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Structure and Function of Myosin

I. Substructure of the myosin molecule.

II. Substrate Conformation and Myosin ATPase.

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Contents

INTRODUCTION

Many vital phenomena are based on the energy-transduction. The motile system is one of the energy-transducing systems, and widely spread over the living organisms, such as muscle, cytoplasm, and cilia. Muscle is an efficient energy-transducing system with highly organized structure. Engelhardt and Ljubimowa (1), in 1939, revealed that myosin possesses an adenosine triphosphatase (ATPase) [EC 3.6.1.3] activity. Furthermore, Szent-Gyorgyi (2) found that the myosin ATPase reaction is activated by F-actin, a polymer of G-actin (actin monomer), at low ionic strength, and that the acto-myosin ATPase reaction is accompanied with "superprecipitation", one of the model of muscle contraction in vitro. It was thus indicated that myosin and F-actin play a dominant role in muscle contraction. A.F.Huxley and Niedergerke (3) and H.E.Huxley et al. (4,5) showed that there are thin and thick filaments in sarcomere, the minimum contractile unit of muscle (Fig. 1). Thick filaments are mainly an assembly of myosin molecules, and thin filaments are mainly a polymer of G-actin (Fig. 2).
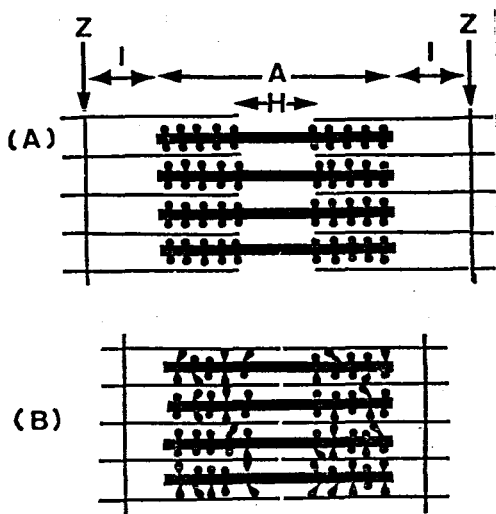
Fig. 1. Schematic representation of a sarcomere in resting (A) and shortening (B) state. Z-lines bound the sarcomeres. Thick filaments have no projections in H-zone. In (B), thin and thick filaments are linked with cross-bridges.
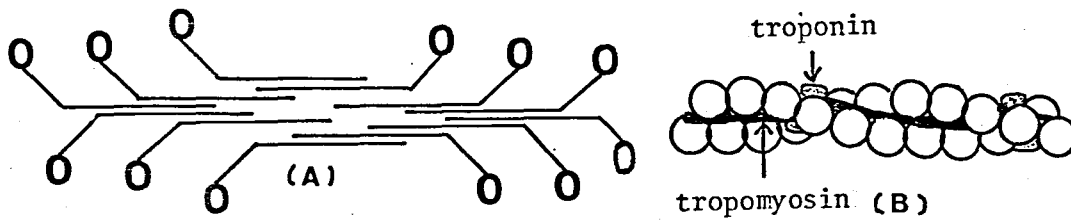
Fig. 2. Models of thick (A) and thin (B) filaments. Myosin molecules assemble at light meromyosin (LMM) portion (cf. Fig. 3). Thin filaments are composed of actim monomers and relaxing proteins, tropomyosin and troponin.

Myosin (MW=4.8x10^5) is a rodlike molecule (160x2nm) with two heads (20x7nm) and a tail (140x2nm) (Fig. 3). Myosin has three
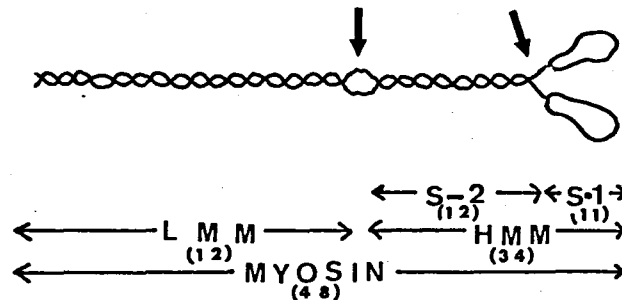
Fig. 3. A schematic representation of the myosin molecule. The number in parentheses are the molecular weight x 10^{-4}. Arrows indicate protease-sensitive regions.

physiologically important functions: (i) forming thick filaments, (ii) catalyzing ATP hydrolysis, and (iii) binding to F-actin. Studies on subfragments* of myosin revealed that the function-(i) is located on the LMM portion, and that the others are on the heads (6,7). Thick filament has projections derived from the heads heads of myosin, and binds to thin filament (F-actin) to form a cross-bridge (cf. Fig. 1). Thick and thin filaments slide past

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* The term, subfragments, is employed here for the proteins ob-
tained through the cleavage of peptide bonds, and the components
without cleavage is called 'subunits' (Fig. 4).
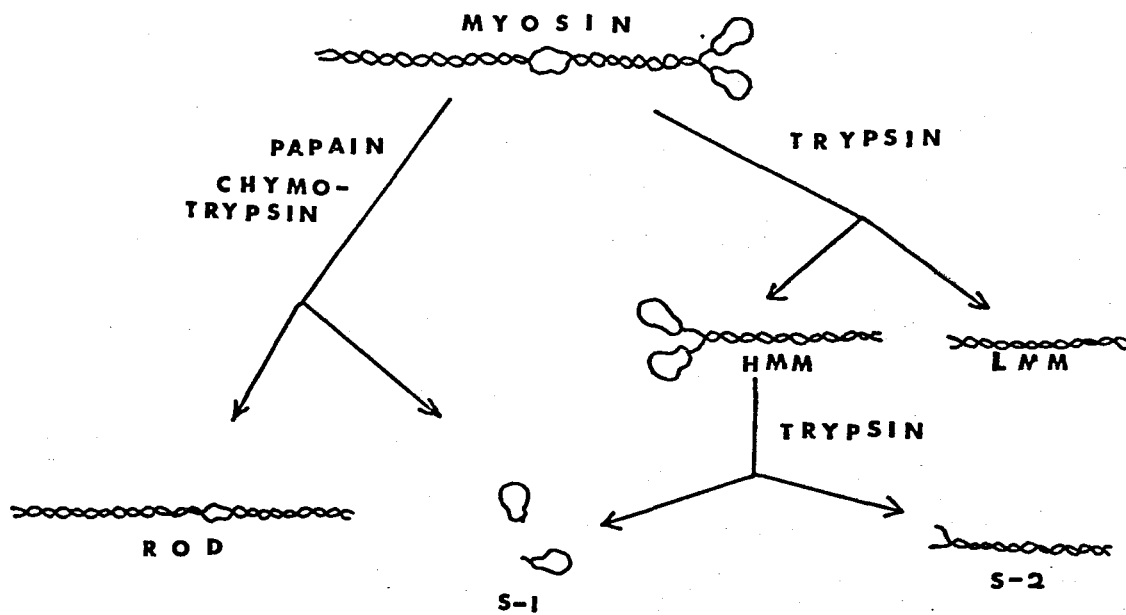
Fig. 4. Various proteolytic fragments of myosin molecule.

each other along the cross-bridges with the hydrolysis of ATP during contraction. The cross-bridge is, therefore, the active site for the force-generation. In other words, myosin binds to F-actin, causes the changes in the conformation of the heads, and dissociates from F-actin during ATP hydrolysis (Fig. 5). Therefore, studies on the myosin ATPase reaction are closely related not only to the contraction mechanism but to the structure-function relationships of a large enzyme molecule.
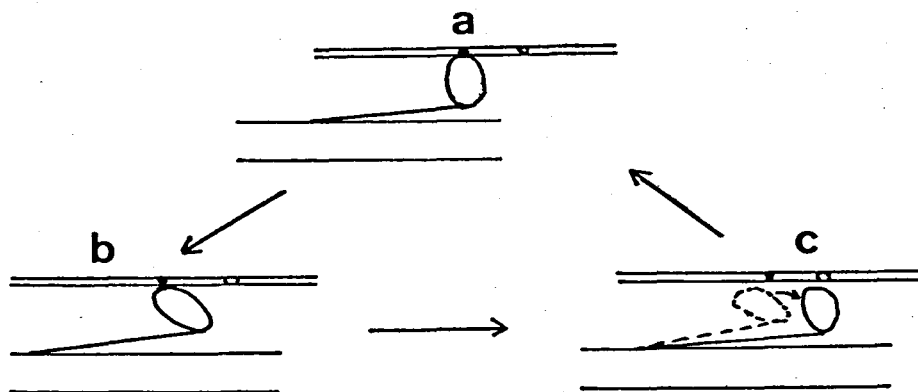
Fig. 5. Model of interaction of the myosin head with actin (H.E. Huxley (1969) Science 164 1356). (a): Attachment of myosin to actin; (b): tilting of the myosin head; (c): detachment of the head.

Tonomura and his coworkers (7,8) present the non-identical two head hypothesis, in which Mg^{2+}-ATP is hydrolyzed differently by two non-identical heads:

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

(2) ${}^{\circ}M + ATP \rightleftharpoons {}^{\circ}MATP \rightarrow {}^{\circ}M + ADP + Pi.$
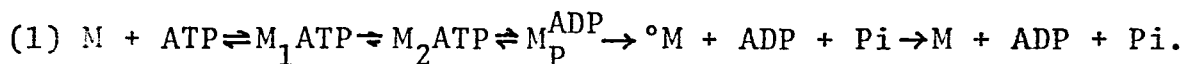
In route-(1), M_2ATP and M_P^{ADP} are fluorescent intermediates and are in a rapid equilibrium. The decomposition of M_P^{ADP} is very slow, and its formation is detected as an initial Pi-burst when the ATPase reaction is stopped by adding trichloroacetic acid. The decomposition of M_P^{ADP} is markedly activated by F-actin, and is thought to be closely related to muscle contraction. On the other hand, route-(2) is supposed to be related to ATP-regulation of the muscle contraction. In contrast to the above hypothesis, Taylor's (9) and Trentham's (10) groups present the identical two head hypothesis, in which Mg^{2+}-ATP is hydrolyzed via single route by two identical heads. Their schemes are basically the same as route-(1) of Tonomura's. It is an important subject to determine whether the two heads are identical or not. Beside the functional differences, the structural differences were also investigated. Weeds and Hartley (11) reported that myosin contains two sets of 22-23 Cys-peptides, and suggested that the two heads of myosin are identical. On the contrary, Ohe et al. (12) reported that treatment of myosin with monoiodoacetamide yielded only one car-boxamidemethylated Cys-peptide, and suggested that the two heads are different in native myosin. However, these studies did not give sufficient information on the difference in the quarternary-structure of the myosin molecule.

The skeletal myosin is composed of two fibrous polypeptides (f-chains) with a molecular weight of about 2×10^5 daltons and four globular polypeptides (g-chains) with molecular weights of about 2×10^4 daltons. Since Tsao (13) found, in 1953, that a concentrated urea solution dissociates myosin into two fractions (Fig. 6), the properties of the subunits have studied in detail.

UREA
ALKALI
GUANIDINE·HCl
SDS

f

g
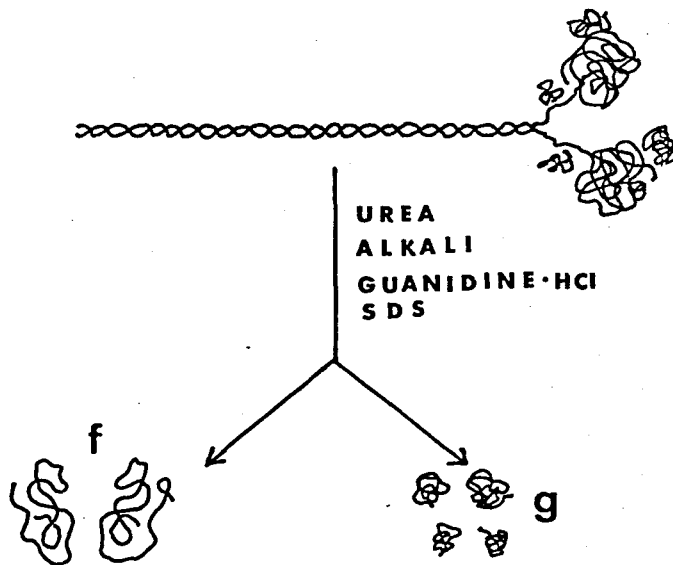
Fig. 6. A schematic structure of subunit composition of myosin molecule. One myosin molecule dissociates into two f-chains and four g-chains.

f-Chains are likely to bear the active site for the ATP hydrolysis, because the chemical modification of f-chains reduces or loses the ATPase activity. On the other hand, the molecular weights and the content of g-chains in myosin had not been established before 1973 (14). It is widely accepted at present that there are three kinds of g-chains with molecular weights of $2.5-2.7 \times 10^4$, $1.74-1.8 \times 10^4$, and $1.4-1.6 \times 10^4$ daltons, respectively. They are called g_1, g_2, and g_3 in the order of their molecular weight in this thesis.

In Part I, I describe the changes in the substructure and functions of myosin after treatment with p-chloromercuribenzoate (CMB) (15). I examined the liberation of g_2 from myosin and

heavy meromyosin, a tryptic digest of myosin (cf. Fig. 4) upon treatment with CMB, and deduced a difference in g_2-binding sites of the myosin molecule.

Use of various ATP analogs on the myosin ATPase reaction is another way to study the reaction mechanism. The naturally occurring nucleoside triphosphates (NTPs) were first examined by Blum (16) and Hasselbach (17) for myosin and actomyosin. Later, Ikehara, Tonomura and their coworkers (18-20) synthesized various kinds of NTP and stressed the importance of the submolecular structure of ATP in the reaction of myosin ATPase. Thereafter, many NTPs are synthesized and their reaction with myosin are studied. On the other hand, the stereochemical conformation of various NTPs are well-defined (21-24). There are two kinds of conformation in both of the natural and the synthetic NTPs concerning the mutual position of the ribose and the base ring, since the glycosyl bond between them is assumed to rotatable. ATP prefers an anti-conformation in solution as well as in crystal (25), that is, the pyrimidine portion of adenine base is placed outside the ribose plane. However, there is little information on the rotation about the glycosyl bond except for a few reports on ATP (26,27). Furthermore, there is no report about the role of the rotation of glycosyl bond of NTPs in their function as the substrate of myosin NTPase.

I synthesized various NTPs of which glycosyl bond is rotatable or not, as judged from CPK-space filling model, and examined initial Pi-burst, actin-activation of myosin NTPase, superprecipitation of actomyosin and myofibrillar contraction using those NTPs. These studies are described in Part II.

REFERENCES

1. Engelhardt,W.A. & Ljubimowa,M.N. (1939) Nature 144 668.

2. Szent-Gyorgyi,A. (1947) "Chemistry of Muscle Contraction," 1st ed. (Academic Press,Inc., New York).

3. Huxley,A.F. & Niedergerke,R. (1954) Nature 173 971.

4. Huxley,H.E. & Hanson,J. (1954) Nature 173 973.

5. Huxley,H.E. (1960) "The Cell," eds. Brachet,J. & Mirsky, A.E. (Academic Press, Inc., New York) vol.4 p.365.

6. Yagi,K. (1972) Adv.Biophys. 8 1.

7. Tonomura,Y. (1972) "Muscle Proteins, Muscle Contraction and Cation Transport," (Univ.Tokyo Press,Tokyo & Univ.Park Press,Baltimore).

8. Inoue,A., Takenaka,H., Arata,T. & Tonomura,Y. (1978) Adv. Biophys. in press.

9. Taylor,E.W. (1973) "Current Topics in Bioenergetics," eds. Sanadi,D.R. & Packer,L. (Academic Press,Inc., New York) vol.5 p.201.

10. Trentham,D.R., Eccleston,J.F. & Bagshaw,C.R. (1976) Q.Rev. Biophys. 9 217.

11. Weeds,A.G. & Hartley,B.S. (1968) Biochem.J. 107 531.

12. Ohe,M., Seon,B.-K., Titani,K. & Tonomura,Y. (1970) J.Biochem. 67 513.

13. Tsao,T.-C. (1953) Biochim.Biophys.Acta 11 368.

14. Sarkar,S. (1973) Cold Spring Harbor Symp. Quant. Biol. 37 14.

15. Hayashi,Y., Takenaka,H. & Tonomura,Y. (1973) J.Biochem. 74 1031.

16. Blum,J.J. (1955) Arch.Biochem.Biophys. 55 486.

17. Hasselbach,W. (1956) Biochim.Biophys.Acta 20 355.

18. Ikehara,M., Ohtsuka,E., Kitagawa,S., Yagi,K. & Tonomura,Y.
     (1964) J.Amer.Chem.Soc. 83 2679.

19. Ikehara,M., Ohtsuka,E., Kitagawa,S. & Tonomura,Y. (1964)
     Biochim.Biophys.Acta 82 74.

20. Tonomura,Y., Imamura,K., Ikehara,M., Uno,H. & Harada,F.
     (1967) J.Biochem. 61 460.

21. Sundaralingam,M. (1969) Biopolymers 7 821.

22. Sundaralingam,M. (1973) "The Jerusalem Symposia on Quantum
     Chemistry and Biochemistry," eds.Bergmann,E.D. & Pullman,B.
     (Academic Press,Inc., New York) vol.5 p.417.

23. Uesugi,S. & Ikehara,M. (1977) J.Amer.Chem.Soc. 99 3250.

24. Ikehara,M., Uesugi,S. & Yoshida,K. (1972) Biochemistry
     11 830.

25. Kennard,O., Isaacs,N.W., Motherwell,W.D.S., Coppola,J.C.,
     Wampler,D.L., Larson,A.C. & Watson,D.G. (1971) Proc.Roy.
     Soc.London A325 401.

26. Millner,Jr.,O.E. & Andersen,J.A. (1975) Biopolymers 14
     2159.

27. Tran-Dinh,S. & Chachaty,C. (1977) Biochim.Biophys.Acta
     500 405.

ABBREVIATIONS (Alphabetical)

ATPase	Adenosine triphosphatase
βME	β-Mercaptoethanol
CD	Circular Dichroism
CMB	p-Chloromercuribenzoate
DEAE	Diethylaminoethyl
DMF	Dimethylformamide
DTNB	5,5'-Dithiobis(2-nitrobenzoate)
DTT	Dithiothreitol
EDTA	N,N,N'N'-Ethylenediamine tetraacetate
EGTA	Ethleneglycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetate
FA-RP	F-actin with relaxing proteins.
HMM	Heavy meromyosin
NDP	Nucleoside diphosphate
NEM	N-Ethylmaleimide
NMR	Nuclear magnetic resonance
NTP	Nucleoside triphosphate
NTPase	Nucleoside triphosphatase
PMR	Proton magnetic resonance
S-1	Subfragment-1 of myosin
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel-electrophoresis
TEAB	Triethylammonium bicarbonate
TPS	2,4,6-Triisopropylbenzene-sulfonyl
Ts	p-Toluene-sulfonyl

PART   I

Substructure of the myosin molecule :

Removal of one g_2-subunit from the myosin

molecule by p-chloromercuribenzoate-treatment.

Since Tsao (1) found that myosin could be dissociated in a concentrated urea solution into two fractions, 92% was polypeptides with the molecular weight of 1.65×10^5 daltons (f-chains) and 8% was polypeptides of 1.6×10^4 daltons (g-chains). The molecular weight of a f-chain was later revised to be about 2×10^5 daltons by Kielley and Harrington (2). Kominz et al. (3) and Wetlaufer and Edsall (4) confirmed that g-chain was released from the myosin molecule in a urea solution. Gershman et al. (5) and Gaetjens et al. (6) found that g-chains were heterogeneous in their mobilities in the gel electrophoresis and amino acid composition, respectively. But their molecular weights had not been accurately measured until the sodium dodecylsulfate-polyacrylamide-gel electrophoresis (SDS-PAGE) technique had been established (7). It is generally agreed at present that myosin from rabbit skeletal white muscle contains three kinds of g-chains with molecular weights of 2.5×10^4, 1.8×10^4, and 1.4×10^4 daltons, in addition to f-chains (8-14). I will call these g-chains g_1, g_2, and g_3, respectively, in decreasing order of molecular weight (12). Hayashi (10) reported the changes of these g-chains during tryptic digestion of myosin and heavy meromyosin (HMM) by SDS-PAGE, and concluded that subfragment-1 (S-1) is composed of g_1'', g_2' (derivatives of g_1 and g_2, respectively), and g_3 itself, besides two kinds of derivatives of f-chains with molecular weights of 5.2-5.5×10^4 and 2.7×10^4 daltons, respectively. Weeds and Lowey (9) reported that the myosin molecule contains one g_1, two g_2, and one g_3, and that g_2 could be removed by treatment with 5,5'-dithiobis(2-nitrobenzoate)(DTNB), without loss of ATPase activity in the steady state. However, it was uncertain which g-chain was essential for ATPase

activity, and the function of the g_2 was also unclear, although some researchers (15-17) had suggested that the g-chains were essential for the expression of ATPase activity.

The ATPase activity of actomyosin is inhibited by high concentrations of ATP (12,18), and this inhibition is markedly affected by Ca^{2+} ions (12,18). On the other hand, Tonomura et al. (18-20) showed that the ATPase activities of actomyosin reconstituted from F-actin and myosin treated with p-chloromercuribenzoate (CMB) and then with β-mercaptoethanol (βME), were scarcely affected by excess substrate or Ca^{2+} ions. Essentially the same results were observed with acto-HMM, even in the presence of regulatory proteins (21). Furthermore, the regulation of the superprecipitation of actomyosin by Ca^{2+} ions disappeared on treatment of the myosin with CMB (20). However, such treatments did not affect the properties of the ATPase of myosin, such as its pH-dependence and activation by Ca^{2+}, EDTA, or CMB (22).

In this Part, I describe about the investigation of Hayashi, I and Tonomura (23) on the changes in the substructure and function of myosin caused by treatment with CMB and the dithiothreitol (DTT) to remove CMB. It was shown that acto-HMM reconstituted from F-actin and HMM prepared from CMB-DTT-treated myosin was resistant to the dissociation induced by ATP, while untreated acto-HMM was almost completely dissociated. About one of the two g_2-chains or its derivative was removed from myosin and HMM, respectively, by treatment with CMB, while the other could not be removed. However, the amino acid compositions of the two g_2 were essentially the same. On CMB-treatment of the myosin, from which S-1 was prepared,

essentially the same suppression of substrate-inhibition of ATPase activity and of dissociation of acto-S-1 by ATP was observed as that seen with actomyosin and acto-HMM. However, the content of g_2' was the same in both CMB-treated S-1 and control S-1. Accordingly, the changes in properties of the actomyosin-type of ATPase and in the dissociation of actomyosin are not attributable solely to the removal of one g_2 from myosin.

EXPERIMENTALS

Materials.------ Myosin was prepared from rabbit skeletal white muscle by the method of Perry (24), with slight modifications (10). Unless otherwise stated, it was further purified by phosphocellulose (Serva Co.) column chromatography (25). HMM and S-1 were prepared by tryptic digestion of myosin and HMM according to the methods of Szent-Györgyi (26) and Hayashi (10), respectively, with slight modifications. G-actin with relaxing proteins was prepared from an acetone powder (27) by the method of Mommaerts (28), with a slight modification. Actin was treated with Dowex 1x4 to remove nucleotides before use.

CMB was purified by precipitating it twice in 1N HCl and dissolving the resultant precipitate in dilute NaOH (29). DTT and βME were purchased from Pierce Chemical Co. (USA) and from Nakarai Chemical Co. (Kyoto), respectively. γ-[^{32}P]-ATP was synthesized enzymatically by the method of Glynn and Chappel (30).

Methods.------ Treatment with CMB; Myosin was treated with CMB essentially as reported by Kitagawa et al. (22), although DTT was used in place of βME to remove the CMB. Myosin (about 15 mg/ml) was incubated with 4.2 moles of CMB per 1.0×10^5 g of protein in 0.5M KCl, 0.05-0.1M Tris-maleate and 5mM EDTA at pH 7.0 and 20°

for 20 min. A fraction soluble at low ionic strength was isolated as g-chains released from myosin by the precipitation of the main core of myosin with 10-15 vol of cold water containing 1mM EDTA at a pH slightly below 7. The fraction of g-chains, which was termed "CMB-sensitive g_2", was exhaustively dialyzed against water and lyophilized. The precipitate of myosin cores was dissolved in approximately 0.5M KCl, 0.1M Tris-HCl, and 5mM EDTA at pH 7.6, and then incubated with a 100-fold molar exess of DTT over CMB at 0° for 1 to 2hr unless otherwise stated. It was next dialyzed against the desired solvent containing 0.5M KCl. The myosin thus obtained is referred to here as "CMB-DTT-treated myosin", whereas that treated as described above but without exposure to CMB is referred to as "control myosin." Unless otherwise stated, "CMB-DTT-treated HMM" and "control HMM" were prepared by the tryptic digestion of CMB-DTT-treated and control myosin, respectively, and "CMB-DTT-treated S-1" and "control S-1" were prepared by the tryptic digestion of CMB-DTT-treated and control HMM, respectively.

Isolation of the various g-chains; CMB-sensitive g_2 was isolated as described above. All g-chains except CMB-sensitive g_2 were separated from CMB-DTT-treated myosin by treatment with 4M urea, following the method of Weeds and Lowey (9). After reduction and carboxymethylation in the presence of 5M guanidine-HCl, the g-chains were subjected to electrophoresis on SDS-gels containing 12.5% acrylamide to separate into g_1, g_2, and g_3 following the method of Wada and Snell (31). The gel was stained with 0.1% Coomassie brilliant blue in 10% trichloroacetic acid and 30% methanol, and the band corresponding to each g-chain was sliced off and smashed into fine pieces. Protein was extracted from the pieces

with 70% formic acid at -10° for 18hr. The gel was removed by passing the extracts through a glass-filter, the formic acid evaporated off _in vacuo_, and the dye extracted with 100% methanol. The preparation of g_2 thus obtained was that referred to as "CMB-resistant g_2." The g-chains, which were subjected to amino acid analysis, were estimated to be approximately 90% pure by electrophoresis.

Estimation of subunit composition by SDS-PAGE; Unless otherwise stated, SDS-PAGE was performed by the method of Weber and Osborn (8), with slight modifications (10). Electrophoretograms were scanned with a densitometer from Fuji Riken Co. (Tokyo) or a Guilford, Model 240 spectrophotometer (USA) at a wavelength of 560 nm. As shown in Fig. 1, the relative area ratios of $g_1 : g_2 : g_3$ estimated from the densitometer tracings were constant when more than about 40µg of unpurified myosin were applied to the SDS-gel. Thus, about 50µg of myosin were usually applied to determine the relative area ratio of the g-chains. The molar ratios of the g-chains and constituents of S-1 were calculated by dividing their weight ratios by their molecular weights, assuming that the weight ratio was equivalent to the area ratio.

