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Effect of Bivalent Cations on the Adenosine Triphosphatase of Actomyosin and its Modification by Tropomyosin and Troponin

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1. After removal of tropomyosin and troponin from the 'natural' actomyosin complex, the adenosine triphosphatase activity of the resulting 'desensitized' actomyosin is stimulated to the same extent by various bivalent cations with an ionic radius in the range 0·65–0·99 Å when tested at optimum concentration of the metal ion in the presence of 2·5 mM-ATP at low ionic strength and pH 7·6. Under identical conditions the adenosine triphosphatase activity of myosin alone is stimulated to an appreciable extent only by Ca^{2+} (ionic radius 0·99 Å). 2. Tropomyosin narrows the range of size of the stimulatory cations by inhibiting specifically the adenosine triphosphatase activity of 'desensitized' actomyosin when stimulated by Ca^{2+} or the slightly smaller Cd^{2+} (ionic radius 0·97 Å). Tropomyosin has no effect on the adenosine triphosphatase activity of 'desensitized' actomyosin when stimulated by the smaller cations, nor on the Ca^{2+}-activated adenosine triphosphatase activity of myosin alone. 3. The adenosine triphosphatase activity of the 'natural' actomyosin system (containing tropomyosin and troponin) stimulated by the smallest cation, Mg^{2+} (ionic radius 0·65 Å), is low when the system is deprived of Ca^{2+} but high in the presence of small amounts of Ca^{2+}. This sensitivity to Ca^{2+} seems to be a unique feature of the Mg^{2+}-stimulated system. 4. The changes in specificity of the myosin adenosine triphosphatase activity in its requirement for bivalent cations caused by interaction with actin, tropomyosin and troponin primarily concern the size of the metal ions. The effects on enzymic properties of myofibrils due to tropomyosin and troponin can be demonstrated at low and at physiological ionic strength.

The effects of Mg^{2+} and Ca^{2+} on the ATPase† activity of actomyosin reflect their specific role in the muscular contraction–relaxation cycle. The high affinity of actomyosin for Mg^{2+} and Ca^{2+} has been predicted by Morales & Botts (1952) and Bozler (1955). Nihei, Morris & Jacobson (1966) suggested that Mg^{2+} and Ca^{2+} modify the enzymic activity of NAM, as it is extracted from muscle, by competing for the same binding sites according to their respective concentrations and to a degree depending on ionic strength. Zn^{2+} was shown to be able to replace Mg^{2+} in stimulating the ATPase activity of myofibrils (Dransfeld & Greeff, 1964) and of NAM (Carvalho & Avivi, 1966). The effect of other bivalent cations has only been studied occasionally, usually in the additional presence of Mg^{2+} or Ca^{2+} (Seidel & Gergely, 1963; Mühlrad, Kovács & Hegyi, 1965).

We have tested the rate of hydrolysis of ATP at low ionic strength by different actomyosin systems in the presence of various bivalent cations; in order of increasing ionic size these were Be^{2+}, Mg^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+}, Cd^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+}.

Studies on the enzymic properties of actomyosin require the preparations to be well defined. NAM still contains minor protein components besides myosin and actin, and its enzymic properties closely resemble those of washed myofibrils. We have also examined myofibrils in which the actin, myosin and other minor protein components are preserved in their original state. During the preparation of DAM some of these additional proteins, e.g. tropomyosin and troponin, are removed and the resulting Mg^{2+}-stimulated ATPase activity is no longer sensitive to Ca^{2+}-chelating agents such as EGTA (Schaub, Hartshorne & Perry, 1967*a*,*b*). In the present paper we describe a procedure which

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† Abbreviations: ATPase, adenosine triphosphatase; NAM and DAM, 'natural' and 'desensitized' actomyosin respectively; EGTA, ethylenedioxybis(ethyleneamino)-tetra-acetic acid.

destroys the EGTA-sensitivity of the Mg^{2+}-stimulated actomyosin ATPase activity of myofibrils without removing other proteins that may be involved in the regulation of the muscle contractility.

The results obtained with the different actomyosin systems and with myosin alone indicate that the effects of bivalent cations in stimulating the ATPase at low ionic strength depend on their ionic size. The range of size of bivalent cations that can stimulate the ATPase becomes larger when tropomyosin and troponin are removed from the actomyosin complex. These regulatory proteins do not change the enzymic properties of myosin, but clearly modify those of the actomyosin complex.

