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Polymorphism of myosin light chains

An electrophoretic and immunological study of rabbit skeletal-muscle myosins

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Antibodies specific for rabbit fast-twitch-muscle myosin LC1F light chain were purified by affinity chromatography and characterized by both non-competitive and competitive enzyme-linked immunosorbent assay (ELISA) and a gel-electrophoresis-derived assay (GEDELISA). The antibodies did not cross-react with myosin heavy chains, and were weakly cross-reactive with the LC2F [5,5′-dithio-(2-nitrobenzoic acid)-dissociated] light chain and with all classes of dissociated light chains (LC1Sa, LC1Sb and LC2S), as well as with the whole myosin, from hind-limb slow-twitch muscle. The immunoreactivity of myosins with a truly mixed light-chain pattern (e.g. vastus lateralis and gastrocnemius) correlated with percentage content of fast-twitch-muscle-type light chains. A more extensive immunoreactivity was observed with diaphragm and masseter myosins, which were also characterized, respectively, by a relative or absolute deficiency of LC1Sa light chain. Furthermore, it was found that the LC1Sb light chain of masseter myosin is antigenically different from its slow-twitch-muscle myosin analogue, and is immunologically related to the LC1F light chain. Rabbit masseter muscle from its metabolic and physiological properties and the content, activity and immunological properties of sarcoplasmic-reticulum adenosine triphosphatase, is classified as a red, predominantly fast-twitch, muscle. Therefore our results suggest that the two antigenically different iso-forms of LC1Sb light chain are associated with the myosins of fast-twitch red and slow-twitch red fibres respectively.

Antibodies directed against myosin light chains have been used as probes for investigating the structural relationship among the several classes of these peptides (Holt & Lowey, 1975*a*,*b*; Silberstein & Lowey, 1977, 1981; Walliman & Szent-Györgyi, 1981) and the information thus gained has complemented that obtained by structural sequence studies (Frank & Weeds, 1974; Weeds, 1976). By this immunological approach, an extensive homology has been demonstrated between the two forms of alkali light chains (LC1F and LC3F) associated with vertebrate fast-twitch-muscle myosin (Holt & Lowey, 1975*b*; Biral *et al.*, 1979), and antigenic differences have been shown between fast-twitch-muscle-type light chains and their slow-twitch-muscle analogues (Gauthier & Lowey, 1979; Margreth *et al.*, 1980; Volpe *et al.*, 1981).

These antigenic differences have been instrumental in studies of myosin polymorphism according to fibre types, by testing antibody specific for the fast-twitch-muscle-type light chains, either on frozen muscle sections (Gauthier & Lowey, 1979) or with the isolated myosins from homogeneous fast-twitch and slow-twitch muscles, by an enzyme-linked solid-phase substrate immunoassay (Volpe *et al.*, 1981).

Two forms of LC1S light chain (LC1Sa and LC1Sb), functionally homologous to fast-twitch-muscle alkali-dissociated light chains, are known to be associated with rabbit slow-twitch-muscle myosin (Weeds, 1976), but their immunological relationship has been little investigated, as compared with their fast-twitch-muscle counterparts. Since preliminary observations made in this laboratory (Margreth *et al.*, 1980), however, indicated that rabbit myosin LC1Sa and LC1Sb light chains might be less

Abbreviations used: ELISA, enzyme-linked immunosorbent assay; GEDELISA, gel-electrophoresis-derived-enzyme-linked immunosorbent assay; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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immunologically interrelated than the LC1F and LC3F light chains, it was considered desirable to investigate this problem in more detail.

In the present investigation, the myosins and all classes of dissociated light chains were investigated for cross-reactivity with antibody specific for rabbit LC1F light chain, in both pure fast-twitch and slow-twitch muscles and in mixed muscles. Besides a non-competitive enzyme-linked immunosorbent assay (Biral *et al.*, 1979; Margreth *et al.*, 1980; Volpe *et al.*, 1981), a competitive immunoassay (two-step ELISA) was used, having a degree of sensitivity comparable with that of competitive radioimmunoassay, which was employed to advantage in myosin studies (Walliman & Szent-Györgyi, 1981).

Our results extend our previous observations by showing that all classes of the dissociated myosin light chains from slow-twitch muscle of the hind-limbs are weakly cross-reactive with anti-(rabbit LC1F) antibody. Furthermore, they provide novel evidence that an antigenically different LC1Sb light chain iso-form, shown to be immunologically related to the LC1F light chain, is associated with the myosin of certain mixed muscles, the masseter in particular.

Materials and methods

Preparative procedure

Myosin and sarcoplasmic-reticulum fragments were isolated from skeletal muscles of male adult New Zealand White rabbits. The muscles used were representative fast-twitch (adductor magnus) and slow-twitch (soleus and semitendinosus) muscles, and the following mixed muscles: crureus, vastus lateralis, gastrocnemius (all hind-limb muscles), flexor digitorum profundus (fore-limb muscle), diaphragm and masseter muscle. Anatomical identification of the muscles was based on the descriptions by Barone *et al.* (1973).

Myosin was prepared from fresh muscle as described previously (Volpe *et al.*, 1981), in the presence of pepstatin (200μg/litre) in the extraction and purification media. Myosin was stored in 5mM-Tris/HCl buffer (pH 7.6) / 25mM-KCl / 0.05mM-dithiothreitol / 50% (v/v) glycerol at −20°C, unless immediately used.

Myosin light chains were isolated and purified by the method of Lowey & Holt (1972), as reported previously (Biral *et al.*, 1979; Volpe *et al.*, 1981). Total light chains were isolated from the myosins of fast-twitch muscle (adductor), slow-twitch muscle (combined soleus and semitendinosus) and masseter muscle, by incubation in 4M-urea/25mM-Tris/HCl buffer (pH 8.0)/2.5mM-dithiothreitol/2.5mM-EDTA for 30min at room temperature. After precipitation of the heavy chains by dilution with 10vol. of water and centrifugation at 20000**g** for 10min, the light chains (about 100mg of protein) were fractionated by DEAE-cellulose (Whatman DE-52) column chromatography and a linear phosphate gradient (0.05–0.35M). The course of the fractionation was followed by continuous monitoring of the absorption at 280nm and 260nm in an LKB Uvicord III spectrophotometer. When separation of light chains appeared to be incomplete, the light chains from the corresponding fractions were precipitated by 80% saturation with $(NH_4)_2SO_4$ and rechromatographed. The degree of purity of the light chains was assessed by disc-gel electrophoresis in the testing range 2–5μg of protein applied per gel. The purified light chains were stored in 50mM-sodium phosphate buffer (pH 7.2)/75mM-NaCl/ 50% (v/v) glycerol at −20°C.

Purified sarcoplasmic-reticulum fragments were isolated from slow-twitch and fast-twitch muscle and from the masseter muscle and were fractionated by sucrose-density-gradient centrifugation, as described by Damiani *et al.* (1981). All fractions were immediately used for enzyme assays, or after being stored in 0.3M-sucrose/1mM-Hepes/NaOH buffer, pH 7.5, at −20°C for polyacrylamide-gel electrophoresis.

