PHYSICO-CHEMICAL STUDY ON "MYOSIN" ATPase*)

By

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Introduction

It has been demonstrated in 1939 by ENGELHARDT and LJUBIMOVA[1] that "myosin", the main component of the structural proteins of muscle, catalyzed the hydrolysis of ATP to ADP and P. Since then many investigations have been carried out on this enzymic action, as the hydrolysis of ATP by "myosin" is considered to be one of the fundamental processes in muscle contraction[2-5].

Recently, the experimental techniques for elucidation of physico-chemical properties of enzymic reaction have highly been developed, and the reaction mechanism of the myosin ATPase has been clarified to some extent. However, many points still remain to be studied, because this enzyme shows very complicated properties such as particular pH-dependence of its activity, activation by various modifiers and complicated effect of divalent cations *etc.*

One of the reasons which have been preventing the unified and systematic explanation of the properties of the "myosin" ATPase is the fluctuation in the enzymic properties of this protein by the method of the preparation. Therefore, it seems to be important for clarification of the reaction mechanism to get grid of the experimental results which are accumulated on the enzyme prepared in one laboratory and by the same method.

In the present report the reaction mechanism of the "myosin" ATPase will be analyzed and its relation to the muscle contraction will be reviewed on the basis of the experimental results recently gathered in our laboratory. In Part I, the properties of the steady ATPase of "myosin" at a high ionic strength will be described. A general reaction scheme will be proposed based on the

*) In this paper the following abbreviations are used: ATP, adenosine triphosphate; ITP, inosine triphosphate; GTP, guanosine triphosphate; UTP, uridine triphosphate; CTP, cytidine triphosphate; ATPase, adenosinetriphosphatase; NTPase, nucleotidetriphosphatase; ADP, adenosine diphosphate; P, inorganic orthophosphate; PP, pyrophosphate; EDTA, ethylenediamine tetraacetic acid; CyDTA, 1,2-cyclohexanediamine tetraacetic acid; PCMB, *p*-chloromercuribenzoate; DNP, 2, 4-dinitrophenol; PMA, phenylmercuric acetate; TNBS, 2, 4, 6-trinitrobenzene sulfonate; TNP, trinitrophenol. "Myosin" will be used when it is necessary to signify the properties common to both of myosins A and B.

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analysis of the experimental results, especially of the effects of divalent cations, pH and modifiers on the enzymic activity. The chemical structure of the active site of the "myosin" ATPase will be depicted in Part II from the effects of TNBS and PCMB on its activity. In Part III the initial rapid splitting of ATP by myosin B and dependence of the velocity of the splitting upon a colloidal state of the protein will be demonstrated. The function of "myosin" ATPase in the muscle contraction will be discussed basing on the results obtained.

Part I. Kinetic Analysis[6]

When "myosin" is dissolved in KCl solution of relatively high ionic strength (0.4–0.6), so-called superprecipitation[3] (see III, ii) of the protein is not observed on addition of ATP. Accordingly, the "myosin" solution at a high ionic strength may be readily accessible to the physico-chemical investigations of the "myosin" ATPase.

In 0.6 M KCl solution, the myosin B ATPase obeys the MICHAELIS-MENTEN relation as usual enzymic reactions[7-10]:

$$v = \frac{V_m}{1+\frac{K_m}{[S]}} \qquad (1)$$

where v is the velocity, [S] the concentration of ATP, V_m the maximum velocity and K_m the MICHAELIS constant. A typical example of the LINEWEAVER-BURK plot[11] of the myosin B ATPase at a high ionic strength is shown in Fig. 1. Then, the properties of steady ATPase are investigated by determining V_m and K_m under various conditions. In the first place the effect of Ca^{++} will be described, since the "myosin" ATPase is affected by divalent cations profoundly and Ca^{++} is the most powerful activator[12]. In Table 1 are summarized the values of V_m and K_m at various concentrations of Ca^{++}. In this Table k_2 stands for the value of V_m per unit weight (5.6×10^5 g)[13] of the ATPase site of myosin B.

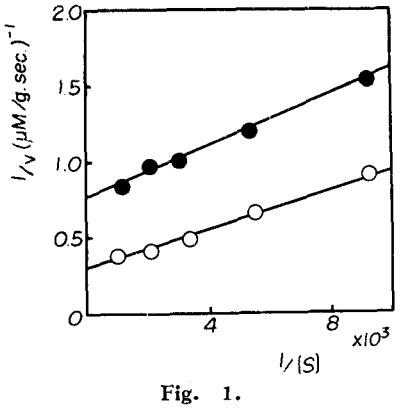

Fig. 1.

Dependence of rate of ATPase on ATP concentration. ○, in presence; ●, in absence of 7 mM Ca^{++}. 0.6 M KCl, pH 6.7, 25°C.

Taking into account the chelation of ATP with Ca^{++}, the mechanism of

TABLE 1. Effect of divalent cation on V_m and K_m at high ionic strength

0.6 M KCl, pH 6.7, 25°C

	V_m (μ moles/g. sec)	k_2 (sec^{-1})		K_m ($\times 10^{-4}$ M)	
		Observed	Calculated	Observed	Calculated
No modifier	1.3	0.73	(0.73)	1.1	(1.1)
Ca^{++} 1.0 mM	2.0	1.1	0.86	1.3	1.26
Ca^{++} 2.8 mM	2.2	1.2	1.2	1.5	1.94
Ca^{++} 7.0 mM	3.2	1.8	1.9	2.1	2.1
(Ca^{++} ∞)	—	2.1	(2.1)	2.4	(2.4)

Ca^{++} activation may be written as follows:

$$S + Ca^{++} \underset{K_{Ca}}{\rightleftharpoons} SCa \tag{2}$$

$$M + SCa \rightleftharpoons MCaS \xrightarrow{k_2} M + P \tag{3}$$

$$M + S \rightleftharpoons MS \xrightarrow{k_2'} M + P \tag{4}$$

In these equations, M stands for the functional unit of myosin B and P for products. K and k are respectively the dissociation constant and the velocity constant of each step. If the MICHAELIS constants of the steps (3) and (4) are signified respectively by K_m and K_m', the steady state kinetics leads to the usual MICHAELIS equations as follows:

$$v = \frac{k_2^\circ}{1 + \dfrac{K_m^\circ}{[S]}} \tag{5}$$

where

$$k_2^\circ = \left\{ k_2 + k_2' \frac{K_m\left(1 + \dfrac{K_{Ca}}{[Ca]}\right)}{K_m'\left(1 + \dfrac{[Ca]}{K_{Ca}}\right)} \right\} \Bigg/ \left\{ 1 + \frac{K_m\left(1 + \dfrac{K_{Ca}}{[Ca]}\right)}{K_m'\left(1 + \dfrac{[Ca]}{K_{Ca}}\right)} \right\} \tag{6}$$

and

$$K_m^\circ = K_m\left(1 + \frac{K_{Ca}}{[Ca]}\right) \Bigg/ \left\{ 1 + \frac{K_m\left(1 + \dfrac{K_{Ca}}{[Ca]}\right)}{K_m'\left(1 + \dfrac{[Ca]}{K_{Ca}}\right)} \right\} \tag{7}$$

Among these constants, K_m' and k_2' can be determined directly from measure-

ments in the absence of Ca^{++}, and K_m and k_2 are given as the saturation values by extrapolation to the high concentration of Ca^{++}. Applying these values and K_{Ca}, which is assumed to be 3×10^{-3}, to the equations (6) and (7), the MICHAELIS constant K_m° and the maximum velocity k_2° were calculated and found to agree satisfactorily with the observed ones in the range of Ca^{++} concentrations investigated. Furthermore, the value of K_{Ca}, 3×10^{-3} M, agrees with the dissociation constant[14] of the complex of ATP with Ca^{++} under the same condition as that of the enzyme assay. It is concluded, therefore, that the acceleration of the "myosin" ATPase by Ca^{++} is due to the higher splitting rate of CaATP than free ATP.

Recently PELLETIER and OUELLET[15] and NANNINGA[16] have carried out quantitative studies on the effect of Ca^{++} upon the "myosin" ATPase. The results of these independent studies agree substantially with ours. The possibility of the activation by the binding of Ca^{++} to "myosin" is indicated by NANNINGA[16], but it seems to be more reasonable to take the above reaction mechanism, partly because the dissociation constant of the Ca^{++} binding to the "myosin"–ATP system which is determined kinetically agrees with that of Ca^{++}–ATP, and partly because this mechanism can explain the inhibition by overoptimal concentrations of divalent cation in the following way. Provided that the pyrophosphate group of ATP associates to the ATPase-active site by mediation of Me^{++}, it may be expected at a high concentration of Me^{++} that ATPase is inhibited due to the combination of Me^{++} both with the substrate and the active site. Actually, ATPase is inhibited by extremely high concentration of Ca^{++} and the relation between the degree of the inhibition and the Ca^{++} concentration obeys the dissociation curve of the first order[9]. The $[Mg^{++}]$–activity curve of ATPase can also be regarded as a sum of two dissociation curves[9], *i.e.*, the dissociation constant of the first step, where Mg^{++} causes inhibition of ATPase at a high ionic strength and activation at a low ionic strength, is much smaller than that of the complex Mg^{++}–ATP, while the dissociation constant of the second inhibitory step is almost equal to that of the complex. Therefore, it may be concluded that, contrary to the case of Ca^{++}, Mg^{++} modifies ATPase by its combination with the enzyme in the first step and it inhibits ATPase by its complex-formation with the substrate in the second one.

From the analysis of dependence of the K_m upon pH (Fig. 2) the ionic species of ATP as the "true" substrate was examined. The constant value of K_m in the range of pH between 6.7 and 8.0 might be explained by assuming that the concentration of the "true" substrate in this range of pH is independent of pH and that the concentration of the active form of the enzyme changes

by reaction with H^+ in such a way that the reaction rate of the binding of substrate with the enzyme and/or the degradation of the enzyme-substrate complex becomes zero[6]. Below pH 6.7, however, K_m apparently increases with decreasing pH. This increase of K_m corresponds to the decrease of the concentration of ATP,[4-14] and hence, the dependence of K_m on pH can be interpreted by assuming ATP^{4-} to be the "true" substrate.

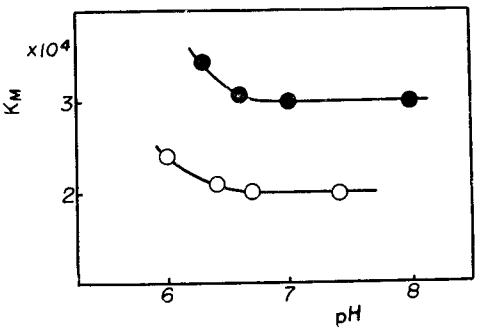

Fig. 2. pH dependence of MICHAELIS constant. ○, 0.6 M KCl, 7 mM Ca^{++}; ●, 0.2 M KCl, 1 mM Ca^{++}. 25°C.

