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Characterization of human muscle myosins with respect to the light chains

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Isolated myosins from human predominantly fast and slow muscles, human neonatal and foetal muscle were examined for light chain composition by one- and two-dimensional electrophoresis. The LC1F, LC2F and LC3F light chains were identical with their counterparts from rabbit fast myosin. Human LC1S was identified by correlative criteria as a single component having a molecular weight slightly lower than, but an electric charge similar to, that of rabbit LC1Sb. Consequently, human LC1S appears to be much less heterogeneous relative to LC1F than is the case with other mammalian species. A high immunological cross-reactivity was likewise observed, with antibody specific to rabbit LC1F, between the isolated myosins from several human mixed muscles and rabbit fast myosin, though reactivity was highest with foetal myosin (having a pure-fast-light-chain pattern).

It is well known that skeletal-muscle myosin is polymorphic with respect both to the heavy and the light chains and that different forms of these peptides are synthesized in fast-twitch and slow-twitch fibres under the regulatory influence from innervating motor neurons (Weeds, 1978).

Investigation of the heavy-chain composition of human muscle myosins provided results consistent with the mixed-fibre population of these muscles, showing differences from muscle to muscle according to the various mixtures of fast and slow myosin (Dalla Libera *et al.*, 1978). On the other hand, conflicting results have appeared concerning the electrophoretic light-chain composition of human myosins (Bailin, 1976; Sreter *et al.*, 1976; Dalla Libera *et al.*, 1978; Samaha & Thies, 1979; Pette *et al.*, 1979*a*; Billeter *et al.*, 1980*b*). The main point of controversy relates to the apparent absence from human muscle myosins of the slow forms of LC1 and LC2 light chains, or at least of peptides with electrophoretic properties identical with the homologous light chains of slow myosins of other mammalian species (Dalla Libera *et al.*, 1978).

We have re-investigated this problem with the greater resolving power of two-dimensional gel electrophoresis (O'Farrell, 1975), by one-dimensional electrophoresis as described by Laemmli (1970), as well as by immunological techniques adapted for this purpose in this laboratory (Biral *et al.*, 1979).

The results reported here suggest that fast, as well as slow, forms of LC1 and LC2 myosin light chains can be characterized in human muscle myosins by these methods and that the extent of structural and immunological homology between the two forms of LC1 and LC2 is greater in the human species than in other mammalian species so far investigated.

Experimental

Tissue sources

Specimens of human adult skeletal muscles were obtained as follows: pectoralis minor from female patients during radical mastectomy; soleus on autopsy 24h after death; samples of latissimus dorsi and of vastus lateralis muscle in the course of orthopaedic surgery. Neonatal muscle (vastus medialis) was autoptic material obtained 24h after death. Foetal muscle (total thigh muscles) was from 13-week-old human foetuses. The muscle with nemaline myopathy was biopsy material from the gastrocnemius of a 3-year-old female patient (Institute of Neurology, University of Milan, Milan, Italy).

The soleus of rabbit, rat and macaque monkey (*Macaca cynomolgus*) (Istituto Superiore di Sanità, Roma, Italy) were used as representative slow-twitch muscles and the adductor magnus of the rabbit as a fast-twitch muscle.

Except when otherwise stated (i.e. autoptic material), fresh muscle specimens were used. The

Abbreviations used: LC, light chain; SDS, sodium dodecyl sulphate; 'ELISA', enzyme-linked immunoadsorbent assay.

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excised muscles were immediately placed in a beaker containing a large volume of 50% (w/v) glycerol solution, immersed in solid CO_2 in a Dewar container. The muscle samples were preserved in the same solution at −20°C for subsequent biochemical analysis, as described previously (Dalla Libera *et al.*, 1978).

