A STUDY OF THE BIOCHEMICAL PROPERTIES OF NATIVE VERTEBRATE SKELETAL MYOSIN FILAMENTS

by

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TO MY FAMILY

Abstract

Chymotryptic S-1 lacking in DTNB LC can be obtained from synthetic rabbit myosin filaments (Weeds and Taylor, 1975; Weeds and Pope, 1977). I have now shown however that chymotryptic digestion of ^2H_2O-H_2O gradients-purified native myosin filaments (Emes and Rowe, 1978) yields S-1 containing the DTNB LC. Our results strongly suggest that this difference between synthetic and native myosin filaments upon subjection to chymotryptic digestion is mostly due to a difference in the myosin conformation within the two types of filaments, since chymotryptic S-1 from filaments formed from solubilised native filaments produced conventional S-1.

Detailed studies of the ATPase activities of native filaments and the derived S-1 (before and after exposure to high ionic strength) have been performed using an isotopic assay (γ-^{32}P ATP). Evidence has been obtained to indicate that DTNB LC before exposure to high ionic strength possesses the potential to repress ATPase activities and it is believed that native DTNB LC plays a role in maintaining the low ATPase activities in relaxed muscle.

ATPase activities of rabbit skeletal myofibrils have been shown to be calcium-sensitive at physiological ionic strength (Lehman, 1977; 1978). Using purified and relaxed filaments, I have shown that these preparations have calcium-sensitive ATPase activities at physiological ionic strength and also at low ionic strengths in the case of purified filaments. My results suggest that DTNB LC before "seeing" high ionic strength is responsible for calcium-sensitivity.

Evidence is presented to suggest that there are at least two populations of myosin heavy chains.

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Contents

Materials

Bromophenol Blue, Coomassie Brilliant Blue R, Trizma Base, N,N,N',N'-tetramethylethylene-diamine (Temed), polyethylene glycol (20,000 M.W.), Coomassie Brilliant Blue G-250, dithiothreitol (DTT), adenosine 5' triphosphate (ATP) (crystalline disodium salt from equine muscle), ethyleneglycol-bis-(β-aminoethyl ether)N,N'-tetraacetic acid (EGTA), dowex resin, guanidine HCl, 2-mercaptoethanol, pyruvate kinase (PK) (from rabbit muscle), phosphoenol pyruvate (PEP) (crystalline monopotassium salt), deoxyribonuclease I (DNAase I) (from bovine pancreas), phosphorylase a (from rabbit muscle), catalase (from bovine liver), bovine serum albumin, lysozyme (from egg white), trypsinogen (from bovine pancreas), pepsin (from hog stomach mucosa), β-lactoglobulin (from bovine milk), bovine serum albumin (cross-linked), hemocyanin (cross-linked), egg albumin and creatine kinase (from rabbit muscle) were from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey; ammonium persulphate, phenylmethane sulphonyl fluoride (PMSF), N,N'-methylene-bis-acrylamide, glycerol, phosphoric acid, ammonium molybdate, dodeca-tungstosilicic acid (silicotungstic acid), acrylamide, α-chymotrypsin (from bovine pancreas), and triton X-100 were from BDH Chemicals Ltd., Poole, England (α-chymotrypsin and acrylamide were also from Sigma and Fisons, respectively) ; glycine,

imidazole, citric acid, urea, diaminoethantetra-acetic acid (EDTA), sodium dodecyl-sulphate (SDS) and 2,5-diphenyloxazole (PPO) were from Fisons Scientific Apparatus Ltd., Loughborough, England; DEAE-Sephadex A-50 and Sephadex G-200 were from Pharmacia (Great Britain) Ltd., London; albumin (human) fraction V was from Calbiochem, Los Angeles, California; New Zealand white rabbits were from Hop Rabbits, Canterbury; adenosine 5'-(γ-^{32}P) triphosphate (triethylammonium salt) was from The Radiochemical Centre, Amersham; all other reagents were of analytical grade or the highest grade commercially available; twice- or thrice-distilled water was used to made up all solutions.

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CHAPTER I

GENERAL VIEWS OR REVIEWS

I. GENERAL VIEWS OR REVIEWS

I.1. The contractile machinery of vertebrate skeletal muscle

The contractile cells of vertebrate skeletal muscles are myofibers surrounded by, and terminating in, connective tissue which is in continuation with the inextensible, collagenous, tendons attached to bones. Each fibre (cylindrical in form) is bounded by the sarcolemma (muscle cell membrane) immediately beneath which are situated many nuclei. The contractile material consists of longitudinally arranged, partially overlapping arrays of thick (myosin) and thin (actin) filaments which form the myofibrils of each myofibre (Hanson and Huxley, 1953). Each myofibril is enveloped by the precisely arranged double-membrane vesicles (with terminal cisternae) of the sarcoplasmic reticulum (SR) and the transverse tubule system (T system) (see Figure 1). Mitochondria are wedged between the invested myofibrils and just beneath the sarcolemma.

In life, myofibrils are transparent as viewed in simple microscopes, but when viewed under polarizing or phase-contrast microscopes they appear to be striated in both the longitudinal and transverse directions. The former results from the parallel arrangement of myofibrils within the myofibre, whilst the later is caused by the

alternations in refractive index and anisotropy along the length of each myofibril. Each myofibril is divided up into segments by thin partitions called Z-lines or Z-discs to form the sarcomeres which are the basic contractile units of the myofibrils. Each sarcomere (see Figure 2) contains a hexagonal array of thick filaments aligned parallel with the long axis of the myofibril and defining the limits of the A-band in the central portion of the sarcomere. The rest of the sarcomere is occupied by I-band. The thick filaments overlap with an array of thin filaments attached to, and ordered by, each of the two Z-lines. In the region of overlap between the thick and thin filaments, cross-bridges extend from the thick filaments towards the thin, as seen from high-resolution electron micrographs (Huxley, 1957). The H-zone in the middle of the A-band is defined by the edges of the thin filaments. Electron micrographs show that there are three to five arrays of transverse bridges connecting each thick filament with its six neighbours in the M-line region of the A-band. There are also filaments parallel to the thick filaments linking each set of M-bridges together, which are responsible to hold the filaments in an ordered array. On either side of the M-line is a region of lower density than the rest of the H-zone, known as the pseudo H-zone, due to the absence of cross-bridges in this region (Knappeis and Carlsen, 1968) (Figure 2). The thin filaments of each sarcomere end on each side of the Z-line in rod-like projections of very

thin filaments (Knappeis and Carlsen, 1962; Franzini-Armstrong and Porter, 1964) and there has been some evidence suggesting that the protein alpha-actinin may be involved in the molecular structure of the Z-line (Masaki et al., 1967; Briskey et al., 1967; Stromer et al., 1969).

In resting muscle there is a potential difference across the sarcolemma (60 mV +ve in the outside) and calcium ions are sequestered in the sarcoplasmic reticulum (SR) by means of a membrane-bound enzyme active transport system, so that calcium concentration in the sarcoplasm is probably less than 10^{-7} or 10^{-8} M (Huxley, 1973a) and that inside the SR is about 10^{-3} M. The T system which is of many repeating tubular invaginations of the sarcolemma runs across the muscle cell at the level of each Z-line and is a specialization of the endoplasmic reticulum (complex net-work of tubular channels, usually expanded into slit-like cavities (cisternae) with more or less flattened vesicles, occuring in the cytoplasm of many eucaryote cells). When a muscle fibre is stimulated via its motor neurone, a wave of electrical depolarization termed an action potential spreads longitudinally along the sarcolemma and this is conveyed to the SR via the T system (see Figure 1) causing the SR to increase in permeability to calcium ions which consequently flood out into the sarcoplasm to activate the troponin-inhibited contractile

apparatus, so that muscle contraction can occur (Ebashi and Endo, 1968; Ebashi, Endo and Ohtsuki, 1969; Weber, 1966; Weber and Herz, 1963; Weber, Herz and Reiss, 1963).

The sliding filament theory of contraction which is now accepted almost universally, was evolved independently and more or less simultaneously by A.F. Huxley and H.E. Huxley (Hanson and Huxley, 1953; Huxley and Hanson, 1954; Huxley and Niedergerke, 1954; Hanson and Huxley, 1955; Huxley, 1957a). According to this theory, the force of contraction is developed by the cross-bridges in the overlap region and active shortening is caused by the movement of the cross-bridges, which causes one filament to slide or creep over the other with the length of each set of filaments remaining constant (Huxley, 1969; Huxley, 1974). The exact molecular mechanism of how the minute cross-bridges achieve the sliding process still remains a subject of debate. However, the most widely held view is that, since the muscle can shorten actively by about 30% (the cross-bridges are spaced roughly 43 nm apart which is only about 5% of the length of half a sarcomere), so each individual cross-bridge must interact cyclically with the thin filament five or six times to cause relative movement between the two sets of filaments (Huxley, 1957a; Huxley, 1974; Huxley, 1969). Hydrolysis of ATP by the myosin molecule (Cain, Infante and Davies, 1962) provides free energy for the sliding process, with actin markedly

activating the rate of this hydrolysis (Hasselbach, 1952; Maruyama and Gergely, 1962; Eisenberg and Moos, 1970).

I.2. Molecular structure and protein components of the filaments

I.2.1. Thin (actin) filaments

Actin, the major constituent of thin filaments, is in a state of dispersed globular monomer, G-actin, in a salt free environment. G-actin has a molecular weight of about 43,000 and its amino acid sequence has been determined (Elzina et al., 1973). Each G-actin molecule contains one molecule of ATP very firmly bound to it, and G-actin molecules are polymerized into a fibrous form of a two-stranded rope, F-actin, by the addition of neutral salts (Kielley and Meyerhof, 1948; 1950), and at the same time , the bound ATP is hydrolysed to ADP.

The thin filaments consist of two F-actin chains wrapped around each other to form an open, double-stranded, helix (see Figure 3). Lying head-to-tail along the grooves of the helix are molecules of tropomyosin to each of which is attached a troponin complex which is of three subunits, troponin-I (TN-I), troponin-C (TN-C) and troponin-T (TN-T). The molecular weight of tropomyosin is about 66,000 (Korn, 1978; Cohen and Cohen, 1972; Cote et al., 1978; 1978a) and

each molecule consists of two apparently identical subunits whose amino acid sequence has been determined (Sodek et al., 1978; Stone and Smillie, 1978).

When the troponin complex is bound to tropomyosin, TN-I (23,000 M.W.) inhibits the actin-induced stimulation of Mg-ATP hydrolysis catalysed by myosin. TN-C (18,000 M.W.) overcomes this inhibition on binding calcium. TN-T (40,000 M.W.) is the subunit which binds TN-I and TN-C to tropomyosin which interacts with actin.

Electron micrographs of negatively stained specimens of actin filaments show a double helical structure of actin filaments (Hanson and Lowy, 1963) and the double helix is right-handed (Depue and Rice, 1965). X-ray diffraction studies on muscle show a meridional reflection with a spacing of 38.5 nm, corresponding to the tropomyosin (or troponin complex) repeat of the actin filaments (Huxley and Brown, 1967) (see Figure 3). Three-dimensional reconstruction of electron micrographs of actin filaments (Moore et al., 1970) indicate the dimensions of actin monomers to be about 5.5 nm x 3.5 nm x 5.0 nm and also give some information about the location of the troponin-tropomyosin complex.

Besides noting that actin monomers contain specific ATPase-activating and myosin-binding sites which are

essential for the sliding of thick and thin filaments over each other during muscular contraction, it is important to realise that there is a reversal of polarity of the actin filaments on either side of the Z-lines, so that actin monomers in the appropriate orientation is able to interact with the cross-bridges of the thick filaments which also have reversed orientation of cross-bridges on either side of the M-line (Figure 2). Evidence for the reversed polarity of actin filaments at the Z-lines has come from the decoration of thin (I-) filaments and I-segments with the myosin subfragments, heavy meromyosin (HMM) and subfragment-1 (S-1) (I.3.). In the electron microscope, the decorated thin filaments are seen to display characteristic arrowhead appearance, pointing in a constant direction, and for the I-segments, the polarity is reversed on either side of the Z-lines (Huxley, 1963). Further evidence has come from the three-dimensional reconstruction of electron micrographs of actin filaments decorated with S-1 (Moore et al., 1970). These show that it is the combination of slewing (45^{o}) and tilting (45^{o}) of the S-1 subunits which are attached to the actin monomers which gives rise to the arrowhead appearance.

I.2.2. Thick (myosin) filaments

The thick filaments are multimolecular aggregates of

the protein, myosin. C-protein and F-protein are examples of other proteins which are associated with the filaments (Starr and Offer, 1971; Offer, 1972). X-ray diffraction studies have shown that myosin cross-bridges (made up of myosin heads) occur at intervals of 14.3 nm along the thick filaments (Huxley and Brown, 1967; Squire, 1973) which are formed by the antiparallel association of myosin molecules (Huxley, 1963) in such a way that all the myosin molecules in one half of the filament are oriented in one direction and all those in the other half are in the opposite direction. The central region of the filament contains no cross-bridges and is packed with myosin rods (Figure 4) of antiparallel myosin molecules.

The number (n) of myosin molecules per 14.3 nm interval along the filament has been investigated by various workers and there has been discrepancy in this value. X-ray diffraction studies (Huxley and Brown, 1967; Squire, 1973) do not give firm indication whether there are two, three or four myosin molecules per 14.3 nm repeat. If there are two myosin molecules per cross-bridge, then n is equal to four and the filament is two-stranded. If there is one myosin molecule per cross-bridge, then n is equal to three and the filament is three-stranded (i.e. there are three cross-bridges per 14.3 nm of filament length). The recent demonstration (Maw and Rowe, 1980) of three sub-filaments within each thick filament has added more

evidence in favour of the three-stranded models in which there are three myosin molecules per 14.3 nm repeat of filament length. Table 1 shows the number of myosin molecules per 14.3 nm interval along the vertebrate skeletal myosin filaments, estimated by various workers, using different methods.

Myosin is the major contractile protein component of the thick filaments and can be extracted from a vertebrate skeletal muscle mince by using a high ionic strength phosphate buffer (Guba and Straub, 1943). Brief extraction periods (about 10 minutes) yield a preparation of myosin containing only small amounts of actin, while prolonged extraction periods will give a preparation enriched in actin (Szent-Gyorgyi, 1945). Synthetic myosin (thick) filaments can be formed by dialysing extracted myosin in true solution down to physiological ionic strength (Weeds and Taylor, 1975; Weeds and Pope, 1977). These synthetic myosin filaments (though not identical) resemble native myosin filaments which can be isolated and purified by $^{2}H_2O$-H_2O gradients (Trinick and Rowe, 1973; Emes and Rowe, 1978; see Chapter II). It is the aim of this thesis to study the biochemical differences between these two types of filaments.

The proposal of the sliding mechanism (Hanson and Huxley, 1953; Huxley and Niedergerke, 1954) has stimulated

the effort to visualize the fine structure of the myosin molecule by electron microscope and other chemical procedures, such as using agents to dissociate into its subunits or splitting the molecule into several fragments by proteolytic enzymes (see Figure 4 and I.3.). In electron micrographs, the myosin molecule is seen to have two "heads" and a long "tail" (Slayter and Lowey, 1967; Lowey et al., 1969; Elliott, Offer and Burridge; 1976; Elliott and Offer; 1978). It is a long (160 nm) and grossly asymmetric molecule because the C-terminal portion is fibrous which is almost completely alpha-helical and is responsible for the formation of the shaft of the myosin filament (Starr and Offer, 1973; Lowey, 1979). Rabbit skeletal muscle myosin (M.W. 470,000) is a hexamer consisting of two heavy chains (M.W. 200,000) and four light chains (Gershman et al., 1969; Lowey and Risby, 1971; Weeds and Lowey, 1971). The presence of two types of heavy chains is indicated by the finding of amino acid substitutions in certain peptide sequences (Weeds, 1967; Starr and Offer, 1973; see Chapter V for more evidence).

The four light chains of the rabbit skeletal muscle myosin can be grouped into two distinct chemical classes. One class can be selectively removed from myosin by reaction with the thiol reagent 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB or NBs_2) without significant effect on the myosin ATPase activity

(Weeds,1969; Weeds and Pope, 1971; Gazith et al., 1970; Weeds and Lowey, 1971). This class has been called the DTNB light chains, and also, based upon its SDS gel electrophoretic mobility, light chain 2 (LC2). The other class of light chains can be liberated from the myosin molecule only by denaturing conditions, such as strong salts (Gershman and Dreizen, 1970) or high pH (Kominz et al., 1959; Gershman et al., 1966), and has been termed the alkali light chains. There are two forms of alkali light chains, termed A1 and A2, or LC1 and LC3 (based upon SDS gel eletrophoretic mobility) which are isotypic variants.

All the four light chains (two moles of DTNB LC and two moles of alkali LC per mole of myosin) are confined to the "heads" (subfragment-1 or S-1) of the molecule, while the heavy chains run the entire length of the molecule (being present in both heads and tail) (see I.3. and Figure 4). The two classes of light chains are distinguished chemically since alkali light chains contain only one thiol group while DTNB light chain contains two. There is also no similarity in the amino acid sequence around the thiol groups in the two classes of light chains (Weeds, 1969). The amino acid sequences of DTNB LC and alkali LC's have been established by Collins (1976), and Frank and Weeds (1974), respectively. Amino acid sequence analysis shows that LC1 and LC3 have an identical sequence over their

C-terminal 141 residues, the N-terminal eight residues of LC3 contain five amino acid substitutions when compared with the corresponding sequence of LC1, and LC1 has an additional 41 residues at its N-terminal end.

The molecular weights estimated by SDS gel electrophoresis for LC1, LC2 and LC3 are 25,000, 18,000 and 16,000, respectively (Weeds and Lowey, 1971; Lowey and Risby, 1971; Sarkar, Sreter and Gergely, 1971). On the basis of amino acid sequence analysis the minimum molecular weights of LC1 and LC3 are 21,000 and 17,000 respectively (Frank and Weeds, 1974). Therefore, it is clear that LC1 has an apparently higher molecular weight when estimated by SDS gel electrophoresis. This abnormal mobility on gels has been attributed to the fact that the N-terminal region of LC1 is rich in basic residues. The complex between dodecyl sulphate and a basic polypeptide may have a lower negative charge density so that the polypeptide adopts a different conformation from the standard complex which is highly charged. In fact, a similar anomaly of molecular weight estimation has been observed with histones and one of the cardiac light chains (Panyim and Chalkley, 1971; Weeds and Frank, 1972).

Experiments based on various criteria have been performed to investigate the functional role of the alkali light chains which have been termed the essential light

chains since their removal result in a total loss of the myosin ATPase activity. Hybridization experiments between heavy and light chains from various muscle types (Dreizen and Richards, 1972) and work based on reversible dissociation and reassociation of the heavy and light chains of myosin with 4 M LiCl (Stracher, 1969; Dreizen and Gershman, 1970; Dow and Stracher, 1971) have suggested that these light chains are involved in the regulation of calcium-ATPase activity of myosin. However, the significance of these experiments is limited by the observation of low recoveries of ATPase activity due to protein denaturation and incomplete dissociation of light and heavy chains (Stracher, 1969; Dreizen and Gershman, 1970; Kim and Mommaerts, 1971; Dreizen and Richards, 1972). The binding of a purine disulphide analogue of ATP to the alkali light chains of myosin leads to a concomitant loss of ATPase activity and actin binding ability (Wagner and Yount, 1975; Wagner and Yount, 1976). Four moles of analogue are incorporated in both alkali light and heavy chains per mole of myosin (with about 1 mole per mole of alkali light chain and none in DTNB LC). However, this incorporation occurs at some second nucleotide binding site and not the ATP hydrolytic (ATPase) site. Experiments of cross-reinnervation of fast and slow muscles in a single animal suggest that alkali light chains are involved in the expression of hydrolytic activity. Physiological changes are accompanied by synthesis of light chains characteristic

of a new muscle type. A partial physiological change from fast to slow muscle is possible by electrical stimulation of the motor nerve to the fast muscle and the modified muscle has a decreased K-EDTA- and Ca-ATPase activities (Sreter et al., 1974; Weeds et al., 1974; Sreter et al., 1973). However, in the modified muscle, not only slow muscle light chains are present, in addition to the original fast light chains, the heavy chains gel pattern is also changed (Sreter et al., 1975; Weeds and Burridge, 1975). Holt and Lowey (1975) using antibodies specific to the alkali light chains have suggested that these light chains have no direct involvement in actin binding or ATPase activity. However, Wagner and Weeds (1977) using recombination and hybridization techniques, involving S-1(A1) and S-1(A2) (Weeds and Taylor, 1975) and excess A1 (LC1) or A2 (LC3) have shown that alkali light chains do not affect ATPase activities in the absence of actin but affect the actin-activated ATPase activity, implying that the myosin "heads" interaction with actin may involve the alkali light chains and confirm that the differences in the actin-activated ATPase activities between the two S-1 isoenzymes, S-1(A1) and S-1(A2), is not caused by the differences in the heavy chains or other effects from chymotryptic cleavage, but due to the particular light chain present.

The unequal molar ratios (1.5 to 2 moles of A1 per

mole of A2) between the two alkali light chains have been taken to imply that there are unequal amounts of two myosin homodimers rather than a myosin population of heterodimers (Lowey and Risby, 1971; Sarkar, 1972; Weeds et al., 1975). It is also possible that there is a mixture of heterodimers and homodimers. Using an immunochemical approach, in which, antibodies specific for the "difference peptide" unique to A1 of chicken breast muscle myosin are coupled to Sepharose to form an immunoadsorbent specific for A1 (LC1) , it is possible to fractionate myosin, heavy meromyosin, and S-1 into two fractions, one rich in A2 (LC3) while the other rich in A1 (LC1). This proves that there are at least two vertebrate skeletal myosin isoenzymes with respect to the alkali light chains (Holt and Lowey, 1977) (see chapter V for more evidence).

Treatment of rabbit skeletal myosin with DTNB (which blocks about 80% of the myosin thiol groups), selectively releases about 1 mole of the DTNB light chains (Weeds and Lowey, 1971; Katoh and Kubo, 1974; Weeds, 1969; Gazith et al., 1970). Treatment of myosin with p-chloromercuribenzoate (PCMB) also releases one mole of DTNB light chain (Hayashi et al., 1973). Immunological studies have shown that there are two types of DTNB light chains (Katoh and Kubo, 1974). However, immunological difference between the two DTNB light chains has not been observed by Holt and Lowey (1975), though they have shown

that antiserum to DTNB light chain also selectively dissociates one mole of the DTNB light chain from myosin, heavy meromyosin and subfragment-1 (Holt and Lowey, 1975a). They have attributed this selectivity difference to the supposition that the remaining light chain is bound more strongly by the heavy chain (i.e. there are different heavy chain binding sites for the two light chains). However, negative co-operativity of dissociation may also be the reason for the selective dissociation of one of the DTNB light chains (Kendrick-Jones et al., 1976; Kendrick-Jones and Jakes, 1976; Bagshaw, 1980).

DTNB light chains have been found to inhibit the binding of S-1 to G-actin (but not DTNB light chains of HMM) (Onodera and Yagi, 1971) and are directly involved in the weakening of the binding of S-1 to F-actin in the presence of calcium (Margossian et al., 1975). However, they have been found to enhance actin and myosin interaction at low free calcium ions (0.05-0.5 μM free calcium; free calcium concentration of living muscle at rest is about 0.1 μM), implying a role in the regulation of the resting tension of the contractile system (Pemrick, 1977). Werber and Oplatka (1974) have shown that though the Mg- and Ca-ATPase activities of DTNB-treated myosin are practically unaffected, the actin-ativated and K-EDTA-ATPase activities are greatly reduced. (It is shown that sulfhydryl reagents are responsible for the decrease

in K-EDTA-ATPase activity (Sekine and Kielley, 1964)). (cf. K-EDTA- and Ca-ATPase activities of S-1 and HMM are not affected by reaction with antisera to light chain 1 or DTNB light chain which dissociate one mole of DTNB light chain from the subfragments whereas actin-activation is reduced in both subfragments (Holt and Lowey, 1975a)). Calcium-sensitivity is also reported to decrease when the actin-activated Mg-ATPase activity of DTNB-treated myosin is measured in the presence of troponin and tropomyosin as compared to non DTNB-treated myosin, suggesting that DTNB light chains have a regulatory role in the vertebrate skeletal thick filaments (Werber and Oplatka, 1974; Werber et al., 1972). On the other hand, dissociation of DTNB light chain from myosin and its subfragments (HMM and S-1) by DTNB light chain antiserum does not affect the calcium-sensitivity of the actin-activated ATPase activity in the presence of troponin and tropomyosin (Holt and Lowey, 1975a). The DTNB light chains of vertebrate skeletal muscle (also cardiac and fast decapod muscles) have been found to have no ability to resensitize purified scallop myosin unless the myosin is complexed with actin and they also do not restore calcium-binding that was lost during desensitization (Kendrick-Jones, 1974; Kendrick-Jones, 1976).

A considerable amount of work has been performed on the binding of divalent cations to the DTNB light chains.

Each DTNB light chain possesses one non-specific site for divalent cations (as compared to scallop and clam myosins which have two specific sites for calcium and two non-specific sites) (Bagshaw and Reed, 1977; Bagshaw and Kendrick-Jones, 1978). It seems that the binding of divalent cations to the light chains involves the interaction of the heavy chains (Okamoto and Yagi, 1976; Higuchi et al., 1978; Kuwayama and Yagi, 1977, 1979) to form a firmer complex (Szent-Gyorgyi, 1975; Kasman and Kakol, 1977; Higuchi et al., 1978). The light chain binding sites are found to have a higher affinity when associated with the heavy chains than when they are dissociated from the myosin (Szent-Gyorgyi et al., 1973; Higuchi et al., 1978; Bagshaw and Kendrick-Jones, 1979). The binding of calcium to the myosin molecule is also found to be pH-sensitive (Wikman-Coffelt, J., 1980). Since the light chains are not pH-sensitive (Stafford and Szent-Gyorgyi, 1978; Chantler and Szent-Gyorgyi,1978), it has been suggested that the pH-sensitivity could be due to the heavy chains which are susceptible to conformational changes with changing pH (Ishigami and Morita, 1977). The binding of calcium and/or magnesium by myosin may be necessary for the myosin structure and this may not have regulatory significance in the vertebrate contractile system (Bagshaw and Reed, 1977; Bagshaw and Kendrick-Jones, 1979; Wikman-Coffelt, 1980).

Morimota and Harrington (1974), based on viscosity and centrifugal studies, have indicated that calcium concentration changes of 10^{-7} to 10^{-5} M causes the myosin heads to fan out radially, suggesting a role for DTNB light chains in the radial heads movement since calcium binds only to DTNB light chains. This is also observed by Alexis and Gratzer (1978) who shows that when calcium binds to DTNB light chains, a considerable conformational change occurs. It is also shown that DTNB light chains promote the interaction of thick (myosin) and thin (actin) filaments in the presence of calcium (Pemrick, 1977). However, the calcium-induced radial fanning of myosin heads is not observed by Mendelson and Cheung (1976). Using actomyosin-threads, Srivastava et al (1980) have shown that DTNB light chains play no role in the tension generation by the threads. Obviously , the latter observation can not reflect the true situation in vivo. On the other hand, electron micrographs show that rabbit F-actin filaments decorated with papain S-1 containing the DTNB light chain, display "barbed" arrowheads, while those filaments decorated with papain S-1 lacking the DTNB light chain, display "blunted" arrowheads (Craig et al., 1980).

Since the finding that DTNB light chains can be phosphorylated (at a single serine residue) by protein kinase (Perrie et al., 1969; Perrie and Perry, 1970; Perrie et al, 1973), work on the effect of phosphorylation on

calcium- and magnesium-binding properties of myosin has been performed. Holroyde et al (1979), using the techniques of Potter and Gergely (1975) for calcium-binding measurements, have found no significant effect of phosphorylation on calcium-binding properties of rabbit skeletal myosin (and porcine left ventricular cardiac myosin). However, there are reports suggesting that phosphorylation may affect calcium-binding to reconstituted myosin (Okamoto and Yagi, 1976) and isolated myosin light chains (Alexis and Gratzer, 1976). These agree with the recent finding of Kardami et al (1980) that phosphorylation regulates the affinity of myosin and isolated DTNB light chains for divalent cations. This has led to the suggestion that there is a physiological role for the phosphorylation-dependent changes in skeletal myosin affinity for calcium. The calcium concentration (about 5 μM) following activation would lead to partial replacement of magnesium which is thought to prevail in skeletal muscle, saturating the divalent cation-binding sites on the myosin. In vitro experiments have indicated that the rate of dissociating and binding of calcium would be too slow to exert control during the early events of muscular contraction (Bagshaw and Reed, 1977). It has been shown that phosphorylation of DTNB light chains can occur rapidly (reaching a maximum in a few seconds in the course of a tetanus) (Barany et al., 1979).

In the present study, using native thick (myosin) filaments, evidence has been obtained to indicate that DTNB light chains may play an important role in the calcium-sensitivity of native myosin filaments ATPase, and in the low Mg-ATPase activity as found in vivo.

I.3. Enzymatic subfragments of myosin

The finding that myosin is susceptible to proteolytic cleavage dates back to the early 1950's when Gergely (1950) and Perry (1951) showed that heavy meromyosin (HMM) and light meromyosin (LMM) could be produced by brief trypsin treatment of the myosin molecule (see Figure 4). Subsequent work showed that these two components could be distinguished ultracentrifugally (Mihalyi and Szent-Gyorgyi, 1953; Szent-Gyorgyi, 1953). This can also be achieved by chymotrypsin (Gergely, 1953; Gergely et al, 1955) or subtilisin (Middlebrook, 1958). HMM can be further digested with trypsin to yield subfragment-1 (HMM S-1 or S-1) (Mueller and Perry, 1961, 1962). S-1 of myosin is also produced by papain (Kominz et al, 1965; Slayter and Lowey, 1967; Nihei and Kay, 1968; Lowey et al., 1969), chymotrypsin (Jones and Perry, 1966; Hotta and Usami, 1967; Onodera and Yagi,1971), subtilisin (Jones and Perry, 1966), and Nagarse (Yazawa and Yagi, 1973). In general, SDS gel electrophoresis shows that tryptic S-1 is more degraded

(Hayashi, 1972; Cardinaud et al., 1973) than papain S-1 (Margossian and Lowey, 1973; Stone and Perry, 1973). However, it is shown that papain digestion of myosin or myofibrils cut both the heavy and light chains into several fragments (Cooke, 1972; Lowey and Holt, 1972). Stone and Perry (1973) have also confirmed that use of papain to digest myosin not only degrades LC1 of the S-1 produced but that several S-1 heavy chain fragments are also produced, one being 26,000 M.W. (corresponding to the 25,000 M.W. band of Cooke, 1972), as analysed by SDS gel electrophoresis. Partial loss of DTNB LC is also observed on exposure of myosin to papain (Lowey et al, 1969), but, if digestion is carried out in the presence of magnesium ions, papain S-1 obtained has a significantly higher proportion of DTNB LC than S-1 which is produced in the absence of magnesium ions (Margossian et al., 1975). (Note that LC1 referred to by Margossian et al is in fact the 26,000 M.W. S-1 heavy chain fragment of Stone and Perry, 1973).

Separation of chymotryptic S-1 into two components was initially made by Yagi and Otani (1974) and this has been improved by Weeds and Taylor (1975), using a DEAE-cellulose column, and these two components have been named S-1(A1) and S-1(A2). Further work on chymotryptic digestion of myosin has shown that divalent cations protect the S-1 site and this is thought to be due to the DTNB

light chains which bind calcium ions (Weeds and Pope, 1977). Digestion of myosin in true solution (0.6 M NaCl) in the absence or presence of divalent cations produces HMM (in the former case, some S-1 may be produced). A gradual transition from HMM production to S-1 is observed with the lowering of ionic strength in the absence of divalent cations (Oda et al., 1980). When the digestion is carried out on synthetic filaments in the presence of divalent cations, HMM is again the product though the DTNB light chains are mostly degraded as shown by SDS gels. However, if digestion is carried out on synthetic filaments in the absence of divalent cations, S-1 lacking the DTNB light chain is produced as shown by SDS gels. Since the separation of this S-1 into S-1(A1) and S-1(A2) (Weeds and Taylor, 1975), more experiments have been performed to study these two components (Wagner and Weeds, 1977). The Ca-ATPase and Mg-ATPase activities of S-1(A1) and S-1(A2) have been found to be identical, but there are differences in the actin-activated ATPase activities. Recombination and hybridization experiments have shown that these differences are due to the different light chain present in the S-1(A1) and S-1(A2) (Wagner and Weeds, 1977). Subfragment-1 (S-1), the individual "head" of the myosin molecule (two "heads" per molecule), is the smallest enzymatic fragment which contains both the ATPase and actin-binding sites. The molecular weight of S-1 is quite variable, depending on the enzyme used for its production. In general, it is about 25%

of the parent myosin molecule. Each S-1 molecule comprises a portion of the main myosin heavy chain whose M.W. has been estimated to be about 93,000, one alkali light chain (A1 or A2) and one DTNB light chain (see I.2.2.).

In the present study, it is shown that digestion of native myosin filaments which are purified by ^2H_2O-H_2O gradients (Trinick, 1973; Trinick and Rowe, 1973; Emes and Rowe, 1978), yields an S-1 population which contains both the alkali light chains and the DTNB light chains, as compared to the S-1 obtained by chymotryptic digestion of synthetic myosin filaments which contains no DTNB light chains. However, if native filaments are dissolved in high ionic strength media and then dialysed back to physiological ionic strength to form synthetic filaments and chymotryptic S-1 from these synthetic filaments again contains no DTNB light chains, showing that high ionic strength has an irreversible effect on myosin, as judged by the chymotryptic S-1 from synthetic and native filaments.

I.4. The regulatory systems of muscular contraction

The work of Heilbrum and Wiercinski (1947) was the first evidence to show that calcium ions play a regulatory role in muscular contraction. The regulatory proteins can only bring about contractile activities if the calcium concentration is

about 10^{-5}-10^{-4} M. Muscular contraction is switched off if the calcium concentration falls below 10^{-7} M and myosin and actin are unable to interact with each other (Ebashi,1961; Weber and Winicur, 1961; Weber and Herz, 1963; Weber, 1966; Weber et al., 1963; I.1.). In the absence of regulatory proteins, ATPase activity and contraction do not require calcium, i.e., calcium control is mediated by the regulatory proteins (Ebashi and Ebashi, 1964).

There are two distinctly different control systems which regulate muscular activities in different animals, one being actin- (or thin filament-) linked and the other being myosin- (or thick filament-) linked. Actin-linked regulation was first shown by Ebashi (1963) in vertebrate striated muscle and myosin-linked regulation in molluscan muscle by Kendrick-Jones et al (1970). In vertebrate striated (skeletal and cardiac) muscle (Weber and Murray, 1973; Perry, 1979), the interaction of actin with myosin is inhibited at about 10^{-7} M calcium concentration by the troponin-tropomyosin complex of the actin- (or thin-) filament. Binding of calcium (10^{-5}-10^{-4} M) to one of the proteins in the complex, troponin C, of the actin-filament, relieves the inhibition (hence actin-linked). Troponin C (M.W. 18,000) has four calcium-binding sites which are analogous to the sites of parvalbumins and calmodulin. These sites have been numbered I, II, III and IV from the N-terminal end of the molecule (Collins et al., 1977). The

first two sites specifically bind calcium while sites III and IV bind both calcium and magnesium (Potter and Gergely, 1975; Leavis et al., 1978). Purified skeletal (or cardiac) muscle myosin has a very low Mg-ATPase activity and this can be activated markedly by purified actin and this activation is independent of calcium. Addition of tropomyosin, troponin T and troponin I inhibits the ATPase activity which is also calcium-independent. In the presence of calcium (10^{-5}-10^{-4} M), addition of troponin C can then only relieve this inhibition (Greaser and Gergely, 1971; Hitchcock et al., 1973; Ebashi, 1974; Brekke and Greaser, 1976). Therefore, in vertebrate striated muscle there is an actin-activated Mg-ATPase activity of myosin (calcium-independent) which is repressed by the binding of the troponin-tropomyosin complex to actin and calcium derepresses the ATPase activity by binding to troponin C (Perry, 1979; Weber and Murray, 1973).

In molluscan muscles, the actin-activated ATPase activity is inhibited at low calcium concentration (10^{-7} M) by a pair of light chains (EDTA or regulatory light chains) on the myosin moleucle and calcium (10^{-5}-10^{-4} M) relieves this inhibition by binding directly to the myosin molecule (hence myosin-linked) (Kendrick-Jones et al., 1970, 1972, 1976; Lehman et al., 1972; Szent-Gyorgyi et al., 1973; Szent-Gyorgyi, 1975). There appears to be two specific calcium-binding sites and two non-specific sites per mole

of scallop (or clam) myosin (Bagshaw and Reed, 1977; Bagshaw and Kendrick-Jones, 1978; Kendrick-Jones et al., 1976; Bagshaw and Kendrick-Jones, 1979). The specific calcium-binding site has been suggested to be shared by the regulatory light chain and the heavy chain or located on the heavy chain (Bagshaw and Kendrick-Jones, 1979). It is interesting to find that isolated regulatory light chains can bind one mole of calcium per mole of light chain, but these binding sites are non specific (Chantler and Szent-Gyorgyi, 1978; Bagshaw and Kendrick-Jones, 1979) and these sites are most probably occupied by magnesium in a physiological environment. It seems that a specific binding site for calcium can only be formed when the regulatory light chain is associated with the heavy chain.

Treatment of scallop myosin, actomyosin or myofibrils with 10 mM EDTA (Szent-Gyorgyi et al, 1973; Kendrick-Jones et al, 1972) at 0^{o} C releases one mole of the EDTA light chain and results in a total loss of calcium-sensitivity, i.e., the preparations are "desensitised" and the actin-activated ATPase activity of myosin requires no calcium. The dissociation of the regulatory or EDTA light chain is reversible, and it recombines with the residual myosin when the magnesium concentration is raised above 10^{-5} M to reform calcium-sensitive scallop myosin. Calcium-binding is reduced by about 40% after treatment with EDTA and is restored when scallop myosin is

reconstituted from "desensitised" myosin and EDTA light chain (Kendrick-Jones et al., 1972). The two regulatory light chains can be dissociated by EDTA (10mM) treatment of scallop myofibrils or myosin at elevated temperature (30-35^{o} C) (Simmons and Szent-Gyorgyi, 1978; Chantler and Szent-Gyorgyi, 1980). Reassociation of the first mole of regulatory light chain with the residual myosin does not restore calcium-sensitivity which can only be restored with the readdition of the second mole of regulatory light chain (Chantler and Szent-Gyorgyi, 1980)

With the development of the very rapid assays to determine whether a muscle possesses a myosin-linked regulatory system or only an actin control system (in addition to isolating native thin filaments and testing these with rabbit myosin to see if they have regulatory properties) (Lehman et al., 1972; Leman and Szent-Gyorgyi, 1975), detailed comparative studies have revealed that actin- and myosin-linked regulation are not restricted to vertebrate striated muscle and molluscan muscles, respectively. Actin-linked regulatory system is also found in mysidacea (opossum shrimps), decapods fast muscles (e.g. tails and claws of lobsters and crabs) and a sipunculid species (peanut worm). In addition to molluscan muscles (e.g. scallops, claws and mussels) which possess only myosin control system, other species, like nemertine worms and echinoderms (sea cucumbers) also have a single

myosin-linked regulatory system. Detailed studies have shown that both actin and myosin control systems do operate simultaneously in most higher invertebrates (e.g. insects, annelids and decapod-crustaceans) (Lehman et al., 1972; Lehman and Szent-Gyorgyi, 1975; Lehman,1977).

The competitive actin-activation assay probes for the presence of a myosin-linked regulatory system in actomyosin or myofibrils by employing excess unregulated (pure) actin, under relaxing conditions. If myosin is regulated, it is unable to interact with the added pure actin in the absence of calcium, i.e. ATPase activity is activated only when calcium is added. If the muscle possesses only an actin control, full activation of the myosin ATPase activity is observed when pure actin is added in the absence of calcium. Whereas full activation is not attained (in the absence of calcium) if a myosin control also operates at the same time. However, care must be taken before a conclusion is reached, since at low ionic strength, ATPase activity can override calcium-sensitivity (Lehman, 1977, 1978). The competitive myosin activation assay can be employed to test for the presence of an actin-linked regulatory system in myofibrils and actomyosin. The presence of an actin control is indicated if the ATPase activity is increased when myosin is added in the presence of calcium, and a lack in calcium-sensitivity indicates the absence of actin control. These competitive tests depend on

the fact that , under relaxing conditions, the myosin and actin are dissociated, so that the added myosin or pure actin can interact with the appropriate protein under investigation.

In addition to the above described myosin-linked regulatory system, there is another form of myosin control system which is found in vertebrate smooth muscle and non muscle cells like macrophages, proliferative myoblasts and platelets (Adelstein and Conti, 1975; Scordilis and Adelstein, 1977; Trotter and Adelstein, 1979). In this form of regulation, phosphorylation of a pair of light chains (M.W. 20,000) on the myosin molecule is required for the actin-activated ATPase activity. Examples of myosins isolated from smooth muscles that require phophorylation of these light chains so that actin-activated Mg-ATPase activity can occur are those from placenta (Huszar and Boiley, 1979), gizzard (Sobieszek and Small, 1977; Onishi and Watanabe, 1979), and stomach (Small and Sobieszek, 1977). Dephosphorylation of the myosin light chains switches off the actin-activated Mg-ATPase activity (Adelstein, 1978; Small and Sobieszik, 1980). Studies using smooth muscle strips and skinned muscle fibres show the same phenomenon (Hoar et al., 1979; Aksoy and Murphy, 1979).

In a myosin phosphorylating system, muscular

contraction is initiated by calcium (10^{-5}-10^{-4} M) which binds to calmodulin, and the binding of calcium-calmodulin to the inactive myosin kinase. The resulting calcium-calmodulin kinase complex catalyzes phosphorylation of the myosin light chains so that the phosphorylated form of myosin can bring about muscular contraction. The myosin kinase is rendered inactive at calcium concentration less than 10^{-7} M because of the dissociation of calmodulin from the kinase. Under these conditions, myosin phosphatase is active, bringing about the dephosphorylation of myosin which results in muscular relaxation.

Cyclic AMP also plays a role in a myosin phosphorylation system. Upon hormonal stimulation of the beta-receptor cells of the tissue, adenylate cyclase is activated and this results in an increase of intracellular level of cyclic AMP and the activation of the protein kinase which is cyclic AMP-dependent. The activated protein kinase catalyzes phosphorylation of myosin kinase and results in weakening the binding of calcium-calmodulin to myosin kinase which is rendered less active upon phosphorylation and therefore causes dephosphorylation of myosin light chains (i.e. relaxation or decrease in the actin-activated ATPase activity) (Krebs and Beavo, 1979; Silver and Disalvo, 1979; see Figure 5). This probably explains the action of epinephrine (adrenaline) which raises the cyclic AMP level and causes relaxation of

vascular and intestinal smooth muscle (Conti and Adelstein, 1980). It has also been suggested that a similar control exists in blood platelets (Hathaway et al., 1980).

It is worth mentioning that the myosin phosphorylating systems also exist in vertebrate striated muscles though their role in these muscles is uncertain; probably playing only a modulating role (Perry, 1979; Adelstein et al., 1979; Stull et al., 1980). Therefore the presence of one type of control system in a particular muscle does not preclude the presence of a second or a third type. More over, the same regulatory control may have a different role in different muscles.

It had generally been accepted that vertebrate skeletal muscle possesses only the actin-linked regulatory system. Solid evidence for this has come from competitive actin-binding assays (Lehman et al., 1972; Lehman and Szent-Gyorgyi, 1975) using conventionally purified myosin or myofibrils (at 20-40 mM NaCl or KCl). However, if myofibrils are assayed at ionic strength near physiological, calcium-sensitivity is observed (Lehman, 1977). Lehman (1978), using washed myofibrils (i.e., myofibrils without the troponin-tropomyosin system), has once again shown that these myofibrils display calcium-sensitivity at physiological ionic strength.

In the present study, using native myosin filaments, purified by ^2H_2O-H_2O gradients (Trinick, 1973; Trinick and Rowe, 1973; Emes and Rowe, 1978) and also relaxed filaments preparation (i.e., preparations containing dissociated thin and thick filaments) (Emes, 1978), further evidence is presented to suggest that vertebrate skeletal muscle ATPase is also myosin-linked calcium-regulated, in addition to the actin-linked regulatory system.

Chapter II looks at the difference between the chymotryptic S-1 from native myosin filaments (before exposure to high ionic strength) and that from synthetic myosin filaments which are formed by dialysing conventionally purified myosin in true solution (high ionic strength) to physiological ionic strength. Chapter III deals with the enzymic properties of conventional myosin (after exposure to high ionic strength) and native myosin filaments (before exposure to high ionic strength) and the derived chymotryptic S-1's. Chapter IV looks at the calcium-sensitivity of purified native myosin filaments and relaxed filament preparations. Evidence is presented in Chapter V to suggest that the two myosin heavy chains of vertebrate skeletal myosin are not identical.

Figure 1

Sarcoplasmic reticulum

Diagrammatic representation of sarcoplasmic reticulum and its association with the transverse tubules and the myofibrils.

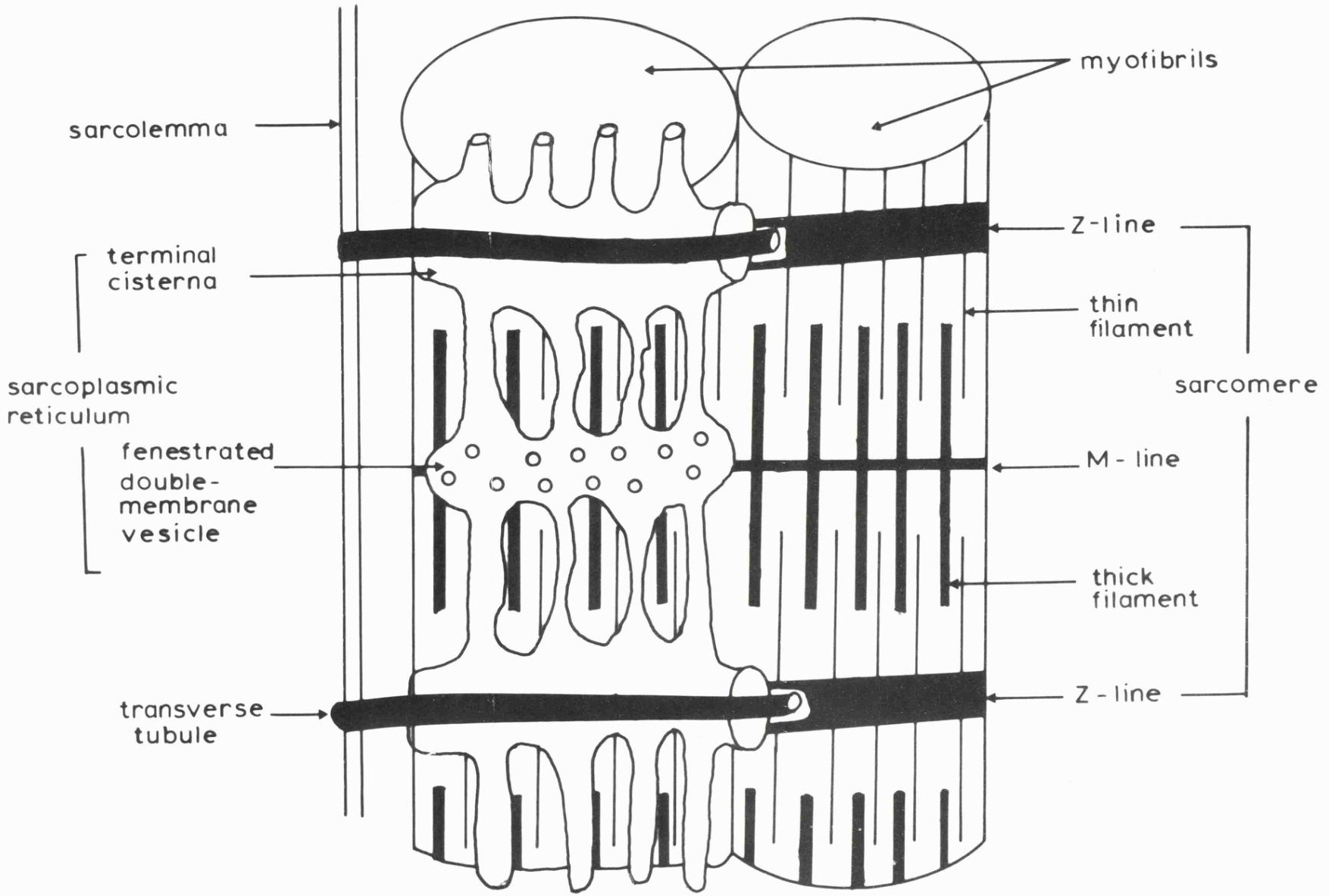

myofibrils
sarcolemma
Z-line
terminal cisterna
thin filament
sarcoplasmic reticulum
sarcomere
fenestrated double-membrane vesicle
M-line
thick filament
Z-line
transverse tubule

Figure 2

Diagrammatic representation of the overlapping arrays of thick and thin filaments in vertebrate skeletal muscle.

Note that the thick filaments are interconnected by the M-line and the thin filaments are interconnected by the Z-line. The H-zone is due to the absence of thin filaments and the pseudo H-zone is due to the absence of cross-bridges. The arrangement of thin and thick filaments in transverse section is also shown.

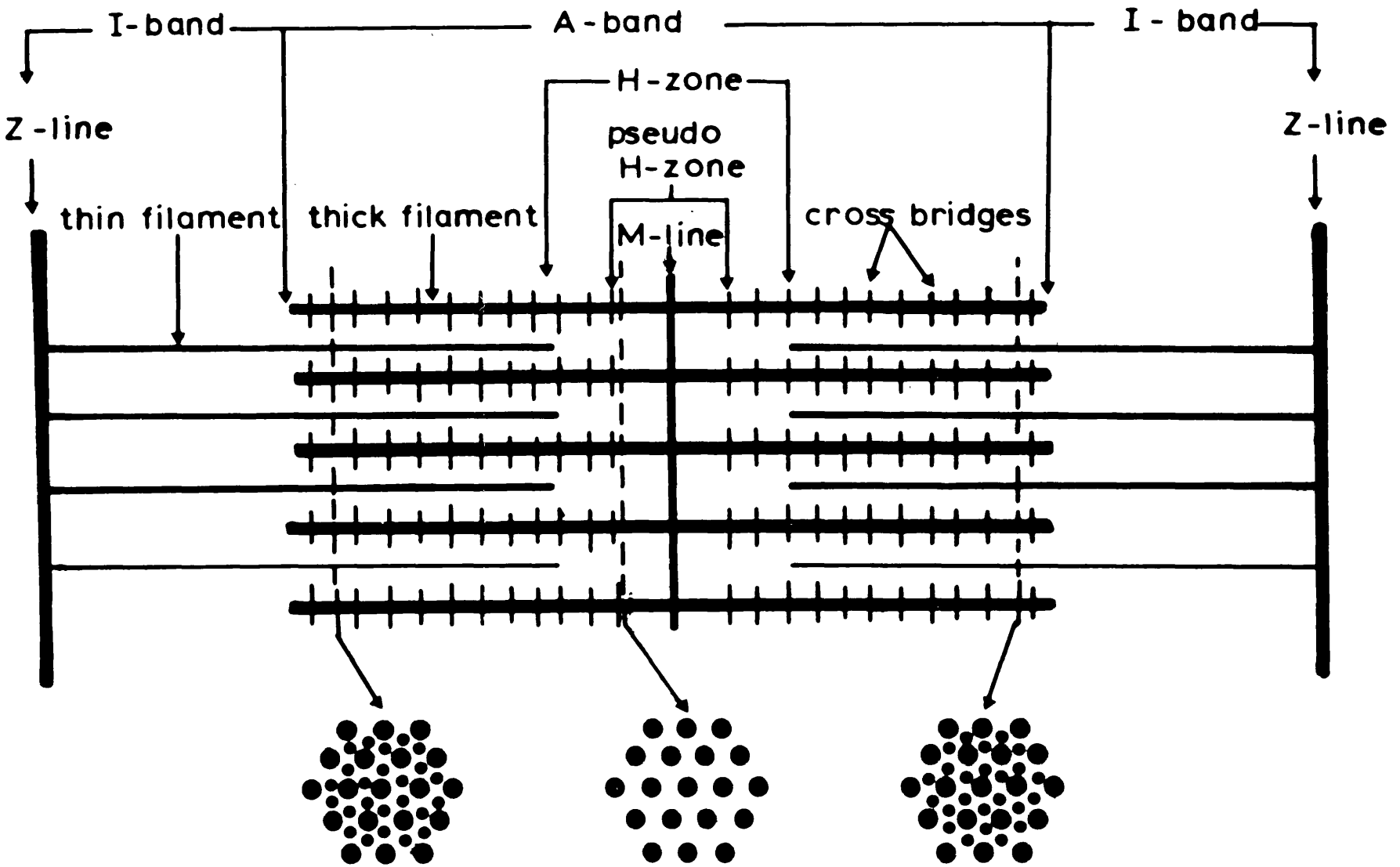

I-band
A-band
I-band
H-zone
Z-line
pseudo
H-zone
Z-line
thin filament
thick filament
M-line
cross bridges

Figure 3

Diagrammatic representation of thin filament structure

The troponin complex is shown as ellipse. F-actin filament containing a double helical arrangement of G-actin monomers is shown as open circles. Tropomyosin molecules which are associated end-to-end lie in the grooves of the double actin helix are represented as ribbons.

