

# Myosin

## *Subunits and their interactions*

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**ABSTRACT** There is fairly general agreement that myosin isolated from rabbit skeletal muscle has a molecular weight of about 500,000. The higher values that have been reported apparently reflect protein aggregation related to the method of preparation. On the basis of present evidence, the myosin molecule has an elongate helical core of two *f* subunits (average weight about 215,000) that extend into a globular head region containing three *g* subunits (average weight about 20,000). Myosin may be dissociated into subunits by a number of methods. In 5 M guanidine, the myosin molecule is dissociated into *f* and *g* subunits, while at pH above 10, the *g* subunits are dissociated from the intact fibrous core of myosin. The dissociation of *g* subunits at pH 10 is accompanied by the loss of both ATPase activity and actin-binding capacity; however, the exact biological significance of the *g* subunits is presently uncertain. In preliminary studies, the *f* subunits appear to contain the sulfhydryl residues currently implicated in myosin ATPase, and there is some indication of allosteric regulation of enzymic activity.

Just six years ago, the New York Heart Association held a symposium entitled "The Myocardium: Its Biochemistry and Biophysics," and the structure of the contractile protein myosin was among the subjects considered (1). It was believed at the time that myosin isolated from rabbit skeletal muscle has a molecular weight of about 420,000 (2-5); that myosin isolated from dog cardiac muscle is half as large (6, 7); and that each myosin molecule contains two fragments of light (L-) meromyosin and one fragment of heavy (H-) meromyosin (8, 9). Several years later, each of these conclusions was untenable. Further data indicated that the molecular weight of skeletal myosin is significantly higher than 420,000 (9-13); that the molecular weight of cardiac myosin is very likely the same as that of skeletal myosin (14-16); and that each myosin molecule contains single fragments of L-meromyosin and H-meromyosin (17-19). We may infer that one should be cautious in interpreting information on this protein, and we can but wonder whether present concepts are to last longer than earlier concepts on myosin.

## MOLECULAR WEIGHT OF MYOSIN

The most elementary property of myosin, its molecular weight, has been rather controversial. In Fig. 1, molecular weight values for rabbit skeletal myosin are plotted against year of publication. The earliest work on myosin largely freed of actin indicated a molecular weight value of 1,500,000 (30). There was a steady fall in reported values of molecular weight to as low as 420,000 in 1958 (2-4). Myosin readily undergoes spontaneous aggregation (31, 32), and some of the early studies were presumably on preparations con-

## MOLECULAR WEIGHT OF RABBIT SKELETAL MYOSIN

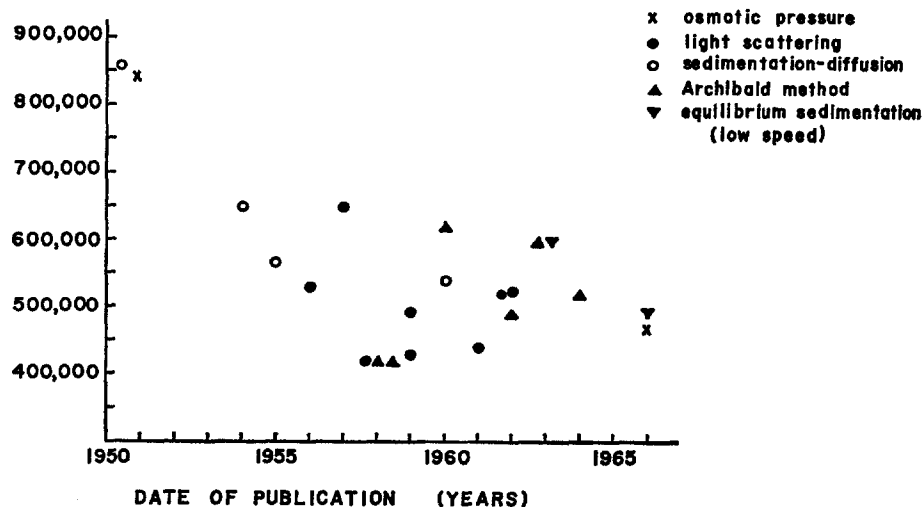


FIGURE 1. Reported values for the molecular weight of rabbit skeletal myosin. X, osmotic pressure (20, 21); ●, light scattering (22, 23, 4, 10, 24, 25, 11, 12); ▲, Archibald method (2, 3, 9, 18, 13, 26); ○, sedimentation-diffusion (20, 27-29); ▼, low-speed sedimentation equilibrium (13, 21).

taining soluble aggregates of myosin. But there was also doubt as to the validity of the 420,000 value (2, 3), based on use of the recently developed Archibald procedure (33). In one study (2), the concentration gradient at the meniscus was poorly defined (34); and in the other (3), the concentration dependence of apparent molecular weight had been neglected (9).

The first study that we might now consider technically satisfactory is that of Holtzer and Lowey, based on light scattering measurements (10). Data reported in 1959 indicated a molecular weight value of 493,000 for myosin (10), and later data a 525,000 value (11). In experiments using the Archibald method, Lowey and Cohen reported a molecular weight of 470,000-500,000 (18).

One might expect these results to be confirmed in other laboratories, but such was not to be the case. Brahm and Brezner (24, 25) determined a molecular weight of 430,000 in light scattering experiments; and, quite differently, Kielley and Harrington (9) proposed a value as high as 619,000, based on Archibald experiments and compiled sedimentation, viscosity, and diffusion data on myosin. The 600,000 value was confirmed in subsequent Archibald and low-speed sedimentation equilibrium experiments (13), and this value seems to have been widely accepted as the correct molecular weight of rabbit skeletal myosin (35–38).

Concurrence was not, however, unanimous. Noting that the Kielley-Harrington data had been obtained on myosin prepared by ammonium sulfate fractionation, Holtzer, Lowey, and Schuster (11) questioned whether the high salt concentration required for the fractionation procedure might lead to aggregation of protein. The point was not further pursued. Indeed, the explanation seemed unlikely following a new light scattering study (12) on myosin prepared by ammonium sulfate fractionation. Gellert and Englander (12) carried out this work with meticulous attention to turbidity, concentration, and refractive increment measurements; and the data indicated a molecular weight value of 520,000 for myosin, in close agreement with the Holtzer-Lowey data (10, 11) on myosin prepared without ammonium sulfate fractionation.

The difference between 500,000 and 600,000 is beyond reasonable error, and the experiments (9–13) were technically more than adequate. It was suggested (36) that the difference in results might be related to the method employed, light scattering giving a 500,000 value and equilibrium centrifugation a 600,000 value. This conclusion would be disheartening, however, since light scattering and equilibrium centrifugation have sound foundations in thermodynamics, and nothing can be more correct than classical thermodynamics. Fortunately, the difficulty was resolved by an unexpected observation.

Gellert and Englander (12) had used the standard Brice-Phoenix cylindrical cell in their light scattering experiments. The cell has a frosted surface designed to eliminate light reflection from the rear surface, and scattering has been calculated assuming negligible back-reflection (39). Tomimatsu and Palmer (40) observed, however, that light is reflected from the frosted surface, and indicated that the proper correction for light reflection is quite different from the customary correction (39). As an example demonstrating the importance of a proper reflection correction, Tomimatsu (41) applied the revised correction to the Gellert-Englander data on myosin, and calculated a molecular weight value of 600,000. Tomimatsu noted that values of molecular weight were now 600,000 for all myosin preparations employing ammonium sulfate fractionation (Fig. 2). The Holtzer-Lowey data (10, 11) had been

obtained with a different kind of scattering cell, and did not require revision (42). A revised correction is apparently required for the data of Brahms and Brezner (24, 25), and would increase the calculated molecular weight value from 430,000 to about 500,000.

Meanwhile, in studies on a conventional preparation of myosin, Mueller (26) determined a molecular weight value of 520,000 from Archibald measurements obtained at multiple field strengths during each experiment. Mueller (26) also demonstrated that the apparent molecular weight is properly referred to the meniscus concentration of protein, and thus clarified discrep-

#### MOLECULAR WEIGHT OF RABBIT SKELETAL MYOSIN

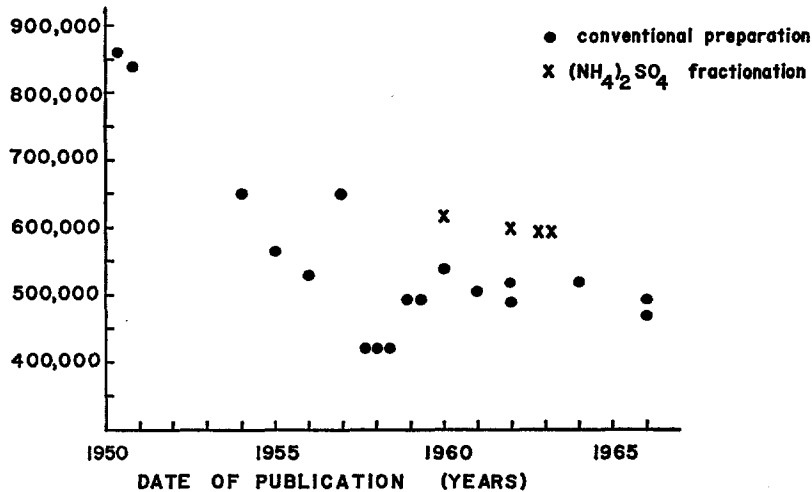


FIGURE 2. Reported values for the molecular weight of rabbit skeletal myosin, based on method of preparation. Light scattering data were corrected for back-reflection (40, 41).

ancies in earlier data, where the apparent molecular weight had been referred to initial protein concentration (18). In other work on conventional preparations of myosin, Tonomura, Appel, and Morales (21) reported a molecular weight of 470,000–510,000, based on osmotic pressure and low speed sedimentation equilibrium.

Thus, neglecting early studies before 1959, there is general agreement that myosin prepared by the conventional Szent-Györgyi procedure has a molecular weight of about 500,000, and that myosin prepared with ammonium sulfate fractionation has a molecular weight of about 600,000. But which value is correct? The 600,000 figure is based on several weight average determinations, and might possibly reflect aggregation of myosin during the purification procedure. The question is open to experimental inquiry, and one refers to the sedimentation velocity data on myosin prepared using am-

monium sulfate fractionation. Unfortunately, none are available in the reported molecular weight studies on rabbit skeletal myosin prepared by this method (9, 12, 13).

In work in our laboratory, myosin prepared by the conventional Szent-Györgyi method usually shows a single sharp peak on sedimentation (Fig. 3 *a, b*). In myosin prepared by ammonium sulfate fractionation, there is a discrete leading peak (Fig. 3 *c*), indicating aggregation of about 5–10% of the total protein (43). Thus, ammonium sulfate fractionation does seem to enhance aggregation of myosin. This effect presumably accounts for the higher (weight average) molecular weight values reported for myosin prepared by this method.