Myosin preparation and isolation of myosin light chains

Unless otherwise stated, all preparative procedures were performed at 4°C. Myosin was prepared from human and skeletal muscles by the method of Barany & Close (1971), under the experimental conditions described in detail previously (Dalla Libera *et al.*, 1978). In most experiments with human muscles, pepstatin (200 μg/litre) was added to the extraction medium and was present through the subsequent steps in the isolation of myosin (Siemankowski & Dreizen, 1978).

Myosin light chains were dissociated by treating the isolated muscle myosin with 4 M-urea and were fractionated by DEAE-cellulose (Whatman DE-52) column chromatography and a linear phosphate gradient, essentially as described by Lowey & Holt (1972). The fractions corresponding to the LC1 material of each type of myosin were pooled and sorted at −20°C for further study.

Electrophoresis

One-dimensional electrophoresis. Electrophoresis of the myosin was performed in vertical slab assemblies (LKB, Bromma, Sweden) by the method of Laemmli (1970), with slight modification (by Dr. U. Carraro of this Laboratory), in an exponential 5–18.5%-polyacrylamide gradient containing 0.375 M-Tris/HCl, pH 8.8, and 0.1% SDS. The electrode buffer contained 5 mM-Tris/0.192 M-glycine, pH 8.3, and 0.1% SDS. The stacking gel of 4.5% (w/v) acrylamide and with a length of 1 cm, contained 0.125 M-Tris/HCl, pH 6.8, and 0.1% SDS. Electrophoresis was performed with a current of 20 mA per slab and was stopped 1 h after the marker dye (0.1% Bromophenol Blue) had reached the bottom of the slab. The samples were solubilized in a small volume of a solution containing 2.3% (w/v) SDS, 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.0625 M-Tris/HCl, pH 6.8. Slabs were stained in a solution containing 0.25% Coomassie Blue in 50% (v/v) methanol/10% (v/v) acetic acid, for 2–3 h at 60°C. The slabs were destained in 40% (v/v) methanol at the same temperature.

Two-dimensional electrophoresis. Isoelectric focusing was performed by the method of O'Farrell (1975), with 1% Ampholines (LKB) (0.8% of pH range 5–7 and 0.2% of pH range 3.5–10). Electrophoresis in the second dimension was performed in a polyacrylamide exponential-gradient gel as described above.

Preparation of antibodies to rabbit LC1F

The LC1F light chain isolated from fast-muscle myosin was used as the immunogen. The purity of each batch of LC1F was controlled both by one- and two-dimensional electrophoresis (see Fig. 7 of Margreth *et al.*, 1980). Preparations containing detectable amounts of contaminating LC2F were discarded. The purified protein (1.5 mg/ml), dissolved in 0.4 M-KCl/0.05 M-potassium phosphate, pH 7.4, was emulsified with an equal volume of Freund's complete adjuvant (DIFCO, Detroit, MI, U.S.A.). Adult hens were immunized as described by Biral *et al.* (1979) by repeated intramuscular injections of the antigen and were bled after 8–9 months from the beginning of the immunization.

Specific antibodies were isolated from immune serum by affinity chromatography, with a column of CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) coupled with rabbit myosin LC1F (5 mg of protein/g of gel) according to the manufacturer's instructions. About 10–15 ml of immune serum in 0.15 M-NaCl/0.016 M-sodium carbonate, pH 8.2, were applied to a column (0.9 cm × 10 cm) of immunosorbent; the bound antibody was eluted with 0.2 M sodium acetate (pH 2.8)/0.3 M-KCl and was dialysed against 0.15 M-NaCl/0.1 M-sodium phosphate (pH 7.2).

Enzyme-linked immunoadsorbent assay ('ELISA')

The immunoassay was performed under conditions identical with those described by Biral *et al.* (1979). Antigen concentrations were kept constant (5 μg/ml) and the concentration of purified anti-LC1F was varied between 0.01 and 10 μg/ml, as described in the legends to the Figures.

