Studies on the ATPases of Fragmented Sarcoplasmic Reticulum of Rabbit Skeletal Muscle

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家兎骨骼筋小胞體切片의 ATPase에 관한 研究

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要 約

토끼의 골격근 小胞體 切片을 遠心分離하여 그 ATPase活性의 生化學的 性質을 Mg^{++}—ATPase와 $(Mg^{++}-Ca^{++})$—ATPase로 구분하여 조사하였다.

$(Mg^{++}-Ca^{++})$—ATPase의 活性은 0°—40°C의 범위, 그리고 pH 6.4—7.6의 범위에서는 Mg^{++}—ATPase보다 훨씬 높다. 이 현상은 온도가 높을수록 더욱 현저하다.

活性化에너지는 온도 0°—40°C의 범위에서는 Mg^{++}—ATPase가 14 kcal/mole, $(Mg^{++}-Ca^{++})$—ATPase가 21 kcal/mole, 그리고 total ATPase가 18 kcal/mole이다. 이 活性化에너지의 값은 pH와 Mg濃度에 무관하다.

이들 효소의 Km의 값은 Mg^{++}—ATPase가 0.36 mM, $(Mg^{++}-Ca^{++})$—ATPase가 2.20 mM, 그리고 total ATPase가 0.86 mM이다.

INTRODUCTION

The fragments of sarcoplasmic reticulum prepared from rabbit skeletal muscle homogenate by fractional centrifugation have been known to possess an ATPase activity which is considered to be closely associated with the active uptake of calcium of this fraction (Ebashi and Lipmann, 1962; Hasselbach and Makinose, 1962).

Various biochemical properties of these fragments (microsomal fraction) have been extensively studied. The membrane fragments require the presence of Mg^{++} for their ATPase activity. The fragments, however, sustain a low rate of ATP hydrolysis in the absence of Ca^{++}. When Ca^{++} is added to the fragments, on the other hand, a burst of extra splitting of ATP occurs for a limited time during which Ca uptake takes place (Hasselbach and Makinose, 1962). This property

suggested that there would be two separate enzymes in the fragmented sarco-plasmic reticulum.

In fact, Hasselbach and Makinose (1963) have shown by kinetic studies and by the use of oxalate that there are at least two ATPase systems in the sarcoplas-mic reticulum fractions. One of these is activated by Ca^{++} and appears to be coupled to the Ca uptake, two Ca being transported per ATP hydrolyzed. The other system is not activated by Ca^{++} and shows up as the residual ATPase. Weber *et al.* (1966) and Duggan (1968) also showed that the enzyme system could be divided into two separate components; basal or residual ATPase and extra ATPase. Meis *et al.* (1970) referred the ATPase activity in the presence of Mg^{++} to as Mg^{++}-dependent ATPase, that observed in the presence of Mg^{++} and Ca^{++} to as total ATPase, and the extra activity induced by Ca^{++} to as Ca^{++}-activated ATPase. Therefore, the basal or residual ATPase seems to be identical to Mg^{++}-ATPase and the extra ATPase to (Mg^{++}—Ca^{++})-ATPase.

Many works on the enzymic activity of the fragments of sarcoplasmic reticulum, however, were done without dealing with these two enzymes separa-tely; their results are those which concerned only with total ATPase. In the present paper, the authors present some biochemical properties of these two enzymes.

MATERIALS AND METHODS

Skeletal muscle (back and leg muscles) of adult rabbits was homogenated in a Waring blender in the tris-maleate buffer (pH 6.8) containing 50 mM KCl and centrifuged as described elsewhere (Ha, 1971 and 1972). The fraction sedimented between 12,000xG and 20,000xG was used throughout the experiments. All preparations were kept in the homogenizing medium (pH 6.8) under 0°~4°C. Preparations not more aged than 72 hours were always used.

The enzyme activity was determined with essentially the same procedure as described earlier (Ha, 1971). The reaction mixture consisted of, unless otherwise specified, 2 mM ATP (disodium salt), 4 mM MgCl$_2$, 0.2 mM CaCl$_2$, 50 mM KCl, all dissolved in 20 mM tris-maleate-NaOH buffer (pH 6.8). The amount of the fragmented sarcoplasmic reticulum as expressed in protein concentration in the reaction mixture was around 1 mg/ml.

