THE BIOCHEMICAL AND STRUCTURAL PROPERTIES OF VERTEBRATE THICK FILAMENTS.

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by

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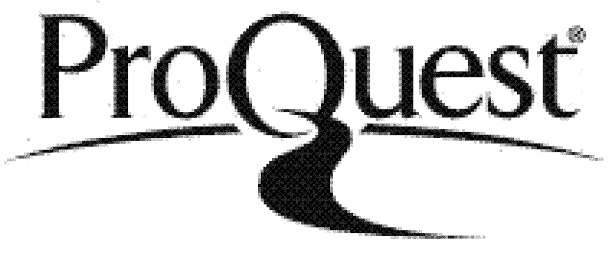

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Abstract

An extraction technique has been developed that removes 50% of the regulatory light chains (RLCs) from native vertebrate myosin filaments. The extraction susceptible population correlates with a chymotryptic resistant population which can be shown to be present. Evidence is provided which suggests that all myosin molecules in native thick filaments are identical and lose one of their two RLCs during the extraction procedure. RLC removal results in the exposure of a 'sticky patch' which interacts with the filament shaft sticking the myosin cross bridge down. The ionic strength dependance of this interaction suggests that it occurs via electrostatic and not hydrophobic residues.

A two fold activation in the myosin ATPase activity can be demonstrated on the addition of calcium to purified native myosin filaments (PNFs). We demonstrate that the RLCs are intimately involved in the calcium sensitivity of the myosin ATPase. We show that after the removal of 50% of the RLCs a 50% reduction in the calcium sensitivity of the ATPase is observed suggesting an activatory role for the RLCs in vertebrate myosin.

Hydrodynamic analysis reveals that PNFs possess a high affinity calcium binding site (Kd = 30 μM) and also a low affinity non specific site. The binding of cations to these sites has a clear effect on the hydrodynamic properties which has been used to study the relationship between them. Such analysis reveals that the Kd of the low affinity site is modulated by calcium binding to the high affinity site. The significance of such interactions and their role in the contractile cycle is discussed.

To
My Mother and Father.

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Chapter I

Introduction

Part A

The basics of muscle structure, function and action.

(I. 1) The structure of vertebrate striated skeletal muscle.

Vertebrate skeletal muscles are usually distinct and relatively large structures. The main structural components of an individual muscle, down to a molecular scale are shown in Figure 1.1. Each muscle is surrounded by a sheath of connective tissue called the epimysium and within each muscle there are bundles of muscle fibres called fasciculi which are joined together by a connective tissue known as the perimysium. Each muscle fibre is in fact a single multinucleate cell which is surrounded by a thin connective sheath (the sarcolemma) which contains the usual plasma membrane as one of its components. The cytoplasmic region of the cell is densely packed with organelles known as myofibrils. The myofibrils possess a characteristic banding pattern and invariably run the entire length of the muscle fibre. The myofibrils are the contractile elements of the muscle fibre and contain the smallest contractile unit of skeletal muscle the sarcomere (See Figure 1. 1).

The sarcomere consists of interdigitating thick, myosin containing and thin, actin containing filaments (Hanson and H. E. Huxley., 1953; H.E. Huxley., 1957). It is believed to be the shortening of the sarcomere by the relative sliding of the two types of filaments that results in contraction. The sliding filament theory of muscular contraction was proposed independently and almost simultaneously by two groups of workers A. F. Huxley and Neidergerke (1954) and H. E. Huxley and Hanson (1954). Both groups had noted that changes in the myofibrilar cross striations occured during muscular contraction or stretching. Hanson and H. E. Huxley (1953) had earlier proposed that the material in the bands was present as submicroscopic rods which the electron micrographs of H. E. Huxley (1957) confirmed to be the case. The sliding filament theory involved the relative sliding of these highly ordered arrays of rods. Further evidence in support of this was produced by H. E. Huxley (1957) who used electron microscopy to demonstrate that the amount of filament overlap was proportional to the extent of contraction, maximum overlap being achieved during maximum contraction. Carlsen et al (1961) proposed that during the contractile process the thick myosin filaments increase in length by up to 10% and that this process was involved in the contractile mechanism. However Page and H. E. Huxley (1963) showed this to be an artifact of electron microscopy preparation and the sliding filament theory of contraction survives to this day as the most widely accepted explanation for the process of muscular contraction. The energy for the sliding process comes from the hydrolysis of ATP by myosin (Cain et al., 1962), the rate of which is markedly activated by myosin / actin interaction (Hasselbach., 1952; Eisenberg and Moos., 1970)(See Section I. 1).

(I. 2) The structure of the thin filaments.

(I. 2. i) Actin.

The thin filaments are composed mainly of a globular protein called actin. In low non physiological ionic strengths actin exists as a globular monomer (G-actin) whose complete amino acid sequence was first determined by Elzinga (1973). Elzinga (1973) showed that the actin molecule consists of a single polypeptide chain containing 374 residues with a molecular weight of 41,785. The N and C - terminal amino acids are acetylated aspartic acid and phenylalanine respectively and the molecule contains an unusual amino acid N-methyl-histidine at position 73. G-actin also binds very tightly one mole of nucleotide (ATP or ADP) and one mole of divalent cation (Mg^{2+} or Ca^{2+})per mole of monomer (Straub and Feuer., 1950).

G-actin molecules are polymerised into a double stranded fibrous form (F-actin) by increasing the ionic strength (Keidley and Meyerhof., 1950). Thin filaments consist of F-actin in the form of a two fold right handed helix with a true repeat of 72 nm and a diameter of about 6nm. The structure of purified F-actin is essentially the same as the structure of thin filaments within myofibrils except that thin filaments contain other (Regulatory) proteins. The regulatory proteins present in native thin filaments are tropomyosin and the troponins (T, I, C) (See Sections I.2.ii - I.2.vi).

(I. 2. ii) Tropomyosin.

Tropomyosin is a long (41 nm) a-helical coiled coil molecule (MW 65,000) consisting of two subunits of which there are two types a (Stone and Smillie., 1980) and ß (Mak et al., 1979). Both chains consist of 284 amino acids, there being 39 difference amino acids between the two.

The tropomyosin molecules lie head to tail along the grooves of the thin filament, forming a continuous strand (Johnson and Smillie., 1977; and Figure 1.1). Each tropomyosin molecule binds 7 molecules of actin and 1 molecule of troponin T. Several workers (H. E. Huxley., 1972; Haselgrove., 1972) have used X-ray diffraction studies to show that tropomyosin can occupy two different positions on the actin filament. One of these positions is on the periphery, which is occupied during relaxation and the other is near the groove, which is occupied during contraction. This movement is believed to play an important role in the regulation of the interaction of myosin with the actin and could well be controlled by the binding of Ca^{2+} to troponin C (See Section I. 6. i).

(I. 2. iii) The Troponins.

Troponin (the troponin complex)actually consists of 3 molecules, and is located on the thin filaments with a periodicity of 38.5 nm. The troponin complex does not bind directly to actin but is in fact located on the tropomyosin molecule (See Figure 1.1). Its possible involvement in regulation was first suggested by Ebashi and Kodama (1965., 1968) who observed that 'native troponin' conferred calcium sensitivity on pure actomyosin. A detailed dissection of the structure and function of

the individual components of the troponin complex has since enabled a detailed model of its mode of action to be proposed (See Section I. 6. i).

(I. 2. iv) Troponin C (TnC).

The amino acid sequence of skeletal troponin C was first described in 1977 by Collins et al (1977) who showed that it was an acidic protein with a molecular weight of 17,965. Analysis of the sequence reveals close homologies with the calcium binding protein parvalbumin. Kretsinger and Barry (1975) used the parvalbumin structure as a basis of a model for TnC and suggested that it contained 4 Ca^{2+} binding domains each of which consisted of the classical helix-loop-helix or EF-hand. Leavis et al (1978) used the apparent calcium binding constants of each site to suggest that sites III and IV were the functional ones and that when calcium was bound at these sites a conformational change occured as revealed by an increase in a-helical content.

(I. 2. v) Troponin I (TnI).

Troponin I is a basic protein that has the ability to bind to TnC and actin, and which has an inhibitory effect on the actin activated ATPase activity of myosin (Schaub and Perry (1977)). Rabbit skeletal TnI has a molecular weight of 20,864 and contains 179 amino acids (Wilkinson and Grand (1975)).

(I. 2. vi) Troponin T (Tnt).

Troponin T has a molecular weight of 30,503 and contains 295 amino acids. TnC binds to tropomyosin at a specific site and it is this specificity that results in the 38.5 nm periodicity of the troponin complex on the actin filament (Ebashi (1972)). Digestion studies have revealed that the C-terminus of TnT is responsible for binding TnI whilst the N-terminus is responsible for actin binding.

(I. 3) The structure and composition of the thick filament proteins.

(I. 3. i) The stucture of the thick filament.

The thick filaments present in the sarcomeres of vertebrate skeletal muscle are composed almost entirely of myosin. Native thick filaments are therefore polymers of myosin molecules which visualisation in the electron microscope has revealed to be of remarkably uniform length and width (1.6μm and 12nm respectively) (H. E. Huxley (1963)).
Native myosin thick filaments consist of a central shaft from which numerous structures project along almost all of its length (See Figure 1.1). These projections are known as cross bridges and are probably formed from the S1/S2 portion of the myosin molecules (see Section I.1.iii). The native thick filaments are bipolar with the molecules in one half of the filament being packed in the opposite direction to those in the other half. The change in polarity occurs at the centre of the filament and is visualised in the electron microscope as the central bare zone region which is so called because it is completely devoid of cross bridge structures (H. E. Huxley (1967)).

The change in polarity at the centre of the filament is essential if the sarcomeres are to shorten by the movement of the thin filaments in each half of the sarcomere towards the centre of the thick filament. A detailed picture of the structure of the native thick filament has proved difficult to obtain and is discussed in more detail in the introduction to Chapters IV and V.

(I. 3. ii) The structure and composition of the myosin molecule.

The detailed structure of the myosin molecule was first visualised in the electron microscope by Lowey et al (1969). The molecule is highly asymmetric consisting of a long slender tail (approximately 150 nm long) (Elliot and Offer., 1978; Walker et al., 1985) with two globular or pear shaped heads (approximately 20 nm long) (Lowey et al (1969), Elliot and Offer (1978) at one end (See Figure 1.2). Analysis of the polypeptide chain composistion using polyacrylamide gel electrophoresis has revealed that the molecule consists of 6 polypepetide chains, two myosin heavy chains (MW 223,000 (Strehler et al (1986)), two DTNB (Dithiobis-nitrobenzoate) (MW 18,000 (Lowey and Risby (1971))) and two alkali light chains ALC1 and ALC2 (MW 21,000 and 17,000 (Frank and Weed (1974))respectively).
The naming of the light chains is for historical reasons related to their method of extraction from the myosin. The DTNB light chain is so called because of the ability to remove it using Dithiobis-nitrobenzoate (DTNB) (Weeds (1969)), however it is also referred to as light chain 2 (LC2) because on SDS gels it runs between ALC1 and ALC2. The DTNB light chain is also referred to as the regulatory light chain because of its role in the regulation of the ATPase in molluscan and smooth

muscle myosins (See Sections I. 6. ii and I. 6. iii). The alkali light chains (ALC1 and ALC2) derived their name from the ability to remove them in alkaline solutions (Gaetjens et al (1968)) although they are sometimes referred to as light chains 1 and 3 respectively. This nomenclature arises from the fact that on SDS gels ALC2 is more mobile than the RLC (LC2) wherease ALC1 is less mobile. However these are not the only techniques available for the removal of the light chains eg. treatment with LiCl or potassium thiocyanate (Gershman and Dreizen (1970)), guanidine HCl (Dreizen et al (1966)), and EDTA (Kendrick-Jones (1974)) all to varying extents remove the light chains from various types of myosin.

In the native myosin molecule the two heavy chains come together to form an a-helical coiled coil tail (Lowey et al., 1969; Weeds and Pope., 1977) and two globular heads each of which contains a pair of light chains one RLC and one ALC (Figure 1. 2). A detailed picture of the structure and relationship of each of these polypeptides has now been obtained and will be discussed in more detail in Chapters III and V.

(I. 3. iii) Proteolytic subfragments of the myosin molecule.

The myosin molecule possesses two regions in its sequence that are particularly susceptible to proteolytic digestion. The presence of these sites has allowed the myosin molecule to be dissected and the properties of the subfragments deduced, thus enabling the function of the various regions of the intact molecule to be determined. Unfortunately the exact position of the cleavage and therefore the relative size of the proteolytic fragments varies depending on the conditions used for digestion. Therefore it is not unusual for several

different workers to quote quite different values for the size and molecular weight of their fragments. The important feature about myosin digestion is however that there are only two sites at which cleavage readily occurs and that alteraction of the conditions does not create new ones. The principle digestion site lies in the myosin tail and cleavage at this point using either trypsin or chymotrypsin yields two subfragments, heavy meromyosin (HMM) and light meromyosin (LMM) (Weeds and Pope (1977)) (See Figure 1.3). LMM has a molecular weight of approximately 150,000, is 90 nm long and does not possess any actin binding or ATPase properties, however it does have the ability to aggregate into filamentous structures at low ionic strengths (Katsura and Noda., 1973b; Yagi and Offer., 1981;). This suggests that it is the LMM portion of the myosin molecule that aggregates to form the shaft of the thick filament. HMM on the other hand has a molecular weight of 350,000 (Margossian and Lowey., 1982), is approximately 80 nm long and possesses actin binding properties and ATPase activity similar to those of myosin but unlike intact myosin it is soluble even in low ionic strengths (Wells and Bagshaw., 1985). The solubility of HMM in low ionic strength is presumably due to the fact that the missing LMM portion contains all of the aggregating properties of the myosin molecule.

Further digestion of HMM using trypsin, chymotrypsin or papain results in cleavage at the other major susceptible site to yield three more subfragments (Lowey et al., 1969) (Figure 1.3). Two of the subfragments are identical possessing a molecular weight of 90,000 (Weeds and Pope., 1977)and they retain the actin binding properties and ATPase activity (Muller and Perry., 1962). These subfragments are the globular heads of the myosin molecule and are called subfragment 1

(S1). SDS gel analysis of the S1 subfragment has revealed that it contains a pair of light chains (one RLC and one ALC) (Chin., 1981)) which are highly susceptible to digestion themselves (Stone and Perry., 1973). In order to retain a significant proportion of the light chains during S1 production it has been shown that the digestion must be carried out in the presence of divalent cation (Margossian et al., 1975; Chin., 1981) which protects them from proteolysis. The third component is the rod like portion of the HMM molecule which is approximately 50 nm long, has a molecular weight of 60,000 (Weeds and Pope., 1977)and is called subfragment-2 (S2). S2 does not possess ATPase activity nor does it aggregate in low ionic strength conditions which suggests that its role in the intact myosin molecule is to hold the S1 heads in a certain position rather than being involved in filamentogenesis.

(I. 4) Myosin ATPase activity and the effects of actin interaction.

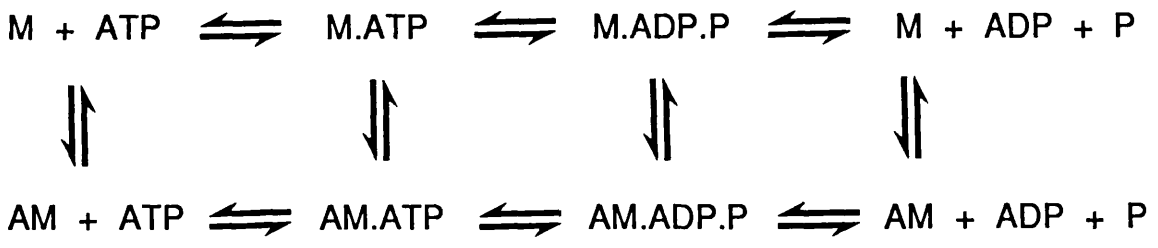
The hydrolysis of ATP by myosin can be simply represented by the following scheme :-

$$M + ATP \longrightarrow M.ATP \longrightarrow M + ADP + P$$

(Where M = Myosin).

However quenched flow studies (Lymn Taylor., 1970) showed that there was a rapid (< 200 ms)burst of phosphate (P)after which point its production rate levels off to a steady state approaching that of the overall ATPase reaction. The rapid build up of P was explained by suggesting the existence of an intermediate M.ADP.P (where M = myosin) that preceeded the rate limiting step therefore Lymn and Taylor proposed the following scheme which also incorporated the

corresponding interactions that must take place in the presence of actin.



The scheme was further complicated by Trentham et al (1972) who proposed that there was at least one slow step before the formation of the M.ADP.P complex. They demonstrated that there was no initial burst of P or ADP release from myosin and that both were similar to the steady state ATPase rate. However in the absence of ATP the production of an M.ADP complex resulted in a much more rapid release of ADP than when the starting components were M.ATP. This discrepancy was explained in terms of the slow formation of intermediate or transient states before the appearance of M.ADP.P. These intermediates could well be conformationally unique configurations of the M.ATP and/or M.ADP.P complexes.

In 1972 Eisenberg et al proposed that two states of myosin were present one of which bound to actin very slowly the other very rapidly, each of these states was termed the refractory and non refractory states respectively. The existence of these states was based on the following evidence. When the ATPase rate of HMM was close to maximal (80%) in the presence of ATP and actin only about 40% of the HMM was bound to actin, wherease 80% of S1 was bound, indicating that at maximum activity only 25% of the heads were bound to actin at

any one instant. The myosin must therefore undergo some slow change before it can bind to actin. Furthermore Chock et al (1976) demonstrated that the rate of recombination of M.ADP.P with actin reaches a plateau with increasing actin concentration as opposed to increasing linearly. Both of these results suggest that the myosin head containing the hydrolysis products is initially incapable of binding to actin and must undergo some sort of internal rearrangement in order to do so. The rate of this rearrangement is obviously equal to the rate of the plateau described by Chock et al (1976) for M.ADP.P recombination with actin.

The ATPase scheme shown over is a relatively simple one which has been considerably extended in recent years to include many more intermediate states. However the original model proposed by Lymn Taylor (1970) still holds true for the major events that are occuring and can readily be used to interpret the gross structural changes that occur in the cross bridges during contraction in terms of chemical activity at the active site (See Section I. 5).

$$\begin{array}{ccccccccccc} M + ATP & \rightleftharpoons & M.ATP & \rightleftharpoons & M^*.ATP & \rightleftharpoons & M.ADP.P & \rightleftharpoons & M.ADP + P & \rightleftharpoons & M + ADP \\ \Updownarrow & & \Updownarrow & & \Updownarrow & & \Updownarrow & & \Updownarrow & & \Updownarrow \\ AM + ATP & \rightleftharpoons & AM.ATP & \rightleftharpoons & AM^*.ATP & \rightleftharpoons & AM.ADP.P & \rightleftharpoons & AM.ADP + P & \rightleftharpoons & AM + ADP \end{array}$$

(I. 5) The changes in cross bridge structure that occur during the contractile cycle.

The crucial finding of the kinetic studies was that ATP weakens the A.M interaction whilst M.ADP.P has a high affinity for actin. These two

findings alone support the proposition that contraction occurs as a result of the cyclical attachment of and release of the myosin heads from the thin filaments. The structural transients that occur between attachment and detachment are obviously of major importance as it is presumably during this phase that force is generated. However the identification of the structural changes that occur during this phase has proved difficult to achieve. Two modes of cross bridge attachment have been consistently observed in the electron microscope and they are those states present in the relaxed and rigor configurations.

In the rigor condition (ie. in the absence of ATP) electron micrographs of muscle sections show that the myosin heads are attached to the thin filaments at a sharp angle (Huxley and Brown., 1967; Wray et al., 1978) of 45 ° in such a way as to give the appearance of numerous arrowheads all pointing away from their nearest Z-line. In the relaxed state (i.e. presence of ATP) however the myosin heads project directly out from the thick filaments and probably attach to the thin filaments at an angle of 90° (H. E. Huxley and Brown., 1967; Wray et al., 1975; Crowther et al., 1985).

The angle of the head in the relaxed state has been generally accepted as representing the beginning of the power stroke whilst the angle in rigor represents the position at the end of the power stroke. In the classical scheme of things these changes have been linked to the biochemical changes to produce a simplistic model for the major events that occur during muscle contraction as shown in Figure 1.4. It must however be emphasised that attempts to visualise conformational changes in the myosin heads during the various stages of the cross bridge cycle in the electron microscope have largely proved unsuccessful (Padron et al., 1988).

Alternative approaches have also failed to produce a common and consistent picure. For instance fluorescent probes such as IAEDANS when covalently linked to the reactive SH1 group of the myosin molecule have revealed that angular reorientation of the S1 relative to the actin filament does occur during various stages of the contractile event (Burghardt et al., 1983; Ajtai and Burghardt., 1987). However spin labels, when attached to the same SH1 group show that no angular reorientation of the S1 occurs (Thomas et al., 1985). One likely explanation for such anomalous results is that during force generation the domains within the myosin heads move relative to one another and that such movements are picked up by one type of probe but not the other.

(I. 6) Regulation of the myosin / actin interaction.

The regulation of the interaction of myosin with actin is obviously of fundamental importance as it is this regulation that will determine whether a muscle is actively contracting or not. Several regulatory systems exist throughout the animal kingdom ranging from solely thin filament linked systems (vertebrate skeletal muscle) to a solely thick filament linked system such as that of molluscan muscles and vertebrate smooth muscles. However there is one common feature to each of these methods of regulation and that is that the trigger for the onset of contraction is the presence of the calcium ion.

(I. 6. i) Regulation in vertebrate skeletal muscle.

The classical mode of regulation in this muscle is entirely thin

filament linked although evidence presented later (See Part B) and recent evidence by other workers (Haselgrove., 1975; Lehman., 1977; Chin., 1980; and Farrow et al., 1988) suggests that this may not be entirely correct and the thick filaments may indeed play a role in regulation. However for now we will only consider the thin filament regulatory system.

When the free calcium levels rise to 10^{-5} M or above calcium binding to TnC results in a conformation change which spin labelling studies (Ohnishi., 1975) have shown is also transmitted to both TnI and TnT which occurs in such a way as to prevent TnI from binding to the actin (Potter and Gergely., 1974). The results of such alterations in the interactions are that TnT is now able to move the tropomyosin strand into the actin groove (See Section I. 2. ii) exposing the myosin binding sites on the actin monomers thus resulting in myosin binding and ATPase activation. This model is known as the steric hindrance model and was first proposed by Potter and Gegerly (1974)). X-ray evidence has clearly demonstrated that tropomyosin occupies two main positions on the thin filament (See Section I. 2. ii). Makawa (1979) used gluteraldehyde fixation of reconstituted thin filaments in either the presence or absence of calcium to demonstrate that tropomyosin could be trapped in one of the two orientations. When the filaments had been fixed in the presence of Ca^{2+} and myosin was added the ATPase became permanently activated, whilst the ATPase of those fixed in its absence was permanently switched off. It must however be emphasised that the mechanism by which the TnT moves the tropomyosin strand away from the myosin binding sites on the actin remains relatively obscure.

(I. 6. ii) Regulation in molluscan muscles.

The thin filament regulatory proteins present in vertebrate skeletal muscles is poorly defined and largely absent from molluscan muscles (Kendrick-Jones et al., 1970; Goldberg and Lehman., 1978). The demonstration by Szent-Gyorgyi et al (1973) that the calcium sensitivity of the molluscan myosin ATPase was completely abolished by the removal of one mole of RLCs/mole myosin by incubation in EDTA at 4 °C was the first demonstration that in this particular muscle type ATPase regulation was entirely myosin linked. Moreover, after RLC removal the ATPase became permanently activated suggesting an inhibitory role for the RLCs. Chantler and Szent-Gyorgyi (1980) later demonstrated that at elevated temperatures (25-30 °C) all of the RLCs (2 moles/mole myosin) could be removed resulting in the permanent activation of the ATPase and loss of calcium sensitivity.

The interesting feature about RLC depleted molluscan myosins is that the RLCs can be reassociated with RLC denuded myosin to stoichiometric amounts with the resultant depression of the ATPase and regain of full calcium sesitivity. The ability of RLC denuded molluscan myosins to bind foreign RLCs has allowed the properties of hybrid myosins to be examined and the role of foreign RLCs be assessed (See Section III. 2). A detailed account of the location, stucture and function of molluscan RLCs, along with those from other muscle types, is given in the introduction to Chapter III and will not be discussed further here.

(I. 6. iii) Regulation in smooth muscle.

Another form of myosin linked control exists in vertebrate smooth muscle and non muscle cells such as macrophages (Adelstein and Conti., 1975; and platelets (Trotter and Aldstein., 1979). In these systems regulation of the ATPase activity is through the RLCs, which is analagous to the situation in molluscan myosins. However in smooth and non muscle cells activation of the ATPase is achieved by phosphorylation of the RLC under the influence of a Ca^{2+} sensitive kinase (Small and Sobieszek., 1977). Dephosphorylation by phosphatase occurs when the calcium concentration drops and results in a decrease in the ATPase activity (Small and Sobieszyik., 1980).

The specific sequence of events is as follows (See Figure 1.5). Contraction is triggered by an increase in the free Ca^{2+} concentration to 10^{-5} M or greater which results in the formation of a calcium-calmodulin complex. The calcium-calmodulin complex binds to and activates a myosin light chain kinase which then phoshorylates the RLCs and causes myosin ATPase activation and contraction. When the Ca^{2+} concentration drops to 10^{-7} M the calmodulin dissociates from the myosin kinase thus inactivating it, allowing phosphatase to dephosphorylate the RLCs and cause relaxation to occur.

The phosphorylation system in smooth muscle can also be influenced by hormonal stimulation of the ß-receptor cells in the cell membrane (Conti and Aldstein., 1980). ß-receptor stimulation results in an increase in the levels of cyclic-AMP by the activation of adenylate cyclase. Cyclic-AMP dependant activation of a protein kinase reults in myosin light chain kinase phosphorylation which weakens the binding of calcium-calmodulin to it. The dissociation of the calcium-calmodulin

from the phosphorylated myosin light chain kinase deactivates the kinase and allows RLC dephoshorylation and muscular relaxation to occur (Krebs and Bearo., 1979; Silver and Disalvo., 1979).

The regulation of the ATPase activity in vertebrate smooth and non muscle myosin is further complicated by the fact that phosphorylation of the RLCs controls filament assembly (Suzuki et al., 1980); Citi et al., 1988). However the exact relationship between the ATPase activity and the degree of filament assembly still remains unclear.

The first to demonstrate that smooth muscle assembly was controlled by phosphorylation was Suzuki et al (1980). They showed that in the absence of Ca^{2+} and at physiological ionic strengths, non-phosphorylated smooth muscle filaments in the presence of calmodulin and myosin light chain kinase were fully depolymerised by the addition of ATP. However the addition of calcium resulted in the activation of the calmodulin/kinase complex causing RLC phosphorylation and myosin filament formation. Similar findings have since been reported with non muscle myosin such as thymus (Scholey et al (1980))and brush border myosin (Citi et al., 1987); Citi and Kendrick-Jones., 1987 and 1988). These results all indicate that in relaxed smooth muscle or non muscle cells non phosphorylated RLCs somehow prevent filament assembly. However following activation the RLCs are phosphorylated by the calcium-calmodulin/kinase complex which causes filament assembly and ATPase activation to occur.

Such a scheme suggests that during relaxation smooth muscle myosin filaments completely depolymerise until they are needed for contraction. However as pointed out by Kendrick-Jones et al (1982) ultrastructural studies have revealed the presence of thick filaments in relaxed smooth muscle cells. Such evidence could be indicative of

the presence of other factors such as copolymerising proteins being present in native filaments and that these help to stabilise the structures in relaxing medium. Indeed Citi et al (1987) demonstrated that non muscle myosin filaments can be stabilised when dephosphorylated in high concentrations of ATP by the incorporation of vertebrate skeletal muscle myosin into the the thick filaments.

Part B.

Does myosin possess distinctive high salt sensitive properties ?

(I. 7) Conventional myosin filaments.

The ability to dissolve myosin into monomeric solution by the exposure of myofilaments to high salt conditions (> 0.5 M KCl)has been exploited in order to develop myosin extraction and purification techniques (Perry., 1955a; Offer et al., 1973; Bremel and Weber., 1975). Once dissolved into monomeric solution the myosin can be separated from the other muscle proteins and re-formed into structures ressembling native thick filaments by dialysis back to physiological ionic strengths (approximately 100 mM KCl). We term filaments prepared in such a fashion as conventional or synthetic myosin filaments (SMFs). Although the structure of these filaments superficially appears to be the same as that of native filaments, closer examination has shown them to be quite different with the exact structure of the SMFs depending on the conditions used for their formation (See Section IV.8). Although filaments of relatively uniform length and diameter can,

under the right conditions be produced (Huxley., 1963; Persechini and Rowe., 1984) they are invariably unipolar or at least do not possess a well defined central bare zone (See Section IV. 8). Such differences have led to speculation that during the extraction and purification procedure the myosin has been irreversibly altered and lost some of its native properties.

(I. 8) The ATPase activity of conventional myosin.

Conventional myosin filaments, the enzymatic subfragments of high salt extracted myosin and its RLCs have been studied under a variety of conditions in an attempt to demonstrate the presence of a myosin linked ATPase regulatory system similar to that found in molluscs. All such attempts have been largely unsuccessful (Weeds., 1969; Pemrick., 1977; Yamamoto et al., 1980)although some workers have demonstrated that vertebrate skeletal myosin is capable of specifically binding calcium (Bagshaw and Reed., 1977; Morimoto and Harrington., 1974)the role of these binding sites was uncertain. Lehman (1978) did suggest the presence of a myosin linked calcium sensitivity in skeletal muscle myofibrils. In this instance the myofibrils were washed in low ionic strength buffers in order to selectively extract the troponin and tropomyosin (Perry., 1977). Once almost complete extraction was achieved the myofibrils still displayed a calcium sensitive ATPase activity which was attributed to the thick filaments. However experiments involving selective troponin and tropomyosin extraction are difficult to interpret and can be misleading because it is possible that residual proteins can still confere actin linked calcium sensitivity. In this way the residual calcium sensitivity

of the actin linked system could be misinterpreted as being evidence of the presence of a myosin linked system.

(I. 9) The need for the production of purified native myosin filaments.

The only way to unambiguously study the biochemistry of thick filaments is to extract and purify these filaments in their native state without the need for high salt exposure. The production of a relaxed filament preparation (See Sections II. 27 and II. 28) has been the first step in all techniques thusfar attempted. A relaxed filament preparation consists mainly of individual thick and thin filaments together with other larger components such as I-segments and A-segments. The large difference in s-value between the thick (140s) and thin (40s) filaments has then been utilised in order to seperate them by ultracentrifugation. However even in relaxing solutions there is still considerable interaction between thick and thin filaments which reults in co-sedimentation and thus prevents complete purification. As a result of this difficulty only a limited number of groups have attempted to purify native filaments (Trinick and Rowe., 1973; Trinick., 1982; Morimoto and Harrington., 1974; Emes and Rowe., 1978b) each of which achieved varying degrees of success. However the most successful of these techniques was the method described by Emes and Rowe (1978b) who achieved >98% purity with respect to actin. Chin (1981) used a modified version of the Trinick and Rowe (1973) method that involves separation on D_20/H_20 relaxing medium gradients and achieved a slightly greater purity than Emes and Rowe, although approximately 1% actin still remained.

(I. 10) Purified native thick filaments do possess salt labile properties.

By purification on D_2O/H_2O gradients Chin (1981) was able to demonstrate for the first time that the ATPase of purified native myosin filaments was indeed calcium sensitive and that after exposure to high ionic strength conditions the calcium sensitivity was lost. However the presence of small amounts of actin in Chins preparations can be used to dispute the suggestion that it is the myosin ATPase that is calcium sensitive. The ATPase activity of conventially prepared myosin whilst being insensitive to calcium is highly sensitive to ionic strength. At low ionic strengths the ATPase activity is high but as it is raised to physiological levels and higher it becomes greatly supressed. However Chin (1981) showed that the ATPase activity of purified native filaments was extremely low and displayed very little ionic strength dependance remaining low even at reduced ionic strenghts. Once the myosin in the purified filaments had been exposed to high salt (> 200 mM)the ionic strength dependance of its ATPase became identical to that of conventionally prepared myosin. Bolger et al (1989a) made further improvements to Chin's purification technique and obtained a greater yield and purity (>> 99% w.r.t. actin) of native thick filaments (See Section II. 29). Using this technique Bolger et al (1989b) have demonstrated that the purified native filaments (PNFs) do indeed display calcium sensitive ATPase activity and that in the presence and absence of pure unregulated actin the addition of 100 μM Ca^{2+} causes a 2-fold activation. The ability to activate the ATPase rate is however completely abolished by transient exposure to high salt (>0.5 M KCl)conditions. Furthermore using a chymotryptic probe technique they have demonstrated the presence of two equally

distributed populations of RLCs one of which is resistant to digestion, the other being susceptible. The same technique when carried out on conventional myosin or transiently high salt exposed PNFs reveals only one population of RLCs which are all susceptible to chymotryptic digestion . This has been interpreted as indicating the presence of a unique conformation or environment for 50% of the RLCs in PNFs and that this is completely and irreveribly lost by exposure to high salt conditions.

(I. 11) The polypeptide chain composition of PNFs.

When the polypeptide chain composition of conventionally prepared SMFs is examined on SDS-PAGE gels proteins other than those associated with the myosin heavy chain, such as the light chains, are not observed to be present. However analysis of the proteins present in PNFs reveals several bands that are not associated with the myosin monomer (See Gel 1.1). Two bands are clearly visible above the myosin heavy chain whose molecular weight must be significantly larger than 200 kD. These bands could possibly represent the high molecular weight protein Titin (Wang and Ramirez-Mitchell., 1983), which itself could well be a part of the end filaments seen at the tips of low ionic strength frayed thick filaments (Trinick., 1982; See Section IV. 6). Immediately below the myosin heavy chain is a triplet of bands one of which is C-protein (See Section IV. 9). C-protein is known to be associated with the cross bridge region of the thick filament (Offer et al., 1973; Pepe and Drucker., 1979) and has been readily incorporated into myosin filaments as they reassemble from monomeric myosin. The other two bands have however not yet been identified. Three minor

bands are present in the 60 - 80 kD region which Bolger et al (1989a) have called filament co-proteins (FCPs)1 to 3 in order of decreasing mobility. Only FCP1 has been tentatively identified as phosphofructokinase (PFK) because of its identical mobility with muscle PFK on SDS-PAGE.

(I. 12) Aims of this thesis.

PNFs possess components and properties that are irreversibly lost after transient exposure to high salt conditions. The work in this thesis was designed to continue to explore the salt sensitive differences between native myosin filaments (PNFs) and synthetic myosin filaments (SMFs). We address the role that the RLCs play in the regulation of the ATPase activity and calcium sensitivity of PNFs and also examine the effect that calcium has on the properties of the cross bridges. Experiments are also carried out in an attempt to determine whether the distinctive properties of native myosin arise from a unique packing arrangement within the thick filament or from a unique tertiary structure of the individual myosin molecules themselves.

Chapter I

Figures and Gels.

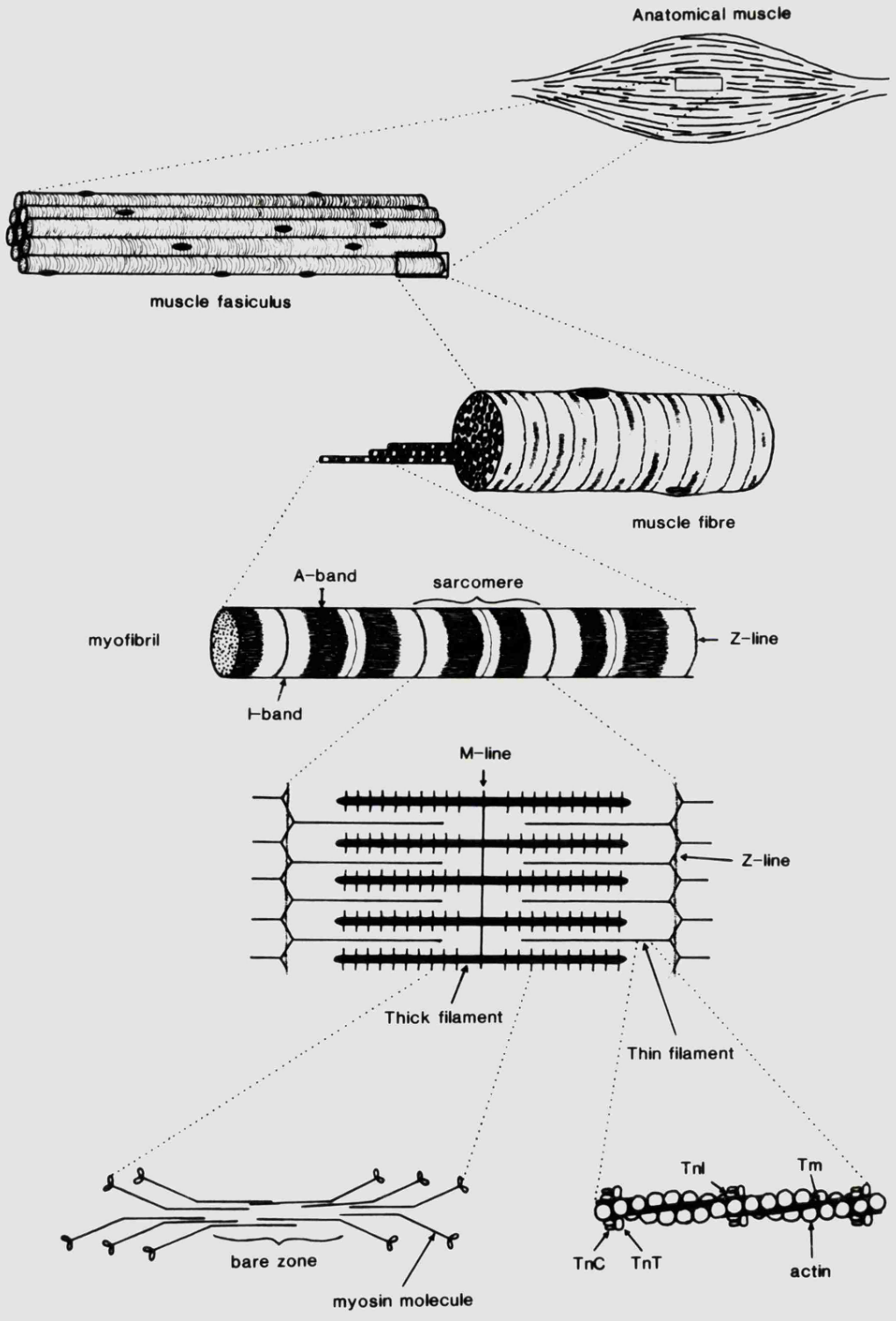

Anatomical muscle
muscle fasiculus
muscle fibre
A-band
sarcomere
myofibril
Z-line
I-band
M-line
Z-line
Thick filament
Thin filament
bare zone
myosin molecule
TnI
Tm
TnC
TnT
actin

Figure 1.1

Diagramatic representation of the various level of organisation present in a vertebrate striated skeletal muscle.

A whole anatomical muscle consists of bundles of muscle fibres called muscle fasiculi, the individual fibres of which are in fact muscle cells which are long multinucleate structures that can be several centimeters long. When viewed in the light microscope using polarised light the muscle fibres possess uniform and highly ordered cross striations from which the muscle type derives its name. The muscle fibres themselves are densely packed with cylindrical organells termed myofibrils which contain the basic unit of contraction called the sarcomere. The sarcomere is delimited by the Z-lines and contains a highly ordered array of interdigitating thick and thin filaments (myofilaments). It is the ordered nature of these filaments that produces the characteristic banding pattern in the myofibril and hence that of the muscle fibre. Contraction occurs when the sarcomere shortens by allowing the two sets of filaments slide over each other in such a direction that the Z-lines are drawn towards the M-line. The thick filaments are bipolar assemblies consisting mainly of myosin whilst the thin filaments are composed mainly of actin in the form of a two fold helix. The energy for the sliding of the filaments comes from the hydrolysis of ATP by the myosin which is markedly activated by its interaction with actin. The thin filaments also contain the so called regulatory proteins which are tropomyosin (Tm), troponin T (TnT), troponin I (TnI) and troponin C (TnC). It is through these proteins that Ca^{2+} exerts its regulatory effect on the contractile process by altering the the degree of interaction between the myosin and actin.

(Adapted from W. Bloom and D. W. Fawcett, A Textbook of Histology, 10th ed., Philadelphia, W. B. Saunders Co., 1975.)

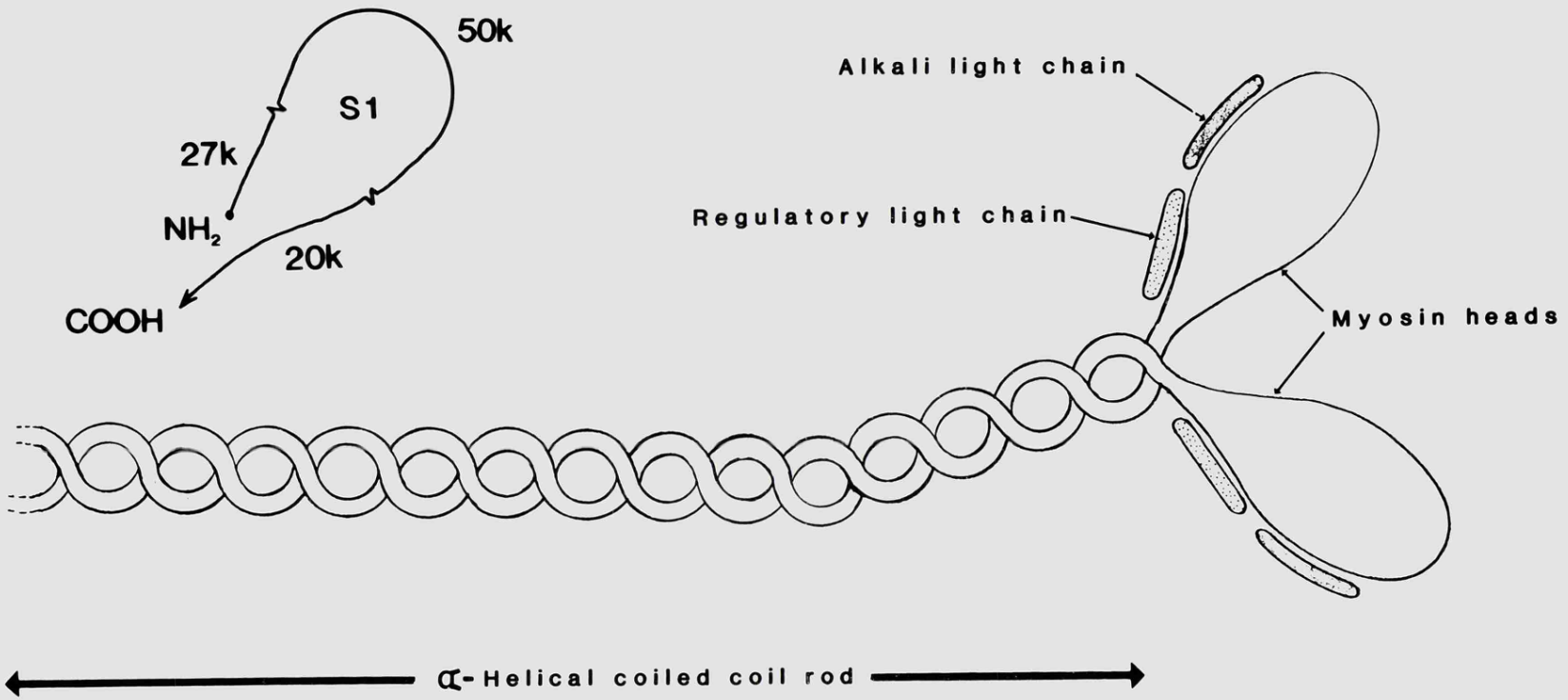

50k
S1
27k
NH2
20k
COOH
Alkali light chain
Regulatory light chain
Myosin heads
α-Helical coiled coil rod

Figure 1.2

Main Figure.

Diagramatic representation of a myosin molecule. The molecule is highly assymetrical consisting of two heavy chains (MW 223 kD) that come together to form a long ∝-helical coiled coil tail and two globular (S1) heads. Each head also contains a pair of light chains one of which is termed an alkali light chain (ALC) the other a regulatory light chain (RLC) and an actin binding and ATP hydrolysing site. The C-terminus of the heavy chain is located at the tip of the tail whilst the N-terminus is located in the head region.

Inset.

Individual S1 myosin heads probably contain a number of heavy chain domains, as shown. Tryptic digestion has revealed the presence of three domains of molecular weight approximately 20k, 50k and 27k in order from the C-terminus, each domain being separated by a short (1 - 2 kD) linker region that is labile to digestion (Holmes and Goody., 1984). Although the structural significance of these domains still remains obscure it is known from cross linking studies (Holmes and Goody (1984)) that actin binds to the 20 and 50k domains but not the 27k.

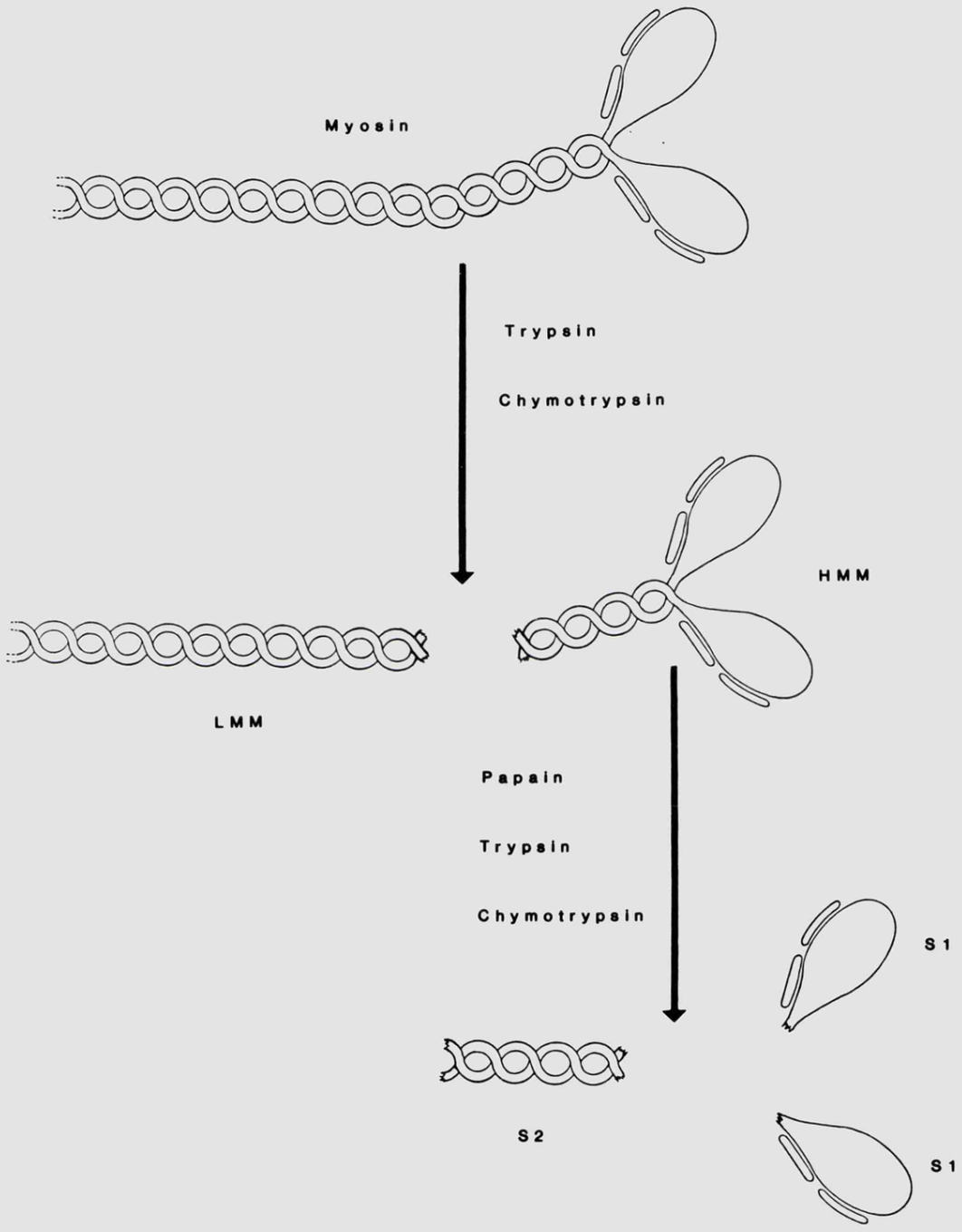

Myosin
Trypsin
Chymotrypsin
HMM
LMM
Papain
Trypsin
Chymotrypsin
S1
S2
S1

Figure 1.3

The cleavage of myosin at its two proteolytic sensitive sites by various enzymes. The exact point of digestion varies slightly depending upon the conditions and particular enzyme being used.

The major proteolytic fragments are shown opposite :-

Heavy meromyosin (HMM) which is readily soluble in low ionic strengths.

Light meromyosin (LMM) which contains the aggregating properties of the myosin.

Subfragment 2 (S2) which is similar in structure to LMM but does not possess the same aggregating properties.

Subfragment 1 (S1) which contains the actin binding and ATP hydrolysing properties of the myosin.

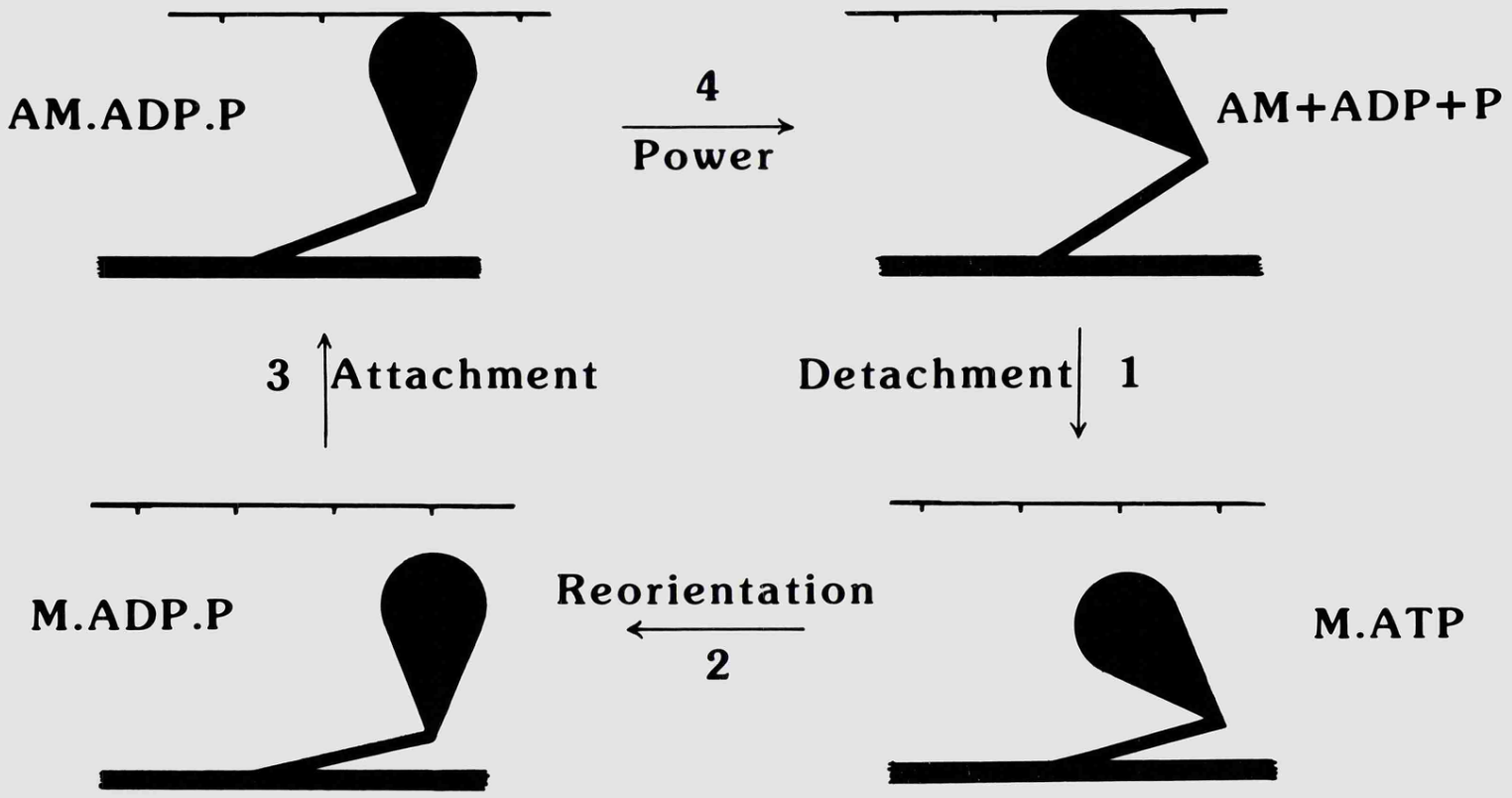

AM.ADP.P
4
Power
AM+ADP+P
3
Attachment
Detachment
1
M.ADP.P
Reorientation
2
M.ATP

Figure 1.4

Showing the probable sequence of structural and chemical events that occur during muscular contraction. The figure shows a myosin S1 head attached to the shaft of the thick filament by the S2 region of the molecule (See Figure 1.3). The thin filament is shown as a thin line with equally spaced graduations representing the myosin binding sites on the actin molecules.

1. Binding of ATP to myosin followed by the release of the head from the thin filament.
2. ATP hydrolysis occurs and is accompanied by a reorientation of the myosin head.
3. The myosin head binds to the thin filament.
4. ATP hydrolysis products are released and the myosin head undergoes another configurational change generating power. The thin filament is moved from right to left.

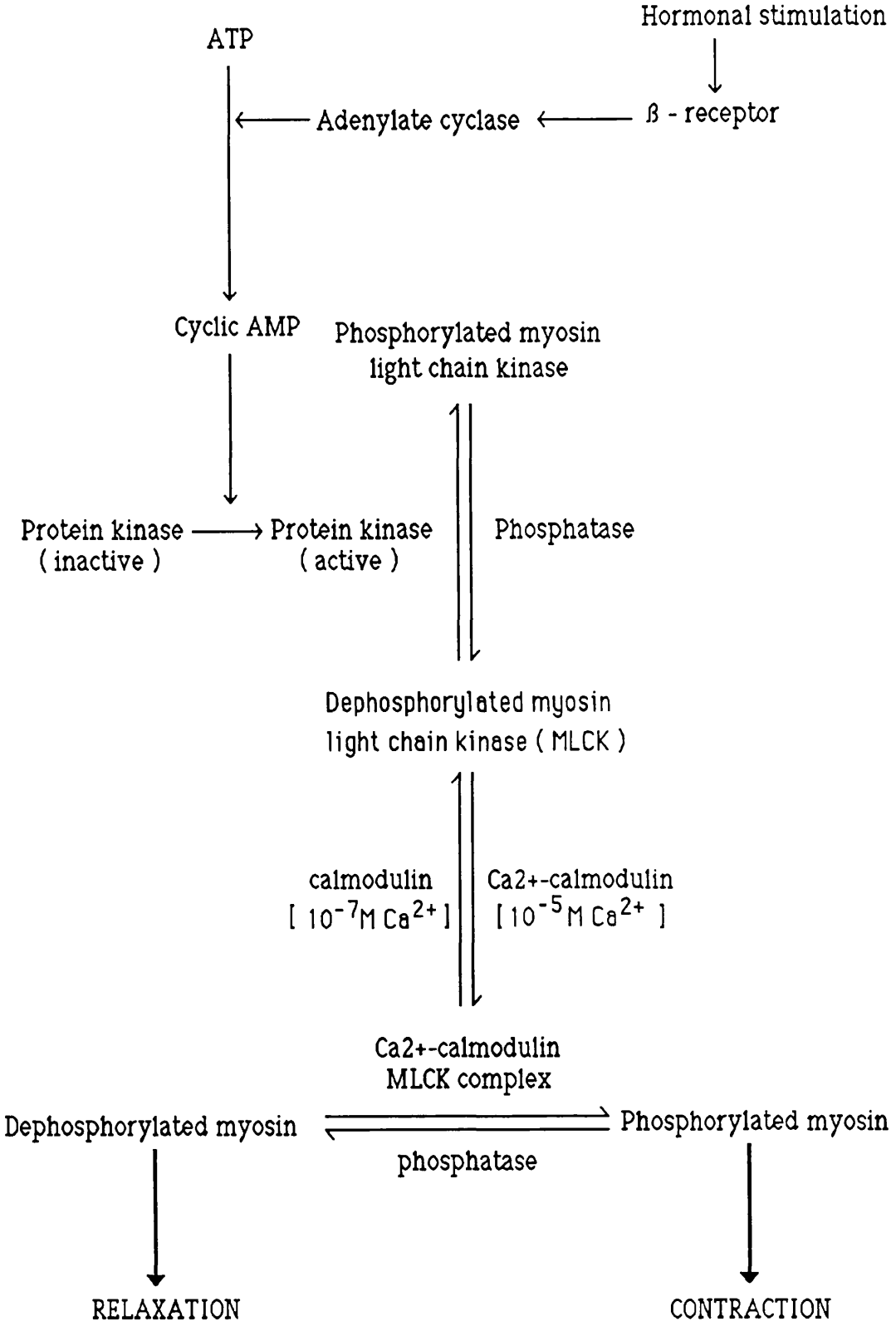
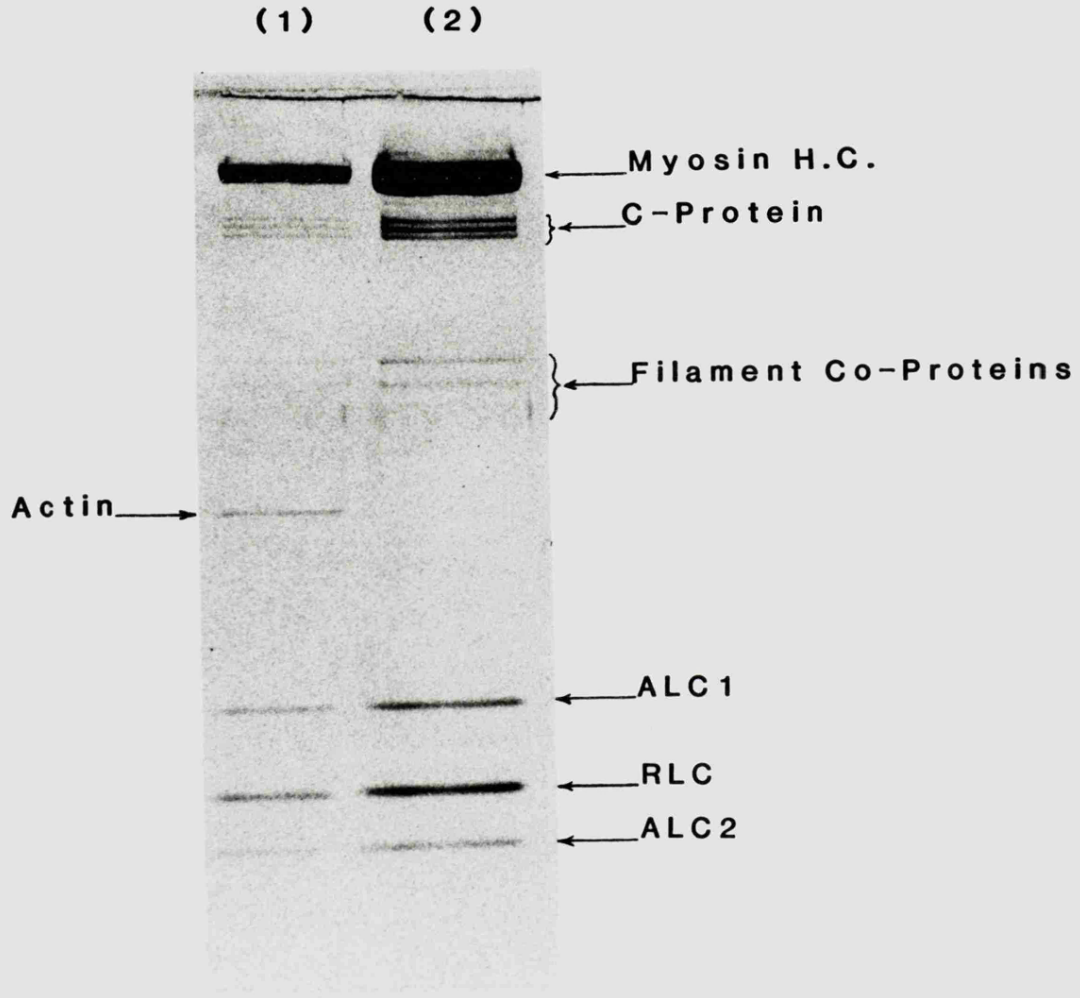

Hormonal stimulation
ATP
Adenylate cyclase
ß - receptor
Cyclic AMP
Phosphorylated myosin light chain kinase
Protein kinase (inactive)
Protein kinase (active)
Phosphatase
Dephosphorylated myosin light chain kinase (MLCK)
calmodulin [10-7M Ca2+]
Ca2+-calmodulin [10-5M Ca2+]
Ca2+-calmodulin MLCK complex
Dephosphorylated myosin
Phosphorylated myosin
phosphatase
RELAXATION
CONTRACTION

Figure 1.5

The events involved in the control of the contraction and relaxation of smooth muscle.


(1)
(2)
Myosin H.C.
C-Protein
Filament Co-Proteins
Actin
ALC1
RLC
ALC2

Gel 1.1

The polypeptide chain composition of purified native filaments (PNFs) as revealed by polyacrylamide gel electrophoresis in SDS.

Lane 1). Native myosin filaments that are slightly contaminated with actin.
Lane 2). PNFs completely free of actin.

Chapter II

Methods

(II. 1) Analytical techniques.

(II. 2) Gradient SDS polyacrylamide gel electrophoresis (SDS PAGE).

The technique of SDS PAGE was essentially that of Laemmli (1970). Slab gels (dimensions 135 mm x 110 mm x 1.5 mm) were produced having a gradient of 7.5% to 20% acrylamide, by mixing solutions A and B (Table 2.1) to give a linear gradient. The top of the gel was covered with isobutanol and allowed to set at room temperature. Once the gel had set the isobutanol was immediately poured off (any delay can cause changes in the gel head resulting in distorted protein bands), the stacking gel (solution C) was then poured on top of the gradient gel and a nine pronged comb inserted to produce sample wells. Samples were prepared by adding 0.6 ml of the protein sample to 0.35 ml of sample buffer (0.1 ml Glycerol, 0.15 ml of 20% SDS, 0.1 ml Tris and 0.2% w/v bromophenol blue, pH 6.8) and 50µl ß-mercaptoethanol, or the appropriate ratios of the above three solutions and plunged into boiling water for 20 seconds. Slow warming of the sample must be avoided at all costs especially if proteolytic enzymes are present. Fairbanks et el (1971) have shown that many proteolytic enzymes are resistant to SDS denaturation, this combined with the increased susceptibility of the exposed peptide bonds of denatured proteins can result in partial

digestion before complete denaturation occurs.
Between 20 µg and 80 µg of protein were loaded into each sample well and run in a Tris/glycine running buffer (2000 mls containing 6 g Tris, 28.8 g glycine, 2 g SDS) at 200 volts, and a constant temperature. The run was stopped when the bromophenol blue marker band reached the bottom of the gel. The gel was removed and stained overnight in 10% acetic acid, 50% v/v IMS, 0.25 g w/v coomassie brilliant blue R-250. The gels were destained in 10% acetic acid until the background was clear.

(II. 3) Gelscan analysis.

Once fully destained the gels were placed on an LKB 2202 ultrascan laser densitometer linked to an Apple 11e microcomputer and an absorbance scan of each track was carried out. The absorbance traces were manipulated using a commercial software package (Gelscan) with which the area under each protein curve was estimated. By comparison of the relative area under each curve with that of a known reference protein, changes in stoichiometry can be followed (See Section III. 12). If an inordinate amount of noise was present in the traces then several scans of the same band were merged using locally written software (Gelmerge by J Marshall., 1986) and the average of these scans was used in the analysis.

(II. 4) Estimation of protein concentration.

Three techniques were employed for the estimation of protein

concentration. The technique used at any one time was determined by how the substances present in the buffers interfered with the assay. The approximate concentration of the protein under examination was also considered when making the choice of assay.

(II. 5) Protein absorbance at 280 nm.

When only small concentrations of nucleotide were known to be present protein concentrations were most conveniently measured using the absorbance at 280nm (A280). An absorbance scan was carried out between 240nm and 340nm using a Unicam SP800B Ultraviolet Spectrophotometer. The scan enabled a baseline to be fitted. The protein concentration was then calculated using the values for the $A^{1\%}_{1cm.280nm}$ of the various myosin fragments shown in Table 2.2.

(II. 7) The BCA protein assay.

BCA protein assay reagent was purchased from the Pierce Chemical Company (Illinois USA). This assay relies on the fact that proteins react with alkaline copper II to produce copper I. The BCA assay reagent reacts with copper I to form an intense purple colour at 562 nm. The key component of the BCA assay reagent is Bicinchoninic acid (BCA) which forms soluble alkali metal salts. The interaction of two molecules of BCA with one cuprous ion results in a purple colour with a maximum absorbance at 562 nm and it is this that is measured in the spectrophotometer.

The standard BCA protocol is as follows. The BCA working reagent is

prepared by mixing solutions A and B (provided by the manufacturer) in the ratio of 50 to 1 respectively. 2mls of the working reagent are then added to 0.1 ml of the unknown protein sample which is then incubated for 30 minutes at 37 °C. After incubation the sample is cooled to room temperature and the absorbance read versus the blank at 562 nm. Using a standard curve the protein concentration can now be estimated.

The final BCA colour does not reach a true endpoint, however the rate of colour formation is dramatically decreased by lowering the reaction temperature from 37 °C to room temperature.

The BCA protein assay (unlike the commonly used Bradford technique) shows only minor protein to protein variation and is not significantly affected by a wide variety of substances that may be present in the protein sample Table 2.3.

(II. 8) The Lowry method of protein concentration determination.

This technique was found to be ideal for protein concentrations below about 0.3 mg/ml. The main drawback of this technique being that it requires constant precise timing and mixing of the various solutions.

The protocol requires the daily production of Folin A reagent (100ml of 2% $NaCO_3$ (w.v), 1ml of 1% $CuSO_4.5H_2O$ (w.v), and 1ml of 2% $(CH(OH).COONa)_2.2H_2O$ (w.v)) and Folin B reagent (Folin Ciocalteu's Reagent) which must be made just prior to use.

The unknown protein sample was made upto 1ml with 1M NaOH and left at room temperature for 10 minutes, after which 145 µl of 3M HCl and 2.5 mls of Folin A reagent was added and the solution left to stand at room temperature for 20 minutes. To the mixture was then added 250

µl of Folin B reagent accompanied by rapid mixing. After leaving the solution to stand at room temperature for a further 45 minutes the absorbance versus a blank was read at 750 nm. The colour development does not reach a true endpoint and so the absorbance must be read at constant time for each sample used.

(II. 9) Sedimentation velocity analysis.

Sedimentation velocity analysis was carried out using scanning absorption optics on an MSE Centriscan-75 Analytical Ultracentrifuge or by classical schlieren optics on an MSE MK II Analytical Ultracentrifuge.

(II. 10) Scanning absorption optics.

For samples containing low concentrations of ATP (< 1 mM) scanning absorption optics at 295 nm were employed. The reason for using 295 nm and not 280 nm for the absorbance scan was that at the higher wavelength the absorption from ATP was considerably reduced whilst that of the protein remained reasonably high.
The samples were loaded into 20mm pathlength cells using a Gilson pipette employing slow controlled filling and evacuation to avoid generating shearing forces. The cells were placed in the appropriate rotor and immediately taken up to the required speed and temperature. Once at the required speed the position of the sedimenting boundary was recorded at fixed time intervals on a chart recorder. Sedimentation coefficients (s) were evaluated by determining the

position of the mid point of the sedimenting boundary for each time point, estimated at 50% of the optical density increment between solvent and plateau. These positions were digitised directly from the recorder traces using an Apple Graphics Tablet linked to an Apple II+ microcomputer. The program used (A. J. Rowe) permits the full precision of the tablet to be used and calculates the s-values corrected for rotor expansion, reference to reference calibration, true rotor speed and as is necessary with scanning systems the true time of recording of the boundary position.

(II. 11) Classical schleiren optics.

Whenever the ATP concentration was high (>1.0 mM) it was not possible to use scanning absorption optics due to the high background absorbance caused by the ATP. In these cases classical schleiren optics were employed to monitor the change in refractive index between the solvent and the sedimenting protein boundary.

Accurately determined volumes of all samples were loaded into 20mm pathlength cells. The samples were loaded by means of a Gilson pipette, once again avoiding shear by slow, controlled filling and evacuation.

Sedimentation coefficients (s) were evaluated by taking photographs of each schlieren pattern at fixed time intervals and determining the radial position of the mid-point of the sedimenting boundary on photographic enlargements using an Apple Graphics Tablet linked to an Apple II+ computer (using a program written by A.J. Rowe, this laboratory). Alternatively the negatives were projected directly on to

the graphics tablet thus eliminating the need for the production of photographic enlargements. The program used calculates the sedimentation coefficients corrected for rotor expansion, reference to reference calibration and rotor speed. The program also yields the average radial dilution and calculates the standard error of the estimate.