Determination of the relative amounts of LC1F and LC1S of human muscle myosin

These were determined on the stained gels by a photographic method and densitometric scanning of the films (46L pancromatic) in an Eppendorf photometer (546 nm filter). The relative area values of each absorption peak were measured both by an integrator system attached to the instrument and by direct planimetry.

Determination of protein concentration

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

Results and discussion

On electrophoresis, as described by Laemmli (1970), of muscle myosins from several mammalian

species, a light-chain pattern characteristic of fast and slow muscle is readily observed (Fig. 1).

Additional differences in the light-chain pattern among slow myosins according to the animal species are also observed under these conditions, unlike under the standard conditions of the Weber & Osborn (1969) method, as has been noted previously (Perrie *et al.*, 1973; Weeds, 1976; Margreth *et al.*, 1980). In particular, the light-chain pattern of slow myosin appears to be either three-banded or two-banded, according to whether LC1S is present as a doublet or as a single band. The LC1Sa component characteristic of slow myosin of the rabbit and the cat (Weeds, 1976), on the other hand, is absent from slow myosin both of the rat and of macaque monkey (Fig. 1).

On the other hand, the electrophoretic light-chain pattern of myosin isolated from human predominantly slow (soleus) and fast (vastus lateralis) muscles is found to be less diverse by any means of comparison (Fig. 1). Firstly an LC1Sa-like component cannot be detected. Secondly, the LC1S and LC1F light chains, tentatively identified as two discrete bands (Fig. 1), are less resolved and are therefore much less heterogeneous as compared with the homologous peptides of, in particular, rabbit muscle myosins. Interestingly, the myosin from human foetal or neonatal muscle exhibits only a single band in the LC1 region, and has electrophoretic properties identical with those of the LC1F light chain of rabbit fast myosin (Fig. 1). Correlative data have provided evidence that the relative proportions of the two peptide bands tentatively identified as the LC1S and LC1F light chains of human adult muscle myosin, do indeed vary in the expected fashion according to the muscle twitch properties (Figs. 1 and 2). Thus by several measurements we obtained a mean percentage value for the peptide component of lower electrophoretic mobility, i.e. LC1S (expressed as a percentage of the total LC1 material $\pm$ S.E.M.), of 68.3 ± 0.4 (12), 63.9 ± 0.3 (12), 35.7 ± 0.4 (6) and 31.5 ± 0.5 (12) for the myosins isolated from soleus, pectoralis minor, latissimus dorsi and vastus lateralis respectively, the values therefore decreasing with muscle contraction time (Buchthal *et al.*, 1973; Buchthal & Schmalbruch, 1970; Eberstein & Goodgold, 1968). A good correlation [even better than was reported for the slow form of troponin I in the human muscle (Cummins & Perry, 1978)] is likewise observed between our present data on LC1S and published histochemical data on the percentage of type 1 fibres in human muscles. For instance, Johnson *et al.* (1973) reported percentages of type I (slow) fibres of

