THE HOMOGENEITY AND MOLECULAR WEIGHTS OF THE MEROMYOSINS AND THEIR RELATIVE PROPORTIONS IN MYOSIN

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SUMMARY

The digestion of myosin by trypsin or chymotrypsin was found to yield ultracentrifugally homogeneous L(ight)-meromyosin. The H(eavy)-meromyosin, however, has a trailing shoulder in the ultracentrifuge. The inhomogeneity observed is variable and due to over-digestion by trypsin, and/or to aggregation of the H-meromyosin. Borate and barbital buffers increase the inhomogeneity while phosphate apparently prevents it to some extent. In high concentrations of phosphate buffer it is possible to obtain digests in which both sub-units are homogeneous.

Measurements of the relative areas of the sedimentation patterns and the application of the radial dilution and JOHNSTON-OGSTON corrections reveal that, exclusive of dialyzable fragments, the digests consist of 25 % L-meromyosin and 75 % H-meromyosin by weight.

Determination of the meromyosin molecular weights by the ARCHIBALD method gave values of 324,000 ± 20,000 and 126,000 ± 2,000 for the heavy and light fragment respectively.

These composition and molecular weight data, and the recently measured molecular weight of myosin lead to the conclusion that myosin is made up of only one molecule of each meromyosin. This LH structure for myosin is also in agreement with a variety of other data, including amino acid analyses and optical rotation.

INTRODUCTION

Previous investigations of the tryptic or chymotryptic digestion of the muscle protein myosin have revealed that the molecule can be split into two components: L(ight)-meromyosin and H(eavy)-meromyosin[1-5]. From sedimentation-diffusion measurements molecular weights of 232,000 and 96,000 were assigned to the H- and L-fragments respectively[6]. Area measurements on the sedimentation patterns of the myosin digest showed 43% L- and 57% H-meromyosin[6]. These figures are consistent with a model of two L- and one H-fragment per myosin molecule (molecular weight ~ 500,000).

However, recent studies of sedimentation, diffusion, and light-scattering have cast doubt on these older figures[7-9]. Since a reconstruction of the parent molecule

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depends so strongly on the molecular weights chosen for the sub-units, we felt it important to measure these by a method free from the difficulties inherent in light-scattering and sedimentation-diffusion measurements. We therefore chose the ARCHIBALD method[10,11]. This procedure has been standardized with known, relatively small protein molecules, and in such cases has been very successful[12]. The method has also been applied to myosin[13,14].

Furthermore, the recent clarification[15–17] of the corrections needed to convert areas measured from sedimentation patterns into percentage composition makes feasible a more precise calculation of the relative proportions of L- and H-meromyosin in the myosin digest than was possible in the earlier work.

This percentage composition is also needed if a correct assignment of the number of sub-unit molecules in the intact myosin is to be made. We therefore redetermined these values, making the corrections now known to be necessary.

In addition, preliminary work[8] had convinced us that the digestion procedure itself required further study. This prompted us to investigate the effect of various conditions of digestion on the homogeneity of the fragments obtained.

EXPERIMENTAL

Materials

Myosin was prepared from rabbit muscle according to the procedure of SZENT-GYÖRGYI[18] and MOMMAERTS[19] with slight modifications, as previously described[8,20]. Great care was taken to keep the protein entirely in the cold (3–4°) during its preparation and storage. Experiments on myosin were performed within 60 h after the rabbit's death. The homogeneity of the preparation was judged at low temperature in the ultracentrifuge which showed a single, shoulder-free peak[8,20]. This test is far from adequate since slight polydispersity in shape or weight (apart from easily detected dimer formation) would not be detected in this manner. However, more sensitive criteria are not yet available.

For the digestion of myosin we used salt-free, twice crystallized trypsin (Worthington Biochemical Corp.) which was dissolved in 0.0025 *M* HCl and standardized at 25° by adding 0.1 ml of 0.05% trypsin solution to 25 ml of 0.00025 *M* benzoyl-L-arginine ethyl ester·HCl, pH 8.0. The hydrolysis of the substrate was followed in a Beckman Model DU spectrophotometer at 253 mμ[21]. In this manner an activity of 1200 trypsin units/mg was obtained where one trypsin unit is defined as that activity causing a rate of change of optical density of 0.001/min.

To stop the enzymic activity immediately, when needed, we used Soybean trypsin inhibitor, five times crystallized from ethanol (Worthington).

Ribonuclease, crystallized from ethanol (Worthington), was employed as a standard for molecular weight determination by the ARCHIBALD method. Before use it was dissolved and dialyzed *vs.* 0.03 *M* phosphate buffer, pH 7, ionic strength 0.1.

Preparation of the meromyosins

We initially prepared the meromyosins by following essentially the digestion procedure outlined by SZENT-GYÖRGYI[8]. Borate buffer (0.1 *M* borate-NaOH; pH 8.8) was added to a 1% stock solution of myosin (in 0.6 *M* KCl) in a 1 to 10 ratio by volume. 0.05% trypsin was stirred into the solution for 10 min at 25.0° ± 0.1°, at

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which time the reaction was stopped with 0.1 % soybean trypsin inhibitor. All reagents were prewarmed to 25° before mixing. The final concentrations of trypsin and trypsin inhibitor were 0.0041 % and 0.0077 %, respectively. Subsequently the procedure was carried out in buffer media different from that described above. These experiments are discussed below.

Purified L-meromyosin was obtained by dialyzing the digest against 10 × its volume of 0.0067 *M* neutral phosphate buffer[8]. The precipitate of L-meromyosin was collected by centrifugation, dissolved in a minimum volume of pH 7, 0.027 *M* K_2HPO_4, 0.019 *M* KH_2PO_4, 0.5 *M* KCl solution, and reprecipitated in the same manner. This re-precipitation was repeated. It is important here to keep the ionic strength above 0.05 *M* to prevent co-precipitation of H-meromyosin.

