(1970), *Eur. J. Biochem. 14,* 486–497.
Bradbury, E. M., Crane-Robinson, C., Goldman, H., Rattle, H. W. E., and Stevens, R. M. (1967), *J. Mol. Biol. 29,* 507–523.
Bradbury, E. M., and Rattle, H. W. E. (1972), *Eur. J. Biochem. 27,* 270–281.
Clark, V. M., Lilley, D. M. J., Howarth, O. W., Richards, B. M., and Pardon, J. F. (1974), *Nucleic Acids Res. 1,* 865–880.
D'Anna, J. A., and Isenberg, I. (1973), *Biochemistry 12,* 1035–1043.
D'Anna, J. A., and Isenberg, I. (1974a), *Biochemistry 13,* 2093–2098.
D'Anna, J. A., and Isenberg, I. (1974b), *Biochemistry 13,* 2098–2104.
Doddrell, D., Glushko, V., and Allerhand, A. (1972), *J. Chem. Phys. 56,* 3683–3689.
Johns, E. W. (1967a), *Biochem. J. 104,* 78–82.
Johns, E. W. (1967b), *Biochem. J. 105,* 611–614.
Kelley, R. I. (1973), *Biochem. Biophys. Res. Commun. 54,* 1588–1594.
Kornberg, R. D. (1974), *Science 184,* 868–871.
Kornberg, R. D., and Thomas, J. O. (1974), *Science 184,* 865–868.
Li, H. J., Wickett, R. R., and Isenberg, I. (1972), *Biopolymers 11,* 375–397.
Luzzati, V., and Nicolaieff, A. (1963), *J. Mol. Biol. 7,* 142.
McDonald, C. C., and Phillips, W. D. (1969), *J. Am. Chem. Soc. 91,* 1513–1521.
Panyim, S., and Chalkley, R. (1969), *Arch. Biochem. Biophys. 130,* 337–346.
Pardon, J. F., and Richards, B. M. (1973), *Biol. Macromol. 6,* 1–70, 305–322.
Pardon, J. F., and Wilkins, M. H. F. (1972), *J. Mol. Biol. 68,* 115–124.
Prothero, J. W. (1966), *Biophys. J. 6,* 367–370.
Richards, B. M., and Pardon, J. F. (1970), *Exp. Cell Res. 62,* 184–196.
Schiffer, M., and Edmundson, A. B. (1967), *Biophys. J. 7,* 121–135.

An Immunological Approach to the Role of the Low Molecular Weight Subunits in Myosin. I. Physical–Chemical and Immunological Characterization of the Light Chains†

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ABSTRACT: The light chains of chicken breast muscle myosin (alkali 1 and 2, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) l.c.) have been isolated in pure form and characterized with respect to amino acid composition, uv and circular dichroism (CD) spectral properties, and molecular weight. Antibodies specific for each of the light chains have been used to demonstrate the similarity of alkali 1 and 2 (mol wt 21,000 and 16,000, respectively), and the distinctness of these from DTNB l.c. (mol wt 18,000). The DTNB l.c. isolated by a variety of methods were all immunologically identical. Significant cross-reactivity was observed between corresponding rabbit and chicken light chains, confirming other indications of homology between these proteins in the two species. The immunological difference between alkali 1 and 2 was largely accounted for by an N-terminal peptide, rich in proline, alanine, and lysine, which is unique to alkali 1. The presence of antibodies to this peptide in anti-alkali 1 serum suggests an immunological approach to the question of how alkali l.c. are distributed in myosin.

All myosins so far studied, whether from vertebrate or invertebrate muscle, or from nonmuscle sources, contain small subunits (mol wt ~20,000) known as light chains (Lowey and Risby, 1971; Sarkar et al., 1971; Lehman et al., 1972; Adelstein and Conti, 1972; Pollard and Korn, 1972). That these are integral components of myosin is implied by their survival of all purification procedures for myosin; moreover, fluorescent antibodies specific for light chains stain only the A-band of myofibrils (Lowey and Steiner, 1972). Since light chains are retained in the proteolytic subfragments heavy meromyosin and subfragment 1, they are presumably localized in or near the globular heads of myosin (Weeds and Lowey, 1971).

As discussed in the following paper (Holt and Lowey, 1975), several lines of evidence suggest that light chains are involved in the hydrolysis of ATP by myosin, although only in the case of invertebrate molluscan myosin is this function understood in any detail. A class of light chain (EDTA l.c.),[1] which can be selectively and reversibly dissociated from scallop myosin, has been shown to be responsible for the myosin-linked Ca-regulation present in this species (Szent-Györgyi et al., 1973). Vertebrate DTNB l.c. can, moreover, substitute for EDTA l.c. in restoring Ca sensitivi-

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[1] Abbreviations used are: EDTA, ethylenediaminetetraacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Gdn·HCl, guanidine hydrochloride.

ty to desensitized scallop myosin (Kendrick-Jones, 1974). A further parallel between these light chains is that the selective dissociating agent, DTNB or EDTA, is capable of removing only about half of the total, or 1 mol/mol of myosin (Kendrick-Jones, 1974; J. Kendrick-Jones, E. M. Szentkiralyi, and A. G. Szent-Györgyi, personal communication).

The fact that no role has been found for DTNB l.c. in vertebrate myosin may result from irreversible modification of myosin by the thiol reagent DTNB. Attempts to define the function of alkali l.c. have suffered from a similar drawback: dissociating conditions are necessarily strong enough that the process is far from reversible even in terms of Ca-ATPase activity. We have therefore undertaken an immunochemical study of the light chains in myosin, based on the observation (Lowey and Steiner, 1972) that antibodies to light chains promote some dissociation of these subunits from the proteolytic subfragment HMM S-1. This approach minimizes the possibility of denaturing the labile myosin structure by making use only of protein–protein interactions. As the first stage in this study, attention has been directed toward characterization of the isolated light chains.

In vertebrate fast muscle, the results from densitometry of sodium dodecyl sulfate gels (Lowey and Risby, 1971) and radiochemical methods (Weeds and Lowey, 1971) agree in suggesting that 4 mol of light chain are present in 1 mol of myosin. These fall into two distinct chemical classes. Reaction of myosin with the thiol reagent DTNB releases a single class of light chain, DTNB l.c. (2 mol/mol of myosin), with little change in Ca-ATPase activity (Gazith et al., 1970; Weeds and Lowey, 1971). The remaining light chains (2 mol/mol of myosin) are liberated only by denaturing conditions, among them exposure to pH 11 (Kominz et al., 1959); for this reason, they are termed alkali light chains. The two classes are distinguished chemically since DTNB l.c. contains two thiol groups whereas alkali l.c. contain only one; the amino acid sequence around the thiol groups, moreover, shows no similarity in the two classes of light chain (Weeds, 1969). Dodecyl sulfate gel electrophoresis reveals that alkali l.c. are present in two size classes: alkali 1, molecular weight 25,000 on dodecyl sulfate gels (21,000 by direct methods) and alkali 2, molecular weight 16,000; the molecular weight of DTNB l.c. is 18,000. Both alkali l.c. contain the same thiol sequence, and for a time it appeared that the smaller alkali 2 might be a fragment of the larger alkali 1. Determination of the complete amino acid sequence (Frank and Weeds, 1974), while showing extensive homology, ruled out this possibility; although 141 C-terminal residues are identical in the two proteins, five of the remaining eight residues of alkali 2 show substitutions relative to alkali 1. The size difference between alkali 1 and 2 consists of 41 N-terminal residues unique to alkali 1. Two forms of DTNB l.c. have also been identified, based not on sequence differences but on whether or not a particular serine residue is phosphorylated (Perrie et al., 1973). Since the two forms can be interconverted enzymatically, and their relative proportions vary with extraction conditions, they bear no obvious relation to the two kinds of alkali l.c. The significance of the phosphorylation remains unclear, although the presence of a phosphorylated light chain in platelet (Adelstein et al., 1973) and invertebrate lobster myosins (J. M. Regenstein, personal communication), as well as rabbit and chicken skeletal myosin, suggests that this may be a general property of myosin.

