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CHARACTERIZATION OF TRYPTIC CLEAVAGE PRODUCTS OF A NONMUSCLE MYOSIN

by

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ABSTRACT

Proteolytic cleavage of muscle myosin has proved useful in elucidating how the molecule functions in the muscle contractile system. In particular, trypsin (used by Andrew Szent-Györgyi in the 1950's) splits muscle myosin into a soluble piece containing the ATPase activity and an insoluble piece containing the aggregating properties of the molecule.

Proteins similar to myosin have been found in eucaryotic cells that exhibit movement, but the manner in which such proteins function to produce the observed contractile locomotion is not clear. With Szent-Györgyi's experiments in mind, we have undertaken to characterize the proteolytic cleavage products obtained when myosin from Dictyostelium discoideum amoeba is digested with TPCK-trypsin.

The products examined on denaturing gels showed five discrete bands and, when examined on native gels, showed two discrete bands; no intact myosin remained. Approximate molecular weights for the denatured products were: 185,000, 165,000, 125,000, 105,000 and 60,000. Time course analysis of denaturing gels showed that the products appeared in the following order: 185,000 MW, 165,000 MW, 125,000 MW, 105,000 MW, and 60,000 MW. Separation by differential solubility in low salt showed the 185,000 and 125,000 MW products to be mainly insoluble, the 165,000 and 105,000 MW products to be mainly soluble, and the 60,000 MW product to be both soluble and insoluble.

Electronmicroscope analysis of intact non-muscle myosin showed it to be similar in shape to muscle myosin, but approximately 200 Å longer in its tail region. Examination of the fractions obtained after cleavage and separation by solubility showed the soluble fraction to be a heterogeneous mixture of predominately single heads, along with assorted head and tail products. The insoluble fraction was found to be double-headed myosin, with a tail portion measuring approximately 300 Å shorter than the parent molecule. Decoration experiments using the soluble fraction and Dictyostelium F-actin showed that the myosin fragment in the soluble fraction decorated F-actin in an arrow-headed manner and was released in the presence of ATP.

Enzymatic assays of the soluble and insoluble tryptic cleavage products showed that the calcium high salt ATPase remained intact, but the actin-activated ATPase was lost.

It appears that non-muscle myosin from Dictyostelium discoideum is more sensitive to trypsin and has a longer tail region than its skeletal muscle counterpart indicating an amino-acid sequence difference and, at least, some conformational difference between the two types of myosin. A model is presented to explain the tryptic cleavage patterns of Dictyostelium discoideum myosin. The model includes two heavy chain cuts, one light chain cut, and a nick in one globular head region.

INTRODUCTION

Investigations into how vertebrate skeletal muscle contracts have been underway for many years. Even as early as the 1600's, when Anton van Leuwenhoek was making his first observations with his newly made microscope, he described (1) the cross striation pattern in skeletal muscle. A more biochemical and finer investigation into the details of muscle cell movement started in 1864 when W. Kühne (2) was able to extract protein material from frozen muscle and so allow for the study of this material. One of the first characteristics elucidated about muscle protein resulted from Jacobsen's demonstration (3) in the 1930's that animal tissue was capable of splitting ATP. Engelhardt and Ljubinova in 1939 (4) then discovered that the protein material extracted from muscle had ATPase activity. Further investigation into the molecular components of the vertebrate skeletal muscle machine resulted in the important finding by Albert Szent-Györgyi and his group in the 1940's that the muscle protein preparation that had been described earlier actually consisted of two discrete proteins in association; myosin, which had the enzymatic activity as well as a structural role, and actin, which could catalyze the myosin ATPase as well as provide structural support (5). Later, in the 1950's H.E. Huxley and Hanson (6) proposed a model for how myosin and actin interacted to produce movement. This "sliding filament" model was based in part on Huxley's earlier finding (7) that there were two kinds of filaments in the isotropic and anisotropic bands seen in muscle through polarized light and that these two filaments

could slide past each other to produce a contracting effect on the cell. The mechanism for the control of contraction of this vertebrate skeletal muscle system was elucidated by Ebashi and co-workers in the 1960's (8). They showed that the Ca^{2+} sensitivity of the actomyosin reaction was dependent on the presence of the regulatory muscle proteins troponin and tropomyosin.

