Epstein, B. N. (1955), *J. Polym. Sci. 16*, 517.
Tanford, C., Kawahara, K., and Lapanej, S. (1966), *J. Biol. Chem. 241*, 1921.
Tanford, C., Kawahara, K., and Lapanje, S. (1967a), *J. Am. Chem. Soc. 89*, 729.
Tanford, C., Kawahara, K., Lapanje, S., Hooker, Jr., T., Zarlengo, M., Salahuddin, A., Aune, K., and Takagi, T. (1967b), *J. Am. Chem. Soc. 89*, 5023.
Volkenstein, M. V. (1963), Configurational Statistics of Polymeric Chains, New York, N. Y., Interscience.

The Substructure of the Myosin Molecule. Production and Properties of the Alkali Subunits*

Dixie W. Frederiksen and Alfred Holtzer

ABSTRACT: The reaction that produces macromolecular fragments from myosin at moderately high pH (~11.0) has been studied in some detail. The fragments so produced have been separated and studied by means of light scattering, viscometry, optical rotatory dispersion, and ultracentrifugation in a variety of both benign and denaturing solvents.

The investigation shows that myosin consists of four polypeptide chains, two small globular chains of average mass near 30,000 amu, which are held in the globular head of native myosin by secondary forces, and two large chains of average mass near 220,000 amu, which form all of the helical tail and a major part of the globular head of native myosin. Neither the light nor the heavy subunit has biological activity, *i.e.*, adenosine triphosphatase activity or actin-combining ability. Upon back-titration to neutral pH, however, the subunits recombine with resulting full recovery of actin binding power and more than 50% recovery of enzymatic activity.

Recent work on the molecular structure of myosin has resolved controversies and brought substructural details into sharper focus. Numerous investigators have attacked the problem of the molecular mass. The result, in most cases, falls between 470,000 and 530,000 amu (Laki and Carroll, 1955; Holtzer and Lowey, 1956, 1959; Lowey and Cohen, 1962; Holtzer *et al.*, 1962; Mueller, 1964; Tonomura *et al.*, 1966; Richards *et al.*, 1967), which undoubtedly represents satisfactory agreement. Thus, it seems wise to use 500,000 amu. Earlier difficulties that led to controversy have been reviewed (Geiduschek and Holtzer, 1958; Holtzer *et al.*, 1962; Mueller, 1964). The molecular length also seems established at near 1600 Å, the molecule being very asymmetric (Holtzer and Lowey, 1956, 1959; Holtzer *et al.*, 1962; Rice, 1961, 1962; Zobel and Carlson, 1963; Huxley, 1963).

Great impetus was provided by the discovery that brief enzymatic digestion of myosin produces, essentially, two macromolecular fragments: light meromyosin and heavy meromyosin (Gergely, 1950, 1953; Mihalyi and Szent-Györgyi, 1953; Mihalyi, 1953). Heavy meromyosin possesses the parental ATPase[1] activity and actin binding capacity whereas light meromyosin is similar to myosin in its solubility (Szent-Györgyi, 1953). A hint of the arrangement of meromyosins in myosin was first obtained by comparing the experimental angular distribution of light scattered by the myosin molecule with the theory for various models; agreement was only obtained for a top-heavy molecule, *i.e.*, with the heavy meromyosin on one end (Holtzer and Rice, 1957). Determination of the molecular weights and relative amounts of the meromyosins established that each myosin molecule comprises one molecule of each meromyosin (Lowey and Holtzer, 1959). Characterization of the meromyosins provided length estimates showing that light meromyosin and heavy meromyosin are asymmetric and are colinearly joined in myosin (Szent-Györgyi, 1953; Geiduschek and Holtzer, 1958).

Purification of light meromyosin,[2] the finding that it is approximately fully α helical, and determination of its intrinsic viscosity led to the proposal that light meromyosin is a two-chain, α-helical, coiled coil (Szent-Györgyi *et al.*, 1960). Detailed studies of hydrodynamic prop-

* From the Department of Chemistry, Washington University, St. Louis, Missouri. *Received April 25, 1968.* This investigation was supported by U. S. Public Health Service Research Grant RG-5488 from the Division of General Medical Sciences, U. S. Public Health Service. During this investigation D. F. held National Institutes of Health Predoctoral Fellowship 5-Fl-GM-30,352.

[1] See *Biochemistry 5*, 1445 (1966).

[2] Some distinction should be made between earlier preparations of light meromyosin, which contained many contaminants, and the highly α-helical protein ("light meromyosin, fraction 1") which can be obtained from the cruder preparations by alcohol precipitation (Szent-Györgyi *et al.*, 1960.) It will be clear from the context which is meant.

erties and of light scattering provided confirmation (Lowey and Cohen, 1962; Holtzer *et al.*, 1962).

Recognition that heavy meromyosin has a lower helix content (~50%) and intrinsic viscosity than light meromyosin, but that the latter property is considerably greater for heavy meromyosin than for globular proteins of comparable helix content, led Cohen (1961) to suggest that heavy meromyosin is itself top-heavy, consisting of a globular region joined to a helix. The model of myosin then becomes a long, two-chain α-helical rod (light meromyosin and the "tail" of heavy meromyosin), topped by a globular region. This idea was confirmed by detailed examination of light-scattering data, which showed that the best fit employed a model of heavy meromyosin having considerable mass near its centroid rather than uniformly distributed (Holtzer *et al.*, 1962), and by visualization of myosin and meromyosin molecules in the electron microscope (Rice, 1961, 1962; Zobel and Carlson, 1963; Huxley, 1963).

While these investigations of the meromyosins were going forward, Mueller and Perry (1960–1962) made the discovery that heavy meromyosin can be further digested enzymatically to yield a globular fragment, heavy meromyosin subfragment 1, that can be isolated and which possesses the ATPase activity and actin binding capacity of myosin. Degradation of heavy meromyosin subfragment 1 accompanying the digestion makes this fragment difficult to characterize. The most recent work on heavy meromyosin subfragment 1 (Mueller, 1965) points to a molecular weight of 112,000–170,000 and a value of 0.55–0.60 for its weight fraction in heavy meromyosin. These data in conjunction with the results of electron microscopy (Slayter and Lowey, 1967) do not as yet lead to an unequivocal assignment of heavy meromyosin subfragment 1 to its precise place in heavy meromyosin, although it certainly arises from the globular region, and evidence favors the existence of two such units in each heavy meromyosin.

The helical tail of heavy meromyosin, or a good part of it, has also been isolated. Acidification of heavy meromyosin solutions (Lowey, 1964) or digestion by a trypsin derivative (Lowey *et al.*, 1967) releases a slowly sedimenting (~3 S) component from which can be isolated a highly (~80%) α-helical particle.[3] The molecule has a mass of 60,000 amu (Lowey *et al.*, 1967), *i.e.*, a length, as a two-chain, coiled coil, of ~400 Å.

The picture revealed by these studies of enzymatic fragments is of two polypeptide chains in an α-helical coiled-coil conformation over an axial distance of ~1300 Å, from which point both chains adopt a globular conformation to form an approximately ellipsoidal head, which on closer examination is shown to be bifurcated (Slayter and Lowey, 1967; see, especially, Figure 7[4]).

In parallel with these experiments on enzymatically produced fragments, studies were made of the influence on myosin of reagents not normally expected to fracture peptide bonds. Kominz *et al.* (1959) describe a slowly sedimenting (~2 S), globular component obtained by exposure of myosin to solutions 0.1 M in Na_2CO_3. This component was isolated and found to have a molecular weight near 30,000. Carbonate is inessential, the effect being a result of exposure to high pH, a fact that is implied, but not explicit, in the work cited and has since been demonstrated (Stracher, 1961). It has also been shown that this fragment originates in the heavy meromyosin region of myosin (Gershman *et al.*, 1966). With the further findings that urea (Wetlaufer and Edsall, 1960), quanidine hydrochloride (Wetlaufer and Edsall, 1960; Dreizen *et al.*, 1966), or temperature elevation (Locker, 1956) also releases small fragments from myosin, it became clear that the myosin molecule contains one or more small, globular subunits in the head region, and that these are probably held there by secondary forces only. These subunits have not always been explicitly accounted for in schematic depictions of the myosin molecule (see, for example, Slayter and Lowey, 1967).

Following this observation by Kominz *et al.* (1959), the fragmentation produced by alkali was studied further. Results of the most thorough investigation (Gershman *et al.*, 1966) indicate that the two-chain picture for myosin is to be modified by addition, in the globular region, of *three* globular polypeptide chains, five chains in all. The choice of three globular subunits arises from the experimental values for their weight percentage in myosin (~12%) and the molecular mass of each (20,000 amu), the latter being at variance with the value (30,000) originally found by Kominz *et al.* Chemical studies, on the other hand, appear to favor four chains in myosin rather than five (Sarno *et al.*, 1965; Weeds, 1967).

The experiments reported here were undertaken to elucidate some of the doubtful points concerning the relationship of these globular fragments to the polypeptide chain structure of myosin. In particular, we hoped to provide further evidence concerning the existence of noncovalently bound subunits and on the number and size of the polypeptide chains in myosin. Finally, it is noteworthy, and surprising, that no experiments bearing on the relationships of the small, globular, alkali subunits to the biological activities of myosin have been reported. We wished also to discover whether these small subunits are essential to such activity, or, perhaps, exhibit it themselves.

Experimental Methods

Reagents. Distilled water was deionized and filtered through activated charcoal before use. $(NH_4)_2SO_4$

[3] The helical particle isolated from such digests has been called heavy meromyosin subfragment 2 (Lowey *et al.*, 1967; Slayter and Lowey, 1967). This nomenclature can be confusing. The same name had earlier been applied by Mueller and Perry (1961a,b) to the entire 3S component in digests of heavy meromyosin produced by treatment with concentrated trypsin. The latter material is undoubtedly much more heterogeneous and much less helical than the component isolated by Lowey and coworkers.

[4] In the figure cited the mass of heavy meromyosin subfragment 1 is given as 120,000 amu. It would be more satisfactory for the mass balance to use 140,000 which, in any case, is almost exactly in the middle of the range 112,000–170,000 recently found for this fragment (Mueller, 1965).

(Mann Research Laboratories, Enzyme Grade) was cleared of particulate matter by dissolution in boiling, distilled, deionized water followed by filtration through a coarse fritted filter. The resulting solution was allowed to cool and to crystallize at 5°; the supernatant, saturated $(NH_4)_2SO_4$ solution was used for subsequent protein precipitations. Guanidine hydrochloride was prepared from the carbonate (Matheson Coleman and Bill) by the method of Anson (1941). All other chemicals were reagent grade.

Protein Preparations. Myosin was prepared from rabbit skeletal muscle as described by Szent-Györgyi (1951) and Mommaerts and Parrish (1951) with slight modifications (Holtzer and Lowey, 1959; Schuster, 1963).

