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Extracellular generation of active oxygen species catalyzed by exogenous menadione in yeast cell suspension

Shiro Yamashoji, Tatsuhiko Ikeda and Kumiko Yamashoji

Institute of King Brewing, Hiraoka, Kakogawa (Japan)

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Luminol chemiluminescence was observed by addition of menadione to yeast cell suspension and was amplified 1000-fold by further addition of Fe-complex. Catalase, superoxide dismutase and ceruloplasmin had inhibitory effects on luminol chemiluminescence, indicating the extracellular generation of active oxygens (H_2O_2 and O_2^-) and reduction of Fe-complex. The generation of H_2O_2 and reduction of Fe-complex were mainly dependent on the activity of NADH : menadione oxidoreductase in the plasma membrane and cytosol fractions. Both luminol chemiluminescence and H_2O_2 production were sensitive to the inhibitory effects of proton conductor, ionophorous antibiotics and ATPase inhibitor rather than the inhibitors of the mitochondria electron transport system. The incubation of glucose with yeast cells caused a parallel increase in luminol chemiluminescence, H_2O_2 production and intracellular NADH concentration. These facts suggest that menadione-catalyzed H_2O_2 production and chemiluminescence are used as the indicators of cell activity to keep the NADH concentration and NADH : menadione oxidoreductase activity which may be sensitive to the change in pH and ion concentrations.

Introduction

Active oxygen species such as O_2^-, H_2O_2 and ·OH are produced in the biological system. For example, xanthine oxidase (EC 1.1.3.22) [1], peroxidase (EC 1.11.1.7) [2] and microsomal NADPH-cytochrome *P*-450 reductase [3] are involved in the generation of active oxygen species. Some important NAD(P)H-dependent flavoenzymes can simultaneously reduce molecular oxygen to generate H_2O_2 and, evently, the ·OH radical [4].

Phagocytes such as polymorphononuclear leukocytes also can produce the active oxygen species (O_2^- and H_2O_2) [5]. The generation of the active oxygen species as a result of respiratory burst activation of polymorphonuclear leukocytes has been considered to be an essential step in the host defence against microorganisms. The active oxygen species are considered to be produced by NADPH-oxidase whose terminator component, cytochrome *b*, is located in both plasma membrane and a granule fraction [6,7]. H_2O_2 produced in cells can be detected on the outside of cells because H_2O_2 can cross the membrane [8].

Recently, H_2O_2 was found to be produced by the eukaryotic cells which were exposed to exogeneous menadione, and menadione-catalyzed H_2O_2 production was proportional to the viable cell number [9]. Furthermore, we found that luminol chemiluminescence was greatly stimulated by the addition of menadione and Fe-complex to the suspension of yeast cells which were chosen as a simple model of eukaryotic cells.

Menadione is known to be reduced to menadiol and semiquinone by NAD(P)H : menadione oxidoreductase [10]. Mitochondria NADH-ubiquinone reductase also reduces menadione to its semiquinone [11]. Menadiol and semiquinone are oxidized by dissolved oxygen, and then O_2^- and H_2O_2 are produced [11,12]. However, little is known about the reduction mechanism of exogenous menadione stimulating H_2O_2 production and luminol chemiluminescence. The present paper demonstrates that menadiol or semiquinone reduces dissolved oxygen to H_2O_2 or O_2^- by shuttling hydrogen

Abbreviations: O_2^-, superoxide anion; FAD, flavin adenine dinucleotide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; NBD, 7-chloro-4-nitrobenzoxadiazole; TFA, 2-tuenoyl-trifluoroacetone; DCCD, dicyclohexyl carbodiimide.

Correspondence: S. Yamashoji, Institute of King Brewing, 2333-2 Kirigaikedani, Shinzaike, Hiraoka, Kakogawa, 675-01 Japan.

or electron between extracellular oxidants and NADH: menadione oxidoreductase located in plasma membranes and cytosol. In addition, the present paper proposes the possibility that menadione-catalyzed H_2O_2 production is an indicator for cell activity.