While much about the skeletal muscle system was learned by taking apart and recombining the various protein components, study of the structure of the individual proteins led to conclusions and discoveries about their capacity to interact and produce movement. Initial observations on the structural makeup of myosin came from Danilevskii's and Weber's optical studies in the 1930's (9) which showed that the molecule appeared to be asymmetric in shape. In the 1950's, Andrew Szent-Györgyi (10) showed by tryptic cleavage of the molecule that there is a correlation between the asymmetric structure of myosin with the asymmetric distribution of its functional properties. By allowing trypsin to digest the myosin for a brief period of time and then dialyzing the resultant cleavage products against low salt, Szent-Györgyi was able to separate the part of the molecule capable of aggregation from the part of the molecule which contained the ATP splitting site and the actin binding site. Later work identified the aggregating region as rod-like and the enzymatic region as a mixture of globular and rod-like. Susan Lowey's work with papain in the 1960's (11) further localized the enzymatic region as being only in the globular heads noting that the previously attached rod demonstrated no observable functional properties. Fig. 1 gives a summary of the cleavage products

obtained from muscle myosin using papain and trypsin along with their nomenclature and molecular weights by the Archibald method using sedimentation velocity (11).

Such structural evidence for myosin along with data on the other components of the skeletal muscle system led to the sliding filament model regulated by Ca^{2+}. Myosin dimers form bipolar thick filaments through their rod-like aggregation regions and the enzymatic globular heads interact with the actin double-stranded thin filaments, made up of actin monomers. The troponin directly interacts with the Ca^{2+} to relay the message to tropomyosin to allow the actin thin filament to interact with myosin and thereby split ATP. Absence of Ca^{2+} negates the message. Since the troponin and tropomyosin are associated with the double-stranded actin thin filament, the muscle skeletal system is under actin-linked Ca^{2+} regulation. Thus, the skeletal muscle system represents a specialized cell for movement with the size and amount of contractile units large enough for light microscope visualization. Such a system affords for easy localization of the contractile apparatus within the cell and easy isolation of the material.

In recent years, proteins similar to the skeletal muscle contractile proteins have been found in many non-muscle, eucaryotic cells such as amoebae (12), blood cells (13), spermatozoa (14), and fibroblasts (15). Since it appears that actin and myosin-like proteins are found in many different and diverse cell types, it would be comforting to generalize their specialized role in muscle cells as molecules of movement to wherever they are found. However, the generalization that actin and myosin-like proteins function to provide

some form of movement wherever they are located has not yet been proved. It does, however, provide an assumption about non-muscle actin and myosin which gives direction to the kinds of questions one can ask about their characteristics. As an example, Clarke & Spudich (12) were able to isolate a myosin-like protein from the eucaryotic amoeba Dictyostelium discoideum using methods similar in principle to those used for extracting rabbit skeletal myosin from striated muscle cells, i.e. purifying an actomyosin complex and utilizing its characteristic solubility properties. The discovery that sucrose would resolubilize the amoeboid actomyosin complex led to the purification of a protein that could interact enzymatically with actin.

Continuing on the assumption that amoeboid myosin was similar to its muscle counterpart, Clarke & Spudich asked further questions about the properties and structures of Dictyostelium myosin and found that there were signs of similarity and signs of differences. Basing their investigations on such muscle myosin assumptions, other workers dealing with myosin-like proteins from non-muscle sources saw many of the same similarities and differences. In this way, a clearer picture of what unites all the myosin-like molecules from different sources is becoming established, although a general definition for the role and mechanism of action of the wide variety of myosins is not yet apparent.

Many of the similarities found between myosins from different sources deal with structure, such as being composed of two heavy chains of identical molecular weight, being composed of non-covalently attached light chains having asymmetric shapes, having the ability to form bipolar thick filaments, and having an ATPase activity stimulated

by actin (12, 16, 17, 18). However, when one gets down to structural specifics, myosins from different sources do differ. The light chains, although similar amongst the various myosins, do have different molecular weights. Furthermore, some myosins have heavy chains that co-migrate on SDS gels with those of muscle myosin, while some myosins have heavy chains that are larger.

With respect to enzymatic activity, one finds that most non-muscle myosins have an actin-activated ATPase not as high as that of muscle. While this difference may not be significant, it does indicate that something different about the structures of non-muscle myosins has an effect on how well they interact with actin and perhaps how they function. Thus, one is again led back to the method of assuming a non-muscle myosin is similar to muscle myosin and asking how the differences between them arise and what effect this difference has on their interaction with actin and their function.

