An Immunological Approach to Myosin Light-Chain Function in Thick Filament Linked Regulation. 1. Characterization, Specificity, and Cross-Reactivity of Anti-Scallop Myosin Heavy- and Light-Chain Antibodies by Competitive, Solid-Phase Radioimmunoassay†

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ABSTRACT: Antibodies specific for the regulatory light-chain (R-LC), "essential" light-chain (SH-LC), heavy-chain, and rod fragment of myosin from the striated adductor muscle of scallop (*Aequipecten irradians*) were prepared and characterized. A competitive, solid-phase radioimmunoassay on microtiter plates, a combination of two systems described earlier by Kuettner et al. [Kuettner, M. G., Wang, A. L., & Nisonoff, A. (1972) *J. Exp. Med. 135*, 579–595] and Klinman et al. [Klinman, N. R., Pickard, A. R., Sigal, N. H., Gearhart, P. J., Metcalf, E. S., & Pierce, S. K. (1976) *Ann. Immunol. (Paris) 127C*, 489–502], was adapted and used for an immunological survey of different myosins and myosin light chains. Anti-myosin light-chain antibodies were specific for the homologous light chain and did not cross-react with the heterologous one, i.e., regulatory and essential light chains of scallop myosin could be distinguished immunologically. These antibodies also had a high degree of species specificity. A partial cross-reactivity was obtained only for the light chains of two closely related molluscan species out of the over thirty invertebrate or vertebrate species tested. Two populations of anti-SH-LC antibodies were found which differed in their ability to abolish regulation of scallop myofibrils and also in their immunological reactivity with cyanogen bromide fragments of the SH-LC. A comparison of the cross-reactivity of the intact SH-LC with its CNBr fragments showed that most antigenic sites of the SH-LC were available to the antibodies. Free light chains and light chains associated with myosin reacted with antibodies in a very similar manner, indicating that the association of the light chains with myosin may not be accompanied by major conformational changes. Antibodies against scallop myosin heavy chain and rod fragment cross-reacted to a variable extent with all invertebrate myosins but with none of the vertebrate species tested. The antibodies did not cross-react with platelet and *Physarum* myosins. The heavy and light chains of myosin from scallop striated adductor, mantle, and foot were found to be immunologically identical, whereas myosin from smooth adductor showed some differences mainly in the heavy-chain portion which forms the subfragment-1 region of the myosin molecule. Heavy and light chains of scallop heart muscle myosin differed significantly from those of striated adductor muscle. Cross-reactivity did not depend on the regulatory properties of myosin.

Myosin molecules consist of a pair of heavy chains and two different pairs of light chains, the regulatory and essential light chains (Lowey & Risby, 1971; Weeds & Pope, 1971; Burridge & Bray, 1975; Kendrick-Jones et al., 1976). The basic structural organization of myosin is conserved throughout evolution. The α-helical regions of the two heavy chains coil around each other to form a 1500 Å long "tail", the other half of the heavy chains forming the globular "head" regions of myosin. The light chains are bound to these globular regions (Weeds & Lowey, 1971).

In contrast to myosins from lobster tail and vertebrate skeletal muscles which require troponin and tropomyosin for calcium regulation (Ebashi & Ebashi, 1964; Lehman & Szent-Györgyi, 1975), a number of invertebrate myosins are regulatory myosins, e.g., they do not require these proteins for regulation. In molluscan muscles, for example, regulation is associated with myosin directly (Kendrick-Jones et al., 1970) and mediated by a pair of elongated (Stafford & Szent-Györgyi, 1978; Alexis & Gratzer, 1978) regulatory light chains (Szent-Györgyi et al., 1973; Kendrick-Jones et al., 1976). Molluscan myosins bind calcium with high affinity and specificity, and their actin-activated Mg^{2+}–ATPase requires calcium even in the absence of troponin or tropomyosin. Removal of regulatory light chains from scallop myosin leads to a loss of regulatory properties, e.g., calcium sensitivity and calcium binding, which are fully restored upon rebinding of these light chains to myosin (Szent-Györgyi et al., 1973; Simmons & Szent-Györgyi, 1978; Chantler & Szent-Györgyi, 1980). Isolated scallop regulatory light chains, however, do not bind calcium with high affinity (Bagshaw & Kendrick-Jones, 1979). Scallop myofibrils from which the regulatory light chains have been removed readily hybridize with regulatory light chains from other species. In mixed hybrids containing one scallop and one foreign regulatory light chain, calcium sensitivity is always regained regardless of the source of the regulatory light chains (Kendrick-Jones, 1974; Kendrick-Jones et al., 1976). Calcium sensitivity of pure hybrids,

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[1] Abbreviations used: RIA, radioimmunoassay; HVE, high-voltage electrophoresis; R-LC, myosin regulatory light chain; SH-LC, myosin "essential" light chain; GarFc, goat anti-rabbit Fc antibody; S1, subfragment-1; CNBr, cyanogen bromide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Gdn·HCl, guanidine hydrochloride; FITC, fluorescein isothiocyanate; OD, optical density; PMSF, phenylmethanesulfonyl fluoride; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; DEAE, diethylaminoethyl; DTT, dithiothreitol; Na-DodSO$_4$, sodium dodecyl sulfate; BSB, borate/saline buffer (0.15 M NaCl and 14 mM boric acid, titrated to pH 8.0 with NaOH); PBS, phosphate-buffered saline (0.15 M NaCl and 7.5 mM phosphate, pH 7.2); EBT, EDTA/boric acid/Tris buffer (0.75 mM EDTA, 25 mM boric acid, and 45 mM Tris, pH 8.6, at 4 °C); wash, 40 mM NaCl, 3 mM NaN_3, and 5 mM phosphate, pH 7.0; Mg^{2+} wash, wash containing 1 mM $MgCl_2$ and 0.1 mM EGTA.

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however, depends on the source of foreign light chains. Regulatory light chains of vertebrate skeletal myosins (DTNB[1] light chains) do not restore calcium sensitivity of ATPase (Chantler et al., 1979; Sellers et al., 1980) or calcium dependence of tension (Simmons & Szent-Györgyi, 1980). Thus, functional information is carried by these light chains and is expressed when combined with scallop heavy chains.

We have used immunological probes to search for structural similarities and differences between various myosin light chains and heavy chains extending previous studies of Silberstein & Lowey (1977). We were particularly interested to see if structural features associated with myosin-linked regulation can be immunologically identified on the regulatory light chains and heavy chains. We also describe a modification of a radioimmunoassay method that was particularly useful for a broad survey of myosins. The second paper of this series will describe in detail the effects of the different antibodies on calcium regulation. Preliminary reports on part of this work have been presented (Wallimann & Szent-Györgyi, 1979; Wallimann, 1980).

Experimental Procedures

Materials. Live animals were obtained from the Marine Biological Laboratory, Woods Hole, MA, and from the Gulf Specimen Co., Panacea, FA. Muscles were dissected and placed for two days at 4 °C in either 50% glycerol or ethylene glycol containing 20 mM NaCl, 0.5 mM $MgCl_2$, 0.05 mM EDTA, 1.5 mM NaN_3, 0.05 mM PMSF, 0.005% sodium diazine, and 2.5 mM phosphate, pH 7.0. The solution was changed several times, and the muscles were then stored at −20 °C.

Regulatory Light Chains. R-LC of myosin from the striated adductor of *Aequipecten irradians* was purified according to Kendrick-Jones et al. (1976). Since the 0 °C EDTA extract of the myofibrils contained in addition to R-LC's minor protein impurities that were difficult to separate from the LC's, this fraction was discarded. Only the light-chain fraction released by subsequent treatment with 10 mM DTNB and 2 mM EDTA in 10 mM Tris, pH 8.0, was purified and used in this study.

Essential Light Chains. SH-LC was obtained from myofibrils from which R-LC's had been previously extracted following the procedure of Kendrick-Jones et al. (1976). Special precautions were taken to avoid any contamination with R-LC. Prior to the treatment with 6 M Gdn·HCl, myofibrils at a concentration of 1 mg/mL were washed twice with 10 mM EDTA at 4 °C (or more recently at 35 °C) and twice with DTNB (see above) to assure complete removal of R-LC. After chromatography on DEAE-Bio-Gel A at pH 6.0 and gel filtration on Sephadex G-100, the SH-LC's were further chromatographed on DEAE-cellulose (Whatman) in 10 mM Tris-HCl and 1 mM DTT at pH 8.3 at 4 °C with a gradient from 0–0.15 M NaCl to remove any trace of R-LC. Later preparations of SH-LC were freed of remaining R-LC by an anti-R-LC IgG affinity column. SH-LC was stored frozen or lyophilized and prior to use reduced with 5 mM DTT in 0.1 mM EDTA, 4–6 M urea, and 10 mM Tris, pH 8.0, for 30 min at 25 °C and then dialyzed against 0.2 mM DTT, 3 mM NaN_3, and 10 mM Tris-HCl, pH 8.0.

Alkylation of SH-LC and CNBr Cleavage. SH-LC was dialyzed against 50 mM phosphate, pH 8.0, containing 0.2 mM DTT and carboxymethylated with iodoacetic acid as described by Szent-Györgyi et al. (1973). The alkylated SH-LC was dialyzed against 1% acetic acid, lyophilized, and dissolved in a minimal volume of 70% formic acid containing 1% phenol, and solid CNBr was added in a 200-fold molar excess over methionine (4 Met/SH-LC). The tubes containing the samples were flushed with nitrogen and then closed off and allowed to stand protected from light at 20 °C for 12–15 h. Completion of the reaction was checked by 15% polyacrylamide gel electrophoresis in the presence of both 4 M urea and 0.1% NaDodSO$_4$. After a 30-fold dilution with distilled water, the samples were lyophilized and dissolved in BSB if used for radioimmunoassays or in pH 2.0 buffer (formic acid/acetic acid/H_2O, 2:8:90) if used for high-voltage electrophoresis.