Two samples of purified H-meromyosin were graciously supplied by Dr. J. GERGELY, one obtained from a trypsin digest, the other from a chymotrypsin digest.

Sedimentation

1. *Area measurements and corrections for the* JOHNSTON-OGSTON *effect*

Schlieren sedimentation patterns were obtained in the Spinco Model E ultracentrifuge. In determining the relative concentrations of two protein components from the areas on the schlieren photograph, it is necessary to make two corrections: the first of these takes into account the familiar radial dilution effect, which arises because of the sector shape of the cell; and the second arises from the "build up" of the slower component that occurs in the region of the cell where it is sedimenting into solution containing the faster component[15–17]. The result of this latter effect is that the concentration of the slower component, as obtained from uncorrected area measurements, is too high, while the concentration of faster component is too low. The reality of this effect has been conclusively demonstrated experimentally[16]. A theoretical examination of the phenomenon by JOHNSTON AND OGSTON[15] led to the correction formula:

$$\frac{C_s^{obs}}{C_s^{\circ}} = \frac{S_f - S_{s,\,mixt.}}{S_f - S_s} \qquad (1)$$

Here C_s^{obs} is the uncorrected concentration of the slower component actually obtained from the area measurement; C_s° is the actual concentration of the slower component; $S_f(S_s)$ refers to the sedimentation coefficient of the faster (slower) component; and $S_{s,\,mixt.}$ is the sedimentation coefficient of the slower component into the mixture containing the faster one. Since no boundary results from the movement of the slower component into the faster, $S_{s,\,mixt.}$ cannot be determined from the schlieren pattern. Various approximations have been used to estimate this quantity. One possibility is to obtain $S_{s,\,mixt.}$ from the sedimentation of the slow component alone at the concentration of the mixture. This is sufficiently accurate only if the components of the mixture have similar dependence of S on concentration. In the case of the meromyosins this condition is not fulfilled; the sedimentation rate of H-meromyosin being much more strongly concentration dependent than that of L-meromyosin.

Since the JOHNSTON-OGSTON effect is large whenever the faster component has a high intrinsic viscosity, the suggestion has been made that the increase in viscosity caused by the presence of the asymmetric molecule is the major factor causing the

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buildup of slow component[16]. Therefore an alternative approximation that has been found useful is:

$$S_{s,\mathrm{mixt}} = S_s/\eta^f_{\mathrm{rel}} \tag{2}$$

where η^f_{rel} is the relative viscosity of the solution of the faster component.

A more detailed correction formula, taking into account both radial dilution and JOHNSTON-OGSTON corrections, has been derived by TRAUTMAN *et al.*[17]. They find the approximate relations:

$$r = \left[\left(\frac{\bar{X}_f^{\mathrm{obs}}}{X_0}\right)^{2(1-\sigma)} - 1\right] \Big/ \left[\left(\frac{\bar{X}_f^{\mathrm{obs}}}{X_0}\right)^{2} - 1\right] \tag{3}$$

and

$$C_s^{\circ} = \left(\frac{\bar{X}_s^{\mathrm{obs}}}{X_0}\right)^{2} C_s^{\mathrm{obs}} \Big/ r \tag{4}$$

in which $\bar{X}^{\mathrm{obs}}$ indicates the "equivalent boundary position which would result if the material were arranged to give infinitely sharp boundaries"; X_0 is the position of the meniscus; $(r-1)$, as is evident from eq. (4), is the "buildup" of the slow component; and σ has been shown empirically[17] to be given to a close approximation by $[S_s]/[S_f]$ where $[S]$ represents the infinite dilution sedimentation constant of the given component. All of the necessary quantities in equations (3) and (4) may be evaluated from the schlieren diagrams and a calculation of the corrected concentrations is possible.

In the present instance the measurements were performed in the following way. Myosin was digested as described above (see also below). The digest was run in the analytical ultracentrifuge at 21°, 59,780 rev. min and a bar angle of 50° (Fig. 1). Photographs were taken at 16 min intervals. The plates were enlarged 8 × and the areas measured with a planimeter. Areas from the upper and lower outlines were averaged. Measurements of the boundary positions were made directly on the plates using a micro comparator. No attempt was made to calculate the "equivalent boundary position"; the position of the maximum ordinate was taken instead. The

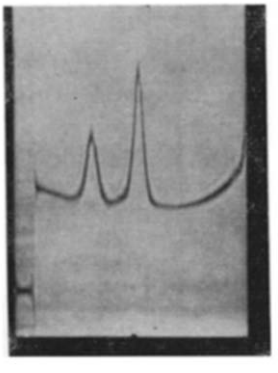

Fig. 1. Trypsin digest of 0.86% myosin in 0.5 *M* KCl, 0.03 *M* phosphate, pH 6.7. Digestion time: 10 min. Ultracentrifuge picture at 59,780 rev./min; 20°; bar angle of 50°; 90 min after attaining full speed.

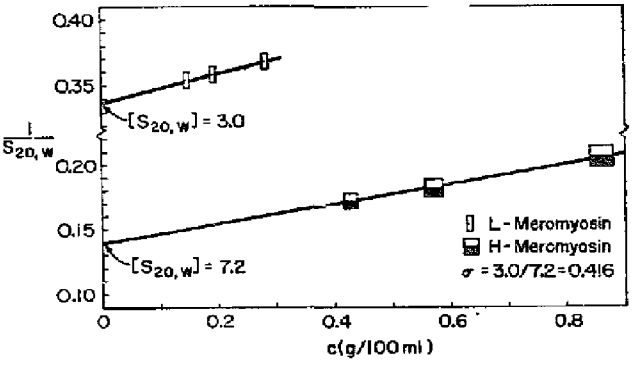

Fig. 2. Reciprocal sedimentation constant of fast and slow peaks in myosin digest as a function concentration. Upper set of points for slow peak plotted against uncorrected concentration of slow component. Lower set of points for fast peak plotted against concentration of total protein.