Electrophoresis

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of myosin was performed (i) by Laemmli's (1970) method, in 15% polyacrylamide 0.75mm-thick slab gels, or (ii) by disc-gel electrophoresis (Weber & Osborn, 1969), in 12.5% polyacrylamide gels buffered at pH 7.0 with either 0.1M-sodium phosphate or 0.4M-sodium borate buffer (Perrie *et al.*, 1973). Two-dimensional electrophoresis, by the procedure of O'Farrel (1975), was performed under conditions identical with those described by Volpe *et al.* (1981).

Two-dimensional electrophoresis of sarcoplasmic-reticulum fragments was performed as described by Michalak *et al.* (1980), with 10% polyacrylamide gels in both dimensions.

Densitometry of the light-chain peak areas was done either by a photographic method in the case of slab gels (Volpe *et al.*, 1981), or by scanning in a Gilford spectrophotometer at 550nm in the case of disc-gels (see under 'GEDELISA' below).

Preparation of antisera and antibody purification

Antibodies to purified rabbit LC1F light chain were raised in adult hens by repeated intramuscular injections of the antigen. The preparation of antisera and purification of antibodies specific for rabbit LC1F light chain by affinity chromatography have been described previously (Volpe *et al.*, 1981). The batch of antibody used in the present study was from

a single bleeding, after 6 months from the beginning of the immunization.

Antibodies against fast-twitch-muscle sarcoplasmic-reticulum Ca^{2+}-activated ATPase were raised in adult hens, by following the immunization schedule reported previously (Damiani *et al.*, 1981). Antisera used in the present study were obtained from a single bleeding, after 70 days from the beginning of the immunization. The immunoglobulin G fraction was purified from the antiserum by an Na_2SO_4 precipitation method (Orlans *et al.*, 1961).

GEDELISA

GEDELISA of the myosin was performed by a method derived from that described by Lutz *et al.* (1978). Electrophoresis of myosin was performed in duplicate, by the procedure of Weber & Osborn (1969), in 12.5% polyacrylamide gels: one gel was stained with Coomassie Blue for densitometric measurements; the other gel was cut into 1.5mm slices, which were incubated for 48h at room temperature in 1 ml of 0.1 M-sodium carbonate buffer (pH 9.6)/0.01% NaN_3, in polystyrene tubes. Binding of anti-(rabbit LC1F) antibody was determined by one-step ELISA, as described below.

One-step (non-competitive) ELISA

The immunoassay, adapted from the method described by Voller *et al.* (1976), was essentially as described by Biral *et al.* (1979). For coating of microtitre wells, antigens were diluted from the respective solution into 0.1 M-sodium carbonate buffer (pH 9.6)/0.01% NaN_3, at a final constant concentration of 5 μg/ml, and allowed to adsorb on the wells at 37°C for 60 min. After a washing with 0.15 M-NaCl / 0.05% Tween 20 / 0.02% NaN_3, 0.2 ml of diluted affinity-purified antibody for rabbit LC1F light chain was added {0.1–10 μg/ml, in PBS buffer [0.1 M-sodium phosphate buffer (pH 7.2)/ 0.15 M-NaCl/0.05% Tween 20/0.02% NaN_3]}. After an incubation period of 60 min at 37°C and a further washing with 0.15 M-NaCl solution, 0.2 ml of diluted affinity-purified anti-(chicken immunoglobulin G) antibodies, conjugated to alkaline phosphatase, in PBS buffer was added. The phosphatase activity bound to the wells was determined by incubation for 60 min at 37°C with *p*-nitrophenyl phosphate and measuring the A_{400} after the reaction had been stopped with alkali.

Two-step (competitive) ELISA

The general conditions were derived from Rennard *et al.* (1980), and, for sarcoplasmic-reticulum Ca^{2+}-activated ATPase, were identical with those described by Damiani *et al.* (1981). For study of myosins and of the dissociated light chains, an anti-(rabbit LC1F) antibody–LC1F light chain system was used. Portions (0.65 ml) of affinity-purified antibody in PBS buffer (10 μg/ml) were premixed overnight at 0–4°C with an equal volume of myosin (5–200 μg/ml) or light chain (0.001–10 μg/ml) solutions, in the same buffer. Samples (0.2 ml) of the mixtures were then added to individual microtitre wells that had been previously coated with LC1F light chain, at a constant coating concentration of 5 μg/ml, and were incubated at 37°C for 30 min. All subsequent steps were performed as in one-step ELISA.

Enzyme assays

ATPase activity was measured by a spectrophotometric enzyme-linked assay (Warren *et al.*, 1974) at 37°C, in a final volume of 3 ml, in the presence of 0.15 mM-NADH, 0.5 mM-phosphoenolpyruvate, 5 units (μmol/min) of pyruvate kinase and 5 units (μmol/min) of lactate dehydrogenase. Basal ATPase activity was measured in a medium of the following composition: 20 mM-histidine/HCl (pH 7.2)/100 mM-KCl/5 mM-$MgSO_4$/2 mM-ATP/0.2 mM-EGTA. The reaction was initiated by adding 10–50 μg of sarcoplasmic-reticulum protein. Extra Ca^{2+}-activated ATPase activity was measured after the addition of 0.2 mM-$CaCl_2$ in the presence of the Ca^{2+} ionophore A23187 (1 μg/ml).

Cytochrome b_5 was determined by differential spectrometry (with dithionite as the reducing agent), as previously described (Salviati *et al.*, 1979).

Protein concentration was determined as described by Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Characterization of antibodies specific for rabbit LC1F light chain

Affinity-purified antibodies to LC1F light chain were tested with fast-twitch-muscle myosin by GEDELISA (Fig. 1*a*) and found to be totally unreactive with the heavy-chain component and with any other electrophoretic component other than the light chains. As determined by this method, the heterologous LC2F light chain was found to be weakly cross-reactive with the immunogen and less so with the LC3F light chain. Similar results were obtained by testing the free light chains for cross-reactivity with these antibodies, by either non-competitive (Fig. 1*b*) or competitive ELISA (Fig. 1*c*). Under the latter conditions, the concentration of LC2F light chain required for 50% inhibition of binding of anti-(rabbit LC1F) antibody to LC1F-coated mitrotitre wells was found to be 50-fold higher, as compared with the homologous light chain. Consequently, these antibodies appeared to be directed mainly to antigenic determinants unique to the alkali-dissociated light chains (LC1F and LC3F) and, only to a limited extent, to structural sequence

