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SEPARATION OF MUSCLE PROTEINS

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CONTENTS

1. Introduction 225
2. Preparation of the material for analysis. Separation and isolation of muscle fibres, myofibrils, myofilaments and muscle particles 226
3. Quantification of muscle proteins 227
4. Fractionation of myofibrillar proteins 228
   4.1. Myosin 230
      4.1.1. Isolation and purification of myosin 230
      4.1.2. Chromatography of myosin 231
      4.1.3. Electrophoresis of myosin 232
      4.1.4. Separation of isoenzymes of myosin 233
      4.1.5. Isolation and separation of proteolytic fragments of myosin 234
      4.1.6. Isolation and fractionation of light chains of myosin 235
      4.1.7. Separation of "marker amino acids" (methyl analogues of histidine, lysine and arginine) in contractile proteins 236
   4.2. Isolation of actomyosin 237
   4.3. Isolation and separation of actin 237
   4.4. Isolation and separation of tropomyosin 238
   4.5. Isolation and separation of the troponin complex 239
   4.6. Minor proteins 241
      4.6.1. M-Protein 241
      4.6.2. Z-Protein 241
      4.6.3. Actinins 241
      4.6.4. C-Protein 242
      4.6.5. Paramyosin 242
5. Conclusions 243
6. Summary 243
References 244

1. INTRODUCTION

The muscle is a specialized tissue, the fibre of which is composed of four fundamental constituents: the sarcolemna, which is the equivalent of the cell membrane; the fibrils, which represent the structural elements for contraction; the sarcosomes (muscle mitochondria), which ensure the supply of energy for muscle contraction; and the sarcoplasm, which contains a variety of soluble proteins and enzymes. Muscle fibrils, the smallest integral contractile unit of the muscle, are composed of thick and thin filaments and their interaction and their reaction with ATP produces the mechanical energy necessary for the shortening of the muscle fibre. The thick filaments

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of the muscle fibre are made up of myosin and C-protein and the thin filaments consist of a highly organized complex of structural (actin) and regulatory (tropomyosin and troponin complex) components. There are also minor proteins in the muscle such as α-actinin, M-protein and Z-protein, the function of which is less clear. Sarcoplasmic proteins form a mixture of proteins which are similar to those present in other cell types and mainly include glycolytic enzymes, myoglobin, myoalbumin and others.

This review deals with the techniques used for the fractionation of mixtures of muscle proteins, for the isolation, quantification and purification of individual contractile proteins, isoenzymic forms of contractile proteins and their proteolytic and other fragments and for the analysis of specific amino acids contained in contractile proteins. Emphasis is placed on skeletal and cardiac muscle. An attempt is also be made to show briefly which problems of muscle biochemistry and physiology have made the greatest progress by using more and more advanced techniques for muscle protein separation.

In recent years, research on contractile proteins has produced a large number of publications, but no review dealing with this subject has as yet been published*.

2. PREPARATION OF THE MATERIAL FOR ANALYSIS. SEPARATION AND ISOLATION OF MUSCLE FIBRES, MYOFIBRILS, MYOFILAMENTS AND MUSCLE PARTICLES

The separation of the constituents of muscle is necessary for various physiological and biochemical studies. Skeletal muscles are composed of a mixture of fibre types. Although the fibre types have been classified according to various terminologies, classification into red and white muscle fibre is frequently used, because it is based on the content of myoglobin and this property is related to other metabolic and contractile properties of the muscle fibre.

The method for the separation of red and white muscle fibres was elaborated by Mowafy *et al.*[3]. Thin cut muscle was exposed to collagenase and the connective tissue was removed mechanically. The sample was then stirred in a Waring blender and individual fibres were thus separated. Red and white fibres were then isolated by centrifugation in a sucrose density gradient.

The method for isolating myofibrils from the skeletal and cardiac muscle was elaborated by Perry[4,5]. Minced muscle is homogenized in a Waring blender (0.08 *M* borate, pH 7.1). From the suspension, myofibrils are obtained by repeated washing and mild centrifugation. Kohn[6] described a method for the isolation of purified myofibrils. Fibre bundles were teased apart with a needle, 0.5% Triton X-100 was added and the tissue was minced with scissors and maintained for 30 min at 25°C. After treatment with collagenase, repeated centrifugation and washing, about 5 mg of myofibrillar protein were obtained per gram of muscle. A very efficient method for the preparation of myofibrils was elaborated by Harbitz *et al.*[7]. This method is based on density gradient centrifugation. From 100 g of minced muscle, 1 g of myofibrils can be obtained.

* During the preparation of this manuscript, a book has been published[1] that deals with general techniques and preparative procedures for isolating muscle proteins, without emphasis on chromatographic and electrophoretic approaches. A brief survey of electrophoretic techniques applicable to contractile and cytoskeletal proteins has also been published[2].

For compositional studies, it is necessary to isolate thick filaments from various muscles. Morimoto and Harrington[8] developed a method for the isolation of thick filaments from rabbit skeletal muscle. Muscles were homogenized in 0.1 *M* KCl with Mg^{2+}, EDTA and dithiothreitol. Myofibrils were isolated and treated with a relaxing medium (0.1 *M* KCl, 10 m*M* $MgCl_2$, 1 m*M* EGTA, 5 m*M* ATP, 7 m*M* potassium phosphate, pH 7.0). Thick and thin filaments were then separated by zone sedimentation in a gradient of glycerol. Obinata *et al.*[9] described a method for the isolation of native thin filaments from embryonic muscle cells.

From subcellular muscle organelles, it is the isolation of ribosomes that is important for studying muscle protein synthesis. Low *et al.*[10] described a method for isolating and fractionating polyribosomes. Cytoplasmic extracts of embryonic chick skeletal muscle were centrifuged through a sucrose gradient and polyribosomes were separated into four size classes, containing from 4 to 80 ribosomes. Zak *et al.*[11] developed a procedure for preparing six subcellular components from a single homogenate of skeletal or cardiac muscles. Emphasis was placed on the retention of both the structural integrity and the biochemical purity of all the isolated organelles. Minced-relaxed muscle (0.1 *M* KCl, 5 m*M* $MgCl_2$, 5 m*M* EGTA, 5 m*M* sodium pyrophosphate, pH 6.8) was homogenized and subsequently fractionated by differential centrifugation. This procedure permits the isolation and purification of the following subcellular organelles: nuclei, ribosomes, myofibrils, mitochondria and rough and smooth surfaced endoplasmic reticulum.

3. QUANTIFICATION OF MUSCLE PROTEINS

There are no major problems in determining the amount of isolated muscle proteins. Proteins can be determined by any analytical method with Folin–Ciocalteu reagent, biuret reagent, the micro-Kjeldahl method or ultraviolet absorption measurement of the protein. It should be mentioned that the UV spectrum (280 nm) of myosin is influenced by nucleotide contamination and that the A_{280nm}/A_{260nm} ratio can serve as a test for the contamination of myosin by nucleotides. For pure myosin preparations this ratio should be over 1.5 (ref. 12).

The determination of the amount of individual proteins after gel electrophoresis is difficult. Densitometric protein quantification cannot be performed if proteins are present in high and low concentrations at the same time. In addition, the proteins vary in their capacity for binding dyes, which is frequently neglected. Coomassie Blue staining is most commonly used. Alternatively a "silver" ($AgNO_3$) solution can be used for protein band staining[13]. This technique is laborious, but more sensitive than the former. Whereas the amount of protein required for detection in the electrophoretic gel is about 1 μg after staining with Coomassie Blue, even 40 ng of protein can be detected with the silver staining. It was shown by Salviati *et al.*[14] that some muscle proteins are relatively more intensely stained with Coomassie Blue than with the "silver" method, whereas for others the relation is the reverse.

Because considerable amounts of tissue are required for the conventional biochemical characterization of contractile proteins in the muscle, very little is known about the constituent contractile proteins during myogenesis. Recently, a solid-phase radioimmunoassay method for quantifying tropomyosin and myosin in embryonic heart homogenates was described[15,16]. The method consists of (1) the production of

a specific antibody to a pure antigen, (2) labelling of the antigen with an appropriate radioisotope that does not alter its antigenicity, (3) saturation of the purified antibody with the labelled antigen, (4) displacement of the bound labelled antigen by the competing antigen in tissue extracts and (5) quantification of the amount of the antigen in these extracts by construction of calibration graphs obtained by adding known amounts of the pure antigen to the assay system.

It is often desirable to know the content of contractile proteins and sarcoplasmic proteins in a muscle. For this purpose the method developed by Helander can be used[17]. Minced muscle is extracted repeatedly with 0.03 *M* potassium phosphate: in this fraction, sarcoplasmic proteins appear, myofibrillar proteins are extracted next with 0.1 *M* potassium phosphate + 1 *M* potassium iodide and the remaining material represents NPN and stroma.

4. FRACTIONATION OF MYOFIBRILLAR PROTEINS

When studying the biochemistry of muscle, it is either necessary to isolate individual muscle proteins or to fractionate the mixture of contractile proteins in order to obtain analytical data. From technical reasons it is not easy to isolate more than two or three individual contractile proteins on a preparative scale at the same time.

