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†

John C. Holt‡ and Susan Lowey*

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

† From the Rosenstiel Basic Medical Sciences Research Center and the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154. *Received March 25, 1975.* This work was supported by grants from the National Science Foundation (GB 38203), the National Institute of Arthritis, Metabolism and Digestive Diseases, U.S. Public Health Service (AM 17350), and the Muscular Dystrophy Associations of America.
‡ Supported by Research Fellowships of the Massachusetts Heart Association (1047-F and 1109-F).

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

the myosin.

Affinity labels (Singer, 1967) have frequently been used to define those residues of the polypeptide chain which are present in the active site of an enzyme. The involvement of alkali l.c. in a nucleotide-binding site (distinct from the hydrolytic site) has been suggested by studies with a purine disulfide analog of ATP (Wagner and Yount, 1975). A total of about 4 mol of analog was incorporated/mol of myosin, with a concomitant loss of ATPase activity. The label was found in both heavy and light chains with about 1 mol/mol of alkali l.c. and none in DTNB l.c.

Experiments of a different kind which suggest that alkali l.c. are involved in the expression of hydrolytic activity concern the innervation of muscles in vivo. Several slow muscle myosins show a characteristic light chain pattern on gel electrophoresis which is distinct from that of fast muscle myosins (Lowey and Risby, 1971; Sarkar et al., 1971). When the nerve connections of fast and slow muscles are interchanged in a single animal, the physiological properties of the muscles are found to change in parallel with innervation (e.g., Close, 1969). A recent study has shown that the physiological changes are accompanied by the appearance of light chains characteristic of the new muscle type (Sréter et al., 1974; Weeds et al., 1974). In another approach, Sréter et al. (1973) brought about a partial physiological change from fast to slow muscle by electrical stimulation of the motor nerve to the fast muscle. The modified muscle was characterized by decreased Ca^{2+}- and EDTA-ATPase activities and a light chain pattern in which slow muscle light chains were present, in addition to the original fast muscle light chains.

The only direct evidence on how light chains function in myosin has come from studies on certain invertebrate species, notably molluscs. The calcium sensitivity of scallop myofibrils is mediated through the thick, myosin-containing filaments (Kendrick-Jones et al., 1970; Szent-Györgyi et al., 1973) and not as in vertebrate striated muscles, through the troponin–tropomyosin complex located on the thin filaments (e.g., Weber and Murray, 1973). The element of the thick filament which responds to calcium is a class of myosin light chain, which can be reversibly dissociated by treatment with EDTA, and is therefore designated EDTA light chain (Kendrick-Jones et al., 1972). Removal of this light chain reduces calcium binding by myosin and abolishes calcium sensitivity of the actin-activated ATPase; recombination fully restores both these functions (Szent-Györgyi et al., 1973). An important insight into the function of vertebrate light chains is provided by the observation that rabbit DTNB l.c. can substitute for the EDTA light chain in restoring Ca sensitivity to scallop myosin desensitized with EDTA (Kendrick-Jones, 1974). Preliminary results (J. Kendrick-Jones, E. M. Szentkiralyi, and A. G. Szent-Györgyi, personal communication) suggest that a number of vertebrate species contain a light chain capable of this partial substitution for EDTA light chain. Comparative studies have shown that myosin-linked calcium regulation, which is widely distributed among invertebrates, often occurs together with, rather than instead of, actin-linked troponin–tropomyosin regulation (Lehman et al., 1972; Lehman and Szent-Györgyi, 1975). The existence of the latter, as in vertebrate muscle, does not therefore preclude the operation of myosin-linked calcium regulation as well.

As an alternative approach to the function of the light chains in vertebrate skeletal myosin, we have undertaken an immunochemical study, based on the observation that antibodies to isolated light chains react with HMM S-1, the globular subfragment of myosin, but do not appear to affect ATPase activity (Lowey and Steiner, 1972). In contrast, reaction with antiserum to whole myosin results in a total loss of activity. Characterization of the purified light chains, and the antisera they elicit, is reported in the preceding paper (Holt and Lowey, 1975). In this paper, we describe the interaction of antisera specific for each of the light chains with myosin and both single-headed (HMM S-1) and double-headed (HMM) subfragments. Analysis of the antigen–antibody precipitate reveals that DTNB l.c. antiserum selectively dissociates this light chain from both the subfragments and myosin. The loss of DTNB l.c. does not affect the calcium sensitivity of the actin-activated ATPase in the presence of troponin and tropomyosin. Ca^{2+}- and EDTA-ATPase activities of the subfragments are unaffected by reaction with antisera to alkali 1 or DTNB l.c. whereas actin activation is reduced in both cases. The most simple interpretation is that antibody which is bound to undissociated light chain interferes with actin binding.

Materials and Methods

The experimental procedures described here are in addition to those detailed in the preceding paper (Holt and Lowey, 1975).

Proteolytic Subfragments of Myosin. HMM S-1[2] was prepared by digesting insoluble myosin (20 mg/ml) in 0.2 *M* ammonium acetate (pH 7.2), with 0.04 mg/ml of papain (Worthington) for 5 min at 12° (Margossian and Lowey, 1973b). The reaction was terminated by addition of iodoacetic acid to a final concentration of 1–2 m*M*. After 10 min at 21°, the mixture was centrifuged at 30,000*g* for 90 min and the supernatant dialyzed against 0.05 *M* Tris-HCl (pH 7.9). HMM S-1 was purified either by chromatography on DEAE-cellulose (Whatman DE-52) in 0.05 *M* Tris-HCl (pH 7.9) (Lowey et al., 1969), or by fractionation between 42 and 57% saturated ammonium sulfate in the presence of 1 m*M* EDTA and 0.2 m*M* dithiothreitol (Margossian and Lowey, 1973b). A low final yield (20% of the theoretical maximum) was accepted since more extensive digestion seemed to result in selective proteolysis of the light chains.

HMM was prepared as described by Margossian and Lowey (1973a) except that a shorter digestion time, 2.5 min, was used, again to minimize degradation of the light chains. The yield of the final 42–57% saturated ammonium sulfate fraction was 25% of theoretical.

Light Chains from Myosin Subfragments. Solid Gdn·HCl and dithiothreitol were added to a solution of HMM or HMM S-1 (10–15 mg/ml) to final concentrations of 5 *M* and 1 m*M*, respectively. After the mixture had stood at 21°, pH 7.9, for a short time the concentration of Gdn·HCl was reduced to 1 *M* or less by rapid dilution into water at 4°. Dialysis against several changes of 0.05 *M* potassium phosphate (pH 6.8) completed the removal of denaturant. The dilute solution of light chains, together with some suspended heavy chains, was then freeze-dried. This procedure did not completely eliminate heavy chain material as evidenced by dodecyl sulfate gel electrophoresis. Since the subfragment total light chain preparations were used only in

[2] Abbreviations used are: HMM, heavy meromyosin; HMM S-1, heavy meromyosin subfragment 1; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N′-tetraacetic acid; Gdn·HCl, guanidine hydrochloride; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

experiments with antisera of defined specificity, the relative proportions in which the different light chains were present was not determined.

This procedure differed from that used to isolate light chains from myosin (Holt and Lowey, 1975), in that Gdn·HCl was removed by dilution rather than by dialysis from the initial 5 *M* concentration. At least for HMM S-1, this modification was essential to the recovery of immunologically reactive material. Presumably if intermediate concentrations of Gdn·HCl (2–4 *M*) were maintained for any length of time, traces of papain, which very readily assumed an active conformation, could digest incompletely folded light chains. A similar process occurs when HMM S-1 is incubated with dodecyl sulfate at 37°, rather than being immediately exposed to 100°; the difference in these two procedures is strikingly revealed by dodecyl sulfate gels (Margossian and Lowey, 1973a).

Actin and Relaxing Proteins. Actin was prepared by the method of Spudich and Watt (1971). All preparations released 0.7–0.9 mol of bound nucleotide per 43,000*g* protein when treated with perchloric acid (Asakura, 1961).

Relaxing factor (tropomyosin–troponin) was extracted from the washed muscle residue with water, after prior extraction of myosin (Ebashi and Ebashi, 1964). Most of the actin was removed by taking the fraction which salted out between 40 and 65% saturated ammonium sulfate. Since this material was found to contain ATPase activity, the solution (in 0.6 *M* KCl, 5 m*M* Tris-HCl (pH 7.5), 0.1 m*M* $CaCl_2$, 0.1 m*M* dithiothreitol, and 10^{-5} *M* *N*-α-tosyllysine chloromethyl ketone) was brought to pH 3 for 15 min at 4° so as to denature myosin. When the salt concentration was lowered to 0.1 *M* KCl by dialysis, 30–40% of the relaxing proteins precipitated and were removed by centrifugation.

Relaxing actin was prepared by polymerizing G-actin in the presence of a twofold weight excess of relaxing factor (Weber and Bremel, 1971). Relaxing factor and G-actin were mixed to give 2 mg/ml of actin, 4 mg/ml of relaxing factor, 0.1 *M* KCl, 5 m*M* Tris-HCl (pH 7.4), and 2 m*M* $MgCl_2$; actin was allowed to polymerize 1–2 hr at 0° before pelletting at 150,000*g* for 2–3 hr. The pellets were rinsed with 0.1 *M* KCl–2 m*M* $MgCl_2$–10 m*M* Tris-HCl (pH 8.4) and allowed to swell overnight in the same solvent. Finally they were gently homogenized in 10 m*M* Tris-HCl (pH 8.4)–2.5 m*M* $MgCl_2$ and dialyzed. The concentration of F-actin was determined from measurements of bound nucleotide, assuming that pure and regulated F-actin derived from the same G-actin contained the same amount of nucleotide. The proportion of total actin which was regulated by Ca^{2+} varied from 65 to 95% in different preparations.