It is with this approach in mind that we have undertaken to study the substructure of the non-muscle myosin from Dictyostelium discoideum by cleaving the intact molecule with trypsin. In light of such work done on muscle myosin, we hoped that breaking down the Dictyostelium discoideum myosin molecule into parts might further clarify where it is similar to and dissimilar to muscle myosin in its properties and how this can be accounted for structurally.

MATERIALS & METHODS

A.) Isolation of Dictyostelium discoideum Myosin

Myosin was extracted and purified from amoebae of Dictyostelium discoideum in three different ways. The first method was described elsewhere (12) with one modification which was to replace the 200-400 mesh agarose column with a 100-200 mesh agarose column resulting in faster separation of the actin and myosin with the same resolution and with higher activity for the actin activation of the myosin. The second method of purification was as described by Mockrin and Spudich (19). The third method of purification was as described in the former reference with the following modifications. Freshly harvested cells were washed in 10mM TEOLA, pH 7.5 and were then disrupted by sonication to greater than 95% breakage 2X for 30 sec at 2 vol/g cells in 40mM $NaPP_i$, 30% sucrose, 0.4mM DTT, and 10mM TEOLA, pH 7.5. The disrupted cells were spun for 15 min at 27,000g. The supernatant fluid (S1) was then spun for 60 min at 105,000 g. The supernatant fluid (S2) including the fluffy layer was dialyzed overnight against 0.1 M KCl, 10mM TEOLA, pH 7.5, 1mM DTT, and 1mM EDTA. The dialyzate was then spun for 30 min at 23,500 g, and the pellet fraction (P3) was treated as in the above reference and loaded onto a 100-200 mesh agarose column. In this modification, the column buffer was changed to 20mM $NaPP_i$, pH 7.5, 10mM TES pH 7.5, 5% sucrose and 1mM DTT. The myosin from the agarose column step was pooled and immediately applied to a DEAE-cellulose

column (DE-52 Whatman Biochemical Ltd.) of appropriate volume such that the total mg of protein applied to the column ran through an equal volume in ml of resin. The column had been previously equilibrated in the agarose column buffer with ten column volumes of the buffer. The non-adsorbing material was washed off with two column volumes of the equilibrating buffer and then the myosin was eluted off in a step of column buffer and 0.15M KCl. The pooled myosin was then worked up as per Mockrin and Spudich (19). All experiments were done with each of the three kinds of myosin.

B.) Isolation of Dictyostelium discoideum Actin

Two Dictyostelium discoideum actin preparations were used in these experiments. "Selective" actin was prepared by the method of Spudich (20), and "nonselective" actin was prepared as per Uyemura (manuscript in preparation). Uyemura's procedure involves filtration through G150 Sephadex, elution from DEAE-cellulose, and ammonium sulfate fractionation.

C.) Isolation of Rabbit Skeletal Actin

Actin was purified from an acetone powder of rabbit striated muscle using the procedure of Spudich and Watt (21).

D.) Cleavage and Separation Conditions

Cleavage of Dictyostelium discoideum myosin was done under conditions similar to those described for muscle myosin by Lowey, Goldstein, Cohen and Luck (22). The myosin in its high salt buffer

(0.43M KCl, 10mM Tris-HCl, pH 7.5, 1mM DTT and 1mM EDTA) was diluted 1:3 or more with 0.04M phosphate buffer, pH 6.5. Thus, the salt concentration was never higher than 0.16M KCl. For time course cleavage experiments, the myosin was diluted to a concentration of 0.5mg/ml, whereas for dialysis experiments the myosin was diluted to a concentration of 1.5 mg/ml. TPCK-trypsin (Worthington Biochemical Corporation) was dissolved in 1mM HCl and added to the myosin at a concentration of either 0.005 mg/mg of myosin or 0.003 mg/mg of myosin. The proteolysis was stopped by addition of soybean trypsin inhibitor (SIGMA Chemical Company) at a concentration of 0.1 mg/0.003 mg of trypsin.

Separation of cleavage products was attained by rapid dialysis for four hours against a low salt buffer containing 0.01M KCl, 0.01M Tris-maleate, pH 6.5, 0.01M $MgCl_2$ and 1mM DTT. The dialyzate was then spun for 15 min at 27,000 g. The supernatant was removed and dialyzed against the same buffer in which the myosin was originally stored, except that the salt concentration was kept at 25mM KCl for the purposes of keeping the salt concentration in the actin-activated ATPase assay low. The pellets were resuspended in the myosin buffer with 0.4M KCl and was dialyzed against this buffer along with the controls from the experiment that were not initially dialyzed against low salt buffer. The pellets were resuspended in one-fourth of the supernatant volume to keep the protein concentration high so that it could be resuspended in high salt and diluted enough for the actin-activated ATPase so that the salt concentration was not inhibitory.