Pioneer studies on the fractionation of muscle proteins were performed with the use of paper electrophoresis. Mariani and Toschi[18] separated myosin and actomyosin using analytical paper electrophoresis. Toschi and Boccacci[19] fractionated muscle extracts with the use of paper electrophoresis and localized adenosine triphosphatase by means of colour development in the presence of ammonium molybdate. Toschi and Bettini[20] employed paper electrophoresis to obtain a correlation between the type of muscle and the protein composition. Toschi and Bettini[21] compared insect leg and wing muscles of five different species. Muscle homogenates were extracted with low and high ionic strength solution and sarcoplasmic and fibrillar proteins were fractionated in these extracts with the use of paper electrophoresis.

Today, for the analytical fractionation of myofibrillar proteins, electrophoresis on polyacrylamide gel is widely used. The mixture of contractile proteins extracted either from a pure preparation of myofibrils or from muscle homogenates devoid of sarcoplasmic proteins by repeated washing with a dilute phosphate buffer (0.05 *M* phosphate, pH 6.8), or another low ionic strength solution, is fractionated using sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (Fig. 1). SDS–polyacrylamide gel electrophoresis can be carried out in 0.1% SDS–100 mM sodium phosphate buffer (pH 7.5)–12% acrylamide according to Weber and Osborn[22], and this method makes it possible to separate myosin heavy chains, actin, tropomyosin, troponin T, I and C and five light chains of myosin. This standard method was modified by Porzio and Pearson[23] to give more effective separations and improved the resolution of myofibrillar proteins. The gel consists of 10% acrylamide incorporating 400 m*M* Tris–glycine (pH 8.8), 0.1 m*M* EDTA, 5% glycerol and 0.1% SDS. With this technique both the heavy chains of myosin and the smaller myofibrillar proteins were resolved as a narrow symmetrical band. Fifteen bands of proteins were separated (molecular mass in the range 300,000–15,000). The pattern of proteins consisted of contractile proteins listed above and, in addition, it contained two M-

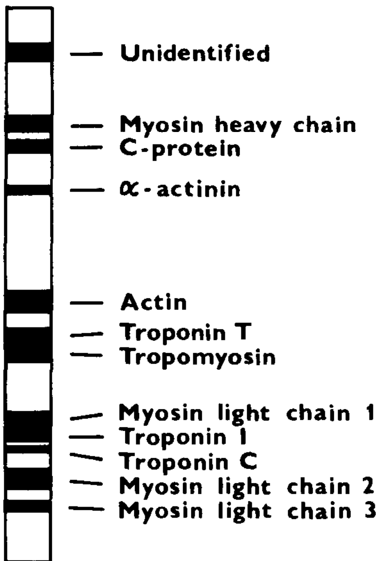

Fig. 1. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis pattern of myofibrillar proteins.

line proteins, C-protein, α-actinin and two unidentified bands.

The higher resolution of contractile proteins of similar molecular mass can be obtained using two-dimensional electrophoresis as elaborated by O'Farrell[24]. Proteins are separated on polyacrylamide gel according to the isoelectric point by isoelectric focusing in the first dimension and according to molecular mass by SDS electrophoresis in the second dimension. This technique has resolved 1100 different components from *Escherichia coli* and, according to the author, it should be capable of resolving a maximum of 5000 proteins. Proteins present in low concentrations and not revealed by staining can be detected and quantified by autoradiography (proteins which constitute 10^{-4}–10^{-5}% of the total proteins). This two-dimensional separation technique is frequently used in the fractionation of contractile proteins and it allows the fractionation of isoforms of individual contractile proteins from samples containing a mixture of muscle fibres, which cannot be separated by one-dimensional techniques.

It is frequently necessary to analyse contractile proteins from single muscle fibres. This can be achieved by SDS–polyacrylamide gel electrophoresis on the micro-scale[25] or by capillary (1 mm I.D.) SDS–polyacrylamide gel electrophoresis[26].

Semi-quantitative detection of individual contractile proteins can also be performed by using the fluorescent antibody technique with antibodies against each structural protein[27].

Studies concerning myofibrillar protein patterns are very useful in determining the composition of proteins in different muscles and under various physiological and pathological conditions such as development and muscle disease, in determining the molecular mass and homogeneity of individual proteins and in following the purity of myofibrillar samples and their contamination with proteolytic or degradative products.

4.1. *Myosin*

4.1.1. *Isolation and purification of myosin*

The procedures for the isolation of myosin are based on the solubility of myosin at high ionic strength and its insolubility at low ionic strength. A widely used method is that of Perry[28]. The preparation is performed at low temperature (2–4°C) and contamination by metal ions must be avoided. Minced muscle is extracted with a solution containing 0.3 *M* KCl, 0.1 *M* KH_2PO_4 and 0.05 *M* K_2HPO_4. By adding 14 volumes of water, myosin is precipitated, then dissolved in a solution of KCl to obtain a concentration of 0.5 *M* KCl. Actomyosin is precipitated at pH 6.6 at 0.3 *M* KCl and removed by centrifugation. After this myosin, is reprecipitated several times by dilution to ionic strength 0.04 and dissolved at ionic strength 0.5.

There are many methods for isolating and purifying myosin, but most include only minor modifications of the standard method. The method used depends on the amount of muscle tissue available and also on the desired purity of the myosin preparation. It is advisable to wash out soluble (sarcoplasmic) proteins from the minced muscle with a dilute phosphate buffer before the extraction of myosin. Because myosin, especially from very young animals or muscles of lower vertebrates, is easily oxidized[29], the isolation should be performed in the presence of 1 m*M* dithiothreitol or 2 m*M* β-mercaptoethanol; 1 m*M* EDTA is frequently added to all solutions to remove heavy metals and enhance the enzymatic activity of myosin[30].

Additional purification of myosin can be achieved by fractionation with ammonium sulphate. Myosin is precipitated in the range of 36–50% saturation[30], but this range is influenced by the actual concentration of myosin and is also not the same in different types of muscles and muscles from various animals. Further purification is achieved by column chromatography (see below). Luchi *et al.*[31] used ammonium sulphate fractionation in the presence of 2.0 *M* LiCl. To remove ribonucleoproteins, the myosin solution is stirred for 2 h at pH 7.6 with DEAE-Sephadex A-50 (ref. 32).

A comparison of five different methods of preparing cardiac myosin was performed[33] and, surprisingly, the method of preparation did not affect the Ca^{2+}-activated ATPase activity of myosin. Shiverick *et al.*[12] described a method for the preparation of highly purified myosin from small amounts of cardiac muscle based on extraction and precipitation by dilution. The content of RNA in the final preparation was 0.03 mg/g of myosin only. The protocol also showed which step is necessary for removing actin contamination (centrifugation of myosin solution in 0.3 *M* KCl, pH 6.8, at 43,000 *g*).

The above methods are used for the preparation of myosin from mammalian skeletal and cardiac muscles and it should be understood that they cannot simply be applied to preparations of myosin from muscles of lower vertebrates or invertebrates. The following references give brief lists of the methods used for the isolation of myosin from muscles of other animals than mammals: fish skeletal muscle myosin[34–36], frog skeletal muscle myosin[37–40] and insect muscle myosin[41–43].

The purity of the myosin preparation is established according to the following criteria: (a) Mg^{2+} ATPase activity at low ionic strength should be very low (it is proportional to actin contamination), (b) the purity on SDS gel electrophoresis and (c) UV spectrum (280–282/269–270 nm ratio); if this ratio is 1.0 or less the myosin

is heavily contaminated by nucleotides, whereas in good preparations this ratio is above 1.4.

4.1.2. Chromatography of myosin

Chromatographic techniques are frequently used for the preparation of myosin as the last step, in order to achieve higher purity. However, the use of chromatography for this purpose has several disadvantages. Eluted myosin has to be concentrated, which together with the chromatographic procedure prolongs the preparation of myosin. In some instances, chromatography is performed at mildly alkaline pH (8.5), but not all myosins are stable under these conditions.

Myosin has been purified[44] on DEAE-cellulose in 0.25 *M* KCl + 5 m*M* Tris–HCl (pH 7.5). The specific ATPase activity was higher than that of the starting material. Harris and Suelter[45] purified myosin on a column of cellulose phosphate and subsequently on a column of DEAE-cellulose. The myosin thus obtained was devoid of AMP deaminase and nucleic acids, which cannot be removed by conventional techniques. The heterogeneity of the myosin preparation was investigated during chromatography on DEAE-cellulose[46]. Myosin was eluted in three fractions (KCl gradient) and only one fraction did not contain polyribonucleotides. This fraction, however, had a lower ATPase activity than that with the ribonucleotide but no explanation was given. Trayer and Perry[47] purified myosin on DEAE-cellulose (KCl gradient, pH 8.2.). By this procedure, nucleoprotein contamination, which is much higher in myosin prepared from embryonic and new-born muscle than from adult muscle, could be completely removed. The ATPase activity of myosin purified by chromatography was 200–400% greater than that of the preparation before chromatography.

DEAE-Sephadex A-50 chromatography with a gradient of KCl was used to separate monomeric myosin from aggregated myosin and other contaminations[48]. DEAE-Sephadex chromatography (KCl gradient) was also used in order to eliminate ammonium sulphate fractionation. The study[49] showed that DEAE-Sephadex chromatography removes contaminant proteins in the range 90,000–150,000. It was also shown in the same paper that the chromatography of myosin on Sepharose 2B is useful for removing actin from myosin preparations. For the separation of the myosin monomer from aggregated myosin and low-molecular-mass contamitants, chromatography on 4 or 2% agarose was used[50].

