Generation of Semiquinone and Oxygen Radicals by the Reaction of Menadione with Reduced Glutathione at Various pH

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Semiquinone radicals of menadione were generated during the reaction of menadione with reduced glutathione (GSH), dependent upon the pH. Under aerobic conditions, cytochrome c was reduced during the reaction, and superoxide dismutase (SOD) inhibited the cytochrome c reduction. The inhibitory effect of SOD was greater at a high pH than at a low pH. In the presence of Fe^{3+} or ethylenediaminetetraacetic acid (EDTA)-Fe^{3+}, deoxyribose was degraded during the reaction of menadione with GSH, dependent upon the pH. Greater amounts of deoxyribose were degraded at a low pH than at a high pH. The reduction of Fe^{3+} or EDTA-Fe^{3+} also depended on the pH, and SOD strongly inhibited the Fe^{3+} reduction, indicating that Fe^{3+} or Fe^{3+}-EDTA was reduced by superoxide. SOD, catalase, mannitol and benzoate inhibited the deoxyribose degradation at various pH values. These results indicate that the menadione semiquinone radical is readily formed at an alkali pH but a hydroxyl radical is predominantly produced near a neutral pH. A hydroxyl radical may be generated *via* an iron-catalyzed Haber–Weiss reaction.

Keywords menadione; glutathione; semiquinone; hydroxyl radical

The metabolism of menadione (I) has been studied by using isolated hepatocytes and microsomes.[1-3] The drug undergoes either one or two electron reductions. The one electron reduction of the drug forms a semiquinone radical and this process can be catalyzed by various flavoenzymes including reduced nicotinamide adenine dinucleotide phosphate (NADPH)–cytochrome P-450 reductase, NADH cytochrome b_5 reductase and NADH-ubiquinone reductase.[2,4,5] Furthermore, menadione has been found to react with protein thiols to cause a decrease in intracellular glutathione level.[1,6] This reaction of menadione with reduced glutathione (GSH) produces semiquinone radicals and some reactive oxygens.[7,8] Semiquinone radicals have been thought to play a critical role in the oxygen damage induced by quinone compounds because in the presence of oxygen, it can be reoxidized to the parent quinone with the concomitant formation of a superoxide.[1,2] Takahashi *et al.*[9] have demonstrated that semiquinone radicals are produced by the reaction of menadione with GSH at a high pH under anaerobic conditions. However, a relation between oxidative damages and pH is unclear.

Iron often mediates oxidative damages because it can catalyze hydroxyl radical (HO·) generation from a superoxide (O_2^-) and H_2O_2 *via* the Haber–Weiss reaction.[10] In the present study, we have investigated a relation between semiquinone radical generation and oxidative damages induced by the reaction of menadione with GSH at various pH values.

Experimental

Materials Menadione, GSH and 2-deoxyribose were obtained from Wako Pure Chemical Industries. Superoxide dismutase (SOD, bovine erythrocytes) and catalase (bovine liver, thymol free) were purchased from Sigma Chem. Co. Ltd. (St. Louis, MO., U.S.A.). Thiobarbituric acid (TBA) was from Merck Japan Co., Ltd. To remove trace metals, all the buffers used here were passed through a column of Chelex-100 twice before use.

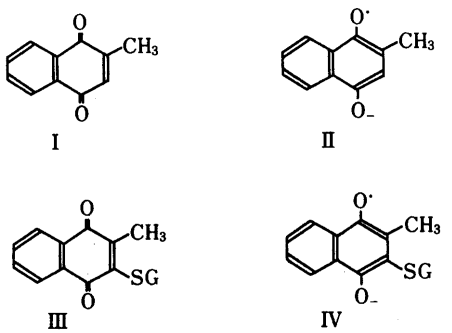
Deoxyribose Degradation The deoxyribose degradation was measured by the method of Halliwell and Gutteridge.[11] To dissolve menadione, organic solvents have generally been used. However, most organic solvents act as a powerful scavenger of HO·. Therefore, the ability as a HO· scavenger of many organic solvents, including ethanol, methanol, dioxane, pyridine, acetone and acetonitrile, was tested in a hydroxyl radical-generating system which consisted of 10 μM Fe^{2+}-ethylenediaminetetraacetic acid (EDTA), 1 mM H_2O_2, 2 mM deoxyribose and 10 mM phosphate buffer at pH 7.4. All the solvents tested scavenged HO·. Among these solvents, however, acetonitrile had the weakest activity as a HO· scavenger. Therefore, acetonitrile was used here to dissolve menadione. In the HO· generating system, 1 and 10% of acetonitrile inhibited deoxyribose degradation about 30 and 50%, respectively.

