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THE UNIVERSITY OF ALBERTA

STUDIES ON THE MYOSIN NUCLEOSIDE TRIPHOSPHATASE

AND THE ACTIN-MYOSIN INTERACTION UNDER THE INFLUENCE OF

CALCIUM AND MAGNESIUM AT LOW CONCENTRATIONS OF POTASSIUM CHLORIDE

by

© EDWARD ARTHUR SUGDEN

A THESIS

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FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Studies on the Myosin Nucleoside Triphosphatase and the Actin-Myosin Interaction under the Influence of Calcium and Magnesium at Low Concentrations of Potassium Chloride" submitted by Edward Arthur Sugden in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

Muscular contraction involves the enzymatic hydrolysis of nucleoside triphosphates by myosin and actomyosin in the presence of the divalent cations Ca^{2+} and Mg^{2+}. The chemical energy thus released is converted into the mechanical energy of contraction. In the present study, the enzymatic activity of myosin and actomyosin and the superprecipitation process have been investigated with the objective of clarifying the roles of the divalent cations and the nature of the actin-myosin interaction in the contractile process.

In the presence of Ca^{2+}, the rate of ATP or ITP hydrolysis by myosin or actomyosin suggests that a relatively weak binding of Ca^{2+} either with the protein or with the substrate molecule is responsible for the activation of the enzymatic activity, or the production of superprecipitated actomyosin particles. 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 daltons of myosin with an apparent association constant, Kapp, of $1.3 \times 10^6 M^{-1}$. However, the second is so weak that its binding parameters cannot be determined by the method used. The first type of Ca^{2+} binding is not observed with N-ethylmaleimide-modified myosin yet this modified myosin shows the Ca^{2+} activation of the adenosine triphosphatase and inosine triphosphatase.

The Mg^{2+} effects on myosin or actomyosin enzymatic activity can be related to a binding reaction of Mg^{2+} to myosin having a

Kapp of $\sim10^6M^{-1}$. A binding of Mg^{2+} occurs at the site in myosin where the Ca^{2+} binds tightly. The value of Kapp for the Mg^{2+} binding calculated by assuming a competition between Ca^{2+} and Mg^{2+} for the same site is $2.1 \times 10^5 - 3.0 \times 10^5 M^{-1}$. A direct measurement of Mg^{2+} binding to myosin shows a Kapp value of $\sim10^6 - 10^7 M^{-1}$. The behaviour of the enzymatic activity of myosin on ATP or ITP in the presence of both Ca^{2+} and Mg^{2+} is consistent with the explanation that the Mg^{2+} inhibition is due to the tight binding of Mg^{2+} to myosin, whereas the Ca^{2+} activation is caused by a weak binding of Ca^{2+} to myosin or by $CaNTP^{2-}$ or by both.

The binding reaction of actin with myosin and its subfragment was studied under the influence of Mg^{2+} and ATP. One actin monomer binds to one subfragment molecule and also to one molecule of myosin. It is explained that if only one of the two sites in myosin react, a second binding would be sterically hindered by the presence of bound actin. Actin binds to myosin with a Kapp of $3.2 \times 10^6 - 5.0 \times 10^6 M^{-1}$ while the maximum Kapp of actin-subfragment is $2.0 \times 10^4 M^{-1}$. The binding experiments and the light scattering analysis on mixtures of actin and myosin suggest that the light meromyosin portion of myosin is necessary for stabilizing the actin-myosin interaction and the superprecipitation of actomyosin.

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LIST OF ABBREVIATIONS

AMP	adenosine 5'-monophosphate
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
DEAE-cellulose	diethylaminoethyl-cellulose
D.T.T.	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycolbis (β-aminoethylether) N,N'-tetraacetic acid
F-actin	fibrous action (polymer of G-actin monomer units)
G-actin	globular actin
HMM	heavy meromyosin
HMM-S1	heavy meromyosin subfragment I (subfragment)
HMM-SII	heavy meromyosin subfragment II
IMP	inosine 5'-monophosphate
ITP	inosine 5'-triphosphate
Kapp	apparent association constant
LMM	light meromyosin
Mw	weight average molecular weight
NEM	N-ethylmaleimide
NDP	nucleoside 5'-diphosphate
NTP	nucleoside 5'-triphosphate
pMB	para-mercuribenzoate
Pi	inorganic phosphate
PP	pyrophosphate

LIST OF ABBREVIATIONS (continued)

$S^o_{20,w}$ intrinsic sedimentation coefficient in water at 20° (in Svedberg units, S)

TCA   trichloroacetic acid

Tris-Cl   tris (hydroxymethyl) amino-methane – chloride

μ ionic strength

I. INTRODUCTION

The investigations of the Russian workers (Ljubimova and Engel-hardt, 1939; Engelhardt et al., 1941) have led researchers to an understanding that the mechano-chemical energy conversion in muscle is founded on the interaction between actin and myosin. Myosin possesses the enzymatic property of hydrolyzing a nucleoside tri-phosphate (NTP) into a nucleoside diphosphate (NDP) and inorganic phosphate:

$$NTP^{4-} + H_2O \rightleftharpoons NDP^{3-} + H^+$$
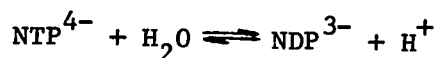
Much of the earlier evidence that this nucleoside triphosphatase (NTPase) activity of myosin is directly related to contractility has come from the experiments with actomyosin, a complex of actin and myosin, obtained as threads by extrusion of its solution in 0.5-0.6M KCl through an orifice into a dilute salt solution (Weber, 1958). Recently, Infante et al., (1965) demonstrated that in isolated living muscle fibers, ATP splitting is proportional to the tension developed. Although the polymerization of monomer actin (G-actin), which forms fibrous polymer (F-actin), is accompanied by hydrolysis of ATP attached to G-actin, this process seems too slow to contribute sig-nificantly to the consumption of ATP during muscle contraction (Hayashi, 1967).

At the time of the discovery of ATPase activity associated to the contractile protein, it was noted that this enzymatic action is affected strongly by certain divalent cations (Bailey, 1954). It is now known that two prominent divalent cations in muscle, Ca^{2+}

and Mg^{2+}, activate actomyosin ATPase. For the ATPase action of myosin, Ca^{2+} remains as an activator, whereas Mg^{2+} becomes an inhibitor. Both Ca^{2+} and Mg^{2+} are also known to be essential for the contraction of living muscle. In the experiments with actomyosin threads, Weber and co-workers found that only Mg^{2+} is necessary for this model system to contract. This prompted Weber (1958) to suggest that Ca^{2+} acts as a deactivator of the "relaxing factor" which inhibits the contractile process unless Ca^{2+} reacts with this factor. In fact, this factor was found (Ebashi and Endo, 1968) to be the proteins tropomyosin and troponin rather than a granular component as Weber had previously thought.

It thus becomes feasible to consider the basic contractile system as being constructed by actin, myosin, Mg^{2+}, and ATP. In this system, the process of ATP hydrolysis catalysed by myosin is coupled to a tension developing step, and the role of Mg^{2+} is apparently in this coupling mechanism. It appears that detailed knowledge of the effects of Mg^{2+} on the system of actin, myosin and ATP helps in understanding the mechanism of energy conversion in muscle contraction.

One of the major problems in studying the actomyosin system has been that both actin (hereafter this term refers to F-actin unless specified otherwise) and myosin are large molecules and are at a state of a hydrated gel, except at high ionic strength, which prevents the application of various physical methods developed for studies of soluble proteins. A frequently used approach to the problem has been to compare the ATPase activity and the macroscopic physical properties such as turbidity and viscosity, of actomyosin suspensions. The

actomyosin gel shows an intensive syneresis, superprecipitation, when it is placed under the conditions which allow muscle fibers to contract, and tends to dissociate if the conditions are favourable for relaxation.

In a previous study of actomyosin suspensions, it was suggested that two types of binding reactions involving Mg^{2+} influence the reactions in a system of actomyosin and ATP: the first is a strong binding of Mg^{2+} to the protein, which accelerates the ATPase activity, and the second is a relatively weak binding to either the protein or ATP or both, which coincides with the increase in the rate of superprecipitation. Considering the fact that both Ca^{2+} and Mg^{2+} activate actomyosin ATPase it seemed of value to see if Ca^{2+} can replace Mg^{2+} in the process of superprecipitation. The results of such an experiment could indicate the specificity of Mg^{2+} in its effects on the actin-myosin interaction.

In the first stage of this study, the effects of Ca^{2+} and Mg^{2+} on myosin ATPase and ITPase were analysed to answer the following questions: do these cations exert their effects through modifying myosin or the substrate or both, and is there any qualitative difference between two cations in their influences on the enzymatic action? Secondly, an attempt was made to assess the efficiency of Ca^{2+}, if it can replace Mg^{2+}, in the reaction of actomyosin leading to superprecipitation. Thirdly, on the purpose of gaining an insight into the mode of interaction between actin and myosin, the Mg-activated ATPase activity was analysed on the assumption that it represents the amount of actomyosin formed. The results were compared with the data obtained

from the experiments in which the enzymatically active subfragment of myosin was used in place of the parent molecule.

Review of Previous Work

In regard to the activation of myosin adenosine triphosphatase by Ca^{2+}, it was proposed that the $CaATP^{2-}$ complex is hydrolysed more rapidly than are other species of ATP (Nanninga, 1957; Blum and Felauer, 1959; Lowenstein, 1960; Tetas and Lowenstein, 1963; Luchi and Kritcher, 1967). According to the observations with nuclear magnetic resonance technique, divalent cations interact mainly with the triphosphate chain of ATP (Hammes et al., 1961; Cohn and Hughes, 1962), which may explain that the lability of the pyrophosphate bond depends on the nature of bound cation. It was also suggested by the same authors (Nanninga, 1959; Blum and Felauer, 1959) that the cations are bound to myosin, modifying its enzymatic activity. Recently, Taylor et al., (1970) analysed in detail the pre-steady state of myosin ATPase action to show that the dissociation of the enzyme-product complex is the rate limiting step of ATP hydrolysis: the dissociation rate of $MgADP^-$-enzyme is slower than that of $CaADP^-$-enzyme or free ADP bound with myosin. In this connection, Lowey and Luck (1969) reported that myosin binds with ADP more tightly in the presence of Mg^{2+} than in the presence of Ca^{2+}, or in the absence of divalent cations.

It was first observed by Singer and Barron (1944) that myosin ATPase is inhibited by a variety of sulfhydryl reagents. Later Kielly and Bradley (1956) showed that a titration of 10-12 of

the 33-36 sulfhydryl groups in one mole of myosin (500,000 daltons) with p-mercuribenzoate (pMB) leads to an activation of the myosin ATPase (see Table 1), whereas further titration results in a progressive inhibition. Sekine et al., in 1962 demonstrated that upon the activation of ATPase by N-ethylmaleimide (NEM), there are primarily two sulfhydryls per myosin molecule which are alkylated. Morales and his colleagues (Morales and Hotta, 1960; Rainford et al., 1964; Warren et al., 1966) interpreted this effect as being due to a local conformational change in the myosin molecule. The modified ATPase is clearly altered in its response to cations and it has been shown by Kitagawa et al., (1961) that pMB-modified myosin loses its tightly bound Ca^{2+} and Mg^{2+}. In this regard, myosin which has been treated with pMB or NEM such that its ATPase is maximal, becomes a valuable tool for exploring the qualitative differences between Ca^{2+} and Mg^{2+} effects on NTP hydrolysis.

It should be noted that there have been reports of differences in the ATPase activity of myosin prepared from red and white skeletal muscle (Sreter et al., 1966; Margreth et al., 1969). It was found that the activity of red muscle myosin is lower, responds more readily to changes in temperature or pH, and requires a higher amount of pMB to show the sulfhydryl effect than white muscle myosin. These observations suggest that there are various species of myosin molecule between muscle types.

The morphological structure of myosin is depicted as a two chain rod, at one end of which there is a part, called the head, where the sites for actin-binding and ATPase are situated (Fig. 1;
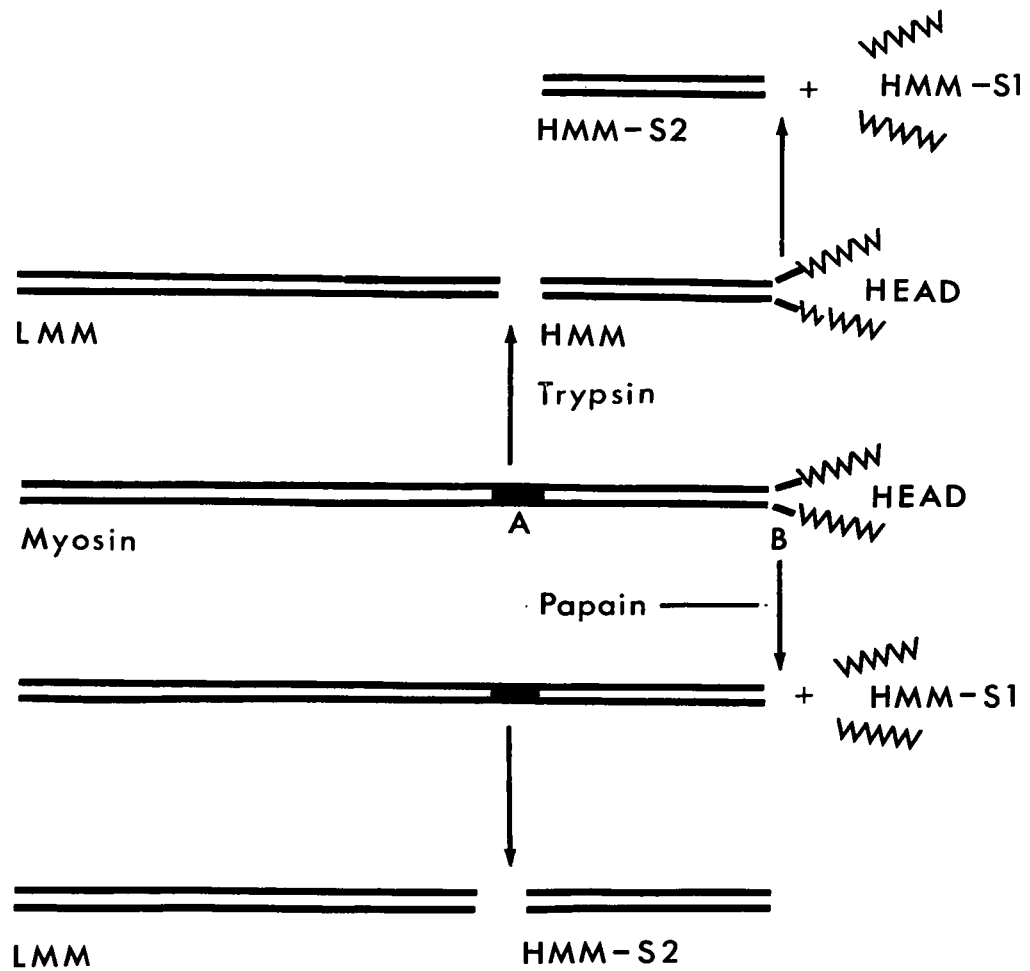
Figure 1: The proteolytic digestion scheme for myosin. Heavy
meromyosin and light meromyosin result from a limited tryptic
digestion, followed by further digestion into HMM-S1 and HMM-S2.
Light meromyosin, HMM-S1, and HMM-S2 result from a single
digestion with papain.

Cohen, 1961; Lowey and Cohen, 1962; Huxley, 1963; Rice et al, 1966; Slayter and Lowey, 1967). Within the myosin molecule are regions which are highly susceptible to proteolytic digestion such that two main fragments, heavy meromyosin (HMM) from the head region (Slayter and Lowey, 1967) and light meromyosin (LMM) result from a limited tryptic digestion (Gergely, 1953; Mihalyi and Harrington, 1959). Further digestion of HMM with trypsin (Mueller and Perry 1961; 1962) or a single digestion with papain (Kominz et al, 1965) gives an ATPase active subfragment (HMM-S1) with a mass of 115,000 daltons. The HMM-S2 depicted in this diagram is believed to be a water soluble helical fragment from the tail portion of HMM (Lowey et al., 1969) which appears as a slow peak in the sedimentation analyses of crude papain digests (Kominz et al., 1965; Lowey et al., 1969).

On the basis of the recoverable mass of the ATPase active material after digestion, it has been repeatedly suggested that there are two or three HMM-S1 units in each HMM unit or in one molecule of myosin as depicted in Fig. 1 (Young et al., 1965; Nauss and Gergely, 1967; Nihei and Kay, 1968). Electron microscopy has also been used to demonstrate two-headed myosin structures (Rice et al., 1966; Slayter and Lowey, 1967). In addition, it has been shown that both HMM and myosin contain two molecules of 3-methylhistidine to the one in HMM-S1 (Johnson et al., 1967). One mole of myosin binds two moles of ADP, or ATP, or pyrophosphate (Kiely and Martonosi, 1968; Schiselfeld and Bárány, 1968; Lowey and Luck, 1969; Nauss et al., 1969; Eisenberg and Moos, 1970), in contrast to a single binding site in subfragment (Young, 1967a; Nauss et al., 1969; Eisenberg and Moos, 1970). This

information supports the earlier idea that the two or three major sub-units of myosin terminate in globular subfragments (Young et al., 1964).

Thus, by proteolytic digestion, the two centers for ATPase and actin-combination can be separated from the parent molecule. The HMM-S1 is soluble in water, while the light meromyosin swells into a hydrated gel, retaining the solubility characteristics of myosin (Mihalyi and Szent-Györgyi, 1953).

When myosin is bound with actin monomer of F-actin, the ATPase activity changes its response to the effect of Mg^{2+} (Banga, 1943; Banga and Szent-Györgyi, 1943). As it was first observed by Szent-Györgyi (1941, 1942), actomyosin gel at low ionic strength (<0.1) and in the presence of Mg^{2+} (0.1mM) undergoes an intensive synaeresis by the addition of ATP, forming a densely packed precipitate (superprecipitation). Systematic studies on the relationship between these processes were initiated by Weber (1958) who pointed out the possibility of superprecipitation being a simple model of muscular contraction.

Regarding the effect of Mg^{2+} on the actin-myosin interaction, two different explanations have been proposed: (1) the formation of $MgATP^{2-}$ complex promotes the ATPase and superprecipitation (Watanabe and Yasui, 1965); and (2) a binding reaction of Mg^{2+} to the protein is responsible for the activation (Geske et al., 1957; Nihei et al., 1966, Nihei, 1967). In the latter proposal it seemed unlikely that the superprecipitation occurred as a mere consequence of the effect of Mg^{2+} on the actomyosin ATPase, since the concentrations of Mg^{2+} required to observe the maximum rate of superpre-

cipitation are well above that needed for the maximum activation of ATPase (Nihei, 1967). In either of the above cases, the proposal has not been tested by direct measurements of binding reactions.

Previously, it was thought that under certain ionic conditions, Ca^{2+} is a necessary factor for the interaction of actin and myosin in the presence of ATP and Mg^{2+} (Weber, 1959). The requirement for Ca^{2+}, however, was explained to be due to tropomyosin and troponin which jointly act as an inhibitory factor of the actin-myosin interaction. The inhibitory effect of these proteins is cancelled by adding Ca^{2+} to the system (Ebashi, 1963; Ebashi and Ebashi, 1964; Ebashi and Kodama, 1966). The same workers have established, however, that with carefully prepared actomyosin, these effects are abolished (see also Perry et al., 1966). Using such preparations, it remains to be tested whether Ca^{2+} can replace Mg^{2+} in the reaction of ATP and actomyosin leading to superprecipitation.

The myosin HMM-S1, like the whole molecule, combines with the actin monomer of F-actin and results in increased Mg^{2+}-ATPase such as to suggest that this property was unadulterated by digestion procedures Eisenberg et al., 1968). It is generally accepted from ultra-centrifugal (Young, 1967b) and ATPase measurements in the presence of Mg^{2+} (Young, 1967a; Rizzino et al., 1968), that the actin monomers of F-actin HMM-S1 combine in a mole to mole ratio.

Contrary to the idea of two actin-combining sites in myosin or heavy meromyosin, Young (1967b) and Rizzino et al., (1970) also found a mole per mole association of the actin monomer to heavy meromyosin. The electron microscopy of Huxley (1963) had forwarded this proposal at an earlier date. However, Szentkiralyi and

Oplatka (1969), studying the increase in the Mg^{2+}-ATPase of HMM when it combines with F-actin, reported that two actin monomers combine with HMM, the second binding being weaker and occurring only at high concentrations of F-actin. Tawada and Oosawa (1969) prepared copolymers and homopolymers of carboxymethylated and natural actin and used them to form modified actomyosin. Their experiments showed that two actin monomers per mole of myosin are required to give the full ATPase of actomyosin. There exists, therefore, a need to clarify the binding reaction between actin and myosin.

The hydrated gel of actomyosin at low KCl concentrations consists of myosin and actomyosin in the form of filaments (Huxley, 1963). These filaments are of the order of microns in length so that it is not unreasonable to view the hydrated actomyosin as a network of actin and myosin filaments (Eisenberg and Moos, 1967) with water in the interfilamentous space. During superprecipitation, the actomyosin gel apparently expels trapped water (Szent-Györgyi, 1951). Recently, a light scattering study revealed changes which were interpreted as being due to a density change (Nihei and Yamamoto, 1969). It was not certain, however, whether just the shrinking of actomyosin was involved or whether there was a conformational alteration in either actin or myosin. It has not been examined whether any physical change can be detected in a mixture of actin and subfragment under conditions favourable for superprecipitation. This actin-subfragment system, which is equivalent to actomyosin except that it lacks the light meromyosin portion, might be useful for investigating the functional significance of the deleted part of myosin.

II. METHODS AND MATERIALS

1. The Preparation of Myosin and Actin

A. The Preparation of Myosin

Myosin was prepared from the back and leg muscles of albino rabbits by the procedure of Tonomura et al., (1966). After the excision of fat deposits and connective tissue, the muscle was homogenized in 3 volumes of a 0.3M KCl solution containing 0.2mM ATP and 0.02M histidine (pH 6.8), in the stainless steel vessel of the Sorvall Omni-mixer (Sorvall Inc., Norwalk, Conn., U.S.A.). The homogenate, after standing for 10 minutes, was centrifuged for 20 minutes at 6,000 rev./min. in the GSA rotor of a Sorvall RC2-B centrifuge. The supernatant was filtered through 4 layers of clean gauze into a chilled graduate cylinder. The volume of filtrate was estimated and this was added to 9 volumes of double distilled water at $4^{\circ}C$ in a large conical container with a clamped opening at the bottom. The precipitated crude myosin sedimented in approximately 45 minutes. The sedimentation was sometimes prolonged and seemed to depend upon fat content and the proportion of leg muscle used. The precipitate was collected from the bottom and centrifuged at 6,000 rev./min. for 20 minutes. The packed precipitate was resuspended in a buffer solution (0.5M KCl, 0.02M histidine, pH 6.8). Then, it was diluted with double distilled water to make the suspension in 0.28M KCl. This was stirred for at least 30 minutes to ensure breaking up of lumps and dissolving the myosin. Insoluble material was removed by centrifugation at 60,000 x g for 30 minutes in the A-211 rotor of an International B-60 preparative

ultracentrifuge (International, Needham Heights, Mass., U.S.A.). The supernatant was passed through 4 layers of gauze and myosin was precipitated at 0.05M KCl. The precipitate was again collected by centrifuging at 6,000 rev./min. for 20 minutes. The dissolution-precipitation cycle was repeated except that the second dissolution was at 0.25M KCl. The myosin prepared showed no turbidity change under the conditions for superprecipitation and no Mg^{2+} activation of the ATPase activity, both indicating the absence of actomyosin contamination. The final myosin precipitate was resuspended in a small volume of 0.5M KCl, 0.02M histidine (pH 6.8), and dialysed against a large volume of the same. Before use this myosin solution was centrifuged at 100,000 x g for 2-3 hours to separate denatured material and fat. The stock myosin solution usually contained 30-50 mg/ml. Table I shows the Ca^{2+}-ATPase activity of several preparations (0.05M KCl, 0.05M tris-Cl, pH 7.4, 10mM $CaCl_2$, 1.0mM ATP, 0.12 mg/ml, 25°C). The average activity of 0.56 μmole Pi/min.mg agrees with that of other workers (Lowey et al., 1969). The observed deviation is likely due to a variation of denatured material in addition to the error of measurement. The state of the myosin sulfhydryl groups did not appear to be severely affected because the p-mercuribenzoate effect was consistent among the preparations tested. The enzymatic activity of myosin was stable for at least two weeks (prep 2 and 3).

Some preparations were stored in 50% glycerol for 1-3 months before use. Pre-cooled glycerol was added slowly with stirring to a measured volume of myosin solution, the presence of air bubbles being avoided. The container was subsequently sealed and stored at -25°C.

Table 1

Ca^{2+}-ATPase Activity of Several Preparations

of Myosin

Date of Preparation	Ca^{2+}-ATPase (μmoles Pi /min. mg)	
	Untreated	pMB-modified*
1. (Oct /66)	0.43	1.05 (2.1)
2. (Nov 17/66)	0.42	1.18 (2.5)
(Nov 22/66)	0.44	---
(Nov 28/66)	0.39	---
3. (Nov 30/66)	0.45	1.25 (2.5)
(Dec 12/66)	0.48	---
4. (Dec /66)	0.52	1.12 (2.5)
5. (Apr /67)	0.62	---
6. (May /67)	0.61	---
7. (Aug /67)	0.46	---
8. (Sept /67)	0.51	---
9. (Jan /68)	0.43	---
10. (Mar /68)	0.56	---
11. (Oct /68)	0.54	---
12. (Nov /68)	0.65	---
13. (Dec /68)	0.86	---
14. (Jan /69)	0.97	---
15. (June /69)	0.61 (glycerol stored)	---
16. (Aug /69)	0.44	---
17. (Dec /69)	0.41	---
	Mean = 0.56 ± 0.16	

ATPase: 0.05M KCl, 0.05M tris-Cl (pH 7.4), 10mM $CaCl_2$, 1.0mM ATP, 0.12 mg myosin/ml, 25°C.

*In brackets is shown the added pMB in moles/10^5 g required to give the maximum ATPase indicated.

For the removal of glycerol, the myosin-glycerol mixture was equili-

brated to $0°C$, and dialysed against 0.5M KCl, 0.02M histidine (pH

7.4). Care was taken to immerse only the lower 50% of the dialysis bag

in the buffer to create an outward hydrostatic pressure opposing the

inward osmotic pressure which may otherwise burst the bag. It was

confirmed that glycerol storage did not adversely affect the ATPase

activity (prep. 15).

B. <u>Chromatographic purification of myosin</u>

Certain myosin preparations were further treated by passing them

through a column of cellulose phosphate and DEAE-cellulose (Diethyl

Amino Ethyl Cellulose) (Sigma Chemical Co., St. Louis, Mo., U.S.A.)

in order to remove impurities of AMP deaminase and nucleic acid.

The procedure presented here is essentially that of Harris and

Suelter (1967), and was simplified to obtain an improved yield. The

cellulose phosphate (0.61meq/gm, medium mesh) and DEAE-cellulose

(0.80meq/gm, medium mesh) were both washed successively with 0.5M

NaOH, 0.5M HCl, 0.5M NaOH, and finally with 5mM EDTA solution. The

cellulose was washed with distilled water after each of these washes

until an indicator reaction showed a pH near neutral. These ion

exchangers were subsequently washed with the eluting buffer (0.2M KCl,

0.02M tris-Cl, pH 7.4) and the suspensions were degassed under re-

duced pressure for 24 hours before being poured into the column. The

column was made by layering cellulose phosphate (50 cm) on top of

DEAE-cellulose (30 cm) in a 2.5 x 100 cm column (Pharmacia, Uppsala,

Sweden). The two exchangers were thus arranged in tandem with

minimal mixing at the interface. The column was fitted with the flow

adapters and then equilibrated with the buffer. Approximately 1 g of myosin at a concentration of 10 mg/ml was pumped through Tygon tubing of small diameter (1mm) onto the top of the column with an LKB 4912A peristaltic pump (LKB-produkter AB, Stockholm, Sweden). The column was then eluted with the buffer at a flow rate of 32 ml/hr. The eluant was monitored with a LKB Uvicord (type 8301A) at 254 mμ. Fractions were automatically collected at a rate of 3 fractions/hr. in an LKB Ultrorac fraction collector (type 7000). Each fraction was then analysed for protein concentration by the biuret reaction; the nucleic acid contamination using the ratio of optical densities measured at 280 and 260 mμ (Layne, E., in Methods of Enzymology, Vol. 3, 1957); the Ca^{2+}-ATPase activity in a reaction mixture containing 0.05M KCl, 0.05M tris-Cl (pH 7.4), 10mM $CaCl_2$, 1mM ATP, 25°C; and the AMP deaminase activity as described below. Previous studies of DEAE-cellulose chromatography have indicated an elution pattern similar to that shown in Fig. 2 (Perry, 1960; Asai, 1963; and Richards et al., 1967). The leading protein peak showed a higher Ca^{2+}-ATPase activity than did the trailing proteins. Approximately 50% of myosin applied on the column was recovered free of AMP-deaminase activity (hatched area, Fig. 2). The column treatment gave protein fractions completely free from nucleic acid contamination.

C. The preparation of an enzymatically active subfragment of myosin

Myosin was digested with an insoluble papain-cellulose complex (Kominz et al., 1965) and the ATPase active fragment was subsequently isolated and purified by gel-exclusion chromatography or by forming its complex with F-actin (Mueller and Perry, 1962). Papain (100 mg

Figure 2: The cellulose phosphate and DEAE-cellulose chromatography of myosin. The myosin was eluted with 0.2M KCl, 0.02M tris-Cl (pH 7.4), 4°C. (●), absorbance at 254 mμ; (O), Ca^{2+}-ATPase at 0.05M KCl, 0.05M tris-Cl (pH 7.4), 10mM $CaCl_2$, 1.0mM ATP, 0.12 mg myosin/ml, 25°C; (Δ), AMP deaminase at 0.1M KCl, 0.01M histidine (pH 6.5), 0.05mM AMP, 30°C; (▲), 280/260 absorbance ratio. On the ordinate is indicated the values for unchromatographed myosin.

