On the Structural Assembly of the Polypeptide Chains of Heavy Meromyosin*

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In a previous study (1), the light meromyosin and heavy meromyosin fragments of rabbit skeletal myosin were isolated after brief trypptic digestion, and their susceptibility to further proteolysis was examined with the pH-stat. It was found that although virtually all of the theoretically susceptible (lysine plus arginine) peptide linkages in light meromyosin were cleaved, only about 25% of such potentially susceptible bonds were hydrolyzed in the heavy meromyosin fragment. This latter finding is reminiscent of a property generally attributable to the compact globular proteins which are relatively resistant to proteolysis and, if the lysine and arginine residues are randomly distributed throughout heavy meromyosin, suggests that this meromyosin consists largely of a trypsin-resistant core amounting to about 270,000 g per mole (based upon a molecular weight for heavy meromyosin of 362,000 g per mole (1)).

This information is pertinent to the observations of Mueller and Perry (2), who showed that trypptic digestion converts a large fraction of the HMM mass into a lower molecular weight and relatively homogeneous particle, which they termed Subfragment 1. Yet the molecular properties of Subfragment 1 [(\(\eta\)) = 0.07 to 0.125 dl per g; \(\delta_{\infty} = 5.95 S\)] would indicate a particle weight not of 270,000 g per mole, but rather in the range 100,000 to 125,000 g per mole (1). On the basis of these considerations and the previously proposed structure for the myosin molecule (3), it was suggested that the effect of trypsin upon HMM was to cause a fragmentation of this meromyosin into three globular, adenosine triphosphatase-active particles of essentially identical mass (1). The purpose of this report is to present further evidence in support of this hypothesis.

EXPERIMENTAL PROCEDURE

Reagents—Rabbit skeletal myosin was isolated as previously described (4). Heavy meromyosin was prepared by treatment of a 16.6 mg per ml solution of myosin in 0.5 M KCl, pH 6.8, with trypsin (crystalline, salt-free, Worthington Biochemical Corporation). The weight ratio of myosin to trypsin in all cases was 250:1 (1). After \(t_0\) seconds of digestion at 20°C, the reaction was stopped by addition of a 2-fold weight excess (inhibitor over trypsin) of soybean trypsin inhibitor contained in 2 ml of 0.2 M NaHCO\(_3\), and the resulting solution was dialyzed against 0.01 M phosphate, pH 7.0, to precipitate LMM together with residual myosin. The supernatant solution was then treated with saturated ammonium sulfate, and the HMM fraction precipitating in the range from 50 to 90% saturation (3) was collected by centrifugation. Following dialysis against 0.1 M Tris-0.05 M KCl, pH 7.6, HMM was separated from trypsin inhibitor and the trypsin-trypsin inhibitor complex by gel filtration with Sephadex G-200 as previously described (1). The HMM eluant fractions were then pooled, and the protein was concentrated by precipitation from a 60% saturated ammonium sulfate solution. The precipitate was redissolved in and dialyzed exhaustively against the desired solvent.

Adenosine triphosphatase activities were determined according to the procedure of Kielley and Bradley (4).

Physical Methods—The Spinco model E analytical ultracentrifuge was used for all sedimentation equilibrium experiments, and rotor temperature was routinely maintained close to 5°C. The Rayleigh interference optical system of the machine, aligned by the procedure of Richards and Schachman (5), was employed throughout. Standard 12-mm, aluminum-Epon double sector centerpieces which had been previously coated with Krylon (an acrylic ester resin obtained from Krylon, Inc., Norristown, Pennsylvania) were used for all runs.

Two general types of sedimentation equilibrium experiments were employed. The first of these utilized 1-mm columns of protein solution layered over FC43 (6) (perfluorotributylamine, Minnesota Mining and Manufacturing Company) together with comparitively low rotor velocities (approximately 6000 r.p.m.). After analysis of the photographic plates (spectroscopic, type II) as previously described (3), molecular weights were calculated from a knowledge of the concentration change across the solution column relative to the initial concentration (determined with the synthetic boundary cell) as described by the equation of Lansing and Kraemer (7).

The high speed equilibrium procedure outlined by Yphantis (8) was also used for molecular weight studies. The essential feature of this technique is that a rotor velocity is selected such that the protein concentration is reduced to a negligible value at the air-liquid meniscus at equilibrium. Consequently, since protein concentration now varies with radius of rotation from essentially zero to a finite level near the base of the solution column, the mass distribution at equilibrium may be determined.
from a single centrifuge run. Moreover, since the initial protein concentrations used in this method are very low (less than 0.1 mg per ml), combination of both low and high speed centrifugation permits molecular weight determinations over a large range of solute concentration. For these experiments, 0.105 ml of a 0.05 to 0.10 mg per ml protein solution was layered over 0.05 ml of FC43 in the conventional double sector cell. The interferograms were measured with a Nikon model 6 optical microcomparator. After alignment of the plate, the vertical fringe displacement (directly proportional to protein concentration, $c_z$) was measured as a function of distance, $x$, from the center of rotation. Values were taken every 0.1 mm (plate coordinate) and were plotted on graph paper (50 x 50 cm) as a function of $x^2$ to permit a choice of the zero reference level near the meniscus (8). In order to correct for any optical inequalities between the reference and solution limbs of the cell, a companion run was made with a cell containing solvent in both limbs, and the resulting interferogram was used to correct the plot of $c_z$ against $x^2$ (8). From a plot of log $c_z$ against $x^2$, the weight average molecular weight may be calculated as a function of $x$ according to Equation 1.