The ATPase activity measured under the presence of both Mg^{++} and Ca^{++} is referred to as the total ATPase, and that measured under the absence of Ca^{++} is referred to as the Mg^{++}-ATPase. The elimination of trace Ca^{++} from the incubation mixture was done by adding 0.5 mM ethyleneglycol-bis-(aminoethyl-ether)-N, N'-tetraacetic acid (EGTA) to the Ca-free mixture. The (Mg^{++}—Ca^{++})-

ATPase activity was obtained by subtracting the activity of Mg^{++}-ATPase from that of total ATPase.

The content of the inorganic phosphate was determined by the method of Allen (1940) modified by Nakamura (1950), and the protein concentration was determined by the biuret method. All chemicals used were of reagent grade. Ion-exchanged, glass-distilled water was used.

RESULTS

1. *The temperature dependency of the enzyme activity.*

The total ATPase activity of the fragmented sarcoplasmic reticulum increases as the temperature increases (Figs. 1~4). Two components of the ATPase system in the fragments, however, differ each other in the pattern of the response to the temperature change. The (Mg^{++}—Ca^{++})-ATPase activity increases sharply
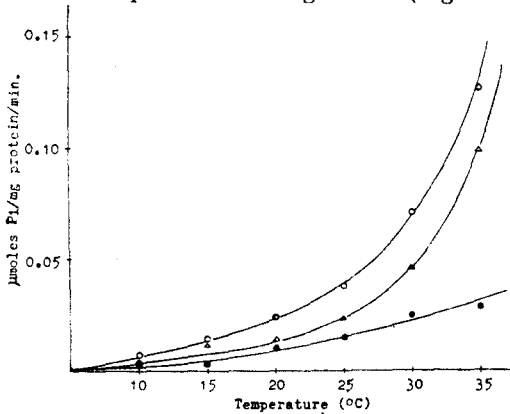
Fig. 1. ATPase activities at various temperatures at pH 6.4. ◯, Total APTase;●, Mg^{++}-ATPase; △, (Mg^{++}—Ca^{++})-ATPase
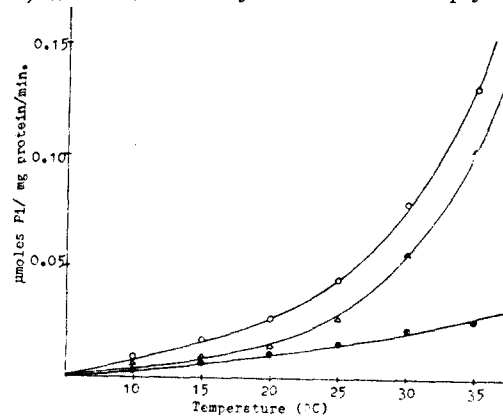
Fig. 2. ATPase activities at various temperatures at pH 6.8. ◯, Total ATPase;●, Mg^{++}-ATPase; △, (Mg^{++}—Ca^{++})-ATPase
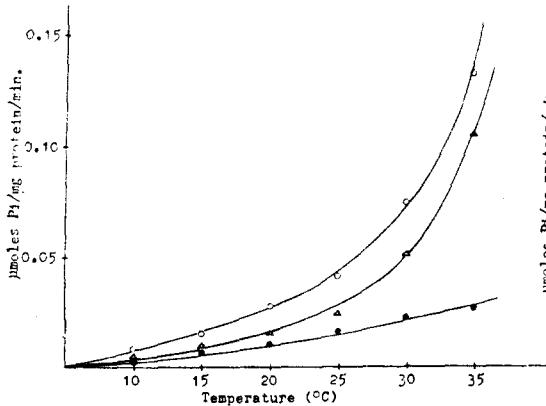
Fig. 3. ATPase activities at various temperatures at pH 7.2. ◯, Total ATPase;●, Mg^{++}-ATPase;△, (Mg^{++}—Ca^{++})-ATPase
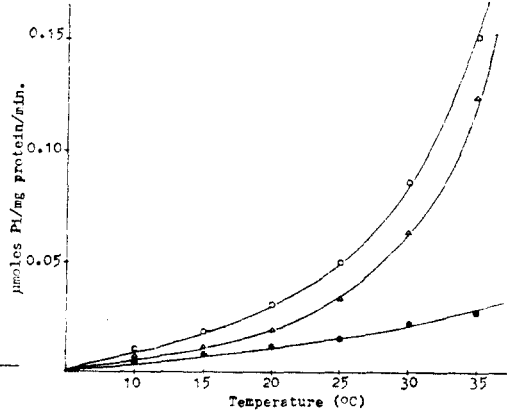
Fig. 4. ATPase activities at various temperatures at pH 7.6. ◯, Total ATPase;●, Mg^{++}-ATPase; △, (Mg^{++}—Ca^{++})-ATPase