Electron Spin Resonance (ESR) Measurement A menadione radical was detected by ESR. The ESR setting was as follows: microwave power, 5 mW; modulation frequency, 100 kHz; modulation field, 1.0 G; receiver gain, 2 × 1000; time constant, 0.3 s; time scan, 100 G/min.

Iron Reduction Reduction of iron was measured by incubating the reaction of menadione with GSH with 100 μM $FeCl_3$ or Fe^{3+}-EDTA in the presence of 100 μM bathophenanthroline sulfonate (BPS). Fe^{3+} and EDTA were premixed and then added to the buffer to enable a chelate to form before the addition of BPS. The formation of the Fe^{2+}-BPS complex was continuously monitored at 530 nm and the rate of iron reduction was calculated from $\varepsilon_{530} = 2.21 \times 10^4\ M^{-1}\,cm^{-1}$.[12]

Results and Discussion

Generation of Menadione Radical As shown in Fig. 1, an ESR signal was obtained during the reaction of menadione with GSH (Fig. 1A), and the signal intensities were extremely dependent upon the pH of the reaction mixture (Fig. 1B). The relative intensity at pH 7.5 was about one one-hundredth that observed at pH 9.0. At pH 7.0, no signals could be detected. The signal corresponded to a menadione semiquinone radical (II), and the spectrum which had a G value of 2.0043 almost agreed with that demonstrated by Takahashi *et al.*[9] Semiquinones are readily oxidized to their quinones by molecular oxygen, which generally prevents the detection of the radicals under aerobic conditions. Indeed, we confirmed that the signal was strongly diminished under aerobic conditions. It can be seen from Fig. 2 that at a fixed menadione concentration at



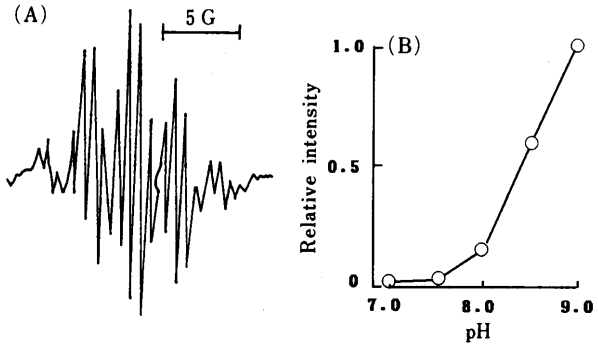


Fig. 1. ESR Signal Formation by the Reaction of Menadione with GSH (A) and the Signal Intensities at Various pH (B)

ESR signals were detected from the reaction mixture containing 1.5 mM menadione, 0.5 mM GSH and 10% acetonitrile in 0.1 M borate buffer under anaerobic conditions. The ESR signal of (A) was obtained at pH 9.0.

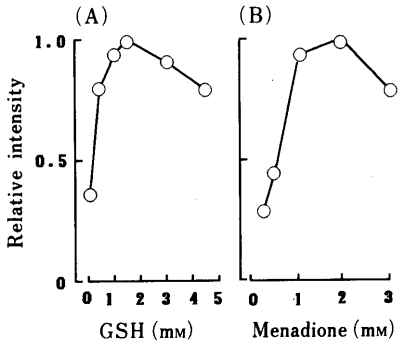


Fig. 2. Effect of Concentrations of the Reaction of Menadione with GSH on the Signal Intensity

The reaction was performed at pH 9.0. Other conditions were the same as that of Fig. 1 except for the concentrations of GSH or menadione.

