

腐植酸水溶液の光照射によって生成する活性酸素の同定

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Original Article

Identification of Reactive Oxygen Species Generated by Irradiation of Aqueous Humic Acid Solution

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Electron spin resonance (ESR) spectroscopy coupled with the spin-trapping or chemical-quenching method was applied to identify the reactive oxygen species generated by irradiation of aqueous humic acid solution. Upon xenon lamp irradiation of aqueous humic acid in the presence of spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), a spin adduct (DMPO-OH) of hydroxyl radical with DMPO was detected. Formation of the DMPO-OH was sensitive to superoxide dismutase, dioxygen and hydroxyl radical scavengers, indicating the formation of superoxide anion and hydroxyl radicals. The irradiation of aqueous humic acid solution in the presence of 2,2,6,6-tetramethyl-4-piperidinol resulted in the formation of 2,2,6,6-tetramethylpiperidine-1-oxyl-4-ol (TEMPOL) nitroxide radical. The ESR signal intensity of the TEMPOL radical was enhanced in deuterium oxide, and reduced in the presence of sodium azide and 1,4-diazabicyclo-[2.2.2]octane (DABCO), indicating the formation of singlet molecular oxygen. The generation of hydrogen peroxide was confirmed spectrophotometrically based on the peroxidase-catalyzed oxidation of leuco crystal violet.

INTRODUCTION

The photochemical transformation of xenobiotics in the aquatic environment takes place through both direct and indirect photochemical processes. Direct photolysis, in which a chemical absorbs incident radiation directly followed by degradation, proceeds in pure water.¹⁾ In indirect photolysis, an additional solute is required as a photosensitizer to transfer the absorbed energy to a chemical. Another sensitization mechanism is that a solute photochemically generates reactive oxygen species, which promote the decomposition of substrates.²⁾ Photosensitizers not only accelerate photolysis rates, but sometimes alter photodegradation pathways to a greater extent.³⁻⁵⁾ Recent studies have shown that naturally-occurring substances, such as humic acid, tryptophan and tyrosine, act as photosensitizers towards some kinds of pesticides in

the aquatic environment.^{3,6,7)} Although the exact processes of sensitization by these substances have not yet been clarified, tryptophan, tyrosine and nitrate are reported to generate a superoxide anion radical, hydrogen peroxide or hydroxyl radical upon UV irradiation in aqueous solution.⁸⁻¹⁰⁾ Humic acid is also reported to sensitize oxygenation by generating singlet molecular oxygen and other photo-reactions of organic chemicals by transferring electron energy.¹¹⁻¹⁴⁾ The generation of other reactive oxygen species from humic acid, however, has not been reported yet.

The present study is to identify reactive oxygen species generated in light-irradiated aqueous humic acid using an ESR coupled with the spin-trapping and spectrophotometric method. The spin-trapping technique involves the addition of a very reactive short-lived free radical to a diamagnetic compound, spin trap, to produce a relatively long-lived

free radical product, spin adduct, which can be easily studied by ESR.¹⁵⁾ In favorable cases, the resulting ESR spectrum allows the identification of the original reactive radical.

MATERIALS AND METHODS

1. Chemicals

Humic acid, hydrogen peroxide, 4-OH TEMP (2,2,6,6-tetramethyl-4-piperidinol), DETAPAC (diethylenetriamine-*N, N, N', N'', N'''*-pentaacetic acid), sodium azide and DABCO were obtained from Wako Pure Chemical Industries, Ltd. DMPO (5,5-dimethyl-1-pyrroline-*N*-oxide), catalase, SOD (superoxide dismutase, from bovine blood) and HRP (horseradish peroxidase, Type VI) were purchased from Sigma, and LCV (leuco crystal violet) from Tokyo Kasei Kogyo Co., Ltd. All the chemicals were used without further purification, excepting DMPO which was distilled under a reduced pressure immediately before use.