Preparative High-Voltage Electrophoresis of CNBr-Fragmented SH-LC. CNBr fragments (1–2 mg) were applied 14 cm from the anode onto Whatman 3MM paper and electrophoresed at 3.5 kV (140 mA) for 35 min in a Savant Model HVE 8036 apparatus. Five ninhydrin-positive spots were eluted with 0.1 M NH_4OH, lyophilized, and dissolved in BSB for radioimmunoassays. A strip of paper from a region where no peptides could be detected was eluted the same way and used as a control for radioimmunoassays.

Purification of Scallop Myosin. Aequipecten myosin was prepared according to a modified procedure of Focant & Huriaux (1976) by ammonium sulfate fractionation of actomyosin in the presence of Mg^{2+}ATP (Stafford et al., 1979). The 45–55% $(NH_4)_2SO_4$ cut was dialyzed and the precipitated myosin washed several times with 40 mM NaCl and 5 mM phosphate, pH 7.0. The myosin was further purified by gel filtration on a Sepharose-4B column (Pharmacia) equilibrated with 0.6 M NaCl, 1 mM ATP, 1 mM $MgCl_2$, 0.1 mM EGTA, 3 mM NaN_3, and 10 mM phosphate, pH 7.5. Myosin was dissolved immediately before chromatography in the above buffer containing 10 mM ATP. The pooled fractions were dialyzed against low-salt buffer and centrifuged. Myosin obtained in this way could be stored for more than 2 months without significant changes in specific activity and calcium sensitivity after dialysis against 40% $(NH_4)_2SO_4$, 1 mM $MgCl_2$, 1 mM ATP, 0.1 mM EGTA, and 10 mM phosphate, pH 7.0, at 4 °C (T. Wallimann and R. Naturman, unpublished observation).

Preparation of Myosin Subfragment-1. S1 was prepared by papain digestion of either intact myofibrils, desensitized myofibrils, or purified myosin in the presence or absence of divalent cations to yield (Ca,Mg)S1 or (EDTA)S1, respectively (Stafford et al., 1979).

Preparation of Myosin-Rod Fragment. The precipitate formed after dialysis of the 45–70% $(NH_4)_2SO_4$ cut of S1 preparations contained mostly myosin-rod, single-headed myosin, some undigested myosin, and traces of actin and tropomyosin. The precipitate was dissolved in 0.6 M NaCl and 5 mM phosphate, pH 7.0, brought to 66% with cold ethanol, and stirred overnight at 4 °C. The precipitate was freed of ethanol by washing it twice with 40 mM NaCl and 5 mM phosphate, pH 6.0. Subsequent resuspension in high-salt buffer resolubilized mostly myosin rod. An additional exposure to acid (to pH 1.0 with HCl and back to 7.0 with NaOH) followed by a high-speed centrifugation, precipitation at low salt, and subsequent lyophilization removed traces of myosin. Chromatography on Sepharose-4B in 0.6 M NaCl and 5 mM phosphate, pH 7.0, yielded pure myosin rod (Figure 9) which was stored frozen in 0.6 M NaCl and 5 mM NaN_3 at pH 7.0.

Immunization Schedule. After the collection of four bleedings used as preimmune sera, female New Zealand rabbits weighing 4–6 lb each were injected at multiple sites into the footpads with 500 μg of antigen (R-LC, SH-LC, myosin, or myosin rod) dissolved in 10 mM Tris buffer, pH

7.5, containing 40 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EGTA, and 1 mM DTT and subsequently emulsified with an equal volume of Bacto complete Freund's adjuvant (Difco Laboratory, Detroit, MI). The buffer was made 0.6 M in NaCl for myosin or myosin rod. Three to four weeks after the first injection a booster with 500 μg of antigen prepared as above with complete Freund's adjuvant was given again at multiple sites into the footpads. After 2 weeks 10–12 bleedings of 50 mL were taken from the ear veins at weekly intervals, stored individually, and tested for titer, specificity, and cross-reactivity. A second booster of 250 μg of antigen emulsified in incomplete Freund's adjuvant was given to some rabbits. Six rabbits were injected with R-LC, six with SH-LC, three with myosin, and three with myosin rod.

Preparation of Antisera, Immunoglobulins, and Specific Antibodies. Drawn blood was made 3 mM in NaN_3, allowed to clot at 37 °C for 2 h, and then kept at 4 °C overnight. After the clot was removed, sera was freed from residual erythrocytes and fat by centrifugation, followed by filtration, and stored at −20 °C. The IgG fraction was then purified by precipitation with $(NH_4)_2SO_4$ at 45% and with Na_2SO_4 at 16% saturation, respectively, chromatographed on DEAE-cellulose equilibrated with 0.0175 M phosphate at pH 6.3, concentrated by $(NH_4)_2SO_4$ precipitation, dialyzed against 40 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EGTA, and 5 mM phosphate, pH 7.0 (referred to as Mg^{2+} wash), and stored frozen.

Specific antibodies were obtained by antigen affinity chromatography. Highly purified R-LC, SH-LC (Figure 1), myosin, or myosin-rod (Figure 9) (50 mg each) were dissolved in 0.5 M NaCl and 0.1 M $NaHCO_3$ at pH 8.3 and coupled to 7.5 g of CNBr-activated Sepharose-4B (Pharmacia) by incubating for 24 h at 4 °C on a gently rotating rotary shaker. The matrix was extensively washed with 1 M NaCl and 0.1 M Tris-HCl, pH 8.0. After passage of 100 mL of antiserum (containing 60–150 mg of specific antibody) through the affinity columns and extensive washing with the coupling buffer, bound antibody was eluted in steps, first with 0.1 M glycine/HCl at pH 2.5 and then at pH 2.0. The eluates were neutralized immediately with 1 M Tris-HCl (pH 8.0), dialyzed, concentrated by vacuum dialysis, tested for antibody activity and specificity, and then stored frozen. An additional fraction of active, high-affinity antibody could be eluted subsequently with 4 M Gdn·HCl. The overall yield after affinity chromatography of precipitable antibody (at antigen equivalence) of the combined eluates was between 65 and 80%.

Double-Immunodiffusion Tests. Gel diffusion (Ouchterlony, 1967) was carried out with 1.5% agar Nobel (Difco) dissolved in either PBS or BSB. For the diffusion of actomyosin and myosin, 0.6 M NaCl and 10 mM Mg^{2+}ATP in 20 mM Tris-HCl at pH 8.0 were used.

Immunoelectrophoresis. Immunoelectrophoresis (Hudson & Hay, 1976) was performed with 1.5% purified agarose (Difco) dissolved in EBT buffer as described earlier (Wallimann et al., 1977).

Quantitative Immunoprecipitin Analysis. To 0.5 mL of either preimmune IgG, anti-R-LC IgG, or anti-SH-LC IgG solution (dialyzed against PBS and clarified by a 30-min centrifugation at 3000*g*) were added increasing amounts of either R-LC or SH-LC, or a mixture of both. After adjustment of the final volume with PBS to 2 mL, the samples were incubated for 2 h at 25 °C and 20 h at 4 °C. The precipitate was washed with PBS, lyophilized, and dissolved in 0.1 N NaOH containing 2% Na_2CO_3. The total protein, antibody titer, and the molar ratio of bound antibody per light chain could be determined by extinction measurements ($OD_{280-320nm}$) using an $E_{280nm}^{1\%,1cm}$ of 14 for IgG and 1.8 and 5.75 for R-LC and SH-LC, respectively, and by assuming that the light chain is quantitatively precipitated at antibody excess.

Immunofluorescence. Preimmune IgG and affinity-purified anti-myosin LC IgG's were conjugated with FITC according to the method described by Fujiwara & Pollard (1976). The fractions having a molar FITC/protein ratio of 2–3 were pooled and used for direct immunofluorescence staining of glycerinated *Aequipecten* myofibrils suspended in Mg^{2+} wash, pH 7.0. Pictures were taken on a standard Zeiss fluorescence microscope with specifications described by Lowey & Steiner (1972), recorded on Ilford HP4 film, and developed with Diafine developer.

Immunoreplicas. To make antibody-containing overlay gels (Wallimann et al., 1978), we mixed 1–1.5 mL of anti-myosin light-chain IgG (5–10 mg/mL) with 1.5 mL of a solution containing 1.5% melted agar Noble in BSB at 50 °C and poured it immediately onto a cover slide seated on an ice-cold aluminum block. After solidification, the gel was transferred on top of a microslab gel (Matsudaira & Burgess, 1978) and incubated in a moist chamber for 24–72 h at 4 °C. This technique was successfully used with anti-myosin heavy-chain and anti-rod antibodies as well. After unprecipitated antibody was washed out with 0.5 M NaCl in BSB for at least 2 days, the overlays were photographed directly under dark-field illumination or were stained with 1% Amido Black in 45% methanol and 10% acetic acid. Gels were similarly stained and photographed after immunodiffusion and immunoelectrophoresis.

