Acta Medica Okayama

Volume 40, *Issue* 3 1986 *Article* 5

JUNE 1996

Generation of active oxygen species by iron nitrilotriacetate (Fe-NTA).

Teruyuki Kawabata* Michiyasu Awai†

Masahiro Kohno‡

*Okayama University,

†Okayama University,

‡Japan Electric Optic Laboratory Ltd,

Generation of active oxygen species by iron nitrilotriacetate (Fe-NTA).*

Teruyuki Kawabata, Michiyasu Awai, and Masahiro Kohno

Abstract

Ferric nitrilotriacetate (Fe3+-NTA) solution showed maximum absorbance at pH 7.5. The iron was in ferric high-spin state and coordinated octahedrally with a relatively symmetric structure and also probably pentagonally. A spin trapping technique employing 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) yielded a DMPO spin adduct of unknown radical with three doublets (DMPO-Z) and a simple nitroxide radical (Y-NO.) in serum from rats injected intraperitoneally with Fe3+-NTA. When the Fe3+-NTA solution was diluted 500-fold with 50 mM NTA solution, DMPO-Z, Y-NO. and an additional signal, DMPO-OH were observed. The DMPO-Z signal was suppressed by a decrease in oxygen tension, alpha-tocopherol and 3-tert-butyl-4-hydroxy-anisole (BHA). The DMPO-OH signal was suppressed in the presence of ethanol and catalase. Fe2+-NTA solution hardly produced DMPO spin adducts. The Fe3+-NTA solution produced a strong DMPO-OH signal in the presence of H2O2. Rose Bengal solution, a singlet oxygen generating system, produced the same DMPO adducts. Fe3+-NTA reacted with oxygen in solution. The oxygen was activated and might be similar to singlet molecular oxygen. In the presence of H2O2, the Fe3+-NTA solution generated a hydroxyl radical. Fe3+-NTA itself generated free radicals, but Fe2+-NTA did not.

KEYWORDS: iron nitrilotriacetate, active oxygen, lipid peroxidation, electron spin resonance, 5, 5-dimethyl-1-pyrroline-N-oxide

---

*PMID: 3017051 [PubMed - indexed for MEDLINE]

Acta Med Okayama 40 (3) 163-173 (1986)

Generation of Active Oxygen Species by Iron Nitrilotriacetate (Fe-NTA)

Teruyuki Kawabata, Michiyasu Awai and Masahiro Kohno*

*Department of Pathology, Okayama University Medical School, Okayama 700, Japan; and *Electron Spin Resonance Application Laboratory, Japan Electric Optic Laboratory Ltd., Tokyo 196, Japan*

Ferric nitrilotriacetate (Fe^{3+}-NTA) solution showed maximum absorbance at pH 7.5. The iron was in a ferric high-spin state and coordinated octahedrally with a relatively symmetric structure and also probably pentagonally. A spin trapping technique employing 5, 5-dimethyl-1-pyrroline-N-oxide (DMPO) yielded a DMPO spin adduct of unknown radical with three doublets (DMPO-Z) and a simple nitroxide radical (Y-NO·) in serum from rats injected intraperitoneally with Fe^{3+}-NTA. When the Fe^{3+}-NTA solution was diluted 500-fold with 50 mM NTA solution, DMPO-Z, Y-NO· and an additional signal, DMPO-OH were observed. The DMPO-Z signal was suppressed by a decrease in oxygen tension, α-tocopherol and 3-tert-butyl-4-hydroxyanisole (BHA). The DMPO-OH signal was suppressed in the presence of ethanol and catalase. Fe^{2+}-NTA solution hardly produced DMPO spin adducts. The Fe^{3+}-NTA solution produced a strong DMPO-OH signal in the presence of H_2O_2. Rose Bengal solution, a singlet oxygen generating system, produced the same DMPO adducts. Fe^{3+}-NTA reacted with oxygen in solution. The oxygen was activated and might be similar to singlet molecular oxygen. In the presence of H_2O_2, the Fe^{3+}-NTA solution generated a hydroxyl radical. Fe^{3+}-NTA itself generated free radicals, but Fe^{2+}-NTA did not.

Key words : iron nitrilotriacetate, active oxygen, lipid peroxidation, electron spin resonance, 5,5-dimethyl-1-pyrroline-N-oxide

Ferric nitrilotriacetate (Fe^{3+}-NTA) is a weak iron chelate compound. Repeated intraperitoneal (i.p.) injections of this solution to rats and rabbits induced an iron overload similar to human hemochromatosis (1). A single i.p. injection of Fe^{3+}-NTA generated a free radical signal in the serum and liver of rats on electron spin resonance (ESR) at 123 K. This free radical was associated with lipid peroxidation in membranes of liver parenchymal cells liberating glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), γ-glutamyltranspeptidase (γ-GTP) (2,3). Increased lipid peroxidation and consequent membrane damage play a role in chronic iron toxicity (4). The hydroxy radical is a powerful oxidant and initiates lipid peroxidation (5,6). Some ferrous chelate compounds produce the hydroxy radical in the presence of H_2O_2 through a Fenton type reaction. On the other hand, it has been suggested that perferryl ions initiate lipid peroxidation through abstraction of hydrogen from polyunsaturated fatty acid (7-9). Bucher *et al.* (10) proposed that a ferrous-dioxygen-ferric chelate complex may initiate lipid peroxidation.

In the propagation of lipid peroxidation, iron compounds react with lipid peroxides

(LOOH) to give alkoxy radicals (LO·) or peroxy radicals (LOO·) (11):

$$LOOH + Fe^{2+}\text{-complex} \rightarrow Fe^{3+}\text{-complex} + OH^- + LO\cdot$$

$$LOOH + Fe^{3+}\text{-complex} \rightarrow Fe^{2+}\text{-complex} + H^+ + LOO\cdot$$

Both alkoxy and peroxy radicals propagate lipid peroxidation by abstracting hydrogen atoms.

The study here clarifies the spectrophotometric and ESR spectrometric structure of Fe^{3+}-NTA, and characterizes the free radicals in the serum of Fe^{3+}-NTA injected rats and the Fe^{3+}-NTA solution itself. We suggest that Fe^{3+}-NTA itself can generate some active oxygens.

Materials and Methods

Materials. 5, 5-Dimethyl-1-pyrroline-N-oxide (DMPO), superoxide dismutase (SOD) (3,000 units/mg protein), catalase (2,000 units/mg protein), 1,4-diazabicyclo [2,2,2] octane (DABCO) and Rose Bengal were purchased from Sigma Chemical Co., Ltd. (St. Louis). α-Tocopherol was obtained from Nakarai Chemicals Co., Ltd. (Tokyo) and 3-tert-butyl-4-hydroxyanisole (BHA) from Tokyo Kasei Kohgyo Co., Ltd. (Tokyo).

