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# CHANGE OF SIZE AND SHAPE OF MYOSIN B BY POLYPHOSPHATE<sup>\*</sup>)

By

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SZENT-GYÖRGYI<sup>1)</sup> and NEEDHAM *et al.*<sup>2)</sup> have found that the viscosity and the flow-birefringence of the solution of the muscle contractile protein, myosin B, decrease on the addition of polyphosphate such as ATP or PP. SZENT-GYÖRGYI<sup>1)</sup> has demonstrated that the complex protein, actomyosin, constituted by F-actin and myosin A, shows similar properties to those of myosin B, and SZENT-GYÖRGYI<sup>1)</sup> and WEBER<sup>3,4)</sup> have investigated the contraction of muscle models by ATP. Through these studies it has been generally accepted that muscle contraction is caused by the interaction between myosin, actin and ATP.<sup>1,3,4)</sup>

The size and shape change of myosin B with ATP or PP, which may be one of the most important reactions in the mechanism of muscle contraction, has been investigated by many workers such as WEBER<sup>3)</sup>, MORALES<sup>5,1)</sup>, GERGELY<sup>7,8)</sup>, MOMMAERTS<sup>9,10)</sup> and us<sup>11)</sup>. But the mechanism of this important reaction remains to be clarified in many points.

Then we have engaged in systematic study on the change of size and shape of myosin B by PP, which produces same effect on myosin B as ATP does but is not hydrolyzed by myosin B.

We have made effort to extract common properties of the myosin B-PP system from the study on as many preparations as possible which are purified in a definite procedure, because some properties of myosin B vary greatly from one preparation to another as all workers in this field have noticed and a conclusion deduced from a few preparations often lacks generality. Moreover, to clarify the reaction mechanism of a complicated system such as the myosin B-PP one it seems to be necessary to attack it by as many different kinds of methods as possible.

To have a reasonably adequate understanding of the reaction mechanism

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<sup>\*</sup>) In this paper the following abbreviations are used: PP, inorganic pyrophosphate; ATP, adenosine triphosphate; PCMB, p-chloromercuribenzoate;  $pK$ ,  $-\log K$ ;  $[ ]$ , concentration.

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between protein and small molecule, we should answer the question put by SCATCHARD<sup>12,13</sup>: How many molecules of small substance can bind to one molecule of protein? How tightly do they bind? Where do they bind on the protein molecule? What is the significance of the binding? In the first part of this paper the results obtained by the methods of light scattering, viscosity, flow birefringence and ultracentrifugal separation will be reported and basing on these results an answer will be presented on the question "What of it?", that is, how molecular size and shape of myosin B does change on binding of PP. In the next part the strength of binding of PP to myosin B and the maximum number of sites of myosin B available to PP will be determined by the equilibrium dialysis method, and the thermodynamic properities of the elementary steps of the reaction between myosin B and PP will be analyzed by means of a transient light scattering method, in other words, the question "Why?" will be solved.

## **PART I. MOLECULAR WEIGHT AND SHAPE OF MYOSIN B**

The molecular mechanism of the change of the size and shape of myosin B by ATP remains unsettled in spite of many efforts already made by several workers and the following two theories are being opposed with each other. SZENT-GYÖRGYI<sup>1)</sup>, WEBER<sup>3)</sup>, GERGELY<sup>7,8)</sup> and many other workers reported experimental results indicating the dissociation of myosin B into myosin A and F-actin by the addition of ATP or PP, MORALES and his associates<sup>5,6)</sup>, however, concluded from light scattering and ultracentrifugal data that the major part of myosin B is elongated by ATP or PP-addition. This controversial situation may be attributable to the following difficulties: myosin B preparation is usually obtained as an extremely polydisperse mixture of very long molecules and the methods to analyze quantitatively such a system of polydisperse macromolecules have not yet fully advanced. Therefore, we made effort to obtain myosin B by the procedures as mild as possible. Myosin B used was extracted from rabbit skeletal muscle with WEBER-EDSALL solution for 24 hours and purified by 3 or 4 times precipitation at 0.2 M KCl and dissolution at 0.6 M KCl. Before use it was ultracentrifuged at  $14,000 \times G$  for 1-2 hours to remove gross impurities. During these procedures the temperature was kept below 3°C.

### **1. Light Scattering<sup>14)</sup>**

The solution of myosin B was clarified by centrifugation for 2.5 hours at  $25,000 \times G$  at the concentration of the protein of 2mg/ml and diluted with the solvent filtered through a millipore filter. The light scattering of myosin B

was measured by a Brice-Phoenix photometer and the scattering envelope was obtained after the ZIMM procedure<sup>15)</sup>.

In Fig. 1 typical examples of light scattering envelopes of myosin B are presented, and in Table 1 the "light scattering" average molecular weight  $\langle M \rangle_z$  and radius of gyration  $\langle r_g^2 \rangle_z^{1/2}$  are listed. The values of  $\langle M \rangle_z$  and  $\langle r_g^2 \rangle_z^{1/2}$  are

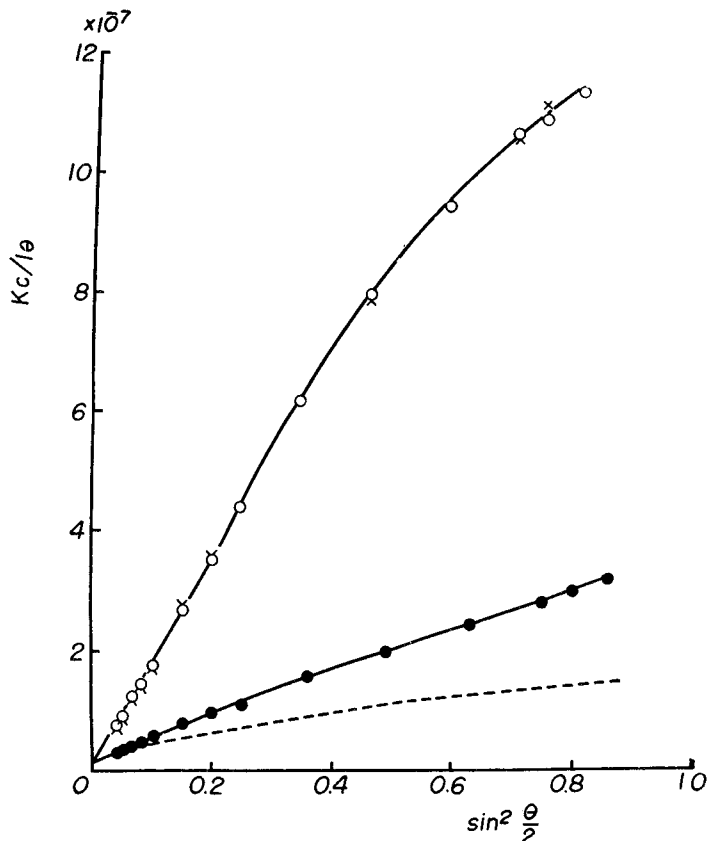


Fig. 1. The reciprocal reduced intensity plot for myosin B (sample No. NM 1). 0.6 MKCl, 1 mM MgCl<sub>2</sub>, pH 7.2, 23°C. ●, control; ○, 0.5 mM PP; ×, 0.5 mM salyrgan. The dotted line represents the reciprocal reduced intensity plot for a monodisperse rod.

respectively  $4.0-18.2 \times 10^7$  g and  $26.8-31.0 \times 10^2$  Å. Here we must point out the possibility of ambiguity in assigning the type of average determined by the light scattering method. BENOTT, HOLTZER and DORY<sup>17)</sup> have shown that the customary interpretation of the slope and intercept of the reciprocal scattering envelope to give the weight-average molecular weight  $\langle M \rangle_w$  and z-average radius

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TABLE 1. The effect of 1 mM PP on the "light scattering" average molecular weight and radius of gyration of myosin B: 0.6 M KCl, 1 mM MgCl<sub>2</sub>, pH 7.2, 23°C.

Prep. No.	Before PP		After PP	
	$\langle M \rangle_l$ $\times 10^{-7} \text{ g}$	$\langle r_g^2 \rangle_l^{1/2}$ $\times 10^{-2} \text{ \AA}$	$\langle M \rangle_l$ $\times 10^{-7} \text{ g}$	$\langle r_g^2 \rangle_l^{1/2}$ $\times 10^{-2} \text{ \AA}$
NM 1	6.65	26.8	6.65	53.6
NM 2	18.2	31.0	18.2	67.8
NM 3	4.6	28.2	4.6	45.7
NM 4	9.1	29.5	—	—

of gyration  $\langle r_g^2 \rangle_z^{1/2}$  is valid only if the scattering is observed at sufficiently low angles, the required angular range decreasing with increasing molecular size. It is clear from their analysis that measurements on myosin B molecule would have to be made in the angular range of 5–10° to eliminate the possible complications caused by polydispersity.

Fortunately, angular distributions,  $P(\theta)$  of our preparations were almost constant, while  $\langle M \rangle_l$  distributed over a wide range (Fig. 2). Therefore, the

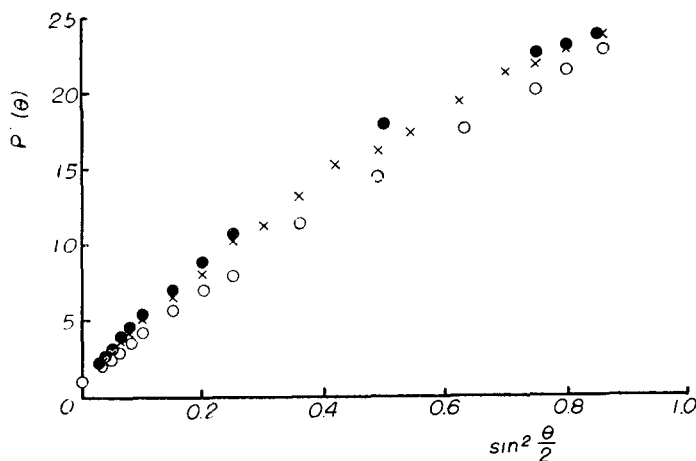


Fig. 2. The reciprocal scattering envelope for three samples. ○, prep. No. NM 1; ●, NM 2; ×, NM 4.

polydispersity of the molecular weight has little effect on the shape of the scattering envelope. This indicates that  $\langle M \rangle_l$  is essentially equal to  $\langle M \rangle_w$ .

As shown in Fig. 1 the scattering envelope deviates from a straight line which corresponds to a random coil, and it curves downward more gently

than in the case of a monodisperse rod<sup>\*</sup>). By adopting the "worm-like" chain model of KRATKY and POROD, PETERLIN<sup>18)</sup> succeeded in relating the degree of downward curvature in the reciprocal envelope to the number and length of the POROD units with which the molecule can be represented. Following the DORY<sup>19)</sup> analysis on deoxyribonucleic acid, the reciprocal envelope of myosin B was compared with the curve calculated from PETERLIN's work and it was shown that the reciprocal scattering envelope of myosin B can be closely approximated by the curve of the "worm-like" chain, of which the number of the POROD units per molecule is 20-30. The persistence length and the effective diameter of the myosin B molecule were then found to be 930-1,080 Å and 6,800-7,900 Å, respectively. This persistence length is longer than those of cellulose trinitrate, 117 Å, and of deoxyribonucleic acid, 500 Å (cf. Ref. 19). This suggests the exceptionally low degree of coiling of myosin B.

Over the range of protein concentration ( $c$ ) from 0.5 to 0.05 mg/ml,  $I_9/c$  was independent of  $c$ . This indicates that the second virial coefficient of myosin B solution is essentially zero. Theoretical considerations<sup>20)</sup> of the meaning of this constant show that in polymer solutions generally it will be approximately equal to the effective volume of the macromolecules if there is no net attraction between the macromolecules. Therefore the very large size of the myosin B molecule would lead to correspondingly large positive values of the second virial coefficient. The fact that these are not found shows that there is fairly strong net attraction between myosin B.