Competitive Solid-Phase Radioimmunoassay. A combination of a modified version of the microtiter plate assay (Klinman et al., 1976) with a competitive radioimmunoassay (Kuettner et al., 1972) led to the development of the competitive solid-phase RIA described in detail as follows: Specifically purified goat anti-rabbit Fc antibody (GarFc, a generous gift from Dr. A. Nisonoff's laboratory) was iodinated with ^{125}I by using the chloramine-T method of Hunter (1970). Later a batch of already iodinated [^{125}I]GarFc (a generous gift from Dr. E. Cannon) was used for the same purpose. The amount of chloramine-T used (12% of protein w/w) was sufficient to give 70% incorporation of iodine by using 600 μCi of ^{125}I (New England Nuclear)/500 μg of GarFc.

Antigens (1 mg/mL *Aequipecten* R-LC or SH-LC dissolved in BSB, total myosin or myosin rod dissolved in BSB containing 0.5 M NaCl) were bound to the U wells of poly(vinyl chloride) microtiter plates (Cooke Laboratories VA No. 1-220-24) by incubating 0.2 mL of antigen for 12 h at 4 °C. The wells were later extensively rinsed with BSB or BSB containing 0.5 M NaCl, and 0.3 mL of 2% horse serum (Miles Laboratories) in BSB was added to each well and incubated for 12 h at 4 °C. After being washed with BSB, the antigen-coated plates were either used directly or stored in BSB for up to 4 days at 4 °C. The antigen concentration may be lowered by a factor of 10 and the incubation time limited to 4–5 h. In addition, if the antigen is stable enough it may be reused several times for the same purpose.

Affinity-purified antibodies were used as primary antibodies. Binding curves, maximal level of binding, and range of linearity between the amount of primary antibodies added and the amount of radioactivity obtained were established with a direct radioimmunobinding assay. A total of 0.2 mL from serial dilutions (1:50–1:5000) of the primary antibodies at 0.5–1 mg/mL was added to individual wells which were rinsed with BSB after an incubation period of 12 h at 4 °C followed by reincubation with an excess of ^{125}I-labeled secondary goat

anti-rabbit Fc antibody ([^{125}I]GarFc). Antibodies were diluted with BSB containing 2% horse serum, and the final concentration of rabbit IgG was kept constant by the addition of rabbit preimmune IgG.

Primary antibodies (0.5–1 mg/mL) were diluted (1:1000–1:3000) for RIA's to reduce binding to 70–80% of the maximum. Aliquots (0.15–0.20 mL) of diluted, specific primary antibody were premixed for 1–3 h at 25 °C with increasing amounts (1 ng–100 μg) of antigens or proteins to be tested for immunological cross-reactivity. The final volume was kept constant by adding BSB containing 2% horse serum. Aliquots (0.2–0.3 mL) of this mixture were then added to the individual wells which were previously coated with antigen. After incubation of the mixture containing primary antibody and the proteins to be checked for cross-reactivity with the solid phase antigen (2 h at 20 °C and 10 h at 4 °C), the wells were each rinsed extensively (20–25 times) with BSB. Thereafter, equal amounts of ^{125}I-labeled GarFc in 0.2 mL were added to each well (approximately 0.1–0.5 μCi/well, representing a 5–10-fold excess over the maximum amount of GarFc bound). After an incubation period of 2 h at 20 °C and 10 h at 4 °C and again excessive rinsing of the wells, the microtiter plate was air-dried and the wells were cut out and counted in a γ counter. Appropriate controls for background and unspecific binding were made by using rabbit preimmune IgG and by omitting either the antigen and/or primary antibody binding steps. Later the incubation times were reduced to 2 h at 25 °C followed by 3 h at 4 °C.

Other Procedures. Protein concentrations were determined either by the Folin–Lowry procedure (Lowry et al., 1951) using bovine serum albumin previously standardized by Kjeldahl nitrogen determination as a standard or by the biuret method (Gornall et al., 1949) using an OD of 0.07 cm^{-1} $(mg/mL)^{-1}$ at 550 nm. For absorbance readings the following values of $E_{280nm}^{1\%,1cm}$ were used after correction for light scattering: myofibrils, 7.2; myosin, 5.3; R-LC, 1.8; SH-LC, 5.75.

Polyacrylamide gel electrophoresis in the presence of 0.1% $NaDodSO_4$ or 8 M urea was performed as described earlier (Kendrick-Jones et al., 1976). Gels were stained with Acid Fast Green prior to densitometry and planimetry. Microslab gel electrophoresis in the presence of 0.1% $NaDodSO_4$ (Matsudaira & Burgess, 1978) and urea microslab gel electrophoresis (the same system but $NaDodSO_4$ replaced by 8 M urea) were used routinely for gel analyses not requiring densitometry and for the microslab immunoreplica technique. These gels were stained with Coomassie Brilliant Blue R-250 or G-250.

Results

Antibodies specific for R-LC, SH-LC, heavy-chain, and rod fragment of scallop (*Aequipecten*) myosin were elicited in rabbits and characterized by double immunodiffusion, immunoelectrophoresis, immunoprecipitation, immunoreplica technique, radioimmunoassay, and affinity chromatography. Their cross-reactivity with actomyosin, myosin, or myosin light chains from a variety of species throughout the animal kingdom was investigated by a competitive, solid-phase radioimmunoassay technique developed for the immunological comparison of myosin and myosin light chains.

Purity of Antigens. Scallop myosin light chains of the highest possible purity were used as immunogens to avoid any cross-contamination of the two types of light chains. The light chains were characterized by polyacrylamide gel electrophoresis in 8 M urea which shows that the two light-chain preparations are not cross-contaminated and in $NaDodSO_4$ which demonstrates the absence of any myofibrillar contaminants

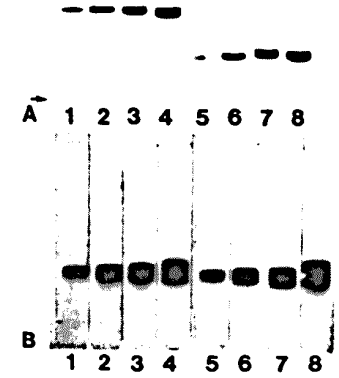

FIGURE 1: Electrophoresis of *Aequipecten* myosin light chains used as antigens for immunization and for purification by affinity chromatography of monospecific antibodies. (A) 8, 20, 50, and 100 μg of R-LC (1–4) and 8, 20, 50, and 100 μg of SH-LC (5–8), after electrophoresis on 10% polyacrylamide disc gels in the presence of 8 M urea. Arrow is indicating the position of the front marker. (B) 5, 20, 50, and 100 μg of R-LC (1–4) and 5, 20, 50, and 100 μg of SH-LC (5–8), after electrophoresis on 10% polyacrylamide disc gels in the presence of 0.1% $NaDodSO_4$.

(Figure 1) or by polyacrylamide gel electrophoresis in native conditions (not shown). Within the test range of 1–100 μg of light chain/gel a single protein band was observed in each case. Since R-LC and SH-LC moved differently on urea–polyacrylamide gel electrophoresis at pH 8.6 (Figure 1A) this test was particularly important to establish their homogeneity. The purity of scallop myosin and myosin rod fragment after Sepharose-4B chromatography is demonstrated by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 9). The protein preparations shown on these figures were used as immunogens and later for the purification of antibodies by affinity chromatography.

Characterization of Antibodies. Both the antibody directed against the R-LC and that directed against the SH-LC alter the behavior of scallop myosin (Wallimann & Szent-Györgyi, 1981). To be certain that these effects were specific, we extensively characterized the antibodies. Their cross-reactivity with myosin and myosin light chains as well as with other related muscle proteins from a variety of species was investigated initially by double immunodiffusion and -electrophoresis and later by solid-phase radioimmunoassay. Double immunodiffusion and -electrophoresis of anti-R-LC and anti-SH-LC antibody showed only one single precipitin line when diffused against pure myosin light chains ranging in concentration from 1 μg/mL–10 mg/mL or against concentrated DTNB and Gdn·HCl extracts of myosin used as starting material for light-chain purification. An example of an immunoelectrophoresis demonstrating the specificity of the two antibodies for the homologous light chain and lack of cross-reactivity with the heterologous light chain from scallop myosin is given in Figure 2. An unexpected high species specificity of these anti-*Aequipecten* myosin light-chain antibodies was observed. Of all the species listed in Table IV and in the legend of Table IV, only the homologous myosin light chains of the closely related molluscs *Placopecten magellanicus* and *Mercenaria mercenaria* showed a limited degree of cross-reactivity. A 10–25 times higher concentration of these light chains was required to get precipitin lines of comparable intensities (insets of Figure 6 and 7). Moreover, R-LC and SH-LC diffused against a mixture of anti-R-LC and anti-SH-LC IgG formed two precipitin lines crossing over without

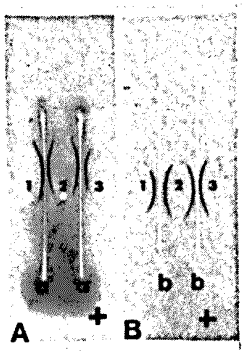

FIGURE 2: Immunoelectrophoresis of *Aequipecten* myosin light chains diffused against rabbit anti-*Aequipecten* myosin light-chain antibodies. (A) 0.25 mg/mL R-LC (1), concentrated extract of Gdn·HCl-treated scallop myofibrils containing both types of myosin light chains (2), and 0.05 mg/mL R-LC (3), diffused against rabbit anti-scallop R-LC IgG (a). (B) 0.05 mg/mL SH-LC (1), concentrated extract of Gdn·HCl-treated scallop myofibrils as above (2), and 0.25 mg/mL SH-LC (3), diffused against rabbit anti-scallop SH-LC IgG (b).

any interaction indicating that the R-LC and the SH-LC of scallop myosin are two immunologically distinct proteins (inset B of Figure 8).