The alkali fragments were prepared from fresh myosin by addition of base to the myosin solution (to pH 10.5–12.0), whereupon the subunits are formed, and by subsequent fractional precipitation with $(NH_4)_2SO_4$. The procedure used is essentially that of Kominz *et al.* (1959) with some modifications; details have been given earlier (Frederiksen, 1967). All operations were performed at 3–10°. In our experiments, the heavy alkali subunit precipitated at 32–40% saturation and was contaminated with about one-third of the total original amount of light alkali subunit.[5] Reprecipitation was therefore necessary. After the third precipitation, however, the heavy alkali subunit is highly aggregated. The precipitate was dissolved in $(KCl)_{0.6}(K[PO_4])_{0.05}(7.2)$[6] and dialyzed, with frequent changes, *vs.* the solvent for a total of 36–72 hr, including at least 12 hr after the first negative test (acidified $BaCl_2$) for sulfate. The light alkali subunit precipitated at 65–70% of saturation and was redissolved and dialyzed as above. The light alkali subunit prepared in this way is generally free of heavy alkali subunit, as evidenced by a single, symmetrical peak in the analytical ultracentrifuge. The solution was then further dialyzed *vs.* a solution of desired ionic composition and was suitable for experiments or for lyophilization and subsequent storage at ~20°. The yield of light alkali subunit was ~0.12 g/g of myosin or ~0.5 g/350 g of wet muscle.

A preparation of actin in 2×10^{-4} M ATP was very generously supplied by Dr. A. Martonosi, Saint Louis University School of Medicine. This protein had been extracted from acetone-dried muscle powder by the method of Feuer *et al.* (1948) and purified by procedures described by Mommaerts (1951) and by Martonosi (1962).

Protein concentrations were routinely determined by measurement of the optical density of a quantitatively diluted stock solution. Optical densities of solutions in 1.0-cm, matched, quartz cells were measured with a Beckman DU spectrophotometer set at a wavelength of 277 mμ. The blank was always the dialysate of the solution whose concentration was being determined.

Micro-Kjeldahl analyses were used to determine the nitrogen content of protein solutions for measurements of the extinction coefficients. A nitrogen factor of 6.2 was used for myosin (Kominz *et al.*, 1954), 6.2 for heavy alkali subunit (as calculated by Frederiksen, 1967), and 6.0 for light alkali subunit (Kominz *et al.*, 1959). The extinction coefficients for myosin, heavy alkali subunit, and light alkali subunit in neutral KCl solution were such as to give 5.87, 5.77, and 4.34, respectively, for the optical densities of 1% solutions of 1-cm path length. The extinction coefficients of light alkali subunit and heavy alkali subunit in concentrated guanidine hydrochloride solutions were determined by the same method that has been used for tropomyosin (Holtzer *et al.*, 1965); the value for light alkali subunit was 4.79 in 5.0 M guanidine hydrochloride, and that for heavy alkali subunit was 5.19 in 6.0 M guanidine hydrochloride and 5.29 in 5.0 M guanidine hydrochloride. The extinction coefficients do not vary with KCl concentration, pH, or β-mercaptoethanol concentration. All three proteins were found to follow Beer's law in all solvents used.

Protein Titrations. Myosin solutions were titrated to desired pH at 4–8° with continuous, vigorous stirring in order to disperse the added base (or acid) immediately. The titration apparatus (Frederiksen, 1967) was designed to minimize surface denaturation by eliminating the introduction of air bubbles into the solution. A Beckman Zeromatic pH meter (9600) was used for all pH measurements; the instrument was standardized with Fisher Certified standard buffer solutions, and was accurate to 0.1 pH unit, but reproducible to ±0.02 pH unit. Myosin (1%) solutions were titrated to the appropriate pH and back with as little as 2-min and no more than 15-min stirring.

Analytical Ultracentrifugation. All sedimentation runs were made in the Beckman Spinco Model E ultracentrifuge operated with schlieren optics. Samples were put into 12-mm standard, single-sector cells and run at 59,780 rpm in the An-D rotor at a bar angle of 60°; with the exception of some experiments with the light alkali subunit performed at 20.0 ± 0.2°, all runs were made at 5.0 ± 0.2°. Sedimentation coefficients were calculated from plots of log (distance) *vs.* time with the usual corrections for solvent viscosity and density.

Viscometry. Viscosity measurements performed on protein solutions in benign solvents were made with Ostwald-Fenske viscometers (Cannon Instrument Co.). Viscometers used for myosin solutions had water flow times of about 200 sec at 0.0°; those used for solutions of the light alkali subunit had water flow times of about 500 sec at 20.0°. Cannon-Ubbelohde semimicro dilution viscometers were used for all experiments performed on proteins in guanidine hydrochloride solutions; these viscometers had water flow times at 20.0° of about 300 sec.

[5] Light alkali subunit and heavy alkali subunit are *presumably* the same (respectively) as the light and heavy alkali fragments isolated by Gershman *et al.* (1966) and referred to by them as the "alkali components" LAC and HAC. However, since light alkali subunit and heavy alkali subunit are obtained as $(NH_4)_2SO_4$ fractions, whereas LAC and HAC were separated by dilution, it is probably wise to retain operational definitions until real proofs of identity are produced.

[6] $K[PO_4]$ is a mixture of K_2HPO_4 and KH_2PO_4. The frequent necessity of referring to complex aqueous solvent media makes shorthand notation desirable. We therefore designate such a solvent by writing the chemical formula (or name) of each component (omitting water) with its molarity as a subscript, followed by parenthetical specification of the pH (Holtzer *et al.*, 1965).

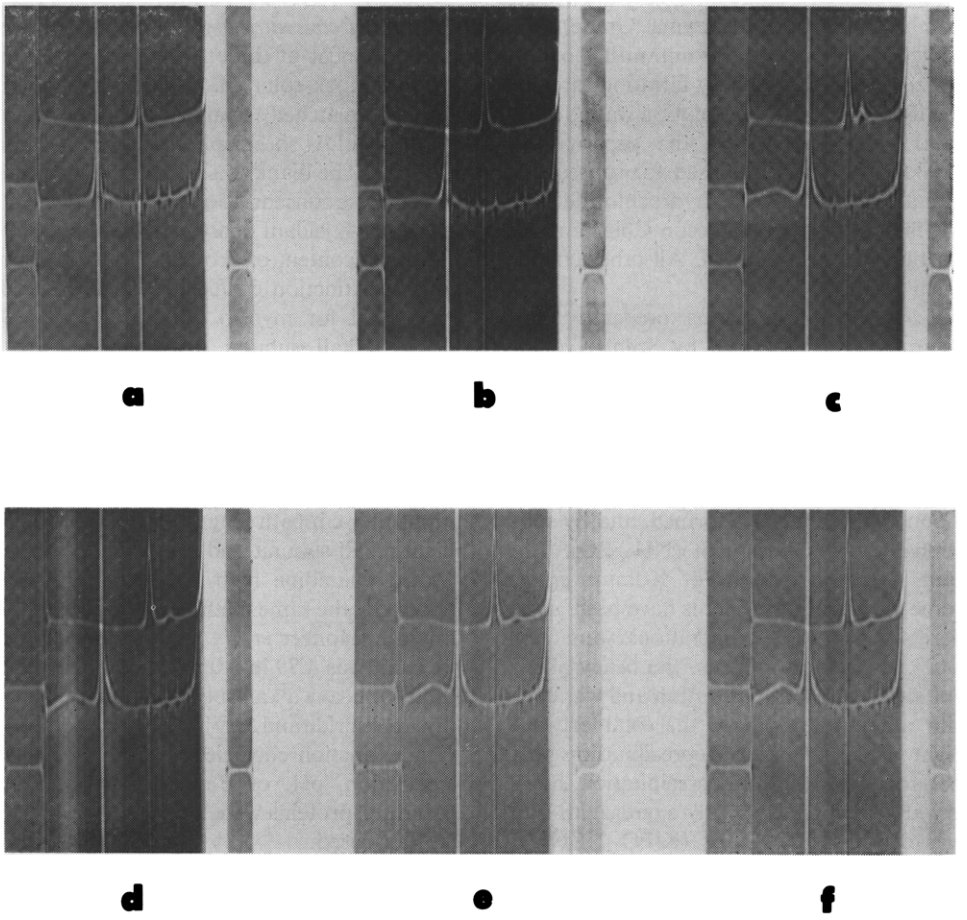

FIGURE 1: Myosin in 0.6 M KCl after titration to various pH values. Run made at 5° and 57,780 rpm with bar angle 60°. In standard cell, 1.0% protein; in wedge cell, 0.3%. Time: 160 min after reaching speed, 220 min after the titration. (a) pH 10.1, (b) pH 10.5, (c) pH 11.0, (d) pH 12.0, (e) pH 12.5, and (f) pH 13.0.

Flow times for myosin solutions were measured at 0.00 ± 0.02°; this temperature was maintained by mounting the viscometer in a vigorously stirred ice–water slurry in the cold room. Viscometry was performed with all other protein solutions at 20 or 25 ± 0.02°; the solutions and viscometers were thermostated with the aid of a Low-Temp bath (Wilkens-Anderson Co., Chicago).

Solvents were cleaned by filtration through a medium fritted-glass filter immediately before use; protein solutions were routinely cleaned by centrifugation for 6–8 hr at 25,000 rpm in the Spinco 40 rotor. The top third of the solution (but not the meniscus) was used. The most dilute protein solution used in this study had a flow time 21 sec greater than that of solvent; the most concentrated solution had a flow time 600 sec greater than that of solvent.

Polarimetry. Optical rotary dispersion was measured with a Rudolph automatic recording spectropolarimeter (Model 260/655/850/810-614) equipped with a high-pressure, high-intensity Xe–Hg lamp. A 0.5-dm cell, a symmetrical angle of 2°, and a wavelength range of 3200–5000 Å were used for all experiments.

The usual equation (Moffitt and Yang, 1956) was used to calculate the parameter $-b_0$, which has been found to be roughly proportional to the α-helix content of the protein; a completely helical protein has $-b_0 \simeq 600°$ in this wavelength region with λ_0 2120 Å, while $-b_0 \simeq 0$ if the molecules have no residues in the α-helical configuration. The IBM-7072 was programmed to give the α-helix content from the optical rotatory dispersion data by determining the best (least-squares) fit to the Moffitt–Yang (1956) plot.

Light Scattering. The techniques and instrumentation employed were as described previously (Schuster, 1963; Holtzer *et al.*, 1962; Holtzer and Lowey, 1959) with minor variations (Frederiksen, 1967).

For particles small compared to the wavelength of the incident light, the dissymmetry of scattering, R_{45}/R_{135} (where R_θ is Rayleigh's ratio for scattering at angle θ), is unity. All the solutions of the light alkali subunit used in these experiments had dissymmetries of less than 1.03. The values determined for R_{45} were from 1.5% smaller to 0.7% larger than the values determined for R_{90} for the same solutions.