2. ESR Experiments

An aqueous humic acid was prepared as follows: humic acid (200 mg) was added to 250 ml of 0.1 M phosphate buffer (pH 8.0) containing 1 mM DETAPAC and then shaken with a mechanical shaker for 24 hr to afford 800 ppm aqueous humic acid. The final pH of the solution was adjusted to pH 7.0 by addition of 0.01 M hydrochloric acid. Sample solution was aspirated into an ESR flat cell (Model ES-LC-110, JEOL) with or without bubbling of nitrogen gas.¹⁶⁾ Samples were irradiated with an Iwaki 200 W Xe arc lamp in the cavity in the presence or absence of DMPO (200 mM) or 4-OH TEMP (100 mM) through a Pyrex filter to cut off the wavelengths of less than 290 nm. The light intensity was 720 $\mu\text{W}/\text{cm}^2$, as measured 1 cm away from the cavity grid using an Ushio radiometer (Model 65).

ESR measurements were carried out under continuous irradiation on a JEOL FE-2XG spectrometer equipped with a TM₁₁₀ cavity operating at 9.4 GHz with a 100 kHz modulation frequency.

3. Assay of Hydrogen Peroxide

Hydrogen peroxide was assayed spectrophotometrically based on the peroxidase-

catalyzed oxidation of LCV.¹⁷⁾ To 1 ml of LCV stock solution (50 mg of LCV in 100 ml of 0.5% hydrochloric acid) in a 10-ml volumetric flask, 1 ml of light-irradiated aqueous humic acid, 0.5 ml of HRP solution (0.25 mg/ml) and 4 ml of 1 M acetate buffer (pH 4.5) were added, followed by dilution to 10 ml with distilled water. After 10 min of incubation at room temperature, absorbance at 596 nm was determined using a Beckman DU-2 ultraviolet-visible spectrophotometer against the reference prepared in the same manner but without irradiation.

RESULTS

1. Detection of Superoxide Anion Radical ($\text{O}_2^{\cdot-}$) and Hydroxyl Radical ($\cdot\text{OH}$)

Irradiation of aqueous humic acid in the presence of DMPO resulted in an ESR spectrum consisting of a quartet with the intensity ratio of 1:2:2:1 as shown in Fig. 1(A). The spectrum was analyzed in terms of the parameters: $a_N = 14.9$ G (Gauss) and $a_H = 14.9$ G. These values are essentially identical to those found for the $\cdot\text{OH}$ spin adduct (DMPO-OH) of DMPO.¹⁸⁾ When the aqueous humic acid containing DMPO was irradiated in the presence of SOD and in a nitrogen atmosphere, the ESR signal intensity reduced as shown in Figs. 1(B) and 1(C), respectively. The findings indicate that $\text{O}_2^{\cdot-}$ was also formed upon irradiation of aqueous humic acid. The spin adduct (DMPO-OOH) of $\text{O}_2^{\cdot-}$ with DMPO was not detected, however. It is likely that DMPO-OOH once formed promptly degrades to DMPO-OH due to instability in this system. The addition of catalase to the reaction mixture prior to irradiation did not change the ESR spectrum of Fig. 1(A), as shown in Fig. 1(D). The hydroxyl radical formation by photolysis of hydrogen peroxide appears to be of little significance under the conditions tested. The ESR spectrum of the DMPO-OH was not observed in the absence of DMPO or humic acid (Figs. 1(E) and 1(F)). No ESR spectra were detected under dark conditions. In addition to the DMPO-OH, another radical showing the ESR spectrum of a triplet of doublets was detected with weak signal intensity as shown in Fig. 1(A). In the ESR spectrum the splitting constant of nitrogen

