Functions of the Two Heads of the Myosin Molecule

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Abbreviations

EGTA,	ethyleneglycol bis(β-aminoethylether)-N,N'-tetraacetate
F-A,	F-actin
HMM,	H-meromyosin
NTP,	p-nitrothiophenyl
PCMB(CMB),	ρ-chloromercuribenzoate
PEP,	phosphoenolpyruvate
PPi,	inorganis pyrophosphate
RP,	relaxing protein
SDS,	sodium dodecyl sulfate
S-1,	myosin subfragment-1
TCA,	trichloroacetic acid

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1. INTRODUCTION

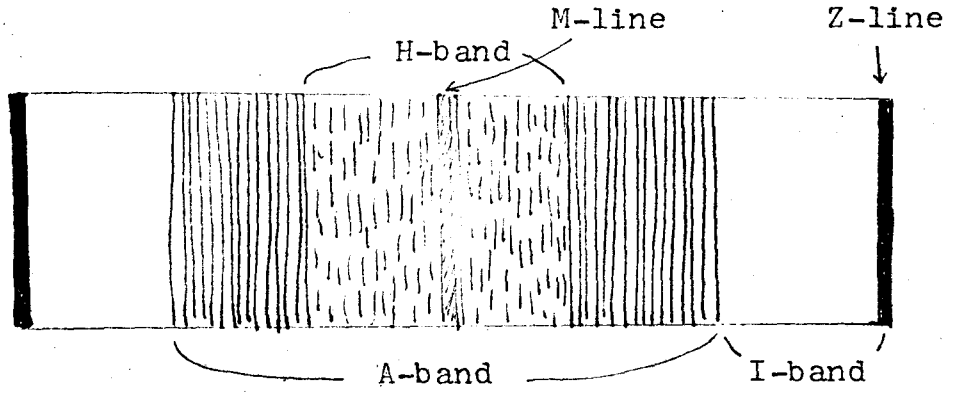
The existence of animal life is characterized by the speed and efficiency of purposeful movement, and the structures which can transduce chemical energy to mechanical work are specialized and highly organized into muschle fiber. The energy transduced into mechanical work in muscle is large. A unique charactor of muscle contraction is its control, since the fastest muscle can attain maximum tension within a few millisecond. The structual basis of these characteristics of muscle contraction is that muscle consists of myofibrils, composed of two kind of filaments made of different proteins, myosin and actin, and that contraction is caused by the interaction between these two filaments

ATP and Ca^{2+} ions are low molecular weight substances, which react with these two protein filaments during muscle contraction. Biochemical studies on metabolishm in muscle suggested that the chemical reaction most likely to provide the energy for contraction is hydrolysis of ATP. Engelhardt[1], Szent-Györgyi[2], Weber[3], and others showed that myosin has ATPase activity[1], which is activated markedly by actin in the presence of Mg^{2+} ions[2], and that glycerol-treated muscle fibers[3], myofibrils[4] and actomyosin threads[5,6] contract on adding ATP, with concomitant hydrolysis of ATP. Therefore, it has generally been accepted that ATP is the energy source of muscle contraction. Using muscle in which creatine kinase, glycolysis and respi-

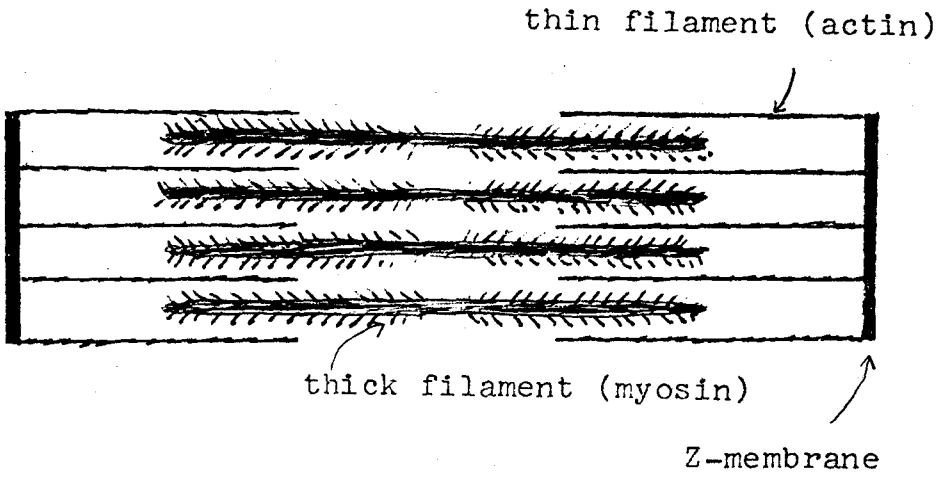
ration were all inhibited, Cain and Davies[7] showed more recently that the amount of ATP decreases during contraction and that the decrease in ATP corresponds to the energy consumption of muscle.

Heilbrunn[8,9] and Kamada and Kinoshita[10] found that muscle contraction is induced when Ca^{2+} ions are injected into the muscle fiber. Later, it was shown that glycerol-treated muscle fibers contract on adding Mg^{2+} -ATP in the presence of a small amount of Ca^{2+} ions and relax on removal of Ca^{2+} ions with a chelate compound[11,12]. Hasselbach and Makinose[13] and Ebashi and Lipmann[14] discovered that Ca^{2+} ions are actively transported in the sarcoplasmic reticulum, coupled with the ATPase reaction. Ebashi[15] discovered that relaxation with removal of Ca^{2+} ions requires the presence of tropomyosin and troponin located on the thin filaments and that troponin is the Ca^{2+} -receptor protein in muscle. These studies showed clearly that muscle contraction is regulated by a trace amount of Ca^{2+} ions[15,16].

Sarcomere is the structual unit of muscle fibers (Fig. 1). A.F. Huxley and Niedergerke[17] and H.E. Huxley[18] and Hanson found that the length of the A-band remains constant when muscle fibers are extended or contracted, and that the length of the H-zone changes by the same amount as the alteration in the length of the sarcomere. Based on these results, it was predicted



(A)



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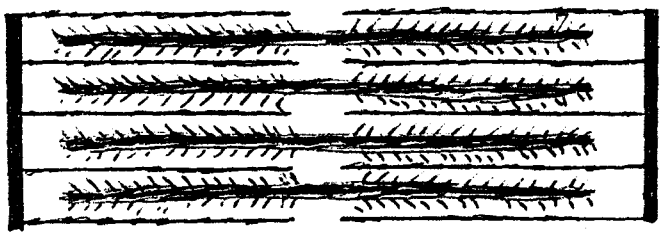


(C)

Fig. 1. Schematic representation of sarcomere,(A), filaments in sarcomere,(B), and its chenge after contraction of muscle

that there must be two kinds of filaments in myofibrils, both having a fixed length. Contraction must therefore occur by means of a change in their positions due to sliding of the filaments. This 'sliding theory' is supported by various lines of evidence. It was found by electron microscopy[19,20] that the thick and the thin filaments are regularly arranged in myofibrils, and the mutual positions of these two filaments change when muscle fibers extend or contract, just as the sliding theory predicts. Furthermore, it was discovered that the thick filaments are mainly composed of myosin and the thin filaments mainly of actin[21-24], and that the development of tension of living muscle[25,26] and the ATPase activity of living muscle[27] and glycerol-treated muscle fibers[28,29] are both proportional to the area of the over-lapping region of these two filaments. Moreover, Fukagawa *et al.* [30] showed that single sarcomeres, which are composed only of the thick and the thin filaments, contract on adding ATP. The fine structure of the sites where tension is developed and ATP is hydrolyzed was investigated by analysis of X-ray diffraction patterns[31], electron microscopy[32], and biochemical studies on the structural proteins[33], *i. e.* myosin, actin and others. From these investigations, it was proposed that the head portions of myosin molecules form projections from the thick filaments, that cross-bridges are formed by the binding of the projections with the

thin filaments, and that sliding of the thin filaments past the thick filaments occurs as a result of the movement of cross-bridges, which is coupled with ATP-splitting.

According to the sliding theory, there are at least three fundamental steps in muscle contraction: (i) the attachment of the projections from the thick filaments to the thin filaments, (ii) movement of the cross-bridges thus formed, which induces sliding of the thin filaments past the thick filaments, and (iii) the detachment of the projections from the thin filaments. Accordingly, studies on the molecular mechanisms of these three fundamental steps have been made from three different directions. One is by study of the fine structure of the contractile apparatus, especially the movement of projections from the thick filaments, using X-ray diffraction, electron microscopy and fluorescence polarization. The second is by physiological studies in the dynamic characteristics of muscle to elucidate the kinetic movement of projections at the molecular level. The third is by biochemical studies on the reactions between myosin, actin and ATP.

The first and second types of experiments were mainly used in early studies on the molecular mechanism of muscle contraction, while the third, which deals with the ATPase reaction, was only recently received attention. The studies on the mechanism of the ATPase reaction have led to discovery of many intermediates

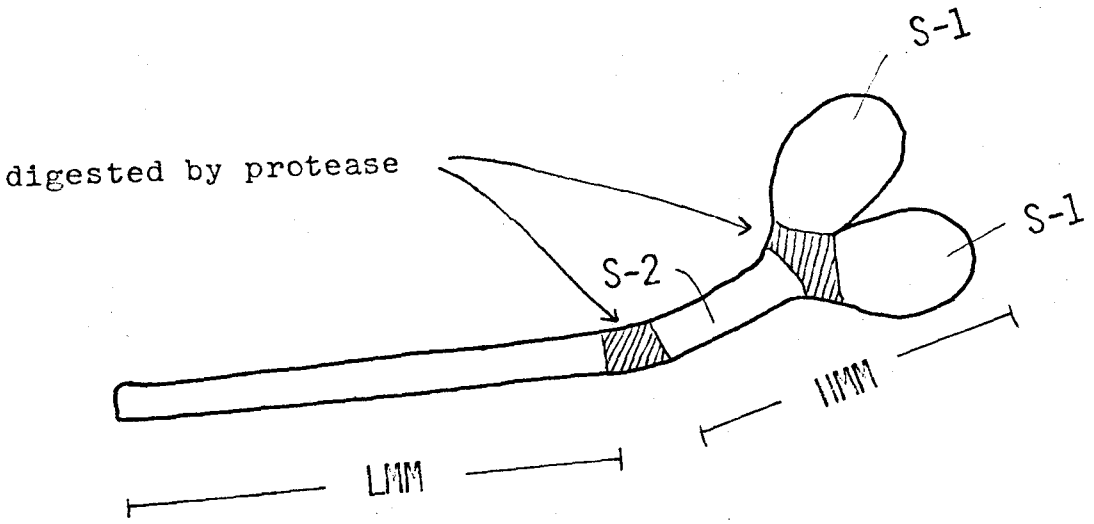


Fig. 2. Schematic representation of myosin molecule

of the myosin-ATPase reaction, such as the reactive myosin-phosphate-ADP complex, and have recently attracted many research workers as one of the most effective ways to clarify the molecular mechanism of muscle contraction,

Physicochemical studies on myosin in solution and electron microscopic studies on myosin molecules showed that myosin has a molecular weight of $4.6 - 4.8 \times 10^5$, with a total length of about 1600Å[34]. Lowey *et al.* [33,34] in electron microscopic studies on myosin and its subfragments using a rotatory shadowing technique showed that the myosin molecule has two separate heads, each with a diameter of 90 Å. Biochemical studies on subfragments the active site of ATPase are located in the head portions which compose the projections from the thick filaments, while the tail portions aggregate at low ionic strength and constitute the back-bone of the thick filaments. It is now well established that the splitting of ATP and development of tension both result from the interaction between the thin filaments, which are mainly composed of F-actin, and the projections from the thick filaments, which are mainly composed of myosin.

A scheme of the myosin molecule and the structures of its various subunits are shown in Fig. 2. One mole of heavy meromyosin, HMM, with a molecular weight of 3.4×10^5 and 1 mole of light meromyosin, LMM, with a molecular weight of 1.2×10^5 are produced by tryptic digestion of 1 mole of myosin[35].

HMM is derived from the head portions and has ATPase activity and ability to bind with actin, while LMM is derived from the tails of myosin molecules and aggregates at low ionic strength. Further tryptic digestion of 1 mole of HMM yields 2 moles of subfragment-1[36], S-1, with a molecular weight of about 11.0 x 10^4 and 1 mole of subfragment-2[37], S-2, with a molecular weight of about 6 x 10^4. S-1 is derived from the head parts of myosin molecules, and has both the active site of ATPase and the binding site for actin. S-2 is considered to function as the hinge which connects the two heads and the tail in the myosin molecule. Furthermore, fluorescent polarization decay studies on myosin and its subfragments[38] have revealed high cending flexibility in the connections between the heads and S-2, and S-2 and the tail of the myosin molecule.

Thus, the myosin molecule has two heads, and the heads split ATP and combine with F-actin. The problem of whether the two heads are identical or not is one of the most improtant ones for elucidation of the molecular mechanism of muscle contraction. The studies on the substructure of myosin, chemical modifications of the active sites and bindings of ATP and its analogues to myosin have provided many evidence that the structures and functions of the two heads of the myosin molecule are different from each other[39,40]. However, although there has been much work on this problem, it is very difficult to obtain conclusive

any evidence[39-43], since it is uncertain whether myosin preparations are homogeneous or not.

Studies on the function of myosin show that myosin molecule has two active sites which are different from each other. Tonomura and Morita[44] measured the binding of myosin with inorganic phrophosphate (PPi), which is a competitive inhibitor of myosin-ATPase, using an equilibrium dialysis method, and showed that 2 moles of PPi bidn to 1 mole of myosin. This result was supported by other workers[45], and a similar result was also reported for the bidning of ADP to myosin[46]. Furthermore, Tonomura and Morita[44] suggested that 2 moles of PPi bind to 1 mole of myosin with different dissociation constants, and that only 1 mole of PPi binds to 1 mole of myosin constituent in actomyosin, and that this binding of PPi causes the dissociation of actomyosin. Morita *et al.* [47,48] have recently shown that in the presence of Mg^{2+} ions at low ionic strength, and especially in the presence of Mn^{2+} ions, the binding constants of 2 moles of ADP with 1 mole of HMM are very different, and the one which binds more strongly induces the change in the UV-absorption of HMM. The heterogeneity of function of the two active sites is also suggested by chemical modifications of the active sites in the myosin molecule[39].

As mentioned above, a basic molecular mechanism of muscle contraction has been provided by the sliding theory, and it is

now well established that the development of tension and hydrolysis of ATP both result from the interaction between the projections from the thick filaments and the thin filaments. A.F. Huxley[49] assumed that there is spontaneous association and ATP-dependent dissociation between a contractile site, capable of oscillating for a certain distance along the backbone of the thick filament, and the binding site on the actin filament. He showed that, given a simple form of probabilities of bonding and cleavage as functions of the position of the contractile site on the myosin filament, the sliding of the two filaments can adequately explain many of the mechanical and thermodynamic properties of contraction discovered by Hill[50]. This work became a starting point for many attempts to explain the mechanical and thermodynamic properties of contraction on the basis of a set of three fundamental reactions; *i. e.*, the attachment of myosin heads to F-actin, the sliding of the two dilaments by the movement of myosin heads, and the detachment of myosin heads from F-actin. Recently, A.F. Huxley and Simmons[51,52] measured the time-course of the tension change after a sudden change in the length of the fiber, and proposed a new molecular kinetic model for muscle contraction. According to this hypothesis, there are more than two states in the myosin head which binds with the actin filament and that the sliding of filament is induced by the transition of the state

of the myosin head bound to the actin filament. However, there is no clear explanation why the myosin molecule has a two headed structure.

Two analysis done by A.F. Huxley have become the basis of many models of contraction proposed later. In these models it is presummed that two heads of the myosin molecule are identical and act independently. However, this situation seems to be improbable from our studies on structure and function of myosin molecule[39,43]. Thus the elucidation of function of two heads of myosin molecule is the central problem for the clarification of molecular mechanism of muscle contration. The studies on the reaction mechanism of myosin-ATPase were started and developed by Tonomura and his colaborator. It was shown by these studies that stoichiometric amount of the reactive myosin-phosphate-ADP complex, M_P^{ADP}, is formed by the reaction of myosin with ATP and its decomposition is ceelerated by F-actin. In the present study, the intermediate of the ATPase reaction catalyzed in each head of the myosin molecule is determined using three methods, kinetical analysis, measurement of amount of ATP and ADP bound to myosin during the ATPase reaction, and separation of two heads (S-1). The result obtained show clearly that M_P^{ADP} is formed in one head and the myosin-ATP complex, M_{ATP}, is formed in the another head of the myosin molecule, and that actomyosin-ATPase reaction is catalyzed through M_P^{ADD} and formation of M_{ATP} is necessary for the Ca^{2+} control of muscle.

In the following section, the step of M_P^{ADP} formation was analyzed by measuring the time courses of Pi liberations after stopping the reaction with TCA and the change in absorption spectrum of myosin during the initial phase of the reaction, and the following mechanism were obtained for formation of M_P^{ADP}:

$$M + ATP \rightleftharpoons M_1ATP \rightleftharpoons M_2ATP \rightleftharpoons M_P^{ADP}$$

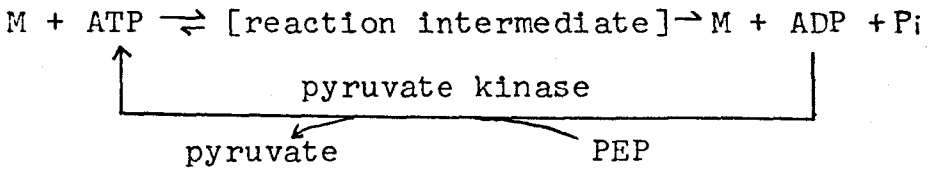
(UV-absorption change) (Initial burst of Pi-liberation)

The decomposition of M_P^{ADP} was measured after forming M_P^{ADP} by adding slightly less than the stoichiometric amount of ATP to amyosin using a rapid-flow dialysis method, and it was shown that the rate of Pi-liberation from M_P^{ADP} was almost equal to that of ADP liberation and that the rates of Pi- and ADP-liberation were unaffected by ATP. The rate of ATPase reaction at the steady state was measured over a wide range of ATP concentration. The rate of ATPase reaction at the steady state was expressed by the sum of two kind of ATPase reaction; one with low K_M and V_{max} values and the other with K_M and V_{max} values of 1 μM and 0.33 min^{-1}. It is concluded that ATPase reaction with low K_M value is that of ATP hydrolysis through M_P^{ADP} (route 1), and ATPase with K_M value of 1 μM is that of simple hydrolysis of ATP through the myosin-ATP complex, M_{ATP}, since M_P^{ADP} is formed extremely rapidly.

$$M + ATP \rightleftharpoons M_1ATP \rightleftharpoons M_2ATP \rightleftharpoons M_P^{ADP} \rightleftharpoons {}^{o}M + ADP + Pi \rightleftharpoons M + ADP + Pi \quad 1)$$

$$M + ATP \rightleftharpoons \underset{ATP}{M} \rightleftharpoons M + ADP + Pi \quad 2)$$

In section 3, the amount of ATP and ADP bound to myosin during the ATPase reaction were measured. An ATP regenerating system was coupled with ATPase reaction to convert free ADP produced to ATP very rapidly:



The amount of bound ADP was measured as the amount of ADP determined by separating nucleotide after stopping the reaction with TCA. The amount of total bound nucleotide to myosin was determined using a rapid-flow dialysis method. Then the amount of bound ATP was determined by substracting the amount of bound ADP from that of total bound nucleotide. The maximum amount of ADP and ATP bound to myosin was 0.6 and about 1 mole, respectively, per mole of myosin. The dissociation constant of binding of ATP was 1 μM which was equal to the K_M value of myosin-ATPase reaction at the steady state at high ATP concentrations (route 2). The binding of ADP to myosin was stronger than that of ATP. The amount of ADP bound to myosin increased rapidly

to 1 mole per mole of myosin in the initial phase of the myosin-ATPase reaction, and it decreased to the steady-state level at rate constant similar to that of decomposition of M_P^{ADP}. These results agreed well to the mechanism of myosin-ATPase reaction shown by kinetic analysis, and showed the existence of stoichiometric amount of M_{ATP}.

In section 4, we examined the separation of S-1 into two fractions: one forms and the other does not form M_P^{ADP}. 0.5 mole of ATP was added to 1 mole of S-1 in acto-S-1 with an ATP regenerating system, and S-1 was separated by centrifugation into two fractions, *i. e.*, S-1 bound with F-actin (precipitate) and S-1 dissociated from F-actin (supernatant). S-1 was devided almost equally between the supernatant and precipitate but the size of initial burst of Pi-liberation (M_P^{ADP} formation) of S-1 in the supernatant was about 0.7 mole/mole of S-1 while that of S-1 in the precipitate was about 0.3 mole/mole of S-1. On repeating the separation procedures the size of the initial burst of S-1 in the supernatant increased, while that of S-1 in the precipitate decreased as anticipated. On the other hand, when actomyosin was dissociated by addition of various amount of ATP, both the size of the initial burst of myosin in the supernatant and that in the precipitate were always equal to the original value, *i. e.*, 0.5 mole/mole of myosin. Therefore it is concluded that two heads of the myosin molecule are dissimilar, and that M_P^{ADP} is formed on the one head and M_{ATP} is formed on

the another heads of myosin.

In section 5, The relationship between the ATPase activity and the amount of ATP and ADP bound to HMM in HMM-F-actin-relaxing protein system was measured both in the presence and absence of trace amount of Ca^{2+} ions. When the amount of ATP added was less than 1 mole/mole of HMM, almost all the nucleotide added was observed as ADP bound to HMM and the ATPase activity of HMM-FA-RP system increased linearly with increase in the amount of ATP added and were independent of the presence of 0.05 mM Ca^{2+}. This result indicate clearly that the reaction intermediate of myosin ATPase, M_P^{ADP} is the intermediate for the actomyosin ATPase reaction, and that the binding of ATP to myosin is not required for expression of full ATPase activity of actomyosin. In the presence of Ca ions, the rate of ATPase activity and the amount of ADP maintained constant with further increase in the amount of ATP added. However, in the presence of EGTA, the ATPase activity decreased with increase in the amount of ATP added and reached the level of HMM ATPase reaction at 3 moles of added ATP/mole of HMM. The amount of ADP bound to HMM decreased with increase in the amount of ATP added and reached to the level (0.5 mole/mole of HMM) of HMM-ATP system. The binding of ATP to HMM was only observed when the amount of ATP added was more than 1 mole/mole of HMM. The amount of bound ATP increased with increase in the amount of ATP added and was about 1.2 moles/mole of HMM at 3 mole of added ATP/mole of HMM. This value was equal

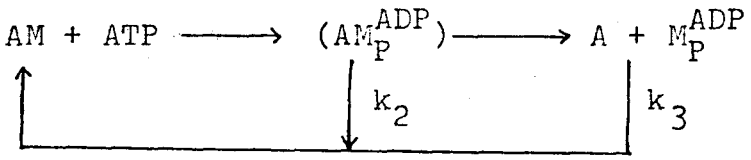
to that observed with HMM-ATP system. The amount of bound ATP was proportional to the extent of inhibition of acto-HMM ATP by removal of Ca^{2+} ions. The binding of ATP to HMM was markedly inhibited by the addition of 0.1 mM $CaCl_2$. It is concluded that formation of the myosin-ATP complex, M_{ATP}, is required for regulation of actomyosin ATPase in the presence of RP by trace amount of Ca^{2+} ions.

The mechanism of the actomyosin-ATPase reactions was studied in the last section. The time course of binding of HMM_P^{ADP} complex formed by adding ATP to HMM with F-actin was measured from the increase in light-scattering intensity, since the rate of formation of HMM_P^{ADP} from HMM and ATP is much higher than that of decomposition of HMM_P^{ADP} even in the acto-HMM-ATPase reaction. The extent of dissociation of acto-HMM in the presence of ATP (α) was also measured, and they were compared with the kinetic parameters of ATP hydrolysis by acto-HMM in the steady state. The rate of the acto-HMM-ATPase activity at the steady state (v_0) is expressed by an equation:

$$v_0 = k_1 + (1 - \alpha)k_2 + \alpha k_3$$

Here k_1, k_2 and k_3 are the rate of HMM-ATPase in the absence of F-actin, the value of maximum activity of acto-HMM-ATPase when the concentration of F-actin was varied, and the rate constant

of binding of HMM_P^{ADP} with F-actin, respectively. This results provides the mechanism that ATP is hydrolyzed in the actomyosin-ATPase reaction via two routes (except myosin-ATPase reaction through $\underset{ATP}{M}$):



It is considered that the hydrolysis of ATP through these two routes are necessary for contraction (39, 40, 43).

2. Kinetic Studies of the Myosin-ATPase Reaction

1. The amount and rate of formation of the reactive myosin-phosphate-ADP complex, M_P^{ADP}, in 0.5 M KCl at pH 7.8 and 0°C was estimated by measuring the rate of the initial burst of Pi-liberation after stopping the reaction by trichloroacetic acid. At ATP concentrations belwo 0.3μM, the values of K_M and V_{max} for formation at M_P^{ADP} were 0.3μM and 0.70 mole Pi/min·4.8×10^5g myosin, respectively, while at ATP concentrations above 0.3μM, the values of K_M and V_{max} were much larger.
2. The rate of rapid change in the UV spectra after adding ATP to H-meromyosin was measured over a range of high concentrations of ATP in 0.2 M KCl at pH 7.8 and 3.7°C. The values of K_M and V_{max} were 0.2 mM and 30 sec^{-1}, respectively.
3. The rate of decomposition of the reactive myosin-phosphate-ADP complex, M_P^{ADP}, was measured using a rapid flow-dialysis method, and the following results were obtained: (i) The rate constants of liberation of radioactive Pi and ADP from M_P^{ADP} were similar to that of ATPase [EC 3.6.1.3] in the steady state at high ATP concentrations, but were much higher than that of ATPase at low ATP concentrations. (ii) They were unaffected by adding a large amount of non-radioactive ATP. (iii) The rate of liberation of ADP from the simple myosin-ADP complex, which was formed by mixing free enzyme and ADP, was too fast to be the rate-determining step in the ATPase reaction.

4. At ATP concentrations above 0.5μM, the values of K_M and V_{max} of myosin-ATPase in the steady state were 1μM and 0.44 mole Pi/min·4.8x10^5g myosin, respectively, while at ATP concentrations below 0.3μM, the rate of the ATPase reaction was almost independent of the ATP concentration and had a V_{max} value of about 1/4 of that obtained at high ATP concentrations.

5. The results of Lymn and Taylor on the dependence of the burst size on the ATP concentration was reexamined in 0.5 M KCl and 10mM $MgCl_2$ at pH 8.0 and 20°C. Both the rate in the steady state and the size of the initial burst of Pi-liberation were found to be independent of the ATP concentration at concentrations of 5 to 200μM.

6. A typical initial burst occurred both at 0 and 20°C, after adding a large amount of ATP to myosin under conditions, in which all the active sites were occupied by ADP.

INTRODUCTION

The initial rapid liberation of inorganic phosphate, which is conventionally called as the initial burst of myosin-ATPase [EC 3.6.1.3], was first observed by Tonomura et al. (53,54) and later confirmed by Sartorelli et al. (55) and Lymn and Taylor (56). Tonomura et al. (54, 57, 58) attributed this initial burst to formation of the reactive myosin-phosphate-ADP complex, M_P^{ADP}. The following results were reported on the formation of M_P^{ADP} : (i) a stoichiometric amount of H^+ is rapidly absorbed by myosin, and then it is liberated just before formation of M_P^{ADP} (59, 60), (ii) NTP binds to myosin in the presence of MgATP and the NTP-myosin thus formed shows no initial burst of Pi-liberation (61-63) and (iii) during the initial phase of the reaction a P-exchange reaction occurs between the intermediate and the terminal phosphate of ATP (64). From these results, they proposed the following reaction mechanism (60),

$$M + S + H^+ \underset{(1)}{\rightleftharpoons} M_1SH^+ \underset{(2)}{\rightleftharpoons} M_2S + H^+ \rightleftharpoons$$

$$\underset{(3)}{} \; M\genfrac{}{}{0pt}{}{\cdot\cdot\cdot ADP}{\sim P} \underset{(4)}{\longrightarrow} M\genfrac{}{}{0pt}{}{\cdot\cdot\cdot ADP}{\cdot\cdot\cdot P}$$

In this scheme, the reactive myosin-phosphate-ADP complex, M_P^{ADP}, consists of two kinds of intermediates, $M\genfrac{}{}{0pt}{}{\cdot\cdot\cdot ADP}{\sim P}$ and $M\genfrac{}{}{0pt}{}{\cdot\cdot\cdot ADP}{\cdot\cdot\cdot P}$.

The former can exchange P with ATP and is called phosphoryl myosin, while the latter cannot exchange P with ATP. After addition of a stoichiometric amount of ATP to myosin, the rate constants of liberation of ADP (53) and of restoration of the change in the UV-spectrum (60) were almost equal to that of ATPase in the steady state, while the rate of slow liberation of H^+ (60, 62) and the recovery process of the initial burst of Pi-liberation (64) were much slower than the rate of the ATPase reaction in the steady state. Therefore, it was concluded that decomposition of $M\!:\!\!\cdot\!\!\cdot^{ADP}_{P}$ occurs in two steps:

$$M\!:\!\!\cdot\!\!\cdot^{ADP}_{P} \xrightarrow[(5)]{k_5} M\cdot\cdot_P + ADP \xrightarrow[(6)]{k_6} M$$

$$+ ADP + Pi + H^+,$$

where k_5 is almost equal to the rate constant (Vm) of the ATPase reaction in the steady state and k_6 is only a fraction of this value. Furthermore, the initial burst of Pi-liberation was completely suppressed by *p*-nitrothiophenylation of myosin, while the ATPase reaction in the steady state was unaffected by this modification (63). These results strongly suggest that M_P^{ADP} is not an intermediate in the main path of ATP-decomposition in the steady state.

To elucidate the mechanism of formation of M_P^{ADP}, the rates

of initial burst of Pi-liberation and change in the UV-spectrum of myosin were measured over a wide range of ATP concentrations. It was found that the value of Km of M_P^{ADP} formation at ATP concentrations below 0.3μM was very low, but apparant values of Km and Vm increased at ATP concentrations above 0.3μM. Saturation of the rates of H^+-liberation and the initial burst of Pi-liberation with increase in the ATP concentration were reported by Finlayson and Taylow (65) and Lymn and Taylor (56), but their results did not follow the Michaelis-Menten equation. On the other hand, Morita (66) reported that the rate of change in the UV spectra of H-meromyosin was proportional to the ATP concentration up to a concentration of 80μM. In the present investigation, we found that the dependence on ATP concentration of the rate of change in the UV spectra of H-meromyosin over a range of high concentrations of ATP followed the Michaelis-Menten equation, and that the values of K_f and V_f were 0.2mM and 30sec^{-1}, respectively, in 0.2 M KCl at pH 7.8 and 3.7°C.

Recently, Taylor et al. (67) measured the rate of decomposition of M_P^{ADP} and that of decomposition of the simple myosin-ADP complex, M-ADP, using a gel-filtration method. They reported (i) that the rate constant of Pi-liberation from M_P^{ADP} was in the same order of magnitude as that of ADP-liberation and was similar to that of ATPase in the steady state, (ii) that the liberation of ADP from M_P^{ADP} was accelerated by ATP, and (iii)

that ADP was liberated from M-ADP with a rate constant comparable to that of ADP-liberation from M_P^{ADP}. However, the difficulty of analysis by transporting the interacting system was indicated by Nicole et al. (68). Therefore, we measured the rates of decomposition of M_P^{ADP} and M-ADP, using a rapid flow-dialysis method (69) developed for measuring enzymatic reactions. We confirmed the result of Taylor et al. that the rate of Pi-liberation from M_P^{ADP} was similar to that of ADP-liberation. However, we could not observe any acceleration of decomposition of M_P^{ADP} on adding high concentrations of ATP. Furthermore, the rate of ADP-liberation from M-ADP was shown to be too fast to be determined by the flow-dialysis method. The conclusion, that the rate constant of ADP-liberation from the simple myosin-ADP complex was much higher than that of the rate-determining step of ATPase, was also drawn by analyzing the initial phase of myosin ATPase in the presence of ADP both at 0 and 20°C.

Recently, Taylor and his collaborators found that the rate of M_P^{ADP} formation is proportional to the ATP concentration from 5 to 100μM (56) and that the rates of liberation of Pi and ADP from M_P^{ADP} are almost equal to the rate of the ATPase reaction in the steady state, as described above and proposed the following scheme:

$$M + S \rightleftharpoons MS \longrightarrow M_P^{ADP} \longrightarrow M + ADP + Pi + H^+.$$

Furthermore, they concluded that the myosin molecule has two hydrolytic sites which interact with each other (56, 67), since their results indicated that the burst size of Pi-liberation increases and approaches tow moles per mole of myosin at an ATP concentration of about 30μM (56).

However, in contrast to the result obtained by them (56), the amount of the initial burst of Pi-liberation and the rate of the ATPase reaction in the steady state were constant and were independent of the ATP concentration, at ATP concentrations above 5 M. Furthermore it was concluded that the rate of the ATPase reaction in the steady state could be expressed as the sum of the rates of two kinds of ATPase reactions: the one with lower values of K_M and V_{max} was a hydrolytic reaction via M_P^{ADP} and the other with higher values of K_M and V_{max} was a simpel hydrolysis of ATP.

EXPERIMENTAL

Myosin was prepared from rabbit skeletal muscle by the method of Perry (70). It was further purified by phosphocellulose column chromatography using the method of Harris and Suelter (71). Fresh preparations of myosin were used throughout. H-Meromyosin was prepared as described by Seikya et al. (72). F-Actin was prepared from an acetone powder oa rabbit skeletal muscle by the method of Mommaerts (73) with slight modifications (74).

The crystalline sodium salt of ATP was purchased from Sigma Chemical Company. ^{32}P-Labelled ATP was synthesized enzymatically by the method of Glynn and Chappel (75). ^{3}H-Labeled ATP and ADP were purchased from the Radiochemical Centre, England, and purified by the method of Cohn and Carter (76).

ATPase was measured as described previously (64), from the time course of ^{32}P-liberation from ^{32}P-ATP. To calculate the rate constant of ATPase, the values of 4.8×10^5g and 3.4×10^5g were adopted as the weights per mole of the ATPase active site of myosin and H-meromyosin, respectively, since we previously showed that the myosin molecule contains one active site for ATPase (60). The reactions were usually made in 0.5 M KCl, 2.5mM $MgCl_2$, and 50mM Tris-HCl at pH 7.8 and 0° The simple mixing apparatus devised by Kanazawa et al. (77) was used to follow the rapid initial reaction. When the reaction time was very long, a correction was made for non-enzymatic hydrolysis of ATP. Change in the UV spectra of H-meromyosin was measured from the absorbance at 293nm in a stopped-flow spectrophotometer with a mixing time of 6-9 msec or 0.9 msec, as described previously (64,66) The concentration of protein was estimated by the biuret reaction calibrated by nitrogen determination.

The rapid flow-dialysis method was developed from that of Colowick and Wamock (69). To follow reactions with considerably high rate constants, a Millipore filter of 0.22μ pore size was used. One sheet of the membrane was used throughout one series

of the experiments, after degassing it in buffer solution. The flow-rate of buffer through the lower chamber was fixed at 56 ml/min. The fluctuation of pressure caused by the pump was decreased by the buffer-action of the enclosed air, and the pressures on both sides of the membrane were maintained at the same level by applying a negative pressure to the efflux tube from the chamber. The temperature was controlled by circulating water. Samples of 5 ml were taken every ten seconds, and their radioactivities of ^{32}P and ^{3}H were measured by the methods described by Nakamura and Tonomura (64) and Taylor et al. (67), respectively.

The rates of diffusion of ^{32}P-phosphate and ^{3}H-ADP through the Millipore filter under the conditions used were 1/200 and 1/600 of the total amount per min, respectively, at 0°C and pH 7.8 in 0.5 M KCl, when the volume of the reaction mixture in the upper chamber was 2 ml. The rate of diffusion of myosin through the membrane was measured both from the decrease in concentration of myosin in the upper chamber after circulation of the buffer for more than 10 hr and from appearance of myosin in the lower chamber at a flow-rate of 2 ml/min. The diffusion rate of myosin through the membrane in 0.5 M KCl at 0°C was less than 1% of that of ^{32}P-phosphate. Figure 3 shows typical time-courses of appearance of ^{32}P-phosphate in the lower chamber. The steady state level was reached within 1 min, and the half value of the steady state level in 34±5 sec. The steady state level was

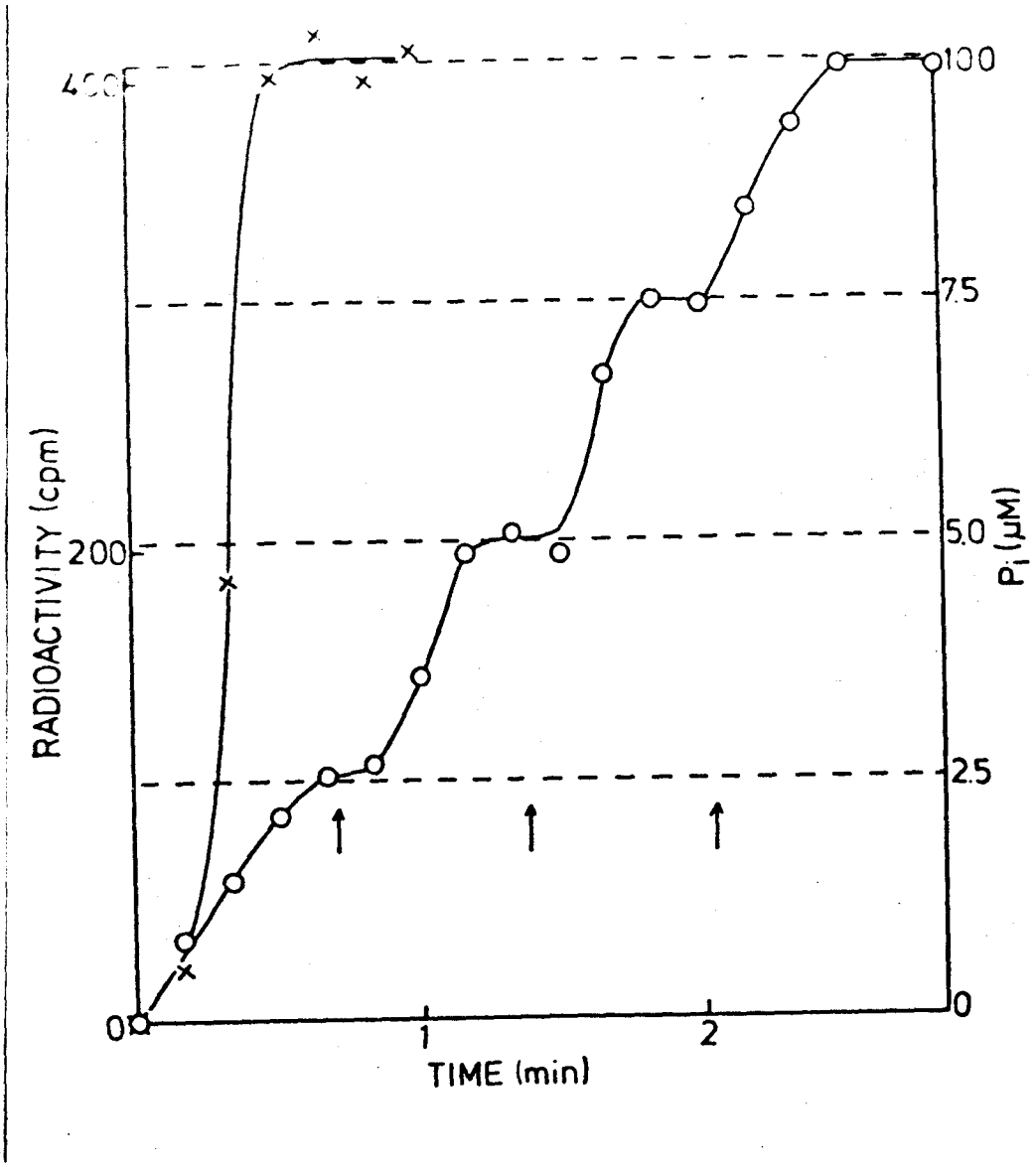


Fig. 3. Time-course of appearance of ^{32}P-phosphate in the lower chamber of the cell after adding a fixed amount of ^{32}P-phosphate to the upper chamber. 0.5 M KCl, 2.5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.8, 0°C. X, 10 μM ^{32}P-phosphate was added to the upper chamber at time 0. O, 2.5 μM ^{32}P-phosphate was added to the upper chamber successively at time 0 and the times indicated by ↑.

proportional to the concentration of Pi in the upper chamber. The time-course of appearance of ^{32}P-phosphate was unaffected by adding 5 mg/ml of myosin, 1mM non-radioactive Pi, 1 mM non-radioactive ATP, or 1 mM ADP (the results not shown). Moreover, neither the KCl concentration nor pH affected the steady state level or the time-course of appearance of ^{32}P-phosphate.

Figure 4 shows the time-course of appearance of Pi, when ^{32}P-phosphate was added exponentially to the upper chamber with fixed rate constants. This figure shows clearly that reactions with half-saturation times of less than about 10 sec could not be followed with this method. To measure the rates of liberation of ADP and Pi from the myosin-phosphate-ADP complex, the time-courses of appearance of ^{32}P-phosphate and ^{3}H-ADP after adding γ-^{32}P-ATP or ^{3}H-ATP to the myosin solution in the upper chamber were compared with the calibration curves, assuming that the liberation of Pi and ADP from M_P^{ADP} occurs as a one step reaction. The reaction was usually started by adding 0.2 ml of γ-^{32}P-ATP solution or ^{3}H-ATP solution to 2 ml of myosin solution in the upper chamber. The rate of liberation of ADP was measured after adding 0.1 ml of non-radioactive ADP or ATP solution to the final concentration of 0.2mM, under conditions where the binding of ADP with myosin was strong.

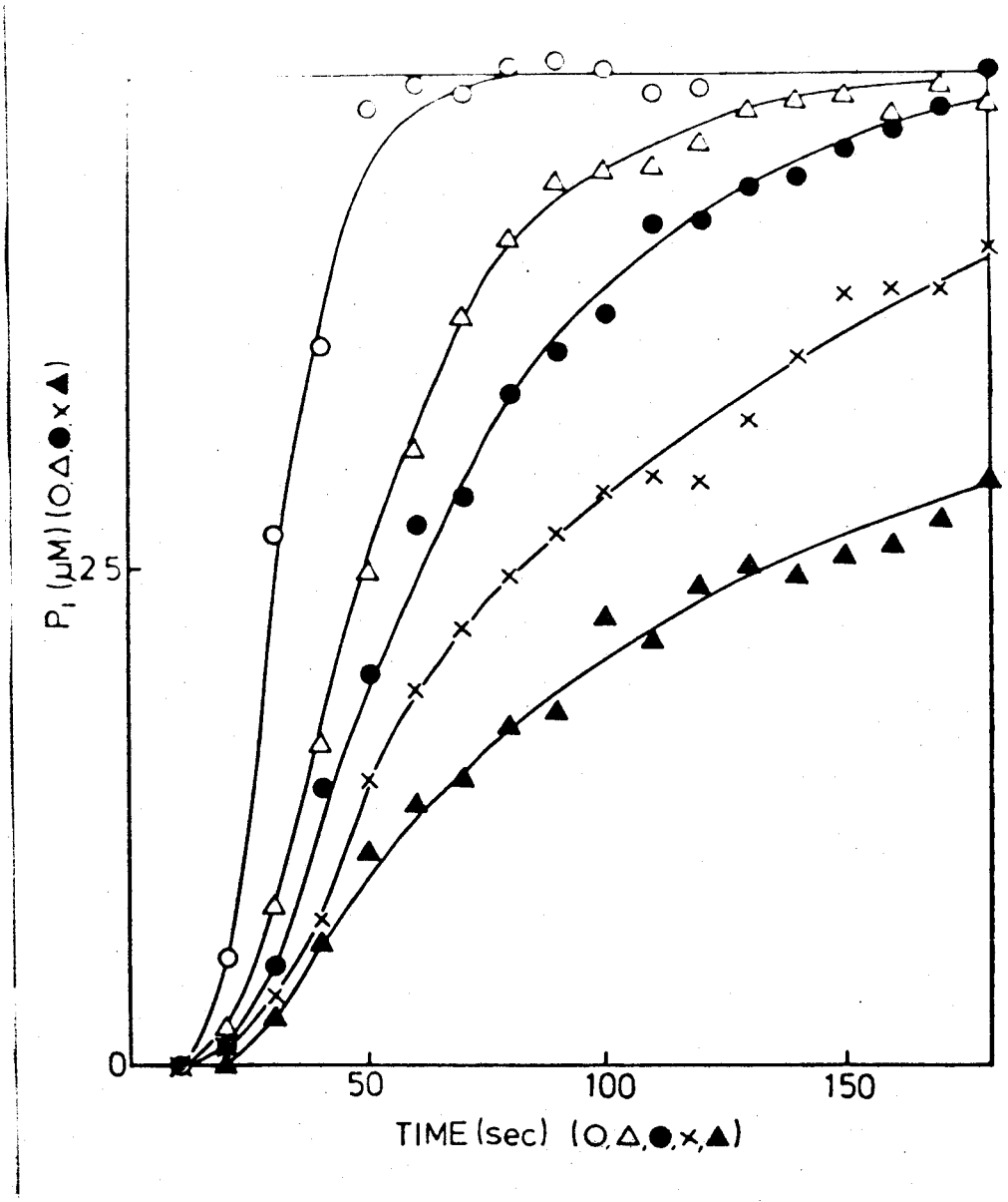


Fig. 4 Time-course of appearance of ^{32}P-phosphate in the lower chamber after adding ^{32}P-phosphate exponentially to the upper chamber. 0.5 M KCl, 2.5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.8, 0°C. ^{32}P-phosphate (final concentration, 5 μM) was added exponentially to the upper chamber with half times of 0(O), 20(Δ), 40(●), 60 (X), and 100 sec (▲), respectively. The concentrations of free Pi, which correspond to the rates of appearance of Pi in the lower chamber, are plotted on the ordinate.