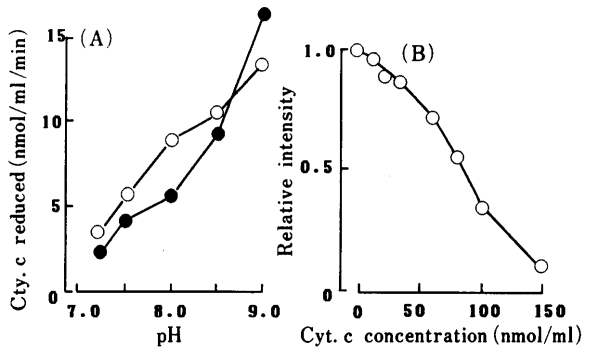


Fig. 3. Reduction of Cytochrome c Induced by the Reaction of Menadione with GSH (A) and Diminution of ESR Signal by Cytochrome c (B).

(A): Reaction mixtures contained 1.5 mM menadione, 0.5 mM GSH, 30 μM cytochrome c and 10% acetonitrile in borate buffer under anaerobic (○) or aerobic (●) conditions. (B): The reaction was performed under anaerobic conditions at pH 9.0. Other conditions were the same as that of (A) except the concentration of cytochrome c.

1.5 mM, the ESR signal intensity of the semiquinone increased with an increase in GSH concentrations up to 1.5 mM. GSH at concentrations above 1.5 mM slightly depressed the signal intensity. At a fixed GSH concentration of 0.5 mM, the semiquinone formation increased with an increase of menadione concentrations up to 2.0 mM. Menadione at concentrations above 2.0 mM depressed the signal intensity. When the ratio of GSH to menadione was greater than 1.0, a different signal from that shown in Fig.

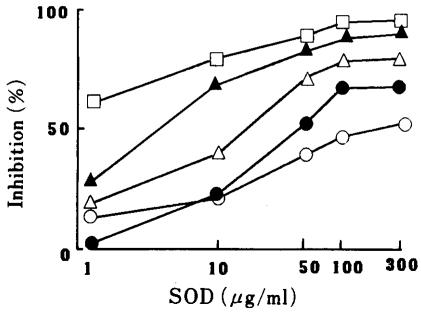


Fig. 4. Inhibitory Effect of SOD on Cytochrome c Reduction Induced by the Reaction of Menadione with GSH under Aerobic Conditions

Various amounts of SOD were added to the reaction mixture containing 1.5 mM menadione and 0.5 mM GSH, 30 μM cytochrome c and 10% acetonitrile in borate buffer. The enzyme was added to the reaction mixture before the start of the reaction. The reactions were started by the addition of menadione. (○), at pH 7.0; (●), at pH 7.5; (△), at pH 8.0; (▲), at pH 8.5 and (□) at pH 9.0.

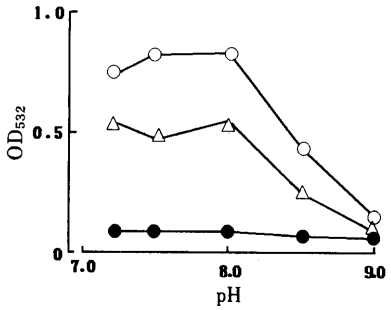


Fig. 5. Degradation of Deoxyribose Induced by the Reaction of Menadione with GSH under Aerobic Conditions

The reaction mixture contained 2.0 mM deoxyribose, 0.15 mM menadione, 0.05 mM GSH and 1% acetonitrile in 10 mM borate buffer in the presence of 10 μM Fe^{3+} (○) or Fe^{3+}-EDTA (△). Closed circle (●) indicates the deoxyribose degradation in the absence of iron. After incubation for 20 min at 37 °C, TBARS formed from deoxyribose were measured.