O.D. 280/260

AMP deaminase (μmole/min·mg) × 10³

ATPase (μmole Pi/min·mg)

O.D.254

FRACTION NO.
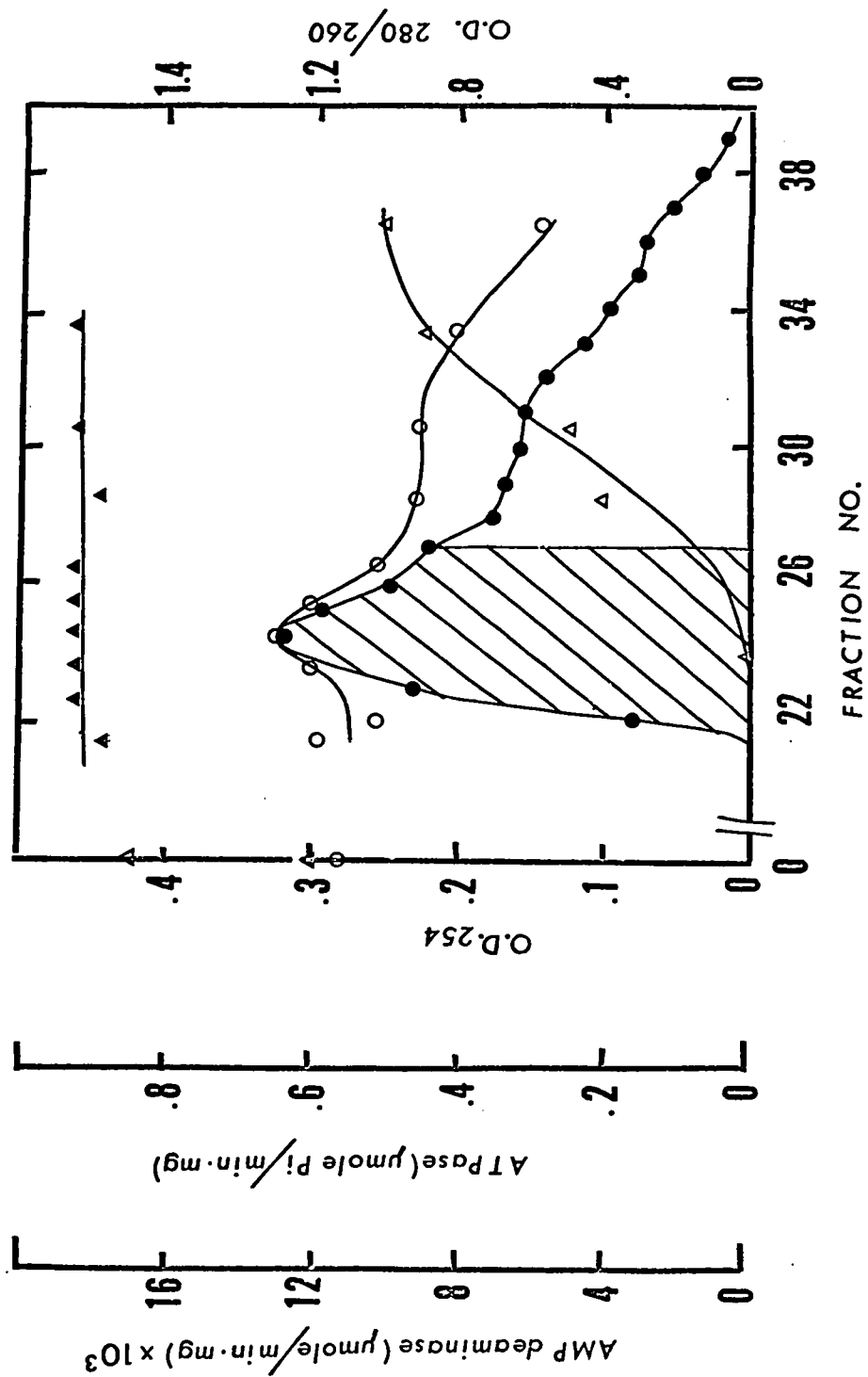
of 2 x crystallized papain in sodium acetate, Sigma Chemical Co.)

was suspended in 50 ml of 5mM EDTA containing 2mM cysteine (pH 7.4),

and was then dissolved by adjusting the pH to 5-5.5 with 0.1N HCl.

The dissolved papain was coupled to diazonium salt of p-aminobenzyl-

cellulose (Bio-Rad Laboratories, Richmond, Calif., U.S.A.) by mixing

it with 0.2 g of the cellulose in 50 ml of 0.05M K_2HPO_4 buffer

(pH 7.0). The diazotized cellulose was prepared according to the

procedure of Campbell et al., (1951). After being stirred slowly

overnight at 4^oC, the papain-cellulose complex was washed 3 times by

centrifugation in the cold with 2mM EDTA, 5mM cysteine (pH 7.4),

to rid it of uncombined papain and fine cellulose particles. The

cellulose complex was finally suspended in an equal volume of the

same solution. The activity of papain-cellulose was standardized

by determining the rate of protein release from myosin (5-10 mg/ml)

in a reaction mixture containing 0.5M KCl, 1mM dithiothreitol

(D.D.T.), and 1mM EDTA (pH 7.0), at 25^oC, using a pH-stat method

(Nihei and Kay, 1968). In this procedure the release of hydrogen

ions during proteolytic hydrolysis was followed by adding 16mM NaOH

to maintain the pH value of the solution using a Radiometer titrator,

type TTTIc, an autoburette, type ABU/a, and a titrigraph, type

SBR 2C. The air phase in the reaction vessel was purged with ultra-

pure Nitrogen (Linde, Union Carbide of Canada) to avoid CO_2 contamin-

ation. The quantity of hydrogen was corrected for the protonation

of α-amino groups (Mihalyi and Harrington, 1959). For the prepara-

tive purpose, a large quantity, ∿500 mg, of myosin was then digested

under the same condition except that 0.02M histidine was present as a

buffer (pH 7.0). The reaction was terminated at intervals by centri-
fugation in a clinical centrifuge and by ultrafiltration through a
Millipore filter of pore size 1.2 μ. The digested myosin was dia-
lysed against 0.01M K_2HPO_4 buffer (pH 7.4), to a final concentration
of 0.05M KCl, and the insoluble fraction was removed by centrifuga-
tion at 100,000 x g for one hour. The supernatant was concentrated
against a bed of Ficoll (Pharmacia) or by filtering through an Amicon
Centriflo cone (Amicon Corp., Lexington, Mass., U.S.A.) at 3,000
rev./min. for 30 minutes in the Sorvall SS-34 rotor (Eisenberg et
al., 1968). This concentrate (approx. 3 ml) was applied by means
of a LKB peristaltic pump to a 2.5 x 30 cm column of G-200 superfine
Sephadex (Pharmacia) which had been pre-equilibrated with 0.05M
KCl, 0.01M K_2HPO_4 (pH 7.4) and 1mM dithiothreitol (D.T.T.). The
column was eluted in a descending fashion with the same buffer solu-
tion at a flow rate of 3 ml/hr. The eluant was monitored using an
optical density at 254 mμ and was collected in fractions by the LKB
apparatus. The fractions containing the active subfragment were
pooled and mixed with a quantity of F-actin. The actin-fragment com-
plex was subsequently collected at 100,000 x g for 2-3 hours. The
pellet was resuspended with the aid of a Teflon pestle in 5mM NaPP,
1mM $MgCl_2$, 0.05M KCl, 0.01M K_2HPO_4 (pH 7.4), and centrifuged at 100,000
x g for 2-3 hours. The supernatant containing the subfragment was
concentrated against Ficoll and was then dialysed against a large
volume of 0.05M KCl, 0.01M K_2HPO_4 (pH 7.4), 1mM Dithiothreitol (D.T.T.).
This stock solution was centrifuged at 100,000 x g for 1 hour to
remove a precipitate. All operations were carried out in the cold.

The fragment was used within one week or stored by freezing at
$-25^{\circ}C$. The enzymatic activity was periodically checked at 0.05M
KCl, 0.05M tris-Cl (pH 7.4), 10mM $CaCl_2$, 1.0mM ATP, $25^{\circ}C$.

D. The preparation of natural actomyosin

Like myosin, actomyosin was extracted from the back and leg
muscles of albino rabbits according to Szent-Györgyi (1951). This
muscle was put through a meat grinder after removal of fat de-
posits and connective tissue and then mixed with 3 volumes of Weber-
Edsall solution (0.6M KCl, 0.01M Na_2CO_3, 0.01M $NaHCO_3$). After a
24 hour extraction in the cold the highly viscous mixture was
diluted with an equal volume of Weber-Edsall solution and centrifuged
at 6,000 rev./min. for 20 minutes in a Sorvall RC2-B centrifuge.
The supernatant containing crude actomyosin was filtered through
4 layers of gauze into a graduate cylinder. By adding enough cold
double distilled water to the filtrate to give a final KCl concen-
tration of 0.25M, actomyosin was precipitated. The precipitate
was collected by centrifugation at 6,000 rev./min. for 20 minutes
and then redissolved in 0.6M KCl. This solution was centrifuged at
30,000 x g for 30 minutes to remove insoluble debris. The super-
natant was filtered again, precipitated, and redissolved in 0.6M KCl.
After the final centrifugation, the middle layer of the centrifuged
solution was collected avoiding fat contamination. Actomyosin gel
was washed with 0.005M tris-Cl (pH 7.4), in order to make the Mg^{2+}-
ATPase activity EGTA-insensitive (Perry et al., 1966). After the
second wash the actomyosin suspension was left standing in this tris
buffer for 72 hours, after which it was centrifuged at 6,000 rev./min.

for 20 minutes, redissolved in 0.6M KCl, 0.02M histidine (pH 7.4),
and then dialysed against a large volume of the same in the cold. In
Table 2 the washed actomyosin was compared to a normal preparation for
EGTA sensitivity, and it is shown that washing makes actomyosin EGTA
insensitive. Apparently the troponin-tropomyosin system has been
removed by this treatment (Perry et al., 1966; Ebashi and Endo, 1968).

E. Modification of the sulfhydryl groups of myosin

   1) pMB-modified myosin

   Myosin at a concentration of 1.33 mg/ml was incubated for 5
minutes at zero degrees in the presence of an appropriate amount of
pMB. Dependence of the enzymatic activity on the pMB concentration
showed an activation and inhibition as observed by Kielly and Bradley
(1956). Maximum activation occured in the presence of 2.5 moles of
$pMB/10^5$ g as was also shown by Mattocks et al. (1967). The activity
was analysed at 0.05M KCl, 0.05M tris-Cl (pH 7.4), 10mM $CaCl_2$, 1.0mM
ATP, 0.12 mg myosin/ml, 25°C.

   2) NEM-modified myosin

   Myosin at a concentration of 10 mg/ml was treated with 2.0
moles of N-ethylmaleimide (NEM)/10^5 g of myosin for 30 minutes
(Perry and Cotterill, 1965). The reaction was stopped by the addi-
tion of a 50 fold excess of β-mercaptoethanol (Sekine and Kielley,
1964). The increase in the activity was analysed at 0.4M KCl,
0.05M tris-Cl (pH 7.4), 10mM $CaCl_2$, 1.0mM ATP, 0.12 mg myosin/ml,
25°C.

F. Extraction and purification of actin

   The acetone powder of muscle (Straub, Szent-Györgyi, 1951) was

Table 2

EGTA Sensitivity of Actomyosin Samples

	ATPase activity (μmoles Pi/min. mg)	Percent of maximum activity in the absence of EGTA
Actomyosin	0.147	100
Actomyosin + EGTA	0.087	59
Washed actomyosin	0.188	100
Washed actomyosin + EGTA	0.174	92
Crude reconstituted actomyosin	0.557	100
Crude reconstituted actomyosin + EGTA	0.378	68
Pure reconstituted actomyosin	0.218	100
Pure reconstituted actomyosin + EGTA	0.232	106

ATPase: 0.006M KCl, 0.05M tris-Cl (pH 7.4), 2.0mM $MgCl_2$, 1.0mM ATP, 1mM EDTA, 1mM EGTA, 0.06 mg actomyosin/ml, 0.06 mg washed actomyosin/ml, 0.20 mg reconstituted actomyosin/ml (3:1 actin: myosin molar ratio), 25°C.

usually prepared from ground (not homogenized) back and leg muscle after the myosin extraction. The muscle was first suspended with constant stirring in 5 volumes of a 0.4% $NaHCO_3$ solution at room temperature for 30 minutes. The fluid was expressed through 4 layers of gauze and the residue suspended in one volume of a solution of chilled 0.01M $NaHCO_3$ and 0.01M Na_2CO_3 for 10 minutes. Then the suspension was diluted with 10 volumes of distilled water at room temperature, and immediately strained through gauze. Three volumes of cold acetone were mixed with the residue at a temperature below 25°C. After stirring for 10 minutes, the acetone was squeezed out and this procedure repeated. The final residue was left to dry at room temperature and the dry fiber was stored at -25°C in a sealed container.

The extraction of actin from the fiber was done with 20 volumes of 0.2mM ATP, 3mM cysteine (pH 8.0), hereafter called the "depolymerizing solution" for 20 minutes at 0°C in order to minimize the tropomyosin contamination (Drabikowski and Gergely, 1962). The first phase of actin purification used the polymerization-depolymerization cycle of Mommaerts (1951). The extracted actin was polymerized with 0.1M KCl, 1mM $MgCl_2$, overnight at 4°C or at room temperature for 1 hour, and then centrifuged at 120,000 x g for 1 hour in an International A-211 rotor. The sides of the centrifuge tubes and the pellets were rinsed with cold water to get rid of fat and excess salt. Then the pellet was suspended in a small volume of the depolymerizing solution (100 ml if 50 g of fiber was originally extracted), and homogenized in a Teflon-glass homogenizer. This

suspension was dialysed overnight against 20 times its volume of depolymerizing solution, followed by centrifugation at 120,000 x g. The resulting G-actin was aspirated from the central portion of the solution to avoid fat. This depolymerization-polymerization cycle was repeated.

The second phase of actin purification involved a partial polymerization step to further eliminate tropomyosin contamination (Martonosi, 1962; Laki et al., 1962). The F-actin was depolymerized as before, except that it was suspended in a smaller volume of depolymerizing solution (30 ml for 50 g of originally extracted fiber) so as to maintain the actin concentration above the critical value (Oosawa et al., 1959) for polymerization (2.5 mg/ml seemed to be ideal). This G-actin was polymerized overnight by dialysis against $0.7mM$ $MgCl_2$, $0.4mM$ ATP, $3mM$ cysteine (pH 8.0). The partially polymerized actin was centrifuged at 70,000 x g for 3 hours. Under these conditions, the tropomyosin contaminated actin was difficult to polymerize, and remained in the supernatant. The pure F-actin sedimented, forming a soft pellet. This polymerization cycle was also repeated. Finally the actin was polymerized with $0.1M$ KCl, $1mM$ $MgCl_2$ and dialysed against $0.1M$ KCl to remove free Mg^{2+}. The low viscosity of actin (Drabikowski and Gergely, 1962) and the lack of a hypersharp peak in the sedimentation of EDTA-denatured actin (Laki et al., 1962), indicated that actin prepared in this way is tropomyosin-free. In Table 2, the lack of an EGTA-sensitivity of reconstituted actomyosin gives an index of purity.

2. Estimation of Protein Concentration

The concentrations of actin, myosin and papain subfragment were routinely determined by the biuret method as standardized with human albumin (Dade Reagents Inc., Miami, Fla., U.S.A.), and calibrated against a micro-Dumas nitrogen determination (Coleman nitrogen analyzer, Model 29), assuming 16.5% nitrogen in myosin (Kominz et al., 1954) and actin (Rees and Young, 1967). Actin and papain subfragment were also determined at 280 mμ using an extinction coefficient of 1149 cm^2/g (Estes and Moos, 1969) and 770 cm^2/g (Young et al., 1965), respectively. The results obtained by the biuret and optical methods agree with each other within the experimental error.

3. Enzymatic Assays

A. Nucleoside triphosphatase

1) Molybdovanadate method for inorganic phosphate (Lecocq and Inesi, 1966)

The reaction was initiated by adding nucleoside triphosphate (NTP) to a constantly stirred mixture containing myosin and other components. Aliquots (1 ml each) of the reaction mixture were taken at intervals and added to 2 ml of the molybdovanadate reagent. The mixture was centrifuged to remove precipitated proteins and the absorbance at 400 mμ was determined after 10 minutes. 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 enzymatic rate determination.

2) <u>Raabe method for inorganic phosphate (Raabe, 1955)</u>

A description is given in the EM-test (E. Merck, Darmstadt, Germany; in the U.S.A., Brink Instruments, Westbury, N.Y.). An aliquot (1 ml each) was added to the molybdate reagent and the resulting phosphomolybdate was reduced to colloidal molybdenum blue. After fifteen minutes a sulfite-carbonate reagent was added to solubilize the protein, and after a further fifteen minutes the blue color was read at 750 mμ.

3) <u>Assay of hydrogen ion release by pH Stat</u>

A solution of 16mM NaOH was standardized with 0.1N HCl (Radiometer). The mixture of components was adjusted to the required pH with the standard NaOH (the titrating system was the same as described for the papain hydrolysis of myosin). The reaction chamber was maintained in a nitrogen (ultrapure, Linde) atmosphere. Myosin was added to initiate the reaction.

B. <u>Adenosine monophosphate deaminase</u>

Myosin was added to a constantly stirred mixture containing 0.1M KCl, 0.01M histidine (pH 6.5), and 0.05mM adenosine monophosphate (AMP) in a thermostated cuvette of 2 cm light path. The specific activity of AMP deaminase (μmoles/min.mg of myosin) was determined in a Zeiss PMQ II spectrophotometer, using the molar coefficient difference between AMP and IMP at 265 mμ of 8.86×10^3 l/cm. mole (Harris and Suelter, 1967).

4. <u>The Binding Reactions of Calcium and Magnesium to</u>

<u>Nucleoside Triphosphates, and Actin and Myosin</u>

A. <u>Preliminary treatment of the proteins and reagents with a</u>
   <u>chelating resin</u>

F-actin and myosin and all reagents were treated with chelex-100 (California Biochemical Corp., Los Angeles, Calif., U.S.A.) to decrease contaminating cations (Offer, 1964). Myosin was dialysed against a large volume of a solution in which chelex-100 was suspended (usually 100 ml of settled chelex in 1 litre of solution), or by passing myosin through a 2.5 x 15 cm column of chelex (Offer, 1964) which had been pre-equilibrated with 0.5M KCl, 0.02M histidine (pH 7.4). F-actin was dialysed against the chelex suspension or against an EDTA solution, the EDTA being subsequently removed by repeated dialysis. KCl, tris-Cl, NH_4OH, and ATP were purified by similarly passing them through a column of chelex which was in the K^+, H^+, NH_4^+, and Na^+ form, respectively. The ability of the resin to remove trace quantities of divalent cations was tested using radioactive Ca^{2+} (Table 3). The Ca^{2+} contamination of myosin and of chelex-100 was determined by the fluorometric technique of Kepner and Hercules (1963) using calcein. For this experiment, Ca^{2+} was extracted from myosin or chelex (2 ml) with 7% (w/v) trichloroacetic acid (TCA) for 24 hr. at room temperature (Mori, 1959). The TCA extract, separated from the proteins by centrifugation, was dried in a sandbath at $75^{\circ}C$ and placed overnight in a desiccator containing a bed of NaOH pellets to remove TCA. The calcein solution (Phillips, 1964) was added directly to the residue and the fluorescence was determined in a Turner Model 110 fluorometer. The results indicated an incomplete removal of Ca^{2+} from myosin (Table 4).

Table 3

The Calcium Purification of Reagents by Chelex-100

	$^{45}Ca^{2+}$ cpm			
Reagent	Untreated	Treated	Background	% Purification
20mM ATP (∿30 ml)	158.7	11.6	9.9	≃100
1M KCl (∿300 ml)	205.4	38.5	39.8	≃100
0.5M Tris (∿200 ml)	71.8	9.4	9.7	≃100

Radioactive $^{45}Ca^{2+}$ was added to each reagent (0.01-0.02 μc $^{45}CaCl_2$/ 10 ml) which was subsequently treated with chelex-100 in a 2.5 x 15 cm column. The radioactivity of 1 ml aliquots was determined in a Tracerlab ultrathin window gas flow counter with 4% efficiency.

Table 4

Calcium Content in Myosin Before and After

Treatment with Chelex-100

Preparation	Chelex Treatment		Ca^{2+} in myosin $\dfrac{(moles \ of \ Ca^{2+})}{(mole \ of \ myosin)}$	
	Ca^{2+} lost from myosin (μmoles)	Ca^{2+} gained by chelex 100 (μmoles)	Untreated	Treated
1. (160 mg.)	0.33	0.30	1.2	0.24
2. (250 mg.)	0.35	0.25	0.90	0.11

Myosin in 0.5M KCl, 0.02M histidine (pH 7.4) was dialyzed against 30 ml of chelex in this buffer. The effect of chelex treatment is indicated by the loss of Ca^{2+} from myosin and the gain by the chelex.

In a separate experiment 1.4 μmoles of Ca^{2+} containing 0.02 μc
$^{45}Ca^{2+}$ was added to 10 ml of myosin (2.6 mg/ml) at $0°C$, which was
then treated with a large volume of chelex. The radioactivity re-
mained in myosin and revealed that 0.1 mole Ca^{2+} per mole myosin was
exchangeable. It was assumed therefore, that the residual Ca^{2+}
bound to myosin should be treated as a part of the bound Ca^{2+} which
dissociates as the total Ca^{2+} concentration decreases.

The TCA extract of chelex-treated myosin and EDTA treated F-actin
was also tested with calmagite (this procedure will be described
in detail in the section on the Mg^{2+} binding to actin and myosin
using calmagite). An aliquot (20 ml each) of 0.1mM calmagite in
NH_4OH-NH_4Cl buffer (μ=0.4, pH 10) was added to the dried TCA extract
and the absorbance difference between 0.1mM calmagite and each sample,
-Δ O.D., was measured at 640 mμ. From Table 5 it is seen that there
is no detectable contamination of magnesium or other cations reacting
with calmagite within the limits of experimental error. From the
estimations of Ca^{2+} contamination in myosin and Mg^{2+} contamina-
tion in myosin and actin, the uncertainty of binding measurements
was estimated as 0.1-0.2 mole per mole of myosin.

B. Calcium ($^{45}Ca^{2+}$) binding to myosin using an ultrafiltration
    technique

The Ca^{2+} binding reaction was measured by ultrafiltration under
the same conditions as for the enzymatic activity. The $^{45}Ca^{2+}$
(535 μc/82.4 μg of Ca^{2+} in 1.4 ml; The radiochemical Centre, Amersham,
Bucks., U.K.) was diluted before use. A 10 ml suspension of myosin
in 0.05M KCl, 0.05M tris-Cl (pH 7.4), containing $^{45}Ca^{2+}$ (0.01 μc)

Table 5

Magnesium Content in EDTA-Treated F-Actin

or Chelex-Treated Myosin

$-\Delta$ O.D.$_{640}$		
Control	EDTA-treated F-actin	Chelex-treated myosin
0.125	0.116	0.137
0.113	0.165	0.123
0.113	0.108	0.141
0.126	0.107	
0.110	0.132	
0.162		
0.124 ± 0.006	0.126 ± 0.024	0.134 ± 0.009

Myosin ($\sim$500 mg) was treated by passing through a 2.5 x 15 cm column of chelex pre-equilibrated with 0.5M KCl, 0.02M histidine (pH 7.4). F-actin (39 mg in 100 ml) was treated by dialysing against 1mM EDTA, 0.1M KCl overnight, and then against 5 x 1 litre changes of 0.1M KCl containing 100 ml chelex. Samples (3.1 mg F-actin, 27.3 mg myosin) were tested. Any contamination would be indicated as a difference in calmagite absorption between sample and control means, the control being TCA-treated dialysate or filtrate of the proteins.

and an appropriate amount of carrier, was filtered through a Millipore filter (100 mμ pore size). Not more than 5% by volume of the reaction mixture was filtered for assay so that the protein concentration was not altered appreciably. Aliquots (0.2 ml each) from the reaction mixture and the filtrate 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. Ten ml of fluor containing 5.5 g of Pre-mix 'M' (Packard Instrument Co., La Grange, Ill., U.S.A.) was added to each vial. Counting was done in a Beckman LS-100 scintillation counter using the variable isoset. External standardization with ^{137}Cs showed no quenching. A counting efficiency of 83% was not changed in a concentration range of $CaCl_2$ up to 0.1mM (same volume as for samples). At the lowest concentration of $CaCl_2$ used in these experiments 0.1 ml of a solution without protein gave 1900±19 counts/min before filtration and 1817±18 counts/min after filtration, the difference of 4.4% suggesting some binding of Ca^{2+} to the membrane. The radioactivity of the reaction mixture and the filtrate, representing the total and unbound Ca^{2+} concentrations, respectively, showed a difference of not less than 50% up to a concentration of $5.0 \times 10^7 M$ added $CaCl_2$, meaning that in this range, the error due to membrane binding would not exceed 10%.

C. **Magnesium binding to actin and myosin**

A technique for determining micromolar concentrations of Mg^{2+} spectrophotometrically using an azo dye, calmagite, was developed. This dye is very soluble and stable in aqueous solutions, unlike eriochrome black T (Lindstrom and Diehl, 1960) which had been used

previously (Kuby et al., 1962; Cantor and Hearst, 1969), and is more sensitive than other dyes (Chauhan and Sarkar, 1969). Both Ca^{2+} and Mg^{2+} react to form a 1:1 complex with calmagite resulting in a color change from blue to red. The absorption spectra of 0.1mM calmagite in NH_4OH-NH_4Cl (μ=0.4, pH 10) and the Mg^{2+} complex of calmagite (Fig. 3) compare favourably with that of Lindstrom and Diehl (1960). The colour change is complete within 15 minutes and remains stable for 4 hours (Chauhan and Sarkar, 1969). It is not expected that the reaction of transition metals (Chauhan and Sarkar, 1969) would present a problem unless there is a high level of contamination in the protein.