Chromatography of myosin preparations on hydroxyapatite was employed for removing protein contamination (Fig. 2)[51–53]. Fractionation was performed in 0.4 *M* KCl and a gradient of phosphate (0.16–0.4 *M*). Chromatography of native myosin from nematode muscles on hydroxyapatite[54] permitted the separation of myosin into fractions containing one or the other myosin heavy chain.

Affinity chromatography of myosin was shown to be a very useful tool for the rapid purification and characterization of myosin from small amounts of muscle tissue. Trayer and Trayer[55] prepared columns with the immobilized ADP derivative N^6-(-aminohexyl)-ADP or 8-(6-aminohexyl)amino-ADP coupled to CNBr-activated Sepharose-4B. With the use of this column chromatography, myosin was isolated directly from the muscle extract in one step.

Affinity chromatography of myosin based on the trapping of actin filaments within agarose gel beads or Sepharose 4B has also been used[56]. Bound protein was

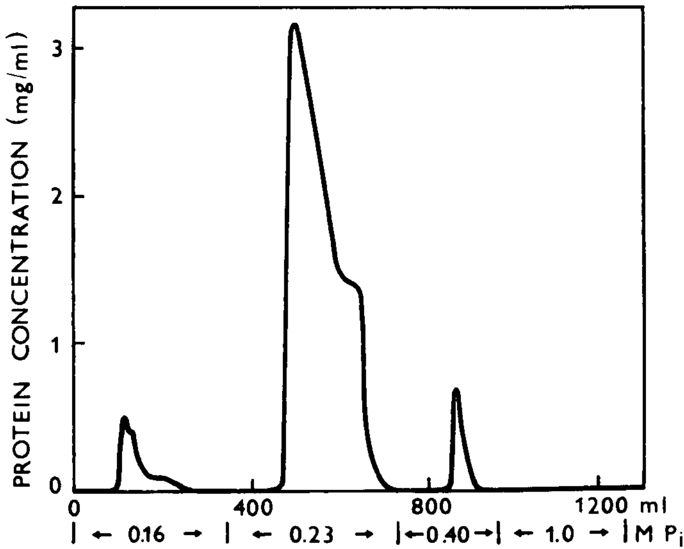

Fig. 2. Chromatography of myosin on a hydroxyapatite column with a four-step elution programme. The first three buffers contained KCl at a concentration of 0.4 *M*. First peak, contaminating proteins; second peak, myosin; third peak, aggregated myosin. Adapted from ref. 51, with permission.

eluted with 10^{-3} *M* PP_i. With this technique, myosin is also obtained from a crude muscle extract in a single step.

Affinity chromatography was used for the purification of myosin variants from heart ventricles of the rabbit. The affinity columns employed for purifying two different myosin variants were prepared by coupling antibody to CNBr-activated Sepharose-4B. The fraction of myosin not retained by the column was washed out with a column buffer and the bound fraction was eluted with 4 *M* guanidine hydrochloride.

4.1.3. Electrophoresis of myosin

Although electrophoresis is one of the most powerful techniques now available for protein analysis, electrophoresis and especially disc electrophoresis have only limited value when studying the whole native myosin molecule, owing to the large size of this protein. Additional problems are created by the low solubility of myosin in the low salt concentrations required for electrophoresis.

These difficulties can only partially be overcome by using dilute polyacrylamide gels. Deyl *et al.*[57] described the separation of a myosin monomer, dimer and three polymers on a 5% gel of acrylamide. The heterogeneity of large myosin subunits was shown on electrophoresis in dilute polyacrylamide gels containing 9 *M* urea[58]. Isoelectric focusing of myosin in dilute polyacrylamide gels was used for the fractionation of large and small subunits of myosin[59]. Nevertheless, classical electrophoretic techniques have never become routine for analysing myosin samples. Extremely important information on the isoenzymes of myosin and subunits of myosin was, however, obtained by applying electrophoresis in the presence of ATP or pyrophosphate and by using SDS electrophoresis (see subsequent sections).

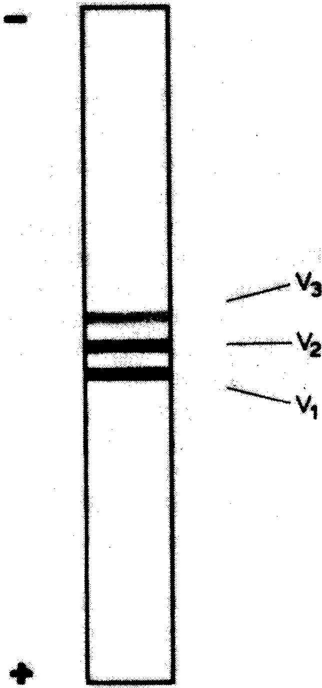

Fig. 3. Schematic representation of gel electrophoresis of native ventricular myosin under non-denaturating conditions. Myosin was prepared from adult rat. Three isozymes are referred as V_1, V_2 and V_3.

4.1.4. Separation of isoenzymes of myosin

Chromatographic and electrophoretic techniques have become exceptionally efficient in demonstrating the existence and in permitting the separation of isoenzymes of myosin and large molecular fragments of these isoenzymes and changes in the proportion of isoenzymes under various physiological and pathophysiological conditions.

D'Albis and co-workers[60–62] succeeded in separating native myosin isoenzymes from skeletal and cardiac muscle on pyrophosphate–acrylamide gel (0.175 *M* KCl, 0.0875 *M* tetrasodium pyrophosphate, pH 8.5, 2.8% gel, electrophoresis for about 48 h in a cold room (Fig. 3). A very similar technique for the separation of three isozymes of myosin was used by Hoh and co-workers[63,64] (20 m*M* tetrasodium pyrophosphate, 10% glycerol, 3% gel). The separated isoenzymes can be characterized by a second electrophoresis after their proteolytic splitting[60].

Isoenzymes of myosin or enzymatically active subfragments of these isoenzymes have also been fractionated by chromatographic techniques. Yagi and Otani[65] separated subfragment-1 of myosin by ion-exchange chromatography (DEAE-cellulose). Two protein components were separated by means of one-step elution using 0.08 *M* KCl–20 m*M* TES buffer (pH 7.5). Weeds and Taylor[66] used chromatography on DEAE-cellulose with a sodium chloride gradient for the same purpose.

The separation of heavy meromyosin isoenzymes was also achieved by affinity chromatography using Sepharose-4B coupled with 6-aminohexyl pyrophosphate

(gradient of sodium pyrophosphate)[67,68] or Sepharose-4B coupled with ADP derivative (Sepharose-4B–adipic acid hydrazide–ADP column, pyrophosphate gradient)[69,70].

Holt and Lowey[71] elaborated an immunochemical method which they used for the fractionation of skeletal muscle myosin and its subfragments into two isoenzymes. This method has the advantage that it makes it possible to obtain isoenzymes on a preparative scale. Antibodies specific for the "difference peptide", which is unique for the alkali 1 light chain of myosin, have been isolated from the antiserum against alkali 1 light chain and coupled to Sepharose-4B to form an immunoadsorbent specific for alkali 1 light chain. When subfragment-1, heavy meromyosin or myosin were passed through this column, one fraction was eluted without retardation, while the other fraction could be eluted with guanidine hydrochloride. The same technique was used with an antibody specific for the alkali 2 light chain[72].

Affinity chromatography has also been exploited for the fractionation of ventricular isomyosins[73]. Ventricular myosin applied to a column with insolubilized anti-beef atrial antibody was fractionated into two isoenzymic forms. Further, affinity chromatography has also been used for isolation of two molecular variants of myosin heavy chain from the rabbit heart (Sepharose-4B with bound antimyosin antibodies)[74].

In addition to the immunochemical technique, all other techniques for fractionating myosin isoenzymes have practically analytical value only and the limited amount of material obtained can be utilized only for techniques such as "peptide mapping" after proteolytic digestion of individual isoforms.

4.1.5. Isolation and separation of proteolytic fragments of myosin

When the structure and function of myosin are to be elucidated, various high- and low-molecular-weight fragments of myosin, arising mostly by splitting the myosin molecule by proteolytic enzymes, have to be isolated and fractionated. The following fragments of myosin can be isolated after proteolytic digestion of myosin under appropriate conditions: heavy meromyosin, subfragment-1, subfragment-2, light meromyosin, the rod of the myosin molecule (these fragments can be obtained owing to their protease-sensitive regions) and peptides, which are obtained mainly from the heavy chain part of the myosin molecule either by prolonged digestion with various proteases or by splitting with cyanogen bromide.

By digestion with trypsin (myosin:enzyme = 100:1) two major fragments of myosin (heavy meromyosin and light meromyosin) can be obtained[75]. This digestion may be arrested with soya-bean trypsin inhibitor. After dialysis of the digest against 10 volumes of water, light meromyosin precipitates and heavy meromyosin remains in solution and can be salted out by ammonium sulphate in the range 40–55% saturation[76,77]. By further digestion with trypsin[77] or with chymotrypsin[78], heavy meromyosin is further degraded into two major components, subfragments-1 and -2. Heavy meromyosin can also be split with water-insoluble trypsin and a helical subunit can be isolated by ethanol fractionation, isoelectric precipitation and gel filtration[79]. Subfragment-1 can be isolated either on the basis of its actin-combining property[77] or more effectively by gel filtration with Sephadex G-200; the major fraction exhibiting ATPase activity is subfragment-1[78]. The treatment of myosin with papain splits the molecule directly into subfragment-1 and a rod-like fragment[80,81]. High-per-

formance liquid chromatography was used to remove impurities from the crude subfragment-1 preparation (Spherogel TSK SW-3000 column)[82].