Amount of bound CMB; Assuming that CMB specifically bound to cysteinyl residues under the conditions used, the residual amount (moles per mole of protein) of myosin, HMM, and S-1 after treatment with DTT were calculated from the following equation, based on the ratio of A_{280nm} to A_{250nm} for CMB-DTT-treated protein $(R_{CMB-DTT})$ and control protein (R_{CON}) in 0.05M sodium phosphate buffer, pH 7.0 (Fig. 2).

$$R_{CMB-DTT} = (\varepsilon_M + 644x) / (\varepsilon_M \times \frac{1}{R_{CON}} + 12270x)$$

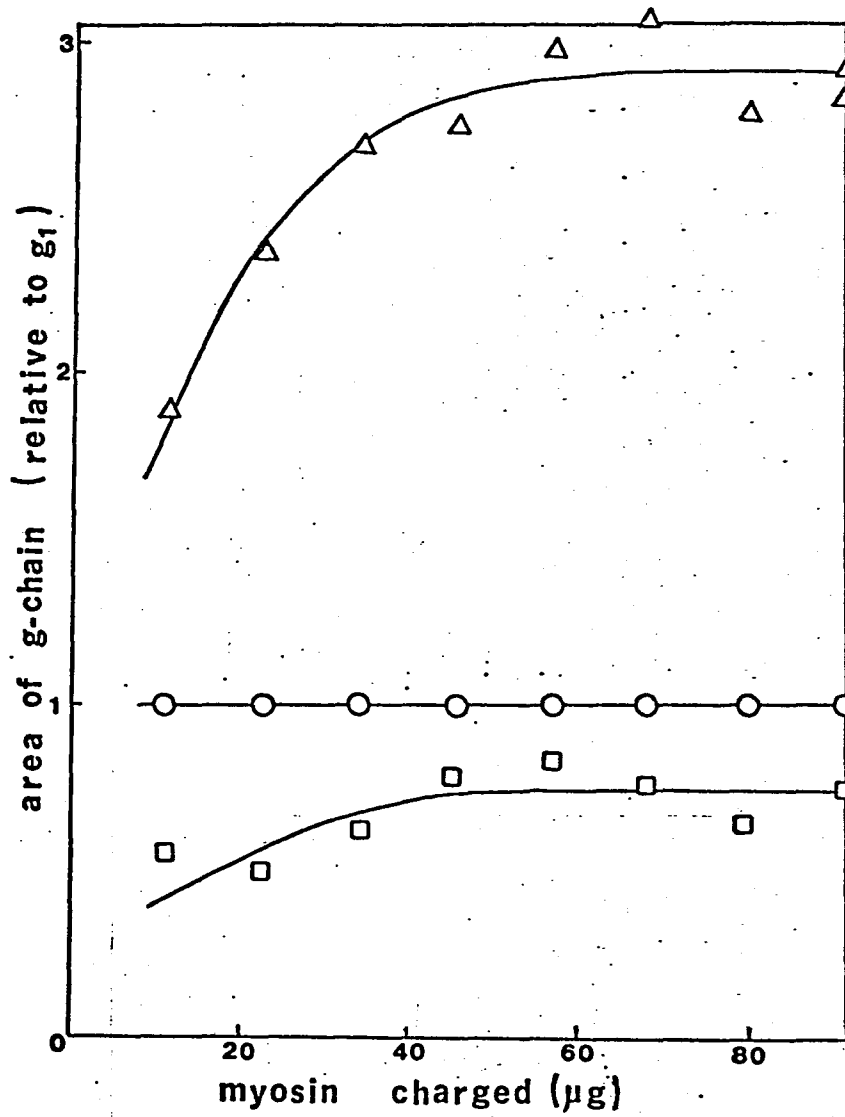Fig. 1. Dependence of stained area of g-chains on the amount of myosin charged. Area under peak for g_2 ($\triangle$) and g_3 ($\square$) relative to that of g_1 ($\bigcirc$) were plotted against the amount of myosin charged.
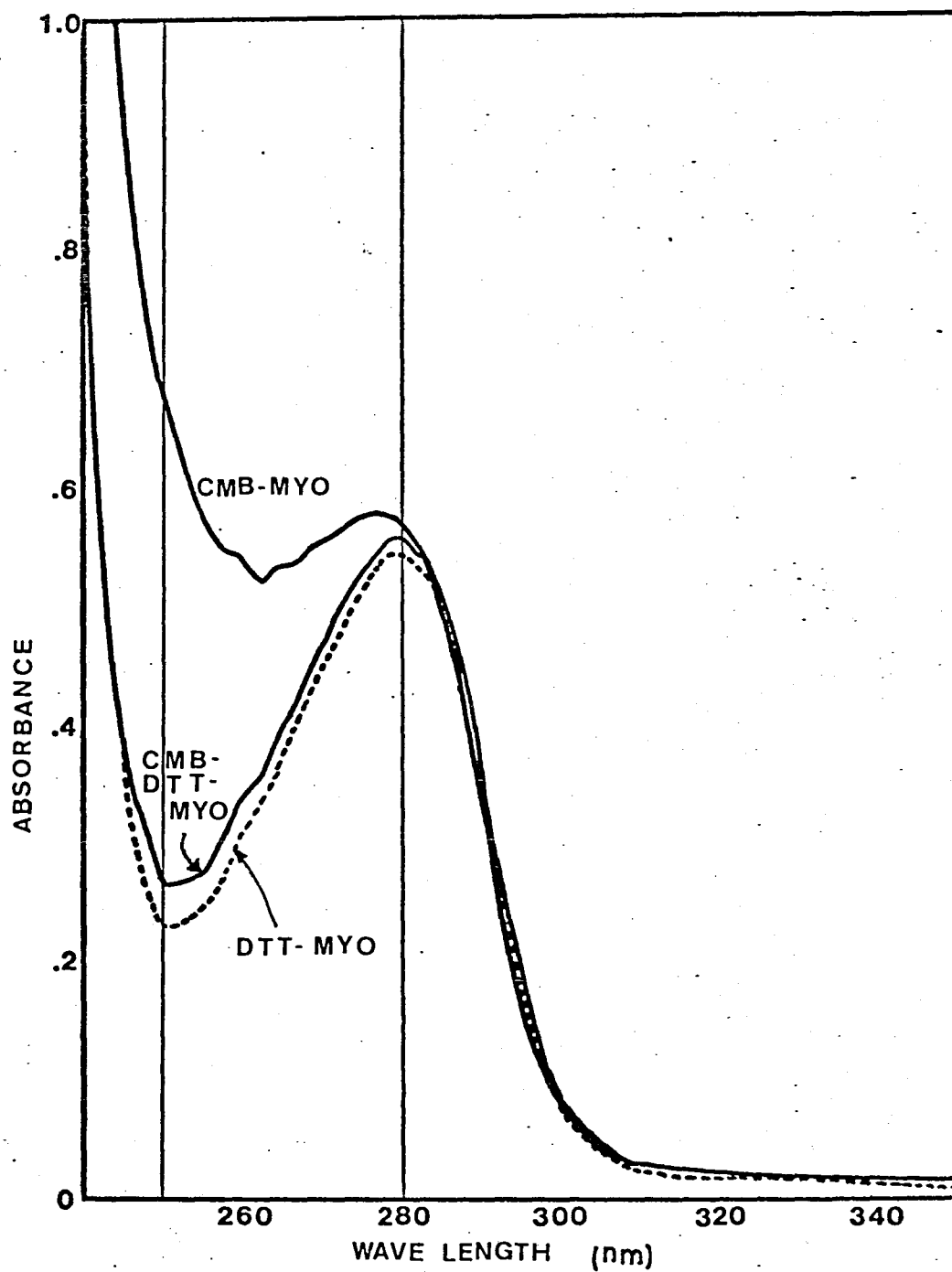
Fig. 2.   Absorption spectra of control-, CMB-, and CMB-DTT-
treated myosin. Amount of bound CMB was estimated from the
ratios of A_{280nm} to A_{250nm}, as mentioned in the text.

where x is the residual amount, ε_M is the molar extinction coefficient of the proteins, and 644 and 12270 are the molar extinction coefficients $(M^{-1} \cdot cm^{-1})$ of CMB-cysteine at 280 and 250nm, respectively (29). Values of 2.33×10^5, 2.20×10^5, and 8.47×10^4 were adopted for the ε_M of myosin, HMM, and S-1, respectively. These values were obtained using the extinction coefficients as described below. All such determinations of optical density were corrected for Rayleigh scattering.

Rapid filtration of acto-HMM and acto-S-1; F-actin (0.4 mg/ml) was mixed with 0.71 mg/ml of HMM in 0.05M KCl, 2mM $MgCl_2$, and 0.02M Tris-maleate at pH 7.0 and 22 to 25°. ATP was added to 2ml samples of the protein solution to give final concentrations of 0.05 to 0.4 mM, and the resulting solutions were immediately shaken and filtered under negative pressure on a Millipore filter of 0.3um pore size, which had been pretreated by passing HMM solution (1 mg/ml) through it three times and then washing it with 20ml buffer solution containing no ATP. Volumes of 0.2 to 1ml of the filtrate were obtained in 10 to 30 sec after adding ATP, and their protein concentration were estimated with Folin-Ciocalteu reagent (32). Acto-S-1 was reconstituted from 0.4 mg/ml F-actin and 0.5 mg/ml of S-1 in the presence of 0.1mM $CaCl_2$ or 1mM EGTA. After adding 0.08 to 0.80mM ATP, the acto-S-1 was subjected to filtration on a Millipore filter of 0.45μ pore size which had not been pretreated with protein solution.

Light-scattering; The angular distribution of light-scattering intensity of acto-HMM was measured at angles ranging from 30 to 135° using a Brice-Phoenix photometer (Model 1000D), and plotted by the method of Zimm (33). Measurements were begun at 30°, and finished

at 135°, within 2 min after adding ATP. The intensities of light scattering at fixed angles of 30° or 40° remained constant for at least 2 min after ATP addition.

ATPase activity; The ATPase activity of acto-S-1 was determined in 0.01 mg/ml S-1 with $1\mu M$ ATP and in 0.1 mg/ml S-1 with more than $10\mu M$ ATP and 1.05 mg/ml F-actin, 1mM $MgCl_2$ in excess of ATP, $10\mu M$ $CaCl_2$ and 0.02M Tris-maleate at pH 7.0 and 25°. The KCl concentration of the reaction mixture was adjusted so as to keep the ionic strength due to KCl, $MgCl_2$, and Na_2ATP constant (0.02), assuming that all the ATP formed a $MgATP^{2-}$ complex with Mg^{2+}. The simple, solenoid-driven mixing appratus devised by Kanazawa et al. (34) was used for measurement of the time course of Pi-liberation. At appropriate times after adding γ-[^{32}P]-ATP to the mixture, the reaction was halted by adding 5% trichloroacetic acid and $50\mu M$ Pi as carrier. The ^{32}Pi liberated from the acto-S-1-γ-[^{32}P]-ATP system was separated by the method of Martin and Doty (35). The ATPase activities of myosin and S-1 in the steady state were determined by measuring the amount of Pi liberated by the method of Martin and Doty (35).

Other methods; Amino acid analysis was performed by the method of Spackman et al. (36) in a Beckman 120B amino acid analyzer (USA). The protein concentrations of myosin, HMM and S-1 in neutral KCl solution were determined using values for $A^{1\%}_{280nm}$ of 4.85, 6.47, and 7.70 (37), respectively, obtained by comparison of the A_{280nm} with the protein concentration estimated by the biuret reaction.

RESULTS

Effects of CMB-treatment on the substructure of myosin, HMM, and

S-1 molecules: --- When myosin preparations were subjected to SDS-

PAGE before chromatography on phosphocellulose, the molar ratio of

$g_1:g_2:g_3$ was estimated from densitometry at $1:4.1:1.3$. After

chromatographic purification, the molar ratio changed to $1:2.4:1.1$

(Fig. 3). The purified myosin was treated twice with a 20-fold

molar excess of CMB, and precipitated by dilution with 12 vol of

deionized water. The myosin molecule was collected by centrifuging,

and pellet and supernatant were separately subjected on SDS-PAGE

after incubation with DTT. The supernatant shows a single band

corresponding to g_2. The molar ratio of g-chains in myosin thus

obtained changed to $1:1.1:0.73$. These results are summarized in

Table I. From them, it was concluded that the myosin molecule

contains two g_2, and that only one of them (CMB-sensitive g_2) is

removed by the CMB-treatment, while the other (CMB-resistant g_2)

is not.

As previously reported by Hayashi (10), g_1 and g_2 were de-

graded by tryptic digestion of myosin to g_1' and g_2', whose masses

were estimated at 2.4×10^4 and 1.6×10^4 daltons, respectively.

HMM was treated with a 14-fold molar excess of CMB under the same

conditions as myosin, and then chromatographed on Sephadex G-100

(Fig. 4). The component in the main fraction showed the molar

ratio of $g_1':g_2':g_3$ was $1:1.0:0.9$, while that in the original HMM

was $1:2.1:0.9$ (Fig. 3 and Table I).

As reported by Hayashi (10), S-1 is composed essentially of f'

and f" derived from f-chain, with molecular weights of 5.2-5.5 x

21
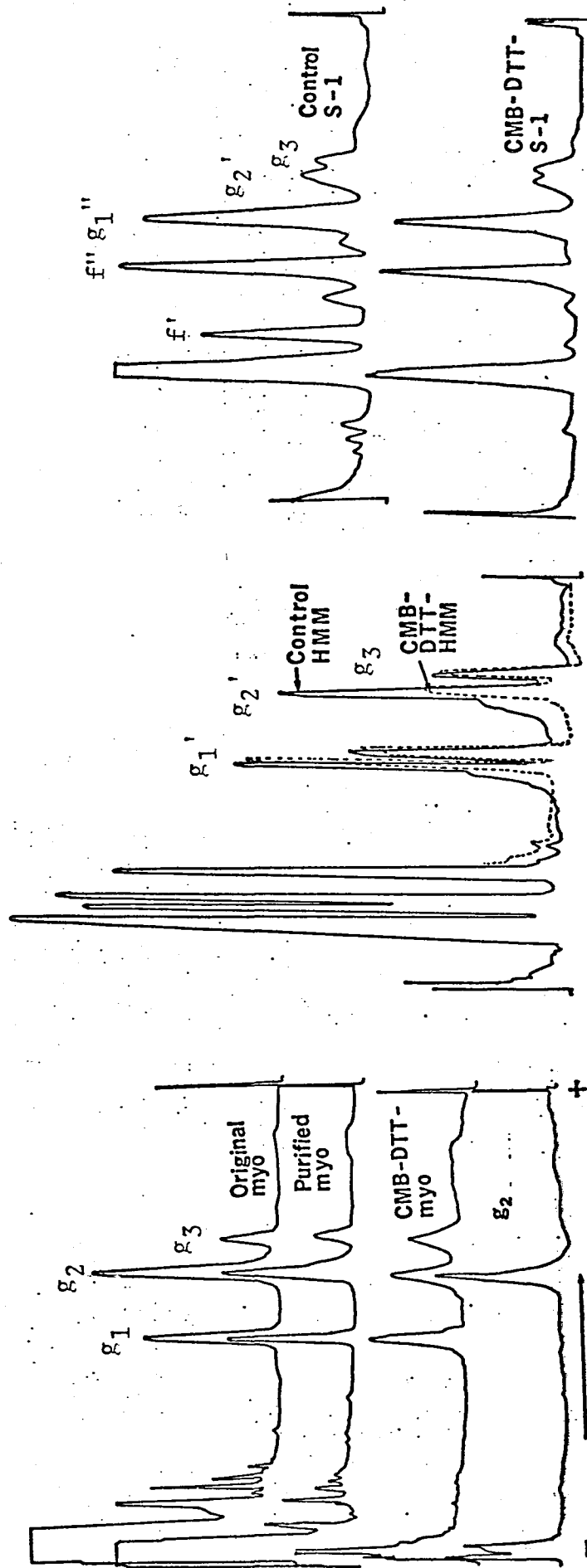
Fig. 3. Densitometer tracings of SDS-PAGE of various myosin and its derivatives. Molar ratios and the content in myosin, HMM, and S-1 were estimated from these electrophoretograms (cf. Fig. 1).

Table I.   Contents of various g-chains in the myosin molecule*.

Proteins	g_1**	g_2**	g_3
Myosin before chromatography	1	4.2	1.3
Myosin after chromatography	1	2.4	1.1
Myosin treated with CMB-DTT	1	1.4	0.83
Myosin treated twice with CMB-DTT	1	1.1	0.73
H-Meromyosin	1	2.1	0.9
H-Meromyosin treated with CMB-DTT	1	1.0	0.9
S-1 purified with actin-binding	2.9	0.87	0.82
Control-treated S-1	2.2	0.81	0.89
CMB-DTT-treated S-1	2.4	0.74	0.76

* Contents are represented in moles/two heads.

** g_1 also represents g_1' for HMM and g_1'' for S-1, and g_2 represents g_2' for both. their molecular weights are not similar as described in the text.
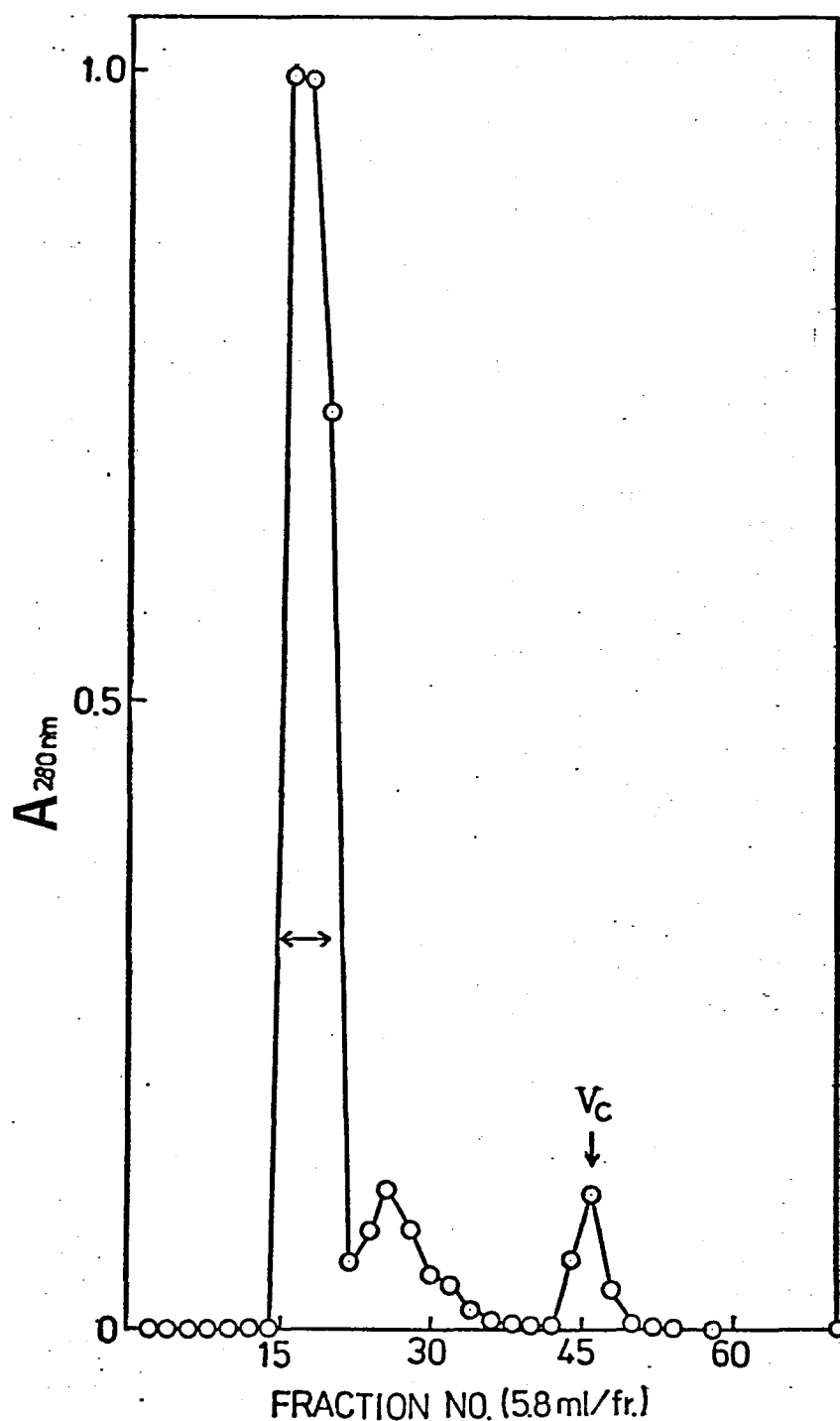
Fig. 4. Sephadex G-100 gel-filtration of HMM treated with CMB. HMM (10mg/ml) was treated with 4 moles of $CMB/10^5$ g of HMM in 50mM KCl, 50mM Tris-maleate, and 5mM EDTA at pH 7.0 and 20° for 20 min. The solution was applied to a column (2.9x41-cm) equilibrated with 50mM KCl, 50mM Tris-HCl, and 0.5mM EDTA at pH 7.6 and 4°. The column was eluted with the same buffer at a flow rate of 23ml/hr, and fractions of 5.8ml were collected. v_c indicates the column volume.

10^4 and 2.7×10^4 daltons, respectively. S-1 also contains g_1'' derived from g_1, g_2' from g_2, and g_3 with molecular weights of 2.1×10^4, 1.6×10^4, and 1.4×10^4 daltons, respectively (Fig. 3 and Table I). As listed in Table I, the molar ratio of these components in CMB-DTT-treated S-1 was essentially the same as that in control S-1. When S-1 was treated with 3.9 moles of CMB under the same conditions used for HMM, and chromatographed on Sephadex G-200, there was no peak other than that of S-1 in the void volume (Fig. 5). Densitometer tracings of SDS-PAGE of S-1 thus obtained showed the molar ratio of $f'':g_2':g_3$ at 2.0:1.0:0.76, which was almost the same as that in control S-1. Therefore, it was concluded that two moles of S-1 contain only one mole of g_2' which is "CMB-resistant g_2."

Amino acid composition of g_1, CMB-sensitive g_2, CMB-resistant g_2, and g_3: --- CMB-sensitive g_2 was isolated after treatment of myosin with CMB as the fraction soluble at low ionic strength, as described above. g_1, CMB-resistant g_2 and g_3 were separated electrophoretically from CMB-DTT-treated myosin which was deficient in CMB-sensitive g_2, as described under "EXPERIMENTALS." As summarized in Table II, the amino acid compositions of the two kinds of g_2 were essentially the same, and similar to those of the two g_2 liberated by DTNB-treatment (9). The compositions of the g_1- and g_3-chains were similar to those of the respective subunits reported by Weeds and Lowey (9).

Effects of CMB-treatment on the Ca^{2+}- and EDTA(K^+)-ATPase activities of myosin and actomyosin;---When myosin was treated with a 20-fold molar excess of CMB, it combined with 16 moles of CMB per 4.8×10^5 g of myosin, and its Ca^{2+}-ATPase activity increased 6.1-
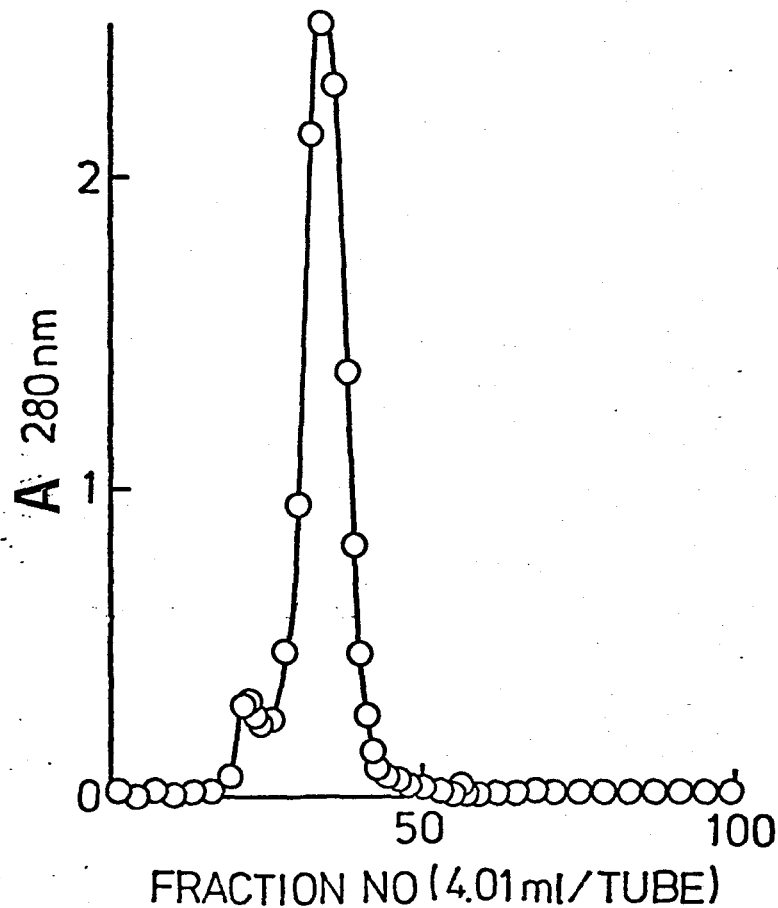
Fig. 5. Sephadex G-200 gel-filtration of S-1 treated with
CMB. S-1 was treated with 3.9 moles of $CMB/1.1 \times 10^5$ g of
S-1, and chromatographed, according to the same method as
described in Fig. 4.

Table II. Amino acid compositions of g-chains of myosin expressed as residues
per 100 residues. Numbers in parentheses are molecular weight $\times 10^{-4}$.

	$g_1 (2.5)$		$g_2 (1.8)$			$g_3 (1.4)$	
	Lowey*	Hayashi	CMB-sensitive	CMB-resistant	DTNB-sensitive	Lowey*	Hayashi
Lys	11.0	9.4	8.9	8.8	9.3	7.6	7.1
His	1.0	0.9	0.7	0.7	0.6	1.2	1.3
Arg	2.2	2.0	3.2	2.8	3.6	2.8	2.4
CMCys	0.5	0.6	0.6	---	1.1	0.6	0.8
Asp	10.3	11.5	14.4	15.3	13.5	12.0	13.1
Thr	4.2	3.8	5.5	5.9	6.0	4.7	4.7
Ser	4.6	3.8	3.2	4.5	3.4	5.4	5.1
Glu	15.3	16.8	16.1	16.2	13.9	15.9	17.4
Pro	6.3	7.3	4.4	4.0	3.6	2.4	2.5
Gly	6.3	6.7	8.1	8.8	7.6	7.9	8.5
Ala	11.9	10.4	9.1	9.2	8.5	8.2	8.1
Val	5.5	5.7	4.3	5.4	5.2	6.7	5.4
Met	3.1	3.3	2.7	2.5	3.7	3.7	3.7
Ile	4.7	4.8	4.7	4.7	5.7	4.6	4.6
Leu	7.2	7.2	5.9	5.7	5.5	8.4	8.0
Tyr	1.6	1.5	1.6	---	1.2	2.0	2.0
Phe	4.2	4.4	6.6	6.2	7.6	5.6	5.2

* These data were reported by Weeds and Lowey. (9).

fold, while its EDTA(K$^+$)-ATPase activity decreased to 14% of the con-
trol value. After treatment with DTT, the residual amount of CMB
was 1.24 moles per 4.8 x 10^5 g (mean of 3 estimations), and the
Ca^{2+}- and EDTA(K$^+$)-ATPase activities became 86 and 89%, respectively,
of the control values. After regeneration by treatment with a 400-
fold molar excess of βME, the residual amount of CMB was only 0.03
mole per 4.8x10^5 g. The S-1 moieties obtained by the tryptic diges-
tion of CMB-DTT-treated myosin contained 0.90 mole of CMB per 2.2x
10^5 g (mean of 2 estimations). The Ca^{2+}- and EDTA(K$^+$)-ATPase activities
of the modified S-1 were 84 and 79%, respectively, of those for
control S-1. These results indicated that g_2 is not essential for
the ATPase activity in the steady state, as suggested by Weeds and
Lowey (9).