In order to determine the molecular weight of the light alkali subunit, the truncated form of the light-scattering expression, $Kc/R_\theta = 1/M + 2Bc$, is satisfactory. In this equation, $K = 2\pi^2 n_0^2[(n - n_0)/c]^2/N_0\lambda^4$, where n and n_0 are the refractive indices of solution and

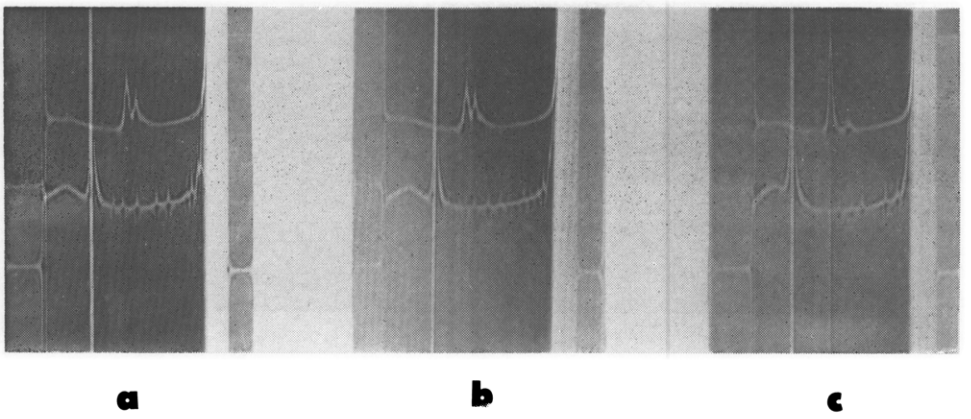

FIGURE 2: Myosin after various times at 5° and high pH after direct titration with KOH. The solvent is 0.6 M KCl. Runs made at 59,780 rpm and 5°, bar angle 60°. In standard cell, 1% protein; in wedge cell, 0.3%. Time 112 min after reaching speed. (a) After 3 days at pH 11.0, (b) after 7 days at pH 11.0, and (c) after 6 hr at pH 13.0.

solvent, respectively, c is the protein concentration in grams per milliliter, N_0 is Avogadro's number, and λ is the wavelength of incident light *in vacuo; M* is the molecular weight of the macromolecular solute, and B is the second virial coefficient.

Protein solutions and solvents were cleaned by pressure filtration through an ultrafine fritted filter (pore size 0.1–1.4 μ). Completely clean solutions (with no visible dust particles) could be obtained in this manner.

Refractometry. The value of the specific refractive index increment, $(n - n_0)/c$, was determined in the manner previously described (Lowey *et al.*, 1963), using a differential refractometer (Brice and Halwer, 1951). Solutions of the light alkali subunit were diluted to near the concentration desired (0.4–1.5%) and dialyzed against solvent for a minimum of 72 hr with no change of dialysate. To avoid concentration changes caused by evaporation, the air spaces in both the dialysis bag and in the dialysis vessel were minimized. The dialysis flask was sealed with Parafilm (Marathon Division, American Can Co.) and a rubber band from the beginning of dialysis until just a few moments before the refractometry cell was to be filled. The dialysis bag was removed from the dialysate and placed in a glass vial; a syringe was then used to transfer solution directly from the bag to the cell. Solvent was similarly transferred from flask to cell. The cell was rimmed with Kel-F 90 grease and sealed with a flat glass plate immediately after the introduction of the solutions. The cell was sealed within 45 sec after the dialysis flask had been opened.

ATPase activity assays were performed by the method outlined by Perry (1960). The reaction was carried out for 5 min at 25°. The final incubation mixture contained 5 mM ATP, 5 mM $CaCl_2$, 50 mM Tris-HCl buffer (pH 7.6), 0.250 ml of dialysate (0.6 M KCl) or protein solution (c 0.1–0.3%) in 0.6 M KCl, and enough KCl solution of such a concentration to bring the final ionic strength to 0.5 and the final volume to 2.250 ml.

The reaction was stopped after 5 min by precipitation of the protein with 1 ml of 15% trichloroacetic acid. The sample was removed from the water bath and the precipitate spun off in a desk-top centrifuge. The resulting supernatant was then assayed for P_i by the phosphomolybdate method of Fiske and Subbarow (1925).

Enzymatic activity is given in μg of P_i liberated per mg of protein per 5 min. Determinations of the activity for protein solutions had average deviations from the mean of about 5% and a range of about 20%.

Actin Binding. The ability to bind actin (Szent-Györgyi, 1951) of myosin, back-titrated myosin, and the alkali subunits was assessed ultracentrifugally. F-actin was mixed with the protein of interest. One aliquot of this mixture was treated with sodium pyrophosphate–$MgCl_2$ (in order to disrupt the protein–actin complex and thus to provide a control for each experiment) while another aliquot of the mixture was diluted to the same degree with protein dialysate. Both aliquots were then run simultaneously in the Model E analytical ultracentrifuge at 5° and 59,780 rpm.

Results

The Effect of pH on the Extent of Reaction. Determination of the minimum pH needed to produce detectable fragmentation of myosin was carried out by direct addition of 1.000 N KOH to rapidly stirred 1% myosin solutions. Protein solutions were titrated to

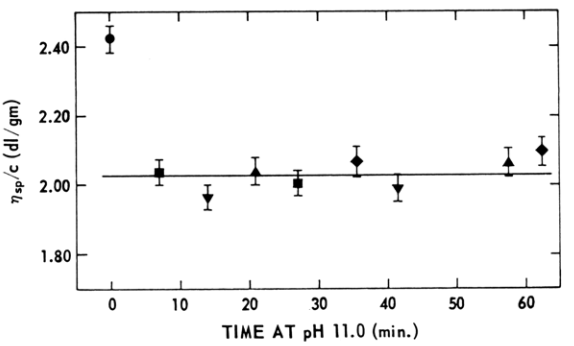

FIGURE 3: The effect of time at pH 11.0 and 0.0° on the viscosity of myosin. Solid circle is native myosin in $(KCl)_{0.6}$(7.0). The other symbols represent myosin in $(KCl)_{0.6}$(11.0), each indicating a different viscometer. Concentration in all cases is 0.092%.

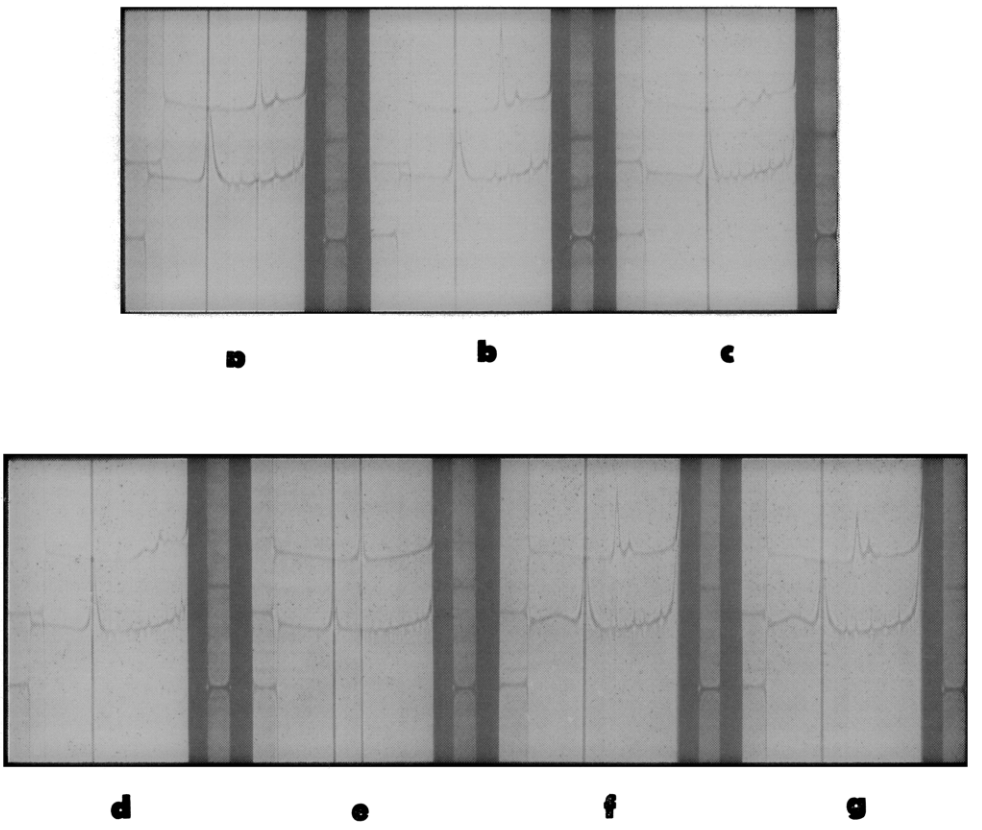

FIGURE 4: Reversal of the myosin dissociation. Myosin titrated to pH 11.0 with KOH, left for the indicated time, back-titrated to pH 7.0 with HCl, and run at 5° and 59,780 rpm with bar angle 60°. In standard cell, 1% protein; in wedge cell, 0.3%. Time as indicated. (a) 10 sec (144 min after reaching speed), (b) 5 min (128 min after reaching speed), (c) 15 min (128 min after reaching speed), (d) 360 min (128 min after reaching speed), (e) after 15 min of vigorous stirring without titration (144 min after reaching speed), (f) after 60 min at pH 11.0, but not back-titrated (144 min after reaching speed), and (g) after 360 min at pH 11.0, but not back-titrated (144 min after reaching speed).

pH 10.1, 10.5, 11.0, 12.5, and 13.0; then aliquots of each were examined in the analytical ultracentrifuge (Figure 1). Two protein concentrations (1.0 and 0.3%) were run at each pH in order, respectively, to show the small, slow peak and to resolve the accompanying fast, hypersharp peak. The first picture of each centrifuge run was taken about 1 hr (±5 min) after titration. As is seen in Figure 1, there is no detectable reaction at pH 10.1, while at pH values of 10.5 or greater, changes are evident. The small peak seen in the schlieren pattern at pH 10.5 is increased at pH 11.0, and only slightly increased again at pH 12.0, but it is not further increased at the two higher pH values. The more dilute solution in each case showed at least two protein peaks (in addition to the small, very slow peak which is not visible at such low concentrations): a predominant, sharp peak, and a much smaller, faster peak. No difference was found between the sedimentation patterns of any of these titrated protein solutions and of a buffered (Na_2CO_3–$NaHCO_3$) solution at the same pH and ionic strength (Frederiksen, 1967).

The Effect of Time at High pH on the Extent of the Reaction. From the experiments described in the previous section it is evident that the reaction has occurred after 1 hr at pH 11.0 (Figure 1). To determine both the effect of longer times at this pH on the reaction products and the minimum time required for the reaction to occur, protein solutions were brought to pH 11.0 and allowed to stand in the cold for specified periods of time; once again, both dialysis *vs.* buffer and direct titration with KOH were used to bring the protein to pH 11.0, with entirely equivalent results.

Figure 2a,b shows schlieren patterns of protein solutions that had been titrated to pH 11.0 and run in the analytical ultarcentrifuge after 3 days and after 7 days at this pH. The light alkali subunit peak did not increase after prolonged periods at high pH. The polydispersity of the heavy alkali subunit, slight in the solution after 1 hr at pH 11.0, was very pronounced after 3 and 7 days; a third, even faster sedimenting heavy alkali subunit peak appeared in the more dilute sample of each run. As the second heavy alkali subunit peak increased and as the third heavy alkali subunit peak was formed with increasing time at high pH, the slowest heavy alkali subunit peak diminished in height. The effect of longer times at even higher pH was studied with the result that myosin solutions titrated to pH 13.0 and left for 1 hr (Figure 1) appeared no different from similar solutions left at that pH for 6 hr (Figure 2c).