In Fig. 3 are shown the effects of various divalent cations, where the ATPase activities are plotted as a function of the ionic radii (r) of the divalent cations. At a high ionic strength (open circles), the bell-shaped curve v versus r has a maximum at $r=0.95$ Å, and deviation of r by 0.35 Å from 0.95 Å causes almost complete suppression of

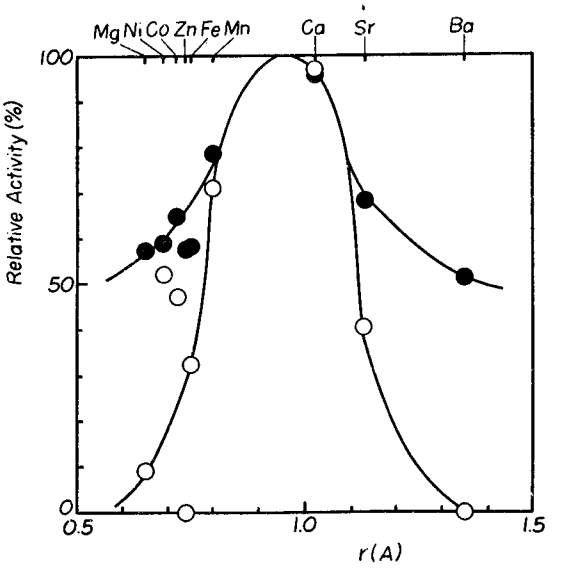

Fig. 3. Relation between ionic radii of divalent cations and ATPase activity. Abscissa indicates ionic radius and ordinate relative ATPase activity defined v at $r=0.95$ Å as 100. ○, 0.6 M KCl, 5 mM Me^{++}; ●, 0.14 M KCl, 1 mM Me^{++}. 1 mM ATP, pH 8.2, 22°C.

the ATPase activity. At a low ionic strength (closed circles), the optimal radius remains at 0.95 Å, but the slope of the curve $v-r$ becomes less sharp, so that the activity is reduced to about 50% of the optimum value by the deviation of 0.35 Å from 0.95 Å. Thus, it can be concluded that the effects of divalent cations depend primarily on their ionic radii and that the seemingly strange dependence on the ionic strength of Mg^{++} effect[12,17] is not characteristic to this cation but common to other divalent cations whose ionic radii are considerably apart from 0.95 Å. The relation between v and r may indicates the close relation between the rate of degradation of the enzyme-substrate complex MS and the conformation of the complex of ATP with divalent cations, since as suggested by MELCHIOR[18] the molecular conformation of ATP is changed markedly by the ionic radius of metal ion which is chelated by ATP.

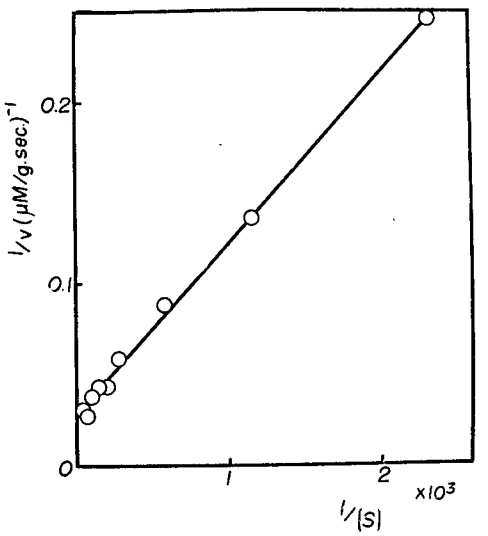

Fig. 4. LINEWEAVER-BURK plot of ATPase in presence of 10 mM EDTA. 0.6M KCl, pH 8.2, 24°C.

The "myosin" ATPase at high ionic strengthes shows a complicated pH-activity curve which has a maximum and a minimum around neutral pH[8,12,17], and the ATPase is activated by various modifiers such as EDTA[19-21], PCMB[22] and DNP[23,24]. The effects of these modifiers share the following common characteristics despite that their chemical structures are not intimately related among them: i) the increase of V_m caused by them is always accompanied by the increase of K_m, ii) in their presence, the pH-activity curve lacks the minimum at neutral pH, and shows a monotoneous increase with pH. In Figs. 4 and 5 typical examples of these effects are shown in the case of EDTA. As shown in Fig. 4, K_m increases from 1.1×10^{-4} to 3.3×10^{-3}M by the addition of 10 mM EDTA. Fig. 5 illustrates the effect of EDTA on the pH dependence of myosin B ATPase. The ratio of the rate in the presence to the one in the absence of EDTA is presented in Fig. 5 as the dotted line which is bell-shaped and has a maximum around pH 8.0. Similar changes in the pH-activity curve were also observed in the presence of sufficient amounts of other modifiers.

If one assumes the following reaction scheme as a general mechanism of the "myosin" ATPase, these facts and other various experimental results can

be explained inclusively.

$$\begin{array}{c} \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} \\ \\ \end{array} \begin{array}{c} M_1S \overset{k_2}{\longrightarrow} M+P \\ \Updownarrow \\ M_2S \end{array} \quad (8)$$

where M stands for the functional unit of the enzyme containing two kinds of the site, 1 and 2. If the equilibrium between M_1S and M_2S is assumed to be attained satisfactorily fast, the reaction velocity can be given by

$$v = \frac{V_m}{1+\frac{K_m}{[S]}} \quad (9)$$

$$V_m = k_2\varepsilon/(1+K) \quad (10)$$

$$K_m = \frac{(k_2+k_{-1})}{k_1}(1+K) \quad (11)$$

where K is the equilibrium constant of the step $M_1S \rightleftharpoons M_2S$ and ε is the total concentration of the site 1. Provided that the modifiers mentioned above bind to or near the site 2, hindering the formation of M_2S but more or less unaffecting the other steps, K should become much smaller than one. Thus both K_m and V_m are increased by the modifiers as expected from the above explanation (cf eq. (10) & (11)). The complicated features of the pH activity curve of ATPase are also well explained by this scheme*). When the formation of M_2S is hindered by the addition of the modifiers, the pH dependence of the "myosin" ATPase reduces to that of $k_{2,m}$**). On the other hand, in the absence of the modifier the dependence of the velocity on pH is a complicated function of the pH dependences of k_2 and K. Consequently, the bell-shaped dotted line in Fig. 5 represents the pH dependence of $(1+K)$. The lines in Fig. 6 are those

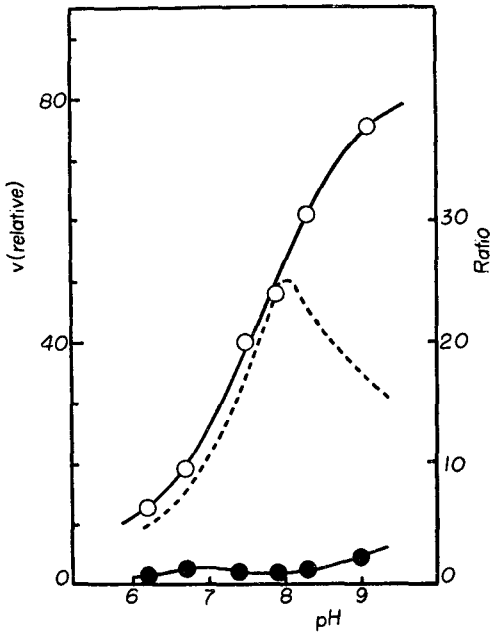

Fig. 5. pH variation of ATPase in presence (○) and absence (●) of 10 mM EDTA. 1 mM ATP, 0.6 M KCl, 24°C. Dotted line indicates ratio of activity in presence to that in absence of EDTA.

*) It was already suggested that the "true" substrate may be ATP^{4-}. Furthermore, essentially the same pH activity curve was obtained in the presence of various concentrations of Ca^{++}. This seems to indicate that the pH dependence of the ATPase is principally controlled by the dissociation of ionic group of the enzyme itself.

**) $k_{2,m}$ is the velocity constant k_2 of the enzyme of which site 2 is occupied by a modifier.

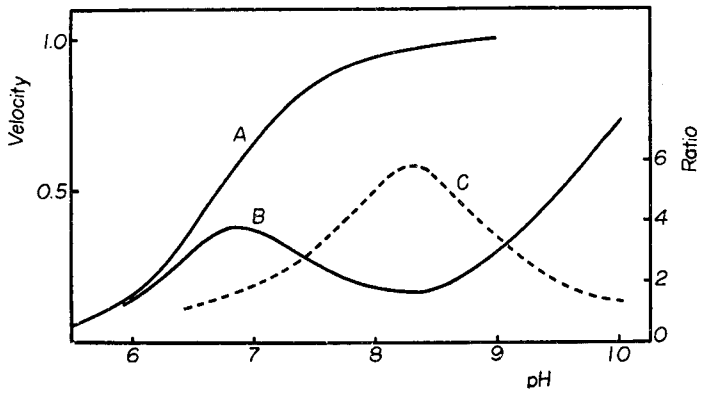

Fig. 6. Theoretical curve of pH-dependence of "myosin" ATPase. Curve A, in presence of activator; curve B, in absence of activator; curve C, ratio of A to B.

calculated by the following equations.

$$k_2 = k_2^{\circ}/(1+[H^+]/10^{-6.9}) \tag{12}$$

$$K = 9/(1+[H^+]/10^{-8.0}+10^{-8.6}/[H^+]) \tag{13}$$

The line (A) is a sigmoid curve of the first order (eq. (12)) and the line (B) is a bell-shaped curve having a maximum at pH 8.3 (eq. (13)), both being the ones most frequently observed in enzyme kinetics. The line (C) represents the ratio of (A) to (B). The relations among these three curves show good correspondence with the curves obtained experimentally. The chemical interpretation of the dependence of K on pH will be given in the following part.

The following scheme has already been proposed by BLUM[25] and one of the present authors[8,26,27] as the reaction mechanism of the interaction between myosin B and ATP:

$$\begin{array}{l} M+S \rightleftharpoons MS \rightleftharpoons M^*S \longrightarrow M^*+P \\ \qquad\quad \downarrow \\ \qquad\quad M+P \end{array} \tag{14}$$

where M* represents the functional unit of myosin B whose size and shape are changed by ATP. Provided that M_1S in (eq. (8)) corresponds to MS in the above scheme and that M_2S is an intermediate complex of the step MS→M*S, the two schemes turn out to be equivalent. The inhibitory effect of EDTA to the formation of M*S might be attributed to the inhibition by EDTA of the formation of M_2S[24]. In one of the previous papers[8,29] it has already been reported that the first step of the size and shape change of myosin B is the breakage of the bond between myosin and actin which is presumably located

in the vicinity of the site 2 by the binding of ATP or PP. Consequently, it may be anticipated that the modifiers can not bind to the site 2 at low ionic strengthes where the binding between myosin and actin is rather strong. As reported by BOWEN and KERWIN[20] and GREVILLE and NEEDHAM[23], at low ionic strengthes the actomyosin or the myosin B ATPase is not activated by the addition of EDTA or DNP.

If one assumes that the process of the physical change of the complex of NTP with the site 2 of myosin B is independent of the species of NTP, then the order of the apparent dissociation constants of NTP's for the physical change must be equal to the one of K_m's of NTPases (cf. eq. (11)). It was already emphasized by several investigators[6,25] that the order of K_m of the "myosin" NTPase (ITP>GTP>UTP>ATP, CTP)[25,30] agrees with that of the apparent dissociation constant of the physical change (ITP>UTP>ATP, CTP)[25,31].

It might also be expected from the equations (10) and (11) that the smaller the value of K_m of a NTPase, the more conspicuously the NTPase is activated by a substance that interacts with the site 2. In fact, EDTA[30,31], DNP and PMA[32] activate ATPase and CTPase profoundly, UTPase slightly, but not ITPase, and PCMB activates ITPase only slightly[6]. The reaction scheme also shows that the pH activity curve of a NTPase, which is sensitive to the above mentioned modifiers, has a maximum and a minimum around neutral pH and that the pH-activity curve of a NTPase which is insensitive to the activators lacks these extremes. As shown in Fig. 7, the pH dependence of ITPase is the curve of sigmoid type, thus satisfactorily fulfilling the explanation. Thus, the proposed reaction scheme offers an adequate explanation for the various experimental results obtained on the "myosin" NTPase. At the present stage, our explanation is only phenomenological and the order of the binding constants of NTP's with the site 2 can not be predicted from their molecular structures, but it might be attributed to their adaptability to the conformation of the site 2.