The specificity of antibodies was further indicated by quantitative immunoprecipitin analysis. A typcial bell-shaped precipitin curve was obtained with anti-R-LC IgG when the amount of precipitate was plotted as a function of the amount of R-LC added (Figure 3A). No precipitate was formed with up to 1 mg of SH-LC, and there was no change in shape nor any shift of the precipitin curve when SH-LC was added simultaneously with the R-LC (not shown here), demonstrating the specificity of the anti-R-LC antibody and the absence of any precipitating or nonprecipitating antibodies directed against the heterologous SH-LC in the anti-R-LC antisera. The estimated maximal molar ratio of antibody bound to R-LC at indefinite antibody excess was ~6 (Figure 3A). Similar results were obtained with the anti-SH-LC antibody except that the maximal molar ratio of antibody bound to SH-LC was slightly lower (Figure 3B).

The immunological identity of R-LC obtained by EDTA or DTNB treatment of scallop myosin could be established by competitive, quantitative immunoprecipitation analysis as well as by double immunodiffusion (not shown). Immunoreplicas of scallop myofibrils after $NaDodSO_4$–polyacrylamide gel electrophoresis show the specificity of the anti-scallop myosin light-chain antibodies for the myosin light chain (Figure 4A). No cross-reactivity with any myofibrillar protein other than light chains was observed. This sensitive technique was successfully applied to polypeptides with a M_r as high as 200 000, e.g., very small amounts of myosin, showing only a faint heavy-chain band after electrophoresis and staining could easily be identified by immunoreplicas containing anti-myosin heavy-chain antibody. Immunoreplicas of myofibrils after polyacrylamide gel electrophoresis in 8 M urea at pH 8.6 show the specificity of the anti-scallop myosin light-chain antibodies for the homologous light chain and the absence of cross-reactivity with the heterologous light chains (Figure 4B).

Both antibodies, after conjugation with FITC, stained the A band of scallop myofibrils. Staining in the region of the H zone which seems to be especially narrow in scallop myofibrils was absent or weak (Figure 5). These observations were confirmed by immunoelectronmicroscopy (R. Craig and T. Wallimann, unpublished observations). Binding of anti-myosin light-chain antibodies was limited to the A band and was not observed in the pseudo H band or in the I band. Binding of these antibodies to the proximal region of the head of isolated

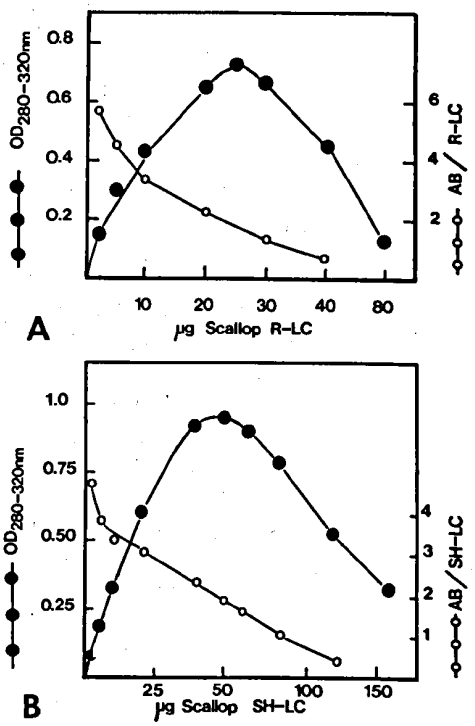

FIGURE 3: (A) Quantitative precipitin analysis of anti-scallop R-LC IgG vs. scallop R-LC. Increasing amounts of R-LC were added to a fixed volume (0.5 mL) of antibody solution. Total protein content of precipitate measured spectrophotometrically [(●) $OD_{280-320nm}$]; molar ratio of antibody bound to R-LC [(○) AB/R-LC]. (B) Quantitative precipitin analysis of anti-scallop SH-LC IgG vs. scallop SH-LC. Increasing amounts of SH-LC were added to a fixed volume (0.5 mL) of antibody solution. Total protein content of precipitate measured spectrophotometrically [(●) $OD_{280-320nm}$]; molar ratio of antibody bound to SH-LC [(○) AB/SH-LC].

myosin molecules could also be observed by electron microscopy of shadow cast material (P. Flicker and T. Wallimann, unpublished experiments).

Both scallop light chains remained freely accessible to the rather bulky antibodies even when bound to myosin. Myosin or S1 reacted with anti-R-LC and anti-SH-LC antibodies in the same way as isolated light chains (see inset A of Figure 8 for a comparison of the precipitin reaction of isolated SH-LC and S1 with anti-SH-LC antibody). Conformational differences, if any, between free and bound light chains were not detectable immunologically by this technique.

Evidence for Two Anti-SH-LC Antibody Populations. Two types of antibody populations were formed in different rabbits when immunized with the same batch of SH-LC. Four out of the six rabbits produced without delay a type of antibody that caused a loss of calcium sensitivity when added to scallop myofibrils. Another rabbit started to produce such antibodies only after a lag period of 4–5 weeks. Rabbit no. 4 was unique in yielding persistently over the entire bleeding period an antibody that had no effect on calcium regulation when added to scallop myofibrils (Wallimann & Szent-Györgyi, 1981). The two anti-SH-LC antibodies (Ab) characterized best and used for this work were the desensitizing type produced by rabbit no. 5 (referred to as R_5-desensitizing anti-SH-LC Ab) and the nondesensitizing type produced by rabbit no. 4 (referred to as R_4-nondesensitizing anti-SH-LC Ab). Both of these antibodies were specific for the SH-LC, did not cross-react with R-LC (Figure 7 and 8), and reacted with pure SH-LC, subfragment-1 (CaMg-S1), and myosin forming completely fused precipitin lines (inset of Figure 8). Obviously, the differences within these two antibody populations were too

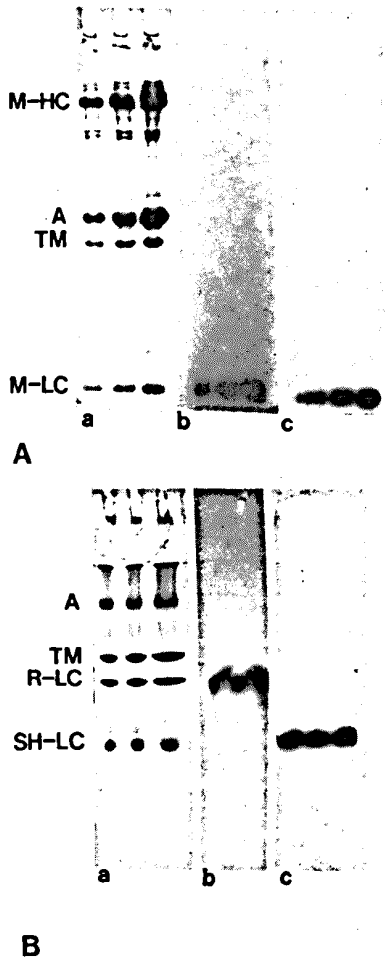

FIGURE 4: (A) Immunoreplica of myofibrils after electrophoresis in $NaDodSO_4$. 12% polyacrylamide microslab gel electrophoresis in 0.1% $NaDodSO_4$ of increasing amounts of scallop myofibrils stained for protein with Coomassie Blue (a); anti-R-LC (b) and anti-SH-LC (c) immunoreplica of (a). Precipitin reaction on immunoreplicas stained with Amido Black after washing out nonprecipitated antibody as described under Experimental Procedures. Myosin heavy chain (M-HC); actin (A); tropomyosin (TM); myosin light chains (M-LC). (B) Immunoreplica of myofibrils after electrophoresis in urea. 12% polyacrylamide microslab gel electrophoresis in 8 M urea at pH 8.6 of increasing amounts of scallop myofibrils stained for protein as in Figure 5 (a); anti-R-LC (b) and anti-SH-LC (c) immunoreplica of (a). Actin (A); tropomyosin (TM); regulatory light chain (R-LC); essential light chain (SH-LC).

subtle to be detected by double immunodiffusion. Immunoelectrophoresis and RIA failed to differentiate between desensitizing and nondesensitizing anti-SH-LC Ab's in their specificity for and reactivity with pure SH-LC, S1, and myosin from scallop. However, differences in the reactivity of these antibodies to various regions of the SH-LC could be demonstrated by competitive, solid-phase RIA analysis of the five CNBr peptides of this LC. The five CNBr peptides separated by high-voltage electrophoresis corresponded to the four methionine residues found in scallop SH-LC (Kendrick-Jones et al., 1976). One of the peptides, however, was very small, moved rapidly with a R_f value of 2.42, and did not react with the antibodies (Table I). The other four peptides individually or in mixture showed a surprisingly high degree of cross-reactivity to SH-LC when tested with both R_4- and R_5-anti-SH-LC IgG. Except for peptide 5 all the others (1–4) were immunologically "active" and were reacting with both antibody populations, whereas a control eluate had no effect. Obvious differences were noted between the two antibody populations.

