Biochem. J. (1973) **135**, 151–164
Printed in Great Britain

A Phosphorylated Light-Chain Component of Myosin from Skeletal Muscle

By W. T. PERRIE, L. B. SMILLIE* and S. V. PERRY
Department of Biochemistry, University of Birmingham, Birmingham B15 2TT, U.K.

(*Received* 9 *March* 1973)

1. The low-molecular-weight components of myosin from rabbit skeletal muscle migrated as four bands on polyacrylamide-gel electrophoresis in 8M-urea but only as three in systems containing sodium dodecyl sulphate. The two bands of intermediate mobility in 8M-urea (Ml_2 and Ml_3) had identical mobilities in sodium dodecyl sulphate. 2. The isolation of pure samples of all four low-molecular-weight components by DEAE-Sephadex chromatography is described. 3. The amino acid compositions of components Ml_2 and Ml_3 were identical. Further analyses showed the presence of 1 mol of phosphate/18500g of component Ml_2 and less than 10% of this amount in component Ml_3. Neither light component contained ribose. 4. Alkaline phosphatase from *Escherichia coli* converted component Ml_2 into Ml_3. Incubation with crude preparations of phosphorylase *b* kinase or protein kinase in the presence of ATP converted component Ml_3 into Ml_2. 5. Phosphorylation of component Ml_3 with the kinases isolated from skeletal muscle and [γ-^{32}P]ATP gave incorporation of ^{32}P only into component Ml_2 whether whole myosin or separated low-molecular-weight components were used. 6. High-voltage electrophoresis at pH 6.5 and pH 1.8 of a chymotryptic digest of ^{32}P-labelled component Ml_2 yielded one major radioactive peptide containing serine phosphate. 7. The amino acid sequence of this peptide was shown to be: Arg-Ala-Ala-Ala-Glu-Gly-Gly-(Ser,Ser(P))-Asn-Val-Phe. This sequence shows no obvious similarity to the site phosphorylated in the conversion of phosphorylase *b* into phosphorylase *a* by phosphorylase *b* kinase. 8. Evidence suggests that *in vivo* all the 18500-molecular-weight light chain is in the phosphorylated form. The extent of dephosphorylation that occurred during myosin extraction depended on the conditions employed.

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate has now firmly established that the light-chain fraction of myosin isolated from rabbit white skeletal muscle consists of three different molecular species with molecular weights in the range 15000–33000 (Paterson & Strohman, 1970; Starr & Offer, 1971; Weeds & Lowey, 1971; Perrie *et al.*, 1972*a*). Earlier electrophoretic studies on myosin under conditions in which its mobility depended on the charge on the protein produced somewhat variable band patterns owing to the relative ease of modification of the individual light-chain components and the effects of different extraction conditions on the composition of the whole light-chain fraction (see Perrie & Perry, 1970).

We have reported (Perrie *et al.*, 1969; Perrie & Perry, 1970) that the light-chain fraction of myosin from rabbit skeletal muscle extracted under specified conditions consistently migrated as four bands on polyacrylamide-gel electrophoresis in 8M-urea at pH 8.6. These have been designated the Ml_1, Ml_2, Ml_3 and Ml_4 light-chain components in order of decreasing electrophoretic mobility under these conditions. In the original investigation (Perrie & Perry, 1970) the relative amounts of the components Ml_1 and Ml_4 which correspond to the light-chain components of molecular weight 15500 and 22500 respectively (Perrie *et al.*, 1972*a*), appeared constant in all preparations of myosin studied. In contrast the relative amounts of components Ml_2 and Ml_3 present were variable, and evidence for their interconversion, possibly by an enzymic system, was obtained. The Ml_3 component possesses a similar electrophoretic mobility to the fraction separated from myosin by treatment with 5,5′-dithiobis-(2-nitrobenzoic acid), the so-called DTNB light-chain fraction (Weeds, 1969; Gazith *et al.*, 1970; Weeds & Lowey, 1971). The Ml_1 and Ml_4 components corresponded to the alkali light chains of Weeds & Lowey (1971).

The work described in the present paper was undertaken to determine the relationship between the Ml_2 and Ml_3 light-chain components of myosin from rabbit skeletal muscle. From the results it is concluded that component Ml_2 is converted into component Ml_3 by the phosphorylation of a single serine residue

* Present address: Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada.

in component Ml_3 by the action of a kinase present in sarcoplasm, by using the γ-phosphate group of ATP as donor. Some aspects of the work have been briefly reported elsewhere (Perrie *et al.*, 1972*a*,*b*).

Methods

Unless otherwise stated all protein preparations were carried out at 4°C.

Preparation of muscle proteins

As a routine myosin was prepared as previously described (Perrie & Perry, 1970). Where different extraction conditions were used they are detailed in the text. For the standard preparation myosin was extracted with a muscle to buffer ratio of 1:3 (w/v). To obtain Ml_2-rich myosin, a muscle to buffer ratio of 1:10 (w/v) was used. The material centrifuged down after the initial extraction of myosin from muscle mince is referred to as muscle residue.

Myofibrils were prepared from mixed rabbit skeletal muscle as described by Schaub & Perry (1971).

Separation of the low-molecular-weight components of myosin

The low-molecular-weight components were prepared by precipitation of the heavy chain with ethanol from myosin dissolved in 5 M-guanidine hydrochloride (Perrie & Perry, 1970). Freeze-dried low-molecular-weight components (500mg) were then suspended in 100ml of 50mM-potassium phosphate (pH6.5)–10mM-β-mercaptoethanol–2mM-EDTA and dialysed against 3 litres of the same buffer. Insoluble material was removed by centrifugation for 15min at 10000*g*, the supernatant made to 18% (v/v) ethanol and left for 3h. The precipitate, which was separated by centrifugation for 15min at 10000*g*, contained mainly components Ml_2 and Ml_3. The ethanol concentration was increased to 26% (v/v) and the precipitate discarded. The supernatant contained mainly components Ml_1 and Ml_4.

The 18%-ethanol precipitate and 26%-ethanol supernatant were separately run on DEAE-Sephadex A-25 columns, under conditions previously described (Perrie & Perry, 1970), to yield pure samples of each of the four low-molecular-weight components. In some cases the low-molecular-weight components were carbamoylmethylated before chromatographic separation, to eliminate the changes in the electrophoretic pattern that occurred with the unmodified light-chain fraction during lengthy chromatographic procedures.

Chemical modification of the light-chain components

Carbamoylmethylation with iodoacetamide was carried out as described by Weeds & Lowey (1971). For maleylation myosin was treated with maleic anhydride under the conditions described by Sia & Horecker (1968).

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel slab electrophoresis was performed in systems containing 8M-urea or 40% (v/v) glycerol as previously described (Perrie & Perry, 1970), by a modification of the method of Akroyd (1967). An upper buffer tank was designed to eliminate the need for cumbersome filter-paper wicks.

The gel (6.25cm×20cm) was cast between the front face of the upper buffer tank and a 22.5cm×7.5cm glass plate held in the vertical position by two spring clips and sealed at the bottom with plasticine. The thickness of the gel was determined by the thickness of 7.5cm×1.25cm Perspex spacers (normally 0.15cm). After removal of the plasticine the upper tank was supported on the lower tank by the two spring clips and buffer was poured into the upper tank to within 0.3cm of the top. For molecular-weight determination by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis, an upper tank with a 15cm deep front (13.7cm gel) was used.

Polyacrylamide-gel slab electrophoresis in the presence of sodium dodecyl sulphate was carried out in the 100mM-sodium phosphate buffer, pH7.0, system described by Weber & Osborn (1969) or in 85mM-Tris–400mM-boric acid buffer, pH7.0. For an identical gel slab, an electrophoresis run in the Tris–borate buffer system required less than one-third of the time needed for the sodium phosphate buffer system. Samples for both types of gel were dissolved in 10M-urea containing 0.1M-β-mercaptoethanol and adjusted to a protein concentration of 2mg/ml for the most intensely staining band. The urea concentration was then decreased to 6M by the addition of 5% (v/v) sodium dodecyl sulphate in 50mM-sodium phosphate buffer, pH7, and the sample incubated for 60min at 50°C.