As shown in Fig. 1 and Table 1, when PP was added into the myosin B solution in the presence of  $Mg^{++}$ , the slope of the plot increased abruptly while maintaining essentially the same intercept, showing thereby the molecular size is greatly increased while the molecular weight remains constant. This result agrees completely with the one obtained previously by MORALES *et al.*<sup>5,6)</sup> However, an unambiguous assignment of the type of the average can not be made in the presence of PP because of a somewhat wide variation of  $P(\theta)$ , but this result strongly suggests that the myosin B molecule elongates on the addition of PP. If we assume, following the GERGELY suggestion<sup>8)</sup> that myosin B consists of 80 per cent of myosin A and 20 per cent of F-actin and it dissociates on the addition of PP completely into myosin A and F-actin, the number and the weight-average molecular weights at the dissociated state will be respectively about  $6 \times 10^5$  g and 10<sup>6</sup> g, both values being extremely lower than  $\langle M \rangle_z$  actually obtained. MORALES and BOTTS<sup>21)</sup> ascribed the elongation of myosin B by ATP

\*) A cylinder model can also give a scattering envelope similar to the observed one<sup>17)</sup>. But this model should be excluded because its molecular volume is far larger than the one expected from the partial specific volume and  $\langle M \rangle_w$  of myosin B.

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or PP to the electrostatic repulsion between polyphosphate molecules absorbed to the protein and the charged groups of the protein. But, we cannot accept the MORALES opinion, partly because SALYRGAN, which has no net charge, produced the same effect on the shape of myosin B as PP did (Fig. 1), partly because in the range of pH from 6.0 to 9.0 the charge of myosin B changes remarkably (according to DUBUISSON and HAMOIR<sup>22</sup>) 35 normals per 10<sup>5</sup> g), while its size and shape remained constant<sup>14</sup>), and partly because the amount of bound PP necessary to the change of myosin B was only 1 mole per 5.6 × 10<sup>5</sup> g protein, as will be mentioned below. As discussed throughly in the following section, it may be more reasonable to assume that the binding of PP breaks the intramolecular bonds of myosin B and looses the structure of the protein and then myosin B is elongated by the electrostatic repulsion between charged segments of the protein chain (see 2nd Part, 5th section).

The light scattering average molecular weight of myosin B increased extremely during the storage for more than 7 days at 0°C. After removal of aggregates, which might be produced during the storage, by ultracentrifugation, both  $\langle M \rangle_l$  and  $\langle r_g^2 \rangle_l^{1/2}$  decreased extremely. When PP was added to such a preparation,  $\langle M \rangle_l$  fell in extreme case to about 63 per cent of the original (Table 2). Since GERGELY<sup>7</sup>) previously reported that  $\langle M \rangle_l$  of myosin B after ultracentrifugation at extremely low concentration decreased on ATP-addition, the

TABLE 2. The effect of 1 mM PP on the light scattering average molecular weight and radius of gyration of myosin B obtained by ultracentrifugation after storage of 7 days or dilution to 0.05 mg/ml: 0.6 M KCl, 1 mM MgCl, pH 7.4, 20°C.

Prep. No.	Before PP		After PP	
	$\langle M \rangle_l$ × 10 <sup>-7</sup> g	$\langle r_g^2 \rangle_l^{1/2}$ × 10 <sup>-2</sup> Å	$\langle M \rangle_l$ × 10 <sup>-7</sup> g	$\langle r_g^2 \rangle_l^{1/2}$ × 10 <sup>-2</sup> Å
NM 3 (ultracentrifugation after storage)	1.5	15	0.95	25
NM 5 (ultracentrifugation after dilution)	3.45	17	1.96	18
NM 6 (ultracentrifugation after dilution)	4.76	17	3.85	21

effect of PP-addition on our preparation after ultracentrifugation at protein concentration of 0.05 mg/ml and at 34,000 × G for 3 hours was investigated. The results, shown in Fig. 3 and summarized in Table 2, showed that  $\langle M \rangle_l$  and  $\langle r_g^2 \rangle_l^{1/2}$  of our sample was decreased considerably by this ultracentrifugation and they were further reduced by PP-addition. Since the heavier components may

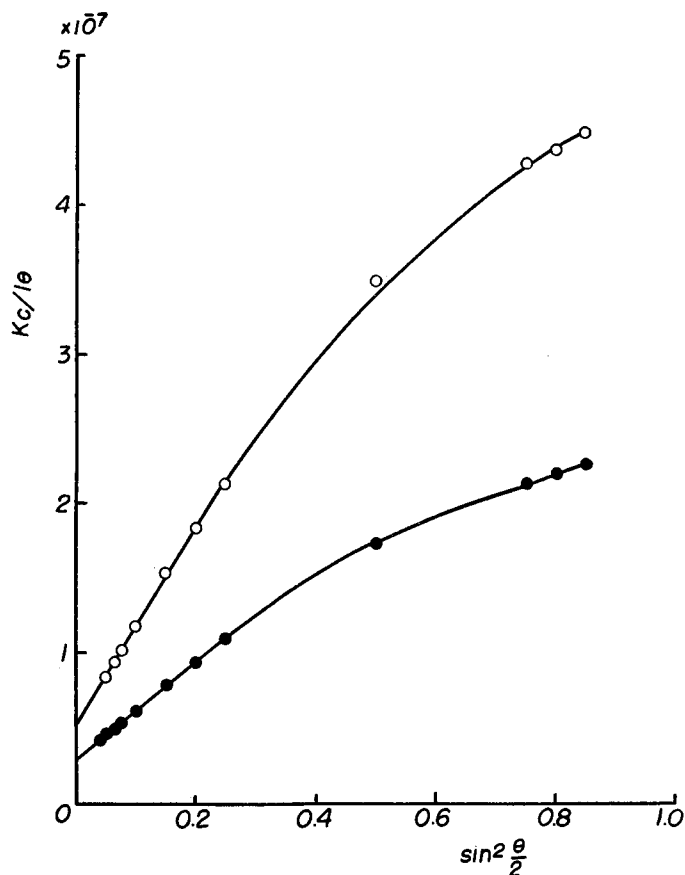


Fig. 3. The reciprocal reduced intensity plot for prep. No. NM 5 after ultracentrifugation at 0.05 mg/ml. 0.6 M KCl, 1 mM  $MgCl_2$ , pH 7.4, 20°C. ●, control; ○, 1 mM PP.

easily be precipitated by ultracentrifugation at extremely low concentration, our results can be well understood if we assume that the lighter components dissociate, while the heavier components do elongate by PP-addition.

## 2. Ultracentrifugal Separation<sup>14)</sup>

Basing on the results of the light scattering, we have deduced that the class of the heavier components of myosin B elongate, while the class of the components with relatively low molecular weight dissociates on PP-addition. Then, the quantities of components which constitute myosin B was determined directly by the ultracentrifugal separation.



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As already reported by A. WEBER<sup>23)</sup>, by the ultracentrifugation for 3 hours at  $10^5 \times G$ , myosin B solution was separated into the water clear layer whose volume was upper two thirds of the total, turbid layer of lower one third and pellet. Pipetting out the upper layer of the second quarter from the surface, we determined the quantity of the protein in the clear layer (light components) and it was found to be about 10 per cent of the total, as shown in Table 3.

TABLE 3. The ultracentrifugal separation of light and heavy components of myosin B preparation ultracentrifugation:  $98,000 \times G$ , 3½ hours, 0.6 M KCl, 1 mM MgCl<sub>2</sub>, pH 7.2-7.4, 3-5°C.

Prep. No.	Before PP		After PP	
	Light comp. (%)	Heavy comp. (%)	Light comp. (%)	Heavy comp. (%)
NM 1	18.0	82.0	41.0	59.0
NM 2	12.0	88.0	24.3	75.7
NM 7	9.3	90.7	33.0	67.0
NM 8	6.3	93.7	23.1	76.9
NM 9	10.0	90.0	30.2	69.8

The intensity of light scattered from the supernatant was lower than 5 per cent of the turbid layer at all angles measured and it did not change on the addition of PP.

On the other hand, when myosin B was ultracentrifuged in the presence of 1 mM PP and 1 mM Mg<sup>++</sup>, it was separated into water clear layer and precipitate, as A. WEBER<sup>23)</sup> already had observed. The quantities of the protein of the water clear layer (light components) of the second quarter layer from the surface was found to be almost identical to the one of the third quarter layer and they were about 30 per cent, as indicated in Table 3. This result is quite different from that of A. WEBER and agrees well with the one of the ultracentrifugal analysis reported by GELLERT *et al.*<sup>5</sup>

The light components must be myosin A or myosin A like protein. This is partly because the salting out analysis<sup>24)</sup> showed contamination of small amount of myosin A in our myosin B, partly because myosin A precipitated scarcely under this centrifugal force and partly because the light scattering of this components did not change on PP-addition. Accordingly, we can conclude that our myosin B is constituted of 10 per cent of myosin A or myosin A like protein and 90 per cent of the heavy components and that about 22 per cent of the latter dissociates by PP in the presence of Mg<sup>++</sup>. SASAKI<sup>24)</sup> has supported this conclusion from his salting-out analysis of the PP-myosin B system.

As previously mentioned, GERGELY<sup>7)</sup> clarified myosin B by the ultracentrifugation at extremely low concentration and observed the decrease of  $\langle M \rangle_i$  of myosin B by the addition of ATP. He concluded that myosin B does dissociate with ATP and the components removed by the ultracentrifugation after dilution are aggregates which do not react with ATP. The present authors cannot, however, accept GERGELY's suggestion, because it is apparent from the following three reasons that the components which do not dissociate are really active ones: (1) our myosin B, of which  $\langle M \rangle_i$  remained constant, exhibited drop of  $I_{90}$  to 35 per cent of the original on the PP-addition, (2) as stated above, it was established by ultracentrifugal separation that the greater part of myosin B did not dissociate by PP, and (3) it was shown by us (see Fig. 2 of ref. 25) that the components which did not dissociate by PP showed ATPase activity equal to the one of the dissociable components.

### 3. Flow Birefringence

From the angular dependence of the light scattering, about 7,500 Å was previously assigned as the length of the main components of myosin B. As well known, measurement of rotary diffusion constant ( $\theta$ ) from flow birefringence ( $\Delta n$ ) is one of the most adequate methods to determine the length of such a long particle. But, since myosin B is degraded at least partially by subjection

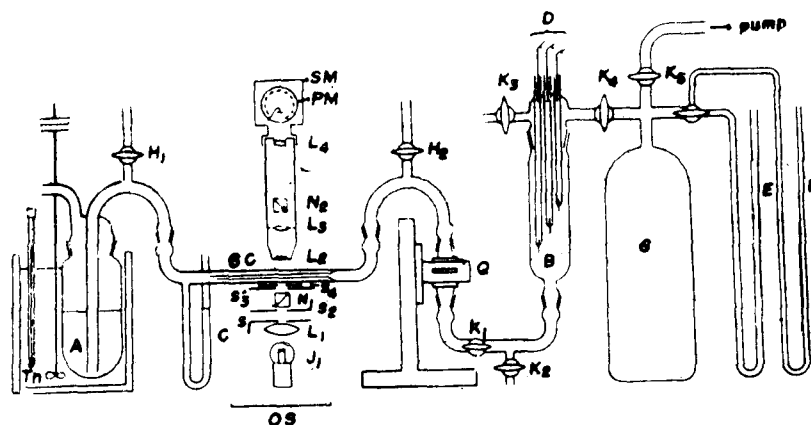
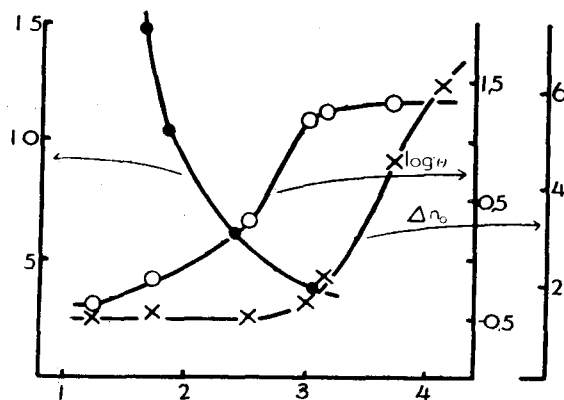


Fig. 4. Apparatus for measurement of flow birefringence and its relaxation. A, reservoir of solution; B, flow meter; D, electrodes of flow meter; E and F, mercuric and water manometer, respectively; GC, sample cell; G, gas reservoir;  $H_1$  and  $H_2$ , cocks to eliminate form in solution;  $K_1$ ,  $K_2$ ,  $K_3$ ,  $K_4$  and  $K_5$ , stopcocks; OS, optical system;  $J_1$ , light source;  $L_1$ , condenser lens;  $L_2$ ,  $L_3$  and  $L_4$ , lenses;  $N_1$  and  $N_2$ , Nicol prisms; PM, photomultiplier;  $S_1$ ,  $S_2$  and  $S_3$ , iris stops;  $S_4$ , rectangular aperture.