Immunological Species. The work with unfractionated serum (Tables I–III; Figures 1–8) employed several bleeds from two rabbits with specificity for DTNB l.c. and many bleeds (over a 2-year period) of a single rabbit immunized with alkali 1. The results shown are typical of those subsequently obtained with sera from at least five rabbits immunized with each antigen. The only exception noted was that one of eight anti-DTNB l.c. sera dissociated appreciably less than 50% of the homologous light chain from HMM S-1, and another reacted poorly with myosin, though well with both subfragments.

"Immune IgG" was isolated from serum by treatment with 40% saturated $(NH_4)_2SO_4$ and chromatography on DEAE-cellulose (Sober and Peterson, 1958; Lowey and Steiner, 1972). The final yield was 0.9–1.2 g of IgG from 120 ml of serum; the yield was markedly lower from the sera of nonimmunized rabbits, 0.5–0.7 g of IgG from the same 120 ml of serum.

"Specific antibody" was isolated from immune serum by adsorption on an immunoadsorbent column. The latter consisted of total light chains released from myosin by 5 *M* Gdn·HCl (Holt and Lowey, 1975) coupled to Sepharose 4B as described by Omenn et al. (1970). Separate portions of this immunoadsorbent were used in the isolation of antibody specific for alkali 1 and DTNB l.c.; the specificity of the antibody preparations therefore depended on the purity of the immunogen initially used, since the immunoadsorbent would bind antibody to any of the light chains. Clarified serum (130 ml) in 0.15 *M* KCl–0.02 *M* potassium phosphate (pH 7.2) was applied at 100 ml/hr to a column (8 × 2 cm) equilibrated in the same buffer at 21°. The column was then washed until the extinction of the effluent was less than 0.1 at 230 nm (~200 ml). Bound antibody was eluted by the application of a step to pH 2; both the column and the eluted protein were returned to pH 7.2 within 10 min. To ensure that all specific antibody was removed from the serum, the cycle of loading, washing, and elution was repeated.

In a typical experiment, 135 mg of antibody was eluted after the first passage of the serum (130 ml) and a further 6 mg after the second; a total of 130 mg of antibody could be precipitated from this volume of serum of equivalence. Although the acid-eluted antibody was fully soluble immediately after neutralization to pH 7.2, as much as 50% of the protein sometimes precipitated over a 12-hr period at 0°. Of the remaining soluble material, moreover, only about 65% was capable of precipitation by light chain antigen. The brief exposure to pH 2 which was responsible for these losses did not affect the properties of the immunoadsorbent in any obvious way.

The Fab fragment of IgG was prepared essentially as described by Porter (1959). IgG (15–20 mg/ml in 0.1 *M* potassium phosphate (pH 7.0)–2 m*M* EDTA) was digested with 1% w/w papain in the presence of 0.1% (14 m*M*) 2-mercaptoethanol. After 60 min at 37°, the solution was made 23 m*M* in iodoacetic acid and incubated a further 20 min at 21 or 37°. The solution was then dialyzed for 48 hr against two changes of 10 m*M* potassium phosphate (pH 7.0) and precipitated Fc removed by centrifugation.

Quantitative Precipitin Analysis. The procedure described with reference to isolated light chains in the preceding paper was followed. Antigens and antisera were dialyzed against 0.3 *M* KCl–0.01 *M* Tris-HCl (pH 8.4) at 4°; when lower concentrations of salt were used there was a tendency for the whole subfragment to precipitate with anti-DTNB l.c. serum. Either the affinity of antibody for the bound light chain was enhanced in low salt, or the subfragment depleted of DTNB l.c. was insoluble at low ionic strength. Antigen–antibody precipitates were washed once with 0.3 *M* KCl–0.01 *M* Tris-HCl (pH 8.4), and twice with water so that essentially no salt remained. They were then dissolved in 0.5 ml of NH_4OH to allow quantitation of specific antibody by an extinction measurement.

Measurements of ATPase Activity. Ca^{2+}-ATPase was measured in 0.225 *M* KCl, 0.05 *M* Tris-HCl (pH 7.9), 2.5 m*M* $CaCl_2$, and 2.5 m*M* ATP. EDTA-activated ATPase was measured in 0.6 *M* KCl–0.05 *M* Tris-HCl (pH 7.9)–4 m*M* EDTA–2.5 m*M* ATP. Actin-activated Mg^{2+}-ATPase was measured in 0–30 m*M* KCl–10 m*M* Tris-HCl (pH 7.9)–2.5 m*M* $MgCl_2$–2.5 m*M* ATP. The concentration of actin was varied from 0.5 to 4.0 mg/ml. When regulated

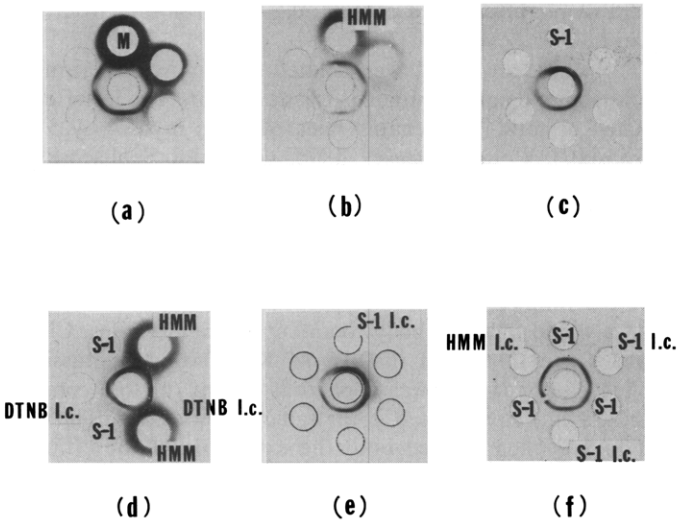

FIGURE 1: Immunodiffusion of myosin and subfragments with antiserum to DTNB l.c. (Upper row) Twofold serial dilutions of (a) myosin (10 mg/ml), (b) HMM (7 mg/ml), and (c) HMM S-1 (4 mg/ml). (Lower row) (d and f) Tests of cross-reactivity; concentrations were: DTNB l.c., 0.07 mg/ml; HMM S-1, 4 mg/ml in (d), 0.05 mg/ml in (f); HMM, 7 mg/ml; S-1 l.c. (total light chains isolated from HMM S-1), ~0.05 mg/ml. (e) Twofold serial dilution from ~1 mg/ml of total light chains isolated from HMM S-1.

actin was used, either 0.1 m*M* $CaCl_2$ or 1 m*M* EGTA was included in the assay medium. Under the conditions used, the Mg^{2+}-ATPase of myosin and its subfragments was negligibly small, so that the values reported are effectively for the activation of this ATPase by actin.

All measurements were carried out at 25 ± 0.1 or 33 ± 0.1° in a volume of 2 ml. For assays at pH 7.0, imidazole-HCl was substituted for Tris-HCl. The reaction was initiated by the addition of ATP and terminated 5–20 min later by making the solution 5% in Cl_3CCOOH (15% Cl_3CCOOH was necessary to precipitate Fab); after removal of the precipitated protein, inorganic phosphate was determined colorimetrically (Fiske and Subbarow, 1925). Each assay mixture contained 10–60 μg (0.05–0.2 nmol) of enzyme, depending on the particular enzyme species and ATPase activity concerned. Not more than 20% of the ATP added was hydrolyzed during the assay. Enzyme activities are reported in specific (μmol of P_i per mg of enzyme per min) or molar (μmol of P_i per μmol of enzyme per sec) units; in the latter case, the molecular weights used were: myosin, 470,000; HMM S-1, 115,000; HMM, 340,000. The molecular weight of actin was taken to be 43,000.

Measurements in the Presence of Antiserum. The ATPase activities of myosin and its subfragments in serum or IgG were close to the values measured in aqueous solution. Over the period of an incubation experiment, typically 100 hr, all activities decreased by about 20%. Serum thus stabilized the enzyme species, since one would normally expect a much more rapid loss of activity from a dilute aqueous solution (~0.1 mg/ml) of myosin or subfragment.

The enzyme–antiserum mixture, on which activity measurements were made, contained of necessity 0.3 *M* KCl (see above). In the case of Ca^{2+}- and EDTA-activated ATPases, measured in 0.225 and 0.6 *M* KCl, this posed no problem. Activation of Mg^{2+}-ATPase by actin, however, is greatly inhibited in the presence of salt (Eisenberg and Moos, 1968). This behavior led to ATPase activities which were too low to be measured, particularly at less than saturating concentrations of actin. The difficulty could not be circumvented by using greater amounts of enzyme in the assay, since the stock enzyme–antiserum solution itself contained 0.3 *M* KCl. The following procedure was therefore adopted. After measurements of Ca^{2+}- and EDTA-ATPase activities, a portion of the enzyme–antiserum mixture was freed of KCl by dialysis into 10 m*M* Tris-HCl (pH 8.4). The volume change, and, hence the concentration change, resulting from dialysis was determined by repeating one or both activity measurements for each sample; these changes amounted to 8–25%. Measurements of actin activation were then made on the salt-free solution. In experiments where measurements were not required at low concentrations of actin, the dialysis step was eliminated and the assay medium contained 10–30 m*M* KCl. No difference between the two systems was observed, beyond the obvious inhibitory effect of KCl on actin activation. The conditions under which particular results were obtained are stated in the text.