Both types of gel were washed for at least 3h in water–methanol–acetic acid (12:7:1, by vol.) before staining for 30min with 0.15% Coomassie Brilliant Blue in the same solvent. Gels were also destained in this solvent. The relatively high concentration of methanol was used to prevent excessive swelling of low-strength gels.

Reaction of myosin with 5,5′-dithiobis-(2-dinitrobenzoic acid)

Myosin was treated with 5,5′-dithiobis-(2-dinitrobenzoic acid) under the conditions described by Weeds (1969).

Enzymic interconversion of the low-molecular-weight components of myosin

(*a*) *By sarcoplasm.* Sarcoplasm, prepared in sucrose solution, as previously described (Perrie & Perry,

1970) was dialysed against two changes of 0.28M-KCl adjusted to pH7.0 with 0.1M-NaOH. A solution of myosin, after the final centrifugation in 0.28M-KCl, was adjusted to pH7.0 with 0.1M-NaOH and to a protein concentration of 10mg/ml. Two 5ml samples of the myosin solution were placed in separate test tubes and 5ml of sarcoplasm, in 0.28M-KCl, was added to one and 5ml of 0.28M-KCl was added to the other. The tubes were mixed, stoppered and incubated in a water bath at 37°C. Samples (1ml) were removed from these tubes at the time of mixing and at further intervals. Each 1ml sample was immediately diluted with 8ml of cold water to precipitate the myosin. The precipitates were dissolved in 8M-urea containing 10mM-β-mercaptoethanol and 20mM-Tris–125mM-glycine buffer, pH8.6, and dialysed against the same buffer, before electrophoresis.

(*b*) *By alkaline phosphatase.* Myosin, suspended in 0.04M-KCl, was added to 0.24M-Tris–glycine buffer, pH8.6, to give a final solution of 10mg of protein/ml in 0.15M-Tris–glycine buffer, pH8.6, containing 5mM-$MgCl_2$. Two 5ml portions of this solution were taken and 0.1ml of a solution of alkaline phosphatase (10mg/ml) in the same buffer was added to one of them. *Escherichia coli* alkaline phosphatase (1mg) (Sigma Chemical Co., St. Louis, Mo., U.S.A.) was added to 5ml of 0.15M-Tris–glycine buffer, pH8.6, in a separate test tube. All tubes were incubated in a water bath at 25°C for 2h. The reaction was stopped by the addition of solid urea to a final concentration of 8M.

(*c*) *By kinases.* A crude protein kinase preparation obtained by the method of Walsh *et al.* (1968) taken to the 32.5%-satd.-$(NH_4)_2SO_4$ precipitate stage was used in some of the earlier experiments. Most of the work was carried out with the phosphorylase *b* kinase preparation (40-precipitate fraction) obtained by the methods of Krebs *et al.* (1964). These preparations were incubated with myosin or light-chain fractions either under the optimum conditions described by the authors or as indicated in the legends or in the text.

Homogenization of muscle in guanidine hydrochloride

The biceps femoris muscle (approx. 10g) was removed from a rabbit (killed by a blow to the back of the neck) as rapidly as possible after death and immediately homogenized in 30ml of 6M-guanidine hydrochloride in a Waring Blendor. A light-component fraction was prepared by lowering the guanidine hydrochloride concentration to 2.5M by the addition of water followed by 2vol. of ice-cold ethanol. After flocculation the precipitate was removed by low-speed centrifugation and the supernatant, which contained the light components, was dialysed extensively against water and freeze-dried. The same muscle from the other leg was removed and kept in ice for 2–3h after death. It was then treated as described above.

Assays

Determination of ^{32}P. Bands were cut from polyacrylamide-gel slabs after destaining. Up to 0.2ml of gel was dissolved in 1ml of 100vol. H_2O_2 by heating at 80°C for 2h. The samples were made to a volume of 10ml with distilled water and radioactivity was measured by the Cerenkov method (Gould *et al.*, 1972) in a Philips model PW4510 liquid-scintillation analyser.

Amino acid and peptide analyses. Amino acid analyses were carried out with the Beckman 120B analyser as described by Wilkinson *et al.* (1972).

Determination of P_i. This was carried out on 1–5mg of purified protein heated with 0.1ml of 5M H_2SO_4 by the procedure of Fiske & Subbarow (1925). The final volume used for the assay was 2.5ml.

Determination of protein concentration. Protein was determined by a Nesslerization method described by Schaub *et al.* (1972).

Determination of ribose. Ribose was determined by the method of Brown (1946).

Cyanogen bromide cleavage of proteins

The method of Gross & Witkop (1962) was used. A portion (10mg) of component Ml_2 or Ml_3 was dissolved in 0.7ml of 99% formic acid and 0.3ml of water and 10mg of cyanogen bromide were added. The solution was left at room temperature for 24h and then taken to dryness in a vacuum dessicator over solid KOH. The fragments obtained were dissolved in 10ml of water and freeze-dried.

Peptide 'mapping' and purification

Purified ^{32}P-labelled component Ml_2 (35mg) prepared as described in Table 3 was dissolved in 3.5ml of 0.02M-*N*-ethylmorpholine–acetate buffer, pH8.0, and digested with 0.7mg of α-chymotrypsin at 37°C for 3h. High-voltage electrophoresis of the digest was done at pH6.5 on Whatman 3MM paper for 45min at 3kV (Michl, 1951; Ryle *et al.*, 1955). A 3cm strip was cut from the paper and electrophoresis in a direction perpendicular to the original performed at pH1.8 for 45min at 3kV. The peptide 'map', after staining with the cadmium–ninhydrin reagent of Heilmann *et al.* (1957), was exposed to X-ray film for 48h. The major radioactive peptide was isolated by cutting out the appropriate band from the pH6.5 electrophoretogram and purifying it by electrophoresis at pH3.5 for 2h at 3kV.

Amino acid sequencing

The dansyl–Edman technique (Gray, 1967) was used for N-terminal and sequence determination of

peptides, except that a 6h hydrolysis of the dansylated peptides was used as a routine. Dns-amino acids were identified by the methods of Woods & Wang (1967) by using their solvents 1, 2 and 3. For further degradation of the phosphorylated peptide, digestion with α-lytic protease of *Myxobacter* 495 [prepared in the laboratory of D. R. Whitaker (1965)] was carried out for 4h at 37°C in 0.05M-*N*-ethylmorpholine–acetate buffer, pH8.0, with an enzyme to peptide molar ratio of 1:50. The mobility of peptides was measured at pH6.5 and relative to aspartic acid. The charge of a peptide was determined by using the mobility–molecular-weight plots of Offord (1966). Carboxyl or amide groups were assigned for asparagine and glutamine on the basis of overall charge and amino acid composition unless otherwise specified.

Results

Effect of extraction conditions on the electrophoresis pattern of the low-molecular-weight components of myosin

In earlier studies (Perrie & Perry, 1970) it was noted that raising the ionic strength and the ratio of volume of extraction buffer to muscle increased the proportion of the Ml_2 to the Ml_3 light chains in myosin preparations from rabbit skeletal muscle. Although changing the conditions in this way increased the ionic strength and lowered the total muscle protein concentration it also increased the phosphate buffer concentration at which the muscle was extracted. This factor seemed of some importance, for if myosin was extracted in the range pH6–7 by solutions that did not contain phosphate, e.g. 0.6M-KCl, the Ml_2 component could not be detected in the myosin extract. If the concentration of potassium phosphate buffer, pH6.5, was varied over the range 0–0.15M and the total ionic strength maintained at approximately 0.6 by the addition of KCl the proportion of the myosin light chain of 18500 molecular weight that was in the Ml_2 form increased as the phosphate concentration rose. At the upper concentration of phosphate 25–35% of the light chain of 18500 molecular weight was present as the Ml_2 component. The addition of 10mM-sodium pyrophosphate, 0.1M-NaF (Fig. 1) or 10mM-ATP to the standard extraction buffer increased the amount of Ml_2 component present above that normally obtained.