DMPO was used as received because there was no difference between commercial DMPO and DMPO purified by vacuum distillation (12).

Preparation of iron chelate solution. Fe^{3+}-NTA solution was prepared just before use by the method of Awai *et al.* (1). Iron stock solution (0.1 M) was made by dissolving ferric nitrate in 1.0 N HCl. NTA solution was made by dissolving disodium nitrilotriacetate (Na_2-NTA) (Eastman Kodak Co.) in distilled water. The NTA solution was added to stock solution, and the pH was adjusted by adding sodium bicarbonate powder under magnetic stirring. The Fe^{3+}-NTA solution was sealed until use. Fe^{3+}-deferoxamine (Desferal, Chiba Geigy Co.) solution was prepared by the same method. The Fe^{2+}-NTA solution was prepared just before use. Ferrous sulfate was dissolved in 50 mM NTA solution containing 30 mM HNO_3. The pH was adjusted with sodium bicarbonate, and the solution was sealed until use. The iron concentration was 17.9 mM (1 mg Fe/ml) in the *in vivo* experiment and 10 mM in the *in vitro* experiments. The molar ratio of iron-to-NTA was 1 : 1 in spectrometry and 1 : 5 in other experiments.

Spectrophotometry and ESR spectrometry. The absorbance of Fe^{3+}-NTA solution was measured at different pH with a Hitachi 230A spectrophotometer. Variation in the pH of the solution before and after use was kept minimized by sealing the cuvette. Corresponding to changes in absorbance, 500 μl of Fe^{3+}-NTA of different pH were placed into ESR tubes, and ESR spectra were recorded at 123 K with a JEOL ESR spectrometer, JES-1XG, equipped with 100 kHz magnetic field modulation.

In vivo experiment. Inbred male Wistar rats (200-300 g body weight) were divided into three groups. Rats of Group I (n=6) received a single i. p. injection of Fe^{3+}-NTA solution (pH 7.3) at 7.5 mg Fe/kg body weight. Rats of Group II (n=3) received a single i. p. injection of an equivalent amount of colloidal iron (Blutal, Dainippon Pharmaceutical Co.). Rats of Group III (n=3) received a single i. p. injection of an equivalent amount of NTA. Blood was taken from the retro-orbital sinus 60 min after injection, and the serum was separated. DMPO (10 μl) was added to 200 μl of serum. One sample (25 μl) of each reaction mixture was taken up into a capillary tube and the ESR spectra were recorded at room temperature.

Spin trapping in diluted Fe^{3+}-NTA solution. The original Fe^{3+}-NTA solution (pH 7.5) was diluted 500-fold with 50 mM NTA solution or distilled water. DMPO (10 μl) was added to 100 μl of diluted solution, and the ESR spectra were recorded 30 sec later.

Effect of oxygen on DMPO-Z. Diluted Fe^{3+}-NTA solution (100 μl, pH 7.5) was placed into two test tubes. One test tube was bubbled with nitrogen, and the other was left standing for 15 min. DMPO (10 μl) was added to each test tube. The reaction mixture (25 μl) was taken up into a capillary tube. ESR spectra were recorded at 1-min intervals for 7 min.

Effects of SOD and other scavengers on DMPO-Z. The concentrations of SOD, DABCO, catalase and ethanol were respectively 3,000

units/ml, 1.0 M, 2,000 units/ml in distilled water and 100 %. The concentrations of α-tocopherol and BHA were both 10 mM in dimethylsulfoxide(DMSO). These scavengers (10 μl) were added to 90 μl of diluted Fe^{3+}-NTA solution prior to addition of 10 μl of DMPO. The reaction mixture (25 μl) was taken up into a capillary tube. After the addition of DMPO, the ESR spectra were recorded at 1-min intervals for 7 min.

Spin trapping in Fe^{2+}-NTA solution. DMPO (10 μl) was added to 100 μl of the original Fe^{3+}-NTA and Fe^{2+}-NTA solutions. The reaction mixture (25 μl) was taken up into a capillary tube. After the addition of DMPO, the ESR spectra were recorded at 1-min intervals for 7 min.

Generation of hydroxyl radical by Fe^{3+}-NTA in the presence of H_2O_2. The original Fe^{3+}-NTA and -deferoxamine solutions (pH 7.3) were diluted 10-fold with distilled water. One hundred microliters of each solution were added to 100 μl of 0.01% H_2O_2 prior to the addition of DMPO. ESR spectra were recorded in a quartz flat cell 30 sec later.

Spin trapping in Rose Bengal solution. One test tube containing 100 μl of Rose Bengal solution (0.05 mg/ml (w/v)) was left standing and the other containing the same amount of the solution was bubbled with oxygen for 15 min. DMPO (10 μl) was added to each test tube. The reaction mixture was placed into a quartz flat cell. ESR spectra were recorded 30 sec after the addition of DMPO.

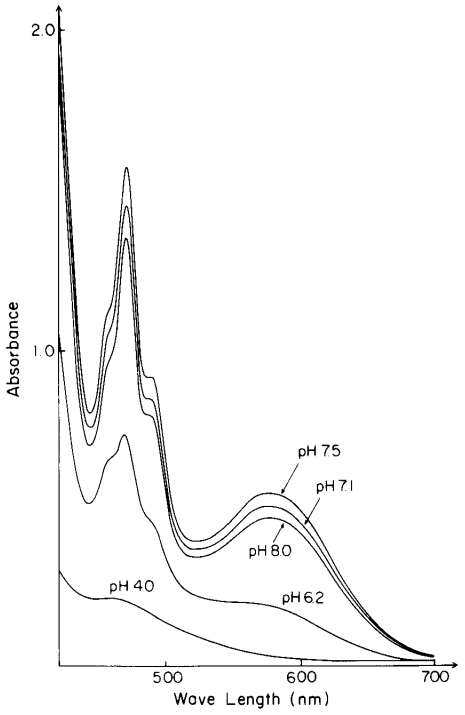


Fig. 1 Absorption spectra of Fe^{3+}-NTA solution at different pHs. The iron concentration was 10 mM, and the ratio of iron-to-NTA was 1 : 1. pH was adjusted with sodium bicarbonate. As the pH of the solution was increased, the Fe^{3+}-NTA solution showed a sharp absorbance at 470 nm with two shoulders at 458 nm and 488 nm and a broad absorbance at 575 nm. Maximum absorbance was at pH 7.5.