METHODS

Desensitized myofibrils. Myofibrils were prepared as described by Perry & Zydowo (1959) from the back and upper-hindleg muscles of adult Wistar rats. Finally the myofibrils were washed twice with 10mM-tris–HCl buffer, pH7·3, and suspended in about 10vol. of this buffer. To render the Mg^{2+}-stimulated myofibrillar ATPase activity insensitive to Ca^{2+}-chelating agents myofibrils, at a concentration of 5–10mg. of protein/ml., were dialysed for 3–4 days against 50vol. of 10mM-tris–HCl buffer, pH7·3, containing EGTA (0·5mM) and $MgCl_2$ (0·2mM). The dialysis fluid was changed daily. Finally the samples were dialysed overnight against 50vol. of buffer solution only to decrease the concentration of EGTA, and subsequently used for enzymic assays. Addition of 30–100mM-KCl to the dialysis medium had no effect, but the inclusion of 0·2mM-$MgCl_2$ facilitated the desensitization; in the absence of $MgCl_2$ the myofibrils had to be dialysed for 1–2 days longer. Incorporation of 0·5mM-dithiothreitol during the dialysis prevented desensitization of the myofibrils.

Actomyosin. NAM was extracted from myofibrils by the method of Perry & Corsi (1958) and when required was converted into DAM as described by Schaub *et al.* (1967*a*).

Myosin. Myosin was prepared by the standard procedure described by Perry (1955).

Tropomyosin. Tropomyosin B was prepared by the method of Bailey (1948).

Sarcoplasmic reticulum. Fragmented sarcoplasmic reticulum was isolated by the procedure of Martonosi & Feretos (1964) and its relaxing activity on myofibrils was tested essentially as described by Baird & Perry (1960).

Determination of protein concentrations. The protein content of the different preparations was determined as a routine by the biuret reaction, which was standardized by ultramicro-Kjeldahl estimations of N (Strauch, 1965).

Assay of ATPase activity. This was carried out as described by Perry (1960), with 0·3–0·6mg. of protein in 2ml. at 25°. The incubation system, complete except for ATP, was equilibrated for 5min. at 25° and the reaction started by the addition of ATP. ATPase activities were determined in 2·5mM-tris ATP–25mM-tris–HCl buffer, pH7·6, containing $MgCl_2$ or $CaCl_2$ (2·5mM). Other stimulatory bivalent cations were used as indicated in the text. When incubations were carried out at different pH values 50mM-tris–maleate buffer solutions of appropriate pH were used. For determination of the EGTA-sensitivity the Mg^{2+}-stimulated ATPase activity was measured in the presence and absence of 0·5mM-EGTA. The Mg^{2+}-stimulated ATPase activity of myofibrils that had been dialysed against EGTA-containing media was measured in the presence of 2·5mM-$MgCl_2$ plus 0·05mM-$CaCl_2$. Specific activities are expressed as μg. of inorganic P released/mg. of protein in 5min.

Reagents. Disodium ATP (Fluka A.-G., Buchs, Switzerland) was converted into tris ATP by the method of Schwartz, Bachelard & McIlwain (1962). EGTA was purchased from J. R. Geigy A.-G. (Basle, Switzerland). All other reagents were of analytical grade. Water was passed through an ion-exchange column [Amberlite IR-120 (H^+ form)–Amberlite IRA-410 (OH^- form) (1:2, w/w)].

RESULTS

Characterization of enzyme preparations. All results reported were obtained from preparations made from rat skeletal muscle. Table 1 summarizes the Mg^{2+}- and Ca^{2+}-stimulated ATPase activities at low ionic strength of preparations of myofibrils, NAM and DAM. Many experiments were also done on preparations from rabbit skeletal muscle. Virtually no differences were observed except that the inhibitory effect of EGTA on the Mg^{2+}-stimulated ATPase activity of NAM from rat muscle was consistently lower (Table 1) than that on the Mg^{2+}-stimulated ATPase activity of NAM from rabbit muscle (about 60–70% inhibition). In both cases, however, the preparations of NAM were washed with 30–50mM-potassium chloride or tris

Table 1. *ATPase activities of myofibrils, NAM and DAM from rat muscle*

ATPase activities were measured as indicated in the Methods section. Results are expressed as means ± S.D. of values for four preparations.