($Q_{10}=3$) with the increase of temperature while the increase in the Mg^{++}-ATPase activity is much slower ($Q_{10}=2$). The patterns of response to the temperature change are the same for both ATPases at different pH. The pH of the reaction mixture in the range of 6.4—7.6 does not seem to influence the activities of both ATPases. Both ATPases show essentially the same tendency of increase with the increase of temperature at all pH measured.

Since the activity of (Mg^{++}—Ca^{++})-ATPase is far greater than that of Mg^{++}-ATPase at higher temperatures, it accounts for the major portion of total ATPase at these temperatures. Table 1 represents the ratio of the two ATPases at various temperatures (pH 6.8).

Table 1. The activity ratio of Mg^{++}-ATPase and (Mg^{++}—Ca^{++})-ATPase at pH 6.8.

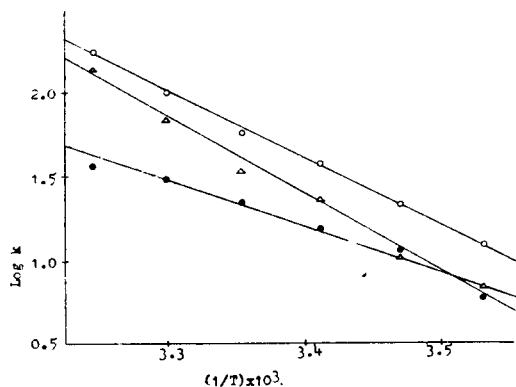| Temperature (°C)	Mg^{++}-ATPase (%)	(Mg^{++}—Ca^{++})-ATPase (%)
10	34	66
15	33	67
20	29	71
25	28	72
30	24	76
35	17	83

Fig. 5. Arrhenius plots for the hydrolysis of ATP by ATPases. The pH of the incubation mixture was 6.8. ○, Total ATPase; ●, Mg^{++}-ATPase; △, (Mg^{++}—Ca^{++})-ATPase
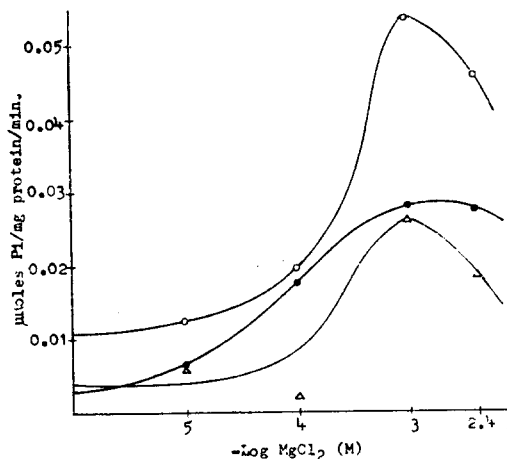
Fig. 6. The effect of Mg^{++} concentration on the ATPase activities. The reaction was carried out at 25°C and under pH 6.8. ○, Total ATPase; ●, Mg^{++}-ATPase; △, (Mg^{++}—Ca^{++})-ATPase

2. The energies of activation of the enzymes.

The data shown in Fig. 2 were used to construct Arrhenius plots for the calculation of apparent energies of activation of the two ATPases. These plots are shown in Fig. 5 and the apparent energies of activation of the ATPases calculated are given in Table 2. It is seen from Table 2 that the energy of activation of $(Mg^{++}-Ca^{++})$-ATPase is much higher than that of Mg^{++}-ATPase at every pH determined (pH 6.4—7.6). The pH of the incubation mixture does not seem to change the energy of activation of either ATPase significantly.