The dye was made in a 10mM aqueous solution and was stored in the dark in a polyethylene bottle (Lindstrom and Diehl, 1960). An aliquot of this stock solution was used to prepare a dilute solution daily (0.1mM calmagite, NH_4OH-NH_4Cl, μ=4, pH 10). The standard curve was constructed by adding the dilute calmagite solution to the samples of $MgCl_2$ (1-10mM in 0.01M KCl, 0.01M histidine, pH 7.4) which had been evaporated to dryness at $75^{\circ}C$ in a vacuum oven. The addition of the dye solution to dry samples caused no change in the dye concentration, and the change in absorbance was measured very accurately by the double beam mode in a Cary 16 spectrophotometer. Since there is a decrease in absorbance upon addition of Mg^{2+}, the position of the reference and the sample are reversed and the readings were recorded as $-\Delta$ O.D. 640. Successful determinations have been also made at 610, 615, and 620 mμ, but with less sensitivity. The experiments shown in Fig. 4 were designed to illustrate the main characteristics of this determination. At the concentration of calmagite used, the $-\Delta$ O.D. at 615 or 640 mμ was essentially linear up to 10μM $MgCl_2$. A certain amount of histidine and/or KCl appeared to be
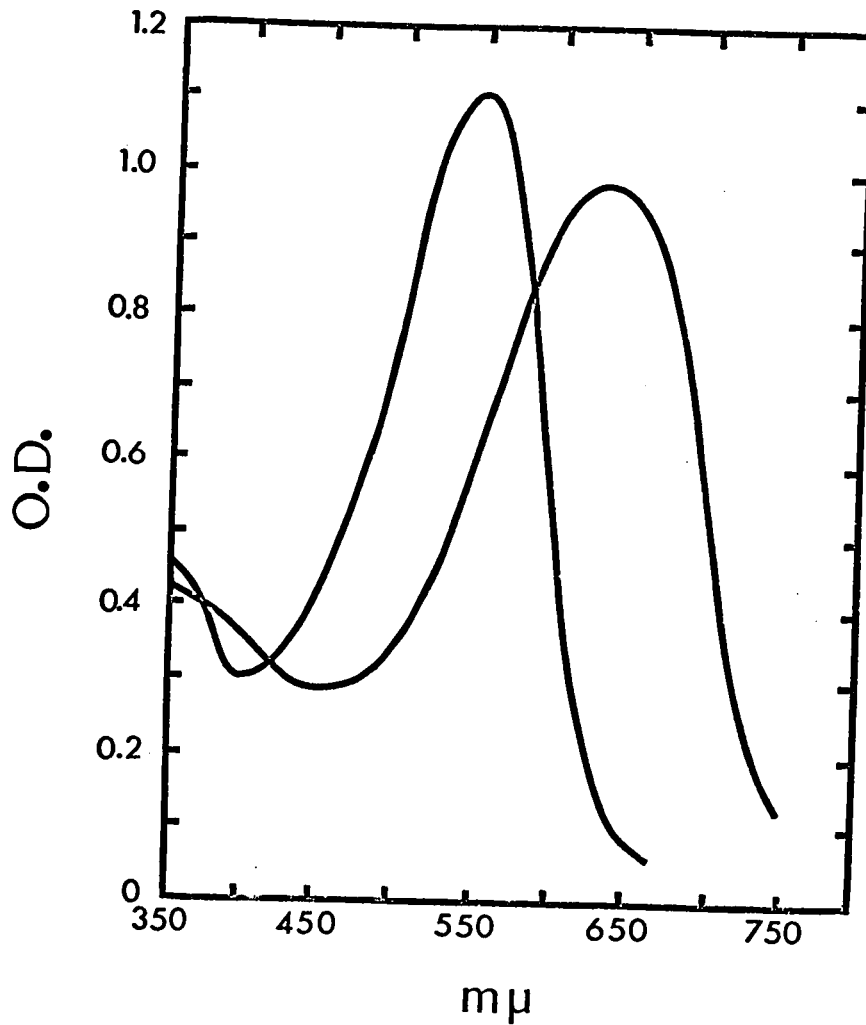
Figure 3. Absorption spectra of calmagite. Calmagite (0.1mM in
NH_4OH - NH_4Cl, μ = 0.4, pH 10) was tested in a 1 cm cuvette of a
Beckman DU-2 spectrophotometer (right-hand peak). The addition
of an excess of $MgCl_2$ (1.0mM) shifted the peak to the left.
The maximum absorbance difference occurs at 610 - 640 mμ.
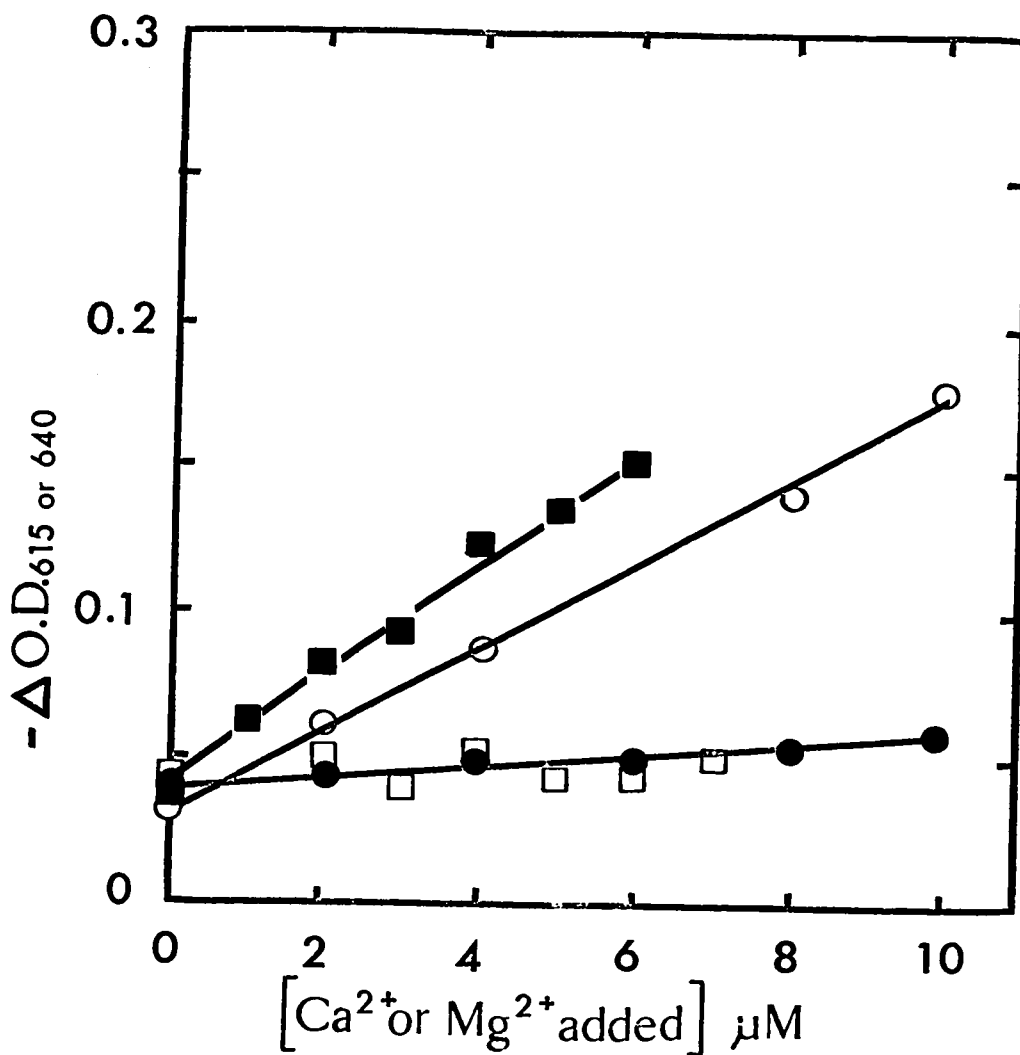
Figure 4: Absorbance changes of calmagite by the addition of Ca^{2+} and Mg^{2+}. Calmagite (0.1mM in NH_4OH – NH_4Cl, $\mu = 0.4$, pH 10) was used. (O), $MgCl_2$ in 0.01M KCl, 0.01M histidine; (●), $CaCl_2$ in 0.01M KCl, 0.01M histidine; (□), $MgCl_2$ in <0.001M KCl, <0.001M histidine. The above determinations were made at 615 mµ. (■), $MgCl_2$ in 0.01M KCl, 0.01M histidine, 640 mµ.

necessary, otherwise the Mg^{2+} did not react with calmagite, probably because of absorption by glass after the sample was dried. Compared with Mg^{2+}, Ca^{2+} did not react to any extent (see also Lindstrom and Diehl, 1960; Chauhan and Sarkar, 1969). The change in absorbance detected without added magnesium did not appear to be due to any contamination in KCl and/or histidine, since no correlation could be observed with varied concentrations of these substances (Table 6). There was no change in pH of the reaction mixture. This unexplained solvent effect is even greater with tris buffer, and due to some degree of fluctuation imposes a technical limitation on this test. In a given set of determinations, however, the solvent effect was consistent between samples and good standard curves were obtained.

In summary, by using the stable, highly sensitive calmagite, and by adding this to a dried sample so as to minimize variations in the concentration of dye, micromolar quantities of Mg^{2+} were determined accurately. This is an improvement of recent, similar, techniques using eriochrome black T (Cantor and Hearst, 1969) or calmagite (Chauhan and Sarkar, 1969).

All glassware was washed with dilute nitric acid and 5mM EDTA (pH 10), followed by a thorough rinse with double distilled water. Myosin in 0.5M KCl, 0.02M histidine (pH 7.4) was dialysed versus a large volume of chelex (100 ml). It was further dialysed to bring the final concentration to 0.01M KCl, 0.01M histidine (pH 7.4). At 5-10 mg/ml, the myosin-gel could be quantitatively pipetted upon rinsing the pipet with the resulting solution.

Table 6

Solvent Effect on Calmagite Absorption

histidine mM	KCl mM	Calmagite mM	$-\Delta$ O.D.$_{610}$	$-\Delta$ O.D.$_{640}$
0.9	<1	0.01	0.0164	
10	12	0.01	0.0985	
10	12	0.01	0.0301	
10	100	0.02	0.0262	
0.6	<1	0.10	0.0336	
10	10	0.10	0.0694	
10	10	0.10		0.0250
10	12	0.10		0.0354
10	17	0.10	0.0970	
10	100	0.10		0.0842

The solvent is the filtrate from the Mg^{2+} binding studies where the original solution contained no added Mg^{2+} or protein. These were dried and analysed in the same way as samples. There was no change in absorbance when KCl and histidine were absent.

The Mg^{2+} binding reaction was determined in an Amicon Model 12

10 ml ultrafiltration cell with a Millipore filter (100 mμ pore size).

All parts of the filtration apparatus were soaked overnight in EDTA.

After assembly, the cell was rinsed thoroughly with 5 changes of

distilled water and a liter of dialysate (0.01M KCl, 0.01M histidine,

pH 7.4). The filtration cell contained 10 ml of the reaction mix-

ture. With constant stirring to prevent concentration at the cell

base, no more than 20% filtrate was expressed using 50 psi of nitro-

gen (ultrapure, Linde). This pressure is too low to affect the

ATPase or the actin-myosin association reaction (Laidler, 1951;

Ikkai and Ooi, 1969). In a given run, standards were also filtered.

Aliquots (1 ml each) of samples and standards were dried at 75°C in

20 ml beakers under a reduced pressure. Then 1 ml of 0.1mM calmagite

in NH_4OH-NH_4Cl (μ=0.4, pH 10) was added. After 15 minutes, the

$-\Delta$ O.D. 640 was determined as described.

D.   <u>Calcium and magnesium binding to adenosine and inosine triphosphates</u>

A technique was employed in which the spectral change of a dye,

8-hydroxyquinoline, was analysed to determine the association con-

stants of Ca^{2+} and Mg^{2+} to ATP or ITP (Burton, 1959) under the condi-

tions of the enzymatic assays. A concentration of 0.8mM and 0.3mM

8-hydroxyquinoline was used for Ca^{2+} and Mg^{2+} binding, respectively.

Burton considered the reaction of the divalent cations, M, with the

nucleoside triphosphate, P, in three possible complexes: MP, M_2P,

and MP_2. Furthermore he considered the possible formation of a

ternary complex between the cation, nucleoside triphosphate, and the

dye. He showed that the association constant K, of MP is given by

$K = 1/\{M\}_{\frac{1}{2}}$ where $\{M\}_{\frac{1}{2}}$ is the free cation concentration when $\{MP\} = \frac{1}{2}\{Po\}$, $\{Po\}$ representing the total concentration of the nucleoside triphosphate. However, the apparent free concentration of Mg^{2+}, $\{M\}_{\frac{1}{2}}'$, was found by Burton to depend on the concentration of $\{Po\}$. Thus K is represented by

$$K = \frac{1}{\{M\}_{\frac{1}{2}}} + k\{Po\}$$

where k is a factor introduced by Burton to account for this dependence, attributed to the formation of a ternary complex, and is expressed by the following equation:

$$k = \frac{\{M\}_{\frac{1}{2}}' - \{M\}_{\frac{1}{2}}}{K\{M\}_{\frac{1}{2}}\{P\}}$$

where $\{P\}$ is the concentration of uncombined nucleoside triphosphate. Here K is found by plotting $\{M\}\frac{1}{2}'$ for various values of $\{Po\}$ and extrapolating to $\{Po\} = 0$. A linear plot of the data was obtained (Fig. 5) in agreement with the findings of Burton (1959) and O'Sullivan and Perrin (1964). A best fit was extrapolated and the value of K obtained from the reciprocal of the ordinate intercept (see Table 7).

5. Superprecipitation of Natural and
Reconstituted Actomyosin

The process of superprecipitation was measured as an increase in turbidity. To a buffer solution being stirred in a 2 cm Zeiss cell was added actomyosin which raised the KCl concentration in the buffer to 0.01-0.02M. The increase in absorbance at 545 mμ, detected by a Zeiss PMQ II spectrophotometer, was recorded on a Texas

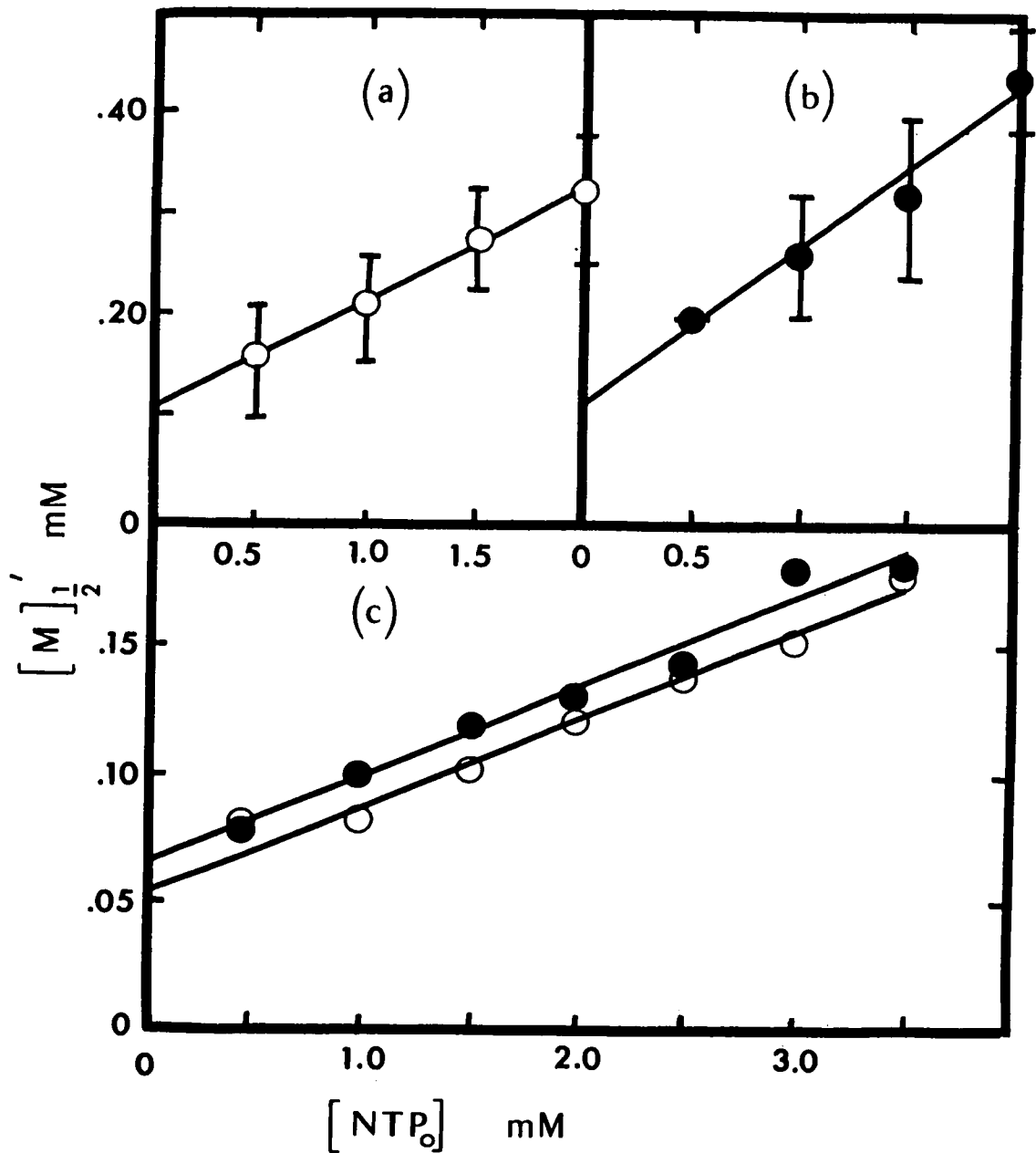Figure 5: Ca^{2+} and Mg^{2+} binding to ATP and ITP measured by the spectral change in 8-hydroxyquinoline. Conditions: 0.05M KCl, 0.05M tris-Cl (pH 7.4), 25°C, 360 mμ. (a) $CaATP^{2-}$, 0.8mM hydroxyquinoline; (b) CaITP, 0.8mM hydroxyquinoline; (c) MgATP (O), and MgITP (●), 0.3mM hydroxyquinoline. Vertical bars indicate standard deviation for several determinations at each concentration of NTP.

Instruments Serva-riter recorder (Levy and Fleisher, 1965; Nihei, 1967).
The rate of superprecipitation was measured from the reciprocal of the
time required for the turbidity change to reach 50% of the total extent
(Nihei, 1967; Nihei and Yamamoto, 1969).

6. Light Scattering of the Actin
Subfragment Complex

The angular distribution of polarized light (436 mµ) scattered
by F-actin in the absence and presence of subfragment was examined
using a Brice Phoenix Series 2000 light scattering photometer
(Phoenix Precision Instrument Co., Phil., Pa., U.S.A.). Light from
a high pressure mercury vapour lamp, type AH3, was either vertically
or horizontally polarized by the polaroid discs. After passing
through a narrow beam diaphragm and being scattered by the solutions,
the intensity of scattered light was measured by a galvanometer and
recorded on the Texas Serva-riter recorder. A pyrex cylindrical
cell, C-101, was cemented to a square glass plate using duPont Duco
cement. To test the correct positioning of the cell, a dilute
solution of fluorescein was viewed through a yellow filter, and the
intensity of green fluorescence was distributed equally in the
angular range from 30 to 135° with 2% deviation (Zimm, 1948).

F-actin was polymerized at 3.4 mg/ml with 1mM $MgCl_2$, 0.01mM
tris-Cl (pH 7.4), which was the solvent condition used for these
measurements. F-actin was dialysed to remove ATP. After dialysis,
F-actin was diluted with the solvent which had been twice filtered
through a Millipore filter (250 mµ pore size). The solutions were

centrifuged in a Sorvall HB-4 swinging bucket rotor at 10,000

rev./min. for 20 minutes to remove dust (Oosawa et al., 1959). The

protein concentration did not change after centrifugation. After

standing 1 hour at room temperature to achieve thermal equilibration

(Oosawa et al., 1959), 20 ml of F-actin suspension was transferred

to the C-101 pyrex cylindrical cell. The myosin subfragment, at 4.7

mg/ml in 0.05M KCl, 0.01M K_2HPO_4 (pH 7.4), 1mM D.T.T. was centrifuged

at 100,000 x g for 2-3 hours before adding to solutions of F-actin.

The data was corrected according to the equation of Tomimatsu and

Palmer (1963) which accounts for extra light arising from back reflection.

7. Sedimentation-Velocity and High-Speed Sedimentation-
Equilibrium Analysis of Myosin Subfragment

Sedimentation-velocity studies of subfragment were carried out

in a Spinco Model E analytical ultracentrifuge at 59,780 rev./min.

with rotor temperature maintained at $20^{\circ}C$.

The molecular weight and homogeneity of subfragment were tested

using the high-speed sedimentation-equilibrium technique of Yphantis

(1964). This technique does not require a separate estimation of

protein concentration and is useful for detecting pauci-dispersion.

Rayleigh interference optics were used for the measurement of solute

distribution in a centrifugal cell. A sample solution (0.11 ml)

and its dialysate (0.12 ml) were placed in a 12mm, double-sector

cell with a Epon-filled centerpiece. The counterbalance of the

An-D rotor was equipped with inner and outer reference holes, the

former having a wire for more precise measurements. The phase bar

angle was 90°. Photographs were taken with Kodak type 11-G high

contrast spectral plates and the fringe displacements were measured

with a Nikon comparator. A density of 1.025 at $4^{\circ}C$ was calculated

from the contribution of individual solution components (0.5M KCl,

0.01M K_2HPO_4, pH 7.4, 0.02-0.1M β-mercaptoethanol).

A plot of the fringe displacement as a function of the distance

from the meniscus indicated zero protein concentration near the

meniscus, a necessary condition for the Yphantis technique (Fig. 6).

8. Chemicals

ATP and ITP were purchased from the Sigma Chemical Co., and

used without further treatment except when chelex treatment was

required. Calcein and calmagite were purchased from the G.F. Smith

Chemical Co., Columbus, Ohio, U.S.A. Other chemicals were of

reagent grade. The stock solutions of $CaCl_2$ and $MgCl_2$ were standard-

ized by titration against EDTA.

III. RESULTS

1. The Effects of Calcium and Magnesium on the

Adenosine Triphosphatase and Inosine Triphosphatase of Myosin*

A. Activation by Calcium

The effects of Ca^{2+} and Mg^{2+} on the enzymatic activity of myosin

were studied at a KCl concentration of 0.05M, the enzymatic activity

of myosin at this concentration not being affected by a variation of

monovalent ion concentrations in the range of ±0.03M. Also, the

*Most of the work described in this section has been published
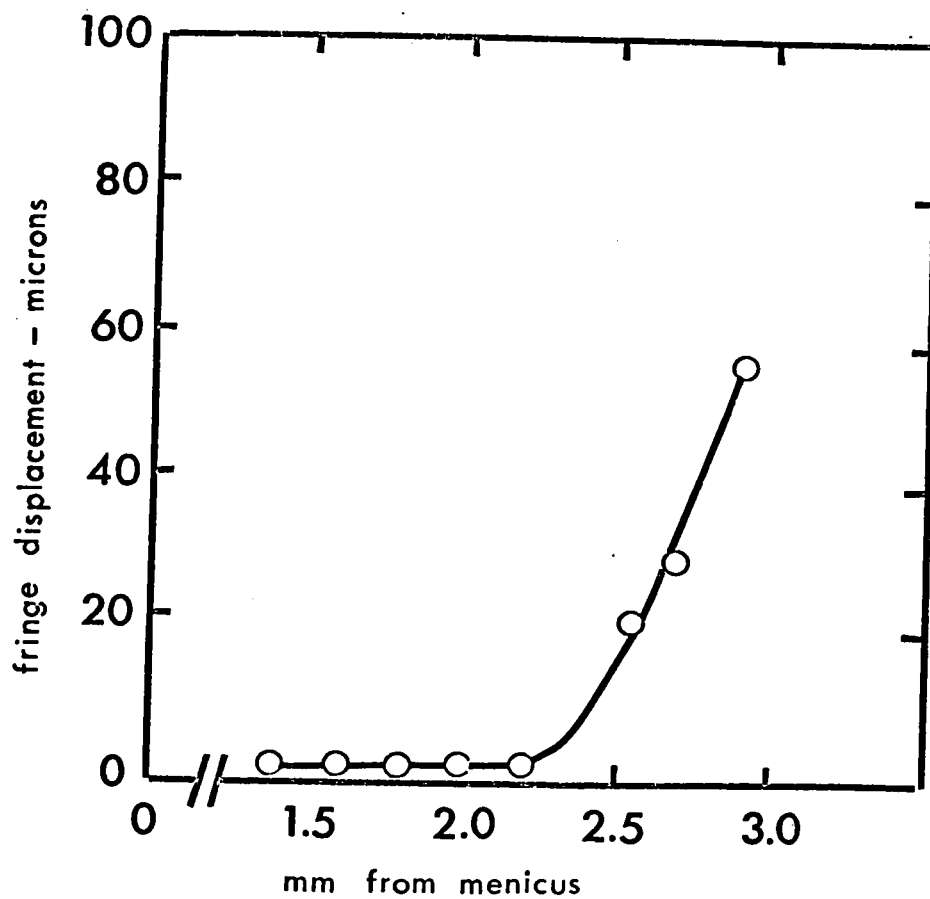(Sugden and Nihei, 1969).

Figure 6. Sedimentation-equilibrium of myosin subfragment (0.5M KCl, 0.01M K$_2$HPO$_4$, pH 7.4, 0.1M β-mercaptoethanol, 1.0 mg/ml, 4°C). Run duration was 24 hours at 18,000 rev./min.

characteristics of myosin ATPase in 0.05M KCl could be compared with those of actomyosin ATPase associated with superprecipitation. As shown in Fig. 7a and 7b, Ca^{2+} at concentrations up to 30mM activates the hydrolysis of ATP and ITP, and further increase in its concentration makes the Ca^{2+} effect inhibitory. For the analysis of Ca^{2+} activation of myosin ATPase or ITPase, the rates of hydrolysis at various concentrations of Ca^{2+} may be expressed as the "relative activity", v, which is:

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

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 activation, and with an intermediate concentration of Ca^{2+}, respectively. This ratio was found to be useful for the analysis of the Mg^{2+} activation of myosin B ATPase (Nihei et al., 1966). Then, to examine the possibility of a binding reaction between Ca^{2+} and myosin being responsible for the activation, the relative activity was plotted against the concentration of the free Ca^{2+}. The free Ca^{2+} was defined as the Ca^{2+} not bound to NTP (ATP or ITP). To calculate the free Ca^{2+} concentration, it was necessary to determine the apparent association constant, Kapp, for $CaNTP^{2-}$ under the experimental conditions. The spectrophotometric method of Burton (1959) was applied (Methods and Materials), and the apparent association constants listed in Table 7 were within the range of values reported by Nanninga (1961), Asai and Morales (1962), and O'Sullivan and Perrin (1964). The relative activity plotted against the Ca^{2+} concentration

Figure 7. ATPase and ITPase activities as a function of the added concentration of Ca^{2+}. (a); (O) 0.1mM ATP; (●), 1.0mM ATP. pMB-treated ATPase: (Δ), 0.1mM ATP; (▲), 1.0mM ATP. ITPase: (b); (□), 0.1mM ITP; (■), 1.0mM ITP. Conditions: 0.05M KCl, 0.05M tris-Cl (pH 7.4), 0.12 mg myosin/ ml in all cases, 25°C.
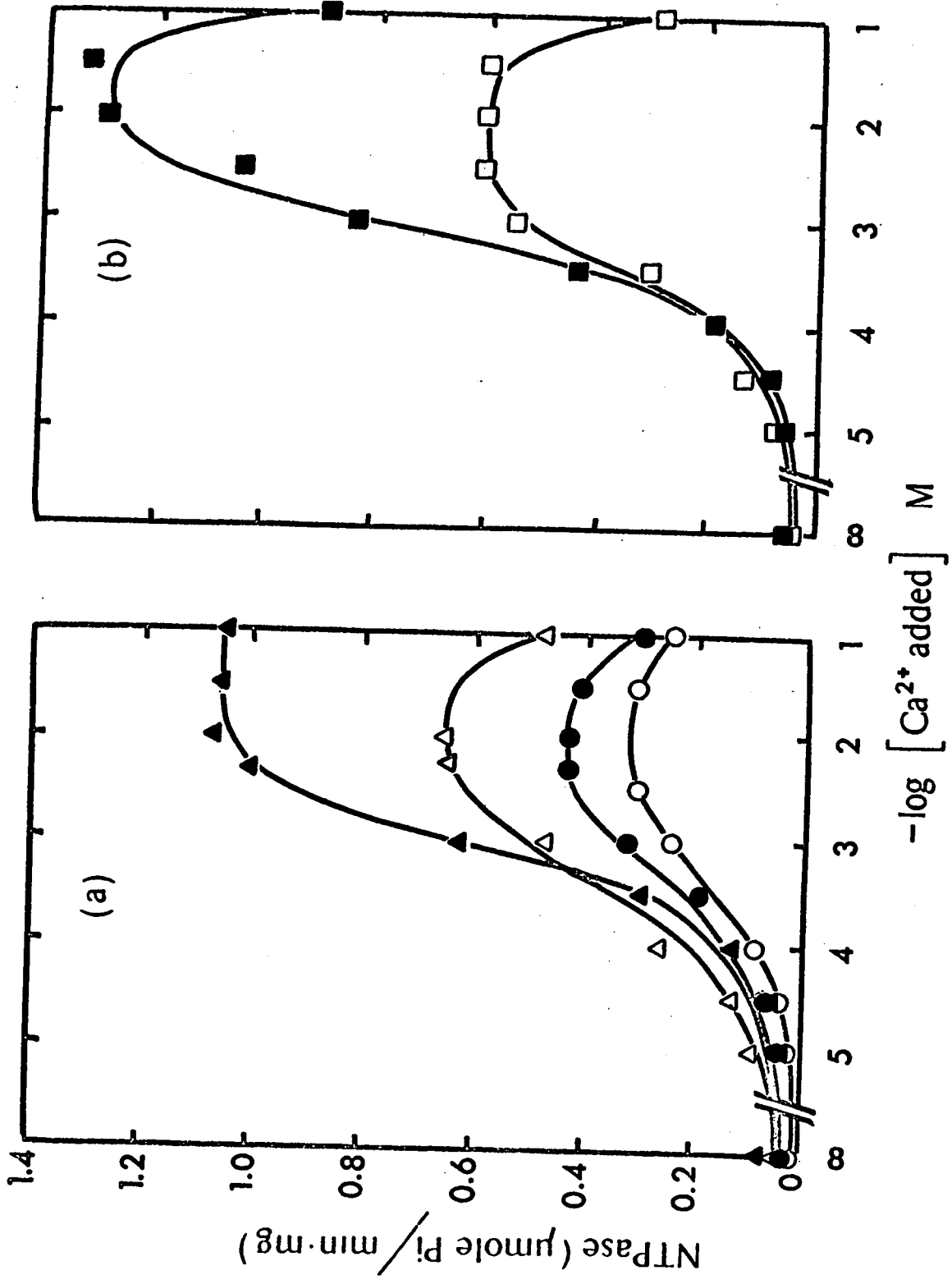
Table 7

Kapp for the Ca^{2+} and Mg^{2+} Complexes with ATP and ITP

as Determined Spectrophotometrically

Complex	Kapp (M^{-1})
$CaATP^{2-}$	$9,300 \pm 1,400$
$CaITP^{2-}$	$8,800 \pm 600$
$MgATP^{2-}$	$18,900 \pm 800$
$Mg\ ITP^{2-}$	$15,400 \pm 300$

Determinations were carried out in 0.05M KCl, 0.05M tris-Cl (pH 7.4), at 25°C. A best fit of the experimental plot (Fig. 3) gave Kapp as the reciprocal of the ordinate intercept (see Methods and Materials).

(Fig. 8) suggests, from the shape of the curve, that a certain Ca^{2+} binding reaction is responsible for the activation. The binding of Ca^{2+} to myosin was therefore studied by a procedure described in Methods and Materials in an attempt to determine whether this was the reaction responsible for activation.