Effects of CMB-treatment on the various properties of myosin,
HMM, and S-1 in the presence of F-actin; --- Superprecipitaion of
actomyosin was followed by measuring the change in turbidity at
660nm after adding 0.16mM ATP to 0.4 mg/ml myosin, 0.1 mg/ml FA-RP,
0.1M KCl, 2mM MgCl$_2$ at pH 7.0 and 20° in the presence of 1mM EGTA
or CaCl$_2$. As shown in Fig. 6, superprecipitation of control acto-
myosin was regulated by Ca^{2+}, while that of CMB-DTT-treated acto-
myosin occurred immediately with or without Ca^{2+} ions. Furthermore,
a clearing response was found only with control actomyosin in the
presence of 1mM EGTA.

Dissociation of acto-HMM after adding ATP was measured by rapid
filtration with a Millipore filter or by a light-scattering method.
Acto-HMM reconstituted from 0.71 mg/ml HMM and 0.4 mg/ml FA-RP was
mixed with or without 0.4mM ATP in 50mM KCl, 2mM MgCl$_2$ at pH 7.0
and 22-25°, and was subjected on a Mollipore filter of 0.3μm under
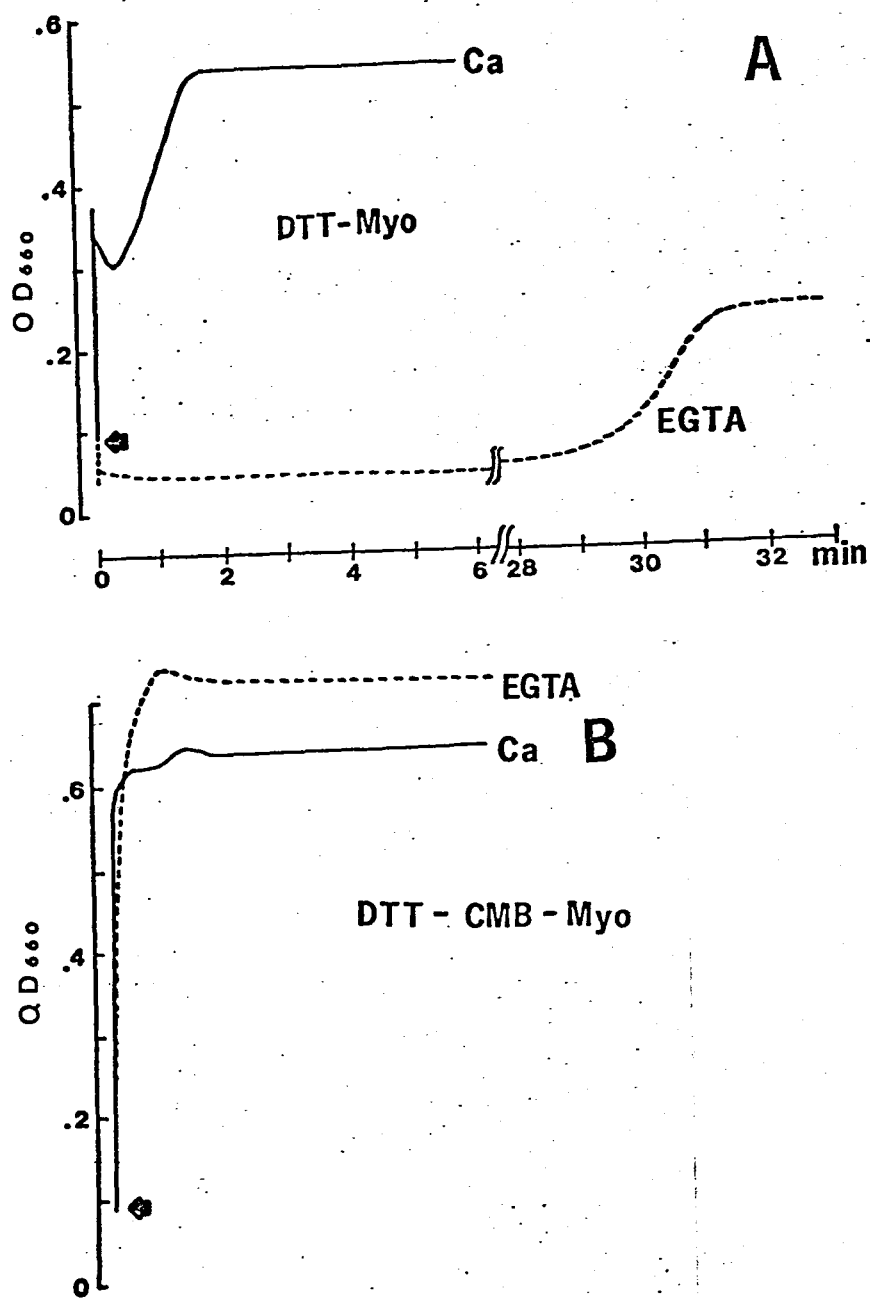
Fig. 6.   Superprecipitation of actomyosin reconstituted from FA-RP and control or CMB-DTT-myosin in the presence or absence of Ca^{2+} ions.   Arrows indicate starting points.   Conditions: 0.4 mg/ml of control (A) or CMB-DTT-treated (B) myosin, 0.1 mg/ml FA-RP, 0.16mM ATP, 2mM $MgCl_2$, 0.1M KCl, and 20mM Tris-maleate at pH 7.0 and 20°. ———— , 0.1mM Ca^{2+}; ——— , 1mM EGTA.

reduced pressure (Fig. 7). Without adding ATP, the concentrations of protein in the filtrate were 9 and 17% of that of HMM applied for control and CMB-DTT-treated acto-HMM, respectively. Addition of ATP to the control acto-HMM increased the amount of protein in the filtrate to almost 100% of that applied. However, in the case of CMB-DTT-treated acto-HMM, the concentration in the filtrate was only 30% of that applied. HMM of the control and CMB-DTT-treated acto-HMM could scarcely pass through the filter on incubation for 40 to 50 min after adding ATP. The protein in solutions containing FA-RP alone scarcely passed through the filter, while almost all the protein in solutions containing control or CMB-DTT-treated HMM alone could pass through. Thus, it seemed that in the presence of excess ATP, 70% of the HMM of CMB-DTT-treated acto-HMM remained bound to FA-RP, while the HMM of control acto-HMM was almost completely dissociated.

The angular distribution of light-scattering intensity of acto-HMM reconstituted from 0.14 mg/ml FA-RP and 0.25 mg/ml HMM was measured in 0.04M KCl, 2mM $MgCl_2$, 0.08mM $CaCl_2$ at pH 7.0 and room temperature. As shown in Fig. 8, the weight-average molecular weight of control-treated acto-HMM (proportional to $R_{\theta\to0}/K_c$) decreased to one fifth of the initial value on adding 0.16mM ATP. On the other hand, the molecular weight of CMB-DTT-treated acto-HMM decreased to one half of the initial value on adding ATP. These results also indicate that the dissociation of acto-HMM by ATP was suppressed significantly by the CMB-DTT-treatment.

Binding shape of acto-HMM after adding 0.6mM ATP was observed by a negatively stained electron micrographs. Acto-HMM was recon-
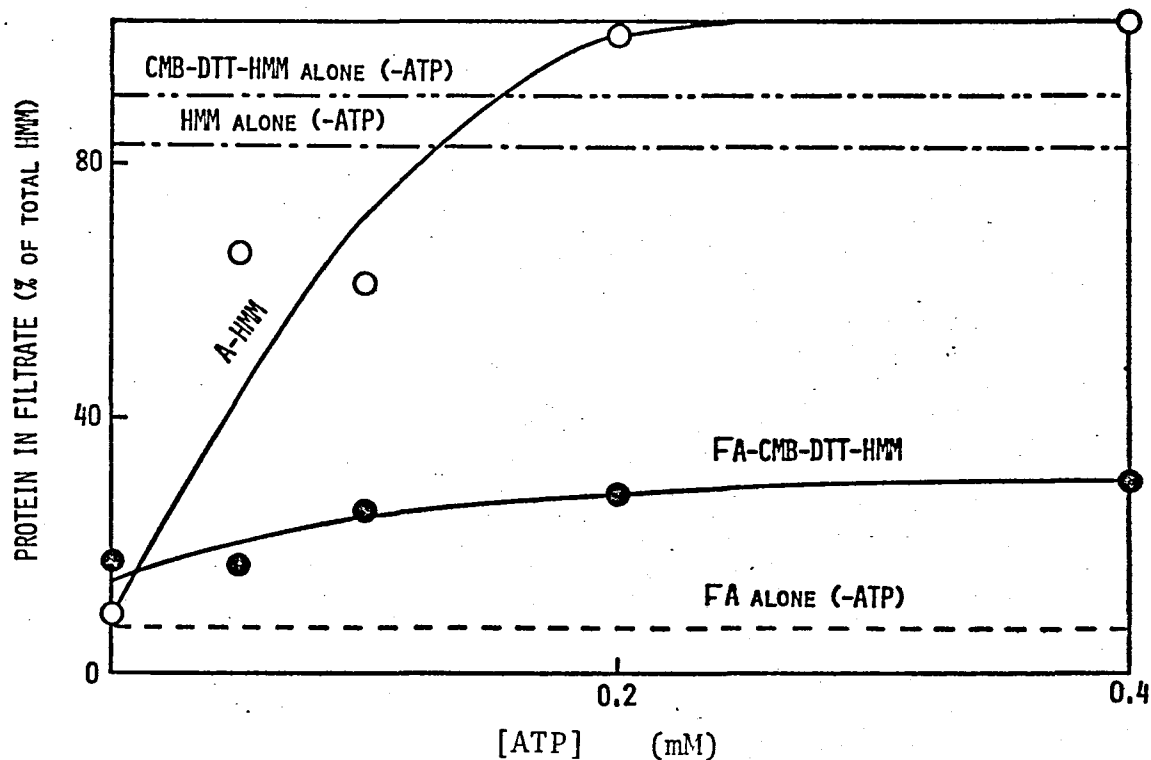
Fig. 7. Rapid filtration of acto-HMM through a Millipore filter (0.3um) after adding ATP. Acto-HMM was reconstituted from 0.4 mg/ml of FA-RP and 0.71 mg/ml of control (O) or CMB-DTT-treated (●) HMM in 2mM $MgCl_2$, 50mM KCl, and 20mM Tris-maleate at pH 7.0 and 22-25°. Various concentrations of ATP were added to the acto-HMM, and the mixture was subjected to filtration. The protein concetrations in the filtrate were plotted against the ATP concentration relative to those of HMM applied. Protein in filtrate of: ---,FA-RP alone without ATP; O , control HMM and ● , CMB-DTT-treated HMM.
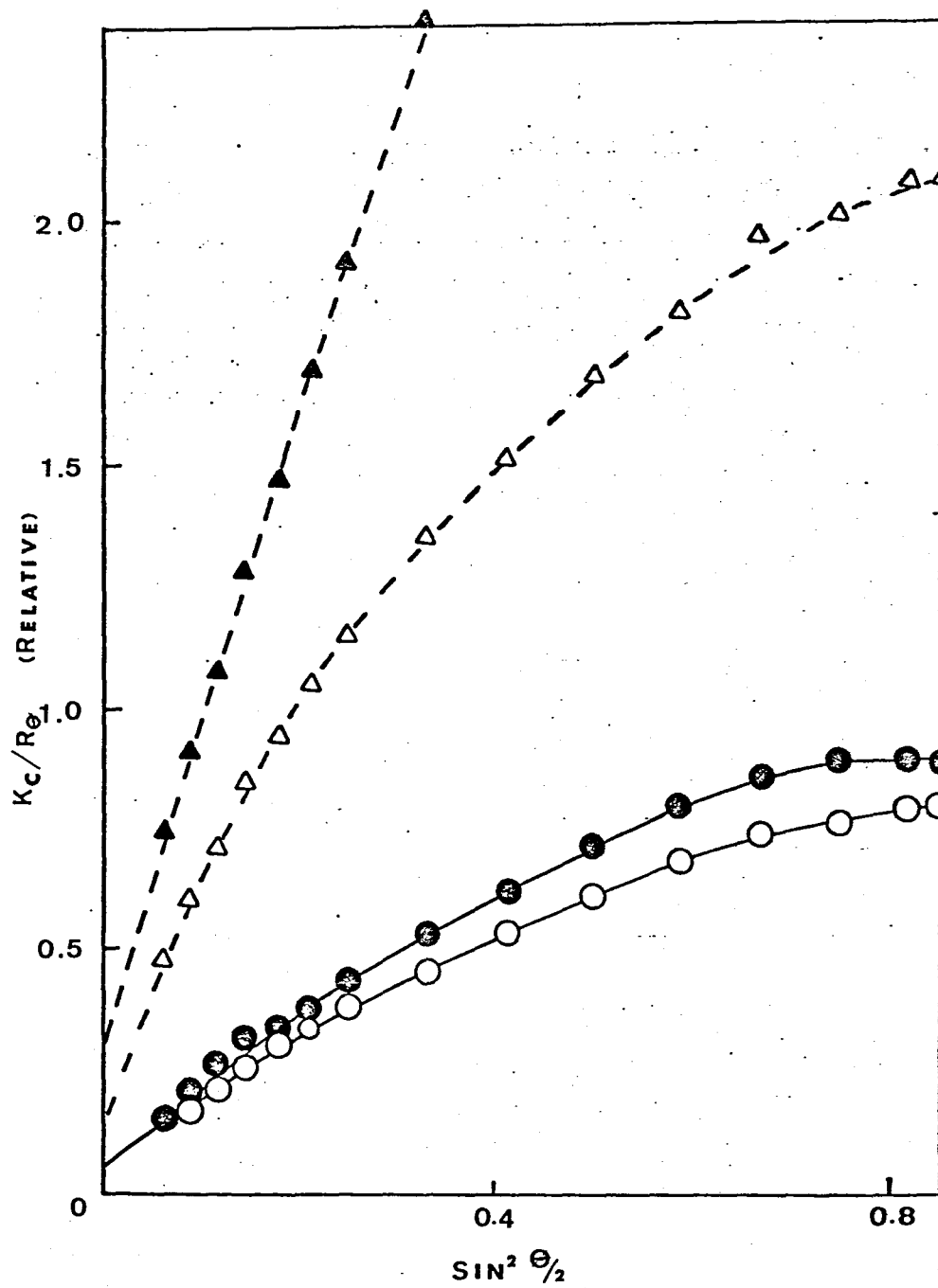
Fig. 8. Changes in angular distribution of light-scattering intensities of acto-HMM on adding ATP. Acto-HMM was reconstituted from 0.25 mg/ml of control (●,▲) or CMB-DTT-treated (O,△) HMM and 0.14 mg/ml FA-RP in the same buffer as for Fig. 7 with 80μM $CaCl_2$ at room temperature. Measurements were begun at an angle of 30° to the incident light and finished at an angle of 135°, within 2 min after adding ATP or water. O,● , no ATP; △,▲ , 0.6mM ATP.

stituted from 0.035 mg/ml FA-RP and 0.065 mg/ml HMM in almost the same solvent as that described above at 20°. An electron micrograph of negatively stained CMB-DTT-treated acto-HMM in the absence of ATP was almost the same as that of control acto-HMM, showing an arrow-head structure. When 0.6mM ATP was added to the control acto-HMM, the HMM dissociated almost completely, and consequently fila-ments of F-actin with no HMM were observed. On the other hand, ATP changed the arrow-head structure of CMB-DTT-treated acto-HMM to a new structure, in which a considerable amount of HMM remained bound to F-actin, and formed randomly oriented projections. These shapes are schematically represented in Fig. 9.

The dependence on ATP concentration of the steady state ATPase activity of acto-S-1 reconstituted from 0.01 or 0.1 mg/ml S-1 and 1.05 mg/ml FA-RP was measured in 1mM $MgCl_2$ with an excess of ATP and 10mM $CaCl_2$ at pH 7.0 and 25°, at constant ionic strength of 0.02 (derived from KCl + $MgATP^{2-}$). As shown in Fig.10, the ATPase activity of CMB-DTT-treated acto-S-1 increased steadily with in-crease in ATP concentration, and reached a saturation value of 37 moles $Pi/min \cdot 10^5 g$ of S-1 at 1mM ATP. The activity of control acto-S-1 was lower than that of CMB-DTT-treated acto-S-1 at con-centrations of ATP above $10^{-5}M$, and the ratio of the activity of the control acto-S-1 to that of the CMB-DTT-treated acto-S-1 de-creased with increase in the concentration of ATP.

Dissociation of acto-S-1 was also measured by the Millipore filtration method described above. Acto-S-1 was reconstituted from 0.4 mg/ml FA-RP and 0.5 mg/ml S-1 in 50mM KCl, and 2mM $MgCl_2$ at pH 7.0. It was mixed with ATP, and subjected to filtration. Fig. 11 shows the dependence on ATP concentration of the S-1 con-
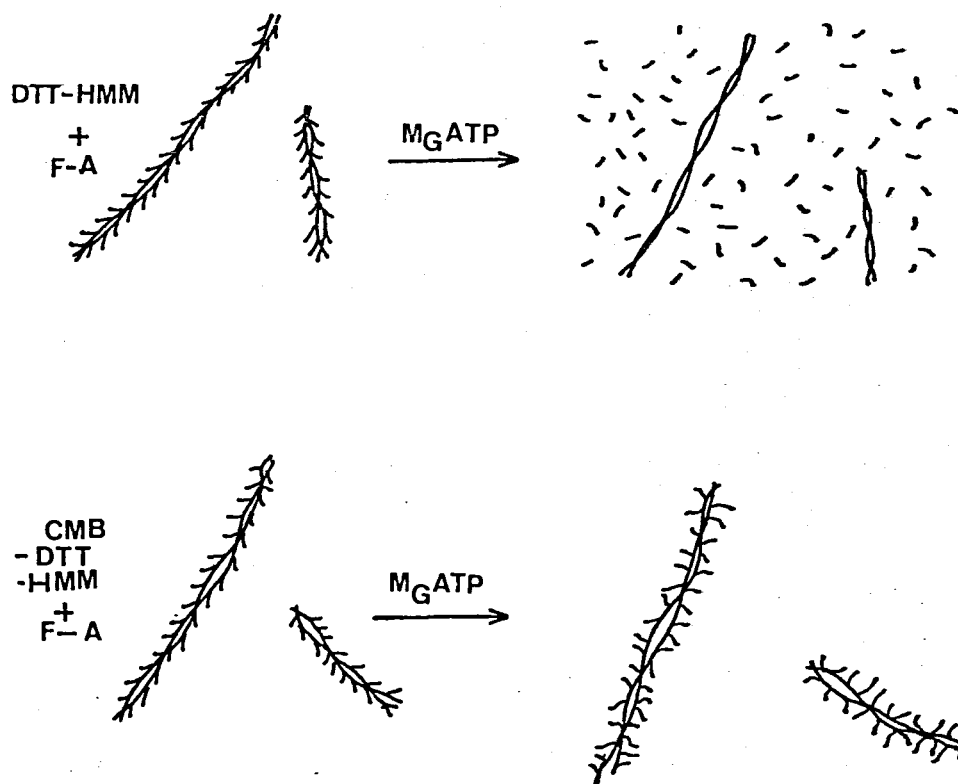
Fig. 9.   Schematic representation of acto-HMM and CMB-DTT-treated acto-HMM in the presence and absence of ATP.   The waved lines represent FA-RP and short lines HMM.
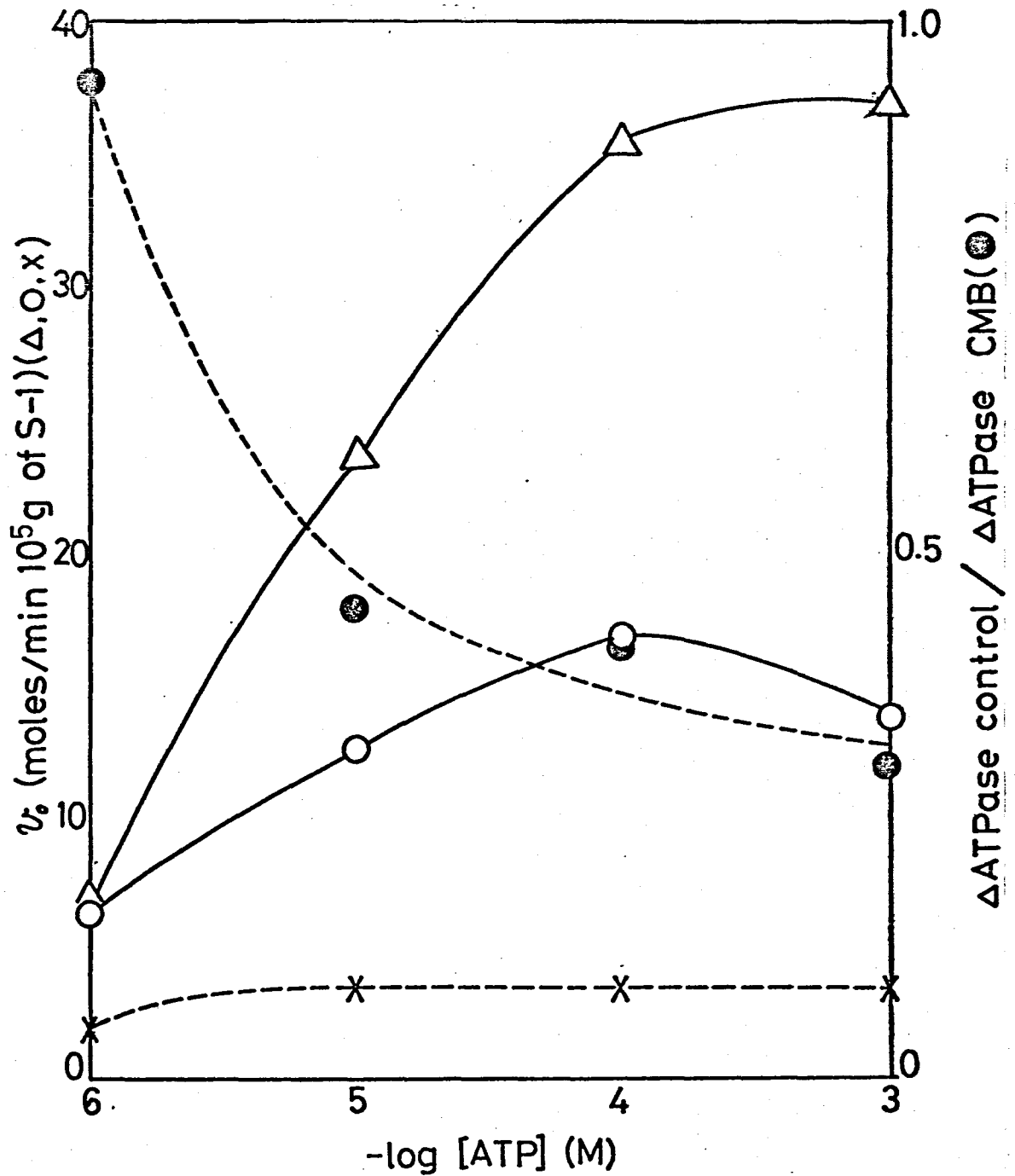
Fig. 10. Dependence on ATP concentration of the ATPase activities of control and CMB-DTT-treated acto-S-1. Acto-S-1 was reconstituted from 0.01 (measurement at 10^{-6}M ATP) or 0.1 mg/ml of control (O) or CMB-DTT-treated (△) S-1. ATPase activities in the steady state were measured with 1mM $MgCl_2$ in excess of ATP, 10 µM $CaCl_2$, and 0.02M Tris-maleate at pH7.0, at an ionic strength of 0.02 (derived from KCl+MgATP^{2-}) and 25°. X, ATPase activity of control S-1 alone; ●, ratio of actin-dependent ATPase of control acto-S-1 (total activity of acto-S-1 minus the activity of acto-S-1 alone) to that of CMB-DTT-treated acto-S-1.
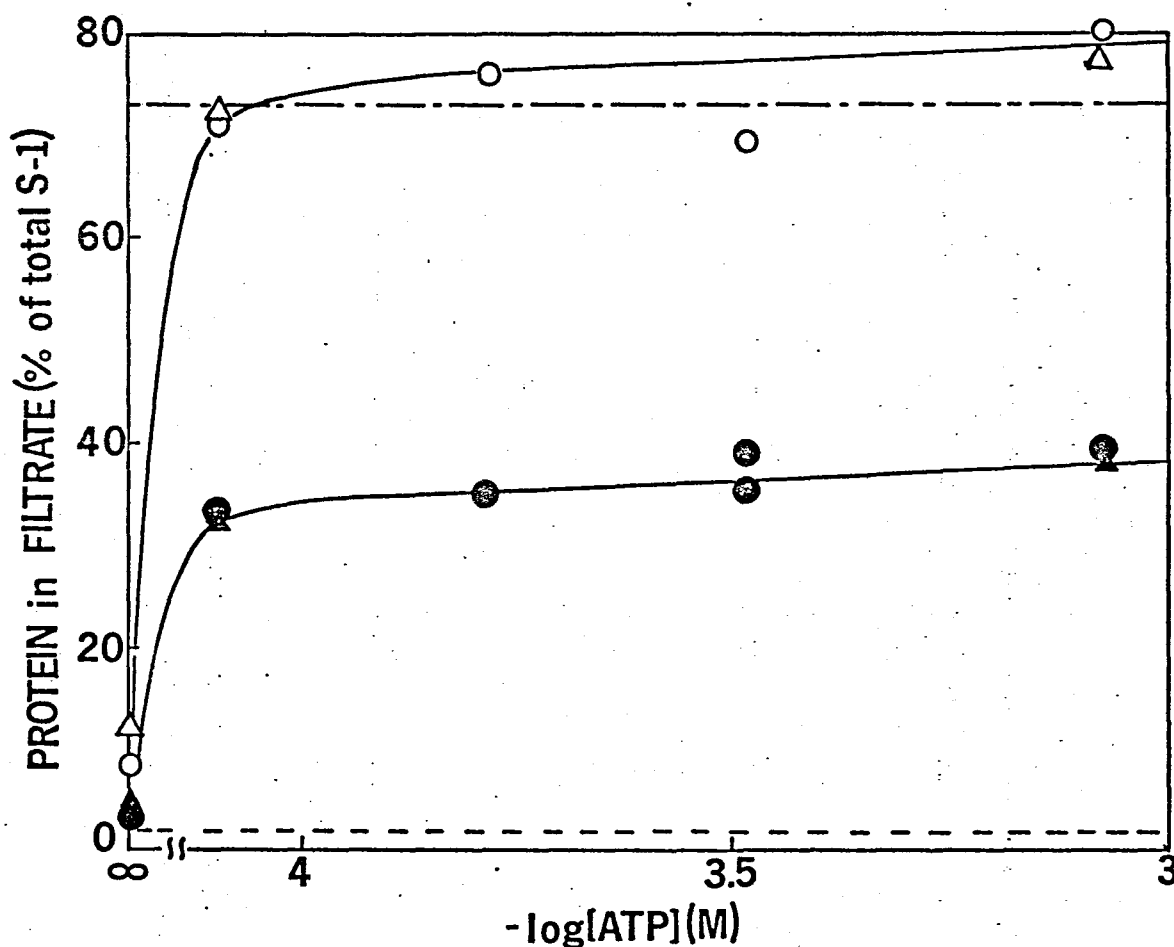
Fig. 11. Rapid filtration of acto-S-1 through a Millipore filter after adding ATP. Acto-S-1 was reconstituted from 0.4 mg/ml of F-actin and 0.50 mg/ml of control (O,Δ) or CMB-DTT-treated ($\bullet,\blacktriangle$) S-1 in the presence of 0.1mM $CaCl_2$ or 1mM EGTA in the same solvent as for Fig. 7 at 18-20°. The acto-S-1 samples were filtered through a Millipore filter (0.45μm) within 5sec after adding various concentrations of ATP. The concentration of protein in filtrate relative to the concentration of S-1 applied were plotted against the concentrations of ATP added. Control acto-S-1: O,0.1mM $CaCl_2$; Δ,1mM EGTA. CMB-DTT-treated acto-S-1: $\bullet$,0.1mM $CaCl_2$; $\blacktriangle$, 1mM EGTA. Protein in filtrate of:---, F-actin alone;—·—, S-1 alone in the presence or absence of ATP.

centration in the filtrates of both types of acto-S-1. Most of
the control S-1 passed through the filter, while only about half
of the CMB-DTT-treated S-1 passed through on adding ATP. The ab-
sence of Ca^{2+} ions did not affect the results. These findings
indicate that the dissociation of acto-S-1 by ATP induced inhi-
bition of ATPase activity, and that the dissociation was suppressed
by CMB-DTT-treatment of the myosin from which the S-1 was prepared.