$$M_{n(x)} = \frac{2RT}{(1 - \phi') \rho^2} \frac{d \ln c_z}{dx^2}$$

where $M_{n(x)}$ is the weight average molecular weight at distance $x$ from the center of rotation; $\rho, \phi, R,$ and $T$ have their usual significance; and $\phi'$ is the apparent specific volume of the protein in a three-component system as defined by Casassa and Eisenberg (9, 10).

In view of the fact that $c_m \ll c_b$ at equilibrium, the number average molecular weight, $M_{n(x)}$, may also be determined and is given by Equation 2 (8).

$$M_{n(x)} = \frac{c_z}{(1 - \phi') \rho^2} \int_{x_m}^{x_b} \frac{dx}{x \gamma x}$$

Similarly, the number average molecular weight over the whole column, $M_n$ (corresponding to the initial solution), is given by

$$M_n = \frac{1}{x_m} \int_{x_m}^{x_b} x \gamma x \ dx$$

where $x_m$ and $x_b$ are the radial coordinates of the meniscus and base of the solution column, respectively. The weight average molecular weight over the whole column, $M_w$, was computed from $M_{n(x)}$, the number average weight corresponding to the bottom coordinate of the solution column. Since $c_m$ becomes essentially zero at equilibrium, the equation of Lansing and Kraemer formally reduces to Equation 2 for $x = b$, and consequently $M_{n(b)} = M_w$ (8). The integrals in Equations 2 and 3 were computed by trapezoidal summation.

Viscosities were measured with a 2-ml Ostwald-type viscometer with water outflow time at 25° of 90 seconds. The average shear gradient was $300 \text{ sec}^{-1}$, and the kinetic energy correction was less than 0.1%.

Polarization of fluorescence was studied by coupling HMM and Subfragment 1 to the fluorescent dye 1-dimethylamino-5-naphthalenesulfonyl chloride (Calbiochem), which had been twice recrystallized from ethanol. The procedure of Weber (11) was used for the coupling reaction with the exception that ethanol rather than acetone was employed as solvent for the dye. The mean degree of conjugation was determined spectrophotometrically by the techniques described earlier (11). Degree of polymerization was measured with a model 1000D Bric-Phoenix light scattering photometer coupled to an external ratio recorder (12, 13); Corning filters Nos. 5970 and 3385 intercepted the excitation and emission beams, respectively. Water from a large external bath was circulated through a specially constructed jacket for temperature control ($\pm 0.1^\circ$) of the protein solution, which was contained in a 1-cm$^2$ quartz cell. Fluorescent intensities were measured normal to the unpolarized excitation beam by inserting a Polaroid disk before the entrance slit of the photocell. The photomultiplier showed no variation in response with plane of polarization. Degree of polarization, $p$, was computed from Equation 4, and the harmonic mean of the rotational relaxation time from Equation 5 (14, 15).

$$p = \frac{I_p - I_o}{I_p + I_o}$$

$$\frac{1}{p} + \frac{1}{5} = \left(\frac{1}{p_0} + \frac{1}{5}\right) \left(1 + \frac{3n}{\gamma h}ight)$$

Here $I_p$ and $I_o$ are fluorescent intensities the electric vectors of which are parallel and perpendicular to the electric vector of the exciting radiation; $p_0$ is the limiting value of $p$ at high $p_0$, the harmonic mean of the principal rotational relaxation times of the macromolecule; and $r_0$ is the lifetime of the excited state. For spherically symmetrical particles,

$$\frac{3}{r_0} = \frac{R}{\eta} = \frac{RT}{\eta V}$$

where $R$ is the gas constant; $T$, absolute temperature; $V$, the molar volume of the macromolecule including any bound solvent; $\eta$, the solvent viscosity; and $\rho_0$, the viscosity time of a sphere of the same volume (14). Since, in general, plots of $1/p + 1/3$ against $T/\eta$ are linear for nonspherical molecules as well, $p_0$ and $p_0$ may be estimated from the slope and intercept of Equation 5, respectively (15). The lifetime of the excited state for the 1-di- methylamino-5-naphthalenesulfonyl chloride-protein conjugates was taken to be $1.2 \times 10^{-8}$ second (16).

