STUDIES ON ADENOSINE TRIPHOSPHATASE SYSTEM IN YEAST: *Saccharomyces cerevisiae*

(I) An Adenosine Triphosphatase in Cytoplasmic Particles from Respiration-Deficient Mutant Yeast

(II) Masking of Hydrolytic Activity of ATPase

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STUDIES ON THE ADENOSINE TRIPHOSPHATASE SYSTEM IN YEAST; Saccharomyces cerevisiae

(I) An Adenosine Triphosphatase in Cytoplasmic Particles from a Respiration - Deficient Mutant Yeast

A soluble factor (F_1) catalysing the transfer of inorganic phosphate from a high-energy intermediate to ADP has been isolated from beef heart mitochondria in a highly purified form by Racker et al. (1). The factor has a high activity hydrolysing ATP but it is essential for recoupling the reverse reaction, phosphorylation to electron transfer in submitochondria particles. From the elegant works of these authors, the ATPase activity of mitochondria has been believed not to be native but to be caused by the environmental changes around F_1 in mitochondrial membrane.

Solubilization of F_1 from the membrane causes some changes of F_1 protein and evokes the hydrolytic activity. Moreover, the solubilization is accompanied with loss of oligomycin sensitivity and cold-stability of the native enzyme. Extensive works have been carried out to elucidate how these changes are caused during solubilization procedure and disclosed other factors involved in the phosphorylation, F_o, CF_o and ATPase inhibitor from the mitochondrial membrane (2,3,4). The factors, F_o and ATPase

inhibitor bind to the solubilized ATPase and the resulting complexes shows the oligomycin sensitivity and cold-stability, respectively. The other factor CF_o has been obtained by the further purification of F_o and demonstrated to inhibite ATPase activity of the enzyme , and therefore to involve the ATPase inhibitor (3).

From yeast mitochondria, several workers have isolated ATPase in a highly purified form (5,6). Schatz reported that the mitochondrial ATPase is also found in the RD-mutant yeast cells and the enzyme is cold-labile oligomycin-insensitive even in the membrane found form (7). The author suggested that these abnormal properties of the enzyme was due to the impaired linkage to the membrane that might be a result of loss or alteration of CF_o or a protein constituent of CF_o. However, the arrangment of these factors, ATPase, ATPase inhibitor and CF_o in the mitochondrial membrane and their relationship to the phosphorylating activity are still ambiguous.

The present paper deals with the oligomycin-sensitive ATPase in RD-mutant yeast cells in disagreement with the observation by Schatz and with some properties of the enzyme comparing with those from wild-type yeast cells.

EXPERIMENTALS

Yeast strains ----- Wild-type strain of haploid Saccharomyces cerevisiae and its respiration-deficient mutant yeast were used.

These strains were kindly supplied by Dr. Hirota*, Depertment of genetics of Osaka University.

Culture of cells ----- The medium for aerobic culture consisted of 1 % yeast extract, 1 % polypeptone and 1 % glucose. In the case of the culture of mutant cells, glucose concentration was increased up to 2 %. The culture medium of 1000 ml in 5000 ml flask was shaken constantly for sufficient supply of oxygen. Cultivation was carried out at 30°C. The cells were harvested by centrifugation at the early period of the stationary phase. The yield was 10 to 13 grams (wet weight) per flask.

Preparation of mitochondria and microsoms ----- The yeast cell walls were digested by snail gut enzyme to make protoplasts susceptible to osmotic shock. There are several reports about the methods to prepare protoplasts using snail enzyme (8,9,10). In this study, the method which was developed by Ohnishi (1o). was employed with minor modification. Yeast cells (100 g) were incubated for 1 hr at 30° C in the medium containing 15 mM cystein, 15 mg pronase, Streptomyces greseus proteinase, and 25 mM Tris - HCl buffer, pH 8.6 in a final volume of 300 ml prior to the snail enzyme treatment. As reported by Burger et al. (9), yeast cells become more susceptible to snail enzyme by this treatment. The resultant protoplasts were suspended in 0.5 M mannitol solution containing 20 mM maleate - Tris buffer, pH 7.0,

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0.2 mM EDTA and 0.05 % bovine serum albumin. This resulted in lysis of the protoplasts by osmotic change. For the fractionation of subcellular organelles, ordinary differential centrifugation was employed. The successive procedures are summerized in Fig. 1. After fractionation of cytoplasmic particles, mitochondria and microsome were suspended in the mannitol solution to give a final protein concentration of 20 to 30 mg per ml. Occationally the preparations were stored at -20°C for about a week without loss of activity.

Preparation of soluble mitochondrial ATPase ----- For the extraction of ATPase the mitochondria preparation (1 g) was centrifuged at 10,000 g for 10 min in order to substitute the medium. The packed pellet was suspended in 50 ml of the extracting medium consisted of 0.1 M Tris - acetate buffer, pH 7.2, 2 mM EDTA and 7 % ethanol to give a protein concentration of 5 mg per ml. The suspension was treated with a sonic oscillator for 2 min in 10 ml batch. The sonicated suspension was centrifuged for 60 min at 105,000 g to remove membrane fraction. The resulting supernatant was used as crude extractof ATPase preparation. Up to this step, all procedures were carried out at 0°C to 4°C. The crude extract was applied to a DEAE-cellulose column equilibrated with 0.1 M Tris - acetate buffer, pH 7.2. After washing the column with the same buffer, ATPase was eluted out with 0.2 M of the buffer at room temperature. To the eluate, Mg^{++} and ethanol

were added at final concentrations of 5 mM and 7 %, respectively, to protect the cold-inactivation. The preparation was used as partially purified ATPase.

Assay of ATPase activity ----- The hydrolytic activity was measured at 25°C in the reaction mixture consisted of 25 mM maleate - Tris buffer, pH 7.0, 2.5 mM $MgSO_4$ and 0.2 mM EDTA. ATPase added was about 0.5 to 1.0 units per ml in the reaction system. The reaction was started by the addition of ATP at a final concentration of 2.5 mM. After the reaction started, 0.5 ml was pipetted out at 0, 1, and 5 min and put into 3 ml of 6 % perchloric acid to stop the reaction. Pi released was determined after Allen (11). When the solution was turbid the color reaction was carried out after the precipitate was spun down.

Protein assay ----- Proteins were determined by the method of Lowry et al. and biuret method using crystalline bovine serum albumin as a standard (12, 13).

Reagents ----- Ordinary chemicals were obtained from commercial products. Oligomycin was dissolved in methanol.

RESULTS

ATP hydrolytic activity and oligomycin sensitivity of membrane - bound enzyme ----- ATPase activities of mitochondria from both strains, wild - type yeast and mutant - yeast are shown in Table 1. Although ATPase activity varied with different

preparation, freshly prepared mitochondrial ATPase from wild - type yeast had a fairly constant specific activity of about 100 mμ moles Pi released per mg protein per min, while the activity in mutant yeast mitochondria was 200 to 300, which was always greater than that from wild - type yeast.

ATPase activity of both the strains was inhibited at a low concentration of oligomycin. By the addition of 10μg oligomycin per mg of mitochondrial protein, ATPase activity in wild-type yeast mitochondria was inhibited to the extent of 70 %, while in the case of mutant type yeast mitochondria, the inhibition rate to the mutant yeast mitochondria suggests that a large part of ATPase is in abnormal form in them, but at least some part is in a similar form to in wild type yeast mitochondria. Actually, as shown in Fig. 2, the activity in both strains mitochondria was found to be inhibited the similar manner at low concentrations of the antibiotics. Thus, although to the mutant yeast mitochondria was found to be inhibition reached at highest 40 to 50 % even at a high concentration of oligomycin, 40μg/mg protein. The half inhibition rate of oligomycin-sensitive activity achieved by the first addition of 1 μg consistently with wild-type yeast mitonchondria. This finding is in disagreement with the results reported by Schatz (7) that ATPase of mitochondria in RD-mutant yeast cells is insensitive to oligomycin even at a high concentration, 80 μg/mg protein.

Extraction of ATPase by sonication ----- Effect of sonication on the release of ATPase from mitochondrial membrane is shown in Table 2. As described in "Experimentals" mitochondriaa in the medium of 0.1 M Tris-acetate buffer were sonicated and particles and soluble fractions were separated by centrifugation. Each fraction was treated with heat in the presence of sodium sulfate to elicite full ATPase activity before the measurement of its activity. About 20 to 30 % of ATPase activity was solubilized from wild-type yeast mitochondria and the enzyme was oligomycin-insensitive, while residual enzyme in particles, like original one, was sensitive to oligomycin. However, from mutant yeast mitochondria, about 90 % ATPase was released and recovered in supernatant fraction. The residual ATPase in the mutant mitochondria fraction was no more solubilized by further sonication and had the some properties as that of microsomal ATPase as will be mentioned latter. From these facts, the ATPase solubilized from mutant cells is very likely to correspond to the mitochondrial enzyme which is loosely bound to mitochondrial membrane in situ, while the residual tightly bound ATPase might be originated microsomes contaminated during preparation.

Another peculiar situation of ATPase in the mutant yeast mitochondria was observed in the effect of Mg^{++} on extracting the enzyme. When Mg^{++} was added to the extracting medium, the recovery in the soluble fraction from wild-type yeast

mitochondria was greatly diminished (Table 3), while about 90 % ATPase was consistently recovered in the supernatant in the case of mutant yeast mitochondria regardless to the presence or absence of Mg^{++}.

