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Kinetic Studies on the Effects of Divalent Cations on the ATPase Activity of the Fragmented Sarcoplasmic Reticulum of Rabbit Skeletal Muscle

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骨骼筋 小胞體의 ATPase活性에 미치는 二價金屬이온의 영향

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적 요

토끼 골격근 小胞體의 ATPase 活性에 미치는 Hg^{2+}, Cu^{2+}, Pb^{2+}, Cd^{2+}, Mn^{2+} 등 2價 陽이온을 영향을 비교 측정하였다.

이들 陽이온은 Mn^{2+}을 제외하고 모두 이 酵素의 活性을 阻害하였다. Mn^{2+}은 低濃度 (12.5—100 μM)에서는 오히려 活性을 증가시켰고, 그 보다 高濃度에서는 극히 약하게 阻害하였다.

Mn^{2+}을 제외한 위의 2價 陽이온들의 阻害能은 Hg^{2+}가 가장 컸고 (阻害係數 Ki=10μM), 그 다음이 Cu^{2+} (Ki=30μM), Pb^{2+} (Ki=120μM), Cd^{2+} (Ki=320μM)의 순이었다.

위의 4종의 陽이온들의 ATPase에 대한 阻害作用을 可逆的 非競爭的 阻害로 판정되었다.

INTRODUCTION

The biological and toxicological roles of many divalent cations have recently been studied in detail. Divalent metal ions may be a part of active sites of enzymes and participate directly in catalysis or may stabilize macromolecular structure of proteins, thereby affecting their function indirectly. They may affect enzymes or membrane, or both, of subcellular or cellular particles to control biological pathways (Vallee and Ulmer, 1972).

Many divalent cations like Pb^{2+}, Cd^{2+} and Hg^{2+} show a strong affinity for ligands such as cysteinyl and histidyl side chains of protein molecules. Hence

these elements can affect a large number of biological sites; they inhibit a large number of enzymes having functional sulfhydryl groups, they bind to and affect the conformation of enzymes, and they disrupt pathways of oxidative phosphory lation, although in each instance the precise reaction depends upon the individual chemical properties of the metal ions (Skilleter, 1975). For instance, alkaline phosphatase of rat liver was inhibited by Cd^{2+} (Sporn et al., 1970) while activated by Hg^{2+} (Nowak, 1969), and the same enzyme from guinea pig urine was activated by Pb^{2+}, (Secchi et al., 1970) while that of rat lung was inhibited (Nowak, 1969).

Kosmider et al. (1964) reported early that the APTase of rabbit skeletal muscle was inhibited by Pb^{2+}, and Peters et al. (1966) subsequently reported that the membrane-bound ATPase activity was also inhibited by exogenous Cu^{2+}. The calcium uptake, as well as the ATPase activity, of the fragmented sarcoplasmic reticulum was also inhibited by $CuSO_4$ (Ha and Kim, 1977).

In an attempt to understand the inhibitory mechanism of divalent cations on the membrane-bound ATPase, the present experiment was done to measure the activity of total ATPase of the fragmented sarcoplasmic reticulum of rabbit skeletal muscle in the presence of Hg^{2+}, Cu^{2+}, Pb^{2+} and Mn^{2+}. The reaction velocities, kinetic parameters and energies of activtion of the enzyme in the presence of these ions were analyzed and compared for each ion. In this paper we report that the cations, except Mn^{2+}, act as reversible noncompetitive inhibitor on the ATP ase of fragmented sarcoplasmic reticulum of rabbit skeletal muscle.

MATERIALS AND METHODS

Framented sarcoplasmic reticulum (FSR) was isolated from rabbit skeletal muscle (back and thigh) by differential centrifugation as described previously (Ha, 1971). The fraction sedimented at 30,000 xG for 60 minutes was collected and suspended in 20 mM tris-maleate buffer containing 50 mM KCl (pH 6.8). The preparation was used for the experiment within 72 hours. Previous studies showed no appreciable contamination of mitochondria in the preparations thus made (Ha, 1975). Protein concentration was measured by Lowry's method (1951) with crystalline rabbit serum albumin as standard. All procedures were carried out at 0–4°C.

