



Title	INTERACTION ON MYOSIN OR ITS SUBFRAGMENTS WITH ACTIN AND ADENOSINE TRIPHOSPHATE
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Citation	大阪大学, 1972, 博士論文
Version Type	VoR
URL	https://hdl.handle.net/11094/30659
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INTERACTION ON MYOSIN OR ITS SUBFRAGMENTS
WITH ACTIN AND ADENOSINE TRIPHOSPHATE

Kikuko TAKEUCHI

PREFACE

From electron microscopic studies on sectioned muscle, Huxley (1) concluded that the contractile element of skeletal muscle consists of two kinds of filaments, the myosin and the actin filaments, which are connected by cross-bridge from the myosin filament, and that during contraction these two kinds of filaments slide without changing the length of the filaments themselves. Furthermore, he (2) concluded from experiments using the negative staining method that the cross-bridges in sectioned muscle represent the globular portion of the myosin molecule (3, 4). The globular portion can be isolated from tryptic digests of myosin as subfragment-1, which retains the ATPase active and actin binding sites of the original myosin (5). The interactions between the globular portions the myosin filaments and the actin filaments produce the actomyosin type ATPase [ATP phosphohydrolase, EC 3.6.1.3] and develop the sliding force (4, 6-9). However, the molecular mechanism of the movement of cross-bridges during contraction remains to be clarified. Since the change in sarcomere length during contraction is much greater than the possible change due to movement of a single cross-bridge, the making and breaking of cross-bridge is a necessary consequence of the sliding-filament model. The reaction sequence of cross-bridge interaction must be cyclic in contraction, and must involve at least the following step: (a) the making of cross-bridge, (b) hydrolysis of ATP, (c) the conformation change or movement of cross-

bridge, and (d) the breaking of cross-bridge.

Tomomura et al. analyzed the reaction mechanism of the myosin-ATP system in detail and have emphasized that phosphorylation of myosin by ATP is the primary biochemical reaction in muscle contraction (10, 11). The molecular model of muscle contraction proposed by him (9, 12, 13) involves the following reaction cycle : (a) the phosphorylation and its conformation change of myosin, (b) binding of myosin with actin which is triggered by Ca^{++} released from the sarcoplasmic reticulum, (c) the transconformation of myosin induced by the dephosphorylation, which accompanies the sliding of actin filament past myosin filament, (d) the breaking of the linkage between myosin and actin by ATP, and (e) the transconformation of myosin to the original state.

However, many essential problems of the interaction of myosin with actin and ATP have been left unsolved. Therefore, detailed investigation on the interaction among them is a necessary prerequisite for a better understanding of the molecular mechanism of the contractile process.

This report consist of the following two parts. The first deals with the reaction of the H-meromyosin - F-actin - ATP system under the various conditions, especially under physiological condition. The second deals with the stoichiometric relation of the bindings of myosin or its subfragments with actin and ATP.

PART I

BINDING OF H-MEROMYOSIN WITH F-ACTIN
AT LOW IONIC STRENGTH

INTRODUCTION

It is well known that the rate of hydrolysis of ATP by myosin is strongly enhanced by F-actin at low ionic strength in the presence of Mg^{++} (14). This type of ATPase is usually called the actomyosin type ATPase. The actomyosin type ATPase has been shown to be a key reaction in muscle contraction (15-17). Therefore, to clarify the molecular mechanism of muscle contraction, it is especially important to analyze the mechanism of interactions among F-actin, myosin and ATP at low ionic strength in the presence of Mg^{++} . However, the system is not suitable to physico-chemical studies, since myosin and actomyosin are insoluble at low ionic strength. H-meromyosin, which was first obtained from a tryptic [EC 3.4.4.4] digest of myosin by Szent-Györgyi (18), retains the biological activity of the original myosin, but it is soluble even at low ionic strength. The effect of F-actin on the H-meromyosin ATPase has been studied at low ionic strength by several investigators (19-21). Leadbeater and Perry (20) first provided evidence that F-actin can increase the enzymic activity of H-meromyosin in the presence of Mg^{++} , though the addition of ATP to acto-H-meromyosin produces a decrease in viscosity of the complex. Yagi et al. (21) have reported that ATP can easily dissociate acto-H-meromyosin at low ionic strength in the presence of Mg^{++} , and consequently the ATPase activity shows a remarkable "substrate inhibition". In these previous studies, the ionic strength in the reaction system was not kept constant, and the activity was determined over narrow ranges of concentrations of ATP and F-actin.

The molecular properties of acto-H-meromyosin were inferred only from the measurements of viscosity and intensity of light-scattering at 90°. Therefore, we have reinvestigated the actin - H-meromyosin - ATP system, and have obtained the following results: at low ionic strength, H-meromyosin combined with F-actin at a weight ratio of 1 : 0.33. The intensity of light-scattering at 90° of acto-H-meromyosin was decreased considerably, but the weight-average molecular weight, $\langle M \rangle_w$, deduced from the angular distribution was only slightly decreased and the z average radius of gyration, $\langle r_g^2 \rangle_z^{1/2}$, was increased several times by the addition of ATP. The ATPase activity and the decrease of light-scattering intensity at 90° of acto-H-meromyosin increased monotonously with increasing ATP concentration to attain certain maximum values. The ATP concentrations necessary to attain half the limiting changes of both the ATPase activity and the light-scattering decreased with increase in the ionic strength and with decrease in the amount of F-actin. The binding of H-meromyosin with F-actin was strengthened by the treatment of H-meromyosin with PCMB* and β -mercaptoethanol (22).

EXPERIMENTAL

Materials - Myosin was prepared from rabbit skeletal muscle

* The abbreviations used are: PCMB, p-chloromercuribenzoate; PEP, phosphoenolpyruvate; PK, pyruvate kinase; EGTA, glycolether-diaminetetraacetic acid.

by the method of Perry (23) with slight modifications. H-meromyosin was obtained according to the method of Szent-Györgyi (18), except for the tryptic digestion at 15°C for 10 minutes and the collection of protein from the 43 to 60 per cent saturated ammonium sulfate fraction. Tightly bound Ca^{++} was removed from H-meromyosin by a treatment with PCMB and β -mercaptoethanol (22). G-actin was prepared from an acetone powder of rabbit skeletal muscle according to the procedure of Mommaerts (24), with slight modifications (25). After removal of free ATP from G-actin solution by the treatment with Dowex 1 x 4, G-actin was polymerized to F-actin by the addition of 1 mM MgCl_2 . F-actin- C^{14} -ADP was prepared with the method described by Nakamura et al. (26). Pyruvate kinase (ATP: pyruvate phosphotransferase, E.C., 2.7.1.40.) was prepared from rabbit skeletal muscle by the method of Teitz and Ochoa (27), and was stored as a suspension in saturated ammonium sulfate solution. It was dialyzed against deionized water before use. ATP and PEP were purchased from Sigma Chemical Co. ATP was used as K_2ATP .

Methods - Light-scattering measurements were made in a Brice-Phoenix photometer (Model 1000D). H-meromyosin and G-actin were clarified by centrifugation at 3.6×10^4 r.p.m. for 3 hours and diluted by the solvent filtered through a Millipore filter. The solution contained 0.05 - 0.1 mg. H-meromyosin per ml., 0.01 - 0.25 mg. F-actin per ml., 10 mM Tris-HCl, 1 mM MgCl_2 in excess of ATP, 20 - 40 μg . pyruvate kinase per ml., and 0.4 mM PEP. All measurements were performed at 25°C. The angles, θ , at which the

measurements were made, ranged from 35 ° to 135°. To correct the back reflection of light-scattering intensity at the angle θ , the equation of Oth et al. (28)

$$(G_{\theta})_{\text{real}} = (G_{\theta})_{\text{exp.}} - 0.045 (G_{180-\theta})_{\text{exp.}}$$

was applied. Here, $(G_{\theta})_{\text{real}}$ and $(G_{\theta})_{\text{exp.}}$ are the real and the experimental intensities of the scattered light at the angle θ , respectively. The value of $(1 + \cos^2 \theta) / (G_{\theta} \times \sin \theta)$ was plotted against $\sin^2 \theta / 2$ according to Zimm (29). All the intensities, G_{θ} , were given only as relative values.

The ATPase activity of acto-H-meromyosin was determined in the coupled system with pyruvate kinase as an ATP generating system, by measuring the time dependence of pyruvate liberation according to the method of Reynard et al. (30). The concentrations of pyruvate kinase and PEP used were so high that the rate of the overall reaction was independent of the rate of the kinase reaction. The reaction mixture was composed of 0.06 - 0.5 mg. H-meromyosin per ml., 0.01 - 1.4 mg. F-actin per ml., 20 - 40 μ g. pyruvate kinase per ml., 0.4 mM PEP, various concentrations of ATP, 10 mM Tris-HCl and 1 mM MgCl_2 in excess of the ATP concentration at pH 7.5 and 25°C. At 2, 4, 6, 8 and 10 minutes or at 0.5, 1, 1.5, 2.0 and 2.5 minutes after the addition of ATP, 1 ml. of the reaction mixture was withdrawn and added to 0.2 ml. of 2.5 mM 2,4-dinitrophenylhydrazine in 3 N HCl. The KCl concentration in the reaction mixture was changed to keep the ionic strength due to KCl, MgCl_2 and K_2ATP constant, assuming that all ATP molecules present were in the form of complex MgATP^{--} . The ionic strengths given in the

figures as due to $\text{KCl} + \text{MgATP}^{--}$ were those derived from KCl , K_2ATP and MgCl_2 , except for that due to 1 mM of free MgCl_2 and other constituents.

The exchange reaction of ADP of F-actin was measured by incubation of 0.15 mg. F-actin- C^{14} -ADP per ml. with 0.45 mg. H-meromyosin per ml. and 1 mM ATP for 1 - 30 minutes, in the presence of 1 mM MgCl_2 , 2.5 mM KCl and 10 mM Tris-HCl at pH 7.5 and 25°C (31). The reaction mixture was filtered through a Millipore filter, and the radioactivity of the filtrate was measured by a Packard Tri-Carb Scintillation Spectrometer (Model 3002).

The concentration of protein was determined by biuret reaction (32) or by the Folin-Ciocalteu reaction (33), after calibration by nitrogen determination.

RESULTS

Light-scattering - As a preliminary experiment, the effect of the pyruvate kinase system on H-meromyosin ATPase was examined. As shown in Fig. 1, the Michaelis constant, K_m , of H-meromyosin ATPase in the presence of 4 mM KCl or 6 mM KCl , 10 mM Tris-HCl, 1 mM MgCl_2 , 0.4 mM PEP and 40 μg . pyruvate kinase per ml. was 3 μM , which was equal to that of myosin ATPase in the presence of Mg^{++} (34-36).

Fig. 2 indicates the increase of light-scattering intensities at 90° when various amounts of F-actin were added to a solution containing 0.16 mg. H-meromyosin per ml. in the presence of 4 mM KCl , 1 mM MgCl_2 and 10 mM Tris-HCl at pH 7.5 and 25°C. The intensity

increased in proportion to the concentration of F-actin until 0.057 mg. F-actin per ml. were reached. Therefore, it was concluded that 3.4×10^5 g. of H-meromyosin binds with 1.21×10^5 g. of F-actin, i.e. 1 mole of H-meromyosin binds with 1 mole of actin dimer (37, 38). Previous studies (39, 40) have also shown that 1 mole of myosin binds with 1 mole of actin dimer.

Fig. 3 shows the decrease of scattering intensity of acto-H-meromyosin on the addition of 0.5 mM ATP. The experimental conditions were as in Fig. 2, except that 0.05 mg. H-meromyosin per ml. were added to various amounts of F-actin in the presence of 0.4 mM PEP and 20 μ g. pyruvate kinase per ml. At low concentrations of F-actin, the scattering intensity decreased on the addition of ATP to the sum of the intensities of H-meromyosin and F-actin but, at high concentrations of F-actin, the intensity after ATP addition was a little lower than the sum. This result cannot be interpreted by the simple dissociation hypothesis of acto-H-meromyosin on the addition of ATP. The dependence on ATP concentration of the light-scattering intensity at 90° of acto-H-meromyosin composed of 0.05 mg. H-meromyosin per ml. and 0.14 mg. F-actin per ml. was measured at three ionic strengthes (Fig. 4). The scattering intensity decreased with increasing the ionic strength. The intensity after the addition of 0.1 mM ATP was independent of ionic strength, and was lower than the sum of the intensities of both proteins. The ATP concentration required to give half the maximum decrease in scattering diminished with increasing ionic strength: 8.19, 6.45 and 3.39 μ M, respectively,

at 5, 30 and 120 mM of the ionic strength due to $\text{KCl} + \text{MgATP}^{--}$. The dependences on ATP concentration of the light-scattering intensities of acto-H-meromyosins composed of 0.05 mg. H-meromyosin per ml. and various amounts of F-actin was measured in the presence of 20 μg . pyruvate kinase per ml., 0.4 mM PEP, 10 mM Tris-HCl, 1 mM MgCl_2 and 5 mM of KCl and MgATP^{--} at pH 7.5 and 25°C (Fig. 5). As is evident from the figure, the ATP concentration necessary to give half the maximum decrease in intensity increased with F-actin concentration: the ATP concentration required to attain half the limiting value were 1.2, 2.4 and 3.63 μM , respectively, in the presence of 0.0167, 0.05 and 0.15 mg. F-actin per ml.

Since the results described above did not support the assumption of simple dissociation of acto-H-meromyosin on the addition of ATP, the angular distribution of scattering intensity of acto-H-meromyosin was measured. In Fig. 6 shows the Zimm plots of acto-H-meromyosin (0.048 mg. H-meromyosin per ml., 0.14 mg. F-actin per ml.) in the presence and the absence of 0.1 mM MgATP^{--} . The value of $\langle M \rangle_w$ increased to about 2 - 3 times the sum of the values for both proteins but the $\langle r_g^2 \rangle_z^{1/2}$ remained almost unchanged on the mixing of H-meromyosin and F-actin. When ATP was added to acto-H-meromyosin, $\langle M \rangle_w$ decreased slightly but $\langle r_g^2 \rangle_z^{1/2}$ increased to 1.8 times that in the absence of ATP. These changes of light-scattering of acto-H-meromyosin on the addition of ATP were very similar to those obtained previously on the myosin B - ATP and the myosin B - PP_i system at high ionic strength (41, 42).

ATPase Activity - Fig. 7 shows the dependence on the KCl

concentration of the ATPase activities of acto-H-meromyosins reconstituted from 0.15 mg. H-meromyosin per ml. and various concentrations of F-actin in the presence of 1 mM MgCl_2 and 1 mM ATP. In the presence of 5 mM KCl, the ATPase activity of acto-H-meromyosin (with a ratio of H-meromyosin to F-actin of 1 : 3) was 550 $\mu\text{moles per minute per g. of H-meromyosin}$. This value was of the same order of magnitude as that obtained on the actomyosin complex. The ATPase activity decreased sharply when the KCl concentration was increased from 5 to 50 mM, and at 100 mM KCl it was 80 $\mu\text{moles per minute per g. of H-meromyosin}$, which was about 4 times as high as that of H-meromyosin. On the other hand, when the ratio of H-meromyosin to F-actin was 3 : 1, the ATPase activity even in the presence of 5 mM KCl was only twice as that of H-meromyosin itself and depended slightly on the KCl concentration.

The dependence on the ATP concentration of ATPase activities of acto-H-meromyosins composed of 0.1 mg. H-meromyosin per ml. and various concentrations of F-actin was also measured. In this experiment, the KCl concentration was fixed at 5 mM, and the ionic strength of the reaction mixture increased with increase in the ATP concentration. As shown in Fig. 8, the ATPase activity increased with the concentration of F-actin. It also increased with the ATP concentration, but only until the concentration reached 0.1 - 1 mM and then it decreased sharply on further increase in the ATP concentration. When the ratio of H-meromyosin to F-actin was 1 : 5, the maximum activity, 1 mmoles per minute per g. of H-meromyosin, was observed at 0.8 mM ATP, but the

activity at 6 mM ATP was only 80 μ moles per minute per g. of H-meromyosin.

In the next experiment, the dependence of the ATPase activity of acto-H-meromyosin on the substrate concentration was investigated under fixed ionic strengths (Figs. 9 & 10). In the case of acto-H-meromyosin produced from 1 part of H-meromyosin and 3 parts of F-actin (Fig. 9), the ATPase activity increased with the ATP concentration, and approached to a maximum value. The maximum activities were 560, 510, 250 and 90 μ moles per minute per g. of H-meromyosin, when the ionic strengths derived from KCl + MgATP⁻⁻⁻ were 5, 10, 30 and 60 mM, respectively. The ATP concentrations necessary for attaining half the limiting value decreased with increase in the ionic strength, similarly to the change in light-scattering intensity of acto-H-meromyosin caused by ATP. They were 10.6, 8.7, 5.2 and 1 μ M, when the ionic strengths due to KCl + MgATP⁻⁻⁻ were 5, 10, 30 and 60 mM, respectively. As shown in Fig. 10, the rate of ATP hydrolysis of acto-H-meromyosin composed of 0.15 mg. H-meromyosin per ml. and 0.05 mg. F-actin per ml. was lower than that given in Fig. 9 over the complete ranges of ionic strength and ATP concentration. In this case, the ATPase activity decreased slightly with increase in the ATP concentration to higher than 10 μ M. The maximum activity was observed at 10 μ M ATP when the ionic strength due to KCl + MgATP⁻⁻⁻ was in the range of 5 to 10 mM but, at ionic strengths higher than 25 mM, the ATP concentration for the maximum rate was lower than 1 μ M.

Fig. 11 shows the dependence on the substrate concentration

of the ATPase activities of acto-H-meromyosins reconstituted from 0.15 mg. H-meromyosin per ml. and various concentrations of F-actin at 5 mM of ionic strength derived from KCl + MgATP⁻⁻⁻. As described previously, the ATPase activity showed a slight inhibition by a high concentration of ATP, especially when the ratio of H-meromyosin to F-actin was high. The maximum values of ATPase activity and the concentrations of ATP for half the maximum activity were 135, 320 and 600 μ moles per minute per g. of H-meromyosin and 21, 3.8 and 7.1 μ M, respectively, in the presence of 0.05, 0.15 and 0.45 mg. F-actin per ml.

The enhancement of the ATPase activity of H-meromyosin by F-actin was measured in the presence of 1 mM ATP and 5 mM KCl (open circles in Fig. 12). At low F-actin concentrations the ATPase activity increased sharply with increasing F-actin concentration, and on further increase of the concentration the activity increased linearly. In another experiment, the enhancement by F-actin of the ATPase activity of 0.1 mg. H-meromyosin per ml. in the presence of 0.1 mM ATP, 1 mM MgCl₂, 5 mM KCl, 10 mM Tris-HCl, 0.4 mM PEP and 50 μ g. pyruvate kinase per ml. at pH 7.5 and 25°C was measured over a wide range of F-actin concentrations (0 - 1.02 mg./ml.). The rate of ATP hydrolysis was found to reach a maximum value, 1.5 mmoles per minute per g. of H-meromyosin, when the F-actin concentration was higher than 0.8 mg. per ml.

To examine whether the enhancement of H-meromyosin ATPase by F-actin is determined by the weight ratio of H-meromyosin to F-actin or by the concentration of F-actin itself, the ATPase activity was

measured at a fixed ratio of myosin to F-actin (1 : 2.8) in the presence of 5, 20 and 100 μ M ATP, while changing the concentration of acto-H-meromyosin (Fig. 13). In the presence of 0.1 mM ATP, the ATPase activity increased with increase in the protein concentration. In the presence of 5 or 20 μ M ATP, the activity increased first with increase in the protein concentration and then decreased gradually on further increase in the concentration. These results show that the enhancement of ATPase depends on the F-actin concentration itself.

Effect of Divalent Cation and Exchange of ADP of F-actin -

To clarify the relation between ATPase activities of acto-H-meromyosin and actomyosin, the effects of divalent cations on the ATPase activity of acto-H-meromyosin and the exchange of ADP of F-actin in the presence of H-meromyosin and ATP were investigated.

The ATPase activity of acto-H-meromyosin was unaffected by 0.1 mM EGTA (one example is shown in Fig. 10). Almost all the measurements in the present investigation were made in the presence of excess Mg^{++} . Therefore, the effect of minute amounts of Mg^{++} on the ATPase activity of acto-H-meromyosin was investigated. Although the effect of Mg^{++} on the ATPase activity varied from preparation to preparation, an inhibition was always observed on the addition of EGTA to the system, and the inhibition was restored by the addition of $MgCl_2$. This result suggests that the ATPase of acto-H-meromyosin is the actomyosin type in the usual sense.

In 1 mM $MgCl_2$ and 10 mM Tris-HCl at pH 7.5 and 25°C, 0.15 mg. F-actin- C^{14} -ADP per ml. was incubated with 0.45 mg. H-meromyosin

per ml. and 1 mM ATP for 30 minutes. About 40 - 80 per cent of C^{14} -ADP of F-actin was released to the reaction medium, but in the absence of ATP the release was observed to be negligibly small. This result indicates that the exchange reaction first reported by Szent-Györgyi (31) is due to the interaction between F-actin and H-meromyosin part and not due to the superprecipitation of actomyosin itself.

