

Enhancement of Hydroxyl Radical Formation from Superoxide Anion Radical in the Presence of Organic Selenium Compounds

Tomofumi Okuno,^a Hitoshi Kawai,^a Tatsuya Hasegawa,^b Hitoshi Ueno,^a and Katsuhiko Nakamuro^{*,a}

^aDivision of Environmental Health, Faculty of Pharmaceutical Sciences, Setsunan University, 45-1 Nagaotoge-cho, Hirakata, Osaka 573-0101, Japan and ^bDepartment of Environmental Biochemistry, Yamanashi Institute of Environmental Sciences, 5597-1 Kenmarubi, Kamiyoshida, Fujiyoshida, Yamanashi 403-0005, Japan

(Received December 4, 2000; Accepted January 31, 2001)

The generation of superoxide anion or hydroxyl radical derived from the organic selenium compounds selenomethionine, selenoethionine, selenocystine, selenocystamine and selenocysteine-glutathione selenenyl sulfide (CySeSG) was investigated by the electron spin resonance (ESR) technique with 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) as a spin trapping agent. The intensity of ESR signals of DMPO-OOH adduct formed by the reaction of the hypoxanthine/xanthine oxidase reaction system with DMPO decreased in the presence of selenomethionine or selenoethionine. However, the decrease of this ESR signal intensity was not due to superoxide anion-scavenging ability of these selenium compounds. When selenomethionine or selenoethionine existed in the superoxide anion generating system at a higher concentration, a new ESR signal was recognized. This signal disappeared with the addition of a hydroxyl radical-scavenging reagent and was similar to the signal of DMPO-OH adduct. The lack of structural change in selenomethionine or selenoethionine following reaction with components of the superoxide anion generating system suggested that these selenium compounds act as a catalyst. Such a phenomenon was not observed in the superoxide anion generating system in the presence of selenocystine, selenocystamine or CySeSG. These findings suggested that coexistence of selenomethionine or selenoethionine under mammalian physiological conditions generating superoxide anion may possibly form a hydroxyl radical.

Key words — selenium, selenomethionine, selenoethionine, electron spin resonance, superoxide anion, hydroxyl radical

INTRODUCTION

Active oxygen species (AOS), namely superoxide anion and hydroxyl radical, have been implicated as substances leading to cell damage.¹⁾ The reactivity of superoxide anion itself may be too low to account for the damage observed in biological systems. Many harmful effects of superoxide anion are believed to be indirect, resulting from its conversion to hydroxyl radical. Hydroxyl radical is known as an AOS which reacts immediately with DNA.²⁻⁶⁾

Selenium is an essential trace element for mammals. Selenium in the form of selenocysteine is a part of the catalytic site of glutathione peroxidase (GPX, EC1.11.1.9)⁷⁾ which is related to detoxifica-

tion of hydrogen peroxide. Moreover, it has been reported that selenocystine and 2-phenyl-1,2-benziselenazol-3(2*H*)-one (ebselen) show GPX-like activity.^{1,8,9)} Dougherty and Hoekstra¹⁰⁾ reported that rats deficient in both selenium and vitamin E are susceptible to oxidative damage, and selenium plays an important role in scavenging AOS. In contrast, several reports suggested the generation of AOS from selenium compounds under certain conditions. Production of superoxide anion by the reaction of selenite with sulfhydryl compounds such as reduced glutathione (GSH) has been reported.¹¹⁻¹⁴⁾ Seko and Imura¹⁵⁾ revealed that selenomethionine may cause oxidative damage derived from the generation of AOS when it is metabolized to selenopersulfide (GSSeH) or selenide (Se²⁻). Hasegawa *et al.*¹⁶⁾ reported that the chemical form of metabolite in small intestine or liver of mice treated with selenocystine is selenocysteine-glutathione selenenyl sulfide (CySeSG). CySeSG is reduced by GSH and/or glu-

*To whom correspondence should be addressed: Division of Environmental Health, Faculty of Pharmaceutical Sciences, Setsunan University, 45-1 Nagaotoge-cho, Hirakata, Osaka 573-0101, Japan. Tel.: +81-72-866-3122; Fax: +81-72-866-3123; E-mail: nakamuro@pharm.setsunan.ac.jp

tathione reductase yielding selenocysteine, which is decomposed to H_2Se by selenocysteine β -lyase.¹⁷⁾ Thus, we hypothesized that organic selenium compounds have both AOS-scavenging action and AOS-generating ability.