	Mg^{2+}-stimulated ATPase activity (μg. of P/mg./5min.)	Inhibition of Mg^{2+}-stimulated ATPase by 0·5mM-EGTA (%)	Ca^{2+}-stimulated ATPase activity (μg. of P/mg./5min.)	Mg^{2+}-stimulated/Ca^{2+}-stimulated ATPase activity ratio
Myofibrils	69·8 ± 5·4	65·5 ± 7·0	26·2 ± 6·9	2·79 ± 0·75
NAM	68·4 ± 4·7	37·0 ± 8·4	27·1 ± 7·0	2·63 ± 0·54
DAM	64·3 ± 9·8	0	82·9 ± 18·5	0·79 ± 0·12

Table 2. *ATPase activities of desensitized myofibrils and of myofibrils protected by dithiothreitol*

Myofibrils were dialysed against 0·5mM-EGTA (causing desensitization) or against 0·5mM-EGTA–0·5mM-dithiothreitol for 3–4 days, and subsequently assayed for ATPase activities as described in the Methods section. The Mg^{2+}-stimulated ATPase activity was measured in the presence of 0·05mM-$CaCl_2$.

	Dialysed against EGTA			Dialysed against EGTA–dithiothreitol		
Preparation no.	Mg^{2+}-stimulated ATPase activity (μg. of P/mg./5min.)	Inhibition of Mg^{2+}-stimulated ATPase by 0·5mM-EGTA (%)	Ca^{2+}-stimulated ATPase activity (μg. of P/mg./5min.)	Mg^{2+}-stimulated ATPase activity (μg. of P/mg./5min.)	Inhibition of Mg^{2+}-stimulated ATPase by 0·5mM-EGTA (%)	Ca^{2+}-stimulated ATPase activity (μg. of P/mg./5min.)
1	70·0	0	31·0	64·5	42	34·1
2	82·9	6	38·4	74·0	45	42·0
3	67·3	0	29·9	61·9	42	38·4
4	101·3	5	21·7	77·1	63	44·4

buffer solutions, under which conditions the EGTA-sensitizing factor remains bound to the actomyosin of rabbit skeletal muscle (Schaub *et al.* 1967*a*).

The EGTA-sensitizing factor could not be removed by washing myofibrils at low ionic strength as was reported for preparations of NAM (Perry, Davies & Hayter, 1966; Schaub *et al.* 1967*a*), neither could myofibrils be desensitized by trypsin treatment (Maruyama & Nagashima, 1967) or by heating at 50° (Ermini & Schaub, 1968) without considerable loss of the Mg^{2+}-stimulated ATPase activity. However, dialysis of myofibrils against EGTA at neutral pH for 3–4 days abolished the EGTA-sensitivity and did not change the ATPase activities (stimulated by Mg^{2+} or Ca^{2+}) at low ionic strength (Table 2). The EGTA-sensitivity was irreversibly lost and could not be restored by subsequent addition of dithiothreitol, although if the latter reagent was present throughout dialysis the EGTA-sensitivity was preserved. When myofibrils were dialysed against buffer solutions of various ionic strengths in the absence of both EGTA and dithiothreitol, their Mg^{2+}-stimulated ATPase activity was lowered invariably to about one-third or less, but was still slightly EGTA-sensitive.

The fact that the Ca^{2+}-stimulated ATPase activity of 'desensitized' myofibrils remained low (Table 2) indicates that tropomyosin was not removed as it is during the washing procedure for preparing DAM. The fact that thiols prevented the desensitization of myofibrils caused by dialysis against EGTA also suggests that the EGTA-sensitizing factor, troponin, was not removed by the latter procedure.

