THE EFFECT OF MONOVALENT SALTS ON THE ACCELERATION OF MYOSIN B ATPASE BY MAGNESIUM

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Knowledge concerning the effect of magnesium on the adenosine triphosphatase (ATPase) activity of myosin B (made by extracting ground rabbit muscle with 0.6 *M* KCl, 0.04 *M* $NaHCO_3$, and 0.01 *M* Na_2CO_3[1]) is based on the results of many independent studies in which limited attention was paid to the concentration and kind of salt accompanying that of magnesium. Two recent reviews[2,3] state that at low concentrations of KCl ($\leqslant$0.05 *M*) magnesium accelerates ATPase activity while at high concentrations it inhibits activity. Of the citations[4–8] used in support of this statement, BANGA's[4] results show that $MgCl_2$ alone increases the activity and that further addition of KCl has no effect. The results of MOMMAERTS AND SERAIDARIAN[5] show that magnesium strongly inhibits the activity of "actomyosin" in reaction mixtures which contain 0.032 *M* $CaCl_2$, 0.12 *M* KCl and 0.05 *M* NaCl. The results of PERRY's investigation[6] with myofibrils prepared with collagenase clearly demonstrate acceleration by $MgCl_2$ in 0.1 *M* borate solution (pH 6.9) and inhibition in solution containing 0.1 *M* borate and 0.1 *M* KCl. HASSELBACH's results[7] show acceleration by $5 \cdot 10^{-5}$ *M* to $5 \cdot 10^{-3}$ *M* $MgCl_2$ at an ionic strength (μ) of about 0.085 but at 0.6–0.9 μ all concentrations of magnesium inhibit an already reduced activity. BLUM[8] also finds inhibition by $MgCl_2$ at 0.6 *M* KCl.

No single study has been made of the effects of $MgCl_2$ on myosin B ATPase over a range of accompanying salt concentrations. This has been done for the present paper by adding in varying concentration the chlorides of potassium, sodium, ammonium and tris (hydroxymethyl) aminomethane and potassium acetate to reaction mixtures containing magnesium. The effect of KCl on the acceleration caused by $CaCl_2$ is also included. Preparations of myosin B and of glycerol-extracted muscle from many rabbits were used as ATPase.

EXPERIMENTAL

Myosin B was prepared by the method of SZENT-GYÖRGYI[1] except that extraction was carried out by continuous stirring in an ice bath for 5 hours. The mixture was then centrifuged and the supernatant fluid removed. The myosin in this fluid was precipitated by diluting it with 20 volumes of water. After the myosin had settled to minimum volume, it was concentrated to a smaller volume (about $^1/_{40}$ of the volume of the diluted supernatant fluid) by 15 minutes of centrifuging at 800–900 *g*. It was then redissolved in 0.6 *M* KCl and stored at 0°. A second precipitation by this procedure did not alter the activity in a qualitative sense and consequently was not done for all preparations. The concentration of myosin B was taken as 6 times the nitrogen content (Kjeldahl).

Glycerol-extracted psoas muscle was also prepared according to SZENT-GYÖRGYI[1]. Homogenates were prepared by blending the extracted muscles at 14° C and then homogenizing with a

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teflon-glass tissue homogenizer which was immersed in ice water. The homogenates were stored either in water, 0.1 *M* KCl or 0.6 *M* KCl at 0°.

Adenosine triphosphate (ATP) was purchased (Sigma crystalline).