The FSR ATPase activity was measured in the reaction medium containing 20 mM tris-maleate buffer (pH. 6.8), 50mM KCl, 4mM $MgCl_2$, 0.1 mM $CaCl_2$, and various concentrations of ATP, enzyme protein, and the metal ions (Hg^{2+}, Cu^{2+}, Pb^{2+}, Cd^{2+} and Mn^{2+} all in the form of chloride) as specified in tables and figures. Otherwise specified the reaction temperature was 35°C.

The reaction was started by adding preincubated FSR to the reaction medium and terminated after 10 minutes incubation by adding cold trichloroacetic acid

in the final concentration of 7%. The reaction medium was then filtered through a Toyo filter paper (No. 2A) to remove FSR protein and the produced inorganic phosphate (Pi) was determined according to Nakamura (1950).

All solutions were prepared with glass distilled deionized water. ATP-2Na was obtained from Schwarz/Mann (USA), tris (hydroxymethyl) aminomethane was purchased from Merck (Germany), and other reagents used were of reagent grade from Wako Pure Chemicals Co. (Japan).

RESULTS

The ATPase activity of FSR measured in the presence of various concentrations of divalant cations is shown in Fig. 1, where the enzyme activity is expressed as relative value to the control. The control was in the range of average 600—800 μmoles Pi/mg protein/min. All cations examined, except Mn^{2+}, apparently showed inhibitory effect on the enzyme activity. The inhibitory effects of the cations increased as the cation concentration increased from 12.5μM to 400 μM. The inhibitory effectiveness at a given concentration of the cation was in the order of $Hg^{2+} > Cu^{2+} > Pb^{2+} > Cd^{2+} > Mn^{2+}$. Thus, the concentration for 50% inhibition was 12.5 (Hg^{2+}), 30 (Cu^{2+}), 120 (Pb^{2+}), 350 (Cd^{2+}) μM.

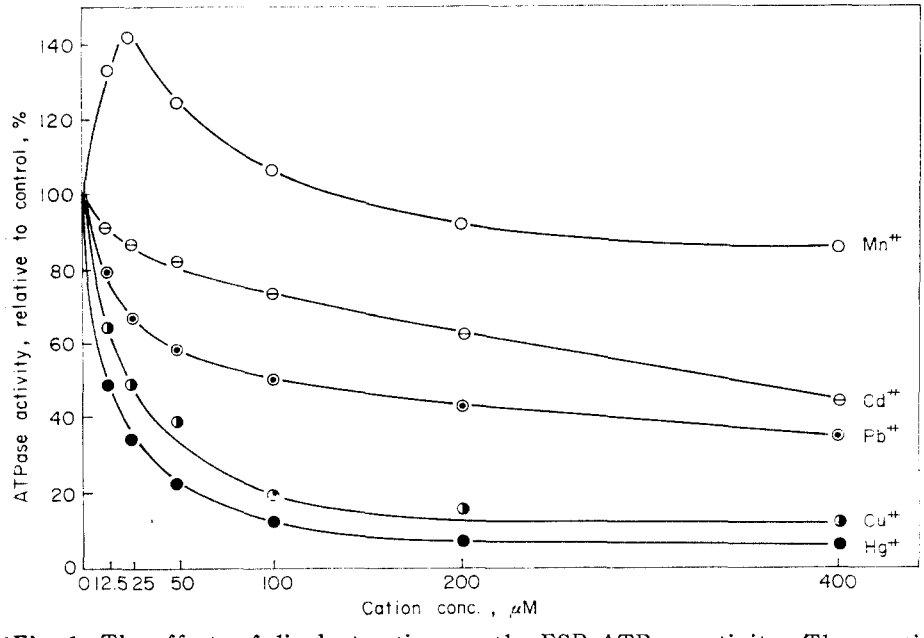

Fig. 1. The effects of divalent cations on the FSR ATPase activity. The reaction medium consisted of 20mM tris-maleate buffer (pH 6.8), 50mM KCl, 4 mM $MgCl_2$, 0.1mM $CaCl_2$, 2 mM ATP, and 0.02 mg protein/ml. The reaction was carried out at 35°C for 10 minutes. Each point represents an average of four determinations.