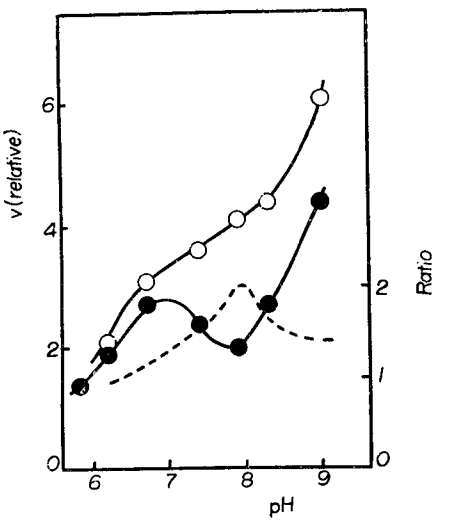

Fig. 7. pH activity curve of ITPase (○) and ATPase (●). 1 mM ITP or ATP, 0.6 M KCl, 23°C. Dotted line indicates ratio of ITPase to ATPase activity.

Part II Chemical Structure of Active Site[33-35]

In the preceding part the reaction mechanism of the ATPase is analyzed principally by the kinetic procedure and it is shown that the "active site" involved in the ATPase activity is constituted by the site 1 which is necessary to the activity and the site 2 which modifies the activity. In Part II the structure of the ATPase-active site will be revealed to some extent chiefly by the method of chemical modifications. It might be premature at the present time to summarize the results and to deduce the chemical picture of the active site, as the results obtained are still fragmental. However, the present authors venture to take this hazardous work, because the elucidation of the chemical structure of the active site is indispensable to elucidate the mechanism of the muscle contraction at a molecular level.

The requirement of intact SH group for the ATPase activity was already suggested by BAILEY and PERRY[36] in their early observations on the effect of several SH reagents on ATPase. Recently KIELLEY and BRADLEY[22] made an

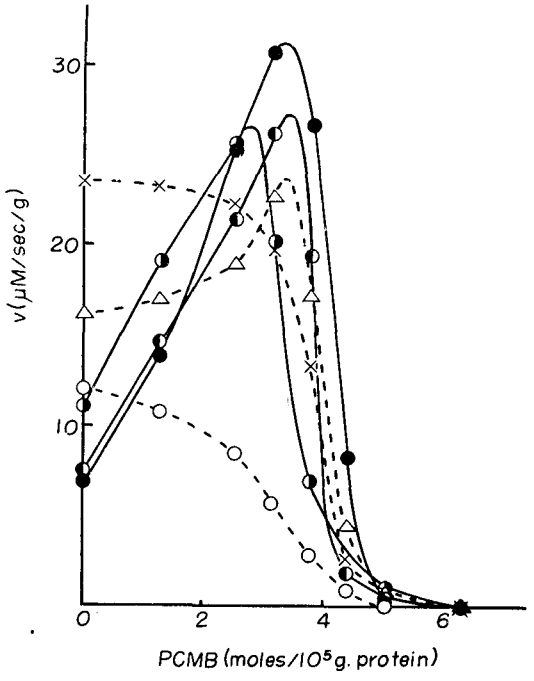

Fig. 8. Effect of PCMB on myosin B ATPase at various pHs. Binding of PCMB; 0.6 M KCl, pH 6.7, 20°C, for 30 min. Assay of ATPase; 0.6 M KCl, 1 mM ATP, 7 mM Ca^{++}, 20°C; ○, pH 5.7; ◑, pH 6.45; ◐, pH 7.3; ●, pH 7.6; △, pH 8.7; ×, pH 9.5,

interesting observation on the effects of PCMB on ATPase, *viz.*, at low concentrations PCMB activates ATPase while at high concentrations it suppresses the activity. More intensive studies on the role of SH group might be useful for the elucidation of the chemical structure of the active site, and hence, in our laboratory the effects of PCMB were further investigated in details[33]. The pH dependence of the effect of PCMB on the myosin B ATPase is shown in Fig. 8. Myosin B was incubated with PCMB at a constant pH (6.7) and the activity was measured at various pHs. In the neutral region the activation was followed by inhibition with the increase of PCMB concentration as already demonstrated by KIELLEY and BRADLEY. From this result and from the data in Part I it seems to be reasonable to presume that the SH group is involved in the site 1 as well as in the site 2 and the SH group of the site 2 is attacked more readily by PCMB than that of the site 1.

Now, we define the degree of the activation by PCMB as the ratio of the observed activity to the one on the straight line connecting the point on the abscissa where PCMB concentration is zero and the point on the ordinate where the activity becomes to be zero. In Fig. 9 the maximum values of the degree of the activation by PCMB thus defined are plotted against pH. If the mechanism developed in the preceding part holds, the pH dependence of the activation by PCMB must be identical with that of $1+K$, where K is the equilibrium constant of $E_1S \rightleftharpoons E_2S$. As clearly seen in Figs. 5 and 9 this requirement is fulfilled satisfactorily. It was also observed that ATPase is activated by PCMB

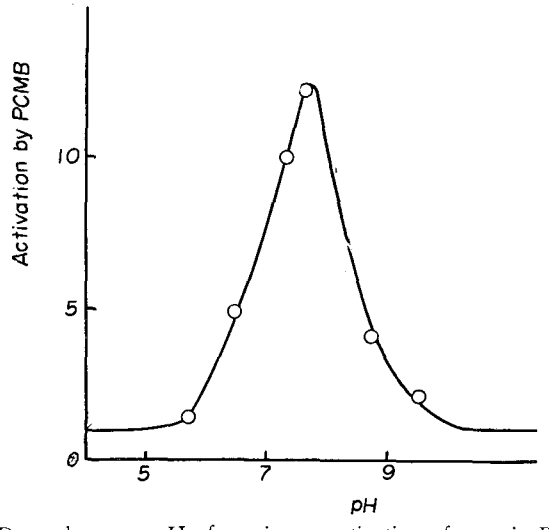

Fig. 9. Dependence on pH of maximum activation of myosin B ATPase by PCMB. 0.6 M KCl, 1 mM ATP, 7 mM Ca^{++}. 20°C.

only slightly in the absence of divalent cation[35]. Therefore, the binding of ATP to the SH group of the site 2 is mediated by divalent cation added.

It is already indicated in the kinetic study that the interaction between EDTA and "myosin" occurrs also at the site 2. KIELLEY and BRADLEY[22] reported that only the inhibition of ATPase is observed by PCMB in the presence of EDTA. FRIESS *et al.*[21] presented some provisional evidences to indicate that EDTA binds to the "intrinsic" divalent cation (probably Mg^{++}) of myosin. KIELLEY and BRADLEY[22], therefore, concluded that the site for activation by PCMB involves the "intrinsic" divalent cation and the SH group. Recently, the relation between the divalent cation and the SH group was studied in our laboratory[35]. In Table 2 are summarized the contents of Ca^{++} and Mg^{++}

TABLE 2. Contents of Ca^{++} and Mg^{++} of myosin A

Preparation	Original myosin A		After PCMB treatment
	Mg^{++} moles/10^5 g	Ca^{++} moles/10^5 g	$Mg^{++} + Ca^{++}$ (moles/10^5 g)
1	0.27	0.45	—
2	0.15	0.40	—
3	0.22	0.37	—
4	0.25	0.48	< 0.05
5	0.30	0.52	< 0.04
6	0.28	0.45	< 0.05
7	0.34	0.51	< 0.05
8	0.23	0.44	—
9	—	—	< 0.10

which cannot be removed by simple washing of myosin A with deionized water. The content of Ca^{++} was higher than that of Mg^{++}. The weight of the protein per mole of the tightly bound Ca^{++} was 2.1×10^5 g. This value agrees with that per mole of the TNBS-specific amino group (see below) and also with the weight per mole of the site available to the PP binding[13]. Furthermore, KIELLEY and HARRINGTON[37] obtained the value of 2.06×10^5 g for the subunit weight of myosin A from their ultracentrifugal study on myosin A in concentrated guanidine-HCl.

Divalent cations (mainly Ca^{++}) tightly bound to myosin A could be removed by washing after the blocking of about half of the SH groups of myosin A by PCMB. This results seems to suggest that the SH groups of the protein are involved in the binding of the divalent cations. However, BLUM[38] has recently suggested that the conformation around the active site of myosin A

ATPase may change by the binding of PCMB. This view may be supported by the following two results; (i) myosin A treated with PCMB is more susceptible to denaturation by urea than the original myosin A[33] and (ii) the ATPase activity in the presence of PCMB increases gradually after the addition of ATP[35]. Therefore, the binding of PCMB to SH groups may cause a conformational change in the myosin A molecule and lead to release the tightly bound divalent cations. The Ca^{++} tightly bound to the protein cannot be the binding site for EDTA. When myosin A was treated by cysteine after the PCMB-treatment, Ca^{++} and Mg^{++} were completely removed from myosin A and the content of the SH group was completely recovered (Fig. 10 and Table 2). As shown in Fig. 11, the ATPase activity of such myosin A was enhanced by EDTA to almost the same extent as the original protein. Therefore, the site 2 does not contain divalent cation and seems to involve a cationic group, to which EDTA, DNP or ATP binds by electrostatic force and/or by hydrogen bonding, as well as the SH group. Recently it has been observed that after binding of 2 moles of TNBS to one mole of myosin A EDTA activation disappears (Fig. 11). As clearly shown later, TNBS binds only to ε-amino group of lysine. Therefore, it is concluded that, when the ε-amino group of lysine is occupied by TNBS, ATP cannot bind to the site 2. In the preceding part, the pH dependence of the ratio of the binding of ATP with the site 2 to that with the site 1 was given by $K=9\,(1+[H^+]/10^{-8.0}+10^{-8.6}/[H^+])$. The first and the second ionization steps may be due respectively to the sulfhydryl group of cysteine and the amino group of lysine*). This means that ATP binds to the site 2 when its ionized state is $-NH_3^+$, $-S^-$. The proposed structure of the site 2-ATP complex will be shown later in Fig. 17.

Although hitherto presented observations permit some supposition regarding the nature of the active site of the ATPase, it is urgently needed at this stage to determine the actual amino acid sequence around the active site. For this purpose SH reagent may be unsuitable, since the mercaptide bond formed is too weak to resist the acid hydrolysis of peptide bond to obtain subfragments. Moreover, as many moles of SH reagent must combine with one mole of myosin A to affect ATPase activity, the determination of the SH group which is involved in the active site is rather difficult. Therefore, attempts were made to use a reagent which bound with myosin A more specifically and more stoichiometrically. TNBS was selected, because it binds only to amino group

*) It is assumed that there is an additional anionic group in the site 2 of which dissociation constant is almost equal to that of the SH group of the site 1 or the dissociation constant of the SH group in the site 1 is much larger than that of the SH group in the site 2 and that the dissociation constant of the cationic group in the site 1 is much smaller than that of the amino group in the site 1 (see Fig. 17).