DISCUSSION

The relative molar ratio of $g_1:g_2:g_3$ in myosin, estimated from the
area of densitometer tracings of electrophoretograms, was 1:2.4:1.1
The amount of g-chains was estimated to be about 12% by gel-fil-
tration in 8M urea or 5M guanidine-HCl (10). These results are
consistent with the conclusions of Weeds and Lowey (9) and Lowey
and Risby (38) that the myosin molecule of rabbit skeletal white
muscle contains one g_1, two g_2, and one g_3. Furthermore, Weeds
and Lowey (9) showed that g_2 could be almost completely removed
from myosin by treatment with DTNB, without loss of ATPase activity
in the steady state. CMB-treatment also specifically removed
about half the g_2 or its derivative (g_2') from myosin and HMM, re-
spectively, although the other half of the g_2 could not be removed.
On the other hand, S-1 prepared from control myosin both contained
one mole of g_2' per two moles (cf. Table I). Furthermore, the g_2'
was not removed even by direct treatment of S-1 with CMB. It thus
seems that the two g_2s in the myosin molecule are not identical as
regards their sensitivity to CMB- and trypsin-treatments, and that
the S-1 does not contain a subunit derived from the g_2 of myosin,
which can be removed by the CMB-treatment. However, the amino acid

compositions of the two g_2s were essentially the same. Thus, the differences in their sensitivities to CMB may be due to their interaction with the other subunits.

Hayashi (10) had previously indicated that S-1 was composed of f' ($5.2-5.5 \times 10^4$) and f" (2.7×10^4) derived from f-chains, g_1" (2.1×10^4) derived from g_1, g_2' (1.6×10^4) derived from g_2, and g_3 itself (1.4×10^4). Thus, a model for the submolecular structure of S-1 was tentatively proposed: Two S-1 molecules were composed of 2f', 2f", $1g_1$", $1g_2$', and $1g_3$. In this study, the contents of these components were estimated from the areas of densitometer tracings of electrophoretograms assuming that the relative weight ratio was equivalent to the relative area ratio. Two moles, i.e. 2.2×10^5 g (10) of S-1 contained 1.7 moles of f', 2.0 moles of f", 2.6 moles of g_1", 0.88 moles of g_2'. and 0.87 moles of g_3 (cf. Table I). However, as described above, the myosin molecule producing two S-1 contained only one g_1 (cf. Table I). Therefore, the above model for the submolecular structure of S-1 must be modified partially as follows: g_1" may be a mixture of one derivative from the g_1 and 1.6 derivatives from f-chains with a molecular weight of about 2.1×10^4 daltons.

When ATP was added to acto-HMM reconstituted from FA-RP and CMB-DTT-treated HMM, only about 30% of the HMM passed through a Millipore filter of $0.3 \mu m$ pore size, whereas most of the HMM of control acto-HMM passed through. The angular distribution of light-scattering intensity of acto-HMM indicated that the weight-average molecular weight of CMB-DTT-treated acto-HMM after adding ATP was much higher than that of control acto-HMM. Electron microscopy also showed that CMB-DTT-treated HMM remained bound to F-actin even

in the presence of ATP, and formed projections oriented more ran-domly to the F-actin axis than in the absence of ATP, while control HMM could not bind to F-actin filaments.

<div style="text-align:center">*   *   *   *   *</div>

Recent studies on the substructure of a myosin molecule revealed that there are at least two populations of isozymes. In 1973, Sarkar (13) precisely determined the content of g-chains in a myosin molecule by the densitometer tracings of SDS-PAGE. According to him, g_1, g_2, and g_3 are involved at 1.35, 2.0, and 0.65 moles per mole of myosin. This non-stoichiometric amount indicated the pre-sence of isozymes in myosin. Actually, there were at least two kinds of myosin, one of which contains g_1 and g_2, and the other g_3 and g_2 (39,40). The properties of these g-chains are also cleared in detail. It is widely accepted that g_1 and g_3 affect the binding of S-1 to F-actin, although these chains contain some fragments of f-chain and g_2, respectively. Moreover, g_2-chains, at least one of them, may exist at the joint between S-1 and the tail (41,42). These subjects are discussed in detail in the recent review by Inoue et al. (18).

Furthermore, Shibata-Sekiya and Tonomura (43) recently showed that CMB still remains in HMM after treating CMB-HMM with an excess amount of βME. They showed that the maximum inhibition of ATP-induced dissociation of acto-HMM is found at 0.5 mole CMB bound per mole of head. The cystein residue bound CMB is found to be located on one head of myosin, which is related to the ATP-regulation of actomyosin ATPase.

REFERENCES

1. Tsao,T.-C. (1953) Biochim.Biophys.Acta <u>11</u> 368.

2. Kielley,W.W. & Harrington,W.F. (1960) Biochim.Biophys.Acta <u>41</u> 401.

3. Kominz,D.R., Carroll,W.R., Smith,E.N. & Mitchell,E.R. (1959) Arch.Biochem.Biophys. <u>79</u> 191.

4. Wetlaufer,D.B. & Edsall,J.T. (1960) Biochim.Biophys.Acta <u>43</u> 132.

5. Gershman,L.C., Dreizen,P. & Stracher,A. (1966) Proc.Natl. Acad.Sci.USA. <u>56</u> 966.

6. Gaetjens,E., Bárány,K., Báilin,G., Oppenheimer,H. & Bárány,M. Arch.Biochem.Biophys. <u>123</u> 82.

7. Weber,K. & Osborn,M. (1969) J.Biol.Chem. <u>244</u> 4406.

8. Sarkar,S., Sreter,F.A. & Gergely,J. (1971) Proc.Natl.Acad. Sci.USA. <u>68</u> 946.

9. Weeds,A.G. & Lowey,S. (1971) J.Mol.Biol. <u>61</u> 701.

10. Hayashi,Y. (1972) J.Biochem. <u>72</u> 83.

11. Perrie,W.T., Smillie,L.B. & Perry,S.V. (1972) Biochem.J. <u>128</u> 105$_p$.

12. Tonomura,Y. "Muscle Proteins,Muscle Contraction and Cation transport." (Univ. Tokyo Press & Univ. Park Press) (1972).

13. Sarkar,S. (1973) Cold Spring Harbor Symp.Quant.Biol. <u>37</u> 14.

14. Yagi,K., Okamoto,Y. & Yazawa,Y. (1975) J.Biochem. <u>77</u> 333.

15. Stracher,A. (1969) Biochem.Biophys.Res.Commun. <u>35</u> 519.

16. Dreizen,P. & Gershman,L.C. (1970) Biochemistry <u>9</u> 1688.

17. Kim.H.D. & Mommaerts,W.F.H.M. (1971) Biochim.Biophys.Acta <u>245</u> 230.

18. Inoue,A., Takenaka,H., Arata,T. & Tonomura,Y. (1978) Adv.

Biophys. in press.

19. Tonomura,Y. & Yoshimura,J. (1960) Arch.Biochem.Biophys. 90 73.

20. Tonomura,Y., Yoshimura,J. & Kitagawa,S. (1961) J.Biol.Chem. 236 1968.

21. Sekiya,K. & Tonomura,Y. (1971) J.Biochem. 69 935.

22. Kitagawa,S., Yoshimura,J. & Tonomura,Y. (1961) J.Biol.Chem. 236 902.

23. Hayashi,Y., Takenaka,H. & Tonomura,Y. (1973) J.Biochem. 74 1031.

24. Perry.S.V. (1955) "Methods in Enzymology," eds.Colowick,S.P. & Kaplan,N.O. (Academic Press, Inc., New York) vol. 2 p582.

25. Harris,M. & Suelter,C.H. (1967) Biochim.Biophys.Acta 133 393.

26. Szent-Györgyi,A.G. (1953) Arch.Biochem.Biophys. 42 305.

27. Straub,F.B. & Feuer,G. (1950) Biochim.Biophys.Acta 4 445.

28. Mommaerts,W.F.H.M. (1952) J.Biol.Chem. 198 445.

29. Boyer,P.D. (1954) J.Amer.Chem.Soc. 76 4331.

30. Glynn,I.M. & Chappel,J.B. (1964) Biochem.J. 90 147.

31. Wada,H. & Snell,E.E. (1972) Anal.Biochem. 46 548.

32. Layne,E. (1955) "Methods in Enzymology," eds.Colowick,S.P. & Kaplan,N.O. (Academic Press, Inc., New York) vol 3 p448.

33. Zimm,B.H. (.948) J.Chem.Phys. 16 1099.

34. Kanazawa,T., Saito,M. & Tonomura,Y. (1970) J.Biochem. 67 693.

35. Martin,J.B. & Doty, D.M. (1949) Anal.Chem. 21 965.

36. Spackman,D.H., Steine,W.H. & Moore,S. (1958) Anal.Chem. 30 1190.

37. Young,D.M., Himmelfarb,S. & Harrington,W.F. (1965) J.Biol. Chem. <u>240</u> 2428.

38. Lowey,S. & Risby,D. (1971) Nature <u>234</u> 81.

39. Holt,J.C. & Lowey,S. (1977) Biochemistry <u>16</u> 4398.

40. Wagner,P.D. (.977) FEBS Letters <u>81</u> 81.

41. Wagner,P.D. & Weeds,A.G. (1977) J.Mol.Biol. <u>109</u> 455.

42. Bagshaw,C.R. (1977) Biochemistry <u>16</u> 59.

43. Shibata-Sekiya,K. & Tonomura,Y. (1977) J.Biochem. <u>80</u> 1371.

PART   II

Substrate conformation and myosin ATPase:

Interactions between stereospecific ATP analogs

and myosin or actomyosin.

The study on the substructure of the myosin molecule was described in Part I. This Part describes the studies on the interactions between various kinds of stereospecific ATP analogs and the contractile proteins from a viewpoint of the conformation of substrate. As pointed out in "Introduction", Mg^{2+}-ATP is hydrolyzed via two different pathways on the two heads of myosin (1,2). Tonomura et al. (3-5) separated the two heads as S-1B and S-1A. S-1B catalyzes the hydrolysis of Mg^{2+}-ATP by the following mechanism:

1. $M + ATP \rightleftharpoons M_1 ATP \rightleftharpoons M_2 ATP \rightleftharpoons M_P^{ADP} \longrightarrow {}^\circ M + ADP + Pi \longrightarrow M + ADP + Pi$
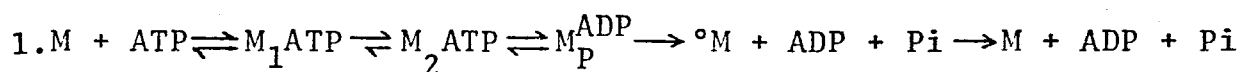
On the other hand, S-1A catalyzes as following:

2. ${}^\circ M + ATP \rightleftharpoons {}^\circ MATP \longrightarrow {}^\circ M + ADP + Pi$

Both $M_2 ATP$ and M_P^{ADP} show an increment in tryptophan fluorescence, and are in a rapid eqiulibrium. The formation of M_P^{ADP} is observed as initial Pi-burst. The slow decomposition of M_P^{ADP} is activated by F-actin.

ATP analogs are useful materials to study the complex mechanisms of myosin ATPase reactions. Many kinds of nucleoside triphosphates (NTPs) have been synthesized and used for studying the interactions with myosin and actomyosin, since Blum (6) and Hasselbach (7) first reported the use of naturally occuring NTPs. However, these analogs did not give precise informations on the ATPase reactions. Ikehara, Tonomura and coworkers (8-10) first synthesized and applied the various kinds of purposeful ATP analogs, named 'intellectual analogs' by Duke and Morales (11), on studying the role of the submolecular structure of ATP. Thereafter, many NTPs were prepared for the studies on the special aspects of the interactions between NTPs and the contractile proteins. These analogs are named 'utilitarian analogs' (11). Adenylyl imidodiphosphate

(AMPPNP) (12) showed that actomyosin can be dissociated on NTP binding without hydrolysis. 1,N^6-Etheno-ATP (εATP) (13) showed that the energy-transferring occurs between NTP and the tryptophan of myosin. 6-Thio-ITP (14) and its dimer (12) showed that there are sites for NTP binding other than the catalytic site. Moreover, adenosine 5'-(2-O-thiotriphosphate) (ATPβS) (15) showed that myosin recognizes the conformation of the triphosphate chain.

The studies on structure of the ATP molecule have markedly progressed in these twenty years. The conformation of ATP in a solution has been analyzed in good detail with various physicochemical techniques, such as NMR and CD. It was thus shown that ATP in a solution preferably exists as the anti-conformer as that in the crystal (16). Relative position of the base to the ribose in nucleosides and nucleotides is expressed by the glycosyl torsion angle, $\mathcal{X}$, as shown in Fig. 1 (17-20). The 0° angle about the glycosyl bond is shown by the N^9-C(8) bond in cis-planar to the O(1')-C(1') bond with respect to rotation about the C(1')-N^9 bond. It is positive in anti-clockwise in Fig. 1, and it has the value of $\pm$180°. Most of naturally occurring nucleosides and nucleotides exist mainly in two regions of $\mathcal{X}$. Each of the regions covers somewhat more than 90° centered at about 30° or -150°, which is called the classical anti- or syn-region, respectively. The region centered at about 120° is called intermediate-region. As mentioned above, ATP exists as the anti-conformer, where five-membered imidazole ring projects over the ribose (cf. Fig.1). Mainly because of the coulombic interactions between the negatively charged phosphate group and the base, nucleotides are conformation-
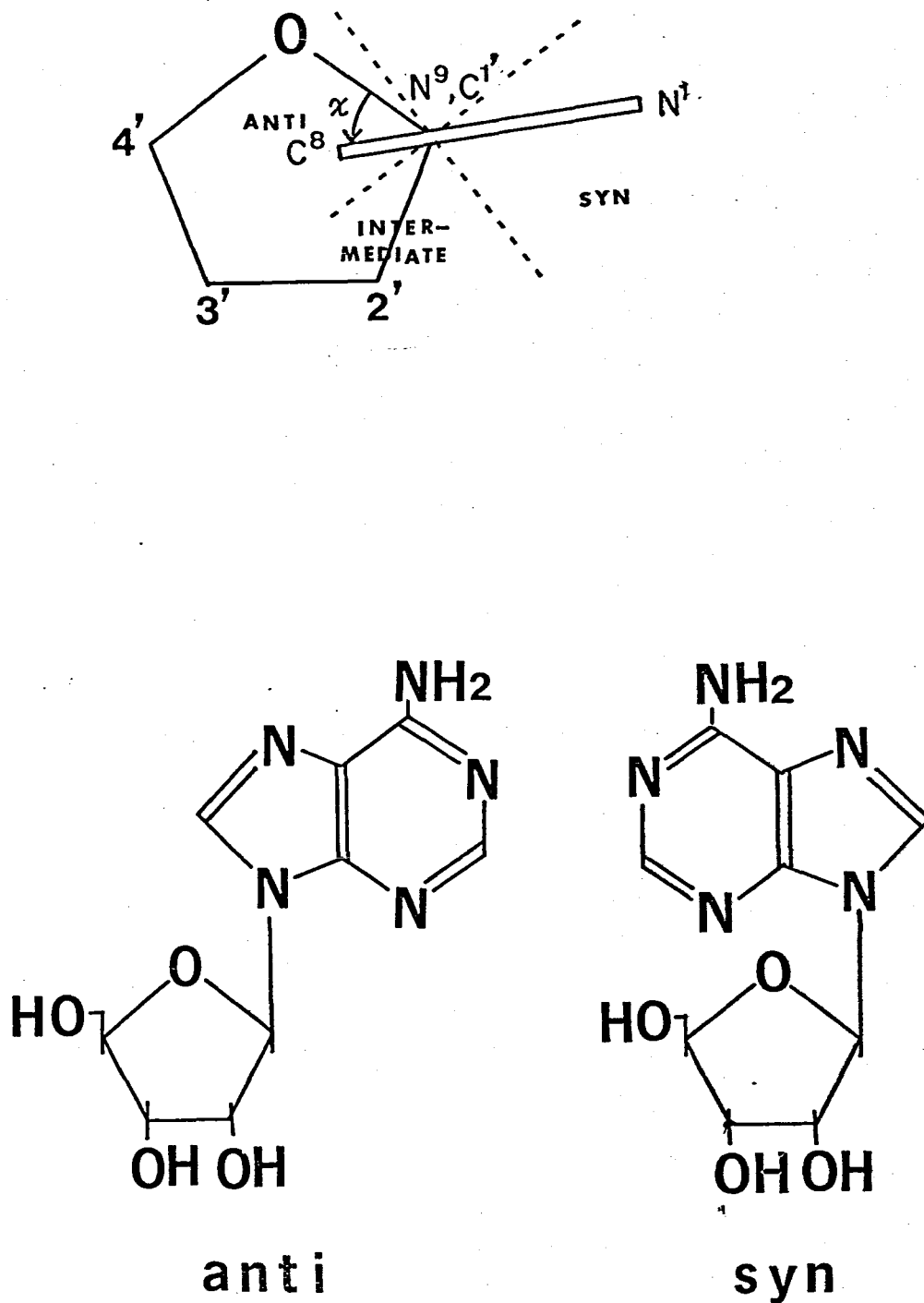
Fig. 1. Representation of $\mathcal{X}$ and syn-anti conformation. Upper: Schematic representation of the glycosyl torsion angl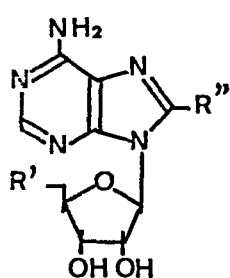e, $\mathcal{X}$. $\mathcal{X}$ is defined as the angle between N^9-C(8) and C(1')-O(1') bonds about the glycosyl bond. Adenosine is overlooked from the adenine portion in this figure. Lower: Schematic representation of adenosine molecules in an anti- and a syn-conformation.

ally far more rigid than the nucleosides. Yathindra and Sundara-
lingam (21) and Millner and Andersen (22) calculated the confor-
mational energies of AMP and ATP, respectively, as the function
of χ. Using their energy maps, it is possible to estimate the
energy differences and the energy barriers between syn-region and
intermediate-region and also anti-region at 3.5-4 and 5-7.5 Kcal·
mole^{-1} for AMP, respectively, and at 20-30 and 25-55 Kcal·mole^{-1} for
ATP, respectively. In both cases of AMP and ATP, the enormous
energy barrier lies around χ=-180 - -120°. Thus, in both AMP and
ATP, full rotation about the glycosyl bond does not seem to occur,
whereas in ATP, fluctuation about the glycosyl bond may be allowed
to occur, because 3',5'-cyclic AMP exists as the rapidly equilib-
rating syn-anti mixture in spite of a high rotational barrier of about
6 Kcal·mole^{-1}, as reported by Hemmes et al. (23). Furthermore,
Tran-Dinh et al. (25) recently reported that ATP exists as an equi-
valent mixture of anti- and syn-conformers in a neutral pH sol-
ution. Such a difficulty in the rotation about the glycosyl bond
can be inferred from the CPK-space filling model, and I mainly
depend on this method to assume difficulty of the rotation of the
stereospecific analogs studied here.

Tavale and Sobell (25) showed in their X-ray studies, that the
adenosine with a bromine atom as the bulky substituent at eighth
position is in a syn-conformation. Later, Sarma et al. (26)
studied the conformation of 8-bromo AMP and 8-methylmercapto AMP
by PMR and ^{31}P-NMR, and Uesugi and Ikehara (27,28) studied the
CD and ^{13}C-NMR of the 8-substituted adenosines with Br, SCH_3,
OCH_3, OH, CH_3, and other groups. It is thus indicated that all

these analogs prefer syn-conformation. Robins et al. (29,30) reported that there were differences in the affinty of 3',5'-cyclic AMP (cAMP) dependent protein kinase for natural and 8-substituted cAMP. Furthermore, Moras et al. (31) found, using X-ray analysis, that two of four subunits of glyceraldehyde-3-phosphate dehydrogenase bind nicotinamide adenine dinucleotide molecules, in which adenosine is an anti-conformer, and that the other two subunits bind syn-conformers.
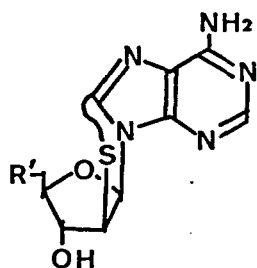
I synthesized fourteen ATP analogs, including 8-substituted and anhydro NTPs, to clarify the relationships between the NTP conformation and nucleoside triphosphatase (NTPase) activities of myosin and actomyosin (Fig. 2). ATP analogs first synthesized in this study are 8-monomethylamino ATP (8-NH·CH$_3$ ATP), 8-monoethylamino ATP (8-NH·C$_2$H$_5$ ATP), 8-dimethylamino ATP [8-N(CH$_3$)$_2$ ATP], 8-methoxy ATP (8-OCH$_3$ ATP), 8-ethoxy ATP (8-C$_2$H$_5$ ATP), 8-methylmercapto ATP (8-SCH$_3$ ATP), 8-oxy ATP (8=O ATP), 8-methyl ATP (8-CH$_3$ ATP), 8,2'-anhydro-8-mercapto-9-β-D-arabinofuranosyladenine 5'-triphosphate (8,2'-S-cyclo ATP), 8,3'-anhydro-8-mercapto-9-β-D-xylofuranosyladenine 5'-triphosphate (8,3'-S-cyclo ATP), and 3'-deoxy ATP (3'-dATP). In addition to these NTPs, 8-bromo ATP (8-Br ATP), 8-azido ATP (8-N$_3$ ATP) and formycin 5'-triphosphate (FTP) were prepared and used for kinetic analyses of their reactions with myosin and actomyosin. From NMR and CD analyses and also from the chemical structure (27,28,32,33), 8-NH·CH$_3$ ATP and 8,3'-S-cyclo ATP are supposed to exist as anti-conformers, 8,2'-S-cyclo ATP as an intermediate-conformer, and the other 8-substituted NTPs as syn-conformers. On the other hand, FTP and 3'-dATP are

R' = $-O-\left[\begin{matrix}O\\ \overset{\|}{-P}-O-\\ OH\end{matrix}\right]_3 H$

R'' = B_R, NH·CH₃, NH·C₂H₅
N(CH₃)₂, OCH₃, OC₂H₅
SCH₃, O, N₃, CH₃.

8-R ATP
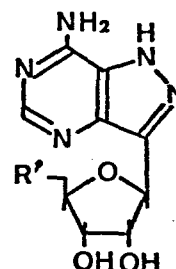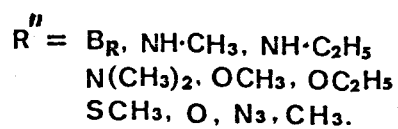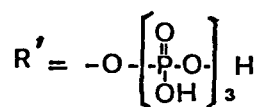
FTP
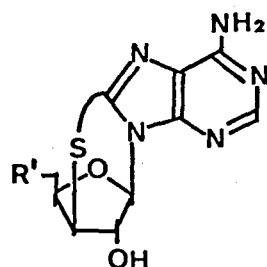
8,2'-S-CYCLO
ATP

8,3'-S-CYCLO
ATP
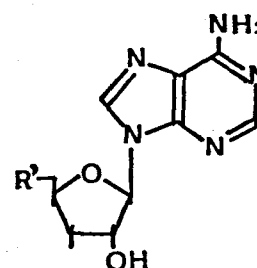
3'- dATP

Fig. 2.   Schematic formulas of synthesized ATP analogs.   8-Substituted analogs are drawn as a syn-conformer, although 8-NH·CH₃ ATP is identified as an anti-conformer.   FTP can exist as both the anti- and syn-conformers.   3'-dATP is supposed to prefer an anti-conformer.

supposed to have an easily rotatable glycosyl bond. Consequently,
all the NTPs with easy rotation about the glycosyl bond were
found to show high EDTA(K^+)-NTPase and low Mg^{2+}-NTPase activities
of myosin, whereas NTPs with difficult rotation show high Mg^{2+}-
NTPase and very low EDTA(K^+)-NTPase activities. Furthermore,
all the NTPs with easy rotation about the glycosyl bond cause
initial Pi-burst, actin-activation of myosin NTPase, superpre-
cipitation of actomyosin and myofibrillar contraction, while
all the NTPs with difficult rotation do not support these phenom-
ena, regardless of whether they have an anti-, intermediate- or
syn-conformation. Moreover, I studied the interactions between
8-Br ATP, which is easily synthesized, and myosin and also acto-
myosin in more detail. 8-Br ATP inhibits the myosin and acto-
myosin ATPase and superprecipitation of actomyosin, but dissociates
acto-S-1 complex as well as ATP. These facts clearly indicate
that the key intermediate for actomyosin NTPase is M_P^{NDP}, but not
M_2NTP, and that the key intermediates for dissociation of acto-
myosin are M_2NTP on head B and $^\circ MNTP$ on head A. Furthermore,
these results indicate that the three-dimensional structure of
the site for binding the base and the ribose change during con-
version from M_2NTP into M_P^{NDP}, on head B.

EXPERIMENTALS

Materials.------ Myosin, HMM, and S-1 were prepared by the methods
of Perry (34), Szent-Györgyi (35), and Hayashi (36), respectively.
Purified G-actin and G-actin with relaxing proteins were prepared
from an acetone powder of rabbit skeletal white muscle (37) by the
methods of Spudich and Watt (38) and Mommaerts (39), respectively.
Actin was treated with Dowex 1x8 to remove nucleotides before use.
Myofibrils were prepared from rabbit psoas muscle by the method
of Perry and Corsi (40) with slight modifications. Samples of
myofibrils contained 5-25 sarcomeres, and the average length of
the sarcomeres was about 2.1μm.

γ-[^{32}P]-ATP was prepared enzymatically by the method of Glynn
and Chappel (41). 8-Substituted adenosines were synthesized by
the methods of Robins et al. (42,43) with slight modifications.
Anhydro adenosines were synthesized by the method of Ikehara et
al. (31) (Fig. 3). Phosphorylation at the 5'-position was followed
to the method of Yoshikawa et al. (44). Obtained AMP analogs were
converted to the morpholidate and triphosphate by the methods of
Moffatt and Khorana (45) with slight modifications.(Fig. 3).
3'-Deoxy adenosine was prepared by Raney Ni reduction of 8,3'-
S-cyclo adenosine (46). 8-Br ATP was synthesized by the method
of Ikehara and Uesugi (47).