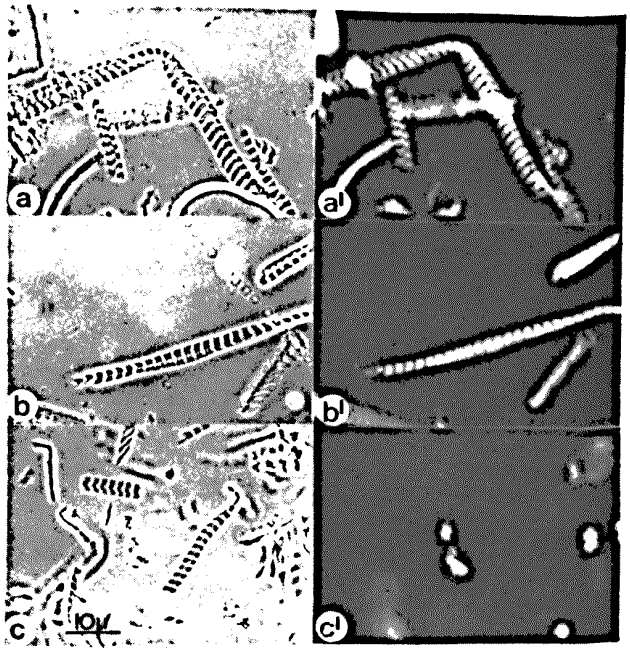

FIGURE 5: Direct immunofluorescence staining of scallop myofibrils with FITC-labeled anti-R-LC IgG (a'), anti-SH-LC IgG (b'), and preimmune IgG (c'). Corresponding phase contrast picture shown on the left (a–c). Bar in (c) represents 10 μm.

The mixture of CNBr fragments competed to a significantly larger extent (90% cross-reactivity) with the intact SH-LC in the case of R_4-nondesensitizing antibody than with R_5-desensitizing antibody (63% cross-reactivity). This difference was also obtained with individual peptides separated by HVE and tested the same way. Peptides 1–4 gave consistently higher values of cross-reactivity with antibodies from rabbit no. 4 than with the ones from rabbit no. 5.

Immunological Survey of Myosin Light Chains by Competitive, Solid-Phase Radioimmunoassay. All findings concerning the specificity of the antibodies and interspecies cross-reactivity of the light chains obtained by immunodiffusion, immunoelectrophoresis, and immunoprecipitation were

Table I: Immunological Cross-Reactivity of CNBr Peptides of SH-LC with Two Types of Anti-SH-LC Antibodies

	R_5-desensitizing anti-SH-LC IgG[a]	R_4-nondesensitizing anti-SH-LC IgG[a]
intact myosin SH-LC	100[b]	100[b]
mixture of CNBr fragments of SH-LC before HVE	63	90
isolated peptide 1 $R_f = 0.63$[c]	32	42
isolated peptide 2 $R_f = 1.08$	20	32
isolated peptide 3 $R_f = 1.71$	27	38
isolated peptide 4 $R_f = 1.94$	11	28
isolated peptide 5 $R_f = 2.42$	0	0
control eluate	0	0
sum of cross-reactivities of isolated peptides (1-5)	90	140

[a] Competitive solid-phase radioimmunoassay (RIA) showing cross-reactivity of anti-SH-LC IgG from rabbit no. 4 (R_4-nondesensitizing anti-SH-LC antibody) and anti-SH-LC IgG from rabbit no. 5 (R_5-desensitizing antibody) with cyanogen bromide (CNBr) fragments of carboxymethylated SH-LC). [b] Numbers indicate percent of cross-reactivity (average of three experiments) and are expressed relative to the maximal value of competition obtained with the intact SH-LC which is taken as 100%. [c] R_f values determined after high-voltage electrophoresis (HVE) at pH 2.0 using e-dinitrophenyllysine as a standard.

Table II: Interspecies Cross-Reactivity of Rabbit Anti-*Aequipecten* Myosin Light-Chain Antibodies

species[a]	common name		anti-*Aequipecten* myosin R-LC IgG		anti-*Aequipecten* myosin SH-LC IgG	
			Ouchterlony[b]	RIA[c]	Ouchterlony[b]	RIA[c]
Mollusca						
Pelecypoda						
Aequipecten irradians	bay scallop	R-LC	+	100	−	0
		SH-LC	−	0	+	100
Placopecten magellanicus	deep sea scallop	R-LC	+	63	−	0
		SH-LC	−	0	+	45
Mercenaria mercenaria	quahog	R-LC	−	0	−	0
		SH-LC	−	0	+	23

[a] Actomyosin, myosin, or isolated myosin light chains from all the other invertebrate and vertebrate species listed in Table IV and the corresponding legend including troponin, DTNB-LC, and alkali LC from chicken and rabbit, and calcium-binding protein from scallop did not cross-react with anti-*Aequipecten* myosin R-LC or SH-LC antibodies in double-immunodiffusion tests and competitive solid-phase radioimmunoassay (RIA). For details see Experimental Procedures as well as Figures 6 and 7. [b] Qualitative cross-reactivity determined by double immunodiffusion of serial dilutions (10–0.05 mg/mL) of myosin light chains (R-LC and SH-LC) from different species against rabbit anti-*Aequipecten* myosin R-LC IgG and SH-LC IgG, judged by the appearance of a single precipitin line (+). [c] Numbers indicate percent cross-reactivity determined by competitive solid-phase radioimmunoassay (RIA) for purified myosin light chains (R-LC and SH-LC) from different species with rabbit anti-*Aequipecten* myosin R-LC IgG and SH-LC IgG. Percent cross-reactivity is expressed relative to the maximal value of competition obtained with the homologous myosin light chain from *Aequipecten* which is taken as 100%.

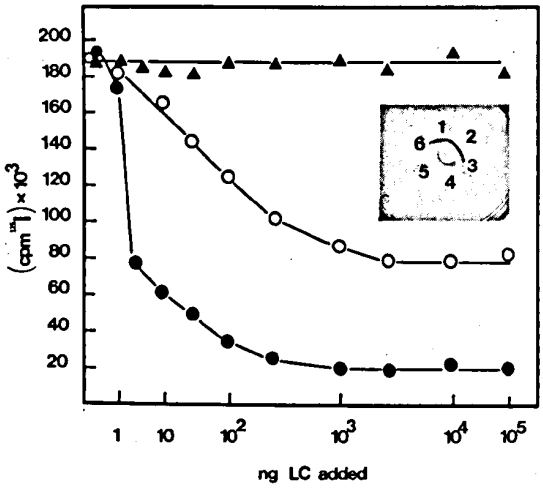

FIGURE 6: Specificity and cross-reactivity of rabbit anti-scallop myosin regulatory light-chain antibody. Competitive solid-phase radioimmunoassay (RIA) consisting of an anti-*Aequipecten* R-LC IgG/*Aequipecten* R-LC system to which increasing amounts (indicated in nanograms) of *Aequipecten* R-LC (●), *Placopecten* R-LC (○), or *Aequipecten* SH-LC (▲) were added competitively. Subsequent binding of ^{125}I-labeled secondary goat anti-rabbit Fc antibody is indicated in counts per minute × 10^3. (Inserted double-diffusion plate) Anti-*Aequipecten* R-LC IgG, in the center well; 0.2 mg/mL *Aequipecten* R-LC (1); 2 mg/mL *Placopecten* R-LC (2); 5 mg/mL *Mercenaria* R-LC (3); 5 mg/mL *Aequipecten* SH-LC (4); 5 mg/mL lobster total light chains (5); 5 mg/mL chicken DTNB-LC (6). Details concerning radioimmunoassay in this and the two following figures are described under Experimental Procedures and Figure 5.

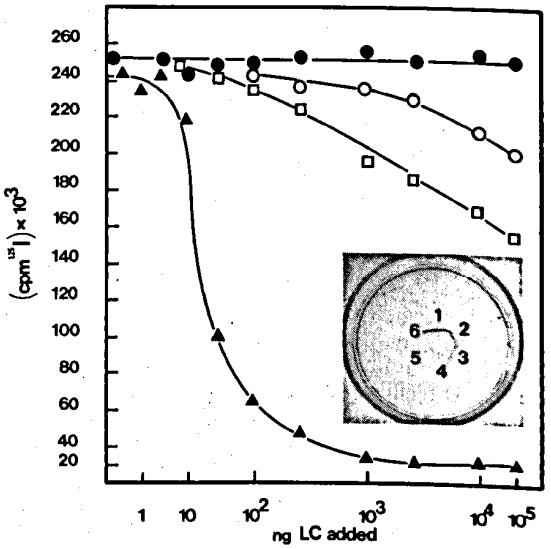

FIGURE 7: Specificity and cross-reactivity of R_5-desensitizing anti-scallop myosin essential light-chain (SH-LC) antibody. (The primary anti-SH-LC antibody used here was obtained from rabbit no. 5 and desensitized scallop myofibrils and myosin). Competitive solid-phase radioimmunoassay (RIA) consisting of an anti-*Aequipecten* SH-LC IgG/*Aequipecten* SH-LC system to which increasing amounts (expressed in nanograms) of *Aequipecten* R-LC (●), *Mercenaria* SH-LC (○), *Placopecten* SH-LC (□), or *Aequipecten* SH-LC (▲) were added competitively. Subsequent binding of ^{125}I-labeled secondary goat anti-rabbit Fc is expressed in counts per minute × 10^3. (Inserted double-immunodiffusion plate) Anti-*Aequipecten* SH-LC, in the center well; 0.2 mg/mL *Aequipecten* SH-LC (1); 2 mg/mL *Placopecten* SH-LC (2); 10 mg/mL *Mercenaria* SH-LC (3); 5 mg/mL *Aequipecten* R-LC (4); 5 mg/mL lobster total light chains (5); 5 mg/mL chicken alkali light chains (6).

confirmed and, in addition, quantitated by RIA. This method was especially suited for comparative immunological work, and its advantages over classical techniques will be discussed later.