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error introduced in neglecting this refinement is entirely negligible as judged by more accurate calculations in one case. The quantity $\sigma = [S_s]/[S_f]$ was obtained by plotting $1/S_f$ *vs.* concentration of total protein and $1/S_s$ *vs.* the uncorrected concentration of slow component (Fig. 2)[17]. The value of r was thus obtained from eq. (3) and the corrected concentration, C°_s, from eq. (4).

2. ARCHIBALD *determination of molecular weights*

The approach to sedimentation equilibrium was observed in the Spinco Model E ultracentrifuge equipped with a slow speed attachment (1/3 reduction), used in runs below 12,590 rev./min. It is important to run at sufficiently low speeds to avoid any separation of the boundary from the meniscus. We followed the procedure of KLAINER AND KEGELES[11], making measurements at short enough times of centrifugation to avoid any redistribution of solute and so obtain the weight-average molecular weight of the original sample. Speeds of 12,590 rev./min and 8,210 rev./min were used for L- and H-meromyosin respectively. Runs on L-meromyosin lasted less than three hours, usually 1.5 to 2 h. H-meromyosin was never run over about an hour.

The temperature of the rotor was regulated at 21° by the temperature control unit which employs a thermistor in the base of the rotor.

The schlieren optical system was used with a phase plate as the diaphragm. Throughout this work, the diaphragm was maintained at 80°; this keeps the curve outlines sharp and minimizes the uncertainty of the curve position at the meniscus. Runs were made with 12-mm cells using a 4° sector, Kel-F centerpiece. To facilitate studying the molecular weight at the cell bottom, 0.1 ml of Dow-Corning No. 555 silicone fluid was first introduced into the cell and the protein solution then added. This oil is sufficiently dense, immiscible with water and inert toward proteins[22]. Nevertheless the extrapolation was extremely difficult and measurements near the cell bottom were only used in a few cases on L-meromyosin, and not at all on H-meromyosin. The concentration of the original sample was determined in arbitrary units in a KLAINER-KEGELES type synthetic boundary cell[11,23]. This is a modified 2° cell, with 2 side holes each taking a volume of 0.07 ml of solvent, the center of the cell being filled to approximately 0.16 ml with solution.

Photographs were made on Kodak Metallographic plates which have long exposure time (~ 15 sec) but are of high resolution and contrast. A full surfaced, swing-out viewing mirror is needed for sufficient light. Measurements of the photographic plates were made with a two-coordinate Gaertner microcomparator designed to read with an accuracy of 1 μ[22].

The concentration of the protein at the meniscus was calculated from the measurements using the equation[11]:

$$C_m = C_0 - \frac{0.01}{F X^2_m} \sum_{n=0}^{n_x} X^2_n Z_n \qquad (5)$$

where C_0 is the original concentration (determined in the synthetic boundary cell); 0.1 cm is the comparator interval along the X axis; F is the enlargement factor; Z_n the ordinate (proportional to concentration gradient, dc/dx); and n_x the number of comparator intervals needed to bring the ordinate to zero (Fig. 3). The solvent correction was checked and found to be negligible.

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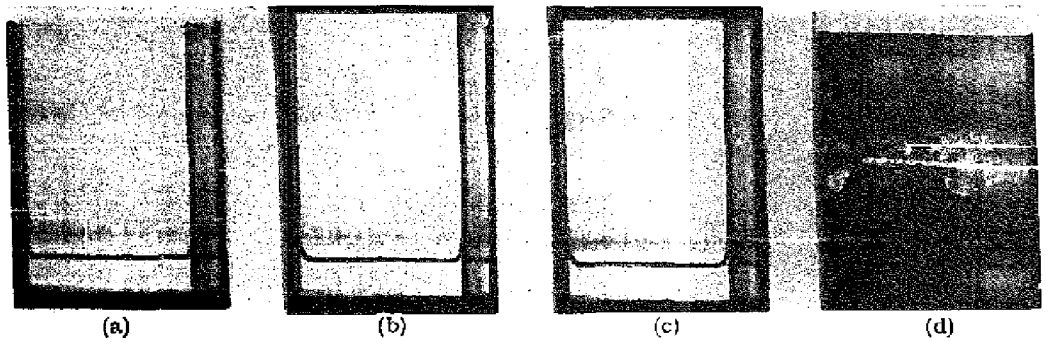

Fig. 3. ARCHIBALD run on H-meromyosin isolated from a three minute trypsin digest. Protein concentration is 0.320% in 0.03 *M* phosphate, pH 7. Speed was 8210 rev./min; bar angle: 80°; temperature: 20°. Times after full speed are: (a) 12 min, (b) 33 min and (c) 65 min. Photograph (d) is a synthetic boundary run for the same solution and conditions.

The weight average molecular weight was then determined from the relation[10]:

$$M_w = \frac{RT}{(1 - \bar{v}\rho)\omega^2} \cdot \frac{(dc/dx)_m}{X_m C_m} \qquad (6)$$

with ω the angular velocity. We did not determine the partial specific volumes, $\bar{v}$, for the meromyosins, but used the value for myosin: 0.728 ml/g[24].