8-Br adenosine. --- Adenosine (Ado, 40 mmoles) was dissolved in
0.25M sodium acetate buffer (pH 4.0, 400ml) by heating, and cooled
to room temperature. Bromine-water saturated at 0° (300ml) was
added to the Ado solution, and the reaction mixture was allowed
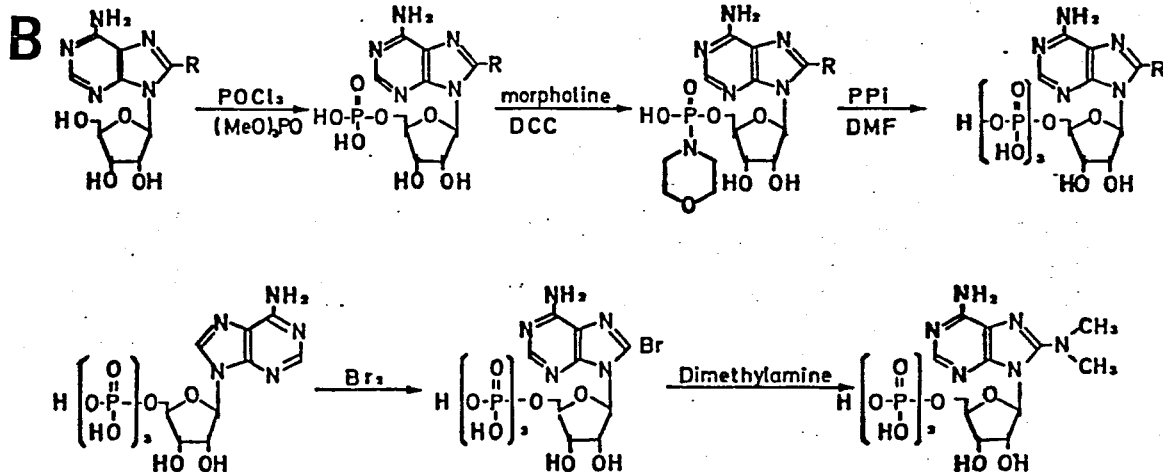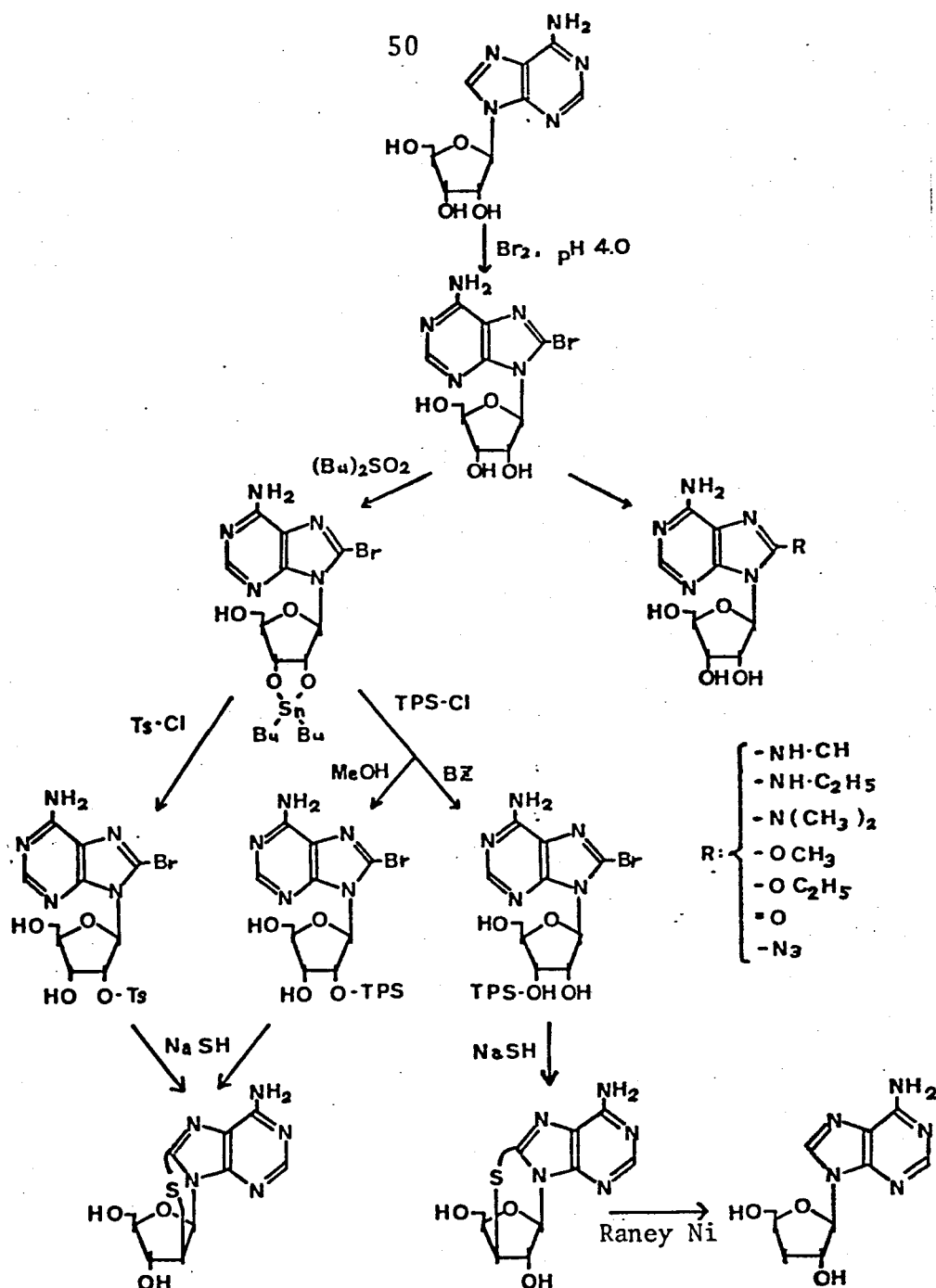to stand overnight at room temperature. The resulting solution

Fig. 3. Synthetic procedures of various adenosine analogs (A) and ATP analogs (B). Detailed conditions are described in the text.

was decolourized by adding 2M NaHSO$_3$ (at most 10ml), and adjusted to pH 6-7 with conc. NaOH solution. After 10hr at 4°, the precipitate was collected by filtration, and dissolved in hot water. Then the solution was treated with activated charcoal and recrystallized from water at 4°. Yellow needle crystal was collected and dried over P$_2$O$_5$ in vacuo at 60-70° with shielding the light to yield 35% of Ado. mp.>200°. Anal. Calcd. for C$_{10}$H$_{12}$N$_5$O$_4$Br: C, 34.70; H, 3.49;N, 20.23; Br, 23.08. Found: C, 34.51; H, 3.55; N, 20.45; Br, 22.86.

8-NH·CH$_3$ Ado. --- 8-Br Ado (3 mmoles) was dissolved in 30% methylamine methanol solution (25ml), and stirred overnight at room temperature. The reaction mixture was evaporated to dryness by a flash evaporator. The obtained solid was dissolved in hot water, and treated with activated charcoal. White powder was collected from water, and dried over P$_2$O$_5$ in vacuo at 60-70° to yield 35-45% of 8-Br Ado. mp.>200°. Anal.Calcd. for C$_{11}$H$_{16}$N$_6$O$_4$: C, 44.59; H, 5.44; N, 28.36. Found: C, 44.41; H, 5.44; N, 28.29.

8-NH·C$_2$H$_5$ Ado. --- 8-Br Ado (3 mmoles) was dissolved in 70% ethylamine solution (25ml), and stirred overnight at room temperature. Pink crystal was collected after the same procedures as 8-NH·CH$_3$ Ado. Yield, 38-49% of 8-Br Ado. mp.>200°. Anal.Calcd.for C$_{12}$H$_{18}$N$_6$O$_4$: C, 46.45; H, 5.85; N, 27.08. Found: C, 46.18; H, 5.80; N, 27.04.

8-[N(CH$_3$)$_2$] Ado. --- 8-Br Ado (3 mmoles) was dissolved in 40% dimethylamine solution (25ml), and stirred overnight at room temperature. White powder was collected after the same procedures as 8-NH·CH$_3$ Ado. Yield, 32-40% of 8-Br Ado. mp.>200°. Anal. Calcd. for C$_{12}$H$_{18}$N$_6$O$_4$: C, 46.45; H, 5.85; N,27.08. Found: C, 46.45;

H, 5.61; N, 27.06.

8-OCH$_3$ Ado. --- 8-Br Ado (3 mmoles) was suspended in freshly prepared anhydrous methanol (25ml) dissolving Na metal (15 mmoles). The suspension was stirred for 16hr at room temperature. The reaction mixture was adjusted to pH 7 with conc. HCl, and the solution was evaporated to dryness, and recrystallized from water at 4° after charcoal treatment. Yield, 24-32% of 8-Br Ado. mp.>200°. Anal. Calcd. for C$_{11}$H$_{15}$N$_5$O$_5$: C, 44.44; H, 5.09; N, 23.56. Found: C, 44.27; H, 5.09; N, 23.67.

8-OC$_2$H$_5$ Ado. --- 8-Br Ado (3 mmoles) was suspended in freshly prepared anhydrous ethanol (25ml) dissolving Na metal (15 mmoles). After the same procedures as 8-OCH$_3$ Ado, pale pink crystal was collected to yield 28-34% of 8-Br Ado. mp.>200°. Anal. Calcd. for C$_{12}$H$_{17}$N$_5$O$_5$: C, 46.30; H, 5.50; N, 22.50. Found: C, 43.55; H, 5.38; N, 22.03.

8=O Ado. --- A suspension of 8-Br Ado (3 mmoles) in anhydrous sodium acetate (2g), acetic anhydride (50ml), and glacial acetic acid (100ml) was heated at 120-140° for 4hr. The reaction mixture was evaporated to dryness, and repeatedly evaporated with ethanol, water and a small amount of pyridine. The resulting caramel was dissolved in water, and extracted with chloroform. Organic phase was dried with anhydrous Na$_2$SO$_4$, and evaporated after filtration. 2% NH$_4$OH-50% ethanol-water (50ml) was added to the caramel and stirred overnight. Then the reaction mixture was evaporated to dryness. After dissolution in hot water, charcoal was added and filtered. The filtrate was cooled to 4°. White powder was collected to yield 27-38% of 8-Br Ado. mp.>200°. Anal.Calcd. for

$C_{10}H_{15}N_5O_5$: C, 42.40; H, 4.63; N, 24.73   Found: C, 41.93; H, 4.63; N, 24.51.

8-N_3 Ado. --- A dimethylformamide (DMF) solution (50ml) of 8-Br Ado (10 mmoles) and sodium azide (15 mmoles) was heated stirring at 65-75° overnight with passing dried nitrogen gas. The reaction mixture was cooled to room temperature, and poured into methylene chloride (1 liter). The precipitate was collected with centrifuging. The pellet was washed with methylene chloride (100 ml), anhydrous methanol (20ml) for three times, and water (20 ml). Pale yellow pellet was dissolved in hot water and treated with activated charcoal. The filtrate was allowed to stand at 4° after removal of charcoal. Pale yellow powder was collected to yield 28-34% of 8-Br Ado. mp.>200°. Anal.Calcd.for $C_{10}H_{12}N_8O_4$: C, 38.96; H, 3.92; N, 36.35. Found: C, 38.32; H, 3.86; N, 34.56.

8-Bromo-[2'(or 3')-triisopropylbenzene sulfonyl] adenosine (48). --- Well dried 8-Br Ado (20 mmoles) was dissolved in dry DMF (180 ml) by heating, and the solution was cooled to -10 to -15° in an ice-salt bath. A DMF (10ml) suspension of sodium hydride (24 mmoles) was added to the 8-Br Ado solution, and stirred for 10 min. After hydrogen gas was not generated, triisopropylbenzene sulfonyl chloride (6.6 g) was added to the suspension, and stirred for 2hr. The reaction mixture was dropped into an aqueous solution (800ml) of sodium bicarbonate (10g) with stirring. After 60 min stirring, the precipitate was collected by filtration and dried over P_2O_5 at below 50°. White powder was first dissolved in methanol (100 ml) and allowed to stand at 4° for 24hr. 8-Br-3'-triisopropylbenzene sulfonyl (TPS) Ado thus obtained was collected by filtration.

The filtrate was evaporated, and the solid was dissolved in benzene (150ml). The mixture was allowed to stand at 4° overnight, and 8-Br-2'-TPS Ado was obtained. Both the Ado derivatives did not yield over 40% of 8-Br Ado.

8-Br-2'-O-tosyl Ado (49). --- A methanol (250ml) solution of 8-Br Ado (10 mmoles) and dibutyltin oxide (10 mmoles) was re-fluxed for 30 min. The solution was evaporated to dryness. 8-Br-2',3'-dibutylstannylene Ado was crystallized from ethanol-acetone mixture. 8-Br-2',3'-dibutylstannylene Ado thus obtained (ca. 5 mmoles) was suspended in a methanol (100ml) solution of triethylamine (75 mmoles) and tosyl chloride (75 mmoles). The solution was stirred at room temperature for 5 min, and evaporated to dryness. After extraction with water-ether, the aqueous phase was condensed and allowed to stand at 4°.

8,2'-S-cyclo Ado. --- A DMF (10ml) solution of 8-Br-2'-TPS Ado or 8-Br-2'-tosyl Ado (1 mmole) and sodium mercaptan (3 mmoles) was heated at 60-70° for 14hr. The solution was neutralized with 1N HCl, and H_2S gas was removed by bubbling N_2 gas. After evaporation to dryness, the solid was dissolved in hot water (10ml) and insol-uble material was removed by filtration. The filtrate was evapo-rated to dryness, and the solid was dissolved in hot water (15ml). After treating with activated charcoal, 8,2'-S-cyclo Ado was crystallized at 4°. mp. 147°. Anal. Calcd. for $C_{10}H_{11}N_5O_3S$: C, 42.70; H, 3.94; N, 24.90; S, 11.40. Found: C, 42.70; H, 3.79; N, 25.02; S, 11.69.

8,3'-S-cyclo Ado. --- A DMF (10ml) solution of 8-Br-3'-TPS Ado (1 mmole) and sodium mercaptan (3 mmoles) was heated at 60-70°

for 14hr. The same procedures for 8,2'-S-cyclo Ado synthesis
was applied. mp. 165°. Anal. Calcd. for $C_{10}H_{11}N_5O_3S$: C, 42.70;
H, 3.94; N, 24.90; S, 11.40. Found: C, 42.70; H, 3.99; N, 25.07;
S, 11.17.

3'-dATP (Cordycepin). --- 8,3'-S-cyclo Ado (1 mmole) was re-
fluxed in water as concentrated as possible. A ethanol suspension
(1ml) of Raney Ni was added to the solution with stirring. The
suspension was filtered and then washed with 2% NH_4OH-50% ethanol-
water. The filtrate and the washed solution were evaporated to
dryness. The solid was dissolved in hot water, and 3'-dAdo was
crystallized at 4°. Anal.Calcd. for $C_{10}H_{13}N_5O_3$: C, 47.81; H, 5.22;
N, 27.87. Found: C, 47.81; H, 5.10; N, 27.89.

8-SCH_3 Ado and 8-CH_3 Ado were kindly supplied by Drs. T.Maru-
yama and W.G.Lymn, respectively, of Osaka University. Formycin
was kindly gifted from Dr. H.Umezawa of Institute of Microbial
Chemistry and Meiji Seika Co.

Nucleoside 5'-monophosphate (NMP). --- The adenosine analog
(1 mmole) was added to a mixture of freshly distilled phosphoryl
chloride (0.37 ml) and trimethylphosphate (2.5 ml) with stirring
at -10 to 0°. After 2.5-3.5hr, the reaction mixture was poured
into a saturated sodium bicarbonate solution at room temperature,
and stirred overnight. The resulting solution was adjusted to
pH 3 with conc.HCl, and desalted with activated charcoal. The
crude NMP solution was purified by DEAE-cellulose column chro-
matography (about 40ml resin/mmole of NMP) with a linear gradient
of 2-100mM triethylammonium bicarbonate (TEAB) buffer at pH 7.5.
The yield of NMP was in the range between 78-85% of the nucleoside.

Nucleoside triphosphate. --- The fractions of NMP were collected and repeatedly evaporated with ethanol-water. NMP (1 mmole) was dissolved in t-butanol (10ml)-water (10ml)-morpholine (4 mmoles), and refluxed. A t-butanol solution (15ml) of dicyclohexylcarbodiimide (DCC, 4 mmoles) was added to the refluxed mixture. After 3 to 4hr, a t-butanol solution of morpholine (2 mmoles) and DCC (2 mmoles) was added further, and the reaction mixture was continued refluxing. When a spot of NMP on paper electrophoresis disappeared, the reaction mixture was evaporated without cooling. A precipitate of dicyclohexylurea was removed by filtration. The resultant solution was extracted with ether. After repeating these procedures, the NMP-morpholidate solution was evaporated to dryness. The caramel thus obtained was dissolved in anhydrous pyridine (10ml), and evaporated four times. Sodium pyrophosphate (PPi, 5 mmoles) was converted to free acid by Dowex 50W (H^+-form), and mixed with tri-n-butylamine (10 mmoles). tri-n-Butylammonium pyrophosphate was dried by repeated evaporation with anhydrous pyridine. Both the pyridine solutions were mixed and evaporated four times. Then, the solvent was changed to DMF, and evaporated once, and the DMF (5ml) solution was allowed to stand for 40hr at room temperature. The reaction mixture was evaporated with water, and pH was adjusted to 8 with conc.NH_4OH. NTP was purified by a column chromatography on Dowex 2x8 (2x20cm) with a linear gradient of 0 - 0.45 M LiCl in 3mN HCl (Fig. 4). The lithium salt of NTP was passed through a column of activated charcoal column (about 30ml). After washing the column with water (2 ℓ), the adsorbed nucleotide was eluted with 2% NH_4OH-50% ethanol-water.
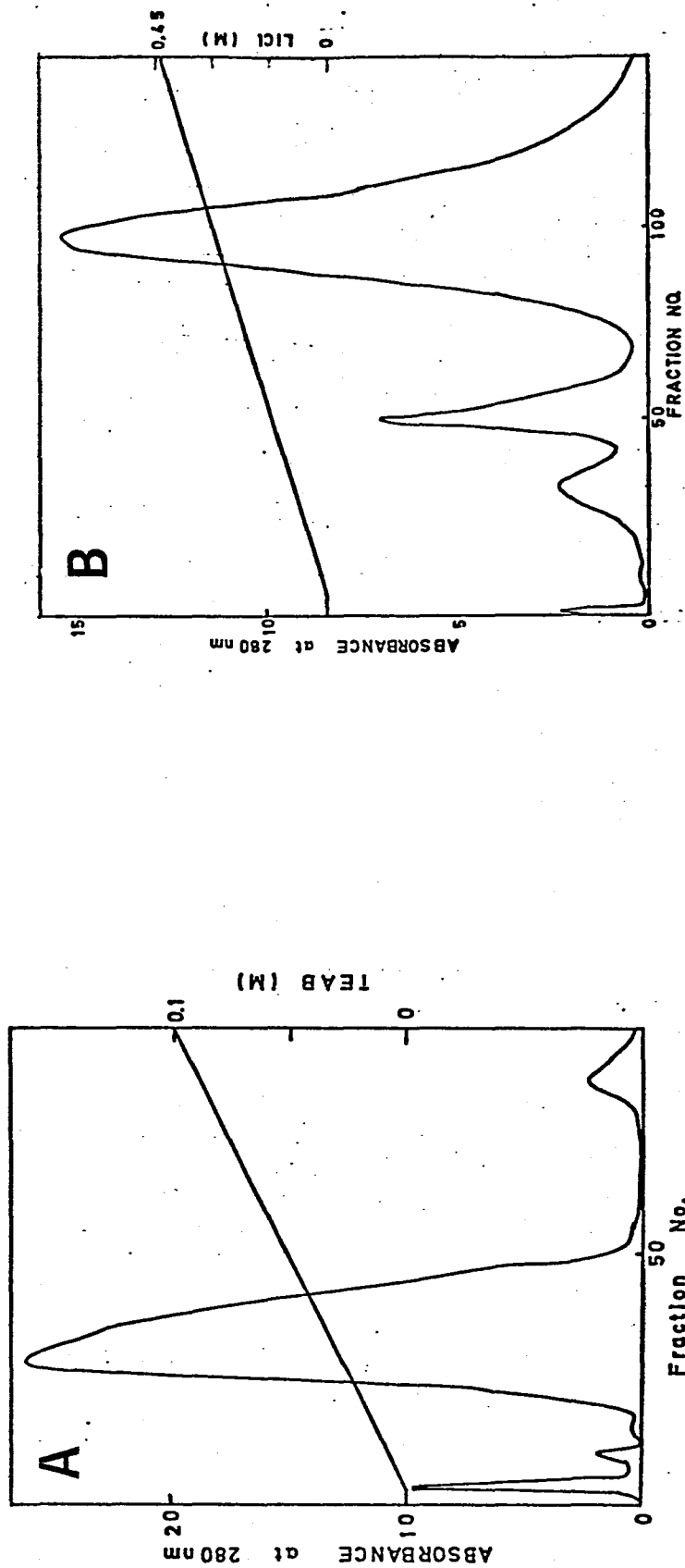
Fig. 4. Elution profiles of NMP on DEAE-cellulose (A) and NTP on Dowex 2x8 (B) column chromatography. A: NMP was subjected on a DEAE-cellulose (Whatman DE-23) column (2x12-cm) chromatography with a linear gradient of 2-100mM TEAB buffer at pH 7.5. Fractions (20 ml) were collected. B: NTP was purified by Dowex 2x8 column (12x20-cm) chromatography with a linear gradient of 0-0.45M LiCl in the presence of 1mN HCl. Fractions (20 ml) were collected.

The solvent was evaporated off, and the residue was dissolved in a small amount of water and passed through a column of Dowex 50W x8 (K^+-form) to yield 50-70% of NMP.

8-[N(CH$_3$)$_2$] ATP was not followed th the above method, because in changed into a different material (λmax=290nm) with Dowex 2x8 and LiCl. Therefore, 8-[N(CH$_3$)$_2$] ATP was purified on DEAE-celllose column with a linear gradient of 0-0.45M TEAB buffer at pH 7.5. 8-[N(CH$_3$)$_2$] ATP was also synthesized by direct substitution of 8-position of 8-Br ATP with dimethylamine. However, some side re-actions occurred with this method.

8-Br ATP. --- Na$_2$ATP (1 mmole) was dissolved in sodium acetate (100ml, pH 4.0) and Br$_2$-water (0° saturated, 10ml) was added. The reaction mixture was allowed to stand at room temperature for 16hr, and extracted hromine with ether. The colour was discharged by adding 2M NaHSO$_3$ (about 5ml). After neutralization with conc. NaOH, the resultant solution was subjected on Dowex 1x4, and eluted with a linear gradient of 0.13-0.25M LiCl in the presence of 3mN HCl. The 8-Br ATP fraction was desalted by the same method for NTP described above. 8-Br-γ-[^{32}P]-ATP was prepared from γ-[^{32}P]-ATP by the same method as unlabelled 8-Br ATP.

All the preparation gave single spot on paper electrophoresis on Toyo 51A filter paper in 50mM TEAB buffer at pH 7.5 and 35 volt xcm^{-1} (Table I) and on descending paper chromatography on Toyo 51A paper with the following solvents: A, isopropanol-conc.NH$_4$OH-water (7:1:2, by volume); B, ethanol-1M ammonium acetate at pH 7.0-water (7:3, by volume); C, n-butanol-acetic acid-water (5:2:3, by volume) (Table I).

ATP was purchased from Kyowa Hakko Kogyo Co. NEM was purchased from Nakarai Chemicals Ltd. and purified by sublimation before use. Other reagents were of analytical grade or chemical grade, and were purified by appropriate methods when necessary.

Table I. Properties of ATP analogs.

	PPC^a (R_f)			PEP^b	λmax	$\varepsilon_M^{H_2O}$
	A	B	C	Rm	nm	x 10^{-3}
8-Br ATP	0.14	0.12	0.22	0.99	263	16.1^c
8-NH·CH$_3$ ATP	0.11	0.07	0.20	0.99	278	18.1
8-NH·C$_2$H$_5$ ATP	0.10	0.08	0.25	0.94	278	18.2
8-N(CH$_3$)$_2$ ATP	0.12	0.17	0.40	0.97	274	17.2
8-OCH$_3$ ATP	0.06	0.11	0.17	0.99	261	14.3
8-OC$_2$H$_5$ ATP	0.03	0.09	0.21	0.97	261	15.1
8-SCH$_3$ ATP	0.17	0.14	0.18	0.97	279	20.7
8=O ATP	0.06	0.13	0.12	1.0	268	12.8
8-N$_3$ ATP	0.14	0.09	0.13	1.01	279	13.2
8-CH$_3$ ATP	0.14	0.10	0.15	0.98	258	15.5
FTP	0.10	0.08	0.12	1.0	294	10.3
8,2'-S-cyclo ATP	0.13	0.09	0.15	0.99	277	23.3
8,3'-S-cyclo ATP	0.11	0.08	0.12	0.92	282	25.4
3'-dATP	0.14	0.11	0.11	0.99	258	15.4
ATP	0.08	0.08	0.11	1.0	258	15.4

a. Paper chromatography. Solvents used are described in the text.
b. Paper electrophoresis. Relative migration ratio was represented by Ado as 0 and ATP as 1.0.
c. Measured at pH 1. λmax=261nm.

Methods.------ The SH$_1$-group of myosin were modified with NEM by the method of Sekine and Yamaguchi (50).

NTPase activity was determined by measuring the amount of Pi liberated by the method of Martin and Doty (51). The amount of ^{32}Pi liberated was measured as described by Nakamura and Tonomura (52).

The time courses of the changes in fluorescence of HMM and light scattering intensity of acto-HMM after adding NTP were measured by a stopped flow method, using the apparatus described by Takemori et al. (53). Fluorescence and light-scattering were recorded in a Hitachi-Perkin Elmer MPF-2A fluorescence spectrophotometer connected to a Hitachi QPD-33 recorder or a direct visual oscillograph (Visigraph FR 301, San-ei Instrument Co.). Fluorescence of HMM was excited at 290 nm, and measured at 340 nm, unless otherwise stated, in 50mM KCl, 5mM $MgCl_2$, and 20mM Tris-HCl at pH 7.8 and 20°. The intensity of light-scattering of acto-HMM was measured at 350 nm in 50mM KCl, 5mM $MgCl_2$, and 20mM Tris-HCl at pH 7.8 and 20°.

Dissociation of acto-S-1 was measured by the rapid filtration method, using a Millipore filter (0.3μm pore size) which had been pretreated with HMM as described in Part I: NTP (0.5mM) was added to protein solution containing 0.7 mg/ml S-1, 0.4 mg/ml FA-RP, 50mM KCl, 2mM $MgCl_2$, and 20mM Tris-maleate at pH 7.0 and 20°, and the resulting solution was immediately shaken and filtered under suction. Protein concentration in the filtrate was estimated with Folin-Ciocalteu reagents (54) calibrated with bovine serum albumin.

The change in turbidity of actomyosin after adding NTP was followed at 660 nm using a Cary 14 spectrophotometer. The experimental conditions are described in "RESULTS AND DISCUSSION."

Shortening of sarcomeres was initiated by adding various concentrations of NTP to a suspension of 4.4 mg/ml of myofibrillar protein in 40mM KCl, 1mM $MgCl_2$, 10μM $CaCl_2$, and 30mM Tris-maleate at pH 7.0 and 20°, and reactions were stopped after various periods by adding an equal volume of 30mM EDTA and 5mM Tris-maleate at pH 7.0 (55). Photographs of myofibrils were taken with an Olympus model PM-7 camera attached to a Nikon phase contrast microscope, and expanded with a Nikon Profile Projector for measuring the length of sarcomeres. The average length of sarcomeres was calculated from the length of 5-15 sarcomeres of 6-15 fibers.

Protein concentration was estimated by the biuret method (56) calibrated by nitrogen determination, unless otherwise stated.