Dodecyl Sulfate Gel Electrophoresis and Densitometry. Samples consisting of antigen–antibody precipitates were washed free of salt and adsorbed serum proteins, dried in air, and dissolved by boiling for 2 min in 200 μl of 1% dodecyl sulfate and 1–5 m*M* dithiothreitol, sometimes with 8 *M* urea. Volumetric fractions of the whole precipitate, corresponding to known amounts of myosin or subfragment, were applied to the gels with a 100-μl Hamilton syringe. The reference sample of unmodified enzyme was dialyzed against 10 m*M* sodium phosphate (pH 7) (+0.4 *M* NaCl for myosin) before an extinction-based concentration measurement. In the case of myosin, determination of the final protein concentration was complicated by the need to remove NaCl by dialysis before electrophoresis. This problem was overcome by making transfers to and from the dialysis bag quantitatively.

Electrophoresis was carried out on 4.5% (heavy chains), 9% (myosin and HMM S-1), or 12% (HMM) polyacrylamide gels as described in the preceding paper (Holt and Lowey, 1975). After destaining, the gels were stored in the dark in 10% acetic acid for 2 days prior to scanning on a Joyce-Loebl microdensitometer. Several gels with different loads were scanned for each protein sample to ensure that conclusions were drawn from the linear range of protein concentration vs. stain intensity. The scans were repeated 2–3 days later, the gels being stored in the meantime in the dark in 10% acetic acid. No appreciable fading of the stained protein bands occurred under these conditions. Areas were determined from the gel traces by means of a planimeter.

Protein Concentrations. These were based on extinction measurements at 280 nm (278 nm for IgG and Fab) corrected for a small amount of scattering by subtraction of the extinction at 340 nm. The coefficients used for a 10-mg/ml solution were: myosin, 5.0; HMM, 6.0; HMM S-1, 8.0; IgG, 14.0; Fab, 17.0; alkali 1, 1.3; alkali 2, 1.9; DTNB l.c., 6.0; subfragment total l.c., 3.5 (an approximate value).

Results

1. Immunological Characterization. Double Diffusion. The reaction of myosin and its subfragments with antisera specific for light chains was investigated by double diffusion in agar. Figure 1a–c shows that all enzyme species (myosin, HMM, and HMM S-1) gave precipitin lines when diffused against antiserum to DTNB l.c. These lines fused both with each other and with those of light chains isolated from the various species (Figure 1d–f). No major antigenic differ-

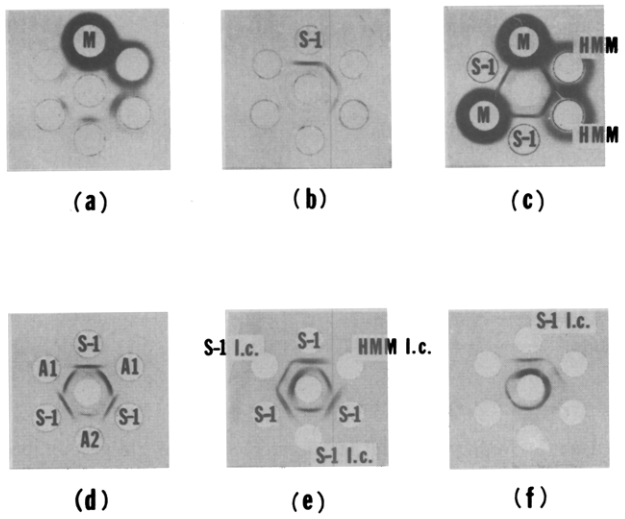

FIGURE 2: Immunodiffusion of myosin and subfragments with antiserum to alkali 1. (a) Twofold serial dilution of myosin (10 mg/ml). (b) Twofold serial dilution of HMM S-1 (4 mg/ml); (c–e) Tests of cross-reactivity; concentrations were: myosin, 10 mg/ml; HMM, 7 mg/ml; HMM S-1, 4 mg/ml; alkali 1, 0.05 mg/ml; alkali 2, 0.01 mg/ml; S-1 l.c. in (e), ~1 mg/ml at top left, and ~0.05 mg/ml at bottom center; HMM l.c., ~0.04 mg/ml. (f) Twofold serial dilution of S-1 l.c. from ~1 mg/ml. The gels are shown after staining for protein because the original patterns, particularly for anti-alkali 1, were sometimes so faint that they could not be photographically reproduced. Examination of the unstained gels showed that the precipitin band for myosin, which is obscured in the stained gel (Figure 2c), fused completely with the bands due to HMM and HMM S-1.

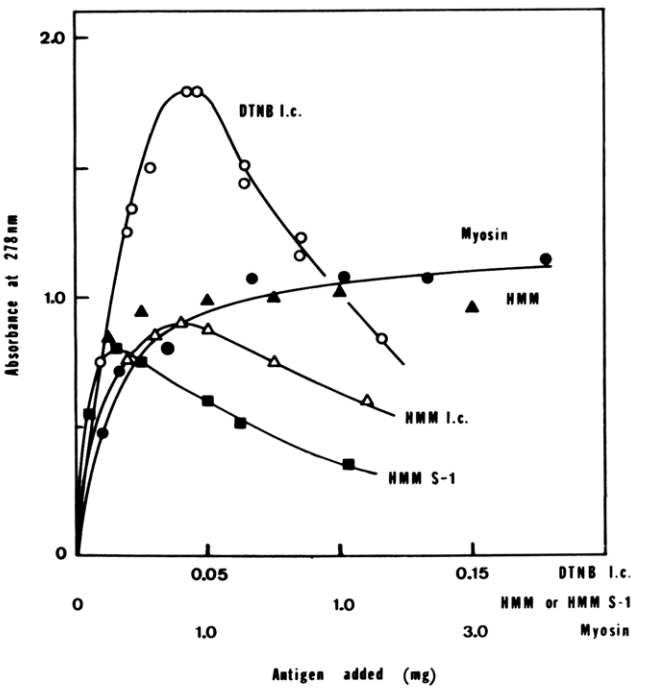

FIGURE 3: Precipitin analysis of anti-DTNB l.c. serum vs. DTNB l.c. (○), myosin (●), HMM (▲), HMM S-1 (■), and total light chains isolated from HMM (HMM l.c., △). The abscissa scale for HMM l.c. is arbitrary, since the concentration of DTNB l.c. is unknown.

ences were therefore apparent between DTNB l.c. in the isolated state and when associated with myosin and its subfragments. Since the light chain preparation derived from subfragments is a mixture of all three light chains plus some heavy chain material, a single precipitin band is strong evidence for the immunological homogeneity of antiserum to DTNB l.c. (Figure 1e).

Reactions of myosin and its subfragments with anti-alkali 1 serum differed from those with DTNB l.c. antiserum in that they were weaker and localized closer to the antigen wells (Figure 2a–c). Furthermore, the type of reaction depended strongly on antigen concentration. At high concentrations (4–10 mg/ml), the precipitin lines due to myosin, HMM, and HMM S-1 all fused (Figure 2c). Light chains derived from HMM S-1 showed two lines at the highest antigen concentration (~1 mg/ml), the outermost line receding into the antigen well on serial dilution (Figure 2f). When HMM S-1 and isolated light chains were tested for cross-reactivity two effects were observed The sharp outer line of HMM S-1 fused only with the faint outer line of the light chain preparation from HMM S-1, and not with the major, inner line (Figure 2e). A diffuse, inner band of HMM S-1 did fuse with light chains present at a sufficiently low concentration (~0.05 mg/ml) to feature only the inner line (Figure 2e). The outer precipitin line of HMM S-1 did not fuse with alkali 1 (Figure 2d). A similar series of diffusion patterns was obtained for the cross-reaction of HMM with alkali 1 and subfragment light chains. These results imply that the principal reaction of HMM S-1 (myosin or HMM) in double diffusion is with a minor component of the antiserum against alkali 1. It is not clear whether this heterogeneity in the antiserum arises from a nonlight chain contaminant in the immunogen or whether the immunogen can exist in two or more conformational states, each giving rise to a distinct population of antibodies (Sachs et al., 1972; Furie et al., 1974).

Quantitative Precipitin Analysis. The precipitation of myosin, HMM, and HMM S-1 with antiserum to DTNB l.c. is shown in Figure 3. The equivalence zone is considerably broader for both myosin and its subfragments than for the immunogen, DTNB l.c. This may reflect, especially for the proteolytic subfragments, a population of molecules heterogeneous in their affinity for antibody, each making a contribution to the total precipitation envelope. The explanation for the plateau in the precipitin curve for myosin is less apparent; possibly the cause lies in the tendency of myosin to aggregate in the solvents routinely used in the immunological tests. However, neither the addition of 50 m*M* phosphate (known for its solvating effect on myosin; Lowey and Holtzer, 1959) nor a fivefold decrease in myosin concentration, achieved by diluting immune serum with nonimmune serum, had any effect on the shape of the precipitin curve. Preliminary measurements on myosin purified by ion-exchange chromatography (Richards et al., 1967) did give some indication of a more normal curve in antigen excess.