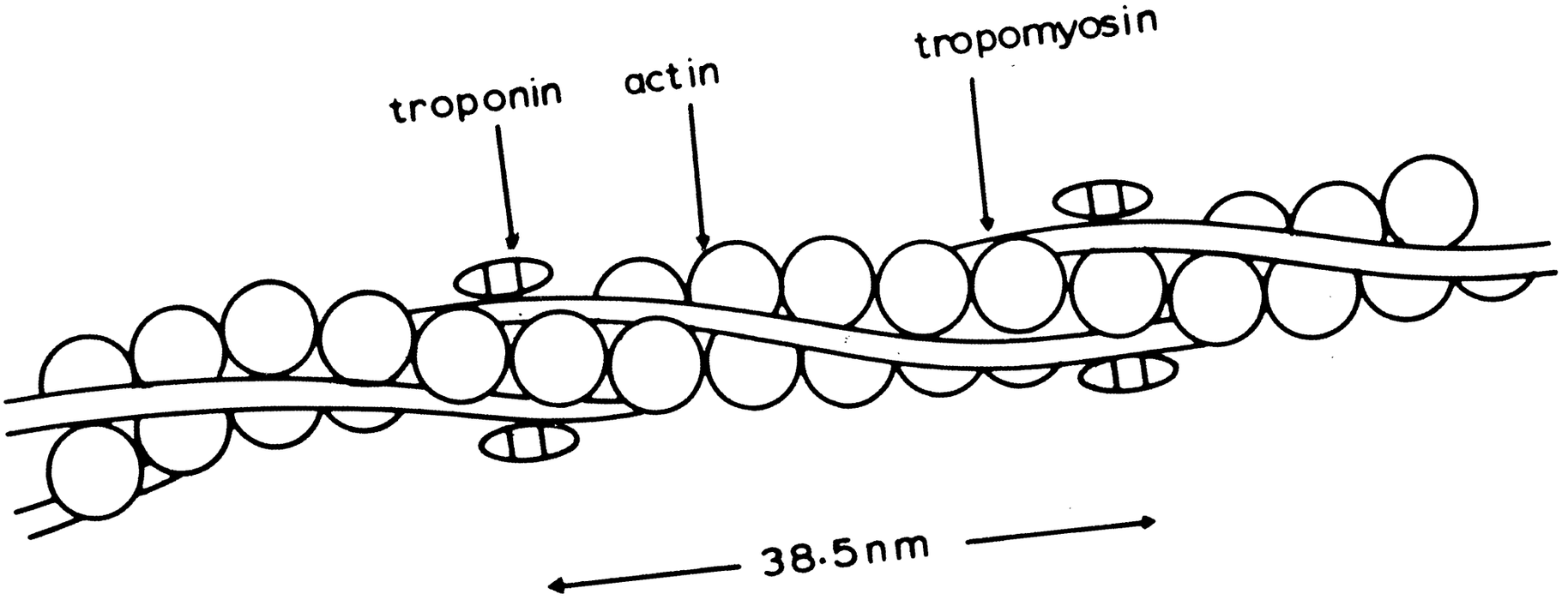

troponin
actin
tropomyosin
38.5nm

Figure 4

Diagrammatic representation of myosin molecule showing points of enzymatic fragmentation

Light meromyosin (LMM) and heavy meromyosin (HMM) can most readily be produced by trypsin or chymotrypsin (at I=0.6) while papain yields mainly subfragment-1 (S-1). Subfragment-2 (S-2) can be produced by further digestion of myosin rod or HMM. S-1 lacking in DTNB light chain can readily be produced by chymotryptic digestion of synthetic myosin filaments (I=0.12) while S-1 containing the DTNB light chain can be obtained by chymotryptic digestion of purified native myosin filaments (Chapter II).

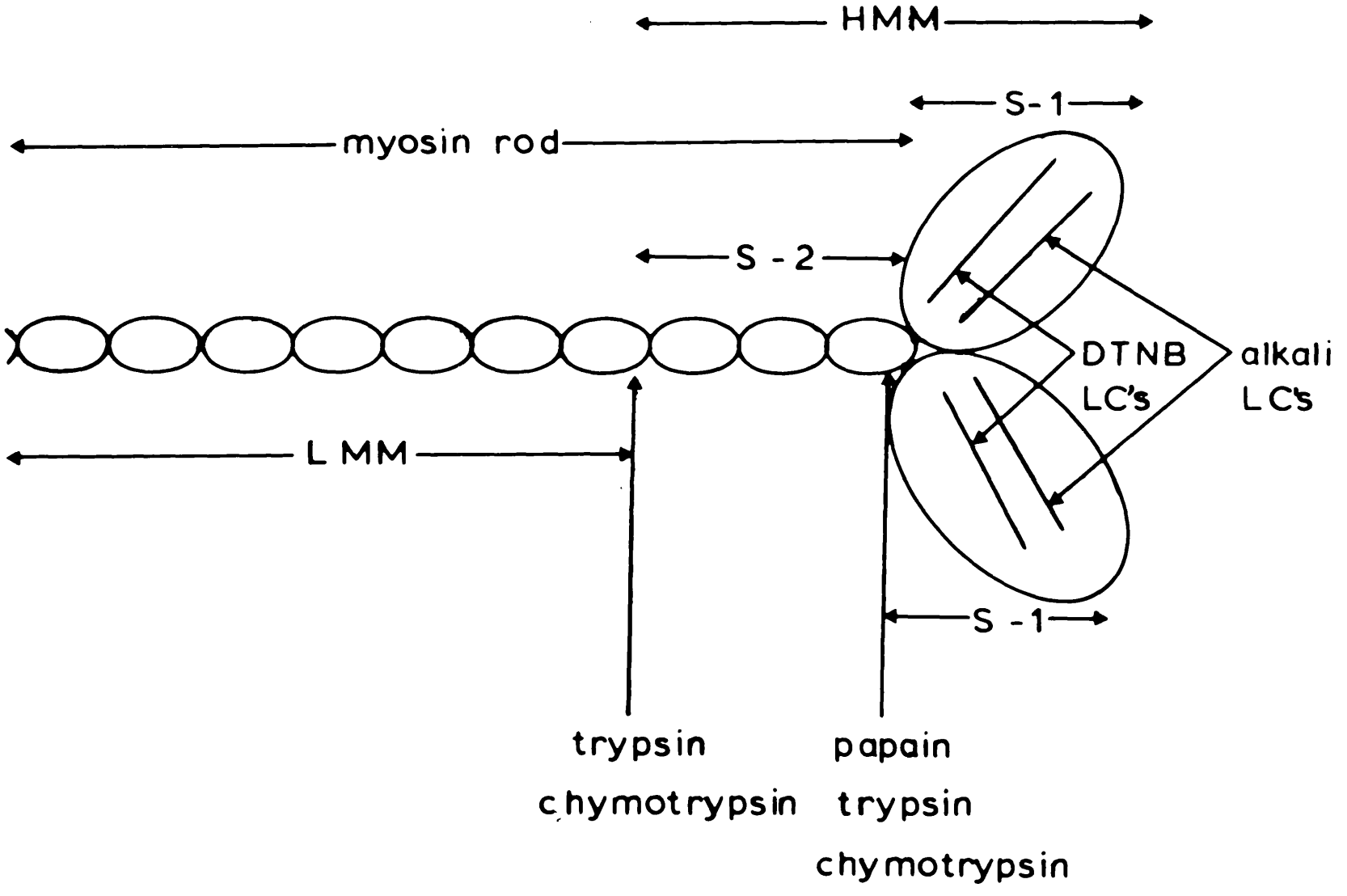

HMM
S-1
myosin rod
S-2
DTNB
LC's
alkali
LC's
LMM
S-1
trypsin
chymotrypsin
papain
trypsin
chymotrypsin

Figure 5

Role of cAMP and calcium in smooth muscle contraction

Calcium-calmodulin complex is formed at 10^{-5} -10^{-4} M calcium and this complex binds to the inactive myosin kinase. The resulting active calcium-calmodulin-myosin kinase catalyses the phosphorylation of myosin light chains so that the phosphorylated form of myosin can interact with actin to bring about muscular contraction. At about 10^{-7} M calcium, calcium and calmodulin dissociate from myosin kinase. which results in an inactive form. Relaxation occurs following the dephosphorylation of myosin catalysed by phosphatase which is independent on calcium for activity. Upon hormonal stimulation of beta-receptor cells, cyclic AMP increases and thus results in the activation of protein kinase which phosphorylates myosin kinase. The phosphorylated myosin kinase has a markedly reduced binding ability to calcium-calmodulin complex and therefore has a reduced potential to phosphorylate myosin to bring about contraction (see text).

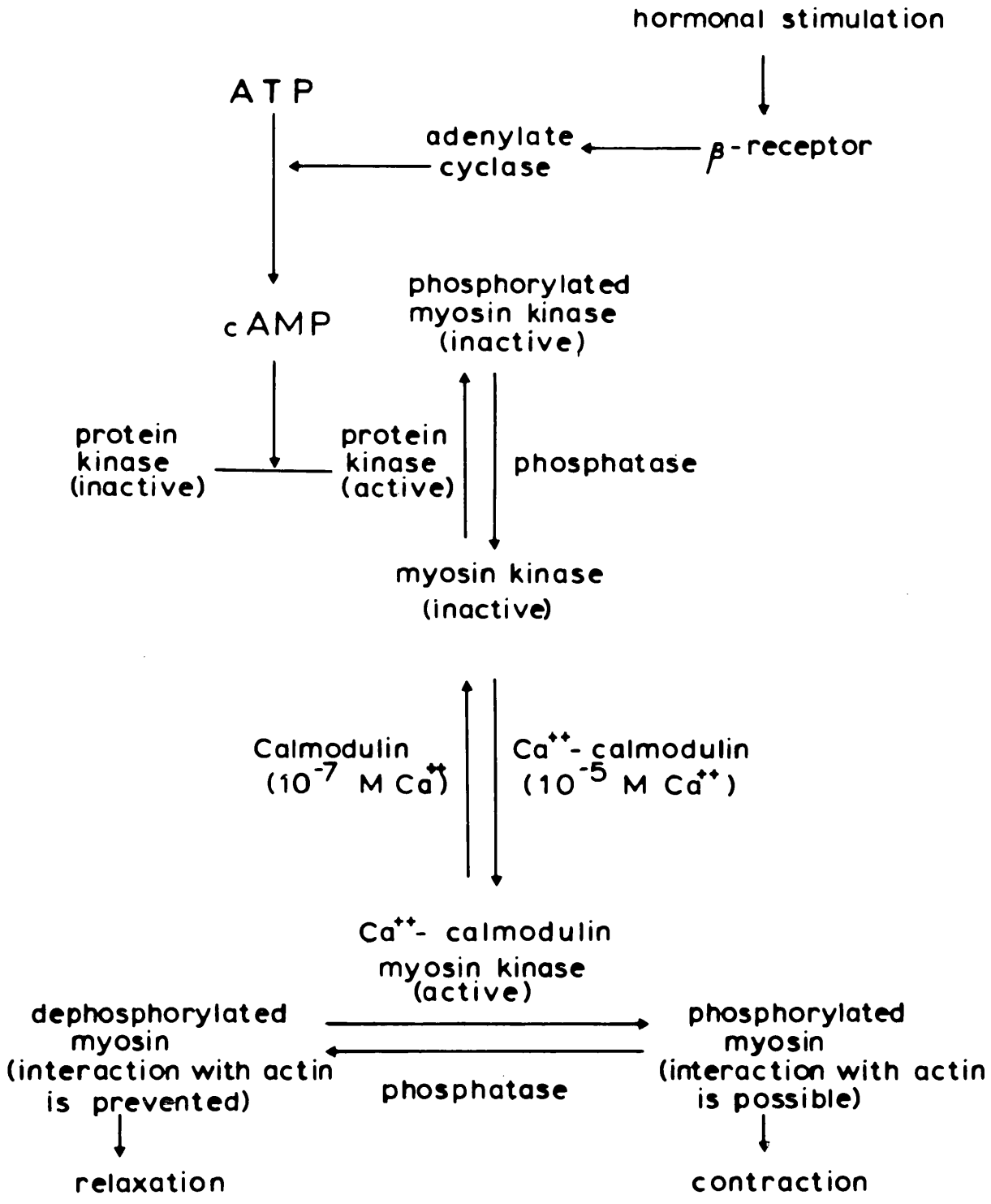

hormonal stimulation
ATP
adenylate cyclase
β-receptor
cAMP
phosphorylated myosin kinase (inactive)
protein kinase (inactive)
protein kinase (active)
phosphatase
myosin kinase (inactive)
Calmodulin (10^{-7} M Ca^{++})
Ca^{++}- calmodulin (10^{-5} M Ca^{++})
Ca^{++}- calmodulin myosin kinase (active)
dephosphorylated myosin (interaction with actin is prevented)
phosphatase
phosphorylated myosin (interaction with actin is possible)
relaxation
contraction

Table 1

Number of myosin molecules (n) per 14.3 nm of filament length in vertebrate skeletal myosin filaments, estimated by different methods

Method	n	Bibliography
(1) From % total protein in fresh muscle and washed fibrils.	4.0	Hasselbach and Schneider, 1951.
(2) Calculation from myosin content and number of myosin filaments per unit volume of muscle.	4.0	Hanson and Huxley, 1957; Huxley, 1960.
(3) From ATP and ADP binding to myofibrillar protein.	3.3-3.7	Weber et al., 1969; Maruyama and Weber, 1972.
(4) ADP binding to myosin.	2.7	Marston and Tregear, 1972.
(5) Quantitative SDS-PAGE of myofibrils.	2.7	Tregear and Squire, 1973.
	3.9	Morimoto and Harrington, 1974.
	2.5	Potter, 1974.
	3.8±0.5	Pepe and Drucker,1979.
(6) Particle counting technique (E/M).	4.3	Morimoto and Harrington, 1974.
(7) Mass per unit length (hydrodynamics).	3.1±0.2	Emes and Rowe, 1978.

(8) Mass per unit length (quantitative electron scattering).	2.7±0.7	Lamvik, 1978.
(9) Fraying of filaments (E/M).	3.0	Maw and Rowe, 1980.

CHAPTER II

CHYMOTRYPTIC DIGESTION

OF NATIVE MYOSIN FILAMENTS

II. CHYMOTRYPTIC DIGESTION OF NATIVE MYOSIN FILAMENTS

II.1. Introduction

Myosin in true solution can be prepared by conventional procedures which solubilises myosin from muscle mince and the final myosin preparation has undergone several precipitation stages and has been exposed to salt of high ionic strength (II.2.1.). Synthetic thick (myosin) filaments can then be formed by dialysing this solubilised myosin down to physiological ionic strength. The ability of myosin molecules to form filamentous aggregates was first observed by Noda and Ebashi (1960) using a flow birefringence technique. These synthetic filaments of vertebrate skeletal myosin resemble native thick (myosin) filaments though they are shorter and the projections (cross-bridges) formed are larger and more irregular than those of the native filaments (Huxley, 1963). Both ionic strength and rate of decrease in ionic strength play an important role in determining the width and length of the synthetic filaments formed. Myosin exists as monomer in a solution of high ionic strength (e.g. 0.6 M KCl) and myosin filament formation begins at an ionic strength of about 0.3 and is completed at physiological ionic strength. Random aggregation of synthetic filaments takes place if the ionic strength is lowered to about 0.04; precipitation of myosin then takes place. Native filaments are of 1.6 μm long and

15 to 17 nm in diameter (Huxley,1963; Kaminer and Bell,1966). Gradual decrease in ionic strength tends to form synthetic filaments of physiological lengths or even longer (Katsura and Noda, 1971; Moos et al., 1975). Though longer filaments have more regularity in their cross bridges (i.e. 14.3 nm axial repeat is often seen; Moos et al., 1975; Moral et al., 1979), they have wider diameters than those of the physiological ones (Moos et al., 1975). Electron microscopic studies have revealed that synthetic filaments of 30 to 50 nm in width and 5 to 15 μm in length can be formed in the absence of ATP or inorganic phosphate and this formation is not influenced by the presence of EDTA, $CaCl_2$ or $MgCl_2$ upto 5 mM. In the presence of ATP or inorganic phosphate, but in the absence of divalent cations, synthetic filaments with length of 0.2 to 0.4 μm and width of 10 to 15 nm are formed. The disruptive effect of ATP and inorganic phosphate is not observed if they are added after filaments are formed. If aggregation is carried out in the presence of ATP or inorganic phosphate and $MgCl_2$, filaments of length 5 to 15 μm and width of physiological dimension (15 to 17 nm) are formed. This shows that $MgCl_2$ counteracts the ATP or inorganic phosphate disruptive effect on synthetic filaments. $CaCl_2$ seems to be more effective in this respect for in the presence of both $CaCl_2$ and ATP or inorganic phosphate, filaments formed are of similar dimensions (30 to 50 nm in width and 5 to 15 μm in length) to those formed in the absence of ATP and

inorganic phosphate (Pinset-Harstrom and Truffy, 1979). It has also been shown that only fresh myosin solution can form long synthetic filaments (15 μm) with physiological diameters (15 to 17 nm) in the presence of millimolar amounts of ATP or inorganic phosphate and magnesium. However, if myosin solution is kept more than several days, shorter and thicker synthetic filaments will be formed. For example, a two week old myosin solution forms filaments with diameter more than 60 nm and length less than 0.5 μm in the presence of 5 mM $MgCl_2$ and ATP. This observation is accompanied by a decrease in DTNB light chain as indicated by SDS gel. Myosin lacking the DTNB light chain also shows the same phenomenon; it can only form short and thick synthetic filaments. Addition of freshly prepared DTNB light chain to aged myosin solution or DTNB-treated myosin does not restore the ability to form thin and long synthetic filaments. These observations seem to imply that DTNB light chains are necessary for filament formation (Pinset-Harstrom and Whalen, 1979).

Myosin Subfragment-1 (S-1) (I.2.2.; I.3.), the individual head of the myosin molecule, containing both the actin-binding and ATPase sites comprises a portion of the myosin heavy chain and two light chains (one DTNB light chain and one alkali light chain) is believed to play a vital role in muscular contraction. Various enzymes (I.3.), such as trypsin, papain and chymotrypsin have been employed

to obtain S-1 from synthetic myosin filaments, monomeric myosin or from myofibrils (Cooke, 1972). S-1 lacking the DTNB light chain can be obtained by chymotryptic digestion of synthetic myosin filaments which are produced by dialysing monomeric myosin down to physiological ionic strength in the absence of divalent cations. However, chymotryptic HMM having most of the DTNB light chain degraded is produced if digestion is carried out in the presence of divalent cations. (Weeds and Taylor, 1975; Weeds and Pope, 1977; I.3.).

In this chapter, a difference between synthetic thick (myosin) filaments and purified native thick (myosin) filaments is shown on subjection to chymotryptic digestion in the absence of divalent cations and in the presence of 20 mM sodium phosphate, pH 7.0, 0.12 M NaCl and 1 mM EDTA. The purification procedure for native myosin filaments involves calcium-depletion of fresh psoas fibres in a sodium-Ringer solution, followed by blending into a Mg-ATP relaxing medium and purification of native myosin filaments on $^{2}H_2O$-H_2O gradients (II.2.7.). The procedure does not involve precipitation stages or exposure to high ionic strength as found in the case of preparing synthetic myosin filaments. Chymotryptic digestion of these purified native myosin filaments (in the same medium as for synthetic filaments) yields an S-1 population containing substantially all the myosin light chains as analysed by

SDS gels. This shows a biochemical difference between synthetic and native myosin filaments when subjected to chymotryptic digestion.

If chymotryptic digestion is carried out on synthetic myosin filaments which are formed by dialysing solubilised native myosin filaments to physiological ionic strength, the S-1 obtained is identical to S-1 from conventional synthetic myosin filaments (i.e. lacking the DTNB light chain) as shown by SDS gels.

Attempts were also made to produce chymotryptic S-1 from unpurified native thick filaments (i.e. preparations containing thick and thin filaments) both in the dissociated and undissociated (rigor) states and from myofibrils. However, results were not obtained by these procedures satisfactory for further experiments to be undertaken.

II.2. Experimental

II.2.1. Preparation of myosin

Young male rabbits were sacrificed by severing the neck and bled. Back and hind leg muscles were removed immediately, chilled and minced in the cold (0-4^{o} C). All subsequent steps were performed in the cold; pre-cooled rotors were employed for centrifugations and all solutions were pre-cooled before use.

Extraction of myosin from muscle mince was carried out in 3-4 volumes of Guba-Straub solution (0.3 M KCl, 0.15 M potassium phosphate buffer, pH 6.5) for 10 minutes with gentle stirring (Szent-Gyorgyi; 1945; Johnson and Rowe, 1960). The extract, after removal of residual mince by centrifugation (6,000 r.p.m., 2 min), was filtered under vacuum through a 2 cm thick filter paper pulp on a Buchner funnel. The precipitated myosin obtained by diluting (about 14 times) the filtrate to I=0.03, recovered by centrifugation (6,000 r.p.m., 3 min), was dissolved in stock buffer (0.55 M KCl, 0.01 M $Na_2HPO_4.12H_2O$, 0.02 M KH_2PO_4, pH 6.7 adjusted with NaOH). The dissolved myosin in stock buffer was diluted to I=0.28 to precipitate the small amount of actomyosin which was removed by centrifugation (18,000 r.p.m., 30 min). After 3-4 reprecipitations at I=0.28 and I=0.03, the myosin was dialysed against stock

buffer overnight. The dialysed myosin solution was cleared by centrifugation (18,000 r.p.m., 30 min) if necessary, before use.

II.2.2. Myosin in glycerol

Myosin solutions which were not used within one week were kept in the cold (-17^{o} C) in 50% (v/v) glycerol. Future use of these solutions were made by dilution to I=0.03 and the precipitated myosin was recovered by centrifugation (6,000 r.p.m., 5 min). After one or two reprecipitations, the myosin was redissolved and dialysed in stock buffer overnight.

II.2.3. Preparation of myosin light chains

Two methods were employed for the preparation of myosin light chains during the course of this study.

II.2.3.1. Use of 2 M LiCl at pH 11.1

Myosin, at about 5-6 mg per ml was incubated with 2 M LiCl, 1 mM DTT and 0.1 M glycine buffer at pH 11.1 and 0^{o} C for 30 min (Gaetjens et al., 1968). The myosin heavy chains were precipitated by adding neutralized 2.5 M potassium citrate to a final concentration of 0.8 M and stirring was continued for another 10 minutes. The precipitated myosin

heavy chains were recovered by centrifugation (12,000 r.p.m., 60 min), and the supernatant containing the light chains was dialysed against water to reduce the salt concentration. Polyethylene glycol was used to concentrate the samples before the dialysis was completed, and finally freeze-dried. The freeze-dried light chains were redissolved in 0.05 M potassium phosphate buffer at pH 8.0 and the residual precipitated heavy chains were removed by centrifugation (18,000 r.p.m., 60 min). After dialysing against water, the light chains were freeze-dried and stored at -17^{o} C for future use.

II.2.3.2. Use of 5 M guanidine HCl

Myosin light chains were prepared by treating myosin with 5 M guanidine HCl followed by ethanol-water fractionation (Perrie and Perry, 1970). Myosin, at 18-20 mg per ml, was incubated in the presence of 5 M guanidine HCl (added as solid), 2 mM EDTA, 0.3 M KCl, 2 mM DTT, and 0.05 M Tris-HCl at pH 7.9 and 20^{o} C, for 1 hour. After cooling to 4^{o} C, the mixture was diluted first with an equal volume of cold water and then with 4 volumes of cold 95% ethanol. After about 20 minutes, the precipitated myosin heavy chains were removed by centrifugation (12,000 r.p.m., 60 min) and the ethanol was removed by dialysing against water. Myosin light chains were then concentrated and freeze-dried (II.2.3.1.). Of the two methods used for the

preparation of light chains, the one employing guanidine HCl was found to be more convenient.

II.2.4. Preparation of actin

Actin was prepared from the muscle residue, after extraction of myosin with Guba-Straub solution (II.2.1.). The muscle residue was brought to room temperature and stirred with 10 volumes of 0.4% $NaHCO_3$ solution for 30 minutes and strained through muslin. The residue was blended with 3-4 volumes acetone and strained through muslin and this was repeated until the acetone washing was clear. The powder was dried overnight on filter paper before use or stored sealed at -17° C for future use.

Actin was extracted from the acetone-dried powder at 0° C for 20 minutes with 20 to 25 volumes of 0.2 mM ATP and 2 mM Tris, pH 7.8. Extraction at 0° C yields actin free of tropomyosin (Drabikowski and Gergely, 1962). After extraction, the contents were filtered (under vacuum) and centrifuged (40,000 r.p.m., 30 min). The supernatant containing G-actin was made 0.1 M in KCl and 1 mM in $MgCl_2$ and allowed to polymerise for more than 2 hours before being centrifuged (40,000 r.m.p., 2.5 hr). The resulting pellet of F-actin was hand-homogenised in 0.2 mM ATP and 2 mM Tris, pH 7.8 and depolymerised by dialysing against the same solution for more than 40 hours with several changes.

This G-actin solution, after being clarified at 40,000 r.p.m., for 20 minutes, was repolymerised in 0.1 M KCl and 1 mM $MgCl_2$. After another cycle of purification, the resulting F-actin was dialysed exhaustively against 0.1 M KCl, 1 mM $MgCl_2$ and 2 mM Tris, pH 7.8. Actin was stored in 0.02% azide at 0^{o} C. SDS gel electrophoresis revealed a single band (II.3.3.; Plate 7, wells 7 and 8) in the preparation.

II.2.5. Preparation of heavy meromyosin

Heavy meromyosin (HMM) was prepared by digestion of soluble myosin for 10 minutes at a concentration of about 10-20 mg per ml in 0.6 M NaCl, 10 mM sodium phosphate, pH 7.0, 1 mM $MgCl_2$, using chymotrypsin at 0.05 mg per ml. The digestion was terminated by addition of 100 mM phenyl methane sulphonyl fluoride (PMSF) in ethyl alcohol to a final concentration of 10^{-4} M. After dialysis to I=0.03, the supernatant containing HMM was recovered by centrifugation (40,000 r.p.m., 30 min) and analysed on SDS gels.

II.2.6. Preparation of relaxed (dissociated) filaments

Relaxed filament preparations containing dissociated thick (myosin or A-) and thin (actin or I-) filaments, and also some A- and I- segments (Trinick and Rowe, 1973) can

be obtained by either blending directly a muscle mince into relaxing medium (0.1 M KCl, 10 mM $MgCl_2$, 1 mM EGTA, 5 mM ATP, 6 mM potassium phosphate buffer, pH 7.0), or by first preparing myofibrils which are then disrupted into filaments by gentle homogenisation (see below) in relaxing medium (Emes, 1977). However, during the course of this study, it was found that only by preparing myofibrils first, could a high yield (at least 50 mg from 2 psoas muscles) of dissociated myosin and actin filaments be obtained. Since the procedure (see below) also involved washing myofibrils in homogenising buffer (0.1 M KCl, 10 mM $MgCl_2$, 1 mM EGTA, 6 mM potassium phosphate buffer, pH 7.0), soluble proteins and debris in the final preparation of filaments were also markedly reduced.

Before preparing myofibrils, rabbit psoas muscle was soaked in a sodium-Ringer solution (0.1 M NaCl, 2 mM KCl, 2 mM $MgCl_2$, 0.1% (w/v) glucose, 1 mM EGTA, 6 mM potassium phosphate buffer, pH 7.0) for 24 hours (Huxley, 1963; Trinick, 1973; Emes, 1977). This solution depletes the calcium level in the muscle, so that the onset of potassium contracture (Hodgkin and Horowicz, 1959) is prevented when the muscle mince (see below) is transferred to physiological potassium concentration as found in homogenising buffer.

To prepare myofibrils, psoas muscle was dissected out

from freshly killed rabbit and teased into strips of about 4 mm in diameter, tied to perspex strips with cotton at about rest length and soaked in sodium Ringer solution for about 24 hours with several changes. After calcium depletion, the muscle strips were teased into smaller strips of about 2 mm in diameter, cut into lengths of about 2 mm and washed in homogenising buffer. The cut pieces of muscle were then homogenised in homogenising buffer in ice using a Chiltern homogeniser at top speed for two 30 second periods. The homogenate was centrifuged (6,000 r.p.m., 1 min) and the pellet was resuspended in homogenising buffer. The centrifugation and resuspension steps were repeated until the supernatant was clear to remove any debris and soluble proteins.

Preparation of relaxed filaments from myofibrils was as follows. Myofibrils suspension in homogenising buffer was centrifuged (6,000 r.m.p., 3 min) and the pellet was redispersed in relaxing medium. The centrifugation and pelleting steps were repeated twice more. The myofibrils were disrupted into thick (A-) and thin (I-) filaments (also some A- and I- segments) by homogenising gently (15 seconds) in relaxing medium using a Chiltern homogeniser at top speed. Residual myofibrils were removed by centrifugation (18,000 r.p.m., 3 min) and the supernatant was a relaxed filament preparation.

II.2.7. Purification of native thick (myosin) filaments on ^2H_2O-H_2O gradients

Relaxed filament preparations contain dissociated thick (myosin) and thin (actin) filaments, and also some segments (Trinick and Rowe, 1973). Separation of thick filaments from thin filaments and other segments can be achieved by employing ^2H_2O-H_2O gradients (Trinick and Rowe, 1973; Emes, 1977; Emes and Rowe, 1978). The principle relies on the difference in $s_{20,w}$ values between the thick (140 S) and thin (40 S) filaments.

Relaxing medium (II.2.6.) containing 10 mM ATP (different from usual, Emes, 1977) was made up in 20% and 100% ^2H_2O and the apparent pH was adjusted to 7.4 (0.4 units higher than the desired value; Glascoe and Long, 1960). Density gradients running from 20-100% ^2H_2O were formed from 26.9 ml of 20% ^2H_2O-relaxing medium and 26.1 ml of 100% ^2H_2O-relaxing medium in Beckman cellulose nitrate tubes (1 1/4" x 3 1/2") in an ice bucket, using a gradient-former.

About 2.5 ml of relaxed filament prparation (II.2.6.) at a concentration of about 5 mg per ml was applied gently to the top of each gradient. Centrifugation was carried out at 5^o C, 24,000 r.p.m., and for 55 minutes, using a 3 x 60 ml swing-out rotor (SW 25.2) in a Beckman L5-65

ultracentrifuge. The gradients were fractionated into 8 equal portions by piercing the bottom of each tube and collected in test-tubes surrounded by ice. Fraction 7 from the top of each gradient was used for the preparation of S-1 (II.2.8.2., II.3.6., II.3.7. ; see II.3.6. for yield of filaments).

II.2.8. Preparation of myosin subfragment-1's (S-1's)

Chymotrypsin was used to prepare S-1's from synthetic myosin filaments, purified native thick (myosin) filaments, unpurified native thick filaments, myofibrils and also from purified native thick filaments which had been exposed to high ionic strength.

II.2.8.1. S-1 from synthetic myosin filaments

Myosin in stock buffer (II.2.1.), at 10-15 mg per ml, was dialysed overnight against digestion medium (0.12 M NaCl, 1 mM EDTA and 20 mM Na_2HPO_4, pH 7.0). Digestion was carried out on the synthetic filaments formed for 10 minutes at 25^{o} C, using chymotrypsin at 0.05 mg per ml (Weeds and Taylor, 1975). After the digestion was terminated by addition of 100 mM phenyl methane sulphonyl fluoride (PMSF) in ethyl alcohol to a concentration of 10^{-4} M, the sample was dialysed to I=0.03. The supernatant containing S-1 was isolated by centrifugation (40,000

r.m.p., 30 min) and analysed by SDS gels (II.3.5.).

II.2.8.2. S-1 from purified native thick (myosin) filaments

Purified native thick (myosin) filament fractions from $^{2}H_2O$-H_2O gradients (II.2.7.) were pooled and concentrated by polyethylene glycol and dialysed exhaustively against "digestion medium" as for synthetic myosin filaments (II.2.8.1.). Chymotryptic digestion of these filaments was carried out in the same manner as for synthetic filaments, except that samples were in 0.4-0.7 ml volumes containing 6-8 mg per ml of filaments. S-1 obtained was analysed by SDS and urea gels (II.2.11.; see II.3.7. for yield of S-1).

II.2.8.3. S-1 from purified native thick (myosin) filaments after exposure to high ionic strength

Purified, concentrated native thick (myosin) filaments (II.2.7.; II.2.8.2.) were dialysed against stock buffer (II.2.1.) overnight or about five hours with stirring. The sample was cleared by centrifugation (50,000 r.p.m., 15 min) and the dissolved myosin in the supernatant was precipitated by dilution and redissolved in stock buffer. Chymotryptic digestion was carried out on the myosin filaments formed by dialysing to physiological ionic strength as for conventional synthetic filaments

(II.2.8.1.). S-1 isolated from the supernatant after dialysis to I=0.03 was analysed by SDS gels (II.3.8.).

II.2.8.4. S-1 from relaxed filament preparations in digestion medium.

Relaxed filament preparation (II.2.6.) was dialysed against digestion medium (II.2.8.1.) overnight before chymotryptic digestion was carried out as for digestion of synthetic myosin filaments as a trial to increase the yield of S-1 from native myosin filaments.

Thick (myosin) and thin (actin) filaments in relaxing medium (II.2.6.) were in the dissociated state, but after dialysis against digestion medium, the components were in the rigor state. Therefore, digestion was essentially carried out in the state where thick and thin filaments were complexed. S-1 isolated in the supernatant after dialysing to low ionic strength was analysed by SDS gels (II.3.4.).

II.2.8.5. S-1 from relaxed filament preparations in relaxing medium

Attempts were also made to increase the yield of chymotryptic S-1 from native myosin filaments by digesting relaxed filaments (as for synthetic filaments) immediatly

after preparation (II.2.6.). Digestion was therefore carried out in the dissociated state of thick and thin filaments.

After the digestion was terminated by PMSF (II.2.8.1.) the contents were centrifuged (65,000 r.p.m., 60 min) using an MSE 8 x 14 ml rotor. The centrifugation at high speed for a relatively brief period was intended to separate the S-1 from the residual thick (myosin) and thin (actin) filaments (also some segments).

Sephadex G-200 columns were used to purify S-1 in the supernatant which contained also other impurities like actin monomers. Separation was carried out both with and without an ATP-regenerating system using column of dimensions 2.5 cm x 55 cm and 5.5 cm x 130 cm. The use of an ATP-regenerating system involved the addition of pyruvate kinase (20 μg per ml) and phosphoenol pyruvate (8 mM) in the relaxing medium during homogenisation and centrifugation steps. This was intended to maintain the ATP level in order to keep the S-1 dissociated from residual actin or thin filaments during its separation in the column. Before applying sample, the whole column was equilibrated with relaxing medium. When the buffer (i.e. relaxing medium) came to level with the gel surface, appropriate amount of sample was applied gently to the top of the column. When all the sample had entered the column,

elution was carried out using the same buffer. Blue dextran was used to determine the frontal volumn. 10 and 20 ml fractions were collected from the small and the large column, respectively, using an LKB automatic fraction collector. Protein concentration of each fraction was estimated (Bradford, 1976; see II.2.10.) and they were analysed by SDS gels to locate the fractions containing S-1.

II.2.8.6. S-1 from myofibrils

Papain digestion of myosin while it is "in situ" in the myofibrils produces S-1 with degraded light chain 1 and DTNB light chain (Cooke, 1972). Following a procedure, similar to that of Cooke, chymotrypsin had been used to digest myofibrils, again in an attempt to produce S-1 in higher yield.

Fresh rabbit hind-leg, back and psoas muscles were minced and suspended in 4 volumes of a 1:1 (v/v) mixture of glycerol:0.1 M KCl, 0.01 M Tris-maleate, pH 7.0. After 30 minutes at 4^{o} C, the muscle mince was diluted with 4 volumes of "stock solution" (0.1 M KCl, 0.01 Tris-maleate, pH 7.0) and homogenised for three 30 second periods using a Chiltern homogeniser at top speed. (Remaining muscle mince was stored at -17^{o} C for not more than a month). After homogenisation, the resulting fibrils were centrifuged

(6,000 r.m.p., 2 min) and resuspended in stock solution a few times to remove debris. The digestion of this fibrils suspension (about 20 mg per ml) was carried out at 25° C for 10 minutes using 0.05 mg per ml chymotrypsin. The digestion was terminated by addition of 100 mM PMSF in ethyl alcohol to a final concentration of 10^{-4} M and the fibrils were washed with stock solution. The S-1 produced was released from the fibrils with 1 mM Mg-pyrophosphate (Mg-PPi) in stock solution. The remaining fibrils were removed by centrifugation (18,000 r.m.p., 15 min) and the supernatant containing S-1 was chromatographed on Sephadex G-200 and 10 ml fractions were collected using an LKB automatic fraction collector. The concentration of each fraction was estimated (II.2.10.) and analysed by SDS gel electrophoresis (II.2.11.1).

II.2.9. Attempt to promote the yield of purified native thick (myosin) filaments by the use of DNAase I

Using the fact that F-actin interacts with deoxyribonuclease I (DNAase I) in a process which eventually leads to the formation of a 1:1 stable complex between G-actin and DNAase I (Mannherz et al., 1975; Hitchcock et al., 1976; Blikstad, 1978), an attempt was made to increase the yield of purified native thick (myosin) filaments by incubating relaxed filament preparations (II.2.6.) with a stoichiometric excess of

DNAase I w.r.t. thin filaments. After more than 5 hours incubation at 0^{o} C, electron microscopy showed that all the thin filaments had been depolymerised (personnal communication, Rowe, A. J.). Purification of native myosin filaments was carried out in the usual manner using $^{2}H_2O$-H_2O gradients (II.2.7.), except that the gradients were also made up in a 10-12% sucrose gradient. After centrifugation (24 r.p.m., 65 min, 5^{o} C), the gradients were fractionated and analysed by SDS gel electrophoresis. The myosin heavy chains of the native thick filaments were found to be nicked at the HMM site due to unidentified enzymes present in the commercial DNAase I. Attempts were therefore made to protect the myosin heavy chains from being nicked by first treating DNAase I with PMSF in propanol before incubation with relaxed filament preparation (PMSF in the final incubation medium was about 6×10^{-4} M). One of the myosin heavy chains was still found to be easily nicked even though DNAase I had been PMSF-treated prior to incubation. (The heavy chains are only nicked, free heavy meromyosin not being produced, as shown by ultracentrifugation; see chapter V for results and further experiments involving DNAase I).

II.2.10. Estimation of protein concentration

For estimation of microgram quantities of protein concentration, the binding of Coomassie Brilliant Blue

G-250 to protein was employed (Bradford, 1976). This method is based on the fact that the red form of dye is converted to the blue form upon binding to protein (Reisner et al., 1975). The binding of the dye to protein shifts the absorption maximum of the dye from 465 to 595 nm and the absorption at 595 nm is monitored. The advantages of this method include (i) high sensitivity, due to high extinction coefficient of the complex formed, (ii) maximum colour intensity is obtained within two minutes, and (iii) the complex remains stable and dispersed in solution for a period of one hour.

The protein reagent was composed of 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol and 8.5% (w/v) phosphoric acid. The procedure for making up this reagent was as follows. 50 mg of Coomassie Brilliant Blue G-250 was dissolved in 25 ml 95% ethanol. To this solution was added 50 ml 85% (w/v) phosphoric acid. The resulting solution was diluted to a final volume of 500 ml.

A standard graph (Figure 6) of protein content (determined gravimetrically) against absorbance at 595 nm was prepared using cytochrome C, egg albumin, human albumin fraction V, bovine serum albumin and lysozyme. To 0.6 ml protein solution in 0.15 M NaCl was added 5 ml protein reagent to give a final volume of 5.6 ml in all samples. After two minutes, the absorbance of the sample in a 3 ml

cuvette was read using a Unicam SP 500 spectrophotometer. The unknown concentration of a sample was read off the standard graph after the addition of 5 ml protein reagent in a final volumn of 5.6 ml.

Protein concentrations were also determined by using an absorption coefficient E(1%,1cm) of 5.6 for myosin (Small et al., 1961), 6.47 for HMM (Young et al., 1964), 7.2 for chymotryptic S-1 (Onodera and Yagi, 1971) and 11.49 for F-actin (Eisenberg and Moos, 1967) at 280 nm.

In addition to the above, protein concentrations were also determined by the biuret method. All procedures gave consistent results within experimental error.

II.2.11. Gel Electrophoresis

II.2.11.1. SDS-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate (SDS) gel electrophoresis systems are employed for the characterization and resolution of the size (M.W.) and number of protein subunits in a protein preparation. In the presence of excess SDS and a thiol reagent (R-SH), such as beta-mercaptoethanol or dithiothreitol (DTT), all disulfide (-S-S-) bonds present in proteins are disrupted, and SDS unravels all intramolecular protein associations and binds

to all regions of polypeptide chains. The resulting highly negative and denatured SDS-carrying protein chains are then resolved electrophoretically in a buffered system containing polyacrylamide gel, SDS and a thiol reagent which maintain the denatured state of the proteins. In the presence of excess SDS, a constant charge (-ve) to mass ratio is generated for all the unraveled polypeptide chains. The polyacrylamide gel acts as a sieve in which the mobility of each polypeptide chain is monitored by the pore size and viscosity of the gel. The mobility of each of the polypeptide chains is a log function of the molecular weight of the polypeptide chain (Weber and Osborn, 1969; see also Figure 10).

The slab gel technique was employed throughout and the system used was essentially that of Laemmli (1970).

Gels of required percentage composition of acrylamide were prepared from a stock solution of 30% (w/v) of acrylamide and 0.8% (w/v) of N, N-methylene-bis-acrylamide (cross-linker). The separation gel was of 0.373 M Tris-HCl, pH 8.8 and 0.1% SDS, in the final concentration. Polymerisation of gel was initiated by the addition of 0.033% (w/v) of ammonium persulphate (a free-radical initiator) and 0.067% (v/v) of N,N,N',N'-tetramethylethylenediamine (TEMED). The stacking gel was of 4% acrylamide, 0.125 M Tris-HCl, pH 6.8, 0.1%

SDS and 10% glycerol, in the final concentration and was polymerised by adding 0.05% (w/v) ammonium persulphate and 0.1% (v/v) of TEMED. Running buffer was of the following composition: 0.025 M Tris base, 0.192 M glycine, 0.1% SDS, pH 8.3. Samples in final concentrations were of 2% (w/v) SDS, 10% (v/v) glycerol, 0.08 M Tris-HCl, pH 6.8, 2 mM PMSF, 0.005% (w/v) Bromophenol Blue, 0.1 M DTT and 0.05-1.25 mg per ml protein. Samples were boiled for 2 minutes before cooling and applying to gels. Electrophoresis was carried out with a constant voltage of 50 mV until the protein samples had gone into the stacking gel. The voltage was then raised to 100 mV and switched off when the Bromophenol Blue marker was about 1 cm from the bottom of the gel. The gel was stained overnight in 56.2% water, 43.8% MeOH and 0.0135% (w/v) Coomassie Brilliant Blue R (7% acetic acid was added before staining) and was destained in the same solution without the dye. Gels were finally fixed and stored in 10% acetic acid.

For dilute protein solutions, cold trichloroacetic acid was added to give a final concentration of 50 mM and left in the cold for at least 20 minutes. The precipitate, recovered by centrifugation (40,000 r.p.m., 5 min) was then used for gel samples. Alternatively, dilute protein solutions were concentrated by polyethylene glycol before preparing for gel samples.

II.2.11.2. Polyacrylamide gel electrophoresis in 8 M urea

As in the case of SDS gel electrophoresis, in the presence of 8 M urea and an excess thiol reagent, all intramolecular protein associations of native proteins are unraveled. However, unlike SDS which binds to all regions of the polypeptide chains (so that the intrinsic charges of the protein chains are negligible as compared to those due to SDS), the intrinsic charges of the polypeptide chains are not altered. Accordingly, urea-polyacrylamide gel electrophoresis systems are often used to resolve polypeptide chains having identical molecular weights but having different intrinsic charges. For example, phosphorylated DTNB light chain has a relatively higher mobility than unphosphorylated DTNB light chain (Perrie et al., 1973) in a urea gel system, but these can not be resolved by SDS gels.

The slab gel technique was employed, but a stacking gel was not used. The system was basically that of Perrie et al (1975) and Head and Perry (1974).

As for SDS gels, gels of required percentage composition of acrylamide were prepared from a stock solution of 30% (w/v) acrylamide and 0.8% (w/v) cross-linker. The separation gel was of 0.375 M Tris-HCl, pH 8.9, 48% (w/v) urea, and 0.012% (v/v) 2-mercaptoethanol.

After adjusting to pH 8.6, the gel was polymerised by the addition of 0.03% (w/v) ammonium persulphate and 0.08% TEMED. Running buffer consisted of 0.059 M Tris base and 0.383 M glycine at pH 8.6. Samples (0.05-1.25 mg per ml protein) in 8 M urea, 0.3 M Tris-HCl, pH 8.9, 4% (v/v) 2-mercaptoethanol and 0.005% (w/v) Bromophenol Blue were incubated for 60 minutes at 37° C before use (5 min incubation was sufficient for myosin light chains; personnal communication, Bagshaw, C. R.). Electrophoresis was carried out at a constant current of 40 mA. The gel was stained and destained in the same manner as for SDS gels (II.2.11.1.).

II.2.12. Microdensitometric scanning of gels

Fully destained gels were scanned (at 560 nm) using a Joyce-Loebl Microdensitometer, with a red filter. The peak areas of the microdensitometer traces were computer-evaluated by using an Apple II with graphics tablet and graphics tablet software. Amounts of protein loaded on gels were such as to give scan response within linearity.

II.2.13. Determination of the molecular weight of S-1 heavy chain

The molecular weight of chymotryptic S-1 heavy chain

from purified native thick (myosin) filaments was determined by SDS-polyacrylamide gel electrophoresis (II.2.11.1.) except that the stock solution contained 0.4% instead of 0.8% N, N-methylene-bis-acrylamide, since this amount of cross-linker had been reported to give satisfactory results for proteins over a wide range of molecular weights (Weber and Osborn, 1969). Protein markers of known molecular weights used were: myosin heavy chain (200,000), phosphorylase a (94,000), bovine serum albumin (66,000), catalase (60,000), egg albumin (45,000), actin (43,000), creatine kinase (40,000). A standard graph of mobility against log of molecular weights of proteins was prepared and the molecular weight of S-1 heavy chain was determined from the graph corresponding to its mobility.

II.2.14. Determination of $s_{20,w}$ value of chymotryptic S-1 from purified native thick (myosin) filaments

The sedimentation coefficient of S-1 from purified native myosin filaments was estimated using an MSE MKII Analytical Ultracentrifuge, the sample being run in a 20 mm double-sector cell, the sectors containing 0.40 ml of sample or solvent. Photographs were taken of the schlieren optical pattern at 200 second intervals, and these photographs were enlarged and the boundary positions measured using a 2-dimensional linear comparator. The logarithm of the radial position (r) of the boundary was

plotted against time (t), and the sedimentation coefficient computed from the relation

$$S = (dlnr/dt)x(1/\omega^2)$$

where ω = rotor velocity in radians/sec, and converted to standard conditions ($s_{20,w}$) by conventional procedures (Bowen and Rowe, 1970).

II.3. Results

II.3.1. Myosin preparation

Glycerolated myosin had been found to contain smaller amount of light chains than fresh myosin. Chymotryptic S-1 from synthetic myosin filaments formed from glycerolated myosin, very often showed considerable deficiency of light chains, as judged by SDS gels. A hypothetical suggestion would be that glycerol "loosens" the non-covalent bonding between the heavy and the light chains and the precipitation stages enhance the reduction in the amount of light chains.

DTNB light chains (LC2) also tended to undergo proteolysis easily during storage. This had been especially observed by Pinset-Harstrom and Whalen (1979) who noticed that the relative intensity of the DTNB light chains band decreased considerably while the LC3 band increased in intensity as shown by SDS gels. Using isoelectric focusing (Whalen et al., 1978), they had confirmed the production of a new peptide at the expense of DTNB light chains in the acidic pH range. Proteolysis began to occur after about 6 days extraction of myosin. Subsequently, fresh myosin was used whenever possible during the course of this study, especially for the preparation of chymotryptic S-1 from synthetic myosin filaments.

II.3.2. Light chain markers

Both procedures (II.2.3.) employed for the preparation of myosin light chains yielded pure light chains as judged by SDS gels (Plate 3). However, if these were stored for more than 7 or 8 weeks at -17° C, minor components running ahead of LC3 were observed in SDS gels. These minor components were believed to be mainly due to the nicking of the DTNB light chains as judged by the relative decrease in intensity of the DTNB light chain band as compared to fresh light chains. Nevertheless, this did not affect their use as markers since the three light chain bands were still prominent.

II.3.3. Purity of actin

The availability of pure actin is useful, if not essential as a marker for SDS gels, but it is of vital importance for the studies of calcium-sensitivity (Chapter IV) of myosin filaments and actin-activated ATPase activities (Chapter III). Following the procedures described (II.2.4.), pure actin was achieved as judged by SDS gels (Plate 7, wells 7 and 8). Pure actin did not keep for more than a week, but the addition of 0.02% azide prolonged the storage time (at 0-4° C) to 3-4 weeks.

II.3.4. Results of various S-1 preparation procedures

Chymotryptic digestion of "relaxed" filament preparations in digestion medium (II.2.8.4.) was carried out when the thick and thin filaments were in the rigor state. S-1 isolated in the supernatant after dialysis to low ionic strength was found to be associated with actin and the troponin complex, as indicated by SDS gel electrophoresis. Tropomyosin was not found to be present with the S-1 in the supernatant. This could either be due to tropomyosin not being released with S-1 into the supernatant or tropomyosin being subjected to proteolysis upon exposure to chymotrypsin. The latter was in fact more likely since SDS gel electrophoresis of the pellet after digestion did not show the presence of tropomyosin. Though S-1 produced in this way showed the presence of DTNB LC, it was contaminated with and obscured by thin filament components.

When chymotryptic digestion of relaxed filament preparations (II.2.8.5.) was carried out immediately after dissociation and upon purification by Sephadex G-200, no satisfactory preparation of S-1 was achieved. In addition to low yield, S-1 was still contaminated with thin filament components, even in the presence of an ATP regenerating system.

Chymotryptic digestion of myofibrils (II.2.8.6.)

yielded a considerable amount of HMM in addition to S-1 (Plate 1). This might be due to the actin-induced protection of the S-1 site (Oda et al., 1980). Chromatography of the digest on Sephadex G-200 showed that S-1 could be separated from HMM (Figure 7 and Plate 2). However, in addition to the low yield of S-1, HMM being the major product, the amount of S-1 light chains was observed to be reduced as compared to those of S-1 from purified native myosin filaments (Plate 3), as shown by SDS gels. The fact that HMM was heavily contaminated with actin (Plate 2) probably indicated that oligomers of actin (resulted from chymotryptic digestion of myofibrils) did run together with HMM during the separation of S-1 from HMM on the Sephadex column.

The above procedures for the production of chymotryptic S-1 involved first the digestion step and then the purification of S-1 obtained. S-1 obtained was always heavily contaminated with thin filament components, and subsequently preparation of a chymotryptic S-1 population containing both the DTNB and the alkali light chains was made by first purifying native thick (myosin) filaments on $^{2}H_2O$-H_2O gradients and then digestion of the purified filaments (II.2.7. and II.2.8.2.). However, other methods involving digestion of myofibrils (II.2.8.6.) or relaxed filament preparations (II.2.8.5.) may be improved (see II.4.1.).