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to shear stress either in the presence or absence of PP (see pages 139 and 140), the physical meaning of the result obtained by a conventional flow birefringence apparatus is rather doubtful. Then we used an apparatus<sup>27)</sup> shown in Fig. 4. The sample cell was a round glass tubing, 38.3 cm in length and 1.20 mm in inside radius, set horizontally on the stage of a Leitz microscope "Panphot". An apparent value of the velocity gradient ( $\langle G \rangle_k$ ) was calculated from the formula of KROEPFELIN<sup>28)</sup>. Relative intensity ( $\Delta n_0$ ) of flow birefringence was obtained by measuring the intensity of light passing through the sample cell and two cross Nicol prisms of the "Panphot". Flow of myosin B solution was suddenly stopped by a knife valve ( $<0.1$  msec) and the relaxation of birefringence was recorded by means of an electromagnetic oscillograph, and the rotary diffusion constant was calculated from the BENOIT equation<sup>29)</sup>.

The dependence on  $\langle G \rangle_k$  of  $\Delta n_0$  and  $\theta$  of myosin B solution at the protein concentration of 5.9 and 1.5 mg/ml are drawn in Figs. 5 and 6, respectively.



**Fig. 5.** Dependence on gradient  $\langle G \rangle_k$  of flow birefringence ( $\Delta n_0$ ) and rotary diffusion constant ( $\theta$ ) at high protein concentration: prep. No. MA 1, 5.94 mg protein/ml; 0.55 M KCl, pH 6.3, 20°C.

As shown in Fig. 7, at low  $\langle G \rangle_k$  the relation of  $\log \Delta n - t$  was not linear, that is, myosin B behaved polydispersedly with respect to rotary diffusion, showing a good correspondence with the ultracentrifugal study<sup>26)</sup>. Irrespective of such polydispersity, a single value of ( $\theta$ ) was plotted in the figure, since the most part of the curve was well approximated by this value. At high  $\langle G \rangle_k$ , the polydispersity of the rotation decreased and a single value of  $\theta$  prevailed (Fig. 8).

The rotary diffusion constant increased with increase of  $\langle G \rangle_k$ , particularly in the range of 100–1,000  $\text{sec}^{-1}$  and  $\theta$  approached to a constant value (18–29  $\text{sec}^{-1}$ ), when  $\langle G \rangle_k$  was higher than 1,500  $\text{sec}^{-1}$ , independent of the protein

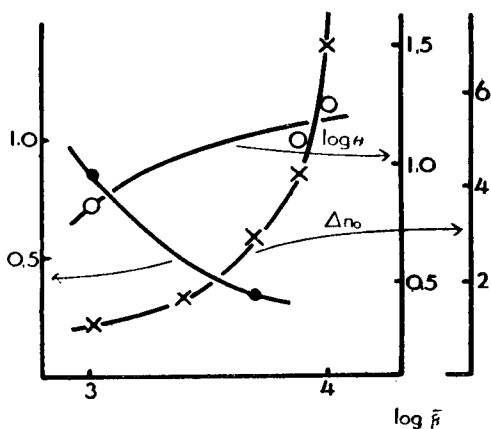


Fig. 6. Dependence on gradient  $\langle G \rangle_k$  of flow birefringence ( $\Delta n_0$ ) and rotary diffusion constant ( $\theta$ ) at low protein concentration; prep. No. MA 2, 1.5 mg protein/ml. 0.6 M KCl, pH 6.7, 20°C. When  $\langle G \rangle_k$  was higher than 4,000 sec<sup>-1</sup>, its value was rather inaccurate because of turbulence of flow.

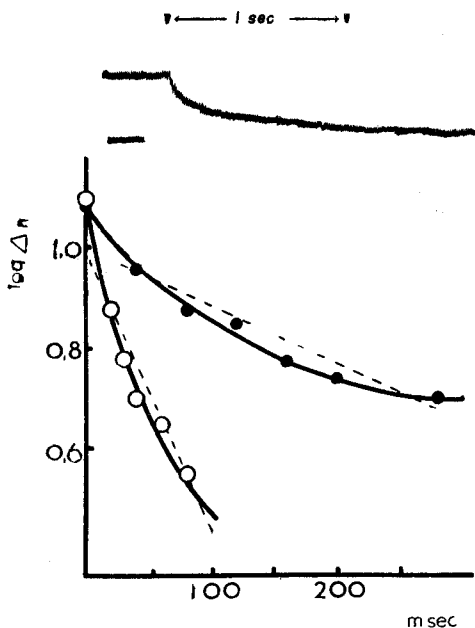


Fig. 7. Relaxation of flow birefringence at low gradient, ●,  $\langle G \rangle_k = 17.45 \text{ sec}^{-1}$ ; ○,  $\langle G \rangle_k = 335 \text{ sec}^{-1}$ . Inset oscillogram is the one of relaxation at  $\langle G \rangle_k = 17.45 \text{ sec}^{-1}$ . Experimental data as in Fig. 5.

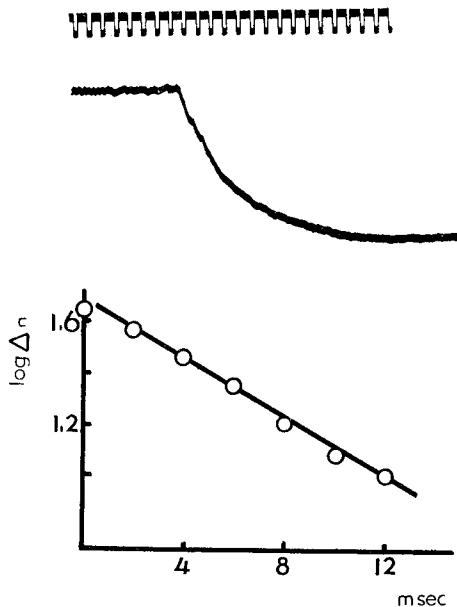


Fig. 8. Relaxation of flow birefringence at high gradient, calculated from the upper oscillogram.  $\langle G \rangle_k = 5,230 \text{ sec}^{-1}$ . Experimental data as in Fig. 5. Time scale 400 cps.

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concentration and  $\langle G \rangle_k$ . The increase of  $\theta$  with  $\langle G \rangle_k$  was found more remarkable at the high protein concentration than at the low one. The length of prolate ellipsoid of axial ratio 100 calculated by the PERRIN equation<sup>30)</sup> from  $\theta$  at high  $\langle G \rangle_k$  was 8,600–10,100 Å. Since a “worm-like” chain is expected to have a smaller value of  $\theta$  than that for a ellipsoid of the same length, the value of  $\theta$  might be in fine correspondence to the length of the “worm-like” chain (about 7,500 Å) obtained by the light scattering method. Consequently, it may be concluded that when the protein concentration is high, a net work is built up in the myosin B solution by the entanglements of the heavier components of the constant length with each other and the entanglements are broken more and more as velocity gradient increases. As described in the 1st section, the light scattering measurement on myosin B also indicated a strong attraction between the heavier components.

As the rotary diffusion constant of myosin B is about  $20 \text{ sec}^{-1}$ , orientation of myosin B must be rather complete when  $\langle G \rangle_k$  becomes higher than several thousand  $\text{sec}^{-1}$ . And yet,  $\Delta n_0$  of myosin B increased remarkably with increase of  $\langle G \rangle_k$ . It exhibited an increase of 4–5 times by increase of  $\langle G \rangle_k$  from 1,000 to 10,000  $\text{sec}^{-1}$ . This increase may be too high to be accounted by the orientation of contaminated myosin A and may indicate that the optical factor of myosin B increases considerably by subjection to high shear stress. On the other hand, NODA and MARUYAMA<sup>31)</sup> have recently concluded from their study on the flow birefringence of myosin B that at low velocity gradient myosin B behaves as rigid particle. These results seem to correspond well with the conclusion of light scattering, that is, myosin B is a “worm-like” chain of exceptionally low degree of coiling.

In one experiment, 1.1 mM ATP were added to myosin B solution (1.5 mg/ml) and the relaxation of flow birefringence was observed at high velocity gradient, where interparticular interaction might be neglected, but as indicated in Fig. 9 it was too fast to be followed by the present apparatus ( $\geq 100 \text{ sec}^{-1}$ ). As described above, PP or ATP does elongate the main components of myosin B, which play a predominant role in light



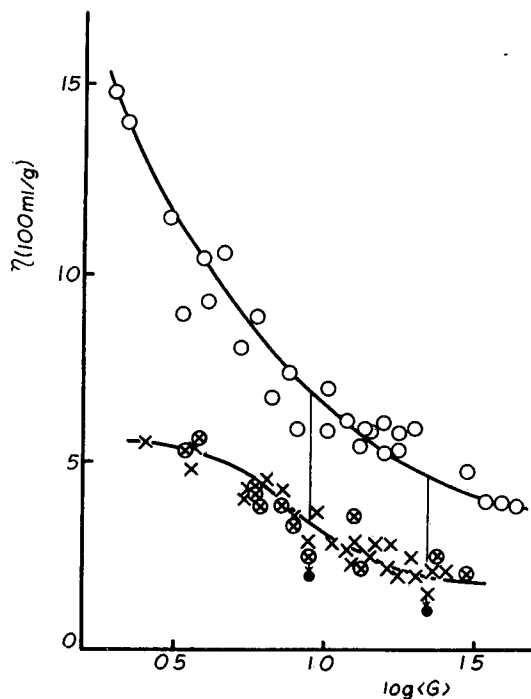
Fig. 9.

Relaxation of flow birefringence in the presence of 1.1 mM ATP, at higher velocity gradient and at lower protein conc. prep. No. MA 3, 1.5 mg protein/ml. 0.6 M KCl, pH 6.7, 20°C. Time scale 400 cps.

scattering and flow birefringence. Accordingly it may be deduced that, by the addition of ATP, (1) the structure of the main particles becomes so loose that it is degraded by subjection to shear stress or (2) the myosin A component on the myosin B particle becomes to be able to rotate rather freely. The results on viscosity, which will be mentioned in the next section, prefers the former as more reasonable explanation than the latter.