Much of the work described above was carried out with rabbit skeletal myosin. In this paper, we describe the isolation of light chains from chicken breast muscle myosin, and their fractionation into pure components. Physical and chemical characterization reveals that the light chains from this source are generally similar to their counterparts in rabbit myosin, although they do elicit a specific antibody titer when injected into rabbits. The antisera prepared in this way confirm the structural relationship between the various light chains implied by chemical studies. Alkali 1 and 2 show extensive cross-reactivity but neither reacts with antiserum to DTNB l.c. The N-terminal peptide unique to alkali 1 accounts for much, though not all, of the antigenic difference between alkali 1 and 2. Chromatographically purified DTNB l.c. released from myosin with the thiol reagent DTNB, 4 *M* urea, or 5 *M* guanidine were all immunologically identical; so too were phosphorylated and nonphosphorylated forms of this light chain. It does not therefore seem that the inability of DTNB to dissociate more than half of the DTNB l.c. from myosin can be ascribed to the existence of two distinct species of this light chain.

Some aspects of this work have already been described in a preliminary report (Lowey and Holt, 1972).

Materials and Methods

Preparation of Proteins. Myosin was prepared from chicken breast muscle as described by Holtzer and Lowey (1959), except that 3 m*M* ATP was included in the initial extracting buffer.

A mixture of all three light chain components ("total light chains") was isolated from myosin in a number of ways, prior to fractionation on DEAE-cellulose in potassium phosphate buffers at pH 6.0. In the procedure initially adopted, myosin (15–20 mg/ml) was incubated with 4 *M* urea, 0.3 *M* KCl, 2 m*M* EDTA, 2 m*M* dithiothreitol, and 0.05 *M* Tris-HCl (pH 7.9), for 30–60 min at 21° (Gazith et al., 1970). After precipitation of heavy chains by dilution into 8 vol of cold water, the released light chains (~0.1 mg/ml) remaining in the supernatant were adsorbed onto a column (5 × 12 cm) of DEAE-cellulose equilibrated in 0.05 *M* Tris-HCl (pH 7.9)–0.1 m*M* dithiothreitol; a step to 1 *M* KCl eluted the light chains in a small volume corresponding to a 10–20-fold increase in concentration (Weeds and Lowey, 1971; Lowey and Holt, 1972).

Alternatively, total light chains were prepared by treating myosin with 5 M Gdn·HCl, followed by ethanol–water fractionation (Perrie and Perry, 1970). Myosin was incubated at 15–20 mg/ml in 5 *M* Gdn·HCl (added as solid), 2 m*M* dithiothreitol, 2 m*M* EDTA, 0.3 *M* KCl, and 0.05 *M* Tris-HCl (pH 7.9), for 60 min or overnight at 21°. The mixture was then cooled to 4° and diluted first with an equal volume of cold water and then with 4 volumes of cold 95% ethanol. After about 15 min at 4°, precipitated heavy chains were removed by centrifugation and ethanol was removed from the supernatant by rotary evaporation at 35–40°.

The antigen for the antiserum to total light chains (Figure 2) was obtained by incubation of myosin at alkaline pH (Gaetjens et al., 1968). Myosin (~5 mg/ml) was exposed to pH 11, 0°, in the presence of 2 m*M* ATP, 0.1 *M* KCl, 1 m*M* dithiothreitol, 2 *M* LiCl, and 0.1 *M* glycine buffer for about 30 min. Heavy chains were precipitated by the addition of neutralized 2.5 *M* potassium citrate to a final concentration of 0.8 *M*. The supernatant containing light chains was dialyzed exhaustively against water and freeze-dried.

DTNB l.c. was selectively dissociated from myosin by reaction with the thiol reagent 5,5′-dithiobis(2-nitrobenzoic acid) (Gazith et al., 1970; Weeds and Lowey, 1971). Myosin (25 mg/ml) was incubated with 10 m*M* DTNB, 1 *M* urea, and 10 m*M* EDTA in the presence of 0.5 *M* KCl and 0.05 *M* Tris-HCl (pH 8.5) for 15 min at 0°. After this period, heavy chains were precipitated by dilution of the mixture into 8 vol of cold water and the supernatant was freeze-dried. The powder, which contained DTNB l.c. and excess DTNB, was dissolved and dialyzed against 0.05 *M* Tris-HCl (pH 7.9)–1 m*M* dithiothreitol until the extinction of the protein was zero at 340 nm, i.e., until all DTNB groups had been removed.

Fractionation of Light Chains. Total light chain mixtures (~250 mg isolated from 3 g of myosin by treatment with 4 *M* urea or 5 *M* Gdn·HCl) were fractionated on a column (2.5 × 60 cm) of DEAE-cellulose (Whatman DE52) equilibrated in 0.08 *M* potassium phosphate (pH 6.0)–0.1 m*M* dithiothreitol (Weeds and Lowey, 1971). Elution of individual light chain components was effected by a linear gradient (total volume 2 l) to 0.27 *M* potassium phosphate (pH 6.0)–0.1 m*M* dithiothreitol. Fractions of 10 ml were collected at a flow rate of 50 ml/hr. The course of the fractionation was followed by measuring extinction at 230 nm. For the analysis of the elution profile by gel electrophoresis and Ouchterlony double diffusion the approximate concentration of individual fractions was estimated from these extinction measurements. Recovery from the column was ~40%.

Preparation of Antisera. Specific antiserum to each of the chicken light chains was elicited in rabbits by immunization with 1.5–2.5 mg of protein in 1 ml of potassium phosphate buffer (pH 7.0) emulsified with an equal volume of complete Freund's adjuvant. Unless otherwise specified, the immunogen was a chromatographically purified light chain, which had been released from myosin by treatment with 4 *M* urea or 5 M Gdn·HCl. Injections were given primarily in the footpads at intervals of 3–4 weeks. After 3–4 immunizations, the specific antibody titer reached a maximum which was shown by quantitative precipitin analysis to correspond to 0.7–2.0 mg of specific antibody/ml of whole serum, the variation occurring between different rabbits. Blood was collected from the ears of the rabbits, and after standing at 22° for several hours and at 4° overnight, the serum was decanted from the clot, clarified, and frozen.

Double Diffusion. Gel diffusion was carried out with 1% agar (purified Difco) dissolved in 0.4 *M* KCl–0.01 *M* potassium phosphate (pH 7.4); 6 ml of agar was placed in a Petrie dish 5 cm in diameter. Antigens were first tested in two-fold serial dilutions from 0.2 mg/ml (light chains) or 5–10 mg/ml (myosin and subfragments). When the development of precipitin lines was complete (3–6 days at 4°), the plates were photographed in dark-field illumination. The gels were then soaked successively in 0.5 *M* KCl and distilled water, dried overnight, and stained with 1% Amido Black in 45% methanol and 10% acetic acid.

Quantitative Precipitin Analysis. The quantitative precipitin reaction was carried out in the standard manner (Kabat, 1961). Antigens and antisera were dialyzed against buffer, usually 0.2–0.3 *M* KCl–10–50 m*M* Tris-HCl (pH 8.4) at 4°, prior to clarification for 30 min at 45,000*g*; in the case of sera, fat was also removed by aspiration. The reaction of antigen and the specific antibody contained in 0.5 ml of antiserum was allowed to proceed at least 24 hr at 4°. When the inhibition of such a reaction was investigated, inhibitor was added to antiserum 30–60 min before antigen since, in an antiserum of particularly high titer, precipitation could be observed within 1 min of adding antigen.

Precipitates were collected by centrifugation at 1300*g* for 40 min or 4300*g* for 12 min, and washed three times with buffer. Finally they were dissolved in 0.5 ml of 0.01 *M* HCl or in 0.5 ml of 1 *M* NH_4OH. The extinction was measured at 278 nm and, in order to make a subtractive correction for scattering, also at 340 nm. No correction was made for antigen contained in the precipitate. The results of quantitative precipitin tests are presented as graphs of antibody precipitated (measured as $A_{278} - A_{340}$) as a function of the increasing weight of antigen added to a fixed volume of serum.