II.3.5. Chymotryptic S-1 from synthetic myosin filaments

Chymotryptic S-1 lacking the DTNB light chain can be produced from synthetic myosin filaments which are formed by dialysing conventional myosin in true solution down to physiological ionic strength (Weeds and Talylor, 1975; Weeds and Pope, 1977; II.2.8.1.). This observation has been confirmed in the present study (Plate 3, well 6).

II.3.6. Purification of native thick (myosin) filaments

Preparations of purified native myosin filaments using $^{2}H_2O$-H_2O gradients of at least 98% pure with respect to the 43,000 molecular weight band seen in SDS gels have been achieved by previous workers (Emes, 1977; Emes and Rowe, 1978). However, the yield (less than 0.5 mg) was too small for the experiments to be carried out during the course of the present study.

Using a Beckman, 3 x 60 ml swing-out rotor (SW 25.2), I have been able to achieve 3-4 mg total yield of purified native thick filaments by pooling 4 runs (II.2.7.). Plate 5 shows the 8 fractions from a $^{2}H_2O$-H_2O gradient. Fractions 7 were pooled and concentrated for the preparation of chymotryptic S-1 (II.2.8.2.).

Figure 8 (i) shows a densitometric scan of SDS gel

electrophoresis of light chains from purified native thick filaments.

II.3.7. Chymotryptic S-1 from purified native thick (myosin) filaments

Fractions from gradients containing purified native thick (myosin) filaments were pooled and concentrated (II.2.8.2.). Chymotryptic digestion of purifed filaments yielded a population of S-1 which contained both the alkali and the DTNB light chains as shown by SDS gels. On the other hand, chymotryptic S-1 from synthetic myosin filaments formed from conventional myosin lacks in DTNB light chain (Plate 3). This finding shows that synthetic myosin filaments are different from native thick filaments when subjected to chymotryptic digestion (see II.4.4.).

The amount of DTNB light chain in the S-1 from native thick filaments varied from preparation to preparation to a certain extent. This was probably due to the variable degree of nicking in each preparation. Figure 8 shows examples of densitometric scans of light chains from various S-1 preparations and from purified native myosin filaments.

The relative molar ratios (Table 2) of light chains in purified native myosin filaments and in the derived chymotryptic S-1 had been estimated from various

preparations. These values (Table 2) were obtained from experiments in which the nicking of DTNB light chains of S-1 preparations was not pronounced as judged by SDS gel electrophoresis.

Some nicking of S-1 HC was also observed occasionally, probably due to some other proteolytic attack (or a combination of these proteolytic enzymes and chymotrypsin). Figure 9 shows S-1 (from purified native myosin filaments) with some nicking (see below) of heavy chains and DTNB light chains. If peaks "a" (M.W. 64,600) and "b" (M.W. 55,100) (Figure 9) were assummed to be due to nicking of S-1 HC and the minor peaks "c" (M.W. 14,400) and "d" (M.W. 12,500) due to nicking of DTNB light chains, then the relative molar ratios of S-1 HC (plus "a" and "b"):DTNB LC (plus "c" and "d"):alkali light chains (LC1 and LC3) would be about 2:2:2. LC3 might also be contaminated with DTNB LC nicking.

The fact that chymotryptic S-1 from purified native myosin filaments was only nicked was shown by ultracentrifugal studies. Ultracentrifugation of this S-1 (Plate 6) showed that the preparation was physically homogeneous and an $s_{20,w}$ value of $5.82 \pm 0.05 \times 10^{-13}$ was obtained. This value agrees well with that of Yang and Wu (1977). Plate 3 also shows the urea gel electrophoresis of S-1.

The total yield of S-1 was about 0.5 mg from filaments pooled from four gradient runs.

II.3.8. Chymotryptic S-1 from purified native thick (myosin) filaments after exposure to high ionic strength

Chymotryptic digestion was carried out on myosin filaments formed from solubilised protein components of purified native myosin filaments after dialysed against high ionic strength solution for about 5 hours or overnight (II.2.8.3.). SDS gel electrophoresis of the S-1 obtained showed that it lacked the DTNB light chain, just like chymotryptic S-1 from conventional synthetic myosin filaments (Plate 3, well 7).

This observation demonstrated that once the protein constituents of native myosin filaments were solubilised, they lost whatever structural feature was responsible in the native filaments for the resistance of the DTNB light chain to chymotryptic digestion.

II.3.9. Molecular weight of chymotryptic S-1 heavy chain of native thick (myosin) filaments

The molecular weight of the S-1 heavy chain obtained by chymotryptic digestion of purified native myosin

filaments was determined by SDS gel electrophoresis.

In order to obtain mobility, the following measurements were made:

(i) dye migration before staining,

(ii) length of gel before staining,

(iii) length of gel after destaining and fixing,

(iv) distance of protein migration after destaining and fixing.

$$\text{Mobility} = [(iv)x(ii)]/[(i)x(iii)]$$

Measurements (ii) and (iii) were included to take into account the swelling of gel. This was of particular importance since gels with lower amount of cross-linker resulted in more swelling (II.2.13.; Weber and Osborn, 1969).

Table 3 shows the mobilities of S-1 heavy chain and proteins of known molecular weights, determined from Plate 7.

The molecular weight of S-1 HC, determined by its

mobility from Figure 10, was found to be 93,300.

II.4. Discussion

II.4.1. Methods of producing S-1 containing the DTNB and the alkali light chains

Preparation of chymotryptic S-1 containing both the DTNB and alkali light chains have been performed on relaxed filament preparations (II.2.8.4. and II.2.8.5.), myofibrils (II.2.8.6.) and purified native myosin filaments (II.2.8.2.). However, procedures involving relaxed filaments and myofibrils did not prove to give satisfactory preparations of S-1. In the case of relaxed filament preparations, once digestion had been carried out, the S-1 produced was heavily contaminated with the protein components of thin filaments and purification by gel chromatography using an ATP regenerating system (II.2.8.5.) did not prove successful. Chymotryptic digestion of myofibrils yielded a considerable amount of HMM, in addition to S-1, the latter being on occasion of good purity but poor yield after separation from HMM using Sephadex G-200 chromatography. SDS gels demonstrated that this S-1 (obtained from glycerolated myofibrils) had a reduced amount of light chains as compared to S-1 from purified native myosin filaments (II.3.7.), probably due to the effect of storage in glycerol (II.3.1.) of the myofibrils or as a result of slow enzymic attack during column purification. The main certain cause of the above

procedures not yielding clean S-1 preparations (i.e. contaminated with thin filament components) was possibly the exhaustion of ATP in the relaxing medium during the relatively long dialysis and centrifugation times, and so allowing the interaction of S-1 and actin filaments.

Subsequently, production of chymotryptic S-1 was accomplished by first purifying native myosin filaments which involved calcium depletion of fresh rabbit psoas muscles, followed by homogenising in a Mg-ATP relaxing medium and purification on $^{2}H_2O$-H_2O gradients (II.2.7.). Chymotryptic digestion was then carried out on the purified filaments. This procedure of preparing S-1 did not involve glycerolation or exposure to high ionic strength and the S-1 obtained was subjected to no further purification steps and therefore avoiding further manipulation of the product.

Even though subsequent experiments (Chapters III and IV) involving chymotryptic S-1 containing the DTNB and alkali light chains were obtained from $^{2}H_2O$-H_2O gradients-purified native myosin filaments, other procedures (mentioned above) involving digestion of relaxed filaments and myofibrils should not be ignored as possible future approaches. Equilibration of a Sephadex G-200 column with magnesium-pyrophosphate (Mg-PPi) in an appropriate solvent prior to chromatography of chymotryptic S-1 from

relaxed filament preparations after centrifugation (II.2.8.5.) might improve both the yield and the purity of S-1. Attempts should be made to use relaxing medium (II.2.6.) containing Mg-PPi instead of Mg-ATP for the preparation of relaxed filaments since Mg-PPi can release both S-1 (Cooke, 1972; II.2.8.6.) and HMM (II.2.8.6.) from myofibrils after enzymatic digestion. Mg-PPi is not only cheaper to use than Mg-ATP in the preparation of relaxing medium and equilibration of Sephadex columns, there may also be a possibility that ATP might solubilize S-1 and other proteins (Cooke, 1972), making purification procedure more difficult.

Although chymotryptic digestion of myofibrils produced a considerable amount of HMM in addition to S-1 with reduced light chains content, it may be possible to increase the yield of S-1 by employing larger Sephadex columns and the use of myofibrils without glycerolation. It seems that the potential of increasing the yield of chymotryptic S-1 might be higher by using myofibrils than purified native myosin filaments or relaxed filament preparations, since the latter can only be prepared successfully from rabbit psoas muscles whereas the former can be obtained from the bulk of rabbit leg and back muscles.

Attempts should also be made to purify S-1 using

affinity chromatography upon Sepharose-N6-(6-Aminohexyl-)ADP or upon a Sepharose-phalloidin-actin column (Winstanley et al., 1979).

In addition to the above possible methods for the purification of chymotryptic S-1, use of pure DNAase I (II.2.9.) to depolymerise thin filaments in relaxed filament preparations (II.2.9.) prior to separation upon ^2H_2O-H_2O gradients might prove to be a successful method in preparing a high yield of purified native myosin filaments. Finally, future work should also include the search for alternative methods for the separation of relaxed myosin and actin filaments (under physiological ionic strength conditions), since these can be produced in a relatively high yield (II.2.6.) and chymotryptic digestion of purified native myosin filaments involves no further exposure to non-physiological environment.

II.4.2. Is our "S-1" really an S-1 ?

In the present study, we have shown that chymotryptic digestion of purified native myosin filaments yields an S-1 containing both the DTNB and the alkali light chains as judged by SDS gel electrophoresis (Plates 3 and 4). However, it was essential to establish that (i) the DTNB light chains present in this S-1 preparation were not due

to contamination by other proteins, such as troponin C which has about the same mobility as DTNB light chain on SDS gels, and (ii) this S-1 is not an HMM with a nick in it.

Chymotryptic digestion of unpurified native myosin filaments (i.e. in the presence of thin filaments) (II.2.8.5.) yielded an S-1 population showing the presence of DTNB light chains but contaminated with other proteins (II.3.4.). It was therefore necessary to check whether this S-1 still contained DTNB light chains when digestion was carried out on purified native myosin filaments (II.2.8.2.). Results from chymotryptic digestion of purified native myosin filaments had confirmed that the S-1 produced contained DTNB light chains in addition to the alkali light chains (II.3.7.), as shown by SDS and urea gels (plates 3 and 4).

The fact that our S-1 is not a "nicked" HMM is supported by the following evidence: (i) The $s_{20,w}$ value of 5.82×10^{-13} agrees well with that of Yang and Wu (1977) whose value has been obtained using chymotryptic S-1 lacking the DTNB light chains. The fact that our value is reconcilable with that of Yang and Wu seems to indicate that the presence of DTNB light chains in our S-1 results in an S-1 which is a more compact unit as compared to S-1 without the DTNB light chains. (ii) The molecular weight of

S-1 HC has been determined to be 93,300 (II.3.9.) which is clearly different from that of the HMM (see plate 4). The absence of an HMM rod of molecular weight about 40,000 further supports that our S-1 is not a nicked HMM (see plates 3 and 4). (However, S-1 HC is slightly nicked; see II.4.3.). (iii) Further solid evidence comes from the demonstration that once purified native myosin filaments are exposed to high ionic strength, the chymotryptic S-1 derived from the reformed myosin filaments shows the absence of DTNB light chains (plate 3). There is no reason to suppose that any impurity of molecular weight equivalent to the DTNB light chain would be lost by high ionic strength treatment.

II.4.3. How intact is our S-1 ?

Although the DTNB light chain and the heavy chain of our S-1 derived from purified native myosin filaments are slightly nicked (Table 2 and Figure 9), this still seems to be the most intact enzymatic myosin subfragment-1 which has ever been produced as compared to tryptic or papain S-1 (I.3.) whose heavy chain and light chains (especially LC1) are heavily degraded, or chymotryptic S-1 from synthetic myosin filaments which has all the DTNB light chain degraded (II.2.8.1.) as demonstrated by SDS gels. Ultracentrifugal studies have shown that although this S-1 is slightly nicked, it is still an intact physical unit

(Plate 6), and the sedimentation diagram shows no evidence of the presence of any other species.

II.4.4. What can we infer from the retention of DTNB LC in the chymotryptic S-1 derived from purified native myosin filaments ?

The present work has demonstrated that synthetic myosin filaments (formed by dialysing myosin in true solution down to physiological ionic strength) can be differentiated from native myosin filaments (not being exposed to high ionic strength) by the absence and presence of DTNB light chains in the derived chymotryptic S-1's from the two types of filaments. If native myosin filaments are solubilised by dialysis against high ionic strength media, the reformed filaments are indistinguishable from conventional synthetic myosin filaments in the sense that the chymotryptic S-1 produced contains no DTNB light chains. This observation demonstrates that whatever structural change that has taken place within the native filaments as a result of exposure to high ionic strength, is irreversible.

The possible explanations for the absence of DTNB light chains in the chymotryptic S-1 from native myosin filaments after high ionic treatment are summarised in Figure 11. Changes that may occur for native myosin

filaments after exposure to high ionic strength are: (i) the packing of myosin molecules in the filaments is altered; native myosin filaments pack with three molecules per 14.3 nm filament length while synthetic myosin filaments pack with 3-7 (Emes and Rowe, 1978), (ii) a change in the tertiary structrue of the myosin molecules, and (iii) a combination of both (i) and (ii).

A change involving the tertiary structure of myosin may result in a translocation of the DTNB light chains or an alteration in the structural association of both the myosin heavy and light chains. The resulting conformation of the myosin and or the mode of packing of myosin in the reformed filaments is to translocate the DTNB light chains to a site susceptible to chymotryptic attack, being otherwise sterically hindered from attack as found in the native form. It is stressed in chapters III and IV that a permanent translocation of DTNB light chains or a conformational change of the tertiary structure of myosin is responsible for the exposure of the ATPase site as a result of high ionic strength treatment, which otherwise is blocked by the DTNB light chains.

It is not possible to decide between alternatives (i) and (ii) above on the basis of the chymotryptic digestion evidence alone. However subsequent evidence (Chapters III and IV) showing that isolated S-1 (from native filaments)

changes its properties on exposure to high ionic strength solution makes it very likely indeed that a change in the tertiary or quaternary structure of the S-1 region is involed (see Figure 28); since S-1 is of course purely in solution and has hence no "packing" properties. Thus although some effect of the specific packing of myosin into the native filaments cannot be excluded as a partial explanation for the chymotryptic digestion results, there exists no very strong reason for advancing such a hypothesis. Translocation of the DTNB light chains on exposure to high ionic strength is in itself a sufficient hypothesis to encompass evidence obtained to date.

The role of DTNB light chains is still a subject of debate (see I.2.2.). For example, they have been found to weaken the interaction between S-1 and F-actin in the presence of calcium (Margossian et al., 1975) and are thought to play a role in the regulation of the resting tension of the contractile machinery (Pemrick, 1977). DTNB light chains may also be involved in the formation of synthetic myosin filaments (Pinset-Harstrom and Whalen, 1979; II.1.) or play an important role in the in vivo conformation of the myosin molecule and the filament. Results from the present study suggest that DTNB light chains which have not been exposed to high ionic strength are responsible for the low Mg-ATPase activity of the thick filaments and S-1 (Chapter III) and they also play a role

in the calcium-sensitivity of the thick filaments (Chapter IV).

In summary, the transformation of native myosin filaments into conventional synthetic myosin filaments via high ionic strength is characterised by at least the following: (i) the mode of packing of myosin in the filaments is altered, (ii) the translocation of the DTNB light chains or the transformation of the overall tertiary structure of the myosin molecule and or the filament, so that the DTNB light chains are exposed to a site susceptible to chymotryptic attack, and (iii) the effect of the high ionic strength modification of the myosin molecule or the filaments being irreversible so that the protein constituents of the filaments have lost their potential to reform into original native filaments as found in vivo. These criteria may serve as a useful probe for the reconstruction studies of myosin filaments from their constituent protein components (Maw and Rowe, 1979).

Figure 6

An example of a standard graph of protein content against absorbance at 595 nm using the method of Bradford (1976)

The proteins used were egg albumin (closed triangle), lysozyme (closed square), bovine serum albumin (closed circle), human albumin fraction V (open square) and cytochrome C (open circle) (II.2.10.).

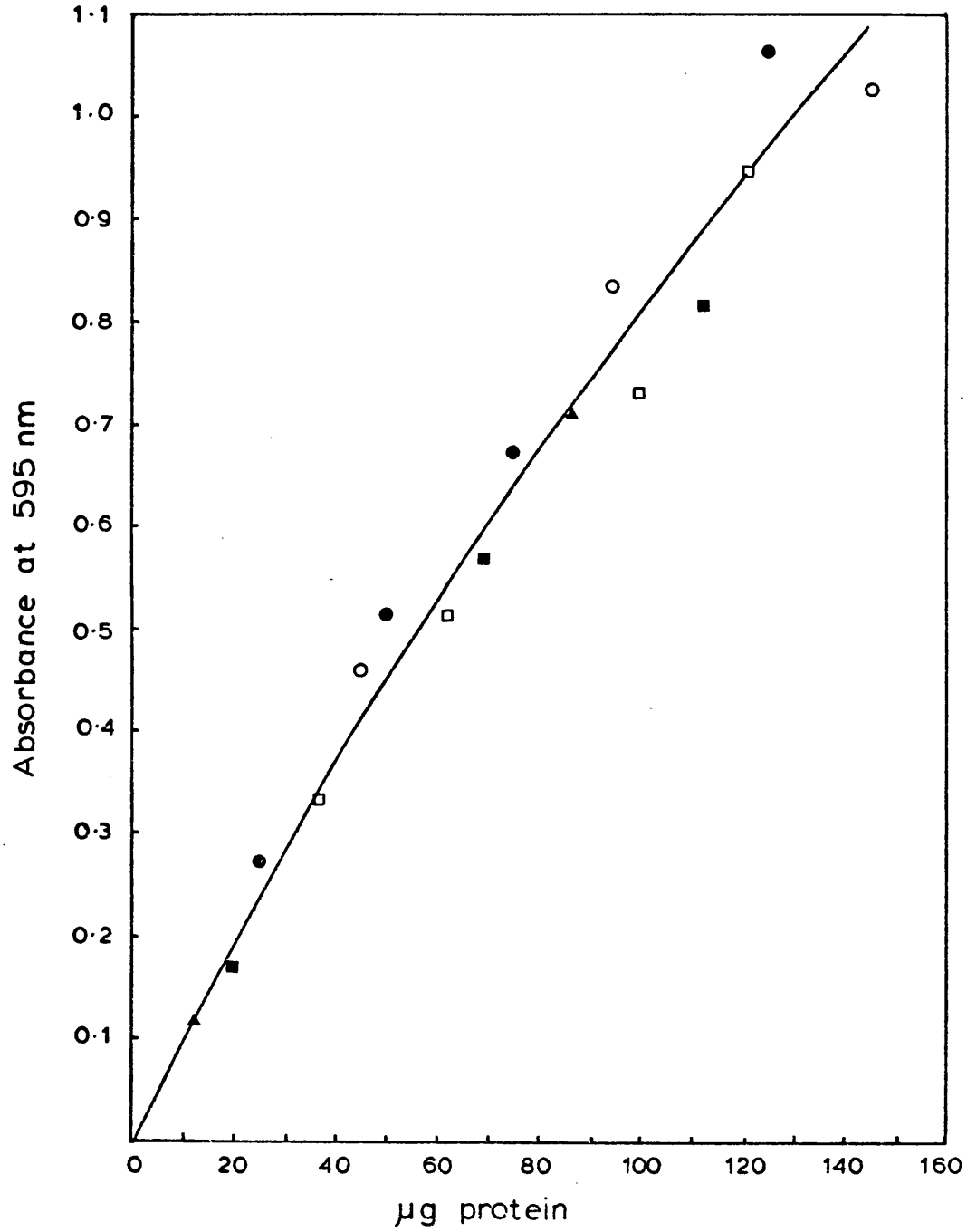

Absorbance at 595 nm
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
20
40
60
80
100
120
140
160
µg protein

Figure 7

Separation of S-1 from HMM (produced by chymotryptic digestion of myofibrils) upon Sephadex G-200

Heavy meromyosin (HMM) and subfragment-1 (S-1) were produced by chymotryptic digestion of myofibrils (II.2.8.6.). Chromatography of the sample was carried out on a 2.5 cm x 55.0 cm column. About 10 ml (about 0.3 mg per ml) protein sample was loaded and the solvent used was 0.12 M KCl, 0.5 mM PPi, 0.5 mM $MgCl_2$ 10 mM Tris-maleate, pH 7.0. Protein concentration of each fraction was estimated as described (II.2.10.). The frontal volume is indicated by an arrow.

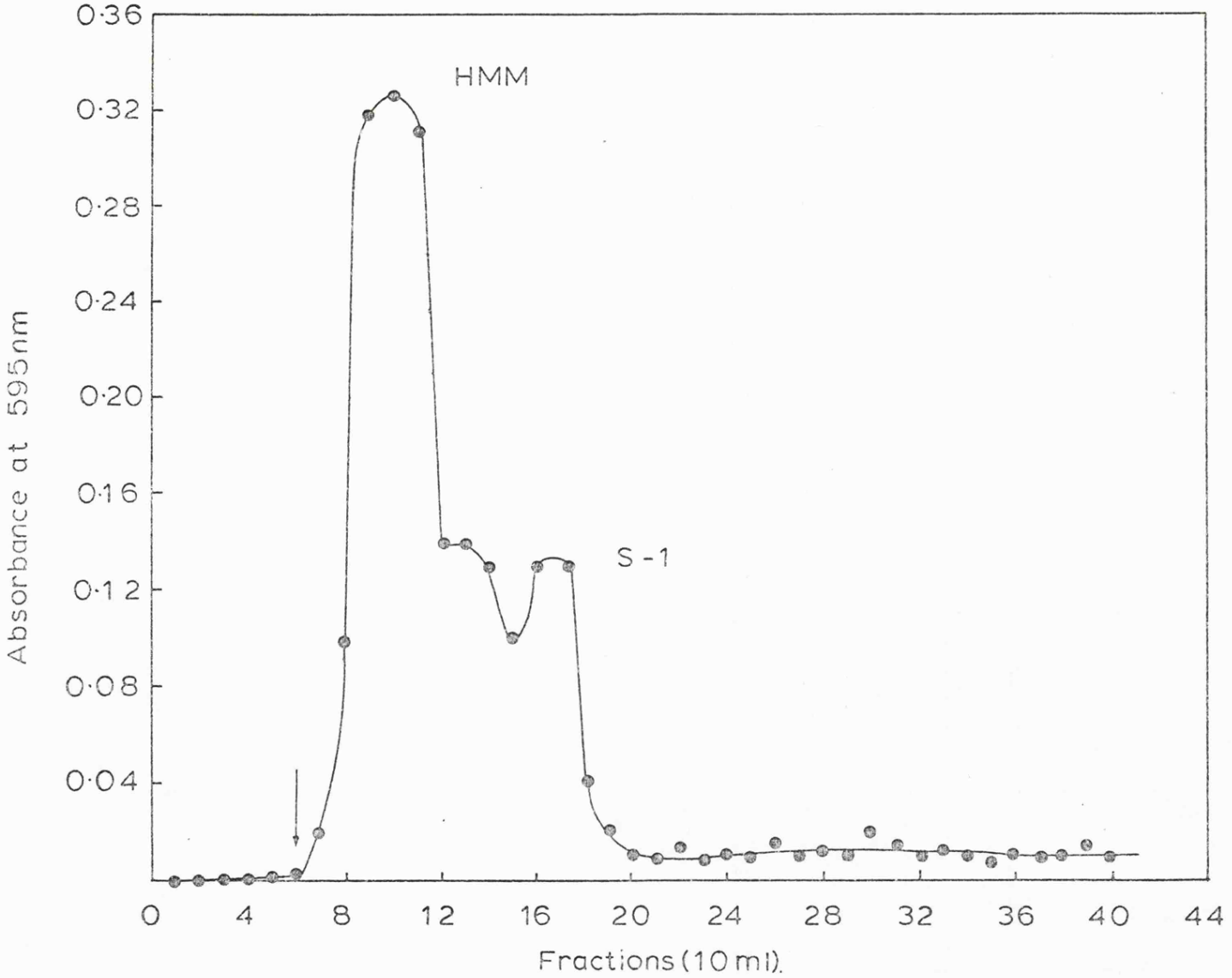

0·36
0·32
0·28
0·24
0·20
0·16
0·12
0·08
0·04
Absorbance at 595nm
HMM
S-1
0
4
8
12
16
20
24
28
32
36
40
44
Fractions (10 ml).

Figure 8

Examples of densitometric scans of SDS and urea gels (14%) electrophoresis of light chains from various preparations

SDS gels: (i) purified native myosin filaments, (ii) subfragment-1 from synthetic myosin filaments, (iii) subfragment-1 from purified native myosin filaments, (iv) subfragment-1 from purified native myosin filaments + purified light chain markers.

Urea gels: (v) subfragment-1 from purified native myosin filaments, (vi) subfragment-1 from purified native myosin filaments + purified light chain markers.

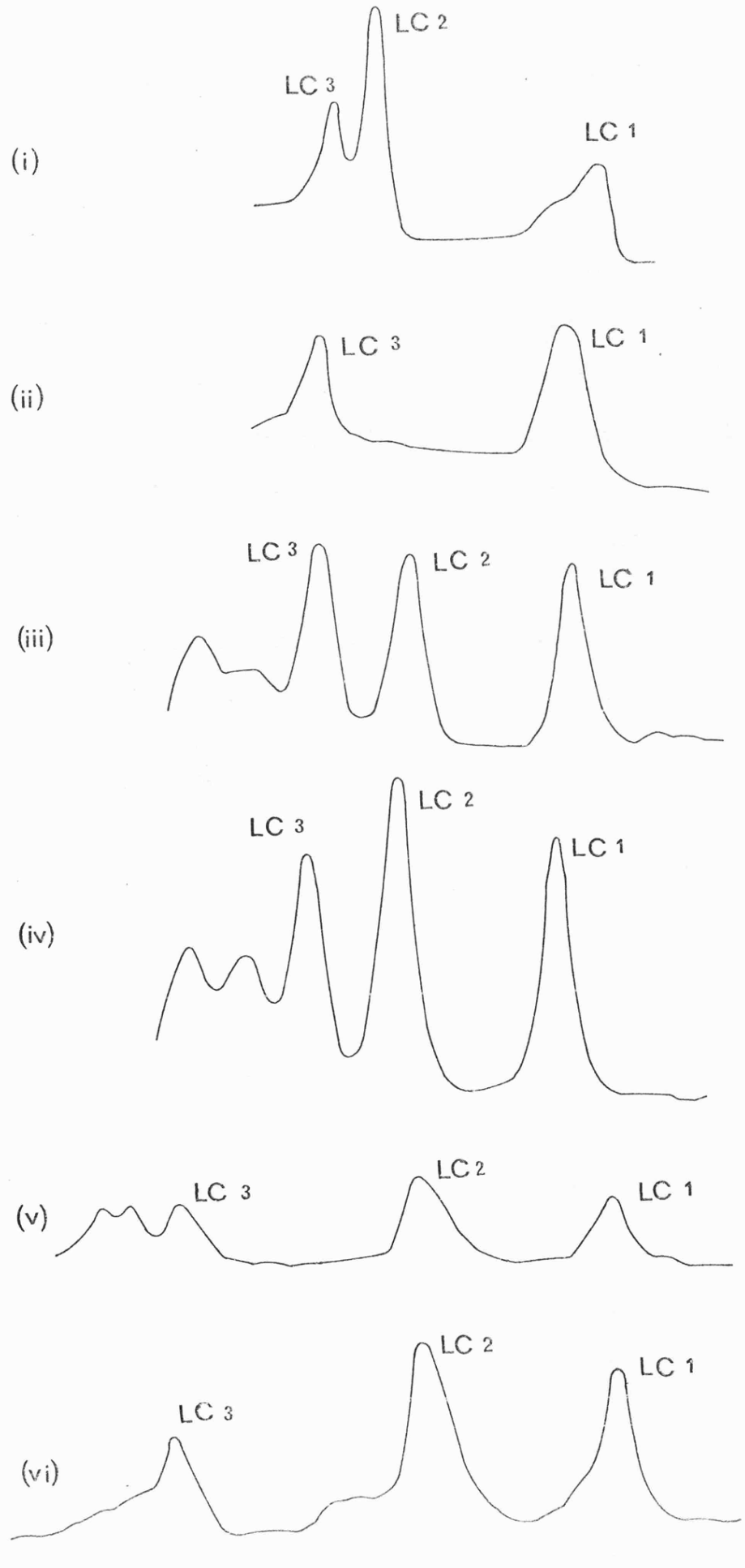

(i)
LC 3
LC 2
LC 1
(ii)
LC 3
LC 1
(iii)
LC 3
LC 2
LC 1
(iv)
LC 3
LC 2
LC 1
(v)
LC 3
LC 2
LC 1
(vi)
LC 3
LC 2
LC 1

Figure 9

Densitometric scan of SDS gel (14%) electrophoresis of subfragment-1 (S-1) from purified native myosin filaments showing some nicking of S-1 HC and DTNB light chains

Peaks "a" and "b" are assummed to be due to nicking of S-1 HC, and "c" and "d" due to nicking of DTNB light chains. LC3 may also be contaminated with nicking of DTNB light chains. Molecular weights of "a", "b", "c" and "d" are estimated (II.3.9.) to be 64,600, 55,100, 14,400 and 12,500 respectively (see II.3.7.).

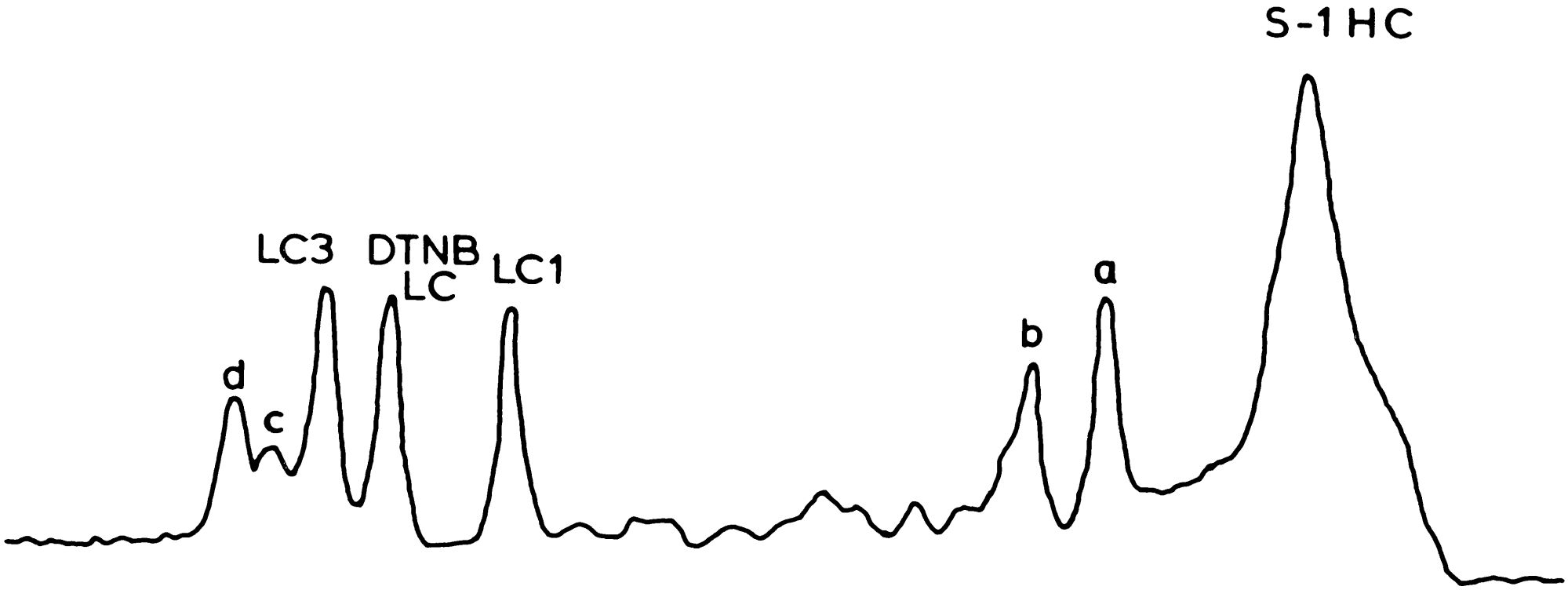

S-1 HC
LC3
DTNB
LC
LC1
d
c
b
a

Figure 10

Mobilities of S-1 HC and proteins of known molecular weights against log of molecular weight (see also Table 3).

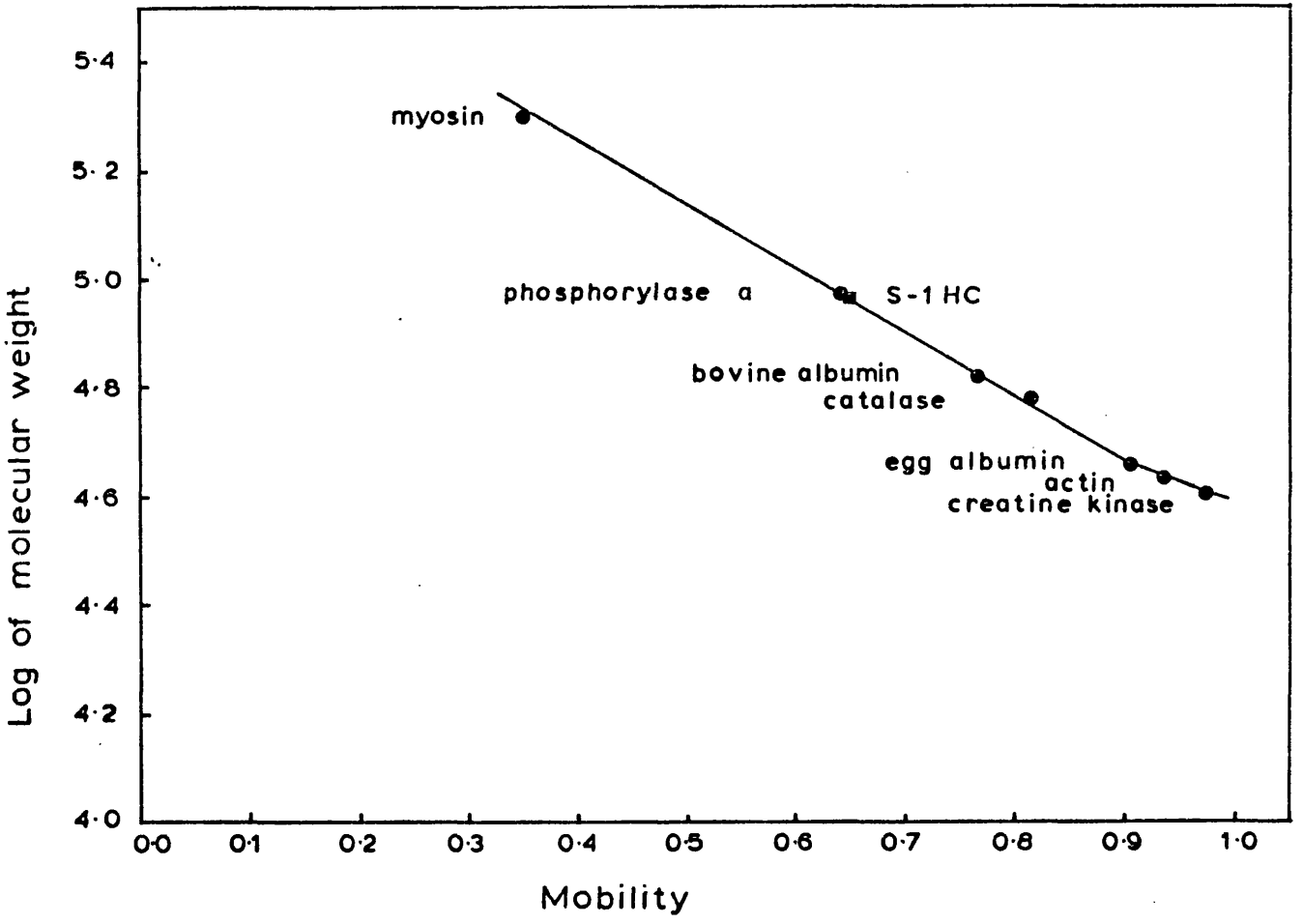

Log of molecular weight
5·4
5·2
5·0
4·8
4·6
4·4
4·2
4·0
0·0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
Mobility
myosin
phosphorylase a
S-1 HC
bovine albumin
catalase
egg albumin
actin
creatine kinase

Figure 11

Possible changes of native myosin filaments after exposure to high ionic strength

NMF: native myosin filaments.

NM: native myosin.

CM: conventional myosin.

SNMF: synthetic myosin filaments formed from native myosin.

SCMF: synthetic myosin filaments formed from conventional myosin.

Native myosin filaments after exposure to high ionic strength result in a change of the mode of myosin packing in the reformed synthetic filaments. The tertiary structure of myosin (especially with respect to DTNB light chains) may or may not be altered (see text).

A: only mode of packing of myosin in the reformed synthetic filaments is altered.

B: both packing and tertiary structure of myosin in reformed synthetic filaments are altered.

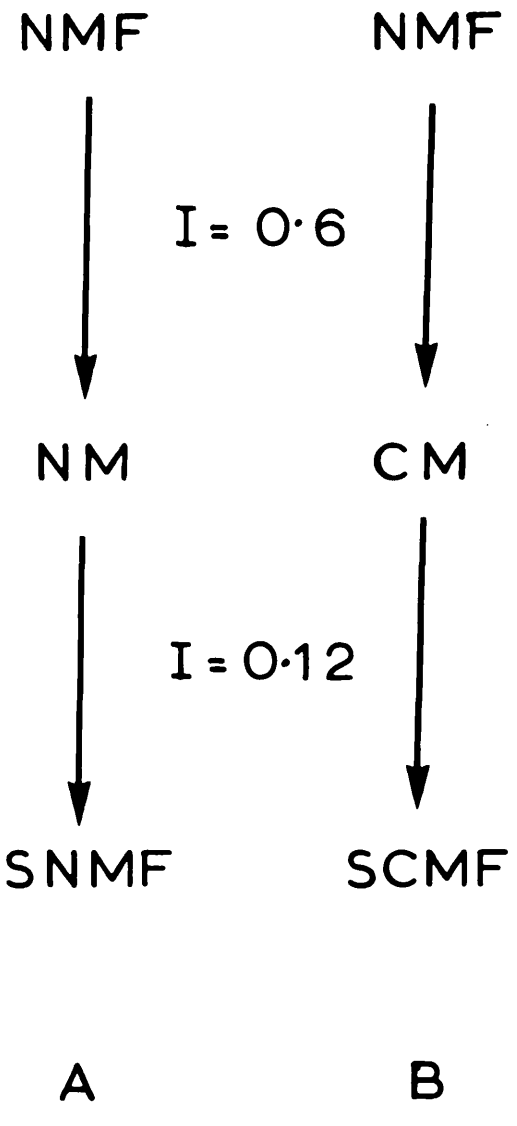

NMF
NMF
I = 0·6
NM
CM
I = 0·12
SNMF
SCMF
A
B

Plate 1

SDS gel (14%) electrophoresis of HMM and S-1 before chromatography upon Sephadex G-200

Heavy meromyosin (HMM) and subfragment-1 (S-1) were obtained by chymotryptic digestion of myofibrils (II.2.8.6.). Heavy chain, actin and light chain have been abbreviated as HC, A and LC, respectively. The positions of HMM HC and S-1 HC were identified by running on the same gel, conventional myosin (II.2.1.), HMM (II.2.5.) and S-1 from synthetic myosin filaments (II.2.8.1.) (not shown) (about 80 µg per well).

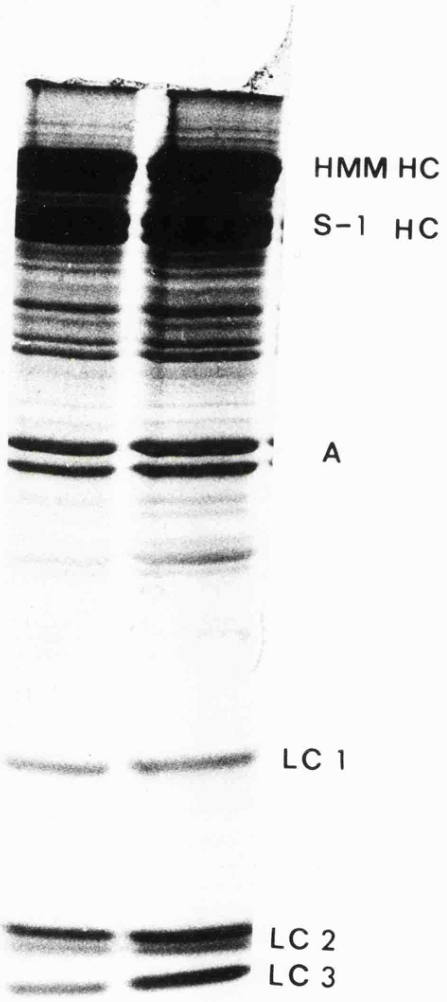

HMM HC
S-1 HC
A
LC 1
LC 2
LC 3

Plate 2

Separation of S-1 from HMM upon Sephadex G-200 (14% SDS gel)

Well 1: fraction 10 of Figure 7 (90 μg).

Well 2: fraction 12 of Figure 7 (80 μg).

Well 3: fraction 14 of Figure 7 (60 μg).

Well 4: fraction 15 of Figure 7 (20 μg).

Well 5: fraction 17 of Figure 7 (30 μg).

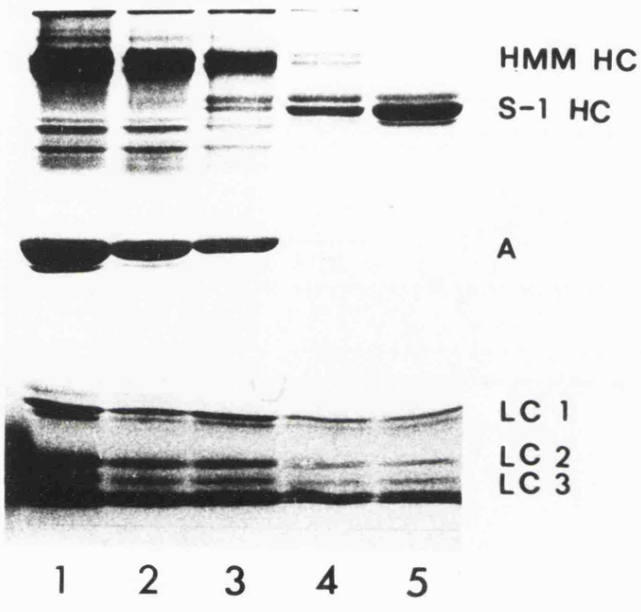

HMM HC
S-1 HC
A
LC 1
LC 2
LC 3
1 2 3 4 5

Plate 3

SDS and urea gels electrophoresis of various chymotryptic subfragment-1's (S-1's)

Wells 1-7: 14% SDS gel.

Wells 8-10: 14% urea gel.

Well 1: S-1 from purified native myosin filaments (PNMF's) (12 μg).

Well 2: S-1 from PNMF'S (15 μg) + light chain markers (9 μg).

Wells 3 and 4: light chain markers (9 μg per well).

Well 5: S-1 from PNMF'S (18 μg).

Well 6: S-1 from synthetic myosin filaments (35 μg).

Well 7: S-1 from filaments formed from solubilised PNMF's (25 μg).

Well 8: S-1 from PNMF'S (12 μg) + light chain markers (10 μg).

Well 9: light chain markers (12 μg).

Well 10: S-1 from PNMF'S (30 μg).

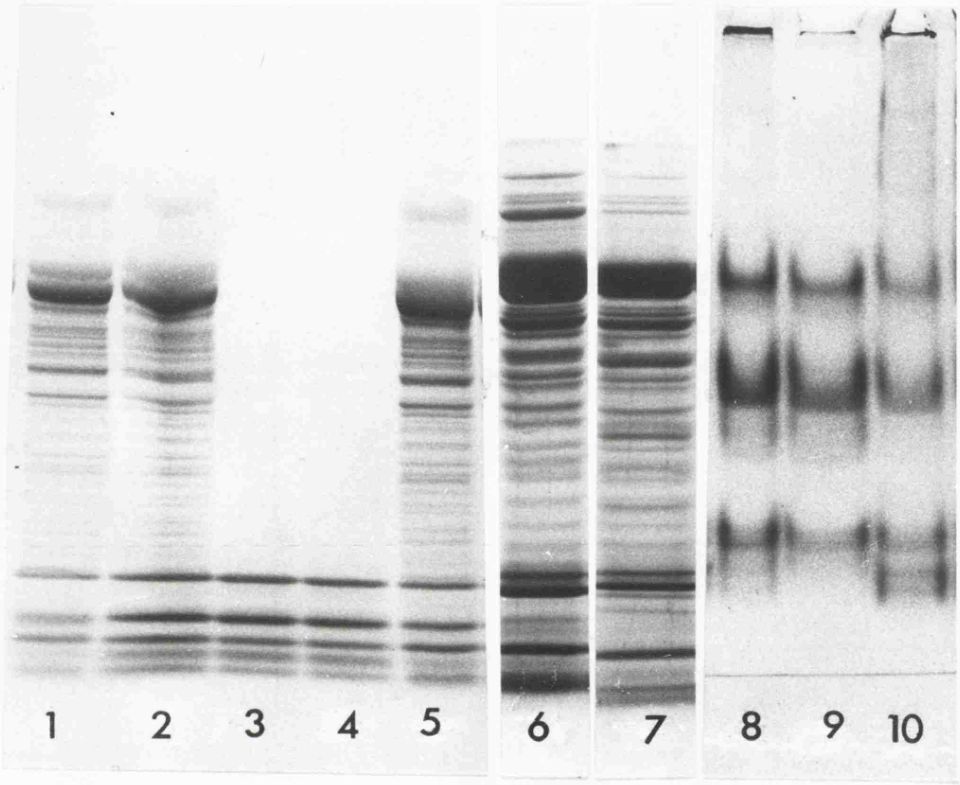

1
2
3
4
5
6
7
8
9
10

Plate 4

SDS gel (14%) electrophoresis of conventional myosin, heavy meromyosin (HMM) and chymotryptic subfragment-1 (S-1) from purified native myosin filaments (PNMF'S)

Wells 1-3: S-1 from PNMF'S (15 μg, 20 μg, 5 μg respectively).

Wells 4-6: conventional myosin (15 μg, 30 μg, 30μg respectively).

Wells 7-8: HMM (25 μg, 25μg, respectively).

The bands are labelled from top of the gel: myosin heavy chain (M HC), heavy meromyosin heavy chain (HMM HC), S-1 heavy chain (S-1 HC), light chain 1, 2 and 3 (LC1, LC2 and LC3).

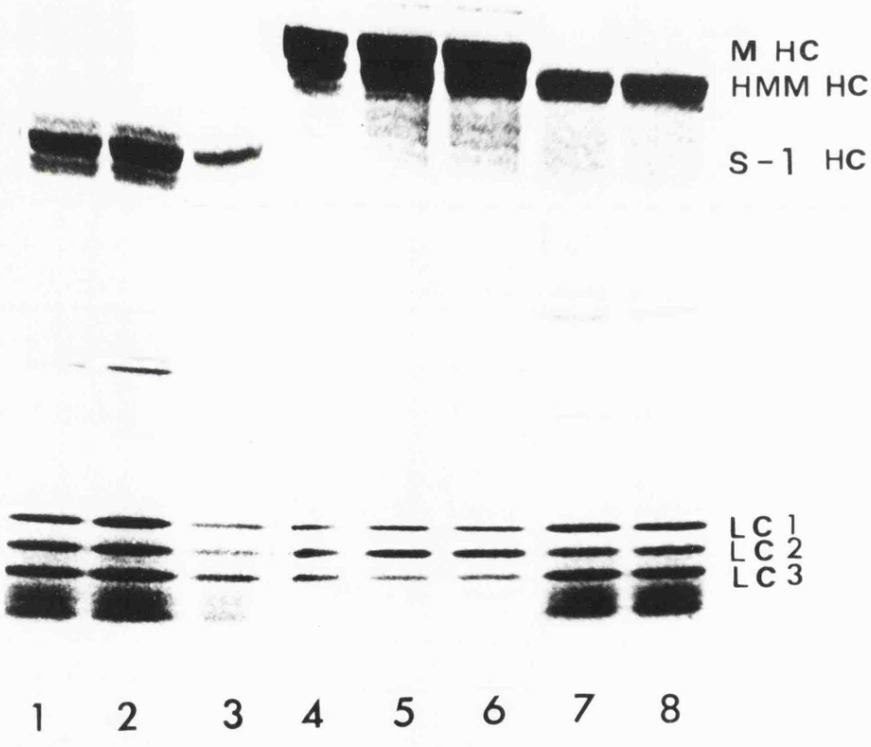

M HC
HMM HC
S-1 HC
LC 1
LC 2
LC 3
1 2 3 4 5 6 7 8

Plate 5

Purification of native thick (myosin) filaments by ^2H_2O-H_2O gradients (14% SDS gel)

Wells 1 to 8 are fraction 1 (20% ^2H_2O) to fraction 8 (100% ^2H_2O), respectively, from a gradient loaded with relaxed filaments. The bands are labelled from top of the gel: myosin heavy chain (HC), B-protein (B), C-protein (C), F-protein (F), actin (A), troponin-T (TN-T), tropomyosin (TM), light chain 1 (LC1), troponin-I (TN-I), light chain 2 (LC2) and light chain 3 (LC3). The thin filament protein components decrease from fractions 1 to 8. Fraction 7 was used for the preparation of chymotryptic S-1 (loading of protein for each well varies from about 60 to 90 μg).

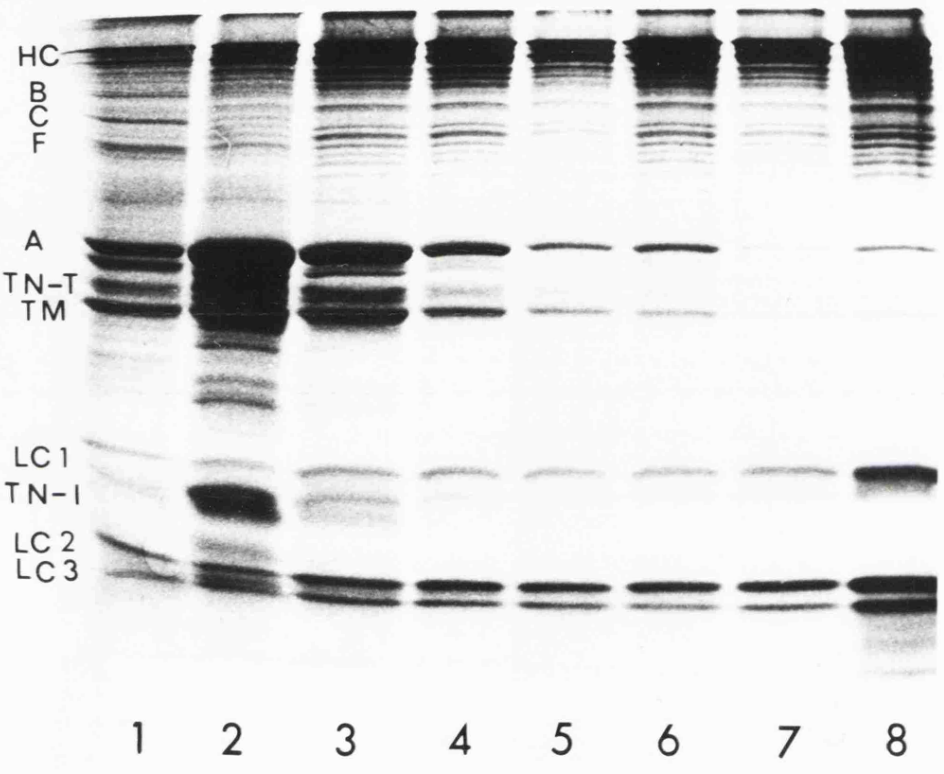

HC
B
C
F
A
TN-T
TM
LC 1
TN-I
LC 2
LC 3
1
2
3
4
5
6
7
8

Plate 6

Optical schlieren pattern of the sedimentation of subfragment-1 from purified native myosin filaments

Direction of sedimentation is shown by the arrow.

Time = 200 x 26 seconds.

Speed = 50,140 r.p.m.

Temperature = 20.12° C.

Solvent was stock buffer (KCl/Pi) diluted to I=0.12, pH 6.8.

Concentration of S-1 = 1 mg per ml.