RESULTS

Dependence on the ATP Concentration of the Rate of Formation of the Reactive Myosin-Phosphate-ADP Complex - The rate of M_P^{ADP} formation, v_f, has previously been measured by us (58) and later by Lymn and Taylor (56). Previously we measured it in the presence of 2 mg/ml of myosin at ATP concentrations above 1.45 μM, while Lymn and Taylor measured it in the presence of 2 mg/ml myosin at ATP concentrations above 5 μM. Therefore, we measured the v_f in a range of ATP concentrations from 0.06 to 5 μM in the presence of 0.03 mg/ml myosin in 0.5 M KCl, 2.5 mM $MgCl_2$ and 25 mM Tris-maleate buffer at pH 7.8 and 0°C. Figure 5 shows a double reciprocal plot of v_f against the ATP concentration, [S]. In this experiment four myosin preparations were used, and the values of v_f relative to that at 0.26 μM ATP were plotted. The values of v_f of these preparations at 0.26 μM ATP were 0.28, 0.35, 0.34 and 0.31 mole/min·4.8×10^5g myosin, and the average value of 0.32 min^{-1} was used in the figure. The figure shows that a plot of v_f^{-1} versus $[S]^{-1}$ gave two straight lines bending at about 0.3 M ATP. At ATP concentrations below 0.3 μM, the maximum value of v_f, $V_{f max}$, was 0.70 mole/min·4.8×10^5g myosin and the value of K_M was 0.3 μM. At ATP concentrations above 0.3 μM, the values of K_M and $V_{f max}$ were too large to measure accurately with our simple mixing apparatus, and

increased in proportion to ATP concentration. The apparent

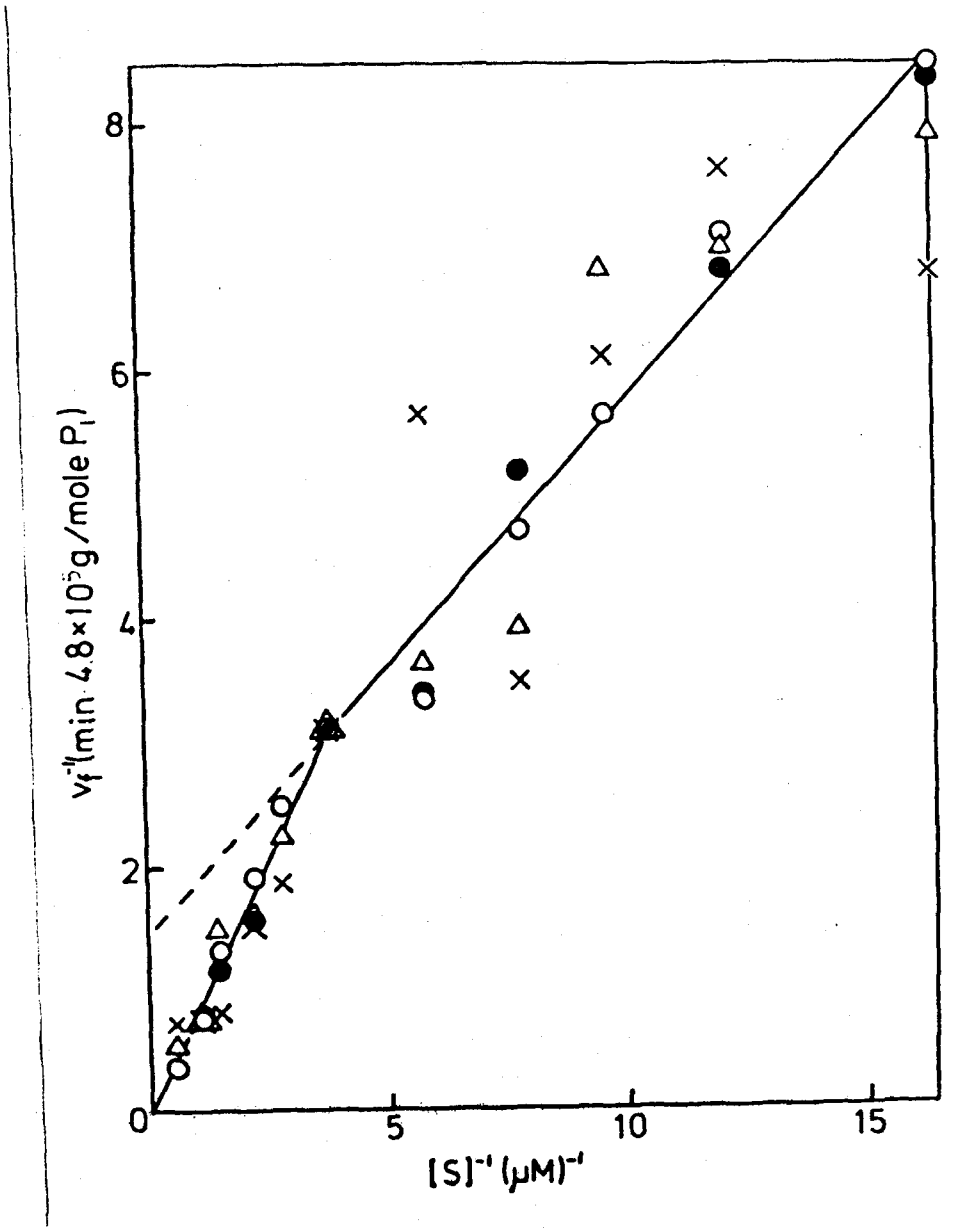


Fig. 5 Lineweaver-Burk plot of the rate of the initial burst of Pi-liberation, v_f, in 0.5 M KCl. 0.03 mg/ml myosin, 0.5 M KCl, 2.5 mM $MgCl_2$ and 25 mM Tris-maleate buffer at pH 7.8 and 0°C. O, ●, X, △, different preparations of myosin, see text.

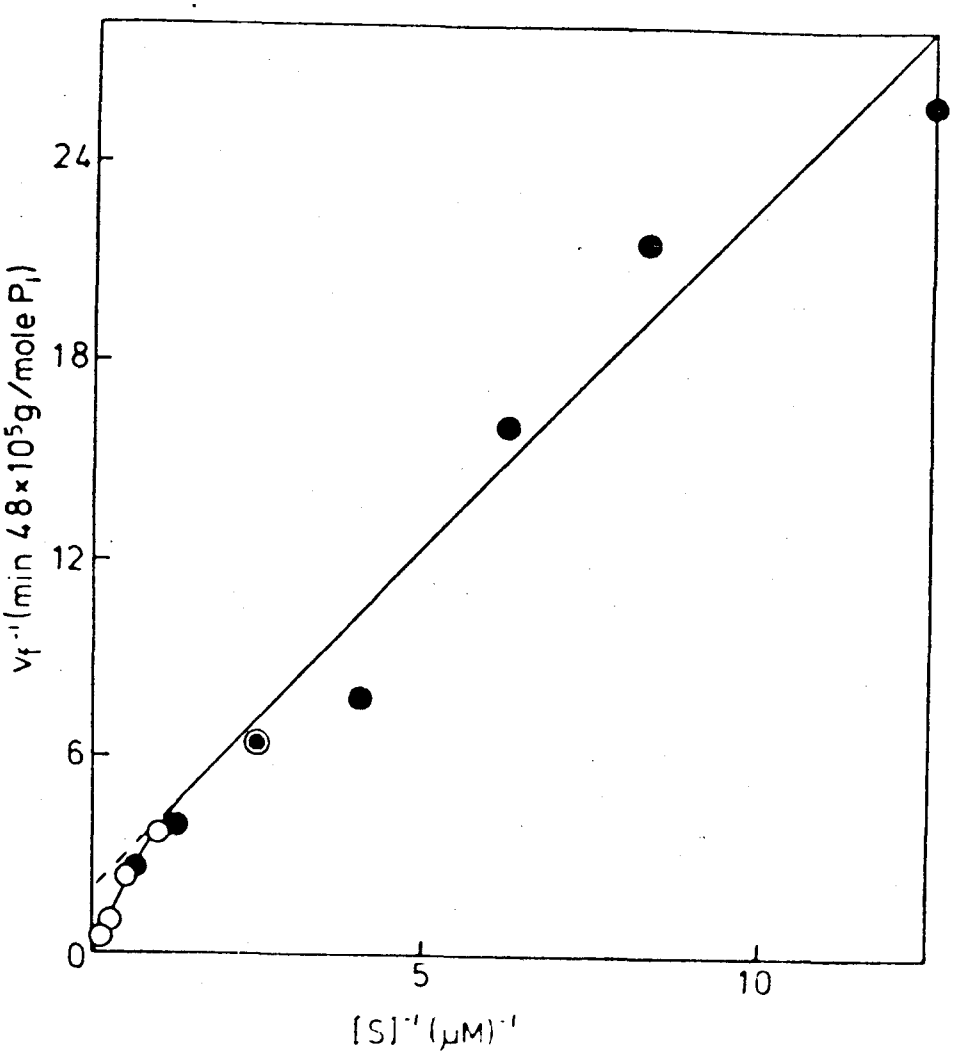


Fig. 6 Lineweaver-Burk plot of the rate of the initial burst of Pi-liberation, v_f, in 1.5 M KCl. 0.03 mg/ml myosin, 1.5 M KCl, 2.5 mM $MgCl_2$ and 25 mM Tris-maleate buffer at pH 7.8 and 0°C. ○, ●, different preparations of myosin.

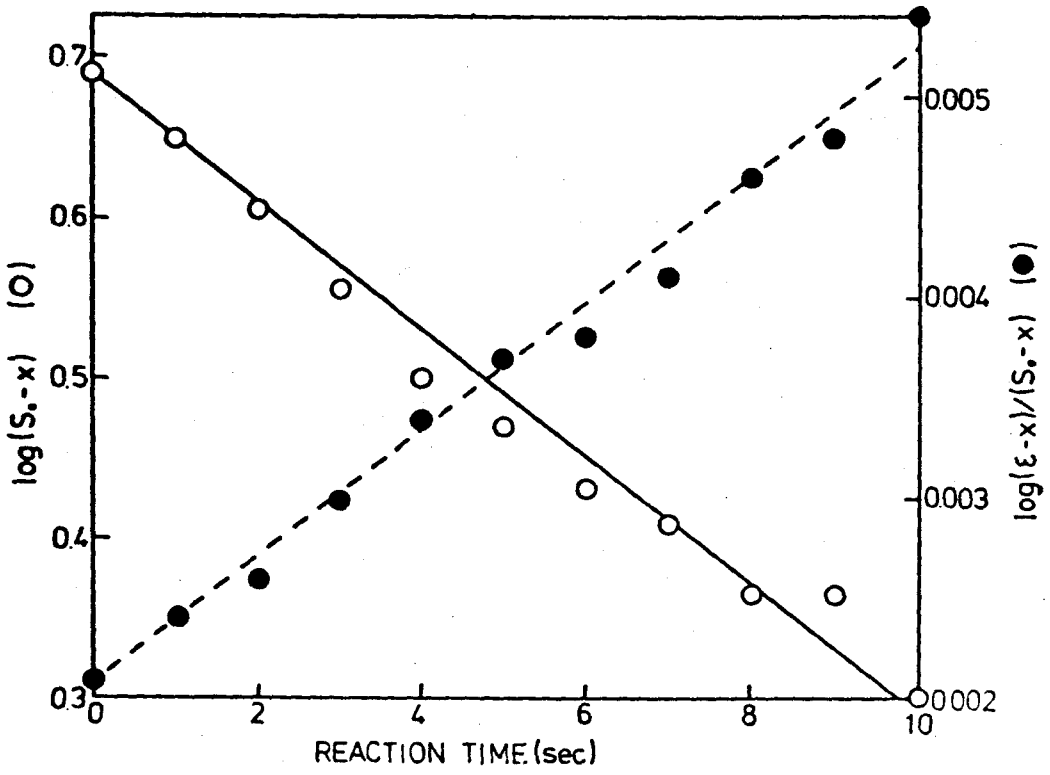


Fig. 7 Time-course of the initial burst of Pi-liberation. 2.52 mg/ml myosin, 5 μM ATP, 0.5 M KCl, 2.5 mM $MgCl_2$ and 25 mM Tris-maleate buffer at pH 7.8 and 0°C. The plot of $\log(S_o - x)$ against t shows a first order reaction and the plot of $\log(\varepsilon - x)/(S_o - x)$ against t shows a second order reaction. S_o, x and ε are the initial concentration of ATP, the concentration of TAC-Pi liberated at time t and the molar concentration of active site.

second order rate constant was about $1.2 \times 10^{6} M^{-1}$ min^{-1}.

As shown in Fig. 6, a plot of v_f^{-1} versus $[S]^{-1}$ in 1.5 M KCl gave two straight lines bending at 1 μM of ATP. In the low ATP concentration range, the values of K_M and $V_{f\,max}$ were 1 μM and 0.43 mole/min·4.8×10^{5}g myosin, while at high concentrations of ATP v_f increased in proportion to the ATP concentration.

The value of v_f after mixing 2.52 mg/ml (5.25 μM) myosin with 5 μM ATP was estimated as 5.9 moles/min·4.8×10^{5}g myosin, which was almost equal to the value of 6.0 moles/min·4.8×10^{5}g myosin obtained in the presence of 0.03 mg/ml (0.0625 μM) myosin (Fig. 5). To examine the order of the reaction of initial Pi-liberation, we plotted $\log(S_0 - x)$ and $\log(\varepsilon - x)(S_0 - x)$ against time, where S_0, x and ε are the initial concentration of ATP, the concentration of TCA-Pi liberated at time t and the molar concentration of active site, respectively. However, we could not decide the order of the reaction from this type of experiment, as shown in Fig. 7.

Dependence of the Rate of Change in the UV Spectra of H-Meromyosin on the ATP Concentration at High ATP Concentrations - we measured the rate of change in the UV spectra of H-meromyosin (3 mg/ml) over the range of ATP concentrations from 20 μM to 5 mM in 0.2 M KCl, 2.5 mM $MgCl_2$, and 50 mM Tris-HCl at pH 7.8 and 3.7°C. Plots of stopped-flow measurements after mixing ATP with H-meromyosin showed an exponential increase in absorbance, without any lag phase. Thus, the semilogarithmic plot of

($\Delta A_{max} - \Delta A$) versus time gave a straight line, where ΔA_{max} and ΔA were the maximum value of increase in the absorbance at 293 nm and the value of increase at each time, respectively. Therefore, the rate of change in the UV spectra, v_f', was estimated from the time for half maximum change, $\tau_{1/2}$. Figure 8 shows a double reciprocal plot of v_f' against the ATP concentration. From this figure 0.2 mM and 30sec^{-1} were obtained as the values of K_M and V_{max}, respectively.

Pi-Liberation from the Myosin-Phosphate-ADP Complex - The rate of Pi-liberation from M_P^{ADP} was measured, using a rapid flow-dialysis method. The rate constant of Pi-liberation from M_P^{ADP} was estimated by comparing the time-couse of appearance of Pi in the lower chamber after adding ATP to myosin at a molar ratio of 1 : 1 with that observed when Pi was added in a predetermined manner in the upper chamber, as described in the "EXPERIMENTAL" (Fig. 4). The reaction was started by adding 0.2 ml of 55 μM ^{32}P-ATP to 2 ml of 2.75 mg/ml myosin solution in the upper chamber. The final concentrations of ATP and myosin were 5 μM and 2.5 mg/ml, respectively. Figure 8 shows the time-courses of appearance of Pi in the lower chamber in 2.5 mM $MgCl_2$ and 50 mM Tris-HCl, pH 7.8 at 0°C at various concentrations of KCl from 0.1 to 1.5 M. Figure 9 shows the dependences on KCl concentration of the rate constant of Pi-liberation from M_P^{ADP}, k_d^p, and that of ATPase in the steady state, K_0^p, at high ATP concentrations, i.e., in 25 μM ATP and 0.1 mg/ml

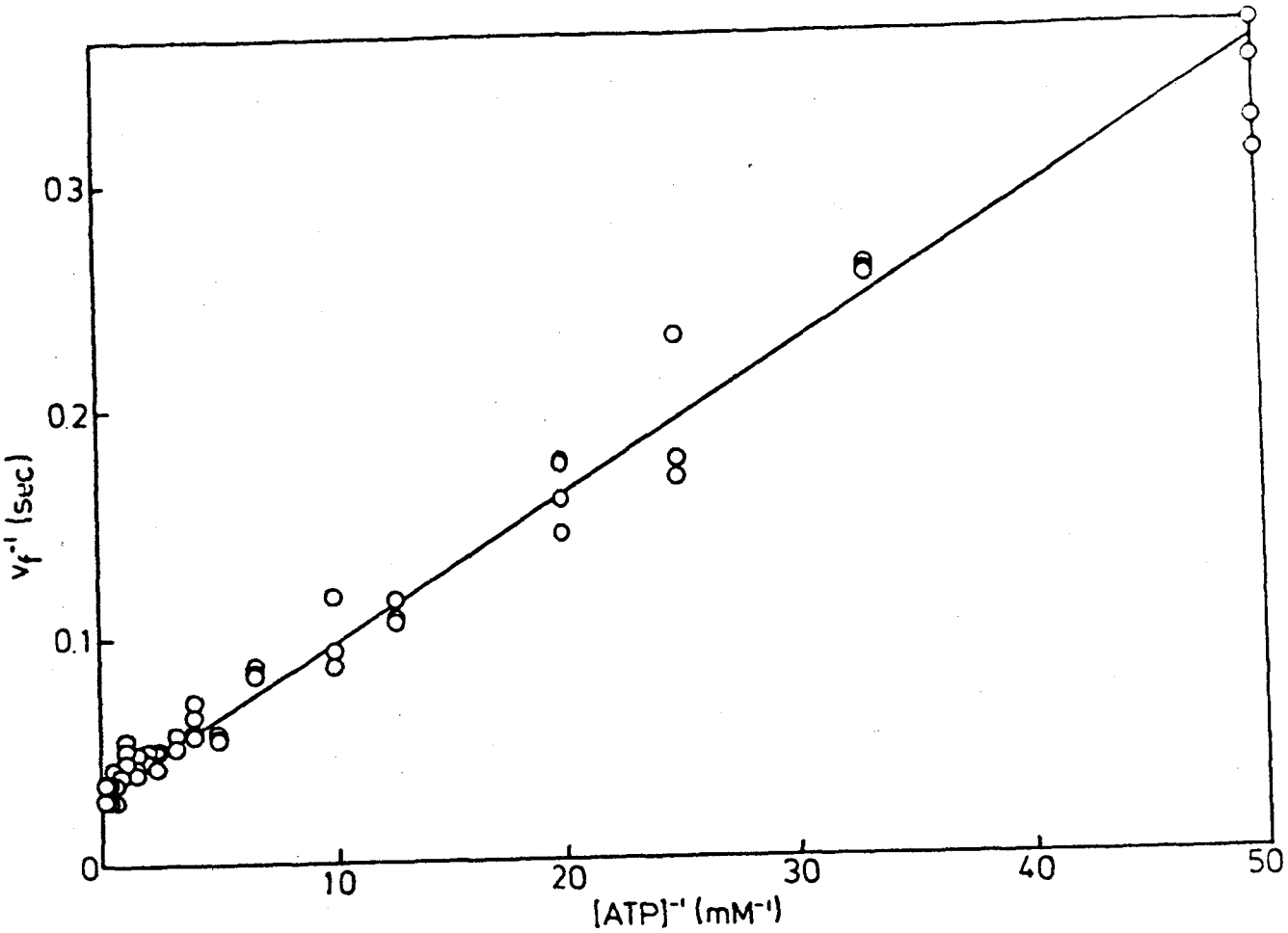


Fig. 8 Lineweaver-Burk plot of the rate of change in the absorbance at 293 nm of H-meromyosin after adding ATP. 3 mg/ml of HMM, 0.2 M KCl, 50 mM Tris-HCl, pH 7.8, 3.7°C. The concentration of $MgCl_2$ is 2.5 mM higher than that of ATP.

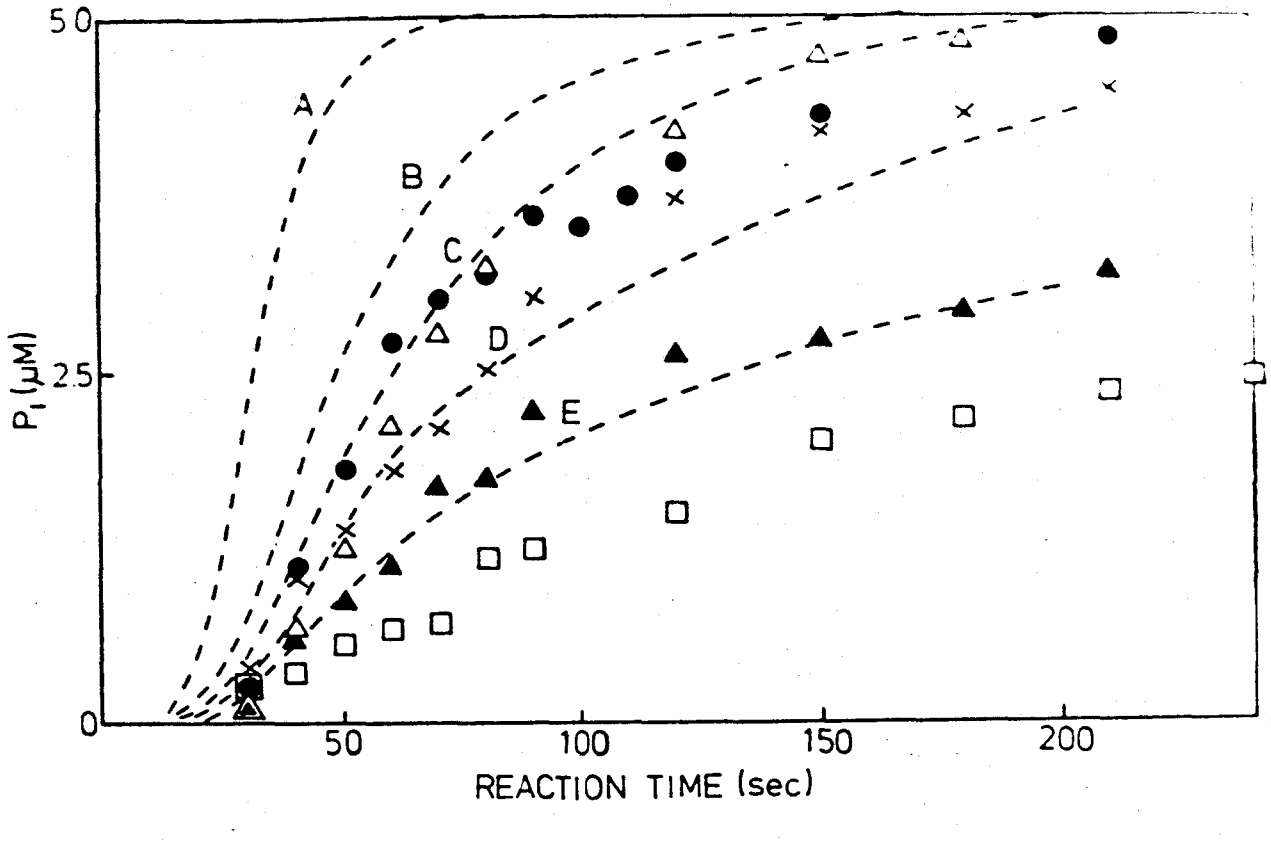
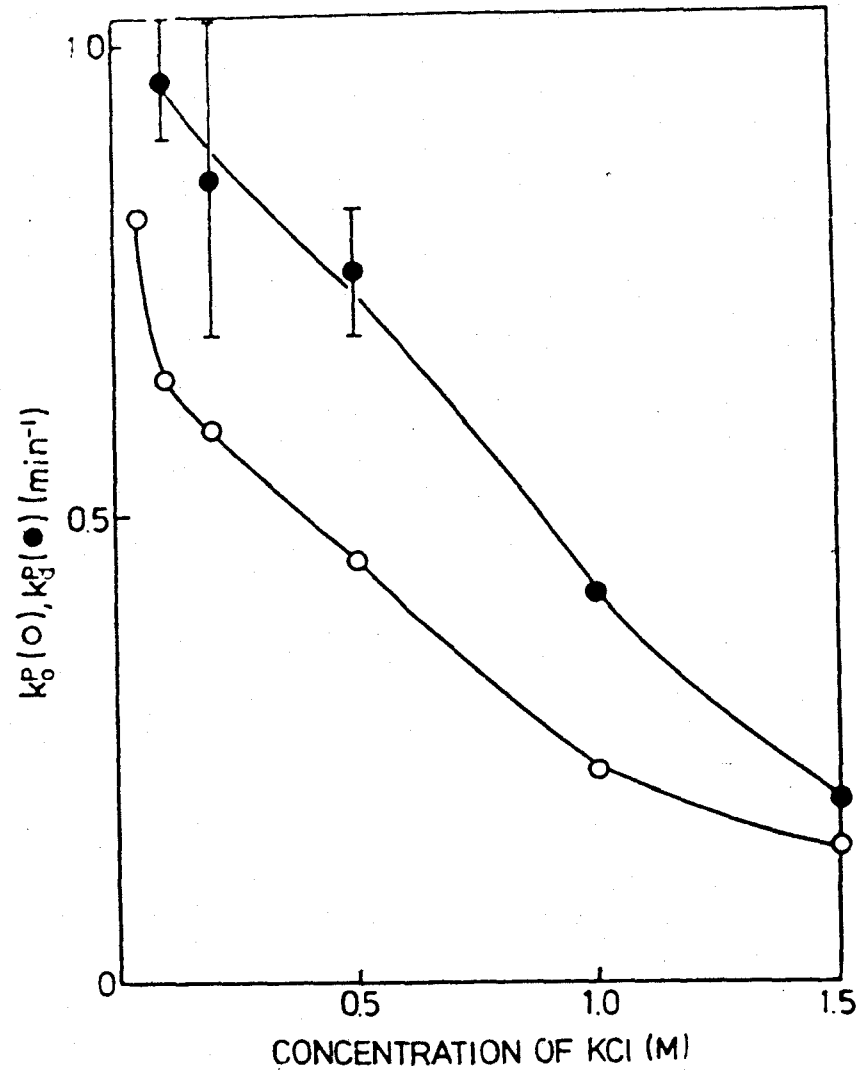


Fig. 9. Dependences on KCl concentration of the rate constant of liberation of phosphate from the myosin-phosphate-ADP complex

(A) Time-courses of appearance of ^{32}P-phosphate in the lower chamber after mixing 5 μM -^{32}P-ATP with 2.5 mg/ml of myosin at different KCl concentrations. 2.5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.8, 0°C. The concentrations of KCl were 0.1 (●), 0.2(△), 0.5(X), 1(▲), and 1.5 M(□), respectively. The broken linew (A-E) show the calibration curves (Fig. 4), when Pi was added to the upper chamber exponentially with half times of 0(A), 20(B), 40(C), 60(D), and 100 sec (E), respectively.



(B) Dependences on KCl concentration of the rate constant of liberation of ^{32}P-phosphate from the myosin ^{32}P-phosphate-ADP complex, k_d^P (●), and that of ATPase in the steady state at high concentrations of ATP, k_0^P (O). 2.5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.8, 0°C. The rate constants of Pi-liberation from M_P^{ADP} were estimated from the results shown in (A) Flags indicate mean square errors.

myosin. The values of k_d^p at various KCl concentrations were slightly higher than those of k_o^p.

Figure 10 shows the dependences on temperature of k_d^p and k_o^p in 0.5 M KCl, 2.5 mM $MgCl_2$, and 50 mM Tris-HCl at pH 7.8. The diffusion of Pi through the membrane at 23°C was about 10% higher than that at 0°C, and the rate constant of Pi-liberation was estimated, making a correction for the effect of temperature on the diffusion rate. As shown in Fig.10 the rate constants of Pi-liberation at various temperatures were similar to those of ATPase in the steady state, but the two did not coincide. Figure 11 shows the dependences on pH of k_d^p and k_o^p in 0.5 M KCl and 2.5 mM $MgCl_2$ at 0°C. The diffusion of Pi through the membrane was unaffected by changing the pH. The dependences on pH of these two rate constants were similar in some respects. The values of the rate constant of Pi-liberation from M_P^{ADP} obtained from three different series of experiments in 0.5 M KCl at pH 7.8 and 0°C were 0.76, 0.80, and 0.95 min^{-1}, respectively. Thus, the rate constant of Pi-liberation from M_P^{ADP} was slightly higher than that of ATPase in the steady state. We previously reported (60, 63) that the rate constant of ADP-liberation is similar to that of ATPase in the steady state. Therefore, it is concluded that the liberations of Pi and ADP from M_P^{ADP} occur concomitantly.

The effects of non-radioactive ATP and ADP on the rate of ^{32}Pi-liberation were measured (Fig. 12). The reaction was started

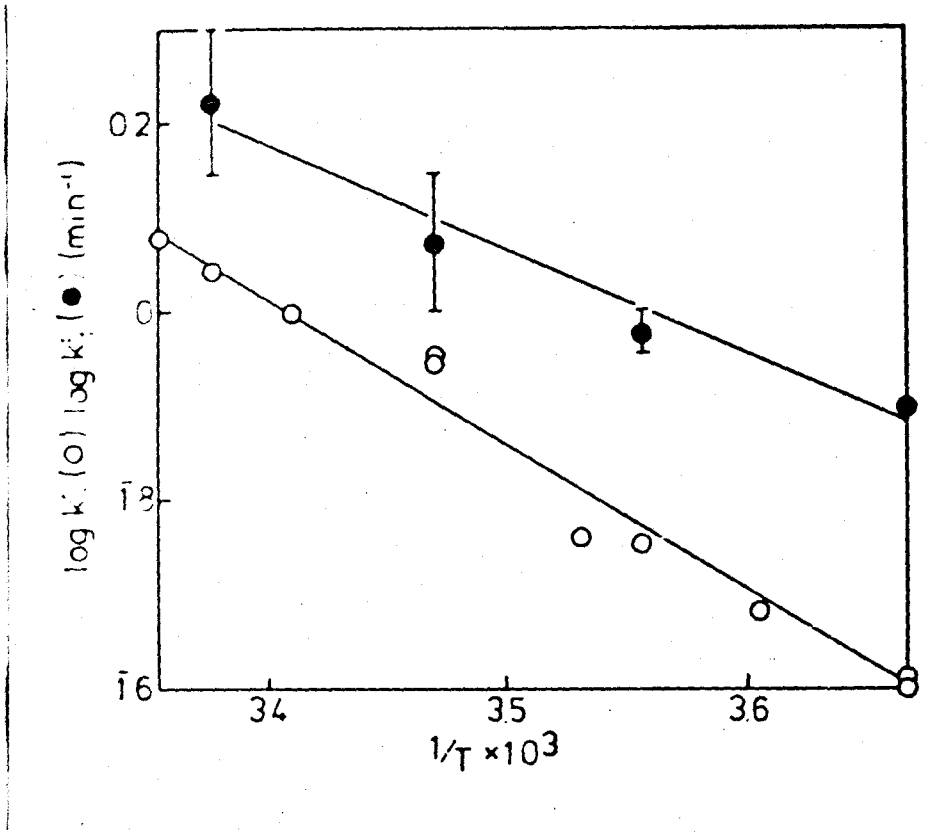


Fig. 10 Dependence on temperature of the rate constant of liberation of ^{32}P-phosphate from the myosin ^{32}P-phosphate-ADP complex, k_d^p (●), and that of ATPase in the steady state at high concentrations of ATP, k_o^p (○). 0.5 M KCl, 2.5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.8. Flags indicate mean square errors.

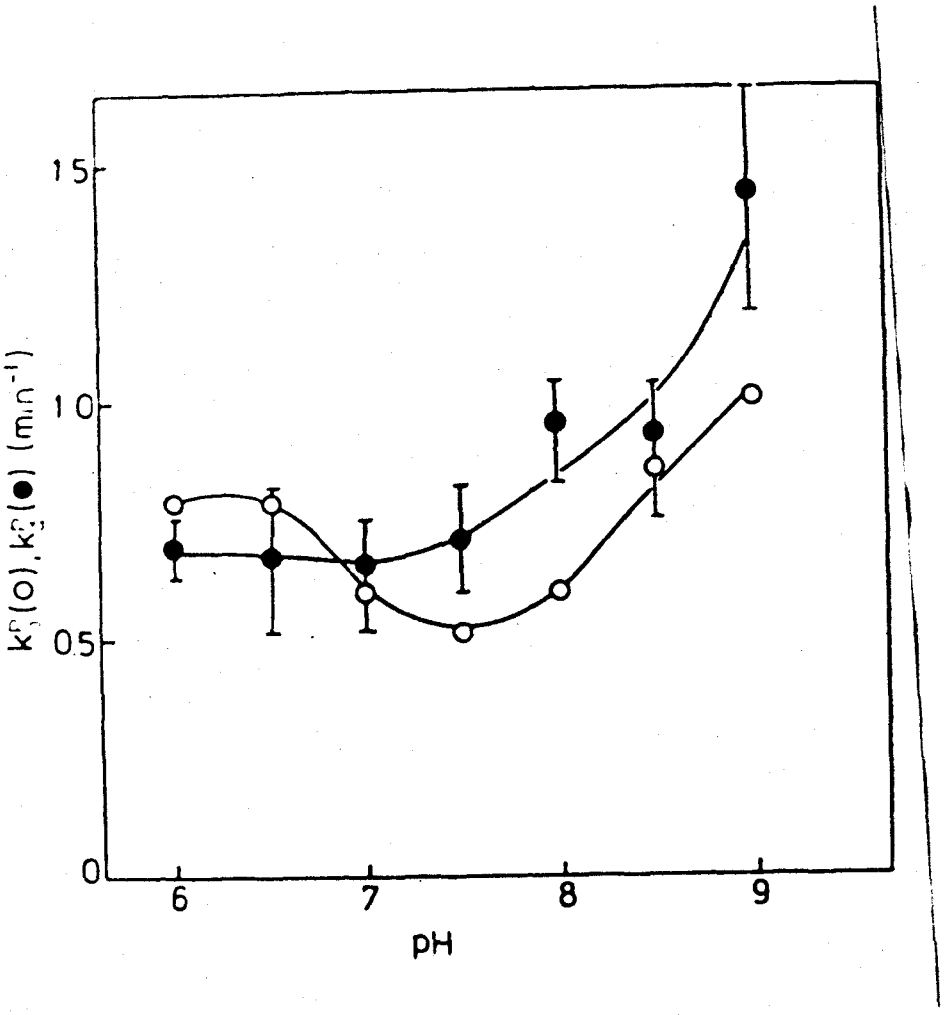


Fig. 11 Dependences on pH of the rate constant of liberation of ^{32}P-phosphate from the myosin-^{32}P-phosphate-ADP complex, k_d^P (●), and that of ATPase in the steady state at high concentrations of ATP, k_0^P (○). 0.5M KCl, 2.5 mM $MgCl_2$, 0°C. 50 mM Tris-HCl (pH 9.0, 8.5, and 8.0), Tris-maleate (pH 7.5 and 7.0) or maleate buffer solution (pH 6.5 and 6.0) was used. Flags indicate mean square errors.

by adding 5 μM ^{32}P-ATP to 2.5 mg/ml of myosin in 0.5 M KCl, 2.5 mM $MgCl_2$, and 50 mM Tris-HCl, pH 7.8 at 0°C, and 18 sec after the start of the reaction, 0.2 mM non-radioactive ATP or ADP was added. But the rate of Pi-liberation was unaffected by adding non-radioactive ATP or ADP.

ADP-Liberation from the Myosin-Phosphate-ADP Complex and the Myosin-ADP Complex - The reaction was started by adding 5 μM ^{3}H-ATP to 2.5 mg/ml of myosin in 0.5 M KCl, 2.5 mM $MgCl_2$, and 50 mM Tris-HCl, pH 7.8 at 0°C. The binding of ADP with myosin was highly dependent on temperature. The constants for competitive inhibition of ADP of the myosin ATPase reaction in the steady state were 1.7, 4.7, and 25 μM, respectively, at 0, 10, and 20°C. Thus, under the conditions used in this experiment, the binding of ADP to myosin was so strong that almost all the ^{3}H-ADP produced by the ATPase reaction remained to be bound to myosin. Therefore, the rate of ^{3}H-ADP-liberation from M_P^{ADP} was measured, after adding 0.2 mM non-radioactive ADP or ATP. As shown in Fig. 13, when 0.2 mM ADP was added 17 sec after the start of the reaction, ^{3}H-ADP was liberated very slowly from M_P^{ADP}, and the rate constant was almost the same as that of ATPase in the steady state at high ATP concentrations, as already reported by us (60, 63). The rate of ^{3}H-ADP- liberation after adding non-radioactive ADP increased with increase in the time after starting the reaction. Thus, when non-radioactive ADP was added 10 min after the start of the reaction, ^{3}H-ADP was liberated too rapidly to be followed by the present method.

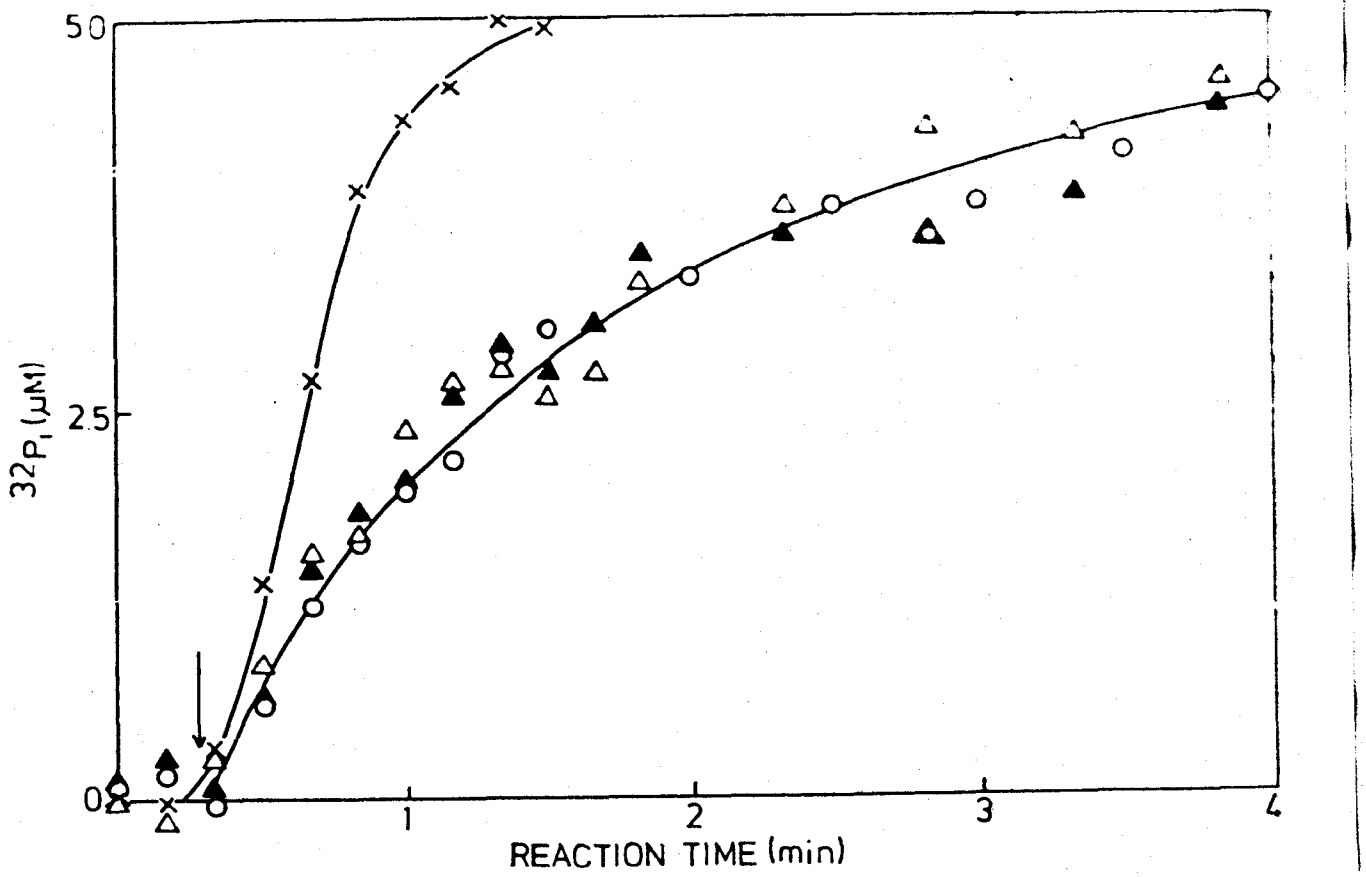


Fig. 12 Effects of ATP and ADP to the rate of liberation of ^{32}P-phosphate from the myosin-^{32}P-phosphate-ADP complex. The reaction was started by adding 5 μM γ-^{32}P-ATP to 2.5 mg/ml of myosin in the upper chamber in 0.5 M KCl, 2.5 mM Tris-HCl, pH 7.8 at 0°C (O,Δ,▲,). 0.2 mM Non-radioactive ATP (▲) or ADP (Δ) was added at the time indicated by ↓. X, Time-course after addition of 5 μM ^{32}P-phosphate to the upper chamber.

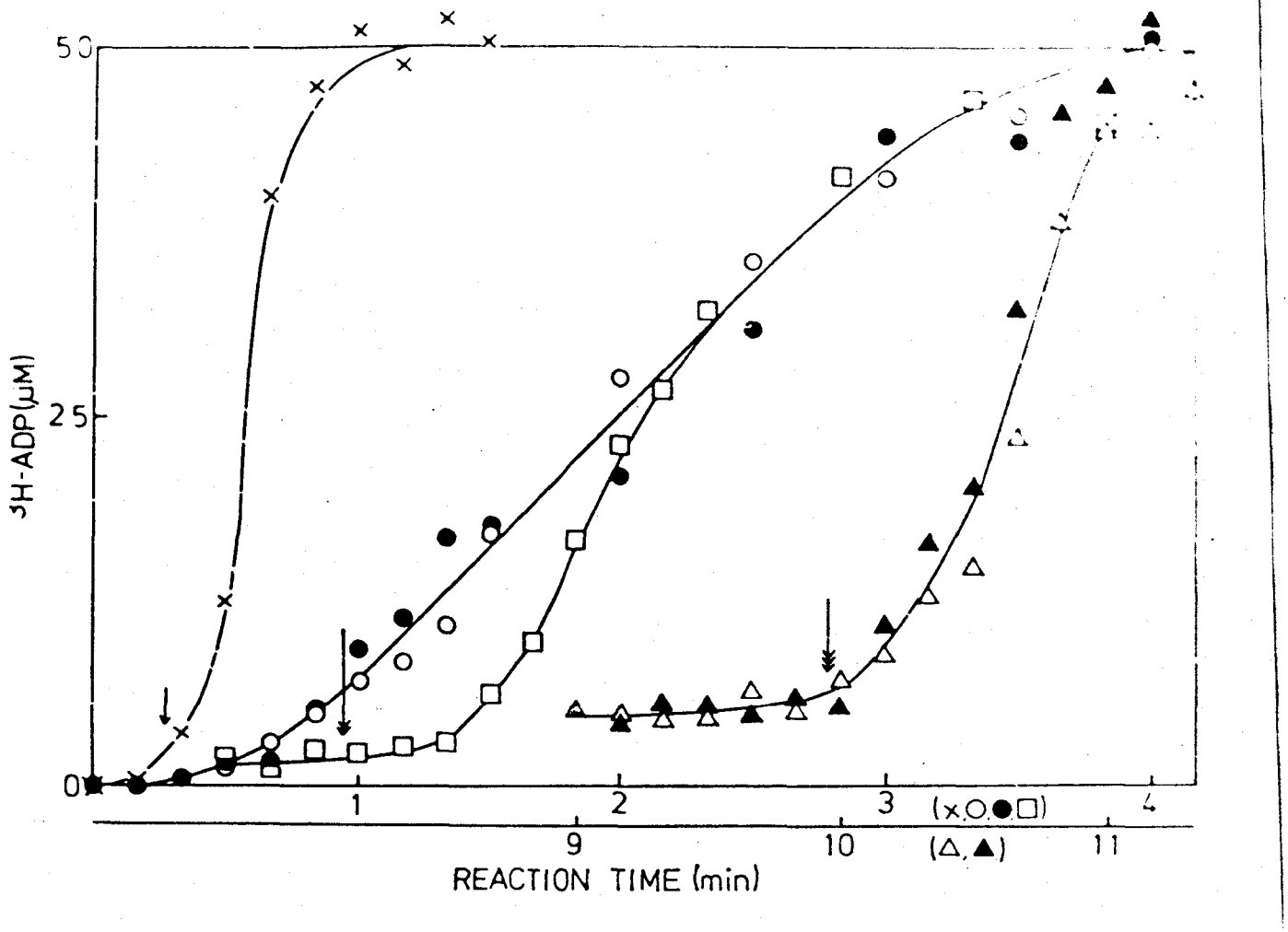


Fig. 13 Time-courses of liberation of ^{3}H-ADP from the myosin-phosphate-^{3}H-ADP complex. The reaction was started by adding 5 μM ^{3}H-ATP to 2.5 mg/ml of myosin in the upper chamber in 0.5 M KCl, 2.5 mM $MgCl_2$, and 50 mM Tris-HCl, pH 7.8 at 0°C (○,●, □, △, ▲). 0.2 mM Non-radioactive ATP (●, ▲) or ADP (○, □, △) was added to the reaction mixture at the times indicated by ↓ (●, ○), ↓↓ (□), and ↓↓ (▲, △). ×, Myosin (2.5 mg/ml) was incubated with 5 μM ^{3}H-ATP for 10 min and then 0.2 mM ADP was added. After 10 min the mixture was poured into the upper chamber, and the time-course of appearance of ^{3}H-ADP in the lower chamber was measured.

The rate of ^{3}H-ADP-liberation was unaffected by adding non-radioactive ATP instead of ADP.

The rate constant of ADP-liberation from the simple myosin-ADP complex was also measured, when about two moles of ADP were added per mole of myosin. In the presence of 10 μM ^{3}H-ADP and 2.5 mg/ml of myosin in 0.5 M KCl, 2.5 mM $MgCl_2$, and 50 mM Tris-HCl, at pH 7.8 and 0°C, the concentrations of free and bound ADP were estimated from the rates of flow-dialysis to be 2.1 and 7.9 μM, respectively. The time-courses of liberation of ^{3}H-ADP after adding 0.2 mM non-radioactive ADP and ATP were measured, but the liberation occurred too fast to be followed by the present method, as shown in Fig. 14.