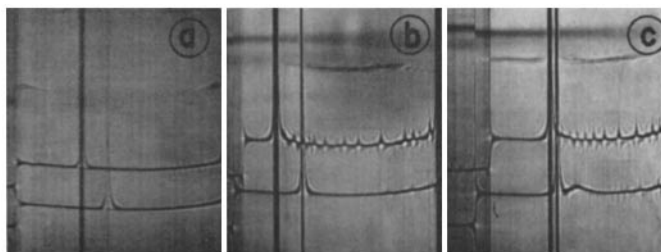


FIGURE 3. Sedimentation velocity of rabbit skeletal myosin in 0.4 M KCl at pH 6.5, 52,640 rpm, 4°C, phase plate angle 70°. *a*, conventional preparation: upper, 9.2 mg/ml; lower, 3.6 mg/ml. *b*, conventional preparation: upper, 26 mg/ml; lower, 4.5 mg/ml. *c*, ammonium sulfate fractionation (40–50% fraction) of same preparation as *b*: upper, 15 mg/ml; lower, 8.5 mg/ml.

One might then question whether spontaneous aggregation may not occur to some extent in all preparations of myosin, and whether the correct molecular weight of myosin is even lower than 500,000. This value is based on number average (osmotic pressure) and weight average methods (light scattering, low-speed sedimentation equilibrium, and Archibald), in which the occurrence of low *n*-mers of myosin would increase the average molecular weight.

The question has been explored in high-speed sedimentation equilibrium experiments by the method of Yphantis (44). The procedure might be expected to resolve low *n*-mers from monomer myosin, and yield a limiting value for monomer molecular weight. Data from one of the experiments (45) are shown in Fig. 4, where concentration is plotted on a logarithmic scale against the square of the radial distance. The theoretical equilibrium time (46) is about 50 hr, at which time the limiting slope (at low concentration) yields a molecular weight value of 470,000. An increase in slope at higher radial distances indicates the presence of low *n*-mers of myosin. At concentrations less than 1 mg/ml (about 4.5 fringes displacement), corrections for non-

ideality are small and would increase the calculated molecular weight by at most 1–2%. On prolonged centrifugation, there is a shift in the concentration distribution towards the bottom of the cell, presumably as a result of continuous aggregation; however, the limiting slope is only slightly increased, yielding a molecular weight value of 500,000 after 90 hr of centrifugation. In several similar experiments, the limiting slope indicates a molecular weight value between 470,000 and 490,000 (45). It should be noted that the limiting slope would not yield a proper value for the monomer molecular weight if significant amounts of low molecular weight contaminants were

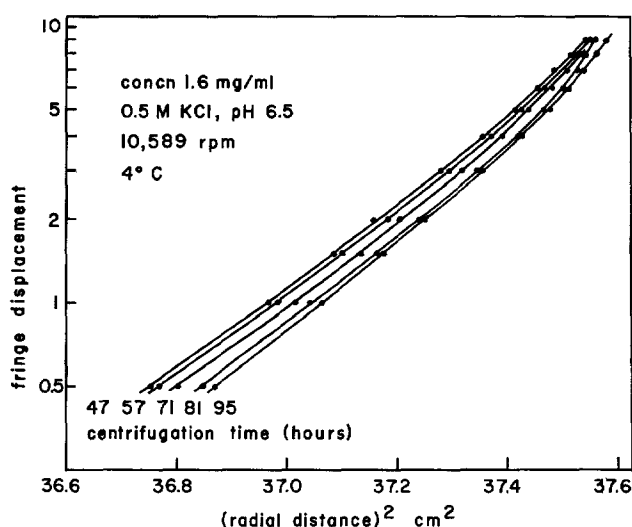


FIGURE 4. Sedimentation equilibrium of rabbit skeletal myosin in 0.4 M KCl at pH 6.5, 10,500 rpm, 4.0°C, double-sector cell with charcoal-filled Epon centerpiece. From Gershman (45).

present. No such material is demonstrable in conventionally prepared myosin in 0.5 M KCl, based on sedimentation equilibrium experiments at rotor speeds as high as 42,000 rpm (45, 47) and sedimentation velocity experiments at high protein concentrations (Fig. 3).

We may reasonably conclude that myosin, at least the protein isolated in the laboratory from rabbit skeletal muscle, has a molecular weight slightly less than 500,000, with uncertainty of 3–4%.

#### SUBUNITS OF MYOSIN

The breakdown of myosin B, natural actomyosin, into smaller components by urea or guanidine was early noted (48, 49), and the effect was found to be a property of myosin itself (30). An interesting study on the subunit composition of myosin was carried out by Tsao (50), working in Kenneth Bailey's labora-

tory at Cambridge. Rabbit skeletal myosin was dissociated in 6.7 M urea. Maximal dissociation required 12–16 months, after which time the protein was fractionated by ammonium sulfate precipitation. The major fraction, about 92% of the protein, seemed to be aggregated in solution at pH 7. At pH 10.7, the component was found to have a particle weight of 164,000–184,000, based on osmotic pressure and fluorescence depolarization measurements. The minor fraction, about 8% of the total protein, had a particle weight of about 16,000 on fluorescence depolarization. Myosin stored in alkaline solution at pH 10.7 for 10 days was found to have an average weight of 170,000 from osmotic pressure measurements.

Tsao (50) concluded that urea detaches a number of small subunits from myosin, and that dilute alkali depolymerizes the main core of myosin into large subunits of 165,000 particle weight. The accepted value, in 1952, for the molecular weight of myosin was 850,000, and Tsao inferred that each myosin molecule contains four to five large subunits.

This work (50) may be criticized on several points. Sedimentation velocity experiments were not reported on native myosin, and the light component isolated in urea solution may possibly represent impurities in the original preparation. Either disulfide-linked aggregation or fragmentation on the hydrolysis of peptide bonds might conceivably occur during the prolonged time required for dissociation. Fluorescence depolarization is ambiguous in determining molecular weight, since approximations are required for molecular shape and volume. Osmotic pressure yields a number average molecular weight, and the unfractionated protein at pH 10.7 may contain heavy and light components, each with particle weight far different from 170,000. Finally, the number of heavy subunits per molecule was based on an incorrect value for the molecular weight of myosin. These are cogent criticisms, and subsequent work has led to quite different conclusions on the subunit structure of myosin. Nevertheless, the general implications of Tsao's work are of great interest.

The question of alkali dissociation of myosin was next studied by Kominz, Carroll, Smith, and Mitchell (51). Preparations of myosin isolated by several methods, including ammonium sulfate fractionation, were dialyzed against 0.1 M sodium carbonate at about pH 10. On sedimentation (Fig. 5), there is a main peak similar to the sharp peak of myosin at pH 7, and also a slow component. From boundary areas corrected for radial dilution, the slow component was found to be 14–18% of the total protein. The main component was not further characterized; however, extensive studies were done on the slow component isolated by ammonium sulfate fractionation. On sedimentation, the purified slow component showed a single peak of 2.3S, and its molecular weight was estimated as 29,000 from sedimentation-diffusion. The diffusion coefficient was determined from boundary spreading measurements during

sedimentation, corrected only for radial dilution, and the calculated molecular weight value is a rough approximation. Although the 29,000 value is nearly twice the molecular weight of the light component isolated in urea solution by Tsao (50), the difference may be within over-all uncertainty, since Middlebrook and Szent-Györgyi (unpublished data; reference 52) reported isolation from myosin in urea solution of a light component with a molecular weight of about 30,000. No details, however, have been published on this observation.

The light component isolated at alkaline pH was found to have a C-terminal group, isoleucine (51). Isoleucine had previously been described as the C-terminal group of myosin (53), and more recently has been found to be the C-terminal group of H-meromyosin (54).

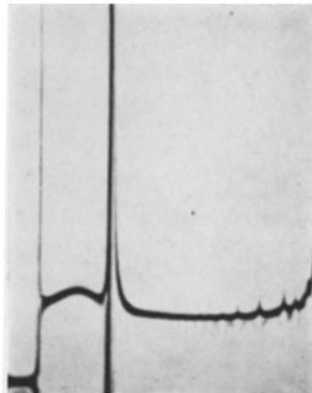


FIGURE 5. Sedimentation velocity of rabbit skeletal myosin at about pH 10. Initial concentration, 9 mg/ml. 59,780 rpm, 25°C, bar angle 60°C. *Figure reprinted by permission from Archives of Biochemistry and Biophysics, 1959, 79:191.*

The amino acid compositions of the light components isolated at alkaline pH and in urea solution were also determined (51). Comparison with the amino acid composition of the meromyosins is of interest (43), and Table I summarizes data on those residues which are significantly different in L-meromyosin and in H-meromyosin. There are obvious similarities in the amino acid composition of the light alkali component, the light urea component, and H-meromyosin, and values essentially overlap for 7 of the 8 residues. Only the tyrosine content of the light components is similar to that of L-meromyosin.

Mueller and Perry (59, 60) have shown that H-meromyosin can be degraded into a smaller fragment, termed subfragment 1, that retains the ATPase activity and the actin-combining property of myosin. Subfragment 1 has an amino acid composition generally similar to that of H-meromyosin, and Jones and Perry (58) have noted that the greatest difference between subfragment 1 and H-meromyosin is in tyrosine content, which is significantly



greater in subfragment 1. The light components, however, have a low tyrosine content.

The data on C-terminal group and amino acid composition suggest that light components are not casual contaminants of myosin and, further, that light components are found in H-meromyosin and may be absent from subfragment 1. Much of these data were obtained after the original work on the light alkali component (51), and their implications were by no means evident in 1960, when a crucial study (9) on the subunit composition of myosin was reported.

Kielley and Harrington (9) found that myosin is dissociated into subunits after dialysis against 5 M guanidine for several days. Solutions of myosin in 5

TABLE I  
AMINO ACID COMPOSITION

All data are given in moles/10<sup>5</sup> g.

Amino acid (Reference)...	L-meromyosin		Light components		H-meromyosin			Subfragment 1		
	(55)	(18)	Alkali (51)	Urea (51)*	(55)	(18)	(56)	(57)	(56)	(58)
Proline	8	7	38	35	29	32	24	26	21	30
Glycine	24	22	55	57	45	50	50	53	51	55
Methionine	14	15	26	27	19	26	28	27	28	29
Phenylalanine	10	6	38	37	40	36	38	43	41	46
Tyrosine	12	10	14	10	21	21	20	26	25	31
Glutamic acid	174	198	126	116	138	137	157	130	131	126
Arginine	51	57	24	24	29	34	30	25	29	32
Histidine	19	20	8	9	12	14	14	13	16	15

\* W. R. Middlebrook and A. G. Szent-Györgyi. Unpublished data cited by Szent-Györgyi (52).