In order to determine a minimum time for the production of light alkali subunit, it is necessary to

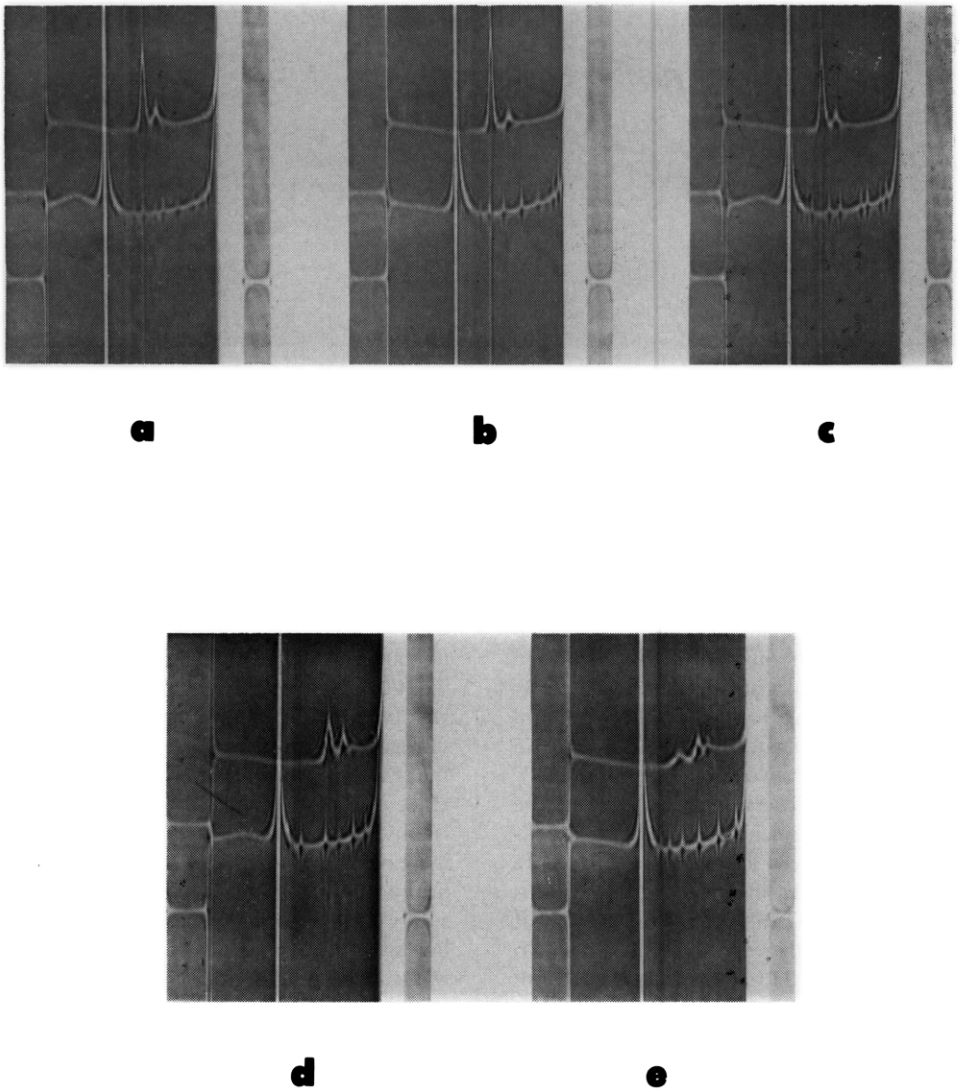

FIGURE 5: The dissociation, reassociation, and redissociation of myosin with changing pH. Run at 59,780 rpm and 5° with bar angle 60°. In standard cell, 1% protein; in wedge cell, 0.3%. (a) Myosin titrated to pH 11.0 (128 min after reaching speed). (b) Myosin titrated to pH 11.0 and back to pH 7.0 after 2 min (128 min after reaching speed). (c) Myosin titrated to pH 11.0, back to pH 7.0 after 2 min, and again to pH 11.0 after 60 min (128 min after reaching speed.) (d) Myosin titrated to pH 11.0, back to pH 7.0 after 60 min, and then again to pH 11.0 after another 60 min (112 min after reaching speed.) (e) Myosin titrated to pH 11.0 and back to pH 7.0 after 60 min (144 min after reaching speed.)

investigate the behavior of some property other than the sedimentation of the protein solution, because the time required to set up a centrifuge run after the titration is of the order of 1 hr. The viscosity of a protein solution, on the other hand, can easily be measured a few minutes after preparation of the sample. Unfortunately, its interpretation is not unequivocal.

The viscosity of the myosin solution before titration was determined; then the viscosity of the titrated myosin was followed at 2-min intervals for 60–90 min. The first measurement could be made within 6–8 min after the titration; by that time, the specific viscosity had already dropped from the value determined for native myosin. The reduced specific viscosity at pH 11.0 was found to be essentially constant with time (Figure 3). After the initial drop in viscosity there is no further change. The possibility of course exists that the rapid viscosity drop is due to some conformation change and that subsequent slower dissociation leads to no further change in viscosity. It is more likely, however, that the drop is caused by dissociation, which, therefore, must be complete within about 6 min.

Evidence for the Reassociation of the Subunits. In a typical series of experiments, 1% myosin solutions were titrated to pH 11.0 with 1.000 M KOH and were allowed to stand for a specified time in an ice–water bath. These solutions were then back-titrated to pH 7.0 with 1.000 M HCl and spun in the analytical ultracentrifuge. The experiment was performed by exposing aliquots to pH 11.0 for 10 sec, 30 sec, 1 min, 2 min, 5 min, 15 min, 30 min, 1 hr, and 6 hr before back-titration (Figure 4). Two control centrifuge runs were also made: untitrated native myosin was vigorously stirred for the 5–7 min ordinarily required for titration and was spun, and myosin was titrated to pH 11.0 and was spun after 1 and 6 hr (*i.e.*, without back-titration) (Figure 4).

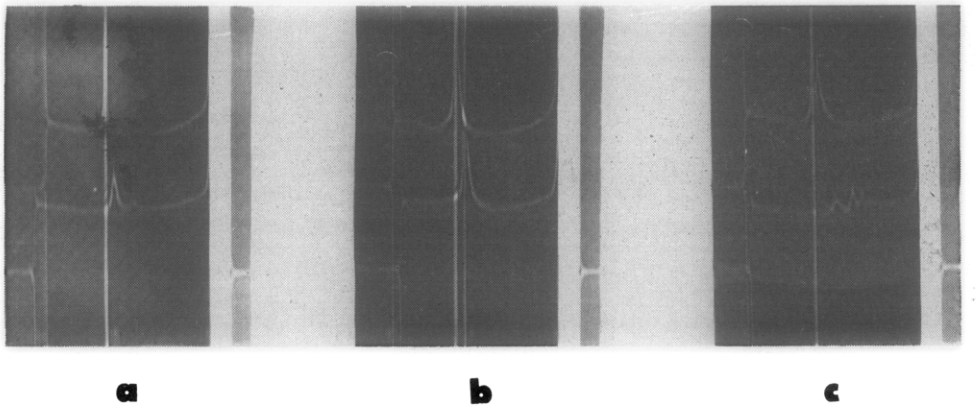

FIGURE 6: Actin binding by myosin, dissociated myosin, and back-titrated (reconstituted) myosin. Runs at 59,780 rpm and 5° with bar angle of 60°. In both standard and wedge cells, 0.5% protein. Time: 96 min after reaching speed. Each cell contains myosin and actin (4:1, w/w); the standard cell has myosin and actin in 0.40 M KCl, while the wedge cell has myosin and actin in $(KCl)_{0.35}(MgCl_2)_{0.001}(Na_4P_2O_7)_{0.01}$. (a) Native myosin. (b) Myosin at pH 11.0. (c) Myosin after back-titration from pH 11.0 to pH 7.0.

The small, slow peak characteristic of the light alkali subunit is not apparent in any of the back-titrated protein solutions. This peak, however, is consistently observed in the solutions at high pH. Those neutral samples that were back-titrated after less than 5 min. at pH 11.0 showed two peaks in the 0.3% (wedge cell) samples (as observed earlier for all the alkaline solutions), and the centrifuge runs for back-titrated solutions that had been left at pH 11.0 for more than 5 min show three peaks in the schlieren patterns for the dilute solutions. The peak height for the slowest of these three decreases with respect to the other two with increasing time (at pH 11.0) before back-titration. The time taken for the addition of acid in the back-titration seems to be directly related to the height of the fastest peak: the longer the titration time, the higher this third peak.

These experiments, which demonstrate the disappearance of light alkali subunit upon back-titration, were extended over several cycles. Myosin solutions were titrated from neutral pH to pH 11.0 and allowed to stand 2 min and for 1 hr. After back-titration to pH 7.0, 1 hr was allowed to elapse; then the protein solution was brought to pH 11.0 for a second time. Analytical ultracentrifugation was performed at the beginning of the experiment and after each titration (Figure 5). Once again, the small slow peak of light alkali subunit is observed to disappear upon back-titration, and the increase in the polydispersity of the peaks attributed to heavy alkali subunit is seen as before. When these solutions were again brought to pH 11.0, the light alkaline subunit reappeared. This back-and-forth procedure was carried out for four cycles, always with the same result.

Because sedimentation experiments clearly indicate that the fragmentation of myosin in weak base can be reversed, it is of interest to investigate the behavior of another property of the protein solution under the same conditions. This property must obviously be one which is sensitive to the protein structure, but it should also be one which is relatively unaffected by the not inconsiderable polydispersity observed in the sedimentation experiments; optical rotatory dispersion is just such a property.

The optical rotation as a function of wavelength was measured for native myosin, giving $-b_0 = 380 \pm 20°$ (the experimental error includes variation from one protein preparation to another and is about double that observed with solutions from the same preparation), and for myosin titrated to pH 11.0, where we found $-b_0 = 320 \pm 20°$. These correspond to respective helix contents of 60 and 51%. When another aliquot of the sample was titrated to pH 11.0 and after a 15-min time lapse back-titrated to pH 7.0, the optical rotatory dispersion of this solution yielded a value of $380 \pm 20°$ for $-b_0$, the same as the value determined for native myosin.

The ATPase activity of myosin before and after titration was also studied (Table I). The activity at pH 7.0 of native myosin was found to be 82 μg of P_i/mg per 5 min, while that of back-titrated myosin was 39

TABLE I: The ATPase Activities of Native Myosin and Back-Titrated Myosin.