Enzymic (ATPase) activity was measured in solutions containing the ions under consideration 0.001 *M*, 0.002 *M* or 0.003 *M* ATP, and 0.185 to 0.27 mg of myosin B per ml. All reactions were in tris buffer, pH 7.5, the concentration of which was 0.02 *M* except when the effect of concentration of tris was being studied. In one experimental procedure (*e.g.* as for Figs. 1–5) 4 ml of reaction mixture were incubated, with shaking, for 4 min. Then the reaction was stopped by adding 1 ml of 20% trichloroacetic acid (TCA). The precipitated protein was removed and a carefully measured portion of the supernatant was analyzed for phosphate by the Fiske-Subbarow method. Blanks for this procedure were prepared by adding the TCA to one reaction mixture before adding substrate. Reactions carried out by the above procedure were tested for maintenance of zero order reaction for the reaction period of 4 min by dephosphorylations carried out as a function of time. This procedure involved incubation of 25.0 ml of reaction mixture from which samples of 4 ml were removed for analysis at intervals of 2 min. Blanks were prepared by removing one sample before adding substrate. To this was added TCA followed by the proper amount of substrate.

Special standardizations were carried out to insure that the color development of the phosphomolybdate complex of the Fiske-Subbarow method was not influenced by high concentration of $MgCl_2$ or NH_4Cl.

RESULTS

The effect of monovalent salts on the acceleration caused by $MgCl_2$ and $CaCl_2$.*

The effect of magnesium on myosin B ATPase is drastically influenced by the concentration of the monovalent salts* KCl, NaCl, K acetate, tris chloride, and NH_4Cl. The effects, except for NH_4Cl, are similar to each other and can be seen by reference to Figs. 1–4. Probably the outstanding feature is that the effect of magnesium varies according to the concentration of accompanying salt. Low concentrations of $MgCl_2$ (*e.g.* $1.7 \cdot 10^{-5}$ *M*, Fig. 1) augment ATPase activity when accompanied by a low

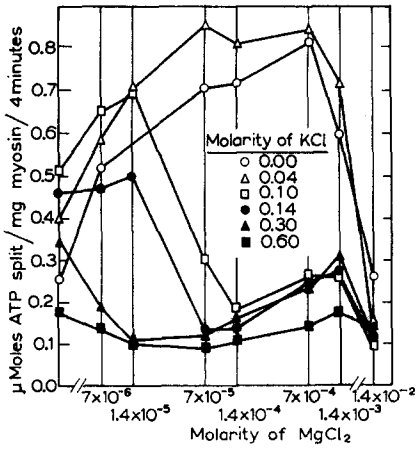

Fig. 1

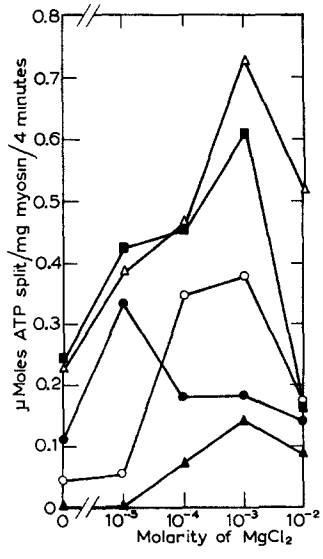

Fig. 2

Fig. 1. The effect of $MgCl_2$ on the dephosphorylation of ATP by myosin B at six concentrations of KCl.
Fig. 2. Ditto Fig. 1 except at five concentrations of NaCl. ○, NaCl absent; △, 0.055 *M*; ■ 0.115 *M*; ●, 0.155 *M*; ▲, 0.30 *M*.

* The term "monovalent salt" applies to the salts such as KCl, NaCl, NH_4Cl, K acetate and tris chloride, all of which have both a monovalent cation and anion. It is abbreviated to Me^+A^-, and $[Me^+A^-]$ means "concentration of monovalent salts".

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[Me+A−], with the exception of NH_4Cl. When accompanied by intermediate [Me+A−], low [$MgCl_2$] augments considerably less and actually inhibits at high [Me+A−]. The effect of increasing the [$MgCl_2$] at any one [Me−A+] also varies with the concentration of Me+A−. At low [Me+A−], especially of KCl (Fig. 1), the addition of magnesium causes two optima of activity, one at 10^{-4} *M* or 10^{-5} *M* and a second at about 10^{-3} *M*. At high [Me+A−], the first optimum disappears and the second persists. At low [Me+A−] the second optimum seems to be superimposed on the acceleration caused by magnesium and then at 0.10 *M* Me+A− is greatly decreased.