Effect of Treatment of H-meromyosin with PCMB and β -mercaptoethanol - The H-meromyosin was treated with PCMB and β -mercaptoethanol under the conditions similar to those for the removal of tightly bound Ca^{++} from myosin (22). The maximum velocity of ATP-splitting of H-meromyosin increased from 20 to 24 μ moles per minute per g. of H-meromyosin and K_m remained constant (4 μ M) before and after the treatment (Fig. 14). Fig. 12 shows the dependence of ATPase activity of 0.176 mg. H-meromyosin per ml. on the concentration of F-actin, before and after the treatment with PCMB and β -mercaptoethanol. As shown in the figure, the enhancement of ATPase activity of acto-H-meromyosin by the treatment was more significant in the presence of low concentrations than in the presence of high concentrations of F-actin. Therefore, the following experiments were performed to see the effect of the treatment on the ATPase activity in the presence of a low concentration of F-actin. Fig. 15 shows the effects of the treatment on the decrease in intensity of scattered light induced by ATP and the rate of ATP hydrolysis of acto-H-meromyosin reconstituted from 3 part of H-meromyosin and 1 part of F-actin. The ATPase activity

was enhanced by the treatment especially in the presence of high concentrations of MgATP^{--} . The maximum values of ATPase activity before and after the treatment were 117 and 380 $\mu\text{moles per minute per g. H-meromyosin}$. The ATP concentration required to attain half the limiting value was increased from 13 to 34 μM by the treatment. The decrease in intensity of light-scattering on the addition of ATP was diminished by the treatment.

In Fig. 16 are given the effects of ionic strength on the ATPase activities of acto-H-meromyosin reconstituted from 0.176 mg. H-meromyosin per ml. and 0.0588 mg. F-actin per ml. before and after the treatment of H-meromyosin with PCMB and β -mercaptoethanol. The rate of ATP hydrolysis by acto-H-meromyosin reconstituted from PCMB- β -mercaptoethanol treated H-meromyosin and F-actin was higher than that of the control, and the activity after the treatment was slightly dependent on ionic strength. Figure 17 shows the enhancement by F-actin of the ATPase activity of 0.176 mg. of treated H-meromyosin per ml. in the presence of various concentrations of MgATP^{--} . The activity increased with increase in the F-actin concentration, and the maximum activities were 380, 500, 610, 930 and 1,270 $\mu\text{moles per minute per g. of H-meromyosin}$ at F-actin concentrations of 0.0315, 0.0587, 0.176, 0.492 and 0.88 mg. per ml., respectively.

DISCUSSION

Gergely and Kohler (39) and Tonomura et al. (40) have reported that the binding ratio of myosin to F-actin is 3.7 - 4 : 1 by

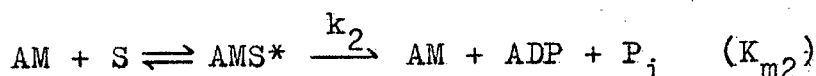
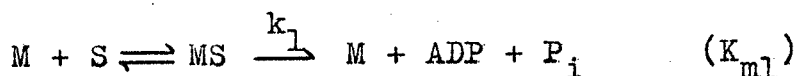
weight. In the present study it was shown that at low ionic strength, H-meromyosin binds to F-actin with a weight ratio of 3.3 : 1. Since the molecular weights of myosin, H-meromyosin and G-actin are taken as 4.8×10^5 (43), 3.4×10^5 (37) and 5.7×10^4 (38), respectively, it is concluded that one molecule of H-meromyosin or myosin can bind with the actin dimer. Nanninga and Mommaerts (34) and Imamura et al. (36) have shown that one mole of ATP combines with one mole of myosin at least at low concentrations of ATP. Furthermore, we (36, 44) have recently reported that one mole of intermediate of the ATPase reaction is formed per mole of myosin or H-meromyosin, and Tokuyama et al. (45) have demonstrated that there is one mole of active center of myosin ATPase in about 4×10^5 g. of myosin. Therefore, we will analyze the reaction mechanism of the H-meromyosin - F-actin - ATP system, assuming that the H-meromyosin molecule contains one ATP-binding site and one site for the binding with the actin dimer.

It was originally reported by Leadbeater and Perry (20) that, at low ionic strength and in the presence of Mg^{++} , H-meromyosin ATPase is activated by the addition of F-actin, and that the viscosity of acto-H-meromyosin in the presence of ATP is equal to the sum of those of H-meromyosin and F-actin. Yagi et al. (21) reported a remarkable "substrate inhibition", which was attributed by them to the dissociation of acto-H-meromyosin induced by ATP. However, as described in the "RESULTS" section, the phenomenon taken as "substrate inhibition" by Yagi et al. (21) must be ascribed to the inhibition of ATPase by an increase in the ionic strength at

higher ATP concentrations. At a fixed ionic strength, and especially in the presence of a high concentration of F-actin, the ATPase activity of acto-H-meromyosin increased monotonously to reach a constant value with increase in the ATP concentration. The result that the ATPase activity of acto-H-meromyosin was scarcely inhibited by EGTA does not support the "substrate inhibition" hypothesis (46). Leadbeater and Perry (20) have concluded from their viscometric studies that acto-H-meromyosin dissociates into F-actin and H-meromyosin on the addition of ATP. However, our results on the angular distribution of light-scattering of acto-H-meromyosin showed that the value of $\langle M \rangle_w$ decreased slightly and the value of $\langle r_g^2 \rangle_z^{1/2}$ increased 1.8 folds on the addition of ATP. It may be noted that the changes in viscosity and light-scattering intensity of acto-H-meromyosin induced by ATP at low ionic strength are very similar to those obtained on the myosin B - ATP system (41) and the myosin B - PP_i system at high ionic strength (42).

Both the rate of ATP hydrolysis and the decrease in light-scattering intensity at 90° of acto-H-meromyosin on the addition of ATP increased monotonously with ATP concentration to reach constant values. The concentrations of ATP necessary for inducing half the maximum changes both in the reduction of light-scattering and the ATPase activity increased with decrease in the ionic strength and with increase in the concentration of F-actin. Furthermore, it was shown that the effect of F-actin on the H-meromyosin ATPase is determined by F-actin concentration but not by

the weight ratio of F-actin to H-meromyosin. On the basis of these results, the following reaction scheme is proposed as the mechanism of reaction between acto-H-meromyosin and ATP:



where M, A and S represent, respectively, the binding units of H-meromyosin and F-actin and $MgATP^{--}$. AMS^* indicates the complex of acto-H-meromyosin with ATP, for which $\langle M \rangle_w$ is almost equal to that of AM, $\langle r_g^2 \rangle_z^{1/2}$ is much larger than that of AM and the light-scattering intensity at 90° is almost equal to that of $A + M$, as described in the "RESULTS" section. Furthermore, the exchange experiment of ADP of F-actin indicated that the state of polymerization of F-actin in AMS^* is different from that of free F-actin. The K , K_{m1} and K_{m2} are, respectively, the dissociation constant of acto-H-meromyosin into F-actin and H-meromyosin, the Michaelis constant of the myosin type ATPase and the Michaelis constant of the actomyosin type ATPase. In our experiments, the concentration of free H-meromyosin was negligibly small because of high a concentration of F-actin used, and k_2 was much larger than k_1 , since the ATPase activity was measured in the presence of 1 mM $MgCl_2$ in excess of ATP. Therefore, the relative activity of ATPase (v/V) and the relative change in the light-scattering intensity at 90° (Δ/Δ_m) are, respectively, given by

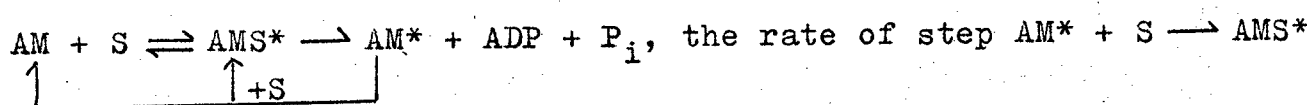
$$v/V = \frac{1}{1 + \bar{K}/[S]} \quad \text{and} \quad \Delta/\Delta_m = \frac{1}{1 + \bar{K}/[S]}$$

$$, \text{where } v = k_2 \varepsilon / \left\{ 1 + \frac{K}{A} \frac{K_{m2}}{K_{m1}} \right\} \text{ and } \bar{K} = k_{m2} / \left\{ 1 + \frac{K}{A} \frac{K_{m2}}{K_{m1}} \right\} .$$

The ATPase activity of acto-H-meromyosin increases with the concentration of F-actin, and in the presence of a fixed amount of F-actin it increases monotonously with increase in the ATP concentration to reach a maximum value. The ATP concentrations necessary for attaining half the maximum decrease in the light-scattering intensity and half the maximum value of the ATPase activity increase with increase in the F-actin concentration. They must decrease with increase in the ionic strength, if the binding of H-meromyosin to F-actin is weakened by increasing ionic strength, as is the case on binding of myosin with F-actin (40). All these conclusions drawn from the reaction scheme are in accord with the experimental results. Furthermore, the effects of treatment of H-meromyosin with PCMB and β -mercaptoethanol were very similar to those obtained when decreasing the ionic strength. This supports our previous conclusion that the binding of myosin to F-actin is strengthened by the treatment of myosin with PCMB and β -mercaptoethanol (47).

However, several phenomena could not be interpreted by the simple reaction mechanism mentioned above. First, when 0.048 mg. H-meromyosin per ml. was mixed with 0.14 mg. F-actin per ml., $\langle M \rangle_w$ increased to 2 - 3 times the value before mixing. If both F-actin and H-meromyosin are homogeneous in their binding capacities and in the size and shape properties, $\langle M \rangle_w$ should increase to 1.4 times the value before mixing. Thus, it may be concluded that F-actin particles are heterogeneous in their binding capacities,

and that H-meromyosin binds co-operatively mainly to those F-actin particles of which have a higher binding strength is higher than others*. Secondly, the concentration of ATP necessary to induce maximum change in light-scattering was smaller than that for attaining half the maximum of the ATPase activity. A probable explanation of such a phenomenon has already been given on the myosin B - ATP system (48), by assuming that in the reaction scheme,



is much larger than that of $\text{AM}^* \longrightarrow \text{AM}$.

SUMMARY

The reaction of the H-meromyosin - F-actin - ATP system was investigated in the presence of various concentrations of ATP, F-actin and KCl in 1 mM MgCl_2 and 10 mM Tris-HCl at pH 7.5 and 25°C. The following results were obtained:

1. The binding ratio of H-meromyosin to F-actin was 3.3 : 1

* This conclusion has recently been confirmed by an electron-microscopic observation on the F-actin - H-meromyosin. F-actin was added to H-meromyosin at a weight ratio of 3 : 1 (F-actin : H-meromyosin) in 5 mM KCl, 1 mM MgCl_2 and 10 mM Tris (pH 7.5). Almost all F-actin fibers were observed to have the following two structures; the one of which binding site were completely occupied by H-meromyosin, and the other, of which binding site were empty.

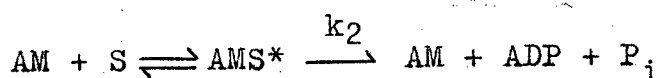
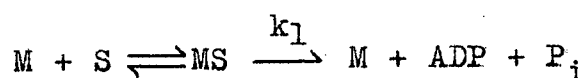
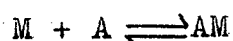
by weight. When ATP was added to acto-H-meromyosin, the intensity of light scattered at high angles decreased to a level lower than the sum of those of H-meromyosin and F-actin. The weight average molecular weight of acto-H-meromyosin estimated from the angular distribution was slightly decreased but the z-average radius of gyration was increased markedly by the addition of ATP.

2. The ATP concentration necessary for obtaining half the maximum change in the intensity of light scattered at 90° by acto-H-meromyosin was increased by decrease in the ionic strength and by increase in the F-actin concentration. At a fixed ionic strength, the ATPase activity of acto-H-meromyosin increased monotonously with the ATP concentration and the so called "substrate inhibition" was not observed. The activity in the presence of sufficient amount of ATP was increased by increase in the F-actin concentration and by decrease in the ionic strength. The ATP concentration necessary for attaining half the maximum activity was increased by decrease in the ionic strength and by increase in the F-actin concentration, and was higher than that for inducing half the maximum change in the intensity of light-scattering. The effect of treatment of H-meromyosin with PCMB and β -mercaptoethanol on the ATPase activity of acto-H-meromyosin was very similar to that observed when decreasing the ionic strength.

3. The ATPase activity of acto-H-meromyosin was unaffected by the addition of EGTA, but inhibited by the addition of EDTA. The inhibition was restored by the further addition of $MgCl_2$. About 40 - 80 per cent of ADP of F-actin was exchanged with

nucleotide in the medium, when H-meromyosin and ATP were added to F-actin.

4. From these results the following reaction scheme is proposed for the reaction between H-meromyosin, F-actin and ATP:



where M and A are the binding units of H-meromyosin and F-actin, and S is $MgATP^{--}$. k_1 and k_2 are the rate constants of ATP splitting by the myosin type and the actomyosin type ATPase, respectively, AMS^* is a complex of acto-H-meromyosin with ATP, which has a weight average molecular weight almost equal to that of AM but a radius of gyration much larger than AM. Furthermore, the polymerization state of F-actin in AMS^* is different from F-actin itself.

PART II

FORMATION OF ACTO-H-MEROMYOSIN AND ACTO-SUBFRAGMENT-1 COMPLEXES
AND THEIR DISSOCIATION BY ADENOSINE TRIPHOSPHATE

INTRODUCTION

There have been many studies on the binding reaction of myosin with actin (14,39,40,49,50). However even the stoichiometric binding ratio on a molecular basis of the actin - myosin interaction still remains controversial. Gergely and Kohler (39), Tonomura et al. (40) and many others (50-54) suggested that one molecule of myosin binds with an actin dimer from viscometric, centrifugal, light-scattering and turbidity measurements. The light-scattering studies of Sekiya et al. (55) also showed that the saturation point of actin - H-meromyosin (HMM*) binding was one mole of HMM per mole of actin dimer. On the other hand, electron microscopic observation by Huxley et al. (2), ultracentrifugal analyses by Young (56) and turbidity measurements by Tawada (57) indicated the binding of one HMM molecule to one actin monomer. Moore et al. (58) analyzed electron micrographs of negatively-stained preparations of the F-actin-subfragment-1 (S-1) complex, using the three-dimensional reconstruction technique of DeRosier and Klug (59), and showed that one S-1 molecule attaches to each of the G-actin units in F-actin. Kinetic analysis of acto-H-meromyosin-ATPase by Szentkiralyi and Oplatka (60) suggested the existence of both types of complex (in molar ratios of HMM : G-actin

* The abbreviations used are: HMM, heavy meromyosin; S-1, subfragment-1; EDTA, ethylenediaminetetraacetic acid; PCMB, p-chloromercuribenzoate; TCA, trichloroacetic acid; PP_i , inorganic pyrophosphate.

of 1 : 1 and 1 : 2), and Rizzino et al. (61) supported the formation of a 1 : 1 complex from kinetic analysis of acto-H-meromyosin-ATPase.

A large amount of recent work on the structure of the myosin molecule (cf. ref. 9) has suggested that the molecule is composed of two identical heavy chains of molecular weight 200,000 (62-67) and two or three light chains of molecular weight 14,000 to 30,000 (67-71) and that it has a double headed structure (72,73), each head yielding one S-1 (72-75). However, it is still uncertain whether the two heads of the myosin molecule are identical (9). Tonomura and his coworkers have studied the mechanism of the myosin-ATPase reaction in the pre-steady state (35,36,44,76-79), and that of dissociation of reconstituted actomyosin induced by ATP in solutions of high ionic strength (77).

In the present study, the binding ratios of HMM and S-1 to F-actin and the amount of ATP necessary to induce the dissociation on their complexes were re-investigated. The pre-steady state of the HMM- and S-1-ATPase reactions were also examined. The results were analyzed on the basis of the molecular structure of myosin and the reaction mechanism of the myosin - actin - ATP system mentioned above.

EXPERIMENTAL

Materials - Myosin was prepared from rabbit skeletal muscle by the method of Perry (23). HMM was prepared as previously described (55), except that protein was obtained from the fraction

precipitated with 45 to 55 per cent saturation of ammonium sulfate containing 0.5 mM EDTA. Ammonium sulfate was passed through a column of Amberlite IR-120 before use. In some cases, HMM were further purified by gel-filtration through a column of Sephadex G-200 equilibrated with 0.05 M KCl, 0.1 M Tris-HCl and 0.5 mM EDTA at pH 7.6 and 4°C. HMM was treated with PCMB and β -mercaptoethanol as described previously (22).

HMM used for preparing S-1 was obtained by digestion of a 15 - 20 mg. per ml. solution of myosin with trypsin (EC 3.4.4.4) (ratio of myosin to trypsin 200 : 1 by weight) for 10 minutes at 22°C. The reaction was stopped by adding 2 fold excess of soybean trypsin inhibitor and the digestion mixture was dialyzed overnight against 6.7 mM phosphate, pH 7.0. The precipitated protein was removed by centrifugation at 80,000 x g for 40 minutes. The supernatant protein fraction was precipitated by 43 to 57 per cent saturation of ammonium sulfate. S-1 was prepared from this HMM essentially according to the procedures of Young et al. (81). HMM was further digested by trypsin (ratio of HMM to trypsin 20 : 1 by weight) in 0.1 M Tris-HCl, 0.05 M KCl and 0.5 mM EDTA at pH 7.6 and 25°C for 20 minutes. The reaction was stopped by adding trypsin inhibitor at a weight ratio of inhibitor to trypsin of 2 : 1. The resulting digest was immediately chilled, applied to a column (6.5 x 70 cm) of Sephadex G-200 which had been equilibrated with 0.1 M Tris-HCl, 0.05 M KCl and 0.5 mM EDTA at pH 7.6 and 4°C, and eluted with the same buffer at a flow rate of 20 - 25 ml. per hour. The fractions in the central portion of the second peak (cf. Fig. 21)

were concentrated by precipitation with 65 per cent saturation of ammonium sulfate or in an ultrafiltration apparatus, and then purified further by chromatography on Sephadex G-200 (5 x 54 cm).

Acetone-dried powder was prepared from rabbit skeletal muscle according to the method of Ebashi and Maruyama (82), unless otherwise stated. G-actin was prepared from an acetone powder by the method of Rees and Young (83), omitting the step of Sephadex G-200 gel-filtration. Some preparations of G-actin were made by the procedures of Drabikowski and Gergely (25). The purification usually included a step of partial polymerization (84). All proteins were clarified by centrifugation at 80,000 x g for 3 hours.

γ - ^{32}P -labelled ATP was prepared from ATP and $^{32}\text{P}_i$ by the method of Glynn and Chappel (85). A crystalline preparation of the disodium salt of ATP was purchased from Kyowa Hakko Kogyo Co. Tokyo. Salt-free twice crystallized trypsin was obtained from Worthington Biochemical Corp. or Sigma Chemical Co., and soybean trypsin inhibitor, type 1-S, was from Sigma Chemical Co.

Methods - Light-scattering intensities of protein solutions were measured at an angle 90° from the incident beam in a Brice Phoenix photometer (Model 1000 D). The solvent was filtered through a Millipore filter. The solution contained 0.01 - 0.30 mg. per ml. HMM or 0.08 mg. per ml. S-1, 0.005 - 0.1 mg. per ml. F-actin, 0.005 - 0.6 M KCl, 0.05 - 1 mM MgCl_2 and 10 mM Tris-HCl at pH 7.5 - 7.6 or 20 mM phosphate at pH 7.0. All measurements were performed at 22 - 23°C. The centrifuge tube contained 5 ml. of a constant concentration of HMM of 2.9 - 3.0 mg. per ml. or S-1 of

2.3 mg. per ml. with increasing concentrations of F-actin (0.04 - 0.9 mg. per ml.) in 3.8 - 100 mM KCl, 0.2 - 1 mM MgCl₂ and 10 mM Tris-HCl at pH 7.6 or pH 8.8 or in some cases 20 mM phosphate, pH 7.0. The mixtures were centrifuged at 130,000 - 165,000 x g for 90 - 100 minutes in a Beckman, model L4, or a Hitachi, 55 P, ultracentrifuge, and the clear supernatant solution, which contained unbound protein (free HMM or S-1) was separated from the small dense pellet, which contained acto-HMM or acto-S-1 complex and F-actin. The protein concentration of the supernatant was determined. The amounts of aggregated HMM (or S-1) and G-actin in the F-actin solution were determined from the values of control tubes not containing other proteins which were centrifuged under the same conditions. The amounts of both were less than 10 per cent of the total protein in the original solution. After correction for these amounts, the number of moles of HMM or S-1 bound per mole of actin monomer, ν , was calculated, and the results were treated by the Scatchard equation (86,87);

$$\frac{\nu}{[\text{HMM}]_{\text{free}} \text{ or } [\text{S-1}]_{\text{free}}} = \frac{1}{K} (n - \nu)$$

where n is the maximum value of ν , K is the intrinsic dissociation constant of the acto-HMM (or S-1) complex, and $[\text{HMM}]_{\text{free}}$ or $[\text{S-1}]_{\text{free}}$ is the molar concentration of free HMM or S-1. The molecular weights of HMM, S-1 and actin monomer were taken to be 3.4×10^5 (37), 1.2×10^5 (81) and 4.6×10^4 (83), respectively. Sedimentation velocity experiments were performed in a Spinco Model E analytical ultracentrifuge with Schlieren optics using a

12 mm aluminium double sector cell at a rotor speed of 56,000 rpm at 14°C.

Changes in light-scattering intensities of protein solutions induced by ATP were measured in 0.05 - 2.0 M KCl, 1 - 10 mM MgCl_2 , 0.2 - 20 μM ATP and 20 mM Tris-HCl at pH 7.6 and 20 - 22°C by the stopped flow method using the mixing chamber described previously (77). The final concentrations of myosin, HMM, S-1 and F-actin were 0.8 mg. per ml., 0.2 - 1.36 mg. per ml., 0.5 mg. per ml. and 0.1 - 0.4 mg. per ml., respectively.