Interspecies Cross-Reactivity of Anti-Scallop Myosin Light-Chain Antibodies. Anti-*Aequipecten* myosin R-LC proved to be highly specific. To test for cross-reactivity, we added myosin light chains (ranging from 1 ng up to 10 μg) in a competitive fashion to anti-*Aequipecten* myosin R-LC antibody in the presence of the homologous light chain. The R-LC of the closely related scallop, *Placopecten*, the only light chain able to compete with the R-LC of *Aequipecten*, showed ~63% cross-reactivity (Figure 6, Table II). No cross-reactivity was detected with the heterologous SH-LC from *Aequipecten* nor with any myosin light chain from ~40 species listed in Table IV and its legend. Troponin from chicken or rabbit and calcium binding protein from scallop did not cross-react either.

In the case of R_5-desensitizing anti-SH-LC antibody, a limited cross-reactivity was obtained with the homologous SH-LC from *Placopecten* and a very weak one with the SH-LC from *Mercenaria*, which are related molluscan species (Figure 7). Again, no cross-reactivity whatsoever could be detected with the heterologous R-LC. The R_4-nondesensitizing type of anti-SH-LC antibody was also highly specific (Figure 8). The results concerning the interspecies cross-reactivity of these anti-*Aequipecten* myosin light-chain antibodies are summarized in Table II. The absence of cross-reactivity be-

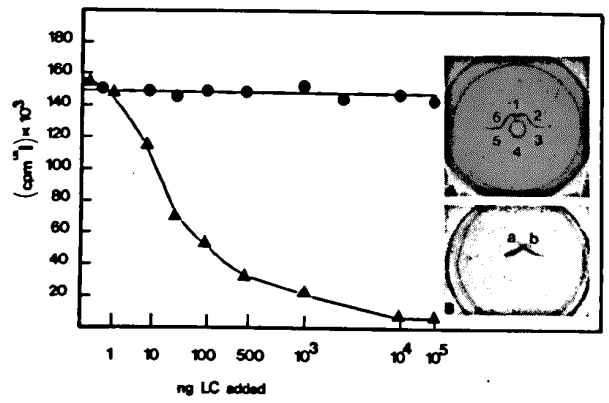

FIGURE 8: Specificity of R_4-nondesensitizing anti-scallop myosin essential light-chain (SH-LC) antibody. (The primary anti-SH-LC antibody used here was obtained from rabbit no. 4 and did not desensitize scallop myofibrils or myosin). Competitive solid-phase radioimmunoassay (RIA) consisting of an anti-*Aequipecten* SH-LC IgG/*Aequipecten* SH-LC system to which increasing amounts (expressed in nanograms) of *Aequipecten* R-LC (●) or *Aequipecten* SH-LC (▲) were added competitively. Subsequent binding of ^{125}I-labeled secondary goat anti-rabbit Fc is expressed in counts per minute $\times 10^3$. (Inserted double-immunodiffusion plates) Comparison of the two types of anti-SH-LC antibodies (A) and immunological nonidentity of R-LC and SH-LC (B). (A) 0.2 mg/mL *Aequipecten* myosin subfragment S1 (Ca,Mg)S1, in the center well; anti-*Aequipecten* SH-LC IgG (R_5-desensitizing antibody) (6); anti-*Aequipecten* SH-LC IgG (R_4-nondesensitizing antibody) (2); a mixture of (2) and (6) (1); 0.07 mg/mL *Aequipecten* SH-LC (3, 5). (B) Mixture of 0.2 mg/mL *Aequipecten* R-LC and SH-LC, in center well; anti-*Aequipecten* R-LC IgG (a); anti-*Aequipecten* SH-LC Ig (b).

tween R-LC and SH-LC from scallop and DTNB-LC of rabbit was also confirmed by noncompetitive, solid-phase RIA where no binding of antibody to heterologous antigens could be observed.

Organ Specificity. Antibodies directed against the myosin R-LC of striated adductor muscle from *Aequipecten* were tested by RIA for cross-reactivity with R-LC or myosin of different organs from the two scallop species *Aequipecten* and *Placopecten.* Judged by the similar extent of cross-reactivity, R-LC of striated adductor, smooth adductor (catch muscle), foot, and mantle of both species seem to be immunologically very similar. However, cross-reactivity of R-LC from the heart muscle of both scallop species was significantly lower (Table III)

Interspecies Cross-Reactivity of Antibodies against Scallop Myosin Heavy Chain. The immunological cross-reactivity of specific antibodies against heavy chain and rod fragment of *Aequipecten* striated adductor myosin was determined by RIA with a wide variety of species from the animal kingdom, including muscles with thick filament linked, as well as with thin filament linked, control of calcium regulation (Table IV).

Anti-total-scallop myosin contained in addition to anti-heavy-chain antibodies antibodies against both light chains (Figure 9) in accordance with the studies of Lutz et al. (1978) on anti-rabbit myosin antibodies. Anti-myosin heavy-chain antibodies, obtained by sequential absorption of anti-total-myosin antibodies with immobilized R-LC and SH-LC followed by affinity chromatography, formed single precipitin lines with myosin, myosin rod, and subfragment-1 from *Aequipecten* but no longer cross-reacted with myosin light chains (Figure 9A,B).

Anti-scallop myosin heavy-chain antibodies obtained from rabbit did cross-react to a variable extent with myosin from all of the over 30 invertebrate species tested (Table IV). The degree of cross-reactivity was mainly dependent on the evolutionary distance of the species in relation to the molluscs,

Table III: Organ Specificity of Antibody against Myosin Regulatory Light Chains from Striated Adductor of *Aequipecten*

species, organs	rabbit anti-*Aequipecten* striated adductor myosin R-LC IgG	
	Ouchterlony[a]	RIA[b]
Aequipecten		
striated adductor	+ (1, 2, 4)	100 (1, 2, 4)
smooth adductor (catch muscle)	+ (1, 2, 3)	92 (1, 2, 3)
foot	+ (1, 3)	89 (1, 3)
mantle	+ (1, 3)	91 (1, 3)
heart	+ (1, 3)	57 (1, 3)
Placopecten		
striated adductor	+ (1, 2, 4)	63 (1, 2, 4)
smooth adductor (catch muscle)	+ (1, 2, 3)	51 (1, 2, 3)
foot	+ (1, 3)	47 (1, 3)
mantle	+ (1, 3)	49 (1, 3)
heart	(+/–) (1, 3)	15 (1, 3)

[a] Qualitative cross-reactivity determined by double immunodiffusion of serial dilutions (5–0.05 mg/mL) of actomyosin (1), myosin (2), crude Gdn-HCl extract containing both myosin light chains (3), or purified R-LC (4) from different organs of the two scallop species *Aequipecten* and *Placopecten* diffused against antibody to R-LC from striated adductor of *Aequipecten.* [b] Numbers indicate percent cross-reactivity between the antibody and the myosin R-LC from different organs of the two scallop species obtained by competitive solid-phase radioimmunoassay (RIA). Percent cross-reactivity is expressed relative to the maximal value of competition obtained with the homologous myosin R-LC from striated adductor of *Aequipecten* which is taken as 100%. (1, 2, 3, and 4 are the same as above.)

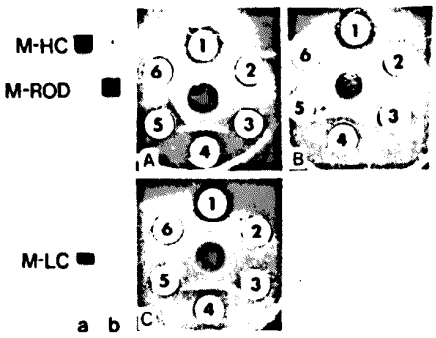

FIGURE 9: Scallop (*Aequipecten*) myosin (a) and myosin rod fragment (b) used for immunization and subsequent purification by affinity chromatography of monospecific antibodies, after $NaDodSO_4$-polyacrylamide gel electrophoresis on 9% and 7.5% gels, respectively. Myosin heavy chain (M-HC); myosin rod fragment obtained by papain digestion of myosin (M-ROD); myosin light chains (M-LC). Characterization of antibodies by double immunodiffusion. (A) Rabbit anti-scallop total myosin antibody, in the center well; 3 mg/mL crude scallop myosin (1); 0.1 mg/mL R-LC (2); 0.1 mg/mL SH-LC (3); 3 mg/mL purified myosin rod (4); 3 mg/mL myosin subfragment-1 [(Ca,Mg)S1] (5); 5 mg/mL crude chicken myosin (6). (B) Anti-scallop total myosin antibody after consecutive absorption on R-LC- and SH-LC-Sepharose-4B affinity columns, in the center well; outer wells (1–5) as described above. (C) Anti-scallop myosin rod antibody, in the center well; outer wells (1–5) as described above.

rather than the presence or absence of thick filament linked regulation within these species. No cross-reactivity at all was found with myosin from any of the vertebrates tested (Table IV legend), and only a low cross-reactivity was observed with myosin from *Protochordata*, the primitive ancestors of vertebrates. These results could be qualitatively confirmed by double immunodiffusion using myosin or actomyosin, dissolved in high salt containing $Mg^{2+}ATP$ (Table IV). It should be noted that within the *Decapoda* cross-reactivity was consistently higher with myosin derived from the claw than from tail or body muscles. This difference is particularly obvious in the

