. It has been reported that the formation of ·OH in a Fenton's reaction was directly confirmed using the spin trapping technique.[31,32] However, the reaction mechanism between iron(II) and H_2O_2 was assumed[33] to be

$$H_2O_2 + Fe^{2+} + H^+ \longrightarrow \cdot OH + Fe^{3+} + H_2O. \quad (1)$$

In our experiment two radical species (·OH and ·H) were observed in a Fenton's reaction used the H_2O_2 of low concentration for the concentration of iron(II). We thus propose the following reaction scheme, whereby both ·OH and ·H are generated:

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+}\text{–}OOH^- + \cdot H \quad (2)$$
$$Fe^{3+}\text{–}OOH^- \longrightarrow Fe^{4+}{=}O^{2-} + \cdot OH \quad (3)$$
$$H_2O_2 + Fe^{2+} \longrightarrow \cdot H + \cdot OH + Fe^{4+}{=}O^{2-} \quad (4)$$

In addition, it can be speculated that the existence of ·OOH generates a reaction between O_2 and ·H. In the case of a high H_2O_2 concentration,[13] ·OH is mainly observed. We therefore propose the follow reaction:

$$H_2O_2 + Fe^{2+} \longrightarrow \cdot OH + Fe^{3+}\text{–}OH. \quad (5)$$

Catalase Reaction. As in the case of a Fenton's reaction, this catalase reaction mechanism with regard to the radical reaction has not been proven experimentally. The proposed catalase reaction mechanism is according to

$$\text{Catalase} + 2H_2O_2 \longrightarrow 2H_2O + O_2. \quad (6)$$

To clarify the mechanism for the oxidation of H_2O_2, the reaction of iron(II), iron(III), or ROOH with CAT and H_2O_2 were assayed under different conditions. Mixtures of just catalase and H_2O_2 gave no signals. However, the ESR spectrum of alkoxyl radical[$(CH_3)_3CO\cdot$] was detected in a reaction between the catalase and ROOH. We therefore propose the existence of the following reaction mechanism:

$$\text{Catalase} + (CH_3)_3COOH \longrightarrow (CH_3)_3CO\cdot + \text{catalase–OH} \quad (7)$$

$$2\,\text{Catalase} + H_2O_2 \longrightarrow 2\,\text{catalase–OH (Compound-II)}^{7)} \quad (8)$$

However, the generation of molecular O_2 in the reaction (Eq. 6) has been confirmed. Unknown factors in the mechanisms in the catalase reaction are as follows:

$$H_2O_2 \longrightarrow 2\cdot OH \quad (9)$$
$$H_2O_2 \longrightarrow \cdot H + \cdot OOH \quad (10)$$
$$\cdot OOH \longrightarrow \cdot H + O_2 \quad (11)$$
$$2\cdot H + 2\cdot OH \longrightarrow 2H_2O \quad (12)$$
$$2H_2O_2 \longrightarrow 2H_2O + O_2 \quad (13)$$

The net equation (Eq. 13) is the same as that of Eq. 6.

Peroxides Reaction.[34] The reaction between iron(II) and ROOH generates three kinds of radicals (RO·, R·, and ROO·). Catalase reacts with ROOH, and generates RO·. Both results show that radical generation is modulated by the redox state and the conformation of the iron sites. The reaction of catalase is expressed as a function of the peroxidase.

These studies confirmed the previous results obtained from indirect assays, which indicated that $O_2^{-\cdot}$, ·OH, ·H, R·, RO·, and ROO· are generated by bio-relative reaction systems. Whether these compounds are the natural microbiological products of the oxidation process or simply intermediates in a complex series of reactions has yet to be determined. The methods employed here allow us to measure the antioxidant potencies of L-ascorbic, D-isoascorbic, sorbic, protocatechuic, and gallic acids on radical species such as $O_2^{-\cdot}$, ·OH, ROO·, and RO·.

References

1) R. J. Korthuis and D. N. Granger, in "Physiology of Oxygen Radicals," ed by A. E. Tayler, S. Matalon, and P. A. Warld, Williams & Wilkins, Baltimore MD (1986), p. 217—249.

2) M. G. Simic, D. S. Bergtold, and L. R. Karam, *Mutat. Res.*, **214**, 3 (1989).

3) M. G. Simic, *Mutat. Res.*, **202**, 377 (1988).

4) I. Fridovich, *Adv. Enzymol.*, **41**, 35 (1974).