Addition of 10mM-EDTA to the standard extraction buffer or lowering the pH to 5.5–6.0 gave a slight increase in the amount of Ml_2 component in the myosin preparation. No effect on the proportion of Ml_2 component present resulted from the addition of 10mM-β-mercaptoethanol to the standard extraction buffer. A slight decrease in the amount of component Ml_2 present in the myosin was obtained when the muscle was extracted at 20°C rather than at 1–2°C as was the normal practice.

When muscle was extracted with different ratios of muscle to standard extraction buffer i.e. that used in Fig. 1*h*, the relative amounts of the Ml_2 and Ml_3 components appeared similar in the myosin present in the soluble extract and that remaining in the muscle residue sedimented by centrifugation at 1500*g* for 15min.

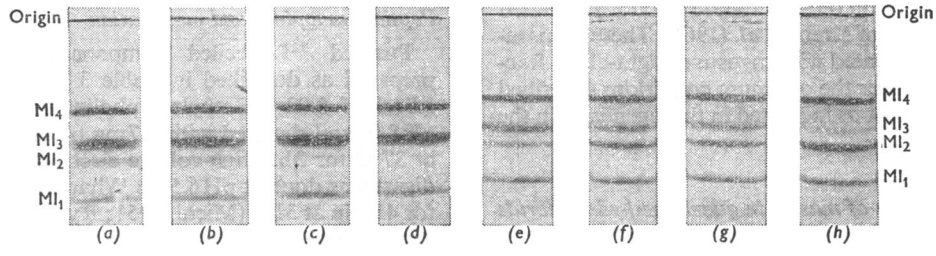

Fig. 1. *Polyacrylamide-gel electrophoresis of myosin extracted from rabbit skeletal muscle under different conditions*

All samples of myosin were dissolved in 20mM-Tris–125mM-glycine (pH8.6)–8M urea–0.1M-β-mercaptoethanol and 100μg in 20–30μl was applied to 10% polyacrylamide (Cyanogum 41) gel in 20mM-Tris–125mM-glycine (pH8.6)–8M-urea. Myosin preparations were extracted from 1 vol. of muscle mince with 3 vol. of buffer indicated below, except for (*h*), and further purified in the normal manner (see the Methods section). Experiments illustrated in (*a*)–(*d*) represent parallel extractions from the same muscle mince; those in (*e*)–(*h*) represent a series of extractions on minced muscle from another animal. (*a*) 0.15M-potassium phosphate buffer, pH6.5, 0.3M-KCl (Guba–Straub solution); (*b*) 0.1M-potassium phosphate buffer, pH6.5, 0.4M-KCl; (*c*) 0.05M-potassium phosphate buffer, pH6.5, 0.5M-KCl; (*d*) 0.6M-KCl; (*e*) 0.15M-potassium phosphate buffer, pH6.5, 0.3M-KCl; (*f*) as (*e*), +10mM-$Na_4P_2O_7$; (*g*) as (*e*), +0.1M-NaF; (*h*) 10vol. of Guba–Straub solution containing 10mM-$Na_4P_2O_7$ and 1mM-$MgCl_2$, 1vol. of muscle.

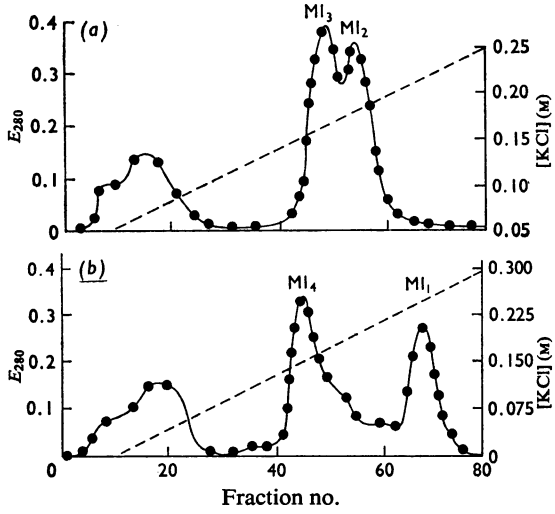

Fig. 2. *Ion-exchange chromatography of the light-chain fractions from myosin isolated from rabbit skeletal muscle*

Portions (100mg) of protein in 50ml of 30mM-KCl–25mM-Tris–HCl buffer (pH7.6)–10mM-β-mercaptoethanol were applied to a column (2.5cm×35cm) of DEAE-Sephadex A-25 equilibrated against the buffer solution. A linear gradient to 0.25M-KCl–25mM-Tris–HCl buffer, pH7.6, was applied for elution of components Ml_2 and Ml_3 and to 0.3M-KCl–25mM-Tris–HCl buffer (pH7.6)–10mM-β-mercaptoethanol for elution of components Ml_1 and Ml_4. Fractions (5ml) were collected. Components were in peaks indicated on the graphs. (*a*) Fraction containing Ml_2 and Ml_3 components obtained by precipitation of light-chain fraction with 18% ethanol (see the Methods section); (*b*) fraction containing Ml_1 and Ml_4 components, supernatant obtained after removal of components Ml_2 and Ml_3 as described in (*a*) by further precipitation with 26% ethanol (see the Methods section). ——, E_{280}; ----, KCl concentration.

Crude myosin extracted from freshly isolated myofibrils usually contained the 18500-molecular-weight component mainly in the Ml_3 form. This was a consequence of the state of the myosin in the myofibrils, for if the relative proportions of components Ml_2 and Ml_3 were estimated from the band patterns produced on dissolving myofibrils directly in 8M-urea and performing the electrophoresis in 8M-urea in 20mM-Tris–125mM-glycine buffer, pH8.6, a similar picture was obtained.

It was apparent from the above studies that none of the extraction conditions employed gave myosin in which all the 18500-molecular-weight light-chain component was present in the Ml_2 form. Extraction with 10vol. of standard buffer to 1vol. of muscle, the procedure otherwise being as described in the Methods section, was finally selected as the method used for preparing myosin of high component Ml_2 content as a compromise between yield of component Ml_2 and convenience. Usually this preparative procedure gave a myosin containing about 60–70% of the 18500-molecular-weight light-chain component in the Ml_2 form (Fig. 1*h*).

Analyses of components Ml_2 and Ml_3

Pure samples of components Ml_2 and Ml_3 were prepared from myosin isolated from mixed rabbit skeletal muscle by first separating the whole light-chain fraction and further splitting this by ethanol precipitation (see the Methods section) into two fractions, one containing components Ml_2 and Ml_3 and the other containing components Ml_1 and Ml_4. Both mixtures were then fractionated by separate column runs on DEAE-Sephadex A-25 (Fig. 2) to give samples of each of the four components that migrated as single bands of different mobility on polyacrylamide-gel electrophoresis in 25mM-Tris–125mM-glycine buffer, pH8.6 (Fig. 3). The individual light chains were obtained by combining the column fractions that were at least 95% pure as judged by the

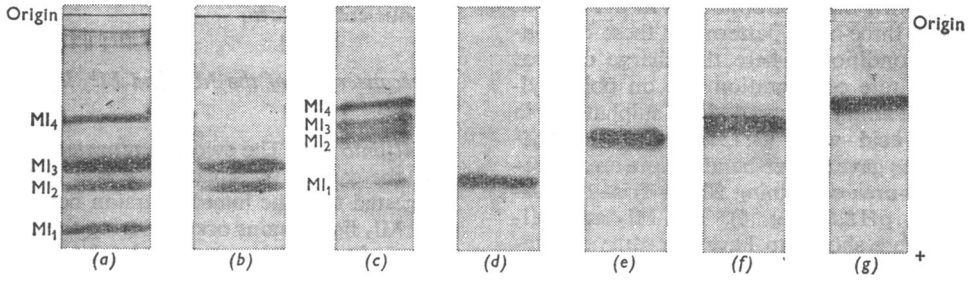

Fig. 3. *Polyacrylamide-gel electrophoresis of light-chain components of myosin from rabbit skeletal muscle.* Samples were applied to 10% Cyanogum 41 gels, in 20mM-Tris–125mM-glycine buffer, pH8.6, in all cases except (*a*) and (*b*) where 8M-urea was also present in the buffer and gel. (*a*) 100μg of whole myosin; (*b*) 5μg of 18% ethanol precipitate of whole light-chain fraction; (*c*) 5μg of whole light-chain fraction of myosin; (*d*) 5μg of component Ml_1; (*e*) 5μg of component Ml_2; (*f*) 5μg of component Ml_3; (*g*) 5μg of component Ml_4.