Results

The absorbance of 10 mM Fe^{3+}-NTA solution was measured at different pHs (Fig. 1). As the pH of the solution increased from 4.0 to 7.5, a sharp absorbance appeared at 470 nm with two shoulders at 458 nm and 488 nm, and a broad absorbance at 575 nm. The absorbance decreased at pH 8.0.

The ESR spectra of Fe^{3+}-NTA solution of different pH were recorded at 123 K (Fig. 2). At pH 4.0, Fe^{3+}-NTA showed a relatively symmetric ESR signal of g=4.3. At pH 5.0, the signal of g=4.3 decreased markedly, but no other signal appeared. At pH 6.0, Fe^{3+}-NTA showed new ESR signals of g=7.1, 6.0 and 2.0, in addition to g=4.3. As the pH of the Fe^{3+}-NTA solotion increased from 6.0 to 8.0, the highest g value shifted from 7.1 to 7.7.

The ESR spectra of DMPO spin adducts formed in rat sera containing DMPO were recorded at room temperature (Fig. 3). Serum one hour after a single i.p. injection of Fe^{3+}-NTA solution had a DMPO spin adduct of unknown radical with three doublets (DMPO-Z ; A_N=15.8, A^{β}_H=23.6) and a simple nitroxide radical (Y-NO·; A_N=17.8). The injection of neither Blutal

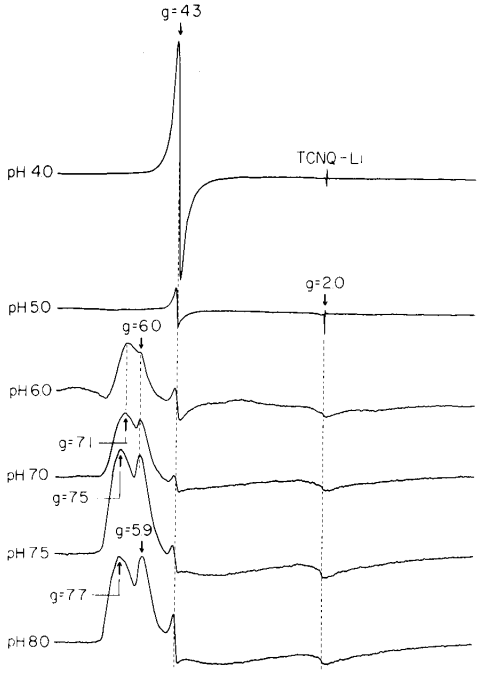


Fig. 2 pH dependence of the ESR spectra of Fe^{3+}-NTA solution at 123 K. The iron concentration was 10 mM, and the ratio of iron-to-NTA was 1 : 1. The pH was adjusted with sodium bicarbonate. ESR spectra were recorded under the following conditions : microwave power, 4.0 mW ; modulation amplitude, 4.0 G ; scanning field, 2,600±2,500 G ; amplitude, 6.3×10 at pH 4.0 and 5.0, and 6.3×100 at pH 6.0, 7.0, 7.5 and 8.0 ; sweep time, 4 min. At lower pHs, the iron in Fe^{3+}-NTA was in the ferric high-spin state and coordinated tetrahedrally with relative symmetry. At higher pHs, iron was in the same spin state, but with octahedral and more asymmetrical coordinations.

nor iron free NTA produced DMPO spin adducts in rat serum.

The ESR spectra of DMPO spin adducts formed in diluted Fe^{3+}-NTA solution containing DMPO were recorded at room temperature (Fig. 4). Fe^{3+}-NTA solution diluted with distilled water produced DMPO-Z (A_N=15.5, A^{β}_H=23.2), Y-NO· (A_N=17.4) and a 1:2:2:1 quartet (A_N=14.9, A^{β}_H=15.6). The last signal had a quartet pattern in the first differential, but a hyperfine splitting constant of $A_N \neq A^{\beta}_H$. The signal decreased rapidly and disappeared 1.5 min after the addition of DMPO. This quartet was not assigned. Fe^{3+}-NTA solution diluted with NTA solution produced DMPO-Z (A_N=15.9, A^{β}_H=23.5), Y-NO· (A_N=17.5) and an additional 1:2:2:1 quartet (DMPO-OH; $A_N=A^{\beta}_H$=15.2). DMPO-Z and Y-NO· were more intense in Fe^{3+}-NTA solution diluted with distilled water than with NTA solution.

In order to study the effect of oxygen in solution on the formation of DMPO-Z, Fe^{3+}-NTA solution was bubbled with nitrogen for 15 min prior to adding DMPO. The relative intensities of the first signal of DMPO-Z to the external standard of MnO were plotted against reaction time (Fig. 5). The production of DMPO-Z was suppressed

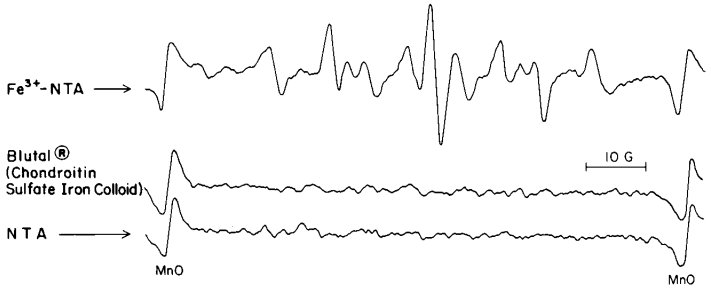


Fig. 3 ESR spectra of DMPO spin adducts formed in rat sera one hour after a single i. p. injection of Fe^{3+}-NTA, Blutal and iron free NTA. Ten microliters of DMPO were added to 100 μl of rat serum. ESR spectra were recorded 1 min after DMPO addition at room temperature under the following conditions : microwave power, 8.0 mW ; modulation amplitude, 2.0 G ; scanning field, 3,283 ± 50 G ; amplitube, 1×1,000 ; sweep time, 30 sec. DMPO-Z and Y-NO· were recorded only in sera of rats injected with Fe^{3+}-NTA. ESR parameters: DMPO-Z(A_N=15.8, A^{β}_H=23.6), Y-NO·(A_N=17.8).

