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## Different characteristics between menadione and menadione sodium bisulfite as redox mediator in yeast cell suspension



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## ABSTRACT

Menadione promoted the production of active oxygen species (AOS) in both yeast cell suspension and the crude enzymes from the cells, but menadione sodium bisulfite (MSB) had little effect on the production of AOS in the cell suspension. MSB kept the stable increase in the electron transfer from intact yeast cells to anode compared to menadione, but the electron transfer promoted by MSB was inhibited in permeabilized yeast cell suspension. Menadione promoted oxidation of NAD(P)H much faster than MSB in permeabilized yeast cell suspension, suggesting the oxidative stress due to consumption of NAD(P)H. The proliferation of yeast cells was inhibited by menadione under aerobic conditions rather than anaerobic conditions, and the inhibitory effect was reduced by superoxide dismutase and catalase. The effect of MSB on the proliferation was much smaller than that of menadione. The above facts suggest that harmless MSB promotes the electron transfer from plasma membrane of yeast cells to anode. On the other hand, harmful menadione might promote the electron transfer from cytosol and plasma membrane to anode and dissolved oxygen.

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## 1. Introduction

The electron transfer between intracellular redox systems and extracellular redox mediators has been applied to the detection of cell viability such as bacteria [1,2], yeast [1,3] and mammalian cells [4,5]. The extracellular redox mediators are required to diffuse into cells and to be reduced by intracellular redox systems. Menadione and 2,3,5,6-TMPD (Tetramethyl-*p*-Phenylenediamine) are considered to be suitable for electron transfer between intracellular redox enzymes and extracellular electrodes because of their hydrophobic property to permeate across the cell membrane [6]. Membrane-permeant menadione is known to be reduced by NAD(P)H:quinone oxidoreductase (called DT-diaphorase, EC 1.6.99.2) utilizing both NADH and NADPH as electron donors in the cells [7]. Thus, the efficiency of electron transport by menadione depends on both the activity of NAD(P)H:quinone oxidoreductase and the intracellular NAD(P)H concentration. On the other hand, hydrophilic menadione sodium bisulfite (MSB) has been proposed not to be superior to menadione because of low efficiency of electron transport of MSB [8].

In the case of electrochemical determination of cell activity the normal cells keep the sustainable current or high redox potential, and the damaged cells lose them [5,8]. If the redox mediators themselves damage the cell viability such as the inhibition of intracellular redox systems and other metabolic systems, the cell

viability may be underestimated. For example, menadione is well known to promote the production of active oxygen species (AOS) in bacteria [1], yeast [1,9] and mammalian cells [10], and AOS may cause the oxidative stress to cell activity. Furthermore, the consumption of intracellular NAD(P)H promoted by menadione may also damage the cell activity.

As mentioned above, menadione has been known to be superior to MSB as redox mediator in spite of the demerit that menadione causes the oxidative stress. However, this study demonstrated that MSB was superior to menadione as redox mediator for electron transfer from yeast cells to anode because MSB promoted the stable electron transfer without oxidative stress compared to menadione. This study discussed the effects of menadione and MSB on the production of AOS, oxidation of NAD(P)H, the cell proliferation and the electron flow to anode in order to clarify the effectiveness of MSB as harmless redox mediator.

## 2. Materials and methods

## 2.1. Yeast strain and growth conditions

Baker yeast cells, *Saccharomyces cerevisiae* IFO2044, were grown in YPD medium (2% glucose, 1% peptone, and 0.5% yeast extract) at 30 °C. These cells were washed by centrifugation after 18 h cultivation, and the cell density was adjusted to the desirable density on the basis of the absorbance at 600 nm.

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## 2.2. Preparation of crude enzymes from yeast cells

After 10 ml of culture medium was cultivated under the above conditions, yeast cells were collected by centrifugation (3000g, 5 min). The freezing and thawing of the pellet were repeated three times, and the pellet was mixed with 0.5 ml of 0.1 M Tris/HCl (pH 7.0) and was sonicated in ice for 1 min. The mixture was centrifuged at  $10,000 \times g$  for 5 min, and the supernatant was used as crude enzymes.