Neither myosin nor its subfragments, nor the light chains isolated from the subfragments, precipitated more than 50–70% of the specific antibody to the DTNB l.c. immunogen. This could mean that some antibodies react with myosin without causing precipitation; alternatively, some of the loss in reactivity of the subfragments and the light chains isolated from them could be a consequence of proteolysis. Unfractionated papain digests of myosin were more effective as inhibitors of the antibody–antigen reaction than in causing precipitation of antibody.

Antiserum to alkali 1 exhibited virtually no precipitin reaction with either myosin or its subfragments (Figure 4). This is consistent with the relatively faint reaction seen in double diffusion between the subfragments and antiserum to alkali 1 (Figure 2). Light chains were undoubtedly

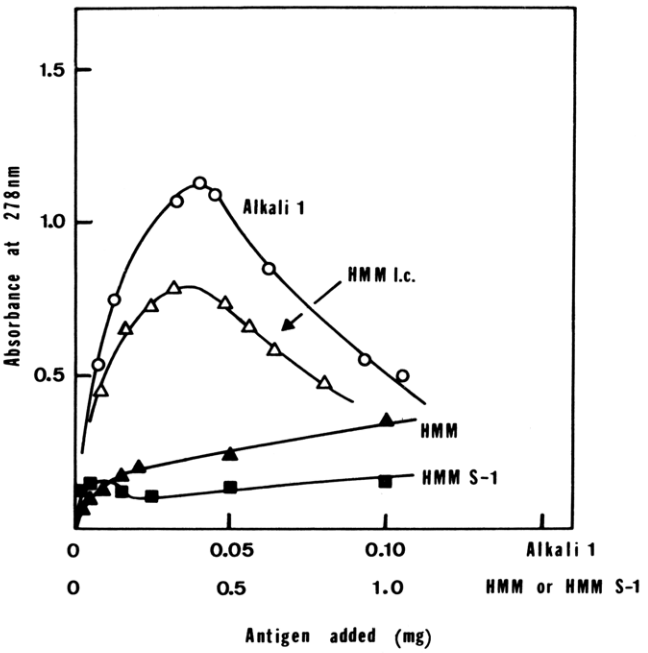

FIGURE 4: Precipitin analysis of anti-alkali 1 serum vs. alkali 1 (○), HMM (●), HMM S-1 (■), and HMM l.c. (△) (see legend to Figure 3).

present in the subfragments since total light chain preparations isolated from HMM or HMM S-1 were able to precipitate about 75% of the specific antibody present, as is shown in Figure 4. The very low affinity of HMM and HMM S-1 for antibodies to alkali l.c. was further demonstrated by their inability, even at very high concentrations, to inhibit the reaction of isolated alkali 1 with homologous antiserum by more than 20%.

2. Analysis of Antigen–Antibody Reaction. The nature of the precipitation reaction between myosin and its subfragments and light chain antisera was studied in two ways. The composition of the antigen–antibody precipitate was examined by dodecyl sulfate gel electrophoresis, while the supernatant remaining after precipitation was characterized by measuring ATPase activities. Experimentally, myosin, HMM, or HMM S-1 was mixed with a large excess of immune serum, and allowed to stand 70–120 hr at 4°. Controls consisted of the same amount of enzyme incubated in nonimmune serum or in immune serum absorbed with an excess of light chain immunogen. A long time period was required for reactions with DTNB l.c. antiserum to be complete, in terms of both light chain and IgG content of the antigen–antibody precipitate. Activity changes were complete after a much shorter time, certainly <40 hr in anti-DTNB l.c. serum, and <1 hr in anti-alkali 1 serum, where essentially no precipitation occurred (J. C. Holt and S. Lowey, unpublished results).

Composition of Precipitates. Dodecyl sulfate gels of myosin, HMM, and HMM S-1, before and after exposure to antiserum, are shown in Figure 5. The left-hand gel of each pair represents a known amount of untreated enzyme species; the right-hand gel shows the precipitate which resulted when the same amount of enzyme was reacted with antiserum specific for DTNB l.c. In addition to the DTNB l.c. are bands related to the subunits of IgG and the proteins of the complement system. The actual amount of DTNB l.c. in the precipitate was estimated by densitometry of matched pairs of stained 10% dodecyl sulfate gels (Figure

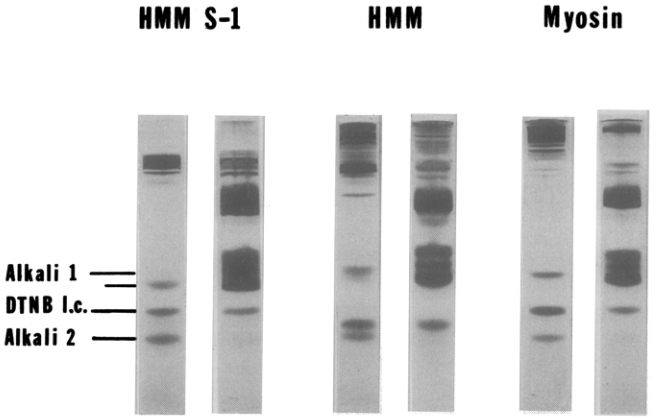

FIGURE 5: Dodecyl sulfate gel electrophoresis of antigen–antibody precipitates from anti-DTNB l.c. serum. The left-hand gel of each pair represents untreated enzyme (myosin, 56 μg; HMM, 47 μg; HMM S-1, 30 μg). The right-hand gel shows the antigen–antibody precipitate which resulted from interaction of this amount of myosin or subfragment with an excess of DTNB l.c. antiserum. All are 9% gels.

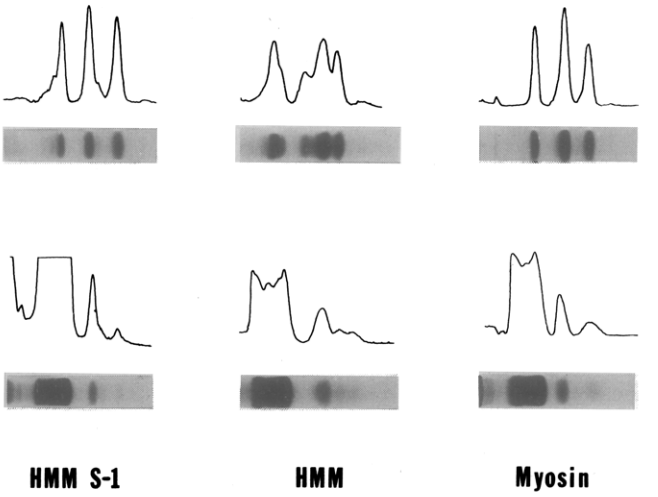

FIGURE 6: Densitometry of antigen–antibody precipitates from DTNB l.c. antiserum. The upper gel and matching densitometer trace of each pair represent a known amount of untreated enzyme: 28 μg of HMM S-1 (10% gel), 37 μg of HMM (12% gel), and 42 μg of myosin (9% gel). Only the light chain region of the gels is shown, with migration from left to right. The lower gel and trace show the antigen–antibody precipitate which resulted from the reaction of a similar amount of enzyme (25 μg of HMM S-1, 38 μg of HMM, and 41 μg of myosin) with antiserum to DTNB l.c. These particular gels gave 55% dissociation of light chain for HMM S-1, 45% for HMM, and 38% for myosin.

6). Results from five experiments for each enzyme species expressed as fraction of DTNB l.c. in the precipitate relative to original concentration were as follows: HMM S-1, 45 ± 5%; HMM or myosin, 47 ● 9%. Densitometry of 5% dodecyl sulfate gels showed that the heavy chain material in the precipitate was 5–15% of that originally mixed with antiserum. (Visual examination of the 10% gels in Figure 5 implies that little heavy chain material was precipitated by antibody.) These data show that a selective dissociation of DTNB l.c. took place in the presence of homologous antibody.

It must be recognized that the figures for dissociated light chain are only approximate. The presence of heavy chain material implies either 5–15% of the light chain was precipitated as whole enzyme or, alternatively, that heavy chains are less stable after removal of light chains, and more inclined toward spontaneous precipitation. Further-

Table I: Activity of HMM S-1 Treated with Light Chain Antisera[a] (μmol of P_i per min per mg).

	Non-immune Serum	anti-DTNB l.c.	anti-Alkali 1	anti-Alkali 2
Ca^{2+}-ATPase	0.93	0.86	0.94	0.94
EDTA-ATPase	4.6	4.2	3.9	4.8
Actin-activated ATPase	8.2	6.7	2.7	4.2

[a] 60 μg of HMM S-1 was incubated 41 hr at 4° with 1 ml of each serum in 0.3 *M* KCl–0.01 *M* Tris-HCl (pH 8.4). Ca^{2+}- and EDTA-ATPase activities were measured directly on the supernatant isolated after this period whereas actin activation was measured after removal of salt by dialysis into 10 m*M* Tris-HCl (pH 8.4) at 4° (see Materials and Methods). Actin concentration was 4.0 mg/ml. In absorbed specific antisera, all activities were the same as in nonimmune serum. Identical results were obtained at a lower antibody excess where 102 μg of HMM S-1 was mixed with 1 ml of serum. The titer of specific antibody in the sera was estimated by quantitative precipitin analysis to be 0.8 mg/ml (alkali 1 and 2) to 1.3 mg/ml (DTNB l.c.).