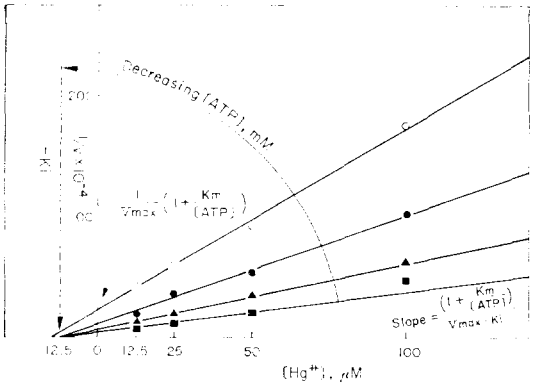

Fig. 2. Dixon plot for inhibition constant (Ki) of Hg^{2+} on the ATP ase activity. The reaction medium consisted of 20 mM tris-maleate buffer (pH 6.8), 50 mM KCl, 4 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.02 mg protein/ml, and the different concentrations of ATP; 1 (○), 2 (●), 4 (▲), 8 (■) mM. The reaction was carried out at 35°C for 10 minutes. Each point represents an average of three determinations.

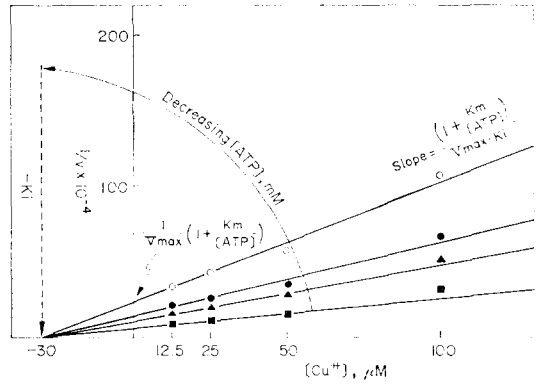

Fig. 3. Dixon plot for inhibition constant (Ki) of Cu^{2+} on the ATP ase activity. The reaction medium consisted of 20 mM tris-maleate buffer (pH 6.8), 50 mM KCl, 4 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.02 mg protein/ml, and the different concentrations of ATP; 1 (○), 2 (●), 4 (▲), 8 (■) mM. The reaction was carried out at 35°C for 10 minutes. Each point represents an average of three determinations.

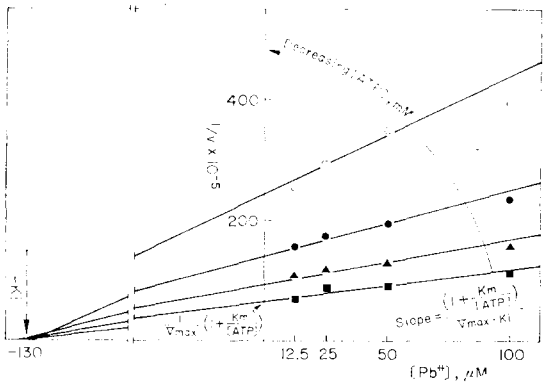

Fig. 4. Dixon plot for inhibition constant (Ki) of Pb^{2+} on the ATP ase activity. The reaction medium consisted of 20 mM tris-maleate buffer (pH 6.8), 50 mM KCl, 4 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.02 mg protein/ml, and the different concentrations of ATP; 1 (○), 2 (●), 4 (▲), 8 (■) mM. The reaction was carried out at 35°C for 10 minutes. Each point represents an average of three determinations.