Optical rotation and rotatory dispersion were measured with a Rudolph model 80 photoelectric spectropolarimeter.

Amino acid analyses were performed with the model 120 B Beckman automatic amino acid analyzer by the procedures of Spackman, Stein, and Moore (17). All protein samples were hydrolyzed with 6 N HCl in sealed evacuated (50 ml) tubes for 24 hours at 110 ± 0.1°.

Concentrations of HMM solutions were measured spectrophotometrically with a Zeiss PMQ II spectrophotometer with the use of an extinction coefficient of 647 cm$^2$ per g ($\lambda = 280$ mp) previously determined from dry weight measurements (1). The extinction coefficient of Subfragment 1, isolated and purified as described below, was determined by the micro-Kjeldahl procedure in conjunction with a value of 16.5 % nitrogen obtained from amino acid analyses. This method gave a value of 770 cm$^2$ per g at $\lambda = 280$ mp.

**RESULTS**

Fig. 1 presents a series of ultracentrifuge schlieren patterns of tryptic digests of HMM as a function of digestion time at 25° with...
a weight ratio of HMM to trypsin of 15:1. This relatively high concentration of trypsin was chosen to allow digestion to proceed rapidly and thereby to minimize aggregation of HMM which had been observed to occur to a significant degree at 25°. It can be seen that the HMM boundary is rapidly transformed under these conditions into a relatively broad major peak, together with a small amount of more slowly sedimenting material. The sedimentation coefficient of the major boundary is essentially identical (s_{20,w} = 5.7 S) with that reported by Mueller and Perry for Subfragment 1 (3).

In the early stages of tryptic digestion a rapidly sedimenting boundary preceding the Subfragment 1 peak is always present in the ultracentrifugal schlieren patterns (e.g. Fig. 1, 5 minutes). This was tentatively identified as undegraded HMM in our initial experiments, but viscosity studies of the reactions made under identical conditions do not support this interpretation. The reduced viscosity of the HMM solution falls from a value of 0.64 to 0.15 dl per g within 1 minute of initiating proteolysis. This represents a decrease of over 90% of the total change in viscosity measured during the complete transformation of HMM to Subfragment 1, suggesting that the most rapidly sedimenting boundary seen in Fig. 1 is not HMM, but represents a much less asymmetrical, high molecular weight intermediate in the degradation pathway from HMM to Subfragment 1. This conclusion is supported by the sedimentation coefficient of this boundary (s_{20,w} = 7.3 S), which is significantly higher than the infinite dilution value for HMM (s_{20,w} = 6.76 S) (1). Further evidence for such an intermediate will be presented below.

To examine the structural relationship between the active fragment and the parent HMM, an attempt was made to establish the weight fraction of HMM which is converted to Subfragment 1 during tryptic digestion. Several velocity sedimentation studies were made as a function of time of tryptic digestion under conditions identical with those detailed in Fig. 1, and the Subfragment 1 boundary areas were determined by planimetry of tracings taken from enlargements of the photographic plates. Areas were corrected for radial dilution. The total area (proportional to initial HMM concentration) was obtained from synthetic boundary experiments and corrected for the contribution of tryspin and trypsin inhibitor (also determined with a synthetic boundary cell). The Subfragment 1 weight fraction of the original HMM mass was then computed and plotted against time as shown in Fig. 2. To minimize the Johnston-Ogston effect, Subfragment 1 boundary areas were estimated after the faster sedimenting peak shown in Fig. 1 had nearly disappeared (approximately 5 minutes). In the calculation of weight fraction it was assumed that Subfragment 1 and HMM have identical refractive index increments. This would seem to be reasonable since most of the HMM mass is transformed into Subfragment 1 and since the refractive index increments of most proteins are closely similar. As shown in Fig. 2, the area of the Subfragment 1 peak declines quite rapidly with time as a result of tryptic hydrolysis, and consequently it is difficult to evaluate precisely the amount of HMM mass converted into this fragment. The initial HMM particle is almost completely degraded within 1 minute of initiation of proteolysis, and the weight fraction of Subfragment 1 produced can thus be closely estimated by extrapolating the curve of Fig. 2 to zero time. This procedure indicates that in the hydrolysis of HMM by trypsin, approximately 80% of the mass is released as Subfragment 1.

