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Biochem. J. (1962) **85**, 431

The Degradation of Heavy Meromyosin by Trypsin

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(*Received* 21 *May* 1962)

Myosin was one of the first proteins with which it could be shown that appreciable proteolytic digestion of the molecule could occur without destruction of its biological activity (Gergely, 1950, 1953; Perry, 1950, 1951). Subsequent extension of these observations led to the isolation of the heavy and light meromyosins (Mihalyi & Szent-Gyorgyi, 1953; Szent-Gyorgyi, 1953; Gergely, Gouvea & Kariban, 1955) and the demonstration that the actin-combining and the adenosine-triphosphatase properties are associated with the former fragment which represents the major portion of the molecule (Lowey & Holtzer, 1959). Clearly a study of the fragments obtained on controlled digestion is of fundamental importance for relating the biological activities of myosin to the ultrastructure of the molecule, yet until studies by Mueller & Perry (1961*a*) heavy meromyosin was considered to be the only fragment which retained the adenosine-triphosphatase activity of myosin. Kakol, Gruda & Rzysko (1961) have produced evidence confirming the findings (Mueller & Perry, 1960, 1961*a*) that heavy-meromyosin preparations contain smaller fragments which possess adenosine-triphosphatase activity.

The present paper, which represents an extension of the investigation by Mueller & Perry (1961*a*), is a study of the tryptic digestion of heavy meromyosin and provides evidence that the ability to combine with actin and to hydrolyse adenosine triphosphate can survive further tryptic digestion. These properties have been shown to be associated with a smaller readily-identifiable fragment which sediments at a slower velocity than does the original heavy meromyosin.

Some aspects of this study have been reported in a preliminary communication (Mueller & Perry, 1961*b*).

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METHODS

Preparation of heavy meromyosin. L-Myosin, prepared as described by Perry (1955), was digested with trypsin at 23° for 10 min. by the method of Szent-Gyorgyi (1953) and immediately dialysed against 10 vol. of 6·7 mM-phosphate Sørensen buffer, pH 7·0. The precipitated light meromyosin was separated by centrifuging; the heavy meromyosin was salted out by $(NH_4)_2SO_4$ in the range 40–55% saturation, dissolved in 0·15M-KCl–20 mM-tris–HCl, pH 7·6, and stored at 1–2°.

Preparation of F-actin. Actin was prepared from rabbit skeletal muscle by the method of Straub (1943); it was purified by isoelectric precipitation with 10 mM-sodium acetate buffer, pH 4·7, or by ultracentrifuging according to the method of Mommaerts (1951).

Digestion of heavy meromyosin. Solutions containing 13·4–14·8 mg. of heavy meromyosin/ml. in 0·15M-KCl–20 mM-tris–HCl, pH 7·6, were generally used and digestion was carried out in a water bath at 25°. Trypsin (salt-free and twice crystallized; Worthington Biochemical Corp.) solutions (10–40 mg./ml.) in 5 mM-HCl were prepared fresh 1–2 hr. before each experiment and appropriate volumes

added to the heavy meromyosin to give final concentrations of 0·5–4·0 mg./ml. as required. Digestion was stopped by the addition of 1·5–2·0 times as much soya-bean inhibitor (Worthington Biochemical Corp.) as trypsin originally added. Stock solutions of 20–60 mg. of soya-bean inhibitor/ml. in 30 or 60 mM-sodium borate buffer, pH 8·5, were also prepared fresh for each digestion. Most experiments were performed with concentrations of 1·0 mg. of trypsin/ml. and 1·5 mg. of inhibitor/ml.

Determination of nitrogen not precipitable with 6·7% trichloroacetic acid. After the addition of inhibitor 9·0 ml. of water was added to 1·0 ml. of the digest and followed by 2·0 ml. of 40% (w/v) trichloroacetic acid. The precipitate was removed by centrifuging and total-nitrogen determinations were performed on samples of the supernatant by the Kjeldahl technique as described by Perry & Zydowo (1959). Similar determinations were also carried out on control samples of undigested heavy meromyosin to which soya-bean inhibitor had been added before the addition of trypsin. The nitrogen not precipitated by trichloroacetic acid in these samples did not significantly exceed the total nitrogen content of the solvent blank. The trichloroacetic acid-precipitable nitrogen arising from heavy meromyosin and its breakdown products in the digest was calculated by subtracting the amount of nitrogen/ml. not precipitated by trichloroacetic acid from the total heavy-meromyosin nitrogen present/ml. before digestion.

Enzyme assays. Adenosine-triphosphatase assays were carried out at 25° and pH 7·6 as described by Perry (1960). Three different concentrations of enzyme from a given sample of digest or fraction were used and specific activities were calculated from the straight-line graphs obtained by plotting the amount of phosphate liberated against the amount of protein present. Specific adenosine-triphosphatase activities were expressed as μg. of phosphorus liberated in 5 min. by 0·248 mg. of heavy-meromyosin nitrogen; this corresponded to a protein solution with $E^{1\,cm.}_{280\,m\mu}$ 1. In some cases the activities of digests were expressed as a percentage of the activity of a control sample in which the inhibitor was added before the trypsin, and the whole exposed to 25° for the same length of time as the digest.

By assuming that all adenosine-triphosphatase activity was associated with the trichloroacetic acid-precipitable fraction of the digest its activity was calculated in some cases according to the relation: 100 × [specific activity of whole digest (% of control)]/[trichloroacetic acid-precipitable protein of digest (% of control)].