If necessary we were able to simultaneously run three samples in the same rotor. However one sample must be loaded in a plane window cell, one in a positive wedge window cell, and the other in a negative wedge window cell. This allows the schlieren pattern from all three cells to be recorded one above the other on the same negative. Unfortunately such a system reults in each trace having the background glare of the other two superimposed over it, thus making analysis difficult. This problem was circumvented by means of an analogue multiplexer (Emes., 1977) or masking system which filters out the background glare from each cell and only allows the signal traces to pass through.

(II. 12) Estimation of the free Ca^{2+} concentration using the fura-2 assay (Grynkiewicz et al., 1985) .

Fura-2 is a fluorescent analogue of the calcium chelators EGTA and BAPTA (Tsien., 1980) . At specific wavelengths its emission fluorescence is altered by the binding of calcium and these changes can be used to calculate the free Ca^{2+} concentration of a protein solution.

The assay involves monitoring the emission fluorescence of a protein solution, containing 0.5 μM fura-2, at 508 nm which is excited at 362

nm and 335 nm respectively. The ratio of the emission fluorescence at each wavelength (emission at 335 nm / emission at 362 nm) in both the absence of calcium and the presence of excess calcium is used to give the minimum (Rmin) and maximum (Rmax) fluorescence obtainable.
The emission spectra of the protein solution of interest, which contains an unknown level of calcium, is then recorded in the presence of 0.5 μM fura-2 at both excitation wavelengths and this ratio gives Rexp. The following equation (Grynkiewicz et al., 1985) can then be used to calculate the free calcium concentration.

$[Ca2+] = Kd (Rexp - Rmin / Rmax - Rexp)$ where Kd = 135 nM

(II. 13) Hydrodynamics of myosin filaments in various divalent cation concentrations.

(a). Analysis of the s-value in the presence of μM Ca^{2+}

Using the fura-2 assay we set the free calcium concentration of our stock PNFs, which had been exhaustively dialysed against 100 mM KCl, 0.2 mM $MgCl_2$ and 15 mM Tris at pH 7.2, to a known level that was below 10μM. The stock PNFs were aliquoted into 1 ml samples and the free calcium concentration of each was adjusted to the desired level (between 0-100 μM) by the addition of the appropriate amount of EGTA or $CaCl_2$. Constant ionic strength was maintained by adding an appropriate amount of KCl. The samples were then analysed in the ultracentrifuge as described in Section II. 11.

(b). Analysis of the s-value in the presence of mM Mg^{2+} only.

The PNFs were dialysed overnight against 100 mM KCl, 15 mM Tris and 0.2 mM $MgCl_2$ at pH 7.2, the free Mg^{2+} concentration was adjusted to the desired level by the addition of $MgCl_2$. Constant ionic strength was maintained by the addition of the appropriate amount of KCl and the samples were analysed in the ultracentrifuge as described in Section II.11.

(c). Analysis of the s-value in the presence of mM Ca^{2+} only

As for Section (b) except PNFs were dialysed overnight against 100 mM KCl, 15 mM Tris and 0.2 mM $CaCl_2$ at pH 7.2 and the free Ca^{2+} concentration was adjusted with $CaCl_2$.

(d). Analysis of the s-value in the presence of mM Mg^{2+} and μM Ca^{2+}

The free calcium concentration of PNFs, which had been dialysed overnight against 100 mM KCl, 0.2 mM $MgCl_2$ and 15 mM Tris at pH7.2, was adjusted to a known level (determined using the FURA-2 assay) that was below 10μM. The appropriate amount of concentrated $CaCl_2$ was then added to ensure a final concentration of 120 μM [Ca^{2+}]. The PNFs were then aliquoted into 1 ml samples and the free Mg^{2+} concentration adjusted to the appropriate values between 0.2 mM and 10 mM and made up to a final volume of 1.2 ml whilst maintaining a constant ionic strength by the addition of KCl. The s-value of the

filaments in the various concentrations of free Mg^{2+} was then estimated as described in Section II. 11.

(II. 14) The estimation of the translational diffusion coefficient (D_{zt}) using quasi elastic light scattering (QLS).

The basic principle of QLS is that, because of the translational motions of a particle in a suspension the scattered intensity in a given direction will fluctuate with time. The intensity fluctuations can be represented in terms of an autocorrelation function $g^2(t)$. As $[g^2(t) -1]^{1/2} = e^{-D_{zt} kt}$ then the dependance of $g^2(t)$ with time can be used to obtain the translational Brownian diffusion coefficient (from the slope of the plot of $\ln [g^2(t) -1]$ against time) and an estimate of the polydispersity (through the z-average variance of the plot).
The principle feature is an autocorrelator, a purpose built computer which performs products of the intensity (as measured by the number of photons, n, received by a photomultiplier) at a time, t, with that at a succession of other times t + bT for between 1 and 64 values of b where T is known as the sample time which is very much less than t. The correlator evaluates the intensity of the autocorrelation function $g^2(t)$ defined by
$g^2(t) = [n(t) n(t+bT)] / [n]2$ as a function of time, t, where the square brackets denotes an average over long times compared with T.
As with all hydrodynamic analysis the values obtained are only the apparent values unless they are extrapolated to zero concentration and in this case zero angle, however neither of these conditions can be satisfied when using PNFs. This is because PNFs cannot be produced at

a concentration greater than 0.5 mg/ml and it becomes difficult to pick up a signal below concentrations of about 0.2 mg/ml. Therefore there is only a limited range within which we can work and this range is not large enough to enable the concentration dependance of D_{zt} to be determined. Extrapolation to zero angle cannot be achieved because of our inability to filter dust out of the samples before use. At angles less than 30 °C the interference of dust in the solution becomes a major problem and severely reduces the signal to noise ration making accurate monitoring of the intensity fluctuation impossible. Any attempt to filter PNFs results in a large loss of the samples concentration (presumably because the filaments stick to the filter) which takes the sample below the measurable limits of the system. Also any filter large enough to allow PNFs to pass through would not be fine enough to stop dust particles, so the filtration procedure would be non effective. For these reasons all measurements were carried out at finite concentrations and at an angle of 90°. Therfore one must emphasise that all estimates of D_{zt} are only apparent values and when used in calculations of the Mr (See Section II. 15) the answer must be by definition an apparent Mr and not the actual value.

The quasi elastic light scattering measurements were performed using Malvern 4700 Light Scattering equipment with a Siemens 40 mW He/Ne laser (Wavelength = 632.8 nm). The beam from the laser was focused onto the centre of a 1 cm x 1 cm cuvette. The cuvette was placed at the centre of a goniometer which allows the scattering angle to be varied from 5° to 90°, however in all of our experiments the angle was fixed at 90°. Scattered light was collected by an EMI photomultiplier via a well collimated pinhole (aperture 100μm and via an

Amplifier-Discriminator to a 64-channel Malvern Autocorrelator (K7032-0S). The digital correlator output was stored on floppy discs and then sent via an Olivetti M24 computer to the University of Cambridge IBM 3081/B computer (via the JANET link) for processing as described above to yield a value for D_{zt} and polydispersity.

(II. 15) The estimation of the apparent molecular weight using the s-value and D_{zt}

The rate of sedimentation of a particle (S or the velocity / unit field) can be described as

$s = dr/dt \ / \ w^2r$ ------- (1)

where w = angular velocity in radians

r = radial position in cell

t = time

Terminal velocity is reached when the acceleration $\equiv$ deceleration and therefore,

$M\ (1- vp\ ).w^2r = F.\ dr/dt$ ------- (2)

where M = Mass of particle (in this case the molecular weight (Mr)

F = the frictional drag

v = partial specific volume of the solute

p = solution density

By substituting (1) in (2)

$s = Mr (1-vp) / F$ ------- (3) and $F = RT / D_{zt}$ ------- (4)

where R = gas constant

T = absolute temperature

D_{zt} = translational diffusion coefficient

By substituting (4) in (3) we obtain the so called Svedberg equation

$Mr = RTs / D_{zt} (1-vp)$ ------- (5)

Therfore as we know the values for v and p for PNFs (Bolger et al., 1989a) the determination of the s-value and D_{zt} for the system enables an estimation of Mr to be obtained. However if s and D_{zt} are not corrected to standard conditions then the calculation will yield only the apparent molecular weight.

(II. 16) Electron microscopy techniques.

(II. 17) Negative staining.

Samples to be negatively stained were diluted to the appropriate concentration (0.1 mg/ml) and a drop was placed onto a freshly glow discharged celloidin/carbon coated copper grid (Square Mesh 300). After allowing a few seconds for the protein to be adsorbed onto the carbon, excess non adsorbed protein was rinsed away using the appropriate diluent buffer. The sample was then vapour fixed by

placing the grid in glutaraldehyde vapour for 1 minute. The grid was rinsed with 2 drops of distilled water and stained with 1 drop of a saturated solution of uranyl acetate. Excess stain was removed by dabbing with a piece of filter paper being careful to leave a thin film of stain on the surface of the grid. Once dry the grid was ready for viewing in the electron microscope.

(II.18) Positive staining.

Samples to be positively stained were diluted to the appropriate concentration (0.1 mg/ml) and a drop was placed onto a celloidin / carbon coated copper grid (square mesh 300). Positive staining does not require the grids to be glow dischsarged. After allowing a few seconds for protein adsorption to occur the excess protein was washed away with a few drops of the appropriate diluent buffer. The sample was then vapour fixed by exposure to glutaraldehyde for 1 minute. After fixation the samples were rinsed with two drops of distilled water and stained by the addition of a drop of 0.5% uranyl acetate which after 5 to 10 seconds was rinsed off with distilled water and allowed to dry. After drying the grid was ready for viewing in the electron microscope.

(II.19) The rotary metal shadowing of PNFs.

The classical method used for the visualisation of of macromolecules by rotary shadowing was to spray the specimens onto freshly cleaved mica, which once dry was covered in carbon by thermal evaporation.

The carbon replica was then floated off onto water, picked up on grids and viewed in the microscope. However we found that technique was not well suited to using large structures such as myosin filaments. The forces involved in the spraying of the sample onto the mica surface had a detrimental effect on the filament structure. Therefore the procedure was modified in such a way that the filaments were not subjected to forces associated with spraying.

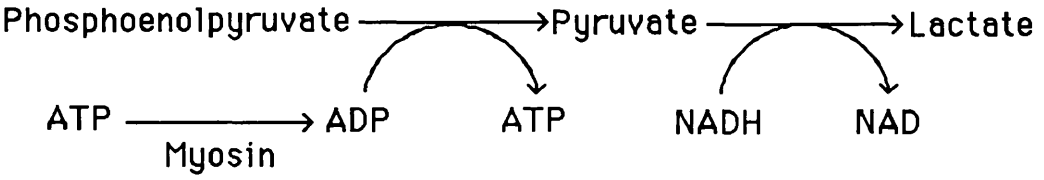
In the new procedure freshly cleaved mica was carbon coated and the carbon floated off onto water. The surface in contact with the water was an exact replica of the surface of the mica and therefore was completely flat. The carbon film was picked up on celloidin - coated copper grids and left to dry in air. A drop containing the specimen in its buffer was placed on the carbon and excess rinsed off with several drops of diluent buffer. The sample was then lightly vapour fixed in glutaraldehyde and excess buffer rinsed off with the appropriate concentration of ammonium acetate (pH 7.0). The grid was then dried down under vacuum and rotary shadowed using the thermal evapouration of platinum. The platinum electrode was held at a distance of 20 cm from the centre of specimen rotation and at an angle of 7 ° to the horizontal.

(II. 20) The determination of the ATPase activity using a continuous (linked enzyme) assay.

The linked enzyme assay allows the ATPase activity of myosin to be followed through a series of reactions which starts with the liberation of ADP by the myosin ATPase and through a series of linked reactions

(see below) ultimately results in the oxidation of NADH to NAD resulting in the loss of absorbance at 340 nm. The important feature of the assay is that the rate limiting step is the release of ADP by the myosin, the other steps in the pathway being fast (Emes and Rowe., 1978b) .

The pathway involved in the linked enzyme assay.



The final assay mixture consisted of no more than 0.1 mg/ml myosin (< 0.4 μM heads), which was made up in 100 mM KCl, 8 mM phosphoenolpyruvate, 50 μg/ml lactate dehydrogenase, 40 μg/ml pyruvate kinase, 6 mM KH_2PO_4 and 10 mM MgATP at pH 7.0. To measure the actin activated MgATPase activity a 10 molar excess of F-actin (See Section II. 24) was added to the above mixture. The assays were invariably started off in the absence of calcium by having 100 μM EGTA in the assay mixture. Once a steady state rate was achieved a 100 μM excess of Ca^{2+} was added in order to obtain the steady state rate in the presence of calcium. The loss in absorbance was followed in either a Perkin Elmer or Unicam SP800B ultraviolet spectrophotometer with the sample temperature being held at 20 °C.
The assay was calibrated by the addition of known amounts of ADP to the assay system (in the absence of myosin), the degree of deflection

per μM of ADP was then calculated. The loss in absorbance during the ATPase experiments was then converted, using this value, to ADP liberation per unit time i.e ATPase activity.

(II. 21) Protein preparation.

(II. 22) Conventional myosin preparation (High salt myosin).

Extraction of myosin from muscle mince was carried out in 3 - 4 volumes of Guba-Straub solution (0.3 M KCl, 0.15 M KH_2PO_4, pH 7.6) for 10 minutes with gentle stirring (Szent - Gyorgyi., 1945) . Johnson and Rowe., 1960) . The residual mince was removed by centrifugation (6000 r.p.m. , 2 minutes) and the extract was filtered under vacuum through 2cm thick filter paper pulp on a Buchner funnel. The filtrate was diluted (14 times) to I = 0.03 to precipitate the myosin which was then recovered by centrifugation at 6000 r.p.m. for 3 minutes. The myosin pellet was then dissolved in stock buffer (0.55 M KCl, 0.02M KH_2PO_4, 0.01 M $Na_2HPO_4.12H_20$, pH 7.6). The dissolved myosin was diluted to I = 0.28 to precipitate actomyosin which was then removed by centrifugation at 6000 r.p.m. for 3 minutes. The myosin was then precipitated at I = 0.03, pelleted and redissolved in stock buffer. The precipitation and redissolving steps were repeated a further 3-4 times. The purified myosin solution was dialysed overnight against several changes of stock buffer. After dialysis the solution was cleared by centrifugation at 18000 r.p.m. for 30 minutes.

(II. 23) The preparation of acetone dried muscle powder.

Actin was prepared from the muscle mince residue, after extraction of the myosin with Guba-Straub solution (See Section II. 22). The muscle residue was brought to room temperature and mixed with 10 volumes of 0.4% $NaHCO_3$ solution which was then gently stirred for 30 minutes and strained through muslin. The solid residue was blended with 3-4 volumes of acetone and strained through muslin which was repeated until the acetone washings remained clear. The residual powder was dried overnight on filter paper and if not used immediately was stored at -20 °C for future use.

(II. 24) The preparation of F-actin.

Actin was extraced 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 at pH 7.8. Carrying out the extraction at 0 °C ensures that the actin is free of tropomyosin (Drabikowski and Gergely., 1962) . After the extraction of tropomyosin the mixture was filtered and centrifuged at 40,000 r.p.m. for 30 minutes. The supernatant containing G-actin had KCl and $MgCl_2$ added so as to yield a final concentration of 100 mM and 1 mM respectively and was left for 2 hours for polymerisation to take place. The solution was then centrifuged at 40,000 r.p.m. for 2.5 hours and the pellet of F-actin was homogenised in 0.2 mM ATP, 2 mM Tris at pH 7.8 and depolymerised by dialysis for 40 hours in 0.2 mM ATP and 2 mM Tris. The resulting solution containing G-actin was clarified by centrifugation at 40,000 r.p.m. for 20 minutes and was repolymerised

in 100 mM KCl and 1 mM $MgCl_2$ as described earlier. After another cycle of depolymerisation and repolymerisation the F-actin preparation was dialysed exhaustively against 100 mM KCl, 1 mM $MgCl_2$ and 2mM Tris at pH 7.8. The purified unregulated F-actin that this preparation yields was stored in 0.02% azide at 0 °C and its integrity checked at regular intervals using SDS gel electrophoresis (See Section II. 2).

(II. 25) Preparation of synthetic myosin filaments (SMFs).

Synthetic myosin filaments were prepared from minced rabbit *latissimus dorsi* muscle using the procedure of Persechini and Rowe (1984). All operations were carried out in a cold room or on ice. Minced tissue was suspended in an MSE Ato-Mix homogeniser for 10 seconds in 800 ml of buffer A (10 mM Tris . HCl, 10 mM $MgCl_2$, 50 mM KCl, 2 mM EGTA, 0.2 mM dithiothreitol, and 3% (v/v) Triton X-100 at pH 7.6). The resulting suspension was sedimented at 3000 g for 10 minutes, the supernatant was discarded and the pellet resuspended in buffer A. This washing procedure was repeated 5 times, followed by 3 washes in buffer B (10 mM Tris . HCl, 1 mM $MgCl_2$, 150 mM KCl, 2 mM EGTA, and 0.2 mM dithiothreitol at pH 7.6). Myosin was extracted from pelleted washed myofibrils by gentle resuspension suspension in 200 ml of a solution containing 0.2 M potassium phosphate, 1 mM $MgCl_2$, 1 mM ATP, and 1 mM dithiothreitol at pH 7.2. This procedure was followed by centrifugation at 14,000 g for 30 minutes. The supernatant was filtered through glass wool and the ATP concentration was raised to 0.5 mM. This solution was then subjected to centrifugation at 150,000g for 4 to 6 hours and the supernatant was diluted 15 fold with

1 mM $MgCl_2$. Myosin was allowed to precipitate overnight. Myosin was pelleted at 3000 g for 15 minutes and then dissolved in buffer C (0.3 M KCl, 10 mM Tris.HCl, and 0.2 mM dithiothreitol at pH 8.0). The final protein concentration should be no greater than 8 mg/ml. Clarification at 25,000 g for 45 minutes yielded a viscous solution of relatively low turbidity. Solubilised myosin was then diluted by stirring into an equal volume of ice cold water. The resulting suspension of crude synthetic myosin filaments was dialysed exhaustively against buffer D (0.15 M KCl, 10 mM Tris.HCl, 1 mM $MgSO_4$ and 0.2 mM dithiothreitol at pH 8.0). Final purification was carried out by centrifugation at 70,000 g for 2 hours in a swinging bucket rotor. The supernatant is a suspension of purified synthetic myosin filaments at a typical concentration of 3 mg/ml. The preparation is stable for 5 days if kept on ice and stored in buffer D. The purity of the synthetic filaments was routinely checked using gradient SDS/gel electrophoresis and the filament length distribution assesed by electron microscopy of negatively stained filaments (See Section II. 17).

(II. 26) Myofibril preparation.

Thin strips (diameter < 3mm) of freshly dissected rabbit or rat psoas muscle were tied at rest length to plastic rods and placed in calcium depleting medium (0.1M NaCl, 2mM $MgCl_2$, 0.1% (w/v) glucose, 1mM EGTA, 6mM KH_2PO_4 , pH 7.0) for 48 hours at 4 °C, with several changes.

After calcium depletion the muscle was chopped into small pieces using a sterile scalpel blade. The chopped muscle was then washed in homogenising medium and myofibrils were produced by subjecting the

muscle to 3 x 30 second full-speed bursts on a MSE Ato-Mix homogeniser in a container packed around with ice. The myofibrils were pelleted by centrifugation at 5000 r.p.m. for 2 mins in an MSE Chilspin with a swing out rotor, the pellet was then resuspended in homogenising medium (0.1M KCl, 10mM $MgCl_2$, 1mM EGTA and 6mM KH_2PO_4 at pH 7.0). To remove undesirable fat from the preparation the pelleting and resuspension steps were repeated a further 2 times or until the supernatant remained clear.

Finally the resuspended preparation was checked for proteolysis using SDS PAGE and the integrity of the myofibrils was checked using the light microscope.

(II. 27) Relaxed filament preparation (Standard protocol).

Myofibrils in homogenising medium were pelleted by centrifugation at 5000 r.p.m. for 2 minutes in a MSE Chilspin and redispersed in relaxing medium (100mM KCl, 10mM $MgCl_2$, 1mM EGTA, 5mM ATP, 6mM KH_2PO_4, pH 7.0). After a further pelleting and resuspension the myofibrils were gently homogenised for two 15 second bursts using a MSE Ato-mix homogeniser at full speed. Residual myofibrils were removed by centrifugation for 20 seconds at maximum speed in a micro centrifuge. The supernatant contained mainly thick (A) and thin (I) filaments along with some intact A- and I- segments.

The relaxed filament preparation was then checked using polyacrylamide gel electrophoresis and electron microscopy for signs of proteolysis or physical damage that can result from excessive homogenisation.

(II. 28) Relaxed filament preparation (for PNF purification).

Relaxed filaments to be used for PNF preparation were prepared in a identical manner to those described in the standard protocol (See Section II. 27) except that disruption of the myofibrils into a relaxed filament preparation was carried out in a relaxing medium containing an elevated ATP (10 mM) and Mg^{2+} (15 mM) concentration. This was found to assist in the process known as "bare zone rupture" (Bolger et al., 1989a) which is an essential part of PNF purification technique.

(II. 29) Preparation of purified native thick filaments (PNFs).

The purification of PNFs takes place on an 100-20% D_2O/H_2O 70 ml gradient. The gradient was made by the gradual mixing of two relaxing medium buffers in a gradient maker. Each relaxing medium contained 100mM KCl, 15mM $MgCl_2$, 1mM EGTA, 10mM ATP and 6mM KH_2PO_4 at pH 7.4 one of which was made up in 20% D_2O the other in 100% D_2O. Once the gradient was formed a RFP (produced as described in Section II. 28) with a concentration of approximately 4 mg/ml was gently loaded on top to give a final loading of 25 mg. The tube was then placed in a 3 x 70 MSE swing out rotor and spun at 21,000 r.p.m. for 1 hour at 7 °C in a superspeed 65 ultracentrifuge. On completion of the run the gradient tube was immediately pierced at the bottom and 7 ml fractions were collected in test tubes. The protein concentration of each tube was then determined, the tube containing the highest concentration (fractions 5 or 6) always contained the PNFs. The integrety of the preparation was then checked in the electron microscope (Plate 1.1)

and on SDS gels and the PNFs were stored at 4 °C*. The procedure described above yields approximately 7mls of PNFs, which are not phosphorylated, with a concentration between 0.4 and 0.5 mg/ml

* N.B. D_2O freezes above 0 °C and therefore PNFs in D_2O buffers should never be placed on ice.

(II. 30) Experimental procedures.

(II. 31) The production of "bare zone assemblages" (BZA's) from rat and rabbit relaxed filament preparations.

The appropriate relaxed filament preparation (See Section II. 27) was observed in the electron microscope using negative staining as described above. The length distribution of the thick filaments was calculated from photograhic enlargements using an Apple Graphics Tablet linked to an Apple II microcomputer. The program used (A.J. Rowe this laboratory) records the length of up to 500 filaments and calculates the average length, maximum length, minimum length, standard deviation and also prints a histogram of the normal and weight averaged length distributions.

The relaxed filament preparation was then dialysed for 18 hours against the BZA buffer (Neiderman and Peters., 1982; (200mM KCl, 3mM $MgCl_2$, 0.25mM EGTA, 0 .25mM EGTA, 1.5mM ATP, 9mM KH_2PO_4 and 1mM DTT at pH 7.0)). The BZA's were then observed by negative staining (See Section II. 17) in the electron microscope and their length distribution calculated as described above.

(II. 32.) The dialysis of PNFs against BZA forming buffer.

PNFs were dialysed for 2 hours against 200mM KCl, 3mM $MgCl_2$, 0.25mM EGTA, 1.5mM ATP, 9mM KH_2PO_4 and 1mM DTT at pH 7.0 in an attempt to form structures analagous to BZAs. The dialysed PNF preparation was then examined in the electron microscope for evidence of the presence of any stable structures.

(II. 33.) The reassembly of thick filaments from rat and rabbit BZAs.

The BZAs produced in Section II. 31 were dialysed against 100mM KCl reassembly buffer (100mM KCl, 3mM $MgCl_2$, 0.25mM EGTA, 1.5mM ATP, 9mM KH_2PO_4 and 1mM DTT at pH 7.0) overnight. The reassembled relaxed filament preparation was then negatively stained and observed in the electron microscope. The length distribution of the reassociated thick filaments was then calculated as described above (See Section II. 31) and compared to that of the control filaments.

(II. 34) Low ionic strength fraying of rat and rabbit thick filaments.

A relaxed filament preparation or PNF preparation was adsorbed onto a celloidin / carbon coated grid and the excess filaments were removed by rinsing with 2 drops of relaxing medium. Thick filament fraying (Maw and Rowe., 1980) was induced by the addition of 2 to 3 drops of low ionic strength fraying medium (2mM Tris pH 7.4 (Trinick., 1982)). The filaments were immediately vapour fixed in glutaraldehyde and negatively stained (See Section II. 17). Once dry they were observed in

the electron microscope and the degree of fraying, fraying pattern and number of subfilaments obtained recorded.

(II. 35) The gentle extraction technique for the removal of regulatory light chains (RLCs) (standard protocol).

A relaxed filament preparation (rabbit or rat) at a concentration of approximately 1mg/ml was incubated at 37 °C in 50mM KCl, 5mM ATP, 20mM EDTA and 5mM KH_2PO_4 at pH 7.4, and samples removed at fixed time intervals. The filaments were placed in chilled eppendorfs and immediately pelleted by spinning in a microcentrifuge for 3.5 minutes at maximum speed. The supernatant containing the extracted RLCs was discarded or prepared for running on a gel, whilst the pellet containing RLC depleted thick filaments was resuspended in relaxing medium or any other appropriate buffer. The amount of RLC removal obtained was then checked using gel and gelscan analysis (See Sections II. 2 and II.3).

(II. 36.) Variations in the gentle extraction procedure using a rabbit RFP.

Several variations in the basic extraction procedure described in Section II. 35 were performed in an attempt to elucidate the vital components in the extraction medium. The variations were as follows;

(a) 120 minutes into the incubation a further addition of 5 mM ATP was made and the extraction was then allowed to proceed as normal. The effect of the addition of ATP on the RLC removal kinetics was then followed as described in Section II. 3.

(b) The extraction was carried out in a buffer identical to the standard

buffer (See Section II. 35) except that it did not contain any ATP. The timecourse of RLC depletion was followed and directly compared with that of the standard technique (See Section II. 35).

(c) The extraction was carried out in a buffer identical to the standard buffer (See Section II. 35) except that it contained 20mM ATP as opposed to 5mM ATP. The timecourse of RLC removal was then followed on a gel and compared directly to that of the standard technique.

(II. 37) The effect of the gentle extraction technique on the RLCs of PNFs.

The incubation conditions and procedure for the gentle extraction of RLCs from PNFs were identical to those used in the standard protocol for RFPs, except for the pelleting procedure used to stop the reaction. In the case of PNFs the pelleting was carried out in an airfuge where the samples were spun in the cold (4 °C) at 50,000 r.p.m. for 5 minutes. The resuspension and preparation for running on a gel were then identical to those when using RFPs (See Section II. 35).

(II. 38) Effect of the gentle extraction technique on the RLCs of conventional high salt exposed myosin (SMFs).

Conventional myosin was prepared (See Section II. 22) and dialysed overnight against 100mM KCl and 5mM KH_2PO_4 at pH 7.0. The filament structure was checked in the electron microscope using negative staining.

To the filaments was added the appropriate volume of a 4x concentration gentle extraction medium so as to produce a final concentration of 50mM KCl, 20mM EDTA, 5mM ATP and 5mM KH_2PO_4 at pH 7.4. The mixture was incubated at 37 °C, filaments were removed at fixed time intervals and separated from their RLCs by centrifugation. The samples were then run on a gel and their RLC content determined at each timepoint using gelscan analysis (See Section II. 3).

(II. 39) Effect of the gentle extraction technique on the RLCs of a high salt exposed rat RFP.

A rat RFP was dialysed overnight against 500 mM KCl, 5 mM $MgCl_2$ and 5 mM KH_2PO_4 at pH 7.0. The preparation was then checked in the e.m. to ensure complete thick filament depolymerisation had occured. The filaments were then reassembled by overnight dialysis against 100 mM KCl pH 7.0 and once again examined in the electron microscope. The reassembled RFP was then subjected to the standard protocol gentle extraction technique (See Section II. 35) and the degree of RLC removal assessed by gel analysis (See Section II. 3).

(II. 40) The effect of RLC depletion on the RLCs of thick filaments reassembled from rabbit BZAs.

BZAs were prepared from a rabbit RFP as described in Section II. 31 which were then reassembled to produce full length filament by dialysis against 100 mM KCl. The reassembled filaments were

examined in the electron microscope to check that complete ressembly had occured and that the filaments were truly full length and displayed a narrow length distribution. The filaments were then subjected to the standard gentle extraction protocol (See Section II.35) and the degree of RLC depletion determined by polyacrylamide gel electrophoresis.

(II. 41) The effect of chymotryptic digestion on the extraction resistant RLC population of a rabbit relaxed filament preparation.

A relaxed filament preparation was was incubated for 30 minutes at 37 °C in a gentle extraction medium. After incubation the RLC depleted filaments were pelleted by centrifugation for 3.5 minutes in a microfuge and the supernatant containing the extracted RLCs was discarded. The RLC extracted filaments were resuspended in homogenising medium followed by a further pelleting and resuspension to ensure that all extracted RLCs were removed. The filaments were then pelleted and resuspended in Weeds buffer (0.12M NaCl, 20mM KH_2PO_4, 1mM EDTA and 5mM ATP, pH 7.0) to give a final concentration of approximately 0.5 mg/ml. The protein concentration was determined using the Lowry technique (See Section II. 8) and 1/200th of this concentration of chymotrypsin was added. The mixture was incubated at 25 °C and samples removed at fixed time intervals. The chymotryptic digestion was stopped at each time point by the immediate addition of a molar excess (w.r.t. chymotrypsin) of Lima bean trypsin inhibitor (LTI). The samples were then prepared for running on a gel and the degree of RLC digestion calculated by gelscan analysis.

Chapter II

Tables and Plates.

TABLE 2.1.

Gel Constituents	Solution A	Solution B	Solution C
	7.5% gradient tube	20% gradient tube	stacking gel
Upper stock	-	-	2.5 ml
Lower stock	2.5 ml	2.5 ml	-
30.8% Acrylamide	2.5 ml	6.7 ml	1.5 ml
Deionised water	5.0 ml	0.5 ml	6.0 ml
Glycerol	-	0.25 ml	-
3.3% APS	60.0 µl	60.0 µl	90.0 µl
TEMED	5.0 µl	5.0 µl	10.0 µl

Table 2.1

The constituents of the appropriate buffers that make up a 7.5 - 20% acrylamide gradient gel.

Table 2.2

Values of $A^{1\%}_{1cm.280nm}$ for the various fragments of myosin

Protein	$A^{1\%}_{1cm.280nm}$
Rabbit myosin	5.4
Rabbit HMM	6.5
Rabbit S1	7.9
Rabbit RLC	5.5
scallop myosin	5.4
Scallop RLC	1.8

Table 2.3

BCA compatible substances

The following substances to the stated level will not interfere (<3% error) with the BCA protein assay reagent.

40% Sucrose	1% SDS
1.0M Glycine	0.1N HCl
10mM Glucose	0.2% Sodium Azide
10mM EDTA	1% Triton X-100

Table 2.2

Examples of the values of $A^{1\%}_{1cm.280nm}$ for the various fragments of myosin.

NB. This technique of protein concentration determination could only be used when the protein samples were completely devoid of nucleotide.

Table 2.3

Some of the BCA compatible substances that do not to a significant extent interfere with the BCA assay. All buffers containing known protein concentrations were checked for interference before use with the BCA reagents.

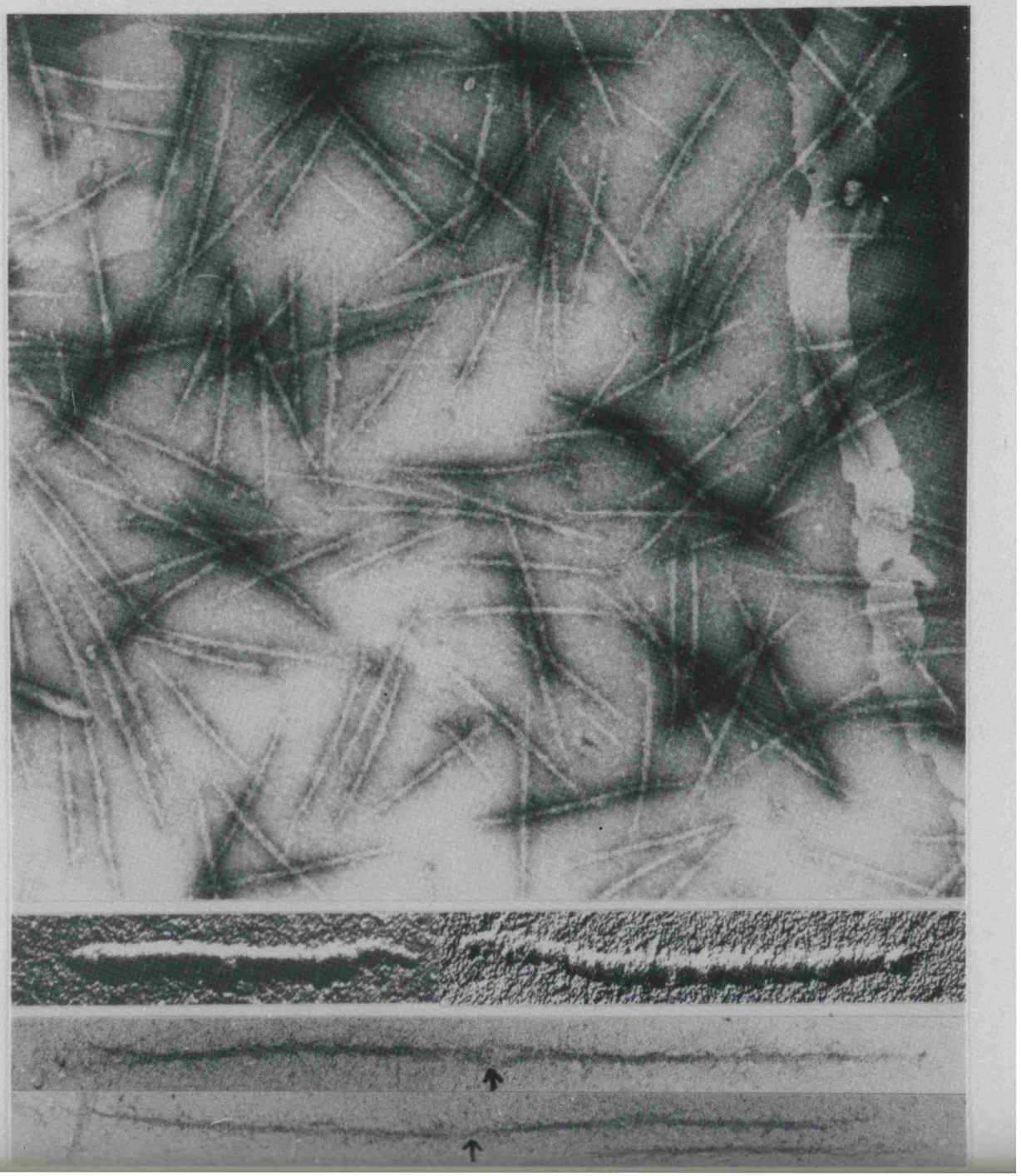


Plate 2.1

a). Negatively stained electron micrograph of PNFs. Note the complete absence of contaminating actin filaments in the background.
Magnification = 40,000x

b). Unidirectionally shadowed PNFs showing the bare zone remnant.
Magnification = 80,000x

c). Positively stained electron micrograph showing full length thick filaments undergoing the process of bare zone rupture to produce two hemifilaments.
Magnification = 80,000x

(Micrographs courtesy of A. J. Rowe)

Chapter III

The role of the regulatory light chains in myosin.

Introduction.

(III. 1) RLC involvement in the ATPase activity of myosin.

Much of the work investigating the function of RLCs has for several reasons been carried out on molluscan myosins which display the apparently unique feature of allowing the reversible removal of their RLCs. This ability was first demonstrated by Szent-Gyorgyi et al (1973) when they showed that the incubation of scallop myosin and myofibrils in the presence of EDTA at 4 °C resulted in the complete desensitisation of the myosin linked ATPase activity to calcium. This was the first evidence for the direct involvement of the RLCs in the calcium sensitivity of the ATPase activity. The extraction at 4 °C removed one mole of RLCs per mole myosin and therefore, they suggested mistakenly that there was only one RLC per myosin molecule. The RLC removal was fully reversed to stoichiometric levels by incubation in the presence of divalent cation. Complete reassociation of the RLCs resulted in the complete recovery (resensitisation) of the calcium sensitivity of the MgATPase of the myofibrils (Szent-Gyorgyi et al., 1973).

These results led Szent-Gyorgyi et al (1973) to suggest that the RLC contained multiple binding sites and was partially bound to both of the

S1 heads of the myosin molecule. Such an arrangement would then allow the single RLC to regulate the ATPase activity of both heads, by holding them in such a position as to prevent them interacting with actin in the absence of calcium. In the presence of calcium the inhibition is relieved. They also pointed out that the single RLC could be bound entirely on one of the S1 heads and exert its regulatory effect on the other head by some form of cooperativity. The idea of cooperativity between heads was supported by the fact that scallop S1 was completely calcium insensitive whilst scallop HMM was fully calcium sensitive which they interpreted as meaning that both heads were needed for regulation.

The number of RLCs per myosin molecule was later corrected to two, when Kendrick-Jones et al (1976) showed that by treating desensitised scallop myofibrils with Dithio bis-nitrobenzoate (DTNB) a further mole of RLC/mole myosin was removed. Treatment with the DTNB caused irreversible damage to the myosin heavy chain and loss of ATPase activity. However the extracted RLCs were identical to those removed by EDTA treatment as judged by their mobility on SDS gels, proteolytic digestion pattern and and their ability to fully resensitise RLC denuded scallop myosin.

The RLCs from all types of myosin so far studied (vertebrate and invertebrate) have shown the ability to bind to desensitised scallop myosin (Kendrick-Jones., 1974; Kendrick-Jones et al., 1976; Chantler and Szent-Gyorgyi., 1980). This suggests that not only do they have a similar structure but that the myosins also posseses a common binding domain. All light chains that have been shown to bind to desensitised scallop myosin are called regulatory light chains (RLCs) even though in

many cases their function in regulation remains obscure (Yamamoto et al., 1980; Moss et al., 1982; Cardinaud., 1987).
Kendrick-Jones et al (1976) also showed that there were two other light chains present which could be removed using guanidine HCl or strong alkali. These light chains were present in the ratio of two moles per mole myosin and did not resensitise scallop myosin, these were the so called ELCs (Wagner and Weeds., 1977; Walliman et al., 1982., Collins et al., 1986).

(III. 2) The production of hybrid myosins.

The discovery of the reversible nature of RLC removal by EDTA at 4 °C (Szent-Gyorgyi et al., 1973) allowed the production of hybrid molecules to take place, in which the scallop RLCs were removed from the myosin and foreign RLCs from other types of myosin were put back on (Chantler and Szent-Gyorgyi., 1978). One result of such work was to show that after the desensitisation of scallop myofibrils at 4 °C the reassociation of rabbit RLCs restored the calcium sensitivity of the ATPase (Kendrick-Jones et al., 1976). However the resensitisation of purified scallop myosin by rabbit RLCs did not restore calcium sensitivity. It was presumed that the actin present in the myofibrils somehow protected the denuded myosin head or held it in a conformation that was favourable for the rebinding of the RLC and the return of calcium sensitivity. Kendrick-Jones et al (1976) provided further evidence in support of this idea. They showed that calcium sensitivity could also be restored to scallop actomyosin preparations. The successful resensitisation of purified scallop myosin had earlier

been reported by Kendrick-Jones (1975), although there were inherent difficulties in the interpretation of this data.

For example it was not certain whether desensitisation caused all of the myosin molecules to lose a single RLC or whether it resulted in a mixed population with some still containing both RLCs, some containing one and some completely devoid of RLCs. If such a mixed population was present, then the use of steady state analysis of the ATPase would reveal little useful information, as the contribution of all of these species would have to be taken into consideration. Wells and Bagshaw (1985) using single turnover experiments later showed that when such mixed populations were present a relatively small population of unregulated molecules can mask the presence of a large population of fully regulated molecules. Therefore, the situation following reassociation of the vertebrate RLCs would be just as confusing and as Kendrick-Jones himself admits complete resensitisation of purified myosin is only rarely observed. So although it appears possible, under certain conditions to fully resensitise the ATPase of desensitised purified scallop myosin, it has never been repeatedly and convincingly demonstrated.

(III. 3) Further evidence for the inhibitory role of molluscan RLCs.

Chantler and Szent-Gyorgyi (1980) later showed that incubation at elevated (25-30 °C) temperatures in the presence of EDTA resulted in the complete loss of all RLCs. Complete RLC depletion once again caused a total loss of the calcium sensitivity of the actin activated ATPase. The removal of the RLCs was fully reversible to

stoichiometric levels by re-incubation in the presence of extracted RLCs and divalent cation. After the complete reassociation of scallop RLCs full calcium sensitivity was restored, once again confirming the inhibitory role of the RLCs in the calcium regulation of the ATPase. They also showed that the removal of all of the scallop RLCs at 25-30 °C (causing complete desensitisation), followed by the reassociation of the regulatory light chains from rabbit myosin did not restore the calcium sensitivity and resulted in the permanent activation of the ATPase activity. This raised the question as to whether the calcium specific binding site of the scallop was due to a unique conformation of the scallop RLC, the scallop heavy chain or both. Kendrick-Jones (1974) showed that scallop RLCs reassociated onto RLC depleted rabbit myosin displayed no myosin linked ATPase activity whatsoever. He suggested that either the rabbit heavy chain was incapable of holding the scallop RLC in the correct conformation to restore calcium sensitivity, or that the scallop RLC had been altered by the extraction technique. The last point seems unlikely as the extracted scallop RLCs have been shown to fully resensitise RLC denuded scallop myosin (Szent-Gyorgyi et al., 1973; Kendrick-Jones et al., 1976; Walliman et al., 1982). Therefore, it seems possible that the lack of calcium sensitivity of rabbit myosin containing scallop RLCs could be due to the heavy chain having a different conformation to that of the scallop.

The situation was further complicated by the result of Sellers et al (1980). They showed that the production of hybrids of scallop myosin containing vertebrate RLCs only, resulted in the complete loss of calcium sensitivity and a permanently switched on actin activated ATPase activity. That is the ATPase remains high both in the presence

and absence of calcium. A completely contradictory result was reported by Scholey et al (1981) and again by Kendrick-Jones et al (1982) when they claimed that such vertebrate RLC hybrids did indeed lose all calcium sensitivity but that the actin activated ATPase was permanently switched off.

It is difficult to reconcile these differences. However, it seems likely that one of the groups may have been using damaged myosin. It was is not clear which group, because both results can be explained if one proposes that they were using damaged myosin. For example when scallop myosin is damaged, by whatever means, the ATPase is usually affected in such a way that it becomes calcium insensitive and permanently switched on (Jackson and Bagshaw., 1988). This would be a convenient explanation for the result obtained by Sellers et al (1980). An equally good explanation for the results of Scholey et al (1981) and Kendrick-Jones et al (1982) can be proposed if one assumes that during the initial RLC depletion a very large proportion of the ATPase activity of the myosin molecules was damaged. Then, after the reassociation of the vertebrate RLCs, only a small proportion of myosin molecules (those that were undamaged) would regain full activity. The ATPase in all cases was followed using steady state assays, which yield the ATPase activity in terms of the total amount of protein present The activity in this situation would therefore be abnormally low and so, create the impression that the ATPase of all of the myosin molecules was permanently switched off. The latter explanation seems unlikely considering the fact that when they removed as many of the hybridised rabbit RLCs as possible (40%) by treatment with EDTA, and replaced them with scallop RLCs, partial recovery of the calcium sensitivity of

the ATPase was observed. The finding (Scholey et al., 1981 and Kendrick-Jones et al., 1982) that scallop hybrids containing 100% vertebrate RLCs have no calcium sensitivity and posses a permanently depressed ATPase are the results that have gained most acceptance and it is these results that are now generally held to be the correct ones. The reason for such confusion stems from the relative insensitivity of steady state kinetics to mixed populations (Wells and Bagshaw., 1985). The only satisfactory way to clear up the confusion would be to use rapid analysis techniques such as stopped flow which enable the contributions from the various species present to be resolved.

(III. 4) The location and properties of the divalent cation binding sites.

In order to obtain a greater understanding of the regulation of the myosin ATPase by calcium, the location the divalent cation binding sites needed to be determined and their characteristics studied. Bagshaw and Kendrick-Jones (1979) used epr studies to compare the Ca, Mg, and Mn binding to several forms of myosin. They concluded that all myosins possess two high affinity divalent cation binding sites (Mn > Ca > Mg) which are non specific. Comparison of the epr spectra of metal binding to the RLC when bound to the heavy chain and when in solution showed them to be the same. Therefore, the non specific sites must be located on the RLC. The fact that the EPR spectra were identical whether the RLC was bound to the heavy chain or not suggests that metal binding to the RLCs' non specific site does not involve ligands from the heavy chain. The binding affinity of the RLCs for divalent cation was however, several orders of magnitude lower when

they were not bound to the heavy chain (Weber et al., 1972; Chantler and Szent-Gyorgyi., 1978). Support for this was provided by Wikman-Coffelt (1979), who showed that when rabbit RLCs associate with the myosin heavy chain the strength of their metal ion binding increases. This was probably through an indirect influence by the heavy chain.

Molluscan myosin was however shown to posses an additional pair of high affinity calcium specific sites (Szent-Gyorgyi et al., 1973; Bagshaw and Kendrick-Jones., 1980). The differece between these sites was emphasised by the fact that in epr studies Mn did not compete for the calcium specific sites on molluscan myosin but it did suppress calcium binding to the non specific sites (Bagshaw and Kendrick-Jones., 1980). Szent-Gyorgyi et al (1973) showed that scallop myosin specifically binds 2 mol calcium / mol myosin with high affinity (10μM) in the presence of 1 mM Mg^{2+}. This has since been confirmed by several workers (Kendrick-Jones et al., 1976; Stafford et al., 1979; Chantler and Szent-Gyorgyi., 1980). It is this site that is believed to be responsible for the calcium response of the scallop myosin ATPase under physiological conditions. Dissociation of the scallop RLCs in the absence of divalent cations causes a loss of the calcium specific sites and results in desensitisation of the ATPase. However, the binding site is not completely lost in the true sense of the word but its affinity is greatly reduced (Chantler and Szent-Gyorgyi., 1980). Reassociation of the RLCs to stoichiometric levels restored both the calcium sensitivity and all of the calcium specific binding sites (Chantler and Szent-Gyorgyi., 1980). This shows a direct relationship between the RLC content and the stoichiometry of

the high affinity calcium specific sites.

Weak non specifc binding sites are present on isolated scallop RLCs and Chantler and Szent-Gyorgyi (1980) showed that these become strong on binding to the myosin heavy chain. The non specific sites are not unique to scallop myosin and have also been studied in vertebrate myosin (Wikmann-Coffelt., 1979;).

(III. 5) The calcium specific sites are located near the S1/S2 hinge.

The exact location of the calcium specific site on molluscan myosin has proved more difficult to determine. Stafford and Szent-Gyiorgi (1979) went some way to resolving this when they showed that the ATPase activity of chymotryptic S1 was completely calcium insenstive (also Szent-Gyorgyi et al., 1973) whereas Myosin, HMM and single headed myosin maintain full sensitivity. The key factor in this paper was that the single headed myosin was calcium sensitive. Therefore, the lack of sensitivity in the S1 preparations could not be put down to the fact that S1 is single headed. The RLCs in their S1 subfragment were observed to be slightly nicked during the digestion. However this was shown not to be responsible for the loss of calcium sensitivity, as exchanging them for fully intact native RLCs (to stoichiometric levels) did not restore the calcium sensitivity. Other evidence to support the idea that the loss of calcium sensitivity was nothing to do with RLC nicking came from the fact that the RLCs on the HMM and single headed myosin were nicked in the same place as those in the S1 yet they still remained fully calcium sensitive.

They therefore proposed that the lack of calcium sensitivity of the S1

was due to the loss of a vital region involved in calcium binding on the S2 portion of the molecule near the S1/S2 hinge. This is where the heavy chain is cleaved during proteolysis. There is also the possibility that the calcium binding site is not lost but is merely damaged during the digestion.

Support for the S1/S2 junction being involved in calcium sensitivity has also come from some recent work involving the use of molluscan abalone (clam) myosin (Asakawa and Azuma., 1988). Digestion of abalone myosin releases S1 subfragments that display a calcium sensitive ATPase activity. The abalone myosin heavy chain is presumably nicked in a unique part of the myosin neck region, which generates an S1 subfragment with a slightly longer neck that still contains enough of the calcium specific binding site to maintain calcium sensitivity.

Weeds and Taylor (1975) proposed that the S1/S2 junction was also important in binding the RLC because of the protection the presence of the light chain offerd the S1/S2 junction during chymotryptic digestion in the presence of divalent cation. The scallop RLC is known to be a long elongated molecule with a length of approximately 100 Å (Stafford and Szent-Gyorgy., 1978; Hartt and Mendleson., 1979). Therefore, it is not unlikely that the RLC plays a role in calcium sensitivity by extending down into the hinge region and interacting with the S1/S2 junction. All RLCs belong to the same family of proteins whose common feature is the presence of domains that are related to the EF hand of parvalbumin (Kretsinger., 1973) and are capable of binding to desensitised scallop myosin. It is therefore, not unreasonable to assume that they all have a similar structure, which sequence analysis

does indeed confirm (Collins., 1976; Matsuda et al., 1977) and would all potentially be capable of binding to the myosin heads in such a conformation as to be able to interact with the S1/S2 junction.
Vertebrate myosin has not, until recently (Chin., 1982; Rowe., 1989) been shown to display calcium sensitive ATPase activity in the absence of regulated actin filaments. The RLCs of vertebrate myosin do however resensitise scallop myosin that has been desensitised at 4 °C (Kendrick-Jones et al., 1976). Scallop myosin desensitised at 30 °C results in the complete loss of all RLCs (Chantler and Szent-Gyorgyi., 1979) and is not resensitised by rabbit RLCs (Scholey et al., 1981; Kendrick-Jones et al., 1982). The fact that the reassociation of rabbit RLCs, to completely RLC denuded scallop myosin, did not restore calcium sensitivity was hardly surprising as rabbit myosin was itself believed to be completely calcium insensitive.

(III. 6) Why are 50 % of the RLC in molluscan myosins more susceptible to removal than the others ?

The reason for the removal of 50% of the RLCs from scallop myosin, causing complete desensitisation of the ATPase, has been addressed by many workers, with two explanations predominating. The first proposes the formation on RLC removal of a so called sticky patch. It is the interaction or interference of this sticky patch with the other RLC containing myosin head, within the same myosin molecule, that causes a complete loss in calcium sensitivity (Bagshaw., 1980). The second possibility is inherently similar to the sticky patch hypothesis. It proposes that the loss of an RLC causes a change in conformation of

the myosin heavy chain (Margossian and Slayter., 1987) which then interacts with the activity of the other head to abolish calcium sensitivity (Chantler and Szent-Gyorgyi., 1980).

Taking into account the above evidence, it is difficult to ignore the possibility of cooperative interaction between the individual myosin heads. It appears that as long as each scallop myosin molecule posseses one scallop RLC full calcium sensitivity is possible, provided the other head contains an RLC (Sellers et al., 1980). The other RLC need not be from a calcium sensitve myosin species. The only requirement is that the foreign RLC is sufficiently similar in structure to be able to bind to the vacant RLC binding site. By binding to this site the foreign RLC presumably covers the so called sticky patch or causes tertiary structure of the myosin head to revert to its native state. Whatever the process is, the effect is to relieve the interaction between the heads, thus allowing the scallop RLC to mediate the calcium sensitivity once more. The fact that one scallop RLC is able to restore full calcium sensitivity to the myosin indicates that it is possibly regulating the ATPase of both heads. This lends further support to the possibility of there being cooperation between the heads.

(III. 7) The role of the alkali light chains.

The alkali light chains of myosin (ALCs), or essential light chains (ELCs) as they are often refered to, have been implicated in the regulation of the ATPase activity (Walliman and Szent-Gyorgyi., 1981; Walliman et al., 1982). Until recently the the ELCs were believed to be essential to the ATPase activity because their removal resulted in the

complete loss of the ATPase activity of the myosin (Frederiksen and Holtzer., 1968; Gershman et al., 1969; Dreizen and Gershman., 1970). However, the conditions needed for the removal of the ELCs were so severe that irreversible damage of the myosin heavy chain also occured. This was held to be the reason for the loss of the ATPase activity.

The most conclusive evidence for the ELCs being regulatory subunits was provided by Walliman and Szent-Gyorgyi (1981), who showed that antibodies specific to the ELC desensitised scallop myosin. Further support for the ELCs involvement in regulation came from several workers who showed that the RLC and ELC were very close together on the myosin head. This was demonstrated by Stafford et al (1979) and Konno and Wantanabe (1985). They showed that the ELC was protected by the RLC from papain digestion and that the presence of the RLC also abolishes the reactivity of the thiol groups on the ELC (Hardwick et al., 1982). Electron microscopy, using antibody labelling, has also shown the close apposition of the RLC and ELC in the neck region of the myosin head (Walliman and Szent-Gyorgyi., 1981; Flicker et al., 1983). Cross linking studies show that the amino terminal portion of scallop RLC, is separated from the ELC by a distance of more than 10Å at rest, but the separation decreases during contraction (Hardwick et al., 1983). The change is apparently due to the movement of of the ELC (Hardwick and Szent-Gyorgyi., 1985) and takes place in the absence of actin (Ashiba et al., 1980; Wells and Bagshaw., 1985). The recent finding (Ashiba and Szent-Gyorgyi., 1985) that the RLC of scallop blocks the exchange of ELC in intact myosin preparations suggests that the ELC lies between the heavy chain and the RLC.

The calcium specific sites on scallop myosin have not been located

exactly. It is however, generally accepted that they lie in the neck region of the S1 head and that the RLC plays a part in the calcium binding. This calcium specific site is not the same as the high affinity non specific binding site found on all RLCs (Bagshaw and Kendrick-Jones., 1980). Although, the removal of the RLC does lower the affinity and selectivity for calcium of the specific sites, they are retained on the desensitised ELC/heavy chain complex (Chantler and Szent-Gyorgyi., 1980). It was therefore suggested that the ELC may be responsible for, or participate in the reduced afinity calcium binding by desensitised scallop myosin.

Collins et al (1986) used sequence analysis to determine the entire scallop ELC amino acid sequence. They found that it was an acidic protein with a molecular weight of 17,616 and a net charge of 18 at physiological pH. They found that the protein had four homologous domains which had evolved from an ancestral calcium binding protein (Baba et al., 1984). Mutations in domains one and two (amino terminal) seemed to eliminate the possibility of calcium binding in that half of the molecule. Although site four could possibly be a functional calcium binding domain, it was felt that domain three was the most likely candidate. The reason for this assumption was that domain three contained an amino acid residue (serine 102) capable of contributing a side chain oxygen at the X-axis of the ancestral site. Scallop ELC is the only one from several myosin species studied (Matsuda., 1983; Matsuda et al., 1981) known to posses such a residue at this point and Collins et al (1986) have suggested that this is a unique and functionally important feature of molluscan ELCs. Vertebrate myosin does not posses such a functional domain three. However, domain one

does appear to retain the possibility of calcium binding (Frank and Weeds., 1974) and as such is the only ELC that does so at that position. Differences between the conformation of the molluscan and vertebrate heavy chain may well result in subtle differences in the interaction with their native light chains. If only small changes in the ligands involved in calcium binding were needed to completely abolish its functionality, then scallop RLCs bound to vertebrate myosin and vica/versa may not be able to interact with the ligands from the foreign heavy chain necessary to produce a proficient calcium binding site. If specific interactions with the ELCs are also required, then perhaps it is not surprising that vertebrate RLCs cannot restore calcium sensitivity to 100% RLC denuded scallop myosin, even if vertebrate myosin is itself calcium sensitive (Chin., 1982; Rowe., 1989).

The ability to selectively remove and reassociate the RLCs from the myosin of invertebrate muscle, with no apparent damage to the other myosin components being caused, has generated a great deal of activity using that particular type of myosin (Szent-Gyorgyi et al., 1973; Kendrick-Jones et al., 1976; Chantler and Szent-Gyorgyi., 1980). A great deal of our knowledge of the function and location of the RLCs has been derived using this system (Szent-Gyorgyi et al ., 1973; Kendrick-Jones et al., 1976; Bagshaw., 1977; Stafford et al., 1979; Chantler and Szent-Gyorgyi., 1980). It is reasonably clear that the RLCs inhibit the ATPase activity of molluscan myosin, the inhibition being relieved by whatever process by the addition of calcium (Kendrick-Jones et al., 1976). The role of the RLCs in vertebrate myosin is however controversial and relatively obscure when compared

to what is known about its role in molluscan myosins.

(III. 8) The role of the RLCs in vertebrate myosins.

Although vertebrate RLCs are very closely related to molluscan RLCs in both sequence and structure (Leger and Elzymya., 1977; Collins., 1976; Kretsinger., 1980) its role in the direct regulation of vertebrate myosin by calcium has not prior to current work been demonstrated. Attempts to understand the role of the RLCs in vertebrate skeletal myosin have gone hand in hand with the study of their function in cardiac myosin. Although there now appear to be many major similarities in their structure and function, it should not be assumed that the role of the RLCs in each type of muscle is the same.

As with molluscan myosin, the development of a technique for the reversible removal of the RLCs, or at least removal that did not damage the rest of the myosin, was essential for the determination of the role of the RLCs within vertebrate myosin. Gazaith et al (1970) and Weeds and Lowey (1971) showed that the RLCs could to a limited extent be removed from vertebrate skeletal myosin using DTNB. Removal under these relatively mild conditions did not significantly affect the Ca ATPase and K EDTA ATPase acvtivities of the myosin. The RLCs in cardiac myosin, on the other hand, are completely unaffected by DTNB treatment and other means of removal have had to be developed. Cardiac myosin proteolytic subfragments (Weeds and Frank., 1972) and skeletal subfragments (Weeds and Taylor., 1975) are devoid of RLCs but were shown to maintain normal ATPase activities. This suggested that the RLCs of skeletal muscle myosin and cardiac myosin were not

necessary for the expression of the ATPase activity.

Experiments to determine the role of the RLCs in skeletal and cardiac muscle myosin have, as mentioned earlier, never been conclusive. This was mainly due to the fact that complete RLC removal had never been achieved. This led to the possibility that sufficient RLCs remained to maintain some of the myosin in its native confomation and thus mask changes that would be obvious if the removal of the RLCs had been complete. The other possibility was that via some form of cooperativity between the heads, the remaining RLCs were able to exert an influence on the RLC denuded heads.

In 1979 Malhotra et al showed for the first time that they could achieve 100% RLC removal from cardiac myosin using cardiac myofibrillar protease, which they showed was highly selective and had a negligible effect on the myosin heavy chain. Complete RLC removal did not affect the Ca2+ ATPase activity, which is identical to the situation in skeletal myosin (Weeds and Taylor., 1975). However it did cause a 25-30% reduction in the K EDTA ATPase and a 50-75% increase in the MgATPase activities. In contrast, the partial removal of the RLCs from skeletal myosin using DTNB has no effect on the K EDTA ATPase (Pemrick., 1977). Malhotra (1979) also showed that the readdition of RLCs to RLC denuded cardiac myosin completely reversed all of the effects caused by RLC removal. Reassociation of skeletal RLCs onto RLC deficient cardiac myosin also caused a complete reversal of the effects of RLC depletion, thus providing further evidence for homology between the RLCs as predicted from sequence information (Leger and Elyimya., 1977). The fact that full reversal was possible, by the reassociation of purified RLCs, suggested that the

changes are not due to sulphydryl modifications and that the removal of the RLC caused either a true conformational change in the active site of the myosin, or a conformational change that indirectly affected the active site.

Support for RLC removal causing a change in the active site came from some elegant proteolysis studies, carried out by Cardinaud (1987) using papain digestion of skeletal myosin in monomeric and filamentous form. Cardinaud (1987) noticed that there was an unsuspected change in the proteolytic digestion pattern when carried out with monomeric and filamentous myosin. The difference observed, was that the digestion of the S1 25-50Kd cleavage point (Applegate and Reisler., 1983; Mornet et al., 1984) was even faster in the filament than the monomer, when a slower cleavage was expected. The S1 25-50kD digestion site had previously been shown to be protected by the presence of nucleotide, indicating a possible relationship between this junction and the ATPase site (Korner et al., 1983; Mahmood and Young., 1984). Therefore as the presence of the RLC in the monomer protected the S1 digestion site, it seemed likely that the RLC was in contact with, or in close approximation to, the ATPase active site.

It was proposed that the reason for the difference in the digestion pattern of the monomeric and filamentous myosin was due to there being an interaction in the filament form between the N - terminus of the RLC and a region on the myosin shaft. This interaction caused the C - terminus of the RLC to move and expose the S1 25-50kD digestion site. The interaction with the shaft was by definition absent in monomeric myosin and therefore the C-terminus of the RLC was not displaced, enabling it to protect the S1 25-50kD digestion site.

The RLC interaction with the shaft may therefore result in a mechanical effect, pulling the myosin heads away from the thin filament. It is also of course possible that the proposed interaction of the RLC with the active site could modulate its affinity for substrate, or product, or even influence the rate of a step in the hydrolysis of the ATP.

(III. 9) RLCs can influence the actin binding properties of myosin

Pemrick (1977) showed that the RLC of skeletal myosin in the presence of calcium and regulated actin enhances the ability of the myosin to interact with the thin filament and that beyond the threshold ratio of myosin to actin (between 5:1 and 2.5:1 A:M) decreases the calcium concentration required to activate the thin filament. The RLC does this by stabilising a particular conformation, which enhances actin interaction as A-M. ADP. P* complexes, thereby increasing the calcium affinity of troponin. This idea is not altogether a new one. Bremel and Weber (1972) and Pemrick and Weber (1976) had already shown that under certain conditions myosin could form complexes with actin that activated the thin filament in the absence of calcium and caused an increase in the affinity of troponin for calcium. With RLC deficient cardiac myosin no such shifts in the calcium requirements of the actin activated ATPase in the presence of regulated actin was observed (Malhotra et al., 1979). An alternative approach by Holt and Lowey (1975a, 1975b), using antibodies against the RLCs of cardiac myosin also showed no effect on the calcium regulation via the troponin-tropomyosin system, but they did observe that the actin

activated ATPase was slightly lowered.

The differences between the results obtained with skeletal and cardiac muscle may reflect fundamental differences in the function of the RLC in each type of muscle. Alternatively it may be entirely due to differences in individual experimental procedure.

The effect of RLC removal from cardiac myosin on the MgATPase activity has been extensively studied. Malhotra et al (1979) reported that the complete removal of the RLCs from cardiac myosin caused a three-fold increase in the V_{max} of the actin activated ATPase. Similar findings were reported (Kuo and Bunerzee., 1982; Bahn et al., 1981) when using cardiac myofibrillar protease to selectively digest the RLC. Both groups found that RLC removal elevated the actin activated MgATPase activities, although to slightly differing extents. Kuo and Bunerzee (1982) found a 50% increase whilst Bahn et al (1981) report a two fold increase. All of these results indicate an increased affinity for actin on the removal of RLCs. It therefore, seems likely that the removal of RLCs from cardiac myosin results in a change in the conformation of the myosin, in such a way as to enhance the interaction with actin. The decreased affinity of native cardiac myosin (with its full compliment of RLCs) for actin strongly suggests a role for the RLC in reducing the actin and myosin interaction.

Contradictory results have been published which suggest that the role of the RLCs in cardiac myosin is to enhance the interaction of myosin and actin. Margossian (1985) demonstrated that removal of the RLCs from dog cardiac myosin, using myopathic hampster protease caused a 40% decrease in Vmax of the actin activated MgATPase. Full reversal of this effect was achieved by the reassociation of intact RLCs. This

ruled out the possibility of the change being caused by proteolysis of the heavy chain.

However, when using skeletal muscle myosin Pemrick (1977) also showed that the removal of 50% of the RLCs using DTNB caused a decreased interaction between actin and myosin. Similarly Margossian (1983) demonstrated a two fold reduction in the actin activated MgATPase of skeletal myosin, when the RLCs had been removed by digestion with hampster protease. Indeed several other workers have reported similar results when looking at the effect of RLC removal on the MgATPase of vertebrate skeletal muscle myosin. Physiological studies on skinned skeletal muscle fibres by Moss et al (1983) have shown that partial removal of 1/3 of the RLCs, using EDTA containing buffers, severely reduced the maximal shortening velocity (Vmax) with only a small effect on the isometric tension. The drop in isometric tension was almost entirely attributed to the loss of the troponins during the RLC removal procedure. The drop in Vmax was the direct result of RLC loss and, it was proposed that the RLC was modulating the kinetics of the interaction of actin with myosin, in such a way as to enhance the ability of the myosin to interact with the actin.

To complicate matters further several workers using in vitro biochemical methods have shown that RLC removal has no effect on the enzymic activity of the ATPase of skeletal myosin and its enzymatic subfragments (Wagner and Weeds., 1977; Maruta et al., 1978; Wagner and Giniger., 1981). However, as Moss et al (1982) pointed out, these studies were carried out at non physiological ionic strengths. Their own studies were done at phsioloigiocal ionic strength in vivo with an intact filament lattice and therfore may bear a more realistic

relationship to their role in vivo. The importance of maintaining as near in vivo conditions as possible was emphasised by Pemrick (1977) who showed that in vitro experiments studying the effect of RLC depletion on the actomyosin ATPase during calcium activation, may require the presence of fully regulated actin filaments.

It therefore appears that the RLCs of both cardiac and skeletal myosin, play a role in modulating the interaction of the myosin with the actin. Although the exact mechanism by which this modulation is achieved appears to be obscure. On the other hand there are many homologies between the RLCs of cardiac and skeletal myosin, in terms of sequence and structure (Leger and Elzyma., 1977; Collins et al., 1976) which may point to them having a similar role. However the overall weight of opinion from the literature suggests, that there is a fundamental difference in their function. The difference appears to be that the RLCs of cardiac myosin reduce the ability of the myosin to interact with actin (Pemrick., 1977; Bahnet al., 1981; Kuo and Bunerzee., 1982) whilst the RLCs of skeletal myosin apparently enhance its ability to interact with actin (Moss et al., 1982; Margossian., 1983; Moss et al., 1983). Conflicting evidence which shows that the RLCs have no influence (Wagner and Weeds., 1977; Wagner and Giniger., 1981) or indeed have exactly the opposite role, to some of those decribed above (Margossian., 1985) may be explained in terms of ionic strength effects.

(III. 10) The aims of this chapter.

The aim of this chapter is to try and clarify the role of the RLCs in native vertebrate myosin. As was the case with scallop myosin, the

ability to remove the RLC was an essential step in the elucidation of their function. Therfore, it was necessary to try and develop a method for the removal of the RLCs from vertebrate myosin, in a manner that did not involve proteolytic digestion or chemical modification of the myosin heavy chain. The technique finally developed does achieve 50% RLC removal, with no apparent damage to the heavy chain being caused. For this reason we have called it the gentle extraction technique.

Chin (1982) was the first to demonstrate that native vertebrate myosin contained two populations of RLCs, one of which was susceptible to chymotryptic digestion, the other resistant. This story was later confirmed by Rowe (1989) who also showed that after exposure to high salt the chymotrypsin resistant population becomes completely susceptible. The presence of the two RLC populations could be indicative of there being two conformationally distinct RLC environments. The gentle extraction technique provided an approach to the removal of one of these RLC populations and to observe the effect that this had on the MgATPase activity and calcium sensitivity of the vertebrate myosin. The gentle extraction technique itself was based on several previously published techniques (Moss et al., 1982; Kasman and Kakol., 1977; Levitsky et al., 1985) none of which gave reproducible results in our hands.

We show that using this gentle extraction technique we can reproducibly remove 50% of the RLCs from native vertebrate myosin, in either relaxed filaments or purified native filaments with no apparent damage to the heavy chain occuring. The RLCs of conventional high salt exposed myosin appear to be completely insensitive to this extraction technique. The effect of varying the conditions used during extraction

is reported and possible reasons for achieving only 50% RLC depletion are put forward.

The role of the RLCs in the regulation of the MgATPase activity is addressed by comparing the MgATPase activity and calcium sensitivity before and after 50% RLC depletion. Evidence is presented which suggests that the RLC plays a role in activating the MgATPase on the addition of calcium, with this ability being lost on RLC removal.

Finally we attempt to show that the two conformationally unique RLC environments observed to be present in rabbit myosin are also present in another type of vertebrate myosin, that of the rat. The similarities and differences between the two types of myosin are then discussed.

Results

Development of the gentle extraction technique.

(III. 11.) Explanatory note.