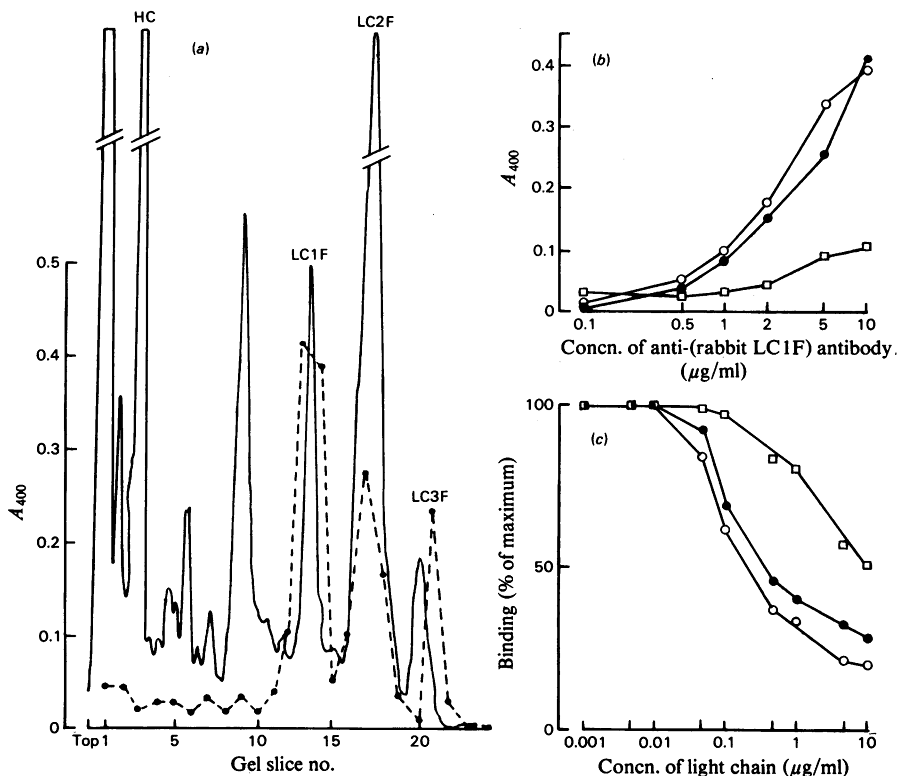

Fig. 1. *Cross-reactivities of anti-(rabbit LC1F) antibody with rabbit fast-twitch-muscle myosin light chains*
(*a*) Protein densitometric profile (——) and GEDELISA (----) of rabbit fast-twitch-muscle myosin, after electrophoresis in 12.5% polyacrylamide gels by the procedure of Weber & Osborn (1969). Two gels, loaded with 50 μg of protein, were run in parallel. For experimental details see the Materials and methods section. Key: HC, myosin heavy chains; LC, myosin light chains, as indicated. (*b*) One-step ELISA of the dissociated LC1F, LC2F and LC3F light chains. Anti-(rabbit LC1F) antibody solution, at the concentrations indicated on the abscissa, was tested in microtitre wells coated with each type of light chain (5 μg of protein/ml). ○, LC1F light chain; □, LC2F light chain; ●, LC3F light chain. (*c*) Competitive ELISA of the dissociated light chains. In the first step, a solution of anti-(rabbit LC1F) antibody (10 μg/ml) was preincubated with an equal volume of light-chain solution, at the protein concentrations indicated on the abscissa. The second step was performed with microtitre wells coated with LC1F light chain (5 μg/ml) (see the Materials and methods section). Each point is the average value of duplicate determinations. Symbols are as in (*b*).

or configurational determinants shared by the functionally heterologous LC2F light chain (Silberstein & Lowey, 1977, 1981; Biral *et al.*, 1979).

The species-specificity of anti-(rabbit LC1F) antibody was assessed by testing the ability of fast-twitch-muscle myosins from several species, relative to that of rabbit fast-twitch-muscle myosin, to compete with immobilized rabbit LC1F light chain for binding of antibody. The results showed that the 50% inhibitory values for rat extensor digitorum longus and rat tibialis anterior were 2.5–2.7-fold higher than for rabbit fast-twitch-muscle myosin, and 2.9- and 8.8-fold higher in the case of human foetal muscle myosin (Volpe *et al.*, 1981) and chicken pectoralis myosin.

Relationship between light-chain composition and immunological reactivities of myosins from several rabbit muscles

The myosin light-chain patterns characteristic of rabbit fast-twitch and slow-twitch muscles of the hind-limbs, as well as of mixed muscles, such as the masseter and diaphragm, have long since been reported (Sreter *et al.*, 1972). The existence of the LC1S light-chain component of slow-twitch myosin as a doublet (LC1Sa and LC1Sb; see Weeds, 1976), and of differences in the ratio of the two components

from muscle to muscle (Margreth *et al.*, 1980; Schachat *et al.*, 1980), attracted attention only recently, however.

A survey of the two-dimensional light-chain patterns of skeletal-muscle myosins of adult rabbits is presented in Fig. 2. On the basis of these results, the several myosins can be classified as follows: (i) myosins with a pure fast-twitch-muscle light-chain pattern (e.g. adductor; Fig. 2*a*), or slow-twitch-muscle light-chain pattern (e.g. soleus; Fig. 2*e*); (ii) myosins with a truly mixed light-chain pattern, i.e. with a full set of light chains characteristic of either main type of myosin (LC1F–LC2F–LC3F and LC1Sa–LC1Sb–LC2S; e.g. crureus; Fig. 2*d*); (iii) myosins with a light-chain pattern as in (ii), but with a relative deficiency either of LC1Sa light chain (e.g. diaphragm; Fig. 2*h*) or of LC1Sb light chain (e.g. flexor digitorum profundus; Fig. 2*f*); (iv) myosins with an LC1F–LC2F–LC3F–LC1Sb–LC2S light-chain complement, i.e. mixed myosins with an absolute lack of LC1Sa light chain (e.g. masseter; Fig. 2*g*).

The percentage of fast-twitch-muscle myosin present in the several preparations, as determined by densitometry of one-dimensional electrophoretic gels, was in agreement with the above classification (Table 1). There was also good agreement between the percentage values of total light-chain material and of LC1F and LC2F light chains. Furthermore, the results showed that both the stoicheiometry of alkali-dissociated light chains (LC1F + LC3F) and of LC2F light chain and the (LC1Sa + LC1Sb)/LC2S molar ratio were considerably stable within the limits of experimental error. Thus the corresponding values for mixed myosins were similar to those found for pure fast-twitch-muscle and pure slow-twitch-muscle myosin [0.83 ± 0.03 (S.E.M., $n = 4$) and 0.77 ± 0.04 (S.E.M., $n = 8$) respectively