Table II: Activity of HMM Treated with Light Chain Antisera[a] (μmol of P_i per min per mg).

	Non-immune Serum	anti DTNB l.c.	anti-Alkali 1	anti-Alkali 2
Ca^{2+}-ATPase	0.65	0.54	0.76	0.65
EDTA-ATPase	3.60	3.15	3.49	3.83
Actin-activated ATPase	4.80	3.13	1.81	1.25

[a] 130 μg of HMM was incubated 42 hr at 4° with 1 ml of each serum. Other details were as in Table I. Pure actin activation was the same whether assayed in the presence or absence of Ca^{2+}. Similar activities to those shown were observed when the incubation period was increased to 100 hr. In absorbed specific antiserum, all activities were the same as in nonimmune serum.

more, we have not accounted for the low molecular weight material seen migrating ahead of the DTNB l.c.

Dodecyl sulfate gels of the very small precipitate which formed when myosin and its subfragments were incubated with antisera to alkali 1 showed no bands in the region of DTNB l.c. or alkali 2 (Lowey and Holt, 1972). Alkali 1, if present, could not be identified due to comigration with the light chain of IgG. However, since alkali 1 and 2 show considerable cross-reactivity, the total absence of alkali 2 implies that appreciable amounts of alkali 1 are unlikely to be contained in the precipitate.

Activity of Supernatants. Tables I and II show the activities observed for HMM S-1 and HMM, respectively, after treatment with various light chain antisera. For DTNB l.c. antiserum, the Ca^{2+}- and EDTA-ATPases were 80–90% of the control values; these figures approach 100% if allowance is made for the loss of about 10% of the subfragment in the antigen–antibody precipitate. When this precipitate was resuspended in the supernatant, the resulting suspension exhibited 100% of the control ATPase. The activity measurements therefore confirmed the results of gel densitometry in suggesting that no more than 10–15% of the heavy chain was present in the precipitate. For antiserum to alkali 1 or 2, where little or no precipitate formed, the Ca^{2+}- and EDTA-ATPase activities of supernatant and suspension were identical, and equal to the control values (Tables I and II).

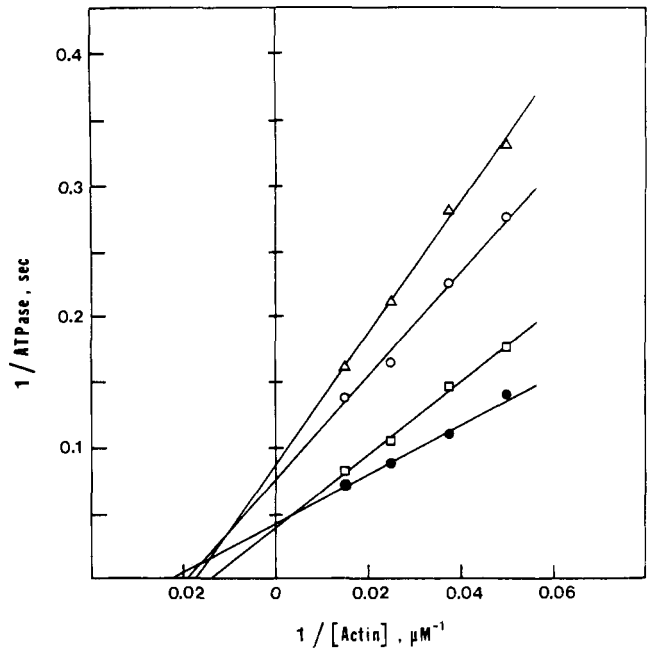

FIGURE 7: Double reciprocal plot of acto-HMM S-1 ATPase vs. actin concentration for HMM S-1 treated with sera specific for alkali 1 (△), alkali 2 (○), and DTNB l.c. (□). The lowest line represents the activity observed in nonimmune serum (●), which was equal to that in absorbed specific antisera; 50 μg of HMM S-1 was added to 0.5 ml of antiserum (titers approximately as in Figure 3 for DTNB l.c., Figure 4 for alkali 1 and 2). After 40 hr at 4°, the suspension was centrifuged and the EDTA-ATPase activity in the supernatant measured. Salt was removed by dialysis against 0.01 m*M* Tris-HCl (pH 8.4); concentration changes were estimated by comparing EDTA-ATPase before and after dialysis.

Differences between HMM S-1 and HMM became more apparent in their actin-activated Mg^{2+}-ATPases in antiserum to DTNB l.c. HMM S-1 was activated by actin to the same extent as the untreated enzyme (Table I). Figure 7 shows the activation of the Mg^{2+}-ATPase as a function of increasing actin concentration. The displacement of the line for treated HMM S-1 from that for the enzyme in nonimmune serum can be explained simply in terms of the small amount of subfragment lost in the antigen–antibody precipitate. HMM treated with antiserum to DTNB l.c. exhibited a significantly lower actin-activated Mg^{2+}-ATPase than the control, even after allowing for the loss of 10% whole subfragment in the precipitate. Activity measurements as a function of actin concentration are shown for several experiments in Figure 8b. At infinite actin concentration, the maximum turnover rate (corresponding to the ordinate intercept) appears to be similar for both treated and control HMM. However, at finite actin concentrations (1–2 mg/ml) the activity of treated HMM was 60–90% of the control value in a series of seven experiments.

In contrast, the actin-activated Mg^{2+}-ATPases in antisera to alkali 1 or 2 were markedly reduced for both HMM S-1 and HMM (Tables I and II). Double reciprocal plots showed clearly that the reduction in activity was due to a decrease in the maximum turnover rate (Figures 7 and 8a). The range of values for activities in the presence of antisera to alkali 1, alkali 2, or a mixture of the two was about 25–45% of the control in a series of seven experiments. The greatest drop in activity corresponded to the greatest excess of antibody over enzyme practically attainable.

Most of the studies involving the effect of antiserum on

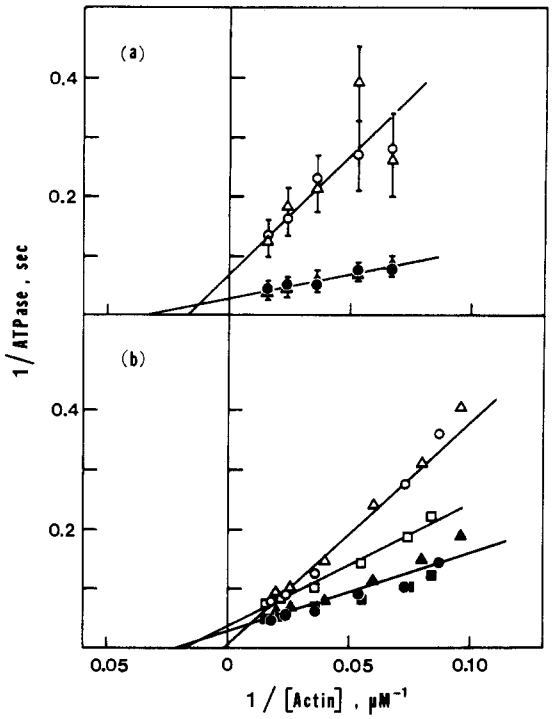

FIGURE 8: Double reciprocal plot of acto-HMM ATPase vs. actin concentration for HMM treated with light chain antisera. (a) HMM in anti-alkali 1 (○) and anti-alkali 2 (△) sera. Filled symbols show the activity in each serum, previously absorbed with an excess of pure light chains; these control activities were experimentally identical with one another and with those measured in nonimmune serum. 35 μg of HMM was incubated 40 hr with 0.5 ml of serum (as in Figure 4) in 0.3 *M* KCl–0.01 *M* Tris-HCl (pH 8.4) at 4°; actin activation was measured after removal of KCl by dialysis. The error bars reflect the uncertainty in determining P_i liberated at these low levels of activity. (b) HMM in anti-DTNB l.c. serum. Two separate experiments are shown. In the first, 50 μg of HMM was incubated 70 hr with 0.5 ml of serum (as in Figure 3) in 0.3 *M* KCl–0.01 *M* Tris-HCl (pH 8.4) at 4°. A supernatant was then isolated and tested for ATPase activity with pure actin (□). In the second experiment, set up in the same way with a different preparation of HMM, measurements were made with both pure (○) and regulated (△) actin. Filled symbols are the same controls as described in (a).

Table III: Calcium Regulation of ATPase Activity[a] (μmol of P_i per min per mg).

	Nonimmune Serum		anti-DTNB l.c. Serum	
	0.1 m*M* $CaCl_2$	1 m*M* EGTA	0.1 m*M* $CaCl_2$	1 m*M* EGTA
HMM S-1	2.36	0.71	1.82	0.55
HMM	1.83	0.58	0.92	0.27
Myosin	0.49	0.06	1.82	0.18

[a] 100 μg of HMM S-1, 120 μg of HMM, and 180 μg of myosin were incubated with 1 ml of serum for 40 hr at 4°. For the subfragments, the enzyme–antiserum supernatant was desalted by dialysis and assayed at 25°, pH 7.9, in 1.2 mg/ml of regulated actin. The myosin-antiserum supernatant was not desalted; measurements were made in 15 m*M* KCl and 0.45 mg/ml of regulated actin at 33°, pH 7.7. The activity of myosin in absorbed antiserum was 1.18 μmol of P_i per min per mg in Ca^{2+} (0.06 in EGTA), intermediate between the values in immune and nonimmune sera.