Effect of tonicity on release of ATPase from mitochondria ----- The mitochondrial ATPase of the mutant yeast, unlike that of wild-type yeast was also found to be released from mitochondria by the incubation in a hypotoni medium in ice and made cold-labile simultaneously. As shown in Fig. 3, this cold lability of the enzyme was not observed in an isotonic mannitol solution (0.5 M) for 20 hrs. Even in the hypotonic medium, however, the enzyme was kept stable in the presence of ethanol and recovered in the supernatant fraction.

Unmaskedness of the mutant mitochondrial ATPase in situ -----

Mitochondrial ATPase has been known to be demasked by disruption of the mitochondrial structure such as aging and sonication (14). In yeast mitochondria, this was also the case and ATPase activity in mitochondria from wild-type yeast cells was found to be increased several fold by heating in 0.5 M sodium sulfate. However, in the case of mutant yeast mitochondria, slight enhancement of the activity was observed by the heat-treatment. As shown in Fig. 4, the enhancement of mutant yeast mitochondria is seen only first 10 min and the activity rather decreases by further incubation. These results indicate that the

mitochondrial ATPase in the mutant yeast cells may be demasked originally in mitochondria.

Properties of ATPase in microsomes----- Since it was prepared by a single differential centrifugation the microsome preparation used in the present investigation was not pure. However, as described above already, mitochondrial ATPase of mutant yeast was released easily from the membrane by sonication and hypotonic treatment. As shown in Table 5, 37 % ATPase activity was released from the microsome fraction and the released ATPase showed the same properties as those of solubilized mitochondrial ATPase. Moreover, the incubation of microsomes in 0.1 M Tris-acetate buffer, pH 7.2 at 0°C for 6 hr resulted in 37 % inactivation of the activity. These results suggest that the released ATPase may come from mitochondria contaminated during preparation. Accordingly the microsome fraction was sonicated to exclude mitochondrial ATPase and the sonicated microsomes were used as microsome preparation.

ATPase was no more solubilized from the microsome by the further sonication. The ATPase activity was neither activated by maleate (Fig. 5) nor sensitive to oligomycin at a low concentration, while the mitochondria enzyme was activated by maleate and sensitive to oligomycin. Moreover, the microsomal ATPase required Mn^{++} for the activity (Table 6), while Mg^{++} was the most effective for the mitochondria enzyme. Mn^{++} was also required

for the ATPase in the wild-type yeast microsomes, which, however, was not so readily separated from the mitochondrial enzyme contaminated as in the mutant microsomes by sonication.

Properties of isolated ATPase from the mutant mitochondria ----- Although between wild-type and mutant yeast mitochondrial ATPase there were differences in the latency of hydrolytic activity and oligomycin sensitivity, both the enzymes were found little distinguishable after they were isolated and purified since isolation and purification were accompanied with loss of the latency and the oligomycin sensitivity of the wild-type mitochondrial enzyme (6). Molecular weight of the mutant mitochondrial enzyme was determined as about 300,000 by Sephadex G-200 gel filtration. As shown in Table 7, no difference was observed in requirment of divalent cation for the enzyme activity, either. These properties, including the cold-lability (Fig. 8), were also very similar, to those of beef heart ATPase.

However, between yeast and beef heart ATPase some differences were observed in the activation by maleate and ADP inhibition. As reported by earlier workers, yeast ATPase, but not beef heart enzyme, is greatly activated in the presence of maleate (6). This was also the case of mutant ATPase. The inhibition of the yeast ATPase activity by ADP was not so great as that of beef heart ATPase and 2.5 mM ADP, instead of 1 mM for beef heart enzyme, was required for the half maximal inhibition (Fig. 9).

These observations on the purified enzyme are in good agreement with those by Schatz that the mitochondrial ATPase itself is not altered even in the RD-mutant yeast.

DISCUSSION

Schatz has isolated mitochondrial ATPase from wild type and mutant yeast cells in a purified form and indicated that the purified enzymes from both the strains are distinguishable neither enzymatically nor immunologically (7). He reported, however, that the mode of attachment of ATPase of mutant yeast to mitochondria membrane was quite different from that of wild-type yeast, viz the ATPase activity associated with the mutant yeast mitochondria was cold-labile and insensitive to oligomycin. He also indicated that the abnormal properties of the mitochondria membrane of mutant yeast were the result of missing or alteration of CF_o itself or a component of CF_o.

McLennon et al. have isolated oligomycin sensitivity conferring protein (OSCP) from beef heart mitochondria, a soluble protein of molecular weight 18,000, which is required for the conferral of oligomycin sensitivity of membrane-bound ATPase (15). The factor has been suggested to be identical with "structural protein" isolated by Conover et al (16). On the other hand, Sherman and Slonimski have suggested that the abnormality in RD-mutant yeast mitochondria is caused by the altered structural protein derived from a mutation of mitochondrial DNA (17).

In the present investigation, however, the mitochondrial ATPase in mutant yeast cells, unless solubilized, was shown to be cold-stable and oligomycin-sensitive. Its difference from the enzyme in wild-type yeast cells was observed in the readiness of the enzyme release by sonication and in hypotonic medium. These observations, contrarily, to Schatz, may indicate that CF_o is neither lost nor altered in the mutuant mitochondria but ATPase and CF_o are disarranged or disordered in the mitochondrial membrane of which structure is greatly altered by mutation. In fact, as will be described eleswhere (18), a protein factor, which strongly inhibit ATPase activity and, therefore, seems to be an ATPase inhibitor involved in CF_o, was found in and isolated from the mutant yeast as well as wild-type yeast mitochondria. The discrepancy between Schatz's and our observations is hardly explained at the moment but might be due to the tonicity of preparation medium. As indicated by earlier workers, the tonicity of the yeast cells are extraordinally high and after the snail enzyme digestion they are readily burst at lower than 0.5 osmolarity. Therefore, at 0.25 M mannitol during preparation, which was used as a preparation, which was used as a preparation medium by Schatz, mitochondria from the mutant yeast cells may be gradually disrupted and concomitantly the mitochondrial ATPase is made cold-labile and oligomycin-insensitive as indicated in Fig. 3.

There is now no doubt for the occurance of ATPase in the mutant yeast mitochondria which lack cytochromes bound tightly to the inner membrane and ATP synthesizing ability, but problem is in abnormality of the enzyme *in situ*. In the present investigation, the abnormality is suggested to be due to disarrangement of the enzyme and CF_o on the membrane altered by mutation. However, organization of these factors on the inner membrane is not revealed even in beef heart and wild-type yeast mitochondria. Therefore, elucidation of this problem requires further investigation.

SUMMARY

Cytoplasmic RD-mutant yeast as well as wild-type yeast cells found to contain the mitochondrial ATPase which is sensitive to oligomycin and, therefore, very similar to that of beef heart mitochondria, F_1. Both the strains also contain the other ATPase, microsomal ATPase which, however, easily distinguished and separated from the mitochondrial enzyme. The mitochondrial enzymes from both the strains are identical each other after purified, so far as enzymatically examined. However, the mode of existence of ATPase in mitochondrial membrane are quite different between the mutant and wild-type yeast cells. In the mutant cells ATPase reveals its hydrolytic activity even in mitochondria and it is readily extracted upon disruption of mitochondrial structure. This environmental

abnormality around ATPase on the mutant mitochondria is also discussed in relation to other factors, ATPase inhibitor and CF_o.

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(18) 主論文 II

TABLE I

Oligomycin Sensitivity of Mitochondrial ATPase

The reaction mixture contained 25 mM maleate-Tris buffer, pH 7.0, 5 mM $MgSO_4$, appropriate amount of mitochondria and indicated amount of oligomycin. The procedure are described in "Experimentals". Mitochondria used in this experiment were prepared freshly before the experiment.

Mitochondria	Oligomycin (µg/mg prot.)	Pi formed (mµmoles/min/mg)	%inhibition
Wild	0	197	—
	10	59	70
Mutant	0	260	—
	1	210	19
	10	180	30

TABLE II

Solubilization of ATPase by Sonication

Mitochondria from wild-type yeast(65 mg) and from mutant yeast cells(26 mg) were suspended in the extracting medium (see "Experimentals") in a final volume of 8 ml and 6 ml, respectively. The suspensions were sonicated for 2 min in ice, and the sonicated suspensions were centrifuged to separate membrane fraction and soluble fraction. Each fraction, the original mitochondrial suspension, sonicated particles and supernatant fraction, was treated at 38°C for 15 min in 0.5 M sodium sulfate containing 20 mM maleate-Tris buffer, pH 7.0, to elicite full activity of the ATPases before the enzyme assay. The reaction system and the procedure were described in "Experimentals".

Fractions	Pi formed (μmoles/min/mg)	Total prot. (mg)	Total activity (μmoles/min)
Wild type yeast			
mitochondria	1.58	65	103
sonicated mitochondria			
supernatant	2.96	9	26.6
residue	2.54	49	124
Mutant type yeast			
mitochondria	0.37	26	11
sonicated mitochondria			
supernatant	1.91	5.4	10.3
residue	0.04	20	0.8

TABLE III

Effect of Mg^{2+} on the Release of ATPase by Sonication

Mitochondria from wild-type yeast (95 mg) were suspended in the extracting medium in a final volume of 15 ml and 5 mM Mg^{2+} was added as indicated. ATPase activity in each fraction was measured as described in Table II.