Sodium Dodecyl Sulfate Gel Electrophoresis. Electrophoresis on polyacrylamide gels containing 0.1% dodecyl sulfate and 0.1 *M* sodium phosphate (pH 7.0) was carried out as described by Weber and Osborn (1969). The total concentration of acrylamide was 9% of which 3.6% was bisacrylamide (Fairbanks et al., 1971). These conditions gave optimum resolution of the different light chain components. An important factor was the quality of dodecyl sulfate used; a technical grade reagent (Matheson Coleman and Bell Cat. No. DX2490, 70% C_{12}, 95% alkyl sulfate) gave better resolution than preparations which were 99% pure dodecyl sulfate.

Protein samples at an ionic strength of 0.1 or less were boiled for 2 min in the presence of 1% dodecyl sulfate and 0.1% 2-mercaptoethanol and applied to the gel. Electrophoresis was carried out for 5 hr at 8 mA/tube (70 × 6 mm). The gels were then simultaneously fixed and stained by diffusion in 25% 2-propanol and 10% acetic acid containing 0.02% Coomassie Brilliant Blue stain (Fairbanks et al., 1971); destaining was also accomplished by diffusion, using 12% 2-propanol and 10% acetic acid.

Urea Gel Electrophoresis. Electrophoresis on 7.5% polyacrylamide gels containing 8 *M* urea and 20 m*M* Tris adjusted to pH 8.6 with solid glycine was carried out as described by Perrie and Perry (1970). Samples were dialyzed against 8 *M* urea, 5 m*M* Tris-glycine (pH 8.6), and 0.1% 2-mercaptoethanol. A current of 2 mA/tube was passed through the gels for 2.5 hr after which they were fixed, stained, and destained by diffusion as described for dodecyl sulfate gels.

Amino Acid Analysis. Samples for analysis were dialyzed exhaustively against 1% acetic acid (alkali 1 and DTNB l.c.) or 0.05 *M* NH_4HCO_3 (alkali 2) and freeze-dried. Hydrolysis of about 1 mg of protein was carried out in 1 ml of 6 *N* HCl containing 1% phenol in evacuated tubes at 105° for 24 and 72 hr. The hydrolysates were evaporated to dryness in vacuo and analyzed in a Beckman Model 121 amino acid analyzer. Cysteine was determined as cysteic acid after performic acid oxidation (Weeds and Lowey, 1971). The results presented are averaged values of not less than four analyses carried out on samples hydrolyzed for 24 or 72 hr, except that serine and threonine are 24-hr values and valine and isoleucine, 72-hr values. Tyrosine was determined only on unoxidized samples, and forms the basis for normalization of the calculations. Tryptophan content was determined spectrophotometrically (Beaven and Holiday, 1952).

Protein Concentrations. The concentrations of dialyzed, clarified protein solutions were determined from extinction measurements at 280 nm, corrected for scattering by subtraction of the extinction at 340 nm. Solutions of light

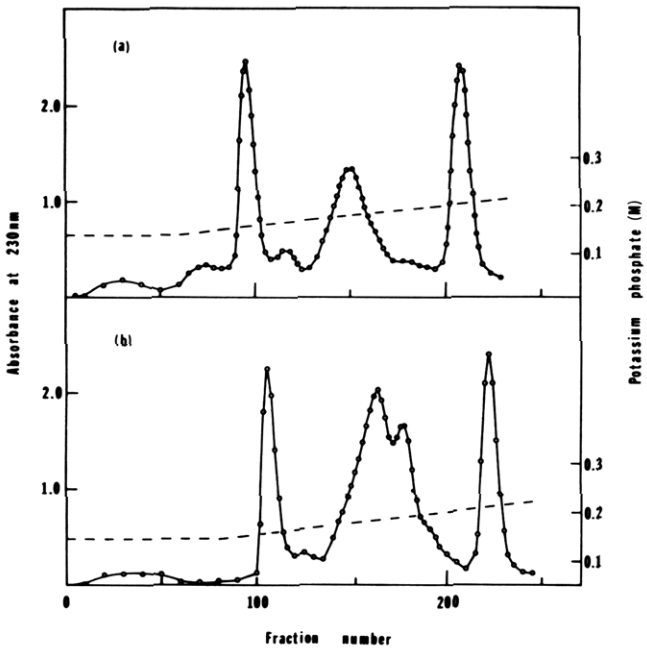

FIGURE 1: Fractionation of light chains dissociated from myosin with (a) 4 *M* urea or (b) 5 *M* Gdn·HCl on DEAE-cellulose. Full details are given in Materials and Methods.

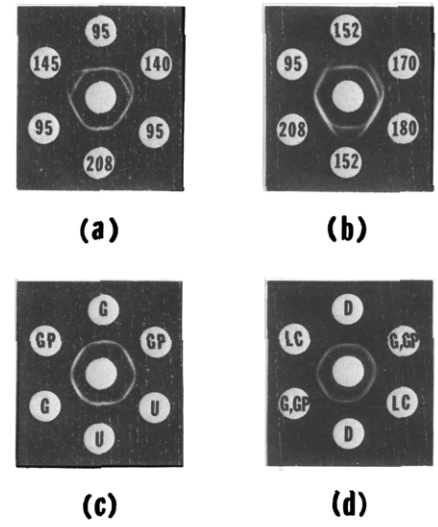

FIGURE 2: Immunodiffusion of light chains. (a and b) Fractions from an elution profile of 4 *M* urea dissociated light chains, renumbered to correspond with the profile in Figure 1a, against an antiserum (center wells) to all light chain components. (c and d) Reaction of phosphorylated DTNB l.c. (abbreviated GP), nonphosphorylated DTNB l.c. isolated with 4 *M* urea (U) or 5 *M* Gdn·HCl (G), the light chain fraction released with DTNB (D), and a total light chain mixture (LC) as in Figure 3a with antiserum to a mixture of Gdn·HCl-dissociated DTNB l.c. (Figure 1b, fractions 148–188). All antigens were present at two concentrations, 0.05 and 0.10 mg/ml, except LC, 0.25 and 0.50 mg/ml.

chains, which had been freeze-dried, usually exhibited an extinction at 340 nm which was about 10% of that at 280 nm. Further high-speed centrifugation decreased both values, with little effect on the relative extinction at the two wavelengths. The following extinction coefficients were used for a 10-mg/ml solution at 280 nm: myosin, 5.0; total light chains, 3.5 (an approximate value); alkali 1, 1.3; DTNB l.c., 6.0; alkali 2, 1.9. These figures were based on nitrogen content determined by the micro-Kjeldahl technique, assuming 16% nitrogen. Total refractive increment measurements made with a synthetic boundary cell in the ultracentrifuge agreed closely with the Kjeldahl figures when a value of 0.185 ml/g was assumed for specific refractive increment.

Circular Dichroism. Spectra were recorded on a Cary 60 spectropolarimeter with a Model 6001 CD attachment. Far-ultraviolet (200–250 nm) CD spectra were obtained using 0.5-mm path-length cells containing 1 mg/ml of protein in 0.05 M potassium phosphate (pH 6.8). Near-ultraviolet (250–300 nm) CD spectra revealed the very weak phenylalanine bands only when the maximum amount of protein compatible with the sensitivity of the instrument was present in the light path. Solutions of 2–5 mg/ml of protein in 1-cm cells (extinction 0.7 at 260 nm) were therefore used. Results are reported as mean residue ellipticity, $[\theta]$, defined as $[\theta] = \theta_{obsd}M/cl$ where M is mean residue weight, taken to be 110, l is path length in dm, and c is concentration in g/100 ml. Percentage α helix was calculated following Greenfield and Fasman (1969) as $100\ ([\theta]_{208} - 4000)/(33{,}000 - 4000)$; in this expression, 100% α helix has $[\theta]_{208} = 33{,}000$ deg cm^2/dmol from which a subtractive correction of 4000 deg cm^2/dmol is made for the contribution of random coil at this wavelength.