Dependence on the ATP Concentration of the Rate of Myosin-ATPase in the Steady State - The dependence on the ATP concentration of myosin-ATPase has been measured by many workers. It has been measured at ATP concentration above 0.5 μM, except in the work reported by Lymn and Taylor (56, 67). Therefore, we measured the rate of ATPase in the steady state over a wide range of ATP concentrations from 0.1 to 5 μM in the presence of 0.003 mg/ml myosin, 0.5 M KCl, 2.5 mM $MgCl_2$ and 50 mM Tris-maleate buffer at pH 7.8 and 0°C. Figure 15 shows results on four myosin preparations. The rates of each preparation are plotted relative to that at 2 μM ATP. The rates of the preparations at 2 μM ATP were 0.36, 0.36, 0.35 and 0.33 mole/min.

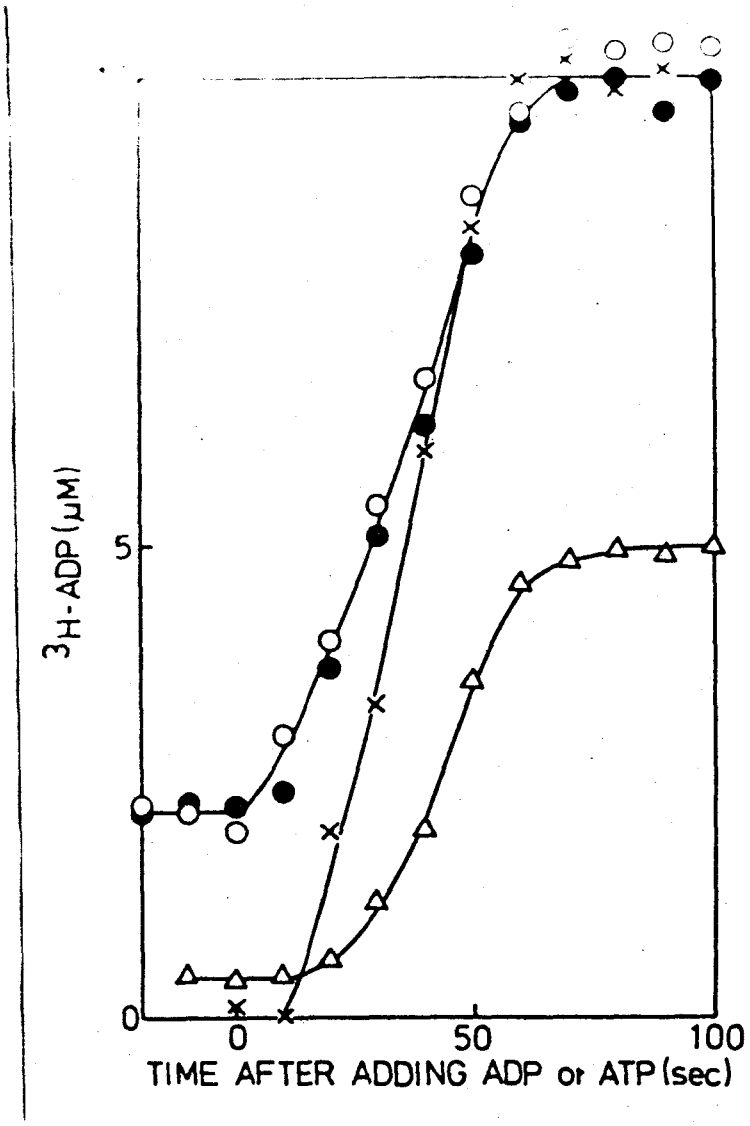


Fig. 14 Time-courses of liberation of ^{3}H-ADP from the myosin-^{3}H-ADP complex. 0.5 M KCl, 2.5mM $MgCl_2$, 50 mM Tris-HCl, pH 7.8, 0°C. A mixture of 2.5 mg/ml of myosin with 10 μM ^{3}H-ADP (●, ○) or 5 μM ^{3}H-ADP (△) was added to the upper chamber, and 0.2 mM non-radioactive ATP (●) or ADP (○, △) was added at time 0. X, After incubation of myosin (2.5 mg/ml) with 10 μM ^{3}H-ADP and 0.2 mM non-radioactive ADP for 10 min, the mixture was poured into the upper chamber, and the time-course of appearance of ^{3}H-ADP in the lower chamber was measured.

4.8×10^5g myosin, and the average value of 0.35 mole/min·4.8×10^5g myosin was used as the rate at 2 μM ATP. At ATP concentrations above 1 μM, the values of K_M and V_{max} were 1 μM and 0.44 mole/min·4.8×10^5g myosin, respectively. At ATP concentrations below 0.3 μM the rate deviated from the straight line obtained at higher ATP concentrations and became almost independent of the ATP concentration. The values of v over all the ATP concentrations used were given by:

$$(\text{moles/min} \cdot 4.8 \times 10^5 \text{g myosin}) = 0.11 + \frac{0.33}{1+1\ M/[S]} .$$

A plot of v^{-1} versus $[S]^{-1}$ in 1.5 M KCl gave a straight line (Fig. 16). The values of K_M and V_m were 0.038 μM and 0.11 mole/min·4.8×10^5g myosin, respectively.

Dependence of the Burst Size and the Rate of the ATPase Reaction on the ATP Concentration - To examine whether Lymn and Taylor's conclusion (56) on the number of active sites of myosin is correct, the size of the initial burst of Pi-liberation and the rate of the ATPase reaction in the steady state were measured in a range of ATP concentrations from 5 to 200 μM under the conditions used by Lymn and Taylor, i.e. in 1 mg/ml myosin, 0.5 M KCl, 10 mM $MgCl_2$ and 0.1M Tris-HCl buffer at pH 8.0 and 20°C. As shown in Fig. 17, the size of the initial burst and the rate of the ATPase reaction in the steady state were independent of the ATP concentration. They were 1.1 mole/4.8×10^5g

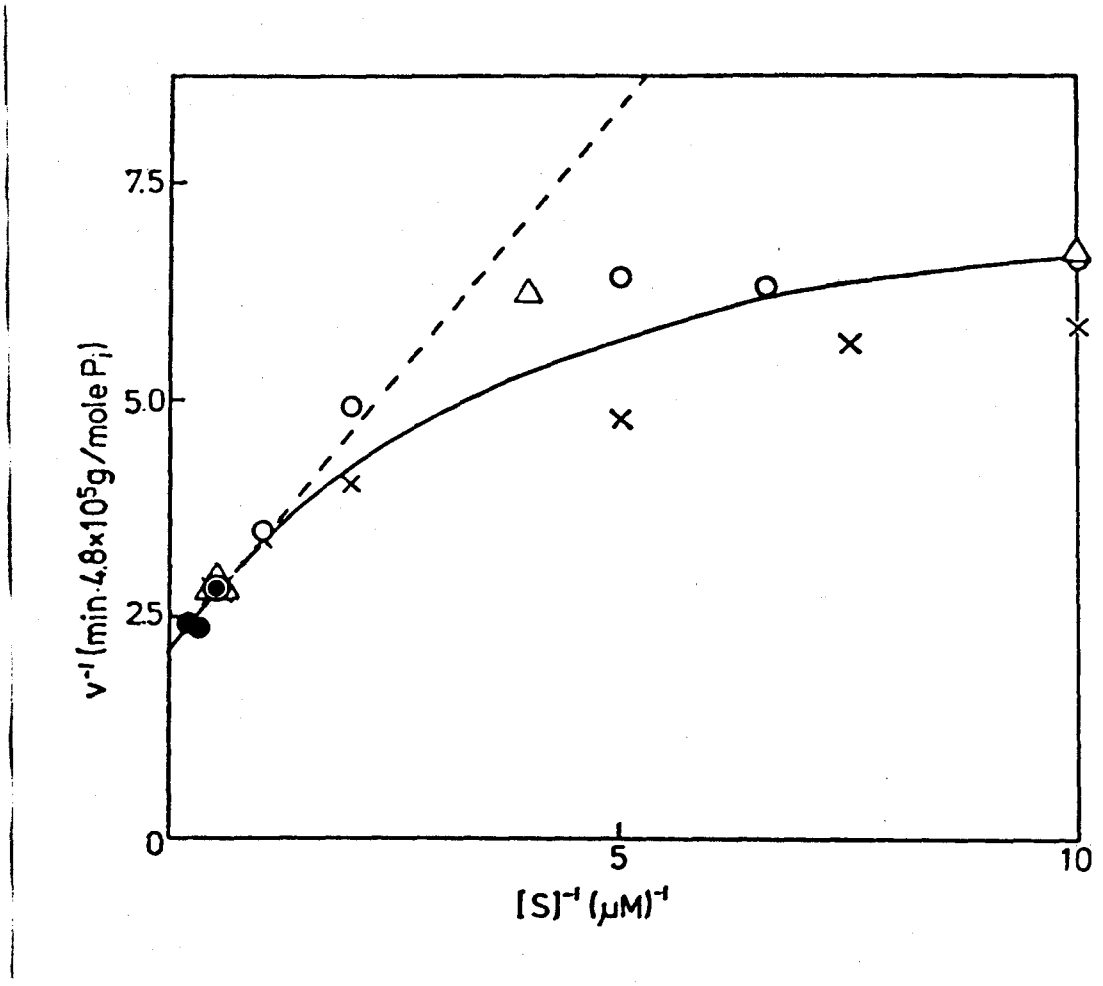


Fig. 15. Lineweaver-Burk plot of the rate of the ATPase reaction in the steady state, v, in 0.5 M KCl. 0.003 mg/ml myosin, 0.5 M KCl, 2.5 mM $MgCl_2$ and 50 mM Tris-maleate buffer at pH 7.8 and 0°C. O, ●, X, Δ, different preparations of myosin; the solid line indicates by:

$$v \text{ (mole/min·4.8 x } 10^5\text{g myosin)} = 0.11 + \frac{0.33}{1+1\ M/[S]} .$$

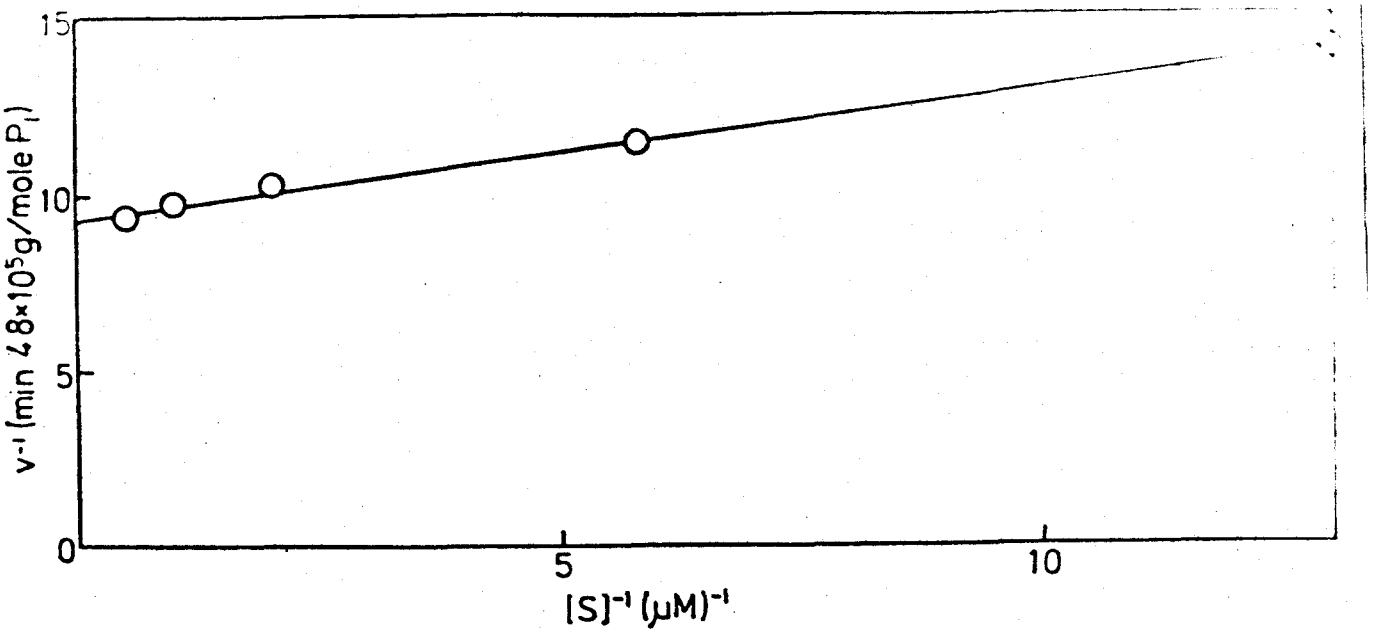


Fig. 16. Lineweaver-Burk plot of the rate of the ATPase reaction in the steady state, v, in 1.5 M KCl. 0.003 mg/ml myosin, 1.5 M KCl, 2.5 mM $MgCl_2$ and 50 mM Tris-maleate buffer at pH 7.8 and 0°C.

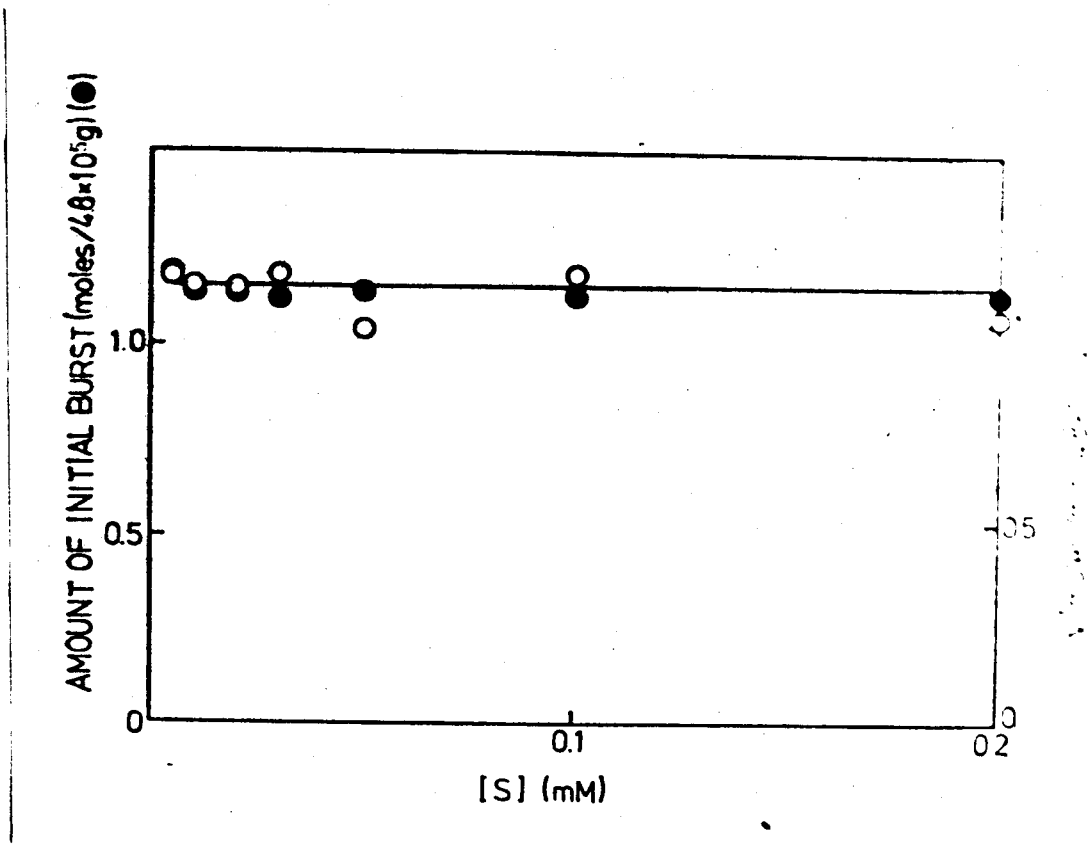


Fig. 17. Dependences on the ATP concentration of the amount of the initial burst of Pi-liberation and the rate of the ATPase reaction in the steady state. 1 mg/ml myosin, 0.5 M KCl, 10 mM $MgCl_2$ and 0.1 M Tris-HCl buffer at pH 8.0 and 20°C. ○, rate of the ATPase reaction in the steady state: ●, amount of the initial burst.

myosin and 1.1 mole/min·4.8×10^5g myosin, respectively.

Initial Burst of Pi-Liberation in the Presence of ADP - The time-course of Pi-liberation was measured after adding 0.1 mM γ-^{32}P-ATP to 2 mg/ml of myosin in the presence of 0.1 mM ADP in 0.5 M KCl, 2.5 mM $MgCl_2$, and 50 mM Tris-HCl, at pH 7.8 at 20°C. Under these conditions almost all the active sites of myosin were occupied by ADP, since the competitive inhibition constant of ADP at 20°C was 25 μM, as stated above. Even under these conditions, a typical initial burst of Pi-liberation was observed, as shown in Fig. 18. The burst size in the presence of ADP decreased with decrease in the temperature, as expected from the finding that the competitive inhibition constant of ADP decreased markedly on lowering the temperature. Thus, an initial burst of Pi-liberation was observed at 10°C, but the size was slightly less than the stoichiometric value (Fig. 18). At 0°C the size of the burst decreased to 14% of the stoichiometric value. Therefore, we measured the time-course of Pi-leberation at 0°C after adding 20 μM of γ-^{32}P-ATP to a mixture of 1.9 mg/ml of myosin and 15 μM ADP in 0.5 M KCl, 2.5 mM $MgCl_2$, and 50 mM Tris-HCl, at pH 7.8. Under these conditions almost all the active sites of myosin were occupied by ADP, since the inhibition constant of ADP at 0°C was 1.7 μM, but as shown in Fig. 19, a typical initial burst of Pi-liberation was observed.

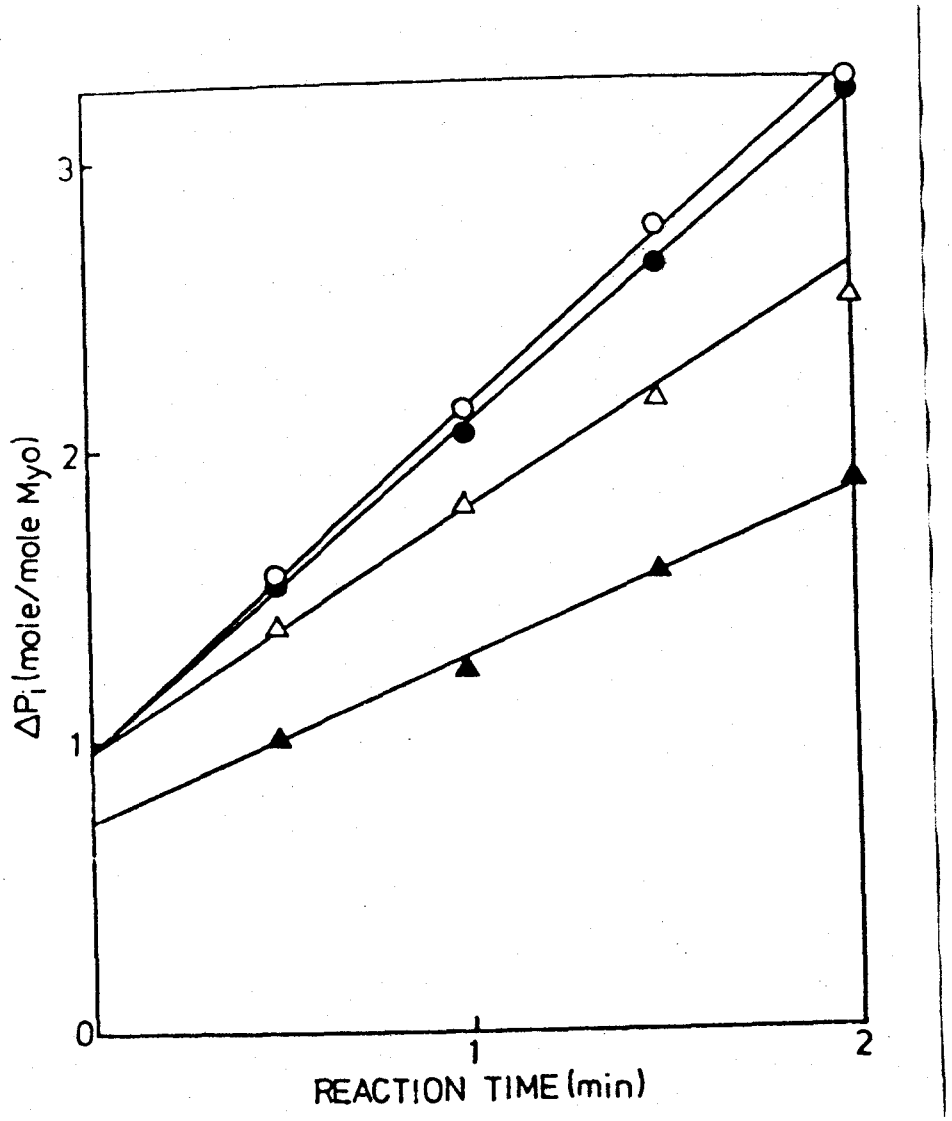


Fig. 18. Time-courses of Pi-liberation from the myosin-ATP system in the presence of ADP at 20 and 10°C. 0.1 mM γ-^{32}P-ATP was added to 2 mg/ml of myosin in the presence (●, ▲) and absence (○, △) of 0.1 mM ADP. 0.5 M KCl, 2.5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.8, 20°C (●, ○) or 10°C (▲, △).

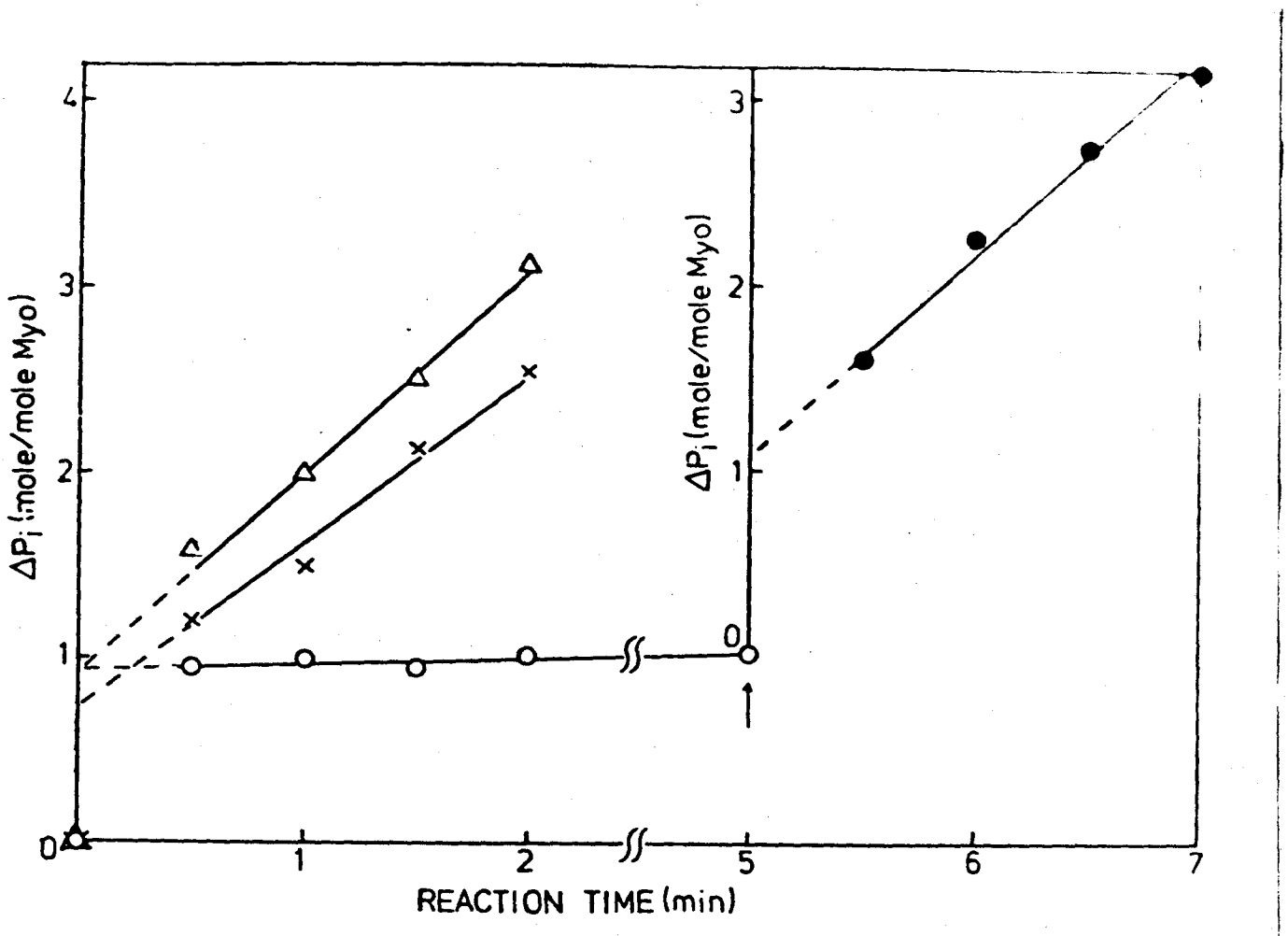


Fig. 19. Time-courses of Pi-libelation from the myosin-ATP system in the presence of ADP at 0°C. 20 M γ-^{32}P-ATP was added to 1.9 mg/ml of myosin in the presence (X) or absence (Δ) of 15 M ADP. 0.5M KCl, 2.5mM $MgCl_2$, 50mM Tris-HCl, 0°C. ○, Time-course of Pi-liberation, after adding 4μM γ-^{32}P-ATP to 1.9mg/ml myosin. ●, Time-course of Piliberation when 20μM γ-^{32}P-ATP was added to myosin 5min after starting the reaction by adding 4 μM γ-^{32}P-ATP.

DISCUSSION

The following mechanism was proposed for formation of the reactive myosin-phosphate-ADP complex, M_P^{ADP}, by our laboratory:

$$M + S + H^+ \underset{(1)}{\rightleftharpoons} M_1SH^+ \underset{(2)}{\rightleftharpoons} M_2S + H^+ \underset{(3)}{\rightleftharpoons}$$

$$M\ {}^{ADP}_{P} + H^+ \underset{(4)}{\rightleftharpoons} M\ {}^{ADP}_{P} + H^+,$$

on the basis of the findings that the amount of the initial burst of TCA-labile Pi-liberation is one mole per mole of myosin (54), that one mole of H^+ per mole of myosin is rapidly absorbed by myosin and is liberated again slightly prior to the initial burst of Pi-liberation (59, 60), and that the rate of change in the UV-spectrum is equal to that of rapid liberation of H^+ (60). They concluded that step 2 is accelerated by ATP itself, since the values of $\tau_{1/2}$, the time for half the final change of rapid H^+-liberation, the change in the UV-spectrum and the initial burst of Pi-liberation were almost independent of the ATP concentration when the latter was lower than the stoichiometric value, but decreased inversely with the ATP concentration when the latter was higher than the stoichiometric one (58). On the other hand, Lymn and Taylor (56) recently proposed a simpler scheme for the formation of M_P^{ADP}, from the finding that the rate of the initial burst of Pi-liberation is almost proportional to the ATP concentration

at concentrations from 5 to 100 μM:

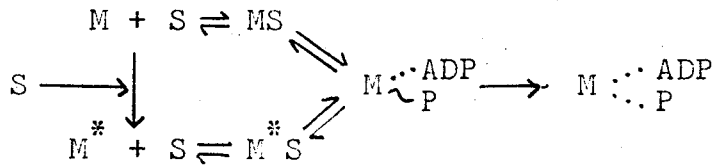
$$M + S \rightleftharpoons MS \longrightarrow M_P^{ADP} .$$

The main differences between these two reaction mechanisms are the presence of phosphoryl myosin, $M \sim_P^{ADP}$, as an intermediate and the dependence of the rate of M_P^{ADP} formation on the ATP concentration. We deduced that phosphoryl myosin was formed from the findings that NTP combines with a carboxyl group of a glutamic acid residue of myosin only in the presence of Mg^{2+} and ATP (63, 78) and that this binding completely suppresses the initial burst of Pi-liberation but does not affect the ATPase reaction in the steady state (63), and that the P-exchange reaction occurs between the reactive myosin-phosphate-ADP complex and the terminal phosphate of ATP during the initial phase of the reaction (64). However, this problem is not related to the present work, and will not be discussed here (see Ref. 39).

As shown in Figs. 5 and 6, the Michaelis constant of M_P^{ADP} formation was 0.3 μM at ATP concentrations below 0.3 μM. Furthermore, at high ATP concentrations, the rate of M_P^{ADP} formation increased with the ATP concentration. These results are consistent with our previous results (58) on the rapid liberation of H^+, the change in the UV-spectrum and the initial burst of Pi-liberation.

To investigate the mechanism of increase in the rate of M_P^{ADP} formation at high ATP concentrations, we measured the rate of the initial burst of Pi-liberation after adding 5 μM ATP to 5.25 μM of myosin. The rate was almost equal to that obtained by adding 5 μM ATP to 0.0625 M myosin. Therefore, if we assume that ATP at high concentrations induces a conformational change in the myosin molecule, this change must not be accompanied by a decrease in the MgATP concentration as substrate of the ATPase reaction. If we adopt this assumption, the reaction mechanism of M_P^{ADP} formation is given by:

(low ATP concentration)



(high ATP concentration)

High concentrations of MgATP induce a change in myosin to a new conformational state, M^*. This conformational change is accompanied by acceleration of $M\sim{}^{ADP}_{P}$ formation from the ES complex. Since our experiments on the rapid absorption and liberation of H^+ were performed in the presence of several M of ATP, the rapid change in H^+ might be attributed to the conformational change in myosin induced by ATP.

Tonomura *et al.* showed that M_P^{ADP} consists of two kinds of intermediates, $M\sim\!\cdot\cdot_P^{ADP}$ and $M\cdot\cdot_P^{ADP}$ (63, 64). Several lines of evidence for the existence of phosphoryl intermediate, $M\sim\!\cdot\cdot_P^{ADP}$, are given in our review articles (39,79). However, $M\sim\!\cdot\cdot_P^{ADP}$ is formed transiently during the initial phase, and the amount of $M\sim\!\cdot\cdot_P^{ADP}$ is negligible for kinetical analyses under the conditions of our experiments (60).

Furthermore, we found that the dependence on ATP concentration of the rate of change in the UV spectra over a high concentration range of ATP follows the Michaelis-Menten equation with values of K_M and V_{max} of 0.2 mM and 30sec^{-1}, respectively, and we could not observe any lag time in the change of the spectra. This indicates the existence of a rapid equilibrium between E+S and the E_1S complex.

It was concluded previously by Tonomura *et al.* that the main route of the ATPase reaction in the steady state is decomposition of ATP by simple phydrolysis, since (i) the rate of slow H^+-liberation (60, 62) and the recovery of the initial burst of Pi-liberation (64) after addition of a stoichiometric amount of ATP to myosin is several times lower than the rate of the ATPase reaction in the steady state, and (ii) p-nitrothiophenylation of myosin suppresses the initial burst of Pi-liberation but does not affect the ATPase activity in the steady state (63). Furthermore, we concluded that the $M\sim\!\cdot\cdot_P^{ADP}$ complex is decomposed via two steps, since the rate of liberation of ADP from $M\sim\!\cdot\cdot_P^{ADP}$

of the same order of magnitude of that of the ATPase reaction
the steady state and is much higher than those of slow H^+-
peration and the recovery of the initial burst (63). Thus,

$$M + S \rightarrow\rightarrow M\begin{matrix}\cdot\cdot ADP \\ \cdot\cdot P\end{matrix} \longrightarrow M_{\cdot\cdot P} + ADP \not\rightarrow$$

$$M + ADP + Pi + H$$

$$M_{\cdot\cdot P} + S \rightleftharpoons M\begin{matrix}\cdot\cdot S \\ \cdot\cdot P\end{matrix} \longrightarrow M_{\cdot\cdot P} + ADP + Pi$$

Taylor and his collaborators (67) recently obtained results
ggesting that the rates of liberation of Pi and ADP from the
DP complex are in the same order of magnitude as that of
Pase reaction in the steady state. They proposed the following
heme:

$$M + S \rightleftharpoons MS \longrightarrow M_P^{ADP} \longrightarrow M + ADP + Pi.$$

eir finding that the rate of liberation of Pi from M_P^{ADP} was
most equal to that of ADP does not agree with the above reaction
chanism. The cause of this difference is not yet clear,
; it is possible that $M_{\cdot\cdot P}$ in the reaction mechanism of Tonomura is
ually a new conformational state of myosin, $^{\circ}M$, which does
; contain P and this conformational state of myosin returns
the original state very slowly:

$$M\begin{matrix}\cdot\cdot ADP \\ \cdot\cdot P\end{matrix} \longrightarrow {}^{\circ}M + ADP + Pi \longrightarrow M + ADP + Pi + H^+.$$

Results of Taylor et al. (67), that the rate constant of Pi-liberation from M_P^{ADP} is similar to that of ADP from M_P^{ADP}, was confirmed. This shows that the intermediate for decomposition of M_P^{ADP} does not contain bound phosphate(°M , not MP).

According to our mechanism, hydrolysis of ATP catalyzed by myosin occurs via two different routes. If they have different K_M and V_{max} values, they can be distinguished by measuring the dependence of the rate of the ATPase reaction in the steady state, v, on the ATP concentration, [S]. As shown in Fig. 15, the rate of the ATPase reaction in the steady state in 0.5 M KCl was given by

$$(\text{mole/min} \cdot 4.8\text{x}10^5\text{g myosin}) = 0.11 + \frac{0.33}{1+1\mu\text{M}/[\text{S}]} \quad .$$

Since the formation of M_P^{ADP} is much faster than its decomposition, the K_M of ATP hydrolysis via M_P^{ADP} and °M° must be lower than the K_M of M_P^{ADP} formation, 0.3 μM. The value of V_{max} of this route must be a small fraction of the whole for ATP hydorlysis, since this is not the main route of hydrolysis. Furthermore, the rate constant of $M_P^{ADP} \rightarrow$ °M + ADP + Pi is much higher than that of °M $\rightarrow$ M, as mentioned above. Therefore, the most stable intermediate in the ATP-hydrolysis via M_P^{ADP} is °M . It must be added that the dissociation constant of the binding of ATP with myosin measured by the luciferin-luciferase method (81) and UV spectroscopy (82) is in the range of a few μM.

This indicates that the Michaelis complex of ATP-hydrolysis in the range of high ATP concentrations ($\gtrsim 1\,\mu M$) contains ATP or ADP, while that in the range of low ATP concentrations ($\ll 1\,\mu M$) contains no mucleotide. Therefore, it seems probable that the route of ATP hydrolysis with a V_{max} of $0.11 min^{-1}$ and $K_M \ll 1\,\mu M$ is the one via M_P^{ADP} [1] and M^o and that the route with a V_{max} of $0.33\ min^{-1}$ and K_M of $1\,\mu M$ is that involving simple hydrolysis of ATP vis MS. Table I summarizes our results on the ratio of the rates of the two routes of ATP hydrolysis under various conditions.

Taylor and his co-workers (56, 65, 67) have tried to explain the complicated properties of myosin-ATPase by assuming the presence of two ATPase sites on the myosin molecule, which interact with each other. However, as described in the previous paper (80), the amount and the rate of the initial burst of Pi-liberation per two moles of subfragment-1 were exactly the same as those per mole of myosin. Furthermore,

---

1 From the rate constant of decomposition of M_P^{ADP} give above and that of M_P^{ADP} formation given in Fig. , we calculated the K_M values of ATP hydrolysis via M_P^{ADP} at ATP concentrations above and below $0.3\,\mu M$ as 0.09 and 0.05 μM, respectively.

Table 1. Comparison of the rate of simple hydrolysis, $V_{}$, with that of decomposition of ATP M_P^{ADP}, $V_{}$.

KCl (M)	$MgCl_2$ (mM)	pH	Temp.(°C)	Method	$V_{}$:V	Reference
0.5	10	8.2	25	H^+ liberation		(60)
1	2	7.5	27	H^+ liberation	1:9	(62)
2.8	10	8.2	25	H^+ liberation		(60)
1.08	5	7.5	0	recovery of burst	1:4	(64)
0.5	2.5	7.8	0	kinetics of steady state	1:3	this paper
1.5	2.5	7.8	0	kinetics of steady state	(?)	this paper

as shown in Fig.17, the burst size of Pi-liberation was constant, being about 1 mole/mole of myosin, and was independent of the ATP concentration even under the conditions used by Taylor et al. (67)

In this paper, we showed that the kinetic properties of M_P^{ADP} are very different from those of the simple M-ADP complex. The UV spectra (83, 84) and the EPR spectra of spin-labeled myosin (85) in the presence of ATP were previously shown to be different from those of the M-ADP complex. On the other hand, Taylor et al. (67) using a gelfiltration method reported that the rate constant of ADP-liberation from the M-ADP complex was comparable with that of M_P^{ADP}, and assumed that the simple M-ADP complex has the same kinetic properties as those of M_P^{ADP}. Malic and Martonosi (86) supported the conclusion of Taylor et al. (67) from measurement of the change in the UV spectra of H-meromyosin after adding ADP. However, the latter authors measured the rate in the absence of ATP. The rate constant of ADP-liberation from M-ADP was also measured by Trentham et al. (89), using SH-analogues of nucleotides. In this paper, we showed both by a rapid flow-dialysis method and by studies on burst kinetics that ADP-liberation from the simple M-ADP complex cannot be the rate-determining step of ATPase either at 0°C or 20°C. Thus, the results in Figs. 13 and 14 indicate that the kinetic properties of the intermediate formed after adding ATP to myosin in a molar ratio of 1 : 1

are very different from those of M-ADP, but that they become similar to the latter after prolonged incubation. The results shown in Figs. 15 and 16 indicate that the simple M-ADP complex cannot be the most stable intermediate for ATP-hydrolysis. We also observed a rapid change in the UV spectra, when 50 μM ATP was added to a mixture of 3.4 mg/ml of H-meromyosin and 20 μM ADP in 0.2 M KCl, 2.5 mM $MgCl_2$, and 50 mM Tris-HCl, at pH 7.8 at 0°C. This also indicates the rapid equilibration of the bindings between myosin and nucleotides. It was also shown that Pi- and ADP-liberation from M_P^{ADP} are unaffected by the existence of a high concentration of ATP, contrary to the result reported by Taylor et al. (67).

3. Binding of Adenosine Di- and Triphosphates to Myosin during the Hydrolysis of Adenosine Triphosphate

The amounts of ATP and ADP bound to myosin during the ATPase reaction [EC 3.6.1.3] were determined in the presence of 2 mM $MgCl_2$ and 50 mM Tris-HCl at pH 7.8, using pyruvate kinase [EC 2.7.1.40] and phosphoenolpyruvate to regenerate ATP and ^3H-labelled ATP as the substrate. The amounts of ADP and total nucleotides bound to myosin were measured, respectively, by thin layer chromatography after stopping the reaction with TCA and by a rappid-flow dialysis method. The following results were obtained; they were all consistent with our original reaction mechanism for myosin-ATPase but in conflict with the oversimplified variant proposed by Taylor et al.

1. Binding of ATP to myosin was only observed when the amount of ATP added was more than about 0.6 mole/mole of myosin. In the presence of both 0.1 and 0.5 M KCl, the maximum amount of ATP bound was about 1 mole/mole of myosin, and the dissociation constant of binding (1 μM in 0.5 M KCl at 0°C) was equal to the K_M value of myosin-ATPase in the steady state at high concentrations of ATP.

2. When a sufficient amount of ATP was added to myosin, 1 mole of ADP bound rapidly to 1 mole of myosin during the initial phase of the reaction. Then, the amount of bound ADP decreased

to the steady state level within a few minutes.

3. The amount of bound ADP in the steady state increased almost linearly with increase in the amount of ATP added, and reached a constant value when the molar concentration of ATP added was higher than that of myosin. The meximum amount of bound ADP was 1 mole/mole of myosin at KCl concentrations above 1 M, and decreased with decrease in the KCl concentration. For example, in 0.125 M KCl at 20°C it was 0.4 mole/mole of myosin. The amount of bound ADP decreased slightly when the temperature was raised from 0 to 30°C.

4. The rate of the ATPase reaction in the steady state was not proportional to the amount of ADP bound to myosin.

INTRODUCTION

Many workers have attempted to measure the amounts of ATP and ADP bound to myosin during the ATPase [EC 3.6.1.3] reaction, since information on this is very valuable in relation to the reaction mechanism of myosin-ATPase and the state of myosin in muscle fibers during contraction and relaxation (39). Bowen and Evans (88), and Schliselfeld and Barany (89) measured the binding of nucleotides to myosin, using a Millipore-filtration method and a gel-filtration method, respectively. The binding of nucleotides to myofibrillar proteins was measured by Bárany and Bárany (90) on living muscle fibers. It was also measured by Marston (91) on glycerol-treated muscle fibers and by Maruyama and Weber (92), on isolated myofibrils. However, these methods had various difficulties and disadvantages, as mentioned in "DISCUSSION," and the results were inconsistent.

We measured the amounts of ATP and ADP bound to myosin during the ATPase reaction by the follwoing methods, which avoid the difficulties and disadvantages inherent in previous methods: An ATP-regenerating system (pyruvate kinase [EC 2.7.1.40] and PEP) was coupled with the myosin-ATPase reaction, and ^{3}H-ATP was used as the substrate. The amount of bound ADP was determined by separating nucleotides using polyethyleneimidecellulose thin layer chromatography, after stopping the reaction with TCA. The amount of bound ATP was determined by

substracting the amount of bound ADP from that of total bound nucleotides, which was measured by a rapid-flow dialysis method. The results obtained were consistent with our previously proposed reaction mechanism for myosin-ATPase (39, 43, 59, 93). According to this mechanism, myosin has two different active sites, one for formation of the reactive myosin-phosphate-ADP complex, M_P^{ADP}, and the other for the myosin-ATP complex, M_{ATP}.

EXPERIMENTAL

Myosin was prepared from rabbit skeletal white muscle by the method of Perry (70). The molecular weight of myosin was taken as 4.8×10^5 (39). Pyruvate kinase was prepared from rabbit skeletal muscle by the method of Tiez and Ochoa (94). Its activities in 0.5 M KCl-2 mM $MgCl_2$ at 0°C and in 0.05 M KCl 2 mM $MgCl_2$ at 20°C and pH 7.8 were 8.5 and 88 moles/min·mg protein, respectively. The K_M value of pyruvate kinase for ADP under the conditions used was about 0.18 mM. No formation of AMP from ADP by adenylate kinase [EC 2.7.4.3] contaminating the myosin or pyruvate kinase preparations was detectable by thin layer chromatography under the experimental conditions used. Protein concentration was determined by the biuret reaction, calibrated by nitrogen determination.

γ-^{32}P-Labelled ATP was synthesized enzymatically by the

method of Glynn and Chappel (75). ^{3}H-Labelled ATP and ADP were purchased from the Radiochemical Centre, Ltd., Amersham, England, and purified by the methed of Cohn and Carter (76). ATP, ADP, and PEP were purchased from Sigma Chemical Co.

The amount of ADP bound to myosin during the ATPase reaction was determined by measuring the amount of ADP remaining in the myosin-ATP system coupled with sufficient amounts of pyruvate kinase and PEP. The reaction was started by mixing 0.1 ml of myosin solution with 0.1 ml of a solution containing pyruvate kinase, PEP and ^{3}H-ATP. After stopping the reaction by addition of 0.2 ml of 10% TCA solution containing 10 mM ATP, ADP, and AMP as carriers, denatured proteins were discarded by centrifuging the reaction mixture at 2,000xg and 0°C for 10 min. The total amount of nucleotides was determined by measuring the radioactivity of a certain volume of the supernatant (usually 7-10 μl) in a Beckman liquid scintillation counter, model LS-150. The scintillation mixture consisted of 10 ml of dioxane-based scintillation mixture (1 g naphthalene, 70 mg 2,5-diphenyloxazole, and 3 mg 1,4-bis[2(4-methyl-5-phenyl-oxazol)]-benzene in 10 ml dioxane), 2.5 ml of 50% ethanol-49% water-1% NH_4OH and 0.35 ml of 0.2 N HCl-0.1 mM ATP. An aliquot of the supernatant (about 30 μl) was placed on a polyethyleneimide (Nakarai, Kyoto)-cellulose thin layer prepared by the method of Randerath and Randerath (95). The layer was treated with methanol for 5 min and dried, and then the chromatogram

was developed with 3.9 M Na-formate buffer at pH 3.4. The Rf values of nucleotides were: ATP, 0.14; ADR, 0.52; AMP, 0.80. The region of the cellulose layer containing the spot of separated nucleotide was put into a centrifuge tube and washed twice with 5 ml of methanol by shaking for 2 min and centrifugation for 10 min at 2,000xg. After drying the cellulose at 60°C, ^{3}H-labelled nucleotide was extracted by incubation with 1 ml of 0.2 N HCl and 0.1 mM ATP at 90°C for 3 hr. The cellulose powder was removed by centrifuging the mixture at 2,000xg for 10 min, and 0.35 ml of the supernatant was added to the scintillation mixture, consisting of 10 ml of dioxane-based scintillation mixture and 2.5 ml of 50% ethanol-49% water-1% NH_4OH. The radioactivity was measured as described above. The extent of separation of nucleotides by these procedures was higher than 98%, and losses of nucleotides were less than 5%.

