THE STRUCTURE OF A-FILAMENTS FROM VERTEBRATE STRIATED MUSCLE AND THEIR RECONSTRUCTION INTO A-BANDS

A Thesis submitted for the Degree of Ph.D by

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March, 1982.
ACKNOWLEDGEMENTS

I would like to thank the following people for their help and kindness during my stay in Leicester.

Dr. Arthur J. Rowe, my supervisor, for the support and advice he has given me with regards my work, this thesis and my career. I am also grateful for the numerous opportunities he has made for me to visit meetings and other laboratories.

Members of the Biochemistry Department at Leicester, especially Dr. Clive R. Bagshaw, Prof. W.V. Shaw, Dr. G. Turnock and Dr. Jill Thompson for their help and useful discussions.

Many thanks also to Evelyn Roberts, Pam Kramer, George McTurk, Chris Townsend, Dr. Muriel Walker, Dave Wright and Stefan Hyman for their friendship and help in the Electron Microscope laboratory.

To other members of Dr. A.J. Rowe's laboratory, past and present, best wishes and good luck.

Dr. H.E. Huxley, Dr. R. Padron, Prof. G.F. Elliott and Dr. Elsa Bartellis for their help with the X-ray diffraction work.

Maxine Clarke, Lawrence Wale and Peter Watt for their friendship and donations of Lethocerus indicus, CAF and labelled rat psoas muscle respectively.

Dr. Carole McKenzie, Dr. Mark Nowel and Dr. Steve Hill, ex-members of the Zoology Department, for their support and friendship.

To the members of Computer Studies Unit for so patiently teaching and helping me to use the word processor.

Special thanks to David Tomley for proof-reading this thesis.

Finally my thanks and best wishes to my mother for her support, and to all the friends who have made my stay in Leicester so enjoyable.
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**Part 2**

Reconstruction of A-Filaments and A-Bands

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CHAPTER 1 INTRODUCTION TO MUSCLE STRUCTURE

Numerous structural studies have been made of vertebrate skeletal (or striated) muscle: the most commonly used muscles being the sartorius and gastrocnemius of frog and the psoas of rabbit. Information gained from vertebrate skeletal muscle from different species is usually considered to be interchangeable.

The primary function of muscle is contraction and it is brought about by the interaction of two proteins – myosin and actin. Hydrolysis of ATP by myosin provides the energy to produce tension. To understand the mechanism of muscular contraction three major problems need to be solved:– 1) How is the splitting of ATP converted into mechanical force? 2) How is this conversion controlled? and 3) What is the molecular structure of the mechanical components and how are these structures assembled and maintained? This present study aims to elucidate and clarify some of the points raised by the third question.

1.1 Muscle Structure

Muscle is composed of multinucleate cylindrical myofibres (which range in diameter from 10 to 100μm and extend up to several centimetres in length) and these in turn are composed of a system of elongated elements 1–2μm in diameter called myofibrils (Figure 1). Light microscopy reveals that each myofibril is subdivided (by regularly spaced dense Z-discs) into repeating units known as sarcomeres. With the development of the phase contrast microscope, vertebrate skeletal muscle was shown to have a striated pattern consisting of two bands alternating down the length of the myofibrils with the bands in register across the fibre. At each end of a sarcomere is a less dense region forming the I-band (so called as it appears isotropic). Between these is the more dense A-band (so called because it appears anisotropic, i.e. having a refractive index which is not the same in every direction). The A-band itself has a central less dense H-zone which in its turn has a central more dense M-line (Figure 1). Hanson and Huxley(1955) found that hypertonic salt solutions dissolved the band structures. Selective removal
of the proteins myosin and actin (by 0.6M KCl and 0.6M KI respectively) from muscle enabled myosin to be identified with the A-band and actin with the I-band. Hasselbach (1953) and Hanson and Huxley (1953) proposed that the material of the anisotropic bands was present as some form of submicroscopic rods.

In the light microscope A.F. Huxley and Niedergerke (1954) and H.E. Huxley and Hanson (1954) noted that changes in the cross-striations of muscle occurred on stretching and contraction. During stretching the sarcomere length increased while a decrease occurred on isotonic contraction. In both cases the length change only affected the width of the I-bands. No change in the band widths resulted from isometric twitches. Electron microscopy of longitudinal sections of muscle fibres demonstrated that the basis of the banding lay in a highly ordered arrangement of myofilaments within each sarcomere. The presence of myosin filaments within the A-band was confirmed and it was postulated that the filaments extending through the I-bands were composed of actin. The I-bands contain only actin filaments and the H-zones only myosin filaments (Figure 1), and between these but within the A-bands are the regions where the myosin and actin filaments overlap. These observations led both groups of workers to independently propose the sliding filament model for contraction. They hypothesised that during muscle contraction the actin filaments were drawn into the A-bands between the myosin filaments. If the relative force between actin and myosin filaments is generated at each of a series of points in the region of overlap in each sarcomere then the tension developed per filament should be proportional to the number of these points and therefore the width of the overlap zone, i.e. isometric tension would decrease with increasing sarcomere length (Huxley and Niedergerke, 1954; A.F. Huxley, 1957). In a more detailed electron microscope study H.E. Huxley (1957) characterised the double hexagonal array of myofilaments (a "thick" filament is surrounded by a hexagonal array of six "thin" filaments). He confirmed that the thick (myosin or A-filaments) and thin (actin or I-) filaments interdigitated (Figure 1) and that the degree of overlap was proportional to the extent of contraction. Furthermore H.E. Huxley observed cross-bridges connecting the two filament types and proposed that these connections formed the molecular basis of contraction. Page (1964) and Page and Huxley (1963) on studying filament length changes proved that the 10% change in A-filament length observed by Carlsen, Knapeis and Buchtal (1961) and predicted by them to be the method by which muscle contracts, arose due to the preparative procedures
used for electron microscopy.

1.2 The Thin Filaments

Actin is a globular protein of molecular weight 41,700 (Elzinga, Collins, Kuehl and Adelstein, 1973). The monomer (G-actin) can polymerize into a filamentous form, F-actin, a right-handed two-start helix which closely resembles the native thin filament. In muscle the thin filaments are approximately 1μm in length and connect to the Z-disc. Synthetic (F-actin) filaments and native I-filaments are unidirectional as shown by myosin or heavy meromyosin decoration (Huxley, Holmes and Brown, 1965). The I-band is bipolar indicating that the Z-disc must produce a reversal in the polarity of the thin filaments in the sarcomere. As in the A-band this property is necessary for the sliding filament model. The characteristic long periodicity of the I-filaments in live muscle as determined by X-ray diffraction is 38.5nm (Huxley and Brown, 1967).

Organized along the backbone to the actin filament are two regulatory proteins - troponin and tropomyosin (Figure 1). Troponin is a globular protein with a molecular weight of 76,000 which binds to the I-filament with an approximate 40nm periodicity (Endo, Nonomura, Masaki, Ohtsuki and Ebashi, 1966). Tropomyosin is a long fibrous protein with a molecular weight of 68,000 and length of approximately 40nm and by aggregating end to end it lies in the grooves of the F-actin helix (Hanson, Lednev, O'Brien and Bennett, 1972).

1.3 Myosin

Myosin has the following properties:– 1) it is a highly asymmetric protein approximately 175nm in length (Elliott and Offer, 1978) consisting of a long tail of coiled-coiled α-helix with two globular heads; 2) the molecular weight is about 470,000, 3) it has ATPase activity and binds actin, and 4) it is soluble at neutral pH and ionic strengths greater than 0.3. Myosin can be split into two fragments by treatment with trypsin (Figure 1): light meromyosin (LMM) and heavy meromyosin (HMM). The LMM portion has a molecular weight of 140,000, a length
of 86.0nm and displays the solubility properties of myosin, being insoluble at physiological ionic strength. HMM on the other hand is soluble even at low ionic strength and has a molecular weight of 340,000. Digestion of HMM by papain forms two further fragment types: HMM subfragment-1, which is the globular head region of myosin (molecular weight 115,000) and contains the ATPase activity and actin-binding ability and HMM subfragment-2, which is a short rod of length 40–50nm. Two HMM subfragment-1 heads are released per HMM molecule on digestion. The trypsin sensitive region between HMM subfragment-2 and LMM is believed to have a more open and flexible structure than the rest of the myosin rod and may act as "hinge region" during cross-bridge formation.

1.4 The Thick Filaments

The myosin filament is a largely rough surfaced bipolar structure of length 1.6μm with a central smooth region, the so called "bare zone" (approximately 150nm wide; Craig and Offer, 1976a), and tapered ends (Huxley, 1963). Because of the solubility properties of the meromyosins previously mentioned, it is generally accepted that the LMM portions polymerize to form the backbone of the filament. The HMM portions lie close to the filament surface in resting muscle (Huxley and Brown, 1967). Huxley (1963), on studying native and synthetic filaments (see Chapter 7.2.1) proposed that the assembly of myosin molecules was initiated by anti-parallel packing in the central region of the filament followed by parallel additions of myosin molecules towards the tapered ends of the filament (Figure 1). The rough surface is attributed to the projecting myosin heads (HMM subfragment-1).

1.5 X-Ray Diffraction of Vertebrate Skeletal Muscle and its Interpretation to Provide a Model for the Molecular Basis of the Sliding Filament Theory

According to results obtained by electron microscopy and X-ray diffraction analysis, the filaments of vertebrate skeletal muscle are arranged in a two-dimensional hexagonal lattice (Figure 1; Huxley, 1957 and 1960; Huxley and Hanson, 1960). In small angle X-ray studies Huxley (1953) observed the (1,0) and (1,1) equatorial
reflections (Figure 2: resulting from the myosin to myosin and actin to myosin filament spacings of the hexagonal myofibrillar lattice). These reflections could be indexed on a hexagonal lattice with a spacing of between 35 to 45nm depending on the degree of stretch of the muscle. Huxley (1952 and 1953) suggested that the lattice has primary (myosin) filaments at the threefold positions, with two secondary (actin) filaments per unit cell.

Comparing living muscle (in which the (1,0) reflection is much stronger than the (1,1)) and rigor muscle at the same sarcomere length, Huxley (1952 and 1953) observed a striking reversal of the relative intensity of the equatorial reflections, i.e. the (1,1) became the stronger reflection in rigor muscle — although the lattice dimensions remained unchanged. The change indicated that a substantial amount of material moves from the vicinity of the thick filaments to the vicinity of the actin-containing filaments (Huxley, 1968).

Interpretation of the axial part of the X-ray diagram of striated muscle has revealed that the reflections give rise to a complex pattern (Figure 3) which may be divided into six main groups (Huxley and Brown, 1967). The first group comprises a system of regularly spaced layer-line reflections based on an axial period of 42.9nm, with a very strong third order meridional reflection at 14.3. These reflections arise from the thick myosin filaments. The distribution of X-ray intensity along the different layer lines is close to that which would be expected from a helical arrangement of cross-bridges on the thick filaments. Huxley and Brown (1967) predicted a 6/2 helix, i.e. one having six bridges in the 43nm repeat, the bridges being arranged in pairs or "crowns" (one bridge on either side of the filament at 14.3nm intervals), with each pair or crown of cross-bridges rotated relative to its neighbours by 120° (Figure 4). However, recent evidence (see Chapter 5.3) suggests that the cross-bridges are arranged on a 9/3 helix, i.e. 3 projecting bridges form each 14.3nm spaced crown (Figure 4).

The X-ray diagrams from actively contracting muscles show large changes in the intensities of the myosin reflections on the original "resting" layer lines (Huxley and Brown, 1967), indicating movement of the cross-bridges. The 14.3nm meridional reflection remains approximately constant in spacing during contraction but tends to decrease somewhat in intensity, indicating some longitudinal movement.
or tilting of the cross-bridges. The off-meridional reflections, arising from the 43nm pitch of the cross-bridge helix, decrease in intensity much more markedly possibly due to a radial movement of the cross-bridges during contraction.

The remaining groups of low-angle axial X-ray reflections arise from the actin filaments and associated proteins (see Huxley and Brown, 1967; Haselgrove and Rodger, 1980). No changes are observed in the spacings of the actin reflections during contraction, and their intensity and sharpness are, if anything, increased.

Interpretation of these X-ray diffraction results (Huxley and Brown, 1967; Huxley, 1968) suggests that in resting muscle the myosin heads lie close to the myosin filament giving strong 43nm layer lines and prominent (1,0) equatorial reflections. During contraction or rigor the myosin heads move away from the thick filament (possibly by bending at the LMM-HMM junction); the myosin heads attach to the actin filaments so forming cross-bridges as seen in electron micrographs of muscle in rigor (Huxley, 1968). The associated movement of mass causes an increase in intensity of the (1,1) equatorial reflection relative to the (1,0).

Once attached to actin, the myosin ATPase activity is greatly increased and it is presumed that during the release of the products of ATP hydrolysis (ADP and phosphate) a conformational change in the myosin molecules occurs causing a movement of the actin filament relative to the myosin filament (Huxley, 1969). Shortening of muscle is permitted by the sliding of the actin filaments over the myosin filaments as predicted by A.F.Huxley and Neidergerke (1954) and H.E.Huxley and Hanson (1954). At the end of the cross-bridge cycle ATP binds to subfragment-1, actin and myosin dissociate and the cross-bridges return to their starting position or proceed on to another cycle.

1.6 The Control Mechanisms

The control mechanisms of muscular contraction operating in vertebrate skeletal muscle are extremely complex and have yet to be clarified. The basic regulating factor is calcium ions. These are released from the sarcoplasmic reticulum on
nervous stimulation and interact with the troponin/tropomyosin complex, exposing the myosin binding sites of the actin filament. During relaxation the calcium ions are actively taken up out of the sarcoplasm, the stimulation is removed from the actin filaments and the myosin heads are no longer able to bind.
THE AIMS OF THIS THESIS

To achieve a better understanding of how muscle contraction occurs it is essential to determine the exact structure of the A-filament i.e. how are the cross-bridges distributed about the six surrounding I-filaments? The answer to this question depends on the A-filament symmetry and the number of cross-bridges per 14.3nm crown, and this cannot be determined by X-ray diffraction.

Elucidating the structure of the A-filament is also vital in determining how A-filament assembly is accomplished in vivo. The mechanism by which the length of the A-filament is determined so precisely (1.57\(\mu\)m in rabbit skeletal muscle; Craig and Offer, 1976b) is unknown. Other details that remain to be solved are, the way in which radial growth of the A-filament is prevented in vivo (particularly since synthetic myosin filaments formed in vitro show a variation in diameter of 10-40nm; Emes and Rowe, 1978a); and how the replacement of structural proteins in living muscle takes place (Koizumi, 1974).

This thesis describes a technique which helps to solve the problem of the A-filament symmetry and a method of re-assembling A-filaments in situ.
Materials

Juvenile, male, New Zealand White rabbits were obtained from Hop Rabbits, Canterbury, and male Wistar/Leicester rats from the Animal Unit of Leicester University. *Lethocerus indicus* was kindly donated by the Agricultural Research Centre, Babraham, Cambridge, and *Pecten maximus* was obtained from the local fish market.

Adenosine 5'-triphosphate (ATP) crystalline sodium salt, ethylene-glycol-bis-(β-amino ethyl ether)N,N'-tetra-acetic acid (EGTA), dithiothreitol (DTT) and Trizma base (TRIS) were purchased from the Sigma (London) chemical Co.Ltd.

Kilogram quantities of D O, 99.8% (v/v), were obtained from the United Kingdom Atomic Energy Authority Research Establishment, Winfrith, Dorset.

The chemicals for “Spurr” (VLD resin NC2012, DER 236, plasticizer NC2304, NSA hardener NC2 109L and DMAE NC2208) were obtained from Polaron Equipment Ltd.. Copper grids (300 square) were purchased from Athene, Smethurst Highlight Ltd. and Agar Aids. Glass and Trufs for knives were obtained from LKB Instruments Ltd..

Dialysis tubing—Visking size 5 24/32" was purchased from Medicell International Ltd.. Conical tip Beem capsules were obtained from EMscope Laboratory Ltd.. Celloldin was obtained from E.Gurr Ltd. and a 0.25% solution prepared in n-butylacetate.

All other chemicals were the highest grade commercially available and were purchased from Fisons Ltd. or British Drug Houses Ltd..

General Equipment

Sections were cut either with an LKB III Ultramicrotome or a Reichert O MU4 Ultracut. A Dupont diamond knife or glass knives prepared with an LKB Knife Maker were used.
Grids were carbon coated in a Nanotech Vacuum Coating Unit.

Two transmission electron microscopes were used to examine specimens, an AEI 802 and a Siemens 102.

Relaxed filament preparations were centrifuged in an MSE Superspeed 50 centrifuge with a 10 x 10ml rotor. Sarcomere lengths were determined using a Spectra-Physics Laser source.
Principal Solutions Used

Secondary Phosphate

0.266M $K_2HPO_4$ (pH 9)

Mammalian Ringer

0.16M NaCl
50mM KCl
1.6mM CaCl$_2\cdot2H_2O$
1.8mM NaHCO$_3$ (pH 7)

Reconstruction Medium

Mammalian ringer
+2mM ATP

Calcium Depletion Medium

0.1M NaCl
2mM MgCl$_2\cdot6H_2O$
2mM KCl
1mM EGTA
0.1% (w/v) Glucose
6mM KH$_2$PO$_4$ (pH 7)

ATP Relaxing Medium

6mM KH$_2$PO$_4$
0.1M KCl
10mM MgCl$_2\cdot6H_2O$
1mM EGTA
5mM ATP
0.5mM DTT (pH 7)
PART 1

A-FILAMENT STRUCTURE AND SYMMETRY
2.1 Introduction

Force is generated in muscle by the active sliding of the thin filaments past the thick filaments. To understand the molecular nature of this process the structures of the two types of myofilament must be determined. While the structure of the thin filament is now well known, that of the thick filament remains, by comparison, poorly understood. Since Huxley (1963) established their bipolar structure, direct electron microscopy of A-filaments isolated from vertebrate skeletal muscle has yielded little further interpretable information about the mode of packing of the myosin molecules within the filament. X-ray diffraction of unfixed muscle (Huxley and Brown, 1967) has demonstrated that the cross-bridges emerge from the myosin filament in an approximately helical array with an axial translation of 14.3 nm and that the myosin molecules within the filament backbone have an axial repeat of 43 nm (see Chapter 1.5). However, the diffraction evidence does not indicate whether the cross-bridge helix is 2-, 3-, or 4-start (Huxley and Brown, 1967; Squire, 1973): that is, whether each “crown” of cross-bridges (one crown per 14.3 nm) contains two, three or four such structures. If the number of myosin molecules per cross-bridge is one, then “n” (the number of myosin molecules per 14.3 nm of filament length) will equal the number of “strands” in the three helical models. Since there appear to be 3 or 4 molecules per 14.3 nm repeat, the two stranded model would need to have the heads of two myosin molecules on each lattice point (Huxley, 1960). i.e. n=4 but the number of cross-bridges per crown is 2. Estimates published for “n” and the methods by which they were determined are shown in Table 1.

One of the first values for “n” for vertebrate skeletal muscle was published in 1951 by Hasselbach and Schneider. They determined the myosin and actin content of washed myofibrils. Using the myosin to actin weight ratio (2.7) so deduced, together with the molecular weight of actin and myosin, the length of the actin filament, the actin monomer repeat distance and the length of the cross-bridge
bearing region of the thick filament they calculated that \( n=4 \). Huxley(1960) estimated that each A-filament was composed of 432 myosin molecules. In 1967 Huxley and Brown, on the basis of X-ray diffraction patterns considered that the cross-bridges lie on a 6/2 helix. Taken together Huxley’s results would imply two myosin molecules per cross-bridge. However he was unable to confirm whether each cross-bridge was composed of heads from 1 or 2 myosin molecules, i.e. \( n=2 \) or 4.

Marston and Tregear (1972) estimated a value of 3 for \( n \) by assuming that each myosin molecule binds two molecules of ADP. An intermediate value of \( n \) (3.5) was determined by Maruyama and Weber(1972) using a similar method.

Four groups of workers (Tregear and Squire,1973; Potter,1974; Morimoto and Harrington,1974a; Pepe and Drucker,1979) estimated various values for \( n \) ranging from 2.5 to 4 using quantitative sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). By separating myofibrils into their components and scanning actin and myosin bands of different loadings, the myosin to actin weight ratios can be calculated. In order to justify their value of \( n=4 \), Pepe and Drucker(1979) compared their methods and results with those in the current literature of this work. They used washed myofibril suspensions and tried varying the washing conditions, concentrations and gel percentages to find the true myosin to actin weight ratio. The washing step was necessary to remove other proteins with a similar molecular weight to actin. Incorporating this step changed the value of \( n \) from 3 to 4. Tregear and Squire’s (1973) and Potter’s(1974) estimates of \( n=3 \) were attributed to incomplete washing. Neither actin nor myosin was preferentially extracted during the washing procedure.

Pepe and Drucker(1979) found the optimum gels to be 6 to 8% and attributed low "\( n \)" values of others (Tregear and Squire,1973) to the use of 10 to 12.5% gels which are unable to resolve actin and closely associated bands. The work of Morimoto and Harrington(1974a), having used 7.5% gels, confirmed their conclusion. However, Pepe and Drucker have not considered that the myosin heavy chain band may not be simple and may instead be a group of closely associated bands or may contain non-muscle myosin: Chin(1981) has found that under certain conditions the myosin heavy chain band can be separated into two distinct components. Furthermore, he has found the ratio for "\( n \)" to be close to 3, using conditions
under which creatine kinase (supposed by Pepe and Drucker to be the major cause of the uncertainty in actin quantitation) was clearly resolved from actin.

Morimoto and Harrington (1974a) confirmed their own estimate of $n=4$ using SDS-PAGE by making a simultaneous evaluation using the particle counting technique. Isolated thick filaments were sedimented onto grids in an analytical ultracentrifuge. After viewing in the electron microscope, the filament numbers of randomly selected unit areas were plotted against the concentration of the filament preparation. Using the weight concentration determined from the slope of the graph the filament molecular weight was calculated ($204 \pm 11 \times 10^6$). Adjusting for the presence of C-protein (see Chapter 11.2) and knowing the molecular weight of myosin ($470 \times 10^6$) they deduced the number of myosin molecules per filament to be 445 $\pm 24$, assuming one myosin molecule per cross-bridge, $n=4$.

Other than the estimate of $n=4$ by Pepe and Drucker (1979), the weight of recent evidence is in favour of $n=3$ (Reedy, Leonard, Freeman and Arad, 1981; Lamvik, 1978; Emes and Rowe, 1978a). Lamvik (1978) used the technique of quantitative electron scattering which determines the filament mass at the microscopic level using scanning transmission electron microscopy (STEM). The mass per unit length of isolated A-filaments was calculated by comparing the amount of electron scattering per unit length of myosin filaments with that of actin filaments and tobacco mosaic virus (T.M.V.) particles for which the mass per unit length are known. A value of $n (=3)$ was deduced using the calculated value for A-filament mass per unit length and the known values of the molecular weight of myosin. Reedy et al. (1981) used a similar method to Lamvik after having treated the rabbit psoas A-filaments with CAF (calcium activated factor; see Chapter 4.5) to remove C-protein; they obtained a value of $n=2.86 \pm 0.34$.

Emes and Rowe (1978a) calculated a value of $n=3$ by hydrodynamic methods. The sedimentation values $(s)$ and concentration dependence coefficients $(K_p)$ of synthetic filaments (Chapter 7.2), determined by analytical ultracentrifugation and
the average length of these filament preparations (0.6 to 0.7μm; Emes and Rowe, 1978a) were used to estimate their molecular weights. Knowing the molecular weight of myosin (the bare zone is usually absent in synthetic filaments and therefore can be ignored) values of \( n \) ranging from 3 to 6 were calculated. This suggested a filament symmetry of 3, the values of 4.5 and 6 arising from lateral aggregation of myosin molecules within the synthetic filaments. The same procedure cannot be applied directly to native filaments, as to date it has been impossible to concentrate native filament preparations sufficiently to achieve the range of filament concentrations required. Therefore synthetic filaments were used as a model to characterize the frictional properties of myosin filaments. Hydrodynamic data of myosin filaments cannot be compared with, or extrapolated from, that of other prolate ellipsoids (cigar shaped molecules) as they exhibit different characteristics. i.e. the frictional drag of an A-filament is considerably higher than would be expected, possibly due to the projecting myosin heads. The method of active enzyme centrifugation was employed to give an accurate estimate for the sedimentation coefficient of native filaments at low concentration, and using the frictional data obtained from synthetic filaments a molecular weight for native filaments of \( 1.2 \times 10^3 \) was deduced. From this value, the molecular weight of myosin and the length of the native filament including the 150nm bare zone, Emes and Rowe (1978a) estimated that 294 myosin molecules are present in each native A-filament and that \( n=3 \).

2.2 Introduction to Fraying

The possibility of determining the vertebrate (and non-vertebrate) A-filament symmetry by direct electron microscopy arose whilst attempting to isolate A-filaments from rat psoas muscle with the aim of using rats as a cheaper alternative to rabbits in studying in vivo myosin turnover by autoradiography (see Chapter 13.4). It was necessary to be able to make rat psoas relaxed filament preparations, and on viewing these in the electron microscope some of the A-filaments appeared to have frayed into three "subfilaments" on either side of the bare zone. The yield of frayed filaments increased on increasing the number of drops of water used to rinse the grid prior to negative staining with uranyl acetate. Grids of relaxed filament preparations were normally prepared by washing with two drops
of 0.1M KCl followed by two drops of distilled water after applying the filament solution. The washing step removes ATP which precipitates with uranyl acetate.

2.3 Trypsin Filaments

Davey and Graafhuis (1976), using controlled tryptic digestion, attempted to remove the heavy meromyosin moieties of chicken native and synthetic thick filaments to expose the underlying aggregated backbone of light meromyosin. In the electron microscope 3 stranded, helically-wound filaments were observed. They suggested that their findings strongly supported the model of Squire (1973), i.e. a triple coil with helical repeat of 130nm. Furthermore each of the 3 strands appeared to be composed of 3 finer filaments, therefore, presumably, the whole filament consisted of 3 sets of 3 coils. The experiments have been repeated here but it has been shown that these coiled filaments are artefacts of trypsin incubation. However, lowering the concentration of trypsin in the digestion medium from 5 mg/ml to 0.05 mg/ml does produce some interesting effects on native A-filaments (see Chapter 5.6).

2.4 Fraying of Scallop A-filaments

The structure of the cross-striated adductor muscle (used for rapidly closing the shell) of scallop (Pecten maximus) has been studied by Millman and Bennett (1976). The thick filaments are arranged in a hexagonal lattice, each filament being surrounded by about 12 thin filaments in the overlap region giving a thin-to-thick ratio of 6:1. Scallop A-filaments are composed of a paramyosin core of approximately 6nm diameter around which the myosin molecules are packed, resulting in a filament backbone diameter of about 21nm. They also show a bare zone and tapering ends similar to vertebrate skeletal filaments. In living, relaxed muscle the projecting myosin heads are symmetrically arranged, with an axial translation of 14.3nm. X-ray and optical diffraction data are consistent with a six-stranded helix. Fraying of fresh scallop A-filaments has been partially successful and preliminary results confirm a six-stranded model.
2.5 Fraying of *Lethocerus* A-filaments

*Lethocerus* flight muscle A-filaments are also bipolar with tapered ends and arranged in a hexagonal lattice. Each A-filament is surrounded by six I-filaments as in vertebrate skeletal muscle. Like scallop A-filaments, insect A-filaments have a core (approximately 10nm diameter) which often appears hollow in transverse sections around which the myosin molecules are packed. Insect A-filaments are composed of myosin and paramyosin but the amount of the latter varies with species. It is believed that paramyosin is not distributed uniformly down the core and its function may differ between species (Bullard, Bell and Luke, 1977). Insect A-filaments are probably joined to the Z-disc by "C-filaments" (Ashhurst, 1977; Auber and Couteaux, 1963; Trombitas and Tigyli-Sebes, 1979; see Chapter 13.1). Generally insect thick filaments are longer than scallop and vertebrate filaments (2.2µm in *Lethocerus* flight muscle) with a similar diameter to scallop filaments (18 to 20nm).

The symmetry of the insect thick filament has not as yet been confirmed. Reedy (1968) predicted a filament structure with a four point helical lattice, as, in thin cross-sections of rigor muscle, cross-bridges projecting from A-filaments formed flared "X" patterns; Wray (1979a and b) has confirmed these findings by X-ray diffraction. Recently Reedy, Leonard, Freeman and Arad (1981) have determined the mass/length ratio of *Lethocerus* and *Musca* A-filaments, by electron scattering measurements with the scanning transmission electron microscope, and so further confirmed a value of 4 myosin molecules per cross-bridge. However, Squire (1972), on adapting his general model for thick filament structure, has postulated a 6-fold symmetry and has provided an explanation for the presence of Reedy's flared "X" configurations (Squire, 1977). Shih-Fang, Ming-Xia and Ming (1966) isolated thick filaments from glycerolated honey-bee (*Apis mellifera*) flight muscle and in the electron microscope these were seen to have longitudinal surface striations; treatment of these A-filaments with urea or guanidine solution caused longitudinal separation into four subfilaments. Recently Aust, Hinkel and Beinbrech (1980) studied electron micrographs of honey-bee flight muscle A-filaments and determined that there were 4 cross-bridges per 14.3nm crown (ie. n=4) and so postulated that these myosin filaments are four-stranded.
By applying the techniques of relaxed filament preparation and induced fraying to *Lethocerus indicus* flight muscle A-filaments, a 4 stranded insect filament model is further supported here.
CHAPTER 3 METHODS

3.1 Glycerolation

Individual strips of rabbit psoas muscle approximately 2 to 3mm in diameter were tied or taped at both ends to perspex rods. The strips were stored in a fresh solution of 50% glycerol (w/v)/50% mammalian ringer at -20°C after soaking in the same solution at 2-4°C for 8 hours.

Before use, the glycerol was washed out by soaking the strips in 15% glycerol (w/v)/85% mammalian ringer at 2-4°C for 30 minutes followed by 1 hour in mammalian ringer.

3.2 Relaxed Filament Preparation

A modified version of Trinick's (1973) and Emes' (1977) methods was developed. Strips of glycerolated or fresh rabbit psoas muscle or fresh rat psoas muscle, 2 to 3mm in diameter were tied or taped at both ends to perspex rods. Fresh scallop (only animals with closed shells were used) adductor muscles were left attached to the shells and subsequently strips of muscle were excised from the periphery only, of the muscle: fresh and glycerolated dorsal longitudinal muscles of the giant tropical water bug Lethocerus indicus were left attached to the carapace. All the prepared samples were soaked in calcium depleting medium for two days at 2-4°C, changing the solution twice daily. A Sorval TC2 Tissue sectioner set to cut every 230μm at force 1 and speed 3 was used to chop the calcium depleted muscle strips. The macerate from each sample was stirred on ice into approximately 5mls. of ATP relaxing medium and washed by discarding the supernatant after spinning at 2,000g for 10 minutes at 5°C. The pellets were resuspended in about 5ml of ATP relaxing medium and homogenised very briefly (5 to 10 seconds) in a 5ml capacity motor-driven, Potter-Elvehjem homogeniser operating at a very slow speed. The homogenate was spun at 3,000-4,000g for 10 to 20 minutes.
at 5°C and the supernatant (containing separated A- and I-filaments released on homogenisation) retained.

This method of isolating A-filaments by making relaxed filament preparations was not entirely successful for scallop and Lethocerus muscles; A-filaments from both were fragmented and scallop myofilaments were also highly aggregated with few free A-filaments and apparently no free I-filaments were present. The scallop preparation was modified by omitting the calcium depletion step. A fresh Lethocerus muscle was divided into three portions and treated thus:- 1) as described above, 2) prior to calcium depletion the muscle was incubated in mammalian ringer with 5mM MgCl₂.6H₂O for 6 hours at 37°C, and 3) the sample was sectioned finely as described above and prior to calcium depletion the macerate incubated for 6 hours at 25°C in mammalian ringer plus 10mM CaCl₂.2H₂O and CAF (calcium activated factor, see Chapter 4.5; kindly donated by Lawrence Wale, A.R.C., Babraham).

Meanwhile a few separated fibres were examined by phase-contrast microscopy after incubating in the following solutions:- Sample (i) mammalian ringer plus 5mM MgCl₂.6H₂O. Sample (ii) concentrated CAF, and Sample (iii) mammalian ringer plus 10mM CaCl₂.2H₂O and CAF. After 3 hours fibres of treatments (ii) and (iii) resembled untreated fibres, but those of sample (i) had completely disintegrated into a homogeneous "soup" (fibre bundles remained intact). After 6 hours incubation sample (ii) remained unchanged but sample (iii) resembled (i), indicating that the CAF preparation was active.

3.3 Electron Microscopy

To provide a reasonable spread of filaments for viewing it was necessary to dilute the relaxed filament preparations, in the order of 5 to 30 times, with ATP relaxing medium. A drop of diluted relaxed filament preparation was applied to a celloidin/carbon-coated copper grid, rinsed with 2 to 3 drops of 0.1M KCl followed by 2–3 drops of distilled water (about pH6.5) and finally negatively stained for 20 to 30 seconds with freshly prepared, saturated, aqueous uranyl acetate.
3.4 Fraying

A-filament fraying was induced by increasing the number of drops of water used in rinsing (up to about 10 drops). The effects on fraying of: 1) substituting the KCl in the relaxing medium and in the wash with ammonium formate (0.2M), and 2) following the water wash with a further KCl rinse, were also studied.

3.5 Purified A-filament Preparation

The method used was that developed by Trinick (1973) and also described by Trinick and Rowe (1973).

Linear D$_2$O/H$_2$O density gradients were formed in 50ml capacity cellulose tubes from 100% D$_2$O ATP relaxing medium (adjusted to pH7.4) and 20% D$_2$O ATP relaxing medium using a Buchler 50ml capacity perspex gradient maker. 3mls. of concentrated fresh rabbit psoas relaxed filament preparation was applied to the tops of each gradient. The samples were spun at 24K and 5°C for 55 minutes in a 3 x 50ml swing-out rotor in a Beckman L2-65 ultra-centrifuge. The gradients were collected as a sequence of 7ml samples after piercing the bottom of the tubes. A-filament fractions were combined and the volume of solution reduced to one third with polyethylene glycol at 4°C.

3.6 Concentrated Tryptic Digestion

The method used here, was described by Davey and Graafhuis (1976). Equal volumes of 10mg/ml trypsin in 0.1M TRIS-HCl pH7.6 and purified rabbit A-filaments were incubated for 40 minutes at 20°C. Digestion was halted by plunging the samples into ice. Samples were washed and stained on grids as described for relaxed filament preparations. In a separate experiment 10mg/ml trypsin was incubated for 40 minutes alone at 20°C and applied to grids for viewing.
3.7 Modified Tryptic Digestion

1mg/ml trypsin in 0.1M TRIS-HCl pH7.6 was added to samples of relaxed filament preparations to a concentration of 0.05mg/ml and these were incubated at 25°C from 2.5 to 40 minutes. Digestion was stopped by adding PMSF in ethanol to give a final concentration of 0.5mM. A 0.05mg/ml trypsin control was also set up and incubated for 40 minutes. Samples were washed and stained on grids as described for relaxed filament preparations.

3.8 Statistical Analysis of isolated A-filaments

A-filament lengths were normally measured from the photographic negatives using a Projectina set at x7 magnification. Some measurements including filament widths were made from enlarged electron micrographs with a calibrated magnifying lens.

To determine the yield of full length filaments in relaxed filament preparations fairly concentrated filament solutions were applied to the grids and areas were randomly photographed at x40 magnification. Approximately 100 filaments were measured from each sample.

Filament mass was assumed to be directly proportional to filament length, and a histogram of mass-weighted frequency was plotted as a function of filament length by computing for each interval of length:

\[ f_{w,i} = (f_{n,i} \cdot l_i / \sum_{i=0}^{q} f_{n,i} \cdot l_i) \cdot 100 \]

where \( f_{w,i} \) = mass-weighted frequency for the i'th length interval,
\( f_{n,i} \) = number weighted frequency of i'th intervals
\( l_i \) = mean length within the i'th interval,
and there are q intervals in length.
This form of plot is appropriate where the original species present (whole filaments) is the largest in size; it ensures that, for example, if 50% of the filaments in a population are broken, 50% of the histogram area will correspond to intact
filament length. A number-weighted plot gives a misleading impression (only 33% of the total area in the appropriate region for the example given).

Although the length of A-filaments in vivo is 1.6μm, full length filaments were taken to be those of 1.4μm and longer as filament tips are frequently lost during preparation or obscured by the stain.

Filament widths of frayed and unfrayed rat A-filaments were determined in the unfrayed central region avoiding the M-line region. The length of the unfrayed region of approximately 1000 frayed rat filaments was also measured.
CHAPTER 4  RESULTS

4.1 Relaxed Filament Preparations

Figures 5a and 5b are length distributions (weight-averaged) of A-filaments isolated from fresh rabbit psoas muscle by the relaxed filament preparation method. With the original version of the method Trinick (1973) obtained a yield of approximately 50% (by number) full length filaments. With a modified homogenisation procedure a 40% yield (weight-averaged) has been obtained (Figure 5a), but on introducing a washing step prior to homogenisation yields of 65% and higher have been achieved (Figure 5b). The washing step was thereafter incorporated throughout. Samples of unwashed and washed relaxed filament preparations are shown in Figures 6 and 7.

A similar yield of full length A-filaments prepared from glycerolated muscle was obtained (63%; Figure 8b). Figures 8a and 8b provide a comparison between plotting a length distribution by number and by weight-averaging i.e. assuming that filament length is a unit of mass. Filaments which appear to be greater than 1.6μm are probably artifacts formed by 2 or more broken filaments joining end-to-end.

4.2 Fraying of Rat A-Filaments

Approximately 1000 frayed rat A-filaments were photographed (Figures 9, 10 and 11) and numerous micrographs of rabbit frayed filaments were made (Figures 12 and 16). In no case was a filament observed that had frayed into more than 3 subfilaments although many which had frayed into only 2 units were seen (Figures 10 and 11). However, in most of these cases it was clear that one of the two subfilaments was thicker than the other. Figure 13b is a length distribution of a population of frayed rat A-filaments and is completely comparable to that obtained with normal unfrayed filaments (Figure 13a). The
number of frayed filaments in randomly selected areas of different populations varied between 32 and 79%.

Due to Pinset-Harstrom and Truffy’s (1979) findings that synthetic myosin filaments have varying diameters when they form in the presence and absence of different ions the diameters of the bare zone (avoiding the M-line) of frayed and unfrayed filaments were measured. It is apparent in Figure 16 that fraying or the cause of fraying has no effect on the diameter of unfrayed regions.