The binding reaction was observed to be hindered at high concentrations of myosin (Table 8). Although there was no detectable difference in the binding measured after 5 and 25 minutes incubation, it is conceivable that the lower binding observed at 2 mg/ml may be due to the anomalous effects seen with high protein concentrations in equilibrium dialysis (Cassel et al., 1969). The precipitated myosin suspension could provide the same barrier to equilibrium approach as does the coating of dialysis membranes with protein. Therefore a concentration of 0.67 mg/ml of myosin was used. The resultant Scatchard (1949) plot suggests that there are at least two types of binding reactions between Ca^{2+} and myosin (Fig. 9). The first type is a binding reaction that occurs at a site present on each 500,000 atomic mass units of myosin and has a Kapp of $1.3 \times 10^6 M^{-1}$. The second type is a weak binding, the binding parameters of which cannot be accurately measured due to the low protein concentration. The results obtained in the presence of ATP show a binding similar to that in the absence of ATP. In this case the plot is made ignoring the $CaATP^{2-}$ formation, which is insignificant within the range of Ca^{2+} concentrations used. When myosin is modified with NEM, which is equivalent to pMB in its effect on the ATPase activity (Kielley and Bradley, 1956), the tight Ca^{2+}
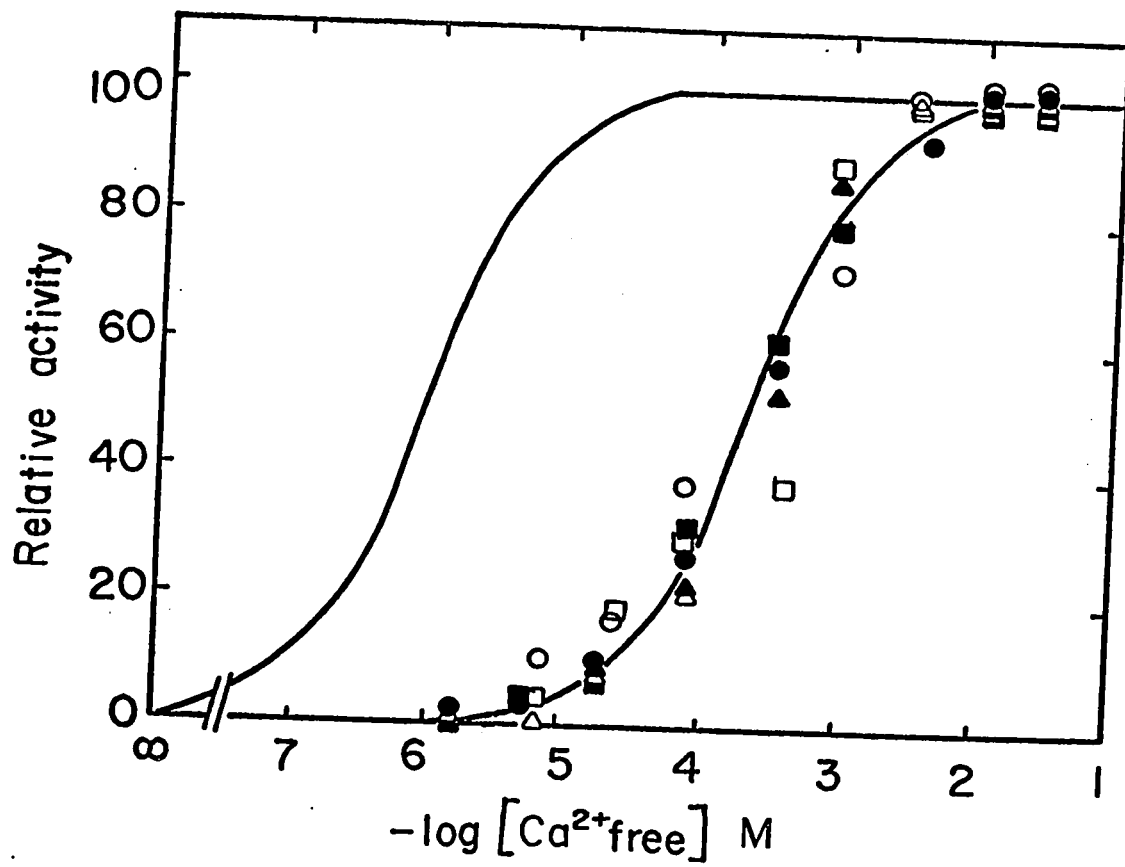
Figure 8: Relative activity as a function of the calculated concentration of free Ca^{2+}. Same conditions and symbols as for Figure 6. The continuous curve to the left depicts the tight Ca^{2+} binding to myosin.

Table 8

Effect of Protein Concentration on the

Binding of Ca^{2+} to Myosin

Conc. of protein (mg/ml)	Time of mixing (minutes)	$\dfrac{\{Ca^{2+}\ bound\}}{\{Protein\}}$ X $\dfrac{1}{\{Ca^{2+}\ free\}}$ $(l.g^{-1})$
0.15	5	1.9
0.33	5	2.1
0.67	5	2.1
2.12	25	0.7
2.05	5	0.7

A fixed amount of Ca^{2+} (0.3 nmole) was added to each 10 ml of myosin in 0.05M KCl, 0.05M tris-Cl (pH 7.4), at 25°C. The assay of Ca^{2+} bound was performed as described in Methods and Materials.

Figure 9. Scatchard plot of Ca^{2+}-binding by native and NEM-modified myosin. Myosin was modified with 2.0 moles of $NEM/10^5$ g. of myosin for 30 minutes and the reaction was stopped with a 50-fold molar excess of β-mercaptoethanol. (O), unmodified myosin, no ATP; (◉), unmodified myosin, 1.0mM ATP; (●), NEM-modified myosin, no ATP. Conditions: 0.05M KCl, 0.05M tris-Cl (pH 7.4), 25°C.
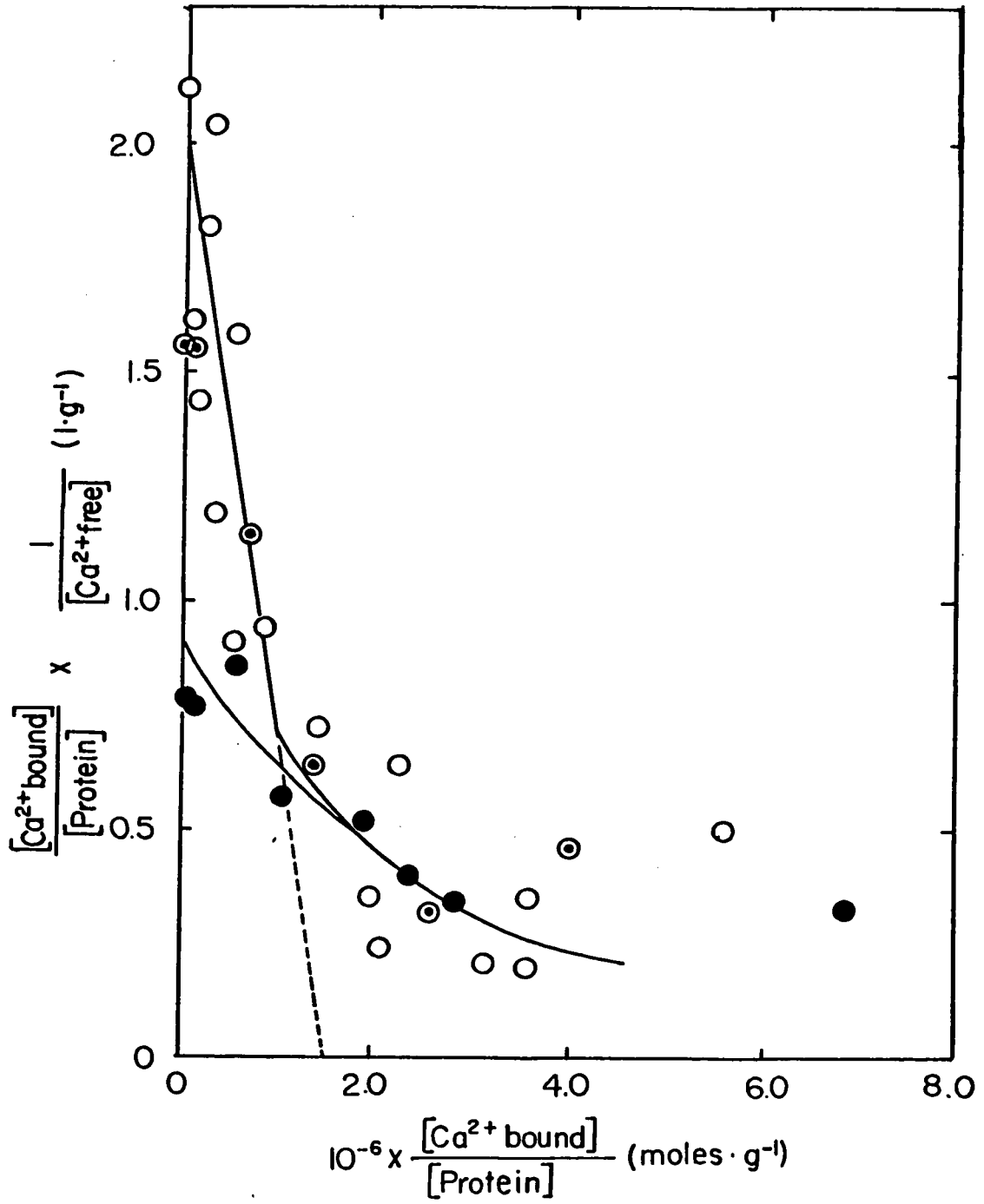
binding is weakened (Fig. 9). This is in agreement with the obser-
vation by Kitagawa et al. (1961) that myosin released the tightly
bound Ca^{2+} and Mg^{2+} when treated with pMB. Despite its decreased
capacity to bind Ca^{2+} tightly, the NEM or pMB-treated myosin retains
the Ca^{2+} activated myosin NTPase.

Although the presence of AMP deaminase, myokinase, or nucleic
acid in myosin does not significantly alter the protein mass or
ATPase characteristics (Byrnes and Suelter, 1965), it is also im-
portant to establish that this contamination does not alter the Ca^{2+}
binding reaction to myosin. A combination of cellulose phosphate
and DEAE-cellulose chromatography removes AMP deaminase and nucleic
acid contamination (Harris and Suelter, 1967; see Methods and
Materials) and there is evidence that myokinase is removed by DEAE-
cellulose chromatography (Richards et al., 1967; Lowey et al., 1969).
A test of the Ca^{2+} binding reaction to this purified myosin shown
in the Scatchard plot of Fig. 10 compares favourably with the data
for unpurified myosin (Fig. 9).

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 faster, or at least leads to a more
rapid release of products, than other species of NTP. If one assumes
that the Ca^{2+} activation is due only to the formation of $CaNTP^{2-}$,
and that the Michaelis-Menten scheme is applicable regardless of the
species of the substrate, an expression of the enzymatic activity,
v, at a given concentration of Ca^{2+} may be written:

Figure 10. Scatchard plot of Ca^{2+} binding by native and NEM-modified myosin purified by chromatography. Same modification and conditions as Figure 9. (O), unmodified myosin; (●), NEM-modified myosin. No ATP in either case.
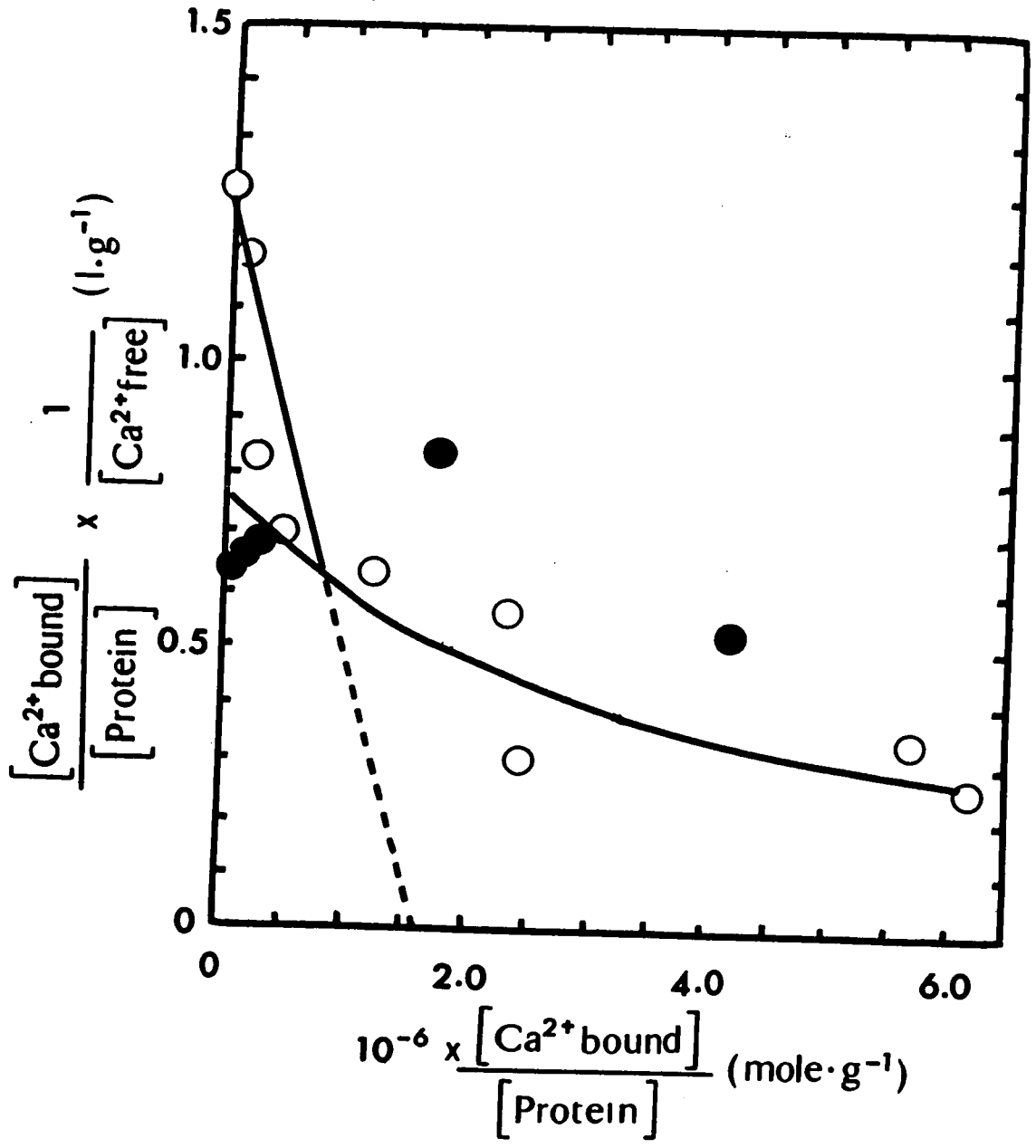
$$\frac{v}{V} = \frac{\dfrac{k_3}{k_3{}^r} \dfrac{\{NTP\}}{Km' + \frac{Km'}{Km}\{CaNTP\} + \{NTP\}} + \dfrac{\{CaNTP\}}{Km + \frac{Km}{Km'}\{NTP\} + \{CaNTP\}}}{\dfrac{\{NTPo\}}{Km' + \{NTPo\}}}$$

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 by using NTP

and $CaNTP^{2-}$ as the substrates, respectively, and Km and Km' are

the corresponding Michaelis constants. $\{NTP\}$, $\{CaNTP\}$, and $\{NTPo\}$

denote the concentrations of Ca^{2+} free NTP, $CaNTP^{2-}$, and total NTP,

respectively. Fig. 11 shows the plot of v/V versus $\{CaNTP^{2-}\}/\{NTP_o\}$.

There are three curves drawn in Fig. 11, each of which is defined

by the values of k_3/k_3', Km and Km'. In the special case where

Km = Km', v/V becomes a linear function of $\{CaNTP\}/\{NTP_o\}$. Small

deviations from linearity shown in Fig. 11 are due to slight differ-

ences in the Michaelis constant. The Km values were estimated from

reciprocal plots of the available data in the concentration range

of 0.1-1.0mM ATP. The values so obtained are higher than determina-

tions made at low concentrations of ATP (Kiely and Martonosi, 1968;

Schiselfeld and Bárány, 1968; Lymn and Taylor, 1970), but there is

little difference between the Km values in the presence or absence

of Ca^{2+} (Table 9; see also Blum, 1955; Schiselfeld and Bárány, 1968).

The experimental results, therefore, illustrate that the Ca^{2+} activa-

tion is due either to a weak Ca^{2+} binding, or to a more efficient

hydrolysis of $CaNTP^{2-}$, or to both of these.

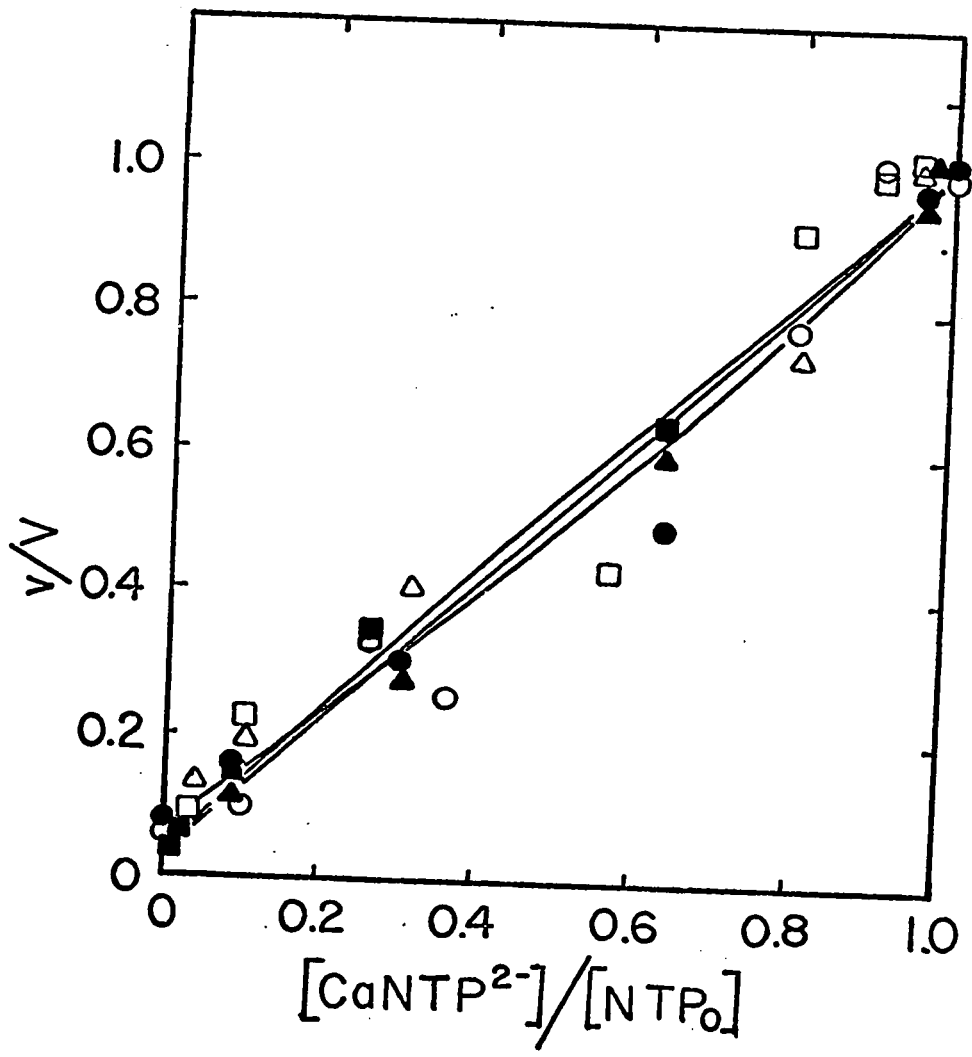Figure 11. Ratio v/V as a function of $\{CaNTP^2\}/\{NTPo\}$. The theoretical curves are from the top: pMB-modified myosin ATPase, native myosin ITPase and native myosin ATPase. The symbols represent the same experimental data as shown in Figure 6.

Table 9

Michaelis Constants for Various Conditions of NTPase

	Km (M)	Km' (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}

Km's were obtained from reciprocal plots of the kinetic data at 0.05M KCl, 0.05M tris-Cl (pH 7.4), and 25°C; Km for activities in the absence of added $CaCl_2$, and Km' for activities at 10mM $CaCl_2$.

B. Inhibition by Magnesium

The ATPase activity as a function of Mg^{2+} concentration added
to the reaction mixture shows that the effective concentration of
Mg^{2+}, which modifies 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 modi-
fication of 10^5 g of myosin, the inhibition by Mg^{2+} becomes un-
observable (Fig. 12). Plotting the experimental results of Fig. 12
in the manner described for Fig. 7 gives two curves (Fig. 13):
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
Kapp of the order of $10^6 M^{-1}$, which is much larger than for the
formation of $MgATP^{2-}$. This suggests that a binding reaction bet-
ween 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 of myosin and
$^{45}Ca^{2+}$. This finding is in agreement with the observed replacement
of the bound Ca^{2+} from myofibrils by Mg^{2+} (Seidel and Gergely,
1963), or the lack of Ca^{2+} binding to myosin in the presence of
2.0mM $MgCl_2$ (Fuchs and Briggs, 1968). Assuming competition between
Ca^{2+} and Mg^{2+} for the site on myosin, the value of Kapp for the
binding of Mg^{2+} to myosin can be estimated as $2.1 \times 10^5 - 3.0 \times 10^5 M^{-1}$ (Table 10). 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 $CaATP^{2-}$
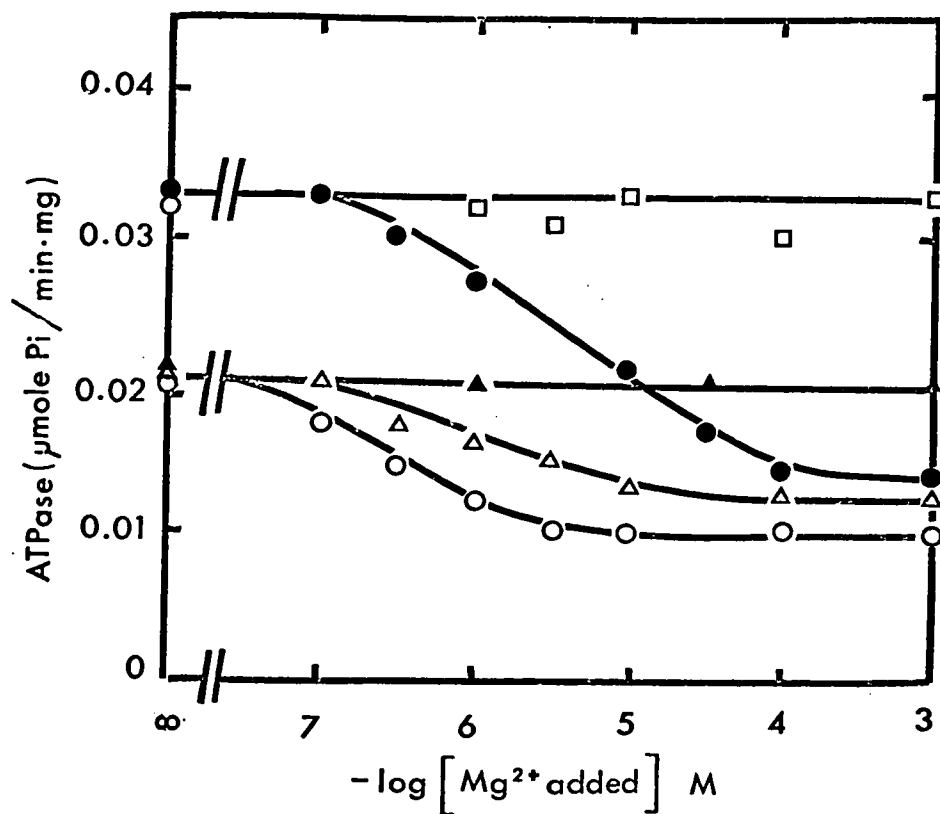can be assumed as negligible. With 1mM Mg^{2+}, a low value of Kapp

Figure 12. Mg^{2+} effect on myosin ATPase untreated or treated with pMB. ATPase: 0.05M KCl, 0.05M tris-Cl (pH 7.4), 25°C. Untreated: (○), 0.1mM ATP; (●), 1.0mM ATP. Treated myosin (1.5 moles pMB/ 10^5 g): (△), 0.1mM ATP. (2.5 moles pMB/10^5 g.): (▲), 0.1mM ATP; (□), 1.0mM ATP. Protein concentration in all cases was 0.33 mg/ml of reaction mixture.
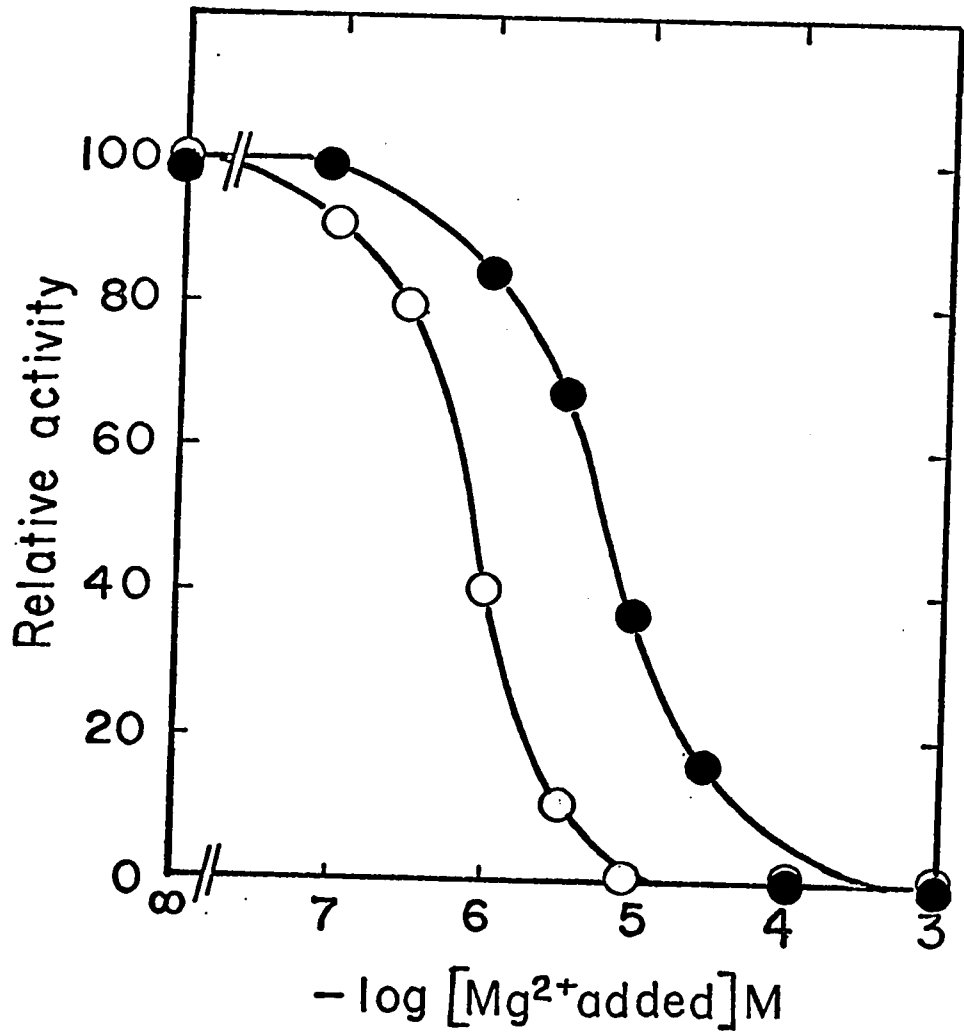
Figure 13. Relative activities for untreated myosin. The symbols are the same as shown in Figure 11. The relative activities were calculated from the data presented in Figure 11.

Table 10

Kapp for Binding of Mg^{2+} to Myosin Calculated From

the Ca^{2+} Binding Reaction

10^8 x {Ca^{2+} added} (M)	10^4 x {Mg^{2+} added} (M)	10^8 x {Ca^{2+} bound} (M)	10^5 Kapp for binding of Mg^{2+} (M^{-1})
3.0	–	2.4	2.2
3.0	0.1	2.0	3.0
3.0	1.0	1.0	3.0
3.0	3.6	0.5	2.1
3.0	10.0	0.2	0.59

Determinations were carried out in 0.05M KCl, 0.05M tris-Cl (pH 7.4) with 1.0mM ATP at 25°C.

is obtained, presumably because of secondary effects of the bound Mg^{2+} on myosin.

