The Effects of Calcium and Magnesium Ions on the Adenosine Triphosphatase and Inosine Triphosphatase Activities of Myosin A

By E. A. SUGDEN AND T. NIHEI

Department of Biochemistry and the Surgical-Medical Research Institute, University of Alberta, Edmonton, Alberta, Canada

(Received 23 December 1968)

1. The effects of Ca^{2+} and Mg^{2+} on the enzymic activity of myosin were studied with myosin preparations treated by the ion-exchange resin Chelex-100. A reaction mixture containing 0.05 M-potassium chloride was chosen in which the effects of univalent ions such as K^+ , Na^+ and Cl^- do not change significantly with small variations in their concentrations. 2. The relationship between the rate of hydrolysis of ATP or ITP and the concentration of Ca^{2+} suggests that a relatively weak binding of Ca^{2+} either to myosin or to the substrate nucleotide is responsible for the activation of the enzymic activity. According to the experiments with an ultrafiltration technique, the binding of Ca^{2+} to myosin proceeds in at least two steps, the first occurring at one site on every 500 000 atomic mass units of myosin with an apparent association constant, $K_{\text{app.}}$, $1.3 \times 10^6 \text{ M}^{-1}$, and the second seeming to be so weak that its binding parameters cannot be determined by the method used. The first type of Ca^{2+} binding is not observable with *N*-ethylmaleimide-modified myosin, yet this modified myosin shows activation by Ca^{2+} of its adenosine triphosphatase and inosine triphosphatase. 3. The inhibition by Mg^{2+} can be related to a binding reaction of Mg^{2+} with myosin having $K_{\text{app.}} \sim 10^6 \text{ M}^{-1}$. Mg^{2+} replaces the Ca^{2+} bound tightly to myosin. The $K_{\text{app.}}$ for Mg^{2+} -myosin binding calculated by assuming a competition between Ca^{2+} and Mg^{2+} for the same site is 2.1×10^5 – $3.0 \times 10^5 \text{ M}^{-1}$. When myosin is modified with a thiol reagent (*p*-mercuribenzoate) at a certain ratio to myosin, the inhibition by Mg^{2+} becomes unobservable. 4. The behaviour of the hydrolytic activity of myosin on ATP or ITP in the presence of both Ca^{2+} and Mg^{2+} is consistent with the explanation that the inhibition by Mg^{2+} is due to the tight binding of Mg^{2+} to myosin, whereas the activation by Ca^{2+} is caused either by a weak binding of Ca^{2+} to myosin or by CaATP^{2-} or by both.

The bivalent cations Mg^{2+} and Ca^{2+} exert qualitatively different effects on myosin NTPase,* presumably reflecting their characteristic roles in muscle contraction. It was proposed that the CaNTP^{2-} complex is dephosphorylated by the action of myosin more rapidly than are other species of NTP (Nanninga, 1957; Blum & Felauer, 1959; Lowenstein, 1960; Luchi & Kritcher, 1967). It was also speculated that, on binding to myosin, Ca^{2+} activates (Nanninga, 1959) and Mg^{2+} inhibits the NTPase action (Blum & Felauer, 1959).

Despite the extensive studies carried out by previous investigators, several basic questions concerning this problem remain unanswered. The question we chose to investigate was whether or

*Abbreviations: NTP, nucleoside triphosphate; NTPase, nucleoside triphosphatase; ATPase, adenosine triphosphatase; ITPase, inosine triphosphatase; PMB, *p*-mercuribenzoate; NEM, *N*-ethylmaleimide.

not Ca^{2+} and Mg^{2+} influence the enzymic activity of myosin by the same mechanism.

In the present work the effects of Ca^{2+} and Mg^{2+} on the ATPase and ITPase of myosin treated by Chelex-100 and, in certain cases, modified with PMB or NEM were studied.