In order to show structural similarities or differences between various myosins, especially their heavy chains, myosin is split by trypsin, chymotrypsin, *Staphylococcus aureus* protease for prolonged periods of time or in high concentrations or treated with cyanogen bromide and peptides are analysed by electrophoresis. When the peptides are fractionated by one-dimensional disc electrophoresis, the modification of gel electrophoresis according to Laemli[83] is frequently used. As 3% stacking gel is used in addition to 8–12.5% resolution gel, much better resolution of peptides occurs during electrophoresis than with the classical technique of Weber and Osborne[22]. However, from the pattern obtained by one-dimensional electrophoresis it is difficult to identify individual peptides clearly. The two-dimensional technique of O'Farrell[24] was applied by Brevet and Whalen[84] for the fractionation of these peptides. Peptides were isoelectrically focused in the first dimension and fractionated in a sodium dodecyl sulphate slab gel (15% acrylamide) in the second dimension.

Rods of myosin can be isolated in good yield from myofibrils by soluble papain digestion. The method is based on purification by alcohol precipitation and gel filtration through Sephadex G-200 (ref. 85).

4.1.6. Isolation and fractionation of light chains of myosin

Light chains of myosin, proteins of the molecular mass in the range 16,000–30,000, are an integral part of the myosin molecule. They differ quantitatively and qualitatively in fast and slow skeletal and in cardiac muscles. Because of this, the electrophoretic profile of light chains of myosin is frequently used as a marker of myosin type.

A variety of methods have been developed for the dissociation of light chains, including treatment with alkaline solutions[86,87], urea[88,89], guanidine hydrochloride[88], LiCl[90], potassium thiocyanate[90], dithiobisnitrobenzoic acid[90] and succinic anhydride[91,92]. The light chains released can be isolated and purified by ammonium sulphate fractionation[92], by hydroxyapatite chromatography[93] or by ethanol precipitation[94].

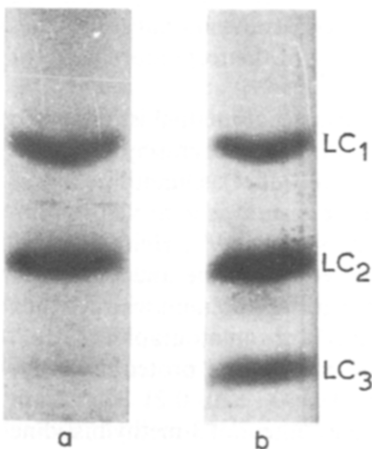

Fig. 4. Sodium dodecyl sulphate polyacrylamide (10%) gel electrophoresis of light chains from (a) newborn and (b) adult rat skeletal myosin.

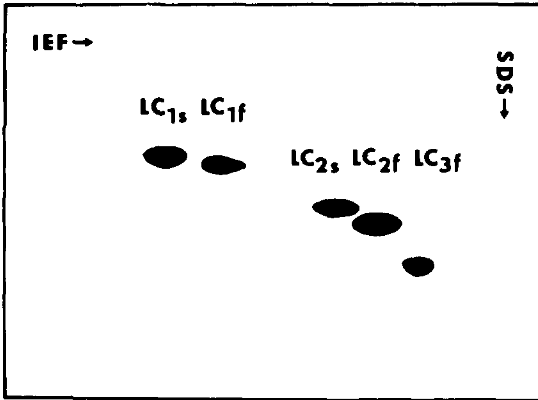

Fig. 5. Two-dimensional polyacrylamide gel electrophoresis of rat skeletal myosin light chains. Light chains of the fast (f) and of the slow (s) type are separated.

Isolated light chains of myosin can be separated by one-dimensional SDS–polyacrylamide gel electrophoresis (6 *M* urea)[95] (Fig. 4) or by isoelectric focusing in polyacrylamide[96]. Better resolution of light chains of myosin, especially when those from various muscle fibres are present, can be achieved by two-dimensional gel electrophoresis[97] (Fig. 5).

Preparative separation of isolated light chains can be performed by chromatography on DEAE-Sephadex A-25 (ref. 98) or DEAE-Sephadex A-50 (ref. 76) or by filtration on agarose beads (Bio-Gel A-1.5 m)[99].

4.1.7. Separation of "marker amino acids" (methyl analogues of histidine, lysine and arginine) in contractile proteins

Some contractile proteins contain methylated amino acids. These methylated amino acids were found in a limited number of proteins, mainly those which take part in contraction. It was shown that the content of 3-methylhistidine and N^G,N^G-dimethylarginine in myosin changes especially during ontogenetic development. The separation of methylated amino acids is not possible with conventional techniques used for amino acid analysis. In this section a list is given of the techniques used for analysis of these amino acids.

Reporter and Corbin[100] determined trimethyllysine, 3-methylhistidine and N^G,N^G-dimethylarginine in hydrolysate of myosin with chromatography on a sulphonated cation-exchange resin (pH 4.26, 0.35 *N* citrate buffer). Quantification of 3-methylhistidine in hydrolysates of muscle actin and myosin was achieved by ion-exchange chromatography on Dowex 50-X8 (pyridine gradient)[101]. Helm *et al.*[102] followed the content of 3-methylhistidine, N^G,N^G-dimethylarginine and trimethyllysine in myosin hydrolysates of various rodent muscles using a column with Aminex A-5 (0.3 *N* citrate buffer, pH 4.26). Zarkadas[103] described a chromatographic method for the determination of ω-N-methylarginine in muscle tissue and protein hydrolysates (Durrum type DC-6A resin or Beckman type AA-10 resin, 0.21 *M* sodium citrate, pH 5.40). Kuehl and Adelstein[104] determined the content of 3-methylhistidine and three methylated lysines in myosin hydrolysate (Beckman AA15 or UR 30 resin, 0.35 *N* citrate buffer). Johnson and Perry[105] analysed actin and various myosins for

the content of 3-methylhistidine (Technicon resin column, 0.35 *M* citrate, pH 5.28). Huszar and Elzinga[106] determined the content of 3-methylhistidine in peptides from muscle myosins by ion-exchange chromatography on Dowex 50-X2.

4.2. Isolation of actomyosin

Actomyosin does not exist in the muscle in the form encountered in solutions of natural or synthetic actomyosin. Therefore, the extraction should proceed through the disintegration of myofibrillar structures, followed by the reassociation of the extracted proteins in a less involved form. Because myosin is completely soluble at high ionic strength of the extraction media, but the actin filaments, on the other hand, appear to be firmly attached to the Z-membranes, the extracted actomyosin is artificial in the sense that the ratio of actin to myosin depends on the extraction conditions and is not the same as in the muscle. When the minced muscle is extracted with a solution of high ionic strength, practically only myosin is extracted initially, and after prolonged extraction myosin is converted into actomyosin[107].

In a number of studies actomyosin had to be isolated in order to obtain information about the ability of the system to split ATP or about the interactions with regulatory proteins (tropomyosin and troponin). Practically all preparations of actomyosin are modifications of the method described by Mommaerts[108]. Minced muscle is extracted for about 24 h with Weber–Edsall solution (0.6 *M* KCl, 0.04 *M* $KHCO_3$, 0.01 *M* K_2CO_3). The extract is clarified by centrifugation and precipitated three times by dilution with water (1:10) and dissolution at high ionic strength.

Actomyosin can also be prepared from myofibrils[109]. A suspension of myofibrils is blended in a Waring blender with an excess of Weber–Edsall solution. The extracted actomyosin is diluted 1:10 with water and the sediment of actomyosin is collected by centrifugation. Actomyosin prepared in this way is called natural actomyosin, and also contains the troponin complex. Troponins can be removed by washing out several times with water, and this preparation is called desentitized actomyosin.

4.3. Isolation and separation of actin

G-actin seems to be a single protein, having no subunits according to recent gel electrophoresis techniques using various denaturating agents. Two methods, different in principle, can be used for the isolation of actin. Myosin has to be removed in advance by extraction with a solution of high ionic strength. The classical method for the isolation of actin is based on the decomposition of the intermolecular linkage in F-actin and extracting it as G-actin. The most helpful agent for breaking the link without denaturing actin is acetone[110,111]. A similar action is induced by potassium iodide[111]. Actin can also be isolated directly from myofibrils. In this case, myosin must be removed completely in advance, *e.g.*, by extraction with a solution of high ionic strength. The attachment of F-actin to the Z-band makes the separation of F-actin difficult. Actin is resistant to trypsin and therefore trypsin can be used for splitting the Z-band. Hama *et al.*[112] succeeded in isolating actin on a large scale and named it natural F-actin.

For the preparation of actin from "acetone powder" two methods are fre-

quently used. The first is that of Feuer *et al.*[113]. Minced muscle is first extracted with Guba–Straub solution (0.3 *M* KCl, 0.15 *M* potassium phosphate buffer, pH 6.5). After washing with water, the muscle residue is dehydrated by washing several times with acetone and then dried (acetone powder). By a similar procedure acetone powder can also be prepared by the method of Carsten and Mommaerts[114].