1A occurred (data not shown). Menadione conjugates with GSH to produce 2-methyl-3-*S*-glutathionyl-1,4-naphthoquinone (thiodione, III) dependent upon the ratio of menadione to GSH.[13] The signal obtained from a high ratio of GSH to menadione corresponded to the 2-methyl-3-*S*-glutathionyl-1,4-naphthosemiquinone (IV) reported by Takahashi *et al.*[9] From Fig. 3 it can be seen that the rate of cytochrome c reduction induced by the reaction of menadione with GSH linearly increased as pH increased, whether under anaerobic or aerobic conditions. As shown in Fig. 3B, the ESR signals of the menadione radical were diminished by the addition of cytochrome c, confirming that the formation of the menadione radical was due to one electron transfer and the menadione radical directly reduced cytochrome c. Under aerobic conditions, similar reductions of cytochrome c were caused, although an ESR signal was not observed. As shown in Fig. 4, the inhibitory effects of SOD on cytochrome c reduction depended on both concentrations of the enzyme and the pH of the reaction mixture under aerobic conditions. At pH 9.0, 1.0 μg/ml of SOD inhibited the cytochrome c reduction about 60%, but at pH 7.5, the same amount of the enzyme did not. At pH 9.0, 10 μg/ml of SOD inhibited the cytochrome c reduction about 80%, but at pH 7.0, 300 μg/ml of the enzyme only inhibited it about 50%. From these results, it is evident that O_2^- is generated during the autoxidation of a menadione radical, but SOD is more effective only at a higher pH.

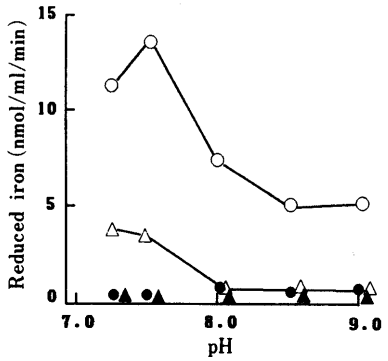


Fig. 6. Inhibitory Effect of SOD on the Reduction of Fe^{3+} or Fe^{3+}-EDTA Induced by Menadione and GSH

The reaction mixture contained 1.5 mM menadione, 0.5 mM GSH, 10% acetonitrile, 1.0 mM BPS and 0.1 mM Fe^{3+} (○) or 0.1 mM Fe^{3+}-EDTA (△) in borate buffer. SOD (100 μg/ml) was added to the reaction mixture in the presence of Fe^{3+} (●) or Fe^{3+}-EDTA (▲) before the start of the reaction.

Winterbourn has reported that cytochrome c reduction by semiquinone radicals under aerobic conditions can be indirectly inhibited by large amounts of SOD.[14] It seems that at a high pH, menadione radicals may rapidly autoxidize to produce O_2^-. However, at near neutral pH, autoxidation of the radical may be low.

Generation of Oxygen Radicals Generation of O_2^- may generate more powerful oxidant HO·. Therefore, we tested the generation of this oxidant during the reaction of menadione with GSH. As shown in Fig. 5, the formation of thiobarbituric acid reactive substances (TBARS) from deoxyribose was scarcely caused by the reaction of menadione with GSH unless Fe^{3+} or Fe^{3+}-EDTA was present. The addition of Fe^{3+} and Fe^{3+}-EDTA markedly increased in the oxidative degradation of deoxyribose dependent upon the pH. Large amounts of TBARS were formed at a lower pH and no appreciable formation of the TBARS was observed at pH 9.0. As shown in Fig. 6, Fe^{3+} and Fe^{3-}-EDTA at near neutral pH were effectively reduced during the reaction of menadione with GSH. SOD strongly inhibited the iron reduction, indicating that the reduction of Fe^{3+} or Fe^{3+}-EDTA was predominantly due to O_2^-. As summarized in Table I, SOD and catalase at various pH inhibited the deoxyribose degradation induced by the reaction of menadione with GSH in the presence of Fe^{3+}-EDTA. Hydroxyl radical scavengers such as mannitol and benzoate also inhibited the deoxyribose degradation. Another hydroxyl radical scavenger, dimethyl sulfoxide (DMSO) was effective only at pH 7.3. These results indicate that HO· is formed *via* an iron-catalyzed Haber–Weiss reaction and the generation of HO· is dependent upon the pH. Baker and Gebicki have reported that the conversion of O_2^- to HO· is more efficient at an acidic pH and that many iron-chelates have maximum catalytic activity near pH 4.8.[15]