METHODS AND MATERIALS

Preparation of myosin A. Myosin A was extracted from the back and leg muscles of albino rabbits by the procedure of Tonomura, Appel & Morales (1966). The protein concentration was determined by the biuret test which was standardized with Labtrol (Dade Reagents Inc., Miami, Fla., U.S.A.). The stock solution of myosin was made in 0.5 M-KCl-0.02 M-histidine buffer, pH 7.4. This was centrifuged at 35000 rev./min. in a no. 40 Spinco rotor for 3 hr. before use. Enzymic activity in the presence of 0.05 M-KCl, 0.05 M-tris-HCl, 10 mM- CaCl_2 and 1 mM-ATP at pH 7.4 and 25° was 0.4–0.5 $\mu\text{mole/min./mg.}$ of protein.

Table 1. Ca^{2+} content of myosin A before and after the treatment with Chelex-100

Myosin A in 0.5M-KCl was dialysed against 30 ml. of regenerated Chelex in 0.5M-KCl. The effect of Chelex treatment is indicated by the loss of Ca^{2+} from myosin and the gain by the Chelex.

Preparation	Chelex treatment		Ca^{2+} in myosin (moles of Ca^{2+} /mole of myosin A)	
	Ca^{2+} lost from myosin (μmole)	Ca^{2+} gained by Chelex-100 (μmole)	Untreated	Treated
1 (160 mg.)	0.33	0.30	1.2	0.24
2 (250 mg.)	0.35	0.25	0.90	0.11

PMB-modified myosin A. Myosin at a concentration of 1.33 mg./ml. was incubated at 0° for 5 min. in 0.5M-KCl-1 mM-tris-HCl buffer, pH 7.0, and an appropriate amount of PMB (Sreter, Seidel & Gergely, 1966). The activity versus PMB concentration curve showed the biphasic relationship first observed by Kielley & Bradley (1956): maximum activity occurred in the presence of 2.5–3.0 moles of PMB/10⁵ g. of myosin, as was also shown by Mattocks, Keswani & Dowben (1967).

NEM-modified myosin A. Myosin at a concentration of 10 mg./ml. was treated with 2.0 moles of NEM/10⁵ g. of myosin for 30 min. (Perry & Cotterill, 1965). The reaction was stopped by the addition of a 50-fold excess of 2-mercaptoethanol (Sekine & Kielley, 1964) and the myosin was dialysed against 0.5M-KCl-0.02M-histidine buffer, pH 7.4.

Enzymic assays. The rate of release of P_i was determined by the molybdovanadate method (Lecocq & Inesi, 1966). The reaction was initiated by adding NTP to a constantly stirred mixture containing myosin and other components. Samples (1 ml.) of the reaction mixture were taken at intervals and added to 2 ml. of the molybdovanadate reagent. A suppression of the colour was encountered when a high protein concentration was used. This difficulty was circumvented by adding 0.05 μmole of K_2HPO_4 /ml. of reaction mixture. This was found to have no effect on the rate. All activities were determined in 0.05M-KCl-0.05M-tris-HCl buffer, pH 7.4, at 25°.

Ca^{2+} -binding reaction. Myosin and all reagents were treated with Chelex-100 (Calbiochem, Los Angeles, Calif., U.S.A.) to decrease cation impurities (Offer, 1964). Myosin was treated by dialysis against a large volume of Chelex-100 suspension. KCl, tris hydrochloride and ATP were purified by passing them through the resin, which was in the K^+ , H^+ and Na^+ forms respectively. Contamination of myosin and Chelex-100 with Ca^{2+} was determined by using Calcein and the fluorimetric technique of Kepner & Hercules (1963). For this experiment Ca^{2+} was extracted from myosin or Chelex (2 ml.) with 7% (w/v) trichloroacetic acid for 24 hr. at room temperature (Mori, 1959). The trichloroacetic acid extract, separated from the residue by centrifugation, was dried in a sand bath at 75°. The Calcein solution (Phillips, 1964) was added directly to the residue and the fluorescence was determined in a Turner model 110 fluorimeter. The results indicated an incomplete removal of Ca^{2+} from myosin by Chelex (Table 1). In a separate experiment, $^{45}\text{Ca}^{2+}$ in 1.4 μmoles of Ca^{2+} was added to 10 ml. of myosin (2.6 mg./ml.) at 0°, which was then treated

with a large volume of Chelex. Some radioactivity remained attached to the myosin, demonstrating that the residual Ca^{2+} was exchangeable. It was therefore assumed that the residual Ca^{2+} bound to myosin is also counted when the binding of Ca^{2+} to myosin is assayed by the method described below.