The present study was undertaken to elucidate the effects of these compounds against superoxide anion or hydroxyl radical generation. We also discuss the mechanisms of hydroxyl radical generation from superoxide anion in the presence of organic selenium compounds.

MATERIALS AND METHODS

Chemicals — Seleno-L-methionine, seleno-D,L-ethionine, seleno-D,L-cystine and selenocystamine were obtained from Sigma (St. Louis, MO., U.S.A.). L-Methionine and D,L-ethionine were purchased from Kanto Chemicals Co., Inc. (Tokyo, Japan); dimethylsulfoxide (DMSO) was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) was from LABOTEC Co., Ltd. (Tokyo, Japan); diethylenetriamine-N,N,N',N'',N'''-pentaacetic acid (DETAPAC) was from Merck (Darmstadt, Germany); hypoxanthine was from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Xanthine oxidase (EC 1.1.3.22, from cow's milk) was obtained from Boehringer Mannheim GmbH (Ingelheim, Germany). All other reagents were analytical grade products.

CySeSG was prepared as previously described.¹⁷⁾

AOS-Generating System — Superoxide anion was generated from a hypoxanthine/xanthine oxidase reaction system.^{18,19)} The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.4), 0.5 mM hypoxanthine, 0.96 mM DETAPAC, an adequate concentration of selenium compounds, 670 mM DMPO and 0.1 units/mL xanthine oxidase. The reaction started with the addition of xanthine oxidase.

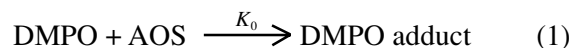
Hydroxyl radical was generated from a Fe^{2+}/H_2O_2 reaction system (Fenton reaction).²⁰⁾ The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.4), 0.25 mM $FeSO_4$, 0.25 mM DETAPAC, various concentrations of selenium compounds, 89 mM DMPO and 0.25 mM H_2O_2 . The reaction started with the addition of H_2O_2 .

Each reaction mixture was transferred to a flat quartz ESR cuvette (LLC-04B, LABOTEC Co. Ltd., Tokyo, Japan), which was fixed to the cavity (ES-

UCX2, JEOL Co., Ltd., Tokyo, Japan) of an ESR spectrometer. After incubation at room temperature for 60 sec, electron spin resonance (ESR) spectra were recorded.

Estimate of the Effects of Selenium Compounds against AOS and Their Generating System

Intensity of the DMPO adduct signal was expressed as the ratio of signal area of the adduct to that of Mn^{2+} at the lowest magnetic field. To estimate the true scavenging activity of selenium compounds by the spin trapping method, experiments were carried out using the modified methods of Prónai *et al.*²¹⁾ and Mitsuta *et al.*²²⁾ If selenium compounds has a scavenging ability against AOS, the competitive reactions are as following:



where S is the selenium compound examined and P is product of the reaction; K_0 is the second-order reaction rate constant for the reaction of DMPO and AOS such as superoxide anion, and K is that for the reaction of selenium compound and AOS. If the selenium compound has a scavenging ability, the following equation can easily be derived from the analysis of the above equations^{20,21)}:

$$I_0/I = X_0/X = 1 + K/K_0 \cdot S/D$$

where I_0 and I are the ESR signal intensity of DMPO adduct in the absence and presence of the selenium compounds, respectively; X and X_0 are the final molar concentration of DMPO adduct with and without the selenium compounds, respectively; S and D are the initial molar concentrations of the selenium compounds and DMPO, respectively. Finally, the ESR intensity ratio (I_0/I) is proportional to S/D . If a selenium compound has scavenging activity against AOS, the linearity between I_0/I and S/D is not disrupted.

To distinguish whether reduction of ESR signal intensity was due to the competition of a selenium compound with DMPO or not, at least two different concentrations of DMPO were used.

ESR Instruments and Condition — Measurements were performed on a JEOL JES-TE300-II ESR spectrometer (Tokyo, Japan) under the following conditions: modulation frequency, 100 kHz; modulation amplitude, 63 μ T; scanning field, 335.5 ± 5 mT; response time, 0.1 sec; sweep time, 2 min; microwave power, 10 mW; microwave frequency, 9.42 GHz; temperature, room temperature.