## 2.3. Determination of protein [11]

Protein concentration of crude enzymes was determined by Bradford method using bovine serum albumin as the standard.

## 2.4. Permeabilization of yeast cells [12]

Yeast cells were collected by centrifugation, and 1 g of pellet was suspended in 4 ml of 0.4 M sorbitol/0.1 M phosphate buffer (pH 7.0). The suspension was mixed with 9 ml of toluene and was agitated at 42 °C for 5 min. The mixture was cooled in ice and was centrifuged at  $10,000g$  for 10 min. The pellet was suspended in 0.4 M sorbitol/0.1 M phosphate buffer (pH 7.0). The suspension was centrifuged at  $10,000 \times g$  for 10 min, and the pellet was diluted with 0.4 M sorbitol/0.1 M phosphate buffer (pH 7.0). The suspension was used as permeabilized yeast cells.

## 2.5. Menadione or MSB-mediated luminol luminescence [1]

Yeast cells grown in YPD medium were washed with 0.1 M Tris/HCl buffer (pH 7.0) after 18 h-cultivation under the above cultivation conditions.

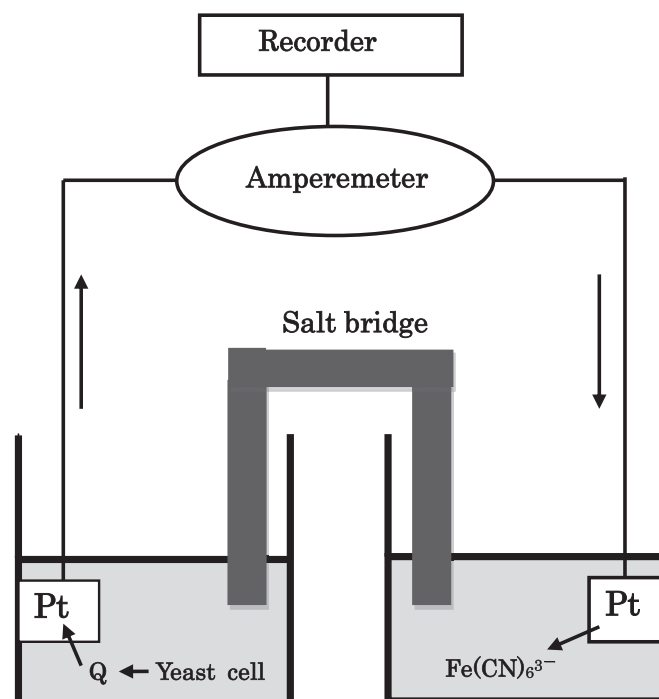
The cell density was adjusted to  $10^7$  cells/ml with 0.1 M Tris/HCl buffer (pH 7.0). The yeast cell suspension (50  $\mu$ l) was mixed with 50  $\mu$ l of menadione or MSB solution containing 0.6 mM menadione or MSB, 20  $\mu$ M  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 1 mM EDTA, and 0.85% NaCl (pH 7.0) and then incubated at 30 °C for 10 min. After the incubation, 100  $\mu$ l of luminol solution containing 5 mM luminol/0.2 M boric acid (pH 9.5) was injected into the mixture, and the chemiluminescence intensity was determined for 5 s.

In the case of crude enzymes extracted from yeast cells, 25  $\mu$ l of crude enzymes (1 mg protein/ml 0.1 M Tris/HCl) was mixed with 25  $\mu$ l of 4 mM NADH/0.1 M Tris/HCl (pH 7.0) and 50  $\mu$ l of the above menadione or MSB solution and then incubated at 30 °C for 10 min. After the incubation, 100  $\mu$ l of the above luminol solution was injected into the mixture, and the chemiluminescence intensity was determined for 5 s.