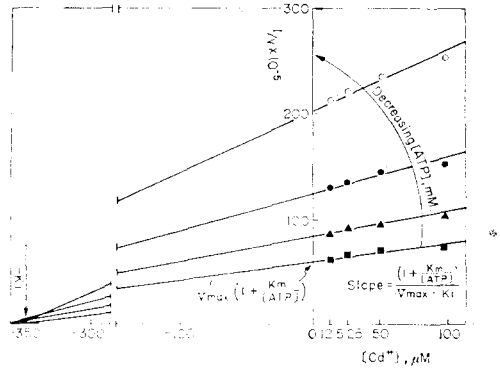

Fig. 5. Dixon plot for inhibition constant (Ki) of Cd^{2+} on the ATP ase activity. The reaction medium consisted of 20 mM tris-maleate buffer (pH 6.8), 50 mM KCl, 4 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.02 mg protein/ml, and the different concentrations of ATP; 1 (○), 2 (●), 4 (▲), 8 (■) mM. The ration was carried out at 35°C for 10 minutes. Each point represents an average of three determinations.

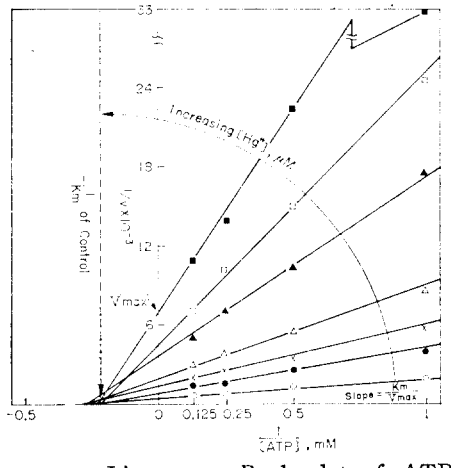

Fig. 6. Lineweaver-Burk plot of ATPase activity at various concentrations of Hg^{2+} and ATP. The reaction mixture consisted of 20 mM tris-maleate buffer(pH 6.8) 50 mM KCl, 4 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 0.02 mg protein/ml. The reaction was carried out at 35°C for 10 minutes. The concentrations of Hg^{2+} were; 0 (○), 12.5 (●), 25 (×), 50 (△), 100 (▲), 200 (□), and 400 (■) uM.

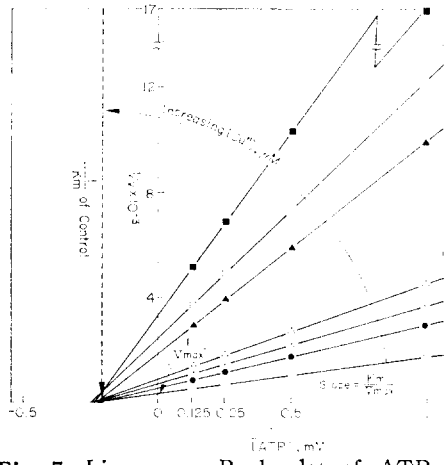

Fig. 7. Lineweaver-Burk plot of ATPase activity at various concentrations of Cu^{2+} and ATP. The reaction mixture consisted of 20 mM tris-maleate buffer (pH6.8) 50 mM KCl, 4 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.02 mg protein/ml. The reaction were carried out at 35°C for 10 minutes. The concentrations of Cu^{2+} was; 0 (○), 12.5 (●), 25 (×), 50 (△), 100 (▲), 200 (□), and 400 (■) uM.

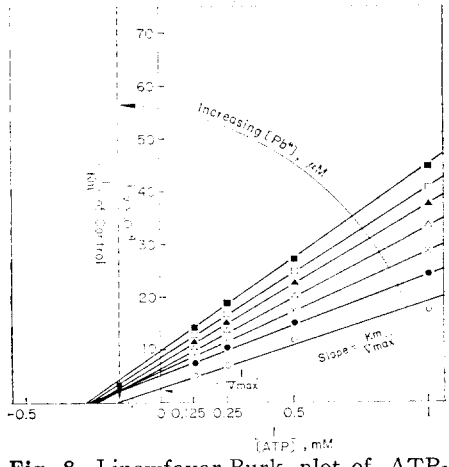

Fig. 8. Linewfavar-Burk plot of ATPase activity at various concentration of Pb^{2+} and ATP. The reaction mixture consisted of 20 mM tris-maleate buffer (pH6.8) 50 mM KCl, 4 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.02 mg protein/ml. The reaction was carried out at 35°C for 10 minutes. The concentrations of Pb^{2+} were; 0 (○), 12.5 (●), 25 (×), 50 (△), 100 (▲), 200 (□), and 400 (■) uM.