#### 4. Viscosity<sup>32)</sup>

The light scattering and the ultracentrifugal separation have indicated that the main components of myosin B elongate on PP-addition. In the case of a monodisperse system of macromolecules, its viscosity should be increased by the elongation of molecules. Accordingly, it is natural, that many workers<sup>1,3)</sup> have considered the decrease of viscosity of myosin B by ATP-addition as the evidence for the dissociation of myosin B. However, for a polydisperse system



**Fig. 10.** The reduced viscosity ( $\eta$ ) of myosin B, prep. No. S 3, as a function of the mean velocity gradient  $\langle G \rangle$  in the presence ( $\times$ ,  $\otimes$ ) and absence ( $\circ$ ) of 1 mM PP, 0.6 M KCl, pH 6.7, 20°C.  $\circ$ ,  $\times$ , 0.626 mg/ml;  $\otimes$ , 0.533 mg/ml. The viscosities observed soon after the flow through capillary are indicated by  $\bullet$ .

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of non-rigid particles with strong inter-molecular interaction such as myosin B, the viscosity must be measured more systematically and be interpreted more carefully in relation to the size and shape of particles.

Then we constructed a viscometer by the method of CLAESSON and LOHMANDER<sup>33</sup>. It consisted of a 9.0 cm length of capillary of radius 0.0318 cm with wide cylindrical tubes of radius 0.54 cm. The movement of the meniscus in the cylindrical tubes was followed by a micrometer in a microscope. The mean gradient  $\langle G \rangle$  was calculated according to the approximation method of KRIEGER *et al.*<sup>34</sup>. The reduced viscosity ( $\eta_{sp}/c$ ) of myosin B was observed to be independent of  $c$  at all velocity gradient, when  $c$  was lower than 1 mg/ml.

In Figs. 10 and 11 typical examples of the relation between reduced viscosity and  $\langle G \rangle$ , in the absence and the presence of PP, are illustrated. The viscosity decreased considerably on the addition of PP at various  $\langle G \rangle$ . However, the most peculiar phenomenon observed was that  $\eta$  was decreased, either in the presence or the absence of PP, by even a very slow flow through the capillary ( $\langle G \rangle \sim 25 \text{ sec}^{-1}$ ) and it returned gradually to the original value during several minutes.

Our results on light scattering and flow birefringence of myosin B have shown that the main components of myosin B are "worm-like" chains of about

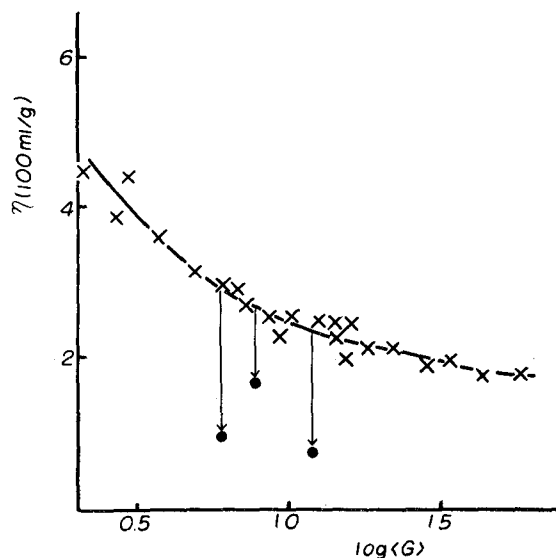


Fig. 11. The reduced viscosity of myosin B (prep. No. S 4) as a function of the mean velocity gradient  $\langle G \rangle$  in the presence of 1 mM PP. 1.2 mg/ml, 0.6 M KCl, pH 6.7, 20°C. The viscosities observed after the flow at 15-35  $\text{sec}^{-1}$  are indicated by ●.

7,500 Å length which do not significantly change at high shear stress and that they do elongate with PP. To compromise with this conclusion, the viscosity of myosin B should be interpreted as follows. In the absence of PP, the reduced viscosity of myosin B should be chiefly attributable to the dissociable components which may have high intrinsic viscosity because of their slender shape and they are degraded even by a low shearing stress and so, the viscosity decreases with the increase of  $\langle G \rangle$ . On the other hand, in the presence of PP, the viscosity may be mainly attributable to the elongated particles, because the dissociable components is dissociated to have a relatively low viscosity. However, in the presence of PP, even the main components became susceptible to degradation by shear stress as shown by the decrease of  $\eta$  (Fig. 11) and also by the increase of the rotary diffusion constant (Fig. 9). Accordingly in order to determine the shape of the elongated particles,  $\eta$  should be observed as low  $\langle G \rangle$  as possible where the particles are not degraded. Unfortunately  $\eta$  at sufficiently low  $\langle G \rangle$  could not be accurately measured by our apparatus, but it was apparent that  $\eta_0$  was higher than 5–9 in the unit of 100 mℓ/g. If we assume that in the presence of PP the viscosity of the elongated components is much higher than the one of the dissociated ones, the intrinsic viscosity at zero gradient of the elongated myosin B components becomes larger than  $(5-9)/0.7=7.15-12.8$  in units of 100 mℓ/g, as the concentration of the elongated particles is about 70 per cent of the total (see the second section). Applying the FLORY-Fox relation<sup>20)</sup> which has been found to apply well to macromolecules of low degree of coiling<sup>19)</sup>,

$$\eta_0 = \frac{2.2 \times 10^{21} \langle r_0^2 \rangle^{3/2}}{P \langle M \rangle_w}$$

and taking  $\langle M \rangle_w$  as  $10^8$ g and  $P$  arbitrarily as 2 the root-mean-square end-to-end distance  $\langle r_0^2 \rangle^{1/2}$  of elongated myosin B becomes longer than 8,200–10,500 Å. In magnitude this corresponds to the value of the radius of gyration  $\langle r_g^2 \rangle^{1/2}$  (4,600–6,800 Å) obtained by the light scattering method.

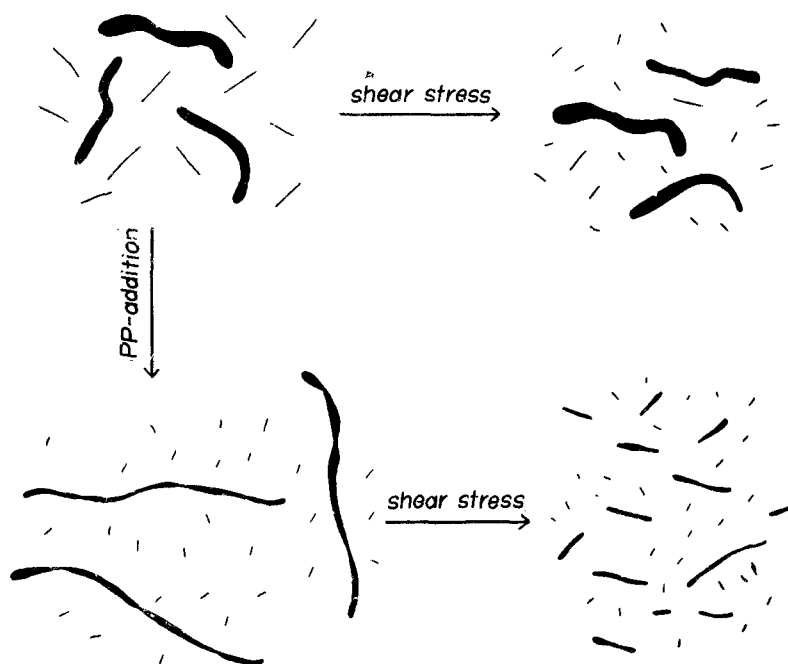
## 5. Summary

Thus it follows from various lines of evidence that the components of our myosin B are as follows: the class of main components takes part of 70 per cent of the total and are the “worm-like” chains of the length of 7,500 Å and of the molecular weights of  $6.6-26.0 \times 10^7$  g. The main components interact with each other rather strongly and at high concentration a net-work is built up in the solution. The class of dissociable components consists of 20 per cent of the total and have much higher viscosity and smaller molecular weights than



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the main components and it disaggregates rather easily by shear stress. The remaining (10%) may be myosin A or myosin A like protein. When PP is added to myosin B, the main components elongate (their radii of gyration become about twice of the original) and its structure becomes so loose that it is degraded by subjection to shear stress. The dissociable components, which is more susceptible to degradation than the main components, dissociate into



**Fig. 12.** Behavior of the heavy components of myosin B under the shearing stress in the presence and the absence of PP. Only schematical. The "worm-like chains" and the shender lines indicate respectively the main and the dissociable components.

the light components by addition of PP. On the basis of all of these findings, the change of myosin B by PP or by shear stress may be depicted as shown in Fig. 12.

However, in closing this part we must notice that the ratio of these components depends, of course, on the method of the preparation: for example, when myosin B was extracted for 5 hours and purified by precipitation at 0.06 M KCl as done by MORALES *et al.*<sup>5,6)</sup> the preparation might be rich in myosin A more than ours, and when myosin B was clarified by ultracentrifugation at low concentration as done by GERGELY<sup>7,8)</sup> the ratio of the dissociable

components to the main components should have been increased by the ultracentrifugation. The apparently different results obtained by various investigators seem, as pointed out by MORALES<sup>6)</sup>, to be due to the difference in the ratio of the three classes of the components whose properties are elucidated here fairly well.

## PART II. THERMODYNAMIC AND KINETIC ANALYSIS

In this part, the binding weight of myosin A and myosin B and the affinity of polyphosphate to the muscle proteins and its reaction mechanisms will be discussed. From the results obtained by the equilibrium dialysis and by the light scattering method, it has been demonstrated that the intensity of light scattered by myosin B is decreased by an almost constant degree every time one PP molecule binds to one site of myosin B and that the degree of binding of PP to myosin B can be determined from the change in scattered light of myosin B. On the basis of these results, we have attempted the transient kinetic analysis of light scattering change of the myosin B-PP system and analyzed the thermodynamic properties of the elementary steps of the PP-myosin B system.

### 1. Equilibrium Dialysis<sup>35)</sup>

In order to obtain informations on the binding weight and on the affinity constant of the binding of ATP or PP to myosin B, several attempts have already been made. The binding of ATP to myosin B was investigated by us<sup>36)</sup> by means of a rapid light scattering method and by MOMMAERTS *et al.*<sup>10,37)</sup> from the change of light scattering in the presence of pyruvate-kinase system. Recently GERGELY<sup>38)</sup> and we<sup>35)</sup> have attacked the problem by means of the KLOTZ equilibrium dialysis method<sup>39)</sup> with PP.

Our procedures were as follows. The cellophane tubings containing the protein solution were dialyzed for P<sup>32</sup> labelled PP solution. At equilibrium, the extent of binding of PP to the protein was calculated from the difference of radioactivities between the external solution of the protein and that of the control solution, with no protein added.

The representative examples on the degree of binding of PP to myosin A are indicated in Fig. 13 and Table 4. These results clearly show that the maximum binding of PP to myosin A is one mole of PP per  $2.3 \times 10^5$  g of the protein. From the Archibald approach to the ultracentrifugal analysis, MOMMAERTS and ALDRICH<sup>40)</sup> and Von HIPPEL *et al.*<sup>41)</sup> have shown that the

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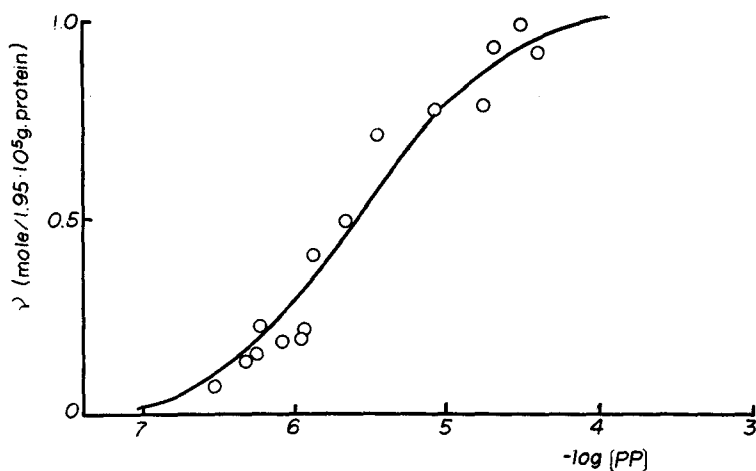


Fig. 13. The binding of PP to myosin A (No. MO 2) as a function of [PP]. 0.6 M KCl, 0.3 mM MgCl<sub>2</sub>, pH 7.5, 5°C. The line represents the theoretical one

$$\nu = \frac{10^{5.9}[\text{PP}](1+2 \cdot 10^{5.2}[\text{PP}])}{1+10^{5.9}[\text{PP}](1+2 \cdot 10^{5.2}[\text{PP}])}$$

TABLE 4. The amount of binding weight of myosin A and the strength of binding of PP to myosinA: pH 7.5, 5°C.