Table 2. The energies of activation of Mg^{++}-ATPase, $(Mg^{++}-Ca^{++})$-ATPase and total ATPase.

	pH	Mg^{++}-ATPase	$(Mg^{++}-Ca^{++})$-ATPase	Total ATPase
Energy of activation	6.4	14.2	21.9	18.5
(kcal/mole)	6.8	13.4	20.6	17.5
	7.2	13.9	20.9	17.8
	7.6	14.1	22.2	18.3

3. The effect of Mg concentration on the enzymes.

The effect of varying the concentration of Mg on the ATPase activity was determined at pH 6.8 and at 25°C. A typical result is shown in Fig. 6, where it is known that the optimal concentration of Mg is about 1 mM for both ATPases. The activity of $(Mg^{++}-Ca^{++})$-ATPase is very low when the Mg concentration is below 0.1 mM. The activity, however, increases sharply when the Mg concentration exceeds 0.1 mM, and drops at higher concentrations. Unlike $(Mg^{++}-Ca^{++})-$
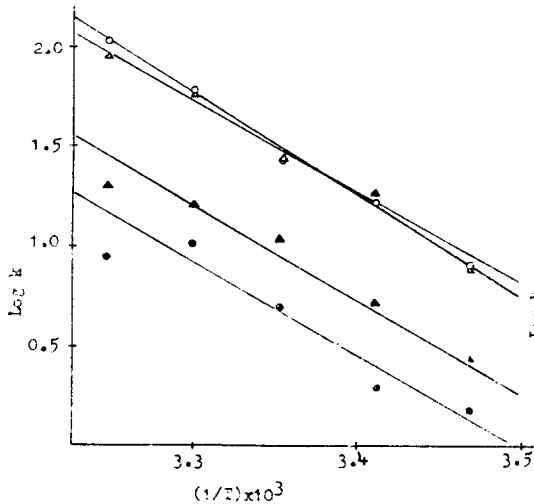

Fig. 7. Arrhenius plots of Mg^{++}-ATPase activity at various concentrations of $MgCl_2$. The reaction was carried out under pH 6.8. ◎, Mg-free; ▲, 0.01 mM; ●, 0.1 mM; ○, 1 mM; △, 4 mM
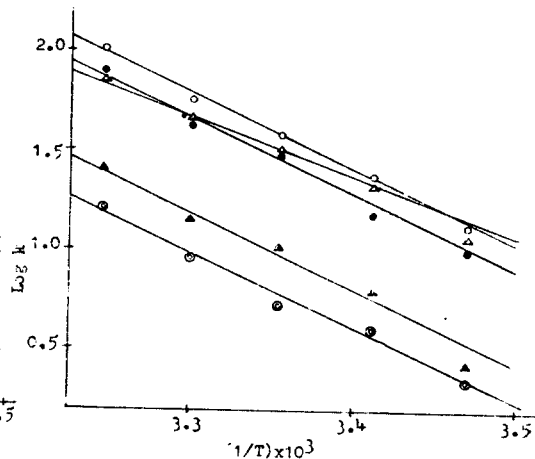
Fig. 8. Arrhenius plots of $(Mg^{++}-Ca^{++})$-ATPase activity at various concentrations of $MgCl_2$. The reaction was carried out under pH 6.8. ▲, Mg-free; ●, 0.01 mM; △, 1 mM; ○, 4 mM

ATPase, the activity of Mg^{++}-ATPase increases rather slowly as the Mg concentration increases. The decrease in the activity of this enzyme at above 1 mM of Mg is also not very remarkable as compared with that of $(Mg^{++}-Ca^{++})$-ATPase. As a result of the sudden increase in $(Mg^{++}-Ca^{++})$-ATPase activity and of the slow increase of Mg^{++}-ATPase activity, the total activity is far greater at 1 mM Mg than at other Mg concentrations. Thus, the response of the enzymic activity to the Mg concentration is different between the two ATPases.

The apparent energies of activation of Mg^{++}-ATPase and $(Mg^{++}-Ca^{++})$-ATPase are not significantly influenced by the change in the concentration of Mg; the energies are essentially the same at various Mg concentrations, as revealed in Figs. 7 and 8.

4. *The Km values of the ATPases.*

To distinguish further $(Mg^{++}-Ca^{++})$-ATPase from Mg^{++}-ATPase, the effect of varying the ATP concentration at a constant Mg level of 4 mM was investigated (Fig. 9). The Km values (Michaelis-Menten constant) for both ATP-ases were read on the Lineweaver-Burk plots and were found to be 0.86 mM for total, 0.36 mM for Mg^{++}—, and 2.20 mM for $(Mg^{++}-Ca^{++})$-ATPase. The difference between two enzymes in the Km values are thus very apparent.