The Ca^{2+} -binding reaction was measured by ultrafiltration under the same conditions as for the enzymic analyses. The $^{45}\text{Ca}^{2+}$ (535 μCi /82.4 μg . of Ca^{2+} in 1.4 ml.; The Radiochemical Centre, Amersham, Bucks.) was diluted before use. A 10 ml. suspension of myosin in 0.05M-KCl-0.05M-tris-HCl buffer, pH 7.4, containing $^{45}\text{Ca}^{2+}$ (0.01 μCi) and an appropriate amount of carrier, was filtered through a Millipore filter tube. No more than 5% of this solution was filtered, so that the protein concentration was not altered appreciably. Samples (0.2 ml.) of both the solution and the supernatant were added to scintillation vials and 0.8 ml. of NCS reagent (Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A.) was added to solubilize the protein. A fluor containing 5.5 g. of Pre-mix 'M' (Packard Instrument Co., La Grange, Ill., U.S.A.)/l. of toluene was prepared. Counting was done in a Beckman LS-100 scintillation counter. External standardization with ^{137}Cs showed no quenching.

ATP and ITP were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A., and used without further treatment. Other chemicals were of reagent grade. The stock solutions of CaCl_2 and MgCl_2 were standardized by titration against EDTA.

RESULTS

Activation by Ca^{2+} . As shown in Fig. 1, Ca^{2+} at concentrations of up to 30 mM activates the hydrolysis of ATP and ITP. The rates of hydrolysis at various concentrations of Ca^{2+} were expressed by the 'relative activity', which is:

$$\frac{100(v - v_1)}{(v_2 - v_1)}$$

where v_1 , v_2 and v denote the rates determined with no added Ca^{2+} , with the optimum amount of added Ca^{2+} to give maximal activity and with an intermediate concentration of Ca^{2+} respectively. This ratio was found to be useful for the analysis of the activation by Mg^{2+} of myosin B ATPase (Nihei, Morris & Jacobson, 1966). Then, to examine the

Fig. 1. Relative activity as a function of the calculated concentration of free Ca^{2+} . ATPase: $\circ$, 0.1 mM-ATP; $\bullet$, 1 mM-ATP. PMB-treated ATPase: $\triangle$, 0.1 mM-ATP; $\blacktriangle$, 1.0 mM-ATP. ITPase: $\square$, 0.1 mM-ITP; $\blacksquare$, 1.0 mM-ITP. Protein concentration was adjusted to 0.12 mg./ml. in all cases.

Table 2. K_{app} for the Ca^{2+} and Mg^{2+} complexes with ATP and ITP as determined spectrophotometrically

Determinations were carried out in 0.05 M-KCl-0.05 M-tris-HCl buffer, pH 7.4, at 25°.

Complex	K_{app} . (M^{-1})
CaATP^{2-}	9300 ± 1400
CaITP^{2-}	8800 ± 600
MgATP^{2-}	18900 ± 800
MgITP^{2-}	15400 ± 300

Table 3. Effect of protein concentration on the binding of Ca^{2+} to myosin

A fixed amount of Ca^{2+} (0.3 nmole) was added to each 10 ml. of myosin solution. The assay of Ca^{2+} bound was performed as described in the Methods and Materials section.