On studying the micrographs of vertebrate filaments it seemed that fraying never proceeded into the central region of the A-filaments. Figure 15 is a distribution of the total length of the unfrayed central region of frayed filaments: the vertical arrows show (from left to right) the distances within the filament corresponding respectively to the width of the M-line, the position of stripe 9, the position of stripe 1 (terminology of Craig and Offer, 1976b; see Figure 1) and the filament tip. The largest peak of the histogram falls between the distances corresponding to the width of the bare zone and the first non-myosin stripe: no filaments frayed within the M-band, i.e. for the majority of the filament population the complete cross-bridge bearing regions of the filament frayed apart. The significance of the failure of the bare zone to fray will be discussed in Chapter 5.3.

The separated subfilaments were generally quite straight or at the most slightly curved (Figures 9, 10, 11 and 12) suggesting that they were packed into the filament in a linear rather than helical fashion. Frequently the subfilaments appear to be joined at the distal points of the filament but this is unlikely to be important as completely separated subfilaments are equally common. Fraying has never been observed within a filament in the absence of fraying of the filament tip. This suggests that fraying commences at the filament tip and proceeds through the filament and this proposal is further supported by the presence of some filaments in which only the filament tips have frayed (Figures 10 and 11). Furthermore the subfilaments do not separate simultaneously; frequently only 2 subfilaments are observed as mentioned before and in other cases 2 subfilaments are still partially bonded together although the third subfilament may have completely frayed apart. The implications of the mode of vertebrate A-filament fraying which have been described will be discussed in Chapter 5.3.
The amount of water washing and the effects on fraying of ammonium formate and potassium chloride were also studied. An experiment was set up as presented in Table 2.

A number of points can be made from these results: 1) an extensive water washing procedure is required to fray the filaments, i.e. the ions of the filament suspending medium must be completely removed. 2) ammonium formate, a volatile solvent, completely destroys the ability of A-filaments to fray whether followed by a water wash or not, and 3) increasing the ionic strength surrounding the filaments after fraying has been induced causes up to 50% of the frayed filaments to reanneal and even in many cases where subfilament association is not completed, the subfilaments have moved closer together and very important, are still linear and lie in parallel (Figure 16). Reassociation of subfilaments appears to occur at the bare zone and travel towards the filament tip, i.e. opposite to the method proposed for fraying.

4.3 Trypsin Digestion

An attempt was made to reproduce the findings of Davey and Graafhuis (1976) who, on digesting isolated A-filaments with concentrated trypsin observed long helical structures with 3-fold symmetry in the electron microscope. Figure 17 is a montage of some of the structures produced on repeating the experiment and they resemble very closely those of Davey and Graafhuis. However, generally these filaments are far longer than native filaments although of similar diameter. One might expect a change in the helical direction towards the centre of the filaments, i.e. to form a bipolar filament.

On incubating 5mg/ml trypsin in the absence of A-filaments similar structures were produced (Figure 18). It was deduced that 5mg/ml trypsin completely digests A-filaments and during the 40 minute incubation at 20°C auto-digestion probably occurs which allows aggregation of the residual trypsin molecules into tightly coiled and generally 3 stranded structures of varying length.

However, the idea of digesting away the A-filament projections to reveal
the underlying filament structure was still plausible and the experiment was repeated with 0.05mg/ml trypsin and followed over a period of time (Figures 19a, b, c, d, e and f). Figure 19a is a micrograph of undigested I- and A-filaments: some of the myosin filaments are frayed at their tips. After 2.5 minutes digestion (Figure 19b) the I-filaments have been completely digested and the A-filaments exhibit a decreased electron density in the region of the M-line. Figure 19c illustrates how after 5 minutes digestion, the bare zone structure has loosened and in some cases disintegrated. After a further 5 minutes digestion (Figure 19d) the structure of the cross-bridge bearing regions of some filaments has started to loosen into subfilaments and in these, the bare zone has reassociated to some extent. Ten minutes later (Figure 19e) the subfilaments appear to have reassembled in most filaments although the bare zone is still generally very diffuse. Some filaments have started to aggregate into A-segment-like structures apparently tightly attached at the bare zone regions. Feathery projections were also sometimes seen similar to those of the synthetic filaments produced by Pinset-Harstrom and Truffy(1979). The filaments of Figure 19f were digested for 40 minutes and are tightly bound into "A-segments"; surface projections are present but there is no evidence for any organized structures.

4.4 Preparation and Fraying of Scallop A-Filaments

The first preparation of filaments made did not show any evidence of I-filaments and the A-filaments were fragmented, as observed by Millman and Bennett(1976), and clumped together. On washing the grids with water some filaments did dissociate into subfilaments (Figure 20). The upper two filaments of the micrographs have dissociated into parallel arrays of 6 subfilaments and the bottom micrograph shows filaments with a 3 stranded linear appearance. The remaining micrograph depicts a filament fragment which appears to consist of subfilaments surrounding a hollow core. Substituting ammonium formate for water did not prevent fraying and washing with 0.1M KCl after the water wash did not cause the subfilaments to reassociate.

Suspecting that autolysis may be occurring during calcium depletion resulting in loss of I-filaments and possibly fragmented and aggregated A-filaments, the
preparation was repeated, omitting the calcium depletion step and homogenisation was reduced to one short plunge of the pestle. The problems were not resolved by these measures.

4.5 Preparation and Fraying of *Lethocerus* A-Filaments

Relaxed filament preparations of fresh and glycerolated muscles were made as described for rabbit and rat muscles. A-filaments from fresh muscle frequently frayed into 4 subfilaments on washing with water (Figure 21). The appearance of the frayed filaments varied considerably from near parallel arrays of completely dissociated short subfilaments, to longer filaments only displaying the initial stages of fraying at the filament tip. Few filaments were full length. Many I-filaments were present and as these resembled the subfilaments, what appeared to be completely dissociated subfilaments, may not have been. The pattern observed in vertebrate A-filaments of fraying commencing at the filament tips and proceeding to the edges of the bare zone was not obvious in *Lethocerus* filaments; fraying may proceed through the bare zone.

Fraying could not be induced in glycerolated A-filaments. Non-frayed *Lethocerus* A-filaments frequently displayed a highly ordered banding pattern in the cross-bridge bearing regions (Figure 22). Preliminary optical diffraction studies of the micrographs revealed that the banding has a 14.5nm repeat. Other interesting features in these micrographs are:- 1) a thin filament cross-linking two thick filaments presumably by the cross-bridges, and 2) a maintained banding pattern in a region which has commenced fraying into 2 subfilaments. Similar micrographs of striated *Lethocerus* A-filaments have been presented by Reedy and Garrett (1977). Optical diffraction revealed that the stripes had a 14.5nm repeat and brief papain digestion (liberates HMM subfragment-1) removed the striping completely. Millman and Bennett (1976) obtained a similar 14.5nm banding pattern in isolated scallop A-filaments and also attributed the effect to projecting heads of myosin molecules.

A subsequent attempt to isolate full length *Lethocerus* A-filaments using CAF was not successful: in the phase-contrast microscope both 1mM Ca$^{2+}$/5mM Mg$^{2+}$
and CAF/10mM Ca$^{2+}$ were successful at destroying the striation pattern of the isolated fibres, presumably by releasing myofilaments from the Z-discs. However, the treatments did not have a noticeable effect on the A-filament length of relaxed filament preparations. CAF, calcium activated factor (Busch, Stromer, Goll and Suzuki, 1972), is a proteolytic enzyme produced in vivo on high (i.e. >0.1mM) calcium and magnesium ion stimulation, which destroys the Z-disc and so disrupts the "C-filaments" (see Chapter 13) connecting the A-filaments of insect striated muscle via the Z-disc. CAF also removes C-protein (Reedy, Leonard, Freeman and Arad, 1981; Chapter 11.2).

Rowe (unpublished) on spreading and fraying Lethocerus A-filaments on holey carbon/celluloid films obtained a higher resolution of the subfilament structure; periodic projections were present, the repeat of approximately 14 to 15nm, being that which would be expected for the cross-bridge projections.
5.1 Vertebrate A-Filaments Fray Into 3 Subfilaments

The structure of the thin filament has been well resolved compared to that of the thick filament: the actin monomers are assembled into two chains, which form a helical coil of pitch 38.5nm, with troponin/tropomyosin complexes associated along the length. To understand the molecular nature of how force is generated in muscle by the active sliding of the thin filament past the thick filaments, it is essential that the myofilament structures are characterised. Surprisingly little is known about the structure of the thick filament considering the number of studies which it has received. The technique of A-filament fraying (Maw and Rowe, 1980a and b) which has now been developed may clarify many of the uncertainties of A-filament structure and provide further information.

Trinick (1981) has since developed the technique of vertebrate A-filament fraying by lowering the surrounding ionic strength of the filaments prior to application on grids. He has found that dissociation into subfilaments is pH sensitive and at a particular ionic strength there is a threshold value of pH (7.3) below which fraying does not occur. In the electron microscope the frayed filaments produced in solution are not identical to those produced on the grid, in that the 3 subfilaments are joined at their tips and frequently attached to a structure which Trinick termed an "end-filament". The end-filaments are approximately 83nm long and 4.8nm wide and are situated perpendicular to the A-filament axis. They are usually seen to be crossed by striations about 4.1nm apart and in some cases the striations can be resolved into pairs of subunits arranged side-by-side and in register along the end-filament. Sometimes, though not invariably, the end-filaments terminate in a globular region which is roughly pear-shaped and with dimensions of about 15nm x 10nm. Although not commented upon, some of the isolated A-filaments of Trinick (1973) also exhibit a filamentous projection with a globular head at the A-filament tips. Trinick has proposed that the end-filaments are located within the A-filament core although detailed analysis of thin cross-sections of A-filaments
in whole muscle (Luther and Squire, personal communication) has not indicated
the presence of such a structure. It is also possible that the constituent protein
of end-filaments is AMP-deaminase since this enzyme binds at the edges of
the A-band (Ashby, Frieden and Bischoff, 1979).

Pepe (1981) has also now produced micrographs of 3 stranded frayed A-filaments
similar to those presented here: fraying was induced whilst washing isolated filaments
with 10mM TRIS-citrate (pH8.0) buffer in order to produce "mini-filaments" (Reisler,
Smith and Seegan, 1980; see Chapter 7.2.4).

5.2 Why Do A-Filaments Fray?

Striated muscle A-filaments fray into subfilaments on washing with distilled
water (but not ammonium formate, pH7, i=0.2) and on rapid dilution to low ionic
strength (Trinick, 1981); a further wash with 0.1M KCl before contrasting produces
almost complete reversal of the effect. The simplest explanation for the phenomenon
of fraying is that at low ionic strength the expansion of the electrical double layer
around the myosin molecules leads to greatly increased repulsive forces (Vervey
and Overbeek, 1948). An increase in net negative charge on the filaments might
be expected to have a similar effect, and with synthetic filaments made from rat
myosin, Pinset-Härström and Truffy (1979) showed that high concentrations of ATP
induces a rather poor tendency to fray. The whole phenomenon may be related
to one of the first observations made on myosin, by Szent-Györgyi (1951), who
showed that prolonged dialysis of myosin solutions at low ionic strength (that is
synthetic filament preparations) against distilled water yielded clear but viscous
solutions.

The reason why thick filaments fray into their component subfilaments is
in itself an intriguing problem as it may throw some light on in vivo A-filament
assembly. However, this discussion will be concerned with the information which
can be obtained from the frayed structures themselves and how this information
may be incorporated and compared with the few known details of A-filament structure.
5.3 A-Filament Models

The main structural features of the vertebrate skeletal A-filament were elucidated by Huxley (1963) and Huxley and Brown (1967). Using the technique of negative staining to observe isolated synthetic and natural filaments in the electron microscope, Huxley (1963) drew the following conclusions: 1) the tails of the myosin molecules form the shaft of the thick filament and that their heads lie on the surface, and 2) the myosin molecules in each half of the filament are arranged with opposite polarities, the region of overlap of the two arrays forming a central bare zone i.e. free of myosin heads. X-ray diffraction of whole muscle has shown that the arrangement of the myosin heads is approximately that of a helix with a repeat of 43nm and a translation of 14.3nm between adjacent levels of heads (or crowns: Huxley and Brown, 1967). Further information derived from electron microscope studies of negatively stained isolated A-segments (assemblies of A-filaments held together at the M-line) by Craig (1977) have shown that: 1) the bare zone is 149nm ± 2nm long; 2) there are perturbations in the packing of the myosin molecules near the centre of the filament and near the ends, due to the initiation and ultimate termination of assembly; and 3) there is a single gap in the 14.3nm cross-bridge array at the third but last position from the filament tips (also shown by labelling muscle with antibodies to subfragment-1: Craig and Offer, 1976a). Electron microscope studies of thin transverse sections through the pseudo H-zone of whole muscle have revealed that in this region the A-filaments have a triangular cross-sectional profile and a 3-fold rotational symmetry of the filament backbone has been postulated (Pepe, 1967 and 1971; Luther, 1978; Luther and Squire, 1978).

Any proposed models of myosin packing within the A-filament must, therefore, account for all these features. Important factors which have yet to be determined and which are vital for model building are: 1) the number of cross-bridges per 14.3nm crown, which need not reflect the backbone symmetry, 2) the myosin molecule environment i.e. whether, within the constant packing region (between the bare zone and filament tip) all the myosin molecules are equivalent; and 3) the fundamental design of the myosin filament which results in a helical array of cross-bridges at the surface of the filament. The axial period of 43nm is a fraction (about 1/4) of the length of the myosin molecule and the filament comprises of several layers of molecules in a radial direction. Therefore the molecules must be tilted slightly.
(or supercoiled as a special case of tilting) to allow the bridges to appear regularly at the surface.

The results which have been obtained here from frayed rabbit psoas filaments suggest that the vertebrate A-filament has 3-fold rotational symmetry as the filaments contain three asymmetric units which appear as subfilaments after fraying. The micrographs presented indicate that these subfilaments are packed in a simple linear array into the A-filaments. Tien-Chin, Yüng-Shui, Tsu-Hsün and Chia-Hsiu (1966) have reported the presence of longitudinal subfilaments within chicken breast muscle A-filaments. It seems implausible that once absorbed onto a carbon film that a coiled structure could become unwound and less likely still, that a coiled structure could reform, on increasing the ionic strength again after washing with distilled water. The evidence does not in itself confirm a value of $n=3$ but it lends strong supporting evidence to other estimates (Emes and Rowe, 1978a; Lamvik, 1978). A brief examination of subfilaments of insect muscle on holey carbon films (Rowe, unpublished) revealed the presence of lateral projections with a regular spacing of approximately 14 to 15nm. Shih-Fang, Ming-Xia and Ming (1966) published micrographs of separated insect subfilaments with regular projections, but the spacing of these was approximately 40nm; it has been subsequently decided that these structures were probably actin filaments, as the backbone was a 2 stranded coil.

Two detailed models of the vertebrate A-filament structure have been published to date: that of Pepe (Pepe, 1967, 1975 and 1981; Pepe, Ashton, Dowben and Stewart, 1981; Pepe and Dowben, 1977) and that of Squire (1973 and 1977). However, neither incorporate all of the features which have been mentioned. Both models are based on a bipolar structure incorporating a bare zone with a triangular cross-sectional profile and with the myosin molecules arranged so that the cross-bridge projections form a helical array of 3x14.3nm. Squire postulates that the light meromyosin rods are in equivalent positions every 14.3nm and Pepe suggests that the light meromyosin moieties are staggered by 28.6nm (Pepe, 1967 and 1971): both of these predictions have been refuted by Craig (1977) who found that the filament backbone has an axial repeat of 43nm. Neither model accounts for the single gap at the ends of the filaments in the cross-bridge array; Pepe does not incorporate any gaps into his model and Squire includes several – near the bare zone and
four gaps at the ends of the filaments. The final requirement, which both models incorporate is a backbone with three fold rotational symmetry. Squire postulates 3 cross-bridges per 14.3nm crown whereas Pepe has built his model with a 2 start cross-bridge helix. Here, what few similarities the two models share, end, and the following postulations have yet to be proven.

The remaining features of how the molecules pack within the A-filament are completely different in the two models. In Squire’s model the myosin molecules are all in equivalent positions within the regions of constant packing, but in Pepe’s model (Pepe et al., 1981) the molecules are not equivalent, and are arranged as dimers (the longitudinal arrays of dimers are termed structural units). Although Pepe’s model has a backbone rotational symmetry of 3 there are 4 myosin molecules per 14.3nm repeat (n=4); the myosin molecules exist as dimers therefore each cross-bridge is formed by two myosin molecules resulting in 2 cross-bridges per 14.3nm repeat. Squire’s filament model has three fold symmetry, 3 myosin molecules per 14.3nm repeat (n=3) and 3 cross-bridges per 14.3nm repeat. The final A-filament characteristic which all models must account for is the mode of molecular packing which enables the myosin heads to project from the filament surface. The model of Pepe packs the myosin tails into a linear array, the maximum tilt of the parallel structural units being 3/4; he postulates that the myosin molecules are kinked at the light/heavy meromyosin junction so enabling the light meromyosin portion to be buried in the filament backbone and the heavy meromyosin subfragment-1 portion to lie on the filament surface. The myosin molecules in Squire’s model A-filament are tilted towards and around the filament axis forming a super-coiled structure in which the myosin molecules gradually emerge from within the filament backbone. Wray (1979a) has proposed a similar model in this respect for crustacean A-filaments.

I have found that native vertebrate A-filaments fray into three subfilaments. Both Squire’s and Pepe’s model A-filaments can theoretically fray into 3, but restrictions on fraying might arise; for example:– 1) the filaments are adsorbed onto carbon films prior to fraying and it seems unlikely that Squire’s coiled structure could unravel. 2) it seems unlikely that a filament, two-stranded with respect to cross-bridge lattice, would fray into 3 subfilaments. If Pepe’s model A-filament were able to fray into 3 subfilaments each subfilament would show an unusual
series of projections when seen with the electron microscope. There would be a 14.3nm repeat but at every third position a projection would be absent (Pepe, 1981). To prove conclusively that n=3 each subfilament must have a completed series of 14.3nm spaced projections.

Since Pepe (Pepe and Dowben, 1977) published his model for the A-filament structure based on the myosin dimer (Harrington and Burke, 1972; Burke and Harrington, 1972) kinetic evidence has been submitted which casts doubt on the existence of such a unit (Emes and Rowe, 1978b). Another factor against Pepe's model is that were his filaments to fray into 3 units they would not be of equal length; in his model the subfilaments are staggered by 57.2nm (4x14.3; Pepe, 1981) and the micrographs presented here clearly demonstrate that the subfilaments all terminate at the filament tip.

The ability of A-filaments to fray into 3 subfilaments provides good evidence for a filament structure with 3-fold symmetry and it is reasonable to assume that the cross-bridges emerge on a three point lattice as predicted by Squire. The subfilaments are likely to be packed in a linear fashion as predicted by Pepe in order to fray apart whilst adsorbed onto a carbon film. Furthermore, in Figures 20 and 21 the subfilaments of frayed scallop and Lethocerus thick filaments and in Figure 16 the rabbit subfilaments (undergoing a reversal of fraying by 0.1M KCl) are extremely straight and almost parallel in some cases. It seems unlikely that these straight subfilaments arose from a helical structure.

A fundamental question remains: are all the myosin molecules in the constant packing region equivalent? The fact that the filament can fray apart into 3 separate structures suggests that the molecules are capable of forming two types of bonds with their neighbours and are therefore not equivalent: one type of bond within the subfilament is presumably more stable than the other type which is formed between subfilaments and is easily broken on lowering the ionic strength of the surrounding medium. However, it seems unlikely that the A-filament should be assembled with different types of myosin molecules although there is increasing evidence for the presence of myosin isozymes (both heavy and light chains isozymes) even within one filament. There is no clear evidence that any non-myosin protein might be involved. It is proposed that the 3 subfilaments arise because fraying
starts at the filament tip, the extreme point of which consists of 3 myosin molecules. As the lateral bonds between these three are disrupted they move apart and 3 tears are initiated which travel through the filament similar to tearing along the warp of a piece of cloth or fraying a blade of grass. These analogies are compatible with the observation that the subfilaments do not fray apart simultaneously (Figures 10 and 11); one might expect that if the bonds between the subfilaments were different to those within the subfilaments that fraying would occur at similar rates between different subfilaments. The initiation of fraying has never been observed within a filament – always at the filament tip (Figures 10, 11 and 21). Furthermore on reversal of fraying by a further wash with 0.1M KCl prior to contrasting, the subfilaments appear to reassociate in an exactly opposite manner to fraying i.e. from the bare zone towards the filament tip (Figure 16). Therefore, it is suggested as that the myosin molecules throughout the region of constant packing are equivalent. This accords with Squire’s view.

The preceding discussion of the interpretations of fraying has dwelt largely on the structure of the cross-bridge bearing region of the A-filament. Fraying which commences at the distal region, can proceed to any point along the filament axis up to a central region of approximately 0.3μm length. Within experimental error this corresponds to the innermost stripe of the A-segment which stains with impure anti-C-protein (that is stripe 9, following the terminology of Craig and Offer.1976b; see Chapter 11.2 and Figure 1). These 43nm spaced stripes reflecting the presence of non-myosin protein including C-protein are associated with the constant packing regions of the A-filament on either side of the bare zone. The fact that fraying does not proceed into the bare zone indicates that the molecular packing arrangement here might differ to that in the cross-bridge bearing regions.

The vertebrate A-filament model designed by Rowe(unpublished) incorporates a twist (1 to 2°) of the subfilaments within the bare zone and this has been supported by some electron microscopy evidence: Trinick and Cooper(1980a) observed that some of the filaments which they subjected to sequential disassembly (see Chapter 7.2.5) had approximately six striations running through the bare zone not quite parallel to the filament axis (similar bare zones are apparent in isolated A-filaments of Trinick.1973). In the clearest example which they obtained the angle
of the striations to the filament axis was $2^\circ$. The presence of this subfilament twist in Rowe's model arises due to the mode of myosin molecule packing within the subfilaments. Rowe postulates that the basic packing unit of the A-filament is a protofilament, 3 of which form a subfilament. Each protofilament consists of a longitudinal staggered array of myosin molecules, the tails of the molecules overlapping by 43nm. To ensure that the heads of the molecules project out of the filament surface, the protofilaments are not arranged in a strictly linear fashion within the subfilament but are slightly slewed i.e. tilted around rather than away from the filament axis. In order to accommodate a change in polarity of the slewed protofilaments within the bare zone it was necessary to propose that the subfilaments twisted in this region by one or two degrees.

As already mentioned, it seems unlikely that a helical structure could unwind once adsorbed on to a carbon film. Therefore it is proposed that fraying terminates at the edges of the bare zone, since this is where linear packing of the subfilament ends, and where a new distinctive packing arrangement associated with the M-line and bare-zone regions begins. In these regions myosin must be packed in a "tail-to-tail" configuration; and the recent findings of Reisler, Smith and Seegan (1980) showing that 16–18 myosin molecules can form a bipolar assembly ("mini-filament"; see Chapter 7.2.4) of length 300nm, which exists under ionic conditions permitting no further assembly, implies that the binding is considerably stronger in this region. On almost any three-stranded model, the number of myosin molecules engaged in tail-to-tail packing will be 18, making the whole hypothesis consistent. Lethocerus filaments on the other hand, show no very clear tendency to fray in a manner which leaves an intact, unfrayed, central region: it would be interesting to know whether Lethocerus myosin forms "mini-filaments".
5.4 Fraying of Scallop and *Lethocerus* A-Filaments

The number of subfilaments into which vertebrate skeletal A-filaments frays, probably reflects the A-filament symmetry and it seemed plausible that the same technique applied to scallop and *Lethocerus* thick filaments will confirm the symmetry of mollusc striated muscle A-filaments (believed to be 6; Millman and Bennett, 1976) and clarify the symmetry of insect flight muscle A-filaments (possibly 6; Squire, 1972 or 4; Reedy, 1968; Aust, Hinkel and Beinbrech, 1980).

The method used for isolating relaxed filaments of vertebrate muscles was not completely suitable for scallop and *Lethocerus* striated muscles and a few attempts at modifying the procedure specifically for these tissues (omitting the calcium depletion step for scallop muscle and treating *Lethocerus* muscles with Mg\(^{2+}\) or CAF prior to homogenisation) were unsuccessful in producing separated, full length A-filaments. However, some isolated A-filament fragments were induced to fray and generally, frayed *Lethocerus* A-filaments resembled rat and rabbit filaments in that fraying occurred from the filament tips (Figure 21). Scallop A-filaments are known to be bipolar with a central bare zone but it was impossible to predict whether fraying of full length A-filaments would resemble those of vertebrate muscles as the filaments were highly fragmented and those which did fray, did so almost completely (Figure 35).

Improved filament preparations and further studies are required before the number of subfilaments produced on fraying of scallop and *Lethocerus* A-filaments can be precisely determined; however, from these initial studies it is proposed that scallop and *Lethocerus* thick filaments fray into 6 and 4 subfilaments respectively (Figures 35 and 36) and if the number of subfilaments reflects the filament symmetry these results support the predictions of Wray (1979a and b), Reedy (1968), Aust et al. (1980) and Millman and Bennett (1976) but not that of Squire (1972).
5.5 Trypsin Filaments

The 3 stranded coiled filaments seen by Davey and Graafhuls (1976) after concentrated trypdic digestion of native and synthetic filaments were easily reproducible (Figure 17). However, incubation of the same trypsin solution in the absence of myosin filaments produced a similar effect (Figure 18). Trypsin molecules in solution normally exist as free globular monomers but it appears that on incubation at 20°C for 40 minutes auto-digestion may occur resulting in a molecule, probably a fibrous α-helical fragment, which is capable of aggregating into long coiled filaments consisting of 3 sets of 3 coiled protofilaments. It is interesting that this trypsin fragment should produce a filament reasonably similar to the native A-filament in that both are 3 stranded and each of the subfilaments is itself composed of 3 protofilaments (Rowe's A-filament model, unpublished). Satir and Satir (1964) proposed a model for the ninefold symmetry of α-keratin and cilia and it is not implausible that this model may hold true for myosin and digested-trypsin filaments. They observed that the basic dimensions of the α-helix (3.6 residues each with a 1.5Å rise, generating a 5.4Å rise of the helix per turn) are such that certain simple repeats of a small number of amino acids within a helical protein can generate nine-fold symmetry. The outer edge of an α-helix viewed end-on is roughly circular (Kendrew, 1963) therefore each amino acid forms an arc subtending 100° in the case of the basic helix (3.6x100°=360°); assuming that the helix consists of 4 repeating amino acids, at each turn of the helix the first amino acid will be displaced by 0.4 arcs. After nine turns of the helix the first amino acid of the repeating sequence will be directly above that of the initial sequence. If this amino acid contains a site e.g. an SH- group which would eventually bind with a side chain, then this site would also be displaced by 0.4 arcs at every turn of the helix and in the end-on view after 9 turns the circle would be divided into 9 equal sectors. The axial repeat unit need not be four; any amino acid repeat which maps out spacings of 0.4,1.6,2.0,2.8 or 3.2 arcs from the original point will suffice.

The myosin molecule structure is largely α-helical and the trypsin molecule is known to possess some α-helical structure which may form the auto-digestion resistant moiety. It is possible, therefore, that the common α-helical structure of the two proteins generates macro structures which have features in common.
5.6 The Effect of Trypsin on A-Filaments

The basic principle of digesting away the heavy meromyosin moieties of thick filaments to reveal the structure of the light meromyosin backbone was still plausible using a far less concentrated trypsin solution. 0.05mg/ml trypsin did not form filaments after 40 minutes incubation at 25°C but it had a noticeable effect on the A-filament structure as observed in the electron microscope at various intervals during the digestion (Figures 19a, b, c, d, e and f). After 2.5 minutes the electron density of the bare zone decreased dramatically (and some subfilament dissociation had occurred) suggesting digestion of a non-myosin protein, perhaps creatine kinase which is known to account for the electron density of the M-line (Turner, Walliman and Eppenberger, 1973). After 10 minutes the cross-bridge bearing regions as well as the bare zone were dissociating into subfilaments (Figure 19d). On further incubation the subfilaments reassociated and structures resembling A-segments were formed (Figure 19e and f). A possible explanation is that partial digestion of the heavy meromyosin has occurred (it cannot be complete as the filaments of Figure 19f still appear to have surface projections) resulting initially in filament dissociation (the DTNB light chain is thought to be involved in A-filament assembly; see Chapter 7.2.2): it is possible that the denatured myosin subfilaments are then capable of reassociating into a slightly different filament configuration. Creatine kinase is believed to form the M-bridges of the M-band (Walliman, Turner and Eppenberger, 1975; see Chapter 13.5) and these in turn are probably involved in the spatial arrangement of the A-filament lattice; removal of creatine kinase may result in A-filament aggregation the points of adhesion being the denuded bare zone.

Unfortunately these results could not be repeated on a subsequent attempt, possibly due to the relaxed filament preparation being several days old. It has since been noted (see Chapter 7.2.2) that filament ageing affects the heavy meromyosin moieties of A-filaments.

5.7 Further Research

The ability to fray A-filaments into their component subfilaments provides
a new and potentially very important method of studying A-filament structure. To
date only preliminary examination by electron microscopy of negatively stained
subfilaments have been made, however, the techniques for high resolution microscopy
are available enabling similar examinations of separated subfilaments as have
been made of isolated myosin molecules (Elliott, Offer and Burridge, 1976; Offer
and Elliott, 1978; Elliott and Offer, 1979) and whole filaments (Craig and Megerman, 1977;
Trinick and Elliott, 1979). These techniques range from:- 1) negative staining with
uranyl acetate after glutaraldehyde fixation of a filament suspension applied to
holey carbon film (Craig and Megermen, 1977). 2) applying the filament suspension
to carbon-coated grids (previously made hydrophilic) and then freeze-drying the
specimen prior to rotary shadowing with platinum (Trinick and Elliott, 1979). to
3) diluting the myosin solution with glycerol or ethylene glycol (30% v/v and 20%
v/v respectively), spraying onto mica and drying in a vacuum at -50°C prior to
rotary shadowing with platinum (Elliott et al., 1976; Elliott and Offer, 1978).

All these techniques have been employed because of the increased preservation
of molecular structure which may be obtained. The freeze-drying techniques in
particular, prevent the cross-bridges from flattening onto the grid surface as
occurs during the normal air-drying procedure used for routine examination by
negative contrasting. Therefore, the use of freeze-drying followed by rotary shadowing
may be particularly useful for determining the cross-bridge register of separated
subfilaments; a technique which has already demonstrated myosin heads emanating
from isolated native A-filament shafts (Trinick and Elliott, 1979).

Further studies of isolated Lethocerus and scallop thick filaments are required
to determine the precise number of subfilaments which are produced on fraying
and the extent of fraying which proceeds into the bare zone. However, before
these studies may be made, improved methods of isolating separated and unbroken
thick filaments must be developed. Lethocerus A-filaments are isolated routinely
by many laboratories using CAF (see Chapter 4.5) suggesting that the lack of
success obtained here was due to a non-active CAF preparation. Furthermore,
Millman and Bennett (1976) obtained filament suspensions from fresh scallop muscle
by a similar method to that used here although the A-filaments of their preparations
were not separated sufficiently for studying individual subfilaments. Although the
average filament length was longer than was achieved in this study, the filaments
were too aggregated to make precise measurements.

The absence of fraying in the bare zone of vertebrate skeletal muscle A-filaments may be an important observation with respect to the A-filament molecular packing, especially when viewed in conjunction with Reisler's stable "mini-filaments" (see Chapter 7.2.4; Reisler, Smith and Seegan, 1980). Further studies of the bare zone structure are necessary to produce a complete picture of vertebrate A-filament structure. The apparent absence of non-frayed bare zones in Lethocerus and scallop filaments may be further evidence that the molecular packing of vertebrate and non-vertebrate striated muscle A-filaments differ. Vertebrate A-filaments may possess a different form of molecular bonding within the bare zone, or there may be an additional component acting as a "glue". An attempt to produce "mini-filaments" from solubilised Lethocerus and scallop myosin may indicate differences between vertebrate and non-vertebrate bare zone structure depending on whether stable, uniform polymers can be synthesised. Similarly, sequential solubilisation of non-vertebrate A-filaments by the method of Trinick and Cooper (1980a; see Chapter 7.2.5) may be used to compare different A-filament structures.

Development of the mild tryptic digestion procedure coupled with high resolution electron microscopy may also reveal further information on the underlying structure of the A-filament backbone.

5.8 Summary

Fraying of vertebrate native A-filaments into 3 subfilaments has provided direct evidence that the A-filament has 3-fold symmetry and implies that the number of cross-bridges per 14.3nm crown is 3. Although Pepe's ingenious model combines a 2-start cross-bridge helix with a type of 3-fold rotational symmetry, postulations have to be made about the non-equivalence in length and in cross-bridge packing of the sub-filaments which do not seem to be experimentally verified. The supposition that the rotational symmetry as reflected by the number of sub-filaments is related to the order of the cross-bridge helix of the filament is further supported by the ability of Lethocerus flight muscle A-filaments to fray into 4 units: a value of 4 cross-bridges per crown having already been proven (Reedy, 1968; Aust, Hinkel
and Beinbrech, 1980). A 6-fold symmetry for scallop adductor A-filaments is also tentatively predicted.

The linear fraying of filaments suggests that the subfilaments are packed in a linear arrangement within the filament and commencement of fraying from the filament tips can be compatible with all the myosin molecules being in equivalent environments at any point along the cross-bridge region of the filament. Failure of fraying to proceed into the bare zone indicates that the subfilament packing in this region may well be different from that in the cross-bridge bearing regions.

Neither Squire's or Pepe's models for the A-filament structure have been able to account for all these findings. Recently Rowe (unpublished) has described a new model based on a structure with 3-fold symmetry composed of 9 protofilaments arranged into 3 subfilaments; the subfilaments are packed in a linear arrangement in the cross-bridge region and twisted by 1 to 2° in the bare zone. At any radial position the myosin molecules are equivalent. The new findings are compatible with this model, and probably with other models having linearly packed sub-filaments, but with perhaps different packing of the myosin molecules within the sub-filaments.

Further extension of this new structural data should lead to a greater understanding of the A-filament properties both biochemical and physical.

Part 2 of this thesis examines a system for in situ A-filament assembly and compares these "reconstructed A-filaments" with the synthetic filaments produced in vitro. Elucidation of the 3-fold symmetry of the native A-filament has enabled a model to be proposed for reconstructed A-filament assembly and this model may reflect a mode of filamentogenesis which operates in vivo.
PART 2

RECONSTRUCTION OF A-FILAMENTS
AND A-BANDS
6.1 Self-Assembly

Organised biological structures, selected for specific functions in dynamic self-reproducing systems, are built up of smaller structures arranged in a definite pattern. The component parts may be synthesized separately by a subassembly process and then associated, following definite rules, to form the organized structure (Caspar, 1964). Different types of control mechanisms are used at the various stages of synthesis and assembly so that biological control can be exercised at each level of organization.

Large organized biological structures can be formed with high efficiency, that is accurately and economically, by making use of the specificity of the non-covalent bond interactions that are possible between the macromolecules. A number of proteins can be reversibly denatured which demonstrates that their stable configuration is determined by the specific pattern of interchain interactions allowed by the amino acid sequence under the particular environmental conditions. Moreover many different protein molecules can reversibly associate in definite structures held together by specific non-covalent bonds between the units. The most significant feature of organized structures built in this way is that their design and stability can be determined completely by the bonding properties of their constituent units. Thus once the component parts are made they may associate themselves without a template or other specific external genetic control.

A biological advantage of a self-assembly design for any large structure is that it can be completely specified by the genetic information required to direct synthesis of the component molecules. For economical use of the genetic information that can be carried by the nucleic acids identical copies of a basic molecule or groups of molecules are used to build large structures. Organized structures such as ribosome particles may be built by self-assembly of different subunits but the maximum sized structure that can be built of units, all of which are different.
such as the protein coats of virus particles, muscle filaments, tendons and cell membranes will necessarily consist of a large number of subunits and the number of different types of subunits will in general be small. In many protein structures there may be only one type of subunit for example the protein coat of tobacco mosaic virus (T.M.V.), actin and myosin filaments of muscle, collagen fibres, microtubules and flagella. In all these the dissociated components can be reassembled under appropriate conditions \textit{in vitro} to produce structures that appear the same or at least similar to the organized structures assembled \textit{in vivo}.

Under near physiological conditions structures assembled \textit{in vitro} which are merely similar, rather than identical to their \textit{in vivo} predecessors, may reflect the necessity for additional control mechanisms other than mechanisms inherent within the structure of the subunit. The "synthetic" structures assembled \textit{in vitro} from the dissociated components of myosin and actin filaments vary considerably from native filaments (Huxley, 1963; Hanson and Lowy, 1963) indicating the presence of an as yet unknown control mechanism(s) \textit{in vivo}. \textit{In vitro} assembly of three other filament-type structures, microtubules, flagella and T.M.V. are far better understood. The control mechanisms have largely been defined and models for assembly proposed (Weisenberg, 1980; Oosawa, Kasai, Hatano and Asakura, 1965; Durham, Finch and Klug, 1971; Durham and Klug, 1971; Butler and Klug, 1971).

6.2 The Assembly of Tobacco Mosaic Virus

Thus T.M.V. consists of a long helically wound filament of RNA embedded in a framework of small identical protein molecules arranged in a helical pattern. \textit{In vitro}, at neutral pH, in the absence of RNA the protein subunits assemble into double layer ring structures termed "disks", each disk consisting of 34 subunits (Durham, Finch and Klug, 1971). The disks assemble by stacking, into cylinders of various lengths. At pH values below 6.5 the helical cylinders found \textit{in vivo} are formed and electron microscopy of intermediary stages shows that the lowering in pH causes a dislocation in the disks to form a helical structure termed a "lock-washer" (Durham, Finch and Klug, 1971). The lock-washers stack to form the helical cylinder. The mode of aggregation of T.M.V. protein subunits is controlled by the state of two abnormally titrating carboxyl groups. \textit{In vitro} aggregation is
determined by the pH (Durham and Klug, 1971).

At neutral pH, in the presence of T.M.V.-RNA, assembly of helical cylinders is initiated by the interaction of the surface of a disk with a special sequence of about fifty nucleotides at the 5' end of the RNA (Butler and Klug, 1971). T.M.V.-RNA reproduces the effect of protonation at acid pH and the disk is transformed into a lock-washer. Subsequent disks are added to the growing helix in this form. The RNA is trapped between the rings of the unrolling preformed disks (Butler and Klug, 1971). This in vitro mechanism is probably that which occurs in vivo. Therefore the helical formation of the T.M.V. protein coat is controlled by the subunit carboxyl groups and these are stabilized either by protonation at acid pH or by interaction with the RNA molecule.