On examination of the gels in this chapter it will become apparent that when using RFPs the amount of protein loaded into each well can vary even though the calculated loading should be identical. This is probably due to the inherant stickyness of RFPs which presumably arises from the interaction of the numerous components of the preparation during incubation at elevated temperatures. Examples of such variations can be seen in many of the gels eg Gel 3.1., Gel 3.6. and Gel 3.11. However no such variation is observed when purified myosin preparations are used (Gel 3.7.), a fact that suggests that it is the multi component composition of RFPs that is the cause of the loading variations.
It was for these reasons that the double loading technique was developed. However for qualitative analysis it is easier to follow the effects of an extraction if all time points are included on the same gel. For this reason the majority of the gels presented in this thesis show the entire extraction timecourse although it must be emphasised that these gels were not used for quantitation.

(III. 12.) Difficulties involved in the accurate determination of RLC loss. (Development of a double loading technique).

The simplest method for determining the amount of RLC removal obtained, by any technique, is to use polyacrylamide gel electrophoresis. However, in order to determine accurately the amount of RLC depletion obtained, by measuring a drop in the intensity of the RLC band, a standard reference protein was needed to compare it with. Ideally this would be one of the polypeptides in the relaxed filament preparation eg ALC1, ALC2, or the Troponins. However, it was important that the protein chosen as the internal reference was not itself affected by the extraction technique. For this reason it was not possible to use ALC1, ALC2 or the thin filament associated proteins (See Sections I. 2.ii to I. 2.vi). This only left us with the two major proteins, actin and myosin, which, for differing reasons are difficult to use for quantitation. Actin is difficult to use due to the fact that creatine kinase runs at almost the same position (Pepe and Drucker., 1977) and therefore, complicates quantitation. The problem with using the myosin heavy chain is that when the sample is loaded for ideal RLC staining, the myosin band is grossly overloaded and therefore can not be used for quantitation.

It was decided however that the myosin heavy chain would be the best standard to use for the internal control by virtue of its stability in the system. A 'double loading technique' was developed which involved loading one well with a concentration of protein that was ideal for RLC staining and an adjacent well with a five fold dilution of the same sample. This dilution would have the effect of bringing the myosin

heavy chain into its ideal loading range. It was then possible to compare the intensity of the RLC band directly with the intensity of the myosin heavy chain and therefore determine the exact amount of RLC depletion that had occured. Control experiments showed that the pipetting and dilution errors were less than 1.5 %.

(III. 13.) Effect of the gentle extraction technique on the RLC content of a rabbit RFP.

Gel 3.1. shows the entire gentle extraction timecourse, each well having a similar loading. A qualitative analysis of the gel suggests that the intensity of the RLC band initially decreases very rapidly, followed by a slow phase showing little change. The absence of any new bands below the RLC shows that no proteolysis has occured even at the longest incubation time of 150 minutes. The fact that the RLC appears intact in the supernatant (Gel 3.2, Lane 3.) also supports this, and there is no evidence to suggest that proteolysis of any of the other proteins has occured. The gel does however, show that other proteins are extracted to varying extents by the incubation conditions, the most notable being ALC1 and the Troponins (See Sections I. 2.iii to I. 2.vi).
Gelscan analysis of the RLC region of a relaxed filament prep before and after 50% RLC depletion (Densitometric analysis 3.1.) shows the degree of RLC removal obtained, and also reveals that there is a slight reduction in the intensity of ALC1.
Gelscan analysis of the double loaded gels of the full gentle extraction timecourse (Graph 3.1.) revealed that after 30 minutes incubation time approximately 50% of the RLCs had been removed (Table 3.1.) and the

rate constant for this removal was 0.13 min^{-1}. The rapid removal phase was followed by a very much slower phase of probably non specific reassociation, with a rate constant of 1 x 10^{-3} min^{-1}.

(III. 14.) Loss of the alkali light chains during the gentle extraction procedure.

The alkali light chains appeared to be susceptible to extraction during incubation in the gentle extraction medium (Gel 3.1. and Gel 3.2.). ALC1 is by far the most susceptible of the two alkali light chains, with upto 15 % being extracted at the longer incubation times. The extraction kinetics take the form of a single term exponential, with a rate constant of 0.10 min^{-1}. The fact that the ALCs are susceptible to extraction under these conditions means that we cannot use them as internal reference proteins in the determination of the degree of RLC depletion obtained. It was for this reason that the double loading technique (See Section III. 12) was adopted, for comparing the RLC band directly with the myosin heavy chain.

Characterisation of the extraction conditions.

(III. 15) Extraction in the absence of ATP.

When a RFP was incubated in an extraction medium containing no ATP (5 mM KH2PO4, 50 mM KCL, 20 mM EDTA, pH 7.4), a limited amount of RLC depletion was obtained (Graph 3.5.). Gelscan analysis revealed

that the removal kinetics for the RLC follow a single term exponential with a rate constant of 0.085 min^{-1}. The maximum amount of removal (23%) occured after 35 minutes incubation time. Extended incubation (up to 260 minutes) did not cause further removal of the RLCs. However it did have an effect on the susceptible thin filament componets such as the troponins. The susceptibility of the troponins was similar to that observed in the presence of ATP, when using the standard gentle extraction medium (See Section III. 13).

(III. 16.) Extraction in the presence of an increased ATP concentration.

If the ATP concentration in the extraction medium is increased to 20 mM, RLC depletion still occurs (Gel 3.3.). However Gelscan analysis (Graph 3.2.) reveals that the degree of RLC removal obtained during incubation was not increased by increasing the ATP concentration. 50% of the RLCs were removed within approximately the first 30 minutes with a rate constant of 0.14 min^{-1}. There then followed a slow reassociation phase with a rate constant of 4.5×10^{-4}. All of these values were identical, within the limits of experimental error, to those obtained using the standard protocol (See Section III.13). ALC1 and the troponins were just as susceptible to extraction as when the standard protocol was used and there was no evidence to suggest that proteolysis had occured.

(III. 17.) Effect of a further addition of ATP after 120 minutes

Gel 3.4. shows the timecourse for a standard extraction, with the addition of 5 mM ATP after 120 minutes. Gelscan analysis (Graph 3.3.) revealed that the extra addition of ATP had no significant effect on the amount of removal, or the removal kinetics. There did not appear to be a significant change in the amount of ALC1 or troponin depletion and there was no evidence of proteolysis.

(III. 18.) Chymotryptic sensitivity of the gentle extraction resistant RLC population.

All of the RLCs remaining after a 50% RLC depletion were found to be chymotrypsin sensitive (Gel 3.5.). All of the RLCs were digested within the first 8 minutes of incubation. Analysis (Graph 3.4.) revealed that the digestion took the form of a single term exponential, with the rate constant for the digestion being 0.78 min^{-1}. The appearance of fragments lighter than ALC2 and the intensification of the ALC2 band suggested that the RLC was not being completely digested but, was initially being nicked into one large fragment (~17kD) and several smaller ones. The reduction in intensity of the ALC2 band at longer digestion times showed that the large (17kD) RLC fragment, produced during the early stages, was itself being further digested as the incubation time proceeded.

(III. 19.) RLC Depletion From Other Systems.

The fact that only 50% of the RLCs could be extracted from the native myosin in rabbit relaxed filaments, was obviously of great interest. However it was felt that it was important to determine whether this was a feature peculiar to rabbit myosin RFPs, or did other systems possess similar properties?

(III. 20.) Effect of the gentle extraction technique on the RLCs of rabbit PNFs.

Incubation of PNFs in the standard gentle extraction medium caused RLC depletion to occur (Gel 3.6.). The lack of any new bands during the entire incubation period demonstrated that proteolytic action was not responsible for their disappearance. The intensification of the RLC band in the supernatant also supported this view.

Removal of the susceptible RLCs occured very rapidly, the removal kinetics take the form of a single term exponential, with a rate constant of 0.26 min^{-1} and maximum extraction apparently occuring after 15 - 20 minutes incubation time. After the extraction of 50% of the RLCs, no further removal occured. There followed a slow phase of reassociation with a rate constant of 0.01 min^{-1}.

Gel 3.6. shows that the technique had no apparent effect on the myosin heavy chain, or on the bands in the C-protein region. The incubation conditions also had a tendency to partially extract the ALC1 band. The amount of ALC1 extraction obtained was similar to that observed in relaxed filament preparations (See Section III.14).

(III. 21.) Effect of the gentle extraction technique on the RLCs of a rat RFP.

Gentle extraction of the RLC from rat relaxed filament preparations was possible (Gel 3.7.). Detailed gelscan analysis revealed that approximately 50 % (Graph 3.8., Table 3.8.) of the the RLCs were susceptible to extraction. The kinetics of the removal could be fitted to a single term exponential with a rate constant of 0.07 min^{-1}, with maximum extraction occuring after approximately 35-40 minutes. At longer incubation times there was no evidence for there being a significant slow reassociation phase, the RLC content remaining constant between 30 and 140 minutes. The appearance of intact RLCs in the supernatant showed that their removal was not due to some form of proteolysis. There was however, evidence to suggest that a small amount of proteolysis of some of the other proteins had taken place. The troponin C band appeared to increase in intensity as the incubation proceeded, whilst there was a corresponding decrease in the intensity of ALC1, along with the appearance of some faint but noticable bands below ALC2. We believe that the ALC1 in rat myosin may be particularly susceptible to proteolysis or heat damage and that it breaks down into a large fragment that runs level with troponin C and also into several very light components (<< 16kD). No new bands appeared or disappeared above the ALC1, suggesting that none of the higher molecular weight proteins had been digested.

(III. 22) Does transient exposure to high salt affect the degree of RLC removal attained ?

The chymotryptic probe experiments (Rowe., 1989) have revealed the presence, in native myosin, of two populations of RLCs, one population occupying a distinctive high salt sensitive environment. The gentle extraction technique has also revealed the presence of a distinctive RLC population (See Section III.13). The next problem addressed was whether the exposure of native myosin to high salt conditions had any effect on the removal of the RLCs using the gentle extraction technique.

(III. 23.) Extraction of the RLCs from a high salt exposed rabbit RFP.

After the transient exposure of a RFP to high salt conditions none of the RLCs appeared to be susceptible to extraction. Gelscan analysis and computer fitting of the data confirmed this, showing that even at incubation times of 150 minutes, the myosin retained its full compliment of RLCs. The incubation conditions had no effect on ALC1 and there was no evidence to suggest that proteolysis had occurred.

(III. 24.) Gentle extraction from SMFs.

The RLCs in an SMF preparation (Persechini and Rowe., 1984) were completely resistant to removal using the gentle extraction technique (Gel 3.8, Graph 3.9) what is more the alkali light chains were also resistant to extraction.

(III. 25.) Gentle extraction from high salt exposed rabbit PNFs.

After transient exposure to high salt conditions (0.5 M KCl , 5 mM Tris pH7.4 for 5 hours) the RLCs of the myosin became completely insensitive to extraction. Gelscan analysis revealed that at the longest incubation time of 140 minutes there was no significant loss of RLCs . The lack of any new bands on the gel showed that no proteolysis had occured. Neither of the alkali light chains, which were known to be susceptible to extraction before high salt exposure, were affected.

(III. 26.) Gentle extraction from a high salt exposed Rat RFP.

Transient exposure of rat relaxed filaments to high salt conditions allowed a large amount of RLC removal to take place (Gel 3.9.). Gelscan analysis (Graph 3.10.) revealed that the degree of removal was approximately 35% greater than that attained with a native rat RFP (Graph 3.7.). The removal kinetics of the RLCs take the form of a single term exponential, with a rate constant of 0.13 min^{-1}. The RLCs appeared intact in the supernatant (Gel 3.10, Lane 1.) and showed no signs of degradation. No new bands appeared on the gel even at the longest incubation times. Therefore, there was no indication that proteolysis had occured.

The alkali light chains did not appear to be extracted during the incubation and were present in similar proportions to those seen in the control.

(III. 27.) Is the removal of RLCs from high salt exposed rat filaments an equilibrium reaction ?

Approximately 85% of the RLCs were removed from a high salt exposed rat RFP by incubation in the gentle extraction medium for 40 minutes at 37 °C (See Section III. 26). When the extracted RLCs were removed from the supernatant by centrifugation and the RLC depleted filaments were subjected to a further gentle extraction for 40 minutes, then a further 72 % of the remaining RLCs were removed (Gel 3.10). This represented a total RLC removal of 95% after two 40 minute incubation periods. Although further incubation periods could not be justified in view of the possibility of heat damage to the myosin, it should be noted that this is the first time that almost all of the RLCs have been removed from a vertebrate myosin, by a non enzymic method, and one that does not involve chemical modification of the myosin. It is surprising that the removal of all of the RLCs was only seen in this study when using high salt exposed rat relaxed filaments.

The effect of RLC depletion on the steady state MgATPase of vertebrate myosin.

(III. 28.) Does the incubation temperature used during the gentle extraction technique have an effect on the steady state MgATPase activity of a rabbit RFP ?

The procedure for the removal of 50% of the RLCs from a relaxed filament preparation involved incubation in an extraction medium at 37 °C for 30 minutes (See Section III. 13). Although 37 °C is a physiological temperature (for vertebrates), the environment of the myosin in a relaxed filament preparation does not mimic physiological conditions exactly. We must therefore consider the possibility that the relatively high temperature and length of incubation involved in RLC depletion may damage the myosin and cause changes in the MgATPase activity and calcium sensitivity. It was therefore important to check that the temperature used for the RLC depletion did not change the steady state MgATPase activity of the myosin.

The calcium sensitivity before and after incubation at 37 °C for 30 minutes in a non RLC extracting buffer (100mM KCl pH 7.4) is shown in assay traces 3.1 and 3.2. The basal rate (absence of calcium) was approximately the same in both cases, 0.25 sec^{-1} before incubation and 0.26 sec^{-1} after. The addition of calcium caused the rate of the control to increase to 2.9 sec^{-1} whilst, the rate after incubation and in the presence of calcium showed a similar increase, rising to 2.9 sec^{-1}. The calcium sensitivity of each sample was therefore almost identical

before and after incubation, with a calcium sensitivity of 11.3 fold and 11.1 fold respectively.

(III. 29.) Effect of 50% RLC Depletion on the MgATPase of a RFP.

After 50% RLC depletion the steady state MgATPase showed considerable variation, in both its basal rate (minus calcium) and the degree of calcium sensitivity remaining. For example, Table 3.12. (4a and 4b) shows a preparation that after 50% RLC depletion, had lost a large proportion of its calcium sensitivity and more importantly showed an elevation in its basal rate, from 0.2 sec^{-1} in the control to 0.5 sec^{-1} after RLC depletion. Table 3.12. (2a and 2b) also shows a preparation that after RLC depletion had an identical basal rate (0.12 sec^{-1}) to that of the control (0.11 sec^{-1}) but showed a 60% reduction in calcium sensitivity. These are just two of the many results obtained using relaxed filament preparations, which showed considerable variation in both the basal and activated rates after RLC depletion. We have found that a consistent story cannot be obtained using a RFP. We believe that the situation is more complicated than expected because the incubation conditions involved in RLC depletion have an effect on the actin linked regulatory proteins (See Section I.2.iii to I.2.vi).
As pointed out in Section III. 13, the gentle extraction technique was to varying extents removing the troponins. Troponin C, the calcium binding protein of the thin filament regulatory system, appeared to be affected the most. Lehman (1978) used the known susceptibility of troponin C to various extraction conditions to completely remove it from the thin filaments and demonstrate the possible existence of a

thick filament linked regulatory system. Therefore any interpretation of the changes occuring in the MgATPase activity and calcium sensitivity after RLC depletion must take into account the fact that the thin filament regulatory system may also be affected and thus, contribute to the observed changes.

Therefore because of the removal of both the RLCs and troponins, it was felt that any attempt to interpret the changes in terms of RLC loss would be masked by the extraction of the thin filament regulatory proteins, which indeed appears to be the case.

(III. 30) Does the incubation temperature used during the gentle extraction technique have an effect on the steady state MgATPase activity of a rabbit PNF preparation ?

Because of the problems involved in interpreting the effect of RLC depletion on the MgATPase of the myosin in a RFP (See Section III. 2), we started using PNFs. PNFs, by definition have none of the thin filament linked proteins present. Therefore any changes observed after depletion could be attributed solely to the loss of the RLCs. However we must be careful to take into account the effect that slight losses of the alkali light chains (See Section III. 14) and especially ALC1 might have on the MgATPase activity.

The possibility of heat damage occuring was even greater when using a highly purified systems such as PNFs. Therefore, it was important to check that the incubation conditions themselves (15 minutes at 37 °C) were not causing changes in the myosins MgATPase.

After 15 minutes incubation at 37 °C in a non RLC extracting medium

containing100 mM KCl pH 7.4, there was no significant change in the MgATPase activity of the PNFs (Table 3.12. preparations 1a and 1b). The basal rate before incubation being 0.059 sec^{-1} and after incubation 0.057 sec^{-1}, with the addition of calcium causing an activation to 0.094 sec^{-1} and 0.091 sec^{-1} respectively. This represented approximately a 1.6 fold increase in the rate in both cases.

It is important to point out that the reported two fold activation of the myosin MgATPase activity on the addition of calcium (See Section III. 29 and Rowe., 1989) was only seen in fresh preparations (Assay trace 3.3). After several days storage the amount of activation observed steadily decreased. However, the effects seen in fresh preparations were always seen in the older ones but the overall magnitude of the effect was proportionally smaller, as post preparation time increased.

(III. 31.) Does 50% RLC depletion affect the intrinsic myosin linked MgATPase activity and calcium sensitivity of a PNF preparation ?

After 50% RLC depletion several changes were observed to occur in the myosin linked MgATPase activity of the PNFs. There invariably appeared to be a very slight but variable drop in the basal rate (- Ca^{2+}) (Table 3.13.). However these small decreases in basal rate were also observed in PNFs incubated in non extracting medium and are probably an indication that there is possibly a small degree of heat damage occuring.

50% RLC depletion did however, have a dramatic effect on the calcium sensitivity of the MgATPase activity, causing a large reduction (Assay traces 3.1 and 3.2, Table 3.13.). Once again the ability to calcium

activate the MgATPase after RLC depletion was slightly variable with the extremes being shown in Table 3.13. The average calcium sensitivity after subjecting the PNFs to the gentle extraction technique was however approximately 50% that of the control value (eg Assay traces 3.1 and 3.2).

We believe that the observed variations in the post RLC depletion calcium sensitivities can be partly explained by errors in the slope determination of the ATPase traces and the possible effects of post incubation aggregation (See Section V. 17). The underlying trend however shows that the loss of 50% of the RLCs from PNFs, resulted in a 50% drop in the calcium sensitivity when compared to that of the control.

(III. 32.) Effect of 50% RLC depletion on the steady state actin activated MgATPase activity of a PNF preparation.

After 50% RLC depletion the basal, actin activated MgATPase activity of a PNF preparation changed very little and although a slight decrease in the rate was most common sometimes a small increase was observed. (Table 3.14.). However, as pointed out above a slight drop in basal rate was also observed after incubation in a non a extracting medium (Table 3.14.), and is therefore possibly an indication that the incubation temperature does affect the myosin to a small degree.

After 50% RLC depletion the amount of calcium activation achieved by the addition of calcium was reduced by approximately 50% (Assay traces 3.5 and 3.6, Table 3.14.). As with the intrinsic MgATPase activity, the drop in calcium sensitivity achieved was slightly variable

(Table 3.14.). However, the average reduction, when compared to that of the control was close to 50%.

Therefore the removal of 50% of the RLCs from vertebrate myosin using the gentle extraction technique, causes a 50% drop in the actin activated MgATPase activity and a slight decrease in the basal rate. The effect of 50% RLC depletion on the MgATPase calcium sensitivity in both the presence and absence of actin is essentially identical, allowing for the differences in rates, in that it reduces the calcium sensitivity by 50%.

Discussion

Development of and conditions necessary for the gentle extraction technique.

(III. 33.) Why is ATP so necessary for extraction to occur ?

The successful development of a technique for the removal of the RLCs from native vertebrate myosin was an essential step in the attempt to elucidate of the role of the RLCs. The gentle extraction technique developed removes a reproducible 50% of the RLCs from native myosin in both RFPs and PNFs. Removal of between 30 and 50% of the RLCs from myosin has been reported by several other groups (Gazith et al., 1970; Kasman and Kakol., 1977; Malhotra., 1979; Margossian et al., 1983). These results are however not directly comparable with ours as they used high salt exposed conventional myosin, or achieved removal by means of chemical modification or proteolysis. The only results directly comparably with our own were those of Moss et al (1982) and Levitsky et al (1985) who both attained RLC extraction from muscle fibres, using EDTA extracting buffers. However, Levitsky et al (1985) reported only that they could remove 50% of the RLCs and not the effects that this had on the properties of the myosin. Moss et al (1982) achieved 30% RLC depletion, which they reported resulted in a 40% drop in the Vmax of the contraction velocity.

The reason that Moss et al (1985) only achieved 30% RLC depletion may be due to one of two causes. Firstly the RLC loss may be related to the type of ALC present on the S1 head (Hozumi et al., 1979). From the

known distribution of these light chains (Weeds and Taylor., 1975) it is possible that only the RLCs on a ALC1 containing head are susceptible to the extraction. Our ability to remove 50% of the RLCs is unlikely to be related to the ALCs, as this figure bears no resemblance to the ALC distribution ratio. Secondly, the use of whole muscle fibres, as opposed to relaxed or purified filaments, may restrict the ability of the bathing extraction buffers to permeate throughout the fibre. We have observed that our extraction technique did not achieve 50% RLC depletion when myofibrils were used as opposed to RFPs or PNFs. The degree of extraction varied from 25 to 40%, perhaps because the extracted RLCs had difficulty diffusing out of the intact filament lattice of the myofibril. The variation in the amount of removal from myofibril preparation to preparation, was probably indicative of the differing degrees of myofibrillar disruption in each preparation.

The presence of ATP in the incubation medium was found to be entirely necessary for complete (50%) extraction to occur. The absence of ATP during incubation resulted in a significant reduction in our ability to remove the RLCs with a maximum depletion of 20% being obtained. It was considered possible that the inability to remove all of the RLCs during a gentle extraction was linked to the hydrolysis and therefore, dissappearance of ATP from the incubation medium. Levitsky et al (1985) reported that the presence of ATP in their extraction medium was essential to achieve 50% RLC depletion but they do not explain why.

In order to determine the role that ATP was playing in the extraction, the use of ATP regenerating reactions, such as those present in the linked enzyme assay (See Section II. 20) was considered. However,

these cannot be used because the necessary enzymes (PK/LDH) need magnesium (Emes., 1977) and the high concentration of EDTA in our buffers chelates free magnesium. The role of the ATP in RLC extraction was therefore, explored by two alternative methods. The first was by increasing the concentration of ATP and the second was by the addition of ATP after 120 minutes. The fact that a four fold increase in the ATP concentration did not significantly alter the extraction kinetics suggests that the inability to remove the final 50% of the RLCs was not due to the exhaustion of ATP. This was further supported when identical extraction kinetics were obtained by the addition of a further 5mM ATP after 120 minutes incubation in the standard extraction medium i.e. after the addition a further burst of RLC depletion was not observed. The ATP appears to be entirely nessecary for complete (50%) extraction to be achieved but the total amount of extraction achieved is not linked to the maximum concentration of ATP. It would therefore appear that for RLC dissociation to occur, the myosin has to be actively turning over ATP. It may be that the hydrolysis cycle at some stage allows the head to adopt a conformation that is conducive to RLC dissociation. The work of Cardinaud (1987) indicated a strong link, spatially, between the ATPase acive site and the RLC. The hydrolysis of ATP at the active site, could therefore, exert an influence on the RLC environment rendering it favourable to RLC extraction. Cardinaud (1987) used enzymic digestion studies to show that the C-terminus of the RLC was probably in close proximity to the ATPase active site. Because the RLC N-terminus was relatively mobile (Walliman et al., 1982; Hardwick et al., 1983). Cardinaud (1987) suggested that the C-terminal domains of the RLC were involved in binding the heavy

chain. Bagshaw and Kendrick-Jones (1980) came to a similar conclusion when using epr to study RLC binding.

The reason for the limited degree (20%) of RLC removal when using a RFP in the absence of ATP could, on the other hand, be due to the formation of rigor complexes. These could confer actin protection on the RLCs, rendering them resistant to the extraction. Actin protection of the RLCs is not a new concept, its occurance has been reported many times when studying the proteolytic digestion patterns of S1, HMM, myosin, actomyosin and myofibrils (Oda et al., 1980; Yamamoto and Sekine., 1986).

The presence of ATP in the incubation medium may only be essential in so far as it stops the myosin from "seeing' the actin and forming rigor complexes. The rate constant for the removal of 50% of the RLCs from the myosin of a RFP is 0.13 min^{-1}, with the maximum extraction being achieved after approximately 120 minutes. The rate constant for RLC removal from PNFs is 0.26 min^{-1}, with maximum extraction being achieved after only 15 minutes. Although the rate constants are slightly different the final degree of extraction was identical (50%). Therfore one presumes that they are both removing the same distinctive RLC populations. The reason for the slower extraction kinetics, when using RFPs, could be due to the myosin's interaction with actin as it hydrolyses ATP i.e. the binding of the myosin heads to the actin results in a conformation which prevents RLC dissociation. It is only when the myosin head is not bound to the actin that the environment of the RLCs is such that it allows the RLC to dissociate. The thin filaments in a relaxed filament preparation are mostly regulated and during extraction would be switched off because of the

presence of EDTA. However, as the thin filament regulatory proteins are also to a small extent stripped off during the gentle extraction procedure, the number of unregulated thin filaments present would increase. This would allow greater myosin/actin interaction to take place, resulting in a reduction in the RLC removal rate because of increased actin protection.

The amount of RLC extraction obtained when using RFPs and PNFs is identical. We therefore feel that although it is highly likely that the ATP does play a part in keeping the myosin off the actin, the degree of extraction in each system is so similar that its main role is probably in maintaining the myosin heads in the optimum conformation for RLC depletion to occur.

(III. 34.) Does the exposure of native myosin to high salt conditions affect the ability to remove 50% of the RLCs using the gentle extraction technique ?

The transient exposure of rabbit PNFs or RFPs to high salt conditions was found to abolish the ability to remove 50% of the RLCs using the gentle extraction technique (See Section III. 23). The thin filament associated proteins present in RFPs however still appeared to be susceptible to removal. Therefore any high salt induced conformational changes that have occured and which prevent RLC removal did not, at least on a qualitative basis, affect the troponins. Exposure to high salt has, it appears, irreversibly altered the conformation of the myosin in such a way as to render the environment of all of the RLCs identical and resistant to extraction. As far as we can determine high

salt exposure has had no irreversible effect on the thin filament and its associated proteins.

The exact location of the change in conformation, caused by high salt exposure, is at this stage unknown but there are two main possibilities. The first is that high salt exposure results in a change in the secondary or tertiary structure, which causes the environment of both RLC populations to become identical and resistant to removal. The second is that the myosin filament itself posesses a unique quarternary structure, which is irreversibly lost on depolymerisation in high salt.

If we first of all consider the possibility of there being a unique secondary or tertiary structure, one can clearly imagine three possibilities for the equal distribution of two unique RLC populations, throughout the native myosin population. In the first there are two populations of myosin molecules which are homodimers, with respect to their RLC content ie. one homodimer population containing a pair of RLCs that were susceptible to gentle extraction, the other population containing a pair of resistant RLCs. During high salt exposure, the population containing the susceptible RLCs undergo a conformation change, that renders all RLCs resistant to extraction. In the second possibility all of the myosins are identical and contain one of each type of RLC (Heterodimers). Upon high salt exposure, the environment of the gentle extraction susceptible RLCs, is altered in such a way that they become completely resistant to removal. The third possibility is that the distribution of extraction susceptible RLCs is completely random and that RLC depletion from one myosin head renders the RLC of the other head resistant to extraction. The random release of RLCs in such a fashion could well be similar to the the random release process from

scallop myosin at 4 °C (Kendrick-Jones et al., 1976). The only way to resolve the problem would be to carry out a similar experiment to those of Kendrick-Jones et al (1976) and reassociate labelled RLCs onto 50% RLC depleted rabbit myosin, followed by a further gentle extraction. The distribution of labelled RLCs removed by the second extraction, would inform us whether the RLC removal process was random or not. However as the conditions necessary for RLC reassociation have not yet been defined using our system, the experiment cannot be conducted.

The possibility of there being a unique myosin filament quarternary structure which is irreversibly lost on depolymerisation during high salt exposure must also be considered. The fact that reassembled (synthetic) thick filaments do not possess the same packing or quarternary structure as native filaments is not altogether surprising. It is well known from the work of several groups that synthetic filaments do not have the same length distribution, width, or mass per unit length (Persechini and Rowe., 1984) as native filaments (Emes and Rowe., 1978) and only rarely do they appear to be bipolar (H. E. Huxley., 1963). Consideration of these points, leads one to suppose that the packing in myosin filaments is altered, after high salt exposure. Such an alteration in packing may result in the loss of one of the unique environments of the RLCs. It is however, difficult to imagine how the packing of myosin tails into the shaft of a thick filament can influence the properties associated with the S1 heads.

The question as to which parts of the myosin filament or molecule are responsible for the distinctive properties of native myosin is addressed in Chapter IV and will not be discussed further here.

However, for the sake of clarity and in the light of results reported in that chapter we will assume that all myosin molecules are identical, with respect to their RLC content (Hetorodimers) and that the distinctive properties of native myosin arise from a unique secondary or tertiary structure and are not the result of a distinctive mode of native filament packing or quarternary structure.

(III. 35.) Do the two RLC populations revealed by the gentle extraction technique correlate with those revealed by the chymotryptic probe ?

The chymotryptic probe technique (Chin., 1982; Bolger et al., 1989b) revealed the presence of two RLC populations, one of which was susceptible to digestion, the other resistant. The gentle extraction technique also revealed the presence of two RLC populations, one susceptible to extraction, the other resistant (See Section III. 13). With these results in mind one would predict that the population of RLCs that were susceptible to chymotryptic digestion, were also susceptible to gentle extraction. However, chymotryptic digestion of the RLCs remaining in a RFP after RLC depletion were all completely chymotrypsin sensitive (See Section III. 18). The entire population was completely digested within 16 minutes, the digestion taking the form of a single term exponential, with a rate constant of 0.78 sec^{-1}.
This apparently anomalous result can be explained in one of two ways. The first is, that the initial process of RLC depletion causes a conformation change within the myosin that renders all of the remaining RLCs chymotrypsin susceptible, whether they were susceptible before extraction or not. The change in conformation may

result from the formation of a sticky patch on the RLC deficient S1 head, which by interaction with the heads still containing RLCs, renders them all chymotrypsin sensitive. The interaction of the sticky patch need not be between two myosin S1 heads, but could be with the shaft. This shaft interaction could then in some way influence the conformation of the myosin heads still containing their RLCs. Another explanation for the apparent disparity between the results is that both techniques are seeing the same two environmentally distinct RLC populations but each technique is, for whatever reason, sensitive to the resistant population of the other.

For example, if one assumes that both techniques reveal the presence of two RLC populations because of their unique environments and that all myosin molecules are the same (Heterodimers) then the following scheme can be envisaged. Removal of one RLC, leaves behind the chymotrypsin susceptible RLC, whereas chymotryptic digestion of intact myosin leaves behind the extraction susceptible one. Unfortunately, the latter point is difficult to confirm as chymotryptic digestion results in the formation of S1 and HMM (Chin., 1982) resulting in the breakdown of filament structure. However, confirmation of the validity of such a model was provided by the work on high salt exposed myosin. This showed that after transient exposure to high ionic strengths, none of the RLCs were susceptible to RLC depletion, whilst they all became chymotrypsin sensitive. This is exactly the result that one would predict from the model proposed above.

It should be emphasised, that during chymotryptic digestion of native myosin it is unlikely that the digested RLCs are being completely removed from the myosin. What appears to happen is that the RLCs are

nicked, initially into several fragments, one approximately 17kD in size. The larger fragment is then nicked into smaller fragments as the incubation proceeds. The nicked RLC fragments however, probably remain bound to the RLC binding site (Cardinaud., 1987) preventing the exposure of a sticky patch. This rules out the possibility of a head containing an exposed sticky patch interferring with an RLC containing head and altering its digestion characteristics. Therefore, the reason for seeing two RLC populations is most likely to be because they occupy two distinctive conformational environments, and is not the result of the interaction of 'sticky patches'.

(III. 36.) Is the native conformation of myosin from other vertebrates irreversibly altered by high salt exposure ?

Rabbit skeletal myosin is widely accepted as being representative in terms of structure and function of all vertebrate skeletal myosins. It is for this reason that the vast majority of vertebrate muscle research is carried out using rabbit myosin. However we felt that it was always possible that the distinctive properties observed to be present in native rabbit myosin (Rowe., 1989) were peculiar to that particular animal. We therefore, attempted to show that this was not the case by subjecting the myosin filaments of another vertebrate (rat) to the gentle extraction technique before and after high salt exposure. Differences in the RLC depletion kinetics would be indicative of a high salt induced conformational having occured.

The results clearly showed that 50% of the population of RLCs present in native rat myosin are completely susceptible to extraction (Gel 3. 7,

Graph 3. 8), the remaining RLCs being entirely resistant to removal. The extraction kinetics are very similar to those observed when using a rabbit RFP, with the maximum degree of extraction occuring after approximately 35-40 minutes. The RLC removal took the form of a single term exponential with a rate constant of 0.07 min^{-1} (as opposed to 0.13 min^{-1} for rabbit). An interesting feature of the removal kinetics was that at the longer incubation times there was no sign of a significant degree of RLC reassociation having occured (cf the rabbit RFPs and PNFs which clearly showed (III. 13 and III. 20) a small amount of apparently non specific reassociation to be occuring at the longer incubation times. This was thought to be due to myosin heat damage). The apparent lack of such reassociation in rat myosin, even after 140 minutes incubation time suggested that rat myosin was more resistant to heat damage and may therefore, be a more stable system to work with in the future.

Nonetheless it was clear that the unique RLC population, observed to be present in native rabbit myosin was also present in native rat myosin, as judged by the RLC depletion kinetics. Therefore, we feel that it is reasonable to assume that other types of vertebrate skeletal muscle myosin possess similar properties.

Having established that native rat myosin has two distinctive RLC populations, transient high salt exposure was shown, as with rabbit myosin, to irreversibly alter this. However, the change observed was the opposite to that which occured when using rabbit myosin. The high salt exposure of rat myosin did not eliminate our ability to remove any of the RLCs but actually enhanced it (Graph 3. 10).

Initially we obtained over 85% extraction by the RLC depletion of high

salt exposed rat myosin. Further experiments however, revealed that the depletion was probably an equilibrium reaction and that removal of the already extracted RLCs from the supernatant, followed by a futher extraction, resulted in even more RLC loss (Section III. 27). If enough washing and incubation cycles were carried out, then presumably complete RLC dissociation would be achieved.

It was concluded that the high salt exposure of rat filaments caused an irreversible change in the environment of 50% of the RLCs and rendered them all susceptible to gentle extraction. Athough this is the opposite change to that observed with rabbit myosin, it does nontheless clearly demonstrate that native rat filaments do possess a high salt sensitive RLC population.

The fact that high salt exposure has the opposite effect on the extraction susceptibilities of rabbit and rat RLCs suggests that it would also have the opposite effect on the chymotrypsin susceptibility of the RLCs. One would therefore predict that after high salt exposure all of the RLCs in the rat myosin would become chymotrypsin resistant. These experiments have, as yet, not been carried out but it would be interesting to see whether the high salt induced conformational change could be picked up in rat RFPs using the chymotryptic probe technique (See Section III. 41).

(III. 37.) The effect of RLC depletion on the MgATPase activity (The role of the vertebrate RLC).

The amount of useful information available about the function of the RLCs from the experiments involving the RLC depletion of rabbit RFPs

was limited. Although changes in the ATPase were observed after RLC removal the changes were variable and in some instances negligible. Examination of the timecourse gels revealed that the technique was not exclusively removing the RLCs. Incubation in the extraction medium was also causing a loss of some of the thin filament associated proteins, especially TnC and TnI. The loss of the thin filament associated proteins during RLC depletion in EDTA buffers, is not unique to our extraction technique. Moss et al (1982 and 1983) reported a significant but reversible loss of TnC during the EDTA treatment of muscle fibres. Such a loss would cause some of the actin filaments to become unregulated resulting in a mixed population of regulated and unregulated actin filaments. Some of the myosin molecules would therefore be able to interact with unregulated actin and permanently display their actin activated MgATPase activity. The rest of the myosin molecules would then only be able to interact with fully regulated thin filaments, which in the absence of calcium would be minimal. When using such mixed populations the presence of a small proportion of unregulated actin/myosin interactions can and does mask the presence of a large population of fully regulated regulated actin/myosin interactions. The masking of regulated scallop myosin ATPase activity by the presence a small proportion of unregulated myosin molecules was demonstrated by Wells and Bagshaw (1985) using limited turnover experiments to resolve the individual species. Furthermore the degree of TnC and TnI extraction, seems to vary slightly from preparation to preparation. The resulting variations in the actin regulation, made interpretation of the effects of RLC depletion difficult, if not impossible to make using steady state

kinetic techniques.

The use of RFPs to look at the MgATPase activities was important however in showing that the incubation temperature of 37 °C was not causing a significant amount of heat damage to the ATPase (See Section III. 28). The MgATPase activities before and after incubation at 37 °C in a non extracting buffer were found to be almost identical. This clearly demonstrated that the incubation temperature required to remove 50% of the RLCs was having no significant effect on the MgATPase activity of the myosin. Chantler and Szent-Gyorgyi (1980) had suggested that incubation at such elevated temperatures was detrimental to the ATPase activity but no indication of this was ever found in our RFPs.

The RLC depletion of PNFs was much more informative and allowed us to propose a role for the RLCs in vertebrate myosin. Once again it, was important to show that the incubation temperature (37 °C) had no adverse effects on the properties of the MgATPase activity. Several control experiments revealed that the intrinsic basal MgATPase and the calcium sensitivity were not significantly affected by incubation at 37 °C for 15 minutes in a non extracting buffer.

As previously mentioned, the gentle extracion technique was not completely specific for removal of the RLCs and did to a limited extent (10 to 15%) extract ALC1. A similar degree of ALC1 removal was reported by Moss et al (1983), when attempting the removal of the RLCs from muscle fibres, using EDTA extraction buffers. The EDTA extraction buffers used by Wickmann-Coffelt (1979) caused a similar level (5-10%) of ALC extraction, which reassociation experiments showed could reasonable be ignored. Although, there was significant

(10 -15%) ALC1 loss from the PNFs during RLC depletion we did not, unfortunately, have a convenient test for the effect of this loss on the MgATPase activity and consequently had to assume, that its effect was negligible (Wickman-Coffelt., 1979; Moss et al., 1983).

After 50% RLC depletion of PNFs, there was invariably a slight drop in the basal rate of the intrinsic MgATPase activity, which possibly indicates that a small degree of myosin heat damage has occured. The fact that a similar drop was also seen after incubation in non extracting mediums (Table 3.13), suggests that the decrease in basal rate was due to a small amount of heat damage and was not a result of RLC removal. Electron microscopy (Flicker et al., 1983; Margossian and Slayter., 1987) has revealed that RLC removal causes a shortening of the S1 heads, which is accompanied by a decrease in the centre to centre distance as revealed by EPR studies (Wells et al., 1983). If such changes in conformation do occur they do not interfere with the basic functioning of the ATPase active site.

Similarly the drop in the basal rate of the MgATPase acivity cannot be due to the slight loss of the ALCs. However the literature is increasingly showing evidence for the involvement of the ALCs in the function of the ATPase activity of myosin (Walliman and Szent-Gyorgyi., 1981) and more specifically in the calcium sensitivity of molluscan myosin (Collins et al 1986). Therefore we cannot rule out the possibility that they might be involved in the calcium sensitivity of the ATPase. Perhaps our assumption that the slight loss of ALC1 during gentle extraction from PNFs was unimportant was a naive one and is a problem that needs addressing more carefully in future work.

The calcium sensitivity of RLC depleted PNFs was reduced by 50% RLC

depletion, to between 30 and 80% of the control value (See Section III.30). The variation in the degree of calcium sensitivity lost by RLC depletion in this case cannot be put down to the loss of the thin filament associated proteins. The variation is probably due to several factors, the main one of which is the limitation in the accuracy with which we can determine the difference in the gradient of two lines, that differ in slope at best (in the control) by two fold. Therefore when the difference in gradient is small, such as after 50% RLC depletion, only a slight discrepancy in gradient estimation can result in significant errors in rate estimations. The length of time post RLC depletion may also have a slight influence because of the increasing time dependent aggregation that is known to occur (See Section V. 23).

These results suggest that the RLCs of native vertebrate myosin are intrinsically linked to both the MgATPase activity and myosin linked calcium sensitivity. As the loss of the RLCs causes a corresponding loss in the ability of calcium to activate, both the intrinsic and actin activated MgATPase activities, we propose that the RLCs in vertebrate myosin are not inhibitory in function and that they play a role in the calcium activation of the MgATPase. By contrast the RLCs of molluscan myosins are entirely inhibitory in function (Bagshaw., 1980), their removal resulting in the permanent activation of the MgATPase activity of the myosin (Szent-Gyorgyi et al., 1973; Chantler and Szent-Gyorgyi., 1980).

This postulated role of the RLCs in the activation of vertebrate myosin is supported by results of several groups who show that RLC removal reduces actin myosin interaction and decreases the actomyosin ATPase activity. Moss et al (1983) show that the Vmax of maximally

Ca-activated skinned vertebrate skeletal muscle fibres decreases as the RLC content of the fibres is reduced by washing in EDTA extraction buffers (Moss et al., 1982). They concluded that the effects on Vmax of RLC removal indicates a role for the RLC in modulating the interaction kinetics of myosin and actin, the presence of the RLC enhancing this interaction. These results are in full agreement with those of Pemrick (1977), who showed that the presence of the RLC enhanced the interaction of the myosin with the actin and reduced the calcium requirement of the thin filaments regulatory proteins. Pemrick (1977) also showed that the removal of 50% of the RLCs (using DTNB treatment) caused the ATPase of the myosin, in the presence of maximally Ca-activated thin filaments to drop by 50%, which is in very close agreement with our results.

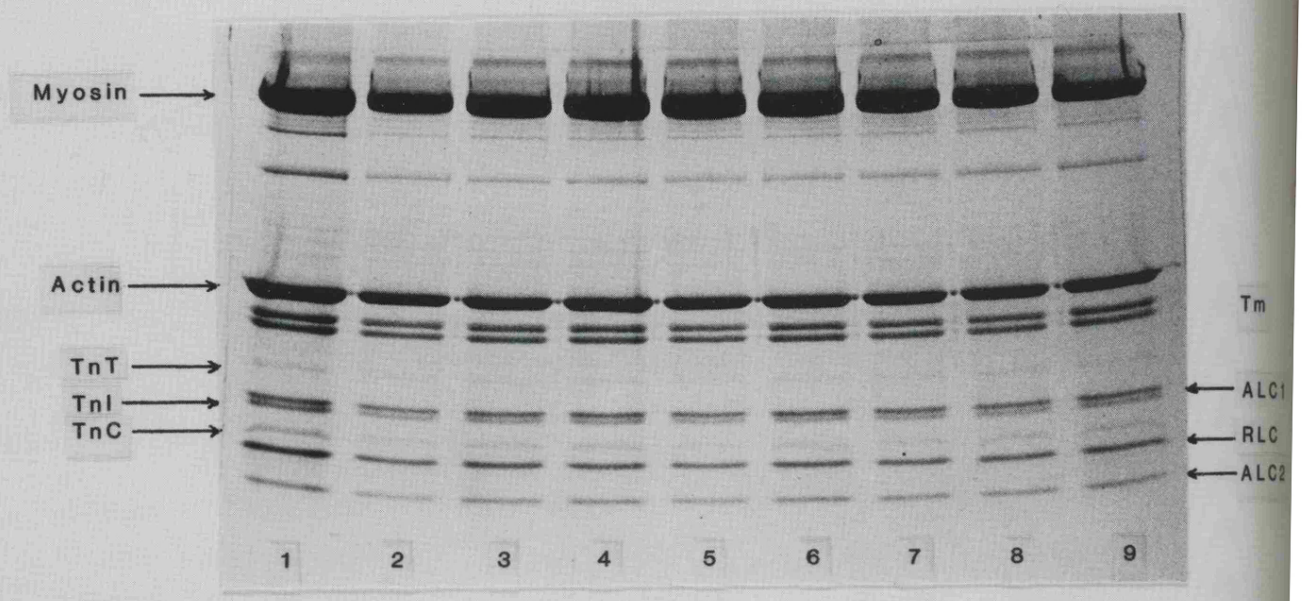
The fact that the removal of 50% of the RLCs from PNFs, causes approximately a 50% decrease in calcium sensitivity suggests that all of the myosin heads are calcium sensitive, the removal of half of the RLCs causing that population of heads to lose their calcium sensitivity. In this case, because the myosin heads still containing their RLCs remain completely functional and posess full calcium sensitivity, we can conclude that the heads within an individual myosin molecule act independently, with respect to their ATPase activity. Support for the independent nature of the myosin heads comes from Stafford et al (1979) who showed that the ATPase of single headed scallop myosin to be calcium regulated. They proposed that both myosin heads were not needed for correct myosin function. Further support for the independent nature of the individual myosin heads came from Mendleson and Cheung (1978), who used fluoresence depolarisation of IA EDANS

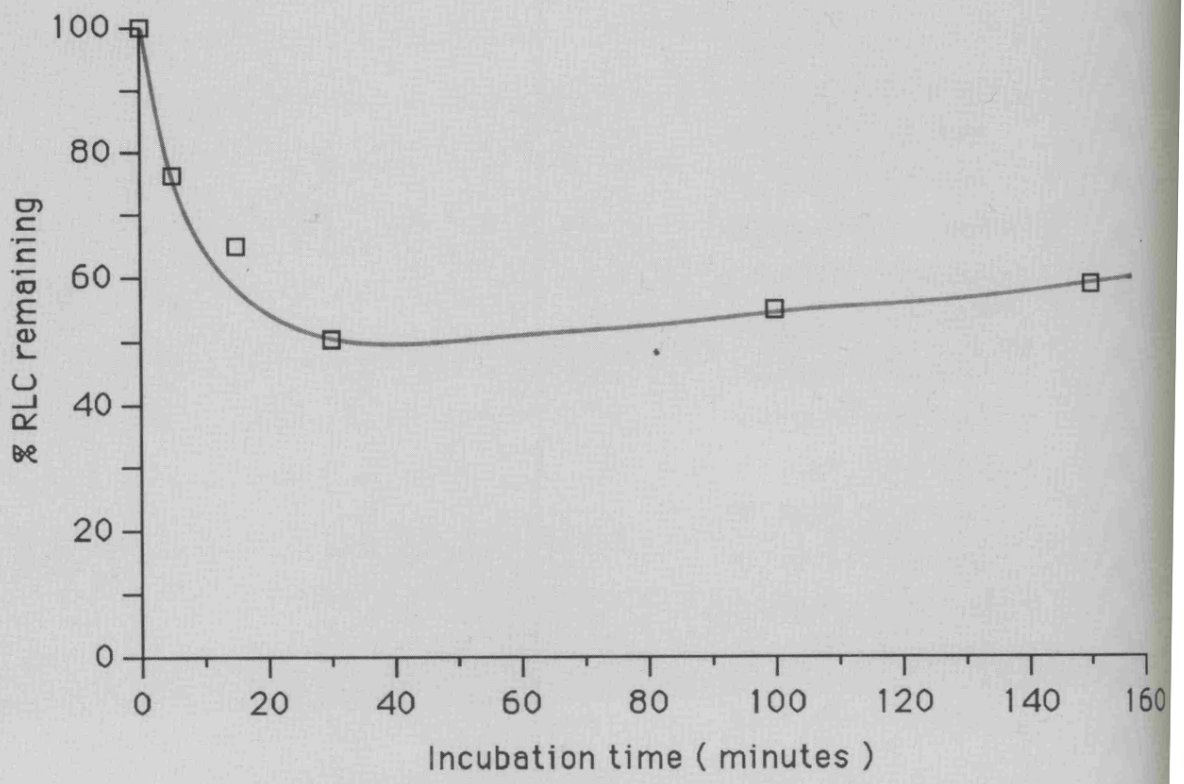
labelled myosin and single headed myosin to show that the S1 moieties pivot independently around the S1/S2 junction. The recent work of Asakawa and Azuma (1988) shows that the enzymatic subfragment S1 of abalone myosin is also calcium sensitive and therefore provides more support for the independent nature of the myosin heads within an individual myosin molecule.

If a sticky patch is present, on the RLC denuded head of an individual myosin molecule, our results indicate that it does not interfere with the function of the other head, which still contains its RLC and displays full calcium sensitivity. Of course, it is always possible that RLC removal does cause sticky patch formation, which interacts with the filament shaft and not other myosin heads. However, Margossian (1987) has recently shown using electron microscopy that after the removal of the RLCs from cardiac myosin monomers there is no evidence to support there being an increase monomer/monomer or head/head interaction, which the presence of numerous sticky patches would presumably cause. This result does not exclusively rule out the possibility of sticky patch formation occuring but does show that under high salt conditions they are not formed between myosin monomers. The possibility of sticky patches being present under physiological conditions and interacting with the filament shaft cannot yet be ruled out, indeed evidence is provided in Chapter V in support of such an interaction.

Chapter III

Gels, Tables, Graphs and Scans.


Myosin
Actin
TnT
TnI
TnC
Tm
ALC1
RLC
ALC2
1
2
3
4
5
6
7
8
9


% RLC remaining
100
80
60
40
20
0
0
20
40
60
80
100
120
140
160
Incubation time (minutes)

Gel 3.1. Showing a standard gentle extraction timecourse using a rabbit RFP.

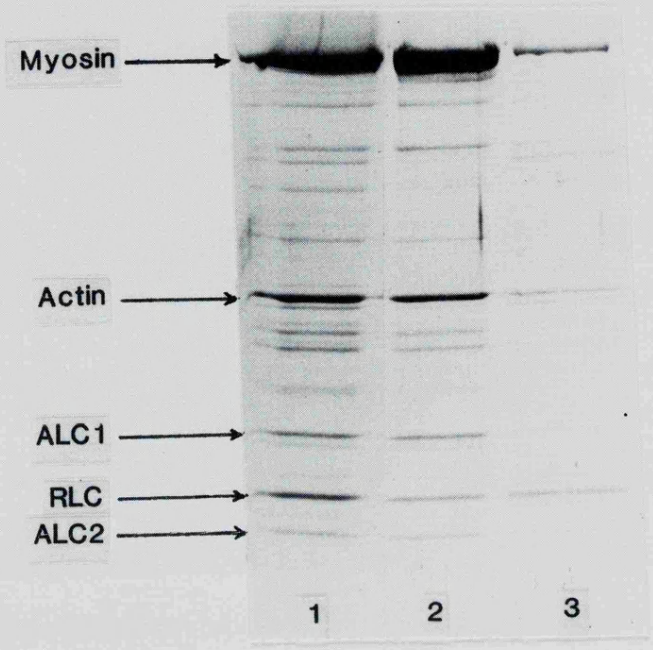
Lane 1. O minutes (control).
Lane 2. 5 minutes
Lane 3. 15 minutes
Lane 4. 30 minutes
Lane 5. 60 minutes
Lane 6. 100 miutes
Lane 7. 150 minutes
Lane 8. 180 minutes
Lane 9. 200 minutes

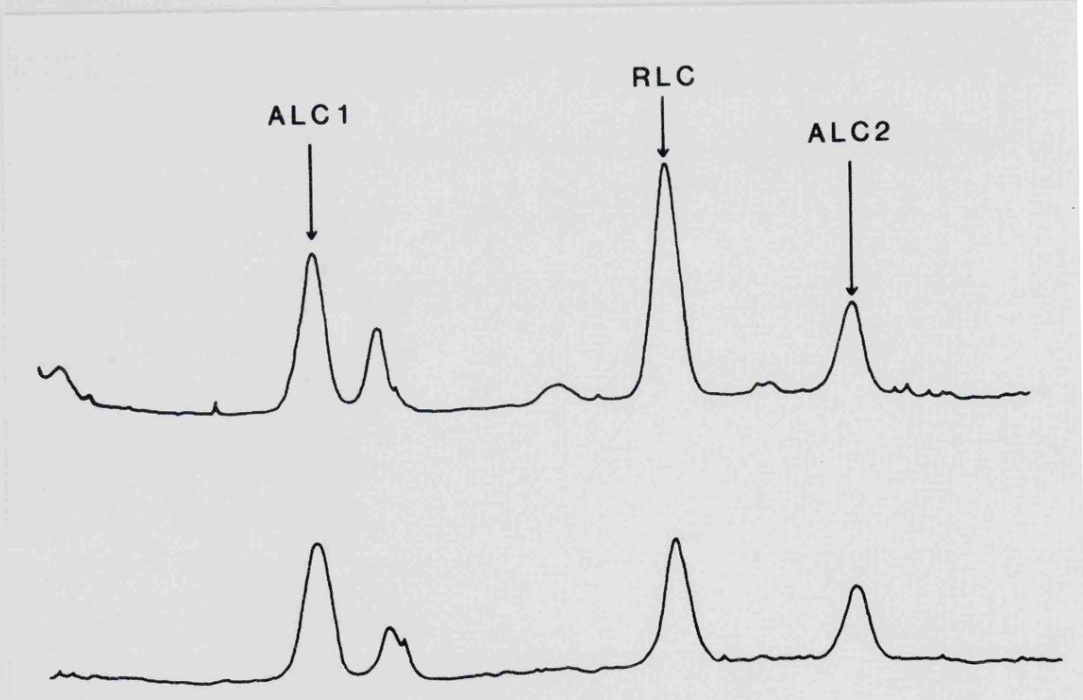
Table3.1. Showing the amount of RLC extraction achieved for each time point as judged by quantitative gelscan analysis of the double loaded gels.

Incubation time (minutes)	% RLC remaining
0	100
5	76
15	65
30	50
100	55
150	59

Graph 3.1. A plot of the data presented in Table 3.1 showing the degree of RLC depletion achieved when using the standard gentle extraction technique on a rabbit RFP.

Rate constant for RLC removal = 0.13 min^{-1}.
Rate constant for RLC reassociation = $1x10^{-3}$ min.
Maximum RLC removal achieved = 49%.


Myosin
Actin
ALC1
RLC
ALC2
1
2
3


ALC1
RLC
ALC2

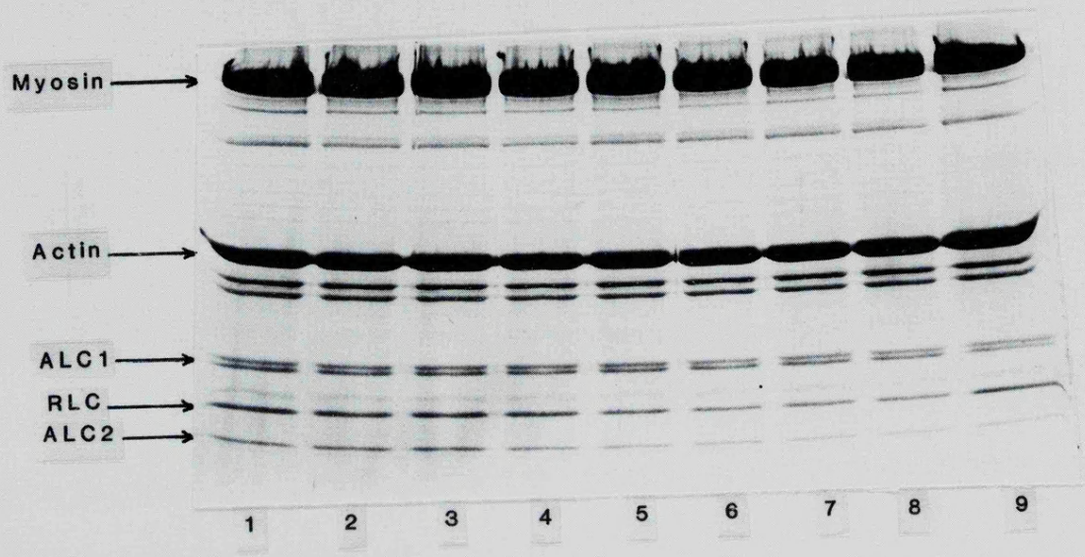
Gel 3.2. Showing that the reduction in the intensity of the RLC band during a gentle extraction timecourse is accompanied by a corresponding intensification of the RLC band in the supernatant.

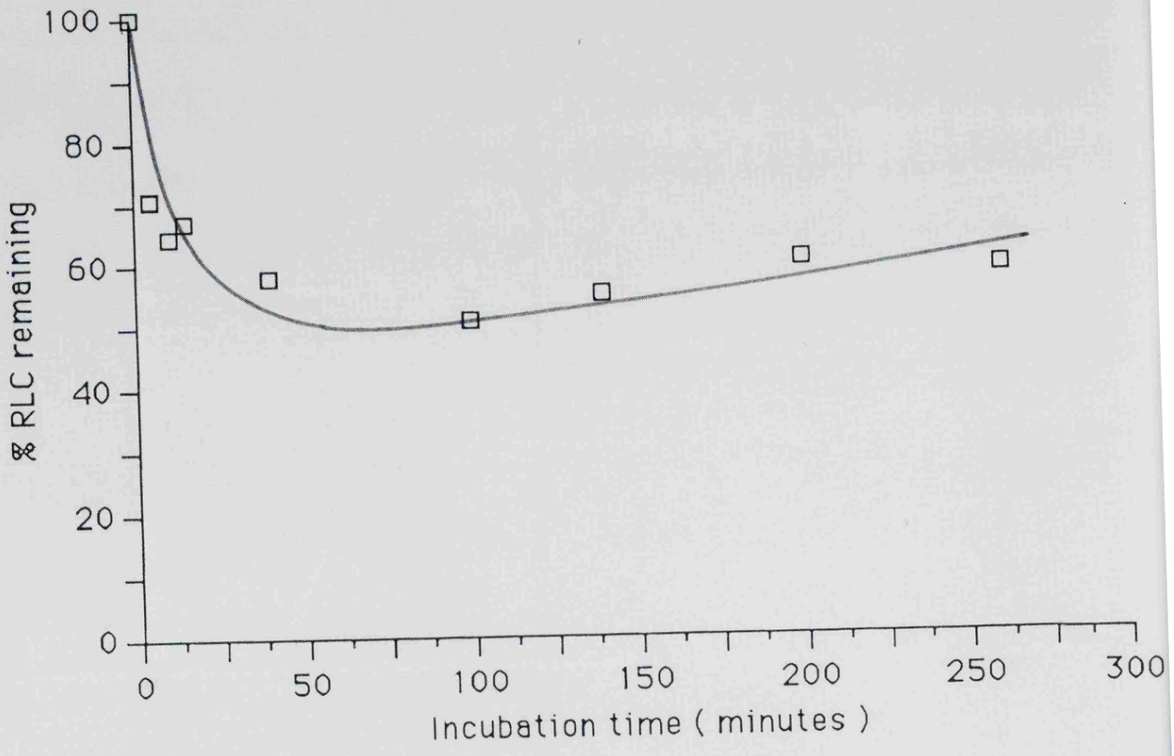
Lane 1. 0 minutes (control).

Lane 2. 30 minutes.

Lane 3. Contents of the supernatant after 30 minutes incubation.

Densitometric analysis 3.1. Showing the results of gelscan analysis of the RLC regions of Lanes 1 (Scan 1) and 2 (Scan 2) of gel 3.2.


Myosin
Actin
ALC1
RLC
ALC2
1
2
3
4
5
6
7
8
9


100
80
60
40
20
0
% RLC remaining
0
50
100
150
200
250
300
Incubation time (minutes)

Gel 3.3. Showing a gentle extraction timecourse using a rabbit RFP carried out in the presence of an increased concentration (20 mM) of ATP.

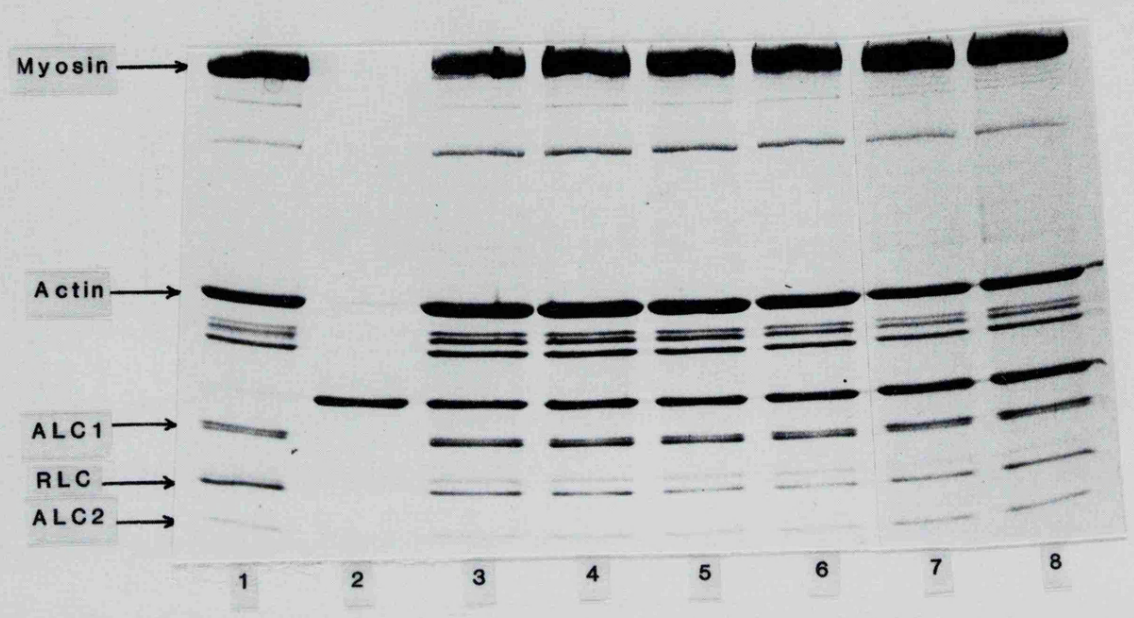
Lane 1. O minutes (control).
Lane 2. 5 minutes
Lane 3. 10 minutes
Lane 4. 15 minutes
Lane 5. 40 minutes
Lane 6. 100 miutes
Lane 7. 140 minutes
Lane 8. 200 minutes
Lane 9. 0 minutes (control)

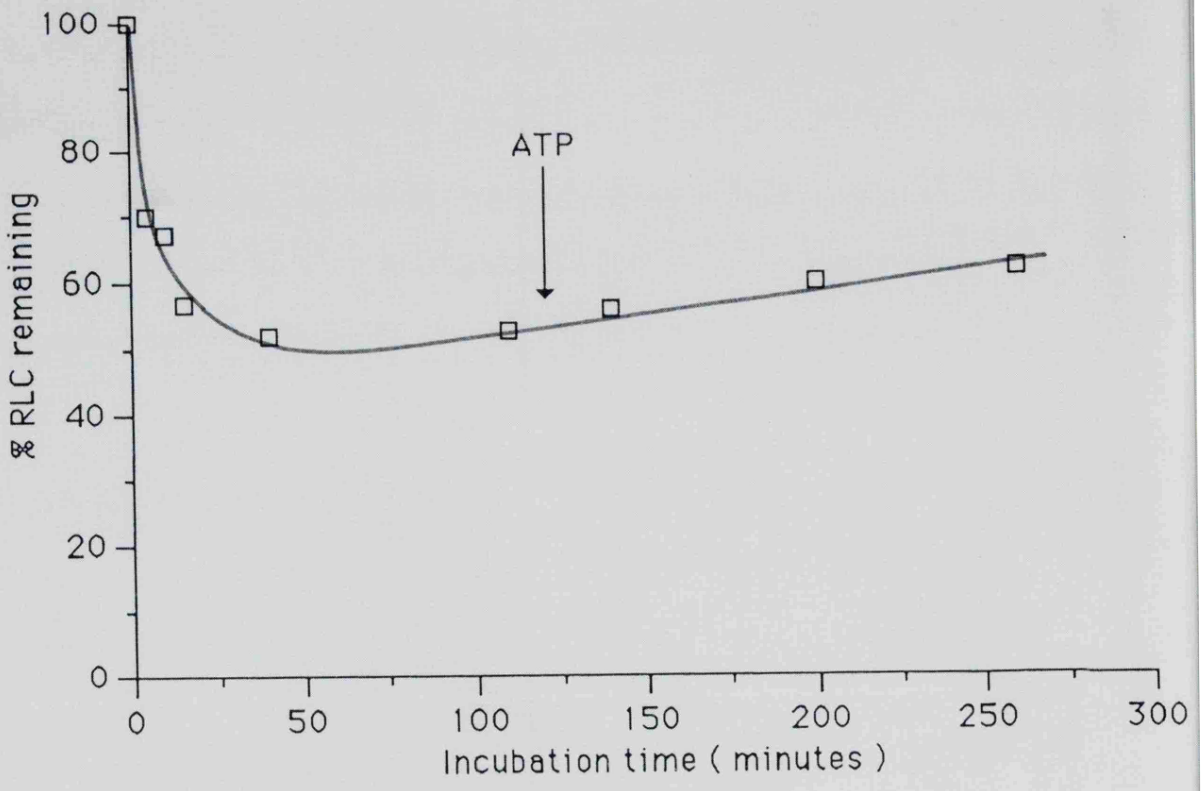
Table 3.2. Showing the amount of RLC extraction achieved for each timepoint as judged by quantitative gelscan analysis of the double loaded gels.

Incubation time (minutes)	% RLC remaining
0	100
5	71
10	64
15	67
40	51
100	52
140	54
200	56
260	59

Graph 3.2. A plot of the data presented in table 3.2 showing the degree of RLC depletion achieved when the incubation is carried out in the presence of 20 mM ATP.

Rate constant for RLC removal = 0.14 min
Rate constant for RLC reassociation = 4.5×10^{-4} min
Maximum RLC removal achieved = 48%


Myosin
Actin
ALC1
RLC
ALC2
1
2
3
4
5
6
7
8


100
80
60
40
20
0
% RLC remaining
ATP
0
50
100
150
200
250
300
Incubation time (minutes)

Gel 3.4. Showing a gentle extraction timecourse using a rabbit RFP with an addition of 5 mM ATP after 120 minutes incubation time.

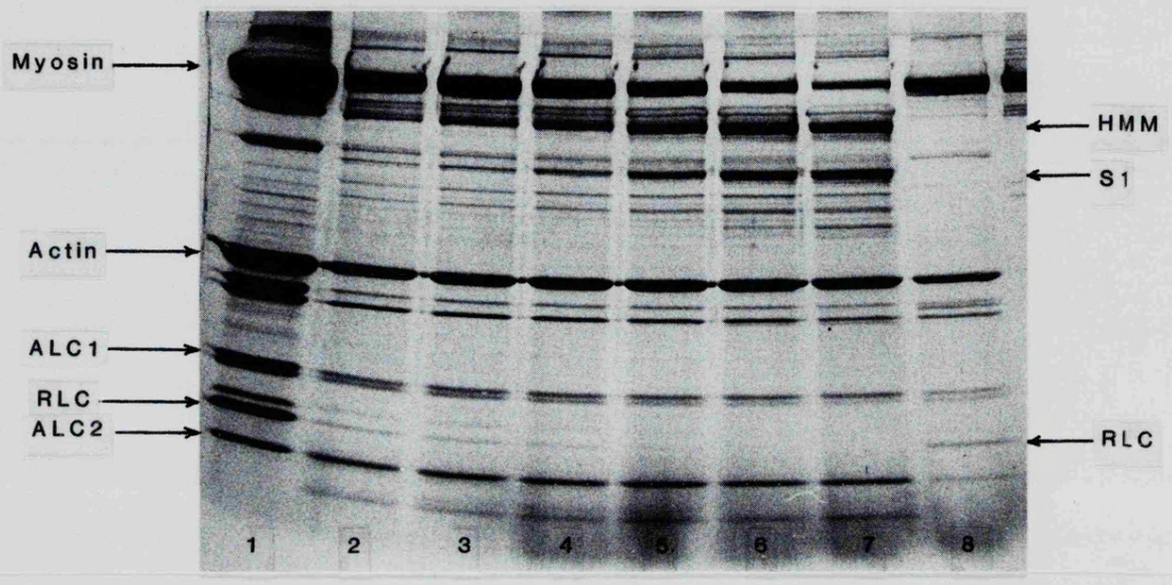
Lane 1. O minutes (control).
Lane 2. Carbonic anhydrase marker
Lane 3. 5 minutes
Lane 4. 15 minutes
Lane 5. 30 minutes
Lane 6. 110 miutes
Addition of 5 mM ATP
Lane 7. 140 minutes
Lane 8. 250 minutes

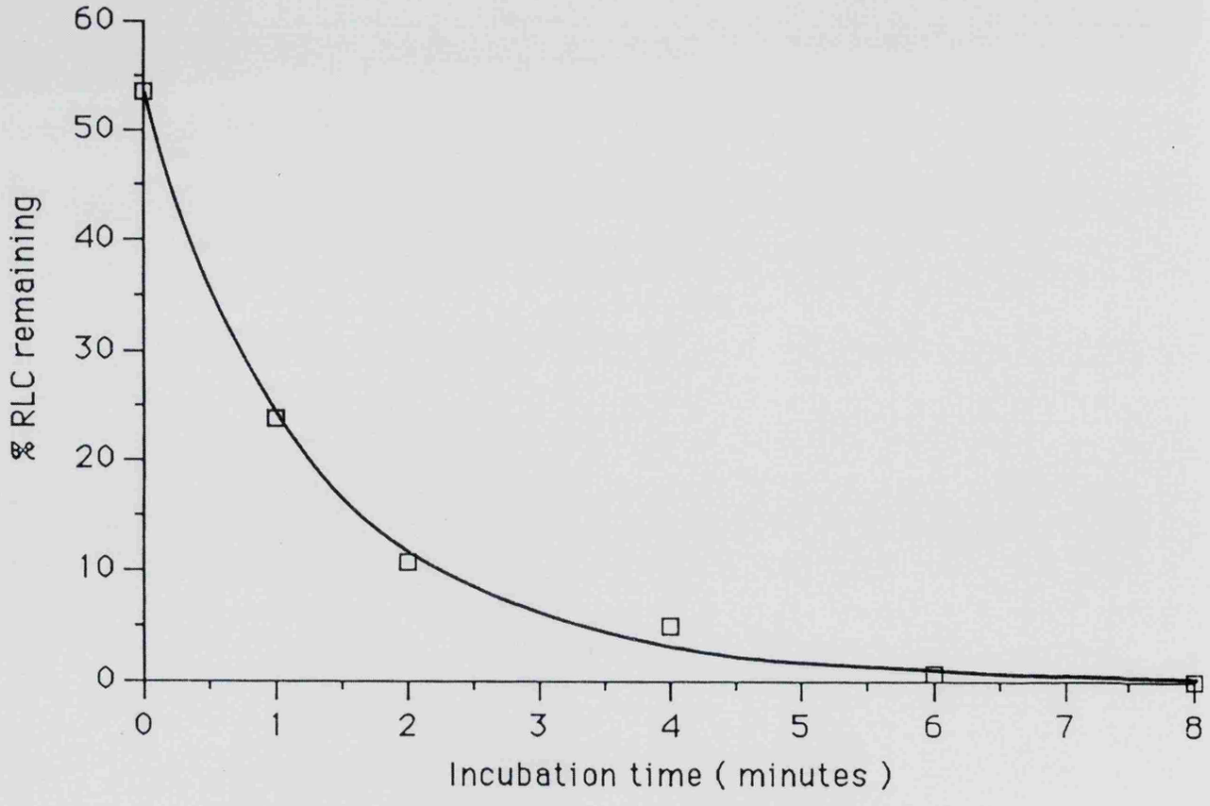
Table 3.3. Showing the amount of RLC extraction achieved for each timepoint as judged by quantitative gelscan analysis of the double loaded gels.