DISCUSSION

In the previous paper (Ha, 1972) the microsomal fraction obtained similarly with the present work was known to have only a slight activity of contaminant mitochondrial ATPase. Further-more, it was also shown in the same paper that the contribution of $(Na^{+}-K^{+})$-ATPase activity in this fraction was

Fig. 9. Lineweaver-Burk plots of ATPase activities. The reaction was carried out at 25°C and under pH 6.8. △, Mg^{++}-ATPase; ○, $(Mg^{++}-Ca^{++})$-ATPase; ●, total ATPase

also negligible. The same result had been reported by Scales and McIntosh (1968). The ATPase activities measured in the present study, therefore, may well be considered to reflect those of Mg^{++}-ATPase and $(Mg^{++}-Ca^{++})$-ATPase.

These two ATPases are remarkably different each other in their temperature dependency as evidenced from Figs. 1—4. The $(Mg^{++}-Ca^{++})$-ATPase is more highly temperature-dependent than is Mg^{++}-ATPase. Consequently, the energy of activation of the former enzyme is much higher than that of the latter one.
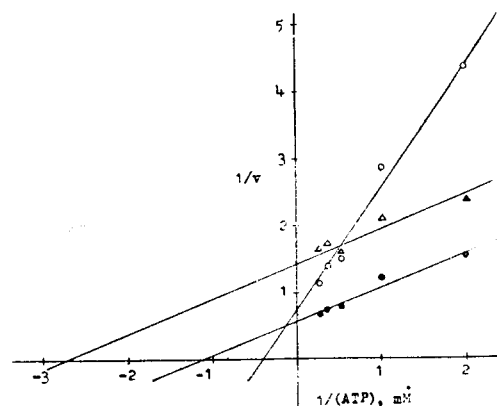
As our skeletal sarcoplasmic reticulum fragments have two distinct ATPases, the cardiac sarcoplasmic reticulum fractions have also been reported to possess $(Mg^{++}-Ca^{++})$-ATPase and Mg^{++}-ATPase (Suko, 1973). The energies of activation of these two cardiac ATPases reported are 14.0 kcal/mole for Mg^{++}-ATPase, 18 kcal/mole for $(Mg^{++}-Ca^{++})$-ATPase, and 17.5 kcal/mole for total ATPase (Suko, 1972). These values are very close with our results (Table 1), though the energy for $(Mg^{++}-Ca^{++})$-ATPase of our preparation is a little higher. It seems that the same ATPase system functions in both skeletal and cardiac sarcoplasmic reticulum in the pumping of Ca.

Shamoo *et al.* (1971) reported that the energy of activation of Mg^{++}-ATPase prepared from the mucosal epithelial cells of the urinary bladder of a freshwater turtle varied from 3.68 to 11.6 kcal/mole, the grand mean value being 7.88 kcal/mole. He explained such variation as due to the possible interaction of the contaminants in the microsome with ATPase, thus changing the value of the energy of activation from one batch of microsomes to another. In our preparation practically the same values were always obtained. If the variation is due to the contaminant interaction, our preparation seems to consist of the two enzymes in relatively pure forms.

Recently, Charnock *et al.* (1971 a) found that the ATPase of renal cortex of rabbit could also be separated into two; one is ouabain-sensitive $(Na^{+}-K^{+})$-ATPase having an energy of activation of 10.2 kcal/mole, and the other is ouabain-insensitive residual ATPase whose energy of activation is 21.2 kcal/mole. They reported that the value for the energy of activation for all reactions below 14°C was 2.2 kcal/mole. Arrhenius plots of these ATPase activities showed that the curves were only linear at temperatures above 25°C. The changes in slope were so abrupt that the curves appeared to have sharp breaks or discontinuities in Arrhenius plots. They speculated that several different conformational forms of the enzyme exist, which have different energies of activation. Similar results were also reported by Sweetman and Griffiths (1971) who observed that, in the reaction catalyzed by a membrane-bound ATPase of *E. coli*, there was a sharp break at 18.8°C in the Arrhenius plot. They considered that this transition temperature indicates either the point at which the lipid of the membrane undergoes a phase change, or a possible conformational change in the enzyme protein, as Charnock *et al.* (1971 a) explained.

The two ATPases in the present study did not show such a break in the Arrehnius plots at any temperature between 0~40°C. Davies and Bragg (1972) also failed to observe this transition point and obtained only a straight line in *E. coli* $(Mg^{++}-Ca^{++})$-ATPase with the energy of activation of 20.7 kcal/mole, which is identical to our value. The ATPases in the fragmented sarcoplasmic

reticulum of rabbit skeletal muscle, therefore, seem not to undergo any confor-
mational change in the temperature range of the present study.