To investigate the mechanism of this transformation, several molecular properties have been examined in parallel. Fig. 3 presents, on the same time scale, the reduced viscosity, degree of fluorescence polarization, ATPase activity, and specific optical
further tryptic degradation of Subfragment 1 which is also seen rapid reduction in asymmetry of HMM. The second and slower phase (with a half-time of 25 minutes) can be attributed to the gestion has a half-time of about 2 minutes and reflect's t'he slopes when log rate is plotted against time (18) and emphasize presented in Fig. 3 yield two linear segments of markedly differing appa rent from Fig. 3. The first, and very rapid, phase of di- the two-phase character of the degradation of HMM which is 

rotation of HMM as a function of time of tryptic digestion. (To decrease the over-all transformation rate and thus to allow a more detailed analysis of the viscosity kinetics, the trypsin to HMM ratio used in the experiments summarized in Fig. 3 is less than that employed for the centrifugal kinetics.) It can be seen that during the initial 10 to 15 minutes of proteolysis, the reduced viscosity declines rapidly to a value approximately one-third that of the initial viscosity, whereas this striking alteration is accom panied by relatively minor changes in polarization of fluorescence, specific ATPase activity, and specific rotation. When taken in conjunction with the data of Fig. 1, these findings indicate that the predominant effect of trypsin upon HMM is to produce a more slowly sedimenting and appreciably less asymmetrical particle (or particles) with a relatively minor alteration in polypeptide chain configuration as judged by optical rotation.

Kinetic analyses of several viscosity studies such as that presented in Fig. 3 yield two linear segments of markedly differing slopes when log rate is plotted against time (18) and emphasize the two-phase character of the degradation of HMM which is apparent from Fig. 3. The first, and very rapid, phase of digestion has a half-time of about 2 minutes and reflects the rapid reduction in asymmetry of HMM. The second and slower phase (with a half-time of 25 minutes) can be attributed to the further tryptic degradation of Subfragment 1 which is also seen in Figs. 1 and 2. The ATPase activity of HMM is retained through the first phase of the reaction (Fig. 3) but declines slowly during the second phase in parallel with the degradation of Subfragment 1 (Fig. 2).

**Isolation and Characterization of Subfragment I—Isolation of Subfragment 1 from undegraded HMM and peptide fragments was accomplished by gel filtration with Sephadex G-200. To establish an appropriate time of digestion such that Subfragment 1 would not be appreciably degraded, proteolysis was stopped at varying times with soybean trypsin inhibitor and the several digest mixtures were applied to a column of Sephadex G-200, 2.8 x 50 cm. Fig. 4 presents a typical elution profile obtained after 15 minutes of digestion and has been divided into three major regions. Peak II, accounting for most of the ATPase activity of the preparation, can be identified as Subfragment 1 (see below). Peaks III, as indicated by the dashed curve, largely represent trypsin-trypsin inhibitor complex, together with the excess trypsin inhibitor used to stop proteolysis. This region also includes peptide fragments released from HMM. Zone I shows anomalous behavior during the formation of Subfragment 1 in that HMM—which invariably appears in the void volume of Sephadex G-200 (1)—is replaced within 1 to 2 minutes of tryptic digestion by an ATPase-active peak which is significantly retarded in its elution (Fig. 4). Qualitatively, it was observed that the area of this peak decreased as a function of digestion time with a concomitant increase in the areas of peaks II and III, and it was of interest to compare the amount of HMM mass transformed into Subfragment 1 (Peak II), as measured chromatographically, with that found from the ultracentrifugation study described earlier. The areas of Peaks II and III were estimated from several Sephadex elution diagrams after correction for the area contributed by trypsin and trypsin-trypsin inhibitor complex. This latter area was determined

![Fig. 3. Changes in reduced viscosity, degree of fluorescence polarization, enzymic activity, and specific optical rotation during tryptic digestion of HMM at 25°. Protein concentration was 10 mg per ml in 0.05 M KCl-0.1 M Tris, pH 7.6. The weight ratio of HMM to trypsin was 78:1. To determine ATPase activity, the digestion reaction was stopped at indicated times with a 2-fold weight excess of soybean trypsin inhibitor. Enzyme activity was measured at 320° (4), and values are expressed as percentage of initial activity of HMM.](http://www.jbc.org/)

![Fig. 4. Fractionation of a tryptic digest of HMM with Sephadex G-200. An 8.3 mg per ml solution of HMM (3 ml) was digested with trypsin (HMM to trypsin ratio, 78:1) for 15 minutes at 25°. The reaction was terminated with a 2-fold weight excess of soybean trypsin inhibitor, and the solution was applied to a column (2.8 x 50 cm) of Sephadex G-200 equilibrated with 0.05 M HC1-0.1 M Tris, pH 7.6. The column was developed at 4° with the same solvent at a flow rate of 20 ml per hour, and 2-ml fractions were collected. O—O, optical density at 280 mµ; C—C, ATPase activity (calcium-activated) measured with 0.05-m M aliquots of the indicated fractions at 320° (4); C—C, optical density profile of a control solution of trypsin and trypsin inhibitor at the same concentrations as those employed in digestion of HMM.](http://www.jbc.org/)
from separate experiments as described in the legend to Fig. 4. The weight fraction of Subfragment 1 (area Subfragment 1/area total products) at digestion times of 5, 10, 20, and 30 minutes was found to be 81, 84, 75, and 70%, respectively. Although these values are certainly less precise than those obtained from the ultracentrifugal patterns, they indicate again that about 75 to 85% of the HMM mass is converted to Subfragment 1.