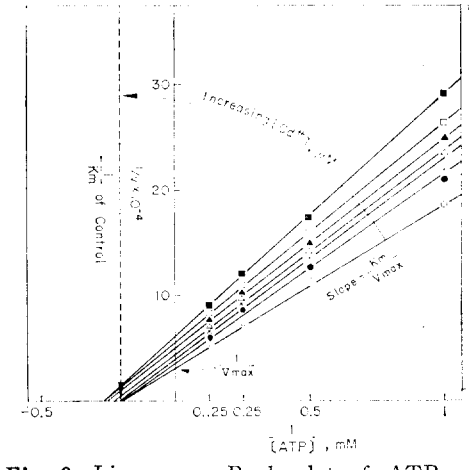

Fig. 9. Lineweaver-Burk plot of ATPase activity at various concentrations of Cd^{2+} and ATP. The reaction mixture consisted of 20 mM tris-maleate buffer (pH6.8) 50 mM KCl, 4 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.02 mg protein/ml. The reaction was carried out at 35°C for 10 minutes. The concentrations of Cd^{2+} were; 0 (○), 12.5 (●), 25 (×), 50 (△), 100 (▲), 200 (□), and 400 (■) uM.

Unlike other cations examined in the present study, Mn^{2+} had a unique property in that it rather increased the ATPase activity in lower concentrations (12.5—100μM) and slightly decreased the activity at higher concentrations. Even at 400 μM where other cations caused more than 50% inhibition of the enzyme activity, Mn^{2+} decreased the activity only 10% or less.

In order to find out the inhibition constant, Ki, of the five cations, the FSR ATPase activity was measured at various concentrations of ATP and cations and the Dixon plot, 1/v versus [ATP], was constructed from the result (Figs. 2, 3, 4 and 5).

The Ki values thus obtained were 10, 30, 120 and 320μM for Hg^{2+}, Cu^{2+}, Pb^{2+} and Cd^{2+}, respectively. These values were very close to the concentration of 50% inhibtion as seen in Fig. 1.

In the Dixon plots for all cations except Mn^{2+}, the slop, $(1+\frac{K_m}{[ATP]})/(V_{max}\cdot Ki)$, and the reciprocal velocity intercept, $\frac{1}{V_{max}}\cdot(1+\frac{K_m}{[ATP]}$, increased as the concentration of ATP decreased due to the decrease of V_{max}.

The ATPase activity measured at various concentrations of ATP and cations was also applied to construct the Lineweaver-Burk, Eadie-Scatchard, Woolf-Augustinsson-Hofstee and Hanes-Woolf plots. Figs. 6—9 show only the Lineweaver-Burk plots for each cation. As can be seen from these figures, the slope, K_m/V_{max}, and the reciprocal velocity axis intercept, $1/V_{max}$, increased as the cation concentration increased in every case of the cation examined, and the plot lines all converged to nearly the same point of $-1/Km$ on the $-1/[ATP]$ abscissa. Thus, the Km's were constant while V_{max}'s decreased as the cation concentration increased. These results suggest that Hg^{2+}, Cu^{2+}, Pb^{2+} and Cd^{2+} all act on the enzyme as a noncompetitive inhibitor. Other plots all gave the same conclusion.

For the purpose of seeing whether the inhibitory action of the cation was reversible or irreversible, the enzyme activity was measured with various concentrations of the FSR protein in the presence of the cations. The concentrations of cations used in this experiment were Ki concentration and 100μM each. Iodoacetamide (200μM) was also employed to compare its reaction mode with those of cations. The results are shown in Fig. 10, where it is revealed that the control and cation-treated groups give lines which converge to the same zero point while that of iodoacetamidetreated one is parallel to the control. These results indicate that the inhibition of the enzyme activity by the cations is reversible while the iodoacetamide inhibition is irreversible.