Figure 20 shows the dependence on the concentration of pyruvate kinase of the amount of ^{3}H-ADP remaining in the reaction mixture, when the reaction was started by adding 3.6 mg/ml of myosin to 18.75 μM ^{3}H-ATP (2.5 moles/mole of myosin) in 1 mM PEP containing 0.5 M KCl, 2 mM $MgCl_2$, and 50 mM Tris-HCl, pH 7.8, at 10 and 20°C, and stopped after 5 min with TCA. As expected, the sum of the amounts of ADP and ATP was equal to the amount of total nucleotides measured before their separation, and the amount of ADP decreased with increase in the

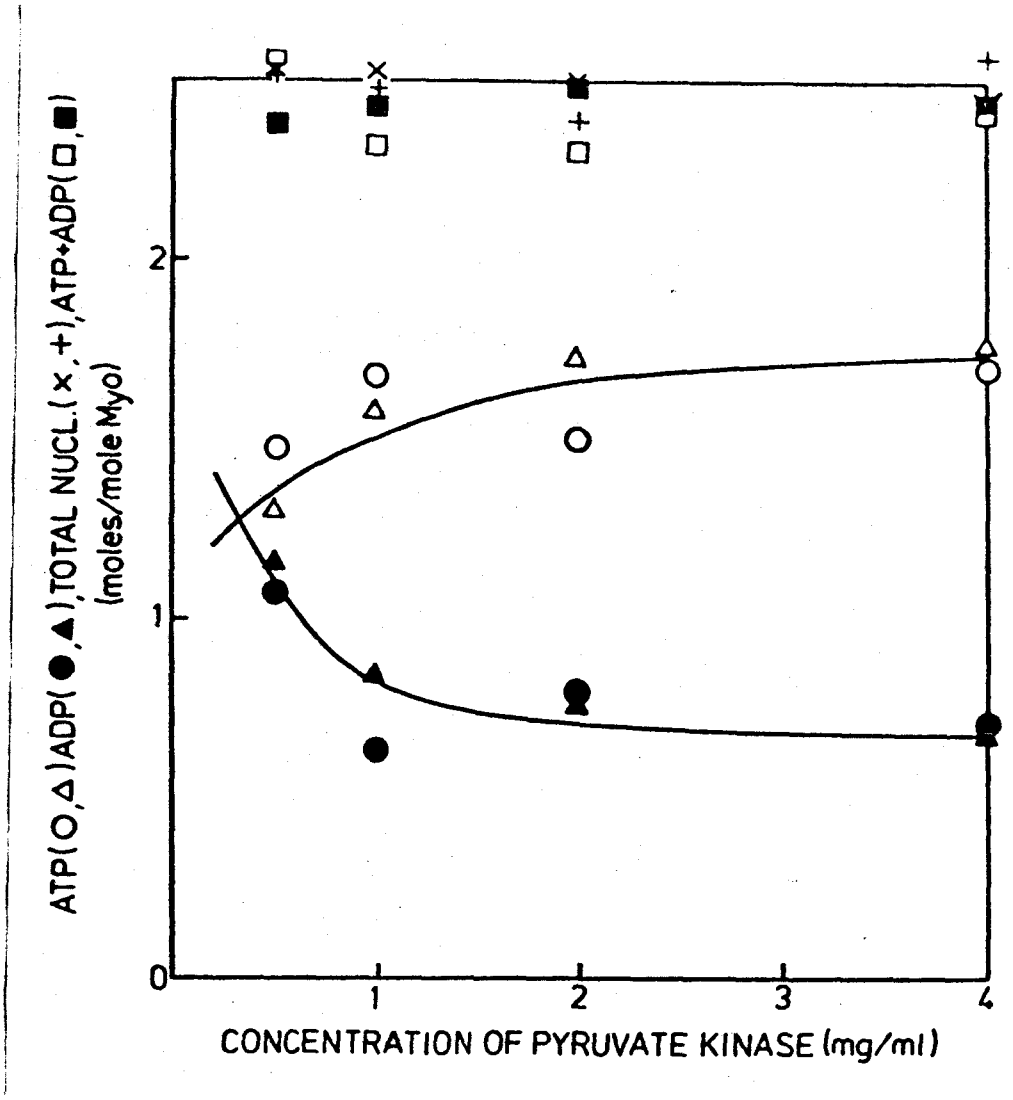


Fig. 20. Dependence of the amounts of ATP and ADP on pyruvate kinase concentration during the ATPase reaction. 3.6 mg/ml myosin, 18.75 μM ^{3}H-ATP, 1 mM PEP, 0.5 M KCl, 2 mM $MgCl_2$, 50 mM Tris-HCl, pH 7/8. Temperature: O, ●, X, □, 10°C; Δ, ▲, +, ■, 20°C. Reaction time, 5 min. The amounts of ATP, ADP, and total nucleotides were measured as described in "EXPERIMENTAL." O, Δ, ATP; ●, ▲, ADP; X, +, total nucleotides; □, ■, ATP + ADP.

concentration of pyruvate kinase. In the presence of 4 mg/ml of pyruvate kinase the amount of ADP approached a constant value of about 0.7 mole/mole of myosin. Therefore, in the following experiments the concentration of pyruvate kinase was fixed at 4 mg/ml.

The binding of total nucleotides to myosin was measured using a rapid-flow dialysis method (69) with several modifications, as described previously (Sec. 2 & Ref. 96). A Millipore filter of 0.22 μm pore size was used. The flow rate of the buffer, which contained KCl, $MgCl_2$, and Tris-HCl, through the lower chamber was fixed at 3.6 ml/min. Two ml of reaction mixture containing 3.6 mg/ml of myosin, 4 mg/ml of pyruvate kinase, 3 or 10 mM PEP, 0.5 or 0.1 M KCl, 2 mM $MgCl_2$, and 50 mM Tris-HCl at pH 7.8 and 0°C was placed in the upper chamber. After adding a definite amount of ^{3}H-ATP to the upper chamber, samples of 10 ml were taken 5 times at 3 min intervals, and their radioactivities were measured. The bindings of ADP and ATP to pyruvate kinase were too small to be detected by the flow dialysis method.

In the experiments presented in Fig. 26, the rate of myosin-ATPase in the steady state, v_0, was measured in 0.5 M KCl containing 2 mM $MgCl_2$ and 50 mM Tris-HCl at pH 7.8 and 0°C. The concentration of myosin was reduced to an extremely low level (0.0024 mg/ml, i.e., 0.005 μM) to avoid any significant change in the concentration of ATP during its reaction with

myosin. The ATPase reaction was started by mixing 1 ml of ^{32}P-ATP solution with 1 ml of myosin solution, and stopped by adding 2 ml of 10% TCA containing 1 mM ATP and 0.1 mM Pi as carriers. The reaction times were 5, 10, 15, and 20 min. The amount of ^{32}Pi liberated was measured as described previously (64).

In the experiments shown in Fig. 27, the rate of the ATPase reaction was determined from the rate of liberation of pyruvate, measured by the method of Reynard et al. (97) under the same conditions as for measurements of the binding of ADP to myosin.

RESULTS

Binding of ADP to Myosin during the ATPase Reaction - The K_M value of myosin-ATPase in the steady state at high ATP concentrations was 1 μM in 0.5 M KCl containing 2 mM $MgCl_2$ and 50 mM Tris-HCl at pH 7.8 and 0°C (cf. Fig. 26). In the follwoing experiments, the concentration of myosin was fixed at 3.6 mg/ml (7.5 μM), which was much higher than the K_M value. The ATPase reaction was coupled with saturating amounts of PEP (1 mM) and pyruvate kinase (4 mg/ml) (cf. Fig. 20). To measure ADP binding in the steady state, the reaction was stopped after 5 min (cf. Fig. 25).

Figure 21 shows the dependence of the amount of ADP bound

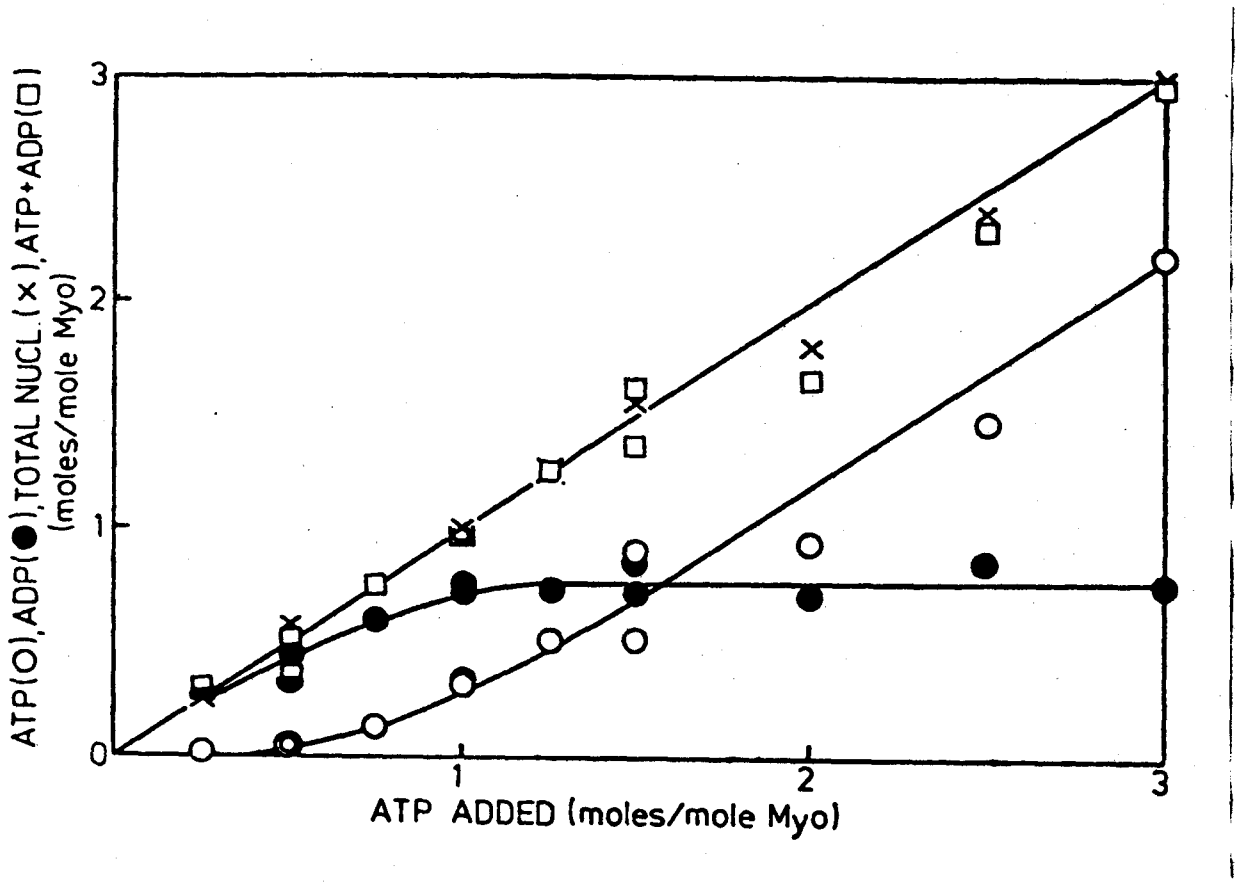


Fig. 21. Dependence of the amounts of ATP and ADP on the amount of ATP added during the ATPase reaction at 0°C. 3.6 mg/ml myosin, 1 mM PEP, 4 mg/ml pyruvate kinase, 0.5 M KCl, 2 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.8, 0°C. Reaction time, 5 min. ○, ATP; ●, ADP; ×, total nucleotides; □, ATP + ADP.

to myosin on the amount of ATP added at 0°C. Even in the presence of saturating amounts of pyruvate kinase and PEP almost all the ATP added was converted into ADP when the amount of ATP added was less than 0.5-1.0 mole/mole of myosin. At ATP concentrations above 1 mole/mole of myosin, the amount of ADP bound to myosin remained constant at 0.70-0.75 mole/mole of myosin. On the other hand, the amount of ATP remaining in the system increased gradually at first, and then linearly with increase in the amount of ATP at ATP concentrations of more than about 1 mole/mole of myosin. The sum of the amounts of ADP and ATP was equal to the amount of total nucleotides added.

The amounts of ADP and ATP remaining in the reaction mixture were also measured at 20°C (Fig. 22). The maximum amount of ADP bound to myosin at 20°C (0.65-0.80 mole/mole of myosin) was almost equal to that observed at 0°C.

The dependence of the amount of bound ADP on the KCl concentration was measured, after mixing 3.6 mg/ml of myosin with 18.75 μM ATP (2.5 moles/mole of myosin) (cf. Figs. 21 & 22). As shown in Fig. 23, at KCl concentrations above 1 M, the amount of ADP bound to myosin during ATP hydrolysis was about 1 mole/mole of myosin, but it decreased with decrease in the KCl concentration. The amounts of ADP bound in 0.125 M KCl were 0.62 and 0.42 mole/mole of myosin, respectively, at 0 and 20°C.

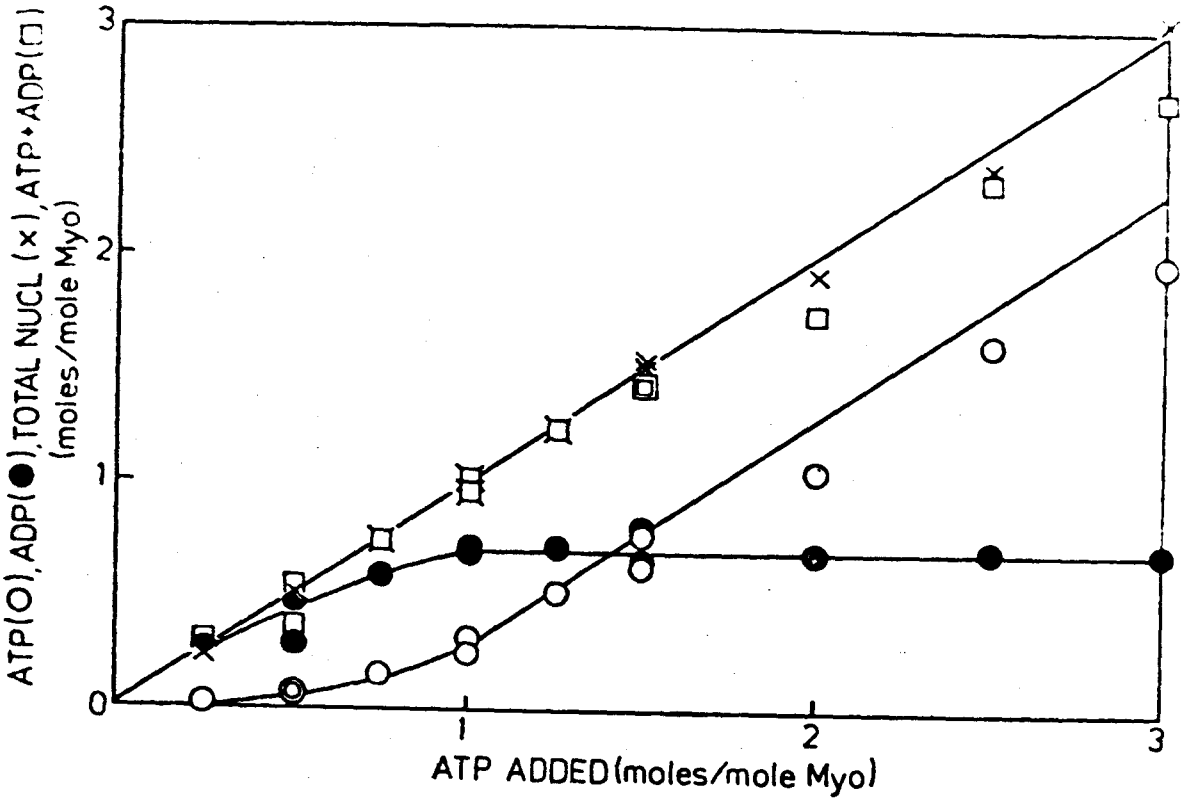


Fig. 22. Dependence of the amounts of ATP and ADP on the amount of ATP added during the ATPase reaction at 20°C. Experimental conditions were as described for Fig. 21, except that the temperature was 20°C. ○, ATP; ●, ADP; ×, total nucleotides; □, ATP + ADP.

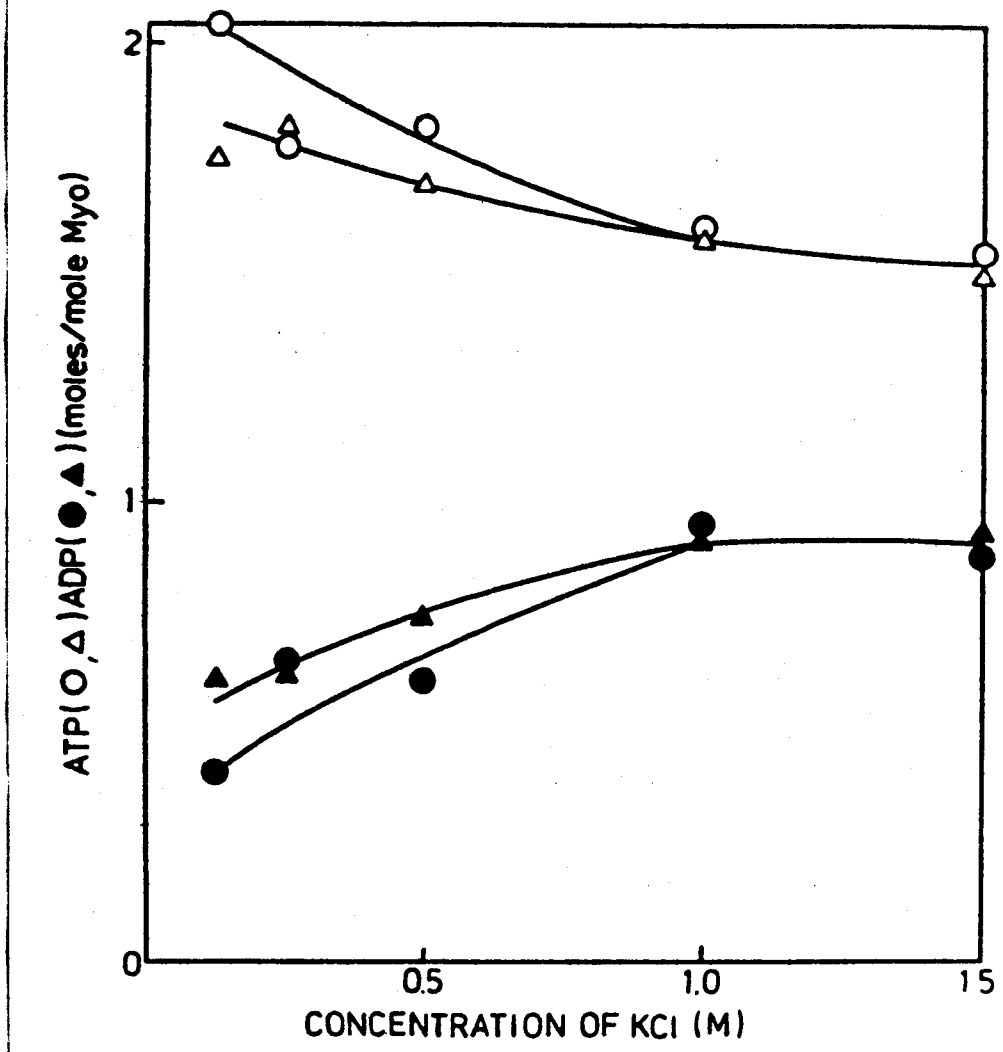


Fig. 23. Dependence of the amount of ADP bound to myosin on KCl concentration during the ATPase reaction. Experimental conditions were as described for Figs. 21 and 22, except that the concentration of ^{3}H-ATP added was 18.75 μM and the concentration of KCl was varied from 0.125 to 1.5 M. Temperature: △, ▲, 0°C; ○, ●, 20°C. ○, △, ATP; ●, ▲, ADP.

Figure 24 shows the dependence of the amount of ADP bound to myosin in 0.5 M KCl on temperature. When the temperature was raised from 0 to 30°C, the amount of bound ADP decreased from 0.85 to 0.65 mole/mole of myosin.

Kinetics of Change in the Amount of Bound ADP - Figure 25 shows the time course of changes in the amount of ADP bound to myosin during the initial phase of the reaction in 0.5 M KCl containing 2 mM $MgCl_2$ and 50 mM Tris-HCl at pH 7.8 and 0°C. In the absence of pyruvate kinase, 1 mole of ADP per mole of myosin was formed rapidly, and then the amount of ADP increased continuously at a rate of 0.4 min^{-1} (cf. Fig. 26). In the presence of 4 mg/ml of pyruvate kinase the initial rapid formation of 1 mole of ADP per mole of myosin was also observed, but the amount of ADP decreased gradually to a steady state level, and 5 min after the start of the reaction the amount of ADP reached 0.65 mole/mole of myosin. The time for half-maximum decrease was about 1 min. In other words, the apparent rate constant for the decrease was about 0.7 min^{-1}.

Relation of the Amount of Bound ADP to the ATPase Activity in the Steady State - Figure 26 shows the Lineweaver-Burk plot of the rate of the myosin-ATPase reaction in the steady state. The rate, v_0, was measured with 0.0024 mg/ml (0.005 μM) myosin in 0.5 M KCl containing 2 mM $MgCl_2$ and 50 mM Tris-HCl at pH 7.8 and 0°C. As described in our previous paper (93), and Section 2, the value of v_0 was given by the sum of the rates of two kinds of ATPase reaction, which had different K_M and V_{max} values.

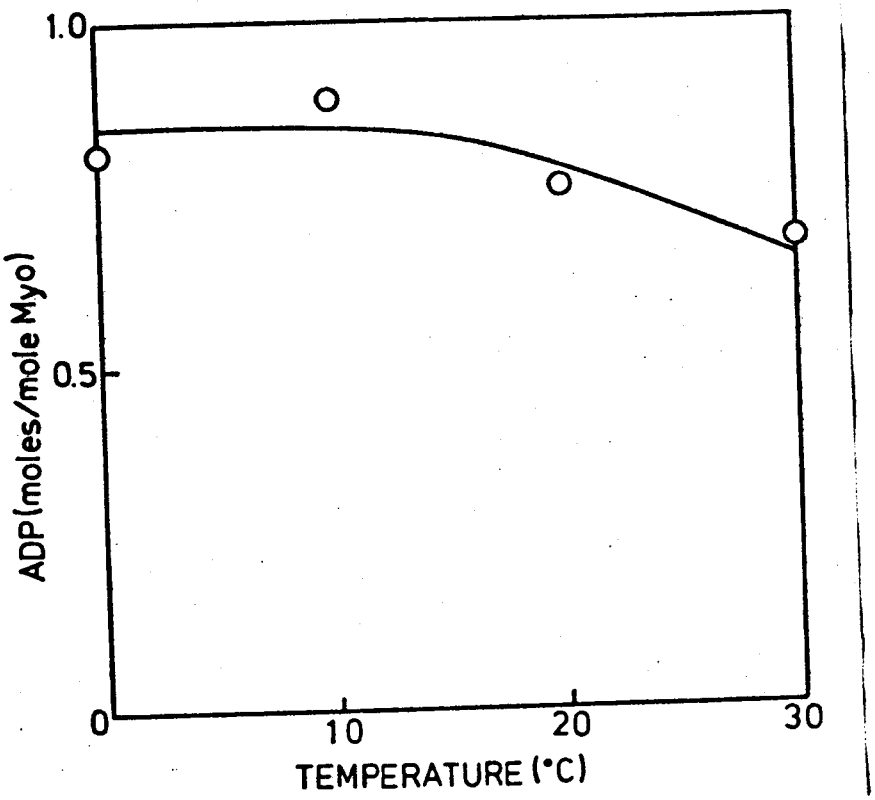


Fig. 24. Dependence of the amount of ADP bound to myosin during the ATPase reaction on temperature. Experimental conditions were as described for Fig. 21, except that the temperature was varied from 0 to 30°C and the concentration of ^{3}H-ATP added was 18.75 μM.

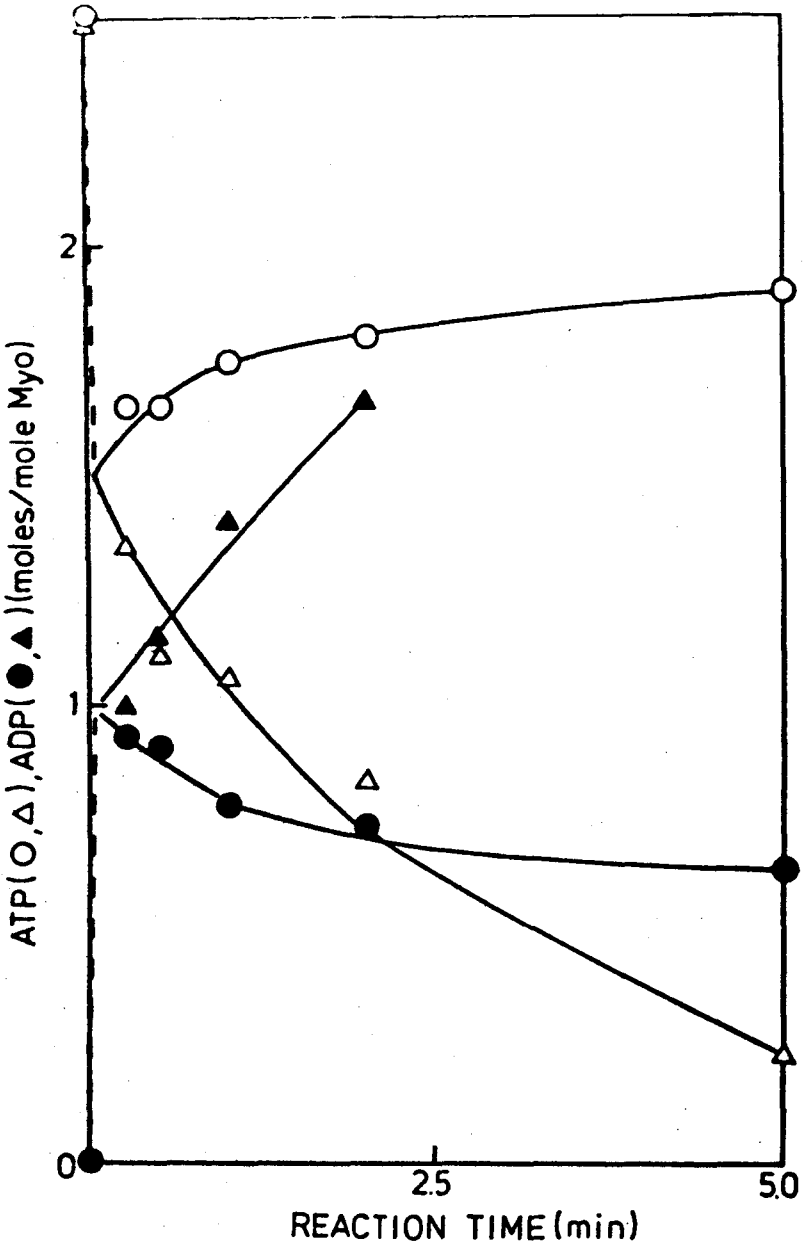


Fig. 25. Time course of changes in the amount of ADP bound to myosin after adding ATP to myosin. Experimental conditions were as described for Fig. 20, except that the concentration of pyruvate kinase was 0(△,▲) or 4 mg/ml (○,●) and the temperature was 0°C. ○,△, ATP; ●,▲, ADP.

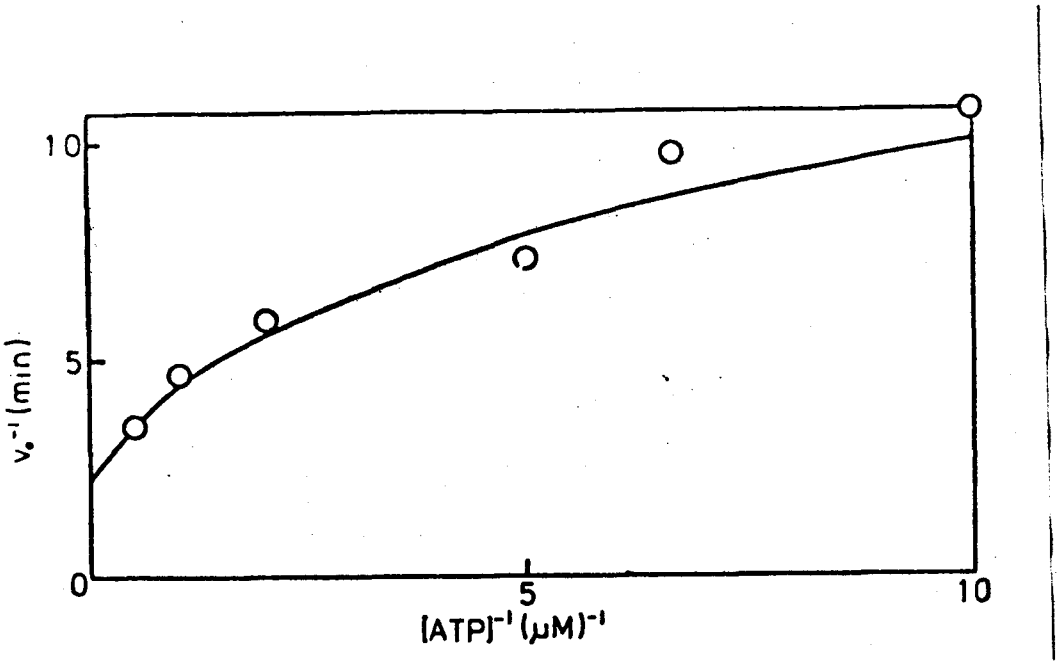


Fig. 26. Double-reciprocal plot of the rate of ATPase reaction in the steady state, v_0, against the concentration of ATP. The reaction mixture contained 0.0024 mg/ml (0.005 μM) of myosin, various concentrations of ^{32}P-ATP, 0.5 M KCl, 2 mM $MgCl_2$, and 50 mM Tris-HCl at pH 7.8. The temperature was 0°C. The line can be expressed as $v_0(min^{-1}) = 0.07 + \{0.33/(1 + 1\mu M/[ATP])\}$.

In the experiments shown in Fig. 26 V_o was given by

$$V_o\ (min^{-1}) = 0.07 + \frac{0.33}{1+1\ M/[ATP]}\ .$$

The dependence of the amount of ADP bound to myosin on the amound of ATP added (cf. Fig. 21) is compared with that of the rate of ATP phdrolysis in the steady state in Fig. 27 The ATPase activity was measured under the same conditions as used in the experiments shown in Fig. 21. The amount of bound ADP during the ATPase reaction remained at a constant level of 0.72-0.85 mole/mole of myosin when the amount of ATP added was higher than 1 mole/mole of myosin, as mentioned above, while the reaction rate of ATPase, V_o, increased from 0.26 to 0.43 min^{-1} when the amount of ATP added increased from 1 to 2 moles/mole of myosin. Thus, the V_o value was not proportional to the amount of bound ADP.

Binding of ATP to Myosin during the ATPase Reaction - The amount of total nucleotides bound to myosin during the ATPase reaction was measured using a rapid-flow dialysis method. Figure 28 shows the results measured with 3.6 mg/ml of myosin and 4 mg/ml of pyruvate kinase in 0.1 or 0.5 M KCl containing 2 mM $MgCl_2$ and 50 mM Tris-HCl at pH 7.8 and 0°C. To maintain the concentration of ATP throughout the measurements, 10 mM and 3 mM PEP were used, respectively, in 0.1 and 0.5 M KCl. The amounts of total nucleotides bound to myosin approached

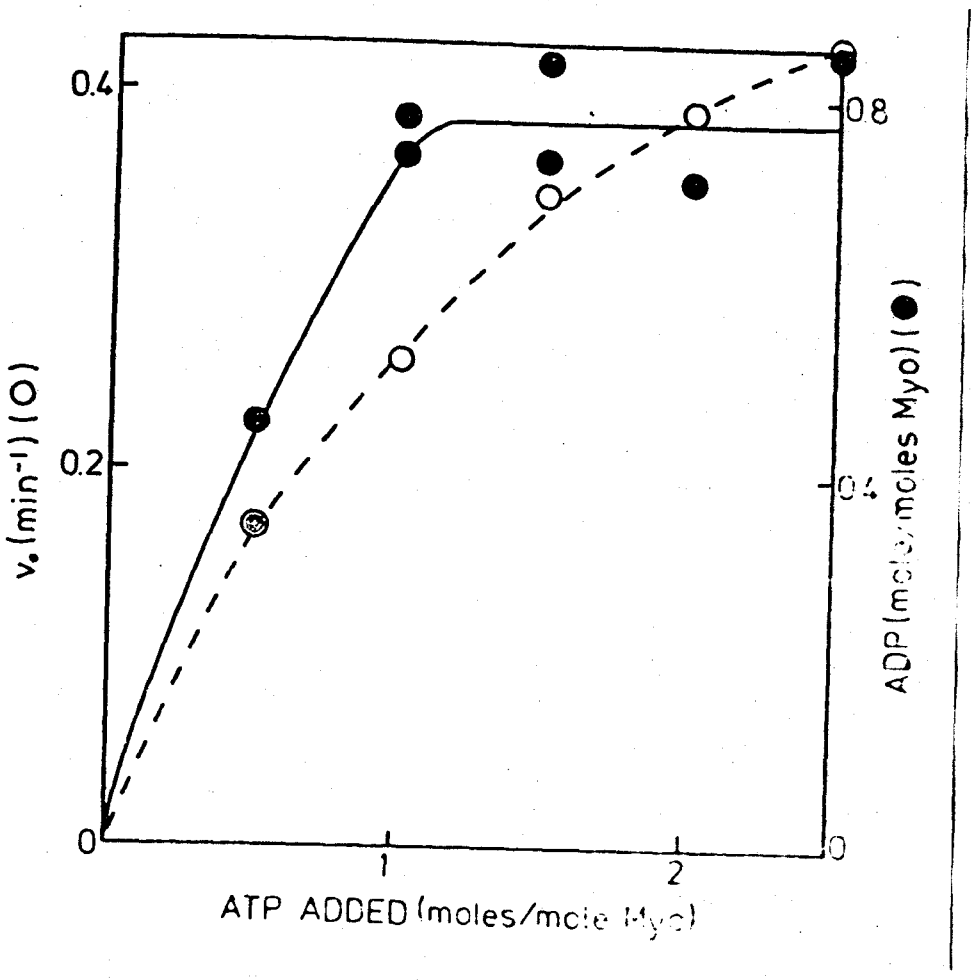


Fig. 27. Dependences of the amount of ADP bound to myosin and the rate of myosin-ATPase in the steady state on the amount of ATP added. The amounts of ADP bound to myosin (●) were those shown in Fig. 21. The rate of the ATPase reaction, v_0, (○) was measured as the rate of liberation of pyruvate under the conditions described in Fig. 21.

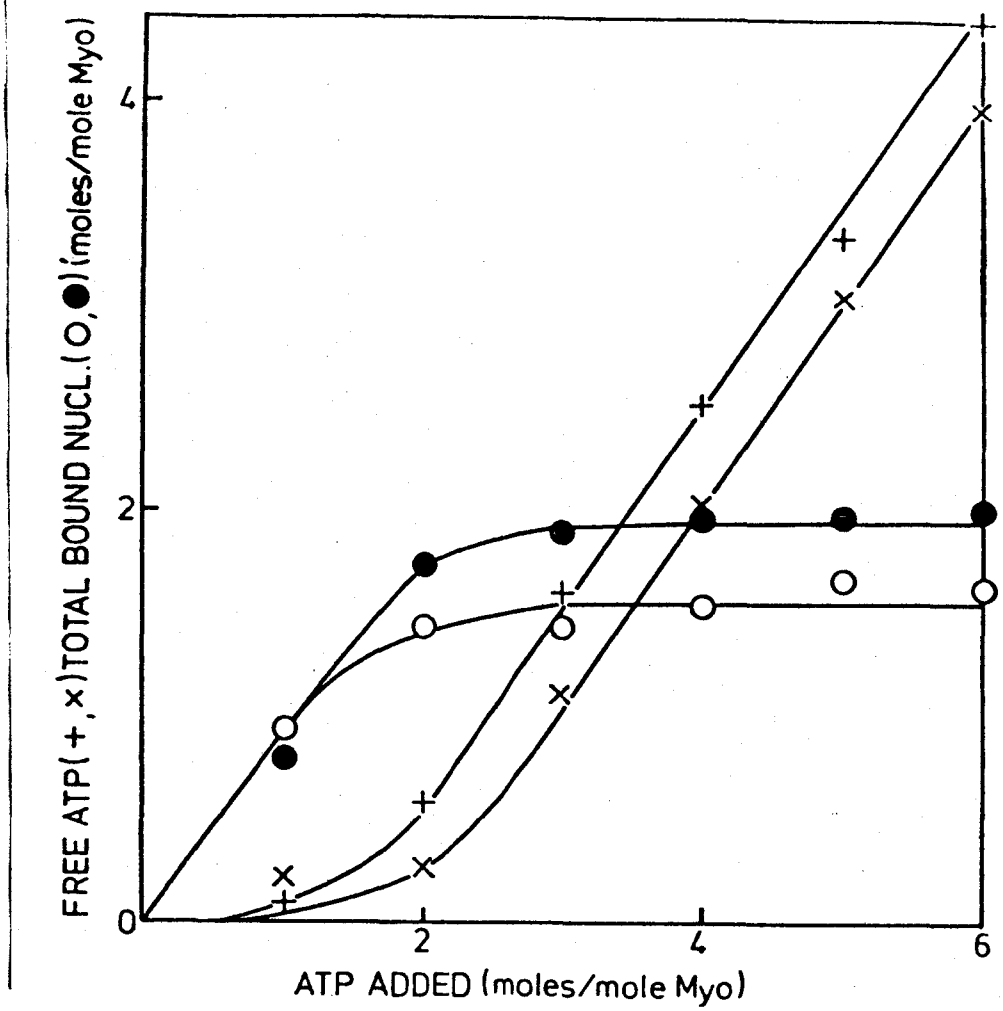


Fig. 28. Dependence of the amount of total nucleotides bound to myosin on the amount of ATP added. The total amount of bound nucleotides was measured by a rapid-flow dialysis method as described in "EXPERIMENTAL." The conditions used were as described for Fig. 21, except that the concentrations of KCl and PEP were 0.5Mand 3 mM (X, ●) or 0.1 M and 10 mM (+, O), respectively. X, +, free ATP; ●, O, total bound nucleotides.

constant levels of 1.45-1.60 and 1.9-2.0 moles/mole of myosin, respectively, in 0.1 and 0.5 M KCl, when the amount of ATP added became higher than 2-3 moles/mole of myosin. Since the amounts of ADP bound to myosin in 0.1 and 0.5 M KCl were 0.55 and 0.8 mole/mole of myosin, respectively (Fig. 23), the amounts of ATP bound to myosin in both 0.1 and 0.5 M KCl were about 1 mole/mole of myosin.

Figure 29 shows an analysis of the results obtained at 0.5 M KCl. For this figure, the amounts of free ATP and total bound nucleotides were measured by a rapid-flow dialysis method, while the amount of bound ADP was obtained from the results shown in Fig. 21 On the other hand, the amount of ATP bound to myosin was calculated from the amount of free ATP assuming that the maximum amount and the dissociation constant of binding of ATP to myosin were 1 mole/mole of myosin and 1 μM, respectively. The amount of total bound nucleotides obtained as the sum of the amount of bound ADP measured and that of bound ATP, as calculated above, was in good agreement with the amount of total bound nucleotides measured directly. It should also be noted that at low concentrations of added ATP almost all the nucleotide bound to myosin was ADP, and ATP bound to myosin appeared only when the amount of ATP added was higher than 0.6 mole/mole of myosin.

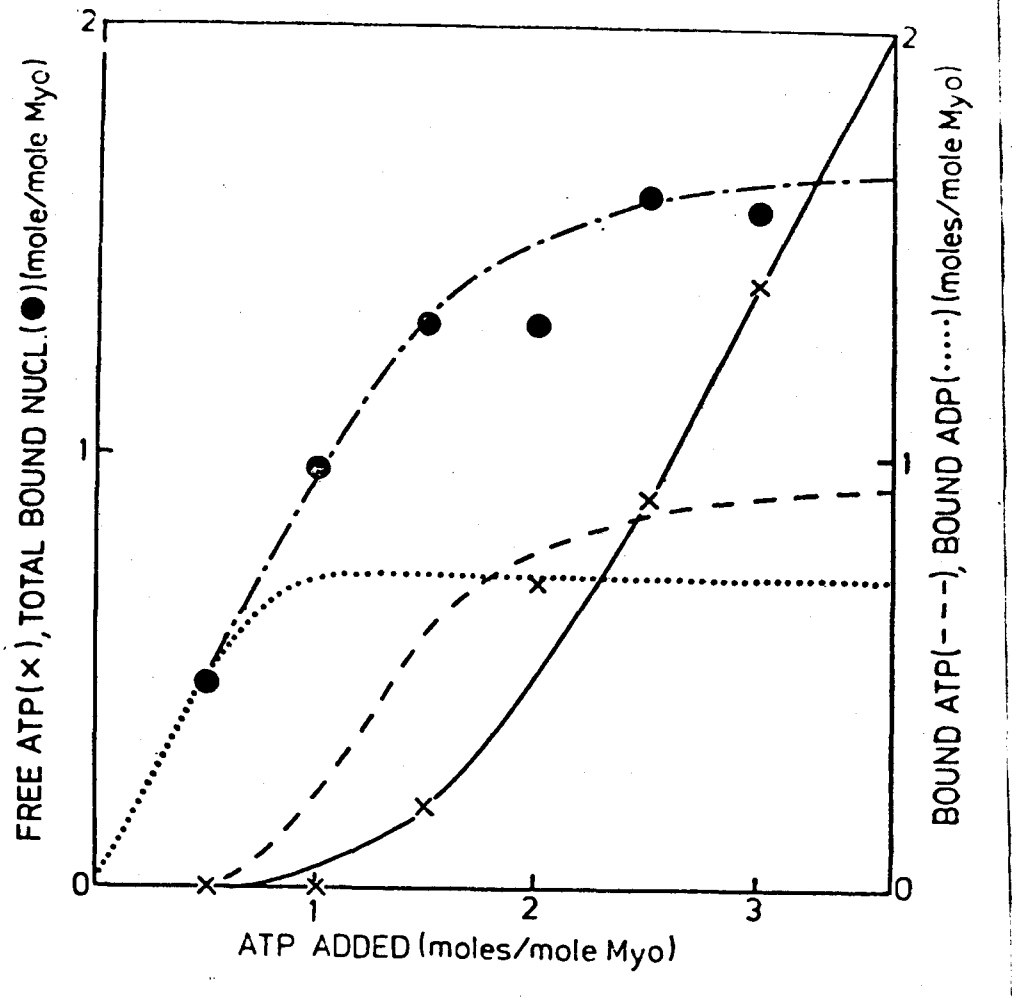


Fig. 29. Dependences of the amounts of ATP and ADP bound to myosin during the ATPase reaction on the amount of ATP added. The amounts of free ATP (X) and total bound nucleotides (●) were measured under the conditions described for Fig. 21, except that the concentration of PEP was 3 mM. The amount of ATP bound to myosin (---) was calculated assuming that 1 mole of ATP binds to 1 mole of myosin with a dissociation constant of 1 μM, using the measured concentration of free ATP (——). The amount of ADP bound to myosin (·····) was obtained from the results shown in Fig. 21. As shown in the figure, the amount of total nucleotides bound to myosin (●) measured by the rapid-flow dialysis method was almost equal to the sum (-·-·-) of the amounts of bound ADP measured and bound ATP calculated as mentioned above.

DISCUSSION

The amount of nucleotide bound to myosin during the ATPase reaction was previously measured by separating or transporting myosin from the reaction mixture. Bowen and Evans (88) measured it in a low concentration of KCl using Millipore filtration to separate bound and unbound nucleotides in the presence of a creatine kinase [EC 2.7.3.2] creatine phosphate system to regenerate ATP. Schliselfeld and Bárány (89) measured it by gel-filtration. These two groups reported the binding of about 2 moles of nucleotides per mole of myosin with high affinity, but they could not determine whether ATP or ADP was bound to myosin.

Bárány and Bárány (90) measured the amount of ADP in living muscle fibers in the relaxed state. They fround that the molar concentration of ADP in living muscle fibers was almost equal to that of actin, and concluded that myosin contains bound ATP but not ADP. However, their measurement might have been affected by the acid-stable adenylate kinase reaction, and it is difficult to estimate accurately the concentration of ADP bound to F-actin in muscle. Maruyama and Weber (92) and Marston (91) measured the amounts of nucleotides bound to myosin in myofibrils and glycerol-treated muscle fibers in the presence of creatine kinase and creatine phosphate, and concluded that 2 moles of ADP were bound to 1 mole of myosin in the

relaxed state. However, it is very difficult to confirm that ATP is regenerated, even in precipitates of myofibrils or in glycerol-treated muscle fibers, sufficiently rapidly that the results are not affected by the rate of its regeneration, and it is almost impossible to ascertain that F-actin does not affect the interaction between myosin and ATP in these systems.

To avoid these difficulties and disadvantages inherent in the previous studies, we used the myosin-ATP system coupled with the pyruvate kinase-PEP system, instead of highly organized muscle models, such as myofibrils and glycerol-treated muscle fibers, and measured the amounts of both ADP and ATP bound to myosin, without separating nucleotides from myosin. Thus, the ATPase reaction was performed in the presence of excess amounts of pyruvate kinase and PEP, and the amount of ADP bound to myosin was determined by separating ATP and ADP using thin layer chromatography after stopping the reaction by the addition of TCA. The total amount of nucleotides bound to myosin was measured using a rapid-flow dialysis method, which did not disturb the steady state of the reaction, and the amount of ATP bound to myosin was obtained as the difference between the total amount of nucleotides and that of ADP bound to myosin. As mentioned in "EXPERIMENTAL," of this section the adenylate kinase activities in the myosin and phruvate kinase preparations and the binding of nucleotides to pyruvate kinase were negligible

under the experimental conditions used. It should also be emphasezed that the ADP contained in the reaction intermediate of the myosin-ATPase reaction is not phosphorylated by pyruvate kinase, becasue the rate of myosin-ATPase measured as liberation of pyruvate on coupling the reaction with the pyruvate kinase system is equal to that measured as liberation of Pi and ADP from ATP in the absence of pyruvate kinase.