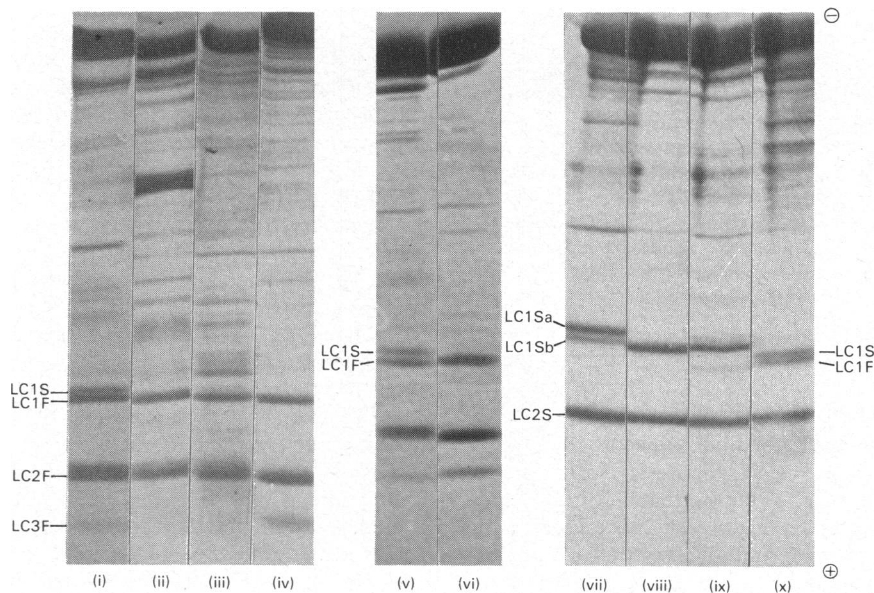


Fig. 1. *SDS/polyacrylamide-gel electrophoresis of skeletal-muscle myosins from several species*
One-dimensional electrophoresis was performed under the experimental conditions described in the Experimental section. Key to muscle myosins: (i) human latissimus dorsi; (ii) human-newborn vastus medialis; (iii) human foetal thigh muscles; (iv) rabbit adductor; (v) human vastus lateralis; (vi) rabbit adductor; (vii) rabbit soleus; (viii) rat soleus; (ix) macaque monkey (*Macaca cynomolgus*) soleus; (x) human soleus. Abut 20μg of protein was applied per gel from (i) to (iv) and about 40μg from (v) to (x).

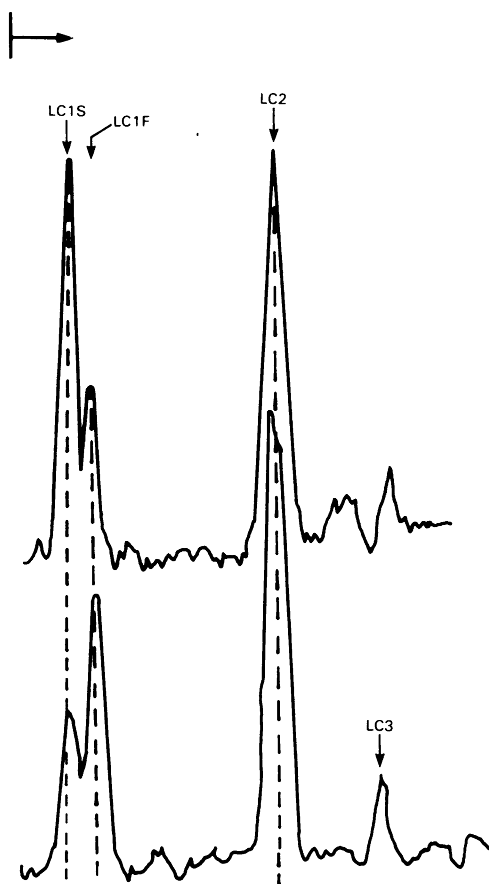


Fig. 2. *SDS/polyacrylamide-gel electrophoresis of myosins from human predominantly slow (soleus) and fast (vastus lateralis) muscles*

Densitometric record of a photography of the electrophoretic gel. Only the light-chain region is shown: (*a*) soleus myosin; (*b*) vastus lateralis myosin. The two LC1 components (LC1F and LC1S) and the LC2 and LC3 bands are indicated. The horizontal arrow indicates the direction of migration.

86% and 36–47% respectively in the soleus and vastus lateralis muscles of adult subjects.