Incubation time (minutes)	% RLC remaining
0	100
5	70
15	57
30	51
110	54
140	57
250	59

Graph 3.3. A plot of the data presented in Table 3.3 showing the degree of RLC depletion achieved before and after the addition of extra ATP.

Rate constant for RLC removal = 0.1 min^{-1}
Rate constant for RLC reassociation = 1 x 10^{-3} min^{-1}
Maximum RLC removal achieved = 49%


Myosin
Actin
ALC1
RLC
ALC2
HMM
S1
RLC
1
2
3
4
5
6
7
8


% RLC remaining
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
Incubation time (minutes)

Gel 3.5. Showing the chymotryptic digestion pattern of a 50% RLC extracted rabbit RFP.

Lane 1. Control RFP with full compliment of RLCs
Lane 2. 1 minutes digestion
Lane 3. 2 minutes digestion
Lane 4. 4 minutes digestion
Lane 5. 6 minutes digestion
Lane 6. 8 minutes digestion
Lane 7. 12 minutes digestion
Lane 8*. 50% RLC depleted RFP

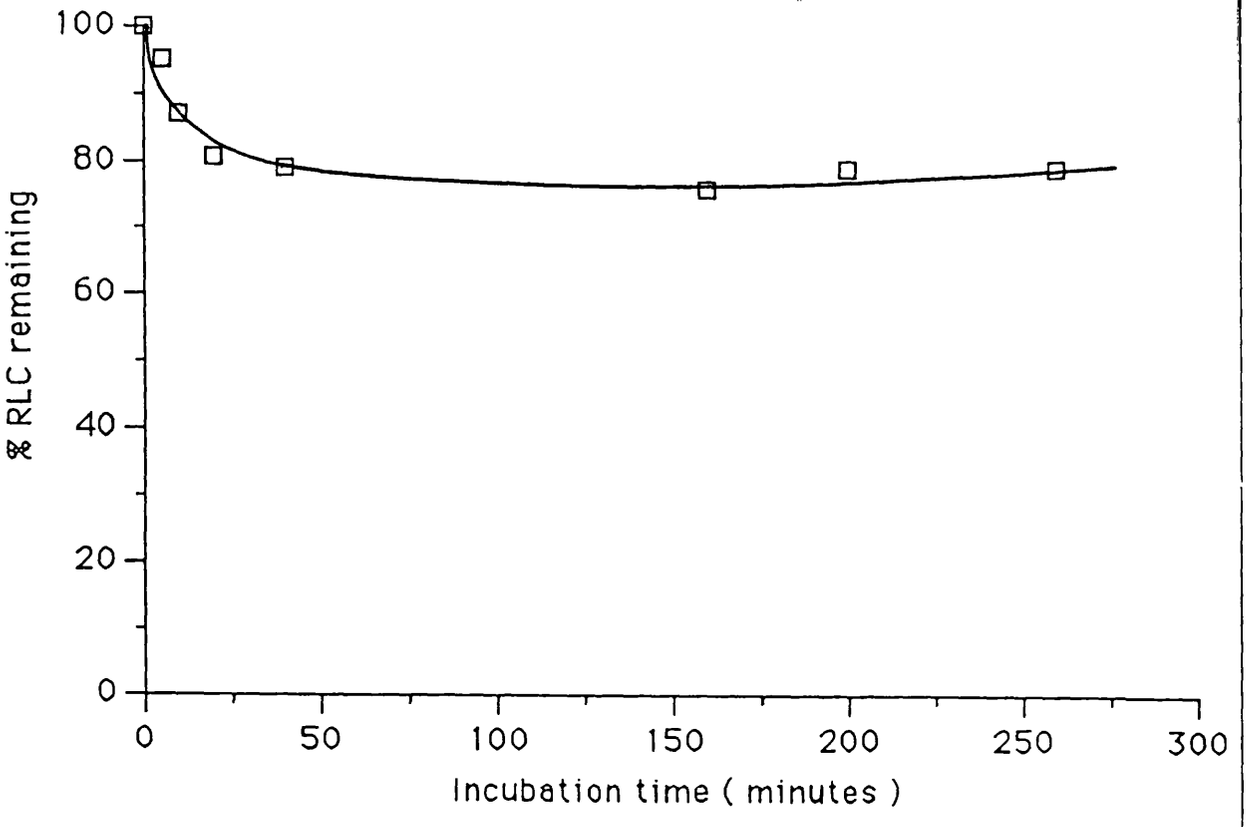
* Lane 8 was the initial sample upon which the chymotryptic digestion was carried out.

Table 3.4. Showing the amount of RLC digestion taking place when a 50% RLC extracted rabbit RFP is subjected to the chymotryptic probe experiment.

Incubation time (minutes)	% RLC remaining
0	53
1	24
2	11
4	5
6	0.8
8	0
12	0

Graph 3.4. A plot of the data presented in Table 3.4. revealing the digestion kinetics of the gentle extraction resistant RLC population.

Rate constant for RLC digestion = 0.78 min^{-1}


100
80
60
40
20
0
% RLC remaining
0
50
100
150
200
250
300
Incubation time (minutes)

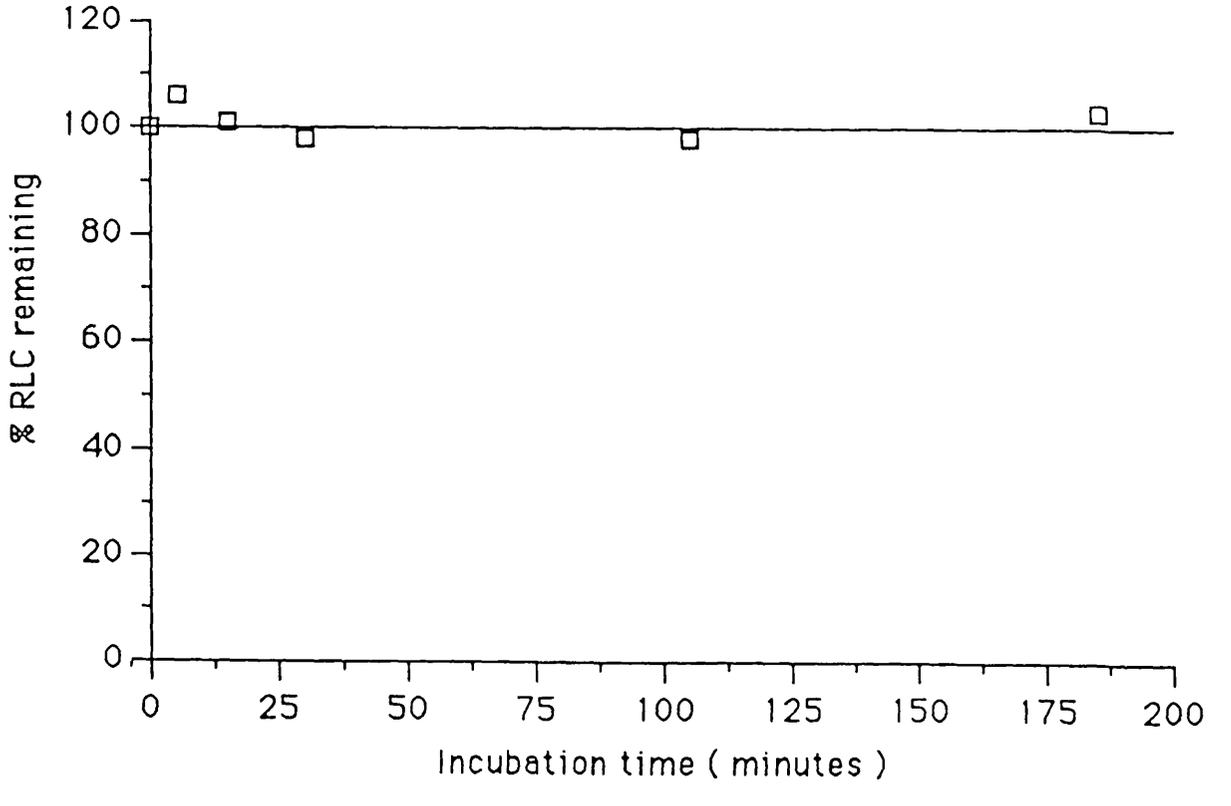

120
100
80
60
40
20
0
% RLC remaining
0
25
50
75
100
125
150
175
200
Incubation time (minutes)

Table 3.5. Showing the amount of RLC removal achieved from a rabbit RFP when the extraction is carried out in the absence of ATP.

Incubation time (minutes)	% RLC remaining
0	100
5	95
15	87
20	81
40	79
160	76
200	79
260	79

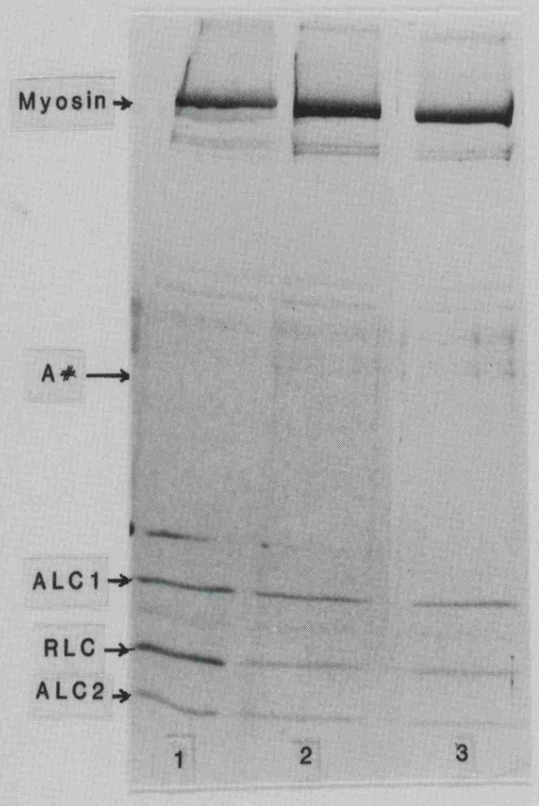
Graph 3.5. A plot of the data presented in Table 3.5. illustrating the effect that the absence of ATP from the incubation mixture has on the RLC removal kinetics.
Rate constant for RLC removal = 0.085 min^{-1}
Maximum RLC removal achieved = 23 %
Note that there does not appear to be a significant amount of RLC reassociation occuring.

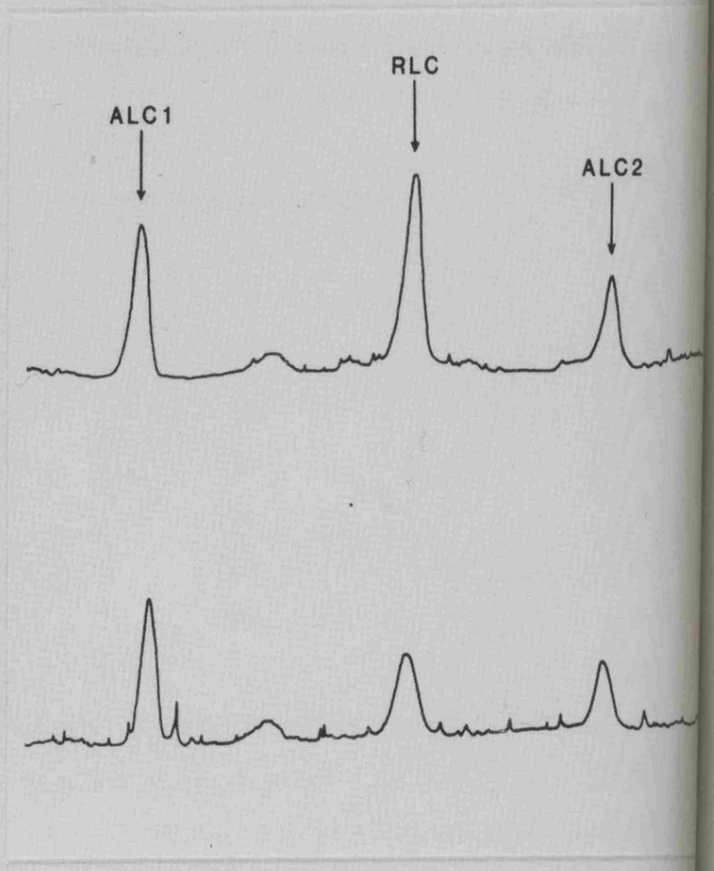
Table 3.6. The gentle extraction susceptibility of the RLCs in a high salt exposed rabbit RFP

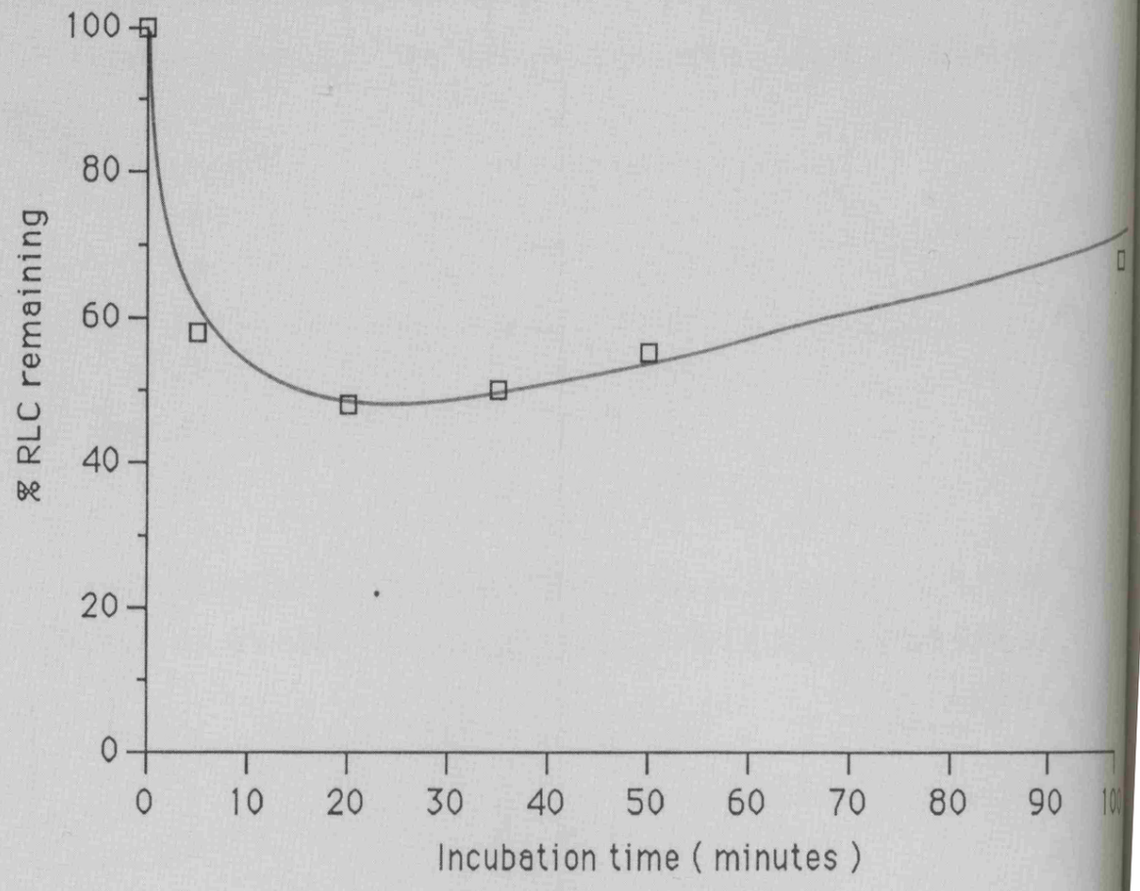
Incubation time (minutes)	% RLC remaining
0	100
5	106
15	101
30	98
105	98
185	102

Graph 3.6 . A plot of the Data presented in Table 3.6. demonstrating that after high salt exposure the entire RLC population in a RFP becomes entirely resistant to removal by the gentle extraction technique.

Maximum RLC removal achieved = 0%


Myosin
A*
ALC1
RLC
ALC2
1
2
3


ALC1
RLC
ALC2


100
80
60
40
20
0
% RLC remaining
0
10
20
30
40
50
60
70
80
90
Incubation time (minutes)

Gel 3.6. Showing the effect of the standard gentle extraction protocol on the RLCs of PNFs.

Lane 1. O minutes (control).
Lane 2. 20 minutes.
Lane 3. 30 minutes.

The band running above ALC1 is contaminant carbonic anhydrase which has diffused across from a heavily overloaded well (not shown) to the left of the control. A* represents the position where actin runs on the gel and emphasises the purity of the PNFs with respect to actin.

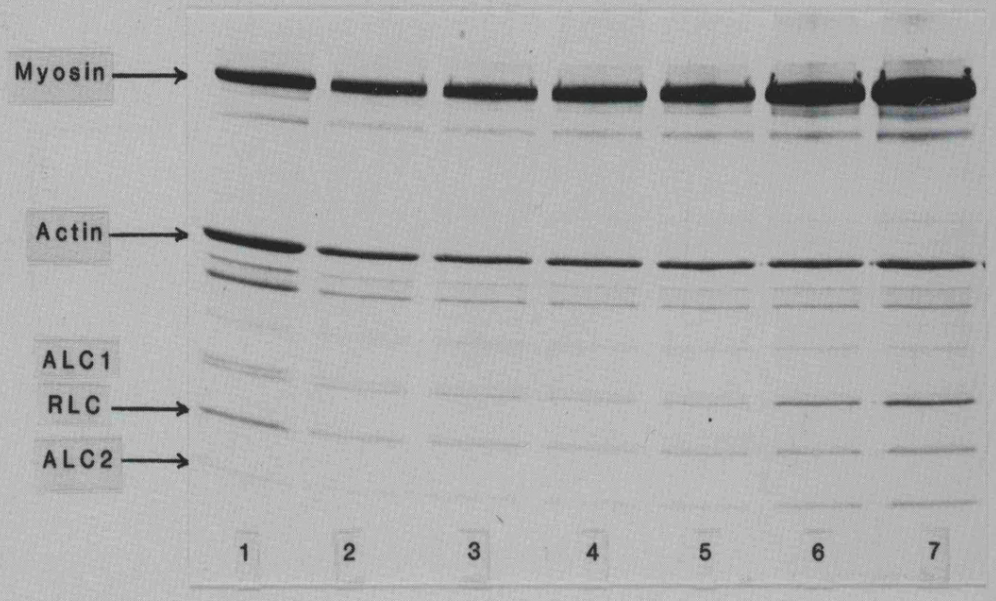
Densitometric analysis 3.2 Gelscans of RLC regions of Lane 1 (Scan 1) and 2 (Scan 2) of Gel3.5.

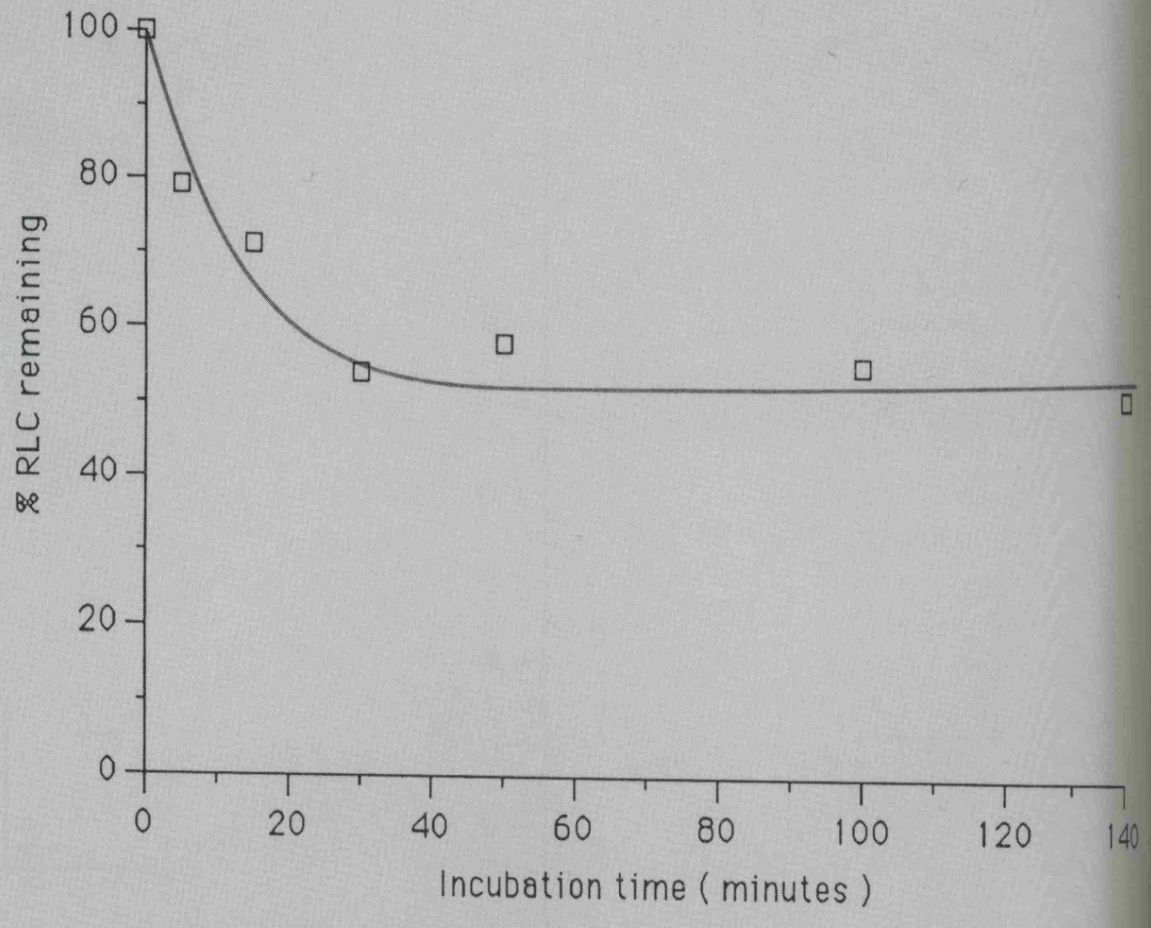
Table 3.7. Showing the amount of RLC removal achieved when PNFs were subjected to the standard gentle extraction protocol as judged by quantitative gelscan analysis of the double loaded gels.

Incubation time (minutes)	% RLC remaining
0	100
5	59
20	49
35	51
50	55
100	65

Graph 3.7. A plot of the data presented in Table 3.7 showing the extraction kinetics of the RLCs in a PNF preparation.

Rate constant for RLC removal = 0.26 min^{-1}
Rate constant for RLC reassociation = 0.01 min^{-1}
Maximum RLC removal achieved = 53%


Myosin
Actin
ALC1
RLC
ALC2
1
2
3
4
5
6
7


100
80
60
40
20
0
% RLC remaining
0
20
40
60
80
100
120
140
Incubation time (minutes)

Gel 3.7. Showing the effect of the standard gentle extraction protocol on the RLCs of a rat RFP.

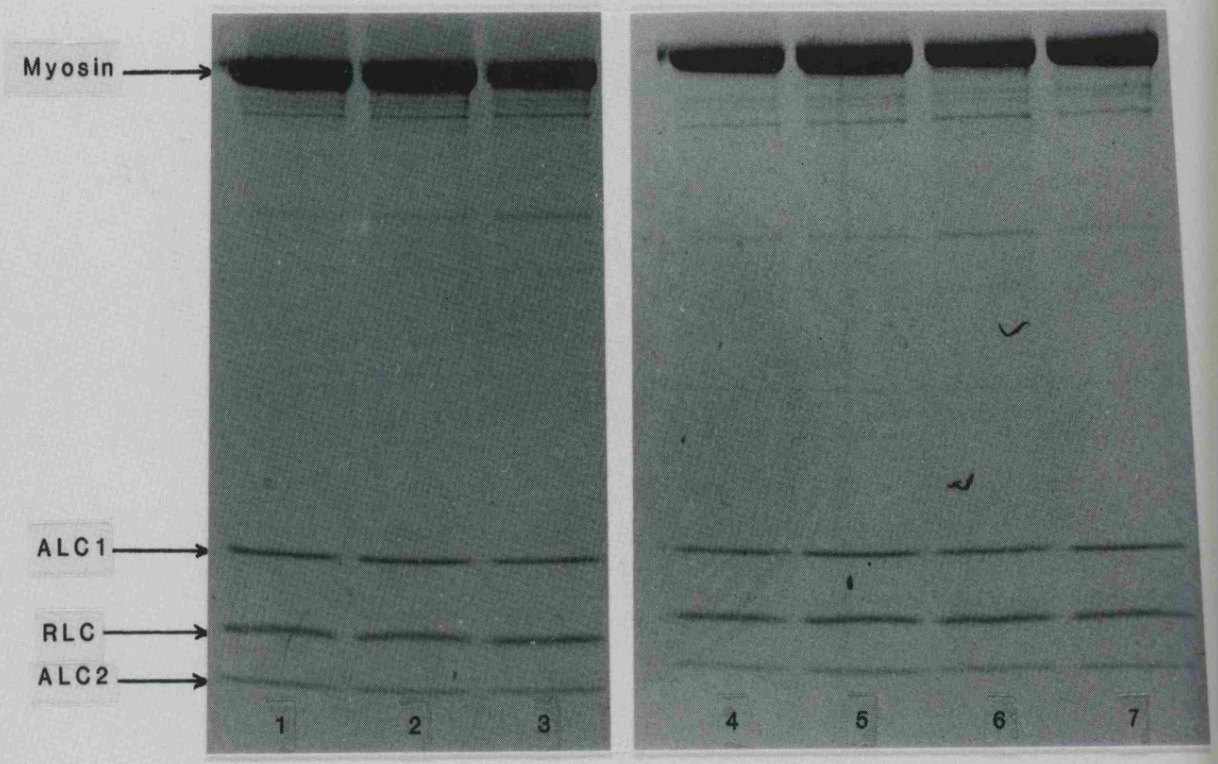
Lane 1. O minutes (control).
Lane 2. 5 minutes
Lane 3. 15 minutes
Lane 4. 30 minutes
Lane 5. 50 minutes
Lane 6. 100 miutes
Lane 7. 140 minutes

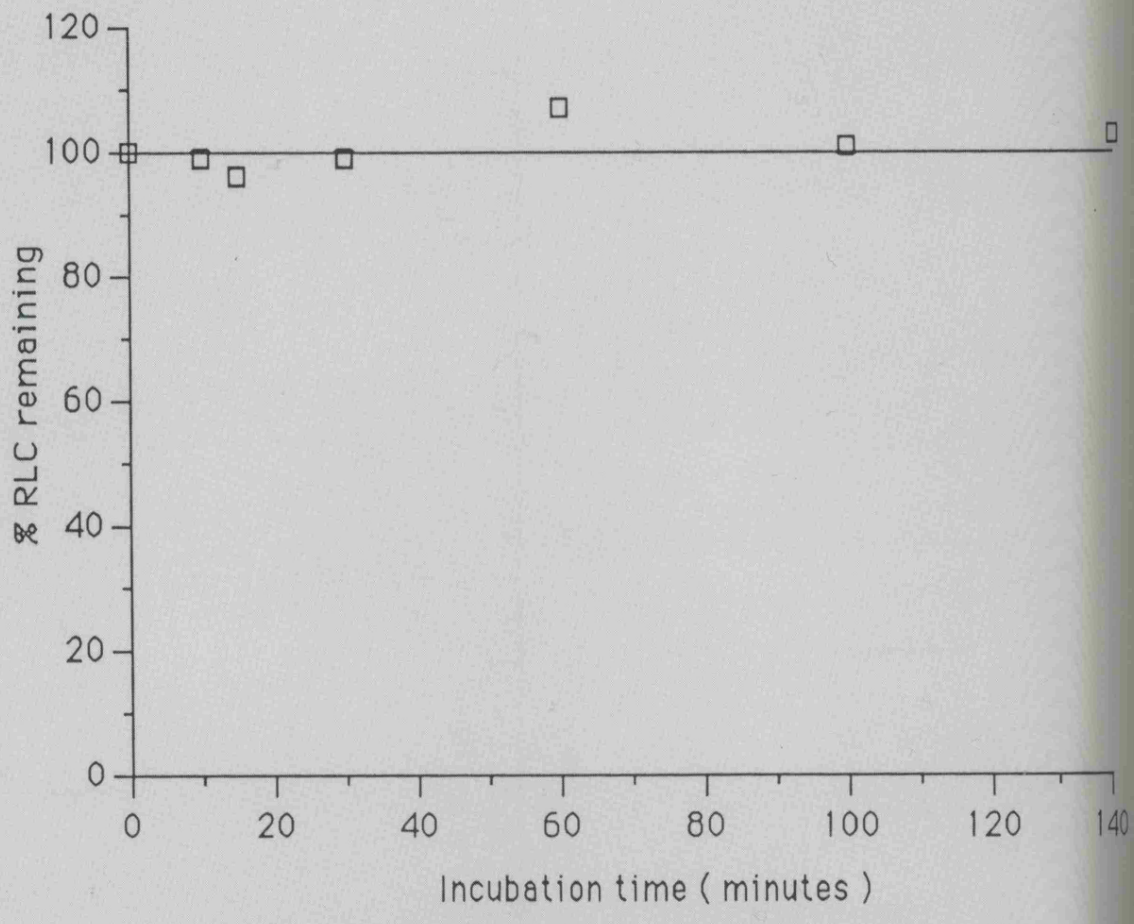
Table 3.8. Showing the amount of RLC removal achieved from a rat RFP as judged by quantitative gelscan analysis of the double loaded gels.

Incubation time (minutes)	% RLC remaining
0	100
5	79
15	71
30	53
50	58
100	52
140	51

Graph 3.8. A plot of the data presented in table 3.8. indicating the extraction kinetics of the RLCs.

Rate constant for RLC removal = 0.07 min^{-1}
Overall RLC removal achieved = 47%


Myosin
ALC1
RLC
ALC2
1
2
3
4
5
6
7


120
100
80
60
40
20
0
% RLC remaining
0
20
40
60
80
100
120
140
Incubation time (minutes)

Gel 3.8. Showing the effect that the standard gentle extraction protocol has on the RLCs of an SMF preparation.

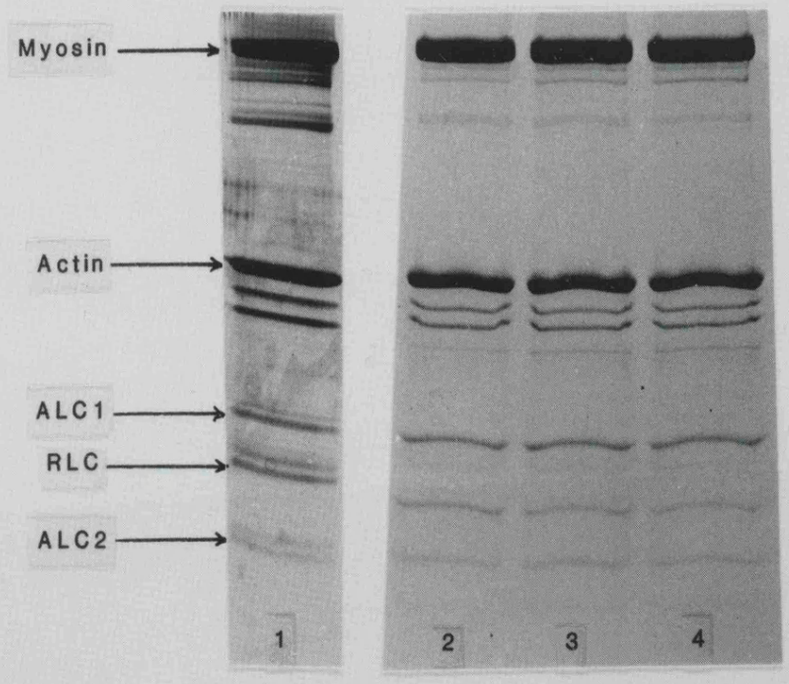
Lane 1. O minutes (control).
Lane 2. 10 minutes
Lane 3. 15 minutes
Lane 4. 30 minutes
Lane 5. 60 minutes
Lane 6. 100 miutes
Lane 7. 140 minutes

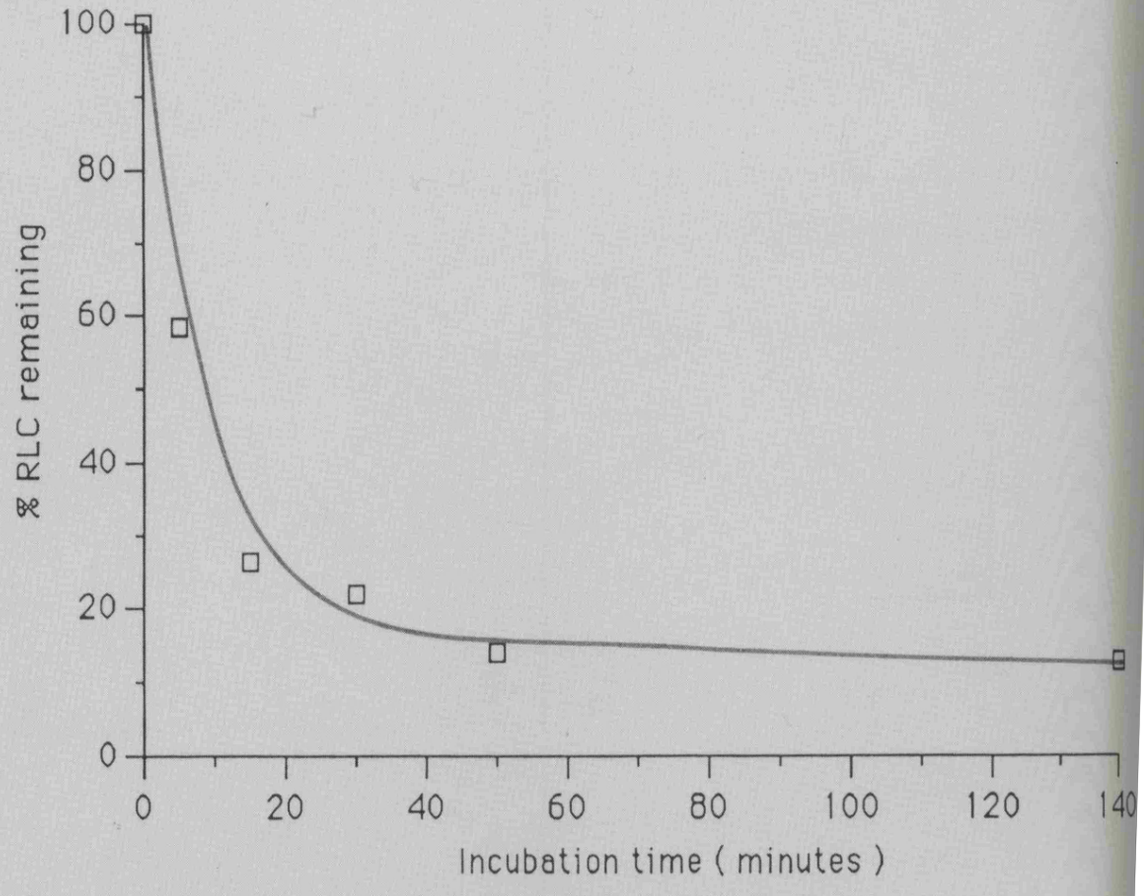
Table 3.9. Showing the RLC content of an SMF preparation at various timepoints in the incubation procedure as judged by gelscan analysis of the double loaded gels.

Incubation time (minutes)	% RLC remaining
0	100
10	99
15	96
30	99
60	107
100	101
140	103

Graph 3.9. Demonstrating that the RLCs of SMFs are totally insensitive to removal using the gentle extraction technique.

Overall RLC removal achieved = 0 %


Myosin
Actin
ALC1
RLC
ALC2
1
2
3
4


% RLC remaining
100
80
60
40
20
0
0
20
40
60
80
100
120
140
Incubation time (minutes)

Gel 3.9. Showing the effect that the gentle extraction technique has on the RLCs in a high salt exposed rat RFP.

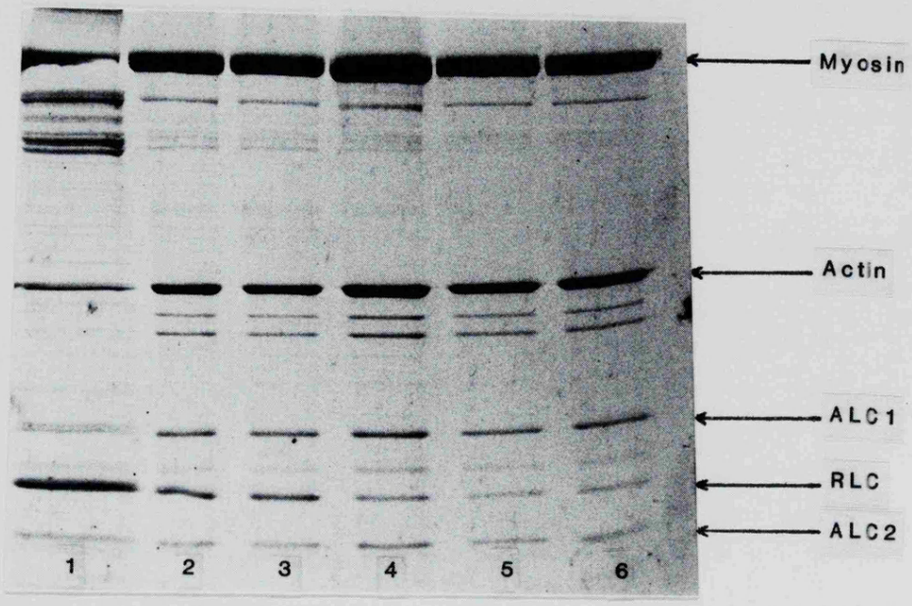
Lane 1. O minutes (control).
Lane 2. 30 minutes
Lane 3. 50 minutes
Lane 4. 140 minutes

Table 3.10. Showing the RLC content of a high salt exposed rat RFP during the incubation timecourse as judged by gelscan analysis of the double loaded gels.

Incubation time (minutes)	% RLC remaining
0	100
5	59
15	27
30	22
50	14
140	12

Graph 3.10. A plot of the data presented in table 3.10. demonstrating that the removal takes the form of a single term exponential.

Rate constant for RLC removal = 0.13 min^{-1}
Overall RLC removal achieved = 85%


Myosin
Actin
ALC1
RLC
ALC2
1
2
3
4
5
6

Gel 3.10. Showing the effect that subjecting a high salt exposed rat RFP to two consecutive 40 minute incubations in the gentle extraction medium has on the RLC content (NB the RLCs extracted from the first incubation were removed before the second incubation proceded.

Lane 1. Contents of the supernatant after 40 minutes incubation.
Lane 2. 0 minutes (control)
Lane 3. 0 minutes (control)
Lane 4. 40 minutes (first incubation)
The RLCs are removed from the supernatant of the sample in lane 4. and the sample is incubated for another 40 minutes.
Lane 5. 40 minutes (second incubation)
Lane 6. 40 minutes (second incubation)

Table 3.11. Showing the RLC content of lanes 3, 4 and 5 (Gel 3.11) when corrected for loading variations.

Lane No	Incubation time (minutes)	% RLC remaining
3	control (0)	100
4	40 (first incubation)	18
5	40 (second incubation)	5

Table 3.12.

	ATPase rate (sec $^{-1}$)			
Preparation	$-Ca^{2+}$	$+Ca^{2+}$	Ca^{2+} activation	% Change *
1a	0.25	2.9	11.3 fold	-
1b	0.26	2.9	11.1 fold	2 % decrease
2a	0.11	0.85	7.6 fold	-
2b	0.12	0.38	3.1 fold	60% decrease
3a	0.26	2.0	7.6 fold	-
3b	0.3	1.7	5.7 fold	25% decrease
4a	0.2	2.3	11.5 fold	-
4b	0.5	1.0	2.0 fold	83% decrease

* % change represents the change in calcium sensitivity achieved after incubation, expressed as a percentage of the change obtained in the appropriate control.

Table 3.12.

Showing the effect of temperature (1a and 1b) and 50% RLC depletion on the steady state MgATPase activity and calcium sensitivity of various rabbit RFPs.

1a : Control RFP.

1b : RFP after incubation at 37 C in a non extracting buffer for 30 minutes.

2a : Control RFP.

2b : RFP after 50% RLC extraction.

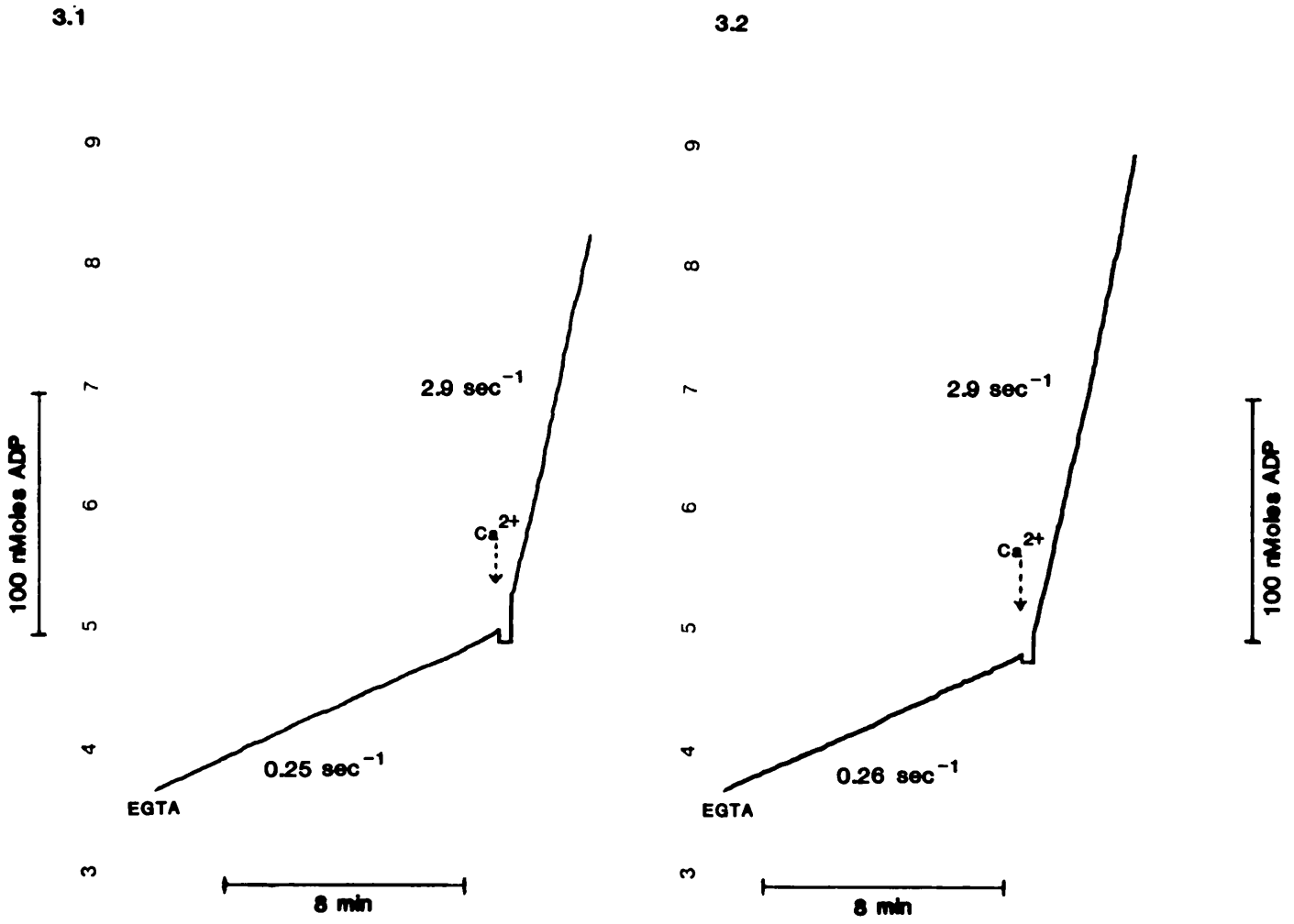
3a : Control RFP.

3b : RFP after 50% RLC extraction.

4a : Control RFP.

4b : RFP after 50% RLC extraction.

ATPase assay traces



ATPase assay traces showing the effect that incubation at 37 °C for 30 minutes has on the steady state ATPase activity of a rabbit RFP. These traces correspond to the rates shown in positions 1a and 1b in Table 3.12.

Assay trace 3.1.
The steady state ATPase activity and calcium sensitivity of a RFP (0.3 mg/ml) before incubation (control).

Assay trace 3.2.
The steady state ATPase activity and calcium sensitivity of a RFP (0.3 mg/ml) after incubation at 37 °C for 30 minutes.

Table 3. 13

	ATPase rate (sec $^{-1}$)			
Preparation	-Ca^{2+}	+Ca^{2+}	Ca^{2+} activation	% change
1a	0.059	0.094	1.6 fold	-
1b	0.057	0.091	1.59 fold	<1 % increase
2a	0.074	0.16	2.1 fold	-
2b	0.076	0.116	1.5 fold	54.5 % decrease
3a	0.06	0.10	1.7 fold	-
3b	0.059	0.08	1.4 fold	43 % decrease
4a	0.08	0.12	1.5 fold	-
4b	0.07	0.09	1.28 fold	44.2 % decrease

* % change represents the change in calcium sensitivity achieved after incubation, expressed as a percentage of the change obtained in the appropriate control.

Table 3.13.

Showing the effect of temperature (1a and 1b) and 50% RLC depletion on the intrinsic steady state MgATPase activity and calcium sensitivity of various PNF preparations.

1a : Control PNF.

1b : PNF after incubation at 37 ^{o}C in a non extracting buffer for 30 minutes.

2a : Control PNF.

2b : PNFs after 50% RLC extraction.

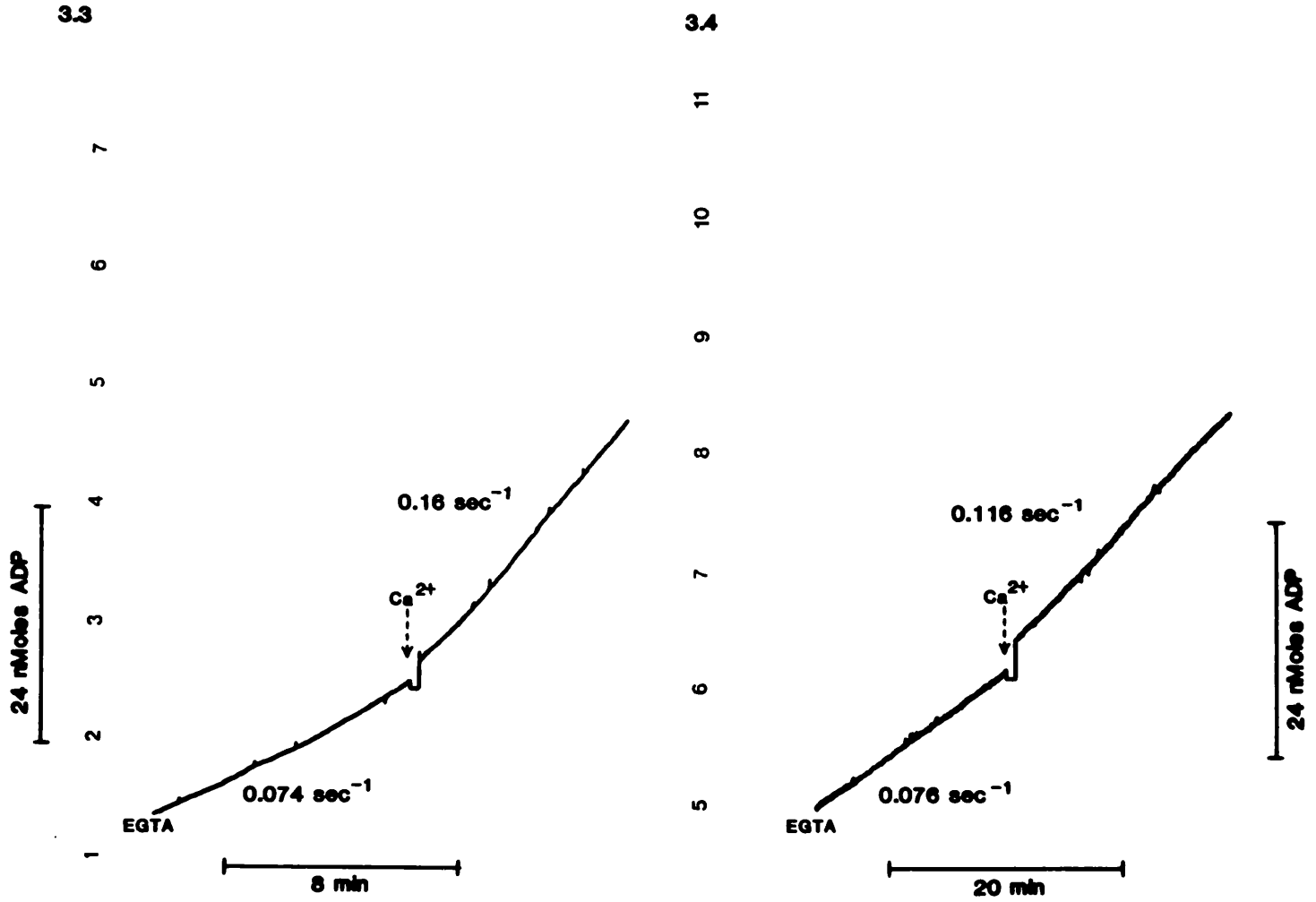
3a : Control PNF.

3b : PNFs after 50% RLC extraction.

4a : Control PNFs.

4b : PNFs after 50% RLC extraction.

ATPase assay traces



ATPase assay traces showing the effect that 50% RLC depletion has on the steady state ATPase activity of a rabbit PNF preparation. Traces 3.3. and 3.4. correspond to the rates shown at positions 2a and 2b in Table 3.13.

Assay trace 3.3.
The steady state ATPase activity and calcium sensitivity of a PNF preparation (0.5 mg/ml) before RLC depletion (control).

Assay trace 3.4.
The steady state ATPase activity and calcium sensitivity of a PNF preparation (0.27 mg/ml) after 50% RLC depletion.

Table 3.14.

	ATPase rate (sec^{-1})			
Preparation	$-Ca^{2+}$	$+Ca^{2+}$	Ca^{2+} activation	% change *
1a	1.2	2.3	1.92 fold	-
1b	1.17	2.3	1.96 fold	2 % increase
2a	1.2	2.2	1.83 fold	-
2b	1.0	1.4	1.40 fold	52 % decrease
3a	1.8	2.3	1.27 fold	-
3b	1.6	1.8	1.13 fold	51 % decrease
4a	1.2	1.92	1.92 fold	
4b	1.13	1.6	1.42 fold	54 % decrease

* % change represents the change in calcium sensitivity achieved after incubation, expressed as a percentage of the change obtained in the appropriate control.

Table 3.14.

Showing the effect of temperature (1a and 1b) and 50% RLC depletion on the actin activated steady state MgATPase activity and calcium sensitivity of various PNF preparations.

1a : Control PNFs.

1b : PNFs after incubation at 37 ^{o}C in a non extracting buffer for 30 minutes.

2a : Control PNFs.

2b : PNFs after 50% RLC extraction.

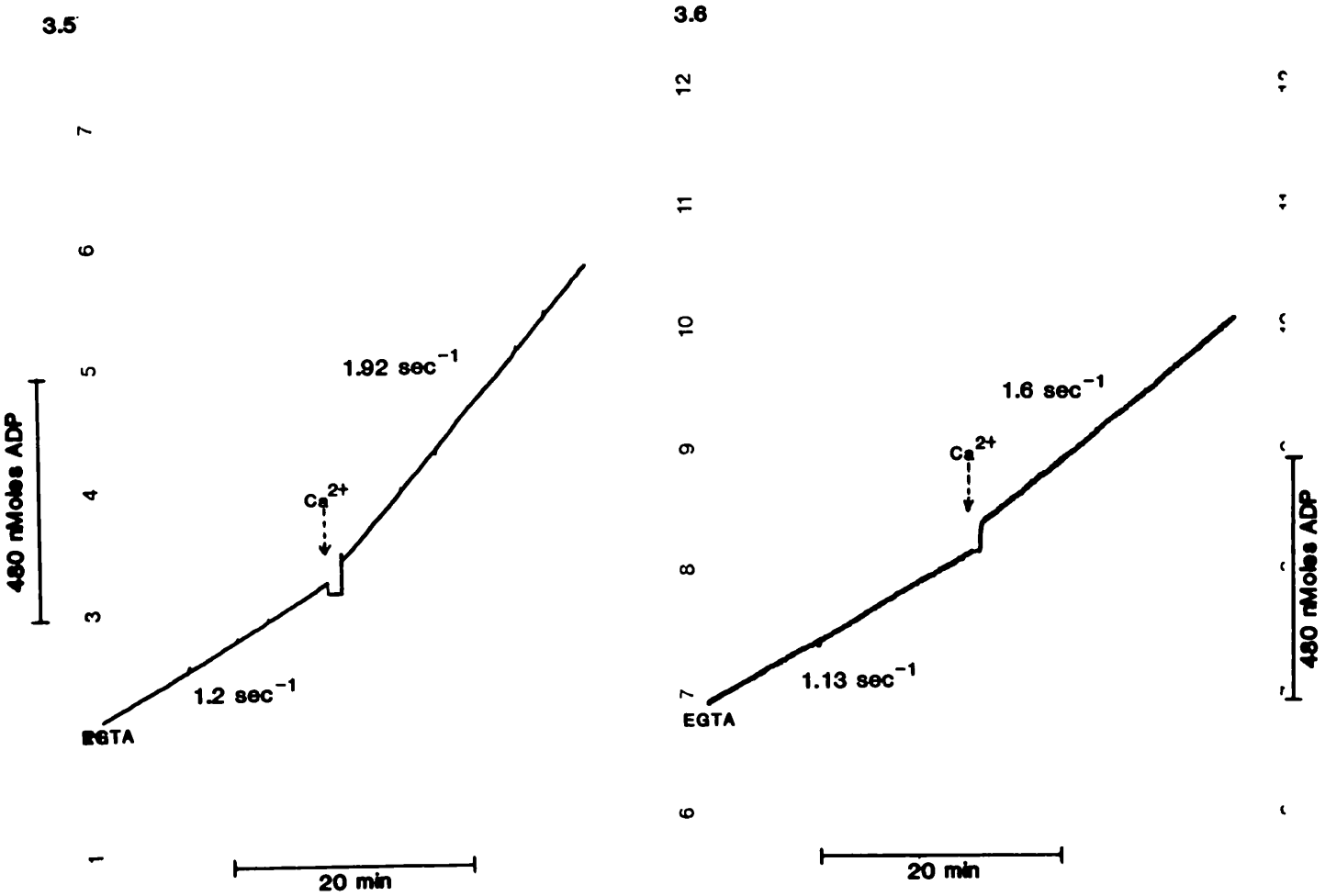
3a : Control PNFs.

3b : PNFs after 50% RLC extraction.

4a : Control PNFs.

4b : PNFs after 50% RLC depletion.

ATPase assay traces



ATPase assay traces showing the effect that 50% RLC depletion has on the steady state actin activated ATPase activity of a rabbit PNF preparation. Traces 3.5. and 3.6. correspond to the rates shown at positions 4a and 4b in Table 3.14.

Assay trace 3.5.
The steady state actin activated ATPase activity and calcium sensitivity of a PNF preparation (0.29mg/ml) before RLC depletion (control).

Assay trace 3.6.
The steady state actin activated ATPase activity and calcium sensitivity of a PNF preparation (0.29mg/ml) after 50% RLC depletion.

Chapter IV

Introduction

The structure of native vertebrate myosin filaments. Can the native filament structure be reconstructed by the assembly of monomeric myosin ?

(IV. 1) The determination of thick filament structure using electron microscopy.

The general arrangement of thick filaments within the sarcomeres of vertebrate striated skeletal muscles has been known for over 30 years (H. E. Huxley and Hanson., 1954; A. F. Huxley and Niedergerke., 1954) (See Introduction Chapter 1). However, the detailed examination of the internal structure of the thick filaments has proved very difficult to achieve. The technique of ultrathin sectioning used for the examination of muscle fibres is incompatible with looking at the internal structure of individual thick filaments.

The alternative approach adopted by H. E. Huxley (1963) was to disrupt muscle fibrils into their constituent filaments and observe their structure using the technique of negative staining (See Section II.17). This classical piece of electron microscopy along with some later X-ray diffraction studies by H. E. Huxley and Brown (1967) laid the

foundations for our present day knowledge of thick filament structure. H. E. Huxley (1963) clearly showed that individual native thick filaments had tapering ends and were bipolar. The length distribution of the filaments was very narrow, with an average length of approximately 1.6 μm. Filaments greater in length than 1.6 μm were clearly two filaments or fragments that were stuck together at the end. Very short thick filaments were present but these showed evidence of being produced by mechanical damage and were probably formed during homogenisation.

The filaments had irregular projections along their entire length except for a small central region which appeared smooth, this region was 0.15 to 0.2 μm in length and was termed the central bare zone (See Figure 1.1). All of these features had previously been observed to be present in ultrathin sections of skeletal muscle (H. E. Huxley., 1957; A. F. Huxley and Neidergerke;1954), which led H. E. Huxley to suggest that the disruption of the muscle fibrils to liberate thick filaments did not alter their structure. The images observed by the negative staining technique were therefore, as far as he could tell, a true representation of thick filament structure in vivo.

H. E. Huxley (1963) proposed that the central bare zone region was the result of antiparallel packing of the myosin tails in that part of the filament (See Figure 1. 1). Antiparallel packing at the centre of the thick filament is essential if bipolar filament formation is to occur. Such a change in polarity at the centre of the filament is essential if the sarcomeres are to shorten in the manner described by the now generally accepted sliding filament hypothesis (H. E. Huxley and Hanson., 1954). The importance of the central bare zone region in

filament assembly will be discussed in more detail later.

(IV. 2) The use of X-rays to elucidate thick filament structure.

X-ray diffraction has been a useful tool in the elucidation of some of the major structural features of native vertebrate thick filaments. The beauty of using X-ray techniques is that they allow one to investigate the filament structure in vivo.

H. E. Huxley and Brown (1967) showed that the in vivo X-ray patterns from resting vertebrate skeletal muscle myosin filaments showed a series of layer lines that were orders of 43nm, and that the third order at 14.3nm had a high intensity on the meridian. They proposed that the layer lines arose from the helical arrangement of the cross bridges with an axial repeat of 43nm and a subunit repeat of 14.3nm. The contribution from the myosin tails to the diffraction pattern is very small and therefore little information about the structure of the filament backbone was obtained. Indeed, little progress has been made since the work of H. E. Huxley and Brown (1967) in the elucidation of the backbone structure using X-ray techniques.

The distribution of intensity along the layer lines is consistent with the cross bridges being further out than the filament backbone. The centre of mass of the cross bridges is believed to be between 17 and 9nm from the filament axis (H. E. Huxley and Brown., 1967; Squire., 1975; Haselgrove., 1980).

H. E. Huxley and Brown (1967) observed meridional intensities on layer lines other than those predicted, if the cross bridges were in an ordered helical array. Perfect helical symmetry of the cross bridges

should, they suggested, give rise to meridional reflections only on every third layer line at orders of 14.3nm. They explained the presence of intensities at unpredicted meridional positions (the so called forbidden intensities) as indicating that the helical symmetry was only approximate. Other workers (Squire et al., 1982; Kensler and Stewart., 1983) have since confimed this finding and Bennett (1981) showed that the forbidden intensities could be explained by a systematic displacement of the cross bridges to either side of a 14.3nm repeat. Yagi et al (1981) and Squire et al (1982) also suggested that the forbidden reflections could derive from a regular axial perturbation of the cross bridges. Stewart and Kensler (1986) used computer processing of electron micrographs to show that the myosin heads were arranged in three strands. They also showed that successive units along each strand were perturbed axially , azimuthally and radially from the positions expected if the structure was perfectly helical. They suggested that the perturbations from the helical structure were a consequence of steric restrictions in the packing of the heads on the thick filament surface. They did however emphasise that it could also reflect an underlying non helical arrangement of the myosin tails (Craig., 1977). Indeed H. E. Huxely and Brown (1967) had already suggested that the rapid fade out of X-ray intensity at relatively low resolution in their X-ray patterns was due to the presence of considerable cross bridge disorder (Thomas and Cooke., 1980).

(IV. 3.) Cross bridge visualisation in the electron microscope.

Direct visualisation of the cross bridges in vertebrate thick filaments, by electron microscopy has been of little use and has yielded only a small amount of information. This is because the techniques used for specimen preparation and observation have, until recently, been unsuccessful in maintaining an ordered array of cross bridges in vertebrate thick filaments. However the arrangement of myosin cross bridges in invertebrate filaments has been studied in some detail and has resulted in greater success (Kensler et al., 1985; Vibert and Craig., 1983). Unfortunately the structure of these filaments differs from that vertebrate in a number of ways. Whereas vertebrate filaments such as rat (Maw and Rowe., 1980), rabbit (Maw., 1980) and frog (Kensler and Stewart., 1983) are typically three standed, arthropod filaments are four stranded (Levine et al., 1982; Kensler et al., 1985) and scallop filaments are seven stranded and arranged over a paramyosin core (Vibert and Craig., 1983).

Although there are obviously major differences in structure between invertebrate and vertebrate thick filaments, the highly ordered state of invertebrate filaments makes them ideal for study in the electron microscope. The high degree of order allows the effects of the techniques used in native filament preparation to be determined with great accuracy. Such studies will hopefully allow better and more precise fixation and staining procedures to be developed, which will hopefully be applied to the more difficult vertebrate system.

Kensler and Levine (1982) used high resolution negative staining techniques to reveal that the cross bridges on the thick filaments from

the Limulus Telson muscle were arranged in a four stranded helix, with twelve cross bridges per turn and an axial repeat of 43.8 nm. They showed that the images observed in the electron microscope were indeed a true representation of the structure in vivo by comparing the optical diffraction pattern of the images with the X ray diffraction patterns . Vibert and Craig (1983) were able to use similar techniques, to show that native scallop filaments possesed 7 fold rotational symmetry and were right handed. Levine and Kensler (1982) had previously demonstrated that the cross bridges of limulus filaments also lie on a right handed helix.

An important aspect of the work of Vibert and Craig (1983) was that they suggested that the two heads of the individual myosin molecules, in the filament were splayed apart axially (Wray., 1975). In 1980 Haslegrove, using less direct methods, had first proposed that there would be a large angle axially between the individual heads of each myosin molecule within a thick filament. Furthermore, he predicted that the angle would be such that one head would point towards the filament tip and the other towards the bare zone. This particular orientation of heads was first visualised by Crowther et al (1985), who confirmed that the individual heads in Tarantula myosin filaments point in opposite directions axially. They clearly demonstrated that the thick filament was four stranded and that the cross bridges lie on a right handed helix, similar to that of the Limulus thick filaments, observed by Levine and Kensler (1982). The resolution of the images obtained by Crowther et al (1985), was good enough to show that the individual myosin heads overlap between one axial level and the next, a finding that was later substantiated by Craig et al (1987). Both groups

of workers proposed that the head-head interaction locked the cross bridges onto the filament shaft in the relaxed state and thus prevented or reduced the interaction with actin.

The fact that such a system of head-head interaction exists in invertebrate thick filaments does not prove its existence in vertebrate filaments. However Stewart and Kensler (1986) have recently demonstrated, using image reconstruction techniques, that such head-head interaction probably exists in frog thick filaments. Unfortunately, because of the greater cross brige dissorder present in vertebrate thick filaments, their images are not as clear as those obtained with invertebrate filaments (Crowther et al., 1985; Stewart et al., 1985; Vibert and Stewart., 1987). As a consequence of the high level of disorder and therefore, reduced resolution Stewart and Kensler (1986) point out that they cannot categorically exclude other possibilities.The X-ray patterns do not unfortunately yield information about the number of cross bridges present at each 14.3nm level (n or the rotational symmetry). This value is obviously of major importance if we are to understand the structure of the thick filament in greater detail and indeed if we are to understand the potential interaction that the thick filament can make with actin filaments in vivo.

(IV. 4.) Estimates for the number of cross bridges per 14.3 nm repeat.

The importance of obtaining an estimate for n has led many workers to turn to more indirect methods, almost all of which involve estimates of the total amount of myosin in the thick filament divided by the number of 14.3nm repeats.

Obviously the simplest arrangement that one can suggest for the

number of myosin molecules per crown is to assume that the number stays constant along the entire filament length. Until recently however it was not altogether certain that this was actually the case, as there was little direct evidence to support the assumption.

Yates and Greaser (1983) used qualitative SDS analysis and found that the myosin content of washed myofibrils was equivalent to 2.5 myosin molecules per crown. They suggested that the reason for the non integer number of myosins was either, that the number per crown changed along the filament length, or that there was a periodic change in the number. Current opinion suggests that such variations do not occur (Lamvik., 1978; Reedy., 1981; Knight et al., 1986) and that the number of myosins per crown is constant along the entire filament length. Nontheless the point raised by Yates and Greaser (1983) was an important one, which forced people to think clearly about the possibility of changes, or variations, in the myosin content of the crowns along the thick filament.

Most workers did however assume a constant number of myosin molecules per crown but this did not result in consistent estimates for n. There were basically two schools of thought, one which suggested that n = 3 and the other n = 4.

Estimations of n = 4 were obtained by several workers. Maruzama and Weber (1972) and Marston and Tregear (1972) used nucleotide binding studies to show that n = 3.5. Their results did however depend on the (apparently reasonable) assumption that only one nucleotide per myosin head was bound. Morimoto and Harrington (1974) used estimates of the mass ratio to show n = 3.9 and also used a technique of filament mass determination which showed that n = 4.3. Pepe and Drucker (1979)

confimed their value for n by using quantitative SDS gel electrophoresis to calculate the actin/myosin ratio in washed myofibrils and show that n = 4.

Experimental techniques using similar principles have however, also yielded eatimates for n = 3. Hanson and H. E. Huxley (1957) and H. E. Huxley and Hanson (1957) used interferrence microscopy and biochemical analysis of intact myosin extracted fibres to obtain a myosin/actin ratio that yields n = 3.2. Tregear and Squire (1973) and Potter (1974) both used quantitative polyacrylamide gel electrophoresis and calculated that n = 2.7 and n = 2.5 respectively.

The non integer results of many of these techniques only emphasises the difficulty in obtaining an unequivical value for n, which mainly revolve around the assumptions used in the calculations.

A different approach by Emes and Rowe (1978b) indicated that n = 3. They used hydrodynamic analysis techniques to determine the molecular weight of myosin filaments. Their estimations used the assumption that native myosin filaments displayed the same frictional properties as certain synthetic filament preparations. Using a procedure defined from their detailed work on synthetic filaments they showed that n = 3.1.

Lamvik (1978) used STEM measurement techniques, to obtain what can be considered a more direct estimate of the number of myosins per crown and found that n = 2.7 . This value was obviously incompatible with estimates of n = 4 and was a strong indication of the filaments possessing a rotational symmetry of n= 3. Reedy (1981), also using STEM measurements, further refined the results obtained by Lamvik (1978) and produced a value of n = 2.9 which is also incompatible with

n = 4. The STEM weighing technique was taken one step further by Knight et al (1986), who carried out similar experiments but attempted to take into account as much of the extra mass of the thick filament as possible. This involved estimations of the contribution made by the M line proteins and C-protein, all of which could be achieved with reasonable accuracy. They did not however take into account the high molecular weight protein titin (Wang., 1979; Trinick et al., 1984) as its exact location and even presence within intact thick filaments is still relatively obscure. They found that n = 3. However the actual value was n = 3.23, and they suggested that the non integer result was due to the presence of titin, which they had ignored in their calculations. The fact that the value of n was consistently high along the entire filament length, suggested that titin was present throughout the filament shaft (Wang., 1979). It also dispelled the suggestion of Yates and Greaser (1983) that the number of myosins per crown varied along the filament length.