Stimulation of different enzyme preparations by bivalent cations at low ionic strength. The different preparations were tested for their ATPase activity under standard assay conditions at low ionic strength, with 2·5mM-ATP, as described in the Methods section. The stimulatory cations were added in a wide range of concentrations. The concentrations that gave optimum activity varied considerably from one cation to another, but for a given cation (except for Ca^{2+}) very similar concentrations brought about optimum stimulation of the ATPase activity of the different preparations, i.e. myofibrils, 'desensitized' myofibrils, NAM and DAM (Table 3). Ca^{2+} fully stimulated the ATPase activity of DAM and myosin at a concentration of 2·5–3mM, although eight- to ten-fold higher concentrations were needed to give optimum stimulation of the ATPase activity of myofibrils and NAM. Further, the ATPase activity of all preparations did not show a distinct optimum when stimulated by Ca^{2+}, but remained high at concentrations up to 20–30mM. All Ca^{2+}-stimulated ATPase activities were tested as a routine with 2·5mM-calcium chloride under standard conditions.

The results shown in Fig. 1 reveal that only bivalent cations with an ionic radius of about

Table 3. *Ionic radii and optimum concentrations of cations stimulating the ATPase of the actomyosin system*

ATPase activities were measured as indicated in the Methods section in the presence of 2·5mM-ATP on myofibrils, desensitized myofibrils, NAM and DAM. Ionic radii are quoted from Pauling (1960).

Cation	Ionic radius (Å)	Optimum concentration (mM)
Be^{2+}	0·31	No activation; >0·8 inhibition
Mg^{2+}	0·65	2–3
Co^{2+}	0·72	0·5–1
Zn^{2+}	0·74	0·2–0·5
Mn^{2+}	0·80	1·5–3
Cd^{2+}	0·97	0·2–0·5
Ca^{2+}	0·99	>20 on myofibrils and NAM; >2·5 on DAM
Sr^{2+}	1·13	No activation
Ba^{2+}	1·35	No activation

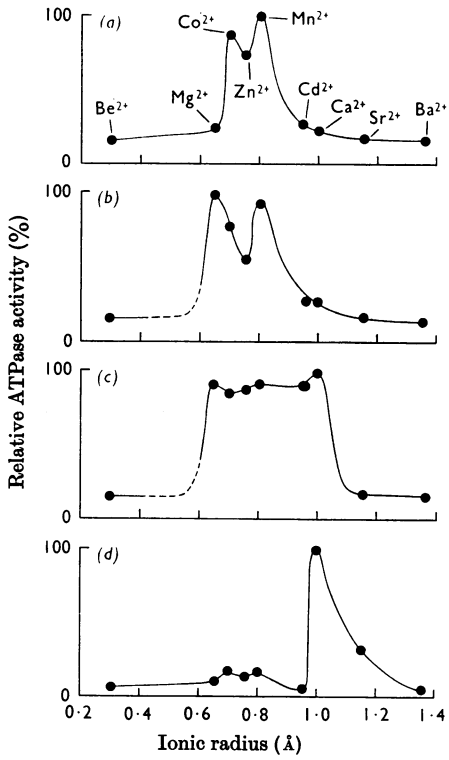

Fig. 1. Stimulation of the ATPase activity of myosin and of different actomyosin systems by various bivalent cations as a function of their ionic radius. Assays were carried out in 2·5mM-tris ATP–25mM-tris–HCl buffer, pH7·6; Mg^{2+}-stimulated actomyosin ATPase activity was measured in the presence of 0·5mM-EGTA; 0·3–0·6mg. of protein was used per assay; metal ions were added in optimum concentration (see Table 3). (*a*) Myofibrils (eight preparations); (*b*) myofibrils desensitized by dialysis against EGTA (four preparations); (*c*) DAM (six preparations); (*d*) myosin (three preparations).

0·7–0·8Å are effective stimulators of the ATPase activity of myofibrils. The curves for preparations of NAM and myofibrils that had been dialysed against EGTA in the presence of dithiothreitol resembled essentially that of myofibrils and are not shown.

The Mg^{2+}-stimulated ATPase activity of all these preparations was dependent on small amounts of Ca^{2+}; the critical concentration of free Ca^{2+} is about 0·1μM (Weber & Winicur, 1961; Portzehl, Caldwell & Rüegg, 1964). Under our test conditions such low concentrations were achieved by chelating contaminating Ca^{2+} with EGTA (0·1mM or higher). In myofibrils that had been dialysed against EGTA and dithiothreitol the contaminating Ca^{2+} concentration was so low that the residual EGTA concentration in the incubation mixture (0·2–0·5μM) was still sufficient to keep the Mg^{2+}-stimulated ATPase activity low, and the latter rose only on the addition of small amounts of Ca^{2+}. The ATPase activity in the presence of Co^{2+}, Zn^{2+} and Mn^{2+} was invariably high and was not influenced either by 0·1mM-EGTA or by addition of Ca^{2+}. Similarly, the Mg^{2+}-stimulated ATPase activity of myofibrils was inhibited by up to 80% by the addition of preparations of sarcoplasmic reticulum; this hardly affected the myofibrillar ATPase activity in the presence of the other stimulatory cations.

