

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

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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 ($R\cdot$), alkoxyl ($RO\cdot$), and then peroxy ($ROO\cdot$) 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 oxygens⁸⁻¹⁰⁾ as of free radicals.¹¹⁻²³⁾

The present study was carried out in order to establish a detection method for such free radicals as $O_2^{\cdot-}$, $\cdot OH$, $\cdot H$, $\cdot CH_3$ ($R\cdot$), $(CH_3)_3CO\cdot$ ($RO\cdot$), and $(CH_3)_3COO\cdot$ ($ROO\cdot$). The six radical-generating systems were: hypoxanthine–xanthine oxidase which generates $O_2^{\cdot-}$ and $\cdot OOH$; iron(II)–hydrogen peroxide which gives $\cdot OH$; iron(III)–hydrogen peroxide, $\cdot OH$; iron(II)–*t*-butyl hydroperoxide and iron(III)–*t*-butyl hydroperoxide, $R\cdot$, $RO\cdot$, $ROO\cdot$, and CAT–*t*-butyl hydroperoxide, $RO\cdot$. 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 $O_2^{\cdot-}$, $\cdot OH$, $ROO\cdot$, and/or $RO\cdot$ 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 Toatsu 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 μ l.

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

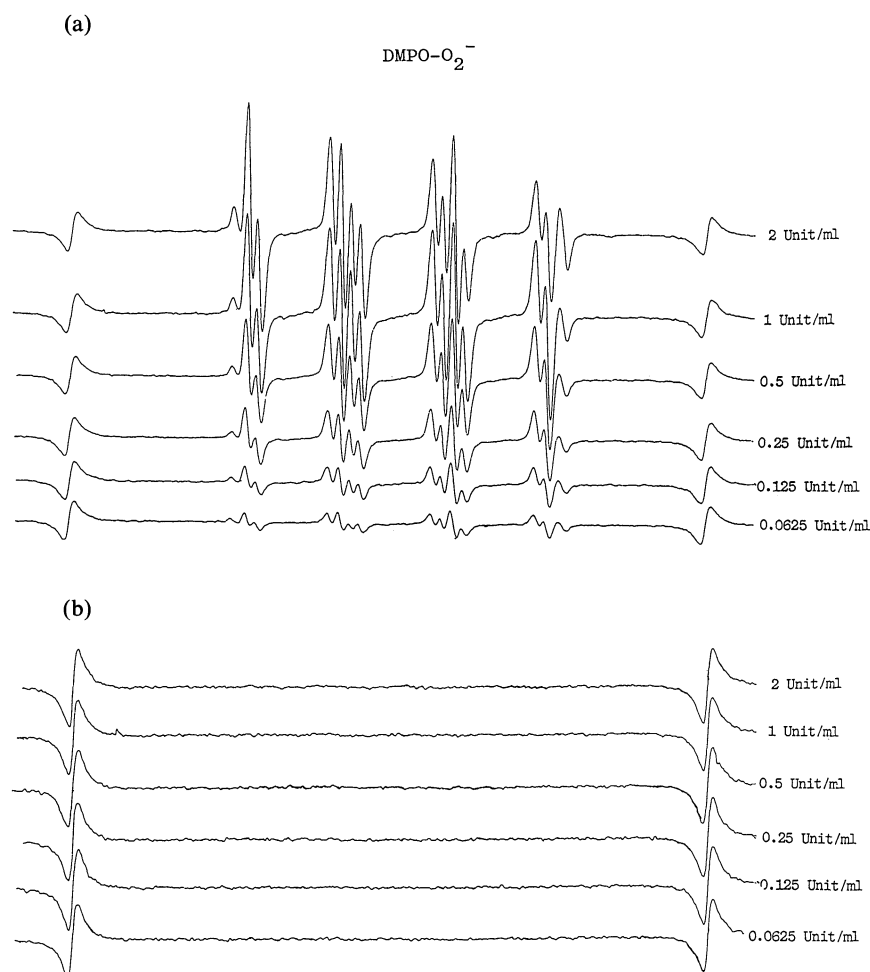


Fig. 1. ESR spectra of spin adducts of $O_2^{\cdot -}$, observed by the use of spin traps DMPO (a) and DNBBS (b). Spectrum (a) recording started 30 s after mixing 50 μ l 2 mM HPX, 35 μ l 5.5 mM DETAPAC, 15 μ l 9.2 M DMPO (a), 50 μ l 0.1—0.5 U ml $^{-1}$ XOD, and 50 μ l PBS. Spectrum (b) was observed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