It is only in the light of recent evidence that a value for the rotational symmetry n = 3 has generally been accepted as approaching the correct value. However, it must be emphasised that this will not be confirmed until direct visualisation of the cross bridge lattice is attained.

The STEM studies of Lamvik (1978), Reedy (1981) and Knight et al (1986) are the most direct methods used to date, for the estimation of n. All workers are however in agreement that n = 3 and other observations in the electron microscope (Maw and Rowe., 1980; Luther and Squire., 1980; Luther., 1981) have since shown the presence of 3 fold rotational symmetry in the filament backbone.

(IV. 5) The axial arrangement of cross bridges as revealed by electron microscopy.

Examination in the electron microscope of the highly ordered bundles of myosin filaments contained within A segments, has revealed much more detail about the axial arrangement of the cross bridges (Hanson et al., 1971). Craig (1977) showed that A segments negatively stained with ammonium molybdate, sometimes revealed axially ordered cross bridges. Craig (1977) used this technique to unambiguously identify the size of the bare zone, which he found to be 149nm +/- 2nm long, which is in good agreement with the earlier estimates of H. E. Huxley (1963), and H. E. Huxley and Brown (1967).

Craig and Offer (1976a) showed that antibodies to the S1 portion of the myosin molecules, labelled the A bands along the whole length of the thick filaments apart from the central bare zone and near the filament tips. Craig (1977) showed that negatively stained A segments contained numerous stain excluding stripes, along the entire thick filament length, which occured with a periodicity of 14.3nm. However, perturbations in the periodicity were observed near the bare zone and filament tips, which suggested that changes in the myosin packing were occuring in these regions.

Sjostrom and Squire (1977) used negatively stained longitudinal cryosections of muscle and observed a greater amount of detail than was previously possible. They also obtained a clear 14.3nm periodicity of stain excluding stripes, which extended from the edge of the bare

zone to the filament tips. The interesting thing was that their sections not only showed perturbations in the periodicity near the bare zone and filament tips but also a gap (i.e. one 'missing' crown) in the periodicity three repeats from the filament tip.

A combination of the data from these three techniques suggests that the cross bridges are present right out to the filament tips and appear as stain excluding bands with a periodicity of 14.3 nm. There are 49 rows of cross bridges in each half of the filament, with a missing row two periods from the end. Perturbations in periodicity near the bare zone and filament tips suggests that the packing of the molecules in these regions is changing.

(IV. 6) The presence of non myosin components within the thick filaments.

Hanson et al (1971) demonstrated that a considerable amount of the structure of the thick filament backbone was revealed by negatively staining A segments with uranyl acetate. They showed that on either side of the bare zone were a series of eleven bands, with a periodicity of 43 nm. Each band consisted of a prominent stain excluding stripe, followed by a complex subsiduary banding although, it was noticed that the subsiduary banding of the first three stripes was slightly different from the rest. Craig (1977) showed, that the prominent stain excluding stripes were due to some of the non myosin components of the thick filament.

Stripes 5 - 11 have been implicated as being due to the presence of C-protein. C-protein was first named by Starr and Offer (1971), who

demonstrated the presence of four contaminant proteins in myosin preparations, all of which they named alphabetically in order of increasing mobility on SDS gels. Offer et al (1973) were the first to purify it and found it to be an elongated monomer, of molecular weight 140,000. It binds tightly to myosin filaments in physiological ionic strengths but not, as demonstrated by Safer and Pepe (1980) to the myosin heads. Antibody labelling studies have shown that C-protein exists as seven discrete stripes, corresponding to stripes 5 - 11 as revealed by the negative staining (Craig., 1977) 43 nm apart (Pepe and Drucker., 1975; Craig and Offer., 1976b) and 8 nm in axial width.

Starr and Offer (1971) had previously shown, using antibody labelling, that stripe three was probably where H-protein was to be found, although the components of stripes 1 - 4 are still relatively obscure and uncertain.

The complex subsiduary bands, present after the prominent stain excluding stripes (1 - 11), have also been observed in LMM paracrystals. The subsiduary bands present in native filaments were therefore implicated as arising from the packing of the LMM backbone within the native filament (Hanson et al., 1971). The differences in the subsiduary banding pattern of the first three stripes (Hanson et al., 1971) suggested that the packing of the myosin tails in that region was different from that in the rest of the filament. This is probably due to the change from the antiparallel mode of packing, present in the bare zone, to the purely parallel packing present in the distal parts of the myosin filament. It is interesting to note that the region of the first three backbone stripes corresponds to the region of cross bridge perturbation reported by Sjostrom and Squire (1977a) and Craig (1977),

which they also put down to the changes in backbone packing occuring in that region.

The strong 43 nm repeat observed by the uranyl acetate staining of A segments (Craig., 1977) is inconsistent with a truly helical tail packing arrangement when one would really expect a 14.3 nm repeat. It is therefore, clear that the departure of the cross briges from true helical symmetry, as revealed by the forbidden meridionals (H.E. Huxley and Brown., 1967), is also displayed by the packing of the tails within the filament backbone. Therefore it seems that even in the region of so called constant packing (stripes 5 - 11) perturbations in the helical packing of the myosin tails within the thick filament are present

(IV. 7) The presence of sub-filaments within the thick filament backbone.

On a larger scale there has been a great deal of evidence for the existence of subfilaments within the thick filament backbone. The most compelling evidence for this came from Maw and Rowe (1980), who showed that native vertebrate thick filaments reversibly fray into three subfilaments when exposed to very low ionic strength. The filaments never fray into more than three subfilaments and each subfilament has a diameter of approximately 6 nm. Fraying never proceeds into the central bare zone region and the filaments often remain attached at the tips. This observation is possibly a further indication of the presence of a different mode of packing in these regions, which previous work has already shown to be the case (Sjostrom and Squire., 1977a; Craig., 1977). The fact that the

filaments could fray when loosely adsorbed onto a carbon substrate, led Maw and Rowe (1980) to suggest that the subfilaments were packed into the intact thick filaments in a linear fashion, rather than winding around the filament axis. Although this was not a completely unambiguous demonstration of the linear packing of the subfilaments, it is hard to see how a coiled structure could reversibly fray once adsorbed onto a carbon substrate.

The presence of 3-fold rotational symmetry within intact native vertebrate myosin filaments was also demonstrated by Luther and Squire (1980) and Luther et al (1981). They both observed the presence of three subfilaments when intact filaments were observed by cross section, at their tips and through the central bare zone region.

(IV. 8) Information obtained from the study of synthetic thick filaments prepared from pure monomeric myosin.

Obviously, the structure of native vertebrate thick filaments is highly complex. In an attempt to simplify the native system many workers have turned to using synthetic myosin filaments which are formed from monomeric myosin. SMFs do not contain any of the non myosin copolymerising components known to be present in native filaments. However the direct interpretation of the results obtained with SMFs in terms of native filaments is fraught with difficulty and has been of only limited value.

Noda and Ebashi (1960) first showed that native filaments could be completely depolymerised in 0.2M KCl at pH 7.3 and reassembled back into synthetic myosin filaments, by reducing the pH to 6.5 or lower.

The SMFs that they produced were approximately 1 μm long.

In 1963 Huxley produced the first electron micrographs of SMFs and revealed that they had similarities in structure to native filaments (See Section I. 7) some even appearing to be bipolar and possesing a bare zone. However, the dimensions of the SMFs were different to those of native filaments, their structure depending on the exact conditions used during their formation. Those produced by rapid dilution were less than 1 μm in length, whilst those produced by dialysis were generally much longer, over 2 μm in length.

Katsura and Noda (1971) and Koretz (1979a) also found that the rapid dilution of high salt myosin caused the formation of a relatively uniform short (< 0.5 μm) population of SMFs, the assembly being complete within 20 ms (Katsura and Noda., 1971). H. E. Huxley's., 1963) finding that slow dialysis caused longer filaments to form, was later demonstrated by Josephs and Harrington (1966) when they obtained filaments between 1 and 12 μm long, whilst Koretz (1979a) obtained filaments up to 25 μm in length.

Pinset-Harstrom and Truffy (1979) were able to produce synthetic myosin filaments at pH7 in the presence of MgATP that were considered to be of physiological diameter (15 to17 nm) but were not of physiological length, typically being 5 to 15 μm long. Katsura and Noda (1973) demonstrated that the filament length was dramatically affected by the pH. They showed that slow dialysis at pH 7 resulted in the longest filaments, whilst changes in pH to either side of 7 always resulted in shorter filaments.

When the salt concentration is lowered below 0.2M the SMFs formed always display a large variation in their length distribution. Under

these conditions the monomer concentration is very low and attains an equillibrium level, whereas the filament length never attains equillibrium and yields SMFs of variable length. By contrast Josephs and Harrington (1968), Katsura and Noda (1971) and Davis (1985) have all shown that a more even distribution of filament lenghts can be obtained by raising the pH or ionic strength. The filaments formed under these conditions are not full length and are always less than 0.7μm long. Under these conditions the monomer concentration remains high and thus allows the filaments to attain an eqiullibrium length.

It has been a major disappointment that few SMF preparations have shown any evidence of axial periodicities when examined by the various diffraction techniques. Whilst their appearance in the electron microscope often very closely resembles that of native filaments, their internal structure is apparently not well ordered or is completely different.

Eaton and Pepe (1974) prepared a population of SMFs by dialysis in 0.3M KCl, that were of short uniform length. At this concentration the myosin monomers only just polymerise but the filaments formed did display the characteristic 43 nm repeat present in native filaments (H. E. Huxley and Brown., 1967). Although these filaments lacked a bare zone they did display the 43 nm repeat along their entire length showing that they possesed a uniform packing. Moos et al (1975), Hinsen et al (1978) and Sobieszek (1972 and 1977) produced SMFs that displayed a 14.3 nm repeat which is the other characteristic repeat of native thick filaments (H. E. Huxley and Brown., 1967).

It is reasonably clear from the papers cited above that many factors can influence the dimensions of SMFs including pH, ionic strength, and

ionic composition of the buffers used. The presence of other non myosin proteins within native thick filaments has led to speculation that they may be involved in the assembly of native myosin filaments. However Pinset Harstrom and Truffy (1979) and Koretz (1979a) have demonstrated that column purified myosin forms SMFs that are indistinguishable from those formed from a crude myosin extract.

(IV. 9) The role of C-protein in thick filament assembly.

H. E. Huxley and Brown (1967) first suggested that a repeating unit of the A-band, now known to be C-protein, may be responsible for determining the uniform length of native thick filaments. The C-protein gave rise to a 44.2 meridional reflection which indicated that it had a slightly different period to that of the myosin. This led them to propose that myosin and C-protein copolymerise forming a vernier system that was particularly stable at the native filament length of 1.6 μm.

The difference in periodicity between the myosin and C-protein has since been brought into question. Rome et al (1973) used optical diffraction of antibody (anti C-protein) labelled myosin filaments to show that the periodicities were the same. Such differences of opinion have yet to be satisfactorily resolved and therefore the exact periodicity of C-protein remains uncertain and is the subject of much debate.

A possible role for C-protein in filament assembly was demonstrated by Koretz (1979b). She showed that if C-protein was added to pure myosin, prior to reassembly at physiological ratios then the SMFs

formed were compact, of narrow but highly uniform diameter and displayed a narrow length distribution. Above and below physiological ratios the filaments displayed a large varyation in diameter and at the higher ratios bulges were present that were probably due to lateral aggregation of myosin molecules. The effect of the addition of C-protein was most dramatic when added prior to reassembly, which led Koretz (1979b) to suggest that it was incorporated into the thick filament structure, rather than forming a collar around it (Starr and Offer., 1978).

Trinick and Cooper (1980) suggested that C-protein played a purely structural role in stabilising the thick filament structure. Evidence for this came from their work on the sequential dissassembly of native filaments at elevated salt concentrations. They found that the disassembly of the area of constant packing (See Section IV. 5), which corresponds to the length of the C-protein containing region, was highly cooperative. They suggested that the sequential dissassembly cooresponds to the release of the C-protein from the filament backbone and therefore that C-protein may be important in thick filament assembly and length determination.

(IV. 10.) How is the length of native thick filaments regulated ?

The exact mechanism by which native thick filaments achieve such a constant and uniform length distribution is still relatively obscure. However various schools of thought have arisen on the subject and several have proposed models for the self limiting assembly process of native thick filaments. There are basically three models for the length

regulation of vertebrate thick filaments.

(IV. 11.) The vernier model.

The first is a vernier process involving copolymerising proteins, the second involves the self limited packing of identical subunits, and the third involves a two component assembly. A brief summary of the main features of these models is given below.

The vernier model proposed by H. E. Huxley and Brown (1967) was based on the earlier work of H. E. Huxley (1963) and the knowledge that thick filaments contained more than one type of protein. Huxley had tentatively suggested that the length determining mechanism could be one in which two protein components, of differing periodicities, came into register after a certain number of repeats. The later and more detailed copolymer, or vernier model of H. E. Huxley and Brown (1967) suggested that the system consisted of two polymers of different proteins. In this case one was obviously myosin and the other was as yet unknown but possibly C-protein. As the molecules copolymerise their initial internal periodicities are different allowing them to bind another monomer thus causing filament growth. The initial difference in internal periodicity was presumably initiated by the unique antiparallel packing present in the central bare zone region. After a certain well defined number of repeats, the internal periodicities of each copolymer come into register and form a stable interaction with each other. The formation of such a stable interaction prevents further monomer addition which results in the termination of filament growth. Although C-protein is a likely candidate for being involved in such a

copolymerisation the work of Koretz (1979b) has already shown that the addition a C-protein in the correct physiological ratio to pure myosin does not result in correct filament assembly. It does however result in the formation of more uniform filaments which suggests that it may play a role in filament assembly but presumably other as yet undefined factors are needed. Interestingly the importance of regulated actin filaments in obtaining correct thick filament dimensions has been pointed out by Persechini and Rowe (1984) and Maw and Rowe (1986) which only serves to emphasises that many complicated interactions may be necessary for native filament assembly to occur.

(IV. 12.) The induced strain model, involving the length dependant dissociation of a myosin dimer coupled to the length independant association.

The second model proposed by Davis (1986) involves the assumption that a myosin dimer is formed and it is this dimer that adds to the growing filament tip. The existence of myosin dimer was first suggested by Godfrey and Harrington (1970) who proposed that in high salt conditions the myosin molecules were in rapid monomer-dimer equilibrium and this was later supported by Katsura and Noda (1971 and 1973). However these findings were contradicted by Emes and Rowe (1978a) who used hydrodynamic techniques to show that the myosin monomer-dimer equilibrium did not occur in high salt conditions. The discrepancy between these results has yet to be satisfactorily resolved.

Davis (1981a and 1981b) used pressure jump experiments, to show that at lower ionic strengths during filament growth the myosin monomers did form parallel dimers as judged by electron microscopy (Davis et al., 1982). The micrographs showed that the dimers formed were all parallel and the myosin molecules in each dimer were staggered by about 44 nm. Davis (1986) suggested that the dimers then add to the growing filament tips at an invariable length independant rate. However, the rate of dissociation of dimers increases with increasing filament length and when the rate of dissociation equals that of association filament growth stops. Davis's experiments were carried out with short uniform synthetic myosin filaments but he did emphasise that the presence of other copolymerising proteins in native filaments could be involved in the fine tuning of the fiament structure. Although dimer formation may indeed be involved in filament growth direct evidence for its existence is still lacking. Furthermore most of the models for filament growth work equally well if dimer formation is ignored and myosin monomers are considered to be the addition units.

(IV. 13) The template model.

The third model proposes that there is a centrally located length regulating protein, similar to that found in invertebrate filaments. Invertebrate thick filaments are known to posses a central core material called paramyosin (Bennet and Elliot., 1981) which is believed to play a central role in filament length determination (Epstein et al ., 1985). However, such a central core protein has never been observed in vertebrate filaments (Emes and Rowe., 1978b and current work) and

therfore, the validity of such a model for vertebrate filament assembly must be brought into question. The fact that filaments fray into three subfilaments (Maw and Rowe., 1980) also suggests that a core protein does not exist because such a protein would posses a complementary charge to that of the subfilaments and therefore, prevent subfilament separation during fraying.

Which of these models if any most closely describes the method of filament assembly in vivo remains unknown. However it seems likely that the combination of a myosin based, self terminating assembly system, fine tuned by copolymerising proteins is employed to obtain the precise length of native thick filaments is involved.

Persechini and Rowe (1984) developed a technique for the production of SMFs that yielded a homogenous population (0.7 μm long) with a near native mass/ unit length. These have since been shown to be unipolar by electron microscopy. A novel feature of their preparation technique, was that the filament reassembly stage takes place in the presence of a small amount of thin filaments. They suggested that the presence of the I filaments was essential in obtaining such a uniform SMF population. Presumably, the actin filaments acted as some sort of template for thick filament formation.

Maw and Rowe (1985) took this idea further when they demonstrated that full length bipolar thick filaments, with the correct mass/ unit length could be reassembled within whole muscle fibres that contained an intact I-Z-I lattice. They tentatively proposed that the myosin monomers initially bind to the actin filaments and gradually condense into hemifilaments. The hemifilaments eventually fuse with BZAs in the M line region, resulting in the formation of full length bipolar thick

filaments. They suggested that the BZAs formed separately, probably by the condensation of myosin monomers onto the remnants of the M line proteins. The hemifilaments produced by Persechini and Rowe (1984) were presumably produced in a similar fashion but were unable to fuse together to form bipolar filaments because of the lack of an intact I-Z-I lattice.

(IV. 14) The aims of this chapter.

The evidence provided in Chapter III demonstrated that PNFs possess several distinctive properties that are irreversibly lost by exposure to high salt. We feel that the abolition of these properties can be explained in terms of the loss of a native myosin conformation or environment. Therefore, the aim of this chapter is to attempt to locate the unique conformation, or environment that is responsible for the disinctive properties of native vertebrate myosin. As we suggested in Chapter III there are clearly three possibilities that spring to mind. The first was that high salt exposure resulted in a change in the secondary or tertiary structure, which caused the environment of both RLC populations to become identical and resistant to removal. The second was that the myosin filament itself posesses a unique quaternary structure, which is irreversibly lost on depolymerisation in high salt. The third possibility was that it was a combination of all types of structure. We felt that the simplest way to approach this problem, would be to see if the changes observed after high salt exposure were due to alterations in the packing of the thick filament.

(IV. 15) The production of full length synthetic thick filaments.

The best way to test the role that the quarternary structure plays in the expression of the distinctive properties was to reconstruct a synthetic thick filament that had an identical structure to that of a native filament. All of the myosin molecules in such "perfectly" assembled SMFs would have been exposed to high salt conditions. However in this introduction we have shown that although our knowledge of native thick filament structure is limited, it is quite clear that the structure of SMFs is different.

(IV. 16) The production of bare zone assemblages (BZAs).

The approach that we have taken involves the use of a technique for the production of BZAs as described by Neiderman and Peters (1982). The formation of BZAs involves exposing native myosin filaments to 200 mM KCl which depolymerises the thick filaments down to BZAs. BZAs are likely to be structurally similar to the min-filaments of Reisler et al (1981) (which contain 18 myosin molecules) but unlike mini-filaments also contain the associated M line proteins.
Dialysis of the BZAs and their solubilised distal myosin molecules back into 100 mM KCl causes filament ressembly to occur (See Section II. 33). The reassembled filaments are remarkably uniform in length and posess an almost identical length distribution and structural morphology to that of native filaments. It appears that the BZAs act as

a nucleation centre for filament growth and that they code for correct filament packing, which results in self limited assembly. Furthermore, the packing must be very similar to that in native myosin because the reassembled filaments are identical in size, within reasonable limits, to native filaments.

The filaments reassembled from BZAs contain high salt exposed myosin but apparently possess a mode of packing that is very similar if not identical to that of native filaments. Therefore, we used this system to test for the presence or absence of the distinctive properties. We chose to test the ability to remove the 50% of the RLCs from the reassembled filaments as an indication of whether the unique properties present in native myosin were still present.

Interpretation of the results obtained still depend upon the assumption that the quarternary structure of the reassembled filaments is identical to that of native filaments. We therefore, attempt to demonstrate that this is correct, by employing the low ionic strength fraying techniques developed by Maw and Rowe (1980). They showed, that native rat myosin filaments fray into three subfilaments when rinsed in low ionic strength media. We demonstrate that rat and rabbit myosin filaments reassembled from BZAs also fray into three identical subfilaments in a similar manner to that observed with native filaments. We suggest that this is good evidence in support of the assumption that filaments, reassembled from BZAs, possess the same quarternary structure as native filaments.

We also attempt to show that the PNFs are indeed unipolar hemifilaments and contain the remnants of the ruptured bare zone. The technique of metal shadowing is employed to produce high resolution

images of individual PNFs which unequivically reveal their unipolar nature. We also show that the PNFs can fray into 3 subfilaments, which are identical to those present in frayed full length native filaments.

Results

Examination of the structure of thick filaments reassembled from BZAs plus monomeric myosin.

(IV. 17) Fraying experiments with rabbit myosin.

All of the experiments in this section were carried out on rabbit myosin filaments from a relaxed filament preparation.

(IV. 18) Electron microscopy of the native myosin filaments in a relaxed filament preparation.

A typical field of negatively stained relaxed filaments is shown in Plate 4.1. The thick filaments are bipolar with tapering ends and sometimes possess a densely staining central bare zone. The central bare zone was not always apparent however it is generally accepted that if both ends of a filament taper the structure is presumed to be bipolar (H. E. Huxley., 1963). Cross bridge structures are rarely seen in negatively stained preparations unless special precautions are taken and our samples were no exeption. The inability to observe cross bridge structures was however unimportant for the determination of the length distribution.

The thick filaments were readily distinguished from the thin (actin) filaments by their much greater diameter and apparent rigidity (Plate

4.1.). The actin filaments were quite flexible and were often attached end to end, to create very long flexible structures (Plate 4.1.).
The average length of the thick filaments was 1.5 μm ± 0.2 and the homogenisation technique used yielded approximately 85% (weight averaged) full length filaments. In this case filaments between 1.3 and 1.6 μm were taken to be full length. Filaments of less than the native length were often present but they were assumed to be broken or damaged and were probably created by the homogenisation conditions used during preparation.

(IV. 19) Fraying of native rabbit filaments.

Native rabbit filaments were readily frayed by rinsing in Trinick's (1982) fraying medium (Plate 4.2.). However the total number of filaments that frayed within each preparation was quite variable. In some instances only 30% of the filaments in a single preparation frayed, whilst in others the figure was over 80%. The variation in the number of frayed filaments was not only seen between different preparations but also between different grids from the same preparation. This result underlines the well known difficulties in obtaining reproducible fraying with rabbit filaments (Maw and Rowe., 1980; J. Cooper, personal communication). However, when the filaments did fray into subfilaments they never frayed into more than three and quite often only into two. Also, as pointed out by Maw and Rowe (1980), when only two subfilaments were observed, one was sometimes but not always thicker than the other.

The characteristic bowed appearance of the subfilaments (Maw and Rowe., 1980; Trinick., 1982) was quite apparent but cross over of the subfilaments was only rarely observed cf. Rowe and Maw (1983). Many of the subfilaments did however appear to be attached at their tips but they were never attached to the tips of other thick filaments.

The tips of the filaments always frayed first. Fraying never started within the main shaft of the filament or near the bare zone region. Furthermore, the central bare zone always remained intact, fraying never proceeded into that region. The average unfrayed clearly visible central region was about 0.33 μm in length, which is rather longer than the length of the BZAs reported in IV. 20.

(IV. 20) The formation of BZAs.

The bare zone asemblages produced by dialysis against 200 mM KCl are shown in Plate 4.3. They have the charateristic dumbbell appearance reported by Neiderman and Peters (1982) and when observed at high magnification (Plate 4.3.) posses a distinctly cross bridge free shaft. The dumb-bell appearance arises from the heads of the myosin molecules protruding and splaying out of the ends of the structure. All of the myosin filaments completely depolymerised down to BZA structures, the average length of which was 0.31 μm ± 0.02 (Plate 4.3.). This value is in close aggreement with that of Neiderman and Peters (0.3 μm) and represents the minimum stable structure at this ionic strength.

The BZAs were highly monodisperse when viewed by electron

microscopy and showed no tendency to aggregate in either an end to end, or side to side manner.

(IV. 21) Reassembly of filaments from the BZAs and distal myosin molecules.

Complete thick filament reassembly was achieved by dialysing BZAs and their distal myosin molecules into 100 mM KCl (See Section II.33) (Plate 4.4). The morphology of the filaments as judged by electron microscopy, was very similar to that of native filaments. The negative staining technique used did not resolve the cross bridge structures or particularly highlight the central bare zone region. However the bipolar nature of the reassembled filaments was implicated by the possession of tapering filament tips (Plate 4.4.). Dialysis back to 200 mM KCL once again resulted in the formation of BZAs with an average length of 0.3 μm ± 0.03. BZA structures are not obtained when synthetic filaments are dialysed in 200 mM KCl cf. Synthetic myosin filaments in 200 mM KCl undergo complete dissassembly to monomeric myosin.

The reassembled filaments had an average length of 1.49 μm ± 0.3 as judged by analysis of their length distribution. This value was similar although not identical to the average length of the native filaments (1.5 μm) (Section IV. 18.).

(IV. 22) Fraying of reassembled myosin filaments.

The reassembled filaments produced in section IV. 21 readily frayed under low ionic strength conditions. However as with the control filaments the amount of fraying observed varied from sample to sample. When fraying was induced no more than three subfilaments were ever observed, although fraying into only two was quite common. When fraying into two subfilaments occured, one of them sometimes appeared thicker than the other.

After fraying the reassembled filament preparations were almost indistinguishable from frayed native filaments. However the degree of bowing induced in the subfilaments was slightly different. The subfilaments of native myosin tend to bow when frayed (Plate 4.2), whereas on a purely subjective basis the bowing of the reassembled myosin subfilaments was not as great (Plate 4.5). The subfilaments were rarely seen to cross but did sometimes appear to be attached at the tips. There was however no tendancy for the tips from one frayed filament to attach to, or interact with those from another filament.

(IV. 23) Fraying experiments with rat myosin.

All of the experiments in this section, were carried out with rat myosin filaments from a relaxed filament preparation.

(IV. 24) Native rat thick filaments in a relaxed filament preparation.

Plate 4.6 shows a typical field of the negatively stained native rat filaments in a relaxed filament preparation. The thick filaments are again easily distinguished from actin filaments which are much narrower in diameter (<10 nm) and do not appear to be as rigid as the thick filaments. Occasionally very long (>1.5 μm) flexible structures 10 nm in diameter were seen which we believe to be actin filaments joined end to end. The thick filaments were bipolar with tapering ends and sometimes displayed a densely staining central bare zone. The yield of full length filaments (between 1.3 and 1.6 μm) was typically greater than 75 to 85% (weight averaged). Analysis of their length distribution showed that the average length of the filaments was 1.5 μm ± 0.2 although, a population of very much smaller filaments was often present.

(IV. 25) Low ionic strength fraying of native rat thick filaments.

Native rat thick filaments readily fray into subfilaments when rinsed in the low ionic strength medium of Trinick (1982). Almost all of the filaments (>95%) were observed to fray (Plate 4.7). The number of frayed filaments did not significantly change even in preparations upto 14 days old. The filaments invariably frayed into three subfilaments and occassionally into two but never more than three. When only two subfilaments were present, one was often but not always thicker than the other.

After fraying the subfilaments (as reported by Maw and Rowe., 1980) invariably appeared slightly bowed but were never sharply bent. The subfilaments rarely crossed over but were often attached at their tips. As reported by Maw and Rowe (1980) fraying was induced near the filament tips (Plate 4.7) and was never observed to be initiated away from the tips, or near the bare zone. Furthermore the central bare zone region was always present in frayed filaments and fraying never proceeded into it. The average length of the unfrayed region when both ends of the filament were frayed was 0.5 μm with a maximum lenght of 0.8 μm and a minimum of 0.26 μm. However when large unfrayed regions were observed it was likely that fraying was incomplete and therefore the structural significance was questionable. Occasionally, after fraying the length of the bare zone appeared to be significantly less than the known length of the intact bare zone (~0.3 μm). This was probably due to variations in the staining protocol rather than an indication of having structural significance. However the presence of short unfrayed regions did appear to increase as the preparations aged and could well be related to some form of proteolytic attack, possibly of the M-line proteins.

(IV. 26) The formation of BZAs from native rat filaments.

BZAs formed by the dialysis of native rat filaments in 200 mM KCl are shown in Plate 4.8. The BZAs were bipolar structures with ill defined ends. At high magnification the central shaft of the BZA is completely devoid of cross bridge structures. However at each end of the shaft the

myosin molecules splay out and produce the so called bottle brush appearance. A histogram of their length distribution (Graph 4.5) revealed an average length of 0.29 μm ± 0.02, which is in good agreement with the value obtained by Neidermann and Peters for the length of rabbit BZAs (0.30 μm). No structures greater than 0.5 μm in length were observed and there was no evidence to suggest that aggregation of the BZAs has occured.

(IV. 27) Filament reassembly from BZAs.

Dialysis of BZAs and their associated distal myosin molecules back to 100 mM ionic strength resulted in the reassembly of thick filament structures (Plate 4.9.). The reassembled filaments were bipolar in nature and had tapering ends, the central bare zone region sometimes stained heavily. The reassembled filaments had an average length of 1.49 μm ± 0.3, as revealed by the length distribution analysis (Graph 4.9.).

(IV. 28.) Fraying of reassembled filaments.

When subjected to the low ionic strength fraying conditions of Trinick (1982) the reassembled rat filaments readily frayed into subfilaments. Almost all (>95%) of the filaments observed on each grid were frayed to some extent, which is in complete aggrement with the amount of fraying observed in the control (See Section IV.25). The reassembled filaments were never frayed into more than three subfilaments but

fraying into only two was quite common. When present the individual subfilaments did not appear to bend as much as those of the control and were usually closely apposed. The close approximation of the individual subfilaments made it difficult to see if they were actually attached at the tips or not but the general impression was that a significant number were (Plate 4.10). Fraying never proceeded into the bare zone region and was initiated at the tips of the filament The length of the unfrayed shaft was variable but was never less than 0.25 μm which is in close agreement with the value obtained with native filaments (IV. 25).

(IV. 29). The polarity of PNFs.

All of the experiments in the following section were carried out using PNFs from rabbit myosin.

(IV. 30) Negatively stained PNFs.

Bolger et al(1989a) has shown that PNFs are created from full length filaments that have ruptured under pressure, in their central bare zone region. Plate 2. 1 shows a typical negatively stained PNF preparation, the average length of the filaments was 0.7 μm ± 0.1. The purity of these filaments with respect to actin can be clearly seen as no thin filament structures are visible in the field of view. Compared with the amount of actin present in a RFP (Plate 4.1) the level of purity is obvious, and has been estimated as at least 99.8% w.r.t. actin

(quantitated by SDS-PAGE).

Information about the unipolar nature of the PNFs was difficult to obtain using the negative staining protocol. However some of the filaments appeared to have one blunt end and one tapering end, which is taken to mean that the filaments are not bipolar. The blunt end was probably the remnant of the ruptured bare zone although the lack of visible detail prevents us from saying more.

(IV. 31) The use of metal shadowing for cross brigde visualisation.

The lack of cross bridge visualisation obtained using conventional negative staining techniques, has not allowed us to convincingly demonstrate the unidirectional nature of the PNFs. However when using native bipolar myosin filaments, high resolution metal shadowing techniques have in the past proved successful for the visualisation of cross bridges. We therefore employed metal shadowing in an attempt to unambiguously demonstrate the unipolar nature of PNFs.

Although rotary or cone shadowing seemed the most appropriate technique to use, we have found that unidirectional shadowing (unpublished observations) can sometimes reveal details lost by the rotary technique. For this reason we employed both unidirectional and rotary shadowing techniques in an attempt to resolve as much detail as possible.

(IV. 32) Unidirectionally shadowed PNFs.

Plate 4. 11 (a + c) shows unidirectionally shadowed PNFs. Plate (a) shows a particularly clear image of a PNF that has projecting cross bridge structures that are clearly visible along most of the filament length. The filament is approximately 0.68 μm long, has a shaft with a diameter of 22 nm and cross bridges that extend up to 30 nm out from the filament shaft. One end of the filament clearly tapers to a point, whilst the other end finishes abruptly. The blunt end of the filament was clearly resolved and appeared to be quite ragged (cf a fractured stick). The ragged end was probably the remnant of the ruptured central bare zone region. Indeed this end was completely devoid of cross bridge structures for approximately 64 nm of its length. This represents almost 40% of the total known length of the central bare zone region in an intact thick filament (See Section IV.5.).

(IV. 33) Rotary (or cone) shadowed PNFs.

Plate 4.12 (a-c) shows rotary shadowed PNFs. The dimensions of all filaments are approximately the same as those observed with unidirectional shadowing. The average length of the rotary shadowed filaments is approximately 0.77 μm. The unipolar nature of the filaments is apparent because the cross bridges (in all cases) point in one direction only. The direction that the cross bridges point is always away from the blunt or ragged end and towards the tapering filament tip. Plate 4.12b shows a particularly clear tapering filament tip whilst

4.12a and 4.12c show that the cross bridges all point in the same direction.

(IV. 34) Low ionic strength fraying of PNFs.

PNFs were induced to fray into subfilaments under low ionic strength conditions (Plate 4.13). We found that the fraying conditions were more critical when using PNFs than when using full length filaments. Rinsing with only one or two drops of fraying medium was found to be ideal. Exposure to the fraying medium for greater lengths of time resulted in dissociation into separate subfilaments, thus making interpretation of the subfilament numbers per PNF impossible.

When fraying was induced the PNFs frayed into a maximum of three subfilaments that sometimes remained intact at both ends. Occasionally the PNFs were frayed at one end only, the other end appearing to be completely intact. When fraying was induced at the extreme tip of a PNF the subfilaments were often completely separated from one another.

All of these fraying patterns were observed in each half of the intact full length native filaments (See Section IV. 19). The fact that some of the frayed PNFs remain intact at one end only suggests that PNFs can still contain a remnant of the bare zone, which we know (See Section IV. 19) is not susceptible to fraying in intact thick filaments.

In some cases the filaments appeared to fray completely along their entire length but the individual subfilaments remained closely apposed and did not appear to be attached to each other even at their extreme

tips. Although it was probable that the closely apposed subfilaments were derived from the same PNF, we could not state this with absolute certainty.

(IV. 35) Can we form BZA structures from PNFs ?

Dialysis of PNFs in 200 mM KCl resulted in complete disassembly of the filaments. There was no evidence for the existence of any structures that were larger in size than the individual myosin molecules. Therefore BZA like structures were not formed by dialysis of PNFs in 200 mM KCl, presumably because of damage to the bare zone induced during preparation. Electron microscopy (See Section IV. 32) has already revealed that the bare zone remnant in the PNFs was quite ragged and therefore probably damaged.

Dialysis of the 200 mM PNF preparation back into 100 mM KCl did not result in the reassembly of filaments with the dimensions of either PNFs or full length native filaments. The filaments produced were of greatly varying lengths and widths and were identical (as far as we could tell using electron microscopy) to the filaments produced by the dialysis of conventionally purified myosin into 100 mM KCl.

(IV. 36) Are end filaments present in any of the frayed preparations?

The end filaments present at the tips of frayed native myosin filaments (Trinick., 1982) were not observed in any of our experiments. However Trinick (1982) reported that they were only observed when the

filaments were frayed in solution and not when adsorbed onto the carbon substrate of the grid. As all of our fraying experiments on PNFs were (of necessity to control the process) done after adsorbtion onto grids, it was not too surprising that end filaments were not observed.

Do reassembled filaments possess the properties associated with native myosin ?

(IV. 37) Are the RLCs in reassembled rabbit filaments susceptible to extraction ?

Gel 4.1 shows an extraction timecourse and reveals that the RLCs of rabbit myosin reassembled from BZAs are completely insensitive to the extraction technique. Gelscan analysis reveals that after 100 minutes in the incubation medium no significant degree of RLC removal occurs (Table 4.1, Graph 4.7). This is identical to the result obtained with the conventional high salt exposed myosin filaments (See Section III. 23).

(IV. 38.) Are the RLCs in reassembled rat filaments susceptible to extraction ?

The RLCs in the reassembled filaments displayed an increased susceptibility to removal by the extraction technique compared to those of native rat filaments (See Section III. 26).
Gelscan analysis reveals that 85% of the RLCs were removed after

approximately 30 minutes incubation time. After 100 minutes incubation time no significant increase in RLC removal was achieved (Table 4.2.). Athough a complete timecourse of RLC depletion from the reassembled filaments was not carried out, comparison with the amount of extraction achieved when using 0.5 M KCl exposed rat myosin (See Section III. 26), at the same incubation times showed the amount of extraction achieved to be the same. Therefore, after 35minutes in the gentle extraction medium there was a 35% increase in the susceptibility of the RLCs when compared to the RLC susceptibility in the native control filaments. Therefore the environment of the RLCs in the rat filaments reassembled from BZAs appears to have undergone the same conformational change as conventional high salt exposed rat myosin although the nature of the change appears to differ from that seen in the rabbit system.

Discussion

(IV. 39) Are PNFs unipolar hemifilaments ?

The negative staining of the PNF preparation (Plate 2.1) does not conclusively demonstrate the unipolar nature of the hemifilaments. The unipolarity of the filaments has been inferred because they are approximately half the length of a native filament and do not display a prominent densely staining bare zone region. The average length of the hemifilaments (0.7 μm) is just under the half the value expected for a native thick filament (1.5 μm). It was therefore important to demonstrate the hemifilaments were created by the process of bare zone rupture (Plate 2.1) which would yield hemifilaments and not by some other means. For example it is conceivable that during preparation the filaments undergo pressure dissociation and reform as a short uniform population of filaments. Although it is highly unlikely that such a process would yield a uniform population of filaments or one which showed fraying into three sub-filaments we needed to eliminate such possibilities.

The unipolar nature of the PNFs is clearly demonstrated by the high resolution metal shadowed images obtained (Plate 4. 11) which show a unipolar hemifilament that contains a ragged bare zone remnant. Plate 4. 11b also clearly shows the central backbone shaft, with crossbridge structures extending out along its entire length, except for the region of the bare zone remnant. The lateral dimensions of the PNF correspond approximately with half the length of measurements made by other

workers using full length thick filaments. The cross bridges extend up to 30 nm from the filament shaft which is slightly greater than the average value of 17 nm predicted by H. E. Huxley and Brown (1967) and Hasselgrove (1980). However it is known that there is considerable dissorder about their mean positions (H. E. Huxley and Brown., 1967; Thomas and Cooke., 1980) which means that many of the cross bridges would be in a highly extended state and could certainly be 30 nm away from the filament shaft. Maw and Rowe (1980) showed that the backbone of native myosin filaments at the central bare zone was about 18 nm across. The PNFs possess a bare zone remnant with a diameter of approximately 22 nm which is similar to this value. The bare zone remnant in PNFs may however be a more open structure and therefore be prone to flattening when drying down onto the e.m. grid. Such a flattening process would increase the apparent diameter of the filament which could well account for the slight over estimate. The blunt end of the hemifilament is quite ragged (Plate 4. 11a) and shows signs of being forcibly snapped. Further experiments (Rowe unpublished observations) have shown that the bare zone rupture probably occurs during purification when the filaments are subjected to pressure in the ultracentrifuge (Plate 2. 1).

The process of bare zone rupture also goes some way to explaining why PNFs are often slightly less than half the length of a full length thick filament. This is because it is likely that that during bare zone rupture some material is lost from that region, indeed Plate 4. 11a does show a ragged bare end to the PNF. The loss of material from the bare zone remnant, combined with the loss of some material from the filament tips during preparation would result in a reduction in the overall length

of the hemifilament. The loss of material from the filament tip is probably caused by mechanical damage incurred by the homogenisation during preparation and was first reported by H. E. Huxley (1963) when looking at the full length filaments in a relaxed filament preparation.

The unipolar nature of the PNFs was also demonstrated by the low ionic strength fraying experiments. The PNFs were consistently frayed into three subfilaments which were identical to the subfilaments observed in frayed full length thick filaments. Fraying was always observed to be initiated at one end of the hemifilament and proceeded down the filament towards the other end. In intact full length filaments a similar fraying pattern (Maw and Rowe., 1980) was observed whereby the fraying always started at the filament tips and proceeded towards the central bare zone. Presumably fraying in the hemifilaments is initiated at the end that was one of the original tips in the intact thick filament. Once fraying has started it then continues down the hemifilament towards the remnants of the bare zone. After fraying, we never observed an intact bare zone in the centre of the PNFs once again demonstrating their unipolarity.

The length of time that the PNFs were exposed to the low ionic strength fraying medium was very critical. If exposure was too long, then the PNFs readily frayed into subfilaments that did not remain attached together. Although, in some instances the three individual subfilaments remained closely apposed they were more often completely separated and determining which subfilaments were related to which was imposible. Presumably the ability of the subfilaments to completely dissociate from each other is related to the process of bare zone rupture described earlier. In full length thick filaments the

central bare zone is completely resistant to fraying and has never been disrupted by exposure to low ionic strength conditions (Also Maw and Rowe., 1980). Exposure to low ionic strength conditions causes the complete separation of the subfilaments in PNFs, suggesting that the bare zone remnant is not able to hold the individual subfilaments together. The inability of the bare zone remnant to hold the subfilaments together during fraying can be simply interpreted in one of two ways. The first is that bare zone rupture damages the remnant, possibly as a result of the loss of myosin or M line proteins. Loss of these proteins could weaken the remnants and render them physically incapable of holding the subfilaments together. The other possibility is that the central bare zone in intact filaments is only resistant to fraying because of the antiparallel packing of the myosin molecules in that region. Bare zone rupture would reduce, or even completely abolish, the presence of antiparallel packing, leaving the bare zone remnant containing almost exclusively the parallel mode of packing. As the parallel mode of packing is known to be distinctively susceptible to fraying (Maw and Rowe., 1980) the bare zone remnants would be incapable of holding the subfilaments together.

The individual subfilaments in a frayed hemifilament do not always appear to be attached at their tips. In many cases the subfilaments are free at the tip that is distal to the bare zone remnant. However, many examples were observed where the frayed tips were still attached although no evidence for the presence of end filaments was ever observed.

The instability of the bare zone remnant in hemifilaments was emphasised by the fact that exposure to 200 mM KCl caused complete

filament disassembly. After the exposure of PNFs to this ionic strength no evidence for the existence of structures ressembling BZAs was found in the electron microscope.

Dialysis of the myosin from disassembled PNFs back into 100 mM KCl caused filament reassembly to occur. However the reassembled thick filaments were not of uniform structure and are very similar in morphology to the synthetic myosin filaments formed from purified monomeric myosin.

(IV. 40) Have we succeeded in obtaining high salt exposed myosin filaments that still possess the native quarternary structure ?

The production of BZAs from full length native myosin filaments and the subsequent reassembly of a uniform population of full length thick filaments as described by Neiderman and Peters (1982) was confirmed using both rabbit and rat thick filaments. The dimensions of the rabbit thick filaments before and after disassembly (Graph 4. 1 and Graph 4. 3 repectively) are very similar to the values reported by Neiderman and Peters (1982). However they did report that only the BZAs formed by raising the pH up to 8 were able to produce native length filaments upon reassembly. They claimed that the reassembled filaments produced from BZAs in 200 mM KCl were slightly shorter than native length. In our hands reassembly onto BZAs formed in 200 mM KCl always resulted in filaments of approximately native length. The similarity between native filaments and reassembled filaments in terms of their average length, narrow length distribution and appearance in the electron microscope is strong evidence in support of

the assumption that the reassembled filaments posses the same quarternary structure as native filaments.

On dialysis into 200 mM KCl rat filaments readily formed bipolar BZA structures (Plate 4. 8). The rat BZAs displayed a similar morphology to those of the rabbit (Plate 4. 3). They possess the clasical double bottle brush appearance described by Neiderman and Peters (1980) and have an average length of 0.29 μm ± 0.05 (n=100), which is slightly shorter than that of the rabbit BZAs which were 0.31 μm ± 0.03 (n=100). Reassembly of full length myosin filaments from rat BZAs was achieved although the reassembled filaments were slightly but not significantly shorter (1.49 μm ± 0.3 (n=100)) than those of the control (1.5 μm ± 0.2 (n=120))

As far as we are aware this is the first time that that BZAs have been formed from rat filaments and subsequently reassembled into full length thick filaments. This once again emphasises the structural similarities between rat and rabbit myosin filaments and demonstrates the validity assuming that most mammalian thick filaments have a similar structure.

As discussed earlier the resistance of the bare zone to disruption under extreme conditions is likely to be due either to the presence of antiparallel packing of the myosin tails in that region, or the presence of the M line proteins. It is however equally likely that the stability of the bare zone is related to both of these factors. The work of Reisler et al (1980) would argue against this because they have produced antiparallel BZA like structures from monomeric myosin, in the complete absence of other proteins. The fact that BZA like structures are stable in the absence of M line proteins would suggest that the

stability of the bare zone was entirely due to the antiparallel packing of the myosin tails. However Reislers mini-filaments are only stable under highly non physiological ionic conditions (*300 mM KCl and pH 8).* The instability of Reislers stub filaments under physiological conditions could be indicative of the need for the presence of M line proteins for complete BZA stability. The fact PNFs do not possess any M line proteins and the bare zone remnant in PNFs is unstable in 200 mM KCl provides further support for the idea that the M line proteins play a role in stabilising the myosin BZAs. It must be emphasised that the mechanism by which the antiparallel packing present in BZAs is achieved in vivo is completely unknown. It is however clear that in vitro, antiparallel packing can only be achieved under extreme (physiologically speaking) conditions. Therefore it is reasonable to assume that other, as yet undefined, factors are involved in forming the antiparallel packing of myosin tails.

The fraying experiments clearly show that the thick filaments reassembled from BZAs readily fray into three subfilaments on exposure to low ionic strength conditions. The subfilaments produced are on average 6 nm across which is the same as for those present in native filaments (Maw and Rowe (1980). When frayed the reassembled filaments are almost indistinguishable from frayed native filaments. However, on a totally qualitative basis the individual subfilaments produced from the reassembled filaments do not appear to separate to the same extent. Fraying of native filaments tends to induce a significant amount of bowing of the individual subfilaments wherease those present in the reassembled ones appear to be more linear. It is however difficult to define the degree of bowing produced and

therefore, to quantitate this difference would be problematic. One must also take into consideration the fact that the fraying conditions on each grid will never be identical and thus make the interpretation of any quantitation obtained uncertain.

The fact that native myosin filaments sometimes remain attached at their tips during fraying led to speculation that there was either a slightly different mode of packing in that region, or that a non myosin component was holding them together.

Trinick (1981) was the first to demonstrate the existence of a structure at the tips of frayed filaments. He called this structure an end filament and suggested that it may be involved in holding the subfilaments together. Throughout the course of this study end filaments were not observed in either rat or rabbit filaments, however this could be due to differences in experimental procedure (See Trinick., 1981). The absence of end filaments in our experiments does not disprove their existence nor does it eliminate the possibility that other proteins may hold the filament tips together.

We know that a slightly different packing arrangement is probably present near the tips of the thick filaments (See Section IV. 5). Perhapse this region is slightly more resistant to fraying in low ionic strength conditions than the areas of constant packing. The presence of such a region would obviously result in the subfilaments remaining attached at their tips.

It was however noticable that the subfilaments did not always remain attached at the tips. Presumably in this instance the fraying conditions were more vigorous forcing the the subfilaments to dissociate, even in the region of distinctive packing at the tips. Or

perhapse, the homogenisation conditions, used during preparation, were particularly severe and caused removal of structures such as the end filaments, allowing complete subfilament separation to occur.

The reassembled filaments frayed in a very similar manner to the native filaments. Sometimes they appeared to remain attached at their tips whilst at others they were completely separated. In both native and reassembled filaments there was no difference in the ratio between number that remained attached and those that did not.

The similarity of these ratios allows us to draw several conclusions. Assuming that end filaments, or other non myosin proteins, are responsible for holding the subfilaments together at their tips in native filaments, then the reassembled filaments must also possess these proteins. This means that during ressembly onto BZAs, other non myosin proteins copolymerise at least in the region of the tip. Such copolymerisation could be indicative of the presence of a vernier length regulating system as proposed by Huxley (1963) (See Section IV. 11).

Conversely if we assume that native subfilaments remain attached at their tips because of the presence of a unique packing at that point (as indicated by the missing crown of cross bridges) then we must assume that the reasembled filaments also possess such a packing arrangement at their tips.

Either of these interpretations indicates that the quarternary structure of the reassembled filaments is similar to that of native myosin filaments in both the rat and the rabbit. Therefore as the reassembled filaments contain high salt exposed myosin but still possess the native quarternary structure, any loss in distinctive properties can probably be solely attributed to a change in the secondary or tertiary structure.

(IV. 41.) Do the reassembled thick filaments possess the distinctive properties associated with native myosin filaments ?

We have already demonstrated that the exposure of native myosin filaments to high salt conditions probably causes a conformational change which manifests itself as an alteration in its properties (Chapter III). Exposure to high salt causes a loss of calcium sensitivity (See Section I. 10), a change in the chymotryptic sensitivity of the RLCs (See Section 1. 10) and a dramatic change in our ability to remove RLCs by gentle extraction (See Section III. 23). We also know that the transient exposure to high salt invariably causes a simultaneous change in all of these properties. Therefore if we observe a change in one of these properties we can assume that the appropriate change has occured in all of the others.

With this in mind, we decided to use the susceptibility of the RLCs to the extraction technique, as an indication of a high salt induced change having occured. The reasons for choosing this property as an indicator were several fold. For instance the effect of the gentle extraction technique has been extensively studied on both rat and rabbit myosin filaments (Chapter III) making interpretation of the results obtained much simpler. By contrast the chymotryptic probe technique has only been used to a limited extent on a few systems and would, therefore require extensive control experiments to be carried out. The effects on the calcium sensitivity cannot be used because PNFs have yet to be produced from native rat filaments and therefore, the presence of a calcium sensitive MgATPase has not been checked.

We have shown that the RLCs in reassembled rabbit filaments are

completely resistant to extraction (Gel 4.1, Table 4.1 and Graph 4.7) whilst there is an increase in the susceptibility of those present in reassembled rat filaments (Table 4.2).

The inability to extract any of the RLCs from reassembled rabbit filaments, even at long incubation times, is identical to the result obtained when using conventional, dimensionally incorrect, high salt exposed rabbit myosin filaments (See Section III. 23). The increased susceptibility (~ 35% after 100 minutes incubation time) of the RLCs, in correctly reassembled rat filaments, corresponds exactly to the increased susceptibility observed when using conventional dimensionally incorrect high salt exposed rat myosin filaments (See Section III. 26). Therefore, it is clear from our results that the distinctive properties of native myosin are not present in correctly reassembled (high salt exposed) myosin filaments.

It is also apparent that the RLCs in reassembled filaments occupy the same conformational environment as those in conventional, polydisperse, SMF preparations. The simplest interpretation of these results is that the quarternary structure of the myosin filaments from the rat and the rabbit is unimportant for the expression of the distinctive properties of native filaments. The results obtained in Sections IV. 22 and IV. 28 clearly demonstrated that the filaments reassembled in the presence of BZAs have as far as we can tell an identical quarternary structure to native filaments. The fact that such filaments display identical properties to those of their respective conventional SMFs, suggests that exposure to high salt conditions has had an effect on the secondary or tertiary structure of the individual myosin molecules. Although in the case of conventional SMFs it has

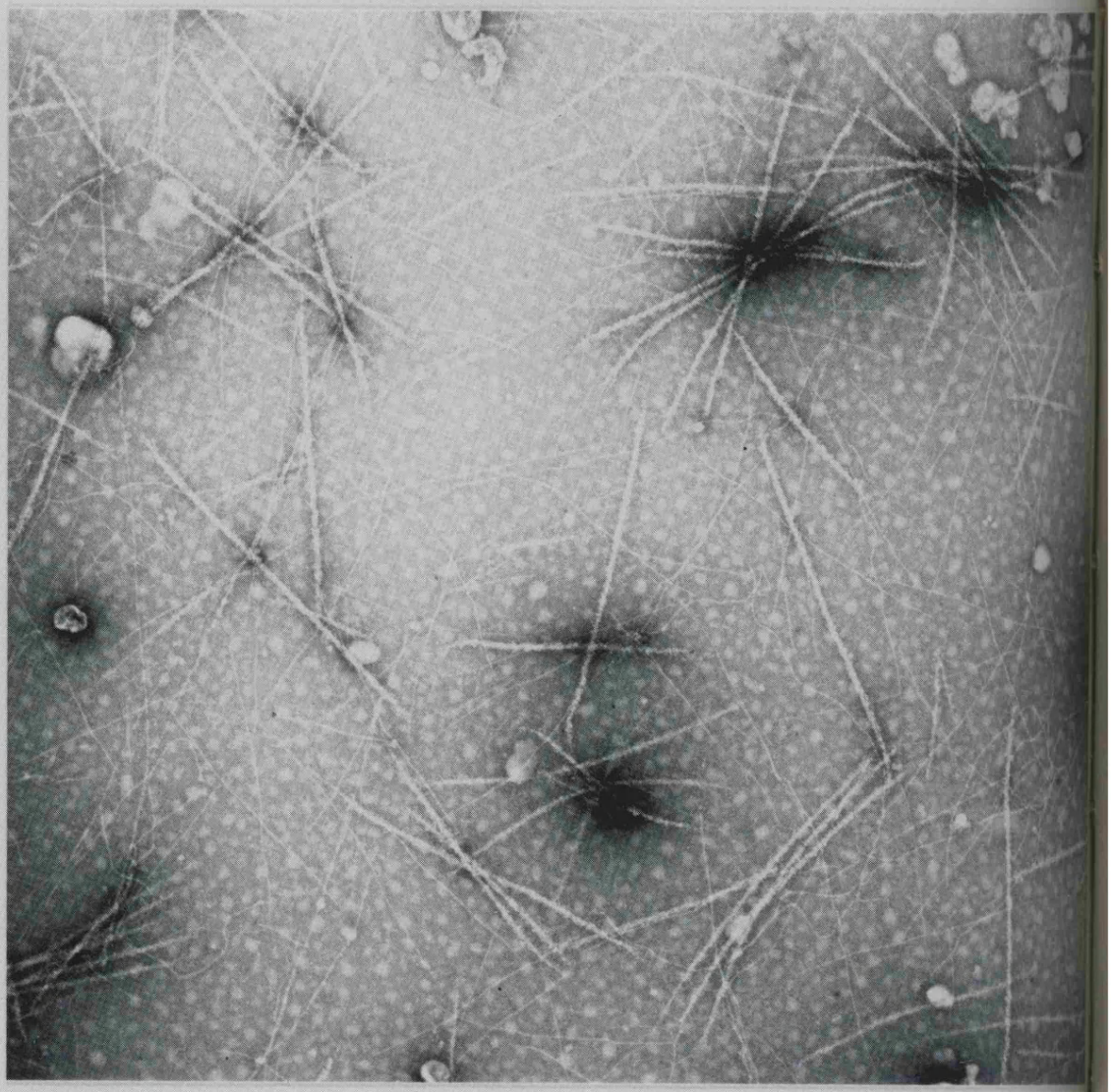
also affected the quarternary structure, such changes are unimportant in terms of the expression of the distinctive properties.

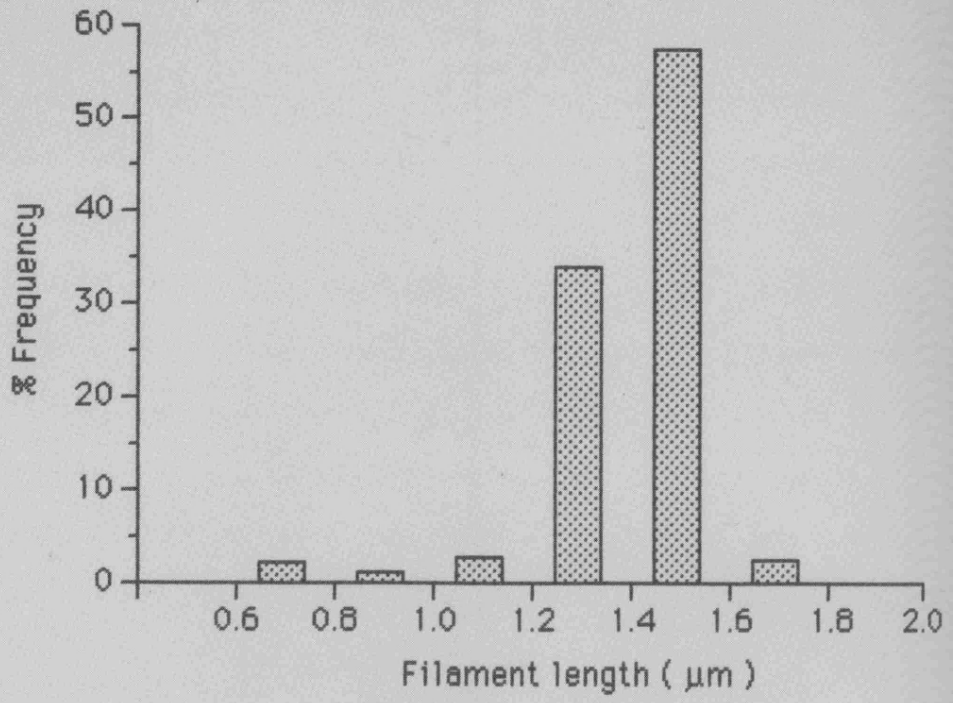
The loss of distinctive properties of myosin filaments by exposure to high salt is clearly a result of some form of conformational change in the secondary or tertiary structure. Unfortunately at this stage we cannot with any certainty, be more specific about the location. However, because high salt exposure affects the environment of the RLC, it would be reasonable to assume that such changes occur in the S1/S2 portion of the myosin molecule (See Section 1. 3ii and 1. 3iii and V. 2). A similar suggestion was made in Chapter III, based on the evidence of the chymotryptic probe and gentle extraction technique. We feel that the evidence presented in this chapter certainly supports this view.

We have also demonstrated that provided BZAs are present during assembly monomeric myosin has the ability to reassemble into filaments possessing a native quaternary structure. The BZAs presumably act as a nucleation centre for correct filament packing. This however raises the question as to how the antiparallel packing present in the BZAs is actually formed. Purified monomeric myosin cannot reproducibly produce antiparallel packing under physiological conditions. Therefore high salt exposure has either altered the myosin's ability to spontaneously produce antiparallel packing but not parallel packing, or some unknown factor that promotes antiparallel packing is lost during the initial depolymerisation stages. Whatever the reason is, it is quite clear that under approximately physiological conditions in vitro the BZA is intimately involved in correct thick filament assembly.

Chapter IV

Gels, Tables, Graphs and Plates.

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% Frequency
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2.0
Filament length (µm)

Plate 4.1

A typical electron micrograph of a negatively stained rabbit relaxed filament preparation (Magnification 34,000 x).

Graph 4.1

A length histogram (weight averaged) showing the distribution of thick filament lengths in a typical rabbit RFP.

Average filament length = 1.5 μm (s.d. 0.2).

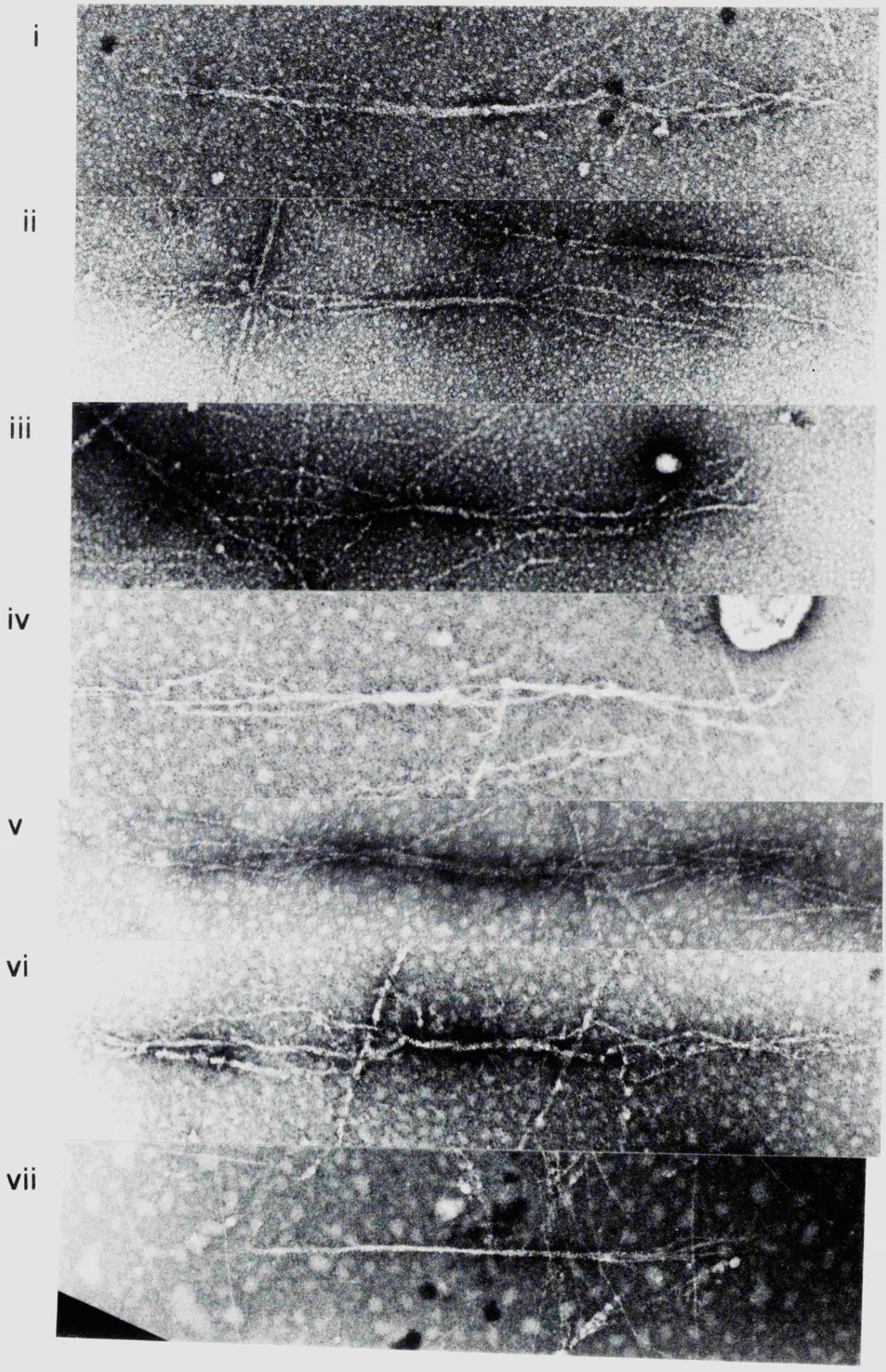
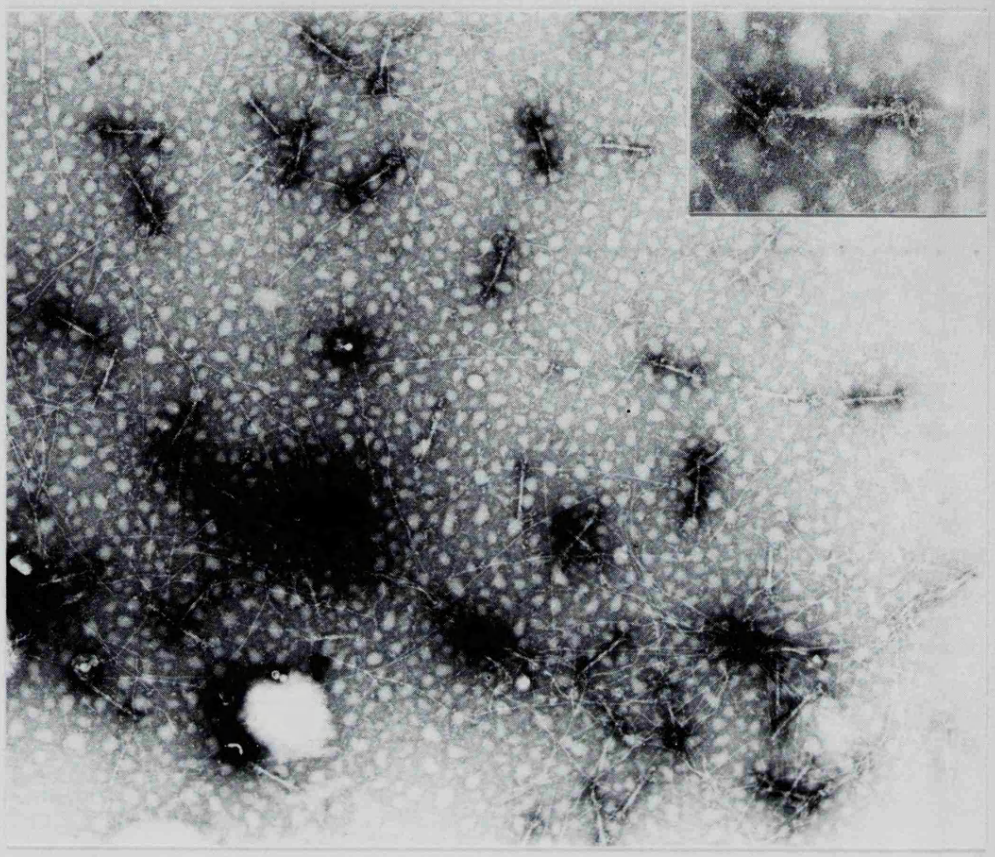

i
ii
iii
iv
v
vi
vii

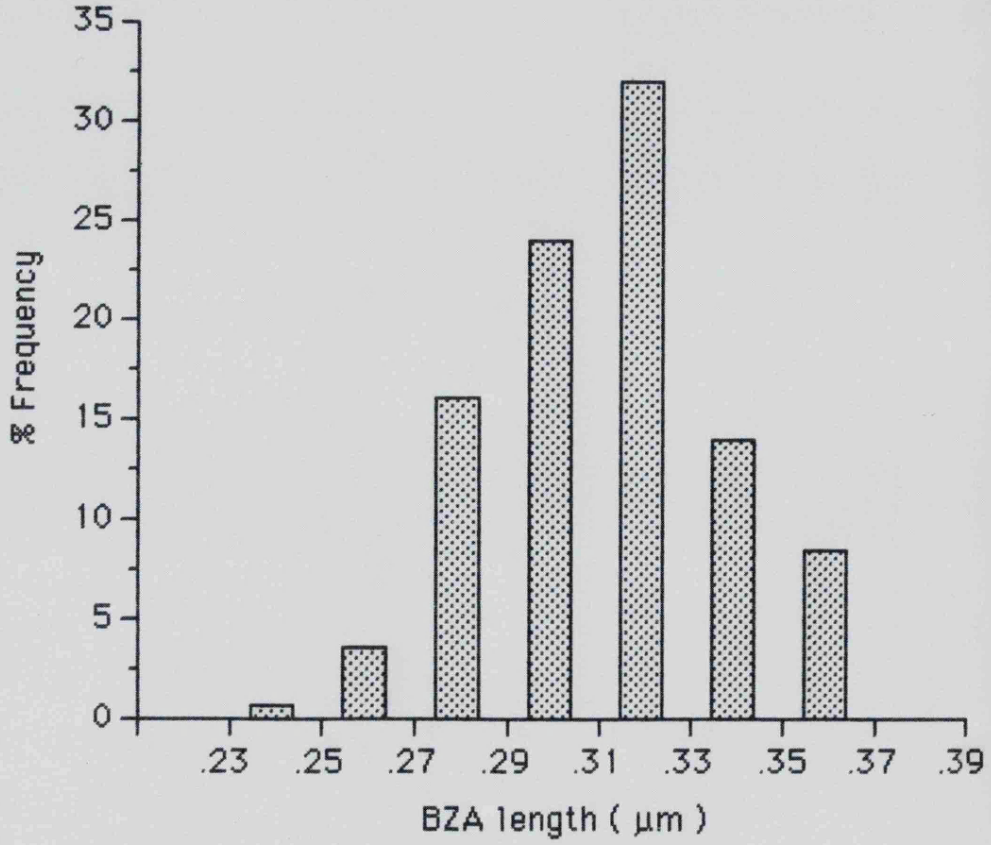
Plate 4.2

A montage of negatively stained rabbit thick filaments that have been frayed into subfilaments by rinsing with low ionic strength buffer.

i - vi. Magnification 73,300 x

vii. Magnification 53,300 x

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BZA length (µm)

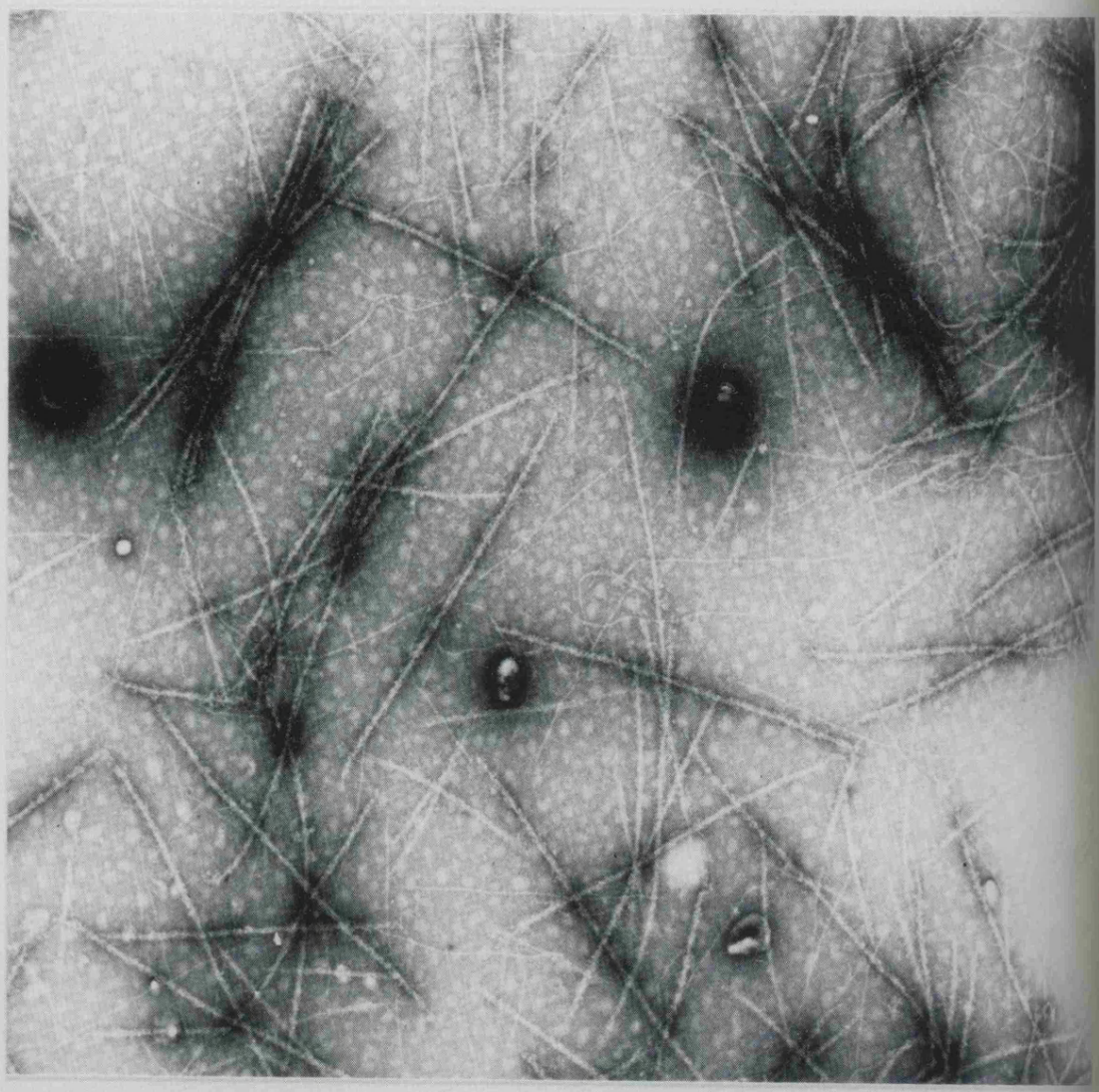
Plate 4.3

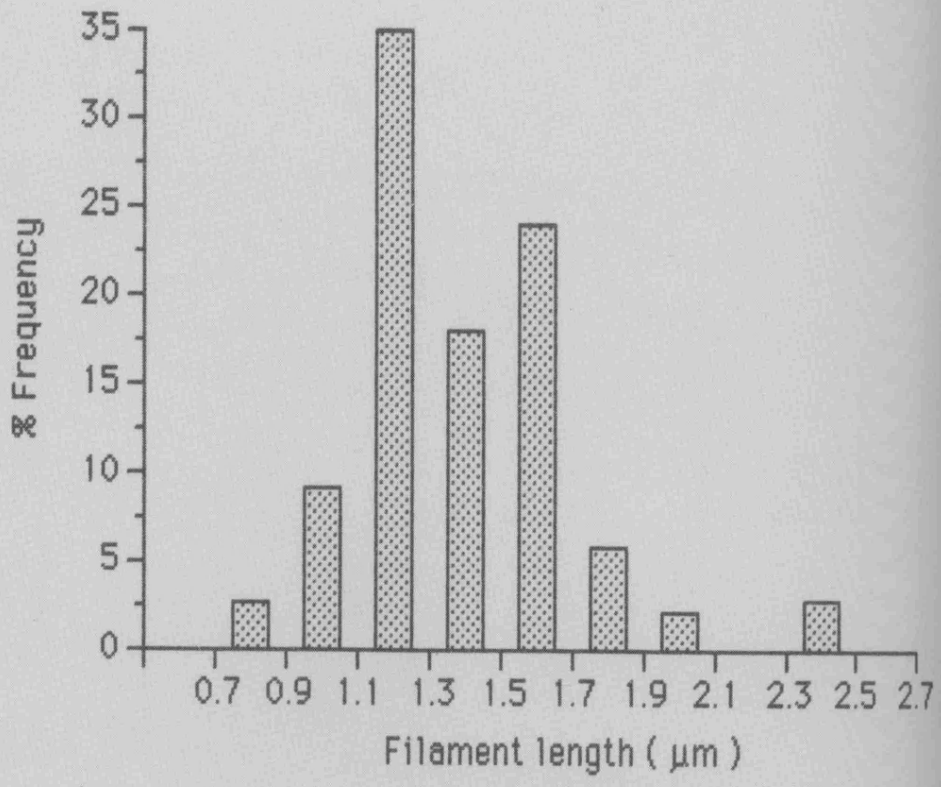
An electron micrograph showing a field of negatively stained rabbit bare zone assemblages (BZAs) produced by the dialysis of a RFP against 200 mM KCL (Magnification 21,750 x).