Chromatography. Diethylaminoethylcellulose was prepared by the method of Peterson & Sober (1956) and the conditions of chromatography were as described by Perry (1960) and Mueller & Perry (1961*a*).

Ultracentrifuging. (*a*) Sedimentation studies were carried out at 20° in the Spinco analytical ultracentrifuge, model E. The composition of the solvent was 0·15M-KCl–20 mM-tris–HCl, pH 7·6, in all experiments. The procedures described by Elias (1960) were employed for the determination of sedimentation coefficients and for the conversion of the experimental sedimentation coefficients into those under standard conditions.

(*b*) Ultracentrifugal analysis of actin combination was carried out according to the method of Mihalyi & Szent-Gyorgyi (1953). F-Actin, purified by the method of Mommaerts (1951), was added to heavy-meromyosin digests after the addition of trypsin inhibitor at the appropriate time. The system was equilibrated by dialysis against 0·15M-KCl–4 mM-$MgCl_2$–20 mM-tris–HCl, pH 7·6. Sedimentation was carried out at 59 780 rev./min. in the presence and absence of 20 mM-ATP and in all cases comparison was made with the original digest to which actin had not been added.

Viscosity measurements. Viscosities were determined with Oswald viscometers in a water bath at 0° or 25°. The viscometers used had a capacity of 3 ml. and outflow times ranging from 35 to 55 sec. for the solvent. Viscosities were calculated relative to the solvent (0·15M-KCl–20 mM-tris–HCl) neglecting the kinematic correction.

Viscometric determinations of actin combination were carried out with an excess of actin, purified by isoelectric precipitation. A sample (2 ml.) of actin solution (4–5 mg. of protein/ml.) was diluted with 3·4 ml. of 0·15M-KCl–4 mM-$MgCl_2$–20 mM-tris–HCl, pH 7·60, and added to 0·6 ml. of the heavy meromyosin which had been digested for various times under standard conditions. The solutions were carefully mixed and 3 ml. was pipetted into the viscometer. After the addition of 1 drop of octan-2-ol the flow-times were measured at 25°. Then 0·09 ml. of 50 mM-ATP solution was added and, after thorough mixing, the flow-times were redetermined. The difference between the logarithm of the relative viscosities in the presence and absence of ATP provided a measure of actin combination (Weber & Portzehl, 1952; Szent-Gyorgyi, 1951).

Isolation of subfragment-1 (actin-combining fraction) from heavy meromyosin. Digestion was carried out under the usual conditions with 1·0 mg. of trypsin/ml. for 15–60 min. Immediately after the addition of trypsin inhibitor the digest was mixed with F-actin. Generally, for 1 ml. of digest, 2 ml. of actin solution containing 2–3 mg. of protein/ml. in 4 mM-$MgCl_2$–0·15M-KCl–20 mM-tris–HCl, pH 7·6, was added. The 'acto-digest' preparation was centrifuged at 100 000*g* for 3·5 hr. in the preparative centrifuge (Spinco model L), the supernatant was discarded and the pellets were resuspended in 40 mM-ATP–0·15M-KCl–20 mM-tris–HCl, pH 7·6. This preparation was centrifuged again at 100 000*g* for 3·5 hr. The pellets, consisting chiefly of F-actin, were discarded and the supernatant, containing the actin-binding components of undigested heavy meromyosin, was dialysed (chiefly to remove ATP) against 0·15M-KCl–20 mM-tris–HCl, pH 7·6. The preparation was completed by repeating the centrifuging at 100 000*g* for 3 hr. and discarding the sediment.

RESULTS

Relationship between adenosine-triphosphatase activity and the nitrogen precipitated by trichloroacetic acid during the tryptic digestion of heavy meromyosin. During the digestion of heavy meromyosin in 0·15M-potassium chloride–20 mM-tris–hydrochloric acid, pH 7·6, with trypsin at concentrations 10–80 times as great as that used in its original preparation from myosin, two stages could be recognized. The rate of increase in nitrogen which was not precipitated by 6·7% (w/v) trichloroacetic acid was most rapid over the first 10 min. and thence afterwards proceeded at a slower but fairly constant rate up to 60 min.

(Fig. 2 of Mueller & Perry, 1961*b*). This general pattern was observed irrespective of the concentration of trypsin used, although, as might be expected, the extent of the digestion within a given time was greater with increasing trypsin concentration. Nevertheless, even after digestion for 60 min. with a trypsin concentration of 4·0 mg./ml., approximately 40 % of the total nitrogen was still precipitable by 6·7 % trichloroacetic acid.

When related to total nitrogen the adenosine-triphosphatase activity of the digest fell progressively with time but did not disappear completely: even after digestion for 60 min. with the highest concentrations of trypsin used (4·0 mg./ml.) considerable enzymic activity remained. If it was assumed that all the enzymic activity was associated with the trichloroacetic acid-precipitable material and the specific activity of this fraction calculated at various stages during digestion, a pattern could be observed which was apparent at all concentrations of trypsin used in this study (Fig. 1). During the first stage of digestion the specific adenosine-triphosphatase activity of the trichloroacetic acid-precipitable protein increased until it was 20–30 % greater than that of the original heavy meromyosin. On further digestion the specific activity fell sharply to values below that of the specific activity of the original heavy meromyosin. Even when the non-precipitable nitrogen has risen to 75 % the remaining trichloroacetic acid-precipitable protein had appreciable specific activity, in some cases 40 % of that of the original heavy meromyosin.