As an attempt to detect the Mg^{2+} binding to myosin directly, the reaction of calmagite with divalent cations was used to measure the concentrations of Mg^{2+} in the filtrate obtained by expressing the buffer solution containing variable amounts of Mg^{2+} through a membrane (100 mμ pore size). The addition of myosin to the buffer was shown to reduce the amount of Mg^{2+} in the filtrate. Assuming that this reduction is caused by the Mg^{2+} binding to myosin, the difference between Mg^{2+} contents between the filtrates obtained from the solutions with and without myosin was plotted as a function of the Mg^{2+} concentration added to the buffer as shown in Fig. 14. The plot in this figure appears to approximate the curve calculated using a value of 3.2 x $10^6 M^{-1}$ for the association constant and that of 0.8 for the number of binding sites per mole of myosin (molecular weight of 500,000 daltons) in a binding reaction between Mg^{2+} and myosin, which follows the mass action law. The two other curves drawn in Fig. 15 depict the range of affinities which cover the results with a slight alteration in the number of binding sites. There is some difference in magnitude between the binding affinities determined in this way and those calculated from the competition with Ca^{2+}, although either set of values show a binding remote from the formation of $MgATP^{2-}$. Fig. 15 shows that the decrease in relative activity follows the amount of Mg^{2+} binding, which was calculated by using Kapp values of $10^6 M^{-1}$ for the Mg^{2+} binding reaction, and 1.89 x $10^4 M^{-1}$ for the formation of $MgATP^{2-}$. On the other hand, the $MgATP^{2-}$ complex may be as good a substrate as ATP^{4-} or $HATP^{3-}$
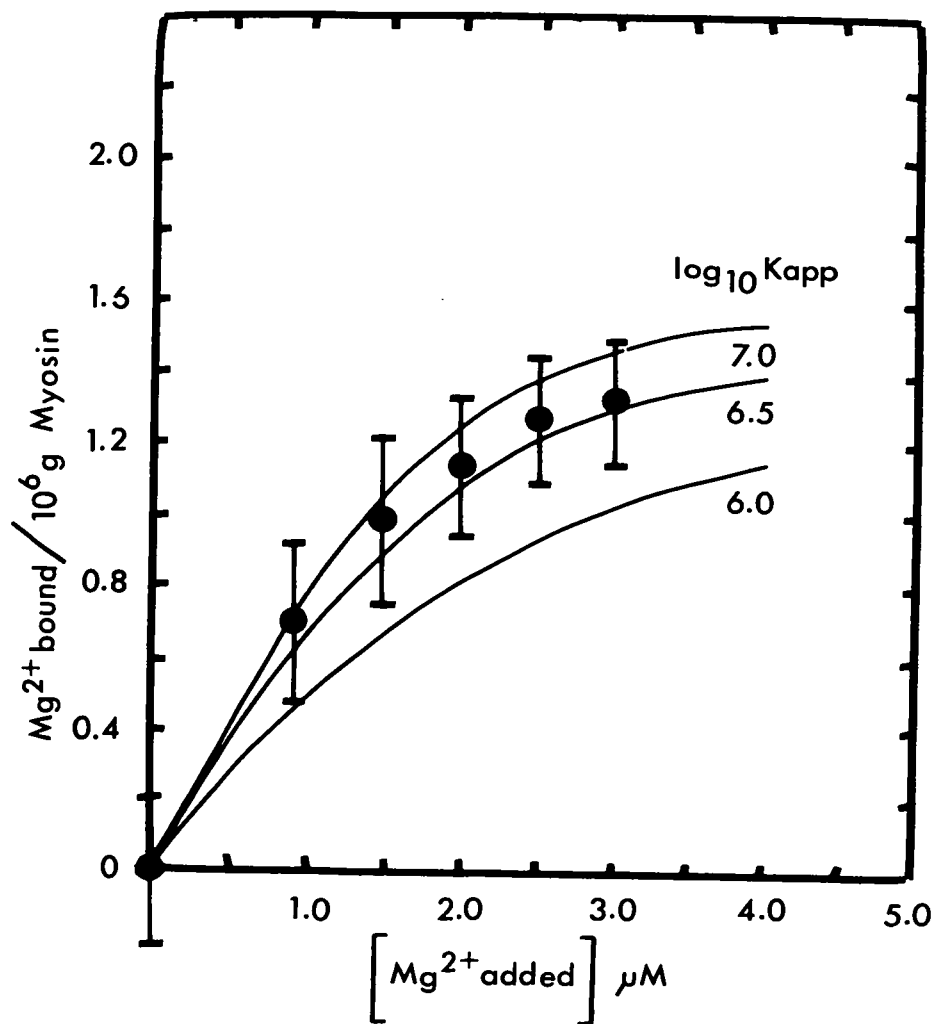
Figure 14: The Mg^{2+} binding reaction to myosin using calmagite. Conditions: 0.01M KCl, 0.01M histidine (pH 7.4), 1.0 mg myosin/ml, 25°C. The points indicate the amount of bound Mg^{2+} and the curves represent the computed formation of bound Mg^{2+} assuming that 0.8 mole Mg^{2+} is binding to one mole of myosin (500,000 daltons). The vertical bar in this figure indicates the sume of the deviations due to the range of duplicates in the determination of free Mg^{2+} and the standard error of the calibration curve (Fig. 3), which is 1.0 ± 0.15 µM Mg^{2+}.
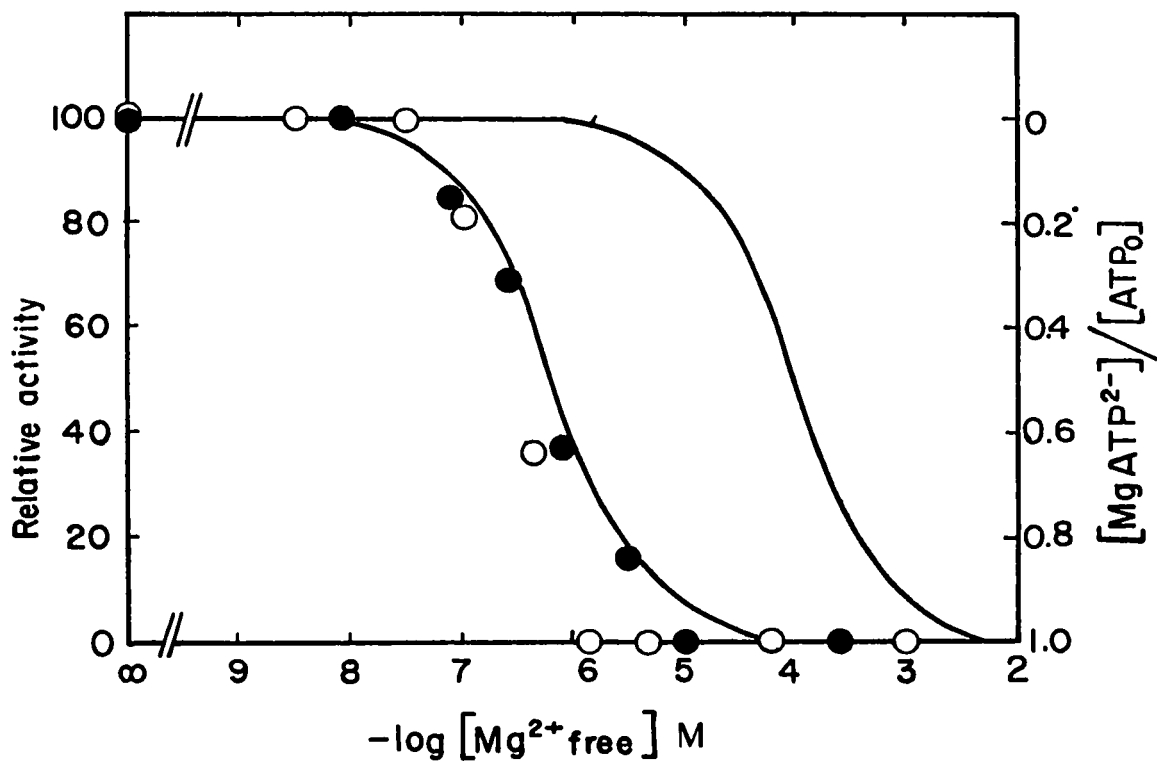
Figure 15. Relative activity of untreated myosin as a function of
the calculated concentration of free Mg^{2+}. The relative activities
were calculated from the data presented in Figure 11. The curve
drawn through the experimental points represents binding of Mg^{2+}
to myosin, and the continuous curve on the right denotes the
formation of $MgATP^{2-}$.

since even when essentially all the ATP molecules are in the form of MgATP^{2-}, the pMB-modified myosin does not show significant inhibition of the ATPase by Mg^{2+} (Fig. 12).

C. <u>Enzymatic Activity in the Presence of Both Calcium and Magnesium</u>

It was suggested above that Ca^{2+} affects the NTPase by a weak binding of Ca^{2+} to myosin, or 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. 16 shows the effect of Mg^{2+} on the ATPase and ITPase activities in the presence of a fixed amount of Ca^{2+}. In Fig. 17, the relative activity as defined in Fig. 8 is plotted against the amount of added Mg^{2+}. The results in Fig. 17 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 1.0mM, the amount of Mg^{2+} required to inhibit the enzymatic 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 enzymatic activity and Mg^{2+} concentration. For instance, in the presence of 0.1mM NTP and 1.0mM 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

Figure 16. Enzymatic activities in the presence of Ca^{2+} and Mg^{2+}. The NTP concentration was 0.1mM in all cases. (a), ATPase; (b), ITPase; (c), modified myosin ATPase (2.5 moles $pMB/10^5$ g. myosin): (O), no added $CaCl_2$; (●), 3.2 x $10^{-5}M$ added $CaCl_2$; (△), 1.0 x $10^{-3}M$ added $CaCl_2$. The protein concentration was 0.33 mg/ ml of reaction mixture in the absence of $CaCl_2$ and 0.12 mg/ml when $CaCl_2$ was added.
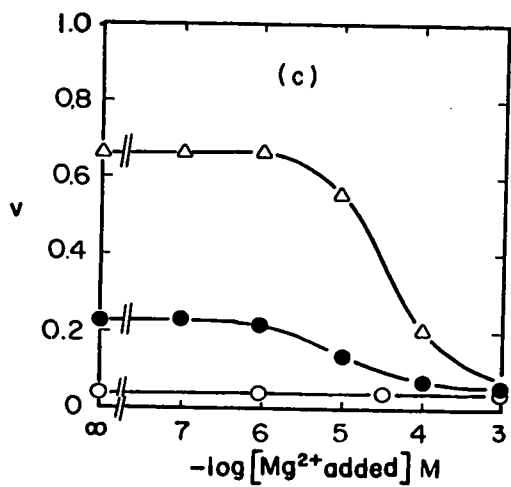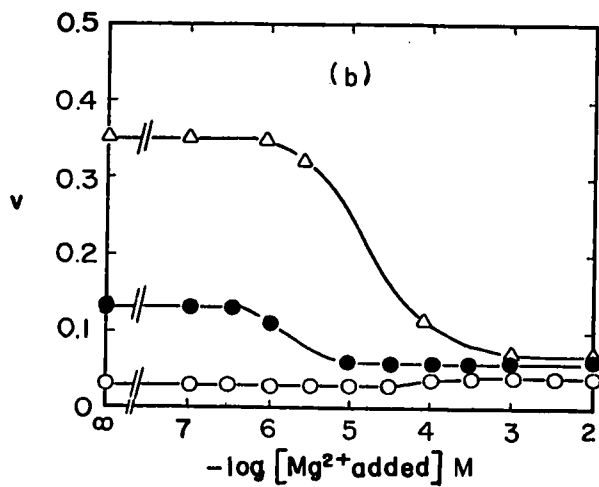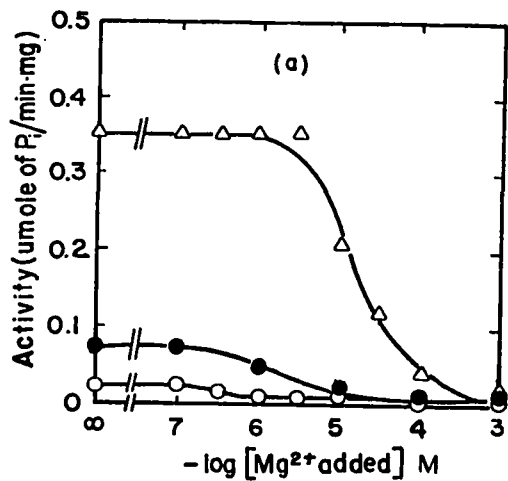
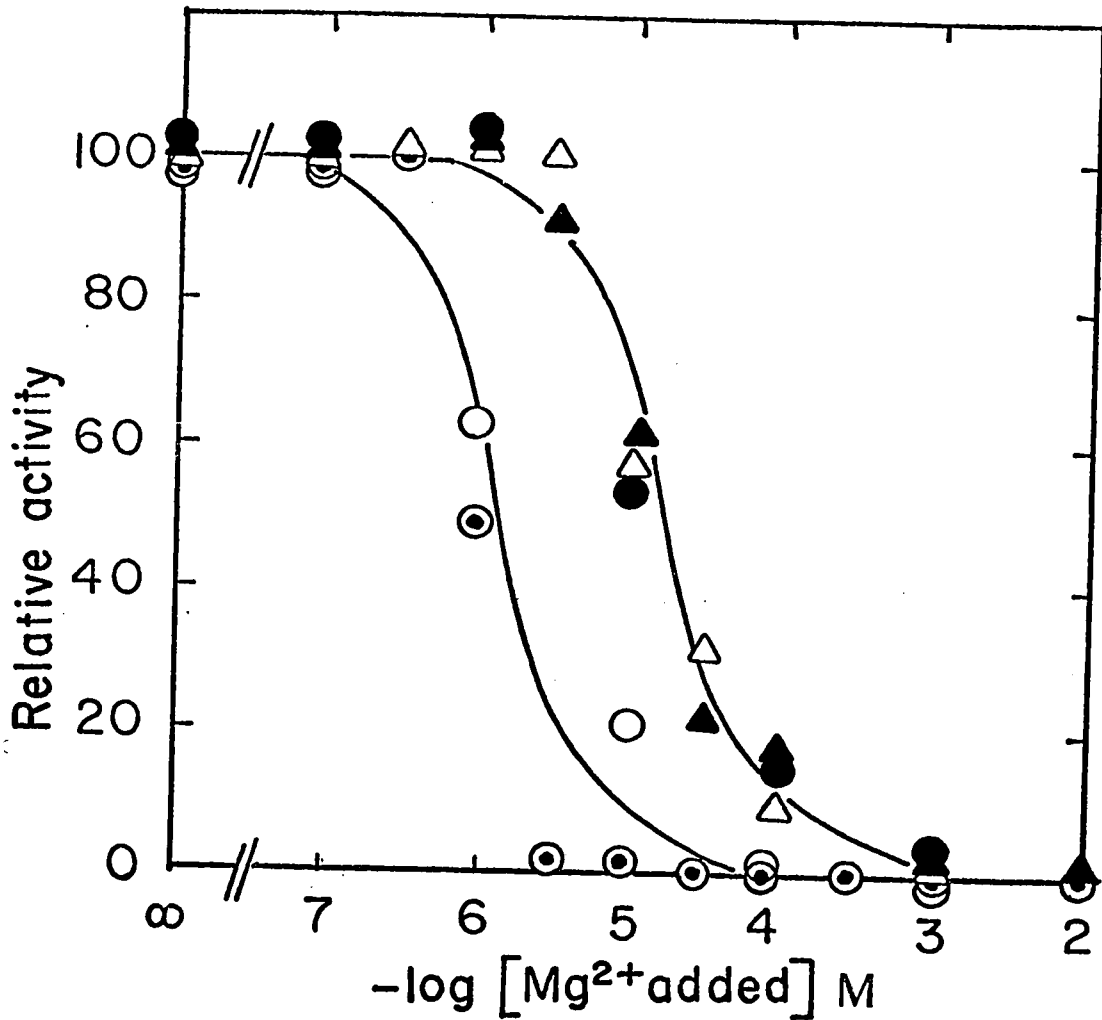Figure 17. Relative activity (calculated from the results shown in
Fig. 15) as a function of the amount of added Mg^{2+}. The NTP con-
centration was 0.1mM in all cases. The circles indicate the value with
$3.2 \times 10^{-5}M$ added Ca^{2+}, and the triangles with $1.0 \times 10^{-3}M$ added
Ca^{2+}. (O), ATP; (⊙), ITP; (△), ATP; (▲), ITP. Modified myosin
(2.5 moles of pMB/10^5 g.): (●), ATP, of myosin ATPase.

by Mg^{2+}, they become susceptible to the Mg^{2+} effect if activated first by Ca^{2+} (Fig. 16b and 16c). The relative activity of ITPase plotted against Mg^{2+} coincides with the results obtained with unmodified ATPase (Fig. 17). However, the pMB-modified ATPase shows some resistence to the inhibitory effect of Mg^{2+}, suggesting a weakened binding of Mg^{2+} to the pMB-modified myosin.

2. The Effects of Calcium and Magnesium on the Actomyosin

Adenosine Triphosphatase and Superprecipitation

A. Activation by Calcium

The effect of various concentrations of Ca^{2+} on the actomyosin ATPase activity was analysed at low concentrations of KCl, using the same procedure as for the analysis of myosin ATPase. The chelating agent, EDTA, was added to the reaction mixture to eliminate the effects of divalent cations when necessary (Nihei, 1967). The time course of ATP hydrolysis in the presence of Ca^{2+} shows a curvature which appears to consist of two phases (Fig. 18). This was not observed with the myosin ATPase. Plotting the reaction rate from both the initial and slow phases of the activity shows a Ca^{2+} activation of the ATPase reaching a maximum at 10mM added Ca^{2+} (Fig. 19), in fair agreement with the Ca^{2+}-ATPase of myosin.

In conjunction with the determinations of ATPase, the rate and extent of superprecipitation were evaluated. From the change in turbidity recorded with time, the rate of superprecipitation was taken as the reciprocal of the time required to reach 50% of the maximum extent ($t\frac{1}{2}$). The extent here is expressed by the difference between the
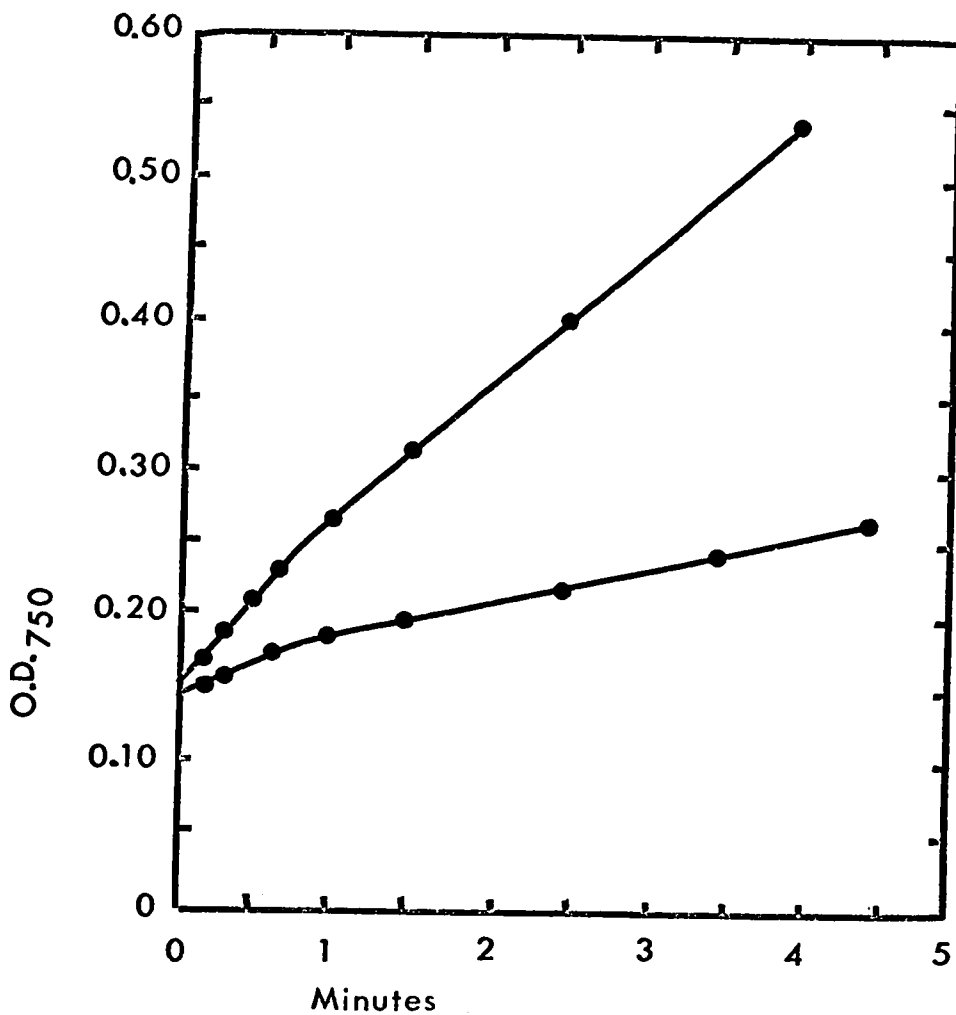
Figure 18. Time course of the Ca^{2+}-ATPase of actomyosin at different concentrations of added $CaCl_2$. Condition: 0.005 - 0.012M KCl, 0.05M tris-Cl (pH 7.4), 1.0mM ATP, 1.0mM EDTA, 0.188 mg actomyosin/ml, 25°C. Lower curve, 1.5 x 10-3M $CaCl_2$, upper curve, 1.0 x 10-2M $CaCl_2$.
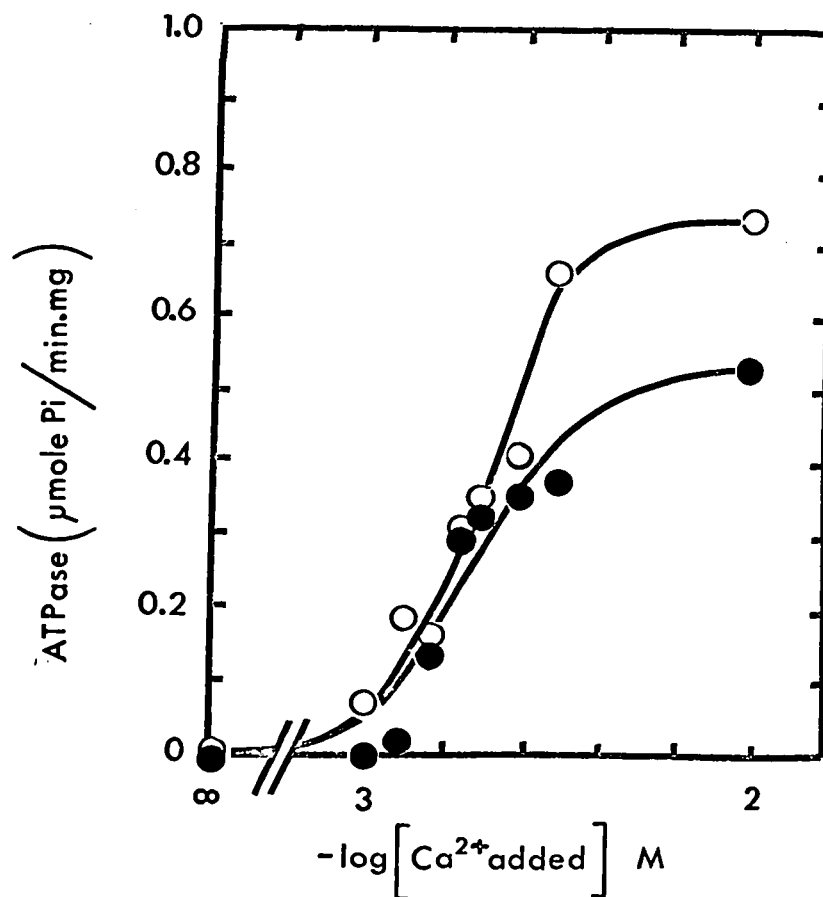
Figure 19: Effects of Ca^{2+} on the ATPase of actomyosin. Same conditions as in Figure 17. (○), ATPase, initial phase; (●), ATPase, late phase.

absorbances of the fully superprecipitated actomyosin and the starting solution. The time course of Ca^{2+} activated superprecipitation resembles the previous results for superprecipitation (Nihei and Yamamoto, 1969), but from the values of the time constant ($t\frac{1}{2}$), the Ca^{2+} activation is clearly more sluggish than the Mg^{2+} effect (Table 11), where the $t\frac{1}{2}$ value is about one second for optimal concentrations of Mg^{2+}.

From a comparison of the rate of superprecipitation with the ATPase activity, it appears that the aggregate formation of actomyosin retards the diffusion of substrate and/or product, which becomes the rate limiting step. The initial activity is not the pre-steady state burst (Kinoshita et al., 1969; Lymn and Taylor, 1970) which occurs at a time in the order of milliseconds.

The experimental data for ATPase and superprecipitation are expressed as relative activity, using the formula previously found successful for the analysis of the myosin NTPase activity. The relative activity was plotted against the concentration of free Ca^{2+} to test the possibility of a Ca^{2+} binding reaction being responsible for the activation. The free Ca^{2+} was calculated as that which is not bound to ATP or EDTA, using the known association constants for ATP (Table 7) and EDTA (Chaberek and Martell, 1959). From a comparison of the plot in Fig. 20 to the myosin ATPase data (Fig. 8), it seems that actin has not altered the nature of the Ca^{2+} effect on myosin. The activation of actomyosin ATPase may thus be interpreted as being a weak Ca^{2+} binding reaction, or a more rapid hydrolysis of the $CaATP^{2-}$ complex. The extent of superprecipitation is about the same at various concentrations of Ca^{2+}, with a slight increase at 10mM $CaCl_2$. This increase

Table 11

The Rate of Superprecipitation of Actomyosin in the Presence of Ca^{2+}.

{Ca^{2+} added} mM	$t\frac{1}{2}$ seconds
1.00	1,070*
1.25	54.3
1.50	18.3
1.75	16.5
2.00	17.4
1.50	12.7
3.00	8.6
9.00	8.0
10.00	8.0

Determinations were carried out in 0.005 - 0.012M KCl, 0.05M tris-Cl (pH 7.4), 1.0mM ATP, 1.0mM EDTA, 0.02 mg actomyosin/ml.

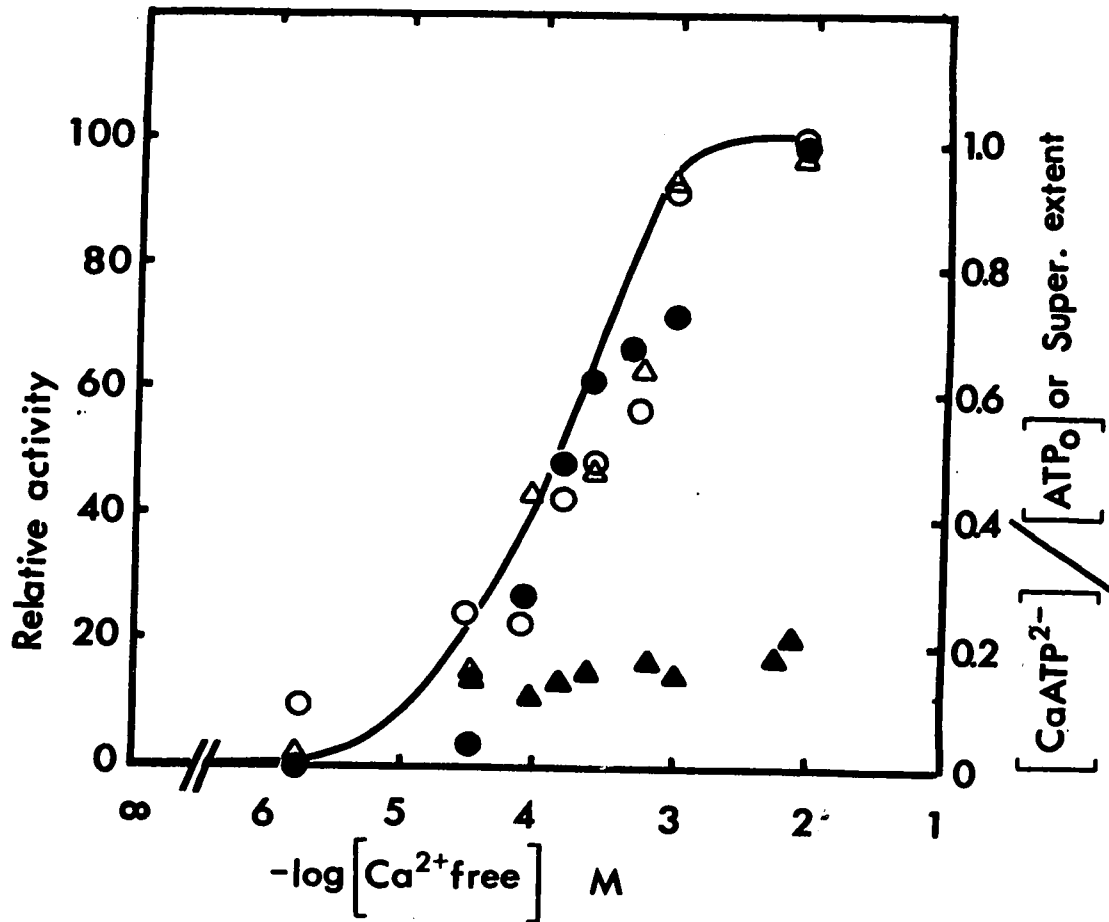*The maximum extent was not recorded, but assumed to be the same as in the presence of 1.25mM $CaCl_2$.

Figure 20:   Relative activity of the effects of Ca^{2+} on the ATPase
and superprecipitation of actomyosin.   (O), ATPase, initial
phase; ($\bullet$), ATPase, late phase; ($\triangle$), rate of superprecipitation;
($\blacktriangle$), superprecipitation extent.

is probably due to a secondary precipitation of myosin by $CaCl_2$ which has been found to occur at this concentration (Szent-Györgyi, 1951).