To check the ARCHIBALD determinations a run was made on ribonuclease. This protein has been found to have a molecular weight in the range 13,000 to 14,000 by chemical analysis[25], sedimentation-diffusion[26] and X-ray crystallography[27] and may be used as a standard in ARCHIBALD work. The most exact value is probably the chemically determined one: 13,895[25]. We obtained a value of 14,300 by the ARCHIBALD method at 22°, with a speed of 25,980 rev./min and using $\bar{v} = 0.700$ ml/g[26]. The agreement is satisfactory.

RESULTS

Meromyosin homogeneity

The original ultracentrifugal analyses of myosin digests were performed[3-5] on material digested in 0.5 *M* KCl–0.05 *M* phosphate, pH 7.1. In subsequent directions for preparing and isolating the meromyosins a borate–KCl buffer, pH 8.8, was recommended[6]. No ultracentrifuge patterns were reported for digestions in the borate medium. To check the course of the digestion in borate we therefore ran these digests in the centrifuge. The result is shown in Fig. 4. One sees a broad, polydisperse leading peak, and a symmetrical slower peak. The trailing L-meromyosin is seemingly homogeneous, and it was thought that perhaps the inhomogeneity of the leading peak might be due in part to some undigested myosin. However, increasing the time of digestion actually broadened the peak further. We then attempted to control the digestion time more carefully and thus avoid overdigestion, by digesting in the cold at 0°. Using the same recipe as at room temperature, 2 h of tryptic action still left some undigested myosin. This was just removed by increasing the digestion time to 3 h. This procedure in the cold sharpened the leading peak slightly, but the preparation still did not approach the homogeneity shown in the literature for phosphate-digested samples.

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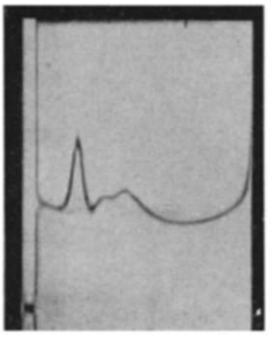
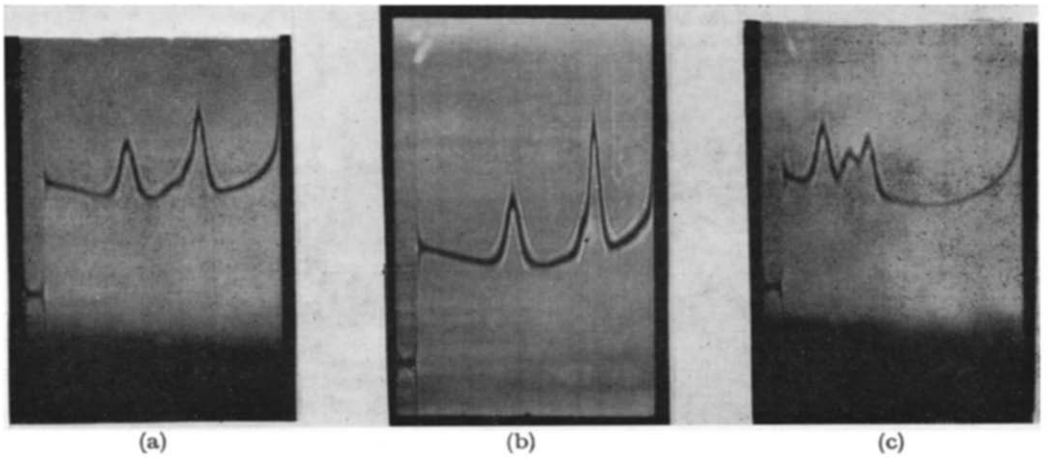


Fig. 4. Trypsin degist of 0.745% myosin in 0.5 *M* KCl, 0.1 *M* borate, pH 8.8. Digestion time: 12 min. Ultracentrifuge photograph at 59,780 rev./min; 24°; bar angle of 45°; 48 min after attaining full speed.

Fig. 5. Trypsin-digested (25°) myosin in 0.5 *M* KCl, 0.03 *M* phosphate, pH 6.7. Ultracentrifuge patterns at 59,780 rev./min (a) Digested for 6 min. Photograph shows 0.700% protein at a temperature of 19.5° and bar angle of 50°, 112 min after reaching full speed. (b) Digested for 10 min; 0.86% protein; 19.5°; bar angle 50°; picture at 136 min. (c) Digested for 40 min; 0.700% protein at 20°, and bar angle of 50°, 74 min after reaching speed.



We then tried a phosphate digestion described in the earlier paper by MIHALYI AND SZENT-GYÖRGYI[3,4]. Myosin dialyzed against 0.03 *M* PO_4, pH 6.7, 0.5 *M* KCl was digested with trypsin for 12 min at 25° and the digestion stopped with soybean trypsin inhibitor. The same protein concentrations were used as before. Under these conditions a much sharper H-meromyosin peak was obtained; however, on close inspection of the peak as it reached the end of the ultracentrifuge cell, a small trailing shoulder could be discerned on the faster peak (Fig. 5b). Neither by decreasing nor increasing the digestion time could this shoulder be eliminated (Fig. 5). However, the shoulder is clearly not myosin, since increasing the digestion time only served to increase the amount of polydispersity. This inhomogeneity of the H-meromyosin may be caused by a fragment of overdigested H-meromyosin or by aggregated meromyosin. There is also the possibility that the shoulder may simply be evidence of polydispersion in shape. For example the unstable molecule may unwind partially, thereby changing its configuration and sedimentation coefficient. From these experiments we concluded that either the pH, the type of buffer, or the nature of the particular myosin sample, was responsible for the wide differences we had observed in the shape of the faster peak. To test this hypothesis we digested myosin at pH 6.7 and 7.1 in 0.03 *M* phosphate; pH 8.0, 0.3 *M* PO_4; and pH 8.0 barbital buffer. The first two showed the same small shoulder. The concentrated-phosphate digest appeared shoulder-free with two homogeneous peaks (Fig. 6). The barbital digest dis-