M guanidine showed, on sedimentation, a single peak with a sedimentation coefficient of 4S. The particle weight of myosin in 5 M guanidine was estimated as 181,000 from sedimentation-diffusion and as 197,000 from sedimentation-viscosity. But the most precise characterization was obtained from Archibald studies, which indicated a strong dependence of apparent molecular weight on initial protein concentration, extrapolating to a molecular weight value of 206,000. This finding, along with a 619,000 value for the molecular weight of intact myosin, led to the conclusion (9) that myosin is composed of three equal weight polypeptide chains that are dissociated by 5 M guanidine.

The three-chain model is a reasonable hypothesis, and one question immediately whether myosin is actually monodisperse in 5 M guanidine. In probing this question, heavy reliance was placed on the Archibald data, particularly on the stability of apparent molecular weight at the meniscus during prolonged centrifugation. Although the finding was considered a good

test for homogeneity (9), this interpretation seems dubious (43). As evident from Archibald studies on intact myosin (26), prolonged centrifugation leads to continuous depletion of protein at the meniscus. If myosin in 5 M guanidine were monodisperse with a positive second virial coefficient, then the fall in meniscus concentration with time should lead to a continuous increase in apparent molecular weight at the meniscus. This was not, however, found to be so. In one experiment, for example, the apparent molecular weight at the meniscus remained at 75,000 during 26 hr of centrifugation (9). The stability

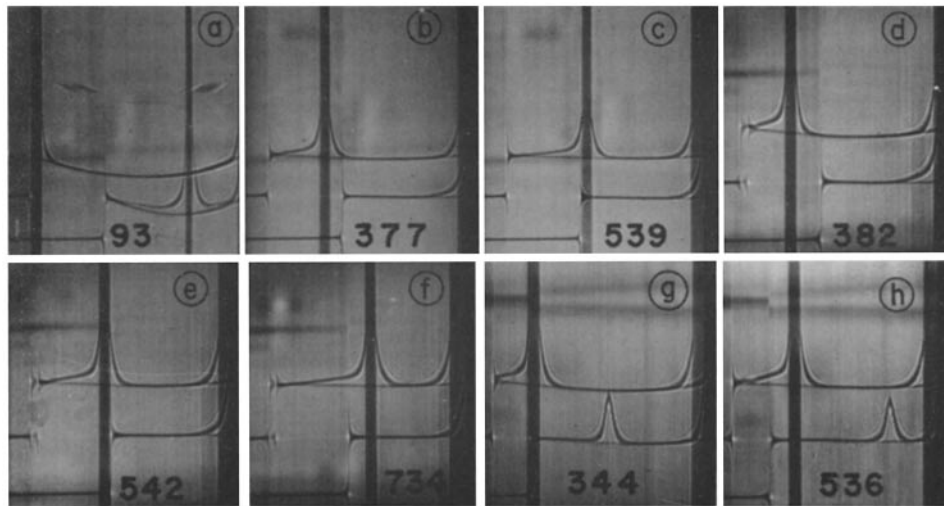


FIGURE 6. Sedimentation velocity of rabbit skeletal myosin in 5 M guanidine·HCl-0.4 M KCl at pH 5.6. 52,640 rpm, 20°C, phase plate angle 70°. *a*, *b*, and *c*, myosin (reduced and carboxymethylated), 11.6 mg/ml; lower patterns from synthetic boundary experiment. *d*, *e*, and *f*, myosin (carboxymethylated), 12 mg/ml; lower patterns from synthetic boundary experiments. *g* and *h*, ammonium sulfate preparation of myosin (reduced and carboxymethylated): upper, 14.3 mg/ml; lower, 4.7 mg/ml. Times of centrifugation are indicated in minutes. Figure 6 *a-c* reprinted by permission from *The Journal of Biological Chemistry*, 1966, 241:443.

of apparent molecular weight at the meniscus throughout this period is rather suggestive that heavy material is preferentially sedimenting from the meniscus, leaving behind an increasing proportion of light material.

In subsequent low-speed sedimentation equilibrium experiments on myosin in 5 M guanidine, single values of weight average apparent molecular weight were calculated from each experiment (13). The data extrapolated to a weight average molecular weight of 197,000. This result, in conjunction with the earlier sedimentation-diffusion, sedimentation-viscosity, and Archibald data (9), provides fairly strong evidence that the *average* molecular weight of myosin in 5 M guanidine is about 200,000. It is much less certain, however, that myosin is monodisperse in 5 M guanidine.

Some question was introduced by Wetlaufer and Edsall (61), who described extensive heterogeneity on sedimentation of myosin in 8 M urea. This was shown to represent incomplete dissociation of myosin, since a single sedimenting boundary was found in 12 M urea (62). Furthermore, a single band was observed on acrylamide gel electrophoresis of myosin in 12 M urea (62). Although Wetlaufer and Edsall (61) also noted that myosin in 5 M guanidine has a slowly sedimenting peak representing about 10% of the total area, this seems

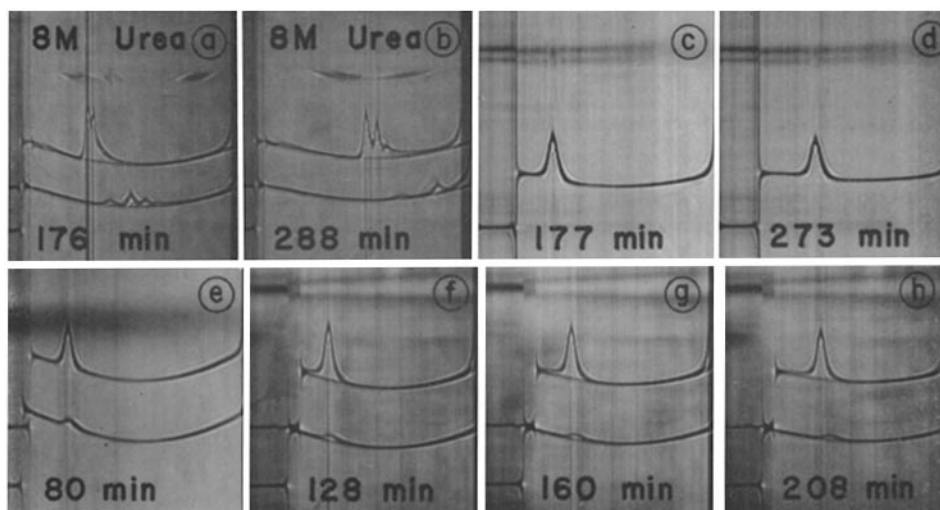


FIGURE 7. Sedimentation velocity of rabbit skeletal myosin (reduced and carboxy-methylated). 52,640 rpm, 20°C, phase plate angle 70°. Solvent is 8 M urea for *a* and *b*, and 5 M guanidine·HCl-0.4 M KCl for others. *a* and *b*: upper, 10.5 mg/ml; lower, 5.0 mg/ml (from Gershman, 45). *c* and *d*, 5.3 mg/ml. *e*: upper, 3.3 mg/ml; lower, 0.97 mg/ml. *f*, *g*, and *h*, ammonium sulfate preparation: upper, 4.1 mg/ml; lower, 0.69 mg/ml.

to have been considered an impurity. Thus Woods, Himmelfarb, and Harrington (13) quite reasonably concluded that the cumulative results indicate myosin to be composed of three polypeptide chains of roughly equal mass, and this conclusion has been widely accepted (35, 36, 38, 63-66).

#### HEAVY AND LIGHT GUANIDINE COMPONENTS

Essential to the three-chain model is proof of the homogeneity of the myosin subunit in 5 M guanidine, and the problem has been explored in our laboratory (67). The sedimentation properties of myosin in 5 M guanidine were found to be essentially identical with those earlier reported (9, 62). The schlieren patterns show a single peak (Figs. 6 and 7), with a sedimentation coefficient of 3.5S (Fig. 8). Yet the schlieren patterns are by no means characteristic of a monodisperse system. The marked concentration dependence of sedimentation (Fig. 8) should result in self-sharpening of the main peak, with negligible

trailing, in a monodisperse system. In the experiments at high concentration (Fig. 6), however, there is obvious trailing behind the main boundary most evident after prolonged centrifugation (*c, f, h*). The trailing is quite suggestive of an additional slow component with a sedimentation coefficient of about 1–2S. At lower protein concentrations (Fig. 7), the trailing is less obvious (*f, g, h*), and, in view of extensive solvent redistribution, the trailing is not at all evident in the absence of a simultaneous solvent base line (*c, d, e*). Previous sedimentation experiments taken to imply homogeneity of myosin in 5 M guanidine seem to have been usually done in single-sector cells at low protein concentrations (62), conditions under which possible heterogeneity is not manifest. Thus the present sedimentation data, while no different from previ-

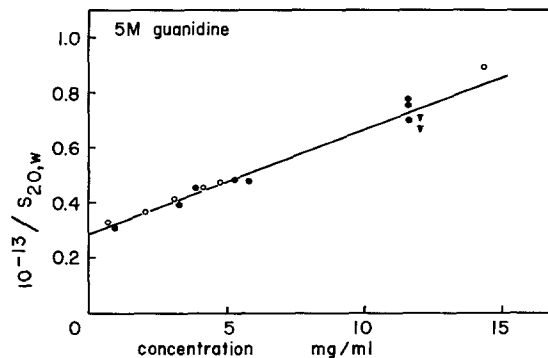


FIGURE 8. Concentration dependence of sedimentation coefficient for rabbit skeletal myosin in 5 M guanidine·HCl–0.4 M KCl. ●, reduced and carboxymethylated myosin, ▼, carboxymethylated myosin; ○, reduced and carboxymethylated myosin from ammonium sulfate preparation. Data include five points from reference 67.

ous data (9, 62), do suggest that myosin is actually polydisperse in 5 M guanidine (67).

The suspicion of heterogeneity is readily confirmed on high-speed sedimentation equilibrium of myosin in 5 M guanidine. In an experiment at 1 mg/ml (Fig. 9), the sigmoid concentration distribution indicates nonideality at high concentration and heterogeneity from an additional light component at low concentration. The two effects are evident in the plot of apparent molecular weight against protein concentration. The early experiments indicated a heavy component with molecular weight between 190,000 and 260,000, and a light component with a molecular weight of the order of 46,000 (67). These are crude estimates, depending largely on schlieren data which demonstrate heterogeneity convincingly but do not very well characterize the light and heavy guanidine components.