Two-dimensional electrophoresis of human muscle myosins (Fig. 3) shows that the LC1F and LC1S light chains have only slightly different pI values (about pH 5.8 and 5.7 respectively). This observation, in addition to the slight differences in apparent molecular weight, accounts for the partial resolution of the two forms of LC1 under any of the electrophoretic conditions used. In co-electrophoretic experiments with rabbit fast and slow myosin (Fig. 3), it was confirmed that whereas rabbit and human LC1F have identical molecular properties, human LC1S has a lower molecular weight compared with rabbit LC1Sb light chain, though pI values appear to be more similar. In agreement with the results in Fig. 1, two-dimensional electrophoresis demonstrates beyond doubt that the LC1S light chain is absent from human-newborn muscle. That is exactly what one should expect from knowledge that genes for fast myosin light chains are the first to be expressed during muscle ontogenesis in several species (Margreth, 1975; Pelloni-Müller *et al.*, 1976; Rubinstein & Kelly, 1978; Pette *et al.*, 1979b).

The two-dimensional light-chain pattern reported here for human muscle myosins is qualitatively similar to that reported briefly by Fardeau *et al.* (1978) for normal deltoid muscle. In identifying the two forms of LC2 (LC2S and LC2F) by this method, one must take into account the existence of each type of peptide in a phosphorylated and dephosphorylated form with different pI values. As shown in Fig. 3, two peptides indeed appear to be present in the LC2F region of neonatal muscle myosin. The two additional peptides of the same molecular weight but with slightly higher pI values that are seen in the LC2 region of adult myosin (Fig. 3, iii) may therefore be interpreted as the corresponding phospho and dephospho forms of LC2S.

As Figs. 1–3 show, and in confirmation of our previous data (Dalla Libera *et al.*, 1978) and other more recent data (Billeter *et al.*, 1980*b*), LC3F, which is a minor light-chain component of human muscle myosins, is present in lower amounts in preparations from predominantly slow muscles (e.g. the soleus and pectoralis minor) and is virtually absent from foetal and neonatal myosin. The latter finding is in agreement with the available information on other species (Margreth, 1975; Sreter *et al.*, 1975; Rubinstein & Kelly, 1978).

Therefore the controversy as to whether the light-chain pattern of human muscle myosins is the same for fast and slow fibres or whether two distinct patterns exist characteristic of each type of fibre (Sreter *et al.*, 1976; Dalla Libera *et al.*, 1978; Samaha & Thies, 1979; Billeter *et al.*, 1980*b*) appears to be resolved in favour of the latter interpretation. The suggestion by Pette *et al.* (1979*a*), on account of the electrophoretic pattern observed with the extracted myofibrillar proteins from human muscle fibres, that LC1F exists as a doublet, is difficult to reconcile with our own and other electrophoretic studies of the myosin from muscle tissue. The solitary nature of these observations, however, means that confirmatory evidence is required before the discrepancy can be understood.

On the other hand, a finding that accounts for our own and others' previous failure to resolve the LC1 material of human muscle myosins into fast and slow forms when the conventional electrophoretic