In 'desensitized' myofibrils the stimulation pattern was similar, except that the Mg^{2+}-stimulated ATPase activity was not Ca^{2+}-dependent and was consistently higher than the Co^{2+}-stimulated ATPase activity. DAM ATPase activity, however, was stimulated by a wide range of bivalent cations, including Cd^{2+} and Ca^{2+} with ionic radii of 0·97 and 0·99Å respectively (Pauling, 1960). Under optimum conditions all these cations stimulated the ATPase activity of DAM, from which tropo-

Table 4. *Inhibitory effect of tropomyosin on the* Cd^{2+}- *and the* Ca^{2+}-*stimulated ATPase activities of actomyosin during conversion of NAM into DAM*

Standard assay conditions were used. NAM suspended in 50mM-KCl–5mM-tris–HCl buffer, pH7·3, was centrifuged for 20min. at 33000*g*, the supernatant discarded and replaced by water. This washing procedure was repeated until the preparation had become desensitized to EGTA.

Preparation	Mg^{2+}-stimulated ATPase activity (μg. of P/mg./5min.)	Inhibition of Mg^{2+}-stimulated ATPase by 0·5mM-EGTA (%)	Cd^{2+}-stimulated ATPase activity (μg. of P/mg./5min.)	Inhibition of Cd^{2+}-stimulated ATPase by 0·10mg. of tropomyosin (%)	Ca^{2+}-stimulated ATPase activity (μg. of P/mg./5min.)	Inhibition of Ca^{2+}-stimulated ATPase by 0·10mg. of tropomyosin (%)
NAM	73·4	33	32·0	1	31·1	0
Washed 1×	78·8	23	42·7	17	32·5	7
Washed 2×	89·8	19	66·9	19	43·5	29
Washed 3×	93·3	15	78·8	37	72·1	43
Washed 4×	84·0	5	114·0	42	79·4	53
DAM	77·9	0	115·0	40	94·8	60

myosin and troponin had been removed, to about the same extent. The ability to stimulate ATPase activity clearly depends on the ionic size, but is restricted to bivalent cations, since univalent and tervalent cations did not stimulate our enzyme preparations. The ATPase activity of myosin was stimulated by Ca^{2+}, and to a smaller extent by Sr^{2+}; the cations that best stimulated the ATPase activity of NAM were not effective.

Effect of tropomyosin on the stimulation of ATPase activity by different bivalent cations. Table 4 shows the enzymic activities of an actomyosin preparation at each step of the washing procedure that converts NAM into DAM. As the EGTA-sensitivity gradually became diminished the ATPase activity stimulated either by Cd^{2+} or Ca^{2+} increased. The addition of tropomyosin (prepared by the method of Bailey, 1948), which possessed no EGTA-sensitizing activity, increasingly inhibited the Cd^{2+}- and the Ca^{2+}-stimulated ATPase activity of the preparations as the conversion proceeded.

Tropomyosin has been reported (Schaub, Perry & Hartshorne, 1967*c*) to inhibit specifically the Ca^{2+}-stimulated ATPase activity of DAM, whereas that of myosin alone was not affected. Fig. 2 shows that the inhibitory effect of tropomyosin on the Ca^{2+}-stimulated ATPase activity of DAM was most apparent at equimolar concentrations of Ca^{2+} and ATP, an excess of either decreasing the effect. The extent of net inhibition remained the same at equimolar concentrations of Ca^{2+} and ATP from 2mM up to over 10mM, although it became proportionally less owing to the increasing amount of ATP hydrolysed. Tropomyosin inhibited both the Ca^{2+}- and the Cd^{2+}-stimulated ATPase activity best when it was added in about equimolar amount to the myosin present in the actomyosin complex of DAM; addition of more tropomyosin did not increase the inhibitory effect. None of the other cations that stimulated the actomyosin ATPase was affected by tropomyosin.