Myosin A No.	Ionic medium	Binding weight × 10 <sup>-5</sup> g	Dissociation constant	
			pK <sub>1</sub>	pK <sub>2</sub>
MO 1	0.6 M KCl, 0.3 mM MgCl <sub>2</sub>	2.45	6.3	5.4
MO 2	0.6 M KCl, 0.3 mM MgCl <sub>2</sub>	1.95	5.9	5.2
MO 3	0.04 M KCl, 0.3 mM MgCl <sub>2</sub>	2.5	6.8	4.7
MO 4	0.6 M KCl, 0.1 mM CaCl <sub>2</sub>	< 3.4	(5.7)	—

molecular weight of myosin A is  $4.2 \times 10^5$  g. This value is supported by us from the light scattering method<sup>(27)</sup>. Therefore, myosin A binds two molecules of PP per one molecule. If we assume that these two sites of myosin A are identical with each other and the binding of PP follows the mass action law, the relation between the extent of binding ( $\nu$ ) and the concentration of PP, [PP] is given by

$$\nu = \frac{[\text{PP}]/K_1 \{1 + 2[\text{PP}]/K_2\}}{1 + [\text{PP}]/K_1 \{1 + [\text{PP}]/K_2\}}$$

where  $K_1$  and  $K_2$  are respectively dissociation constant of the binding of PP to the free protein and the one to the myosin A-PP complex. The  $\nu$  values calculated using  $K_1$  and  $K_2$  showed the satisfactory agreement with the experimental ones. However, there is a contradiction between results of GERGELY and his associates<sup>38)</sup> and us: they have reported that myosin A binds only one mole of PP per  $5 \times 10^5$  g of the protein. It is supposed that they examined the binding at too low concentrations of PP to find the second binding of PP.

GERGELY and co-workers<sup>38)</sup> have reported that PCMB and EDTA inhibit the binding of PP to myosin A. It is also supported by our deduction<sup>43,44)</sup> from the kinetical analysis of ATPase that those substances bind to the site of light scattering change of the protein and decrease the stability of the Michaelis complex of ATPase. They have also established that actin does not bind with PP. YAGI<sup>45)</sup> previously suggested this point from the analyses of ATP response to myosin B containing the different amounts of actin. Recently the contents of  $Mg^{++}$  and  $Ca^{++}$  of myosin A throughly dialysed in advance was measured by KITAGAWA<sup>46)</sup> in our laboratory by the method of YANAGISAWA<sup>47)</sup>. It was observed that myosin A contained about 2 moles of  $Ca^{++}$  tightly bound to one myosin A molecule, and this  $Ca^{++}$  was released completely by PCMB, while the content of  $Mg^{++}$  was much smaller than  $Ca^{++}$ . Therefore it may be reasonable to conclude that PP binds to "intrinsic"  $Ca^{++}$  bound tightly to the SH group of myosin A.

Typical examples of the relations between the degree of binding of PP to

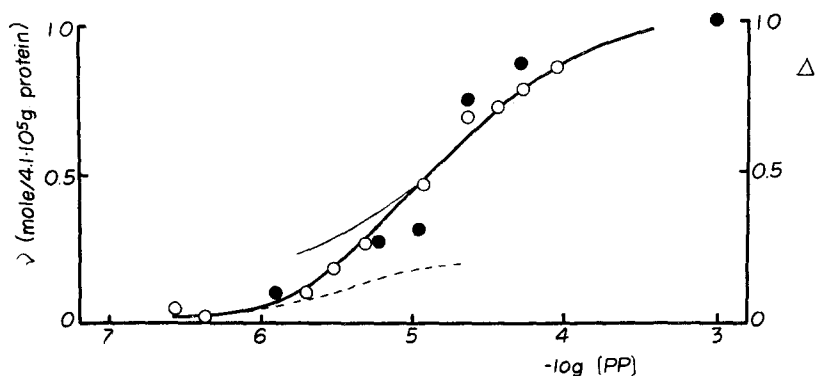


Fig. 14. The binding of PP to myosin B (No. NM 13) as a function of  $[PP]$ , 0.6 M KCl, 0.3 mM  $MgCl_2$ , pH 7.5, 5°C. ○, extent of binding; ●, degree of change in light scattering. The fine solid line represents the theoretical one:  $\nu = \frac{1}{1 + 10^{-4.7}/[PP]}$ . The dashed line illustrates the binding curve due to minor component.

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myosin B and of the change in light scattering by PP are illustrated in Figs. 14, 15 and 16.

Figs. 14 and 15 show respectively the binding of PP of the first and the second order in the presence of  $Mg^{++}$ . Fig. 16 illustrates the one in the persence of  $Ca^{++}$ . Although the binding of PP to myosin B mostly followed

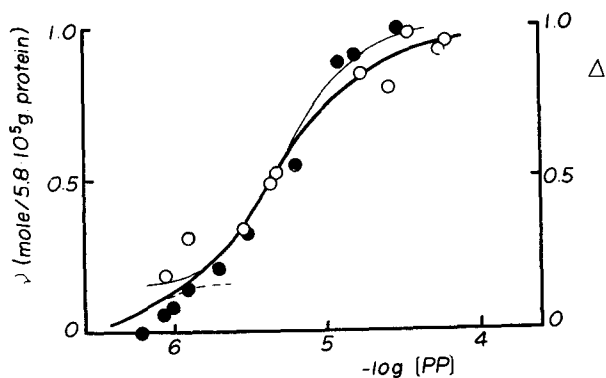


Fig. 15. The binding of PP to myosin B (No. NM 15) as a function of [PP]. 0.6 M KCl, 0.3 mM  $MgCl_2$ , pH 7.5, 5°C. ○, extent of binding; ●, degree of change in light scattering. The fine solid line represents the theoretical one:  $\nu = \frac{1}{1 + (10^{-5.25}/[PP])^2}$ . The dashed line illustrates the binding curve due to minor component.

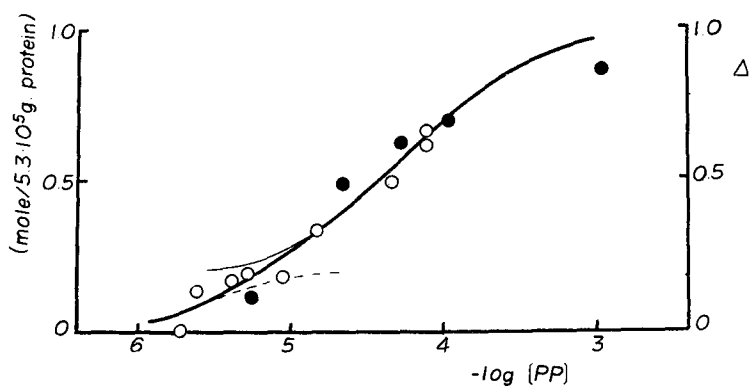


Fig. 16. The binding of PP to myosin B (No. NM 17) as a function of [PP]. 0.6 M KCl, 0.1 mM  $CaCl_2$ , pH 7.5, 5°C. ○, extent of binding; ●, degree of change in light scattering. The fine solid line represents the theoretical one:  $\nu = \frac{1}{1 + 10^{-4.25}/[PP]}$ . The dashed line illustrates the binding curve due to minor component.

the dissociation curve of the first or the second order, a minor heterogeneous binding was observed under the range of low concentration of PP. Assuming the minor component has the same molecular weight and the same binding capacity as those of myosin A, we can estimate the content of this component from the binding curves. As listed in Table 5, its content was almost as same as that of the light component estimated by the ultracentrifugal separation. The order and the  $pK$  of the binding reaction of PP to the main components of myosin B preparations are also listed in Table 5. Although the values differed

TABLE 5. The amount of binding weight of myosin B and the strength of binding of PP to myosin B: 0.6 M KCl, pH 7.5, 5°C.

Myosin B No.	Divalent cation	Binding weight × 10 <sup>-5</sup> g	Dissociation constant				Minor comp. (%)
			chemical		optical		
			order	$pK$	order	$pK$	
NM 9	0.3 mM MgCl <sub>2</sub>	5.8	1	5.1	1	5.1	10 <sup>*</sup> )
NM 13	0.3 mM MgCl <sub>2</sub>	4.4	1	4.7	1	4.8	10
NM 14	0.3 mM MgCl <sub>2</sub>	5.0	2	4.4	2	—	10
NM 15	0.3 mM MgCl <sub>2</sub>	6.5	2	5.25	2	5.25	7.5
NM 16	0.3 mM MgCl <sub>2</sub>	5.0	2	5.5	2	5.25	15
NM 17	0.1 mM CaCl <sub>2</sub>	5.9	1	4.25	1	4.3	10
NM 18	0.1 mM CaCl <sub>2</sub>	5.8	1	4.15	1	4.15	10 <sup>*</sup> )

\* ) Value suggested from data of ultracentrifugal separation.

with the experimental conditions and the preparations, the  $pK$ 's for myosin B were generally larger than that of myosin A.

The most significant features of the results are as follows. First, the order and the  $pK$  of the binding reaction of the major components of myosin B agree with those calculated from the degree of light scattering change, that is, the plot  $\Delta/\Delta_0$  versus [PP], where  $\Delta$  and  $\Delta_0$  indicate the decreases of scattered light caused by the addition of PP of certain and of sufficiently high concentration respectively. This fact clearly shows that the units of myosin B behave as if they are almost independent of each other, in respect to the change of light scattering. In other words the intensity of light scattered by myosin B is decreased by an almost constant value every time one PP molecule binds with one myosin B unit and each site has the same intrinsic affinity for PP. Thus the binding of PP to myosin B can be measured by means of the light scattering. Second, the maximum binding of PP to the main component is one mole of PP per  $5.6 \times 10^5$  g of the protein, irrespective of the order and  $pK$

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of the binding. As myosin B is mostly composed by myosin A (more than three quaters), it should be concluded that only one of the two sites of myosin A is available to PP when myosin A is the constituent of myosin B. This contradicts with the claim<sup>7,8)</sup> that myosin B dissociates into myosin A and actin completely by the addition of PP or ATP.

**2. Change of Light Scattering at Equilibrium State<sup>(8)</sup>**

On the basis of the results described above, we can investigate the mechanism of the binding of PP to myosin B more easily by the method of light scattering technique than by equilibrium dialysis. MOMMAERTS<sup>9)</sup> already investigated the interaction between myosin B and polyphosphate by viscometry. As

TABLE 6. Apparent dissociation constants for the myosin B-pyrophosphate system.