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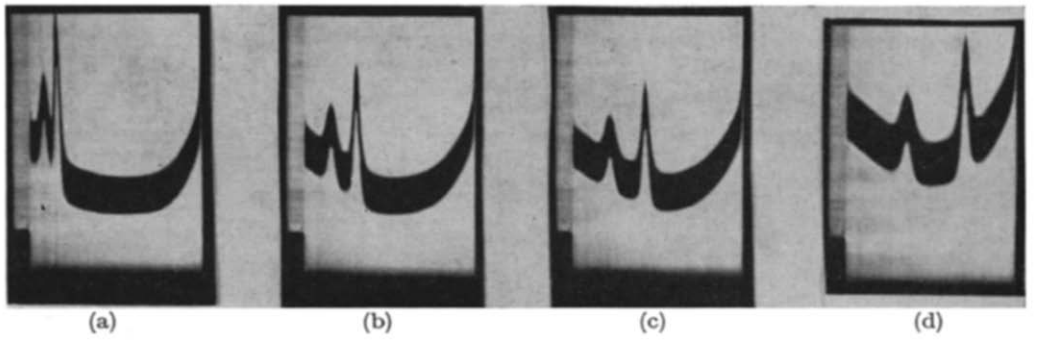

Fig. 6. Ten-minute trypsin digest in 0.3 *M* phosphate, pH 8.0. Speed: 59,780; conc.: 0.55% protein; temp. of run: 20°; (a) bar angle 50°, 31 min (b) bar angle 50°, 55 min (c) bar angle 50°, 79 min (d) bar angle 40°, 103 min.

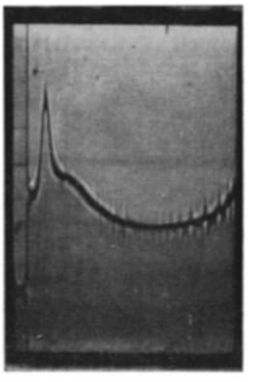

Fig. 7. Twelve-minute trypsin digest in 0.1 *M* barbital, pH 8.0. Speed 59,780 rev./min; protein conc. 0.70%; temperature of run 27°; bar angle, 50°; 30 min.

played no sharp leading peak at all, only a broad, rapidly-sedimenting peak (Fig. 7). We also repeated the borate digestion with the same result as before.

These experiments seem to indicate that the buffer is important in the digestion while the pH is not critical. We were forced to use such a high phosphate concentration at pH 8 since 0.03 *M* PO_4, pH 8 has too low a buffer capacity to maintain a constant pH when trypsin breaks the peptide linkages of myosin. Enough protons are liberated during the digestion to cause a pH drop of one unit. Since 0.3 *M* phosphate is the only medium in which we obtained homogeneous H-meromyosin we infer that the high phosphate ion concentration is responsible. We have previously noted that phosphate ions slow the side-to-side aggregation of myosin and seem to stabilize the molecule[20,21]. Phosphate may exert a similar stabilizing effect on H-meromyosin.

However, although homogeneous H-meromyosin was obtained from two separate myosin preparations in 0.3 *M* PO_4, it was not obtained in this medium on two other occasions, the same trailing shoulder being evident as in 0.03 *M* phosphate. As far as we were able to ascertain the same conditions prevailed in all the experiments, with only slight concentration changes in the myosin depending on the yield of the preparation. At present we can offer no explanation for the variability of the faster peak. It appears that H-meromyosin is quite unstable, or very sensitive to tryptic action or both*. Phosphate ions seem to favor stability during the digestion, while

* A study of the small, dialyzable peptides formed during the tryptic digestion of myosin has been made by Dr. P. F. SPAHR of the Biological Laboratories, Harvard University, Cambridge, Mass. By means of paper electrophoresis and paper chromatography he found that a considerable number of peptides were released on partial or complete digestion. The mobilities and R_F values

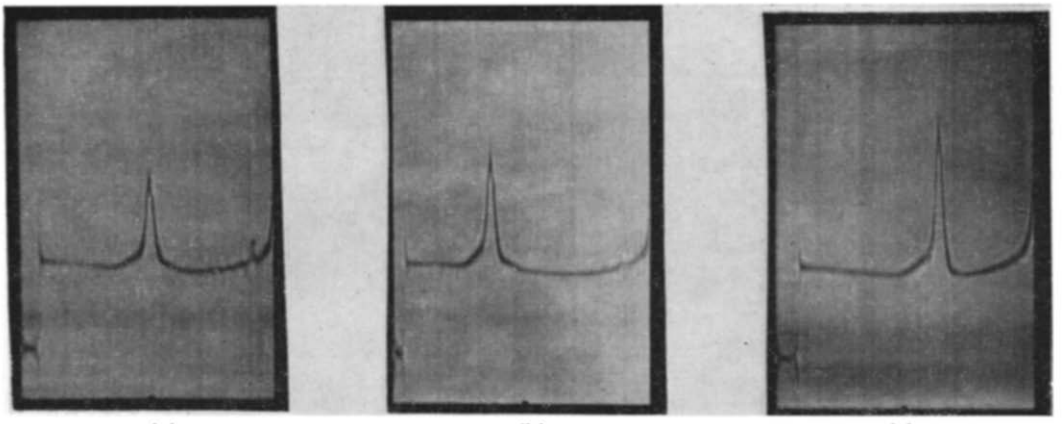

Fig. 8. H-meromyosin isolated from myosin digested for three minutes at 25° by trypsin. Speed in all cases :59,780 rev./min; solvent: 0.03 *M* phosphate, pH 7.0; bar angle 50°. (a) Protein concentration of 0.32 %; run temperature: 20°; time: one hour. (b) Protein concentration 0.32 %; run temperature: 10°; time: 40 min. (c) H-meromyosin isolated from analogous chymotryptic digest. Protein concn. 0.483 %; run temperature: 20°; time: 78 min.

borate and barbital do not. In contrast to the unstable nature of H-meromyosin, L-meromyosin seems quite resistant to trypsin. The shape and symmetry of the L peak is uninfluenced by altering the buffer medium.