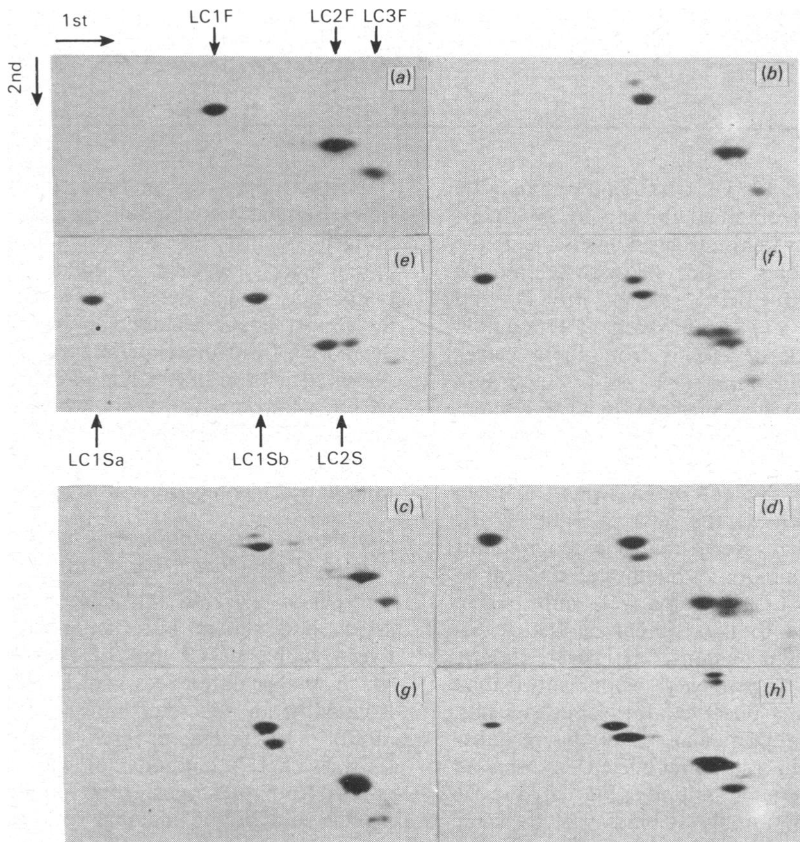

Fig. 2. *Two-dimensional gel electrophoresis of myosins from several rabbit muscles*
Isoelectrofocusing (first dimension) and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (second dimension) were performed as reported in the Materials and methods section, on about 40 μg of myosin. Key to muscle myosin: (*a*) adductor; (*b*) vastus lateralis; (*c*) gastrocnemius; (*d*) crureus; (*e*) soleus; (*f*) flexor digitorum profundus; (*g*) masseter; (*h*) diaphragm. Only the light-chain region is shown.

Table 1. *Immunological cross-reactivity and light-chain composition of rabbit muscle myosins*
Immunological data were calculated from inhibition curves obtained with the several myosins by competitive ELISA. The 50%-inhibitory level corresponds to the concentration of myosin required to inhibit by 50% the maximum binding of anti-(rabbit LC1F) antibody to microtitre wells coated with the homologous light chain. The 50%-inhibitory level for soleus myosin is the extrapolated value obtained by linear-regression analysis of the data in Fig. 4(*b*). The percentage values of fast-twitch-muscle light chains (LC1F + LC2F + LC3F) and the other values were calculated from densitometric measurements on one-dimensional electrophoretic gels, after staining with Coomassie Blue, and are expressed as means ± S.E.M. when more than two determinations were performed (numbers of determinations in parentheses).

Muscle	Immunological cross-reactivity of myosin [50%-inhibitory level (μg/ml)]	Content of light chains (%)		
		Fast-twitch-muscle light chains	LC1F / (LC1F + LC1Sa + LC1Sb) ratio	LC2F / (LC2F + LC2S) ratio
Hind-limb muscles				
Adductor magnus	100	100 (4)	100	100
Vastus lateralis	124	85.6 ± 0.5 (3)	79	87
Gastrocnemius	125	93·4 ± 1.3 (3)	91	95
Crureus	160	18.4 ± 1.1 (3)	12	19
Soleus	1450	0 (8)	—	—
Fore-limb muscle				
Flexor digitorum profundus	147	38.3 (2)	32	42
Other muscles				
Masseter	113	52.9 ± 3.9 (4)	42	55
Diaphragm	133	70.1 ± 1.5 (3)	65	73

(cf. Lowey & Risby, 1971)]. On the other hand, the experimental values obtained for the LC1F/LC3F molar ratio (fast-twitch-muscle myosin) and the LC1Sa/LC1Sb molar ratio (slow-twitch-muscle myosin) were 2.80 ± 0.18 (S.E.M., $n = 4$) and 0.90 ± 0.13 (S.E.M., $n = 8$) (cf. Weeds, 1976; Julian *et al.*, 1981). Major deviations from these values were observed with masseter and diaphragm myosins, which were characterized by a low content of LC3F light chain (about 5% of total fast-twitch-muscle light chains) and respectively by the absence and a low content of LC1Sa light chain (Fig. 2). As shown by the data in Table 1, the concentration of the several myosins required for 50% competitive inhibition of binding of anti-(rabbit LC1F) antibody to LC1F-coated wells appeared to by inversely related to the content of fast-twitch-muscle myosin light chains. However, 'extra' immunological cross-reactivity with anti-(rabbit LC1F) antibody was observed for some myosins, masseter myosin in particular. Both the response curve obtained with the immobilized myosins at various concentrations of antibody (Fig. 3*a*) and the pattern of inhibition of antibody binding by the same antigens in the competitive immunoassay (Fig. 3*b*) revealed a close immunological relationship between fast-twitch-muscle myosin and the myosins of the masseter muscle and diaphragm, greater than that expected on the basis of the observed differences in light-chain composition.

Furthermore, by plotting the percentage inhibition of antibody binding by a constant amount of antigen against the percentage content of fast-twitch-muscle myosin of mixtures of pure fast-twitch-muscle and pure slow-twitch-muscle myosin, an almost linear relationship was obtained. Major deviations from the experimental curve were again observed with masseter and diaphragm myosins, as well as with crureus myosin, whereas the percentage inhibition by the myosins of flexor digitorum profundus, vastus lateralis and gastrocnemius muscle was in conformity with predicted values.

Immunological relationship between the different classes of slow-twitch-muscle myosin light chains

Previous work in this laboratory (Volpe *et al.*, 1981) had shown wide antigenic differences between rabbit LC1F and LC1Sa light chains, and much smaller differences with LC1Sb light chain, as isolated from masseter muscle (Margreth *et al.*, 1980). The extent of cross-reactivity with anti-(rabbit LC1F) antibody of the dissociated light chains from the myosins of hind-limb slow-twitch muscle and of the masseter muscle was therefore re-investigated, by both non-competitive and competitive ELISA.