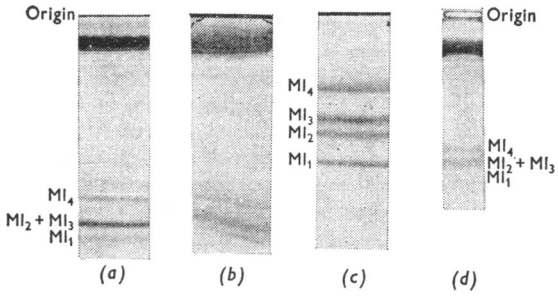

Fig. 4. *Comparison of electrophoretic patterns in the presence of sodium dodecyl sulphate and of urea with myosin isolated from rabbit white skeletal muscle*

(*a*) 200μg of whole myosin on 7% Cyanogum 41 gels in 85mM-Tris–400mM-boric acid buffer, pH7.0, 0.05% sodium dodecyl sulphate; (*b*) 100μg of maleylated myosin on 10% polyacrylamide gels in 20mM-Tris–125mM-glycine buffer (pH8.6)–8M-urea; (*c*) 100μg of myosin under same conditions as (*b*); (*d*) 100μg of myosin on 7% polyacrylamide gels in phenol–acetic acid–water (2:1:1, by vol.).

intensity of staining of electrophoretograms of each fraction. In the runs illustrated in Fig. 2 the myosin light components were reduced with 100mM-β-mercaptoethanol before fractionation. Under these circumstances u.v.-absorbing material that was not protein was eluted from the DEAE-Sephadex at low KCl concentrations. If all β-mercaptoethanol was removed before chromatography, e.g. when carboxymethylated light chains were separated, no non-protein u.v.-absorbing material was eluted. On electrophoresis in 100mM-phosphate buffer, pH7.0, or 85mM-Tris–400mM-boric acid buffer, pH7.0, containing 0.1% sodium dodecyl sulphate, components Ml_2 and Ml_3 moved as single bands of apparently identical mobility corresponding to a molecular weight of 18500.

Myosin from which a four-band light-chain pattern could be obtained on electrophoresis in 8M-urea at pH8.6 gave a three-band pattern for these components under conditions where the charge did not determine the rate of migration e.g. on polyacrylamide gels containing sodium dodecyl sulphate, or in phenol–acetic acid–water (2:1:1, by vol.); maleylated myosin also gave a three-band picture on electrophoresis in 8M-urea containing 20mM-Tris–125mM-glycine buffer, pH8.6 (Fig. 4). The Ml_2 and Ml_3 components were shown to have the same electrophoretic mobility in sodium dodecyl sulphate, pH 7.0, and in urea, pH8.6, after maleylation.

Amino acid analysis

The amino acid compositions of the Ml_2 and Ml_3 components were identical so far as could be judged from the analytical results but were clearly different from those of the Ml_1 and Ml_4 components, which also showed significant differences in composition between each other (Table 1). The analyses of the Ml_2 and Ml_3 components were in reasonable agreement with the light-chain fraction isolated from myosin of skeletal muscle by treatment with 5,5′-dithiobis-(2-nitrobenzoic acid), the so-called DTNB light chain (Weeds, 1969; Gazith *et al.*, 1970). A noticeable feature was the relatively high phenylalanine content that was reflected in the characteristic u.v.-absorption spectrum. The spectra of the Ml_2 and Ml_3 components were identical and different from those given by the Ml_1 and Ml_4 components, which also could not be distinguished from each other (Fig. 5). There was no evidence from the u.v. spectra of the presence of significant amounts of the purine or pyrimidine nucleotides in any of the light-chain components of myosin.

Total phosphorus analysis

Analysis for total phosphorus showed that the Ml_2 component contained approx. 1mol of phosphorus/18500g (Table 2). Less than 10% of this amount was present in the Ml_3 component and similar amounts of phosphorus were detected in the Ml_1 and Ml_4 components. It was not possible to decide whether these low values of phosphorus in components Ml_1, Ml_3 and Ml_4 were significant. The phosphorus was clearly tightly bound to the Ml_2 component, for it survived isolation and electrophoresis in strong urea and precipitation of the protein with 5% (w/v) trichloroacetic acid at 20°C. It possessed considerable acid stability, for initial treatment with 70% (v/v) formic acid for 24h at 20°C followed by dialysis and adjustment of the pH to 7.0 did not remove the phosphorus. These preliminary experiments suggested that the phosphorus was covalently linked to the protein, but the absence of ribose in the Ml_2 component and the appearance of the u.v. spectrum indicated that it was not present in the nucleotide form.

Interconversion of the Ml_2 and Ml_3 light-chain components

Ml_2 into Ml_3. The evidence reported in the present and the earlier investigation (Perrie & Perry, 1970) suggested that the interconversion between the Ml_2 and Ml_3 light chains occurs during the extraction of myosin from whole muscle. It has been noted (Perrie & Perry, 1970) that once the myosin has been isolated from muscle homogenates and partially purified, interconversion ceases. This implied that the whole homogenate contained systems, possibly enzymic, that were responsible for the process. Preliminary experiments in which the whole light-component frac-

Table 1. *Amino acid analyses of the light-chain components of rabbit white skeletal muscle*

Analyses were carried out on each preparation as described by Wilkinson *et al.* (1972) in duplicate after hydrolysis for 12h and 40h and the values for serine and threonine are extrapolated to zero time. The values for valine, leucine and isoleucine were taken from the average of the values obtained after 40h hydrolysis. Results are averaged for numbers of preparations indicated and expressed as residues per molecule.

Component ...	Ml_1	Ml_2	Ml_3	Ml_4	Ml_3*
Mol.wt. ...	15500	18500	18500	22500	18500
No. of preparations ...	2	5	5	2	
Lysine	10.65	15.90	15.64	20.61	15.77
Histidine	1.79	1.23	1.07	2.17	1.15
Arginine	4.56	6.26	6.48	5.37	6.37
Aspartic acid	16.18	22.95	23.33	21.78	23.14
Threonine	7.18	9.38	9.67	8.15	9.52
Serine	6.75	5.38	5.50	8.67	5.44
Glutamic acid	22.28	23.56	22.74	32.42	23.15
Proline	3.06	5.32	5.70	14.01	5.51
Glycine	11.76	12.91	13.45	13.27	13.18
Alanine	12.60	15.66	15.24	25.96	15.45
Valine	8.93	9.08	9.52	12.71	9.30
Methionine	5.92	6.29	6.51	7.04	6.40
Isoleucine	6.65	9.01	9.09	9.20	9.05
Leucine	11.35	9.34	9.56	15.30	9.45
Tyrosine	2.75	2.55	2.17	2.76	2.36
Phenylalanine	8.41	11.60	12.09	8.61	11.85

* Average of values for components Ml_2 and Ml_3 analysed independently and taken as composition of Ml_3.

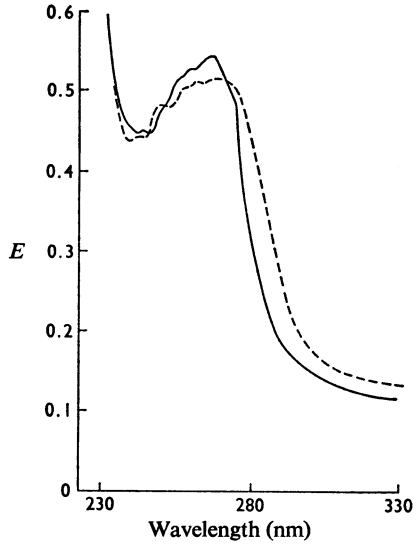

Fig. 5. *U.v. spectra of the light-chain components of myosin*

Spectra were obtained on pure samples of individual components isolated by chromatography on DEAE-Sephadex A-25 (see the Methods section). Samples were measured in 20mM-Tris–HCl, pH8.5, components Ml_1, Ml_2 and Ml_3 (each 1mg/l) and Ml_4 (1.3mg/ml). ——, Components Ml_1 and Ml_4; – – –, components Ml_2 and Ml_3.