To determine the effect of the cation on the energy of activation of the enzyme, the ATPase activity was measured at various temperatures between 10 and 40°C in the presence of various concentrations of the metal ions.

As shown in Fig. 11, the ATPase activity increased as the temperature increased with a Q_{10} value of approximately 2.0. The presence of cation obviously decreased the Q_{10} value and the decrement was greater at higher cation concentrations (Table 1).

The energy of activation of the ATPase in the presence of various cations of

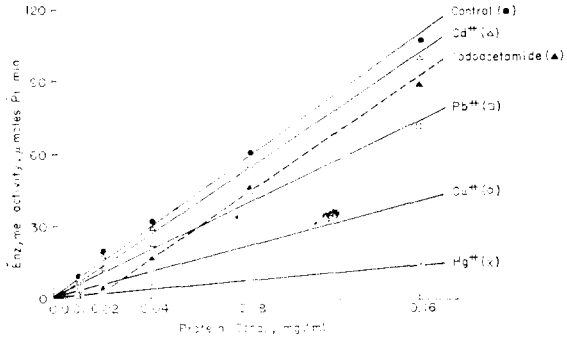

Fig. 10. The effects of divalent cations (each 100 μM) and iodoacetamide (200μM) on the ATPase activity. The reaction mixture consisted of 20 mM tris-maleate buffer (pH 6.8), 50 mM KCl, 4 mM $MgCl_2$, 2 mM ATP, and 0.1 mM $CaCl_2$. The reaction was carried out at 35°C for 10 minutes.

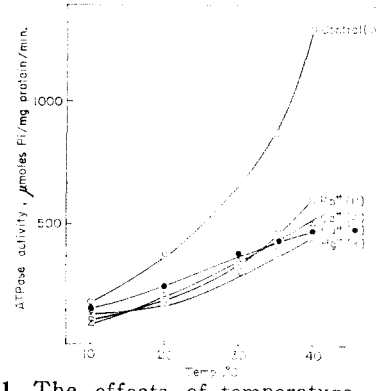

Fig. 11. The effects of temperature on the ATPase activity in the presence of divalent cation. The concentration of each cation was that of Ki. The reaction mixture consisted of 20 mM trismaleate buffer (pH 6.8), 50 mM KCl, 4 mM $MgCl_2$, 0.1 mM $CaCl_2$, 2 mM ATP, and 0.02 mg protein/ml.

Table 1. Q_{10} of the ATPase activity in the presence of divalent cations.

Ion conc., μM / Ions	0	10	25	50	100	200	500
Hg^{2+}	2.02	1.55	1.41	1.39	1.18	1.04	1.10
Cu^{2+}	2.20	1.53	1.46	1.37	1.13	1.15	1.12
Pb^{2+}	2.04	1.84	1.76	1.70	1.63	1.65	1.69
Cd^{2+}	2.02	1.95	1.89	2.02	1.92	1.81	1.37
Mn^{2+}	2.06	2.05	2.29	2.21	1.90	1.92	1.96

Table. 2. Energies of activation (Kcal/mole) of the ATPase in the presence of divalent cations.

Ion conc., μM / Ions	0	10	25	50	100	200	500
Hg^{2+}	19.50	16.23	17.13	16.50	15.14	14.54	14.29
Cu^{2+}	18.60	17.18	16.93	16.36	15.20	14.85	14.47
Pb^{2+}	18.90	18.39	18.25	18.12	17.85	17.89	18.00
Cd^{2+}	19.21	19.06	18.98	18.54	18.94	18.42	17.97
Mn^{2+}	19.19	19.20	20.75	20.25	18.67	19.14	19.31

various concentrations was calculated from the Arrhenius plot constructed form the temperature data and is show in Table 2. The ATPase of the present experiment had an energy of activation of approximately 19 Kcal/mole (far left column of Table 2). The presence of any metal ion, except Mn^{2+} had an effect to decrease the activation energy though the degree of decrease was not very significant.