	Native Myosin	Titrated to pH 11, Then to pH 7
Sample	μg of P_i/mg of Myosin per 5 min	μg of P_i/mg of Myosin per 5 min
1	84.4	38.7
2	80.2	33.9
3	79.0	38.7
4	78.2	38.7
5	84.3	44.1
6	83.7	
	81.6	38.8

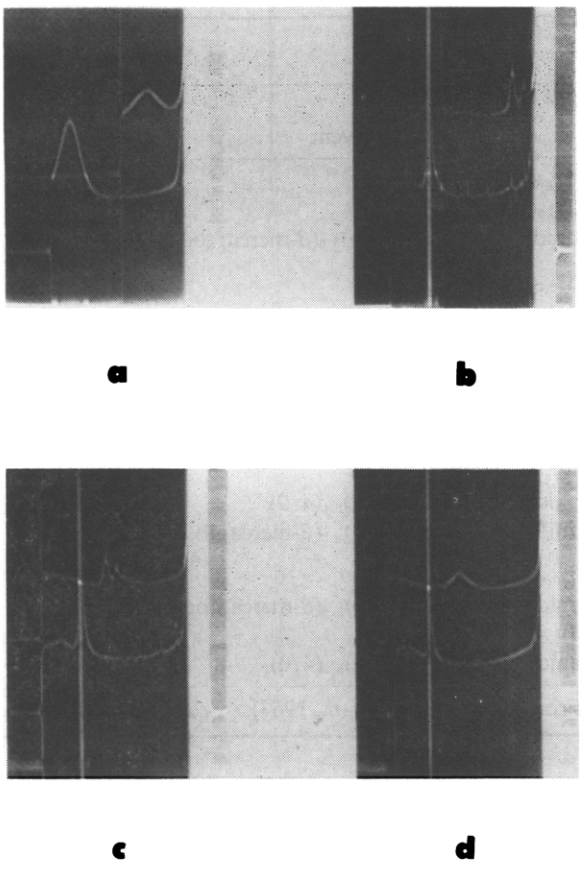

FIGURE 7: The recombination of light alkali subunit and heavy alkali subunit. Run at 59,780 rpm and 5° with bar angle 60°. In standard cell, 1% protein; in wedge cell, 0.3%, unless otherwise indicated. Time: 96 min after reaching speed, unless otherwise indicated. (a) Light alkali subunit in $(KCl)_{0.6}(KOH)_{0.01}(12.0)$. In standard cell, 0.9% protein; in wedge cell, 0.7%. (b) Heavy alkali subunit, fractionation 1, in $(KCl)_{0.6}(KOH)_{0.01}(12.0)$. (c) Light alkali subunit–heavy alkali subunit mixture (12:88 w/w) in $(KCl)_{0.6}(KO)_{0.01}$ (12.0). (d) Light alkali subunit–heavy alkali subunit mixture (12:88 w/w) from (c), back-titrated to pH 7.0 with HCl. Time: 65 min after reaching speed.

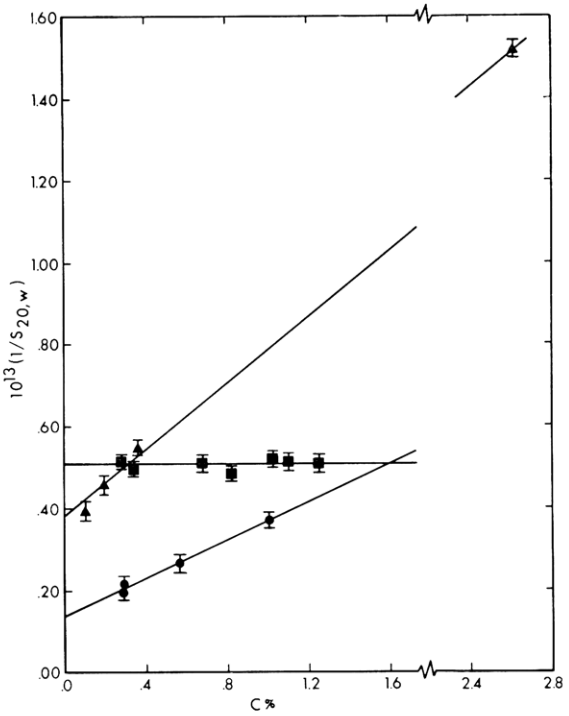

FIGURE 8: Intrinsic sedimentation coefficient of the alkali subunits. Circles, heavy alkali subunit peak in $(KCl)_{0.6}$-$(KOH)_{0.01}(12.0)$, plotted *vs.* total protein concentration; $[s_{20,w}]$ = 7.35 S. Triangles, heavy alkali subunit in (guanidine hydrochloride)$_{5.0}$(β-mercaptoethanol)$_{0.1}(4.9)$; $[s_{20,w}]$ = 2.63 S. Squares, light alkali subunit in $(KCl)_{0.6}(7.0)$; $[s_{20,w}]$ = 1.97 S.

μg of P_i/mg per 5 min. Thus, upon back-titration to pH 7.0 after 10 min at pH 11.0 and 5°, myosin recovers about 50% of the original enzymatic activity.[7] Yet, neither fragment alone has any activity at pH 7.0.

The actin-combining abilities of myosin at pH 11.0, and back-titrated myosin, as well as of the native (pH ~7) protein, are shown in Figure 6. Native myosin combines with actin to form actomyosin (as expected); myosin at pH 11.0, *i.e.*, myosin fragments at pH 11.0, do *not* complex with the F-actin; back-titrated myosin (*i.e.*, titrated to pH 7.0 after 10 min at pH 11.0, 5°), except for the aggregates mentioned earlier, combines with actin just as native myosin does.

After the alkli subunits had been separated by means of $(NH_4)_2SO_4$ fractionation, their recombination was studied in greater detail. The light alkali subunit and its heavy counterpart were mixed in the approximate weight ratio of 12:88 at pH 12.0. The relative proportions by weight of the subunits in myosin is anticipated here; the determination of this ratio is described below. Because of the increasing polydispersity with each successive fractionation of alkaline myosin, once-fractionated heavy alkali subunit was used in this experiment. The mixture was then titrated with HCl back to pH 7.0. Analytical ultracentrifugation was performed on the separated subunits, the synthetic mixture at pH 12.0, and the back-titrated mixture (Figure 7).

The light alkali subunit is homogeneous in the ultracentrifuge. The heavy alkali subunit, however, is slightly contaminated (2–3%) by the smaller protein. The high pH mixture of the two (Figure 7) is indistinguishable from the previously described myosin solution at pH 12.0 (Figure 1d). Upon back-titration, the peak due to the small subunit is decreased, its magnitude being approximately that of the contaminating light subunit in the original once-fractionated heavy fragment.

Analysis of the Composition of Myosin in Terms of the Alkali Subunits. The percentage composition of light

[7] More recent experiments (Cross and Holtzer, unpublished) have yielded larger recoveries (70-80%) and demonstrated that the percentage recovered is independent of the duration at high pH up to 100 min. Since it is clear from ultracentrifuge runs that the dissociation is complete after 60 min at high pH, the recovery of ATPase is real and cannot simply be a consequence of incomplete dissociation in the 10-min experiments. The evidence for recovery is thus independent of the 6-min minimum time determined by the (somewhat equivocal) viscosity measurements.

TABLE II: Some Physical Properties of the Alkali Subunits.

Property	Light Alkali Subunit	Heavy Alkali Subunit	Solvent
$[s_{20,w}]$ (S)	1.97 ± 0.04		$(KCl)_{0.6}$(7.0)
		7.3	$(KCl)_{0.6}$(12.0)
		2.6	(Guanidine hydrochloride)$_{5.0}$(β-mercaptoethanol)$_{0.1}$(4.9)
$-b_0$ (deg)	230	305	$(KCl)_{0.6}$(7.0)
	230		$(KCl)_{0.6}$(11.0)
		42	(Guanidine hydrochloride)$_{5.0}$(β-mercaptoethanol)$_{0.1}$(4.9)
$[\eta]$ (dl/g)	0.087		$(KCl)_{0.6}$(7.2)
	0.087		$(KCl)_{0.1}(K[PO_4])_{0.1}$(7.2)
	0.087		$(KCl)_{0.3}(K[PO_4])_{0.1}$(8.0)
	0.087		$(K[PO_4])_{0.15}$(7.2)
	0.272		(Guanidine hydrochloride)$_{5.0}$(4.9)
	0.272	1.093–1.075	(Guanidine hydrochloride)$_{5.0}$(β-mercaptoethanol)$_{0.1}$(4.9)
M	29,000[a]		$(KCl)_{0.6}$(7.0)
	31,600[b]	232,000–238,000[b]	(Guanidine hydrochloride)$_{5.0}$(β-mercaptoethanol)$_{0.1}$(4.9)
	33,600[c]		$(KCl)_{0.3}(K[PO_4])_{0.1}$(8.0)
	31,600[b]		(Guanidine hydrochloride)$_{5.0}$(4.0)

[a] From the equation of Scheraga and Mandelkern (1953). [b] From eq 1 (Tanford *et al.*, 1967). [c] From light scattering.

alkali subunits and heavy alkali subunits in myosin was determined by a series of quantitative fractionations of the reaction mixtures (Frederiksen, 1967). The procedure followed was essentially that described above for the preparation and separation of the subunits except that the volumes and concentrations of the original myosin solution and of each protein fraction were carefully determined. The results of these experiments, assuming that the protein not recovered (which was less than 5%, presumably lost to dialysis bags, spoons, and other equipment) contained light alkali subunits and heavy alkali subunits in the same proportion as protein that *was* recovered, show myosin to be 11.2% light alkali subunits. Using a round-number molecular weight for myosin of 500,000 (Holtzer *et al.*, 1962; Mueller, 1964), the mass of light alkali subunits contained in one myosin molecule is computed to be approximately 56,000 amu while that for heavy alkali subunits is about 444,000 amu.

Properties of the Heavy Alkali Subunit. The sedimentation behavior of heavy alkali subunit was studied in both benign and denaturing media. As indicated in Figure 8 and Table II the intrinsic sedimentation coefficient, $[s_{20,w}]$, varies from 7.3 in $(KCl)_{0.6}(KOH)_{0.01}$(12.0) to 2.63 S in (guanidine hydrochloride)$_{5.0}$(β-mercaptoethanol)$_{0.1}$(4.9).

Optical rotatory dispersion experiments were performed on the heavy alkali subunit in order to estimate the fraction of the protein in the α-helical conformation. At pH 7.2 in 0.6 M KCl the helix content of heavy alkali subunit is about 51% as estimated from a $-b_0$ of 305°. A similar experiment was performed on this protein in 5.0 M guanidine hydrochloride–0.1 M β-mercaptoethanol; the helix content of the heavy alkali subunit in this solvent is 5.5–6.0%.

Because the aggregation always present in samples of heavy alkali subunit in benign media makes interpretation of viscosity measurements impossible, such experiments were not performed on this protein. Rather, the viscosity of heavy alkali subunit was determined for the subunit in guanidine hydrochloride-β-mercaptoethanol solutions (Figure 9), in which all aggregation should be suppressed. Furthermore, the results should be unaffected by the presence of small amounts of the much smaller light alkali subunit. From the empirical equation given by Tanford *et al.* (1967) for proteins in such media

$$[\eta] = 0.00716n^{0.66} \quad (1)$$

where $[\eta]$ is the intrinsic viscosity (in deciliters per gram) of the protein and n is the number of amino acid residues per molecule, the molecular weight of heavy alkali subunit can be calculated. The results ($[\eta]$ = 1.093–1.075 dl/g) indicate that there are 1985 to 2035 amino acid residues per heavy alkali subunit molecule in this denaturing, reducing solvent. Since the average residue weight of the heavy alkali subunit is 117.3 (Frederiksen, 1967), the molecular weight of heavy alkali subunit in (guanidine hydrochloride)$_{5-6}$(β-mercaptoethanol)$_{0.1}$(4.9) is 232,000–238,000.