The activity of $(Mg^{++}-Ca^{++})$-ATPase in our preparation far exceeds that of
Mg^{++}-ATPase as shown in Table 1. Similar results were also reported by Suko
(1973) in the cardiac sarcoplasmic reticulum fractions, where $(Mg^{++}-Ca^{++})$-ATPase
represented about 90% of the ATPase activity assayed in the presence of 5 mM
azide. This ratio was nearly constant in the temperature range of 10~30°C. He
observed that azide did not influence the Ca uptake of Ca^{++}-dependent ATP
splitting by the cardiac sarcoplasmic reticulum, however the Ca^{++}-independent
ATPase activity was partially inhibited. The action of azide on the ATPases
seems to be different in the cardiac and skeletal muscles since azide has no
effect on the ATPases of skeletal muscle microsomes (Ha, 1972). Furthermore,
the ratio of the activities of the two ATPases is also different in the cardiac
and skeletal muscles since in our skeletal preparations the ratio varies at
different temperature; the higher the temperature, the greater is the relative
activity of $(Mg^{++}-Ca^{++})$-ATPase.

As to the effects of Mg^{++} and Ca^{++} on the ATPase activity, Shami and Radde
(1972) found that adding increasing amounts of Mg to 5 mM Ca inhibited ATP
hydrolysis and that adding increasing amounts of Ca to 5 mM Mg consequently
stimulated the hydrolysis. In the present study, the optimal concentration of Mg
was found to be 1 mM when the Ca concentration was 0.2 mM. When the Mg
concentration exceeds 1 mM, both ATPase activities were more or less inhibited.
The ionic effects have been reported by many different workers differently and
it seems that, probably because of the difference in the preparation of the
microsomes and in the composition of the reaction mixtures, no conclusion could
be yet established. Though Shami and Radde (1972) consider that Ca^{++} and
Mg^{++} compete for the same binding site on the enzyme, they did not distinguish
the two ATPases.

The energies of activation of the two ATPases are not altered by the change
in the concentration of Mg as shown in Figs. 7 and 8. This result is, in some
respects, identical to that reported by Charnock et al. (1971 b) that the
concentrations of Na and K seemed not to alter the energy of activation of $(Na^+$
$-K^+)$-ATPase. They found that the ratio of Na and K neither altered the energy
value, though the activity was affected. In our preparation, the ratio of Ca and
Mg did not alter the energy value either (data not presented).

The Km values of the ATPases in the skeletal muscle microsomes seem to be
dependent on the ionic composition of the reaction mixture and on the
concentration of ATP, though Panet et al. (1971) reported that 5 mM Mg did
not affect the affinity of the ATPase for ATP. Duggan (1968) obtained the Km

value for the Mg^{++}-ATPase as 2×10^{-4} M which is somewhat lower than ours. He reported that $(Mg^{++}-Ca^{++})$-ATPase has two Km values, 2×10^{-6} M and 2×10^{-4} M, indicating two different activities; one with a high affinity for ATP and the other requiring as high a concentration of ATP as Mg^{++}-ATPase. In our experiment, the value for $(Mg^{++}-Ca^{++})$-ATPase is 2.2 mM which is much higher than that reported by Duggan. Inesi and Watanabe (1967) and Yamamoto and Tonomura (1967) also reported three Km values for the rabbit microsomal ATPase system, though their results differ from those of Duggan (1968).

SUMMARY

Fragmente dsarcoplasmic reticulum of rabbit skeletal muscle was prepared and biochemical properties of its ATPase activity were studied. The ATPase of the fragments could be distinguished as $Mg^{++}-$ATPase and $(Mg^{++}-Ca^{++})$-ATPase.

The activity of $(Mg^{++}-Ca^{++})$-ATPase was predominant over that of Mg^{++}-ATPase in the temperature range of $0 \sim 40°C$ and in the pH range of 6.4~7.6. At higher temperatures the predominance of $(Mg^{++}-Ca^{++})$-ATPase was far greater.

The apparent energies of activation were 14 kcal/mole for Mg^{++}-ATPase, 21 kcal/mole for $(Mg^{++}-Ca^{++})$-ATPase, and 18 kcal/mole for total ATPase. Changes in pH and Mg concentration did not alter the energies of activation of these ATPases.