Sedimentation and viscometric studies of heavy-meromyosin digests. Undigested heavy meromyosin sedimented as a very sharp boundary and, at concentrations of 13·4–14·8 mg./ml., the bar persisted throughout the ultracentrifuge runs (Fig. 2*a*). The heavy meromyosin was not purified by chromatography on diethylaminoethylcellulose for these studies, and consequently a small amount of slower-sedimenting components could be detected behind the sharp heavy-meromyosin boundary (Mueller & Perry, 1961*a*). The trypsin and trypsin inhibitor used for the digestion experiments made only a small contribution to the sedimentation pattern in the form of a flat peak superimposed on the slower impurities mentioned above (Fig. 2*b*).

The first stage of digestion (Figs. 2*c* and 2*d*) was characterized by the rapid disappearance of the original heavy-meromyosin peak and the concomitant production of a slower-sedimenting symmetrical boundary. The transformation into this new component, referred to below as subfragment-1, was completed within about 10 min. when 1·0 mg. of trypsin/ml. was employed for digestion. During this stage the total adenosine-triphosphatase activity of the whole digest decreased at a slower rate than did the amount of trichloroacetic acid-precipitable nitrogen.

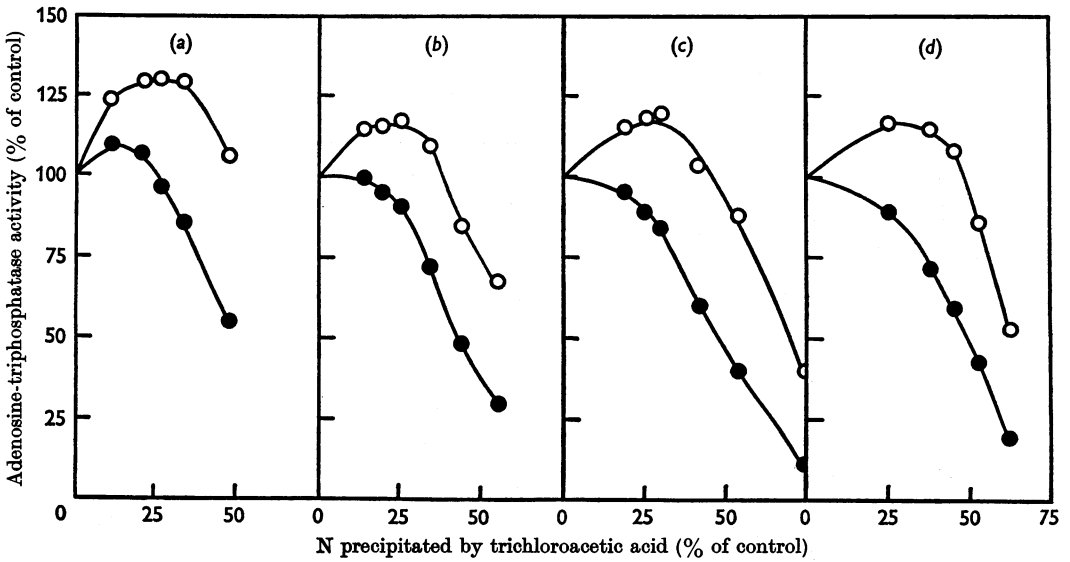

Fig. 1. Relationship between adenosine-triphosphatase activity and trichloroacetic acid-precipitable nitrogen during the tryptic digestion of heavy meromyosin. Heavy meromyosin [13·4 mg./ml., except in Expt. (*d*) where the concentration was 14·5 mg./ml.] in 0·15 M-KCl–20 mM-tris–HCl, pH 7·6, was digested at 25° with trypsin at concentrations of: (*a*) 0·5 mg./ml., (*b*) 1·0 mg./ml., (*c*) 2·0 mg./ml., (*d*) 4·0 mg./ml. ●, Specific activity of the whole digest; ○, specific activity of trichloroacetic acid-precipitable fraction.

On further digestion the peak of subfragment-1 was slowly transformed into an unsymmetrical boundary of still lower sedimentation velocity, due to the appearance of a new component, subfragment-2. Small amounts of a component of sedimentation characteristics similar to those of subfragment-2 could also be detected during the conversion of heavy meromyosin into subfragment-1, suggesting that slow formation of subfragment-2 also occurred at this stage of digestion (cf. Tables 1 and 2). As the transformation of subfragment-1 into subfragment-2 progressed there also appeared an increasing amount of very slowly sedimenting material which did not separate from the meniscus and caused elevation of the base-line. During this stage the total adenosine-triphosphatase activity of the whole digest decreased at a faster rate than the amount of trichloroacetic acid-precipitable nitrogen.