In further experiments on myosin in 5 M guanidine, the use of higher protein concentrations and higher rotor speeds has led to better resolution of the light

component, and analysis of fringe patterns at widely different field strengths has permitted more exact characterization of the heavy component (44, 45, 47). Fig. 10 shows data from an experiment at three rotor speeds over a 12-fold range of field strength. At 37,010 rpm ( $\omega_2$ ), the concentration distribution (upper graph) indicates a light component with a molecular weight of about

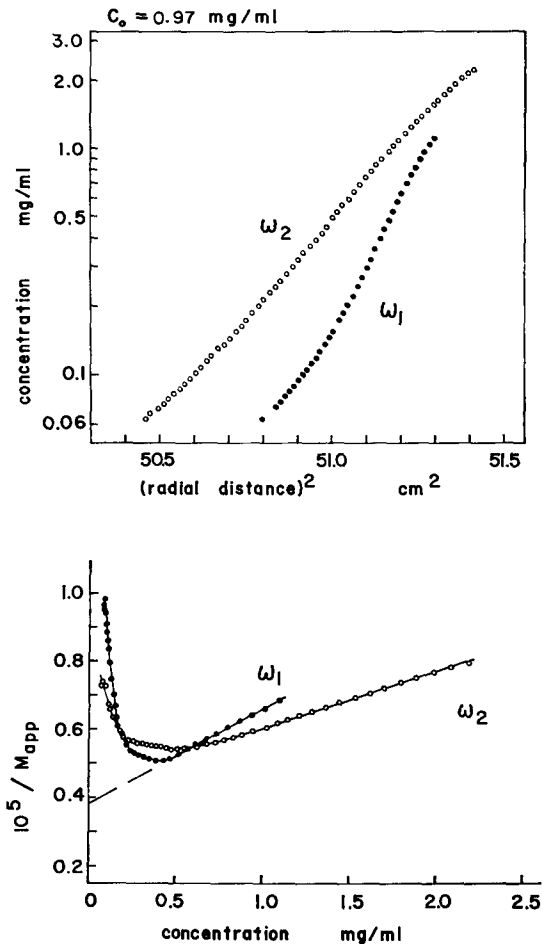


FIGURE 9. Sedimentation equilibrium of rabbit skeletal myosin (reduced and carboxymethylated) in 5 M guanidine-HCl-0.4 M KCl. Initial concentration, 0.97 mg/ml.  $\omega_1$ , 29,500 rpm;  $\omega_2$ , 23,150 rpm. 4.0°C. Figure reprinted by permission from *The Journal of Biological Chemistry*, 1966, 241:443.

21,000. Assuming ideal behavior of the light component, its proportion was estimated as 11% of the total protein. On subtracting the contribution of light component from the total concentration, the residual material has a molecular weight of  $1.9 (\pm 0.3) \times 10^5$ . At 29,500 rpm ( $\omega_1$ ), after correcting meniscus concentration from the  $\omega_2$  data, the concentration distribution is consistent with a light component of molecular weight about 21,000, and a residual heavy component of molecular weight  $2.0 (\pm 0.1) \times 10^5$ . At 10,650 rpm ( $\omega_3$ ), after subtracting the contribution of light component based on the

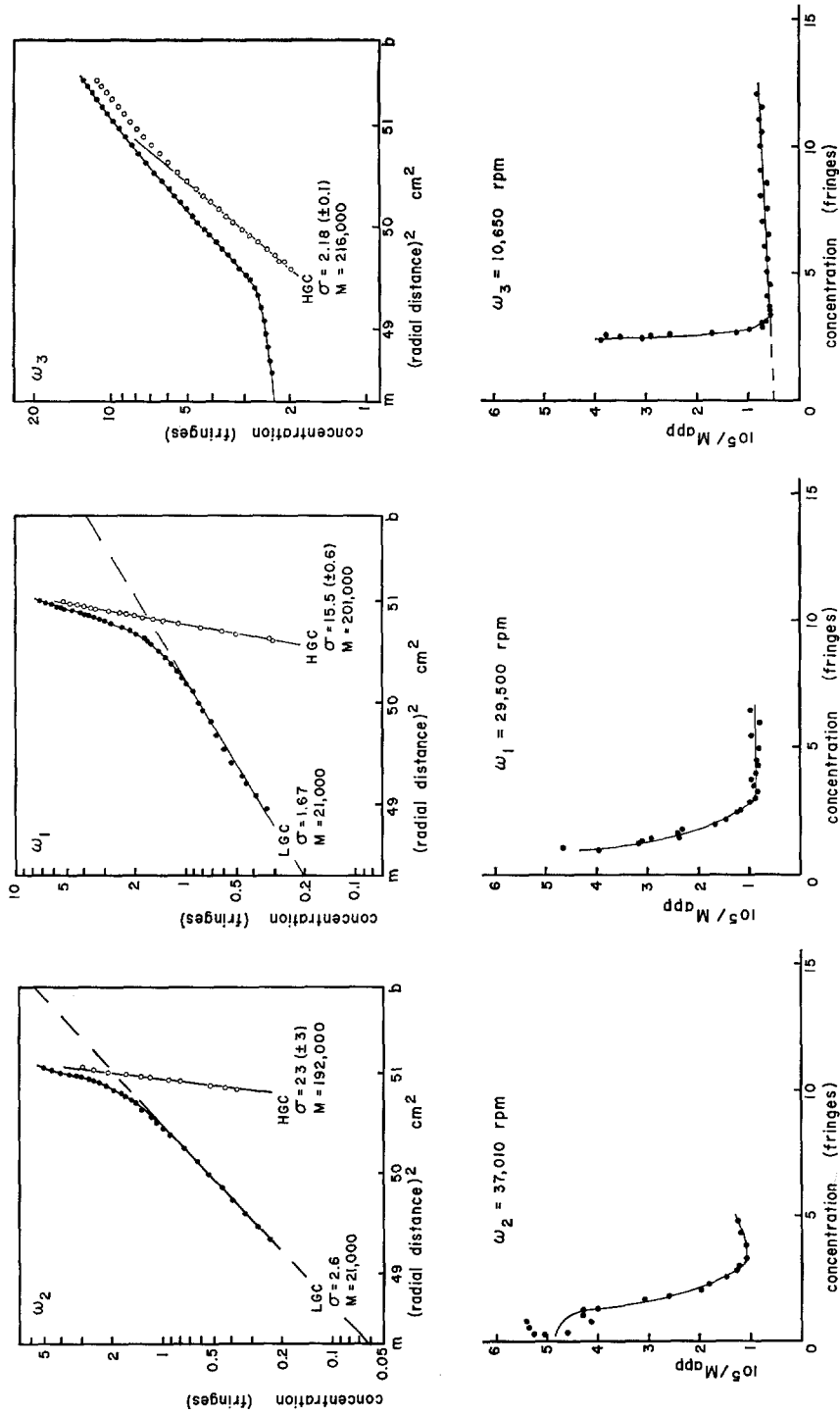


FIGURE 10. Sedimentation equilibrium of rabbit skeletal myosin centerpiece. Rotor speeds are indicated. LGC, light guanidine (ammonium sulfate preparation, reduced and carboxymethylated) component; HGC, heavy guanidine component;  $m$ , meniscus; in 5 M guanidine  $\cdot$  HCl-0.4 M KCl at pH 5.5. Initial concentration,  $b$ , bottom. Apparent molecular weight values were calculated 2.5 mg/ml. 4.0°C. Double-sector cell with charcoal-filled Epon using an Olivetti-Underwood 101 computer.

$\omega_2$  data, the residual heavy component has a limiting molecular weight of  $2.2 (\pm 0.1) \times 10^5$ . The molecular weight values of the heavy component determined at  $\omega_1$  and  $\omega_3$  are the more reliable, in view of the high  $\sigma$  value (approximately 23) at  $\omega_2$ .

The lower graphs (Fig. 10) show the concentration dependence of apparent molecular weight. At the higher speeds, the predominant contribution is from light guanidine component, while at low speed ( $\omega_3$ ), there is extensive admixture of heavy and light guanidine components. Extrapolation of the high concentration data ( $\omega_3$ ) to infinite dilution yields a value somewhere between the weight average molecular weight for the entire system and the molecular weight of the heavy guanidine component; the extrapolated value is  $2.0 (\pm 0.1) \times 10^5$  in this experiment.

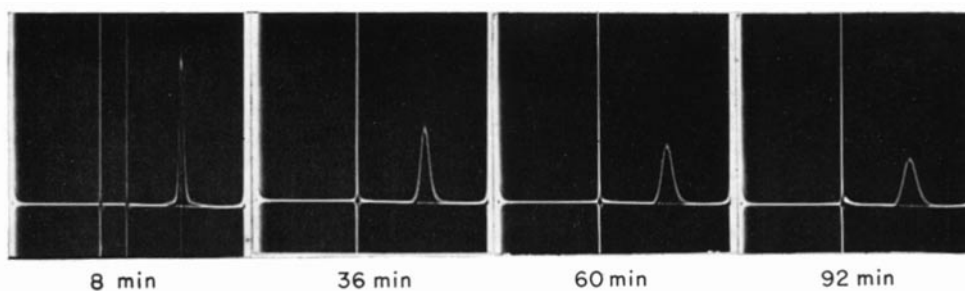


FIGURE 11. Synthetic boundary experiments on rabbit skeletal myosin (reduced and carboxymethylated) in 5 M guanidine·HCl-0.4 M KCl. Initial concentration, 5.4 mg/ml. 5563 rpm, 4°C, phase plate angle 70°. Times from formation of boundary are indicated.

Data from a number of experiments indicate identical polydispersity in 5 M guanidine for myosin prepared with and without prior ammonium sulfate fractionation. The proportion of light component, analyzed as in Fig. 10 (45, 47), is about 10–15% of the total protein, a value somewhat less than the preliminary estimate of 17% (67). Most work was done on reduced and carboxymethylated myosin, in order to protect exposed sulfhydryl groups; heterogeneity was found without prior reduction, indicating that light and heavy guanidine components are not linked by disulfide bonds.

Studies were also done on the diffusion properties of myosin in 5 M guanidine. The schlieren patterns, shown for a synthetic boundary experiment at 5.4 mg/ml (Fig. 11), indicate a symmetrical boundary that might perhaps be taken to imply homogeneity of the protein. A more refined analysis of the boundary, based on the method of Creeth and Gosting (68), indicates that the protein distribution is actually heterogeneous (Fig. 12). In the lower graphs, the parameter  $Z^* \sqrt{t}$  is plotted against radial distance. A monodisperse system would be linear throughout, whereas the data indicate a small

amount of rapidly diffusing component on the shoulders of the boundary. The preliminary data from a number of experiments indicate values for the diffusion coefficient,  $D_{20,w}$ , of  $1.2\text{--}2.0 \times 10^{-7}$  cm<sup>2</sup>/sec for the main component, and  $5\text{--}8 \times 10^{-7}$  cm<sup>2</sup>/sec for the rapidly diffusing component. In addition, the proportion of rapidly diffusing component diminishes with increase in the initial protein concentration, suggesting that at high concentration (12–20 mg/ml) myosin may not be fully dissociated in 5 M guanidine under the low field conditions obtained in the synthetic boundary experiments.