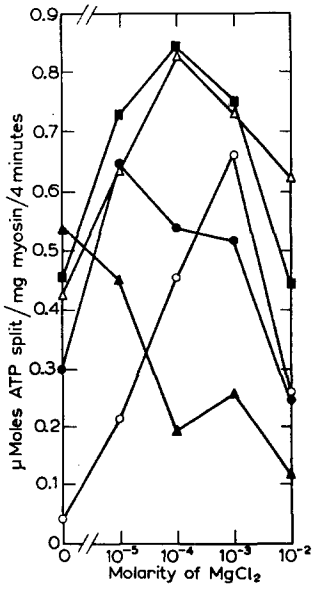

Fig. 3. The effect of $MgCl_2$ on the dephosphorylation of ATP by myosin B at five concentrations of K acetate. ○, K acetate absent; △, 0.055 *M*; ■, 0.115 *M*; ●, 0.155 *M*; ▲, 0.30 *M*.

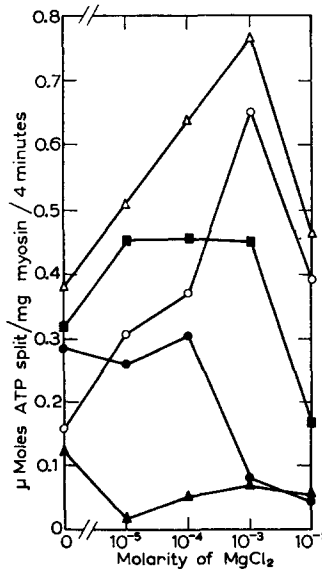

Fig. 4. Ditto Fig. 3 except at five concentrations of tris (hydroxymethyl) amino methane, pH 7.5. ○, 0.02 *M*; △, 0.0675 *M*; ■, 0.125 *M*; ●, 0.20 *M*; ▲, 0.30 *M*.

The existence of two such optimal concentrations has not been noted in earlier studies[4–8] and both optima do not exist in this study for those reaction mixtures containing NH_4Cl or $CaCl_2$. The second rise in activity by $MgCl_2$ at intermediate and high concentrations of KCl has been confirmed by dephosphorylations done as a function of time (Fig. 5). The greatest slope in the time plots, and, therefore, rate of dephosphorylation, was for the reaction done in $1.4 \cdot 10^{-3}$ *M* $MgCl_2$.

The effect of NH_4Cl, as Me+A− (Fig. 6), on the Mg-effect is different from that of the other salts. At 0.055 *M*, increases of $MgCl_2$ at first cause a marked inhibition followed by a small increase in activity, which resembles the increase that occurs in the presence of 0.001 *M* $MgCl_2$ when other Me+A− salts are present. Above 0.055 *M* NH_4Cl, increases of [$MgCl_2$] cause increasingly greater inhibition of the ATPase activity in a manner similar to that which occurs when $CaCl_2$ is put into reaction mixtures.

The optimum concentration of $CaCl_2$ for myosin B ATPase has long been known to be about 10^{-2} *M*. Fig. 7 shows that the optimum concentration is not changed by

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additions of KCl, but the activity of the enzyme in the presence of 0.01 *M* $CaCl_2$ is sharply reduced by the addition of 0.3 *M* KCl. The effect of $CaCl_2$ is different from that of $MgCl_2$ in that low concentrations do not inhibit the activity under any conditions. The strong accelerative effects of $CaCl_2$, however, are sharply inhibited by additions of $MgCl_2$ (Fig. 8). This inhibition demonstrates the strong antagonism between calcium