No release of orthophosphate from ATP was observed in ATP–heavy alkali subunit mixtures; the subunit is not an ATPase. Similarly, heavy alkali subunit does not complex actin. Thus, this component

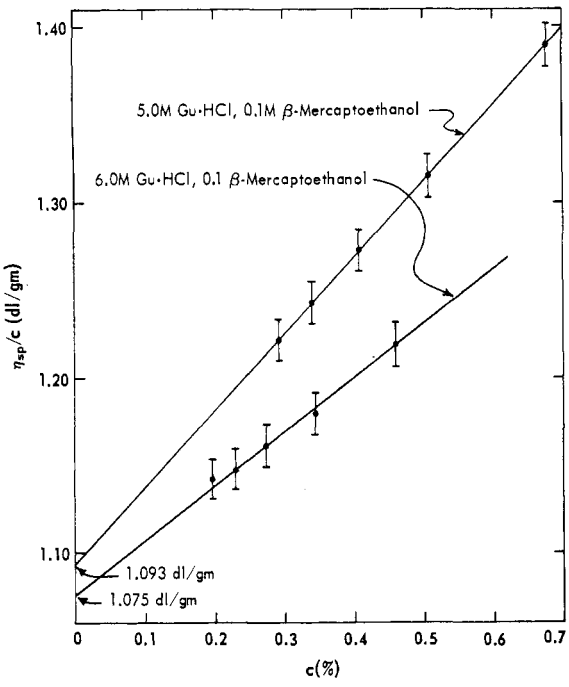

FIGURE 9: Viscosity of heavy alkali subunit in guanidine hydrochloride–β-mercaptoethanol. Upper line is for heavy alkali subunit in (guanidine hydrochloride)$_{5.0}$(β-mercaptoethanol)$_{0.1}$(4.9); $[\eta]$ = 1.09 dl/g, k' = 0.37. Lower line is for heavy alkali subunit in (guanidine hydrochloride)$_{6.0}$(β-mercaptoethanol)$_{0.1}$(4.9); $[\eta]$ = 1.08 dl/g, k' = 0.27.

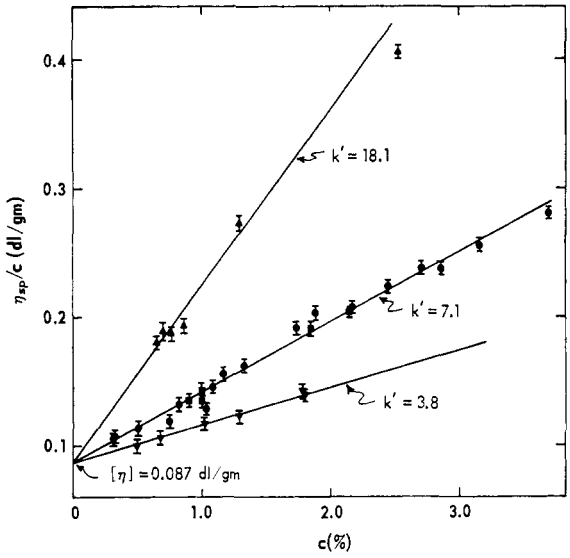

FIGURE 10: Viscosity of light alkali subunit in various solvents. $[\eta]$ = 0.087 dl/g for all. Triangles with apex down, (KCl)$_{0.3}$(K[PO$_4$])$_{0.1}$(8.0); k' = 3.8. Squares, (KCl)$_{1.0}$(K[PO$_4$])$_{0.1}$(7.2); k' = 7.1. Triangles with apex up, (K[PO$_4$])$_{0.15}$(7.2); k' = 18.1. Circles, (KCl)$_{0.6}$(7.2); k' = 7.1.

of myosin does not retain any of the biological activities of the native protein.

Properties of the Light Alkaline Subunit. Because the light alkali subunit originates from the heavy meromyosin end of myosin (Gershman *et al.*, 1966), it is of particular interest in view of its possible role at or near the active site(s) of myosin. Although the light alkali subunit and several other similar small macromolecular components of myosin have been isolated and studied in a number of laboratories, consistent results have not been obtained. The present study includes, in addition to determination of a number of hydrodynamic parameters of the molecule, three independent determinations of its molecular weight and an investigation of some of its chemical properties.

The intrinsic sedimentation coefficient of light alkali subunit in 0.6 M KCl (pH 7.0) is 1.97 ± 0.04 S (Figure 8 and Table II). This value is slightly lower than that reported by Kominz *et al.* (1959) for the subunit in an alkaline medium.

In order to determine the amount of helix in the light alkali subunit, optical rotatory dispersion measurements were performed on the purified protein at pH 7.0 and 11.0. A least-squares fit to the Moffitt plot yielded a $-b_0$ of 230 ± 10° for the subunit in both media. Using $-b_0$ = 600° for 100% helix, this corresponds to an α-helix content of ~37%.

Since the light alkaline subunit, in contrast to its heavy counterpart, could be isolated free of contaminating aggregates, viscosity measurements on light alkali subunit are meaningful in benign solvents as well as denaturing ones. Figure 10 shows the viscosity of light alkali subunit in several benign solvent systems; in each case, the intrinsic viscosity is 0.087 dl/g. If the concentration dependence of η_{sp}/c is expressed by $\eta_{sp}/c = [\eta] + k'[\eta]^2c$, then k' varies from 18.0 in (K[PO$_4$])$_{0.15}$(7.2) to 3.8 in (KCl)$_{0.3}$(K[PO$_4$])$_{0.1}$(8.0). It should be noted that there is no known physical significance of k' for proteins in aqueous salt solutions.

Substitution of these viscosity and sedimentation data for benign media into the Scheraga–Mandelkern (1953) equation for ellipsoids of revolution should provide a fairly reliable estimate of molecular weight for this nonasymmetric macromolecule. This relation is $M^{2/3} = N_0[s_{20,w}][\eta]^{1/3}\eta_0/(1 - \bar{v}\rho_0)\beta_e$, where M is the molecular weight; N_0, Avogadro's number; $[s_{20,w}]$, the intrinsic sedimentation coefficient corrected to water at 20°; ρ_0, the density of water at 20°; η_0, the viscosity of water at 20°; $[\eta]$ the intrinsic viscosity of macromolecular solute; $\bar{v}$, the partial specific volume (which we assumed to be the same for light alkali subunit and myosin); and β_e is taken as 2.14 × 10^6 and thus corresponds to a prolate ellipsoid with an axial ratio of about 2.5. The dependence of β_e upon the asymmetry is such that an axial ratio of 8.0 would only decrease the calculated molecular weight by 10%. The molecular weight of light alkali subunit as determined by this method is 29,000.

In both 5 M guanidine hydrochloride and in 5 M guanidine hydrochloride–0.1 M β-mercaptoethanol, denaturing media, the intrinsic viscosity of light alkali subunit is 0.272 dl/g and k' is 0.455 (Figure 11). This quantity, coupled with the average residue weight of this subunit (128.4 g/(residue mole); Kominz *et al.*, 1959), may be used in eq 1 to yield a second estimate of the molecular weight of the light alkali subunit; we find M = 31,600. The identity of the intrinsic viscosity for solutions of light alkali subunit in concen-

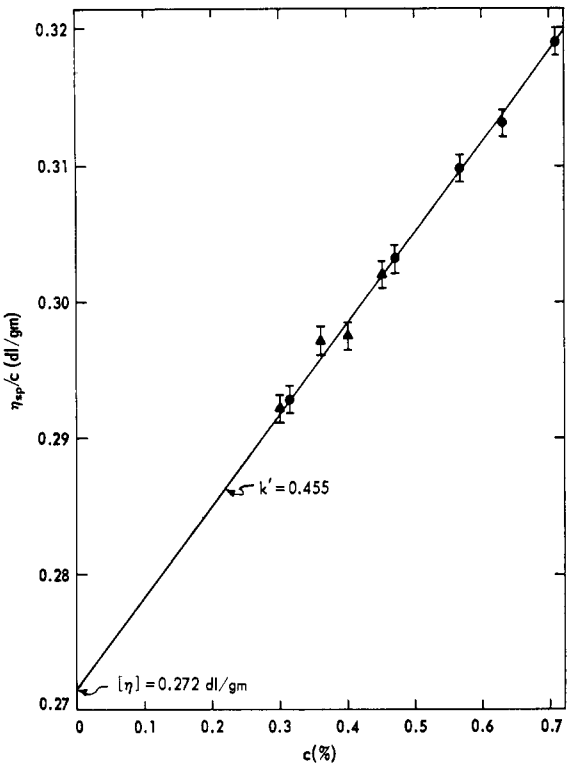

FIGURE 11: Viscosity of light alkali subunit in denaturing media. Circles, (guanidine hydrochloride)$_{5.0}$(4.9). Triangles, (guanidine hydrochloride)$_{5.0}$(β-mercaptoethanol)$_{0.1}$(4.9). For both, $[\eta]$ = 0.272 dl/g and k' = 0.455.

trated guanidine hydrochloride with and without β-mercaptoethanol provides direct evidence that the subunit molecule consists of a single polypeptide chain and has no large loops held together by disulfide groups.

To determine the molecular weight of light alkali subunit conclusively, a series of light-scattering experiments was carried out; the intensity of scattered light was measured as a function of scattering angle and of protein concentration (Figure 12). The independence of Kc/R_θ on scattering angle is characteristic of particles small compared with the wavelength of incident light. Also, light alkali subunit exhibits a second virial coefficient close to zero. The intercept of the extrapolation of c/R_θ to zero angle and zero concentration (or, in this case the average of c/R_θ at all angles and concentrations) is 55.55 g cm^2 and is directly proportional to the reciprocal of the molecular weight of the solute species. Independent measurement of $(n - n_0)/c$, the refractive index increment, yielded values of 0.182 ± 0.002 ml/g, essentially the same as myosin (Holtzer *et al.*, 1962); the value of K determined from this parameter and known constants is 5.362×10^{-7} mole g^{-2} cm^{-2}. Thus, the molecular weight of the light alkali subunit as determined from light-scattering data is 33,600. All the physical properties of the light alkali subunit determined in this study are summarized in Table II.

In addition to the physical properties already discussed, a few particularly relevant aspects of the chemical behavior of light alkali subunit have been investigated: the ATPase activity, the actin-combining

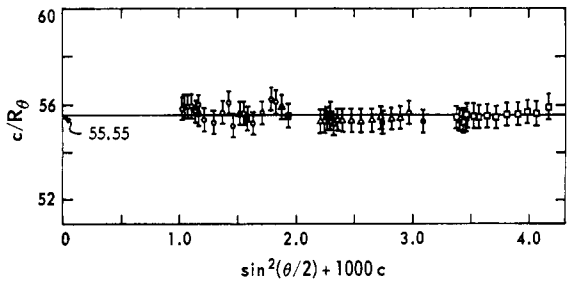

FIGURE 12: Light scattering of light alkali subunit in (KCl)$_{0.3}$-(K[PO$_4$])$_{0.1}$(8.0). Protein concentrations: open circles, 0.096%; filled triangles, 0.102%; filled squares, 0.143%; open triangles, 0.214%; filled circles, 0.259%; open squares, 0.331%.

power, and the solubility of the subunit as a function of ionic strength and pH.

Like heavy alkali subunit, and unlike myosin itself, the smaller alkali subunit does not catalyse the hydrolysis of ATP and does not combine with actin (Figure 6). These negative results give evidence for the hypothesis that both the light alkali subunit and the heavy alkali subunit are integral parts of the active site(s) of myosin *i.e.*, that each are essential for the biological activities of the native protein.

Although earlier investigators have reported that the light alkali subunit is soluble in solutions of ionic strength as low as 0.1 (Connell and Olcott, 1961; Kominz *et al.*, 1959), it was observed during the course of this investigation that the small protein is soluble in 0.1 M KCl only above pH 10 and that the protein is acceptably soluble in neutral media only if the ionic strength of the solvent is at least 0.3. The best benign solvents for light alkali subunit were found to be KCl solutions, phosphate buffered at pH 7.2–8.0, with ionic strength at least 0.6. The concentration dependence of η_{sp}/c of light alkali subunit solutions in these media is less than that at lower ionic strengths, and the light alkali subunit in these solvents passes through ultrafine frits without the clogging sometimes observed at lower ionic strengths.