Effect of ionic strength and pH *on the stimulation of ATPase activity by different cations.* The ATPase activity of DAM decreased with increasing ionic strength in the presence of all stimulatory cations except Ca^{2+}, reaching half its original activity at a concentration of about 60mM-potassium chloride. The Ca^{2+}-stimulated ATPase activity was less sensitive to ionic strength, being decreased only by 50% by 500mM-potassium chloride. In myofibrils

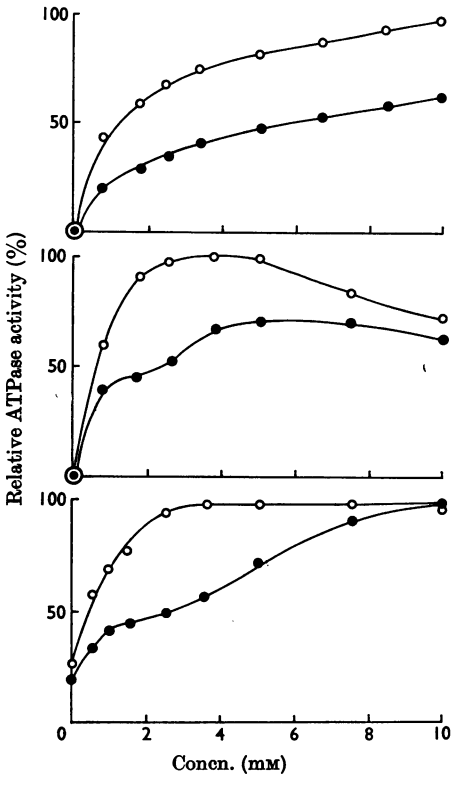

Fig. 2. Inhibitory effect of tropomyosin on the Ca^{2+}-stimulated ATPase activity of DAM as a function of concentration of the ATP and of the cation. Assays were carried out in 25mM-tris–HCl buffer, pH7·6; 0·40mg. of DAM protein was used per assay. ●, With 0·68mg. of tropomyosin; ○, without tropomyosin. (*a*) Concentration of equimolar $CaCl_2$ and ATP varied; (*b*) 2·5mM-$CaCl_2$ and concentration of ATP varied; (*c*) 2·5mM-ATP and concentration of $CaCl_2$ varied.

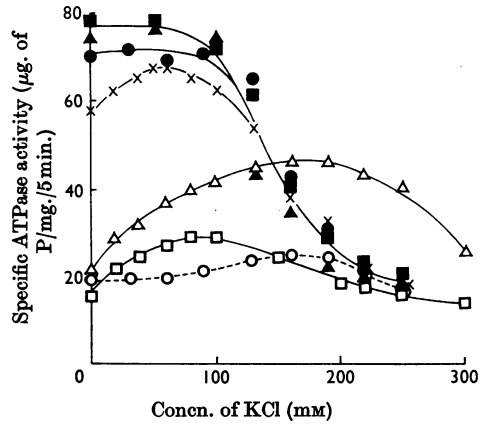

Fig. 3. Effect of KCl concentration on myofibrillar ATPase activity stimulated by different cations. Assays were carried out in 2·5mM-tris ATP–25mM-tris–HCl buffer, pH7·6; 0·53mg. of myofibrillar protein was used per assay. ●, 2·5mM-$MgCl_2$+0·05mM-$CaCl_2$; ○, 2·5mM-$MgCl_2$+0·5mM-EGTA; ▲, 0·5mM-$CoSO_4$; ×, 0·2mM-$ZnCl_2$; ■, 1·5mM-$MnCl_2$; □, 0·25mM-cadmium acetate; △, 2·5mM-$CaCl_2$.