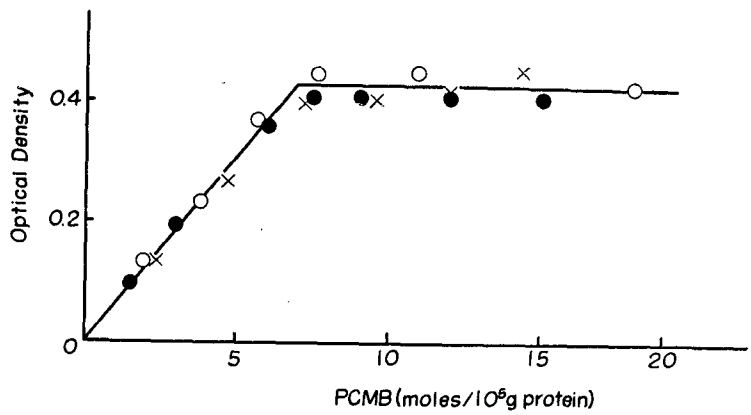

Fig. 10. Content of SH group measured by binding of PCMB. ○, control; ×, myosin A; after binding of PCMB (4moles/10^5 g protein) SH group was recovered by cysteine (160 moles/10^5 g protein) and then cysteine was washed out; ●, myosin A; after treatment with PCMB and then cysteine, cysteine was dialyzed out. Ordinate: increase of extinction at 252 mμ.

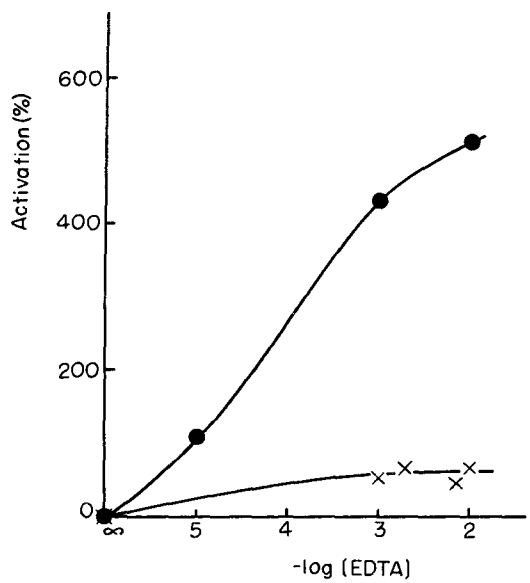

Fig. 11. Percentage of activation of myosin A ATPase by EDTA. ●, myosin A; after binding of PCMB (4 moles/10^5 g protein) SH group was recovered by cysteine and then cysteine was removed by washing. ×, myosin A; 2.08 moles of TNBS bound with 4.2×10^5 of the protein. 0.6 M KCl, pH 7.0, 20°C.

of a protein even at neutral pH and at low temperatures[39]. Furthermore, the bond of TNBS with amino group is resistant to acid hydrolysis of peptide bond and it has quantitatively been determined by a specific absorption at 345 mμ[39].

It was already observed by TURBA and KUSCHINSKY[40] and BÁRÁNY and BÁRÁNY[41] that activity of the myosin A ATPase is inhibited by the modification of the amino group. In these studies, however, the ATPase activity disappeared when a large number of the reactive groups in myosin A were modified by the reagents. Also there remains some doubt whether the modified group is actually the amino group of the protein. On the other hand, the modification of myosin A by TNBS exhibits very specific and stoichiometric effect on ATPase[34].

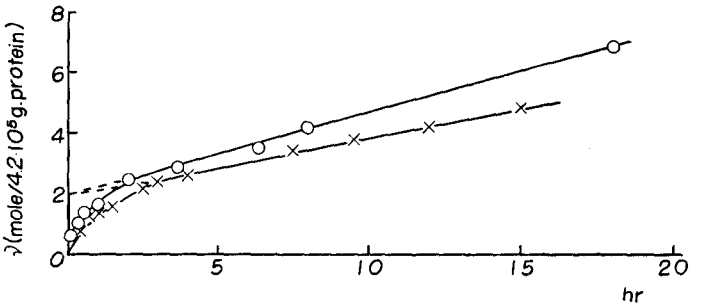

Fig. 12. Time course of binding of TNBS with myosin A. Ordinate, moles of bound TNBS per mole of myosin A. ○, 33 μM fresh myosin A, 330 μM TNBS; ×, 15.5 μM aged myosin A (50 hr. after death of rabbit), 166.7 μM TNBS. 0.6 M KCl, pH 7.5. 0°C.

In Fig. 12 is shown the time course of the combination of TNBS with myosin A. As clearly seen in this figure, the binding of 2 moles of TNBS to one mole of fresh or aged myosin A*) (molecular weight, 4.2×10^5 g)[42,43] occurred rapidly and thereafter the combination increased slowly and linearly with time. This implies that two amino groups in one myosin A molecule were attacked specifically by TNBS. When a SH reagent such as Mersaryl or PCMB was added beforehand into myosin A solution, the velocity of the binding of TNBS was depressed especially in the first rapid phase (Fig. 13). To determine the binding site of TNBS, the acid-hydrolyzate of TNP-myosin A was separated into the water-soluble and the ether-soluble fractions. The water soluble fraction was chromatographed with tert-amylalcohol mixture. The position of the TNP-hydrolyzate on the paper chromatogram agreed perfectly

*) "Fresh" or "aged" myosin A refers to the one which is used within 24 hrs. or more than 50 hrs. after death of rabbit, respectively.

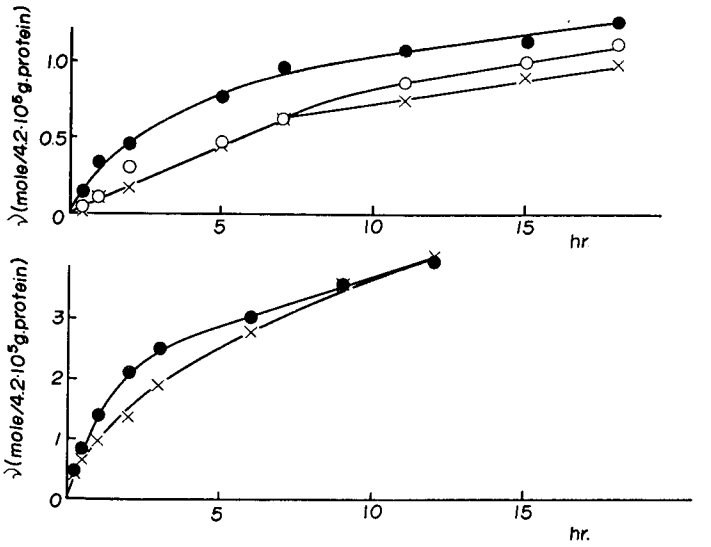

Fig. 13. Effect of SH reagents on TNBS binding. A: ●, 5 μM fresh myosin A, 20 μM TNBS; ○ and ×, ditto plus 12.5 moles PCMB and 12.5 moles Mersalyl/10^5 g protein respectively. B: ●, 15.5 μM fresh myosin A, 155 μM TNBS; ×, ditto plus 12.5 moles Mersaly/10^5 g protein.

TABLE 3. Content of ε-TNP-lysine in TNP-myosin A

(moles/4.2×10^5 g)

Amount estimated from absorption of TNP-myosin A	0.36	0.80	1.07	2.25	4.62	6.46
Amount of isolated TNP-lysine	0.4	0.9	1.1	2.3	4.7	6.4

with the position of ε-TNP-lysine. As presented in Table 3 the amount of TNP-lysine was in good agreement with that estimated by the absorption at 345 mμ of TNP-myosin A. In the ether soluble fraction, α-TNP-alanine, -valine, -threonine and -leucine were detected. The total amount of these α-TNP -amino acids, however, was only a few per cent of that of ε-TNP-lysine. These α-TNP-amino acids seem to originate from impurities remained in the preparation of myosin A. Therefore it is concluded that in 367 lysine[44] residues in the myosin A molecule there are 2 lysine residues which are specifically attacked by TNBS and that these lysine residues are located in the vicinity of the SH group.

As shown in Fig. 14, the ATPase activity at a high concentration of ATP decreased to about 30% of the original value in proportion to the amount of bound TNBS up to 2 moles per mole of myosin A. This diminished activity

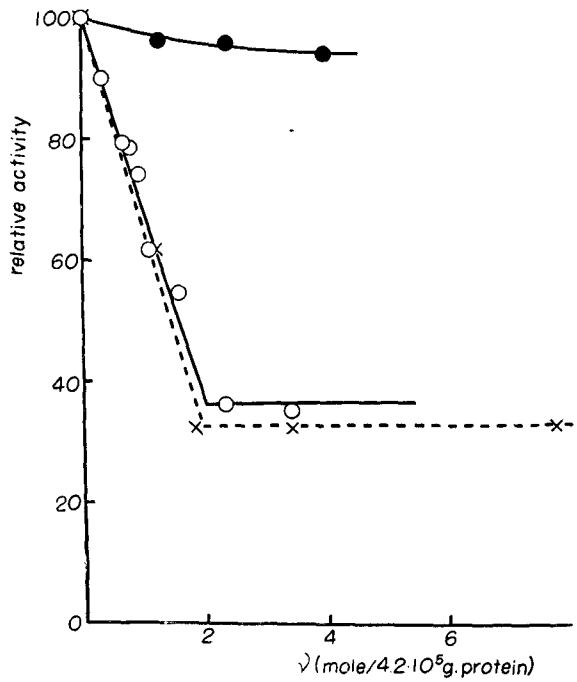

Fig. 14. ATPase activity of TNP-myosin A. Percentage of activity is plotted against degree of binding of TNBS. ○ and ×, two different preparations of fresh myosin A; ●, aged myosin A (one weak after death of rabbit).

was kept constant on further binding of TNBS up to 8 moles per mole of myosin A. The MICHAELIS constant of the compound of fresh myosin A with TNBS was lower than 2×10^{-6} M, while the value of the control was 1.6×10^{-4} M (Fig. 15). This may be due to a stabilization of the MICHAELIS complex by the formation of an intramolecular conjugate of TNP with ATP on the surface of the protein, since a strong attraction can be expected between TNP and the adenine base of ATP[45]. The determination of the amino acid sequence around the ATPase-specific amino group is now in progress.

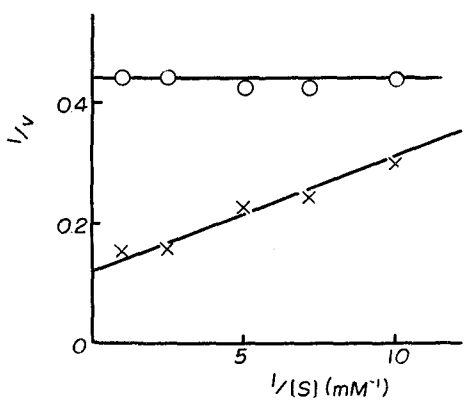

Fig. 15.
LINEWEAVER-BURK plot of ATPase of TNP-myosin A. ×, control; ○, TNP-myosin A (2.82 moles of bound TNBS/mole of myosin A). 7 mM Ca^{++}, 0.3 M KCl, pH 7.4, 25°C. Unit for v; μ moles P/sec/g protein.

The foregoing results were obtained by the reaction between TNBS and fresh myosin A. It is well known that myosin A polymerizes during storage even at 0°C[46]. The action of TNBS on polymerized myosin A was quite different from that on fresh myosin A. As seen in Fig 14, the ATPase activity of aged myosin A was not affected significantly by the binding of TNBS, while polymerized myosin A was disaggregated on the binding of TNBS (Fig. 16). The masking of the amino group specific to ATPase by the storage might be due either to the change in the conformation around the ATPase active site, as suggested from the change in the properties of ATPase during storage[10], or to the steric effect due to the polymerization of myosin A.