The sedimentation coefficients of the components observed on progressive digestion of heavy meromyosin are shown in Table 1. During the earlier course of digestion, subfragment-1 sedimented about 1s more slowly than the surviving heavy-meromyosin boundary. It was apparently retarded by the presence of the heavy-meromyosin boundary, for after the disappearance of the latter sedimentation of the subfragment-1 component speeded up to a fairly constant velocity of approximately 5·5s. Likewise during the latter stage the

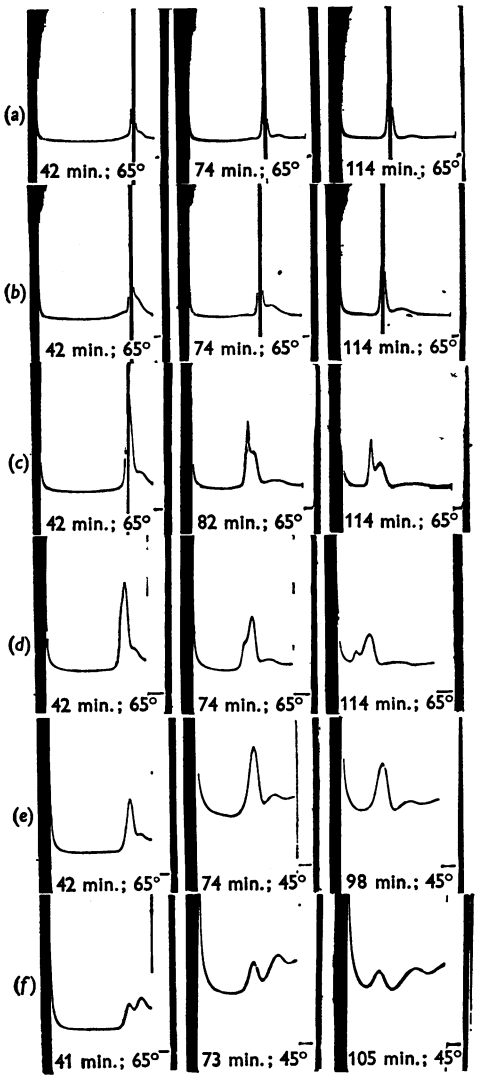

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Table 1. *Sedimentation coefficients of the components in digests of heavy meromyosin*

Details are given in the text. The sedimentation constants in parentheses are of doubtful accuracy because of difficulties in making measurements due to the small amount of subfragment-2 present.

Digestion time (min.)	$S^{\circ}_{20,w}$ (s) Heavy meromyosin	Sub-fragment-1	Sub-fragment-2
1·5	5·85	4·95	(2·95)
2·5	5·96	4·96	(2·87)
5·0	6·01	5·11	(2·61)
7·5	*	4·94	(2·52)
10	†	5·36	(2·53)
30	Absent	5·49	2·33
60	Absent	5·45	2·37
180	Absent	5·50	2·33

* Present but difficult to measure.
† Presence doubtful.

Fig. 2. Sedimentation pattern of heavy meromyosin after tryptic digestion. Meromyosin (13·4–14·4 mg./ml.) in 0·15 M-KCl–20 mM-tris–HCl, pH 7·6, was digested at 25° with 1·0 mg. of trypsin/ml. The digestion was stopped at the times indicated with 1·5 mg. of trypsin inhibitor/ml. and ultracentrifuging was carried out immediately. The times and bar angles are indicated on the photographs. (*a*) Heavy meromyosin alone (14·1 mg./ml.); (*b*) heavy meromyosin + trypsin inactivated by inhibitor; (*c*) digestion for 1·5 min.: 9% of N not precipitated; specific adenosine-triphosphatase of whole digest 100, of precipitable N 110; (*d*) digestion for 5 min.: 17% of N not precipitated; specific adenosine-triphosphatase of whole digest 95, of precipitable N 113; (*e*) digestion for 10 min.: 24% of N not precipitated; specific adenosine-triphosphatase of whole digest 88, of precipitable N 116; (*f*) digestion for 60 min.: 47% of N not precipitated; specific adenosine-triphosphatase of whole digest 54, of precipitable N 100. The specific adenosine-triphosphatase activities of the whole digest or the fraction precipitated by 6·7% (w/v) trichloroacetic acid are expressed as percentages of the specific activities of the undigested controls.

subfragment-2 component exhibited a fairly constant velocity of about 2·3s. The constant sedimentation velocities of the subfragment-1 and subfragment-2 peaks throughout the second stage of digestion suggested a similar and not very pronounced concentration-dependence of $S^{\circ}_{20,w}$ for both components.

The small flat peak of sedimentation velocity similar to that of subfragment-2 observed during the first stage of digestion was probably largely due to inhibitor and trypsin. As digestion proceeded this peak was gradually obscured by the slightly slower-sedimenting subfragment-2 boundary produced by the further digestion of subfragment-1.

Digestion proceeded consistently as described above with fresh heavy-meromyosin preparations, but, when the preparations had been stored for some time, digestion appeared to progress more rapidly and occasionally atypical sedimentation patterns were obtained. This behaviour is illustrated in Fig. 3 (*a*), where the four boundaries that are apparent have been identified in order of decreasing sedimentation velocity as: (1) an aggregation product travelling faster than the usual heavy-meromyosin boundary, (2) the characteristic sharp peak of undigested heavy meromyosin, (3) subfragment-1 and (4) subfragment-2. The fastest component was presumably an aggregation product as it was apparent as a faster component sedimenting in front of the typical heavy-meromyosin peak in undigested controls (Fig. 3*b*).

Disappearance of the heavy meromyosin during the first stage of digestion was accompanied by a rapid decrease in the intrinsic viscosity to about 25–30 % of that of the original undigested preparation. Only a slight further decrease of viscosity occurred in the second stage of digestion. A representative experiment was illustrated by Mueller & Perry (1961*b*).