Materials and Methods

Organism and growth conditions. *Saccharomyces cerevisiae* strain IFO 2044 was grown aerobically at 30°C in a medium containing 1% malt extract, 0.5% yeast extract and 2% glucose (pH 5.8). The anaerobic conditions were performed by bubbling nitrogen through the medium. The cells were harvested in the initial stationary phase of growth, washed twice and suspended in 50 mM imidazole-nitrate (pH 7.0). The viable cell number was determined using 4% trypan blue stain and hemicytometer.

Preparation of protoplasts, plasma membrane and mitochondria. Yeast protoplasts were prepared as reported in Ref. 13. The protoplasts were suspended and homogenized in 0.5 M mannitol, 1 mM EDTA, 10 mM Tris-HCl (pH 7.4) and 0.1% bovine serum albumin. The homogenate was centrifuged at $2000 \times g$ for 5 min, and the pellet was used for the purification of plasma membrane according to the method described in Ref. 14. The supernatant was centrifuged at $8000 \times g$ for 10 min, and the pellet was used for the purification of mitochondria according to the method described in Ref. 15.

Solubilization of membrane proteins. Purified plasma membrane was incubated in the solution containing 1% Triton X-100, 1 M KCl, 2.5 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonylfluoride and 0.1 M Tris-HCl (pH 7.0) overnight. Solubilized proteins were obtained from the supernatant after the centrifugation at $105\,000 \times g$ for 1 h.

Gel filtration of solubilized proteins. Proteins solubilized were applied to a Sepharose CL-6B column (3×92 cm), and eluted with 0.1 mM phenylmethylsulfonylfluoride and 0.1 M Tris-HCl (pH 7.0). Each fraction volume was 6.3 ml. Protein concentration was determined by the Coomassie brilliant blue G-250 dye-binding method using Bio-Rad dye reagent [16]. Elution of enzymes was monitored by measuring the enzyme activity to produce H_2O_2. Elution of flavoproteins was monitored by measuring fluorescence emission at 530 nm with excitation at 450 nm. The molecular mass of the enzyme eluted was measured by gel filtration on a Sepharose CL-6B column (3×92 cm). β-Amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12.4 kDa) were used as molecular mass markers.

Assay of NAD(P)H: menadione oxidoreductase activity. The activity of NAD(P)H: menadione oxidoreductase was assayed in 50 mM imidazole-nitrate (pH 7.0), 1 mM NAD(P)H, and 0.5 mM menadione. The reaction mixture was incubated at 30°C for 2 min. H_2O_2 produced was determined with chemiluminescent assay.

Assay of H_2O_2. 1 ml of the reaction mixture was introduced into the vial for the photomultiplier, and 1 ml of the solution containing 60 mg bis(2,4,6-trichlorophenyl)oxalate and 10 mg pyrene in 100 ml of acetonitrile was injected into the above vial through the tube [9]. The chemiluminescence intensity after the injection was automatically counted for 5 s.

Assay of luminol chemiluminescence. Chemiluminescent assay was performed in a 0.2 M Tris-HCl (pH 7–9.5) or 0.2 M borate (pH 10–10.5) containing yeast cells, luminol (0.6 mg/ml), 0.5 mM menadione and 5 mM EDTA. The chemiluminescence intensity was counted after the addition of 0.1 mM $FeCl_3$ into the above reaction mixture. The temperature of the reaction mixture was kept at 30°C. Measurement of light intensity was carried out with a model ATP-237 lumicounter (Toyo Kagaku Sangyo, Tokyo).

Assay of intracellular NADH concentration. Yeast cells were washed twice with distilled water, heated in 0.1 M KOH at 70°C for 7 min, and centrifuged at $10\,000 \times g$ for 10 min. The supernatant was used for the determination of NADH concentration according to the method described in Ref. 17.

Assay of ferricyanide reduction. The rate of ferricyanide reduction was determined by recording the decrease in the absorbance at 400 nm reference to that at 500 nm. The millimolar extinction coefficient was 1.0 [18,19].

Chemicals. All reagents were of analytical grade.