E.) Protein Determination

Protein concentration was determined by the method of Schaffner and Weissmann (23). Crystallized bovine albumin (Pentex, Miles Laboratories) was used as a standard.

F.) Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

The discontinuous buffer system of Laemmli (24) was used according to the procedure of Ames (25).

G.) Gel Electrophoresis of Native Proteins

The method was developed from that described by Hoh (26). Polyacrylamide gels containing 3% acrylamide were polymerized in the presence of 10% glycerol. The continuous buffer system contained 0.025M Tris-HCl, 0.19 glycine, 10mM $NaPP_i$ and 10% glycerol and was brought, after dilution from a 10X stock, to pH 8.3 at 4^oC. The gels were prerun for one hour, then samples were diluted to 50% glycerol and were applied to the gels which were then run for 24 hours.

H.) Assay of ATPase Activity

ATPase activity was measured using (γ-^{32}P) ATP, essentially as described previously (12). Standard reaction mixtures for measuring actin-activated myosin ATPase contained 1mM (γ-^{32}P) ATP, 25mM $CaCl_2$ and 10mM KCl, 5 µg of myosin sample, 0.2 mM $CaCl_2$ and 10 µg of actin in 0.1 ml total volume. The actin-activated myosin ATPase activity was calculated by subtracting the activity found for myosin alone.

I.) Electron Microscopy

The method for high resolution shadowing was refined by Dr. Don Fischman from a procedure essentially following Offer's methods. The sample is dialyzed overnight at a concentration of 50 μg/ml against a buffer containing 0.3 M ammonium formate, 30% glycerol overnight with two changes of buffer. The sample is sprayed onto a freshly cleaved surface of mica and then, under vacuum, is unidirectionally shadowed with platinum followed by a coating of carbon. The carbon replica of the molecule is then floated off in a water dish and picked up on a clean copper grid.

The method for S1 decoration of actin was essentially as described by Spudich, Huxley and Finch (27).

RESULTS

A.) TIME COURSE OF CLEAVAGE WITH TPCK-TRYPSIN

After 7.5 minutes of proteolysis of filamentous aggregates of Dictyostelium discoideum myosin with a concentration of TPCK-trypsin of 0.006 mg/mg myosin, five discrete bands on denaturing gels and two discrete bands on non-denaturing gels were observed; no intact myosin remained. In contrast, muscle myosin cleaved under the same conditions gave three discrete bands on denaturing gels and three discrete bands on non-denaturing gels with intact myosin as the topmost band in each case. Approximate molecular weights for the denatured Dictyostelium discoideum myosin cleavage products were obtained on 5% SDS-gels using Rf values for internal standards, including the muscle myosin cleavage products identified as intact heavy chain (200,000), heavy meromyosin (130,000), and light meromyosin (70,000). Such values yielded the following weights for the five Dictyostelium discoideum myosin products: 185,000, 165,000, 125,000 105,000, and 60,000.

A time course analysis of this cleavage for the Dictyostelium discoideum myosin using gel scans of SDS tube gels is shown in Fig. 2. After 2.5 minutes, a major band of molecular weight 185,000 was obtained along with smaller amounts of other components and some intact heavy chain running at 210,000. By five minutes of cleavage, all intact heavy chain was gone and two bands became prominent with molecular weights of 165,000 and 125,000. At ten minutes, five discrete bands

were present. By twenty minutes, only the 105,000 and 60,000 products were observed, the others having been presumably degraded to lower molecular weight products observed as small, light intensity bands running near the dye front. At forty minutes, very little protein was visible on the gel. In the case of muscle myosin, after 2.5 minutes there were already faint bands of the HMM and LMM products which became more intense at five minutes and ten minutes as the intact heavy chain diminished in intensity. By twenty minutes, the intact chain was a barely visible band, as was the HMM while the LMM became less discrete. By forty minutes, no intact muscle myosin was left, and the HMM band was very faint while the LMM was even more diffuse.