Chromatographic behaviour of heavy meromyosin digests. Digestion of heavy meromyosin by trypsin profoundly modified its chromatographic behaviour on diethylaminoethylcellulose. Whereas the main component of heavy-meromyosin preparations was held on the diethylaminoethylcellulose in 0·15M-potassium chloride–20 mM-tris–hydrochloric acid, pH 7·6 (Mueller & Perry, 1960, 1961*a*), digestion with 0·5 mg. of trypsin/ml. for 60–180 min. at 25° modified the protein so that 68–72 % of the digest was not held under these conditions (Fig. 4).

Significant adenosine-triphosphatase activity was present in both fractions although the maximum specific activity was obtained at the front of the first peak. In fact the specific activity in this first fraction was considerably higher than that of the whole digest and sometimes even higher than that of the original heavy meromyosin. The indications of enzymic heterogeneity in each fraction that were suggested by the distribution of specific enzymic activity along the eluted peaks were supported by evidence of polydispersity obtained with the ultracentrifuge (Mueller & Perry, 1961*b*). These studies indicated that the more readily eluted peak consisted of subfragment-1 together with smaller amounts of subfragment-2 than were present in the whole digest. Some concentration of subfragment-1 occurred at the front of the first peak, for a relatively homogeneous boundary of sedimentation coefficient similar to that of subfragment-1 could

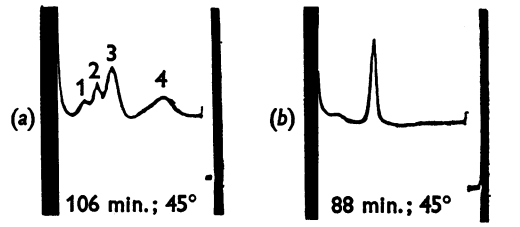

Fig. 3. Atypical sedimentation pattern obtained on tryptic digestion of an aged heavy-meromyosin preparation. The heavy-meromyosin preparation was stored for 24 days at 0° before digestion. A sample was centrifuged immediately after digestion. The significance of the peaks 1–4 is indicated in the text. The bar angles and times of centrifuging are indicated on the photographs. (*a*) Digested for 1·5 min. with 1·0 mg. of trypsin/ml.; (*b*) undigested control sample.

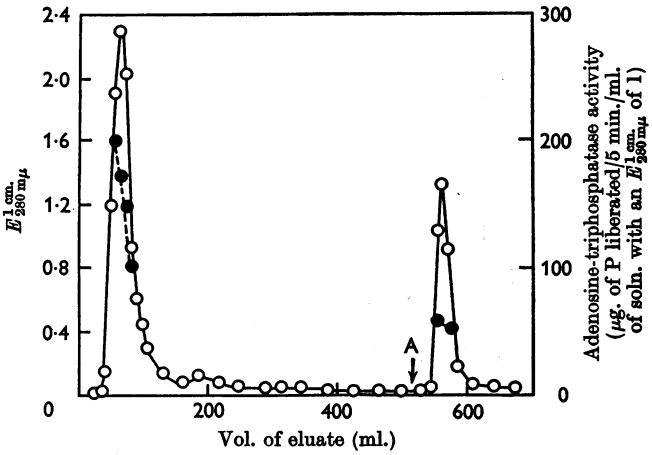

Fig. 4. Chromatography on diethylaminoethylcellulose of heavy meromyosin after digestion with trypsin. The heavy meromyosin was digested for 180 min. at 25° with 0·5 mg. of trypsin/ml. in 0·15M-KCl–20 mM-tris–HCl, pH 7·6. A sample (20 ml.) of the digest was applied to a column (2 cm. × 20 cm.) equilibrated against the buffer. The step to 0·35M-KCl–20 mM-tris–HCl, pH 7·6, was made at point A. ○, $E^{1\,cm.}_{280\,m\mu}$; ●, specific adenosine-triphosphatase activity.

be obtained by ultracentrifuging this fraction. Subfragment-1 appeared to be largely absent from the material subsequently eluted by the stepwise application of 0·35M-potassium chloride–20 mM-tris–hydrochloric acid, pH 7·6. This fraction consisted predominantly of subfragment-2 together with at least one faster minor component, the nature and identity of which was not certain.

Interaction of products of digestion with actin. Combination of heavy meromyosin with actin produces the viscous acto–heavy-meromyosin complex which is dissociated by ATP. This property survived digestion of the meromyosin with trypsin under the conditions used although the viscosity drop obtained by the addition of ATP fell off with the duration of digestion (see Fig. 5). As was observed with other properties studied during the digestion, an initial phase of rapid change in the viscosity response was followed by a slower one, the transition becoming apparent after digestion for about 10 min., when the transformation of heavy meromyosin into subfragment-1 was completed.

To determine more directly and specifically which subfragment retained the capacity to combine with actin, the interaction was studied in the analytical ultracentrifuge. The addition of F-actin to meromyosin that had been digested to the first stage (Fig. 6*a*) resulted in the very rapid sedimentation of subfragment-1 and undigested heavy meromyosin as actin complexes. Subsequent addition of ATP caused dissociation of both actin complexes; subfragment-1 and heavy meromyosin sedimented independently and at the rates measured in the absence of actin. The addition of F-actin to meromyosin digested to the second stage (Fig. 6*b*) resulted again in rapid sedimentation of subfragment-1 as an actin complex, whereas subfragment-2 manifested no appreciable actin-combining activity and sedimented at its original rate. The sloping of the base-line in this diagram was shown to be due to the presence of ATP, for it was obtained in control experiments in which the buffer containing 20 mM-ATP alone was centrifuged. The viscosity changes associated with these reactions were, however, much smaller than with heavy meromyosin or myosin.