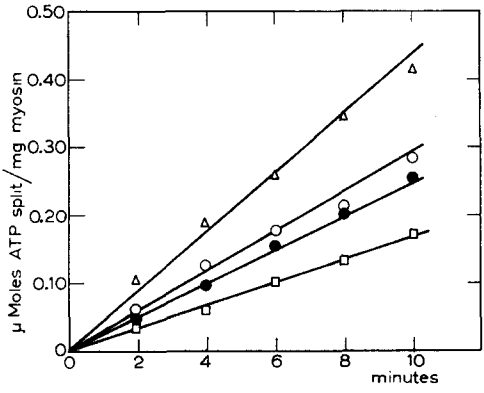

Fig. 5. Fig. 5. The effect of four concentrations of $MgCl_2$ on the dephosphorylation of ATP by myosin B in reactions done as a function of time. ○, $MgCl_2$ absent; □, $1.4 \cdot 10^{-5}$ *M*; △, $1.4 \cdot 10^{-3}$ *M*; ●, $1.4 \cdot 10^{-2}$ *M*.

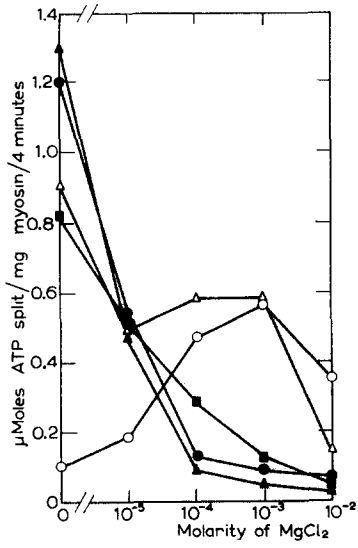

Fig. 6. The effect of $MgCl_2$ on the dephosphorylation of ATP by myosin B at five concentrations of NH_4Cl. ○, NH_4Cl absent; △, 0.055 *M*; ■, 0.115 *M*; ●, 0.155 *M*; ▲, 0.30 *M*.

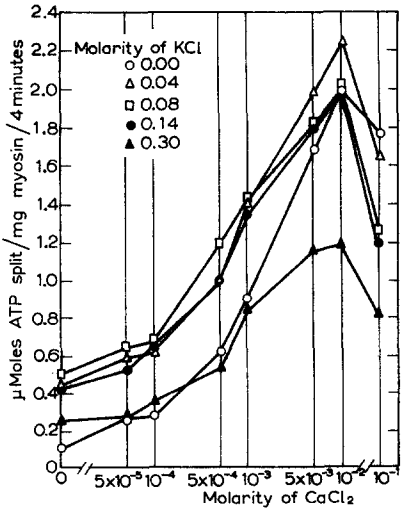

Fig. 7. The effect of $CaCl_2$ on the dephosphorylation of ATP by myosin B at five concentrations of KCl.

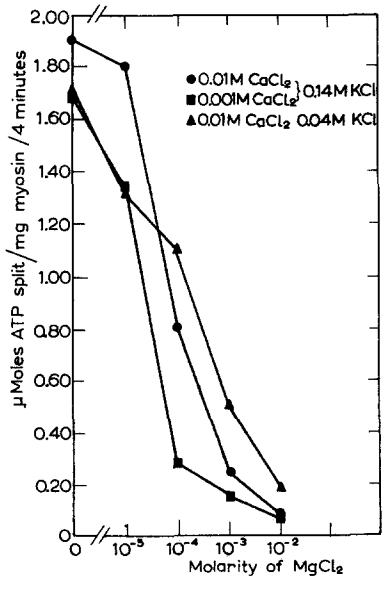

Fig. 8. The effect of additions of $MgCl_2$ upon the augmentation of dephosphorylation by $CaCl_2$.

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and magnesium and affords an example of the accelerative effects of magnesium being reduced at low ionic strength.