A time course cleavage was done again on aggregated myosin with TPCK-trypsin at a concentration of 0.0015 mg/mg myosin and followed on a 3% continuous, non-denaturing, tube gel system. This showed (Fig. 3) that at five minutes intact heavy chain was cleaved to a faster running band of equal intensity. By ten minutes, no intact heavy chain was left. By twenty minutes, two bands were apparent and of equal intensity.

B.) SEPARATION OF 7.5 MINUTE CLEAVAGE PRODUCTS BY DIFFERENTIAL SOLUBILITY

In an effort to separate and characterize the Dictyostelium discoideum myosin cleavage products, myosin which was cleaved for 7.5 minutes with TPCK-trypsin at a concentration of 0.005 mg/mg myosin was dialyzed against a low salt buffer (0.01 M KCl). After a short, low-speed spin the pellet was resuspended in high salt buffer,

the supernatant was dialyzed against the same buffer and samples were examined by gel electrophoresis on non-denaturing gels, the control gave the expected pattern (the control was myosin incubated with pre-mixed trypsin-inhibitor complex on ice, then dialyzed, spun and treated as the experimental sample). The control sample before dialysis ran as intact myosin, the supernatant had nothing and the pellet ran the same as the sample before separation. The cleaved sample before dialysis ran as in the first experiment as apparently two bands running faster than intact myosin. In a preliminary experiment, the supernatant appeared to have only the faster running band and the pellet only the other. A 5% SDS gel was run with the same sample as above. Again the control gave the expected results; the control sample before dialysis ran as intact heavy chain, the supernatant had nothing and the pellet ran as the sample before dialysis. The cleaved myosin before dialysis ran as described above; five bands with molecular weights of 185,000, 165,000, 125,000, 105,000 and 60,000. These products separated as follows (see Table 1, quantitative analysis of gel scans): the supernatant contained primarily the 165,000 (88% of total), 105,000 (89% of total), and the 60,000 (64% of total) molecular weight products. The pellet contained primarily the 185,000 (75% of total), 125,000 (72% of total) and some 60,000 (36% of total) molecular weight products. It appeared then that the 165,000 and 105,000 molecular weight pieces were mainly soluble in low salt, while the 185,000 and 125,000 molecular weight pieces were mainly insoluble. The 60,000 molecular weight piece appeared as both soluble and insoluble. The average protein distribution by

weight after dialysis against low salt buffer was 6% in the supernatant and 94% in the pellet for the control and was 50% in the supernatant and 50% in the pellet for the experimental. The calculated protein distribution was based on the total protein recovered after dialysis and resuspension.

Since the denaturing gel discussed above was 5% acrylamide and not small-pored enough to observe the light chains, the experiment was also analyzed on a 12% gel (Fig. 4). The control ran with the intact myosin at the top part of the gel and the two intact light chains of 18,000 and 16,000 molecular weight for both the pellet and the sample before dialysis. Again, nothing was in the control supernatant. The cleaved myosin samples showed that one of the light chains had been cleaved. In the sample before dialysis, most of the cleavage products seen on the 5% gel were near the top of the gel except for the 60,000 molecular weight fragment and a newly observed component that ran at 25,000 molecular weight. The 16,000 molecular weight light chain also appeared and comigrated with the control. However, the 18,000 molecular weight light chain was apparently cleaved to a band running at about 17,000 molecular weight. These four discrete bands observed before dialysis were separated as follows (see Table 1, quantitative analysis of gel scans): the supernatant contained the 60,000 (64% of total), 25,000 (99% of total), 17,000 (60% of total) and 16,000 (65% of total) molecular weight bands; whereas the pellet contained only the 60,000 (36% of total), 17,000 (40% of total) and 16,000 (35% of total) molecular weight bands. Note, that as explained before, the calculated protein distribution was based on the total

protein recovered after dialysis and resuspension steps. Analysis of gel scans before and after separation gave the following net recoveries: 60,000 - 77%; 25,000 - 87%; 17,000 - 66%; 16,000 - 70%.