The results obtained may be summarized as follows. (i) About 1 mole of ATP binds to 1 moel of myosin during the ATPase reaction, and the dissociation constant of binding is equal to the K value for myosin-ATPase in the steady state over the range of high concentrations of ATP (Figs. 26 & 29). (ii) The amount of bound ADP is 1 mole/mole of myosin in the initial phase of the reaction, and then decreased on a steady state level with a rate constant of 0.7 min^{-1} (Fig. 25). (iii) Until the amount of ATP added reaches 0.6 mole/mole of myosin, almost all the nucleotide bound to myosin is ADP, and ATP bound to myosin appears only when the amount of ATP added is more than about 0.6 mole/mole of myosin (Figs. 21,22 & 29). (iv) At high concentrations of KCl, the amount of ADP bound to myosin in the steady state if 1 mole/mole of myosin, and it decreases to a value lower than the stoichiometric amount with decrease in the KCl concentration (Fig. 23). (v) The rate of myosin-ATPase in the steady state is not proportional to the amount of bound ADP (Fig. 27).

Previously, we presented several lines of evidence for two non-identical active sites in myosin, one for formation of the reactive myosin-phosphate-ADP complex, M_P^{ADP}, and the other for formation of the myosin-ATP complex, M_{ATP} (cf. Sec. 2). We proposed the following scheme for the myosin-ATPase reaction:

$$M + ADP \rightleftharpoons M_1ATP \rightleftharpoons M_2ATP \rightleftharpoons M_P^{ADP} \longrightarrow$$

$$^{\circ}M + ADP + Pi \longrightarrow M + ADP + Pi \qquad (1)$$

$$^{\circ}M(M \text{ or } M_P^{ADP}) + ATP \rightleftharpoons {}^{\circ}M_{ATP} \longrightarrow {}^{\circ}M + ADP + Pi \qquad (2)$$

By measuring the rate constants of the elementary steps, we showed that in 0.5 M KCl the route of ATP hydrolysis with a low V_{max} value and an extremely low K_M value is route (1), i.e. via M_P^{ADP} and °M, and that the route with a high V_{max} value and a K_M value of 1 μM is route (2), i.e., simple hydrolysis of ATP via M_{ATP} (cf. Fig. 26). All the results obtained here are consistent with this reaction mechanism, since as shown previously the rate of formation of M_P^{ADP} is much higher than that of its decomposition, and both the size of the initial burst of Pi liberation and the minimum amount of ATP necessary to saturate the initial burst are 1 mole/mole of myosin (58). If we assume an equilibrium between M_1ATP, M_2ATP, and M_P^{ADP} in the above sequence (98), this indicates that the equilibrium shifts strongly to the M_P^{ADP} side, and that the amounts of both M_1ATP and M_2ATP in the steady state

can be neglected. The result (ii) that the apparent rate constant of decrease in the amount of bound ADP during the initial phase is 0.7 min^{-1} is also consistent with our previous result (Sec. 2) that the rate constant of step $M_P^{ADP} \rightarrow$ °M + ADP + Pi is 0.7 min^{-1}. We previously concluded that in the presence of high concentrations of KCl (1.5-2.8 M), route (1) is the main path of ATP hydrolysis and step $M_P^{ADP} \rightarrow$ °M + ADP + Pi is rate-determining, while in low concentrations of KCl, route (2) is the main path of ATP hydrolysis and step °M → M is rate-determining in route (1). The result (iv) is consistent with these conclusions.

However, the amount of bound ADP in 0.5 M KCl at 0°C, calculated as the ratio of the rate constant of step $M_P^{ADP} \rightarrow$ °M + ADP + Pi to that of step °M → M is 0.2-0.3 mole/mole of myosin, which is rather lower than the value measured (0.76 mole/mole of myosin). Even if we assume that both the concentration of pyruvate kinase and the reaction time are not sufficient and that a sufficient increase in each of these factors gives 90% of the observed value, the true value of bound ADP would become 0.62 mole/mole of myosin. Therefore, the difference between the observed value and that calculated from the kinetic constants might be attributable to the binding of ADP to sites of myosin other than that of M_P^{ADP}.

Several years after the proposal of our original reaction mechanism, Taylor et al. (56, 57) proposed the following

simplified variant of our mechanism. Myosin has two identical active sites, and ATP is decomposed via one route, in which M_P^{ADP} is the main intermediate:

$$M + ATP \rightleftharpoons MATP \rightarrow M_P^{ADP} \rightarrow M + ADP + Pi.$$

Various defects of this mechanism have been discussed in our previous papers (93, 96), reviews (40, 43) and Sec. 2. In addition, the results (i) - (v) obtained here are all inconsistent with the mechanism of Taylor et al., since according to their mechanism the maximum amounts of ATP and ADP bound to myosin during the ATPase reaction in the steady state should be about 0 and 2 moles/mole of myosin, respectively, and the rate should be proportional to the amount of bound ADP.

The present results agree substantially with those of Bárany and Bárany (90) on living muscle fibers, but are in conflict with those of Marston (91) for glycerol-treated muscle fibers and of Maruyama and Weber (92) for isolated myofibrils. This suggests that in the experiments of Marston, Maruyama, and Weber the rate of regeneration of ATP in fibers was in sufficient or that the muscle was not in a truly relaxed state, although in the experiments of Marston (91) development of tension was very low.

4. Separation of Subfragment-1 of H-Meromyosin into Two Equimolar Fractions with and without Formation of the Reactive Enzyme-Phosphate-ADP Complex

H-Meromyosin (HMM) was digested with insoluble papain [EC 3.4.4.10]. Neither the size of the initial burst of Pi-liberation (0.5 mole/mole of myosin head) nor the Mg^{2+}-ATPase [EC 3.6.1.3] activity of HMM in the steady state was affected by this treatment. Acto-S-1 was obtained by mixing F-actin with HMM digested with insoluble papin (HMM-S-1).

The size of the initial burst of Pi-liberation of acto-S-1 was 0.35 mole/mole of S-1 at an ATP concentration of 0.5 mole/mole of S-1, and was 0.5 mole/mole of S-1 at ATP concentrations above 1 mole/mole of S-1.

F-Actin (2 mg/ml) was mixed with 3.4 mg/ml (20 μM) of HMM-S-1 in 50 mM KCl, 2 mM $MgCl_2$, and 10 mM Tris-HCl at pH 7.8 and 0°. After adding 10 μM ATP (0.5 mole/mole S-1) with 4 mg/ml pyruvate kinase and 12 mM PEP, the reaction mixture was centrifuged at 1.6×10^5 g and 0° for 40 - 60 min. The amounts of S-1 bound to F-actin in the precipitate and of S-1 dissociated from F-actin in the supernatant were measured by SDS-gel electrophoresis. The sizes of the initial bursts of Pi-liberation of S-1 in the supernatant and the precipitate were about 0.7 and 0.3 mole/mole S-1, respectively. When the separation procedures were repeated, the size of the initial burst of S-1 in the super-

natant increased to 0.8 mole/mole of S-1, while that of S-1 in the precipitate decreased to 0.25 mole/mole of S-1.

Myosin was separated into two fractions, one bound to F-actin (precipitate) and the other dissociated from F-actin (supernatant), by centrifuging actomyosin in the presence of various concentrations of ATP with an ATP-regenerating system at high ionic strength. At all the ATP concentrations used, the myosin in the precipitate and the supernatant gave the same sizes of initial burst (0.5 mole/mole of myosin head) as that of myosin before the separation.

INTRODUCTION

Our studies on the substructure of myosin, chemical modifications of the active sites of myosin ATPase [EC 3.6.1.3], and the binding of ATP and its analogues to myosin have provided strong evidence that the structures and functions of the two heads of the myosin molecule are different. These studies have been described in our review articles (40, 43), but a brief explanation of these studies seems in order. Ohe et al.(99) and Tokuyama et al.(100) showed that maximal changes in the ATPase activity of myosin occur by specific binding of 1 mole of monoiodoacetamide and trinitrobenzene sulfonate per mole of myosin. More recently, Shibata-Sekiya and Tonomura (101) reported that the substrate inhibition of actomyosin ATPase in the presence of the relaxing protein and the absence of Ca^{2+} ions is blocked by the binding of 1 mole of p-chloromercuribenzoate per mole of myosin. Tonomura and Morita (44) previously reported that 2 moles of PPi bind to 1 mole of myosin with different binding constants, and that only one of these induces the dissociation of actomyosin. More recently, Morita (102) showed that 2 moles of ADP bind to 1 mole of HMM with different binding constants, and that only the one of these two moles of ADP which binds the more strongly induces a change in the UV spectrum of H-meromyosin. Furthermore, our transient kinetic studies (93, 103) showed

that myosin has two non-identical actives sites, one for formation of the reactive myosin-phosphate-ADP complex, M_P^{ADP}, and the other for formation of the myosin-ATP complex, M_{ATP}.

On the basis of these studies, we (Ref. 40, 43 and Sec. 2, 3) proposed the following scheme for the myosin ATPase reaction:

$$M + ATP \underset{(1)}{\rightleftharpoons} M_1ATP \underset{(2)}{\rightleftharpoons} M_2ATP \underset{(3)}{\rightleftharpoons} M_P^{ADP} \underset{(4)}{\rightleftharpoons} {}^{\circ}M + ADP + Pi \underset{(5)}{\rightleftharpoons} M + ADP + Pi$$

$$M({}^{\circ}M, M_P^{ADP}, \cdots) + ATP \underset{(6)}{\rightleftharpoons} M \underset{(7)}{\longrightarrow} M + ADP + Pi$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad ATP$$

where °M is myosin with a changed conformation, which shows no initial burst of Pi-liberation, i.e.formation of M_P^{ADP}, on adding ATP. We concluded that there is one active site for formation of M_P^{ADP} in the myosin molecule, since both the size of the initial burst of Pi-liberation and the amount of the initial rapid liberation of H^+ were 1 mole per mole of myosin (54, 59, 93). Recently, we (103) measured the amounts of ATP and ADP bound to myosin during the transient and steady state phases, and obtained further support for the mechanism.

We showed that M_P^{ADP} is the intermediate of the actomyosin ATPase reaction and F-actin greatly accelerates the regeneration of myosin (M) from M_P^{ADP} (Ref. 39, 40, 43 and Sec. 5). Recently, we (Ref. 104 and Sec. 5) measured the ATPase activity and the amounts of ATP and ADP bound to HMM in the FA-HMM-relaxing

fetal fast muscle (107), cardiac muscle (108), and smooth muscle[3] are all 1 mole per mole of myosin, and the rates of the initial bursts of myosins from the different types of muscle are of the same order of magnitude.

Our studies on myosin ATPase did not indicate whether the active sites of the two routes of the myosin ATPase reaction are both located on the same head or whether they are located on different heads of the myosin molecule. On the other hand, our studies on the dissociation of actomyosin and acto-S-1 induced by ATP suggested that M_P^{ADP} is formed on one head and M_{ATP} on the other. We showed that actomyosin dissociates into myosin and F-actin on adding 1 mole of ATP (109) or PPi (44) per mole of myosin, that the rate of dissociation of actomyosin at low concentrations of ATP is slightly lower than that of formation of M_P^{ADP}, and that the extent of dissociation of actomyosin is the same as that of formation of M_P^{ADP} (109, 110). Lymn and Taylor (111) later reported that at very high concentrations of ATP, the rate of dissociation of actomyosin by ATP is much higher than that of formation of M_P^{ADP}. These results suggest that the dissociation of actomyosin is induced either

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3. Takeuchi, K. & Tonomura, Y., in preparation.

protein system both in the presence and absence of Ca^{2+} ions, and showed that formation of M_{ATP} is not required for the ATPase activity of actomyosin but that it is required for the inhibition of actomyosin ATPase by removal of trace amounts of Ca^{2+} ions in the presence of the relaxing protein[2].

Thus, the separation of the two heads of the myosin molecule into two equimolar fractions with and without formation of M_P^{ADP} was urgently required to establish our proposal. Sarkar (105) recently reported that the three light chains of myosin are present in the molar ratio of 1.3, 2 and 0.7, and Star and Offer (106) showed that the N-terminal amino acid sequence in myosin is not homogeneous. They suggested that myosin is composed of at least two isozymes with slightly different chemical structures. However, it seems rather improbable that the two routes of myosin ATPase shown above are catalyzed by different species of isozymes of myosin ATPase, since the sizes of the initial burst of Pi-liberation of myosins prepared from skeletal adult fast muscle (93, 103), skeletal

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2. At present, many workers are of the opinion that the two heads of the myosin molecule have the same structure and function. The experimental bases for this opinion were critically discussed in our article (40).

by formation of M_P^{ADP} on one head(B) or by formation of M_{ATP} on the other head (A) of the myosin molecule (Fig. 30).

Takeuchi and Tonomura (112) previously reported that the size of the initial burst of S-1 is 0.5 mole/mole S-1, and that complete dissociation of acto-S-1 is induced by adding 1 mole of ATP per mole of S-1. Therefore, they (112) suggested that acto-S-1 is dissociated when one S-1 forms M_P^{ADP} and the other forms M_{ATP} (Fig. 30), while actomyosin is dissociated by formation either of M_P^{ADP} on one head or of M_{ATP} on the other head because of mutual interactions between the two heads of the myosin molecule, as mentioned above (112).

The reaction mechanism for dissociation of acto-S-1 given in Fig. 30 indicates a means for separating S-1 into two fractions which do and do not form M_P^{ADP}, since the Michaelis constant for formation of M_P^{ADP} is usually smaller than that for formation of M_{ATP} (40, 43). Thus, in this study 0.5 mole of ATP was added to 1 mole of S-1 in acto-S-1 with an ATP-regenerating system, and S-1 was separated by centrifugation into two fractions, i.e. S-1 bound with F-actin (precipitate) and S-1 dissociated from F-actin (supernatant). S-1 was divided almost equally between the supernatant and the precipitate, but the size of the initial burst of Pi-liberation of S-1 in the supernatant was about 0.7 mole/mole S-1, while that of S-1 in the precipitate was about 0.3 mole/mole S-1. This result was in good accordance with the result that

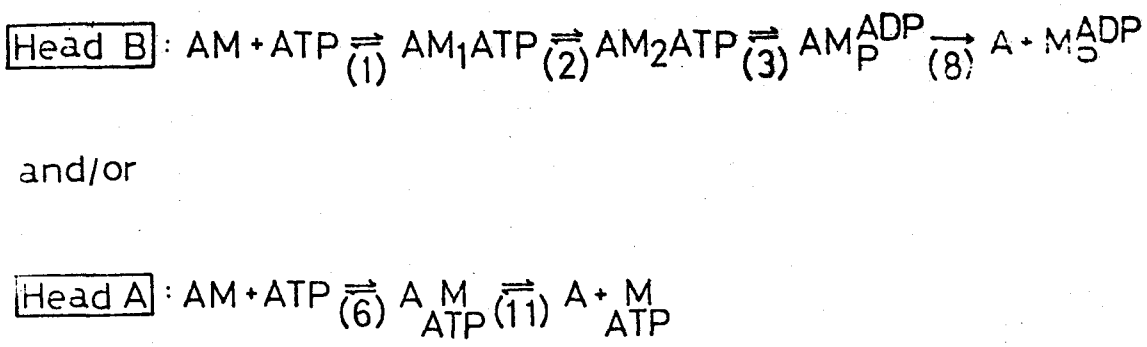

Fig. 30. Mechanism of dissociation of actomyosin by ATP. Because of mutual interactions between the two heads of the myosin molecule, actomyosin dissociates either by formation of M_P^{ADP} on one head (B) or by formation of M_{ATP} on the other head (A). On the other hand, the S-1 are independent of each other, and one type of S-1 (S-1B) dissociates from FA on formation of M_P^{ADP}, while the other type of S-1 (S-1A) dissociates on formation of M_{ATP}.

the size of the initial burst of acto-S-1 at 0.5 mole ATP/mole of S-1 was 0.35 mole/mole S-1. On repeating the separation procedures, the size of the burst of S-1 in the supernatant increased, while the that of S-1 in the precipitate decreased, as anticipated.

On the other hand, when actomyosin was dissociated by additions of various concentrations of ATP, both the size of the initial burst of myosin in the supernatant and that in the precipitate were always equal to the orginal value, i.e., 0.5 mole/mole myosin.

EXPERIMENTAL

Myosin was prepared from rabbit skeletal white muscle by the method of Perry (70). HMM was prepared by tryptic digestion of myosin using the method of Szent-Györgyi (113). The molecular weights of myosin and HMM were taken as 4.8×10^5 and 3.4×10^5, respectively (39). Purified G-actin was prepared from an acetone powder of rabbit skeletal muscle as described by Spudich and Watt (114). After removal of free ATP from the G-actin solution by treatment with Dowex 1-4, G-actin was polymerized to F-actin by addition of 50 mM or 0.5 M KCl, 2 mM $MgCl_2$, and 10 or 50 mM Tris-HCl at pH 7.8.

Pyruvate kinase (PK) [EC 2.7.1.40] was prepared from rabbit skeletal muscle by the method of Tiez and Ochoa (93).

Crystalline papain [EC 3.4.4.10] was purchased from Worthington Biochemicals Co., Freehold, N.J., U.S.A., and was made insolube as described by Lowey et al. (33), except that reduction of the thiol residues of papin with cysteine was omitted. The concentration of protein was determined by the biuret reaction, calibrated by nitrogen determination.

γ-^{32}P-Labelled ATP was synthesized enzymatically by the method of Glynn and Chappel (75). ATP and PEP were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.

The esterase activity of papain was measured from the decrease of absorbance of cresol red at 560 nm using α-N-benzyl-L-arginine ethyl ester as substrate. Myosin and HMM were digested with insoluble papain, which was activated by addition of 2 nM EDTA and 2 mM DTT. The digestion was stopped by removal of the insoluble papain by centrifugation at 10^4xg and 0° for 10 min or by filtration through gause. The ATPase activity was measured as described previously (93), using γ-^{32}P-labelled ATP as substrate.

SDS-gel electrophoresis of the reaction mixture containing HMM-S-1, FA and PK was performed as follows. The reaction mixture (50 μl) was added to a mixture of 200 μl of 8 M urea and 1 % SDS with 50 μl of β-mercaptoethanol. The combined mixture was heated at 80° for 20 min, and then 50 μl of 50 % glycerol and 0.025 % bromphenol blue were added. Twenty μl of the mixture were applied to a 10 % polyacrylamide gel in the presence of 0.1 % SDS, and electrophoresis was performed by the method of Weber

and Osborn (115), with slight modifications (116). After electrophoresis, the gel was stained at 45° for 1 hr with 0.2 % Coomassie brilliant blue. The electrophoretogram was scanned with a densitometer, Fuji-OX model F.D.A. IV. SDS-gel electrophoretograms of myosin, FA and PK were obtained in the same way as that of HMM-S-1.

RESULTS

Preparation of HMM-S-1 — Myosin (24 mg/ml) was digested with 8 μg/ml of insoluble papain in 0.5 M KCl, 2 mM EDTA, 2 mM DTT, and 50 mM Tris-HCl at pH 7.8 and 0°. At intervals the digestion of myosin was stopped by removing the insoluble papain by centrifugation at 10^4xg for 10 min. The supernatant was diluted 10-fold with cold water, and proteins that were insoluble at low ionic strength were removed by centrifugation at 10^4xg and 0° for 10 min. The concentration of protein in the supernatant and its ATPase activity in 0.5 M KCl, 2 mM $MgCl_2$, and 50 mM Tris-HCl were measured at pH 7.8 and 0°. As shown in Fig. 31, the concentration of protein in the supernatant increased to a saturation value of about 1.7 mg/ml at 15 min after starting the digestion. The time course of increase in the ATPase activity of the supernatant was the same as that of increase in protein concentration, and reached a maximal value of about 0.19 mole/mole of total head/min after about 15 min of digestion. When

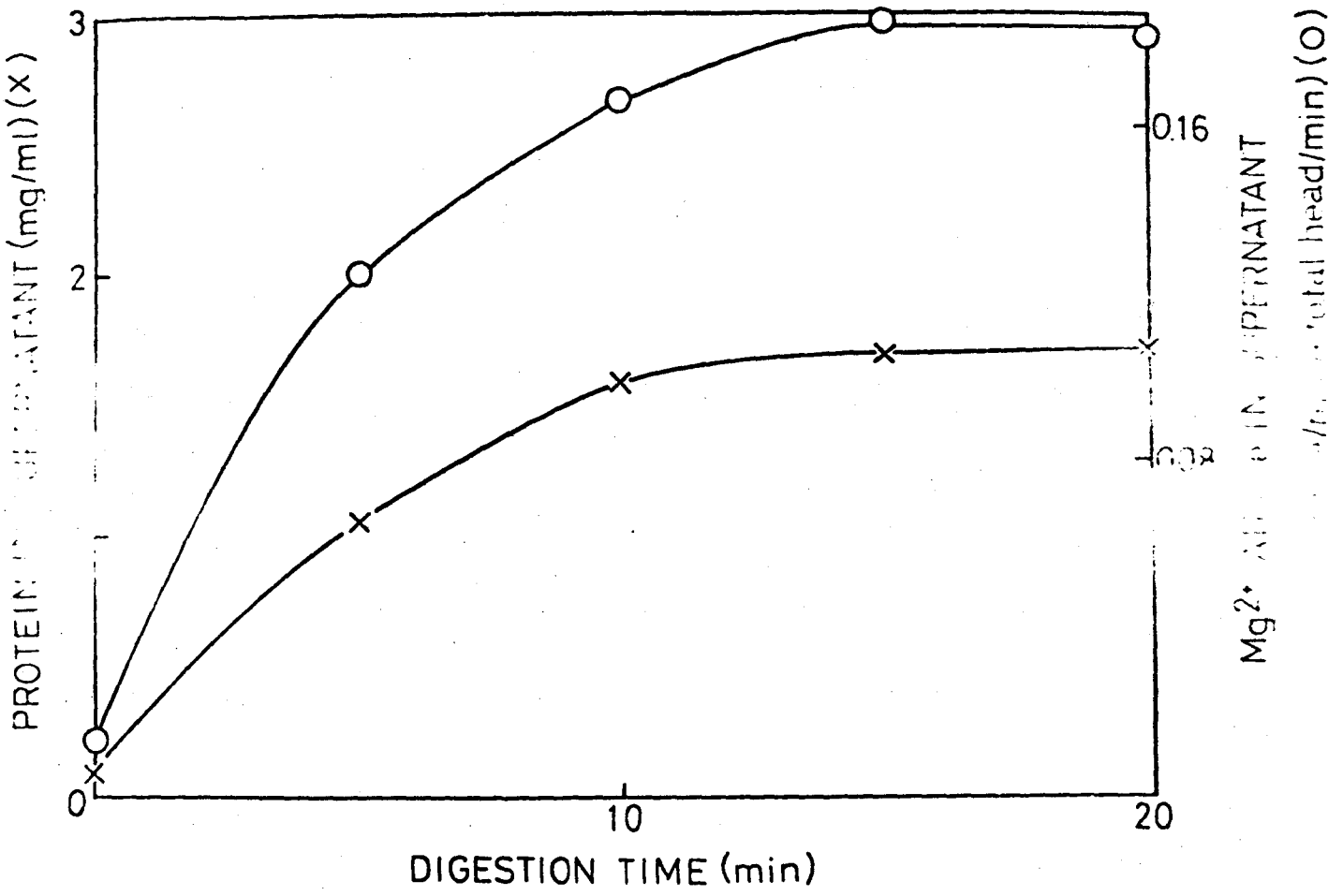

Fig. 31. Time course of digestion of myosin with insoluble papain. Myosin (24 mg/ml) was digested with 8 μg/ml insoluble papain in 0.5 M KCl, 2 mM EDTA, 2 mM DTT, and 50 mM Tris-HCl at pH 7.8 and 0°. The digestion was stopped by removal of insoluble papain by centrifugation at 10^4 x g and 0° for 10 min. The supernatant was diluted 10-fold with cold water, and proteins insoluble at low ionic strength were removed by centrifugation at 10^4 x g and 0° for 10 min. The concentration of protein in the supernatant was measured by the biuret reaction. The ATPase activity was measured in 50 μM [γ-^{32}P]ATP, 0.5 M KCl, 2 mM $MgCl_2$, and 50 mM Tris-HCl at pH 7.8 and 0°. O, protein concentration; X, Mg^{2+}-ATPase activity in the supernatant. The activity is given as mole of ATP hydrolyzed/mole of head of HMM before digestion with insoluble papain/min.

the concentration of insoluble papain was increased 2-fold, or the temperature at the digestion was increased to 10°, both the protein concentration and the ATPase activity in the supernatant reached a maximal level within 5 min. The esterase activity of insoluble papain in 50 mM KCl was about 1.4 times as high as the activity in 0.5 M KCl (1.6 unit/mg).

Therefore in following work, the digestion was started by adding 17 μg/ml (1/1000) of insoluble papain to 17 mg/ml HMM in 50 mM KCl, 2 mM EDTA, 2 mM DTT, and 50 mM Tris-HCl at pH 7.8 and 0°, and was stopped after 10 min by removing the insoluble papain by filtration through gauze. In this paper, the papain digest of HMM is called HMM-S-1, and the molar concentration of S-1 was calculated assuming that 2 moles of S-1 are produced by digestion of 1 mole of HMM (MW = 3.4×10^5). Therefore, 3.4 mg HMM-S-1 contained 20 ηmoles of S-1.

Figure 32 shows the effect of digestion of HMM with insoluble papain on the ATPase activity in the steady state. The ATPase activity was measured in 3.4 mg/ml HMM or its digest, 50 μM [γ-^{32}P]ATP, 0.5 M KCl, 2 mM $MgCl_2$, and 50 mM Tris-HCl at pH 7.8 and 0°. The ATPase activity decreased 5 % on digestion with incoluble papain for 10 min and decreased 20 % on digestion for 25 min. Figure 33 shows the time course of Pi-liberation after adding ATP to HMM before and after digestion of HMM with insoluble papain for 10 min. Neither the size of the initial burst of Pi-liberation (0.5 mole/mole head) nor the rate of the ATPase reaction in the steady state was affected by the digestion.

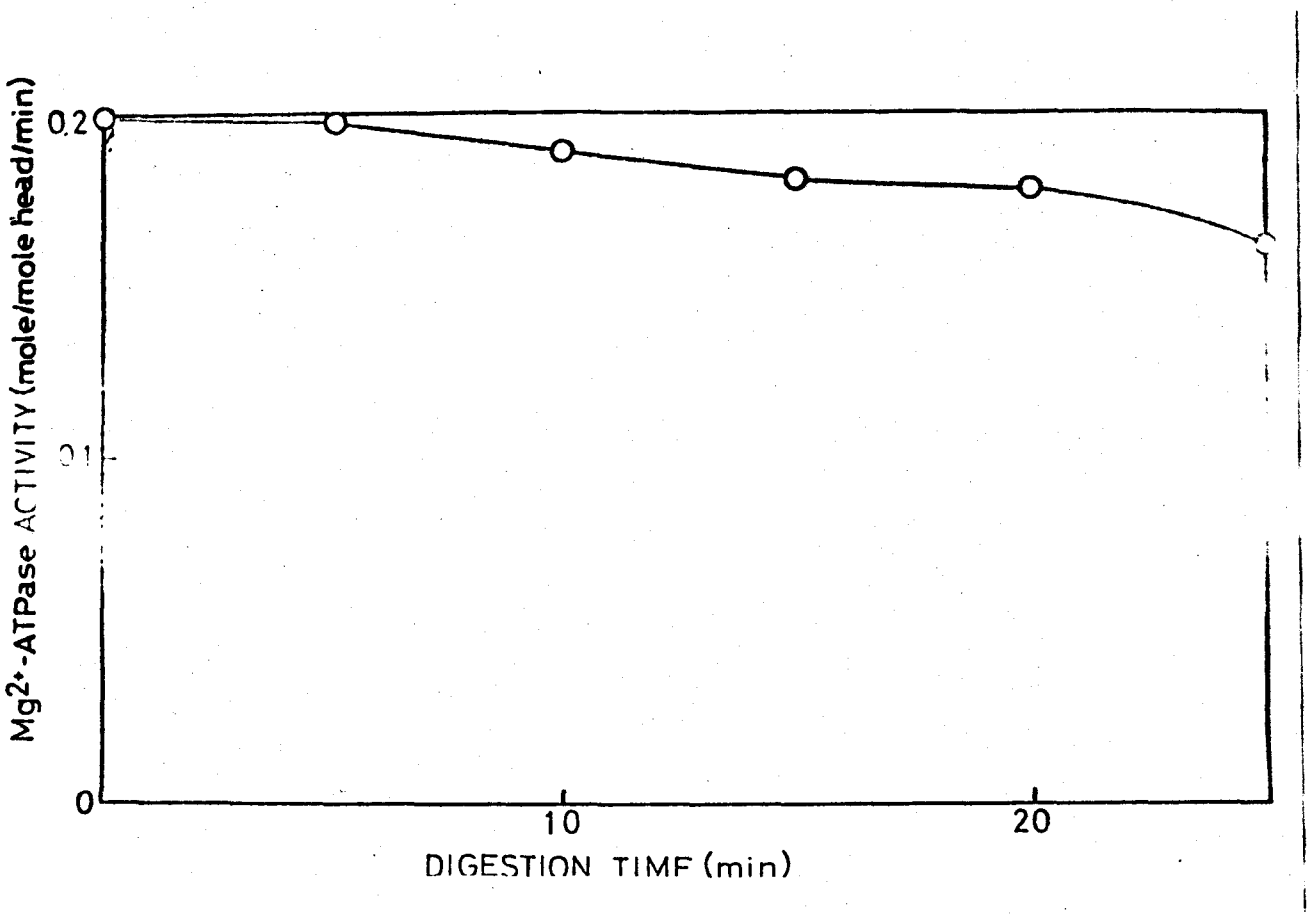

Fig. 32. Decrease in the Mg^{2+}-ATPase activity of HMM during digestion with insoluble papain. HMM (17 mg/ml) was digested with 17 μg/ml of insoluble papain in 50 mM KCl, 2 mM EDTA, 2 mM DTT, and 50 mM Tris-HCl at pH 7.8 and 0°. The digestion was stopped by removing the insoluble papain by filtration through gauze. The Mg^{2+}-ATPase activity was measured under the conditions described for Fig. 31, and is given as mole/mole of head/min.

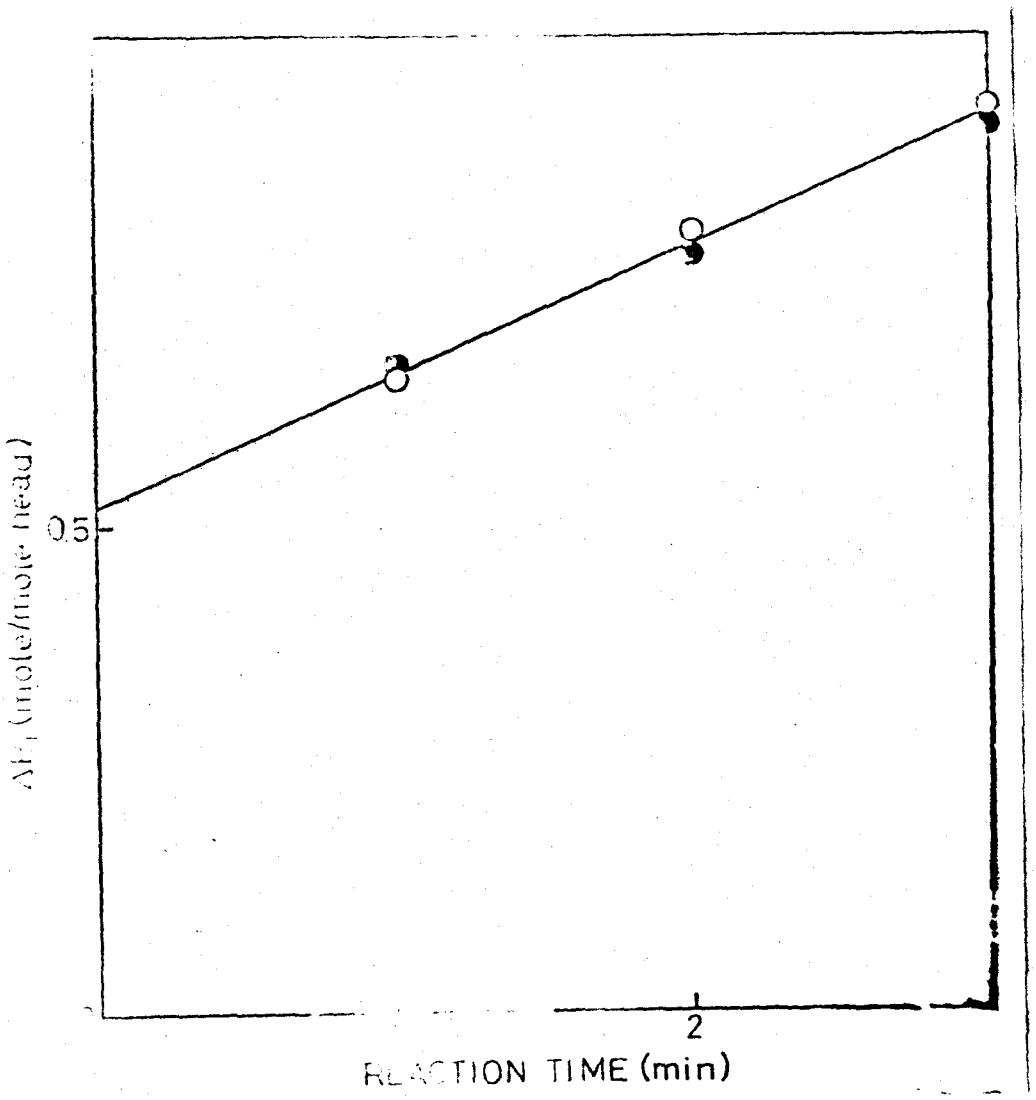

Fig. 33. Burst size of Pi-liberation of HMM before and after digestion of HMM with insoluble papain. The ATPase reaction was measured under the same conditions as for Fig. 31. ○, HMM before digestion; ●, HMM-S-1 obtained by digestion of HMM with insoluble papain for 10 min under the same conditions as for Fig. 32.

Measurement of the Amount of HMM-S-1 by SDS-gel Electrophoresis — As mentioned later, HMM-S-1 was separated into two fractions by centrifuging a mixture containing 3.4 mg/ml HMM-S-1, 2 mg/ml FA, various concentrations of ATP and an ATP-regenerating system (4 mg/ml PK and 12 mM PEP). Myosin was separated under the same conditions, except that 4.8 mg/ml of myosin were used in place of HMM-S-1. Thus, the samples of HMM-S-1 and myosin separated contained high concentrations of FA and PK, and the concentrations of S-1 and myosin were calculated from the areas of the peaks of the protein components in the SDS-gel electrophoretogram.

Figure 34 shows densitometer tracings of SDS-gel electrophoretograms of the supernatant and the precipitate obtained by centrifuging acto-S-1 in the presence of 0.5 mole ATP per mole of S-1. Peaks shaded with oblique lines were used for measurement of the amount of S-1. Our PK preparation was impure, and peaks, I, II, IV, and V were attributed to proteins in the PK preparation. Peak III was due to actin. The papain digest of HMM was separated into one fraction which combined with F-actin, i.e., S-1, and an other, which did not combine with FA, i.e. S-2. The SDS-gel electrophoretogram of S-2 showed a main peak at peak I and several minor bands, which overlapped the peaks of S-1. However, the sum of the areas of the minor bands of S-2 was less than 5 % of that of HMM-S-1. Furthermore, no significant difference was observed between the electrophoretograms of S-1 in the supernatant and the precipitate.

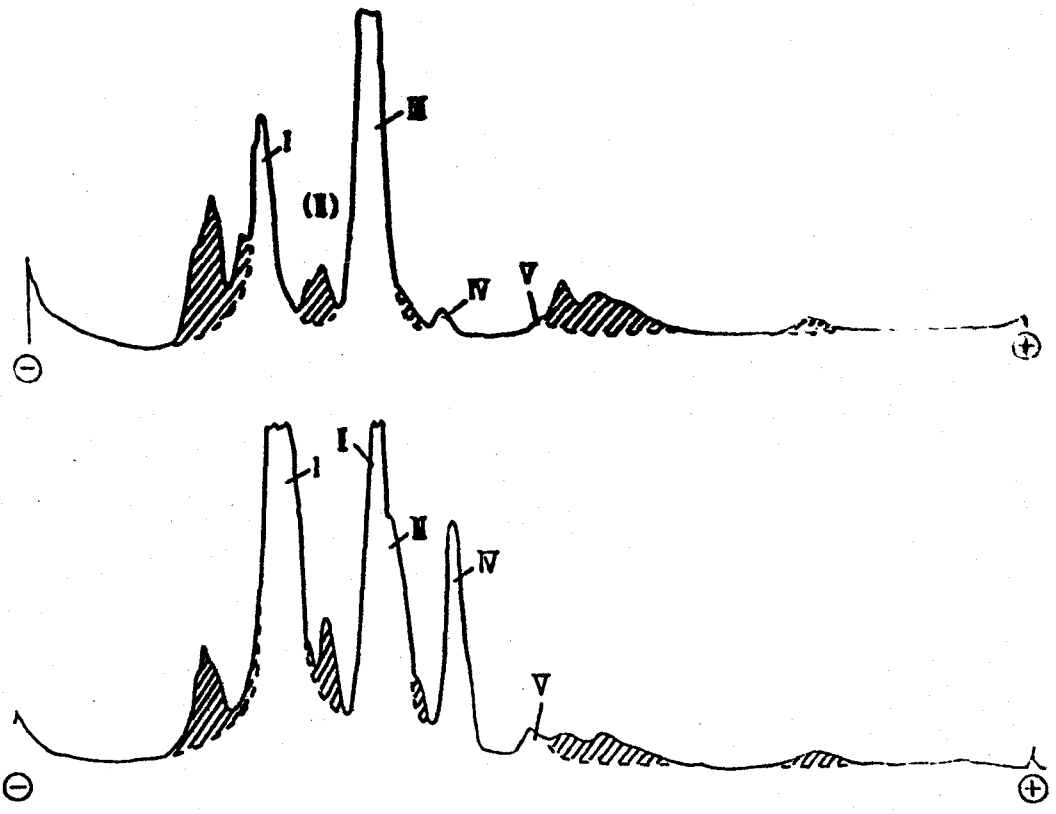

Fig. 34. Densitometer tracings of SDS-gel electrophoretograms of the supernatant and the precipitate obtained by centrifugation of acto-S-1 in the presence of 0.5 mole of ATP per mole of S-1. HMM-S-1 was obtained by digestion of 17 mg/ml HMM with 17 μg/ml of insoluble papain for 10 min under the same conditions as for Fig. 32. ATP (10 μM) and an ATP-regenerating system (4 mg/ml PK + 12 mM PEP) were added to the reaction mixture (total volume 5 ml) containing 3.4 mg/ml (20 M) HMM-S-1 and 2 mg/ml FA in 50 mM KCl, 2 mM $MgCl_2$, and 10 mM Tris-HCl at pH 7.8 and 0°. The mixture was ultracentrifuged at 1.6 x 10^5 x g and 0° for 60 min. The volumes of the supernatant and the precipitate were adjusted to 5 ml with buffer solution. A sample (2.86 μl) of the supernatant or precipitate was applied to SDS-polyacrylamide disk gel, and electrophoresis was performed as mentioned in the "EXPERIMENTAL". Upper figure, precipitate; lower figure, supernatant. Peak I is due to S-2 and a protein component in the PK preparation. Peaks II, IV, and V are due to proteins in the PK preparation, and peak III is due to action. The areas of peaks of S-1 indicated by oblique lines were measured (Fig. 35) to calculate the amount of S-1.

Figure 35 shows the dependence of the total area of the peaks assigned to S-1 in Fig. on the concentration of HMM-S-1 applied to the gel. A linear relationship was obtained between the concentration of HMM-S-1 and the total area, but its slope varied slightly in different experiments. Therefore, to measure the amount of S-1 in the reaction mixture, SDS-gel electrophoresis of the mixture was performed simultaneously with electrophoreses of known amounts of HMM-S-1.

Figure 36 shows the SDS-gel electrophoretogram of 5 μl of mixture containing 4.8 mg/ml myosin, 2 mg/ml FA, and 4 mg/ml PK. Peak I was due to heavy chains of myosin, peaks II, III, V, and VI were due to proteins in the PK preparation, and peak IV was due to actin, as mentioned above. The concentration of myosin was measured from the areas of the peaks due to the light chains of myosin (shaded with oblique lines). The relationship between the total area of light chains and the concentration of myosin is shown in Fig. 35. As expected, the area of light chains was proportional to the amount of myosin applied.

Dependence of the Amount of the Initial Burst of Pi-Liberation of Acto-S-1 on ATP Concentration — One of the most important factors in determining the extent of separation of S-1 into two fractions by the method used in thispaper is the dependence of the size of the initial burst of Pi-liberation of acto-S-1 on the amount of ATP added. The dependence of the size of the initial burst of S-1 on the concentration of ATP added was

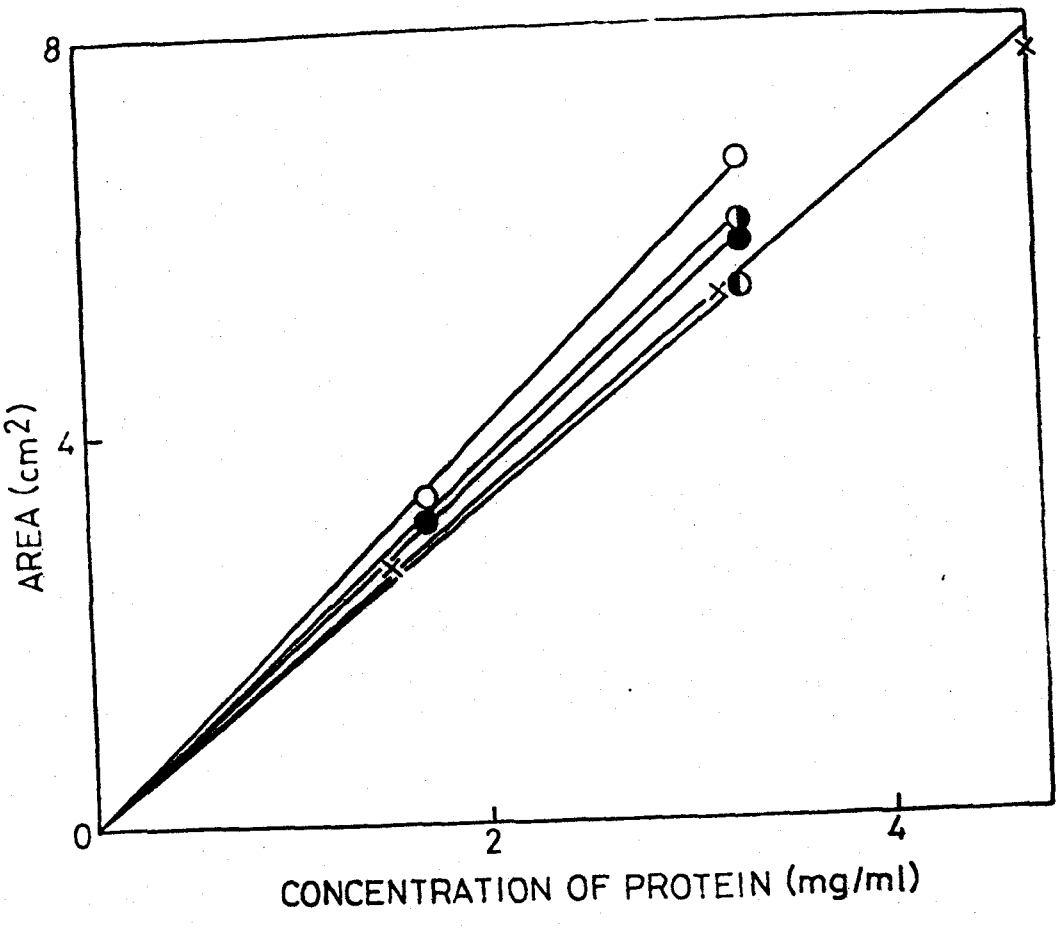

Fig. 35. Relationship between the concentration of myosin or papain digest of HMM, *i.e.* HMM-S-1, and the area of the electrophoretogram. HMM-S-1 was obtained by digestion of 17 mg/ml HMM with 17 μg/ml of insoluble papain for 10 min under the same conditions as for Fig. 32. ○, ◐, ◑, ●; Various concentrations of HMM-S-1 (2.86 μl) were applied to SDS-polyacrylamide disk gel. The total area of the peaks of S-1 (Fig. 34) was plotted against the concentration of HMM-S-1 applied to the gel. Different symbols show the results of different experiments using different preparations of HMM-S-1. X, Various concentrations of myosin (5 μl) were applied to the gel. The total area due to light chains of myosin, indicated by oblique lines in Fig. 36, was plotted against the concentration of myosin applied to the gel.

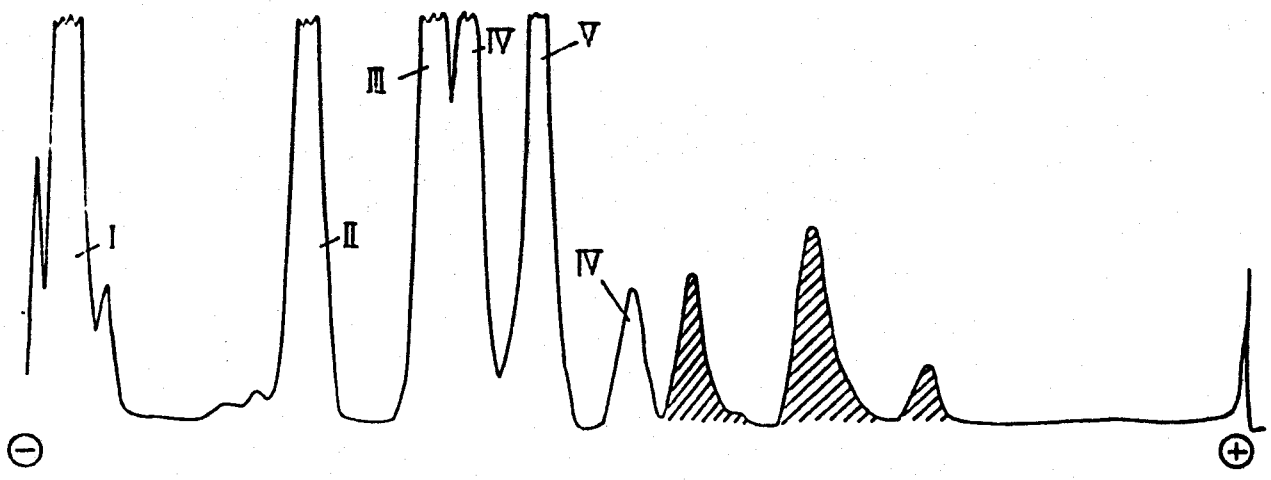

Fig. 36. Densitometer tracing of SDS-gel electrophoretogram of a mixture of myosin with F-actin and pyruvate kinase preparation. Myosin (final concentration, 4.8 mg/ml) was mixed with 2 mg/ml FA and 4 mg/ml PK in 0.5 M KCl, 3 mM PEP, 2 mM $MgCl_2$, and 10 mM Tris-HCl at pH 7.8, and 5 μl of the mixture were applied to an SDS-polyacrylamide disk gel. Peak I is due to myosin heavy chains, peaks II, III, V, and VI are due to protein in the PK preparation, and peak IV is due to actin. The concentration of myosin was calculated from the total area of peaks of myosin light chains indicated by oblique lines.

previously measured by Hayashi and Tonomura (117). However, they used S-1 obtained by tryptic digestion of HMM, and measured the dependence in the absence of FA and at high ionic strength.