The results in Fig. 4 clearly show that all classes of light chains from slow-twitch-muscle myosin are antigenically different from the LC1F light chain, with a decreasing cross-reactivity for the LC1Sb,

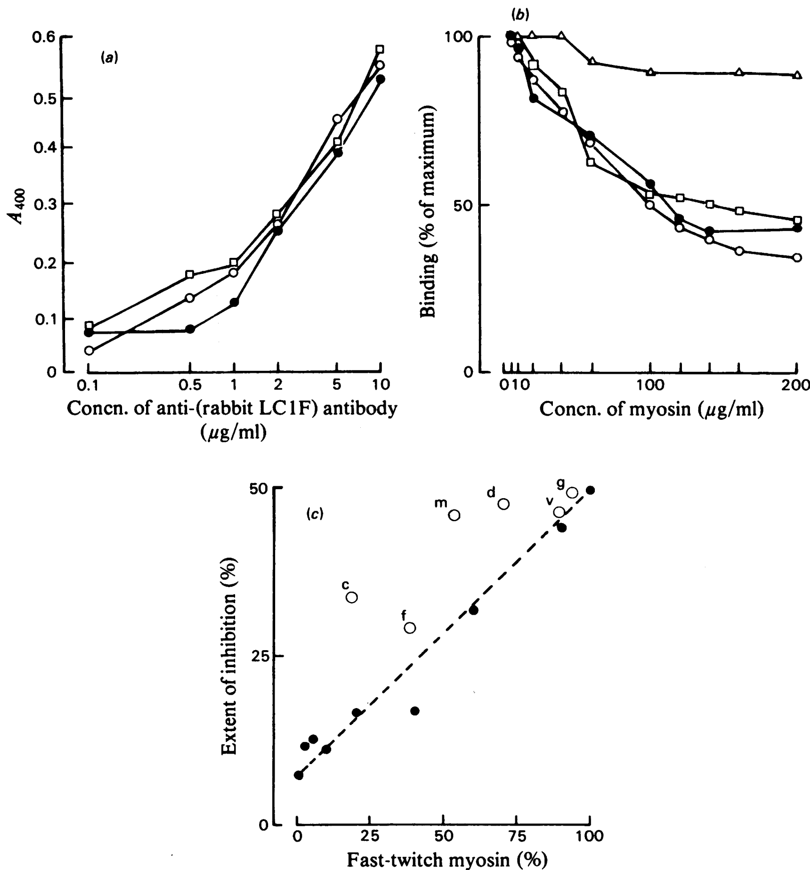

Fig. 3. *Cross-reactivities of anti-(rabbit LC1F) antibody with myosins from several skeletal muscles by non-competitive and competitive ELISA*

(*a*) One-step ELISA. Microtitre wells were coated with antigen at a protein concentration of 5μg/ml. The test was performed as described in the Materials and methods section, with anti-(rabbit LC1F) antibody at the concentrations indicated on the abscissa. Key to myosin: ○, adductor; □, diaphragm; ●, masseter. (*b*) Competitive ELISA. Myosins were preincubated with anti-(rabbit LC1F) antibody at the concentrations indicated on the abscissa. The second step was performed as described for Fig. 1(*b*). Each point is the average value of duplicate determinations. Key to myosin: ○, adductor; □, diaphragm; ●, masseter; △, soleus. (*c*) Competitive ELISA of the myosins of mixed muscles and of mixtures of pure fast-twitch-muscle and pure slow-twitch-muscle myosin. The immunoassay was performed as described for (*b*) except that the concentrations of antibody and of antigen were both kept constant (10μg/ml and 100μg/ml respectively). In the assay of the mixtures, the percentage of fast-twitch-muscle myosin was varied from 0% to 100% total protein. The percentage content of fast-twitch-muscle myosin in the several myosin preparations was calculated from the data in Table 1. The corresponding percentage inhibition values of antibody binding are reported on the abscissa. ----, Experimental curve obtained with mixtures of fast-twitch-muscle and slow-twitch-muscle myosin. Key to muscle: c, crureus; d, diaphragm, f, flexor digitorum profundus; g, gastrocnemius; m, masseter; v, vastus lateralis.

LC1Sa and LC2S light chains, in agreement with our previous observations on the light chains bound to myosin (Volpe *et al.*, 1981). Furthermore, it appears that the LC1Sb light chain from slow-twitch-muscle myosin is much less cross-reactive with LC1F light chain than is its masseter analogue. Thus (Fig. 4*b*) it was found that the concentration of slow-twitch-muscle LC1Sb light chain required for 50% inhibition of binding of anti-(rabbit LC1F) antibody to immobilized LC1F light chain was about 20-fold higher than the corresponding concentration of masseter LC1Sb light chain.

The different classes of slow-twitch-muscle type light chains investigated for immunological cross-

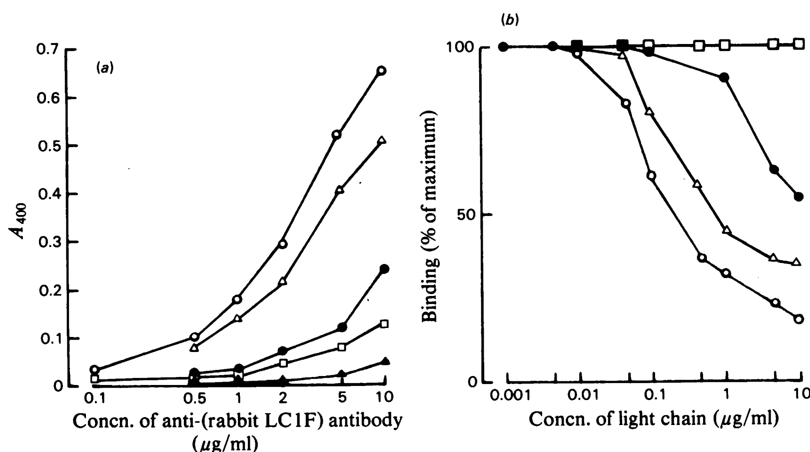

Fig. 4. *Cross-reactivities of anti-(rabbit LC1F) antibody with the homologous light chain and with the dissociated LC1S and LC2S light chains from slow-twitch muscle (soleus and semitendinosus) and masseter myosin*

(*a*) One-step ELISA. Assay conditions were as described for Fig. 1(*b*). ○, LC1F light chain; △, LC1Sb light chain from masseter myosin; ●, LC1Sb light chain from slow-twitch-muscle myosin; □, LC1Sa light chain from slow-twitch-muscle myosin; ▲, LC2S light chain from slow-twitch-muscle myosin. (*b*) Competitive ELISA. Assay conditions were as described for Fig. 1(*c*). Symbols are as in (*a*).