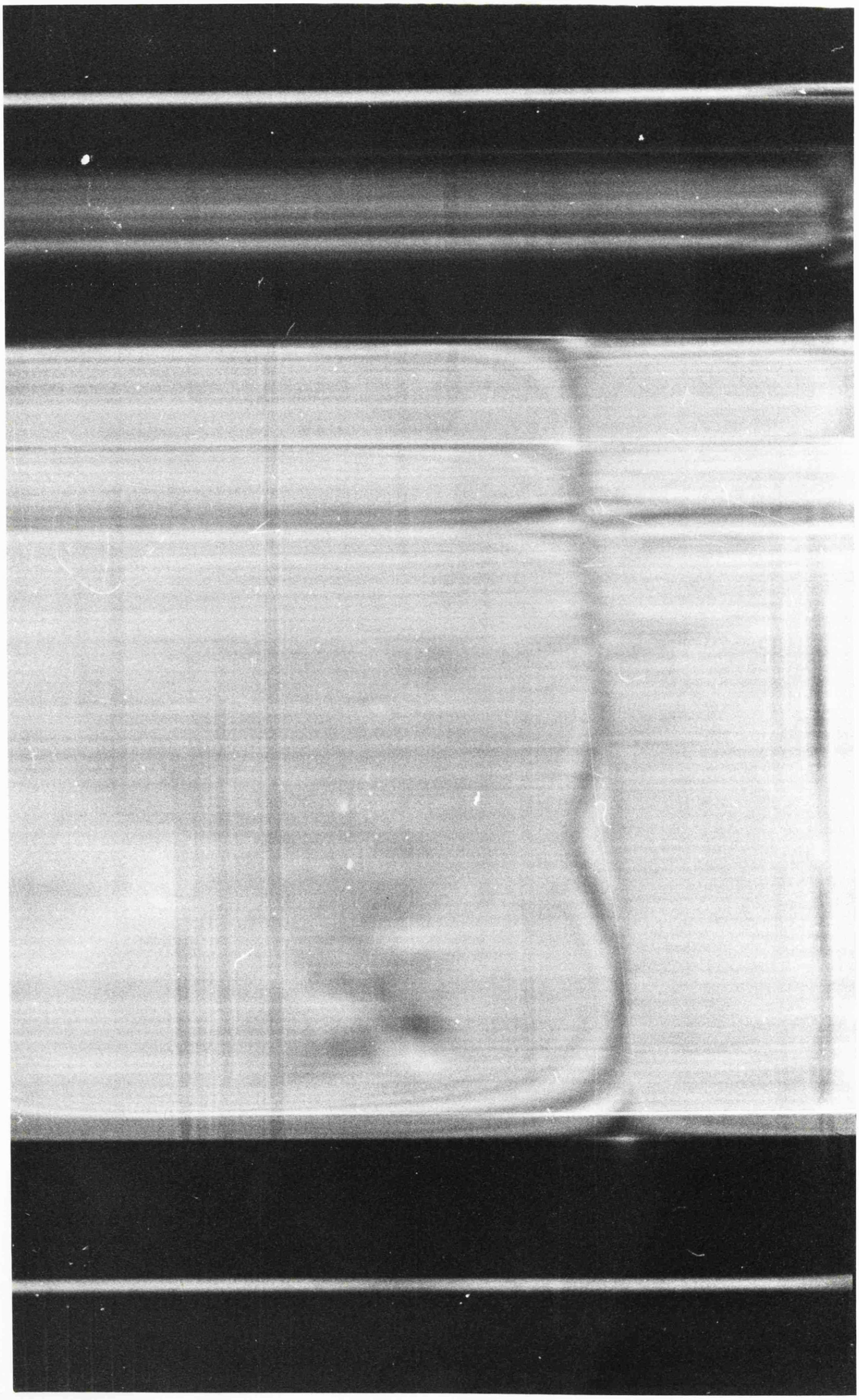


Plate 7

SDS gel (14%) electrophoresis (with half the normal amount of cross-linker) of S-1 HC from purified native myosin filaments and proteins of known molecular weights (Table 3)

Well 1: myosin (20 μg).

Well 2: S-1 HC (7 μg).

Well 3: phosphorylase a (7 μg).

Well 4: albumin (highest M.W. band) (25 μg).

Well 5: catalase (7 μg).

Well 6: egg albumin (7 μg).

Well 7: actin (7 μg).

Well 8: actin (7 μg).

Well 9: creatine kinase (14 μg).

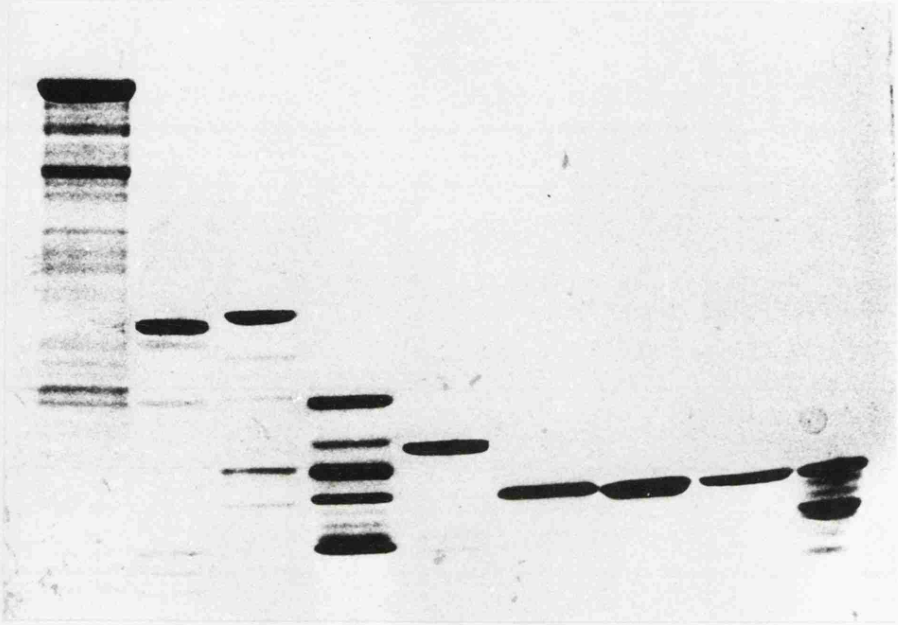

1
2
3
4
5
6
7
8
9

Table 2

The relative molar ratios of light chains in purified native myosin filaments and in the derived chymotryptic S-1

The results for filaments are normalised with respect to 2 moles of DTNB light chains while those for the S-1 are normalised with respect to 1.31 moles of LC1. These results indicate some nicking of DTNB light chains and that LC3 is slightly contaminated, probably due to nicking of DTNB light chains. Results were obtained by optical scanning of SDS gels as described (II.2.12.). Numbers in brackets indicate number of estimations.

	LC1	DTNB LC	LC3
Filaments (7)	1.31±0.05	2	0.70±0.06
S-1(6)	1.31	1.70±0.20	0.80±0.01

Table 3

Mobilities and known molecular weights of proteins for the determination of the M.W. of S-1 HC from purified native myosin filaments (see also Figure 10 and II.3.9.)

Protein	M.W.	Log M.W.	Mobility
Myosin HC (rabbit muscle)	200,000	5.301	0.345
Phosphorylase a (rabbit muscle)	94,000	4.973	0.639
S-1 HC	93,300	4.970	0.645
Bovine serum albumin	66,000	4.820	0.765
Catalase (bovine liver)	60,000	4.778	0.815
Egg albumin	45,000	4.653	0.902
Actin (rabbit muscle)	43,000	4.633	0.93
Creatine kinase (rabbit muscle)	40,000	4.603	0.974

CHAPTER III

ENZYMOLOGY OF NATIVE MYOSIN FILAMENTS AND THE DERIVED CHYMOTRYPTIC S-1

III. ENZYMOLOGY OF NATIVE MYOSIN FILAMENTS AND THE DERIVED CHYMOTRYPTIC S-1

III.1. Introduction

The discovery of myosin as an ATPase by Engelhardt and Ljubimova (1939) opened a new era in muscle biochemistry. The two heads (I.3.) of the myosin molecule are involved in the transformation of chemical energy into mechanical energy which is essential for muscular contraction. Each head (subfragment-1 or S-1) possesses not only an enzymatic site which binds ATP and subsequently hydrolyses it to liberate energy necessary for muscular contraction, but has also an actin binding site which is essential for the transmission of mechanical force. Studies of the ATPase activities not only characterise the enzyme myosin and its subfragments (I.3) but also shed light on the mechanism of interaction of myosin and actin in the presence of Mg-ATP to bring about muscular contraction.

The ATPase activities of myosin and its proteolytic subfragments have generally been investigated in the presence of magnesium-ATP complex (Mg-ATPase), calcium (Ca-ATPase), EDTA (K-EDTA-ATPase) and actin (actin-activated Mg-ATPase).

Under physiological ionic conditions (i.e.

approximately, 1-3 mM Mg^{++}, 0.0001 mM Ca^{++}, 120 mM K^{+} and 0.0001 mM H^{+}) in the resting state, myosin has a very low ATPase activity which is due to the presence of magnesium in the myoplasm. Calcium and potassium are known to activate myosin ATPase activity in vitro, but their concentrations are too low to derepress the magnesium effect (Szent-Gyorgyi, 1951; Mommaerts and Green, 1954; see also later). The inhibitory effect of magnesium has also been studied in the presence of calcium and at physiological [KCl], using actomyosin and myosin (Mommaerts and Seraidarian 1947; Mommaerts and Green, 1954). The study of "Mg-Ca antagonism" has shown that at a Mg:Ca ratio near unity, almost complete ATPase inhibition is observed for myosin and inhibition exceeding 95% is observed for actomyosin. Since the Mg:Ca ratio in muscle is well over unity, it has been concluded that myosin ATPase activity is inhibited by magnesium in the resting state of muscle. It has been suggested that the formation of a cyclic ternary complex involving the two essential sulfhydryl groups, SH_1 and SH_2 of myosin and Mg-ATP is responsible for the low ATPase activity. The binding of actin at or near the SH_1 group breaks the inhibitory cyclic complex and results in ATPase activation (Burke et al., 1973). Using chymotryptic myosin subfragment-1 (S-1) and tryptic heavy meromyosin (HMM), it has been observed that the low Mg-ATPase activities of these fragments at low ionic strength (10 mM KCl) decrease further as the [KCl] increases (Hozumi and

Tawada, 1973).

It is appropriate to mention at this point the activating effect of potassium on ATPase activities since ATPase activities are generally measured in the presence of KCl. In the absence of other salts, myosin ATPase has a well defined optimum (about 150 n moles Pi per minute per mg protein) at 0.20 to 0.25 M KCl, while actomyosin has a wider optimal range at about the same KCl concentration. This optimum is shifted to 0.08-0.10 M KCl and to much higher ATPase activities in the presence of 1 mM $CaCl_2$ (Mommaerts and Seraidarian, 1947; Mommaerts and Green, 1954).

Despite the fact that calcium has been known for a long time as an activator of myosin ATPase (Banga, 1942; Engelhardt and Ljubimova, 1942; Szent-Gyorgyi, 1951; Mommaerts and Green, 1954), its mechanism of activation is not yet well understood. Ca-activated ATPase activities have been considered to have no physiological significance since the degree of activation is very low at physiological concentrations (10^{-7}-10^{-4} M) and more over it is very strongly inhibited by magnesium. The activating effect of calcium (1-10 mM) on actomyosin and myosin ATPases is observed both in the absence and in the presence of KCl, but this effect is depressed in the presence of KCl concentrations greater than physiological (Mommaerts and

Seraidarian, 1947).

The effect of monovalent cations and EDTA or other chelating agents on myosin ATPase activities has been investigated by various workers. As mentioned, potassium activates myosin ATPase while sodium can not, but it depresses the K-activated ATPase (Mommaerts and Green, 1954). Lithium ion resembles sodium, but it has a slight activating effect (Kielley et al., 1956) while rubidium and ammonium ions have similar effects of potassium. Myosin ATPase activity has been found to be markedly activated by EDTA in the presence of high potassium chloride concentrations at a slightly alkaline pH, while at low [KCl] there is no activation even in the presence of 10 mM EDTA (Bowen and Kerwin, 1954; Friess, 1954). At high [KCl], EDTA begins to accelerate ATPase activity at about 0.1 mM, and about 4-fold activation is reached when [EDTA] is about 1-10 mM. The inhibitory action of low NaEDTA concentration (10^{-5} M) at both low (0.02M) and high (0.6M) KCl concentrations has been attributed to the removal of calcium from the assay medium and this seems to be supported by the fact that CaEDTA does not inhibit while MgEDTA, like NaEDTA, inhibits myosin ATPase at low KCl concentrations; calcium replaces magnesium in MgEDTA (Bowen and Kerwin, 1954). The activation by EDTA (1-10 mM) at high KCl concentrations (0.6 M) seems to be due to the deprivation of magnesium, since the extent of activation by

other chelating agents parallels their Mg-binding capacities (Ebashi et al., 1960; Martonosi and Meyer, 1964; see also Offer, 1964 and Muhlrad et al., 1964). However, it still remains unexplained why K-EDTA-ATPase is markedly depressed at high EDTA concentrations (1-10 mM) in the presence of low KCl concentrations (0.02 M KCl), i.e. K-EDTA-ATPase activity is only pronounced at both high KCl and EDTA concentrations (Bowen and Kerwin, 1954). This cast considerable doubt on the supposition that K-EDTA-ATPase is solely due to the removal of magnesium by the chelating agent. Could it not be possible that EDTA together with KCl has some direct activating effect on the hydrolysis of ATP by the enzyme (Friess, 1954; Bowen and Kerwin, 1954) ?

The effects of ions other than magnesium on myosin ATPase activities mentioned above have no direct physiological significance. However, actin-activated Mg-ATPase activity of myosin has been considered to be the biochemical manifestation of the basic process of muscular contraction in vitro. Magnesium is not only essential for the dissociation of actin from myosin and its subfragments by ATP, especially at low ionic strength (Eisenberg and Moos, 1968; Eisenberg et al., 1968), but is also required for the actin activation of the ATPase activities of myosin (Barron et al., 1966), HMM (Maruyama and Watanabe, 1962; Eisenberg and Moos, 1968) and S-1 (Eisenberg et al., 1968). It is worth noting that, for example, acto-HMM is about 100

times more dissociated in the presence of both magnesium and ATP than in the absence of ATP or magnesium (Eisenberg and Moos, 1968).

One of the most important findings in the studies of the steady-state ATPase activity is the linear double reciprocal plots of actin-activated ATPase activities against actin concentrations which were first obtained for HMM by Eisenberg and Moos (1968). Since then, kinetic studies of this sort have been widely employed for the ATPase studies of the soluble proteolytic subfragments of myosin (Eisenberg et al., 1968; Eisenberg and Moos, 1970) and the myosin itself (Burke et al., 1974) over a wide range of ionic strength and temperature (Rizzino et al., 1970; Barouch and Moos, 1971; Hozumi and Tawada, 1973). As mentioned by Eisenberg and Moos (1968), the validity of this kind of plot depends on the fact that the binding sites on the actin filaments for myosin and its subfragments are acting independently and that the added actin and free actin concentrations are essentially the same. The inverse of the intercept on the ordinate of a double reciprocal plot yields the maximum actin-activated ATPase rate (Vmax) at infinite actin concentration, that is, when the enzyme is fully complexed with actin. The intercept on the abscissa gives the apparent association constant (Ka) of actin with the enzyme, in the presence of Mg-ATP. Further work of Eisenberg and Moos (1970) also

showed that double reciprocal plots of HMM ATPase activities against actin concentration at varied ATP concentration and ATPase activities against ATP concentration at varied actin concentration are linear, suggesting that the system is in the form of simple Michaelis kinetics and can be interpreted in terms of the steady-state kinetics of a simple enzyme-substrate-modifier model (London, 1968).

In general, Vmax obtained from linear double reciprocal plots of ATPase against actin concentration at low ionic strength is about 100-200 fold greater than the ATPase activity of HMM or S-1 in the absence of actin, that is, comparable to the ATPase observed in working muscle (Eisenberg and Moos, 1968, 1970; Eisenberg et al., 1968) and that actin is very much more dissociated from S-1 or HMM in the presence of Mg-ATP. While steady-state kinetic studies were in progress, studies on the presteady-state hydrolysis of ATP by myosin and its subfragments were also carried out by other workers. It has been found that when ATP is bound to myosin, it is hydrolysed to ADP and Pi on the surface of the enzyme. This hydrolysis of ATP has been termed the initial Pi burst because it is much faster than the release of the products Pi and ADP from the surface of the enzyme (Tokiwa and Tonomura, 1965; Tonomura et al., 1969; Lymn and Taylor, 1970; Tonomura and Inoue, 1975). Further presteady-state kinetic studies have shown that the initial Pi burst is much slower than the dissociation of

actin from HMM (Lymn and Taylor, 1971). On the basis of these findings, Lymn and Taylor (1971) proposed the kinetic scheme as shown in Figure 12 A. As can be seen, the initial Pi burst (M.ATP---→ M.ADP.Pi) takes place after the dissociation of actin (A.M.ATP---→ M.ATP) from the S-1 (M) by ATP. Actin then activates the release of ADP and Pi from the enzyme (A.M.ADP.Pi---→ A.M) which is faster than without actin (M.ADP.Pi---→ M). Figure 12 A1 (based on Lymn and Taylor model) hypothesises that in the absence of ATP, S-1 binds to the actin filament at an angle of 45^{o}, but as soon as ATP binds to S-1 it is detached from the actin filament. S-1 can only reattach to the actin filament after the initial Pi burst has taken place, at a 90^{o} angle. Following reattachment to the actin filament, ADP and Pi are released from the S-1 which rotates back to a 45^{o} angle, simultaneously causing relative sliding of filaments. Therefore, this model provides a possible mechanism for the cyclic attachment and deattachment of S-1 with the actin filament during muscular contraction.

As mentioned, in the Lymn and Taylor model, ATP is hydrolysed only when S-1 is dissociated from the actin filament, that is, A.M.ATP---→ A.M.ADP.Pi does not take place. This assumption has been based on observation at very low actin concentration when the ATPase activity is very much lower than Vmax. It has been suggested that (Lymn and Taylor, 1971) at high actin concentration, nearly all

the myosin should be complexed with actin filament when the ATPase is near Vmax and that the rate limiting step is the release of ADP and Pi which is much slower than the initial Pi burst. However, contrary to this suggestion, it has been shown that at high actin concentration (5° C) when the ATPase is close to Vmax, a substantial fraction of S-1 or HMM is dissociated from actin (Eisenberg et al., 1972; Eisenberg and Kielley, 1972; Fraser et al., 1975; Mulhern and Eisenberg, 1976). Subsequently, the refractory state model has been proposed to account for this observation (Figure 12 B., excluding steps a, b and c; Eisenberg and Kielley, 1972). In this model, the refractory state, M.ADP.Pi[RS] and the nonrefractory state, M.ADP.Pi[NS] have been introduced as compared to the Lymn and Taylor model. The transition from refractory to nonrefractory state which is the rate limiting step in the model, occurs only after S-1 is dissociated from actin by ATP. Presteady-state kinetics studies at 5° C show that when stoichiometric ATP is added to the complex of acto-S-1 at high actin concentration, actin is found to dissociate rapidly from S-1 (A.M.ATP---→ M.ATP) and the rebinding of actin to S-1 is a much slower process (M.ADP.Pi[RS]---→ M.ADP.Pi[NS]---→ A.M.ADP.Pi[NS]) (Chock et al., 1976). These data are therefore in favour of the refractory state model.

According to the above refractory state model, all

HMM or S-1 is rapidly dissociated from actin upon the binding of ATP even at high actin concentration, that is, all the S-1 or HMM is in the form M.ADP.Pi[RS] when the steady state ATPase activity approaches Vmax. However, studies carried out at 25° C (Marston, 1978; Wagner and Weeds, 1979) have shown a substantial amount of binding between actin and S-1 as compared to the relatively small but significant amount of binding observed at 0° C which had also always been observed by previous workers (Eisenberg and Kielley, 1972; Fraser et al., 1975; Mulhern and Eisenberg, 1976). This binding between S-1 or HMM and actin at high actin concentration could be due to the incomplete dissociation of S-1 or HMM from actin (A.M.ATP---→ M.ATP) or it could be due to the rebinding of actin to the enzyme (A.M.ADP.Pi---→ A.M). Using stopped-flow studies, Stein et al (1979) have distinguished between these two forms of binding and confirmed the binding between actin and S-1 in the presence of ATP at high actin concentration.

According to the Lymn and Taylor model, ATP hydrolysis occurs only after S-1 is dissociated from actin (M.ATP---→ M.ADP.Pi), and that the ATPase activity should decrease as A.M.ATP accumulates at high actin concentration (Lymn, 1974), that is, the equilibrium is shifted from M.ATP to A.M.ATP. However, no decrease in ATPase activity is observed at actin concentration up to 0.2 mM (about 8.6

mg per ml), when all S-1 should be essentially complexed with actin to give A.M.ATP (Stein et al., 1979). On the basis of this observation, that is, no inhibition of ATPase at high actin concentration, Stein et al have proposed that hydrolysis of ATP can also take place before S-1 is dissociated from actin (A.M.ATP---→ A.M.ADP.Pi; Figure 12B.) which seems to occur at more or less the same rate as the step M.ATP---→ M.ADP.Pi (Figure 12B.). According to Stein et al (1979), the refractory state M.ADP.Pi[RS] can also bind actin with the same rate as M.ATP is bound to actin. This has been based on the observation that the turbidity of the mixture of actin-S-1-ATP remains constant as the initial Pi burst occurs. The binding of actin to M.ADP.Pi[RS] is also suggested by O^{18} exchange studies (Sleep and Boyer, 1978). Although actin binds to M.ADP.Pi[RS], in order to explain the observation that the amount of actin needed for half-maximal binding to M.ADP.Pi[RS] is about four times greater than the amount required for half-maximul ATPase activity, it is still necessary to assume the existence of the transition from refractory to nonrefractory state.

Therefore, in the modified refractory state model of Stein et al (1979), three additional steps (a, b and c in Figure 12B.) are included as compared to the original refractory state model of Eisenberg and Kielley (1972). In the Lymn and Taylor model, release of Pi is the rate

limiting step while in the modified refractory state model of Stein et al, the slowest step is the transition from the refractory to the nonrefractory state which occurs at about the same rate whether actin is dissociated from the S-1 or not (Figure 12B.).

The models mentioned above have been based on presteady-state and steady-state kinetic studies using S-1 and HMM, the soluble proteolytic myosin subfragments. Though it is generally considered that the aggregation of myosin at low ionic strength has precluded quantitative kinetic modeling from data resulted from myosin (Maruyama and Gergely, 1962, 1962a; Eisenberg and Moos, 1967), there is no justifiable definitive evidence for this. Linear double reciprocal plots of actomyosin ATPase activities against actin concentrations at KCl concentrations varying from 0.05 M to 0.15 M have been obtained (Burke et al., 1974). The present study also includes linear double reciprocal plots using conventional myosin and purified native myosin filaments.

Studies of Mg-, Ca- and actin activated Mg-ATPase activities of myosin and its subfragments have mostly been carried out at very low ionic strengths, mainly due to the fact that activities are generally high at low ionic strengths. Comparison of the salt-(ionic strength-)dependence of Mg-ATPase activities of tryptic HMM

and chymotryptic S-1 indicate that there may be no interaction between the two heads of HMM for Mg-ATPase activities. However, interaction between the two heads may exist in the presence of actin as shown by the difference in ionic strength-dependence of actin-activated Mg-ATPase activities between HMM and S-1 (Hozumi and Tawada, 1973).

In the present work, using a rapid and sensitive isotopic assay ([γ-^{32}P]ATP), the ionic strength-dependence of various ATPase activities (Mg-, Ca-, K-EDTA- and actin-activated ATPase activities) of native myosin filaments and the derived chymotryptic S-1 containing the DTNB light chains have been compared with those of conventional myosin and its chymotryptic S-1 without the DTNB light chains. Studies of ionic strength-dependence of ATPase activities may indicate the relation between the physical states of proteins (myosin and S-1) and their activities since the physical properties (e.g. the lengths and widths of synthetic myosin filaments) may depend on ionic strength (II.1.) Evidence is presented to show that high ionic strength (0.6 M KCl) treatment of native myosin filaments irreversibly modifies the quaternary structure with respect to the DTNB light chains. It is concluded that DTNB light chains play an important role in the ionic strength-dependence of ATPase activities and that they are responsible for the low ATPase activities of native myosin filaments and the derived S-1 before exposure to high ionic strength.

III.2. Experimental

III.2.1. Preparation of proteins for ATPase studies

Unregulated actin (i.e. pure actin without the troponin and tropomyosin components) was prepared from acetone-dried powder of muscle mince after extraction of myosin with Guba-Straub solution. The procedure (II.2.4.) described yielded pure actin as judged by SDS gels. Pure actin was also checked to have no ATPase activity before using for actin-activated ATPase studies. Pure F-actin was stored in 0.02% azide at 0-4^{o} C and used within three weeks of preparation.

Conventional myosin and chymotryptic subfragment-1 (S-1) of synthetic myosin filaments were prepared from fresh rabbit back and hind leg muscles (see II.2.1. and II.2.8.1., respectively).

Preparation of $^{2}H_2O$-H_2O gradient-purified native myosin filaments and chymotryptic S-1 from purified native myosin filaments were made from fresh rabbit psoas muscles (II.2.7. and II.2.8.2.). For accurate ATPase studies and to avoid product inhibition of ATPase activities by nucleotides, it was essential that traces of nucleotides in the final preparations of native myosin filaments and S-1 were kept to a minimum. Initially , dowex resin (AG 1x8,

200-400 mesh, Cl-form) was employed for the removal of nucleotides from the preparations. However, this was proved to be not appropriate, owing to the fact that purified myosin filaments adhered to the resin strongly and the final yield of the product was less than 20% and still containing considerable amount of nucleotide (as checked by absorbance at 259 nm). Instead, using boiled and well stretched dialysis tubing, it was possible to get rid of all traces of nucleotides, as judged by absorbance at 259 nm. Purified myosin filaments were dialysed against stock buffer whose ionic strength had been reduced to 0.12 (so as to keep the filaments at physiological ionic strength) by employing dialysis tubing which had been boiled in water for a few minutes and well stretched. After more than 30-40 hours of dialysis, with many changes, no traces of nucleotides could be detected. Further manipulations of this kind did not involve exposure of myosin filaments and the derived chymotryptic S-1 to high ionic strength.

Myosin was also prepared from purified native myosin filaments as follows. Purified filaments were dialysed against stock buffer for about five hours with stirring or overnight (II.2.8.3.) to solubilise the protein components of the filaments. After clearing by centrifugation (50,000 r.p.m., 15 min), the myosin in the supernatant was recovered by dilution and the precipitate was redissolved in stock buffer. Chymotryptic S-1 was also prepared from

myosin filaments formed from this myosin solution after dialysing to physiological ionic strength (as for chymotryptic S-1 from conventional synthetic filaments, II.2.8.1.).

Chymotryptic S-1 of purified native myosin filaments which had not been exposed to high ionic strength before was dialysed against stock buffer for about 5 hours or overnight.

The ionic strength-dependence of the Mg-, Ca-, K-EDTA- and actin-activated Mg-ATPase activities of the above protein preparations were analysed by an isotopic ([γ-^{32}P] ATP) method (Seals et al., 1978) or a pH-stat with an automatic titrator.

III.2.2. Media for the assays of ATPase activities

The ATPase activity was assayed in a total volume of 0.1 ml in the case of the isotopic method and in a total volume of 8 ml when using the pH-stat, at 25^{o} C and pH 7.5 with the following media: (i) Mg-ATPase, 0.0-0.6 M KCl, 4 mM $MgCl_2$, 1 mM ATP, 20 mM Tris-HCl, and 0.1 mg per ml of protein; (ii) Ca-ATPase, 0.0-0.6 M KCl, 10 mM $CaCl_2$, 1 mM ATP, 20 mM Tris-HCl (1 mM Tris-HCl in case of pH-stat assay), and 0.05-0.10 mg per ml of protein; (iii) K-EDTA-ATPase, 0.0-0.6 M KCl, 5 mM EDTA, 1 mM ATP, 20 mM

Tris-HCl (without Tris-HCl in case of pH-stat assay) and 0.05-0.10 mg per ml protein; (iv) actin-activated Mg-ATPase, 0.0-0.6 M KCl, 5 mM $MgCl_2$, 1 mM ATP, 12.5 mM Tris-HCl, 2 mM DTT, 0.05-0.10 mg per ml protein and actin concentration from 0.5 mg per ml to 7.5 mg per ml.

III.2.3. Methods of assaying ATPase activities

Two methods of assays were employed for ATPase studies. The isotopic ($[\gamma\text{-}^{32}P]ATP$) method was used for Mg-ATPase studies and for samples, such as purified native myosin filaments and the derived chymotryptic S-1, which could only be prepared in minute quantities. The pH-stat assay was employed for the studies of Ca- and K-EDTA-ATPases of conventional myosin and chymotryptic S-1 of synthetic myosin filaments, where relatively large amount of proteins could be obtained quite conveniently. Only the isotopic assay was used for Mg-ATPase studies, since the pH-stat method was found to be not sensitive enough. Results obtained from both methods where applicable were identical within experimental error.

III.2.3.1. The isotopic assay

This assay is based on the procedure of Seals et al (1978), in which the release of ^{32}Pi from $[\gamma\text{-}^{32}P]$ ATP is extracted as phosphomolybdate complex by organic solvents,

and quantitated by scintillation counting. The rapid assay procedure minimizes hydrolysis of ATP due to exposure to acidic conditions. It is possible to design experiments so that 10^{-15} to 10^{-7} moles of ^{32}Pi can be extracted from aqueous solution. Less than 1% of unhydrolyzed ATP is extracted but this can easily be corrected by counting blanks containing no enzymes. The enzyme sample was incubated with ATP and a tracer amount (0.25-0.5 μCi) of [γ-^{32}P] ATP in a final volume of 0.1 ml, in a 1.5 ml capacity "Eppendorf cup" with cap and the reaction was terminated by the addition of SDS. Experiments and assay time were designed and adjusted so that only about 20% of the ATP was hydrolyzed. Usually, a 3-5 minutes incubation time was found to be appropriate. The fact that only 5-10 μg protein was sufficient for each assay, this method made possible the detailed studies of the ATPase activities of the purified native myosin filaments and chymotryptic S-1 from these filaments, and other protein samples which could only be prepared in minute quantities.

The reaction was started by addition of 0.01 ml 10 mM ATP solution and incubated for an appropriate time at 25° C, and was terminated by adding 0.1 ml 2% SDS. Since no further hydrolysis of ATP occurs after the addition of SDS, all necessary incubations could be conveniently completed before the extraction of ^{32}Pi liberated (Seals et al., 1978). Extraction of liberated ^{32}Pi was initiated by

addition of 0.1 ml molybdate reagent (molybdate reagent was always freshly prepared by mixing 2 vol of 10% (W/V) ammonium molybdate, 1 vol of 5 M H_2SO_4, and cooled before adding 1 vol of 0.1 M silicotungstic acid) and 0.8 ml of benzene:isobutanol (1:1). Samples were capped and mixed vigorously on a Vortex mixer for 10 seconds (20 or 30 sec did not extract more ^{32}Pi) and the aqueous and organic phases were separated by centrifugation (1 min) using a micro-centrifuge (precipitated protein was collected at the interface between the aqueous and organic phases). In order to minimize the acid and molybdate-catalysed hydrolysis of ATP after the addition of molybdate reagent, the time of extraction for each sample was reduced by performing the extraction and centrifugation steps in groups of six to eight samples. 0.1 ml or 0.2 ml of the organic phase, containing liberated ^{32}Pi, was removed into 4 ml of scintillant for counting in a Packard Tri-Carb liquid scintillation spectrometer (scintillant was prepared by dissolving 4 g 2,5-Diphenyloxazole (PPO) in 700 ml toluene and made up to 1 l by triton X-100). An appropriate blank was prepared in the same manner for counting. In order to determine the specific activity of the enzyme (n moles Pi per min per mg protein), besides knowing the amount of enzyme during incubation, it was also necessary to determine a standard count (per min) for the [γ-^{32}P] ATP used at the same time. This was done by counting 0.01 ml of the 10 mM [γ-^{32}P] ATP (100 n moles) in 4 ml scintillant.

Each assay was done in triplicate or more.

The specific ATPase activity of the enzyme was determined by the relationship,

$(AxBxC)/(DxExF)$

where,

A = n moles of $[\gamma\text{-}^{32}P]$ ATP ($\equiv {}^{32}Pi$),

B = sample count (per min) after blank correction,

C = reciprocal of fraction of organic phase counted,

D = standard count (per min) from A,

E = time (min) of incubation,

F = amount (mg) of enzyme during incubation.

III.2.3.2. The pH-stat assay

Assays of Ca- and K-EDTA-ATPases of preparations which could be obtained in relatively good yields (III.2.3.) were carried out in a pH-stat with an automatic titrator (REA 160 titrigraph and TTT80 titrator,

Radiometer, Copenhagen) by following proton liberation with NaOH (0.02 M) as titrant (see III.2.2. for conditions and media of assays). Moles of H^+ liberated were assumed to be equivalent to moles of ATP hydrolysed.

Usually, the reaction was initiated by the addition of appropriate amount of enzyme and the time of assay was from 3-7 minutes. To determine the specific activity of the enzyme (n moles Pi per min per mg protein), the rate of H^+ liberated was first converted into rate of ATP hydrolysis by titrating 0.01 ml 0.1 M HCl (1 µmole) with 0.02 M NaOH.

III.2.4. Studies on the effect of ^2H_2O on ATPase activities

In order to investigate whether ^2H_2O has any effect on the ATPase activites of myosin, the Mg-, Ca- and K-EDTA-ATPase activities of myosin (II.2.1.) were compared with those of the same myosin (II.2.1.), but the stock buffer in which the myosin was dissolved during the procedure of preparation was made up in 100% ^2H_2O. The isotopic assay (III.2.3.1.) was employed for these studies.

III.3. Results

The results presented in this chapter were obtained by the isotopic method (III.2.3.1.) unless otherwise specified. At each ionic strength, ATPase activities of three to four preparations were determined and the average taken is shown in the figures. Conditions (in legends to figures) were of final concentrations.

III.3.1. Comparison of the ATPase activities of purified native myosin filaments and the derived chymotryptic S-1 with those of conventional myosin and chypmotryptic S-1 from synthetic myosin filaments

The Mg-, Ca-, K-EDTA- and actin-activated Mg-ATPase activities of purified native myosin filaments (II.2.7.) and the derived chymotryptic S-1 (II.2.8.2.) which had not been exposed to high ionic strength, was compared with those of conventional myosin (II.2.1.) and chymotryptic S-1 from synthetic myosin filaments (II.2.8.1.), and it was found that the former were very much less than those of the latter. This can best be appreciated by comparing and examining the following:

III.3.1.1. Mg-ATPase activities

The Mg-ATPase activities, of both purified native

myosin filaments (Figure 13, open circle) and the derived S-1 (Figure 14, closed square) at physiological ionic strength was found to be about 100-fold less than those of conventional myosin (Figure 13, closed circle) and S-1 from synthetic myosin filaments (Figure 14, open square). Thus for the first time that we appear to have a preparation of myosin and of S-1 whose Mg-ATPase activities are as low as that of resting muscle in vivo (Kushmerick and Paul, 1976; Ferenczi et al., 1978; see III.4.)

III.3.1.2. Ca-ATPase activities

The Ca-ATPase activity of conventional myosin (Figure 15, open circle) was about 200-fold that of purified native myosin filaments (Figure 16) which had not been exposed to high ionic strength, when measured at physiological ionic strength. It is also interesting to note that there was a maximum of activity at about 180 mM KCl for purified native filaments (Figure 16) as compared to the steady decrease in activity for conventional myosin (Figure 15, open circle) as the [KCl] was increased.

There was also a marked difference between the Ca-ATPase activities of S-1 from synthetic myosin filaments (Figure 17, open square) and those of S-1 (Figure 18, open circle) from native myosin filaments which had not been exposed to high ionic strength. As for conventional myosin

and native myosin filaments, a difference of about 200-fold in activities was also observed when measured at physiological ionic strength.

III.3.1.3. K-EDTA-ATPase activities

The K-EDTA-ATPase activities of purified native myosin filaments (Figure 19) which had not been exposed to high ionic strength during the course of preparation were found to be about 1000-fold lower than those of conventional myosin (Figure 20) when assayed at 600 mM KCl.

When determined at 600 mM KCl, the K-EDTA-ATPase activities of S-1 (Figure 21, open triangle) from synthetic myosin filaments were more than 100-fold greater than those of S-1 (Figure 22, open triangle) from purified native myosin filaments.

III.3.1.4. Actin-activated Mg-ATPase activities

Actin-activated Mg-ATPase activity is defined as the difference between the ATPase activity in the absence of actin and in the presence of actin, the latter being 100-200 fold greater than that of the former for conventional myosin and S-1's from synthetic myosin filaments. Reciprocals of the actin-activated ATPase activities were plotted against the reciprocals of actin

concentrations (see III.1.). Linear double reciprocal plots of this kind yield Vmax (maximum actin-activated ATPase) from the reciprocal of the intercept on the ordinate and Ka (apparent association constant of actin with the enzyme in the presence of Mg-ATP) from the intercept on the abscissa. In the present study, Vmax's and Ka's were obtained as a function of KCl concentrations.

Figures 23 and 24 show the ionic strength-dependence of Vmax's and Ka's of purified native myosin filaments and conventional myosin, respectively. The Vmax's (Figure 23, open circle) of purified native myosin filaments were much lower than those of conventional myosin (Figure 24, closed circle), for example, there was about 400-fold difference at physiological ionic strength.

The Ka (Figure 23, open triangle) of actin with purified native myosin filaments increased rather steadily as the [KCl] increased from 30 mM to 180 mM and it fell off as the [KCl] increased further. This was rather different from the Ka (Figure 24, closed triangle) of conventional myosin which seemed to decrease slightly as the [KCl] increased from about 30 mM and then increased steadily with [KCl]. This seems to correlate well with the results of Burke et al (1974) in that the Ka they found for synthetic actomyosin increases as the [KCl] increased from 50 mM to 150 mM, though the absolute values are not identical. The

Ka of tryptic HMM has also been found to decrease as the [KCl] in the assay medium increases from about 30 mM to 100 mM, but it increases sharply as the [KCl] approaches 300 mM. (Hozumi and Tawada, 1973).

Though the Vmax of S-1 from purified native myosin filaments (Figure 25, open circle) at physiological ionic strength was more than 10-fold (or about 6-fold on mole basis; Table 5) that of the native myosin filaments (Figure 23, open circle), it was about 17-fold (or about 14-15 fold on mole basis; Table 5) lower that of S-1 from synthetic myosin filaments (Figure 26, closed circle).

The Ka of S-1 from purified native myosin filaments (Figure 25, open triangle) was found to decrease from 20 mM KCl to about 140 mM KCl, but it increased sharply at 300 mM KCl, as compared to the Ka of S-1 from synthetic myosin filaments (Figure 26, closed triangle) which remained more or less constant from about 20 mM KCl to 300 mM KCl and then increased steadily up to more than 600 mM KCl.

The above results show that the ATPase activities of purified native myosin filaments and the derived chymotryptic S-1 which have never been exposed to high ionic strength during the course of preparation are relatively much lower than those of conventional myosin and S-1 from synthetic myosin filaments. (These much lower

activities are not due to dead enzyme! ; III.4.2.1.) This shows that high KCl concentrations not only have a pronounced effect on the chymotryptic digestion of native myosin filaments and synthetic myosin filaments produced by dialysis of myosin in true solution down to physiological ionic strength (Chapter II), but also have the potentiating effect of elevating the low ATPase activities of native myosin filaments and the derived chymotryptic S-1 (see III.3.2.). Since chymotryptic S-1 from native myosin filaments contains substantially all the myosin light chains as compared to chymotryptic S-1 from synthetic myosin filaments which lack the DTNB light chains (Chapter II), it seems very likely that DTNB light chains (before exposure to high ionic strength) are responsible for the low ATPase activities and that the DTNB light chains ability to depress ATPase activity is abolished by high ionic strength in the case of conventional myosin which contains DTNB light chains, but has elevated ATPase activity as compared to native myosin filaments which have not "seen" high ionic strength. The fact that DTNB light chains do play a role in depressing ATPase activities is further supported by the pronounced activation of ATPase activities of S-1 containing the DTNB light chains when subjected to high ionic strength treatment (III.3.2.) and the convergence of the ATPase activities of conventional myosin and S-1 from synthetic myosin filaments and those of myosin and S-1 from the solubilised myosin of purified

native myosin filaments after exposure to high ionic strength (III.3.3.).

III.3.2. Effect of high ionic strength on ATPase activities

An examination of the above results would indicate the possibility of high ionic strength being responsible for the differences between the ATPase activities of native preparations (before exposure to high ionic strength) and conventional preparations (after "seeing" high ionic strength), since other factors (such as pH) are not variants in the preparation of these proteins. It was decided to check for the effect of high ionic strength on ATPase activities.

III.3.2.1. Mg-ATPase activities

The Mg-ATPase activities of both the myosin (compare Figure 13, open circle and open triangle) from purified native myosin filaments and the chymotryptic S-1 (containing the DTNB light chains) (Figure 14, closed square and closed circle) of purified native myosin filaments were found to be activated from more than 10 to 30-fold at physiological ionic strength after high ionic strength treatment. These observations seem to suggest that DTNB light chains after having "seen" high ionic strength tend to lose in part the depressing effect of Mg-ATPase

activities, not only at physiological ionic strength, but at all ionic strength studied (Figures 13 and 14).

III.3.2.2. Ca-ATPase activities

The Ca-ATPase activities of chymotryptic S-1 (containing the DTNB light chains) of purified native myosin filaments were also found to be ionic strength-sensitive, though it was not as marked as that of Mg-ATPase activities (III.3.2.1.). There was about a 4 to 5-fold increase at physiological ionic strength (Figure 18).

III.3.2.3. K-EDTA-ATPase activities

When the K-EDTA-ATPase activities of S-1 from purified native myosin filaments before exposure to high ionic strength were compared with those of the same S-1 (still containing the DTNB light chains) after high ionic strength treatment, it was observed that the relative degree of ionic strength-activation was much higher at low KCl concentrations than at 600 mM KCl, though the absolute activities were highest for both at 600 mM KCl (Figure 22).

III.3.2.4. Vmax of S-1 from purified native myosin filaments

Though the ionic strength-activation of Vmax of S-1 from purified native myosin filaments was not as pronounced as ATPase activities mentioned above, a significant activation was also observed, more so at low ionic strength than at high ionic strength (Figure 25).

The activation of ATPase activities mentioned above has been observed after the sample has been exposed to high ionic strength by dialysis against stock buffer for 5 hours or overnight. These results clearly demonstrate that high ionic strength (0.6 M KCl) can activate the low ATPase activities of purified native myosin filaments and the derived S-1, probably by affecting the DTNB light chains in such a way that they tend to lose their inhibiting property (see III.4.2.3.). Further evidence also comes from the small, but significant steady increase of ATPase activities for preparations which have not been exposed to high ionic strength during the course of preparation, as the KCl concentration in the assay media increases from low to about more than physiological range. This can be seen by examining (i) Figure 13, open circle; Mg-ATPase activities of purified native myosin filaments, (ii) Figure 14, closed square; Mg-ATPase activities of S-1 from purified native myosin filaments, (iii) Figure 16; Ca-ATPase activities of purified native myosin filaments, (iv) Figure 23, open circle; Vmax of purified native myosin filaments, (v) Figure 25, open circle; Vmax of S-1

from purified native myosin filaments.

III.3.3. Convergence of ATPase activities of conventional preparations and those of the preparations after exposure to high ionic strength

The possibility that DTNB light chains are responsible for the low ATPase activities of purified native myosin filaments and the derived S-1 (containing the DTNB light chains; Chapter II) is not only indicated by ionic strength-activation mentioned above, but is also supported by the convergence of ATPase activities of conventional myosin and its S-1 and the ATPase activities of myosin prepared from the solubilised myosin of native myosin filaments and the S-1 (containing no DTNB light chains, Chapter II) of myosin filaments formed from the solubilised myosin of native myosin filaments. This convergence can best be observed by examining the following.

(i) Mg-ATPase:

The Mg-ATPase activities of S-1 (with no DTNB light chains) from myosin filaments formed from solubilised myosin of native myosin filaments after exposure to high ionic strength, had been found to be similar to the Mg-ATPase activities of S-1 from conventional synthetic

myosin filaments, in the manner of their ionic strength-dependence, that is, the ATPase activities decreased steadily as the [KCl] increased from low to high (Figure 14, open circle, open square).

(ii) Ca-ATPase:

The convergence of Ca-ATPase activities of conventional preparations and preparations after exposure to high ionic strength can be seen from (a) conventional myosin (Figure 15, open circle) and myosin of purified native myosin filaments after exposure to high ionic strength (Figure 15, open triangle), and (b) S-1 from conventional synthetic myosin filaments (Figure 17, open square) and S-1 from myosin filaments formed from solubilised myosin of purified native myosin filaments (Figure 17, open triangle); both S-1's contained no DTNB light chains (Chapter II).

(iii) K-EDTA-ATPase:

The K-EDTA-ATPase activities of both conventional myosin and S-1 from conventional synthetic myosin filaments increased steadily as the [KCl] increased from about 60 mM to 600 mM (Figure 20, open triangle and Figure 21, open triangle), in a similar manner to those of myosin from solubilised native myosin filaments (Figure 20, open

circle) and S-1 from myosin filaments formed from solubilised native myosin filaments (Figure 21, open circle), except that the ATPase activities were unusually activated at physiological ionic strength for preparations which were not prepared by conventional means.

(iv) Vmax:

The convergence of the ionic strength-dependence of Vmax can be seen from (a) conventional myosin and myosin from solubilised native myosin filaments after exposure to high ionic strength (Figure 24, closed circle and open circle), and (b) S-1 from conventional synthetic myosin filaments and S-1 from myosin filaments formed from solubilised purified native myosin filaments; both S-1 contained no DTNB light chains (Figure 26, closed circle and open circle).

III.3.4. Effect of $^{2}H_2O$ on ATPase activities

Results (Table 4) from the studies on the effect of $^{2}H_2O$ on ATPase activities show that $^{2}H_2O$ has practically no effect on the ATPase activities.

III.4. Discussion

III.4.1. General considerations

Using a rapid and sensitive isotopic method (III.2.3.1.), detailed studies of the ionic strength-dependence of the ATPase activities of $^{2}H_2O$-H_2O gradients-purified native myosin filaments (Chapter II) and the derived chymotryptic S-1 containing the DTNB light chains (native preparations) have been made and compared with the ATPase activities of conventional myosin and chymotryptic S-1 (lacking the DTNB light chains) of synthetic myosin filaments (conventional preparations). The pH-stat assay (III.2.3.2) has also been employed for ATPase studies and consistent results have been obtained by the two methods. However, detailed studies of native preparations which can only be prepared in small quantities, have been made possible only by the use of the isotopic assay which required only a minute amount of enzyme (5-10 μg) for each assay as compared to the pH-stat method which requires about 100 times more protein for each assay. The very low ATPase activities (III.3.1.) of native preparations are not only activated by high ionic strength (0.6 M KCl) treatment (III.3.2.), but convergence of ATPase activities are also observed for conventional preparations and native preparations after the latter have "seen" high ionic strength media, especially for S-1 lacking the DTNB

light chains (III.3.3.).

Using conventional preparations and native preparations after exposure to high ionic strength, the results presented above (III.3.) have shown that the Mg-ATPase and Ca-ATPase activities and also the Vmax decrease as the ionic strength increases and that the K-EDTA-ATPase activities are most pronounced at about 0.6 M KCl. It is generally considered that increasing the ionic strength decreases the apparent association constant (Ka) of the enzyme for actin, and therefore decreases the ATPase activity (Eisenberg and Moos, 1970). However, there are also exceptions, for instance, using HMM, it has been found that the Ka increases sharply while the ATPase activities decrease sharply as the KCl concentrations are increased from about physiological to 300 mM (Hozumi and Tawada, 1973). This phenomenon is also observed in the present study, in cases of S-1 from purified native myosin filaments (Figure 25, open circle and open triangle), solubilised myosin of purified native myosin filaments (Figure 24, open circle and open triangle), and S-1 from myosin filaments formed from solubilised myosin of native myosin filaments (Figure 26, open circle and open triangle), as the KCl concentrations increase from about 300 mM to 600 mM.

While the ATPase activities of conventional

preparations (exposed to high ionic strength) and native preparations after exposure to high ionic strength behave in a similar manner, the ATPase activities of native preparations before exposure to high ionic strength are very low and these low ATPase activities are seen to increase significantly though slightly as the ionic strength in the assay media increases (III.3.2.), showing that ionic strength-activation of ATPase activities is obvious even during the brief incubation time (3-5 minutes) of the assay. However, the failure of these activities to rise during the assay to the level seen after prolonged incubation at ionic strength I=0.60; implies that the time constant for the ionic strength effect must be of the order of at least several minutes.

III.4.2. Why are the ATPase activities of native preparations extremely low ?

The most striking finding during the course of the enzymological studies is the extremely low ATPase activities of the purified native myosin filaments and the derived chymotryptic S-1 containing the DTNB light chains before exposing to high ionic strength (native preparations). These observed low ATPase activities could be due to several factors: (i) "dead enzyme", (ii) contamination by actin which has been reported to depress the Ca-ATPase of myosin (Nanninga, 1964) and HMM (Yagi et

al., 1965) in the absence of magnesium, and that actin reduces the K-EDTA-ATPase of myosin (Bowen and Kerwin, 1954), and (iii) the inhibitory effect of DTNB light chains before exposure to high ionic strength.

III.4.2.1. Are these low ATPase activities due to "dead enzyme" ?

Before any significance of the observed low ATPase activities can be claimed, one of the important questions to ask is whether the low ATPase activities of native preparations arise from some feature of our preparation procedure which has led to irreversible loss of catalytic activity at the active sites. There are at least two possibilities which should be considered: (i) does ^2H_2O affect the ATPase activities of the enzyme? and (ii) has the enzyme otherwise denatured during the course of preparation?

^2H_2O has been reported to reduce enzyme rates (Bender and Hamilton, 1962; Thomson, 1963), however, it is known that it does not affect the ATPase activities of actomyosin at about pH 7 (Hotta and Morales, 1960). The fact that ^2H_2O does not affect the ATPase of myosin has also been confirmed in the present study. Practically identical ATPase activities (Table 4) have been obtained for myosin which is prepared either by using stock buffer made up in

water or in $^{2}H_2O$.

Denaturation by other agencies can be rather unequivocally ruled out by further experiments (III.3.2. and III.3.3.) which together show that myosin initially prepared as native filaments, and the derived chymotryptic S-1 can be essentially converted into forms having a conventional level of ATPase activities by limited exposure to high ionic strength.

III.4.2.2. Could the low ATPase activities of native preparations be due to the contamination by actin ?

Though it has been reported that actin reduces Ca-ATPase activities in the absence of added magnesium, this observation is evident only at very high ratios of actin to myosin (Nanninga, 1964; Yagi et al., 1965). The following evidence shows that the low ATPase activities are not due to contamination by actin: (i) SDS-gel electrophoresis confirms that our preparations of native myosin filaments are at least 98% pure with respect to the 43,000 M.W. actin band (Emes, 1977; Emes and Rowe,1978; the purity could be higher with respect to actin since the 43,000 M.W. band is likely to be due to or contaminated by creatine kinase which is thought to be associated with the M-line), (ii) the extremely low Mg-ATPase activities of our native preparations (native myosin filaments and the

derived S-1) before "seeing" high ionic strength unequivocally show the purity of native preparations with respect to actin since actin activates Mg-ATPase activities, (iii) the marked activation of Mg- (III.3.2.1.), Ca- (III.3.2.2), and K-EDTA-ATPase activities (III.3.2.3.) of S-1 containing the DTNB light chains by high ionic strength treatment (without further precipitation steps), suggests that the low ATPase activities of native preparations are due to the presence of DTNB light chains before exposure to high ionic strength treatment (native DTNB light chains) rather than the contamination by actin (see III.4.2.3.).