On the basis of this behaviour a method for the routine preparation of subfragment-1 by differential centrifuging from second-stage digests (see the Methods section) was developed. The degree of purification and separation achieved by this method is illustrated in Fig. 7, which shows the

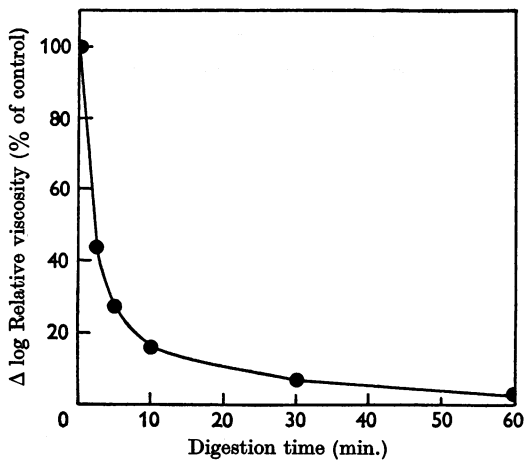

Fig. 5. Effect of tryptic digestion of heavy meromyosin on its interaction with actin as indicated by the viscosity change produced by the addition of adenosine triphosphate to the complex. Heavy meromyosin was digested under standard conditions with 1·0 mg. of trypsin/ml. The reaction was stopped by the addition of 1·5 mg. of trypsin inhibitor/ml. The viscosities were measured on 3 ml. samples, each containing 0·3 ml. of digested heavy meromyosin (14·8 mg./ml.), 1·0 ml. of F-actin (4·0 mg./ml.) and 0·15M-KCl–3·6 mM-$MgCl_2$–20 mM-tris–HCl, pH 7·6, before and after the addition of ATP (final concn. 1·5 mM).

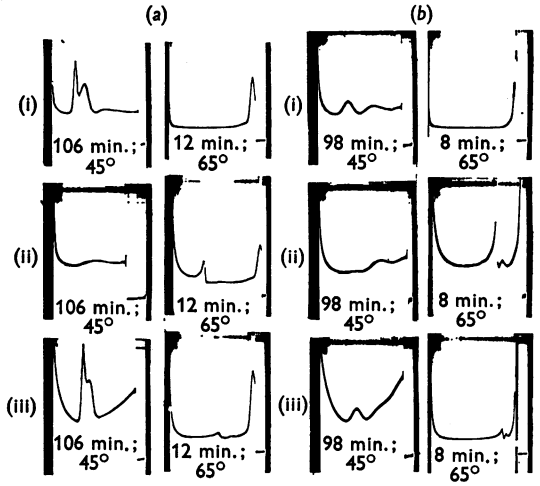

Fig. 6. Combination of actin with components of the tryptic digest of heavy meromyosin. The digestion and centrifuging were carried out under standard conditions. The times and bar angles are indicated on the photographs. (*a*) Heavy meromyosin was digested for 1·5 min., 0·6 ml. of F-actin (26 mg./ml.) added to 3 ml. of the digest and the mixture dialysed against 0·15M-KCl–4 mM-$MgCl_2$–20 mM-tris–HCl, pH 7·6. (i) Digest alone to which an equivalent volume of solvent was added instead of actin; (ii) digest + actin; (iii) digest + actin + ATP (final concn. 20 mM). (*b*) Heavy meromyosin was digested for 60 min., 0·9 ml. of F-actin (20 mg./ml.) added to 3 ml. of the digest and the mixture dialysed as in (*a*). (i) Digest alone as in (*a*) (i); (ii) digest + actin; (iii) digest + actin + ATP (final concn. 20 mM).

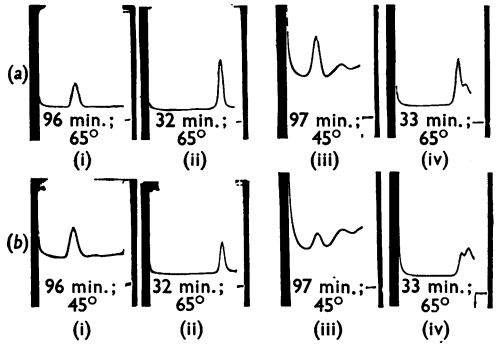

Fig. 7. Sedimentation of subfragment-1 isolated from digests of heavy meromyosin by combination with actin. Heavy meromyosin was digested under standard conditions for the times indicated and subfragment-1 isolated as described in the Methods section. Ultracentrifuging was carried out in 0·15M-KCl–20 mM-tris–HCl, pH 7·6. The times and bar angles are indicated on the photographs. (*a*) Heavy meromyosin digested for 15 min. (i) and (ii) Isolated subfragment-1; (iii) and (iv) original heavy-meromyosin digest. (*b*) Heavy meromyosin digested for 60 min. (i) and (ii) Isolated subfragment-1; (iii) and (iv) original heavy-meromyosin digest.