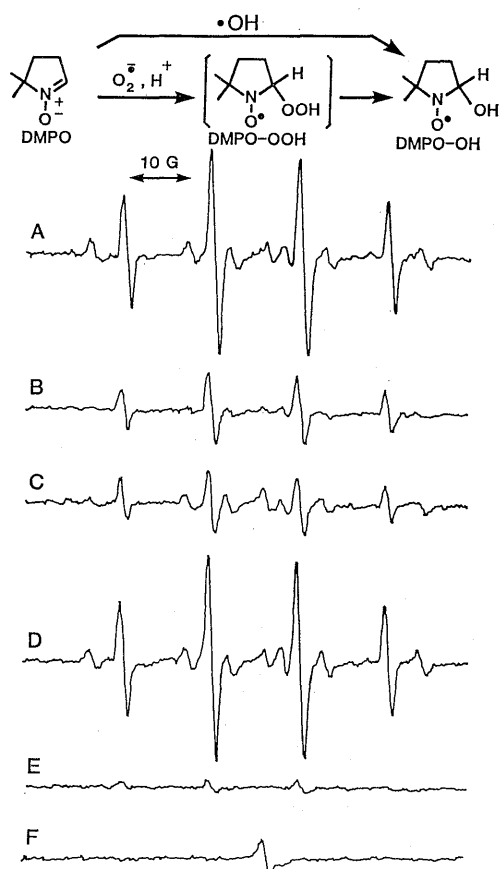


Fig. 1 ESR spectra obtained during irradiation of aqueous humic acid in the presence of DMPO. Scanning started 7 min after irradiation: (A) under aerobic conditions; (B) in the presence of SOD (0.4 mg/ml); (C) in a nitrogen atmosphere; (D) in the presence of catalase (0.06 mg/ml); (E) in the absence of humic acid; (F) in the absence of DMPO. Spectrometer setting: 10 mW, microwave power; 1 G, modulation amplitude; 0.3 sec, time constant; 2500, receiver gain; 100 G/4 min, scan rate.

($a_N = ca. 16.3$ G) was smaller than that of β -proton ($a_H = ca. 23.1$ G), suggesting the formation of DMPO adduct with the carbon-centered radical.¹⁹⁾

DMPO-OH can be formed directly by reacting DMPO with $\cdot\text{OH}$. To examine the formation of $\cdot\text{OH}$ by irradiation of aqueous humic acid, a series of kinetic competition experiments were carried out according to the method of Buettner *et al.*²⁰⁾ When an $\cdot\text{OH}$

Table 1 Effects of $\cdot\text{OH}$ scavengers on the formation of DMPO-OH^{a)} during irradiation of aqueous humic acid.

| Competitor | Rate constant with $\cdot\text{OH}$ ^{b)} ($10^9 \text{ M}^{-1} \text{ sec}^{-1}$) | DMPO-OH formed (relative %) |
|-----------------------------|--|-----------------------------|
| None | — | 100 |
| Ethanol (378 mM) | 1.9 | 86 |
| Dimethyl sulfoxide (103 mM) | 7.0 | 90 |
| Sodium formate (248 mM) | 2.9 | 90 |

^{a)} The rate constant of DMPO with $\cdot\text{OH}$ has been reported to be $3.4 \times 10^9 \text{ M}^{-1} \text{ sec}^{-1}$.²¹⁾

^{b)} The rate constants have been taken from Ref. 20).

scavenger, such as ethanol, dimethyl sulfoxide, or sodium formate, was added to the spin-trapping mixture prior to irradiation, the ESR signal intensity of DMPO-OH decreased by 10–14%, confirming that $\cdot\text{OH}$ had been produced by irradiation of aqueous humic acid (Table 1).

2. Detection of Singlet Molecular Oxygen ($^1\Delta_g\text{O}_2$ or $^1\text{O}_2$)

It is reported that sterically hindered amines, such as 4-OH TEMP and 2,2,6,6-tetramethyl-4-piperidone, selectively react with $^1\text{O}_2$ to form the corresponding nitroxide radicals.^{22,23)} When aqueous humic acid was irradiated in the presence of 4-OH TEMP, an ESR spectrum consisting of a triplet with $a_N = 17.03$ G was observed as shown in Fig. 2. The ESR parameters were in good accord with those of the TEMPOL nitroxide radical (2,2,6,6-tetramethylpiperidine-1-oxyl-4-ol).²⁴⁾ Although the signal had been weak in the ESR spectrum before irradiation, the intensity was significantly enhanced with irradiation. More TEMPOL radical was formed in D_2O buffer than in H_2O possibly because the lifetime of $^1\text{O}_2$ is more than tenfold longer in D_2O than in H_2O .²⁵⁾ In this case, the pD of the solution was maintained at 7, since the formation of TEMPOL is dependent on pH or pD.¹⁸⁾ In the presence of a $^1\text{O}_2$ quencher, such as sodium azide or DABCO, the radical formation was efficiently suppressed (Fig. 3), indicating that