B. Activation by Magnesium

The Mg^{2+} activation of actomyosin ATPase occurs at concentrations of Mg^{2+} lower than was required for the Ca^{2+} activation (Fig. 21). It is important to measure the activity at low KCl concentrations, since at 0.1M KCl, added Mg^{2+} in the concentration range of 0.1-1.0mM inhibits the ATPase. In addition to natural actomyosin, a mixture consisting of a 10 molar excess of actin over myosin was used. Under the conditions of the experiment, over 80% of the myosin is bound to actin, as will be shown in the section on actin-myosin combination. Using the same mixture of actin and myosin, the ATPase activity was determined at various concentrations of ATP with an excess of Mg^{2+} (5.0mM). From a reciprocal plot of the experimental data, it is observed that the activity follows Michaelis-Menten kinetics (Fig. 22) with a value for Km of 0.05mM, compatible with that previously estimated by Schiselfeld et al., (1970). The relative activity of actomyosin ATPase is calculated from the results of Fig. 21, as previously described. The plot of Fig. 23 suggests that the binding of Mg^{2+} to actomyosin is responsible for the activation as in the case of the inhibition of myosin. These findings are also in fair agreement with those of Nihei (1967). In consequence, an attempt was made to determine the Mg^{2+}-binding to actomyosin. This was unsuccessful however, in that too much deviation occurred in the experimental data to make any conclusions about the magnitude of the binding constant or the number of sites. According to the result shown in Fig. 24, there is no detectable binding of Mg^{2+} to
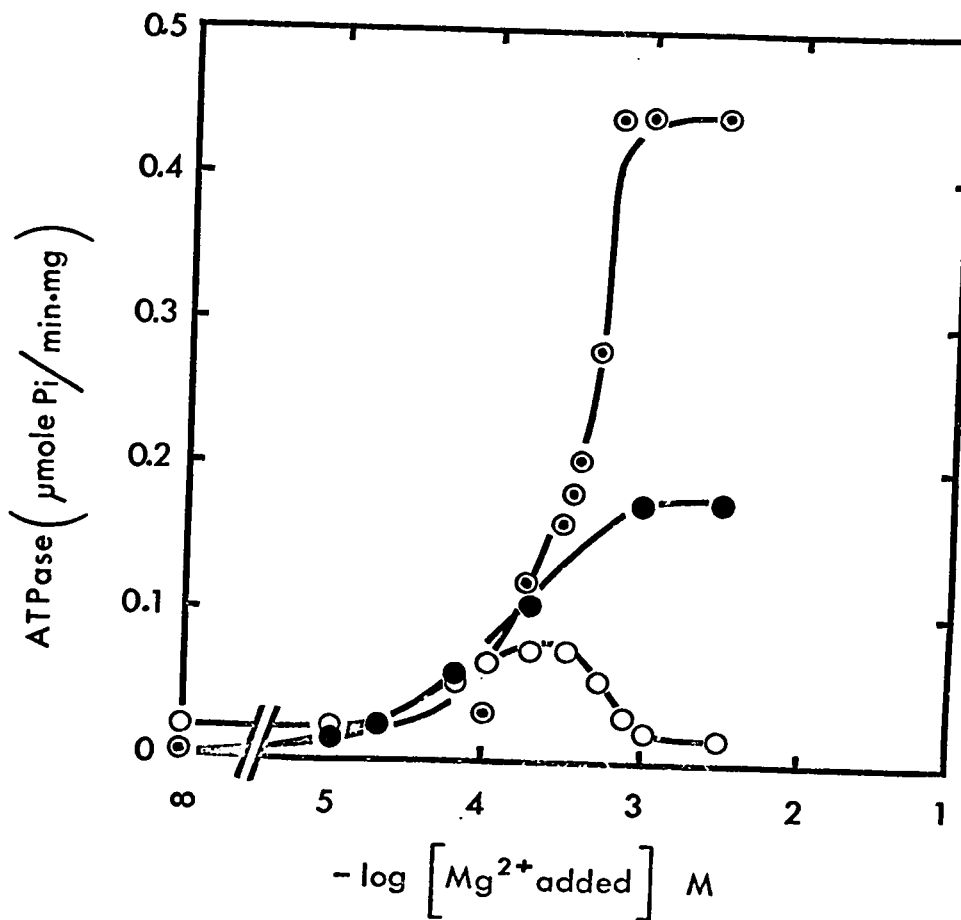
Figure 21: The effect of Mg^{2+} on actomyosin at different KCl
concentrations. ATPase: 0.02M tris-Cl (pH 7.4), 1.0mM EDTA,
25°C. Natural actomyosin, 1.0mM ATP, 0.19 mg/ml: (○),
0.1M KCl; (●), 0.012M KCl. Reconstituted actomyosin, 0.1mM
ATP, 0.11 mg actin/ml, 0.09 mg myosin/ml (10:1 actin to myosin
molar ratio, activity calculated per mg. myosin): (◉), 0.014M
KCl.
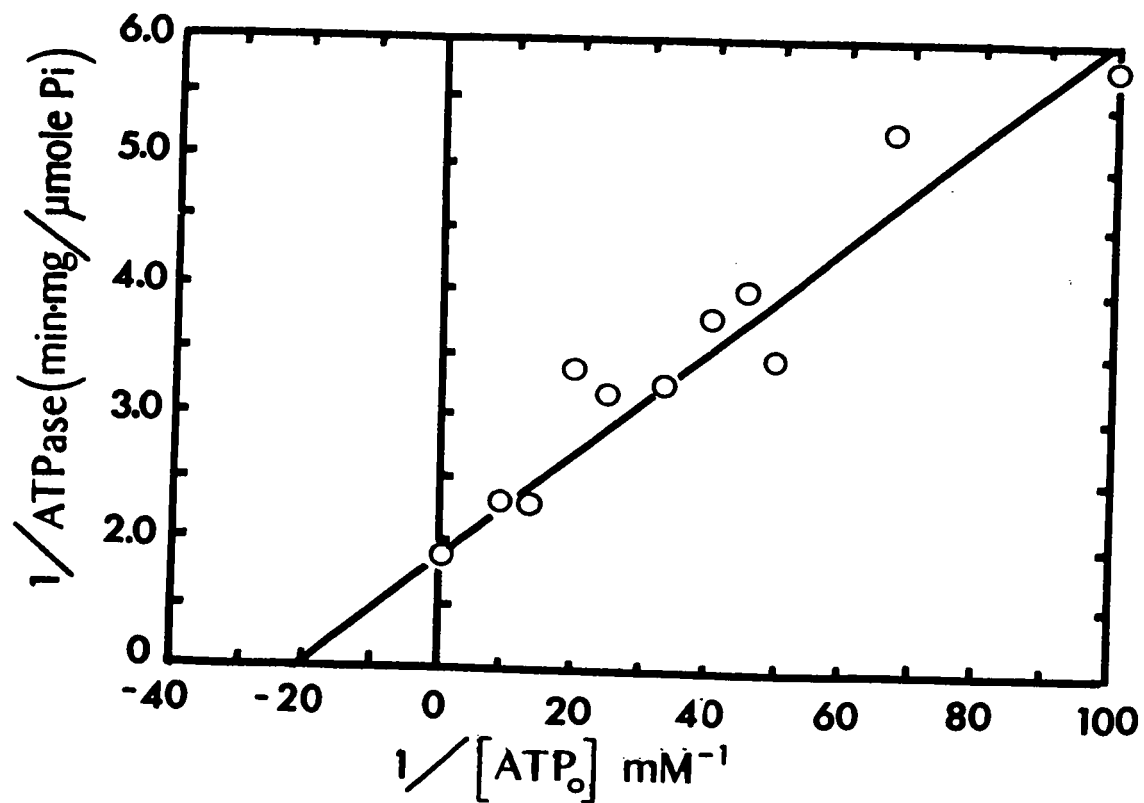
Figure 22: Reciprocal plot of actomyosin ATPase. Conditions: 0.014M KCl, 0.02M tris-Cl (pH 7.4), 5.0mM $MgCl_2$, 1.0mM EDTA, 0.11 mg/ml, 0.09 mg myosin/ ml, 25°C.
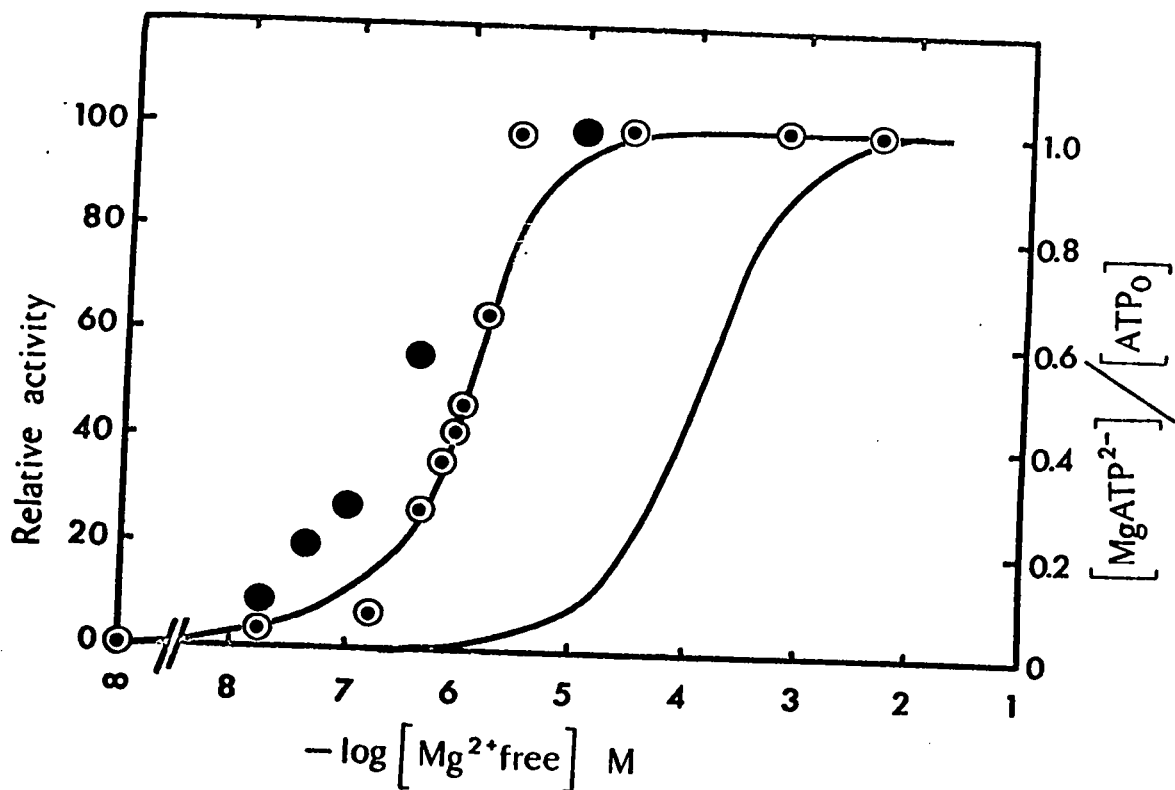
Figure 23: Relative activity of actomyosin as a function of the calculated free Mg^{2+}. The symbols are the same as in Figure 20. The curve drawn through the experimental points represents the binding of Mg^{2+} to actomyosin and the continuous curve to the right depicts formation of $MgATP^{2-}$.
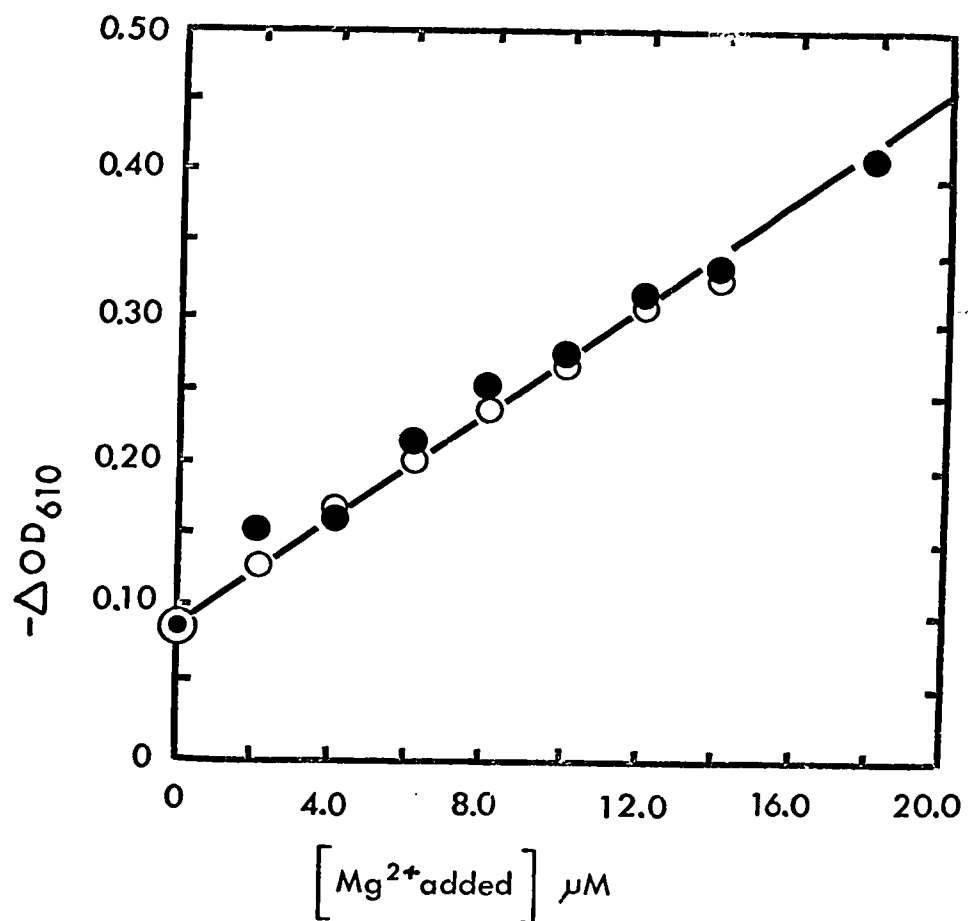
Figure 24: The Mg^{2+} binding reaction to F-actin using calmagite. Conditions: 0.1M KCl, 0.01M histidine (pH 7.4), 0.28 mg F-actin/ml, 25°C. (O), total Mg^{2+}; (●), free Mg^{2+} in filtrate.

F-actin within the range of Mg^{2+} concentrations studied. Therefore any detectable binding which occurs in a mixture of actin and myosin can be regarded as that occurring to myosin.

3. The Preparation of the Papain-Hydrolyzed
Subfragment of Myosin

Mueller and Perry (1961, 1962) demonstrated for the first time, that the heavy meromyosin fragment prepared by tryptic digestion of myosin, can be further hydrolyzed by trypsin to yield a subfragment of smaller molecular weight which is capable of binding with actin and of hydrolyzing ATP. The observations of the tryptic subfragment with hydrodynamic techniques and electron microscopy indicated that it is a globular particle with a molecular weight ranging from 100,000-170,000 (Mueller and Rice, 1964; Young et al., 1965; Mueller, 1965). As mentioned earlier, it has been reported that the subfragment comes from the thickened end of the myosin molecule. In recent years, the papain digestion of myosin has been shown to release the subfragment from myosin in a single digestion step (Kominz et al., 1965). By using an insoluble cellulose-papain complex, the digestion is easily terminated by centrifugation or ultrafiltration. The light meromyosin portion of the digest can be separated by centrifugation at low ionic strength and the subfragment may be subsequently isolated from the supernatant by Sephadex chromatography (Nihei and Kay, 1968; Lowey et al., 1969), or by the centrifugation of an actin-subfragment complex (Mueller and Perry, 1962; Tada et al., 1969).

The size of the subfragment from the papain digestion of myosin has been estimated as ranging from 110,000-140,000 daltons (Kominz et al., 1965; Nihei and Kay, 1968; Lowey et al., 1969).

As suggested by Lowey et al., (1969), the population of active

subfragment (HMM-S1) molecules is likely to be heterogeneous due to an under- or over-digestion of the tail region of myosin or an internal proteolysis of active subfragment. Also, the active subfragment is well known for its tendency to aggregate.

Jones and Perry (1966) reported that prolonged digestion with trypsin attacks the actin-combining site without affecting the ATPase activity, leading to the conclusion that these two properties of myosin are due to different sites on the subfragment molecule.

The experiments described in this section were carried out to obtain a homogeneous preparation of the myosin subfragment which could be used for the detailed analysis of interactions between actin and the subfragment under various experimental conditions.

In the early stage of the experiments, the subfragment was prepared from digested myosin which had released four equivalents of H^+ per 10^5g of protein mass during 10 minutes of reaction. This process was shown to convert one-third of the myosin mass into a chromatographically separable single component from which the ATPase activity (Nihei and Kay, 1968) of the parent molecule can be recovered. Thereafter, the hydrolysis slowed down, presumably because less-susceptible regions remained for proteolytic attack (Mihalyi and Harrington, 1959). In some experiments, preparations were made using a longer digestion period.

After the removal of insoluble light meromyosin by centrifugation, the digest was concentrated and chromatographed in a descending fashion on a Sephadex G-75 column in order to see if a significant proportion of the digested mass could permeate into

this gel. However, this seemed unlikely, since Fig. 25 shows that the majority of the protein material emerges in the void volume with a small amount of heterogeneous material appearing in later fractions. On the other hand, the chromatographic analysis on a column of Sephadex G-200 superfine exhibited a slight resolution of the main peak (Fig. 26). The protein concentration for these fractions was determined at 280 mμ, where the subfragment extinction coefficient was taken as 770 cm^2/g (Young et al., 1965). The right hand peak of the chromatogram (hatched area), representing lighter material, was found to contain most of the ATPase active component.

Rechromatography of the pooled fractions (hatched area) resulted in the pattern depicted in Fig. 27, showing most of the protein in a single peak, but also showing a certain amount of heavier material. From previous studies on papain-digested myosin, a single peak isolated by chromatography in this way was found to show two peaks in the ultracentrifuge (Kominz et al., 1965). One of these two components was subsequently shown to combine with actin, hydrolyze ATP and sediment with S$^o_{20}$,w of 2.7 (Lowey et al., 1969). The latter is believed to represent the tail portion of heavy meromyosin (Lowey et al., 1969) and has a high helical content. However, it is water soluble like subfragment and could only be removed from the mixture by chromatographic recycling which was found to be quite tedious. On the other hand, if F-actin was added to a myosin digest, the resulting actin-subfragment could be separated by centrifugation. Thus the subfragment separation should be accomplished in a single step (Mueller and Perry, 1962). In conjunction with this procedure, it was attempted
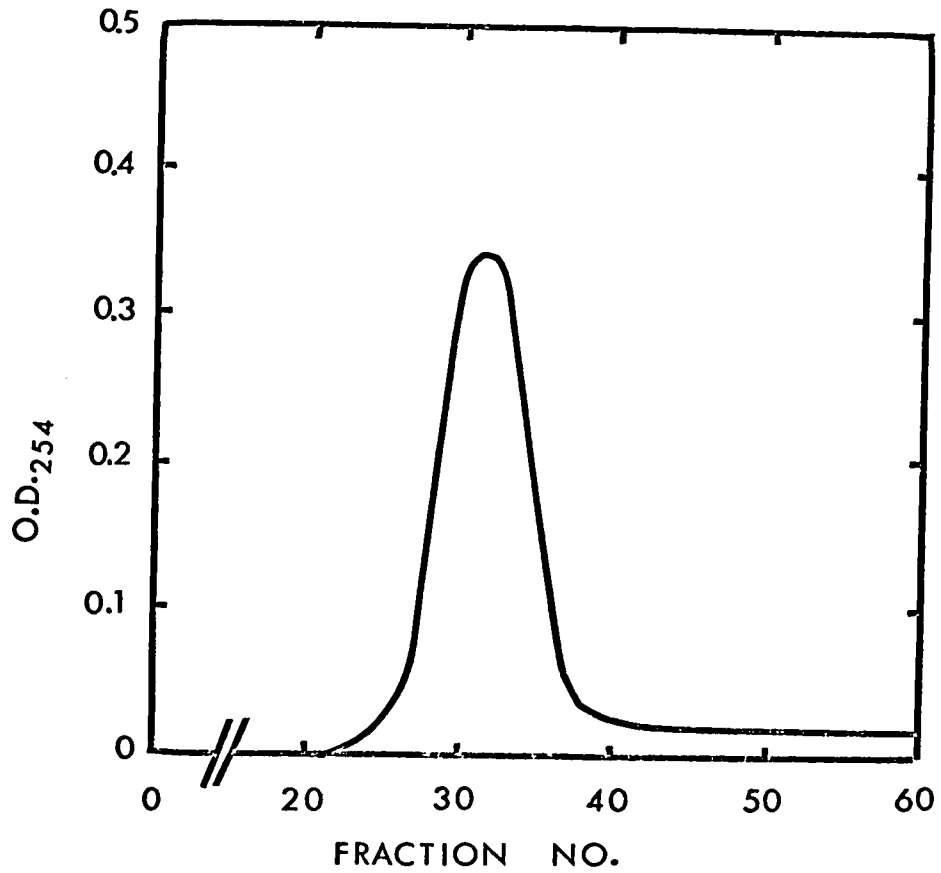
Figure 25: Chromatography of a papain digest of myosin on a Sephadex G-75 column. Applied 3 ml of concentrated digest to a 2.5 x 37 cm column pre-equilibrated with 0.05M KCl, 0.01M K_2HPO_4 (pH 7.4), 1mM D.T.T., $4^{\circ}C$, with a descending elution of 2.6 ml/hr where 20 minute fractions were taken.
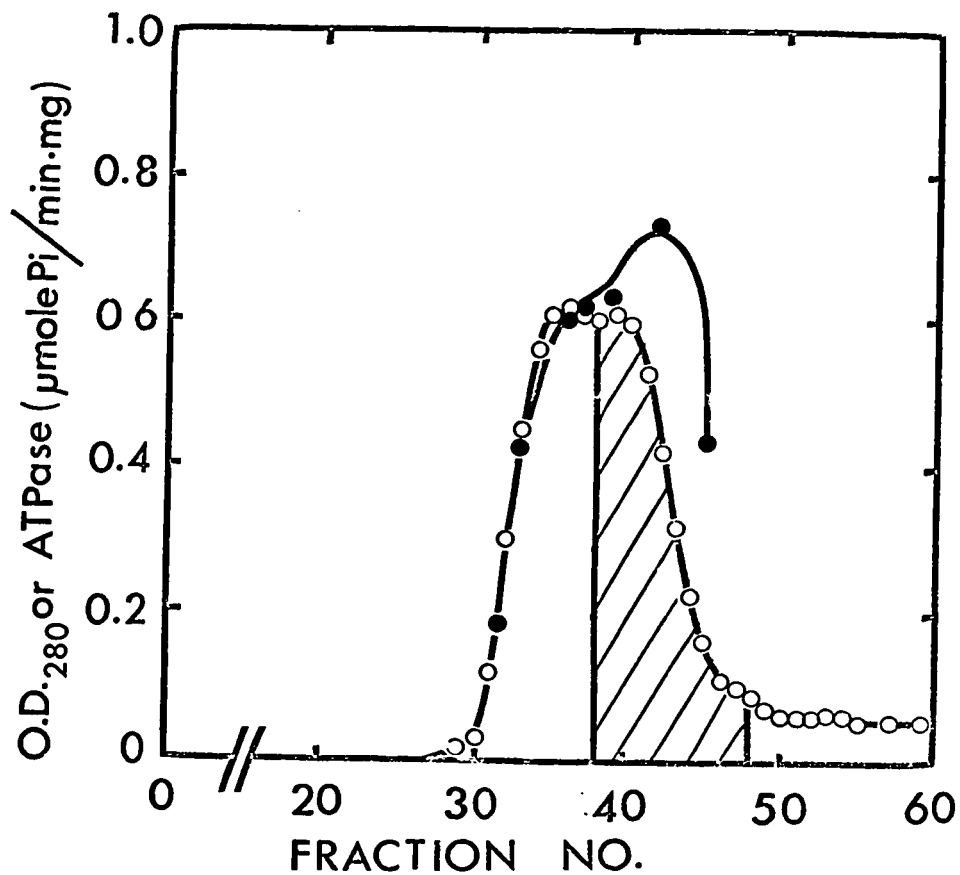
Figure 26: Chromatography of a papain digest of myosin on a Sephadex G-200 superfine column. Applied 3 ml of concentrated digest to a 2.5 x 30 cm column pre-equilibrated with 0.05M KCl, 0.01M K_2HPO_4 (pH 7.4), 1mM D.T.T., 4^oC, with a descending elution of 3 ml/hr where 1 hour fractions were taken. (O), $O.D._{280}$ from which protein concentration was calculated using 770 cm^2/g (Young, 1967b); (●), ATPase at 0.05M KCl, 0.05M tris-Cl (pH 7.4), 10mM $CaCl_2$, 1.0mM ATP, 25^oC.

Figure 27: The second chromatography on Sephadex G-200 superfine. Applied the pooled fractions from Fig. 26 (hatched area) (3 ml) to a 2.5 x 30 cm column which was pre-equilibrated and eluted as in Fig. 26.

to detect a component in the supernatant material which possesses ATPase activity but is not capable of combining with actin. According to the results listed in Table 12, successive additions of F-actin to the pooled fractions of the main peak of Fig. 27 revealed that all the ATPase active material in these fractions was removed by F-actin.
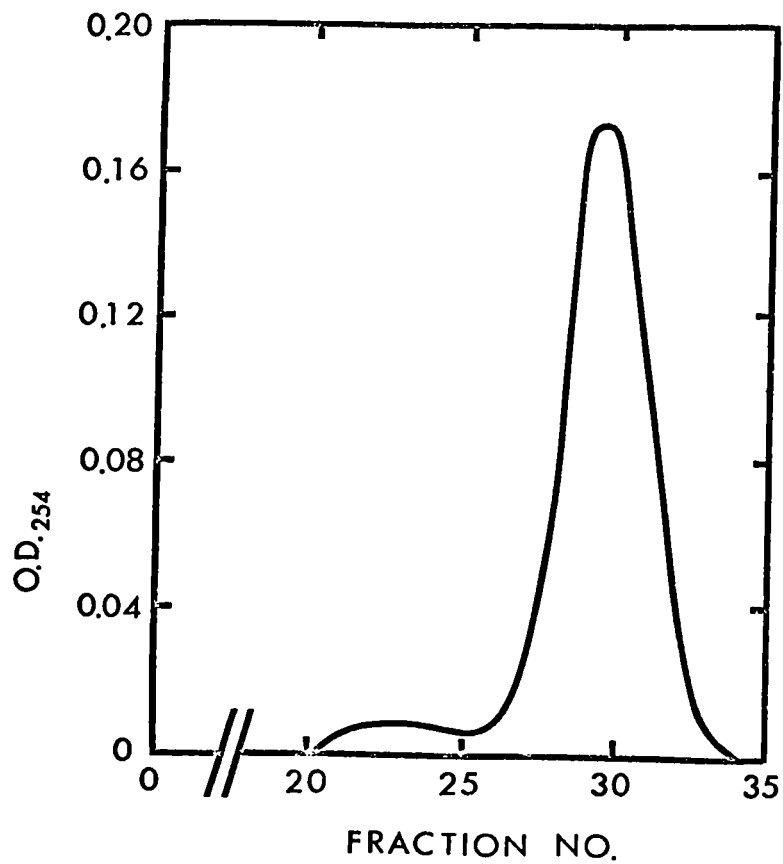
The subfragment was subsequently dissociated from F-actin by Mg-pyrophosphate (MgPP) and dialysed against 0.5M KCl, 0.01M K_2HPO_4 (pH 7.4), 1mM dithiothreitol (D.T.T.). Sedimentation studies revealed the presence of two components (Fig. 28, Plate I), but when the subfragment was treated with 0.1M β-mercaptoethanol, a single component was observed (Plate II). β-mercaptoethanol was found to equally effective at 0.02M. The observed S value of the single component in Plate II was found to be 5.0 at a protein con-centration of 7.1 mg/ml and at 20°C which is in agreement with the results of Jones and Perry (1966) for active subfragment.

In the absence of more comprehensive date, the sedimentation of the various species observed in Plate I of Fig. 28 was not extensively investigated, but it seems likely that the more rapidly sedimenting peak could be due to an aggregate, such as a dimer, and that this aggregation is due to disulfide formation. This is also evident from the presence of heavier material in the chromatography of the sub-fragment prepared through the actin binding step (Fig. 29).

In order to determine the molecular weight of subfragment, a high-speed sedimentation-equilibrium analysis of samples treated with β-mercaptoethanol was carried out according to Yphantis (1964). For this experiment, the centrifugal speed was chosen such that the concentration at the meniscus was negligible in comparison to the initial concentra-

Table 12

Recovery of Subfragment as the Actin-Subfragment Complex

Centrifugation	ATPase (μmole Pi/min. mg)	Protein remaining in supernatant (mg/ml)
Initial	1.30	1.18
1	0.34	0.62
2	0.08	0.57

An aliquot (7.6 mg of 4.4 mg/ml) of F-actin was added to the pooled fractions of the main peak (Fig. 27) (14 mg). The addition of F-actin to the supernatant was repeated thrice. After each centrifugation at 120,000 x g for 1 hour, the Ca^{2+}-ATPase activity in the supernatant was determined at 0.05M KCl, 0.05M tris-Cl (pH 7.4), 10mM $CaCl_2$, 1.0mM ATP, 0.05-0.10 mg subfragment/ml, at 25°C.