The Ca^{2+} -activated ATPase activities of HMM and S-1 were determined in 0.05 mg. per ml. protein, 1.0 M KCl, 7 mM CaCl_2 , 1 mM ATP and 50 mM Tris-maleate at pH 7.0 by the method described previously (44). EDTA-activated ATPase was measured under the same conditions, except that 3 mM EDTA was used instead of CaCl_2 . Mg^{2+} -activated ATPase activities were measured in 0.1 - 0.5 mg. per ml. HMM or S-1, 0.03 - 1 mM ATP, 3.9 - 5 mM KCl, 0.5 - 2 mM MgCl_2 and 10 - 20 mM Tris-HCl at pH 7.6 and 25°C. The ATPase activities of S-1 obtained from Sephadex G-200 were measured in 0.25 M KCl, 5 mM CaCl_2 , 1 mM ATP and 50 mM Tris-HCl at pH 7.6 and 25°C. To measure the amounts of the initial burst of $^{32}\text{P}_i$ -liberation from the HMM-AT ^{32}P and the S-1-AT ^{32}P systems, the reaction was initiated by the addition of 0.5 ml. of ^{32}P -ATP solution (0.03 - 0.1 mM) to 0.5 ml. of reaction mixture containing 2 mg. per ml. protein. Both solutions contained 0.0039 - 0.5 M KCl, 2 mM MgCl_2 and 20 mM Tris-HCl at pH 7.6. The reaction was stopped by adding 1 ml. of 10 per cent TCA, 1 mM cold ATP and 50 μM cold P_i as carriers.

$^{32}\text{P}_i$ -liberation was determined as described previously (76).

The method of negative staining described by Huxley (2) was used with slight modifications. A drop of solution containing 0.045 mg. per ml. HMM, 0.03 mg. per ml. F-actin, 5 mM KCl, 1 mM MgCl_2 and 10 mM Tris-HCl (pH 7.5) was placed on a carbon coated, plastic microgrid which had been perforated, washed with the solvent, and stained with 0.5 - 1.0 per cent uranyl acetate. The preparation was examined in a JEM-7 electron microscope using an accelerating voltage of 80 KV. A histogram of the theoretical random distribution of HMM on the actin filament was obtained from the following equation. It was assumed (1) that a maximum of 55 HMM molecules attached per 1μ length of F-actin filament are observable by electron microscopy, (2) that the sites are not influenced by neighboring site and (3) that the intrinsic affinity constant of all the sites for HMM are identical:

$$P_k = \binom{n}{k} \binom{Nn - n}{M - k} / \binom{Nn}{M}$$

where P_k is the probability that k molecules of HMM bind to n sites on 1μ of F-actin filament, N is the total lengths of the F-actin filament, n is the number of binding sites of HMM on 1μ of F-actin filament and M is the total number of HMM molecules. If N is very large, as in our experiments, P_k can be represented approximated by the following equation

$$P_k = \binom{n}{k} (1 - Q)^{n-k} Q^k$$

where Q is M/Nn . If the maximum binding ratio of HMM to F-actin is 3.6 : 1 by weight, Q is 1.5/3.6 in solution contained 0.045 mg.

per ml. HMM and 0.03 mg. per ml. F-actin.

The concentrations of myosin and actin were determined by the biuret reaction calibrated by the micro-Kjeldahl procedure, and those of HMM and S-1 were determined using values for $E_{1\text{cm}}^{1\%}$ at 280 nm and pH 7.6 of 6.47 and 7.70, respectively (81).

RESULTS

Stoichiometry of the Combination of HMM with Actin - Figure 1 shows a typical saturation curve of light-scattering measurements, when various concentrations of HMM from 0.01 to 0.2 mg. per ml. were added to 0.02 mg. per ml. of F-actin in 5 mM KCl, 1 mM MgCl_2 and 10 mM Tris-HCl at pH 7.5 and 25°C. The light-scattering intensity increased with the concentration of HMM and the saturation endpoint was at an HMM to actin ratio of 4.2 : 1 by weight. Therefore, the maximum weight of F-actin which can be bound by 3.4×10^5 g. of HMM is 8.1×10^4 g., i.e., 1 mole of HMM binds with 1.8 moles of actin monomer. The saturation curve of F-actin bound with HMM was often sigmoidal (○, Fig. 18) as previously observed for actin-myosin binding (40). When increasing concentrations of HMM were added to a fixed concentration of G-actin (0.02 mg. per ml.) in 5 mM KCl and 3 mM Tris-HCl at pH 7.5 and 22°C, the saturation curve of the light-scattering intensity was not sigmoidal but linear (●, Fig. 18).

Results on the binding ratios of various actin preparations and HMM obtained by light-scattering measurements under different conditions are summarized in Table I. Acetone dried powders were

prepared by the method by Straub (88) and also by that of Ebashi and Maruyama (82) to remove contaminating α -actinin. G-Actin was prepared by the method of Ress and Young (83) or by the method of Drabikowski and Gergely (25), and tropomyosin and denatured G-actin contained in the actin preparation were removed by partial polymerization with 0.6 mM MgCl_2 (84). The binding ratio was not affected by the method of preparation. Moreover it was not affected by the extraction temperature (0°, 20°C), actin concentration (0.02 - 0.06 mg. per ml.), KCl concentration (0.005 - 0.6 M), MgCl_2 concentration (0 - 1 mM) or pH (7.0, 7.5). The treatment of HMM with PCMB- β -mercaptoethanol, which strengthens actin-HMM binding in the presence of ATP (55), did not change the maximum binding ratio of HMM to actin. The average of the values in 21 different experiments was 1 : 4.07 \pm 0.085 by weight (actin : HMM), i.e., 1.8 moles of actin monomer : one mole of HMM.

Unbound HMM cannot be estimated directly by light-scattering measurements, so it was measured by ultracentrifugal analysis. Scatchard plots for the binding of HMM with F-actin are given in Fig. 19. The values were not very accurate, but the apparent intrinsic dissociation constants obtained from the slopes were 2.6×10^{-7} M at 3 - 4°C (0.34 - 6.8 mg. per ml. HMM, 0.046 - 0.46 mg. per ml. F-actin, 0.1 M KCl, 1 mM MgCl_2 , 10 mM Tris-HCl, pH 8.8 and 3 mg. per ml. HMM, 0.05 - 0.6 mg. per ml. F-actin, 0.1 M KCl, 1 mM MgCl_2 , 10 mM Tris-HCl, pH 7.6) and 1.6×10^{-7} M at 22°C (3 mg. per ml. HMM, 0.05 - 0.6 mg. per ml. F-actin, 0.1 M KCl, 1 mM MgCl_2 , 10 mM Tris-HCl, pH 7.6). These values were much lower

than those reported previously by other workers (56,61). These small dissociation constants indicate that nearly all the added HMM is bound to F-actin in light-scattering experiments. The intercept of the lines with the abscissa indicates the maximum binding number of 0.46 - 0.52 mole of HMM per mole of actin monomer, i.e., the binding of one mole of HMM to 1.9 - 2.2 moles of actin monomer, which is almost equal to that obtained from light-scattering experiments.

When F-actin was saturated with HMM, a maximum of 55 bound HMM molecules per 1 μ of F-actin filament could be counted from electron micrographs. Figure 20A indicates the distribution of the numbers of bound HMM molecules per 1 μ of F-actin filament, when 0.03 mg. per ml. of F-actin was mixed with 0.045 mg. per ml. of HMM in 5 mM KCl, 1 mM MgCl₂ and 10 mM Tris-HCl at pH 7.5 and room temperature. In the actin preparations shown in Fig. 3A, the histogram had a peak at about 16 - 20 molecules per μ of F-actin, which coincided rather well with that for the random distribution, assuming that the maximum binding ratio of HMM to F-actin is 3.6 : 1 by weight and that the sites of binding of HMM to F-actin are independent and identical (see "EXPERIMENTAL"). However, some actin preparations showed a broad distribution with two peaks at 6 - 10 molecules and at 25 - 30 molecules per μ of F-actin, as shown in Fig. 20B.

Purity and Stability of Subfragment-1 - Tryptic digests of HMM were prepared as described in the "EXPERIMENTAL" and fractionated on a column of Sephadex G-200 (Fig. 21). Four distinct peaks were

obtained. The first peak emerged near the void volume and was probably formed from undigested HMM and rod-like fragments of HMM, since the size of this peak decreased with increase in the extent of digestion and the Ca^{2+} -activated ATPase activity of this peak was lower than that of undigested HMM. The second peak contained high ATPase activity and was precipitated with 65 per cent saturation of ammonium sulfate. When this fraction was rechromatographed on Sephadex G-200, a single peak appeared with a small shoulder near the void volume (Fig. 22). Almost all the Ca^{2+} -ATPase activity was associated with the main peak. The sedimentation diagram of the main peak showed a symmetrical single boundary and a slower sedimenting component(s) with a broad peak at the base-line (Fig. 23).

The apparent sedimentation constant at 20°C was 5.5 - 5.7 S. Therefore, the material in the second peak in Fig. 22 was S-1. The third peak contained trypsin and trypsin inhibitor, since its size varied with the amounts of trypsin and inhibitor used. The fourth peak may be composed of peptide fragments of HMM. The amount of material in the second peak was 47 - 57 per cent of the original amount of HMM. After rechromatography, S-1 was stored in 0.05 M KCl, 20 mM Tris-HCl and 0.5 mM EDTA at pH 7.6 and 0°C. As shown in Fig. 23, the Ca^{2+} - and EDTA-activated ATPase activities and the sedimentation pattern did not change significantly during storage for 24 days. When myosin was digested to HMM, its Ca^{2+} -activated ATPase activities usually increased about 1.4 - 1.5 fold on a weight basis. This corresponds to a 30 per cent reduction in

myosin mass with no change of the active sites. The Ca^{2+} -activated ATPase activity of HMM and S-1, in 1 M KCl and 7 mM CaCl_2 at pH 7.0 and 25°C, were, respectively, 255 and 350 - 400 $\mu\text{moles P}_i$ per minute per g. of protein, i.e., 1 : 1.4 - 1.6. The Mg^{2+} -activated ATPase activities of HMM and S-1, in 4 - 5 mM KCl and 0.5 - 2 mM MgCl_2 at pH 7.6 and 25°C, were, respectively, 20.0 - 24.6 and 31.5 - 62 $\mu\text{moles P}_i$ per minute per g. of protein, i.e., 1 : 1.6 - 2.5. The ATPase activity ratios of HMM to S-1 were almost equal to the value of 1 : 1.4, calculated assuming that the active site of ATPase is not affected by digestion and that one HMM molecule yields two S-1 units (74,75).

Stoichiometry of the Combination of S-1 with Actin - Figure 24 shows the change of light-scattering intensity when various amounts of F-actin (0.005 - 0.1 mg. per ml.) were added to 0.08 mg. per ml. of S-1. The light-scattering intensities increased with increase in the concentration of actin, and reached a saturation point. From this point, it was calculated that 1.2×10^5 g. of S-1 binds with 4.6×10^4 g. of F-actin, i.e., one mole of S-1 binds with one mole of actin monomer.

Using different Mg^{2+} concentrations (0.2 or 1 mM), KCl concentrations (3.8 - 100 mM) and temperatures (2 or 16.5°C), various concentrations of F-actin (0.023 - 0.93 mg. per ml.) were added to 2.3 mg. per ml. of S-1 in 10 mM Tris-HCl at pH 7.6 and the amounts of free S-1 were obtained by centrifugation. Figure 25 shows Scatchard plots of the results of 4 experiments. It was difficult to determine the dissociation constant, since the

results were not accurate and values varied from 3.8×10^{-7} to 1.22×10^{-6} M. However, the limiting binding ratio was calculated to be 0.9 - 1.08 mole of S-1 per mole of actin monomer, which agrees well with the value obtained by the light-scattering method.

Stoichiometric Liberation of P_i in the Initial Phase - On the initial rapid liberation of P_i from the myosin-ATP system, Tonomura et al. indicated that one mole of P_i was liberated per 4×10^5 g. of myosin when the Mg^{2+} concentration was above 1 mM (35,36,44, 76-79). The amounts of initial rapid P_i -liberation from the HMM-ATP (36,75) and S-1-ATP (75,89) system were about 1 and 0.5 mole per mole of protein, respectively. Previous experiments were made at high ionic strengths, so I reinvestigated the initial phase of P_i -liberation with the HMM and S-1 ATP-system using a low ionic strength like that used in experiments on the binding of HMM and S-1 to F-actin. Figure 26 shows the time-course of P_i -liberation from the HMM-ATP system measured with 1 mg. per ml. of HMM, 0.03 - 0.1 mM ATP, 2 mM $MgCl_2$, 20 mM Tris-HCl and 0.005 or 0.5 M KCl at pH 7.6 and 25°C. The amounts of the initial burst in 0.005 and 0.5 M KCl were 1.0 and 1.2 mole per 3.4×10^5 g. of HMM, respectively. The time-courses of P_i -liberation from the S-1-ATP system with 1 mg. per ml. of S-1, 0.03 - 0.1 mM ATP, 2 mM $MgCl_2$ and 20 mM Tris-HCl are shown in Fig. 27. The amounts of the initial burst measured in 0.0039 or 0.5 M KCl were both 0.5 mole per 1.2×10^5 g. of S-1.

Dissociation of the Acto-myosin, Acto-HMM and Acto-S-1 Complexes by ATP - Various concentrations of ATP (0 - 10 μ M) were added to the complexes formed from 0.8 mg. per ml. of myosin and

0.4 mg. per ml. F-actin in 1 M KCl, 10 mM MgCl₂ and 20 mM Tris-HCl at pH 7.6 and 20 C, and the extents of decrease in light-scattering intensities were measured (Fig. 28). The light-scattering intensities decreased linearly with the ATP concentration, until the latter reached 1.75 μ M, i.e., 1 mole per 4.0×10^5 g. of myosin, and remained constant at higher ATP concentrations. The stiochiometric value was equal to that of the previous study (77).

Figure 29 shows the amount of decrease in light-scattering intensity after adding various concentrations of ATP from 0 to 20 μ M to the acto-HMM complex reconstituted from 1.36 mg. per ml. HMM and 0.368 mg. per ml. of F-actin in 1 mM MgCl₂, 20 mM Tris-HCl and 0.1 or 0.5 M KCl at pH 7.6 and 21°C. The light-scattering intensities decreased linearly with increasing the ATP concentration and the minimum amount of ATP required to obtain the maximum decrease in light-scattering intensity of the complex both in 0.1 and 0.5 M KCl was 9.1 μ M, i.e., 2.27 moles per 3.4×10^5 g. of HMM. Table II summarizes the minimum amounts of ATP required for the maximum decrease in light-scattering intensity of the acto-HMM complex under various conditions (0.2 - 1.36 mg. per ml. HMM, 0.1 - 0.368 mg. per ml. F-actin, 0.1 - 1.0 M KCl, 1 - 5 mM MgCl₂). The average of these values was 2.20 moles per 3.4×10^5 g. of HMM, and the value decreased slightly with increase in the concentrations of MgCl₂, KCl and protein, as previously reported for the actomyosin-ATP system (77).

On addition of various concentrations of ATP (0.5 - 10 μ M) to the complexes formed from 0.5 mg. per ml. of S-1 and 0.192 mg.

per ml. of F-actin in 1 mM MgCl_2 , 20 mM Tris-HCl and 0.05 - 0.5 M KCl at pH 7.6 and 22°C, the light-scattering intensity of acto-S-1 decreased with increase in the ATP concentration and the maximum decrease was reached at one mole of ATP per 1.2×10^5 g. of S-1 (Fig. 30). The relative amount of decrease in light-scattering intensity was unaffected by increase in the KCl concentration from 0.05 to 0.5 M.

DISCUSSION

Comparing the results obtained in the present work, which are summarized in Table III, with those obtained previously, it can be concluded that myosin (40) and HMM have two binding sites for actin, whereas subfragment-1 has only one site. Moreover the amounts of ATP required for dissociation of the acto-HMM and acto-S-1 complexes are two and one mole per mole of HMM and S-1, respectively, while dissociation of the actomyosin complex is induced by one mole of ATP per mole of myosin.

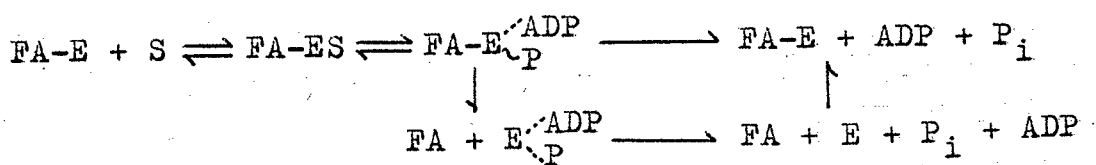
Gergely and Kohler (39) and Tonomura et al. (40) suggested that one myosin molecule binds with one actin dimer from light-scattering measurements. On the other hand, Young (56) reported a binding ratio of one HMM molecule per actin monomer from ultracentrifugal analyses. Ultracentrifugal methods can be used for direct estimation of the amount of unbound reactant in a multi-component system but interpretation of results may be complicated by the effect of pressure on the equilibrium of a system. As described in the "RESULTS", the ratio of the binding of HMM to

actin obtained by the ultracentrifugal method agreed well with that obtained by the light-scattering method, but it did not agree with that obtained by Young. This discrepancy could not be attributed to contamination of our F-actin preparation by significant amounts of impurities, since on centrifugation of our preparation the amount of proteins in the supernatant was less than 10 per cent of the total protein concentration of the F-actin, and since the ratio was not affected by the method of purification of actin (Table I). Recently, Rizzino et al. (61) calculated from kinetic analyses of acto-HMM-ATPase that the stoichiometry of actin-HMM binding is one-to-one. Szentkiralyi and Oplatka (60) concluded from kinetic analyses of ATPase that HMM and F-actin can form two types of complex and the stoichiometry of the complex obtained is affected by the ratio of actin to HMM. However, the molecular mechanism of the acto-HMM-ATPase reaction is much more complicated than is usually thought (cf. ref. 9), and I think that it is very difficult to deduce the stoichiometry from kinetic studies on ATPase. Furthermore, it is possible that the interactions between actin and HMM differ in the presence and absence of ATP. Further experiments are required on this problem. Experiments on the binding of S-1 to F-actin indicated a stoichiometry of one mole of S-1 per mole of actin monomer. Previously, it was reported that all the S-1 binds with actin (5). Thus it is concluded that both the heads of the myosin molecule have an actin binding site. Although the Scatchard plot seems to be almost linear, the affinity constant is too large to distinguish whether the two sites are

identical or not.

Electron microscopic observation of actin-HMM binding showed that there are two different kinds of distribution of HMM bound to F-actin. In one type of acto-HMM, HMM molecules seem to bind to F-actin independently, whereas in the other type HMM molecules seem to bind to actin co-operatively. The former type of binding was usually observed using F-actin contaminated with regulatory protein(s), while the latter type was seen using purified actin. The mechanism of the change in distribution of binding of HMM on F-actin, which is probably induced by regulatory protein(s), requires clarification.

The amounts of the initial burst of P_i -liberation with HMM and S-1 were one mole per 3.4×10^5 g. of HMM and 0.5 mole per 1.2×10^5 g. of S-1, respectively. Thus, only one of the 2 heads of myosin and HMM has a site for the initial burst of P_i -liberation. Onishi and Tonomura (77) proposed the following mechanism (9,77) for the dissociation of reconstituted actomyosin by ATP;



The dissociation of the actomyosin complex (FA-E) is induced by formation of myosin-phosphate-ADP ($\text{E} \begin{array}{c} \text{ADP} \\ \diagup \text{P} \end{array}$). To clarify the molecular mechanism of the reactions studied in this paper, it is essential to know the submolecular structure of myosin. As discussed in detail in recent monograph (9), there is much evidence to support the view that the two heads of the myosin molecule have identical

structure and function. For example, nucleotide and PP_i binding studies indicated that in binding of two moles of ligand per mole of myosin or HMM the two sites having essentially the same affinities (74,90-92). However, the fact that the bindings of two moles of nucleotide or PP_i per mole of myosin have identical dissociation constants under special conditions is not sufficient to provide that the two binding sites are identical. From kinetic analyses of ATPase Eisenberg and Moos (93) concluded that the myosin, HMM and S-1 molecules, respectively, had 2, 2 and 1 binding sites for ATP per molecule of protein. The possibility that the subunits were identical was also supported by the results of chemical modification. Sekine et al. (94,95) demonstrated that the EDTA-ATPase activity of myosin is completely suppressed by modification of one mole of sulfhydryl group in each of the subunit chains of 2.0×10^5 g. of myosin. Murphy and Morales (96) concluded that myosin has two ATPase sites from results of affinity labelling of the site by 6-mercapto-9- β -D-ribofuranosylpurine 5'-triphosphate. Furthermore, Seidel et al. (97) indicated the presence of two rapidly reacting groups per myosin molecule by titration of myosin with the spin label, N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidiny1) iodoacetamide. However, the reaction mechanism of ATPase is rather complicated (cf. ref. 9 for review) and the myosin molecule is composed of two identical heavy chains and 2-3 light chains. Therefore, these results cannot be accepted as conclusive evidence that the two heads are identical.

On the other hand, some years ago Tonomura and Morita (98)

reported that two mole of PP_i combined with one mole of myosin and had different dissociation constants and that one mole of PP_i combined with one mole of myosin in actomyosin. Morita (99,100) showed that the two nucleotide binding sites in HMM have different affinities for ATP and ADP, only one site being involved in the generation of the difference spectrum. Furthermore, Tonomura and his coworkers showed that the binding of one mole of reagents such as p-nitrothiophenol (78), trinitrobenzenesulfonate (45), diazonium-1-H-tetrazole (101) or monoiodoacetamide (75,102) to a specific site per mole of myosin induces a maximum change in enzyme activity. Takashina (103) recently reported that the ATPase activity of myosin was completely inhibited by reaction of one molecule of the fluorescent dye, 1-dimethylaminonaphthalene-5-sulfonyl chloride, per 3.6×10^5 g. of HMM. The best evidence that the two heads are not identical is the existence of one phosphorylating site on the myosin molecule, i.e., only one of the two heads has this site (75). Recent support for the idea that the heads are not identical has also come from investigation of the light-chains in the myosin molecule. Paterson and Strohman (104) reported that the myosin molecule is composed of two identical heavy chains and two-non-identical component proteins of the low molecular weight with approximate molecular weights of 18,500 - 19,500 and 32,000 - 33,000. More recently, Sarker and Cooke (105) showed that the myosin molecule contains three light chains with molecular weights of 25,500, 17,000 and 15,200. Hayashi (106) obtained similar results and obtained circumstantial evidence for the non-identity

of the two heads from studies on the submolecular structure of S-1.