HPLC/ICP-MS Analysis — A 0.1 ml aliquot of each reaction mixture was applied to the Asahipak GS-220 HQ column (7.6×300 mm, Showa denko Co., Ltd., Tokyo, Japan), and the column was eluted with 20 mM Tris-HCl buffer (pH 7.4) at a flow rate of 0.5 ml/min. The eluate was introduced directly into the ICP-MS (HP-4500, Yokogawa, Musashino, Japan) to detect selenium (m/z 82).

RESULTS

The scavenging effects of selenium compounds against superoxide anion were examined by the signal intensity of ESR. As shown in Fig. 1, the relative intensity of DMPO-OOH adduct derived from superoxide anion generated in the hypoxanthine/xanthine oxidase system decreased when it coexisted with selenium compounds except for selenocystine. The relative intensity of DMPO-OOH adduct with the addition of selenomethionine and selenoethionine was dramatically decreased. With the addition of 5 mM selenomethionine and 5 mM selenoethionine, the relative intensity decreased by $99.0 \pm 1.1\%$ and $99.5 \pm 0.3\%$, respectively. As shown in Fig. 2, however, no decrease of the ESR signal intensity from the DMPO-OOH was recognized in the presence of the same concentration of methionine and ethionine.

It was recognized that the relative intensity of DMPO-OH signals derived from hydroxyl radical generated in the Fenton reaction was little influenced by the selenium compounds tested (data not shown).

The next experiment was carried out to eluci-

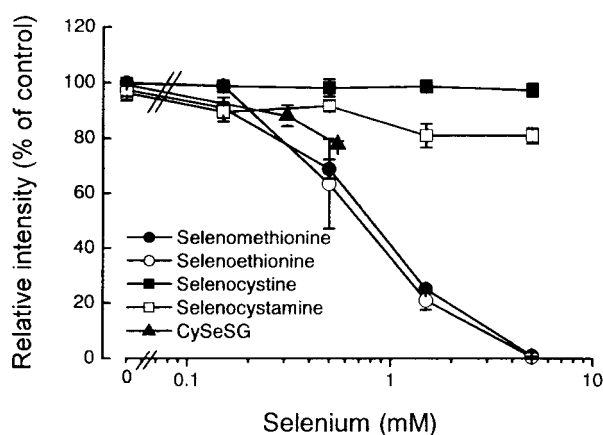


Fig. 1. Effect of Organic Selenium Compounds on Relative Signal Intensity of DMPO-OOH Adduct Formed from Superoxide Anion and DMPO

Superoxide anion was generated by hypoxanthine/xanthine oxidase system. Data are expressed as mean \pm S.D. ($n = 3$ to 5).

date the true scavenging ability of selenomethionine and selenoethionine against superoxide anion. If a selenium compound has scavenging activity against superoxide anion generated in the hypoxanthine/xanthine oxidase system, the linearity between I_0/I and S/D ought not to be disrupted at either of the two concentrations of DMPO as mentioned above. However, as shown in Fig. 3, the slope between I_0/I and S/D was disrupted at these two different concentrations of DMPO.

Figure 4 shows the ESR signals of DMPO adduct formed from the reaction mixture of DMPO and hypoxanthine/xanthine oxidase reaction system in the presence of 5 mM selenomethionine or 5 mM

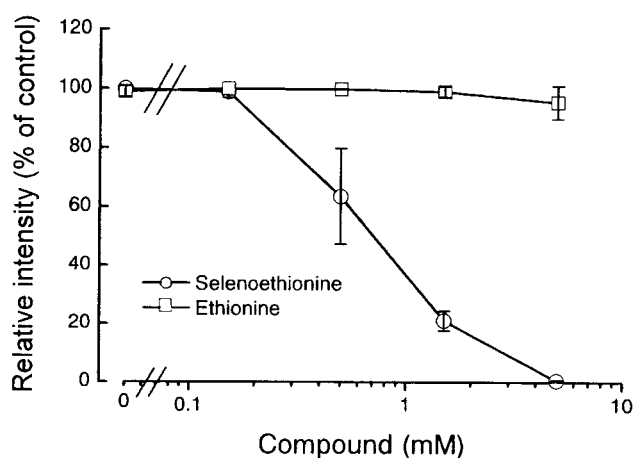
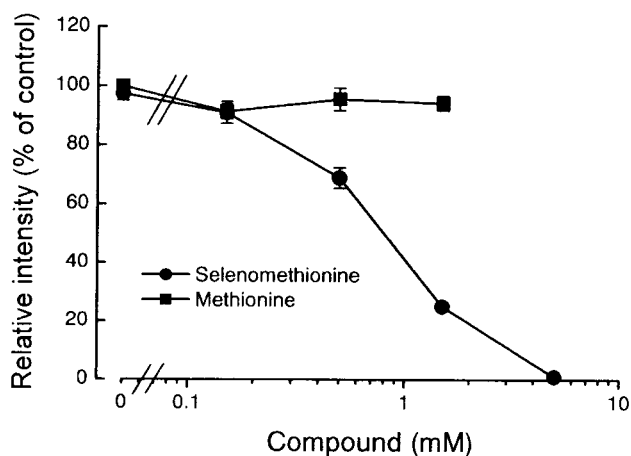