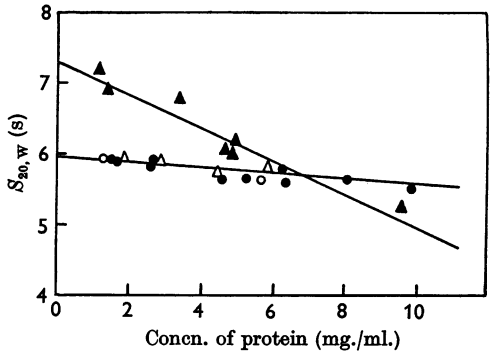

Fig. 8. Effect of concentration on the sedimentation coefficients of heavy meromyosin and of subfragment-1 isolated from it after various periods of digestion with trypsin. Ultracentrifuging was carried out in 0·15M-KCl–20 mM-tris–HCl, pH 7·6. ▲, Heavy meromyosin; ●, subfragment-1 isolated after digestion for 15 min.; ○, subfragment-1 isolated after digestion for 30 min.; △, subfragment-1 isolated after digestion for 60 min.

sedimentation behaviour of subfragment-1 isolated as the actin-combining fragment of heavy meromyosin digested for 15 and for 60 min. Although the fractions isolated after various digestion times up to 60 min. showed very similar sedimentation behaviour, the yields fell off progressively with time. On plotting against concentration the sedimentation coefficients of subfragment-1, isolated after various times, all points fell on a straight line (Fig. 8). Extrapolation of zero protein concentration gave a sedimentation constant of 5·95S. The sedimentation of subfragment-1 was much less concentration-dependent than that of heavy meromyosin (Fig. 8).

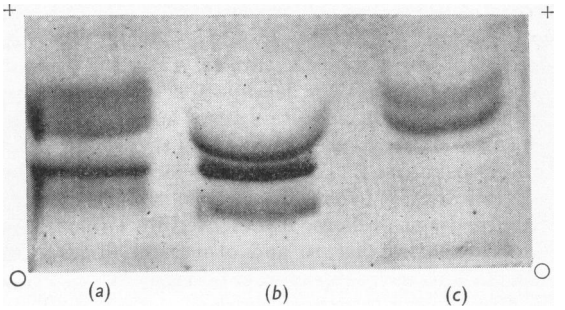

Fig. 9. Starch-gel electrophoresis of myosin and the products of tryptic digestion. Electrophoresis was carried out at 0° in 22 mM-borate, pH 9·1, containing urea (final concn. 8M). The origin is indicated by ○—○. (*a*) Heavy meromyosin; (*b*) myosin; (*c*) subfragment-1.

Zone electrophoresis in urea on starch gel. Evidence of the close chemical similarity of the subfragment-1 fraction obtained after different periods of digestion was provided by electrophoretic studies in 8M-urea at 0° under the conditions described by Smithies (1955). Little electrophoretic movement of myosin, heavy meromyosin and subfragment-1 could be observed in 22 mM-sodium borate buffer, pH 9·1, but satisfactory electrophoresis was obtained on the addition of urea (final concentration 8M) to the system. The pattern obtained consisted of two major bands followed by a slower minor band. The electrophoretic pattern was clearly different from the original heavy meromyosin, which is shown together with that of myosin for comparison in Fig. 9.

DISCUSSION

Whereas the initial attack on myosin by trypsin gives rise to two components which appear simultaneously as digestion proceeds, tryptic action on heavy meromyosin resulted in the replacement of the latter by a single new major component. This new component, subfragment-1, which sedimented more slowly than heavy meromyosin, in turn was transformed into a still slower one, subfragment-2; material not precipitated by trichloroacetic acid, presumably of low molecular weight, was produced during both transformations.

The transformation of subfragment-1 into subfragment-2 occurred largely in the second stage of digestion, after the heavy meromyosin had disappeared. It is likely, however, that this second transformation begins earlier, probably as soon as

subfragment-1 is formed (Table 1), but, since it is a very slow process, it contributes little to the sedimentation diagrams obtained during the first stage of digestion. A similar situation, namely that slow secondary degradation of the primary products occurs simultaneously with their rapid formation, seems to apply in the tryptic digestion of myosin itself. The reactions occurring during digestion may be summarized as in Table 2. Undoubtedly the scheme represented by Table 2 is an oversimplified picture and other undefined transformations including aggregation may be occurring.

The slower transformations (*b*), (*c*) and (*d*) (see Table 2) which occur during meromyosin formation give rise to the 'H1 fraction' separated from standard heavy-meromyosin preparations by chromatography on diethylaminoethylcellulose (Mueller & Perry, 1961*a*). By a study of its sedimentation in the ultracentrifuge, of its adenosine-triphosphatase activity and of its chromatographic and electrophoretic behaviour, this fraction has been shown to be identical with material isolated under similar conditions from heavy-meromyosin digests (Mueller, 1961). The heterogeneity of meromyosin preparations described by Lowey & Holtzer (1959) and Fryar & Gibbs (1960) may also be accounted for in the same way.

Heavy meromyosin contains a total of about 126 lysine and arginine residues per 100 000 g. weight (Kominz, Hough, Symonds & Laki, 1954), and if the peptide bonds linking the residues were all equally sensitive to hydrolysis the products of extensive tryptic breakdown would not be expected to possess a high degree of homogeneity. Nevertheless, the sedimentation characteristics of the boundaries identified in the digest suggest two types of fragment different in size and shape from each other but similar in themselves.