We measured the time course of ^{32}Pi-liberation after adding various amounts of [γ-^{32}P]ATP to acto-S-1 under the conditions used for separation of S-1. The size of the initial burst of Pi-liberation was obtained by extrepolating the time course of Pi-liberation to time 0. As shown in Fig. 37, the sizes of the initial bursts of Pi-liberation were 0.205, 0.35, and 0.5 mole/mole of S-1, respectively, when 0.25, 0.5, and 1 mole ATP were added per mole of S-1. The burst size remained at 0.5 mole/mole of S-1 with further increase in the ATP concentration above 1 mole/mole of S-1.

Dissociation of Acto-S-1 Induced by ATP and Separation of S-1 into two Fractions — ATP at various concentrations was added with 4 mg/ml PK and 12 mM PEP to a mixture of 3.4 mg/ml (20 μM) HMM-S-1 (papain digest of HMM) and 2 mg/ml FA in 50 mM KCl, 2 mM $MgCl_2$, and 10 mM Tris-HCl at pH 7.8 and 0°. The reaction mixture (5 ml) was centrifuged at 1.6×10^5xg and 0° for 40 - 60 min. The supernatant and the precipitate were each diluted to 5 ml with the buffer solution, and the concentration of S-1 was measured as mentioned above (*cf.* Fig. 34). The ATPase activity was measured 24 hr after the separation. During the 24 hr, all the PEP and ATP were converted into pyruvate and ADP. PK and pyruvate did not interfer with the

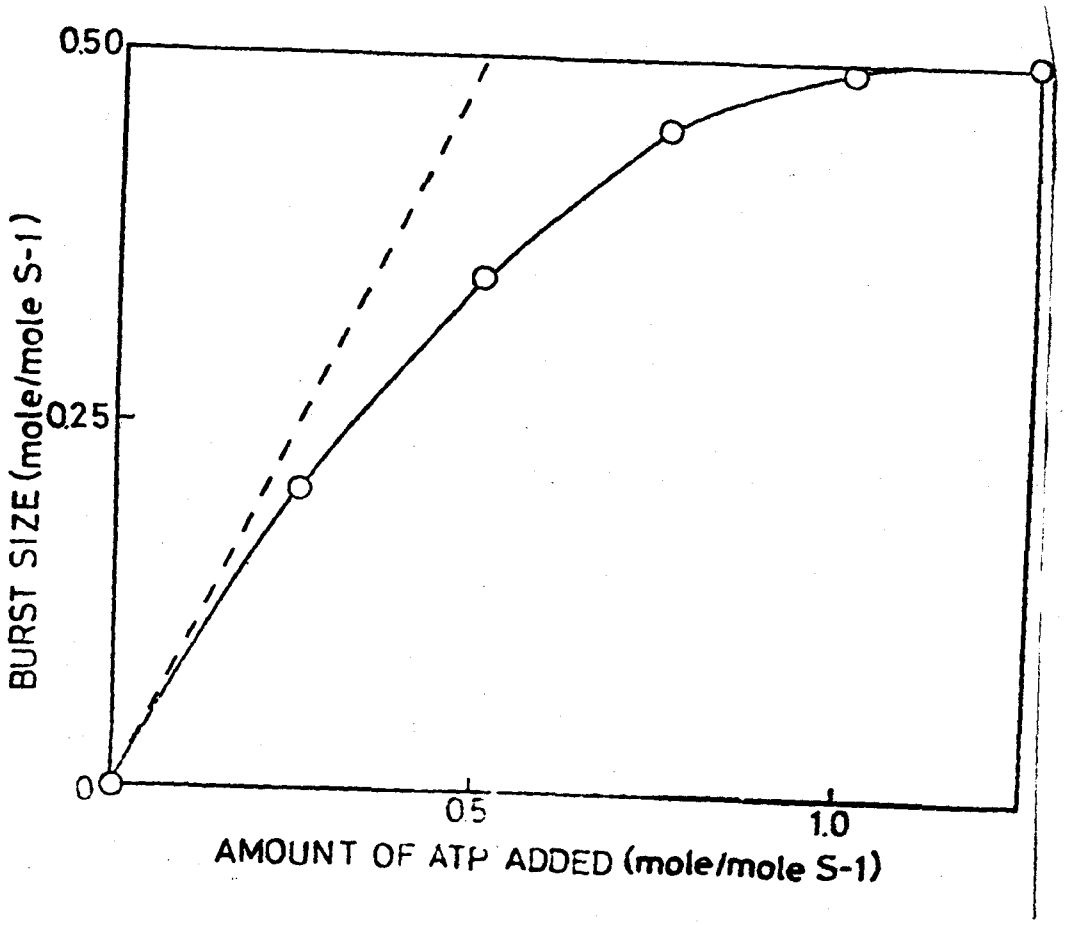

Fig. 37. Dependence on ATP concentration of the size of the initial burst of Pi-liberation of acto-S-1. The time course of ^{32}Pi-liberation was measured by adding various concentrations of [γ-^{32}P]ATP to 3.4 mg/ml (20 μM) HMM-S-1 (papain digest of HMM), 2 mg/ml FA, and 4 mg/ml PK is 50 mM KCl, 2 mM $MgCl_2$, and 10 mM Tris-HCl at pH 7.8 and 0°. The size of the initial burst of Pi-liberation was obtained by extrapolating the time course of ^{32}Pi-liberation in the steady state to time 0. The broken line indicates the dependence when the initial burst of Pi-liberation occurs by a stoichiometric reaction of 1 mole of ATP with 2 moles of S-1 in acto-S-1.

measurement of ATPase, since the equilibrium of the reaction, PEP + ADP $\rightleftharpoons$ pyruvate + ATP, is greatly to right side. The size of the initial burst of Pi-liberation was measured in 20 μM γ-^{32}P-ATP, 0.5 M KCl, 2 mM $MgCl_2$, and 50 mM Tris-HCl at pH 7.8 and 0°.

The results are summarized in Table 2. The amounts of S-1 in the supernatant at 0, 10, and 50 M ATP were 12, 53, and 89 %, and their recoveries were 102, 101, and 95 %, respectively, of the total S-1 before centrifugation. The recoveries of the initial burst of Pi-liberation were 94, 98, and 92 %. The sizes of the initial burst of the precipitate at zero ATP concentration and of the supernatant at 50 μM ATP were both 0.47 mole/mole S-1, and this value is equal to that before the centrifugal separation, as expected. On the other hand, when the ATP concentration was 10 μM, *i.e.* 0.5 mole/mole S-1, the amounts of S-1 in the supernatant and the precipitate were 53 and 48 % of the total S-1 before the separation, while the size of the initial burst of the supernatant and that of the precipitate were 0.68 and 0.27 mole/mole of S-1, respectively.

Figure 38 shows the time courses of Pi-liberation of the supernatant and the precipitate obtained by centrifugal separation at 0.5 mole ATP/mole of S-1, together with that for HMM-S-1 before the separation. The size of the initial burst before the separation was 0.48 mole/mole of S-1 for all the HMM-S-1 preparations used. After centrifugal separation, the size of the

Table 2. Separation of S-1 into two fractions by dissociation of acto-S-1 with ATP. HMM-S-1 was obtained by digestion of 17 mg/ml of HMM with 17 μg/ml of insoluble papain in 2 mM EDTA, 2 mM DTT, 50 mM KCl, and 50 mM Tris-HCl at pH 7.8 and 0° for 10 min. After adding various concentrations of ATP to 5 ml of mixture containing 3.4 mg/ml (20 μM) HMM-S-1 (papin digest of HMM), 2 mg/ml FA, 4 mg/ml PK, and 12 mM PEP in 50 mM KCl, 2 mM $MgCl_2$, and 10 mM Tris-HCl at pH 7.8 and 0°, the reaction mixture was centrifuged at 1.6 x 10^5 x g and 0° for 60 min. The amounts of S-1 in the supernatant and the precipitate were measured by SDS-gel electrophoresis, as described in the "RESULTS". 24 hr later, the size of the initial burst of Pi-liberation was measured in 20 μM [γ-^{32}P] ATP, 0.5 M KCl, 2 mM $MgCl_2$, and 50 mM Tris-HCl at pH 7.8 and 0°. The total amount of S-1 and its burst size before the centrifugal separation were 100 nmoles and 0.5 mole/mole S-1, respectively. Therefore, the recoveries of S-1 by the separation at 0, 10 and 50 M ATP were 102, 101, and 95 %, while the recoveries of the burst size were 94, 98 and 92 %, respectively.

ATP added (μM)	Fraction	S-1 (nmole)	Burst size (nmole)	Burst size (mole/mole S-1)
0	Sup	12	5	
	Ppt	90	42	0.47
	Total	102	47	0.46
10	Sup	53	36	0.68
	Ppt	48	13	0.27
	Total	101	49	0.49
50	Sup	89	41.5	0.47
	Ppt	6	4.5	
	Total	95	46	0.48

initial burst of the supernatant was 0.72 ± 0.037, while that of the precipitate was 0.275 ± 0.042 mole/mole S-1 (Fig. 39). These results are in good accordance with results showing that the size of the initial burst at 0.5 mole ATP/mole of S-1 was 0.35 mole/mole of S-1 (Fig. 37), and that at this ATP concentration 50 % of the S-1 was dissociated from F-actin (Table 2). On the other hand, the rate of the ATPase reaction in the steady state of the reaction mixture before the separation and in the supernatant and the precipitate after the separation were 0.172 ± 0.112, 0.194 ± 0.026, and 0.206 ± 0.04 min^{-1}, respectively (Fig. 39).

Table 3 summarizes the results obtained when the separation procedures were repeated. The second separation procedures were performed using the same amount of ATP as that of the size of the initial burst of the supernatant or the precipitate obtained by the first centrifugation. The size of the initial burst of the supernatant increased from 0.71 to 0.8 mole/mole of S-1 and that of the precipitate decreased from 0.32 to 0.25 mole/mole of S-1 on repeating the separation procedures. In the second separation the recoveries of S-1 in the supernatant and precipitate were 93 and 83 %, respectively of the S-1 obtained by the first separation. The recoveries of the size of the initial burst were equal to those of protein. The burst sizes in the supernatant and the precipitate obtained by repeating the separation were nearly equal to those calculated assuming

Fig. 38. Time courses of Pi-liberation in the ATPase reaction of two fractions of HMM-S-1 obtained by ultracentrifugation of acto-S-1 in the presence of 0.5 mole ATP per mole of S-1. HMM-S-1 was obtained by digestion of 17 mg/ml HMM-S-1 with 17 μg/ml of insoluble papain papain for 10 min under the same conditions as for Fig. 32. ATP (10 μM) was added with 4 mg/ml PK and 12 mM PEP to mixture containing 3.4 mg/ml (20 μM) HMM-S-1 and 2 mg/ml FA in 50 mM KCl, 2 mM $MgCl_2$, and 10 mM Tris-HCl at pH 7.8 and 0°. The final mixture was centrifuged at 1.6 x 10^5 x g and 0° for 40 - 60 min. 24 hr later, the ATPase activity was measured in 20 μM [γ-^{32}P]ATP, 0.5 M KCl, 2 mM $MgCl_2$, and 50 mM Tris-HCl at pH 7.8 and 0°. ○, ◐, ◑, ●, before separation; △, ◭, ◮, ▲, supernatant; □, ◧, ◨, ■, precipitate. The amount of Pi-liberation is expressed as mole/mole S-1. The results of four series of experiments (○, △, □; ◐, ◭, ◧; ◑, ◮, ◨; ●, ▲, ■) are shown. Open and solid arrows indicate the values of the size of the initial burst before and after separation of S-1, expected from the dependence of the burst size on ATP concentration given in Fig. 37.

Fig. 39. Relationship between the ATPase activity in the steady state and the size of the initial burst of Pi-liberation. The results given in Fig. 37 are replotted. ○, before separation; △, supernatant; □, precipitate. ⟷ indicates the standard means of errors.

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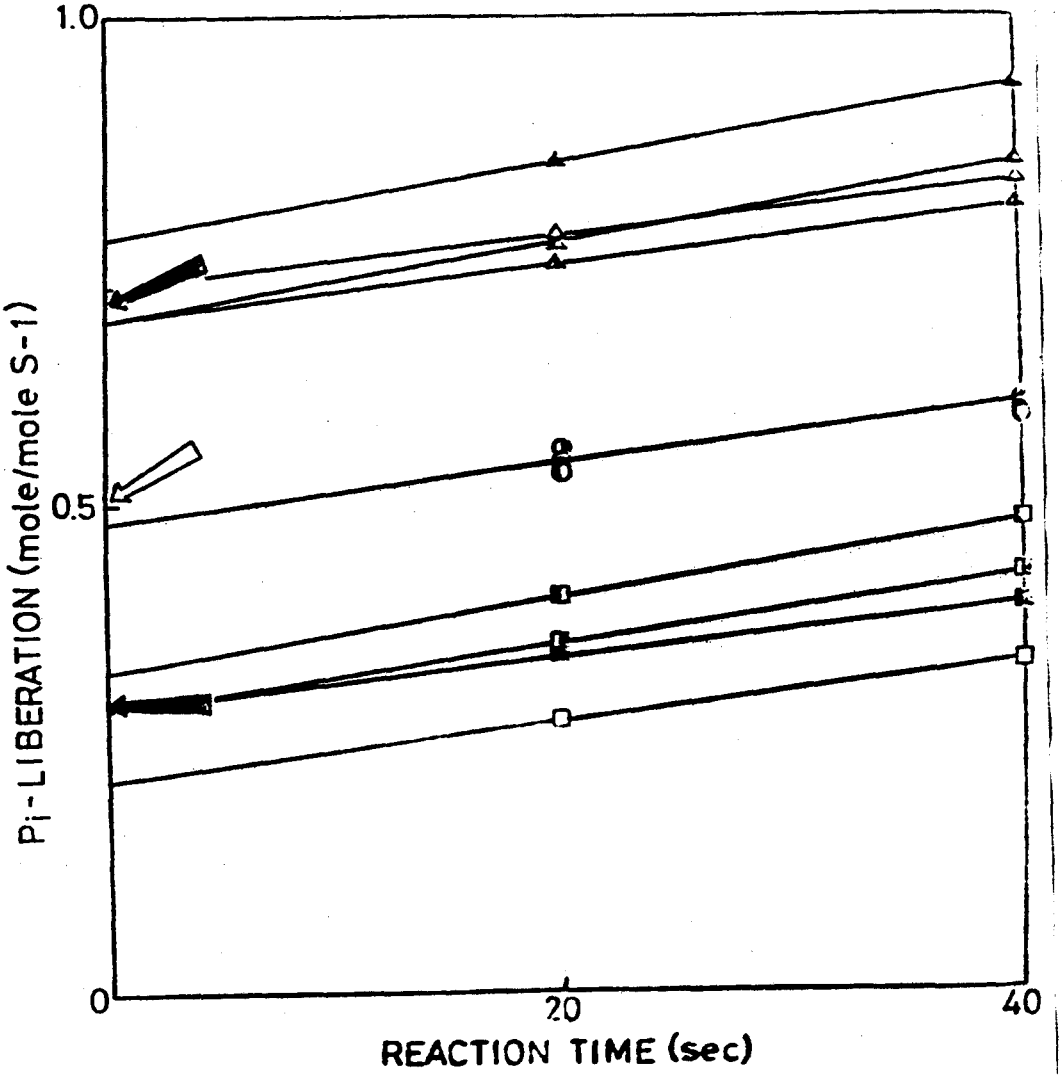
1.0
0.5
0
20
40
Pi - LIBERATION (mole/mole S-1)
REACTION TIME (sec)

39

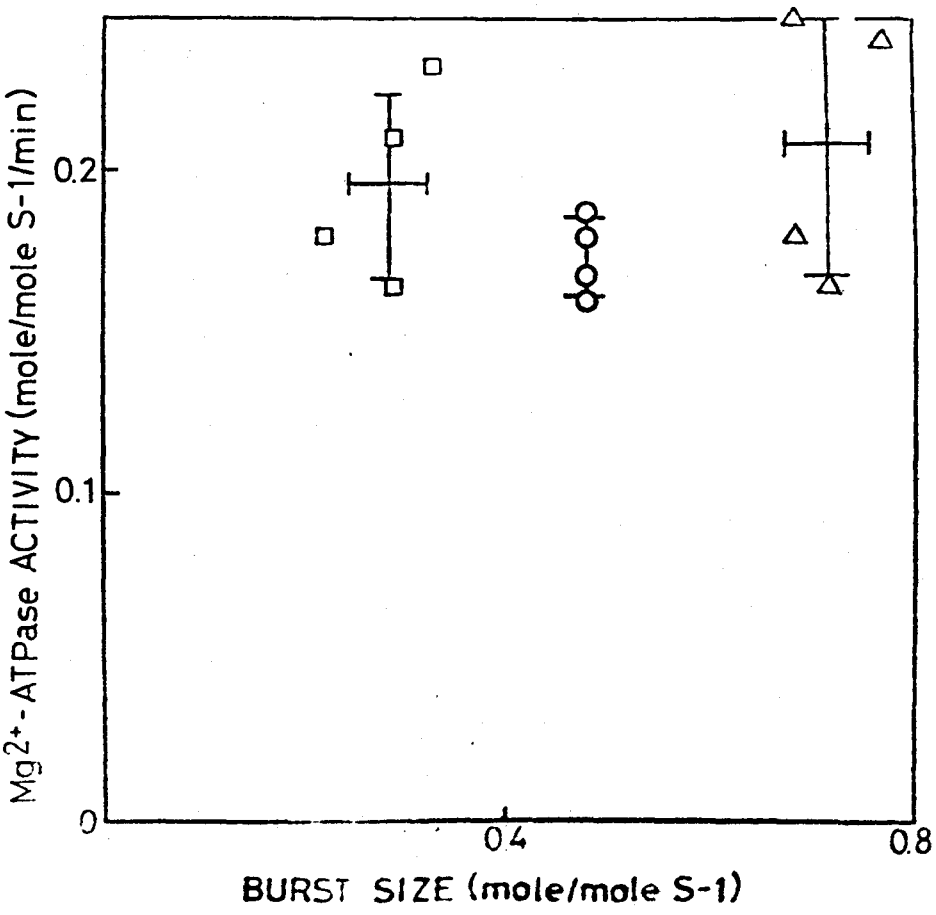
0.2
0.1
0
0.4
0.8
Mg2+ - ATPase ACTIVITY (mole/mole S-1/min)
BURST SIZE (mole/mole S-1)

Table 3. Separation of S-1 into two fractions by repeating the partial dissociation of acto-S-1 with 0.5 mole ATP per mole of S-1. (A): 5 ml of mixture containing 3.4 mg/ml HMM-S-1 and 2 mg/ml FA were separated into two fractions by ultracentrifugation in the presence of 10 μM ATP, as described for Table 2. The total amount of S-1 and its burst size before separation were 105 nmoles and 0.5 mole/mole S-1, respectively. Therefore, the recoveries of S-1 and of burst isze were 84 and 87 %, respectively. (B): To the supernatant obtained in (A), final concentrations of 2 mg/ml FA, 4 mg/ml PK, and 12 mM PEP were added and the total volume of the mixture was adjusted to 6.25 ml with buffer solution. The mixture contained 43 mole S-1, and its total burst size was 30.5 nmoles. After adding 4.9 μM ATP (1 mole/mole of burst size), the reaction mixture was separated by ultracentrifugation, as described for Table 2. The recoveries of S-1 and of burst size were both 93 %. (C): To the precipitate obtained in (A), final concentrations of 2 mg/ml FA, 4 mg/ml PK, and 12 mM PEP were added, and the volume of the mixture was adjusted to 6.25 ml with buffer solution. The mixture contained 45 nmoles S-1, and its total burst size was 14.5 nmoles. After adding 2.3 μM ATP (1 mole/mole of burst size), the mixture was separated by ultracentrifugation, as described for Table. 2. The recovery of S-1 and that of the burst size were 82 and 83 %, respectively.

(A)

ATP added (μM)	Fraction	S-1 (nmole)	Burst size (nmole)	Burst size (mole/mole S-1)
10	Sup	43	30.5	0.71
	Ppt	45	14.5	0.32
	Total	88	45	0.51

(B)

ATP added (μM)	Fraction	S-1 (nmole)	Burst size (nmole)	Burst size (mole/mole S-1)
4.9	Sup	30	24	0.80
	Ppt	10	4.5	0.45
	Total	40	28.5	0.71

(C)

ATP added (μM)	Fraction	S-1 (nmole)	Burst size (nmole)	Burst size (mole/mole S-1)
2.3	Sup	19	7.5	0.39
	Ppt	18	4.5	0.25
	Total	37	12	0.30

that the fraction of S-1 in the supernatant which forms M_P^{ADP} increases from 50 to 70 % in one separation, *i.e.* 0.82 and 0.18 mole/mole of S-1, respectively, for the supernatant and the precipitate of the second separation.

Dissociation of Actomyosin with ATP — Various concentrations of ATP and an ATP-regenerating system (4 mg/ml PK and 12 mM PEP) were added to a mixture of 4.8 mg/ml of myosin and 2 mg/ml FA in 0.5 M KCl, 2 mM $MgCl_2$, and 50 mM Tris-HCl at pH 7.8 and 0°. Then the mixture was centrifuged at 1.6×10^4 xg and 0° for 60 min, and the supernatant and the precipitate were diluted with the buffer to 5 ml. The amounts of myosin in the supernatant and the precipitate were measured by SDS-gel electrophoresis (*cf.* Fig. 35). Twenty four hr after the separation, the ATPase activity was measured in 20 M [γ-^{32}P] ATP, 0.5 M KCl, 2 mM $MgCl_2$, and 50 mM Tris-HCl at pH 7.8 and 0°. The results are summarized in Table 4. Even when no ATP was added, 25 % of the myosin was present in the supernatant. This was attributed to a low degree of polymerization of actin at high ionic strength, since the SDS-gel electrophoretogram of the supernatant revealed the presence of actin at a rather high concentration. When the amount of ATP added was increased to 0.5, 1 and 2 moles/mole myosin, the concentration of myosin in the supernatant increased to 70, 85 and 92 % of the total amount of myosin before the centrifugal separation. The recoveries of the size of initial burst and of myosin were higher

Table 4. Dissociation of actomyosin by ATP and the burst size of myosin dissociated from F-actin and bound to F-actin. The reaction mixture contained 4.8 mg/ml myosin (20 μM mysoin head), 2 mg/ml FA, 4 mg/ml PK, and 3 mM PEP in 0.5 M KCl, 2 mM $MgCl_2$, and 50 mM Tris-HCl at pH 7.8 and 0° in a volume of 5 ml. After adding various concentrations of ATP, the mixture was centrifuged at 1.6 x 10^5 x g and 0° for 60 min. The amounts of myosin in the supernatant and the precipitate were measured as described in the "RESULTS". The size of the initial burst of Pi-liberation was measured, as described for Table 2. The total amount of myosin head and its burst size before centrifugation were 100 nmoles and 0.46 mole/mole head, respectively.

ATP added (μM)	Fraction	Myosin head (nmole)	Burst size (nmole)	Burst size (mole/mole head)
0	Sup	25	10	0.42
	Ppt	70	30	0.43
	Total	95	40	0.42
5	Sup	70	32.5	0.46
	Ppt	30	15	0.50
	Total	100	47.5	0.48
10	Sup	85	40	0.47
	Ppt	3	(0)	-
	Total	88	40	0.45
20	Sup	92	42.5	0.46
	Ppt	2	(0)	-
	Total	94	42.5	0.45

than 86 %. In sharp contrast with the case of acto-S-1, the sizes of the initial burst of both the supernatant and the precipitate separated at all ATP concentrations used were always in the range of 0.40 - 0.50 mole/mole of myosin head.

In Fig. 40 the time courses of Pi-liberation of the supernatant and the precipitate are compared with that of the mixture before centrifugal separation. The supernatant and the precipitate were obtained by centrifugation of actomyosin in the presence of 0.5 mole ATP/mole of myosin head. The proportions of myosin in the supernatant and the precipitate were 70 and 30 %, respectively (Table 4), and their sizes of their initial bursts were 0.46 and 0.50 mole/mole of myosin head, which were almost equal to the original value before centrifugal separation, *i. e.* 0.46 mole/mole of myosin head. The rates of the ATPase reaction in the steady state of the supernatant and the precipitate were 0.18 and 0.20 min^{-1}, respectively, which were also almost equal to the original value, *i. e.* 0.18 min^{-1}.

DISCUSSION

S-1 was separated into two fractions by centrifugation of acto-S-1 in the presence of various concentrations of ATP with an ATP-regenerating system. One fraction was precipitated with F-actin and the other was dissociated from F-actin. When 0.5 mole ATP was added per mole of S-1 in acto-S-1, the S-1

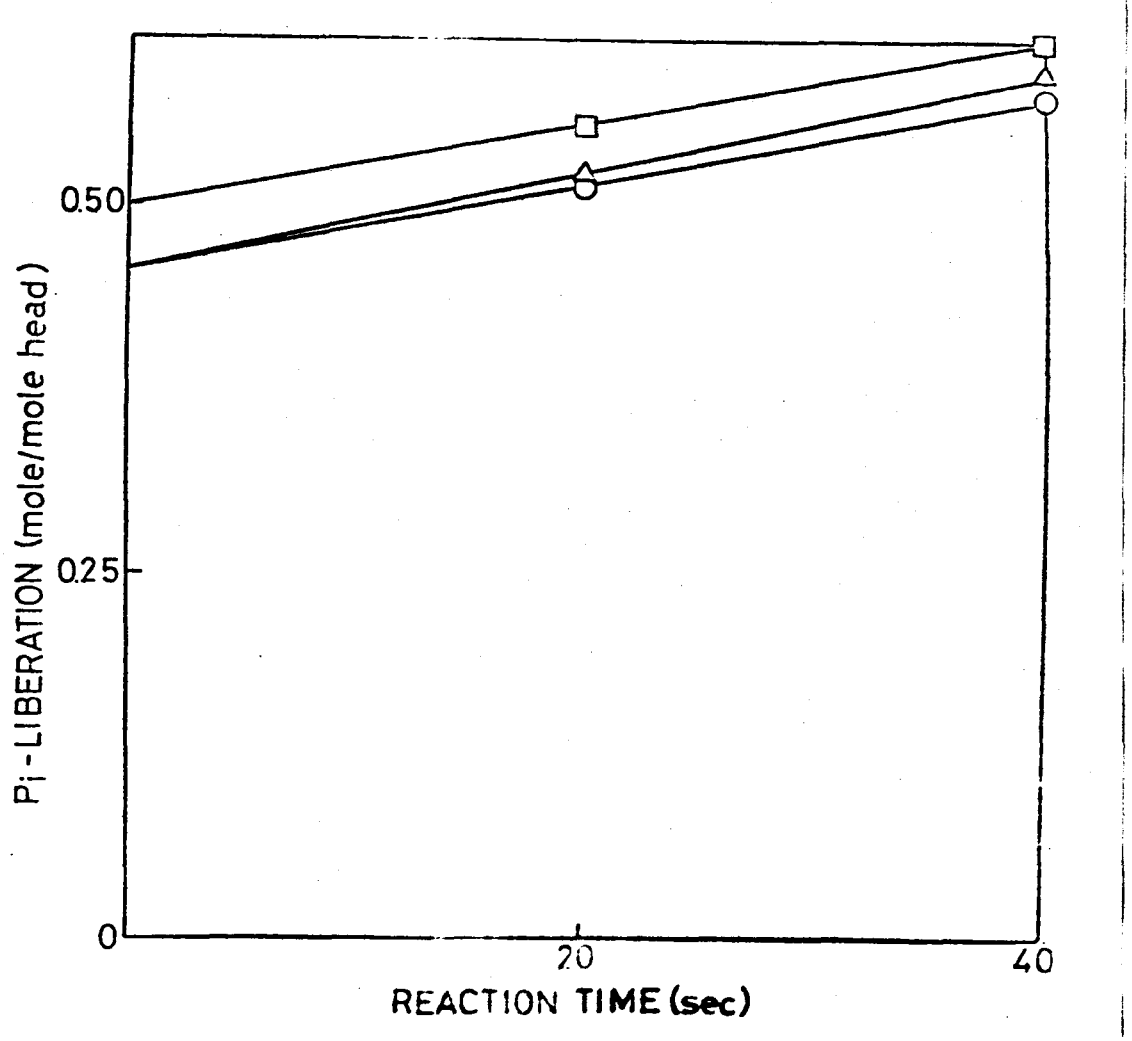

Fig. 40. Time courses of Pi-liberation in the ATPase reaction of the two fractions of myosin obtained by ultracentrifugation of actomyosin in the presence of 0.25 mole of ATP per mole of myosin head. ATP (5μM) was added with 4 mg/ml PK and 3 mM PEP to reaction mixture containing 4.8 mg/ml myosin and 2 mg/ml FA in 0.5 M KCl, 2 mM $MgCl_2$, and 50 mM Tris-HCl at pH 7.8 and 0°. The mixture was centrifuged at 1.6 x 10^5 x g and 0° for 60 min. The ATPase activity was measured under the same conditions as for Fig. 37. The amount of Pi-liberation is expressed as mole/mole of myosin head. ○, before separation; △, supernatant; □, precipitate.

became equally divided between the supernatant and the precipitate (*cf.* Table 2), and the sizes of the initial burst of Pi-liberation (formation of M_P^{ADP}) of the supernatant and the precipitate were about 0.7 and 0.3 mole/mole S-1, respectively (Table 2 & Fig. 37). An repeating the separation procedures, the size of the initial burst of the supernatant increased to 0.8 mole/mole S-1 and that of the precipitate decreased to 0.25 mole/mole S-1 (Table 3). On the other hand, when acto-myosin was centrifuged in the presence of ATP, the sizes of the initial burst of the supernatant and the precipitate were always about 0.5 mole/mole of head (*cf.* Table 4).

Several problems had to be solved before the concluding that S-1 consisted of two equimolar fractions, one of which formed M_P^{ADP} on adding ATP and the other of which did not. The first problem was about estimation of the amount of S-1 in the reaction mixture. Since the mixture contained high concentrations of FA and PK, the amount of S-1 was measured from the areas of the peaks of S-1 on an SDS-gel electrophoretogram. The amount of S-1 estimated from the areas of several specified peaks was equal to that estimated from the total area of the peaks shown in Fig. 34 . Furthermore, the recoveries of S-1 throughout the separation procedures were equal to the recoveries of the size of the initial burst, and were higher than 90 %.

The second problem was about preparation of S-1. HMM-S-1 was obtained by digestion of HMM with insoluble papain for 10 min.

The size of the initial burst of Pi-liberation and the rate of the ATPase reaction in the steady state were only slightly affected by digestion with insoluble papain under the conditions used (*cf.* Figs. 32 & 33). The time used for digestion of HMM in this study was sufficient to digest myosin to HMM and/or S-1, as described in the "RESULTS." It was concluded that almost all the HMM was digested to S-1, since digestion of myosin with papain to S-1 and long rods occurs more rapidly than digestion to HMM and L-meromyosin (33). Furthermore, the increase in light-scattering intensity of the HMM-S-1 obtained on its binding with FA was much less than that of HMM and was similar to that of purified S-1. In this study the papain digest of HMM, HMM-S-1, was used without further purification, to exclude problems of the recovery and denaturation of S-1 during purification procedures. Therefore, the problems remained of contamination of S-1 with S-2, FA and PK. It was found that contamination with these proteins did not affect measurement of the amount of S-1 by gel electrophoresis, as mentioned above and also in the "RESULTS." Contamination of HMM-S-1 with S-2 or undigested HMM did not invalidate the conclusion, although it reduced the extent of separation of S-1, since S-2 was present in the supernatant and HMM was not separated into fractions by the method used in this work (*cf.* Fig. 40). If 20 % of the HMM remained undigested and half of it was precipitated by centrifugation, the amount of formation of M_P^{ADP} in the supernatant should

decrease from 0.7 to 0.66 mole/mole of myosin head.

The third problem was on ultracentrifugal separation. In this work S-1 was separated by centrifugation in the presence of 0.5 mole ATP per mole of S-1 in acto-S-1 into two equimolar fractions with burst sizes of 0.7 and 0.3 mole/mole S-1. The main reason for this incomplete separation was that the size of the initial burst of acto-S-1 on adding 0.5 mole of ATP/mole of S-1 was 0.35 and not 0.5 mole/mole of S-1 (*cf.* Fig. 37), as mentioned in the "RESULTS." Furthermore, the burst sizes of the supernatant and the precipitate obtained by repeating the separation procedures were in good agreement with those calculated assuming that the fraction of S-1 in the supernatant which forms M_P^{ADP} increases from 50 to 70 % in one separation, as mentioned in the "RESULTS" (*cf.* Table 3). However, it is possible that there was insufficient regeneration of ATP in the precipitate because of the concentration of S-1, and that this reduced the extent of the separation. Thus, we can conclude that S-1 contains two equimolar fraction, only one of which forms M_P^{ADP} on adding ATP.

As mentioned in the "INTRODUCTION", several authors (105, 106) reported that myosin has at least two isozymes. However, the size of the initial burst was 1 mole/mole of myosin and the rate of the initial burst was of the same order of magnitude for myosins of all types of muscles examined. This suggests that isozymes of myosin have similar properties, at least with

regard to formation of M_P^{ADP}. The possibility that the myosin preparation was a mixture of myosin which did not form M_P^{ADP} and that which formed one mole of M_P^{ADP} per mole of head was exculded by the results shown in Table 4 and Fig. 40. At high ionic strength and in the presence of a high concentration of Mg^{2+} ions, the extent of dissociation of actomyosin induced by ATP increased linearly with increase in the ATP concentration. The extent of dissociation was equal to that of formation of M_P^{ADP}, and actomyosin dissociated completely at an ATP concentration of 1 mole/mole of myosin (109). As seen in Table 4, the sizes of the initial bursts of Pi-liberation of myosin bound to F-actin and of that dissociated from F-actin were both 1 mole/mole of myosin (0.5 mole/mole head) at all ATP concentrations used for dissocation of actomyosin. Thus, we can exclude the possibility that myosin consists of two isozymes with and without the abilities to form one mole of M_P^{ADP} per mole of head.

Furthermore, the results that the ATPase activities in the steady state of the two fractions of S-1 obtained by centrifugal separation were almost equal and were independent of the size of the initial burst of Pi-liberation (Fig. 39) could be explained by our mechanism that the active sites for two routes of myosin·ATPase shown in "INTRODUCTION" are located on different heads of myosin and the rate constant of both

routes is of the same order of magnitude[4]. They are inconsistent with the mechanism of Taylor *et al.* (56, 67) that M_P^{ADP} is the obligatory intermediate for the myosin-ATPase reaction in the steady state, and also with the mechanism that the active sites are both located in the same head of the myosin molecule, since according to these mechanisms the rate of ATPase in the steady state must be proportional to the size of the initial burst. Therefore, it is concluded that the two heads of the myosin molecule are dissimilar, and that M_P^{ADP} is formed on the one head (B) and M_{ATP} is formed on the other head (A) of myosin. It should be noted that this conclusion is in good accrodance with our previous studies (40 - 43) on the structures and functions of myosin and S-1, as described in the "INTRODUCTION".

We showed previously that M_P^{ADP} is the reaction intermediate of actomyosin ATPase, and that formation and decomposition of M_P^{ADP} is coupled with development of tension (40, 43, 105), while the formation of M_{ATP} participates in the regulation of

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4. If the ratio of the rate of ATPase *via* M_P^{ADP} to that *via* M_{ATP} is 1 : 2, the ratio of the rates of ATPase in the steady state per mole of S-1 in the supernatant, the precipitate, and HMM-S-1 before separation is 0.87 : 1.13 : 1.

muscle contraction by trace amounts of Ca^{2+} ions (105). Thus, the results presented here correlate our reaction mechanism of myosin ATPase with the two headed structure of the myosin molecule.

5. The Amounts of Adenosine Di- and Triphosphates Bound to H-Meromyosin and the Adenosinetriphosphatase Activity of the H-Meromyosin-F-Actin-Relaxing Protein System in the Presence and Absence of Calcium Ions: The Physiological Functions of the Two Routes of Myosin Adenosinetriphosphatase in Muscle Contraction

The rates of the ATPase [EC 3.6.1.3] reaction of the H-meromyosin-F-actin-relaxing protein system were measured in 2 mM $MgCl_2$, 50 mM KCl and 10 mM Tris-HCl at pH 7.8 and 20° in the presence and absence of 0.05 - 0.1 mM Ca^{2+} ions. The concentrations of H-meromyosin (HMM) and the F-actin-relaxing protein (F-A-RP) complex were 3.4 and 3 mg/ml, respectively, and the ATPase reaction was coupled with 4 mg/ml of pyruvate kinase [EC 2.7.1.40] and 1 or 20 mM phosphoenol pyruvate to regenerate ATP. The amount of ADP bound to HMM during the ATPase reaction was determined by measuring the amount of ADP remaining in the reaction mixture. The amount of ATP bound to HMM was determined by subtracting the amount of bound ADP from the total amount of nucleotides bound to HMM, which was measured by a rapid flow-dialysis method. The following results were obtained.

1. The ATPase activity of the HMM-F-A-RP system increased linearly with increase in the amount of ATP added, and was independent of the presence of 0.05 mM Ca^{2+}, when the amount

of ATP added was less than 1 mole/mole of HMM. In the presence of 0.05 mM Ca^{2+}, the ATPase activity reached a maximal level when 1.2 - 1.5 mole of ATP was added per mole of HMM, and maintained this level even at 3 moles of added ATP/mole of HMM. In the presence of 3 mM EGTA, the ATPase activity decreased with increase in the amount of ATP added, with from 1.5 mole to 3 moles of ATP/mole of HMM, and reached the level of the HMM ATPase reaction at 3 moles of added ATP/mole of HMM. Similar results were observed, when the concentration of HMM was maintained at 3.4 mg/ml and the concentration of the F-A-RP complex was decreased from 3 to 1 or 0.5 mg/ml.

2. When the amount of ATP added was less than 1.2 mole/mole of HMM, almost all the added nucleotide was bound to HMM as ADP. In the presence of 0.1 mM Ca^{2+}, the amount of ADP bound to HMM remained constant in the ATP concentration range of 1.5 to 3 moles/mole of HMM, while in the presence of 3 mM EGTA the amount of ADP bound to HMM decreased with increase in the ATP concentration above 1.5 mole/mole of HMM. At 3 moles of added ATP/mole of HMM, 0.5 moles of ADP were bound per mole of HMM, this being equal to that observed with the HMM-ATP system.

3. The binding of ATP to HMM was only observed when the amount of ATP added was more than 1 mole/mole of HMM. In the presence of EGTA, the amount of bound ATP increased with increase in the amount of ATP added, and was about 1.2 mole/mole of HMM at 3 moles of added ATP/mole of HMM. This value was equal to that

observed with the HMM-ATP system. The amount of bound ATP was proportional to the extent of inhibition of acto-HMM ATPase by removal of Ca^{2+} ions. The binding of ATP to HMM was markedly inhibited by the addition of 0.1 mM $CaCl_2$.

It is concluded from these results that the reactive myosin-phosphate-ADP complex, M_P^{ADP}, is the reaction intermediate of actomyosin ATPase and the ATPase does not require binding of ATP to the complex, and that formation of the myosin-ATP complex, M_{ATP}, is required for regulation of actomyosin ATPase in the presence of RP by trace amounts of Ca^{2+} ions.

INTRODUCTION

Previously we (*cf.* Sec. 2 - 4 and Ref. 39, 43, 105) showed that myosin has two non-identical active sites, one for formation of the reactive myosin-phosphate-ADP complex, M_P^{ADP}, and the other for formation of the myosin-ATP complex, M_{ATP}. We proposed the following scheme for the myosin ATPase[EC 3.6.1. 3] reaction:

$$M + ATP \rightleftharpoons M_1ATP \rightleftharpoons M_2ATP \rightleftharpoons M_P^{ADP} \rightarrow {}^{\circ}M + ADP + Pi \rightarrow M + ADP + Pi \quad (1)$$

$$M({}^{\circ}M, M_P^{ADP}, \cdots) + ATP \rightleftharpoons \underset{ATP}{M} \rightarrow M + ADP + Pi \quad (2).$$

We (39, 43, 93, 118) showed that M_P^{ADP} is the intermediate of the actomyosin ATPase reaction and F-actin greatly accelerates the regeneration of myosin (M) from M_P^{ADP}. However, previous experiments (93, 118) did not exclude the possibility that the intermediate of actomyosin ATPase, M_P^{ADP}, might contain bound ATP or that its decomposition might be accelerated by ATP. Therefore, we measured the relationship between the ATPase activity and the amounts of ADP and ATP bound to HMM during the ATPase reaction, when the HMM-F-A-RP-ATP system was coupled with the pyruvate kinase system. The results obtained showed clearly that the ATPase activity of acto-HMM is proportional to the amount of M_P^{ADP} and does not require binding of ATP to HMM.

The second aim of this work was to clarify the physiological function of the reaction of myosin ATPase via M_{ATP}, *i.e.* route (2), in muscle contraction. For this propose, we measured the relationship between the amount of ATP bound to HMM and the extent of inhibition of the ATPase activity of the HMM-F-A-RP system by removal of trace amounts of Ca^{2+} ions. The results indicated that formation of the myosin-ATP complex, M_{ATP}, is required for control of actomyosin ATPase in the presence of RP (the troponin-tropomyosin complex) by trace amounts of Ca^{2+} ions (15, 119, 120).

EXPERIMENTAL

H-Meromyosin (HMM, MW = 3.4×10^5) was prepared from rabbit skeletal white muscle by the method of A.G. Szent-Györgyi (108). Purified G-actin was prepared as described by Spudich and Watt (114), and the G-actin-relaxing protein (RP) complex was prepared as actin extracted at room temperature from acetone powder of rabbit skeletal white muscle as described by Mommaerts (73). Free ATP was removed from the G-actin solution by treatment with Dowex 1-4. G-Actin (3.75 or 3.6 mg/ml) was added to reaction mixture containing 4.25 or 4.08 mg/ml HMM and 5 or 4.8 mg/ml pyruvate kinase at 0°, and polymerized to F-actin by incubating the mixture at 20° for 5 min. Pyruvate kinase was prepared from rabbit skeletal muscle as described by Tiez and

Ochoa (94). The activities of pyruvate kinase preparations were 90 - 160 moles/min/mg protein in 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, 1 mM ADP, and 1 mM phosphoenol pyruvate (PEP) at pH 7.8 and 20°. The activity was not inhibited by 0.05 - 0.1 mM $CaCl_2$. The K_m value for ADP was about 0.2 mM. No adenylate kinase activity [EC 2.7.4.3] was detected in the HMM and pyruvate kinase preparations (103). Protein concentration was determined by the biuret reaction, calibrated by nitrogen determination. ^3H-Labelled ATP was purchased from the Radiochemical Centre, Ltd., Amersham, England, and purified by the method of Cohn and Carter (76). ATP and PEP were purchased from Sigma Chemical Co.

The reaction mixture contained 3.4 mg/ml HMM, 3 mg/ml F-A-RP, 2 mM $MgCl_2$, 50 mM KCl and 10 mM Tris-HCl at pH 7.8 and 20°, unless otherwise stated. The ATPase reaction was coupled with 4 mg/ml pyruvate kinase and 1 mM PEP to regenerate ATP. The reaction was started by adding 0.5 ml of ATP solution to 2 ml of the protein solution, and the rate of the ATPase reaction was determined from the rate of liberation of pyruvate, as described previously (97).

The amount of ADP bound to the protein during the ATPase reaction was determined as the amount of ADP remaining in the reaction mixture, when the ATPase reaction was coupled with 4 mg/ml of pyruvate kinase and 1 mM PEP (103). The reaction was started by adding 50 μl of solution containing ^3H-labelled

ATP and PEP to 250 μl of the protein solution, and was stopped by adding 100 μl of 20 TCA containing 20 mM ATP, ADP and AMP as carriers. ^{3}H-ADP was separated by polyethyleneimido cellulose thin layer chromatography (95), and measured as described previously (103).

The amount of total nucleotides bound to the protein was measured using a rapid flow-dialysis method, as described previously (96). The flow-rate of the buffer through the lower chamber was 12 ml/min, and samples of 10 ml were taken at 1 min intervals for 5 min. In flow-dialysis experiments, 20 mM PEP, instead of 1 mM PEP, was used to maintain the concentration of free ATP.

RESULTS

The ATPase Activity of the HMM-F-A-RP System in the Presence and Absence of Ca^{2+} — The ATPase activity of the HMM-F-A-RP system was measured in 2 mM $MgCl_2$, 50 mM KCl and 10 mM Tris-HCl at pH 7.8 and 20°. The concentration of HMM was 3.4 mg/ml, *i.e.* 10 μM, and F-actin extracted at room temperature (3 mg/ml) was used as the F-A-RP complex. At low concentrations of HMM, the ATPase activity in the presence of 0.05 mM Ca^{2+} showed a maximal value at about 2 μM ATP, and the activity in 3 mM EGTA was inhibited almost completely by 3 - 5 μM ATP. These results were essentially the same as those of

Sekiya and Tonomura (121). Thus, the concentration of HMM used (10 μM) was higher than the concentrations of ATP required to saturate the ATPase activity in the presence of Ca^{2+} ions and to inhibit the activity in the absence of Ca^{2+} ions.