Variations from preparation to preparation

The response of activity to $MgCl_2$ differs from that described above for some preparations of myosin B. This is illustrated by comparison of Figs. 1 and 9. All the myosins were prepared by identical procedure except that different rabbits were used. The type of myosin used in the experiments of Fig. 9 occurs infrequently. It is relatively unresponsive to $MgCl_2$ in the absence of KCl and the additions of KCl to the reaction

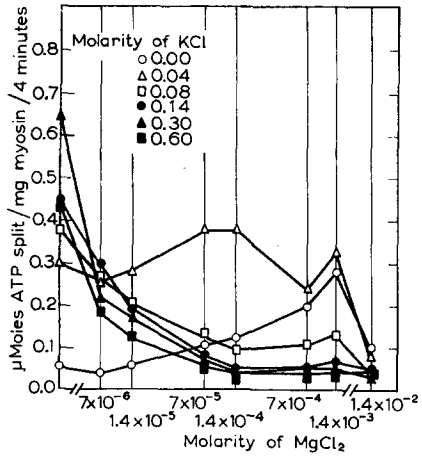

Fig. 9. The effect of $MgCl_2$ on the dephosphorylation of ATP by myosin B of a type of preparation which responds differently to the addition of salt.

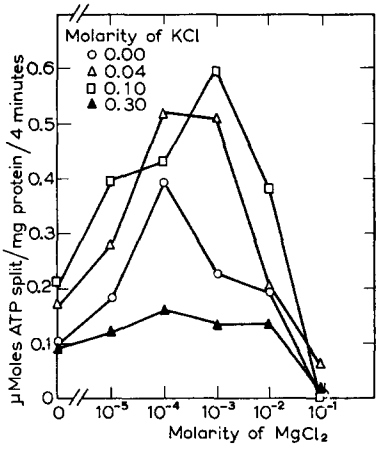

Fig. 10. The effect of $MgCl_2$ at four concentrations of KCl on the dephosphorylation of ATP by homogenized glycerol-extracted psoas muscle.

mixtures affect the response to $MgCl_2$ in a manner similar to that of NH_4Cl (compare Figs. 6 and 9). When KCl only is added to the reaction mixtures containing the unresponsive myosin B the optimum concentration is between 0.2 *M* and 0.3 *M*. The optimum concentration of KCl for other myosins is about 0.1 *M*. Other investigators have reported 0.1 *M* to 0.15 *M*[4,6]. Additional differences between these two kinds of myosin will be given in the DISCUSSION.

Homogenized glycerol-extracted treated psoas fibers

Another form of myosin which acts as ATPase is homogenized glycerol-treated rabbit psoas muscle[10]. When the concentration of KCl is increased in the reaction mixtures of homogenized psoas muscle and ATP, the activity of the ATPase increases to a maximum at about 0.1 *M* KCl and then decreases as the concentration is increased up to 0.6 *M*. This decrease has been noted previously by SARKAR *et al.*[10], but in their experiments low concentrations of KCl were not suboptimal. The activity is increased by additions of magnesium[10] and the magnitude of that effect is influenced by the concentration of KCl (Fig. 10) as it was with myosin B, except that the concentration of $MgCl_2$ which was optimum increased rather than decreased with addition of KCl. The decrease in response to magnesium in the presence of 0.3 *M* KCl is like that obtained by CHAPPELL AND PERRY for non-glycerol-treated fibrils[11].

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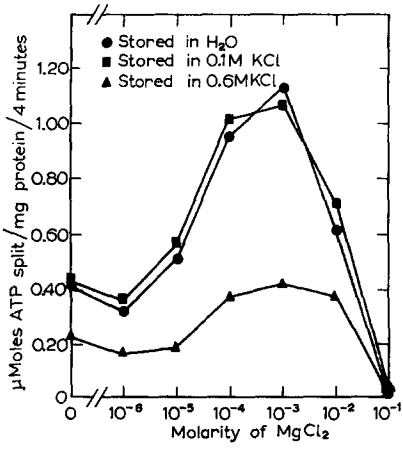

Fig. 11. The influence of storage of homogenized psoas fibers in water *vs.* solutions of KCl on ATPase activity of myosin B. All reactions carried out in 0.1 *M* KCl.

The above results were obtained by the use of fibers which were homogenized and then stored in aqueous suspension at 0°, as was done in a previous study of the ATPase activity of this material[12]. The suggestion has been made[3] that such storage results in deterioration of the myosin which decreases the response to added salts.