Analytical Ultracentrifugation. Molecular weights were determined by sedimentation equilibrium in a Beckman Model E ultracentrifuge equipped with interference optics. Experiments were carried out at 20° in 12-mm double sector cells with Epon centerpieces and sapphire windows, filled to a column height of 3 mm. Since aggregation was indicated by the high extinction of light chain solutions at 340 nm, 5 *M* Gdn·HCl, 1 m*M* dithiothreitol, and 0.05 *M* potassium phosphate (pH 6.8) was used as a dissociating solvent.

To avoid the need for an independent measurement of the initial concentration (~0.5 mg/ml), which is unreliable in 5 *M* Gdn·HCl, the meniscus depletion technique of Yphantis (1964) was employed, at a rotor speed of 52,000 rpm. A second procedure adopted was to use an "intermediate" speed (26,000 rpm), and to determine the small, finite meniscus concentration analytically from the equilibrium pattern (J. M. Creeth and J. C. Holt, unpublished; Gratzer et al., 1972). A plot of $j(r)/(r^2 - a^2)$ vs. $1/(r^2 - a^2)\ \int_a^r rj(r)dr$, where $j(r)$ is observed fringe number, r is radial position, and $r = a$ at the meniscus, yields a straight line with ordinate intercept y_o and slope m. The absolute concentration at the meniscus in fringes, $J(a)$, is then given by $J(a) = 2m/y_o$. Molecular weights were calculated from plots of log $J(r)$ vs. r^2 by means of the relation $M_w = 2RT/(1 - \phi'\rho^0)\ d \ln J(r)/dr^2$. The apparent specific volume, ϕ', was taken to be 0.73 and the solvent density, ρ^0, measured pycnometrically, was 1.128.

Results

1. Preparation of Light Chains. A typical fractionation profile of light chains dissociated from myosin by treatment with 4 *M* urea is shown in Figure 1a. The composition of individual fractions was examined by electrophoresis on polyacrylamide gels containing 0.1% dodecyl sulfate and by double diffusion in agar against a serum containing antibodies to all light chain components (Figure 2). The immunological analysis augmented the results of dodecyl sulfate gels by showing that pure light chains gave single, sharp precipitin lines, whereas mixtures showed evidence of more than one precipitin reaction. Pure alkali 1 was readily obtained from the first peak, fractions 90–101; the second

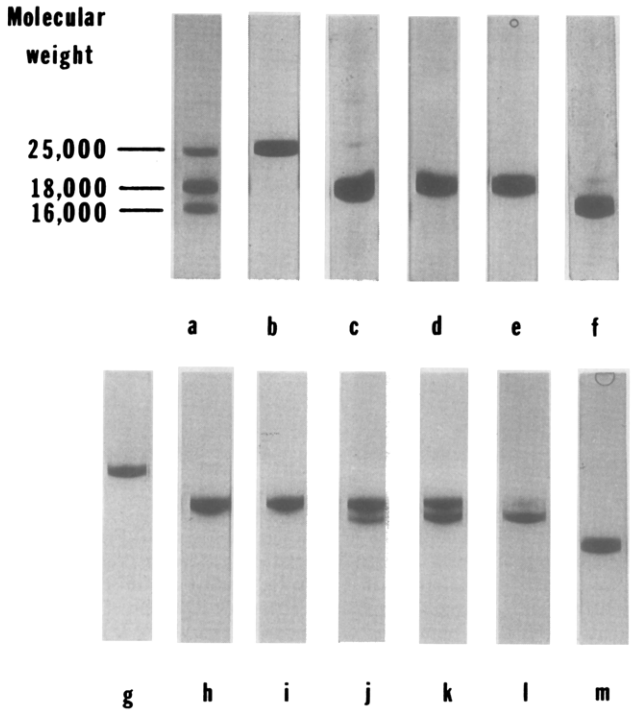

FIGURE 3: Gel electrophoresis of light chains isolated with 5 *M* Gdn·HCl and fractionated as in Figure 1b. (Upper row) 9% dodecyl sulfate gels: (a) total light chains; (b) pooled fractions 102–112; (c) 148–162; (d) 163–172; (e) 173–188; (f) 218–228. Loads of 10–15 μg were applied to all gels. (Lower row) 7.5% urea gels: (g–m) fractions 105, 150, 162, 168, 173, 180, and 222, respectively, from the elution profile in Figure 1b. The load applied was approximately 5 μg.

peak consisted mainly of DTNB l.c., although the pure protein (fractions 150–166) could be obtained only by discarding the broad leading edge which contained traces of alkali 1; the third peak (fractions 201–216) yielded alkali 2, sometimes with a trace of DTNB l.c.

A similar elution profile (Figure 1b) was obtained for light chains which had been dissociated from myosin with 5 *M* Gdn·HCl (Perrie and Perry, 1970). The Gdn·HCl-dissociated light chains differed, however, in several ways from those released by 4 *M* urea: dodecyl sulfate gels of pooled fractions (Figure 3) showed a higher degree of purity than was routinely obtained with 4 *M* urea light chains, particularly for DTNB l.c. This light chain was also obtained in about threefold greater yield and was resolved on the column into two components. Gel electrophoresis in the presence of 8 *M* urea (Figure 3g–m) showed that these were the phosphorylated and nonphosphorylated DTNB l.c. identified by Perrie et al. (1972). Similar electrophoretic analysis of the fractionated 4 *M* urea light chains failed to reveal any significant amount of the phosphorylated species. That this was a real difference between the two methods of isolation was confirmed by dividing a single myosin preparation into two parts and carrying out the dissociation with 4 *M* urea and 5 M Gdn·HCl in parallel; fractionation profiles very similar to those in Figure 1a and b were obtained for the two procedures.

Occasionally, in the case of 4 *M* urea dissociated light chains, additional peaks appeared at fractions 120–130 and 180–190 (Figure 1a). These were shown by double diffusion in agar and gel electrophoresis to be mixtures of all three light chains. Since 0.1 m*M* dithiothreitol was present throughout, they were presumably caused by noncovalent aggregation. Light chains isolated with 5 *M* Gdn·HCl were free of these aggregates and indeed the leading edge of the DTNB l.c. peak was sometimes free of the alkali 1 contaminant consistently observed in 4 *M* urea dissociated light chains. In general, optimum results were obtained when (a) the myosin was less than 1 week old, (b) all manipulations were carried out as fast as practicable, and (c) freeze-drying was avoided as a means of concentrating the dilute light chain containing supernatant resulting from precipitation of heavy chains at low ionic strength.

Table I: Amino Acid Composition of Myosin Light Chains.

Amino Acid	DTNB Light Chain	Alkali 1	Alkali 2	Alkali 1 – Alkali 2
Lys	16.0	18.8	11.2	8
His	1.1	2.0	1.9	
Arg	5.9	4.1	4.0	
Cys	1.9	0.90	0.81	
Asp	23.7	20.9	20.5	
Thr	8.9	7.9	7.8	
Ser	5.9	5.5	5.5	
Glu	22.0	27.6	24.3	3
Pro	7.2	12.6	4.1	8
Gly	12.6	11.9	11.5	
Ala	13.1	23.2	11.0	12
Val	8.8	9.8	8.8	1
Met	5.9	5.7	6.0	
Ile	10.1	9.2	7.5	2
Leu	9.6	13.4	12.3	1
Tyr	2.0	1.0	1.0	
Phe	11.8	9.9	9.8	
Trp	0.96	0.23	0.02	
Mol wt	18,500	20,000	16,500	

2. Characterization of Light Chains. Chemical Composition. Table I shows the amino acid composition of the isolated light chains and, in the last column, the composition difference between alkali 1 and 2. With the exception of tyrosine and tryptophan, all values are very similar to those given by Weeds and Lowey (1971) for the light chains of rabbit skeletal myosin. Moreover, for the rabbit, Weeds and Frank (1972) have shown that the unusual composition difference exists almost entirely as an N-terminal peptide, present in the larger alkali 1, and absent in the smaller alkali 2.