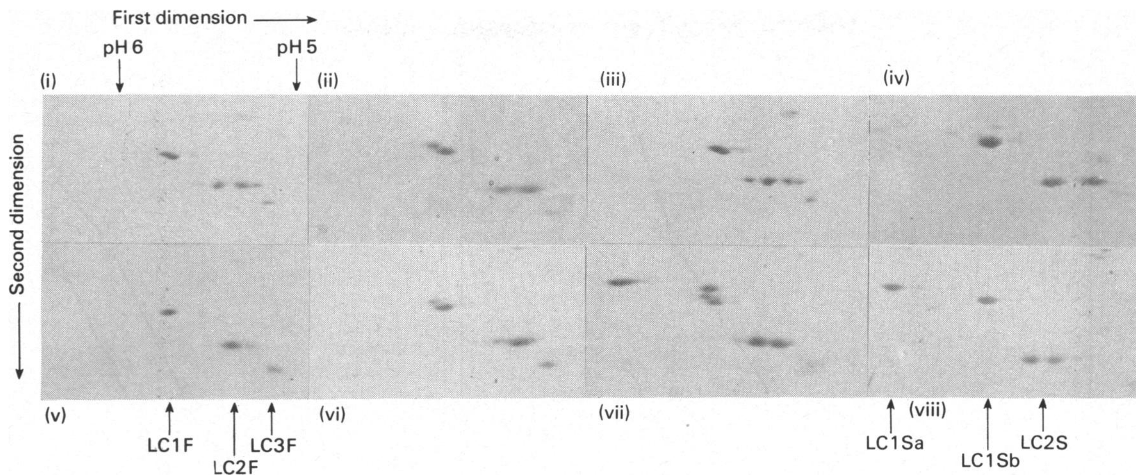


Fig. 3. *Two-dimensional gel electrophoresis of myosins from individual human and rabbit skeletal muscles and co-electrophoretic peptide 'maps'*

Only the light-chain region is shown, over the pH range indicated. Key to muscle myosins: (i) human vastus lateralis; (ii) human latissimus dorsi; (iii) human pectoralis minor; (iv) human newborn vastus medialis; (v) rabbit adductor; (vi) co-electrophoresis of (ii) and (v); (vii) co-electrophoresis of (ii) and (viii); (viii) rabbit soleus. The positions of the light chains from rabbit fast myosin (LC1F, LC2F, LC3F) and from rabbit slow myosin (LC1Sa, LC1Sb, LC2S) are indicated. About 20μg [or 40μg of protein in the case of (iv)] was applied per gel.

method of Weber & Osborn (1969) is used is that, strikingly, the two forms appear to be so slightly dissimilar by molecular weight as to escape identification by this method. This suggests that the structural interrelatedness between LC1F and LC1S of human muscle myosins is even more extensive than that known to exist for instance between LC1F and LC1S of rabbit muscle myosins (Weeds, 1976; Margreth *et al.*, 1980).

We have sought some clue to resolve this problem by an immunological approach. We had previously shown that LC1Sa from rabbit slow myosin has a low degree of immunological cross-reactivity with LC1F relative to LC1Sb, both from the same species (Margreth *et al.*, 1980). By the same type of immunoassay we have now found that the overall LC1 material isolated from the myosins of human pectoralis and vastus lateralis muscles, though containing different percentages of fast and slow forms of this light chain, had a similar reactivity with anti-(rabbit LC1F) and greater than that found with rabbit LC1Sa (Fig. 4).

From the knowledge that antibodies specific for myosin alkali light chains (i.e. LC1F and LC3F) are poorly reactive with myosin by double-diffusion in agar (Holt & Lowey, 1975), though they can bind to myosin in frozen muscle sections (Gauthier *et al.*, 1978) and because of the greater sensitivity of micro-'ELISA' immunoassay compared with immunoprecipitation (Biral *et al.*, 1979), we have further investigated the immunological reactivities of rabbit and human myosins with antibody specific for rabbit LC1F. As shown in Fig. 5, the antibody reacted strongly with rabbit fast myosin by micro-'ELISA', but reacted weakly with rabbit slow myosin. On the other hand, the myosin from human vastus lateralis was highly cross-reactive with rabbit fast myosin, (Fig. 5), and more than was expected from the mixed light-chain composition of the vastus-lateralis-muscle myosin. Results rather similar to those with vastus-lateralis myosin were obtained with the myosin from human pectoralis muscle (Fig. 6), though there was a tendency to find a decreased immunological reactivity relative to rabbit fast myosin. On the other hand, the extent of cross-reactivity between human myosins and rabbit fast myosin was found to be highest for foetal myosin (Fig. 6), which is consistent with the observed light-chain pattern of this myosin. Since human normal muscle with a homogeneous population of type I slow fibres does not exist, but a pathological condition is known, namely nemaline myopathy, leading to a large predominance of these fibres (Engel, 1977), the myosin from one such case was included in this series, the results being shown in the same Fig. 6. The relatively low cross-reactivity of this myosin with rabbit fast myosin appeared to correlate with its electrophoretic light-chain pattern,