Evidence from several different methods hence demonstrates heterogeneity of myosin in 5 M guanidine, and further suggests that myosin dissociates into a

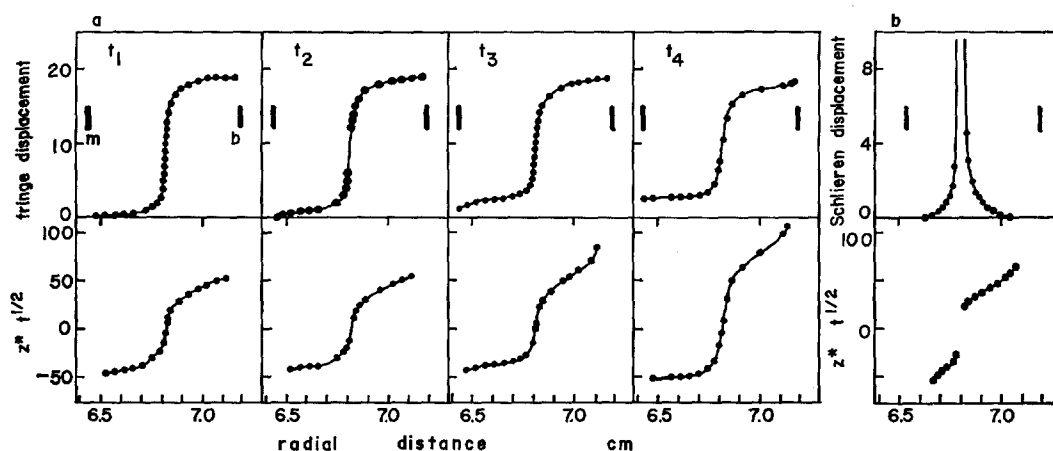


FIGURE 12. Boundary spreading of rabbit skeletal myosin in 5 M guanidine·HCl-0.4 M KCl, analyzed from synthetic boundary experiments, at 5563 rpm, by the method of Creeth and Gosting (68). *a*, initial concentration, 4.6 mg/ml; 8, 10, 18, and 40 min after formation of boundary. *b*, initial concentration, 19.1 mg/ml; 73 min after formation of boundary. *m*, meniscus; *b*, bottom.

heavy component with molecular weight slightly over 200,000 and a light component (about 10–15% of the protein) with a molecular weight of the order of 20,000. Assuming a molecular weight of 500,000 for myosin, there would seem to be two heavy subunits and about two to four light subunits per myosin molecule (43). These values are approximate, since heavy and light guanidine components were not fully resolved by any of the methods so far described.

#### HEAVY AND LIGHT ALKALI COMPONENTS

One returns with interest to the dissociation of myosin at alkaline pH. There are several questions. Is the low molecular weight component described at alkaline pH (51) a casual contaminant of myosin? Is the material the same as the light component in 5 M guanidine? Does the myosin core depolymerize at



alkaline pH (50)? After all, the sedimentation patterns of the main component (Fig. 5) appear rather similar to those of native myosin (Fig. 3). Guanidine and urea solutions, which act on water structure and hydrophobic and hydrogen bonds (69, 70), are clearly different dissociating conditions from alkaline pH, which acts through electrostatic charging of basic residues.

These considerations prompted further studies on the properties of myosin at alkaline pH (45, 47). Sedimentation velocity experiments indicate consistent dissociation of a slow component from myosin at pH above 10 (Fig. 13). The schlieren patterns are similar to those earlier reported (51; see Fig. 5); there is a self-sharpened main peak and a discrete slow peak (Fig. 13 *b, c*). The sedimentation properties of myosin at alkaline pH are notably different from those of myosin in 5 M guanidine (62, 67; see Figs. 6 and 7), where the main and trailing boundaries closely overlap.

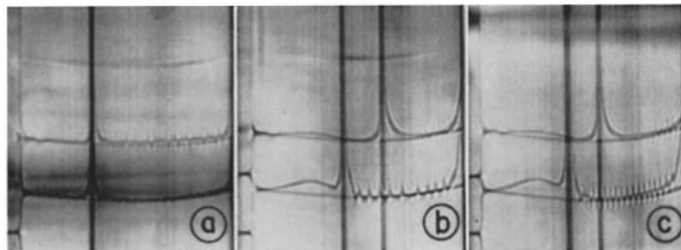


FIGURE 13. Sedimentation velocity of rabbit skeletal myosin at alkaline pH. 56,100 rpm, 4°C, phase plate angle 70°. *a*: upper, pH 9.5, 10.7 mg/ml; lower, pH 6.5, 13.7 mg/ml. *b*, pH 11.0: upper, 9.0 mg/ml; lower, 17.5 mg/ml. *c*, pH 12.5: upper, 9.0 mg/ml; lower, 18.4 mg/ml. Figure reprinted by permission from *Proceedings of the National Academy of Sciences*, 1966, 56:966.

The heavy alkali component has a marked concentration dependence, with a sedimentation coefficient of 5.7S, and the light alkali component has a sedimentation coefficient of 1.6S (Fig. 14). From area measurements corrected only for radial dilution, the proportion of light alkali component is about 17%, a value within the range of 14–18% reported previously (51). The concentration dependence of sedimentation might be expected, however, to cause a significant Johnston-Ogston effect (71), and, indeed, the proportion of light alkali component is found to be only 12.2% of the total protein after appropriate correction for both radial dilution and the Johnston-Ogston effect (71, 72).

The heavy and light alkali components are readily characterized on high-speed sedimentation equilibrium (47). In the experiment shown in Fig. 15, the graph of total protein indicates obvious heterogeneity at 13,340 rpm. On equilibrium centrifugation at higher rotor speeds (35,690 rpm and 42,010 rpm), the predominant material is light component, with a molecular

weight of 19,300; its proportion was estimated as 10.4% of the total protein. The contribution from light alkali component was subtracted from the total concentration for the data at 13,340 rpm, and the residual heavy alkali component has a limiting molecular weight of 418,000.

From data in several experiments at pH 11–12.5, the light alkali component has a molecular weight of 20,000 and comprises about 11.6% of the total protein, and the heavy alkali component has a molecular weight of  $4.3 (\pm 0.2) \times 10^5$ . The sedimentation velocity and sedimentation equilibrium data hence

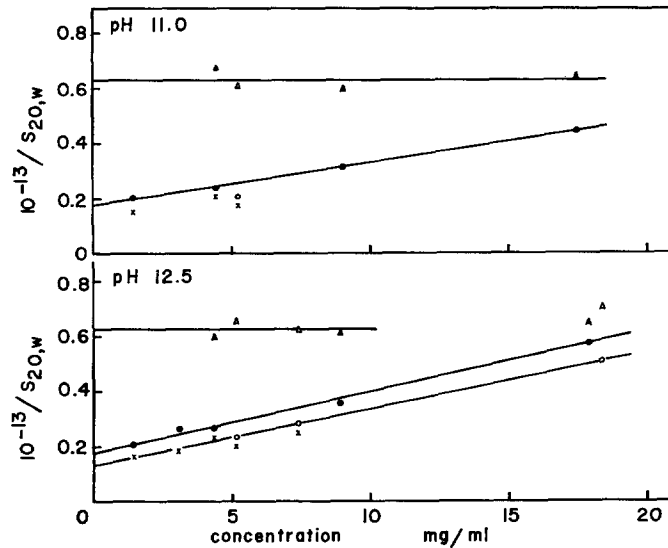


FIGURE 14. Concentration dependence of sedimentation coefficient for rabbit skeletal myosin at alkaline pH.  $\Delta$ , light alkali component;  $\circ$ , heavy alkali component;  $\blacktriangle$ , carboxymethylated light alkali component;  $\bullet$ , carboxymethylated heavy alkali component;  $\times$ , aggregated heavy alkali component. *Figure reprinted by permission from Proceedings of the National Academy of Sciences, 1966, 56:966.*

indicate that, at pH above 10, a light component is detached from the intact main core of the myosin molecule. If myosin has a molecular weight of 500,000 and contains 12% by weight of light alkali component, of mol wt 20,000, there are three light subunits on the average per myosin molecule (45, 47).

It now becomes critical to fractionate heavy and light alkali components and to characterize them in some detail. In fractionating the alkali components of myosin, our aim was to obtain total recovery and to minimize spontaneous aggregation of the heavy alkali component. A straightforward solubility method was developed (47), analogous to the original method of A. G. Szent-Györgyi (8) for separation of the meromyosins. At pH 11–12.5, myosin is soluble at ionic strength as low as 0.05. On decrease to neutral pH, the

protein remains soluble at 0.5 ionic strength; but on decrease to 0.05 ionic strength, there is prompt precipitation of a major component. The results from the first use of the fractionation procedure are shown in Fig. 16 *a*. The precipitate obtained at low ionic strength and neutral pH was dialyzed at pH 8, and on sedimentation contained a heavy component, but no light compo-

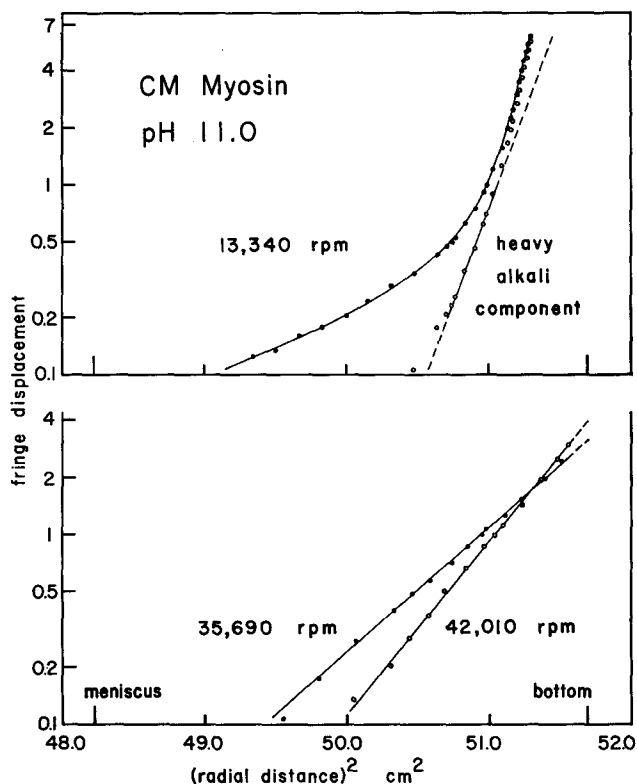


FIGURE 15. Sedimentation equilibrium of rabbit skeletal myosin at pH 11.0. Initial concentration, 3 mg/ml. 4.0°C. Upper, 13,340 rpm; lower, 35,690 rpm and 42,010 rpm. Figure reprinted by permission from *Proceedings of the National Academy of Sciences*, 1966, 56:966.

nent. The supernatant was lyophilized and then dialyzed at pH 8, and showed a small peak, taken to represent light alkali component.