Spectra. The high content of phenylalanine in relation to tyrosine and tryptophan results in the appearance of fine structure in the ultraviolet absorption spectra of the light chains (Figure 4; see also Horváth and Gaetjens, 1972). The maxima in the 260-nm region are typical of the phenylalanine spectrum, which predominates in the case of the alkali light chains. The presence of even a single tryptophan and two tyrosine residues in the DTNB l.c. is sufficient to overshadow the phenylalanine contribution and give a more typical protein absorption spectrum.

The near-ultraviolet CD spectrum in the 250–270-nm region has a similar appearance for all three light chains, regardless of their aromatic chromophores (Figure 5). It would appear that the transitions of tyrosine and tryptophan do not contribute strongly in this region of the CD spectrum, and one observes primarily the phenylalanine dichroism. The CD spectrum was not changed appreciably in 5 *M* Gdn·HCl and is therefore an intrinsic property of the residues and independent of the conformation of the protein.

An estimate of α-helix content was made from the magnitude of the trough at 208 nm in the CD spectrum, Figure 5 (Greenfield and Fasman, 1969). The values found were

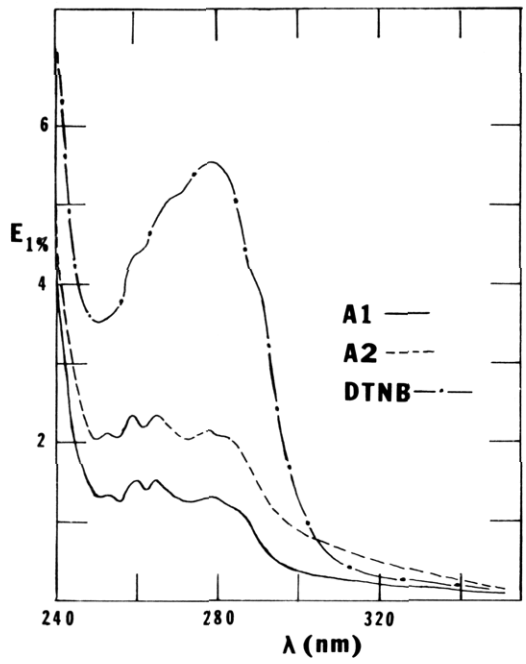

FIGURE 4: Ultraviolet absorption spectra of isolated light chains. Reproduced from Lowey and Holt (1972).

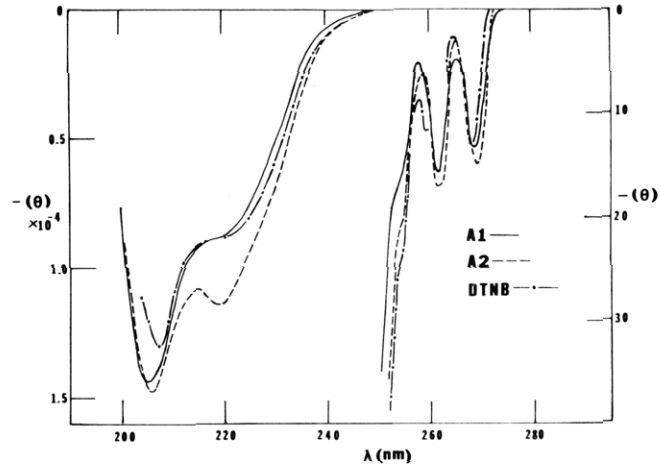

FIGURE 5: CD spectra of isolated light chains. Reproduced from Lowey and Holt (1972).

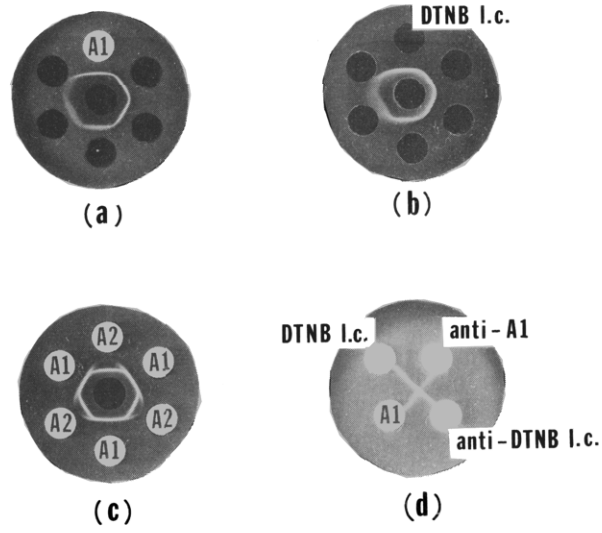

FIGURE 6: Immunodiffusion of light chains. (a) Twofold serial dilution from 0.2 mg/ml of alkali 1 against homologous antiserum; (b) twofold serial dilution from 0.4 mg/ml of DTNB l.c. against homologous antiserum; (c) alkali 1 and 2 (0.05 mg/ml) against anti-alkali 1 serum; (d) DTNB l.c. and alkali 1 (both 0.07 mg/ml) against homologous antisera.

30% for DTNB l.c. to 35% for alkali 1 and 2. Far-ultraviolet optical rotatory dispersion (ORD) measurements (not shown) yielded similar results: 35% (DTNB l.c.) to 40% (alkali 1 and 2) α helix; the mean residue rotation for 100% α helix was taken to be $-15{,}000$ deg cm^2/dmol at 233 nm (Greenfield et al., 1967).

Molecular Weight. Molecular weights are usually assigned to myosin light chains on the basis of their mobility on dodecyl sulfate gels. For chicken breast muscle myosin, these values are shown in Figure 3a. Minimum chemical molecular weights (Table I) confirm those deduced from dodecyl sulfate gels except in the case of alkali 1. In order to reconcile the discrepancy, sedimentation equilibrium experiments were carried out on each of the light chains dissolved in 5 *M* Gdn-HCl–1 m*M* dithiothreitol. Equilibrium was first established at 26,000 rpm, where the finite meniscus concentration could be determined as described in Materials and Methods. The rotor speed was then increased to 52,000 rpm until a new equilibrium was established under conditions of meniscus depletion (Yphantis, 1964). In this way, two independent analyses were applied to the results of sedimentation equilibrium experiments. The molecular weights obtained were: alkali 1, 20,000; DTNB l.c., 20,000; alkali 2, 17,500. Comparison with the values mentioned above suggests that alkali 1 differs from most proteins (Weber and Osborn, 1969) in that its relative mobility on dodecyl sulfate gels is not proportional to molecular weight.

3. Immunological Properties. Antisera prepared by immunizing rabbits with each of the chicken myosin light chains were characterized by double diffusion in agar and by quantitative precipitin analysis. Figure 6 shows that when serial dilutions of DTNB l.c. or alkali 1 were diffused against the homologous antiserum, essentially a single precipitin band was observed. The lower patterns represent cross-reactivity tests: with anti-alkali 1, the precipitin line due to alkali 2 formed a spur of partial identity with that due to the immunogen. In contrast, alkali 1 and DTNB l.c. were immunologically distinct by this criterion since the precipitin lines from homologous antigen–antibody reactions crossed (Figure 6d).