III.4.2.3. Native DTNB light chains are responsible for the low ATPase activities of native preparations

Despite the fact that a substantial amount of work has been carried out on the DTNB light chains of vertebrate skeletal myosin, the role of these light chains is still a subject of debate and controversy (see I.2.2.). Using myosin and DTNB-treated myosin, it has been shown that the Mg-, Ca-, and K-EDTA-ATPase activities are independent of the DTNB light chains, while the superprecipitation of actin and DTNB-treated myosin occurs more slowly than that of actin and non-treated myosin. The actin-activated ATPase activities and Ka have been found to decrease for DTNB-treated HMM as compared to non-treated HMM (Hozumi and

Hotta, 1978). However, these and all other observations on DTNB light chains have been based on conventional myosin which has been exposed to high ionic strength during the course of preparation. On the other hand, Malhotra et al (1979) have shown that removal of LC2 (corresponding to the DTNB light chain of rabbit skeletal muscle) from canine cardiac myosin does not affect the Ca-ATPase activities of myosin, while the Mg- and actin-activated ATPase activities are increased considerably as compared to myosin with the intact of LC2. Reassociation of LC2 or rabbit DTNB light chains with LC2 deficient canine cardiac myosin lowers the ATPasse activities close to those of the myosin with the intact of LC2.

The present study strongly suggests that DTNB light chains of vertebrate skeletal muscle before exposure to high ionic strength possess the ability to maintain the Mg-ATPase activities of native preparations (purified native myosin filaments and the derived S-1 containing the DTNB light chains) to a level as low as those of the resting muscle. This is the first demonstration of this known physiological property in a purified in vitro system. In addition, it is interesting to find that the Ca-, K-EDTA- and actin-activated ATPase activities are also low for the native preparations and these low activities can be activated by high ionic strength treatment (III.3.2.) and removal of DTNB light chains further activates the ATPase

activities similar to those of coventional preparations (conventional myosin and S-1 without the DTNB light chains from synthetic myosin filaments) (III.3.3.).

The evidence that DTNB light chains are responsible for the low ATPase activities of native preparations comes from the high ionic strength activation (III.3.2.) and convergence (III.3.3.) phenomena. This is best illustrated by the ionic strength-activation of S-1 containing the DTNB light chains and removal of these light chains results in even higher activities, similar to those of the conventional S-1 without the DTNB light chains. Figure 27 diagrammatically represents the ionic strength-activation of S-1 (containing the DTNB light chains) at physiological ionic strength and this activation is further elevated in the absence of DTNB light chains, reaching that of the conventional preparation (see Figure 14). This phenomenon can equally well be seen by comparing and examining other ATPase activities of S-1 with or without the DTNB light chains: (i) Ca-ATPase: Figures 17 and 18, (ii) K-EDTA-ATPase: Figures 21 and 22, and (iii) Vmax, Figures 25 and 26. The fact that high ionic strength has the potential effect to cause a permanent conformational change of myosin especially with respect to DTNB light chains, so that ATPase activities are markedly elevated is further supported by Figures 13, 15, 20, and 24.

It has been suggested (Chapter II) that the difference in the chymotryptic digestion pattern of native myosin filaments and synthetic myosin filaments is either due to the alteration of the myosin packing into filaments or due to an irreversible conformational change of the myosin molecule or due to both as a result of exposure to high ionic strength. In the present chapter, more evidence is therefore presented to show that the quaternary structure of native myosin (Chapter II) can be altered irreversibly by high ionic strength treatment so that the DTNB light chains are translocated or conformationally changed so that they are susceptible to chymotryptic attack and at the same time they lose their potential to maintain low ATPase activities. Figure 28A shows how in native myosin and in the absence of calcium, the DTNB light chain might be in a position so that the hydrolytic site (ATPase site) of the S-1 is blocked so as to result in low ATPase activities. High ionic strength treatment however, switches on the ATPase activities irreversibly to the level found in conventional preparations, by irreversibly exposing the hydrolytic site at the expense of a relative movement or conformational change of the DTNB light chain (Figure 28B). In vivo, in the presence of actin and activating calcium concentration (10^{-5}-10^{-4} M), the ATPase activity is "turned" on as the DTNB light chain is switched from position A to B (Figure 28). However, this switching on of ATPase activity is reversible so that muscle is relaxed at

non-activating calcium concentration (10^{-7} M). This probably explains the observed low actin-activated ATPase activities in the absence of calcium during the course of this study. Therefore, it seems that ionic strength activation is similar to calcium-activation in vivo except that the former is permanently switched on due to the loss of the DTNB light chain "control" after exposure to high ionic strength. Evidence that DTNB light chains (before exposure to high ionic strength) do play a role in muscular activity is presented in Chapter IV.

III.4.3. ATPase activities at specific ionic strengths

Table 5 shows the ATPase activities (both expressed as n moles Pi per min per mg enzyme and moles Pi per myosin ATPase site per second) at physiological ionic strength (0.12 M KCl) and at 0.6 M KCl.

As described (III.3.1.1.) the Mg-ATPase activities of purified native myosin filaments and the derived S-1 were extremely low. The present results (Table 5, rows 2 and 5; column b: Mg-) show that preparations before exposure to high ionic strength media have Mg-ATPase activities (25° C) several fold lower than that obtained for resting frog muscle at 0° C (Kushmerick and Paul, 1976; Ferenczi et al., 1978) and about 70-90 fold lower than conventional preparations (Table 5, rows 1 and 4, column b: Mg-). This

discrepancy may be explained (III.4.2.3.) by assuming that once the preparations are exposed to high ionic strength, the ability of the DTNB light chains to repress ATPase activities is lost. One may therefore assume that resting muscle has negligible ATPase activities. The turnover rate per myosin ATPase site of about 0.0024 per second for muscle at rest might be mainly due to other ATPase (Ferenczi et al., 1978). Therefore, the present result (Table 5) of about 0.0006 ± 12% per second per ATPase site might be the true ATPase activity in resting vertebrate skeletal muscle.

The results of Mg- and Ca-ATPase activities of conventional preparations (Table 5, rows 1 and 4) and native preparations (Table 5, rows 2 and 5) closely resemble each other when expressed as moles Pi per myosin ATPase site per second. This seems to indicate that there is no cooperativity between the two S-1's of the myosin molecule in cases of Ca- and Mg-ATPase activities. A similar comparison for K-EDTA- and actin-activated Mg-ATPase activities (Table 5) indicates that they might be positive cooperation in case of the conventional preparations (Table 5, rows 1 and 4) and negative cooperation in case of native preparations (Table 5, rows 2 and 5), at the specific ionic strengths (Table 5).

It is interesting to find that the actin-activated

Mg-ATPase activities (Vmax) of the native preparations are very low as compared to conventional preparations (Table 5). This may be due to the inhibitory effect of native DTNB light chains (III.4.2.3.). The observed low activity may also be due to tracer amount of calcium which is present in commercial KCl (Bagshaw, 1980) as the incubation media contained no EGTA (III.2.2.).

III.4.4. Summary

Up to the present chapter, the following points can be observed concerning the effect of high ionic strength on native myosin filaments and the possible modification of myosin quaternary structure in relation to ATPase activities.

(i) High ionic strength media not only transform native myosin filaments into conventional myosin as judged by the S-1 produced by chymotryptic digestion of the two types of filaments (Chapter II), but also have striking effect on the elevation of ATPase activities of native preparations as shown by the high ionic strength activation (III.3.2.) and convergence (III.3.3.) phenomena.

(ii) The low ATPase activities of native preparations are maintained by the DTNB light chains which sterically block the hydrolytic (ATPase) site.

(iii) The low ATPase activities of native preparations are permanently activated by high ionic strength (0.6 M KCl) treatment, probably by inducing irreversible translocation or conformational change of the DTNB light chains so that the hydrolytic site of S-1 is exposed for ATP hydrolysis, implying that all conventional myosin preparations have therefore artifactually elevated ATPase activities due to exposure to high ionic strength during the course of preparation.

(iv) The extremely low Mg-ATPase activities of native myosin filaments and the derived chymotryptic S-1 are the first demonstration of this known physiological property in a purified in vitro system, implying that the role of DTNB light chain is to "switch-off" the ATPase activity in the resting muscle.

Figure 12.

Kinetic models for the hydrolysis of ATP by S-1 and acto-S-1

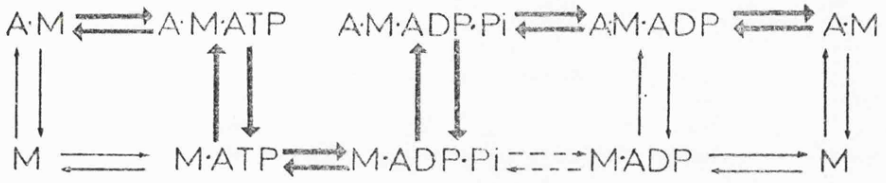
A. Lymn and Taylor Model.

A1. Cross bridge cycle based on Lymn and Taylor model.

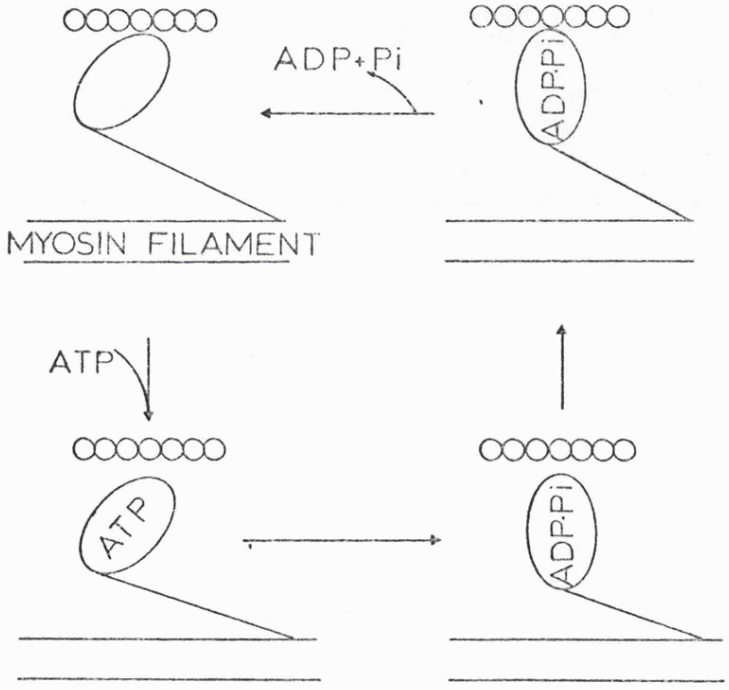
B. Refractory state model of Stein et al (1979); in the original refractory state model of Eisenberg and Kielley (1972), steps a, b, and c are not included.

Heavy solid arrows represent the pathways of ATP hydrolysis in the presence of actin and the dashed arrows represent the rate-limiting steps in the absence of actin. A: actin monomer; M: S-1; [RS]: refractory state; [NS]: nonrefractory state. Note that M.ADP.Pi [RS] may transform directly to M.ADP and that M.ADP.Pi [NS] is a branch of the cycle in the absence of actin (Chock et al., 1976), and the superscripts used to indicate comformational changes of the myosin have also been omitted. According to Goody et al 1977), the binding of ATP and the release of products involves the formation of a collision intermediate followed by a conformational change.

A.


A·M
A·M·ATP
A·M·ADP·Pi
A·M·ADP
A·M
M
M·ATP
M·ADP·Pi
M·ADP
M

A1.


ADP+Pi
ADP·Pi
MYOSIN FILAMENT
ATP
ATP
ADP·Pi

B.

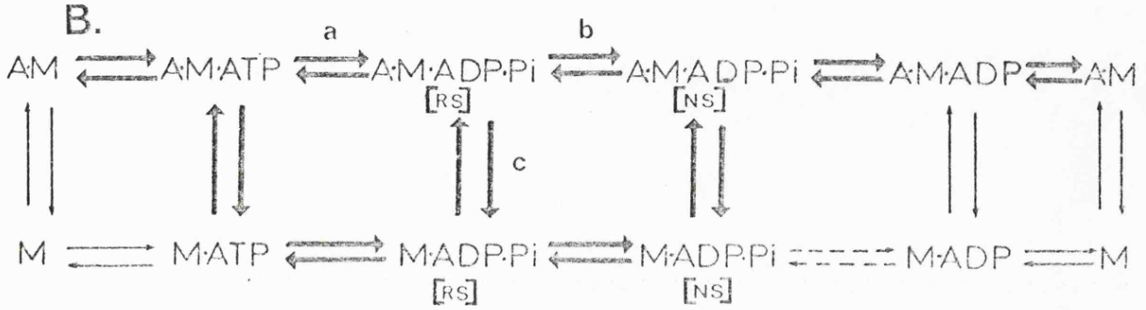

a
b
A·M
A·M·ATP
A·M·ADP·Pi
[RS]
A·M·ADP·Pi
[NS]
A·M·ADP
A·M
c
M
M·ATP
M·ADP·Pi
[RS]
M·ADP·Pi
[NS]
M·ADP
M

Figure 13

Comparison of the ionic strength-dependence of the Mg-ATPase activities of conventional myosin, native myosin filaments and myosin from solubilised filaments

Closed circle: conventional myosin.

Open circle: purified native myosin filaments.

Open triangle: myosin from solubilised native myosin filaments, prepared by dissolving purified native myosin filaments at high ionic strength by dialysis against stock buffer for 5 hours or overnight; the solution was cleared by centrifugation and the myosin precipitated by dilution was pelleted and redissolved in stock buffer.

Conditions: 4 mM $MgCl_2$, 1mM ATP, 20 mM Tris-HCl, pH 7.5, 0.1 mg per ml enzyme, and [KCl] varied as shown.

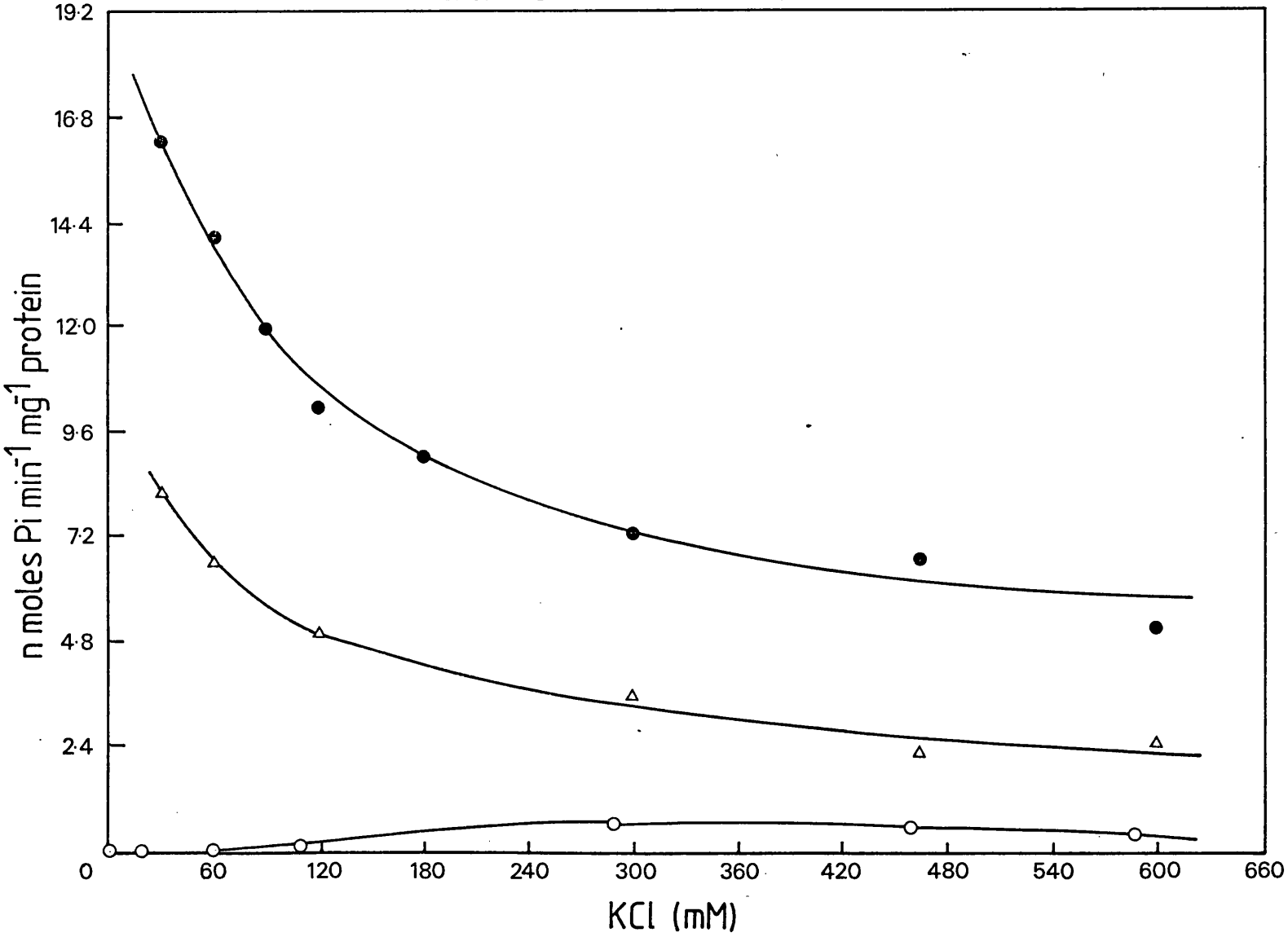

19·2
16·8
14·4
12·0
9·6
7·2
4·8
2·4
0
n moles Pi min-1 mg-1 protein
60
120
180
240
300
360
420
480
540
600
660
KCl (mM)

Figure 14

Comparison of the ionic strength-dependence of Mg-ATPase activities of S-1 from synthetic and purified native myosin filaments, and of S-1 from filaments formed from solubilised native myosin filaments, and the ionic strength-activation of Mg-ATPase activities of S-1 from native myosin filaments

Open square: S-1 from synthetic myosin filaments.

Closed square: S-1 from purified native myosin filaments.

Open circle: S-1 from myosin filaments formed from solubilised purified native myosin filaments (see legend to Figure 13).

Closed circle: S-1 from purified native myosin filaments after this S-1 had been exposed to high ionic strength for 5 hours or overnight by dialysis against stock buffer.

Conditions: same as those of Figure 13.

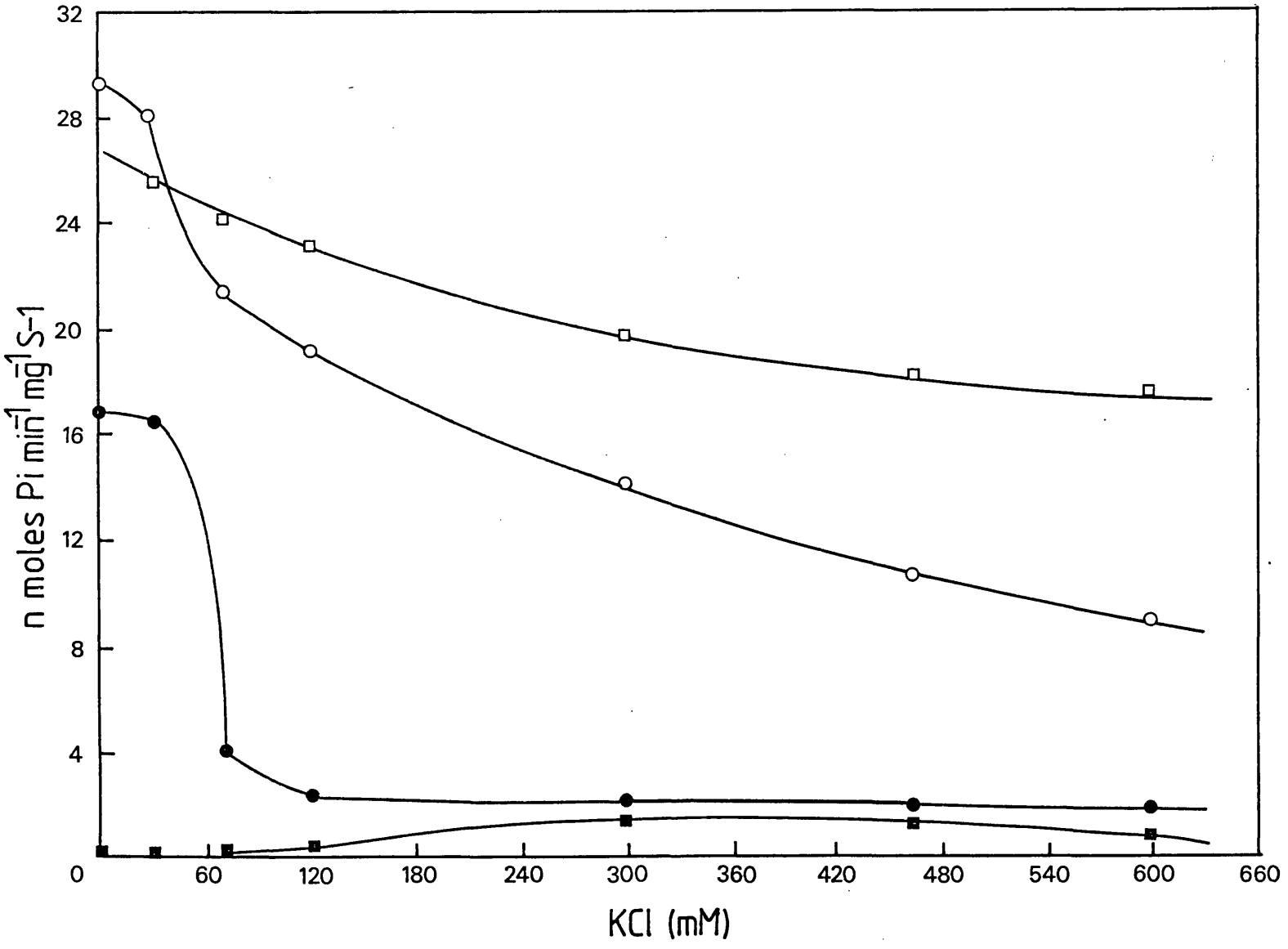

32
28
24
20
16
12
8
4
0
60
120
180
240
300
360
420
480
540
600
660
n moles Pi min⁻¹ mg⁻¹ S-1
KCl (mM)

Figure 15

Ionic strength-dependence of Ca-ATPase activities of conventional myosin and myosin from solubilised purified native myosin filaments

Open circle: conventional myosin (pH-stat method).

Open triangle: myosin from solubilised purified native myosin filaments (see legend to Figure 13).

Conditions: 10 mM $CaCl_2$, 1mM ATP, 20 mM Tris-HCl, pH 7.5, 0.05 mg per ml conventional myosin or 0.1 mg per ml myosin from solubilised purified native myosin filaments, and [KCl] varied as shown.

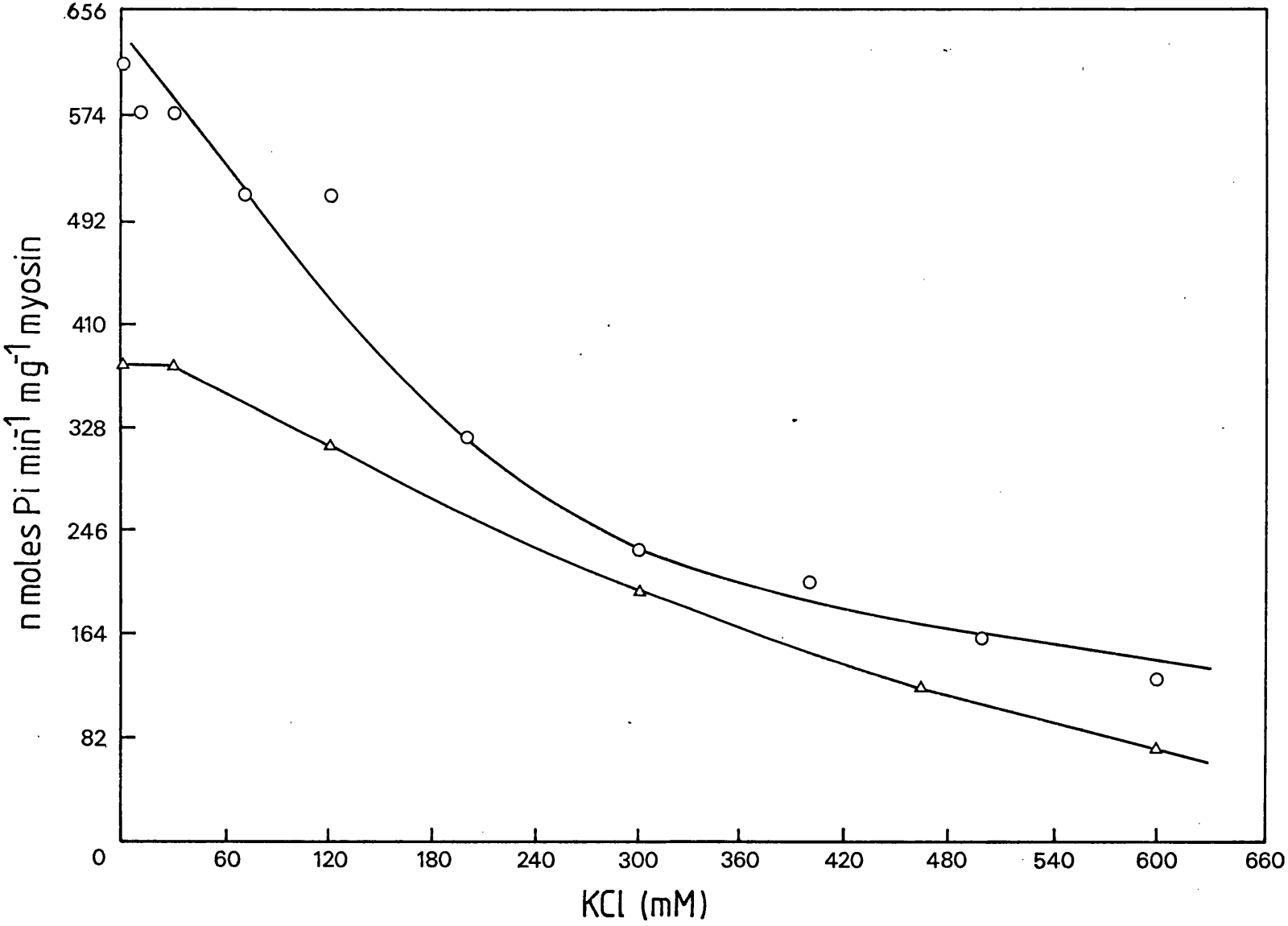

656
574
492
410
328
246
164
82
0
60
120
180
240
300
360
420
480
540
600
660
n moles Pi min-1 mg-1 myosin
KCl (mM)

Figure 16

Ionic strength-dependence of Ca-ATPase activities of purified native myosin filaments

Conditions: 10 mM $CaCl_2$, 1mM ATP, 20 mM Tris-HCl, pH 7.5, 0.1 mg per ml enzyme and [KCl] varied as indicated.

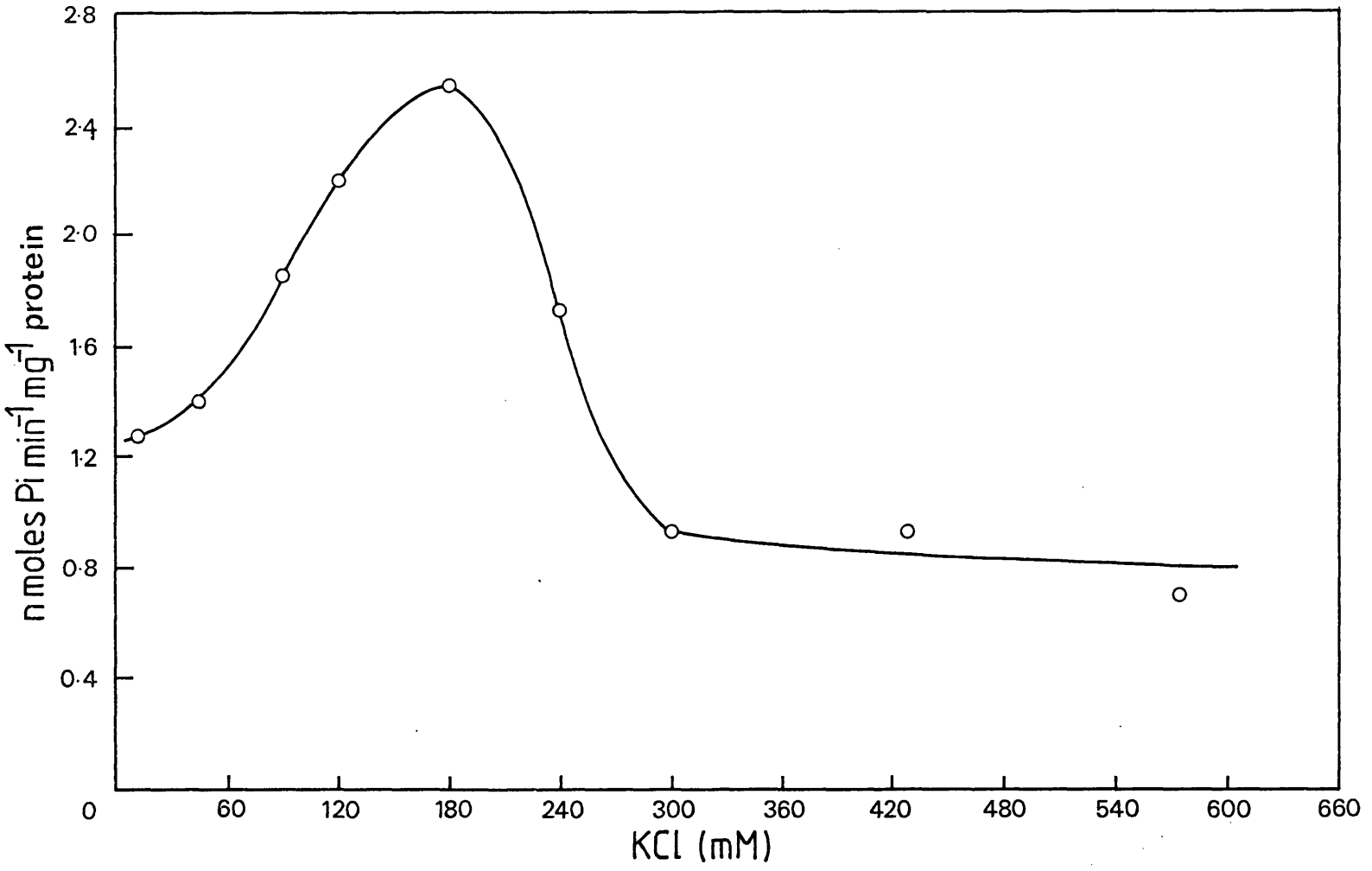

2·8
2·4
2·0
1·6
1·2
0·8
0·4
0
n moles Pi min⁻¹ mg⁻¹ protein
60
120
180
240
300
360
420
480
540
600
660
KCl (mM)

Figure 17

Ionic strength-dependence of Ca-ATPase activities of S-1 from synthetic myosin filaments and of S-1 from filaments formed from solubilised native myosin filaments

Open square: S-1 from synthetic myosin filaments (pH-stat assay).

Open triangle: S-1 from filaments formed from solubilised purified native myosin filaments (see legend to Figure 13).

Conditions: 10 mM $CaCl_2$, 1 mM ATP, 20 mM Tris-HCl, pH 7.5, 0.1 mg per ml enzyme, and [KCl] varied as indicated.

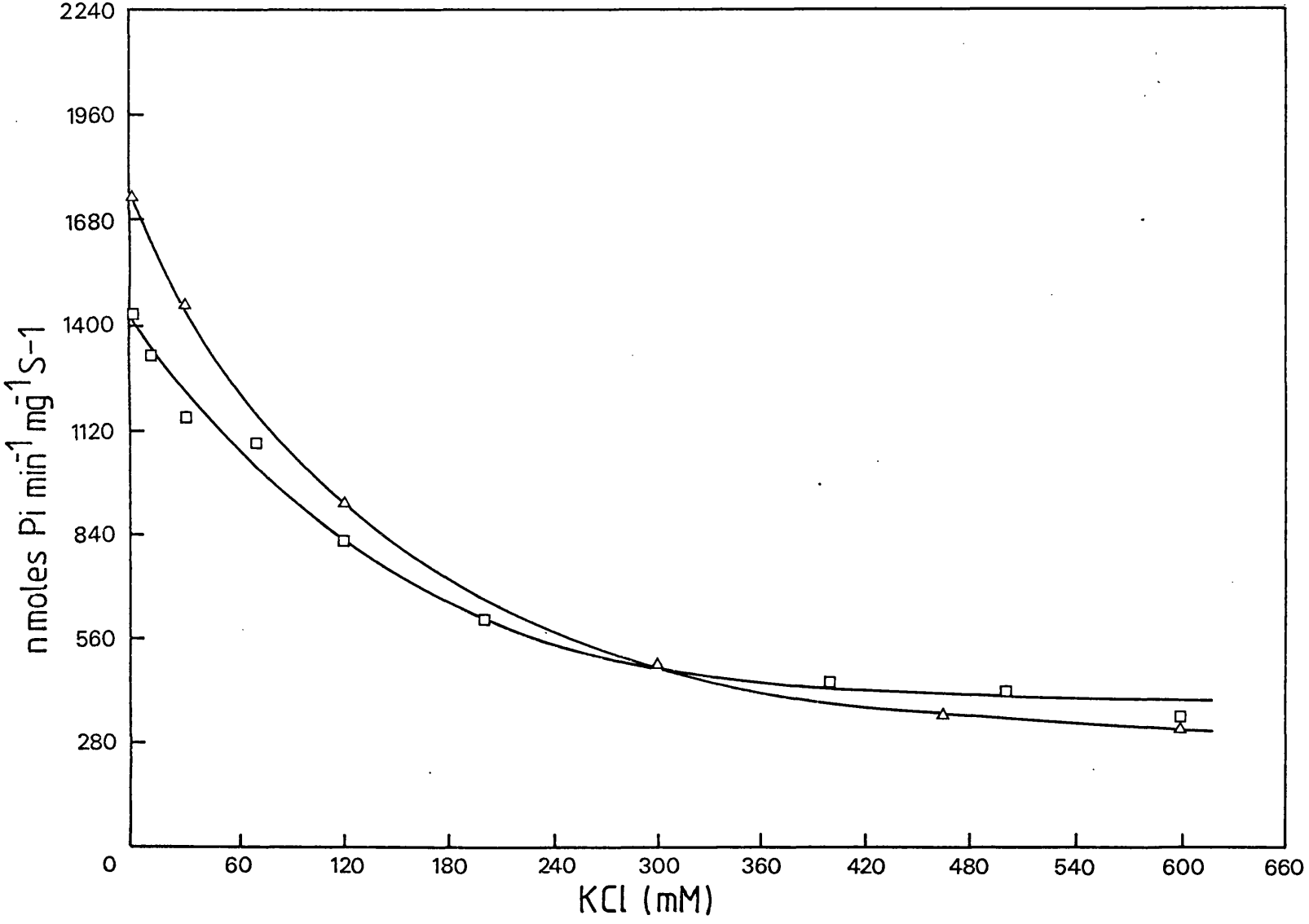

2240
1960
1680
1400
1120
840
560
280
0
60
120
180
240
300
360
420
480
540
600
660
n moles Pi min-1 mg-1 S-1
KCl (mM)

Figure 18

Ionic strength-dependence of Ca-ATPase activities of S-1 after exposure to high ionic strength

Open circle: S-1 from purified native myosin filaments.

Closed circle: S-1 from purified native myosin filaments but this S-1 had been exposed to high ionic strength by dialysis against stock buffer for 5 hours or overnight.

Conditions: same as those of Figure 17.

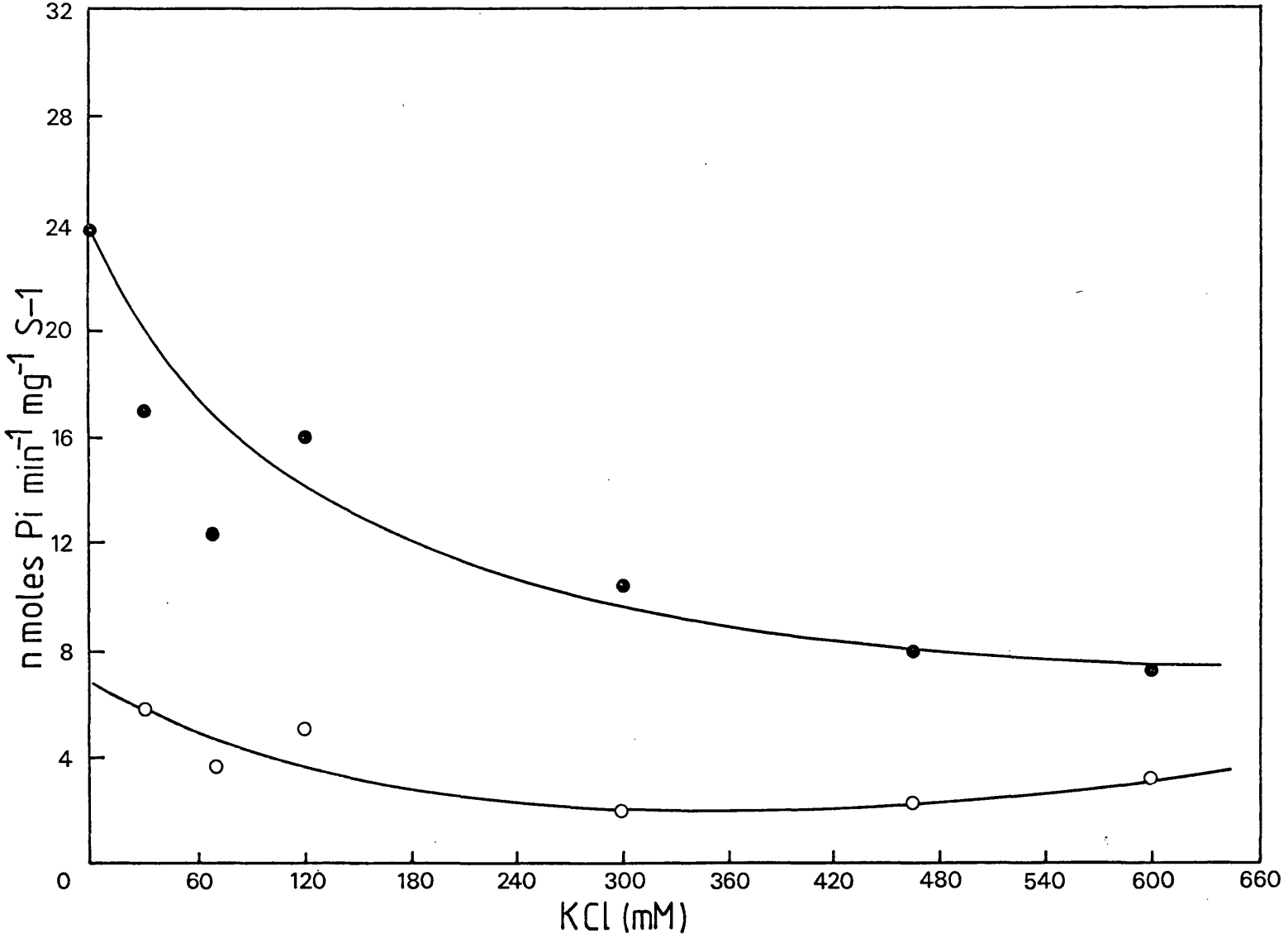

32
28
24
20
16
12
8
4
0
n moles Pi min-1 mg-1 S-1
60
120
180
240
300
360
420
480
540
600
660
KCl (mM)

Figure 19

K-EDTA-ATPase activities of purified native myosin filaments

Conditions: 5 mM EDTA, 1 mM ATP, 20 mM Tris-HCl, pH 7.5, 0.1 mg per ml enzyme, and [KCl] varied as indicated.

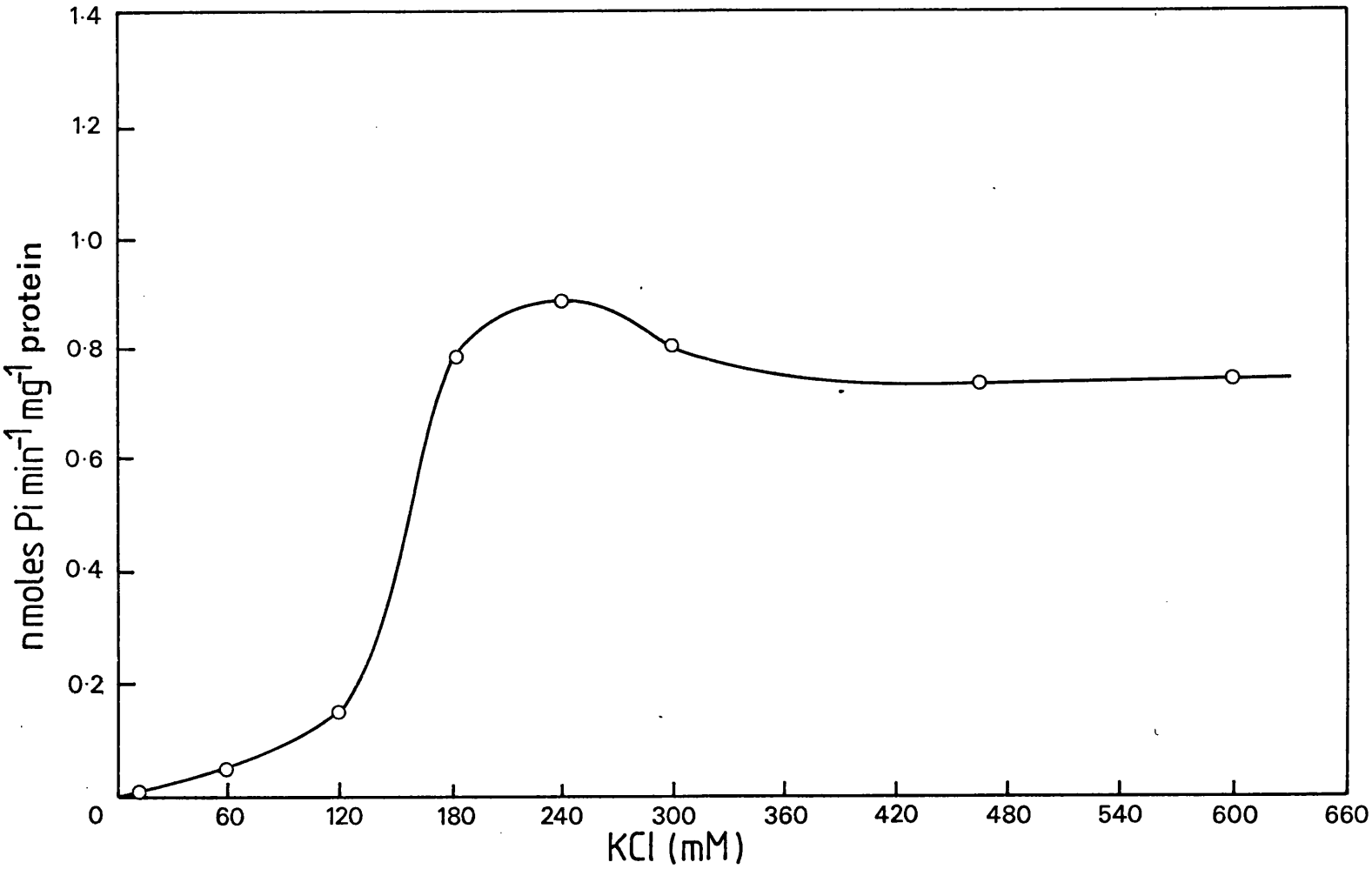

1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
nmoles Pi min⁻¹ mg⁻¹ protein
60
120
180
240
300
360
420
480
540
600
660
KCl (mM)

Figure 20

K-EDTA-ATPase activities of conventional myosin and myosin from solubilised native myosin filaments

Open triangle: conventional myosin (pH-stat assay).

Open circle: myosin from solubilised purified native myosin filaments (see legend to Figure 13).

Conditions: same as those of Figure 19, except no Tris-HCl was used in case of the pH-stat assay.

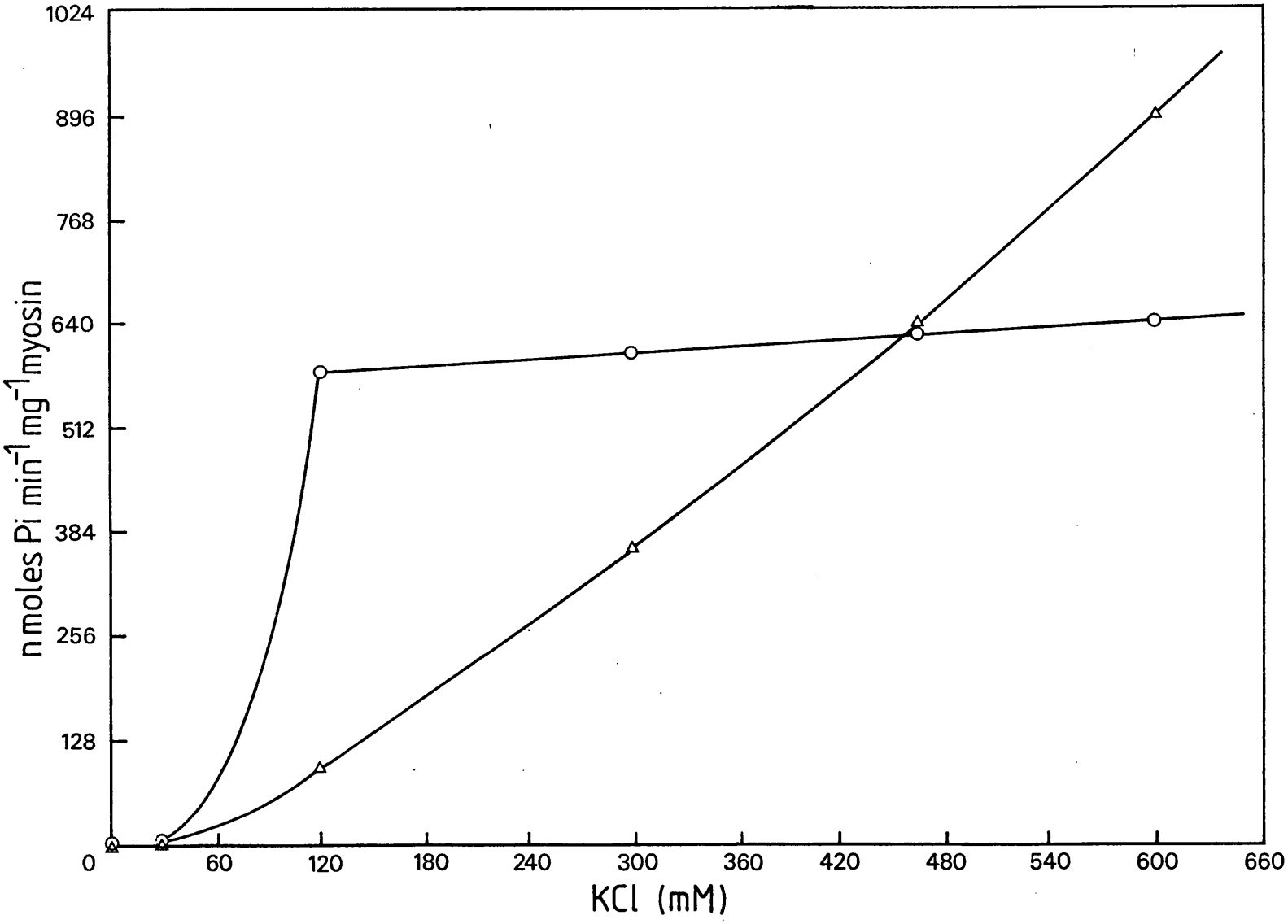

1024
896
768
640
512
384
256
128
0
nmoles Pi min-1 mg-1 myosin
60
120
180
240
300
360
420
480
540
600
660
KCl (mM)

Figure 21

K-EDTA-ATPase activities of S-1 from synthetic myosin filaments and S-1 from filaments formed from solubilised native myosin filaments

Open triangle: S-1 from synthetic myosin filaments.

Open circle: S-1 from filaments formed from solubilised native myosin filaments (see legend to Figure 13).

Conditions: 5 mM EDTA, 1 mM ATP, 20 mM Tris-HCl, pH 7.5, 0.05 mg per ml enzyme, and [KCl] varied as shown.

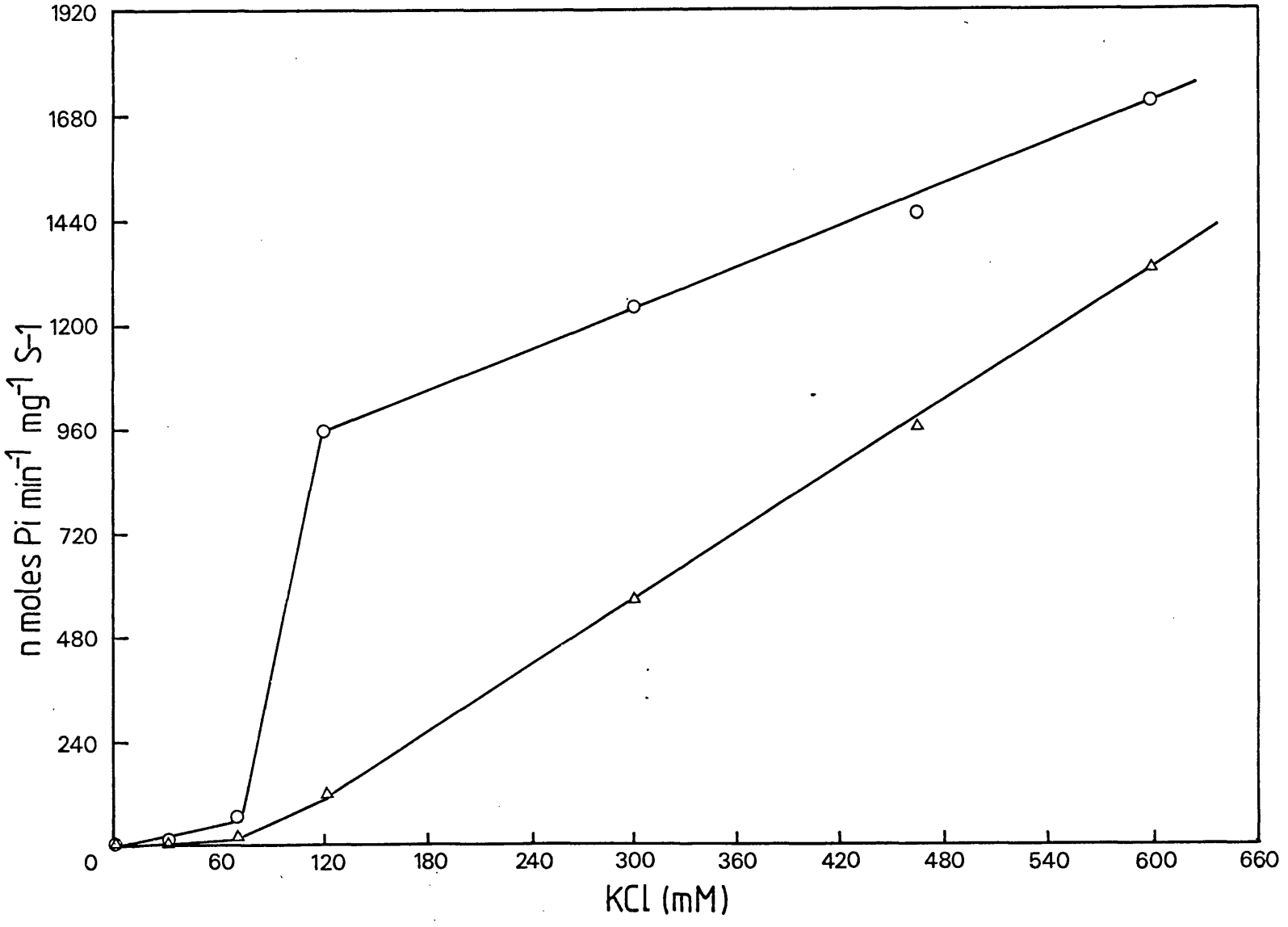

1920
1680
1440
1200
960
720
480
240
0
n moles Pi min-1 mg-1 S-1
60
120
180
240
300
360
420
480
540
600
660
KCl (mM)

Figure 22

K-EDTA-ATPase activities of S-1 from purified native myosin filaments and the same S-1 after exposure to high ionic strength

Open triangle: S-1 from purified native myosin filaments.

Closed circle: same S-1 but after exposure to high ionic strength by dialysis against stock buffer for 5 hours or overnight.

Conditions: same as those of Figure 21 except 0.1 mg per ml enzyme was used.