Concn. of protein (mg./ml.)	$\frac{[\text{Ca}^{2+} \text{ bound}]}{[\text{Protein}]} \times \frac{1}{[\text{Ca}^{2+} \text{ free}]} \text{ (l.g.}^{-1}\text{)}$
0.15	1.9
0.33	2.1
0.67	2.1
2.12	0.7
2.05	0.7

possibility of a binding reaction between Ca^{2+} and myosin being responsible for the activation, the relative activity was plotted against the concentration of free Ca^{2+} . The free Ca^{2+} was defined as the Ca^{2+} not bound to NTP. To calculate the concentration of free Ca^{2+} , it appeared necessary to determine the apparent association constant,

Fig. 2. Scatchard plot of binding Ca^{2+} by native and NEM-modified myosin. Myosin was modified with 2.0 moles of NEM/10⁵ g. of myosin for 30 min. and the reaction was stopped with a 50-fold excess of 2-mercaptoethanol. $\circ$, Unmodified myosin, no ATP; $\odot$, unmodified myosin, 1 mM-ATP; $\bullet$, NEM-modified myosin, no ATP.

K_{app} , of CaNTP^{2-} under the experimental conditions. This was done by the method of Burton (1959) and the results listed in Table 2 were within the range of values reported by Nanninga (1961), Asai & Morales (1962) and O'Sullivan & Perrin (1964). The relative activity plotted against the free Ca^{2+} concentration (Fig. 1) suggests that a certain Ca^{2+} -binding reaction is responsible for the activation. The binding of Ca^{2+} to myosin was therefore studied by the procedure described in the Methods and Materials section, in an attempt to determine whether this was the reaction responsible for activation.

Since the binding reaction was observed to be hindered at high concentrations of myosin (Table 3), a concentration of 0.67 mg. of myosin/ml. was used. The resultant Scatchard (1949) plot suggested that there are at least two types of binding reactions between Ca^{2+} and myosin (Fig. 2). The first type is a binding reaction that occurs at a site present on each 500 000 atomic mass units of myosin and that has K_{app} . $1.3 \times 10^6 \text{ M}^{-1}$, and the second type is a weak binding, the binding parameters of which cannot be accurately measured owing to the low protein concentration used in this experiment. The results obtained in the presence of ATP indicate a binding similar to that in the absence of ATP. In this case the plot is made by ignoring the formation

of CaATP^{2-} , which is insignificant within the range of Ca^{2+} concentration used. When myosin is modified with NEM, which is equivalent to PMB in its effect on the ATPase activity (Kielley & Bradley, 1956), the tight Ca^{2+} binding is weakened (Fig. 2). This is in agreement with the observation by Kitagawa, Yoshimura & Tonomura (1961) that myosin releases the tightly bound Ca^{2+} and Mg^{2+} when treated with PMB. Despite its decreased capacity to bind Ca^{2+} tightly, the NEM- or PMB-treated myosin retains the Ca^{2+} -activated myosin NTPase.

Although the above results indicate that the Ca^{2+} bound tightly to myosin does not cause the activation, and that the weak Ca^{2+} binding to myosin may be the cause, it remains to be tested whether or not CaNTP^{2-} is hydrolysed by myosin more rapidly than other species of NTP. If one assumes that the activation by Ca^{2+} is due only to the formation of CaNTP^{2-} , and that Michaelis-Menten kinetics are applicable regardless of the species of substrate, an expression of the enzymic activity, v , at a given concentration of Ca^{2+} may be written:

$$\frac{v}{V} = \frac{\frac{k_3}{k'_3} \left(\frac{[\text{NTP}]}{K'_m + \frac{K'_m}{K_m} [\text{CaNTP}] + [\text{NTP}]} \right) + \left(\frac{[\text{CaNTP}]}{K_m + \frac{K_m}{K'_m} [\text{NTP}] + [\text{CaNTP}]} \right)}{\frac{[\text{NTP}_0]}{K'_m + [\text{NTP}_0]}} \quad (1)$$

where V is the velocity when all the substrate molecules are in the form of CaNTP^{2-} , k_3 and k'_3 are the rate constants of the step at which the enzyme-substrate complex forms the products with

Fig. 3. Ratio v/V as a function of $[\text{CaNTP}^{2-}]/[\text{NTP}_0]$. The theoretical curves are, from the top: PMB-modified myosin ATPase, native myosin ITPase and native myosine ATPase. The symbols represent the same results as shown in Fig. 1.