Inset. A typical BZA at high magnification (65,000 x).

Graph 4.2

A length histogram (weight averaged) showing the distribution of BZA lengths in a typical preparation. Average length = 0.31 μm (s.d. 0.02).

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% Frequency
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Filament length (µm)

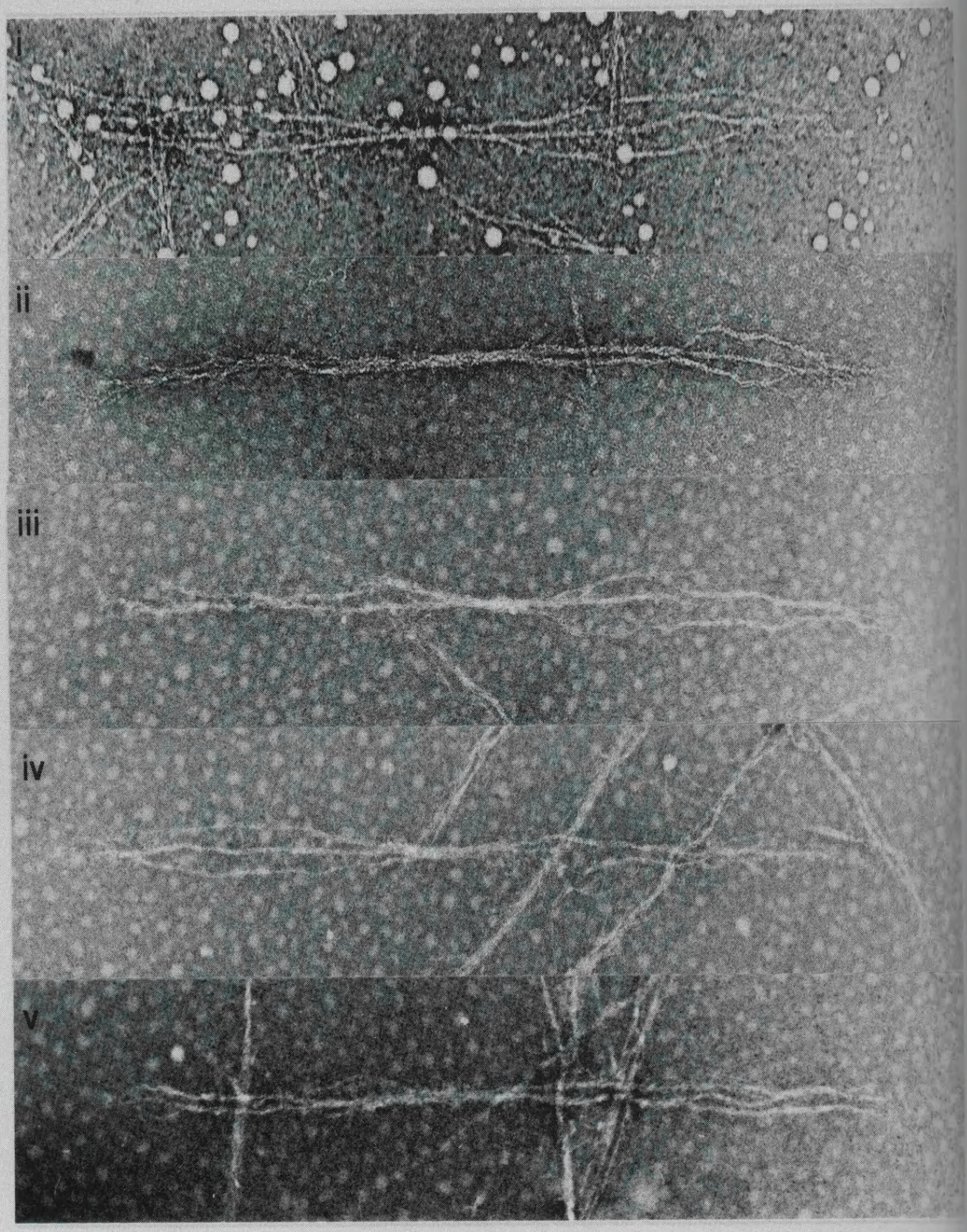
Plate 4.4

A typical field of negatively stained reassembled rabbit thick filaments which were obtained by the dialysis of BZAs and their associated distal myosin molecules into 100 mM KCl buffer (Magnification 34,000 x).

Graph 4.3

A length histogram (weight averaged) showing the distribution of thick filament lengths after reassembly from BZAs.

Average length = 1.49 μm (s.d. 0.3).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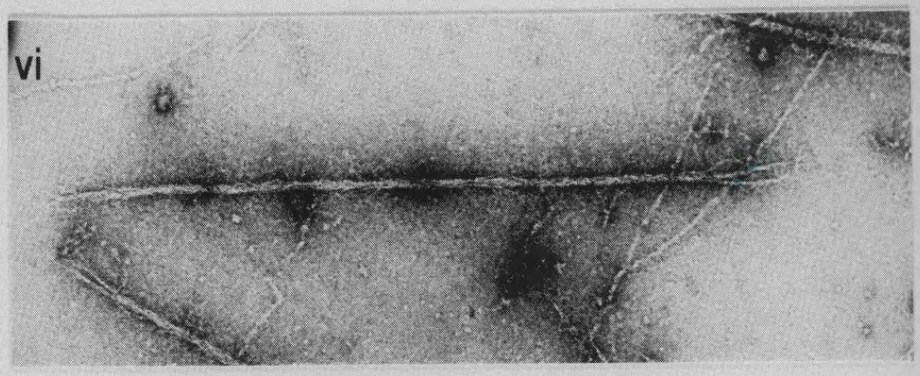
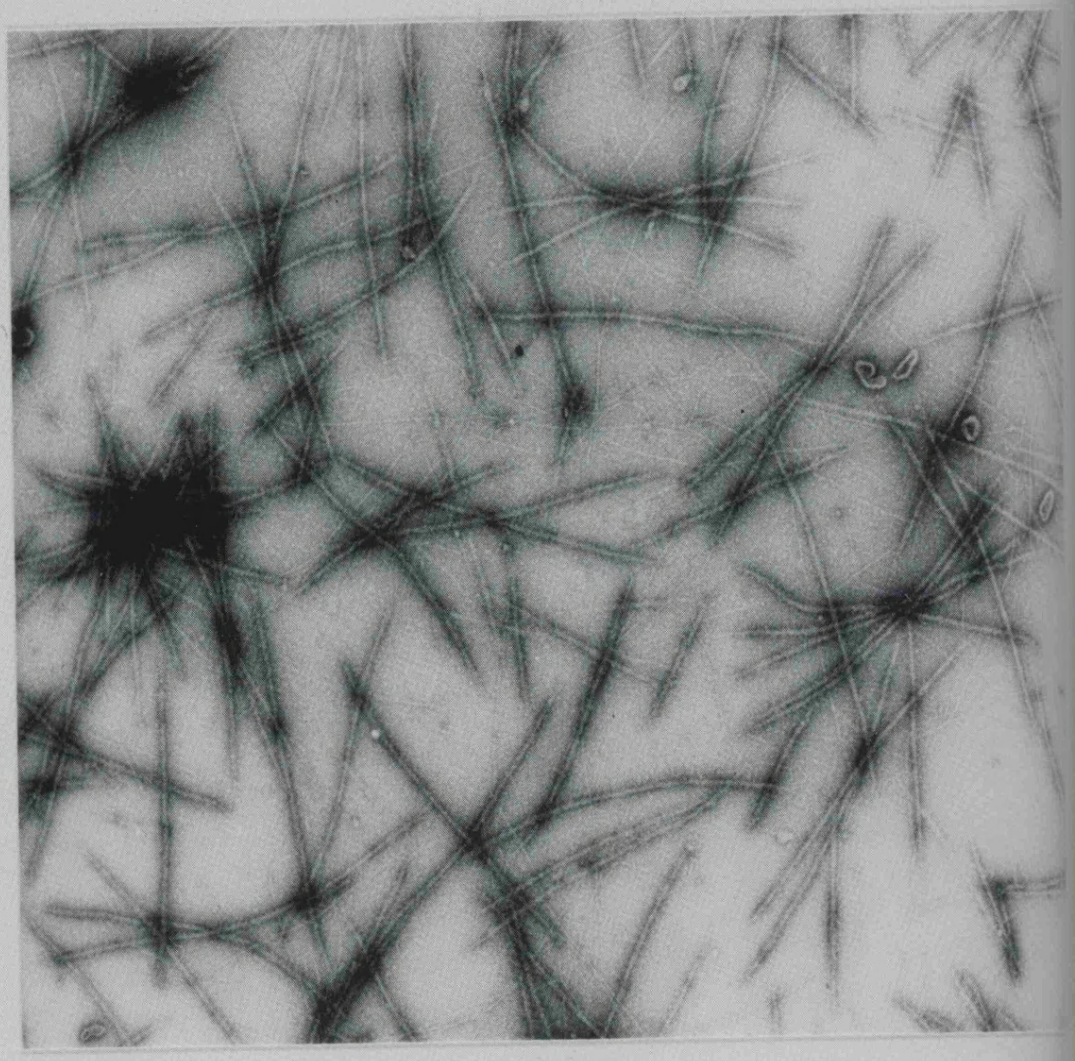

vi

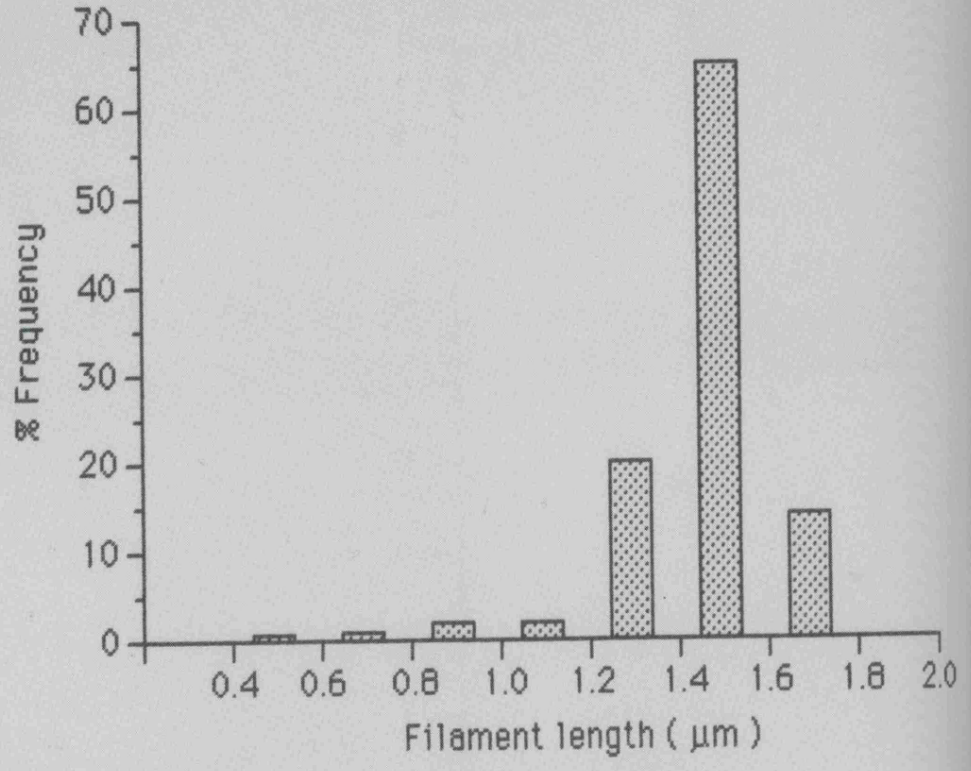
Plate 4.5

A montage of negatively stained reassembled rabbit thick filaments that have been frayed in to sub-filaments by rinsing with low ionic strength buffer.

i - v. Magnification (70,000 x).

vi. Magnification (50,000 x).

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% Frequency
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1.6
1.8
2.0
Filament length (µm)

Plate 4.6

A typical electron micrograph of a negatively stained rat relaxed filament preparation (Magnification 30,500 x).

Graph 4.4

A length histogram (weight averaged) showing the distribution of thick filament lengths In a typical rat RFP.

Average length = 1.5 μm (s.d. 0.2).

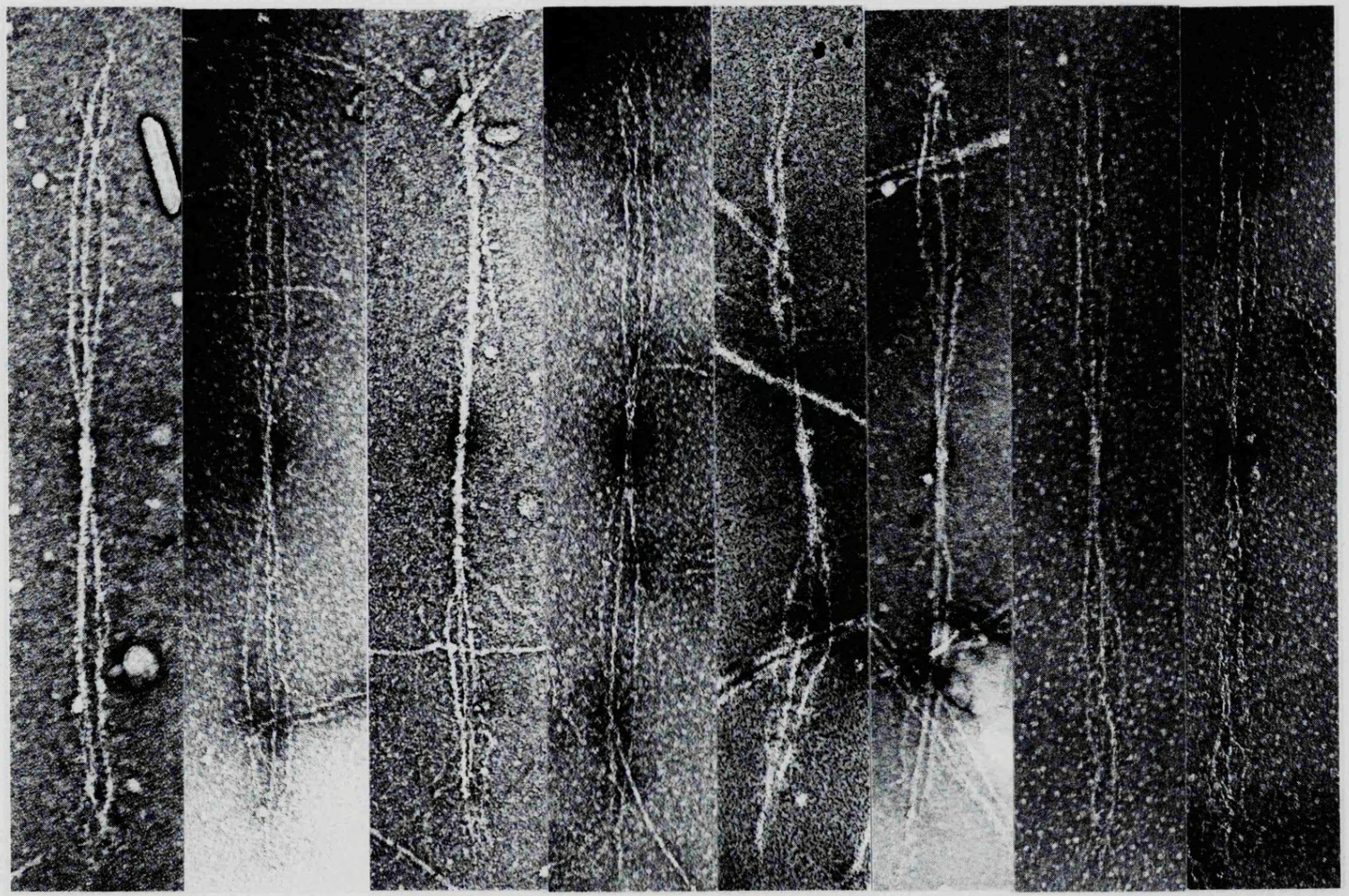
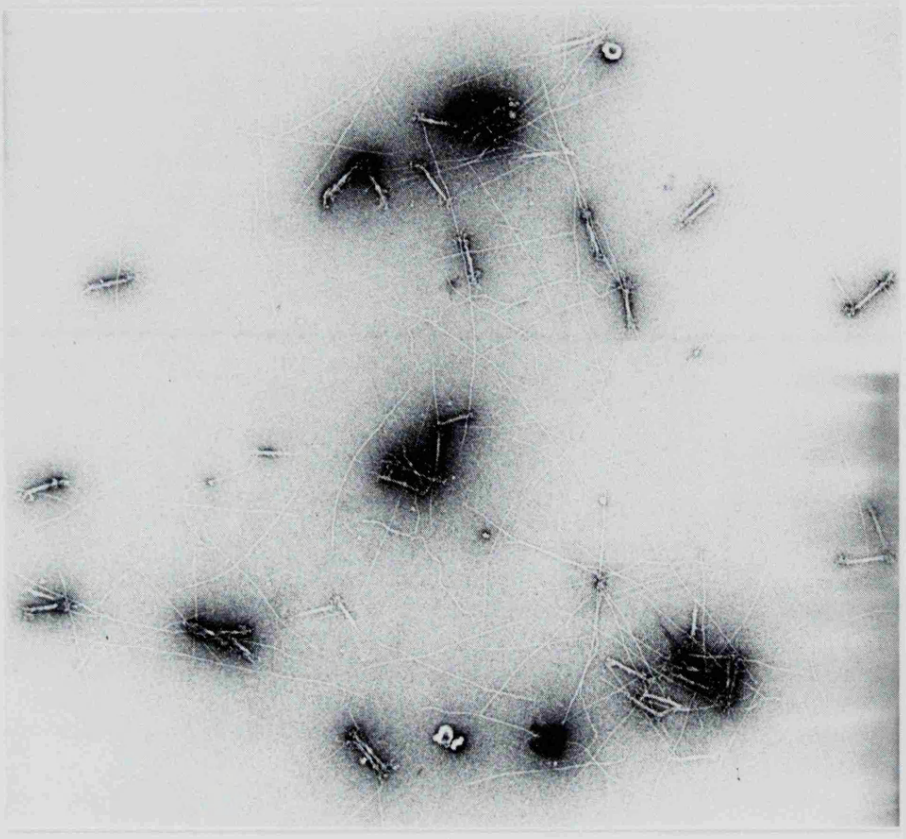
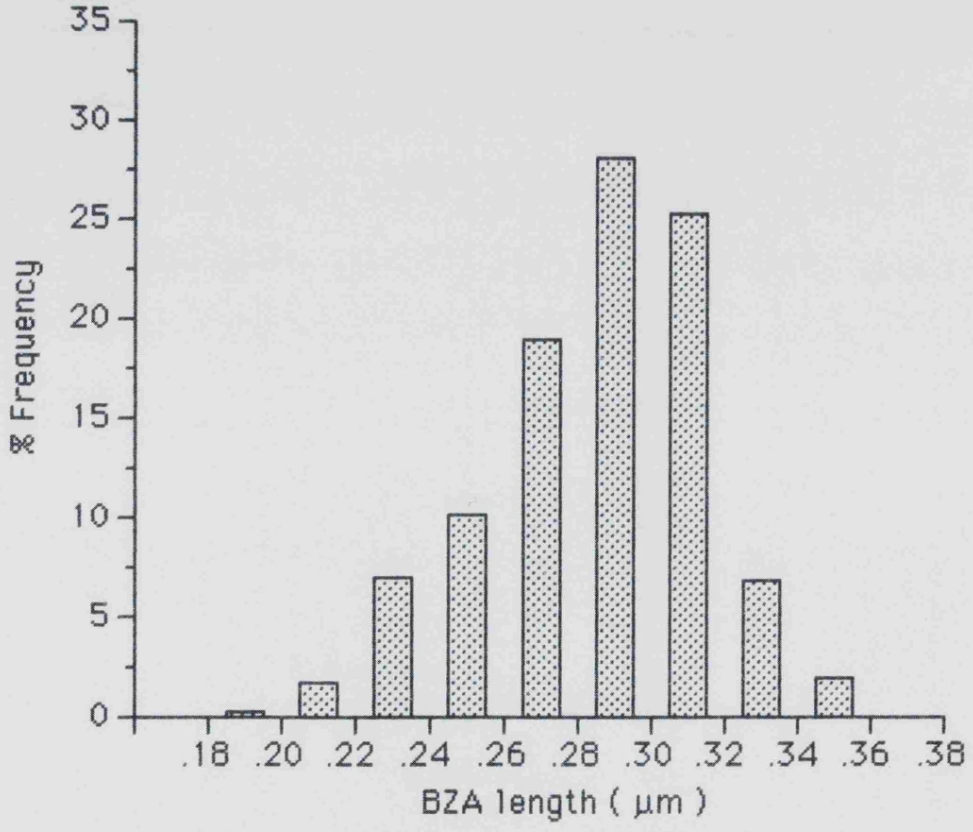


Plate 4.7

A montage of negatively stained rat thick filaments that have been frayed into sub-filaments by rinsing with low ionic strength buffer (Magnification 74,000 x).

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BZA length (µm)

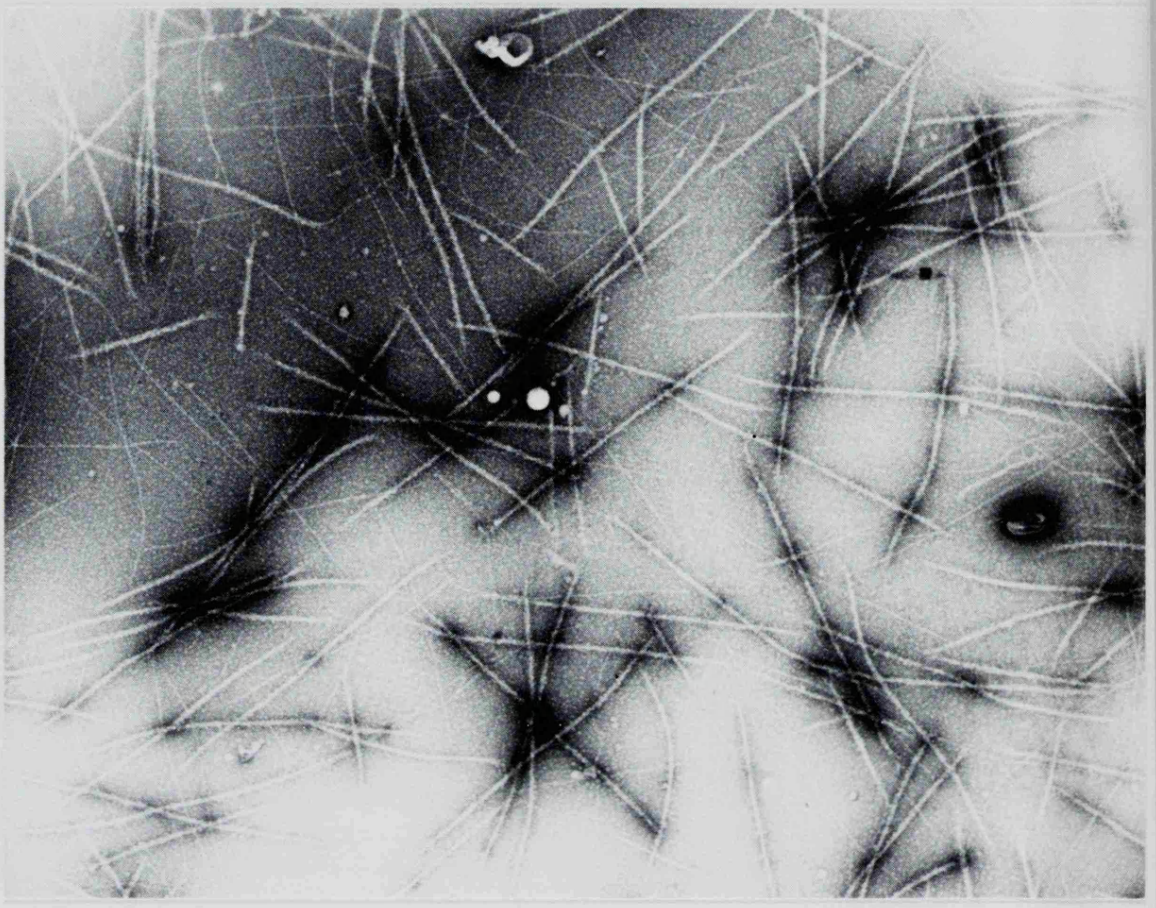
Plate 4.8

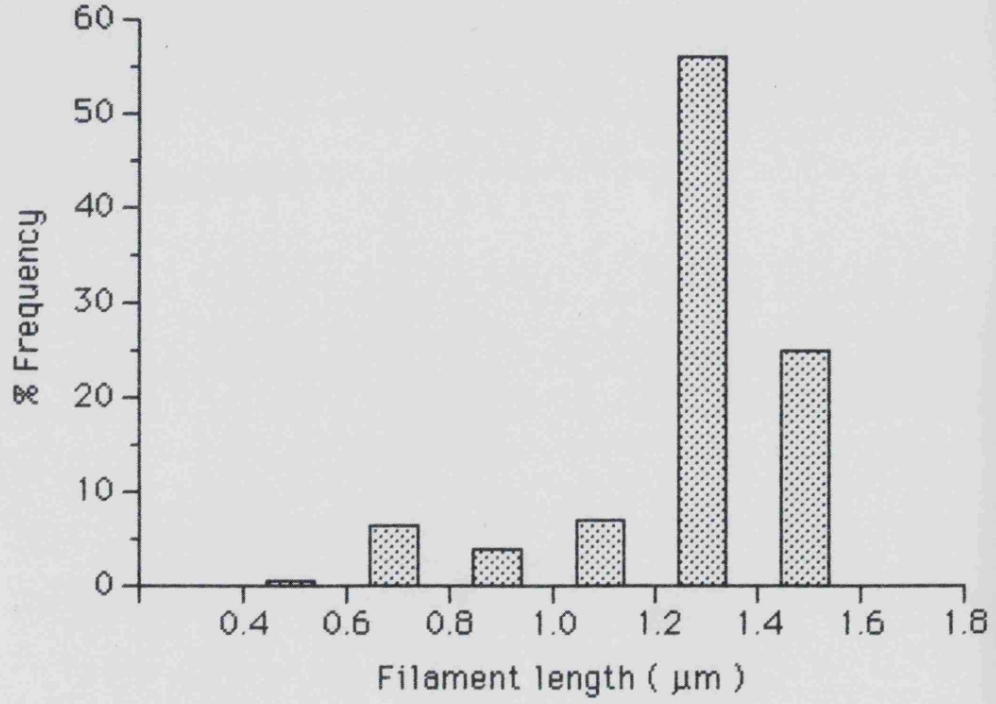
An electron micrograph showing a field of negatively stained rat BZAs produced by the dialysis of a RFP against 200 mM KCl (Magnification 21,750 x). Note the similarity between the structure of the rat BZAs and that of the rabbit BZAs shown in Plate 4.2.

Graph 4.5

A length histogram (weight averaged) showing the distribution of BZA lengths in a typical rat preparation.

Average length = 0.29 μm (s.d. 0.03).

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50
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20
10
0
% Frequency
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
Filament length (µm)

Plate 4.9

A typical field of negatively stained reassembled rabbit thick filaments which were obtained by the dialysis of BZAs and their distal myosin molecules into 100 mM KCl buffer (Magnification 30,500 x).

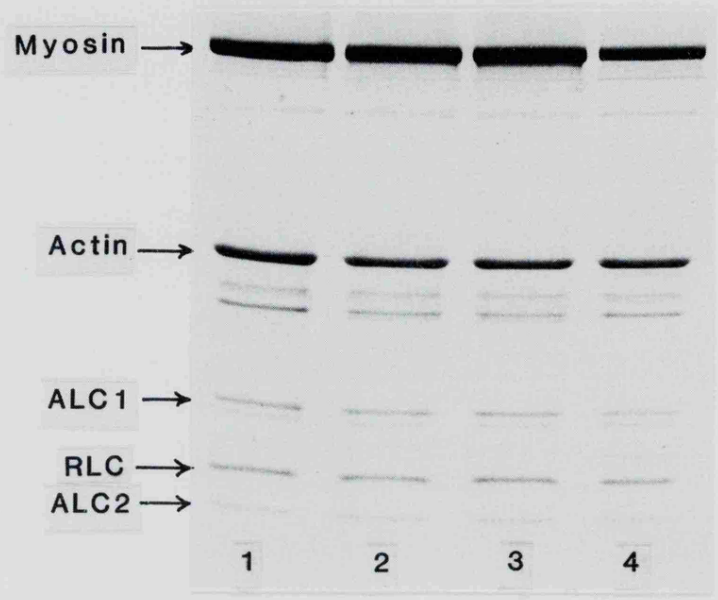
Graph 4.6

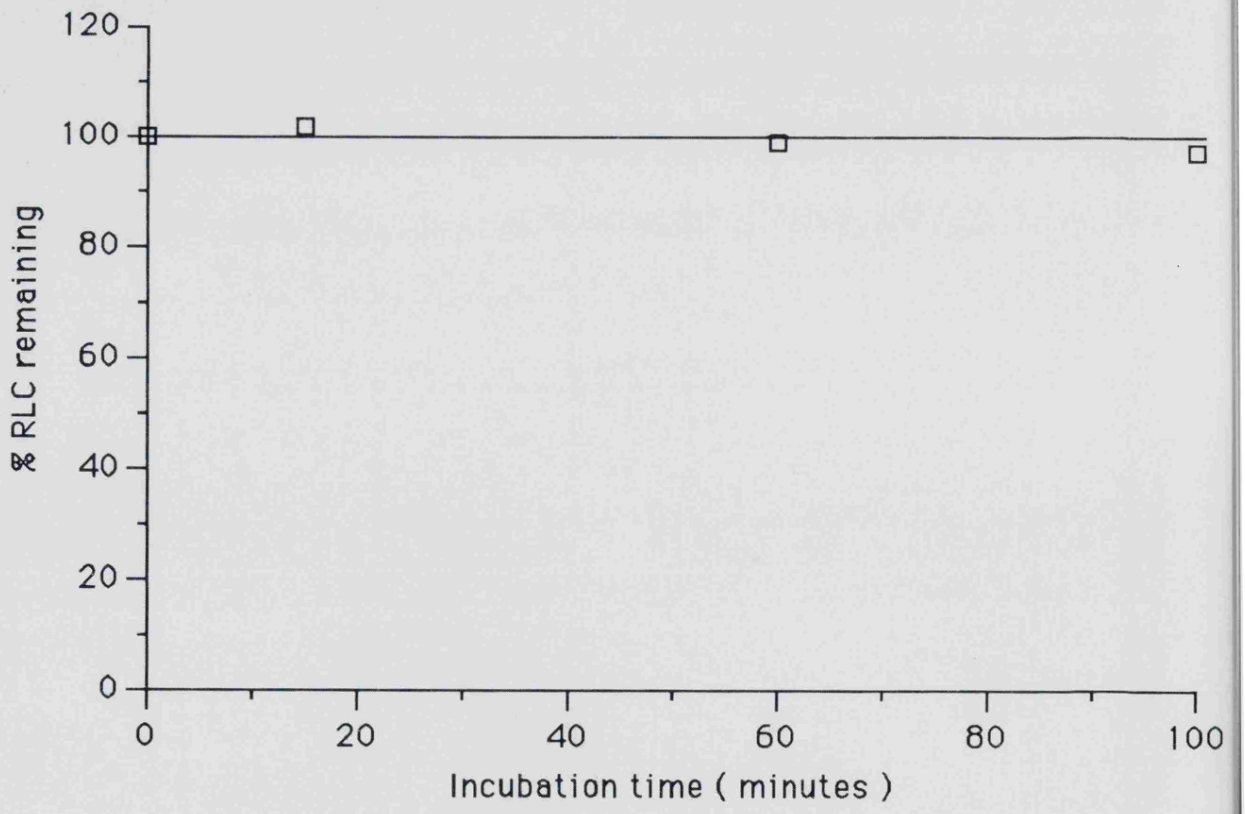
A length histogram (weight averaged) showing the distribution of thick filament lengths after reassembly from BZAs.

Average length = 1.49 μm (s.d. 0.3).

Plate 4.10

A montage of negatively stained reassembled rat thick filaments that have been frayed into sub-filaments by rinsing with low ionic strength buffer (Magnification 78,000 x).


Myosin
Actin
ALC1
RLC
ALC2
1
2
3
4


120
100
80
60
40
20
0
% RLC remaining
0
20
40
60
80
100
Incubation time (minutes)

Gel 4.1. Showing the effect of the gentle extraction technique on the RLCs of rabbit thick filaments reassembled from BZAs.

Table 4.1. Showing the results of gelscan analysis of the samples shown in Gel 4.1

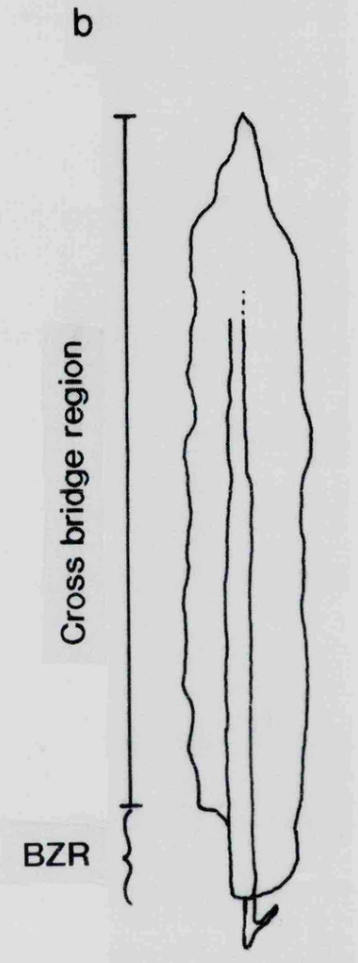
Incubation time (minutes)	% RLC remaining
0	100
15	102
60	99
100	97

Graph 4.7. A plot of the data presented in Table 4.1. showing that all RLCs a resistant to removal by the gentle exteraction technique after reassembly from BZAs.
Overall removal achieved = 0%

Table 4.2. Showing the effect of the gentle extraction technique on the RLCs of rat thick filaments reassembled from BZAs as compared to the values obtained when using filaments that had been exposed to 0.5 M KCl.

Incubation time (minutes)	% RLC remaining	
	BZA*	0.5 M KCl**
0 (control)	100	100
30	25	22
100	15	13 (approx.)

BZA* = Filaments that have been reassembled from BZAs in 200 mM.
0.5 M KCl** = Filaments that have been reassembled from 500 mM KCl.
In both cases a similar degree of RLC depletion is achieved whether the filaments have been exposed to 200 mM KCl or 500 mM KCl.


b
Cross bridge region
BZR


c

Plate 4.11

a). High resolution unidirectionally shadowed PNF. The cross bridge region is clearly visible as is the bare zone remnant (BZR) and tapering filament tip. (Magnification 155,000 x).

b). Diagramatic representation of the filament shown in 4.11a. indicating the position of the bare zone remnant, filament tip and cross bridge region.

c). A lower reolution unidirectionally shadowed PNF clearly showing a tapering filament tip at on end and a blunt one at the other thus confirming the unidirectional nature of PNFs. (Magnification 145,000 x).

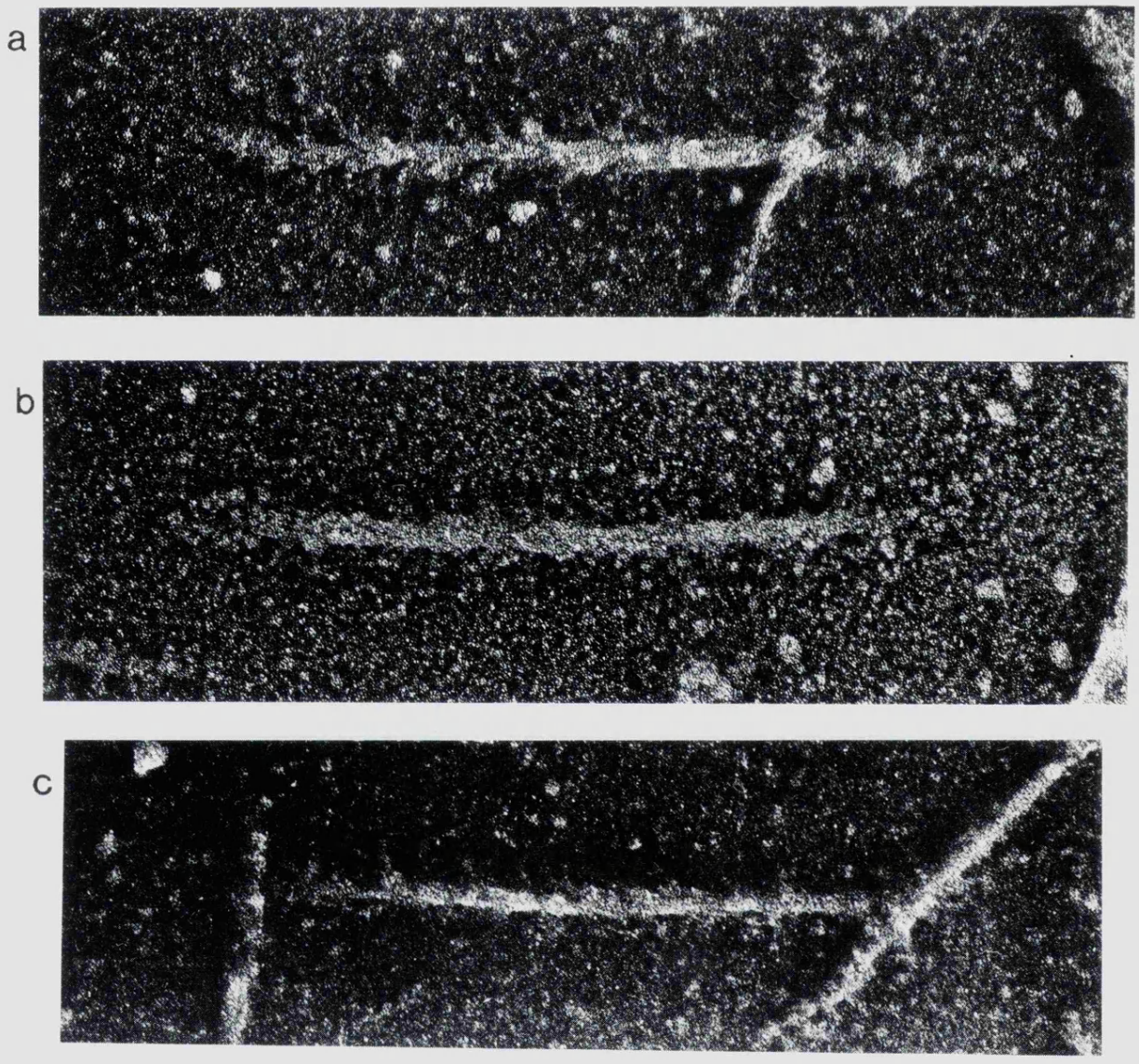

a
b
c

Plate 4.12

a). A rotary shadowed PNF showing cross bridge structures projecting along almost the entire length of the filament shaft towards the filament tip. Note that the cross bridges point in one direction only suggesting that the filament is unipolar. (Magnification 145,000 x).

b). A rotary shadowed PNF clearly displaying a tappering filament tip at one end and a blunt bare zone remnant at the other. (Magnification 145,000 x).

c). A rotary shadowed PNF showing cross bridges pointing in on direction only i.e. towards the tappering tip. (Magnification 145,000 x).

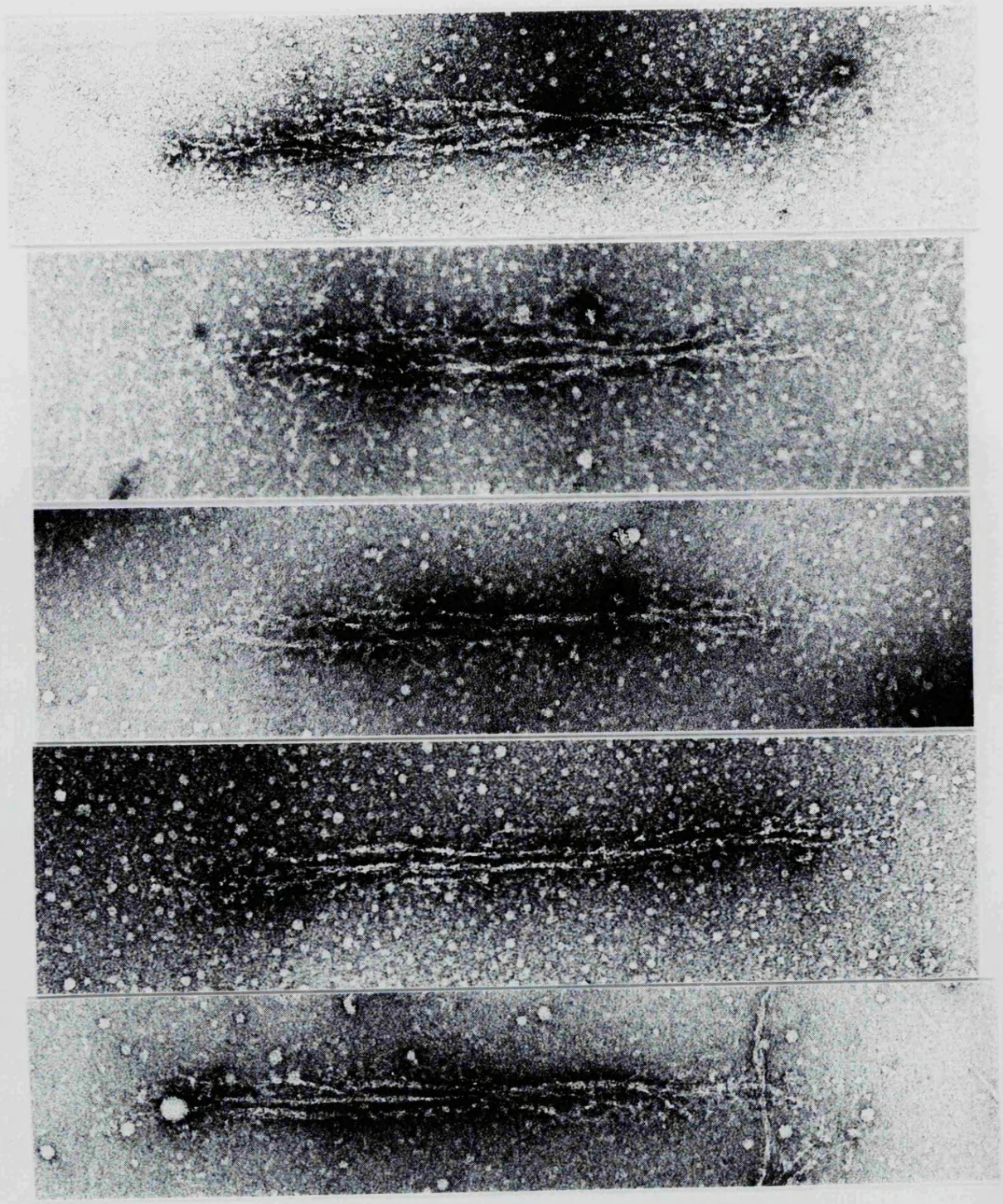


Plate 4.13

A montage of negatively stained PNFs that have been frayed into subfilaments by exposure to low ionic strength conditions (Magnification 143,000 x).

Chapter V

Introduction

The structural function of the RLCs and factors that affect properties of myosin cross bridges

(V. 1) The effect of RLC removal on myosin structure (does it result in 'sticky patch' formation ?).

The role of the RLCs in the regulation of muscular contraction has been extensively studied in all major muscle and non muscle systems. However its exact role in most is still relatively obscure (See Introduction to Chapter III) although in molluscan myosins at least it is largely responsible for the calcium sensitive properties of the ATPase (See Sections I. 6.ii and III. 3). The role of the RLCs in vertebrate skeletal muscle myosin is not as clear although there is increasing evidence to suggest that it too is intimately involved in the activity of the ATPase active site (See Section I. 6.i and III. 8).

Not only do the RLCs have a role in the ATPase activity of most myosins but it also appears that they play a part in maintaining the native myosin heavy chain composition and furthermore are possibly involved in myosin filamentogenesis.

(V. 2) Location of the RLCs.

Ever since Slayter and Lowey (1967) used platinum shadowing to show that myosin molecules consisted of two nearly spherical or globular heads (7.0 nm in diameter) attached to a long slender tail (140 nm long) many workers have tried to determine the exact location of the light chains on the myosin heads. Later and better techniques of shadowing and negative staining showed that the myosin heads were pear shaped rather than spherical, the myosin S1 heads being widest at the end distal to the point of attachment to the rod portion (Elliot and Offer.,1978. The interpretation of the S1 being pear shaped was supported by physico-chemical studies which showed that isolated S1 subfragments were not spherical (Mendelson et al.,1973); Kretzschmar et al.,1978. Estimates for the dimensions of the S1 head have come from several independant sources and are all in close agreement with the values obtained by Elliot et al (1976) and Elliot and Offer (1978) that the S1 is 19 - 19.5 nm in its longest dimension and 6 - 6.5 nm across at its widest point. Walker et al (1985) used an improved negative staining technique to obtain a length of 19 nm and a maximum width of 6 nm. Winkelmann et al (1985) used crystallographic analysis of S1 prepared by papain digestion in the presence of Mg^{2+} to show that isolated S1 was 18 nm long and 7nm wide. Flicker et al (1983) used rotary shadowing techniques, similar to those of Elliot and Offer (1978), and demonstrated that the S1 portion of intact scallop myosin was 19.5 nm long and 8 nm across at the widest point. They suggested that their estimate for the width was slightly greater than obtained by other workers because their particular preparation technique caused a

greater degree of head flattening during the drying down of the specimen.

Electron microscopy combined with antibody labelling has proved useful in localising the position of not only the RLC but also the ELCs. Flicker et al (1983) showed that the RLCs and ELCs were both present in the neck region of intact myosin molecules and that antibodies to both bound up to 100 Å from the S1/S2 hinge. This was the first visual demonstration of the close proximity of each type of light chain. Earlier evidence for this had been provided by Stafford et al (1979) who showed that the RLC protected the ELC from papain digestion. Walliman et al (1982) and Hardwick et al (1983) had also shown that both types of light chain could be cross linked with bifunctional reagents up to 8 Å long which suggested that there was considerable light chain overlap. Winkelmann and Lowey (1985) took the antibody labelling one step further when they demonstrated that an epitope within the amino terminal third of the RLC was located close to the S1/S2 hinge. However the amino terminus (residues 1 to 8) of the ELC was visualised further up the myosin head and was separated by 50 Å from the RLC epitope.

(V. 3) Effect of the RLCs on myosin heavy chain conformation.

Flicker et al (1983) also carried out extensive studies into the effect of RLC removal on the structure of scallop myosin. They found that the removal of the RLCs by incubation in EDTA caused a change in S1 head shape. The heads become more rounded as opposed to pear shaped and appear to shorten by 6 nm to 13.5 nm whilst their maximum width

remains virtually unaltered.

A similar degree of head shortening was observed when isolated S1 subfragments were examined with and without their RLCs. Flicker et al (1983) found that CaMgS1 containing its full complement of light chains possess a similar pear shape to the S1 in intact myosin molecules. The length of the CaMgS1 (17.5 nm) was similar to that of the S1 in intact native myosin (19.5 nm). The slight difference in length was probably due to the difficulty in locating the narrow end of the isolated S1 whith any certainty whereas, in an intact myosin molecule the end of the S1 is precisely located at its junction with the S2 rod. By contrast EDTA-S1 containing no RLC was more spherical or globular in appearance with a maximum length of 13.5 nm, which is identical to the longest dimension of the rounded heads of RLC depleted myosin.

These results led Flicker et al (1983) to suggest that the RLCs were located in the neck region of the myosin and that they were involved in maintaining head shape. It is still uncertain whether the loss in the ability to visualise the S1 neck after RLC removal is a consequence of a true conformation change or just a result of the loss of mass from that region. However, after RLC depletion from intact myosin, the heads not only lose their pear shape but also sometimes move much closer to the S1/S2 junction on the LMM causing the heads to abut, a finding that was also reported by Vibert and Craig (1982). This was interpreted as being a result of a local collapse of the myosin heavy chain in the neck region and led them to suggest that the RLC was involved in the stabilisation of the heavy chain ELC complex. This was supported by the fact that EDTAS1 did not contain a narrow heavy chain extension

suggesting that such a structure had collapsed down onto the main body of the S1. The molecular weight of the myosin heavy chain in both CaMgS1 and EDTAS1 was the same lending further support to the notion that the EDTAS1 heavy chain extension had collapsed.

The apparent shortening of the heads after RLC removal observed by Flicker et al (1983) was called into question by Margossian and Slayter (1987). They used myopathic hamster protease to digest the RLCs of cardiac myosin selectively and although the heads did appear to become more spherical they suggested that this was a direct result of a loss of the mass of the RLC from the neck region and not a loss of heavy chain structure. They clearly visualised a rod like connection (10 nm long) between the globular head, which itself was 8.5 nm in diameter, and the myosin tail. The combined length of the head and rod region was the same as the total length (18.9 nm) of myosin heads still containing their RLCs. At no stage did they observe a collapse of the rod like connection after RLC depletion suggesting that it was a stable structure even in the absence of the RLC. It is important to remember that we cannot rule out the possibility that there are slight differences in heavy chain structure between various types of myosin. Therefore it is possible that the removal of the RLCs from scallop myosin does cause a reduction in the local heavy chain stability (Flicker et al (1983)) whereas RLC loss from cardiac myosin does not (Margossian and Slayter.,1987.

(V. 4) Effect of RLC removal on the actin binding properties of myosin.

RLC removal has also been shown to affect the binding of proteolytic subfragments of myosin to actin filaments. This was first demonstrated by Moore et al (1970) who showed that rabbit skeletal S1 minus its RLC still bound to actin but instead of producing the usual barbed shape arrowheads the S1 decorated the filaments in a way that produced blunted arrowheads.

Craig et al (1980) used scallop HMM and S1 to decorate actin filaments and demonstrated that in the absence of RLCs the arrowheads in both cases were blunted. Vibert and Craig (1982) were the first to show that RLC deficient HMM from rabbit skeletal myosin also produced blunted arrowheads when bound to actin. Furthermore Margossian and Slayter (1987) have since produced identical results but this time using cardiac myosin.

All of these results suggest that whether the myosin is from a calcium sensitive species or not the loss of the RLCs apparently affects the mode of attachment of the S1 heads to the actin. However it is not clear whether the attachment to actin is altered by the presence or absence of the extra mass of the RLC ie. is it a steric effect, or is it a result of an altered heavy chain conformation caused by the removal of the RLC ? Margossian and Slayter (1987) have shown that RLC reassociation results in the formation of sharply barbed arrowheads similar to those of the control. Although this does not tell us why RLC loss causes such changes in actin binding, it does show that if they are a result of a change in heavy chain conformation the change is fully reversible. Whatever the explanation might be, such changes in actin

binding may in future be incorporated into models describing the role of the RLCs in vertebrate muscle myosins.

(V. 5) Does RLC removal expose sticky patches ?

It has been suggested that the removal of the RLCs from myosin results in the formation of a 'sticky patch' (See Section III. 6). It is clear that the removal of an RLC must expose its binding site on the heavy chain and that the exposed residues, in their native conformation, would have a higher affinity for protein than solvent. The affinity of the exposed binding site for protein could conceivably result in head aggregation. However such a process could only occur if the exposed site maintained its conformation and did not collapse after RLC depletion which the work of Flicker et al (1983) suggested was real a possibility. If sticky patches were formed, Bagshaw (1980) suggested that they must contain a large proportion of charged residues (to complement the large proportion in the RLC) and that the known temperature dependance of RLC dissociation (Kendrick Jones et al.,1975; Chantler and Szent-Gyorgyi.,1980 indicated that hydrophobic interactions were probably of minor importance.

However, Bennett et al (1984) suggested that at least part of the RLC binding site involved hydrophobic residues. They used 8-anilino-1-napthalenesulphonate (ANS), a fluorescent compound that binds to hydrophobic residues, as a fluorimetric probe for the RLC binding site on scallop myosin. They showed that after RLC removal the amount of bound ANS increased as indicated by an increase in fluorescence, whilst RLC reassociation caused a corresponding drop in

in ANS fluorescence. The increased fluorescence after RLC removal was explained by the exposure of hydrophobic residues within the vacant RLC binding site/sticky patch. The increased binding of ANS was ambiguous with respect to the sticky patch hypothesis (Bagshaw.,1980, which results in head clumping as such intramolecular clumping would presumably prevent the hydrophobic residues from reacting with ANS. However if the binding site of the RLC was multivalent then head clumping would occur whilst still leaving some hydrophobic residues fully exposed and available for ANS binding.

Chantler and Szent-Gyorgyi (1980) suggested that the reassociation of RLCs onto 30 °C EDTA extracted scallop myosin was a negatively cooperative process. Negative cooperativity implies that the binding of one RLC to a single myosin molecule within a population prevents another RLC from binding until all other myosin molecules possess one RLC. They put such a negatively cooperative process down to an interference of the exposed RLC binding site with the other intact head. Such intramolecular interactions have never been observed directly in the electron microscope, although Wells and Bagshaw (1983) claimed to have observed such interactions using saturation EPR spectroscopy. They demonstrated that the rotational correlation time (t) of the S1 heads within intact native scallop myosin was almost identical to that of isolated S1. This indicated that the heads in intact myosin move independently of each other, which had previously been demonstrated by Thomas et al (1975) using skeletal muscle myosin. However when one RLC was removed there was a 2.5 to 4.0 fold increase in t which they suggest is an indication of the association of one head with the

other. Bagshaw (1980) had previously proposed that such head clumping did occur as a result of sticky patch formation on the RLC denuded head. However he suggested that such interaction did not physically trap the remaining RLC but merely perturbed the dissociation constant of the remaining RLC in such a way as to render it more resistant to extraction.

Intermolecular head clumping of RLC deficient skeletal muscle myosin was first visualised in the electron microscope by Pastra - Landis and Lowey (1984, 1986). Using rotary shadowing they demonstrated that after the removal of all of the RLCs (using immuno affinity chromatography) the myosin molecules tended to aggregate. The oligomeric structures consisted mainly of dimers or trimers and were all attached at or near the S1/S2 neck region. We know from the work of Flicker et al (1983), Walliman et al (1982) and Winkelmann and Lowey (1985) that the RLC binding site is near the S1/S2 hinge and therefore aggregation of RLC deficient myosin molecules in this region is indicative of the presence of a sticky patch. Pastra - Landis and Lowey (1985) also found that the aggregation was abolished by the addition of purified RLCs to stoichiometric levels. This supported the assumption that RLC loss does not cause a large change in the myosin heavy chain conformation as suggested by Flicker et al (1983) because purified RLCs can still recognise and associate to their vacant binding site. Interestingly no evidence of intramolecular head aggregation was observed; however it was pointed out that the conditions used for preparation favoured the dissociated state and procedures such as chemical cross linking may be necessary for its visualisation.

The formation of sticky patches by RLC removal was brought into

question by Margossian and Slayter (1987). They used rotary shadowing of skeletal and cardiac myosin after RLC removal by myopathic hampster protease and found no evidence for inter or intramolecular aggregation. They did observe myosin clusters in the RLC deficient preparations but their frequency was identical to that of the control. Detailed measurement of head/head distances both before and after RLC removal revealed no change, suggesting that the head shortening described by Flicker et al (1983) and sticky patch formation described by Pastra-Landis and Lowey (1984, 1985) was not occuring. The lack of aggregation in their RLC deficient `samples was emphasised by the ease with which the myosin entered non denaturing gels and the absence of a fast moving boundary during sedimentation velocity runs (Margossian.,1985.

(V. 6) Role of the RLCs in thick filament assembly.

The RLCs are not only believed to be involved in processes related to the myosin heads i.e. regulation of ATPase activity and heavy chain conformation but they are also suspected to be intimately involved in myosin filament assembly. Much of this evidence, although not all, has come either from smooth muscle or non muscle myosin containing systems.

Scholey et al (1980) were the first to demonstrate that regulation of non muscle myosin assembly in thymus and platelets was controlled by phosphorylation of the RLCs. RLC phosphorylation is carried out by the calcium activation of a specific calmodulin dependant kinase (Dabrowska et al.,1978 and results in filament assembly at

physiological ionic strengths and in the presence of MgATP. Similar findings were obtained with smooth muscle myosin by Suzuki et al (1978) and Trybus (1982) who showed that phosphorylation of the RLC caused myosin filament assembly even in the presence of 3 mM ATP.

It is generally accepted that the activation of the ATPase activity in non muscle and smooth muscle by the interaction of the myosin with actin was regulated by phosphorylation of the RLCs (Scholey et al.,1980; Kendrick-Jones et al.,1987. The observation by Suzuki et al (1978), Scholey et al (1980) and Trybus et al (1982) that filament assembly was also regulated by phosphorylation led to the suggestion that force generation may be controlled by filament assembly and disassembly.

However the process of filament assembly and disassembly in smooth and non muscle myosin is not a simple monomer polymer one. The situation is complicated by the presence of two monomer conformations one of which is an extended structure (6S) the other being folded (10S) (Trybus et al.,1982; Craig et al.,1983). The 10S molecules, which in the terminology of Kendrick Jones et al (1987) are assembly incompetent, only exist when the RLCs are dephosphorylated, unfolding to give the 6S (assembly competent) monomer occurs during phosphorylation (Craig et al.,1983). The possibility that the 10S monomer can directly polymerise to form filaments without the need to go through the 6S conformation, although unlikely, cannot be completely eliminated. Citi et al (1987) have recently demonstrated that the presence of a small number of phosphorylated skeletal muscle myosin molecules can stabilise non phosphorylated non muscle myosin filaments. They suggested that this was achieved by some form of

cooperativity within the filaments although an alteration in the 10S - 6S equilibrium could not be ruled out. This may have an important function in vivo where the cooperative effects of phosphorylation may help maintain stable myosin filaments even at low levels of RLC phosphorylation.

By contrast the role that the RLCs play in vertebrate skeletal muscle assembly is poorly understood and little research has been carried out in this area.

Pinset-Harstrom and Whalen (1979) were the first to suggest that the RLC was involved in filament formation. They showed that during the ageing of a myosin preparation selective proteolysis of the RLC occured, the proteolysis being remarkably specific having little or no effect on other components. The selective protelysis of the RLC was shown to reduce the myosins ability to form long thin filaments. The filaments formed after such a modification of the RLCs were always much shorter and fatter than those of the control. They also found that removal of the RLC with DTNB had the same effect although heavy chain modifications may also have occured. These results suggested that the RLC had a role in myosin filament assembly although it was difficult to predict how changes in the S1/S2 hinge region could affect the packing of the myosin tails. However the work of Cardinaud (1987) (See Section III. 8) has clearly demonstrated a strong interaction between the RLCs N-terminus and the shaft of the thick filament. Such interactions could quite conceivably be involved in producing the correct packing of LMM tails within the filament shaft.

Margossian et al (1983) also looked at the effect that RLC removal had on the production of myosin minifilaments (Reisler et al.,1980).

Although they found that minifilaments could be produced from both RLC deficient and RLC reconstituted myosin there were subtle differences in their structures and properties.

The RLC deficient filaments were much more viscous than those of the control and in the analytical ultracentrifuge a large amount of aggregation was observed with most material sedimenting at 218s. A peak sedimenting at 25.6s was present and later identified as being the RLC deficient minifilaments. Reassociation of RLCs caused a reduction in viscosity, a disappearance of the 218s boundary and a drop in minifilament s-value to 23.5s, which approaches that of the control (20.6s). They suggested that the difference in s-value was not significant and probably due to concentration differences, although it is possible that it is due to the presence of hydrophobic patches on the RLC denuded heads sticking the cross bridges down.

In the electron microscope the minifilaments of Margossian et al (1983) did strongly resemble the minifilaments of Reisler et al (1980). Both the control and RLC recombined filaments had similar dimensions, being approximately 3200 Å long and possessing a well defined bare zone 1800 Å long. By contrast RLC deficient minifilaments were slightly longer (3500 Å) and possessed a bare zone 2000 Å in length and 9.3 nm across. All of these values are significantly greater than those of the control, the width of the bare zone being 40% greater (6.7nm in control). All of these factors led Margossian et al (1983) to suggest that the modification of the myosin by RLC removal does influence the assembly of minifilaments, and presumably would influence full length filament formation. It is also possible that the minifilaments are less stable after RLC depletion which may result in partial dissociation

during drying down or negative staining. Such effects could account for the observed increase in filament dimensions after RLC removal.

(V. 7) What effect does divalent cation have on the cross bridges ?

Does calcium in μM concentrations affect the cross bridge disposition?

Haselgrove (1970, 1975) used X-ray diffraction techniques to look at the thick filament structure in muscle fibres during contraction and in rigor and demonstrated the possible presence of a thick filament linked regulatory system. He showed that muscles contracting isometrically at sarcomere lengths between 2 and 3 μm have identical diffraction patterns. The most striking feature of each diffraction pattern was the large uniform decrease in the intensity of the myosin layerlines. These changes were identical for all sarcomere lengths and indicated a movement of the cross bridges away from their resting positions on the filament shaft. At sarcomere lengths of 3.0 μm or more fewer than half the cross bridges can interact with actin filaments whereas at 2.0 μm all of the cross bridges can interact with the actin. Because the diffraction patterns produced during contraction are similar, irrespective of sarcomere length, the same proportion of cross bridges probably move in all cases suggesting that the initial cross bridge movement was independent of the degree of actin interaction.
It was however possible that there was some form of cooperativity between the cross bridges and that those able to interact with actin were able to influence those in the region of non overlap. In this way a

slight degree of overlap would allow the effect of a small amount of actin interaction to be propagated to the cross bridges that were not in contact with actin. However H. E. Huxley (1972) apparently demonstrated that under total non overlap conditions (Sarcomere lengths above 3.6 μm) when no cross bridges were able to interact with actin filaments the movement of the cross bridges was not dependent on the presence or interaction with actin. He was however cautious to point out that even at these long sarcomere lengths a small amount of actin interaction could possibly still be present and influence cross bridge movement.

The results obtained with the diffraction patterns of filaments in rigor also supported the idea that cross bridge movement was independent of actin. In this instance H. E. Huxley (1972) showed that none of the thick filaments in rigor sarcomeres at rest length or in non-overlap (>3.7μm) contained cross bridges in their rest positions i.e. they had all moved away from the thick filament shaft. The cross bridges in the non-overlapped sarcomeres had therefore moved into a rigor conformation without the need for actin interaction. The absence of actin interaction at non overlap lengths is emphasised by the fact that there is no enhancement of the actin layer lines, presumably because the cross bridges cannot label the thin filaments. By contrast, at rest length sufficient cross bridges are attached to the actin filament to give rise to a series of layer lines indexing on the actin structure.

Haselgrove (1970, 1975) suggested that the movement of the cross bridges was associated with a change in the backbone packing of the thick filaments. This was based on the observation that during isometric contraction at lengths between rest and non overlap the

decrease in myosin layer line intensity was accompanied by a 1% increase in the 143 Å and 72 Å reflections. These reflections are meridionals and are associated with the axial spacing in the filament backbone. The increase in axial spacing was not due to an elastic strain induced during isometric contraction because it is also seen in the rigor configuration when no tension is developed. The fact that the lengthening is also seen in the non overlap filaments eliminates the possibility that it is caused by the interaction with actin.

Haselgrove (1975) concluded that the extensive movement of the cross bridges away from the filament shaft, which occurs during contraction or in rigor, is accompanied by a specific structural change in the backbone structure as indicated by the 1% increase in the axial repeat of the 143 Å and 72 Å reflections. He speculated that the changes in the filament backbone may have a functional role and that such changes may be involved in regulating the cross bridge disposition. X-ray evidence supports the idea that the initial movement of the cross bridges is independent of the actin filaments; however the cause of the initial movement itself is not obvious. He stated that the change in the axial repeat of the backbone allows or possibly causes the cross bridges to move away from the filament shaft. In other words before myosin can interact with actin the thick filaments must adopt an activated state which releases the cross bridges from their resting position on the filament shaft.