## 2.6. Current determination

Acrylic plastic box (25  $\times$  40 mm square and 30 mm high) were used for the reaction mixture of 5 ml. Cathodic and anodic reaction mixtures were linked with salt bridge (saturated potassium chloride and 5% agar), and the current between cathode and anode was determined with amperemeter linking to recorder. The both cathode and anode were Pt electrodes 5 mm in diameter. The cathodic reaction mixture was composed of 0.1 M potassium phosphate (pH 7.0) and 1 mM potassium ferricyanide, and the anodic reaction mixture was composed of 0.1 M potassium phosphate (pH 7.0) and yeast cells ( $7 \times 10^6$  cells/ml) in 5 ml. Both reaction mixtures were shaken at 30 °C, and the reaction was started by the addition of 0.05 ml of 30 mM menadione or MSB to 5 ml of anodic reaction mixture.

In the case of permeabilized yeast cells, the anodic reaction mixture was composed of 4.5 ml of 0.4 M sorbitol/0.1 M phosphate buffer (pH 7.0), 0.25 ml of permeabilized yeast cells



**Fig. 1.** Disposition of containers, electrodes, salt bridge and amperemeter. Arrows show the direction of electron flow from yeast cells to  $\text{Fe}(\text{CN})_6^{3-}$ . Q represents menadione or MSB.

( $2 \times 10^9$  cells/ml), 0.25 ml of 6 mM NADH and 0.05 ml of 30 mM menadione or MSB.

Fig. 1 shows the arrangement of containers, electrodes, salt bridge, amperemeter and recorder.

## 2.7. Oxidation of NAD(P)H catalyzed by menadione or MSB in permeabilized yeast cells

The reaction mixture was composed of 950  $\mu$ l of 0.4 M sorbitol/0.1 M phosphate buffer (pH 7.0), 20  $\mu$ l of permeabilized yeast cells (6 mg protein/ml 0.1 M phosphate buffer), 25  $\mu$ l of 0.15 mM NAD(P)H and 5  $\mu$ l of 30 mM menadione or MSB. The reaction was started by menadione or MSB at 20 °C, and the absorbance at 340 nm was determined. Oxidation rate of NAD(P)H was calculated on the basis of molar extinction coefficient of 6270 at 340 nm for NADH and NADPH.

## 2.8. Fluorescence due to NAD(P)H in cell suspension

The concentration of NAD(P)H in yeast cell suspension was determined by fluorescence (ex. 365 nm, em. > 430 nm). The effect of menadione or MBS on intracellular NAD(P)H was determined after the addition of 0.02 ml of 30 mM of menadione or MSB to 2 ml of yeast cell suspension ( $7 \times 10^6$  cells/ml 0.1 M phosphate buffer), and the aeration was performed for 5 s.

## 2.9. Growth rate

The growth rate of yeast cells was determined on the basis of the increase in the absorbance at 600 nm. The initial absorbance of yeast cell suspension was 0.1, and the absorbance after 9 h-cultivation under the aerobic and anaerobic conditions was 1.98 and 1.65, respectively. The growth inhibition by menadione or MSB under the aerobic or anaerobic conditions was estimated on the basis of the above absorbance.

### 3. Results

#### 3.1. Production of AOS mediated by menadione or MSB

Menadione has been known to generate AOS by transporting electron from intact yeast cells to dissolved oxygen. However, MSB has been little clarified about the action as redox mediator in yeast cell suspension. Fig. 2 shows that menadione promotes luminol luminescence due to AOS in both yeast cell suspension and crude enzymes extracted from yeast cells. MSB had little activity to promote luminol luminescence in yeast cell suspension, but had smaller activity to promote luminol luminescence than menadione in crude enzymes as shown in Fig. 2. These facts suggest that menadione is membrane-permeant redox mediator and that MSB is non membrane-permeant redox mediator. Luminol luminescence promoted by menadione or MSB in crude enzymes was inhibited by superoxide dismutase rather than catalase as shown in Fig. 3. This fact indicates that menadione and MSB generate superoxide anion rather than hydrogen peroxide when menadione and MSB are present in yeast cells.