6.3 The Assembly of Microtubules

The controlling mechanisms of microtubule assembly are also now well established (Welsenberg, 1980). Microtubules are long cylinders composed of tubulin subunits which form 13 longitudinal protofilaments. Tubulin assembles into rings or spiral shaped aggregates, which break down into oligomers before incorporation (Welsenberg, 1974). A round of microtubule elongation starts with the addition of a single oligomer (a protofilament segment) to the end of a microtubule. Subsequent additions occur by lateral association of oligomers to those previously bound and these additions are stabilized by co-operative interactions between the subunits of the oligomers. Addition of the 13th oligomer completes the round of assembly and free subunits fill in any slots remaining. Microtubule initiating sites in vivo are the centrosomes and kinetochores.

Microtubules will assemble from pure tubulin in high protein and magnesium ion concentrations (Lee and Timasheff, 1977), or in the presence of microtubule stabilizing agents such as glycerol (Lee and Timasheff, 1977). All microtubule assembly systems require GTP hydrolysis during polymerization (Welsenberg, Deery and Dickinson, 1976). In vivo, microtubules are associated with MAP's (Microtubule associated proteins of high molecular weight, also termed HMW proteins) and a group of low molecular weight proteins called Tau (Weingarten, Lockwood, Hwo and
Kirschner, 1975): both types of proteins promote microtubule assembly in vitro by inducing the formation of rings and oligomers (Weingarten et al., 1975; Sloboda, Dentier and Rosenbaum, 1976) and are incorporated stoichiometrically throughout the length of the microtubule. The predominance of Tau or MAP's in microtubule preparations depends on the method of microtubule protein isolation and purification (Timasheff and Grisham, 1980). One component of Tau is a heat stable protein termed TAP (tubulin associated protein; Lockwood, 1978). Runge, Detrich and Williams (1979) found that a very similar protein to TAP was released with others on boiling of a 10nm filament (intermediate filaments; see Chapter 13.2) fraction and this protein also stimulated microtubule assembly.

This suggests that MAP's are the true components and promoters of microtubule assembly in vivo and TAP or Tau are probably contaminants released from intermediate filaments e.g. microfilaments during tubulin preparation. However in vivo, Tau may serve to link 10nm filaments to microtubules as the two types of filaments are frequently found in close association. It is also possible that 10nm filaments regulate the assembly of microtubules and vice versa via Tau, as it has been frequently noted that on disruption of the 10nm filament network the number of microtubules increases and disruption of microtubules by colchicine increases the number of 10nm filaments (Ishikawa, Bischoff and Holtzer, 1969).

6.4 The Assembly of Flagella

The in vitro assembly of flagella, the tubular motile structures of bacteria, has been studied by Oosawa, Kasai, Hantano and Asakura (1965) and Asakura, Eguchi and Iino (1964, 1966). Electron micrographs of negatively stained flagella reveal a beaded surface structure. Both a hollow core (Kerridge, Horne and Glauert, 1962; Asakura, Eguchi and Iino, 1964) and an apparently impenetrable centre (Lowy and Hanson, 1964; Lowy and McDonough, 1964) have been described. Disc electrophoresis and sedimentation studies of flagella components reveal the presence of only one protein, flagellin (molecular weight of 20,000–40,000; Asakura et al., 1964; Weibull, 1948; Erlander, Koffler and Foster, 1960). The helix content is approximately 40% (Yaguchi, Foster and Koffler, 1964) and one could picture the molecule as some kind of tadpole-shaped structure with a globular head forming the external
beaded appearance) and a short coiled–coiled tail perhaps constituting the core.

Bacterial flagella disassemble on heating; however, cooling of a flagellin solution does not promote the assembly of flagella. Polymerisation of flagellin requires the presence of "nuclei" or "seeds" i.e. flagellar fragments produced by sonication of flagella (Asakura, Eguchi and lino, 1964) and once assembly is initiated it proceeds until the monomer is depleted. This co-operative nucleation followed by growth is equivalent to the growth of crystals, which also require a seed to initiate crystallization of soluble components. Theoretically the crystallization of flagella is predictable due to their tubular structure. As seen in the assembly of microtubules the monomers assume a minimum free energy state, which, in the case of flagellin is through two or more different types of bonds with four or more neighbouring molecules (Oosawa and Kasai, 1962).

Flagellin monomers have the intrinsic ability to form two types of flagella - normal (long and slightly wavy) and curly (short with accentuated waves). The structure of the nucleation fragment controls the configuration which the assembling monomers adopt whether they are isolated from normal or curly flagella. This indicates that the difference in free energy between the two types of structures is very small (Asakura, Eguchi and lino, 1966). Transformation between the two types of structures can be brought about by changing the solvent conditions i.e. by changing the ATP concentration. When basal bodies are isolated from bacteria and incorporated into flagellin solutions to act as seeds, electron microscopy reveals that the structures formed are very different from flagella; thin rods are produced which resemble bacterial pill in appearance and in their resistance to heat. Therefore different seeds are capable of initiating assembly of different polymers from identical constituents and in the same medium.

6.5 The Control of Assembly

T.M.V., flagella and microtubule assembly can be reproduced in vitro to produce "synthetic" structures which are morphologically identical to those in vivo, however, this is not the case as yet for myosin and actin filaments (see
Chapter 7.2): the dimensions of "synthetic" myofilaments are incorrect and although the dimensions can be varied by altering the ionic strength or pH of the medium (Kaminer and Bell, 1966) the correct dimensions have never been consistently achieved. Until myofilaments resembling native filaments can be produced in vitro, the control mechanisms which operate in vivo are unlikely to be resolved as has been achieved for the assembly of T.M.V., flagella and microtubules.

The ordered, self-assembling structures that have been most intensively studied are regular crystal lattices. The maximum possible size of a crystal is in principle unlimited. Biological structures have well-determined dimensions which have been selected for specific functions and the assembly designs are intrinsically self-limited. Self-limitation may result from co-ordinated interactions between two or more different types of components i.e. growth of the T.M.V. helix is terminated when the RNA molecule is completely packaged (Butler and Klug, 1971). Or self-limitation may be an essential property of the stable bonding pattern of a single type of subunit. Myosin may be assembled by either of these mechanisms. Synthetic filaments are produced in the absence of any other component (Koretz, 1979a; Moos, Offer, Starr and Bennett, 1975) suggesting that the molecules are assembled so that each molecule or group of molecules is not in an identical environment forming a pattern of contacts to establish a self-termination mechanism i.e. the angle that each molecule makes with the axis of the filament decreases and as more units are added a point is reached where further growth is unstable (Cohen, 1964). However, synthetic filaments must have a slightly different termination mechanism from that used in vivo and a second component may be required to assemble filaments of the correct dimensions. The likely candidate (Huxley and Brown, 1967; see Chapter 11.2) is called "C-protein", a non-myosin protein of A-filaments (Starr and Offer, 1971), but addition of C-protein to an assembling synthetic filament system still does not produce filaments of the correct width or length (Koretz, 1979b) as will be discussed in Chapter 7.2.1. The situation for invertebrate A-filaments is slightly different. These filaments have a central core material of paramyosin (Bennett and Elliott, 1981) which is thought to establish the length of the thick filament in a manner analogous to T.M.V.-RNA (MacKenzle, Jnr. and Epstein, 1980).

Synthetic I-filaments are usually longer than 1μm (Hanson and Lowy, 1963) and probably also require a length regulating protein i.e. α-actinin (Maruyama, 1965).
α-actinin has recently been found to increase the viscosity of actin solutions (Singh, Goll, Robson and Stromer, 1981) and subsequent studies indicated that α-actinin may act by altering the actin monomer structure (Singh, Goll and Robson, 1981). The length regulating factor of actin filaments may be myosin filaments themselves (Kikuchi, Noda and Maruyama, 1969; Kawamura and Maruyama, 1969) as G-actin forms shorter polymers in the presence of synthetic myosin filaments.

6.6 The Energetics of Assembly

The nature of biological organization, in both its static structural aspect and its dynamic functional aspect, is governed by the physical principle of minimum energy embodied in the law of thermodynamics. All chemical reactions in living cells proceed in the direction that leads to a decrease in free energy. The microtubule nucleating sites assemble subunits into minimum free energy configurations and the length of the microtubule may be determined by the structure of the nucleating site and the time required to reach a minimum free energy configuration (Weisenberg, 1980). Additional regulation of microtubule assembly and stability may be by modification of MAP's such as phosphorylation (Sloboda, Rudolph, Rosenbaum and Greengard, 1975) so altering the properties of MAP interactions with tubulin or oligomers. The minimum free energy state of T.M.V. protein subunits is in the disk form as the protein is obtained in monomeric form only in fairly extreme conditions, for example, at low temperature, low concentration, low ionic strength and high pH (Durham, Finch, and Klug, 1971). The bipolar aggregation of the myosin molecule tails in the centre of a thick filament and their subsequent parallel aggregation away from the centre (Huxley, 1963) must also represent a minimum free energy configuration as synthetic filaments formed in the absence of an energy source or control mechanism also adopt the bipolar configuration.

It is clear then, that many biological structures are built of identical structural units by a self-assembly process and the possible structural designs are limited by the principles which govern the subunit construction. Additional control mechanisms may also operate in structures built by self-assembly. Furthermore any ordered structure built of identical units must have some type of well-defined symmetry. Specific bonding between the units necessarily leads to a symmetrical structure.
since there is only a limited number of ways in which each unit can be connected to its neighbours to form the maximum number of most stable bonds. The symmetry of the A-filament is discussed in Part 1.

Myosin has an additional and unusual assembly problem due to the unique position of myosin among proteins (Cohen, 1964); it is the only naturally occurring fibrous molecule within the α-class of fibrous proteins with enzymatic activity (required for muscular contraction). This functional division is reflected in the form of the molecule and its aggregates, the rod-like α-helical portion of myosin being orientated along the filament and the globular bridge containing the "active site" projecting on the outside. Therefore construction of myosin filaments must differ from that of other fibrous systems such as collagen or fibrin for example, where the exact size of the aggregates is not critical in their functioning and where the role of the protein is purely structural.
As has been seen in Part 1 a greater understanding of A-filament structure has been achieved although the precise molecular packing arrangements have still to be elucidated. However, very little progress has been made in determining the method by which myosin molecules are assembled into A-filaments and how these in turn are assembled in the A-bands. \textit{In vivo} filamentogenesis occurs: 1) In the developing myotube of the embryo, 2) in myofibrillogenesis during growth, and 3) in regeneration of diseased muscle and protein turnover. It is unclear whether the same mechanism occurs in all these conditions. Other than fixation, sectioning and electron microscopy of developing myotubes and muscle fibers no morphological examinations of \textit{in vivo} myofibrillogenesis have been made. Some attempts at \textit{in situ} filamentogenesis (Tawada, Yoshida and Morita, 1976; Isenor, 1976; Tanaka and Tanaka, 1979; Maw and Rowe, 1979; see Chapter 7.3) and numerous studies of \textit{in vitro} filamentogenesis i.e., in the absence of other myofibrillar components, have been published (Huxley, 1963; Emes and Rowe, 1978a; Reisler, Smith and Seegar, 1980; see Chapter 7.2).

7.1 Myosin Solubilisation

A pre-requisite of filament assembly is a solution of the protein monomer which has been extracted from an intact muscle sample or for high yields from a muscle homogenate. Actin filaments are selectively solubilised and extracted by 0.6M KI and dialysis or dilution of a G-actin solution back to physiological ionic strength proceeds the production of F-actin filaments (Hanson and Lowy, 1963; Huxley, 1963). Myosin solutions have also been traditionally made by solubilisation and extraction by a high ionic strength solution i.e., \( \lambda > 0.3 \) and composed primarily of KCl. Three such solutions have commonly been used: 1) Guba–Straub (0.3M KCl, 0.2M Na$_2$HPO$_4$, pH 6.5; Guba and Straub, 1943), 2) Weber–Edsall (0.6M KCI, 0.04M KHCO$_3$, 0.01M K$_2$CO$_3$; Meyer and Weber, 1933), and 3) Hasselbach–Schneider (0.47M KCI, 1mM MgCl$_2$, 10mM Sodium pyrophosphate, 0.1M phosphate, pH 6.4; Hanson and Huxley, 1955). Potassium chloride is an effective myosin solubiliser.
and extractor but tends to disrupt other myofibrillar components, notably the Z-line and tropomyosin (Mitsuka, Yamada and Shimizu, 1979). Mihalyi and Rowe (1966) compared the effects of Guba–Straub, Weber–Edsall and a high ionic strength secondary phosphate solution (0.266M K$_2$HPO$_4$, I=0.6) on myosin extraction. Long extraction times with KCl resulted in actomyosin being extracted rather than myosin and the concentration of actomyosin extracted rose as the concentration of intracellular ATP fell. Guba and Straub first noticed, in 1943, that the presence of 0.2M phosphate inhibits the formation of actomyosin and in secondary phosphate solution, even after intracellular ATP is completely depleted, actomyosin is not extracted. Either, phosphate ions, like ATP, stabilise actomyosin dissociation or the fact that as ATP levels decrease, actomyosin extraction increases is a coincidence and phosphate ions inhibit the latter in any condition. During phosphate extraction of muscle homogenates the level of ATP rises during the first two hours extraction time, which may be due to phosphate activation of a rephosphorylating mechanism. ATP levels fall to zero after about 10 hours. It was inferred by Mihalyi and Rowe (1966), that phosphate ions have a stabilising effect on actin filaments or their attachment to the Z-lines.

Potassium chloride in the presence of pyrophosphate is capable of nearly 100% myosin extraction whereas secondary phosphate solution only extracts 70% of solubilised myosin (Isenor, 1976). For myosin assembly in vitro potassium chloride solubilisation and extraction is normally employed, whereas for attempted reconstruction of A-filaments in situ, where myosin extraction is not desirable but an intact remaining myofibrillar network is, secondary phosphate solution would appear to be more suitable. In vitro myosin assembly has been studied by many workers, one of the most important original works having been published by Huxley (1963) in which he termed his myosin polymers "synthetic" A-filaments. Synthetic filaments are produced by diluting or dialyzing the high ionic strength myosin solution back to a low ionic strength i.e. below I=0.3. The rate of dilution or dialysis and the presence of various ions has a pronounced effect on the morphology of the resulting filaments.

7.2 The Assembly of Synthetic Myosin Filaments
7.2.1 Effects of the Solvent Environment and C-Protein

Noda and Ebashi (1960) performed one of the first studies of synthetic filaments. They determined the length of polymers produced on lowering the ionic strength or pH of a myosin solution by flow birefringence. They found that myosin was fully dissociated by 0.2M KCl, at pH7.3 or higher and polymerised into structures approximately 1μm in length at pH6.5 or lower. Furthermore, during intermediate states of aggregation, addition of ATP caused dissociation. Huxley's (1963) detailed electron microscope study of natural and synthetic myosin and actin filaments from striated muscle showed that synthetic myosin filaments resembled native filaments in that they were bipolar and had tapered ends. Along the length of the filaments on each side of the central zone, which was sometimes bare, were numerous projections, probably corresponding to the cross-bridges. However, the synthetic filaments did not exhibit the same dimensions as native filaments, tending to be shorter and thicker, and the dimensions within a synthetic filament population were not consistent. Emes and Rowe (1978a) determined that the average length of synthetic filaments prepared by a particle dilution technique was 0.6 to 0.7μm and the filament mass/unit length was always greater or equal to that of native filaments. Koretz (1978a) studied electron micrographs of synthetic filaments by optical diffraction and found that they, like native filaments, exhibited a 14.3nm subunit and 43nm axial repeats.

Since Huxley's detailed account, numerous studies have been made of synthetic filaments in an effort to elucidate the factors involved in determining the filament dimensions. A resume will be given here of a collation of papers. Unpurified myosin, extracted (usually with a KCl solution) from homogenates, is normally used in synthetic filament preparations. Pinset-Härström and Truffy (1979) and Koretz (1979a), found that column purification of extracted myosin prior to assembly, did not affect the synthetic filaments produced. I.e., there is not a component extracted with myosin which affects assembly. Koretz (1979b) added C-protein (see Chapter 11.2) to myosin prior to assembly in different ratios; she found that at a physiological ratio i.e., 1 C-protein:3-4 myosin, the synthetic filaments were highly organized, compact, of uniform diameter within each filament and that the distribution of widths within the population was narrow. The helical cross-bridge repeat of 14.3nm as determined by optical diffraction was present but the helical
myosin repeat of 43nm was replaced by one of 114.4nm. Below physiological concentrations of C-protein, a broad distribution of filament diameters resulted and the meridional spacing had narrowed from 14.3nm$^{-1}$ to 28.6nm$^{-1}$ indicating occasional subunit level absences. Some evidence for a 43nm repeating distance was present. Above physiological concentrations of C-protein, the filaments were not of uniform diameter and had bulges probably due to lateral aggregation of excess myosin molecules. This suggests that C-protein molecules have binding sites for 3 or 4 myosin molecules. C-protein may exert a structural effect by slipping between adjacent myosin rods. The effect of C-protein is more marked on addition prior to assembly rather than afterwards. Contrary to Craig and Offer(1976b) and Starr and Offer(1978), Koretz(1979b) suggests C-protein is partially incorporated into the filament rather than forming a collar-like distribution around the filament.

Various other factors of the assembly medium affect the dimensions of synthetic filaments: for example the ionic concentration, pH, temperature, magnesium ion concentration and hydrostatic pressure. Kaminer and Bell(1966) obtained 1.6µm long synthetic myosin filaments in vitro in near physiological conditions but they were much wider than naturally occurring ones. The method by which the assembly medium is produced also affects filament assembly. The ionic strength of the solubilised myosin solution can either be diluted to promote aggregation, or the ionic strength can be lowered gradually by dialysis. Koretz(1979a) and Katsura and Noda(1971) found that by rapid dilution short filaments resulted with thick bare zones and clusters of heads at both ends. By slow dialysis to a low ionic strength, the myosin aggregated into filaments up to 25µm long (Koretz, 1979a). Filament production was not found to be affected by the final myosin concentration down to 5µg/ml (Katsura and Noda, 1971). Josephs and Harrington(1966) found that the myosin monomer-polymer equilibrium below l=0.3 (the point at which myosin starts to assemble) was affected by the final KCl concentration (large thick filaments at 0.2M KCl, clumps of needle-shaped filaments at 0.05M) and they also found that the temperature at which assembly occurred was important.

The effect of pH of the assembly medium was monitored by Josephs and Harrington(1966 and 1968) and by Katsura and Noda(1973). The latter group found that with slow dialysis the longest filaments were produced at pH7 and
on either side of this the filament length decreased. Josephs and Harrington suggested that the filament length was also affected by the hydrostatic pressure which is capable of altering the monomer–polymer equilibrium. Recently Davis (1981) has made a study of the effects of hydrostatic pressure and found that an increase in pressure shortens the average filament length although the length distribution remains similar. These effects of hydrostatic pressure would be important in sedimentation velocity and density-gradient experiments as explained by Kegeles, Rhodes and Bethune (1967) and TenEyck and Kauzman (1967). Furthermore, pressure effects on interacting systems can provide additional information on the underlying mechanisms of association.

Pinsent–Härdström and Truffy (1979) found that synthetic filaments assembled in the presence of Mg\(^{2+}\)ATP were of physiological diameter, i.e., 15 to 17nm and 5 to 15μm in length, but those assembled in Ca\(^{2+}\)ATP were extremely thickened (30 to 50nm in diameter). The effect of ATP and inorganic phosphate in the assembly medium and on synthetic filaments was also studied by Pinsent–Härdström and Truffy (1979): it was found that millimolar concentrations of ATP or phosphate added to a myosin solution prior to decrease in ionic strength produced branched twig-like structures on dilution (0.2 to 0.4μm in length), and these were thought to arise from lateral aggregation with incomplete overlapping of a small number of molecules (Kamler, 1969). However ATP and phosphate added to assembled filaments produced no effect as noted by Huxley (1963) who used 0.1 to 1mM ATP. This is in disagreement with Oriol–Audit, Lake and Reisler (1981) who found similar concentrations (1 to 5mM) of ATP or pyrophosphate ions dissociated synthetic filaments, as did Harrington and Himmelfarb (1972). ATP can interact with the tail of the myosin molecule (Harrington and Himmelfarb, 1972) as well as the head region.

7.2.2 The Effect of Myosin Light Chains

Pinsent–Härdstrom and Whalen (1979) monitored the effects of myosin ageing on synthetic filament production. They found that storing solubilized myosin from several days to two weeks produced progressively shorter and thicker filaments in the presence of Mg\(^{2+}\)ATP, suggesting a partial degradation or denaturation
of the myosin molecule. The phenomenon was found to be accompanied by a progressive proteolysis of light chain 2, the so called DTNB light chain (Weeds, 1969) as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis and isoelectric focusing in the presence of urea. The light chain bands were never completely lost, but they become progressively less dense on the gels. Removal of light chain 2 by DTNB produced the same effect on synthetic filament assembly as aging and the effect was not reversible by adding back light chain 2. These results suggest that light chain 2 plays a role in filament formation, and, indeed magnesium ions which are also required to produce long bipolar filaments with physiological diameters (Pinset-Häström and Truffy, 1979), competitively bind to light chain 2 under physiological conditions (Bagshaw, 1977). It was suggested (Pinset-Häström and Whalen, 1979), that as light chain 2 is part of the subfragment-1/subfragment-2 hinge region (Margossian, Lowey and Barshop, 1975) that this region may be involved in controlling the packing of the myosin tails in the filament shaft. Alternatively, loss of light chain 2 may cause a steric hindrance of the specific interaction of the tails as myosin rods (filaments produced from myosin molecules minus subfragment-1, i.e., in the absence of light chain 2) are able to form large regular paracrystals (Moos, Offer, Starr and Bennett, 1975) and are solubilised by ATP, whereas the disintegrating effect of ATP on normal synthetic filaments decreases with ageing.

7.2.3 A Myosin Filament Elongating Protein?

Katsura and Noda (1973) did not find any change in the length of synthetic filaments after partial removal of light chains, but an unknown factor was removed from solubilised myosin by DEAE-Sephadex A50 and on its removal far shorter filaments were produced. Longer filaments were regained on addition of the factor to the purified myosin. They termed this factor "myosin filament elongating protein" (MFEP). The isolated MFEP was found to be heterogeneous and on purification (or possibly due to aging) there was some denaturation (loss of capacity of filament elongation and myosin binding). MFEP was soluble in 0.6M KCl and 0.6M KI which solublise myosin and actin respectively, but only partially soluble in 5mM TRIS-buffer (pH8.2) which removes the electron dense M-line material (Samosudova, 1966; Morimoto and Harrington, 1972). It was postulated that MFEP may be the 165,000 dalton
M-protein of Masaki and Takaito (1972) as both bind very strongly to myosin (see Chapter 13.5) but the molecular weights are dissimilar.

7.2.4 "Mini-Filaments"

Reisler, Smith and Seegan (1980) dialysed solubilized myosin against 10mM citrate/TRIS buffer (pH 8) and produced what they termed "mini-filaments". These were stable and sedimentation velocity, diffusion and viscosity measurements determined that there were 16 to 18 myosin molecules per mini-filament. In the electron microscope the filaments were seen to have a homogeneous size distribution of about 0.3μm. were bipolar and the bare zone was 160 to 180nm in length (similar to native filament, 150nm; Huxley, 1963) and 8nm wide. The filaments possessed Mg\(^{2+}\)ATPase and Ca\(^{2+}\)ATPase activities and were dissociated by ATP, but not by Mg\(^{2+}\)ATP. These filaments may be equivalent to those produced by Huxley (1963) at 0.05M KCl, as Reisler found that on increasing the KCl concentration from 0.04M to 0.2M KCl further polymerization occurred; the rate of polymerization depended on the ionic strength, temperature and pH.

Oriol-Audit, Lake and Reisler (1981) studied the effect of the subfragment-1 heads on aggregation by comparing mini-filaments with mini-rods (made from myosin chymotryptically digested to remove subfragment-1). Both were found to be dissociated by 1-5mM ATP and phosphate and the ion binding to both filaments were identical. It was concluded that myosin heads play no role in destabilization and therefore assembly.

7.2.5 Interpretation of Synthetic Filament Studies

What interferences can be made concerning the physiological assembly of myosin filaments from the studies of synthetic filaments? The aim of in vitro studies of synthetic filaments is to gain structural information of myosin packing and myosin assembly. The bare zone is probably the earliest stage of myosin assembly and occurs by anti-parallel association and growth proceeds by parallel addition of myosin molecules (Huxley, 1963). This evokes different bonding interactions and
stabilities in the different regions. Part of, or the entire central region may represent a self-assembly nucleation unit (Katsura and Noda, 1973) and kinetic studies of filament formation show typical lag and growth phase characteristic of this type of reaction (Reisler, Smith and Seegan, 1980). Mini-filaments may constitute the nucleus of the growing filament (Reisler et al., 1980).

Recently Trinick and Cooper (1980b) compared the reassembly of wholly and partially depolymerised native thick filaments. When separated native thick filaments were completely dissolved at $l=0.5$ and then dialysed to $l=0.13$ the resulting synthetic filaments had a wide range of lengths between 0.5$\mu$m and 6.0$\mu$m. Similar experiments (Trinick and Cooper, 1980a) were performed on native filaments depolymerised to 0.4$\mu$m ($l=0.25$) and 1.3$\mu$m ($l=0.2$). On dialysis back to $l=0.13$ the filaments which had shortened to 0.4$\mu$m had a narrow range of lengths with a mode of 1.0$\mu$m. The filaments which had been reduced to 1.3$\mu$m did not show any tendency to elongate after 18 hours dialysis at $l=0.13$. These results indicate that although myosin is capable of self-assembly, a factor other than a shortened myosin filament as a template is required to produce filaments morphologically identical to native filaments. Furthermore, this other factor is probably not an integral part of the native thick filament.

Conflicting evidence has been presented regarding the involvement of the myosin heads in synthetic filament assembly and therefore presumably in their involvement in A-filament assembly in vivo. 1) Pinset-Härfström and Whalen (1979) reported that ageing of myosin or removal of light chain 2 by DTNB produces shorter and thicker filaments. 2) Moos, Offer, Starr and Bennett (1975) found that myosin rods (myosin molecules minus subfragment-1 i.e. LMM plus subfragment-2) aggregate to form large paracrystals. 3) Oriol-Audlt, Lake and Reisler (1981) concluded that subfragment-1 plays no part in myosin assembly as mini-filaments and mini-rods are essentially identical with respect to size and dissociation properties. 4) Subfragment-2 is almost certainly involved in determining the size of myosin filaments as there is a limit to the dimensions of synthetic filaments but not to light meromyosin paracrystals (Szent-Györgyi, Cohen and Philpott, 1960).

Chin (1981) has recently discovered that myosin extraction with high salt (i.e. KCl) or possibly secondary phosphate solution (see Chapter 7.1) alters the configuration
of the DTNB light chain (light chain 2) binding site. However, Chin has also found that myosin solubilised in the presence of actin is protected from the effects of high salt on the DTNB light chain. Myosin solubilised within a myofibrillar network and not subsequently extracted reassembles, on lowering the ionic strength, into filaments of apparently physiological dimensions (reconstructed filaments; see Chapter 10.2). These results strongly suggest that the DTNB light chain and its binding configuration are involved in myosin filament assembly. If this hypothesis is correct, the inability to produce myosin filaments of native dimensions in vitro, may be due entirely or partly to the effect of the myosin solubilising medium initially used to obtain a myosin solution. The properties or the assembly medium may still have some effect on the assembly process, but the assembly conditions may not be as critical as so far supposed. All studies of synthetic filament assembly have used high salt extracted myosin. The DTNB light chain is found in subfragment-1; removal of myosin heads may have no effect on filament assembly as the DTNB light chain of solubilised myosin is probably not in its native state. To solve the problem of filament length determination, Cohen(1964) and Casper(1964) have proposed the idea of “a self-limiting packing arrangement of identical subunits” i.e. all myosin molecules are not in an identical environment and the binding constant of the monomer varies along the filament length. It is possible that it is only the specific interaction of myosin molecules which regulates filament size and structure the final dimensions being determined by the precise nature of the myosin molecule. For example subfragment-2 confers upon the myosin molecule packing a self-limiting mechanism, as the packing of light meromyosin is not self-limiting (Szent–Györgyi, Cohen and Philpott,1960). Subfragment-1 may act as a fine control of the self-limiting mechanism, therefore any alterations in the myosin head structure (i.e. the DTNB light chain) may produce filaments of different dimensions. However, the effect of environmental conditions and non-myosin proteins such as MFEP and C-protein may still not be ruled out. Furthermore, another mechanism which may affect myosin filament assembly has been proposed—phosphorylation.

Myosin from smooth and non-muscle cells only forms synthetic bipolar filaments, stable in millimolar Mg^{2+}ATP, under physiological conditions if the myosin is phosphorylated (Scholey, Taylor and Kendrick-Jones,1980). Non-phosphorylated myosin produces short filaments easily dissociated by Mg^{2+}ATP. Frederiksen(1980) has suggested that phosphorylated striated muscle myosin may produce more stable
and/or ordered myosin filaments than non-phosphorylated myosin. However, the
mode of non-muscle and smooth muscle myosin filament assembly may be completely
different from striated filament assembly. Hinssen, D’Haese, Small and Sobieszek(1978)
found that synthetic non-muscle and smooth muscle myosin filaments resembled
those found in vivo and the filaments tended to have an asymmetric appearance,
which they attributed to an antiparallel myosin dimer building unit. These structures
are not found in striated muscles.
7.3 The Reassembly of Myosin Filaments in situ - Reconstruction

As has been seen, reassembly of myosin filaments in vitro has been intensively studied, however, A-filament and A-band reconstruction in situ have not been widely attempted and the aims of the experiments were not so much to reassemble A-filament but to return the ability to contract to myosin extracted fibres. One of the first such experiments was performed by Opiatka, Gadasi and Borejdo(1974) who irrigated "ghost myofibrils" (striated rabbit fibrils from which myosin has been completely extracted) with subfragment-1 and heavy meromyosin. On addition of 5mM Mg\(^{2+}\)ATP, 90% of the ghost myofibrils contracted, as observed by phase microscopy. It was concluded that mechanochemical force can be generated by the interaction of isolated myosin heads with actin in the presence of Mg\(^{2+}\)ATP and that the filament shaft (light meromyosin) is not required. The work was followed by Mitsuka, Yamada and Shimizu(1979), who repeated the experiments and deduced that as myosin active fragments do not form filaments in fibres, there must be some element(s) which transmits the contractile force along the whole fibre i.e. connectin filaments (Maruyama, Natori and Nonomura,1976; see Chapter 13.1). They also suggested that shortening of fibres with heavy meromyosin is caused by a co-operative effect among acto-heavy meromyosin molecules incorporating "metachronal" rotations of heavy meromyosin molecules on thin filaments. Rotations of myosin heads may also cause shortening in the case of subfragment-1 solutions.

Tawada, Yoshida and Morita(1976) were perhaps the first group to attempt to reassemble complete A-filaments and they did so by irrigating ghost myofibrils with a solution of whole myosin. To ensure that myosin extraction was complete beforehand, muscle fibres, skinned with glycerol, were treated with a non-ionic detergent, Triton X-100, after Hasselbach-Schneider extraction. On irrigation of these ghost myofibrils with 3.2mg/ml myosin solution and Mg\(^{2+}\)ATP, contraction was completely recovered but only 10% of the original isometric tension (i.e. of untreated muscle) was regenerated. It was presumed that the weak tension was due to the irregular packing of A- and I-filaments as in the electron microscope.
the reconstituted fibres were seen to have some A-filaments running from Z-line to Z-line in parallel with the I-filaments, but the packing was very poor. The ability to contract was completely lost in extracted fibres and only partially regained after irrigation with heavy meromyosin or a solution of 11.4mg/ml myosin. The latter result was interpreted such that the myosin molecules were inhibited from penetrating the fibres due to the high viscosity of the myosin solution. On irrigating with a solution of 3.2mg/ml or even 11.4mg/ml, one would not expect 100% reassembly of A-filaments, as the intracellular myosin concentration on solubilisation of A-filaments in situ would be approximately 100mg/ml (based on Huxley and Hanson’s calculation in 1957 that myosin constitutes 50% of the total myofibrillar protein).

Tanaka and Tanaka (1979) chemically skinned molluscan catch muscle fibres, extracted myosin with 0.6M KCl and found no tension generated on subsequent addition of Mg$^{2+}$ATP. After irrigation of the ghost fibres with molluscan or rabbit psoas myosin one tenth to one third of the original tension was recovered. The irrigated myosin which had penetrated the fibres was subsequently extracted and it was found that during irrigation approximately 50% of the original myosin content had been reassembled. Therefore it seems that irrigation of ghost fibres with heavy meromyosin and subfragments-1 can recover contraction (Oplatka, Gadasi and Borejdo, 1974; Mitsuka, Yamada and Shimizu, 1979) but intact myosin is required for tension regeneration (Tawada, Yoshida and Morita, 1976; Tanaka and Tanaka, 1979).

In 1976, a project student in this laboratory, Isenor, studied the ability of rabbit psoas fibres to contract after myosin extraction with secondary phosphate solution, 0.266M $K_2HPO_4$ (Mihalyi and Rowe, 1966). Contraction and tension generation were regained on addition of Mg$^{2+}$ATP but in this case without previously irrigating extracted fibres with myosin or myosin fragments. Electron microscopy revealed that myosin extraction by secondary phosphate was not complete; residual myosin had reassembled into filaments and these in turn formed into regular A-bands. Samples examined after phosphate treatment but prior to addition of Mg$^{2+}$ATP, did not possess A-bands or A-filaments. The "reconstructed" A-filaments and A-bands were well ordered and of uniform length, however, the "reconstructed" A-filament length was approximately one third of native filaments i.e. the A-bands were about 0.6μm wide compared to 1.6μm. It was concluded that secondary phosphate solution
completely solubilises intracellular myosin but only extracts two thirds (as shown by analytical methods) and on return to a physiological ionic strength and addition of Mg^{2+}ATP the residual myosin is capable of reassembly into filaments. Furthermore, filament assembly must occur simultaneously to produce a complete filament population of a uniformly decreased length, rather than one third of the physiological number of full length filaments.

It was postulated that by preventing myosin extraction by surrounding the muscle strips with a tightly apposed artificial membrane (made from dialysis tubing, to prevent loss of macromolecular components but allow entry of phosphate ions; see Figure 23) full length filaments might be reconstructed. Controlled dialysis back to physiological ionic strength, followed by addition of Ca^{2+} and ATP (Figure 24) has resulted in a good degree of reconstruction of 1.6μm A-filaments, correctly located with respect to the M-line as judged by electron microscopy (Maw and Rowe, 1979). Reconstruction has since been studied by light microscopy and X-ray diffraction which will be discussed here. Pre-depletion of muscle calcium ions, pre-glycerolisation and solubilisation at long sarcomere lengths (3.7μm) all abolish the complete reconstruction of rabbit psoas A-filaments.

Whilst the studies of rabbit psoas muscle reconstruction which were in progress two other project students in the laboratory have been working on reconstruction: Clode(1981) used rat psoas and Byrne(1981) frog gastrocnemius, both in the absence of a limiting membrane. Both types of muscle were capable of reconstruction. Rat reconstructed A-filaments were shorter than native filaments due to the absence of a limiting membrane during phosphate extraction; however the sarcolemma of frog gastrocnemius was found to prevent myosin extraction as full width A-bands were produced in the absence of an artificial membrane. The aim of the experiments using frog muscle was to determine the amount of contraction which was recovered on the addition of ATP. However, because the sarcolemma was still intact ATP could not penetrate into the muscle. Surprisingly, the muscles were excitable and were, therefore, stimulated electrically enabling the tension developed during isometric contraction to be measured. Reconstructed muscle was found to have recovered its ability to create tension completely when compared with control muscle. The important points here are that: 1) phosphate treatment only temporarily disrupted the sarcolemma (the muscles gradually became paler on losing myoglobin, therefore.
the sarcomere must have become leaky to a certain extent), and
2) on return to a physiological ionic strength solution, not only did the A-bands reconstruct,
but the sarcolemma reformed and the muscle was once again excitable.

The ability of native A-filaments to fray into 3 subfilaments was described in Part 1: reconstructed A-filaments also fray into 3 units as described here and these structures resemble very closely those of native filaments, suggesting that the structure and molecular packing mechanism of reconstructed A-filaments resembles those of native filaments.
Rabbit psoas muscles were used throughout this work. Usually the animals were killed by slitting the throat following a blow to the back of the neck but occasionally a lethal dose of anaesthetic was given. The muscles were dissected out and stored on ice until required (up to one hour).

8.1 The Basic Reconstruction Procedure

Strips, the length of the muscle and approximately 3mm in diameter, were teased from the psoas and each one inserted with the aid of a spatula into a 150cm. length of presoaked 24/32" visking tubing. Excess tubing was taken up between two glass slides clamped together resulting in muscle strips with neatly fitting artificial membranes (Figure 23). The ends of the tubing and muscle strip were sealed off by tying with cotton thread. Four of these so called "reconstruction assemblies" were required for each experiment.

One assembly (the control, designated as sample C) was left in mammalian ringer throughout the experiment whilst the other three (samples 1, 2 and 3) underwent myosin solubilisation in 2 or 3 changes of secondary phosphate solution for 16 to 20 hours (Figure 24). Sample 1 remained in phosphate while samples 2 and 3 were transferred to mammalian ringer for 1.5 hours. The ringer was changed once. Finally the artificial membrane of sample 3 was slit along the length of the muscle (to allow entry of ATP) and the assembly was incubated in reconstruction medium for 1 hour. The whole procedure was carried out at 2-4°C.

8.2 Extensions of the Basic Procedure

To study the method by which A-filaments are reconstructed it was necessary to impose different conditions on the muscle prior to and during the reconstruction procedure i.e. reconstruction at different sarcomere lengths, after pre-glycerolation
and/or pre-calcium ion depletion (with and without the return of calcium ions after phosphate treatment). The differences between reconstruction of fresh muscle initially in a relaxed, contractile or rigor state and the effect of magnesium ions on the secondary phosphate action were also explored.