Table IV: Activity of HMM in Sera and IgG Fractions Specific for Alkali 1[a] (Percentage of Control Activity).

Expt No.	Material	Ca^{2+}-ATPase 30 m*M* KCl	Ca^{2+}-ATPase 225 m*M* KCl	Actin-Activated ATPase (30 m*M* KCl
1	Serum 12 (0.5 mg)	110	125	50
2	Serum 12 (1.0 mg)	118	134	29
3	Antibody 12 (1.1 mg)	116	130	10
4	Antibody 12 (2.1 mg)	116	110	10
5	Serum 14 (0.7 mg)	84	86	41
6	Serum 14 (1.4 mg)	59	64	23
7	IgG 14 (2.4 mg)	73	57	50
8	Fab 14 (11.5 mg)	44	40	81
9	IgG + Fab 14	47	42	72

[a] HMM (100 μg) was mixed with the amount of specific antibody given in parentheses, either directly or in the form of serum or immune IgG, and incubated 1–8 hr at 4° in 0.3 *M* KCl (sera) or 0.13 *M* KCl (otherwise)–10 m*M* Tris-HCl (pH 8.4). The mixture was assayed for activity without removal of any precipitate which formed. The range of absolute activities exhibited by different HMM preparations in nonimmune serum or IgG was (100% values expressed in μmol of P_i per mg per min at 25°): Ca^{2+}-ATPase, 2.0–3.3 (30 m*M* KCl) or 0.9–1.5 (225 m*M* KCl); actin activated ATPase, 2.0–3.0 (2 mg/ml of actin). At 33° (experiments 7, 8, and 9), the activities were about twofold greater. In experiments 7, 8, and 9, the effects of IgG 14, Fab 14, and a combination of the two were tested under identical conditions.

activity were made on the subfragments of myosin rather than on myosin itself. One reason for this choice is that the specific activity of myosin, particularly the actin-activated ATPase, is considerably lower than that of HMM or HMM S-1; thus activity measurements are difficult to make at the low concentrations of enzyme dictated by the requirement of antibody excess over antigen. Another reason was the unusual effect of antiserum on the activity of myosin: while Ca^{2+}- and EDTA-ATPases were experimentally equal to the control values in nonimmune serum (Horváth and Gaetjens, 1972) or absorbed immune serum, the actin-activated ATPase was enhanced rather than inhibited by antiserum to DTNB l.c. Moreover, absorbed immune serum no longer behaved as a nonimmune serum. It appeared that interaction of myosin with anti-DTNB l.c. serum caused an increase in activity even in the presence of a large excess of pure DTNB l.c. These effects were not seen when activity measurements were carried out at pH 7.0 rather than pH 7.9, or when immune IgG was used instead of serum.

3. Effect of DTNB l.c. on Ca Regulation. Since removal of DTNB l.c. had little or no effect on any of the ATPase activities of HMM or HMM S-1, the ability of Ca^{2+} to regulate actin activation through the relaxing proteins was investigated. As shown in Figure 8b, regulated actin in the presence of Ca^{2+}, and pure actin were identical in their activation of treated and untreated HMM. When Ca^{2+} was chelated by EGTA, pure actin activation was unaffected for both enzyme samples whereas activation by regulated actin was greatly reduced (Table III). The relative drop in activity was the same for HMM in immune and nonimmune sera, being a function only of the proportion of the actin which was regulated. Similar results were observed for HMM S-1, HMM, and for myosin. In each case, calcium regulation through the troponin–tropomyosin system was the same for the control as for the enzyme species depleted of DTNB l.c.

The possibility that Ca^{2+} might influence the interaction between HMM and DTNB l.c. was examined by incubating HMM in DTNB l.c. antiserum not only under the standard conditions, but also, in parallel, in 0.1 m*M* $CaCl_2$, 1 m*M* EGTA, and 1 m*M* EDTA. Although light chain dissociation was not quantitated, the fact that all four samples yielded the same amount of antigen–antibody precipitate

argues against a difference in the amount of antigen involved.

4. Origin of Changes in ATPase Activity. The results of activity measurements showed that light chain antisera shared the general property of inhibiting the actin-activated ATPase activity, without affecting greatly Ca^{2+}- or EDTA-ATPases. Similar results were obtained with immune IgG and purified antibody specific for alkali 1 (Table IV) and DTNB l.c. (not shown), indicating that the inhibition was not caused by a nonspecific effect of serum on enzyme. The fact that only actin activation was affected suggests that antibodies bound to the subfragment sterically prevented the binding of actin. Several observations supported this interpretation: when an excess of free light chain was added to absorb the specific antibody, the inhibition of activity was partially reversed. In the case of alkali 1, displacement of bound antibody is the only possible explanation for the recovery of activity; for DTNB l.c., some of which had been removed from the subfragment, a reassociation with heavy chain may also have occurred.

An alternative way to displace bound antibody was to raise the salt concentration used in the assay of actin-activated ATPase activity. The influence of ionic strength on the interaction between HMM and antibodies to alkali 1 was demonstrated by the rapid appearance of a precipitate when these components were mixed in 0.1 *M* as opposed to 0.3 *M* KCl. Because actin activation is reduced with increasing ionic strength (Eisenberg and Moos, 1968), it was difficult to measure ATPase activity under these conditions. Nevertheless, the indications were that the antibody-induced inhibition was reduced at 100–200 m*M* KCl. This raised the possibility that Ca^{2+}- and EDTA-ATPases were constant simply because, at the high ionic strength used for these assays (225 and 600 m*M* KCl, respectively), little or no interaction with antibody occurred.[3] Parallel measurements of Ca^{2+}-ATPase carried out at 30 and 225 m*M* KCl showed that this was not the case (Table IV). Under identical assay conditions light chain antibodies inhibited activation by actin but not the hydrolysis of Ca^{2+}-ATP.

In order to determine more directly whether inhibition by antibody represented a specific modification of the actin-binding site, or a general exclusion of actin from an antibody-linked aggregate, ATPase measurements were made in the presence of the univalent antibody fragment, Fab. Not only is this fragment smaller than an IgG molecule in terms of steric hindrance, but it is also incapable of producing any lattice formation by virtue of its valency. Since initial experiments with Fab revealed little effect on the actin-activated ATPase, it was necessary to demonstrate that it was indeed binding to HMM. Table IV, experiments 7 and 8, shows the activities observed in the presence of IgG and Fab, respectively; experiment 9 shows the activity in a mixture containing the same amounts of IgG and Fab which had been tested individually. Since the inhibition seen with IgG alone occurred to a lesser extent in the mixture, Fab was apparently able to compete successfully with IgG for antigenic sites on HMM. While Fab produced little change in the actin-activated ATPase of HMM, the Ca^{2+}-ATPase was for the first time appreciably affected. This observation does not necessarily conflict with earlier results on the constancy of the Ca^{2+}-ATPase, since the very high excesses of Fab used represent significantly different experimental conditions.

[3] Preliminary gel filtration experiments using Sepharose 4B demonstrate that interaction does in fact occur in both 0.3 and 0.6 *M* KCl. When HMM was mixed with only one-third as much specific antibody as was used for studies of ATPase activity, complexes containing 1–5 mol of antibody/mol of HMM were found. In 0.3 *M* KCl, sedimentation velocity experiments suggested that essentially all of the HMM in such a mixture with specific antibody was present as heterogeneous high molecular weight material.

Discussion

Immunology. Antisera were prepared by immunization with light chains which had been isolated from myosin and fractionated by ion-exchange chromatography (Holt and Lowey, 1975). In myosin and its subfragments, however, the light chains may exist in a different conformation as a consequence of their association with heavy chains. Such a difference between free and bound light chains is one explanation for the relative lack of reactivity observed with myosin and anti-alkali l.c. sera. Alternatively, these light chains may be largely buried in myosin so that they are inaccessible to IgG. Free and bound DTNB l.c., on the other hand, show extensive cross-reactivity, both by immunodiffusion and quantitative precipitin analysis. The conformations of DTNB l.c. in the two states appear therefore to be very similar.

The reaction with DTNB l.c. antiserum was of particular interest because it resulted in the selective dissociation of much of this light chain from myosin as well as the subfragments. A finding of this kind was implied by the work of Lowey and Steiner (1972) with antiserum to a mixture of light chains: a precipitate formed when HMM S-1 was mixed with antiserum, but ATPase activity remained in the supernatant, suggesting that the light chain had been separated from the hydrolytic site located primarily on the heavy chains. Moreover, immunodiffusion of myosin and anti-myosin serum yielded several precipitin lines, which were shown to include the separate reactions of light and heavy chains (Lowey and Steiner, 1972). The use of antisera to individual light chains, and analysis of the antigen–antibody precipitate on dodecyl sulfate gels has allowed us to show directly that DTNB l.c. dissociates in the presence of homologous antiserum, whereas alkali l.c. do not. To our knowledge, the dissociation of one subunit from a multisubunit enzyme by antibodies to the subunit has not previously been described. The closest analogy appears to be with removal of the prosthetic heme group from myoglobin by antibodies to apomyoglobin (Crumpton, 1966). An example in which true subunits are involved concerns the heavy and light chain components of Fab. Antibodies to the light chain reacted only with free light chain, and were dissociated from the immune precipitate by addition of heavy chain (Yagi et al., 1968).