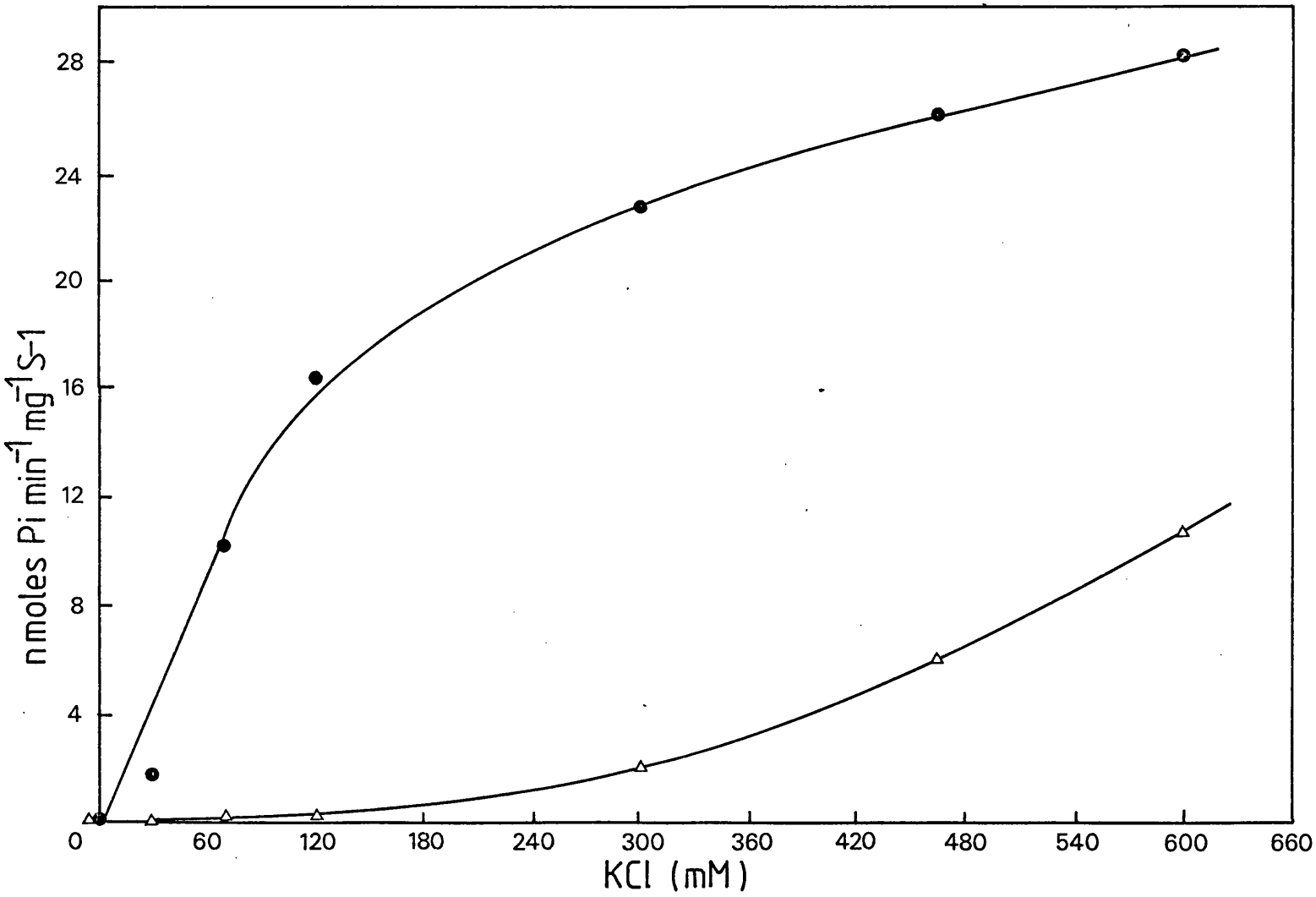

28
24
20
16
12
8
4
0
60
120
180
240
300
360
420
480
540
600
660
nmoles Pi min-1 mg-1 S-1
KCl (mM)

Figure 23

Ionic strength-dependence of Vmax and Ka of purified native myosin filaments (actin activation)

Open circle: Vmax.

Open triangle: Ka.

Conditions: 5 mM $MgCl_2$, 1 mM ATP, 12.5 mM Tris-HCl, pH 7.5, 2 mM DTT, 0.1 mg per ml enzyme, actin concentrations varied from 0.5 - 7.5 mg per ml, and KCl concentrations varied as indicated.

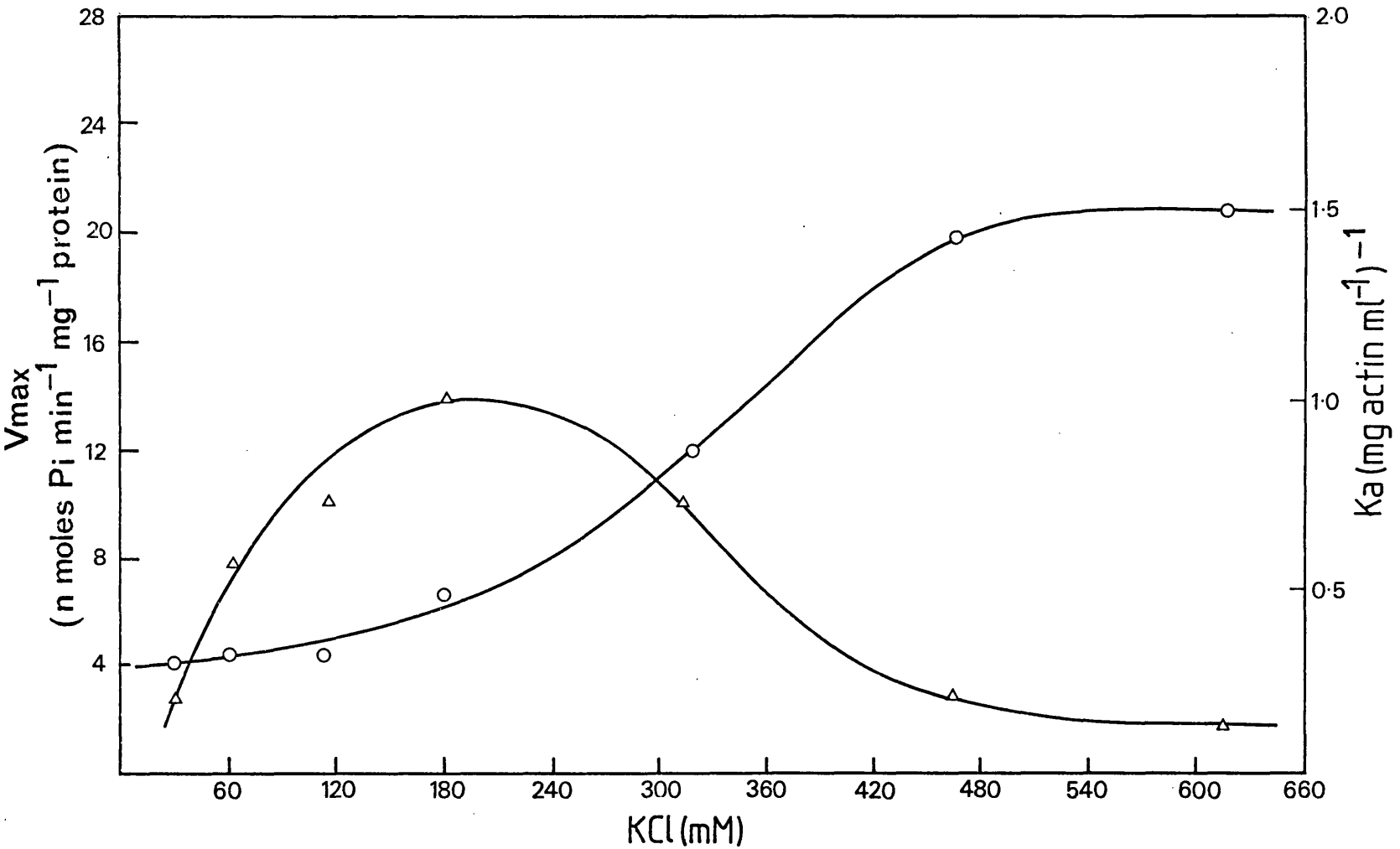

28
24
20
16
12
8
4
Vmax
(n moles Pi min^{-1} mg^{-1} protein)
2·0
1·5
1·0
0·5
Ka (mg actin ml^{-1})$^{-1}$
60
120
180
240
300
360
420
480
540
600
660
KCl (mM)

Figure 24

Ionic strength-dependence of Vmax and Ka of conventional myosin and myosin from solubilised native filaments (actin activation)

Closed circle, closed triangle: Vmax and Ka (respectively) of conventional myosin.

Open circle, open triangle: Vmax and Ka (respectively) of myosin from solubilised purified native myosin filaments (see legend to Figure 13).

Conditions: same as those of Figure 23.

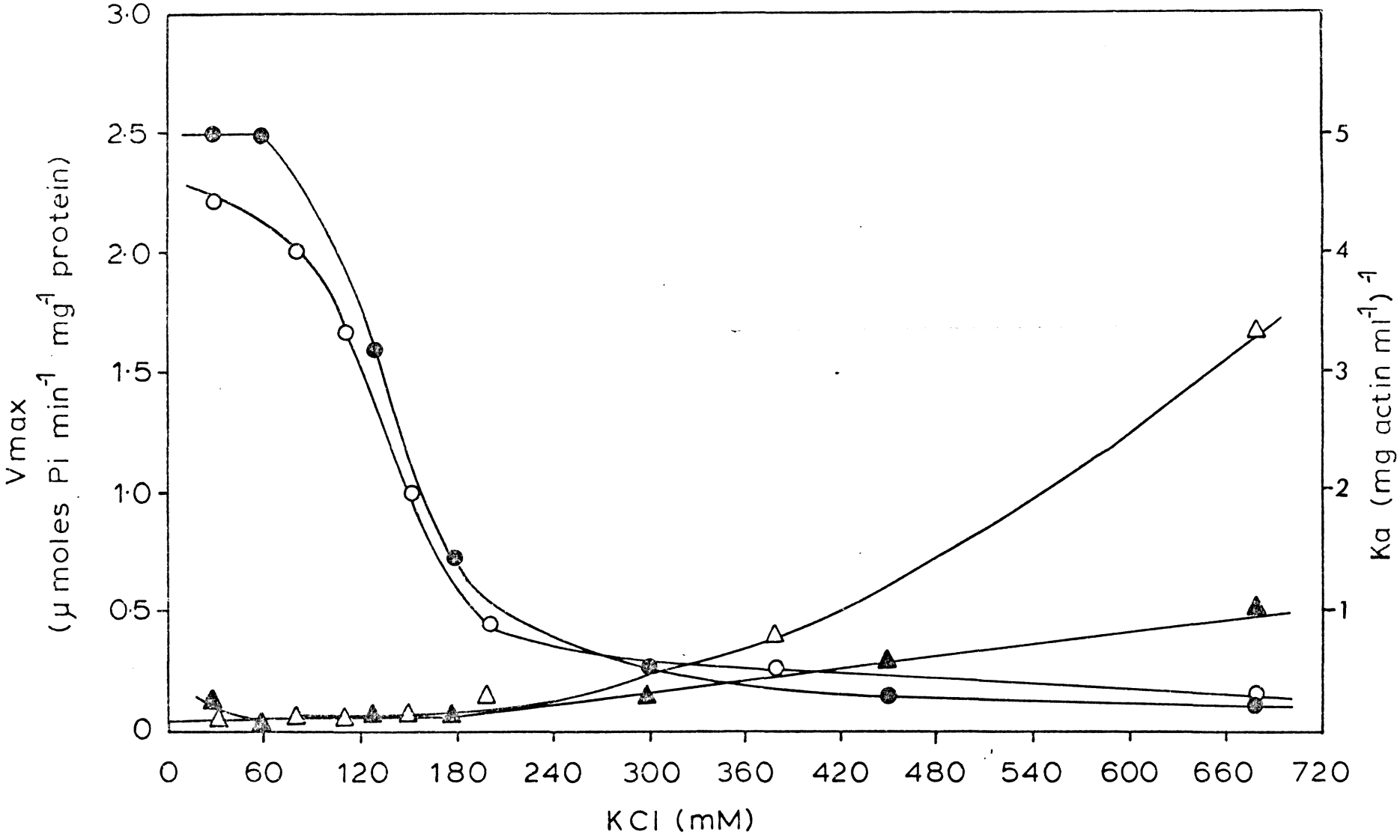

3·0
2·5
2·0
1·5
1·0
0·5
0
Vmax
(μ moles Pi min-1 mg-1 protein)
5
4
3
2
1
Ka (mg actin ml-1)-1
0
60
120
180
240
300
360
420
480
540
600
660
720
KCl (mM)

Figure 25

Ionic strength-dependence of Vmax and Ka of S-1 from purified native myosin filaments and the same S-1 after exposure to high ionic strength (actin activation)

Open circle, open triangle: Vmax and Ka (respectively) of S-1 from purified native myosin filaments.

Closed circle, closed triangle: Vmax and Ka (respectively) of the same S-1, but after exposure to high ionic strength by dialysis against stock buffer for 5 hours or overnight.

Conditions: same as those of Figure 23.

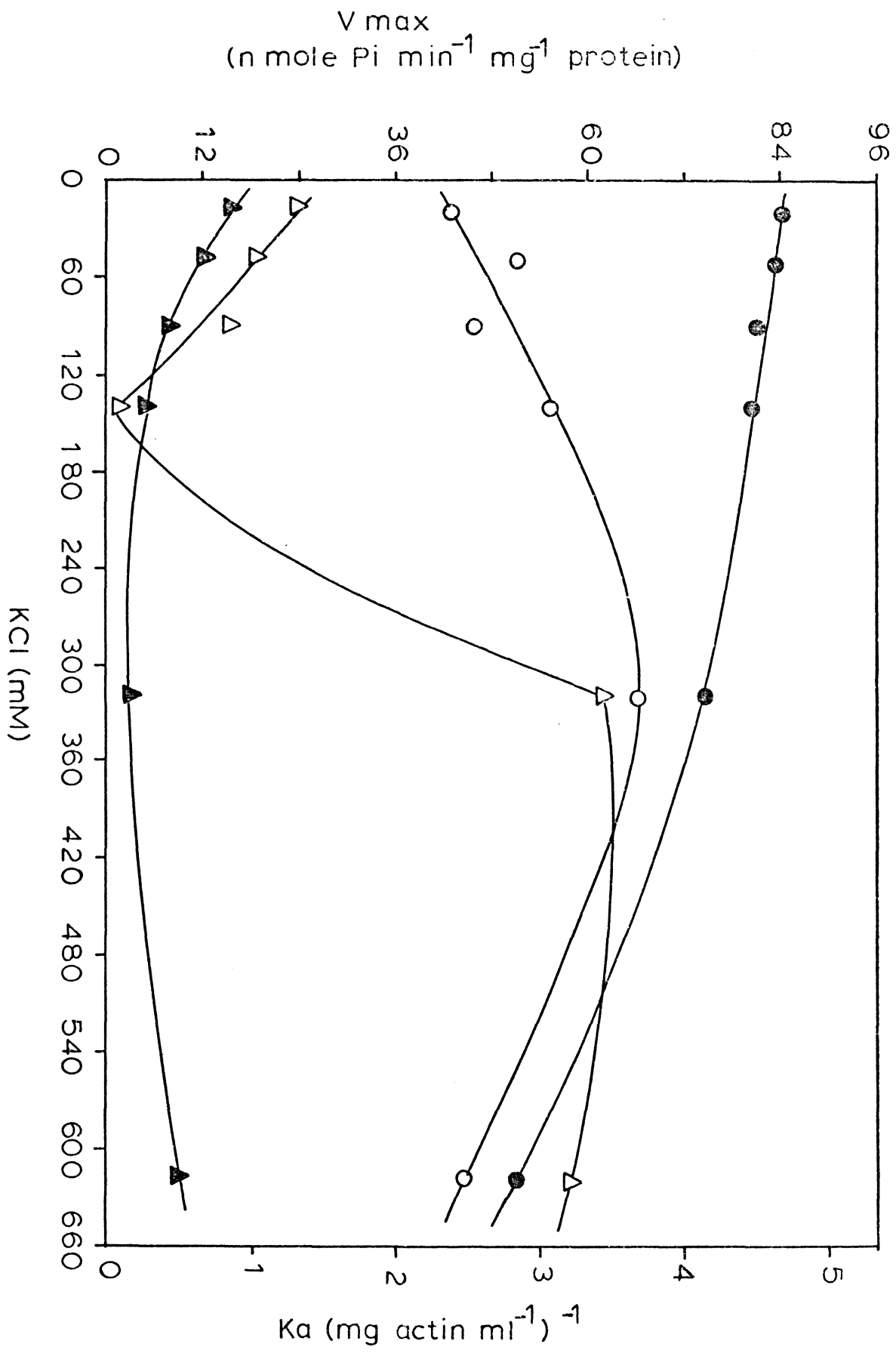

V max
(n mole Pi min^{-1} mg^{-1} protein)
0
12
36
60
84
96
KCl (mM)
0
60
120
180
240
300
360
420
480
540
600
660
0
1
2
3
4
5
Ka (mg actin ml^{-1}) $^{-1}$

Figure 26

Ionic strength-dependence of Vmax and Ka of S-1 from synthetic myosin filaments and of S-1 from filaments formed from solubilised native myosin filaments(actin activation)

Closed circle, closed triangle: Vmax and Ka (respectively) of S-1 from synthetic myosin filaments.

Open circle, open triangle: Vmax and Ka (respectively) of S-1 from filaments formed from solubilised purifed native myosin filaments (see legend to Figure 13).

Conditions: same as those of Figure 23, except 0.05 mg per ml enzyme was used.

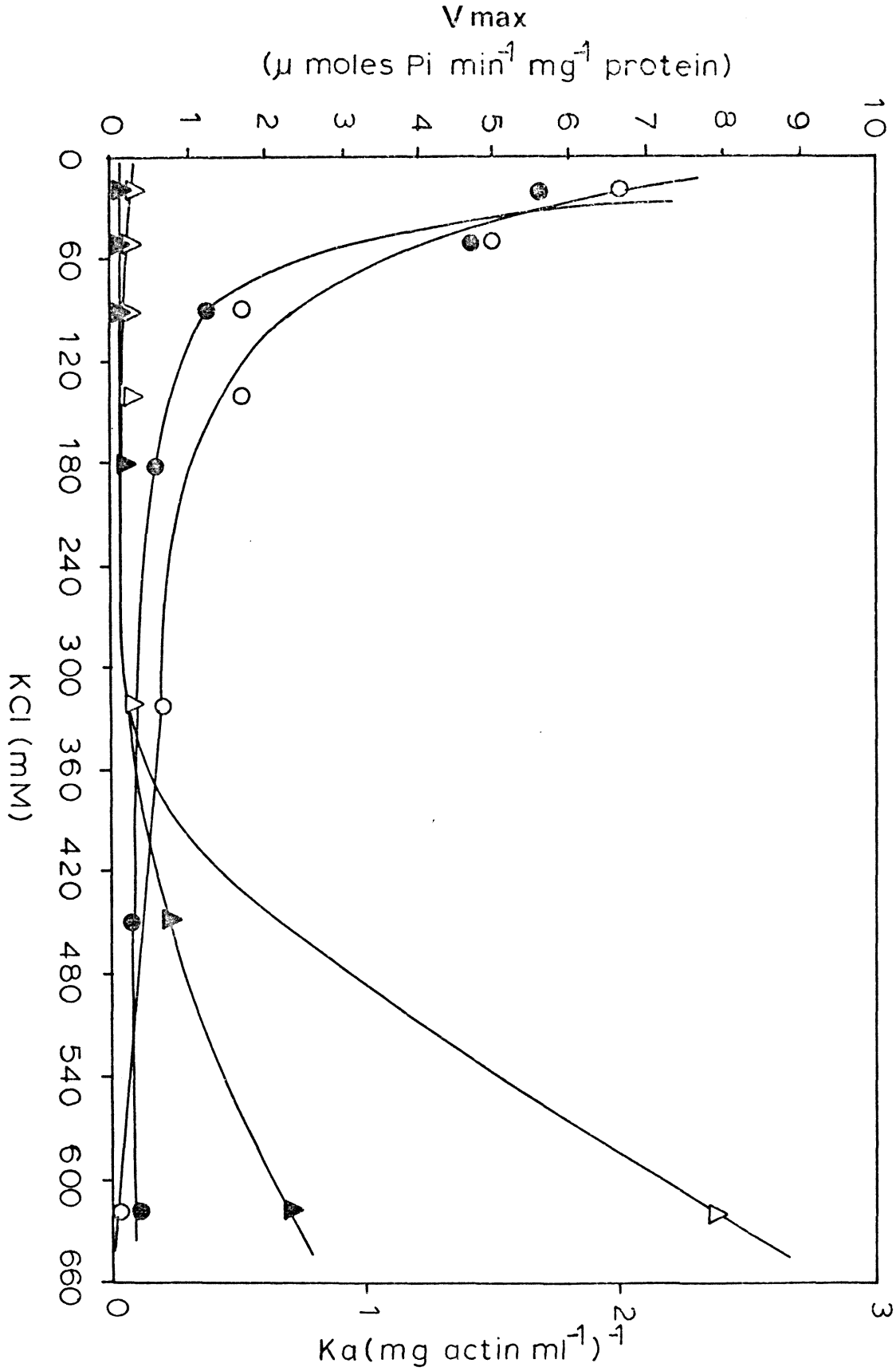

Vmax
(μ moles Pi min-1 mg-1 protein)
0
1
2
3
4
5
6
7
8
9
10
KCl (mM)
0
60
120
180
240
300
360
420
480
540
600
660
Ka (mg actin ml-1)-1
0
1
2
3

Figure 27

Diagrammatic representation of Mg-ATPase activities activation due to high ionic strength and removal of DTNB light chains at physiological ionic strength

A: chymotryptic S-1 (containing the DTNB light chains) of purified native myosin filaments.

B: the same S-1 as A above, but after dialysis against stock buffer for 5 hours or overnight.

C: S-1 (lacking in DTNB light chains), prepared from myosin filaments formed from solubilised native myosin filaments,

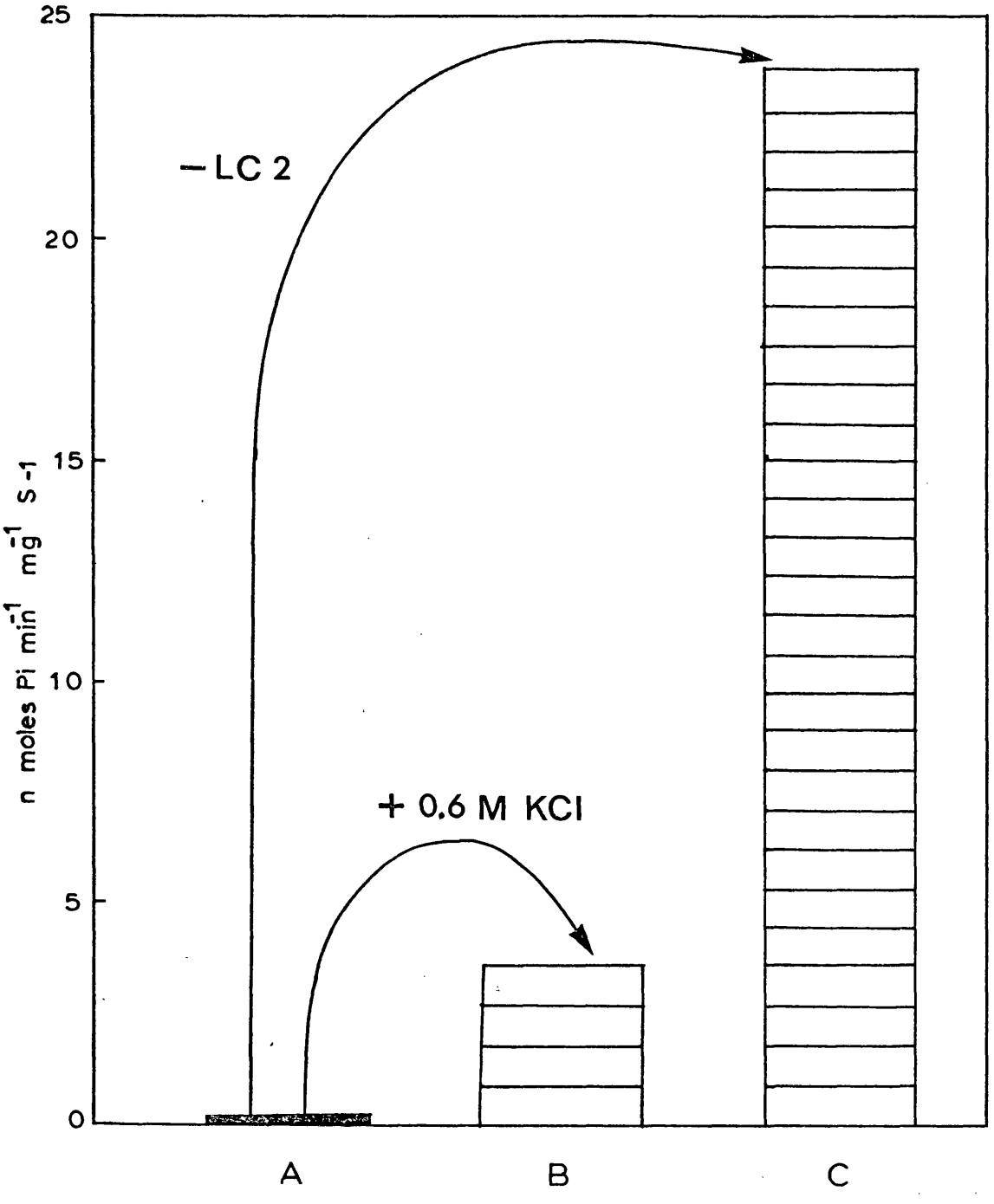

25
20
15
10
5
0
n moles Pi min-1 mg-1 S-1
– LC 2
+ 0.6 M KCl
A
B
C

Figure 28

Diagrammatic representation of S-1 showing relative movement or conformational change of DTNB light chain as a result of high ionic strength treatment or calcium-activation in vivo

In B, DTNB LC is susceptible to chymotryptic attack while in A, it is not.

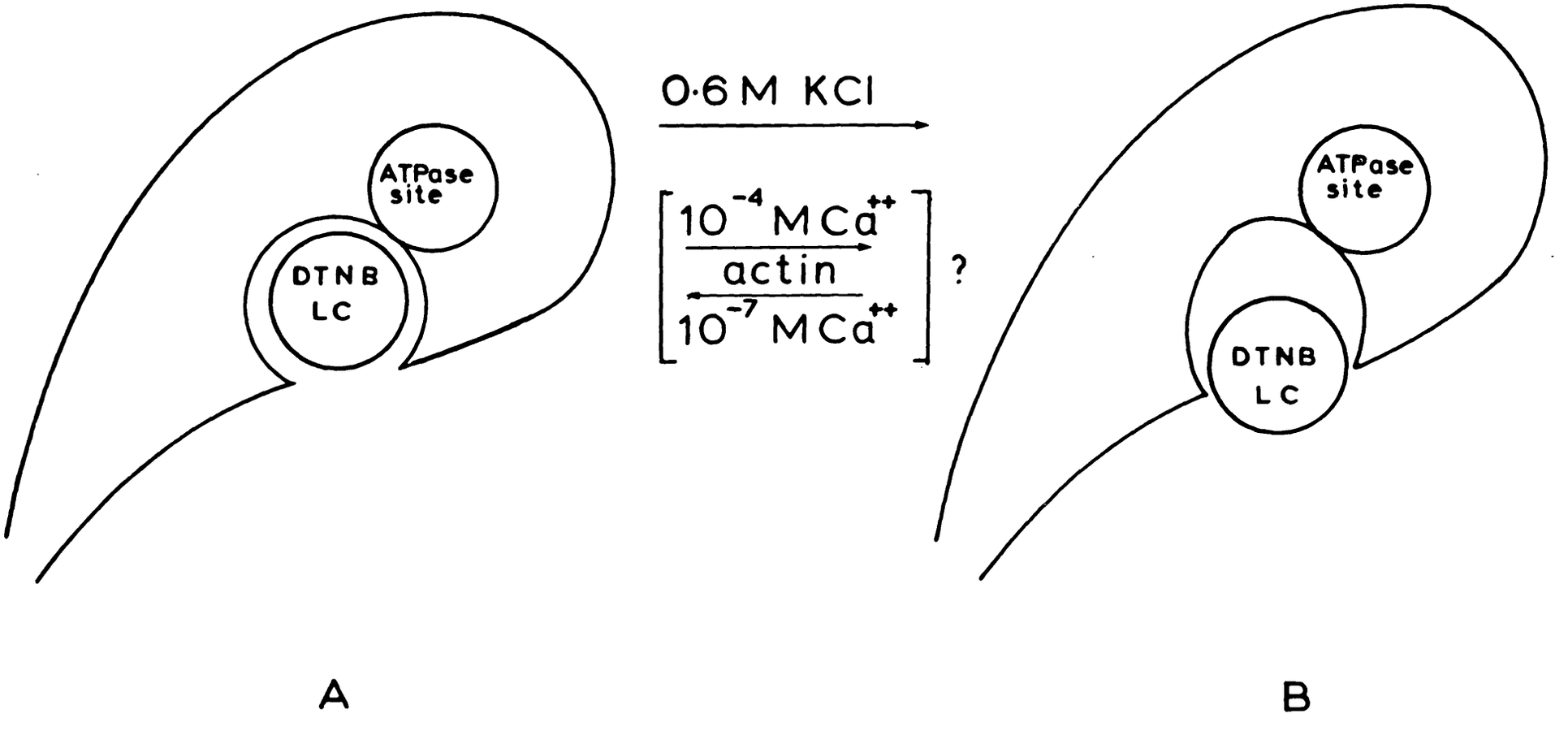

ATPase site
DTNB LC
0·6 M KCl
10^{-4} M Ca^{++}
actin
10^{-7} M Ca^{++}
?
ATPase site
DTNB LC
A
B

Table 4

ATPase activities of myosin with and without treatment with ^2H_2O

ATPase activities (n moles Pi per min per mg protein) were determined using the isotopic assay (III.2.3.1.). Standard errors of ATPase activities were estimated by applying standard statistical procedures to multiple estimates (normally 3-4) of each value, and were found to lie in the range ±0.5% to ±4.0%.

-: not treated with ^2H_2O.

+: treated with ^2H_2O.

ATPase activities

	Mg^{++}-ATPase		Ca^{++}-ATPase		K^{+} EDTA-ATPase	
mM KCl	-	+	-	+	-	+
90	14.5	15.0	500.7	501.4	50.1	51.2
150	10.2	10.4	390.5	392.0	120.4	122.3
660	4.3	4.2	140.2	139.0	1200.0	1210.0

Table 5

Mg-, Ca-, K-EDTA- and actin-activated Mg-ATPase activities of various preparations at specific ionic strengths

Values are estimated for various preparations of myosin and subfragment-1 (S-1) (cf. values of ATPase activities in Figures 13-26). Activities in columns "a" are expressed in n moles Pi per minute per mg enzyme and those in columns "b" are expressed in moles Pi per myosin ATPase site per second; assuming molecular weight values as shown below. The rows in the table (1-7) show data for:

1. CM: Conventional myosin (470,000 M.W.).

2. PNMF: Purified native myosin filaments (assuming 97% myosin).

3. MPNMF: Myosin from solubilised purified native myosin filaments, prepared as described in the legend to Figure 13.

4. CS-1: S-1 (lacking DTNB LC) from conventional synthetic myosin filaments (average M.W. 112,600; based on a 2:1 molar ratio of LC1 (21,000 M.W.):LC3 (17,000 M.W.); S-1 HC M.W. 93,000.

5. NS-1: S-1 (containing DTNB LC) from purified native myosin filaments (average M.W. 131,600; based on a 2:1 molar ratio of LC1:LC3; DTNB LC M.W. 19,000.

6. NS-1(I): Same S-1 as 5, but after exposure to high ionic strength.

7. CS-1(I): S-1 (lacking DTNB LC) from filaments formed from solubilised purified native myosin filaments at high ionic strength; M.W. same as 4.

Standard errors of ATPase activities were estimated (see also legend to Table 4) and found to lie in the range ±0.5% to ±5%, unless otherwise specified.

* : ±8%.

**: ±12%.

ATPase activities

Sample	Mg^{++}-ATPase (0.12 MKCl) a	b	Ca^{++}-ATPase (0.12 M KCl) a	b	K^{+} EDTA-ATPase (0.6 M KCl) a	b	Actin-activated Mg^{++}-ATPase (Vmax; 0.12 M KCl) a	b
1. CM	10.80	0.040	434.12	1.70	896.00	3.51	1810	7.09
2. PNMF	0.14	0.0006**	2.20	0.009*	0.74	0.003*	4.94	0.020
3. MPNMF	4.90	0.020	72.35	0.280	640.00	2.51	1512	5.92
4. CS-1	23.30	0.044	823.53	1.55	1320	2.48	923	1.73
5. NS-1	0.23	0.0005**	3.76	0.008*	11.10	0.024	53.65	0.12
6. NS-1(I)	2.40	0.005*	14.12	0.031	28.00	0.06	81.20	0.18
7. CS-1(I)	19.30	0.036	922.35	1.73	1708	3.21	1692	3.18

CHAPTER IV

CALCIUM-SENSITIVITY OF VERTEBRATE SKELETAL MYOSIN FILAMENTS ATPase

IV. CALCIUM-SENSITIVITY OF VERTEBRATE SKELETAL MYOSIN FILAMENTS ATPase

IV.1. Introduction

As described (see I.4.), there are two basic forms of regulatory controls which operate during muscular contraction in various animals, one being actin-linked and the other being myosin-linked. In the actin- (or thin filament-) linked regulatory system, the interaction of myosin with actin is prevented by the troponin-tropomyosin complex of the actin filament in the absence of calcium (10^{-7} M) and this inhibition is relieved by calcium (10^{-5}-10^{-4} M) which binds to troponin C of the troponin complex, so that muscular contraction can take place. In the myosin- (or thick filament-) linked control system, inhibition of actin and myosin interaction is mediated through a pair of light chains on the myosin molecule in the absence of calcium. Calcium relieves this inhibition by binding to a pair of light chains on the myosin molecule (EDTA or regulatory light chains in case of molluscan muscle).

Competitive actin- and myosin-activation assays (Lehman et al., 1972; Lehman and Szent-Gyorgyi, 1975; see I.4.) have shown that actin- and myosin-linked regulations are not restricted to vertebrate striated and molluscan

muscles, respectively. Studies of this sort have revealed that myosin and actin controls can operate simultaneously in most higher invertebrates (I.4.).

There is another form of myosin-linked control system which does not involve direct binding of calcium to the myosin molecule, but depends on phosphorylation of a pair of 20,000 M.W. light chains on the myosin molecule and this phosphorylation which is indirectly dependent on calcium, is the prerequisite for muscular contraction. This form of control system is found in vertebrate smooth muscle and non muscle cells (see I.4.), and is more complex than the systems which involve troponin C and myosin regulatory light chains as described above (see also I.4.). This complex phosphorylating system involves not only calmodulin, but also hormonal control of cyclic AMP (see I.4.). It has been reported that for chicken gizzard smooth muscle myosin, the serine residue 13 of the light chain near the N-terminal end is phosphorylated (Jakes et al., 1976). It is also worth noting that myosin phosphorylating systems are also found in skeletal and cardiac muscles though their role is uncertain (Perry, 1979; Adelstein et al., 1979; Stull et al., 1980). It has been shown that for rabbit skeletal muscle, serine residue 15 near the N-terminal end of the DTNB light chain is phosphorylated (Perrie et al., 1973).

In addition to the phosphorylation system, other forms of regulatory controls have also been proposed for smooth muscle contraction. For example, using guinea pig vas deferens myosin, Chacko et al (1977) have shown that actin-activated ATPase activity requires phosphorylation of myosin, catalysed by calcium-dependent kinase, but unlike other smooth muscle systems, the actin-activated ATPase activity of purified phosphorylated myosin is lowered in the absence of calcium, suggesting that in some smooth muscles, muscular contraction is regulated by both direct binding of calcium to myosin and phosphorylation of myosin light chains. Using bovine stomach, aorta, and chicken gizzard smooth muscle, the leiotonin system has been proposed to exist in smooth muscle contraction (Mikawa et al., 1978; Ebashi et al., 1978). These investigators have shown that in the absence of tropomyosin, leiotonin A (80,000 M.W.) and leiotonin C (18,000 M.W.), actin-activated myosin ATPase activity does not occur and more over, activation by leiotonin does not involve phosphorylation of myosin (Ebashi et al., 1978). Leiotonin C binds calcium, but is distinct from calmodulin. Marston et al (1980) have also suggested that there are proteins analogous to troponin which may act to confer calcium regulation on the actin-activated myosin ATPase activity in smooth muscle.

Myosin-linked regulation has been extensively studied

in molluscan muscle, in which the effect was first observed by Kendrick-Jones et al., (1970). This type of regulation has been shown to occur in a range of other muscles (e.g. sea cucumbers, nemertine worms, insects, decapod-crustaceans and annelids, Lehman et al., 1972; Lehman and Szent-Gyorgyi, 1975; Lehman, 1977; see also I.4), but detailed studies have yet to be performed. Like rabbit skeletal myosin, scallop myosin consists of two heavy chains and four light chains. Two of these light chains have been termed essential since they cannot be removed without irreversibly destroying the activity of the myosin and the other two light chains are termed regulatory (or EDTA) as they are involved in the regulation of muscular contraction. To date, calcium control of muscular contraction in scallop adductor muscle is solely a property of the thick filament which is in possession of the calcium switch on the myosin molecule (Lehman et al., 1972; Kendrick-Jones. et al., 1972, 1976; Szent-Gyorgyi, 1975; Simmons and Szent-Gyorgyi, 1978; Chantler and Szent-Gyorgyi, 1980). It is known that each scallop myosin molecule possesses two highly specific calcium-binding sites (Szent-Gyorgyi et al., 1973, Kendrick-Jones et al., 1976; Bagshaw and Kendrick-Jones, 1979; see also I.2.2. and I.4.) and these sites are lost upon complete removal of the regulatory light chains. Scallop myofibrils whose regulatory light chains have been removed are found to have two non-specific divalent cation binding sites of lower

affinity and these sites are absent in intact myofibrils (Chantler and Szent-Gyorgyi, 1980), suggesting that specific calcium binding sites only exist when the regulatory light chains are associated with the heavy chains (cf. the regulatory (or DTNB) light chains of rabbit skeletal myosin which are found to have a higher affinity for divalent cations when they are in association with the parent heavy chains; I.2.2.; note that DTNB light chains, at least as studied in conventional myosin, possess only non-specific divalent cation-binding sites).

Vertebrate skeletal muscle had generally been thought to possess only the actin-linked regulatory system for muscular contraction. Previous work, employing conventional purified myosin, using the competitive actin-binding assay (Lehman and Szent-Gyorgyi, 1975; Lehman, 1977) failed to detect calcium-dependent ATPase activity of the myosin at all ionic strengths studied. However, using rabbit skeletal myofibrils, Lehman (1977) was able to show that the ATPase activities of these myofibrils were calcium-sensitive when the assay was carried out near physiological ionic strength. Calcium-sensitivity was found to decrease markedly as the ionic strength of the assay medium was lowered. Using yet another preparation of myofibrils which had been extensively washed in low ionic strength buffers so as to dissociate the troponin-tropomyosin complex from the actin filaments, Lehman (1978) had again demonstrated

the presence of a myosin-linked calcium regulatory system in vertebrate skeletal muscle. This observation was also made at physiological ionic strength.

Although Lehman's results apparently show that the ATPase activities of vertebrate skeletal myofibrils both in the presence and in the absence of the troponin-tropomyosin complex can be calcium-sensitive (especially at physiological ionic strength) the inference of the existence of thick filament-linked calcium regulation in skeletal muscle has not been widely accepted; principally perhaps because of the well-established results using conventionally purified myosin, in which no calcium-sensitivity has been shown.

Evidence from the previous chapter shows that native myosin filaments and the derived chymotryptic S-1 which have not been exposed to high ionic strength possess the ability to maintain physiologically low Mg-ATPase activities. It has been concluded that DTNB light chains play a vital role in the "switching off" of the Mg-ATPase activity in the relaxed muscle. More over, when native myosin filaments are exposed to high ionic strength, the resulting myosin loses its distinctive properties and behaves like conventional myosin: that is, the potential of the DTNB light chains to repress ATPase activities appears to be lost.

Could it not be then that calcium-sensitivity of the actin-activated Mg-ATPase activity of filaments is also a property which exists, but which is lost on exposure to high ionic strength ? The availability of native myosin filaments in highly purified form provides a unique opportunity for investigating this issue, in a preparation free of the many components inevitably present in myofibrillar system as employed by Lehman. It was therefore decided to check for the presence of calcium-sensitivity of vertebrate skeletal myosin Mg-ATPase using purified native myosin filaments (II.2.7.) and pure (unregulated) actin (II.2.4.). In addition, the competitive actin binding assay can readily be applied to our relaxed filament preparations (II.2.6.), providing an alternative approach to this question.

IV.2. Experimental

IV.2.1. Preparation of proteins

Relaxed filament preparations containing dissociated thick and thin filaments in a Mg-ATPase relaxing medium were prepared from fresh rabbit psoas muscle as described previously (II.2.6.). Purified native myosin filaments were prepared from relaxed filament preparations using $^{2}H_2O$-H_2O gradients (II.2.7.). Conventional myosin was prepared from rabbit back and hind leg muscle by extraction of muscle mince using Guba-Straub solution as already described (II.2.1.).

IV.2.2. Methods of assays of ATPase activities and assay media

ATPase activities of purified native myosin filaments and conventional myosin were assayed using the isotopic ([γ-^{32}P] ATP) method (see III.2.3.1.) in the following medium: 0.7 mM ATP, 1 mM $MgCl_2$, 0.1 mM EGTA (or 0.1 mM EGTA + 0.2 mM $CaCl_2$), 20 mM Tris-HCl, pH 7.5, 0.05 mg per ml actin, 0.15 mg per ml purified native myosin filaments or conventional myosin, and the [KCl] was varied from 20 mM to about 600 mM, in a final volume of 0.1 ml assay solution.

ATPase assays of relaxed filaments preparation were carried out using the pH-stat method (III.2.3.2.) in the following medium: 0.5 mM ATP, 1 mM $MgCl_2$, 0.1 mM EGTA (or 0.1 mM EGTA + 0.2 mM $CaCl_2$), pH 7.5, the concentration of relaxed filaments was from 0.15-0.45 mg per ml (more protein was used when the ATPase activity was low, but ratio of pure actin to relaxed filaments was kept at 1:3), and the [KCl] was varied from 20 mM to 600 mM, in a final volume of 8 ml assay solution.

IV.2.3. Determination of calcium-sensitivity

Calcium-sensitivity was determined by comparing the specific ATPase activities (n moles Pi per minute per mg enzyme) in the presence of 0.1 mM EGTA containing 0.2 mM $CaCl_2$ and in the presence of 0.1 mM EGTA alone, by the following relationship (Lehman, 1977, 1978):

Calcium-sensitivity = 100(A-B)/A, where

A = ATPase in the presence of calcium,

B = ATPase in the absence of calcium.

IV.3. Results

IV.3.1. Conventional myosin and pure actin

Studies of the ATPase activities of conventional myosin in the presence of pure actin (II.2.4.) have indicated virtually the complete lack of calcium-sensitivity of conventional myosin (Table 6); confirming the known general observation (e.g. Lehman, 1977). This experiment was essential to make sure that the pure actin used was unregulated (i.e. containing no traces of troponin-tropomyosin complex) for further experiments involving purified native myosin filaments (IV.3.2.) and relaxed filament preparations (IV.3.3.). However, it seems that the highly sensitive isotopic assay used was able to show some residual calcium-sensitivity at physiological ionic strength (Table 6).

IV.3.2. Calcium-sensitivity of purified native myosin filaments

Using purified native myosin filaments and pure actin (checked to be unregulated; IV 3.1.), results (Table 7) show that calcium-sensitivity is observed at low ionic strengths and at physiological or near physiological ionic strengths, as compared to relaxed filaments which do not show calcium-sensitivity at low ionic strengths (IV.3.3.).

From the results, one can assume that at low ionic strength, in the presence of pure actin but in the absence of calcium, the "switch", DTNB light chain on the S-1, is on the "off" position so that the hydrolytic site (ATPase site) is not exposed (position A of Figure 28, Chapter III). However, this is switched on (Figure 28 B, Chapter III) as soon as calcium (10^{-5}-10^{-4} M) is introduced. Almost a 100% calcium-sensitivity is observed at physiological ionic strength (Table 7).

The ATPase activities increase as the ionic strength increases from physiological in the absence of calcium (Table 7). Once again, the fact that high ionic strength can turn on the ATPase "switch" of native myosin molecules is indicated (Chapter III) (note that native myosin filaments are exposed to high KCl concentration for a few minutes only during assay at high ionic strength).

ATPase activity at high ionic strength in the presence of calcium is markedly reduced (Table 7). This is also observed for relaxed filament preparations (Table 8) and conventional myosin (Table 6). This agrees with the finding that the actin-activated myosin ATPase activity is inhibited at high ionic strength in the presence of calcium (Oda et al., 1980).

IV.3.3. Relaxed filaments and pure actin

The competitive actin-binding ATPase assay (Lehman and Szent-Gyorgyi, 1975; see also I.4.) was employed in the present study. Pure actin (which was checked to be unregulated; IV.3.1.) was mixed with relaxed filaments containing dissociated myosin and actin filaments both in the presence and in the absence of calcium. If the muscle possesses only an actin regulatory system, full activation of myosin ATPase activity is observed when pure actin is added in the absence of calcium, since the myosin filaments are free to interact with the added pure actin. However, full activation is not observed in the absence of calcium if a myosin regulatory control operates simultaneously (see also I.4.).

The results (Table 8) show that relaxed filaments in the presence of pure actin have a complete lack of calcium-sensitivity at low ionic strength (20 mM KCl) while calcium-sensitivity is observed as the ionic strength increases to physiological (Table 8). These results support the view that the interaction of myosin and actin at low ionic strength increases tremendously so that calcium-sensitivity is over-ridden (Lehman, 1977, 1978). Table 8 also shows the calcium-sensitivity of filaments ATPase activities after exposure to high ionic strength for about two hours at physiological ionic strength assay

medium, though the sensitivity is slightly decreased as compared to filaments not being exposed to high ionic strength. This observation seems to suggest that actin-filaments have some sort of protective effect on myosin filaments against high ionic strength treatment, since conventional myosin (which has already "seen" high ionic strength media) has practically no detectable calcium-sensitivity (IV.3.1.).

In general, the ATPase activities are seen to decrease as the KCl concentration in the assay media is increased. It is also interesting to note that "full" calcium-sensitivity is observed at 600 mM KCl due to the complete lack of ATPase activity in the absence of calcium (Table 8).

IV.4. Discussion

IV.4.1. General comments and observations

Results from the present study strongly suggest the presence of a myosin-linked calcium regulatory system in vertebrate skeletal muscle. The methods of approach were similar to those of Lehman (1977, 1978), in the sense that the competitive actin-binding assay and pure (unregulated) actin were used. However, the use of fresh relaxed filament preparations and purified native myosin filaments have distinct advantages over the use of crude myofibrils or low ionic strength washed myofibrils (to remove the troponin-tropomyosin system): (i) the use of freshly prepared relaxed filaments avoids the necessity of solubilising myofibrils at high ionic strength and the pre-mixing with pure actin before ATPase assays are carried out to ensure that actin and myosin are able to interact, since relaxed filaments are in the form of dissociated myosin and actin filaments, (ii) the use of purified native myosin filaments (II.2.7.) does not involve exposure of filaments to non-physiological ionic environment and also avoids unnecessary manipulations of the sample such as successive days of homogenisation, sedimentation, resuspension and dialysis steps, as found in the preparation of washed myofibrils (Lehman, 1978).

The ATPase activities (expressed as moles Pi per myosin ATPase site per second) of purified native myosin filaments (Table 7) are considerably lower than those of the relaxed filament preparations (Table 8) when measured at low ionic strength and in the presence of calcium. (The ATPase activities of relaxed filament preparations (Table 8), both in the presence and in the absence of calcium and at low ionic strengths are comparable with those of Lehman (1977) using myofibrils at about the same low ionic strengths). This seems to indicate that fully regulated thin filaments (as in the case of relaxed filament preparations) are necessary to switch on more completely the ATPase activities of myosin filaments in the presence of calcium and at low ionic strengths. However, at physiological ionic strengths, the ATPase activities of relaxed filaments in the presence of calcium (Table 8) is at least three-fold less than that of myofibrils washed to remove tropomyosin and troponin (Lehman, 1978). This may reflect a difference between the DTNB LC conformation in the two preparations. However, it still remains to be explained why the ATPase of purified myosin filaments (Table 7) is about three-fold higher than that of the relaxed filaments (Table 8) in the presence of calcium and at physiological ionic strength when expressed as moles Pi per myosin ATPase site per second.

The most distinct difference between the results

obtained for relaxed filaments and purified filaments is that calcium-sensitivity in the latter case is also observed at low ionic strengths. These results show that pure actin cannot switch on the ATPase activity of purified myosin filaments in the absence of calcium (Table 7) at low ionic strengths. However, a high ATPase activity is observed for relaxed filaments (containing actin-filaments) in the presence of pure actin and in the absence of calcium at low ionic strength (Table 8). The calcium-insensitivity at low ionic strength in the case of relaxed filaments (Table 8) could be due to at least two factors: (i) the strong interaction of actin filaments and myosin filaments which are only well dissociated at physiological or greater ionic strengths in a Mg-ATP relaxing medium, that is, calcium-sensitivity is over-ridden, (ii) the fraying of myosin filaments into three sub-filaments at low ionic strength (Maw and Rowe, 1980) with the enhancement of the interaction between these sub-myosin filaments and actin filaments.

IV.4.2. Possible role of DTNB light chain for calcium-sensitivity

In addition to Lehman's observation (using myofibrils, both containing and lacking the troponin-tropomyosin system), the present study has once again demonstrated that native myosin filaments before

exposure to high ionic strength display calcium-sensitive ATPase activities, as compared to the conventionally prepared myosin which lacks calcium-sensitive ATPase activity at all ionic strengths tested (Table 6, 7, and 8; Lehman, 1978). This observation naturally favours the supposition that calcium-sensitivity of conventional myosin ATPase has been lost during the process of purification (Haselgrove, 1975; Lehman, 1977).

However, there has been no suggestion or indication of how or why calcium-sensitivity is abolished through preparation procedures. It is also uncertain on the question of the possible subunit(s) of myosin which might be responsible to confer calcium-sensitivity to native myosin filaments ATPase.

Alkali light chains are clearly involved in the ATPase sites of the myosin molecule, since their removal results in the concomitant loss of ATPase activity (Kominz et al., 1959; Gershman et al., 1966; Gershman and Dreizen, 1970; see also I.2.2.). Though the EDTA light chains of molluscan muscles have been firmly established to be the regulatory light chains (Simmons and Szent-Gyorgyi, 1978; Chantler and Szent-Gyorgyi, 1980), the role of the DTNB light chains (LC 2) of vertebrate skeletal muscle is still uncertain. Nevertheless, it has been suggested that DTNB light chains might play a role in the regulation of

muscular contraction (Werber, et al., 1972; Morimoto and Harrington, 1974; Haselgrove, 1975).

There are at least two reasons why one could envisage the DTNB light chains of vertebrate skeletal muscle playing a role in the regulation of muscular contraction: (i) each DTNB light chain of conventionally prepared myosin is known to possess a high calcium affinity site though it is non-specific (Bagshaw and Kendrick-Jones, 1979), (ii) evidence from the previous Chapter shows that DTNB light chain which has not been "damaged" by high ionic strength possesses the ability to inhibit Mg-ATPase activity at physiological ionic strength to a level similar to that found in resting muscle, as demonstrated by native myosin filaments and the derived chymotryptic S-1 containing the DTNB light chains (Chapter III). This low physiological Mg-ATPase activity can be activated not only by media of high ionic strength, but even more by the removal of DTNB light chains, as shown by chymotryptic S-1 of conventional myosin (Chapter III).

One can, therefore, assume that DTNB light chains of vertebrate skeletal muscle, before exposure to high ionic strength, do possess the potential to play a role in the "switching-off" of ATPase activity in resting muscle in the absence of calcium. This is achieved possibly by the DTNB light chain which sterically blocks the ATPase (hydrolytic)

site in the absence of calcium (Chapter III, Figure 28A). However, this potential of the DTNB light chain is easily abolished by high ionic strength media, so that native myosin filaments after exposure to high ionic strength (as is true for conventionally purified myosin) display no calcium-sensitivity in ATPase activities. That is, the DTNB light chains of myosin are irreversibly translocated or conformationally changed by high ionic strength treatment, so that the ATPase site is permanently exposed, resulting in calcium-insensitivity. (It is possible, however, that actin can have a protective effect on the DTNB light chains from being "damaged" by high ionic strength; IV.3.3.).