NTP and CaNTP^{2-} as substrates respectively, and K_m and K'_m are the corresponding Michaelis constants. $[\text{NTP}]$, $[\text{CaNTP}]$ and $[\text{NTP}_0]$ denote the concentrations of Ca^{2+} -free NTP, CaNTP^{2-} and total NTP respectively. Fig. 3 shows the plot of v/V versus $[\text{CaNTP}^{2-}]/[\text{NTP}_0]$. There are three curves drawn in Fig. 3, each of which is defined by the values of k_3/k'_3 , K_m and K'_m . In the special case where $K_m = K'_m$, v/V becomes a linear function of $[\text{CaNTP}]/[\text{NTP}_0]$. Small deviations from linearity shown in Fig. 3 are due to slight differences in the Michaelis constants (Table 4). Therefore it may be speculated from the experimental results that the activation by Ca^{2+} is due either to a weak binding of Ca^{2+} , or to a more effective hydrolysis of CaNTP^{2-} , or to both of these.

Inhibition by Mg^{2+} . The NTPase activity as a function of Mg^{2+} concentration added to the reaction mixture shows that the effective concentration of Mg^{2+} for modification of the ATPase is lower than that of Ca^{2+} . If myosin is treated with PMB the inhibitory effect of Mg^{2+} becomes less significant, and when 2.5 moles of PMB are used for the modification of 105g. of myosin the inhibition

by Mg^{2+} becomes unobservable. Plotting the experimental results in the manner described for Fig. 1 gives two curves (Fig. 4): one for the values with 0.1mM-ATP and the other for those with 1.0mM-ATP. The curves appear to represent a binding reaction with K_{app} of the order of 10^6M^{-1} , which is much larger than that for the formation of MgATP^{2-} . This suggests that a binding reaction between Mg^{2+} and myosin is responsible for the inhibition by Mg^{2+} . In fact, an experiment revealed that the amount of Ca^{2+} bound to myosin decreases when Mg^{2+} is added to a mixture containing myosin and $^{45}\text{Ca}^{2+}$. Assuming competition between Ca^{2+} and Mg^{2+} for the site on myosin, the value of K_{app} for the binding of Mg^{2+} to myosin can be estimated as 2.1×10^5 – $3.0 \times 10^5 \text{M}^{-1}$ (Table 5), which is in

Table 4. *Michaelis constants for NTPases under various conditions*

K_m values were obtained by double-reciprocal plots of the kinetic data.

	K_m (M)	K'_m (M)
Unmodified ATPase	6×10^{-5}	4×10^{-5}
Unmodified ITPase	1.5×10^{-4}	1.5×10^{-4}
PMB-modified ATPase	1.1×10^{-4}	9×10^{-5}

reasonable agreement with the value of 10^6M^{-1} derived from the kinetic data. Under the experimental conditions the total Ca^{2+} concentrations are so low that the calculated amount of Ca^{2+} reacting with the weak binding sites of myosin or forming CaATP^{2-} is found to be negligibly small. With 1mM-Mg^{2+} a low value of K_{app} is obtained, presumably because of secondary effects of the bound Mg^{2+} on myosin. Fig. 5 shows that the relative activity follows the amount of binding of Mg^{2+} to myosin, and this is calculated by using K_{app} values 10^6M^{-1} for the Mg^{2+} -myosin binding and $1.89 \times 10^4 \text{M}^{-1}$ for the formation of MgATP^{2-} . On the other hand the MgATP^{2-} complex may be as good a substrate as ATP^{4-} or HATP^{3-} , since even when essentially all the ATP molecules are in the form of MgATP^{2-} the PMB-modified myosin does not show significant inhibition by Mg^{2+} of the ATPase.