8.2.1 Sarcomere Length Determination

Generally the muscle strips (once inserted into the membranes) were eased manually to approximately "rest length" i.e. the length assumed by the psoas muscle in the body when a relaxed rabbit is laid on its back with the legs stretched. Elliott, Lowy and Worthington (1963) determined the sarcomere spacing as 2.7 ± 0.2 μm at rest length. For accurate sarcomere length determinations, laser diffractometry was used. This procedure occurred after inserting the muscle strips into visking tubing prior to clamping between glass slides. The ends of each strip were held in a sliding clamp enabling the muscle to be stretched whilst in the path of a laser beam, until the distance between the diffracted spots corresponded to the desired sarcomere length (Figure 25). Two sarcomere lengths were selected - approximately 2.6 and 3.8 μm.

8.2.2 Pre-Glycerolation

Individual strips of muscle (approximately 3mm in diameter) were attached at both ends to perspex rods with double sided adhesive tape or cotton thread and incubated in 50%(w/v) glycerol / 50% mammalian ringer at 2-4°C for about 8 hours. The samples were transferred to fresh solution prior to storing at -20°C. Storage times varied from one day to several weeks.

When required the strips were soaked in 15%(w/v) glycerol / 85% mammalian ringer at 2-4°C for 30 minutes and then in mammalian ringer for 1 hour.
8.2.3 Calcium Ion Depletion

Calcium ion depletion prior to reconstruction was carried out on muscle strips (fresh or glycerolated) attached to perspex strips. In order to calcium deplete reconstructed and phosphate treated samples for relaxed filament preparations the muscle strips were left within the reconstruction assemblies but the artificial membranes were slit to allow entry of EGTA.

Calcium depletion was carried out as described in Part 1 (Chapter 3.2) i.e. several changes of calcium depleting medium for 2 days at 2-4°C.

Reconstruction of pre-calcium depleted fresh muscle was attempted with and without the presence of calcium ions in the mammalian ringer and reconstruction medium after secondary phosphate treatment.

8.2.4 Reconstruction of Muscle in Relaxed and Rigor States

Myosin solubilisation by secondary phosphate solution of fresh muscle was normally commenced within one hour of excision i.e. the muscle would still be in an excitable state. Mihayli and Rowe (1966) found that during secondary phosphate extraction of muscle mince, ATP levels rose during the first 2.5 hours of extraction and ATP was not in fact exhausted until after 10 hours extraction. Muscle assemblies were left for 16 to 20 hours in secondary phosphate to allow complete myosin solubilisation. If the process is monitored by low angle X-ray diffraction the myosin equatorial reflections are lost after about two hours (see Chapter 9.1.5). Therefore myosin filaments are almost certainly solubilised by secondary phosphate solution before the intracellular ATP is depleted.

To compare the effects of the degree of cross-bridge binding on reconstruction, some muscle strips were assembled in a rigor state and some in a relaxed state. In a rigor state all the myosin heads are attached to actin filaments (Cooke and Franks, 1980; Lovell and Harrington, 1981), a small proportion are attached during contraction (Huxley and Brown, 1967), and the heads are completely free in a relaxed fibre. Rigor was induced by soaking the muscle strips for 2 days.
at 2-4°C in mammalian ringer and relaxation was induced by incubating some of these strips in mammalian ringer with 10mM MgCl₂·6H₂O and 2mM ATP for 1.5 hours prior to extraction. Mg²⁺ATP releases actin-myosin bonds (Huxley, 1968).

8.2.5 The Effect of Magnesium Ions on the Action of Secondary Phosphate Solution

Myosin containing filaments bind negative ions, especially chloride and phosphate, so increasing the filament charge. Magnesium ions (but only in the presence of phosphate) decrease phosphate induced charge. Elliott (1980) suggests that these negative ions bind to myosin filaments (and other fibrous proteins) on networks (“clusters”; Loeb and Saroff, 1964) of charged amino acid side chains set up between molecules as they assemble. These ion-binding “clusters” might be an important feature of fibrous protein assembly. As high concentrations of phosphate ions solubilise myosin filaments it was interesting to see if the effect was prevented by the presence of magnesium ions. Elliott (1980) found that 3mM magnesium ions appreciably decreased the charge induced by 0.1M phosphate. 10mM MgCl₂·6H₂O was added to the regular 0.266M K₂HPO₄ solution to test this hypothesis.

8.3 Low Angle X-Ray Diffraction

Strips of muscle were set up in assemblies, phosphate extracted and reconstructed as described. The glass slides were removed before X-ray diffraction. Two sets of experiments were carried out and these will be described separately.

8.3.1 Long Exposure - Equatorials

A perspex cell was specially designed which could accommodate the reconstruction assembly (minus glass slides) allowing a small area of muscle to be irradiated
in the centre of the strip, whilst being held rigid and squashed (between 1.5mm x 6mm mylar windows) from a thickness of 3mm to 1.5mm (Figure 26). The rest
of the muscle strip was bathed in the appropriate solution. No attempt was made to keep the cell cold.

These experiments were done at the Open University Research Centre, Oxford using an Elliott Bros. X-Ray Generator GX-6. Exposure times varied from 4 to 6 hours. To cover a wide range of intensities 2 pieces of Ilford Industrial G film were used to record the diffraction patterns. The specimen-to-film distance was approximately 17cm. Fresh muscle, set at a relaxed sarcomere length was examined and recordings were made of samples C1.2 and 3.

Contact prints made from the exposed films were used as negatives to obtain final prints.

8.3.2 Short Exposure - Equatorials and Meridionals

The same cell (Figure 26) was used to record equatorial reflections but for meridionals the muscles were removed from the assembly and fitted into a specially designed cell of H.E.Huxley, normally used for recording frog meridionals. This cell had larger mylar windows enabling a greater area to be sampled. These experiments used an Elliott Rotating Anode X-Ray Generator GX-13 with slit source at the M.R.C. Laboratory of Molecular Biology, Cambridge.

Again only fresh muscle was examined, but for these experiments muscle strips were set at two different sarcomere lengths - resting (approximately 2.5µm) and extended (approximately 3.7µm). A frog muscle in rigor was also used as a control and a collagen recording was made to calibrate the spacings.

The samples were irradiated for approximately one minute and the diffracted X-rays collected by a multi-channel analyser. The data collected from each channel was displayed on an oscilloscope and a permanent recording of the trace from each sample was made by photographing the screen. Background traces (no sample) were also recorded, using approximately the same total number of recorded counts.
To minimise background noise from the meridional recordings, the heights of all channels to the left of the backstop were measured on an Apple Digitising Tablet, the curve and background were then smoothed (using a Chebychev orthogonal polynomial of order 15, evaluated by means of a BASIC program on the Apple II Microcomputer) and the smoothed background subtracted.

8.4 Preparation of Tissue for Microscopy

The muscle strips were left within the assemblies, the artificial membranes of samples C.1 and 2 having been slit along the length of the muscle as was previously done to sample 3 prior to the addition of ATP. Each assembly was fixed for 4 hours at 2-4°C in glutaraldehyde (25% stock solution) diluted to 4% with the appropriate solution i.e. mammalian ringer or secondary phosphate. The samples were then chopped as described in Figure 27 for post-fixation (1-1.5 hours at room temperature) in osmium tetroxide (2% stock solution) diluted to 1% with the appropriate solutions. The fixatives were then rinsed away with mammalian ringer or secondary phosphate solution. A series of alcohols was used to dehydrate the samples: 15 minutes each in 50, 70, 90 and 100% ethanol and finally 2 periods of 30 minutes in absolute ethanol. Three changes of propylene oxide within 15 minutes were used to wash out the ethanol and provide a miscible exchange for the embedding medium, a low-viscosity epoxy resin, Spurr (Spurr, 1969). Infiltration proceeded over a 2 day period through a series of propylene oxide/spurr mixtures - 25, 50, 75 and 100% (v/v) spurr. The samples were then surface embedded to ensure they were horizontally orientated (Figure 27) and baked overnight at 70°C. The beam capsules were easily removed from the glass slides by standing for a few hours on solid carbon dioxide.

Surface embedding in this manner provided correctly orientated samples for longitudinal sectioning. For transverse sectioning the samples were re-embedded or glued onto old "blocks" as shown in Figure 27.
8.4.1 Light Microscopy

Glass knives were used for cutting thick sections for light microscopy. Blocks containing the large samples i.e. the complete diameter of the original muscle strip were trimmed to give a very large cutting face (approximately 3 x 4mm) through the widest part of the sample (Figure 27). 0.5μm thick sections were cut, mounted onto glass slides and stained with toluidine blue. The sections were viewed in a Zeiss Photomicroscope set up for bright field illumination. Areas throughout the width of the muscle strips were photographed using an objective aperture of 63x magnification and oil immersion.

8.4.2 Transmission Electron Microscopy

Blocks were trimmed to give a surface face no larger than 0.5 x 0.5mm. Sections were cut either with a freshly prepared glass knife or a diamond knife and only sections which were silver to pale gold (approximately 80nm thick) or grey to silver (approximately 60nm thick) respectively were selected. After stretching with chloroform they were picked up on uncoated 300 square mesh copper grids. Thicker sections were stained with a saturated solution of alcoholic uranyl acetate for 20 minutes and thinner sections for 30 minutes. All were post-stained in Reynold's (1963) lead citrate for 2 minutes.

Generally an accelerating voltage of 80kV was used to view and photograph the specimens, with 60kV for samples of very low contrast. The most satisfactory magnifications for photography were 10,000x for longitudinal sections and 20,000x for transverse sections.
Reconstruction of rabbit psoas muscle A-filaments and A-bands has been attempted after a number of imposed conditions in addition to the use of fresh muscle at a resting sarcomere length (approximately 2.6μm):– 1) Fresh muscle stretched to a long sarcomere length (about 3.8μm). 2) Pre-calcium depleted muscle (with and without return of calcium ions). 3) Pre-glycerolated muscle. 4) Muscle pre-calcium depleted and pre-glycerolated. 5) Muscle in rigor, and 6) Muscle allowed to go into rigor and then relaxed with Mg^{2+}ATP. The effect of magnesium ions on the action of secondary phosphate has also been examined.

The following techniques were used to study treated muscle samples to obtain qualitative and quantitative assessments of reconstruction:– 1) Transmission electron microscopy (T.E.M.) of longitudinal and transverse sections of embedded samples. 2) Light microscopy of thick sections. 3) T.E.M. of isolated myofilaments, and 4) X-ray diffraction of fresh samples. T.E.M. of thin sections provides the best form of qualitative assessment but it samples such small areas that quantitative methods were required to examine a greater proportion of the muscle strip i.e. techniques 2,3 and 4. Only T.E.M. of embedded samples has been used to examine each of the conditions imposed on reconstruction and only fresh muscle at a relaxed sarcomere length has been examined by all the techniques.

N.B. Sample C = untreated control. 1 = phosphate treated. 2 = phosphate/mammalian ringer treated, and 3 = phosphate/mammalian ringer/mammalian ringer + 2mM ATP treated (see Figure 23).

9.1 Fresh Muscle at a Resting Sarcomere Length

"Fresh" implies here that the muscle was assembled and put into secondary phosphate solution as soon as possible after being removed from the rabbit. The sarcomere length was either set at resting (approximately 2.6μm) by laser diffraction
or the muscle was stretched gently to the length at which it assumed in the body (Elliott, Lowy and Worthington, 1963; see Chapter 8.2.1).

Reconstruction of fresh muscle at a resting sarcomere length has been assessed by all the methods mentioned and was used as a control for most of the other imposed experimental conditions. Therefore this experiment has been repeated many times and in each case the loss of A-filaments during secondary phosphate extraction has been confirmed before studying the reconstructed samples.

9.1.1 Transmission Electron Microscopy

Figure 28 shows a typical electron micrograph of a longitudinal section of vertebrate striated muscle: "striated" due to the alternate light and dark bands throughout the length of the muscle. The A-bands appear darker as the A-filaments being thicker than the I-filaments stain more densely. At the overlap between the A- and I-bands (at the edges of the A-band) the density increases further. Three stripes are apparent in the centre of the A-bands: the least dense and therefore paler outer stripes indicate the edges of the bare zone or pseudo H-zone and the dark dense centre stripe in the M-line - dense because of the presence of many additional non-myosin proteins (M-proteins; see Chapter 13.5).

Bisecting the I-band is one heavily staining stripe with 2 much fainter stripes - one on either side. The former is the Z-line or Z-disc, the latter the "N-lines" (Yarom and Meirl, 1971; see Chapter 13.1 and Figure 1) and as in the case of the M-line these are darker than the I-band due to additional non-actin proteins being present. At the ends of each Z-line are groups of membranous vesicles which are cross-sections of the sarcoplasmic reticulum cisternae and T-tubule systems.

The sarcomere length (the distance between neighbouring Z-discs in one fibril) in this micrograph is approximately 2.8μm - a "long" resting length.

Sample C Figure 29a shows the same features as Figure 28 but at a higher magnification. Individual A-filaments are easily discernable and the M-line is seen
to be a thickening at the midpoint of each filament and in some areas can be differentiated into 3 stripes (Knappeis and Carlsen,1968; Sjöström and Squire,1977a and b; see Chapter 13.5).

Sample 1 Figure 29b is a micrograph of a muscle sample which has been treated with secondary phosphate solution. Striations, although less obvious, are still apparent. The narrow dense bands, bisecting the I-bands, are the Z-discs. Between the I-bands are less dense regions where the A-bands were originally. The I-bands, being denser than the background, now appear as the darker striations. They are denser than a normal I-band and the background is not clear, presumably due to the high concentration of solubilised myosin present, which also causes the severe lack of contrast. The entire A-bands have been solubilised and the N-lines have also disappeared.

Sample 2 Replacement of the secondary phosphate solution by mammalian ringer, a physiological ionic strength medium, has a dramatic effect. In Figure 29c it can be seen that the heavily staining A-bands have returned, including the darker M-line. The contrast is still low and the filaments are not easily distinguishable, probably due to the presence of solubilised myosin still present in the cytoplasm. All the A-bands have reconstructed uniformly to full length (1.6μm). The N-lines are still absent.

Sample 3 The addition of 2mM ATP to the mammalian ringer appears to “tidy-up” the reconstructed A-filaments as can be seen in Figure 29d. The most obvious difference from Figure 29c is that the contrast has improved, suggesting that most of the solubilised myosin has reassembled. The N-lines have reappeared and the Z-disc is less thick. The A-bands and M-lines appear somewhat less uniform than in Figure 29c but this could be due to the section being cut slightly off the horizontal.

Figure 30 is another example of a thin section of control fresh muscle. The wavy appearance of the fibres is due to local contraction occurring in other areas of the muscle causing resting fibres to compress longitudinally below slack length (Brown, Gonzalez-Serratos and Huxley,1981)
Figures 31a and b are of phosphate extracted muscle at slightly different sarcomere lengths. In micrograph 8c the l-bands are pulled well apart showing clear areas of background in between, whereas in Figure 31b the sarcomere length is so short that the l-bands overlap producing the darker zone between the Z-discs.

Figures 32a and b are micrographs of reconstructed muscle – the former before addition of ATP and the latter, after. However, the two samples appear very similar suggesting that the effect of ATP is minimal (see Chapter 10.2.5).

Figure 33 is a montage of examples of all four samples with the sarcomeres aligned enabling the disappearance of the A-bands in the phosphate treated sample, and their consequent reassembly on return to a physiological ionic strength medium, to be followed.

9.1.2 Transverse Sections

The use of transverse sections of muscle enables the regular packing order of the A-filaments to be examined more thoroughly. Very thin sections were cut i.e., grey (approximately 60nm thick), to minimise obscuring caused by slight tilting within the sample. All transverse sections were cut from the same blocks as longitudinal sections previously presented.

Sample C Figures 30 and 34 show electron micrographs of longitudinal sections of fresh relaxed muscle and transverse sections cut from similar areas. The transverse sections span nearly a whole sarcomere as the fibres in the left hand corners are one sarcomere out of register with those in the right hand corners. Some forms of sarcomere displacement arise from the helicold topology of sarcomeres within striated fibres (Rudel and Thaer, 1981); others may be due to localised stretching. So, starting in the top left of Figure 34a the section cuts through an l-band where the thin filaments appear to be in small clusters and in fact in the longitudinal section this can be seen too. Towards the centre of the micrograph the fibres become denser as the filament mass and filament number increases as the section.
passes first through a region of A/I overlap and then into the pseudo H-zone of an A-band. In the A/I overlap areas each thick filament is surrounded by 6 thin filaments and in the A-band the A-filaments form an hexagonal array. Small patches of the A-band appear denser still; these are the M-line regions and the M-bridges are easily visible connecting each A-filament to 6 others and so causing the increased density. Moving towards the lower right of the micrograph the section again passes through a region of A/I overlap and then back into the I-band.

Sample 1 The micrograph of Figure 29b is a typical longitudinal section of phosphate extracted muscle. Figures 35a and b are micrographs of the corresponding transverse sections. Although each fibril is still slightly out of register with its neighbours i.e. the sections are cutting through different parts of the sarcomere all the fibril cross-sections appear similar because, as can be seen in the longitudinal section from one Z-disc to another there are only I-filaments. In the top right hand corner of the transverse section of Figure 35b is an area of Z-disc in cross-section. These areas are very distinctive due to a basket-weave effect caused by the regular rectangular packing within the Z-disc which varies from the hexagonal packing of the rest of the I-band (Knappels and Carlsen, 1962; Reedy, 1964).

As the transverse sections are so thin individual I-filaments are easily distinguishable but the sections are slightly obscured and of low contrast due to the massive amounts of solubilised myosin everywhere.

The most important feature of the micrographs are the uniformity of filament type due to the absence of A-filaments.

Sample 2 Reconstruction brings a return of the A-bands in longitudinal sections (Figure 29c) and the different fibre cross-section types in transverse sections (Figures 36a and b). The micrographs still lack contrast, probably due to the presence of non-assembled myosin or other disrupted components, e.g. lipoproteins, but the A-filaments are very obvious in the cross-sections. In fact the hexagonal pattern in the pseudo H-zone is very strong indicating that the reconstructed filaments are regularly packed. In longitudinal sections the M-line density has returned but the presence of M-bridges in the cross-sections is not clear.
Sample 3 On addition of ATP to the mammalian ringer the excess myosin may be assembled as the contrast has improved in Figure 37a. There is a strong hexagonal pattern in the pseudo H-zone and M-line, and many M-bridges have reformed. The double hexagonal array in the A/I overlap region is not quite as clear as in the control (Figure 37b) but otherwise the two areas are almost identical.
9.1.3 Light Microscopy

Figure 38a is a micrograph of a typical thick section cut for light microscopy. Individual fibres show up as long dark patches, the peripheral ones being slightly darker than the innermost, due to the incomplete penetration of osmium tetroxide during fixation. The dense specks along the edges of the fibres are nuclei. A schematic representation of the section is made in Figure 38b and it has been divided into 6 regions. Small areas were photographed randomly within the section and then the micrographs subdivided into the 6 regions.

Sample C. Six micrographs of control muscle are presented in Figure 39; areas A and F are from the periphery of the muscle strip and C and D from the centre.

The peripheral fibres of Figures 38a and 39A appear distorted and twisted. This probably arises when stretching and inserting the muscle into the artificial membrane. In all the areas the distinctive striation of vertebrate skeletal muscle is obvious. The wider dark bands are the A-bands with a paler H-zone bisecting them and in between are the lighter I-bands. In turn bisected by the narrow dense Z-disc. The A/I band overlaps are apparent as very dark edges to the A-bands.

Sample 1. Six areas traversing the muscle strip of a phosphate extracted sample are shown in Figure 40. The striation pattern here is very different from the control and varies through the sample. In all the micrographs the narrow dense stripes are the Z-discs; tiny non-staining areas around the Z-discs are probably where the membraneous vesicles have been disrupted. The dark A-bands and paler H-zones are absent throughout the muscle strip. In areas A and F the sarcomere length is longer than through the rest of the sample and this probably arises due to the excess stretching and distortion caused during assembly of the muscle. This results in a different striation pattern in the peripheral fibres, as a new stripe is introduced into the pattern - that of the clear background between the I-bands.
seen here as pale areas of varying widths. The variation in width of the gaps between the I-bands confirms the hypothesis of localised distortion and uneven stretching occurring within the peripheral muscle fibres during assembly. Areas B to E are of much shorter and uniform sarcomere lengths.

Sample 2. On removal of the secondary phosphate solution and addition of mammalian ringer, the striation pattern changes dramatically as seen in Figure 41, except in area A. Here the thin dark stripes are the Z-discs and they can be seen to bisect the I-bands, but these are again only separated by clear background of varying widths. This area has not reconstructed probably because of the excess distortion and stretching destroying the I-band alignment; this will be discussed later in Chapter 10.2.2. The remaining areas show a very similar striation pattern to the control with the dark A-bands, bisected by the pale H-zones, separated by the lighter I-bands with their dense Z-discs. The contrast varies between the areas, probably due to varying amounts of solubilised myosin still present in the background.

Area E has a striation pattern similar to the control but the edges of the A-bands and the H-zones are indistinct, suggesting that complete reconstruction has not occurred. Occasionally, a fibre was seen in the centre of a reconstructed muscle strip which had not reconstructed indicating that the secondary phosphate solution had penetrated the muscle completely but not mammalian ringer. The presence of partially and non-reconstructed fibres will be discussed in Chapter 10.2.2.

Sample 3. The sections of reconstructed samples treated with ATP (Figure 42) were very similar to those of sample 2, except that the contrast had improved suggesting that most of the solubilised myosin had reassembled. Some peripheral fibres have still failed to reconstruct but central non-reconstructed fibres were not seen.

A montage of all four samples is shown in Figure 43, showing the progression from the control to reconstructed muscle through a phosphate extracted sample. The constant features throughout are the Z-discs and between these, the A-bands and H-zones which are seen to disappear during phosphate treatment and reappear.
9.1.4 X-Ray Diffraction - Long Exposure Equatorials

The equatorial reflections of striated muscle arise from the principal lateral spacings of the myofilament lattice (Figure 2). The longer spacing arises from the rows of myosin filaments only, and results in the (1.0) reflection. The rows which incorporate both myosin and actin filaments have a smaller spacing, and hence give rise to a reflection (1.1) at a larger reciprocal distance.

In the diffraction patterns of Figure 44, the reflections appear as lines rather than spots, due to the use of a slit source rather than a spot source. The patterns on one side of the backstops should be mirror images of the other side, but in these experiments the apparatus was slightly misaligned giving rise to an unevenly placed backstop so allowing part of the undeflected beam to come through on one side. Therefore the following discussion of the results refers to the reflections present in the left hand side of the patterns only.

The relative intensities of the lines reflect the cross-bridge positions (Huxley and Brown, 1967; see Chapter 1.5): relaxed muscle produces a stronger (1.0) reflection as the myosin heads lie close to the filament backbone and in rigor, the movement of myosin heads towards the actin filaments, effectively increases the thin filament mass so producing a stronger (1.1) reflection. The relative intensity of the (1.1) reflection also increases on decreasing sarcomere length (Huxley and Brown, 1967).

Sample C. The diffraction pattern of Figure 44a is of control muscle; two distinct diffracted spots are present, the inner (1.0) and the outer (1.1). The sarcomere length of the muscle strip was at resting ie. 2.6μm. As the (1.1) spot is slightly more intense than the (1.0) the muscle is probably not in a relaxed condition.

Sample 1. After incubation in secondary phosphate overnight, the equatorial reflections completely disappear (Figure 44b). The loss of a (1.0) reflection indicates a
disruption of the regular hexagonal A-filament lattice. One might expect an actin to actin spacing to remain after phosphate extraction, giving rise to a weak (1,1) but bearing in mind the disorganisation of the I-band seen in electron micrographs after phosphate treatment any ordered lattice structure has probably been lost although the I-filaments are still present. Similarly, the absence of A-bands in electron micrographs suggests that the disappearance of a (1,0) reflection is due to myosin solubilisation, and not merely disorganisation of the A-filament lattice.

Sample 2. Figure 44c is a diffraction pattern of a phosphate extracted muscle after return to mammalian ringer. This muscle sample received the same exposure as the control sample and similar exposure times were used in printing therefore the relative intensities of the reflections in Figures 44a and c may be compared. The return of the (1,0) and (1,1) reflections indicates that not only has myosin assembly occurred, but that the reconstructed A-filaments are correctly aligned with respect to the I-filaments. The number of reconstructed A-filaments and their packing arrangement must be very similar to that found in untreated muscle as the diffraction spots are in similar positions and of similar intensities.

Sample 3. The spot intensities of Figure 44d are also similar to the control sample indicating that the complement of reconstructed A-filaments resembles that of native filaments. However the (1,0) and (1,1) reflections cannot be individually distinguished, indicating that although the A-filaments are present, they are not as uniformly packed as in the control and sample 2.

9.1.5 Short Exposure Equatorials

The diffracted X-rays were picked up by a multi-channel analyser and the height of each displayed spot reflects the intensity of the corresponding reflection. Therefore these traces are equivalent to a cross-sectional view of the diffraction patterns of Figure 44; the backstop here is represented as a deep trough in the middle of the trace. The traces on either side of the backstop should be mirror images; anomalies arise due to misalignment of the apparatus, background noise, or electronic faults which on occasion were present in certain (right hand)
channels.

Two sets of results are presented (Figures 46 and 47), one photographed at a higher instrumental magnification than the other.

Sample C. Figures 46a and 47a are the same diffraction pattern except that the latter was photographed at a higher magnification. The patterns show two peaks not present in the background diffraction pattern (Figure 45), which consists of a smooth curve rising up to the backstop. The outer (1,1) reflection of the sample is a larger peak than that of the inner (1,0) reflection i.e., the intensity of the (1,1) is greater. This implies that much of the myosin mass is associated with the actin filaments i.e., many cross-bridges are attached. As the X-ray diffraction pattern was made about 18 hours after excision from the animal the muscle must be in rigor and the diffraction pattern is therefore consistent.

Sample 1. Figures 46b and 47b are patterns from different muscle samples. Other than a small peak on the left hand side of the trace of Figure 46b, which may be "noise" or the remainder of a (1,0) peak, the traces are quite smooth and similar to the background. Secondary phosphate treatment has completely removed the characteristic diffraction pattern peaks of control muscle.

The pattern of Figure 47b was produced by a muscle strip which had been in secondary phosphate for approximately 20 hours. The spurious peak of Figure 46b may be the remnants of a (1,0) reflection as this sample had only been in secondary phosphate solution for 2 hours. Two hours previously a diffraction pattern was made of untreated muscle and leaving the muscle within the cell the reservoir was filled with secondary phosphate solution. The diffraction pattern prior to extraction resembled that of Figure 47a suggesting that myosin solubilisation occurs very rapidly i.e. within a few hours.

Sample 2. The two diffraction patterns of Figures 46c and 47c are from 2 different muscle strips, both of which have been phosphate treated and then returned to mammalian ringer. The patterns are remarkably similar. Right of the backstop only the (1,1) reflection is present, whereas on the left is a (1,1) peak and a small (1,0). As in sample 1 (Figure 46b), the (1,0) peak may be noise, especially
as it is so similar to that found in Figure 47b. Nevertheless the (1,1) reflections have definitely reappeared, implying that the solubilised myosin has reassembled into filaments. The absence or only partial return of the (1,0) reflections suggests that the reconstructed myosin filaments are not as regularly packed as in control muscle.

Sample 3. The X-ray diffraction patterns of Figures 46d and 47d were produced by 2 different muscle strips after incubation in reconstruction medium. The sample of Figure 46d is in fact the same sample of Figure 47c which, after having been irradiated, was left within the cell, bathed in reconstruction medium for 2 hours and then irradiated again. Careful comparison of Figures 46d and 47c reveals that the (1,1) reflections have become stronger and the (1,0) reflections have reappeared, suggesting that the addition of ATP has improved the reconstructed A-filament packing. This may arise due to the ATP releasing the actomyosin bonds and so removing any imposed constraints on the assembled A-filaments. The sample of Figure 47d does not appear to have regained a (1,0) reflection although the (1,1) reflections are as strong as in the control.

9.1.6 Short Exposure Meridionals

After assessing that reconstruction of A-filaments has occurred and that they are packed with a high degree of order by recording the equatorial reflections, X-ray diffraction patterns of the meridionals were made. These reflections (see Chapter 1.5) assess the order of packing of the myosin molecules within the filaments by recording the 14.3nm axial repeat. As the 14.3nm peaks are quite small and are almost indistinguishable from background noise, it was essential to compare the traces with those of the background. This was done by digitising the height of each spot on the left of the backstop, normalising to a constant total count to facilitate comparisons, and smoothing the curves with a Chebychev program (Chapter 8.3.2). The background (Figure 48a; also smoothed) was then subtracted and a narrow range of results (approximately six on either side of the 14.3nm channel) was plotted.

An X-ray diffraction pattern of collagen was also made (Figure 48b); the
repeat distances are known and therefore the exact position of the 14.3nm spacing within the pattern was determined. As a further control a fresh frog muscle in rigor was also irradiated (Figure 48c), as these are constantly being measured at the M.R.C. laboratories.

9.1.7 Calibration of Meridional X-Ray Diffraction Patterns

Collagen
The X-ray diffraction pattern of a collagen sample (rat tail in ringer's solution, kindly donated by Dr. Raul Padron, M.R.C. Laboratory of Molecular Biology, Cambridge) is shown in Figure 48b. The lattice dimension of rat tail collagen is 67nm. In Figure 48b the first reflection is not apparent, being lost in the scatter around the backstop. The second order peaks form a shoulder to the backstop scatter; the most obvious reflections are those of the third order (22.3nm⁻¹). The distance between the third order reflections was used to calibrate the muscle patterns.

Frog Control
The X-ray diffraction pattern of a frog muscle in rigor is presented in Figure 48c. It is very difficult to determine the 14.3nm reflection; smoothing the left hand curve and then subtracting the smoothed background (Figure 48a) produced the curve of Figure 50, the peak of which falls on the 14.3nm position as calibrated from the collagen spacings.

9.1.8 Changes in the Meridional Pattern During Reconstruction

The meridional X-ray diffraction patterns of samples 1, 2 and 3 are presented in Figure 49. The control pattern was found to be obscured by background noise on analysis and has not been included. X-ray patterns from frog and rabbit muscle are generally agreed to be indistinguishable, therefore, the frog pattern has been used as a control. After smoothing and subtracting the smoothed background (Figure
the 14.3nm reflections became apparent (Figure 50). On phosphate extraction the reflection is reduced to a level not significantly greater than background (the small residual peak in intensity, dubiously significant in magnitude, is not in fact centred around 14.3nm⁻¹). Some increase in the peak height occurs on return to mammalian ringer indicating that myosin assembly has occurred with some degree of order. On addition of ATP to the mammalian ringer the intensity of the 14.3nm reflection is greatly increased and is now comparable to the frog control. Electron microscopy of samples 2 and 3 (Figures 29c and d) and the equatorial reflections (Figures 47c and d) suggest that the effects of ATP are minimal. The implications are that the effect of ATP on the A-band structure is indeed minimal and that ATP affects the myosin molecule packing within the reconstructed filament and so produces a more uniform helical array of projecting myosin heads.

9.1.9 Relaxed Filament Preparations

Relaxed filament preparations of reconstructed muscle were made using the same method as described in Part 1 (see Chapter 3.2) except that the muscle samples were left in the assemblies during calcium depletion. The washing step was also included. Some care was taken to use approximately the same weight of muscle and the same volume of solution for each sample in order that the dilutions of filament preparations required for viewing could be roughly compared. Significantly different dilutions were found to be required for different samples in order to produce approximately the same density of filaments per unit area.

Relaxed filament preparations were also made of phosphate treated samples, although as has been seen no A-filaments were detected in the whole muscle. Filaments can be isolated from these preparations but they are much shorter than native or reconstructed filaments and occur in lower yields. The reason why they are present will be discussed in Chapter 10.1.4.

Relaxed filament preparations are used for quantitative assessments of reconstruction. Electron microscopy of embedded samples only allows examination
of small areas of the whole muscle strip and light microscopy and X-ray diffraction
only sample one narrow portion of the muscle. In making filament preparations
the whole tissue is used and therefore filaments throughout the sample are collected.
This means that although only a fraction of the preparation is viewed it will be
representative of the entire filament population.

Figure 52 shows four histograms, one for each sample of a reconstruction
experiment. To compile the histograms, randomly chosen areas of filaments were
photographed at 10,000x magnification and the filament lengths measured with
a Projectina at 7x magnification. The filament lengths were taken as a unit of
mass (Chapter 3.8) and the percentage of total filament mass plotted against the
filament length. 1.4µm or greater was taken to be the length of an essentially
complete filament, since although the length of a native filament is 1.6µm (Huxley,1963),
filament tips tend to be either non-visualised in the stain or sheared off during
homogenisation. Average lengths derived from this procedure will be weight-averaged.

Sample C. A fifteen times dilution of the relaxed filament preparation was required
for easy viewing and measuring (Figure 51a). Full length filaments made up nearly
70% of the total filament mass (Figure 52a). A few filaments appeared to be longer
than 1.6µm. These probably arise due to broken filaments adhering at their ends
which may become "sticky" on breakage.

Sample 1. Only a five times dilution was required for viewing, suggesting that
this relaxed filament preparation was approximately 3 times less concentrated than
the control. Furthermore only about 30% of the total mass of these were full
length (Figure 52b). The average filament length was approximately 0.6µm which
is also the average length of a population of synthetic filaments (Emes and Rowe,1978a).

Sample 2. A fifteen times dilution was required to give an equivalent concentration
for viewing as used for samples C and 1. Therefore, this sample must have a
concentration of filaments similar to the control and 3 times that of the phosphate
treated sample. However the proportional yield of the full length filaments is equivalent
to that of sample 1 i.e., about 30% (Figure 52c). This implies that on removal
of secondary phosphate and on return to a physiological ionic strength medium, the solubilised myosin reassembles into roughly the same number of A-filaments as found in untreated muscle but they tend to be shorter than native filaments.

Sample 3. Again a fifteen times dilution is required for viewing (Figure 51b) indicating that a full complement of reconstructed filaments is present. Just over 30% of the total filament mass is composed of full length filaments which is a small improvement on sample 2 but probably not significant (Figure 52d).

Comparing the results of sample C and 3, it seems that during reconstruction a full complement of filaments is reassembled but only about 50% of these are full length.

N.B. These results correspond very well to the quality of reconstruction as assessed by electron microscopy, being achieved at the same time (Figure 32c). More recently, the reconstructed A-bands (Figures 32b) have borne a far closer resemblance to control A-bands and it is possible that a higher yield of full length filaments could be achieved now.

9.1.10 Fraying of Reconstructed A-Filaments

The micrographs of Figure 53 show several frayed reconstructed A-filaments. In the upper plate, two filaments are lying side-by-side and due to their close association it is difficult to distinguish how many subfilaments are present, but it is clear that reconstructed A-filaments fray into at least two units on either side of the bare zone. The filament of the lower micrograph is not full length and one end has failed to fray. However, the frayed region clearly demonstrates 3 subfilaments and resembles very closely the rabbit frayed filaments presented in Part 1 (Figure 12).

The correlation between the number of subfilaments produced on fraying and filament symmetry and structure has already been discussed (Chapter 5.3). As reconstructed A-filaments appear to fray in a similar manner it seems that
the same discussion may be applied. Therefore, fraying of reconstructed A-filaments provides an initial clue to their structure and the evidence suggests that the structure of reconstructed A-filaments closely resembles that of native filaments. Fraying of synthetic myosin filaments with distilled water washing has not been attempted but Pinset-Härström and Truffy (1979) found that ATP (which would induce a net negative charge on the filament) in the surrounding medium produced synthetic filaments with a feathery appearance. These poorly frayed synthetic filaments do not resemble the frayed filaments presented here and if ATP does have a similar effect to water washing this suggests that the structure of synthetic filaments may be quite dissimilar to that of native and reconstructed A-filaments.
9.2 Fresh Muscle at an Extended Sarcomere Length

Muscle strips were stretched after insertion into the artificial membrane by the use of a sliding clamp and the sarcomere lengths determined by laser diffraction. It was attempted to stretch the muscle to the point where A- and I-filaments no longer overlapped i.e. a sarcomere length of 3.8\mu m. Reconstruction of stretched muscle has been assessed by electron microscopy of embedded samples and relaxed filament preparations, and by X-ray diffraction.

9.2.1 Electron Microscopy – Longitudinal Sections

Sample C. The sample of Figure 54a has been stretched to an extended sarcomere length (approximately 3.8\mu m) but is otherwise untreated. Extensive stretching tends to disrupt the regular striation pattern such that the edges of the bands become jagged. The M-lines are often obscured. Knappels and Carlsen (1962) have also described the virtual disappearance of M-lines on extensive sarcomere stretching. Stretching also pulls the N-lines away from the Z-disc towards the A-bands (Page, 1968).

Sample 1. Secondary phosphate treatment solubilised the myosin filaments producing the effect shown in Figure 54b. The A-bands and M-lines have completely disappeared as have the N-lines; the I-bands and background are very dense with solubilised myosin. Note the sharp edges of the I-bands.

Sample 2. Return of fresh muscle at a relaxed sarcomere length to mammalian ringer after phosphate extraction promotes reconstruction of A-filaments and A-bands, but as can be seen in Figure 54c, at extended sarcomere lengths this does not occur. However the micrograph is quite different from that of Figure 54b, in that the background is no longer a homogeneous solution of myosin molecules. Scattered aggregates of myosin are now present in an otherwise clear background and many of the aggregates are associated with the I-bands.
Sample 3. Addition of 2mM ATP to the mammalian ringer has a profound effect on the myosin aggregates (Figure 54d). The background is still vaguely granular but most of the myosin appears to have taken the form of short filaments projecting from the l-bands. These filaments reach a maximum distance of approximately 0.5μm (equivalent to the total length of 3 myosin molecules) from the l-bands and never extend from one l-band to another. Neither M-lines or N-lines have reappeared. The implications of the effect of reconstruction medium on the extracted stretched muscle will be discussed in Chapter 10.3.2.

9.2.2 Tranverse Sections

Sample C. Figure 55a is another micrograph of fresh extended muscle and Figure 55b a transverse section cut from the same block. In the latter plate the section cuts through an entire sarcomere; the areas in the top left and bottom right are l-bands. Only cross-sections of l-bands and A-bands are present; there is no overlap region — a distinct demarcation line is present. The A-filaments have largely maintained their regular hexagonal packing but the M-line region is completely obscured, which correlates with the longitudinal sections.