#### 3.2. Effects of menadione and MSB on electron transfer from yeast cells to anode

Fig. 1 shows the electron flow from yeast cells to potassium ferricyanide in the presence of redox mediator. Current was little observed in the absence of menadione or MSB (data not shown) and increased after the addition of menadione or MSB as shown in Fig. 4. After the rapid increase in current was observed after the addition of menadione, current was maximized 4 min later and then slowly decreased with time. On the other hand, MSB kept the constant increase in current, and current enhanced by MSB was the same as current enhanced by menadione 8 min later as shown in Fig. 4. From the results of Figs. 2 and 4, most of MSB is expected to be reduced by redox system in plasma membrane because of poor membrane permeation of MSB.

Menadione showed the maximum current after 4 min-incubation with permeabilized yeast cell suspension as shown in Fig. 5 and showed the peak of current as observed in yeast cell suspension (Fig. 4). On the other hand, MSB-mediated current was about one-quarter menadione-mediated current after 8 min-

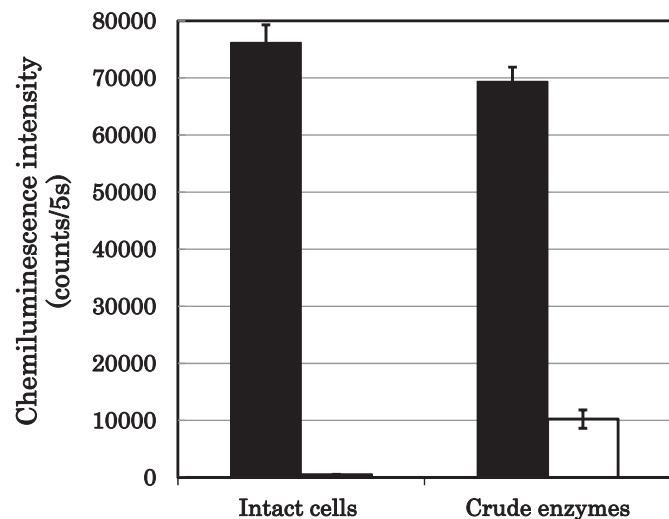


Fig. 2. Luminol luminescence mediated by menadione or MSB in intact yeast cell suspension or crude enzymes of yeast cells. Black bars represent menadione-mediated luminescence, and white bars represent MSB-mediated luminescence. Small bars show the standard deviation. Each value is the mean of four different determinations.

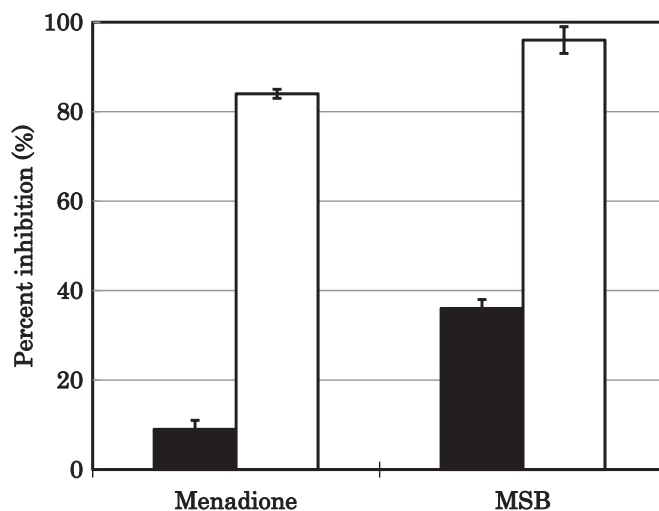


Fig. 3. Effects of catalase and superoxide dismutase (SOD) on luminol luminescence mediated by menadione or MSB in crude enzymes of yeast cells. Black bars represent the addition of catalase (28 U), and white bars represent the addition of SOD (21 U). Small bars show the standard deviation. Each value is the mean of four different determinations.