Alkali Light Chains. The antisera were further characterized by quantitative precipitin reactions carried out in solution. The upper solid curves in Figure 7a and b represent the reaction of alkali 1 and alkali 2, respectively, with homologous antiserum. The lower solid curves in each case show the extent of cross-reactivity, alkali 2 with anti-alkali 1 and vice versa. As noted earlier, a single peptide can be isolated from alkali 1, which represents almost all of the composition difference between alkali 1 and 2 (peptide C.1/36 of Frank and Weeds, 1974). We have used this "difference peptide", prepared for us from chicken alkali 1 by Dr. Alan G. Weeds, to analyze the immunological relationship between alkali 1 and 2. Since the molecular weight of the peptide is ~4000, its efficiency in precipitating antibody by cross-linking is low. The peptide was therefore tested as an inhibitor of the precipitin reaction between specific antiserum and an equivalent amount of light chain immunogen. About 50% of the specific antibody in alkali 1 serum was absorbed by large amounts of difference peptide (Fig-

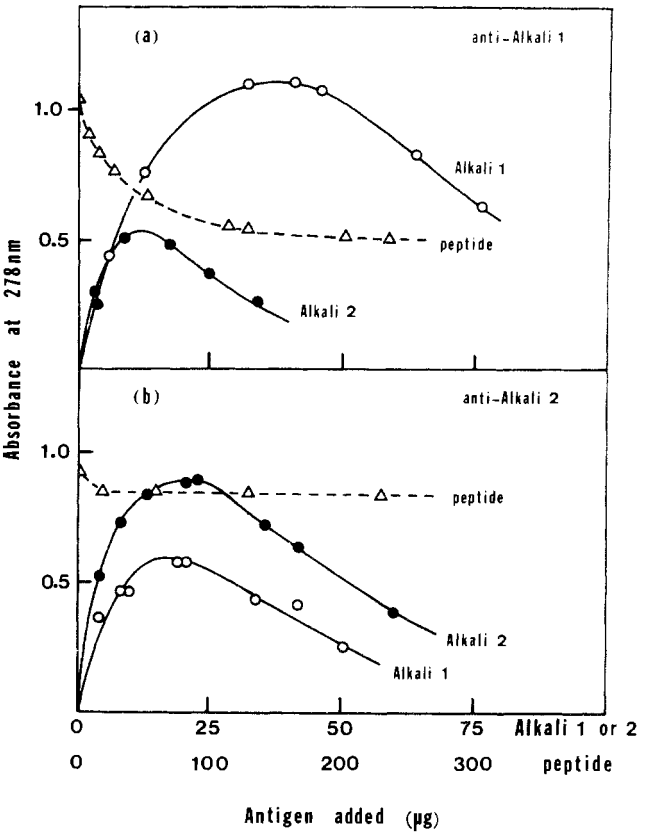

FIGURE 7: Precipitin analysis of anti-alkali 1 and 2 sera. (a) Reaction of 0.5 ml of anti-alkali 1 with alkali 1 (○) and alkali 2 (●). (△) Effect of difference peptide on the reaction between 0.5 ml of serum and 35 μg of alkali 1. (b) Reaction of 0.5 ml of anti-alkali 2 with alkali 1 (○) and alkali 2 (●). (△) Effect of difference peptide on the reaction between 0.5 ml of serum and 20 μg of alkali 2.

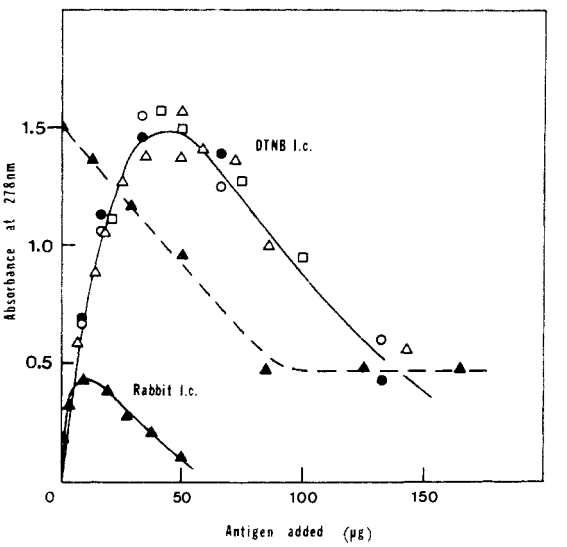

FIGURE 8: Quantitative precipitin reactions with antiserum to DTNB l.c. The immunogen for this antiserum was a mixture of all DTNB l.c. dissociated by 5 *M* Gdn·HCl (fractions 148–188 in the profile of Figure 1b). Antigens tested were: chromatographically purified DTNB l.c. initially dissociated by the thiol reagent DTNB (□), 4 *M* urea (△), and 5 *M* Gdn·HCl (○, phosphorylated and ●, nonphosphorylated) dissociated DTNB l.c. Total light chains isolated from rabbit myosin with 5 *M* Gdn·HCl (▲) were tested both as precipitating antigens (—) and as inhibitors of the reaction between 0.5 ml of serum and 40 μg of chicken DTNB l.c. (- - -). Since the concentration of DTNB l.c. in these total light chain preparations is unknown, the abscissa scale is arbitrary.

ure 7a, dashed curve), thus accounting for the difference in the precipitin reactions of alkali 1 and 2 with anti-alkali 1 serum. The purity of the peptide could be inferred from its lack of effect on the reaction of alkali 2 with homologous antiserum (Figure 7b). We conclude from these results that the sequentially homologous regions of alkali 1 and 2 are very similar in conformation. The inability of alkali 1 to precipitate all of the antibody in anti-alkali 2 serum (Figure 7b, solid curves) may represent a conformational difference, or simply the masking of antigenic determinants by the additional peptide.

DTNB Light Chain. The reaction of DTNB l.c. with homologous antiserum is shown in Figure 8. The immunogen for this serum was the whole DTNB l.c. fraction isolated from myosin with 5 *M* Gdn·HCl (fractions 148–188 in the elution profile of Figure 1b). Since this dissociative procedure was nonspecific and high in yield, the anti-DTNB l.c. antiserum had the broadest specificity experimentally available. Differences between DTNB l.c. fractions obtained by more selective dissociation could be sought by their ability to react with only some of the specific antibody present. Figure 8 therefore includes the reaction of immunogen, phosphorylated and nonphosphorylated DTNB l.c., 4 *M* urea-dissociated DTNB l.c., and DTNB l.c. selectively dissociated with the thiol reagent DTNB. Within the experimental limits of this test, all DTNB l.c. preparations were immunologically identical. The same result was obtained when this series of antigens was tested against two other antisera, one elicited by immunization with 4 *M* urea-dissociated DTNB l.c. and the other with a total light chain mixture isolated from myosin at pH 11 (Gazith et al., 1970; Lowey and Steiner, 1972). The identity of the different DTNB l.c. fractions was further demonstrated by fusion of the precipitin lines formed during double diffusion in agar (Figure 2c and d). The DTNB l.c. released by 5 *M* Gdn·HCl or by the reagent DTNB appeared therefore to be the same (cf. Katoh and Kubo, 1974).

Rabbit Light Chains. In the inhibition experiments described above with reference to alkali l.c., it was found that difference peptides derived from either rabbit or chicken alkali 1 could be used with equal effect. This implied that the antiserum produced in rabbits by immunization with chicken alkali 1 contained antibodies reactive with a rabbit, or self, protein. In order to show this directly, a total light chain preparation was isolated from rabbit myosin and tested by quantitative precipitin analysis. The rabbit total light chains showed 20–30% cross-reactivity with antisera specific for both DTNB l.c. (Figure 8), alkali 1 (Figure 9), and alkali 2 (not shown). The inhibition experiments with difference peptide had, however, suggested that 50% of the antibody in alkali 1 serum was recognized by that peptide alone. Since inhibition reveals interactions of lower affinity than does the quantitative precipitin test, it was not surprising to find that the rabbit light chains showed greater cross-reactivity when examined as inhibitors (Figures 8 and 9, dashed lines). The immunological similarity between the two species was clearly not confined to the difference peptide, since rabbit DTNB l.c., as well as alkali 2, exhibited cross-reactivity with its chicken homolog.

It was recognized that genetic differences between the rabbits from which the serum and the light chain were derived might account for this apparent breaking of immunological tolerance. For this reason, one of the three rabbit light chain preparations examined was isolated from the same rabbit as the anti-DTNB l.c. serum with which the re-

sults of Figure 8 were obtained. Since this particular preparation precipitated antiserum in the same way as the two other unrelated preparations, the ability of rabbit light chains to react with rabbit antiserum did not seem to depend on genetic variations between rabbits.