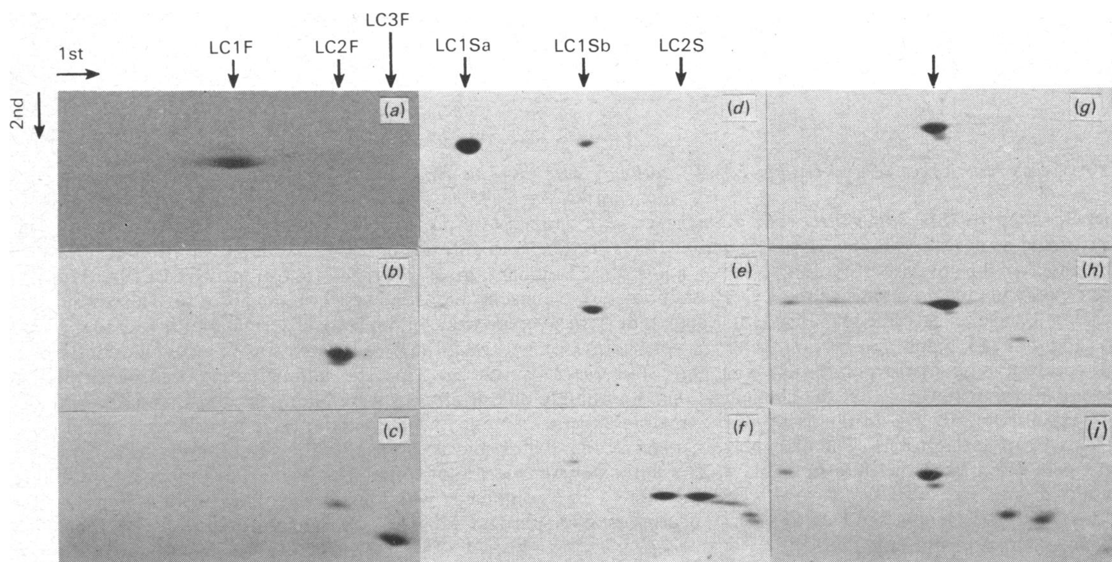

Fig. 5. *Two-dimensional gel electrophoresis of the dissociated light chains from fast-twitch-muscle and slow-twitch-muscle myosin and masseter myosin*

Fast-twitch-muscle myosin light chains (4μg of protein): (*a*) LC1F light chain; (*b*) LC2F light chain; (*c*) LC3F light chain. Slow-twitch-muscle myosin light chains (4μg of protein): (*d*) LC1Sa light chain; (*e*) LC1Sb light chain; (*f*) LC2S light chain. Masseter-muscle myosin light chain (4μg of protein); (*g*) LC1Sb light chain. Co-electrophoresis of masseter-muscle LC1Sb light chain (2μg of protein) and slow-twitch-muscle myosin LC1Sb light chain (2μg of protein) (*h*); co-electrophoresis of 30μg of masseter-muscle myosin (only the light-chain region is shown) with 4μg of slow-twitch-muscle myosin LC1Sb light chain (*i*). The positions of the light chains of fast-twitch-muscle and slow-twitch-muscle myosin are labelled as indicated, and that of masseter-muscle LC1Sb light chain by arrow only. Slow-twitch-muscle LC2S light chain (*f*) is detected as two spots, probably corresponding to the phosphorylated and dephosphorylated forms of this light chain (compare with the soleus myosin light-chain pattern in Fig. 2*e*).

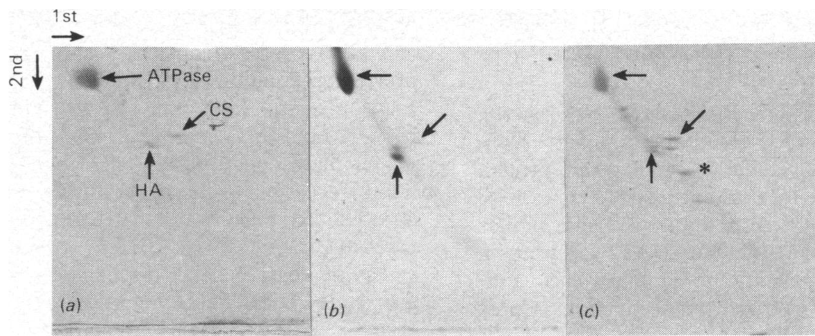

Fig. 6. *Two-dimensional electrophoresis of isolated sarcoplasmic reticulum from fast-twitch* (*a*), *masseter* (*b*) *and slow-twitch* (*c*) *muscle of the rabbit*

Two-dimensional electrophoresis of the sarcoplasmic-reticulum fragments was performed in the gel system of Michalak *et al.* (1980), by the procedure of Weber & Osborn (1969) in the first dimension, and by the procedure of Laemmli (1970) in the second dimension (see the Materials and methods section). The amount of protein loaded per gel was 20μg (*a*), 30μg (*b*) and 40μg (*c*). Key: ATPase, Ca^{2+}-activated ATPase (mol.wt. 100000); CS, calsequestrin (mol. wt. 64000); HA, high-affinity Ca^{2+}-binding protein (mol.wt. 55000). Unlabelled peptides are seen at positions near the high-affinity Ca^{2+}-binding protein (*b* and *c*) and immediately below the calsequestrin spot (only in *c*). A peptide specific to slow-twitch-muscle sarcoplasmic reticulum (mol.wt. 45000) is marked with an asterisk (*c*).

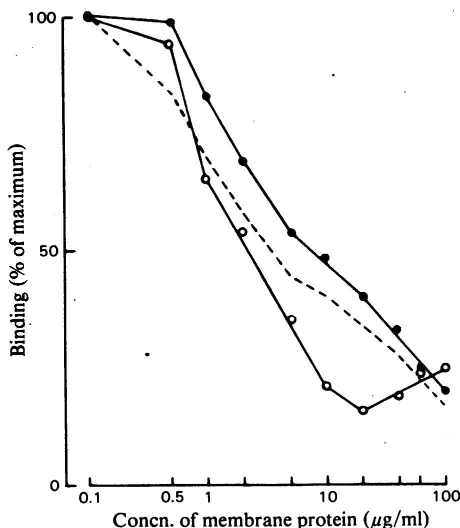

Fig. 7. *Cross-reactivities of anti-(rabbit fast-twitch-muscle Ca^{2+}-activated ATPase) antibody with membrane-bound Ca^{2+}-activated ATPase of sarcoplasmic-reticulum fragments from fast-twitch muscle and masseter muscle*

Anti-(rabbit fast-twitch-muscle Ca^{2+}-activated ATPase) antibodies were tested for cross-reactivity with light sarcoplasmic-reticulum fragments from fast-twitch-muscle and masseter muscle, by competitive ELISA, as described by Damiani *et al.* (1981) (see the Materials and methods section). Portions (0.65ml) of antibody solution were mixed with an equal volume of diluted sarcoplasmic-reticulum suspension (0.1–100μg of sarcoplasmic-reticulum protein/ml, as indicated) in PBS buffer, pH 7.2. The second step was performed in microtitre wells coated with the immunogen at a coating concentration of 5μg of protein/ml. Densitometry of one-dimensional sodium dodecyl sulphate/polyacrylamide-gel electrophoretograms (Laemmli, 1970) of the sarcoplasmic-reticulum fragments showed that the ATPase protein accounted for 82% and 69% of total protein of fast-twitch muscle and masseter muscle respectively. ○, Inhibition curve with the sarcoplasmic-reticulum fragments from fast-twitch muscle; ●, inhibition curve with the sarcoplasmic-reticulum fragments from masseter muscle, and ---- the same curve normalized to the ATPase content of fast-twitch-muscle sarcoplasmic-reticulum fragments, by using a correction factor of 0.8.

reactivity with LC1F light chain could not be isolated at the same degree of high purity as the latter type of light chain. Nevertheless two-dimensional electrophoresis (Fig. 5) revealed a low degree of cross-contamination of hind-limb muscle LC1Sa light chain with the LC1Sb light chain (Fig. 5*d*), and, to a smaller extent, of masseter LC1Sb light chain with the LC1F light chain (Fig. 5*g*). On the other hand, co-electrophoresis of the two antigenically different forms of LC1Sb light chain (Fig. 5*h* and Fig. 5*i*) confirms (Margreth *et al.*, 1980) that the two forms are identical by the criteria of both molecular weight and pI.