Results

Luminol chemiluminescence

Upon addition of menadione to yeast cell suspension containing luminol, chemiluminescence was amplified and increased with an increase in pH. Further addition of Fe-EDTA complex caused 1000-fold amplification at pH 9.5 as shown in Fig. 1. The chemiluminescence intensity was proportional to viable cell number and disappeared upon heating of yeast cells (data not shown). These facts show that menadione-catalyzed luminol chemiluminescence requires iron complex and intact yeast cells. The catalytic actions of iron complexes on menadione-catalyzed luminol chemiluminescence depend on the kinds of chelating reagent. For example, ferricyanide-catalyzed chemiluminescence was inhibited by catalase rather than superoxide dismutase, and the chemiluminescence catalyzed by the Fe-EDTA complex was inhibited by superoxide dismutase rather than catalase as shown in Fig. 2. The catalytic action of the Fe-ADP complex was inhibited

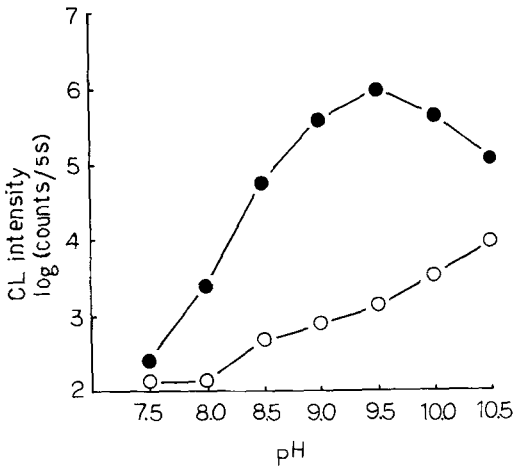

Fig. 1. Luminol chemiluminescence (CL) catalyzed by yeast cells. The reaction conditions were described in Materials and Methods. Cell density was $8 \cdot 10^6$ cells/ml. (○) 0.5 mM menadione (●) 0.5 mM menadione, 0.1 mM $FeCl_3$ and 5 mM EDTA.

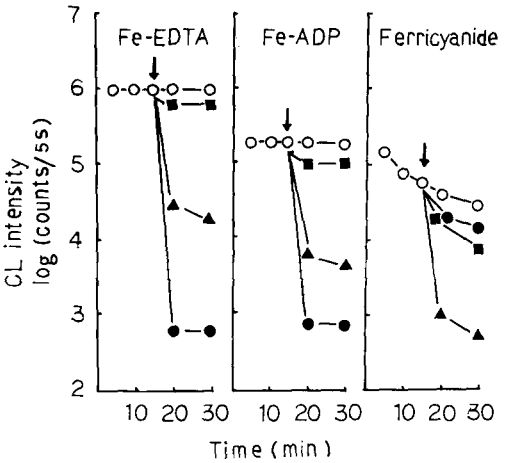

Fig. 2. Effects of catalase, superoxide dismutase and ceruloplasmin on luminol chemiluminescence in the presence of yeast cells ($7 \cdot 10^6$ cells/ml), 0.5 mM menadione and Fe-complex at pH 9.5. The concentrations of Fe-complex were as follows: Fe-EDTA, 0.1 mM $FeCl_3$ and 5 mM EDTA; Fe-ADP, 0.1 mM $FeCl_3$ and 25 mM ADP; ferricyanide, 0.1 mM. The reaction conditions of luminol chemiluminescence are described in Materials and Methods. ○, no addition; ●, superoxide dismutase (20 U/ml); ▲, catalase (420 U/ml); ■, ceruloplasmin (6 U/ml). Arrows represent the additions of above enzymes.

by superoxide dismutase rather than catalase and was similar to that of the Fe-EDTA complex. Ceruloplasmin having ferroxidase activity had also an inhibitory effect on luminol chemiluminescence catalyzed by menadione and iron complexes. When superoxide dismutase, catalase and ceruloplasmin were boiled, these enzymes had no inhibitory effect on luminol chemiluminescence, indicating that these enzymes had the enzyme activities at alkali pH. The above facts show that O_2^-, H_2O_2 and the reduced form of the iron complex are extracellulary produced in yeast cell suspension containing menadione and that the production ratio of O_2^- and H_2O_2 depends on the kinds of iron complex.