Fig. 2. Effect of Selenium and Sulfur Compound on Relative Signal Intensity of DMPO-OOH Adduct Formed from Superoxide Anion and DMPO

Superoxide anion was generated by hypoxanthine/xanthine oxidase system. Methionine is not dissolved at 5 mM. Data are expressed as mean \pm S.D. ($n = 3$ to 5).

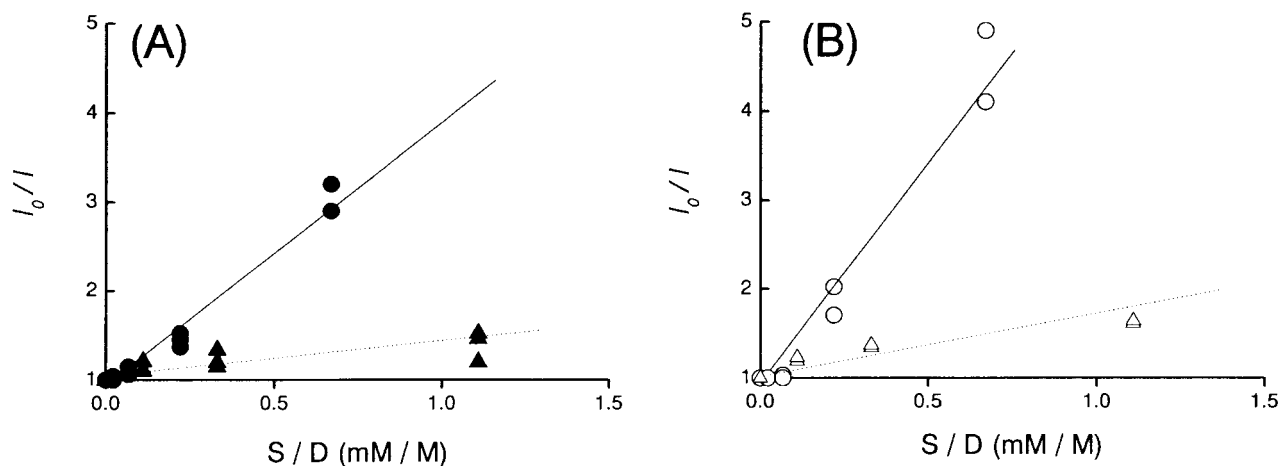


Fig. 3. Analysis of Competitive Reaction of Superoxide Anion with DMPO and Selenomethionine (A) or Selenoethionine (B)
 ●, ○: 670 mM DMPO; ▲, △: 134 mM DMPO. Superoxide anion was generated by hypoxanthine/xanthine oxidase system.