Most of the previously reported sedimentation pictures of H- and L-meromyosin have shown the peaks during the early stages of the ultracentrifuge run. Consequently, it is impossible to judge the homogeneity of such peaks, since the shoulder we have observed only shows up towards the end of the run (Fig. 5b). However, in one published case in which the run was carried rather further than usual (ref.[4], Fig. 4a) a slow shoulder does begin to appear. It is not likely, therefore, that our method of myosin preparation is the cause of the inhomogeneity.

We now turn from consideration of the digests to the homogeneity of the protein fragments isolated from them. H-meromyosin separated from our digests showed, as expected, a pronounced trailing shoulder. Because of the absence in the literature of ultracentrifuge patterns sufficiently far down the cell to show up the existence of inhomogeneity, and to check the sinister possibility that our technique in preparing myosin was at fault, we obtained two different samples of H-meromyosin from Dr. J. GERGELY. One of these had been obtained from a trypsin digest, the other from a chymotrypsin digest. Both preparations show trailing shoulders, whether run at room temperature or in the cold (Fig. 8).

Whether a completely homogeneous H-meromyosin preparation can be prepared using the present enzymic digestion procedure is uncertain. Work is in progress

of most of these peptides were independent of the extent to which the myosin had been digested, between 30 and 100% digestion. This is compatible with the view that the digestion is of an all-or-none character. However there also occurs a secondary slower digestion process, which is evident from the fact that some components in the electrophoretic and chromatographic patterns change in relative concentration as digestion proceeds, and some new components appear. In addition to these small dialyzable peptides there is at least one non-dialyzable component which is distinct from L- or H-meromyosin. One possible interpretation of these observations would involve a progressive hydrolysis of H-meromyosin. This would be compatible with the heterogeneity of H-meromyosin as observed in the ultracentrifuge.

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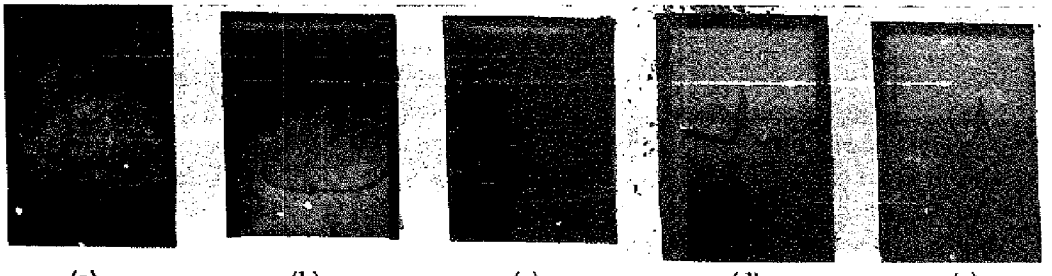

Fig. 9. L-meromyosin isolated from myosin digested for twelve minutes at 25° by trypsin. Run in 0.6 *M* KCl at 59,780 rev./min. (a), (b) and (c) show 0.423% protein run at 10°, with bar angles of 50°, 60° and 50° at 1 min, 22 min, and 86 min, respectively, showing the absence of a leading peak. Towards the end of the run, L-meromyosin always looks homogeneous as shown by (d) and (e) taken for a preparation that had a small leading peak. Photographs for 0.40% protein at 27.5° with a bar angle of 45°. Times were: (d) 145 min and (e) 193 min.

to purify H-meromyosin by ion exchange methods*. Unfortunately, H-meromyosin is an extremely unstable protein and it may not be possible to obtain it in a homogeneous form if, indeed, it ever exists as such. It is very sensitive, not only to over-digestion by trypsin, but also to heat; it denatures rapidly on exposure to room temperature.

L-meromyosin, on the other hand, is relatively insensitive to tryptic action. However, if the ionic strength is allowed to fall below 0.05 during the precipitation, small amounts of H-meromyosin may be coprecipitated. This may explain the small leading peak that appears in preparations allegedly pure (ref.[6], Fig. 5). When all precautions of choice of buffer and ionic strength of precipitation are observed, a very homogeneous peak is obtained (Fig. 9). Furthermore, the preparation is very stable. When L-meromyosin was left at room temperature for 23 h it remained homogeneous, in contrast to the behavior of myosin and H-meromyosin.

A study of the rate of sedimentation of L-meromyosin as a function of concentration gave a value of 2.99 Svedberg units for the intrinsic sedimentation constant. This agrees with the value determined by SZENT-GYÖRGYI: 2.86 Svedberg.

Meromyosin composition of myosin

The results obtained from area measurements on the diagrams of the run illustrated by Fig. 1, and the corrections applied according to equations (3) and (4) are summarized in Table I. The value of σ obtained from the plates and the plot of Fig. 2 was 0.416. This agrees well with the value of 0.410 calculated from the data obtained by SZENT-GYÖRGYI in studies of the isolated components. Column 11 of Table I gives the per-cent L-meromyosin (~33%) corrected only for radial dilution. The "buildup" of slow component (Column 5) is seen to be appreciable and this is reflected in the values of the last column, showing the % L-meromyosin corrected both for radial dilution and JOHNSTON-OGSTON effect. The corrected values are constant from frame to frame; the determinations on two separate digests also agreed well and showed

* Attempts have been made by Dr. P. F. SPAHR to purify the H-meromyosin using ion-exchange chromatography on DEAE cellulose. Under certain conditions a sharp chromatographic peak was obtained. However, when analyzed in the ultracentrifuge this peak still showed a trailing shoulder. This shoulder might be due to a material similar in properties to H-meromyosin, since it is eluted under the same chromatographic conditions.