Experiments using homogenized fibers, after storage in water and in 0.1 *M* KCl, show that storage in 0.1 *M* KCl has little or no effect (Fig. 11). The results of Fig. 11 indicate no effect of storage in 0.1 *M* KCl but in other experiments psoas homogenate stored in 0.1 *M* KCl was either a little more or a little less active than that stored as aqueous suspension. Storage in 0.6 *M* KCl, however, does decrease the activity of this type of myosin ATPase (Fig. 11). The response to $MgCl_2$ for material stored in either 0.1 *M* or 0.6 *M* KCl is qualitatively the same as for material stored in aqueous suspension; however, it is to be noted that the homogenate used in the experiments of Fig. 11 is of about twice the activity as that of Fig. 10.

For the above experiments homogenates were made of six preparations of glycerol-treated psoas muscle which had been stored in 50% glycerol at —20° for times varying from two weeks to 20 months. The maximal activities produced by additions of $MgCl_2$ were at 10^{-3} *M* $MgCl_2$ for each preparation and varied from 0.4 to 1.3 μM ATP split/mg protein/4 min but there was no definite correlation of the activity with age (*cf.* ref. [13]). One old preparation (16 months) had an activity of 1.1 μM split/mg/4 min.

The effect of concentration of ATP

In the above experiments the concentration of ATP was 0.001 *M* at which concentration the enzyme was saturated and with which 0.001 *M* $MgCl_2$ forms $5.6 \cdot 10^{-4}$ *M* MgH_2ATP (using the association constant of 3000 M^{-1} (SMITH AND ALBERTY[9]) for the formation of complex between magnesium and ATP^{-4} at 0.2 μ). Since the concentration of ATP is known to have some effect on myosin ATPase[7], experiments such as those for Fig. 1 were done with 0.002 *M* and 0.003 *M* ATP at each of 0.04 *M* and 0.14 *M* KCl. Again using 3000 M^{-1} as the association constant[9], 0.001 *M* $MgCl_2$ forms 7.8 and $8.6 \cdot 10^{-4}$ *M* MgH_2ATP with 0.002 *M* and 0.003 *M* ATP. In spite of these large differences in the concentration of MgH_2ATP in the reaction mixtures the activity of the enzyme was the same as it was with 0.001 *M* ATP. The response of the Mg-effect to increased $[Me^+A^-]$ with 0.002 *M* ATP was exactly like that obtained with 0.001 *M* ATP (Fig. 1). With 0.003 *M* ATP the response to 0.14 *M* KCl was like that of 0.3 *M* KCl with 0.001 *M* ATP (Fig. 1).

The effect of NH_4Cl

The effect of NH_4Cl on myosin B ATPase in the absence of other salts is of possible

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significance. In the experiments for Fig. 6 the minimum concentration of NH_4Cl, with no $MgCl_2$ added, was 0.055 *M* and that concentration increased the myosin B ATPase activity about 9-fold over that existing with no NH_4Cl added. In experiments on the effect of varying concentrations of NH_4Cl (no other salt, except 0.02 *M* tris chloride, pH 7.5) 0.015 *M* NH_4Cl increased the activity 5-fold and concentrations comparable to those of Fig. 6 showed the same degree of increase of activity as those shown in Fig. 6.

DISCUSSION

These experiments and results represent a study of the effects of magnesium and of calcium on myosin B ATPase through a wide range of ionic strengths produced by the addition of several salts. The results, in general, uphold the statements by MORALES *et al.*[2] and by PERRY[3], based on several studies, that at low salt concentrations the addition of magnesium increases the activity of myosin B ATPase and at high salt concentrations it decreases the activity; however, our results show that these statements need modifying to include the small, but definite, increases in rate of dephosphorylation caused by 10^{-3} *M* $MgCl_2$ at high $[Me^+A^-]$. Our results show that it is the moderate ($1 \cdot 10^{-4}$ *M*), rather than the high ($1 \cdot 10^{-3}$ *M*), concentrations of $MgCl_2$ which inhibit myosin B ATPase at high $[Me^+A^-]$.