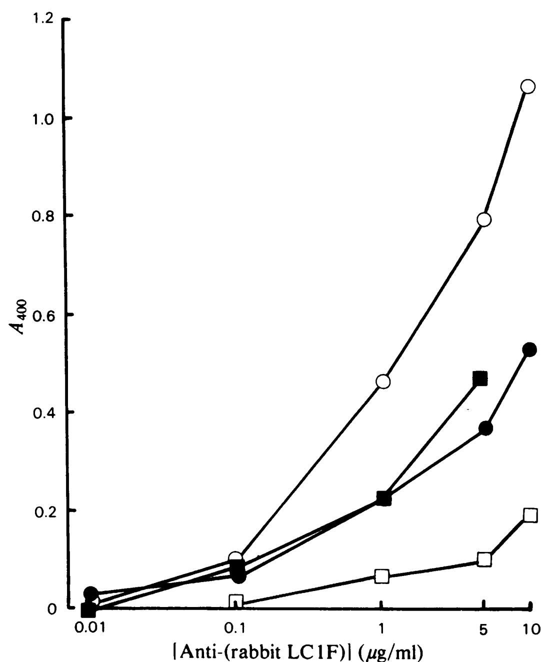


Fig. 4. *Enzyme-linked immunosorbent assay (micro-'ELISA') of myosin light chains*

The micro-'ELISA' test was performed as described in the Experimental section, with anti-(rabbit LC1F) antibody at the concentrations indicated on the abscissa, in plate wells coated with antigen solution at a concentration of 5μg/ml. The phosphatase activity bound to the wells was determined by incubation for 30min at room temperature with *p*-nitrophenyl phosphate and by measuring the A_{400} after stopping the reaction with alkali. ○, LC1F light chain from rabbit fast myosin; □, LC1Sa light chain from rabbit slow myosin; ●, total LC1 material from human vastus lateralis myosin; ■, total LC1 material from human pectoralis minor myosin.

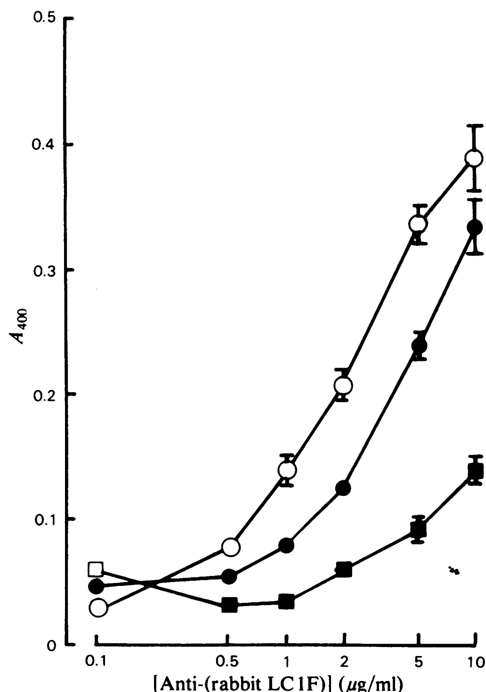


Fig. 5. *Enzyme-linked immunosorbent assay (micro-'ELISA') of rabbit fast and slow myosins and of human vastus lateralis myosin*

The micro-'ELISA' test was performed as described in the Experimental section, with anti-(rabbit LC1F) antibody in plate wells coated with myosin solution (5μg/ml). Absorbance values are given as means ± S.E.M.; S.E.M. values smaller than the symbols are not drawn. All determinations were performed with the same preparation of anti-LC1F antibody and of anti-(chicken immunoglobulin)–alkaline phosphate conjugate. Key to muscle myosins: ○, rabbit adductor ($n = 6$); ■, rabbit soleus ($n = 6$); ●, human vastus lateralis ($n = 7$).

consisting of two bands, the slowest moving band probably being identical with the LC1S component of normal human myosins (Fig. 7).