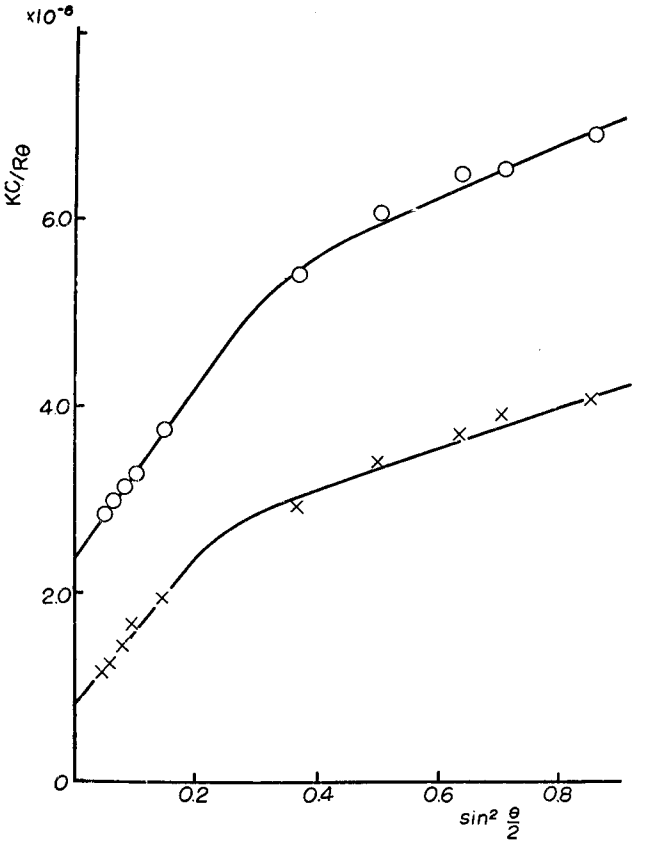

Fig. 16. Zimm plot of light-scattering of aged myosin A (two days after death of rabbit). ×, control; ○, TNP-myosin A (1.56 moles of bound TNBS/mole of myosin A). 0.5 M KCl, pH 6.5, 18.5–20°C.

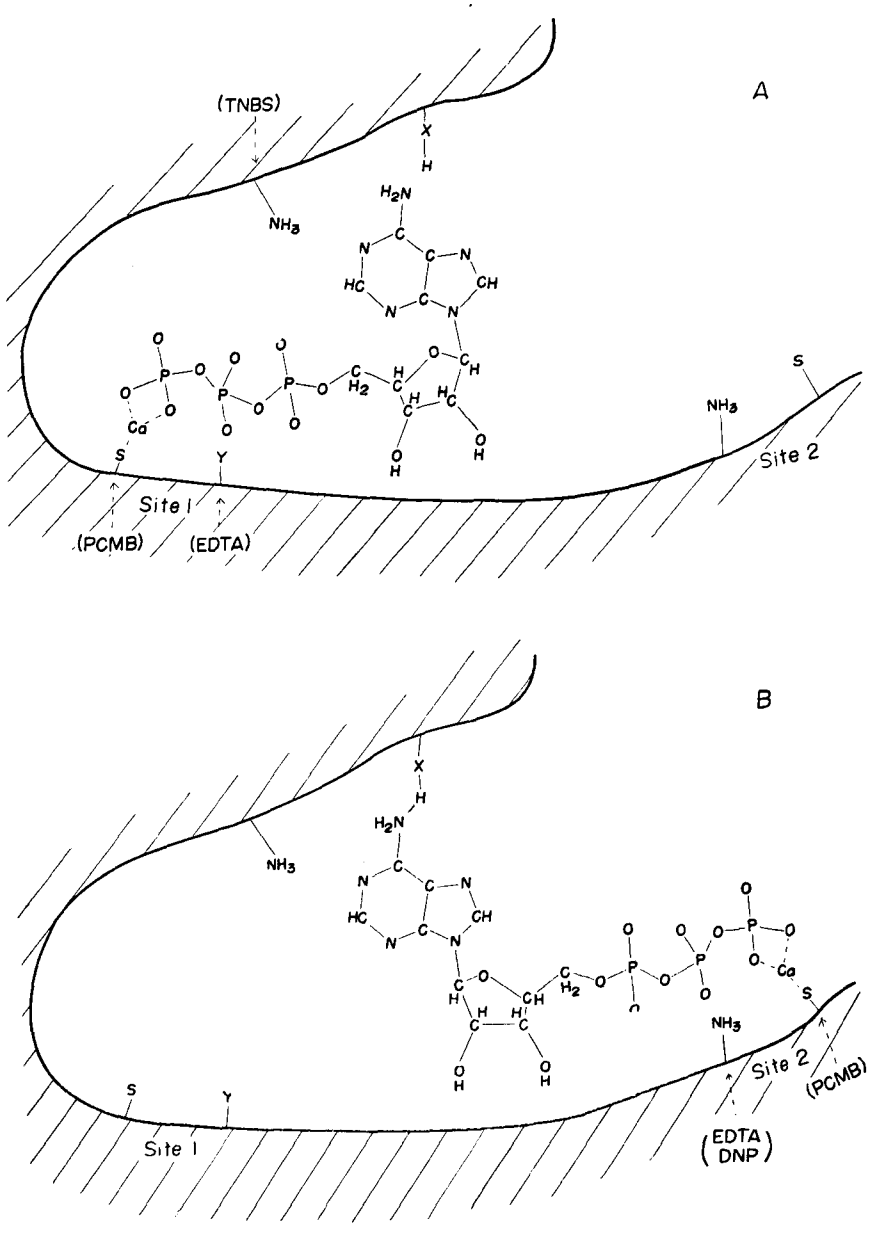

Fig. 17. A model of active site of myosin ATPase.
A: complex of ATP with site 1.
B: complex of ATP with site 2.
Only schematic.

From the results of the preceeding investigations the following figure might be proposed as a model of the chemical structure of the ATPase active site of myosin A (Fig. 17). The PP part of NTP binds by the mediation of Me^{++} with the site 1, which contains one SH group and one guanidum group, and the terminal phosphate bond is hydrolyzed. On the other hand the binding of PP part with the site 2, which includes one SH group and an amino group, stabilizes the MICHAELIS complex, while it changes the size and shape in the case of actomyosin or myosin B. Consequently, the occupation of the site 2 by PCMB or EDTA *etc.* brings about the activation of ATPase activity and the inhibition of the change in size and shape of myosin B by ATP*). The binding capacity of the site 2 with PP group is profoundly dependent on the structure of the nucleoside, and the binding strength is in the decreasing order of CTP, ATP, UTP, GTP and ITP. It may be reasonable to assume[47] that the nucleoside binds by hydrogen binding at N or O of the position 6 of the base with an unspecified site X of myosin A, which is located near the TNBS specific lysine residue, and the attraction between TNP and adenine base may cause the inhibition of ATPase and the inhibition of the binding of ATP with the site 2. The fact[34] that the binding of actin with myosin is not influenced by the combination of TNBS seems to indicate that the TNBS-specific lysine residue is located near the site 1 but at some distant from the site 2 which may be involved in the actin-myosin binding site.

Part III ATPase and Muscle Contraction

In the preceding two parts the kinetic properties and the chemical structure of the active site of the "myosin" ATPase were described. However, the relation between the "myosin" ATPase and the muscle contraction still remains to be discussed. Since the discovery of this enzymic reaction by ENGELHARDT, the significance of the "myosin" ATPase in the muscle contraction has long been the subject of repeated investigations, and there have been many reports on this problem such as by WEBER[4], MORALES[5] and BOWEN[48]. However, two phenomena, which have very important connections with this problem, did not seem to attract sufficient attention of the previous workers. One is the initial rapid P liberation from ATP by "myosin" and the other is the dependence

*) KIELLEY & BRADLEY[27] reported that, when EDTA is used as a modifier, ATPase is inhibited by the binding of PCMB. This may be due to the binding of EDTA to the cationic group, probably the guanidine group (denoted as Y in Fig. 17; see the footnote of p. 103) of the site 1 which can occur only when the site 1 is already occupied by a reagent, for when EDTA occupies the site 2, another one cannot bind to the site 1 because of electrostatic repulsion between the two EDTA molecules.

of the actomyosin ATPase on the colloidal state of the protein.

i Initial ATPase[49,50]

It has been observed by A. WEBER and HASSELBACH[51] that the rate of liberation of P from the actomyosin-ATP system is, in an earlier stage, several times higher than the constant value ultimately attained at a stationary state. Since then it has been expected that more intensive investigation of this phenomenon will throw some new light on the process of dephosphorylation of ATP in living muscle. However, it is almost impossible to measure the initial velocity of ATPase by the usual kinetic procedure. In our laboratory attempt was made to construct an apparatus with which the rate of the ATPase reaction during early few seconds could be measured.

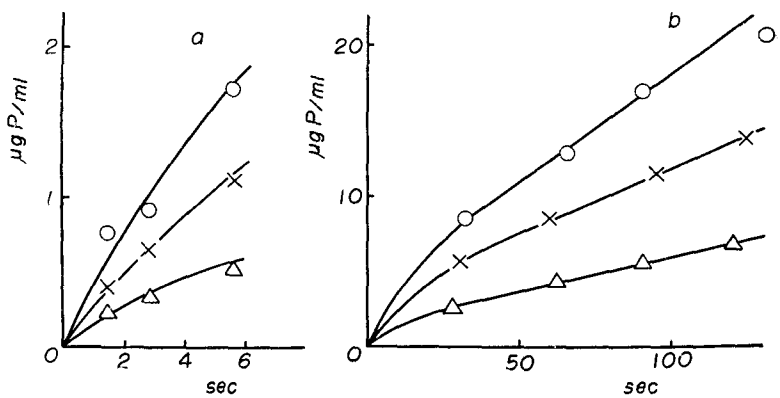

Fig. 18. Dependence of rate of liberation of P on amount of enzyme. ○, 1.8 mg; ×, 1.2 mg; △, 0.6 mg myosin B/mℓ. 1 mM ATP, 0.6 M KCl, 7 mM Ca^{++}, pH 6.7, 23°C.
(a), initial phase; (b), steady phase.

As shown in Fig. 18, the velocities in the initial phase as well as in the steady state are proportional to the concentration of the protein. Thus, the quantity of the initial burst, *i.e.*, the intercept of the ordinate obtained by extrapolating the linear P-liberation to zero time, was proportional to the amount of the enzyme. Fig. 19 shows that the time course of the P-liberation measured by radioactivity with ^{32}P labelled ATP as substrate agrees with that measured colorimetrically. These two results show clearly that the inorganic P liberated during the initial rapid phase is originated from the ATP added, and it is impossible to ascribe this effect to a displacement of P from the protein or to the presence of a labile impurity in the ATP preparation. The initial rapid liberation of P was observed not only in 0.6 M KCl but also in 0.055 M KCl in the presence of Mg^{++}. It was also observed in the case of myosin A and

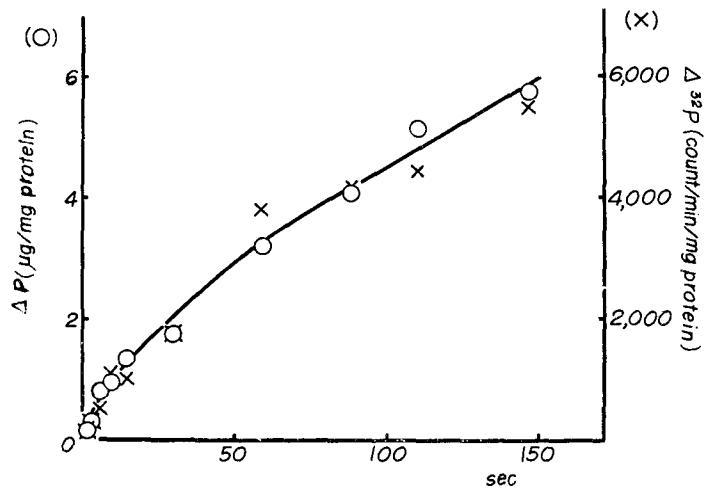

Fig. 19. Rate of liberation of P, determined both by colorimetry (○) and radioactivity (×). 1 mM ^{32}P-labelled ATP, 0.6 M KCl, 9 mM Ca^{++}, pH 6.7, 24°C.