The difference between the effect of potassium, sodium and tris salts and of NH_4Cl shows that the effect of monovalent salts on the Mg-effect is not one of mere ionic strength (μ). The kind of salt has an effect. This is again demonstrated by the inhibitory effect of $CaCl_2$ on the Mg-effect (Fig. 8) regardless of μ in the reaction mixture. Also, the persistence of the effect when K acetate is substituted for KCl and the modification of the effect by NH_4Cl, indicate that the effect of Me^+A^- is due to the cation rather than the anion. Apparently, the size of the cation, as demonstrated by the similarity of the effect of tris chloride, KCl and NaCl, has no effect.

The effects of Me^+A^- and of the divalent cations, calcium and magnesium, on each other represent an intricate balance of synergisms and antagonisms of cations. Low concentrations of magnesium are dominated by potassium while high concentrations of magnesium dominate the inhibitive effects of potassium (Figs. 1 and 3) and there is a reappearance of a favorable effect at 10^{-3} *M* $MgCl_2$. Ammonium ion (Fig. 6) dominates the Mg-effect more completely than K-ion and in the presence of ammonium ion the reappearance of the favorable Mg-effect (Figs. 1, 3) does not occur. Calcium, on the other hand, dominates the effect of potassium regardless of the concentration of the latter (Fig. 7), but the strong augmentative effect of calcium is sharply diminished by magnesium (Fig. 8).

The inhibition of myosin B ATPase by intermediate concentrations of $MgCl_2$ in the presence of high concentrations of such salts as KCl has been explained as the result of dissociation of the myosin B into actin and myosin A ([3], p. 45). $MgCl_2$ depresses the ATPase activity of myosin A[6]. Dissociation of myosin B into these two components occurs, according to SZENT-GYÖRGYI[1], only when the myosin is in solution; hence, the inhibition of myosin B ATPase at high salt concentration. PERRY reported one circumstance (that of 0°C with the proper salt conditions) in which myosin B is insoluble, and therefore not dissociated, and the ATPase activity is depressed by magnesium. The experiments described here with 0.1 *M* NH_4Cl (Fig. 6) and a solution

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of 0.01 *M* $CaCl_2$ – 0.04 *M* KCl (Fig. 8) are another instance in which magnesium inhibits myosin B ATPase without dissociation into myosin A and actin. The basis for stating that myosin B is not dissociated is that extrusions (threads) of myosin B made in 0.1 *M* NH_4Cl, or in 0.01 *M* $CaCl_2$ – 0.04 *M* KCl remain firm and intact, even in the presence of 0.001 *M* ATP. The inhibition rather than the acceleration by magnesium occurs in the presence of NH_4Cl (Fig. 6) and especially in the presence of $CaCl_2$ (Fig. 8). Both kinds of reaction mixtures are of low μ.

The results from the experiments with various concentrations of ATP indicate that the Mg-effect is due to a complex of magnesium with myosin rather than with ATP. Assuming that the plots of this paper can be used as criteria for the binding of cations, inspection of Figs. 1 and 7 suggests that the Mg-effect and the Ca-effect depend upon different types of bonds between cation and protein. A possible explanation* of this phenomenon is that both calcium and magnesium bind to negatively charged groups on the myosin, but that calcium is held by purely electrostatic bonds and that magnesium is held in part by covalent-type bonds as well as by electrostatic bonds.

Fig. 7 shows that the curves relating activity to calcium concentration indicate decreasing activity with increasing [KCl] but that the shape of the curves and the position of the maximum is not altered. This observation is in agreement with the supposition that Ca-ion and K-ion bind to myosin in essentially similar fashion (electrostatic bonding) and that the potassium competes with the calcium for sites on the myosin, thus decreasing the number of sites available to the calcium. Electrostatic bonding also appears to be the predominant bond involved between myosin prepared from glycerol-treated psoas and magnesium (Fig.10).