Discussion

The Nature of the Dissociation of Myosin at Moderately High pH. As seen above, if we assume that the viscosity drop reflects dissociation only, the reaction must proceed to completion at 0–5° within 6 min (the minimum time required to measure the decrease in viscosity of the protein solution) after the solution is brought to pH 10.5–11.0. Furthermore, although dissociation is accompanied by the complete loss of ATPase activity (Stracher, 1961) and ability to bind actin and probably by a small decrease in helix content, the enzymatic activity is recovered in good part and other properties in full after the myosin solution is returned to neutral pH. The incomplete recovery of ATPase activity may be due to the intervening aggregation observed in all solutions of myosin at high pH and in all preparations of heavy alkali subunit. Longer times at high pH neither increase the yield of alkali subunits

nor produce new, smaller fragments of the native myosin; the only effect of prolonged periods at pH 11–12 is to enhance somewhat the extent of aggregation of the heavy alkali subunit. Thus, the reaction in question is fast, is accompanied by no other detectable dissociation, and can be reversed.

The possibility that the linkages in question are covalent must be considered. Disulfide bonds are a possibility; the breakage at pH 11 might be ascribed to a β-elimination reaction (Nozaki and Tanford, 1967; Cecil and McPhee, 1959; Bohak, 1964). However, this type of reaction would not ordinarily be expected to occur so rapidly under the present conditions. Furthermore, dissociation apparently can be made to occur at neutral pH (using urea or guanidine hydrochloride; see Wetlaufer and Edsall, 1960), making it unlikely that disulfide bonds are involved, a conclusion already reached by Dreizen *et al.*, (1966). Peptide bonds are another possibility. Although peptide bonds are indeed hydrolyzed by base, they are much more difficult to break than, for example, ester linkages; prolonged heating under reflux with strong base is usually required to effect cleavage, hardly the conditions described here as sufficient to fragment myosin. Nevertheless, it might be argued that the bond(s) involved in the dissociation reaction are unusually labile and thus sensitive to hydrolysis in the cold at only moderately high pH. While such an argument is difficult to refute, there are apparently no precedents for such rapid hydrolysis of a peptide bond in weak base, even the most susceptible ones requiring considerably more drastic treatment for breakage (Sine and Hass, 1967). Furthermore, such a bond, once broken, would certainly not be expected to re-form under the conditions which readily reverse the dissociation reaction of myosin. These arguments, however, do not preclude the possibility that such an extremely sensitive peptide bond *and* secondary forces hold the native macromolecule intact, and that upon "reversing" the reaction the covalent bond does not re-form, but that the secondary forces are themselves sufficient to hold the reconstituted, although slightly modified, myosin in, roughly, the native conformation.

The hypothesis that only secondary forces are involved explains the facts quite readily. The effect of raising the pH of the solvent medium is to increase the net negative charge of the macromolecule. If the light alkali subunits and heavy alkali subunits exist in native myosin as noncovalently linked polypeptide chains, then an increase in the net negative charge could result in the production of electrostatic repulsion effects strong enough to release them. The production of small subunits from myosin upon acetylation, carboxymethylation, or succinylation (all of which increase the net negative charge by chemical substitution) has been observed in several laboratories (Locker and Hagyard, 1967; Middlebrook, 1962; Oppenheimer *et al.*, 1966) in support of this view.

The rapid dissociation of myosin under extremely mild conditions and its reassociation are, therefore, better explained by the hypothesis that only secondary forces are involved. Thus, the light alkali subunit and heavy alkali subunit may be considered to be bound together noncovalently, but specifically, in the heavy meromyosin portion of myosin to form the active region for both ATPase activity and actin binding.

There is evidence, although not very strong, that the dissociation of myosin into the light alkali subunits and heavy alkali subunits is accompanied by a conformation change in one or both subunits. The measurements of b_0 give helix contents for light alkali subunit (37%) and heavy alkali subunit (51%) at neutral pH that would suggest, when proper account is taken of their relative weight proportions in myosin and of the differing residue weights, that myosin should be about 50% helical. In fact, b_0 studies of myosin at neutral pH lead to an estimate of 60%. However, at pH 11, where myosin is dissociated into its subunits, the value drops to 50%. Although it is doubtful that b_0 values can be so literally interpreted, these data do suggest that assemblage of the subunits into myosin is associated with some increase in helix content.

The Subunit Structure of Myosin. The small macromolecular fragment (light alkali subunit) released by myosin at pH values in excess of 10.5 has been shown from the optical rotatory dispersion studies presented here to be a globular protein containing only about one-third of its residues in the α-helical conformation. The molecular weight of the light alkali subunit in both benign and in denaturing reducing solvents has been found to be 32,000 $\pm$ 2000. The latter result is essentially the same as found by Kominz *et al.* (1959), but disagrees with the later work of Gershman *et al.* (1966). Since the method employed by us to prepare light alkali subunit follows the earlier group of workers, whereas Gershman *et al.* chose a different method, the possibility exists that the two preparations are not the same, perhaps reflecting underlying heterogeneity of the globular components of myosin. We were unable to check this, however, since in our hands the dilution method of Gershman *et al.* (1966) failed to yield appreciable quantities of the light alkali component.

We believe it unlikely that the higher value we find for the molecular weight is a result of contamination with: (1) heavy alkali subunit, since there is no sign of such contamination in the ultracentrifuge; there is agreement between the molecular weights determined from the $[s]$–$[\eta]$ combination in benign media and from $[\eta]$ in denaturing media, the former being more sensitive to traces of a highly asymmetric component; and, most important, there is no angular dependence of the light scattering. The latter is much more sensitive to contamination by a massive, highly extended component than is the molecular weight, (2) light alkali subunit aggregates, since, again, agreement is manifest among measurements in benign, denaturing, and denaturing–reducing media, (3) globular impurities, since the two most likely candidates can be ruled out, myokinase because its low molecular weight (20,000) makes it incapable of causing the desired effect, and deaminase because our myosin preparations possess no such activity (L. Cross and and A. Holtzer, unpublished data), and because the high reported molecular weight of this enzyme is doubtless due to association, which the agreement found here for various media precludes. Under

the circumstances, we will proceed by accepting our own value of (in round numbers) 30,000, while recognizing that further investigation is desirable.

The agreement of molecular weights in the two solvent types means that this subunit must exist as single polypeptide chains. Although the light alkali subunit originates from the heavy meromyosin end of myosin (Gershman *et al.*, 1966), it does not by itself contain the active region(s) for ATPase activity or for actin binding; it exhibits none of the specific activity associated with native myosin, heavy meromyosin, or heavy meromyosin subfragment 1. Since myosin (M = 500,000) contains 11–13% light alkali subunit by weight, there must be *two* such subunits in each myosin molecule. The percentage composition found in our work agrees with that obtained in a quite different way by Gershman *et al.* (1966). The difference between their conclusion (three globular units) and ours (two) therefore hinges on the molecular weight determinations. Finally, since there is no evidence for heterogeneity in solutions of the light alkali subunit, it is probably best, for the present, to use Occam's razor and to assume that the two light alkali subunit chains belonging to a given myosin molecule are the same, or at least very similar.

Although the heavy subunit has not been as extensively studied as its light counterpart, some information about the character of this molecule has been established here that corroborates earlier work done by different methods (Dreizen *et al.*, 1967). Based on a rounded molecular weight of 220,000 in concentrated guanidine hydrochloride with reducing agent (β-mercaptoethanol) present and on the 88–89% by weight of heavy alkali subunit in myosin, our conclusion, in agreement with earlier expectations that most of the myosin molecule is double stranded (Lowey and Cohen, 1962; Holtzer *et al.*, 1962) and with later measurements on HAC[5] (Dreizen *et al.*, 1967), is that there must be *two* heavy alkali subunit chains per myosin molecule. Assuming these two chains are the same, or very similar, several possibilities remain for the structure of heavy alkali subunit. (1) The chains might not be bound to one another in "native" heavy alkali subunit (*i.e.*, as it comes from myosin at pH 10.5–11.0); then each would constitute a heavy alkali subunit molecule with M = 220,000. (2) The two chains could be bound together (in benign solvent) by noncovalent, secondary forces in a heavy alkali subunit with M = 440,000. Or (3) the chains might be bound in nondenatured, nonreduced heavy alkali subunit by one or more disulfide linkages.

The first of these possibilities is rather unlikely; since light alkali subunit comes from the globular head of myosin, heavy alkali subunit necessarily contains the entire light meromyosin portion of myosin; light meromyosin is a very stable portion of the native molecule, consisting of two α-helices twisted about one another; thus, if heavy alkali subunit itself were a single chain, the light meromyosin end of myosin should also be single under alkaline conditions. Light meromyosin is not nearly this sensitive to changes in pH; it does not behave in this manner (Lowey, 1965). The third possibility is ruled out by the finding that the molecular weight of the heavy alkali component is halved in 5 M guanidine hydrochloride (Dreizen *et al.*, 1967). Thus, the second possibility for the binding of the chains in heavy alkali subunit can be chosen with some certainty. This would make heavy alkali subunit analogous to tropomyosin and paramyosin (Olander *et al.*, 1967).

Since heavy alkali subunit is ineffective as an ATPase and since it cannot bind actin, this fragment does not contain all the active regions of the native molecule.

The picture of the myosin molecule (Slayter and Lowey, 1967) can now be modified in light of information from studies of the dissociation of the native protein in weak base. Myosin consists of *four* polypeptide chains: a pair of short chains (*i.e.*, two light alkali subunit molecules) of average mass 30,000 amu (in round numbers) held in the native molecule by secondary bonds, and a second pair of long chains (heavy alkali subunit) with average mass 220,000 amu.

The two short chains originate entirely from somewhere within the globular portion of myosin. Although these light alkali subunit chains are so small that their dimensions are indiscernible from light-scattering or electron microscope studies, the molecular weight and the partial specific volume (*ca.* 0.71–0.73 ml/g for nearly all proteins) indicate a molecular volume of 3.8 $\times 10^4$ Å^3; this corresponds to a sphere of radius ~20 Å. The longer (heavy alkali subunit) chains comprise all of the light meromyosin, all of the helical part of heavy meromyosin, and ~80% of the globular part of the parent molecule.

Because the separated fragments of myosin retain none of the original ATPase activity or actin binding power inherent in the native protein, the light alkali subunit *and* heavy alkali subunit, held in combination by noncovalent bonds, must form the centers of the specific activities in myosin. This result further emphasizes a fundamental difficulty, alluded to above, concerning the relationship of heavy meromyosin subfragment 1 to myosin. Considerations of mass balance would seem to favor the interpretation that the molecular unit of heavy meromyosin subfragment 1 is, essentially, the globular piece of *one* of the long myosin chains, *i.e.*, that two such particles are produced from a single myosin molecule (Slayter and Lowey, 1967). Since heavy meromyosin subfragment 1 retains biological activity, the present work would require that this fragment also include one small globular chain, assuming, again, that two *identical* long and two *identical* short chains constitute a myosin molecule. This would imply, in the simplest view, *two* enzymatic sites per myosin molecule, a conclusion that, it has been pointed out (Mueller, 1965), is at variance with the experimental finding that there is only one such site (Gergely *et al.*, 1951; Nanninga and Mommaerts, 1960). The same is true of the actin binding capacity; both heavy meromyosin and heavy meromyosin subfragment 1 have been found to bind one actin monomer unit each (Young, 1967). Either some of the experimental results are incorrect[8] or their interpretation has been over-

[8] It has been pointed out to us that there are reports, of recent vintage, indicating a value for pyrophosphate binding that is

simplified. It may be, for example, that only one actin or one ATP is bound, *at a time* (Young, 1967), or that the long pair or short pair of chains, or both, are not pairwise identical. Hypotheses, at this stage, are not in short supply; evidence is.