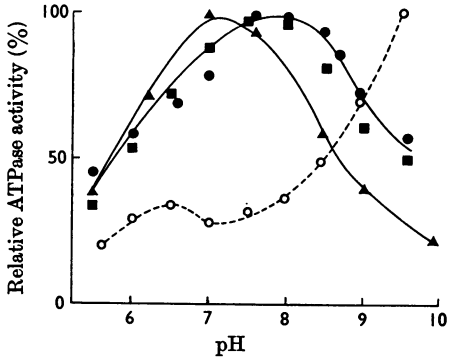

Fig. 4. Effect of pH on the ATPase activity of DAM stimulated by different cations. Assays were carried out in 2·5mM tris ATP–50mM-tris–maleate buffer; 0·3–0·6mg. of DAM protein was used per assay. ■, 2·5mM-$MgCl_2$; ▲, 0·2mM-cadmium acetate; ●, 2·5mM-$CaCl_2$; ○, 2·5mM-$CaCl_2$+500mM-KCl.

the high ATPase activity stimulated by Mg^{2+}, Co^{2+}, Zn^{2+} and Mn^{2+} was more resistant to ionic strength (Fig. 3), and a concentration of about 180mM-potassium chloride was needed to decrease it by half. Fig. 3 also shows the low ATPase activity of myofibrils in the presence of Cd^{2+} and Mg^{2+}+EGTA at different ionic streng ths. The Ca^{2+}-stimulated ATPase activity was optimum at concentrations of potassium chloride at which the ATPase activities stimulated by the other cations were decreasing. It seems likely that at these high ionic strengths the modification of the enzymic centre of myosin by actin becomes abolished and the ATPase of the actomyosin is converted into the myosin type.

This is supported by the pH-dependence of the Ca^{2+}-stimulated ATPase activity of actomyosin preparations at low and at high ionic strengths (Fig. 4). In DAM, where the Ca^{2+}-stimulated ATPase activity at low ionic strength was as high as that stimulated by the other cations, the pH optimum was between pH 7 and 8. In the presence of 500mM-potassium chloride, however, where only Ca^{2+} showed appreciable stimulation, the highest enzyme activity occurred at above pH 9 and had a subsidiary optimum at pH 6·5; this is characteristic of an ATPase of the myosin type.

DISCUSSION

In our experiments on preparations of myofibrils, actomyosin and myosin the greatest differences in ATPase activities, when stimulated by various bivalent cations, occur at low ionic strength. Ca^{2+} is virtually the only cation that stimulates myosin ATPase to an appreciable extent. Through the interaction with actin the enzymically active centre of myosin becomes accessible to stimulation by a wider range of bivalent cations. All cations with an ionic radius in the range 0·65–0·99 Å are as potent as Mg^{2+} as stimulators of the ATPase activity of the purified actomyosin system of DAM, provided that each cation is tested at the optimum concentration and metal ion/ATP ratio. The different optimum concentrations of the various cations that bring about the same maximal rate of hydrolysis of ATP by DAM may reflect the different affinities for the cations of the protein as well as of ATP. The complex of ATP with the metal ion, which is thought to serve as the true substrate (Weber, 1959; Kitagawa & Tonomura, 1960), also differs slightly in its shape according to the ionic size (Melchior, 1954). However, the similar velocity of liberation of inorganic phosphate as well as the similar dependence on pH and on ionic strength indicate that all cations of the appropriate size catalyse the hydrolysis of ATP by the actomyosin type of enzyme (Tonomura, Kitagawa & Yoshimura, 1962).

Nihei & Tonomura (1959), using myosin B, reported a rather different pattern of cation stimulation. However, they added all cations at the same concentration in the presence of a constant amount of ATP, and the measurements were carried out on preparations of myosin B containing actin and other proteins.

The inhibitory effect of tropomyosin on the ATPase activity of DAM in the presence of the two largest stimulatory cations is reflected in the corresponding low activities of NAM and myofibrils. The stoicheiometric ratio of tropomyosin to myosin in DAM at which the optimum inhibitory effect occurs indicates that the former protein modifies the actomyosin complex so that the range of size of the stimulatory cations becomes restricted again. It has in fact been shown that tropomyosin combines with DAM under the conditions of the enzyme assays (Schaub *et al.* 1967*c*). It is unlikely that tropomyosin inhibited the stimulation of DAM by Cd^{2+} or Ca^{2+} by lowering the concentration of the free cations in the reaction mixture, for there were 500–5000ions/mol. of tropomyosin.

Troponin, on the other hand, can modify the actomyosin complex in such a way that the ATPase activity becomes decreased in the presence of the smallest stimulatory cation, namely Mg^{2+}, in the absence of Ca^{2+}. The effect of troponin, however, seems to depend on the presence of tropomyosin (Ebashi & Kodama, 1966; Hartshorne & Mueller, 1967), so that the latter protein also has a role in the EGTA-sensitizing activity. This modification of the actomyosin complex is known to be reversed by small amounts of Ca^{2+}. The sensitivity of the myofibrillar ATPase activity *in vivo* to changes of the concentration of free Ca^{2+} is a unique property

of the Mg^{2+}-stimulated system, for the stimulatory effect of the other cations does not depend on Ca^{2+}.