RESULTS AND DISCUSSION

NTPase in the steady state. --- Table II summarizes the rates of hydrolysis of ATP analogs by myosin measured at a fixed concentration of analogs (0.3mM) in 0.5M KCl at pH 7.8 and 20° in the presence of 5mM EDTA, 10mM $CaCl_2$ or 5mM $MgCl_2$. All the NTPs with easy rotation about the glycosyl bond were hydrolyzed by myosin very rapidly in the presence of EDTA, but very slowly in the presence of Mg^{2+} ions. On the other hand, all the NTPs with difficult rotation about the glycosyl bond were hydrolyzed by myosin rapidly in the presence of Mg^{2+} ions, whereas in the presence of EDTA, NTPs of syn-conformation were not hydrolyzed and those of anti- and intermediate-conformation hydrolyzed very slowly. ITP, 6-keto analog, is not hydrolyzed in the presence of EDTA, but very readily in the presence of Mg^{2+} ions (2). These facts indicate that the effects of EDTA and Mg^{2+} ions on myosin NTPase are

Table II. Myosin NTPase Activity

0.3 mM NTP, 0.5 M KCl, 20 mM Tris-HCl, pH 7.8, 20°

Rotation about glycosyl bond	NTP	Modifier		
		EDTA	Ca^{2+}	Mg^{2+}
Easy	natural			
	ATP	397	126	1.2
	synthetic			
	2'-dATP	219	68	1.0
	3'-dATP	174	81	2.7
	FTP	37	78	1.9
anti	8-NH·CH_3 ATP	5.5	54	12
	8,3'-S-cyclo ATP	7	27	117
intermediate	8,2'-S-cyclo ATP	3	18	58
syn	8-Br ATP	0	200	45
Difficult	8-NH·C_2H_5 ATP	0	61	4.2
	8-N(CH_3)$_2$ ATP	0	111	54
	8-OCH_3 ATP	0	31	11
	8-OC_2H_5 ATP	0	80	60
	8-SCH_3 ATP	0	16	15
	8=O ATP	0	148	72
	8-N_3 ATP	0	164	39
	8-CH_3 ATP	0	50	21

The values are expressed as min^{-1} (MW of myosin = 4.8×10^5 daltons).

affected by both the conformation of NTP and the substituents at the 6-position. There was no general relationship between Ca^{2+}-NTPase activity and the NTP conformation and also the properties of the substituent.

The modification of the specific SH-group of myosin, SH_1, activates the Mg^{2+}-ATPase and lower the EDTA(K^+)-ATPase activities (57). The results obtained with the stereospecific analogs resemble to that of SH_1-modified myosin ATPase reaction. Fig. 5 shows the time courses of Pi-liberation after adding 0.5mM ATP and 8-Br ATP to 0.48 mg/ml of SH_1-modified myosin in 50mM KCl, 5mM $MgCl_2$, and 20mM Tris-maleate at pH 7.0 and 20°. It is clearly shown that the effects of 8-substituted ATP analogs are not due to their modification of SH_1-group of myosin, because myosin isolated by repeated precipitations after reaction with excess 8-Br ATP had the same Mg^{2+}-ATPase activity as untreated myosin. Furthermore, 8-Br ATPase of myosin was strongly inhibited by modification with NEM, in contrast to ATPase.

The Michaelis constant (K_m) and the maximum velocity (V_m) were estimated from the double-reciprocal plot of HMM-NTPase against the NTP concentration in 50mM KCl and 5mM $MgCl_2$ at pH 7.8 and 20° (Figs. 6,7), and their values are summarized in Table III. Both the K_m and V_m values for NTPs with difficult rotation were much larger than those for NTPs with 6-NH and easy rotation, regardless of the conformation. Figs. 6 and 7 also show the double-reciprocal plots of the change in tryptophan fluorescence ($\Delta F/F$) of HMM induced by 8-Br ATP and 8-OC_2H_5 ATP. F is the fluorescence intensity of HMM before NTP addition, and ΔF is the increment of fluor-
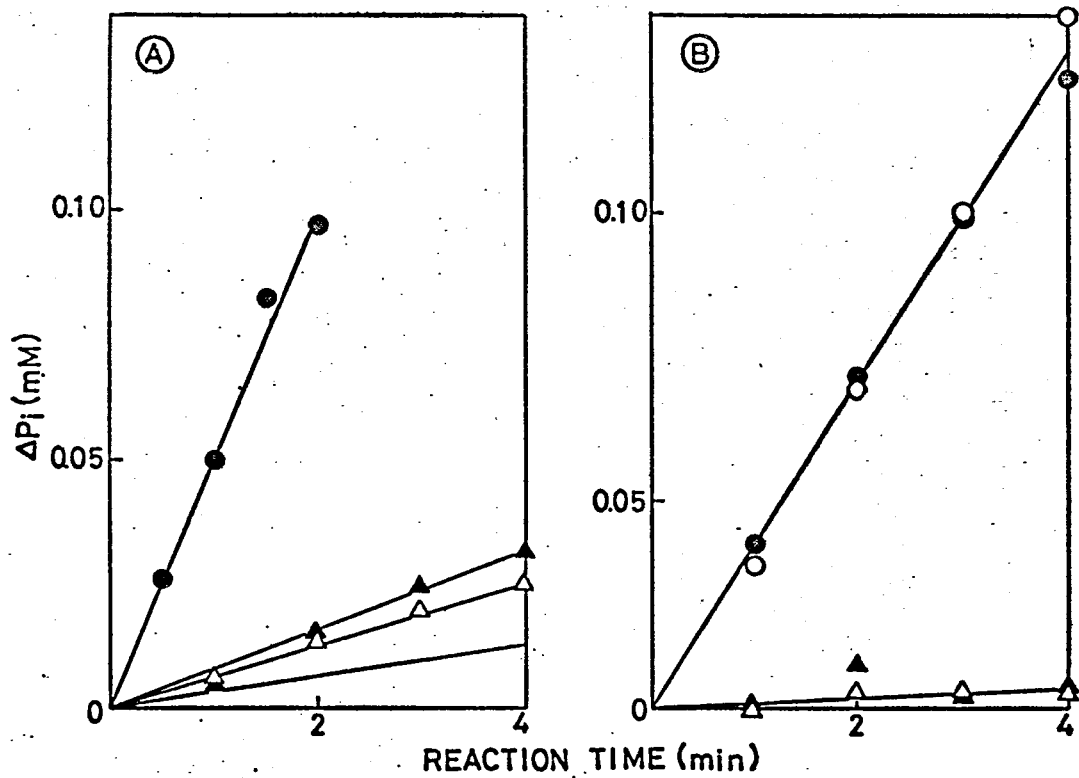
Fig. 5.   Effects of modification of SH_1 group   and addition of
FA on myosin ATPase and 8-Br ATPase activities.   50mM KCl,
5mM $MgCl_2$, 20mM Tris-maleate, pH 7.0, 20°.   A: 0.5mM ATP; B:
0.5mM 8-Br ATP.   O , 0.48 mg/ml myosin; ● , 0.48 mg/ml myosin
+0.2 mg/ml FA; △ , 0.48 mg/ml SH_1-modified myosin; ▲ , 0.48
mg/ml SH_1-modified myosin + 0.2 mg/ml FA.   The lowest line in
A shows the time course of Pi-liberation from ATP with 0.48
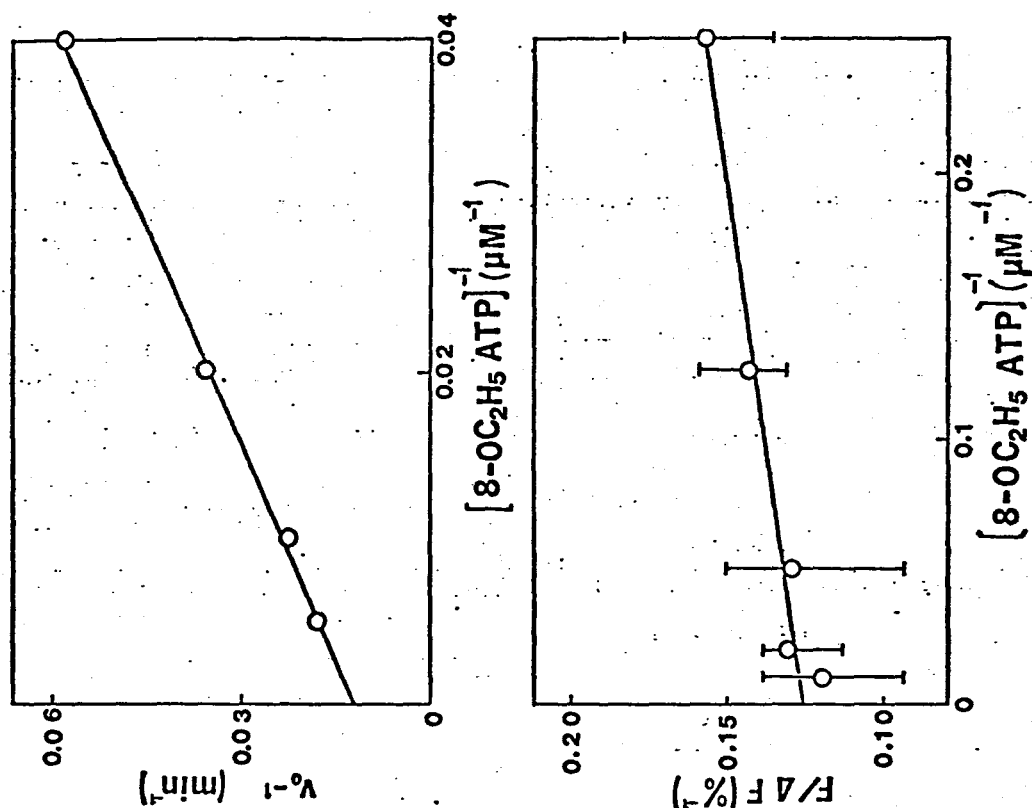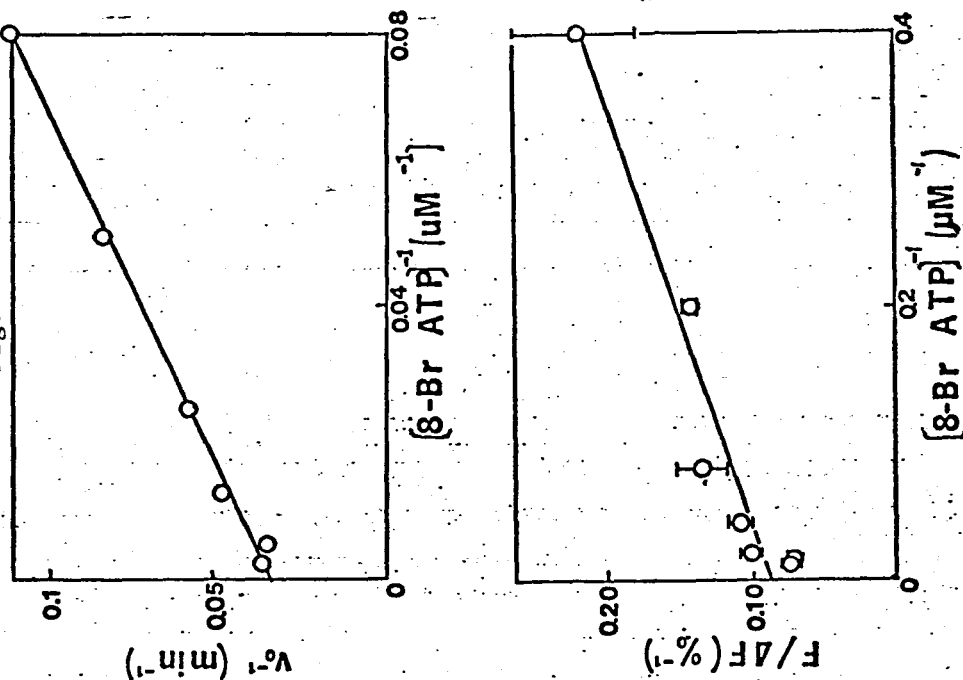mg/ml myosin.

Fig. 6 & 7. Dependences on the 8-Br ATP (left) and 8-OC$_2$H$_5$ ATP (right) concentrations of the NTPase in the steady state and of the increment in tryptophan fluorescence of HMM induced by NTP. For both the figures; Upper: Double-reciprocal plot of the NTPase in the steady state versus NTP concentration. 0.1 mg/ml HMM, 50mM KCl, and 5mM MgCl$_2$ at pH 7.0 and 20°. Lower: Double-reciprocal plot of the increment in tryptophan fluorescence versus the NTP concentration. 0.17 mg/ml HMM, 50mM KCl, and 5mM MgCl$_2$ at pH 7.8 and 21-22°. Excitation at 290nm; emission at 340 nm. F indicates the intensity of tryptophan fluorescence of HMM before NTP addition. Bars indicate standard means of errors.

Table III.   HMM-NTPase Activity and Change in Trp Fluorescence

50 mM KCl, 5 mM MgCl$_2$, 20 mM Tris-HCl, pH 7.8, 20°.

Excitation at 290 nm; emission at 340 nm.

Rotation about glycosyl bond	NTP	NTPase activity		Fluorescence change	
		V_m (min^{-1})	K_M (μM)	$\Delta F_m/F$ (%)	K_{fl} (μM)
Easy natural	ATP	4	<1	17	<0.1
	ITP*	36	0.8	7.1	- ‡
	εATP+	7.8	0.93	5.9#	14.8
anti	8-NH·CH$_3$ ATP	50	121	9.5#	25
	8,3'-S-cyclo ATP	500	132	12	4
intermediate	8,2'-S-cyclo ATP	263	53		
Difficult syn	8-Br ATP	21	27		
	8-N(CH$_3$)$_2$ ATP	70	200		
	8-OCH$_3$ ATP	very large	very large		
	8-OC$_2$H$_5$ ATP	95	115	5.0	2.9
	8-SCH$_3$ ATP	23	500	8.1	1.1
	8=O ATP	76	89	17	- ‡
	8-N$_3$ ATP	35	52	10.6	6.2

* 50 mM KCl, 2 mM MgCl$_2$, pH 7.8, 20°. A. Inoue, Y. Tonomura and S. Watanabe, J. Biochem., 77, 1135-1145 (1975).

+ 1 M KCl, 2 mM MgCl$_2$, pH 7.5, 20°. H. Onishi, E. Ohtsuka, M. Ikehara and Y. Tonomura, J. Biochem., 74, 435-450 (1973).

‡ F/ΔF vs [XTP]$^{-1}$ non-linear.

Excited at 300 nm. Emitted light was filtered with 310 nm-filter. $\Delta F_m/F$ of ATP was 10 %.

escence after it. Table III summarizes the estimated maximum increment of fluorescence ($\Delta F_m/F$) and the concentration for the half maximal change (K_{fl}). $\Delta F_m/F$ values varied with the NTP structure as already reported by Werber et al. (58). The K_{fl} values for NTPs with difficult rotation about the glycosyl bond were much larger than the value for ATP. The double-reciprocal plots for 8-NH·CH$_3$ ATP and 8-SCH$_3$ ATP were not linear, and the K_{fl} values could not be estimated. Table III clearly shows that for every NTP with difficult rotation about the glycosyl bond, the K_m value is larger than the K_{fl} value.

Dissociation of acto-HMM and acto-S-1. --- It is well known (59,60) that ATP induces dissociation of acto-HMM and acto-S-1. Figure 8 shows time courses of change in light-scattering intensity of acto-HMM reconstituted from 0.34 mg/ml HMM and 0.09 mg/ml FA after adding various concentrations of 8-Br ATP in 50mM KCl, 5mM MgCl$_2$, and 10mM Tris-HCl at pH 7.6 and 20°. The time for half-maximum decrease in light-scattering intensity, $\tau_{1/2}$, for acto-HMM after adding 8-Br ATP was 0.75 sec for 6.25μM, 0.6 sec for 12.5μM, and 0.4 sec for both 25 and 37.5μM 8-Br ATP. The intensity remained at the lowest level, LS_c, for some time, and then gradually increased. Figure 9 shows semilogarismic plots of the time courses of recovery of light-scattering intensity. The apparent first-order rate constants of recovery, k_r, for the two highest concentrations of 8-Br ATP were almost equal, i.e. 0.17 sec^{-1} for 25μM, and 0.15 sec^{-1} for 37.5μM 8-Br ATP. They were larger than the k_r value for 6.25μM ATP, i.e. 0.082 sec^{-1}.

The extent of dissociation of acto-HMM reconstituted from

Fig. 9. Recovery from reduced light-scattering intensity as a function of time after adding NTP. Experimental conditions were as in Fig. 8. ○, 37.5 μM 8-Br ATP; ●, 25μM 8-Br ATP; ×, 6.25μM ATP. LS_c is the extent of maximum decrease in light-scattering intensity at each NTP concentration.
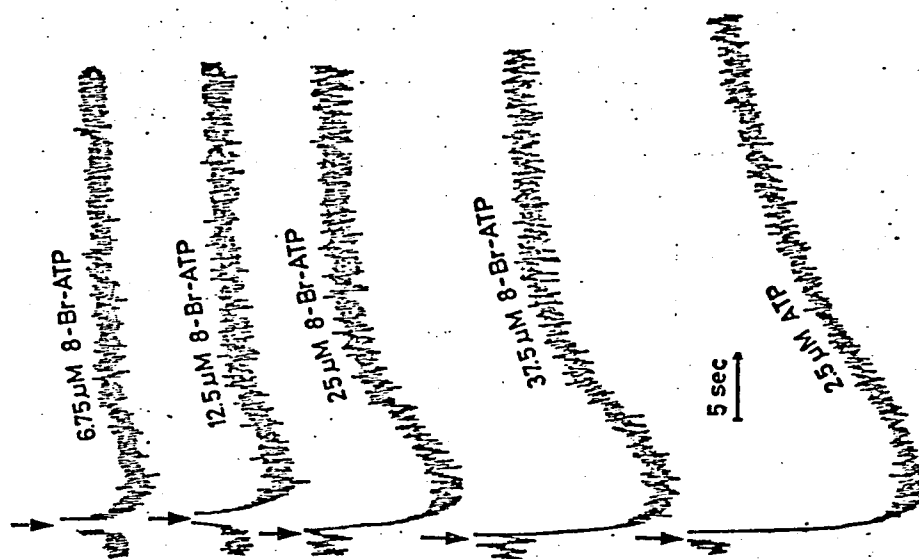

Fig. 8. Time course of changes in the light-scattering intensity of acto-HMM after adding 8-Br ATP. 0.34 mg/ml HMM, 0.09 mg/ml FA, 50mM KC1, 5mM $MgCl_2$, 10mM Tris-HC1, pH 7.6, 20°. Arrow indicates the point of flow-stop.
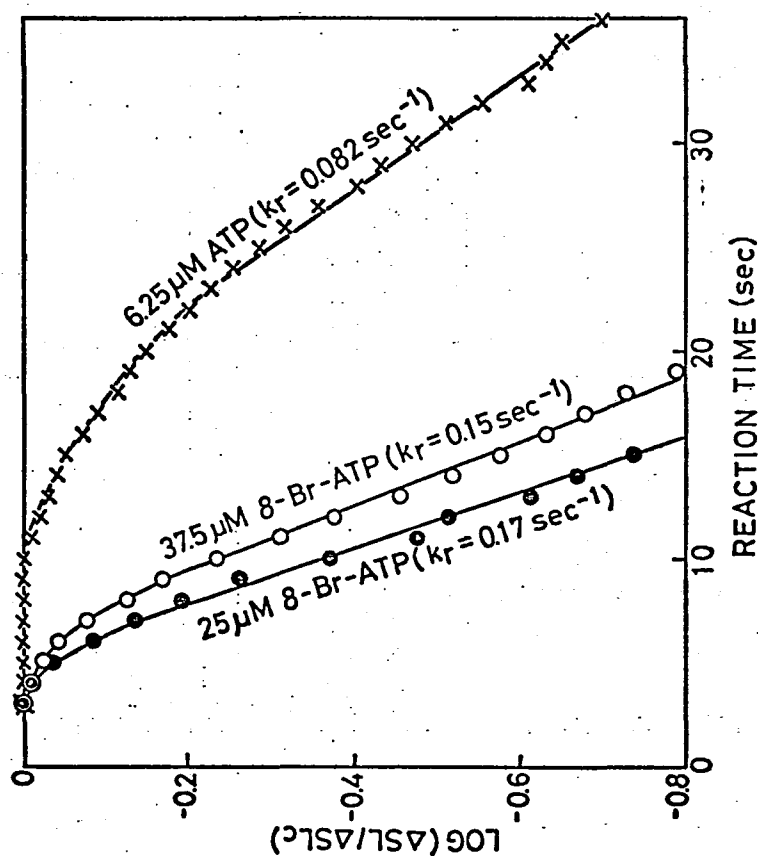
0.34 mg/ml HMM and 0.09 mg/ml FA-RP induced by NTP was measured
in 50mM KCl and 5mM $MgCl_2$ at pH 7.8 and 20°. The double-reciprocal
plot of the relative extent of decrease in light-scattering (ΔLS)
induced by NTP ($\Delta LS_{NTP}/\Delta LS_{ATP\rightarrow\infty}$) against the NTP concentration
(Fig. 10) showed that the maximum decrease induced by a suf-
ficient amount of NTP is constant, regardless of its structure.
The dependence of the extent of dissociation of acto-HMM on the
concentration of NTP followed the Michaelis-Menten equation. The
concentrations (K_{dis}) required for half maximal dissociation are
summarized in Table IV. Clearly, NTPs with easy rotation about
the glycosyl bond generally dissociated acto-HMM at low concen-
tration, whereas most NTPs with difficult rotation showed large
K_{dis} values, regardless of whether they had an anti-, intermediate-
or syn-conformation.

The extent of dissociation of acto-S-1 reconstituted from 0.7
mg/ml S-1 and 0.4 mg/ml FA-RP after adding 0.5mM NTP was measured
by the rapid filtration method in 50mM KCl and 2mM $MgCl_2$ at pH 7.0
and 20°. The results are summarized in Table V. In the case of
S-1 alone, 93% of the applied protein passed through the filter,
whereas only 1.3% of FA-RP did. Even when no protein was applied,
7-8 μg/ml of protein appeared in the filtrate, since the HMM used
for pretreatment of the membrane was partially removed. The extent
of dissociation, α, was calculated from the protein concentration
in the filtrate of acto-S-1 relative to the sum of the protein
concentrations in the filtrates of buffer alone, S-1, and FA-RP
alone. The α value was 19.6% in the absence of NTP, and 85.4% and
86.8% in the presence of ATP and 8-Br ATP, respectively. Thus,
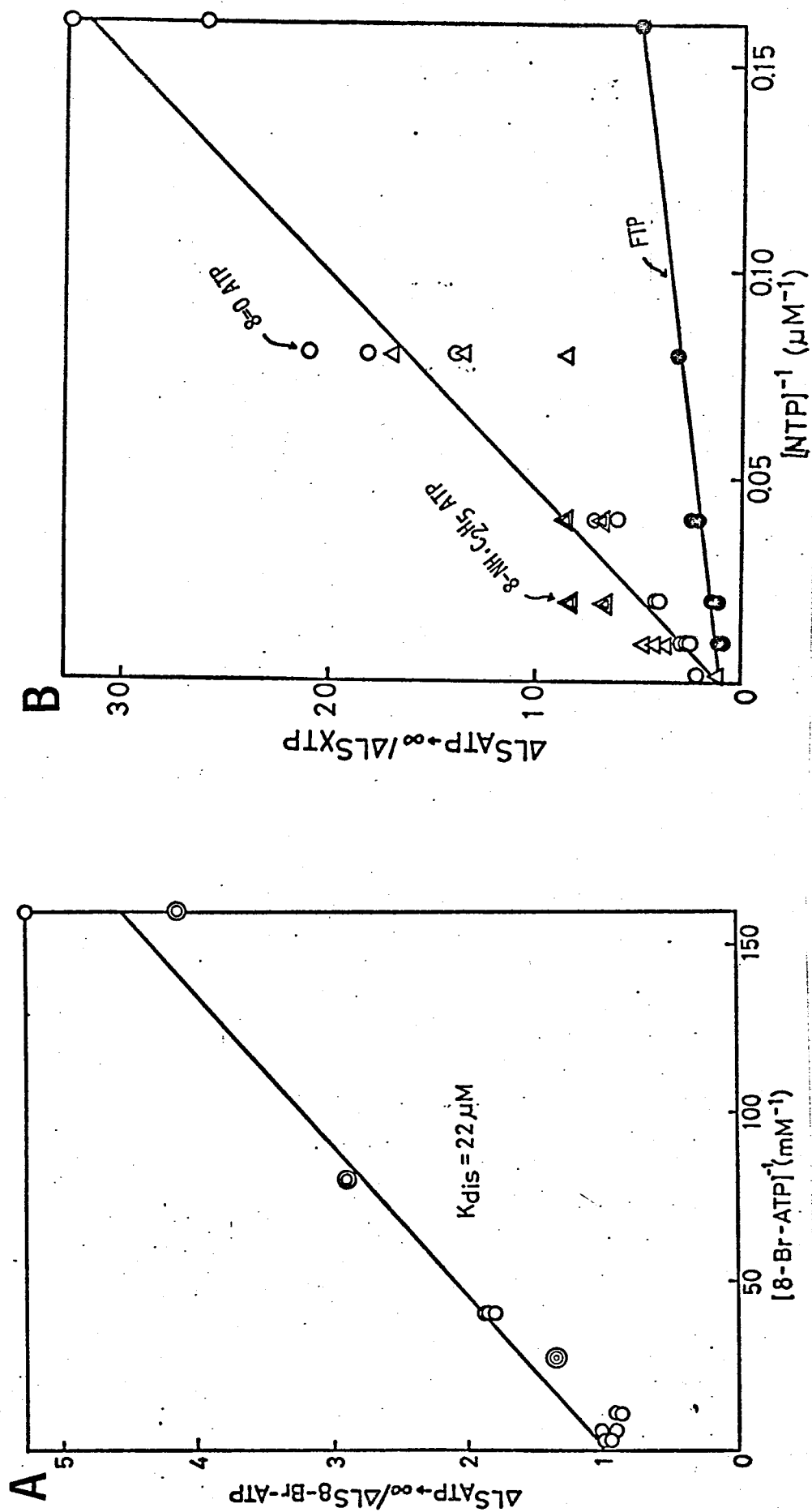
Fig. 10. Double-reciprocal plots of the relative decrease in light-scattering intensity of acto-HMM, $(\Delta LS_{NTP}/\Delta LS_{ATP\to\infty})$, versus the concentration of NTP. Experimental conditions were as in Fig. 8. ΔLS_{NTP} represents the decrease in light-scattering intensity after NTP addition, and $\Delta LS_{ATP\to\infty}$ the maximum decrease after sufficient ATP addition. A: 8-Br ATP; B, FTP ($\bullet$); 8=0 ATP ($\bigcirc$); and 8-NH·C_2H_5 ATP ($\triangle$).

Table IV.   Dissociation of Acto-HMM Induced by NTP

0.34 mg/ml HMM, 0.09 mg/ml FA^{RT}, 50 mM KCl, 5 mM MgCl$_2$, 20 mM Tris-HCl, pH 7.8, 20°.

Rotation about glycosyl bond		NTP	K_{dis} (μM)
natural		ATP	< 1
Easy		2'-dATP	< 5*
		ITP	0.5 - 1[+]
	synthetic	3'-dATP	< 5*
		FTP	25
	anti	8-NH·CH$_3$ ATP	250
Difficult		8,3'-S-cyclo ATP	>100 ‡
	intermediate	8,2'-S-cyclo ATP	>100 ‡
	syn	8-Br ATP	22
		8-NH·C$_2$H$_5$ ATP	200
		8-OCH$_3$ ATP	143
		8-SCH$_3$ ATP	61
		8=O ATP	200

The extent of the decrease in light scattering intensity attained the same level in all NTP.