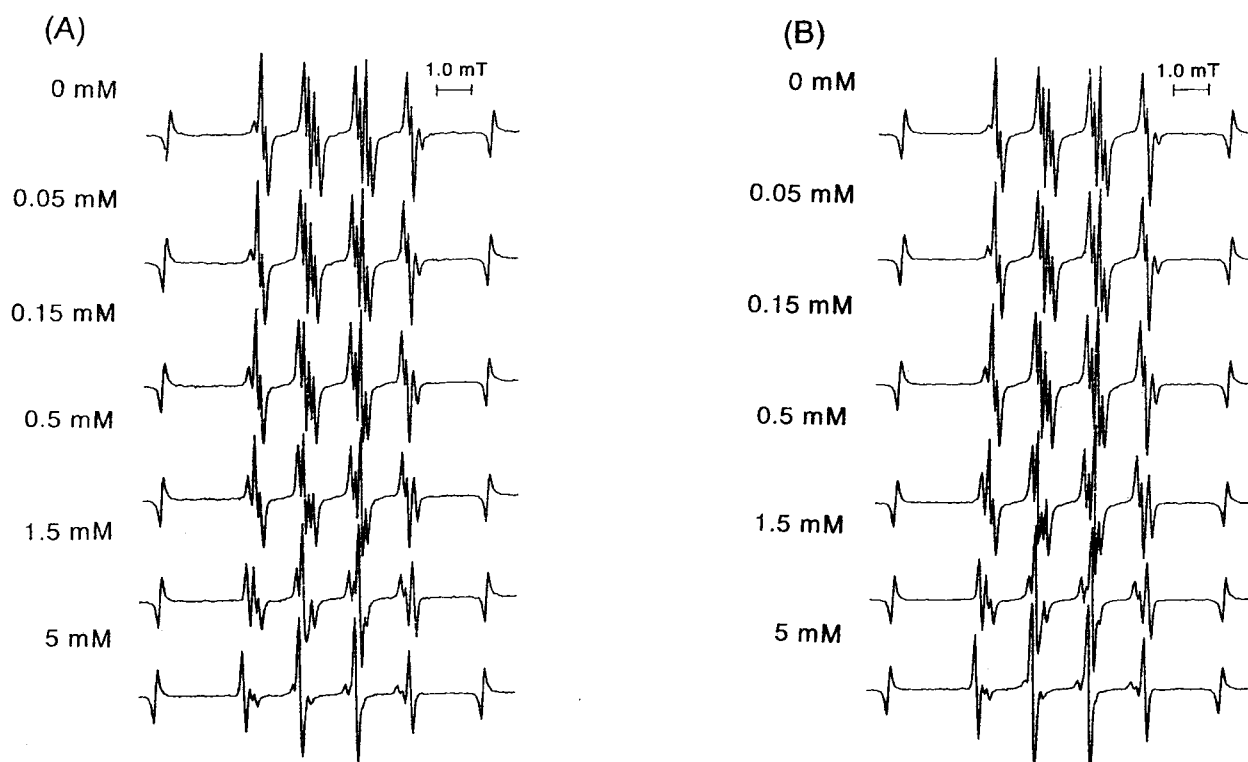


Fig. 4. ESR Signals of DMPO-OOH Adduct Formed from Superoxide Anion and DMPO in Presence of Selenomethionine (A) or Selenoethionine (B)
 Superoxide anion was generated by hypoxanthine/xanthine oxidase system.

selenoethionine. The observed ESR signal differed from the DMPO-OOH adduct derived from superoxide anion. The newly appearing ESR signals were similar to the signal of DMPO-OH adduct derived from hydroxyl radical. The generation of hydroxyl

radical was confirmed from disappearance of the DMPO-OH adduct after addition of various hydroxyl radical-scavenging reagents. As shown in Fig. 5, the new ESR signal intensity shown in control was significantly decreased by the addition of 0.5 M thio-