Enzymic activity in the presence of both Ca^{2+} and Mg^{2+} . It was suggested above that Ca^{2+} affects the NTPase by a weak binding of Ca^{2+} to myosin or by the formation of CaATP^{2-} or by both, whereas Mg^{2+} inhibits the NTPase by binding tightly to

myosin. If so, Mg^{2+} should inhibit the Ca^{2+} -activated NTPase without causing a significant change in the amount of Ca^{2+} bound weakly to myosin or to NTP. Fig. 6 shows the effect of Mg^{2+} on the ATPase and ITPase activities in the presence of a fixed amount of Ca^{2+} . In Fig. 7 the relative activity as defined in Fig. 1 is plotted against the amount of added Mg^{2+} . The results in Fig. 7 suggest that the inhibition is not caused by a decrease in the amount of CaNTP^{2-} or by the formation of MgNTP^{2-} . As the Ca^{2+} concentration is increased from 0.032mM to 1mM , the amount of Mg^{2+} required to inhibit the enzymic activity also increases. This is interpreted as being due to the competition between Ca^{2+} and Mg^{2+} for the binding site in myosin, but not for complex-formation with nucleotides, since the calculated changes in amounts of cation-nucleotide complexes do not follow the relationship between enzymic activity and the Mg^{2+} concentration. For instance, in the presence of 1mM-NTP and 1mM-Ca^{2+} , the addition of 0.1mM-Mg^{2+} causes 90% inhibition of the relative activity, whereas the decrease in CaNTP^{2-} concentration is less than 30%, and the formation of MgNTP^{2-} amounts to only 7–8% of the total nucleotide present.

Although the ITPase and the PMB-modified ATPase are not inhibited by Mg^{2+} , they become susceptible to the Mg^{2+} effect if activated first by

Fig. 4. Relative activity of unmodified myosin as a function of the concentration of added Mg^{2+} . ATPase: O, 0.1mM-ATP ; ●, 1.0mM-ATP . Protein concentration was 0.33mg./ml. of reaction mixture.

Fig. 5. Relative activity of untreated myosin as a function of the calculated concentration of free Mg^{2+} . The symbols are the same as in Fig. 4. The curve drawn through the experimental points represents binding of Mg^{2+} to myosin and the continuous curve to the right depicts formation of MgATP^{2-} .

Table 5. K_{app} for binding of Mg^{2+} to myosin calculated from the Ca^{2+} -binding reaction

Determinations were carried out in $0.05 \text{M-KCl-0.05M-tris-HCl}$ buffer, pH 7.4, with 1mM-ATP at 25° .

$10^8 \times [\text{Ca}^{2+} \text{ added}] (\text{M})$	$10^4 \times [\text{Mg}^{2+} \text{ added}] (\text{M})$	$10^8 \times [\text{Ca}^{2+} \text{ bound}] (\text{M})$	$10^{-5} \times K_{\text{app}}$ for binding of $\text{Mg}^{2+} (\text{M}^{-1})$
3.0	—	2.4	
3.0	0.1	2.0	2.2
3.0	1.0	1.0	3.0
3.0	3.0	0.5	2.1
3.0	10.0	0.2	0.59

Fig. 6. Enzymic activities in the presence of Ca^{2+} and Mg^{2+} . The NTP concentration was 0.1 mM in all cases. (a) ATPase; (b) ITPase; (c) modified myosin ATPase ($2.5 \text{ moles of PMB}/10^5 \text{ g.}$): $\circ$, no added CaCl_2 ; $\bullet$, $3.2 \times 10^{-5} \text{ M}$ added CaCl_2 ; $\triangle$, $1.0 \times 10^{-3} \text{ M}$ added CaCl_2 . The protein concentration was 0.12 mg./ml. of reaction mixture in the absence of CaCl_2 and 0.33 mg./ml. when CaCl_2 was added.