Table IV: Interspecies Cross-Reactivity of Rabbit Anti-*Aequipecten* Myosin Heavy-Chain Antibody[a]

anti-*Aequipecten* myosin heavy-chain IgG[a]				
Ouchterlony[b]	RIA[c]	species[d]	common name	muscle dissected
		Mollusca		
		Pelecypoda		
+	100 (1, 2)	*Aequipecten irradians*	bay scallop	striated adductor
+	97 (1, 2)	*Placopecten magellanicus*	deep sea scallop	striated adductor
+	95 (1, 2)	*Mercenaria mercenaria*	quahog	pink adductor
+	87 (1, 2)	*Spisula solidissima*	surf clam	adductor
		Cephalopoda		
+	87 (1, 2)	*Loligo paelei*	squid	ventral retractor pharynx
		Gasteropoda		
+	84 (1)	*Lunatia heros*	moon shell	foot
+	77 (1)	*Busycon canalicatum*	whelk	foot
		Brachiopoda		
+	75 (1)	*Glottidia pyramidata*	lampshell	pedunculus
		Annellida		
+	62 (1)	*Nereis virens*	clam worm	body wall
+	51 (1)	*Glycera* sp.	blood worm	body wall
		Sipunculida		
+	48 (1)	*Golfingia gouldi*	peanut worm	body wall
		Arthropoda		
		Crustacea		
		Cirripedia		
+	63 (1)	*Balanus nubilis*	giant acorn barnacle	depressor
		Amphipoda		
+	60 (1)	*Talorchestia longicornis*	beachhopper	body
		Decapoda		
+	65 (1, 2)	*Homarus americanus*	lobster	crusher claw
+	51 (1, 2)	*Homarus americanus*	lobster	tail
+	57 (1)	*Pagurus pollicaris*	hermit crab	claw
+	52 (1)	*Pagurus pollicaris*	hermit crab	tail
+	51 (1)	*Libinus emarginata*	spider crab	carapace
+	47 (1)	*Cancer irroratus*	rock crab	claw
+	39 (1)	*Cancer irroratus*	rock crab	body
+	46 (1)	*Uca pugnax*	black fiddler crab	claw
+	33 (1)	*Penaeus setiferus*	edible shrimp	body
		Insecta		
+	59 (1, 2)	*Gryllus domesticus*	cricket	leg
+	51 (1, 2)	*Lethocerus cordofanus*	giant waterbug	leg, thoracic
		Chelicerata		
+	52 (1, 2)	*Eurypelma* sp.	tarantula	leg
+	47 (1, 2)	*Limulus polyphemus*	horseshoe crab	telson muscle
		Echinoderma		
+	26 (1, 2)	*Thyone briareus*	sea cucumber	lantern retractor
		Protochordata		
		Hemichordata		
(+/–)	20 (1)	*Saccoglossus kowaleskii*	acorn worm	proboscis muscle
		Urochordata		
(+/–)	21 (1)	*Ciona intestinalis*	sea squirt	pharyngial muscle
		Cephalochordata		
(+/–)	15 (1)	*Branchiostoma floridae*	amphioxus	body

[a] Rabbit anti-*Aequipecten* myosin heavy-chain antibody (anti-M-HC IgG) obtained by consecutive absorption of anti-total-myosin antibody (purified by affinity chromatography) with both R-LC and SH-LC. For details see Experimental Procedures and Figure 9. [b] Qualitative cross-reactivity determined by double immunodiffusion of actomyosin (1) or myosin (2) from different species, diffused against anti-M-HC IgG in the presence of 10mM Mg^{2+}ATP and judged by the appearance of a single precipitin line (+). A very faint, hardly visible precipitin reaction is indicated by (+/–). [c] Numbers indicate percent cross-reactivity determined by competitive solid-phase radioimmunoassay (RIA) of actomyosin (1) or myosin (2) from different species with anti-M-HC IgG. Percent cross-reactivity is expressed relative to the maximal level of competition obtained with the homologous myosin from *Aequipecten* which is taken as 100%. Similar values of cross-reactivity (usually 10–20% higher) were obtained with rabbit anti-*Aequipecten* myosin rod antibody which was purified by affinity chromatography as well. [d] No cross-reactivity in Ouchterlony tests or with RIA could be observed with nonmuscle myosin from *Physarum polycephalum* (slime mold) and human blood platelets nor with actomyosin (1) and myosin (2) from all the following vertebrate species: *Raja clavata* (skate), dorsal (1, 2); *Mustelus canus* (dogfish), body (1); *Lophius americanus* (goosefish), body (1); *Rana catesbiana* (bullfrog), leg (1, 2); *Gallus domesticus* (chicken), skeletal (1, 2), heart (1, 2), and gizzard (1, 2, phosphorylated and unphosphorylated); *Mus musculus* (mouse), skeletal and heart (1, 2); *Oryctolagus cunniculus* (rabbit) skeletal (1, 2).

case of the lobster, *Homarus americanus*.

Organ Specificity of Anti-Scallop Myosin Heavy-Chain Antibodies. Cross-reactivity of specific antibodies against the heavy chain and rod fragment of *Aequipecten* striated adductor myosin was also tested with myosins from different organs of the same species by RIA (Table V). Myosin from striated adductor, mantle, and foot all gave very similar, high values of cross-reactivity with both anti-heavy-chain and anti-rod antibody indicating immunological identity of these myosin. Myosin from the smooth adductor (catch muscle) showed a significantly lower value with anti-heavy-chain and a somewhat reduced value with anti-rod antibody indicating some differences between the myosins of smooth and striated adductor muscles. The myosin heavy chain from scallop heart muscle differed considerably. The differences between heavy chains were particularly great in the region that forms the myosin

Table V: Organ Specificity of Antibodies against Myosin Heavy Chain and Myosin Rod from Striated Adductor Muscle of *Aequipecten*

	anti-striated-adductor myosin heavy chain IgG[a]	anti-striated-adductor myosin rod IgG[a]
striated adductor (1, 2)	100[b]	100[b]
mantle (1)	97	99
foot (1)	89	95
smooth adductor (1, 2) (catch muscle)	78	93
heart (1)	55	80

[a] Rabbit anti-*Aequipecten* striated adductor myosin heavy-chain antibody and myosin-rod antibody, both affinity purified, tested by competitive solid-phase radioimmunoassay (RIA) for intraspecies cross-reactivity against actomyosin (1) and myosin (2) from different organs of *Aequipecten*. [b] Numbers indicate percent cross-reactivity determined by RIA and are expressed relative to the maximal value of competition obtained with the homologous myosin from striated adductor which is taken as 100%.

heads, since anti-myosin-rod antibodies showed a much higher degree of homology in their cross-reactivity with myosins from different organs than the anti-myosin total heavy-chain antibodies (Table V).

Discussion

Competitive Solid-Phase Radioimmunoassay. The system described has some advantages over the classical radioimmunoassays and appears to be convenient, fast, and particularly well suited for comparative immunological studies. In this method a single batch of secondary antibody is radiolabeled. Antigens, primary antibodies, or the proteins tested for cross-reactivity remain unmodified, thus eliminating possible artifacts due to conformational changes caused by the iodination procedure. The proteins tested do not have to be pure. Crude cell extract may be readily assayed, provided that the antigenic sites are freely accessible. Cross-reactivity is based on the specific binding of primary antibody to the solid-phase antigen. Bound antibody may then be quantitated by measuring the subsequent binding of radiolabeled secondary antibody. Once a well-characterized and highly specific primary antibody/antigen system is available, e.g., anti-myosin light-chain antibody and myosin light chains, any protein irrespective of size and purity can be tested for immunological cross-reactivity by competing with the antigen-coated wells for the antibody. Nonspecific precipitates caused by denaturation of proteins, and coprecipitated radio-labeled proteins, often a problem in conventional RIA systems, are completely removed in this assay by extensive washing of the wells. The method is specific, is sensitive with a high signal to noise ratio, and allows quantitation of myosin light chains in the nanogram range (Figures 6–8). The only protein used in microgram quantities is the primary antigen that has to be bound to the microtiter plate U wells. Although one may use crude IgG or even antisera, provided the specificity of the primary antibody is ascertained, it is preferable to use affinity-purified antibodies to reduce nonspecific background. However, apart from a slightly higher background, no difference in the cross-reactivity was observed, in this study, when antisera or crude IgG's were used instead of affinity-purified antibodies.

We have tested the cross-reactivity of some 40 different myosin and myosin light-chain species. When available, cross-reactivities of purified myosin and myosin light chains were compared with those of crude myosin or actomyosin extracts. The same relative values of cross-reactivity were obtained with crude extracts and purified components (Tables I and IV).

Immunological Comparison of Myosin Light Chains. Antibodies against R-LC and against SH-LC were specific to the homologous light chains in the different tests used in this study. Neither antibody cross-reacted with the heterologous light chain, troponin C of rabbit and chicken, or with the calcium-binding protein of scallop. There was also a considerable species specificity. Cross-reactivity was limited to light chains from two closely related molluscan species (Table II and legend; Figures 6 and 7).

The results confirm and extend some preliminary observations obtained by immunodiffusion with anti-scallop R-LC (Kendrick-Jones & Jakes, 1977). The specificity of the anti-scallop light-chain antibodies in our studies was considerably greater than that of the rabbit antibody directed against chicken DTNB-LC obtained by Silberstein & Lowey (1977) and Silberstein (1978). Their rabbit anti-chicken DTNB-LC antibody showed a very weak cross-reactivity with scallop R-LC and rabbit troponin-C and a more extensive cross-reactivity with the heterologous alkali LC from chicken and even rabbit. The differences in cross-reactivity and the appearance of autoimmune antibodies (Holt & Lowey, 1975; Silberstein & Lowey, 1977) may in part be due to differences in the schedules of immunization and to the different species of LC's used as antigens.