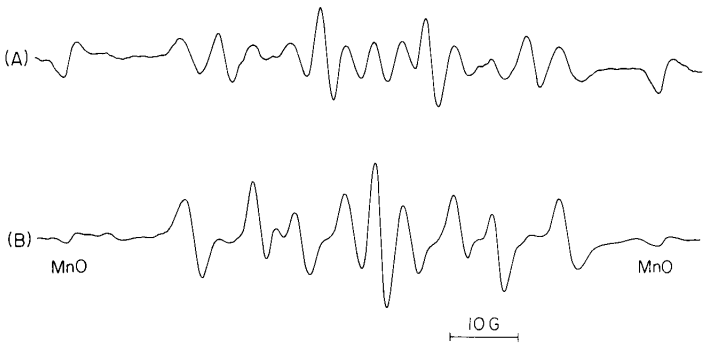


Fig. 4 ESR spectra of DMPO spin adducts formed in Fe^{3+}-NTA solution containing DMPO. The iron concentration of the original Fe^{3+}-NTA solution was 10 mM; the ratio of iron-to-NTA was 1 : 5, and the pH was 7.3. The original solution was diluted 500-fold with 50 mM NTA solution (A) and distilled water (B). Ten microliters of DMPO were added to 200 μl of each diluted solution. ESR spectra were recorded 30 min after the addition of DMPO at room temperature under the following conditions: microwave power, 8.0 mW; modulation amplitude, 2.0 G; scanning field, 3,345±50 G; amplitude, 3.2×100 in (A) and 2×100 in (B); sweep time, 30 sec. In (A), DMPO-Z (A_N=15.9, A_H^β=23.5), Y-NO· (A_N=17.5) and DMPO-OH (A_N=A_H^β=15.2) were produced. In (B), DMPO-Z (A_N=15.5, A_H^β=23.2) and Y-NO· (A_N=17.4) were produced. The intensities of DMPO-Z and Y-NO· were weaker in (A) than in (B).

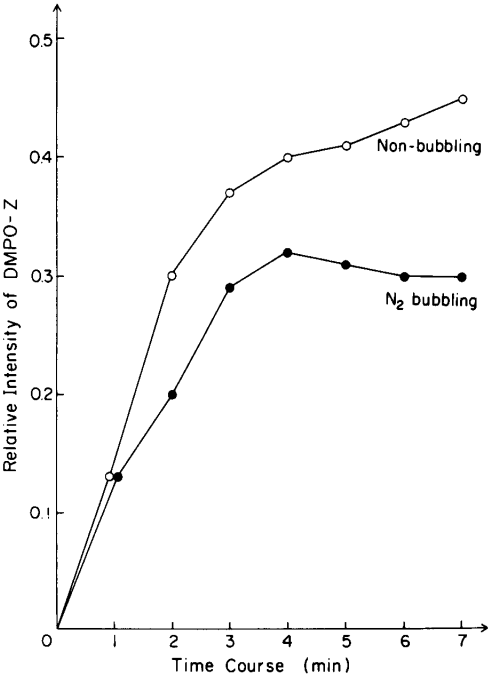


Fig. 5 Effect of oxygen in the solution on the formation of DMPO-Z. The original Fe^{3+}-NTA solution was diluted 500-fold with 50 mM NTA solution. One hundred microliters of diluted solution were placed into 2 test tubes. One test tube was bubbled with nitrogen (●——●), and the other was left standing (○——○) for 15 min. After addition of DMPO, ESR spectra were recorded at 1-minute intervals for 7 min. The relative intensities of the first signal of DMPO-Z to the external standard of MnO were plotted against reaction time. ESR spectra were recorded at room temperature under the following conditions: microwave power, 4.0 mW; modulation amplitude, 2.0 G; scanning field, 3,361±50 G; amplitude, 1×1,000; sweep time, 30 sec. The production of DMPO-Z was suppressed by nitrogen bubbling.

after nitrogen bubbling.

Fig. 6 shows the effects of SOD, DABCO, α-tocopherol and BHA on the formation of DMPO-Z in solution containing Fe^{3+}-NTA and DMPO. SOD and DABCO had no effect on the relative intensities of DMPO-Z (Fig. 6A), but α-tocopherol and BHA suppressed DMPO-Z production (Fig. 6B). Since DMSO suppressed DMPO-OH production, DMPO-OH was not detected. The effects of ethanol and catalase on the formation of DMPO-Z in solution containing Fe^{3+}-NTA and DMPO were also examined. Ethanol suppressed DMPO-OH production partially, and catalase completely suppressed it (Fig. 7).