adducts obtained in the hypoxanthine–xanthine oxidase reaction by using two kinds of spin traps, DMPO and DNBBS, 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 $DMPO-O_2^{\cdot -}$ reported previously.⁸⁾ Using the spin trap DNBBS instead of DMPO, no signals were observed under the same conditions (Fig. 1b). However, the addition of DNBBS (60 mM) reduced the signal intensity of $DMPO-O_2^{\cdot -}$. From the experimental results for changing the concentration of DNBBS, the value of 50% inhibition (ID_{50}) of $DMPO-O_2^{\cdot -}$ was

measured to be 0.3 mM. The reaction rate constant (k_2) of DNBBS with $O_2^{\cdot -}$ in a hypoxanthine–xanthine oxidase reaction were determined to be $3.9 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ 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, $6.6 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ 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 DNBBS 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 DNBBS. 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 DNBBS, respectively, in reactions between 0.1 mM iron(II) chelated by DETAPAC (0.1 mM) and H_2O_2 (1 mM) in pure water.

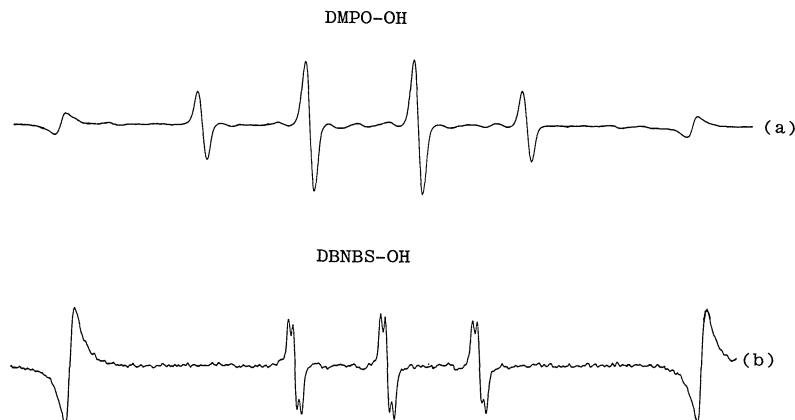


Fig. 2. ESR spectra of spin adducts of $\cdot\text{OH}$ observed by the use of spin traps DMPO (a) and DNBNS (b). Spectra recording started 30 s after mixing 75 μl 0.1 mM iron(II) chelated by 0.1 mM DETAPAC before use [iron(II)-DETAPAC], 75 μl 1 mM H_2O_2 , 50 μl 60 mM DMPO and 60 mM DNBNS, in 50 μl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

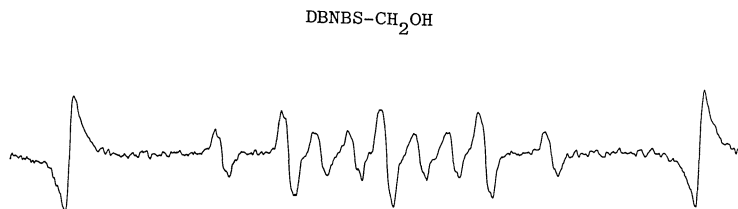


Fig. 3. ESR spectra of spin adducts of $\cdot\text{CH}_2\text{OH}$ after mixing 75 μl 0.1 mM iron(II)-DETAPAC, 75 μl 1 mM H_2O_2 , 15 μl 60 mM DNBNS and 50 μl 1.6 mM methanol.

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 DNBNS, 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 DNBNS- O_2^- . In order to confirm that this radical species was DNBNS-OH, the methanol, which is a specific scavenger of $\cdot\text{OH}$, 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 (DNBNS- CH_2OH ; $a_N=1.36$ mT, $a_H=0.91$ mT and $a_{H\beta}=0.06$ mT),^{20,27)} is detected as is shown in Fig. 3. In addition, a concomitant increase in the intensity of the DNBNS-OH signal is observed with increasing amount of added H_2O_2 (Fig. 4). Thus, $\cdot\text{OH}$ can be trapped not only by DMPO, but

also by DNBNS. The rate constant (k_2) for the reaction of DNBNS with $\cdot\text{OH}$ is determined to be $2.2 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$, as measured from its competitive reaction with DMPO in trapping $\cdot\text{OH}$ radicals. In this treatment, $k_2=3.4 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ ¹⁹⁾ was used as the rate constant for the reaction of DMPO to trap $\cdot\text{OH}$. From these values, DNBNS is more sensitive than DMPO for detecting $\cdot\text{OH}$.