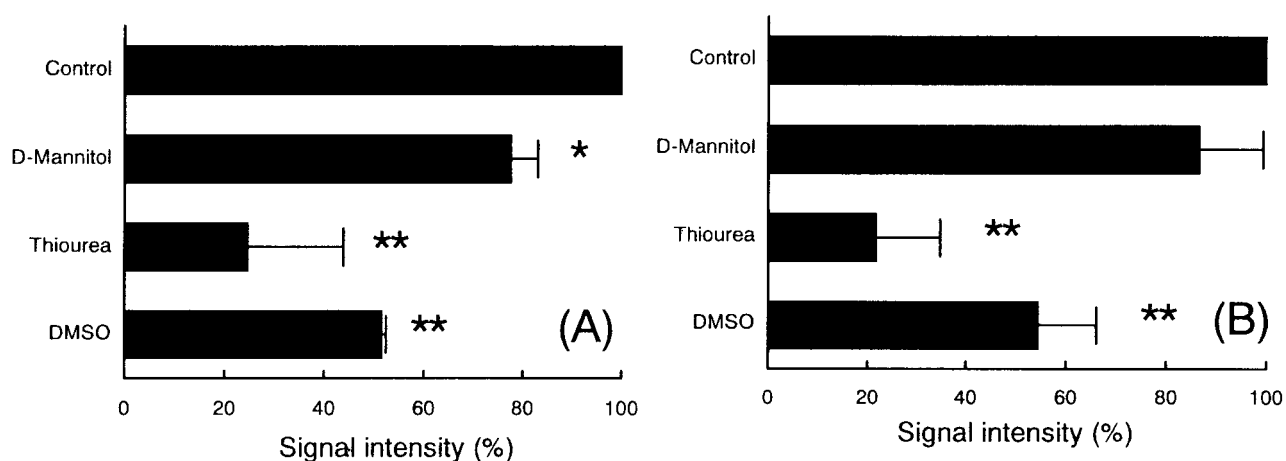


Fig. 5. Reduction of Signal Intensity of DMPO Adduct in the Presence of Various Hydroxyl Radical-Scavenging Reagents

(A) Selenomethionine. (B) Selenoethionine. Control is ESR signal intensity of DMPO adduct formed from the reaction mixture of DMPO and hypoxanthine/xanthine oxidase system in the presence of selenomethionine or selenoethionine. Data are expressed as mean \pm S.D. ($n = 3$). Asterisks indicate experimental values that are significantly different from control values (* $p < 0.05$, ** $p < 0.01$).

urea ($p < 0.01$) or 3.5 M DMSO ($p < 0.01$). No new selenium-containing product except for the initial selenium compound formed by the reaction of selenomethionine or selenoethionine with components in the superoxide anion generating system was detected by HPLC-ICP/MS (data not shown).

DISCUSSION

The ESR-spin trapping technique using DMPO has been highly evaluated and widely used for the identification of such AOS as superoxide anion and hydroxyl radical. This investigation of effect of organic selenium compounds against superoxide anion and hydroxyl radical showed that the ESR signal intensity of DMPO-OOH adduct formed from the hypoxanthine/xanthine oxidase reaction system was markedly decreased in the presence of selenomethionine or selenoethionine. In our experiments, the DMPO concentration (670 mM) was sufficiently high to trap almost all of the superoxide anion.²²⁾ Therefore, these results may suggest that selenomethionine and selenoethionine have the scavenging ability against superoxide anion (Fig. 1). It was further found that these phenomena are due to chemical structures of these two compounds which contain selenium (Fig. 2). In contrast, the selenium compounds tested had no scavenging ability against hydroxyl radical.

The true superoxide anion-scavenging ability of selenomethionine and selenoethionine was determined using different concentrations of DMPO and,

as a result, the slope between I_0/I and S/D was different (Fig. 3). These results suggested that the decrease of DMPO-OOH signal intensity may be caused by inhibition of the superoxide anion generating system, disproportional reaction of superoxide anion or reduction from DMPO-OOH adduct to DMPO-OH adduct. When the higher concentration (5 mM) of selenomethionine or selenoethionine was present in the superoxide anion generating system, the newly ESR signal was recognized (Fig. 4). The ESR signal intensity was significantly decreased in the presence of the three different hydroxyl radical-scavenging reagents (Fig. 5), supporting that hydroxyl radical is generated in the reaction mixture. However, there is a possibility that the ESR signal intensity of DMPO-OH adduct was not completely eliminated by hydroxyl radical-scavenging reagents, because this adduct immediately appeared with the reduction of the DMPO-OOH adduct derived from superoxide anion (Fig. 5). Additionally, as the new ESR signal disappeared with the addition of excess superoxide dismutase and catalase (data not shown), the existences of superoxide anion and selenium compound are thought to be important for generation of hydroxyl radical. The structural change of selenomethionine or selenoethionine by the reaction with components in the superoxide anion generating system was not identified. Therefore, it is possible that these selenium compounds acted as a catalyst rather than according to a chemical reaction. The finding that hydroxyl radical was generated by addition of selenomethionine or selenoethionine to the superoxide anion generating system led us to specu-