ESR spectra of DMPO spin adducts formed in Fe^{3+}-NTA and Fe^{2+}-NTA solu-

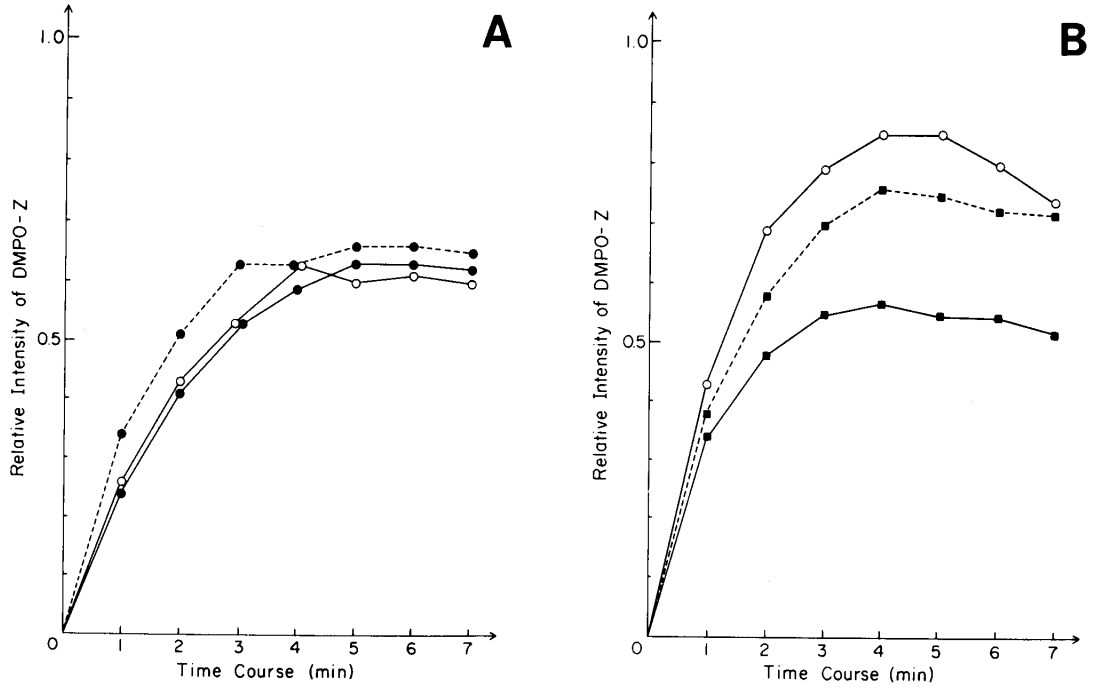


Fig. 6 Effect of SOD and DABCO (A), and α-tocopherol and BHA (B) on formation of DMPO-Z in solution containing Fe^{3+}-NTA and DMPO. Ten microliters of scavenger solution were added to 90 μl of diluted Fe^{3+}-NTA solution ($\times$500) with 50mM NTA solution prior to addition of 10 μl of DMPO. ESR spectra were recorded at 1-minute intervals for 7 min. The relative intensities of DMPO-Z were plotted against reaction time. SOD (●——●) and DABCO (●---●) had no effect on the formation of DMPO-Z. α-Tocopherol (■——■) and BHA (■---■) suppressed DMPO-Z production compared to the control (○——○).

tions were recorded at room temperature (Fig. 8). DMPO-Z was produced in Fe^{3+}-NTA solution, but there was no DMPO spin adduct formed in Fe^{2+}-NTA solution. These iron NTA solutions (10 mM) were further diluted with NTA solution. The diluted solutions formed the same spin adducts in both ferric and ferrous states (data not shown).

The ESR spectra of DMPO spin adducts formed in iron chelate solution containing H_2O_2 and DMPO were recorded at room temperature (Fig. 9). Fe^{3+}-NTA solution strongly produced a 1:2:2:1 quartet with $A_N = A^{\beta}_H = 15.0$ (DMPO-OH) in the presence of H_2O_2. Fe^{3+}-deferoxamine produced no DMPO spin adduct.

The ESR spectra of DMPO spin adducts formed in Rose Bengal solution containing DMPO with and without oxygen bubbling were recorded at room temperature (Fig. 10). Rose Bengal solution produced the same DMPO-Z ($A_N = 16.1$, $A^{\beta}_H = 23.2$), DMPO-OH ($A_N = A^{\beta}_H = 15.0$) and Y-NO· ($A_N = 17.8$) as those formed in Fe^{3+}-NTA solution. The intensities of DMPO-Z and DMPO-OH were increased by oxygen bubbling.

Discussion

The Fe^{3+}-NTA solution showed a sharp absorbance at 470 nm with two shoulders at 458 nm and 488 nm and a broad absorbance at 575 nm. The absorbances increased rapidly between pH 4.0 and pH 6.0, and were

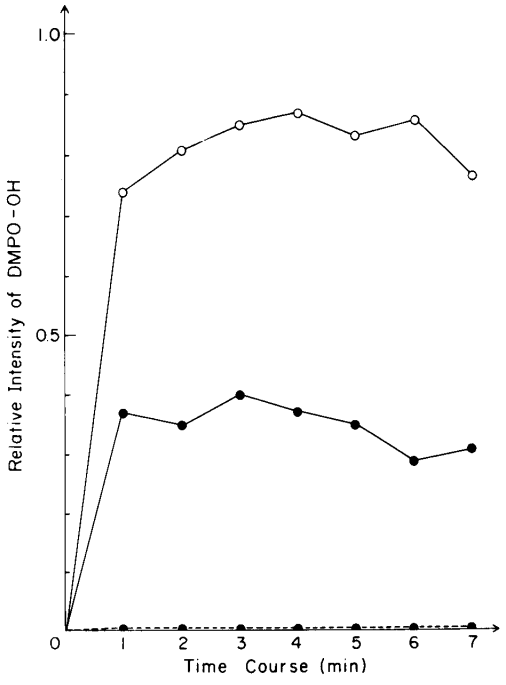


Fig. 7 Effect of ethanol and catalase on the formation of DMPO-OH in solution containing Fe^{3+}-NTA and DMPO. The procedure was the same as that described in the legend for Fig. 6. The relative intensities of the second signal of DMPO-OH were plotted against reaction time. Ethanol (●——●) suppressed DMPO-OH production partially, and catalase (●- - -●) completely suppressed it compared to the control (○——○).

maximum at pH 7.5. NTA has three dissociation constants of $pK_1=3.03$, $pK_2=3.07$ and $pK_3=10.70$. As the pH of the Fe^{3+}-NTA solution increased, the acetyl group dissociated and chelated ferric ion. At pH 8.0, hydroxy ions compete with acetate ions, and the Fe^{3+}-NTA chelate compound might have been unstable.

The ESR data at 123 K showed that Fe^{3+}-NTA had a relatively symmetric signal of $g=4.3$ at pH 4.0 and 5.0. Non-heme iron proteins such as transferrin, pyrocatechase (cathecol-1, 2-oxygenase) and rubredoxin produce ESR signals in the vicinity of $g=4.28$, as long as iron ions are in the ferric high-spin state (13-15). The coordinating structure of the iron atom in non-heme protein is not well known, but iron atoms are thought to be cooridinated tetrahedrally by 4 electronegative ligands (16). Thus, Fe^{3+}-NTA at pH 4.0 and 5.0 might be coordinated tetrahedrally in the ferric high-spin state. As the pH of Fe^{3+}-NTA solution increased, Fe^{3+}-NTA showed a signal of $g_{XY}=6.0$ and $g_Z=2.0$, and a signal of the highest g value. Most heme proteins

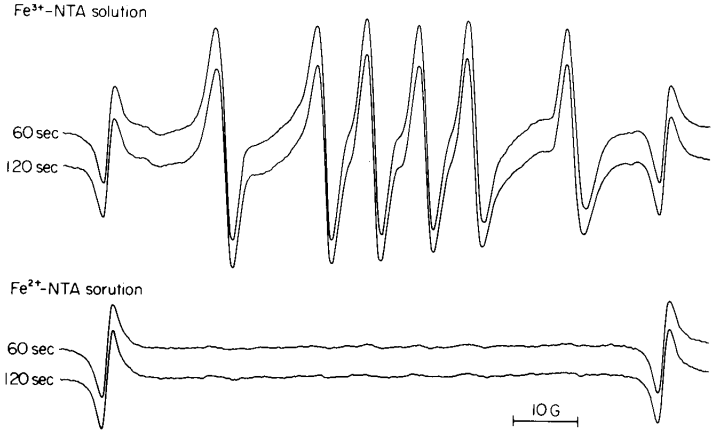


Fig. 8 ESR spectra of DMPO spin adducts in Fe^{3+}-NTA and Fe^{2+}-NTA. The iron concentration was 10 mM, and the molar ratio of iron-to-NTA was 1 : 5. The pH was adjusted to 7.5. Ten microliters of DMPO were added to 100 μl of each solution. Twenty five microliters of reaction mixture were taken up into a capillary. ESR spectra were recorded at room temperature 60 and 120 sec after the addition of DMPO under the following conditions: microwave power, 4.0 mW; modulation amplitude, 2.0 G; scanning field, $3,361\pm50$ G; amplitude, 6.3×100; sweep time, 30 sec. Fe^{3+}-NTA produced a high quantity of DMPO-Z, but Fe^{2+}-NTA hardly produced DMPO spin adducts.