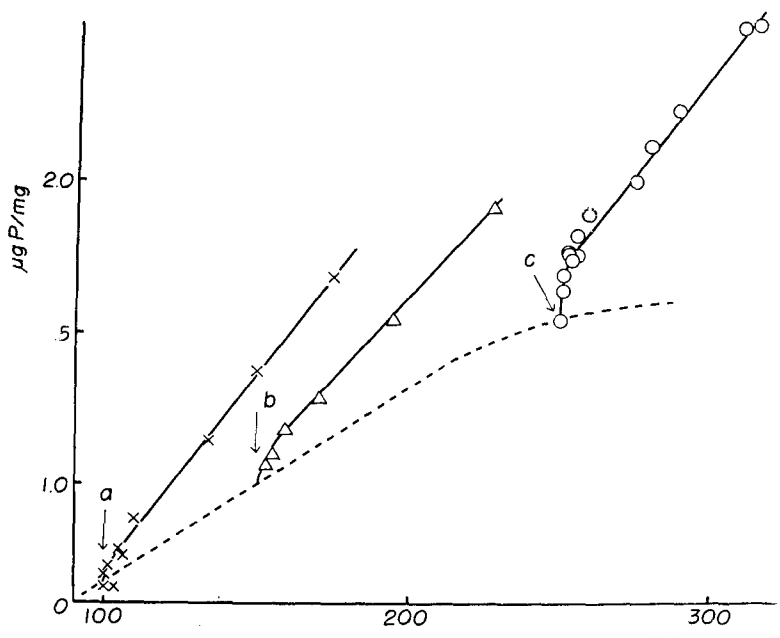

Fig. 20. Recovery of initial phase. Dotted line, hydrolysis of 50 μM ATP. Arrows, additions of 1 mM ATP when concentrations of residual ATP are (a) 25 μM, (b) 18 μM and (c) 0 μM. 0.6 M KCl, 1 mM Mg^{++}, pH 6.7, 22°C.

H-meromyosin[52].

When ITP was the substrate, the quantity of the initial burst in the presence of Mg^{++} was found to be about 20–50% of that of the case of ATP hydrolysis. In the case of ATP, the moles of P liberated during the initial phase were always several times as great as those of the ATP binding site[13]. This fact indicates that the initial burst is not a consequence of formation of a enzyme-P or a enzyme-ADP complex as an intermediate of a conventional MICHAELIS-MENTEN type per. se., because this supposition would unequivocally demand that one mole of P per one mole of the ATP binding site should be liberated during the initial phase. The fact, that the kinetic constants of the steady ATPase did not change when the initial burst was suppressed by several treatments, also supported this view (see below).

The same initial rapid splitting of ATP was repeatedly obtained against a fresh ATP if the old ATP had been completely hydrolyzed. The relationship between the remaining quantity of ATP preincubated and the initial burst of

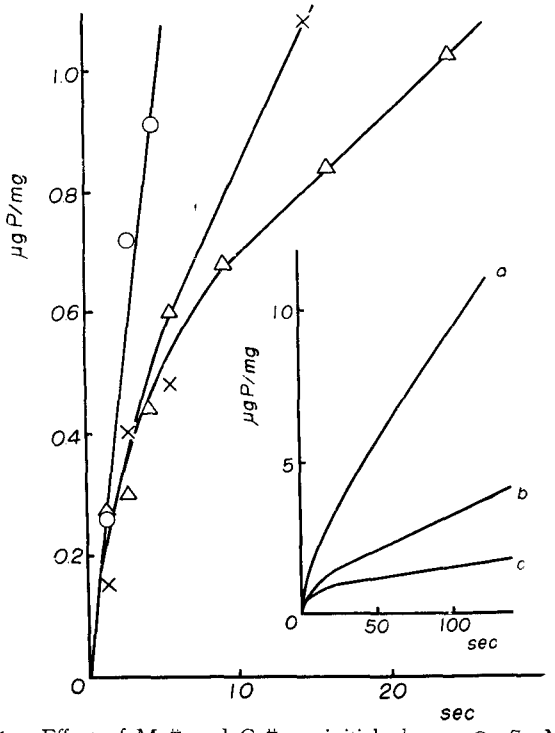

Fig. 21. Effect of Mg^{++} and Ca^{++} on initial phase. ○, 7 mM Ca^{++}; △, 4.5 mM Mg^{++}; ×, no divalent cation. 1 mM ATP, 0.6 M KCl, pH 6.7, 22°C. Inserted figure, steady phase, (a), 7 mM Ca^{++}; (b), no divalent cation; (c), 4.5 mM Mg^{++}.

P-liberation is illustrated in Fig. 20. The concurrent recovery of the burst to the normal level was obtained as soon as the preincubated ATP was exhausted, thus indicating that the enzyme is restored reversibly with considerable quickness to its original state which is capable of producing the initial burst of P-liberation.

The marked activation by Ca^{++} and inhibition by Mg^{++} [12] which together form one of the characteristic properties of the steady ATPase, did not appear at the initial stage. As shown in Fig. 21, in 0.6 M KCl the initial rate was changed only slightly by the addition of Ca^{++} or Mg^{++}.

The contents of Ca^{++} and Mg^{++} in the myosin B preparation were 1.2–1.8 moles and 1.4–1.6 moles per 10^5 g of protein, respectively. After exhaustive dialysis of myosin B solution against 0.6 M KCl, the quantities of Ca^{++} and Mg^{++} decreased to 0.28–0.34 mole and 0.27–0.32 mole par 10^5 g, respectively. The initial burst was abolished almost completely by the depletion of these dialyzable cations, but after this treatment no appreciable change in the velocity of the steady ATPase was observed (Fig. 22). The restoring effect of several divalent cations on the initial burst, which had been reduced by the dialysis, was investigated. The reduced burst restored to almost the same quantity as that before the dialysis by the addition of a minute amount of Mg^{++}, Mn^{++} or Sr^{++} but not by a large amount of Ca^{++}. In view of these observations the distinction between the initial and the steady ATPases seems to be not simply the matter of rate but should be attributed to more substantial difference.

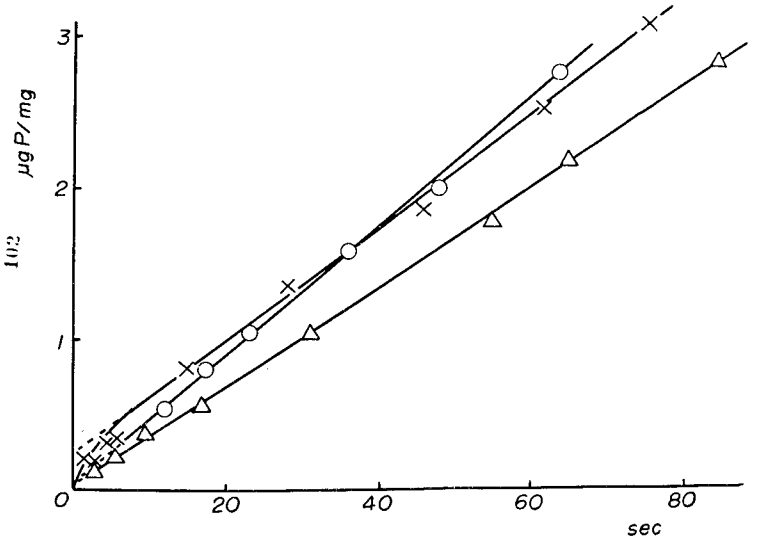

Fig. 22. Effect of dialysis on initial phase. ×, before dialysis; △, after dialysis; ○, 50 μM Ca^{++} added after dialysis. 1 mM ATP, 0.6 M KCl, pH 6.7, 21°C.

The initial burst disappeared completely on the addition of EDTA. The minimum concentration of EDTA causing the disappearance of the initial phase was too low to activate the steady ATPase and to cause an appreciable decrease in the concentration of dialysable Mg^{++} contaminated in the reaction mixture and was of the same order of magnitude with the ATP-binding site. Therefore, the effect of EDTA is not accounted for merely by the removal of dialyzable Mg^{++} by its chelation. The initial ATPase might be suppressed by some complex formation among the protein and EDTA.

In order to clarify further the reaction mechanism of the initial phase, the sequence of the addition of ATP and Mg^{++} was altered in several ways in the presence of a minute amount of EDTA as illustrated in Fig. 23. It is evident

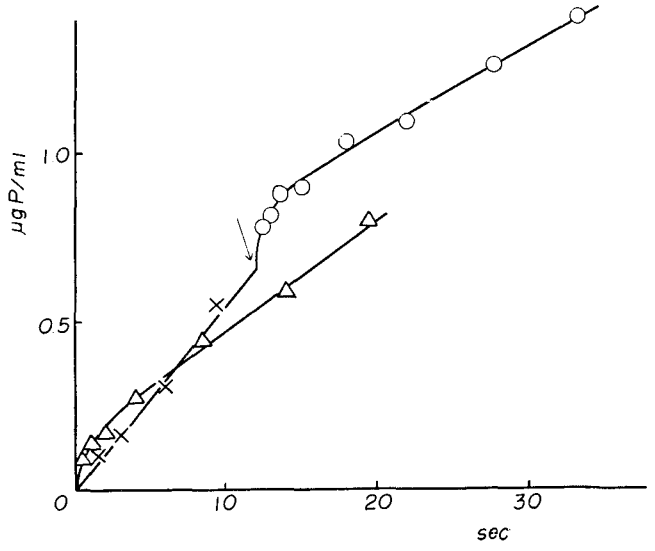

Fig. 23. Initial burst of P-liberation by adding Mg^{++} to a reaction mixture containing EDTA and ATP. ×, 50 μM EDTA and 1 mM ATP at zero time; arrow, 1 mM Mg^{++} added. △, 50 μM EDTA and 1 mM ATP; 1 mM Mg^{++} added at zero time. 0.6 M KCl, pH 6.7, 1°C.

from this figure that the initial burst occurs immediately only when Mg^{++} is present in sufficient quantity together with ATP, regardless of the order and the time of the addition of Mg^{++} and ATP. This observation, along with the fact that almost the same ATPase activity to that at the steady state could be obtained from onset of the reaction by treatments which abolished the initial burst, excludes the following hypothesis, *i.e.*, the transition of dephosphorylation rate is accompanied by the change in the enzyme itself from a highly active

state (E) to a less active one (E^*). If this mechanism holds, it seems reasonable to expect that a treatment which brings about disappearance of the transition of the activity, such as the addition of EDTA or the dialysis, would retard or accerelate extremely the step of the transition from E to E^*. The concept that EDTA or the dialysis retards the step $E \rightarrow E^*$ is in contradiction with the fact that the activity of ATPase, which is almost equal to that at the steady state, could be obtained by these treatments from the onset of the reaction. The other alternative, *i.e.*, the accerelation of the step $E \rightarrow E^*$ is not compatible with the observation of the appearance of the additional burst of P-liberation by the addition of sufficient amount of Mg^{++} to the reaction mixture preincubated with EDTA and ATP, because according to this supposition the conversion from E to E^* should be completed by the action of EDTA before the addition of Mg^{++}. Thus, neither situation was realized.