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 ($\cdot\text{H}$ and $\cdot\text{OH}$) 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 ($\text{R}\cdot$), *t*-butoxyl ($\text{RO}\cdot$), and *t*-butylperoxyl ($\text{ROO}\cdot$) radicals (Fig. 6). However, when DNBNS was used, only the spin adduct of methyl radical (DNBNS- CH_3 ; $a_N=1.37$ mT and $a_{\text{CH}_3}=1.35$ 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

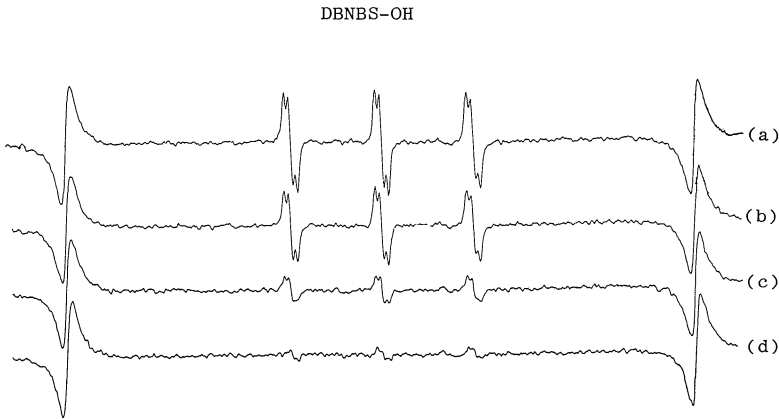


Fig. 4. ESR spectra of spin adducts of $\cdot\text{OH}$ observed by the use of spin trap DBNBS. 75 μl 0.1 mM iron(II)-DETAPAC, 75 μl of (a) 1 mM, (b) 0.5 mM, (c) 0.25 mM and (d) 0.125 mM H_2O_2 concentration, 15 μl 60 mM DBNBS, in 50 μl pure water.

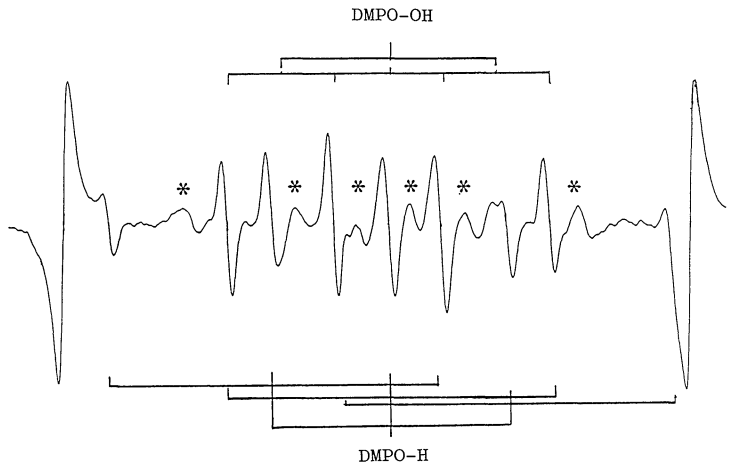


Fig. 5. ESR spectra of spin adducts of $\cdot\text{OH}$ and $\cdot\text{H}$ after mixing 75 μl 0.1 mM iron(II)-DETAPAC, 75 μl 0.01 mM H_2O_2 , 15 μl , 0.92 M DMPO, in 50 μl pure water. The signal with "*" is the adduct of carbon center radical.

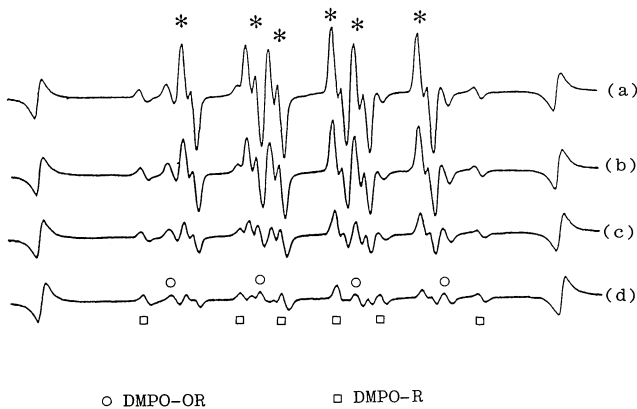


Fig. 6. ESR spectra observed in the reaction between 0.1 mM iron(II)-DETAPAC and ROOH of (a) 10 mM, (b) 5 mM, (c) 2.5 mM, (d) 1.25 mM ROOH concentration. The signal with "*" is the adduct of $\text{ROO}\cdot$.