Location of NADH: menadione oxidoreductase

The production of H_2O_2 and reduction of ferricyanide were observed in the mixture containing menadione, NAD(P)H and plasma membrane or cytosol fraction prepared from yeast cells (Table I). NADH was a more specific reductant for menadione-catalyzed H_2O_2 production than NADPH. The production of H_2O_2 was greatly stimulated by the addition of both NADH and menadione compared to the reduction of ferricyanide. These facts suggest that the production of H_2O_2 is sensitive to the intracellular NADH concentration and NADH: menadione oxidoreductase activity.

The NADH: menadione oxidoreductase producing H_2O_2 was mainly located in the pellet obtained at $2000 \times g$ and the supernatant obtained at $8000 \times g$, which were prepared from protoplasts of yeast cells grown aerobically or anaerobically (Table II). The pellet obtained at $8000 \times g$ had a low activity of NAD(P)H: menadione oxidoreductase, suggesting the low activity of NAD(P)H: menadione oxidoreductase in mitochondria. As shown in Table II, aerobic growth

TABLE I

H_2O_2 production and ferricyanide reduction by plasma membrane and cytosol fractions

The reaction mixture contained 50 mM imidazole-nitrate (pH 7.0), 1 mM NAD(P)H, 0.5 mM menadione, 0.8 mM ferricyanide and plasma membrane (230 μg/ml) or cytosol fraction (290 μg/ml). Assays of H_2O_2 and ferrocyanide were performed after the incubation at 30°C for 2 min. Values are mean ± S.E. of duplicate determinations.

Additions	H_2O_2 production (nmol/min per mg protein)		Ferricyanide reduction (nmol/min per mg protein)	
	P [a]	C	P	C
NADH	9± 0	1±0	1066±43	493±21
NADPH	9± 0	1±0	467±19	266±12
NADH + menadione	1623±55	145±6	2933±76	488±19
NADPH + menadione	165± 6	79±3	476±18	254±10

[a] P and C represent plasma membrane and cytosol fractions, respectively.

TABLE II

Activity of NAD(P)H:menadione oxidoreductase in the different fractions prepared by centrifugation from yeast cells grown aerobically or anaerobically

Fractionation by centrifugation and assay of H_2O_2 are described in Materials and Methods. Values are mean ± S.E. of duplicate determinations.

Additions	Enzyme activity (H_2O_2 μmol/min per g wet cells)					
	aerobic growth			anaerobic growth		
	A[a]	B	C	A	B	C
NADH + menadione	9.0 ± 0.1	0.6 ± 0.1	4.7 ± 0.3	2.1 ± 0.2	0.2 ± 0	11.1 ± 0.5
NADPH + menadione	1.8 ± 0.1	0.1 ± 0	4.7 ± 0.2	1.8 ± 0.1	0.1 ± 0	7.7 ± 0.3

[a] A, B and C represent the 2000 × *g* sediment, the 8000 × *g* sediment and the 8000 × *g* supernatant, respectively.

conditions caused the induction of NADH : menadione oxidoreductase located in the pellet at 2000 × *g* and the decay of this enzyme located in the supernatant at 8000 × *g*. The NADPH : menadione oxidoreductase activity was not affected by the change in growth conditions.

Table III shows the specific activity of NAD(P)H : menadione oxidoreductase of plasma membrane and cytosol fractions prepared from yeast cells grown aerobically. The specific activity of NADH : menadione oxidoreductase in plasma membrane and mitochondria fractions was much higher than that of NADPH : menadione oxidoreductase in those fractions. The specific activity of NAD(P)H : menadione oxidoreductase in the cytosol fraction was much lower than that in the plasma membrane and mitochondria fractions.

On the basis of the results shown in Table II, NADH : menadione oxidoreductase located in plasma membrane is considered to play an important role in the production of H_2O_2 and luminol chemiluminescence catalyzed by intact yeast cells. Furthermore, NADH : menadione oxidoreductase in plasma membrane may face cytosol because proteinase inhibited NADH : menadione oxidoreductase activity in the plasma membrane fraction rather than protoplasts as shown in Table IV.

TABLE III

Specific activity of NAD(P)H:menadione oxidoreductase in plasma membrane, mitochondria and cytosol fractions

The preparation of each fraction and assay of enzyme activity are described in Materials and Methods. Values are mean ± S.E. of duplicate determinations.