Figure 31 shows various models of the interaction of HMM with actin and ATP. In the first model (Fig. 31 (a), (b)), the two heads are assumed to be non-identical: both heads have actin binding sites but only one head has a phosphorylating site while the other has a simple binding site for ATP. This model is further divided into two types in which the heads show co-operative and independent binding with F-actin. In Fig. 31 (a), only one of two heads (S-1a) has a phosphorylating site, and when the protein-phosphate-ADP complex, $E \cdot \overset{ADP}{P}$, is formed on this site, the two heads dissociate from actin co-operatively. In Fig. 31 (b), the bindings of the two non-identical heads to actin are independent, and these two heads (S-1a and S-1b) are dissociated from F-actin by the formation of $E \cdot \overset{ADP}{P}$ and $E \cdots S$ (simple binding with ATP), respectively. The two sites have different affinities for ATP, but the affinity constants may be too large to be distinguishable from each other under our experimental conditions.

In contrast, if the two heads are identical, the affinity constants of the actin binding sites of the two heads should be equal and the affinities of their binding sites for ATP should be the same. Fig. 31 (c) shows the case of heads on which interaction is co-operative. When either of the two heads forms $E \cdot \overset{ADP}{P}$ and its binding with actin is broken, the other head co-operatively dissociates from actin. When the interaction of the two heads are independent (Fig. 31 (d)), both heads form $E \cdot \overset{ADP}{P}$ and dissociate from actin.

As described in the "RESULTS", HMM binds with an actin dimer, and S-1 with an actin monomer. Furthermore, the amounts of the initial burst of P_i -liberation, i.e., $E_{P_i}^{ADP}$ were one mole per mole of HMM and 0.5 mole per mole of S-1. The amounts of ATP necessary to dissociate the acto-HMM and actin-S-1 complexes were two moles per mole of HMM and one mole per mole of S-1. These results can be most easily explained by model (b), and are inconsistent with model (d), but they do not exclude models (a) and (c). If model (b) is accepted for acto-HMM, model (a) seems to be the most reasonable for the interaction between myosin, actin and ATP, since in this case the amount of ATP necessary for dissociation is one mole per mole of myosin. However, further work is required to establish the validity of this model and the further characterization of the structure and function of the different light chains in the myosin molecule would be very valuable.

SUMMARY

1. The binding ratios of heavy meromyosin, HMM, and subfragment-1, S-1, of myosin to F-actin were measured by light-scattering and ultracentrifugal methods. The ratios were one mole of HMM per mole of actin dimer with a dissociation constant of $1.6 - 2.6 \times 10^{-7}$ M and one mole of S-1 per mole of actin monomer. They were not affected by the method of preparation of actin or the KCl and $MgCl_2$ concentrations. The distribution of HMM on the F-actin filament was analyzed by electron microscopy. Some acto-H-meromyosin preparations showed a random distribution of HMM on F-actin,

while others showed a distribution suggesting co-operative binding of HMM to F-actin.

2. The amounts of the initial burst of P_i -liberation from the HMM-ATP and the S-1-ATP systems were one mole and 0.5 mole per mole of protein, respectively. The values were not affected by ionic strength.

3. The decrease in light-scattering intensity after adding ATP to acto-myosin, acto-HMM and acto-S-1 complexes was measured by a stopped flow method. The minimum amounts of ATP required for the maximum decrease in light-scattering intensity were 1.0, 2.2 and 1.0 moles per mole of myosin, HMM and S-1, respectively.

4. From these results it was concluded that each of the two head parts of HMM has one binding site for actin, and that the binding of one site with actin is dissociated by formation of the HMM-phosphate-ADP complex, while the binding of the other site is broken by simple binding of ATP.

ACKNOWLEDGMENTS

I wish to thank Prof. Y. Tonomura for his valuable comments and continuous encouragement through these works.

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TABLE I. Ratio of combination of actin and H-meromyosin. The binding ratio was obtained from light-scattering measurements at pH 7.5 and room temperature.

Actin prep. ¹⁾	Ext. temp.	Actin (mg/ml)	KCl (mM)	Conditions	Weight ratio (actin : HMM)
S.D.	0-2°C	0.03	5	1 mM MgCl ₂ , 10 mM Tris-HCl	1 : 3.50
S.D.	0-2	0.03	200	1 mM MgCl ₂ , 10 mM Tris-HCl	1 : 3.44
S.D.	0-2	0.03	5	1 mM MgCl ₂ , 10 mM Tris-HCl	1 : 5.33
S.D.	0-2	0.03	200	1 mM MgCl ₂ , 10 mM Tris-HCl	1 : 5.33
S.D.	0-2	0.03	600	1 mM MgCl ₂ , 10 mM Tris-HCl	1 : 5.00
S.R.	0-2	0.03	5	1 mM MgCl ₂ , 10 mM Tris-HCl	1 : 4.34
S.R.	0-2	0.03	5	1 mM MgCl ₂ , 10 mM Tris-HCl	1 : 4.00
S.R.	0-2	0.03	200	1 mM MgCl ₂ , 10 mM Tris-HCl	1 : 4.00
S.R.	0-2	0.03	5	1 mM MgCl ₂ , 10 mM Tris-HCl	1 : 3.66
S.R.	0-2	0.03	5	1 mM MgCl ₂ , 10 mM Tris-HCl (PCMB-HMM)	1 : 3.66
E.R.	0-2	0.03	5	10 μ M MgCl ₂ , 10 mM Tris-HCl	1 : 4.83
E.R.	0-2	0.02	5	10 μ M MgCl ₂ , 10 mM Tris-HCl	1 : 4.25
E.R. (G-actin)	0-2	0.02	0	4 mM Tris-HCl	1 : 4.00
E.R. (G-actin)	0-2	0.02	5	10 μ M MgCl ₂ , 10 mM Tris-HCl	1 : 4.00
E.D.	20	0.02	5	10 μ M MgCl ₂ , 10 mM Tris-HCl	1 : 4.00
E.D.	20	0.02	5	10 μ M MgCl ₂ , 10 mM Tris-HCl	1 : 4.25
E.D. (G-actin)	20	0.02	5	3 mM Tris-HCl	1 : 3.70
E.D.	0-2	0.06	5	30 μ M MgCl ₂ , 10 mM Tris-HCl	1 : 3.83
E.D.	0-2	0.04	5	30 μ M MgCl ₂ , 10 mM Tris-HCl	1 : 3.75
E.D.	0-2	0.02	5	30 μ M MgCl ₂ , 10 mM Tris-HCl	1 : 3.65
E.D.	20	0.02	100	1 mM MgCl ₂ , 20 mM phosphate (pH 7.0)	1 : 4.00
Av.					1 : 4.07 \pm 0.0846

¹⁾ S, Acetone dried powder prepared by the method of Straub with slight modifications; E, acetone dried powder prepared by the method by Ebashi and Maruyama; D, G-actin prepared by the method of Drabikowski; R, G-actin prepared by the method of Rees and Young with slight modifications.

TABLE II. Minimum amount of ATP required for the maximum decrease in light-scattering intensity of acto-HMM.

20 mM Tris-HCl, pH 7.6, 21 - 23°C

Exp.	HMM (mg/ml)	F-actin (mg/ml)	KCl (M)	MgCl ₂ (mM)	mole ATP
					3.4 x 10 ⁵ g of HMM
1	1.36	0.368	0.1	1	2.27
2	1.36	0.368	0.5	1	2.27
3	1.36	0.368	0.5	2	2.00
4	0.68	0.184	0.1	1	2.45
5	0.68	0.184	0.1	5	2.00
6	0.20	0.10	0.1	2	2.38
7	0.20	0.10	0.5	2	2.14
8	0.20	0.10	1.0	2	2.14

TABLE III. Complex-formation of myosin and its subfragments with F-actin and dissociation of complexes on addition of ATP

Protein	Binding with F-actin (mole of actin monomer/mole of protein)	Initial Burst (mole/mole of protein)	Amount of ATP necessary to dissociation (mole/mole of protein)
Myosin	2 ¹⁾	1 ²⁾	1
HMM	2	1	2
S-1	1	0.5	1

1) The value measured by Tonomura et al. (40).

2) The value measured in our laboratory (35,36,44,76-79).

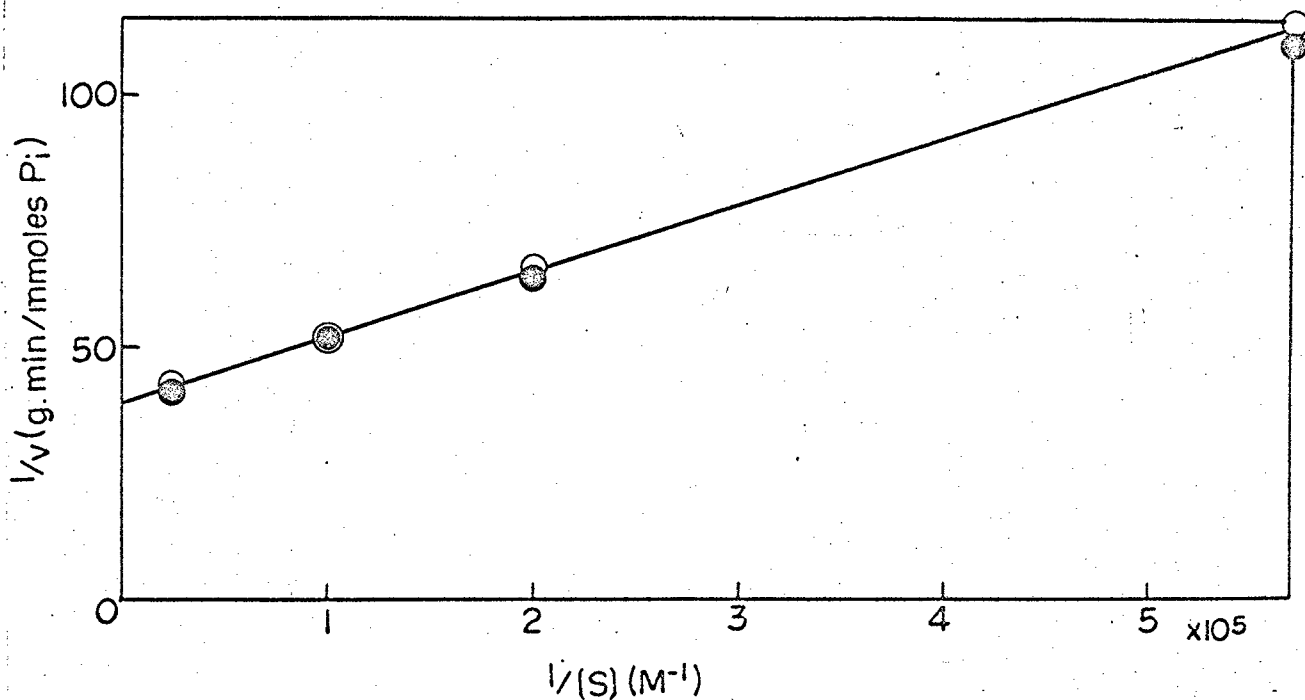


Fig. 1. Lineweaver-Burk plot of H-meromyosin ATPase coupled with a ATP generating system.

0.2 mg. H-meromyosin/ml., 40 μ g. pyruvate kinase/ml., 0.4 mM PEP, 1 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 25°C.

KCl concentration: \bigcirc , 6; \bullet , 4 mM.

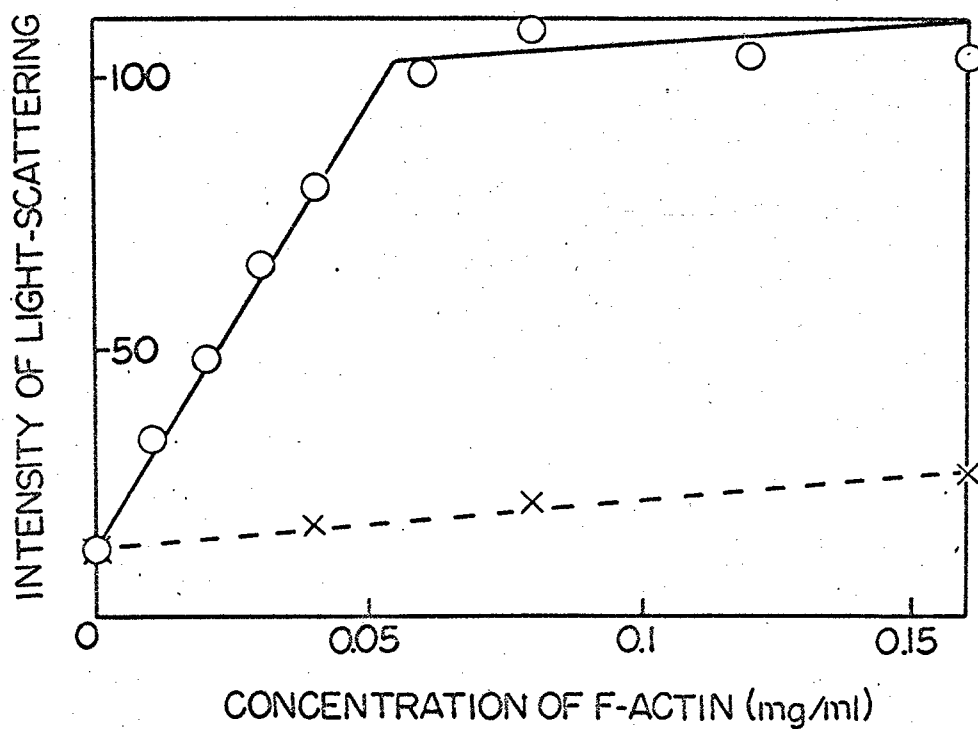


Fig. 2. Binding of F-actin to H-meromyosin measured by increase in light-scattering intensity at 90° .

0.16 mg. H-meromyosin/ml., 4 mM KCl, 1 mM MgCl_2 , 10 mM Tris-HCl, pH 7.5, 25°C . \bigcirc , Acto-H-meromyosin; \times , sum of H-meromyosin and F-actin.

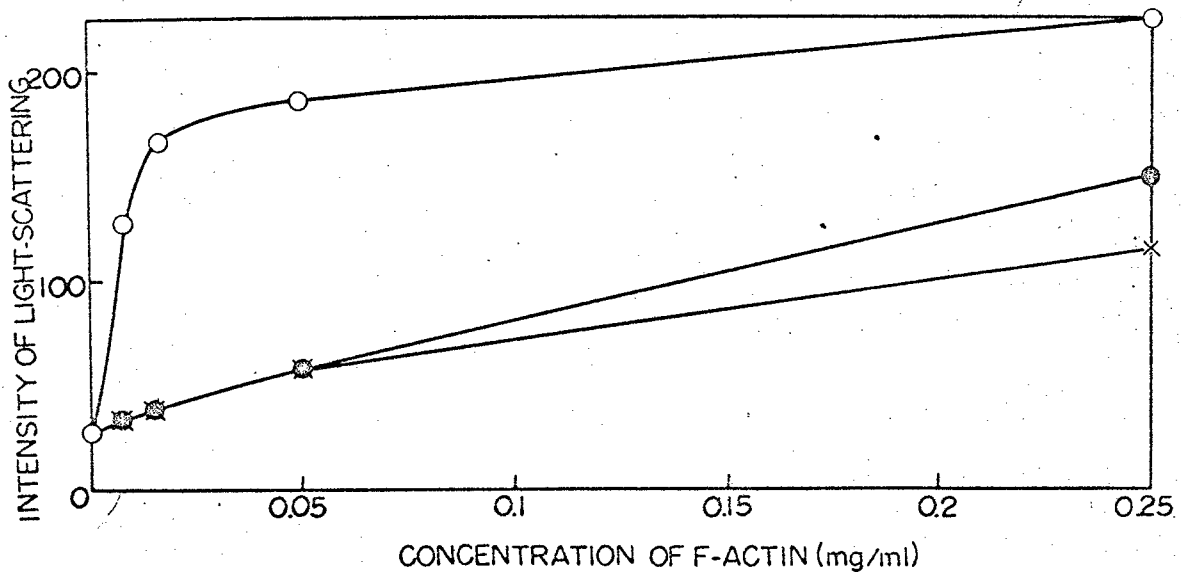


Fig. 3. Decrease in light-scattering intensity at 90° of acto-H-meromyosin by the addition of ATP.

0.05 mg. H-meromyosin/ml., 20 μ g. pyruvate kinase/ml., 0.4 mM PEP, 5 mM KCl, 1 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 25°C.

○ , After mixing.

● , Before mixing of H-meromyosin and F-actin.

× , On the addition of 0.5 mM ATP to acto-H-meromyosin.

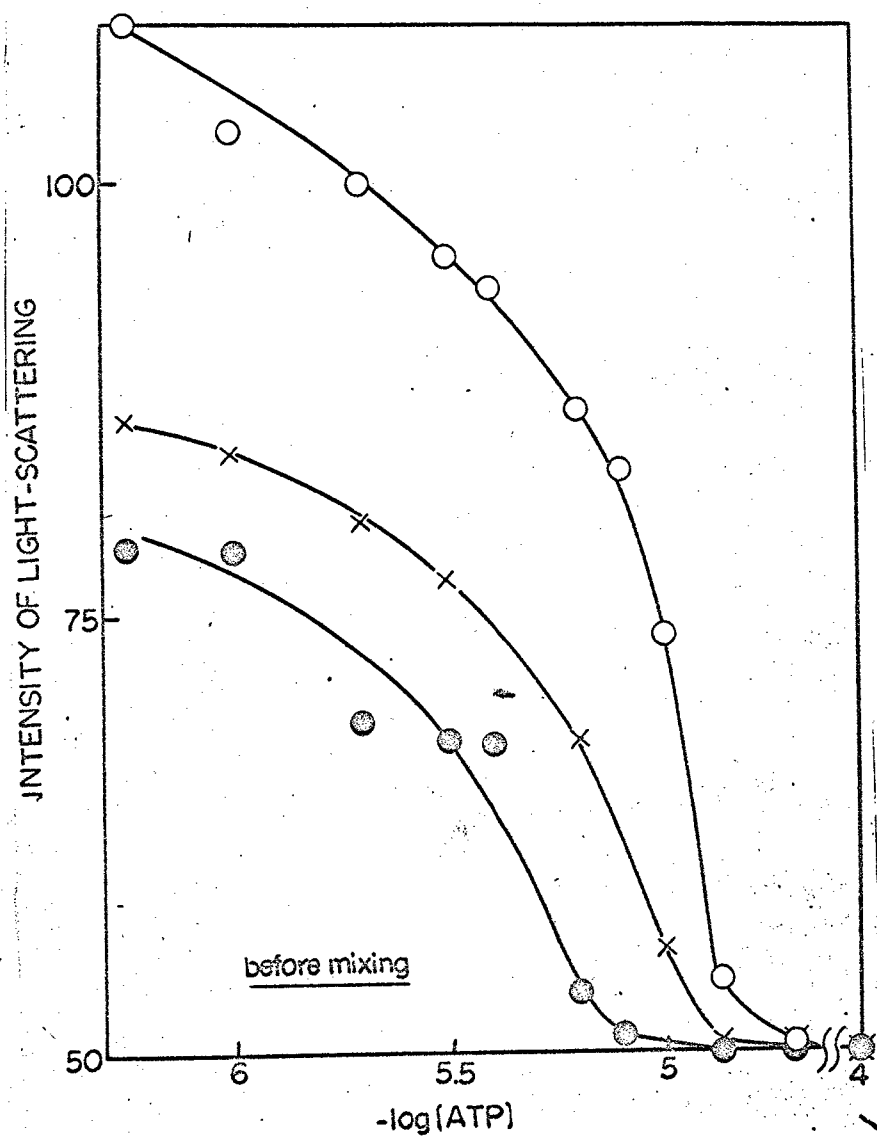


Fig. 4. Effect of ionic strength on decrease in light-scattering intensity at 90° of acto-H-meromyosin induced by ATP.

0.05 mg. H-meromyosin/ml., 0.14 mg. F-actin/ml., 30 μ g. pyruvate kinase/ml., 0.4 mM PEP, 1 mM MgCl_2 , in excess of ATP, 10 mM Tris-HCl, pH 7.5, 25°C .

KCl concentration: \bigcirc , 5; \times , 30; \bullet , 120 mM.