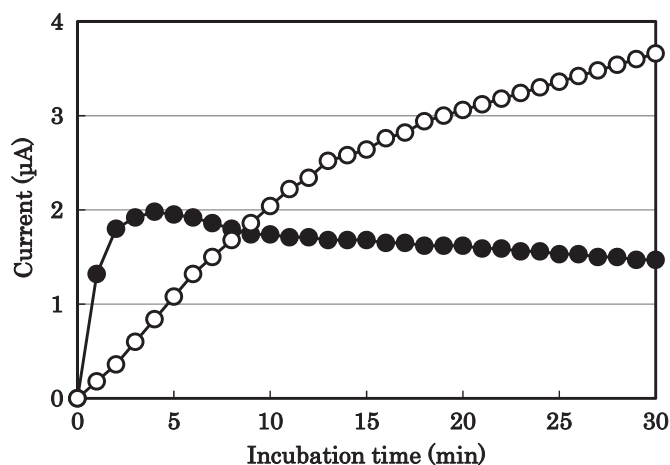


Fig. 4. Menadione or MSB-mediated current from yeast cells to  $\text{Fe}(\text{CN})_6^{3-}$ . Each symbol is shown as follows;  $\bullet$ , menadione-mediated current;  $\circ$ , MSB-mediated current. Each symbol represents the mean of three different determinations, and the standard deviation was less than 5% of the mean.

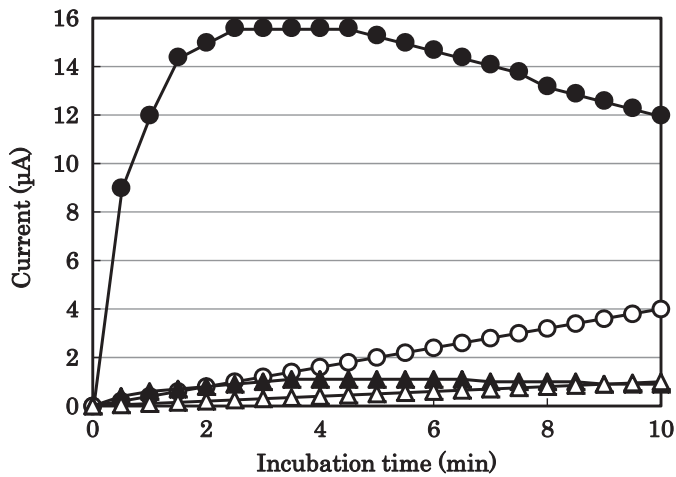
incubation with permeabilized yeast cells (Fig. 5), indicating the inhibition of MSB-mediated current by the destruction of plasma membrane.

MSB-mediated current in the suspension of yeast cells and permeabilized yeast cells was estimated to be  $29 \mu\text{A}$  and  $4 \mu\text{A}/10^8$  cells, respectively, after 10 min-incubation. On the other hand, menadione-mediated current in the suspension of yeast cells and permeabilized yeast cells was estimated to be  $25 \mu\text{A}$  and  $12 \mu\text{A}/10^8$  cells, respectively, after 10 min-incubation. As permeabilized yeast cells inhibited current mediated by MSB rather than menadione, most of menadione might be reduced by the redox system of cytosol rather than plasma membrane, and most of MSB might be reduced by plasma membrane redox system.

The addition of superoxide dismutase (2100 U/5 ml) and catalase (2800 U/5 ml) had little effect on current mediated by 0.3 mM menadione under the conditions of Fig. 5 (data not shown).