Mihalyi & Harrington (1959) have suggested that the appearance of distinct subfragment species on progressive digestion of myosin is due to the presence of sections of the polypeptide chain which are more randomly oriented than other parts of the molecule and hence more susceptible to proteolytic digestion. Thus the undigested fragments corresponding to the meromyosins will contain the regions of the polypeptide chain less susceptible to proteolysis. This concept may be extended to the degradation scheme presented here, by assuming further regions differing in the randomness of orientation and liability to proteolytic attack. The three transformations (*a*), (*c*) and (*d*) (see Table 2) would result from cleavage in regions of decreasing randomness within the myosin molecule, and it follows that the parts of the molecule associated with reactions (*c*) and (*d*) are located within the heavy-meromyosin fragment.

The apparent failure of Mihalyi & Szent-Gyorgyi (1953) to observe further transformation of the meromyosins into components with identifiable boundaries may probably be attributed to the low concentrations of trypsin (about 0·05 mg./ml.) and myosin (about 6·0 mg./ml.) used for the digestion. Under these conditions the subfragment concentration is probably too low to produce peaks in the sedimentation diagrams, and the presence of light meromyosin was a further complicating feature.

In a study on the tryptic digestion of myosin (16·0 mg./ml.) Mihalyi & Harrington (1959) noticed the appearance of 'intermediate components' in their sedimentation diagrams. They considered that degradation of heavy meromyosin, aggregations of light meromyosin or a combination of both these changes might be occurring. Close inspection of the sedimentation diagrams published by these authors reveals a small peak, which in all probability corresponds to the subfragment-1 described in the present paper, but as in Mihalyi & Szent-Gyorgyi's (1953) study interpretation is complicated by the presence of light meromyosin.

The present study indicates that little or no adenosine-triphosphatase activity is lost when a portion of about 25 % of the heavy meromyosin is transformed to products which are not precipitated by 6·7 % (w/v) trichloroacetic acid. If all enzymic activity is assumed to be associated with the trichloroacetic acid-precipitable fraction its specific activity is at a maximum at this point in the digestion. When these findings are considered together with the sedimentation studies, it be-

Table 2. *Summary of reactions occurring during digestion of myosin and heavy meromyosin by trypsin*

Reactions during digestion of myosin by a low concn. of trypsin	Reactions during digestion of heavy meromyosin by a high concn. of trypsin	
	First stage	Second stage
(*a*) *Myosin → heavy meromyosin + light meromyosin	—	—
(*b*) Light meromyosin → ?	—	—
(*c*) Heavy meromyosin → subfragment-1	*Heavy meromyosin → subfragment-1	—
(*d*) Subfragment-1 → subfragment-2	Subfragment-1 → subfragment-2	*Subfragment-1 → subfragment-2
Production of low-molecular-weight peptides	Production of low-molecular-weight peptides	Production of low-molecular-weight peptides

* Major transformations.

comes apparent that the phase of digestion in which the specific activity of the trichloroacetic acid-precipitable fraction rises corresponds to the transformation of heavy meromyosin into subfragment-1. Completion of this phase is marked by the maximum of the curves in Fig. 1 and the main product is the new enzymically active component, subfragment-1. In line with the degradation scheme discussed above, the low-molecular-weight products produced during digestion would represent the region with the most randomly ordered polypeptide chains within the heavy-meromyosin molecule. Further, it seems to follow that this random region is enzymically inert, and that the active centres of the myosin molecule are localized in regions in which the arginine and lysine residues are less susceptible to tryptic attack and represented by subfragment-1 of the heavy-meromyosin molecule. This fragment also possesses the actin-binding capacity characteristic of the parent molecule, for it was not possible to dissociate these two activities into different proteolytic fragments, or to destroy one and maintain the other. The transformation of subfragment-1 into subfragment-2 was associated with the loss of the actin-binding and enzymic activities, but it could not be definitely ruled out from the available data that a low level of activity in either case survived the degradation of subfragment-1.

Further characterization of the size and shape of subfragment-1 must await studies now in progress. Nevertheless the low dependence of the sedimentation coefficient on concentration suggests a less asymmetric molecule than that of heavy meromyosin.

SUMMARY

1. On digestion of heavy meromyosin by trypsin two different and characteristic stages could be recognized.

2. In the first and rapid phase heavy meromyosin was transformed into subfragment-1 which was homogeneous in the ultracentrifuge and retained the adenosine-triphosphatase and actin-combining properties of the original heavy meromyosin. During this transformation 25% of the total nitrogen became non-precipitable with 6·7% (w/v) trichloroacetic acid and the intrinsic viscosity decreased by 70%.

3. In the slow second phase of digestion, subfragment-1 was transformed into a more heterogeneous fraction, subfragment-2. This transformation was associated with the loss of enzymic and actin-combining activities. The formation of non-precipitable nitrogen gradually increased, yet there was relatively little change of intrinsic viscosity during this phase of the digestion.

4. A method of isolation of subfragment-1 based on its actin-combining property is described.

5. Subfragment-1 appears to be less asymmetric than heavy meromyosin and sediments more slowly in the ultracentrifuge ($S^{\circ}_{20,w}$ = 5·95s).

This work was done during the tenure by H.M. of an Established Investigatorship of the American Heart Association. We are indebted to the National Science Foundation for a grant (NSF-G10938) which has defrayed in part the expenses incurred in this research, and to Mrs G. Mueller for skilled technical assistance. Our thanks are also due to Mrs G. A. Gilbert, Department of Chemistry, and Miss A. P. Ratcliff, Department of Experimental Pathology, for carrying out numerous ultracentrifuge runs on our behalf, and to Professor M. Stacey and Professor J. R. Squire for putting these facilities in their respective Departments at our disposal.

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