Protein No.	Temp. °C	pH	Divalent cation added mM		Order	<i>pK</i>
MY 1	5-6	7.5	10	Mg	2→1	5.25→5.2
MY 1	5-6	7.5	1	Mg	2→1	5.1 →5.1
MY 1	5-6	7.5	0.1	Mg	2	4.7
MY 1	5-6	7.5	0.02	Mg	2	4.5
MY 1	5-6	7.5	0.015	Mg	2 (?)	4.35
MY 1	5-6	7.5	0.01	Mg	1	3.6
MY 2	5-6	7.5	10	Mg	2	4.8
MY 2	5-6	7.5	0.1	Mg	2	3.9
MY 2	5-6	7.5	0.01	Mg	2	3.4
MY 2	16.5	7.5	10	Mg	2	4.5
MY 2	16.5	7.5	10	Mg	2	4.4
MY 2	5-6	6.7	10	Mg	2	4.45
MY 2	5-6	6.7	0.1	Mg	1	3.6
MY 3	6	7.5	10	Mg	2	4.7
MY 3	6	7.5	0.1	Mg	2	4.45
MY 3	6	7.5	0.01	Mg	1	3.9
MY 2	5	7.5	0.1	Ca	1	4.2
MY 2	5	7.5	0.01	Ca	1	4.2
MY 2	17	7.5	0.1	Ca	1	3.2
MY 2	17	7.5	0.01	Ca		2.7
MY 3	5	7.5	0.1	Ca	1	3.7
MY 3	5	7.5	0.01	Ca	1	3.7

his results were, however, only qualitative, the degree of light scattering change,  $\Delta/\Delta_c$  versus [PP], was measured quantitatively and more systematically under the various concentrations of  $Mg^{++}$  or  $Ca^{++}$ .

As shown in Table 6 and Fig. 17, the decrease in the scattered light of myosin B caused by PP was enhanced remarkably by divalent cations. In the presence of sufficiently high concentration of  $Mg^{++}$ , the relation between the degree of scattering drop,  $\Delta/\Delta_c$ , and [PP] was given by as a dissociation curve

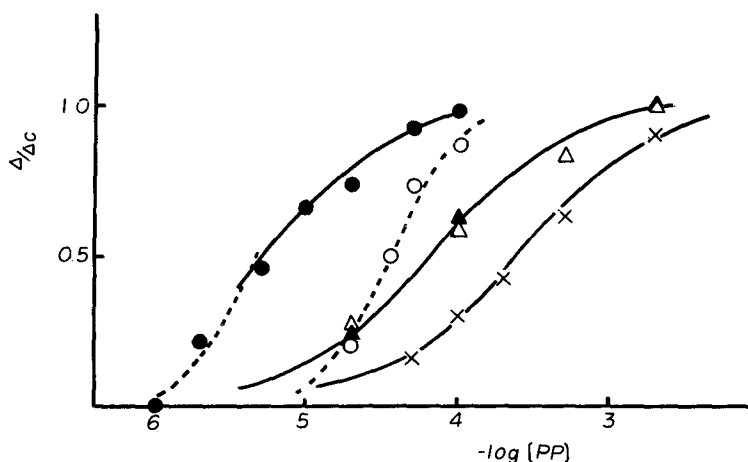


Fig. 17. Relation between the grade of light scattering change ( $\Delta/\Delta_c$ ) and [PP]. 0.6 M KCl at 5-6°C, 0.2 mg protein/ml. The solid line and the dotted lines correspond, respectively, to  $\Delta/\Delta_c = 1/2 + 1/2 \tanh (2.303 (1/2) \log [PP]/K)$  and  $\Delta/\Delta_c = 1/2 + 1/2 \tanh (2.303 \log [PP]/K)$ . ●, prep. No. MY 1, 10 mM  $MgCl_2$ , pH 7.5; ×, prep. No. MY 2, 10 mM  $MgCl_2$ , pH 6.7; ○, prep. No. MY 2, 0.1 mM  $MgCl_2$ , pH 6.7; ▲, prep. No. MY 2, 0.1 mM  $CaCl_2$ , pH 7.5; △, prep. No. MY 2, 0.01 mM  $CaCl_2$ , pH 7.5.

of the first order. When  $[Mg^{++}]$  was moderately high, the relation  $\Delta/\Delta_c - [PP]$  was described as a dissociation curve of the second order and when  $[Mg^{++}]$  was sufficiently low, the relation was again given as the first order one. On the other hand, in the presence of  $Ca^{++}$ , the relation followed a dissociation curve of the first order independently of the concentration of the cation and its  $pK$  was also independent of  $[Ca^{++}]$ .

As has been shown by the equilibrium dialysis, the size and shape change of myosin B by PP is constantly caused by the binding of PP to the site. Then, as the most reasonable mechanism for the interaction between PP and myosin B, the following one was proposed by us<sup>(8)</sup>,



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where M and M\* represent the unchanged and changed unit of myosin B respectively and Me is divalent cation added. In the step (1) PP binds to the "intrinsic" Ca<sup>++</sup> bound tightly to the SH group of the protein (see the previous section). In the step (2), Ca<sup>++</sup> added binds more strongly with the protein than Mg<sup>++</sup> does, and on the other hand, in the step (3) the binding of PP with Mg<sup>++</sup> absorbed on the protein is fairly strong but the binding with Ca<sup>++</sup> is weak and almost imperceptible. As mentioned in the previous section, even in the case when  $\Delta/\Delta_c$  versus [PP] given as a dissociation curve of the second order, the number of the sites for PP binding was same as that obtained in the case when  $\Delta/\Delta_c$  versus [PP] followed a first order one. Therefore, the number of binding site of the cation (MMe) in the above mechanism must be much smaller than that of PP directly related to the shape change (M\*PP). It is of course possible to suggest other mechanisms for interpretation of the second order dissociation curve in the presence of Mg<sup>++</sup>. For example, the attraction between PP molecules bound to the two neighbouring sites may be so strong that the curve  $\Delta/\Delta_c$  versus [PP] follows a second order dissociation curve. But our mechanism seems to be preferable than such one, because it can explain the effects of Mg<sup>++</sup> and Ca<sup>++</sup> more consistently, as thoroughly described in one of our previous paper<sup>(8)</sup>.

The number of binding of ATP to myosin B cannot be determined by the equilibrium dialysis method, as ATP is hydrolyzed by myosin B. The minimum quantity of ATP necessary to cause the maximum change in light scattering was measured in the presence of various concentration of Mg<sup>++</sup>, which inhibits ATPase and intensifies the strength of the binding of ATP. Since high concentration of Mg<sup>++</sup> formed precipitate with ATP, the limiting value at sufficiently high concentration of Mg<sup>++</sup> was inferred by extrapolation and estimated to be one mole of ATP per  $4-6 \times 10^5$  g of protein as shown in Fig. 18. This value agrees well with that of PP obtained by the equilibrium dialysis. As PP causes the same change in myosin B as ATP does<sup>1,3)</sup>, ATP and PP may bind to the same site of myosin B.

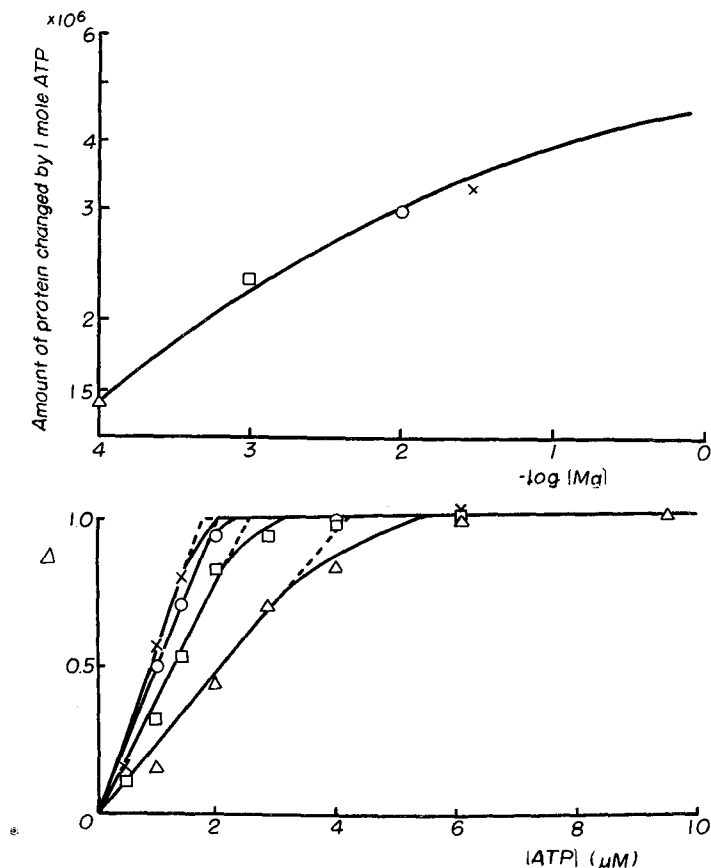


Fig. 18. The minimum amount of ATP necessary to maximum change in light scattering. Myosin B, No. NM 19. 0.6 mg protein/ml. 0.6 M KCl, pH 6.9, at 15°C.  $\Delta$ , 0.1 mM  $MgCl_2$ ;  $\square$ , 1 mM;  $\circ$ , 10 mM;  $\times$ , 30 mM

### 3. Transient Change of Light Scattering. I<sup>49)</sup>

In 1952, one of the present authors<sup>36)</sup> already studied the change in scattered light of myosin B by ATP by a rapid scattering method and put forward the following mechanism for the shape change of myosin B by ATP:



The shape of myosin B is changed by ATP (S) through an intermediate complex, but the shape of MS is not changed. It was also concluded at that time that the first step is not affected by divalent cations. However, the rate of scattering change by ATP was so rapid that a technique of rapid mixing used was some-

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what unsatisfactory and correction due to hydrolysis of ATP should be made.

As well known, PP is not decomposed by myosin B. Furthermore it was found that the rate of change in the scattered light by PP is much slower than in the case of ATP. Taking advantage of these facts the mechanism of the shape change of myosin B by PP was studied under various conditions.

As described above, in the presence of  $\text{Ca}^{++}$  the relation  $\Delta/\Delta_0$  against  $[\text{PP}]$  was given by a dissociation curve of the first order and its  $pK$  was almost independent of  $[\text{Ca}^{++}]$ . Then to simplify the analysis the myosin B-PP system has studied mostly in the presence of  $\text{Ca}^{++}$ . Typical examples of the decrease in the scattered light after the addition of PP are shown in Fig. 19. During an early period, the plots of  $\ln(1-\Delta/\Delta_m)$  versus  $t$  followed the straight lines where  $\Delta_m$  denotes the maximum value of  $\Delta$ , which is observed when the dielectric constant of the medium is sufficiently low (see below). Furthermore the plot of the reciprocal slopes of these lines ( $1/v$ ) against  $1/[\text{PP}]$  gave a straight line

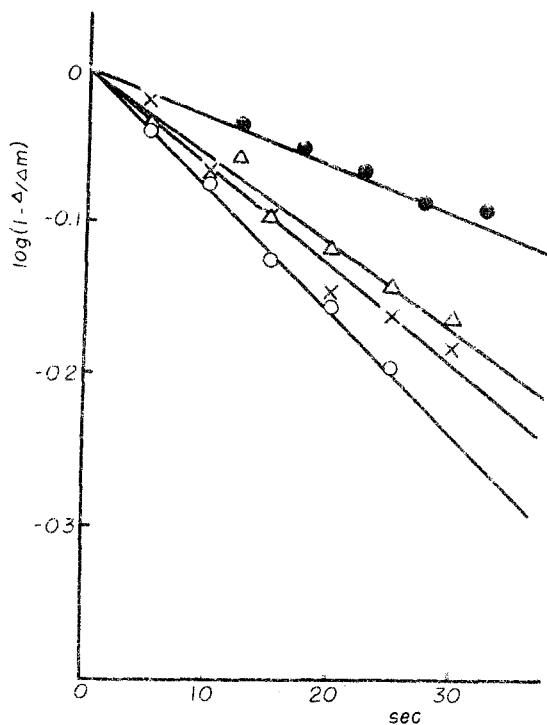


Fig. 19. Time course of the decrease in scattered light after the addition of PP. Prep. No. MO 2, 0.6M KCl, 0.5 mM  $\text{CaCl}_2$ , 2 per cent dioxane, pH 7.88, 5°C. Concentrations of PP:  $\circ$ , 1 mM;  $\times$ , 0.7 mM;  $\Delta$ , 0.5 mM;  $\bullet$ , 0.2 mM.