At the end of the HMM → Subfragment 1 reaction, a small but variable amount of enzymically inactive material was often observed to emerge within the void volume of Sephadex G-200 (Fig. 4). The origin of this fraction is not clear, but it may arise from a time-dependent aggregation of HMM, from degradation products of the digestion mixture at 25°, or both.

The small, ATPase-active fraction which falls in Zone I of Fig. 4 appears to be related to the intermediate in the pathway from HMM to Subfragment 1 which was suggested from the centrifugation and viscosity studies. As mentioned above, this peak is slightly retarded in its elution position and, unlike HMM, does not appear within the void volume of Sephadex G-200. When material from the central portion of this peak was concentrated and examined in the ultracentrifuge, a single boundary was observed with sedimentation characteristics (s20,w = 7.3 S at a concentration of 1.4 mg per ml) essentially identical with those of the faster sedimenting peak of Fig. 1 (s20,w = 7.2 S). Moreover, the reduced viscosity of this fraction (ηp/c = 0.30 dl per g at a concentration of 1.4 mg per ml) was found to be significantly less than that of HMM ([η] = 0.58 dl per g (1)). Preliminary molecular weight determinations of this fraction by high speed equilibrium sedimentation gave values in the range 300,000 to 320,000 g per mole, and a molecular weight of 290,000 was estimated from the Scheraga-Mandelkern equation (19). Taken together, this information strongly suggests that a high molecular weight, ATPase-active intermediate of low particle asymmetry is rapidly produced during the tryptic degradation of HMM and is a precursor of Subfragment 1.

The fractions corresponding to Peak II were pooled and con-

![Fig. 5](image-url)  
**Fig. 5.** Ultra centrifugal schlieren patterns of purified Subfragment 1. Protein concentration was 13 mg per ml in 0.05 M KCl-0.1 M Tris, pH 7.6; temperature, 3.0°; bar angle, 75°; rotor velocity, 99,780 r.p.m. Pictures were taken at indicated times after reaching full speed.
eraly give somewhat lower precision than does the low speed method (8), the weight average molecular weights (\(M_w = 117,000\) to 121,000 g per mole) and the number average molecular weight (\(M_n = 115,000\) g per mole) are in good agreement, suggesting that the Subfragment 1 particles comprise a rather narrow molecular weight distribution. Within the experimental precision of these measurements, the molecular weight was found to be independent of protein concentration over a 100-fold range. This finding is consistent with the viscosity measurements in that it points to a small second virial coefficient and thus to a rather low axial ratio for the active fragment. Another estimate of the molecular weight of Subfragment 1 was obtained from a combination of \(z_v\), \(c_v\), and \(c_w\) according to the Sehersch-Mandelkern equation (19). This calculation yields \(M = 114,000\) g per mole. Several different Subfragment 1 preparations were employed in the molecular weight studies cited above and represent samples obtained after widely varying reaction times (in the range 10 to 40 minutes of tryptic digestion) under the conditions summarized in the legend to Fig. 4. No trend in particle size was detected with time of digestion, in agreement with the observation of Mueller and Perry that the sedimentation coefficient of Subfragment 1 is also invariant with time of proteolysis (2).

High Speed Equilibrium Sedimentation of HMM—In an earlier study with low speed equilibrium centrifugation, the molecular weight of HMM isolated under the same conditions used in the present work was found to be 362,000 g per mole (1). As discussed at that time, solutions of HMM exhibited not only appreciable dependence of molecular weight upon concentration but also detectable upward curvature of log \(c\) against \(x^2\) plots, indicative of particle heterogeneity. Consequently it was difficult to evaluate the apparent molecular weight at infinite dilution. (See Williams et al. (20) for a discussion of the problems inherent in extrapolation of apparent molecular weight of heterogeneous systems to infinite dilution.) A second difficulty encountered in evaluation of \(M_w\) arises from the fact that some type of extrapolation procedure is required to evaluate \(c_m\) and \(c_w\), and, in the case of heterogeneous systems, this introduces some uncertainty in estimation of concentrations at the ends of the solution column. The recently developed high speed equilibrium method minimizes this difficulty and also possesses the advantage that molecular weights may be determined at extremely low protein concentrations, thus minimizing the error in evaluating the molecular weight at infinite dilution. Table II presents several determinations of both \(M_w\) and \(M_n\) for HMM in the concentration range from 0.08 to 0.11 mg per ml, giving an average value of \(M_w\) (405,000 g per mole) significantly greater than \(M_n\) (386,000 g per mole). No attempt has been made to extrapolate these values to infinite dilution of solute, and both number and weight average molecular weights could be slightly higher than those reported here.