DISCUSSION

The ATPase system of FSR of skeletal muscle has been well known to consist of Mg^{2+}-activated ATPase and Mg^{2+} plus Ca^{2+} -activated ATPase. Many kinetic studies on this enzyme system have been done for each component separately. In this experiment, however, the two enzymes were not distinguished and every measurement was done on the total activity because the apparent ATPase activity revealed by FSR is the sum of these two components.

The effects of metal ions on the FSR ATPase have been studied by many investigators. The enzyme activity was reported to be inhibited by La^{3+} (Yamada and Tonomura, 1972), by Cu^{2+} (Peters, 1966; Ha and Kim, 1977) and by Hg^{2+}, Cu^{2+}, La^{3+}, Li^{+}, Cd^{2+}, Pb^{2+}, Zn^{2+} and Co^{2+} (Kim et al., 1978).

The results of the present investigation revealed that the FSR ATPase activity (total activity) was inhibited by many metal divalent ions such as Hg^{2+}, Cu^{2+}, Pb^{2+} and Cd^{2+} (Fig. 1). These results are generally in good agreement with previous report from this laboratory (Kim et al., 1978). In the present experiment, the concentration of the metal ions for 50% inhibition was found to be 12.5, 30, 130, and 350μM for Hg^{2+}, Cu^{2+}, Pb^{2+}, Cd^{2+}, respectively, which are very close to the inhibition constant (Ki) for each ion.

The inhibition of the enzyme activity by these ions was suggested from the Dixon plot to be noncompetitive one. Other plots including the Lineweaver-Burk plot also gave the same suggestion. Therefore, it is tentatively concluded that ATP molecules as substrate and the metal ions as inhibitors bine to the enzyme molecule at different sites and that the "ATP-ATPase-metal complex" is catalytially incative.

Since the stronger the affinity of the metal ions to the enzyme molecule the more the complex may be incative, it might be thought that the percent inhibition and the value of Ki of each ion would reflect the affinity of the ion to the enzyme molecule and then the order of the affinity would be $Hg^{2+}>Cu^{2+}>Pb^{2+}>Cd^{2+}$. Gurd and Wilcox (1956) reported the behavior of a simple amino acid, having an alpha amino and carboxyl group as a ligand, toward metal ions. According to this report, the association constants are in the order of $Hg^{2+}>Cu^{2+}>Ni^{2+}>Pb^{2+}>Zn^{2+}>Co^{2+}>Cd^{2+}$. This order of association constants is well matched

with the present result.

The noncompetitive inhibiton of the enzyme activity by the metal ions was reversible one. When tlhe enzyme activity was measured with different enzyme concentrations in the presence of 200μM of iodoacetamide (Fig. 10), the activity was less than that of control by a constant amount throughout all the enzyme concentrations measured, suggesting that a fixed portion of the enzyme was blocked by the alkylating agent. Thus the plot in the presence of iodoacetamide was parallel to that of control. Iodoacetamide is an alkylating agent that forms a covalent bond irreversibly with a sulfhydryl groups of ATPase and thereby inactivates the enzyme. The effect of iodoacetamide seems to be a noncompetitive inhibition because V_{max} decreases in the presence of iodoacetamide while K_m remains unchanged. Unlike iodoacetamide, the metal ions did not give a parralel plot but showed one that all converged to the zero point (Fig. 10), suggesting that the inhibition is reversible It has been known that divalent metal ions such as Hg^{2+}, Pb^{2+}, Cu^{2+} and Cd^{2+} all show strong affinity for ligands such as cysteinyl and histidyl side chains of proteins (Vallee and Ulmer, 1972). Since the purified ATPase protein has 3% of cysteine and 1% of histidine (Martonosi and Halpin, 1971), it is suggested that the metal ions employed in the present study bind irreversibly to these ligands of the enzyme forming a mercaptide.

The reaction of Hg^{2+} with S-S bond was reported by Brown and Edwards (1969) and the existence of S-Hg-S bond in the protein has been demonstrated by EDTA and carboxymethylation of the -SH groups thus released (Burstein and Sperling, 1970). This reversible reaction supports the present conclusion.