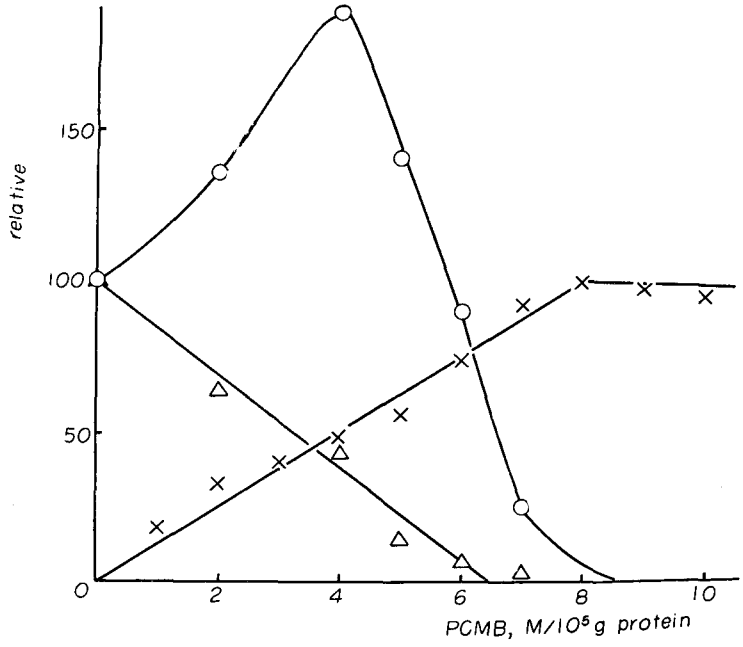

Fig. 24. Decrease of quantity of initial burst with binding of PCMB to myosin B. ×, increase of extinction at 255 mμ; ○, rate in steady phase; △, quantity of initial burst. 1 mM ATP, 0.6 M KCl, 5 mM Ca^{++}, pH 6.7, 20°C.

In Fig. 24 is presented the relationship between the quantity of PCMB bound to SH group of myosin B and the quantity of the burst, together with the activity of the steady ATPase. About 8 moles of PCMB were bound to

10^5 g of myosin B and a linear decrease of the quantity of the burst proceeded together with the binding of PCMB to SH groups.

Taking these facts into consideration, the assumption most likely at present is the transphosphorylation from ATP to the enzyme. The transphosphorylation must occur much more rapidly than the steady ATPase at several sites of myosin molecule and the dephosphorylation must be much slower than the steady ATPase. The additional high dephosphorylation rate can be observed until the slower steady state rate of the transfer reaction is attained, when transferred P is detached by the denaturation by trichloroacetic acid and is measured together with P from ATP by the usual ATPase. Since transphosphorylation requires generally Mg^{++} and intact SH group of the enzyme, this mechanism might be adequate to explain the abolishment of the initial burst of P-liberation by the addition of EDTA, SH reagent or dialysis.

The effectors which have been shown to inhibit the initial burst of P-liberation (such as EDTA, PP[52], Oxarsan[53] and PCMB) also behave as inhibitory reagents on the contraction of the muscle models[4,54]. The effect of divalent cations upon the recovery of the diminished initial burst of the dialyzed myosin B is quite compatible with their effects on the contraction of the dialyzed muscle models. Isolated myofibrils[55] and glycerol-treated muscle[56] which are depleted of Mg^{++} by dialysis have been unable to contract on the addition of ATP without further supplement of Mg^{++} or Mn^{++} which cannot be replaced by Ca^{++}. It has recently observed[47] that 9-(4′-hydroxybutyl)-6-aminopurine 4′-triphosphate, 9-(3′-hydroxypropyl)-6-aminopurine 3′-triphosphate and 9-(2′-hydroxyethyl-6-aminopurine 2′-triphosphate decrease the intensity of light scattered by myosin B to the same extent as ATP and are hydrolyzed by myosin B at similar rates to that of ATP but they cannot contract myofibrils and 9-(2′-hydroxyethyl)-6-aminopurine 2′-triphosphate does not have the initial burst. In addition the smaller quantity of the initial burst observed with ITP, 20–50% of the quantity for ATP, is in agreement with the lesser degree of tension development of the muscle model by ITP used in place of ATP[57]. These parallelisms lead to the assumption that the initial burst of P-liberation from ATP is one of the necessary conditions for the muscle contraction.

It has been established by the thorough study of HILL[cf.58,59] that the initial heat in muscle activity consists of the heat of activation and the heat of shortening. The rate of production of the heat of activation reaches its maximum value before the earliest detectable sign of the muscle response. The heat of activation is not affected by the shortening or the performance of work. It is little influenced, if at all, by the length of muscle. Recently MOMMAERTS[60] observed that the breakdown of creatine phosphate during the

muscle contraction is divided into the following two parts: the one independent of and the other dependent on the amount of shortening. As described above, the initial burst of ATP splitting is one of the necessary reactions for the muscle contraction. Furthermore this phenomenon is observed both with actomyosin and myosin at low ionic strengthes as well as at high ionic strengthes. These facts seems to indicate the close correlation between the initial ATPase and the heat of activation; immediately after the excitation of muscle, myosin may be phosphorylated during the initial phase of the ATP splitting, and the heat accompanied with this phosphorylation may be observed as the heat of activation.

ii ATP ase at low ionic strength[61]

At low ionic strengthes (below 0.15) myosin B does not dissolve and it is superprecipitated by the addition of a small amount of ATP[3], but it is dissolved by the addition of a large amount of ATP (clearing responce)[62]. This change in the colloidal state of myosin B produces a large effect on its ATPase activity.

When the concentration of KCl is low, the rate of the ATP splitting does not obey the MICHAELIS-MENTEN relation and it decreases with increasing the substrate concentration. The inhibition of ATPase by excess substrate has been reported by HASSELBACH[63], WEBER[4] and PERRY and GREY[64]. However, the mechanism of the inhibition by excess substrate of the myosin B ATPase remains unclarified. Recently in our laboratory this phenomenon has been studied extensively. The inhibition by excess substrate is influenced markedly by the ionic condition. In Fig. 25 is shown the dependence on the concentration of KCl of the relation between v and [S]. As seen in this figure, the MICHAELIS-

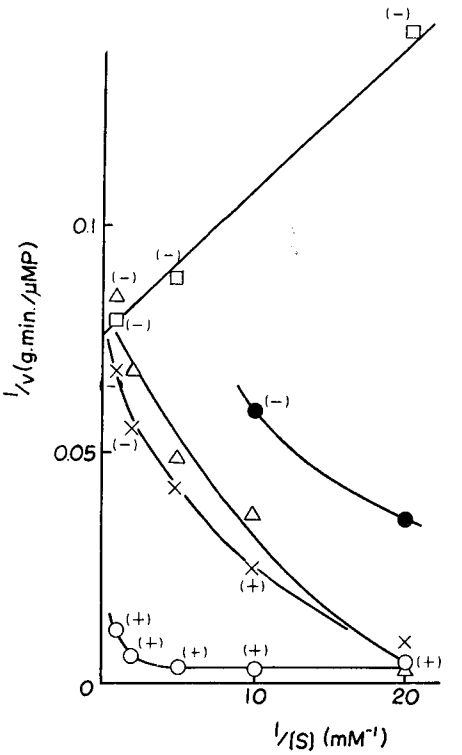

Fig. 25.

Dependence on ionic strength of myosin B ATPase. KCl concentration: ○, 0.03 M; ×, 0.075 M; △, 0.105 M; ●, 0.12 M; □, 0.15 M. Designations + and − represent superprecipitation and non-superprecipitation, respectively. Mg^{++} was 1 mM in excess of ATP, pH 7.0, 25°C.

MENTEN relation was attained when the concentration of KCl was above 1.5 M, but the substrate inhibition appeared when the concentration reduced to 0.12 M. The ATP level, at which the substrate inhibition commenced, was increased by the decrease of the KCl concentration, and the rate at sufficiently high ATP concentration approached to the rate in 0.15 M KCl at which substrate inhibition could not be observed. When KCl was replaced by choline chloride, NH_4Cl or LiCl at the same concentration (0.075 M), the inhibition by excess substrate was scarecely affected. On the other hand, on the addition of 0.12 M of salt such as NH_4Cl, NaCl or choline chloride to 0.075 M KCl, the simple MICHAELIS-MENTEN relation was obtained as in the case of high concentration (above 0.15 M) of KCl, though V_m and K_m varied slightly with the change of salt. When ITP was the substrate, no substrate inhibition was observed under various conditions.

The inhibition by excess substrate was influenced markedly by concentration and species of divalent cation. The experiments mentioned above were carried out in the presence of Mg^{++}. The inhibition was never observed in the presence of Ca^{++}, Co^{++} or Ba^{++}, but occurred in the presence of Mn^{++}, though to much less extent than in the presence of Mg^{++}. The relation between the rate of the ATP splitting and the concentration of the Mg–ATP complex is shown in Fig. 26, where the concentration of Mg–ATP was calculated using 10^4 as the binding constant of Mg^{++} to ATP^{4-}[14]. When the total concentration of ATP exceeded 10^{-4} M, the rate decreased abruptly with the increase of the ratio of Mg–ATP to total ATP.

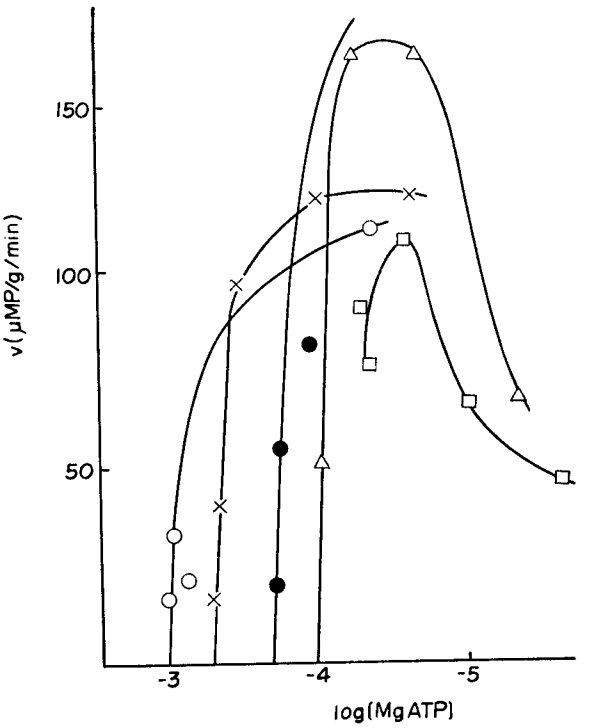

Fig. 26. ATPase activity as a function of concentration of Mg–ATP. Total concentration of ATP: ○, 1.0 mM; ×, 0.5 mM; ●, 0.2 mM; △, 0.1 mM; □, 0.05 mM. 0.075 M KCl, pH 7.0, 25°C.