Table 2. *Total phosphate content of light-chain components of myosin isolated from rabbit white skeletal muscle*

Light chains were isolated as described in the Methods section and as illustrated in Fig. 2. Determinations were carried out in duplicate on the numbers of preparations indicated in parentheses and the results were averaged.

Light-chain component	Mol.wt.	Content (mol of P/mol)
Ml_1	22500	0.06 (2)
Ml_2	18500	1.08 (4)
Ml_3	18500	0.09 (4)
Ml_4	15500	0.10 (2)

tion of myosin was incubated with a sarcoplasmic extract (see the Methods section) for several hours supported this view. A decrease in the amount of Ml_2 component and a complementary increase in the Ml_3 component was obtained under these conditions, although more prolonged incubation decreased the intensity of all the bands, probably owing to the endogenous catheptic activity of the sarcoplasm.

The most effective method for interconversion of component Ml_2 into Ml_3 was by incubation with a pure alkaline phosphatase of *E. coli* at pH8.6. Complete conversion of the Ml_2 into the Ml_3 component

was achieved in this way both with the separated light components (Fig. 6) and also with whole myosin. Similar results were obtained with an alkaline phosphatase from sheep intestinal mucosa but some degradation of the light-chain components occurred, probably owing to the contamination of this phosphatase preparation with proteolytic enzymes.

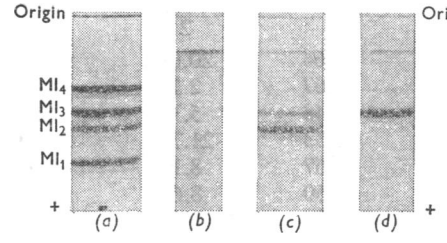

Fig. 6. *Gel electrophoresis of Ml_2 component from rabbit skeletal myosin after incubation with alkaline phosphatase from E. coli*

Samples were applied to 10% Cyanogum 41 gels in 20mM-Tris–125mM-glycine buffer (pH 8.6)–8M-urea. (*a*) 15μg of light-chain fraction of myosin; (*b*) 5μg of alkaline phosphatase from *E. coli*; (*c*) 15μg of component Ml_2+1.5μg of phosphatase at zero time; (*d*) as (*c*), after incubation for 2h at 25°C. See the Methods section for conditions.

Ml_3 into Ml_2. The combined evidence of the extraction, chemical, electrophoretic and enzymic studies strongly suggested that component Ml_2 was a phosphorylated form of the Ml_3 component. Attempts to phosphorylate the Ml_3 component were therefore carried out with a partially purified protein kinase fraction from the sarcoplasm of rabbit skeletal muscle (Walsh *et al.*, 1968). When the precipitate obtained with 32.5%-satd. $(NH_4)_2SO_4$ was incubated under the conditions described in Fig. 7 significant incorporation of ^{32}P was obtained in the Ml_2 component. The amounts of radioactivity in components Ml_1, Ml_3 and Ml_4 were much lower and of doubtful significance. Further purification of this protein kinase gave a preparation that was less effective in converting the Ml_3 into the Ml_2 component. This suggested that the protein kinase was not the enzyme responsible for the phosphorylation of the myosin light chain. Incubation of the isolated component Ml_3 with a phosphorylase *b* kinase preparation (see the Methods section) in 50mM-sodium glycerophosphate buffer (pH 6.5)–10mM-magnesium acetate – 10mM-NaF – 2mM-EGTA [ethanedioxybis(ethylamine)tetra-acetate], however, produced extensive conversion into component Ml_2 as judged by the appearance of the polyacrylamide gels.

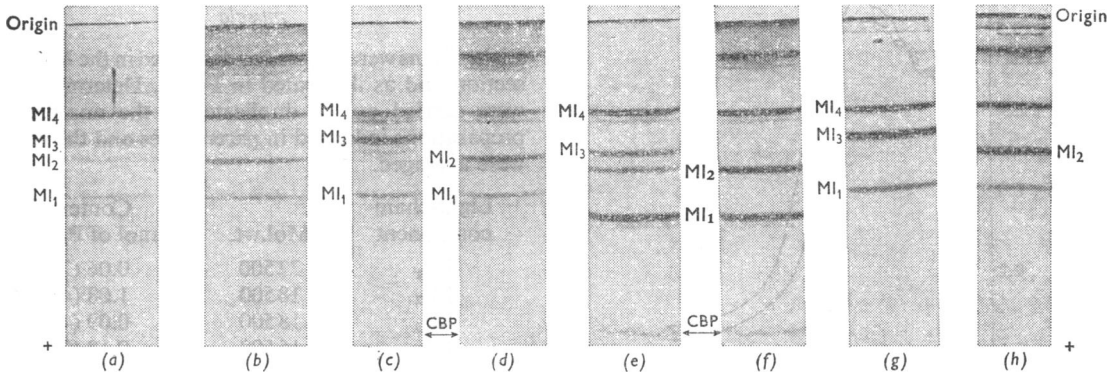

Fig. 7. *Polyacrylamide-gel electrophoresis of light-chain fractions of myosin after incubation with a kinase mixture*

Samples were incubated for 15min with 50mM-sodium glycerophosphate buffer (pH 6.5)–10mM-magnesium acetate–10mM-NaF–2mM-EGTA, 10μM-[γ-^{32}P]ATP (50μCi/μmol) and 0.2mg/ml of a mixture of equal amounts (estimated on an N basis) of phosphorylase *b* kinase and protein kinase. Myosin was added to a final concentration of 10mg/ml and whole light components to 1mg/ml. Electrophoresis was done on 10% Cyanogum 41 gels in 20mM-Tris–125mM-glycine buffer (pH 8.6)–8M-urea; 10μg of protein was applied to gels illustrated in (*a*)–(*f*) and 100μg to gels (*g*) and (*h*). In the crude light-chain fraction of myosin isolated directly from myofibrils, illustrated in (*e*) and (*f*), the very fast band (C B P) is the calcium-binding protein of the troponin complex (Perry *et al.*, 1972). (*a*) Light-chain fraction of myosin from rabbit skeletal muscle; (*b*) as (*a*) after incubation; (*c*) light-chain fraction of myofibril from rabbit skeletal muscle showing components Ml_1, Ml_3 and Ml_4 each with 'satellite' bands; (*d*) as (*c*) after incubation; (*e*) light-chain fraction of myofibrils from chicken breast muscle; (*f*) as (*e*) after incubation; (*g*) whole rabbit white skeletal-muscle myosin extracted with 0.6M-KCl; (*h*) as (*g*) after incubation.

Table 3. *Incorporation of ^{32}P into the Ml_3 light-chain component of myosin from rabbit white skeletal muscle*

A sample (20mg) of component Ml_3 that was free from component Ml_2 was incubated with [γ-^{32}P]ATP of known specific radioactivity for 60min under the conditions described in Fig. 7. The reaction was stopped by the addition of solid urea to give a concentration of 8M and component Ml_2 was separated by chromatography on DEAE-Sephadex as illustrated in Fig. 2. The specific radioactivity was determined on a sample taken from the top of the peak which migrated as a single band on electrophoresis in 8M-urea–20mM-Tris–125mM-glycine buffer, pH8.6.