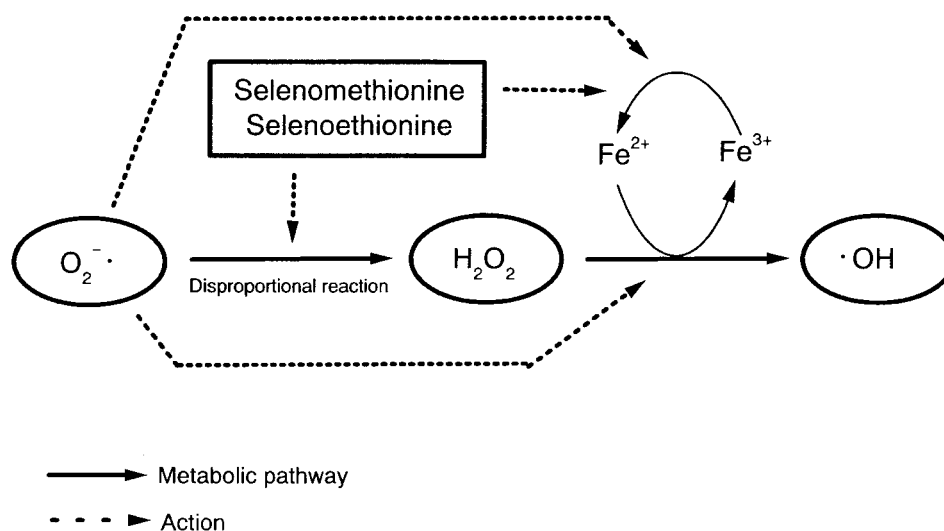


Fig. 6. Possible Mechanisms in the Generation of Hydroxyl Radical

late on a mechanism whereby these selenium compounds play a catalytic role as electronic donor in a disproportionation reaction from superoxide anion to hydrogen peroxide or the Fenton reaction from hydrogen peroxide to hydroxyl radical as shown in Fig. 6. On the other hand, selenocystine, selenocystamine or CySeSG which has a –Se–Se– or –Se–S– bond in their structure was not related to the generation of hydroxyl radical. These selenium compounds may not promote the disproportionation reaction. But, many studies suggested that selenocystine and selenocystamine which have a –Se–Se– bond in their structure have GPX-like activity.^{1,8,9)} Since hydrogen peroxide is scavenged by selenocystine and selenocystamine even though it is generated from superoxide anion, hydroxyl radical is not believed to be produced in the presence of these compounds.

In the reactions of DETAPAC-chelated ferrous iron with hydrogen peroxide at pH 7.4 by Yamazaki and Piette,²³⁾ hydroxyl radical generated in proportion to the amount of the iron existed in the reaction mixture. They indicated that, at trace ferrous iron concentrations, hydroxyl radical was formed according to the Fenton reaction. As the mechanism of hydroxyl radical generation, we assumed that a little ferrous iron contained in the hypoxanthine/xanthine oxidase reaction system reacted with hydrogen peroxide produced by disproportionation reaction of superoxide anion to form hydroxyl radical.

Although the superoxide anion and hydrogen peroxide at physiological levels are a poorly reactive species,²⁴⁾ they may be generated during acci-

dental²⁵⁾ or purposeful^{26–29)} metabolism in all aerobic cells of mammals. Gardner and Fridovich³⁰⁾ proposed that the toxicity of superoxide anion and hydrogen peroxide are involved in their conversion to a more reactive hydroxyl radical, which may cause DNA damage.^{2–6)} On the other hand, a living body has an AOS-elimination enzyme as a defense mechanism to AOS. GPX catalyzes the reduced reaction of hydrogen peroxide or organic hydroperoxides with GSH, and plays an important role in the protection of a cell from oxidative damage. Both experimental and clinical selenium deficiency cause a decrease of GPX activity^{31,32)}, resulting in enhancement of the toxicity induced by AOS.

In conclusion, these results indicated that when selenium compounds selenomethionine and selenoethionine exist in physiological conditions generating superoxide anion and/or hydrogen peroxide, hydroxyl radical may be generated. It has been reported that the toxicity of selenium compound is controlled by an iron-chelating reagent,³³⁾ suggesting that hydroxyl radical generation may be related to selenium toxicity in mammals. In this *in vitro* study, however, hydroxyl radical was generated when selenomethionine or selenoethionine was present above the clinical concentration. Further study is required to learn in detail how these two compounds are involved in the generation of hydroxyl radical from superoxide anion.

Acknowledgements The authors thank Mr. M. Kiguchi, Ms. H. Takagishi, Ms. T. Yamazaki and Ms. H. Morimoto for expert technical assistance.

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