Understandably, interpretation of the pattern of immunological reactivity of human adult myosins is complicated by the observation that the reactivity for specific antibodies varies between free light chains and these components in myosin (Holt & Lowey, 1975) and that common antigenic determinants are shared by LC1F and LC3F as well as by LC1F and LC2F (Silberstein & Lowey, 1977; Holt & Lowey, 1977; Biral *et al.*, 1979). Also, it cannot be said with enough confidence at the present time whether the light chains associated with myosin in muscle with nemaline myopathy are a truly normal type of slow forms of light chains (see also Dalla Libera *et al.*, 1978).

With these reservations, though, it may be suggested that the convergence of the myosins from human mixed muscles, whether predominantly slow or fast muscles, toward a pattern of immunological reactivity approaching that of pure fast myosin, entails more than one factor: (i) the structural and antigenic interrelatedness between fast and slow forms of human myosin light chains, as well as between human light chains and the homologous light chains of rabbit fast myosin; (ii) the hypothetical existence (Billeter *et al.*, 1980*a*) in human muscle fibres of hybrid light-chain forms of myosin (i.e. myosins containing light chains both of the fast and slow form, and which would be expected to have

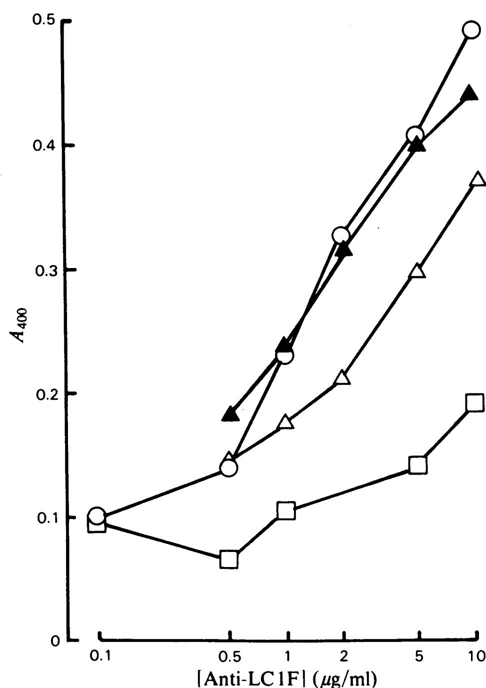
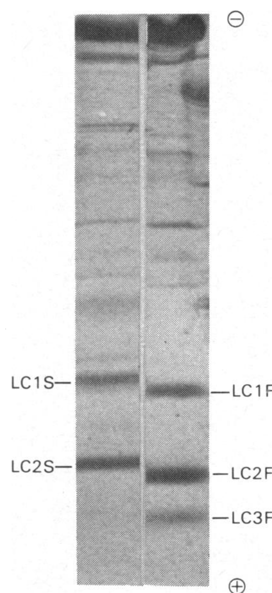


Fig. 6. *Enzyme-linked immunosorbent assay (micro-'ELISA') of rabbit fast myosin and of human myosins*
Absorbance values for several myosins were obtained by using rabbit fast myosin as a standard. Experimental conditions were as in Fig. 5, but with different preparations of both rabbit fast myosin and anti-LC1F antibody and of anti-(chicken immunoglobulin)–alkaline phosphatase conjugate. Key to muscle myosins: O, rabbit adductor; ▲, human foetal muscle; △, human pectoralis; □, gastrocnemius with nemaline myopathy.



an immunological reactivity intermediate between that of pure fast or pure slow myosin isoenzymes).

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Fig. 7. *SDS/polyacrylamide-gel electrophoresis of myosins from human and rabbit skeletal muscles*
Experimental conditions were as in Fig. 1. Key to muscle myosins: (i) human gastrocnemius with nemaline myopathy; (ii) rabbit adductor. About 25 μg of protein was applied per gel.

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