Recently the results of Haselgrove (1975) using actively contracting muscle stretched to non overlap have been disputed. Yagi and Matsubara (1980) showed that specimens stretched to non overlap that did not produce tension displayed no decrease in the intensity of the 42.9 nm

(Myosin) layer line. This suggests that in the absence of actin interaction no cross bridge movement is observed after calcium addition. They did however note that some muscles when stretched to apparently non overlap lengths still developed tension. The development of tension was they suggested a result of disorder induced in the muscle during the stretching process and that such disorder could possibly explain the results obtained by Haselgrove (1975) at non overlap lengths. Similar findings were obtained by H. E. Huxley et al (1980) who showed that in most of their non overlap experiments no decrease in the intensity of the first layer line was observed. Those that did show a decrease in intensity were also explained in terms of actin interaction caused by the stretch induced disorder of the sarcomeres. Furthermore Mendelson and Cheung (1976) demonstrated the absence of a direct effect of calcium on cross bridge movement away from the thick filament shaft of a synthetic filament preparation. They used fluorescence depolarisation to show that the rotational Brownian motion of the S1 heads was hindered when the myosin was contained within the filament structure and that the inhibition was not relieved by the addition of calcium

These results provide us with a dilemma that has yet to be resolved which is that the recent results from activated muscle at non overlap lengths are at variance with those from muscles in rigor. The results from the rigor experiments at non overlap are unambiguous in their interpretation. They clearly show that actin interaction is not required for the cross bridges to undergo a disordering and form a rigor conformation similar to that obtained during full overlap. The results during activation however seem to suggest that no cross bridge

movement is observed without the interaction with actin. The reason for these differences is not yet clear and the interpretation of the results is somewhat uncertain and is still subject to re-examination.

(V. 8) Why do dually regulated contractile systems exist ?

Lehman et at (1972) have demonstrated that several types of invertebrate muscles contain dual actin and myosin linked regulatory systems, so the presence of such a system in vertebrate skeletal muscle would not be unique. However the question that arises is why a dual regulatory system is necessary at all? It could be that the actin linked regulation on its own is not efficient enough. If the contractile mechanism is based solely on thin filament regulation then why do the cross bridges move away from them and take up specific positions on the thick filament shaft during relaxation?

Lehman et al (1972) have shown that in vitro inhibition of the ATPase of the myosin from solely actin linked systems is less successful than from dually regulated. Perhaps a system that physically holds the cross bridges near or on the thick filament shaft during relaxation and thus prevents interaction with actin is highly desirable. Craig et al (1987) have recently demonstrated that phosphorylation of the RLCs in tarantula thick filaments results in a high degree of cross bridge order on the filament backbone. They suggested that this was possibly part of an extra regulatory system involved in keeping the myosin away from the actin filaments during relaxation.

The presence or absence of a myosin linked regulatory system in

vertebrate skeletal myosin has been the subject of great debate for many years (Kendrick-Jones.,1974; Morimoto and Harrington.,1974 and is something that we address further in this chapter.

(V. 9) Does vertebrate skeletal muscle possess calcium specific binding sites ?

The presence of a myosin linked regulatory system in molluscan myosins is well established (Szent-Gyorgyi et al.,1973; Kendrick-Jones et al.,1976; See Section I. 6.ii) and has been extensively studied. The main reason for such intense activity using molluscan myosin has been the ability to dissociate the RLCs reversibly using EDTA (Szent-Gyorgyi et al 1973). Bagshaw and Kendrick-Jones (1979) used EPR spectroscopy to show that all molluscan myosin molecules contained a pair of high affinity non specific divalent cation binding sites and that in addition they possessed a pair of calcium specific sites. They also demonstrated that the non specific sites reside entirely on the RLC whilst the specific calcium binding sites involve ligands from both the RLC and myosin heavy chain. They showed that purified vertebrate myosin contains only one pair of divalent metal ion binding sites that are non specific and located entirely on the RLC. They do however point out that native skeletal myosin may indeed possess calcium specific binding sites which are lost during the initial stages of extraction and purification.

Morimoto and Harrington (1974) had earlier developed a technique for the extraction and purification of vertebrate skeletal myosin filaments

in their native state and apparently demonstrated the presence of a high affinity calcium specific binding site.

By looking at the effect that calcium had on the sedimentation coefficient of their purified filaments they showed that its presence in μM concentrations caused an increase in s-value. They obtained a titration curve for the change in s-value and showed that the maximum change was obtained in the presence of 10 μM free calcium and that the binding site was of high affinity and had a Kd = 3 μM. In the absence of calcium, elevating the free magnesium concentration had no detectable effect on s-value, suggesting the effects observed with calcium resulted from specific binding. Raising the free magnesium concentration to 5 mM did however cause an increase in the calcium concentration required to fully saturate the sites indicating that there was some competition between Ca^{2+} and Mg^{2+}.

(V. 10) Location of the specific sites.

Morimoto and Harrington (1974) located the specific calcium binding site on the RLC and suggested that interaction with ligands from the heavy chain had no effect on the affinity. However Bagshaw and Reed (1977) used EPR spectroscopy to show that the so called calcium specific site described by Morimoto and Harrington (1974) was analagous to the non specific Mg^{2+} binding site present on molluscan RLCs (See Section III. 4). They suggested that in vivo the sites would be saturated with Mg^{2+} and that the binding of calcium to this site would be too slow for it to act as an effective switch during the early

stages of contraction. The slow kinetics of calcium binding and release from the RLC mean that it is incapable of competing with the switch on the troponin system. They did not entirely rule out the presence of a myosin linked regulatory system but suggested that if it was present it was lost during the myosin extraction process. This point was later supported by Bagshaw and Kendrick-Jones (1979) who also showed that rabbit myosin does bind some calcium in the presence of Mg^{2+}. However the binding occurs at the site on the RLC which is analagous to the Mg^{2+} site in molluscan myosins. Once again they did not categorically state that there were no calcium specific sites presnt and suggested that they could have been lost during preparation.

The changes in s-value caused by calcium binding reported by Morimoto and Harrington (1974) were interpreted by them as being the result of some sort of conformational change within the thick filaments. The increase in s-value was consistent with a conformation change that resulted in a reduction in particle assymmetry. However they did point out that it was difficult to interpret these results in terms of an alteration in the organisation of the cross bridges along the filament length. The increase in s-value is obviously consistent with a decrease in the frictional coefficient. However what alterations in cross bridge disposition were necessary to cause such changes was not known, indeed they felt that the effect that cross bridge movement had on the frictional coefficient was too complicated in terms of both magnitude and direction to predict.

An interesting feature of their work was that the high affinity calcium binding site was present in SMFs, suggesting that the site was not sensitive to high salt exposure. Furthermore SMFs that contained

C-protein gave identical results to those that did not, thus eliminating the possibility that C-protein was involved in calcium binding.

(V. 11.) The influence of the low affinity binding sites on the hydrodynamic properties of myosin filaments.

Persechini and Rowe (1984) used sedimentation techniques to look at the effect that the binding of divalent cation to the low affinity sites had on the s-value of a particularly homogeneous preparation of SMFs. They found that 3 mM free magnesium caused a maximum increase of 15% in s-value and a titration curve of the sites revealed a Kd = 1.5 mM. The increase in s-value caused by binding divalent cation was interpreted in terms of a change in cross bridge extension resulting in a change in frictional coefficient and therefore s-value. However the exact change in frictional coefficient resulting from a change in cross bridge disposition is difficult to predict (Morimoto and Harrington.,1974; Emes and Rowe.,1978b; Persechini and Rowe.,1984; Rowe and Maw.,1983. Emes and Rowe (1978) had demonstrated that the frictional coefficient of myosin filaments was anomalously (~70%) high compared to the value predicted when using prolate elipsoids as the classical hydrodynamic model. They suggested that the frictional anomaly probably arose from the presence of the projecting cross bridges and therefore that changes in the properties of the cross bridges would presumably affect this parameter and consequently alter the s-value.

(V. 12) Why do myosin filaments possess anomalous hydrodynamic properties ?

Several explanations for the presence of such a frictional anomaly have been put forward all of which are entirely hypothetical and unsupported by experimental evidence. Nevertheless it is only by exploring such possibilities that an eventual answer will be found.

Persechini and Rowe (1984) suggested that the frictional anomally was at least partially due to distance that the cross bridges projected out from the thick filament shaft. The further out they projected the greater the frictional drag and therefore the smaller the s-value. This idea could possibly be countered by an alternative argument which suggests that the extended cross bridges would entrap an envelope of water and therefore become less asymmetrical and as such display properties approaching those predicted by modeling using a prolate elipsoid. Such a particle would presumably sediment at an s-value nearer to that predicted by classical hydrodynamic theory.

Emes and Rowe (1978b) had earlier suggested that the anomalous frictional coefficient of myosin filaments resulted from properties induced by the helical array of cross bridges on the filament surface. The helical array could they suggested cause a screw effect, which would increase the drag of the filaments in a similar way to that of an idling propellor on a boat.

Rowe and Maw (1983) put forward an alternative argument which proposed that shear during sedimentation induced cyclical changes in the cross bridge lattice resulting in extra energy dissipation and a decrease in s-value. However it is still unclear as to the effect, in

terms of both magnitude and direction, that such cyclical oscillations would have on the frictional coefficient.

Which if any of these suggestions is correct is still the subject of much debate. However the fact remains that by monitoring the anomalous properties of thick filaments we have a useful probe for observing changes in crossbridge properties.

(V. 13) Effect of divalent cation on cross bridge disposition.

Persechini and Rowe (1984) used the assumption that the anomalous properties were due to cross bridge extension and suggested that the binding of divalent cation to the low affinity sites caused the cross bridges to move closer to the filament shaft resulting in a 15% decrease in frictional coefficient and therefore a 15% increase in s-value. It can be predicted from this result that that the binding of divalent cation should cause a reduction in the rate of proteolytic attack at the HMM/LMM hinge. Borejdo and Werber (1982) had earlier reported such an effect and had determined a Kd for the site of 1.4 mM, which is in close agreement with the value obtained by Persechini and Rowe (1984).

The exact role of the non-specific low affinity binding sites has however remained relatively obscure. Persechini and Rowe (1984) suggested that because the free magnesium concentration *in vivo* is probably held fairly constant it does not itself modulate cross bridge disposition. Although it is generally accepted that the free Mg^{2+} concentration in vivo does not fluctuate there is still some dispute over its equilibrium concentration although most estimates fall within

the 1 - 3 mM region (Hess et al.,1982; Blatter et al.,1987). Persechini and Rowe (1984) concluded that physiological levels of free Mg^{2+} probably maintained the cross bridge lattice in a critical configuration which was modulated by other as yet unknown factors (See Discusion Section V. 44).

The aim of this chapter is to look at the effect that RLC removal has on the properties of thick filament structure. The literature shows that the RLCs are located in the neck region of the S1 head but it is not clear as to whether removal of the RLC results in the formation of so called sticky patches. Much of the recent evidence now suggests that although RLC removal must result in the exposure of the binding site, the site itself is not sticky and does not cause aggregation as judged by electron microscopy and hydrodynamic techniques (Magossian and Slayter.,1987. Probes for the exposed site have however revealed the presence of hydrophobic residues (Bennet et al.,1984. The presence of these residues does not in itself mean that the exposed site will be sticky and interact with other proteins because RLC binding could result mainly from specific charge interactions. Here we attempt to look at the effect RLC removal has on the cross bridges of intact native myosin filaments as judged by electron microscopy and hydrodynamics. One can clearly imagine that if the removal of an RLC exposes some sort of sticky patch then the cross bridges may interact with the filament shaft or other cross bridges. Such interactions may be visible in the electron microscope or may alter the hydrodynamic properties of the filaments. We believe that the evidence presented in chapter III demonstrates that the removal of 50% of the RLCs from native myosin causes each myosin molecule to lose one RLC. If this is in fact true

then all of the cross bridges in a thick filament would be affected by RLC loss and effects should easily be picked up.

Some sort of sticky patch on the S1 heads of the cross bridges would have plenty of oportunity to interact with the filament shaft because several workers (McClachlan and Karn.,1982 and 1983; Morris and Chen Lu.,1987) have shown that the myosin tails which make up the shaft possess alternating bands of charged residues. If sticky patches did indeed result in an interaction between the cross bridge and the shaft of the filament then the hydrodynamic properties of the filaments would be considerably affected. As mentioned earlier the frictional anomaly is believed to be due to the presence of the cross bridges (Emes and Rowe.,1978b; Persechini and Rowe.,1984) and results in an s-value that is 70% smaller than predicted using classical hydrodynamic theory. We now suggest that if the cross bridges interact, with each other or the filament shaft after RLC depletion we should see a reduction in the frictional anomaly.

We demonstrate that after RLC depletion from PNFs there is approximately a 50% increase in s- value as judged by sedimentation velocity experiments, which is also accompanied by the appropriate change in diffusion coefficient as judged by quasi elastic light scattering. Electron microscopy of the filaments shows that the cross bridges <u>appear</u> to be stuck to the filament shaft and that filament disassembly in 200 mM KCl is inhibited by the prior removal of the RLCs.

Results

Electron microscopy.

(V. 14) The effect of 50% RLC depletion on the myosin cross bridges of rabbit thick filaments.

In our hands myosin cross bridges are easiest to visualise in the electron microscope when a positive staining technique (See Section II.18) is used as opposed to the negative staining protocol described earlier (See Section II. 17). Plate 5.1 shows a typical positively stained full length myosin filament with numerous cross bridges projecting from the filament shaft. The filament is obviously bipolar as judged by its tapering ends although a well defined central bare zone region is not present as this is always difficult to see using this technique.

Plate 5.2 shows positively stained thick filaments after 50% RLC depletion. After 50% RLC depletion the most striking feature of the filaments structure is that both the full and approximately half length filaments appear to be smooth rods that are completely devoid of cross bridge structures. The full length RLC depleted filaments are similar in length (1.6 μm) to those of the control (1.6 μm), although they do appear to be of a more uniform width (0.10 - 0.13 nm) along their entire length as compared to the control filaments whose diameter varies considerably from 0.10 to 0.19 nm.

(V. 15) Can 50% RLC depleted rabbit and rat thick filaments from a RFP be frayed using low ionic strength conditions ?

When subjected to low ionic strength fraying medium (Maw and Rowe.,1980; Trinick.,1982;) RLC depleted rabbit and rat thick filaments were seen to fray into subfilaments (Plates 5.3, and 5.4 respectively). The fraying pattern in both cases was very similar to that of their respective control filaments (Rabbit IV. 19, Rat IV. 25). The filaments never frayed into more than three subfilaments whilst fraying into two was common. When three subfilaments were present the average diameter of each was 7.0 nm which is not significantly different from the diameter of those observed when native filaments are frayed. Fraying never proceeded into the central bare zone region and the sub-filaments were often attached at their tips but were rarely seen to cross. After fraying the smallest intact bare zone observed in both rabbit and rat filaments was approximately 0.26 um long which is similar to the value obtained when native filaments are frayed (See Section IV. 19 and IV. 25).

(V. 16) Can RLC depleted rat thick filaments from a RFP form BZAs ?

Rat filaments have been shown to form structures ressembling rabbit BZAs when dialysed for 18-24 hours against 200 mM KCl (See Section IV. 26). The rat BZAs formed after dialysing for this length of time were 0.29 μm long which is slightly shorter than the value for rabbit BZAs (0.31 μm, See Section IV. 20) produced under identical conditions. Such a difference is possibly indicative of a slightly different packing

arrangement within the two types of myosin filament. A possible difference in packing between rat and rabbit filaments was further emphasised by the observation that a uniform population of rabbit BZAs (0.31 μm long) could be formed by dialysis against 200 mM KCl for only 2 hours, whilst rat filaments failed to completely depolymerise and produced a wide distribution of lengths (average = 0.34 μm). Only after >18 hours dialysis was a uniform population of rat BZAs obtained. Dialysis of rat and rabbit filaments for longer than 18 hours failed to produce further decreases in length suggesting that the minimum stable structure had been attained. The difference in the rate at which rat and rabbit BZAs are formed may reflect a difference in the rate at which the myosin monomers dissociate possibly because of differences in packing.

After 50% RLC depletion of rat filaments the BZAs produced by 18 -24 hours dialysis were significantly longer than those of the control with an average length of 0.36 um. Filament like structures having a length of up to 0.8 um were also present in the preparation (Plate 5.5) suggesting complete depolymerisation had not occured although it was equally possible that RLC depleted BZAs had stuck together end to end.

<u>The effect of RLC depletion on the hydrodynamic properties of PNFs.</u>

<u>(V. 17) Sedimentation velocity analysis (using schleiren optics) of PNFs before and after 50% RLC depletion.</u>

The cells containing the control PNFs and the 50% RLC depleted PNFs both showed sharp single schleiren peaks. A monomer boundary was not

detected and no evidence of filament aggregation was seen in any of the runs. Detailed analysis of the traces revealed that the control filaments had an s-value of 125s ± 1.3 whilst the RLC depleted filaments sedimented much more rapidly at 186s ± 2.13. This represents a 48% increase in the s-value of PNFs after 50% RLC depletion. The s-values were determined at a finite concentration of 0.29 mg/ml and therefore would be slightly larger when corrected to zero concentration.

(V. 18) Can Quasi elastic light scattering (QLS) pick up changes in cross bridge conformation.

(V. 19) Control experiments.

We felt that although the sedimentation velocity analysis revealed a large change in the s-value of PNFs after RLC depletion it would be useful to observe the hydrodynamic effects by some other means. Quasi elastic light scattering (QLS) is a highly sensitive technique for determining the D_{zt} of individual molecules and biomolecular assemblies. We know from the Svedberg equation that M S/D (See Section II. 15) and therefore any changes in s-value that occur as a result of RLC depletion should be accompanied by an equivalent change in D_{zt} provided the mass remains constant.

We know from the work of Persechini and Rowe ((1984) and See Section V. 20) that the addition of 3mM divalent cation to SMFs results in a 15% increase in s-value. Therefore a corresponding increase in D_{zt} should also occur which can be picked up by QLS. It must be emphasised that

the D_{zt} will only be an apparent one as we are, for reasons described in Section II. 39, unable to extrapolate this value to zero angle and concentration.

(V. 20) Determination of the s-value of SMFs in 0.2 mM and 3.0 mM Mg^{2+}

SMFs sediment as a single sharp boundary in both 0.2 mM and 3.0 mM Mg^{2+}. The low magnesium sample at a finite concentration of 0.5 mg/ml had an s-value of 176 s ± 6.0 which increased to 196.6 s± 6.0 in the presence of 3 mM Mg^{2+}. This represents an 11% increase in s-value on the addition of 3 mM divalent cation. When extrapolated to zero concentration the change in s-value caused by the addition of divalent cation was only 8%. However as reported by Persechini and Rowe (1984) the magnitude of the magnesium effect varied from preparation to preparation, and therefore a direct comparison with their results is not necessarily valid. The important finding here was that we did observe a change in s-value by increasing the [Mg^{2+}] from 0.2 to 3.0 mM and that the observed change was in the right direction.

(V. 21) Determination of the D_{zt} of SMFs in 0.2 mM and 3.0 mM Mg^{2+}

The D_{zt} of each SMF sample (0.2 and 3.0 mM $Mg^{2+)}$ at a finite concentration of 0.5 mg/ml was calculated from the slope of the respective plots (Graph 5.2 and Graph 5.3) of their auto-correlation function against time. The D_{zt} of the SMFs in 0.2 mM Mg^{2+} was ($1.67 \times 10^{-12} \pm 0.02$) m^2 sec^{-1} and those in 3 mM Mg^{2+} possessed a D_{zt} of

(1.92 x 10^{-12} ± 0.02) m^2 sec^{-1}. This represents a 15% increase in D_{zt} on raising the divalent cation concentration from 0.2 to 3.0 mM Mg^{2+}. Such an increase is, allowing for the assumptions involved, entirely consistent with the 11% increase in s-value (See Section V. 20) obtained using sedimentation velocity analysis at the same concentration and under identical conditions.

(V. 22) Calculation of the apparent molecular weight of SMFs in high and low magnesium.

As shown in Section II. 15 a determination of the apparent molecular weight can be achieved if the s-value and D_{zt} are known. Using the Svedberg equation (See Section II. 15) and the appropriate values of S and D_{zt} the apparent molecular weight of the SMFs in 0.2 mM and 3.0 mM Mg^{2+} was calculated to be Mr = 9.0 x 10^7 and Mr = 9.2 x 10^7 respectively. Allowing for the approximate nature of the calculation the apparent molecular weight at each divalent cation concentration has not changed and therefore any alteration in the s-value or D_{zt} must be due to changes in the frictional coefficient and not a loss or gain of filament mass.

(V. 23) Changes in the D_{zt} of PNFs after 50% RLC depletion.

Plots of the autocorrelation function against time for PNFs before and after 50% RLC depletion (Graph 5.4 and Graph 5.5 respectively) were used to obtain an estimate for the apparent D_{zt} of each sample. The

coefficients obtained revealed that the process of RLC depletion resulted in an increase in D_{zt} from a value of ($1.95 \times 10^{-12} \pm 0.07$) m^2 sec^{-1} for the control filaments to ($3.30 \times 10^{-12} \pm 0.5$) m^2 sec^{-1} after RLC extraction. This represents a 59% increase in D_{zt} as a result of the removal of 50% of the RLCs which correlates with the observed 48% increase in s-value reported in Section V. 17.

The linear nature of the auto-correlation plot (Graph 5.4) obtained for native PNFs indicates that the preparation is highly monodisperse. However after 50% RLC depletion the plot becomes slightly curved (Graph 5. 5) suggesting that the preparation is slightly more polydisperse than the control. Indeed RLC depleted filaments left at room temperature for 28 hours displayed a significant drop in D_{zt} accompanied by a further large increase in the degree of polydispersity.

(V. 24) Calculation of the apparent molecular weight of PNFs before and after 50% RLC depletion.

Using the values of S and D_{zt} obtained in Sections V. 17 and V. 23 the apparent molecular weight of the PNFs before and after 50% RLC depletion can be calculated using the Svedberg equation (See Section II. 15). The apparent molecular weight of the control filaments was found to be 57 MDaltons whilst the RLC denuded filaments displayed an apparent molecular weight of 50.5 MDaltons.

The recent work of Bolger et al (1989) has determined the extrapolated molecular weight of PNFs to be 75 MDaltons and if one assumes that each PNF contains approximately 150 myosin molecules (Emes and Rowe.,1978b;) then each filament will lose 150 RLCs during the

extraction procedure. The removal of 150 RLCs each with a mass of 18 KDaltons represents a loss of 2.7 MDaltons which is 3.6% of the PNFs total molecular weight. Therefore the apparent molecular weight of the control PNFs can be corrected for the loss of 50% of the RLCs which gives a final value of 55 MDaltons. Allowing for the approximate nature of the calculation we feel that the molecular weight of the control filaments, after correction for RLC loss (55 MDaltons), and that of the filaments that have actually been RLC depleted (50.5 MDaltons) are the same. Therefore the changes in s-value and D_{zt} observed in Sections V. 17 and V. 20 can be attributed to some sort of conformation change and not to a large change in the molecular weight.

(V. 25) Do RLC depleted myosin molecules in monomeric solution interact ? (Do sticky patches exist ?).

The following sedimentation velocity experiments were carried out on PNFs that after 50% RLC depletion were dissolved in high salt. If sticky patches were exposed by RLC depletion aggregation of the myosin molecules should occur and result in the formation of fast moving boundaries ahead of the monomeric myosin. The experiment was carried out once in the presence of H_2O and again in the presence of D_2O to eliminate any differences that may be caused by the use of the different solvents.

(V. 26) The determination of the s-value of myosin molecules in 60% D_2O (before and after RLC depletion).

Both the control and RLC depleted monomeric myosin showed single sharp schleiren traces and the lack of any faster moving boundaries indicated that no aggregation had occured. The s-value of the control myosin was 4.108 s ± 0.041 and after RLC depletion this decreased to 4.033 s ± 0.084. This represents approximately a 2% change in s-value however the large standard errors obtained are indicative of the difficulties involved in measuring small differences in s-value between samples. Although the drop in s-value is consistent with the loss of the mass of a single RLC (See Discussion) the large errors involved mean that we cannot attach any significance to this change.
The s-value of the control myosin is lower than the accepted value of monomeric myosin (approximately 5.52s, Emes and Rowe.,1978a) however this can be accounted for in two ways. The first is that the experiments were carried out in D_2O with no correction for the increased density and viscosity, and the second is that the analysis was carried out at a finite concentration of 0.3 mg/ml. Allowing for these factors would increase the s-values of both samples to approximately the expected level. However as we are only looking for relative and not absolute changes in s-value this does not matter, so the direct comparison of the s-values is perfectly valid.

(V. 27) The determination of the s-value of myosin molecules in 100% H_2O (before and after RLC depletion).

As with the samples in D_2O (See Section V. 26) no signs of aggregation were seen in the presence of 100% H_2O either before or after RLC depletion. The control myosin had an s-value of 5.2s ± 0.039 whilst the RLC depleted myosin had an s-value of 5.014s ± 0.031 which represents a 3.6% decrease. In this instance the standard errors are small and the change in s-value is significant. Although no aggregation of the RLC depleted myosin was observed the drop in s-value is consistent with the change expected if one RLC/myosin molecule had been removed by the extraction proceedure (See Discusion).

The effect of divalent cation on the hydrodynamics of PNFs.

(V. 28) Do μM levels of free calcium have an effect on the s-value of PNFs ?

All of the PNF samples with free calcium concentrations between 0-100 μM displayed sharp single schlieren peaks (See Schleiren Traces 5.1 and 5.2) and showed no tendency to aggregate. The exact s-values obtained along with the relative changes for each concentration are shown in Table 5.5.

Notwithstanding the difficulties involved in looking at small differences in s-value we can say that the addition of 100 μM free calcium causes a significant increase of approximately 5% in s-value. A plot of the % change in s-value against free Ca^{2+} (Graph 5.6) reveals

that the site has an approximate Kd = 30 μM (pCa = 4.5) and reaches a plateau somewhere in the region of 60 μM free calcium. Possible errors in the estimation of the free calcium concentration would always be of a sign leading to underestimation of the Kd.

Do PNFs possess a low affinity binding site similar to that found in SMFs ?

(V. 29) The effect of mM Mg^{2+} on the s-value of PNFs (in the absence of Ca^{2+}).

Analysis of the sedimentation of PNFs in the presence of mM Mg^{2+} under the same conditions as those used by Persechini and Rowe (1984) for SMFs, has revealed the presence of a low affinity Mg^{2+} binding site (Graph 5.7). At all Mg^{2+} concentrations used there was no sign of aggregation and the PNFs sedimented as a single sharp boundary. The titration curve for this site is almost identical to the one reported for SMFs (Persechini and Rowe.,1984;) with a maximum 16% increase in s-value being obtained by the addition of 5 mM Mg^{2+}. Examination of the titration curve revealed that the site has a dissociation constant (Kd) of 1.6 mM which correlates with the value of 1.5 mM reported for SMFs by Persechini and Rowe (1984).

(V. 30) The effect of mM Ca^{2+} on the s-value of PNFs (in the absence of Mg^{2+}).

Sedimentation analysis of PNFs in the presence of mM Ca^{2+} also revealed the presence of a low affinity binding site with the filament s-value increasing at higher cation concentrations (See Graph 5.8). A maximum increase of 16% in s-value was observed in the presence of 5mM Ca^{2+} with no evidence of aggregation having taken place at any cation concentration. The site apparently displayed a lower affinity for Ca^{2+} than for Mg^{2+} and had a Kd = 3.4 mM (c.f. for Mg2+ Kd = 1.6 mM).

(V. 31) The Effect of mM Mg^{2+} on the s-value of PNFs (in the presence of 100 µM Ca^{2+}).

The results obtained in Sections V. 29 and V. 30 above suggest that the low affinity binding site apparently has a lower affinity for Ca^{2+} (Kd = 3.4 mM) than for Mg^{2+} (Kd = 1.6 mM). However, we felt that an alternative explanation was available if we considered the possibility that calcuim binding to the high affinity site had an influence on, and reduced the affinity of, the mM site for divalent cation. We attempt to answer this question by following the titration of the low affinity site for mM Mg^{2+} in the presence of 100 µM Ca^{2+} (Table 5.8). Interaction between the high and low affinity sites should result in a shift in the Mg^{2+} titration curve.

Analysis of the sedimentation data for PNFs in the presence of mM Mg^{2+} and 100 µM Ca^{2+} shows that the Kd of the low affinity binding site has

been shifted from Kd = 1.6 mM to Kd = 3.6 mM and the maximum change in s-value occurs in the presence of approximately 5 mM Mg^{2+} (Graph 5.9) Both of these values are in close aggreement with the values obtained in the presence of mM Ca^{2+} (See Section V. 30). All of the samples sedimented as single sharp boundaries and no signs of aggregation was seen in any of the runs.

Discussion

(V. 32) Does RLC removal result in sticky patch formation ?

As discussed in the introduction opinion is divided over the issue of whether sticky patch formation occurs as a result of RLC removal (See Section V. 5). We feel that the results presented in this chapter may go some way to explaining many of the ambiguities reported in the literature and hopefully provide a unifying working hypothesis about some of the properties of the exposed RLC binding site.

(V. 33) The effects of RLC depletion on myosin filaments.

From the results presented in Chapters III and IV it seems likely that all of the myosin molecules within the native filaments are identical in that they all lose one RLC during the depletion procedure (See Section III. 13). Indeed, as no evidence has yet been presented that suggests the presence of two conformationally distinct populations of myosin, this seems to be a reasonable assumption. With this information in mind we can reasonably predict that any changes in the properties of the cross bridges that resulted from sticky patch formation should appear in all of them.

When positively stained native myosin filaments were viewed in the electron microscope numerous cross bridges were clearly visible (Plate 5.1). However after RLC depletion all filaments appeared to be completely devoid of cross bridge structures (Plate 5.2). We know from

the evidence provided by the gels of the extraction timecourse (Gel 3.1) that proteolysis had not taken place, so the cross bridges are still present. It therefore seems likely that all of the cross bridges have moved down close to or actually stuck down on to the thick filament shaft (See Figure 5.1). Our initial interpretation of these results was that RLC removal did indeed result in the formation of some sort of sticky patch, and it was the interaction of the exposed binding site with the filament backbone that prevented the cross bridges adopting extreme radial positions and therefore prevented their visualisation in the electron microscope (See Figure 5.1).

The fact that all of the cross bridges were apparently stuck down onto the filament shaft was we felt strong evidence in support of the idea that all myosin molecules had lost a single RLC and therefore possesed a single sticky patch with which interaction with the filament shaft was possible. We feel that the micrographs do not support the idea that head clumping occurs in which the exposed RLC binding site on one of the S1 heads sticks to the other RLC containing S1 head within the same cross bridge (See Figure 5.1). If this process was occuring to any great extent then we would not expect to see a dramatic difference in structure between the control and RLC depleted filaments when viewed in the electron microscope. Head clumping would not prevent cross bridge visualisation but would probably result in the observation of a larger more massive S1 region of the cross bridges because of the close apposition of the two S1's. Therefore any sticky patch interactions that are occuring are likely to be between the RLC denuded S1 head of the myosin molecule and the shaft of the thick filament itself.

However one must be careful when making such a simplistic interpretation of the images obtained in the electron microscope. Although we know the apparent lack of cross bridges after RLC depletion is not a result of proteolysis (See Section III. 13) there are several other explanations that might be put forward to explain their absence from the micrographs. The first is that the removal of the RLCs causes a complete loss of the secondary or tertiary structure of the myosin heavy chain in the S1 and possibly S2 region, leaving just the primary structure which itself would not readily be visible in the E.M. There are two reasons why such large structural changes seem highly unlikely: the first being that it is difficult to conceive how the loss of an RLC from the S1 head of an individual myosin molecule could affect the structure of the RLC containing head; the second that the isolated unravelling of the heavy chain of the RLC deficient head would only partially affect the image obtained in the E.M. as the S2 and the intact S1 would still be visible. Loss of the tertiary or secondary structure of the cross bridges is even less likely when the effects of RLC depletion on the ATPase activity (See Section III. 31 and III. 32) are taken into account. RLC depletion does not have a dramatic effect on the basal ATPase activity of native myosin it merely reduces the ability of the myosin linked ATPase to be calcium activated. If RLC depletion was indeed causing loss of the secondary and tertiary structure of the S1 heads one would not expect ATPase activity to remain.

(V. 34) Hydrodynamic evidence for the existence of sticky patches.

Sedimentation velocity analysis and QLS of PNFs has revealed a large increase in the s-value and D_{zt} after 50% RLC depletion (Sections V.17 and V. 23 respectively). Aggregation has been ruled out as the cause of these increases as the RLC depleted filaments sedimented as a single sharp boundary. QLS analysis did however reveal a small increase in polydispersity after RLC depletion, as revealed by the slight departure from linearity in the slope of the plot of auto-correlation function against time (See Graph 5.5). However this departure was relatively small and was not large enough to account for the observed 59% increase in D_{zt}. The increase in polydispersity that occurs after RLC depletion is probably indicative of a small degree of aggregation (although not enough to be picked up by schleiren optics) arising from the interaction of sticky patches between filaments (interfilament interaction).

Analysis of the micrographs of RLC depleted filaments suggested that the main interaction of the cross bridges is with the shaft of the filament to which they belong (intra-filament interaction) as judged by their smooth appearance. Although some degree of filament aggregation was observed after RLC depletion, the frequency of such interactions was identical to that in the control preparations. However the conditions used for electron microscopy may not favour the aggregated state and therefore its absence in the electron microscope is not conclusive evidence against its existence.

One can clearly imagine that if sticky patches were formed after RLC depletion and there was an interaction, possibly a specific one, with

the shaft of the thick filament then the cross bridges may be able to interact with the shaft of other thick filaments. Even if the majority of the cross bridges, possibly for steric reasons, were stuck on to their own filaments shaft (intrafilament interaction) some would probably be able to interact with the corresponding sites on the shaft of other filaments (interfilament interaction) and therefore cause some degree of aggregation to occur.

(V. 35) Are the changes in s-value and D_{zt} after RLC depletion consistent with the cross bridges being stuck down on the filament shaft ?

From the work of Emes and Rowe (1978b) we know that myosin filaments have an anomalously high (70%) frictional coefficient which Persechini and Rowe (1984) believed to be due to the presence of the cross bridges and that the magnitude of this anomaly was determined by the radial position of the cross bridge. If we accept this to be the case then sticking all of the cross bridges down on to the filament shaft should result in the abolition of the frictional anomaly and therefore up to a 70% increase in s-value. Sedimentation and QLS analysis reveals a 49% and 59% increase in s and D_{zt} respectively after 50% RLC depletion. Obviously this could be interpreted as indicating the formation of filament dimers. However the calculation of the filaments molecular weight before and after RLC depletion (carried out in Section V. 24 using the appropriate values of s and D_{zt}) reveals, that allowing for the RLC loss no significant change occurs. This effectively eliminated the possibility of dimer formation and therefore

the changes in s and D_{zt} are entirely consistent with the abolition of a large portion of the frictional anomaly. We feel that the abolition of this anomaly is probably a result of the cross bridges being stuck down on to the filament shaft by sticky patches.

The question now arises as to why the sticking down of the cross bridges results in approximately a 50% increase in s-value. It has recently become apparent that the explanations for frictional incriment described in the introduction (See Section V. 12) are not sufficient to explain why myosin filaments possess such an anomaly (Rowe., 1989). Although, the suggestion of Persechini and Rowe (1984), that it is caused by the radial projection of the cross bridges is simple and conceptually makes sense, modeling studies have revealed that such an explanation cannot account for the entire anomaly (Rowe and Maw., 1983). Rowe (1989) has suggested that the frictional anomaly could arise from the presence of elastic elements within the myosin cross bridge structure probably located in the S1/S2 region of the myosin molecules.

The suggestion that there is elasticity within the components of striated muscle is not a new one. Over 50 years ago Hill (1938) suggested a three element model for the cross bridges based on experiments looking at the length tension relationships of whole muscle fibres. His model was based on the results of quick release experiments, in which the muscle is held at constant length whilst developing a tetanic force appropriate for that length. The muscle is then streched at constant force which results in a rapid length change followed by a slower phase until the appropriate length for the applied tension is reached. Such a rapid length change accompanying a sharp

change in load is consistent with the definition of a spring which has a unique length for every tension applied. His model therefore consisted of a contractile component in parallel with a parallel elastic component both of which were in series with a series elastic component (SEC).

A. F. Huxley and Simmons (1971a, 1973) modified this model by suggesting that the components responsible for the elasticity were not just passive visco elastic elements but also contained active tension generators. Evidence for this came from the fact that the active element in the cross bridge was capable of taking up approximately 60 Å of shortening whilst still maintaining tension. Furthermore analysis of the tension curves at different sarcomere lengths revealed that this was dependent on filament overlap and therefore implied that the elasticity truely resides in the cross bridges.

The above description of some of the mechanical properties of muscle illustrates that the concept of elasticity in the cross bridges is supported by evidence from mechanical studies. For a detailed description of this area there are two review articles by White and Thorson (1973) and A. F. Huxley (1974) that summarise many of the major mechanical models that incorporate elastic elements.

It is now however generally accepted that there is an instantaneous elasticity somewhere in the cross bridges. A. F. Huxley and Simmons (1971b) and later A. F. Huxley (1974) suggested that this elasticity resided in the S2 region of the cross bridge although he did point out that the compliance need not be located in the S2 region at all. Indeed Tawada and Kimura (1984) have recently used Dimethylsubarimidate (DMS) in muscle fibres to cross link the S2 portion of the cross bridges

to the filament shaft and showed that this did not significantly affect the compliance. Therefore they suggested that the elastic elements of the cross bridges resided in the proximal domain of the S1 head and that this domain acts as a stretchable spring. Holmes and Goody (1984) have reported flexibility between the individual domains of the S1 head which could possibly be linked to the elastic properties of the cross bridges.

Although Rowe's theory of the hydrodynamics of elastically linked oligomers does not conclusively define the hydrodynamic properties of thick filaments it does provide the bounds within which such a particle could reasonably be expected to fit. The question now arises as to why the sticking down of the cross bridges, presumably by a sticky patch, results in a 50% increase in s-value ? If one accepts that the so called elastic element of the cross bridge is in the S1/S2 region of the myosin molecule and that the removal of the RLC from a myosin head creates a sticky patch near the S1/S2 hinge which sticks the head to the shaft then it is reasonable to suggest that after RLC depletion the elastic element is no longer free to act elastically. In such a case the process of sticking the head to the myosin shaft would effectively remove the elastic properties of that particular cross bridge. The other S1 head in the cross bridge (which still contains its RLC) would also presumably lose its elasticity. Although if a significant amount of the elastic element was located in the S1 portion of that head as suggested by Tawada and Kimara (1984) it may still display limited elastic properties.

If the S1 heads still containing their RLCs were able to display some elasticity then we would still expect the filaments to possess some of

the frictional anomaly which they do indeed seem to do. Using the values of Emes and Rowe (1978b) we can predict that if all of the cross bridges were stuck down on to the filament shaft and displayed no elastic properties there should be a 70% increase in s-value and of course a corresponding increase in D_{zt}. Our results do show a large increase in s-value and D_{zt} (50% and 60% respectively) after RLC depletion, which although short of the maximum change predicted is not significantly different given the limitations of the theory involved. Whatever the exact reason for the increase in s-value and D_{zt} after RLC depletion is we feel that the results obtained in this chapter strongly suggest that the exposed RLC binding site is in some way sticky. The majority of the exposed RLC binding sites interact with the shaft of the filament to which they belong (intrafilament interaction) and effectively stick the cross bridges down. However some interfilament interactions can take place and do cause a small degree of aggregation as indicated by the QLS results (See Section V. 23).

(V. 36). Is the interaction of the exposed RLC binding site with the filament shaft via hydrophobic or electrostatic interactions ?

Although the presence or absence of a sticky patch after RLC removal is still a controversial one those workers who feel that there is suggest that it probably interacts via hydrophobic residues (Bagshaw., 1980; Wells and Bagshaw., 1983; Bennet et al., 1984). Furthermore Bennett et al (1984) showed that on the removal of the RLCs from scallop myosin hydrophobic residues are exposed (See Section V. 5) which could possibly form part of such a hydrophobic sticky patch. However if the

exposed RLC binding site interacted through hydrophobic residues one would expect to observe the aggregation of solublised RLC deficient myosin monomers in 0.5M KCl. The increase in ionic strength would not significantly affect hydrophobic interactions (if they were present) as they arise from the increase in entropy obtained when water molecules are liberated from them. The entropy increase that arises from the liberation of D_2O is slightly greater than for H_2O (Trinick and Rowe., 1973) and therefore any hydrophobic interactions that were present would be stronger in D_2O. However if electrostatic interactions were present the situation would be somewhat different. An increase in salt concentration would increase the dielectric constant (D) of the solvent and as electrostatic force is inversely proportional to D the interactions would become weaker eventually causing bond disruption. It is this mechanism that enables electrostatic interactions to be salted out.

We have shown that after solublisation in 0.5M KCl the myosin from RLC depleted PNFs does not self interact. No interaction between the RLC deficient myosin molecules was observed whether the sedimentation was carried out in 100% H_2O or 60% D_2O containing buffers. In both cases the control myosin sedimented at the predicted rate but after RLC removal a small drop in s-value was observed in both solvents. In the case of the D_2O containing buffer the drop in s-value was 2% but due to the large errors involved in the s-value estimation we could not attach any significance to it. By contrast in the 100% H_2O containing buffer there was a 3.6% decrease in s-value which was significant. We feel that because no evidence of monomer interaction was observed at this ionic strength it is a strong indication that

hydrophobic residues are not involved in sticky patch formation. This view was also expressed by Bagshaw (1980) who suggested that the exposed binding site would possess a large number of charged residues to balance the high charge of the RLCs. Also Kendrick Jones et al (1980) and Chantler and Szent-Gyorgyi (1980) suggested that because of the nature of the temperature dependance of such interactions hydrophobic residues were not involved in sticky patch interaction. This does not mean to say that hydrophobic residues are not present in the exposed RLC binding site for they undoubtably are as shown by Bennett et al (1984). Indeed these residues may participate to a small extent in a part of the interaction with the filament shaft but the major interaction is it would seem electrostatic.

(V. 37) What electrostatic interactions can be made with the shaft of thick filaments ?

The sequence of the myosin LMM rod is well known and several workers have shown that the rod contains bands of alternating charged residues (McLachlan and Karn., (1983a, 1983b); Chen Lu and Wong., 1985; Morris and Chen Lu., 1987). These bands of residues are so well defined that they have been used to propose a model for the packing of the myosin tails within the thick filament shaft (McLachlan and Karn., 1983b; Morris and Chen Lu., 1987). From such models we can predict that the surface of the shaft of the thick filament would also possess highly ordered bands of charged residues. Clearly if the removal of an RLC results in the exposure of a binding site containing numerous

charged residues such a site would be able to interact with the bands of charged residues on the filament shaft. Indeed there could well be a sterically favourable specific interaction that the exposed binding site is able to make on the shaft. Furthermore various workers have demonstrated that small changes in the charge of the intact cross bridges are sufficient to alter their interaction with the filament shaft. For instance the binding of the head to the shaft is apparently weakened by increased salt concentration (Levine et al., 1986), lowering the ATP concentration (Haselgrove., 1975), lowering the Mg^{2+} concentration (Persechini and Rowe., 1984) and by increasing the pH (Levine et al., 1986) all of which suggests that ionic interactions exist between the cross bridges and filament shaft. If RLC removal exposed a charged area on the myosin head as we have suggested then there is no reason why it too should not interact with the filament shaft.

(V. 38) All native myosin molecules lose a single RLC during the gentle extraction procedure.

When one considers that an RLC (MW 18 KD) is approximately 3.5% of the total mass of a myosin molecule, a 3.6% drop in s-value as observed in 100% H_2O containing buffer is entirely consistent with the loss of the mass of one RLC per myosin molecule. Further support for this comes from the fact that the RLC depleted myosin sediments as a single boundary wherease if some of the population had lost 2 RLCs whilst others had lost none and some only one we would expect to see up to 3 boundaries. Obviously such a decrease in s-value could be

indicative of a change in the conformation of the myosin head after RLC depletion. Indeed several workers have suggested that the removal of the RLC destabilises the myosin heavy chain and causes a local collapse in the structure or decrease in head to head distance (Vibert and Craig., 1982; Flicker et al., 1983; See Page 121). However Slayter and Margossian (1987) have produced high resolution micrographs of RLC deficient myosin heads that shows no local collapse of the heavy chain structure and therefore suggest that no conformation change has occured. They also reported that after the removal of the RLCs sedimentation velocity analysis showed that no interaction between the myosin monomers occured. They interpreted this as an indication that sticky patches were not produced by the exposure of the RLC binding site. This is probably a perfectly justified statement if one assumes that the binding site consists mainly of hydrophobic residues. However they do not consider the possibility that the exposed site may interact via electrostatic interactions and that high salt concentrations could mask their presence. They do not look at the effect of RLC depletion at lower ionic strengths which on reanalysis may indeed reveal that interaction does takes place.

(V. 38) RLC depletion affects the ability to form BZAs.

RLC depleted thick filaments do indeed partially depolymerise in 200 mM KCl buffers, however the structures remaining are on average 60 nm longer than the BZAs formed in the control preparations (See section IV. 26). The presence of some very long (0.8 μm) filaments

(Plate 5.5) in the RLC depleted sample leads one to the conclusion that RLC depletion has affected the ability of the myosin monomers to dissociate from the filaments. In Section V. 32 we suggested that electrostatic interactions between the exposed RLC binding site and the filament shaft might exist. The apparently greater stability of RLC depleted filaments in 200 mM KCl may be a result of these extra interactions which could act as restraining rods and hinder dissociation from the filament.

Obviously it is possible that the process of RLC depletion causes the BZAs to aggregate end to end and it could be this process that reults in the appearance of long filaments. Although we cannot conclusively rule this idea out the images obtained in the electron microscope suggest that this is not the case (Plate 5.5). Nontheless if this process was occuring it is obviously a result of RLC depletion and would suggest that there was a fairly specific interaction between the ends of RLC deficient BZAs. However Margossian and Slayter (1987) did not report end to end interaction between RLC deficient minifilaments, although their conditions were considerably different to those used here.

A way of resolving which interpretation is correct would be to gradually take the BZAs above 200 mM and observe the point at which they dissappear. Presumably if the longer filaments were assemblies of BZAs they would be present until all of the BZAs disassembled. However if they were partially depolymerised filaments increasing the [KCl] should result in their gradual disassembly until the entire preparation contained a homogeneous population of BZAs.

(V. 40) 50% RLC depletion has no effect on the ability of the filaments to fray into subfilaments.

The fact that 50% RLC depleted full length thick filaments fray into three subfilaments when exposed to low ionic strength conditions indicates that RLC depletion does not cause changes in the packing of the thick filament shaft. If sticky patches are present after RLC depletion they are not able to stabilise the three subfilaments under low ionic strength conditions and prevent them from separating. On the other hand the sticky patches could be interacting with the shaft of the sub filament to which they belong. In this instance a strong sticky patch/shaft interaction could not influence subfilament separation.

The effects of divalent cation on myosin cross bridges.

(V. 41) PNFs possess a high affinity Ca^{2+} binding site that is irreversibly lost on high salt exposure.

The results presented in Section V. 28 clearly demonstrate that PNFs possess a high affinity calcium binding site that is fully saturated by 60 μM free Ca^{2+} and posesses a Kd = 30 μM. Morimoto and Harrington (1974) also reported the presence of such a site and that it was saturated at even lower levels of free Ca^{2+} (10 μM) and possessed a Kd = 3 μM. The reason for the differences between their results and our own is however not yet clear. When the high affinity site in PNFs is fully saturated a 5% increase in s-value is observed which represents a slight movement of the cross bridges towards the filament shaft.

Morrimoto and Harrington (1974) observed a similar although smaller (2%) increase in the s-value of their filaments.

Presumably the high affinity calcium binding site is responsible for, in some way, priming the cross bridges ready for contraction to take place. It is difficult to see how the movement of the cross bridges towards the filament shaft would be an advantage during the early stages of contraction as one would predict that a movement away from the shaft and towards the thin filaments would be needed to enhance the possibility of actin interaction.

Although the true function of the high affinity calcium binding site is at this point difficult to specify it is important to remember that Persechini and Rowe (1984) found no evidence for the presence of such a site in SMFs. This clearly suggests that the high affinity sites present in native myosin have been lost during the SMF preparation. As the calcium sensitivity of the ATPase and RLC susceptibility to the gentle extraction technique are not present in SMFs (Bolger et al (1989b)) it is likely that they have all been lost for the same reason i.e. that each of these properties are susceptible to high salt exposure.

Morrimoto and Harrington (1974) suggested that the high affinity calcium binding site was present in both native and synthetic myosin filaments which obviously contradicts the results presented here and those of Persechini and Rowe (1984). However Morrimoto and Harringtons (1974) results with synthetic filaments have never been convincingly repeated and when one considers the fact that they suggest that their full length native myosin filaments had an s-value of 55s (cf. PNFs 140s, SMFs 190s) one must question the validity of some of their results.

(V. 42) PNFs possess a low affinity Mg^{2+} binding site that is similar to that found in SMFs.

In the previous chapters we have described many examples of the salt labile properties of native myosin filaments from both rabbit and rat filament preparations. Persechini and Rowe (1984) had previously reported a low affinity non specific divalent cation binding site in SMFs that caused a 15% increase in s-value when fully saturated. As they had used synthetic filaments we felt that the properties of this site may have been altered by the high salt exposure that is inherent in the preparation of such filaments. We did indeed find that PNFs possessed a low affinity binding site for Mg^{2+} which was fully saturated by 5 mM free Mg^{2+} and had a Kd = 1.6 mM (See Section V. 29). Allowing for experimental error these values are identical to those reported by Persechini and Rowe (1984) for the low affinity binding site present in SMFs. The similarity of the titration curve of the site for SMFs (Persechini and Rowe., 1984) and PNFs (Graph 5.7) suggests that we are observing the effects of Mg^{2+} binding to the same site in both preparations. It therefore appears that the low affinity site is not an artifact created by high salt exposure and its affinity for Mg^{2+} is apparently not altered by exposure to high salt conditions.

(V. 43.) This site has a lower affinity for Ca^{2+} than Mg^{2+}

The low affinity site in SMFs binds Mg^{2+} and Ca^{2+} equally well (Persechini and Rowe., 1984) and is therefore non specific. However when one looks at the titration curve for Ca^{2+} binding to the low

affinity site of PNFs the situation is quite different (See Graph 5.8). In this instance the site is saturated at the higher concentration of 5 mM Ca^{2+} (cf. 3 mM for SMFs) and displays a Kd = 3.4 mM as compared to a Kd = 1.6 mM for Mg^{2+} (See Section V. 30).

This can be interpreted in one of two ways. The first is that the low affinity site in PNFs is more specific for Mg^{2+} than Ca^{2+} and that upon exposure to high salt conditions a conformation change occurs in the site which increases its affinity for Ca^{2+} from Kd = 3.4 mM to Kd = 1.6 mM. If such a change occurs it does so in such a way as to have no effect on the affinity for Mg^{2+} whose Kd = 1.6 mM in PNFs and Kd = 1.5 mM in SMFs. The other possibility is that the binding of calcium to the high affinity site in PNFs (V. 28) results in an alteration in the low affinity site which shifts its Kd for divalent cation from Kd = 1.6 mM to Kd = 3.4 mM. If this was the case one would expect that in the presence of 100 μm Ca^{2+*} the titration curve for the binding of mM Mg^{2+} would be displaced to the same extent as the titration curve for mM Ca^{2+} i.e. the dissociation constant should be shifted from Kd = 1.6 mM to Kd = 3.4 mM.

Graph 5.9 clearly shows that when the high affinity binding site is saturated with calcium in the presence of mM Mg^{2+} a change in the Kd of the low affinity site is observed which is consistent with a reduction in its affinity for Mg^{2+}. The affinity of this site is now approximately identical for both mM Ca^{2+} and mM Mg^{2+} (See Graph 5.10) and in that respect the site is similar to the one obtained in SMFs which also displays non specific binding. However the actual Kd of the non specific sites in PNFs (Kd ~ 3.5 mM) is reduced after the high salt exposure which is nesecary to prepare SMFs (Kd = 1.5 mM).

It is difficult to say whether high salt exposure results in the loss of the high affinity site and also in some way alters the conformation of the low affinity site for calcium, or whether the effect is entirely attributable to the loss of the high affinity site only. Whatever the case may be it is clear that we have demonstrated the presence of another distinctive property of native myosin which is irreversibly lost on exposure to high salt conditions.

* As under these conditions the high affinity site would be fully saturated.

(V. 44) Why do the high affinity sites influence the properties of the low affinity sites ?

Persechini and Rowe (1984) suggested that the low affinity sites were not a way of regulating cross bridge disposition *per se* but were in fact a way of holding the cross bridges in an optimum conformation ready for modulation by some other as yet unknown factor. The reasons for suggesting this were two fold. The first was that free calcium concentrations *in vivo* never reached the millimolar levels needed to influence the site and secondly the concentration of free magnesium was held fairly constant between 1 and 3 mM. If Mg^{2+} was regulating the cross bridges via the low affinity site then large very rapid changes in its concentration would be necessary and such changes have never been observed.

We conclude that the other unknown factor affecting cross bridge configuration described by Persechini and Rowe (1984) could be the

high affinity binding site present in PNFs. Using such a site the cross bridge disposition could be regulated using the low affinity sites, not by changing the free Mg^{2+} concentration, but by altering the Kd of the low affinity site itself.

(V. 45) What role does the interaction between the high and low affinity sites play in contraction ?

It would probably be of great benefit if the cross bridges themselves were sensitive to calcium and that in response to its presence they flared out from the shaft of the cross bridge to engage the actin binding sites on the thin filaments. Such was the scheme proposed by Haselgrove (1970, 1975), a conclusion which has since been brought into question by several workers (H. E. Huxley., 1972; H. E. Huxley., 1980; Yagi and Matsubara., 1980). However we feel that the evidence presented in this chapter strongly supports the suggestion that the coss bridges do indeed possess a myosin linked regulatory system that controls the properties of and/or the disposition of the cross bridges. We have combined all of the data obtained and propose that the following mechanism for the control of the myosin cross bridges may well exist in vertebrate skeletal muscle. Once again it must be emphasised that the changes in the properties of the cross bridges are interpreted as representing their relative displacement from the filament shaft (Persechini and Rowe., 1984). This interpretation has been adopted purely because it lends itself to diagramatical representation, however any or all of the possibilities discussed earlier (See Section V. 12) may well be involved in the overall effect.

(V. 46) A model for cross bridge movement induced by divalent cation.

The literarture is somewhat at variance as to the exact concentration of free Mg^{2+} in vivo and estimates range from 1 to 3 mM (Hess et al., 1982; Blatter et al., 1987). Due to the absence of a well accepted value we will for arguments sake adopt a value of 2 mM free Mg^{2+} throughout the following discusion although values of between 1 to 3 mM are still acceptable and will have a similar but probably reduced effect.

Consider the situation at rest (Figure 5.2) where the free calcium levels are 10^{-7} M or less and the myosin binding sites on the thin filaments are blocked by the tropomyosin. In this instance the cross bridges will be held mid way between the fully up and down configuration (Point A, Figure 5.2) by the binding of Mg^{2+} to the low affinity sites i.e. the low affinity sites are holding the cross bridges in an optimum configuration as first described by Persechini and Rowe (1984). As the contractile signal arrives and depolarises the cell membrane Ca^{2+} floods into the cell and its concentration rises to above10^{-5} M saturating the high affinity calcium binding site (Kd = 30 μM). The binding of calcium to the high affinity site modulates the Kd of the low affinity site and swithches it from Kd = 1.5 mM to Kd = 3.5 mM. The Mg^{2+} concentration however remains constant and because of the new Kd of the low affinity sites this causes the cross bridges to flare out from the shaft of the thick filament (Point B, Figure 5.2) and engage the myosin binding sites on the by now activated thin filaments. We know that such changes in the location of the cross bridges under

the influence of calcium have never been convincingly observed in muscle fibres using diffraction techniques. However if we think of these changes not in terms of alterations in the cross bridge displacement but as changes in the elasticity then a slightly different picture emerges. Classical diffraction techniques would be incapable of detecting changes in the elasticity of a certain region of the cross bridge. Diffraction only reveals a time averaged picture of the position of the various components so although it is possible that changes in elasticity may well affect the rate at which the cross bridges cycle between the 'heads up' and 'heads down' configuration the changes in average position would not be at a detectable level.

(V. 47.) How does the elasticity change during the initiation of contraction ?

According to Rowe's (1989) theory on the hydrodynamics of elastically linked oligomers a particle with a high elasticity will have a anomalously low s-value and as the elasticity is reduced so the s-value increases until it approaches that predicted by classical hydrodynamic analysis. The anomalously low s-value of myosin filaments can therefore be explained in terms of the presence of elastic elements somewhere in the cross bridge (See Section V. 12). The increase in s-value observed after the addition of millimolar divalent cation can therefore be interpreted as representing a decrease in the elasicity of the cross bridge.

Re-analysis of Figure 5.2 enables us to describe the changes in terms of alterations in the elasticity of the cross bridges rather than their

radial disposition. If one accepts that in high divalent cation concentrations the cross bridges have a reduced elasticity and in low concentrations a high elasticity then the following scheme can be proposed. At rest the the in vivo magnesium concentration produces an intermediate elasticity in the cross bridges (Point A, Figure 5.2). On binding calcium to the high affinity site the Kd of the low affinity site is altered and results in an increase in the elasticity of the cross briges (Point B, Figure 5.2).

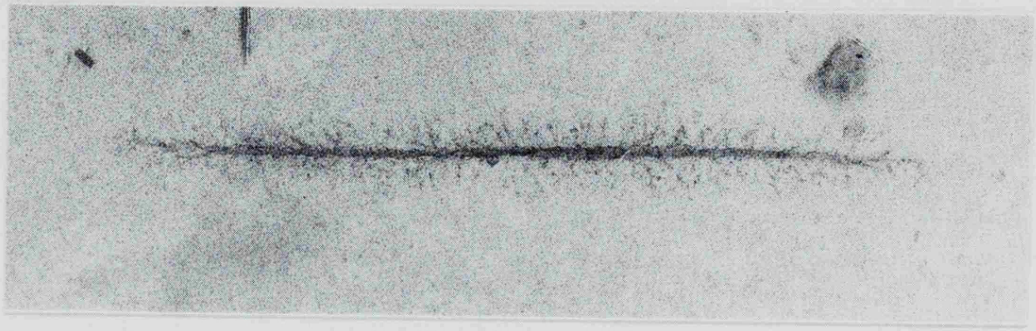
(V. 48) Why does the elasticity increase during contraction ?

We have already discussed that during relaxation it would be favourable for the cross bridges to be held down onto the filament shaft and away from the thin filaments. Indeed, evidence has been presented which suggests that such a system may well exist (Craig et al., 1987; Haselgrove., 1975). Obviously if the myosin heads are to be held away from the actin filaments it would be desirable to reduce the degrees of freedom that are available to the cross bridge. One way of doing this would be to decrease the elasticity of the cross bridges which could well limit the number of actin molecules that the heads are able to interact with. As contraction was initiated the elasticity of the cross bridges could then be increased and enable a larger number of myosin binding sites within the thin filaments to be targetted by the myosin heads.

Chapter V

Graphs, Tables, Plates, Figures and Schleiren traces.

5.1



5.2

Plate 5.1. (Magnification 71,330 x)

Electron micrograph of a positively stained full length rabbit thick filament from a RFP.

Cross bridge structures are clearly visible along the entire length of the thick filament and although a well defined central bare zone is not visible the filament is assumed to be bipolar because of its tappering ends.

Plate 5.2. (Magnification 63,250 x)

Electron micrograph of a positively stained full length rabbit thick filament from a RFP after 50% RLC depletion.

Cross bridge structures are completely absent along the entire length of the filament, the shaft of which appears to be of a more uniform diameter than that of the control filament. Two thick filament fragments are also shown neither of which reveal any cross bridge structures. These short filaments were probably produced by the homogenisation needed to produce an RFP and could well be hemifilaments.

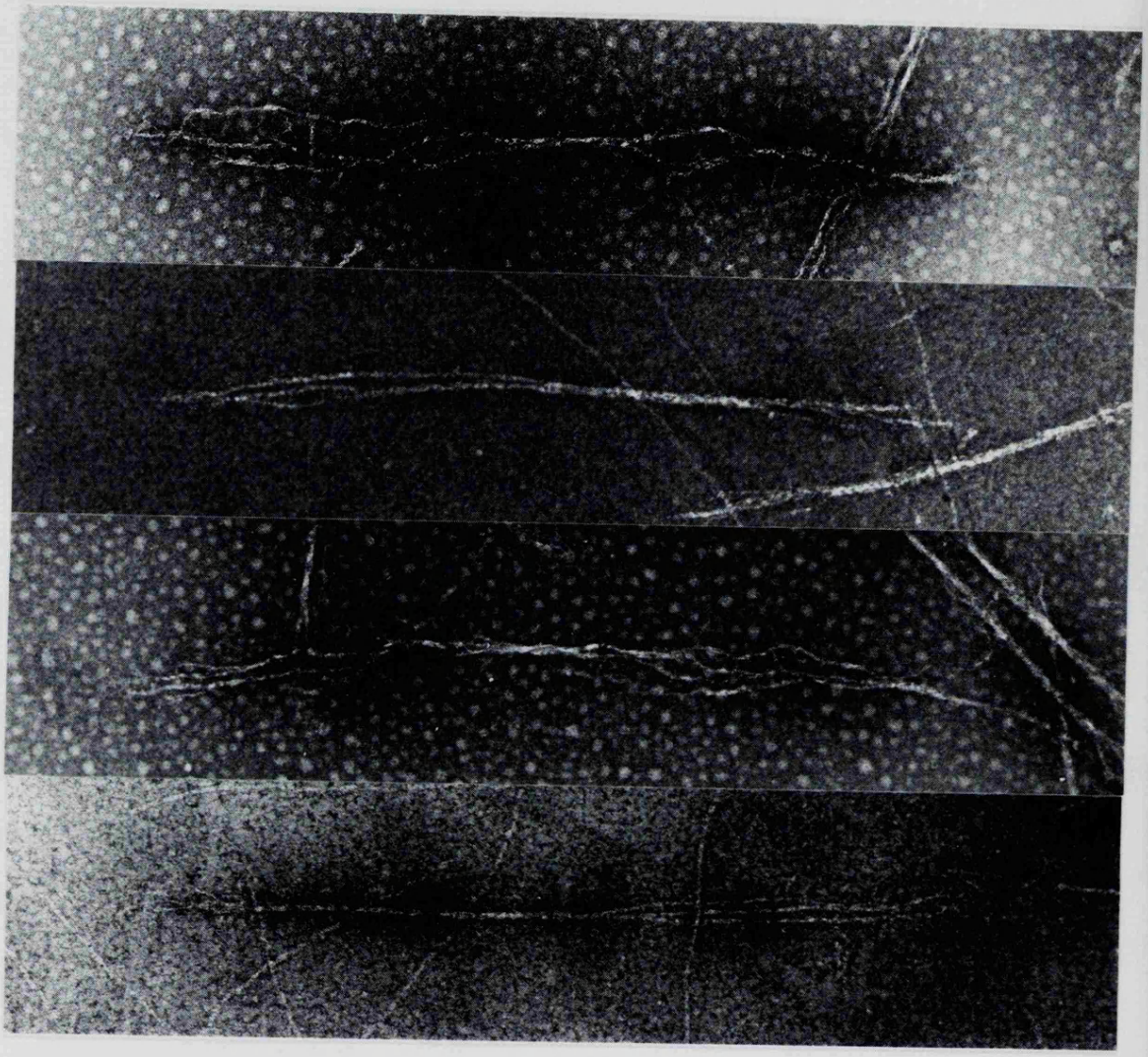


Plate 5.3

A montage of negatively stained 50% RLC depleted rabbit thick filaments that have been frayed into sub-filaments by rinsing with low ionic strength buffer.
(Magnification 66,600 x)

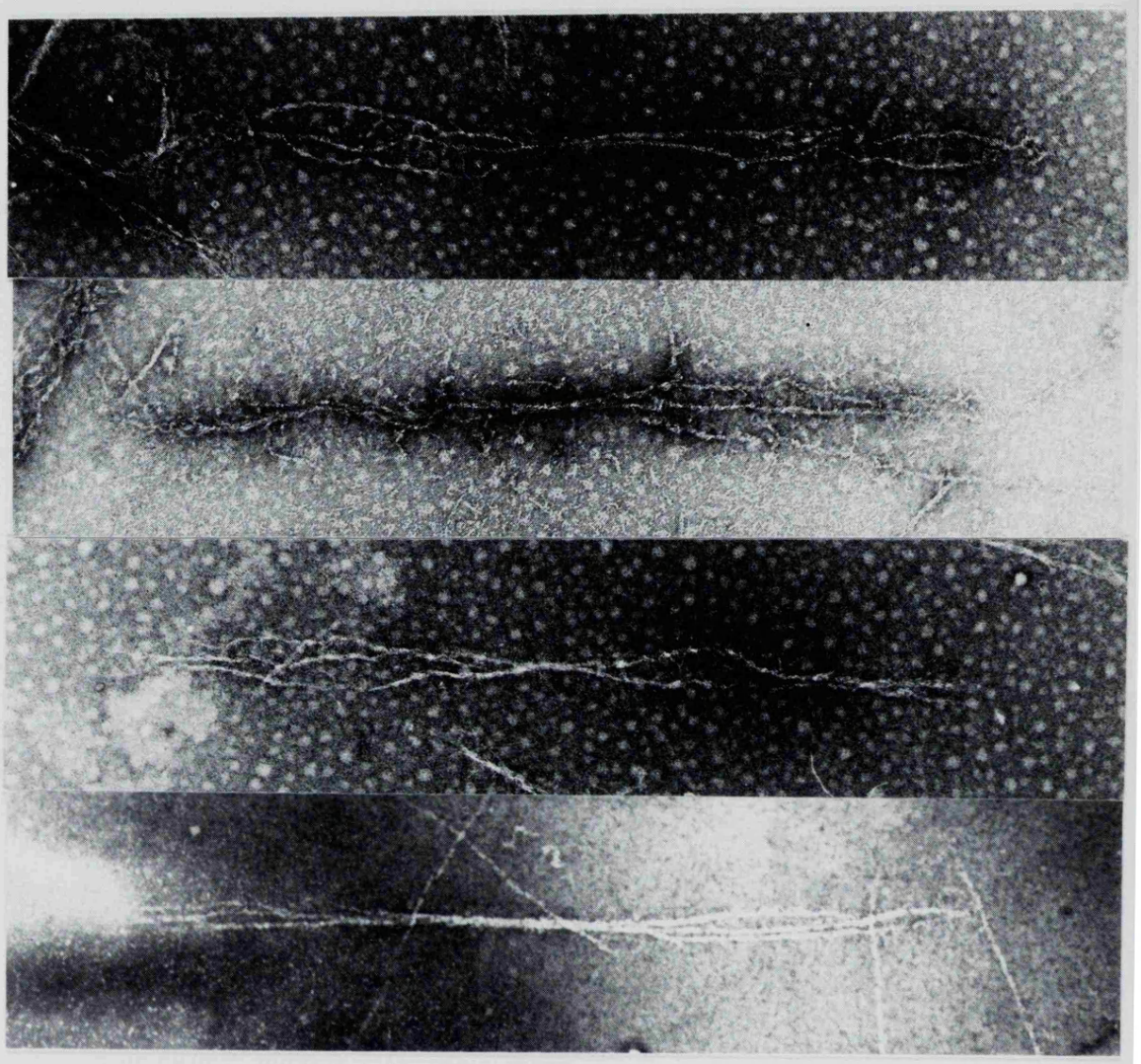
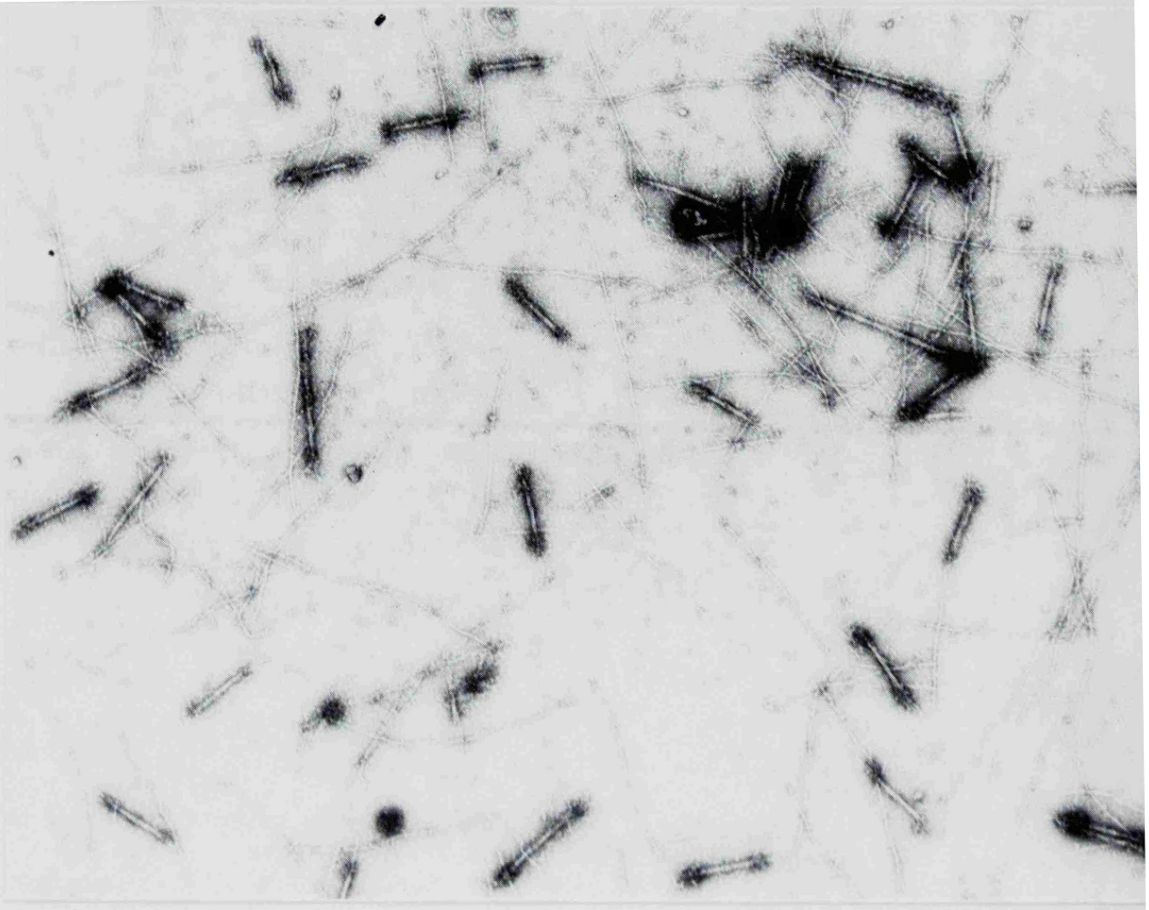


Plate 5.4

A montage of negatively stained 50% RLC depleted rat thick filaments that have been frayed into sub filaments by rinsing with low ionic strength buffer (Magnification 66,600 x).