The specificity of anti-light-chain antibodies is in contrast with the similarities in the properties and sequences of different regulatory light chains, with the similar sequences between the two kinds of light chains, and with the related sequences of calcium-binding proteins such as troponin C and parvalbumin. Foreign regulatory light chains bind to scallop myofibrils, from which their own regulatory light chains have been previously removed, with comparable affinities to scallop regulatory light chains (Sellers et al., 1980). SH-LC's may also be partially replaced by rabbit alkali light chains in the presence of chaotropic agents (T. Wallimann and R. Naturman, unpublished observations). Rabbit DTNB light chains (Coffee & Bradshaw, 1973; Collins, 1976) and scallop and gizzard regulatory light chains show sequence homologies particularly in the N-terminal third of these molecules which are nearly identical (Kendrick-Jones & Jakes, 1977). Sequence homologies among the two types of light chains and the different calcium-binding proteins led to the proposition that these proteins are evolutionarily related and have a common ancestry (Weeds & MacLachlan, 1974; Kretsinger & Nockolds, 1973; Collins, 1976; Weeds et al., 1977). The species specificity of the light-chain antibodies may indicate that the constant regions (possibly the attachment sites and divalent cation binding sites) are too well conserved and therefore were not antigenic under our short-term, low-dosage immunization conditions. The antibodies may have been directed exclusively against the variable, species-specific domains of the light-chain regions that lack homology. Comparative studies of alkaline phosphatase (Cocks & Wilson, 1969) and of cytochrome *c* (Margoliash et al., 1970) have shown that a few changes in amino acid sequences, when occurring at a critical place, may result in large structural alterations that may have important immunochemical effects by significantly affecting immunological cross-reactivity.

Differences between Anti-SH-LC Antibodies. Two different antibody populations were elicited in different rabbits by the same batch of SH-LC. Although both antibodies reacted specifically with SH-LC, only one of the two antibody populations interfered with regulation. Additional differences were also observed (Wallimann & Szent-Györgyi, 1981). The extent of cross-reactivity toward CNBr peptides of SH-LC

differed in the two batches of anti-SH-LC antibodies. In the case of R_4-nondesensitizing anti-SH-LC antibody, most of the antibody reacted with CNBr peptides, and only a relatively minor fraction of the antibody required the integrity of the antigen. By contrast, only 60% of the desensitizing R_5-anti-SH-LC antibodies reacted with CNBr peptides, and 40% required the native conformation of the SH-LC (Table I). The desensitizing action of the R_5-antibody population may be related to antibodies formed against native structures. The sum of the cross-reactivities of the isolated peptides exceeded the amount obtained with the unfractionated mixture of the CNBr fragments or even the value obtained with intact SH-LC. The presence of one or more similar antigenic sites in more than one peptide fragment may explain this discrepancy.

The reason for the different immune response resulting in these two populations of anti-SH-LC antibodies is not known. However, it is not unusual that differences in the genetic makeup of the immunized animals can cause elicitation of antibodies which differ significantly in their specificity, affinity, and uniformity, even when the same batch of immunogen is used. The presentation and the fate (e.g., degradation, oxidation, etc.) of the immunogen within the immunized animal can also be very important. The relative absence of antibodies within R_4 antiserum exclusively directed against the native SH-LC suggests that for some reason the SH-LC may have been degraded more rapidly to smaller peptides which in turn elicited the bulk of antibodies. It should also be noted that scallop SH-LC's containing three cysteine residues (Kendrick-Jones et al., 1976) are readily oxidized and form either intra- or intermolecular disulfide bonds (T. Wallimann, unpublished observations) which may also affect the immune response.

Structural Aspects. Both R-LC's and SH-LC's attached to myosins can each bind up to three antibody molecules (Wallimann & Szent-Györgyi, 1981). Antibody binding to free light chains based on the extrapolation of precipitin curves appears to be even somewhat greater. The extended asymmetric shape of the R-LC having a length of ~100 Å (Stafford & Szent-Györgyi, 1978; Alexis & Gratzer, 1978; Hartt & Mendelson, 1979) may explain how such a small molecule (M_r = 17 500) can accomodate several large antibodies. Similarity in antibody uptake and in stability against denaturing agents between the two light chains suggests that the SH-LC may also have an elongated structure. [The immunological reactivity of both light chains remain unchanged by EDTA, DTNB, urea, Gdn·HCl, trichloroacetic acid, or acetone treatment (T. Wallimann, unpublished observations).]

A number of studies have indicated that the conformation of the light chains when bound to myosin is similar to the conformation of the isolated light chains (Holt & Lowey, 1975; Kendrick-Jones & Jakes, 1977; Stafford & Szent-Györgyi, 1978). Such findings are supported by the observation that anti-scallop myosin LC antibodies form a single precipitin line with subfragment-1 (S1), heavy meromyosin, and myosin, although R-LC in (Ca,Mg)S1 is modified by the loss of a 1500-dalton peptide. This line fuses with the one formed with isolated LC indicating the immunological identity of bound and free LC (reaction with S1 shown in Figure 8A). Furthermore, the degree of immunological cross-reactivity measured by RIA was similar with myosin and with free light chains demonstrating that the antigenic determinants of bound LC are accessible to the antibodies. The fact that antibodies against LC, elicited either by injecting isolated LC or total myosin, had indistinguishable immunological characteristics (not shown) and did exert similar effects on the actin-activated Mg^{2+}–ATPase activity (Wallimann & Szent-Györgyi, 1981) also confirms the close structural similarity of these light chains in the isolated and the bound stage.

Immunological Comparison of Myosins: (*1*) *Organ Specificity of Myosins.* Both the light chains and heavy chains of the striated adductor, mantle, and foot muscles are very similar. There are moderate differences between the light and heavy chains of smooth and striated adductor; the differences are extensive in the case of heart myosin light chain and heavy chain (Tables III and V). The dissimilarities among heavy chains are mainly located on the subfragment-1 portion, since anti-myosin-rod antibodies show a much higher degree of homology than the anti-total-myosin heavy chain antibodies. The generally larger (by 10–30%) cross-reactivity of myosins with anti-rod antibody indicates that this region of the molecule has been better conserved during evolution. It is clear that myosin isozymes are also found in scallop and that heart myosin significantly differs from the myosin of striated adductor. The functional implications of the immunological differences, however, are not yet clear.

(*2*) *Species Specificity of Myosins.* The light chains of *Aequipecten* cross-reacted only with the homologous light chain of two closely related species (*Placopecten* and *Mercenaria*). The light-chain antibodies are thus sensitive tools to establish the identity of a particular myosin but are not suitable for determining partial homologies. R-LC's that restore calcium regulation when hybridized with desensitized scallop myofibrils are not distinguishable by immunological means from the R-LC's that do not restore regulatory functions (cf. Kendrick-Jones et al. (1976); Chantler et al., 1979; Sellers et al., 1980).

The anti-heavy-chain and anti-rod antibodies both cross-reacted with a wide variety of invertebrate myosins but did not cross-react with any of the vertebrate or nonmuscle myosins (*Platelet* and *Physarum*) (Table IV and legend). Interestingly, myosins of hemichordates (*Amphioxus*, *Balanoglossus*, and *Ciona*) or echinoderms showed only a very limited cross-reactivity (<20%). Invertebrate myosins thus possess antigenic sites not present in vertebrates and nonmuscle systems.

The extent of cross-reactivity among invertebrate myosins depended mainly on the evolutionary distance of the species in relation to the molluscs and did not obviously depend on the presence of a particular regulatory system (Table IV). The cross-reactivities of crusher claw myosin of lobster or of claw myosins of other decapods exceeded the cross-reactivities of myosins derived from the tail muscles of the same animals. However, the type of regulatory system present in these muscles is under dispute and has not yet been satisfactorily resolved (Lehman & Szent-Györgyi, 1975; Lehman, 1977, 1978; Sellers et al., 1980). Since most of the specific regulatory calcium-binding sites reside on the heavy chain (Bagshaw & Kendrick-Jones, 1979; Chantler & Szent-Györgyi, 1980), it may not be unreasonable to find antibodies specific to these sites. Antibodies associated with the calcium switch may only be demonstrated, however, if both regulatory and nonregulatory myosins within a species were found.

General Conclusions. Regulatory light chains of all myosins have highly conserved domains (attachment sites to myosin, divalent metal binding sites) which therefore may be less antigenic. The anti-R-LC antibodies are too specific to differentiate between functional and nonfunctional regulatory light chains. The structure of scallop myosin LC when bound to myosin is similar to the structure of isolated LC. Antigenic sites of both light chains are readily available for reaction with

antibody, suggesting that the SH-LC, like the R-LC, may be an extended asymmetric molecule. Heart muscle myosin of scallop differs greatly from adductor, mantle, and foot muscle myosins. The differences between smooth and striated adductors are "small". Antibodies against scallop heavy chains and myosin rods do not cross-react with vertebrate or non-muscle myosins but cross-react to a variable extent with all the invertebrate myosins tested. Cross-reactivity does not depend in an obvious manner on the regulatory properties of the different myosins.

While this paper was in preparation, a similar RIA system was described and successfully applied for the serological analysis of chromosomal components by Romani et al. (1980).

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