Expt. no.	Component Ml_2 (mg)	Radioactivity (c.p.m.)	Content: P (μM)	Content: (mol of P/18500g)
1	4.36	30440	0.29	1.23
2	0.48	3487	0.029	1.12

Table 4. *Incorporation of ^{32}P into the light-chain components of myosin from skeletal muscle after incubation with mixed kinase preparation*

Results were obtained by cutting out bands corresponding to components indicated from actual gels illustrated in Fig. 7, except for the experiment (asterisked) with isolated rabbit myosin light components in which 100μg of protein was applied to the polyacrylamide gel. Otherwise 10μg of light components or 100μg of whole myosin was applied to gels. Slices were digested and ^{32}P measured as described in the Methods section. The listed values of c.p.m. are not corrected for background, which was usually 40–50c.p.m. when a slice of polyacrylamide gel alone was digested and radioactivity measured.

	Component	Incorporation (c.p.m.)
Isolated light components		
Rabbit white skeletal myosin (Fig. 9*b*)	Ml_1	50 60*
	Ml_2	129 849
	Ml_3	64 73
	Ml_4	52 65
Rabbit white skeletal myofibrils (Fig. 9*d*)	Ml_2	124
	Ml_3	53
Chicken white skeletal myofibrils (Fig. 9*f*)	Ml_2	99
	Ml_3	47
Whole myosin		
Rabbit white skeletal myosin (Fig. 9*h*)	Ml_2	1554
	Ml_3	74

In the experiment illustrated in Fig. 7 the mixture of kinases was incubated at pH6.5 in the presence of EGTA and Mg^{2+} to activate the phosphorylase *b* kinase and so that the myosin ATPase* activity was kept low and thus to maintain the concentration of [γ-^{32}P]ATP. Despite the low Ca^{2+} concentration considerable phosphorylation occurred both of myosin and of isolated light components. In another experiment with the mixed kinase system used for the experiments illustrated in Fig. 7 the amount of phosphate transferred from [γ-^{32}P]ATP of known specific radioactivity to a purified sample of component Ml_2 was determined. The results were compatible with the incorporation of 1mol of phosphate/18500g of Ml_2 component (Table 3). Incorporation was also obtained under the more usual conditions for phosphorylase *b* kinase activity, i.e. in the presence of Ca^{2+} at pH8.6. It was not necessary to separate Ml_3 component from myosin for phosphorylation to occur. When phosphorylase *b* kinase was incubated with a preparation of whole native rabbit myosin, the light-chain fraction of which contained only components Ml_1, Ml_3 and Ml_4, the Ml_3 component was virtually completely converted into the Ml_2 component, in which all the radioactivity was localized (Fig. 7*g*,*h* and Table 4). In some circumstances each of the light chains was accompanied by a faster, minor band, which we term a 'satellite' band (Fig. 7*c*). These 'satellite' bands were apparently some type of oxidation product, as they disappeared on treatment with β-mercaptoethanol. It was noted that on phosphorylation both component Ml_3 and its 'satellite' band could be converted into double-banded component Ml_2, presumably Ml_2 and its 'satellite' band (Fig. 7*c*,*d*).

* Abbreviation: ATPase, adenosine triphosphatase.

Myosin isolated from fowl skeletal muscle by using the standard extraction procedure shows four bands similar to the Ml_1, Ml_2, Ml_3 and Ml_4 light chains of rabbit skeletal myosin (Perrie & Perry, 1970). Although of slightly different electrophoretic mobility the Ml_2 and Ml_3 components of fowl white skeletal muscle appeared to be similar to their rabbit counterparts, for phosphorylation of fowl component Ml_3 to Ml_2 was shown to occur on incubation with phosphorylase *b* kinase from rabbit muscle and [γ-^{32}P]ATP (Table 3 and Fig. 7).

The electrophoretic behaviour of the light chains of rabbit cardiac myosin is significantly different from those of skeletal muscle (Perrie & Perry, 1970; Lowey & Risbey, 1971; Weeds & Pope, 1971; Sreter *et al.*, 1972). When isolated rabbit cardiac-muscle light components were incubated with the phosphorylase *b* kinase and [γ-^{32}P]ATP, under conditions that gave marked incorporation into the Ml_2 component of skeletal-muscle myosin at pH6.5, very little protein or radioactivity could be detected in the region at which the Ml_2 component of skeletal myosin migrated on electrophoresis of the light chains of rabbit cardiac myosin in 8M-urea, pH8.6. On electrophoresis in 45mM-Tris–400mM-boric acid buffer, pH7.0, containing 0.05% sodium dodecyl sulphate of the whole light-chain fraction of cardiac myosin after incubation with the phosphorylase *b* kinase system, radioactivity was detected in a minor band of mobility corresponding to a molecular weight of about 28000. The extent of incorporation was much less than that obtained with the Ml_2 component from rabbit skeletal muscle.

State of phosphorylation of myosin in vivo

From the results described above it was not possible to decide unequivocably whether the myosin in intact rabbit skeletal muscle possessed the light chain of molecular weight 18500 in the fully phosphorylated form or whether the phosphorylation occurred during the homogenization and general disorganization of the tissue associated with the preparation of myosin. Certainly if the former were the case rapid dephosphorylation occurred when extraction was carried out in the absence of added phosphate. Evidence was obtained that some phosphorylation of myosin could occur during extraction for if skeletal-muscle mince was extracted with 3vol. of 0.5M-KCl–0.1M-NaF in the presence of [γ-^{32}P]ATP, a small but significant fraction of the total radioactivity could be detected in the Ml_2 component.

When steps were taken to minimize enzymic activity by homogenizing skeletal muscle in 6M-guanidine hydrochloride within 1min of death (see the Methods section) only the Ml_2 component could be detected by the usual electrophoretic analysis of the whole muscle light-chain fraction (Fig. 8). If the intact muscle was kept in ice for 2h before similar treatment, component Ml_3 was present as well as the Ml_2 component.

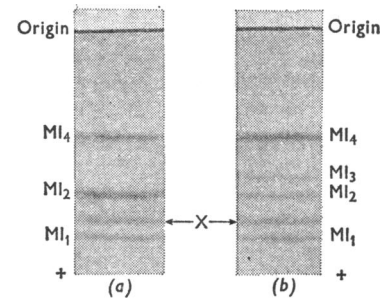

Fig. 8. *Polyacrylamide-gel electrophoresis of light-chain components of myosin isolated from rabbit biceps femoris immediately after death*

Whole muscle was homogenized in guanidine hydrochloride (see the Methods section), and electrophoreses in 10% Cyanogum 41 gel in 20mM-Tris–125mM-glycine buffer (pH8.6)–8M urea. Band X is not a myosin light-chain component but is either of sarcoplasmic origin or derived from the troponin complex and not removed by the fractionation procedure when applied to whole muscle. (*a*) 10μg of light-chain fraction isolated at death; (*b*) 10μg of light-chain fraction isolated 2h *post mortem*.

Isolation and analysis of the phosphate-containing peptide

Cyanogen bromide treatment. Electrophoresis, in 20mM-Tris–125mM-glycine buffer, pH8.6, on a 10% (w/v) polyacrylamide gel containing 40% (v/v) glycerol, of component Ml_3 after treatment with cyanogen bromide gave six main and one minor bands. After phosphorylation of the light-chain component with [γ-^{32}P]ATP and phosphorylase *b* kinase an additional band was observed. When gels were sliced so that radioactivity could be measured, ^{32}P was present only in the additional band. It was concluded that after phosphorylation and subsequent cyanogen bromide treatment the phosphorylated (derived from component Ml_2) and the corresponding dephosphorylated cyanogen bromide peptide (derived from component Ml_3) were present. This could be either due to incomplete phosphorylation of the original light component or partial breakdown of the phosphorylated peptide during the cyanogen bromide treatment.

Peptide 'mapping'. A chymotryptic digest of ^{32}P-labelled Ml_2 showed a single major radioactive spot which corresponded to a ninhydrin-positive peptide

Table 5. *Amino acid composition and sequence information on phosphate containing peptide isolated from a chymotryptic digest of purified light-chain component Ml_2*

Peptides 2a, 3a, 3b and 4 were isolated from a *Myxobacter* α-lytic protease digest of the chymotryptic peptide. The symbol → represents the identification of the DNS-amino acid at the appropriate step of the Edman degradation. Mobilities at pH6.5 were calculated relative to free aspartic acid ($m = -1$).