Sample 3. Transverse sections of reconstructed extended muscle (Figure 56b) have a completely different appearance from untreated samples (Figure 55b) and from reconstructed relaxed muscle (Figure 37a). The two dense wide bands of Figure 56b are cross-sections through the l-bands and between these (and in the top right hand corner) the clear areas are the sites originally occupied by the A-bands. Running through the l-bands are the dense zones of the Z-discs, recognisable by the characteristic basket-weave appearance (Reedy, 1964). Individual l-filaments are difficult to distinguish as they are probably disorientated and obscured by myosin molecules. However dense dark spots are present within the edges of the l-bands and in clusters between the l-bands: these are cross-sections of myosin filaments. In longitudinal sections it was not apparent whether the short filaments which projected from the l-bands originated at the edge or within the l-bands. The presence in tranverse sections of thick filament cross-sections well within the l-bands suggests that there is considerable overlap of l-filaments and reconstructed A-filament fragments, although prior to phosphate extraction (Figure
55b) there was no A-/l-filament overlap. The lack of a continuous spread of A-filament cross-sections between the l-bands confirms the absence of fully reconstructed A-filaments in longitudinal sections. The implications of these results are discussed in Chapter 10.3.2.

9.2.3 X-Ray Diffraction - Short Exposure Equatoriala

Sample C. Extended but otherwise untreated muscle produced the equatorial diffraction pattern of Figure 57a. Two peaks on either side of the backstop are present. The pattern resembles that of unstretched muscle (Figure 47a) except that the (1,0) peaks are slightly obscured, probably due to misalignment of the A-filament lattice caused by stretching. The (1,1) peaks are far less pronounced, which can be accounted for by the decrease in A-/l-filament overlap.

Sample 1. Unfortunately the diffraction pattern of the phosphate treated sample is not symmetrical (Figure 57b). The peak on the right of the backstop may indicate the presence of a myosin to myosin filament (1,0) spacing, but the absence of any such peak left of the backstop suggests that it is background noise. Therefore, in the absence of any other peaks it seems that the A-bands have been completely removed.

Sample 2. The (1,0) and (1,1) reflections have returned on subsequent incubation in mammalian ringer (Figure 57c). As the pattern is not symmetrical and the fault appears to lie on the right of the backstop (Figure 57b), only the peaks on the left will be discussed. The trace was photographed at a lower magnification than the control but nevertheless it is obvious that the (1,0) reflections are of comparable magnitude and the (1,1) peaks are greater in sample 2 than in the control. These results indicate that reconstruction of A-filaments has occurred and that they are correctly aligned within the A-band lattice and furthermore the A-filaments must overlap with the l-filaments to produce such a strong (1,1) reflection. The sarcomere length of this sample was approximately $3.7\mu m$ therefore the reflections probably arise from the packing of A-fragments, rather than complete A-filaments, as suggested by electron microscopy.
Sample 3. The diffraction pattern of this sample (Figure 57d) is very similar to that of sample 2 (Figure 57c); the introduction of ATP has not had a noticeable effect.

9.2.4 Short Exposure Meridionals

The same procedure of digitising, smoothing and subtracting the smoothed background was applied to the extended samples as described for the rest length muscles (Chapter 9.1.6). The diffraction patterns are presented in Figure 58 and the net results in Figure 59. Meridional reflections of extended muscle appear to be very similar to non-stretched samples throughout the reconstruction procedure, except that the peaks are slightly smaller, although in the same positions. The return of the 14.3nm reflection of reconstructed extended muscle confirms the presence of A-filament structure and the decrease in peak height compared with the non-extended sample 3, may reflect the presence of less A-filament structure which would arise if only short A-filament fragments were present, rather than full length A-filaments.

9.2.5 Relaxed Filament Preparations

The procedure for making and analyzing relaxed filament preparations of extended muscle was the same as that for used for muscle at rest length.

Sample C. Figure 60a is a length distribution (weight-averaged) for control extended muscle. The yield of full length filaments (50%) is not as high as in the relaxed sample, which may be due to the excess stress that was imposed. A fifteen times dilution of the filament preparation was required for viewing.

Sample 1. A much lower dilution was required for viewing – only five times indicating that the filament preparation was three times less concentrated than the control. The average filament length was about 0.6μm (Figure 60b) and only 20% of the filament population consisted of full length filaments. The explanation for the presence of filaments in a phosphate treated sample is given in Chapter 10.1.4.
Sample 2. The number of full length filaments in this sample was extremely low (2%; Figure 60c) and only a five times dilution was required for viewing. This confirms the electron microscopy evidence that there are few reconstructed A-filament fragments and no full length filaments present in these samples.

Sample 3. The absence of full length A-filaments in electron micrographs is confirmed by the virtual absence of full length filaments in the total filament population (1.3%; Figure 60d). Although filament fragments were observed in the micrographs of embedded samples an increased dilution compared with that used for sample 1 was not required for viewing. The filament fragments may have been lost in the washing step prior to homogenisation.

9.3 Pre-Glycerolated Muscle

Many of the original X-ray diffraction and length/tension experiments and synthetic filament preparations used pre-glycerolated muscle. The experimental results were found to improve with increased storage time in glycerol; the mechanisms by which muscle contraction is controlled were largely unknown and prolonged glycerol treatment releases all but the insoluble myofibrillar matrix from the tissue so eliminating unknown interfering effects. Elucidation of the control mechanisms operating in muscle now enables fresh muscle to be used, for example, knowing that Mg\(^{2+}\)ATP releases actin–myosin bonds in the absence of calcium ions enables native myofilaments to be isolated using the relaxed filament preparation procedure. Glycerolation induces rigor by disrupting the sarcoplasmic reticulum so releasing calcium ions and subsequently washing out intracellular ATP.

The effect of pre-glycerolation on A-filament reconstruction was studied by electron microscopy of embedded samples and isolated filaments.
9.3.1 Electron Microscopy

Sample C. A micrograph of pre-glycerolated, but otherwise untreated muscle is shown in Figure 61a. The sarcomere length is extremely short due to the glycerol induced rigor; the A- and I-filaments completely overlap. All the bands are distinct and resemble fresh muscle except the N-lines which have moved away from the Z-discs and are very prominent. There are comparatively few sarcoplasmic reticulum and T-tubule vesicles.

Sample 1. The A-bands and M-lines seen in Figure 61a are now absent (Figure 61b) and the background is dense with solubilised myosin.

Sample 2. In Figure 61c there is, as yet, no sign of reconstruction. A-bands, M-lines and N-lines are still absent. The background has become more granular, possibly due to myosin clumping.

Sample 3. Pre-glycerolated muscle does not reconstruct (Figure 61d). The muscle appears very similar to phosphate treated samples.

Failure of pre-glycerolated muscle to reconstruct was confirmed in a later experiment as shown in Figures 62a and 62b. The addition of ATP in sample 3 (Figure 62b) does appear to have caused some clumping of solubilised myosin compared to sample 2 (Figure 62a).

9.3.2 Relaxed Filament Preparations

Sample C. A large dilution (12 times) was required to produce a filament concentration suitable for viewing. Approximately 80% of the total filament mass was composed of full length filaments (Figure 63a).

Sample 1. The complete absence of A-filaments in Figure 61b is confirmed by the low dilution (4 times) required and the small percentage (about 3%) of full length filaments present (Figure 63b). The average filament length was slightly longer than 0.6μm.
Sample 2. Again only a low dilution (4 times) was required to produce the desired dilution for viewing and the yield of full length filaments was only about 1% (Figure 62a). These results confirm the lack of reconstruction observed in electron micrographs (Figure 61c).

Sample 3. Addition of ATP had little effect on that of mammalian ringer (Figure 63d). A 4 times dilution was made for viewing and 10% of the filaments released were full length.

9.4 Glycerolation of Pre-Calcium Depleted Muscle

Muscle strips were calcium depleted, and glycerolated prior to assembly for reconstruction. Mammalian ringer used in these experiments was prepared without calcium ions.

9.4.1 Electron Microscopy

Sample C. The sample of Figure 64a was stored in glycerol after calcium depletion but was otherwise untreated. Calcium depletion has prevented the muscle from contracting on incubation in glycerol.

Sample 1. A-bands, M-lines and N-lines are all absent as expected (Figure 64b). It is unlikely that phosphate extraction caused the decrease in sarcomere length resulting in overlapping I-bands; the muscle may have been assembled at a short sarcomere length originally.

Sample 2. Return to mammalian ringer has not promoted reconstruction although some aggregation of free myosin has occurred (Figure 64c).

Sample 3. Addition of ATP has no additional effect on that of mammalian ringer as seen in the micrograph of Figure 64d.
9.4.2 Relaxed Filament Preparations

All the samples were calcium depleted again after reconstruction.

Sample C. As with fresh and glycerolated muscle a relaxed filament preparation of calcium depleted, glycerolated muscle (Figure 65a) produced a high concentration (10 times dilution) of full length filaments (70%).

Sample 1. Confirming the prediction from the micrograph, Figure 64b, a low dilution was required for viewing (3 times) and there were few full length filaments (11%; Figure 65b).

Sample 2. A 4 times dilution was made and only 1 to 2% of filaments were full length (Figure 65c).

Sample 3. The low concentration of filaments (3 times dilution) and small yield of full length filaments (14%; Figure 65d) confirms the lack of reconstruction observed in Figure 64d.

9.5 Pre-Calcium Depleted Fresh Muscle

Fresh muscle was calcium depleted prior to assembly for reconstruction. Calcium ions were omitted from mammalian ringer used in these experiments. The samples were only examined by electron microscopy of embedded tissue.

Sample C. Figure 66a is a micrograph of pre-calcium depleted fresh muscle. The short sarcomere length is not the result of calcium depletion: calcium depletion does not affect the morphology of striated muscle.

Sample 1. Phosphate treatment solubilises the A-bands and M-lines and removes the N-lines as expected (Figure 66b).

Sample 2. The absence of calcium ions inhibits reconstruction. Figure 66c is
very similar to sample 1 (Figure 66b).

Sample 3. Addition of ATP has no further effect although some myosin clumping has occurred (Figure 66d).

9.6 The Return of Calcium Ions to Phosphate Extracted, Calcium Depleted, Fresh Muscle

Calcium depletion prior to assembly and phosphate extraction was carried out as described above, but samples 2 and 3 were subsequently treated with mammalian ringer incorporating calcium ions.

Sample 2. The return of calcium ions to the mammalian ringer after phosphate extraction has a profound effect. In the absence of calcium ions, sample 2 (Figure 66c) resembles sample 1 (Figure 66b). On addition of calcium ions reconstruction is promoted. Figures 67a, b and c are three micrographs of 3 different areas within sample 2. In Figure 67a the edges of the l-bands have become denser and small filaments can be seen to project from them and sometimes traverse to the neighbouring l-band. Furthermore between the l-bands are often areas of M-line - sometimes without A-filaments being present, although the M-lines do have a striped appearance, which seems to be composed of the lateral association of very short filaments (approximately 140nm in length - equivalent to the length of one myosin molecule).

The micrograph of Figure 67b shows the filamentous projections from opposite l-bands coming into contact with the M-line and so joining up. In Figure 67c the reconstruction appears to be complete. The A-band edges lack uniformity and the inter-l-band widths of Figure 67a vary, possibly because the sections have been cut slightly off the horizontal. The widths of the A-bands vary from 1.4\mu m to 1.6\mu m and the narrower bands may represent intermediate stages of reconstruction. M-lines and N-lines have also reappeared in reconstructed fibres.

Sample 3. Unfortunately sections cut from sample 3 did not show reconstructed
A-bands (Figure 67d) although presumably such areas as seen in sample 2 are present elsewhere. This reflects a disadvantage of electron microscopy. A relaxed filament preparation producing a high yield of filaments would have confirmed the presence of A-filaments elsewhere in the sample.

9.7 Reconstruction of Fresh Muscle Initially in a Rigor, Relaxed or Contractile State

Fresh muscle used in the experiments so far described (i.e. muscles set at resting and extended sarcomere lengths) has been in a potentially excitable state. Intracellular ATP levels would still be high as the muscle strips were assembled and incubated in secondary phosphate solution well within 2 hours of excision and ATP levels are not depleted until after 10 hours phosphate extraction (Mihalyi and Rowe, 1966). Magnesium ions are not incorporated in the reconstruction procedure thereby preventing relaxation.

To determine what effect the initial state of muscle has on reconstruction, fresh muscle was given various treatments prior to phosphate extraction. The phosphate treated samples will be described first (including a sample to which magnesium ions were added), and then the corresponding reconstructed samples. Interpretations of the results will be discussed in Chapter 10.3.3.

9.7.1 Phosphate Extracted Samples

Figure 68a is a section from a sample which has been previously described - fresh muscle at a resting sarcomere length. Note the even dense staining of the i-bands. The sample of Figure 68b was incubated in mammalian ringer for two days prior to secondary phosphate treatment i.e., the muscle was in rigor. The i-bands look distinctly different: the edges of the bands are much denser than the areas on either side of the Z-discs. Figure 68c is a micrograph of a section cut from a muscle sample which had been allowed to go into a state of rigor in mammalian ringer for 2 days, but, prior to phosphate extraction was incubated in mammalian ringer with 10mM magnesium ions and 2mM ATP thereby
inducing a relaxed state. The I-bands are now evenly stained throughout. Finally, Figure 68d is a section from a sample to which 10mM magnesium ions were added to the secondary phosphate solution. The A-bands and M-lines have clearly been solubilised.

9.7.2 Reconstructed Samples

Figure 69a is a micrograph of fresh muscle at a relaxed sarcomere length. The A-bands and M-lines have completely reassembled. Reconstruction also occurs in muscle previously in rigor prior to phosphate extraction (Figure 69b) but the A-filament lattice, packing is not as uniform as in Figure 69a and the M-lines are not as well defined. Furthermore, the N-lines have not reappeared. However inducing a relaxed state with Mg$^{2+}$ATP after rigor-mortis has occurred inhibits subsequent reconstruction (Figure 69c) although the background is full of aggregated myosin.
Reassembly of A-filaments in vitro from their solubilised components is not a new idea. Huxley (1963) produced so called "synthetic" myosin filaments by selectively solubilising and extracting myosin (using a high ionic strength solution) from muscle homogenates. On lowering the ionic strength of the solution, myosin polymerises into filaments. However, electron microscopy reveals that these filaments are not of regular length or thickness (Emes and Rowe, 1978a). Their average length is 0.6 to 0.7 μm compared to 1.6 μm of native filaments (Huxley, 1963).

Furthermore the extracting media were not totally selective for myosin; Z-discs tended to be disrupted too (Mihalyi and Rowe, 1966). On testing a range of extracting solutions, Mihalyi and Rowe (1966) found that a 0.266 M K$_2$HPO$_4$ solution would completely solubilise myosin and is capable of extracting up to 70% (Ilsenor, 1976) without disrupting or affecting any other intracellular components. The secondary phosphate solution was initially used on muscle homogenates.

In 1976 Ilsenor applied the technique of myosin solubilisation and extraction with secondary phosphate solution to strips of muscle. After several hours extraction, he observed the contraction which could be generated by the strips on return to a physiological ionic strength medium in the presence of 2 mM ATP. Simultaneously, blocks were made of the phosphate extracted and stimulated samples. Examination of sections revealed that, as expected, the A-bands had been completely solubilised in the former samples but, after treatment with mammalian ringer/ATP "mini A-bands" had reformed. These were approximately 1/3 of the original A-band width, indicating that secondary phosphate solution extracts 70% of solubilised myosin. Presumably the remaining 30% preferentially assembles a complete complement of filaments but at 1/3 of their original length rather than into 1/3 of the number of full length filaments.

So the possibility of reconstructing full length filaments arose by preventing myosin extraction from taking place i.e., by putting a tightly apposed artificial membrane
around the muscle strip which would be permeable to small ions such as phosphate and impermeable to solubilised myosin. Comparing these experiments of the production of reconstructed filaments with those of Huxley's synthetic filaments, it was obvious that the same mechanism was in operation i.e., myosin is solubilised in a high ionic strength solution and reassembles on lowering the ionic strength, either by dilution or replacement by a physiological ionic strength medium such as mammalian ringer.

It was assumed that the presence of 2mM ATP in the mammalian ringer was required for reconstruction, but for ATP to gain access to the muscle it is necessary to slit the artificial membrane. The assemblies were preincubated in mammalian ringer to wash out the phosphate solution before slitting the membranes and adding ATP. Since then, it has been seen that mammalian ringer alone is sufficient to produce reconstruction of A-filaments, but, as will be discussed later (Chapter 10.2.5), ATP may improve the molecular packing of reconstructed filaments.

10.1 The Evidence for Disassembly

Before qualitative and quantitative determinations of reconstruction could be performed, it was essential to prove that secondary phosphate solution was completely solubilising all the A-filaments. In all experiments, the first sample to be analysed was the phosphate treated one and in all except one of the experiments, virtually complete phosphate extraction was achieved.

The micrographs of Figure 70 are samples from the experiment which gave anomalous results. Figure 70a is a phosphate treated fresh muscle. Figure 70b a phosphate/mammalian ringer treated fresh muscle. Figure 70c a pre-calcium depleted, phosphate/reconstruction medium treated muscle and Figure 70d a pre-glyceroiated/phosphate/mammalian ringer treated muscle. Clearly the samples of Figures 70c and 70d have been successfully phosphate extracted, but in Figure 70a, using the same phosphate solution at the same time, the sample still possesses A-bands and consequently throws doubt on the presence of genuinely reconstructed muscle in the sample of Figure 70b.
The sample of Figure 70a seems to be in an intermediary stage between the control and completely extracted muscle. The contrast is extremely low, the A-bands almost being obscured, suggesting that the filaments are only partially solubilised. It is interesting to note that myosin solubilisation appears to occur along the length of filament rather than the filaments being disassembled from either end, as the residual A-band is still full width i.e., 1.6μm.

The reason why this sample has not completely phosphate extracted, may be found in studying the muscle samples of Figures 70c and d. Muscle strips subjected to glycerol, calcium depletion and phosphate treatments tend to swell (Mihalyi and Rowe, 1966; Godt and Maughan, 1977). The artificial membrane surrounding the muscle during reconstruction is usually fairly closely apposed, but not too tight allowing room for the muscle to swell. It is possible that in this experiment, the muscle strips were very tightly bound in their artificial membranes, preventing swelling. The pre-glycerolated and pre-calcium depleted samples would already be swollen prior to assembly, allowing easy access of phosphate ions but the fresh samples probably took up phosphate and swelled to a certain point, but then the constraints of the membrane stopped further swelling and therefore further phosphate uptake, resulting in only partially phosphate treated samples.

The quality of reconstructed A-bands and filaments improved markedly from the first reconstruction experiment of fresh relaxed muscle to the last; a major reason for which, may be the increasing adeptness of assembling the muscle strips and causing the least amount of unnecessary stretching and distortion. Simultaneously, the ease of assembling the muscle into the artificial membrane improved producing a tighter membrane. After the failure to phosphate extract in this experiment, the membranes in future experiments were not so tightly apposed to the muscle and complete phosphate extraction occurred thereafter.

Nevertheless, it seems probable that the upper fibre of sample 2 in Figure 70b is truly reconstructed, as the A-filament packing is too irregular to be regarded as being identical to the control muscle. However, the lower fibre may be reconstructed or the filaments may be native. Occasionally, in thick muscle strips peripheral fibres are trapped between the glass slides and the A-bands of these fail to solubilise.
10.1.1 Electron Microscopy

The technique which has been used throughout for determining the loss of A-filaments and their reassembly during the reconstruction procedure has been transmission electron microscopy (T.E.M.). As pointed out before, this technique is excellent for observing the fine detail of specimens, but cannot be representative of the whole sample. For this reason other techniques were also employed, namely light microscopy, X-ray diffraction and relaxed filament preparations.

In the areas studied by T.E.M. the absence of A-filaments after phosphate treatment was very obvious. The normal striated appearance of control muscle was completely altered to a less well defined striation pattern as the densely staining regular A-bands were lost and the normally lightly staining l-bands became predominant. Sections of phosphate treated samples were always low in contrast, presumably because of the high concentration of solubilised myosin in the cytoplasm trapped within the artificial membrane. In addition to the most noticeable effect of phosphate treatment i.e. the loss of the distinctive A-bands - M-lines and N-lines also disappeared. In many samples, the l-bands took on a "wheat sheaf" appearance; the l-filaments splaying out from the constricting Z-discs, very similar to the myosin extracted fibres of Etlinger, Zak and Fischman (1976) who used a high salt relaxing buffer.

10.1.2 Light Microscopy

The use of light microscopy enabled a section through the widest part of the muscle strip to be examined, thereby sampling a range of fibres from the periphery through to the centre, to ensure (by direct visual observation) that phosphate extraction occurred throughout. Although individual A-filaments cannot be seen, it is possible to observe the loss of the characteristic striated pattern of control muscle after phosphate treatment and the appearance of a new pattern (similar to the myosin extracted myofibrils of Huxley and Hanson, 1954). Osmium tetroxide stains the M- and Z-lines equally as seen in T.E.M. but toluidine blue only stains the latter, therefore, in the light microscope the M- and Z-lines can be distinguished easily. During the change in the striation pattern one can assume
that it is the A-bands which are lost, as it is the bands which are bisected by the dark staining lines which remain in the phosphate treated samples.

In the light microscope the A-bands were seen to be absent throughout the sections from phosphate extracted tissue, confirming that secondary phosphate solution completely penetrates the muscle strip. In only one muscle strip did some A-bands fail to solubilise; these were in a few peripheral fibres which had probably become trapped between the glass slides on assembly.

10.1.3 X-Ray Diffraction

X-ray diffraction is a similar method to light microscopy in that it will sample the complete width of the muscle. In some respects it is perhaps more conclusive evidence, being applied to the fresh tissue rather than after exhaustive fixation, dehydration and embedding, the effects of which, on cell structure are not entirely known. The combination of X-ray diffraction with electron microscopy provides an ideal probe for noting myofibrillar structural changes. Electron microscopy assesses morphological changes occurring during reconstruction and X-ray diffraction is able to compare the actual packing of the myosin molecules within reconstructed filaments, as well as the packing of the filaments into the A-bands. Furthermore, thin sections of reconstructed muscle only sample one or two layers of filaments, making it very difficult to judge the packing arrangement of the whole A-segment. The structure of myofibrils can be compared to a crystal; the regular packing of myosin and actin molecules within their filaments and the regular arrangement of the filaments within the myofibrillar network of the sarcomere being equivalent to the repeating unit cell of a crystal. Any change in the structure of the myofilaments or myofibrillar network will be detected by X-ray diffraction.

X-ray diffraction of phosphate extracted samples confirmed the loss of A-bands as seen in T.E.M. and L.M.: the absence of the (1,1) and (1,0) equatorial reflections as seen in control muscle confirmed that regularly packed A-filaments were no longer present. The A-bands may still be present but in a disordered state. The loss of the 14.3nm meridional reflection strongly suggests however the disassembly of the A-filaments, and hence of the A-band, in agreement with electron microscopy.
Some residual order, attributable to myosin must in a sense remain, as there is insufficient space within the I-filament lattice for all the myosin molecules to completely randomise in rotational orientation. Such residual order, would however, be of a "liquid crystal" form, not related to filament structure. It is concluded that the absence of visible thick filaments in longitudinal and transverse sections of phosphate treated muscle in the electron microscope, combined with the drastic change in the typically observed striation pattern of rabbit psoas muscle (the I-bands are now the most prominent structures) and the loss of A-filament and A-band X-ray reflections, together imply that by the standards of currently available criteria, all the distinctive structure of the myosin filaments has been completely disassembled.

10.1.4 Relaxed Filament Preparations

The use of relaxed filament preparations gives a quantitative assessment of the A-filament population. Unlike viewing a muscle section, this filament preparation is a method of sampling the whole A-filament population within the muscle sample. The method used to sample reconstructed muscle is the same as that described in Part I (see Chapter 4.1). Filament lengths and filament concentrations (see Chapter 3.8 and 9.1.9) have been assessed.

Myosin filaments cannot be seen in fixed phosphate treated samples and yet they are released during relaxed filament preparations. However, these filaments are very short (average lengths of 0.5 to 0.8μm) and a much lower dilution (approximately one third of that required for control muscle) is required for viewing. Solubilised myosin aggregates at neutral pH on lowering of the ionic strength (the basic principle in the production of synthetic and reconstructed filaments), therefore, on treating phosphate extracted samples with the physiological ionic strength media utilised in relaxed filament preparations one would expect myosin polymerisation to occur. Nevertheless, pre-calcium depleted and pre-glycerolated muscle fail to reconstruct on return to a physiological mammalian ringer and yet myosin filaments are released during relaxed filament preparations. Two conclusions may be drawn from these observations:- 1) it is unlikely that "relaxed" (i.e. from a relaxed filament preparation) filaments are assembled on treating non-reconstructed
samples with calcium depletion medium and therefore must be produced on homogenisation of the samples into ATP relaxing medium, and 2) at least two different species of myosin filaments can be assembled from solubilised myosin on lowering of ionic strength: those produced within a myofibrillar network and those produced in solution. Presumably "relaxed" filaments resemble, or are identical to synthetic filaments, as both are assembled in the absence of a complete myofibrillar network and their average filament lengths are similar (0.6 to 0.8μm; see Chapter 7.2.1 and 9.1.9). Washing the chopped muscle samples prior to homogenisation may account for the low concentrations of "relaxed" filaments released from non-reconstructed muscles (approximately one third of control or successfully reconstructed samples), as 70% of phosphate solubilised myosin is readily extracted (Isenor,1976; see Chapter 7.3).

The conclusions imply that there are several fundamental differences between the assembly conditions and morphology of synthetic and reconstructed filaments. The only characteristics which synthetic and native or reconstructed filaments have in common is that they possess tapered ends and surface projections, and synthetic filaments occasionally have a bare zone (Huxley,1963). Otherwise synthetic filaments are of varying length, the average length being approximately one third of that of native filaments and of varying width (Emes and Rowe,1978a).

It is possible that there are two modes of myosin filament assembly; the first requires a myofibrillar network to produce filaments resembling those found in vivo (see Chapter 11.1), while the second, which does not produce homogeneous filament populations, occurs in solution. The l-filament lattice may have two effects on the assembly of full length myosin filaments: 1) the presence of actin may protect the DTNB light chain from the effects of secondary phosphate (Chin,1981; see Chapter 7.2.2), possibly enabling inherent self-limiting mechanism of myosin packing to operate, and 2) the l-filament lattice may act as a template for myosin filament assembly. Neither effect can operate in solution in the absence of l-filaments.

The presence of filaments in relaxed filament preparations of phosphate treated and non-reconstructed samples is predictable. However, their lengths and concentration are indicative of whether they are native, reconstructed or synthetic.
Filaments. Phosphate treated and non-reconstructed samples produce low concentrations of short filaments.

10.1.5 The Action of Myosin Solubilising Solutions

High ionic strength solutions solubilise myosin filaments but the reason why is largely unknown. Chloride and phosphate ions are "adsorbed" to myosin filaments and not actin filaments (Elliott, 1980) and, as the ion concentration increases the filaments become increasingly charged i.e., in 5mM KCl, $4 \times 10^4 \text{e} \mu\text{m}^{-1}$ to $12 \times 10^4 \text{e} \mu\text{m}^{-1}$ in a 100mM (I=0.1) solution although Elliott has not found further increases in filament charge on increasing I to 0.6. The maximum charge achieved is in a solution containing about 5:1 chloride:phosphate. All the myosin extracting media contain one or both of these ions but the two types of ions have differing effects (Mihalyi and Rowe, 1966). During myosin extraction with any solution, myosin alone is extracted until the ATP levels are depiected, from then on potassium chloride (but not phosphate solutions) proceeds to extract actomyosin. This is thought to be because ATP stabilises the actin filaments or their attachment to the Z-discs and phosphate and ATP competitively bind actomyosin. Phosphate has a very low binding constant, but it may maintain the effect of ATP in this way. Elliott (1980) has postulated that chloride and phosphate ions bind to "clusters" (Loeb and Saroff, 1954) of charged amino acid side chains set up between the molecules as they assemble together and that these clusters are an important feature of fibrous protein assembly and therefore probably disassembly too. However, neither Elliott (1980) nor Loeb and Saroff (1954) describe physical effects caused by ion binding.

The binding of ATP and phosphate ions to myosin filaments may be an important element in contractile events (Rome, 1967;Thomas, Seidel Gergely and Hyde, 1975; Mendelson and Cheung, 1976; Sutoh, Chiao and Harrington, 1978) and the manner in which they cause filament dissociation may reflect the method by which filaments assemble. Binding of pyrophosphate to myosin and rod mini-filaments produces a decrease in the helical content as monitored by circular dichroism (Oriol-Audit, Lake and Relsier, 1981). Subfragment-1 remains unchanged and is obviously not involved, as myosin rods and filaments pack in a similar manner.
However, there is a 10% $\alpha$-helix coil transition in subfragment-2 on binding pyrophosphate which is possibly relevant to contraction and myosin packing. Pyrophosphate also dissociates light meromyosin paracrystals but without structural change. Therefore, it appears that subfragment-2 is the region of structural perturbation during disassociation of myosin molecules by pyrophosphate and this structural change in subfragment-2 may be involved in the cross-bridge cycling mechanism in contraction as predicted by Tsong, Karr and Harrington (1979) possibly by releasing the cross-bridges from the filament surface. Physiological concentrations of phosphate ions may be involved in contraction in vivo whilst high concentrations disassemble A-filaments in vitro. High salt induces repulsive electrostatic interactions in myosin filaments so causing dissociation and phosphate ions increase the charge of myosin filaments (Elliott, 1980). Lowering the ionic strength of a native filament preparation produces a different form of electrostatic repulsion which initiates fraying (see Chapter 5.2). These results emphasise the importance of charge interactions in the functional behaviour and structure of thick filaments.

Trinick and Cooper (1980a) have studied the sequential disassembly of isolated native rabbit psoas A-filaments using potassium chloride. They found that by using incremental rises in salt concentration that there were three quite separate stages of depolymerisation. These were thought to reflect constraints imposed on the disassembly process by variations in the packing of myosin and by the presence of other thick filament proteins. For example, after a rise in salt concentration from 100mM ($\approx$0.131) to 170mM KCl there was a gradual reduction in the filament lengths from 1.6$\mu$m to approximately 1.3$\mu$m which is equivalent to the loss of the filament tips (about 0.2$\mu$m long). At 170mM KCl two distinct and equal populations of filaments co-existed of lengths 0.75$\mu$m and 1.3$\mu$m which implied that the transition from one state to the shorter involved co-operative unpacking of a large number of molecules of roughly equivalent environments i.e., the C-protein region (see Chapter 11.2.1). Above 170mM KCl, only shorter filaments existed and the length fell smoothly to a minimum value of about 0.2$\mu$m at a KCl concentration of 300mM. At higher concentrations no filaments remain.

A residual structure of 0.2$\mu$m would be equivalent to a bipolar filament with only one or two registers of myosin heads at each end. based on 149nm bare zone length (Craig, 1977) and 19nm for the myosin head length (Elliott and Offer, 1978).
Such structures would consist of 6 or 12 myosin molecules, assuming 3-fold symmetry (Squire, 1973; Maw and Rowe, 1980a). These structures appear to be smaller than the "mini-filaments" of Reisler, Smith and Seegan (1980; see Chapter 7.2.4). A direct comparison might prove informative.

Ishiwata (1981) observed, by phase-contrast microscopy, melting from both ends of A-bands in glycerolated myofibrils treated with increasing concentrations of KCl. As reported by Trinick and Cooper (1980a), Hasselbach (1953) and Huxley and Hanson (1954) 0.3M KCl did not induce complete disassembly - narrow A-bands remained but these could be removed by 0.5M KCl or addition of 10mM pyrophosphate (Hanson and Huxley, 1957). KCl concentrations less than 0.3M only produced partial "melting" of filament ends; higher concentrations were required to produce further melting which corresponds with the sequential disassembly observed by Trinick and Cooper (1980a) and supports the suggestion that the myosin molecule packing varies throughout the A-filament and is probably more stable towards the centre than at the ends.

It seems likely that secondary phosphate solution would act in the same way on A-filaments in situ, as potassium chloride does on isolated filaments. However, intermediary stages of narrow A-bands have not been seen probably because the ionic strength of 0.266M K$_2$HPO$_4$ ($\mu$=0.7) vastly exceeds that of 0.3M KCl ($\mu$=0.3) and therefore the in situ filaments would be solubilised completely very rapidly. Etinger, Zak and Fischman (1976) found that pre-glycerolated rabbit psoas fibres were completely extracted within two hours with 0.5M KCl. Presented here (Figures 70a, 71a and 71b) are sections from two phosphate treated samples showing structures which may be intermediary states. The sample of Figure 70a seemed to be only partially solubilised but rather than the A-bands being shorter they are just less dense implying that disassembly occurs along the length of the filament simultaneously. The micrographs of Figure 71 show residual M-lines (incorporating very short filaments) between the I-bands. This occurrence corresponds well with Trinick's data as he found that even in the short 0.2μm filaments the central M-line thickening remained. However, the filaments in the micrograph are only 0.05 to 0.08μm long. The structure may represent the myomesin complex (Eppenberger, Perriard, Rosenberg and Strehler, 1981) which is discussed later (see Chapter 13.5).
Finally Elliott (1980) found that 3mM magnesium ions could decrease the thick filament charge induced by phosphate ions from about $12 \times 10^4 \text{e} / \mu m^{-1}$ to $8 \times 10^4 \text{e} / \mu m^{-1}$. Prior to phosphate extraction of a fresh muscle sample 10mM magnesium ions were added to the secondary phosphate. As seen in Figure 68d, myosin solubilisation still occurred. Possibly the magnesium concentration was not sufficient to cancel the phosphate ion induced filament charge, or it may be that phosphate ion absorption is not involved in the solubilisation of myosin filaments.

10.1.6 Conclusion

In conclusion it is apparent that the method by which phosphate or potassium chloride dissociates myosin filaments is uncertain. It is possible that an increased filament charge which develops on increasing ion concentration is involved, or a structural change occurring in subfragment-2 on pyrophosphate binding may be important. However, the latter effect cannot be totally responsible for filament disassembly (although it may be pertinent in contraction) as pyrophosphate also dissociates light meromyosin paracrystals. Nevertheless, the majority of evidence suggests that disassembly occurs by a process reflecting the different stages of molecular packing occurring within the filament. This is probably initiated at the filament tip, the region of antiparallel packing being the most resistant to dissociation.

10.2 The Evidence for Reconstruction

A combination of methods has conclusively shown that myosin filaments are solubilised completely after treatment of muscle strips with secondary phosphate solution. The same methods were used to assess reconstruction, and reconstructed muscle was compared with untreated samples. The following discussion will be limited to fresh muscle at a resting sarcomere length, as all other samples either failed to reconstruct, or there were some differences in the type of reconstruction. These will be discussed later (see Chapter 10.3).
10.2.1 Electron Microscopy

Of all methods for assessing reconstruction of A-bands, T.E.M. is one of the most important and the most critical. In many cases the reconstructed A-bands are indistinguishable from control. Longitudinal sections showed the characteristic striation pattern of A- and I-bands, M-, Z- and N-lines and transverse sections of reconstructed muscle demonstrate the typical hexagonal lattice and M-bridge structures. However, micrographs of control and the best reconstructed samples are identifiable since granular I-bands cause a decrease in contrast in the latter. The granular nature probably results from the presence of some unsolubilised myosin and disrupted membraneous structures.

10.2.2 Light Microscopy

In the light microscope, reconstructed samples were indistinguishable from control muscle except for one factor, the presence of a few non-reconstructed fibres. These were generally peripheral fibres (Figures 41A and 42A), but in one sample a central fibre was still lacking A-bands (not shown). The peripheral fibres probably failed to reconstruct owing to localised stretching and distortion. Extended muscle fails to reconstruct and additional distortion could disalign the I-segments making reconstruction even less possible. The presence of a non-reconstructed central fibre, suggests that that particular muscle strip was not completely penetrated by mammalian ringer, but it is further evidence that the muscle strip had previously been completely phosphate extracted. In Figure 41, strip E is lacking H-zones and the edges of the A-bands are rather diffuse, suggesting that this fibre had not completed reconstruction of its A-bands.

10.2.3 X-Ray Diffraction

X-ray diffraction of reconstructed muscle confirms the return of A-filaments and indicates the extent to which they pack within the A-bands by the intensity of the equatorial (1,1) and (1,0) reflections. The most important property of X-ray diffraction, which the other techniques do not possess, is the ability to assess
the molecular packing within the reconstructed A-filaments. Comparing the axial X-ray diffraction patterns of control and reconstructed samples, one can deduce that not only have the A-filaments reassembled into a well ordered arrangement within the A-bands, but the myosin molecules have assembled into myosin filaments with the same spatial arrangements as native filaments (see Figures 49 and 50).

10.2.4 Relaxed Filament Preparations

Relaxed filament preparations of the control and reconstructed samples of fresh muscle, at a resting sarcomere length, required the same dilution for viewing indicating that a full complement of A-filaments had been reconstructed (Figures 51 and 52). Compared with untreated muscle, the reconstructed sample yielded a population with 50% full length filaments, which is compatible with the electron microscopy evidence (Figure 32c) presented at the same time. The quality of reconstruction has since been improved and a relaxed filament preparation would probably yield a similar percentage of full length filaments as the control sample.

10.2.5 The Effect of ATP in the Reconstruction Medium

The effect of ATP on reconstruction is unclear. Isenor (1976) originally added ATP to mammalian ringer, in the absence of magnesium ions, in order to stimulate contraction of phosphate extracted fibres. The first attempts at reconstruction within an artificial membrane showed marked improvements in A-band reconstruction after addition of ATP (Figures 72a and 32c, samples 2 and 3 from the same experiment). In more recent experiments, other than an increase in contrast of ATP treated sections, the A-bands have appeared very similar. A few exceptions have been noted, but these may be due to the use of separate muscle strips. For example: 1) the A-bands of Figure 29d appear tidier than those of 29c and less solubilised myosin is present, 2) after ATP treatment the (1.1) and (1.0) reflections could not be individually distinguished in one sample (Figure 44d) and yet in another sample on a different occasion (Figure 46d). ATP apparently caused the
reappearance of the (1.0) reflection, and 4) ATP produced an increase in the intensity of the 14.3nm reflection (Figure 50). Therefore, the effect of ATP on reconstruction, if any, is to improve the molecular and filament packing within the A-filaments and A-band lattice respectively, possibly via myosin molecule binding (see Chapter 7.2.1).

10.2.6 Conclusion

Combining the evidence obtained, further supports the hypothesis that reconstructed A-filaments and A-bands resemble those found in vivo. Reappearance of the equatorial reflections and the well-ordered hexagonal pattern of transverse sections are both evidence for a well-packed A-filament lattice. Longitudinal sections and relaxed filament preparations indicate that reconstructed A-filaments are the length of native filaments, and they are therefore, presumably, the right width because a) the lattice number, as seen in transverse sections, is correct, b) the molecular packing, as judged by the meridional reflections, is good, and c) the total amount of myosin must remain constant; a constant width, therefore, follows as a logical necessity.