Although this experiment suggests that fractionation is total, further work indicated residual light alkali component in the precipitate fraction. The procedure was modified to include three fractionation cycles on the precipitate (Table II). About half the residual light component is removed with each fractionation cycle, and after three cycles the proportion of light alkali component in the precipitate fraction is reduced to less than 1%.

On sedimentation, the purified heavy alkali component has properties similar to those of unfractionated heavy alkali component (Fig. 16). On

sedimentation equilibrium, the heavy alkali component in first-cycle precipitate has a molecular weight of about  $4.3 \times 10^6$ . Further purification leads to extensive aggregation of the heavy alkali component, and the limiting molecular weight is of the order of 1,000,000 in third-cycle precipitate.

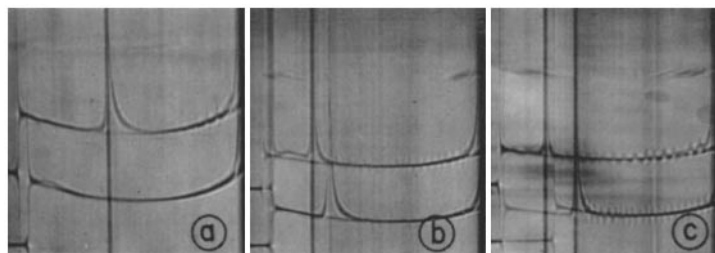


FIGURE 16. Fractionation of heavy alkali component at 4.0°C. *a*: upper, first-cycle precipitate, at pH 8; lower, first-cycle supernatant, at pH 8; 59,780 rpm, phase plate angle 65°. *b*, first-cycle precipitate, at pH 11: upper, 10 mg/ml; lower, 5 mg/ml; 50,740 rpm, phase plate angle 70°. *c*, third-cycle precipitate, at pH 11: upper, 17 mg/ml; lower, 9.4 mg/ml; 50,740 rpm, phase plate angle 70°. From Gershman (45).

The supernatant fractions containing light alkali component were found to be monodisperse in sedimentation velocity experiments, with a sedimentation coefficient of 1.9S. A molecular weight of 20,000 was determined by high speed sedimentation equilibrium. On electrophoresis on cellulose acetate, the

TABLE II  
FRACTION OF LIGHT ALKALI COMPONENT  
FROM SEDIMENTATION VELOCITY

Carboxymethylated myosin. Calculations as in reference 47. Data from Gershman (45).

Fraction	Corrected for	Corrected for
	radial dilution	radial dilution and Johnston-Ogston effect
	%	%
Myosin, pH 11.0	17.2	12.4
1st-cycle precipitate, pH 11.0	10.3	6.5
2nd-cycle precipitate, pH 11.0	4.5	3.1
3rd-cycle precipitate, pH 11.0	0.7	0.6
Myosin from pH 11.0 to pH 8.0 for		
1 hr	15.3	13.4
2 days	8.8	8.0

light alkali component is heterogeneous, with usually a fast band, a slow band that may be split, and occasionally an intermediate band. The C-terminal group was found to be isoleucine, as previously described for myosin (53, 54), H-meromyosin (54), and the light alkali component (51).

Our interest now focuses on the structure of the heavy alkali component.

After dialysis against 5 M guanidine, the first-cycle precipitate shows on sedimentation a main peak similar to that of unfractionated myosin in 5 M guanidine, and a marked loss of trailing material (Fig. 17). On high-speed sedimentation equilibrium, the first-cycle precipitate contains a 22,000 mol wt component, representing only 6% of the total protein. After correction for the light component, the residual heavy guanidine component has a molecular weight of 210,000. We take these findings to indicate that the light components liberated by alkaline pH and by guanidine are identical, and that the heavy alkali component is dissociated by guanidine into two subunits of comparable weight (45, 47).

Further work indicates that light meromyosin has a single peak on sedimentation at pH 8 and pH 11, but that on dialysis of H-meromyosin to pH 11, a discrete slow component becomes evident, representing somewhat less than

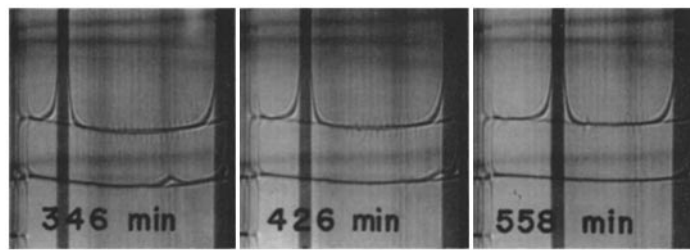


FIGURE 17. Sedimentation velocity of first-cycle precipitate in 5 M guanidine·HCl-0.4 M KCl at pH 5.7. 52,640 rpm, 4.0°C, phase plate angle 70°. Upper, 15 mg/ml; lower, 3 mg/ml. Times of centrifugation are indicated. From Gershman (45).

19% of total H-meromyosin. High-speed sedimentation equilibrium indicates that at pH 11.0, H-meromyosin contains a 22,000 particle weight material (about 19%). Both H-meromyosin and L-meromyosin contain protomyosin-like material (73) of 6000 particle weight (about 10%) and a predominant heavy component (particle weights over 200,000 for H-meromyosin and 100,000 for L-meromyosin).

#### SUBUNIT STRUCTURE OF MYOSIN

The findings thus indicate that a number (three, on the average) of light subunits are dissociated at pH above 10 from the head (H-meromyosin) end of the myosin molecule. The light alkali component is monodisperse on sedimentation but is electrophoretically heterogeneous, has an average molecular weight of 20,000 and a frictional ratio of 1.4, and is soluble at ionic strength from 0 to 0.5 M at pH 7-12.5. The light subunits have hydrodynamic and solubility properties characteristic of a globular protein, and have been termed globular or *g* subunits (47, 74). The *g* subunits have an amino acid composition similar to that of H-meromyosin, and have the same C-terminal

end group, isoleucine. The heavy alkali component comprises most of the myosin molecule, having a molecular weight of about 430,000, a frictional ratio of 3.6, and the solubility properties of intact myosin. The heavy alkali component is dissociated by guanidine into two subunits of average molecular weight about 210,000–220,000. These have been termed fibrous or *f* subunits.<sup>1</sup>

The general structure of the myosin molecule, as first shown by Rice (75), is that of a rodlike protein, about 1400 Å long and 20 Å in diameter, that terminates in a globular enlargement, 200 Å long and 50 Å in diameter (76–

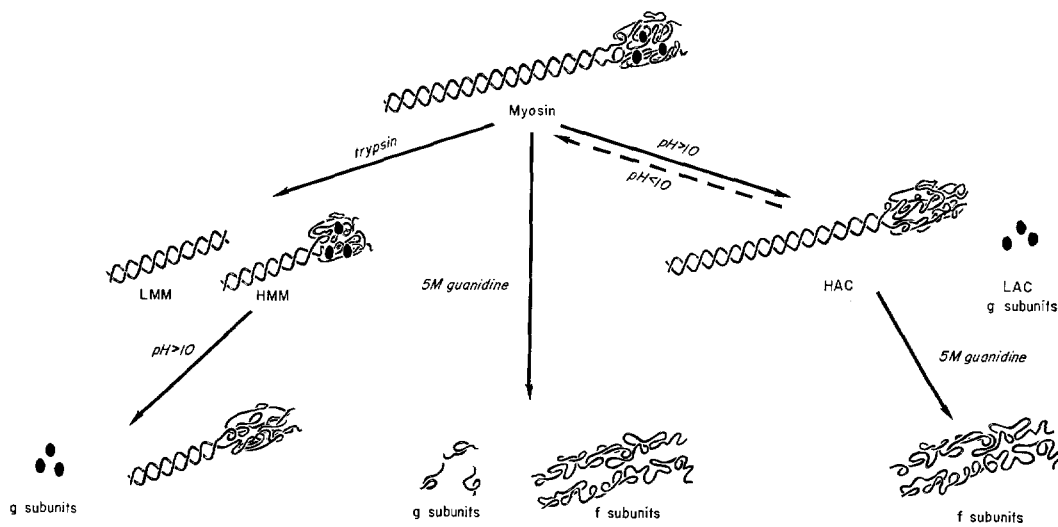


FIGURE 18. A schematic diagram for the subunit structure and interactions of myosin. LMM, light meromyosin; HMM, heavy meromyosin; LAC, light alkali component; HAC, heavy alkali component. From Gershman (45).

79). Rotatory dispersion measurements indicate that L-meromyosin has a high helical content (18). From preliminary X-ray diffraction findings and structural information on the meromyosins, primarily on L-meromyosin, Lowey and Cohen have proposed that the rodlike portion of myosin has a two-chain,  $\alpha$ -helical, coiled coil conformation (18). This interpretation is supported by further X-ray evidence that a number of  $\alpha$ -fibrous proteins, including tropomyosin and paramyosin as well as myosin, may have a two-chain  $\alpha$ -helical structure (80, 81). The Lowey-Cohen model (18) is ambiguous as to the subunit composition of myosin, and does not attempt any interpretation of the substructure of the globular head region.

<sup>1</sup> The terms fibrous (*f*) and globular (*g*) have been chosen (47, 74) in preference to the terms heavy (H) and light (L), which are used to describe the meromyosins (8), the guanidine components (67), and the alkaline components (47, 74) of myosin.

The present evidence on the dissociation of myosin into subunits may be considered within this general picture. It would seem that myosin (mol wt about 490,000) has an axial helical core composed of two *f* subunits (average weight about 215,000) that extend into a globular head region also containing three *g* subunits (average weight about 20,000) (45, 47, 74). The globular head region presumably contains about 60% of the total mass of the myosin molecule. The proposed structure for myosin, as well as the subunit interactions on which the model is based, is summarized schematically in Fig. 18.

In 5 M guanidine, the myosin molecule is dissociated into *f* and *g* subunits. According to the proposed model, the system would have a weight average molecular weight of 191,000 on complete dissociation. Interestingly, the early studies (9, 13) on myosin in 5 M guanidine indicated molecular weight values from 181,000 to 206,000 by a number of essentially weight average methods (43). Thus, although originally interpreted to imply a three-chain structure for myosin (9, 13), these extensive data actually support the present model (Fig. 18), in view of the revised molecular weight of myosin.

The proposed subunit structure (Fig. 18) is thus consistent with the basic experimental findings that led to the earlier models for myosin, namely, the multichain axial skeleton and small subunits proposed by Tsao (50); the three-chain structure proposed by Kielley, Harrington, et al. (9, 13, 62); and the coiled coil double helix terminating in a globular head, proposed by Lowey and Cohen (18).