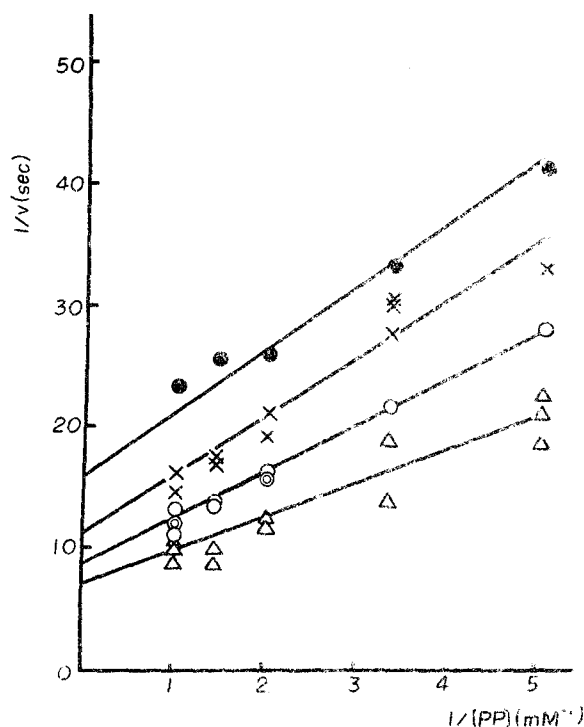
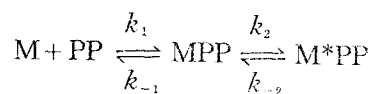


Fig. 20. Reciprocal slope of the time course of the decrease in scattered light against reciprocal concentration of PP. Prep. No. MO 4, 0.6 M KCl, 4 per cent dioxane, pH 7.88, 5°C. Concentration of CaCl<sub>2</sub>: Δ, 0.7 mM; ○, 0.5 mM; ×, 0.3 mM; ●, 0.1 mM.

as illustrated in Fig. 20.

The reaction between PP and myosin B is reversible as demonstrated previously<sup>50)</sup> and the unit of myosin B behaves as if independent of each other. Thus if the unit of myosin B is represented by M, the above results are well explained by the following mechanism:



where  $k_i$  represents the velocity constant of each step. A similar mechanism has already been proposed by us for the ATP-myosin B system. Thus during early period where the reverse reaction of the step 2, can be neglected, we obtain for the time course of decrease of light scattering

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$$-\ln\left(1 - \frac{\Delta}{\Delta_m}\right) = \frac{k_2}{1 + \frac{K_1}{[\text{PP}]}} t$$

where  $K_1 = \frac{k_{-1} + k_2}{k_1}$ . Accordingly,  $k_2 / \left(1 + \frac{K_1}{[\text{PP}]}\right)$  is given as the slope of the line of  $\ln(1 - \Delta/\Delta_m)$  versus  $t$  (Fig. 19), and  $K_1/k_2$  and  $1/k_2$  respectively as the slope and the intercept on the ordinate of the slope of  $\left(1 + \frac{K_1}{[\text{PP}]}\right) / k_2$  against  $1/[\text{PP}]$  (Fig. 20). The  $K_1$  and  $k_2$  values for various preparations in the presence of  $\text{Ca}^{++}$  are listed in Table 7. Though  $k_2$  increased with increase of  $[\text{Ca}^{++}]$ ,  $K_1$  was almost independent of  $[\text{Ca}^{++}]$  (see Table 7, lines 4-7). This indicates that  $k_{-1}$  is much larger than  $k_2$  and  $K_1$  can be reduced to  $k_{-1}/k_1$ , *i. e.* the dissociation constant of the step 1. At equilibrium,  $\Delta/\Delta_m$  is given by the following equation:

TABLE 7. Dissociation and velocity constants of the change in the light scattering of myosin B on PP-addition: 0.6 M KCl, pH 7.88, 5°C,  $D = 32.56$ .

Protein No.	Conc. of $\text{Ca}^{++}$ mM	$K_1 \times 10^4$ (M/l)	$K_2 \times 10$	$k_2 \times 10$ (sec <sup>-1</sup> )	$k_{-2} \times 10^3$ (sec <sup>-1</sup> )
MO 1	0.01	2.54	1.06	0.94	10
MO 2	0.5	12.5	0.46	1.43	6.6
MO 3	0.1	3.0	0.91	0.64	5.9
MO 4	0.1	3.5	1.2	0.53	6.3
MO 4	0.3	4.4	1.04	1.01	10.5
MO 4	0.5	4.4	1.04	1.30	13.5
MO 4	0.7	4.4	1.04	1.79	18.6
MO 5	0.5	5.7	0.62	1.0	6.2
MO 6	0.5	12.0	0.36	0.90	3.2

$$\Delta/\Delta_m = v = \frac{1/(1 + K_2)}{1 + \frac{K_1 K_2}{(1 + K_2)[\text{PP}]}}$$

where  $K_2$  is  $k_{-2}/k_2$ . This equation reduces to a simple approximation when  $K_2$  becomes sufficiently small with decrease of dielectric constant, namely,  $\Delta/\Delta_m = \frac{1}{1 + \frac{K_1 K_2}{[\text{PP}]}}$ . These equations show that the dissociation constant of the

over-all reaction,  $K$ , obtained from the relation  $\Delta/\Delta_c$  versus  $[PP]$  is equal to  $K_1K_2/(1+K_2)$ .  $K_2$  can be calculated from  $K$  using  $K_1$  determined by the transient kinetics. If the light scattering due to MPP is practically identical to the one due to  $M$ , as assumed above,  $\Delta_c/\Delta_m$  must be equal to  $1/(1+K_2)$ .  $K_2$  and  $\Delta_c/\Delta_m$  were determined under various dielectric constants of the medium. As shown later (Table 9), the observed values of  $\Delta_c/\Delta_m$  were in fair agreement with the calculated ones  $1/(1+K_2)$ , showing thereby that the assumption on the unchanged shape of MPP is correct.

KARUSH<sup>51)</sup> and LUMRY and EYRING<sup>52)</sup> already pointed out that when a configurational change of the protein occurs accompanying with the binding of a small molecule to the protein, the experimental results will include the thermodynamic change associated with the change in the configuration. In our case, the configurational change of myosin B could be clearly followed and the thermodynamic changes due to the configurational change could be separated from

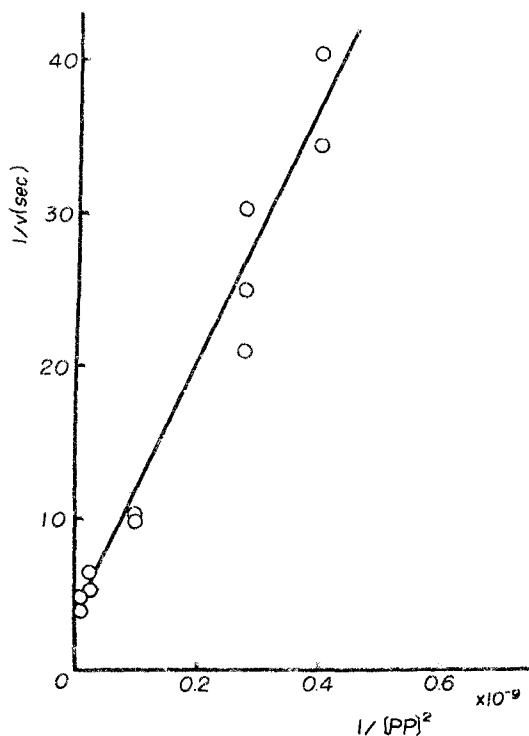


Fig. 21. Reciprocal slope of the time course of the decrease in scattered light against reciprocal of  $[PP]^2$ , when  $\Delta/\Delta_c$  versus  $[PP]$  followed a dissociation curve of the second order. Prep. No. MO 7, 0.6 M KCl, 10 mM  $Mg^{++}$ , pH 7.88, 5°C.

the one associated with the binding.

When, in the presence of  $Mg^{++}$ , the relation  $\Delta/\Delta_c$  versus [PP] followed a dissociation curve of the first order, the equations derived above were also applicable. The value of  $K_1$  was almost equal to the value in the presence of  $Ca^{++}$ , while  $k_2$  was much larger than in the presence of  $Ca^{++}$ . For example, the values of  $K_1$  of prep. No. MO 7 in the presence of 0.5 mM  $Ca^{++}$  and 0.1 mM  $Mg^{++}$  were respectively 4.2 and  $4.8 \times 10^{-4}$  M. This confirms the result previously obtained on the ATP-myosin B system. On the other hand, when  $[Mg^{++}]$  was high,  $\Delta/\Delta_c$  versus [PP] followed the second order dissociation curve and the plot  $1/v - 1/[PP]^2$  was found to be linear (Fig. 21), thus indicating that in this case the binding step was the second order with respect to [PP].

#### 4. Transient Change of Light Scattering. II<sup>(9)</sup>

As shown above, when the concentration of  $Ca^{++}$  was higher than 0.1 mM, both  $K_1$  and  $K_2$  were independent of  $[Ca^{++}]$ . Then in the presence of sufficiently high concentration of  $Ca^{++}$ , the intrinsic thermodynamic properties of the system saturated by  $Ca^{++}$  could be obtained. In Table 8, the enthalpy changes of the elementary steps calculated by the Arrhenius equation together with the free energy changes calculated from the dissociation constants are summarized.

TABLE 8. Thermodynamic functions of the elementary steps of the reaction of PP with myosin B. 0.6 M KCl, pH 7.88. The values of  $\Delta F$  and  $\Delta S$  are the ones at 5 °C,  $D=82.56$ .

Prep. No.	Ca <sup>++</sup> mM	Step 1				Step 2			
		$\Delta F$ , (Kcal)	$\Delta F_{el}$ , (Kcal)	$\Delta H$ , (Kcal)	$\Delta S$ (cal/deg)	$\Delta F$ , (Kcal)	$\Delta F_{el}$ , (Kcal)	$\Delta H$ , (Kcal)	$\Delta S$ (cal/deg)
MO 2	0.5	-3.7,	11.6,	- 9.7,	-20	-1.7,	-44,	-12.1,	-37,
MO 3	0.1	-4.7,	9.6,	- 7.8,	-11.3	-1.15,	-21,	-8.6,	-26.8
MO 4	0.3-0.7	-4.3,				-1.25,			
MO 5	0.5	-4.1,	12.4,	-10.0,	-21	-1.5,	-39,	-10,	-31.