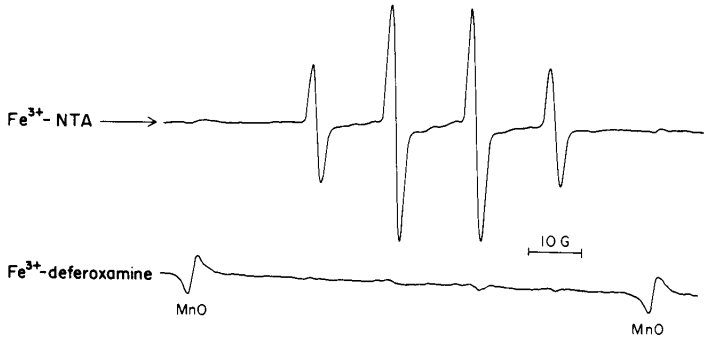


Fig. 9 ESR spectra of DMPO spin adducts formed in iron chelate solutions containing H_2O_2 and DMPO. Iron chelate solution was diluted 10 fold with distilled water. One hundred microliters of 0.01% H_2O_2 solution were added to 100 μl of diluted iron chelate solution, and 10 μl of DMPO were added to the reaction mixture. ESR spectra were recorded at room temperature 30 sec after the addition of DMPO under the following conditions: microwave power, 4.0 mW; modulation amplitude, 2.0 G; scanning field, 3,286± 50 G; amplitude, 3.2×10. Fe^{3+}-NTA solution intensively produced a DMPO-OH ($A_N=A_H^\beta=15.0$) in the presence of H_2O_2. Fe^{3+}-deferoxamine produced no DMPO spin adduct.

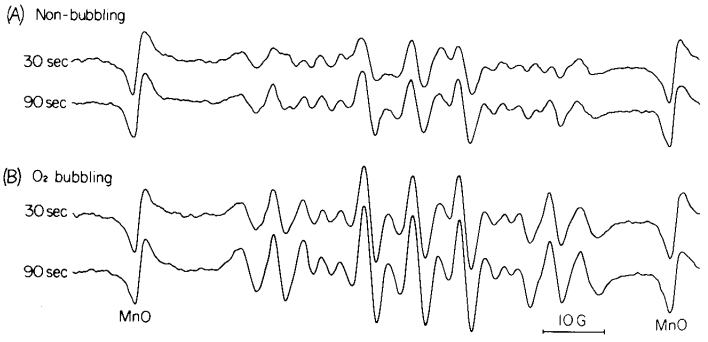


Fig. 10 ESR spectra of DMPO spin adducts formed in Rose Bengal solution containing DMPO with and without O_2 bubbling. The concentration of Rose Bengal was 0.05 mg/ml in distilled water. Two hundred microliters of the solution were placed into 2 test tubes. One test tube was allowed to stand without O_2 bubbling (A) and the other was bubbled with O_2 (B) for 15 min prior to the addition of 10 μl of DMPO. ESR spectra were recorded 30 sec and 90 sec after the addition of DMPO. ESR spectra were recorded at room temperature under the following conditions: microwave power, 8.0 mW; modulation amplitude, 2.0 G; scanning field, 3,345±50 G; amplitude, 1×1,000; sweep time, 30 sec. The solution produced the same DMPO adducts as Fe^{3+}-NTA, and these signals were increased by O_2 bubbling. ESR parameters: DMPO-Z($A_N=16.1$, $A_H^\beta=23.2$), Y-NO· ($A_N=17.8$), DMPO-OH($A_N=A_H^\beta=15.0$).

such as myoglobin and hemoglobin produce $g_{XY}=6$ and $g_Z=2$ in the ferric high-spin state (17-21). In the case of heme proteins, the iron atom is coordinated approximately octahedrally by 6 electronegative ligands. Theoretically, it may be assumed that:

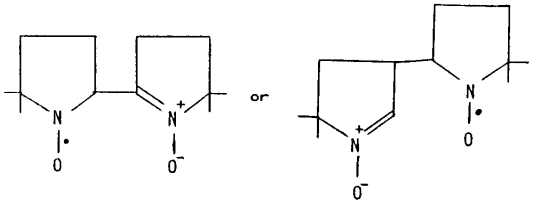
$$g_X, g_Y=6\pm 24E/D;\quad g_Z=2.0,$$

where D and E are parameters induced in spin Hamiltonian (16). According to this formula, the splitting of $g_{XY}=6.0$ might occur at pH 7.5, and Fe^{3+}-NTA solution

might show signals $g_X=7.5$ and $g_Y=4.5$. In our experiment, the signal of $g_Y=4.5$ was not clearly identified.

Therefore at higher pH, the iron of Fe^{3+}-NTA was in the ferric high-spin state and coordinated both octahedrally and in a structure of anisotropic g value. Clegg *et al.* (22) and Yoneda *et al.* (23) reported a pentagonal structure for Fe^{3+}-NTA. It is probable that Fe^{3+}-NTA solution is a mixture of octahedral and pentagonal structures.

By spin trapping, DMPO-Z appeared in the serum of rats injected with Fe^{3+}-NTA and in Fe^{3+}-NTA solution itself (Figs. 3, 4). Finkelstein *et al.* (24) reported that in phosphate buffer, iron EDTA oxidized DMPO into a nitroxide, $A_N=15.3$, $A_H^{\beta}=22.0$, which we termed DMPO-Z. The same signal was also observed in Tris buffer containing iron in the absence of EDTA. They suggest that the spectrum was due to formation of a DMPO dimer:



However, they did not discuss the mechanism of this oxidation in detail.