#### 3.3. Oxidation of NAD(P)H promoted by menadione or MSB in permeabilized yeast cells

Fig. 6 shows that menadione and MSB oxidize NADH rather

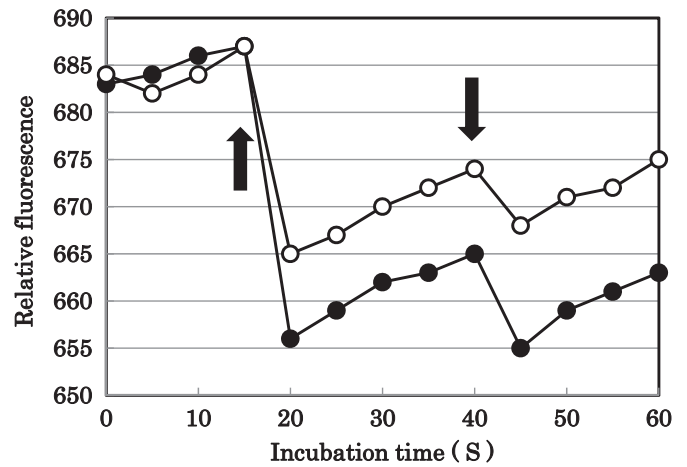


**Fig. 5.** Menadione or MSB-mediated current from permeabilized yeast cells to  $\text{Fe}(\text{CN})_6^{3-}$ . Each symbol is shown as follows: ●, menadione-mediated current in the presence of NADH; ○, MSB-mediated current in the presence of NADH; ▲, menadione-mediated current in the absence of NADH; △, MSB-mediated current in the absence of NADH. Each symbol represents the mean of three different determinations, and the standard deviation was less than 5% of the mean.

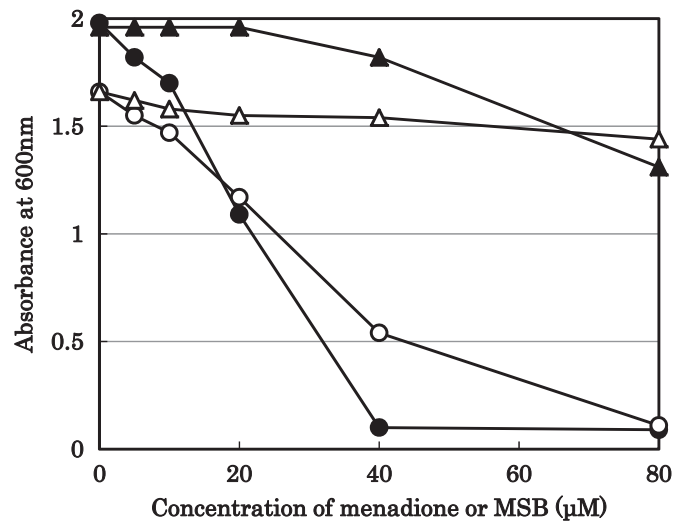
than NADPH in the presence of permeabilized yeast cells and that oxidation rate of NADH by menadione is 13 times faster than that by MSB. The great difference in oxidation rate of NAD(P)H between menadione and MSB suggests that the rapid consumption of NAD(P)H by menadione results in the inhibition of the sustainable current and that the slow consumption of NAD(P)H by MSB results in the stable increase in current.

The ratio of the intact yeast cells in suspension of permeabilized yeast cells was estimated to be 7% on the basis of the difference in menadione-mediated current between the presence and the absence of NADH (Fig. 5).

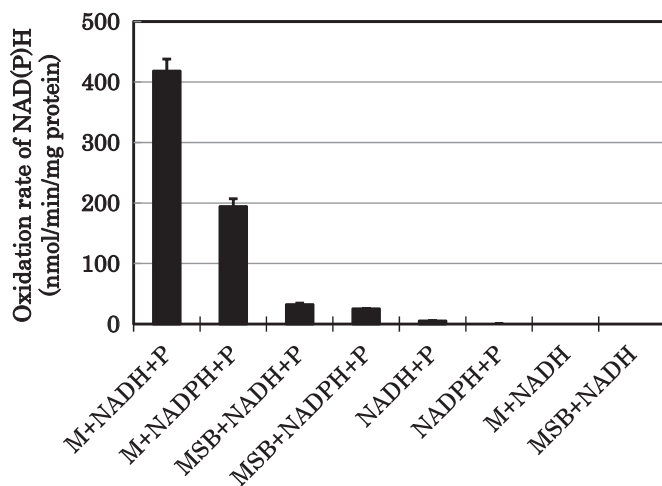
Fig. 7 shows the effects of menadione and MSB on oxidation of NAD(P)H in yeast cells. Fluorescence intensity due to NAD(P)H in the presence of menadione was lower than that in the presence of MSB, and the difference in fluorescence intensity between menadione and MSB was increased by the repeat of aeration (Fig. 7). These facts also suggest that oxidation of NAD(P)H promoted by menadione is faster than that by MSB in yeast cells.