The PP-myosin B system was markedly affected by the dielectric constant ( $D$ ) of the medium. As shown in Fig. 22 and Table 9,  $\Delta_c$  approached to the limiting value,  $\Delta_m$  and the dissociation constants  $K_1$  and  $K_2$  decreased as  $D$  decreased, and moreover, their dependence on  $D$  was not changed by using either dioxane or acetone to reduce  $D$ . The electrostatic free energy of a reaction is given by:

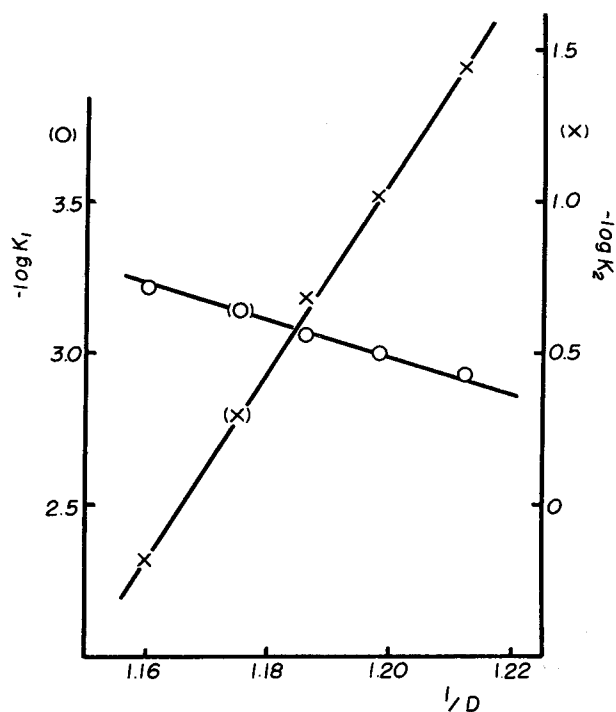


Fig. 22. The plots of  $\log K_1$  and  $\log K_2$  against  $1/D$ . Prep. No. MO 6. 0.6 M KCl, 0.5 mM CaCl<sub>2</sub>, pH 7.88, 5°C. O,  $\log K_1$ ; X,  $\log K_2$ . The bracket indicates that  $D$  is decreased by addition of acetone. Other values are the one in the dioxane-water mixture.

TABLE 9. Dependence on dielectric constant of the dissociation and velocity constants of the myosin B-PP system: Prep. No. MO 6, 0.6 MKCl, 0.5 mM Ca<sup>++</sup>, pH 7.88, 5°C.

$D$	Solvent	$K_1$ (M)	$K_2$	$k_2$ (sec <sup>-1</sup> )	$k_{-2}$ (sec <sup>-1</sup> )	$\Delta_e/\Delta_m$ (%)	
						obs.	cal <sup>*)</sup>
86.3	H <sub>2</sub> O	$6.03 \times 10^{-4}$	1.51	$8.4 \times 10^{-3}$	$1.27 \times 10^{-2}$	44	40
84.8	2.4% acetone	$7.15 \times 10^{-4}$	$5.06 \times 10^{-1}$	$1.87 \times 10^{-2}$	$9.47 \times 10^{-3}$	70	67
84.3	2% dioxane	$8.85 \times 10^{-4}$	$2.06 \times 10^{-1}$	$2.86 \times 10^{-2}$	$5.74 \times 10^{-3}$	86	84
83.5	3% dioxane	$1.0 \times 10^{-3}$	$9.54 \times 10^{-2}$	$5.0 \times 10^{-2}$	$4.76 \times 10^{-3}$	96	92
82.56	4% dioxane	$1.15 \times 10^{-3}$	$3.58 \times 10^{-2}$	$9.01 \times 10^{-2}$	$3.47 \times 10^{-3}$	100	98

\*) Calculated from the eq.:  $\Delta_e/\Delta_m = \frac{1}{1+K_2}$ .



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$$\frac{\ln K}{(1/D_e)} = \frac{\Delta F_{el}}{RT}$$

where  $D_e$  is the "effective" dielectric constant of the reaction. If  $D_e$  is proportional to the bulk dielectric constant ( $D$ ) of the medium,  $\Delta F_{el}$  can be calculated from a slope of the plots of  $\ln K$  versus  $1/D_e$ . There was some theoretical basis to show that this situation was satisfied and in fact, as shown in Fig. 22, the plot of  $\log K$  against  $1/D$  was linear. Therefore, the electrostatic free energy changes were calculated by this equation and the values are listed in Table 8.

According to the experimental results the electrostatic free energy change associated with the step 1 was considerably large positive value (about 10-12 Kcal). This indicates that the binding of PP is not promoted by its electrostatic attraction with myosin B, but contrarily, PP combines with myosin B by a covalent linkage, breaking some intramolecular electrostatic bond of the protein. Furthermore, the step of the change of shape (step 2) associates distinctly large decrease of electrostatic repulsion ( $-21 \sim -44$  Kcal) and decrease of entropy ( $-27 \sim -37$  cal/deg). This seems to support the MORALES and BOTTS theory<sup>19,53</sup> that a balance between electrostatic and entropic forces determines the length of the myosin B particles. However, as described above, the binding of PP breaks the intramolecular bonds and loosens the structure of myosin B. The

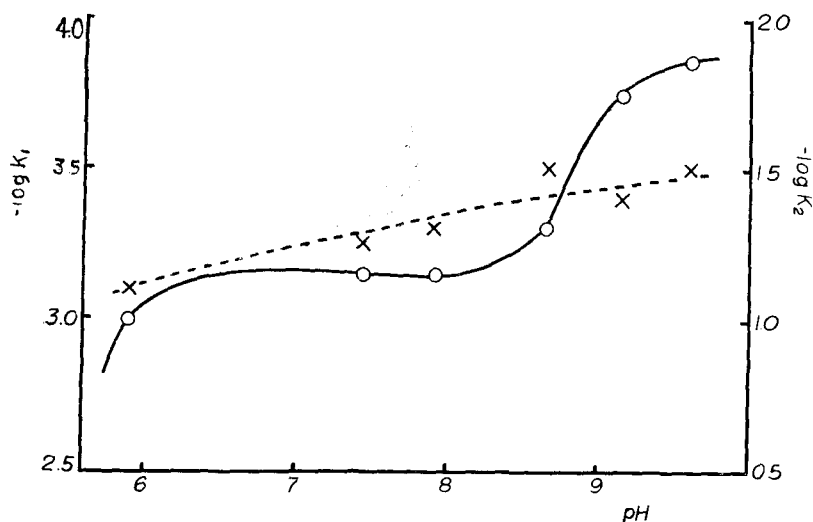
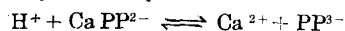


Fig. 23. Dependence on pH of the dissociation constants of the myosin B-PP system. Prep. No. MO 8, 0.54 M KCl, 0.4 mM  $\text{CaCl}_2$ , 4 per cent dioxane, 5°C. O,  $\log K_1$ ; X,  $\log K_2$ .

\*) The increase of  $K_1$  below pH 6.5 may be due to the dissociation reaction of PP:



electrostatic repulsion for the elongation of myosin B is not the one between absorbed PP and the charges of the protein but it must be the repulsion between intrinsic charges of the protein.

The pH effects on  $K_1$  and  $K_2$  were investigated to get information on the nature of the intramolecular bond which is broken in the first step and of the groups which contribute electrostatic repulsion in the second step. As shown in Fig. 23,  $K_2$  was slightly decreased by increase of pH from 6.0 to 9.5. Therefore it seems likely that the electrostatic repulsion of the step 2 may be attributable dominantly to the one between the carboxyl groups of the protein which are dissociated completely at pH above 6.0. On the other hand,  $K_1$  was decreased abruptly by increase of pH from 8.5 to 9.5\*, thus indicating that the bond broken in the first step is the one between an cationic group, most probably an amino group, whose  $pK$  is about 9.0, and an unspecified anionic one.

### 5. Summary

The results by the equilibrium dialysis have demonstrated that myosin B changes its shape by the binding of PP to one of the two of myosin A of which

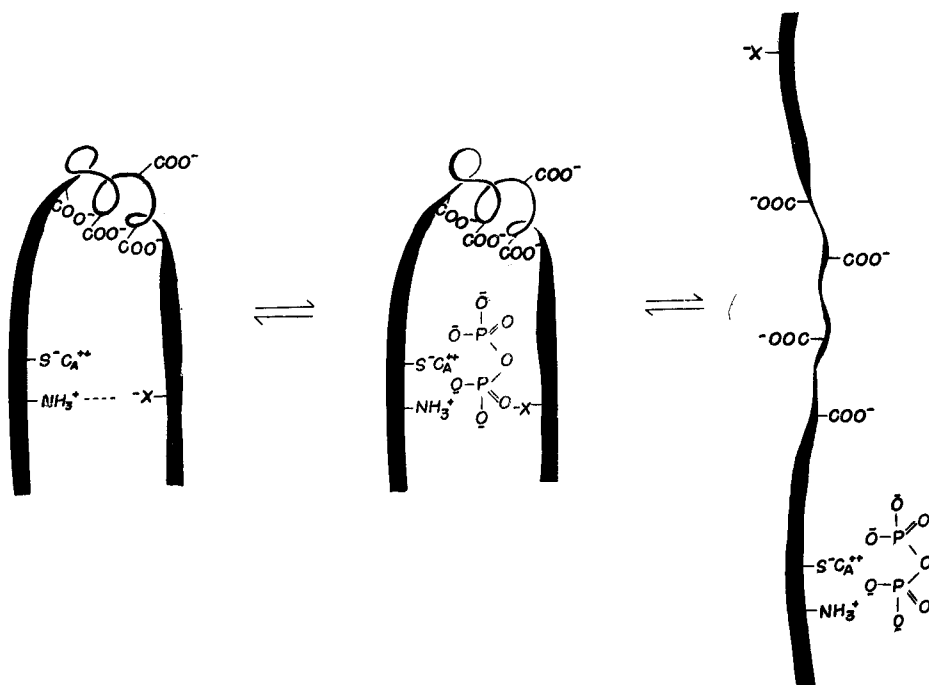


Fig. 24.

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myosin B constitutes. The binding site of PP may be the "intrinsic"  $\text{Ca}^{++}$  bound tightly to the SH group of the protein. It is also concluded that the units of myosin B behave in regard to the change of light scattering as if they are nearly independent of each other and the intensity of light scattering of myosin B is decreased by an almost constant degree every time one PP molecule binds to one myosin B unit. Basing on this conclusion, the reaction mechanism of the interaction between PP and myosin B has been analyzed by means of light scattering method. The reaction between PP and myosin B is described in terms of two steps that is, myosin B changes its shape through an unchanged intermediate complex with PP. In the first step, the binding of PP to myosin B is associated with cleavage of a intramolecular electrostatic bond, probably between an amino group and an unspecified anionic group, of the protein, and in the second step the myosin B-PP complex changes its shape accompanying the decrease of entropy and electrostatic repulsion between the carboxyl groups. A model for the shape change of myosin B deduced from our results are schematically shown in Fig. 24.

### **CONCLUDING REMARKS**

We have reviewed our studies on the interaction between myosin B and polyphosphate. In the first part, the molecular size and shape of myosin B have been elucidated both in the presence and the absence of PP or ATP by the methods of light scattering, flow birefringence, viscosity and ultracentrifugal separation. In the second part, the reaction mechanism of the shape change of myosin B by PP has been analyzed by the equilibrium dialysis and light scattering method and also kinetically by the transient light scattering method.

These various experimental results seems to the writers to elucidate fairly well the physicochemical properties of this biologically interesting reaction. According to MORALES<sup>53)</sup>, muscle contraction is caused by the shape change of myosin B itself and by the energy of binding of ATP to the protein. On the other hand, WEBER and HUXLEY<sup>cf. 54)</sup> have suggested that the interforce between F-actin and myosin is reduced by binding of polyphosphate and muscle contracts by energy of hydrolysis of ATP. To solve this question it may be necessary to get detailed information on myosin B-ATPase, which will be treated in a following paper.

Recently, size and shape changes of proteins, such as the dimerization of mercaptalbumin, the conversion of fibrinogen to fibrin and the polymerization of insulin, have aroused keen interest of many workers. As EDSALL said in his review<sup>55)</sup> on the dimerization of albumin, even when the biological significance of the reaction is obscure, the method of approach in this field is more

important than the results obtained and the future harvests to be reaped in this field should be far greater than that have yet been gathered in.

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