Release of DTNB l.c. by so mild a treatment as interaction with specific antibody suggests that its association with heavy chain is weak. If one takes a dynamic view of protein conformation, free and bound DTNB l.c. exist in equilibrium with one another, and the reaction of antibody is with free light chain. Dissociation then proceeds as the equilibrium concentration of free light chain is maintained at the expense of bound light chain. A model of conformational equilibria in protein antigens has been developed by Anfinsen and his colleagues (Sachs et al., 1972a,b), based on their immunological studies of staphylococcal nuclease. Antibodies to native nuclease reacted with isolated fragments whose average conformations were known to be different from homologous regions of the native molecule; similarly, antibodies to the isolated fragments were capable of binding

to native nuclease (Furie et al., 1974). These cross-reactions were interpreted in terms of a conformational equilibrium in the antigen so that native nuclease could spontaneously generate a determinant characteristic of the isolated fragment, and vice versa.

An alternative interpretation, which is operationally hard to distinguish, is that antibody to one conformational state (free light chain) is capable of binding with lower affinity to a similar, though nonidentical, structure (bound light chain). As a consequence of this initial reaction, the antigen refolds to yield the precise conformation against which the antibody was elicited. In this case, dissociation results from a conformational change actively induced by specific antibody.

The amount of DTNB l.c. dissociated by antibody was ~50% for all the enzyme species. Our preliminary report (Lowey and Holt, 1972) that all of this light chain was dissociated from HMM S-1 could not be confirmed in these more extensive experiments. Recent studies with the thiol reagents DTNB and *p*-chloromercuribenzoate have also demonstrated about 50% dissociation of DTNB l.c. from HMM or myosin (Hayashi et al., 1973; Kendrick-Jones, 1974; A. G. Weeds, personal communication). In molluscan myosin, moreover, only 1 mol out of 2 mol of EDTA l.c./mol of myosin can be liberated with EDTA (J. Kendrick-Jones, E. M. Szentkiralyi, and A. G. Szent-Györgyi, personal communication). Specific antibody therefore parallels the action of selective chemical dissociating agents, which produce only partial release of DTNB l.c. or EDTA l.c. The mechanism of dissociation is not necessarily the same in the two cases; while antibody must produce its effect by direct interaction with the light chain, DTNB or EDTA may cause dissociation by modifying the heavy chain structure. An example of this is found in scallop myosin: DTNB is capable of releasing (irreversibly) EDTA l.c. even though this light chain contains no sulfhydryl groups (Szent-Györgyi et al., 1973).

No immunological evidence could be found for different species of DTNB l.c. so it does not seem that the light chain which failed to dissociate was intrinsically different from that which did. Similarly, experiments with light chains released almost quantitatively from myosin with 5 *M* guanidine minimize the possibility that the antisera lacked altogether antibodies reactive with a class of DTNB l.c. (Holt and Lowey, 1975). Indeed, the presence of antibodies bound to undissociated DTNB l.c. in the treated HMM was implied by activity measurements (see below). The chemical identity of dissociable and nondissociable DTNB l.c. has been inferred from amino acid composition data (Hayashi et al., 1973). Both immunological and chemical methods suggest, therefore, that a single species of DTNB l.c. can exist in either the free or bound state. Preliminary evidence for interconversion between the two states has come from experiments in which DTNB-treated myosin was mixed with labeled, free DTNB l.c. and subjected to a second reaction with DTNB. The light chain which remained bound after the second reaction consisted of both labeled and unlabeled species (J. Kendrick-Jones, personal communication). The fact that only one of the 2 mol of DTNB l.c. present in myosin or HMM can be dissociated under mild conditions suggests that the remaining light chain is bound more strongly. The most simple explanation for this is different heavy chain binding sites for the two light chains.

Enzyme Activity. Interaction of enzymes with specific antibody may result in either an increase or a decrease in the activity of the enzyme. The former implies that a conformational change has occurred, whereas a loss of activity may result simply from the presence of bound antibody in the active site (Celada and Strom, 1972). Ribonuclease exhibited both effects, depending on the predominant antibody population in a particular serum (Cinader, 1967). Inactive forms of β-galactosidase (Rotman and Celada, 1968; Messer and Melchers, 1970) and penicillinase (Pollock, 1964) isolated from mutant bacteria were found to become active in the presence of antibodies to the wild-type enzyme. If, however, binding occurs in or near the active site, inhibition of activity must be expected. Such is the case with antibodies to staphylococcal nuclease (Sachs et al., 1972b) and, indeed, antibodies to myosin or HMM S-1 abolish activity (Lowey and Steiner, 1972). Our results show that, in the case of myosin, the fraction of the antibody population responsible for inhibition is not that directed toward light chains.

The ability of HMM S-1 to hydrolyze ATP was unaffected by removal of DTNB l.c. This subfragment contains only half as much DTNB l.c. per mole active sites as myosin, probably due to proteolysis by papain (Weeds and Lowey, 1971; densitometry of dodecyl sulfate gels also supports this conclusion). Thus, the antibody-treated HMM S-1 contains not more than 25% of the DTNB l.c. originally present in myosin. A similar result has been reported by Kendrick-Jones (1974), who prepared HMM S-1 from DTNB-treated myosin and found full actin-activated ATPase despite the absence of DTNB l.c. from the dodecyl sulfate gel pattern of the subfragment.

Little DTNB l.c. is lost when HMM is prepared by tryptic digestion of myosin (Weeds and Lowey, 1971; Hayashi et al., 1973), although most of this light chain in HMM has a slightly greater mobility on dodecyl sulfate gels than it does in myosin (Figure 5). HMM from which about half the DTNB l.c. had been dissociated by specific antibody exhibited the same Ca^{2+}- and EDTA-activated ATPase activities as the untreated enzyme; activation by actin was, however, decreased by 10–40%. The likeliest explanation for this loss in activity is that antibody molecules were bound to the fraction of DTNB l.c. which did not dissociate, and interfered with actin binding. The constancy of the actin-activated ATPase of HMM S-1 may reflect the fact that for this subfragment only 25% of the DTNB l.c. remained. Even when 50% of the light chain was available to bind antibody, as in HMM, the decrease in activity was not always significant.

Alkali 1 or 2 antisera produced no change in Ca^{2+}- or EDTA-activated ATPase activities of either subfragment. The very small precipitate which formed during incubation in these sera, and which was removed before activity measurements were made, could not therefore have been active enzyme. The inhibition of actin-activated ATPase activity consistently observed in these sera again suggests that IgG bound to alkali l.c., none of which dissociated, sterically prevented actin binding. The relatively small effect of monovalent Fab, specific for alkali 1, shows that the light chain need not be located in the actin binding site itself. While this possibility cannot be ruled out (actin might displace the more weakly binding Fab, but not IgG), the results are more suggestive of a cross-linked network, which excludes actin nonspecifically, as it would any macromolecule.

The loss of Ca^{2+}-ATPase observed at very high excesses of Fab does not necessarily result from an interaction with

antibodies to alkali 1. The double diffusion reactions of this antiserum unexpectedly revealed two precipitin lines (Figure 2). This observation implies that the principal immunodiffusion reaction of myosin and its subfragments is with a minor component of the antiserum to alkali 1, perhaps directed toward the heavy chains. The possibility that such antibodies were present raises a question about the significance of activity measurements in anti-alkali 1 serum. Two lines of evidence suggest that the effects seen were due to the major population of anti-alkali 1 antibodies rather than to a lesser population of heavy chain antibodies. First, anti-alkali 2 produced the same effects on ATPase activity but showed no evidence of the second immunodiffusion reaction (J. C. Holt and S. Lowey, unpublished results). Second, antibody purified from anti-alkali 1 serum duplicated the effect of the serum on ATPase activity (Table IV) whereas the double precipitin reaction was observed only with whole serum. Altogether, antibodies or antisera which depended on 5 *M* Gdn·HCl-dissociated light chains (as opposed to those isolated with 4 *M* urea or alkali), whether at the immunization or immunoadsorption stage, seemed not to show the outer precipitin line. In view of the possibility that contaminating antibodies were present, as well as the preliminary nature of the experiments with Fab, the conclusion that alkali l.c. are not located in the actin binding site must be considered tentative.

Implications for the Function of Light Chains. Calcium regulation of vertebrate actomyosin by the troponin-tropomyosin complex was not altered when about 50% of the DTNB l.c. was dissociated by specific antibody. Both myosin and the subfragments, moreover, retained full Ca^{2+}- and EDTA-ATPase activity. The recent conclusion (Werber and Oplatka, 1974) that DTNB-treated myosin is less sensitive to Ca^{2+} regulation by troponin-tropomyosin than the untreated control is weakened by the considerable loss of EDTA- as well as actin-activated ATPase activity which occurred in their experiments. The implication is that treatment with DTNB not only released the light chain but also denatured the myosin (cf. Léger and Marotte, 1975). This is known to be the case with other myosins, such as lobster or scallop, where DTNB causes preferential dissociation of one light chain component; the loss of activity is complete and irreversible (Szent-Györgyi et al., 1973; Regenstein and Lowey, 1974).