C.) ELECTRONMICROSCOPIC ANALYSIS OF CLEAVAGE PRODUCTS

The above separated cleavage products were further characterized by electronmicroscopic examination. To determine the general shape of the molecules in the supernatant and pellet fractions relative to the intact myosin, the samples were prepared by heavy metal shadowing. Initial comparison of intact muscle myosin and intact Dictyostelium discoideum myosin indicates that the two appear similar in size and shape (Fig. 5). The reported value for the length of the muscle myosin tail region is 1400 Å (28), whereas the length of the D.d. myosin tail measures 1590 Å. This difference may not be statistically significant or could reflect the fact that Dictyostelium discoideum myosin has a heavy chain that is about 10,000 molecular weight heavier than the heavy chain of muscle myosin on SDS discontinuous gels. Approximate calculations from the apparent difference in length would indicate the D.d. heavy chain should be about 30,000 molecular weight heavier than muscle myosin in its native dimer form. This is based on the assumption that the weight distribution in the tail region is everywhere the same. The muscle rod length of 1400 Å divided into the 110,000 SDS molecular weight of a single rod fragment gives a muscle unit of 78.6 MW/Å of single rod fragment. This factor multiplied by the 190 Å longer length of the dimer rod region of D.d. myosin as measured in the electron micrographs gives an approximate MW of 15,000 for the D.d. myosin single rod over that of the muscle myosin. This value is not significantly different from the estimated difference in molecular weights of D.d. myosin and muscle myosin on SDS gels.

Upon examination of the supernatant fraction, it was observed that there was a heterogenous mixture of predominantly single-headed myosin (60%), single-headed myosin with tail particles (16%), double-headed myosin (15%), and tail fragments (9%). The pellet fraction appeared to be double-headed myosin with smaller tail portions than the intact myosin by about 28 Å (shorter tails measured 1308 Å).

In order to further characterize the supernatant fraction, which seemed to indicate the presence of soluble HMM and HMMS-1-like fragments, decoration of Dictyostelium discoideum F-actin by the supernatant fraction in high salt was done. The result (Fig. 6) was a decoration pattern similar to the arrow-headed pattern obtained when muscle HMM S-1 is added to muscle F-actin except that there is randomly interspaced tail-like extensions from the D.d. F- actin. This probably is a result of the presence in the supernatant fraction of fragments with tail regions.

To show that this decoration was not due to a non-specific association between the soluble cleavage products and the actin, ATP was added to the high salt supernatant sample to a concentration of 10 mM before addition to the F-actin. In this case (see insert to Fig. 6), there was no decoration pattern observed which is indicative of a specific binding as in the case of muscle HMM S-1 to muscle F-actin.

D.) ANALYSIS OF CLEAVAGE PRODUCTS BY ENZYMATIC ACTIVITY

The specific activity of the Ca^{2+} high salt ATPase of the supernatant and pellet fraction from the cleaved myosin was about the same as the activity of the intact control myosin, control myosin after dialysis and the cleaved myosin before dialysis (Table 2). This indicates that the Ca^{2+} high salt ATPase of the Dictyostelium discoideum myosin was not destroyed by the proteolytic cuts made in the molecule. Such data for the supernatant fraction after cleavage also indicates that the rod portion of the molecule is not necessary for the Ca^{2+} high salt ATPase since the electron-micrographs of this fraction indicated that 60% of the sample was single headed. This information is consistent with the characteristics known about the Ca^{2+} high salt ATPase site in the muscle myosin molecule. The control supernatant had no activity, as expected.

Assays for the actin-activated ATPase of the cleaved myosin supernatant fraction along with the control assays done for intact Dictyostelium discoideum myosin, intact muscle myosin, muscle HMM, and muscle HMM S-1 are shown in Fig. 7. The myosin that was not cleaved and the control pellet obtained after dialysis showed normal activation. The supernatant control had no activity. (Note control pellet and supernatant not shown on graph.) However, the cleaved myosin before dialysis as well as the supernatant and the pellet fractions after dialysis had very poor actin-activated ATPase activities. Since the activity of the experimental fractions was about the same, only the supernatant fraction is shown in graph B

to contrast with the control soluble fractions of muscle myosin in graph A (HMM and HMM S-1). This data indicates that while the Dictyostelium discoideum myosin molecule is like the muscle myosin molecule in that trypsin cleavage does not effect the Ca^{2+} high salt ATPase of the molecule, trypsin cleavage of the D.d. myosin essentially destroys the actin-activated ATPase of the intact molecule.

Discussion

A.) Model:

Although there is not enough evidence to prove conclusively how the Dictyostelium discoideum myosin is being cleaved, one possible model is suggested and explored with respect to the data obtained and predictions it makes for further experiments. This model proposes that the molecule is cleaved at two points in the tail region and is cleaved at one of its light chains associated with the head region. The model further offers the argument that the cut in the light chain allows a nick in the globular portion of the heavy chain. The ramifications of such a nick in the globular head region are discussed in detail.