There are additional findings consistent with an essential twofold axial symmetry of myosin (43). First, a myosin molecule of 500,000 weight would contain 37 sulfhydryl residues (9, 62). On tryptic digestion, there seem to be 15 different sulfhydryl peptides consistently (82, 83), and an additional 9 sulfhydryl residues irregularly (in less than half the samples) (82). Although these results were originally interpreted as evidence for threefold symmetry of myosin, they are clearly in accord with a basic twofold symmetry, slightly perturbed by additional *g* subunits. Second, a fragment similar to L-meromyosin in molecular weight, amino acid composition, and rotatory dispersion is obtained from myosin treated with alkaline copper cyanide (84, 85). Kominz and Lewis (85) found this fragment to be reversibly dissociated by guanidine or urea into two apparently equal weight chains. Third, the maximal myosin-actin binding ratio of 3.7-4.0 by weight (86-89) indicates binding of one myosin molecule per two actin molecules. This molar ratio may reflect either steric limitations on the binding of myosin to F-actin, or the occurrence of two actin-combining sites on each myosin molecule.

The question arises whether *g* subunits are an essential part of myosin or are merely tenaciously bound impurities that just happen to be found in myosin preparations. In our own work, the most convincing evidence has been the stoichiometric yield of light alkali component, a finding that would

be rather unusual for a casual contaminant. The data from a number of laboratories on the C-terminal group and the amino acid composition (Table I) also suggest that *g* subunits are generally present. In addition, there is evidence that conditions other than urea (45, 50, 61, 62; unpublished data of Middlebrook and Szent-Györgyi, 52), guanidine (50, 61, 62, 67), and alkaline pH (47, 51) may dissociate light components from myosin, with properties rather similar to *g* subunits. Such dissociating conditions presently include succinylation (90, 91), acetylation (92), carboxymethylation of lysine residues

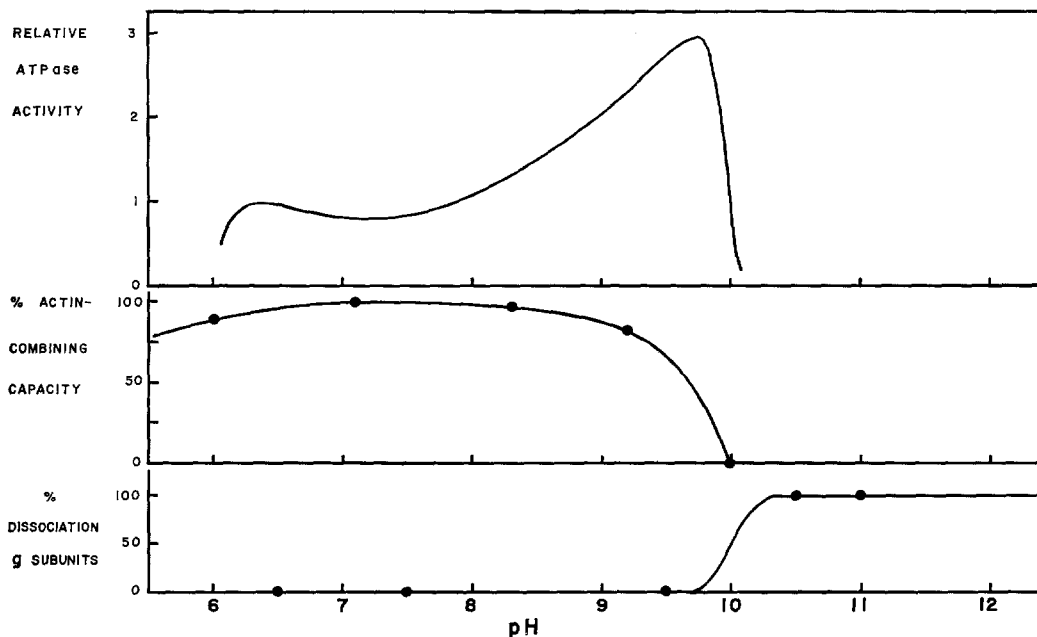


FIGURE 19. pH dependence of myosin ATPase activity (from Mommaerts and Green, 95), actin-combining capacity (data from Engelhardt, 97), and *g* subunit dissociation. From Gershman (45).

(92), and cationic detergent (93). Thus the available evidence strongly suggests that *g* subunits are a part of the myosin molecule as isolated in the laboratory.

Of interest, then, is the role, if any, that *g* subunits have in the biological activity of myosin. Since the *g* subunits are found in H-meromyosin, they might possibly be involved in the ATPase activity or the actin-combining property of myosin. Fig. 19 shows well-known data on the pH dependence of these two properties. Myosin ATPase has a maximum at pH 6-7, a sharp rise on increase in pH, and an abrupt fall at pH 10 (94-97). The actin-combining capacity of myosin also has a striking pH dependence (96, 97). On increase in pH from 7 to 9, there is a fall in actin binding of the order of 15%, and on further increase above pH 10, there is a total loss of actin-binding capacity.



Thus the myosin molecule undergoes on abrupt and drastic change at pH 10, with loss of ATPase activity, loss of actin binding, and dissociation of *g* subunits. All three phenomena are presumably related, perhaps independently, to the ionization of basic residues like tyrosine and lysine at hydrogen ion concentrations about pH 10, and electrostatic charging of the entire myosin molecule.

One might expect electrostatic interactions to be reversible, and it is notable that neither ATPase nor actin binding is regenerated on return of pH to neutrality (96). What happens to the *g* subunits on return to neutral pH? A solution of myosin at pH 11 was titrated to pH 8 (Table II). Within several hours the proportion of *g* subunits was 13%, but after storage for 2 days at 4°C the proportion of *g* subunits was only 8%, suggesting that at neutral pH the *g* subunits may slowly reassociate with the heavy alkali component (45). Prolonged storage at 4°C, however, has not been found to lead to regeneration of ATPase activity or actin-binding capacity, even after final reduction with  $\beta$ -mercaptoethanol (45).

Thus, the behavior of alkaline-treated myosin is not solely dependent on reversible electrostatic interactions. According to present concepts (35, 98, 99), all noncovalent interactions within proteins are determined by the primary structure and the solvent environment, and the irreversibility that is demonstrated by myosin would be explained by alkaline hydrolysis of side-chain groups. It is nevertheless possible that, in contrast to changes in simple globular proteins like ribonuclease or hemoglobin, there may be changes of tertiary structure or subunit interactions in a complex structural protein like myosin that are not readily reversible in dilute solution. The failure to regenerate ATPase activity after guanidine treatment of myosin, even after reduction with  $\beta$ -mercaptoethanol (100), then takes on added significance.

Studies have also been carried out to determine whether *g* subunits are present in subfragment 1. On separation by gel diffusion on Sephadex G-200 at alkaline pH, *g* subunits are found to be present in H-meromyosin and seem to be absent in subfragment 1 (101). This finding, however, should be interpreted with caution since, in the conversion of H-meromyosin to subfragment 1, the *g* subunits may be degraded by trypsin and may not be evident as intact subunits on subsequent gel diffusion at alkaline pH. This matter is under present investigation.

At least two different sulfhydryl residues in myosin have been shown to be essential for ATPase activity. Reaction of these groups with specific sulfhydryl reagents leads, in one case, to the activation of myosin ATPase (102) and, in the other, to its inhibition (103). Recent studies indicate that these sulfhydryl residues are located in the *f* subunits and not in the *g* subunits (101). One might expect these groups to be present in subfragment 1. Surprisingly, however, preliminary studies suggest that they are lost in going from H-meromyosin to subfragment 1 (101). In addition, subfragment 1 is much less

sensitive to activating reagents such as EDTA,  $\text{Ca}^{++}$ , and *N*-ethylmaleimide than is H-meromyosin or myosin (101). These results suggest that the active site residing in subfragment 1 may be regulated by sulfhydryl residues or other groups located elsewhere in the myosin molecule. Furthermore, the failure to regenerate ATPase activity after dissociation of myosin by guanidine or alkaline pH may indicate that subunit interactions too are involved in enzymic functioning.

These final considerations are unquestionably speculative but do raise important questions, and the story of myosin is just beginning.

*Note Added in Proof* Recent experiments (45) indicate that *g* chains are dissociated from rabbit skeletal myosin in concentrated solutions of lithium, iodide, and thiocyanate salts at neutral pH at 4°C. Gross structural changes in the main core of the myosin molecule may also occur, and these are now under study. The subunit structure remains intact in concentrated KCl, NaCl, and ammonium sulfate solutions. Interestingly, the *g* chains dissociate under salt conditions earlier shown to result in irreversible loss of ATPase activity but in little change in helical content of myosin (Y. TONOMURA, K. SEKIYA, and K. IMAMURA. 1962. *J. Biol. Chem.* **237**:3110). The new findings strongly support the present subunit concept for myosin.

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## Discussion

*Dr. Eric Gaetjens:* In connection with the work of Oppenheimer et al.<sup>1</sup> and Gershman et al.<sup>2</sup> which demonstrated that rabbit skeletal myosin contains low molecular weight protein components, we wish to report that rabbit cardiac myosin and chicken gizzard myosin also contain low molecular weight components.

Fig. 1, Discussion, shows the sedimentation pattern of rabbit cardiac myosin when untreated, when treated with 0.2 M K<sub>2</sub>CO<sub>3</sub> for 1 hr at 25°, and after succinylation. Whereas the untreated myosin sediments as a single peak, the carbonate-treated myosin and the succinylated myosin show, in addition to a main peak, a slow sedimenting component.

<sup>1</sup> OPPENHEIMER, H., K. BÁRÁNY, G. HAMOIR, and J. FENTON. 1966. Polydispersity of succinylated myosin. *Arch. Biochem. Biophys.* **115**:233.

<sup>2</sup> GERSHMAN, L. C., P. DREIZEN, and A. STRACHER. 1966. Subunit structure of myosin. II. Heavy and light alkali components. *Proc. Natl. Acad. Sci. U.S.* **56**:966.

Fig. 2, Discussion, shows the electrophoretic pattern on polyacrylamide gel of the isolated small component from rabbit skeletal myosin (1), rabbit cardiac myosin (2), and chicken gizzard myosin (3). All these myosins yield multiple bands on the gel with different mobilities.