Actin is isolated from acetone powder by extraction with 20 volumes of a solution containing 0.2 m*M* ATP, 2 m*M* Tris–HCl buffer (pH 7.6) and 2 m*M* $MgCl_2$ for polymerization and clarified by high-speed centrifugation.

There are several modifications of these methods. A typical method for the extraction and purification of G-actin is that of Mommaerts[115]. Drabikowski and Gergely[116] described a method for isolating actin and removing contaminating tropomyosin. G-actin can be purified by gel filtration on a Sephadex G-150 column[117]. SDS–polyacrylamide gel electrophoresis and Sephadex filtration on a G-200 column can be used for testing the homogeneity of actin preparation and the evaluation of impurities, respectively[118]. Gel filtration chromatography on Sephadex G-150 and SDS–polyacrylamide gel electrophoresis were utilized in the detection of a factor in conventional muscle actin preparation which inhibits actin filament self-association[119].

Actin is an ubiquitous protein; the amino acid sequences of actins derived from many different sources resemble each other, although several replacements of amino acid residues occur. The heterogeneity of actin preparations from various sources had not been reported until recently. Whalen *et al.*[120] found that actin exists in three forms possessing similar biochemical properties and identical molecular weights but with slightly different isoelectric points. Purified actins were distinguished on gel using isoelectric focusing into α-, β- and γ-actin; their ratios differed in muscle cultures of different ages and in adult muscle tissue[120].

4.4. Isolation and separation of tropomyosin

Tropomyosin was first isolated and crystallized by Bailey[121]. Tropomyosin is very stable towards organic solvents such as alcohols, acetone, ethers, acids and alkalis and heat, and is therefore more easily purified and crystallized than other muscle structural proteins. The principle of Bailey's technique for the isolation of tropomyosin is as follows: minced muscle is washed three times with ethanol and three times with diethyl ether and the residue is dried at room temperature. The powder is then extracted overnight with 1 *M* KCl–0.1 m*M* $CaCl_2$ at pH 8.0. The next steps in the isolation of tropomyosin are isoelectric precipitation and ammonium sulphate fractionation[122]. The supernatant is acidified to pH 4.6 and precipitated tropomyosin is dissolved in water at pH 7.0. Extracted tropomyosin is purified by ammonium sulphate fractionation. Tropomyosin is precipitated between 40 and 70% ammonium sulphate saturation. To obtain more purified tropomyosin, the isoelectric precipitation at pH 4.6 in 1 *M* KCl and fractionation with ammonium sulphate can be repeated, collecting the precipitate obtained in a narrower range of ammonium sulphate saturation (53–60%).

Tropomyosin was also isolated from the material obtained during troponin preparation[123]. The pH 4.6 precipitate obtained in the course of the preparation of troponin was redissolved in 1 *M* KCl and the pH was adjusted to 7.0. The precipi-

tation was repeated at pH 4.6 and, after neutralization, tropomyosin was obtained by fractionation with ammonium sulphate (the precipitate between 53 and 60% saturation was retained).

When the amount of tissue is limited and large amounts of nucleic acids are present (embryonic muscle), the tropomyosin–troponin complex can be first isolated by chromatography on a column of DEAE-cellulose and from this complex tropomyosin is purified by ammonium sulphate fractionation and isoelectric precipitation[124].

For the purification of tropomyosin, chromatography on a Sephadex G-50 column, hydroxyapatite chromatography (gradient of sodium phosphate in 0.5 *M* NaCl) or carboxymethyl-cellulose chromatography (in 8 *M* urea with an eluting gradient of 10 to 300 m*M* sodium chloride, pH 4.0) have also been used[125].

Skeletal muscle tropomyosin was resolved by polyacrylamide gel electrophoresis in the presence of SDS into two components (α- and β-tropomyosin)[122] and into four components on isoelectric focusing in the presence of urea (α^1, α^2, β^1, β^2). The two main subunits (α and β) were also separated by chromatography on CM-cellulose in the presence of urea at pH 4.0 (Fig. 6). Montarras *et al.*[126] analysed various tropomyosin preparations by two-dimensional gel electrophoresis and were able to distinguish tropomyosin α-subunit from slow, fast and cardiac muscle.

4.5. Isolation and separation of the troponin complex

Electrophoretic and chromatographic techniques have been exceptionally useful in proving the existence of three components of the troponin complex and in effectively isolating of some of its components. Troponin was first isolated as an acidic protein from "native tropomyosin"[127,128] by Ebashi's group. In 1968, Hartshorne and Mueller[129] fractionated Ebashi's troponin into two components. A number of studies were carried out to clarify the troponin components and, by 1972, it had been established that a troponin molecule consists of three component molecules:

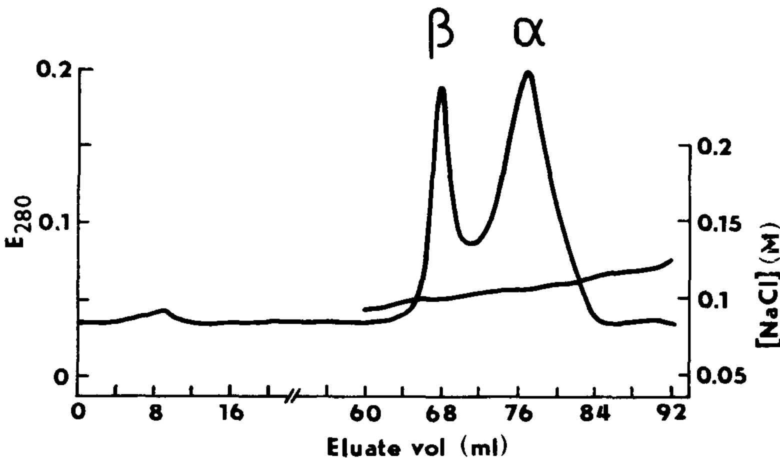

Fig. 6. Chromatography of rabbit skeletal muscle tropomyosin on CM-cellulose. The column was equilibrated against 50 m*M* sodium formate buffer (pH 4.0) 8 *M* urea. A linear gradient of NaCl from 0 to 0.2 *M* was applied after 20.0 ml had been eluted. Adapted from ref. 122, with permission.

troponin C, I and T. Several methods have been reported for the preparation of troponin[130–134]. All of them isolate native tropomyosin first, with subsequent separation of troponin. A method was elaborated for isolating troponin directly from the muscle residue[135]. The technique of Tsukui and Ebashi[136] is very convenient and rapid. It consists in extraction with lithium chloride, isoelectric precipitation of tropomyosin and several ammonium sulphate fractionations. Greaser *et al.*[137] has proposed a technique using extraction in 1 *M* KCl, ammonium sulphate fractionation in the range of 30–50% saturation and separation of the components on a hydroxyapatite column.

Several techniques for the separation of the troponin components (troponin T, C and I) have been published, including SDS gel electrophoresis (Fig. 7) and DEAE-Sephadex chromatography[123,138–147].

For the isolation of troponin I and C from small amounts of tissue, a rapid technique was elaborated[148,149] which permits their isolation directly from whole muscle extracts by affinity chromatography. Troponin I or C was coupled to CNBr-activated Sepharose 4B. The muscle extract was applied to the column of the Sepharose–protein matrix. Unbound proteins were eluted from the column by washing with 8 *M* urea–1 m*M* $CaCl_2$–20 m*M* Tris–HCl (pH 7.8). Bound protein was eluted with the same solution containing 10 m*M* EGTA instead of Ca^{2+}. The column with troponin I coupled to the Sepharose is thus used for troponin C isolation and the troponin I bound column for troponin C isolation.

The separation techniques have also been applied in studies of the interaction of troponin components. Head and Perry[146] investigated the interaction of troponin C with Ca^{2+} and with troponin I, following the mobility of protein complexes on polyacrylamide gel electrophoresis in the presence of urea. Katayama[150] overcame

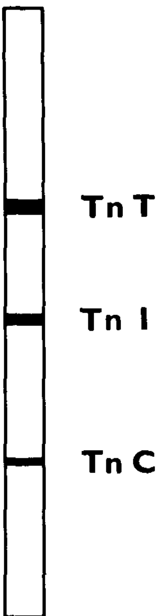

Fig. 7. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of cardiac troponin. Tn = troponin.

experimental difficulties in studying the interaction of troponin components in a solution and followed the interaction of troponin fragments with other proteins using affinity chromatography. Troponin I was immobilized on a CNBr-activated Sepharose 4B matrix and this provided evidence for the strong binding of troponin I to troponin T and also to troponin T fragments obtained by chymotryptic digestion.

4.6. *Minor proteins*

4.6.1. *M-Protein*

The M-band protein was isolated by Masaki and co-workers[151,152] from minced muscle, which was washed repeatedly with 0.1 *M* KCl to wash out soluble proteins. The residue was extracted with Hasselbach–Schneider's solution (0.6 *M* KCl, 0.01 *M* sodium pyrophosphate, 0.1 *M* phosphate, pH 6.4) and the extract was fractionated with ammonium sulphate. The method of Eaton and Pepe[153] is based on extracting myofibrils with 5 m*M* Tris–HCl buffer (pH 8.0) and purifying the extract by isoelectric precipitation. During discontinuous polyacrylamide gel electrophoresis the isolated protein was separated into components A and B.