Figure 41 shows the dependences of the ATPase activity of the HMM-F-A-RP system on the amount of ATP added. In the presence of 0.05 mM Ca^{2+}, the ATPase activity increased linearly with increase in the amount of ATP added, and reached a maximal level at 1.2 - 1.5 mole of added ATP/mole of HMM. The activity remained constant in the ATP concentration range of 1.5 to 3 moels/mole of HMM. These results were unaffected by decreasing the concentration of pyruvate kinase from 4 to 2 or 1 mg/ml. In the presence of 3 mM EGTA, the ATPase activities at ATP concentrations below 1 - 1.2 mole/mole of HMM were equal to those in the presence of 0.05 mM Ca^{2+} ions. However, at ATP concentrations above 1.5 mole/mole of HMM, the activity decreased with increase in the amount of ATP added, and was reduced to nearly the level of HMM ATPase when 3 moles of ATP were added per mole of HMM.

Figure 42 shows the dependences of the ATPase activity on the amount of ATP added at various concentrations of the F-A-RP complex. The activity increased with increase in the concentration of the F-A-RP complex in the presence of either 0.05 mM Ca^{2+} or 3 mM EGTA. It should be noted that both the amount of added ATP required for the maximal activity of ATPase in

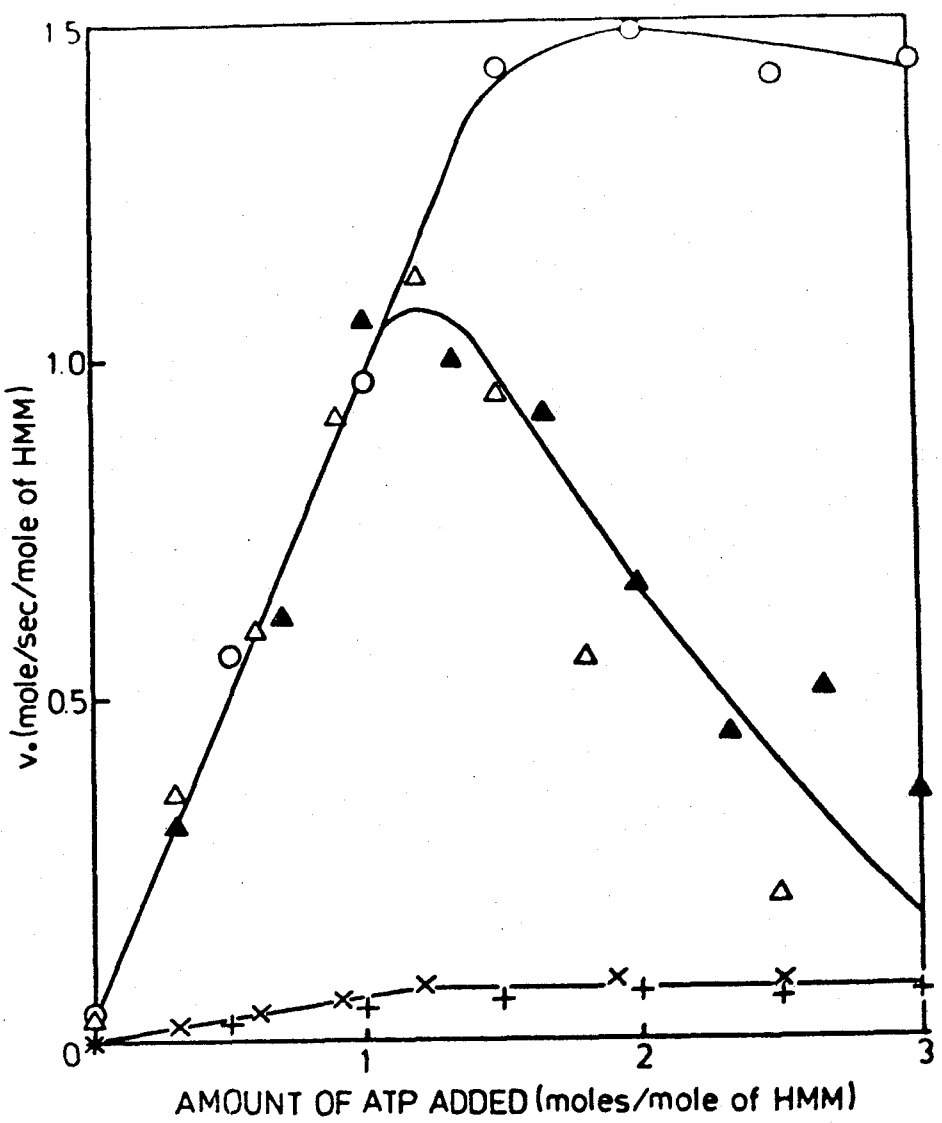


Fig. 41. Dependences of the ATPase activity of the HMM-F-actin-RP system on the amount of ATP added in the presence and absence of Ca^{2+} ions. 3.4 mg/ml HMM, 3 mg/ml F-A-RP, 4 mg/ml pyruvate kinase, 1 mM PEP, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 7.8, 20°C. ○, 0.05 mM $CaCl_2$; △, ▲, 3 mM EGTA. ×, +, ATPase activity of HMM alone. Different symbols are for different preparations of HMM.

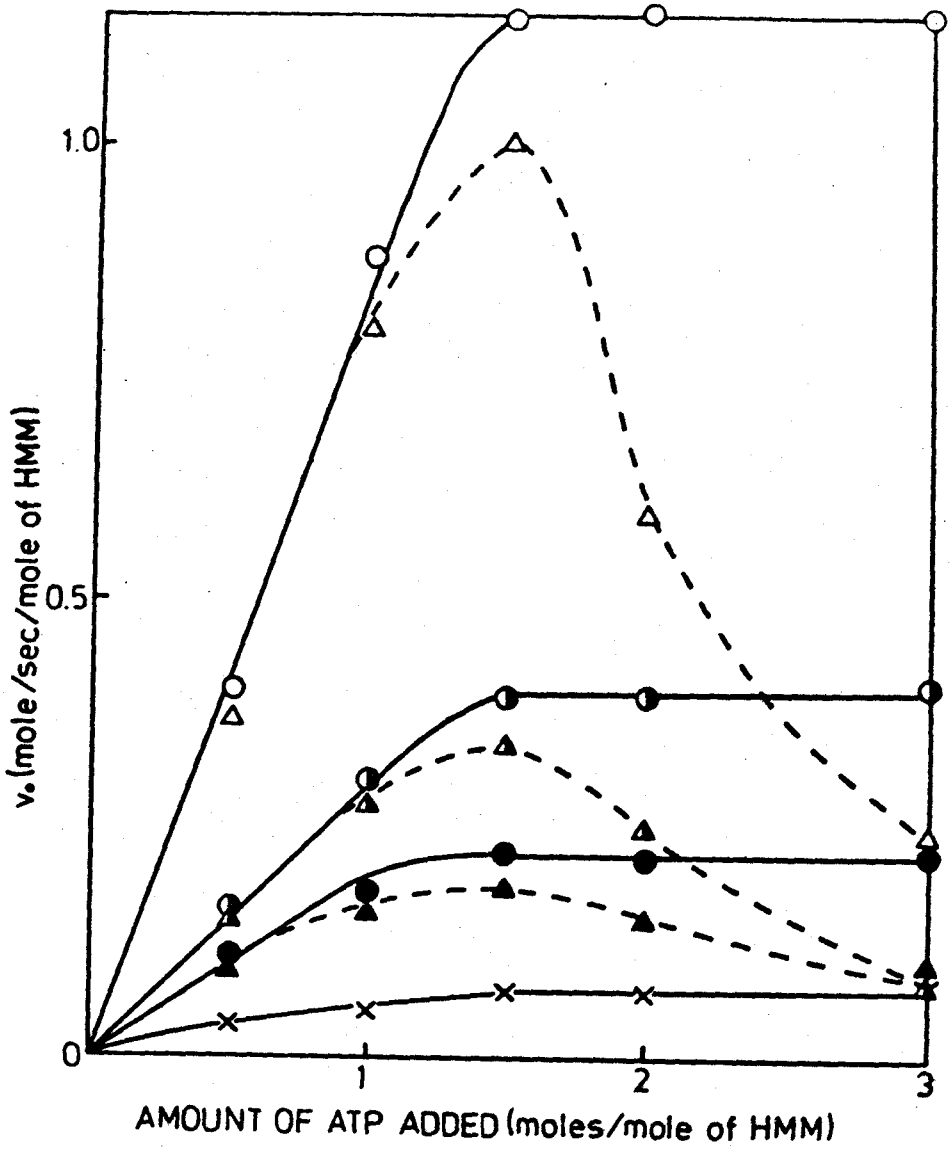


Fig. 42. Dependences of the ATPase activity of the HMM-F-actin-RP system on the amount of ATP added at various concentrations of the F-A-RP complex. Experimental conditions were as for Fig. 41, except that the concentrations of F-A-RP were 0 (X), 0.5 (●, ▲), 1 (◑, ◭) and 3 mg/ml (○, △). ○, ◑, ●, 0.05 mM $CaCl_2$; △, ◭, ▲, 3 mM EGTA.

0.05 mM Ca^{2+} and the amount required for inhibition of ATPase to the level of HMM ATPase in 3 mM EGTA were unaffected by reducing the concentration of the F-A-RP complex from 3 to 1 or 0.5 mg/ml.

Relation between the Amount of ADP Bound to HMM and the ATPase Activity of the Actomyosin Type — Figure 43 shows the dependences of the amount of bound ADP during the ATPase reaction on the amount of ATP added in the presence and absence of a minute amount of Ca^{2+} ions. In the presence of either 0.1 mM $CaCl_2$ or 3 mM EGTA, almost all the ATP added was converted to ADP bound to the protein, when the amount of ATP added was less than 1 mole/mole of HMM. The amount of bound ADP reached a maximal value of about 1.2 mole/mole of HMM, when about 1.2 moel of ATP was added per mole of HMM. In the presence of 0.1 mM Ca^{2+}, the amount of bound ADP remained constant with further increase in the amount of ATP added to 3 moles of ATP/mole of HMM. In the presence of 3 mM EGTA, the amount of bound ADP decreased from 1.2 to 0.5 mole/mole of HMM with increase in the amount of ATP added from 1.2 to 3 moles of ATP/mole of HMM. The amount of bound ADP observed at 3 moles of added ATP/mole of HMM was equal to that when the same amount of ATP was added to HMM in the absence of the F-A-RP complex, as described later.

The amount of ATP remaining in the reaction mixture was less than 0.1 mole/mole of HMM at 1 mole of ATP added/mole of

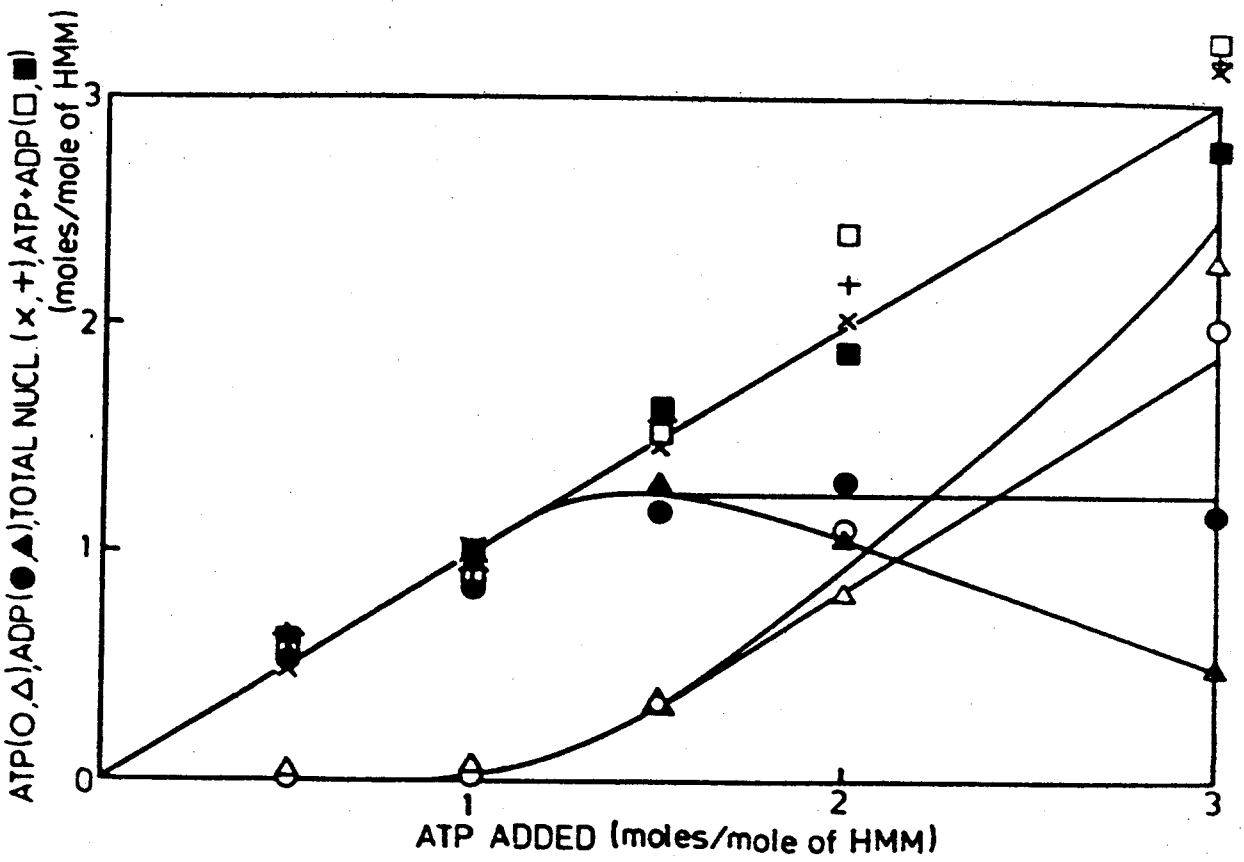


Fig. 43. Dependences of the amounts of ADP and ATP on the amount of ATP added during the ATPase reaction of the HMM-F-actin-RP system in the presence and absence of Ca^{2+} ions. Experimental conditions were as for Fig. 41. ○, ●, ×, □, 0.1 mM $CaCl_2$; △, ▲, +, ■, 3 mM EGTA. ○, △, amount of ATP; □, ■, amount of ADP; ×, +, amount of total nucleotides; □, ■, ATP + ADP.

HMM, and it increased with increase in the amount of ATP added above 1.2 mole/mole of HMM. The amount of ATP remaining in the reaction mixture was more in the presence of EGTA than in the presence Ca^{2+} ions. The sum of the amounts of ADP and ATP was equal to the amount of total nucleotide added.

The amounts of bound ADP in the presence of 3 mM EGTA were also measured at various concentrations of the F-A-RP complex (Table 5). In the absence of the F-A-RP complex, the amount of bound ADP was 0.45 - 0.53 mole/mole of HMM, when the amounts of ATP added were 1 and 3 moles/mole of HMM. This value was equal to that observed when 3 moles of ATP/mole of HMM were added to the HMM-F-A-RP system in the presence of 3 mM EGTA. Furthermore, the amounts of bound ADP at 1 and 3 moles of added ATP/mole of HMM were unaffected by decreasing the concentration of the F-A-RP complex from 3 to 1 mg/ml. Therefore, it was concluded that ADP binds to HMM in the HMM-F-A-RP system.

Figure 44 shows the dependence of the amount of bound ADP on the amount of ATP added when 3 mg/ml of purified F-actin were used, in place of the F-A-RP complex. Even in the presence of 3 mM EGRA, the amount of ADP bound to HMM did not decrease with increase in the amount of ATP added to 3 moles of ATP/mole of HMM, when RP was omitted from the reaction mixture.

Relation between the Amount of ATP Bound to HMM and the Extent of Inhibition of Acto-HMM ATPase Induced by Removal of

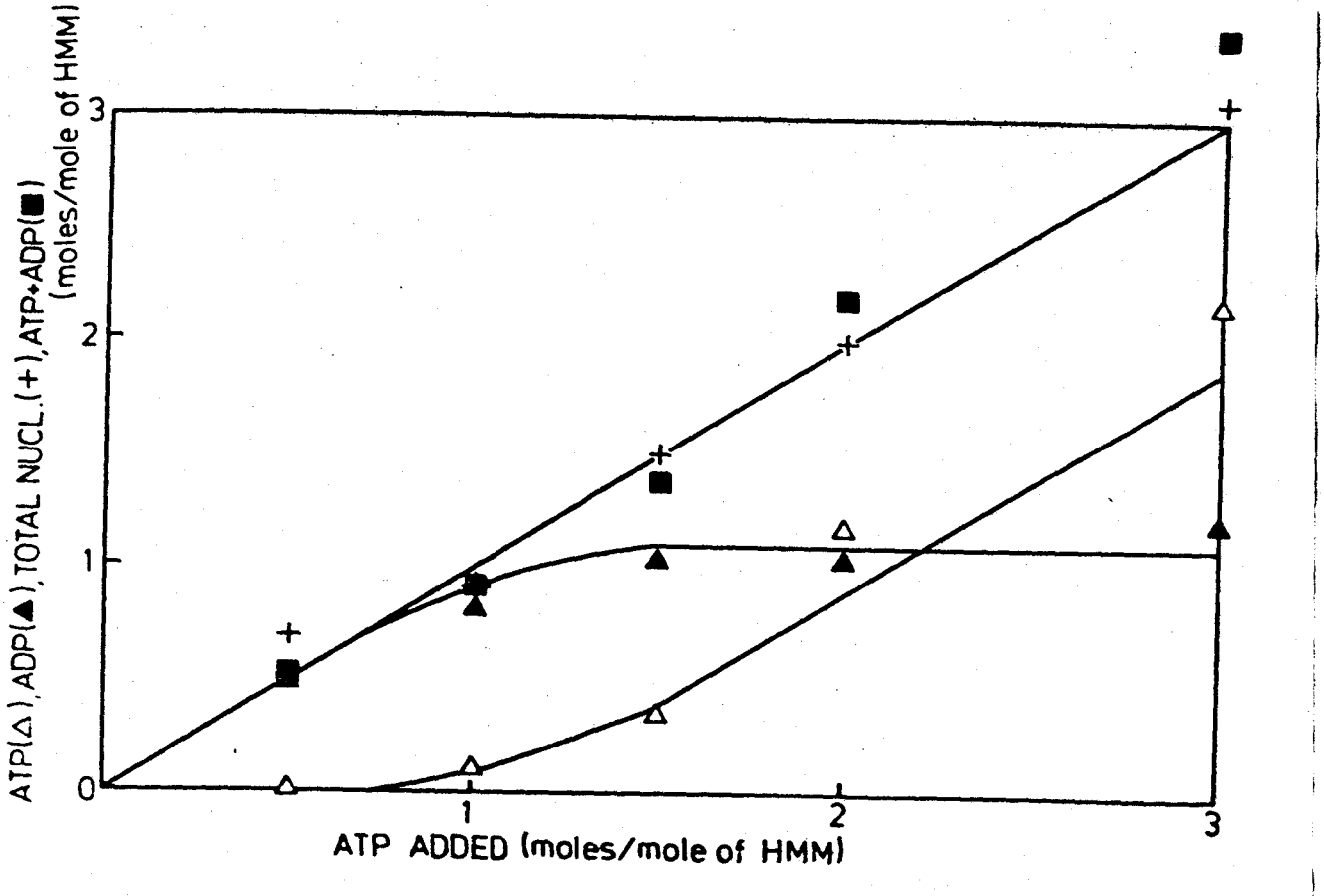


Fig. 44. Dependences of the amounts of ADP and ATP on the amount of ATP added during the HMM-ATPase reaction activated by purified F-actin in the presence of 3 mM EGTA. Experimental conditions were as for Fig. 41, except that 3 mg/ml of purified F-actin were used. △, amount of ATP; ▲, amount of ADP; +, amount of total nucleotides; ■, ATP + ADP.

Table 6. Amounts of ADP and ATP bound to HMM in the presence of various concentrations of the F-A-RP complex.

3.4 mg/ml HMM, 4 mg/ml pyruvate kinase, 1 mM PEP, 3 mM EGTA, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 7.8, 20°. Amounts of nucleotides are expressed as moles/mole

F-A-RP (mg/ml)	ATP added	ADP remaining	ATP remaining	ADP + ATP	Total nucleotide
3	1	0.98	0.03	1.01	1.08
		0.90	0.07	0.97	1.10
1	1	0.94	0.04	0.98	1.05
0	1	0.45	0.58	1.03	1.03
		0.49	0.50	0.99	1.09
3	3	0.58	2.40	2.98	3.06
		0.69	2.42	3.11	3.03
1	3	0.40	2.71	3.11	3.00
0	3	0.45	2.43	2.88	2.95
		0.53	2.56	3.09	3.07

Ca^{2+} Ions — The dependences of the amounts of ATP and ADP bound to the HMM-F-A-RP system on the amount of ATP added were measured in the presence of 0.1 mM Ca^{2+} or 3 mM EGTA (Table 6). The amount of bound ATP was obtained by subtracting the amount of bound ADP from the total amount of nucleotides boudn to the protein.

When 1 mole of ATP was added per mole fo HMM, the amounts of bound ATP in the presence of 0.1 mM Ca^{2+} and of 3 mM EGTA were 0 - 0.055 and 0.002 mole/mole of HMM, respectively. In the presence of 3 mM EGTA, the amounts of bound ATP were 0.42 - 0.56 and 1.23 - 1.24 mole/mole of HMM, respectively, when the amounts of added ATP were 2 and 3 moles/mole of HMM. The amount of boudn ATP at 3 moles of added ATP/mole of HMM (1.23 - 1.24 mole/mole of HMM) was equal to that observed in the absence of the F-A-RP complex (1.22 mole/mole of HMM).

It is noteworthy that the amount of bound ATP was proportional to the extent of inhibition of the acto-HMM ATPase activity by removal of trace amounts of Ca^{2+} ions. The extent of inhibition was defined as $(v_{Ca} - v_{EGTA})/v_{Ca}$, where v_{Ca} and v_{EGTA} represent the F-actin dependent ATPase activity in the presence of Ca^{2+} and that in the presence of EGTA, respectively. The extents of inhibition were 0 - 0.07, 0.5 - 0.65 and almost 1, respectively, at 1, 2 and 3 moles of added ATP/mole of HMM (*cf.* Figs. 41 & 42).

The amounts of bound ATP were also measured in the presence

Table 5. Amounts of ATP bound to HMM of the HMM-F-A-RP system in the presence and absence of a minute amount of Ca^{2+} ions.

3.4 mg/ml HMM, 3 mg/ml F-A-RP, 4 mg/ml pyruvate kinase, 20 mM PEP, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 7.8, 20°. Amounts of nucleotides are expressed as moles/mole of HMM.

Protein	Modifier	ATP Added	Bound Nucleotides			Free ATP[b]
			Total	ADP[a]	ATP	
HMM-F-A-RP	3 mM EGTA	1	0.92 0.77	0.865 ± 0.102 (2)	0.055 0	0.08 0.23
		2	1.62 1.48 1.53	1.06 ± 0 (2)	0.56 0.42 0.47	0.38 0.52 0.47
		3	1.77 1.78	0.54 ± 0.057 (2)	1.23 1.24	1.23 1.22
HMM-F-A-RP	0.1 mM $CaCl_2$	1	0.83	0.823 ± 0.131 (4)	0.002	0.17
		2	1.37	1.218 ± 0.127 (4)	0.152	0.63
		3	1.74	1.283 ± 0.72 (4)	0.457	1.26
HMM	3 mM EGTA	3	1.72	0.50	1.22	1.28

[a] Numbers in parentheses indicate numbers of experiments, performed in the presence of 1 mM PEP (*cf.* Figs. 43 & 44).

[b] ATP added - total bound nucleotide.

of 0.1 mM Ca^{2+}. They were 0.002, 0.15 and 0.46 mole/mole of HMM, respectively, at 1, 2 and 3 moles of added ATP/mole of HMM. The values at 2 and 3 moles of added ATP/mole of HMM were much less than those observed in the presence of 3 mM EGTA.

DISCUSSION

Previously we (43) concluded that the reactive myosin-phosphate-ADP complex, M_P^{ADP}, is the reaction intermediate in the actomyosin ATPase reaction, and that F-actin accelerates the decomposition of M_P^{ADP} without affecting its formation. Various lines of evidence for this conclusion were presented in the monograph of Tonomura (39) and our reviews (40, 43). Especially, we (93) showed that the rate of formation of M_P^{ADP} is almost equal to the rate of actomyosin ATPase at low ATP concentrations. We (Ref. 118 and Sec. 6) also showed that acceleration of decomposition of M_P^{ADP} by F-actin occurs *via* two routes, *i. e.* the direct decomposition of F-A-M_P^{ADP}, F-AM_P^{ADP}. $\longrightarrow$ F-A-M + ADP + Pi, and the dissociation of actomyosin into F-actin and M_P^{ADP} and their recombination, F-A-M_P^{ADP} $\longrightarrow$ F-A + M_P^{ADP} $\longrightarrow$ F-A-M + ADP + Pi. However, from these studies we could not exclude the possibility that the binding of ATP to M_P^{ADP} might be required for expression of the full activity of actomyosin ATPase (Sec. 5).

Therefore, we studied the relation between the amount of

bound ADP and the ATPase activity, using a high concentration (10 μM) of HMM. In the presence of 0.05 - 0.1 mM $CaCl_2$, the ATPase activity was proportional to the amount of bound ADP, as shown in Figs. 41 and 43. In particular, when 1 mole of ATP was added per mole of HMM, the ATPase activity was nearly equal to the maximal value, and the amount of bound ADP was about 1 mole/mole of HMM, while the amount of ATP bound to the protein was less than 0.06 mole/mole of HMM (Table 6). Since the amount of bound ADP was independent of the concentration of the F-A-RP complex (Table 5), we concluded that ADP binds to HMM in the protein complex. Furthermore, we (103) showed previously that in the presence of excess amounts of pyruvate kinase and PEP, ADP binds to HMM in the state of M_P^{ADP}. Thus, the results obtained here indicate clearly that the reaction intermidiate of myosin ATPase, M_P^{ADP}, is the intermediate for the actomyosin ATPase reaction, and that the binding of ATP to myosin is not required for expression of the full ATPase activity of actomyosin.

In the presence of 3 mM EGTA, the ATPase activity decreased with increase in the concentration of ATP added above 1.5 mole of ATP/mole of HMM, and decreased to the level of HMM alone at 3 moles of added ATP/mole of HMM (Fig. 41). The amount of bound ADP also decreased to the level observed with the HMM-ATP system (Fig. 43 and Table 5). When F-actin freed from RP was used, the amount of bound ADP did not decrease with increase in the amount

of ATP added even in the presence of 3 mM EGTA (Fig. 44). These results indicate that acto-HMM only dissociates when a high concentration of ATP was added in the presence of RP and the absence of Ca^{2+} ions (15).

Both the maximal amount of bound ADP and the amount of added ATP necessary for the meximal level of ATPase activity were higher than 1 mole/mole of HMM, *i.e.* 1.2 - 1.5 mole/mole of HMM. However, the dependence of the ATPase activity on the amount of ATP added was not affected by changing the concentration of the F-A-RP complex (Fig. 42), and no incorporation of labelled nucleotides into F-actin was observed using a rapid-flow dialysis method. Furthermore, the amount of bound ADP during the HMM ATPase reaction (103) or the ATPase reaction of the HMM-F-A-RP system in the dissociated state (Fig. 43 and Table 5) was more than the value calculated from the rate constants of steps, $M_P^{ADP} \rightarrow {}^{\circ}M + ADP + Pi$ and ${}^{\circ}M \rightarrow M$, as discussed previously (103). These results may be attributed to the binding of ADP to a site(s) of myosin other than that for M_P^{ADP} formation.

The most important result obtained in this study was that the amount of binding of ATP to HMM was in good proportion to the extent of inhibition of the ATPase activity of the HMM-F-A-RP system by removal of trace amounts of Ca^{2+} ions (Figs. 41 & 42, Table 6). The binding of ATP to HMM was concluded to occur in the reaction of myosin ATPase *via* route (2), *i.e.*, as M_{ATP}, since both the amount of ATP bound to HMM and the ATPase

activity of the HMM-F-A-RP system at high ATP concentrations were equal to those observed with the HMM-ATP system, and since the binding of ATP to HMM in the latter system occurs at the active site for route (2), as reported previously (103). Furthermore, it should be noted that the binding of ATP to HMM was markedly inhibited by addition of a trace amount of Ca^{2+} ions (Table 6).

According ot the mechanism mentioned above, ATP hydrolysis

M should be inhibited by tight binding of myosin to F-actin.
ATP
Actually, Barron *et al.* (122) previously showed that the ATPase activity of myosin was markedly inhibited by F-actin when Mg^{2+} ions were removed by 1, 2-diaminocyclohexanetetraacetate. This is because the dissociation of actomyosin by ATP was inhibited by removal of Mg^{2+} ions (123) and ATP hydrolysis by myosin in the absence of Mg^{2+} ions occurred mainly *via* route (2) (53).

The maximal amount of ATP bound to HMM was slightly higher than 1 mole/mole of HMM (Table 6). The following two explanations of this may be considered. One is that ATP binds not only to the active site for route (2) but also to the site for route (1) when HMM is in the state of °M. The other is that ATP binds to a site(s) other than the active sites of the ATPase reaction, since Bowen and Evans (124) reported that myosin has two kinds of ATP binding site, one with high affinity and the other with low affinity. The latter mechanism seems rather improbabale, since we (103) previously showed that the amount of total nucleo-

tides bound to myosin was independent of the amount of ATP added in the range from 3 to 6 moles of ATP/mole of myosin.

We (118) showed previously that during the acto-HMM ATPase reaction, ATP is hydrolyzed *via* two routes, *i.e.* by direct decomposition of acto-HMM_P^{ADP} and by dissociation of acto-HMM into F-actin and HMM_P^{ADP} and their recombination. We (37, 43, 60) presented several lines of evidence to indicate that the direct decomposition of acto-HMM_P^{ADP} is the path for activation of the myosin molecule, in other words the movement of myosin heads. We (39, 43, 60, 109, 110) also concluded that the dissociation of actomyosin induced by formation of M_P^{ADP} (*cf.* Fig. 42) is the reaction step for detachment of myosin heads from the actin filament in the contraction cycle, which is independent of the presence of RP and Ca^{2+} ions. On the other hand, the inhibition of ATPase by formation of M_{ATP} only occurred in the presence of RP, and formation fo M_{ATP} was markedly inhibited by adding trace amounts of Ca^{2+} ions. Thus, it is concluded that muscle relaxes when the dissociation of actomyosin is induced by formation of M_{ATP} on removal of Ca^{2+} ions bound to troponin (15). Shibata - Sekiya and Tonomura (125) previously reported that the Ca^{2+}- sensitivity of ATPase of the HMM-F-A-RP complex is completely inhibited by modification of one specific sulfhydryl group in the HMM molecule. This result and the result reported here clearly indicate that the site for the Ca^{2+} -control is located on one of the two heads of the myosin molecule.

6. Direct Evidence for the Two Route Mechanism of the Acto-H-meromyosin-ATPase Reaction

The time-course of binding of the reactive HMM-phosphate-ADP complex with F-actin was measured from the increase in light-scattering intensity. The reaction medium contained 2 mM $MgCl_2$, 50 mM KCl and 10 mM Tris-HCl, pH 7.8 at *ca* 20°C. The time-course followed first order kinetics, and its apparent rate constant, k_3, increased with increase in the concentration of F-actin, *i.e.*, it creased from 0.16 to 0.41 sec^{-1} with increase in F-actin concentration from 0.1 to 0.75 mg/ml. The reaction was not affected by treatment of HMM with CMB and β-mercaptoethanol.

The rate of acto-HMM-ATPase in the steady state, v_o, increased with increase in ATP concentration, and reached a maximum at 5 μM ATP. Further increase in the ATP concentration was inhibitory, and in ATP concentrations above 50 μM the rate was constant and independent of the ATP concentration. On the other hand, when HMM treated with CMB and then with β-mercaptoethanol was used, the value of v_o increased steadily with increase in the concentration of ATP, without showing any substrate inhibition, and reached a definite maximum value. The dependence of Δv_o (total activity minus HMM-ATPase activity) at high concentrations of ATP on the concentration of F-actin was measured. It was found that the dependence obeys the Michaelis-Menten equation, and that the maximum value of Δv_o, k_2, is 4.25 sec^{-1}, whereas the

rate of HMM-ATPase (k_1) in the absence of F-actin in the steady state is 0.095 sec^{-1}.

At high concentrations of ATP, the relation between k_1, k_2, k_3 and v_o can be expressed by an equation:

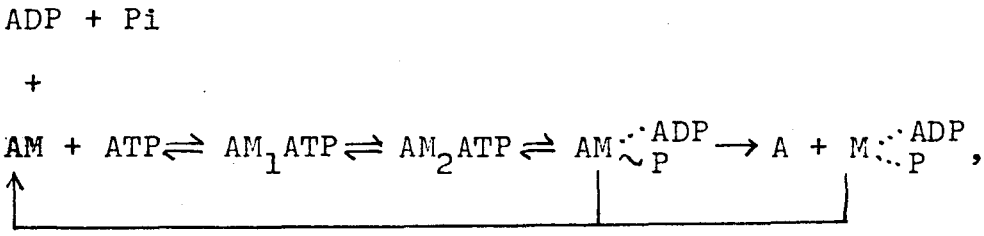
$$v_o = k_1 + (1 - \alpha)\, k_2 + \alpha k_3$$

Here α is the extent of dissociation of acto-HMM in the presence of ATP, which was measured by the Millipore filtration method and by the light-scattering method under limited conditions. Treatment of HMM with CMB and β-mercaptoethanol affected the value of α only, *i. e.*, it decreased the extent of dissociation of acto-HMM, without altering the three rate constants, k_1, k_2 and k_3.

These results provide direct evidence for the two route mechanism of the actomyosin-ATPase reaction, and are inconsistent with the one route mechanism.

INTRODUCTION

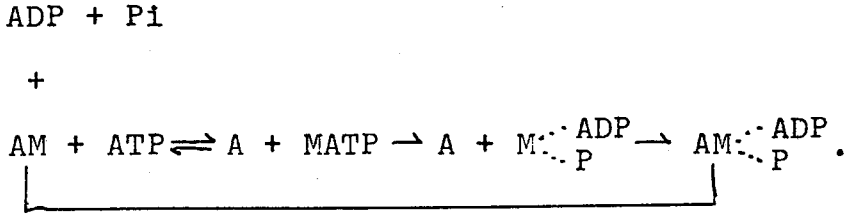
Previously we (40, 43, 93, 60, 110) showed that the reactive myosin-phosphate-ADP complex, M_P^{ADP}, is the reaction intermediate in the actomyosin-ATPase reaction, and that F-actin accelerates its decomposition without affecting the rate of its formation. In 1968, Tonomura et al.(39, 109) proposed the following reaction mechanism for actomyosin-ATPase:



where A and M are actin and myosin, respectively, and $M\sim_P^{ADP}$ and $M\cdots_P^{ADP}$ are phosphoryl myosin and the myosin-phosphate-ADP complex, respectively. $M\sim_P^{ADP}$ and $M\cdots_P^{ADP}$ are collectively called the reactive myosin-phosphate-ADP complex. This mechanism will be called the two route mechanism in thispaper. It is based on the mechanism of myosin-ATPase deduced from analyses of the pre-steady state of myosin-ATPase over wide ranges of experimental conditions (40, 43). The experimental bases for the above mechanism were also provided by the relationship between the rate of formation of M_P^{ADP} and dissociation of actomyosin (109, 110), and by the effects of chemical modifications of myosin (especially p-nitrothio-phenylation) on the myosin- and acto-myosin-ATPase reaction (60, 110). Details of these experimental

results were described by Tonomura (39).

However, three years later in 1971, Lymn and Taylor (111) proposed the following simplified mechanism based on their studies on myosin-ATPase (67):



This mechanism, which will be called the one route mechanism in this paper, was deduced from the following two results. They (111) measured the rate of dissociation of acto-HMM after addition of ATP over a wide range of ATP concentrations, using the light-scattering method (126, 127). They reported that the rate of dissociation of acto-HMM was similar to that of formation of HMM $\overset{ADP}{P}$ in a low concentration range of ATP, but that in high ATP concentrations, the rate of formation of HMM$\cdots\overset{ADP}{P}$ approached a plateau, while the rate of the dissociation increased almost linearly with increase in the ATP concentration. Therefore, they concluded that the dissociation of acto-HMM occurs by formation of an HMM-ATP complex. Furthermore, they (111) measured the rate of binding of F-actin with the HMM-ADP complex, on the assumption that the HMM-ADP complex has the same kinetic properties as HMM$\cdots\overset{ADP}{P}$. They found that the rate was of the same order of magnitude as that of the acto-HMM-ATPase reaction in the steady state.

The two mechanisms for the reaction of ATP with actomyosin can be distringuished in the following three differences. (i) The dissociation of actomyosin is caused by formation of $M\cdot\cdot\cdot{}^{ADP}_{P}$ in the two route mechanism, whereas it is caused by formation of the M-ATP complex in the one route mechanism. (ii) Since $M\,{}^{ADP}_{P}$ is formed very rapidly in the presence of high concentrations of ATP, in the one route mechanism the rate-determining step of actomyosin-ATPase is expected to be the binding reaction of F-actin with $M\cdot\cdot\cdot{}^{ADP}_{P}$. On the other hand, in the two route mechanism the rate of ATP-hydrolysis by actomyosin in the steady state is supposed to be much higher than that of the binding step, at least in the presence of high concentrations of F-actin. (iii) From the one route mechanism it can be expected that the rate of ATP-hydrolysis by purified acto-HMM increases with increase in the ATP concentration and approaches a plateau. In the two route mechanism, the rate of actomyosin-ATPase is expected to increase with increase in the ATP concentration and reach a maximum, and then decrease with further increase in the ATP concentration to a definite but small value, since the conversion of $M\cdot\cdot\cdot{}^{ADP}_{P}$ to $M\cdot\cdot\cdot{}^{ADP}_{P}$ is accelerated by high concentrations of ATP. Especially, in the presence of low concentrations of F-actin and high concentrations of ATP, the rate of actomyosin-ATPase is almost the same as the sum of the rate of binding of F-actin with $M\cdot\cdot\cdot{}^{ADP}_{P}$ and that of myosin-ATPase.

As pointed out in our recent review (40, 43), the conclusion

of Lymn and Taylor (111) that the dissociation of acto-HMM occurs by formation of the HMM-ATP complex is inconsistent with the finding of us and Taylor *et al* . that after adding ATP to myosin or HMM no initial lag phase is observed either in the change in the UV spectrum (95) or in the initial rapid H^+-liberation.[4] (60, 65). Furthermore, it is now well established (40, 43) that the HMM-ADP complex is not the most stable intermediate in the HMM-ATPase reaction.

In the present study, we measured both the rate of binding of HMM_P^{ADP} with F-actin and the extent of dissociation of acto-HMM in the presence of ATP, and compared them with the kinetic parameters of ATP-hydrolysis by acto-HMM in the steady state. The results obtained provided conclusive evidence for the two route mechanism, but were inconsistent with the one route mechanism.

EXPERIMENTAL

Myosin was prepared from rabbit skeletal muscle by the

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4. According to their reaction mechanism of myosin-ATPase, the lag time is 20 - 30 msec.

method of Perry (70). HMM was prepared by tryptic digestion of myosin, using the method of Szent-Györgyi *et al.* (128). HMM was treated with CMB and then with β-mercaptoethanol to remove CMB (129). G-Actin was extracted from an acetone powder of rabbit skeletal muscle at 0°C for 20 min, and purified by a plymerization procedure (114) in 0.6 M KCl at 0°C. After removal of free ATP from the G-actin solution by treatment with Dowex 1-4, G-actin was polymerized to F-actin by addition of 50 mM KCl, 2 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.8. Pyruvate kinase [EC 2.7.1.40] was prepared from rabbit skeletal muscle by the method of Tiez and Ochoa (94). Protein concentration was determinated by the buret reaction, which was calibrated by nitrogen determination. The molecular weights of HMM and actin monomer were taken as 3.4×10^5 and 4.5×10^4, respectively (39). γ-^{32}P-Labelled ATP was synthesized enzymatically by the method of Glynn and Chappel (75). ATP and PEP were purchased from Sigma Chemical Co.

The standard reaction mixture contained 50 mM KCl, 2 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.8 at 20°C. The HMM-ATPase reaction was stopped by adding 5 percent TCA containing 0.1 mM cold Pi as carrier, and the amount of ^{32}P liberated from ^{32}P-labelled ATP was determined as described previously (64). In measurement of acto-HMM-ATPase the pyruvate kinase system was used as an ATP-regenerating system, and the amount of pyruvate produced was measured by the method of Reynard *et al.* (97).

The time-course of binding of F-actin with HMM_P^{ADP} was pursued in a stopped-flow apparatus (98) combined with a Hitachi MPF-2A spectrofluorometer. The observation chamber was of a cylindrical shape with a diameter of 0.2 cm. Before measurements, HMM solution was clarified by centrifugation, and other solutions were filtered through a Millipore filter (pore size, 0.45μ). Degassed F-actin solution was placed in one syringe of the flow system. A mixture of 2 ml of HMM solution and the same volume of ATP solution was placed in the other syringe of the flow apparatus. The reaction was started by mixing equal volumes of the two solutions, and the light-scattering intensity of the reaction mixture was recorded. The time interval between mixing ATP with HMM and starting the reaction was varied.

The time-course of change in the UV spectrum of HMM induced by ATP was followed by measuring the change in optical density at 293 nm, using a stopped-flow method, as described by Morita (66).

The extent of dissociation, α, of acto-HMM in the presence of ATP was measured by the following two methods. (i) A Millipore filter (pore size, 0.3 - 0.45μ) was treated with HMM solution to avoid non-specific adsorption of proteins to the membrane. Acto-HMM solution in the presence of ATP and the ATP-regenerating system was placed on the filter, and then rapidly filtered with suction. In the absence of ATP acto-HMM did not pass through the membrane, while in the absence of F-actin HMM passed through completely. F-Actin also passed through the membrane to some extent. The amount of HMM in the filtrate was

estimated by densitometric scanning of an SDS-gel electrophoretogram (116) of the filtrate. (ii) The extent of decrease in light-scattering intensity in the steady state after mixing acto-HMM with ATP was measured at 90° to the incident light. The extent of decrease in light-scattering intensity in the presence of low concentrations of actin (0.125 mg/ml) and HMM (0.25 mg/ml) and high concentrations of ATP (0.2 - 0.33 mM) was usually taken as the value for 100 % dissociation of acto-HMM, since by Millipore filtration method acto-HMM seemed to be completely dissociated under these conditions.

RESULTS

Initial Burst of Pi-Liberation from the HMM- and Acto-HMM-ATP System — It is generally agreed that the initial burst of TCA-labile Pi-liberation is due to the formation of M_P^{ADP} (39). Nakamura dnd Tonomura (64) suggested that M_P^{ADP} is composed of two intermediates, $M\sim\cdot\cdot{}_P^{ADP}$ and $M\cdot\cdot{}_P^{ADP}$. However, $M\sim\cdot\cdot{}_P^{ADP}$ is formed transiently in the intial phase of the reaction, and its amount is negligible compared with that of $M\cdot\cdot{}_P^{ADP}$ under the usual experimental conditions.

Imamura *et al.* reported (130) that the size of the initial burst of Pi-liberation by HMM is one mole per mole of HMM, when a sufficient amount of ATP is added. However, the dependence of the initial burst on the ATP concentration has not yet been reported. Therefore, we have measured the dependence this time.

Figure 45 shows results obtained under the standard conditions, *i.e.*, in 50 mM KCl, 2 mM $MgCl_2$ and 10 mM Tris-HCl, pH 7.8 and at 23°C in the presence of 0.136 mg/ml HMM. The size of the initial burst of Pi-liberation increased with increase in the ATP concentration, and reached 1 mole per mole of HMM when the molar concentration of ATP was twice that of HMM. The burst size remained constant with further increase in the ATP concentration. It decreased slightly when 0.2 mg/ml F-actin was added or when HMM was treated with CMB and then with β-mercaptoethanol. When 1 mole and 2 moles of ATP per mole of HMM were added to HMM (0.136 mg/ml) in the presence of 0.2 mg/ml F-actin, the sizes of the initial burst of Pi-liberation were 0.65 and 0.97 mole per mole of HMM, respectively. Takeuchi and Tonomura (112) previously reported that 2 moles of ATP per mole of HMM were necessary to cause complete dissociation of acto-HMM. Therefore, the above result that 2 moles of ATP per mole of HMM were necessary for the maximum amount of initial burst is consistent with our mechanism (109, 110) that the dissociation of acto-HMM upon addition of ATP occurs after formation of HMM$\therefore^{ADP}_{P}$.

