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Hydroxyl Radical produced by the Reaction of Superoxide Ion with Hydrogen Peroxide: Electron Spin Resonance Detection by Spin Trapping

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The spin trap, 5,5-dimethyl-1-pyrroline-1-oxide has been used to detect the OH radical produced by the Haber-Weiss reaction between superoxide ion and hydrogen peroxide. In the presence of a small amount of H_2O_2, the OH radical adduct can be detected by electron spin resonance spectroscopy, while, H_2O_2 being added excessively, the HO_2 radical adduct can be detected.

Keywords——ESR; spin trapping; 5,5-dimethyl-1-pyrroline-1-oxide; superoxide ion; hydroxyl radical; hydroperoxy radical; Haber-Weiss reaction

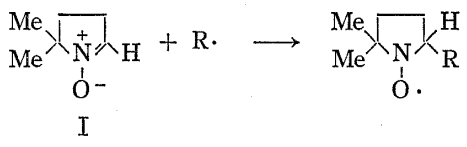
It is well known that superoxide ion, O_2^-, which is an active species of molecular oxygen, disproportionates spontaneously to yield H_2O_2 and O_2 (equation (1)).[2]

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad (1)$$

In the presence of H_2O_2, O_2^- reacts with H_2O_2 to yield the OH radical (Haber-Weiss reaction).[3] The Haber-Weiss reaction has been proposed as the main path of the formation of the OH radical in biological systems producing O_2^-.[4] Considerable evidences that the OH radical is produced from O_2^- are demonstrated by employing the scavengers of the OH radical.[4a,b] Production of the OH radical seems to be inhibited by catalase,[4a,b] suggesting that H_2O_2 is required for the reaction.[5] On the contrary, Fee, *et al.* have reported that H_2O_2 had little effect on the rate of decay of O_2^- in an aqueous solution.[6]

We have been studing the reactivities of O_2^-.[7] The present study is attempted to demonstrate that the OH radical is produced by the Haber-Weiss reaction. For this purpose, we have employed 5,5-dimethyl-1-pyrroline-1-oxide (I; DMPO) as a spin trap. A spin trap reacts with short-lived free radicals, *e.g.* the OH radical, to yield a spin adduct having a longer lifetime. The resultant spin adduct can be characterized by electron spin resonance (ESR) spectroscopy which gives an information about the original free radical.

Recently Bolton, *et al.* have applied this technique to biological systems and detected OH[8a] and O_2^- or HO_2 radical[8a,b] adduct of DMPO in an aqueous solution. DMPO reacts rapidly with the short-lived free radical (R·) as follows:



I (2)

1) Location: *Anagawa, Chiba-shi, 280, Japan.*
2) I. Fridovich, *Accts. Chem. Res.*, **5**, 321 (1972).
3) F. Haber and J. Weiss, *Proc. Roy. Soc. London Ser. A*, **147**, 332 (1934).
4) *a*) I. Fridovich, *Ann. Rev. Biochem.*, **44**, 147 (1975); *b*) W. Bors, M. Saran, F. Lengfelder, R. Spottl and C. Michel, *Curr. Top. Rad. Res. Q.*, **9**, 247 (1974); *c*) B. Halliwell, *New Phytol.*, **73**, 1075 (1974).
5) E.K. Hodgeson and I. Fridovich, *Biochim. Biophys. Acta*, **430**, 182 (1976).
6) G.J. McClune and J.A. Fee, *FEBS Lett.*, **67**, 294 (1976).
7) T. Ozawa, A. Hanaki, and H. Yamamoto, *FEBS Lett.*, **74**, 99 (1977).
8) *a*) J.R. Harbour, V. Chow, and J.R. Bolton, *Can. J. Chem.*, **52**, 3594 (1974); *b*) J.R. Harbour and J.R. Bolton, *Biochem. Biophys. Res. Comm.*, **64**, 803 (1975).