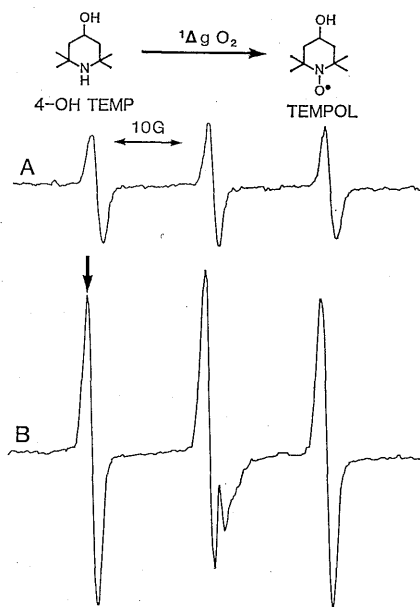


Fig. 2 ESR spectra of TEMPOL in aqueous humic acid in the presence of 4-OH TEMP before irradiation (A) and after 5-min irradiation (B) under aerobic conditions.

Spectrometer setting: 10 mW, microwave power; 1 G, modulation amplitude; 0.3 sec, time constant; 2500, receiver gain; 100 G/4 min, scan rate.

$^1\text{O}_2$ had been generated from the light-irradiated aqueous humic acid.

3. Detection of Hydrogen Peroxide

Hydrogen peroxide increased with time during irradiation, accounting for $14 \mu\text{M}$ after 1 hr of irradiation (Fig. 4). The formation of hydrogen peroxide was not detected when aqueous humic acid was irradiated in a nitrogen atmosphere. This indicates that the oxygen atom of hydrogen peroxide is derived from dissolved dioxygen in the aqueous solution.

DISCUSSION

Irradiation of aqueous humic acid resulted in the formation of reactive oxygen species such as superoxide, hydroxyl radical, singlet molecular oxygen and hydrogen peroxide. The formation and trapping scheme of the reactive oxygen species is summarized in Fig. 5. Light absorption promotes a humic acid molecule to

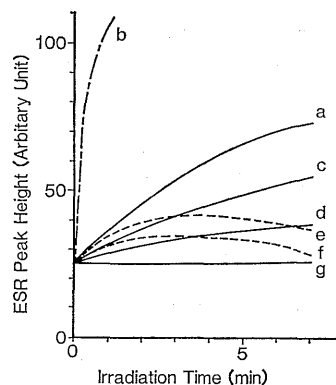


Fig. 3 Effects of deuterium oxide, sodium azide and DABCO on the formation of singlet molecular oxygen during irradiation of aqueous humic acid under aerobic conditions.

Magnetic field was fixed at the position indicated by the arrow in Fig. 2 (B): (a) in phosphate buffer, pH 7.0; (b) in D_2O phosphate buffer, pD 7.0; (c) and (d) same as (a) but in the presence of sodium azide at 1 mM and 5 mM, respectively; (e) and (f) same as (a) but in the presence of DABCO at 1 mM and 5 mM, respectively; (g) in the absence of humic acid. Spectrometer settings were the same as Fig. 2.

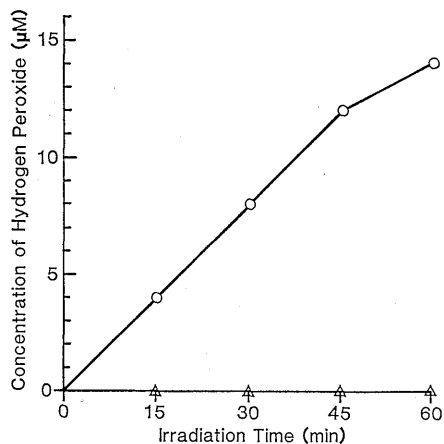


Fig. 4 Formation of hydrogen peroxide by irradiation of aqueous humic acid.