Additions	Specific activity (H_2O_2 μmol/min per mg protein)		
	plasma membrane	mito-chondria	cytosol
NADH + menadione	1.6 ± 0.2	1.4 ± 0.1	0.1 ± 0
NADPH + menadione	0.2 ± 0	0.1 ± 0	0.1 ± 0

Gel filtration of NAD(P)H:menadione oxidoreductase

NAD(P)H : menadione oxidoreductase in plasma membrane was solubilized with Triton X-100 and KCl, and the supernatant obtained after the centrifugation at 105 000 × *g* was applied to a Sepharose CL-6B column. The elution of NAD(P)H : menadione oxidoreductase from the column yielded one peak of the enzyme activity (Fig. 3). The molecular mass of NAD(P)H : menadione oxidoreductase in the peak was estimated to be about 80 kDa, and was similar to that of NADH : menadione oxidoreductase (70 kDa) in bakers' yeast cells [20]. Misaka et al. reported that NAD(P)H : menadione oxidoreductase from bakers' yeast had FAD as the prosthetic group, but they did not confirm the location of this enzyme in yeast cells [20]. NADH : menadione oxidoreductase solubilized from plasma membrane fraction may also be a flavoprotein because the elution peak of enzyme activity overlapped with the peak of fluorescence resulting from flavoprotein.

Inhibition of H_2O_2 production and luminol chemiluminescence

Table V shows that nystatin, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and 7-chloro-4-nitro-

TABLE IV

Effect of proteinase on H_2O_2 production by plasma membrane fraction and protoplasts

Plasma membrane fraction and protoplasts were incubated with proteinase in 50 mM imidazole-nitrate (pH 7.0) at 30°C for 30 min. After the incubation with proteinase, 0.5 mM menadione and 1 mM NADH were added to plasma membrane fraction or protoplasts suspension. The reaction mixture was kept at 30°C for 2 min, and then H_2O_2 produced was assayed. Values are mean ± S.E. of duplicate determinations.

Additions	Percent inhibition (%)	
	plasma membrane	protoplasts
Alkaline proteinase (35 U/ml)	85 ± 4	8 ± 1
Papain (6 U/ml)	81 ± 5	9 ± 1

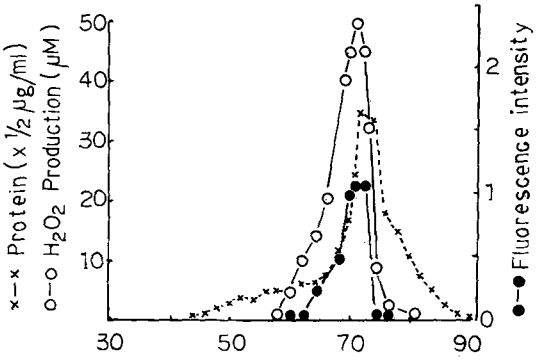

Fig. 3. Gel filtration of NAD(P)H: menadione oxidoreductase solubilized from plasma membrane fraction. The conditions of gel filtration were described in Materials and Methods.

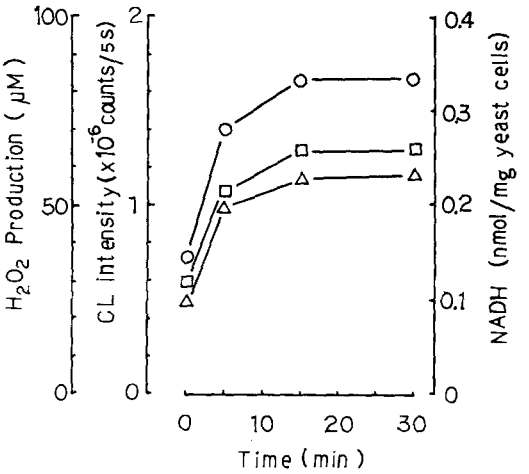

Fig. 4. Effect of glucose on H_2O_2 production, luminol chemiluminescence, and intracellular NADH concentration. The reaction was started by addition of 50 mM glucose to yeast cell suspension ($2.6 \cdot 10^7$ cells/ml) in 50 mM imidazole-nitrate (pH 7.0). H_2O_2 production, luminol chemiluminescence and NADH concentration were assayed at the time marked by each symbol in Fig. 4, according to the methods described in Materials and Methods. ○, luminol chemiluminescence in the presence of 0.5 mM menadione, 0.1 mM $FeCl_3$ and 5 mM EDTA at pH 9.5; □, NADH concentration; △, H_2O_2 production in the presence of 0.5 mM menadione at pH 7.0.