**Fig. 7.** Effects of menadione or MSB on fluorescence due to intracellular NAD(P)H. Each symbol is shown as follows: ●, fluorescence in the presence of menadione; ○, fluorescence in the presence of MSB. Two black arrows mean the aeration for 5 s.



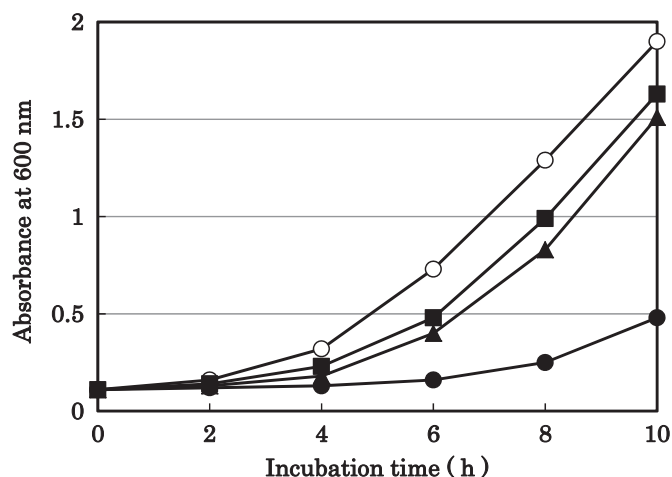
**Fig. 8.** Effects of menadione or MSB on the proliferation of yeast cells. Each symbol is shown as follows: ●, effect of menadione under aerobic conditions; ▲, effect of MSB under aerobic conditions; ○, effect of menadione under anaerobic conditions; △, effect of MSB under anaerobic conditions.



**Fig. 6.** Oxidation of NAD(P)H promoted by menadione or MSB in permeabilized yeast cells. M and P represent menadione and the permeabilized yeast cells, respectively. Large bars and small bars represent the average and the standard deviation, respectively. Each value is the mean of four different determinations.

### 3.4. Inhibitory effect of menadione or MSB on proliferation

Fig. 8 shows that the inhibitory effect of menadione on the proliferation of yeast cells is greater than that of MSB under both anaerobic and aerobic conditions. The aerobic conditions enhanced the inhibitory effect of menadione rather than MSB (Fig. 8). The inhibitory effect of menadione on the proliferation might depend on the generation of AOS rather than oxidation of NAD(P)H under the aerobic conditions, because the difference in generation of AOS between menadione and MSB was much greater than the difference in oxidation rate of NAD(P)H between them as shown in Figs. 2 and 8. Furthermore, this speculation might be supported by the fact that the addition of superoxide dismutase (6300 U/5 ml) and catalase (8400 U/5 ml) reduced the inhibitory effect of menadione on the proliferation of yeast cells as shown in Fig. 9. The extracellular AOS generated by menadione might be scavenged by these non membrane-permeant antioxidant enzymes during the cell proliferation.



**Fig. 9.** Effects of superoxide dismutase and catalase on proliferation of yeast cells in the presence of menadione under aerobic conditions. Each symbol is shown as follows: ○, menadione-free; ●, 30 μM menadione; ▲, 30 μM menadione + superoxide dismutase (6300 U/5 ml); ■, 30 μM menadione + catalase (8400U/5 ml).

#### 4. Discussion

Menadione is known to be uptaken to yeast cells and to makes a complex with glutathione. This complex was identified as thiodione, (2-Methyl-3-glutathionyl-1,4-napthoquinone), and a flux of thiodione was estimated to be about 30,000 molecules/s/cell [13]. A constant efflux of thiodione from the cells was limited by uptake of menadione, and the efflux through the glutathione-conjugated pump was an order of magnitude faster [13]. The efflux of the complex of menadione and glutathione might contribute to the decrease in electron transfer from yeast cells to anode. However, the efflux of menadione by glutathione-conjugate pump might be little involved in the decrease in current, because the time-dependent change in menadione-mediated current in suspension of permeabilized yeast cells was the same as that in suspension of yeast cells (Figs. 4 and 5).