A further simple but important conclusion follows. Under conditions which can be identified, the A-filament and A-band of skeletal muscle are structures which can self-assemble from their pre-formed components. In this respect they resemble the growing number of biological structures which have been shown to possess this inherent property (see Chapter 6.1).
10.3 Factors which Partially or Totally Inhibit Reconstruction

Reconstruction of full length A-filaments fails to occur in muscle which has been a) pre-glycerolated and/or pre-calcium depleted, b) stretched to an extended sarcomere length, and c) muscle which was in a relaxed state prior to phosphate treatment.

10.3.1 Pre-Glycerolation and Pre-Calcium Depletion

50% glycerol treatment of muscle produces "skinned fibres". The high concentration of glycerol causes osmotic shock disrupting cell membranes and releasing much of the intracellular soluble components. Glycerol is not thought to affect the insoluble myofibrillar network. Therefore it appears that for reconstruction to occur a soluble component of the cell, either associated with the myofilaments, or free in the cytoplasm is required. However, this component is not necessary for the production of synthetic filaments from extracted myosin of pre-glycerolated muscle homogenates (Figure 63d; Huxley, 1963). As has been said, synthetic filaments differ from reconstructed and native ones in that they are generally shorter and thicker, but they are still formed by the initial anti-parallel association of myosin tails followed by parallel addition of myosin molecules. It was also found that non-reconstructed pre-glycerolated muscle produced synthetic filaments on relaxed filament preparation. Structures resembling synthetic filaments are never seen in electron micrographs of non-reconstructed pre-glycerolated sections (Figure 61d and 62b).

Similar arguments apply to the inability of pre-calcium depleted muscle to reconstruct (Figure 66d) and yet synthetic filaments are found in relaxed filament preparations (results not presented).

It can be concluded that a) calcium ions (involved directly or via a calcium dependent system) and b) a soluble component (extracted by glycerol) are required
for in situ A-filament reconstruction but not for in vitro assembly.

10.3.2 Effect of Extended Sarcomere Length

At extended sarcomere lengths, reconstruction of full length A-filaments does not occur, but short filaments are produced as seen by electron microscopy (Figure 54d and 56a). Transverse sections of these filaments (Figure 56b) indicate that they originate well within the I-band and project out of it by approximately 0.25μm. It cannot be said whether the filaments isolated from the relaxed filament preparation (Figure 60d) are those observed in thin sections or synthetic filaments, as the short reconstructed filaments would probably be approximately the same length as synthetic ones. A more detailed study of the isolated filaments to reveal whether the myosin molecules are assembled unidirectionally or in a bipolar arrangement would differentiate between the two types, as synthetic filaments are bipolar and these are probably not.

The presence of these short reconstructed A-filaments was also detected by X-ray diffraction. The axial reflection which disappeared on phosphate treatment (Figures 58 and 59) reappeared on return to mammalian ringer, indicates that the myosin in these short filaments is regularly packed with a 14.3nm repeat. The equatorial reflection patterns also demonstrated the loss of the (1,1) and (1,0) spacings on phosphate treatment and their reappearance in reconstruction medium (Figure 57). Interestingly, the reappearance of the (1,0) reflection was much weaker than in the corresponding relaxed sample, indicating that although the myosin/actin spacings were present again, the myosin/myosin spacings were not completely regained, which is in accordance with the absence of myosin filaments in the non-overlap region.

The areas beneath the 14.3nm and (1,0) peaks in the reconstructed samples were almost the same as controls, which suggests that almost the full complement of short A-filaments had been reconstructed. The electron micrographs and relaxed filament preparation give the impression of only a small number of filaments, but the microscopy work was done in the initial stages of this reconstruction project and the X-ray diffraction in the latter and as mentioned before the quality of
reconstruction improved greatly with time.

The presence of short A-filaments within the I-bands of muscle set at an extended sarcomere length, provides one of the most important clues about the method by which muscle reconstructs A-filaments and, furthermore, reconstructs them to the correct length. The myosin molecules appear to reassemble within the I-bands (perhaps using the I-filaments as templates) and subsequently project out of the I-bands. Presumably if the sarcomere length is not extended the filament halves can meet and so form a full length filament.

It therefore seems that for the production of reconstructed A-filaments, calcium ions, one or more soluble compounds and an intact I-band lattice are required and for full length filaments the muscle must be at a relaxed sarcomere length. Conversely the I-filament lattice may physically obstruct the assembly of synthetic filaments: the only difference between a system which produces reconstructed filaments and one which produces synthetic filaments, is the presence of a residual myofibrillar network in the former i.e., pre-glycerolated and pre-calcium depleted samples.

10.3.3 Relaxed and Rigor States

However, soluble components and calcium ions are present in fresh relaxed muscle which, after going into a rigor state was induced to relax with Mg$^{2+}$ATP prior to phosphate treatment. Reconstruction still failed to occur. Reconstruction does occur when the muscle is in a rigor or contractile state (fresh muscle left for two days before use or used immediately on excision); in both cases, myosin heads are attached to the actin filaments, almost 100% attachment in the former case (Cooke and Franks, 1980; Lovell and Harrington, 1981) and approximately 30% attachment in the latter. In relaxed muscle there are no attached cross-bridges. At an extended sarcomere length, where the A- and I-filaments do not overlap, there must also be no attachment; however, extended muscle is still contractile. During phosphate treatment of extended muscle disassembled myosin molecules will migrate and attach to actin filaments owing to the presence of high levels of intracellular ATP and calcium ions. Free myosin molecules in relaxed samples
cannot bind to actin filaments because of the relaxing presence of magnesium ions with ATP.

Secondary phosphate solution is capable of extracting up to 70% of solubilised myosin, implying that 30% of cross-bridge attachments are resistant to phosphate action (A.J. Rowe, unpublished). Reconstruction of myosin extracted muscle produces A-bands 1/3 of the native width (Isenor, 1976). Furthermore, l-filaments isolated from phosphate extracted muscle are only sparsely decorated with myosin molecules (Clode, 1981) compared with the typical arrowhead appearances previously demonstrated (Moore, Huxley and DeRosier, 1970; Craig, Szent-Györgyi, Beese, Flicker, Vibert and Cohen, 1980). It is interesting that the myosin decoration of Clode's l-filaments has a regular spatial distribution (approximately 40nm) rather than being randomly distributed. This implies that certain sites on the actin filaments may have a higher affinity for myosin binding than others. The periodicity of the distribution of the binding sites is similar to that of the troponin complex.

The phosphate treated sample (previously in rigor) of Figure 68b, shows an interesting effect, namely the "shadow" of an A-band, presumably indicating the presence of individually attached myosin molecules in their original pre-solubilisation positions. The effect is not present in the pre-relaxed sample (Figure 68c) nor in a sample previously in a contractile state, presumably because approximately 70% of the myosin molecules were not originally attached and these would disperse into the l-band camouflaging any shadows. Pre-glycerolated samples also, on occasion show the shadow effect (Figure 62a).

**10.3.4 Summary**

Thus, 1) phosphate solubilised myosin in fresh muscle at a relaxed sarcomere length, on return to a physiological medium, assembles into A-filaments and A-bands resembling those of untreated muscle (reconstruction); 2) fresh muscle previously stretched to the point of no A/I overlap produces short A-filament fragments on reconstruction and these are found largely within the l-bands; 3) phosphate extracted myosin i.e. in the absence of an l-filament lattice, assembles into short and often thickened "synthetic" or "relaxed" filaments.
Within the muscle fibre, myosin solubilised in the presence or absence of intracellular ATP (i.e. muscle in a contractile or rigor state) is capable of reassembly into A-filaments; however, myosin solubilised in the presence of Mg\(^{2+}\)ATP (i.e. muscle in a relaxed state) cannot reassemble within the myofibril. These results suggest that assembly of reconstructed A-filaments requires the presence of intact I-filament lattices and that the presence of actin may protect the solubilised myosin DTNB light chain from phosphate denaturation (see Chapter 10.1.4; Chin, 1981). For full length A-filaments, the I-filament lattices must be longitudinally orientated and positioned at a resting sarcomere length. Furthermore, it appears that not only do the I-bands act as a framework within which reconstruction occurs, but the I-filaments may act as templates, because myosin assembly a) fails to occur (relaxed sample), or b) produces malformed filaments (synthetic) if a percentage of the myosin molecule population is not attached to actin filaments, and c) synthetic actin filament assembly in the presence of synthetic myosin filaments produces tightly associated hexagonal complexes of thick and thin filaments (Hayashi, Silver, Ip, Cayer and Smith, 1977; see Chapter 13.3).
11.1 The Evidence from Reconstruction

It is very tempting to compare the assembly of myosin molecules into reconstructed A-filaments with synthetic filaments as both are produced on lowering of ionic strength. However, these comparisons must be tentative as other conditions of assembly vary, for example, only synthetic filaments can be produced from pre-glycerolated or pre-calcium depleted muscle and only reconstructed filaments are formed in situ. Reconstructed filaments so closely resemble native filaments in electron micrographs (see Chapter 9.1.1 and 9.1.2 and Figure 29), in their X-ray diffraction properties (see Chapter 9.1.6 and Figure 50) and in their ability to fray into 3 subfilaments (see Chapter 9.1.10 and Figure 53) that it would not be too presumptive to assume that the mode of reconstruction could resemble that occurring in vivo.

The environmental control mechanisms (e.g. ionic conditions, pH) of assembly, operating in vivo, in vitro, and in situ are probably similar. As discussed in Chapter 7.2, different groups of workers produced synthetic filaments with different characteristics and these are probably attributable to the employment of different assembly conditions. Kaminer and Bell (1966) studied the effect of ionic strength and pH on growth of synthetic filaments (produced by rapid dilution of a 0.6M KCl myosin solution). They found that at lower concentrations i.e. 0.1 to 0.2M KCl, and neutral pH (6.5 to 7.0) long (up to 1.8µm) tapered filaments were formed, which resembled native filaments. At higher ionic concentrations and pH i.e. 0.2 to 0.3M KCl and pH 7.5 to 8.0, very short filaments were formed and free monomer was also present. Filaments of intermediate size (0.44µm average length) with a relatively smooth bare zone and gross irregular projections at the ends were produced predominantly at pH 8.0 in 0.1M KCl. Interconversions of the filament types was effected readily by rapidly changing the pH or ionic concentration. The transition from a population of short to one of long filaments after a slight drop in pH is reminiscent of the sequential disassembly of A-filaments described by Trinick.
Is reconstruction of A-filaments a reverse process of high ionic concentration Induced disassembly? One might expect that as phosphate ions are washed out by mammalian ringer and the ionic concentration and pH, gradually fall to l=0.12 and pH7 short filaments would assemble and gradually increase in length. However, evidence from intermediate stages of reconstruction suggests alternative mechanisms may operate. Two processes may be involved: Independent assembly of the M-line and A-filament halves (Figure 73) and growth from the M-line into the l-bands (Figure 72).

Figure 71a and b are sections of a phosphate treated muscle where it seems that the M-line is the last structure to disassemble. Figure 72a is a reconstructed sample in which the M-line appears to be the first structure assembled. In the section of Figure 72b, the M-lines have almost completely reconstructed and on either side of them there is the appearance of some growth of A-filaments. The growth of A-filaments away from the M-line is almost complete in Figure 72c. N-lines seem to reappear very early in the reconstruction process (Figure 72b).

The micrographs of Figure 73, by contrast, demonstrate how reconstruction of the A-bands can apparently start in the l-bands and terminate with the addition of the M-line. Figure 73a is a section of extended muscle demonstrating A-filament fragments projecting from the l-bands. Complete reconstruction presumably cannot occur, as the l-bands are too far apart. In Figure 73b, the sarcomere length is approximately 2.7μm and a few A-bands can be seen to have reconstructed completely e.g. just left of the centre. On the right hand edge of the micrograph is an A-band which has reconstructed A-filaments but as yet does not show a prominent M-line. Other A-bands have not completely reformed and only show a few traversing A-filaments. However, the assembled A-filament fragments are very obvious at the edges of the l-bands. The apparent width of the l-bands in the areas where the A-bands are not complete in this micrograph are about 2.5μm. This is 0.3μm wider than the normal width (2.2μm), therefore the half A-bands must originate in the l-bands but project 0.15μm (the approximate length of one.
myosin molecule) out of them which is compatible with the presence of projecting filaments in Figure 73a.

In the area right of the centre in Figure 73b (in which complete A-filaments are absent) the half A-bands are about 0.6µm in width and the distance from the outer edge of the A-band in one I-band, to that in the other, is 1.6µm i.e. the width of a normal A-band. The gap between the A-bands yet to be spanned is 0.4µm. On either side of this myofibril are ones in which the half A-bands are almost touching, but the total width of the A-band is only about 1.4µm, although the half filaments are still 0.6µm. This implies that the narrowing gap is not due to the filament projections growing towards the centre. Determining the widths of the non-overlapped I-bands, reveals that the half A-filaments may be sliding along the I-filaments towards each other, as these exposed I-bands are wider (about 1.2µm) than in the neighbouring fibril (non-overlapped I-band width approximately 1.1µm) where the half A-bands are well separated.

So it seems possible that the 0.6µm A-filament fragments, once assembled in the I-bands, slide along the I-filaments until the opposite halves meet and join together. The extreme right hand A-band of Figure 73b in which the M-line has not returned, is about 1.5µm wide and normal A-bands are 1.6µm (as in the completely reconstructed A-band in the micrograph) implying that on joining up, the A-filaments proceed to grow at their tips until they are full length and meanwhile the M-line appears. In Figure 73c the A-bands are all at approximately the same stage of development i.e. half filaments joining up and the M-line appearing.

As to the question of whether reconstruction is initiated in the I-bands or at the M-line, the situation is further confused by the micrographs of Figure 67. Here the two processes appear to occur simultaneously. Figure 67a demonstrates fibrils in which the M-lines have assembled and are seen to compose of very short filaments (0.1 to 0.2µm long) - similar to the residual M-line of Figure 71a. Simultaneously in the I-bands, half A-filaments are assembling, sometimes projecting out of the I-band and of varying lengths as assumed from the uneven width of the dark edge of the I-band. In Figure 67b the half filaments have grown within the I-bands and many opposite halves have joined up, presumably within
the M-line. The process is almost complete in Figure 67c, although the edges of the A-bands are still very irregular but in this case that may be due to sectioning at a slight angle or it may be that the individual A-filaments are growing from their tips and have not yet reached full length.

There is one major unanswered question in these hypotheses – what is/are the length determining mechanism(s)? The mini A-bands of Isenor (1976) were approximately 1/3 of the width of native A-bands. This might imply that the filament length is determined by the amount of myosin that is available; the complete complement of filaments starts to grow and when the myosin runs out they stop growing. But one would not then expect all the filaments to be the same length, as they seem to reconstruct at slightly different rates. If a specific length determining factor existed, one might expect either 1/3 of the filaments but all full length or at least some full length filaments. In the presence of the full complement of solubilised myosin and if the myosin concentration were the length determining factor in Figure 73b, one would expect to see some very long filaments and others much shorter.

As A-bands do not assemble simultaneously, the A-filament length determining factor is unlikely to be the intracellular myosin concentration, or an inherent feature of myosin self-assembly (mini A-bands would not then be produced). This suggests that another intracellular component is involved and one which is not associated with the A-filament, as solubilised, unpurified A-filaments do not assemble into full length structures in vitro (Trinick, 1973; Emes and Rowe, 1978a). The essential component may be an integral part of the myofibrillar structure i.e. the I-bands. These possibilities will be discussed in Chapter 14.1.

11.2 The Possible Effects of C-Protein

Huxley and Brown (1967) noted in their X-ray studies the presence of additional protein component(s) associated with thick filaments. The axial periodicity of this component (approximately 44nm) being greater than that of myosin (43nm), they speculated that the additional component(s) may be involved in a vernier length determining mechanism in the thick filament. The hypothesis was supported by
Squire, Sjöström and Luther (1976) who made a detailed study of striated muscle by cryomicrotomy. Offer, Moos and Starr (1973) identified one of the proteins as C-protein when it was discovered as a contaminant of myosin preparations. Using pure C-antibody, Offer (1976) demonstrated that C-protein is found in seven stripes (Figure 1) on either side of the M-line. However, with the same technique, Craig and Offer (1976b) determined that the C-protein stripes were 43 nm apart (later confirmed by Craig, 1977) and therefore, cannot act as a vernier control.

Synthetic filaments assembled in the presence of physiological concentrations of C-protein do not resemble native A-filaments although a more homogeneous population of filaments results (Koretz, 1979b; see Chapter 7.2.1). C-protein may not be involved in length determination, or another factor may be required in addition to C-protein, or the myosin molecules may have been altered during solubilisation (see Chapter 7.2.5). C-protein also binds to actin filaments, but far more weakly than to myosin. The physiological significance of this is unknown (Moos, Mason, Besterman, Feng and Dublin, 1978).

11.2.1 A Hypothetical Role for C-Protein

It is possible that C-protein may still influence A-filament length. C-protein is confined to a limited region of the thick filament and in cross-sections it is seen to be distributed around the A-filaments (Craig and Offer, 1976b). The thick filament is a bipolar structure with tapered ends. In consequence there will be changes in the myosin molecular packing as one proceeds from the region of anti-parallel interactions in the middle of the filament, to parallel interactions away from the middle and again as one proceeds to the tapered ends. However, the molecular packing could be approximately constant in the middle of each half of the filament (Pepe, 1967; Squire, 1973), the seven stripes of C-protein defining the extent of the constant packing region. The outermost C-protein stripe may then indicate a change in myosin packing, which would ultimately cause the filament to taper and end. On this hypothesis the presence of C-protein and filament length determination are directly related. However, if C-protein surrounds the filament rather than being incorporated, this would suggest that the myosin packing is regulated by another mechanism and that C-protein is added later.
This hypothesis, in which an exactly defined incorporation of C-protein into the filament structure inhibits termination, cannot at present explain all observed features (why only 9 stripes, in particular?), but it does have attractive features. It predicts that in the absence of C-protein, filaments synthesized from pure myosin should start to terminate immediately, from the region of anti-parallel packing; and hence yield "synthetic" filaments of length (central region) + 2 x (stripe 1 to filament tip) i.e. about 0.7µm. This is precisely the sort of length seen in synthetic filaments of mass/unit length nearest to physiological (Emes and Rowe, 1978a). Although we must postulate a very defined and ordered mechanism for C-protein incorporation to explain the failure of C-protein to produce true A-filaments in vitro, the whole theory of reconstruction (see Chapter 14.1) requires such a mechanism to operate with the I-filament lattice providing a structural framework. The hypothesis may, therefore, merit further consideration.

11.3 The Role of Paramyosin

The invertebrate A-filament protein, paramyosin, has been found to be necessary in vivo for determination of the thick filament length and diameter of the body-wall muscle cells of the nematode C. elegans (MacKenzie and Epstein, 1980). Besides paramyosin, there are two types of myosin present, A and B (derived from distinct heavy chain genes). To test the hypothesis that paramyosin is the length controlling factor, 3 mutant types were chosen each failing to produce either myosin A, myosin B or paramyosin. Filaments isolated from mutants failing to produce one of the myosin species were long and narrow and resembled native filaments. However, in the absence of paramyosin the filaments isolated were abnormally short and wide. Myosin and paramyosin may, therefore, act in a vernier mechanism to determine thick filament length in invertebrate muscles (Huxley, 1963; Huxley and Brown, 1967).

Analysis of the protein composition of isolated, purified rabbit striated muscle thick filaments reveals that myosin and C-protein are the major components (Morimoto and Harrington, 1973 and 1974a; Trinick, 1973; Emes, 1977). No additional protein which might fill the role of a core protein has been found. C-protein of vertebrates is not equivalent to paramyosin, the latter forming a core within myosin filaments.

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and the former probably being situated on the surface of the thick filaments in distinct regions on either side of the H-zone.
CHAPTER 12

RECONSTRUCTION OF RAT PSOAS MUSCLE

A System in which other Intermediate Stages of Reconstruction are Observed

Some interesting results were obtained by a project student (Clode, 1981) in this laboratory. A. Clode studied the reconstruction of rat psoas muscle, and various intermediate stages were observed. The section of Figure 74a was from fresh control muscle and it appears very similar to fresh rabbit psoas muscle. After phosphate extraction and return to reconstruction medium, four different types of areas were seen to be present in the muscle (Figures 74b, d, g and h). Transverse sections were also cut from the same block and again the areas within the muscle varied (Figures c, e, f and i), although one cannot say which transversely cut area corresponds to which longitudinally cut area.

The tissue of Figure 74b is at a very long sarcomere length (about 3.6μm) and short reconstructed filaments project out of the l-bands. The sarcomere length is much shorter in Figure 74d (about 2.7μm), but complete reconstruction has still failed to occur, although some A-filaments may traverse from one l-band to another. Both sections have clumps of material lying in the background. In transverse sections (Figure 74b and e, long and short sarcomere lengths respectively) A-filaments are present in the l-bands but do not cross to the neighbouring l-bands and the background is filled with the amorphous clumps.

Reconstruction has obviously occurred to some extent in Figures 74g and h, and yet the sarcomere length is quite long (about 3.3μm). The A-band is not quite full width in Figure 74h at about 1.5μm (probably because reconstruction was not carried out within an artificial membrane) and the general appearance is of highly stretched muscle.

The striation appearance of Figure 74g is very unusual. An M-line appears to be present and many filaments traverse through it, sometimes from one l-band
to another although they are far fewer in number than in a normal A-band resulting in the I-bands remaining the darker striations in the pattern. On either side of each M-line is a dense band of seemingly structureless material which may be associated with the filaments.

Figure 74f and l are tranverse sections demonstrating filaments traversing from one I-band to another. In the former the A-filaments are sparsely scattered in no regular arrangement and they originate from well within the I-bands. The background still contains a lot of amorphous material. In Figure 74l the I-bands have been somewhat distorted but between them are distinct clumps of A-filaments often showing M-bridges and a regular hexagonal arrangement but the A-filaments do not appear to cross from one I-band to another in very large numbers suggesting that this area corresponds to Figure 74g.

The morphology of untreated rat psoas muscle resembles very closely that of rabbit psoas. Rat and rabbit striated muscle A-filaments probably have similar structures as both fray into 3 subfilaments in low ionic strength media (see Part 1). Fresh rat muscle is also capable of reconstructing A-filaments and A-bands after phosphate treatment. However, some differences between rat and rabbit reconstruction exist: 1) rat psoas appears to be capable of complete A-filament reconstruction at much longer sarcomere lengths than rabbit, and 2) intermediate stages of reconstruction seem to differ. An apparently non-myosin filamentous network exists in the pseudo H-zone of rat myofibrils prior to complete reconstruction whereas in rabbit samples intermediate stages frequently demonstrate a seemingly structureless pseudo H-zone (see Figure 73b).
CHAPTER 13  THE CYTOSKELETAL NETWORK AND ITS RELATIONSHIP TO MYOFIBRILLOGENESIS

The preliminary discussion in Chapter 12 raises the following important issues:

1) the total structure of the muscle filament system in vitro, 2) the possible existence of cytoskeletal structures not normally visualised and possibly resistant to dissolution in strong salt solutions, and 3) the question of the manner in which myosin filaments are synthesised de novo and subsequently turned over in the living muscle. Before presenting a model for the type of reconstruction which we have achieved (Chapter 14.1) the current state of our knowledge in these important but often imperfectly understood areas will be reviewed.

13.1 Superthin Filaments

It is possible that the filaments traversing between the l-bands of Figure 74g are not A-filaments but may be other types of structure involved in A-filament assembly. There has been a lot of work done on non-myofibrillar filament systems but the evidence is very inconclusive and unco-ordinated. The earliest speculation on the presence of other structural proteins was by Huxley and Hanson (1954 and 1955) who proposed the existence of very fine filaments which connected the Z-discs and which they referred to as "S-filaments". On solubilisation and extraction of myosin and actin filaments, a material was left which prevented the Z-discs from separating as viewed by phase microscopy. Furthermore, extended myofibrils after extraction, still sprang back to a little below rest length although with little power (Huxley and Hanson, 1954; Hanson and Huxley, 1955). Later A.F. Huxley (1957) expressed reservations about the existence of S-filaments, there being no firm evidence.

The presence of another filament system was also postulated to provide structural continuity by Ming-Xia and Shih-Fang (1963) and Carlsen, Fuchs and Knappels (1965a), who were working on glycerolated honey bee striated muscle and rabbit psoas muscle respectively. It was found that on stretching fibres to a point of no A-/l-band overlap, the fibres were capable of ATP-induced isotonic...
...shortening and regained their normal morphology.

In 1962, Sjöstrand observed fine filaments, thinner than actin-containing thin filaments, which appeared when muscle was stretched to the point where a gap appeared between I and A-bands. He termed these "gap filaments". A similar observation was made by Carlsen, Fuchs and Knappels (1965b); they postulated that gap filaments attached the ends of the I-filaments to the ends of A-filaments. Locker and Leet (1975) also observed gap filaments in highly stretched muscle (beef) and suggested that they attached one end of an A-filament to its nearest Z-disc i.e. half the A-filaments would then be attached to one end of the sarcomere and half to the other.

Auber and Couteaux (1963) and Garamvolgyi (1965) studying insect fibrillar muscle, described the presence of "C-filaments" which connected the ends of the myosin-containing thick filaments to the Z-discs. Recently Trombitas and Tlgyi-Sebes (1979) isolated native A-filaments from honey bee flight muscle, which are connected at one tapered A-filament end to another i.e. two filaments from consecutive sarcomeres. Occasionally, the "connecting" filaments, as termed by Pringle (1967), were seen to bifurcate at the level of the former Z-disc and join with two different thick filaments from the adjacent sarcomere. These connecting filaments probably have a specific physiological specialisation in insect flight muscle, as the myofibrils have very short I-bands in the resting state and the muscle acts under nearly isometric conditions. The presence of connecting filaments in insect flight muscle is not disputed, but they are probably not the same as gap filaments of vertebrate muscle, as the latter are thought to extend right through the sarcomere, rather than from one A-filament to another (Walcott and Ridgway, 1967).

McNeill and Hoyle (1967) and Walcott and Ridgway (1967) did some detailed work on these superthin filaments using muscle from a variety of different species, invertebrate and vertebrate. Similar results were obtained in all cases, but here, those of rabbit psoas will be mainly focused on. The filaments are about 2.5nm thick and are found in the H-zone, A-band and I-bands as well as bridging the gap between actin and myosin filaments in greatly stretched muscle. In some thin longitudinal sections of non-stretched invertebrate muscle these so called...
"T-filaments" are seen interdigitating with the myofibrillar components, but in vertebrate muscle they are seen mostly after extensive stretching and in transverse sections. McNeill and Hoyle (1967) described cross-sections of filaments within the M-bridges of the M-band. These were later described as "M-filaments" (see Chapter 13.5) and were thought to be confined to the M-band (Knappels and Carlsen, 1968). McNeill and Hoyle (1967) did not observe T-filaments in any other region of the A-band in thin sections, but after stretching, they were seen as gap filaments. Densitometric measurements across a region of gap filaments and across an I-band demonstrated that the latter consists of alternating I- and T-filaments. So it seems that T-filaments stretch from one Z-disc to the next, do not connect A or I-filaments and are highly elastic.

Using the Guba-Straub method of Hanson and Huxley (1953), Walcott and Ridgway (1967) myosin extracted some of the muscle samples used by McNeill and Hoyle (1967). The underlying T-filament network was examined by electron microscopy. The micrographs of myosin extracted rabbit psoas muscle obtained were very similar to those described here (Figures 29b and 31) and thin filaments between the I-bands were not observed. A low pH (5.0) lipid solvent i.e. acetone were also used to extract the A-band (the M-line remains, presumably stabilised by the cross-connecting bridges between the remaining myosin rods). The actin filaments clumped somewhat due to the low pH, but between the I-bands and the M-line, many thin filaments were seen. Although the micrographs presented do not look like that of Figure 74g, descriptions of both would be similar.

Extraction of myosin and actin from glycerolated rabbit psoas to observe the complete underlying superthin filament system by transmission electron microscopy was done by dos Remedios and Gilmore (1978). They used modified Hasselbach-Schnelder solution, followed by 0.6M KI, a similar method to that used by Hanson and Huxley (1953). Myosin extraction of fibrils on grids, followed by negative staining, revealed regular Z-discs and I-band brushes with an H-band that appeared to contain filaments of approximately the same diameter as actin-containing thin filaments. The M-band had also been extracted. Hasselbach-Schnelder solution followed by 0.6M KI, produced structures consisting of Z-discs connected by a filamentous material of variable diameter (2 to 7nm) and a lace-like appearance. The amount of material joining the Z-discs appeared
to be approximately the same as that crossing the H-band. dos Remedios and Gilmour (1978) found that their superthin filament nets resembled those of Maruyama and colleagues (1977b) who extracted myosin and actin from frog skeletal muscle and later from glycerolated cardiac myofibrils (Toyoda and Maruyama, 1978). 1% sodium dodecyl sulphate treatment of the cardiac filament nets removed material from the centre of the Z-discs and superthin filaments were seen to run through the electron opaque gap suggesting that these nets run through the Z-disc. Complete removal of the Z-discs with 0.1M NaOH left continuous nets; removal of the Z-disc also decreased the tension which the nets were able to develop (Maruyama, Natori and Nonomura, 1976). Recently, Ramirez-Mitchell and Wang (1981), having removed all myosin and actin (using 0.6M KI) from rabbit striated muscle myofibrils, also observed a residual three dimensional filamentous network. The filaments or filament bundles connected longitudinally successive residual Z-structures of the same and adjacent myofibrils. Secondary filament bridges were also present, appearing to connect the midpoint of longitudinal filaments (at the M-line position) with membranes and the midpoints and Z-discs of adjacent structures.

Maruyama, Natori and Nonomura (1976) termed the protein of superthin filaments "connectin". Connectin is insoluble in the normal myofibrillar extracting solutions but can be removed by strong alkali and 6M urea (Maruyama, Natori and Nonomura, 1976; dos Remedios and Gilmour, 1978).

Surprisingly, indirect immunofluorescence demonstrated that although connectin was present in the A- and I-bands and especially at the A/I junction the Z-discs were not stained at all. It is possible that the nets are connected to the Z-disc network (Knappels and Carlsen, 1962; Reedy, 1964) and there is also some evidence that the nets are connected to the N2-line on either side of the Z-discs (Locker and Leet, 1976). The N2-line protein from rabbit psoas has recently been identified by PAGE (Wang and Williamson, 1980) and found to have a molecular weight of approximately 5-6x10^5. The N-lines may be an intracellular storage site of rapidly released. calcium as calcium precipitates of pyroantimonate can be formed here as well as in the triads (Yarom and Meiri, 1971 and 1972), and X-ray microanalysis results are compatible with a higher calcium concentration in this region (Hillman, 1975).
Connectin is an elastic protein found in muscle from many different organisms and in other cell types (Maruyama, Murakami and Ohashi, 1977a). It has been postulated that connectin is responsible for the passive elasticity and mechanical continuity of muscle (Maruyama et al., 1976 and 1977b). Connectin may also be involved in maintaining A-filaments at the centre of the sarcomeres as attachment to myosin filaments cannot be ruled out and part of the connectin net is extracted with myosin by Hasselbach–Schneider solution (Maruyama, Kimura, Ohashi and Kuwano, 1981). Connectin may be the parallel elastic component predicted by physiologists to explain muscle function (Hill, 1950; Jewell and Wilkie, 1958; Tameyasu and Sugi, 1979). The elastic nature of connectin is due to the cross-links of polypeptide chains. This is the same as in collagen, the elastic protein of connective tissues (Fujii, Kimura and Maruyama, 1978). Connectin is, however, distinctly different from collagen in amino acid composition (Kimura, Akashi and Kubota, 1978) and is resistant to collagenase (Maruyama et al., 1977a). Connectin is one of the highest molecular weight single chain proteins known (about $10^6$, Wang et al., 1979).

As has already been mentioned, Figure 74g fits the description of rabbit psoas muscle (of Walcott and Ridgway, 1967) from which the A-bands but not the M-lines have been extracted, suggesting that the filaments running between the I-bands and through the M-line in Figure 74g may be connectin filaments. Similarly a transverse section of highly stretched beef muscle presented by Locker and Leet (1975), looked remarkably similar to Figure 74i. The description that follows is of Locker's sections but it is also applicable for Figure 74i: "A transition from A-band to Z-disc can be seen across the plate. A densely packed region, which we identify with the residual overlap of the thick filaments, can be distinguished from less densely packed thick filaments corresponding to the extension zone of the A-band. The gap region between A and I-bands are sparsely filled with thin filaments consisting of a dense core, surrounded by wisps of less dense material". The section in Figure 74i passes from an I-band through a gap region to an A-band.

Comparing the Figures 74g and h the A-filaments of the latter form distinct A-bands, whereas the filaments in the former appear to become lost in the I-bands, although the actual filament numbers are not so different. This further suggests that the filaments of Figure 74g may not be A-filaments.
The fact that connectin filaments are not seen in phosphate treated samples could be because they are obscured by the solubilised myosin, although Figure 54b does have a sense of linearity in the background. Furthermore connectin filaments were not seen in Walcott and Ridgway's (1967) Guba-Straub treated muscle strip. Extraction of myosin from isolated fibrils on grids followed by negative staining, does reveal connectin filaments (dos Remedios and Gilmour, 1978) suggesting that complete myosin extraction and not merely solubilisation of fibrils is required. The rat muscle samples of Figure 74 did not have an artificial membrane and therefore some myosin would have been extracted during phosphate treatment.

Unfortunately, recent evidence throws doubt on the existence of superthin filaments: they may in fact be Intermediate filaments (see Chapter 13.2). Electron microscopy studies of the residual filamentous networks have assigned filament diameters of 2–7nm (dos Remedios and Gilmour, 1978) and 8–20nm (Ramirez-Mitchell and Wang, 1981) and the component proteins have been termed connectin (Maruyama, Natori and Nonomura, 1976) and “titin” (Wang, 1981) respectively. Maruyama et al. (1981) found that the protein “titin” isolated by Wang, McClure and Tu (1979) is in fact the same protein as connectin as shown by electrophoretic mobility on 2–3% polyacrylamide gels. amino acid composition and localization in myofibrils. as determined by indirect immunofluorescence. Both proteins were found to contribute to a major fraction of the total fibrillar protein: connectin approximately 5% (Maruyama, Natori and Nonomura, 1976) and titin, approximately 12% (Wang, 1981).

Whether connectin and titin are the same protein, or whether they form a class of intermediate filaments rather than superthin filaments has yet to be determined. Nevertheless, a three dimensional filamentous network other than the myofilaments appears to exist in myofibrils and this network may play a significant mechanical role in muscle tension generation (Ramirez-Mitchell and Wang, 1981).

13.2 The Intermediate Filaments

Besides the connectin network, there is one other non-myofibrillar filament
system known to be associated with the myofibrils and involved in myofibrillogenesis — that of the intermediate filaments (Nunzi and Franzini-Armstrong, 1980; Bennett, 1979; Anderton, 1980; Granger and Lazarides, 1978 and 1979; Lazarides and Hubbard, 1976; Small and Sobieszek, 1977; Lazarides, 1978 and 1980; Lazarides and Granger, 1978). These filaments are so called because of their diameter (10 nm) which is intermediate between those of actin filaments (6 nm), myosin filaments (16 nm) and microtubules (25 nm).

Intermediate filaments are a fibrous organelle of the eukaryotic cytoskeleton and they have roles in motility, maintenance of organelle distribution and cellular architecture (Nunzi and Franzini-Armstrong, 1980). They generally act as mechanical integrators of intracellular space. There are five subclasses of these filaments and each is biochemically and immunologically distinct:— 1) Keratin (tono) filaments — found in epithelial cells and cells of epithelial origin. 2) Desmin filaments — found in smooth, skeletal and cardiac muscles. 3) Vimentin filaments — present in mesenchymal cells and cells of mesenchymal origin. 4) Neurofilaments — found in neurones and 5) Glial filaments — present in glial cells.

Lazarides and Hubbard (1976) isolated an intermediate filament protein from glycerolated chicken smooth muscle. The protein had a molecular weight of 50,000 and was soluble in urea but insoluble in high salt solutions which normally extract the myofibrillar components. Immunofluorescence showed that the protein had close associations with the Z-discs resulting in filament "rings" and was present between adjacent Z-discs of different myofibrils. Fluorescent antibodies also stained filaments which ran in close association with the plasma membrane and between the myofibrils. Lazarides and Hubbard (1976) proposed that the protein forms a three dimensional matrix of filaments, interconnecting the myofibrils and plasma membrane at the level of the Z-discs. This would provide mechanical integration of the contractile apparatus. They called the protein "desmin" which is derived from the Greek word for "bond". Muscle fibres lacking in 10 nm filaments (by specific proteolysis) still contracted in ATP, therefore the desmin network is not essential in contraction. Small and Sobieszek (1977) extracted actomyosin from calcium depleted guinea pig smooth muscle and found the cell ghosts consisted of a network of 10 nm filaments. The filaments often many microns in length. They termed their 55,000 molecular weight protein "skeletin" but Lazarides (1978) found that skeletin is in

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fact desmin.

Lazarides and Granger (1978) used markers to localise hydrophobic membrane sites of mitochondria, the sarcoplasmic reticulum and the T-system of glycerolated chicken skeletal muscle. They found that the markers were associated with the Z-discs and occasionally extended from one Z-disc along the myofibril to the Z-disc of the next sarcomere. Treatment of muscle fibres with strong KCl and KI to extract myosin and actin (Lazarides and Granger, 1978; Granger and Lazarides, 1978) left a filament network which maintained the remaining Z-discs in a honeycomb pattern. The stability of the sheets indicated the strength of the connections between the adjacent myofibrils. Polyacrylamide gel electrophoresis (PAGE) of these sheet preparations revealed that they consisted of actin, desmin, $\alpha$-actinin, some myosin and some tropomyosin. Immunofluorescence staining, located desmin at the periphery of the Z-discs and $\alpha$-actinin within the discs. Actin is found throughout. Myosin and tropomyosin are found loosely attached on both surfaces of the discs. Desmin, with or without actin, links the Z-discs of adjacent myofibrils and has a coincident distribution with the T-system. Desmin filaments may mediate filament/membrane interactions i.e. anchoring the triad to the Z-disc. The collar-like distribution of desmin may also help maintain the structure of the Z-disc. By linking the Z-discs of neighbouring myofibrils and linking the contractile apparatus to the membrane system, desmin would help maintain transverse order within the muscle fibre, especially, during muscle contraction as, during sarcomere shortening, the cross-striations remain aligned and the specific location of the membranes relative to the sarcomere is preserved (Nunzi and Franzini-Armstrong, 1980).