benzoxadiazole (NBD) had greater inhibitory effects on both luminol chemiluminescence and H_2O_2 production than other ionophorous antibiotics, ATPase inhibitor and inhibitors of the mitochondria electron transport system. Rotenone and 2-tuenoyl-trifluoroacetone (TFA) had a small inhibitory effect on luminol chemiluminescence, but antimycin A and dicyclohexyl carbodiimide (DCCD) inhibited both luminol chemiluminescence and H_2O_2 production. These facts suggest that menadione-catalyzed luminol chemiluminescence and H_2O_2 production are sensitive to the actions of proton conductor (CCCP), ionophorous antibiotics (nystatin, nigericin and valinomycin) and inhibitors of the proton pump (stilbestrol and DCCD) rather than the inhibitors of the mitochondria electron transport system (rotenone and TFA). As DCCD is known to have no effect on NADH: quinone oxidoreductase in yeast mitochondria [21], DCCD acting as carbonyl modifying reagent may inhibit the proton translocation by binding to a proteolipid subunit of ATP synthase [22,23]. Antimycin A also may inhibit the membrane functions rather than the electron transport in complex III in mitochondria because the contribution of mitochondria to menadione-catalyzed H_2O_2 production is considered to be very small according to the results in Table II.

TABLE V

Inhibitory effects of protonophore, ionophoric antibiotics, inhibitors of ATPase and inhibitors of the mitochondria electron transport system

Assay of H_2O_2 and luminol chemiluminescence were described in Materials and Methods. Values are mean ± S.E. of duplicate determinations.

Additions	Percent inhibition (%)	
	H_2O_2 production	luminol chemiluminescence [a]
20 μM CCCP	92±3	94±3
20 μM NBD	97±2	99±0
20 μM Nystatin	93±4	99±1
10 μM Valinomycin	61±3	69±5
20 μM Nigericin	39±2	56±4
20 μM Antimycin A	44±2	50±4
40 μM DCCD	44±2	46±3
20 μM Rotenone	0±0	17±2
40 μM TFA	0±0	0±0
20 μM PCMS	0±0	7±0
20 μM Diethylstilbestrol	28±2	72±3

[a] The assay of luminol chemiluminescence was performed at pH 9.5.

Effect of glucose

Yeast cells had low activity to produce H_2O_2 and to promote luminol chemiluminescence in the presence of menadione after the growth of yeast cells reached to the stationary state. The addition of glucose to yeast cells after the incubation in distilled water for 3 days caused the increase in intracellular NADH concentration, H_2O_2 production and luminol chemiluminescence, showing the proportionality among these changes (Fig. 4). These increases reached to the maximum state within 30 min after the addition of glucose. These facts suggest that glucose is rapidly metabolized by glycolysis producing NADH and that both H_2O_2 production and luminol chemiluminescence catalyzed by menadione are dependent upon the intracellular NADH concentration.

Discussion

The present paper demonstrates the presence of NADH: menadione oxidoreductase in plasma mem-

brane. This enzyme required NADH rather than NADPH and reduced dissolved oxygen to H_2O_2. The molecular mass of NADH : menadione oxidoreductase solubilized from plasma membrane was estimated to be about 80 kDa, and this enzyme seems to be flavoprotein on the basis of the elution pattern on gel filtration (Fig. 3). Though flavin has been considered to be a redox carrier in plasma membrane of yeast cells [24,25], flavin has not been identified as a component of NADH : menadione oxidoreductase in plasma membrane. Fig. 3 suggests that flavin is a component of NADH : menadione oxidoreductase in yeast plasma membrane. NADH : menadione oxidoreductase may be tightly bound to the plasma membrane which faces cytosol, because this enzyme was not completely solubilized with Triton X-100 and KCl, and proteinase inhibited the enzyme activity of the plasma membrane fraction rather than protoplasts.