The informations obtained by the ESR spectrum can be used to identify R·, because both the β-proton hyperfine splitting and the nitrogen hyperfine splitting of the nitroxide spin adduct are very sensitive to the nature of R·.[9]

By using DMPO, we can demonstrate that the OH radical is, in fact, generated in the Haber-Weiss reaction.

Experimental

ESR measurements were carried out on a JEOL-PE-IX spectrometer (X-band) with 100 kHz field modulation. ESR spectra of reaction solutions were measured in a quartz flat cell at room temperature. The O_2^- solution was obtained from electrochemical reduction of oxygen in acetonitrile by using tetra-*n*-propylammonium perchlorate as a supporting electrolyte.[7] DMPO was a gift from Dr. Y. Kirino, The University of Tokyo. Acetonitrile used was spectroquality grade from Wako Pure Chemical Industries (Osaka, Japan). Other chemicals used were reagent grade.

Results and Discussion

Mixing O_2^- with H_2O_2 causes the Haber-Weiss reaction to yield the OH radical (equation-(3)). The OH radical may react with H_2O_2 to yield the HO_2 radical (equation(4)), the concentration of which increases with an increase of H_2O_2 concentration. The HO_2 radical formed is in equilibrium with O_2^- ($pK_a=4.4\pm0.4$[10])(equation(5)).

$$O_2^- + H_2O_2 \longrightarrow O_2 + OH\cdot + OH^- \quad (3)$$

$$OH\cdot + H_2O_2 \longrightarrow H_2O + HO_2\cdot \quad (4)$$

$$HO_2\cdot \rightleftarrows H^+ + O_2^- \quad (5)$$

We attempted to investigate the above mechanisms by use of DMPO as a spin trap.

Figure 1 represents the ESR spectrum obtained when the O_2^- solution was added to an acetonitrile solution containing DMPO. This spectrum can be analyzed in terms of the parameters: $A(N)=14.20$ G and $g=2.0058$. The observed hyperfine splitting constant is in agreement neither with that reported for O_2^- or HO_2 adduct to DMPO ($A(N)=14.3$ G, $A^\beta(H)=11{,}7$ G, $A^\gamma(H)=1.25$ G and $g=2.0061$),[8] nor with that of the oxidation product of DMPO, namely 5,5-dimethylpyrrolidone-2-oxyl-1($A(N)=7.1$ G, $A^\gamma(H)=4.2$ G and $g=2.0065$).[11] Then, a radical species observed in Fig. 1 is assumed to be a reduction product of DMPO, although its structure can not be elucidated at present time.

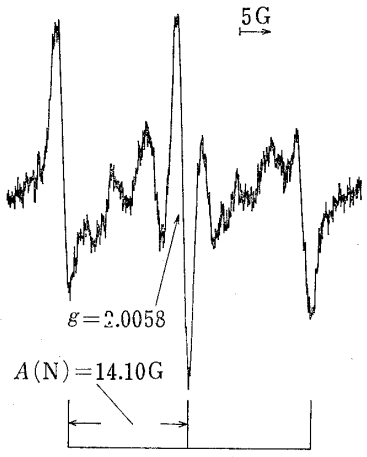


Fig. 1. The ESR Spectrum observed by the Reaction of O_2^- with DMPO in Acetonitrile

Reaction conditions were as follows: 5.4 mM O_2^-, 50 mM DMPO. Instrument settings: microwave 10 mW; modulation amplitude 0.5 G; time const. 0.3 sec.; scan time 8 min.