Peptide in *Myxobacter* protease digest	pH6.5 mobility	Calculated net charge	Analyses (molar ratios) and partial sequences
	−0.38	>−1 <−2	Arg- Ala-Ala- Ala- Glx-Gly-Gly- Ser- Ser- Asx -Val- Phe 1.33 0.90 0.90 0.90 1.10 0.93 0.93 0.80 0.80 1.36 0.85 1.18 → → → → → → → → → → → →
2a	0	0	Arg-(Ala,Ala,Ala,Glu,Gly) 0.64 1.06 1.06 1.06 1.06 1.11 →
3a	−0.66	−1	Ala-(Glu,Gly) 0.77 1.09 1.14 →
3b	−0.63	<−2	Gly-(Ser,Ser(P),Asn,Val,Phe) 0.99 0.96 0.97 0.78 1.23 1.07 →
4	−0.71	>−2 <−3	Ala-(Glu,Gly,Gly,Ser,Ser(P),Asn,Val,Phe) 1.08 1.08 1.00 1.00 0.85 0.86 1.26 1.08 0.86 →
			1 2 3 4 5 6 7 8 9 10 11 12
		Final sequence ...	Arg-Ala-Ala-Ala-Glu-Gly-Gly-(Ser,Ser(P))-Asn-Val-Phe

on a 'map' produced by high-voltage electrophoresis of the digest at pH6.5 and 1.8 (see the Methods section). Its mobility relative to aspartic acid at pH6.5 was −0.38 and relative to serine at pH1.8 was +0.47. The peptide was further purified by electrophoresis at pH3.5 where it migrated with the neutral amino acids. Two minor radioactive peptides were also detected but were estimated to represent less than 20% of the total radioactivity. The major radioactive peptide was eluted and 0.04 μmol was hydrolysed in 1M-HCl for 20h at 110°C. The presence of serine phosphate was confirmed by high-voltage electrophoresis of the hydrolysate at both pH6.5 and 3.5. In both systems a ninhydrin-positive spot was found with the same mobility as standard serine phosphate.

The amino acid composition of the peptide was determined and its sequence elucidated by the dansyl–Edman method (Table 5). When the products of the *Myxobacter* α-lytic protease digestion of 0.2 μmol of the peptide were subjected to electrophoresis at pH6.5 and 3.5, four peptides, 2a, 3a, 3b and 4, were recovered in adequate amounts and purity for amino acid and N-terminal analysis. The sequence results are summarized in Table 5. Assuming approximately 1.5 charges for serine phosphate at pH6.5 the mobility values for all peptides are consistent with the assignment of residue 5 as glutamic acid and of residue 10 as asparagine. The identity of the serine residue, either 8 or 9, to which the phosphate is attached has not been established.

Discussion

No completely satisfactory evidence has yet been presented for the existence of a labile phosphorylated derivative of myosin as an intermediate in the enzymic hydrolysis of ATP. Nevertheless in the past some effort has been directed to the detection of such an intermediate, and Tonomura *et al.* (1962) in particular have favoured a mechanism of hydrolysis of ATP involving the phosphorylation of myosin. It seems unlikely that myosin in which the 18500-molecular-weight light chain is phosphorylated corresponds to such an intermediate, for there is evidence that this light-chain component can be removed without loss of enzymic activity (Weeds, 1969; Gazith *et al.*, 1970; Weeds & Lowey, 1971). The phosphorylated form of myosin described in the present paper is also much more stable than that postulated by Kitagawa & Tonomura (1962), for example.

The phosphorylation of myosin appears to be a highly specific process involving a unique amino acid sequence in each of the two molecules of the light chain of molecular weight 18500 present in each molecule of myosin. Thus peptide 'mapping' of a

chymotryptic digest of the ^{32}P-labelled protein yielded only a single major radioactive peptide in which the phosphate was covalently attached to a serine residue. The amino acid sequence of this peptide shows no obvious similarity to the site phosphorylated in the conversion of phosphorylase *b* into *a* by phosphorylase *b* kinase (Nolan *et al.*, 1964). Although much of the enzymic work reported here is preliminary it is clear that there exists in skeletal-muscle tissue an enzymic system capable of transferring the γ-phosphate groups of ATP to a specific serine residue of the 18500-molecular-weight light-chain component.

The protein kinase of muscle does not appear to be the enzyme responsible, for although crude preparations of this enzyme phosphorylated the light-chain component of myosin in the presence of EGTA, further purification gave a preparation which was less effective in converting the MI_3 into the MI_2 component. If phosphorylase *b* kinase has a high specificity for the amino acid sequence at the phosphorylation site of phosphorylase *b*, it would seem unlikely that this kinase is responsible for the phosphorylation of the 18500-molecular-weight light-chain component of myosin, since the amino acid sequences in question bear no obvious similarity. Nevertheless more purified preparations of phosphorylase *b* kinase than those used in this study, which have been shown to phosphorylate the '37000 component' of the troponin complex (Perry & Cole, 1973), also phosphorylate the 18500-molecular-weight light chain of myosin from white skeletal muscle of the rabbit (Perrie *et al.*, 1973). The facts suggest that partially purified preparations of phosphorylase *b* kinase can phosphorylate the light chain of myosin, but further investigation is necessary to identify precisely the enzyme that is concerned in phosphorylating this component.

Irrespective of the identity of the enzyme involved the results obtained indicate that myosin is fully phosphorylated in the intact resting muscle. More careful study of whole functioning muscle is required, however, to determine whether cyclical changes in phosphorylation occur during the contraction–relaxation cycle. Much of the enzymic work in the present investigation was carried out with the isolated light components; nevertheless phosphorylation and dephosphorylation of the intact myosin molecule can occur when it is either in solution or localized in the myofibril on the A filament. This is evident from the experiments with the kinase preparations and the fact that myosin present in the intact myofibril is found in various states of phosphorylation.

Clearly an enzyme or enzymes exist in skeletal muscle for dephosphorylation of the phosphorylated light chain. It is assumed that this may be the protein phosphatase present in skeletal muscle that dephosphorylates phosphorylase *b* kinase (Krebs *et al.*, 1959), phosphorylated components of the troponin complex (England *et al.*, 1972) and possibly other phosphorylated protein in muscle. At this stage the existence of a protein phosphatase specific for myosin cannot be excluded, for in at least one case an enzyme specific for a phosphorylated protein has been reported (Siess & Wieland, 1972). In all cases in which phosphatase inhibitors such as P_i, NaF, ATP, sodium pyrophosphate etc. were added to the extraction medium the relative amount of component MI_2 to MI_3 increased.

Even though the light chain that is phosphorylated is apparently not essential for ATPase activity, light chains of similar electrophoretic mobility to those present in myosin from rabbit skeletal muscle occur in the myosin from skeletal muscles of other species examined. The cardiac myosin light chains are clearly different and the significance of the low incorporation of ^{32}P into the faint band corresponding to a molecular weight of about 28000 has yet to be determined. We believe that it is a contaminant rather than one of the light-chain components of cardiac myosin, which we report (Perrie *et al.*, 1973) as possessing molecular weights of 19000 and 24000. It is more probably a proteolytic fragment of the troponin-T component of the troponin complex which has been shown to be phosphorylated by phosphorylase kinase (Perry & Cole, 1973; W. T. Perrie, L. B. Smillie & S. V. Perry unpublished work). In the rabbit at least, cardiac myosin as isolated by our procedures does not contain a component of 18500 molecular weight that is readily phosphorylated by the phosphorylase *b* kinase from skeletal muscle. It is possible that the light-chain component of cardiac muscle of similar molecular weight to the component of 18500 molecular weight in skeletal-muscle myosin is normally isolated in the fully phosphorylated form and therefore does not incorporate additional phosphate when incubated with phosphorylase *b* kinase and [γ-^{32}P]-ATP. Until this possibility is eliminated it cannot be concluded with certainty that the light chain of molecular weight of 18000–19000 that occurs in a phosphorylated form is a special feature of the myosin present in skeletal muscle.