From the viscosity and chromatography data presented above, it seems clear that transformation of HMM to a high molecular weight intermediate occurs very rapidly after the addition of trypsin and presents another obstacle in the evaluation of the

2 Low speed equilibrium centrifugation experiments on Subfragment 1 isolated from a single passage through Sephadex invariably exhibited upward curvature of log \(c\) against \(x^2\) plots. Rechromatography as described above in all cases yielded solutions characterized by linear plots.

3 In the molecular weight calculations for Subfragment 1, the \(\phi\) (apparent specific volume) found for HMM (1) of 0.720 ml per g has been used.

### Table I

**Molecular weight of Subfragment 1**

<table>
<thead>
<tr>
<th>Sample</th>
<th>(M_w)</th>
<th>(M_n)</th>
<th>(M_o)</th>
</tr>
</thead>
<tbody>
<tr>
<td>32</td>
<td>117,000</td>
<td>121,000</td>
<td>119,000</td>
</tr>
<tr>
<td>33</td>
<td>117,000</td>
<td>115,000</td>
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<td>120,000</td>
</tr>
<tr>
<td>36</td>
<td>90,000</td>
<td>102,000</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>129,000</td>
<td>123,000</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>115,000</td>
<td>117,000</td>
<td>121,000</td>
</tr>
</tbody>
</table>

### Table II

**Molecular weight of heavy meromyosin from high speed equilibrium sedimentation**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration</th>
<th>(M_w)</th>
<th>(M_n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>0.08</td>
<td>379,000</td>
<td>392,000</td>
</tr>
<tr>
<td>38</td>
<td>0.10</td>
<td>377,000</td>
<td>395,000</td>
</tr>
<tr>
<td>47</td>
<td>0.30</td>
<td>379,000</td>
<td>403,000</td>
</tr>
<tr>
<td>48</td>
<td>0.30</td>
<td>376,000</td>
<td>411,000</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>386,000</td>
<td>405,000</td>
</tr>
</tbody>
</table>

*“true” particle weight of HMM. Isolation of this meromyosin from a myosin digest after any time period would be expected to yield a partially degraded particle. This could explain why there has been such a wide variation in reported values for the molecular weight of this meromyosin (see References 1, 21, and 22). In view of the heterogeneity problem and the results presented in Table II, it seems reasonable to assume that the weight of HMM prepared after 1 minute of digestion probably lies in the range 380,000 to 400,000 g per mole.*

**Enzymic Activity of Subfragment 1 and HMM**—Mueller and Perry observed that Subfragment 1 retained both the ATPase and actin-binding properties of HMM (2). On the assumption of a molecular weight of 115,000 g per mole for this particle, the specific ATPase activity per mole was determined and is shown in Table III together with the specific molar ATPase activity of HMM. These data reveal that the specific activity (calcium-activated) of Subfragment 1 is one-half to one-third that of HMM, whereas the EDTA-activated ATPase activity is one-third to one-quarter of that of HMM.

**Polarization of Fluorescence Measurements**—Fig. 7 presents plots of \(1/p + 1/3\) against \(T/\rho\) for both HMM and Subfragment 1, and the rotational relaxation times derived from these plots are given in Table III. Within the temperature range 8-35°,
TABLE III
Molecular properties of heavy meromyosin and Subfragment 1

<table>
<thead>
<tr>
<th>Preparation</th>
<th>$M_n$</th>
<th>$M_w$</th>
<th>$\tilde{M}$</th>
<th>$\tilde{g}$</th>
<th>$\alpha$</th>
<th>$\nu$</th>
<th>$\nu^b$</th>
<th>ATPase activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMM</td>
<td>386,000</td>
<td>405,000</td>
<td>6.47</td>
<td>0.580</td>
<td>37°</td>
<td>284</td>
<td>$1.22 \times 10^7$</td>
<td>Calcium</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$3.7 \times 10^9$</td>
</tr>
<tr>
<td>Subfragment 1</td>
<td>115,000</td>
<td>119,000</td>
<td>5.75</td>
<td>0.086</td>
<td>33</td>
<td>287</td>
<td>$1.16 \times 10^7$</td>
<td>$1.5 \times 10^9$</td>
</tr>
</tbody>
</table>

* Rotary dispersion Drude constant obtained from a plot of $\lambda^2 [\alpha]$ against $[\alpha]$ in the range 600 to 350 m$^{-1}$.$^b$ The solvent was 0.05 M KCl-0.1 M Tris, pH 7.6.

$^b$ Harmonic mean of the principal rotational relaxation times at 25°.$^c$ ATPase activities are expressed as micromolar concentration of Pi per mole per 5 minutes at 37°.$^d$ Values were measured in the presence of $1 \times 10^{-3}$ M ATP-$5 \times 10^{-3}$ M CaCl$_2$ (0.05 M KCl) or $1 \times 10^{-3}$ M EDTA (0.4 M KCl)-0.05 M Tris-histidine, pH 7.6 (4).