Menadione has a strong antioxidant activity and prevents lipid peroxidation induced by various model systems.[16,17] On the other hand, HO· is a powerful initiator of lipid peroxidation.[18] As summarized in Table II, 0.1 mM of menadione was without effect but at 1.0 mM, menadione slightly inhibited the deoxyribose degradation induced by Fe^{2+}-EDTA and H_2O_2. The inhibitory activity was to the same extent as that exhibited by mannitol and DMSO. However, it is unlikely that menadione alone would act as a scavenger of HO· in a cell because of the low concentration of menadione that can be achieved in a cell. By contrast, menadione could degrade deoxyribose during the reaction with GSH in the presence of Fe^{3+}-EDTA. Evidently, menadione acts as a generator but not a scavenger of HO·.

Wefers and Sies[19] have found that chemiluminescence occurs during the reaction of menadione with GSH, and they thought that the chemiluminescence results from singlet oxygen because it emits at a near-infrared level. They have recently suggested that the singlet oxygen generates from an intermediate oxygen addition product such as a glutathione peroxysulphenyl radical resulting from the reaction of O_2^- with GSH.[20] In the present study, deoxyribose was degraded only when Fe^{3+} or Fe^{3+}-EDTA were present. Although the data is not shown, replacement of GSH with NADH gave the same results. From these findings, it seems that singlet oxygen may not be involved in deoxyribose degradation.

The present study demonstrated that during the reaction of menadione with GSH, a semiquinone radical readily formed at an alkali pH, but more powerful oxidants were predominantly generated at near neutral pH. Oxygen radicals damage various biological components including lipids, proteins and nucleic acids. However, since menadione is a strong inhibitor of lipid peroxidation,[15,16] it may be toxic to nucleic acids and protein rather than lipids. It has been demonstrated that the diminution of protein thiols decreases the viability of cells.[1]

TABLE I. Effect of Oxygen Radical Scavengers on the Deoxyribose Degradation at Various pH

Scavengers	OD_{532}				
pH	7.3	7.5	8.0	8.5	9.0
None	0.540	0.421	0.531	0.294	0.092
SOD[a]	0.247	0.258	0.248	0.228	ND[b]
Catalase[a]	0.324	0.310	0.328	0.261	ND[b]
Mannitol	0.411	0.320	0.341	0.271	ND[b]
Benzoate	0.154	0.200	0.216	0.192	ND[b]
DMSO	0.463	0.593	0.538	0.439	ND[b]

Reaction mixtures contained 2 mM deoxyribose, 0.15 mM menadione, 0.05 mM GSH, 1% acetonitrile and 10 μM EDTA-Fe^{3+} in borate buffer. The reactions were started by the addition of menadione. Various scavengers (100 mM) were added to the reaction mixture before the start of the reaction. Each value represents the mean of triplicate experiments. *a*) 100 μg/ml. *b*) Not determined.

TABLE II. Effect of Menadione on Deoxyribose Degradation Induced in Hydroxyl Radical-Generating System

Scavengers	OD_{532}
1. None	3.186
2. 1% acetonitrile	2.419
3. 2+menadione (0.1 mM)	2.575
4. 10% acetonitrile	0.778
5. 4+menadione (1 mM)	0.720
6. 4+mannitol (10 mM)	0.655
7. 4+DMSO (10 mM)	0.614

Hydroxyl radical-generating system consisted of 2.0 mM deoxyribose, 10 μM EDTA-Fe^{2+} and 1.0 mM H_2O_2 in 3.0 ml of 10 mM phosphate buffer at pH 7.4. The reaction was started by the addition of Fe^{2+}-EDTA. After incubation for 3 min at 37 °C, 30 μl of 30% trichloroacetic acid and 1.0 ml of 0.6% TBA were added. Other conditions were same as that of Table I. Each value represents the mean of duplicate experiments.

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