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TABLE I

PERCENTAGE COMPOSITION OF MYOSIN DIGEST

Frame	$Area_s$	X_S *(cm)*	$\left(\frac{X_S}{X_0}\right)^2 C_S^{obs}$	*(7–1)100*	C°_S	$Area_F$	X_F	$\left(\frac{X_F}{X_0}\right)^2 C_F^{obs}$	C°_F	*% L-mero**	*% L-mero (corr)***
2	135	6.142	146	37%	107	257	6.322	292	331	33.2	24.4%
3	126	6.194	139	31%	106	243	6.424	287	320	32.6	24.9%
4	121	6.254	135	18%	114	227	6.535	278	299	32.7	27.6%
5	112	6.316	128	32%	97	212	6.650	267	298	32.4	24.6%
6	110	6.356	128	36%	95	199	6.723	257	290	33.2	24.6%
7	108	6.425	127	34%	95	187	6.850	251	283	33.6	25.1%
Avg.											25.2 ± 0.3***

S = L-meromyosin; F = H-meromyosin; $\sigma = 0.413$; $X_1 = 5.922$ cm.

* Corrected for radial dilution only.

** Corrected for radial dilution and JOHNSTON-OGSTON effect.

*** The estimate of probable error does not take account of the fact that about 1% of the total protein consists of residual enzyme.

an average value of 25.5 % L-meromyosin, and 74.5 % H-meromyosin. These values differ substantially from those obtained previously. In fact, even the figure obtained for the percentage of L-meromyosin, making only the radial dilution correction, is 35 % lower than the value used hitherto. If both corrections are omitted our plates show about 40 % of the material as the slower component. Since no details of the corrections made are given in the report of the earlier work, it may be inferred that this is the cause of the discrepancy.

Since the JOHNSTON-OGSTON effect is so important, it is necessary to consider the justification for such a correction. HARRINGTON AND SCHACHMAN have shown the effect of a fast component on different slow components by studying artificial mixtures in which the concentrations of the individual components were known[16]. They found that the area of the slow component in a mixture could increase by as much as 300% over that of a comparable concentration of the slow component alone. They also showed that the magnitude of the anomaly depended on the proximity of the sedimentation constants of the individual components; as the ratio of the sedimentation constants decreased from 14.4 to 1.09 the magnitude of C_s^{obs}/C°_s increased from 1.0 to 2.7. The mixture most closely approximating ours contained fibrinogen ($S_F = 5.3$) and β-lactoglobulin ($S_s = 2.7$). In that case they observed $C_s^{obs}/C^\circ_s = 1.5$. For comparison, in one of our experiments ($S_F = 4.9$ and $S_s = 2.7$) we found a value of 1.3 for this ratio. It thus appears that natural mixtures of L- and H-meromyosin occur in a range where the JOHNSTON-OGSTON correction has been well established both theoretically and empirically, and we would expect calculations based on equations (3) and (4) to express accurately the percent composition of these mixtures.

Furthermore, if one assumes, as a first approximation, that $S_{s,\,mixt.} < S_s$ because of the viscosity increment of the fast component, one can calculate $S_{s,\,mixt.}$ by dividing S_s by the relative viscosity of the solution containing the fast component (eq. (2)). The correction derived in this manner has been found empirically to agree with that from the more exact formulation[16,17]. SZENT-GYÖRGYI's data yield a value of 1.19 for the relative viscosity of an H-meromyosin solution having 0.58 g/dl. Therefore we have from eq. (2) and the data at the highest concentration (0.86% *total protein*) of Fig. 2: $S_{s,\,mixt.} = 2.71/1.19 = 2.28$ S. Substituting this value into eq. (1) we get: $C_s^{obs}/C_s^\circ = 1.20$. If we apply this ratio to column four of Table I (which has already been corrected for radial dilution) we obtain a value of 27.6% L-meromyosin, in close accord with the value more elaborately derived previously. The use of successive approximations in calculating the viscosity correction brings the results even closer.

We may conclude from these comparisons that the JOHNSTON-OGSTON correction is necessary to give true results in these mixtures and that the current value, 25% L-meromyosin, is to be preferred over the older figure of 43% L.

One obvious means of checking the JOHNSTON-OGSTON correction would be to take known concentrations of purified L- and H-meromyosin and determine their areas in artificial mixtures. This procedure has the drawback that the H-meromyosin is difficult to obtain in pure form and aggregates quickly.

Meromyosin molecular weights

A typical schlieren photograph of the approach to sedimentation equilibrium

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and a synthetic boundary cell photograph are shown in Fig. 3. The data for both meromyosins are plotted as M_W *vs.* time in Fig. 10. Since no trend in molecular weight values appeared with varying protein concentration, we may assume that the second virial coefficient in both cases is zero.

For L-meromyosin, which has been shown to be stable and homogeneous, there is likewise no dependence of apparent molecular weight on time. The average molecular weight obtained for this sub-unit is 126,000 ± 2000.

As expected from the stability and homogeneity studies reported above, the data for H-meromyosin are scattered over about a 30% range, and in the case of the H-meromyosin isolated from the trypsin digest there does appear to be a trend upwards of molecular weight with time. However, the arithmetic average of all the values (solid line, Fig. 10) gives exactly the same molecular weight as the value deduced from the intercept at zero time of the least-squares line (dashed line of Fig. 10) through the data for the trypsin digest; and the value so obtained is only 3.5% higher than the average of the chymotrypsin values alone (dot and dashed line of Fig. 10). We therefore may accept the figure 324,000 ± 20,000 as the molecular weight of H-meromyosin.