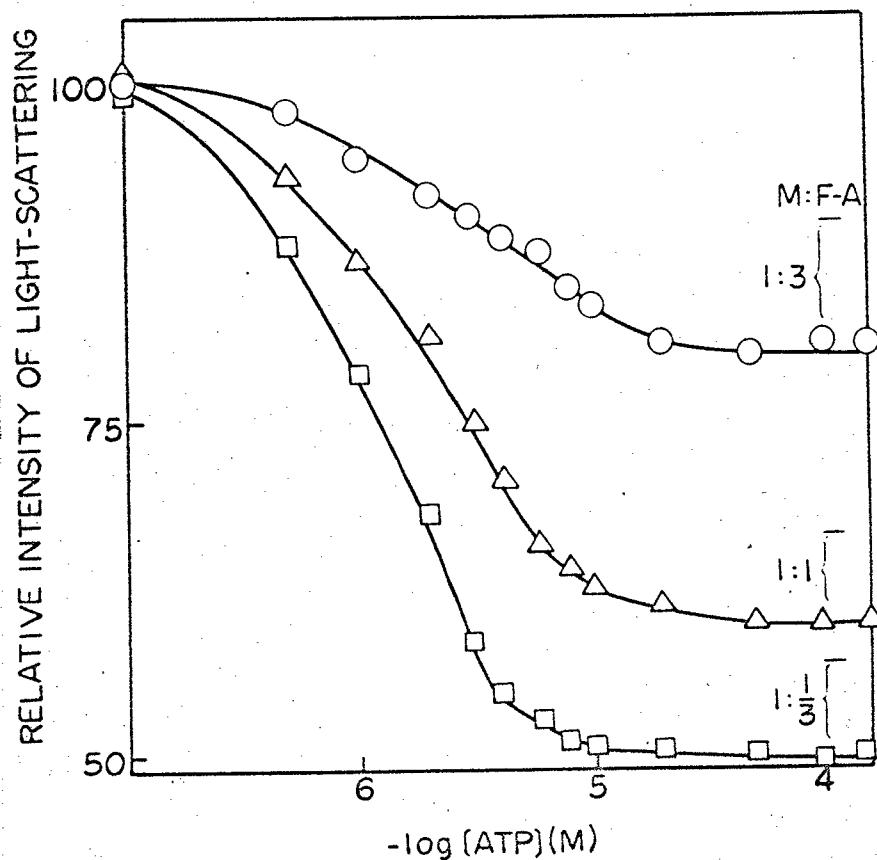


Fig. 5. Effect of ratio of H-meromyosin to F-actin on decrease in light-scattering intensity at 90° of acto-H-meromyosin induced by ATP.

Bars represent sum of intensities due to both proteins.
 0.05 mg. H-meromyosin/ml., 20 μ g. pyruvate kinase/ml., 0.4 mM PEP, 5 mM KCl, 1 mM $MgCl_2$ in excess of ATP, 10 mM Tris-HCl, pH 7.5, $25^\circ C$.

Ratios of H-meromyosin to F-actin: \bigcirc , 1 : 3; \triangle , 1 : 1;
 \square , 1 : 0.33.

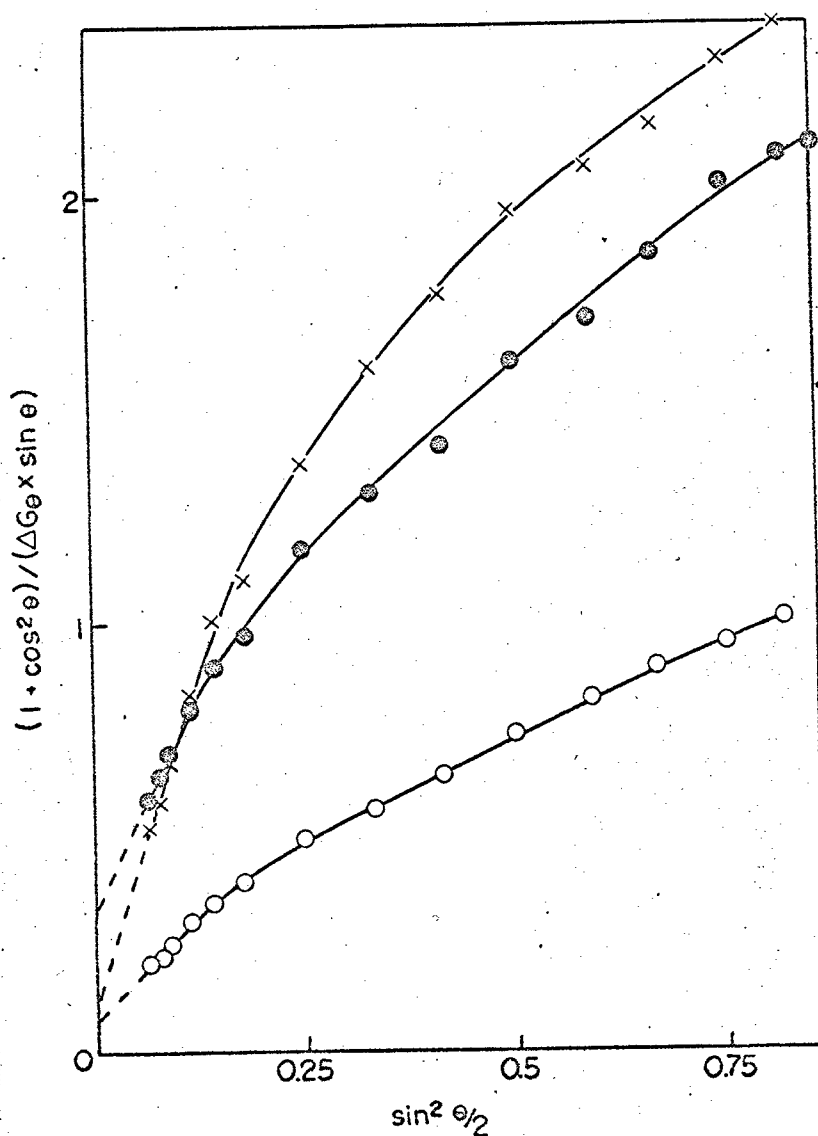


Fig. 6. Change in angular distribution on scattering intensity of acto-H-meromyosin on the addition of ATP.

0.048 mg. H-meromyosin/ml., 0.14 mg. F-actin/ml., 30 μ g. pyruvate kinase/ml., 0.4 mM PEP, 5 mM KCl, 1 mM MgCl_2 , 10 mM Tris-HCl, pH 7.5, 25°C.

- , Acto-H-meromyosin after mixing.
- , Before mixing of H-meromyosin and F-actin.
- × , On the addition of 0.1 mM MgATP^{--} to acto-H-meromyosin.

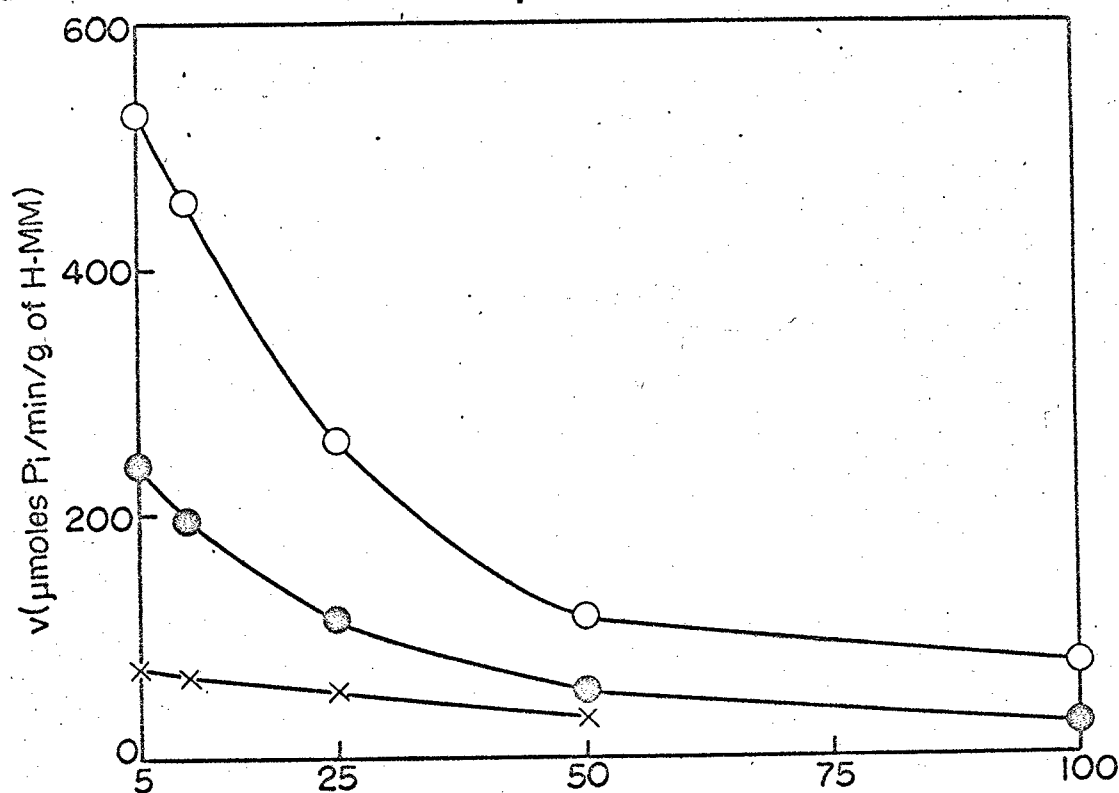


Fig. 7. Effect of KCl concentration on ATPase activity of acto-H-meromyosin.

0.15 mg. H-meromyosin/ml., 20 μg. pyruvate kinase/ml., 0.4 mM PEP, 1 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 25°C.

Ratios of H-meromyosin to F-actin: ○ , 1 : 3; ● , 1 : 1;
 × , 1 : 0.33.

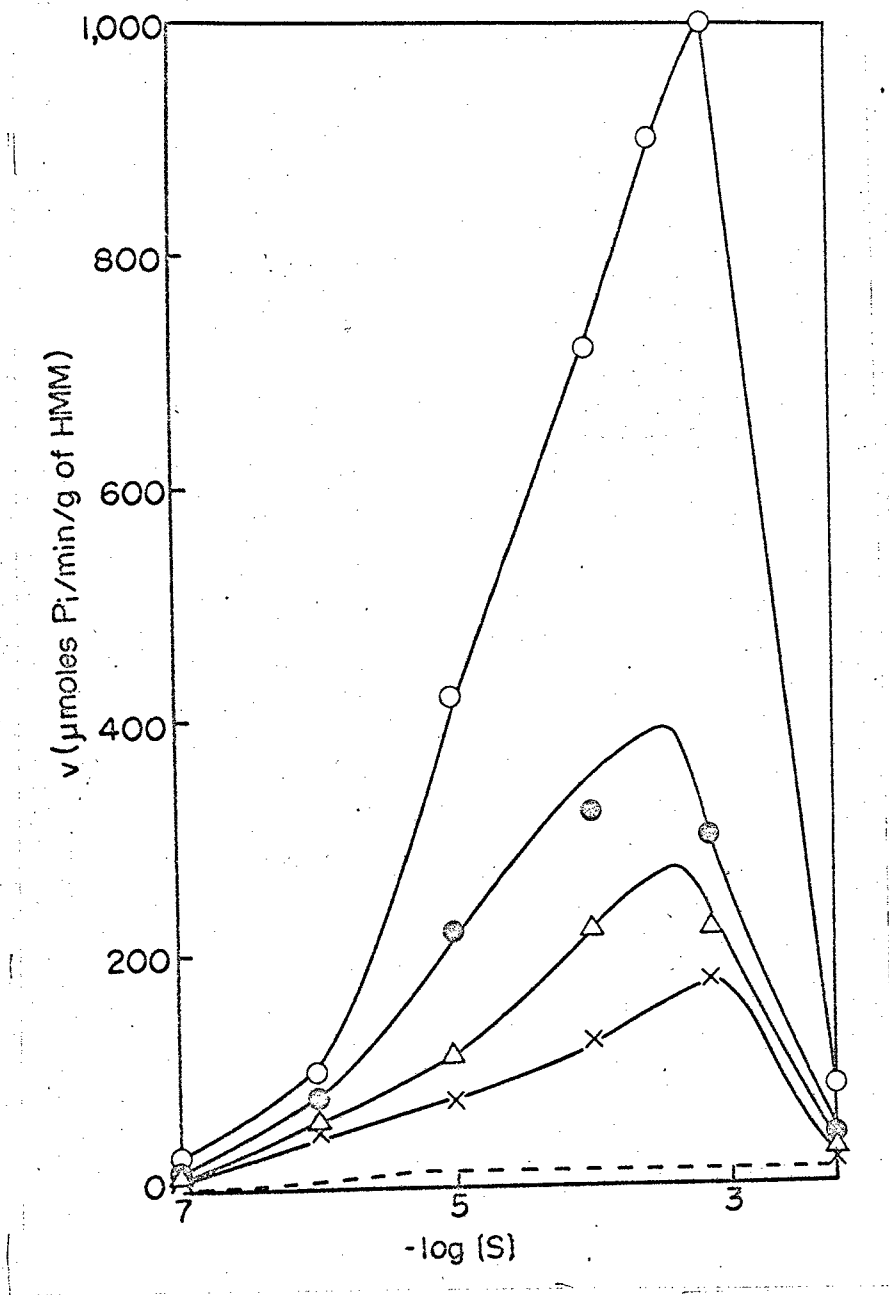


Fig. 8. Dependence on substrate concentration of ATPase activity of act-H-meromyosin.

In this experiment ionic strength was increased with increase in ATP concentration. 0.15 mg. H-meromyosin/ml., 30 μ g. pyruvate kinase/ml., 0.4 mM PEP, 5 mM KCl, 1 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 25°C.

Ratios of H-meromyosin to F-actin: \bigcirc , 1 : 3; \bullet , 1 : 1; \triangle , 1 : 0.33; \times , 1 : 0.167; -----, H-meromyosin alone.

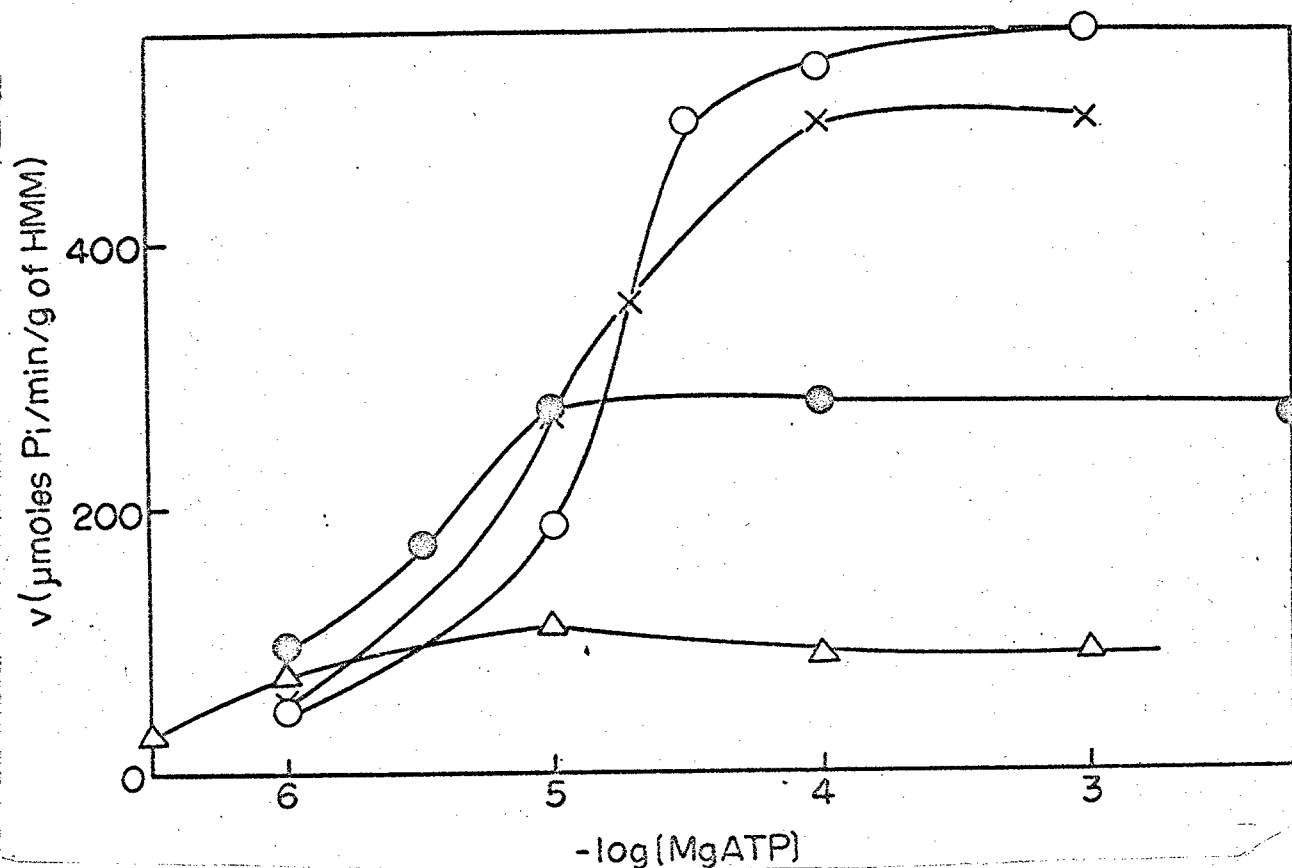


Fig. 9. Dependence on substrate concentration of ATPase activity at fixed ionic strength of acto-H-meromyosin (ratio of H-meromyosin to F-actin 1 : 2.8).

0.176 mg. H-meromyosin/ml., 0.5 mg. F-actin/ml., 15 μ g. pyruvate kinase/ml., 0.4 mM PEP, 1 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 25°C.

Ionic strengths due to KCl + $MgATP^{--}$: \bigcirc , 5; \times , 10;

\bullet , 30; \triangle , 60 mM.

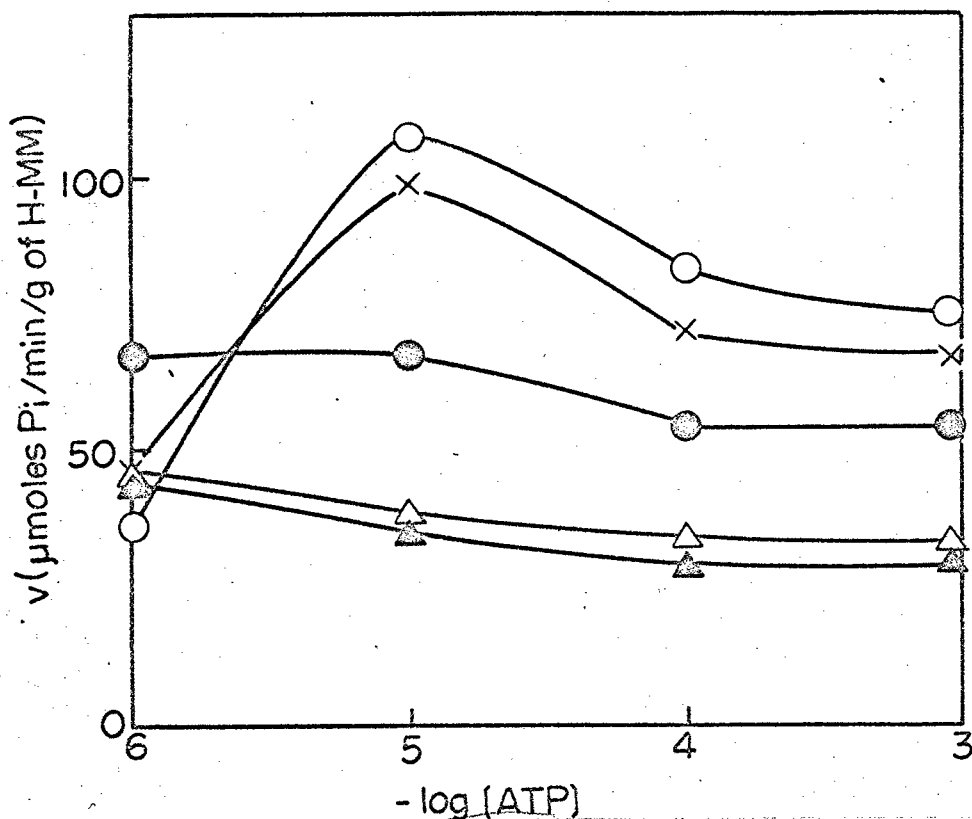


Fig. 10. Dependence on substrate concentration of ATPase activity at fixed ionic strength of acto-H-meromyosin (ratio of H-meromyosin to F-actin 3 : 1).

0.15 mg. H-meromyosin/ml., 0.05 mg. F-actin/ml., 20 μg. pyruvate kinase/ml., 0.4 mM pep, 1 mM MgCl_2 , 10 mM Tris-HCl, pH 7.5, 25°C.

Ionic strengths derived from KCl + MgATP^{--} : ○ , 5; × , 10; ● , 25; △ , 50; ▲ , 50 mM + 0.1 mM EGTA.

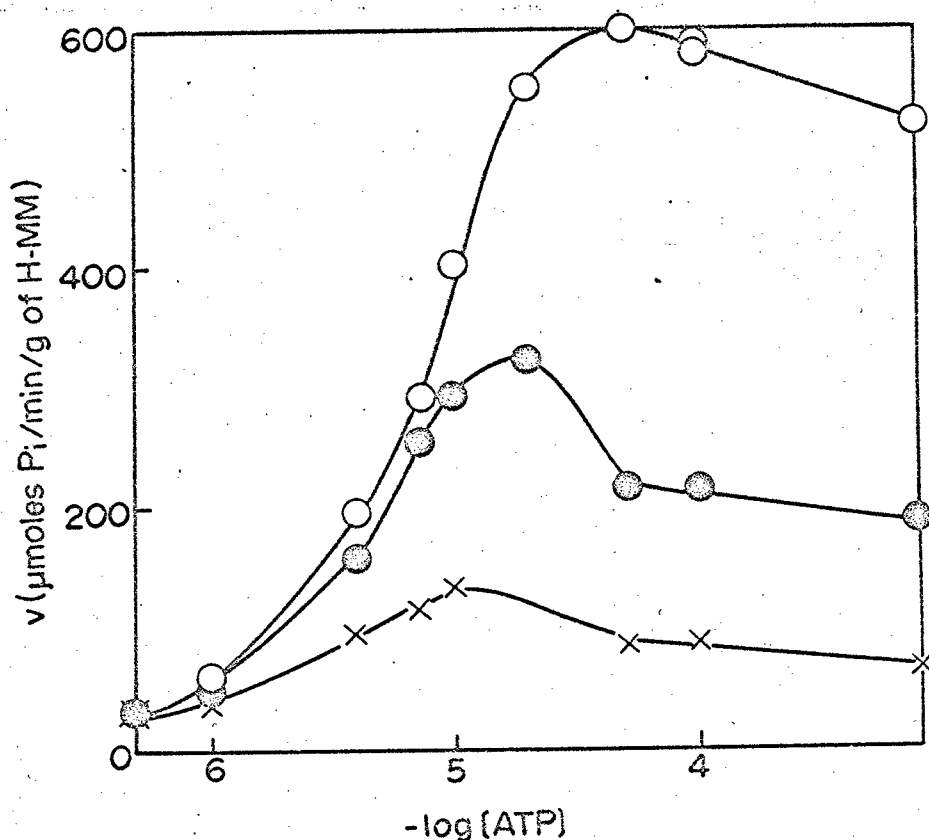


Fig. 11. Dependence on substrate concentration of ATPase activity of acto-H-meromyosins with various concentrations of F-actin.