Discussion

The two methods used to isolate light chains from myosin offered a slight difference of approach. With 4 *M* urea, the aim was to use the mildest dissociating conditions consistent with release of the subunits in order that the conformations of free and bound light chains be as similar as possible. Treatment with 5 *M* Gdn·HCl and a reducing agent, on the other hand, eliminated all noncovalent structure in both heavy and light chains (Tanford, 1968) so that the latter, upon removal of the denaturant, adopted stable conformations dictated solely by their amino acid sequences. Reversible denaturation of the urea-dissociated light chains with 5 *M* Gdn·Cl showed that, in practice, the two procedures yielded conformationally similar light chains (Lowey and Holt, 1972). The immunological identity of all species of DTNB l.c. (Figures 2c and d and 8) further supports this conclusion. The isolation of the light chains from myosin could not therefore have been accompanied by greater conformational changes than those induced by 4 *M* urea. The degree of similarity between free and bound light chains is considered in more detail elsewhere (Holt and Lowey, 1975). The low yield of DTNB l.c. from the 4 *M* urea procedure may result either from coprecipitation of released light chain with heavy chains or from incomplete dissociation in this mild denaturant. The use of 5 *M* Gdn·HCl not only increased the overall yield of DTNB l.c. but also allowed isolation of the phosphorylated fraction of this light chain (Perrie et al., 1973).

A further possible difference between the two isolation procedures has only recently been recognized: alkali 1 derived from 4 *M* urea dissociated light chains may be contaminated by small amounts of a water-soluble heavy chain fragment which copurifies with the light chain. The existence of such a contaminant is suggested by immunodiffusion of unpurified total light chain preparations from the proteolytic subfragments of myosin against anti-alkali 1 serum (Holt and Lowey, 1975). If the myosin preparation from which light chains are to be isolated contains traces of proteolytic activity, incubation in a mild denaturant (4 *M* urea) at room temperature maximizes the likelihood of this activity being expressed. Immediate adjustment of the solution to 5 *M* Gdn·HCl, however, may be expected to inactivate almost all proteases before cleavage of the protein substrate can occur. An analogy may be drawn with the incubation of a protein sample, known to contain protease activity, in dodecyl sulfate at 37° as opposed to 100° (Margossian and Lowey, 1973).

The amino acid compositions of the light chains from chicken breast muscle myosin are very similar to those of the corresponding subunits in rabbit skeletal myosin. All have a high content of phenylalanine, but the contribution of this amino acid to the uv absorption spectra is clearly visible only in the case of chicken alkali 1 and 2. These light chains are unique in containing only 1 tyrosine residue and no tryptophan. Troponin C also has a high ratio of phenylalanine to tyrosine such that a spectrum similar to that of chicken alkali l.c. is observed (Schaub et al., 1972). A more definitive demonstration that these proteins may be structurally related has come from a comparison of their sequences, which show a high degree of homology (Collins, 1974; Weeds and McLachlan, 1974).

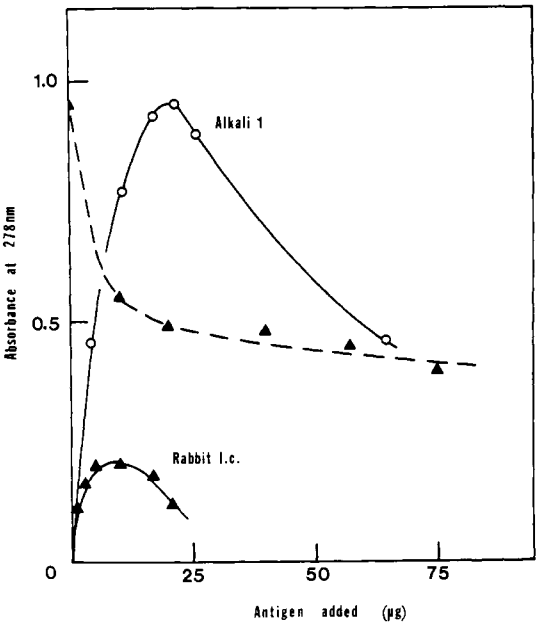

FIGURE 9: Cross-reactivity between rabbit and chicken alkali l.c. (—) Precipitation reactions (○, chicken alkali 1; ▲, rabbit total l.c.); (- - -) inhibition by rabbit total l.c. of the reaction between 0.5 ml of serum and 20 μg of alkali 1. The abscissa scale for rabbit l.c. is arbitrary (see Figure 8).

Sequence studies on rabbit alkali 1 and 2 have shown that the major difference between these light chains is a single peptide, rich in alanine, proline, and lysine (Frank and Weeds, 1974). Preliminary results suggest that a similar peptide, accounting almost exactly for the composition difference between alkali 1 and 2 (Table I), occurs in chicken alkali 1 (A. G. Weeds, personal communication). The unusual composition of this peptide may be responsible for the incorrect molecular weight (25,000) estimated for alkali 1 from dodecyl sulfate gel electrophoresis. Direct methods, based on amino acid composition and sedimentation equilibrium, yield molecular weights of 19,000–21,000 for this light chain. In the case of rabbit alkali 1, the molecular weight calculated from the amino acid sequence is 20,700 (Frank and Weeds, 1974), whereas the gel estimate is 25,000. Alkali 2, where the difference peptide is absent, and DTNB l.c., show no anomaly on dodecyl sulfate gels.

The α-helix content of all three light chains is in the range of 30–40%, typical of globular proteins. Since the antigenic properties of proteins depend to a large extent on conformation (e.g., Arnon and Maron, 1971; Sachs et al., 1972a), the immunological results provide a conformational basis for the division of light chains into the two classes, alkali l.c. and DTNB l.c., first defined by chemical studies (Weeds, 1969; Gazith et al., 1970). Alkali 1 and 2 showed extensive cross-reactivity, but they did not react with antisera specific for DTNB l.c. Similar results have been obtained using antisera to a mixture of light chain components (Horváth and Gaetjens, 1972; Lowey and Holt, 1972). About half of the antibody population elicited by alkali 1 was directed against the difference peptide, while the balance of the antibodies reacted equally well with alkali 1 or 2. It is not clear whether the conformations of alkali 1 and 2 are identical, since alkali 1 failed to cross-react completely with anti-alkali 2 serum. Although far-uv CD spectra imply the same α-helix content for the two alkali l.c., the trough

at 220 nm (Figure 5) may be indicative of a small structural difference.

DTNB l.c. released from myosin with the thiol reagent DTNB, 4 *M* urea, or 5 *M* Gdn·HCl all reacted equally well with antisera elicited by the urea- or Gdn·HCl-dissociated immunogen, as well as with an antiserum to alkali-dissociated total light chains. The phosphorylated and nonphosphorylated forms of this light chain were also immunologically identical, their spectral and chemical identity already having been established (Perrie et al., 1973). There is thus no evidence to suggest that more than one type of DTNB l.c. may exist. We did not observe two immunological classes of DTNB l.c. as reported by Katoh and Kubo (1974). This conclusion is important for an understanding of why DTNB or specific antibody is capable of dissociating only half of the DTNB l.c. from myosin or its subfragments (Holt and Lowey, 1975).

The significance of the two closely related, but distinct alkali l.c. found in vertebrate fast muscle has yet to be determined. The possibilities are twofold: either they represent the two nonidentical heads of a single myosin molecule or, alternatively, they may be derived from two isozymic populations of myosin, in which each molecule contains 2 mol of the same alkali l.c.; formally, one must recognize a third possibility, consisting of a combination of the first two. Densitometry of dodecyl sulfate gels has given ambiguous results for the relative amounts of alkali 1 and 2; in fast muscles from the rabbit, the amounts were unequal, whereas in chicken breast muscle, they were equal (Lowey and Risby, 1971; Sarkar, 1972; J. C. Holt, B. Smith, and S. Lowey, unpublished results). Even though such factors as the constancy of dye uptake by the different proteins were determined, and bound dye quantitated both by elution and densitometry, these results cannot be considered definitive. For rabbit myosin, however, quantitative radioactive labeling of the thiol groups, followed by separation of the light and heavy chains on dodecyl sulfate gels, has allowed a more rigorous determination of the stoichiometry. In a selected region of the psoas, as well as the standard preparation of mixed back and leg muscles, appreciably different amounts of alkali 1 and 2 were found, 1.35 and 0.68 mol/mol of myosin, respectively (Weeds et al., 1975). The available evidence therefore favors the isoenzyme hypothesis, although an alternative approach would clearly be of interest.