Based on the idea that the small component can be released at alkaline pH values, myosin was prepared from rabbit skeletal muscle by three different methods. (a) The myosin was extracted from the minced muscle with Weber-Edsall solution for 7 min



FIGURE 1. Sedimentation of rabbit cardiac myosin.

in the cold and purified by 3-fold fractionation with ammonium sulfate between 30 and 45% saturation at pH 10. (b) Prior to the extraction of the myosin, the muscle was washed 20 times, each time with 20 volumes of 0.03 M KCl at pH 10, and the residue was extracted with 0.6 M KCl-2 mM ATP, pH 9.5, for 10 min. Traces of

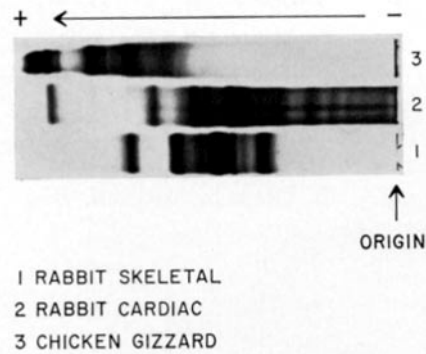


FIGURE 2. Electrophoretic patterns on polyacrylamide gel of isolated small component of various myosins.

actomyosin were removed from this myosin by conventional methods. (c) Actomyosin was prepared by extraction of the minced muscle with Weber-Edsall solution for 24 hr and purified by 3-fold precipitation at 0.12 M KCl, pH 9.5. Myosin was prepared from this actomyosin by the dissociation procedure of A. Weber.<sup>3</sup>

Fig. 3, Discussion, shows that the small protein components can be isolated from myosin prepared by these three different methods. Moreover, the electrophoretic pattern of the small components are very similar.

<sup>3</sup> WEBER, A. 1956. The ultracentrifugal separation of L-myosin and actin in an actomyosin sol under the influence of ATP. *Biochim. Biophys. Acta.* 19:345.

In view of the reported differences between myosin from the newborn rabbit and adult rabbit,<sup>4, 5</sup> it was of interest to look for similar small protein components in the myosin from the newborn rabbit.

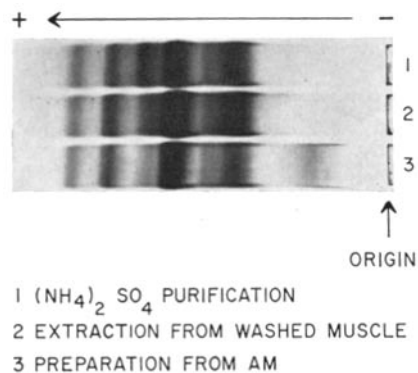


FIGURE 3. Electrophoretic patterns of myosin prepared by three different methods. *AM*, actomyosin.

Fig. 4, Discussion, compares the electrophoretic pattern of small components from newborn and adult rabbit myosin. Myosin from the newborn rabbit shows a pattern similar to that of the adult rabbit except for the absence of one band.

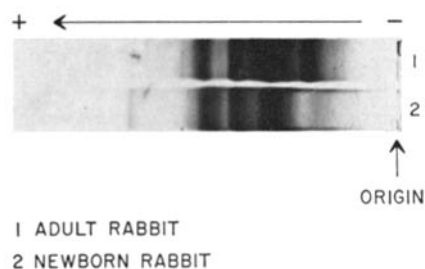


FIGURE 4. Electrophoretic patterns of small component of myosin from two different sources.

In conclusion, the data show that myosin prepared by different methods and from different sources contains tightly bound small protein components. However, the role of these components in the myosin molecule remains to be elucidated.

This work was done in collaboration with Dr. Michael Bárány.

*Dr. Dreizen:* These are interesting findings, although I am concerned whether some of the electrophoretic differences in the light component isolated from succinylated myosin may result from the succinylation procedure itself. Unless the same

<sup>4</sup> PERRY, S. V., and D. J. HARTSHORNE. 1963. The proteins of developing muscle. *In* The Effect of Use and Disuse on Neuromuscular Functions. E. Gutmann and P. Hnik, editors. Elsevier Publishing Company, Amsterdam. 491.

<sup>5</sup> BÁRÁNY, M., A. F. TUCCI, K. BÁRÁNY, A. VOLPE, and T. RECKARD. 1965. Myosin of newborn rabbits. *Arch. Biochem. Biophys.* 111:727.

residues identically are succinylated in all myosin molecules, one might find some charge variation in originally homogeneous material. This has been a problem in our own work on the light alkali component, where electrophoretic heterogeneity seems to be more extensive, certainly more striking, after prior carboxymethylation of sulfhydryl groups.

Also, I would be somewhat cautious in identifying *all* low molecular weight material found in preparations of myosin from different kinds of muscle and from different species with the fairly well defined *g* subunits of rabbit skeletal myosin. This protein is known to adsorb trace amounts (less than 1%) of adenylic deaminase and RNA, and different preparations may contain other impurities, perhaps not even bound to myosin. The phylogenetic story may be further complicated by weight heterogeneity of the *g* subunits, if they do turn out to be a biologically important part of myosin. It is difficult, for example, to interpret the extensive low molecular weight heterogeneity found in cod myosin preparations.<sup>6</sup>

*Dr. Perry:* I wonder if I may ask Dr. Dreizen a question. You rather imply that the two main chains are identical. Have you any evidence that they are? They are certainly similar in molecular weight.

*Dr. Dreizen:* No, the evidence only points to the absence of gross heterogeneity of the *f* chains. Our sedimentation equilibrium experiments in 5 M guanidine indicate that the *f* chains have an average weight of about 210,000–220,000, but the present data could not differentiate two chains each of slightly different weight, say, 240,000 and 190,000. On sedimentation in 5 M guanidine, if we neglect the trailing component, the main boundary is a single peak; and on gel electrophoresis of myosin in 12 M urea, only a single band has been reported. The sulfhydryl labeling experiments are somewhat ambiguous as to the subunit composition of myosin, yet would certainly indicate extensive symmetry within the myosin molecule. All these findings are quite suggestive that *f* chains are similar to each other. Whether or not they are identical is an open question.

*Dr. F. A. Sreter:* We have recently<sup>7</sup> compared myosins prepared by the classical dilution procedure of Szent-Györgyi with myosin prepared by the LiCl-ammonium sulfate procedure introduced by J. M. Marshall. Since the latter procedure involved the use of EDTA, we also studied preparations extracted and precipitated according to Szent-Györgyi but with all solutions having 1 mM EDTA; finally we fractionated myosin with EDTA-ammonium sulfate without LiCl being added. The upshot of all this is that whenever EDTA was present throughout the preparative procedure, both the Ca-activated and EDTA-activated ATPase activities were almost doubled, compared with the classical Szent-Györgyi preparation. Ammonium sulfate or LiCl had no effect on the activity. I would add that the differences between myosins from white and red muscles were still present in these highly active preparations. It is also interesting that the slowly sedimenting component found in succinylated<sup>1</sup> conventional myosins was found to be present in the white and red muscle myosins prepared in the presence of EDTA.

<sup>6</sup> CONNELL, J. J., and H. S. OLCOTT. 1961. *Arch. Biochem. Biophys.* **94**:128.

<sup>7</sup> SRETER, F. A., B. NAGY, and J. GERGELY. 1966. *Intern. Biophys. Congr., 2nd, Vienna.* 35.



*Dr. Dreizen:* Most of our experiments involving ammonium sulfate fractionation were done by the Keilley-Bradley method, which does involve the use of EDTA. These preparations of myosin showed the same low molecular weight heterogeneity as our usual preparations obtained by the Szent-Györgyi procedure (without EDTA) and also one preparation obtained by ammonium sulfate fractionation without EDTA (the procedure used by Tsao). As you noted, EDTA activation seems to be a general property of myosin ATPase, but there does not seem to be any relationship between the presence of EDTA and the dissociation of light component.

*Dr. Bárány:* I would like to make a general comment relating to the talk of Dr. Dreizen. It was misleading to the audience to mention only the names of the Bárány in connection with the succinylation of myosin. Dr. Oppenheimer started this project at our Institute with the cooperation of Dr. Kate Bárány. I joined this project only lately.

Concerning the comments made by Dr. Sreter, I also want to mention that according to our experience, the presence of EDTA during the purification of myosin does not decrease the amount of the light component in myosin.

*Dr. E. Glen Richards:* We have spent several years trying to purify myosin by a column procedure using DEAE-Sephadex, and we were very unhappy to find that myosin prepared by this method also exhibited this low molecular weight component, because we had hoped that we had the best myosin available.

The second comment relates to the sedimentation equilibrium experiments that were mentioned here. I think that the use of three significant figures is overly optimistic for such mixtures.

*Dr. Dreizen:* No need to be unhappy. I think you have very good myosin indeed.

The molecular weight values for the light alkali component are obtained under high field conditions, where the system is essentially monodisperse (see Fig. 15). These calculations are fairly accurate, and the error in molecular weight for the light alkali component is about 2 or 3%, neglecting possible uncertainty in partial specific volume. Three significant figures are needed to express molecular weight values of the order of 20,000 to this accuracy. The molecular weight values for the heavy alkali component and for both guanidine components are based on multicomponent analyses, and are less accurate, of course. I believe that we have generally expressed these data in two significant figures, and apologize for any slips. We have been careful, in any case, to indicate some estimate of uncertainty for each experiment.

*Question from the Floor:* Having used alkali solubilization for carotene for many years, and knowing that this solubilization breaks covalent bonds, I wonder if this effect could explain the heterogeneity as demonstrated by large and small particles. That is, do you need to explain the phenomena by a subunit concept, when you are just breaking a series of covalent bonds?

*Dr. Dreizen:* We did find the same low molecular weight component in 5 M guanidine, and similar low molecular weight components seem to be released from myosin on succinylation, acetylation, and carboxymethylation. None of these methods involves alkaline treatment of myosin. The last three procedures add negatively charged groups to free amino groups on the protein, and are comparable in a way with the

ionization of basic residues at pH 10. Despite a preliminary report<sup>8</sup> on dissociation of succinylated myosin to about the 100,000 weight average level, I would guess that the main core of two *f* chains remains intact during the various chemical substitution procedures. The reported sedimentation patterns, certainly, are similar to those obtained at pH 11.

It is possible that alkaline hydrolysis may result in covalent changes in some of the side chains, and these changes might account for some of the electrophoretic heterogeneity of the light component and also the irreversible loss in biological activity of myosin. I rather doubt that the light alkali component results from non-specific fragmentation of the original peptide chains. The light component is relatively homogeneous in weight and has the C-terminal end group attributed to myosin and H-meromyosin, and its proportion is constant over prolonged periods in alkaline solution, over a range from pH 10.5 to pH 12.5. These findings suggest that fragmentation, if it occurs, would be at unique sites close to one end of the myosin molecule, although the release of three fragments from two main chains would be somewhat troublesome to explain. In any case, this kind of fragmentation of peptide chains has not been found over the same pH range in other proteins, hemoglobin, for example. It is conceivable, I suppose, that an unusual peptide bond may be involved or that a side-chain covalent bond (not a disulfide bridge) might hold light and heavy fragments together in native myosin. These are speculative considerations, and I think that the present evidence would favor *g* subunits being discrete chains rather than peptide fragments.

<sup>8</sup> OPPENHEIMER, H., K. BARANY, J. FENTON, and O. MASLIVEC. 1964. *Abstr. Intern. Congr. Biochem., 6th, New York*, 662.