Fraction	Total activity (μmoles/min)	
	without Mg^{2+}	with Mg^{2+}
Mitochondria	185	185
Sonicated mitochondria		
supernatant	48	27
residue	133	143

TABLE V

Removal of Mitochondrial ATPase from Microsome

Experiment I; Freshly prepared microsomes were sonicated and the sonicated microsomes were separated to membrane fraction and soluble fraction by centrifugation. ATPase activities of the membrane fraction, the soluble fraction and the original microsomes were measured as described in "Experimentals".

Experiment II; Freshly prepared microsomes were kept in hypotonic medium (0.1 M Tris-acetate buffer, pH 7.2) at 0°C for 6 hrs. ATPase activities of the incubated microsomes and the original microsomes were measured as described in "Experimentals".

Fractions	Total activity (μmoles Pi formed/min)	
Exp. I.		
Microsome	22.5	
Sonicated microsome		
supernatant	8.4	37 % solubilized
precipitate	14.0	
Exp. II		
Microsome	3.15	
Microsome incubated in cold	1.98	37 % inactivated

TABLE VI

Effect of Metal Ion on ATPase Activity of Microsomal Fraction

The reaction mixture consisted of 25 mM maleate-Tris buffer, pH 7.0, 5 mM metal ion indicated and 2.5 mM ATP.

Metal ion (conc. 5 mM)	Activity(mμmoles/min/mg)	
	Wild	Mutant
None	16	12
Mn^{2+}	50	41
Mg^{2+}	44	28
Co^{2+}	30	20
Ca^{2+}	20	15

TABLE VII

Metal Ion Requirement for ATPase Activity

The reaction mixture was consisted of 25 mM maleate-Tris buffer, pH 7.0, 2.5 mM ATP and 5 mM indicated metal irons.

Metal ion (conc. 5 mM)	Activity(μmoles/min/mg)	
	Wild	Mutant
None	0.43	0.02
Mg^{2+}	13.3	10.1
Mn^{2+}	8.6	6.6
Co^{2+}	9.0	7.5
Ca^{2+}	4.7	0.2

Fig. 1 Preparation of Mitochondria and Microsomes

Preparation of Mitochondria and Microsomes

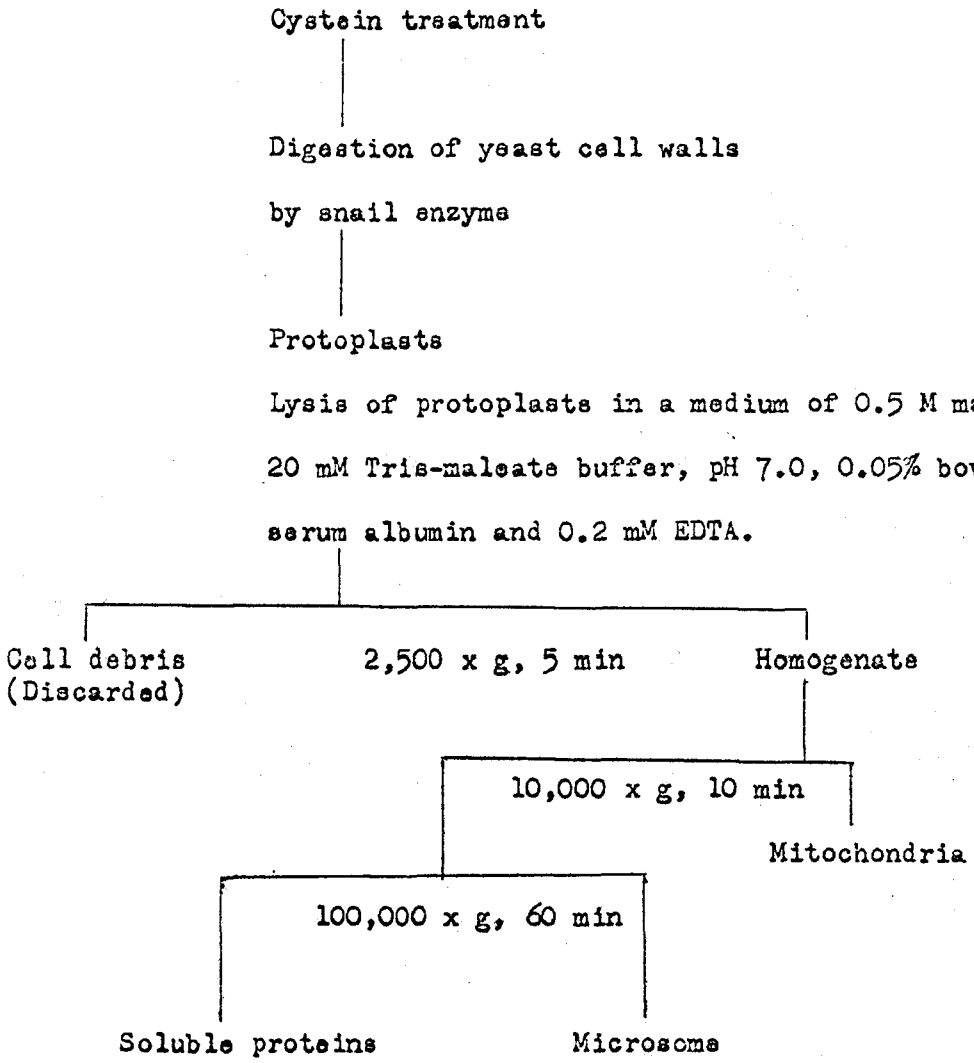


Fig. 2 Effect of Oligomycin Concentration on ATPase Activity

Mitochondria from the mutnt yeast(0.32 mg) and from the wild-type yeast(0.18 mg) were incubated in the reaction mixture of 0.5 ml for 10 min at 25 °C with oligomycin indicated. The reaction was started by the addition of 2.5 mM ATP at a final concentration. Reaction was stopped with 3 ml of 6 % PCA and Pi was determined on the whole reaction mixture.

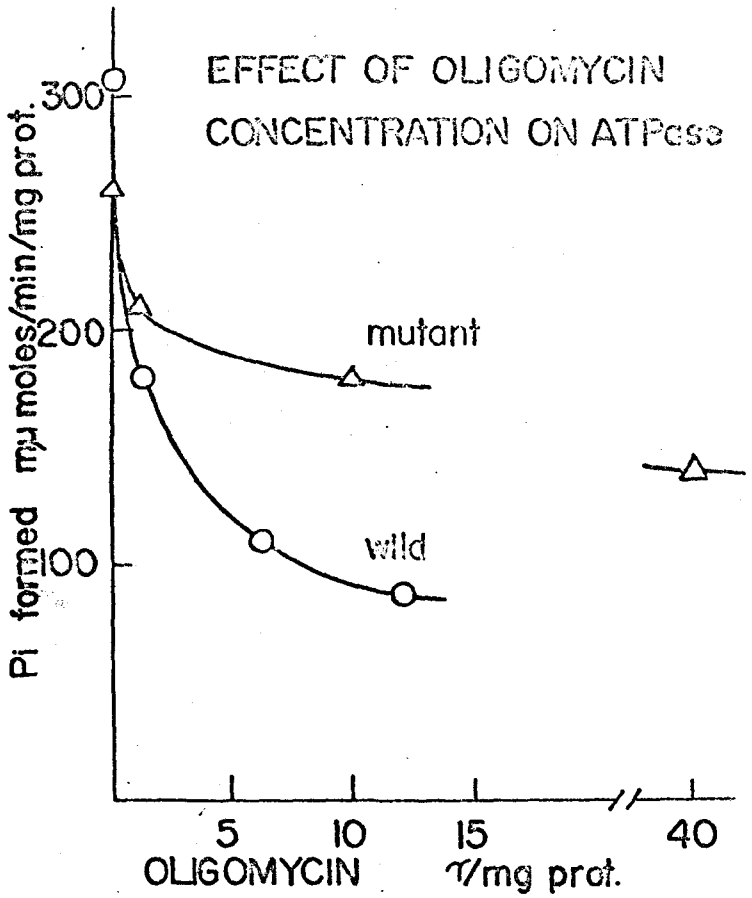

EFFECT OF OLIGOMYCIN
CONCENTRATION ON ATPase
Pi formed mμ moles/min/mg prot.
300
200
100
mutant
wild
5
10
15
40
OLIGOMYCIN γ/mg prot.

Fig. 3 Release of ATPase from Mitochondrial Membrane in Iso- and Hypotonic Medium

Mitochondria from mutant yeast were centrifuged and suspended in the medium indicated. Protein concentration was 5 mg per ml in each case. This suspension were kept at 0°C, and at indicated time an aliquot of suspension was pipeted out and ATPase activity was measured.