5) J. M. McCord and I. Fridovich, *J. Biol. Chem.*, **244**, 6049 (1969).

6) N. Shimizu, K. Kobayashi, and K. Hayashi, *J. Biol. Chem.*, **259**, 4414 (1984).

7) I. Yamazaki, *Protein, Nucleic Acid Enzyme*, **33**, 2934 (1988).

8) E. Finkelstain, G. M. Rosen, E. J. Raukckman, and J. Paxton, *Mol. Pharmacol.,* **16**, 676 (1979).
9) E. Finkelstain, G. M. Rosen, E. J. Raukckman, and J. Paxton, *Arch. Biochem. Biophys.,* **200**, 1 (1980).
10) I. Ueno, M. Kohno, K. Mitsuta, Y. Mizuta, and S. Kanegasaki, *J. Biochem.,* **105**, 905 (1989).
11) M. Makino, M. M. Mossoba, and P. Riese, *J. Am. Chem. Soc.,* **102**, 4994 (1980).
12) M. Makino, M. M. Mossoba, and P. Riese, *J. Phys. Chem.,* **87**, 1369 (1983).
13) M. Makino, M. M. Mossoba, and P. Riese, *FEBS Lett.,* **100**, 23 (1979).
14) K. Mitsuta, Y. Mizuta, M. Kohno, H. Hiramatsu, and A. Mori, *Bull. Chem. Soc. Jpn.,* **63**, 187 (1990).
15) I. Ueno, M. Kohno, K. Yoshihira, and I. Hirano, *J. Pharm. Dyn.,* **7**, 563 (1984).
16) I. Ueno, M. Kohno, K. Yoshihira, and I. Hirano, *J. Pharm. Dyn.,* **7**, 798 (1984).
17) H. Hiramatsu and M. Kohno, *JEOL News,* **23A**, 7 (1987).
18) H. Miyagawa, T. Yoshikawa, T. Tanigawa, N. Yoshida, S. Sugino, M. Kondo, H. Nishikawa, and M. Kohno, *J. Clin. Biochem. Nutr.,* **5**, 1 (1988).
19) T. Tanigawa, *J. Kyoto Pref. Univ. Med.,* **99**, 133 (1990).
20) Y. Mizuta, K. Mitsuta, and M. Kohno, Proceeding of the 4th Biennial General Meeting of the Society for Free Radical Research, Kyoto, 1988, Abstr., pp. 9—13.
21) A. Mori, R. Edamatsu, M. Kohno, and S. Ohmori, *Neuroscience,* **15**, 371 (1989).
22) S. Uchida, M. Hiramatsu, A. Mori, G. Monaka, I. Nishioka, M. Niwa, and M. Ozaki, *Med. Sci. Res.,* **15**, 831 (1987).
23) F. Hayase, S. Hirashima, G. Okamoto, and H. Kato, *Agric Biol. Chem.,* **53**, 3383 (1989).
24) T. Hatano, R. Edamatsu, M. Hiramatsu, A. Mori, Y. Fuzita, T. Yasuhara, T. Yoshida, and T. Okuda, *Chem. Pharm. Bull.,* **37**, 2016 (1989).
25) E. G. Janzen, D. E. Nutter, and E. D. Davis, *Can. J. Chem.,* **56**, 2237 (1978).
26) N. B. Nazhat, G. Yang, R. E. Allen, D. R. Blake, and P. Jones, *Biochem. Biophys. Res. Commun.,* **166**, 807 (1990).
27) T. Ozawa and A. Hanaki, *Biochem. Biophys. Res. Commun.,* **136**, 657 (1986).
28) A. Samuni, A. Samuni, and H. M. Swartz, *Free Radicals Med. Biol.,* **7**, 37 (1989).
29) G. R. Buettner, *Free Radicals Med. Biol.,* **3**, 259 (1987).
30) B. Kalyanaraman, C. Mottley, and R. P. Mason, *J. Biol. Chem.,* **258**, 3855 (1983).
31) I. Yamazaki and L. H. Piette, *J. Biol. Chem.,* **265**, 13589 (1990).
32) J. M. C. Gutteridge, L. Maidt, and L. Poyer, *Biochem. J.,* **269**, 169 (1990).
33) C. C. Winterbourn, *Free Radicals Med. Biol.,* **3**, 33 (1987).
34) R. A. Floyd, *Biochim. Biophys. Acta,* **756**, 204 (1983).