If calcium-specific binding sites are essential for regulation, one would predict the presence of these sites on native myosin, but these specific sites can easily be lost by experimental manipulations, especially by exposure to high ionic strength as compared to the specific calcium-binding sites on molluscan myosin which are still present after conventional purification procedures. On the other hand, calcium-specific binding sites may not be essential for regulation in vertebrate skeletal muscle for this myosin-linked control system may just act as a modulating device, once the actin-linked regulatory system is switched on (Lehman, 1978).

However, whether specific calcium binding sites are

essential or not, it may be possible that binding of calcium to the DTNB light chains of myosin is a prerequisite for activation of muscular contraction in living muscle. That is, only upon calcium-binding, can the ATPase (hydrolytic) site on the S-1 be exposed (Chapter III, Figure 28) and the subsequent hydrolysis of ATP for muscular contraction. Therefore, one may assume that before muscular contraction can occur, calcium (at activating concentration) must be bound to, (i) the DTNB light chain to expose the ATPase site, (ii) troponin C to allow the tropomyosin to shift close to the centre of the groove of actin filament, exposing the site on the actin filament for the binding of S-1.

When calcium concentration falls below that of activation, the ATPase site is sterically blocked by the DTNB light chain (Chapter III, Figure 28) and at the same time tropomyosin molecules rotate away from the centre of the actin filament groove and physically block the attachment of S-1 to actin. Evidence for the steric blocking model involving the troponin-tropomyosin system has come mainly from X-ray diffraction studies (Haselgrove, 1973; Huxley, 1973) and studies on actin paracrystals (Wakabayashi et al., 1975). Thesse studies have indicated that tropomyosin shifts its relative position on the actin filament upon calcium-binding to troponin.

The results from the present and the previous chapters, therefore, strongly suggest that DTNB light chains may play an important role in the regulation of muscular contraction in vertebrate skeletal muscle. However, the exact mechanism by which this control operates and how it is coupled to the actin-linked regulatory system, requires further experimental verification using relaxed filament preparations and purified native myosin filaments. These experiments may include phosphorylation and cation binding studies in relation to ATPase activities and calcium-sensitivity.

IV.4.3. Future experiments

In order to investigate further the role of DTNB light chains, future experiments involving purified native myosin filaments and the derived S-1 should include the following: (i) divalent metal ion binding experiments, (ii) phosphorylation experiments in relation to metal ion binding, ATPase activities and calcium-sensitivity.

IV.4.3.1. Divalent metal ion binding experiments

Work on the binding of divalent cations to DTNB light chains has shown that each DTNB light chain possesses a non-specific site for divalent cations (Bagshaw and Reed, 1977; Bagshaw and Kendrick-Jones, 1978) and that binding of

divalent cations to the light chains involves also the myosin heavy chains in order to form a firmer complex and therefore an enhanced affinity for divalent cations is observed when the heavy chains are associated with the light chains (Higuchi et al., 1978; Bagshaw and Kendrick-Jones, 1979; see also I.2.2.). However, these results have been obtained using conventional preparations after exposure to high ionic strength media. The rate of binding and dissociating of calcium or magnesium in vivo might be high enough to account for activation, but this is uncertain (Bagshaw and Reed, 1977).

It, therefore, seems essential to perform experiments involving divalent cations (especially calcium and magnesium) binding to purified native myosin filaments and the derived chymotryptic S-1 (containing the DTNB light chain), in order to elucidate the rate and binding constants of these ions, and to see if calcium-specific binding sites are present or not in these native preparations which have not been exposed to high ionic strength treatment.

IV.4.3.2. Phosphorylation experiments

Phosphorylation of DTNB light chains is shown to occur in vivo in skeletal (frog) (Barany and Barany, 1977) and cardiac muscle (Holroyde et al., 1979a), but further

experiments have yet to be performed to clarify its relation to metal ion binding, ATPase activities and also calcium-sensitivity. Since it is shown that DTNB light chain can be phosphorylated at the serine residue 15 near the N-terminal end of the light chain (Perrie et al., 1973), work on the relation of phosphorylation to magnesium and calcium binding of myosin has been performed, however, it is still a subject of controversy (Okamoto and Yagi, 1976; Alexis and Gratzer, 1976; Holroyde et al., 1979; Bagshaw and Kendrick-Jones, 1979; see also I.2.2.). This might be due to the variable degree of intact of preparations or as a result of experimental manipulations. Since purified native myosin filaments and the derived S-1 resemble more closely to living muscle, it is worth while exploring this subject further using these native preparations.

Using conventionally purified rabbit skeletal myosin, it has been shown that phosphorylation of the DTNB light chains increases the actin-activated Mg-ATPase activity of myosin in the presence of calcium (μM) and this is independent of the regulatory proteins, troponin and tropomyosin (Pemrick, 1980). However, further work concerning the role of phosphorylation in relation to ATPase activities and calcium-sensitivity should be performed (in the absence and in the presence of a range of calcium concentration), using purified native myosin

filaments and the derived S-1.

In summary, biochemical studies of $^{2}H_2O$-H_2O gradients-purified native skeletal myosin filaments and relaxed filament preparations demonstrate the likely existence of a myosin-linked calcium regulatory system in vertebrate skeletal muscle. The reason that previous studies employing conventional myosin failed to demonstrate this system is probably due to the fact that this myosin-linked regulatory system is operative only in intact native filaments as found in vivo and that the DTNB light chain regulatory ability is easily lost upon purification procedure, especially due to exposure to high ionic strength media. However, more experiments are needed to clarify the possible functional role of DTNB LC.

Table 6

Calcium-sensitivity of conventional myosin

ATPase activities were obtained by the isotopic method (IV.2.2.). Standard errors of ATPase activities were in the range ±0.5% to ±3.5% (see also legend to Table 4).

a: n moles Pi per minute per mg myosin.

b: moles Pi per myosin ATPase site per second; based on myosin M.W. 470,000.

ATPase activities

mM KCl	$-Ca^{++}$ a	$-Ca^{++}$ b	$+Ca^{++}$ a	$+Ca^{++}$ b	Ca^{++} - sensitivity
22	420	1.65	426	1.67	1.4
40	350	1.37	354	1.39	1.1
68	300	1.18	306	1.20	2.0
118	250	0.98	260	1.02	3.8
298	80	0.31	50	0.20	-60.0
598	20	0.08	12	0.05	-67.0

Table 7

Calcium-sensitivity of purified native myosin filaments

ATPase activities were determined by the isotopic method (IV.2.2.). Standard errors of ATPase activities were in the range ±0.5% to ±4.0% (see also legend to Table 4), unless otherwise specified.

*: ±0.05 n moles Pi per minute per mg filaments.

ATPase activities

mM KCl	$-Ca^{++}$ a	$-Ca^{++}$ b	$+Ca^{++}$ a	$+Ca^{++}$ b	Ca^{++} - sensitivity
22	0.0 *	0.0	74.7	0.30	100.0
40	0.0 *	0.0	86.6	0.35	100.0
68	0.0 *	0.0	114.6	0.46	100.0
118	4.3	0.04	125.2	0.51	96.0
298	15.1	0.06	200.4	0.81	92.5
598	40.8	0.16	24.7	0.10	-65.2

Table 8

Calcium-sensitivity of relaxed filament preparations

ATPase activities were obtained by the pH-stat assay (IV.2.2.), using freshly prepared relaxed filament preparations and relaxed filaments after exposure to high ionic strength (0.6 M KCl) for more than two hours by adding 2 M KCl solution. Standard errors of ATPase activities were in the range ±0.5% to ±4% (see also legend to Table 4), unless otherwise specified.

*: ±0.05 n moles Pi per minute per mg filaments.

a: n moles Pi per minute per mg relaxed filaments.

b: moles Pi per myosin ATPase site per second; based on myosin M.W. 470,000 and 60% of myosin in the preparations.

ATPase activities

Sample	mM KCl	$-Ca^{++}$ a	$-Ca^{++}$ b	$+Ca^{++}$ a	$+Ca^{++}$ b	Ca^{++} - sensitivity
Fresh	20	389.5	2.54	389.5	2.54	0.0
	80	70.1	0.46	215.1	1.40	67.4
	120	8.26	0.05	30.69	0.20	73.1
	600	0.0*	0.0	20.1	0.13	100.0
After exposure to high ionic strength	20	323.5	2.12	260.4	1.70	-24.2
	80	95.7	0.62	225.6	1.47	57.6
	120	10.52	0.07	34.19	0.22	69.2
	600	0.0*	0.0	25.25	0.16	100.0

CHAPTER V

STUDIES ON THE HETEROGENEITY OF MYOSIN HEAVY CHAINS

V. STUDIES ON THE HETEROGENEITY OF MYOSIN HEAVY CHAINS

V.1. Introduction

It is now firmly established that vertebrate skeletal myosin consists of two heavy chains and four light chains. The light chains can be classed into DTNB light chains (or LC2) and alkali light chains (LC1 and LC3) (see I.2.2. for a more detailed description). It is generally considered that there are two moles of DTNB light chains and two moles of alkali light chains per mole of myosin. However, unequal molar ratios between the two alkali light chains (1.5 to 2 moles of LC1 or A1 per mole of LC3 or A2) have been observed (Lowey and Risby, 1971; Sarkar, 1972; Weeds et al., 1975). This observation has stimulated research into the possible existence of myosin isoenzymes with respect to alkali light chains and has been taken to imply that there are at least two populations of myosin homodimers although the existence of a mixture of homodimers and heterodimers can not be excluded, that is, both of the two "heads" of myosin contain either LC1 or LC3 or one head of the myosin contains LC1 and the other head contains LC3 (each head is believed to contain one DTNB light chain).

Further evidence for the existence of myosin isoenzymes with respect to the alkali light chains has come from experiments of other workers. Based on the fact that

LC3 (A2) has a different actin affinity from that of LC1 (A1), it has been possible to obtain populations of myosin and HMM enriched in LC1 or LC3, by dissociation of rabbit skeletal myosin from F-actin by pyrophosphate and subsequent centrifugation (Alexis and Gratzer, 1976) and by chromotography of chymotryptic HMM of rabbit skeletal myosin on a column of Sepharose-bound glutaraldehyde cross-linked F-actin-tropomyosin complex (Trayer et al., 1977). (Note that Trayer et al have found that LC1 has a higher affinity than LC3 for actin while the opposite has been observed by Alexis and Gratzer). These observations have indicated the existence of homodimers of myosin with respect to the alkali light chains.

Further evidence that homodimers of myosin do exist comes from fractionation studies by an immunochemical approach (Holt and Lowey, 1977) and fractionation of HMM by an ADP affinity column (Wagner, 1977). Using antibodies specific for the "difference peptide" of LC1 (A1) of chicken breast muscle myosin, Holt and Lowey (1977) were able to fractionate not only rabbit skeletal myosin but also its chymotryptic and papain subfragments (HMM and S-1) into two populations, one enriched in LC1 (A1) and the other enriched in LC3 (A2). Using a Sepharose 4B adipic and hydrazide-ADP column, Wagner (1977) achieved the separation of chymotryptic HMM of both rabbit and chicken skeletal myosin into three fractions, one enriched in LC1, one

enriched in LC3 and another containing both LC1 and LC3, giving some indication the presence of both homodimers and heterodimers (myosin with one head containing LC1 and the other LC3).

The existence of a mixture of homodimers and heterodimers with respect to the alkali light chains is further demonstrated by the work of Hoh (1978) using fast-twitch pectoralis muscle of chicken and the use of adult rabbit fast-twitch longissimus dorsi muscle myosin (Hoh and Yeoh., 1979). Using pyrophosphate polyacrylamide (4%) gel electrophoresis (Hoh et al. 1976; Hoh, 1978), these workers have separated these native myosins into three components termed FM1, FM2, FM3, in the order of decreasing mobility. SDS gel electrophoresis of these components has revealed the following approximate light chain composition: FM3 contains only LC1 and DTNB LC (2:2), FM2 contains LC1, DTNB LC and LC3 (1:2:1), and FM1 contains only DTNB LC and LC3 (2:2). This clearly demonstrates the existence of heterodimers and homodimers with respect to the light chains in vertebrate skeletal myosin.

Whereas it is quite well established that isoenzymes of myosin with respect to alkali light chains do exist as homodimers and heterodimers in vertebrate skeletal muscle, the evidence for isoenzymes regarding myosin heavy chains is more tenuous. Evidence for the presence of two

chemically different myosin heavy chains has been presented in the form of amino acid substitutions in the N-terminal (head) chymotryptic peptides (Weeds, 1967; Starr and Offer, 1973; Pope et al., 1977) of rabbit skeletal myosin. The two different N-terminal peptides are (Starr and Offer, 1973):

N-acetyl-Ser-Ser-Asp-Ala-Asp-Met-Ala-(Ile, Phe),

N-acetyl-Ser-Ser-Asp-Ala-Asp-Met-Ala-(Val, Phe).

The ratio of the isoleucine-containing peptide to the valine-containig peptide is about 1.8:1. Based on this information and the ratio of alkali light chains, it has been suggested that there may be two populations of myosin, each having two identical alkali light chains and two heavy chains with the same N-terminal sequence (Starr and Offer, 1973). However, analysis of the amino acid compositions of S-1(A1) and S-1(A2) heavy chains shows the presence of both valine and isoleucine in S-1(A1) and S-1(A2) species. This demonstrates that there is no selectivity in the association of a particular heavy chain with an alkali light chain (Pope et al., 1977). Therefore, the picture of isoenzymes becomes much more complex when both the heavy and light chains are taken into account at the same time.

In the present study using rabbit psoas muscle , more evidence is presented to show that the two myosin heavy

chains can be differentiated during digestion by an unidentified proteolytic enzyme(s), presumably of pancreatic origin, found in a commercial preparation of DNAase I.

The present work has originated from an attempt to promote the yield of purified native myosin filaments by the use of DNAase I (see II.2.9.). In order to further the studies of the biochemical or biophysical properties of native myosin filaments and the derived chymotryptic S-1 (containing the DTNB LC), greater yield of purified native myosin filaments is essential. Experiments which may increase the yield of these preparations have been described (II.4.1.).

One of the reasons for the low yield (II.3.6.) of purified native myosin filaments (when using $^{2}H_2O$-H_2O gradients) is most likely to be the residual interaction between the myosin and actin filaments during their sedimentation down the gradients, and as a result only one of the eight fractions from each gradient is used for the production of S-1 (II.3.6.). If the thin filaments in the relaxed filament preparations are depolymerised prior to gradient fractionation, a considerably higher yield of native myosin filaments should be obtained, since the depolymerised thin filaments, in the form of actin monomers will be retained at the top of the gradient. DNAase I which

is known to depolymerise F-actin (see II.2.9.) was incubated with relaxed filaments and electron microscopic studies showed that all the thin filaments were depolymerised after about 5 hours incubation at 0° C (II.2.9.).

However, SDS gel electrophoresis showed that one of the myosin heavy chains was still preferentially attacked by an unidentified enzyme(s) in the DNAase I preparation which had been PMSF treated (II.2.9.). It was therefore decided to investigate further the difference between the two myosin heavy chains using DNAase I before and after PMSF treatment.

V.2. Experimental

V.2.1. Preparation of relaxed filaments

Relaxed filaments containing dissociated thick myosin and thin actin filaments in a relaxing medium were prepared according to the previously described procedure (II.2.6.).

V.2.2. Incubation of relaxed filaments with DNAase I not treated with PMSF

Relaxed filaments (7-8 mg per ml) were incubated for about 5 or 21 hours with DNAase I (5 mg per ml) which had not been treated with PMSF. After incubation, the sample was analysed by SDS gels (II.2.11.1.).

Relaxed filaments (7-8 mg per ml) after dialysed overnight to I=0.6 was also incubated as above.

V.2.3. Incubation of relaxed filaments with PMSF-treated DNAase I

Prior to incubation with relaxed filaments, DNAase I was treated with 100 mM PMSF in ethyl alcohol and relaxed filaments (7-8 mg per ml) were then incubated with the PMSF-treated DNAase I for 20 hours with concentrations of DNAase I varied from 0.1-5 mg per ml and containing about

0.5 mM PMSF in the final incubation media. The samples were analysed by SDS gels (II.2.11.1.) after incubation (plate 9).

V.2.4. Kinetic studies on the attack of myosin heavy chains by the unidentified enzyme(s) of DNAase I preparation

Use of the unidentified enzyme(s) in the commercial preparation of DNAase I was further made to investigate the heterogeneity of myosin heavy chains of the native myosin filaments. Relaxed filaments (7-8 mg per ml) prepared as usual (V.2.1.) were incubated with PMSF-treated (V.2.3.) DNAase I with concentrations of 1 mg per ml DNAase I and 0.5 mM PMSF in the final incubation medium. At specified time intervals (5-530 minutes) (Plate 10), 0.1 ml sample was removed and the reaction was terminated by the addition of sufficient SDS and boiled for several minutes. The SDS-treated and boiled samples were analysed by SDS gel electrophoresis (II.2.11.1.). The molar ratios of myosin heavy chains (200,000 M.W.) and HMM heavy chains (132,000 M.W.) were quantitated (II.2.12.) and the mole fraction of HMM heavy chains was plotted against time of incubation (Figure 30).

Exactly the same procedure as above was also followed for relaxed filaments whose ionic strength had been raised to about 0.6 by dialysis against stock buffer overnight.

Therefore, incubation with PMSF-treated DNAase I was essentially carried out when myosin was in true solution.

V.2.5. Ultracentrifugal studies of myosin from thick filaments after incubation with PMSF-treated DNAase I

Relaxed filaments (7-8 mg per ml) after incubation with PMSF-treated DNAase I (1 mg per ml) for 20 hours (V.2.3.) were made 0.6 M in KCl and 10 mM in Mg-ATP, pH 7.0, before analysed by an MSE MK II Analytical Ultracentrifuge (II.2.14.). Photographs were taken of the schlieren optical pattern at 501 second intervals (see Plate 12 and Plate 9, wells 4 and 5). The same sample was also examined by an MSE Centriscan (Figure 31). Essentially, myosin (in true solution) was sedimented in the presence of thin (I-) filaments and some I-segments which, however, were pelleted rapidly before the first schlieren peak of myosin was observed.

The same sample above (but incubation had been prolonged to 36 hr) after being made 0.6 M in KCl and 10 mM in Mg-ATP, was centrifuged at 50,000 r.p.m. for 2 hours to get rid of the I-filaments and I-segments. The supernatant (containing attacked myosin heavy chains) was then dialysed to I=0.03 against diluted stock buffer. The precipitated attacked myosin was recovered by using a micro-centrifuge and dissolved in stock buffer. This sample of myosin (1 mg

per ml) was again analysed by an MSE MK II Analytical Ultracentrifuge (II.2.14.). Photographs were taken of the schlieren optical pattern at 500 second intervals (see Plate 13). This sample was then analysed by SDS gel electrophoresis (Plate 10, well 11).

V.3. Results

V.3.1. Effect of proteolytic enzyme(s) in DNAase I on myosin heavy chains

When relaxed filaments before exposure to high ionic strength were incubated with DNAase I not treated with PMSF, it was found that all the myosin heavy chains were attacked at the HMM junction, as shown by SDS gels (Plate 8, well 3). The heavy chains were only "nicked"; free heavy meromyosin (HMM) and light meromyosin (LMM) not being produced as demonstrated by ultracentrifugation (V.3.4.). Practically the same result was obtained when relaxed filaments were dialysed to high ionic strength prior to incubation with DNAase I (Plate 9, well 9). These results showed that myosin in the filamentous or monomeric form behaved in the same manner when subjected to the enzymatic attack due to the presence of unidentified enzyme(s) in the commercial DNAase I preparation.

V.3.2. Partial attack of myosin heavy chains

When relaxed filaments were incubated with PMSF-treated DNAase I, partial attack of myosin heavy chains was observed. The concentration of DNAase I (therefore the concentration of unidentified enzyme(s)) was varied to see the effect on the nicking of the myosin heavy

chains. Plate 9 (wells 2-8) shows that the myosin heavy chains were attacked less rapidly as the concentration of DNAase I was decreased from 5-0.1 mg per ml in the incubation medium (I=0.12).

Relaxed filaments whose ionic strength had been raised to 0.6 were also incubated with PMSF-treated DNAase I and partial attack of myosin heavy chains was also observed (Plate 9, wells 10 and 11). Therefore, filamentous myosin and myosin in true solution behaved in the same manner when incubated with DNAase I not treated with PMSF (V.3.1.) or when incubated with PMSF-treated DNAase I (in the former case, all the myosin heavy chains were attacked while in the latter case partial attack was observed).

V.3.3. Preferential digestion of part of the heavy chain complement of myosin

In order to investigate the kinetic behaviour of the digestion of the myosin heavy chains by the unknown enzyme(s), the time of incubation of relaxed filaments (I=0.12) with PMSF-treated DNAase I was varied from 5-530 minutes (V.2.4.). It can be seen from Plate 10 (wells 1-10) that the intensity of the myosin heavy chain band decreases while that of the HMM heavy chain and LMM bands increases as the time of incubation increases.

In order to quantitate the molar ratios of the myosin heavy chains and HMM heavy chains at each time of incubation, the loadings on the gels were reduced to give linear scaning response (II.2.12.) (Plate 11, wells 3-12). When the mole fraction of HMM heavy chain was plotted against time of incubation a biphasic action of the enzyme(s) was observed, an initial very rapid reaction being followed by a much slower, second phase of digestion. When this slower second phase was back-extrapolated to zero time, about half of the myosin heavy chains was found to be preferentially attacked initially (Figure 30, 0.12 I).

When exactly the same procedure was carried out for relaxed filaments after exposure to high ionic strength (V.2.4.), a similar set of results was obtained (Figure 30, 0.6 I) as for relaxed filaments not being exposed to high ionic strength (Figure 30, 0.12 I).

These results, therefore, demonstrated that about half of the myosin heavy chains were preferentially attacked initially, irrespective of whether the myosin was in the filamentous or monomeric form. However, more myosin heavy chains were attacked (but only very slowly) as the time of incubation was increased, implying that there are at least two populations of myosin heavy chains, one of these is preferentially attacked while the other is not, since the similarity of the digestion curve in true

solution to that observed when myosin is in the filamentous form would seem to preclude the biphasic response arising from differential availability of the two heavy chains in the fully assembled filamentous form.

V.3.4. The myosin heavy chains were only "nicked"

In order to investigate whether the myosin was only "nicked" and as an attempt to check for the presence of homodimers and/or heterodimers (see Figure 29), ultracentrifugal studies were performed (V.2.5.). If the myosin is not held together by non-covalent bonding after enzymatic attack at the HMM-LMM junction (in the case of the dimer H1.H1), then ultracentrifugation would reveal three peaks (with HMM sedimenting first, followed by myosin and and LMM) when about 50% of the myosin is attacked. If three peaks are revealed, then it is certain that the homodimer, H1.H1, does exist, (giving rise to the HMM and LMM peaks), but it would be uncertain whether the myosin peak is due to the other dimer, H2.H2, or the presence of the heterodimer, H1.H2 or a mixture of the latter two.

When the ionic strength of the relaxed filaments after incubation with PMSF-treated DNAase I was raised to 0.6 and the sample was examined by ultracentrifugation (V.2.5.), only one component was observed (Plate 12). (Note that the thin (I-) filaments and I-segments were pelleted

before the first schlieren peak was taken). The $s_{20,w}$ value obtained for this single, homogeneous component, was $4.33 \pm 0.025 \times 10^{-13}$, a value typical for intact myosin at this level of concentration (Emes and Rowe, 1978), indicating that although the myosin was attacked at the HMM-LMM junction, it was only nicked and the myosin sedimented as a single physical component. The fact that only one peak was observed strongly indicates the existence of the heterodimer, H1.H2, in which the H1 chain was attacked preferentially, initially. However, prolonged incubation with DNAase I (see below and V.4.1) when more than 50% of the myosin heavy chains was attacked, ultracentrifugation still revealed only one peak, indicates that homodimers, H1.H1 and H2.H2 might also be present. This shows that although the two heavy chains of the myosin might be nicked, they are still held together as a physical unit. SDS gel electrophoresis showed the presence of myosin and HMM heavy chains and also light meromyosin (LMM) (Plate 9, wells 4 and 5), in addition to the light chains.

When the myosin in the native filamentous form was solubilised at high ionic strength, precipitated, and redissolved in stock buffer (V.2.5.), again a homogeneous single component was observed (Plate 13), however, the $s_{20,w}$ value was $8.02 \pm 0.13 \times 10^{-13}$ (about $9\text{-}10 \times 10^{-13}$ when extrapolated to zero concentration) (see V.4.1. for possible explanation).

V.4. Discussion

V.4.1. What can we infer from an $s_{20,w}$ value of about 8 x 10^{-13} ?

Ultracentrifugal analysis of the myosin resulting from a prolonged (36 hr) incubation of relaxed filaments with DNAase I (V.2.5.), showed the presence of a single homogeneous component (V.3.4.; Plate 13). The $s_{20,w}$ value of this sample was found to be 8.02±0.13 x 10^{-13} at a concentration of about 1 mg per ml. There might be several possibilities what this sample might be: (i) HMM, (ii) myosin dimer and (iii) conformer of native myosin.

There are at least two reasons why this homogeneous single component is not likely to be an HMM: (i) an $S_{20,w}$ value of about 8 x 10^{-13} is too high for HMM at such a concentration, and (ii) the absence of LMM as indicated by the schlieren peak pattern of the sample.

The possibility that this component is a myosin dimer is rather high, since such an $s_{20,w}$ value is reconcilable with that of the dimer at such a concentration (Johnson and Rowe, 1961; Emes and Rowe, 1978). However, it is worth noting that no one has ever produced such a "clean" dimer preparation.

Finally, it is possible that this homogeneous component may be a conformer of native myosin. Although a change in $s_{20,w}$ from about 6 S (Emes and Rowe, 1978) to 9-10 S is very large, myosin has previously been shown to be susceptible to rather large changes in this parameter (Johnson and Rowe, 1961; Emes and Rowe, 1978). The appropriate value for a compact sphere would be about 18 S: a 9-10 S myosin would be a very assymmetrical particle. Therefore, it seems provisionally that this increase in $s_{20,w}$ value might probably reflect the possibility that the nicked myosin after dissolution at high ionic strength, prolonged incubation and other further manipulations (V.2.5.), has indeed adopted a conformation different from that of the native myosin. Since this myosin is nicked, it is also probable that the increase in $s_{20,w}$ value might be due to an increase in flexibility at the HMM-LMM junction of the myosin molecule.

V.4.2. Possible myosin isoenzymes of vertebrate skeletal muscle

Using the PMSF-treated unidentified enzyme(s) in the commercial preparation of DNAase I, the present study has demonstrated the likely presence of two different myosin heavy chains in the relaxed filament preparations of rabbit skeletal psoas muscle, as judged by their susceptibility to enzymatic attack. About half (V.3.3.) of the myosin heavy

chains is preferentially attacked (nicked) to form heavy meromyosin (HMM) and light meromyosin (LMM) initially, while the rest of the heavy chains is also attacked, but only very slowly as the time of incubation with PMSF-treated DNAase I increases (V.3.3.). This observation is true whether the myosin is in the filamentous form (I=0.12) or in the monomeric form (I=0.6) (Figure 30), indicating that the preferential attack of about half of the heavy chains initially, does not depend on the physical state of the myosin.

Results from ultracentrifugation (V.3.4.) have shown that although the heavy chains are nicked, the myosin is still held together by non-covalent bonding as one physical component during centrifugation, otherwise, more than one schlieren peak would be observed. SDS gel electrophoresis reveals the presence of myosin heavy chains, HMM heavy chains, light meromyosin and the alkali and DTNB light chains (Plates 8-11).

If one accepts the presence of two different myosin heavy chains which are in a 1:1 ratio, then the possible isoenzymes with respect to the heavy chains would be the homodimers, H1.H1 and H2.H2 in about equal proportion, or the heterodimer H1.H2 or a mixture of the two homodimers and the heterodimer in a 1:2:1 ratio (H1 represents the myosin heavy chain preferentially attacked while H2

represents that which is attacked only slowly). Our result of a 1:1 ratio of H1:H2 is irreconcilable with the 1.8:1 ratio of the isoleucine-containing peptide to the valine-containing peptide of the two myosin heavy chains (Starr and Offer, 1973; V.1.). The ratio 1.8:1, however, has been obtained from only the two relatively small N-terminal peptides of the myosin heavy chains. Moreover, it is not known which myosin heavy chain (H1 or H2) contains which N-terminal peptide (the isoleucine-containing or the valine-containing peptide). Further experiments would be necessary to clarify this. For example, preparative SDS-polyacrylamide gels could be used to prepare more H2 which is then isolated for the analysis of amino acid composition in the N-terminal chymotryptic peptide (Starr and Offer, 1973).

Nevertheless, if one accepts the following information: (i) the existence of a mixture of homodimers and heterodimers (A1.A1, A2.A2 and A1.A2; A1 represents alkali light chain 1 and A2 represents alkali light chain 2) with respect to the alkali light chains, (ii) the presence of two different myosin heavy chains (H1 and H2), and (iii) assuming the absence of selectivity in the association of an alkali light chain with a particular heavy chain (Pope et al., 1977; V.1.), then, the possible isoenzymes of vertebrate skeletal myosin would be those shown in Table 9.

Although it is quite clear that the differences in the actin-activated ATPase activities between S-1(A1) and S-1(A2), S-1(A1) being the lower, are due to the presence of a particular alkali light chain (Weeds and Taylor, 1975; Wagner and Weeds, 1977), further experiments would be necessary to elucidate the role of isoenzymes.

In conclusion, more evidence is presented in the present study to support a difference between the two myosin heavy chains, however, more work is yet needed to be done to clarify the functional role of myosin isoenzymes with respect to both the heavy chains and the alkali light chains.

Figure 29

Diagrammatic representation of the possible isoenzymes of myosin with respect to the heavy chains

H1: myosin heavy chain preferentially attacked by enzyme(s) in DNAase I.

H2: myosin heavy chain which is attacked only very slowly after prolonged incubation with DNAase I (V.3.3.); however, although both heavy chains are attacked, they are still held together as a physical unit (V.3.4.), due to non-covalent bonding and/or a slight difference between the two points of attack of the two myosin heavy chains.

Arrows indicate the sites of attack at the HMM-LMM junction.

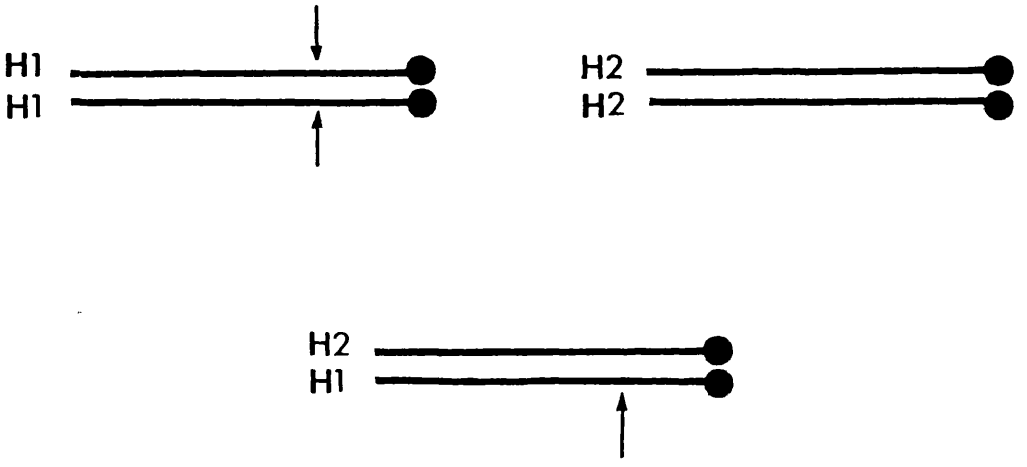

H1
H1
H2
H2
H2
H1

Figure 30

Kinetic analysis of digestion of myosin heavy chains by the enzyme(s) in DNAase I preparation

0.12 I: digestion pattern obtained at physiological ionic strength.

0.6 I: digestion pattern obtained at ionic strength = 0.6.

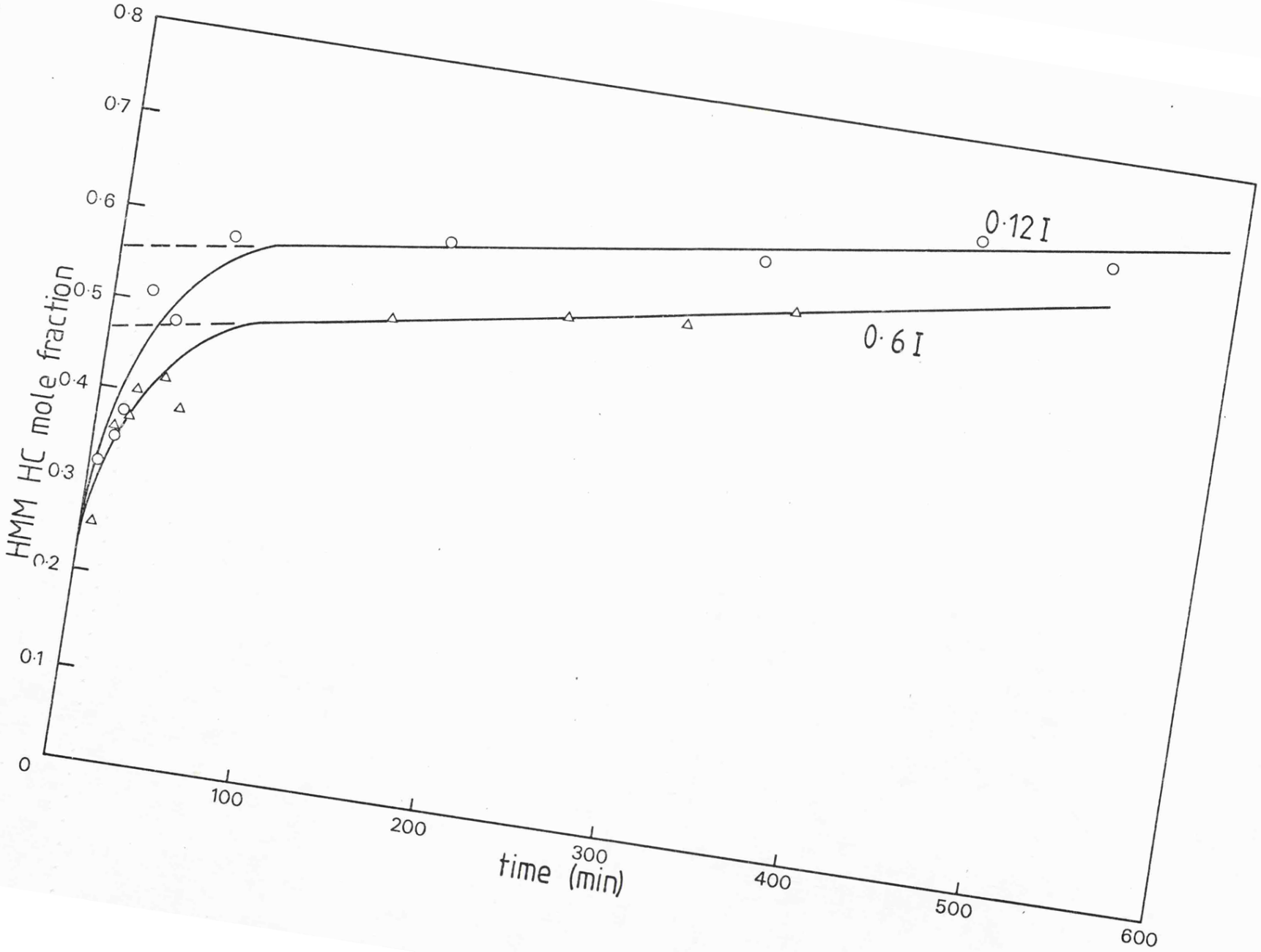

0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
HMM HC mole fraction
0·12 I
0·6 I
100
200
300
400
500
600
time (min)

Figure 31

Sedimentation diagram of nicked myosin before removal of thin filament components, obtained by refractometric scanning optics in the MSE Centriscan 75.

Sedimentation is from left to right.

Speed: 50,000 r.p.m.

Temperature: 7.0° C.

Solvent and protein concentration: same as Plate 12.

Large arrow: boundary.

Small arrow: meniscus.

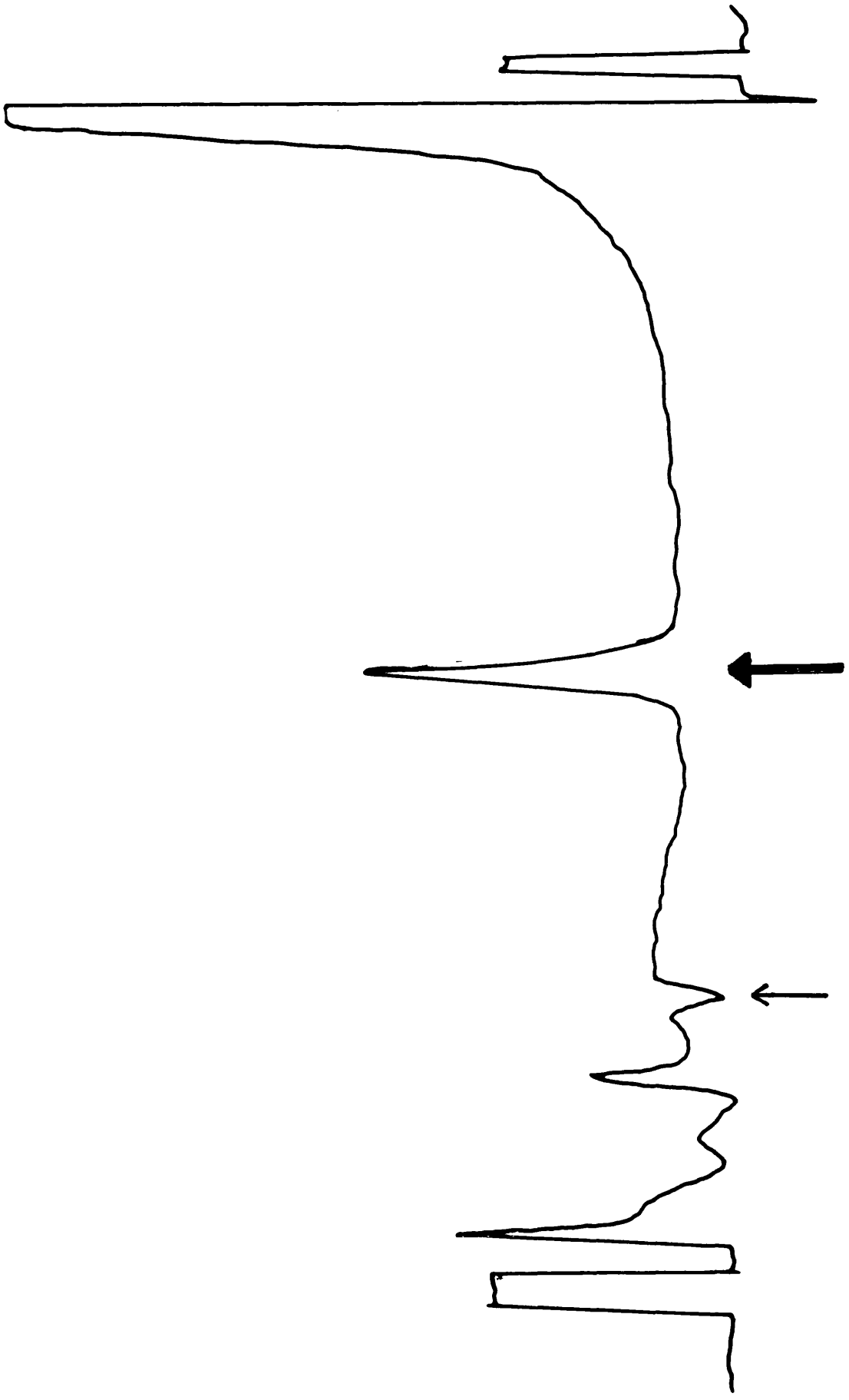


Plate 8

SDS gel (14%) electrophoresis showing both the myosin heavy chains being attacked by the enzyme(s) in DNAase I

Wells 1, 8, 9 and 10: relaxed filaments (35 μg per well).

Wells 2 and 11: DNAase I (45 μg per well).

Well 3: relaxed filaments not exposed to high ionic strength, incubated for 21 hours with DNAase I (5 mg per ml) not treated with PMSF (80 μg).

Well 4: relaxed filaments not exposed to high ionic strength, incubated for 24 hours with PMSF-treated DNAase I (5 mg per ml) (80 μg).

Wells 5, 6, 7, and 12: same as well 4 but incubated for 6 hours.

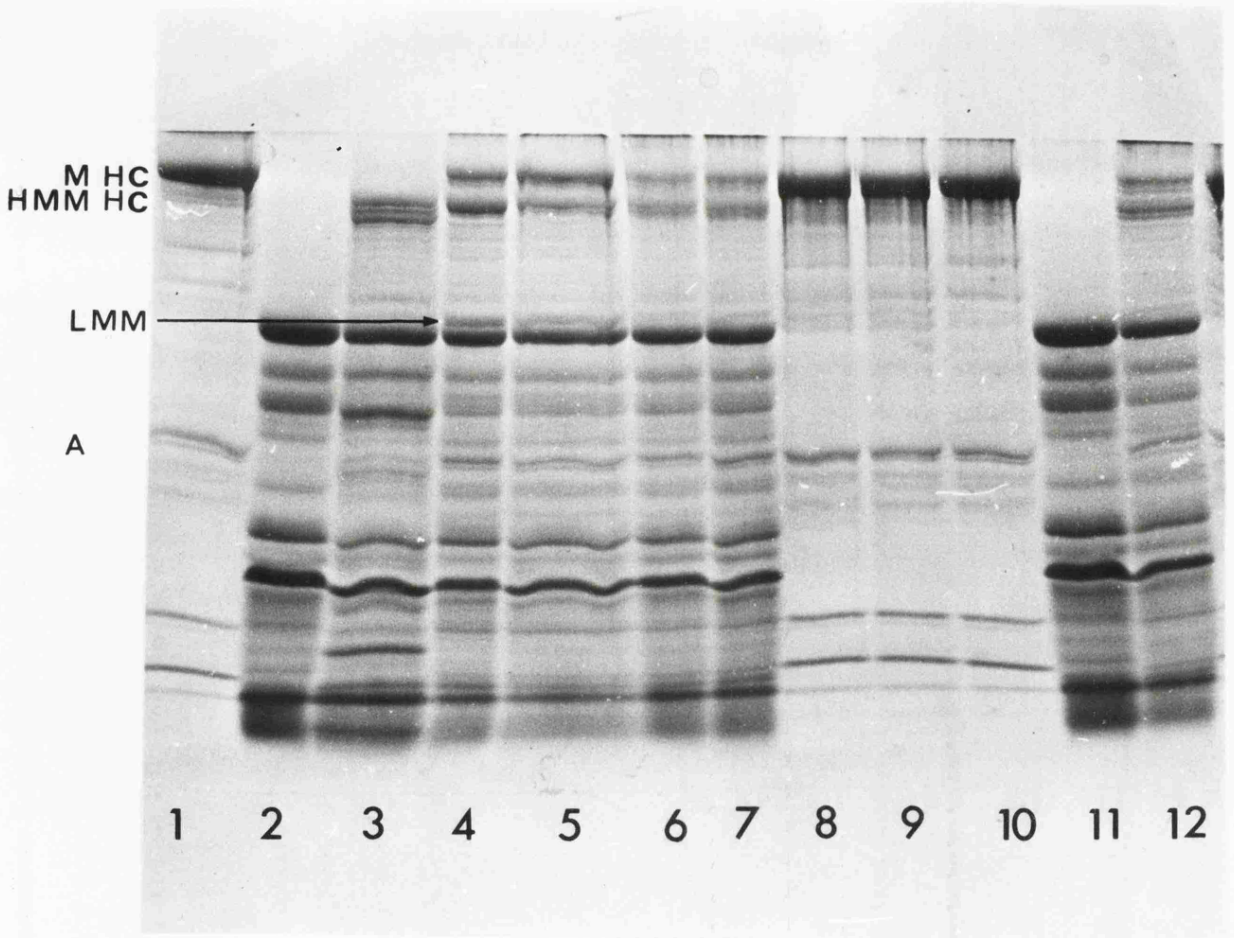

M HC
HMM HC
LMM
A
1
2
3
4
5
6
7
8
9
10
11
12

Plate 9

Effect of DNAase I concentration on the degree of attack of myosin heavy chains (14% SDS gel)

Well 1: DNAase I (45 μg).

Well 2: relaxed filaments not exposed to high ionic strength, incubated for 20 hr with PMSF-treated DNAase I (5 mg per ml) (80 μg).

Well 3: same as well 2, but with 3 mg DNAase I per ml.

Wells 4 and 5: same as well 2, but with 1 mg DNAase I per ml. This sample was used to obtain Plate 12.

Wells 6 and 7: same as well 2, but with 0.5 mg DNAase I per ml.

Well 8: same as well 2, but with 0.1 mg DNAase I per ml.

Well 9: relaxed filaments after exposure to high ionic strength, incubated for 20 hr with DNAase I (5 mg per ml) not treated with PMSF (90 μg).

Wells 10 and 11: same as well 9, but with PMSF-treated DNAase I.

Wells 12 and 13: relaxed filaments (75 μg per well).

Note the presence of LMM which is barely separated from DNAase I as the DNAase I concentration decreases, but this separation is well shown in Plate 10.

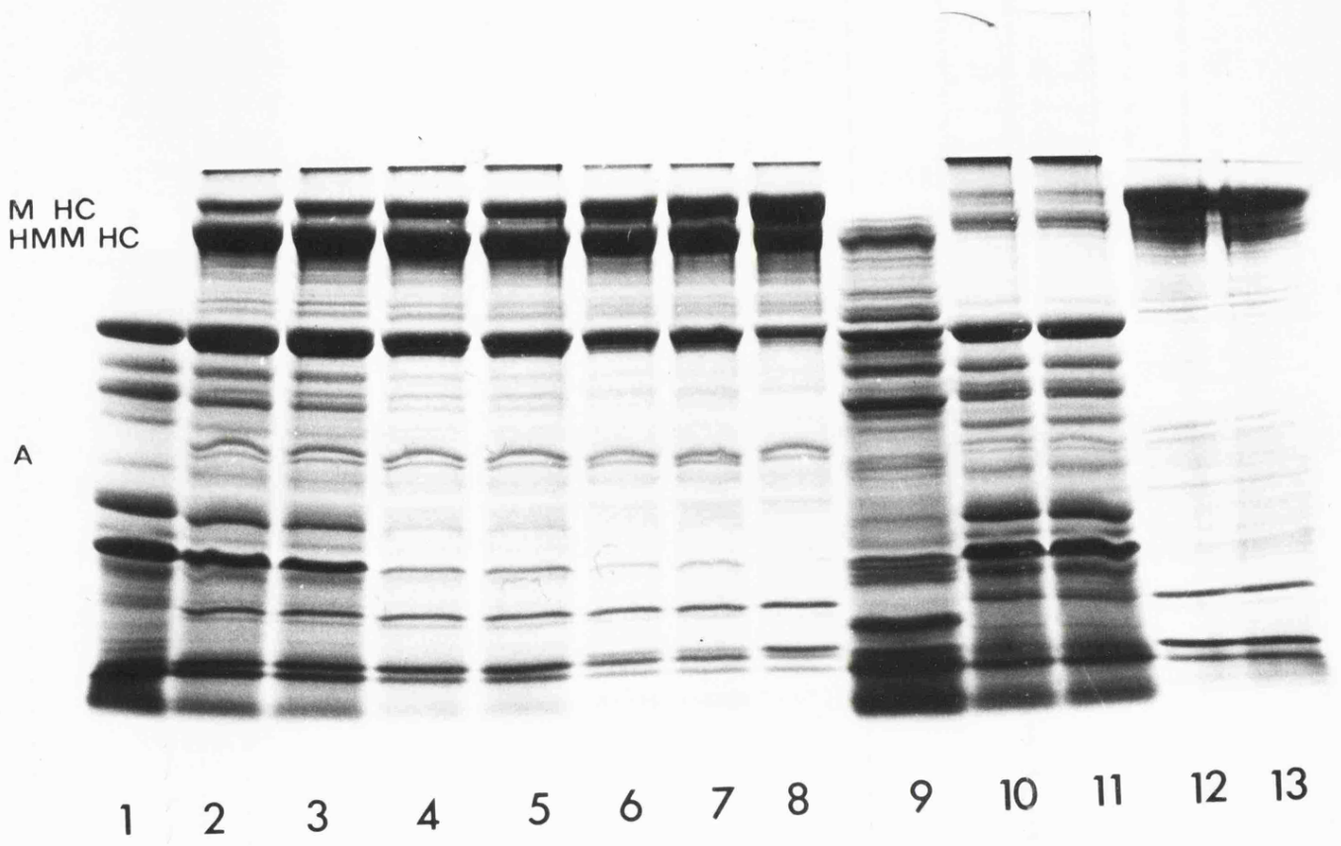

M HC
HMM HC
A
1
2
3
4
5
6
7
8
9
10
11
12
13

Plate 10

Degree of attack of myosin heavy chains as a function of time of incubation with DNAase I (14% SDS gel)

Wells 1-10: relaxed filaments not exposed to high ionic strength, incubated with PMSF-treated DNAase I (1 mg per ml) (80 µg per well); time of incubation for wells 1 to 10 was 5, 10, 15, 20, 35, 60, 175, 345, 460, 530 minutes, respectively (see V.2.4.).

Well 11: relaxed filaments, after incubation with PMSF-treated DNAase I (1 mg per ml) for 36 hours, and thin (I-) filaments and I-segments had been removed by centrifugation; note the presence of mainly HMM HC and LMM (60 µg) (see V.2.5.)

Well 12: same as well 11, but before removal of thin (I-) filaments and I-segments (120 µg).

Well 13: DNAase I (45 µg).

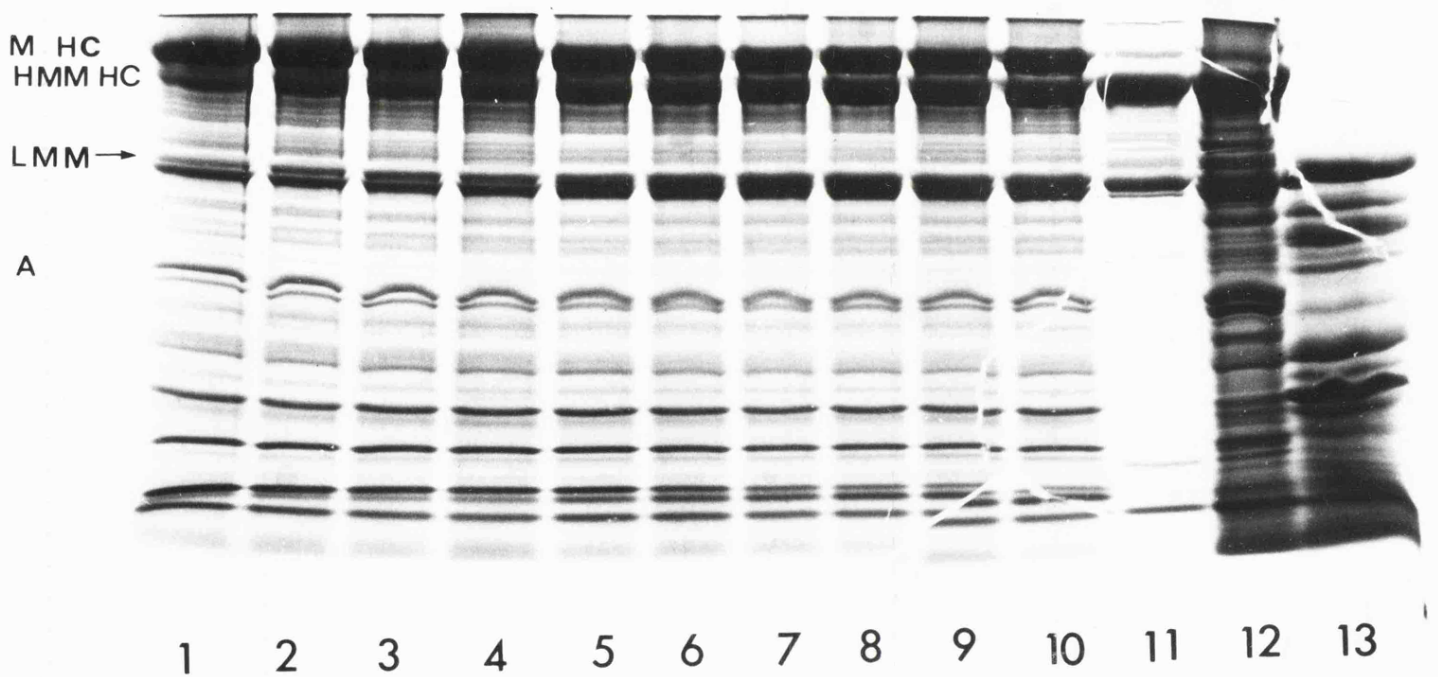

M HC
HMM HC
LMM
A
1
2
3
4
5
6
7
8
9
10
11
12
13

Plate 11

Same as Plate 10 except the loadings were reduced for scanning (14% SDS gel)

Well 1: relaxed filaments (80 μg).