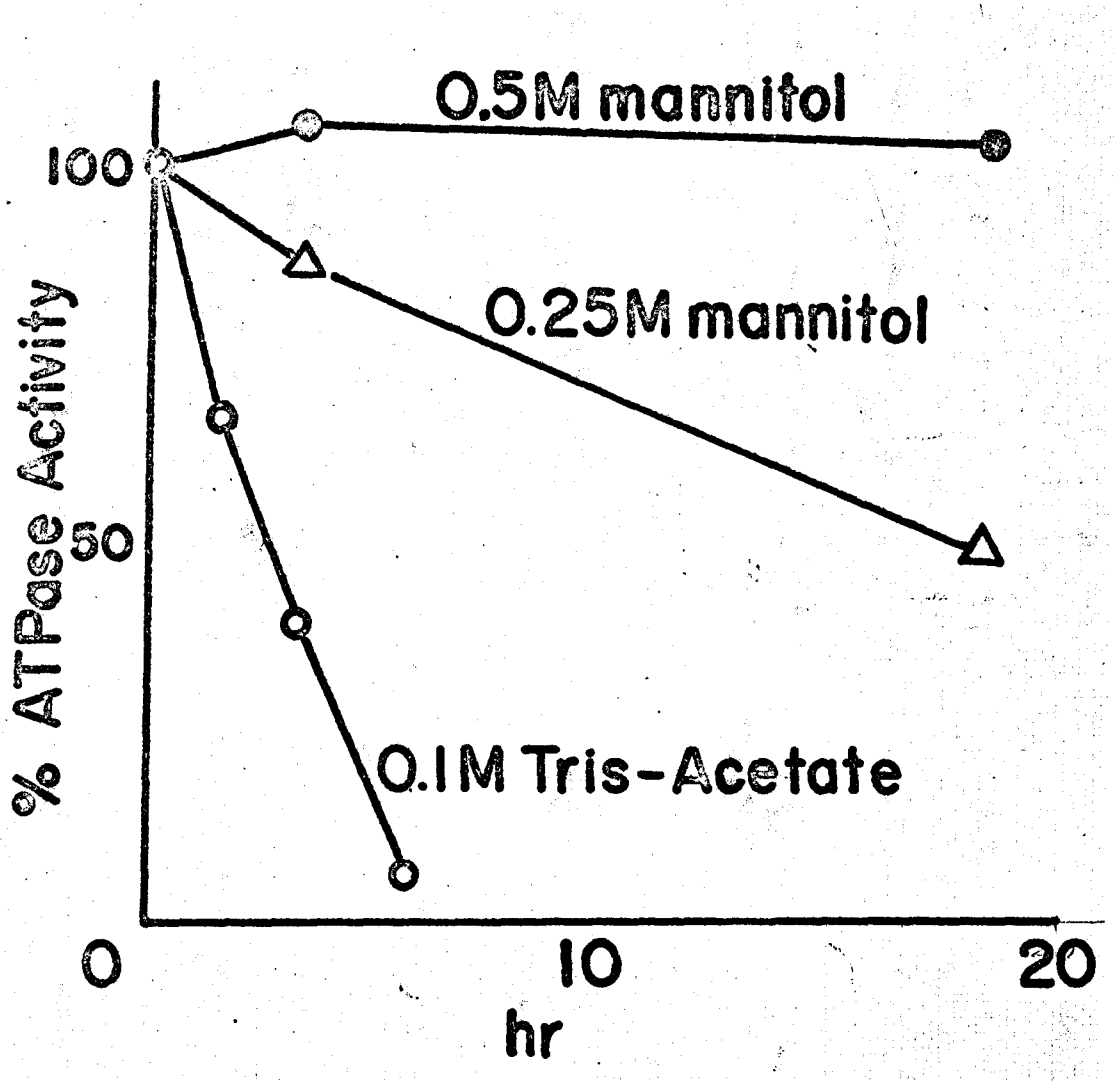

0.5M mannitol
0.25M mannitol
0.1M Tris-Acetate
100
50
0
% ATPase Activity
10
20
hr

Fig. 4 Activation of Membrane Bound ATPase by Heat Treatment

Mitochondria from the wild-type yeast(0.217 mg) and from the mutant yeast were suspended in the 0.5 M sodium sulfate solution containing 25 mM maleate-Tris buffer, pH 7.0, in a final volume of 2.0 ml and kept at 38°C. At the times indicated 0.027 ml aliquots of the suspension were withdrown, and added to 0.5 ml of the ATPase assay mixture. Pi was measured on the whole reaction mixture.

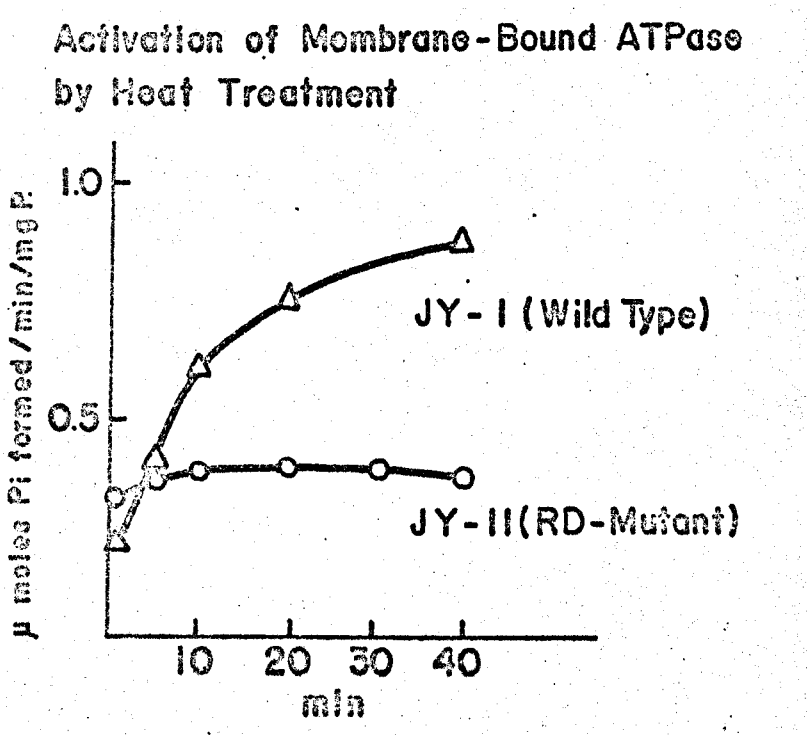

Activation of Membrane-Bound ATPase
by Heat Treatment
μ moles Pi formed / min/mg P
1.0
0.5
10
20
30
40
min
JY-I (Wild Type)
JY-II(RD-Mutant)

Fig. 5 Effect of Maleate on ATPase Activities of Mitochondria and Microsomes

The mitochondria and microsomes were obtained from mutant yeast cells. The microsomal fraction was sonicated to remove the ATPase from contaminated mitochondria as described in text. The reaction mixture contained 25 mM Tris-acetate buffer, pH 7.2, 2.5 mM $MgSO_4$ and a different concentration of Na-maleate buffer, pH 7.2.

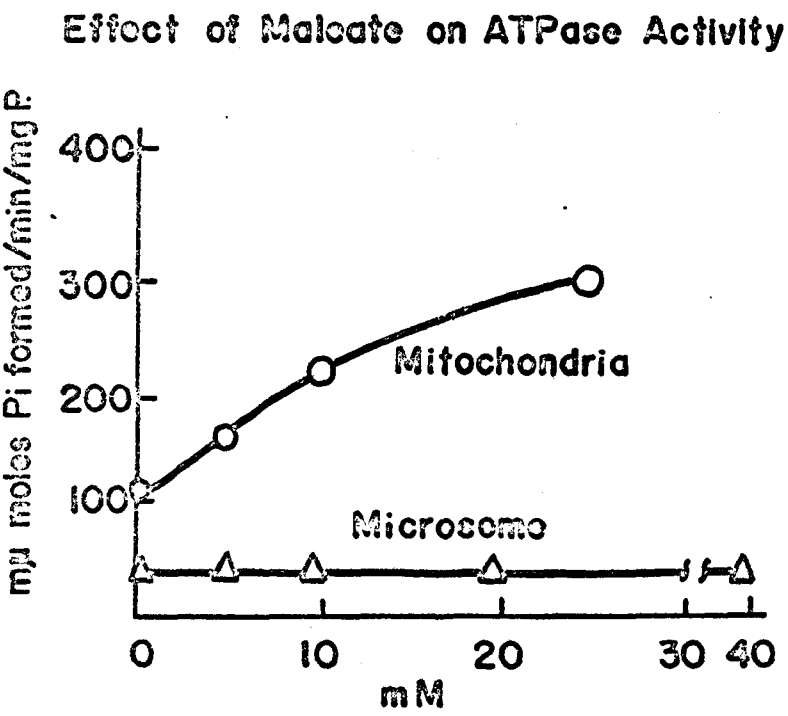
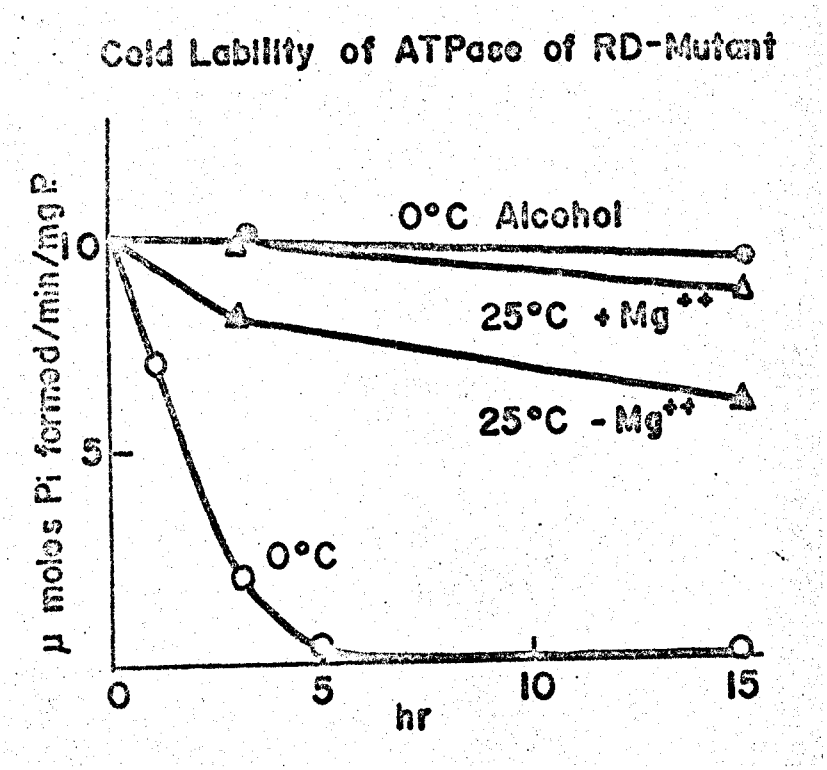

Effect of Malcate on ATPase Activity
mμ moles Pi formed/min/mg P
400
300
200
100
Mitochondria
Microsome
0
10
20
30
40
mM

Fig. 8 Effect of Temperature on the Stability of ATPase

The ATPase (20 to 30 μg) from wild-type yeast and from mutant yeast were added to 0.2 ml of 25 mM maleate-Tris buffer, pH 7.0, at 0°C. At the time indicated 0.027 ml samples were pipeted out and added to 0.5 ml of the assay mixture described in "Experimentals". Released Pi was determined on the whole reaction mixture.