In the other examples of enzymes that have been reported to exist in phosphorylated forms a regulatory function for the enzymic modification has been suggested. For example phosphorylation increases the enzymic activity of phosphorylase *b* kinase (Krebs *et al.*, 1966) but decreases the activity of pyruvate dehydrogenase (Linn *et al.*, 1969). The limited studies so far carried out (Perrie & Perry, 1970), however, indicate that phosphorylation of the light chain of 18500 molecular weight does not significantly affect the Ca^{2+}-stimulated ATPase of myosin. In the cell, however, the myofibrillar ATPase is of the Mg^{2+}-stimulated actomyosin type and regulated by complex interactions with the troponin complex and with

tropomyosin. It is possible that, in fast muscle at least, phosphorylation of the myosin may modify the complex interactions involved in this system.

Preliminary reports (Bailey & Villar-Palasi, 1971; Stull *et al.*, 1972; Pratje & Heilmeyer, 1972; Perry & Cole, 1973) have indicated that the inhibitory protein and the '37000 component' of the troponin complex can also be phosphorylated, but as yet no role has been ascribed to this process. The implications of the existence of mechanisms for the phosphorylation and dephosphorylation of several of the protein components of a system that is regulated by changes of the Ca^{2+} concentration are obvious. In mammals the troponin components are all localized on the I filament in association with actin, but it is becoming clear that the troponin-type system associated with actin is not the only type of regulatory mechanism in muscle. The work of Lehman *et al.* (1972) has shown that in molluscan adductor muscle a light-chain component of myosin can play a regulatory role involving Ca^{2+} binding. There is no evidence so far of phosphorylation in this system and none relating the light chain of molluscan myosin that regulates the actomyosin activity in molluscan muscle to the phosphorylated light chain of myosin in higher animals. It is worth speculating, however, that the molluscan system may represent a primitive regulatory mechanism which in the higher animals has been supplanted or possibly complemented by the I-filament-localized system. Certainly Ca^{2+} binding by myosin would be increased by phosphorylation of the light chain, but further investigation is necessary to decide if indeed this system is involved in the regulation of the myofibrillar ATPase.

Note added in proof (*received* 19 *July* 1973)

An enzyme preparation has now been isolated from crude phosphorylase *b* kinase that is neither identical with the latter enzyme nor with protein kinase but that possesses activity in phosphorylating the M_3 light-chain component of myosin. This enzyme requires Ca^{2+} for full activity and has provisionally been named 'myosin light-chain kinase'. Unlike phosphorylase *b* kinase it does not phosphorylate troponin (E. M. V. Pires, M. A. W. Thomas, H. A. Cole & S. V. Perry, unpublished work).

The work was supported in part by research grants from the Medical Research Council, the Muscular Dystrophy Associations of America Inc., and during the tenure of a Medical Research Council of Canada Visiting Scientist award to L. B. S.

References

Akroyd, P. (1967) *Anal. Biochem.* **19**, 339–410
Bailey, C. & Villar-Palasi, C. (1971) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **30**, 1147
Brown, A. H. (1946) *Arch. Biochem. Biophys.* **11**, 269–278
England, P. J., Stull, J. T. & Krebs, E. G. (1972) *J. Biol. Chem.* **247**, 5275–5277
Fiske, C. H. & Subbarow, Y. (1925) *J. Biol. Chem.* **66**, 375–400
Gazith, J., Himmelfarb, S. & Harrington, W. F. (1970) *J. Biol. Chem.* **245**, 15–22
Gould, J. M., Cather, R. & Winget, G. D. (1972) *Anal. Biochem.* **50**, 540–548
Gray, W. R. (1967) *Methods Enzymol.* **11**, 469–475
Gross, E. & Witkop, B. (1962) *J. Biol. Chem.* **237**, 1856–1860
Heilmann, J., Barollier, J. & Watzke, E. (1957) *Hoppe-Seyler's Z. Physiol. Chem.* **309**, 219–220
Kitagawa, S. & Tonomura, Y. (1962) *Biochim. Biophys. Acta* **57**, 416–418
Krebs, E. G., Graves, D. J. & Fischer, E. H. (1959) *J. Biol. Chem.* **234**, 2867–2873
Krebs, E. G., Love, D. S., Bratvold, G. E., Trayser, K. A., Meyer, W. L. & Fischer, E. H. (1964) *Biochemistry* **3**, 1022–1033
Krebs, E. G., Delange, R. J., Kemp, R. G. & Riley, W. D. (1966) *Pharmacol. Rev.* **18**, 163–171
Lehman, W., Szent-Gyorgyi, A. G. & Kendrick-Jones, J. (1972) *Cold Spring Harbor Symp. Quant. Biol.* **37**, 319–330
Linn, T. C., Pettit, F. H. & Reid, L. J. (1969) *Proc. Nat. Acad. Sci. U.S.* **62**, 234–241
Lowey, S. & Risbey, D. (1971) *Nature (London)* **234**, 81–85
Michl, H. (1951) *Monatsh. Chem.* **82**, 489–498
Nolan, C., Novoa, W. B., Krebs, E. G. & Fischer, E. H. (1964) *Biochemistry* **3**, 542–551
Offord, R. E. (1966) *Nature (London)* **211**, 591–593
Paterson, B. & Strohman, R. C. (1970) *Biochemistry* **9**, 4094–4105
Perrie, W. T. & Perry, S. V. (1970) *Biochem. J.* **119**, 31–38
Perrie, W. T., Perry, S. V. & Stone, D. (1969) *Biochem. J.* **113**, 28P–29P
Perrie, W. T., Smillie, L. B. & Perry, S. V. (1972*a*) *Biochem. J.* **128**, 105P–106P
Perrie, W. T., Smillie, L. B. & Perry, S. V. (1972*b*) *Cold Spring Harbor Symp. Quant. Biol.* **37**, 17–18
Perrie, W. T., Thomas, M. A. W. & Perry, S. V. (1973) *Biochem. Soc. Trans.* **1**, 860–861
Perry, S. V. & Cole, H. A. (1973) *Biochem. J.* **131**, 425–428
Perry, S. V., Cole, H. A., Head, J. F. & Wilson, F. J. (1972) *Cold Spring Harbor Symp. Quant. Biol.* **37**, 251–262
Pratje, E. & Heilmeyer, L. M. G. (1972) *FEBS Lett.* **27**, 89–93
Ryle, A. P., Sanger, F., Smith, L. F. & Kitai, R. (1955) *Biochem. J.* **60**, 541–565
Schaub, M. C. & Perry, S. V. (1971) *Biochem. J.* **123**, 367–377
Schaub, M. C., Perry, S. V. & Hacker, W. (1972) *Biochem. J.* **126**, 237–249
Sia, C. L. & Horecker, B. L. (1968) *Biochem. Biophys. Res. Commun.* **31**, 731–737
Siess, E. A. & Wieland, O. H. (1972) *Eur. J. Biochem.* **26**, 96–105
Sreter, F. A., Sarkar, S. & Gergely, J. (1972) *Nature (London)* **239**, 124–125
Starr, R. & Offer, G. (1971) *FEBS Lett.* **15**, 40–44

Stull, J. T., Brostrom, C. O. & Krebs, E. G. (1972) *J. Biol. Chem.* **247**, 5272–5374
Tonomura, Y., Kitagawa, S. & Yoshimura, J. (1962) *J. Biol. Chem.* **237**, 3660–3666
Walsh, D. A., Perkins, J. P. & Krebs, E. G. (1968) *J. Biol. Chem.* **243**, 3763–3764
Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
Weeds, A. G. (1969) *Nature (London)* **223**, 1362–1364
Weeds, A. G. & Lowey, S. (1971) *J. Mol. Biol.* **61**, 701–725
Weeds, A. G. & Pope, B. (1971) *Nature (London)* **234**, 85–88
Whitaker, D. R. (1965) *Can. J. Biochem.* **43**, 1935–1945
Wilkinson, J. M., Perry, S. V., Cole, H. A. & Trayer, I. P. (1972) *Biochem. J.* **127**, 215–228
Woods, K. R. & Wang, K.-T. (1967) *Biochim. Biophys. Acta* **133**, 369–370