* The relative decrease in light scattering intensity were 87 and 65 % with 5 μM of 2'-dATP 3'-dATP, respectively.

[+] A. Inoue, Y, Tonomura and S. Watanabe, J. Biochem., 77, 1135-1145 (1975).

‡ The relative decrease did not exceed 20 % with both 8,2'- and 8,3'-S-cyclo ATP even at 100 μM.

Protein	Nucleotide Added (0.5 mM)	Protein in Filtrate* (µg/mL)	α (%)
None	None	7.6	
S-I	None	657	
F-A^{RT}	None	4.9	
F-A^{RT}	8-Br-ATP	I.I	
Acto-S-I	None	I3I	I9.6
Acto-S-I	ATP	572	85.4
Acto-S-I	8-Br-ATP	58I	86.8

Table V. Dissociation of acto-S-1 on adding NTP. 0.7 mg/ml S-1, 0.4 mg/ml FA-RP, 50mM KCl, 2mM MgCl$_2$, 20mM Tris-maleate, pH 7.0, 20°. NTP (0.5mM) was added to the protein solution, and the resulting solution was filtered through a Millipore filter (0.3µm) pretreated with HMM. The extent of dissociation, α, was calculated from the protein concentration in the filtrate of acto-S-1 relative to the sum of those for no protein, S-1, and FA-RP.

acto-S-1 was dissociated as completely by adding 8-Br ATP as by
adding ATP. As described in 'Introduction' for this Part, S-1
is an equimolar mixture of two fractions (3-5): one which shows
an initial Pi-burst (S-1B), and one which does not but has
ATPase activity via route-2 (cf. 'Introduction') (S-1A). Therefore,
this result indicates that acto-S-1A dissociates on the formation
of $^\circ$MNTP, and that acto-S-1B dissociates on the formation of
M_2NTP. This agrees with the results obtained with AMPPNP, which
is not hydrolyzed by myosin, but which dissociates actomyosin
completely and causes changes in the fluorescence of myosin by the
formation of an intermediate, M_2AMPPNP, in route-1 (cf. 'Intro-
duction') (Fig. 11).

Actomyosin NTPase and initial Pi-burst. --- The effects of 0.5
mg/ml FA-RP on hydrolysis of 0.3mM NTPs by 0.1 mg/ml myosin were
observed in the presence of 2mM $MgCl_2$ and 20μM $CaCl_2$ in 40mM KCl
at pH 7.8 and 20° (Figs. 5, 12, and Table VI). Hydrolysis of ATP,
3'-dATP and FTP by myosin were markedly activated by FA-RP, where-
as hydrolysis of every type of NTP with difficult rotation about
the glycosyl bond by myosin were not activated or inhibited by
FA-RP. The apparent inhibition was found to be competitive;
addition of FA-RP increased the K_m value without changing the V_m
value for hydrolysis by myosin of NTP with difficult rotation
(Fig. 13). It is widely accepted that the main intermediate of
actomyosin NTPase is a myosin-P-NDP complex (M_p^{NDP}), the formation
of which is observed as an initial Pi-burst (1,2,62,63). As
shown in Table VI, the size of the initial Pi-burst was 0.5-0.6
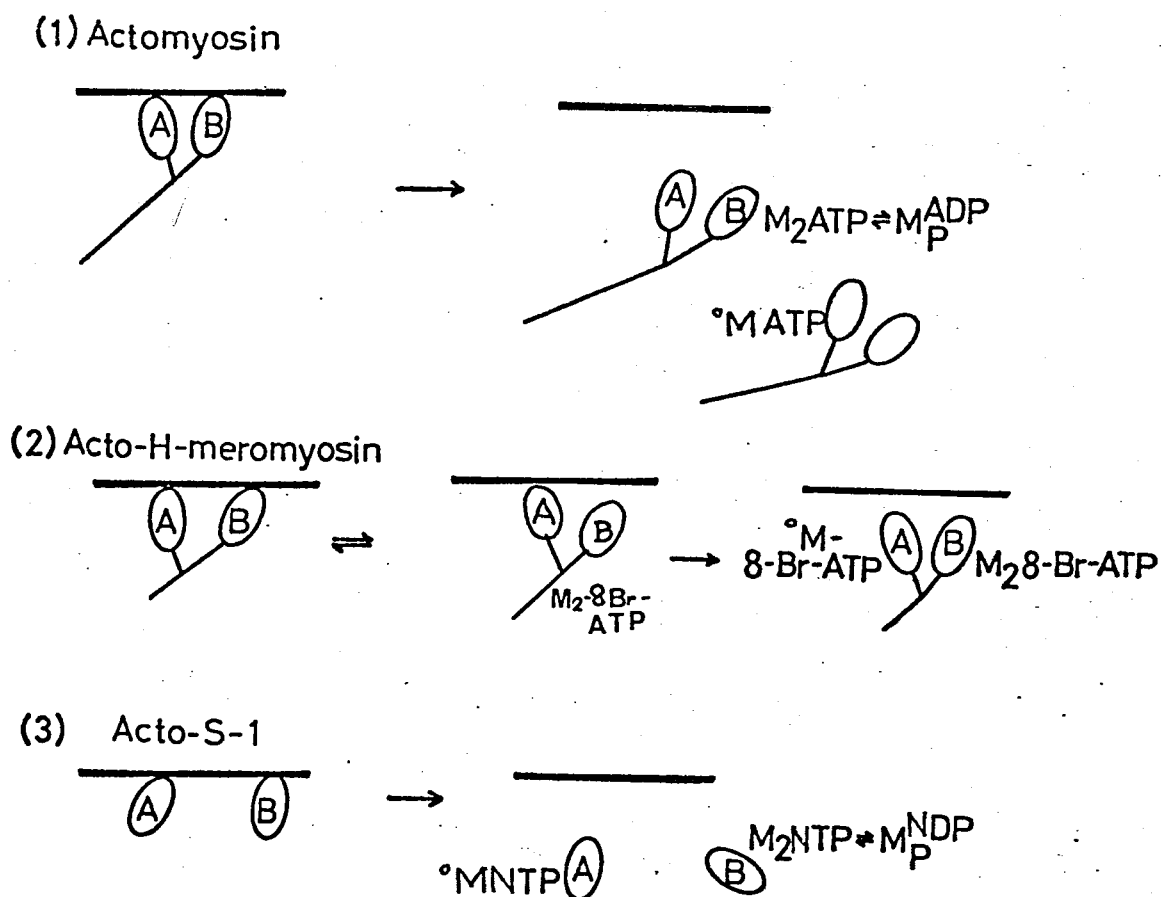mole per mole of myosin head for all NTPs with easy rotation about

(1) Actomyosin

(2) Acto-H-meromyosin

(3) Acto-S-1

Fig. 11. Schematic models of dissociation of actomyosin, acto-HMM, and acto-S-1 complexes. As already snown by Takeuchi and Tonomura (60), actomyosin dissociates on binding 0.5 mole of ATP/mole of myosin head. On the contrary, acto-HMM and acto-S-1 reqire 1 mole of ATP/mole of head for dissociation. 8-Br ATP binds to head B with higher affinity than to head A. Detailed discussion is described in the text.
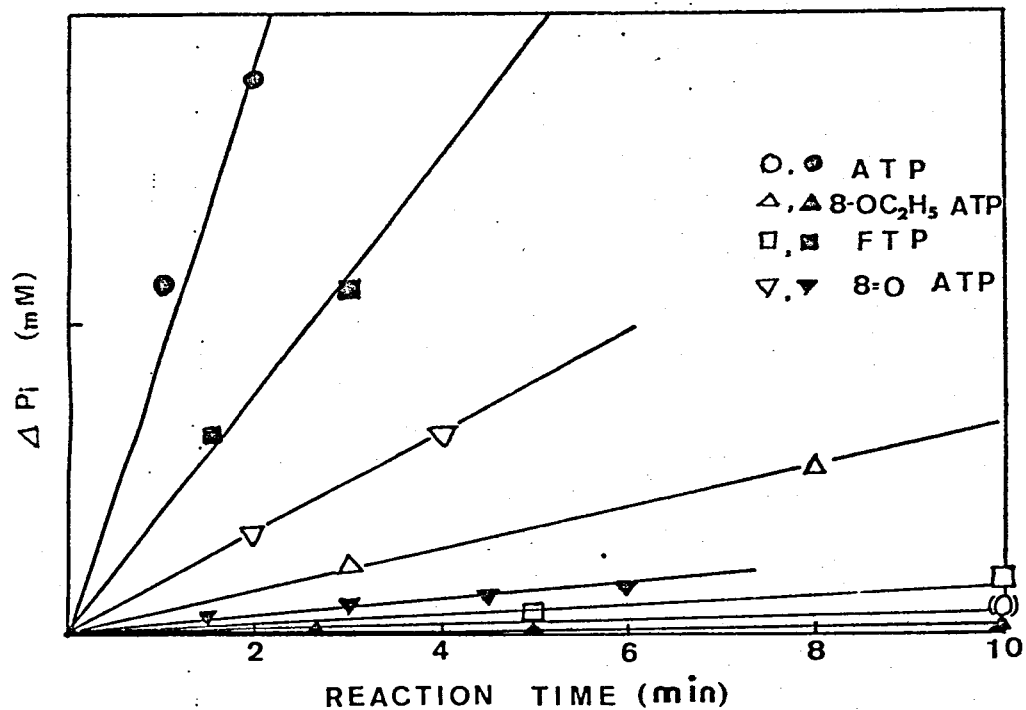
Fig. 12. Effects of FA on the myosin NTPase activity. 0.1 mg/ml myosin, 40mM KCl, 5mM $MgCl_2$, 10mM Tris-HCl, pH 7.6, 20°. NTPase activities were measured in the presence (closed symbols) and absence (open symbols) of 0.5 mg/ml FA.
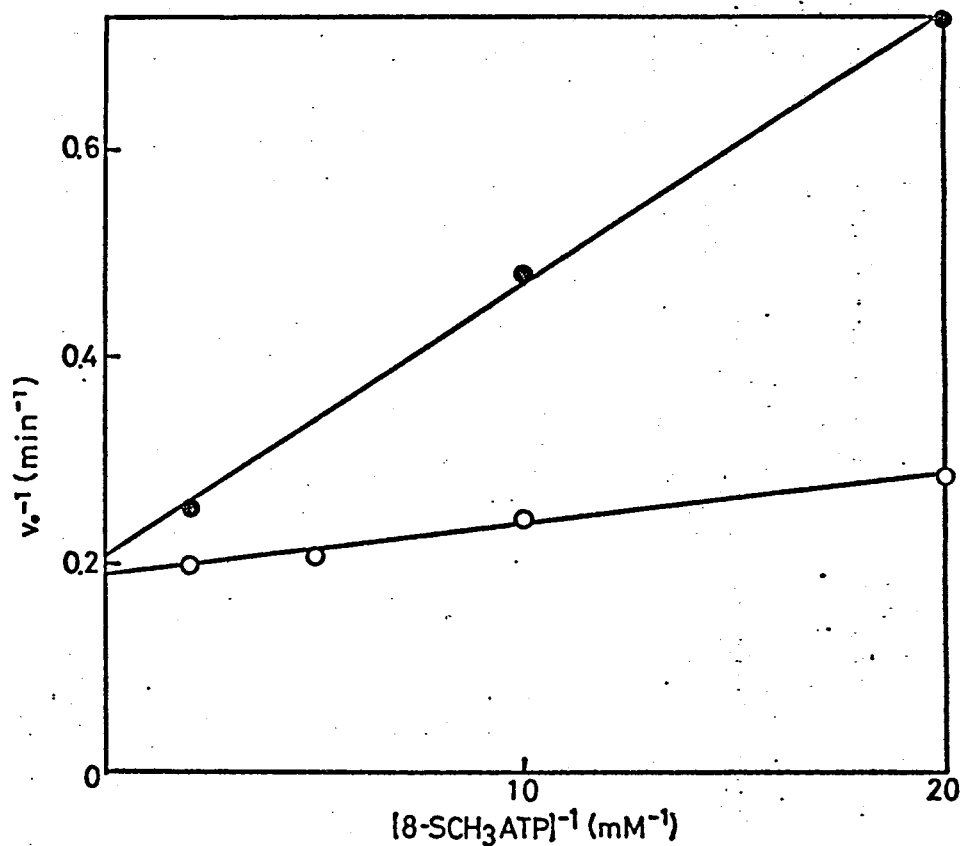

Fig. 13. Double-reciprocal plots of 8-SCH$_3$ ATPase activity of myosin in the presence and absence of FA versus the concentration of 8-SCH$_3$ ATP. 0.1 mg/ml myosin, 50mM KCl, 2mM $MgCl_2$, 20μM $CaCl_2$, 20mM Tris-HCl, pH 7.8, 20°. Activities were measured in the presence (●) and absence (○) of 0.4 mg/ml FA-RP.

Table VI.   Actomyosin NTPase Activity

0.1 mg/ml myosin, 0.3 mM NTP, 20 mM Tris-HCl, pH 7.8, 20°

Rotation about glycosyl bond		NTP	P_i-burst size (mole/mole of head)	NTPase activity (min^{-1})		
				50 mM KCl 5 mM $MgCl_2$	0.5 mg/ml FARP 2 mM $MgCl_2$, 20 μM $CaCl_2$	0.5 mg/ml FARP, 40 mM KCl, 40 mM $CaCl_2$
Easy	natural	ATP	0.5 - 0.6	3.5		359
		2'-dATP		2.6		
	synthetic	ITP*	0.5			
		εATP+	0.6			
		3'-dATP		4.8		233
		FTP	0.5‡	8.8		227
Difficult	anti	8-NH·CH₃ ATP	0	36	0.5	
		8,3'-S-cyclo ATP	0	115	43	
	intermediate	8,2'-S-cyclo ATP	0	58	8	
	syn	8-Br ATP	0	37	37	
		8-NH·C₂H₅ ATP	0	5.8	4.5	
		8-N(CH₃)₂ ATP		39	11	
		8-OCH₃ ATP	0	4.4	2	
		8-OC₂H₅ ATP		32	0.7	
		8-SCH₃ ATP	0	4.8	4.4	
		8=O ATP		76	14	
		8-N₃ ATP		35	10	

* 0.1 M KCl, 2 mM $MgCl_2$, pH 7.8, temp > 20°. A. Inoue, K. Kikuchi and Y. Tonomura, J. Biochem., 82, 783-800 (1977).

+ 1 M KCl, 2 mM $MgCl_2$, pH 7.5, 20°. H. Onishi, E. Ohtsuka, M. Ikehara and Y. Tonomura, J. Biochem., 74, 435-450 (1973).

‡ H. Onishi, personal communication.

the glycosyl bond, whereas Pi-burst was not found for any type of NTPs with difficult rotation (Figs. 14,15).

Superprecipitation of actomyosin and contraction of myofibrils. --- As is well known, Mg^{2+}-ATP induces superprecipitation of actomyosin at low ionic strength, and this can be followed by measuring the turbidity of an actomyosin suspension (1). Figure 16 shows the time courses of increase in turbidity at 660 nm when 0.5mM NTP was added to an actomyosin suspension reconstituted from 0.8 mg/ml myosin and 0.2 mg/ml FA in 0.1M KCl, 1mM $MgCl_2$ at pH 7.0 and 20°. Superprecipitation occurred rapidly on adding NTP with easy rotation about the glycosyl bond, but did not occur on adding NTP with difficult rotation.

Myofibrils contract on adding Mg^{2+}-ATP, and with sufficient amount of Mg^{2+}-ATP contraction bands are formed. Contraction of myofibrils was observed after adding NTP to 4.4 mg/ml of myofibrillar protein in 40mM KCl, 1mM $MgCl_2$, 10μM $CaCl_2$ at pH 7.0 and 20°. Figure 17 shows the time courses of change in sarcomere length after adding various concentrations of Mg^{2+}-ATP (A) and Mg^{2+}-8-Br ATP (B). The sarcomere length before adding NTP was 2.14±0.15μm . As shown in Fig. 17A, the sarcomere length scarcely changed on adding 2.5, 5, or 10μM ATP, but shortening was clearly observed on adding 20μM ATP, and the length decreased to 1.23± 0.335μm on incubation for 120 sec. When 100μM ATP was added, the sarcomere length decreased rapidly, and reached 1.11±0.135μm within 20 sec. In contrast, no shortening was observed on addition of 8-Br ATP, even at a concentration of 200μM, as shown in Fig. 17B.
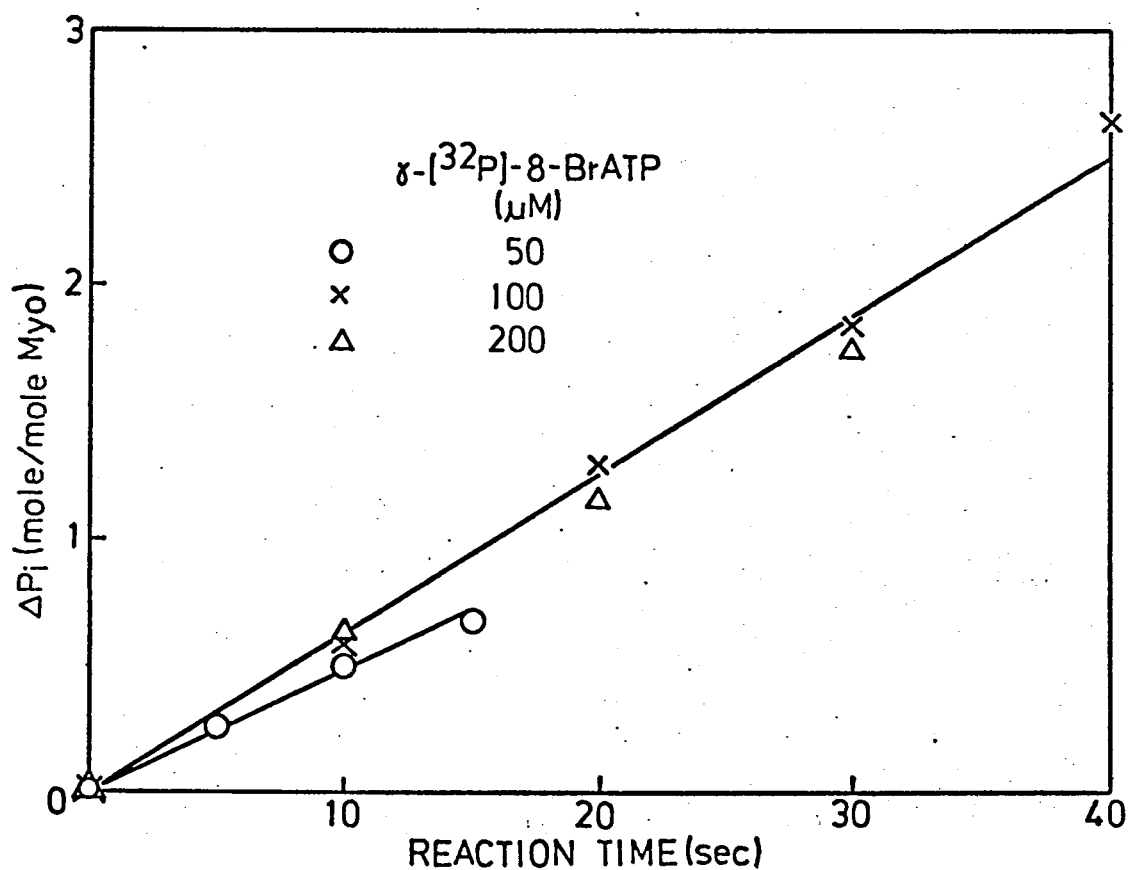
Fig. 14. Time course of ^{32}Pi-liberation from myosin-8-Br ATP system. 2.4 mg/ml myosin, 0.5M KCl, 5mM $MgCl_2$, 20mM Tris-HCl, pH 8.0, 0-1°. The concetrations of 8-Br-γ-[^{32}P] ATP added were indicated in the figure.
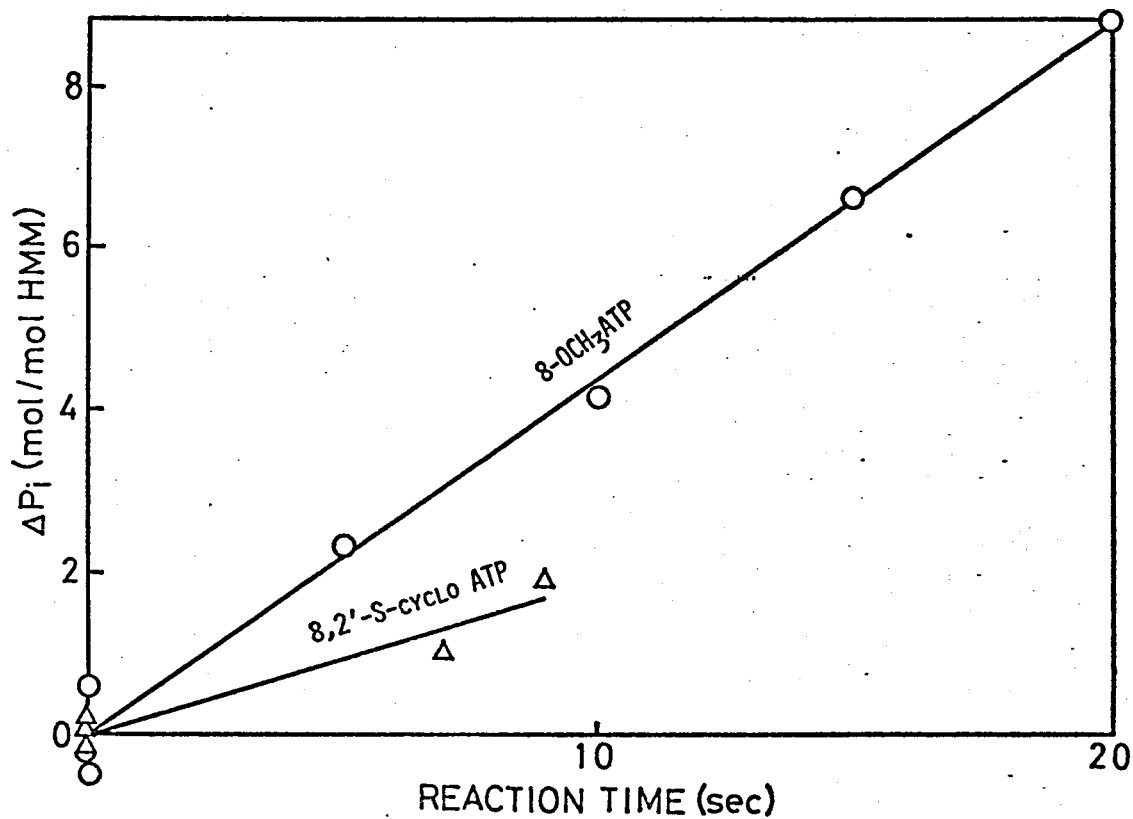

Fig. 15. Time course of Pi-liberation from HMM-NTP systems. 0.3mM NTP, 6.8 mg/ml HMM, 0.5M KCl, 5mM $MgCl_2$, 20mM Tris-HCl, pH 7.8, 0-1°. NTPs used were indicated in the figure.

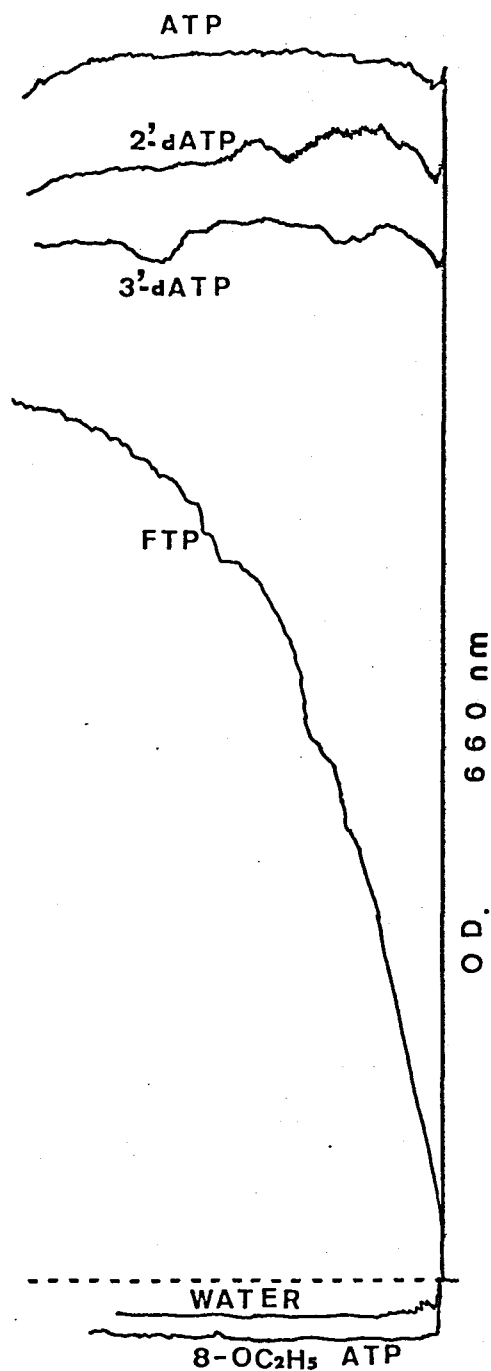Fig. 16. Superprecipitation of actomyosin induced by 0.5mM of ATP, 2'-dATP, 3'-dATP, FTP, and 8-OC_2H_5 ATP. 0.8 mg/ml myosin, 0.2 mg/ml FA-RP, 0.1M KCl, 1mM $MgCl_2$, 10mM Tris-maleate, 10μM $CaCl_2$, pH 7.0, 20°. Superprecipitation of actomyosin was followed the change in turbidity at 660nm. Dotted line represents the level before NTP addition, and water addition shows the decrease in turbidity by dilution.