Thus, although some of the details of the molecular structure of myosin have been resolved, several important problems remain: (1) the homogeneity of light alkali subunit and heavy alkali subunit, *i.e.*, whether there are two pairs of identical chains or not; (2) the macromolecular structure of the large double-stranded heavy alkali subunit; (3) the exact positions of the light alkali subunit chain in the myosin molecule; and (4) the nature of the active sites of myosin, heavy meromyosin, and heavy meromyosin subfragment 1. Unequivocal answers to these questions may make it possible, at last, for the molecular studies of myosin and the ultrastructural studies of muscle (Huxley and Brown, 1967; Huxley, 1963) to converge and produce a unified and accurate picture of muscular contraction.

References

Anson, M. L. (1941), *J. Gen. Physiol. 24*, 399.

Bohak, Z. (1964), *J. Biol. Chem. 239*, 2878.

Brice, B. A., and Halwer, M. (1951), *J. Opt. Soc. Am. 41*, 1033.

Cecil, R., and McPhee, J. R. (1959), *Advan. Protein Chem. 14*, 255.

Cohen, C. (1961), *J. Polymer Sci. 49*, 144.

Connell, J. J., and Olcott, H. S. (1961), *Arch. Biochem. Biophys. 94*, 128.

Dreizen, P., Gershman, L., Trotta, P., and Stracher, A. (1967), *J. Gen. Physiol. 50*, 85.

Dreizen, P., Hartshorne, D. J., and Stracher, A. (1966), *J. Biol. Chem. 241*, 443.

Feuer, G., Molnar, F., Pettko, E., and Straub, F. B. (1948), *Acta Physiol. Akad. Sci. Hung. 1*, 150.

Fiske, C. A., and Subbarow, Y. (1925), *J. Biol. Chem. 66*, 375.

Frederiksen, D. W. (1967), Ph.D. Thesis, Washington University, St. Louis, Mo.

Geiduschek, E. P., and Holtzer, A. (1958), *Advan. Biol. Med. Phys. 6*, 431.

Gergely, J. (1950), *Fed. Proc. 9*, 176.

Gergely, J. (1953), *J. Biol. Chem. 200*, 543.

Gergely, J., Martonosi, A., Gouvea, M. A. (1951), Sulfur in Proteins, New York, N. Y., Academic, p 297.

Gershman, L. C., Dreizen, P., and Stracher, A. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 966.

Holtzer, A., Clark, R., and Lowey, S. (1965), *Biochemistry 4*, 2401.

Holtzer, A., and Lowey, S. (1956), *J. Am. Chem. Soc. 78*, 5954.

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

Holtzer, A., Lowey, S., and Schuster, T. (1962), The Molecular Basis of Neoplasia, Austin, Texas, University of Texas, p 259.

close to two per molecule (Nauss and Gergely, 1967). Values for ATP binding may also need revision.

Holtzer, A., and Rice, S. (1957), *J. Am. Chem. Soc. 79*, 4847.

Huxley, H. E. (1963), *J. Mol. Biol. 7*, 281.

Huxley, H. E., and Brown, W. (1967), *J. Mol. Biol. 30*, 383.

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

Kominz, D. R., Hough, A., Symonds, P., and Laki, K. (1954), *Arch. Biochem. Biophys. 50*, 148.

Laki, K., and Carroll, W. R. (1955), *Nature 175*, 389.

Locker, R. H. (1956), *Biochim. Biophys. Acta 20*, 514.

Locker, R. H., and Hagyard, C. (1967), *Arch. Biochem. Biophys. 120*, 454.

Lowey, S. (1964), *Science 145*, 597.

Lowey, S. (1965), *J. Biol. Chem. 240*, 2421.

Lowey, S., and Cohen, C. (1962), *J. Mol. Biol. 4*, 293.

Lowey, S., Goldstein, L., Cohen, C., and Luck, S. M. (1967), *J. Mol. Biol. 23*, 287.

Lowey, S. and Holtzer, A. (1959), *Biochim. Biophys. Acta 34*, 470.

Lowey, S., Kucera, J., and Holtzer, A. (1963), *J. Mol. Biol. 7*, 234.

Martonosi, A. (1962), *J. Biol. Chem. 237*, 2795.

Middlebrook, W. R. (1962), *in* The Biochemistry of Muscular Contraction, Boston, Mass., Little, Brown.

Mihalyi, E. (1953), *J. Biol. Chem. 201*, 197.

Mihalyi, E., and Szent-Györgyi, A. G. (1953), *J. Biol. Chem. 201*, 189, 211.

Moffitt, W., and Yang, J.-T. (1956), *Proc. Natl. Acad. Sci. U. S. 42*, 596.

Mommaerts, W. F. H. M. (1951), *J. Biol. Chem. 188*, 559.

Mommaerts, W. F. H. M., and Parrish, R. G. (1951), *J. Biol. Chem. 188*, 545.

Mueller, H. (1964), *J. Biol. Chem. 239*, 797.

Mueller, H. (1965), *J. Biol. Chem. 240*, 3816.

Mueller, H., and Perry, S. V. (1960), *Biochim. Biophys. Acta 40*, 187.

Mueller, H., and Perry, S. V. (1961a), *Biochem. J. 80*, 217.

Mueller, H., and Perry, S. V. (1961b), *Biochim. Biophys. Acta 50*, 599.

Mueller, H., and Perry, S. V. (1962), *Biochem. J. 85*, 431.

Nanninga, L. B., and Mommaerts, W. F. H. M. (1960), *Proc. Natl. Acad. Sci. U. S. 46*, 1155.

Nauss, K., and Gergely, J. (1967), *Fed. Proc. 26*, 727.

Nozaki, Y., and Tanford, C. (1967), *J. Am. Chem. Soc. 89*, 742.

Olander, J., Emerson, M. F., and Holtzer, A. (1967), *J. Am. Chem. Soc. 89*, 3058.

Oppenheimer, H., Barany, K., Hamoir, G., and Fenton, J. (1966), *Arch. Biochem. Biophys. 115*, 233.

Perry, S. V. (1960), *Biochem. J. 74*, 94.

Rice, R. V. (1961), *Biochim. Biophys. Acta 52*, 602.

Rice, R. V. (1962), *in* Biochemistry of Muscle Contraction, Gergely, J., Ed., Boston, Mass., Little, Brown, p 41.

Richards, E. G., Chung, C.-S., Menzel, D. B., and Olcott, H. S. (1967), *Biochemistry 6*, 528.

Sarno, J., Tarendash, A., and Stracher, A. (1965), *Arch. Biochem. Biophys. 112*, 378.

Scheraga, H. A., and Mandelkern, L. (1953), *J. Am. Chem. Soc. 75*, 179.
Schuster, T. (1963), Ph.D. Thesis, Washington University, St. Louis, Mo.
Sine, H. E., and Hass, L. F. (1967), *J. Am. Chem. Soc. 89*, 1750.
Slayter, H. S., and Lowey, S. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 1611.
Stracher, A. (1961), *J. Biol. Chem. 236*, 2467.
Szent-Györgyi, A. (1951), The Chemistry of Muscular Contraction, 2nd ed., New York, N. Y., Academic.
Szent-Györgyi, A. G. (1953), *Arch. Biochem. Biophys. 42*, 305.
Szent-Györgyi, A. G., Cohen, C., and Philpott, D. E. (1960), *J. Mol. Biol. 2*, 133.
Tanford, C., Kawahara, K., and Lapanje, S. (1967), *J. Am. Chem. Soc. 89*, 729.
Tonomura, Y., Appel, P., and Morales, M. (1966), *Biochemistry 5*, 515.
Weeds, A. G. (1967), *Biochem. J. 105*, 25c.
Wetlaufer, D. B., and Edsall, J. T. (1960), *Biochim. Biophys. Acta 43*, 132.
Young, M. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 2393.
Zobel, C. R., and Carlson, F. D. (1963), *J. Mol. Biol. 7*, 78.

Circular Dichroism and Optical Rotatory Dispersion of α-Gliadin*

Donald D. Kasarda, John E. Bernardin, and William Gaffield

ABSTRACT: The circular dichroism spectrum of α-gliadin, a wheat protein, was measured in 10^{-3} M HCl (pH 3), 10^{-5} M HCl (pH 5), and 10^{-5} M HCl plus 0.005 M KCl (pH 5). Bands at 222, 208, and *ca.* 191 mμ indicate the presence of some helical structure. The ellipticity 11,300 (deg cm²)/dmole^{-1} at 222 mμ for pH 5 solutions suggests about one-third helical structure. Although the protein aggregates when a small amount of salt is added at pH 5, no corresponding change was found in the circular dichroism associated with the peptide bonds. We infer that no major conformational change occurs when the protein aggregates. Bands associated with side-chain optical activity at 277 and 294 mμ became more intense when the protein aggregated. This change suggests that the environment of aromatic side chains changes in a specific way during interaction between protein subunits.

Optical rotatory dispersion and circular dichroism measured at various temperatures between 4 and 90° showed the molecule to be conformationally stable below about 30°. Above this temperature conformation changed progressively, the helical content decreasing, but even at 90° about 65% of the helical structure present at 25° remained intact.

Recently, Bernardin *et al.* (1967) separated α-gliadin from a mixture of wheat proteins by means of its specific aggregation properties. That is, increasing the salt concentration of aqueous solutions at pH 5 to only 0.005 M in KCl causes the protein to aggregate. The aggregated form can be collected by ultracentrifuging at about 133,000*g*. The particle weight of this aggregated α-gliadin must be in the millions. Electron micrographs of the aggregated protein show long fibrils with a uniform diameter of about 80 Å (Kasarda *et al.*, 1967). Similar fibrils may exist in solution. We have used circular dichroism and optical rotatory dispersion to study the conformation of dissolved α-gliadin and to look for changes in conformation upon aggregation.

For most experiments we used three solvent conditions: pH 3 (10^{-3} M HCl), pH 5 (10^{-5} M HCl), and pH 5 with KCl (10^{-5} M HCl–0.005 M KCl). At pH 3, α-gliadin dissolves readily, is insensitive to shearing forces, and is relatively insensitive to added salt; its apparent molecular weight is about 50,000 (Bernardin *et al.*, 1967). At pH 5 the protein dissolves slowly, is very sensitive to shearing forces (becomes turbid), aggregates at low salt concentrations, and precipitates or gels if the ionic strength is more than about 0.01. We have studied effects of temperature (4–90°) on solutions in these solvents and have also studied optical properties of solutions in 8 M urea, 90% D_2O, and 70% aqueous ethanol.

Materials and Methods[1]

The α-gliadin was prepared by the method of Ber-

* From the Western Regional Research Laboratory, Agricultural Research Service, U. S. Department of Agriculture, Albany, California 94710. *Received May 8, 1968.*

[1] Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.