The distribution of alkali 1 and 2 in myosin can be studied by immunochemical methods. Although antisera to these light chains show extensive cross-reactivity, the antibodies in anti-alkali 1 serum which react with the difference peptide are capable in principle of providing a marker specific for alkali 1. Several conditions must be fulfilled if this approach is to be successful. The peptide as it occurs in myosin must be available for reaction with IgG, and its antigenic structure must resemble that in isolated alkali 1, the immunogen. Isolation of the appropriate antibody fraction from anti-alkali 1 serum requires that the peptide be coupled to a matrix (for use as an immunoadsorbent) in such a way that it retains the ability to bind antibody. Several examples of the fractionation of antibodies using peptide fragments suggests that this approach is feasible (e.g., Sachs et al., 1972b). If all myosin molecules react with antibody to the difference peptide, then all must contain alkali 1, but alkali 2 need not always accompany alkali 1 in a heterodimer. If, however, a reactive (alkali 1) and a nonreactive (alkali 2) population can be demonstrated, then the isoenzyme hypothesis will have been verified in the most direct way, by fractionation of the two populations. These studies on isolated myosin could readily be extended to the next level of organization, the myofibril, by making use of fluorescent-labeled antibody.

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References

Adelstein, R. S., and Conti, M. A. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37,* 599.

Adelstein, R. S., Conti, M. A., and Anderson, W., Jr. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3115.

Arnon, R., and Maron, E. (1971), *J. Mol. Biol. 61,* 225.

Beaven, G. H., and Holiday, E. R. (1952), *Adv. Protein Chem. 7,* 319.

Collins, J. H. (1974), *Biochem. Biophys. Res. Commun. 58,* 301.

Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971), *Biochemistry 10,* 2606.

Frank, G., and Weeds, A. G. (1974), *Eur. J. Biochem. 44,* 317.

Gaetjens, E., Bárány, K., Bailin, G., Oppenheimer, H., and Bárány, M. (1968), *Arch. Biochem. Biophys. 123,* 82.

Gazith, J., Himmelfarb, S., and Harrington, W. F. (1970), *J. Biol. Chem. 245,* 15.

Gratzer, W. B., Creeth, J. M., and Beaven, G. H. (1972), *Eur. J. Biochem. 31,* 505.

Greenfield, N., Davidson, B., and Fasman, G. D. (1967), *Biochemistry 6,* 1630.

Greenfield, N., and Fasman, G. D. (1969), *Biochemistry 8,* 4108.

Holt, J. C., and Lowey, S. (1975), *Biochemistry,* following paper in this issue.

Holtzer, A., and Lowey, S. (1959), *J. Am. Chem. Soc. 81,* 1370.

Horváth, B. Z., and Gaetjens, E. (1972), *Biochim. Biophys. Acta 263,* 779.

Kabat, E. A. (1961), in Kabat and Meyer's Experimental Immunochemistry, 2nd ed, Springfield, Ill., Charles C. Thomas.

Katoh, N., and Kubo, S. (1974), *Biochim. Biophys. Acta 365,* 301.

Kendrick-Jones, J. (1974), *Nature (London) 249,* 631.

Kominz, D. R., Carroll, W. R., Smith, E. N., and Mitchell, E. R. (1959), *Arch. Biochem. Biophys. 79,* 191.

Lehman, W., Kendrick-Jones, J., and Szent-Györgyi, A. G. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37,* 319.

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

Lowey, S., and Steiner, L. A. (1972), *J. Mol. Biol. 65,* 111.

Lowey, S., and Risby, D. (1971), *Nature (London) 234,* 81.

Margossian, S. S., and Lowey, S. (1973), *J. Mol. Biol. 74,* 301.

Perrie, W. T., and Perry, S. V. (1970), *Biochem. J. 119,* 31.

Perrie, W. T., Smillie, L. B., and Perry, S. V. (1973), *Biochem. J. 135,* 151.

Perrie, W. T., Smillie, L. B., and Perry, S. V. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37,* 17.

Pollard, T. D., and Korn, E. D. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37,* 573.

Sachs, D. H., Schechter, A. N., Eastlake, A., and Anfinsen, C. B. (1972a), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3790.

Sachs, D. H., Schechter, A. N., Eastlake, A., and Anfinsen, C. B. (1972b) *J. Immunol. 109,* 1300.
Sarkar, S. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37,* 14.
Sarkar, S., Sréter, F. A., and Gergely, J. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 946.
Schaub, M. C., Perry, S. V., and Häcker, W. (1972), *Biochem. J. 126,* 237.
Szent-Györgyi, A. G., Szentkiralyi, E. M., and Kendrick-Jones, J. (1973), *J. Mol. Biol. 74,* 179.
Tanford, C. (1968), *Adv. Protein Chem. 23,* 122.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Weeds, A. G. (1969), *Nature (London) 223,* 1362.
Weeds, A. G., and Frank, G. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37,* 9.
Weeds, A. G., Hall, R., and Spurway, N. C. S. (1975), *FEBS Lett. 49,* 320.
Weeds, A. G., and Lowey, S. (1971), *J. Mol. Biol. 61,* 701.
Weeds, A. G., and McLachlan, A. D. (1974), *Nature (London) 252,* 646.
Yphantis, D. A. (1964), *Biochemistry 3,* 297.

An Immunological Approach to the Role of the Low Molecular Weight Subunits in Myosin. II. Interaction of Myosin and Its Subfragments with Antibodies to the Light Chains†

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ABSTRACT: Immunological methods, in parallel with measurement of ATPase activity, have been used to characterize the reactions of antibodies specific for light chains with myosin and its water-soluble proteolytic subfragments, heavy meromyosin (HMM) and subfragment 1 (HMM S-1). Antiserum to the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) light chain undergoes a precipitation reaction with all of the enzyme species, in which half of the homologous light chain is selectively dissociated. The results suggest that the incomplete dissociation reflects the way in which the light chain is bound, rather than the existence of two distinct species of DTNB l.c. Little reaction was observed with antisera to alkali-released light chains, indicating that these components in myosin and the subfragments are either largely buried or else conformationally different from the isolated light chains used as immunogens. None of the antisera produced significant changes in Ca^{2+}- or EDTA-ATPase activities. Moreover, calcium regulation through the troponin-tropomyosin system was unaffected by removal of DTNB l.c. from myosin, as well as from the subfragments. The absolute level of actin-activated ATPase activity was, however, consistently lower in the presence of light chain antisera (or purified IgG and antibody) than in aqueous buffer or nonimmune serum. For both alkali and DTNB l.c. antisera, this loss in activity seemed to result from steric hindrance of actin binding by antibody bound to undissociated light chain. Experimental conditions which would be expected to weaken such an antigen–antibody interaction, as well as the use of monovalent Fab in place of IgG, decreased the inhibition of activity. Altogether the activity measurements suggest that the light chains, particularly DTNB l.c., are probably not integral parts of either the hydrolytic or actin-binding sites.

The initial studies which implicated light chains in the enzymic function of myosin sought to show that activity was lost upon removal of the light chains, and restored when they were recombined with the heavy chain core. The methods which were successful in dissociating light chains included high pH (Kominz et al., 1959; Gershman et al., 1966), strong salts (Gershman and Dreizen, 1970), and the thiol reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (Weeds, 1969; Gazith et al., 1970). Selective removal of DTNB l.c.[1] had little effect on Ca^{2+}- or EDTA-ATPase activity, so that its requirement for the hydrolysis of ATP remained unproven (Weeds and Lowey, 1971). Alkali l.c., on the other hand, appeared to be essential since their removal resulted in a total loss of activity. The significance of this observation is, however, limited by the fact that only part of the activity was recovered when light chains were added back to the inactive heavy chains (Stracher, 1969; Dreizen and Gershman, 1970; Kim and Mommaerts, 1971). The interpretation of activity changes is therefore complicated by the possibility that the strong dissociating conditions necessary to release light chains may also have irreversibly denatured

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[1] For nomenclature and properties of light chains, see preceding paper (Holt and Lowey, 1975).