Vimentin, the intermediate filament subunit of mesenchymal origin, is also found (co-existing with desmin) in muscle cells (Granger and Lazarides, 1979; Granger, Gard and Lazarides, 1979). The two proteins have the same distribution and vimentin is probably also involved with lateral registration of sarcomeres. The ratio of vimentin to desmin changes from high to low during skeletal myogenesis (see Chapter 13.3).

Besides vimentin, two new intermediate filament proteins have been identified in muscle cells: filamin (molecular weight 250,000, Gomer and Lazarides, 1981) and synemin (molecular weight 230,000, Granger and Lazarides, 1980; Muguruma.
Kobayashi, Fukazawa, Ohashi and Maruyama, 1981). Synemin, apart from its molecular weight, has very similar properties to vimentin and desmin, copurifying with them from avian smooth and skeletal muscle and having the same distribution i.e. wavy filaments in the cytoplasm of multinucleate myotubes and one week after myoblast fusion, forming networks of filaments at the Z-disc peripheries. Synemin is also not extracted by non-ionic detergent or high salt.

Recently (Pierobon-Bormioli, 1980) has used various fixation procedures on rat diaphragm to improve preservation of the 10nm intermediate filament systems. Filaments were seen to run at the Z-, M- and N- levels (Pierobon-Bormioli, 1980; Granger and Lazarides, 1978) and were attached on the plasma membrane at "dense plaques" (similar to the distribution of the titin network; see Chapter 13.1). Thus, besides forming an intracellular skeleton, there are strong suggestions from ultrastructural images that the transverse intermediate filament systems form a tight structural integration with the Z-, M- and N-lines.

Numerous desmin filaments are found in embryonic muscle cells and they may be involved in myofibrillogenesis (Nunzi and Franzini-Armstrong, 1980; Lazarides, 1978 and 1980; Bennett, Fellini and Holtzer, 1978; Small and Sobieszek, 1977; Ishikawa, Bischoff and Holtzer, 1968; Bennett, Fellini, Toyama and Holtzer, 1979). Ishikawa et al. (1968) used mitotic inhibitors to arrest myotubes, including presumptive myoblasts, in metaphase. 10nm filaments, 2-2.5μm in length were present in the cytoplasm, but A- and I- filaments did not appear until a few hours after mitosis had occurred. Intermediate filaments were present throughout myogenesis, although there was no evidence for their participation in myofibril formation. Antibodies to desmin labelled cytoplasmic networks in embryonic chick cardiac cells (Lazarides, 1978) but were not associated with newly forming Z-discs or sarcomeres (Lazarides, 1980). As myofibrils begin to appear in myotubes, immunofluorescence shows the presence of longitudinally orientated 10nm filament bundles between the striated myofibrils (Lazarides and Hubbard, 1976; Bennett et al., 1978 and 1979; Lazarides, 1980; Nunzi and Franzini-Armstrong, 1980). As the myotubes mature, there is a redistribution of the intermediate filaments, resulting in filament rings round the myofibrils at the level of the Z-disc (Bennett et al., 1979; Nunzi and Franzini-Armstrong, 1980; Lazarides, 1980) so bringing them into register. The intermediate filament network may also direct the deposition of the membrane system to the Z-disc. Bennett
et al. (1979) found that the transverse 10nm filaments were of a different antigenic type from that of the longitudinal filaments.

In conclusion, the intracellular intermediate filament network characteristic of all eukaryotic cells is also present in striated muscle cells where a cytoskeleton may not be thought to be necessary because of the myofilament network. Whether the intermediate filament network still functions in striated muscle cells, as a cytoskeleton is unclear, but it also appears to have the additional structural function of integrating the transverse elements of the myofibrils. Intermediate filaments probably also play an important role in myofibrillogenesis (see Chapter 13.3). Several intermediate filament proteins have been identified but their precise cellular localisations have yet to be determined; the different proteins may form filaments with different structural roles.
13.3 Myofibrillogenesis and the Role of Self-Assembly

Two classical reviews of myofibrillogenesis have been presented by Fischman (1970 and 1972): a summary of his conclusions incorporating more recent findings is presented here.

The events of skeletal muscle development take place in three principal cell types: 1) the "presumptive myoblast", where transformation of undifferentiated mesenchymal cells can result in these mononucleated, actively dividing myoblasts which contain no detectable myofibrils. 2) the myoblast, a post-mitotic cell capable of synthesising myofibrillar proteins, and 3) the multinucleated myotube produced by aggregation and cytoplasmic fusion of myoblasts. The myotubes contain the bulk of myofibrillar material in embryonic muscle.

The precise sequence of events in filamentogenesis and myofibrillogenesis is still uncertain, but the following is an outline of what is currently known (see reviews by Fischman, 1970 and 1972).

Within the cytoplasm of chick skeletal muscle myoblasts, 2 filament populations are present, 10nm and 6nm in diameter. The latter are found primarily near the pseudopodial process of a myoblast in long sheets or bundles, running in the longitudinal axis of the cell beneath the plasma membrane. The 6nm diameter filaments, the so called cortical filaments are probably composed of actin or an actin-like protein, as they are decorated by heavy meromyosin (ishikawa, Bischoff and Holtzer, 1969). The cortical filaments are distinguishable from another class of 6nm cytofilaments called "microfilaments" which are found in networks within the pseudopodia as only the latter are disrupted by the drug cytochalasin B (Wessells et al., 1971). Microfilaments are also decorated by heavy meromyosin (Goldman and Knipe, 1972). The 10nm diameter filaments are not decorated by heavy meromyosin and are probably desmin and/or vimentin filaments as described previously (Chapter 13.2). Both 6nm and 10nm filaments are found in other mononucleate cells (fibroblasts, macrophages etc.) and are therefore not specific
to myofibrillogenesis. Mononucleate cells which form muscle, have a predominantly bipolar configuration whereas non-muscle cells i.e. fibroblasts, are triangular in shape (Konigsberg, 1963).

In the long cylindrical multinucleate myotubes there are three types of cytofilaments, 6nm, 10nm and 16nm (Ishikawa, 1968). The latter are probably A-filaments and they are often found at the sites of myofibril assembly i.e. beneath the plasma membrane. There may be two types of 6nm filaments, the myofibrillar actin filaments and the actin-type filaments associated with the cell cortex.

A variety of evidence suggests that the elongate shape of myotubes is not determined by the longitudinally orientated developing myofibrils. E.g. 1) microtubules are also present in the cell cortex, orientated in the same direction as the 6nm filaments. Bischoff and Holtzer (1968) found that colchicine, which depolymerises microtubules (Borisy and Taylor, 1967), caused fragmentation and rounding-up of myotubes and within these were malpositioned myofibrils. E.g. 2) trypsin treatment of embryonic muscle cell cultures produced a similar effect on the myotubes and myofibrils and caused the microtubules to become irregular, shortened and smaller in number. The trypsin was thought (Fischman, 1970) to destroy the cell-substrate adhesion, so cell attachment may also be important in maintaining the elongated myotube shape and the longitudinal orientation of developing myofibrils.

The method by which actin and myosin molecules assemble into I- and A-filaments respectively, is unknown. Both types of monomers are capable of self-assembly in vitro into synthetic filaments (Huxley, 1963; Hanson and Lowy, 1963). However, synthetic filaments are not identical to native filaments (see Chapter 10.1.4). The large non-membrane bound polysomes which synthesise the polypeptides of the myosin molecules (Allen and Pepe, 1965) are not involved in filament assembly as they are not preferentially found near myofibrils. Intermediate stages of thick filament assembly have not been seen (Allen and Pepe, 1965). Thick filaments incorporated into newly developing myofibrils are always 1.6µm long (Fischman, 1967).

Fischman (1967) made a detailed electron microscope study of developing muscle cells in chick embryo legs. He found that initially far more actin than
myosin filaments were produced, a ratio of 7:1. Allen and Pepe (1965) also found that I-filaments were produced in large numbers before myosin filaments. The free myofilaments become aligned in the long axis of the cells predominantly beneath the plasma membrane prior to aggregation into hexagonally packed arrays. The 6nm filaments of the cell cortex may be involved in aligning I-filaments in the longitudinal axis, as I-filaments within the core of the myotube are randomly dispersed (Fischman, 1967).

Once the thick and thin filaments have polymerised, they orientate along the longitudinal axis of the cell, predominantly in the cell cortex and there are many microtubules in close association, which may be involved in producing the correct alignment. The thin filaments are distributed in a hexagonal pattern around the thick filaments as seen in cross-sections in the absence of Z- and M-lines (Allen and Pepe, 1965; Fischman, 1967). Individual sarcomeres with double hexagonal arrays of myofilaments can result in the absence of any cross-striated sarcomere pattern.

Therefore, the Z- and M-lines are probably not essential for sarcomere production. This suggests that the double hexagonal lattice of myofilaments is determined by an inherent six fold axial symmetry of each thick filament reflecting the 6/2 helical distribution of its cross-bridges (Huxley and Brown, 1967). Thin filaments aligned in parallel with thick filaments and attached to the cross-bridges, would of necessity be disposed to six fold symmetry (Fischman, 1967). Such a placement of thin filaments would produce the maximal number of thick to thin bonds and therefore the most stable configuration for the filament bundles (Allen and Pepe, 1965). The cross-bridges are now believed to be arranged with a 9/3 helical distribution (see Part 1), but as described in Chapter 14.1 a six fold symmetry of thick and thin filaments may still be attained if each myosin head in a crown of cross-bridges attaches to one actin filament; each cross-bridge thereby cross-linking two actin filaments.

The 6nm filaments and microtubules present in the cell periphery may be involved in myofilament and myofibril alignment, although some myofibrils do assemble away from the plasmalemma and sarcoplasmic reticulum. Myofibril assembly is not dependent on protein synthesis (Fischman, 1970) and may be a self-assembly
process analogous to the assembly of T.M.V. protein coats (Etlinger and Fischman, 1972; see Chapter 6.2).

Self-assembly of a hexagonal lattice (probably involving the cross-bridges), has been observed in vitro. Hayashi, Silver, Ip, Cayer and Smith (1977) polymerised G-actin in the presence of synthetic rabbit myosin filaments (1.2 to 2.2 μm in length). They observed, in the electron microscope, closely associated complexes of six actin filaments lying parallel to one myosin filament. In transverse sections the actin filaments formed a hexagonal array around the thick filament. Heavy meromyosin decoration demonstrated that the synthetic actin filament were bipolar. The entire complex is therefore analogous to an in vivo sarcomere. It was suggested that the complex involves a unidirectional polymerization of G-actin, probably specified by the actin–myosin interactions. Thus, the cross-bridges may act as organizing centers for polymerization.

Longitudinal growth of myofibrils, by addition of new sarcomeric units, probably requires the deposition of Z-discs (Fischman, 1967) to act as a cementing substance to bind together the thin filament ends. The Z-disc may be developed from amorphous dense material found within bundles of thin filaments. Besides γ-actinin (95,000 chain weight, Masaki, Endo and Ebashi, 1967) and 10–S actinin (43,000 chain weight, Sugita, Masaki and Ebashi, 1974), a new structural protein has been located in the Z-disc (Ohashi and Maruyama, 1979). These workers have extracted and purified a protein of molecular weight 55,000, from chicken skeletal muscle Z-discs. It has a different amino acid composition from desmin and is distinguishable from all other known muscle proteins. Immunofluorescence indicates the protein is only present in the Z-discs. The protein forms a powder-like suspension on standing in solution; when negatively stained and viewed in the electron microscope, thin filaments approximately 2 nm in diameter forming two types of lattice structures resembling those of the native Z-disc are seen. Z-disc structure has been described by Knappels and Carlsen (1962), Reedy (1964) and Ullrick, Toselli, Salde and Phaer (1977).

10–S actinin is a mixture of actin and eu-actinin (Kuroda and Masaki, 1980). Eu-actinin is a newly described structural protein of striated muscle Z-discs of molecular weight 42,000 (Kuroda, Tanaka and Masaki, 1981). Eu-actinin strongly
interacts with actin and α-actinin and is a tightly integrated component of the Z-disc; it provides nucleation sites for actin, suggesting that eu-actinin may bind to the Z-disc end of actin filaments. α-actinin is known to gelate F-actin (Maruyama and Ebashi, 1965).

The actin-binding protein "filamin" (Gomer and Lazarides, 1981) is present in myoblasts and early fused cells associated with α-actinin-containing filament bundles as shown by double immunofluorescence. One day after cell fusion, but before development of α-actinin containing Z-disc striations, filamin disappears from cells. Later, several days after the Z-discs appear filamin reappears, localized at the Z-disc and visible just before vimentin or desmin become associated; filamin has the same distribution as vimentin and desmin, forming networks surrounding each Z-disc. Filamin may be involved by binding to actin (Granger and Lazarides, 1978) in the transition of desmin and vimentin filaments to the Z-discs. Antibodies to α-actinin and antibodies to desmin and vimentin demonstrate that these two domains assemble sequentially during myogenesis (Gard and Lazarides, 1980), α-actinin first being localized in punctate pattern along the actin filament bundles. Several days later desmin and vimentin become associated with the periphery of the α-actinin Z-discs (Gard and Lazarides, 1980) after having been present as a cytoplasmic filamentous network.

Filamin also reduces actin-activated ATPase activity (Davies, Bechtel and Pastan, 1977). Therefore it is possible that around the time of filamin disappearance, myosin/actin filament interaction and activation of myosin ATPase by actin, become vital for sarcomere assembly. Removal of filamin must be a pre-requisite for subsequent assembly of sarcomeres, as Z-discs appear one day later. Reappearance and subsequent removal of filamin suggests that it is required for the final assembly of the Z-disc, but it is not necessary or may interfere in sarcomere morphogenesis. The Z-disc is probably assembled in 2 distinct stages.

Samosudova, Larin, Enenko and Shungskaya (1981) have suggested that the Z-disc has a membraneous origin. Electron micrographs of chick embryo myotubes showed invaginating plasma membrane included in the formation of the sarcotubular system and thin filaments were seen attached to the sarcoplasmic reticulum at sites of the future Z-discs.
Williams and Goldspink (1971) found that increase in fibre length during normal growth was accompanied by an increase in sarcomere number and new sarcomeres are added to the ends of fibres rather than being inserted (Griffen, Williams and Goldspink, 1971).

Myofibrils appear to increase in width by addition of new myofilaments at their periphery. After the start of myofibril assembly, polysomes are often found around the circumference, suggesting newly synthesised fibrillar proteins are added at the periphery of the enlarging hexagonal lattice (Fischman, 1967). Newly incorporated tritiated leucine is found in the periphery of myofibrils of developing rat diaphragm (Morkin, 1970) and these labelled filaments are released first from disrupted myofibrils in filament preparations (Etlinger, Zak, Fischman and Rabinowitz, 1975) suggesting that they are also fairly loosely incorporated into the lattice. There must be a limiting factor on myofibrillar diameter; Goldspink (1970) has found fibre numbers increase by longitudinal splitting and this may be triggered when a certain myofibrillar diameter is reached. Post-embryonic growth and differentiation of striated muscle has been reviewed by Goldspink (1972).

The time of deposition of the sarcoplasmic reticulum and the T-system around the myofibrils and their role, if any, in myofibrillogenesis is uncertain. The T-tubules develop as invaginations of the plasma membrane, and cisternae of the endoplasmic reticulum bud off from the sarcoplasmic reticulum (Ishikawa, 1968). Fischman (1967) has stated that the sarcotubular system is not involved in myofibril assembly. It is probably incorporated into the muscle fibres after the cross-striation pattern has been assumed and the network of desmin filaments is probably involved in these stages.

13.4 Myosin Turnover in vivo

Myosin turnover is an even less well understood phenomenon. Electron microscopy of adult muscle does not show structural aspects of myofibrillogenesis. Etlinger et al. (1975) have postulated that new myosin filaments are added to the periphery of myofibrils, but where are the filaments which they replace removed from? Dreyfus, Kruh and Schapira (1960) intravenously administered C14 to rats and isolated...
myofibrils and myosin from their skeletal muscle. They found that the life of the label in each was about 30 days. A 30 day life-span for myosin was confirmed by McManus and Mueller (1966), but later still, Garlick (1969) and Everett Prior and Zak (1981) found the half-lives of rat myosin to be 6 and 5.5 days respectively. Dreyfus et al. (1960) suggest that on completion of a myofibril the myosin filaments are stable and after about 30 days the whole myofibril is destroyed and replaced with a newly formed one. Possibly the newly formed filaments added to the periphery of an existing myofibril form a new myofibril after longitudinal splitting and the original myofibril is then disassembled.

Unfortunately, myofibrillar turnover may be far more complicated than imagined, as Koizumi (1974) discovered that the turnover rates of individual rabbit skeletal muscle structural proteins varied considerably. Based on a myosin turnover rate of 29 days (McManus and Mueller, 1966), Koizumi calculated that M-protein, troponin, tropomyosin, α-actinin, 10S-actinin and actin have turnover rates of 11, 12 to 16, 22, 24, 44 and 75 days respectively. Furthermore, Vellick (1956), proposed that light meromyosin and heavy meromyosin are renewed individually, the former having a turnover rate of 20 days and the latter 80 days. It seems unlikely that the myosin heavy chains would be synthesised in two portions, but the results indicate that the light and heavy chains may be replaced separately.

Some preliminary investigations of in vivo filamentogenesis and myosin turnover have been made by attempting to incorporate radioactive labels into assembling A-filaments, and analysing the uptake by autoradiography. The aim was to observe whether newly synthesised myosin molecules were incorporated individually into A-filaments (and whether incorporation occurred at the filament tips or in the bare zone), or if complete A-filaments were disassembled and subsequently reassembled (in which case whole filaments would be radioactively labelled).

In one experiment rats were given one pulse of 3H-leucine intravenously (0.2μCi/g body weight) and the animals were sacrificed after a series of intervals ranging from 10 minutes to 2 hours. Relaxed filament preparations were made of the psoas muscles. The aim was to make autoradiograms of the filament preparations, a) separated on polyacrylamide gels (in order to determine the ratio of activity incorporated into the different components of the thick filaments and myosin molecules).
and b) spread on grids for electron microscopy. However on mixing small portions of the preparations with scintillation fluid and counting in a scintillation counter, the specific activities were found to be barely above background. The experiment would be feasible if the dosage of radioactivity could be increased approximately one thousand fold in order to expose autoradiograms. An attempt was then made to incorporate a tritiated amino acid in vitro, by incubating thin strips of psoas in a petri dish containing a culture medium of salts, glucose and 10μCi/ml ³H-leucine. Uptake rose throughout a 2 hour period as determined by scintillation counting, but still the specific activities of the filament preparations were not high enough for electron microscopy or gel autoradiography. The uptake might be improved by using a culture medium containing all the amino acids minus leucine plus very high activity ³H-leucine.

13.5 The Role of the M-Line in Myofibrillogenesis

The M-line is a region of increased electron density in the centre of the sarcomere of striated muscle. Its function is believed to be the maintenance of the thick filaments in register (Strehler, Pelloni, Helzmann and Eppenberger, 1980), as the less regular alignment of A-filaments in slow fibres. It is consistent with the absence of an M-line. The M-line network may also guide the sliding I-filaments during contraction.

The M-line of chicken skeletal muscle has at least two principal components. One component is the muscle type isoprotein of creatine kinase (Herasymovych, Mani and Kay,1978; Turner, Wallman and Eppenberger,1973; Morimoto and Harrington,1972); strong evidence that creatine kinase is an integral element in the M-line structure has been found by the use of immunological and electron microscopy techniques (Kundrat and Pepe,1971; Wallman, Turner and Eppenberger,1977; Wallman, Kuhn, Pelloni, Turner and Eppenberger,1977). The second component of the M-line, as determined by antibody and ultrastructural evidence, is the 165,000 molecular weight protein (Masaki and Takaiti,1974; Trinick and Lowey,1977; Mani and Kay,1978a; Herasymowych, Mani, Kay, Bradley and Scraba,1980; Strehler and Eppenberger,1979 and 1980), termed M-protein or more recently "myomesin" (Eppenberger, Perriard, Rosenberg and Strehler,1981). Myomesin
is also present in myofibrils lacking a visible M-line e.g. chicken heart muscle (Strehler, Pelloni, Helzmann and Eppenberger, 1980) and in which M-line creatine kinase is absent, indicating that creatine kinase is almost certainly responsible for the electron-dense structural material forming the visible M-line, besides having an enzymatic role (Wallman, Turner and Eppenberger, 1975). Models for skeletal muscle M-line structure based on electron microscope investigations, have been described (Knappels and Carlsen, 1968; Pepe, 1975; Luther and Squire, 1978). High resolution electron microscopy, using ultrathin cryosections showed that the M-line is actually composed of substripations, the number of striations varying among different muscle types (Sjöström and Squire, 1977a and b). Three structural elements have been identified: primary and secondary (Y-shaped) M-bridges and M-filaments (Huxley, 1972; Knappels and Carlsen, 1968; Mani and Kay, 1978b; Sjöström and Squire, 1977b). The M-filaments lie parallel to the thick filaments in the M- and H-zones (Franzinl-Armstrong and Porter, 1964); the two filament types are interconnected by transverse arrays (producing three or five predominant substripations in longitudinal sections) of M-bridges. Page (1965) considered that the M-bridges observed in transverse sections of the M-line might arise from the points at which the myosin molecules abut tail-to-tail.

Unfortunately, there is conflicting evidence about the properties of myomesin (or M-protein). Whereas creatine kinase is easily extracted by low ionic strength solutions (Samosudova, 1966; Morimoto and Harrington, 1972; Perry and Corsi, 1958), myomesin can only be extracted quantitatively under conditions where the myofibrils are completely destroyed (Strehler, Pelloni, Helzmann and Eppenberger, 1980). This property of myomesin suggested that the protein has a very high affinity for myosin and this was supported by use of the high-speed sedimentation equilibrium technique (Mani and Kay, 1978a and b). However, using an increased range of concentrations with the same technique, J.L.Woodhead and S.Lowey (personal communication), found in vitro reactions of myomesin with myosin or its subfragments are in fact, weak. M-line proteins appear to be firmly bound to the myofibril in situ, suggesting that either, other proteins are required to produce binding or, the M-proteins are modified during isolation and purification. J.L.Woodhead and S.Lowey have made an electron microscope examination of isolated M-protein and found that it is an elongated molecule (approximately 35nm by 4nm) of similar dimensions to the M-filaments. M-filaments are bound to A-filaments by M-bridges: presumably
isolated M-filament protein would only react with myofibrils in vitro in the presence of the M-bridge protein.

Evidence obtained from immunofluorescence studies revealed a synchronous appearance of M-protein and other myofibrillar proteins, such as myosin and actin, in myogenic cell cultures (Strehler and Eppenberger, 1979). Antibodies against myomesin stain a tranverse structure, within the nascent H-zone, of small myofibrils consisting of only a few myofilaments (Eppenberger, Perriard, Rosenberg and Strehler, 1981). It is impossible to detect any myomesin when only single randomly arranged myofilaments are present, suggesting that myomesin plays a key role in structural organisation in myofibrillogenesis (Strehler and Eppenberger, 1980). The M-line or more specifically myomesin (as the electron dense band i.e. creatine kinase, is not observed until later in myofibrillogenesis) may be a prerequisite for myofibrillar assembly i.e. myofibrillogenesis and reconstruction.
14.1 A Description of the Model

As discussed earlier (see Chapter 11.1), it is possible that the l-filament lattice provides a template (or "nucleation site": see Chapter 6.4) and length controlling factor for A-filament reconstruction. A model is now proposed (see Figure 75) by which myosin filaments could be assembled during reconstruction, based on the following facts and assumptions:

1) There is a numerical relationship between the number of myosin molecules per A-filament and the number of repeating units within the l-filaments of a contractile unit. The ratio of thick to thin filaments in the striated muscle hexagonal lattice is 1 to 2, therefore, a contractile unit consists of one A- and two l-filaments.

Each l-filament may be divided up into repeating unit cells each comprising 1 tropomyosin, 1 troponin and 13 actin molecules. There are 24 such unit cells in each half of an l-filament (assuming l-filaments are bisected by the Z-disc) based on the troponin repeating distance determined by anti-troponin antibody labelling (Ohtsuki, Masaki, Nonomura and Ebashi, 1967). Therefore, there are 48 unit cells per actin filament and 96 per contractile unit.

A-filaments cannot be divided into unit cells as the molecular packing and non-myosin component distribution varies throughout. However, the number of myosin molecules per A-filament has now been estimated with greater accuracy. Combining the cryosectioning results of Sjöström and Squire (1977a and b) with their own antibody labelling results, Craig and Offer (1976a) concluded that: a) the myosin cross-bridges in each half of an A-filament occur on an approximate 14.3nm sequence but that there is a gap on the third row ("crown": see Chapter 1.5) in from the end. and b) the number of occupied rows in each half is 49 (this implies a thick filament length of 1.57μm as in the same study the bare
zone width was measured as 150nm) and there are 98 occupied rows in a complete A-filament.

Therefore, each contractile unit consists of 98 myosin crowns and 96 actin unit cells; almost a one-to-one relationship.

2) Subfragment-1 decoration demonstrates that myosin binding can occur at every pair of actin monomers along the l-filament (Craig, Szent-Gyorgyi, Beese, Flicker, Vibert and Cohen; 1980). However, based on the l-filament decoration obtained by Clode(1981; see Chapter 10.3.3) it is assumed in the model that each l-filament has 48 selective myosin binding sites, the number and periodicity of which are dictated by the troponin binding sites.

3) It has also been assumed, due to the numerical relationship, that in the model, the actin unit cells code for the myosin molecules in the A-filament.

4) Development of the numerology suggests that 3 cycles of myosin assembly occur to produce a full length A-filament. For example:- a) The heads from 3 myosin molecules constitute each crown (see Part 1; thus making a total of 294 myosin molecules per filament). Therefore, within each contractile unit there are 98x3 myosin cross-bridges and 96 myosin binding sites. b) After 3 cycles of actin-myosin binding, 6 myosin molecules remain. c) The cross-bridge portion of each myosin molecule is capable of attaching to two actin filaments simultaneously (Offer and Elliott, 1978; Trinick and Offer, 1979 and 1980; Freundlich, Luther and Squire, 1980; Borejdo and Oplatka, 1981) and thereby cross-linking them. This property is complemented by (d). d) The l-filament possesses 2-fold symmetry and as demonstrated by subfragment-1 decoration can bind 2 myosin heads at any point along the filament. It is not essential for the model that myosin binding causes cross-linking of actin filaments, but it would produce a more stable actin-myosin complex.

5) Reconstruction of extended muscle produces short myosin filament fragments as shown by longitudinal and transverse sections (Figures 54d, 56a and 56b) and X-ray diffraction (Figures 57d and 58d). These fragments initiate in the l-band and project into the pseudo H-zone space suggesting the opposite to the proposal.
that A-filaments assemble from the bare zone towards the filament tips. Thus, A-filaments appear to grow from the filament tip, within the I-filament lattice, and towards the M-line.

Reconstruction of individual subfilaments has not been demonstrated: myosin extraction prior to reconstruction results in mini A-bands (Isenor, 1976; see Chapter 7.3). These facts suggest that the triple cycling mechanism of actin–myosin binding proposed, produces not 3 individual subfilaments per cycle, but full length filaments assembled in three distinct stages. During myosin solubilisation approximately one third of the myosin molecules (Isenor, 1976) remain attached to the actin filaments (Clode, 1981), and it is suggested that on lowering the ionic strength, these molecules assemble to form filament fragments within the I-bands. These fragments then migrate towards the pseudo H-zone vacating the myosin binding sites on the actin filaments, which are then filled again with free myosin molecules which in turn assemble and migrate out of the I-band. A third cycle of myosin binding and assembly depletes the solubilised myosin pool and completes the half A-filaments being assembled within each I-band.

Hayashi, Silver, Ip, Cayer and Smith (1977; see Chapter 13.3) assembled synthetic actin filaments in the presence of synthetic myosin filaments and postulated that the cross-bridges act as organizing centres for polymerization of G-actin and that the assembly would occur unidirectionally towards each end of the myosin filament. Presumably, if this hypothesis were correct, the opposite could also be feasible, i.e. myosin heads attaching to actin monomers orientate the myosin molecules so that on complete assembly a bipolar filament is produced.

It is proposed that the six residual myosin molecules of each contractile unit are not assembled within the I-bands but are incorporated into a developing M-line. Strehler and Eppenberger (1980) and Eppenberger, Perriard, Rosenberg and Strehler (1981) have found that the M-line protein myomesin (see Chapter 13.5) has a high affinity for myosin and is present within the nascent H-zone in the very earliest myofibrils (as demonstrated by immunofluorescence) before any M-line is visible and only a few myofilaments are present. Some of the micrographs presented here (e.g. Figure 67a) show the presence of an M-line incorporating very short filaments (0.1 to 0.2 μm) prior to complete filament reconstruction. Presumably,
the six myosin molecules would assemble in a bipolar arrangement, 3 on either side, forming a mini-filament similar to those of Reisler, Smith and Seegm (1980).

Reconstruction of a full length A-filament is thus completed when the assembled A-filament halves either slot into a developed M-line or an M-line structure may be developed between opposing half filaments when they are close together; intermediary stages show an absence of M-line (Figure 73) as often as its presence (Figures 67a and 72b).

In conclusion, the model proposes that the complete process of reconstruction is as follows: approximately 30% of pre-phosphate treatment cross-bridges remain intact whilst the A-filament backbone is disassembled. On return to a physiological ionic strength medium, attached and correctly orientated (by the I-filaments) myosin molecules assemble and the filament fragments are moved out of the I-bands by a cross-bridge cycling mechanism. Meanwhile the M-line is assembled. The remaining solubilised myosin completes 2 further cycles of binding, assembly and migration. One cycle of myosin assembly would produce the mini A-bands of Isenor (1976); a second cycle would be inhibited by the lack of further myosin. How the filament fragments build into a complete unit is uncertain. Two cycles of A-filament assembly, prior to migration out of the I-band, are possibly represented by Figure 73b in which the half A-bands are approximately 2/3 of a normal half A-band width.

14.2 Implications of the Model and the Possible Role of the Cytoskeletal Network

A similar model of A-filament assembly may be utilised during myotube development and in A-filament turnover. However, in the latter case, gaps in the A-filament lattice are not observed in thin sections (longitudinal or transverse). During filamentogenesis a large number of full-length I-filaments are present in the cytoplasm before A-filaments are detectable (Allen and Pepe, 1965; Chapter 13.3) and the ratio of I- to A-filaments is initially 7:1 (Fischman, 1967; Chapter 13.3), which is similar to the ratio in the hexagonal complexes of synthetic actin.
filaments produced by Hayashi et al. (1977; Chapter 13.3). If A-filament assembly incorporates a cross-bridge cycling mechanism, the whole process may have similar controls to the contraction/relaxation cycle; this theory is supported by the following evidence:— a) myosin filaments may only be present in smooth muscle during contraction, i.e. contraction and relaxation occurs simultaneously with A-filament assembly and disassembly and both processes are regulated by calcium ions (Shoenberg, 1969), and b) pyrophosphate induced disassembly of A-filaments accompanies a structural change in subfragment-2 (Oriol-Audit, Lake and Reisler, 1981; see Chapter 10.1.5) and this change may also be important in cross-bridge cycling (see Chapter 1.5).

Variations on this theme, probably caused by the conditions which were imposed on the muscle prior to phosphate treatment will now be discussed.

Inducing a relaxed state by the incorporation of Mg\(^{2+}\)ATP after rigor has developed inhibits reconstruction. This is explained by the model as cross-bridge attachment is inhibited in the presence of Mg\(^{2+}\)ATP and without attaching to the actin filaments the myosin molecules would be unable to assemble in the correct conformation and alignment.

Stretching fresh muscle to a sarcomere length where the A and I-filaments no longer overlap (about 3.7\(\mu\)m) also inhibits complete reconstruction. Filament fragments are still assembled within the I-bands and project out of the I-bands but the opposite halves are unable to meet as the gap to be spanned is too large. In this sample and in the fresh relaxed sample the M-lines have not developed indicating that close proximity of the A-filaments halves may be required.

One might expect the half filaments of extended muscle to project much further out of the I-bands. It is possible that the maximum sarcomere length at which reconstruction can occur is much less than 3.7\(\mu\)m as the further the filament halves are moved out of the I-band the more likely they are to sway about in the space between the I-bands and become disorientated. Therefore, we predict that the maximum sarcomere length at which reconstruction could occur is about 2.7\(\mu\)m (the sum of the widths of actin band at 2.2\(\mu\)m, the developing M-line
about 0.2µm and 2x0.16µm for the length of the myosin molecule tails projecting out of each l-band). This agrees with results of rabbit fibres. However, the reconstructed sample of Clode (1981, Figure 74h) has a sarcomere length of about 3.2µm which suggests that the filament halves must project approximately 0.3µm out of each l-band. It is possible that connectin filaments are involved in guiding the A-filament halves out of the l-bands towards the M-line and connectin may be involved in the correct positioning and assembly of the M-line. However if this were so one would expect extended rabbit psoas to reconstruct as the connectin nets are highly elastic and allow rabbit psoas to be stretched to a sarcomere length of approximately 4µm without ill effect (dos Remedios and Gilmour, 1978).

Muscle which has been allowed to go into a state of rigor prior to phosphate treatment should be able to reconstruct according to the model and so it does (Figure 69b). All the cross-bridges are attached (Huxley and Brown, 1967; Cooke and Franks, 1980; Lovell and Harrington, 1981) but phosphate tends to detach up to two thirds of these (Mihalyi and Rowe, 1966) so effectively the muscle is in a similar state to fresh muscle once in phosphate. However, the reconstructed M-line in this muscle is not as well defined as in fresh muscle possibly implying some post-mortem changes have occurred. The effects of post-mortem storage on muscle are discussed in the Appendix.

The lack of reconstruction after calcium ion depletion (Figure 66d) may be predictable from the model. Calcium depletion effectively induces a relaxed state as in the absence of calcium ions the myosin heads are barred from binding to the actin filaments. Therefore, one would expect a similar effect by calcium depletion on reconstruction as the presence of Mg\(^{2+}\)ATP prior to phosphate treatment. However, as calcium ions are an important factor in many cell processes, calcium depletion could also affect some other factor necessary for reconstruction. In this case the latter argument can be partly dismissed as reconstruction occurs on return of calcium ions in the mammalian ringer after phosphate treatment (Figure 67a, b and c). Nevertheless, calcium depletion may have some effect on another important factor as the reconstructed A-bands are not as well ordered as in fresh muscle: or possibly the presence of ATP is required to produce perfectly ordered A-bands but unfortunately in this experiment the area chosen in sample
3 had failed to reconstruct at all. Morimoto and Harrington (1974b) found that the DTNB light chain binds calcium ions in vitro and at physiological levels of Ca$^{2+}$ the thick filaments undergo a conformational change. They suggested that this provides evidence for a Ca$^{2+}$-sensitive regulatory mechanism at the level of the thick filament. Calcium ion depletion may induce a reverse conformational change which affects filament assembly.

According to the model, pre-glycerolated muscle should also reconstruct, as glycerol treatment induces rigor. Glycerolation also disrupts the cell membranes releasing soluble components one of which may be required for reconstruction. Morphologically glycerolated muscle fibres resemble fresh fibres (Figures 61a and 28) except that they are contracted as described by Hattori and Takahashi (1979). Other filamentous systems which may be involved in reconstruction are also preserved during glycerol treatment i.e. connectin (dos Remedios and Gilmour, 1978) and desmin (Lazarides and Granger, 1978) but disruption of their membranous connections may result in the loss of the precise degree of three dimensional order necessary for reconstruction.

The model described provides an explanation for the mode of reconstruction: it incorporates the idea of myosin filaments being aligned within the I-bands by utilising the binding properties of myosin and actin similar to the predicted method by which the hexagonal packing is developed during myofibrillogenesis (Flschman, 1970). However, this model does not account for the change in myosin packing along the filament.

In this respect a possible role for C-protein has been suggested earlier (see Chapter 11.2.1). It would be interesting to know whether C-protein becomes randomised in location during A-filament solubilisation. In any case, the highly ordered system for filament assembly now postulated could also account for a rather exact mode of incorporation of C-protein into the filament structure. Although no specific evidence can be adduced, it would be satisfying if some other system (sites on intermediate filaments?) could be found to code for the number of C-protein molecules available for incorporation: the termination of our A-filament assembly mechanism after three cycles would then be accounted for.
Finally it must be asked whether the type of reconstruction which we have
induced is related in any simple manner to the mode of filament synthesis or repair found in the living myofibril. Clearly the conditions which have been used as the starting point for reconstruction (high ionic strength) are artificial. However, just as the use of a non-physiological pH enables the reconstruction of T.M.V. protein coat to be achieved in vitro by mimicking the action of the T.M.V.-RNA, and glycerol and high magnesium mimic the action of MAP's in microtubule assemble (see Chapter 6.3), so the use of very high myosin concentrations held in true solution may mimic a natural state in which newly synthesised myosin is liberated at effectively very high local concentration in the region of filament formation. Whilst knowledge of the spatial details of myosin synthesis is currently minimal (see Chapter 13.3) it must follow from the biochemical properties of myosin as a protein, that it can hardly be transported any significant distance by simple diffusion through a cytoplasmic medium in which spontaneously polymerises.

If the synthesis of molecular myosin occurs in the region of the developing lattice, then the model which has been described above could account for its incorporation into A-filaments. In addition, the same model could account for filament turnover, on the basis of an as yet poorly identified mechanism for filament removal. On the other hand, if the synthesis of molecular myosin occurs in a region removed from the developing lattice, then the model described could apply only to filament turnover. A mechanism for transporting the assembled filaments to the lattice would then be required and the cytoskeletal network (see Chapter 13.1 and 13.2) could well be involved. Present evidence does not allow a choice to be made between these alternatives. Our reconstruction experiments presumably do not involve synthesis of molecular myosin, but rather the assembly of filaments from pre-existent myosin. They are therefore logically closer to a filament turnover system than to myofibrillogenesis. In either case the cytofilaments, which seem to be particularly visible in rat fibres in which the A-bands have been solubilised (Chapter 12), are likely to play an important role, and this could be a fruitful area for future investigation.

14.3 Further Research

The technique of reconstructing myosin filaments in situ (from solubilised
myosin) which resemble native A-filaments as determined by electron microscopy and X-ray diffraction may enable a wide range of A-filament properties to be examined. However before any assumptions are made, the precise details of reconstructed A-filament properties must be elucidated. This report has shown that reconstructed A-filaments are bipolar, approximately 1.6μm in length, capable of fraying into 3 subfilaments and the myosin molecules are assembled to produce a 14.3nm axial translation: all these properties are found in native A-filaments. Byrne(1981) has demonstrated that reconstructed frog muscle can generate tension and the tension developed is equivalent to that produced by untreated muscles. Reconstructed A-bands are frequently indistinguishable from native A-bands, both in longitudinal and transverse sections i.e. reconstructed A-filaments adopt the native hexagonal filament lattice and are correctly aligned with respect to the M-line.