In a number of invertebrates, regulation of the actin-activated ATPase is mediated through the light chains of myosin, either exclusively, or in concert with the actin-linked regulation found in vertebrate muscle (Lehman and Szent-Györgyi, 1975). Scallop myosin, for example, binds Ca^{2+} with high affinity (10^{-6}–10^{-7} *M* free Ca^{2+}), comparable to that of troponin in thin filament regulation. Vertebrate myosin, however, binds Ca^{2+} only at a higher concentration, 10^{-5} *M* (Bremel and Weber, 1975). Although this binding may well involve DTNB l.c. (Morimoto and Harrington, 1974), the difference in pCa between the two systems argues against a functional identity.

The binding of Ca^{2+} to vertebrate myosin is accompanied by small changes in the hydrodynamic properties of myosin filaments (Morimoto and Harrington, 1974) and in the tryptophan fluorescence of free DTNB l.c. (Werber et al., 1972). Haselgrove (1975), moreover, has reported that activation of muscle causes an initial movement of the cross-bridges which is independent of a direct interaction with actin. While DTNB l.c. does not appear to play a primary role in the calcium sensitivity of vertebrate muscle, it may be the site of a direct influence of Ca^{2+} on thick filaments (Miller and Tregear, 1970; Huxley, 1972; Margossian and Lowey, 1975). The picture has been further complicated by the recent suggestion, based on studies of sequence homologies, that alkali l.c. may also be capable of binding calcium (Collins, 1974; Weeds and McLachlan, 1974). Our activity measurements imply that none of the light chains is an integral part of the hydrolytic site. While there is ample evidence that antibodies bind to light chains in the subfragments, only under the most extreme conditions of antibody excess is the hydrolysis of ATP affected directly. This is in contrast to anti-myosin serum, which abolishes ATPase activity. Preliminary results with Fab, and the constancy of acto-HMM S-1 ATPase, respectively, suggest that neither alkali nor DTNB l.c. are present in the actin binding site. Taken together, therefore, the evidence favors a regulatory function for the light chains rather than their direct involvement in hydrolysis or actin binding.

Acknowledgments

We thank Ms. Angela Holt for careful technical assistance and Dr. Lisa Steiner for many helpful discussions.

References

Asakura, S. (1961), *Arch. Biochem. Biophys. 92,* 140.
Bremel, R. D., and Weber, A. (1975), *Biochim. Biophys. Acta 376,* 366.
Celada, F., and Strom, R. (1972), *Q. Rev. Biophys. 5,* 395.
Cinader, B. (1967), in Antibodies to Biologically Active Molecules, Cinader, B., Ed., Oxford, Pergamon Press, p 85.
Close, R. (1969), *J. Physiol. (London) 204,* 331.
Collins, J. H. (1974), *Biochem. Biophys. Res. Commun. 58,* 301.
Crumpton, M. J. (1966), *Biochem. J. 100,* 223.
Dreizen, P., and Gershman, L. C. (1970), *Biochemistry 9,* 1688.
Ebashi, S., and Ebashi, F. (1964), *J. Biochem. (Tokyo) 55,* 604.
Eisenberg, E., and Moos, C. (1968), *Biochemistry 7,* 1486.
Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971), *Biochemistry 10,* 2606.
Fiske, C. H., and Subbarow, Y. (1925), *J. Biol. Chem. 66,* 375.
Furie, B., Schechter, A. N., Sachs, D. H., and Anfinsen, C. B. (1974), *Biochemistry 13,* 1561.
Gazith, J., Himmelfarb, S., and Harrington, W. F. (1970), *J. Biol. Chem. 245,* 15.
Gershman, L. C., and Dreizen, P. (1970), *Biochemistry 9,* 1677.
Gershman, L. C., Dreizen, P., and Stracher, A. (1966), *Proc. Natl. Acad. Sci. U.S.A. 56,* 966.
Gershman, L. C., Stracher, A., and Dreizen, P. (1969), *J. Biol. Chem. 244,* 2726.
Haselgrove, J. C. (1975), *J. Mol. Biol. 92,* 113.
Hayashi, Y., Takenaka, H., and Tonomura, Y. (1973), *J. Biochem. (Tokyo) 74,* 1031.
Holt, J. C., and Lowey, S. (1975), *Biochemistry,* preceding paper in this issue.
Horváth, B., and Gaetjens, E. (1972), *Biochim. Biophys. Acta 263,* 779.
Huxley, H. E. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37,* 361.
Kendrick-Jones, J. (1974), *Nature (London) 249,* 631.

Kendrick-Jones, J., Lehman, W., and Szent-Györgyi, A. G. (1970), *J. Mol. Biol. 54,* 313.
Kendrick-Jones, J., Szentkiralyi, E. M., and Szent-Györgyi, A. G. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37,* 47.
Kim, H. D., and Mommaerts, W. F. H. M. (1971), *Biochim. Biophys. Acta 245,* 230.
Kominz, D. R., Carroll, W. R., Smith, E. N., and Mitchell, E. R. (1959), *Arch. Biochem. Biophys. 79,* 191.
Léger, J. J., and Marotte, F. (1975), *FEBS Lett. 52,* 17.
Lehman, W., Kendrick-Jones, J., and Szent-Györgyi, A. G. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37,* 319.
Lehman, W., and Szent-Györgyi, A. G. (1975), *J. Gen. Physiol. 66,* 1.
Lowey, S., and Holt, J. C. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37,* 19.
Lowey, S., and Holtzer, A. (1959), *J. Am. Chem. Soc. 81,* 1378.
Lowey, S., and Risby D. (1971), *Nature (London) 234,* 81.
Lowey, S., Slayter, H. S., Weeds, A. G., and Baker, H. (1969), *J. Mol. Biol. 42,* 1.
Lowey, S., and Steiner, L. A. (1972), *J. Mol. Biol. 65,* 111.
Margossian, S. S., and Lowey, S. (1973a), *J. Mol. Biol. 74,* 301.
Margossian, S. S., and Lowey, S. (1973b), *J. Mol. Biol. 74,* 313.
Margossian, S. S., and Lowey, S. (1975), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34,* 671.
Messer, W., and Melchers, F. (1970), in The Lactose Operon, Beckwith, J. R., and Zipser, D., Ed., Cold Spring Harbor Monographs, p 305.
Miller, A., and Tregear, R. T. (1970), in Contractility of Muscle Cells and Related Processes, Podolsky, R. J., Ed., Englewood Cliffs, N.J., Prentice-Hall, p 205.
Morimoto, K., and Harrington, W. F. (1974), *J. Mol. Biol. 88,* 693.
Omenn, G. S., Ontjes, D. A., and Anfinsen, C. B. (1970), *Nature (London) 225,* 189.
Pollock, M. R. (1964), *Immunology 7,* 707.
Porter, R. R. (1959), *Biochem. J. 73,* 119.
Regenstein, J. M., and Lowey, S. (1974), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33,* 1581.
Richards, E. G., Chung, C.-S., Menzel, D. B., and Olcott, H. S. (1967), *Biochemistry 6,* 528.
Rotman, M. B., and Celada, F. (1968), *Proc. Natl. Acad. Sci. U.S.A. 60,* 660.
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), *Biochemistry 11,* 4268.
Sarkar, S., Sréter, F. A., and Gergely, J. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 946.
Singer, S. J. (1967), *Adv. Protein Chem. 22,* 1.
Sober, H. A., and Peterson, E. A. (1958), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 17,* 1116.
Spudich, J. A., and Watt, S. (1971), *J. Biol. Chem. 246,* 4866.
Sréter, F. A., Gergely, J., and Luff, A. L. (1974), *Biochem. Biophys. Res. Commun. 56,* 84.
Sréter, F. A., Gergely, J., Salmons, S., and Romanul, F. (1973), *Nature (London), New Biol. 241,* 17.
Stracher, A. (1969), *Biochem. Biophys. Res. Commun. 35,* 519.
Szent-Györgyi, A. G., Szentkiralyi, E. M., and Kendrick-Jones, J. (1973), *J. Mol. Biol. 74,* 179.
Wagner, P. D., and Yount, R. G. (1975), *Biochemistry 14,* 1908.
Weber, A., and Bremel, R. D. (1971), in Contractility of Muscle Cells and Related Processes, Podolsky, R. J., Ed., Englewood Cliffs, N.J., Prentice-Hall, p 37.
Weber, A., and Murray, J. M. (1973), *Physiol. Rev. 53,* 612.
Weeds, A. G. (1969), *Nature (London) 223,* 1362.
Weeds, A. G., and Lowey, S. (1971), *J. Mol. Biol. 61,* 701.
Weeds, A. G., and McLachlan, A. D. (1974), *Nature (London) 252,* 646.
Weeds, A. G., Trentham, D. R., Kean, C. J. C., and Buller, A. J. (1974), *Nature (London) 247,* 135.
Werber, M. M., Gaffin, S. L., and Oplatka, A. (1972), *J. Mechanochem. Cell Motil. 1,* 91.
Werber, M. M., and Oplatka, A. (1974), *Biochem. Biophys. Res. Commun. 57,* 823.
Yagi, Y., Rutishauser, U. S., and Pressman, D. (1968), *Immunochemistry 5,* 67.