Binding of the H-Meromyosin-Phosphate-ADP Complex with F-Actin — Tonomura *et. al* (109, 110) reported previously that actomyosin dissociates into F-actin and myosin upon additon of 1 mole ATP per mole of myosin in 0.6 M KCl, and that recombination of myosin with F-actin occurs after liberation of ADP from M$\therefore^{ADP}_{P}$. In this study, we followed the time-course of binding of F-actin with HMM $^{ADP}_{P}$ by measuring the increase in light-

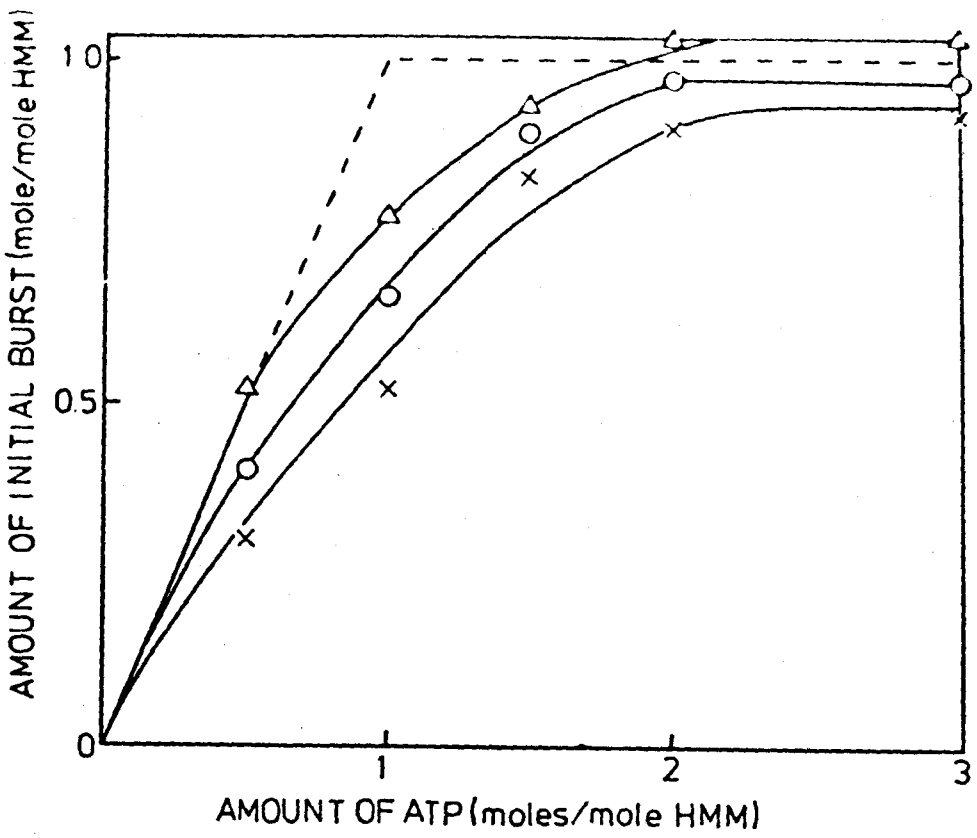

Fig. 45. Dependence on ATP concentration of the size of the initial burst of Pi-liberation of HMM. 0.136 mg/ml HMM, 50 mM KCl, 2 mM $MgCl_2$ and 10 mM Tris-HCl at pH 7.8 and 23°C. Δ, untreated HMM; O, untreated HMM + 0.2 mg/ml F-actin; X, CMB-β-mercaptoethanol-treated HMM.

scattering intensity.

Figure 46A shows the time-course of increase in light-scattering intensity upon addition of F-actin to HMM under the experimental conditions. The addition of F-actin was made at various time intervals after addition of ATP to HMM in a molar ratio of 1:1. The final concentrations of F-actin, HMM and ATP were 0.1 mg/ml, 0.3 mg/ml and 0.87μM, respectively. In the figure the ratio of Δ_t(increment in light-scattering intensity at tiem t) to $\Delta\infty$(increment in light-scattering intensity after completion of the binding) is plotted against the time for the preincubation of HMM with ATP before addition of F-actin. When F-actin was mixed with HMM in the absence of ATP or at a sufficiently long time after addition of ATP to HMM, the intensity of light-scattering increased very rapidly, and $\tau_{1/2}$, the time for reaching a half maximum intensity, was estimated to be 0.4 sec^{-1}. On the other hand, when F-actin was mixed with HMM within 30 sec after adding ATP to HMM, $\Delta_t/\Delta\infty$ increased very rapidly in the initial phase of the reaction, and then more slowly. Figure 2B shows a plot of log $(\Delta\infty - \Delta_t)/\Delta\infty$ against the reaction time. The plot consists of two straight lines. The slope of the initial rapid decrease in log $(\Delta\infty - \Delta_t)/\Delta\infty$ was similar to the slope observed when F-actin was mixed with HMM in the absence of ATP. The slope of the subsequent slow decrease was independent of the length of time between mixing ATP with HMM and addition of F-actin to the HMM-ATP system. The rate constant determined from the slope in the slow phase was 0.16 sec^{-1}.

Fig. 46. Time-course of increase in light-scattering intensity after adding F-actin to an equimolar mixture of ATP and HMM. The reaction was started at time 0 by adding an equimolar amount of ATP to HMM, and after various intervals F-actin solution was added. The final reaction mixture contained 0.3 mg/ml HMM, 0.87 μM ATP, 0.1 mg/ml F-actin, 50 mM KCl, 2 mM $MgCl_2$ and 10 mM Tris-HCl at pH 7.8 and 19°C.

A: —, — - — -, untreated HMM, - - -, — -- — --, CMB-β-mercaptoethanol-treated HMM. The arrow indicates the time when F-actin was added. ● , the size of increase in light-scattering intensity during the initial rapid phase. — - — -, — -- — --, F-actin was added after ATP had been completely hydrolyzed by HMM.

B: Semilogarithmic plot of time-course of increase in light-scattering intensity after adding F-actin to an equimolar mixture of ATP and HMM.

Fig. 47. Semilogarithmic plot of time-course of increase in light-scattering intensity after adding a high concentration of F-actin to an equimolar mixture of ATP and HMM. The experimental conditions were as described in Fig. 46, except that the F-actin concentrations were 1 mg/ml (X) and 0.5 mg/ml (O).

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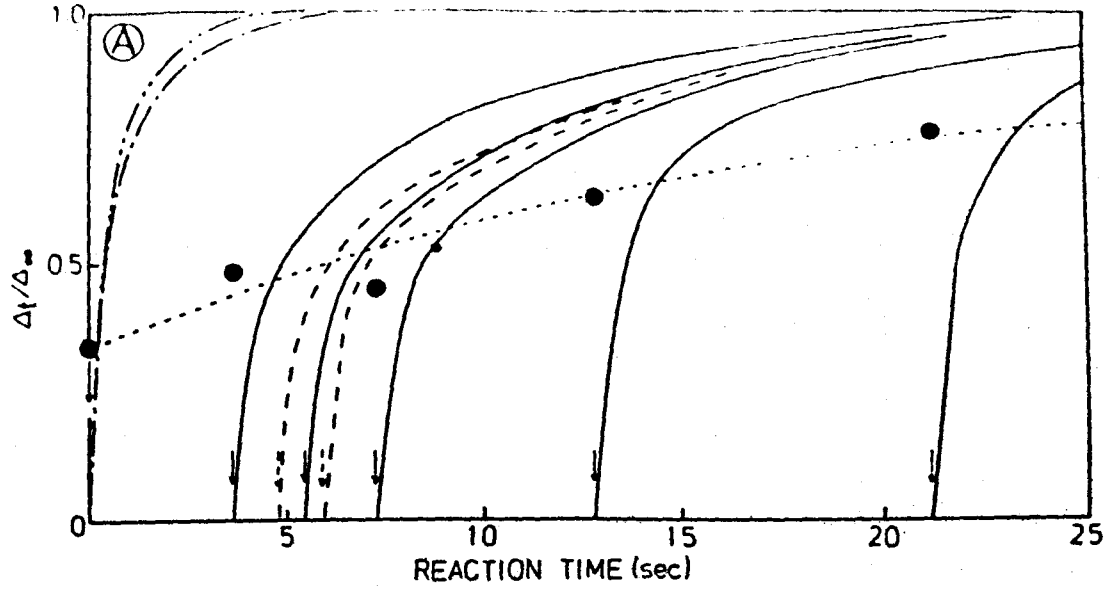
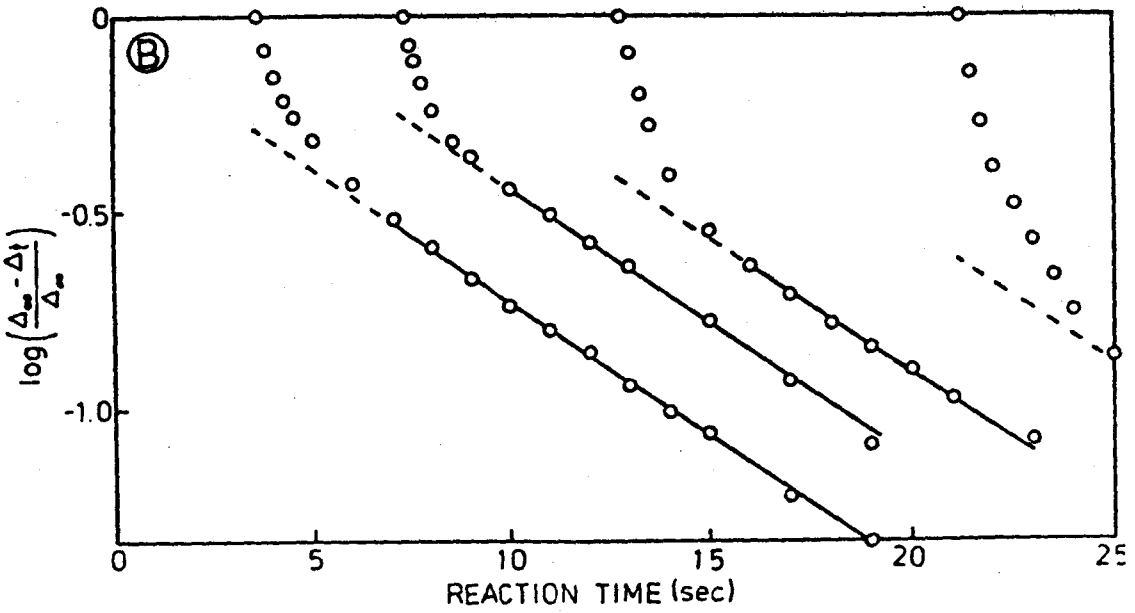
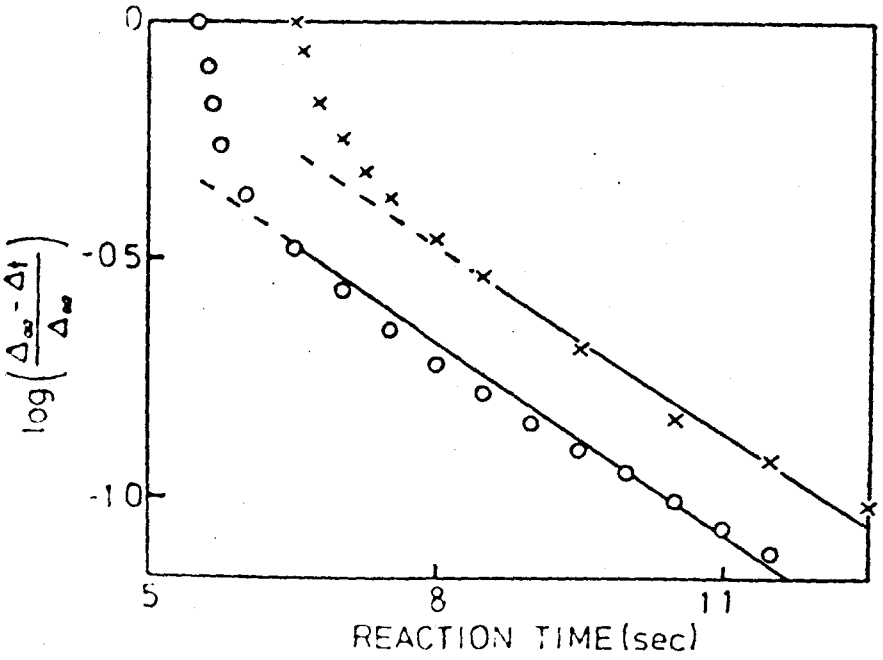

A
1.0
0.5
0
Δ_t/Δ_∞
5
10
15
20
25
REACTION TIME (sec)


B
0
-0.5
-1.0
$\log\left(\frac{\Delta_\infty - \Delta_t}{\Delta_\infty}\right)$
0
5
10
15
20
25
REACTION TIME (sec)

47


0
-0.5
-1.0
$\log\left(\frac{\Delta_\infty - \Delta_t}{\Delta_\infty}\right)$
5
8
11
REACTION TIME (sec)

The size of the rapid increment in light-scattering, obtained by extrapolating the straight line in the slow phase to the time when F-actin was added to the HMM-ATP system, is plotted as filled circles in Fig. 46. The percentage of the rapid phase increased gradually from 35 to 100 with increase in the interval between the time for mixing ATP with HMM and that for adding F-actin. The rate constant of this increase was 0.048 sec. Tonomura *et al.* (60) reported previously that the change in the UV-spectrum of myosin induced by ATP was reversed when ADP was released from M_P^{ADP}. Therefore, the rate constant of ADP-liberation from HMM_P^{ADP} was estimated from the time-course of decay in the change of optical density at 293 nm after addition of ATP to HMM in a molar ratio of 1:1 (10 μM), using the stopped-flow method. The rate was estimated to be 0.05 - 0.06 sec^{-1}, which was almost equal to that (0.048 sec^{-1}) of increase in the size of the initial rapid increment. However, the rate was lower than that (0.095 sec^{-1}) of the HMM-ATPase reaction in the steady state at high ATP concentrations.

These results can be interpreted as indicating that the initial, rapid increase in light-scattering intensity is due to the binding of F-actin with HMM (not with HMM$\therefore_P^{ADP}$), and that the slow increase is due to the binding of F-actin with HMM$\therefore_P^{ADP}$. Figure 47 shows a plot of log $(\Delta\infty - \Delta_t)/\Delta\infty$ versus time, when the concentration of F-actin was increased to 0.5 and 1.0 mg/ml. The rate constant for the F-actin-binding with HMM$\therefore_P^{ADP}$ increased

with increased in the F-actin concentration, *i.e.*, it was 0.16 and 0.32 sec^{-1} respectively, in the presence of 0.1 and 0.5 mg/ml of F-actin. However, the rate constant did not increase with further increase in the concentration of F-actin to 1.0 mg/ml.

Sekiya and Tonomura (84) previously reported that when HMM which had been treated with CMB and then with β-mercaptoethanol was used, the ATPase activity of HMM in the steady state was greatly accelerated even by addition of low concentrations of F-actin. Therefore, we measured the binding of HMM:$\cdot\cdot^{ADP}_{P}$ with F-actin, using HMM pre-treated with CMB and then with β-mercaptoethanol. As shown in Fig. 46A, the time-course of increase in light-scattering intensity after adding F-actin was not altered by treatment of HMM with CMB and -mercaptoethanol.

ATP was added to HMM in a molar ratio of 2:1, and the time-course of increase in light-scattering intensity after mixing F-actin with the HMM-ATP system was measured. As shown in Fig. 48, when F-actin was added to the HMM-ATP system shortly after mixing ATP with HMM, no initial rapid phase in the increase in light-scattering intensity could be detected, but the size of the rapid phase increased gradually with increase in time interval between mixing ATP with HMM and adding F-actin to the HMM-ATP system. This result is in accord with the previous observation (112) that 2 moles of ATP per mole of HMM are necessary to cause complete dissociation of acto-HMM. When the concentrations of HMM and F-actin were 0.136 mg/ml and 0.15 mg/ml, respectively,

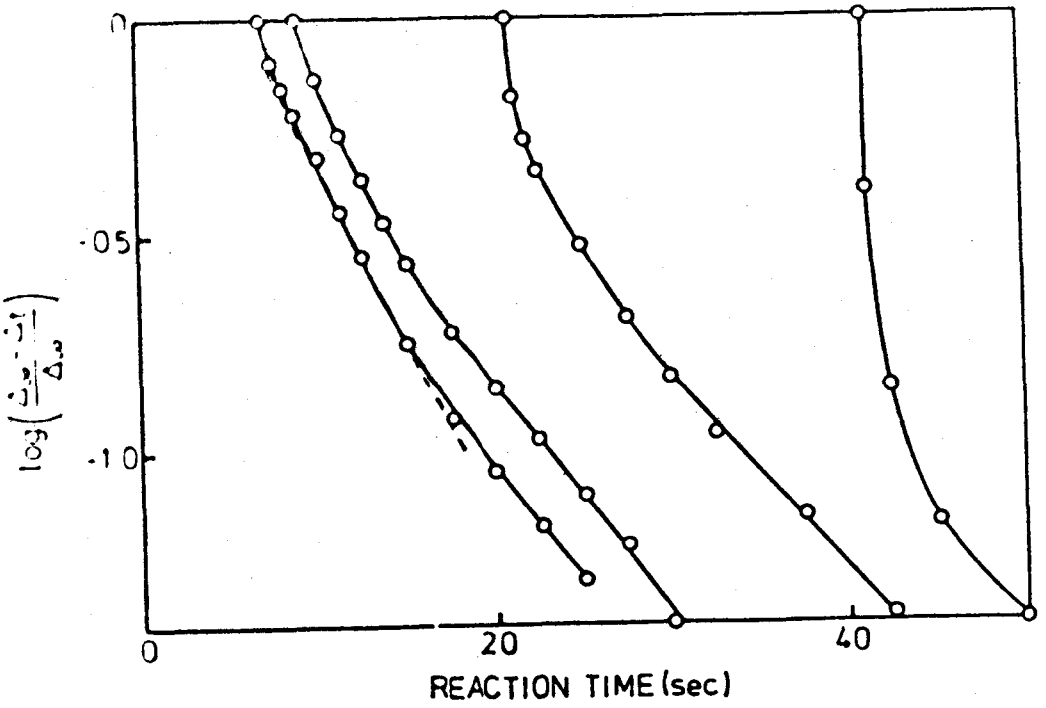


Fig. 48. Semilogarithmic plot of time-course of increase in light-scattering intensity after adding F-actin to a mixture of ATP andHMM in a 2:1 molar ratio. The final reaction mixture contained 0.15 mg/ml F-actin, 0.136 mg/ml HMM, 0.8 μM ATP, 50 mM KCl, 2 mM $MgCl_2$ and 10 mM Tris-HCl at pH 7.8 and 23°C. The apparent rate constant, k_3, was calculated from the part shown by a broken line.

the plot of log $(\Delta_\infty - \Delta_t)/\Delta_\infty$ versus time in the slow phase gave a curved line, and the rate constant determined from a straight line which covered most of the time-course, was about 0.19 sec^{-1}.

Figure 49 shows the time-course when the F-actin concentration was increased to 0.3 and 0.75 mg/ml. The increase in light-scattering intensity in the slow phase followed first order kinetics at both F-actin concentrations, but the rate constant of binding of HMM$\cdot\cdot^{ADP}_{P}$ with F-actin increased with increase in the F-actin concentration. Thus, the rate constants were 0.24 and 0.41 sec^{-1}, respectively, for 0.3 and 0.75 mg/ml F-actin.

ATPase Activity of Acto-H-Meromyosin in the Steady State— The dependence on ATP concentration of the rate of acto-HMM-ATPase in the steady state, v_o, under the standard conditions is shown in Fig. 50. As reported previously (97), when acto-HMM was reconstituted from 0.2 mg/ml F-actin and 0.17 mg/ml HMM pretreated with CMB and then with β-mercaptoethanol, the value of v_o increased with increasing ATP concentration, without showing any substrate inhibition, and was constant at ATP concentrations above 0.3 mM. On the other hand, the value of v_o of acto-HMM reconstituted from 0.2 mg/ml F-actin and 0.17 mg/ml untreated HMM increased with increasing ATP concentration, and reached a maximum at 3 μM. Further increase in ATP concentration was inhibitory, but the rate became constant and was independent of the ATP concentration when the concentration of ATP was above

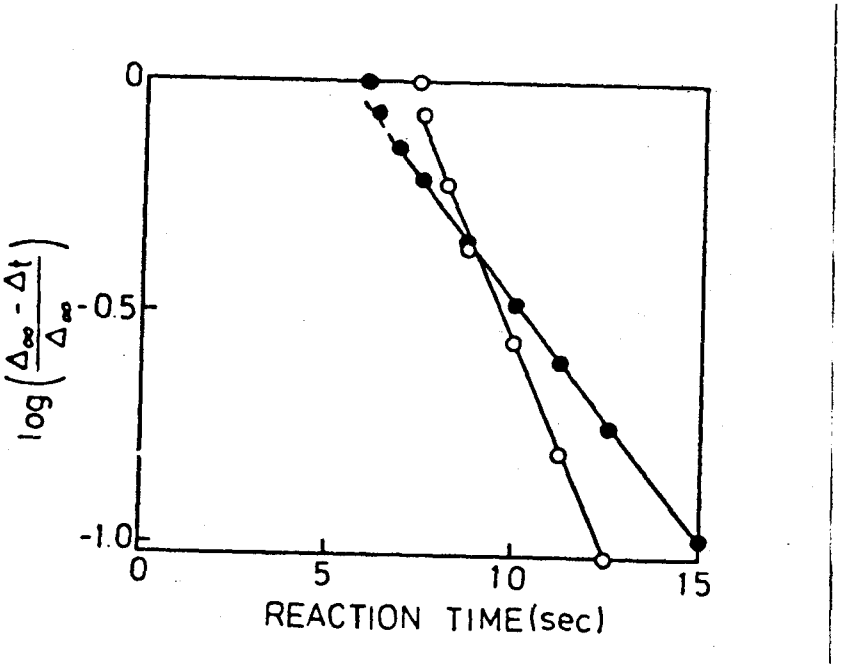


Fig. 49. Semilogarithmic plot of time-course of increase in light-scattering intensity after adding a high concentration of F-actin to a mixture of ATP and HMM in a 2:1 molar ratio. Experimental conditions were as described in Fig. 48, except that the F-actin concentrations were 0.75 mg/ml (○) and 0.3 mg/ml (●).

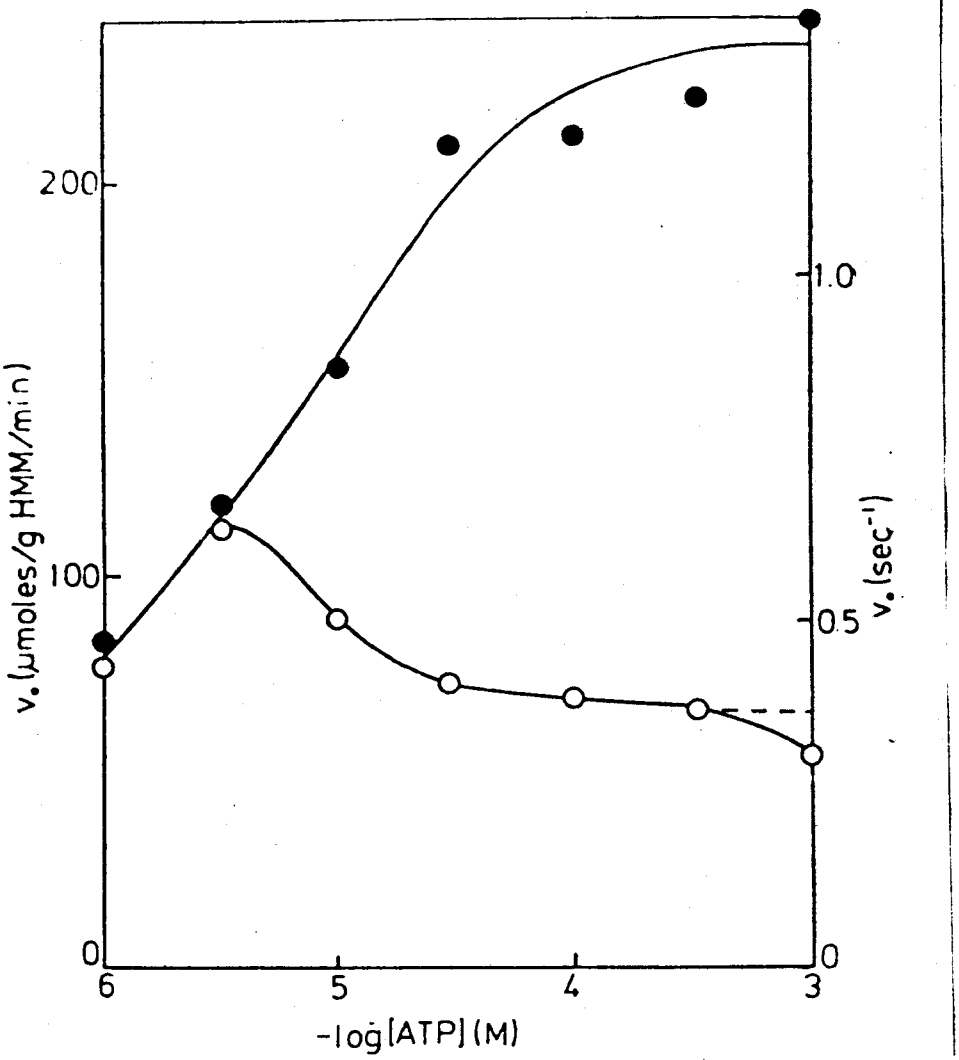


Fig. 50. Dependence on ATP concentration of the rate of acto-HMM-ATPase activity in the steady state. 0.17 mg/ml HMM, 0.2 mg/ml F-actin, 50 mM KCl, $MgCl_2$ = 2 mM + [ATP], 0.15 mg/ml pyruvate kinase, 0.5 mM PEP and 10 mM Tris-HCl buffer at pH 7.8 and 23°C. ●, acto-HMM reconstituted from F-actin and HMM pre-treated with CMB and β-mercaptoothanol; ○, acto-HMM reconstituted from F-actin and untreated HMM.

50 μM.[5] Figure 51 shows the dependence of the acto-HMM-ATPase activity, v_o, on the F-actin concentration in the presence of a high concentration of ATP (0.33 mM). In this figure, the rate constant of binding of F-actin with HMM$\cdot\cdot\cdot_P^{ADP}$ is also plotted against the concentration of F-actin. The value of v_o for acto-HMM reconstituted from F-actin and untreated HMM increased with increase in F-actin concentration. When the concentration of F-actin was low, the rate constant of acto-HMM-ATPase in the steady state was almost equal to $k_1 + k_3$, the sum of the rate constant of HMM-ATPase and that of binding of F-actin with HMM $_P^{ADP}$, but when the concentration of F-actin was high, the rate constant of acto-HMM-ATPase was much higher than $k_1 + k_3$. At 1.2 mg/ml F-actin, the rate constant was 3.8 times higher than $k_1 + k_3$.

Figure 52 shows a double reciprocal plot of the F-actin-activated ATPase activity of HMM, Δv_o (total activity minus activity of H-MM alone), *versus* the F-actin concentration. A linear relationship was obtained, and the maximum value of Δv_o and the concentration of F-actin for half-saturation were 4.25

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5. The slight decrease in v_o at ATP concentrations above 1 mM may be due to increase in ionic strength caused by increasing the concentration of ATP itself.

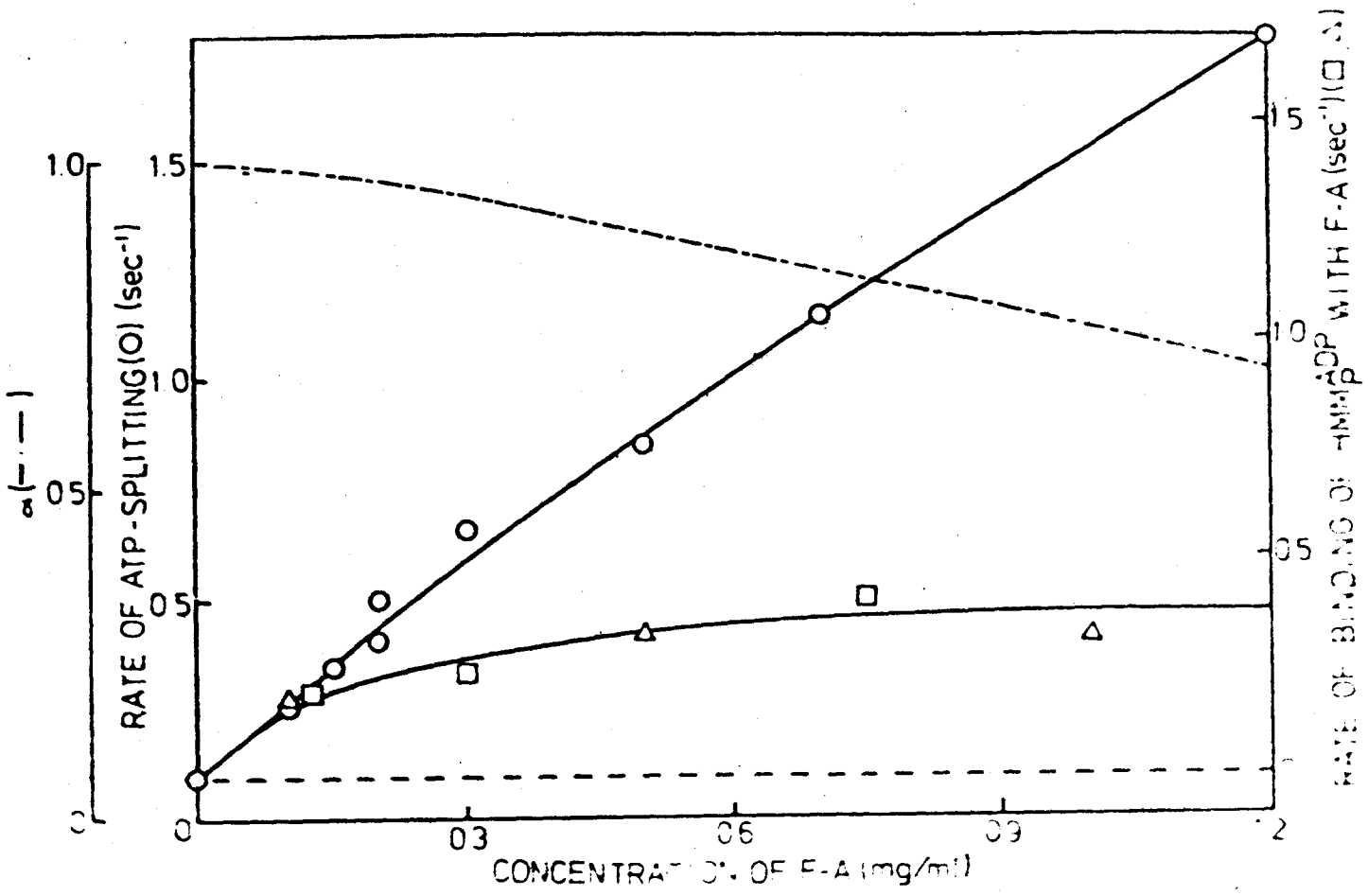

Fig. 51. Dependences on F-actin concentration of the rate of acto-HMM-ATPase, the rato of binding of F-actin with the myosin-phosphate-ADP complex, HMM$\cdot\cdot\cdot{}^{ADP}_{P}$ and the extent of dissociation of acto-HMM in the steady state, α. ATPase activity was measured under the conditions described in Fig.50 . ○, ATPase activity of acto-HMM; △, □, rato of binding of HMM $^{ADP}_{P}$ with F-actin. △ and □ were obtained from the results shown in Figs. 46 and 47and Figs.48 and 49, respectively. — - — -, α, extent of dissociation of acto-HMM, as described in the text.

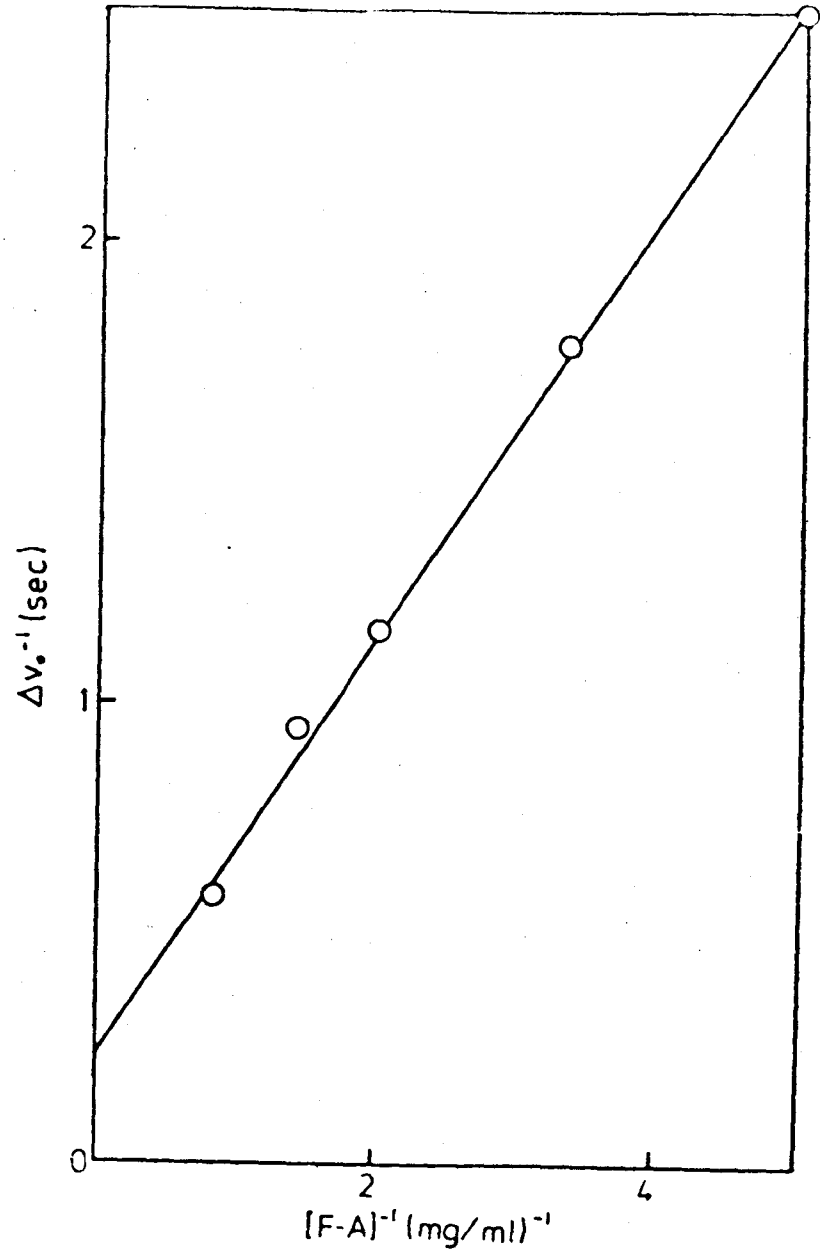

Fig. 52. Double reciprocal plot of the rate of the acto-HMM-ATPase reaction in the steady state against the concentration of F-actin. Experimental conditions were as described in Fig. 50. v_o = total ATPase activity of acto-HMM minus that of HMM alone.

sec^{-1} and 2 mg/ml, respectively.

Relationship between ATPase Activity and Extent of Dissociation of Acto-H-Meromyosin — The extent of dissociation, α, of acto-HMM which was reconstituted from untreated, or CMB-β-mercaptoethanol-treated HMM was measured by the Millipore filtration method in the presence of 0.2 - 0.3 mM ATP. As listed in Table 7, the extent of dissociation, α, of acto-HMM reconstituted from 0.25 mg/ml of untreated HMM and 0.125 mg/ml of F-actin was estimated to be 1.0 in the presence of 0.2 mM ATP, 0.1 mg/ml pyruvate kinase, and 1.2 mM PEP. However, the value of α decreased to 0.72, when CMB-β-mercaptoethanol-treated HMM in place of untreated HMM and 0.2 mg/ml instead of 0.125 mg/ml of F-actin were used.

The rate of ATP-hydrolysis was compared with the extent of ATP-induced decrease in light-scattering intensity of acto-HMM reconstituted from 0.125 mg/ml of F-actin and 0.25 mg/ml of HMM. The HMM used was the mixture of untreated and CMB-β-mercaptoethanol-treated HMM in various weight ratios. As shown in Fig. 53, a linear relationship was observed between the rate of ATP-hydrolysis, v_o, and the decrease in the relative extent of ATP-induced decrease in light-scattering intensity.

DISCUSSION

When the ATP concentration was lower than 5 μM, the rate of the acto-HMM-ATPase reaction in the steady state, v_o, increased

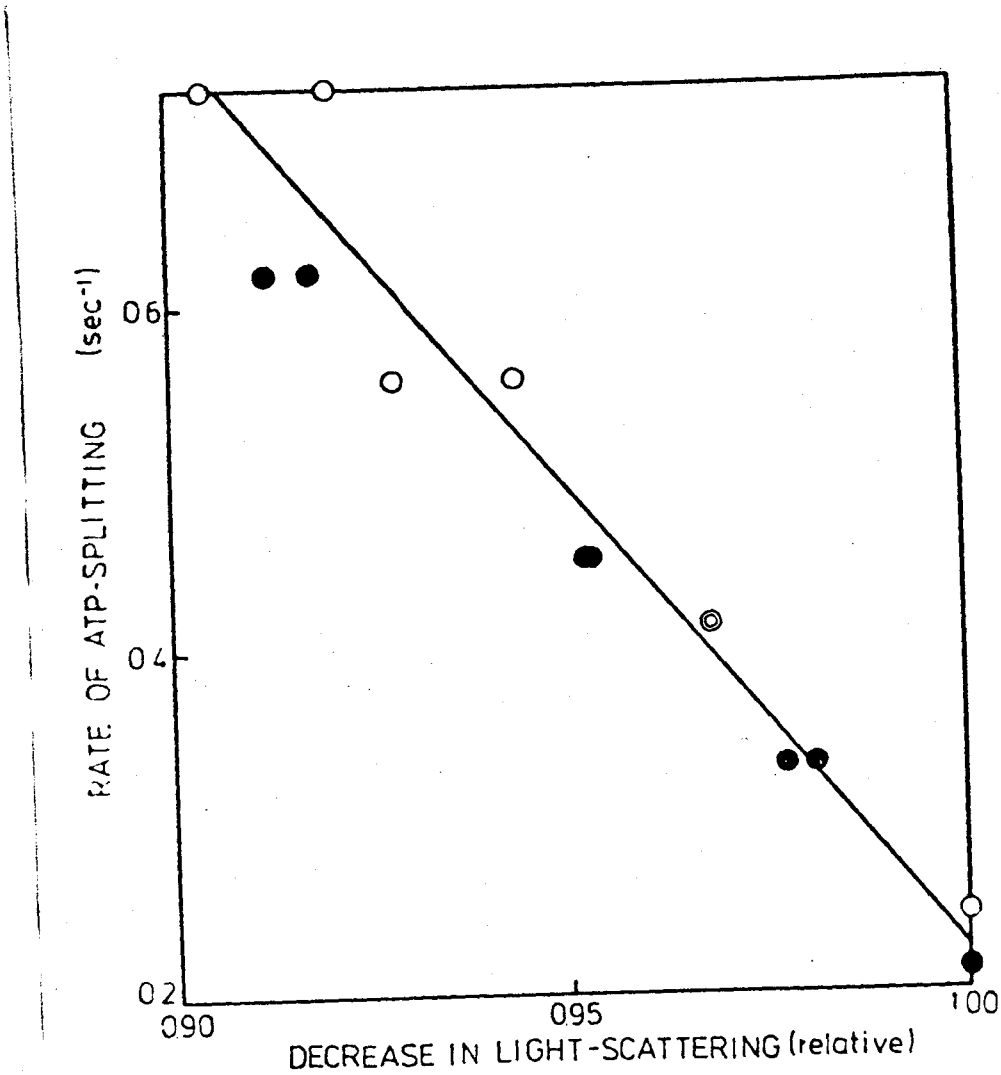

Fig. 53. Relation between the rate of ATPase in the steady state and the extent of decrease in light-scattering intensity of acto-HMM. 0.2 mg/ml HMM, 0.125 mg/ml F-actin, 0.2 mM ATP, 1.2 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-maleato buffer, 1 mM PEP, and 0.07 mg/ml pyruvate kinase at pH 7.2 and 24°C. Mixtures of untreated HMM and CMB- -mercaptoethanol-treated HMM were used. ○ and ● indicate results obtained, using different preparations. of HMM.

Table.7. Extent of dissociation of acto-H-meromyosin, α, in the presence of ATP. 50 mM KCl, 2 mM $MgCl_2$, 0.33 mM ATP, 10 mM Tris-HCl, pH 7.8, 20°C.

F-Actin (mg/ml)	Treatment of HMM	v_o (sec^{-1})	α		
			v_o	Millipore Filtration[a)]	Light-Scattering[a)]
0.125	-	0.295	0.995	1.0	1.0
0.125	CMB-DTT	0.575	0.93		0.91
0.20	CMB-DTT	1.33	0.75	0.72	
0.40	CMB-DTT			0.5	

a) measured at pH 7.2.

with increase in ATP concentration. A further increase in the ATP concentration caused inhibition, but the rate became constant and independent of the ATP concentration, when the concentration of ATP was above 50 μM (Fig. 50). The inhibition of acto-HMM-ATPase by high concentrations of ATP can be explained on the two route mechanism as due to acceleration of step, $M\therefore_{P}^{ADP} \rightarrow M\therefore_{P}^{ADP}$, by high concentrations of ATP (97). According to the two route mechanism, the rate of ATP-hydrolysis by acto-HMM in the steady state at high ATP concentrations is given by

$$v_o = k_1 + (1-\alpha)K_2 + \alpha k_3$$

where k_1, k_3 and k_2 are the rate constant of HMM-ATPase in the steady state, that of binding of HMM $\therefore_{P}^{ADP}$ with F-actin, and that of ATP-hydrolysis catalyzed by undissociated acto-HMM, i.e., acto-HMM$\therefore_{P}^{ADP} \rightarrow$ acto-HMM + ADP + Pi, respectively. The value α is the extent of dissociation of acto-HMM in the presence of ATP. The value of k_1 was 0.095 sec^{-1}. The value of k_3 was measured, as shown in Figs. 46 - 49, and the dependence of k_3 on the F-actin concentration is shown in Fig. 52.[6] The rate

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. When ATP was mixed with HMM in a molar ratio of 1:1 or 2:1, ATP remained partially unhydrolyzed, without conversion to HMM $\therefore_{P}^{ADP}$. However, we assumed that the value of k_3 obtained was not affected by the ATP remaining unhydrolyzed, since (i)

constant of the ATPase reaction catalyzed by undissociated acto-HMM, k_2, can be estimated by extrapolation of the double reciproacal plot of v_o *versus* F-actin concentration to a sufficiently high concentration of F-actin. The value thus obtained was 4.25 sec^{-1} (Fig. 52). The value of α was calculated from the estimated values of k_1, k_2, k_3 and v_o. The dependence of α calculated from v_o on the F-actin concentration is shown in Fig. 52. In Table 7, the values of α measured by the Millipore filtration method are compared with those calculated from v_o and the three rate constants by the equation given above. Thus, the values of measured by the Millipore filtration method were in good agreement with those calculated from v_o. It is also reasonable to assume that the extent of decrease in light-scattering intensity of acto-HMM induced by ATP is proportional to α when the concentration of F-actin is low and the interaction between proteins

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* the size of the decrease in light-scattering intensity was proportional to the amount of HMM $\overset{ADP}{P}$ produced (109, 110), and since (ii) the rate constant obtained when ATP was mixed with HMM in a 1:1 molar ratio was equal to that when ATP was mixed with HMM in a 2:1 molar ratio. The second statement is true in spite of the fact that the ratios of the amount of ATP remaining unhydrolyzed to that of HMM $\overset{ADP}{P}$ were 0.35 : 0.65 and 1:1, respectively, in these two cases.

is negligible. On the other hand, since $k_3 \gg k_2$, v_o is proportional to $(1 - \alpha)$, when α approaches 1. Accordingly, the relation between v_o and α given above is supported by the observed relation of the ATPase activity to the extent of decrease in light-scattering intensity (Fig. 53). We found that the extent of decrease in ligh-scattering intensity decreases with increase in the concentration of F-actin, *i.e.*, the relative extents were 1.0, 0.91, 0.80, 0.70 and 0.67, respectively, when the concentrations of F-actin were 0.1, 0.2, 0.35, 0.5 and 0.65 mg/ml in the presence of 0.25 mg/ml HMM. However, the quantitative evalution of α from the extent of decrease in light-scattering intensity was impossible in the presence of high concentrations of F-actin, because interactions between F-actin and/or acto-HMM became too strong to be avoided. Thus, all the terms in the above equation derived from the two route mechanism can be measured separately, and the equation was verified by direct measurements of all these terms, although we could measure only under limited conditions. On the other hand, these results were inconsistent with the one route mechanism.

Leadbeater and Perry (131) first reported that the ATPase activity of HMM is stimulated by a low concentration of F-actin, in spite of the complete dissociation of acto-HMM. This is because in the presence of high concentrations of ATP and low concentrations of F-actin, the ATPase activity of acto-HMM is equal to $k_1 + k_3$, *i.e.*, the rate constant of HMM-ATPase plus

that of binding of HMM$\cdot\cdot\cdot{}^{ADP}_{P}$ with F-actin. On the other hand, in the presence of high concentrations of F-actin, the activity varies almost linearly with $(1-\alpha)$, *i.e.*, the extent of binding of F-actin with HMM in the presence of ATP, as already assumed in the analyses done by Hisenberg and Moos (132) and us (133), since k_2 is much higher than k_3.

Previously Tonomura and Yoshimura reported (82, 129) that the ATPase activity fo acto-HMM reconstituted from F-actin and HMM pre-treated with CMB and β-mercaptoethanol increases with increase in the ATP concentration, without showing any substrate inhibition, and that both the rate of HMM-ATPase, k_1, and the rate in the presence of sufficiently high concentrations of F-actin, k_2, were unaffected by treatment of HMM with CMB and β-mercaptoethanol. The present study showed that the rate of binding of F-actin with HMM$\cdot\cdot\cdot{}^{ADP}_{P}$, k_3, was also not affected by treatment of HMM with CMB (Fig. 46). Therefore, according to the two route mechanism, the acceleration of the acto-HMM-ATPase reaction by CMB-treatment must be attributed to decrease in α, as confirmed by direct measurements of α (Table 7), and electron-microscopy of acto-HMM in the presence of ATP (133). Furthermore, the concentration of F-actin for half saturation of acto-HMM-ATPase reconstituted from CMB-β-mercaptoethamol-treated HMM was 0.3 mg/ml, which was much lower than that (2 mg/ml) for untreated acto-HMM (Fig. 52). However, it is uncertain whether preparations of acto-HMM are homogeneous and the rate of step,

AHMM$\sim^{ADP}_{P}$ → A + HMM$\cdot^{ADP}_{P}$, is suppressed by CMB-treatment of HMM or whether preparations of CMB-treated acto-HMM are heterogeneous and consist of at least two fractions (the ratio of which depends on the concentration of F-actin) one catalyzing ATP-hydrolysis mainly via direct decomposition of AHMM$\sim^{ADP}_{P}$ and the other via A + HMM $^{ADP}_{P}$.

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