0.15 mg. H-meromyosin/ml., 35 μg. pyruvate kinase/ml., 0.4 mM PEP, 1 mM MgCl_2 , 10 mM Tris-HCl, pH 7.5, 25°C. Ionic strength due to $\text{KCl} + \text{MgATP}^{--}$ was 5 mM.

Concentrations of F-actin: ○ , 0.45; ● , 0.15; × , 0.05 mg/ml.

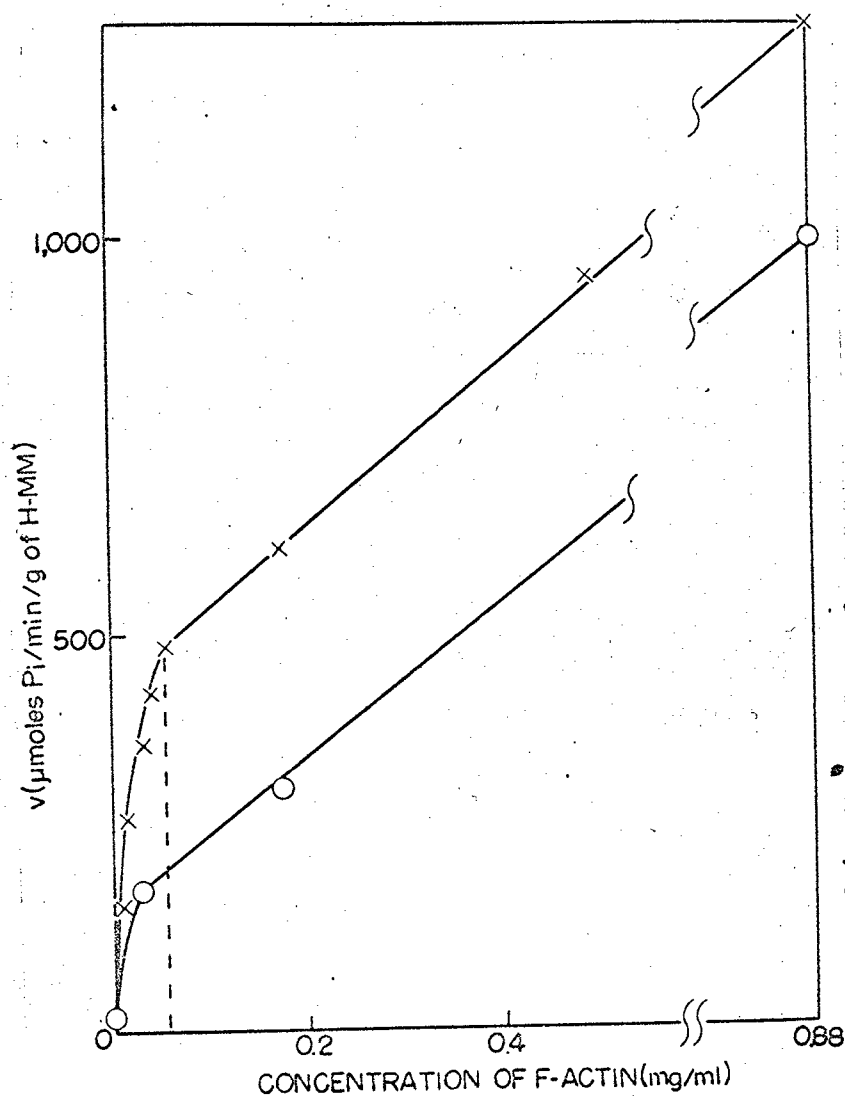


Fig. 12. Enhancement of ATPase activity of H-meromyosin by F-actin before and after treatment of H-meromyosin with PCMB and β -mercaptoethanol.

0.176 mg. H-meromyosin/ml., 40 μ g. pyruvate kinase/ml., 0.4 mM PEP, 1 mM ATP, 5 mM KCl, 1 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 25°C.

○ , Before treatment of H-meromyosin with PCMB and β -mercaptoethanol; x , After treatment of H-meromyosin with PCMB and β -mercaptoethanol.

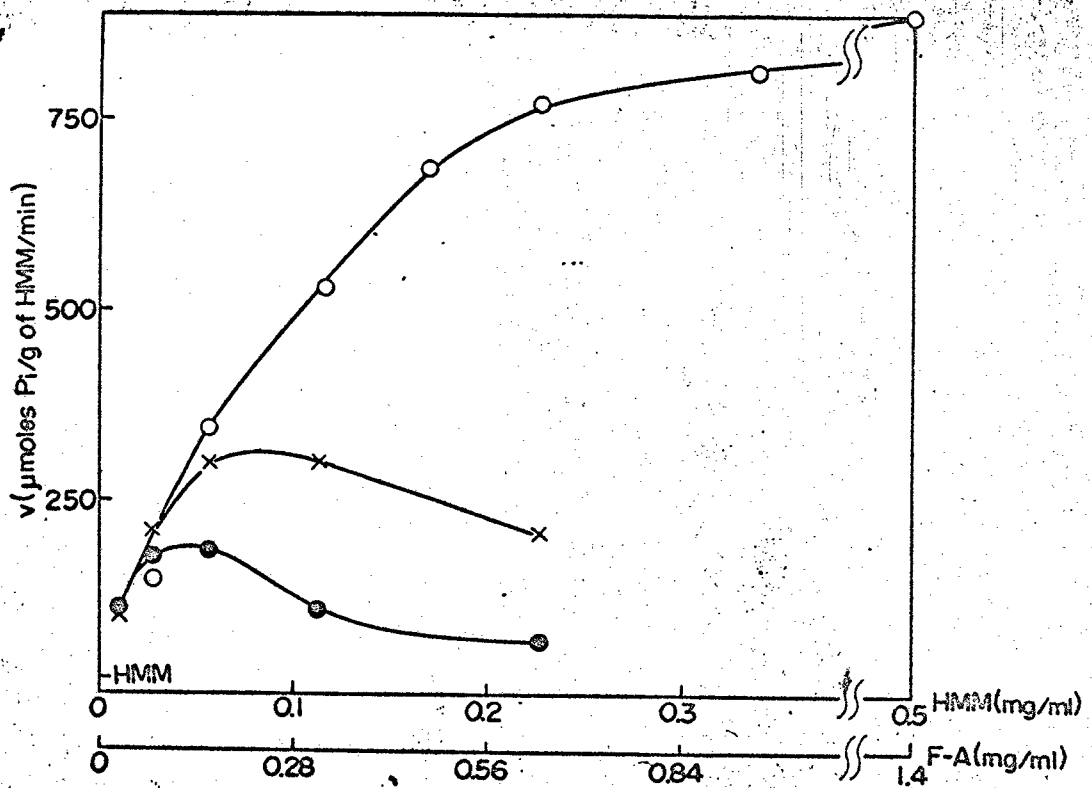


Fig. 13 Dependence on protein concentration of specific activity of acto-H-meromyosin at constant ratio of H-meromyosin to F-actin (1 : 2.8).

20 μg. pyruvate kinase/ml., 0.4 mM PEP, 5 mM KCl, 1 mM MgCl₂, 10 mM Tris-HCl, pH 7.5, 25 C.

Concentration of ATP: ○ , 100; × , 20; ● , 5 μM.

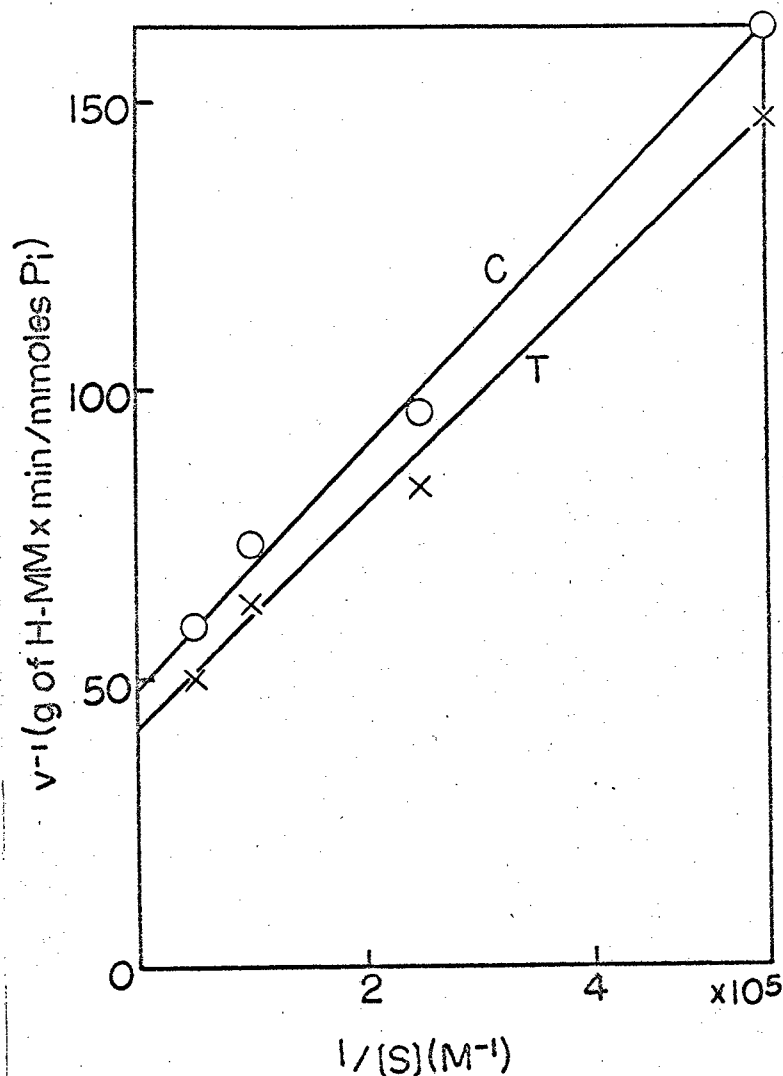


Fig. 14. Effect of treatment of H-meromyosin with PCMB and β-mercaptoethanol on Lineweaver-Burk plot of H-meromyosin ATPase.

0.2 mg. H-meromyosin/ml., 40 μg. pyruvate kinase/ml., 0.4 mM PEP, 5 mM KCl, 1 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 25°C.

○ , Before treatment; × , After treatment with PCMB and β-mercaptoethanol.

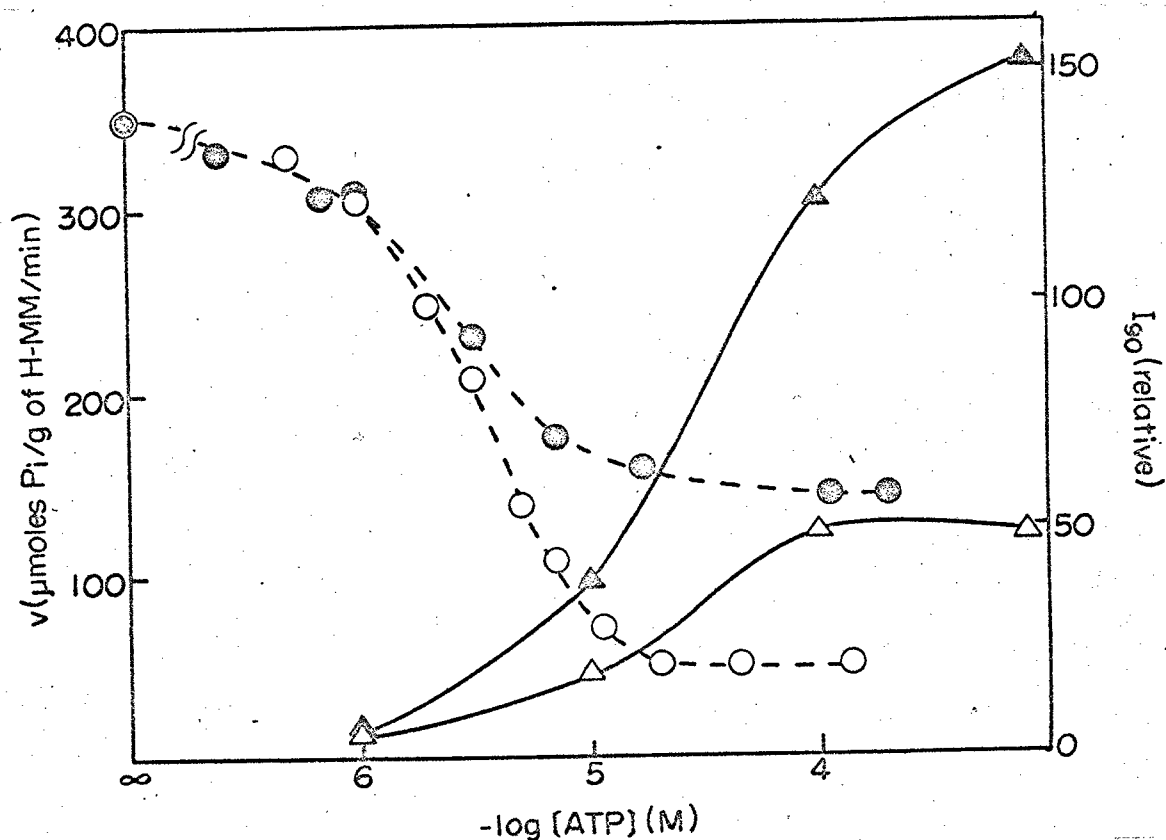


Fig. 15. Effect of treatment of H-meromyosin with PCMB and β -mercaptoethanol on ATP-dependences of ATPase activity and decrease in intensity of light-scattering at 90° of acto-H-meromyosin.

0.15 mg. H-meromyosin/ml., 0.05 mg. F-actin/ml., 28 μ g. pyruvate kinase/ml., 0.4 mM PEP, 5 mM KCl+MgATP⁻⁻⁻, 1 mM MgCl₂, 10 mM Tris-HCl, pH 7.5, 25°C.

- , ● , Intensity of light-scattering at 90° .
- △ , ▲ , ATPase activity.
- , △ , Control H-meromyosin.
- , ▲ , H-meromyosin treated with PCMB and β -mercaptoethanol.

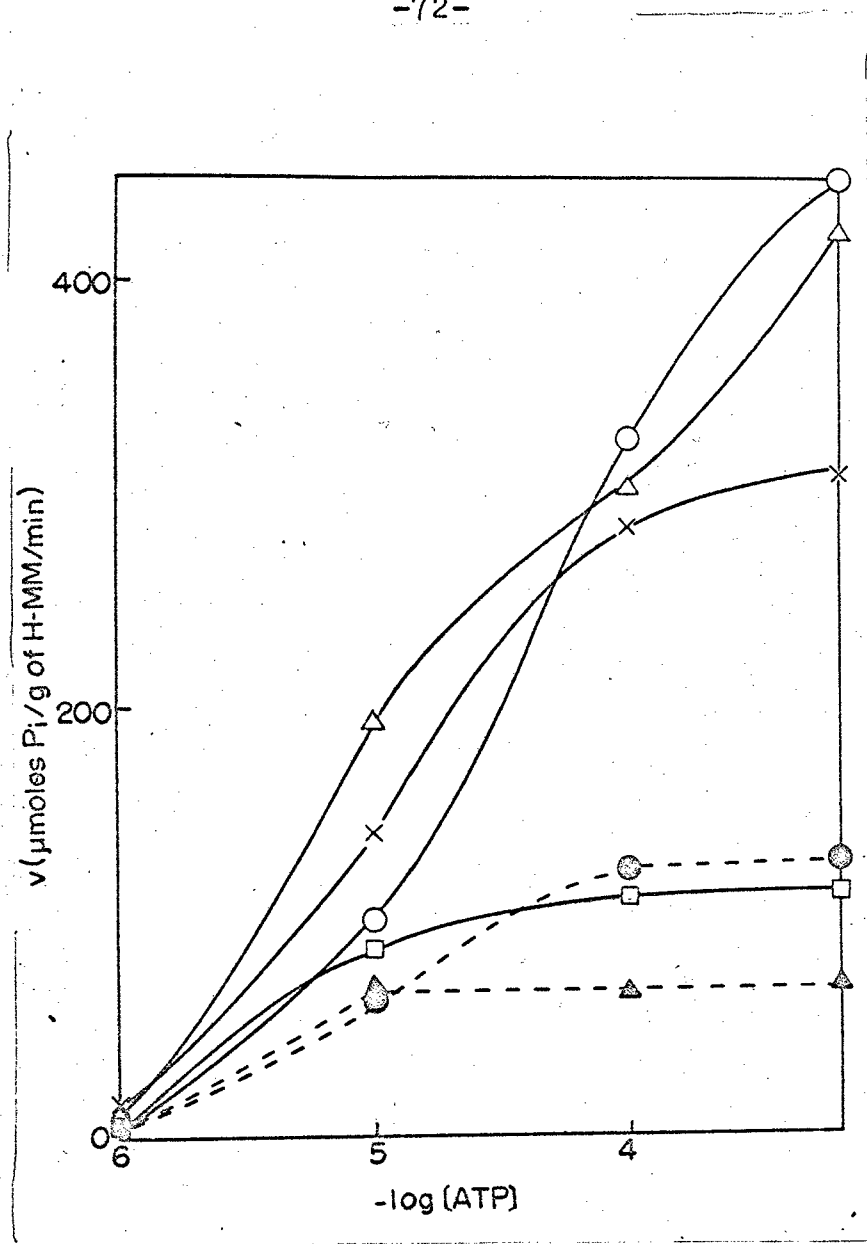


Fig. 16. Dependence on ATP concentration of ATPase activity of acto-H-meromyosin reconstituted from F-actin and H-meromyosin treated with PCMB and β -mercaptoethanol.

0.176 mg. H-meromyosin/ml., 0.0588 mg. F-actin/ml., 40 μ g. pyruvate kinase/ml., 1 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 25°C.

Treated H-meromyosin: ○ , 5; △ , 30; × , 60; □ , 120 mM KCl+MgATP⁻⁻⁻.

Original H-meromyosin: ● , 5; ▲ , 30 mM KCl+MgATP⁻⁻⁻.

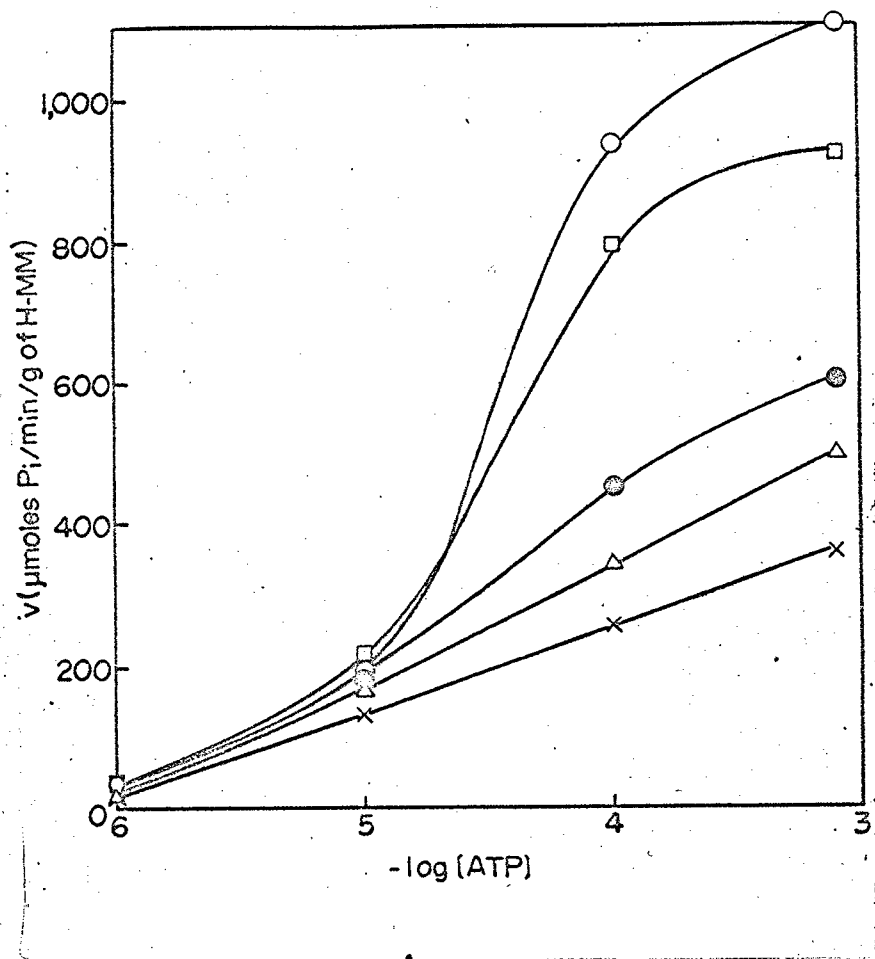


Fig. 17. Dependences on ATP concentration of ATPase activities of acto-H-meromyosins reconstituted from 0.176 mg./ml of H-meromyosin treated with PCMB and β -mercaptoethanol and various concentrations of F-actin.