Figure 28: Sedimentation of the myosin subfragment. Conditions: 0.5M KCl, 0.01M K_2HPO_4 (pH 7.4), 7.1 mg/ml, 20°C. Plate I, 1mM D.T.T.; Plate II, 0.1M β-mercaptoethanol. Kel F cell, bar angle is 55° for first picture and 50° for remaining pictures. Time after reaching full speed (59,780 rev./min.) is indicated under each frame.
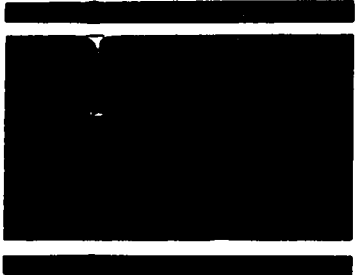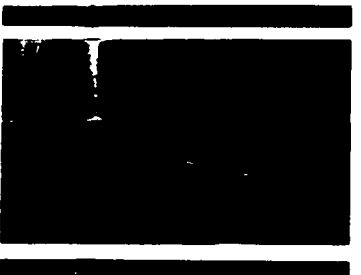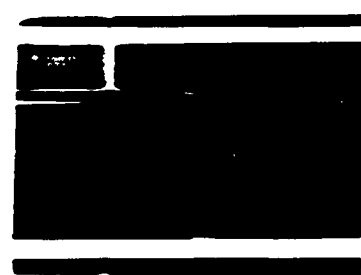
PLATE I

10
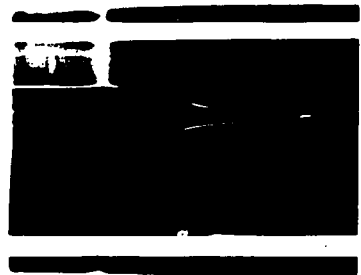
26

42

58

74

PLATE II

10

26

42

58

74
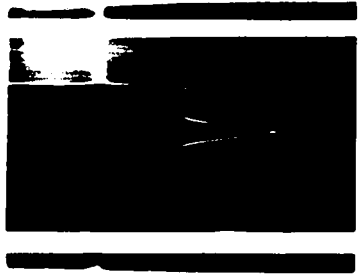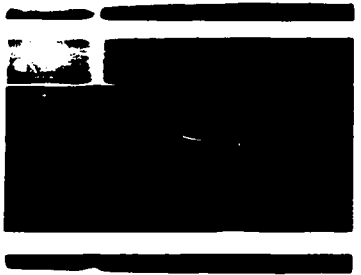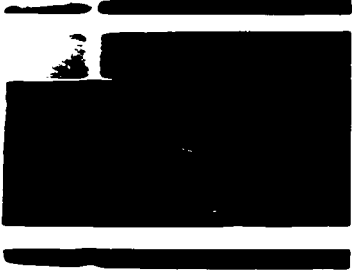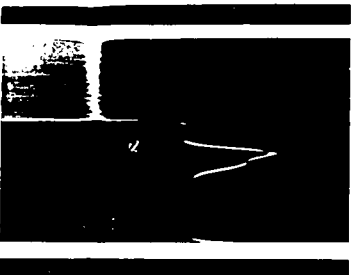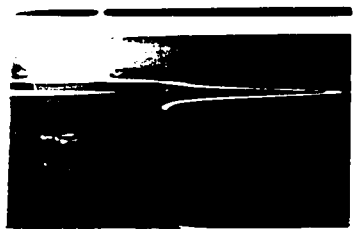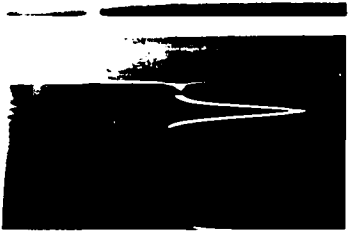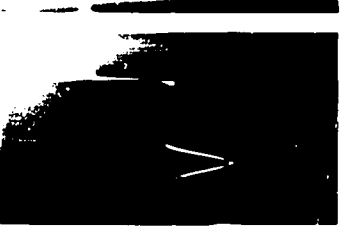
PLATE I
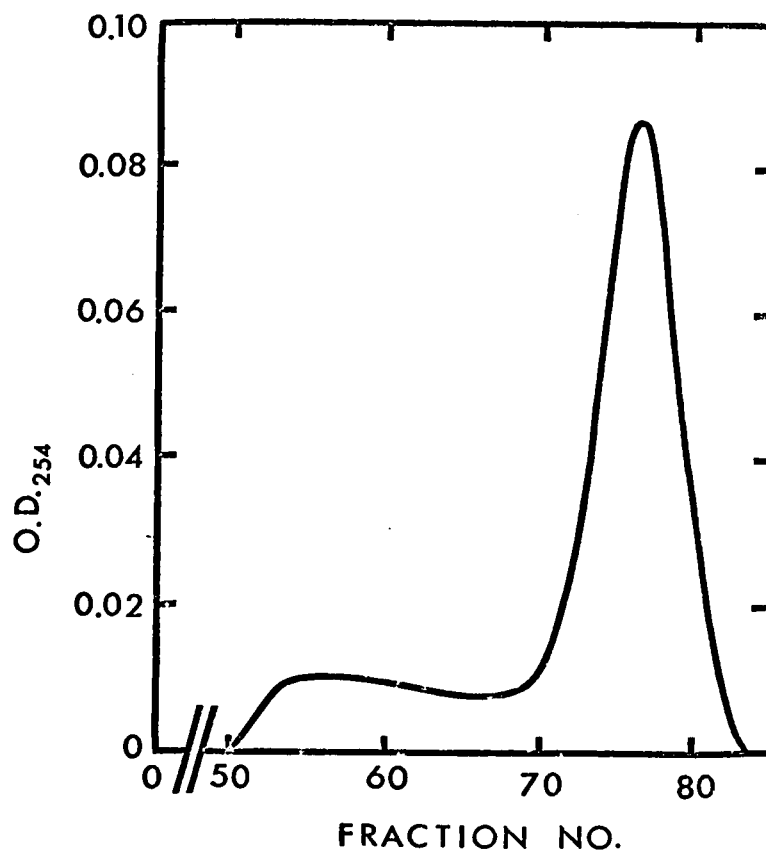
Figure 29. Chromatography of the actin-isolated myosin subfragment.
An aliquot (3 ml) was applied to the top of a 2.5 x 30 cm column
which was pre-equilibrated and eluted as in Fig. 26 except that 20
minute fractions were taken.

tion (see Fig. 5, Methods and Materials). In Fig. 30, a representative

plot of the change in log (natural) fringe displacement is shown as

a function of R^2, where R is the distance in cm from the center of the

rotation to the position of the considered fringe displacement. At the

highest protein concentration tested (1.8 mg/ml) a linear plot is ob-

tained without detectable heterogeneity. There is no indication of a

lighter species at or near the meniscus region. In all, five determina-

tions of the weight average molecular weight were carried out on two

different preparations of fragment (Table 13), assuming a partial spec-

ific volume of 0.74 ml/g (Mueller, 1965; Kominz et al., 1965). All

the determinations, including those at different centrifugation times,

show linear plots of log fringe displacement vs R^2 with an equivalent

molecular weight within experimental error, indicating that equilibrium

was attained by 24 hours at 18,000 rev./min. Preparation No. 2 was

digested for approximately twice the time as preparation No. 1 with

no apparent change in the molecular weight. Similar findings by

Lowey et al., (1969) support the idea that the subfragment molecule

is somehow protected from further papain digestion. There is no

observed concentration dependency within the range investigated, so

that the values for weight average molecular weight (Yphantis, 1964)

can be averaged to give a value of 116,000 ± 5,000, in excellent agree-

ment with previous results (Nihei and Kay, 1968; Lowey et al., 1969;

Tada et al., 1969). The findings reported here suggest that a prominent

factor in the discrepancy for reported values of the subfragment mole-

cular weight may be due to aggregation. Upon treatment with enough

sulfhydryl reducing agent, the subfragment appears homogeneous within
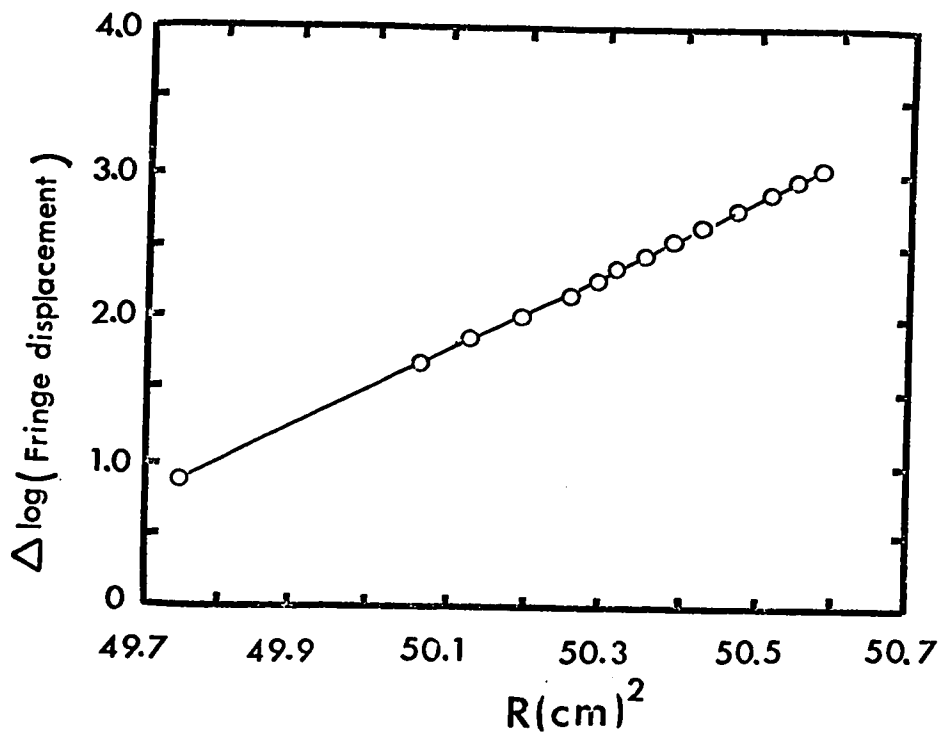
Figure 30. Sedimentation-equilibrium of the myosin subfragment.
Conditions: 0.5M KCl, 0.01M K_2HPO_4 (pH 7.4), 0.02M β-mercapto-
ethanol, 1.8 mg/ml. Run for 30 hours and 18,000 rev./min. at
4°C. The change in log fringe displacement (microns) was plotted
as a function of R^2, the distance in cm from the center of
rotation.

Table 13

The Sedimentation-Equilibrium of the Papain-Hydrolyzed

Myosin Subfragment

Myosin Digest	Run No.	Digestion Time in minutes	Protein Conc. (mg/ml)	Time of run in hrs	Mw app
1	1	10	0.5	30	120,000
	2	$(4H^+/10^5$ gm myosin)	1.0	24	110,000
	3		1.8	30	120,000
	4		1.8	46	120,000
2	5	19-27	0.9	30	110,000
					116,000±5,000

The preparations were made by the centrifugation of the actin plus myosin digest mixture. Conditions: 0.5M KCl, 0.01M K_2HPO_4 (pH 7.4), 4°C. All samples contained 0.02M β-mercaptoethanol. Density of solvent was 1.025 g/ml determined from the contribution of each dissolved species.

the range of experimental error shown in Table 13 although it cannot be ruled out that some heterogeneity exists due to an incomplete digestion or an internal proteolysis.

Table 14 depicts the increase in Ca^{2+}-ATPase at the various stages of subfragment preparation. Despite the considerable evidence of two ATPase active sites on myosin (see Introduction), the specific activity at subfragment is 3-4 times that of myosin, which is about twice as high as expected. This discrepancy may be due to the sulfhydryl reducing agents β-mercaptoethanol and D.T.T. used in the preparation of subfragment, which may increase the Ca^{2+} or Mg^{2+} activated ATPase in the same way that pMB or NEM does (Kielly and Bradley, 1956; Perry and Cotterill, 1965; see also Discussion).

Table 14

The Ca^{2+}-ATPase of Myosin Subfragment

Sample	ATPase (μmole Pi/min. mg.)		
	1	2	3
Myosin	0.44	0.47	0.41
Crude digest after separation of fraction unsoluble at 0.05M KCl	0.95		
Pooled fractions from first chromatogram (Hatched area Fig. 26)	1.30		
Actin isolated by centrifugation of an actin subfragment complex		1.62	1.65
Ratio of the activity of subfragment to myosin		3.4	4.0

Determined at successive stages in the preparation at 0.05M KCl, 0.05M tris-Cl (pH 7.4), 10mM $CaCl_2$, 1.0mM ATP, 0.12 mg myosin/ml, 0.05-0.10 mg/ml subfragment/ml at 25°C.

4. The Interactions of F-actin with Myosin and Subfragment

From the available information to date, the interaction between actin and myosin is considered as the molecular basis of muscle contraction. In this interaction, a part of myosin that can be separated as heavy meromyosin from the rest of the molecule makes contact with actin. It has been shown by many investigators that there are two sets of sites capable of binding with actin and catalyzing ATP hydrolysis in one unit of heavy meromyosin. The subfragment of myosin prepared through papain digestion possesses these sites. The detailed information concerning the interaction of this subfragment and actin was thought to give some insight into the mechanism of the actin-myosin interaction.

Previously, the affinity of actin to myosin was estimated assuming that the extent of the complex formation is proportional to the increment of viscosity or turbidity of the mixture of the two proteins. However, it was found not to be possible to measure the binding reaction at low concentrations of KCl (<0.1M KCl) since actomyosin becomes a hydrated gel capable of superprecipitation and the viscosity or turbidity increase associated with this process is distinct from that due to actomyosin formation.

An early investigation established the close relationship between the actin combining property of myosin and the ATPase activity (Bailey and Perry, 1947). It is known that the addition of F-actin to myosin results in an increase in the Mg^{2+}-ATPase at low concentrations of KCl (<0.1M). It is thus possible to use this property as a criterion of actin-myosin binding. This requires a consideration of the equilibria

for a mixture of F-actin, myosin Mg^{2+} and ATP. Accordingly, Botts and Morales (1953) and Botts (1958) formulated a kinetic scheme considering the actomyosin formation in the presence of a large molar excess of substrate.

Both KCl and ATP tend to dissociate F-actin from myosin, heavy meromyosin, or subfragment as reflected by a viscosity decrease or a decrease in the Mg^{2+}-ATPase activity (Gergely, 1956; Eisenberg and Moos, 1967; Eisenberg et al, 1968; Tawada and Oosawa, 1969; Rizzino et al., 1970). The experiments for the binding reaction between F-actin and myosin or subfragment have been conducted at low concentration of KCl and at high levels of ATP such that there would be almost complete substrate saturation of the myosin and actomyosin enzyme systems. The concentration of 1.0mM ATP used for the present analyses of the Mg^{2+}-ATPase of a mixture of actin and myosin is twenty times as high as the Km value for Mg^{2+}-ATPase (0.05mM, Fig. 21). For the measurement of activity in an actin-subfragment system the concentration of ATP was 0.5mM (Eisenberg et al., 1968; Szentkiralyi and Oplatka, 1969). This concentration seemed to saturate the enzyme with ATP since at increased concentration, e.g. 2.0mM, the results were the same as that at 0.5mM ATP (Eisenberg et al., 1968). An excess of $MgCl_2$ was added such that most of ATP was in the form of $MgATP^{2-}$, and consequently there was sufficient concentration of free Mg^{2+} to give maximum activation of the actomyosin and actin-subfragment ATPase.

The rate of ATP hydrolysis, v, catalyzed by a mixture of myosin and actomyosin which are saturated by ATP, can be expressed by the

following equation:

$$v = k_3\{M\} + k_3'\{AM\}$$

where k_3 and k_3' are the rate constants of the step at which the enzyme-substrate complex forms the products, and the concentrations of myosin and actomyosin are represented by $\{M\}$ and $\{AM\}$, respectively. This equation may also be written as:

$$v = k_3 (\{Mo\} - \{AM\}) + k_3' \{AM\}$$

where $\{Mo\} = \{M\} + \{AM\}$.

In order to test the binding reaction, the Mg^{2+}-ATPase activity was determined in various mixtures of F-actin and myosin: (1) different concentrations of F-actin were added to a constant concentration of myosin, and (2) the concentration of F-actin was held constant while myosin was varied. These two sets of data can be used to estimate the number of actin binding sites per mole of myosin.

A. Actin-Myosin Binding

In Fig. 31, the Mg^{2+}-ATPase activity was plotted against the ratio of F-actin and myosin. Under the experimental condition tested, no ATPase activity was detected in F-actin. The ratio of F-actin to myosin in Fig. 31a was calculated on the basis of a molecular weight of 500,000 daltons for myosin (Tonomura et al., 1966) and of 60,000 daltons for actin monomer (Kay, 1960; Lewis et al., 1963; Krans et al., 1965). Recently, the molecular weight of chromatographically purified actin showed a value of 46,000 daltons (Rees and Young, 1967), which does not, however, alter the order of magnitude of the estimated binding constant for the actin-myosin interaction (Fig. 31b).
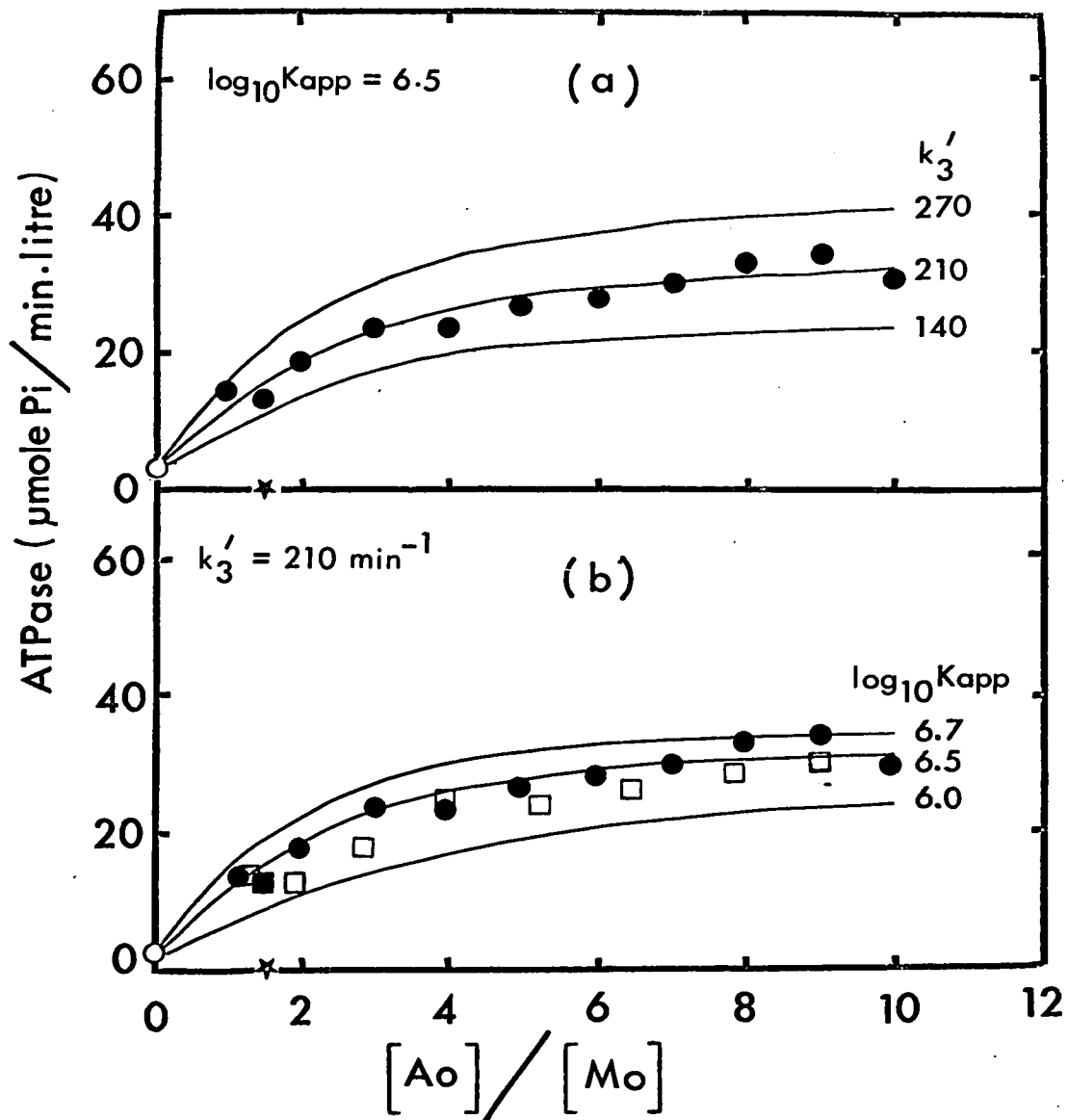
Figure 31: The relationship between Mg^{2+}-ATPase and the actin to myosin ratio. The reaction mixture contained 0.010M KCl, 0.02M tris-Cl (pH 7.4), 5.0mM $MgCl_2$, 1.0mM ATP, 0.090 mg myosin/ml. The reaction at 25°C was started by adding ATP and aliquots were taken for Pi measurements at time intervals of 15 seconds. The values for v were computed assuming a mole per mole association with actin. The values of the constants used for the calculations are described in the text. (O), myosin alone; (★), F-actin alone; (●) and (□), F-actin plus myosin assuming an actin M.W. of 60,000 and 46,000 daltons, respectively.

It is evident that there are two ATPase sites per myosin molecule. In view of the role of actin-myosin interaction in the contractile phenomenon, it is important to see whether or not both of these sites can be modified independently. The experimental results, however, were consistent with two assumptions: (1) only one of the ATPase sites in myosin can be modified with actin thereby giving rise to the increased Mg^{2+}-ATPase activity; and (2) the binding of one mole of actin to one mole of myosin affects both sites simultaneously. This was also shown with heavy meromyosin, which possesses two potential ATPase sites (Szentkiralyi and Oplatka, 1969; Rizzino et al., 1970). If one mole of actin bound to myosin results in the modification of only one of two sites, leaving the other site free, this free site should be considered to give the ATPase activity of free myosin. Since the activity of free myosin in the presence of Mg^{2+} is very low (see Table 15), the consideration of a remaining free ATPase site did not give a detectable difference in the calculated curves from those shown in Fig. 31.

For the calculations, the formation of actomyosin for different values of the apparent association constant was computed as a function of the actin to myosin ratio, where it was assumed that one actin binds to each molecule of myosin (Fig. 32). The assumed values of Kapp for actomyosin formation were then entered into the equation for v, and using an APL program, were used to calculate a set of curves corresponding to various values of k'_3.

At $\log_{10}$Kapp value of 6.5, the actomyosin formation approximates the experimental data for a vlaue of k_3' equal to 210 min^{-1} as shown in Fig. 31a, and from Fig. 31b, it is apparent that other values of
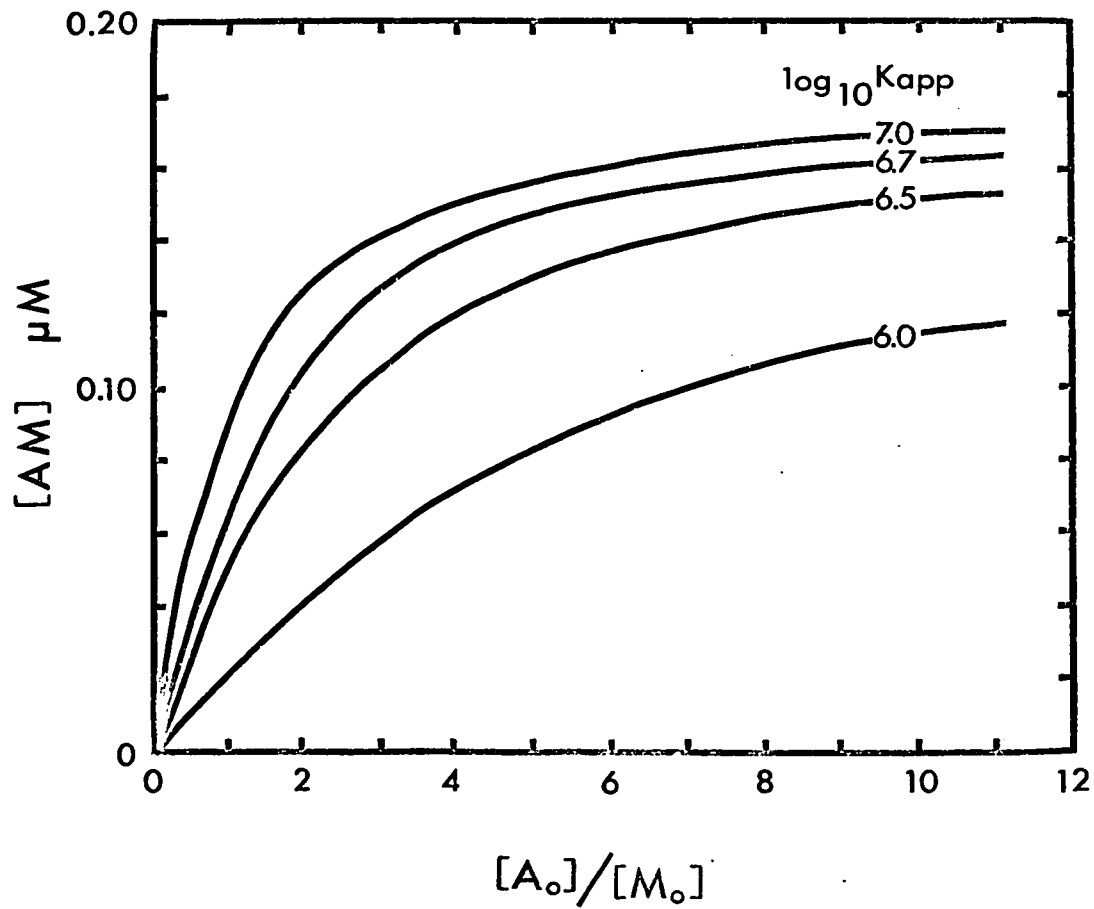
Figure 32: Computed formation of actomyosin where 0.18µM myosin is available for actin combination.

$\log_{10}$ Kapp would not correspond as well to the data.

The increase in the activity when a varied amount of myosin is added to actin is shown in Fig. 33. The activity due to myosin alone was found to be proportional to the myosin concentration within the range investigated. Theoretical curves for the velocity of the Mg^{2+}-ATPase were computed as above where a $\log_{10}$Kapp of 6.7 and k_3' of 270 min^{-1} were found to agree with the experimental results.

B. Actin-Subfragment Binding

The above relationships between the increase in the Mg^{2+}-ATPase and the formation of actomyosin may also be used for analysing the binding reaction between actin and the subfragment where actin is expected to affect the single ATPase site of each subfragment (Young, 1967a and b; Eisenberg et al., 1968). In Fig. 34, the Mg^{2+}-ATPase activity is also seen to increase with the addition of actin to subfragment, except that it is apparent that the binding is much weaker. These data were plotted assuming a molecular weight of 116,000 daltons for subfragment. Fig. 34b indicates also that a molecular weight of 46,000 daltons for actin does not appreciably alter the plot. Various values of k_3' at a $\log_{10}$Kapp value of 160 min^{-1} approximates the data most closely. However, the experiments were not able to show the exact change in slope with increasing levels of added F-actin. As a result, there is no reason to exclude the possibility that the actin-subfragment binding constant is lower (Fig. 34b) with a corresponding higher value of k_3' being required to fit the data. It is unlikely however, that the binding exceeds $2 \times 10^4 M^{-1}$, since a higher association constant of complex formation would show

Figure 33: The relationship between Mg^{2+}-ATPase and the myosin to actin ratio. The reaction mixture contained 0.008-0.024M KCl, 0.02M tris-Cl (pH 7.4), 5.0mM $MgCl_2$, 1.0mM ATP, 1.0mM EDTA, 0.018 mg F-actin/ml. The reaction at 25°C was started by adding ATP and aliquots were taken for Pi measurements at time intervals of 15 seconds. The values for v were computed as in Fig. 31, and the symbols are also the same. The values of the constants used for the calculations are described in the text.

Figure 34: The relationship between the Mg^{2+}-ATPase and the actin to subfragment ratio. The reaction mixture contained 0.025M KCl, 0.02M tris-Cl (pH 7.4), 1.0mM $MgCl_2$, 0.5mM ATP, 0.032 mg subfragment/ml. The reaction at 25°C was started by adding ATP and aliquots were taken for Pi measurements at time intervals of 1 minute. The values for v were computed assuming that there is one active site per mole of subfragment which combines with actin. The values of the constants used for the calculations are described in the text. (O), subfragment alone; (★), F-actin alone; (●) and (□), F-actin plus myosin assuming an actin M.W. of 60,000 and 46,000 daltons, respectively.

a curvature incompatible with the data. Other authors have claimed

to have determined the exact strength for the binding reaction

within the same range of actin or subfragment concentrations used

in these experiments (Eisenberg et al., 1968; Lowey et al., 1969).

However, their use of a reciprocal plot is questioned because the

slight changes of the ordinate intercept would show large variations

in the value of the maximum velocity and the value for the free

concentration of F-actin required for the calculation would not be

equal to the total concentration of actin used for the reciprocal

plot.

The corresponding experimental results for the addition of

subfragment to F-actin were found to agree quite well with the

values of the association constant and k_3' for F-actin addition

to subfragment (Fig. 35). It is also shown that the Mg^{2+}-ATPase

activity of subfragment alone is proportional to the subfragment

concentration.

The values of Kapp and k_3' which are appropriate to explain

the experimental data are summarized in Table 15. The good agree-

ment between the two sets of values for the binding reaction bet-

ween F-actin and myosin or F-actin and subfragment is compatible

with a mole per mole association in either case.