Cold Lability of ATPase of RD-Mutant
μ moles Pi formed/min/mg P
10
5
0°C Alcohol
25°C + Mg++
25°C - Mg++
0°C
0
5
10
15
hr

Cold Lability of ATPase of Wild Type Yeast

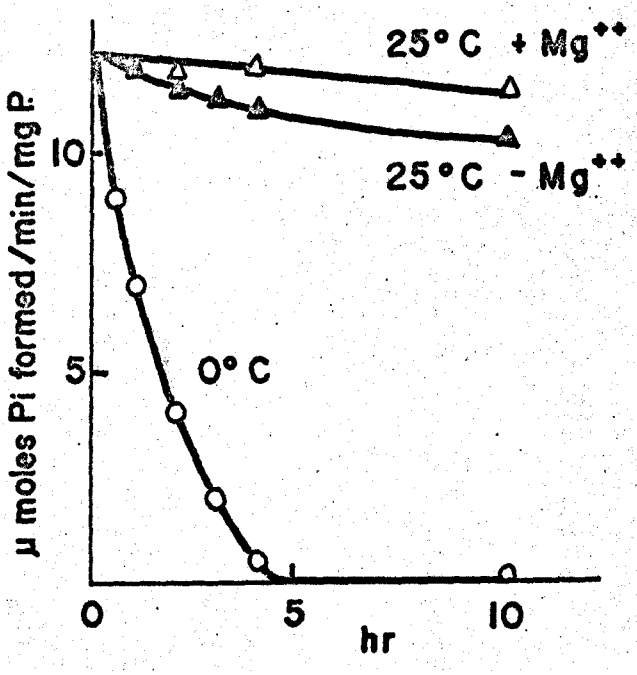
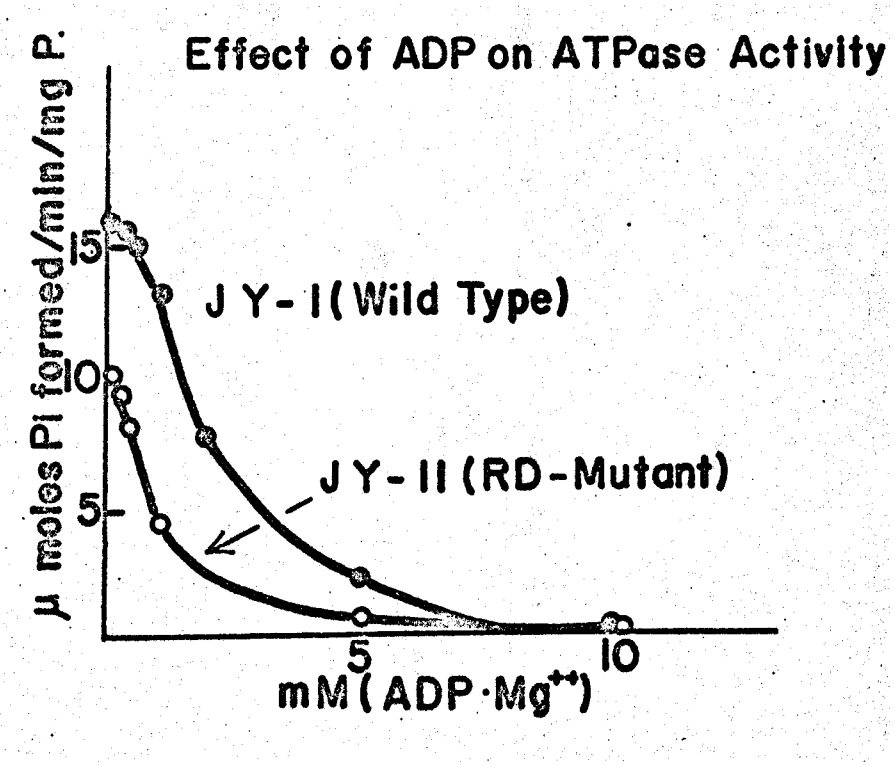


Fig 9 Effect of ADP on ATPase Activity

The ATPase from wild-type yeast and from mutant yeast were added to 2.0 ml of the reaction mixture containing the indicated concentration of ADP and Mg^{2+}. The reaction was initiated by the addition of 2.5 mM ATP. Procedures for ATPase assay were the same as described in "Experimentals".


Effect of ADP on ATPase Activity
μ moles Pi formed/min/mg P.
15
10
5
JY-I (Wild Type)
JY-II (RD-Mutant)
5
10
mM (ADP·Mg++)

STUDIES ON THE ADENOSINE TRIPHOSPHATASE SYSTEM IN YEAST; *Saccharomyces cerevisiae*

(II) Masking of hydrolytic activity of ATPase

Coupling factor 1 (F_1) catalysing terminal phosphorylation step in mitochondria has been isolated from beef heart in a highly purified form (1). The purified F_1 had a high activity to hydrolyse ATP and was cold-labile and insensitive to oligomycin, whereas the F_1, *in situ*, had no activity to hydrolyse ATP and was cold-stable and sensitive to oligomycin. Racker et al carried out studies to elucidate the curious properties of F_1. Their efforts resulted in the isolation of F_o, CF_o and ATPase inhibitor from the mitochondrial membrane (2,3,4). The F_o and phospholipids bound CF_o confer oligomycin sensitivity to solubilized F_1 and the ATPase inhibitor blockes a hydrolytic activity of the F_1 accompanying restoration of cold stability.

On the other hand, several workers reported the isolations of factors similar to F_1 without concomitant ATPase activity from beef heart mitochondria (5,6). Sandi et al isolated factor-A, latent ATPase which could be converted into demasked ATPase by heat-tr eatment and found that the factor-A contained ATPase inhibitor and ADP (7). Sone et al prepared latent

ATPase from yeast mitochondria and found that Mg^{++} prevented the conversion of latent ATPase into demasked one (8).

In present investigation, a protein factor similar to the ATPase inhibitor was isolated from yeast mitochondria. The factor interacted with purified ATPase by the aid of Mg^{++} and ADP and masked the hydrolytic activity of the enzyme. These facts Mg^{++} and ADP as well as the protein factor had particular roles in latency of hydrolytic activity of ATPase.

On the other hand, Vambutas et al purified latent ATPase (CF_1) from spinach chloroplasts, which is required for recoupling photophosphorylation of subchloroplast particles (9). The authors have indicated the ATP hydrolysing activity of CF_1 is elicited by trypsin accompanying a loss of the coupling activity. The similar observation that demasking of ATPase activity resulted in a loss of coupling activity was also reported in a bacterial phosphorylation system (10). From these finding, it is likely that masked form of ATPase represents a functional form as coupling factor and, therefore, studies on the latency of ATPase activity may provide additional insight into problems of terminal phosphorylation system. This paper describes masking-demasking changes of yeast mitochondrial ATPase caused by the co-operative works of a protein factor, ADP and Mg^{++}.

EXPERIMENTAL

Materials ----- Crystalline trypsin was kindly supplied by Dr. Yano, Labolatory for Protein Research, Osaka University. The other chemicals were commercial products.

Strains, culture of cells and preparation of mitochondria ----- A wild-type strain of Saccharomyces cerevisiae and its RD-mutant yeast cells were grown aerobically and mitochondria fraction was obtained by the same treatment as described in a preceding paper (11).

Preparation of ATPase and "heated-extract" ----- The mitochondria (1 g) were suspended in 50 ml of 0.1 M Tris-acetate buffer, pH 7.2 containing 2 mM EDTA and 7 % ethanol. Each suspension of 10 ml was sonicated for 2 min at 0°C. Membrane fraction was removed by centrifugation at 105,000 g for 60 min. The resulting supernatant was charged on DEAE-cellulose column and ATPase was eluted out with the buffer of 0.2 M. The eluate was dialyzed against 20 mM Tris-acetate buffer, pH 7.2, containing 7 % ethanol and 5 mM Mg^{++}. Unless otherwise stated, this dialyzate was used as ATPase preparation.

Aliquots of the supernatant was heated at 80°C for 5 min and denatured proteins, precipitate, were span down. The resulting supernatant was also dialyzed against the 20 mM Tris-acetate buffer at room temperature. The dialyzate was used as "heated-extract" preparation.

Treatment for demasking of ATPase activity ----- Masked ATPase activity was demasked after the method reported by Sone et al. Masked ATPase was heated at 38°C for 15 min in a medium containing 0.5 M Na_2SO_4 and 20 mM maleate-Tris buffer, pH 7.0.