The Km values of these ATPases were found to be 0.36 mM for $Mg^{++}-$ ATPase, 2.20 mM for $(Mg^{++}-Ca^{++})$-ATPase, and 0.86 mM for total ATPase.

REFERENCES

Allen, R.J.L., 1940. The estimation of phosphorus. *Biochem. J.* **34** : 858.

Charnock, J.S., D.M. Doty, and J.C. Russell, 1971 a. The effect of temperature on the activity of $(Na^{+}-K^{+})$-ATPase. *Arch. Biochem. Biophys.* **142** : 633.

Charnock, J.S., D.A. Cook, and R. Casey, 1971 b. The role of cations and other factors on the apparent energy of activation of $(Na^{+}-K^{+})$-ATPase. *Arch. Biochem. Biophys.* **147** : 323.

Davies, P.L., and P.D. Bragg, 1972. Properties of a soluble Ca^{++}-and Mg^{++}-activated ATPase released from *E. coli* membranes. *Biochim. Biophys. Acta* **266**(1) : 273.

Duggan, P.F., 1968. Calcium-independent adenosine triphosphatase activity in frog muscle microsomes. *Life Sci. Biochem. Gen. Mol. Biol.* **7** (24 Part 2) : 1265.

Ebashi, S., and F. Lipmann, 1962. Adenosine triphosphate-linked concentration of calcium ions in a particulate fraction of rabbit muscle. *J. Cell Biol.* **14** : 389.

Ha, D. B., 1971. Studies on the calcium uptake and ATPase activity of the fragmented sarcoplasmic reticulum. *Korean J. Zool.* **14** : 43.

Ha, D.B., 1972. The effects of caffeine on the ATPase activity and the calcium uptake of the fragmented sarcoplasmic reticulum of rabbit skeletal muscle. *Korean J. Zool.* **15** : 163.

Hasselbach, W., and M. Makinose, 1962. ATP and active transport. *Biochem. Biophys. Res. Commun.* **7** : 132.

Hasselbach, W., and M. Makinose, 1963. Ueber den Mechanismus des Calcium-transoortes durch die Membranen des sarkoplasmatischen Reticulums. *Biochem. Z.* **339** : 94

Inesi, G., and S. Watanabe, 1967. Temperature-dependence of ATP hytdrolysis and calcium uptake by fragmented sarcoplasmic membranes. *Arch. Biochem. Biophys.* **121** : 665.

Meis, L. De, B.M. Rubin-Alschul, and R. D. Machano, 1970. Comparative data of Ca^{++} transport in brain and skeletal muscle microsomes. *J. Biol. Chem.* **245** : 1883.

Nakamura, M., 1950. Colorimetric determination of phosphorus. *J. Agr. Chem.* (Japan) **24** : 1.

Panet, R., U. Pick, and Z. Selinger, 1971. The role of calcium and magnesium in the adenosine triphosphatase reaction of sarcoplasmic reticulum. *J. Biol. Chem.* **246** : 7349.

Scales, B., and D.A.D. McIntosh, 1968. The effect of propranolol and its optical isomers on the radiocalcium uptake and adenosine triphosphatases of skeletal and cardiac sarcoplasmic reticulum fractions. *J. Pharmacol. Exptl. Therapy* **160** : 261.

Shami, Y., and I.C. Radde, 1972. The effect of the Ca^{++}/Mg^{++} concentration ratio on placental $(Ca^{++}-Mg^{++})$-ATPase activity. *Biochim. Biophys. Acta* **255** : 675.

Shamoo. A.E., T.P. Schilb, and W.A. Brodsky, 1971. The effect of temperature on Mg^{++}- and (Na^+-K^+)-ATPases. *Biochim. Biophys. Acta* **225** : 254.

Suko, J., 1973. The effect of temperature on Ca^{++} uptake and Ca^{++} activated ATP hydrolysis by cardiac sarcoplasmic reticulum. *Experimentia* **29** : 396.

Sweetman, A. J., and D.E. Griffiths, 1971. *Biochem. J.* **121** : 117.

Weber, A., R. Herz, and I. Reiss, 1966. Study of the kinetics of calcium transport by isolated fragmented reticulum. *Biochem. Z.* **345** : 329.

Yamamoto, T., and Y. Tonomura, 1967. Reaction mechanism of the Ca^{++} dependent ATPase of sarcoplasmic reticulum from skeletal muscle. 1. Kinetic studies. *J. Biochem.* (Tokyo) **62** : 558.