Well 2: DNAase I (45 μg).

Well 3-12: same as Plate 10 wells 1-10 except the loadings were reduced for scanning (20 μg per well).

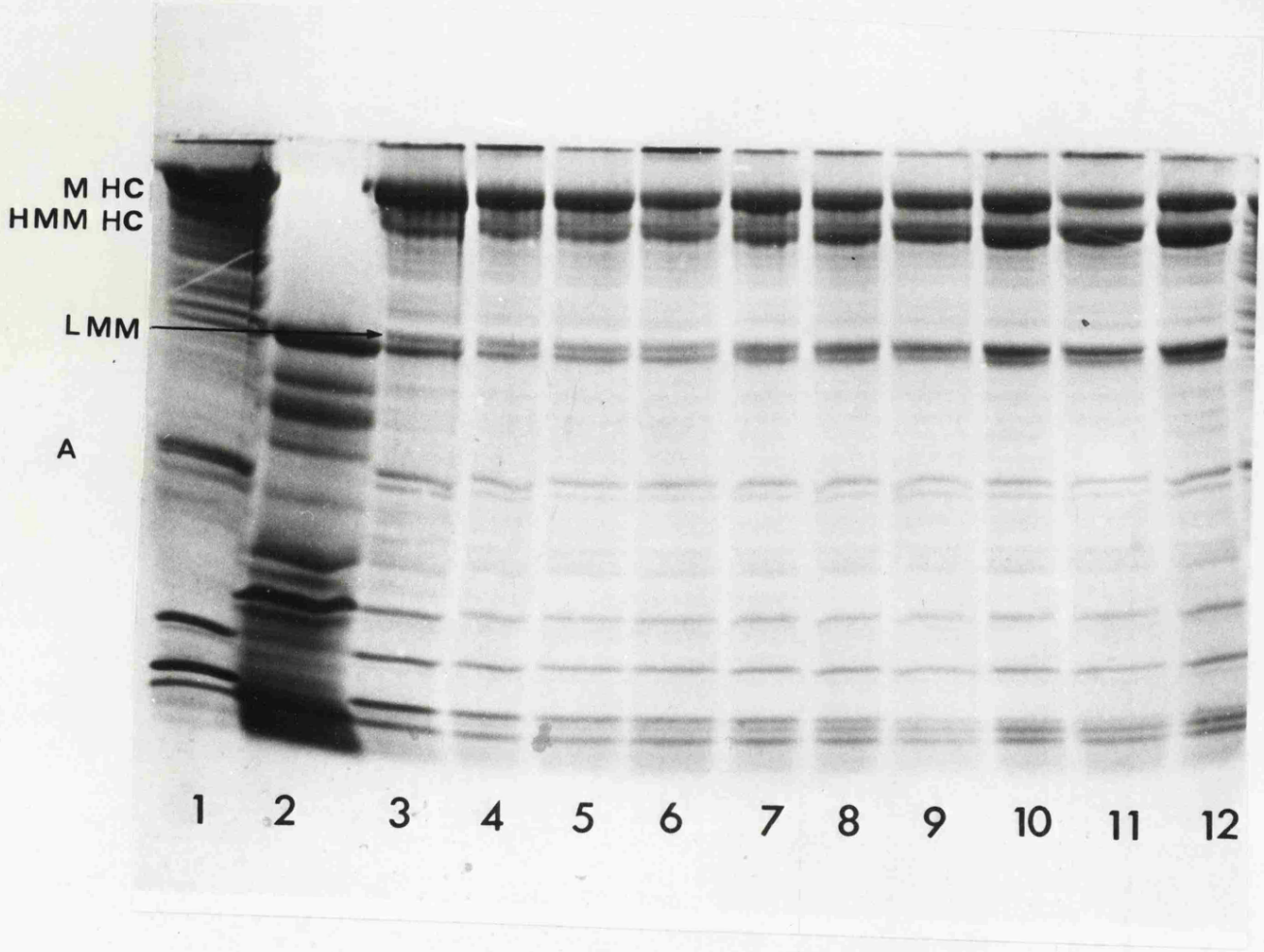

M HC
HMM HC
LMM
A
1
2
3
4
5
6
7
8
9
10
11
12

Plate 12

Optical schlieren pattern of the sedimentation of nicked myosin before removal of thin-filament components (see V.2.5.)

Direction of sedimentation is shown by the arrow.

Time = 501 x 12 seconds.

Speed = 50,090 r.p.m.

Temperature = 20° C.

Solvent: relaxing medium (II.2.6.) made 0.6 M in KCl and 10 mM Mg-ATP, pH 7.0.

Concentration of protein = 7-8 mg per ml.

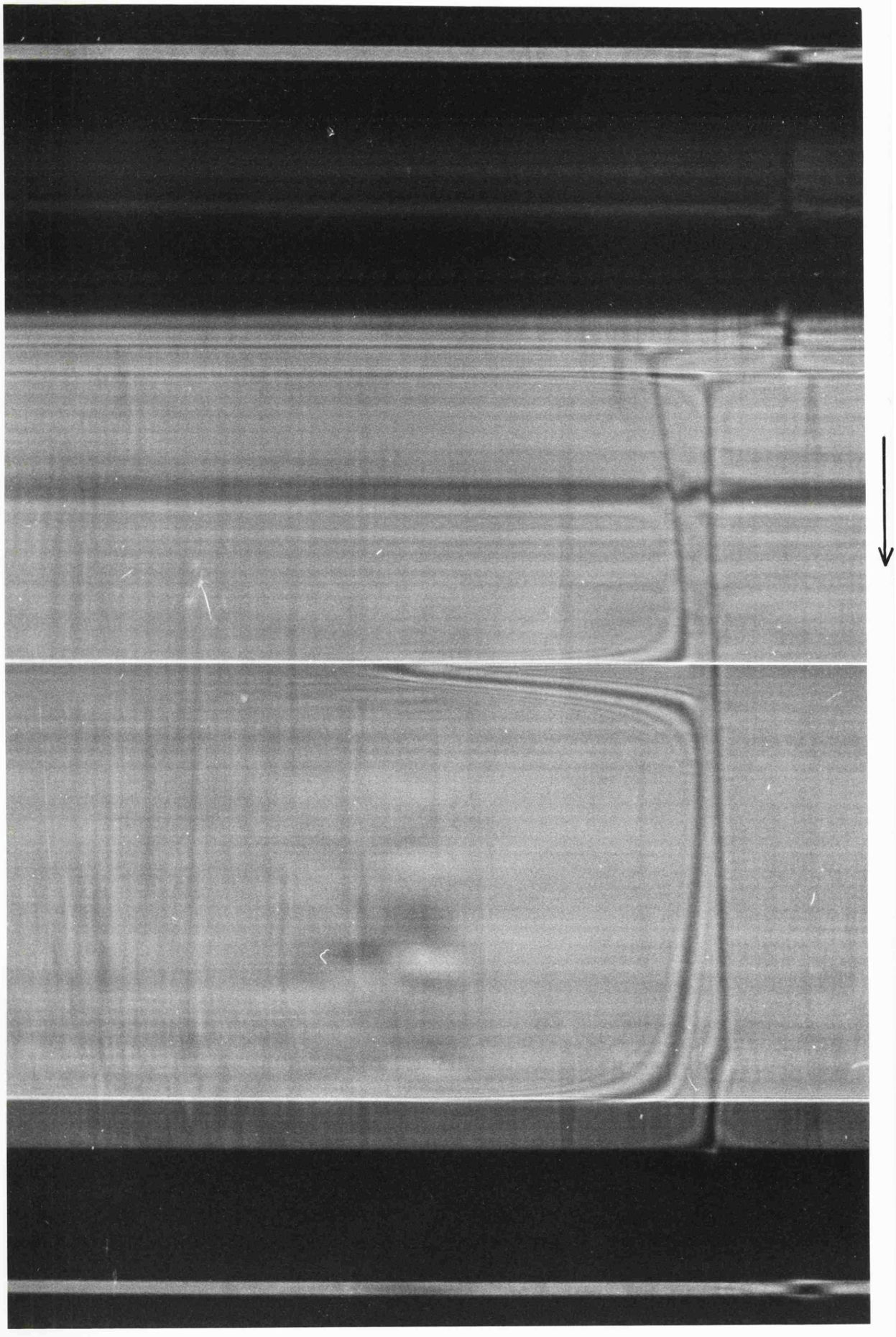


Plate 13

Optical schlieren pattern of the sedimentation of nicked myosin after removal of thin-filament components (see V.2.5.)

Direction of sedimentation is indicated by the arrow.

Time = 500 x 10 seconds.

Speed = 49,190 r.p.m.

Temperature = 20.5° C.

Solvent: stock buffer (II.2.1.).

Concentration of protein = 1 mg per ml.

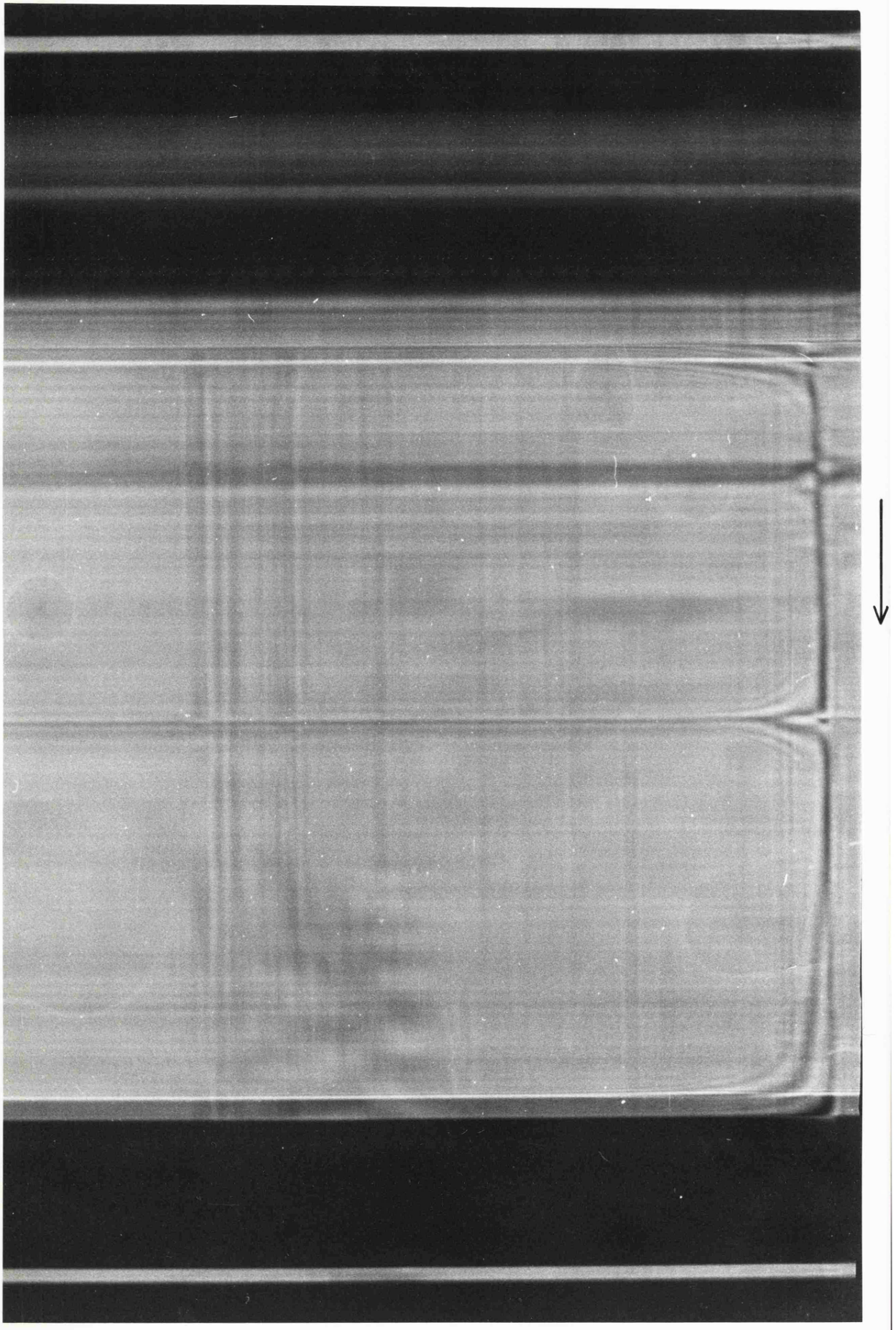


Table 9

Possible myosin isoenzymes of vertebrate skeletal muscle (see text)

A1: alkali light chain 1.

A2: alkali light chain 2.

H1 and H2: different myosin heavy chains.

A1.A1 H1.H1	A1.A2 H1.H1	A2.A2 H1.H1

A1.A1 H1.H2	A1.A2 H1.H2	A1.A2 H2.H1	A2.A2 H1.H2

A1.A1 H2.H2	A1.A2 H2.H2	A2.A2 H2.H2

CHAPTER VI

CONCLUDING REMARKS

VI. CONCLUDING REMARKS

This thesis concerns a study of the biochemical properties of rabbit skeletal myosin filaments which have not been subjected to non-physiological ionic strengths. These properties have been compared with those of conventionally purified myosin. Results from the present study show that native myosin filaments can be distinguished from synthetic myosin filaments by at least the following criteria: (i) a difference in the chymotryptic digestion pattern to obtain S-1, (ii) differences in ATPase activities, and (iii) presence and absence of calcium-sensitive ATPase activities.

S-1 containing the DTNB light chain can be obtained by chymotryptic digestion of purified native myosin filaments while chymotryptic digestion of synthetic myosin filaments (which are formed from monomeric myosin after dialysis to physiological ionic strength) yields S-1 lacking in DTNB light chain (Chapter II). Chymotryptic S-1 from filaments formed from solubilised native myosin filaments also lacks in DTNB light chain, that is, identical to S-1 from conventional synthetic myosin filaments. It has been concluded from these observations that once the native filaments components are solubilised at high ionic strength, not only their potential to reform native filaments is abolished, but the quaternary structure

of myosin is also irreversibly altered (especially with respect to the DTNB light chains), as judged by the resulting filaments whose DTNB light chains are rendered susceptible to chymotryptic attack.

The most striking finding during the course of enzymological studies is the extremely low Mg-ATPase activities of native myosin filaments and the derived S-1 which can be activated by high ionic strength treatment and the removal of DTNB light chain in the case of S-1 (Chapter III). Our results have been found to be lower than that for frog resting muscle (see III.4.3.). The higher value for intact muscle (see III.4.3.) may be due to the presence of other ATPases which are active in muscle at rest. Our extremely low value (III.4.3.), therefore strongly suggests that our preparations of native myosin filaments are free of other non-myosin ATPases. Once the native myosin filaments are solubilised at high ionic strength, the resulting myosin and S-1 have elevated ATPase activities which are comparable to those of conventional preparations. This activation of ATPase activities is irreversible and has been attributed to the permanent loss of the inhibitory effect of DTNB light chain as a result of an irreversible conformational change or a translocation of DTNB light chain; the ATPase site is permanently exposed (Figure 28).

Results from calcium-sensitivity experiments (Chapter

IV) have shown that calcium-sensitive ATPase activities are only displayed by myofibrils and native myosin filaments which have never been exposed to high ionic strength. It is arguable that myofibrillar calcium-sensitive ATPase activities might be due to other ATPases which are associated with the myofibrils and are calcium-sensitive under the assaying conditions. However the fact that vertebrate skeletal muscle ATPase activity is calcium-sensitive is unequivocally demonstrated by the purified native myosin filaments calcium-sensitive ATPase activities.

Since conventional myosin (exposed to high ionic strength) shows a complete lack of calcium-sensitive ATPase activity, it has been concluded that DTNB light chain (before exposure to high ionic strength) plays a role in the calcium-sensitivity of vertebrate skeletal myosin ATPase activity. It is believed that calcium must bind to both troponin C (TN-C) and the DTNB light chain for the activation of muscular contraction. However, the rates of binding and dissociating of calcium to and from the DTNB light chains might be slower than those for TN-C. This has been shown to be so for conventional myosin (Bagshaw and Reed, 1977). However, further experiments (Chapter IV) are essential to elucidate the rate constants of calcium binding to and dissociating from the DTNB light chains of native myosin filaments. If it is assumed that the binding

and dissociating rate constants of calcium for DTNB LC are slower than those of calcium for TN-C, it may be possible (at least in part) to explain the post-tetanic potentiation phenomenon (Gage and Hubbard, 1966) and the latent phase of contraction after stimulation. On such a hypothesis, following stimulation, calcium is first bound to TN-C before the binding of calcium to the DTNB light chains, and only when calcium is also bound to the DTNB light chains, can the interaction of myosin heads (S-1's) with the actin filaments take place. During a tetanus, calcium is bound to both TN-C and the DTNB light chains, but the release of calcium from the DTNB light chains is slower than that from the TN-C when the stimulus is terminated. Therefore , at this stage (i.e. before calcium is dissociated from the DTNB light chains), just after a tetanus, the muscle is more excitable (probably due to the fact that calcium has still not been released from the DTNB light chains) when subjected to a stimulus, giving rise to post-tetanic potentiation. However, this and other hypotheses await further experimental verification.

The present work has also shown that there are at least two different myosin heavy chains which can be distinguished by the enzyme(s) in the DNAase I preparation (Chapter V). This observation has been obtained using relaxed filaments and solubilised native myosin filaments (in the presence of actin filaments).

The conclusions which can be drawn from the present study are as follows:

(i) The effect of chymotryptic digestion of purified native myosin filaments is to yield S-1 in which the DTNB LC is substantially retained. Similar digestion of synthetic filaments (made from conventionally purified myosin) or filaments formed from the solubilised components of native myosin filaments destroys DTNB LC completely. It follows that myosin in native myosin filaments differs from myosin in synthetic filaments in packing and in quaternary structure, especially with respect to the DTNB light chains.

(ii) The extremely low Mg-ATPase activities of purified native myosin filaments and the derived S-1 is the first demonstration of this known physiological phenomenon in a purified in vitro system.

(iii) The myosin-DTNB LC binding is sensitive to high ionic strength - as employed in all conventional myosin preparations. The latter have therefore an artifactually elevated Mg-ATPase activity.

(iv) The function of DTNB LC is at least partly to "switch-off" the Mg-ATPase activity in resting muscle, and

calcium switches on this activity in the presence of actin - i.e. skeletal muscle is dually regulated.

(v) Conventional myosin and myosin from solubilised native myosin filaments, and S-1 from synthetic myosin filaments and filaments formed from solubilised native filaments, all have similar ATPase activities though not identical.

(vi) Evidence has been obtained to show that there are at least two populations of myosin heavy chains - suggesting that the question of isoenzymes is much more complex when both the heavy and the light chains are taken into consideration at the same time.

(vii) It is believed that high ionic strength is the major factor which is responsible for the biochemical differences obtained between conventional and native myosin, though other factors may not be excluded.

BIBLIOGRAPHY

Adelstein, R. S. (1978), Trends Biochem. Sci. 3: 27-30.

Adelstein, R. S. and Conti, M. A. (1975), Nature 256: 597-598.

Adelstein, R. S., Scordilis, S. P. and Trotter, J. A. (1979), In Methods and Achievements in Experimental Pathology. ed. G. Jasmin, M. Cantin, 8: 1-41. Basel, Switzerland: S. Karger.

Aksoy, M. O. and Murphy, R. A. (1979), The Physiologist 22: 2 (Abstr.).

Alexis, M. N. and Gratzer, W. B. (1976), Biochem. 17: 2319-2325.

Alexis, M. N. and Gratzer, W. B. (1976), Febs. Letters 67: 119-121.

Alexis, M. N. and Gratzer, W. B. (1978), Biochem. 17: 2319-2320.

Bagshaw, C. R. and Reed, G. H. (1977), Febs. Letters 81: 386-390.

Bagshaw, C. R. and Kendrick-Jones, J. (1979), Biochem. Soc. Trans. 6: 1262-1264.

Bagshaw, C. R. and Kendrick-Jones, J. (1979), J. Mol. Biol. 130: 317-336.

Bagshaw, C. R. (1980), J. Musc. Res. Cell Motility 1: 255-277.

Banga, I. (1942), Studies Inst. Med. Chem. Univ. Szeged. 1: 27-36.

Barany, K. and Barany, M. (1977), J. Biol. Chem. 252: 4752-4754.

Barany, K., Barany, M., Gillis, J. M. and Kushmerick, M. J. (1979), J. Biol. Chem. 254: 3617-3623.

Barouch, W. W. and Moos, C. (1971), Biochim. Biophys. Acta 234: 183-189.

Barron, S., Eisenberg, E. and Moos, C. (1966), Science 151: 1541-1542.

Bender, M. L. and Hamilton, G. A. (1962), J. Amer. Chem. Soc. 84: 2570-2576.

Blikstad, I., Markey, F., Carlsson, L., Persson, T. and Lindberg, U. (1978), Cell 15: 935-943.

Bowen, W. J. and Kerwin, T. D. (1954), J. Biol. Chem. 211: 237-247.

Bowen , T. J. and Rowe, A. J. (1970), In An Introduction to Ultracentrifugation p. 34; University Press, Aberdeen.

Bradford, M. M. (1976), Anal. Biochem. 72: 248-254.

Brekke, C. J. and Greaser, M. L. (1976), J. Biol. Chem. 251: 866-871.

Bremel, R. D. and Weber, A. (1975), Biochim. Biophys. Acta 376: 366-374.

Briskey, E. J., Seraydarian, K. and Mommaerts, W. H. E. M. (1967), Biochim. Biophys. Acta 133: 424-434.

Burke, M., Reisler, E. and Harrington, W. F. (1973), Pro. Nat. Acad. Sci. 70: 3793-3796.

Burke, M., Reisler, E., Himmelfarb, S. and Harrington, W. F. (1974), J. Biol. Chem. 249: 6361-6363.

Cain, D. F., Infante, A. A. and Davies, R. E. (1962), Nature 196: 214-217.

Cardinaud, R., Dassin, E. and Pelletier, F. (1973),

Biochem. Biophys. Res. Commun. 52: 1057-1063.

Chacko, S., Conti, M. A. and Adelstein, R. S. (1977), Proc. Nat. Acad. Sci. 74: 129-133.

Chantler, P. D. and Szent-Gyorgyi, A. G. (1978), Biochem. 17: 5440-5448.

Chantler, P. D. and Szent-Gyorgyi, A. G. (1980), J. Mol. Biol. 138: 473-492.

Chock., S. P., Chock, P. B. and Eisenberg, E. (1976), Biochem. 15: 3244-3253.

Cohen, I. and Cohen, C. (1972), J. Mol. Biol. 68: 383-387.

Collins, J. H. (1976), Nature 259: 699-700.

Collins, J. H., Greaser, M. L., Potter, J. D. and Horn, M. J. (1977), J. Biol. Chem. 252: 6356-6362.

Conti, M. A. and Adelstein, R. S. (1980), Fed. Proc. 39: 1569-1573.

Cooke, R. (1972), Biochem. and Biophys. Res. Commun. 49: 1021-1028.

Cote, G. P., Lewis, W. G. and Smillie, L. B. (1978), Febs. Letters 91: 237-241.

Cote, G. P., Lewis, W. G., Pato, M. D. and Smillie, L. B. (1978a), Febs. Letters 94: 131-135.

Craig, R., Szent-Gyorgyi, A. G., Beese, L., Flicker, P., Vibert, P. and Cohen, C. (1980), J. Mol. Biol. 140: 35-55.

Depue, Jr. R. H. and Rice, R. V. (1965), J. Mol. Biol. 12: 302-303.

Dreizen, P. and Richards, D. H. (1972), Cold Spring Harbor Symp. Quant. Biol. 37: 29-45.

Dow, J. and Stracher, A. (1971), Proc. Nat. Acad. Sci. 68: 1107-1110.

Drabikowski, W. and Gergely, J. (1962), J. Biol. Chem. 237: 3412-3417.

Dreizen, P. and Gershman, L. C. (1970), Biochem. 9: 1688-1693.

E bashi, S. (1974), Essays Biochem. 10: 1-36.

Ebashi, S., Ebashi, F. and Fujie, Y. (1960), J. Biochem.

47: 54-59.

Ebashi, S. (1961), J. Biochem. 50: 236-244.

Ebashi, S. (1963), Nature 200: 1010.

Ebashi, S. and Ebashi, F. (1964), J. Biochem. 55: 604-613.

Ebashi, S. and Endo, M. (1968), Prog. Biophys. Mol. Biol. 18: 123-183.

Ebashi, S., Endo, M. and Ohtsuki, I (1969), Quant. Rev. Biophys. 2: 251-384.

Ebashi, S., Mikawa, T., Hirata, M. and Nonomura, Y. (1978), Ann. NY Acad. Sci. 307: 451-461.

Eisenberg, E. and Moos, C. (1967), J. Biol. Chem. 242: 2945-2951.

Eisenberg, E. and Moos, C. (1968), Biochem. 7: 1486-1489.

Eisenberg, E. Zobel, C. R. and Moos, C. (1968), Biochem. 7: 3186-3194.

Eisenberg, E. and Moos, C. (1970), J. Biol. Chem. 245: 2451-2456.

Eisenberg, E., Dobkin, L. and Kielley, W. W. (1972), Biochem. 11: 4657-4660.

Eisenberg, E. and Kielley, W. (1972), Cold Spring Harbor Symp. Quant. Biol. 37: 145-152.

Elliot, A., Offer, G. and Burridge, K. (1976), Proc. R. Soc. B. 193: 45-53.

Elliot, A. and Offer G. (1978), J. Mol. Biol. 123: 505-519.

Elzinga, M., Collins, J. H., Kuehl, W. M. and Adelstein, R. S. (1973), Proc. Nat. Acad. Sci. 70: 2687-2691.

Emes, C. H. Ph.D. thesis (1977), University of Leicester.

Emes, C. H. and Rowe, A. J. (1978), Biochim. Biophys. Acta 537: 125-144.

Engelhardt, W. A. and Ljubimova, M. N. (1939), Nature 144: 668-669.

Engelhardt, W. A. and Ljubimova, M. N. (1942), Biokhimia. 7: 205-231.

Ferenczi, M.A., Homsher, E., Simmons, R.M. and Trentham, D. R. (1978), Biochem. J. 171: 165-175.

Frank, G. and Weeds, A. G. (1974), Eur. J. Biochem. 44:317-334.

Franzini-Armstrong, C. and Porter, K. R. (1964), Z. Zellforsch. Mikrosk. Anat. 61: 661-672.

Fraser, A. B., Eisenberg, E., Kielley, W. W. and Carlson, F. D. (1975), Biochem. 14: 2207-2213.

Friess, E. T. (1954), Arch. Biochem. Biophys. 51: 17-23.

Gaetjens, E., Barany, K., Bailin, G., Oppenheimer, H. and Barany, M. (1968), Arch. Biochem. Biophys. 123: 82-96.

Gage. P. W. and Hubbard, J. I. (1966), J. Physiol. 184: 353-375.

Gazith, J., Himmelfarb, S., and Harrington, W. F. (1970), J. Biol. Chem. 245: 15-22.

Gergely, J. (1950), Fed. Proc., Fed. Amer. Soc. Exp. Biol. 9: 176.

Gergely, J. (1953), J. Biol. Chem. 200: 543-550.

Gergely, J., Gouvea, M. A. and Karibian, D. (1955), J. Biol. Chem. 212: 165-177.

Gershman, L. C., Dreizen, P. and Stracher, A. (1966), Proc. Nat. Acad. Sci. 56: 966-973.

Gershman, L. C., Stracher, A. and Dreizen, P. (1969), J. Biol. Chem. 244: 2726-2736.

Gershman, L. C. and Dreizen, P. (1970), Biochem. 9: 1677-1687.

Glascoe, P. K. and Long F. A. (1960), J. Phys. Chem. 64: 188-190.

Goody, R. S., Hofmann, W. and Mannherz, H. G. (1977), Eur. J. Biochem. 78: 317-324.

Greaser, M. L. and Gergely, J. (1971), J. Biol. Chem. 246: 4226-4233.

Guba, F. and Straub, F. B. (1943), Studies Inst. Med. Chem. 3: 46-48.

Hanson, J. and Lowy, J. (1963), J. Mol. Biol. 6: 46-60.

Hanson, J. and Huxley H. E. (1953), Nature 172: 172: 530-532.

Hanson, J. and Huxley, H. E. (1955), Symp. Soc. Exp. Biol.

9: 228-264.

Hanson, J. and Huxley, H. E. (1957), Biochim. Biophys. Acta 23: 250-260.

Haselgrove, J. C. (1973), Cold Spring Harbor Symp. Quant. Biol. 37: 341-352.

Haselgrove, J. C. (1975), J. Mol. Biol. 92: 113-143.

Hasselbach, W. (1952), Z. Naturf. 7b: 163-174.

Hasselbach, W. and Schneider, G. (1951), Biochem. Z. 321: 462-475.

Hathaway, D. R., Eaton C. R. and Adelstein, R.S. (1980), In The Regulation of Coagulation ed. K. G. Mann, F. B. Taylor, Jr. pp. 271-276. New York: Elsevier/North-Holland.

Hayashi, Y., Takenaka, H. and Tonomura, Y. (1973), J. Biochem. 74: 1031-1047.

Head, J. F. and Perry, S. V. (1974), Biochem. J. 137: 145-154.

Heilbrum, L. V. and F. J. Wiercinski (1947), J. Cellular Comp. Physiol. 29: 15-32.

Higuchi, M., Fabian, F., Wandzilak, T., Wason. D. T. and Wikman-Coffelt, J. (1978), Eur. J. Biochem. 92: 317-323.

Hitchcock, S. E., Carlsson, L. and Lindberg, U. (1976), Cell 7: 531-542.

Hitchcock, S. E., Huxley, H. E. and Szent-Gyorgyi, A. G. (1973), J. Mol. Biol. 70: 825-836.

Hoar, P. E., Kerrick, W. G. L. and Cassidy, P. (1979), Science 204: 503-506.

Hodgkin A. L. and Horowicz P. (1959), J. Physiol. 148: 127-160.

Hoh, J. F. Y. (1978), Febs. Letters 90: 297-300.

Hoh, J. F. Y., McGrath, P. A. and White, R. I. (1976), Biochem. J. 157: 87-95.

Hoh, J. F. Y. and Yeoh, G. P. S. (1979), Nature 280: 321-323.

Hozumi, T. and Hotta, K. (1978), J. Biochem. 83: 671-676.

Hozumi, T. and Tawada, K. (1973), J. Biochem. 74: 385-387.

Holroyde, M. J., Potter, J. D. and Solaro, R. J. (1979), J. Biol. Chem. 254: 6478-6482.

Holroyde, M. J., Small, D. A. P., Howe, E. and Solaro, R. J. (1979a), Biochim. Biophys. Acta 587: 628-637.

Holt, J. C. and Lowey, S. (1975), Biochem. 14: 4600-4609.

Holt, J. C. and Lowey, S. (1975a), Biochem. 14: 4609-4620.

Holt, J. C. and Lowey, S. (1977), Biochem. 16: 4398-4402.

Hotta, K. and Morales, M. F. (1960), J. Biol. Chem. 235: PC 61.

Hotta, K., and Usami, Y. (1967), J. Biochem. 61: 415-423.

Huszar, G. and Bailey, P. (1979), Am. J. Obstet. Gynecol. 135: 718-726.

Huxley, A. F. (1957a), Progr. Biophys. Biophys. Chem. 7: 255-318.

Huxley, A. F. (1974), J. Physiol. 243: 1-43.

Huxley, A. F. and Niedergerke, R. (1954), Nature 173: 971-973.

Huxley, H. E. (1957), J. Biophys. Biochem. Cytol. 3: 631-648.

Huxley, H. E. (1960), In The Cell 4: 365, Academic Press, New York and London.

Huxley, H. E. (1963), J. Mol. Biol. 7: 281-308.

Huxley, H. E. (1969), Science 164: 1356-1366.

Huxley, H. E. (1973), Cold Spring Harbor Symp. Quant. Biol. 37: 361-376.

Huxley, H. E. (1973a), Nature 243: 445-449.

Huxley, H. E. and Brown, W. (1967), J. Mol. Biol. 30: 383-434.

Huxley, H. E. and Hanson, J. (1954), Nature 173: 973-976.

Ishigami, F. and Morita, F. (1977), J. Biochem. 81: 297-303.

Jakes, R., Kendrick-Jones, J. and Northrop, F. (1976), Febs. Letters 70: 229-234.

Johnson, P. and Rowe, A. J. (1960), Biochem. J. 74:

432-440.

Johnson, P. and Rowe, A. J. (1961), Biochim. Biophys. Acta 53: 343-360.

Jones, J. M. and Perry, S. V. (1966), Biochem. J. 100: 120-130.

Kaminer, B. and Bell, A. L. (1966), J. Mol. Biol, 20: 391-401.

Kardami, E., Alexis, M., de la Paz, P. and Gratzer, W. (1980), Eur. J. Biochem. 110: 153-160.

Kasmam, K. and Kakol, I. (1977), Biochim. Biophys. Acta 491: 509-514.

Katsura, I and Noda, H. (1971), J. Biochem. 69: 219-229.

Kendrick-Jones, J. (1974), Nature 249: 631-634.

Kendrick-Jones, J. (1976), J. Mol. Biol. 104: 747-775.

Kendrick-Jones, J. and Jakes, R. (1976), In International Symposium on Myocardial Failure (eds. Rieker, G., Weber, A. and Goodwin, J.), pp. 28-40. Munich: Tergenese.

Kendrick-Jones, J., Lehman, W. and Szent-Gyorgyi, A. G. (1970), J. Mol. Biol. 54: 313-326.

Kendrick-Jones, J., Szentkiralyi, E. M. and A. G. Szent-Gyorgyi (1972), Cold Spring Harbor Symp. Quant. Biol. 37: 47-53.

Kendrick-Jones, J., Szentkiralyi, E. M. and Szent-Gyorgyi, A. G. (1976), J. Mol. Biol. 104: 747-775.

Kielley, W. W., Kalckar, H. M. and Bradley, L. B. (1956), J. Biol . Chem. 219: 95-101.

Kielley, W. W. and Meyerhof, O. (1948), J. Biol. Chem. 176: 591-601.

Kielley, W. W. and Meyerhof, O. (1950), J. Biol. Chem. 183: 391-401.

Kim, H. D., and Mommaerts, W. F. H. M. (1971), Biochim. Biophys. Acta. 245: 230-234.

Knappeis, G. G. and Carlsen, F. (1962), J. Cell Biol. 13: 323-335.

Knappeis, G. G. and Carlsen, F. (1968), J. Cell. Biol. 38: 202-211.

Kominz, D. R., Carroll, W. R., Smith, E. N. and Mitchell, E. R. (1959), Arch. Biochem. Biophys. 79: 191-199.

Kominz, D. R., Mitchell, E. R., Nihei, T. and Kay, C. M. (1965), Biochem. 4: 2373-2382.

Korn, E. D. (1978), Proc. Nat. Acad. Sci. 75: 588-599.

Kotoh, N. and Kubo, S. (1974), Biochim. Biophys. Acta. 365: 301-304.

Krebs, E. G. and Beavo, J. A. (1979), Ann. Rev. Biochem. 48: 923-959.

Kushmerick, M. J. and Paul, R. (1976), J. Physiol. 254: 693-709.

Kuwayama, J. and Yagi, K. (1977), J. Biochem. 80: 25-33.

Kuwayama, J. and Yagi, K. (1979), J. Biochem. 85: 1245-1255.

Laemmli, U. K. (1970), Nature 227: 680-685.

Lamvik, M. K. (1978), J. Mol. Biol. 122: 55-68.

Leavis, P. C., Rosenfeld, S. S., Gergely, J., Grabarek, Z.

and Drabikowski, W. (1978), J. Biol. Chem. 253: 5452-5459.

Lehman, W. (1977), Biochem. J. 163: 291-296.

Lehman, W. (1978), Nature 274: 80-81.

Lehman, W., Kendrick-Jones, J. and Szent-Gyorgyi, A. G. (1972), Cold Spring Harbor Symp. Quant. Biol. 37: 319-330.

Lehman, W. and Szent-Gyorgyi, A. G. (1975), J. Gen. Physiol. 66: 1-30.

London, W. P. (1968), Bull. Math. Biophys. 30: 253-277.

Lowey, S. (1979), In Fibrous Proteins: Scientific, Industrial and Medical Aspects ed. D. A. D. Parry, L. K. Creamer, 1: 1-25. New York: Academic.

Lowey, S. and Holt, J. C. (1972), Cold Spring Harbor Symp. Quant. Biol. 37: 19-28.

Lowey, S. and Risby D. (1971), Nature 234: 81-85.

Lowey, s., Slayter, H. S., Weeds, A. G. and Baker, H. (1969), J. Mol. Biol. 42: 1-29.

Lymn, R. W. (1974), J. Theor. Biol. 43: 313-328.

Lymn, R. W. and Taylor, E. W. (1970), Biochem. 9: 2975-2983.

Lymn, R. W. and Taylor, E. W. (1971), Biochem. 10: 4617-4624.

Malhotra, A., Huang, S. and Bhan, A. (1979), Biochem. 18: 461-467.

Mannherz, H. G., Barrington-Leigh, J., Leberman, R. and Pfrang, H. (1975), Febs. Letters 60: 34-38.

Margossian, S. S. and Lowey, S. (1973), J. Mol. Biol. 74: 313-330.

Margossian, S. S., Lowey, S. and Barshop, B. (1975), Nature 258: 163-166.

Morimoto, K. and Harrington, W. F. (1974), J. Mol. Biol. 88: 693-709.

Marston, S. B. (1978), Febs. Letters 92: 147-151.

Marston, S. B., Trevett, R. M. and Walters, M. (1980), Biochem. J. 185: 355-365.

Marston, S. B. and Tregear, R. T. (1972), Nature New Biol.

235: 23-24.

Martonosi, A. and Meyer, H. (1964), J. Biol. Chem. 239: 640-647.

Maruyama, k. and Weber, A. (1972), Biochem. 11: 2990-2998.

Maruyama, K. and Watanabe, S. (1962), J. Biol. Chem. 237: 3437-3442.

Maruyama, M. and Gergely, J. (1962), J. Biol. Chem. 237: 1095-1099.

Maruyama, M. and Gergely, J. (1962a), J. Biol. Chem. 237: 1100-1106.

Masaki, T., Endo, M. and Ebashi, S. (1967), J. Biochem. 62: 630-632.

Maw, M. C. and Rowe, A. J. (1979),J. Ultrastruct. Res. 69: 142-143.

Maw, M. C. and Rowe, A. J. (1980), Nature 286: 412-414.

Mendelson, R. and Cheung, C. (1976), Science 194: 190-192.

Middlebrook, W. R. (1958), Proc. Int. Congr. Biochem. 4th,

1958 vol 17: p.84.

Mihalyi, E. and Szent-Gyorgyi, A. G. (1953), J. Biol. Chem. 201: 189-196.

Mikawa, T., Nonomura, Y., Hirata, M., Ebashi, S. and Kakiuchi, S. (1978), J. Biochem. 84: 1633-1636.

Mommaerts, W. F. H. M. and Green, I. (1954), J. Biol. Chem. 208: 833-843.

Mommaerts, W. F. H. M. and Seraidarian, K. (1947), J. Gen. Physiol. 30: 401-422.

Moos, C., Offer, G., Starr, R. and Bennett, P. (1975), J. Mol. Biol. 97: 1-9.

Moore, P. B., Huxley, H. E. and DeRosier, D. J. (1970), J. Mol. Biol. 50: 279-295.

Morel, J. E., Pinset-Harstrom, I. and Bardin, A. M. (1979), Biol. Cell. 34: 9-16.

Morimoto, K. and Harrington, W. F. (1974), J. Mol. Biol. 80: 693-705.

Morimoto, K. and Harrington, W. F. (1974), J. Mol. Biol.

83: 83-97.

Mueller, H. and Perry, S. V. (1961), Biochem. J. 80: 217-223.

Mueller, H. and Perry, S. V. (1962), Biochem. J. 85: 431-439.

Muhlrad, A., Fabian, F. and Biro, N) A. (1964), Biochem. Biophys. Acta 89: 186-188.

Mulhern, S. A. and Eisenberg, E. (1976), Biochem. 15: 5702-5708.

Nanninga, L. B. (1964), Biochim. Biophys. Acta 82: 507-517.

Nihei, T. and Kay, C. M. (1968), Biochim. Biophys. Acta 160: 46-52.

Noda, H. and Ebashi, S. (1960), Biochim. Biophys. Acta 41: 386-392.

Oda, S., Oriol-Audit, C. and Reisler, E. (1980), Biochem. 19: 5614-5618.

Offer, G. W. (1964), Biochim. Biophys. Acta 89: 566-569.

Offer, G. (1972), Cold Spring Harbor Symp. Quant. Biol. 37: 87-96.

Okamoto, Y. and Yagi, K. (1976), J. Biochem. 80: 111-120.

Onishi, H. and Watanabe, S. (1979), J. Biochem. 85: 457-472.

Onodera, M. and Yagi, K. (1971), J. Biochem. 69: 145-153.

Panyim, S. and Chalkley, R. (1971), J. Biol. Chem. 246: 7557-7560.

Pemrick, S. M. (1977), Biochem. 16: 4047-4054.

Pemrick, S. M. (1980), J. Biol. Chem. 255: 8836-8841.

Pepe, A. F. and Drucker, B. (1979), J.Mol. Biol. 130: 379-393.

Perrie, W. T. and Perry, S. V. (1970), Biochem. J. 119: 31-38.

Perrie, W. T., Perry, S. V. and Stone, D. (1969), Biochem. J. 113: 28p-29p.

Perrie, W. T., Smillie, L. B. and Perry, S. V. (1973),

Biochem. J. 135: 151-164.

Perry, S. V. (1951), Biochem. J. 48: 257-264.

Perry, S. V. (1979), Biochem. Soc. Trans. 7: 593-617.

Pinset-Harstrom, I. and Truffy, J. (1979), J. Mol. Biol. 134: 173-188.

Pinset-Harstrom, I. and Whalen, R. G. (1979), J. Mol. Biol. 134: 189-197.

Pope, B. J., Wagner, P. D. and Weeds, A. G. (1977),J. Mol. Biol. 109: 470-473.

Potter, J. D. (1974), Arch. Biochem. Biophys. 162: 436-441.

Potter, J. D. and Gergely, J. (1975), J. Biol. Chem. 250: 4628-4633,

Reisner, A. A., Nemes, P. and Bucholtz, C. (1975), Anal. Biochem. 64: 509-516.

Rizzino, A. A., Barouch, W. W., Eisenberg, E. and Moos, C. (1970), Biochem. 9: 2402-2408.

Sarkar, S. (1972), Cold Spring Harbor Symp. Quant. Biol.

37: 14-17.

Sarkar, S., Sreter. F. A. and Gergely, J. (1971), Proc. Nat. Acad. Sci. 68: 946-950.

Scordilis, S. P. and Adelstein, R. S. (1977), Nature 268: 558-560.

Seals, J. R., McDonald, J. M., Bruns, D. and Jarett, L. (1978), Anal. Biochem. 90: 785-795.

Sekine, T. and Kielley, W. W. (1964), Biochim. Biophys. Acta 81: 336-345.

Silver, P. and Disalvo, J. (1979), J. Biol. Chem. 254: 9951-9954.

Simmons, R. M. and Szent-Gyorgyi, A. G. (1978), Nature 273: 62-64.

Slayter, H. S. and Lowey, S. (1967), Proc. Nat. Acad. Sci. 58: 1611-1618.

Sleep, J. A. and Boyer, P. D. (1978), Biochem. 17: 5417-5422.

Small, P. A., Harrington, W. F. and Kielly, W. W. (1961),

Biochim. Biophys. Acta 49: 462-470.

Small, J. V. and Sobieszek, A. (1977), Eur. J. Biochem. 76: 521-530.

Small, J. V. and Sobieszek, A. (1980), Int. Rev. Cytol. 64: 241-306.

Sobieszek, A. and Small, J. V. (1977), J. Mol. Biol. 112: 559-576.

Sodek, J., Hodges, R. S. and Smillie, L. B. (1978), J. Biol. Chem. 253: 1129-1136.

Squire, J. M. (1973), J. Mol. Biol. 77: 279-323.

Sreter, F. A., Elizinga, M. and Mabuchi, K. (1975), Febs. Letters 57: 107-111.

Sreter, F. A., Gergely, J. and Luff, A. L. (1974), Biochem. Biophys. Res. Commun. 56: 84-89.

Sreter, F. A., Gergely, J., Salmons, S. and Romanul, F. (1973), Nature New Biol. 241: 17-19.

Srivastava, S., Cooke, R. and Wikman-Coffelt, J. (1980), Biochem. Biophys. Res. Commun. 92: 1-7.

Stafford, W. F. and Szent-Gyorgyi, A. G. (1978), Biochem. 17: 607-614.

Starr, R. and Offer, G. (1971), Febs.Letters 15: 40-44.

Starr, R. and Offer, G. (1973), J. Mol. Biol. 81: 17-31.

Stein, L. A., Schwarz, R. P., Chock, P. B. and Eisenberg, E. (1979), Biochem. 18: 3895-3909.

Stone, D. and Perry, S. V. (1973), Biochem. J. 131: 127-137.

Stone, D. and Smillie, L. B. (1978), J. Biol. Chem. 253: 1137-1148.

Stracher, A. (1969), Biochem. Biophys. Res. Commun. 35: 519-525.

Stromer, M. H., Hartshorne, D. J., Mueller, H. and Rice, R. V. (1969), J. Cell Biol. 40: 167-178.

Szent-Gyorgyi, A. G. (1945), Acta Physiol. Scand. 9:suppl. 25.

Szent-Gyorgyi, A. G. (1951), In Chemistry of Muscular Contraction, 2nd rev. ed. Academic Press, N. Y.

Szent-Gyorgyi, A. G. (1953), Arch. Biochem. and Biophys. 42: 305-320.

Szent-Gyorgyi, A. G. (1975), Biophys. J. 15: 707-723.

Szent-Gyorgyi, A. G. (1975), J. Supramol. Struct. 3: 348-353.

Szent-Gyorgyi, A. G., Szentkiralyi, E. M. and Kendrick Jones, J. (1973), J. Mol. Biol. 74: 179-203.

Thomson, J. F. (1963), IN Biological Effects of Deuterium McMillan, New York.

Tokiwa, T. and Tonomura, Y. (1965), J. Biochem. 57: 616-623.

Tonomura, Y. and Inove, A. (1975), In MTP International Rewiew of Science, Biochem. Ser. I ed. E. Racker, 3: 121-161, London: Butterworth.

Tonomura, Y., Nakamura, H., Kinoshita, N., Onishi, H. and Shigekawa, M. (1969), J. Biochem. 66: 599-618.

Trayer, H. R., Winstanley, M. A. and Trayer, I. P. (1977), Febs. Letters 83: 141-144.

Tregear, R. T. and Squire, J. M. (1973), J. Mol. Biol. 77: 279-290.

Trinick, J. A. (1973), Ph.D. thesis. Leicester University.

Trinick, J. A. and Rowe, A. J. (1973), MSE Application Information A9.

Trotter, J. A. and Adelstein, R. S. (1979), J. Biol. Chem. 254: 8781-8785.

Wagner, P. D. (1977), Febs. Letters 81: 81-85.

Wagner, P. D. and Weeds, A. G. (1977), J. Mol. Biol. 109: 455-473.

Wagner, P. D. and Weeds, A. G. (1979), Biochem. 18: 2260-2266.

Wagner, P. D. and Yount, R. G. (1975), Biochem. 14: 5156-5162.

Wagner, P. D. and Yount, R. G. (1976), J. Biol. Chem. 251: 5424-5426.

Wakabayashi, T., Huxley, H. E., Amos, L. A. and Klug, A. (1975), J. Mol. Biol. 93: 477-497.

Weber, A. (1966), Current Topics Bioenergetics 1: 203-254.

Weber, A. Herz, R. and Reiss, I. (1963), J. Gen. Physiol. 46: 679-702.

Weber, A. and Herz. R. (1963), J. Biol. Chem. 238: 599-605.

Weber, A., Herz, R. and Reiss, I. (1969), Biochem. 8: 2266-2271.

Weber, A. and Murray, J. M. (1973), Physiol. Rev. 53: 612-673.

Weber, A. and R. Herz (1963), J. Biol. Chem. 238: 599-605.

Weber, A., R. Herz, and I. Reiss (1963), J. Gen. Physiol. 46: 679-702.

Weber, A. and Winicur, S. (1961), J. Biol. Chem. 236: 3198-3202.

Weber, K. and Osborn, M. (1969), J. Biol. Chem. 244: 4406-4412.

Weeds, A. G. (1967), Biochem. J. 104: 44p.

Weeds, A. G. (1969), Nature 223: 1362-1364.

Weeds A. G. and Burridge, K. (1975), Febs. Letters 57: 203-208.

Weeds, A. G. and Frank, G. (1972), Cold Spring Harbor Symp. Quant. Biol. 37: 9-14.

Weeds, A. G., Hall, R. and Spurway, N. C. S. (1975), Febs. Letters 49: 320-324.

Weeds, A. G. and Lowey, S. (1971), J. Mol. Biol. 61: 701-725.

Weeds, A. G. and Pope, B. (1971), Nature 234: 85-88.

Weeds, A. G. and Pope, B. (1977), J. Mol. Biol. 111: 129-157.

Weeds, A. G. and Taylor R. S. (1975), Nature 257: 54-56.

Weeds, A. G., Trentham, D. R., Kean, C. J. C. and Buller, A. J. (1974), Nature 247: 135-139.

Werber, M. M., Gaffin, S. L. and Oplatka, A. (1972), J. Mechanochem. Cell Motility 1: 91-96.

Werber, M. M. and Oplatka, A. (1974), Biochem. Biophys. Re. Commun. 57: 823-830.

Whalen, R. G., Butler-Browne, G. S. and Gros, F. (1978), J. Mol. Biol. 126: 415-431.

Wikman-Coffelt, J. (1980), Biochem. J. 185: 265-268.

Winstanley, M. A., Small, D. A. P. and Trayer, I. P. (1979), Eur. J. Biochem. 98: 441-446.

Yagi, K., Nakata, T. and Sakakibara, I. (1965), J. Biochem. 58: 236-242.

Yagi, K. and Otani, F. (1974), J. Biochem. 76: 365-373.

Yamamoto, K. and Sekine, T. (1977), J. Biochem. 82: 1435-1441.

Yang J. T. and Wu, C-S. C. (1977), Biochem. 16: 5785-5789.

Yazawa, Y. and Yagi, K. (1973), J. Biochem. 73: 567-580.

Young, D. M., Himmelfarb, S. and Harrington, W. F. (1964), J. Biol. Chem. 239: 2822-2829.

Abstract

A STUDY OF THE BIOCHEMICAL PROPERTIES OF NATIVE VERTEBRATE SKELETAL MYOSIN FILAMENTS

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Chymotryptic S-1 lacking in DTNB LC can be obtained from synthetic rabbit myosin filaments (Weeds and Taylor, 1975; Weeds and Pope, 1977). I have now shown however that chymotryptic digestion of $^{2}H_2O$-H_2O gradients-purified native myosin filaments (Emes and Rowe, 1978) yields S-1 containing the DTNB LC. Our results strongly suggest that this difference between synthetic and native myosin filaments upon subjection to chymotryptic digestion is mostly due to a difference in the myosin conformation within the two types of filaments, since chymotryptic S-1 from filaments formed from solubilised native filaments produced conventional S-1.

Detailed studies of the ATPase activities of native filaments and the derived S-1 (before and after exposure to high ionic strength) have been performed using an isotopic assay (γ-^{32}P ATP). Evidence has been obtained to indicate that DTNB LC before exposure to high ionic strength possesses the potential to repress ATPase activities and it is believed that native DTNB LC plays a role in maintaining the low ATPase activities in relaxed muscle.

ATPase activities of rabbit skeletal myofibrils have been shown to be calcium-sensitive at physiological ionic strength (Lehman, 1977; 1978). Using purified and relaxed filaments, I have shown that these preparations have calcium-sensitive ATPase activities at physiological ionic strength and also at low ionic strengths in the case of purified filaments. My results suggest that DTNB LC before "seeing" high ionic strength is responsible for calcium-sensitivity.

Evidence is presented to suggest that there are at least two populations of myosin heavy chains.