$^e$ Reference 1.

$^f$ Weight average molecular weight of Subfragment 1 represents the average of values from low and high speed equilibrium sedimentation.

**Fig. 7.** Fluorescence polarization of HMM and Subfragment 1. The solvent was 0.1 M phosphate, pH 7.0. O-O, HMM; concentration, 3 mg per ml. Mean degree of conjugation, 4.6 moles of 1-dimethylamino-5-naphthalenesulfonyl chloride per mole of HMM. ●-●, Subfragment 1; concentration, 4 mg per ml. Degree of conjugation, 4 moles of 1-dimethylamino-5-naphthalenesulfonyl chloride per mole of subfragment. Degrees of labeling were determined spectrophotometrically as described in the text.

the Perrin equation is obeyed by both conjugates, and values of $p_0$ of 0.26 were calculated from the intercepts, in agreement with that found for the limiting polarization of 1-dimethylamino-5-naphthalenesulfonyl chloride ($p_0 = 0.25$ to 0.27) (11). As may be seen from Table III, the harmonic mean of the principal relaxation times of Subfragment 1 is identical, within experimental error, with that of HMM.

**Amino Acid Analyses**—Amino acid analyses of duplicate samples of HMM and Subfragment 1 are given in Table IV, where values for each residue have been computed on the basis of 100,000 g of protein exclusive of tryptophan and half-cystine. The data for HMM are in good agreement with those reported earlier by Lowey and Cohen (21) and by Kominz, Hough, Symonds, and Laki (23) and, with a few exceptions, are closely similar to the results for Subfragment 1.

**DISCUSSION**

Since the ultracentrifugation and chromatographic results presented above indicate that approximately 80% of the HMM mass is transformed into Subfragment 1, the molecular weight of this particle should be of the order of 304,000 to 320,000 g per mole (based on a molecular weight for HMM of 380,000 to 400,000 g per mole). Direct determination of the molecular weight yields values between 114,000 and 120,000 g per mole. It is therefore concluded that the brief action of trypsin upon HMM releases three particles of virtually identical mass, and, in view of the finding that the ATPase activity of HMM remains essentially constant during production of Subfragment 1 (Fig. 3) and that the molar ATPase activity of HMM is 2 to 4 times that of Subfragment 1 (Table III), it seems likely that each of these particles possesses an active site for the hydrolysis of ATP.

Earlier studies on the structure of native myosin (24–26) have indicated that this molecule is composed of three polypeptide chains, each of which is folded into an identical tertiary structure with 3-fold axis of rotational symmetry in the HMM region (3). The present results are consistent with the view that each of the three Subfragment 1 particles represents a folded, globular...
Fig. 8. A, schematic diagram of the tryptic hydrolysis of myosin. The dimensions shown for myosin and the meromyosin segments are taken from several sources: LMM (1, 21, 30); HMM (3, 21, 29, 30); enzyme-sensitive region (1, 3). Although the three polypeptide chains are presumed to be identical (20, 27), they are shaded differently. B, proposed relationship of myosin to the interfilament cross-bridge of the myofibril. Only 1 myosin molecule of a thick filament is shown. G-Actin monomers are represented diagrammatically as spheres, and the general structure of the F-actin filament is taken from Hanson and Lowy (31).

From a study of the kinetics of peptide bond cleavage of each of the meromyosins during tryptic digestion, the rates of proteolysis of LMM and HMM were found to be essentially identical, suggesting (1) that a portion of the HMM structure may resemble the rodlike \( \alpha \)-supercoil configuration proposed for LMM (3, 21). On the basis of x-ray diffraction and optical rotatory dispersion studies, Lowey and Cohen (21, 28) have also proposed that HMM consists of a rodlike, \( \alpha \)-helical, fibrous core attached to a less helical, globular region or regions. These ideas are particularly relevant to the electron microscopic studies of Rice (29) and Huxley (30), which reveal the HMM particle as a globular structure attached to a rodlike "tail" segment.\(^5\) Taken together, the preceding evidence supports the concept that HMM is an asymmetrical, coiled coil structure of three \( \alpha \)-helices, each of which terminates in a tightly folded, enzymically active tertiary structure. The rapid fall in viscosity observed during the initial stages of tryptic hydrolysis of HMM (Fig. 3) could thus reflect destruction of the rodlike tail segment. In support of this reasoning, electron micrographs of brief tryptic digests of HMM are essentially devoid of tail segments and reveal the predominating particle to be globular with diameter about one-half as large as that of the HMM region of myosin.

A particularly surprising feature of the HMM structure is that the globular fragments are quite easily dissociated from one another during tryptic digestion, indicating that once the "tail" segment has been removed, the interchain packing forces are weakened.