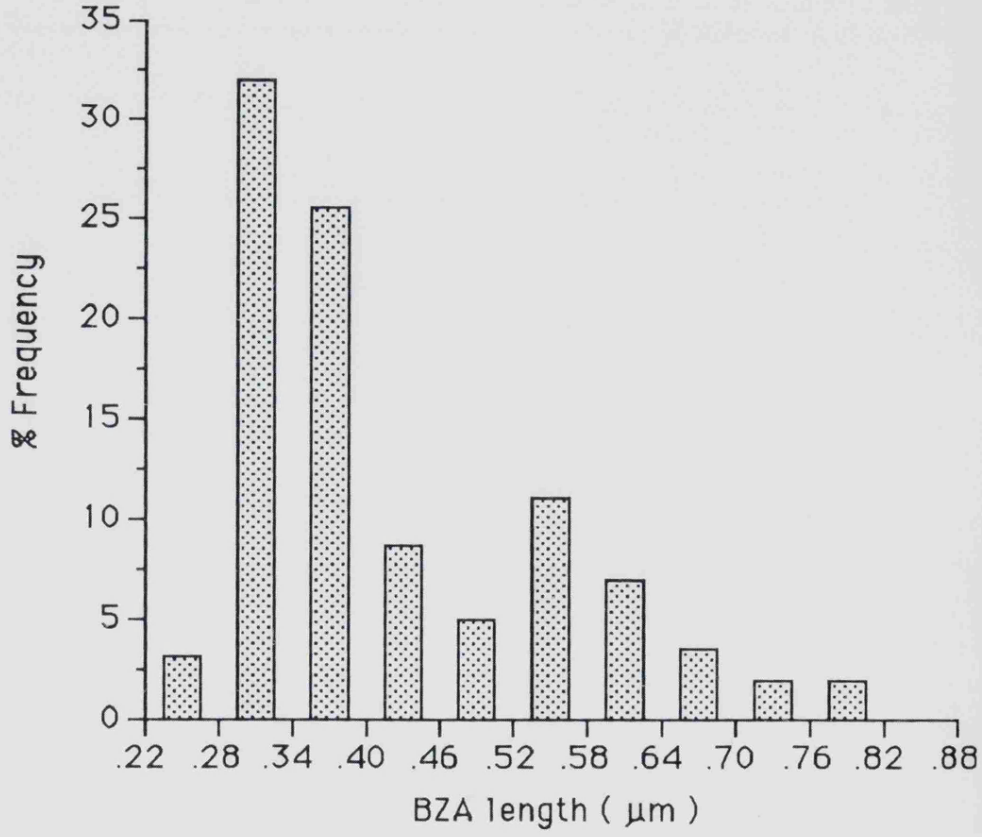
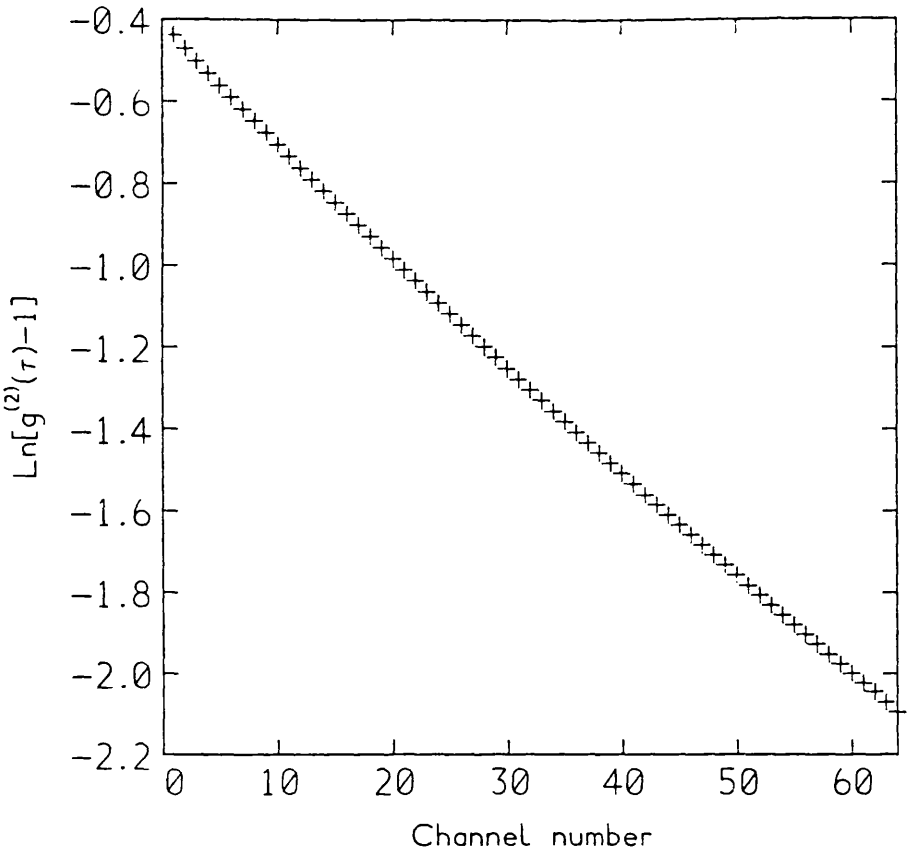

35
30
25
20
15
10
5
0
% Frequency
.22 .28 .34 .40 .46 .52 .58 .64 .70 .76 .82 .88
BZA length (µm)

Plate 5.5

An electron micrograph showing a field of negatively stained RLC depleted rat filaments after dialysis in 200 mM KCl for 18 hours (Magnification 34,100 x). Notice the presence of several long filaments which in some cases were 0.8 μm in length. Control filaments under identical conditions formed a very uniform population of BZAs (average length 0.29 μm) as shown in Plate 4.8 and Graph 4.5.

Graph 5.1

A length histogram (weight averaged) showing the distribution of filament lengths from an RLC depleted rat RFP after dialysis against 200 mM KCl.
Average length = 0.36 μm (s.d. = 0.1).


-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
-1.6
-1.8
-2.0
-2.2
0
10
20
30
40
50
60
Ln[g(2)(τ)-1]
Channel number

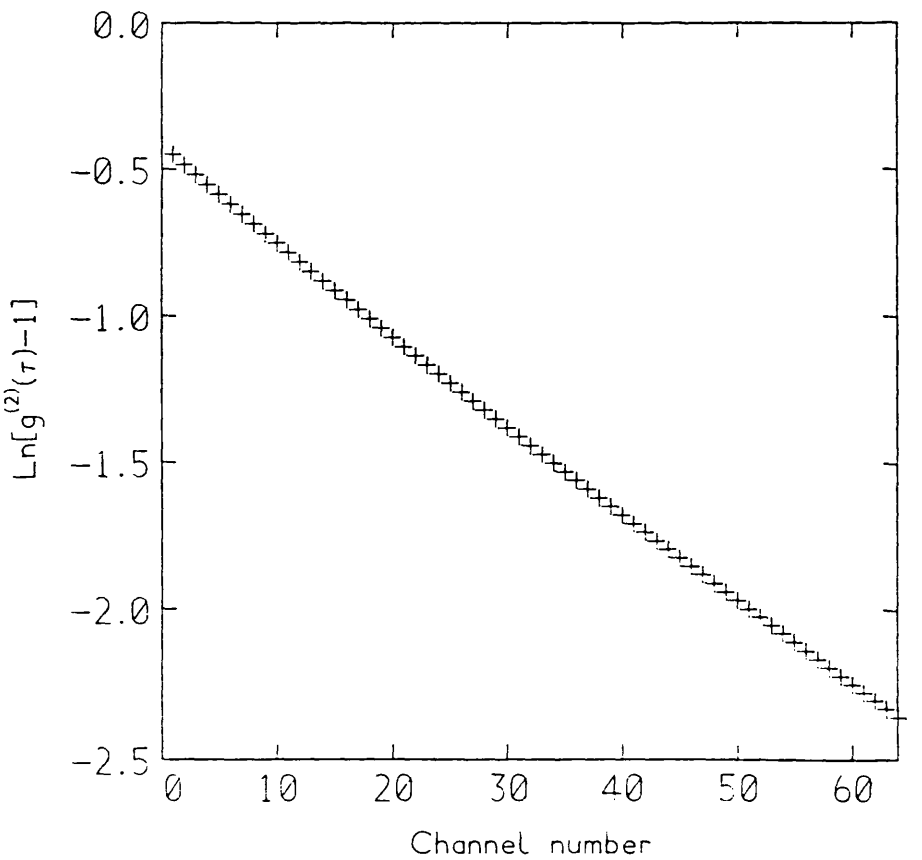

0.0
-0.5
-1.0
-1.5
-2.0
-2.5
0
10
20
30
40
50
60
Ln[g(2)(τ)-1]
Channel number

Table 5. 1. The effect of 3 mM Mg^{2+} on the s-value of an SMF preparation*

Mg^{2+} concentration (mM)	s-value	% change in s-value
0.2 (control)	176.0 ± 5.0	-
3.0	197.0 ± 5.0	+11

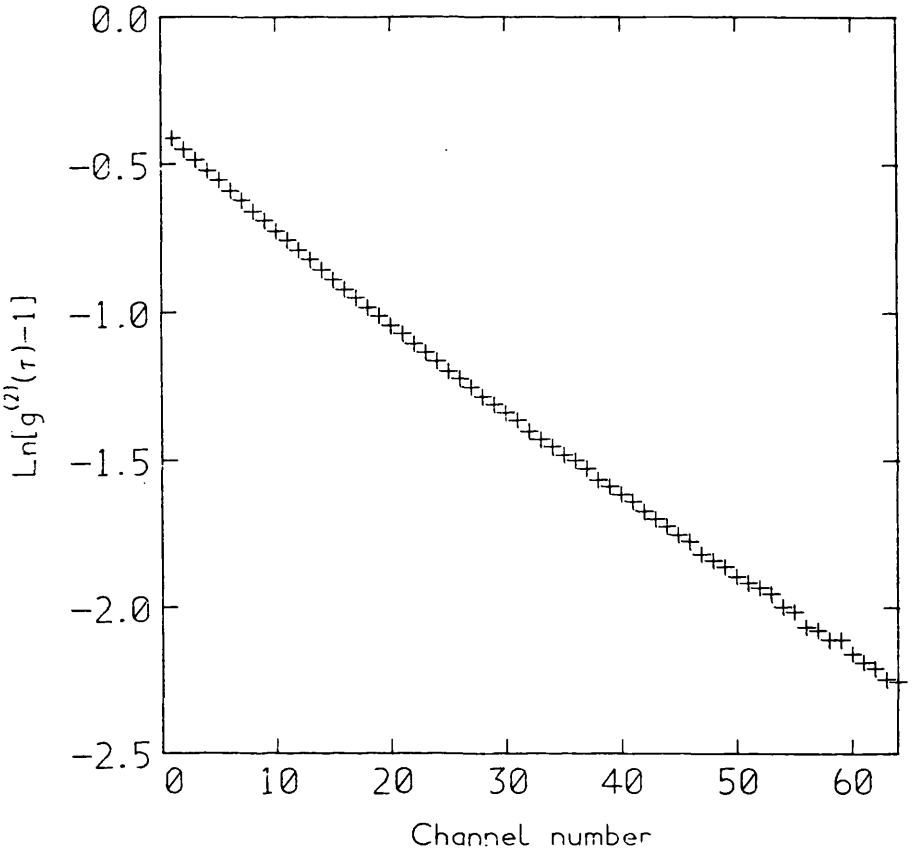
Graph 5. 2. A plot of the autocorrelation function against channel number for SMFs in 0.2 mM Mg^{2+} from which the D_{zt} is estimated to be (1.67 ± 0.02) m^2 sec^{-1} (See Table 5.2). The linear nature of the plot indicates that the preparation is highly monodisperse and free from aggregation.

Graph 5. 3. A plot of the autocorrelation function against channel number for SMFs in 3 mM Mg^{2+} from which the D_{zt} is estimated to be (1.92 ± 0.02) m^2 sec^{-1} (See Table 5.2). The increase in D_{zt} is not a result of filament aggregation as the plot has remained linear.

Table 5. 2. The effect of 3 mM Mg^{2+} on the Dzt of an SMF preparation*

Mg^{2+} concentration (mM)	D_{zt} (m^2 sec^{-1})	% change in s-value
0.2 (control)	1.67 ± 0.02	-
3.0	1.92 ± 0.02	+15

* The same SMF preparation at 0.5 mg/ml was used for both the determination of the s-value and the Dzt.


0.0
-0.5
-1.0
-1.5
-2.0
-2.5
0
10
20
30
40
50
60
Ln[g(2)(τ)-1]
Channel number

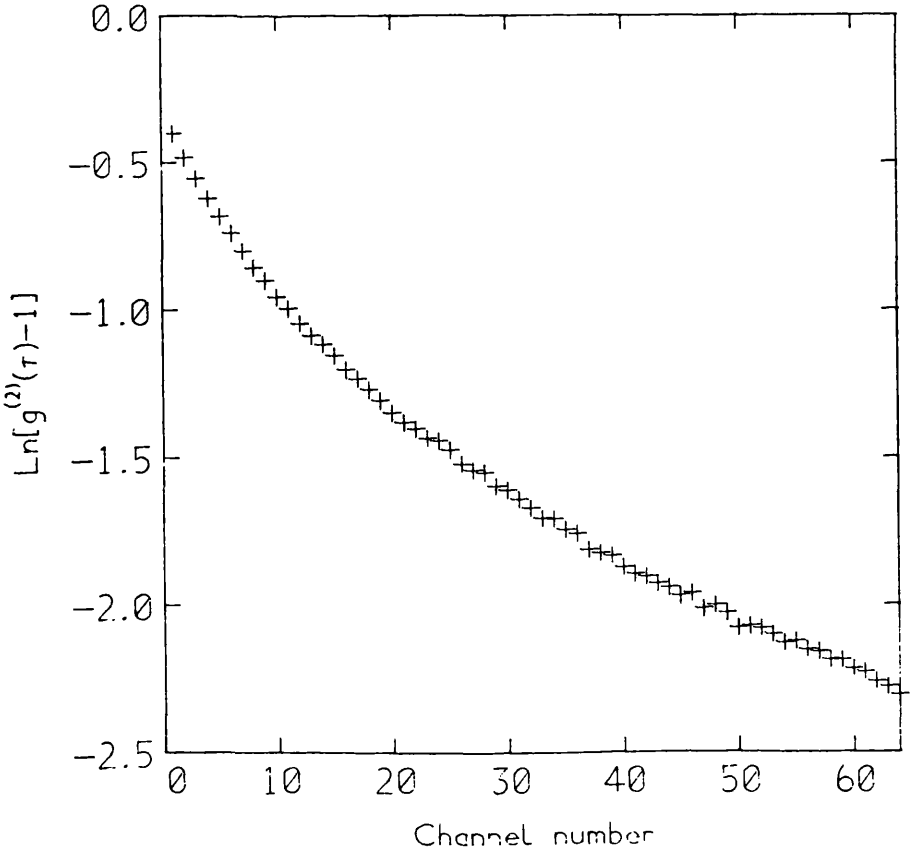

0.0
-0.5
-1.0
-1.5
-2.0
-2.5
0
10
20
30
40
50
60
Ln[g(2)(τ)-1]
Channel number

Table 5. 3. The effect of 50% RLC depletion on the s-value of PNFs (0.4 mg/ml).

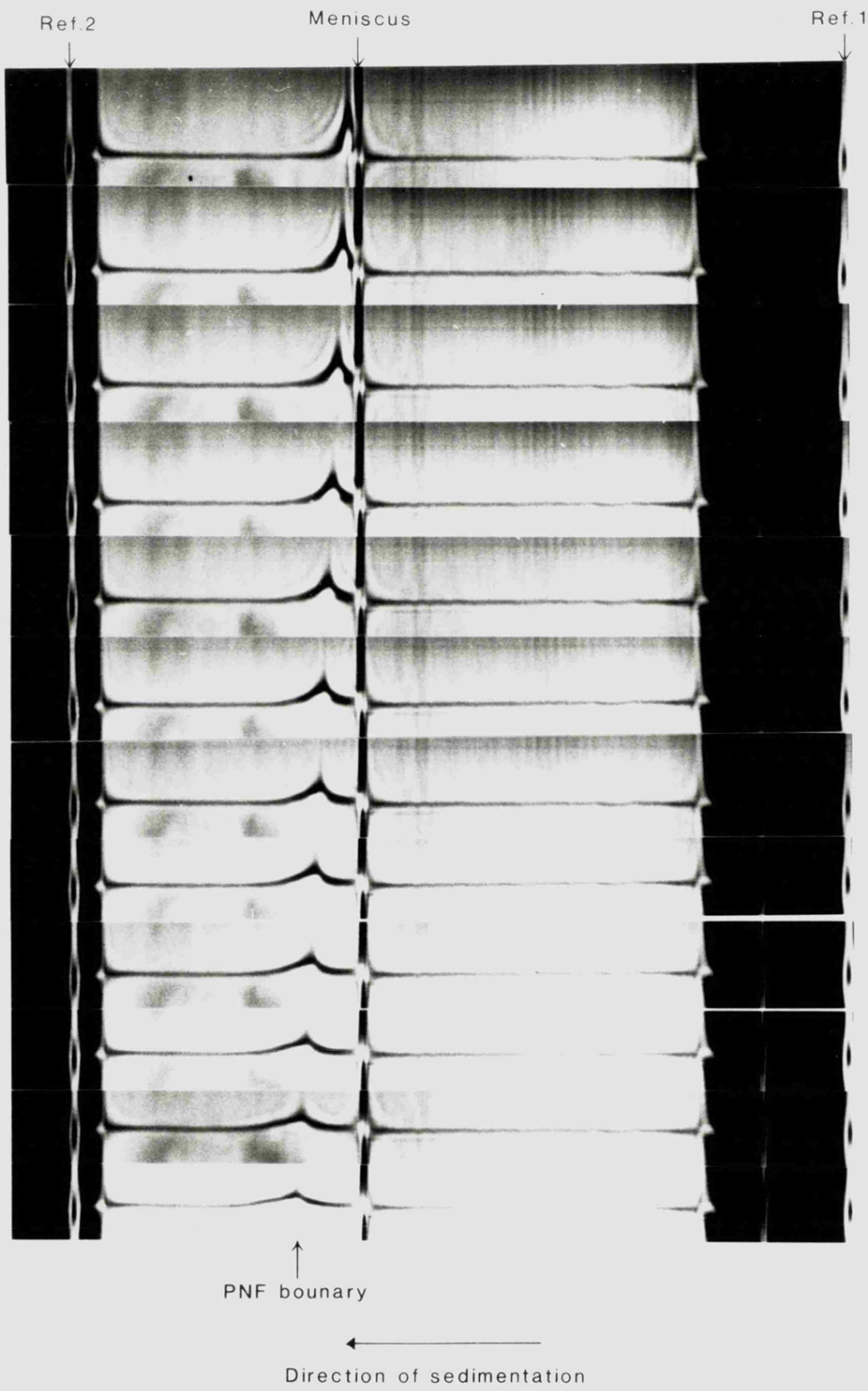
Sample used	s-value	% change in s-value
Control PNFs	126 ± 5.0	-
PNFs -50% of RLCs	189 ± 3.0	+49

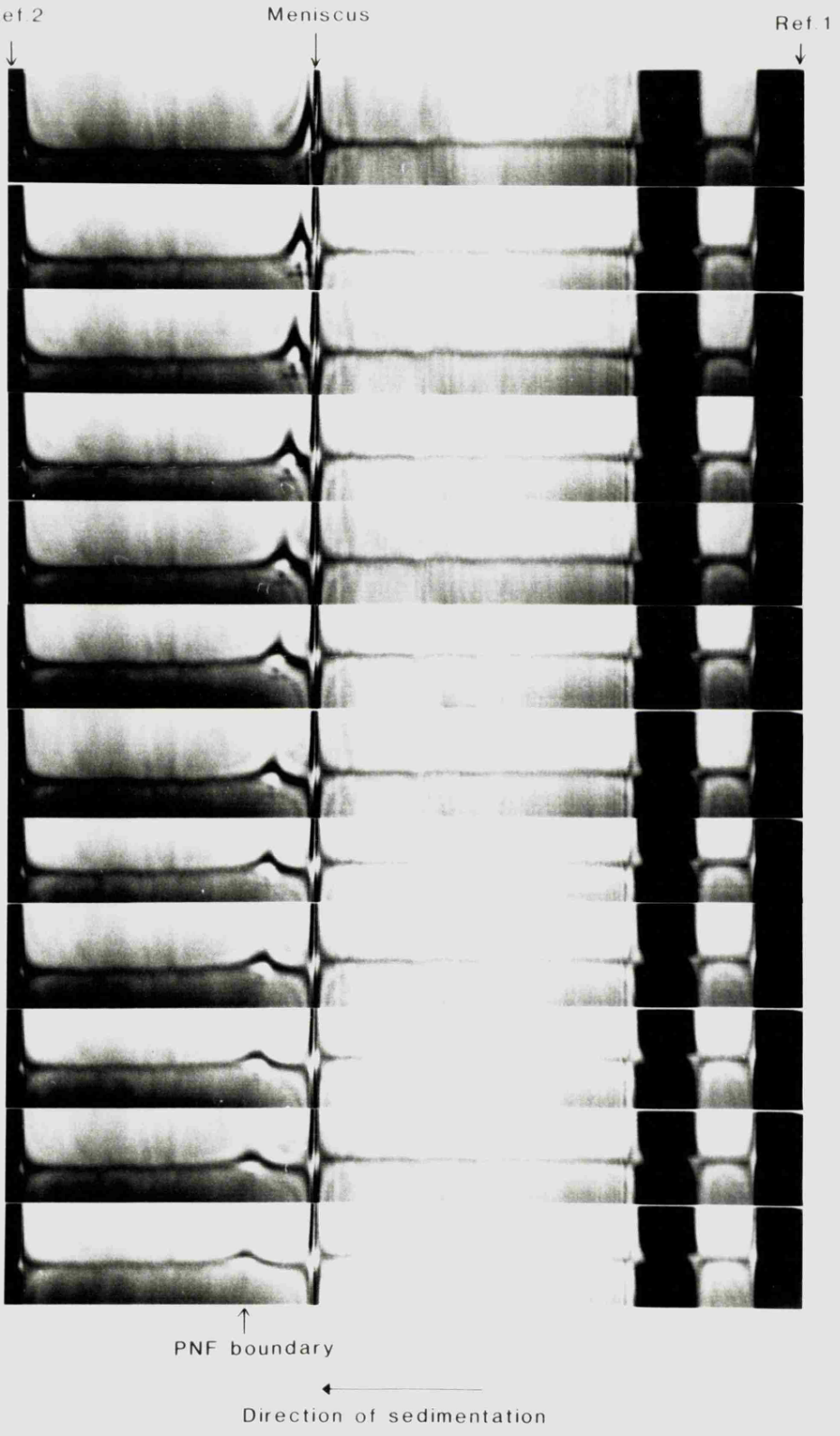
Graph 5. 4. A plot of the autocorrelation function against channel number of PNFs from which the D_{zt} is estimated to be (1.95 ± 0.02) m^2 sec^{-1} (See Table 5.4). The linear nature of the plot indicates that the preparation is highly monodisperse and free from aggregation.

Graph 5. 5. A plot of the autocorrelation function against channel number for 50% RLC depleted PNFs from which the D_{zt} is estimated to be (3.30 ± 0.5) m^2 sec^{-1} (See Table 5.4). Their is a slight increase polydispersity as indicated by the departure of the plot from linearity. This is inicative of the presence of a small amount of aggregation, however this was not large enough to be detected by sedimentation velocity analysis once again emphasising the sensitivity of QLS to small amounts of aggregation

Table 5. 4. The effect of 50% RLC depletion on the D_{zt} of PNFs (0.4 mg/ml)

Sample used	D_{zt}	% Change
Control PNFs	1.95 ± 0.07	-
PNFs -50% of RLCs	3.30 ± 0.5	+59


Ref.2
Meniscus
Ref.1
PNF bounary
Direction of sedimentation


Ref.2
Meniscus
Ref.1
PNF boundary
Direction of sedimentation

Schleiren trace 5.1. Showing the sedimentation of PNFs on the absence of Ca^{2+} (+EGTA).

Experimental details :-
Concentration = 0.3 mg/ml
Speed = 4990 r.p.m.
Temperature = 19 ^{o}C
Photograph time intervals = 360 seconds
Calculated s-value = 149.6 s (S.E. = 1.03)

Schleiren trace 5.2. Showing the sedimentation of PNFs on the presence of 100 μM free Ca^{2+}.

Experimental details :-
Concentration = 0.3 mg/ml
Speed = 4990 r.p.m.
Temperature = 19 ^{o}C
Photograph time intervals = 360 seconds
Calculated s-value = 157.6 s (S.E. = 2.01)

PNFs do possess a high affinity Ca^{2+} binding site.
Comparison of the s-value obtained in trace 5.1. with that obtained in 5.2. reveals that Ca^{2+} binding to the high affinity site has an effect on the s-value of PNFs. The graph clearly demonstrates that the addition of 100 μM Ca^{2+} results in approximately a 5 % increase in s-value.

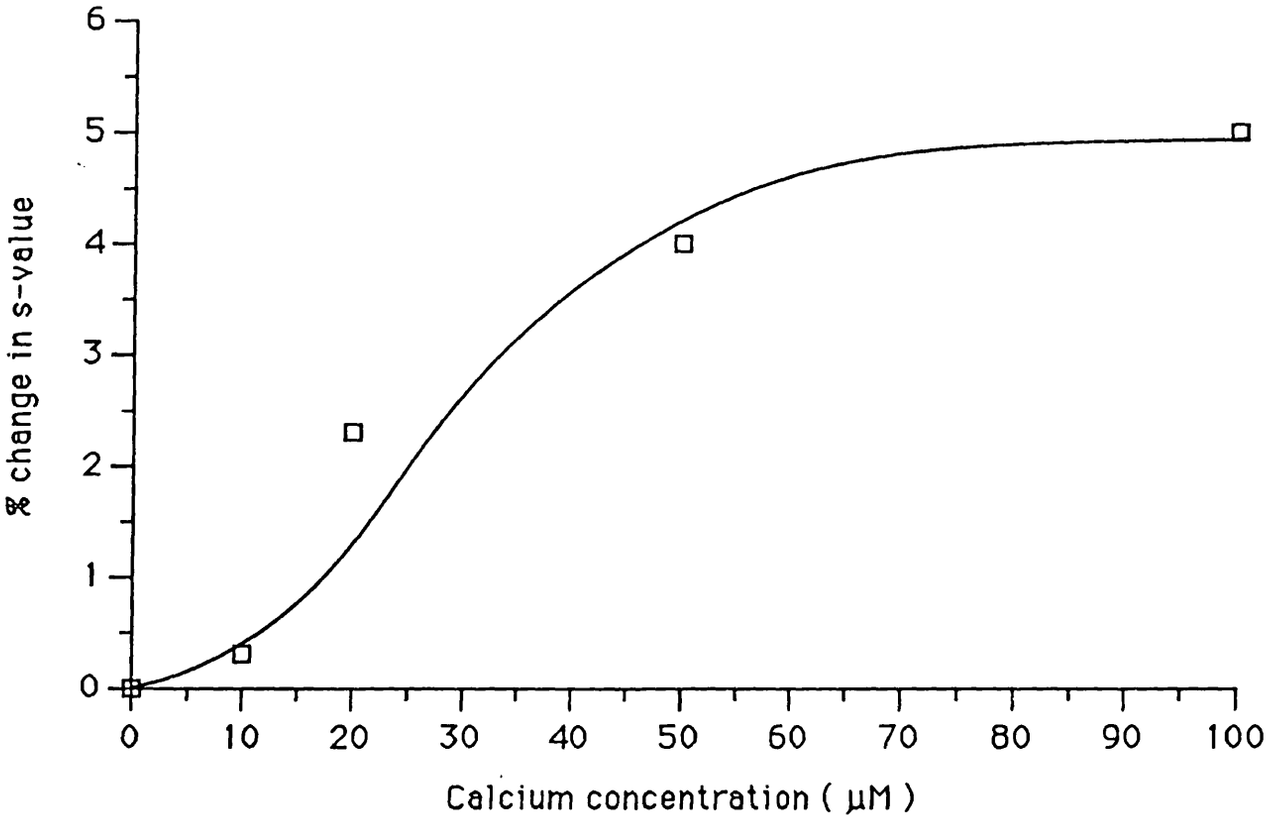

% change in s-value
0
1
2
3
4
5
6
0
10
20
30
40
50
60
70
80
90
100
Calcium concentration (µM)

Table 5.5

The effect that μM Ca^{2+} has on the s-value of PNFs in the presence of 0.1 mM Mg^{2+}.

Ca^{2+} concentration (μM)	S/S_0	% change in s-value
0 (control)	1.000	0
10	1.003	+0.3
20	1.024	+2.3
50	1.042	+4.0
100	1.053	+5.0

Experimental details :-

Concentration = 0.3 mg / ml

Speed = 4990 r.p.m.

Temperature = 19 ^{o}C

S0 = control s-value

S = experimental s-value

Graph 5.6. A plot of the data presented in Table 5.5. showing the titration curve for the binding of Ca^{2+} to the high affinity sites in PNFs.

Approximate value for the dissociation constant K_d = 25 - 30 x 10^{-6} M.

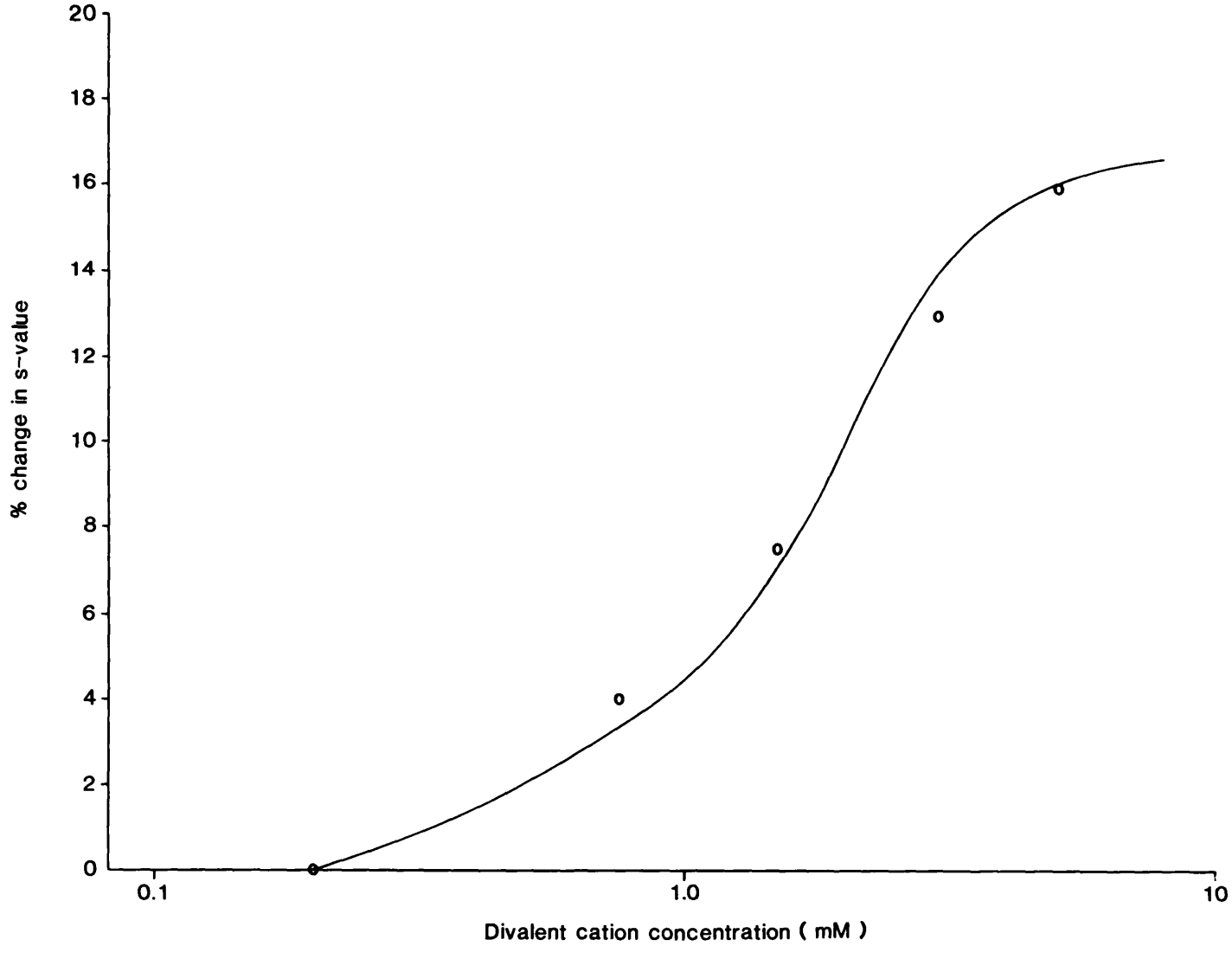

20
18
16
14
12
10
8
6
4
2
0
% change in s-value
0.1
1.0
10
Divalent cation concentration (mM)

Table 5.6

The effect that mM Mg^{2+} has on the s-value of PNFs in the absence of free Ca^{2+}.

Mg^{2+} concentration (mM)	S/S_0	% change in s-value
0.2 (control)	1.000	0
0.75	1.043	+4.1
1.5	1.081	+7.5
3.0	1.132	+12.7
5.0	1.190	+16.0

Experimental details :-

Concentration = 0.29 mg / ml

Speed = 5040 r.p.m.

Temperature = 20 ^{o}C

S0 = control s-value

S = experimental s-value

Graph 5.7. A plot of the data presented in Table 5.6. showing the titration curve for the binding of Mg^{2+} to the low affinity sites in PNFs when carried out in the absence of Ca^{2+}.

Approximate value for the dissociation constant $K_d = 1.6 \times 10^{-3}$ M.

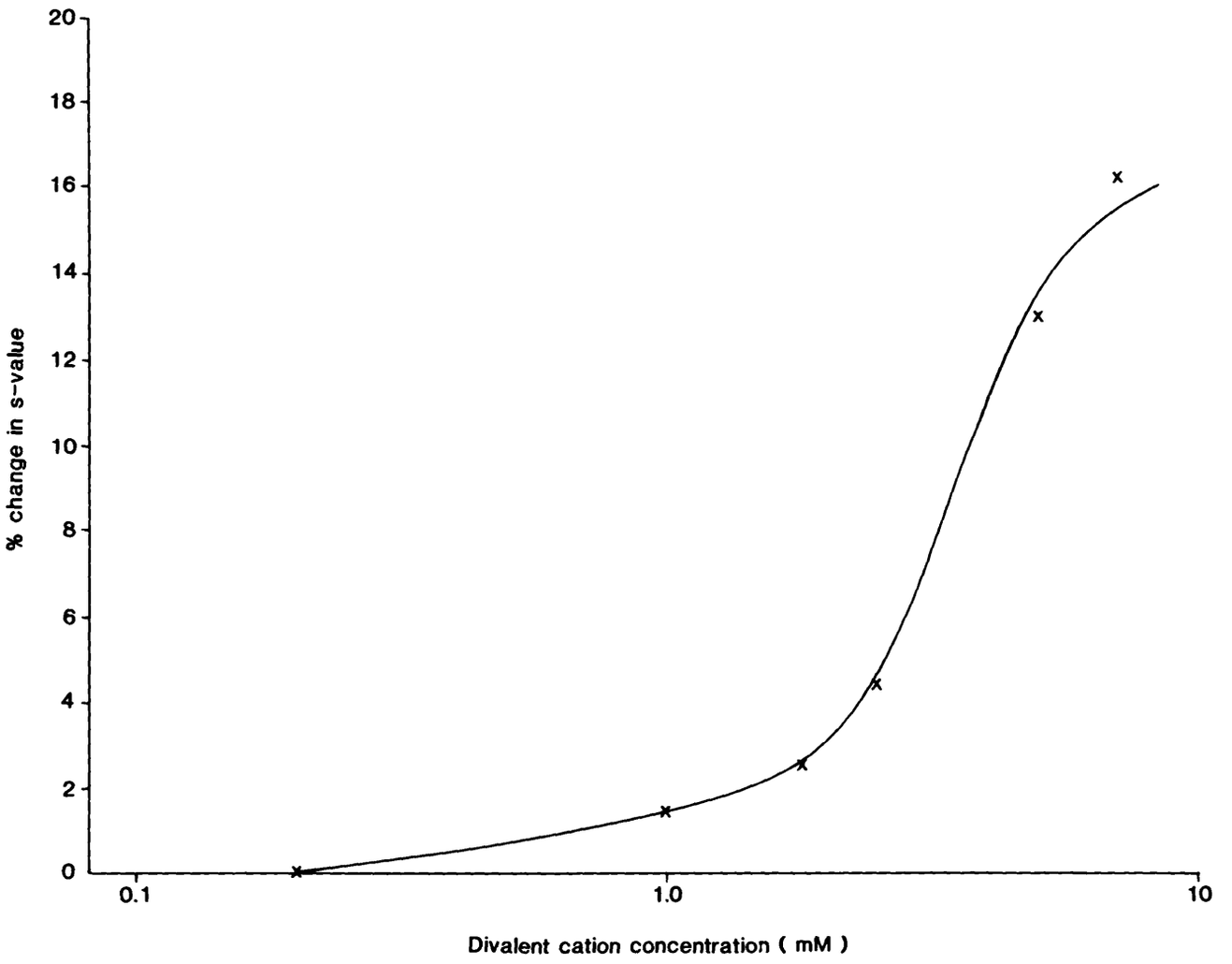

20
18
16
14
12
10
8
6
4
2
0
% change in s-value
0.1
1.0
10
Divalent cation concentration (mM)

Table 5.7

The effect that mM Ca^{2+} has on the s-value of PNFs in the absence of free Mg^{2+}.

Ca^{2+} concentration (mM)	S/S_0	% change in s-value
0.2 (control)	1.000	0
1.0	1.014	+1.4
1.8	1.026	+2.5
2.5	1.046	+4.4
5.0	1.154	+13.0
7.0	1.195	+16.3

Experimental details :-

Concentration = 0.28 mg / ml

Speed = 4990 r.p.m.

Temperature = 20 ^{o}C

S0 = control s-value

S = experimental s-value

Graph 5.8. A plot of the data presented in Table 5.7. showing the titration curve for the binding of Ca^{2+} to the low affinity sites in PNFs when carried out in the absence of Mg^{2+}.

Approximate value for the dissociation constant $K_d = 3.4 \times 10^{-3}$ M.

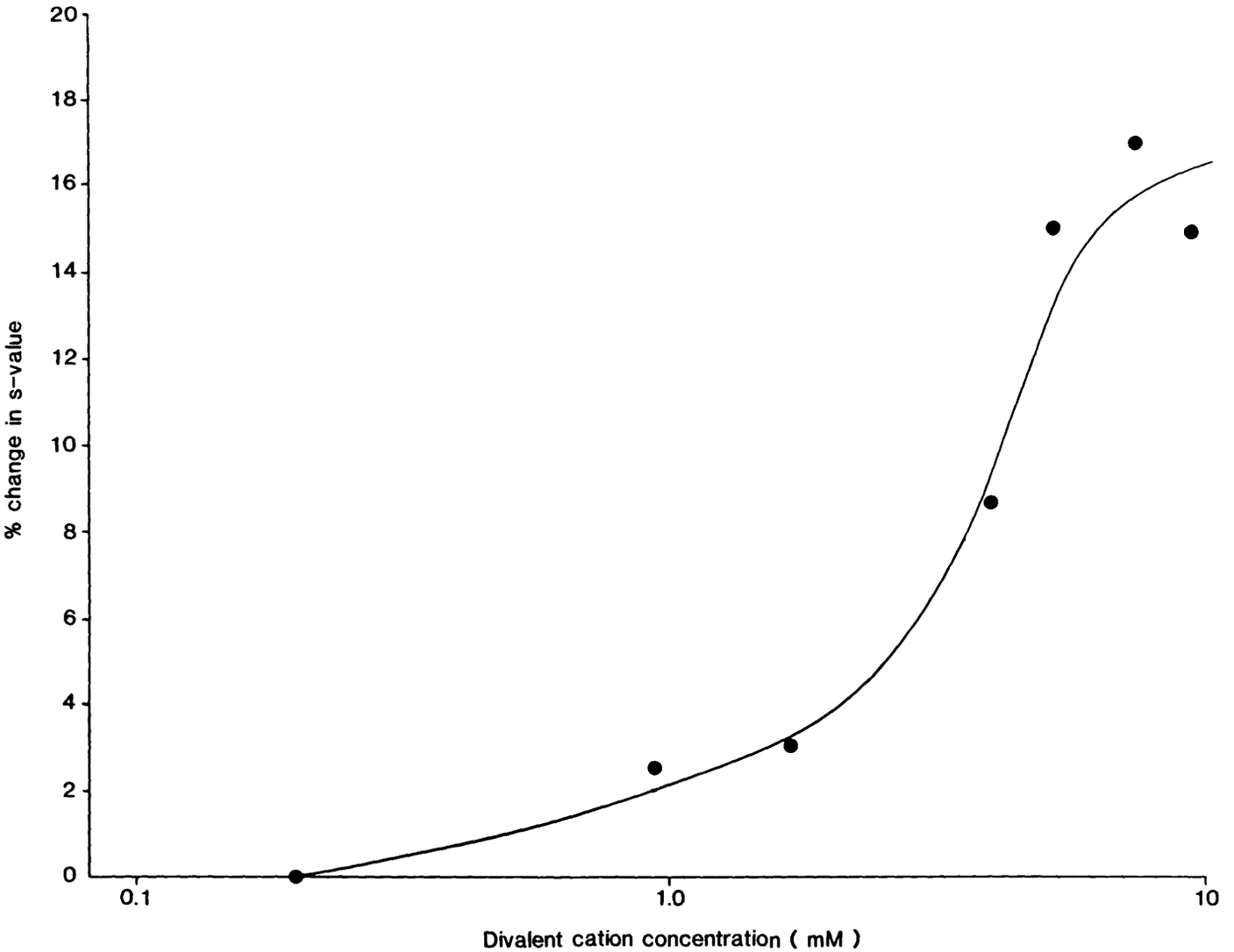

20
18
16
14
12
10
8
6
4
2
0
% change in s-value
0.1
1.0
10
Divalent cation concentration (mM)

Table 5.8

The effect that mM Mg^{2+} has on the s-value of PNFs in the presence of 100 μM free Ca^{2+}.

Mg^{2+} concentration (mM)	S/S_0	% change in s-value
0.2 (control)	1.000	0
0.95	1.026	+2.6
1.7	1.032	+3.1
4.2	1.098	+8.9
5.2	1.178	+15.1
7.2	1.205	+17.0
9.2	1.177	+15.0

Experimental details :-

Concentration = 0.28 mg / ml

Speed = 5000 r.p.m.

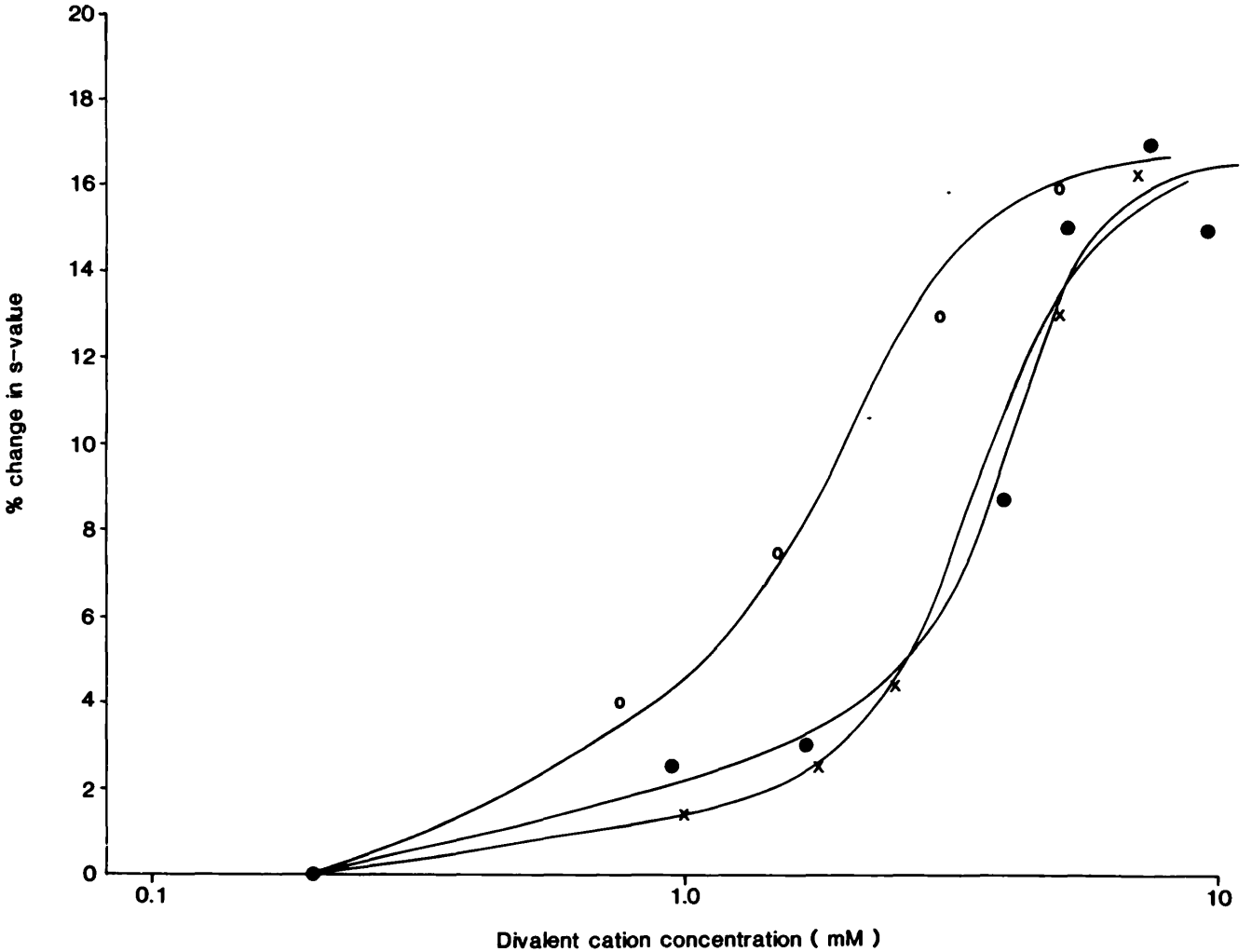
Temperature = 20 ^{o}C

S0 = control s-value

S = experimental s-value

Graph 5.9. A plot of the data presented in Table 5.8 showing the titration curve for the binding of Mg^{2+} to the low affinity sites in PNFs when carried out in the presence of 100 μM Ca^{2+}.

Approximate value for the dissociation constant $K_d = 3.6 \times 10^{-3}$ M.


20
18
16
14
12
10
8
6
4
2
0
% change in s-value
0.1
1.0
10
Divalent cation concentration (mM)

Graph 5.10. A superimposition of Graphs 5.7, 5.8 and 5.9 to show the effect that calcium binding to the high affinity site has on the characteristics of the low affinity site.

o : Titration curve in the presence of mM Mg^{2+} only. (Kd = 1.6×10^{-3} M)

X : Titration curve in the presence of mM Ca^{2+} only. (Kd = 3.4×10^{-3} M)

● : Titration curve in the presence of mM Mg^{2+} and μM Ca^{2+}. (Kd = 3.6×10^{-3} M)

The titration curve for the low affinity site in the presence of mM Mg^{2+} only is essentially identical to the curve obtained by Persechini and Rowe (1984) when using SMFs. The graph clearly shows a shift in the Kd of the low affinity site of PNFs when calcium is present. Persechini and Rowe (1984) however found no evidence of such an effect in SMFs.

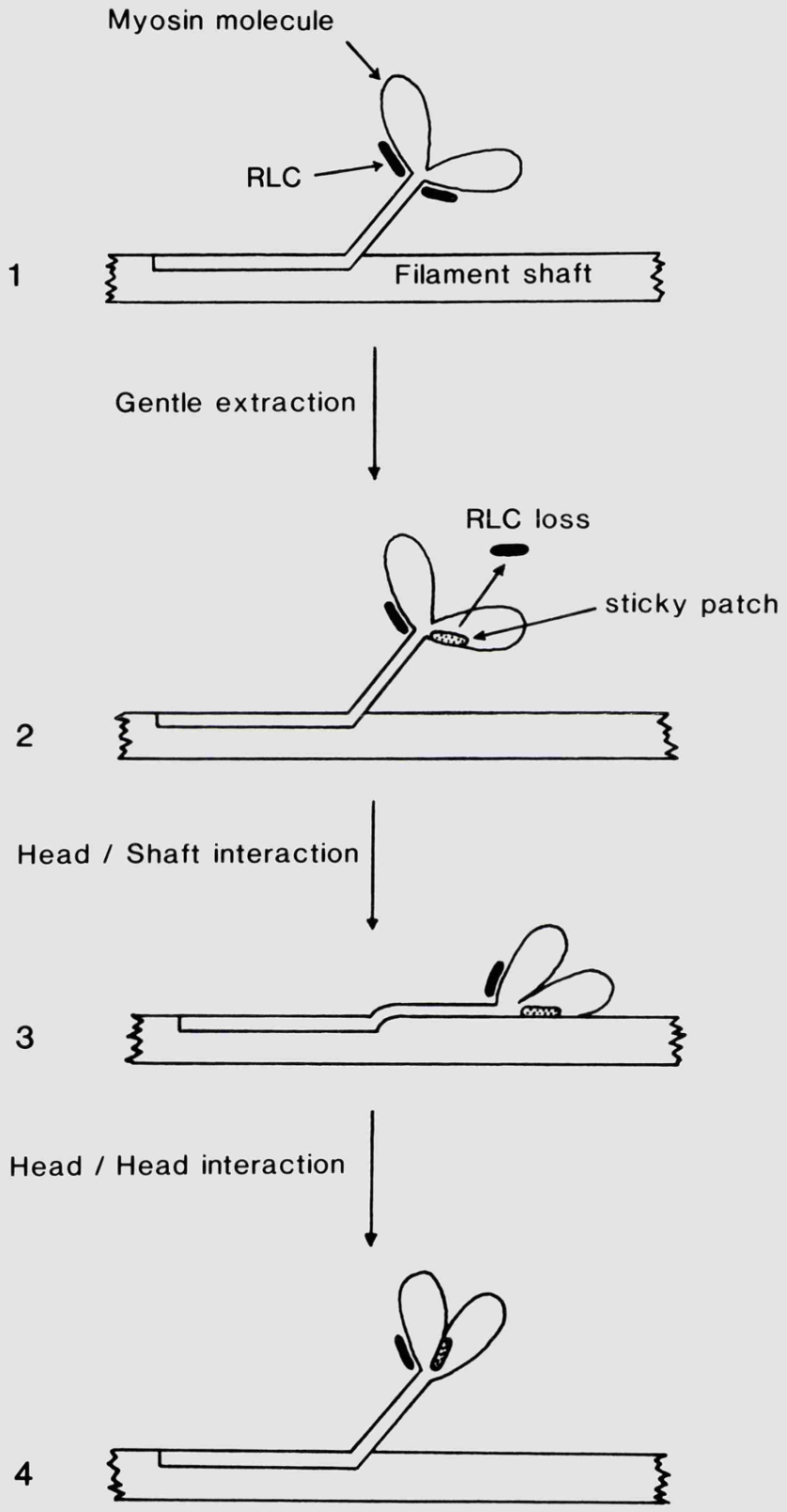

Myosin molecule
RLC
1
Filament shaft
Gentle extraction
RLC loss
sticky patch
2
Head / Shaft interaction
3
Head / Head interaction
4

Figure 5.1

Diagramatic representation of the possible effects that RLC removal has on the cross bridges in a myosin thick filament.

1. A myosin molecule (cross bridge) containing its full compliment of RLCs* projects from the shaft of the thick filament.

2. Incubation in the gentle extraction medium results in the loss of one RLC from the cross bridge. The exposed RLC binding site on the denuded head forms a 'sticky patch'.

3. The exposed RLC binding site interacts with the thick filament shaft and sticks the cross bridge down. Such a process would prevent cross bridge visualisation in the electron microscope.

4. The interaction of the exposed RLC binding site with the other S1 head would not prevent cross bridge visualisation in the electron microscope and therefore we feel that such an interaction does not, to any great extent, take place.

* For clarity the alkali light chains are not shown in this diagram.

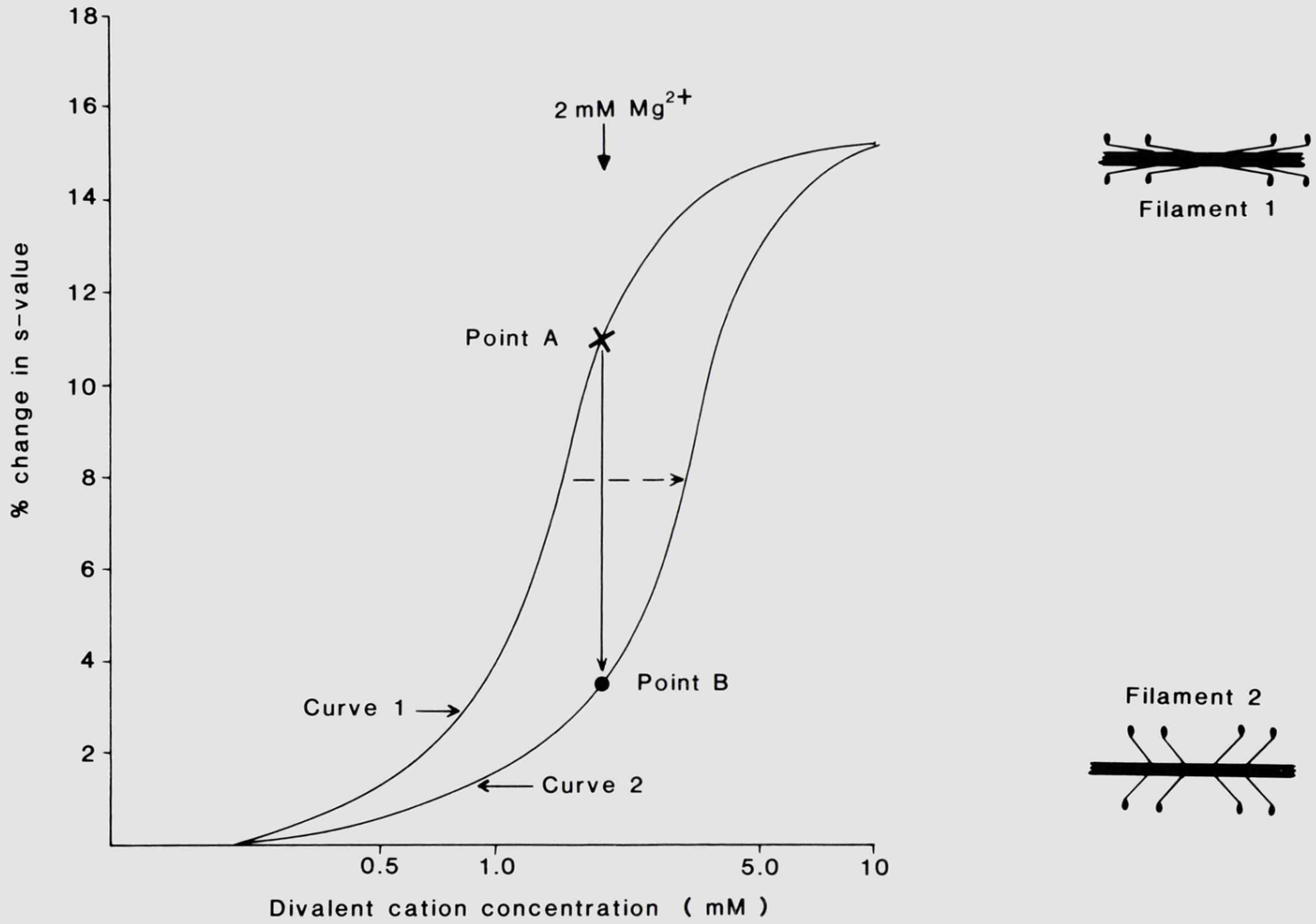

18
16
14
12
10
8
6
4
2
% change in s-value
2 mM Mg2+
Point A
Point B
Curve 1
Curve 2
0.5
1.0
5.0
10
Divalent cation concentration (mM)
Filament 1
Filament 2

Figure 5.2

Schematic representation of the effect that calcium binding to the high affinity sites has on the low affinity sites and what this possibly means in terms of cross bridge disposition.

Curve 1 : Titration curve for the low affinity sites in the absence of free calcium (i.e. muscle at rest).

Curve 2 : Titration curve for the low affinity sites in the presence of 100 μm calcium (i.e.during contraction).

Point A : Position of cross bridges assuming 2 mM free Mg^{2+} (no calcium).

Point B : Position of cross bridges assuming 2 mM free Mg^{2+} (+ calcium).

A diagramatic representation of the radial disposition of the cross bridges at high (Filament 1) and low (Filament 2) divalent cation concentrations is shown to the right of the graph.

Chapter VI

Concluding remarks.

The main conclusions to be drawn from the present study are;

i.

That native vertebrate skeletal muscle myosin posseses two distinct but equally distributed populations of RLCs (See Chapter III). This was first demonstrated by Chin (1981) who showed that each population had a different susceptibility to chymotryptic digestion. We have now developed a technique (the gentle extraction technique) for the removal of one of these populations using mild, non-enzymic conditions which have allowed the role of the RLCs in filament assembly (See Section V. 6 and V. 16) and in regulating the ATPase activity (See Section III. 31 and III. 32) to be investigated.

The extraction technique is equally successful at removing 50% of the RLCs from both rabbit and rat RFPs and rabbit PNFs. Moreover transient exposure to high salt conditions radically alters the ability to remove the RLCs using this technique. After high salt exposure the RLCs in rabbit preparations become completely resistant to extraction and behave in an identical manner to the RLCs present in SMFs that have been prepared from purified monomeric myosin. However the RLCs in rat preparations become completely susceptible to removal and under the appropriate conditions all of the RLCs can probably be removed.

Although the response of the rabbit and rat myosin to high salt

exposure, as judged by the susceptibility of their RLCs to extraction, is different the important point to be made is that there is an effect and that it is irreversible.

ii.

We have shown that it is possible to reconstruct full length myosin filaments from BZAs and their distal myosin molecules, and that the reassembly is equally successful with rat and rabbit filaments (See Chapter IV). In both cases the reassembled filaments fray into 3 sub filaments when exposed to low ionic strength conditions in an almost identical manner to the native control filaments. We have taken this as an indication that the reassembled filaments have a similar if not identical quaternary structure to that of native filaments. This, combined with the fact that the reasembled filaments contain RLC populations that are identical to those found in conventional SMFs (which do not possess the native quarternary structure), implies that the loss of distinctive properties is due to a change in the tertiary and not the quaternary structure.

iii.

The RLCs are intimately involved in the regulation of the myosin ATPase activity by calcium (See Section III. 28 - 32). After the removal of 50% of the RLCs from rabbit PNFs the calcium sensitivity of the ATPase was reduced by approximately 50%. However there was very little effect on the basal rate, suggesting that the RLCs are directly involved in switching on the ATPase in response to calcium and are not necessary for the basic functioning of the hydrolytic site.

Similar results were obtained in the presence and absence of pure unregulated actin although the absolute magnitude of the ATPase activity was much higher in the presence of actin.

Hydrodynamic studies have revealed that all myosin molecules are identical in that each myosin molecule loses a single RLC during the gentle extraction procedure. As the removal of 50% of the RLCs results in a 50% loss in calcium sensitivity and each molecule loses a single RLC this probably means that the S1 heads that still retain their RLCs also retain their calcium sensitivity. Therefore each myosin S1 head in the intact molecule is equally calcium sensitive and is able to act independently of the others.

iv.

The removal of the RLCs from PNFs using the gentle extraction technique does indeed expose a 'sticky patch' which hydrodynamic and electron microscopical analysis reveals sticks the RLC depleted S1 heads down onto the thick filament shaft (See Section V. 14). However sedimentation velocity analysis of RLC depleted myosin in monomeric solution shows that head-head interaction does not occur, which has been confirmed using electron microscopy of the thick filaments. Furthermore the apparent ionic strength dependence of the properties of the so called sticky patch suggests that it does not interact via hydrophobic residues but rather through electrostatic interactions.

v.

PNFs have been shown to possess a high affinity (Kd = 30 μM) calcium binding site and when this site is saturated a 5% increase in s-value is

observed (See Section V. 28). Changes in the s-value have been tentatively interpreted in terms of alterations in the cross bridge properties (ie. elasticity and radial disposition). A low affinity divalent cation binding site (similar to that present in SMFs as described by Persechini and Rowe (1984)) was found to be present in PNFs with up to approximately a 15% increase in s-value occuring with increasing cation concentration. However unlike the site in SMFs its Kd is modulated by calcium binding to the high affinity site.

We have used the interaction between the high and low affinity sites to propose a mechanism by which the properties of the cross bridges can be altered during contraction and relaxation, in such a way as to favour or hinder the extent to which the cross bridges can interact with the thin filaments.

Recent studies into the mechanical physiology of skinned rabbit psoas have shed further light into the role of calcium in the activation of skeletal muscle and on the possibility of the presence of dual regulatory systems. Farrow et al (1988) looked at the effect that calcium ions had on the unloaded contraction velocity (Vmax) and also made measurements of parameters linked to the cross bridge cycling rates. They found that their results were consistent with the suggestion that calcium activation initially occurs via the classical switch on the thin filament followed at higher calcium concentration, when the thin filament is fully switched on, by a modulation of the steady state kinetics of cross bridge interactions. This has been supported by Brenner (1988), who measured force redevelopment after a period of isotonic shortening along with the force, stiffness and ATPase activity during isometric contraction, and demonstrated that

calcium modulated the cross bridge turnover kinetics but did not alter the number of cross bridges that were cycling. Brenner (1988) suggested that changes in turnover kinetics may represent a further mechanism of regulation in addition to that which involves changes resulting from calcium binding to TnC (i.e. another mechanism for the modulation of contractile function). Although he does not explicitly say that there may be a thick filament linked regulatory system it is difficult to envisage where an additional regulatory system could reside other than on the thick filament.

These results directly contradict earlier studies (Brenner., 1980; Gulati and Podolsky., 1981) who favour the idea that the kinetics of cross bridge interaction are fixed and that the cross bridges are either switched in or out of the cross bridge cycle, a process known as 'recruitment'. Indeed Brenner (1988) is careful to point out that at 25-30% maximal calcium activation his measurements are not sensitive enough to eliminate the possibility that recruitment is taking place: in which case he suggests that a dual mechanism might exist, with recruitment initially bringing the cross bridges into the cycle followed by an alteration in the interaction kinetics at higher calcium concentrations.

It is evident that mounting evidence, both physiological and biochemical now supports a model for activation and modulation of contractile function that is more sophisticated than a simple switch residing on the thin filaments. One can clearly now speculate that the changes in cross bridge elasticity that we believe may occur under the influence of divalent cation (See Section V. 35) could well be one of or indeed the mechanism by which the kinetics of cross bridge interaction

are modulated.

Future prospects.

In this thesis evidence has been provided that demonstrates the presence of a calcium sensitive thick filament linked regulatory system in vertebrate skeletal muscle that is probably mediated via the RLCs. However all of our experiments have been carried out on PNFs that are not phosphorylated, that is the phosphorylatable RLC has in all cases been in the dephosphorylated state. Studying the properties of PNFs when in their phosphorylated state would seem to be the next logical step to take. Over the past decade there has been increasing evidence to suggest that phosphorylation of the RLCs in vertebrate myosin may in fact be an important process in the regulation of the contractile event.

Initially, Barany and Barany (1977) observed an increase in the level of RLC phosphorylation during the onset of contracture. They noted that the RLCs in frog muscle fibres underwent an 85% increase in the level of phosphorylation. Barany et al (1979) later showed that the amount of phosphorylation present followed the rise in tension and for short periods of tetani dephosphorylation followed relaxation. However after a long tetanic contraction dephosphorylation lagged behind relaxation, which they suggested may be related to a delay in the reduction of calcium levels or indeed to 'post tetanic potentiation'.

The phenomenon of post tetanic potentiation was first described by Brown and Euler (1938) and later in greater detail by Close and Hoh (1968) who demonstrated that after repeated stimuli a transient

increase in peak isometric twitch tension was observed. Manning and Stull (1979) confirmed this finding using rat soleus muscle and also demonstrated that the post tetanic twitch tension decreased at a rate comparable to that of RLC dephosphorylation. Manning and Stull (1982) also found that the amount of RLC phosphorylation and peak twitch tension in both fast and slow twitch muscle increased rapidly in a temporal fashion after the cessation of stimulation. They suggested that although RLC phosphorylation was not essential for contraction to occur, it may play a role in the potentiation of peak twitch potential.

The phosphorylation of the RLCs could conceivabley affect twitch potentiation by altering the affinity of the myosin for actin. Such a possibility was substantiated by Szczensa et al (1987) who showed that skeletal muscle HMM had a 2.5x weaker affinity for F-actin when its RLCs were phosphorylated. Such alterations in affinity could well be indicative of a slightly altered myosin conformation when phosphorylated. Indeed electron microscopy has shown that phosphorylated HMM decorates F-actin with blunted arrow heads whilst dephophorylated HMM produces the classical barbed arrowheads (Stepkowski et al., 1985) strongly suggesting an altered myosin conformation. The change in the actin decoration pattern is very similar to that observed when the RLCs are completely removed fom HMM which also produces blunted arrow heads (Craig et al., 1980), See Section III. 9). However the significance of this similarity in terms of myosin conformation still remains unclear.

Biochemical studies have been somewhat at variance over the issue of the effect that RLC phosphorylation has on the ATPase of vertebrate skeletal myosin. Pemrick (1977) suggested that phosphorylation is a

modulator of the ATPase and results in up to a 181% increase in its activity whilst others (Morgan et al., 1976; Pires and Perry., 1977; Stull et al., 1980) have found no effect of phosphorylation on the ATPase activity. Such differences of opinion still exist today and although it is difficult to reconcile such differences in the in vitro results the great weight of evidence from the work on intact muscle fibres suggests that phosphorylation of the RLCs plays an important role in the contractile mechanism and as such may represent yet another form of thick filament regulation. Persechini and Stull (1984) provided a possible explanation for the variation in the biochemical results when they showed that in myosin prepared by conventional means (high salt extraction) there was a rapid loss of phosphorylation effects and after 5 days they had completely disappeared. Therefore unless all groups were using very fresh myosin of exactly the same age the interpretation and comparison of results may not be strictly valid.

It should in principle be possible by the use of PNFs for the biochemistry of the effects of RLC phosphorylation to be studied in filaments whose quaternary structure is identical to that of the filaments in the intact muscle lattice, and the effects of phosphorylation may not decline as rapidly in PNFs as when using conventionally purified myosin. After all native myosin is irreversibly altered by high salt exposure so it is possible that the effects of phosphorylation are lost or altered by the same process.

The work carried out to date, in the absence of phosphorylatory mechanisms, has served to clarify the possible direct role of the RLCs in the modulation of contraction in vertebrate skeletal muscle. A definition of the more indirect additional effects of phosphorylation

should enable us to construct a relatively complete model for thick filament associated modulation of force generation in the muscle systems responsible for movement, maintenance of posture and cardiac function in the vertebrates.

Bibliography

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