Morimoto and Harrington[154] isolated the M-line protein from skeletal muscle by extraction with 5 m*M* Tris buffer (pH 7.4) and removal of contaminants by precipitation at pH 5.0 followed by their absorption on added DEAE-cellulose in 50 m*M* Tris buffer (pH 8.6). However, it is still not clear whether the M-protein constitutes all of the M-line proteins.

4.6.2. *Z-Protein*

Z-Protein, a protein which presumably constitutes the lattice structure of the Z-lines, was characterized by Ohashi and Maruyama[155,156]. The protein was isolated from chicken skeletal myofibrils, from which actin, β-actinin and other proteins had been extracted with 0.6 *M* KI. The residue was further extracted with KI solution for 24 h at 4°C. The extract was fractionated with ammonium sulphate and the fraction precipiated between 20 and 40% saturation was retained. The protein was purified by chromatography on a DEAE-cellulose column. Affinity chromatography was used to show that interaction of Z-protein with actin and α-actinin does not occur. It is thought that some other protein(s) is (are) necessary for linking actin filaments to the lattice structure of the Z-line.

In the Z-line other minor regulatory proteins are also located but these are only partially characterized. One of them is a protein of molecular mass 220,000, isolated by Muguruma *et al.*[157]. This protein was extracted from I–Z–I brushes prepared from skeletal muscle by extraction with 0.6 *M* KI containing 6 m*M* $Na_2S_2O_3$, ammonium sulphate precipitation and three column chromatographic procedures in succession (Sepharose CL-4B, DEAE-cellulose and Bio-Gel A-50 m columns).

4.6.3. *Actinins*

Three species of actinins have been characterized since 1965 (α-, β- and γ-actinin). α-Actinin was prepared by Ebashi and Ebashi[158] by a method derived from actin preparation, and included extraction, ethanol treatment, isoelectric precipitation and ammonium sulphate fractionation. The preparation of chicken α-actinin was described by Masaki and Takaiti[159]. Minced muscle was extracted repeatedly with 0.6 *M* KCl, 10 m*M* sodium pyrophosphate, 1 m*M* $MgCl_2$, 0.1 *M* potassium

phosphate buffer (pH 6.4). The residue was extracted with 1 m*M* $NaHCO_3$ and fractionated with ammonium sulphate. Robson *et al.*[160] used DEAE-cellulose chromatography for the purification of α-actinin.

β-Actinin was prepared by Maruyama[161]. The starting material was actin prepared by extraction with potassium iodide. The supernatant was fractionated with ammonium sulphate and purified by DEAE-Sephadex chromatography[162].

γ-Actinin was isolated from the extract of native thin filaments of skeletal muscle[163] by salting out with ammonium sulphate and DEAE-Sephadex and Sephadex G-200 chromatography.

4.6.4. C-Protein

When the SDS gel electrophoresis of crude preparations of myosin was examined by Starr and Offer[164], several protein bands were seen in addition to those of myosin. One of them corresponded to the C-protein and was isolated by Offer *et al.*[165] from ammonium sulphate purified myosin. When this myosin was chromatographed on a DEAE-Sephadex column, myosin and C-protein were separated (Fig. 8) and the latter was further purified by ammonium sulphate precipitation. Reinach *et al.*[166] separated C-protein from posterior latissimus dorsi muscle of the chick into two components by hydroxyapatite chromatography

4.6.5. Paramyosin

Techniques for the isolation of paramyosin from inverterbrate muscles take advantage of the protein's resistance to denaturation by organic solvents and its unique solubility properties. For the highest yields, it is useful to use mucles.that have higher paramyosin to myosin ratios. Some of these tissues also contain intrinsic proteases or those from bacterial parasites, so appropriate precautions should be taken to avoid proteolysis of paramyosin.

Paramyosin is a protein found in molluscan muscle, annelids, in the arthropod *Limulus* and in flight muscles of insects. Paramyosin was isolated from molluscan "catch" muscles[167,168] either by ethanol fractionation or from the sediment obtained

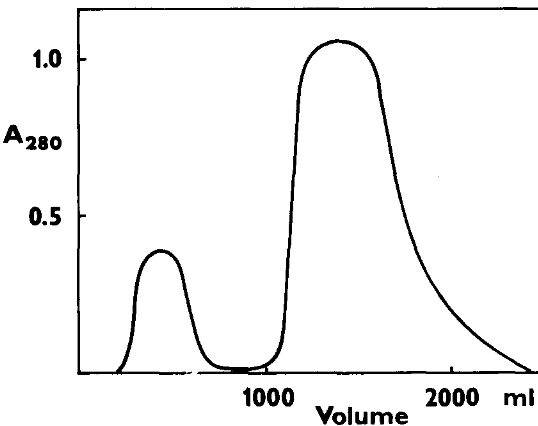

Fig. 8. Chromatography of myosin purified by ammonium sulphate fractionation. DEAE-Sephadex A-50 column, gradient of KCl. C-protein is the main component of the small peak and myosin is eluted as the second peak.

after myosin extraction. The sediment was re-extracted with 0.6 *M* KCl and paramyosin precipitated by the dilution–precipitation method. Paramyosin from insect flight muscles was isolated by Bullard *et al.*[169] by extraction of muscle ether powder, which is usually used for tropomyosin preparation.

5. CONCLUSIONS

This review was intended to survey some representative methods used for the separation of muscle proteins. It was hardly possible to list all the methods elaborated in the past. Instead, an attempt was made to outline the general approaches used in investigating the physiology and biochemistry of muscle proteins. Logically, we started with the fractionation of muscle components, continued with methods used for the fractionation and separation of muscle proteins and finally mentioned some techniques used for the separation of various protein fragments.

During the last few decades, considerable progress has been made in muscle biochemistry and the methodological progress has been very useful in obtaining a number of very basic contributions in this field. It is understandable that each technique has its limitations and that the choice of the methods employed thus depends on the origin of the material, choice of animal, the amount and purity of protein required and other factors.

How much the new separation techniques have helped in studying muscle proteins can be illustrated by the number of newly discovered contractile proteins and also by the fact that the amount of muscle tissue required for the isolation of a particular protein has decreased very considerably, so that it is possible today to obtain important information even from one muscle fibre. Chromatographic and electrophoretic techniques have been very useful in these studies. Affinity chromatography especially has become a very efficient approach in this respect, owing to its speed and the possibility it provides for isolating proteins, which often cannot be obtained with other techniques. Examples of new discoveries of contractile proteins that may be mentioned here are the discovery of the existence of troponin T, I and C, the proof of the existence of myosin isoforms and the finding of methylated amino acids in myosin. With the help of new techniques it has also been shown that practically all contractile proteins exist in polymorphic forms. The contractile apparatus of the muscle changes under various functional demands. One of the most important contributions in this area was the discovery that the ratio of cardiac myosin isoforms changes during development and under pathophysiological conditions.

New techniques of muscle protein investigation have recently made fundamental contributions to our ideas about muscle structure and function and it may be expected that more light will be shed on this intriguing problem in the near future. New ideas and also new separation techniques and micro methods will be necessary for this to be achieved.

6. SUMMARY

This review covers various methods used in the separation and isolation of individual muscle contractile proteins. It is shown which methods have been most useful for the separation of contractile proteins and their fragments and in extending our knowledge of muscle biochemistry and physiology.