The difference in electron transfer from yeast cells to anode between menadione and MSB is expected to depend on the difference in the production of AOS and oxidation of NAD(P)H between them.

For example, oxidation of the reduced menadione by dissolved oxygen might inhibit the electron transfer from the reduced menadione to anode, and the rapid oxidation of NAD(P)H by menadione might result in the inhibition of sustainable electron transfer. On the other hand, MSB might be reduced by yeast cells without oxidation of the reduced MSB by dissolved oxygen, and slow oxidation of NAD(P)H by MSB might result in the stable increase in electron transport. The difference in the production of AOS between menadione and MSB also might relate to the inhibition of proliferation, because MSB with low productivity of AOS had smaller inhibitory effect on the proliferation than menadione with high productivity of AOS (Figs. 2 and 8), and the inhibitory effects of menadione and MSB on the proliferation were enhanced under the aerobic conditions rather than the anaerobic conditions (Fig. 8). In fact the inhibitory effect of menadione on the proliferation was reduced by superoxide dismutase and catalase under the aerobic conditions (Fig. 9). Some studies reported that the various cellular damage such as morphological change, protein oxidation and lipid peroxidation were induced by the incubation of menadione with yeast cells [14–16]. Menadione might induce cell death through the generation of oxidant stress in multiple subcellular compartments [17].

Rapid Oxidation of intracellular NAD(P)H mediated by menadione is also considered to be involved in the oxidative stress.

Though menadione was proposed to be reduced by NADPH produced in the pentose phosphate pathway [3], menadione and MSB promoted oxidation of NADH rather than NADPH (Fig. 6) [18]. As the addition of glucose to yeast cell suspension promoted both menadione-mediated luminol chemiluminescence and the production of NADH [9], menadione might oxidize NADH produced by glycolysis rather than by NADPH produced by pentose phosphate pathway. Other study suggested the involvement of pentose phosphate pathway and/or glycolysis in yeast cells [8]. In mammalian cells reduction of menadione might be reduced by NADPH of pentose phosphate pathway and NADH of glycolysis [5].

MSB might be reduced by plasma membrane redox system, because electron transfer mediated by MSB was inhibited by permeabilization of yeast cells (Figs. 4 and 5). As MSB promoted oxidation of NADH rather than NADPH in permeabilized yeast cells (Fig. 6), MSB as well as extracellular  $\text{Fe}^{3+}$  might be reduced by the electron transfer across plasma membrane redox system which could reduce the extracellular and non membrane-permeant  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  [19–25] by oxidizing NADH rather than NADPH [9,26].

Reduction of menadione and MSB by NAD(P)H is considered to be catalyzed by NAD(P)H:quinone reductase. This enzyme is a flavoprotein catalyzing two-electron reduction of quinones to hydroquinones, using either NADH or NADPH as electron donor [7,18]. Hydroquinone is considered to be produced without the accumulation of a dissociated semiquinone [7]. According to this mechanism one-electron reduction of dissolved oxygen by menadiol might generate superoxide anion. Hydrogen peroxide might be generated by dismutation of superoxide anion and/or two-electron reduction of dissolved oxygen by menadiol.

From the above results and discussion, this study is summarized as follows. Menadione is membrane-permeant redox mediator and can't keep the stable electron transfer from yeast cells to anode by promoting both the production of AOS and oxidation of NAD(P)H. On the other hand, MSB is non membrane-permeant redox mediator and mediates the stable electron transfer from yeast cells to anode rather than the production of AOS after reduction of MSB by plasma membrane redox system.

The above different characteristics between menadione and MSB acting as redox mediators will contribute to the studies on mitochondrial diseases [27] and cancer therapy [28–30], because menadione and MSB are expected to be useful tool to study the mechanism of diseases resulting from the disorder of cellular redox systems.

#### Conflict of interest

I have no conflict of interest about this paper because this study was independently performed within the limited role and budget, and is not involved in any patents.

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