When the O_2^- solution was added to the solution containing DMPO and 1% (v/v) H_2O_2,[12] the ESR spectrum in Fig. 1 had almost disappeared and a new ESR spectrum as shown in Fig. 2 was observed. This spectrum can be analyzed in terms of the parameters: $A(N)=14.10$ G, $A^\beta(H)=12.29$ G and $g=2.0060$. On increasing the H_2O_2 concentration to 5% (v/v) in the presence of DMPO, another ESR spectrum different from Fig 2 appears after the reaction (Fig. 3). Figure 3 is composed of two

9) E.G. Janzen and J. I-Ping Liu, *J. Mag. Resonance*, **9**, 510 (1973).
10) G. Czapski and B.H.J. Bielski, *J. Phys. Chem.*, **67**, 2180 (1963); K. Sehested, O.L. Rasmussen, and H. Fricke, *J. Phys. Chem.*, **72**, 626 (1968).
11) R.A. Floyd and L.M. Soong, *Biochem. Biophys. Res. Comm.*, **74**, 79 (1977).
12) This acetonitrile solution did not give any ESR signal.

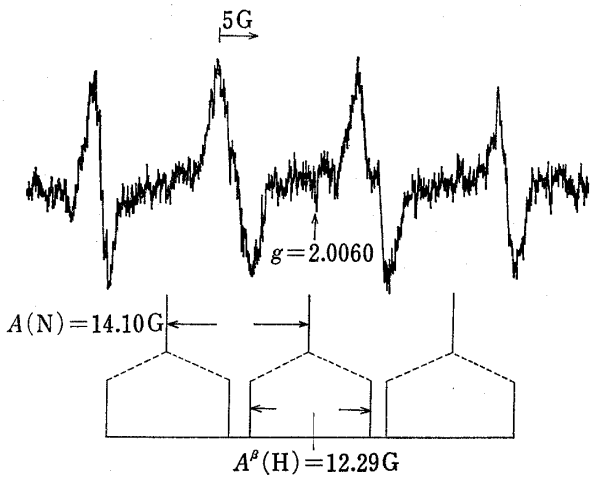


Fig. 2. The ESR Spectrum observed by the Reaction of O_2^- with H_2O_2 in the Presence of DMPO

Reaction conditions were as follows: 5.2 mM O_2^-, 1% (v/v) H_2O_2 and 10 mM DMPO.
Instrument settings; see in Fig. 1.

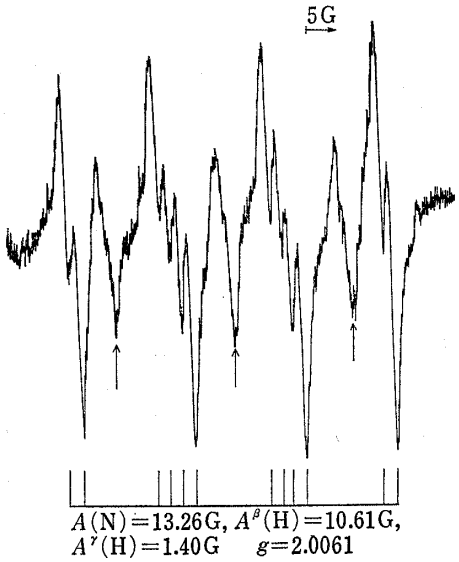
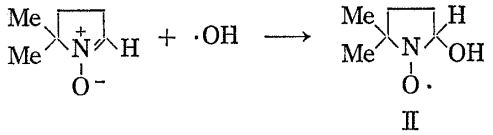


Fig. 3. The ESR Spectrum obtained by the Reaction of O_2^- with H_2O_2 in the Presence of DMPO

Reaction conditions were the same as described under Fig. 2 except that the concentration of H_2O_2 varied to 5% (v/v).
Instrument settings; see in Fig. 1.
The signal denoted by the arrows is identical to the signal in Fig. 1.

spectra: the main spectrum is characterized by $A(N)=13.26$ G, $A^\beta(H)=10.61$ G, $A^\gamma(H)=1.25$ G and $g=2.0061$, and another (showed by the arrows) is the same as the spectrum in Fig. 1.