Protein profile of masseter-muscle sarcoplasmic reticulum and immunological properties of Ca^{2+}-activated ATPase

Two-dimensional electrophoresis (Michalak *et al.*, 1980) of sarcoplasmic-reticulum fragments from rabbit masseter muscle shows an overall protein composition similar to that of fast-twitch muscle sarcoplasmic reticulum (Fig. 6), as well as a similar prominence of the Ca^{2+}-activated ATPase protein, in agreement with its immunoreactivity with anti-(fast-twitch-muscle sacroplasmic-reticulum Ca^{2+}-activated ATPase) antibody (Fig. 7). Thus it was found that the concentration of masseter-muscle sarcoplasmic-reticulum protein required for 50% inhibition of binding of antibody was about 1.6-fold higher compared with the purified Ca^{2+}-activated ATPase from fast-twitch-muscle. In agreement with these immunological data and other earlier data (Margreth *et al.*, 1974*a*,*b*), extra Ca^{2+}-activated ATPase activity, measured in the presence of ionophore A23187, was 6.2 μmol of P_i/min per mg of protein for masseter-muscle sarcoplasmic reticulum and 10.9 μmol of P_i/min per mg of protein for fast-twitch-muscle sarcoplasmic reticulum. Furthermore, measurements of cytochrome b_5 (a specific marker of slow-twitch-muscle sarcoplasmic reticulum; Salviati *et al.*, 1979, 1981) showed that the cytochrome concentration in masseter-muscle sarcoplasmic reticulum was as low as 0.06 nmol/mg of protein.

Discussion

The low degree of species-specificity of our affinity-purified anti-(rabbit LC1F) antibodies, their high specificity for antigenic determinants unique to fast-twitch-muscle myosin alkali-dissociated light chains (LC1F and LC3F) and weak cross-reactivity of these antibodies with the functionally heterologous 5,5′-dithiobis-(2-nitrobenzoic acid)-dissociated light chain (LC2F) appear to be accounted for by: (i) the long immunization period; (ii) the greater sensitivity of solid-phase immunoassays, of competitive ELISA in particular, as compared with other immunoassays (cf. Silberstein & Lowey, 1977, 1981; Biral *et al.*, 1979; Volpe *et al.*, 1981; Walliman & Szent-Györgyi, 1981).

With regard to the still controversial problem of the extent and nature of differences in immunological reactivity of myosin light chains with specific antibodies, when free or bound to myosin (Holt & Lowey, 1975*b*; Silberstein & Lowey, 1981; Walliman & Szent-Györgyi, 1981), our present results stress the importance of the experimental conditions as the major cause of these differences. Thus we found that these differences are by far greater as determined by competitive than by non-competitive ELISA (Volpe *et al.*, 1981; present results). It is reasonable to assume that, under the prevailing conditions in the competitive immunoassay, i.e. in high-ionic-strength PBS buffer at pH 7.2, myosin is mainly in the monomeric form. Under these conditions the light chains may be less freely accessible to the antibody than when assaying immobilized myosin after treatment with sodium carbonate solution at pH 9.6, which could itself cause at least partial dissociation of the alkali-dissociated light chains, i.e. in the one-step immunoassay (see the Materials and methods section).

However, it is remarkable that, under either condition used, our antibodies were able to distinguish between pure fast-twitch-muscle and pure slow-twitch-muscle myosins, to the extent predicted from the overall immunological reactivity of the respective dissociated light chain. This finding supports the interpretation that, although the exposure of the light chains on myosin to the antibody is influenced by the experimental conditions, the specific determinants involved do not vary and are also the same involved in antibody binding to the free light chains, i.e. possibly structural sequence determinants. Given the above correlation, the observation of high immunological cross-reactivity with anti-(rabbit LC1F) antibody of some 'mixed' myosins, of masseter myosin in particular, must take into account both peculiarities in the light-chain composition and the association with these myosins of an LC1Sb light chain having extensive homology with fast-twitch-muscle myosin LC1F light chain. However, the absence from masseter myosin of the LC1Sa light chain (Margreth *et al.*, 1980; Schachat *et al.*, 1980; present results) does not explain the 'extra' immunological reactivity of this myosin (see under 'Immunological relationship between the different classes of slow-twitch-muscle myosin light chains' in the Results section), nor does the very absence of this light chain constitute a basic difference between myosins of mixed and pure slow-twitch muscles (Julian *et al.*, 1981; Pinter *et al.*, 1981; Carraro *et al.*, 1981).

Masseter myosin LC1Sb light chain and its slow-twitch muscle analogue appear to have the same molecular weight and isoelectric point (Margreth *et al.*, 1980; present results). Thus, although the observed antigenic differences imply subtle structural differences between the two forms, it would be unwarranted, at the present time, to assign them to the existence of specific amino acid sequences.

The immunological interrelatedness of masseter LC1Sb light chain and fast-twitch-muscle LC1F light chain deserves comment within the more general context of myosin polymorphism in relation to fibre types. In skeletal muscles of the guinea pig, three main types of fibres have been described, i.e.

fast-twitch white, fast-twitch red and slow-twitch red fibres (Peter *et al.*, 1972). In the rat diaphragm, Gauthier & Lowey (1979) demonstrated a high degree of immunological cross-reactivity between the myosins of fast-twitch white and fast-twitch red fibres, including all classes of light chains. They proposed that fast-twitch-red-fibre myosin either differed from fast-twitch-white-fibre myosin by the proportion of LC1F and LC3F homodimers (see also Dalla Libera *et al.*, 1980), or that it was a third type of myosin.

These observations are pertinent to the interpretation of our immunological findings, since it has long been recognized that rabbit masseter muscle is a highly aerobic but predominantly fast-twitch muscle (Paukul, 1904). That is further supported by enzymic studies of the isolated mitochondria and soluble sarcoplasmic fraction (Margreth *et al.*, 1974*a*,*b*), and by the catalytic and immunological properties of the Ca^{2+}-activated ATPase and other properties of the sarcoplasmic-reticulum fragments, as shown in the present work. From the latter results, it may be estimated that rabbit masseter muscle is 60–70% composed of fast-twitch fibres. All that, however, leaves open the question of the relative proportion of fast-twitch white and fast-twitch red fibres, which cannot be distinguished with respect to Ca^{2+}-transport activity (Fiehn & Peter, 1971).