Assay of ATPase activity and proteins ----- All procedures were the same as described in the preceding paper (11).

RESULTS

Alternate conversion of ATPase between demasked and masked form ----- Freshly prepared crude extract from wild-type yeast mitochondria contained masked ATPase concomitant with demasked one, while crude extract from mutant yeast mitochondria had demasked enzyme originally (Table 1). The masked ATPase of wild-type yeast was converted into demasked one by heat-treatment (see "Experimentals") or by prolonged aging in Mg^{++} free medium. On the contrary, the demasked ATPase thus obtained could not be converted into the masked one until the demasked ATPase was incubated at 0°C for 15 hrs in a medium containing ADP and Mg^{++}. As shown in Table 2, ATPase activity of the aged extract was greatly decreased during the 15 hrs incubation in the medium containing 0.1 M Tris-acetate, pH 7.2, 5 mM $MgSO_4$, 5 mM ADP and 7 % ethanol. The ATPase thus converted into masked form by the incubation again demasked by the heat-treatment. The column of non-treated and treated in Table 2. represents an apparent and total ATPase activities of the

incubated enzyme. The ratio of the apparent and total ATPase activities increased greatly, when the extract was incubated with ADP and Mg^{++}, while little increment was obserbed when ADP or Mg^{++} was omitted from the incubation medium. This was also the case in mutant yeast.

The time course of the masking is shown in Fig. 1. Both Mg^{++} and ADP were added to the crude extract from wild-type mitochondria, ATPase of which was demasked. The ATPase activity of the crude extract was then gradually masked. The rate of conversion was not fast compared with that of the F_1-ATPase inhibitor complex formation. In that case, masking of F_1 by ATPase inhibitor completed within 10 min (4). The masking process of yeast ATPase rather resembled that of the reversible thermal activation and inactivation processes of ATPase activity of factor-A (5).

Effect of heated-extract on masking of ATPase activity ----- As has been reported by Sone et al, masked ATPase obtained from yeast mitochondria was converted into demasked one at a step of DEAE-cellulose column of purification procedure (8). In this study, it was found that the purified ATPase obtained at this step was no longer returned to masked enzyme by the incubation with Mg^{++} and ADP. It is probable, therefore, that some component is missed from crude extract at the step of DEAE-cellulose column. As shown in Table 3, however, heated-

extract obtained from the crude extract (see "Experimentals") could convert the purified ATPase, demasked ATPase, into masked one. In this case, ADP and Mg^{++} were also essential. The degree of masking was proportional to the amount of heated-extract added (Fig. 2).

Properties of heated-extract ----- The masking activity of heated extract was susceptible to trypsin and was dializable (Table 4). The activity was recovered in precipitate at pH 3.0. It is, therefore, likely that the heated-extract containes dializable protein factor which is stable to heating. But, at present, further purification of this factor has not been done.

Interaction of the factor and ATPase was sensitive to the concenteation of salt (Fig. 3). Almost complete interferance was observed when 0.5 M Tris-acetate buffer was present. The similar observation has been reported in the complex formation of F_1-ATPase inhibitor. As shown in Table 5, nucleotides specificity for the masking was tested. Among them, ADP was the most effective and CDP partially took place in ADP.

It has been reported that the age of the solution of F_1 influences on the interaction of F_1 and ATPase inhibitor and Pullman et al, therefore, carried out the masking experiment within 30 min after preparing of F_1 solution (4). In our case, however, the age of ATPase solution did not influence on the masking process.

Although the factor in the heated-extract and ATPase inhibitor have quite similar properties each other, there are some differences between them at least in three points. These are the absolute requirment of Mg^{++} and ADP for heated-extract, aging effect for ATPase inhibitor and time-consuming conversion for heated-extract. At present, although it is still not clear whether the factor and ATPase inhibitor are similar substance that participate similar function in ATPase system, it seems that the investigation on the functions of Mg^{++} and ADP provide a clue to this problem.

Protective effect of Mg^{++} on proteolytic inactivation of ATPase activity ----- In the present investigation, it was found that susceptibility of ATPase molecule to trypsin was markedly changed by incubation with Mg^{++}. As shown in Fig. 4A, ATPase activity of the incubated ATPase was more resistant to trypsin than that of non-treated enzyme. When Mg^{++} added just before the trypsin digestion, the effect of Mg^{++} was not observed. The time for preincubation could not shorten even when, besides Mg^{++}, ADP and heated-extract were added but in this case, the ATPase activity of partially masked enzyme seemed more resistant to trypsin than that of Mg^{++}-treated enzyme (Fig. 4B). It is, therefore, conceivable that Mg^{++}, ADP and heated-extract cause some conformational changes on the ATPase molecule.

DISCUSSION

In the present investigation, a protein factor that resembles ATPase inhibitor was isolated from yeast mitochondria. As has been reported, the ATPase inhibitor obtained from beef heart mitochondria makes complex with F_1 accompanying a masking of hydrolytic activity of the F_1 (4). Our factor also can convert demasked ATPase into masked one. Both ATPase inhibitor and our factor are heat-stable protein. It has been reported that the interaction of ATPase inhibitor and F_1 is markedly sensitive to the concentration of salt (4). This was also the case in our factor (Fig. 3). These data suggest that the ATPase inhibitor and our factor are similar each other.

However, as described elsewhere, the ATPase inhibitor interacted directly with F_1, while our factor interacted with ATPase by the co-operative works with Mg^{++} and ADP. On the roles of Mg^{++} and ADP in mitochondrial ATPase system, it has been reported by Sone et al that Mg^{++} prevents cold-inactivation and demasking of ATPase obtained from yeast mitochondria (8). It was also found, in the present investigation, that the masked ATPase was resistant to trypsin. It is generally accepted that conformational changes of protein molecule have influence on the susceptibility to proteolytic degradation of the molecule. Therefore, it is conceivable that masking of yeast mitochondrial ATPase by Mg^{++} and ADP in the presence of the protein factor

may be a reflection of some conformational changes of ATPase molecule. Sanadi et al isolated from beef heart mitochondria factor-A, latent ATPase, which was a complex of F_1 and ATPase inhibitor containing one mole of ADP, which was released by heating. The authors have indicated, with respect to conformational changes of ATPase molecule, that heat-treatment greatly evokes ATPase activity accompanying conformational changes of the factor-A, moreover, that at the next cooling stage, the demasked ATPase turned into masked one and that this demasking-masking changes are cycled (5).

From these observations, it is tempting to speculate that the role of the protein factor of yeast mitochondria is not merely to mask hydrolytic site of ATPase but it is concerned in a dynamic conformational changes of ATPase molecule which regulate a terminal phosphorylation step by the co-operative works with Mg^{++} and ADP. The same question should be thrown on the mechanism of ATPase inhibitor of beef heart mitochondria.

SUMMARY

A protein factor that resembled ATPase inhibitor obtained from beef heart mitochondria was found in and isolated from mitochondria of yeasts, both wild-type and mutant strains. The factor could change demasked ATPase into masked one by the aid of Mg^{++} and ADP. Possible roles of the factor, ADP and

Mg^{++} in a terminal phosphorylation step were discussed in the relation to the conformational changes of ATPase molecule.

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(11) 主論文(I)

TABLE I

Activation of ATPase in Mitochondrial Crude Extract of Wild-type and Mutant Yeast by Heat Treatment

ATPase activities of freshly prepared crude extract from wild-type and mutant yeast were measured before and after treatment at 38°C for 15 min in the medium containing 0.5 M Na_2SO_4 and 25 mM Tris-maleate buffer, pH 7.0. Assay procedure was the same as "Experimentals" except that the volume of reaction mixture was 0.5 ml and released Pi was determined on the whole reaction mixture.

Treatment	Activity (μmoles/min/mg)	
	Wild	Mutant
None	2.0	1.9
38°C, 15min	3.4	1.9

TABLE II

Effect of Mg^{2+} and ADP on the Conversion of Demasked ATPase into Masked One

$MgSO_4$ and ADP (final conc. 5 mM) were added to the demasked ATPase in 0.1 M Tris-acetate buffer, pH 7.2, and 7 % ethanol (aged crude extract), and kept at 0°C for 15 hrs. 0.1 ml of the incubated crude extract was pipeted out and added into the same volume of 0.5 M sodium sulfate solution containing 20 mM maleate-Tris buffer, pH 7.0. The mixture was kept at 38°C for 15 min. The ATPase activity was measured before and after the heat treatment, respectively, by the same method described in text.

Additions	Pi formed/min/mg protein	
	Non-treated	Treated
	μmoles	
Wild-type yeast		
None	1.21	1.15
Mg^{++}	1.55	2.40
ADP	1.18	2.03
Mg^{++}, ADP	0.21	1.90
Mutant type yeast		
None	1.50	—
Mg^{++}	1.16	—
ADP	0.90	—
Mg^{++}, ADP	0.25	—

TABLE III

Effect of Heated-Extract on Masking of ATPase Activity

ATPase(25 μg) was incubated in 0.1 M Tris-acetate buffer, pH 7.2, containing 7 % of ethanol for 15 hrs at 0 °C with 45 μg of heated-extract, 5 mM Mg^{2+} and ADP as indicated, in a final volume of 0.3 ml. ATPase activity was measured as in Table I. In this case ATPase was dialized against 20 mM Tris-acetate buffer, pH 7.2, containing 7 % ethanol only.