In myofibrils the different effects of the interaction of the four proteins on the ATPase activity can clearly be seen in the presence of 100–120mM-potassium chloride, which brings the ionic strength of the incubation medium close to the physiological one (about I 0·15). At present the changes in specificity of the cation requirement of myosin ATPase activity that are brought about by its interaction with actin and the other proteins seem to concern the ionic size of the metal ions rather than their chemical properties.

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REFERENCES

Bailey, K. (1948). *Biochem. J.* **43**, 271.
Baird, G. D. & Perry, S. V. (1960). *Biochem. J.* **77**, 262.
Bozler, E. J. (1955). *J. gen. Physiol.* **38**, 735.
Carvalho, A. P. & Avivi, Y. (1966). *Arch. Biochem. Biophys.* **113**, 617.
Dransfeld, H. & Greeff, K. (1964). *Pflüg. Arch. ges. Physiol.* **281**, 365.
Ebashi, S. & Kodama, A. (1966). *J. Biochem., Tokyo*, **60**, 733.
Ermini, M. & Schaub, M. C. (1968). *Hoppe-Seyl. Z.* **349**, 1266.
Hartshorne, D. J. & Mueller, H. (1967). *J. biol. Chem.* **242**, 3089.
Kitagawa, S. & Tonomura, Y. (1960). *J. Res. Inst. Catalysis Univ. Hokkaido*, **8**, 91.
Martonosi, A. & Feretos, R. (1964). *J. biol. Chem.* **239**, 648.
Maruyama, K. & Nagashima, S. (1967). *J. Biochem., Tokyo*, **62**, 392.
Melchior, N. C. (1954). *J. biol. Chem.* **208**, 615.
Morales, M. F. & Botts, J. (1952). *Arch. Biochem. Biophys.* **37**, 282.
Mühlrad, A., Kovács, M. & Hegyi, G. (1965). *Biochim. biophys. Acta*, **107**, 567.
Nihei, T., Morris, M. & Jacobson, A. L. (1966). *Arch. Biochem. Biophys.* **113**, 45.
Nihei, T. & Tonomura, Y. (1959). *J. Biochem., Tokyo*, **46**, 305.
Pauling, L. (1960). *The Nature of the Chemical Bond*, 3rd ed., p. 518. Ithaca: Cornell University Press.
Perry, S. V. (1955). In *Methods in Enzymology*, vol. 2, p. 582. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Perry, S. V. (1960). *Biochem. J.* **74**, 94.
Perry, S. V. & Corsi, A. (1958). *Biochem. J.* **68**, 5.
Perry, S. V., Davies, V. & Hayter, D. (1966). *Biochem. J.* **99**, 1C.
Perry, S. V. & Zydowo, M. (1959). *Biochem. J.* **71**, 220.
Portzehl, H., Caldwell, P. C. & Rüegg, J. C. (1964). *Biochim. biophys. Acta*, **79**, 581.
Schaub, M. C., Hartshorne, D. J. & Perry, S. V. (1967*a*). *Biochem. J.* **104**, 263.
Schaub, M. C., Hartshorne, D. J. & Perry, S. V. (1967*b*). *Nature, Lond.*, **215**, 635.
Schaub, M. C., Perry, S. V. & Hartshorne, D. J. (1967*c*). *Biochem. J.* **105**, 1235.
Schwartz, A., Bachelard, H. S. & McIlwain, H. (1962). *Biochem. J.* **84**, 626.
Seidel, J. C. & Gergely, J. (1963). *J. biol. Chem.* **238**, 3648.
Strauch, L. (1965). *Z. clin. Chem.* **3**, 165.
Tonomura, Y., Kitagawa, S. & Yoshimura, Y. (1962). *J. biol. Chem.* **237**, 3660.
Weber, A. (1959). *J. biol. Chem.* **234**, 2764.
Weber, A. & Winicur, S. (1961). *J. biol. Chem.* **236**, 3198.