Harbour *et al.* (25) and Finkelstein *et al.* (26) reported that DMPO traps superoxide radical with hyperfine splitting constants (hfsc) of $A_N=14.3$, $A^{\beta}_H=11.7$, $A^{\gamma}_H=1.25$ and $A_N=14.2$, $A^{\beta}_H=11.6$, $A^{\gamma}_H=1.2$. Both $O_2^{\cdot-}$ and HOO· added to DMPO and the spin adducts gave the same hfsc (25). The superoxide radical spin adduct (DMPO-OOH) of DMPO is relatively unstable in aqueous solution, and the half-life of the ESR signal was approximately 80 sec at pH 6 and 35 sec at pH 8 (27). One new signal of $A_N=15.31$, $A_H^{\beta}=22.0$ increased chronologically (28). In our study, the DMPO-OOH signal which appeared in the KO_2 or xanthine-xanthine oxidase system was not detected. The DMPO-Z which we detected in the Fe^{3+}-NTA system had a hfsc similar to the signal of $A_N=15.31$, $A_H^{\beta}=22.0$, which increased chronologically in the xanthine-xanthine oxidase system.

Hydroxyl radical reacts with ethanol to produce α-hydroxyethyl radical. This secondary radical can then react with DMPO to produce a DMPO-hydroxyethyl which gives a hfsc similar to DMPO-Z. Janzen and Liu (29) suggest that the large A_N and A_H^{β} values for the hydroxyethyl spin adduct are probably due to an increase in spin density on nitrogen and a decrease in the β-CH dihedral angle caused by intramolecular hydrogen bonding from hydroxy hydrogen to nitroxide oxygen. They also suggest that DMPO-OOH does the same, but which species exists in which solvent is unclear (30).

In our study, oxygen in solution is essential to the production of DMPO-Z. Antioxidants, such as α-tocopherol and BHA, suppressed DMPO-Z production, which is in accord with data using the thiobarbituric acid method (3). In this sense, Fe^{3+}-NTA activated oxygen to some active oxygen. Nevertheless, Fe^{3+}-NTA produced neither free-state $O_2^{\cdot-}$ nor its protonated form, ·OOH. Ferrous chelate compounds react with oxygen and form perferryl ion ($Fe^{3+}\cdots O_2^{\cdot-}$), as proposed by Hochstein *et al.* (7, 31). If the perferryl ion reacted with DMPO to produce DMPO-OOH with intramolecular hydrogen bonding, as proposed by Janzen *et al.* (30), Fe^{2+}-NTA solution would produce DMPO-Z more intensively than Fe^{3+}-NTA solution. However, Fe^{2+}-NTA solution produced no DMPO spin adduct. In addition, this proposal of perferryl ion has been criticized on thermodynamic grounds by

Koppenol (32).

The other possibility is that Fe^{3+}-NTA forms Fe^{3+}-NTA dioxygen complex, in which oxygen is in the state of $^1\Delta_g$. When the oxygen molecule approaches to transition metals, such as ferrous ion, the oxygen sometimes has the same spin state as $^1\Delta_g$ through back donation of one electron between the π^* orbital of oxygen and d orbital of the iron. Rose Bengal solution, a popular singlet oxygen generating system, also produced DMPO-Z. α-Tocopherol, an antioxidant and a scavenger of singlet molecular oxygen, suppressed DMPO-Z production. Since singlet oxygen is a strong oxidant, it oxidizes DMPO and generates a DMPO dimer, as reported by Finkelstein *et al.* (24). However the production of DMPO-Z was not suppressed by DABCO, which suggests that DABCO is not as effective as α-tocopherol in scavenging singlet oxygen in the solution.

Harbour *et al.* (25) reported that the hydroxyl spin adduct of DMPO showed a 1:2:2:1 quartet of $A_N = A_H^\beta = 15.3$. Reported values vary in magnitude of hfsc, but all investigators have found a 1:2:2:1 pattern. However, the presence of DMPO-OH does not necessarily mean that the hydroxyl radical is trapped (33) and that DMPO produces a DMPO-OH spin adduct through sequences other than ·OH trapping in the presence of a Fe^{3+}(24) or Fe^{2+}(34)-phosphate complex. In our study the addition of ethanol inhibited the production of DMPO-OH. This means that DMPO trapped the hydroxyl radical directly. In addition, DMPO-OH was inhibited by catalase. In the Fe^{3+}-NTA system, the hydroxyl radical is generated directly and/or through H_2O_2.

In our Fe^{3+}-NTA system, iron is ferric, not ferrous. Fe^{2+}-NTA produced no DMPO spin adducts in high concentration. The ferrous ion might inhibit the oxidation of DMPO by its own oxidation. When the Fe^{2+}-NTA solution was diluted, the ferrous ion might have been readily oxidized and have changed to ferric ion. Therefore, we could find no difference between Fe^{3+}-NTA and Fe^{2+}-NTA solution.

Fe^{3+}-NTA intensively produced a hydroxyl radical in the presence of H_2O_2. Fe^{3+}-NTA also has the same characteristics as some other iron chelate compounds.

Acknowledgments. The authors are grateful to Dr. Y. Fujita of Kurashiki Central Hospital for his helpful advice and kind support. This research was supported by a Grant-in-Aid for Scientific Research, No. C-60570159, from the Japanese Ministry of Science, Education and Culture.

Parts of these results were presented at the 7th International Conference on Proteins of Iron Storage and Transport held at the University of Science and Technique, Lille, France, June 30-July 5, 1985.

References

1. Awai M, Narasaki M, Yamanoi Y and Seno S: Introduction of diabetes in animals by parenteral administration of ferric nitrilotriacetate. Am J Pathol (1979) **95**, 663-674.
2. Yamanoi Y, Matsuura R and Awai M: Mechanism of iron toxicity in the liver and pancreas after a single injection of ferric nitrilotriacetate. Acta Haematol Jpn (1982) **45**, 1229-1235.
3. Matsuura R, Yamanoi Y and Awai M: Mechanism of iron toxicity in the rat liver after a single injection of ferric nitrilotriacetate (Fe-NTA); in Structure and Function of Iron Storage and Transport Proteins, Urushizaki, Aisen and Listowsky eds, Elsevier Science Publichers B.V., Drysdale (1983) pp 481-484.
4. Jacobs A: The pathology of iron overload; in Iron in Biochemistry and Medicine, Jacobs and Worwood eds, Academic Press, London (1980) pp 427-459.
5. Fong KL, McCay PB, Poyer JL, Keele BB and Misra H: Evidence that peroxidation of lysosomal membranes is initiated by hydroxyl radicals produced during flavin enzyme activity. J Biol Chem (1973) **248**, 7792-7797.
6. King MM, Lai EK and McCay PB: Singlet oxygen production associated with enzyme-catalyzed lipid peroxidation in liver microsomes. J Biol Chem (1974) **250**, 6496-6502.
7. Hochstein P, Nordenbrand K and Ernster L: Evidence for the involvement of iron in the ADP-activated peroxidation of lipids in microsomes and mitochondria. Biochem Biophys Res Commun (1964)