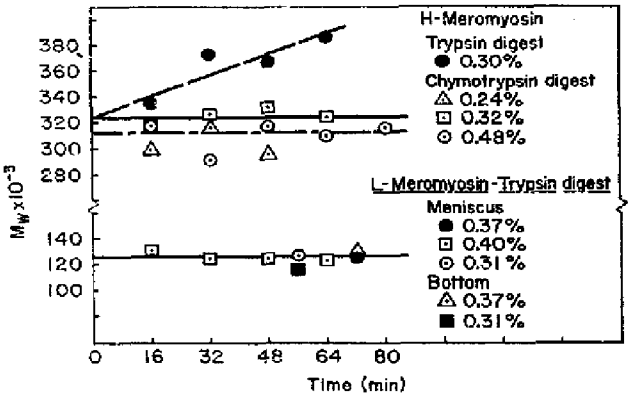

Fig. 10. Archibald measurements of meromyosin molecular weights as a function of time and protein concentration. The dashed line is the least squares fit of the data for the H-meromyosin from trypsin digest only. The solid line is the average of all H-meromyosin determinations, and the dot-dash line is the average of data from the chymotrypsin digest only.

The comparison of these molecular weights with hitherto accepted values has some puzzling features. SZENT-GYÖRGYI reported 96,000 and 232,000 for L- and H-meromyosin respectively, by the sedimentation-diffusion method[6]. However, in that study, a value of 0.748 ml/g was used for the partial specific volume. To be comparable to our own measurements, these must be corrected to the value: $\bar{v} = 0.728$. Making this correction we find 89,000 for the molecular weight of Light-meromyosin and 215,000 for the heavier fragment. The current values represent an increase of 30–35% in both cases. This increase, it will be seen below, is of vital importance in any attempt to reconstruct the myosin molecule from its fragments. Since we find quantitative agreement with the earlier determination of the intrinsic sedimentation constant of both meromyosins the difference must arise in the determination of diffusion constant. It must be admitted that the measurement of diffusion constant for substances with molecules that are both asymmetric and unstable is a difficult matter, even with the highest precision apparatus. In the study under discussion, the diffusion curves obtained are not shown, but the sedimentation patterns show a leading shoulder for both meromyosins and the further possibility of a trailing shoulder in the H-meromyosin case. Consequently, it is hard to understand why

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inhomogeneities did not appear in the diffusion experiments. Admitting these deficiencies of the sedimentation-diffusion method, however, it is difficult to explain the fact that the results in this case led to molecular weights that are too *low*! In contrast, some recent sedimentation-diffusion experiments by GERGELY and coworkers[7] gave molecular weights of 120,000 for L-meromyosin and 340,000 for H-meromyosin. The latter value was also obtained by light-scattering. Although these experiments have not yet been reported in sufficient detail to provide an explanation for the disparity in diffusion constants measured in the different laboratories, their work and ours may tentatively be taken as mutually corroborative.

DISCUSSION

On the basis of these values of the meromyosin molecular weights, the currently accepted reconstruction of myosin from its sub-units (L_2H) would require a myosin molecular weight of over $6 \cdot 10^5$, taking into account that the digestion releases about 6% of the total material in the form of dialyzable fragments[2,6]. In contrast, the most recent determinations of the molecular weight of intact myosin show values between $4.2 \cdot 10^5$ and $4.9 \cdot 10^5$[13,14,20]. It is apparent that only one molecule of each meromyosin can be accommodated in an aggregate of such low total mass. Correcting again for the small fragments lost, our meromyosin molecular weights would then require a value of $4.7 \cdot 10^5$ for the mass of intact myosin. This agrees quantitatively with the measured value for the parent myosin. Furthermore, if our percentage data are correct, L-meromyosin should make up about one-fourth of the mass of myosin (less 6% dialyzable fragments). This is likewise seen to be correct, within experimental error.

Amino acid analyses of myosin and the meromyosins have also been used to justify the older formula (L_2H). We must inquire how well the structure we propose, (LH), fits these data. On examination of the data of KOMINZ *et al.*[28] we find that only in the cases of arginine and phenylalanine is the agreement worse than for the L_2H model, and even in those cases it is doubtful if the disagreement is serious. Apparently the amino acid compositions of the meromyosins are too similar to allow for any great sensitivity in using this criterion. The same is true of the optical rotation: within the limits of error allotted to the measurements the optical activities of one L- and one H-meromyosin also add up to that of myosin[29].

Although measurements of the molecular lengths of the meromyosins are in preliminary stages only, it might be added that light-scattering studies show a length of about 900 Å for L-meromyosin[30] and 800 Å for H-meromyosin[7]. The aggregate length, 1700 Å, agrees quantitatively with the value for myosin[20], suggesting an end-to-end arrangement.

It is clear that these considerations serve to terminate the budding controversy over the question of whether the meromyosins are arranged in myosin in the order LLH or LHL. The structure LLH had been proposed from evidence deduced from the angular scattering envelope[31], while end-group determinations have been interpreted as requiring the LHL arrangement[32]. In this connection it should be added that the newer LH structure is in agreement with the measured angular scattering envelope of myosin[33].

Perhaps a word of warning is necessary at this stage. It must be emphasized that the Heavy-meromyosin is ultracentrifugally inhomogeneous, and both fragments

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are immunologically inhomogeneous[34]; so although the myosin molecule is undoubtedly divided roughly into two different parts, it would appear either that partial enzymic digestion does not separate these uniquely, or that the dividing line is itself not entirely sharp.

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