Hasselbach and Elfvin (1967) indicated a difference between the outer and inner surface of sarcoplasmic reticulum. When FSR vesicles were opened by osmotic shock and both sides of vesicles exposed to Hg-phenylazoferritin, this label reacted prevalenty with certain specific-SH groups on the outer surface of the FSR.

Inesi and Asai (1968) also suggested that the ATPase protein was more accessible from the outer surface than from the inner side of the FSR. Taking account of these reports into consideration, Hg^{2+} seems to react with and incativate the ATPase protein by forming one of the following complexes:

Hg-S-(ATPase)-S-Hg, (ATPase)-S-Hg-S-(ATPase), or (ATPase)$\begin{matrix} \diagup \\ \\ \diagdown \end{matrix}\begin{matrix} S \\ | \\ Hg \\ | \\ S \end{matrix}$.

The similar explanation may also be possible for Cu^{2+} and Pb^{2+} inhibiton. According to Singer (1978), Pb^{2+} not only binds to sulfhydryl groups but also straddles two cysteine sulphurs to form mercaptides and the resulting conformational distribution of polypeptide chains accounts for the Pb^{2+} inhibition of the enzyme system. Rausa and Calapaj (1970) also reported that cysteine reversed the Pb^{2+} inhition of

the erythrocyte (Na^++K^+)-ATPase while the corresponding membrane acetylcholinesterase is unaffected.

In the present experiment, Cd^{2+} also seemed to act as a reversible noncompetitive inhibitor on the FSR ATPase. Recently Siegel and Fogt (1979) reported that Cd^{2+} together with Pb^{2+}, Cu^{2+} and Hg^{2+} reduced the binding of ^{3}H-ouabain to (Na^++K^+)-ATPase in *E. electrocus* electroplex microsomal preparations in the presence of Mg^{2+} and ATP.

The effect of Mn^{2+} on the FSR ATPase was significantly different from other divalent metal ions in that it rather activated the enzyme activity at lower concentrations (12.5—100μM) and only slightly inhibited at higher concentrations (200—400μM). The mechanism of this effect is presently unknown and should wait for further studies. Mn^{2+} can substitute Mg^{2+} which is an essential ion for the activation of ATPase (MacLennan et al., 1974). The association constant of Mn^{2+} to alpha amino and carboxyl groups as a ligand is slightly higher than Mg^{2+} and Ca^{2+} as described above (Gurd and Wilcox, 1956). Mn^{2+} is known to activate and induce conformational change of enolase (Brewer and Weber, 1966).

The energy of activation of the FSR ATPase determind in the present experiment was about 19 Kcal/mole. This is practically the same with those of Suko (1973) and Ha et al. (1974) which are 18 and 20 Kcal/mole, respectively. The presence of Hg^{2+}, Cu^{2+}, Pb^{2+} or Cd^{2+} lowered the energy, Hg^{2+} and Cu^{2+} sharply and the rest two mildly.

SUMMARY

The effects of divalent cations, Hg^{2+}, Cu^{2+}, Pb^{2+}, Cd^{2+}, and Mn^{2+} on the total ATPase activity of the fragmented sarcoplasmic reticulum isolated from rabbit skeletal muscle were investigated.

The inhibitory effects of the cations on the enzyme activity increased as the concentrations of the ions increased with the order of efficiency of $Hg^{2+}>Cu^{2+}>Pb^{2+}>Cd^{2+}>Mn^{2+}$ in the concentration range between 10 and 500μM.

The 50% inhibition for each ion was almost identical with the inhibition constant (Ki) value for each ion. The Ki's were 10, 30 130, and 350μM for Hg^{2+}, Cu^{2+}, Pb^{2+}, and Cd^{2+}, respectively. Mn^{2+} seemed to be an activator at lower concentrations and an inhibitor at higher concentrations. The presence of the cations did not change the Km values, suggesting that the ions act as a reversible noncompetitive inhibitor on the FSR ATPase.

The energy of activation of the enzyme was approximately 19 Kcal/mole. The presence of the ions decreased the value slightly.

A possible mechanism for the reversible noncompetitive inhibitory effect of the cations was discussed.

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