Addition	µmoles Pi formed/min/mg prot.	
	Non-treated	Treated
None	10.8	10.5
ADP	10.8	11.0
ADP, Mg^{2+}	11.2	12.0
Heated-Extract	10.6	10.8
Heated-Extract, Mg^{2+}	11.9	12.2
Heated-Extract, Mg^{2+}, ADP	5.0	12.0

TABLE IV

Effect of Trypsin, Dialysis on Heated-Extract

Trypsin digestion of heated-extract: Heated-extract (209 µg) was incubated at 25°C with 50 µg trypsin for several hours, in a final volume of 0.3 ml. The digested heated-extract was heated at 80°C for 5 min to in activate residual trypsin activity.

Dialysis: 1 ml of heated extract was dialyzed against 200 ml Tris-acetate buffer, pH 7.2, for 15 hrs at room temperature.

25 µg ATPase was incubated with trypsin digested or dialyzed heated-extract of 45 µg protein for 15 hrs at 0°C in the buffer of 50 mM Tris-acetate, pH 7.2, containing 5 mM Mg^{2+}, 5 mM ADP and 7 % ethanol.

Treatment	µmoles Pi formed/min/mg prot.	
	Non-treated	Treated
None	4.3	10.5
Trypsin digestion	12.2	12.5
Dialysis	12.5	12.8

TABLE V

Effect of Nucleotides on Masking

20 µg ATPase was incubated with 40 µg heated-extract and 50 mM indicated nucleotides in a final volume of 0.3 ml. Buffer used was 50 mM Tris-acetate, pH 7.2, containing 7 % ethanol and 5 mM Mg^{2+}. The other incubation conditions were the same as described elsewhere.

Addition	μmoles Pi formed/min/mg prot.
None	12.0
ADP	8.7
AMP	12.8
CDP	10.8
UDP	12.0

Fig. 1 Masking of ATPase Activity by ADP and Mg^{2+}

Mitochondrial crude extract was incubated in a medium containing 10 mM ADP, 10 mM Mg^{2+}, 7 % ethanol and 100 mM Tris-acetate buffer, pH 7.0, at 0°C. At the indicated times an aliquot of the extract was pipeted out and ATPase activity was measured.

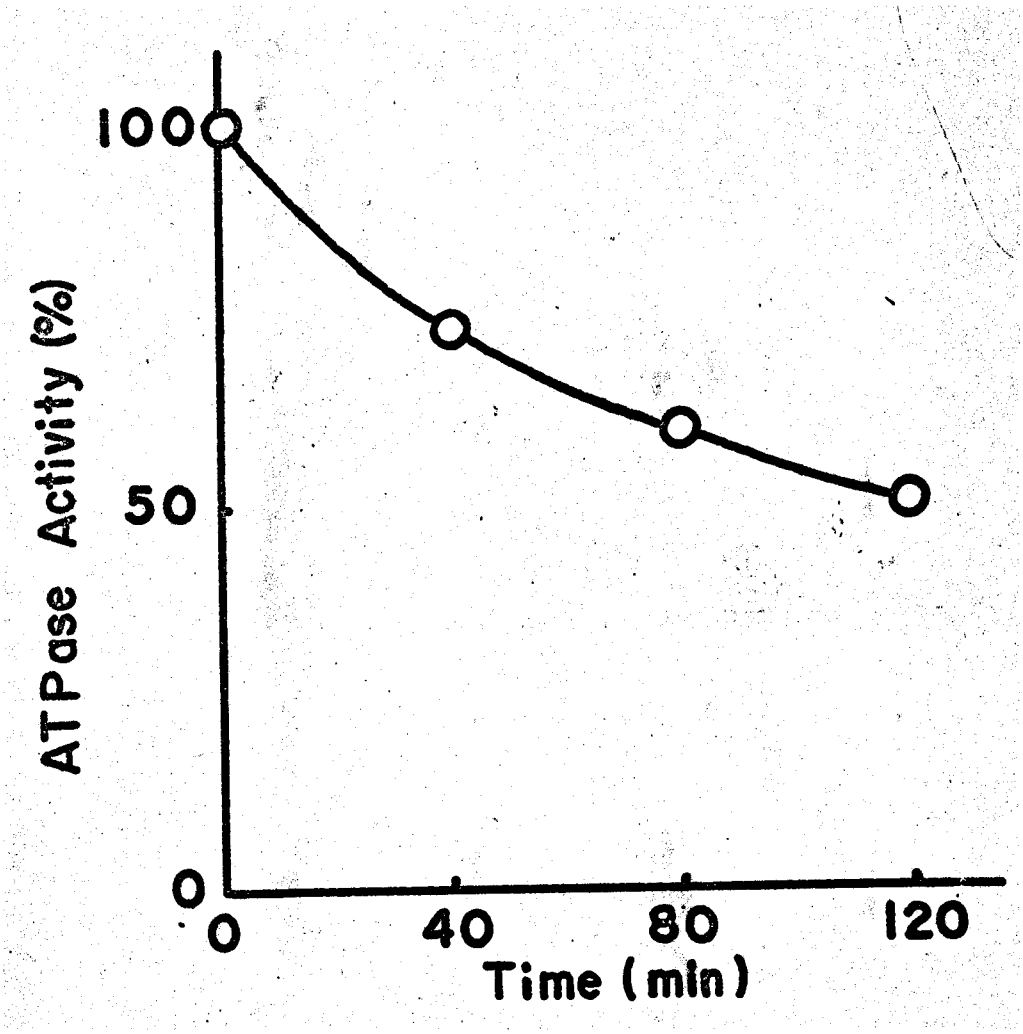

100
50
0
ATPase Activity (%)
0
40
80
120
Time (min)

Fig. 2 Effect of Heated-Extract Concentration on ATPase Activity

25 μg ATPase was incubated for 15 hrs with 5 mM ADP, 5 mM Mg^{2+} and indicated amount of heated-extract in 0.4 ml of 50 mM Tris-acetate buffer, pH 7.2, containing 7 % ethanol. Residual ATPase was measured as described in text.

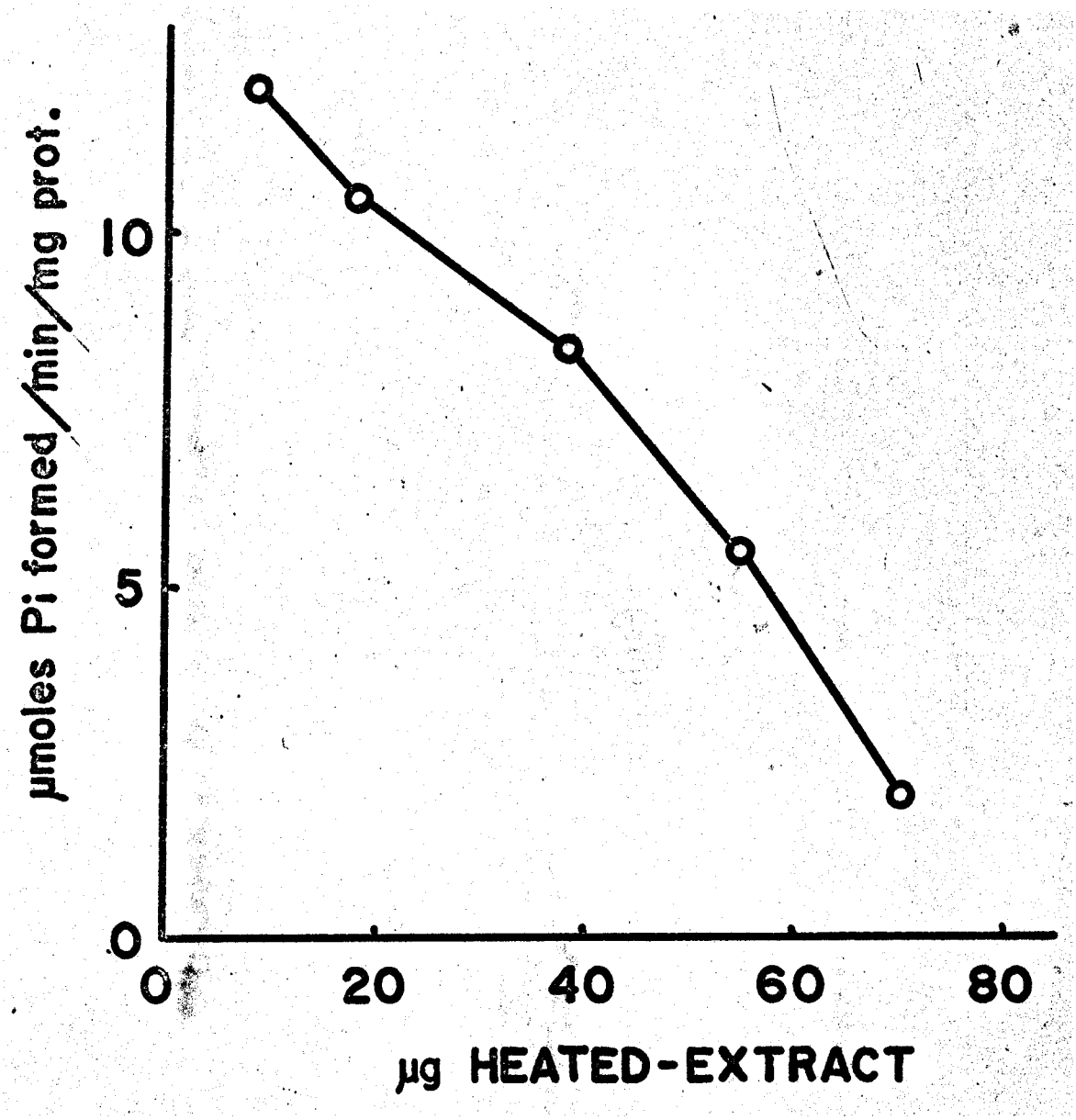

µmoles Pi formed/min/mg prot.
10
5
0
0
20
40
60
80
µg HEATED-EXTRACT

Fig. 3 Effect of Buffer Concentration on Masking of ATPase Activity

25 μg ATPase was incubated for overnight at 0°C in the 0.5 ml solution containing 36 μg heated-extract, indicated concentration of Tris-acetate buffer, pH 7.2, 10 mM ADP, 10 mM Mg^{2+} and 7 % ethanol. ATPase activity was measured as described in Table I.