Fig. 7. Relative activity (calculated from the results shown in Fig. 6) as a function of the amount of added Mg^{2+} . The circles indicate the value with $3.2 \times 10^{-5} \text{ M}$ added Ca^{2+} , and the triangles with $1.0 \times 10^{-3} \text{ M}$ added Ca^{2+} . Untreated myosin: $\circ$, 0.1 mM-ATP ; $\odot$, 0.1 mM-ITP ; $\triangle$, 0.1 mM-ATP ; $\blacktriangle$, 0.1 mM-ITP . Modified myosin ($2.5 \text{ moles of PMB}/10^5 \text{ g.}$): $\bullet$, 1.0 mM-ATP .

Ca^{2+} (Figs. 6b and 6c). The relative activity of ITPase plotted against added Mg^{2+} coincides with the results obtained with unmodified ATPase (Fig. 7). However, the PMB-modified ATPase shows some resistance to the inhibitory effect of Mg^{2+} , suggesting a weakened binding of Mg^{2+} to the PMB-modified myosin.

DISCUSSION

The experiments described in the present paper were planned to clarify the similarities and differences between the effects of Ca^{2+} and Mg^{2+} on the

myosin NTPase reaction. For this reason, it was desirable to eliminate the effect of univalent ions, such as K^+ and Cl^- . However, variation of the potassium chloride concentration between 5 and 100 mM did not alter the characteristics of effects of bivalent cations on the enzymic activity of myosin.

It has been an open question whether Ca^{2+} or Mg^{2+} or both bind tightly to myosin. The experimental results reported in this work suggest that both Ca^{2+} and Mg^{2+} can bind tightly to myosin, although only the binding of Mg^{2+} shows a direct influence on the enzymic activity.

It is difficult to determine whether the activation by Ca^{2+} is due to the formation of CaNTP^{2-} or to the weak binding of Ca^{2+} to myosin. Luchi & Kritcher (1967) observed that there are 8 Ca^{2+} -binding sites/ $500\,000$ atomic mass units of myosin, with $K_{\text{app}} \cdot 1.8 \times 10^4 \text{ M}^{-1}$. This type of binding is shown in Fig. 2 as the weak binding reaction, which in our experiments was not extensively studied. If we compare the results obtained for formation of CaNTP^{2-} with the K_{app} value obtained by Luchi & Kritcher (1967) for the weak Ca^{2+} -myosin binding, the difference between them is too small to decide which value is likely to best fit the kinetic data. This was observed earlier by Nanninga (1959) in his kinetic experiments. The fact that, in the presence of Ca^{2+} , ITPase and PMB-modified myosin ATPase become liable to the effect of Mg^{2+} suggests that the tight binding of Ca^{2+} is 'indirectly' involved in the activation, so that when the site for this tight binding of Ca^{2+} is occupied by Mg^{2+} the activation does not occur.

If the inhibitory effect of Mg^{2+} is assumed to be caused by MgATP^{2-} , which is a potent inhibitor of the ATPase and has a high affinity for myosin, it

is necessary to introduce an unrealistic value of the association constant for the binding of MgATP^{2-} to myosin, since the results in Fig. 4 indicate that when the concentration of MgATP^{2-} reaches 10^{-7}M the relative activity shows a 50% inhibition. Therefore the binding of Mg^{2+} to myosin appears most likely to cause the inhibition (see Blum & Felauer, 1959). The activation by Mg^{2+} of myosin B ATPase is also caused by the binding of Mg^{2+} to the protein with a K_{app} value similar to that estimated for the Mg^{2+} -myosin binding (Nihei *et al.* 1966; Nihei, 1967). The fact that there is a remaining ATPase activity of myosin at increased concentrations of Mg^{2+} (Fig. 6) may be related to the Mg^{2+} -activated myosin B ATPase.

With regard to the effect of Mg^{2+} on the activity of myosin treated with a thiol reagent, the observations reported here are in general agreement with those reported by Blum & Felauer (1959), Morales & Hotta (1960) and Perry & Cotterill (1965). Apparently the modification of the thiol groups of myosin is accompanied by certain structural changes, which in turn alter the enzymic characteristics such as the sensitivity toward the inhibitory effects of univalent ions (Warren, Stowring & Morales, 1966) and Mg^{2+} . The experiments with substrates of different chemical structure also illustrate that the enzyme activity is related to protein structure: the inhibition by Mg^{2+} is not observed with the ITPase unless it is assayed in the presence of Ca^{2+} , and the hydrolysis of ribose triphosphate has been shown to be activated by Mg^{2+} (M. F. Morales, personal communication).