Some other fundamental properties of reconstructed A-filaments have still to be determined:— 1) the precise dimensions of isolated filaments i.e. the filament diameter and length of bare zone, 2) the physical parameters such as the sedimentation coefficient: this reflects the size and conformation of the particle in solution, 3) the presence and location of non-myosin proteins such as C-protein. It is assumed that at least the M-protein, creatine kinase, is present in reconstructed A-bands as M-bridges are seen in transverse sections, and 4) the biochemical properties e.g. ATPase activities, magnesium and calcium sensitivity.

On determining the precise physical and biochemical properties of reconstructed A-filaments as compared to native A-filaments this method of in vitro A-filament assembly may be used to study in vivo A-filament properties; for example filamentogenesis. Some possible requirements of reconstruction have already been determined such as a soluble component of the cytoplasm which is removed on glycerolation, calcium ions, correctly orientated I-band lattices and actin-myosin bonding. By altering other conditions of the reconstruction system other requirements may be determined. Return of calcium ions after phosphate extraction and reconstruction of muscle in rigor produced some incompletely reconstructed A-bands which may represent intermediate stages of the process: further studies on intermediate stages either induced by altered conditions or by halting reconstruction after a series of time intervals may help to elucidate the assembly process. Further studies of
mini A-bands (Isenor, 1976) may prove useful and the involvement of connectin
ets should also be determined especially as post-mortem ageing affects the network
and storing muscle for 2 days prior to reconstruction affects the quality of assembled
A-filaments (Figure 69b). The involvement of M-line proteins should also be investigated;
Morimoto and Harrington (1972) have published a method of removing the M-line
component creatine kinase. It may be possible to reproduce in vivo some of the
conditions which affect reconstruction and so study the effects of these conditions
on myofibrillogenesis in cell and tissue cultures.

Due to the problems arising from radioactive labelling of assembling filaments
(see Chapter 13.4), another method of following filamentogenesis was devised,
although it has not been attempted. Around the time of birth (the exact time varies
with species) changes occur in myofibrillogenesis: foetal light chains are replaced
by adult light chains and these two types of molecules are antigenically different.
It is possible that A-filament turnover and assembly (or at least light chain turnover
depending on whether the light and heavy chains of myosin molecules are renewed
together or independently) may be followed at the time of birth by incorporating
labelled antibodies to adult light chains. This method would also eliminate confusion
caused by M- and C-protein labelling which needed to be resolved by gel analysis
in the autoradiography experiments.

14.4 Summary

A technique has been developed for in situ resynthesis of 1.6μm A-filaments
and well organised A-bands from their solubilised components. Electron microscopy
and X-ray diffraction indicate that unlike synthetic filaments, reconstructed A-filaments
resemble native filaments in morphology and structure although both are produced
on lowering the ionic strength of a myosin solution. Complete reconstruction is
inhibited at long sarcomere lengths and by Mg^{2+}ATP induced relaxation,
pre-calcium ion depletion and pre-glycerolation; synthetic filaments are produced
on extracting the myosin from these non-reconstructed systems. Therefore it appears
that calcium ions, a soluble component and correctly orientated I-bands at a
resting sarcomere length are required to produce reconstructed A-filaments but
not synthetic filaments. Examination of reconstructed A-filament assembly and structure
may provide a more useful model to examine native A-filament structure and filamentogenesis than do synthetic filaments.

A model for A-filament reconstruction has been postulated based on the implications of the results presented both in Part 1 and 2. This model supposes that the I-filament lattice plays an essential role in A-filament assembly, and is partly based upon observed numerical relationships between the molecular parameters of the two types of filaments.
BIBLIOGRAPHY


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


POST-MORTEM CHANGES OF MUSCLE

Our reconstruction studies have of necessity been performed on post-mortem muscle. After excision from the animal frog muscle may be kept alive (i.e. metabolising) for several hours under suitable conditions. The rabbit muscle used in reconstruction is not maintained in a viable state and is regarded as post-mortem tissue on excision from the animal. Very few studies have been made on post-mortem changes of muscle (Hattori and Takahashi, 1979; Takahashi and Saito, 1979; Takahashi, Nakamura and Inoue, 1981). The most obvious change after death is stiffening of skeletal muscles, rigor mortis, which is induced by loss of ATP (Bate-Smith and Bendall, 1947) and post-mortem contraction of myofibrils (Bendall, 1951). Mihalyi and Rowe (1966) found in rabbit psoas that ATP is depleted after approximately ten hours in muscle homogenates at about 2°C implying that at this temperature rigor occurs approximately ten to 12 hours after death. In rigor, all the myosin heads are bound to actin (Cooke and Franks, 1980; Lovell and Harrington, 1981) forming rigor complexes (Reedy and Holmes, 1965). Subsequently, the rigor is resolved and the muscles become soft with time post-mortem; approximately 2 days at 10°C storage of rabbit psoas (Takahashi et al., 1981). This finding indicates that post-rigor storage causes a modification of the actin/myosin interaction, giving rise to a loosening or weakening effect on the myofibrillar structure.

Morphological aspects and SDS-PAGE profiles of separated thick and thin filaments remain unchanged during post-mortem storage of muscles (Takahashi et al., 1981). Homogenisation of post-rigor muscle causes the myofibrils to break up into fragments of 1-4 sarcomeres (Hattori and Takahashi, 1979) and the dissociation of thick and thin filaments is far easier to induce by ATP than when in rigor (Takahashi et al., 1981). Both of these effects are thought to be caused by calcium ion induced degradation of the Z-disc. CAF (calcium activated factor) is known to degrade Z-discs in vivo in the presence of more than 0.1mM Ca^{2+} (Busch, Stomor, Goll and Suzuki, 1972) and releases γ-actinin but iodoacetate (CAF inhibitor) does not inhibit myofibril fragmentation (Hattori and Takahashi, 1979).
of myofibrils with 10mM CaCl\(_2\) caused release of 12% of the total myofibrillar proteins after homogenisation and release of Z-disc components of 54,000 (possibly the structural protein of Ohashi and Maruyama, 1979) and 76,000 daltons (Hattori and Takahashi, 1979) but not \(\alpha\)-actinin. In freshly prepared myofibrils the dissociation of thick from thin filaments caused by ATP in post-rigor muscle was caused by the addition of a protein which is released from myofibrils (probably from Z-discs) on Ca\(^{2+}\)-treatment (Takahashi et al., 1981).

It is possible that in post-mortem muscles, Ca\(^{2+}\) ions at about 0.1 mM (Hattori and Takahashi, 1979) bind to the Z-disc constituents and induce some loosening of the Z-disc structure. Additional degradation would follow due to the tension developed during the contraction at rigor. Therefore dissociation of thick and thin filaments is easily induced by ATP in myofibrils prepared from post-rigor muscles, because the rigor bond between actin and myosin is modified by the protein released from post-mortem degradation of Z-discs.

Muscle fibres stored in glycerol have an increased tendency to fragment into sarcomere lengths of 1-4 even if no Ca\(^{2+}\) was added and this effect was completely inhibited by 5mM EDTA. Therefore, glycerolation must increase the effect of Ca\(^{2+}\) on the Z-disc (Hattori and Takahashi, 1979). Glycerolation is widely used to preserve muscle fibres.

Takahashi and Saito (1979) have also studied the post-mortem changes in skeletal muscle connectin. Vertebrate skeletal muscle loses its elasticity and becomes plastic with post-mortem time. Myofibrils from fresh rabbit psoas and chicken breast were prepared on grids, extracted with KCl and KI and the Z-discs removed with 0.1M NaOH: the disappearance of connectin nets with post-mortem time coincided with approximately 30% of the muscle elasticity being lost from the myofibrils. Therefore 70% of fresh muscle elasticity results from the myosin/actin interactions. In chicken muscle, the connectin nets and the corresponding elasticity were lost after one day post-rigor and 7 days post-rigor in rabbit myofibrils. It was suspected that connectin nets were destroyed by a similar Ca\(^{2+}\)-activated mechanism as described by Hattori and Takahashi (1979). As the connectin nets are linked to the Z-disc this is a reasonable supposition.
The structure of vertebrate A-filaments has long been disputed and various models have been proposed. However, detailed knowledge of the structure is critical for further research and understanding of the mode of muscular contraction and filamentogenesis.

A procedure is described here for isolating native A-filaments and by lowering the ionic strength of the surrounding medium inducing these filaments to fray into their component subfilaments. I have found that rat and rabbit A-filaments fray into 3 subfilaments; scallop probably frays into 6 and Lethocerus into 4, and these results confirm previous predictions. In all cases the subfilaments appear to be arranged in a linear rather than in a coiled fashion within the cross-bridge region of the filament.

A method is also described for reconstructing A-filaments and A-bands from their solubilised components in situ. Complete reconstruction is inhibited at long sarcomere lengths and by Mg^{2+}ATP induced relaxation, by pre-calcium ion depletion and by pre-glycerolation.

A model for in situ A-filament assembly is proposed, derived from observations of A-filament reconstruction and from the information gained of A-filament structure.
Volume 2

TABLES AND FIGURES
## Table 1

**ESTIMATES BY VARIOUS WORKERS FOR "n", THE NUMBER OF MYOSIN MOLECULES PER 14.3nm REPEAT IN THE A-FILAMENT OF VERTEBRATE SKELETAL MUSCLE**

<table>
<thead>
<tr>
<th>METHOD</th>
<th>ESTIMATED VALUE FOR &quot;n&quot;</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inference from observed symmetry (E.M)</td>
<td>3</td>
<td>Maw and Rowe, 1980a and 1980b</td>
</tr>
<tr>
<td>Quantitative SDS-PAGE of myofibrils</td>
<td>3.8±0.5</td>
<td>Pepe and Drucker, 1979</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>Potter, 1974</td>
</tr>
<tr>
<td></td>
<td>2.7</td>
<td>Tregear and Squire, 1973</td>
</tr>
<tr>
<td></td>
<td>3.9</td>
<td>Morimoto and Harrington, 1974a</td>
</tr>
<tr>
<td>Mass per unit length determined by hydrodynamics</td>
<td>3.1±0.2</td>
<td>Emes and Rowe, 1978a</td>
</tr>
<tr>
<td>Mass per unit length determined by quantitative electron scattering</td>
<td>2.7±0.7</td>
<td>Lamvik, 1978</td>
</tr>
<tr>
<td></td>
<td>2.86±0.34</td>
<td>Reedy, Leonard, Freeman and Arad, 1981</td>
</tr>
<tr>
<td>Particle counting (E.M.)</td>
<td>4.3</td>
<td>Morimoto and Harrington, 1974a</td>
</tr>
<tr>
<td>Nucleotide binding to myofibrillar protein</td>
<td>3.5±0.2</td>
<td>Maruyama and Weber, 1972</td>
</tr>
<tr>
<td>Calculation from myosin content and density of A-filaments per unit volume of muscle</td>
<td>4</td>
<td>Huxley, 1960</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hanson and Huxley, 1957</td>
</tr>
<tr>
<td>Calculation from myosin and actin content of myofibril</td>
<td>4</td>
<td>Hasselbach and Schneider, 1951</td>
</tr>
<tr>
<td>Nucleotide binding to myosin filaments</td>
<td>2.7</td>
<td>Marston and Tregear, 1972</td>
</tr>
</tbody>
</table>
**Table 2**

**Solvent Composition and Its Effect on Fraying**

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<thead>
<tr>
<th>Composition of Relaxing Medium</th>
<th>First Grid Wash</th>
<th>Second Grid Wash</th>
<th>Third Grid Wash</th>
<th>Percentage of Frayed Filaments</th>
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</thead>
<tbody>
<tr>
<td><strong>Rat Filaments</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP relaxing medium</td>
<td>2-3 drops</td>
<td>4-5 drops</td>
<td>-</td>
<td>79%</td>
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<tr>
<td>0.1M KCl</td>
<td>H₂O</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;</td>
<td>2-3 drops</td>
<td>1-2 drops</td>
<td>-</td>
<td>0%</td>
</tr>
<tr>
<td>0.1M KCl</td>
<td>H₂O</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;</td>
<td>2-3 drops</td>
<td>-</td>
<td>-</td>
<td>0%</td>
</tr>
<tr>
<td>0.1M KCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot; minus KCl plus 0.2M Am.F.</td>
<td>2-3 drops</td>
<td>4-5 drops</td>
<td>-</td>
<td>1%</td>
</tr>
<tr>
<td>0.2M Am.F.</td>
<td>H₂O</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot; minus KCl plus 0.2M Am.F.</td>
<td>2-3 drops</td>
<td>1-2 drops</td>
<td>-</td>
<td>0.7%</td>
</tr>
<tr>
<td>0.2M Am.F.</td>
<td>H₂O</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot; minus KCl plus 0.2M Am.F.</td>
<td>2-3 drops</td>
<td>-</td>
<td>-</td>
<td>0%</td>
</tr>
<tr>
<td>0.2M Am.F.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Rabbit Filaments</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;</td>
<td>3 drops</td>
<td>5 drops</td>
<td>-</td>
<td>62%</td>
</tr>
<tr>
<td>0.1M KCl</td>
<td>H₂O</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;</td>
<td>3 drops</td>
<td>5 drops</td>
<td>4 drops</td>
<td>32%</td>
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<tr>
<td>0.1M KCl</td>
<td>H₂O</td>
<td>0.1M KCl</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Am.F. = Ammonium Formate
Figure 1
A schematic representation of the structure of vertebrate striated muscle showing:

a) a short segment of a single myofibril,
b) a longitudinal section of the protein filaments (so called "myofilaments") within a myofibril,
c) cross-sections of the protein filaments in different regions of the myofibril

d) an A-filament demonstrating the M-line, C-protein and cross-bridge repeats,
e) the packing of myosin molecules in the centre portion of an A-filament,
f) two representations of a myosin molecule showing subfragments and polypeptide chains
and g) a short segment of an I-filament.
Figure 2
The hexagonal filament lattice (top) showing the (1,0) and (1,1) axes. Below are two equatorial X-ray diffraction patterns from rabbit psoas muscle (sarcomere length 2.2 \mu m) showing the (1,0) and (1,1) reflections from the hexagonal filament lattice.

a) Live muscle.

b) Rigor (glycerolated) muscle.

Note the large change in the relative intensities of the two reflections.

Photographs by courtesy of H.E. Huxley.
(1,1) Axis
A- and I-Filaments

(1,0) Axis
A-Filaments only

a

b
**Figure 3**

X-ray diffraction patterns of living muscle at a resting sarcomere length. The longitudinal axis of the muscle is vertical. The diagonal line is an artifact, arising from the system of monochromators. The scales (reciprocals) show the spacing on the specimen that would give a first-order spectrum in the position indicated.

a) This pattern has been exposed so as to show the transverse spacing between thick filaments, i.e. the equatorial diffraction pattern.

b) Increased exposure shows longitudinal (meridional) spots arising from the myosin filaments, and horizontal (layer) lines from the cross-bridges, i.e. a so-called "meridional" diffraction pattern.

Photographs by courtesy of H.E.Huxley.
Figure 4
The arrangement of cross-bridges on the A-filament.
a) The 6:2 helical arrangement of cross-bridges in frog sartorius muscle originally deduced from low-angle X-ray patterns.
b) The 9:3 helical arrangement of cross-bridges.
Figure 5

Histograms of the length distribution (weight-averaged) of A-filaments from relaxed filament preparations of fresh rabbit psoas muscle.

a) Preparation not washed prior to homogenisation of myofibrils.

b) Preparation washed to eliminate broken filaments.

Note the reduction in the population of short filaments in the washed preparation.
71 filaments measured.
113 filaments measured.
Figure 6
Electron micrograph of a negatively stained, unwashed, relaxed filament preparation of fresh rabbit psoas muscle.
Note the number of broken filaments.
×27,000.

Figure 7 Electron micrograph of a negatively stained, washed, relaxed filament preparation of fresh rabbit psoas muscle.
Note the absence of broken filaments.
×27,000.
Figure 8a
A histogram of the A-filament lengths from a relaxed filament preparation of washed, glycerolated, rabbit psoas muscle.

Figure 8b Using the same population of A-filaments presented in Figure 8a, this histogram shows the A-filament length distribution after weight-averaging.
110 filaments measured.
Figures 9, 10 and 11
Electron micrographs of negatively stained "frayed" A-filaments prepared from rat psoas muscle.
Figures 9 (second and third row) and 11 include some small A-segments.

Figure 9. x50,000 and x90,000.

Figure 10. x73,600.

Figure 11. x58,600.
(Figure 9 previously published. Maw and Rowe, 1980a)
Figure 12
Electron micrographs of negatively stained "frayed" A-filaments prepared from fresh rabbit psoas muscle.
X80,300.
Figure 13

Histograms of (weight-averaged) length distributions of populations of
a) unfrayed rabbit A-filaments.
and b) frayed rat A-filaments.
73 filaments measured.
~1000 filaments measured.
Figure 14
Histograms of the widths (×68,700) of the bare-zones, avoiding the M-line region, of frayed (left) and unfrayed (right) rat A-filaments.
100 filaments measured.

```
<table>
<thead>
<tr>
<th>Filament Number</th>
<th>Total</th>
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<tbody>
<tr>
<td>1</td>
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</tr>
<tr>
<td>2</td>
<td>40</td>
</tr>
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<td>3</td>
<td>36</td>
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<td>4</td>
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<td>5</td>
<td>28</td>
</tr>
<tr>
<td>6</td>
<td>24</td>
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<table>
<thead>
<tr>
<th>Filament Number</th>
<th>Total</th>
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<tbody>
<tr>
<td>11</td>
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<td>12</td>
</tr>
<tr>
<td>13</td>
<td>8</td>
</tr>
</tbody>
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Figure 15
A histogram demonstrating the distribution of the total length of the unfrayed central region of frayed rat A-filaments. The vertical arrows show (from left to right) the distances corresponding respectively to the M-line, stripe 9, stripe 1 (terminology of Craig and Offer, 1976: see Figure 1) and the filament tip.
~1000 filaments measured.
Figure 16
Electron micrographs of negatively stained rabbit A-filaments showing reversal of fraying (b) on washing with 0.1M KCl after washing with distilled water to induce fraying (a).
x32,300.
Figure 17
Electron micrographs of negatively stained filaments resulting from incubation of rabbit A-filaments with 5mg/ml trypsin for 40 minutes at 20°C.
x97,000.

Figure 18 Electron micrographs of negatively stained filaments produced from incubation of 5mg/ml trypsin for 40 minutes at 20°C.
x97,000.
Electron micrographs of negatively stained rabbit A-filaments.

a) prior to incubation with trypsin,

and after incubation with 0.5mg/ml trypsin at 20°C for:

b) 2.5 minutes (arrows indicate decreased electron density in M-line region).

c) 5 minutes (arrows indicate loosening of structure in M-line region).

d) 10 minutes (arrows indicate reassociation of M-line region and dissociation of cross-bridge bearing regions).

e) 20 minutes (arrows indicate reassociation of subfilaments, association of filaments into A-segments, and feathery projections).

f) 40 minutes (note the tightly associated A-segments).

a) and b) x14,400.

c), d) and e) x98,400

f) x108,300.
Figure 20
Electron micrographs of negatively stained scallop (*Pecten maximus*) A-filaments after washing with distilled water. Arrows indicate six subfilaments.

$\times70,500$.

Figure 21 Electron micrographs of negatively stained frayed *Lethocerus indicus* A-filaments. The relaxed filament preparation was made from fresh muscle. Note the presence of four subfilaments.

$\times57,800$.

Figure 22 Electron micrographs of negatively stained *Lethocerus* A-filaments isolated from glycerolated muscle.

$\times114,800$. 
Figure 23
A diagram of the reconstruction assembly. A strip of rabbit psoas muscle (3–4 mm in diameter) is inserted into presoaked visking tubing. The sliding clamp allows the muscle to be stretched to the desired sarcomere length. Glass slides are clamped on either side of the tubing to create a tightly apposed membrane around the muscle. Finally the ends of the tubing and muscle are tied close to the glass slides and then released from the clamp.
The reconstruction procedure. Four muscle assemblies are required for each experiment. The control (C) is incubated in mammalian ringer throughout. Three assemblies are incubated in secondary phosphate solution for approximately 16 hours. One assembly then remains in phosphate (sample 1) whilst the remaining two (samples 2 and 3) are incubated in mammalian ringer for 1.5 hours. Finally, the membrane of sample 3 is slit and the assembly incubated in mammalian ringer plus 2mM ATP.
4 Muscle Assemblies

C

Mammalian ringer

CONTROL

1

Secondary phosphate

2

Mammalian ringer

3

$+2_{mM}ATP$
Sarcomere length determination. The striated nature of vertebrate skeletal muscle enables it to act as a diffraction grating when placed in the path of a laser beam. The distance between the refracted spots is inversely proportional to the sarcomere length.
\[ \lambda = \text{Wavelength of Laser} \]
\[ d = \text{Sarcomere Length} \]

\[ \frac{d}{l} = \frac{\lambda}{s} \]

\[ \lambda = 628\text{nm} \quad l = 5\text{cm} \]

\[ d(\mu) = 0.628 \mu \times 5 \times 10^4 \mu \]
\[ s \times 10^3 \mu \]

\text{e.g.} \quad \text{when} \quad d = 2.6\mu \quad s = 12.1\text{mm} \quad \text{and} \quad d = 3.6\mu \quad s = 8.7\text{mm} \]
Figure 26
A diagram of the perspex cell designed to hold a reconstruction assembly in X-ray diffraction experiments.
Figure 27

A diagramatic representation of the procedure followed during fixation, embedding and sectioning of muscle in reconstruction experiments.
Glutaraldehyde Fixation

LIGHT MICROSCOPY

ELECTRON MICROSCOPY

Osmium Fixation, Dehydration and Infiltration.

Surface Embedding

'S Blocks'

Trimming

Glued Re-embedded

Sections

Longitudinal Transverse
Figure 28

An electron micrograph of a thin, longitudinal section of fresh rabbit psoas muscle at a resting sarcomere length. The wide dense bands are the A-bands and between these are the less dense I-bands. Bisecting the I-bands are three dense stripes (not clearly defined in this micrograph), the Z-disc and two N-lines. The N-lines lie on either side of the more prominent Z-disc. The region between one Z-disc and the next in a myofibril is termed a sarcomere.

The A-bands show several striations. The dense central stripe is the M-line on either side of which is a narrow pale region, the pseudo H-zone. The M-line and pseudo H-zone correspond to the bare-zone of the A-filaments. The dense regions at the edges of the A-bands are the overlap zones, i.e. the regions where the A-filaments of the A-bands and the I-filaments of the I-bands overlap and interdigitate. The central, non-overlapping regions of the A-bands are the true H-zones.

Between the myofibrils at the levels of the Z-discs are cross-sections of membrane-bound vesicles. These form parts of the intercellular membrane networks, the "sarcoplasmic reticulum" and the "T-system". x16,300.
Figure 29
Electron micrographs of thin longitudinal sections of fresh rabbit psoas muscle at a resting sarcomere length during different stages of reconstruction (see Figure 24).
a) Sample C, the control.
b) Sample 1.
c) Sample 2.
d) Sample 3.
X19,000.

Figure 30 An electron micrograph of a thin longitudinal section of fresh rabbit psoas muscle at a shorter sarcomere length (approximately 2μm) showing a wavy appearance.
X19,000.

Figure 31 Electron micrographs of thin longitudinal sections of fresh rabbit psoas muscle after incubation in secondary phosphate solution (i.e. sample 1). Note the absence of A-bands.
a) Sarcomere length approximately 2.7μm (x7,600).
b) Sarcomere length approximately 2.2μm. The i-bands are touching and slightly overlapping (x12,000).

Figure 32 Electron micrographs of thin longitudinal sections of reconstructed fresh rabbit psoas muscle. Note the return of A-bands.
a) Sample 2.
b) and c) Sample 3.
X19,000.
Figure 33
A montage of sampled longitudinal sections demonstrating the solubilisation and reconstruction of A-bands in fresh muscle at a resting sarcomere length. From top to bottom, samples C to 3. x21,000.
Figure 34
Electron micrographs of thin transverse sections of untreated (sample C) fresh rabbit psoas muscle at a resting sarcomere length. Cross-sections of I-bands, overlap regions (insert of b) and H-zones (with small areas of M-line; insert of a) are visible.
a) x30,400 (insert x70,200).
b) x38,000 (insert x87,800).

Figure 35 Electron micrographs of thin transverse sections of phosphate treated (sample 1) fresh rabbit psoas muscle at a resting sarcomere length. Only cross-sections of I-filaments are present. The characteristic basket-weave pattern of the Z-disc (arrow) is present in b.
x38,000.

Figure 36 Electron micrographs of thin transverse sections of sample 2. The characteristic hexagonal pattern of the A-filaments has reappeared.
a) x38,000.
b) x57,000.

Figure 37 Electron micrographs of thin transverse sections of fresh rabbit psoas muscle at a resting sarcomere length.
a) Sample 3 (insert of M-line, x87,800).
b) Sample C.
Note the similarity between untreated and reconstructed muscle.
x38,000.
Figure 38

Light microscopy.

a) A thick (approximately 0.5μm) longitudinal section of fresh rabbit psoas muscle at a resting sarcomere length (x43).

b) A diagramatic representation of a thick section divided into six regions (A to F) through the muscle strip. The regions A and F form the periphery of the strip.

Figure 39 Light micrographs of thick longitudinal sections of fresh rabbit psoas muscle (sample C) at a resting sarcomere length. Top to bottom, regions A to F.

x1,500.

Figure 40 Light micrographs of thick longitudinal sections of phosphate treated (sample 1) fresh rabbit psoas muscle at a resting sarcomere length. Note the change in striation pattern due to the loss of A-bands.

x1,500.

Figure 41 Light micrographs of thick longitudinal sections of sample 2. Note the return of A-bands.

x1,500.

Figure 42 Light micrographs of thick longitudinal sections of sample 3.

x1,500.
Figure 43
A montage of thick longitudinal sections of the four stages of a reconstruction experiment viewed in the light microscope demonstrating the solubilisation and reconstruction of the A-bands.

x1.500.
X-RAY DIFFRACTION RESULTS

Figure 44 Equatorial X-ray diffraction patterns of rabbit psoas muscle (sarcomere lengths approximately 2.5µm).

a) Sample C.
b) Sample 1.
c) Sample 2.
d) Sample 3.

Note the disappearance of the (1,0) and (1,1) reflections in sample 1 and their reappearance in sample 2 and 3.

Figures 45 - 49 These photographs show channel counts from a position sensitive detector. The camera was aligned to give either equatorial or meridional reflections. The number of counts in each channel (represented by the height of each spot) indicate the intensities of the diffracted signals. The trough in the middle of each pattern corresponds to the backstop. These equatorial and meridional patterns are equivalent to densitometric scans along the equators of Figures 2a and b, 3a and 44 and along the meridian of Figure 3b respectively.
Figure 45
A photograph of the background trace with the camera aligned to give equatorial reflections.

Figure 46 The equatorial X-ray diffraction patterns of fresh rabbit psoas muscle at a resting sarcomere length during different stages of reconstruction. The traces were photographed at low magnification.

a) Sample C.
b) Sample 1.
c) Sample 2.
d) Sample 3.

Note the absence of the (1,0) and (1,1) reflections in sample 1 and their complete return in sample 3.

Figure 47 The equatorial X-ray diffraction patterns of fresh rabbit psoas muscle at a resting sarcomere length during different stages of reconstruction. With the exception of sample C these patterns were produced from a different experiment to that presented in Figure 46, and the traces were photographed at a higher vertical magnification.

a) Sample C.
b) Sample 1.
c) Sample 2.
d) Sample 3.
In Figure 48 and 49 the camera is aligned to give meridional reflections.

**Figure 48**

a) The background trace.
b) The meridional X-ray diffraction pattern of a rat tail collagen sample.
c) The meridional X-ray diffraction pattern of rigor frog muscle.

**Figure 49**
The meridional X-ray diffraction patterns of fresh rabbit psoas muscle at a resting sarcomere length during different stages of reconstruction. Sample C is not presented.
b) Sample 1.
c) Sample 2.
d) Sample 3.
Figure 50

A graph of the meridional X-ray diffraction data obtained from Figure 49. The curves were plotted by subtracting a smoothed background trace from the smoothed traces of Figure 49. The channels were calibrated from the known spacings of the collagen pattern.

Note the reduced 14.3\(^{-1}\)nm peak height of sample 1, the slight increase of sample 2 and the marked increase in sample 3.
Samples

- 3
- Frog
- 2
- 1

Centre of Primary Beam Stop =
Channel Number 42
Figure 51

Electron micrographs of negatively stained relaxed filament preparations of fresh rabbit psoas muscle.

a) Sample C.

b) Sample 3.

x27,000.
Figure 52
Histograms of the length distribution (weight-averaged) of A-filaments from relaxed filament preparations of fresh rabbit psoas muscle during different stages of reconstruction.

a) Sample C.
b) Sample 1.
c) Sample 2.
d) Sample 3.
73 filaments measured.

101 filaments measured.
52

176 filaments measured.

226 filaments measured.
Figure 53
Electron micrographs of negatively stained "frayed", reconstructed, rabbit psoas A-filaments.
x77,000.
Figure 54
Electron micrographs of thin longitudinal sections of fresh rabbit psoas muscle stretched to a sarcomere length of approximately 3.7μm at different stages of a reconstruction experiment.

a) Sample C.
b) Sample 1.
c) Sample 2.
d) Sample 3.

Note the absence of A-bands in samples 2 and 3 and the presence of short filaments projecting from the I-bands in (d).
x19,000.

Figure 55 Electron micrographs of thin sections of untreated, fresh, rabbit psoas muscle (sample C) stretched to a sarcomere length of 3.7μm.

a) Longitudinal section. x19,000.
b) Transverse section. x47,500.

Note the absence of A-/I-filament overlap regions in (b).

Figure 56 Electron micrographs of thin sections of fresh, rabbit psoas muscle stretched to a sarcomere length of 3.7μm after attempted reconstruction (sample 3).

a) Longitudinal section. x19,000.
b) Transverse section. x19,000.

Note the presence of dense filament cross-sections at the regions where the edges of the I-bands are sectioned in (b).
X-RAY DIFFRACTION RESULTS

See explanation preceding Figure 45.

Figure 57 The equatorial X-ray diffraction patterns of fresh rabbit psoas muscle stretched to a sarcomere length of approximately 3.7μm during different stages of reconstruction. The background trace is presented in Figure 45.

a) Sample C.
b) Sample 1.
c) Sample 2.
d) Sample 3.

Figure 58 The meridional X-ray diffraction patterns of fresh rabbit psoas muscle stretched to a sarcomere length of approximately 3.7μm during different stages of reconstruction.

a) Background.
b) Sample 1.
c) Sample 2.
d) Sample 3.
Figure 59
A graph of the meridional X-ray diffraction data obtained from Figure 58. The curves were obtained by subtracting a smoothed background trace from the smoothed traces of the samples.
Note the comparative peak heights of samples 1, 3 and sample 3 of Figure 50.
14.3 nm\(^{-1}\)

Samples

- 3 (of Fig. 50)
- 3
- 2
- 1

Relative Counts per Channel

Channel Number

Centre of Primary Beam Stop =

Channel Number 42
Figure 60
Histograms of length distributions (weight-averaged) of A-filaments from relaxed filament preparations of fresh rabbit psoas muscle during a reconstruction experiment after stretching the muscle strips to sarcomere lengths of approximately 3.7μm.

a) Sample C.
b) Sample 1.
c) Sample 2.
d) Sample 3.
109 filaments measured.

113 filaments measured.
104 filaments measured.

81 filaments measured.
Figure 61

Electron micrographs of thin longitudinal sections of rabbit psoas muscle at a resting sarcomere length during different stages of attempted reconstruction after glycerolation.

a) Sample C.
b) Sample 1.
c) Sample 2.
d) Sample 3.

Note the absence of A-bands and short filaments in samples 2 and 3.

x12,000.

Figure 62

Electron micrographs of thin longitudinal sections of pre-glycerolated rabbit psoas muscle after a further attempt at reconstruction.

a) Sample 2.
b) Sample 3.

x19,000.
Figure 63

Histograms of length distributions (weight-averaged) of A-filaments from relaxed filament preparations of pre-glycerolated rabbit psoas muscle during different stages of reconstruction.

a) Sample C.
b) Sample 1.
c) Sample 2.
d) Sample 3.
123 filaments measured.

77 filaments measured.
c) 133 filaments measured.

Total Filament Mass

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<thead>
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<th>Mass (μm)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
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<td>4</td>
</tr>
<tr>
<td>0.6</td>
<td>6</td>
</tr>
<tr>
<td>0.8</td>
<td>8</td>
</tr>
<tr>
<td>1.0</td>
<td>10</td>
</tr>
<tr>
<td>1.2</td>
<td>8</td>
</tr>
<tr>
<td>1.4</td>
<td>6</td>
</tr>
<tr>
<td>1.6</td>
<td>4</td>
</tr>
<tr>
<td>1.8</td>
<td>2</td>
</tr>
</tbody>
</table>

---

d) 137 filaments measured.

Total Filament Mass

<table>
<thead>
<tr>
<th>Mass (μm)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>2</td>
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<tr>
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</tr>
<tr>
<td>0.6</td>
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<td>1.0</td>
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<tr>
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<td>1.6</td>
<td>4</td>
</tr>
<tr>
<td>1.8</td>
<td>2</td>
</tr>
</tbody>
</table>
Figure 64

Electron micrographs of thin longitudinal sections of rabbit psoas muscle during different stages of attempted reconstruction after glycerolation and calcium ion depletion.

a) Sample C.
b) Sample 1.
c) Sample 2.
d) Sample 3.

x12,000.
Figure 65

Histograms of length distributions (weight-averaged) of A-filaments from relaxed filament preparations of pre-glycerolated and pre-calcium ion depleted rabbit psoas muscle during different stages of reconstruction.

a) Sample C.
b) Sample 1.
c) Sample 2.
d) Sample 3.
69 filaments measured.

92 filaments measured.
139 filaments measured. c)

115 filaments measured.

d)
Figure 66
Electron micrographs of thin longitudinal sections of rabbit psoas muscle pre-depleted of calcium ions, during different stages of reconstruction in the absence of calcium ions.

a) Sample C.
b) Sample 1.
c) Sample 2.
d) Sample 3.
x19,000.

Figure 67 Electron micrographs of thin longitudinal sections of rabbit psoas muscle pre-depleted of calcium ions, after reconstruction in the presence of calcium ions.

a), b) and c) Sample 2.
d) Sample 3.
x19,000.
Figure 68
Electron micrographs of thin longitudinal sections of rabbit psoas muscle at a resting sarcomere length after incubation in secondary phosphate solution (sample 1).
Initial state of muscle:-
 a) Fresh muscle (i.e. in a contractile state).
 b) Muscle in rigor.
 c) Fresh muscle but with addition of 10mM magnesium ions to the phosphate solution.
 d) Relaxed muscle (muscle relaxed from rigor by the addition of 2mM ATP and 10mM Mg$^{2+}$).
Note the increased density at the edges of the I-bands in (b).
X19,000.

Figure 69 Electron micrographs of thin longitudinal sections of rabbit psoas muscle at a resting sarcomere length after reconstruction.
Initial state of muscle:-
 a) Fresh muscle (i.e. in a contractile state).
 b) Rigor muscle.
 c) Relaxed muscle (as in Figure 68d).
Note the less regular reconstruction of A-bands in (b) and the absence of reconstruction in (c).
X19,000.
Figure 70

Electron micrographs of thin longitudinal sections of rabbit psoas muscle during an anomalous reconstruction experiment.

a) Sample 1 (fresh muscle).
b) Sample 2 (fresh muscle).
c) Sample 3 (pre-calcium ion depleted muscle).
d) Sample 2 (pre-glycerolated muscle).

Note the presence of partially solubilised A-bands in (a).
x19,000.
Figure 71
Electron micrographs of thin sections of phosphate treated (sample 1) fresh rabbit psoas muscle.

a) Longitudinal section.
b) Transverse section.

Note the presence of residual M-line structure (arrows).

$\times 19,000$.

Figure 72
Electron micrographs of thin longitudinal sections of intermediate stages of reconstruction observed in fresh rabbit psoas muscle at a resting sarcomere length.

a) Sample 2.
b) Sample 3.
c) Sample 3.

Note the apparent growth of A-bands from the M-line (arrows).

$\times 19,000$. 
Figure 73
Electron micrographs of thin longitudinal sections of intermediate stages of reconstruction of rabbit psoas muscle.
a) Sample 3 of fresh muscle previously stretched to a long sarcomere length.
b) Sample 2 of fresh muscle at a resting sarcomere length.
c) Sample 3 of rigor muscle at a resting sarcomere length.

Note in these cases the apparent growth of A-bands from the edges of the I-bands, and adjacent A-band regions with and without M-line (arrows).

x19,000.
Figure 74

Electron micrographs of thin sections of fresh rat psoas muscle. Muscle samples and blocks prepared by Anne Clode of Leicester University.

a) Longitudinal section of Sample C.

b), d), g) and h) Longitudinal sections of Sample 3.

c), e), f) and i) Transverse sections of Sample 3.

Note the intermediate stages of reconstruction in longitudinal sections and the appearance of corresponding stages in transverse sections, i.e. b) and c), d) and e) or f), g) or h) and i).

All x19,000 except (i), x38,000.
Figure 75
A schematic representation of a model for vertebrate A-filament reconstruction.
During phosphate treatment one third of the solubilised myosin is bound to I-filaments.

On return to a physiological ionic strength medium the bound myosin assembles into filament fragments.

The A-filament fragments are released from the I-filaments and half of the remaining solubilised myosin becomes attached.

The newly attached myosin assembles with the A-filament fragments.

The lengthened fragments are released from the I-filaments and moved out of the I-bands.

The remaining solubilised myosin attaches to the I-filaments. Meanwhile an M-line is assembled.

Assembly of the half A-filaments is completed.

The half A-filaments slot into the M-line to form complete A-filaments and, therefore, A-bands.
Figure 76
Electron micrographs of thick (approximately 0.15µm) longitudinal sections of fresh rabbit psoas muscle.

a) Sample C.
b) Sample 3.
x 19,000.

Under optimum conditions, as judged by transmission electron microscopy, reconstruction of A-filaments and A-bands is complete and the structures appear similar to native. Examination of these sections in a scanning transmission electron microscope (S.T.E.M.) would help to determine the order of packing of the A-filaments in the A-bands after reconstruction.