14, 323-328.
8. Svingen BA, Buege JA, O'Neal FO and Aust SD: The mechanism of NADPH-dependent lipid peroxidation. J Biol Chem (1979) **254**, 5892-5899.
9. Nakano M and Noguchi T: Mechanism of the generation of singlet oxygen in NADPH-dependent microsomal lipid peroxidation; in Biochemical and Medical Aspects of Active Oxygen, Hayaishi and Osada eds, University of Tokyo Press, Tokyo (1977), pp 29-43.
10. Bucher JR, Tien M, and Aust SD: The requirement for ferric in the initiation of lipid peroxidation by chelated ferrous iron. Biochem Biophys Res Commun (1983) **111**, 777-784.
11. O'Connell MJ and Garner A: Radiation-induced generation and properties of lipid hydroperoxide in liposome. Int J Radiat Biol (1983) **44**, 615-625.
12. Bonnet R, Brown RFC, Clark VM, Sutherland IO and Todd A: Experiments towards the synthesis of corrins. Part II The preparation and reaction of Δ^1-pyrroline-1-oxide. J Chem Soc (Lond) Pt II (1959) 2094-2102.
13. Aisen P, Leibman A and Zweier J: Stoichiometric and site characteristics of the binding of iron to human transferrin. J Biol Chem (1977) **253**, 1930-1937.
14. Nakazawa T, Kojima Y, Fujisawa H, Nozaki M, Hayaishi O and Yamano T: Studies on the mechanism of action of pyrocatechase by electron spin resonance spectroscopy. J Biol Chem (1965) **240**, 3224-3226.
15. Orme-Johnson WH: Iron-sulfur proteins: structure and function. Ann Rev Biochem (1973) **42**, 159-204.
16. Kotani M and Watari H: A theoretical survey of EPR spectra due to high spin ferric ions in proteins, in Magnetic Resonance in Biological Research, Fanconi ed, Gordon & Beach Press, New York (1971), pp 75-96.
17. Bennet JE, Gibson JF and Ingram DJE: Electron resonance studies of hemoglobin derivatives. I. Heam-plane orientation. Proc R Soc (Lond) (1957) A240, 67-82.
18. Helcke GA, Ingram DJE and Slade EF: Electron resonance studies of hemoglobin derivatives. III. Linewidth and g-valve measurements of acid-metmyoglobin and of metmyoglobin azide derivatives. Proc R Soc Ser B (1968) **169**, 275-288.
19. Hayashi A, Shimizu A, Yamamura Y and Watari H: Electron spin resonance of haemoglobin. M. Iwate and M. Osaka. Biochim Biophys Acta (1965) **102**, 626-628.
20. Hayashi A, Suzuki T, Shimizu A, Morimoto H and Watari H: Changes in EPR spectra of M-type abnormal haemoglobins induced by deoxygenation and their implication for the haem-haem interaction. Biochim Biophys Acta (1967) **147**, 407-409.
21. Iizuka T and Kotani M: Analysis of thermal equilibrium between high-spin and low-spin states in ferrimyoglobin complexes. Biochim Biophys Acta (1969) **181**, 275-286.
22. Clegg W, Powell AK and Ware MJ: Structure of trisodium bis(nitrilotriacetato)ferrate(III) pentahydrate, $Na_3[Fe\ N(CH_2CO_2)_{3\ 2}]\cdot 5H_2O$. Acta Cryst (1984) C**40**, 1822-1824.
23. Yoneda H: Personal communication. Department of chemistry, Hiroshima university.
24. Finkelstein E, Rosen GM and Rauckman EJ: Spin trapping of superoxide and hydroxyl radical: Practical aspects. Arch Biochem Biophys (1980) **200**, 1-16.
25. Harbour JR, Chow V and Bolton JR: An electron spin resonance study of the spin adducts of OH and HO_2 radicals with nitrones in the ultraviolet photolysis of aqueous hydrogen peroxide solution. Can J Chem (1974) **52**, 3549-3553.
26. Finkelstein E, Rosen GM and Rauckman EJ: Spin trapping of superoxide. Mol Pharmacol (1979) **16**, 676-685.
27. Buettner GR and Oberley LW: Considerations in the spin trapping of superoxide and hydroxyl radical in aqueous systems using 5, 5-dimethyl-1-pyrroline-1-oxide. Biochem Biophys Res Commun (1978) **83**, 69-74.
28. Finkelstein E, Rosen GM and Rauckman EJ: Spin trapping. Kinetics of the reactin of superoxide and hydroxyl radicals with nitrones. J Am Chem Soc (1980) **102**, 4994-4999.
29. Janzen EG and Liu JI-P: Radical addition reactions of 5, 5-dimethyl-1-pyrroline-1-oxide. ESR spin trapping with a cyclic nitrone. J Mag Resonance (1973) **9**, 510-512.
30. Janzen EG: A critical review of spin trapping in biological system; in Free Radicals in Biology, Vol. 4, Pryor ed, Academic Press, New York (1980) pp 115-154.
31. Hochstein P: Nucleotide-iron complexes and lipid peroxidation: mechanisms and significance. Isr J Chem (1981) **21**, 52-53.
32. Koppenol WH: Chemical and biochemical aspects; in Oxy Radicals and Their Scavengers Systems, Cohen and Greenwald eds, Elsevier Press, Amsterdam (1983), pp 27-68.
33. Rosen GM and Rauchman EJ: Spin trapping of superoxide and hydroxyl radicals; in Oxygen Radicals in Biological Systems, Methods in Enzymology, Packer ed, Vol. 105, Florida (1985) pp 198-209.
34. Tero-Kubota S, Ikegami Y, Kurokawa T, Asaki R, Sugioka K and Nakano M: Generation of free radicals and initiation of radical reactions in nitrones-Fe^{2+}-phosphate buffer systems. Biochem Biophys Res Commun (1982) **108**, 1025-1031.

Received: Jannary 31, 1986
Accepted: March 29, 1986

Reprint requests to:
Teruyuki Kawabata
Department of Pathology
Okayama University Medical School
2-5-1 Shikata-cho, Okayama 700, Japan