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

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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 nitrooxide 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

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

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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 nitrooxide 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, a-tocopherol and 3-tert-butyl-4-hydroxyanisole (BHA). The DMPO-OH signal was suppressed in the presence of ethanol and catalase. Fe$^{3+}$-NTA solution hardly produced DMPO spin adducts. The Fe$^{3+}$-NTA solution produced a strong DMPO-OH signal in the presence of H$_2$O$_2$. 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$_2$O$_2$, the Fe$^{3+}$-NTA solution generated a hydroxyl radical. Fe$^{3+}$-NTA itself generated free radicals, but Fe$^{3+}$-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$_2$O$_2$ 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):

\[
\text{LOOH} + \text{Fe}^{2+}-\text{complex} \rightarrow \\
\text{Fe}^{3+}-\text{complex} + \text{OH}^- + \text{LO}.
\]

\[
\text{LOOH} + \text{Fe}^{3+}-\text{complex} \rightarrow \\
\text{Fe}^{2+}-\text{complex} + \text{H}^+ + \text{LOO}.
\]

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

The study here clarifies the spectrophotometric and ESR spectrometric structure of Fe³⁺-NTA, and characterizes the free radicals in the serum of Fe³⁺-NTA injected rats and the Fe³⁺-NTA solution itself. We suggest that Fe³⁺-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-hydroxanisole (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. Fe³⁺-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 nitritriacetate (Na₂-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 Fe³⁺-NTA solution was sealed until use. Fe³⁺-deferoxamine (Desferal, Chiba Geigy Co.) solution was prepared by the same method. The Fe³⁺-NTA solution was prepared just before use. Ferrous sulfate was dissolved in 50 mM NTA solution containing 30 mM HNO₃. 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-NTA was 1:1 in spectrometry and 1:5 in other experiments.

Spectrophotometry and ESR spectrometry. The absorbance of Fe³⁺-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 Fe³⁺-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 Fe³⁺-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 (Bulinal, 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 Fe³⁺-NTA solution. The original Fe³⁺-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 Fe³⁺-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 Fe³⁺-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 Fe³⁺-NTA solution. DMPO (10 µl) was added to 100 µl of the original Fe³⁺-NTA and Fe⁴⁺-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 Fe⁴⁺-NTA in the presence of H₂O₂. The original Fe⁴⁺-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% H₂O₂ 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.

Results

The absorbance of 10 mM Fe⁴⁺-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 Fe⁴⁺-NTA solution of different pH were recorded at 123 K (Fig. 2). At pH 4.0, Fe⁴⁺-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, Fe⁴⁺-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 Fe⁴⁺-NTA solution 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 Fe⁴⁺-NTA solution had a DMPO spin adduct of unknown radical with three doublets (DMPO-Z; Aₙ=15.8, A₉=23.6) and a simple nitroxide radical (Y-NO⁻; Aₙ=17.8). The injection of neither Blutal
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 ($A_n=15.5$, $A_d^o=23.2$), Y-NO· ($A_n=17.4$) and a 1:2:2:1 quartet ($A_n=14.9$, $A_d^o=15.6$). The last signal had a quartet pattern in the first differential, but a hyperfine splitting constant of $A_n \neq A_d^o$. 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 ($A_n=15.9$, $A_d^o=23.5$), Y-NO· ($A_n=17.5$) and an additional 1:2:2:1 quartet (DMPO-OH; $A_n=A_d^o=15.2$). 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

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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; modulation, 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.

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 iron free NTA solution. 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; amplitude, 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 ($A_n=15.8$, $A_d^o=23.6$), 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\(_{xx}=15.9\), A\(_{yy}=23.5\)), Y-NO\(_{2}\) (A\(_{xx}=17.5\)) and DMPO-OH (A\(_{xx}=A_{yy}=15.2\)) were produced. In (B), DMPO-Z (A\(_{xx}=15.5\), A\(_{yy}=23.2\)) and Y-NO\(_{2}\) (A\(_{xx}=17.4\)) were produced. The intensities of DMPO-Z and Y-NO\(_{2}\) 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 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 (×500) with 50 mM 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 (○—○).

ions 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$^{3+}$-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$_2$O$_2$ and DMPO were recorded at room temperature (Fig. 9). Fe$^{3+}$-NTA solution strongly produced a 1:2:2:1 quartet with $A_N$ = $A_H$ = 15.0 (DMPO-OH) in the presence of H$_2$O$_2$. 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, $A_H$ = 23.2), DMPO-OH ($A_N$ = $A_H$ = 15.0) and Y-NO$_2$ ($A_N$ = 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 (○—○).

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±50 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$_2$O$_2$, and DMPO. Iron chelate solution was diluted 10 fold with distilled water. One hundred microliters of 0.01% H$_2$O$_2$ solution were added to 100 $\mu$l of diluted iron chelate solution, and 10 $\mu$l 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 ($A_s=A_d=15.0$) in the presence of H$_2$O$_2$. 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 $\mu$l 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($A_s=16.1$, $A_d=23.2$), Y-NO$_2$, $A_s=17.8$, DMPO-OH($A_s=A_d=15.0$).

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, \( A_n = 15.3, A_n^0 = 22.0 \), 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:

![Diagram of 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 \( A_n = 14.3, A_n^0 = 11.7, A_n^0 = 1.25 \) and \( A_n = 14.2, A_n^0 = 11.6, A_n^0 = 1.2 \). Both \( O_2^- \) 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 \( A_n = 15.31, A_n^0 = 22.0 \) 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 \( A_n = 15.31, A_n^0 = 22.0 \), which increased chronologically in the xanthine-xanthine oxidase system.

Hydroxyl radical reacts with ethanol to produce \( \alpha \)-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 \( A_n^0 \) values for the hydroxyethyl spin adduct are probably due to an increase in spin density on nitrogen and a decrease in the \( \beta \)-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 \( \alpha \)-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 \( O_2^- \) nor its protonated form, \( \cdot \text{OOH} \). Ferrous chelate compounds react with oxygen and form perferryl ion (Fe\(^{3+}\)-... O\(_2^+\)), 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\(^{3+}\)-NTA solution would produce DMPO-Z more intensively than Fe\(^{3+}\)-NTA solution. However, Fe\(^{3+}\)-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 $\text{Fe}^{3+}$-NTA forms $\text{Fe}^{3+}$-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 $\pi^*$ orbital of oxygen and d orbital of the iron. Rose Bengal solution, a popular singlet oxygen generating system, also produced DMPO-Z. $\alpha$-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 $\alpha$-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 $\Delta u_1=\Delta u_2=15.3$. 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 $\cdot$OH trapping in the presence of a $\text{Fe}^{3+}$ (24) or $\text{Fe}^{2+}$ (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 $\text{Fe}^{3+}$-NTA system, the hydroxyl radical is generated directly and/or through $\text{H}_2\text{O}_2$.

In our $\text{Fe}^{3+}$-NTA system, iron is ferrous, not ferrous. $\text{Fe}^{2+}$-NTA produced no DMPO spin adducts in high concentration. The ferrous ion might inhibit the oxidation of DMPO by its own oxidation. When the $\text{Fe}^{2+}$-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 $\text{Fe}^{3+}$-NTA and $\text{Fe}^{2+}$-NTA solution.

$\text{Fe}^{3+}$-NTA intensively produced a hydroxyl radical in the presence of $\text{H}_2\text{O}_2$. $\text{Fe}^{3+}$-NTA also has the same characteristics as some other iron chelate compounds.

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