A linear calibration curve was obtained by photometric analysis in the range of $0.6 \mu\text{M}$ to $80 \mu\text{M}$ of hydrogen peroxide. ○: aerobic conditions, Δ: anaerobic conditions.

its first excited singlet state, $^1\text{HA}^*$.²⁶⁾ Singlet excited states, with lifetimes usually only on the order of 10 nsec or less, are far too short-

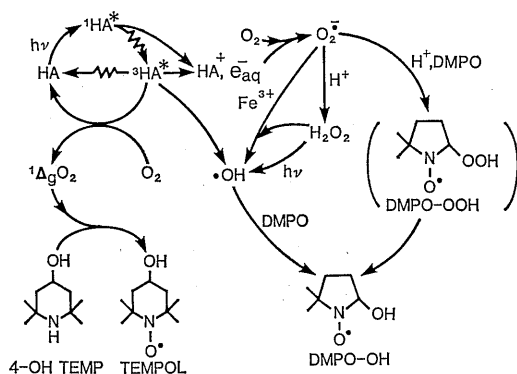
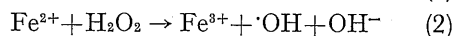
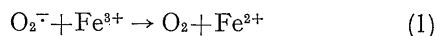


Fig. 5 Formation and trapping scheme of reactive oxygen species during irradiation of aqueous humic acid.

HA stands for humic acid.

lived to significantly interact with organic substances or dissolved dioxygen. $^1\text{HA}^*$ decays in part by undergoing intersystem crossing to the excited triplet states, $^3\text{HA}^*$, whose lifetimes are considerably longer. $^3\text{HA}^*$ can be deactivated *via* pathways including decay to the ground state together with heat formation and energy transfer to dissolved dioxygen or other substrates. Energy transfer rapidly progresses only if the triplet energy of a sensitizer equals to or exceeds that of an energy acceptor. The triplet state energy of humic acid is around 250 kJ/mol, more than the energy required to excite dioxygen, *i.e.*, 94 kJ/mol, to singlet molecular oxygen.²⁶⁾ $^1\text{HA}^*$ and $^3\text{HA}^*$ seem to generate hydrated electron,⁸⁾ which rapidly forms the superoxide anion radical through the reaction with dioxygen, at a rate constant of $2 \times 10^{10} \text{ M}^{-1} \text{ sec}^{-1}$.²⁷⁾ The formation of hydrated electron is also suggested by flash photolysis studies of humic chromophores.²⁸⁾ Although the ESR spectrum of the DMPO-OOH was not observed in the present study, there is the report that DMPO-OOH decomposes to DMPO-OH in phosphate buffer (pH 7.8).²⁹⁾ The half-life of DMPO-OOH formed in a light-riboflavin-DETAPAC system is pH dependent, ranging from 27 sec at pH 9 to 91 sec at pH 5 with a value of 58 sec at pH 7.³⁰⁾ Hydrogen peroxide is produced through the dismutation of superoxide.³¹⁾ The hydroxyl radical can be

generated by the reaction of superoxide with metal ions, followed by the reaction of hydrogen peroxide with reduced metal ions, the metal-catalyzed Haber-Weiss reaction,³²⁾ as shown in Eqs. (1) and (2).



Although a chelator DETAPAC, which can eliminate the interfering effect of metals,³¹⁾ was included in the reaction mixture in the present study, ferric ions ligated by humic acid may contribute to the Haber-Weiss reaction. The hydroxyl radical is also formed by photolysis of hydrogen peroxide but photolysis did not contribute significantly to the production of hydroxyl radical because the effect of catalase was weak. The hydroxyl radical would also be produced through homolytic cleavage of humic acid molecule upon irradiation.

Pesticides can be substrates for reactive oxygen species. Allethrin, phenothrin and tetramethrin react with singlet molecular oxygen to yield hydroperoxides by an ene mechanism, resmethrin yielding an endoperoxide.³³⁻³⁵⁾ Pyrimidine fungicides, such as ethirimol and dimethirimol and thiabendazole, degrade in the presence of singlet molecular oxygen in solution.^{36,37)} The hydroxyl radical enhances the decomposition of thiobencarb, molinate, aldrin and bromobutide *via* radical addition, hydrogen transfer or electron transfer.^{38,39)} Fenprothrin is decomposed by a peroxy radical to form a hydroxylated derivative at the 4-position of phenoxy group.⁴⁰⁾ Although degradation of pesticides by a superoxide anion radical has not been reported yet, superoxide undergoes efficient oxidation and reduction with organic and inorganic substrates having reduction or oxidation potentials superior to those of superoxide.⁴¹⁾ Superoxide also undergoes nucleophilic reactions with a number of substrates possessing reactive leaving groups and act as a ligand in a variety of metal complexes, since it is a weakly reactive radical and base.⁴¹⁾ Under certain conditions, superoxide produces a highly reactive hydroxyl radical.