Assuming that the two sites in myosin bind with two actin mono-

mers would involve introducing unrealistic values of k_3', whereas

it doesn't seem likely that only one site in myosin can react with

actin. Rather, it may be speculated that because of the large

size of the actin monomer ($50\overset{o}{A}$) and its arrangement in the double-
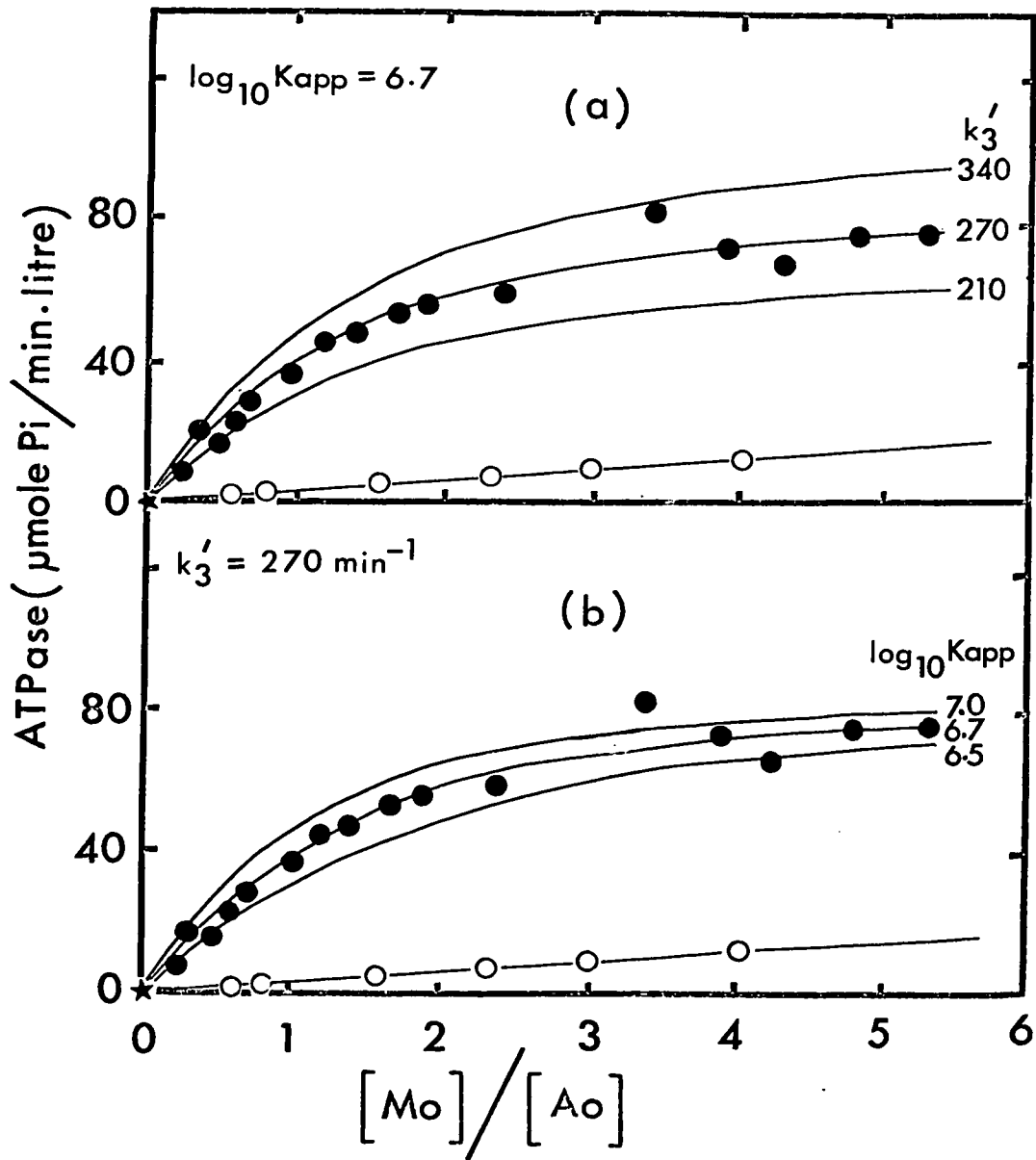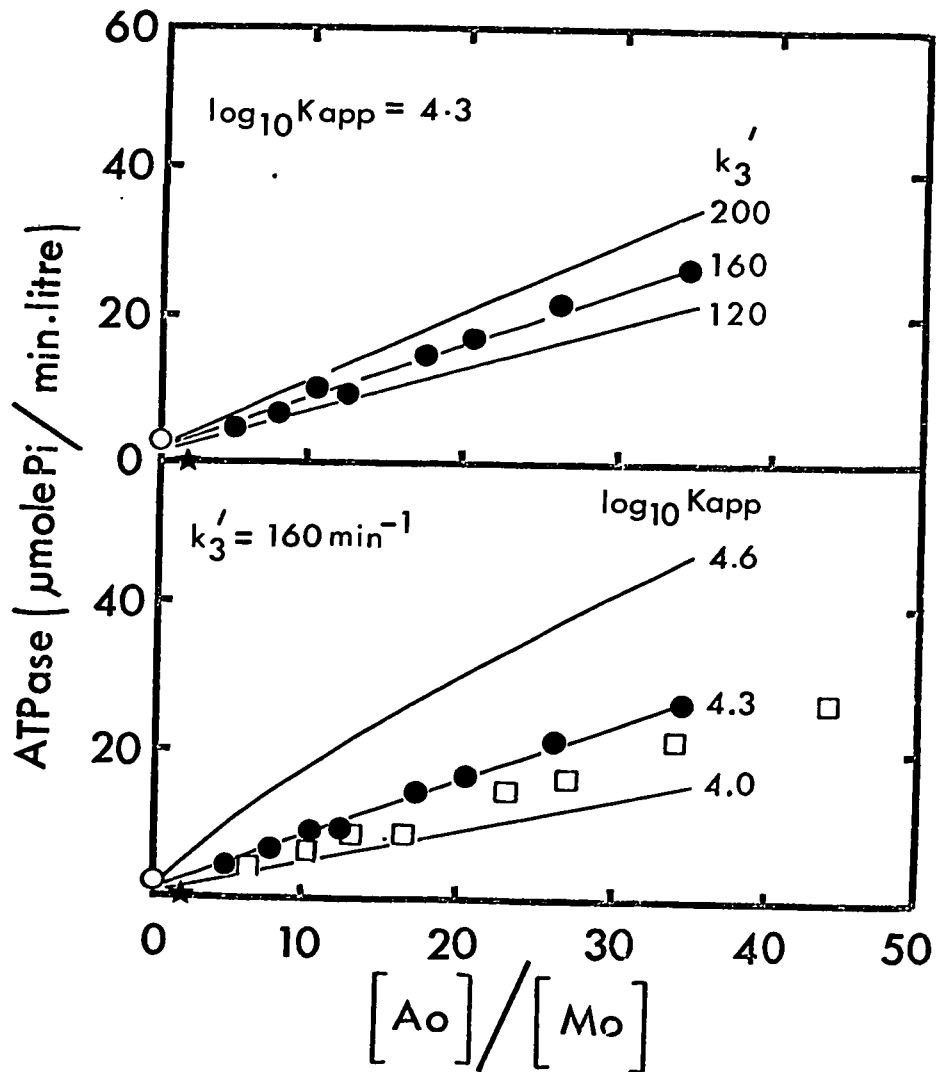
Figure 35: The relationship between the Mg^{2+}-ATPase and the sub-fragment to actin ratio. The reaction mixture contained 0.025M KCl, 0.02M tris-Cl (pH 7.4), 1.0mM $MgCl_2$, 0.5mM ATP, 0.017 mg F-actin/ml. The reaction at $25^{\circ}C$ was started by adding ATP and aliquots were taken for Pi measurements at time intervals of 1 minute. The values of v were computed as in Fig. 34, and the symbols are also the same. The values of the constants used for the calculations are described in the text.
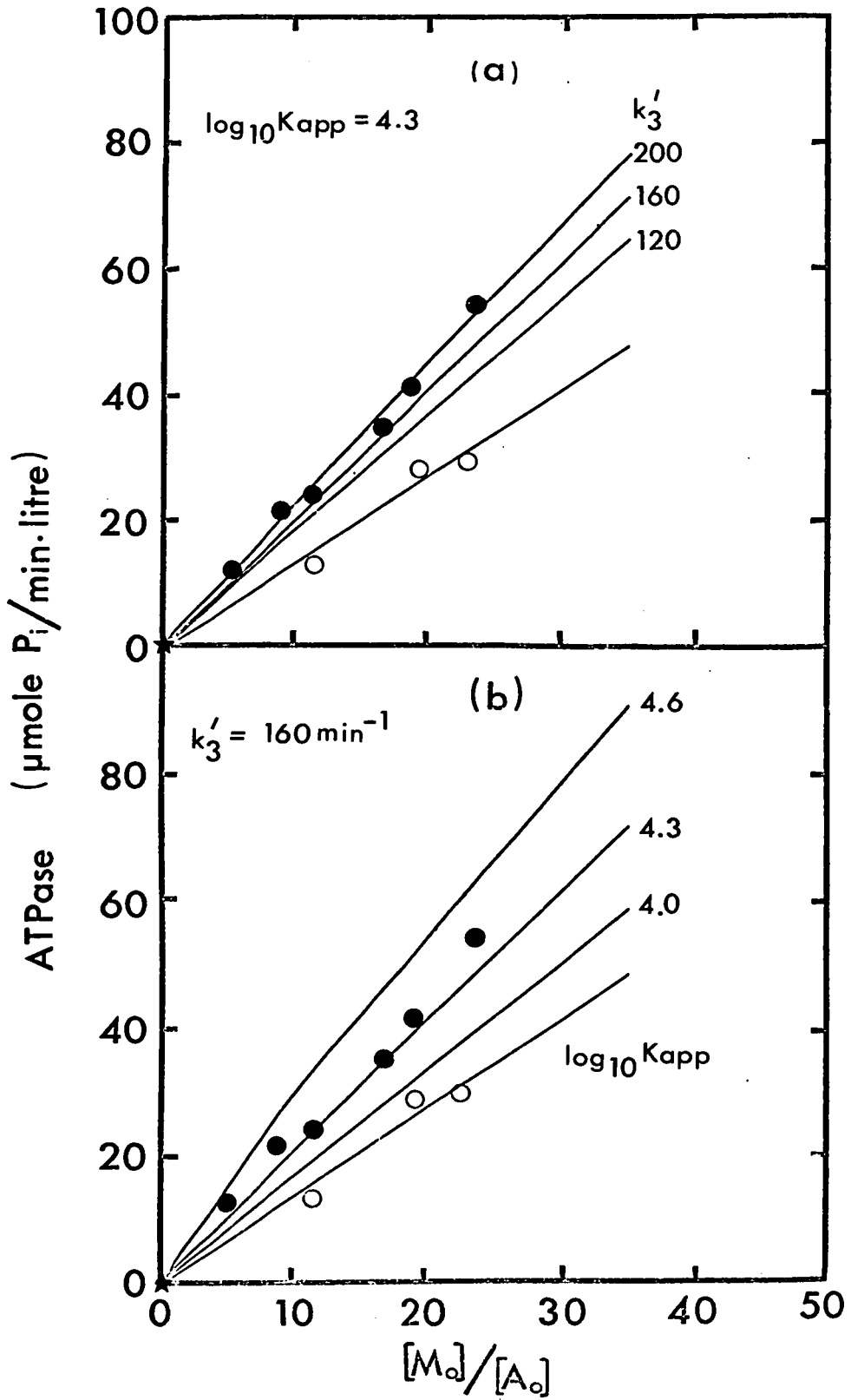
Table 15

The Actin-Myosin and Actin-Subfragment

Binding Parameters

	$\log_{10}$ Kapp	k_3' (min^{-1})	k_3 (min^{-1})
Actin added to myosin	6.5	210	5.2*
Myosin added to actin	6.7	270	---
Actin added to subfragment	4.3	160	4.5*
Subfragment added to actin	4.3	160	---

The values of k_3 and k_3' represent the rate constants for the breakdown of the enzyme-substrate complex for free myosin or subfragment and the corresponding actin-complexes, respectively.

* - determined from the Mg^{2+}-ATPase of myosin or subfragment

stranded, helically-wound F-actin polymer (Hanson and Lowy, 1963),
and due to the close arrangement of the myosin sites in the 50$\overset{o}{A}$
wide head portion of myosin, a binding of one actin monomer would
produce a steric hindrance to the second binding site.

5. A Study of the Actin-Subfragment Interaction

by Light Scattering

It was reported recently that in addition to the turbidity
and volume changes of actomyosin gel (Levy and Fleisher, 1965;
Sekine and Yamaguchi, 1966; Nihei, 1967), the polarization ratio
of light scattered by the gel suspension can be used as an indi-
cator phenomenon of the superprecipitation process (Nihei and
Yamamoto, 1969). Hence, it seemed of interest to examine whether
or not a change in the light scattered by a mixture of F-actin and
the subfragment of myosin can be detected under the conditions
which induce superprecipitation.

For this purpose, it was important to establish that actin
was in the polymerized (F-actin) state under the conditions of the
experiment. As reported by Oosawa et al., (1959), G-actin polymer-
izes only above a certain critical concentration of K+, Mg^{2+}, and
ATP. Above the critical concentration, however, the ratio of F-actin
to G-actin at equilibrium is independent of protein concentration.

For an understanding of the following light scattering
experiments, it is necessary to consider certain basic principles.
The angular distribution of scattered light is expressed as,

$$\frac{Kc}{I\Theta} = \frac{1}{MP(\Theta)} + 2BcP(\Theta)$$

where $I\Theta$ is the Rayleigh Ratio of the scattering intensity, M is the
molecular weight, $P(\Theta)$ is the interference factor accounting for the
interaction of radiations from different parts of the molecule, B is
the protein interaction term and c is the protein concentration.
Using the optical constants included in K, it is possible, by plotting

Kc/IΘ versus $\sin^2(\Theta/2)$ and extrapolating to zero protein concentration and zero angle, to quantitatively evaluate the above parameters (Zimm, 1948). The molecular weight could thus be determined, since, with this extrapolation, B approaches zero and P(Θ) becomes unity. The magnitude of the slope term at various angles in the Zimm plot is proportional to the P(Θ) factor, which depends upon the size and shape of the scattering particles. In addition, any change in this slope can signify heterogeneity, since there is a greater contribution to the scattering from large particles at low angles.

A large polymer such as F-actin would thus have a large P(Θ) factor and give a high scattering intensity at low angles. The Zimm plot of Fig. 36 is observed to slow a steep slope indicating the presence of F-actin (these light scattering intensities were measured with vertically and horizontally polarized light at various angles and a sum of the intensities at each angle was used to express the intensity of unpolarized light plotted in Fig. 36 and Fig. 37). It is predicted that a depolymerization of F-actin to G-actin monomers would result in a significant change in the slopes of Fig. 36 due to proportionally less scattering at low angles. However, the slopes are not different within the range of protein concentrations used, suggesting that actin remains in the polymerized state under the experimental conditions. The change in slope between 60-90° indicates that large size particles, which should be treated according to the Mie (1908) theory, contribute to the scattering. The polydispersity here becomes more obvious at higher protein concentrations.

For the study of the angular scattering of actin-subfragment

complex, the scattering intensities (unpolarized light) were plotted
as a function of the scattering angle (Fig. 37). A single preparation
of F-actin was used for this analysis because it seemed likely that
changes in the size distribution of the F-actin polymers would affect
scattering to a large degree. A solution of F-actin at 0.17 mg/ml
was centrifuged at 20,000 r.p.m. for 20 minutes in a Sorvall rotor to
remove dust. This solution was stirred by a magnetic stirrer
fitted under the scattering cell, and the measurement was repeated.
Fig. 37 shows the light scattering from F-actin which is independent
of stirring even at low angles. An aliquot of the stock solution
of subfragment (0.05M KCl, 0.01M K_2HPO_4, pH 7.4 1mM D.T.T., 4.7
mg/ml) was added with stirring to a solution of F-actin until the
final concentration reached 0.26 mg subfragment/ml. The concentration
of F-actin in this solution was 0.16 mg/ml, which gave an approximately
equimolar ratio of the subfragment to actin. The observed 3-fold
increase in the scattering intensity at 30°, compared to an 8-fold
increase at 130°, was expected since the small spherical subfragment
molecule would not show the high scattering at low angles where F-actin
dominates the scattering. For the measurement of light scattering
in the presence of 0.1mM ATP, the solution was stirred for 10
minutes after the addition of ATP. Under this condition the ATPase
activity was found to be 0.33 μ mole Pi/min.mg compared to an
activity of 0.19 μmole Pi/min.mg for the subfragment alone. After
10 minutes at this rate of hydrolysis, ATP should be virtually
exhausted. The scattering intensities were determined and plotted
as unpolarized light at the various angles but no change was detected
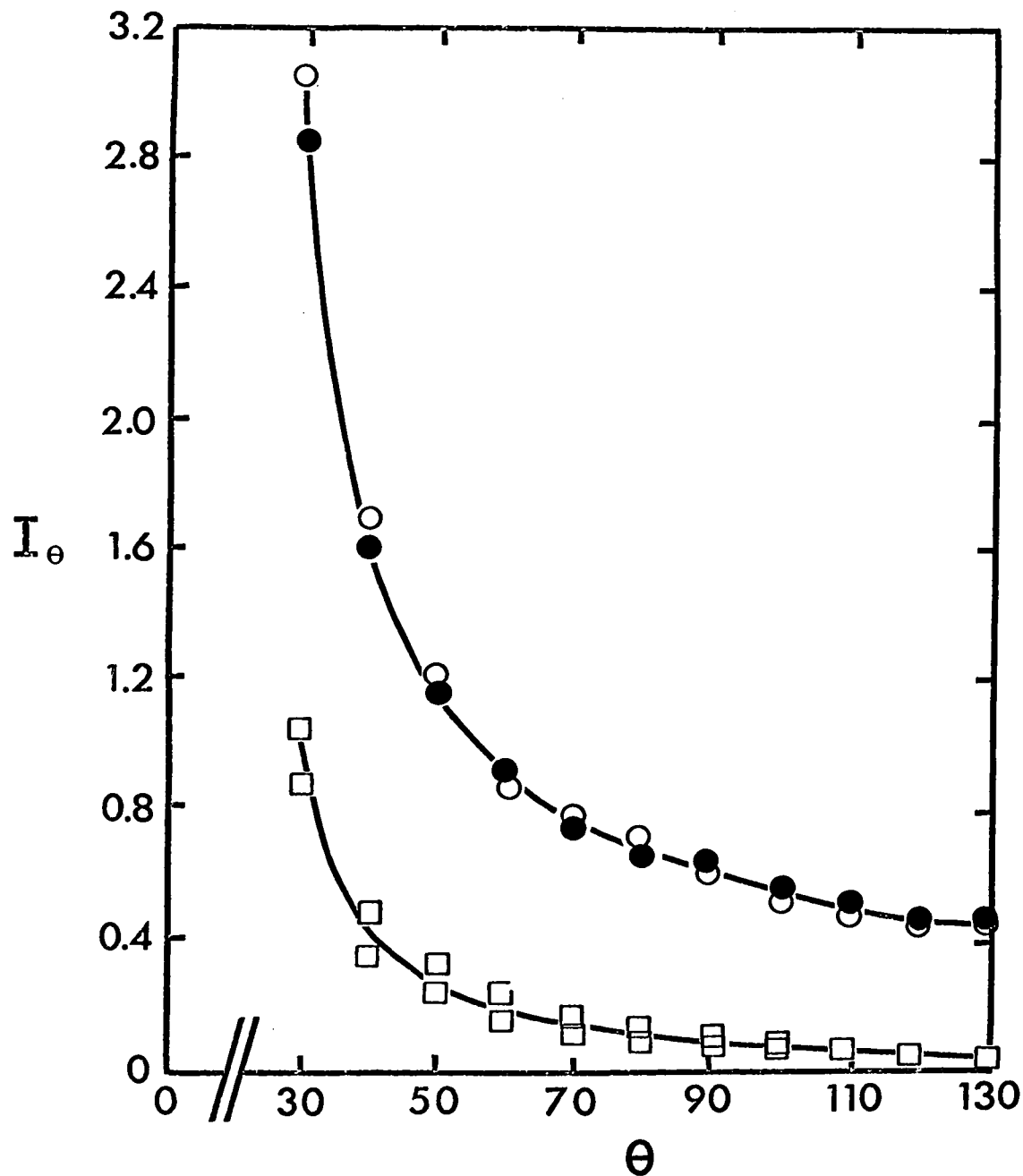
Figure 37:   Light scattering intensity from solutions of F-actin and actin-subfragment mixtures.   1.0mM MgCl$_2$, 0.01M tris-Cl (pH 7.4), 25-30°C.   (□), duplicates of F-actin scattering at 0.17 mg/ml; (○), 0.16 mg/ml of F-actin plus 0.26 mg/ml of myosin subfragment; (●), this same actin-subfragment mixture after reaction with 0.1mM ATP.

in this or in either of the polarized components. The subfragment

binding to F-actin was examined by centrifuging the actin-subfragment

mixture at 110,000x g for 1 hour. The supernatant contained only

0.14 mg protein/ml, i.e. approximately 50% of the subfragment

remained bound to F-actin.

As observed by light scattering, the interaction of ATP with

the actin-subfragment in the presence of Mg^{2+} does not show a

lasting effect on the scattering of unpolarized light or on the

polarization ratio which can be observed with actomyosin. The unique

changes of superprecipitation are apparently brought about by the

characteristic ability of light meromyosin to form a hydrated gel.

Without the formation of gel, the actin-myosin complex may be regarded

as a simple dissociation-association system.

DISCUSSION

The work presented in this thesis has its immediate origin in the observations by the research group of H. Weber that Mg^{2+} is necessary for the actin-myosin system to convert the energy stored in ATP into mechanical work. As an approach to the problem of how Mg^{2+} acts in the contractile system, a comparative analysis of the effects of Mg^{2+} and Ca^{2+} on the reactions among actin, myosin, and ATP or ITP has been performed.

The question posed was whether the binding of divalent cations with myosin causes the change in enzymatic characteristics of this protein or whether the formation of cation-substrate complexes is responsible for changes in the rate of pyrophosphate bond splitting. The range of Mg^{2+} concentration in which the rate of ATP hydrolysis changes appears to indicate that a binding of Mg^{2+} to myosin modifies the enzymatic property of this protein. The measurement of Mg^{2+}-myosin binding performed using an ultrafiltration technique suggests that one atomic equivalent of Mg^{2+} binds with a mole of myosin with an affinity constant having a value of the order of $10^6 M^{-1}$, which agrees with the kinetic data. With Ca^{2+} as a modifier of myosin ATPase, an analysis of the rate of ATP hydrolysis as a function of the Ca^{2+} concentration also suggests a binding reaction involving Ca^{2+}, which seems to coincide with the formation of $CaATP^{2-}$. Observations on the binding of $^{45}Ca^{2+}$ to myosin illustrate that there are at least two types of Ca^{2+} binding to myosin; one is similar to the Mg^{2+} binding in the number of ions bound and in affinity, and the other has an affinity constant of the same order as that found in the $CaATP^{2-}$

formation. The latter binding reaction was reported by Luchi and

Kritcher (1967) as occurring at 8 binding sites per mole of myosin

with an affinity constant estimated as $1.8 \times 10^4 M^{-1}$. In this re-

gard there are several reports (Nanninga, 1957; Blum and Felauer,

1959; Lowenstein, 1960; Tetas and Lowenstein, 1963) which support

the idea that $CaATP^{2-}$ is hydrolysed more rapidly by myosin than other

ionic species of ATP. On the other hand, the weak binding of Ca^{2+}

to myosin cannot be ruled out as the cause of the Ca^{2+} activation of

myosin ATPase (Nanninga, 1959). It seems reasonable, therefore, to

state that both Ca^{2+} and Mg^{2+} can bind tightly to myosin, although

only the binding of Mg^{2+} shows a direct influence on myosin ATPase.

There is, however, some evidence that the Ca^{2+} bound tightly

to myosin affects its enzymatic activity indirectly. As shown in

the experiments with ITP or pMB-modified myosin, the Ca^{2+} activation

of the enzymatic activity is nullified by the addition of small

amounts of Mg^{2+}, which apparently is the result of Mg^{2+} replacing

Ca^{2+} at the tight binding site.

Concerning changes in the chemical nature of ATP caused by a

bound cation, $MgATP^{2-}$ does not seem to differ from $HATP^{3-}$ and ATP^{4-}

in the capacity to act as a substrate for ATPase. For instance, the

maximum Mg^{2+} effect can be attained by adding 0.01 mM Mg^{2+} to a reac-

tion mixture containing 0.1 mM ATP (Fig. 15). Under this condition

less than 10% of total ATP is complexed. Further additions of Mg^{2+}

increase $MgATP^{2-}$ without affecting the kinetic parameters of myosin

ATPase. However, the Ca^{2+} bound to ATP may affect the hydrolytic pro-

cess as discussed above. In this connection, Lymn and Taylor (1970)

pointed out the possibility that Ca^{2+} accelerates the rate at which the product is released from the active site in myosin. The experimental data obtained in this work and by other researchers have not made clear, however, whether the Ca^{2+} binding to myosin or the $CaATP^{2-}$ formation is responsible for the Ca^{2+} activation of myosin NTPase.

The relationship between the actomyosin ATPase activity and the concentration of Ca^{2+} or Mg^{2+} was shown to be remarkably similar to that observed for myosin ATPase, except that Mg^{2+} acts as an activator for actomyosin ATPase. This may seem to indicate that myosin reacts with these divalent cations without being affected by the bound actin. It is probable however, that F-actin contains certain amounts of Mg^{2+}, since it is usually polymerized in the presence of Mg^{2+}, and it may bind with the added cation, as the affinity of Mg^{2+} or Ca^{2+} to G-actin has been reported by Maruyama and Gergely (1962), to be of the same order of magnitude as that to myosin.

If the assay of actomyosin ATPase is performed in the absence of EDTA, a limited level of activity is observable even without added divalent cations. Upon addition of EDTA, ATP hydrolysis becomes undetectable. Although it is not clear whether or not EDTA chelates the divalent cation bound to actin, the fact is that at a KCl concentration below 0.1 M, actomyosin requires divalent cations to catalyse ATP hydrolysis, whereas monovalent cations alone can activate myosin ATPase.

Superprecipitation apparently involves several steps before reaching the final stage (Nihei and Yamamoto, 1969). A detection method for the physical changes in this process may follow any one or a combination of those steps. Although turbidity changes of actomyosin

suspensions have often been used to detect aggregate formation, the rate and extent of superprecipitation so measured depend on the optical arrangement of the measuring device. The method used in this work is the one which gives the turbidity change following the first order kinetics at actomyosin concentrations lower than 0.05 mg/ml. The extent of superprecipitation with different ATP concentrations is constant at a fixed concentration of actomyosin. If the concentration of ATP is much reduced, the turbidity change ceases at the time of ATP exhaustion. According to a previous study by Nihei and Yamamoto (1969), the rate measured by the method used represents that of actomyosin gel dehydration. The experiments showed that both Mg^{2+} and Ca^{2+} activate superprecipitation maximally at 3-4 mM. The rate of ATP hydrolysis in the presence of 4 mM Ca^{2+} is 0.7-0.8 μmole/min.mg protein, accompanied by the turbidity change at a rate giving a $t_{\frac{1}{2}}$ of about 8 seconds, whereas with 4 mM Mg^{2+}, the rate becomes 0.4-0.5 μmoles/min.mg, and the turbidity changes at a rate indicating a $t_{\frac{1}{2}}$ of about one second. It may be restated that if actomyosin is to undergo superprecipitation in the presence of a sufficient amount of Ca^{2+}, it needs about 60 moles of ATP hydrolysis to convert one mole of myosin into the superprecipitated state, whereas in case Mg^{2+} is the activator, actomyosin requires only 5 moles of ATP to be split per mole of myosin in completing the change. These observations suggest that the actin-myosin system is better adapted to Mg^{2+} in the process of coupling ATP hydrolysis to mechanical work.

It was thought then that if one could ascertain the quantitative relationship between actin and myosin in the formation of

actomyosin, it might be of value in understanding the nature of superprecipitation. In carrying out the experiments for this purpose, it has become apparent that since myosin is a large molecule with two active centers, a simpler system than actomyosin can be analysed on the basis of an uncomplicated chemical equilibrium. For this reason, attempts have been made to obtain homogeneous preparations of an active subfragment (HMM-S1) from myosin digested by papain. The subfragment prepared by the technique described in the Results appeared to be reasonably homogeneous as observed in the ultracentrifuge experiments, except that it tends to form aggregates unless it is in a solution containing β-mercaptoethanol. In the presence of 0.02 M of this reagent, the subfragment showed a higher ATPase activity (0.21 μmole Pi/min.mg) in a reaction mixture containing 1.0mM ATP, 1.0mM $MgCl_2$, 0.05M tris-Cl (pH 7.4), and 1.0mM D.T.T. at 25° than in the absence of β-mercaptoethanol (0.11 μmole Pi/min.mg). It may be that this reagent blocks the sulfhydryl groups of the subfragment by forming disulfide bonds, in which case the ATPase is increased as in the case of the pMB-treated myosin.

The analysis of the Mg^{2+} activated ATPase as a function of the ratio of actin to myosin or its subfragment is based on the assumption that in the presence of ATP and Mg^{2+}, actin and myosin or its active subfragment are at equilibrium with their complex as can be expressed by the scheme:

n (actin) + myosin (subfragment ⇌ actomyosin (-subfragment)

where n is an integer, and actin denotes a monomer incorporated in F-actin. If myosin, its subfragment, actomyosin, and acto-subfragment

possess distinct ATPase characteristics, in particular the rate con-
stants of the slowest step, and if the total concentrations of actin
and myosin or its subfragment are known, the equilibrium constant of
the above reaction can be determined. The rate constant of myosin
ATPase can be experimentally measured, whereas that of actomyosin
(-subfragment) ATPase cannot be determined unequivocally, since it is
difficult to secure the condition under which the amount of free
myosin (or subfragment) becomes negligibly small. Hence assumed
values of the rate constant for actomyosin (-subfragment) ATPase were
used in the calculation of the relationship between the ATPase acti-
vity and the ratio of actin to myosin or subfragment. As described
in the Results, both myosin and its subfragment appear to bind with
F-actin at the molar ratio of one to one where monomer actin is
taken as the molecular unit. Previously, Young (1967b) and Rizzino
et al. (1970) reported a mole per mole association of actin to heavy
meromyosin, although Szentkiralyi and Oplatka (1969) observed the
second actin binding to heavy meromyosin at high concentrations of
actin. It is generally agreed among several workers (Eisenberg et
al, 1968; Szentkiralyi and Oplatka, 1969; Rizzino et al., 1970; see
also Table 15) that the affinity of actin to heavy meromyosin or to
active subfragment (HMM-S1) is much weaker than to myosin.

There is little doubt that at least two active centers exist
in a molecule of myosin. It is not unreasonable to assume that
these centers influence each other in their reactions with other
molecules. The observations that a binding reaction of either Ca^{2+}
or Mg^{2+} to myosin with relatively high affinity occurs at a site per

mole, suggest the influence of one cation on two active centers.

There has been a discrepancy in the values of the Michaelis constant, K_m, for the myosin ATPase obtained in a high range of ATP concentration ($K_m \simeq 10^{-4}M$) and in low range ($K_m \simeq 10^{-7}M$). In studies of the proton release during the transient phase of myosin ATPase two reaction rates depending on the substrate concentration were recorded (Finlayson and Taylor, 1969). These may also mean that some interactions exist between the active centers of myosin (Lymn and Taylor, 1970). In the absence of evidence that the myosin subfragment is functionally heterogeneous in its binding with actin, the mole per mole binding between actin and myosin could be explained in terms of interactions between the two active centers in myosin. It should be pointed out that there could be two types of interactions; one is the competition between two active centers for one actin monomer and the other is the co-operation of the centers to attract actin. To determine which type of interaction is more feasible, one may consider the affinity of myosin to actin, which is stronger than that of the myosin subfragment. This phenomenon, however, is in accord with either type of the active center interaction, since the competition between two in the same molecule does not necessarily reduce the attractive force applied to the whole molecule.

It may be that the experimental conditions used in this study are far from physiological reality, under which myosin is unable to react with actin as it does in vivo. In fact, the ATPase activity of actomyosin as a function of the KCl concentration gives a bell-shaped curve (Fig. 21). In living muscle, ionic strength is approximately

0.15, and the concentration of ATP is kept constant at about 4-5mM. Under physiological conditions, the active centers of myosin and actin could be at equilibria more dynamic than the simple equilibrium assumed in analyzing the data.

In closing, the following conclusions can be stated: the binding reactions of Ca^{2+} to myosin are quantitatively similar to that of Mg^{2+}, though the effects of these cations on myosin ATPase appear qualitatively different. In the actomyosin system, Mg^{2+} as an essential factor for superprecipitation can be replaced by Ca^{2+}. Considering the greatly reduced efficiency of Ca^{2+} in this role, the interaction of actin and myosin as the basic reaction of muscular contraction seems to possess a high specificity, if not absolute, in its requirement for Mg^{2+}. In addition, the small quantity of Ca^{2+} relative to Mg^{2+} present in living muscle makes it nearly impossible for Ca^{2+} to act directly on the actin-myosin system in vivo. Secondly, the interaction of actin and myosin as observed in the dependence of Mg^{2+}-ATPase on the ratio of actin to myosin shows that only one actin monomer can bind to a mole of myosin. The binding of myosin subfragment (HMM-S1) to actin also indicates the same extent, i.e., a mole per mole binding. In view of the evidence currently available, the interaction between two active centers in a molecule of myosin appears to have become an important factor in understanding the mechanism of actin-myosin interaction.

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