The following two explanations have been already presented for the substrate inhibition of the actomyosin ATPase. The one is the suggestion of GESKE *et al.*[65], that the inhibition is due to an inhibitory effect of free ATP. The other is the claim of PERRY and GREY[64], that the inhibition is attributable to the removal of Mg^{++} by its chelation with ATP. The results presented in Fig. 26 indicate that both explanations are unapplicable at least to our cases and it is likely that the inhibition is due to the increase of the amounts of Mg-ATP complex. In Fig. 27 is shown the effect of temperature. At low

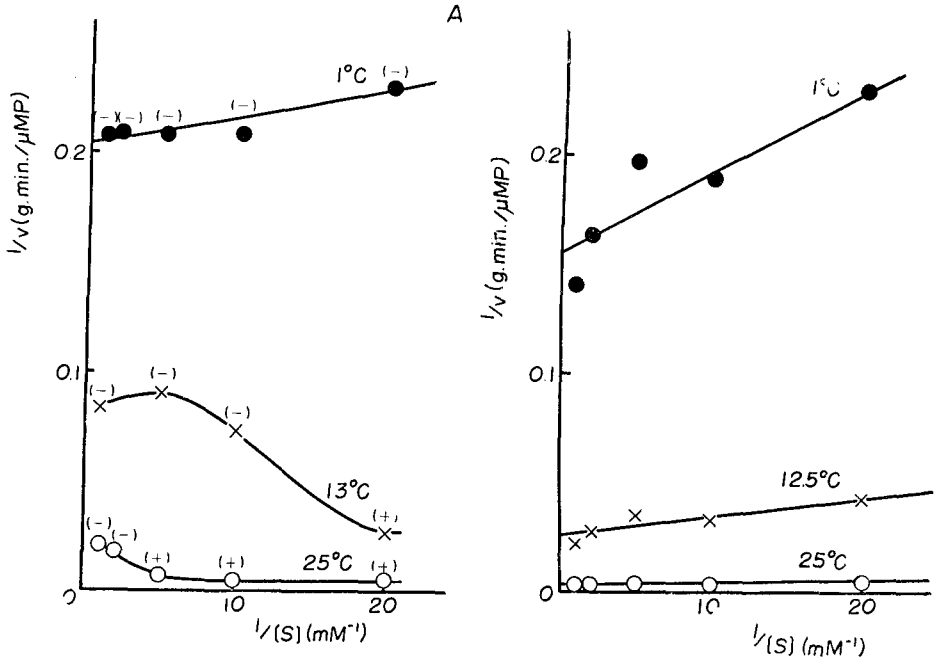

Fig. 27. Dependence on temperature of ATPase at low ionic strength. A: ATPase activity was measured by usual procedure. ○, 25°; ×, 13°; ●, 1°. Designations + and − indicate superprecipitation and non-superprecipitation of myosin B, respectively. B: ATPase activity was measured after dialysis of superprecipitated myosin B. ○, 25°; ×, 12.5°; ●, 1°. Mg^{++} was 1 mM in excess of ATP. 0.075 M KCl, pH 7.0.

temperature (0°C) the superprecipitation was not observed in the wide range of the ATP concentration and the MICHAELIS-MENTEN relation was obtained. However, when the temperature was raised, the superprecipitation occurred at low ATP concentrations and the corresponding velocities were observed to be

much higher than those at high ATP concentrations, where no superprecipitation was observed. The superprecipitated myosin B by the addition of relatively low concentrations of Mg–ATP, remained superprecipitated during dialysis against 0.075 M KCl. After these treatments the activity of ATPase was measured by the addition of ATP. In this case no substrate inhibition was observed even at relatively high temperatures; the ATPase activity increased with increasing ATP concentration. In the presence of an appropriate concentration of ATP, the superprecipitation commenced after several minutes the addition of ATP. In this case the rate of the ATP hydrolysis increased abruptly accompanied by the appearance of the superprecipitation. Thus it is evident that the ATPase activity of myosin B is significantly controlled by the colloidal state of the protein, and that the ATPase activity of superprecipitated myosin B is markedly higher even under the same condition than that of non-superprecipitated one. Hence the substrate inhibition seems to be caused by the prevention of the superprecipitation by a high concentration of Mg–ATP.

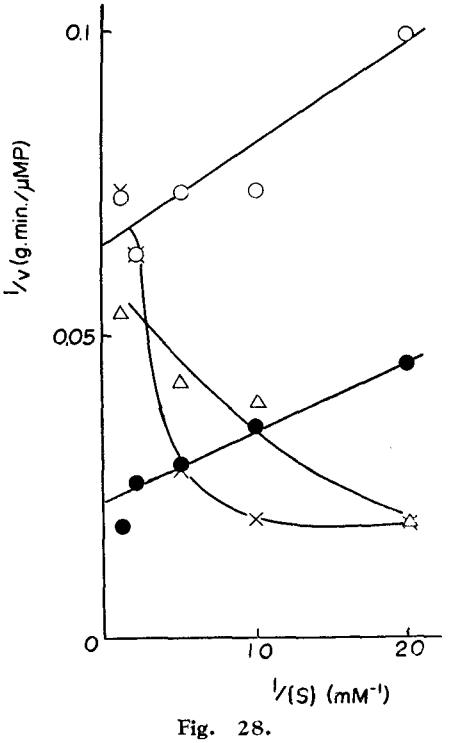

Fig. 28.
LINEWEAVER-BURK plot of synthetic actomyosin ATPase. Ratio of myosin A to F-actin: ○, ∞; △, 5; ×, 2.5; ●, 0.4. Mg^{++} was 1 mM in excess of ATP. 0.075 M KCl, pH 7.0, 25°C.

Since ATPase activity of actomyosin is much higher than that of myosin both in the presence of Mg^{++} (Fig. 3), SZENT-GYÖRGYI[3] and HASSELBACH[63] interpreted the substrate inhibition by the transition from the actomyosin ATPase to the myosin ATPase with the increase of the concentration of Mg–ATP. This explanation was supported by the following results. In Fig. 28 are shown the ATPase activities of synthetic actomyosins formed by mixing pure myosin A with various amounts of F-actin. When the ratio of actin to myosin was 1 : 2.5 a typical substrate inhibition was observed and the activity approached to that of myosin A with increase of ATP concentration. However, the inhibition by excess substrate was not observed not only on myosin A but also on actomyosin whose content of actin was very high.

Furthermore, several additional evidences exist to support the above interpretation. As mentioned above, the ATP level where the substrate inhibition commences decreases with increase of the KCl concentration, and the ATP level also decreases at low temperature, where myosin B seems to be susceptible to dissociation even in the absence of ATP[66]. The substrate inhibition is observed in the presence of Mg^{++} or Mn^{++} which enhances more powerfully the size and shape change of myosin B by PP than of Ca^{++} or Ba^{++}[13]. This is not observed when the substrate is ITP, whose effect on the size and shape of myosin B is much weaker than that of ATP[25].

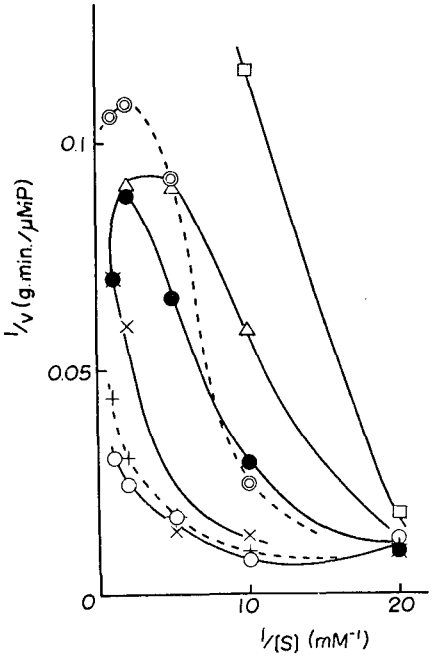

Fig. 29.

Effect of chelate compounds on substrate inhibition of myosin B-ATPase. EDTA concentration: ○, zero; ×, 50 μM, ●, 0.1 mM; △, 0.5 mM; □, 1 mM. CyDTA concentrations: +, 0.2 mM; ◎, 2 mM. Mg^{++} was 1 mM in excess of EDTA or CyDTA plus ATP. 0.075 M KCl, pH 7.0, 25°C.

Finally, the close connection of the substrate inhibition of ATPase and the relaxation of muscle model may be indicated by the following results. In the presence of Mg^{++}, EDTA remarkably promoted the substrate inhibition (Fig. 29), and this EDTA effect was suppressed by a small amount of Ca^{++}. CyDTA and PP also promoted the substrate inhibition but their effects were much weaker than that of EDTA. As mentioned above, in the presence of Mn^{++} the degree of the substrate inhibition was much lower than that in the presence of Mg^{++}, but no substrate inhibiton was observed when ITP was the substrate. The relaxing effect of chelate compounds has already been reported by several workers[54,56]: the relaxing effect of EDTA is more remarkable in the presence of Mg^{++} than of Mn^{++} and it is greatly inhibited by the addition of a small amount of Ca^{++}. It has also been observed that in the presence of ITP the relaxing effect of EDTA does not appear and the effect of CyDTA is far weaker than that of EDTA. These results perfectly agree with our results on the inhibition of ATPase by excess substrate.

As beautifully shown by HILL[58,59], the shortening heat is proportional to

the amount of shortening of muscle and as shown by MOMMAERTS[60] one part of breakdown of creatine phosphate in the muscle contraction is intimately related to the extent of shortening. According to the sliding model of HANSON and HUXLEY[67-70] the quantity of the formation of actomyosin from actin and myosin is proportional to the degree of shortening. In company with the formation of actomyosin by the sliding of myosin filament through actin filament the splitting rate of ATP increases, as in the physiological medium the rate by actomyosin is far greater than that by myosin. The proportionality of the shortening heat to the amount of shortening may indicate that in living muscle a definite number of ATP molecules are splitted at each actomyosin ATPase site in the contracted state and thereafter a regulator for relaxation may operate.

As mentioned above, the heat of activation seems to be due to the initial ATPase, and the shortening heat may be originated from the remarkable enhancement of ATPase by the transition from the myosin to the actomyosin type. The sum of the heat in the initial process is 3 millical per gram of muscle. The work which an isolated muscle of frog or toad can perform under optimal condition may be as high as 40% of the total energy given out during the initial process[58,59]. According to POLLISAR[71], 15% of contractile unit is transformed into shortened state in the single twich. In a physiological medium the free energy of ATP hydrolysis is about 12 Kcal[72], the weight per one mole of the ATPase active site is 2.1×10^5 g for myosin A[13], and a fresh muscle contains about 10% of myosin[73]. Therefore, about 6 moles of ATP per one mole of the ATPase active site of myosin A are splitted during the initial process of shortening.

Conclusion

The reaction mechanism of the "myosin" ATPase is clarified by the kinetic analysis of the steady state (Part I) and by the change in ATPase by the chemical modification of "myosin" (Part II); ATP is splitted by its binding to the site 1, which involves a cysteine, and the MICHAELIS complex of ATPase is stabilized by the binding to the site 2, which involves a cysteine and a lysine. By this scheme, reasonable explanations are given not only on the pH activity curve and the activation by various reagents of ATPase, but also on the properties of NTPase. From the binding of TNBS with myosin A, the presence of a TNBS-specific lysine residue near the active site 1 is clearly established. In Part III the initial rapid splitting of ATP is demonstrated. The initial burst of the ATP splitting is shown to have an intimate relation with the muscle contraction and may correspond to HILL's heat of activation. At low ionic strengthes the rate of the ATP splitting by actomyosin depends on the change

of colloidal state caused by Mg–ATP. In the superprecipitated state ATP is splitted several ten times as high as in the clearing state. The enhancement of the ATPase activity by the change of the colloidal state corresponds probably to the additive shortening heat of HILL.

Thus the reaction mechanism of ATPase and the role of ATPase in the muscle contraction is clarified to a considerable degree. However, many problems still remain to be studied. We are now undertaking the determination of the sequence of amino acid around the lysine residue specific to ATPase and the clarification of conformation of the active site using ATP analogue as substrate[47] and organic solvents as modifiers of ATPase. We are also continuing the analysis on the denaturation of myosin A-ATPase[74] to clarify the conformation of the active site. The identification of the assumed phosphorylated compounds in the initial ATPase may be most important to elucidate the role of ATPase in the muscle contraction. Recently LEVY and KOSHLAND[75,76] observed that the phosphate produced by the splitting of ATP in H_2O^{18} with "myosin" had an O^{18}-content greatly in excess of the value to be expected for simple cleavage of the terminal bond. The comprehension of this fact in the reaction mechanism of "myosin" ATPase seems also to be one of the important points in the further investigations.

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