On the basis of the foregoing, it may be reasoned that the myosin of masseter-muscle fast-twitch red fibres contains a specific iso-form of LC1Sb light chain, antigenically different from that associated with the myosin of slow-twitch red fibres, and characterized by extensive homology with fast-twitch-muscle myosin LC1F light chain. Thus the actual immunological properties of the isolated LC1Sb light chain from the masseter muscle would be the average properties of the mixture of the two forms, each derived from a different type of fibre. Though this interpretation would fit some of the results obtained by Gauthier & Lowey (1979), there are, however, several contraindications, such as recent immunological evidence suggesting that myosin heterogeneity between fast-twitch white and fast-twitch red muscle fibres, in the rat and the guinea pig at least, apparently concerns solely the heavy chains (Pierobon-Bormioli *et al.*, 1981).

Furthermore, caution must be exercised against extrapolating the current nomenclature of fast-twitch-muscle and slow-twitch-muscle types of myosin light chains to the twitch properties of muscle fibres, and that particularly so in the case of so-called fast-twitch red fibres. Thus Young & Davey (1981) were able to identify a subpopulation of 'fast-twitch' fibres in bovine skeletal muscle having a light-chain pattern consisting of only the LC2F and LC3F light chains, whereas the LC1F component appeared to be substituted by a light chain electrophoretically identical with the LC1S light chain of slow-twitch-muscle myosin. The coexistence in 'intermediate' fibres of rabbit skeletal muscle of the light chains characteristic of each main type of myosin had been described previously by Lutz *et al.* (1979).

Therefore an alternative possibility must be considered, i.e. that the two antigenically different forms of myosin LC1Sb light chain in rabbit muscle relate to the existence in some muscles, e.g. the masseter, of sub-types of red fibres containing myosin heterodimers with respect to the LC1F light chain and either of the two forms of LC1Sb light chain. Further study of the myosin of individual muscle fibres and of their twitch properties, in combination with immunological studies, might help to subject the several working hypotheses to direct experimental test.

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References

Barone, R., Pavaux, C., Blin, B. C. & Cuq, P. (1973) *Atlas d'Anatomie du Lapin*, pp. 56–63, Masson, Paris

Biral, D., Dalla Libera, L., Franceschi, C. & Margreth, A. (1979) *J. Immunol. Methods* **31**, 93–100

Carraro, U., Dalla Libera, L. & Catani, C. (1981) *J. Muscle Res. Cell Motil.* **2**, 335–342

Dalla Libera, L., Sartore, S., Pierobon-Bormioli, S. & Schiaffino, S. (1980) *Biochem. Biophys. Res. Commun.* **96**, 1662–1670

Damiani, E., Betto, R., Salvatori, S., Volpe, P., Salviati, G. & Margreth, A. (1981) *Biochem. J.* **197**, 245–248

Fiehn, W. & Peter, J. B. (1971) *J. Clin. Invest.* **50**, 570–573

Frank, G. & Weeds, A. G. (1974) *Eur. J. Biochem.* **44**, 317–334

Gauthier, G. F. & Lowey, S. (1979) *J. Cell Biol.* **81**, 10–25

Holt, J. C. & Lowey, S. (1975*a*) *Biochemistry* **14**, 4600–4609

Holt, J. C. & Lowey, S. (1975*b*) *Biochemistry* **14**, 4609–4620

Julian, F. J., Moss, R. L. & Waller, G. S. (1981) *J. Physiol. (London)* **311**, 201–218

Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685

Lowey, S. & Holt, J. C. (1972) *Cold Spring Harbor Symp. Quant. Biol.* **37**, 19–28

Lowey, S. & Risby, D. (1971) *Nature (London)* **234**, 81–85

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Lutz, H., Ermini, M., Jenny, E., Bruggman, S., Joris, F. & Weber, R. (1978) *Histochemistry* **57**, 223–235

Lutz, H., Weber, R., Billeter, R. & Jenny, E. (1979) *Nature (London)* **281**, 142–144

Margreth, A., Salviati, G., Mussini, I. & Carraro, U. (1974*a*) in *Explanatory Concepts in Muscular Dystrophy* (Milroath, A. T., ed.), pp. 406–415, Excerpta Medica, Amsterdam

Margreth, A., Salviati, G., Carraro, U. & Mussini, I. (1974*a*) in *Calcium Binding Proteins* (Drabikowsky, W., Strzelecka-Golaszewska, H. & Carafoli, E., eds.), pp. 519–545, Elsevier/North-Holland, Amsterdam, and Polish Scientific Publishers, Warsaw

Margreth, A., Salviati, G., Dalla Libera, L., Betto, R., Biral, D. & Salvatori, S. (1980) in *Plasticity of Muscle* (Pette, D., ed.), pp. 193–208, Walter de Gruyter, Berlin and New York

Michalak, M., Campbell, K. P. & MacLennan, D. H. (1980) *J. Biol. Chem.* **255**, 1317–1326

O'Farrel, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021

Orlans, E., Rose, M. E. & Marrack, J. R. (1961) *Immunology* **4**, 262–277

Paukul, E. (1904) *Arch. Anat. Physiol. Physiol. Abt.* 100–120

Perrie, W. T., Smillie, L. B. & Perry, S. V. (1973) *Biochem. J.* **135**, 161–164

Peter, J. B., Barnard, R. J., Edgerton, V. R., Gillespie, C. A. & Stempel, K. E. (1972) *Biochemistry* **14**, 2627–2633

Pierobon-Bormioli, S., Sartore, S., Dalla Libera, L., Vittadello, M. & Schiaffino, S. (1981) *J. Histochem. Cytochem.* **29**, 1179–1188

Pinter, K., Mabuki, K. & Sreter, F. A. (1981) *FEBS Lett.* **128**, 336–338

Rennard, S. I., Berg, R., Martin, G. R., Fordart, J. M. & Robey, G. P. (1980) *Anal. Biochem.* **104**, 205–214

Salviati, G., Betto, R., Salvatori, S. & Margreth, A. (1979) *Biochim. Biophys. Acta* **574**, 280–289

Salviati, G., Salvatori, S., Betto, R. & Margreth, A. (1981) *Biochem. J.* **197**, 515–518

Schachat, T. A., Bronson, D. D. & McDonald, O. V. (1980) *FEBS Lett.* **122**, 80–82

Silberstein, L. & Lowey, S. (1977) *Biochemistry* **16**, 4403–4408

Silberstein, L. & Lowey, S. (1981) *J. Mol. Biol.* **148**, 153–189

Sreter, F. A., Sarkar, S. & Gergely, J. (1972) *Nature (London) New Biol.* **239**, 124–125

Voller, A. D., Bidwell, O. & Bartlett, A. (1976) in *Manual of Clinical Immunology* (Rose, N. R. & Friedmann, H., eds.), pp. 506–512, American Society of Microbiology, Washington

Volpe, P., Biral, D., Damiani, E. & Margreth, A. (1981) *Biochem. J.* **195**, 251–258

Walliman, T. & Szent-Györgyi, A. G. (1981) *Biochemistry* **20**, 1176–1189

Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G. & Metcalfe, J. C. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 622–626

Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4410

Weeds, A. G. (1976) *Eur. J. Biochem.* **66**, 157–173

Young, O. A. & Davey, C. L. (1981) *Biochem. J.* **195**, 317–327