The question posed at the outset of this work is how Ca^{2+} and Mg^{2+} exert qualitatively different effects on the enzymic activity of myosin. The experiments described here have explored three possible binding reactions that may influence the activity: two types of cation-myosin binding reactions and the complex-formation between a nucleotide and a cation. Apparently the inhibition by Mg^{2+} is due to the tight binding to myosin, whereas the activation by Ca^{2+} is caused by either the weak binding to myosin or CaATP^{2-} , or both.

The authors thank Professor M. F. Morales for his valuable communication. This work was supported by grants from the Medical Research Council of Canada, Life Insurance Medical Research Fund and the Muscular Dystrophy Association of Canada. E.A.S. is a recipient of a pre-doctoral fellowship from the Muscular Dystrophy Association of Canada.

REFERENCES

- Asai, H. & Morales, M. F. (1962). *Arch. Biochem. Biophys.* **99**, 383.
- Blum, J. J. & Felauer, E. (1959). *Arch. Biochem. Biophys.* **81**, 285.
- Burton, K. (1959). *Biochem. J.* **71**, 388.
- Kepler, B. L. & Hercules, D. M. (1963). *Analyt. Chem.* **35**, 1238.
- Kielley, W. W. & Bradley, L. B. (1956). *J. biol. Chem.* **218**, 653.
- Kitagawa, S., Yoshimura, J. & Tonomura, Y. (1961). *J. biol. Chem.* **236**, 902.
- Lecocq, J. & Inesi, G. (1966). *Analyt. Biochem.* **15**, 160.
- Lowenstein, J. M. (1960). *Biochem. J.* **75**, 269.
- Luchi, R. J. & Kritcher, E. M. (1967). *J. Pharmacol.* **158**, 540.
- Mattocks, P. W., jun., Keswani, G. B. & Dowben, R. M. (1967). *Biochemistry*, **6**, 3751.
- Morales, M. F. & Hotta, K. (1960). *J. biol. Chem.* **235**, 1979.
- Mori, K. (1959). *Arch. Biochem. Biophys.* **83**, 552.
- Nanninga, L. B. (1957). *J. phys. Chem.* **61**, 1144.
- Nanninga, L. B. (1959). *Biochim. biophys. Acta*, **36**, 191.
- Nanninga, L. B. (1961). *Biochim. biophys. Acta*, **54**, 330.
- Nihei, T. (1967). *Nature, Lond.*, **215**, 886.
- Nihei, T., Morris, M. & Jacobson, A. L. (1966). *Arch. Biochem. Biophys.* **113**, 45.
- Offer, G. W. (1964). *Biochim. biophys. Acta*, **89**, 566.
- Perry, S. V. & Cotterill, J. (1965). *Biochem. J.* **96**, 224.
- Phillips, R. E. (1964). *Manual of Fluorometric Clinical Procedures*, p. 23. Palo Alto, Calif.: G. K. Turner Associates.
- O'Sullivan, J. S. & Perrin, D. D. (1964). *Biochemistry*, **3**, 18.
- Scatchard, G. (1949). *Ann. N.Y. Acad. Sci.* **51**, 660.
- Sekine, T. & Kielley, W. W. (1964). *Biochim. biophys. Acta*, **81**, 336.
- Sreter, F. A., Seidel, J. C. & Gergely, J. (1966). *J. biol. Chem.* **241**, 5772.
- Tonomura, Y., Appel, P. & Morales, M. F. (1966). *Biochemistry*, **5**, 515.
- Warren, J. C., Stowring, L. & Morales, M. F. (1966). *J. biol. Chem.* **241**, 309.