\(^4\) In view of the finding from gel filtration studies (Fig. 4) that Subfragment 1 emerges as a single ATPase-active fraction, together with the fact that no weight class heterogeneity (by both velocity and low and high speed equilibrium sedimentation) was detected, the three particles have been assumed to be identical. This conclusion is supported (a) by the finding that the subunit polypeptide chains of myosin are ultracentrifugally (3, 24-26) and electrophoretically (25) identical and (b) by peptide mapping studies of tryptic digests of myosin, which indicate that the tryptic peptides are chemically identical (27). The possibility remains, however, that undetected differences do exist and therefore that not all three Subfragment 1 particles are enzymically active.

\(^5\) It should be noted that the diameter of the tail segment seen in electron microscopic preparations of HMM is appreciably less than that of LMM (29). However, the HMM preparations examined so far in the electron microscope were obtained after 10 to 20 minutes of tryptic digestion of myosin (28, 30). On the basis of pH-stat kinetics, about 50% of the susceptible bonds of HMM have been cleaved at this time of isolation (1).
insufficient to hold these particles together. In fact, it seems possible that these segments have some freedom of movement relative to each other since the polarization of fluorescence data summarized in Fig. 3 and Table III show that conversion of HMM into Subfragment 1 occurs with no change in degree of polarization and that the rotational relaxation times of HMM and Subfragment 1 are essentially identical. A decrease in molecular weight from 380,000 to 115,000 g per mole would be expected to produce an appreciable decrease in polarization if HMM behaves in solution as a single rigid macromolecular unit, and this is evidence that the three polypeptide chains constituting HMM are united together by relatively strong forces in the "tail" or fibrous section, but may enjoy a relatively independent existence from one another in the globular region. The optical rotation and optical rotatory dispersion constant of Subfragment 1 ([α]_{c}^{20} - 33° (λ = 287 mμ)) are virtually identical with those of HMM, consistent with the idea that very little change in chain configuration of the globules occurs during dissociation. The proposed structural relationship of HMM and Subfragment 1 to the myosin molecule is illustrated in Fig. 5A.

The length and diameter of Subfragment 1 may be estimated by assuming this particle to be an anhydrous prolate ellipsoid of revolution. From the molecular weight, apparent specific volume, and intrinsic viscosity, the major and minor axes of this ellipsoid from the Sihaba equation (32) would be of the order of 250 Å and 30 Å, respectively. (The Scheraga-Mandelkern treatment (19) gives major and minor axes of 260 Å and 38 Å, respectively.) It will be noted that a length of 250 Å would be sufficient to accommodate the length (approximately 180 Å) of the lateral cross-bridges which unite the thick (myosin-containing) and thin (actin) filaments in electron microscopic preparations of the myofibril (30).

Huxley has inferred from his electron microscopic studies (30) that a single lateral cross-bridge corresponds to the HMM segment of myosin. Since the length of an individual HMM particle is of the order of 600 ± 200 Å based on both hydrodynamic (21) and electron microscopic measurements (29, 30), it would appear that only that segment of HMM consisting of the three active globules acts as a cross-bridge and that the fibrous rodlike section of HMM lies within the thick filament as indicated schematically in Fig. 5B. The loose packing of these segments, together with their apparent freedom of relative movement, may play an important role in the mechanism whereby thin filaments slide past thick filaments (30, 33, 34) during the contractile event.

**SUMMARY**

The kinetics of trypsin digestion of heavy meromyosin (HMM) has been examined in the ultracentrifuge. From an analysis of the sedimentation patterns it was found that in the early phase of digestion approximately 80% of the mass of HMM is converted into a slower sedimenting, relatively homogeneous component (Subfragment 1: Reference 2) together with lower molecular weight peptides. This process is accompanied by large changes in viscosity (η_{0}c = (0.3%)) falls from 0.60 dl per g (ω 0.10 dl per g). Subfragment 1 was isolated from trypsin digests by gel filtration of Sephadex G-200 and its molecular properties were determined. Velocity sedimentation and low and high speed equilibrium sedimentation studies revealed a relatively high degree of particle homogeneity (M_{w} = 117,000 to 121,000 g per mole; M_{w} = 115,000 g per mole), while viscosity measurements ([γ] = 0.080 dl per g) indicated a low particle asymmetry.

The specific enzymic activity (molar basis) of Subfragment 1 was found to be one-fourth to one-third that of HMM, and polarization of fluorescence measurements of fluorescent dye-protein conjugates yielded essentially the same rotational relaxation time as that determined for HMM. Specific optical rotation and rotatory dispersion constants and amino acid analyses of Subfragment 1 are also closely similar to these properties of HMM.

These results have been interpreted as indicating that the effect of trypsin upon HMM is to cause a fragmentation of this meromyosin into three globular, adenosine triphosphatase-active particles of essentially identical mass, and a model for the HMM segment of myosin is proposed.

**REFERENCES**