0.176 mg. treated H-meromyosin/ml., 40 μ g. pyruvate kinase /ml., 0.4 mM PEP, 5 mM KCl, 1 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 25°C.

F-actin concentration: \times , 0.0315; Δ , 0.0587; \bullet , 0.176;
 \square , 0.492; \circ , 0.88 mg/ml.

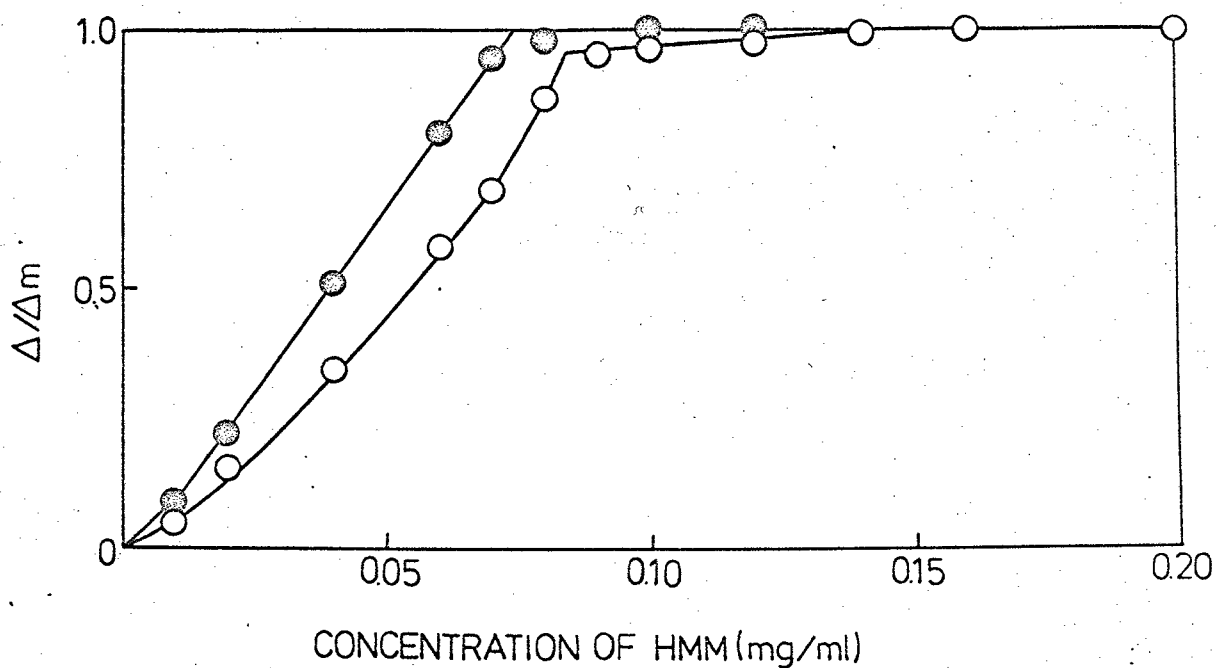


Fig. 18. Increase in the light-scattering intensity of actin solution on adding various amounts of H-meromyosin.

Measurements were performed in 5 mM KCl at pH 7.5 and 25°C.

○ , 0.01-0.20 mg/ml H-meromyosin, 0.02 mg/ml F-actin, 1 mM $MgCl_2$, 10 mM Tris-HCl.

● , 0.01-0.12 mg/ml H-meromyosin, 0.02 mg/ml G-actin, 3 mM Tris-HCl.

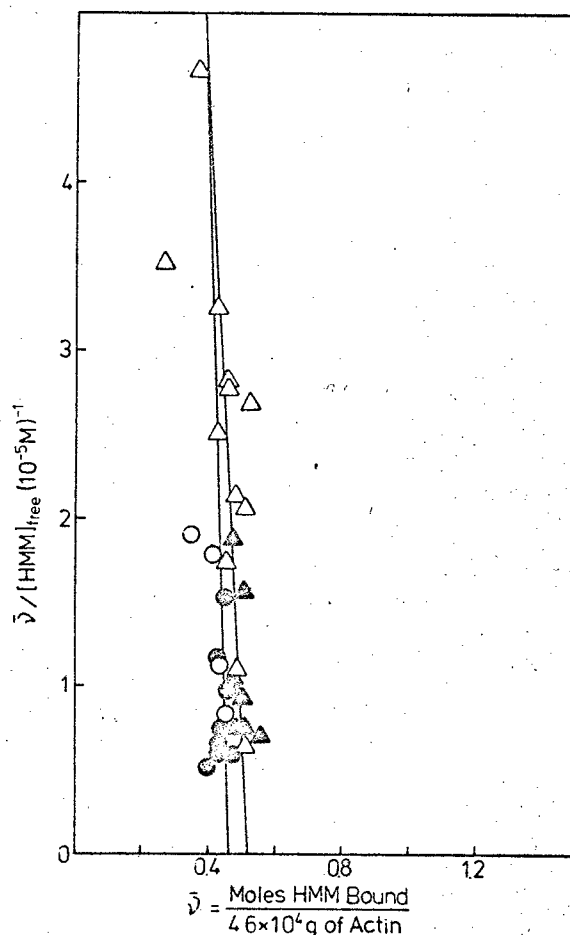


Fig. 19. Scatchard plots of binding of HMM to F-actin obtained by ultracentrifugal analysis.

Acto-HMM samples were reconstituted from a constant concentration of HMM(or F-actin) and various concentrations of F-actin (or HMM). The four different symbols represent different preparations. All mixture contained 0.1 M KCl and 1 mM $MgCl_2$. ○ , 2.9 mg/ml HMM, 0.3-0.9 mg/ml F-actin, 20 mM phosphate, pH 7.0, 6-8°C; ● , 3.0 mg/ml HMM, 0.05-0.6 mg/ml F-actin, 10 mM Tris-HCl, pH 7.6, 22°C; ▲ , 3.0 mg/ml HMM, 0.05-0.6 mg/ml F-actin, 10 mM Tris-HCl, pH 7.6, 3-4°C; △ , acto-HMM reconstituted from 1.02 mg/ml of HMM and 0.138 mg/ml of F-actin, 10 mM Tris-HCl at pH 8.8 and 4°C.

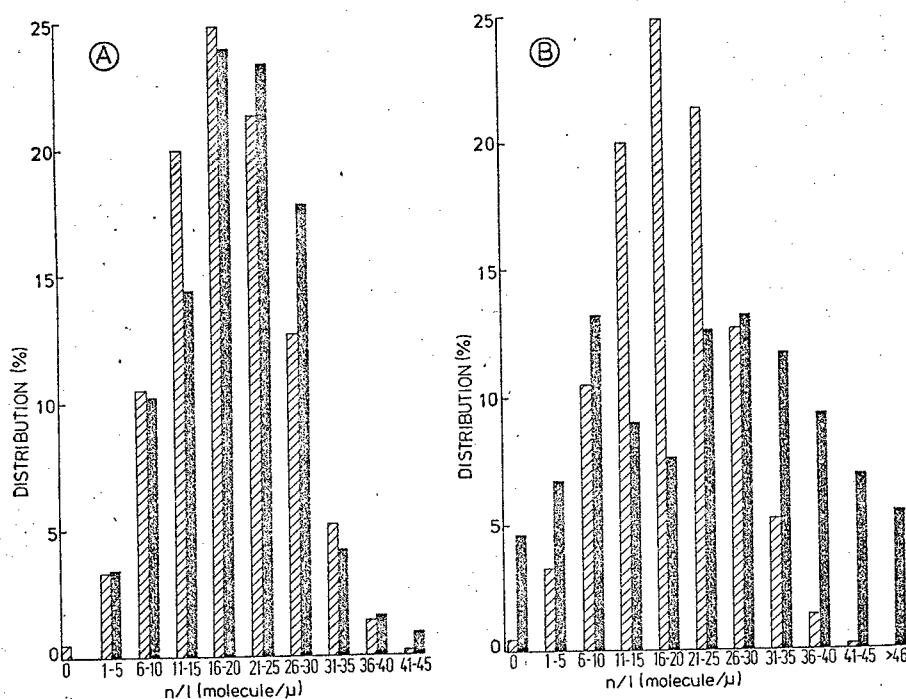


Fig. 20. Distribution of number of HMM molecules bound per 1 u of F-actin filament.

The numbers were determined from direct visualization of the HMM molecules by electron microscopy. 0.045 mg/ml HMM, 0.03 mg/ml F-actin, 5 mM KCl, 1 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 23°C.

, calculated assuming a random distribution and a maximum binding ratio of HMM to F-actin of 3.6 : 1 by weight; , observed distribution. (A) and (B) represent different preparations. Total length of F-actin: (A) $l=209$ u; (B) $l=194$ u. Note the two types of character of acto-HMM: (A) independent binding of HMM to F-actin, (B) cooperative binding.

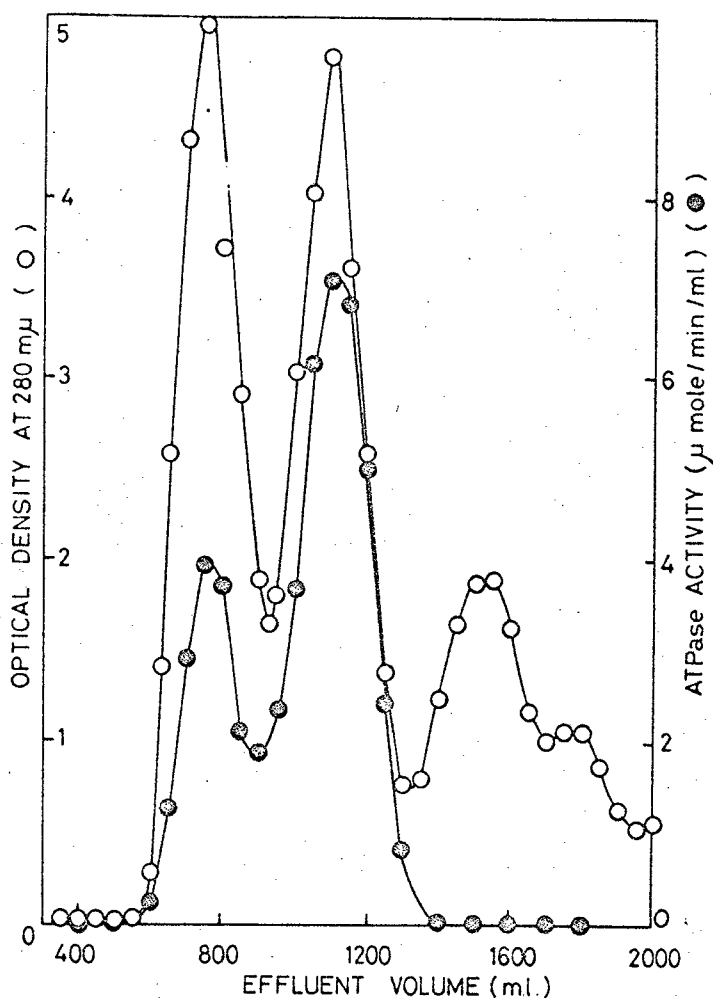


Fig. 21. Fractionation of tryptic digest of HMM on Sephadex G-200.

HMM was digested with trypsin(HMM to trypsin, 20 : 1) for 20 min at 25°C. The digest was applied to column(6.5 x 70 cm) of Sephadex G-200 which had previously been equilibrated with 0.1 M Tris, 0.05 M KCl and 0.5 mM EDTA at pH 7.6 and 4°C. The column was eluted with the same solvent at a flow rate of 10 ml/hr, and 10 ml fractions were collected. ATPase activities were measured in 1 mM ATP, 0.25 M KCl, 5 mM CaCl_2 and 50 mM Tris-HCl at pH 7.6 and 25°C. ○ , E_{280} ; ● , Ca^{2+} -activated ATPase(μmole Pi/min/ml of fraction).

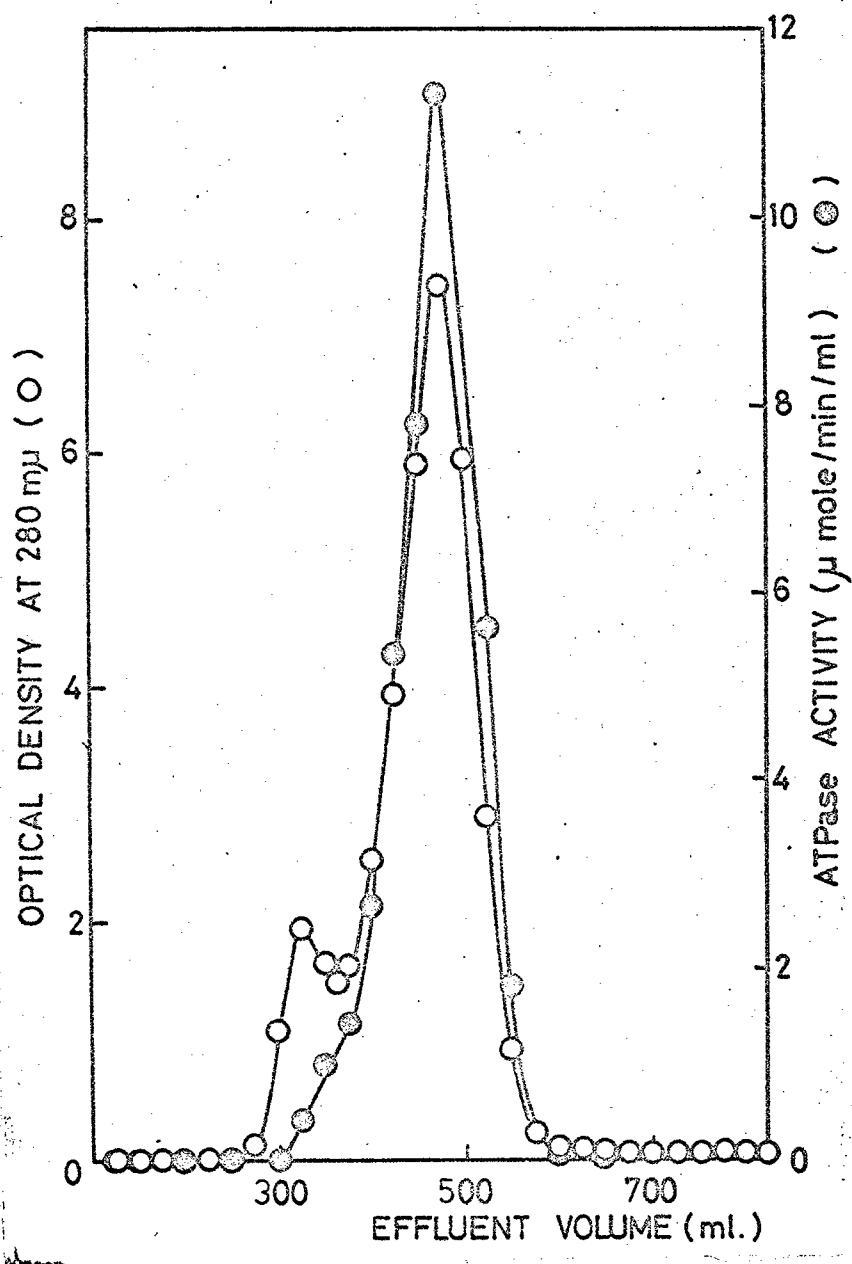


Fig. 22. Rechromatography of S-1 on Sephadex G-200.

Fractions from the central portion of the second peak in the chromatogram shown in Fig. 4 were concentrated and rechromatographed on a column(5 x 54) of Sephadex G-200. The conditions of elution and ATPase assays were as for Fig. 4. 5 ml fractions were collected.

○ , E_{280} ; ● , Ca^{2+} -activated ATPase activity.

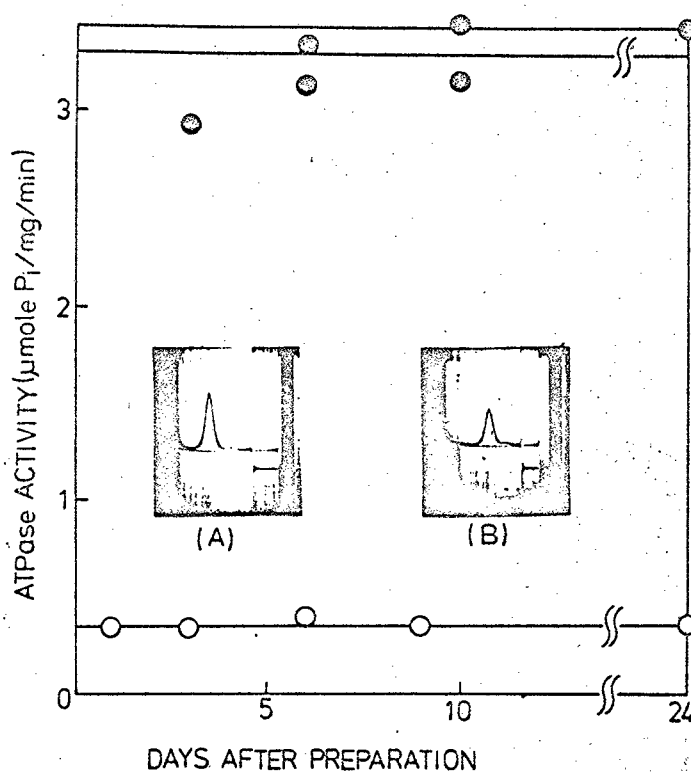


Fig. 23. Changes in ATPase activity and the ultracentrifugal pattern of S-1 during storage in 0.05 M KCl, 0.5 mM EDTA and 20 mM Tris-HCl at pH 7.6 and 0°C.

Ca²⁺-activated ATPase activities were measured in 0.05 mg/ml S-1, 1 mM ATP, 1 M KCl, 7 mM CaCl₂ and 50 mM Tris-maleate at pH 7.0 and 25°C. EDTA-ATPase activities were used instead under the same conditions, except that 3 mM EDTA was used instead of CaCl₂. Ultracentrifugation was performed in 50 mM KCl, 0.5 mM EDTA and 20 mM Tris-HCl at pH 7.6 and 14°C. Bar angle 60°, rotor speed 56,100 rpm. (A) 7 days after rechromatography. The picture was taken 80 min after reaching full speed with 11 mg/ml S-1. (B) 1 day after rechromatography. The picture was taken 90 min after reaching full speed with 6.6 mg/ml S-1. ○, Ca²⁺-ATPase activity; ●, EDTA-ATPase activity.

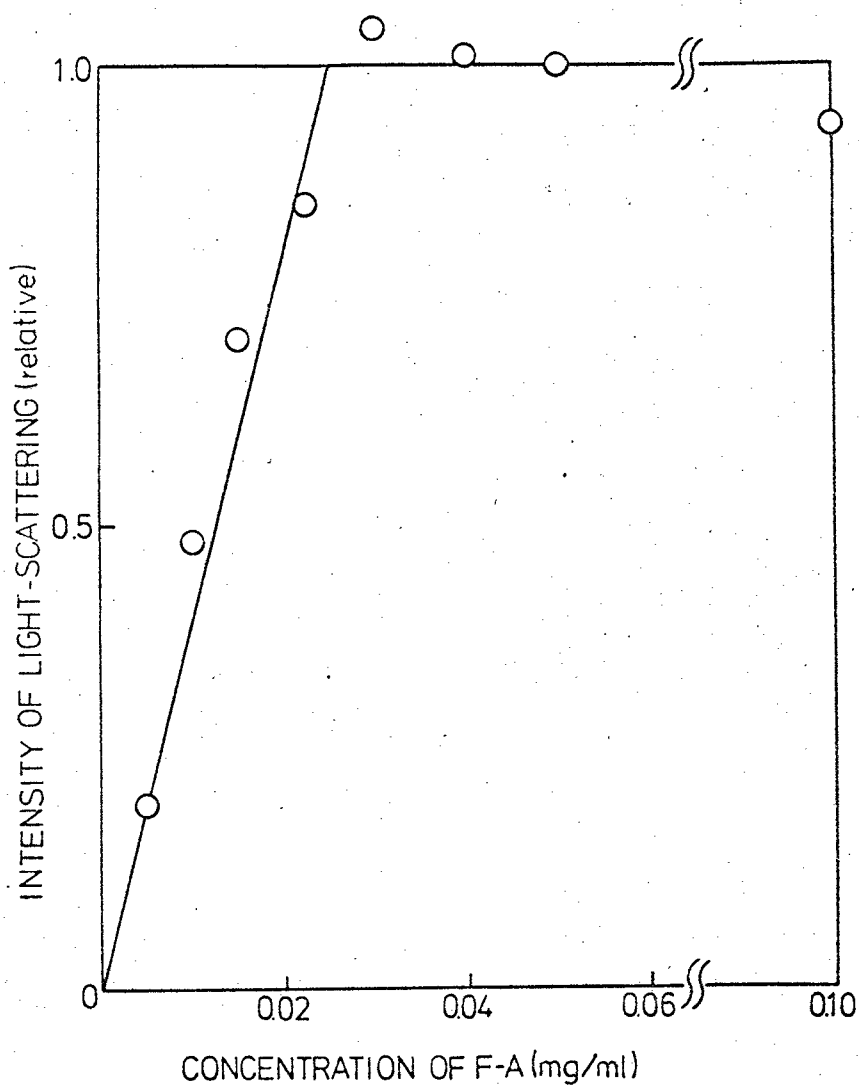


Fig. 24. Change in the light-scattering intensity of S-1 solution on addition of various amounts of F-actin.