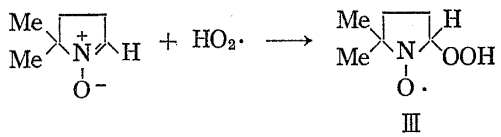
These ESR spectra are due to the radical species produced by equations (3), (4) and (5). Thus, with a small amount of H_2O_2, the OH radical is a main radical species and in the presence of DMPO, it can be trapped as follows:



(6)

II

Radical (II) is assumed to be responsible for the spectrum as shown in Fig. 2. On increasing the H_2O_2 concentration, the OH radical may react with H_2O_2 rather than be trapped by DMPO, and the HO_2 radical formed may also be trapped by DMPO as follows:



(7)

III

Radical (III) is assumed to be responsible for the spectrum in Fig. 3. In this case, the same ESR signal as Fig. 1 was observed, because O_2^- may be in equilibrium with the HO_2 radical (eq. (5)).

In studies of the ultraviolet photochemical dissociation of H_2O_2 in the presence of DMPO in an aqueous solution, Harbour, *et al.*[8] were able to obtain the ESR spectrum consisting of 1:2:2:1 quartet with hyperfine splittings of $A(N)=A^\beta(H)=15.3$ G and $g=2.0060$ at a small concentration of H_2O_2, while at a high concentration of H_2O_2 the ESR spectrum with hyperfine splittings of $A(N)=14.3$ G, $A^\beta(H)=11.7$ G, $A^\gamma(H)=1.25$ G and $g=2.0061$ was observed. The former signal was identified as the DMPO–OH radical adduct (II) and the latter was assigned as the DMPO–HO_2 radical adduct (III).

Our results are in rather good agreement with that of Harbour, *et al.*, by taking into consideration that the different solvents are used. However, they have reported that in the HO_2 radical adduct, it is still uncertain as to whether DMPO traps O_2^- directly (followed by protonation) or traps the HO_2 radical which is in equilibrium with O_2^-.[8b] In this point, our results suggest that DMPO traps the HO_2 radical in equilibrium with O_2^- directly, but does not trap O_2^-. This is supported by the fact that, when the O_2^- solution is added to an acetonitrile solution containing a small amount of HCl and DMPO, the same ESR spectrum as shown in Fig. 3[13] can be observed.

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13) The ESR spectrum in Fig. 3 was, of course, not observed when HCl was added to the mixture of O_2^- and DMPO.

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Reaction of 1-Methoxyimino-6-nitrohexa-2,4-diene, Resulting from the Reaction of N-Methoxypyridinium Iodide with Nitromethane

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Chemical transformations of 1-methoxyimino-6-nitrohexa-2,4-diene (II), resulted from the reaction of N-methoxypyridinium iodide (I) with nitromethane, were examined in relation to the nitro-aci-nitro prototropy. II reacted with diazomethane to afford methyl 1-methoxyimino-hexa-2,4-diene-6-acinitronate (III), with acetic anhydride and with benzoyl chloride to give O-acetate and O-benzoate of 1-methoxyimino-hexa-2, 4-diene-6-hydroxamic acid (VI) respectively. The ultraviolet spectra of them were correlated to their structures.

Keywords——N-methoxypyridinium salt; pyridine ring opening; nitro-acinitro prototropy; nitroalkane; hydroxamic acid; UV spectra

In the previous paper,[2)] it was found that the reaction of N-methoxypyridinium iodide (I) with nitromethane in the presence of sodium ethoxide proceeded *via* nucleophilic attack of nitromethane anion at the 2-position and subsequent N-C bond scission of the pyridine nucleus affording 1-methoxyimino-6-nitrohexa-2,4-diene (II). This paper deals with the reactions of II with some reagents and the spectral data of the products.

Treatment of II with ethereal diazomethane afforded a fairly stable monomethylated compound, mp 122—123° (dec.), $C_8H_{12}N_2O_3$, the structure of which was determined as depitched in Chart 1, since the infrared (IR) spectrum did not show any nitro stretching band but $\nu^{+}_{N=C}$ at 1608 cm^{-1} and the nuclear magnetic resonance (NMR) spectrum in deuteriochloroform ex-

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2) H. Takayama and T. Okamoto, *Chem. Pharm. Bull.* (Tokyo), **26**, 2422 (1978).