The activity of NADH : menadione oxidoreductase in plasma membrane and cytosol was larger than that in mitochondria, and H_2O_2 production was proportional to the intracellular NADH concentration increased by the addition of glucose into yeast cell suspension. These facts suggest that NADH : menadione oxidoreductase producing H_2O_2 is mainly located in plasma membrane and cytosol, and the production of H_2O_2 is controlled by the concentration of NADH which is produced by cytosol glycolysis system. Furthermore, it was found that the aerobic growth conditions enhanced the activity of NADH : menadione oxidoreductase in the pellet (at $2000 \times g$) containing plasma membrane.

Reduction of menadione catalyzed by NAD(P)H : menadione oxidoreductase has been considered to result in the production of relatively stable hydroquinone accompanied by a stoichiometric oxidation of NADH and no O_2 consumption [10]. Furthermore, this enzyme has been considered to inhibit the formation of toxic semiquinones which are readily autooxidized with the formation of O_2^-. However, it was reported that menadiol produced by NAD(P)H : menadione oxidoreductase could generate O_2^- and H_2O_2 during autoxidation of menadiol in oxygenated buffer and that menadiol yielded 14% O_2^- and 86% H_2O_2 during autoxidation [11]. Other NAD(P)H-oxidase flavoproteins, one-electron transfer enzymes, can generate semiquinones whose oxidation results in the formation of O_2^- and H_2O_2 [4]. The present paper demonstrates that NADH : menadione oxidoreductase in yeast plasma membrane and cytosol catalyzes the formation of O_2^- and H_2O_2. The extracellular production of O_2^- and H_2O_2 catalyzed by exogenous menadione suggests that menadione permeates into the cell to be reduced by NADH : menadione oxidoreductase and that the resultant menadiol and semiquinone permeate into the outside of cell to reduce extracellular dissolved oxygen to

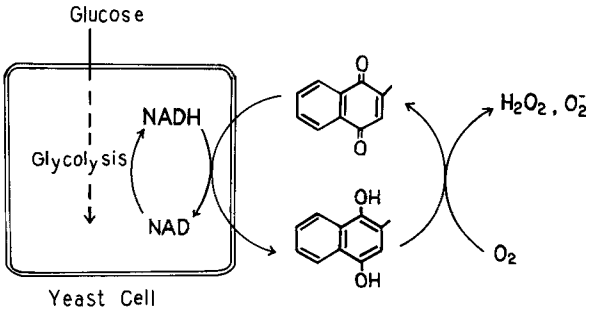

Fig. 5. Proposed mechanism for the production of H_2O_2 and O_2^- catalyzed by exogeneous menadione in yeast cell suspension.

O_2^- and H_2O_2. Extracellular Fe-complex is also considered to be reduced by menadiol and semiquinone, because luminol chemiluminescence was inhibited by ceruloplasmin having ferroxidase activity. These results suggest that exogenous menadione acts as hydrogen or electron mediator between extracellular oxidants and intracellular NADH : menadione oxidoreductase.

The activity of NADH : menadione oxidoreductase may be sensitive to the change in pH and ion concentrations, because proton conductor and ionophorous antibiotics inhibited both luminol chemiluminescence and H_2O_2 production. For example, nystatin and valinomycin are known to form the pore in plasma membrane of yeast cells and to cause the release of ions from the cells. Though exogenous menadione is known to be reduced by yeast mitochondria supplemented with NADH [26] and to act as an electron carrier between NADH and cytochromes c and c_1 in respiratory-deficient box mutant mitochondria [27], the inhibitors of mitochondria electron transport system have little effect on both luminol chemiluminescence and H_2O_2 production. The little effects of these inhibitors may result from the fact that NADH : menadione oxidoreductase activity is low in the $8000 \times g$ sediment containing mitochondria compared to the $2000 \times g$ sediment containing plasma membrane and the $8000 \times g$ supernatant containing cytosol.

The above results and discussion suggest the mechanism as shown in Fig. 5 and that the extracellular production of H_2O_2 and O_2^- catalyzed by exogenous menadione is the signal for the detection of cell viability to keep the activity of NADH : menadione oxidoreductase and the concentration of NADH resulting from glycolysis.

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