The measurements were performed in 0.08 mg/ml S-1, 0.005-0.1 mg/ml F-actin, 0.1 M KCl, 50 μ M $MgCl_2$, 25 μ M EDTA and 10 mM Tris-HCl at pH 7.6 and 23°C.

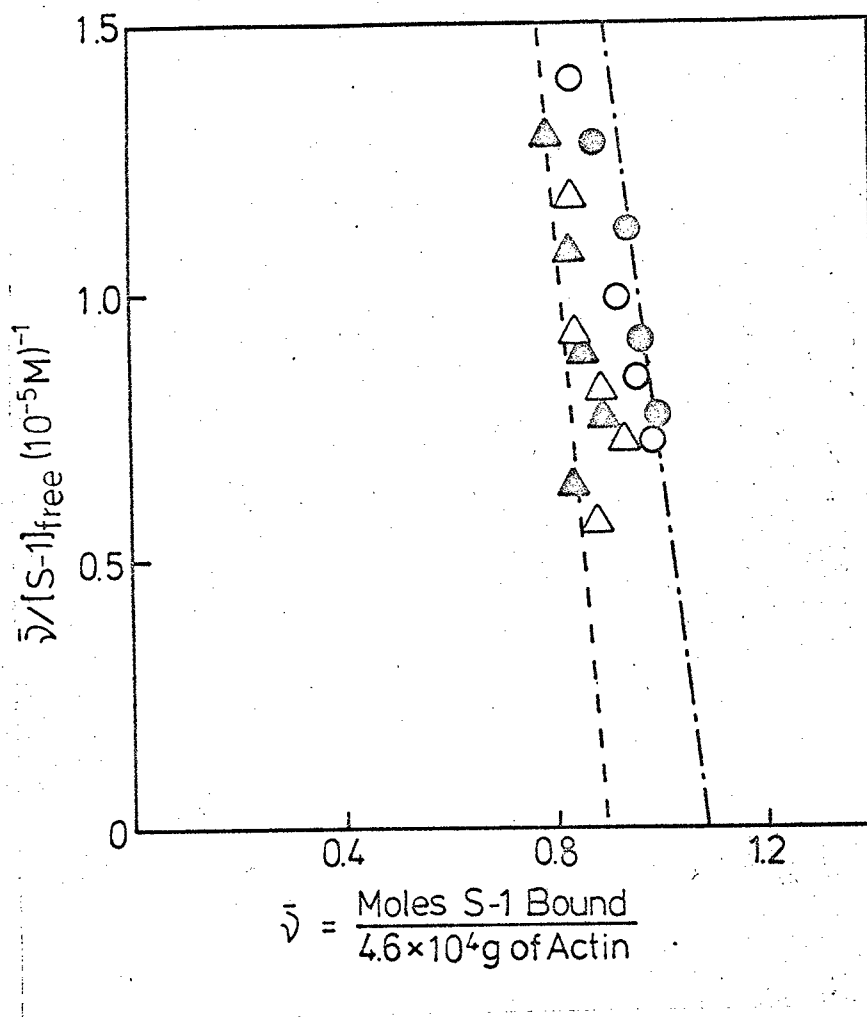


Fig. 25. Scatchard plots of binding of S-1 to actin from ultracentrifugal analysis.

Acto-S-1 complexes were reconstituted from a constant concentration of S-1 (2.3 mg/ml) and various concentrations of F-actin (0.115-0.69 mg/ml). The four symbols represent different preparations. All the mixtures contained 10 mM Tris (pH 7.6).

- , 100 mM KCl, 1 mM $MgCl_2$, 16.5°C.
- , 100 mM KCl, 0.2 mM $MgCl_2$, 2°C.
- △ , 10 mM KCl, 0.2 mM $MgCl_2$, 16.5°C.
- ▲ , 3.8 mM KCl, 1 mM $MgCl_2$, 16.5°C.

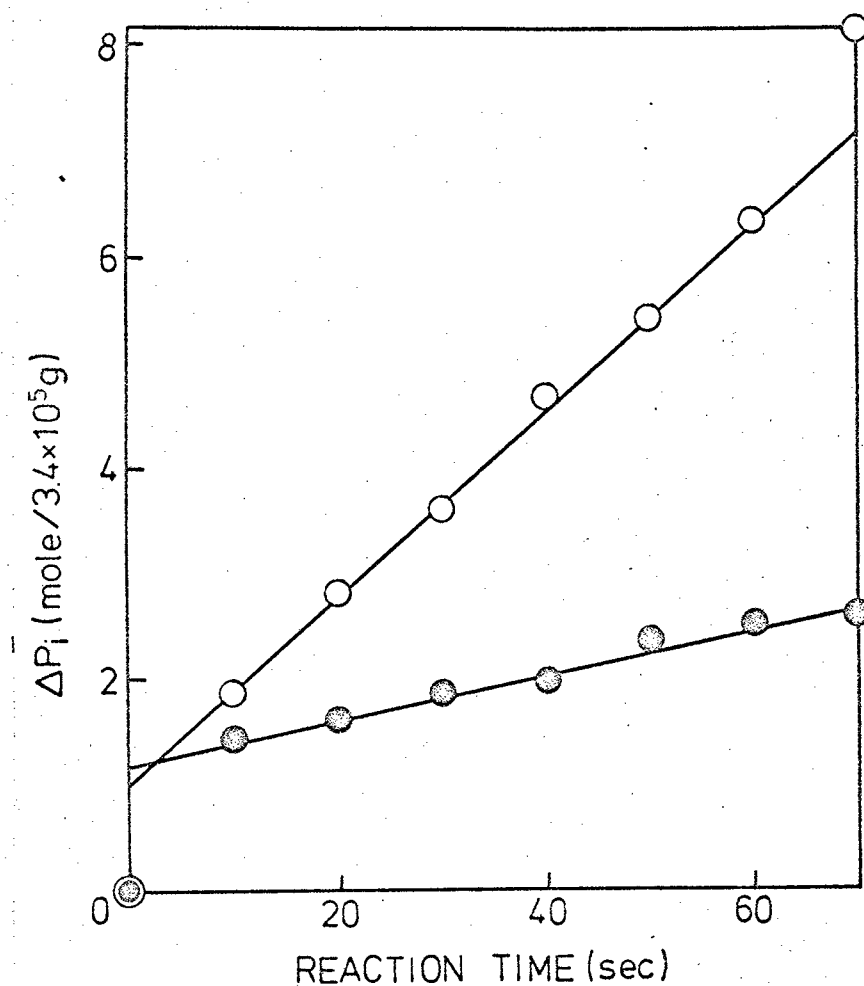


Fig. 26. Time-courses of Pi-liberation from the HMM-ATP system at two ionic strengths.

ATPase activities were measured in 1 mg/ml HMM, 0.1 mM or 30 μ M ATP, 2 mM $MgCl_2$ and 20 mM Tris-HCl at pH 7.6 and 25°C.

○ , 5 mM KCl.

● , 0.5 M KCl.

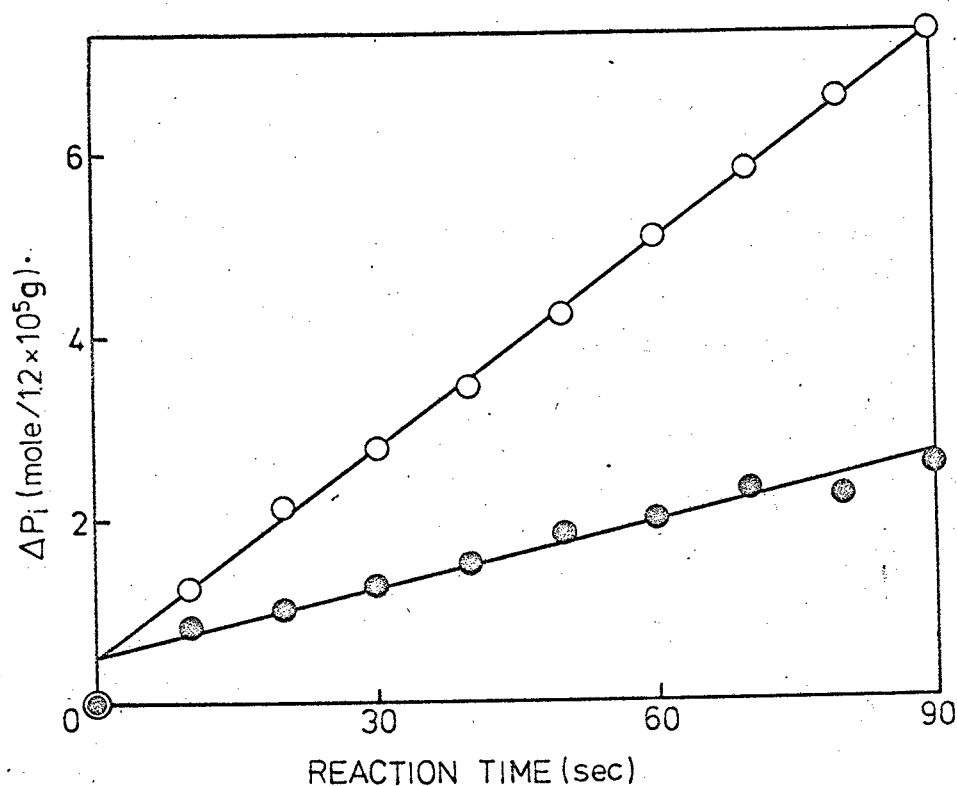


Fig. 27. Time-courses of P_i -liberation from the S-1-ATP system at two ionic strengths.

ATPase activities were measured in 1 mg/ml S-1, 0.1 mM or 30 μ M ATP, 2 mM $MgCl_2$ and 20 mM Tris-HCl at pH 7.6 and 25°C.

○ , 3.9 mM KCl.

● , 0.5 M KCl.

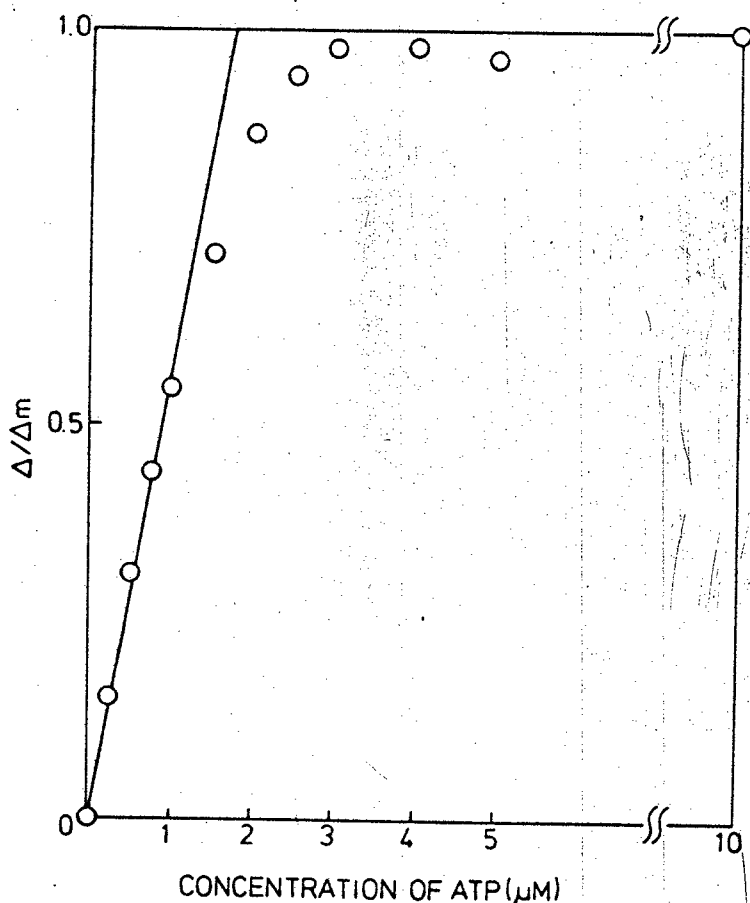


Fig. 28. Decrease in light-scattering intensity of actomyosin on adding various concentrations of ATP.

Light-scattering intensities were measured in 0.8 mg/ml myosin, 0.4 mg/ml F-actin, 1 M KCl, 10 mM MgCl_2 and 20 mM Tris-HCl at pH 7.6 and 20°C.

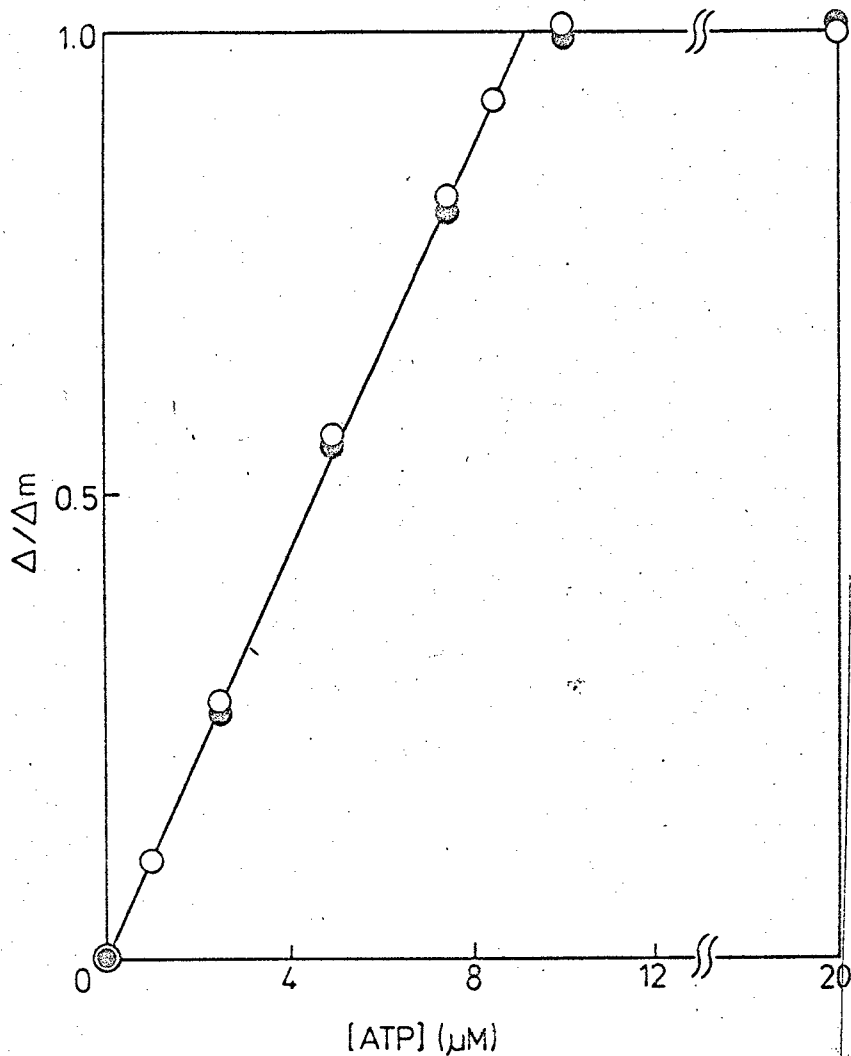


Fig. 29. Decrease in light-scattering intensity of acto-HMM on adding various concentration of ATP.

Light-scattering intensities were measured in 1.36 mg/ml HMM, 0.368 mg/ml F-actin, 1 mM $MgCl_2$ and 20 mM Tris-HCl at pH 7.6 and 21°C.

KCl concentration: ○ , 0.5 M; ● , 0.1 M.

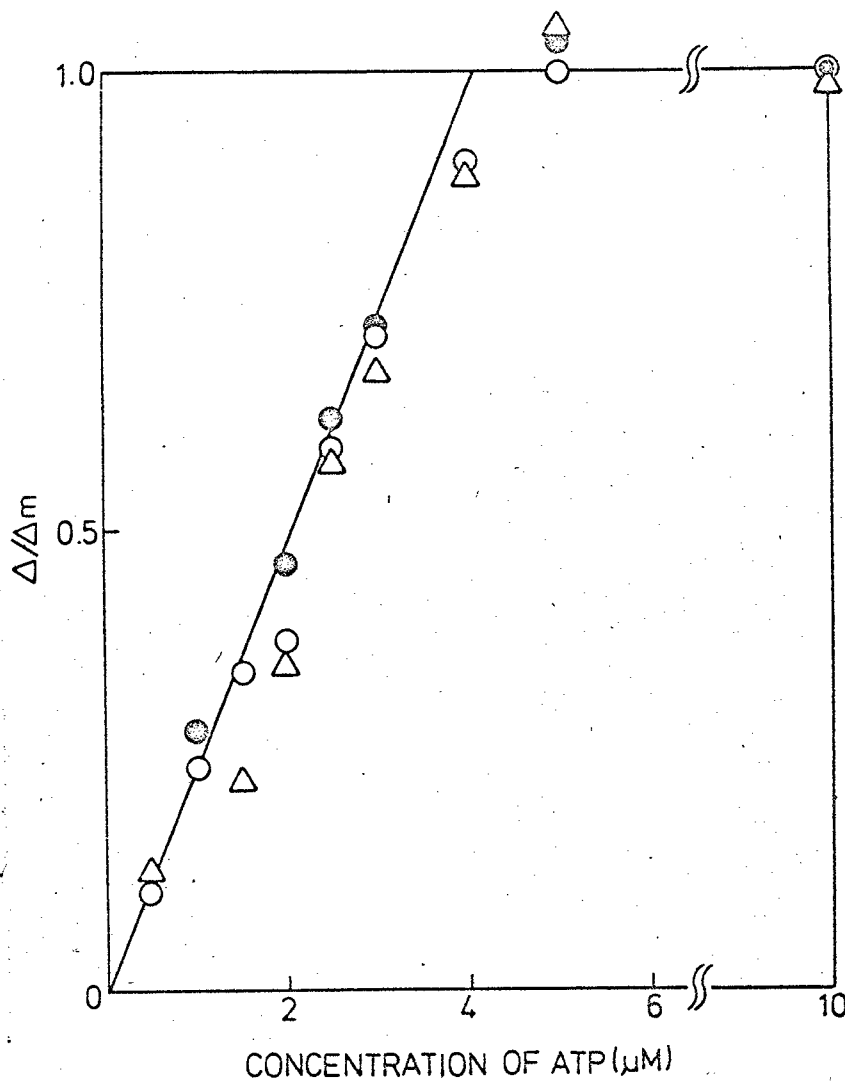


Fig. 30. Decrease in light-scattering intensity of the acto-S-1 complex on adding various concentrations of ATP.

Light-scattering intensities were measured in 0.5 mg/ml S-1, 0.192 mg/ml F-actin, 1 mM MgCl_2 and 20 mM Tris-HCl at pH 7.6 and 22°C.

KCl concentration: ○ , 50 mM; ● , 0.1 M; △ , 0.5 M.

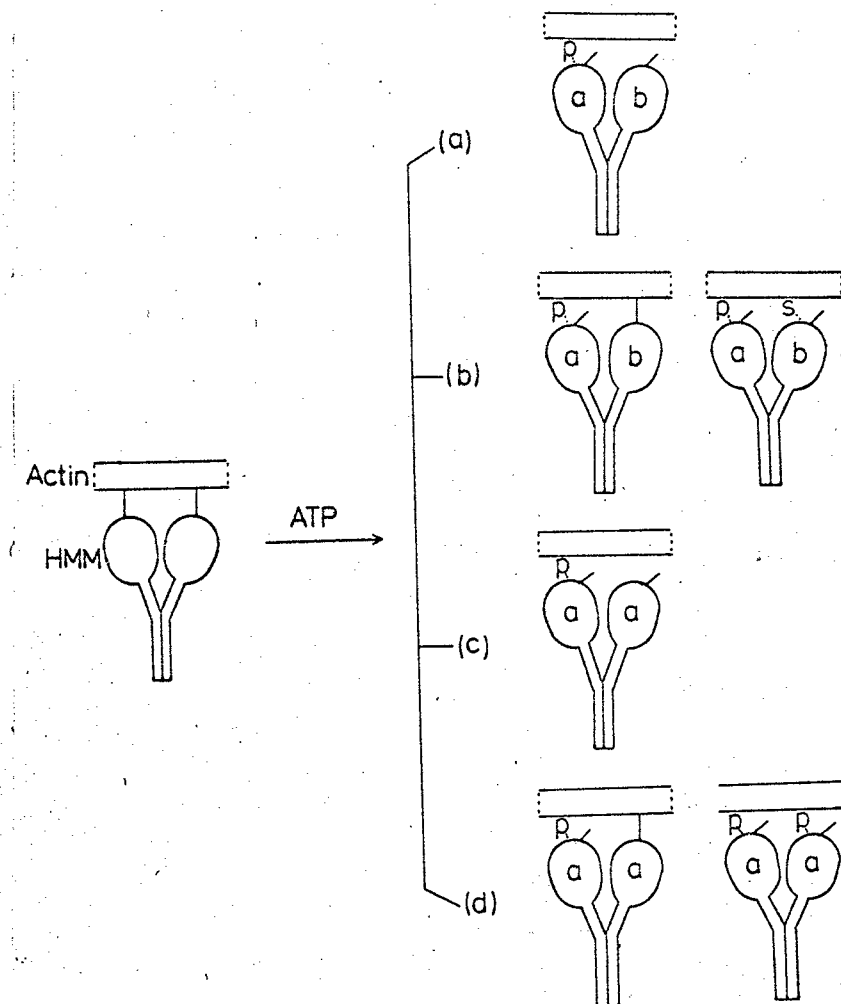


Fig. 31. Schematic representation of the interaction of HMM with actin and ATP(S).

P, protein-phosphste-ADP complex; S, simple binding of ATP. Globular portions show the two S-l parts(A, B) of the HMM molecule. For explanation see text.