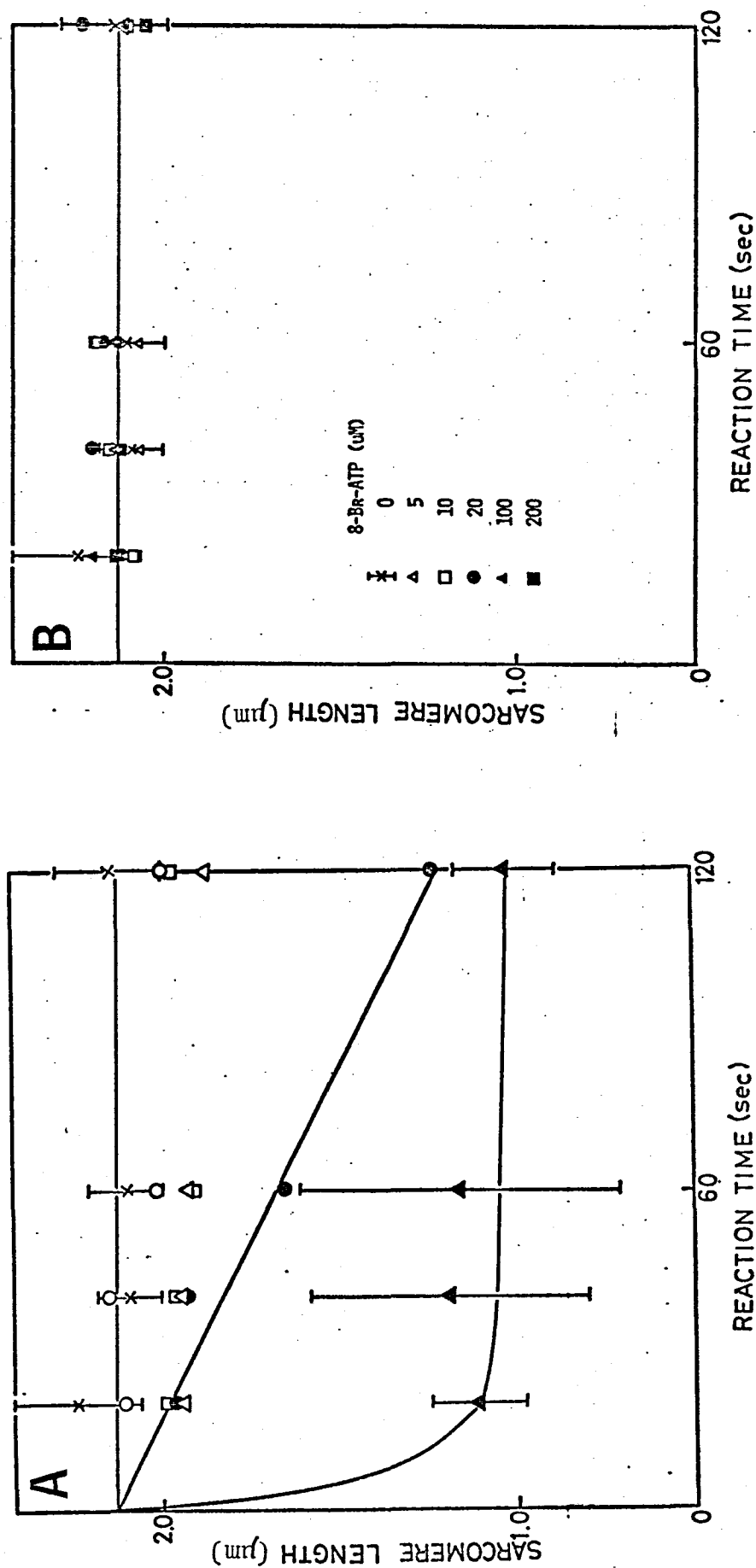Fig. 17. Time courses of shortening of myofibrils after adding
NTP. 4.4 mg/ml myofibrillar protein, 40mM KCl, 1mM MgCl$_2$, 10μM
CaCl$_2$, 30mM Tris-maleate, pH 7.0, 20°. The average sarcomere
length was measured as described in "EXPERIMENTALS." NTP added:
A, ATP; B; 8-Br ATP. Concentrations of NTP: ✗, 0; ○, 2.5μM; □,
5μM; △,10μM; ●, 20μM; ▲, 100μM; ■, 200μM. The bars indicate the
standard means of errors.
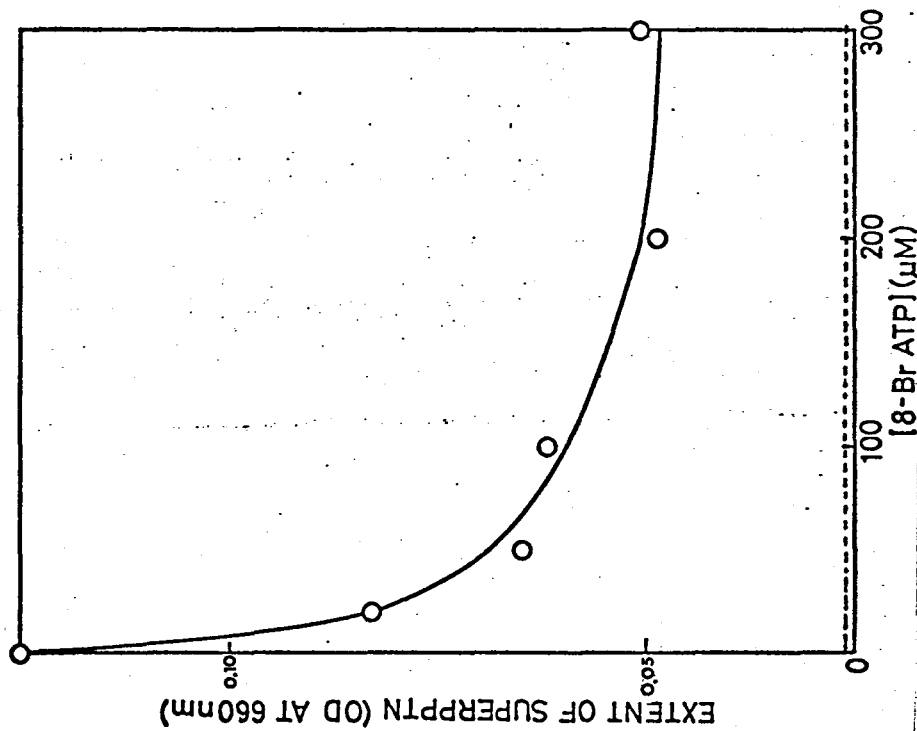
Fig. 19. Inhibitory effect of 8-Br ATP on the superprecipitation of actomyosin induced by ATP. 0.2 mg/ml myosin, 0.05 mg/ml FA-RP, 10μM ATP, 0.1M KCl, 1mM MgCl$_2$, 10μM CaCl$_2$, 10mM Tris-maleate, pH 7.0, 20°. The extent of superprecipitation was obtained as the final value of 660nm at the highest plateau relative to the value of the clearing response of actomyosin. The broken line indicates the absorbance at 660 nm before adding ATP (cf. Fig. 16).

Fig. 18. Inhibition of ATPase activity of HMM and acto-HMM by 8-Br ATP. 0.1 mg/ml HMM, 2.5μM γ-[^{32}P]-ATP, 40mM KCl, 2mM MgCl$_2$, 20μM CaCl$_2$, 20mM Tris-maleate, pH 7.0, 20°. ATPase activities were measured in the presence (O,●) and absence (△,▲) of 0.6 mg/ml FA-RP. Concentrations of 8-Br ATP: O,△,0; ●,▲, 150μM.

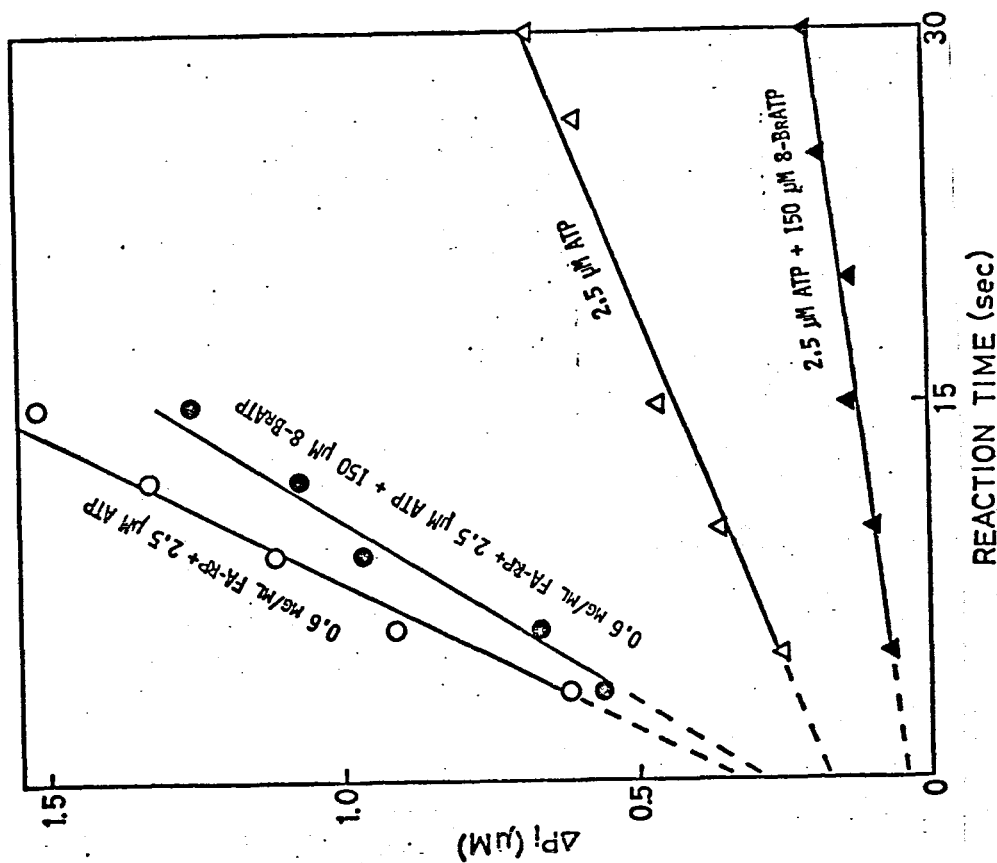Table VII summarizes the efficiency of NTPs for supporting the contraction of myofibrils and/or superprecipitation of acto-myosin. Both natural and synthetic NTPs with easy rotation about the glycosyl bond supported myofibrillar contraction and superprecipitation of actomyosin, as expected from marked acceleration with FA-RP of their hydrolyses by myosin (Table VI). On the other hand, all the NTPs with difficult rotation did not support superprecipitaion of actomyosin, regardless of their conformation.

Inhibition of ATPase by 8-Br ATP. --- Figure 18 shows the effects of 150μM 8-Br ATP on the time courses of ^{32}Pi-liberation after adding 2.5μM γ-[^{32}P]-ATP to 0.1 mg/ml HMM in 40mM KCl, 2mM MgCl$_2$, and 20μM CaCl$_2$ at pH 7.0 and 20° in the presence and absence of 0.6 mg/ml FA-RP. The rate constant of HMM ATPase decreased from 3.4 to 1.1 min^{-1} on adding 8-Br ATP. Thus, HMM ATPase in the presence of 2.5μM ATP was inhibited 68% by adding 150μM 8-Br ATP. Figure 19 shows the inhibition by 8-Br ATP of the superprecipitation of actomyosin induced by ATP. ATP (10μM) together with various concentrations of 8-Br ATP was added to actomyosin (0.2 mg/ml myosin and 0.05 mg/ml FA-RP) in 0.1M KCl, 1 mM MgCl$_2$, and 10μM CaCl$_2$ at pH 7.0 and 20°. The extent of superprecipitation decreased to a steady level of 40% with increase in 8-Br ATP, and the concentration of 8-Br ATP for the half-maximum effect was 20μM. These results indicate that 8-Br ATP and ATP bind at the same site of myosin. However, superprecipitaion of actomyosin was partially inhibited (60%) by adding sufficient ATP. Furthermore, 20μM 8-Br ATP was required for half-

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Table VII.   Relation between superprecipitation of actomyosin and glycosyl torsion of NTP

Rotation about glycosyl bond		NTP	Supported contraction of myofibrils and/or superprecipitation of actomyosin
Easy	natural	ATP, 2'-dATP*, ITP*, UTP*, TTP*, GTP+, CTP+	Yes
	synthetic	FTP‡, 3'-dATP‡, εATP§, N^6-monomethyl ATP*, N^6-dimethyl ATP*, 2',3'-0-diacetyl ATP*, 2',3'-0-isopropylidene ATP*, 6-thio ITP∥	
Difficult	anti	8-NH·CH$_3$ ATP‡, 8,3'-S-cyclo ATP‡	No
	intermediate	8,2'-S-cyclo ATP‡	
	syn	8-Br ATP, 8-NH·C$_2$H$_5$ ATP‡, 8-N(CH$_3$)$_2$·ATP‡, 8-OCH$_3$ ATP‡, 8-OC$_2$H$_5$ ATP‡, 8-SCH$_3$ ATP‡, 8=0 ATP‡, 8-N$_3$ ATP‡, 8-CH$_3$ ATP‡	

* Tonomura, Y. (1972) Muscle Proteins, Muscle Contraction and Cation Transport (Univ. Tokyo Press, Tokyo & Park Univ. Press, Baltimore).

+ Hasselbach, W. (1956) Biochim. Biophys. Acta 20, 355-368.

‡ Only superprecipitation was observed.

§ Mowery, P. C. (1973) Arch. Biochem. Biophys. 159, 374-377.

∥ Murphy, A. J. & Morales, M. F. (1970) Biochemistry 9, 1528-1532.

maximum inhibition of the superprecipitaion of actomyosin after adding 10μM ATP, while the actomyosin ATPase activity in the presence of 2.5μM ATP only decreased 20% on adding 150μM 8-Br ATP. These results indicate that the mechanism of superprecipitaion is complex.

CONCLUSION

The following reaction scheme is generally accepted for myosin ATPase which is coupled with tension development during muscle contraction (1,2,62,63, and "Introduction"):

$$M + ATP \rightleftharpoons M_1ATP \rightleftharpoons M_2ATP \rightleftharpoons M_p^{ADP} \longrightarrow \longrightarrow M + ADP + Pi$$
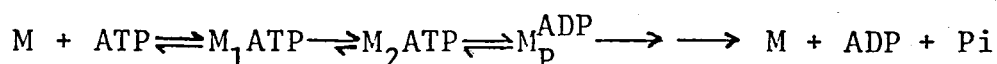
Formation of M_1ATP is not accompanied by a fluorescence change of tryptophan. As described in 'Introduction' of this Part, M_2ATP and M_p^{ADP} are accompanied by an increment of tryptophan fluorescence on adding NTP, and these intermediates are in a rapid equilibrium. The M_p^{ADP} formation is observed as a Pi-burst, and its decomposition is activated by FA. Actin-activated ATPase of myosin is closely related to muscle contraction (1,2,62,63).

In summary, the results obtained in this work indicated that the key intermediate for actomyosin NTPase is M_p^{NDP}, not M_2NTP, that hydrolysis of 8-Br ATP via route-2 is not affected by FA, and that difficulty of rotation about the glycosyl bond affects two characteristic properties of Mg^{2+}-NTPase reactions of myosin and actomyosin (Table VIII). First, all the NTPs with difficult rotation about the glycosyl bond did not support initial Pi-burst, actin-activation of myosin NTPase and superprecipitation of actomyosin, although they increased the tryptophan fluorescence of HMM (Tables III,IV,VI, & VIII). The properties common to NTPs

Table VIII. Summary of the interactions between stereospecific ATP analogs and myosin NTPase.

ATP ANALOGS	MYO-NTPASE IN THE STEADY STATE*		REACTION ON HEAD B	EFFECT OF F-A ON MYO-NTPASE
	EDTA (K^+)	Mg^{2+}		
ROTATION ABOUT GLYCOSYL BOND IS EASY	VERY HIGH	VERY LOW	$M + NTP \rightleftharpoons M_I NTP \rightleftharpoons M_2 NTP$ $\rightleftharpoons M_P^{NDP} \dashrightarrow M + NDP + P_I$	MARKED ACCELERATION
ROTATION ABOUT GLYCOSYL BOND IS DIFFICULT	NEGLIGIBLY SMALL	VERY HIGH	$M + NTP \rightleftharpoons M_I NTP \rightleftharpoons M_2 NTP$ $\not\rightleftharpoons M_P^{NDP}$	COMPETITIVE INHIBITION

* FOR ATP ANALOGS WITH 6-NH OF THE BASE.

ATP analogs used in this study are classified into two groups. Effects of EDTA and Mg^{2+} ions on the myosin NTPase activities are opposite between NTPs with easy rotation about the glycosyl bond and with difficult rotation. Furthermore, NTPs with easy rotation about the glycosyl bond showed initial Pi-burst, actin-activation of myosin NTPase, and contraction of myofibrils, whereas NTPs with difficult rotation showed no effect on these phenomena. However, NTPs with difficult rotation showed the increment of tryptophan fluorescence From these facts, it was concluded that NTPs with easy rotation about the glycosyl bond formed both the $M_2 NTP$ and M_P^{NDP}, whereas NTPs with difficult rotation formed only $M_2 NTP$.

with difficult rotation did not depend on the confomation and substituents. Second, in the case of NTPs with 6-NH and in the presence of Mg^{2+} ions, all the analogs with difficult rotation about the glycosyl bond were hydrolyzed by myosin much faster than NTPs with easy rotation (Table II). The K_m values for hydrolysis of NTPs with difficult rotation were larger than K_{fl} values estimated from the change in tryptophan fluorescence (Table III). Furthermore, NTPs with difficult rotation about the glycosyl bond were hardly hydrolyzed in the presence of EDTA (Table II).

There are two possible explanations for these results. If the two heads of myosin are identical and reaction intermediates of myosin NTPase are formed sequentially, as asserted by Taylor's (62) and Trentham's (63) groups, no occurrence of initial Pi-burst and actin-activation of myosin NTPase for NTP with difficult rotation indicates that the conversion from M_2NTP into M_p^{NDP} is rate-limiting. However, this assumption is inconsistent with the fact that K_m values were larger than K_{fl} values for NTPs with difficult rotation about the glycosyl bond (Table III). On the other hand, there are many experimental results indicating that the two heads of myosin are not identical (1,2), and have shown that myosin subfragment-1 (S-1) is an equimolar mixture of S-1B forming M_p^{ADP} and S-1A forming a MATP complex by separation of two S-1 fractions (3-5). Accordingly, all the results obtained with NTPs with difficult rotation about the glycosyl bond can be explained by assuming that these NTPs are hydrolyzed mainly on head A (S-1A), and that M_2NTP formed on head B (S-1B) is not con-

verted into M_p^{NDP}. The non-identical two head hypothesis is also consistent with the fact that NTPs with difficult rotation can not be used as a substrate for EDTA(K^+)-NTPase, which is previously shown as being catalyzed by head B (64).

Furthermore, two recent studies clearly showed that 8-Br ATP is hydrolyzed only on head A. Inoue et al. (64) showed that the steady-state hydrolysis of Mg^{2+}-8-Br ATP by HMM appeared immediately after its addition to M_p^{ADP} which had been formed on head B, indicating that hydrolysis of 8-Br ATP does not require decomposition of M_p^{ADP}, which takes several minutes under the conditions used. Takashi et al. (65) measured the binding of 8-Br ATP to S-1 from the decrease in fluorescence of 4,4'-bis-(1-anilinonaphthalene-8-sulfonate) bound to the active sites, and showed that S-1 behaves like an equimolar mixture of two components, each with its own dissociation constant for the binding of 8-Br ATP. These two dissociation constants were almost equal to the K_m and K_{fl} values of Mg^{2+}-8-Br ATPase of HMM. These two observations support the assumption cited here.

In the above discussion, I tacitly assumed that the two characteristic properties mentioned above are due to difficulty of rotation about the glycosyl bond between the base and the ribose. However, all the NTPs with difficult rotation were prepared by modification of the adenine moiety at the 8-position. Therefore, it is possible that the two properties resulted from 8-substitution itself, although they did not depend on the species of substituents. FTP, which appears to be easily rotatable about the glycosyl bond according to various physicochemical methods (66), showed the properties characteristic to NTPs with easy ro-

tation, although the $\backslash$CH group at the 8-position of ATP is re-

placed by $\underset{\mathbin{/\mkern-6mu/}}{\backslash}$N: and $\overset{\backslash\mkern4mu/}{\underset{|}{N}}$ at the 9-position by $\overset{\backslash\mkern4mu/\mkern-4mu/}{\underset{|}{C}}$. Furthermore,

contraction of myofibrils was supported by ITP (6-keto purine),

GTP (2-amino-6-keto purine), CTP (2-keto-4-amino pyrimidine), UTP (2,4-di-

keto pyrimidine), and TTP (2,4-diketo-5-methyl pyrimidine) (1,

2,7, and Table VII). It is thus highly probable that the above

two properties are due to difficulty of rotation about the gly-

cosyl bond rather than 8-substitution. Therefore, it is concluded

that, in the case of NTP with difficult rotation, M_2NTP can not

be converted into M_P^{NDP} since the three-dimensional structure of

the active site for binding the base and the ribose changes

during the conversion of M_2NTP into M_P^{NDP}.

        *   *   *   *   *   *

A part of these studies was published in 1976, and it appears

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Proc.Natl.Acad.Sci.USA.

REFERENCES

1. Tonomura,Y. (1972) "Muscle Proteins, Muscle Contraction and Cation Transport," (Univ. Tokyo Press & Univ. Park Press).

2. Inoue,A., Takenaka,H., Arata,T. & Tonomura,Y. (1978) Adv. Biophys. in press.

3. Inoue,A. & Tonomura,Y. (1976) J.Biochem. 79 419.

4. Inoue,A. & Tonomura,Y. (1976) J.Biochem. 80 1359.

5. Shibata-Sekiya,K. & Tonomura,Y. (1976) J.Biochem. 80 1371.

6. Blum,J.J. (1955) Arch.Biochem.Biophys. 55 486.

7. Hasselbach,W. (1956) Biochim.Biophys.Acta 20 355.

8. Ikehara,M., Ohtsuka,E., Kitagawa,S., Yagi,K. & Tonomura,Y. (1961) J.Amer.Chem.Soc. 83 2679.

9. Ikehara,M., Ohtsuka,E., Kitagawa,S. & Tonomura,Y. (1964) Biochim.Biophys.Acta 82 74.

10. Tonomura,Y., Imamura,K., Ikehara,M., Uno,H. & Harada,F. (1967) J.Biochem. 61 460.

11. Duke,J.A. & Morales,M.F. (1973) "Organization of Energy-Transducing Membranes," eds. Nakao,M. & Packer,L. (Univ. Tokyo Press) p143.

12. Yount,R.G. (1975) Adv.Enzymol. 43 1.

13. Onishi,H., Ohtsuka,E., Ikehara,M. & Tonomura,Y. (1973) J.Biochem. 74 435.

14. Murphy,A.J. & Morales,M.F. (1970) Biochemistry 9 1528.

15. Eckstein,F. & Goody,R.S. (1976) Biochemistry 15 1685.

16. Kennard,O., Isaacs,N.W., Coppola,J.C., Kirby,A.J., Warren,S.,

Motherwell,W.D.S., Watson,D.G., Wampler,D.L., Chenery,D.H.,
Larson,A.C., Kerr,K.A. & Riva di Sanserverino,L. (1970)
Nature 225 333.

17. Donohue,J. & Trueblood,K.N. (1960) J.Mol.Biol. 2 363.

18. Sundaralingam,M. & Jensen,L.H. (1965) J.Mol.Biol. 13 930.

19. Sundaralingam,M. (1969) Biopolymers 7 821.

20. Sundaralingam,M. (1973) "The Jerusalem Symposia on Quantum Chemistry and Biochemistry," eds. Bergmann,E.D. & Pullman, B. (Academic Press, New York) vol. 5 p 417.

21. Yathindra,N. & Sundaralingam,M. (1973) Biopolymers 12 297.

22. Millner,Jr.,O.E. & Andersen,J.A. (1975) Biopolymers 14 2159.

23. Hemmes,P., Oppenheimer,L. & Jordan,F. (1976) J.Chem.Soc., Chem.Commun. 929.

24. Tran-Dinh,S. & Chachaty,C. (1977) Biochim.Biophys.Acta 500 405.

25. Tavale,S.S. & Sobell,H.M. (1970) J.Mol.Biol. 48 109.

26. Sarma,R.H., Lee,C.-H., Evans,F.E., Yathindra,N. & Sundaralingam, M. J.Amer.Chem.Soc. 96 7337.

27. Uesugi,S. & Ikehara,M. (1977) J.Amer.Chem.Soc. 99 3250.

28. Ikehara,M., Uesugi,S. & Yoshida,K. (1972) Biochemistry 11 830.

29. Muneyama,K. Bauer,R.J., Shuman,D.A., Robins,R.K. & Simon,L.N. (1971) Biochemistry 10 2390.

30. Khwaja,T.A., Boswell,K.H., Robins,R.K. & Miller,J.P. (1975) Biochemistry 14 4238.

31. Moras,D., Olsen,K.W., Sabesan,M.N., Buehner,M., Ford,G.C. & Rossmann,M.G. (1975) J.Biol.Chem. 250 9137.

32. Ikehara,M. (1969) Accounts Chem.Res. 2 47.

33. Evans,F.E. & Kaplan,N.O. (1976) J.Biol.Chem. 251 6791.

34. Perry,S.V. (1955) "Methods in Enzymology," eds. Colowick, S.P. & Kaplan,N.O. (Academic Press, Inc., New York) vol. 2 p582.

35. Szent-Györgyi,A.G. (1953) Arch.Biochem.Biophys. 42 305.

36. Hayashi,Y. (1972) J.Biochem. 72 83.

37. Straub,F.B. & Feuer,G. (1950) Biochim.Biophys.Acta 4 445.

38. Spudich,J.A. & Watt,S. (1971) J.Biol.Chem. 246 4866.

39. Mommaerts,W.F.H.M. (1952) J.Biol.Chem. 198 445.

40. Perry,S.V. & Corsi,A. (1958) Biochem.J. 68 5.

41. Glynn,I.M. & Chappel,J.B. (1964) Biochem.J. 90 147.

42. Long,R.A., Robins,R.K. & Townsend,L.B. (1967) J.Org.Chem. 32 2751.

43. Holmes,R.E. & Robins,R.K. (1965) J.Amer.Chem.Soc. 87 1772.

44. Yoshikawa,M., Kato,T. & Takenishi,T. (1969) Bull.Chem. Soc.Japan 42 3505.

45. Moffatt,I.G. & Khorana,H.G. (1961) J.Amer.Chem.Soc. 83 649.

46. Ikehara,M. & Tada,H. (1967) Chem.Pharm.Bull. 15 94.

47. Ikehara,M. & Uesugi,S. (1969) Chem.Pharm.Bull. 17 348.

48. Ikehara,M. & Kaneko,M. (1970) Tetrahedron 26 4251.

49. Wagner,D., Verheyden,J.P.H. & Moffatt,J.G. (1974) J.Org. Chem. 39 24.

50. Sekine,T. & Yamaguchi,M. (1963) J.Biochem. 54 196.

51. Martin,J.B. & Doty,D.M. (1949) Anal.Chem. 21 965.

52. Nakamura,H. & Tonomura,Y. (1968) J.Biochem. 63 279.

53. Takemori,S., Nakamura,M., Suzuki,K., Katagiri,M. & Nakamura,
    T. Biochim.Biophys.Acta 284 382.

54. Layne,E. (1955) "Methods in Enzymology," eds. Colowick,
    S.P. & Kaplan,N.O. (Academic Press, Inc., New York) vol. 3
    448.

55. Takahashi,K., Mori,T., Nakamura,H. & Tonomura,Y. (1965)
    J.Biochem. 57 637.

56. Gornall,A.G., Bardmill,C.J. & David,M.M. (1949) J.Biol.Chem.
    177 751.

57. Kielley,W.W. & Bradley,L.B. (1956) J.Biol.Chem. 218
    653.

58. Werber,M.M., Szent-Györgyi,A.G. & Fasman,G.D. (1972) Bio-
    chemistry 11 2872.

59. Onishi,H., Nakamura,H. & Tonomura,Y. (1968) J.Biochem. 64
    769.

60. Takeuchi,K. & Tonomura,Y. (1971) J.Biochem. 70 1011.

61. Bagshaw,C.R., Eccleston,J.F., Eckstein,F., Goody,R.S.,
    Gudfreund,H. & Trentham,D.R. (1974) Biochem.J. 141 351.

62. Taylor,E.W. (1973) "Current Topics in Bioenergetics," eds.
    Sanadi,R.O. & Packer,L. (Academic Press, Inc., New York)
    vol.5 p.201.

63. Trentham,D.R., Eccleston,J.F. & Bagshaw,Q.R. (1976) Quart.
    Rev.Biophys. 9 217.

64. Inoue,A., Kikuchi,K. & Tonomura,Y. (1977) J.Biochem. 82
    783.

65. Takashi,R., Tonomura,Y. & Morales,M.F. (1977) Proc.Natl. Acad.Sci.USA 74 2334.

66. Prusiner,P., Brennan,T. & Sundaralingam,M. (1973) Biochemistry 12 1196.