The steady-state concentrations of reactive oxygen species in natural fresh waters, reaction

Table 2 Steady-state concentrations and reactivities of reactive oxygen species in the aquatic environment.

| Reactive oxygen species | Conc. [M] | Reactive substrate ^{a)} | Rate const. ^{a)} [M ⁻¹ sec ⁻¹] | Minimum half-life of substrate [hr] |
|--|--------------------------------------|----------------------------------|---|-------------------------------------|
| ¹ A _g O ₂ | ~10 ^{-12b)} | Furan, Trialkylamine | ~10 ⁸ | 1.9 |
| ROO· | ~10 ^{-9c)} | Hydroquinone | ~10 ⁶ | 0.19 |
| ·OH | ~10 ^{-16d)} | Phenol Hydroquinone | ~10 ¹⁰ | 19 |
| H ₂ O ₂ | 10 ⁻⁸ ~10 ^{-5e)} | — | Slow | — |

^{a)} Ref. 42), ^{b)} Ref. 43), ^{c)} Ref. 44), ^{d)} Ref. 45), ^{e)} Ref. 14).

rate constants of the most reactive substrates, and resultant disappearance half-life values are shown in Table 2. Singlet molecular oxygen, peroxy radical and hydroxyl radical seem to play an important role in degradation of the most reactive chemicals with half-lives of less than one day. Since hydrogen peroxide produces ·OH under sunlight³⁸⁾ or by the Fenton-type reaction, it would also be an important oxygen species in the natural aquatic environment, despite of its low reactivity.

Besides in natural waters, the formation of reactive oxygen species has been reported in solid and gas phases. Singlet molecular oxygen is detected in irradiated soils⁴⁶⁾ and on irradiated inorganic metal oxides such as silica gel, aluminum oxide and magnesium oxide.⁴⁷⁾ Superoxide is also been detected on irradiated metal oxide surfaces.⁴⁸⁾ In a gas phase, the presence of singlet molecular oxygen, hydroxyl radical, superoxide, hydrogen peroxide, atomic oxygen (³P, ¹D) and ozone is reported.⁴⁹⁾

Although photosensitization by electronic energy transfer from an additional solute is important for indirect photolysis processes, the reactive oxygen species generated by photolysis of naturally-occurring substances can also accelerate the transformation of chemicals in the environment to a greater extent, in some cases resulting in rapid decomposition of compounds that are stable to direct photolysis.

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要 旨

腐植酸水溶液の光照射によって生成する活性酸素の同定

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腐植酸水溶液の光照射によって生成する活性酸素を ESR(電子スピン共鳴)により検討した。スピントラップ剤 DMPO (5,5-dimethyl-1-pyrroline-*N*-oxide) 存在下, 腐植酸水溶液に光照射すると OH ラジカルと DMPO とのスピニアダクト (DMPO-OH) の ESR スペクトルを検出した。DMPO-OH の生成量はスーパーオキシドジスルターゼや OH ラジカルスカベンジャー存在下および嫌気条件下で抑制されたことより, スーパーオキシドアニオンラジカルおよび OH ラジカルの生成が明らかとなった。さらに, 2,2,6,6-tetramethyl-4-piperidinol 存在下の光照射では対応するニトロキシドラジカルが検出された。その生成量は重水中で増大するのに対して, アジ化ナトリウムや DABCO(1,4-diazabicyclo[2.2.2]octane) 存在下で抑制されたことより, 一重項酸素分子の生成が推察された。また, 光照射した腐植酸水溶液はペルオキシダーゼ存在下で leuco crystal violet を酸化することから, 過酸化水素の生成も示唆された。これらの活性酸素は自然環境下における農薬の光分解にきわめて重要な役割を果たすものと考えられる。