REFERENCES

1 D. W. Frederiksen and L. W. Cunningham (Editors), *Methods Enzymol.*, 85B (1982) 1–724.
2 P. Rubenstein, in Z. Deyl (Editor), *Electrophoresis —A Survey of Techniques and Applications, Part B, Applications* (*Journal of Chromatography Library*, Vol. 18B), Elsevier, Amsterdam, Oxford, New York, 1983, pp. 172–176.
3 M. Mowafy, L. L. Kastenschmidt and R. G. Cassens, *J. Anim. Sci.*, 34 (1972) 563.
4 S. V. Perry, *Biochem. J.*, 51 (1952) 495.
5 S. V. Perry, *Biochem. J.*, 64 (1956) 184.
6 R. R. Kohn, *Exp. Cell Res.*, 49 (1968) 206.
7 O. Harbitz, E. Slinde, H. Kryvi and G. K. Totland, *Anal. Biochem.*, 125 (1982) 105.
8 K. Morimoto and W. F. Harrington, *J. Mol. Biol.*, 77 (1973) 165.
9 T. Obinata, I. Hayashi and D. A. Fischman, *Devl. Growth Differ.*, 16 (1974) 105.
10 R. B. Low, J. N. Vournakis and A. Rich, *Biochemistry*, 10 (1971) 1813.
11 R. Zak, J. Etlinger and D. A. Fischman, in B. Q. Banker, R. Przybylski, J. P. van der Meulen and M. Victor (Editors), *Proc. Int. Symp. Research in Muscle Development and the Muscle Spindle*, Excerpta Medica, Amsterdam, 1972, pp. 163–177.
12 K. T. Shiverick, L. L. Thomas and N. R. Alpert, *Biochim. Biophys. Acta*, 393 (1975) 124.
13 C. R. Merrill, D. Goldman, S. A. Sedman and M. H. Ebert, *Science*, 211 (1981) 1437.
14 G. Salviati, R. Betto and D. D. Betto, *Biochem. J.*, 207 (1982) 261.
15 L. F. Lemanski, X. Joseph and M. K. Iyengar, *J. Cell. Biol.*, 67 (1975) 239a.
16 P. B. Moore and L. F. Lemanski, *J. Muscle Res. Cell Motility*, 3 (1982) 145.
17 E. Helander, *Acta Physiol. Scand.*, 41 (1957) Suppl. 141, p. 1.
18 A. Mariani and G. Toschi, *Rend. Ist. Super. Sanità*, 16 (1953) 148.
19 G. Toschi and M. Boccacci, *Boll. Soc. Ital. Biol. Sper.*, 30 (1954) 67.
20 G. Toschi and S. Bettini, *Boll. Soc. Ital. Biol. Sper.*, 32 (1956) 905.
21 G. Toschi and S. Bettini, *Rend. Ist. Super. Sanità*, 21 (1958) 678.
22 K. Weber and M. Osborn, *J. Biol. Chem.*, 144 (1969) 4406.
23 M. A. Porzio and A. M. Pearson, *Biochim. Biophys. Acta*, 490 (1977) 27.
24 P. H. O'Farrell, *J. Biol. Chem.*, 250 (1975) 4007.
25 O. A. Young and C. L. Davey, *Biochem. J.*, 195 (1981) 317.
26 D. Pette and U. Schnez, *Histochemistry*, 54 (1977) 97.
27 T. Masaki and Ch. Yoshizaki, *J. Biochem. (Tokyo)*, 71 (1972) 755.
28 S. V. Perry, *Methods Enzymol.*, 2 (1955) 582.
29 J. Dow and A. Stracher, *Biochemistry*, 10 (1971) 1316.
30 F. A. Sreter, J. C. Seidel and J. Gergely, *J. Biol. Chem.*, 241 (1966) 5772.
31 R. J. Luchi, E. M. Kritcher and H. L. Conn, *Circ. Res.*, 16 (1965) 74.
32 A. G. Weeds and B. S. Hartley, *Biochem. J.*, 107 (1968) 531.
33 J. Vierling, J. L. Roberts, C. Conway and R. Heazlitt, *Biochim. Biophys. Acta*, 160 (1968) 53.
34 J. J. Connell, *Biochem. J.*, 58 (1954) 360.
35 G. Hamoir, H. A. McKenzie and M. B. Smith, *Biochim. Biophys. Acta*, 40 (1960) 141.
36 I. M. Mackie and J. J. Connell, *Biochim. Biophys. Acta*, 93 (1964) 544.
37 M. A. Ferenczi, E. Humsher, D. R. Trentham and A. G. Weeds, *Biochem. J.*, 171 (1978) 155.
38 A. Giamdalvo and P. Dreizen, *Biochim. Biophys. Acta*, 537 (1978) 466.
39 B. Pliczka, A. Szpacenko and H. Strzelecka-Golaszewska, *Int. J. Biochem.*, 10 (1979) 343.
40 B. Focant and F. Huriaux, *J. Muscle Res. Cell Motility*, 1 (1980) 61.
41 K. Maruyama, J. W. S. Pringle and R. T. Tregear, *Proc. R. Soc. London, Ser. B*, 169 (1968) 229.
42 B. Bullard and M. K. Reedy, *Cold Spring Harbor Symp. Quant. Biol.*, 37 (1973) 423.
43 W. Lehman, J. Kendrick-Jones and A. G. Szent-Györgyi, *Cold Spring Harbor Symp. Quant. Biol.*, 37 (1973) 319.
44 S. V. Perry, *Biochem. J.*, 74 (1960) 94.
45 M. Harris and C. H. Suelter, *Biochim. Biophys. Acta*, 133 (1967) 393.
46 E. F. Baril, D. S. Love and H. Herrmann, *J. Biol. Chem.*, 241 (1966) 822.
47 I. P. Trayer and S. V. Perry, *Biochem. Z.*, 345 (1966) 87.
48 E. G. Richards, C. S. Chung, D. B. Menzel and H. S. Olcott, *Biochemistry*, 6 (1967) 528.
49 I. Pinset-Härström and E. Ehrlich, *FEBS Lett.*, 34 (1973) 227.
50 E. F. Rossomando and K. A. Piez, *Biochem. Biophys. Res. Commun.*, 40 (1970) 800.

51 S. M. Mozersky, J. D. Pettinati and S. D. Kolman, *J. Chromatogr.*, 65 (1972) 387.
52 S. M. Mozersky, R. E. Guggor, J. D. Pettinati and D. J. Kolman, *J. Chromatogr.*, 100 (1974) 192.
53 S. M. Mozersky, K. M. Valentine, R. E. Guggor and M. Tunick, *J. Chromatogr.*, 207 (1981) 395.
54 F. H. Schachat, H. E. Harris and H. F. Epstein, *Cell*, 10 (1977) 721.
55 H. R. Trayer and I. P. Trayer, *FEBS Lett.*, 54 (1975) 291.
56 A. Grandmont-Leblanc and J. Gruda, *Can. J. Biochem.*, 55 (1977) 949.
57 Z. Deyl, V. Pelouch, H. Málková and J. Rosmus, *J. Chromatogr.*, 41 (1969) 423.
58 J. R. Florini and R. P. Brivio, *Anal. Biochem.*, 30 (1969) 358.
59 J. R. Florini, R. P. Brivio and B. M. Battelle, *Anal. Biochem.*, 40 (1971) 345.
60 A. d'Albis, C. Pantaloni and J. J. Bechet, *Eur. J. Biochem.*, 99 (1979) 261.
61 A. d'Albis, C. Pantaloni and J. J. Bechet, *FEBS Lett.*, 106 (1979) 81.
62 A. M. Lompre, J. J. Mercadier, C. Wisnewsky, P. Bouveret, C. Pantaloni, A. d'Albis and K. Schwartz, *Dev. Biol.*, 84 (1981) 286.
63 J. F. Y. Hoh, *FEBS Lett.*, 90 (1978) 297.
64 J. F. Y. Hoh, P. A. McGrath and P. T. Hale, *J. Mol. Cell. Cardiol.*, 10 (1978) 1053.
65 K. Yagi and F. Otani, *J. Biochem. (Tokyo)*, 76 (1974) 365.
66 A. G. Weeds and R. S. Taylor, *Nature (London)*, 257 (1975) 54.
67 H. R. Trayer, M. A. Winstanley and I. P. Trayer, *FEBS Lett.*, 83 (1977) 141.
68 K. Yagi, S. Matsuda and H. Kowayama, *J. Biochem. (Tokyo)*, 82 (1977) 1463.
69 M. Yoshida and F. Morita, *J. Biochem. (Tokyo)*, 79 (1976) 1049.
70 P. D. Wagner, *FEBS Lett.*, 81 (1977) 81.
71 J. C. Holt and S. Lowey, *Biochemistry*, 16 (1977) 4398.
72 L. Silberstein and S. Lowey, *J. Mol. Biol.*, 148 (1981) 153.
73 S. Sartore, L. D. Libera and S. Schiaffino, *FEBS Lett.*, 106 (1979) 197.
74 R. A. Chizzonitte, A. W. Everett, W. A. Clark, S. Jakucic, M. Rabinowitz and R. Zak, *J. Biol. Chem.*, 257 (1982) 2056.
75 J. Gergely, *J. Biol. Chem.*, 200 (1953) 543.
76 A. G. Szent-Györgyi, *Arch. Biochem. Biophys.*, 42 (1953) 305.
77 H. Mueller and S. V. Perry, *Biochem. J.*, 85 (1962) 431.
78 J. M. Jones and S. V. Perry, *Biochem. J.*, 100 (1966) 120.
79 S. Lowey, L. Goldstein and S. Lick, *Biochem. Z.*, 345 (1969) 248.
80 S. Lowey, H. S. Slayter, A. G. Weeds and H. Baker, *J. Mol. Biol.*, 42 (1969) 1.
81 C. Oriol, M. F. Landon and N. van Thoai, *Biochem. Biophys. Res. Commun.*, 51 (1973) 1023.
82 A. P. Toste, *J. Chromatogr.*, 197 (1980) 207.
83 U. K. Laemli, *Nature (London)*, 227 (1970) 680.
84 A. Brevet and G. Whalen, *Biochimie*, 60 (1978) 459.
85 E. Gaetjens, K. Barany, G. Bailin, H. Oppenheimer and M. Barany, *Arch. Biochem. Biophys.*, 123 (1968) 82.
86 D. R. Kominz, W. R. Carroll, E. N. Smith and E. R. Mitchell, *Arch. Biochem. Biophys.*, 79 (1959) 191.
87 P. Dreizen, D. J. Hartshorne and A. Stratcher, *J. Biol. Chem.*, 241 (1966) 443.
88 S. Lowey and J. C. Holt, *Cold Spring Harbor Symp. Quant. Biol.*, 37 (1972) 19.
89 L. C. Gersham and P. Dreizen, *Biochemistry*, 9 (1970) 1677.
90 A. G. Weeds, *Nature (London)*, 223 (1969) 1362.
91 H. Oppenheimer, K. Barany, G. Hamoir and J. Fenton, *Arch. Biochem. Biophys.*, 120 (1967) 108.
92 H. Oppenheimer, K. Barany, G. Hamoir and J. Fenton, *Arch. Biochem. Biophys.*, 115 (1966) 233.
93 P. Appel, E. G. Richards and H. S. Olcott, *J. Chromatogr.*, 76 (1973) 351.
94 W. T. Perrie and S. V. Perry, *Biochem. J.*, 119 (1970) 31.
95 R. Dabrowska, J. Sosinski and W. Drabikowski, *FEBS Lett.*, 79 (1977) 295.
96 P. Cummins, *Biochem. J.*, 205 (1982) 195.
97 R. G. Whalen, G. S. Butler-Browne and F. Gros, *J. Mol. Biol.*, 126 (1978) 415.
98 J. Wikman-Coffelt, R. Zelis, C. Fenner and D. T. Mason, *Prep. Biochem.*, 3 (1973) 439.
99 G. Klotz, J. J. Leger, F. Marotte and H. Mendes, *Biochim. Biophys. Acta*, 532 (1978) 369.
100 M. Reporter and J. L. Corbin, *Biochem. Biophys. Res. Commun.*, 43 (1971) 644.
101 L. N. Haverberg, H. N. Munro and V. R. Young, *Biochim. Biophys. Acta*, 371 (1974) 226.
102 R. Helm, Z. Deyl and O. Vančíková, *Exp. Gerontol.*, 12 (1977) 245.
103 G. G. Zarkadas, *Can. J. Biochem.*, 56 (1978) 952.