Table 1. Hyperfine Coupling Constants of Spin Adduct of Radicals, $\text{O}_2^{\cdot-}$, $\cdot\text{OH}$, $\cdot\text{H}$, $\text{R}\cdot$, $\text{RO}\cdot$, and $\text{ROO}\cdot$.^{a)}

Spin adduct.	a_N	a_H^β	a_H^γ
DMPO- $\text{O}_2^{\cdot-}$	1.41	1.14	0.13
DMPO- $\cdot\text{OH}$	1.48	1.48	
DMPO- $\cdot\text{H}$	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 $\text{RO}\cdot$ is observed (Fig. 8). The spin adducts of $\text{R}\cdot$, $\text{RO}\cdot$, and $\text{ROO}\cdot$ were identified using the hfcc values listed in Table 1.^{29,30)} As shown in Figs. 7 and 8, the spin adducts of $\text{ROO}\cdot$ and $\text{RO}\cdot$ changed, depending on each concentration of iron(II), CAT and ROOH.

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

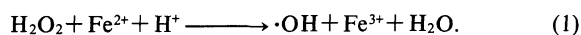
Compound	$O_2^{\cdot-}$ ^{a)}	$\cdot OH$ ^{b)}	$ROO\cdot$ ^{c)}	$RO\cdot$ ^{d)}
L-Ascorbic acid	93.3	70.3	0	0
D-Isoascorbic acid	100.0	100.0	0	0
Sorbic acid	0	0	0	5.8
Protocatechuic acid	63.6	0	10.3	3.0
Gallic acid	96.5	0	0	28.5

Measurement conditions for generating $O_2^{\cdot-}$, $\cdot OH$, $RO\cdot$, $ROO\cdot$ 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) $\cdot OH$, 0.1 mM iron(II)-DETAPAC+1 mM H_2O_2 +0.92 M DMPO. c) $ROO\cdot$, 1.0 mM iron(II)-DETAPAC+5 mM ROOH+0.07 M DMPO. d) $RO\cdot$, 1.0 mM iron(II)-DETAPAC+5 mM ROOH+0.07 M DMPO.

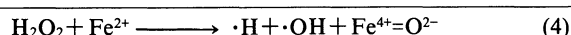
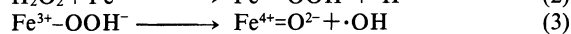
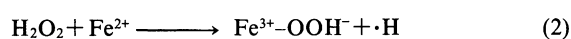
acid $\gg$ L-ascorbic acid = D-isoascorbic acid = 0%. In the case of $ROO\cdot$, only protocatechuic acid show the function.

Reaction Mechanisms

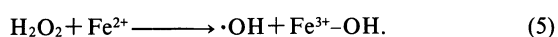
The Fenton's Reaction. It has been reported that the formation of $\cdot OH$ in a Fenton's reaction was directly confirmed using the spin trapping technique.^{31,32)} However, the reaction mechanism between iron(II) and H_2O_2 was assumed³³⁾ to be



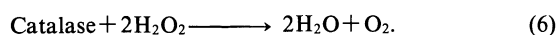
In our experiment two radical species ($\cdot OH$ and $\cdot H$) were observed in a Fenton's reaction used the H_2O_2 of low concentration for the concentration of iron(II). We thus propose the following reaction scheme, whereby both $\cdot OH$ and $\cdot H$ are generated:



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

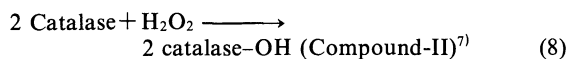
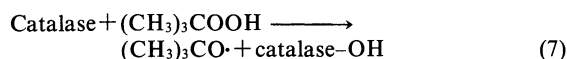


Catalase Reaction. As in the case of a Fenton's reaction, this catalase reaction mechanism with regard to the radical reaction has not been proven experimentally. The proposed catalase reaction mechanism is according to

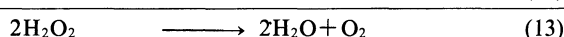
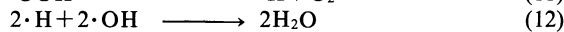
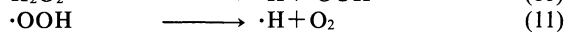
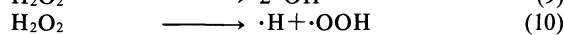
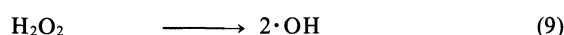


To clarify the mechanism for the oxidation of H_2O_2 , the reaction of iron(II), iron(III), or ROOH with CAT and

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:



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:



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 ($RO\cdot$, $R\cdot$, and $ROO\cdot$). Catalase reacts with ROOH, and generates $RO\cdot$. 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 $O_2^{\cdot-}$, $\cdot OH$, $\cdot H$, $R\cdot$, $RO\cdot$, and $ROO\cdot$ 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 $O_2^{\cdot-}$, $\cdot OH$, $ROO\cdot$, and $RO\cdot$.

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