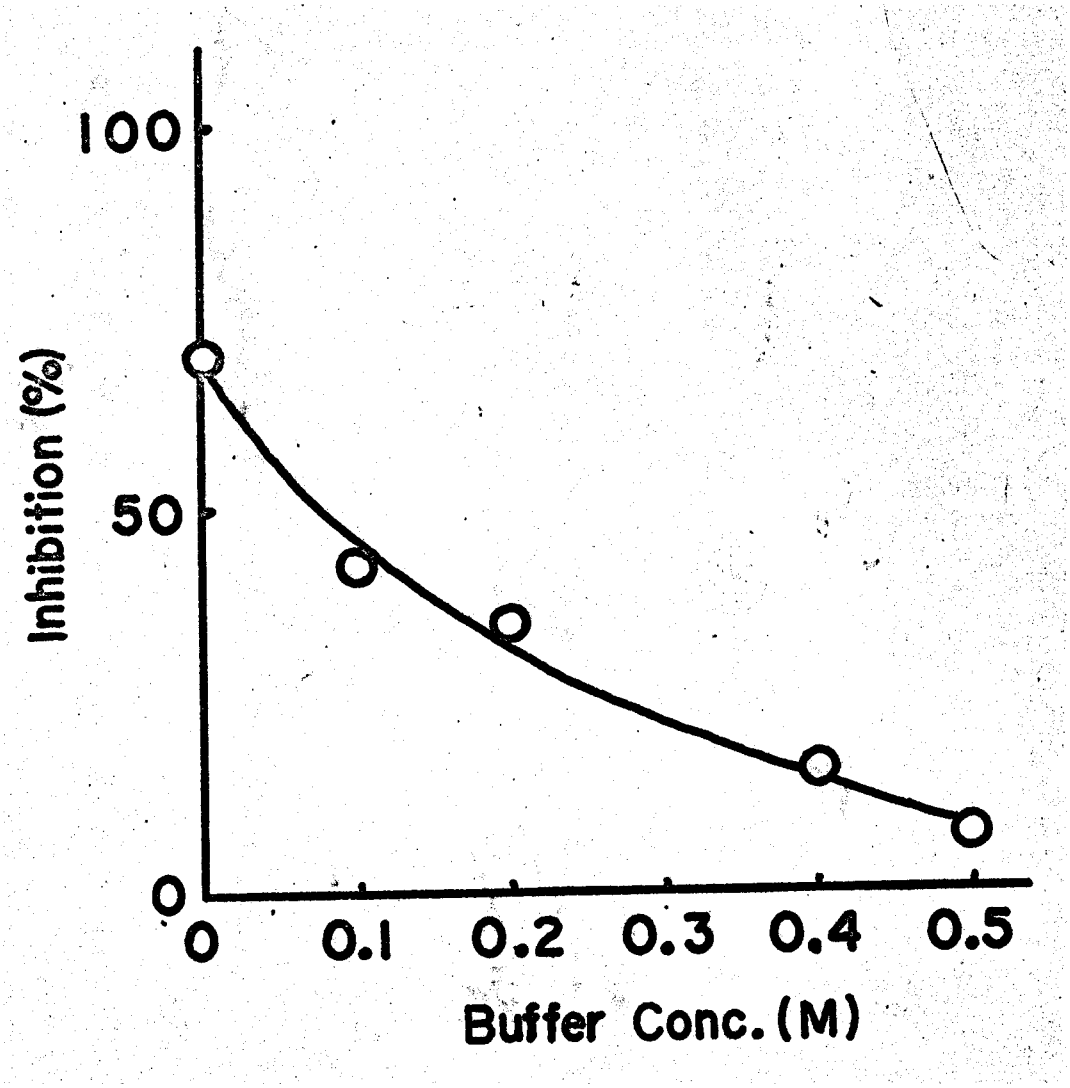
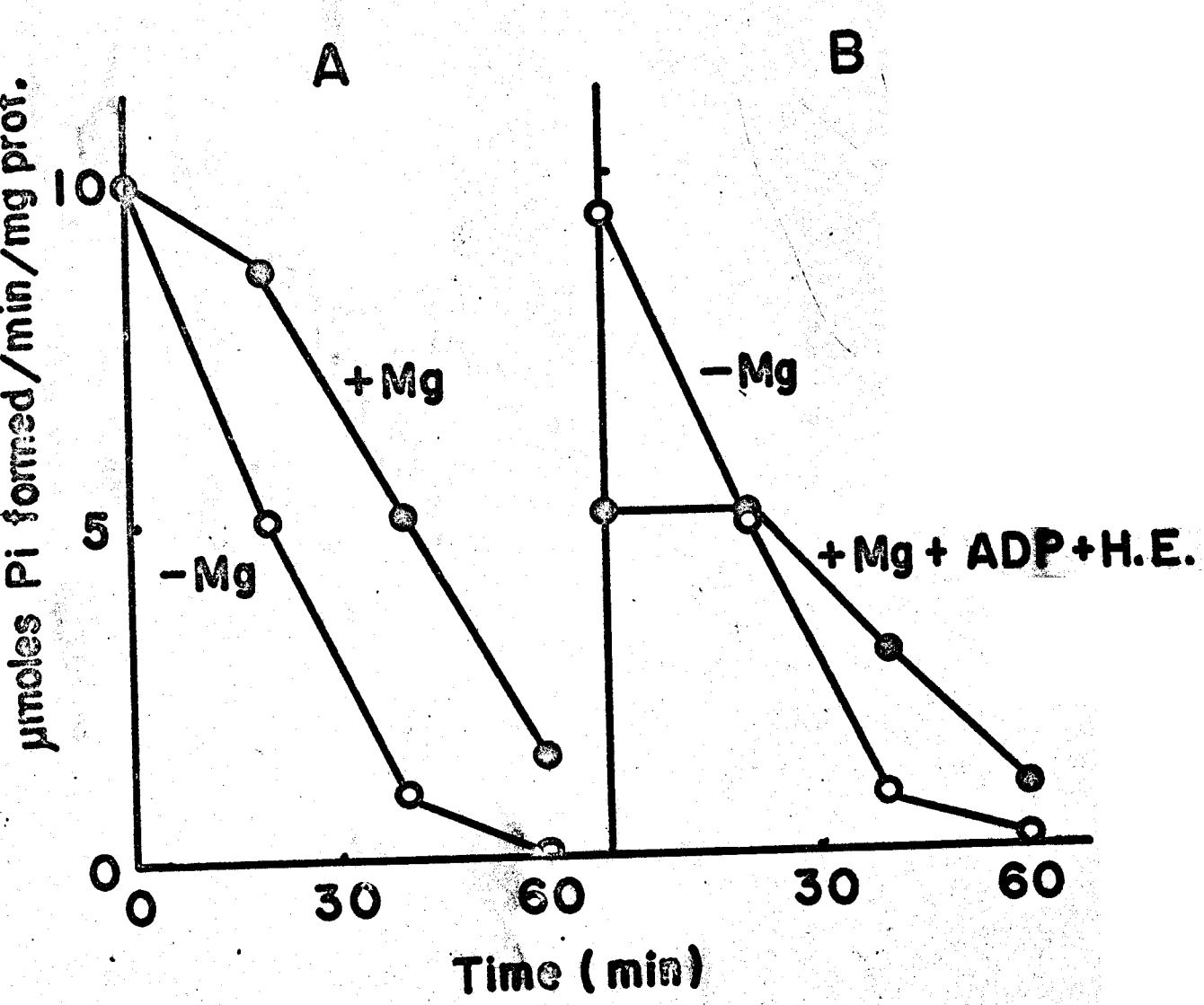

100
50
0
Inhibition (%)
0
0.1
0.2
0.3
0.4
0.5
Buffer Conc. (M)

Fig. 4 Susceptibility of ATPase to Trypsin

(A) ATPase (50 μg) in 25 mM maleate-Tris buffer, pH 7.0, containing 1 mM Ca^{2+} and 5 mM Mg^{2+} was digested by trypsin. At the indicated time, 0.027 ml of the ATPase mixture was withdrown and the activity was measured.

Before the digestion the ATPase were preincubated in a medium of 5 mM Mg^{2+} and Mg^{2+} free, and they were disignated as $+Mg^{2+}$ and $-Mg^{2+}$, respectively.

(B) ATPase and heated-extract-ADP-Mg^{2+} treated ATPase were digested by trypsin as described in (A). ATPase activity was measured at indicated time as in the case of (A).


A
B
µmoles Pi formed/min/mg prot.
10
5
0
+Mg
−Mg
−Mg
+Mg + ADP + H.E.
0
30
60
30
60
Time (min)