104 W. M. Kuehl and R. S. Adelstein, *Biochem. Biophys. Res. Commun.*, 37 (1969) 59.
105 P. Johnson and S. V. Perry, *Biochem. J.*, 119 (1970) 293.
106 G. Huszar and M. Elzinga, *J. Biol. Chem.*, 247 (1972) 745.
107 T. Haga, K. Maruyama and H. Noda, *Biochim. Biophys. Acta*, 94 (1965) 226.
108 W. F. H. M. Mommaerts, in J. V. Warren (Editor), *Methods in Medical Research*, Vol. 7, Yearbook Publisher, Chicago, 1958, p. 1.
109 M. Schaub and S. V. Perry, *Biochem. J.*, 123 (1971) 367.
110 F. B. Straub, *Stud. Inst. Med. Chem. Univ. Szeged*, 2 (1942) 31.
111 A. G. Szent-Györgyi, *J. Biol. Chem.*, 192 (1951) 361.
112 H. Hama, K. Maruyama and H. Noda, *Biochim. Biophys. Acta*, 102 (1965) 249.
113 G. Feuer, F. Molnar, E. Pettko and F. B. Straub, *Hung. Acta Physiol.*, 1 (1948) 150.
114 M. E. Carsten and W. F. H. M. Mommaerts, *Biochemistry*, 2 (1963) 28.
115 W. F. H. M. Mommaerts, *J. Biol. Chem.*, 198 (1952) 445.
116 W. Drabikowski and J. Gergely, *J. Biol. Chem.*, 237 (1962) 3412.
117 M. Kuroda, *J. Biochem. (Tokyo)*, 92 (1982) 1863.
118 J. A. Spudich and S. Watt, *J. Biol. Chem.*, 246 (1971) 4866.
119 S. MacLean-Fletcher, T. D. Pollard, *Biochem. Biophys. Res. Commun.*, 96 (1980) 18.
120 R. G. Whalen, G. S. Butler-Browne and F. Gros, *Proc. Nat. Acad. Sci. U.S.*, 73 (1976) 2018.
121 K. Bailey, *Biochem. J.*, 43 (1948) 271.
122 P. Cummins and S. V. Perry, *Biochem. J.*, 133 (1973) 765.
123 M. L. Greaser and J. Gergely, *J. Biol. Chem.*, 246 (1971) 4226.
124 R. K. Roy, J. D. Potter and S. Sarkar, *Biochem. Biophys. Res. Commun.*, 70 (1976) 28.
125 E. Eisenberg and W. W. Kielley, *J. Biol. Chem.*, 249 (1974) 4742.
126 D. Montarras, M. Y. Fiszman and F. Gros, *J. Biol. Chem.*, 256 (1981) 4081.
127 S. Ebashi and A. Kodama, *J. Biochem. (Tokyo)*, 58 (1965) 107.
128 S. Ebashi and A. Kodama, *J. Biochem. (Tokyo)*, 59 (1966) 425.
129 D. J. Hartshorne and H. S. Mueller, *Biochem. Biophys. Res. Commun.*, 31 (1968) 647.
130 S. Ebashi and F. Ebashi, *J. Biochem. (Tokyo)*, 55 (1964) 604.
131 K. Arai and S. Watanabe, *J. Biol. Chem.*, 243 (1968) 5670.
132 B. Yasui, F. Fuchs and F. N. Briggs, *J. Biol. Chem.*, 243 (1968) 735.
133 D. J. Hartshorne and H. Mueller, *Biochim. Biophys. Acta*, 175 (1969) 301.
134 M. C. Schaub and S. V. Perry, *Biochem. J.*, 115 (1969) 993.
135 S. Ebashi, T. Wakabayashi and F. Ebashi, *J. Biochem. (Tokyo)*, 69 (1971) 441.
136 R. Tsukui and S. Ebashi, *J. Biochem. (Tokyo)*, 73 (1973) 1119.
137 M. L. Greaser, M. Yamaguchi, C. Brekke, J. Potter and J. Gergely, *Cold Spring Harbor Symp. Quant. Biol.*, 37 (1972) 235.
138 W. Drabikowski, V. Rafalowska, R. Dabrowska, A. Szpacenko and B. Barylko, *FEBS Lett.*, 19 (1971) 259.
139 W. Drabikowski, R. Dabrowska and B. Barylko, *Acta Biochim. Pol.*, 20 (1973) 181.
140 W. Drabikowski, E. Nowak, B. Barylko and R. Dabrowska, *Cold Spring Harbor Symp. Quant. Biol.*, 37 (1972) 245.
141 S. Ebashi, *J. Biochem. (Tokyo)*, 72 (1972) 787.
142 D. J. Hartshorne and P. Dreizen, *Cold Spring Harbor Symp. Quant. Biol.*, 37 (1972) 225.
143 I. Staprans, H. Takahashi, M. P. Russel and S. Watanabe, *J. Biochem. (Tokyo)*, 72 (1972) 723.
144 J. M. Wilkinson, S. V. Perry, H. A. Cole and I. P. Trayer, *Biochem. J.*, 127 (1972) 215.
145 R. Dabrowska, M. Dydynska, A. Szpacenko and W. Drabikowski, *Int. J. Biochem.*, 4 (1973) 189.
146 J. F. Head and S. V. Perry, *Biochem. J.*, 137 (1974) 145.
147 S. V. Perry and H. A. Cole, *Biochem. J.*, 131 (1974) 733.
148 H. Syska, S. V. Perry and I. P. Trayer, FEBS Lett., 40 (1974) 253.
149 J. F. Head, R. A. Weeks and S. V. Perry, *Biochem. J.*, 161 (1977) 465.
150 E. Katayama, *J. Biochem. (Tokyo)*, 85 (1979) 1379.
151 T. Masaki, O. Takaiti and S. Ebashi, *J. Biochem. (Tokyo)*, 64 (1968) 909.
152 T. Masaki and O. Takaiti, *J. Biochem. (Tokyo)*, 71 (1972) 355.
153 B. L. Eaton and F. A. Pepe, *J. Cell. Biol.*, 55 (1972) 681.
154 K. Morimoto and W. F. Harrington, *J. Biol. Chem.*, 247 (1972) 3052.
155 K. Ohashi and K. Maruyama, *J. Biochem. (Tokyo)*, 85 (1979) 1103.
156 K. Ohashi and K. Maruyama, in S. Ebashi, K. Maruyama and M. Endo (Editors), *Muscle Contraction, Its Regulatory Mechanisms*, Japan Science Society Press, Tokyo, 1980, p. 497.

157 M. Muguruma, K. Kobayashi, T. Fukuzawa, K. Ohashi and K. Maruyama, *J. Biochem. (Tokyo)*, 89 (1981) 1981.
158 S. Ebashi and F. Ebashi, *J. Biochem. (Tokyo)*, 58 (1965) 712.
159 T. Masaki and O. Takaiti, *J. Biochem. (Tokyo)*, 66 (1969) 637.
160 R. M. Robson, D. E. Goll, N. Arakawa and M. H. Stromer, *Biochim. Biophys. Acta*, 200 (1970) 296.
161 K. Maruyama, *Biochim. Biophys. Acta*, 94 (1965) 208.
162 K. Maruyama, S. Kimura, T. Ishii, M. Kuroda, K. Ohashi and S. Muramatsu, *J. Biochem. (Tokyo)*, 81 (1977) 215.
163 M. Kuroda and K. Maruyama, *J. Biochem. (Tokyo)*, 80 (1976) 315.
164 R. Starr and G. Offer, *FEBS Lett.*, 15 (1971) 40.
165 G. Offer, C. Moos and R. Star, *J. Mol. Biol.*, 74 (1973) 653.
166 F. C. Reinach, T. Masaki and D. A. Fischman, *J. Cell. Biol.*, 96 (1983) 297.
167 C. Cohen, *J. Mol. Biol.*, 56 (1971) 223.
168 A. G. Szent-Györgyi, C. Cohen and J. Kendrick-Jones, *J. Mol. Biol.*, 56 (1971) 239.
169 B. Bullard, B. Luke and L. Winkelman, *J. Mol. Biol.*, 75 (1973) 359.