The effect of $[Me^+A^-]$ on the Mg-myosin system is quite different. Here, there is not only a decrease in activity with increasing [KCl] (Fig. 1) but the shape of the curves changes considerably; the optimum magnesium concentration at low [KCl] is considerably lower than the optimum at high [KCl]; the transition in Fig. 1 occurs between 0.04 *M* and 0.14 *M* KCl. These data can be explained by the postulation of a bond of covalent nature between Mg-ion and myosin at low [KCl] or other salt. At about 0.14 *M* KCl on the other hand, the bonding of covalent nature is greatly reduced and disappears between 0.14 *M* and 0.30 *M* KCl. Also between 0.14 *M* and 0.30 *M* the nature of the bond which does exist appears to be entirely electrostatic thus resembling the bond between Ca-ion and protein, and is affected by the concentration of KCl as is that bond. The fact that the optimum concentration of magnesium for splitting ATP under electrostatic binding conditions is lower than for calcium, can be attributed to the higher charge concentration on the ion with the smaller radius.

The variations in activity of these myosin preparations and their responses to magnesium appear to be similar to the variations of activity reported by BLUM[14] at the Naval Medical Research Institute. Their variations, however, occurred in reaction mixtures containing calcium. In our experiments we found no variation when calcium was included in the reaction mixture. Analyses of our preparations of myosin for magnesium and calcium show that the ratio of magnesium to calcium was considerably higher in those preparations in which the ATPase was accelerated by addi-

* The authors are greatly indebted to Dr. GUNTHER EICHHORN for discussions which led to these postulations of the role of the protein-magnesium and protein-calcium bonds in the splitting of ATP by myosin B.

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tions of magnesium (Fig. 1) than in those which responded only slightly or not at all to magnesium (Fig. 9). Preparations of the first kind, after two precipitations, contained about 5.4 micro-equivalents (μequiv.) of magnesium and 5.0 μequiv. of calcium per g of protein. Preparations of the second type contained about 0.48 μequiv. magnesium and 7.0 to 19.0 μequiv. of calcium per g of protein. Analyses of myosin preparations for these two divalent cations will be extended as myosins of the two types are obtained.

The myosins of preparations which did not respond to additions of magnesium appear to bind magnesium by covalent-type of bond to a smaller extent than do the myosins which respond to magnesium. Binding of magnesium by an electrostatic type of bond appears to be of about the same extent by both types of myosin.

Another difference between these two kinds of myosin was the rapidity with which they settled after precipitation by dilution with 19 volumes of water (final [KCl] = 0.03 M). The proteins of preparations of which the ATPase was augmented by magnesium settled rapidly (30 to 60 min) to a small volume while those of which the ATPase responded only slightly to magnesium required many hours to settle to the same volume.

SUMMARY

The effects of magnesium and of calcium on the ATPase activity of myosin B have been investigated in the presence of a range of concentrations of monovalent salts ($[Me^+A^-]$). At zero or low $[Me^+A^-]$ $MgCl_2$ increases the ATPase activity; at intermediary $[Me^+A^-]$ low concentrations of $MgCl_2$ depress activity while high concentrations of $MgCl_2$ accelerate slightly; at high $[Me^+A^-]$, high concentrations of $MgCl_2$ have a small accelerative tendency. The rise in activity at high $[Me^+A^-]$ and the second rise at intermediate $[Me^+A^-]$ are caused by 10^{-3} M $MgCl_2$ and have not been observed in other investigations. The inhibition caused by magnesium is not clearly due to dissociation of myosin B into actin and myosin A. The effects of salts on the Mg-effect does not appear to be one of ionic strength because the kind of salt has an effect. The response of myosin B ATPase is inconsistent from preparation to preparation. Differences in myosin B preparations are shown. The possibility of covalent and electrostatic bonding of magnesium to myosin is discussed.

The effects of magnesium on the ATPase of glycerol-extracted muscle are not as complex as on that of myosin B.

Ammonium chloride in the absence of other metallic cations increases the activity of myosin B ATPase.

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