The cleavage sites in the heavy chain of the myosin molecule are schematicized in the first part of Fig. 8. Illustration 1) shows the intact myosin dimer complete with the two sets of light chains non-covalently associated with the head region. The arrow pointing to the tail region marks the site of the first cut. This cut splits the molecule into two pieces: 2a) a smaller molecule that retains the insoluble properties in low salt of the parent molecule and 2b) a short intact piece of the tail that is soluble at low salt. The next cut, as indicated by the arrow in 2a), is further along the tail region, and this results in an even shorter molecule 3a) which is now soluble in low salt. The piece of tail region cut off is assumed to be degraded to small peptides (3b).

The cleavage of the Dictyostelium discoideum myosin light chain is diagrammed in the second part of Fig. 8. For reasons that will be explained in the SDS analysis of the cleavage mechanism, it is assumed that the 18,000 MW light chain is being cleaved in both the 2a) and the 3a) cleavage products from the first part of Fig. 8. The result of this light chain cut (indicated by solid arrows) is that a nick is made in one of the globular regions (indicated by the broken line) producing the final cleavage products which are insoluble 2a'), soluble 3a'), and the small soluble piece of tail 2b) from the first part of Fig. 8.

This model agrees with the preliminary data obtained from the native gel analysis (see Fig. 3 for primary data). The first new band seen on the non-dissociating gel represents the 2a') cleavage product while the second faster running new band represents the 3a') cleavage product. The 3b) degraded piece of tail would not be visible on such a gel and neither would the 2b) piece of tail since the gel pore size is so large and the gel is run for so long. The preliminary experiment indicating that the first new native band is insoluble in low salt while the second new band is soluble also agrees with the model.

The SDS analysis of the cleavage mechanism in terms of the proposed model is diagrammed in Fig. 9. It is proposed that after ten minutes cleavage the three products 2a'), 2b), and 3a') result. The fragments that result when these products are put on a dissociating gel are indicated on the right side of Fig. 9, along with their SDS MW and solubility characteristics in low salt (see

Fig. 2 and Table 1 for primary data). The insoluble cleavage product 2a') would pellet when dialyzed against low salt. When exposed to the dissociating conditions of SDS the 16,000 MW light chain would run separately as well as the other light chain now cleaved to 17,000 MW (the 1000 MW piece cleaved off the light chain would not be picked up on the 12% gel). Furthermore, the heavy chain would dissociate from dimer to monomer and one of these monomers would further dissociate from the nick in the head region (this nick is not enough to cause the dissociation under native conditions). This heavy chain dissociation results in the 185,000 MW piece of shorter tail and head region, the 125,000 MW piece of tail with the nicked head gone, and the 60,000 MW head region resulting from the nick.

Both the 2b) and 3a') cleavage products are soluble and would therefore remain in the supernatant after dialysis against low salt and low speed spin. On an SDS gel system these products would also dissociate to many more fragments. Product 3a') would have its two light chains running separately as the 16,000 MW band and the 17,000 MW band (again, the cleaved 18,000 MW light chain results in the 17,000 MW band and the other 1000 MW piece would not be observed on the 12% gel). The heavy chain dimer of 3a') would also dissociate into the 165,000 MW short tail with head monomer and into the 105,000 MW tail and 60,000 MW head region from the nicked monomer (this nick in the head region is not detected under native conditions). The 2b) soluble tail product from the first heavy chain cut would also dissociate into two tail monomers both running at 25,000 MW on the SDS gel. This analysis explains the

12% SDS gel of Fig. 4 where the supernatant slot shows the four soluble bands running at 60,000 MW (nicked head region), 25,000 MW (soluble tail cleavage product), 17,000 MW (cleaved 18,000 MW light chain), and 16,000 MW (other uncleaved light chain). The slot showing the pellet fraction in Fig. 4 also agrees with the model showing bands running at 60,000 MW (nicked head region), 17,000 (cleaved 18,000 MW light chain) and 16,000 MW (other uncleaved light chain).

The time course gel scan also agrees with the SDS analysis of the proposed cleavage scheme in Fig. 9. Since the scan was only of the 5% time course gel only the heavy chain cleavage can be directly followed although the time course of the light chain cleavage can be inferred. The first cut is made at the tail region producing product 2a) (see Fig. 8) which appears as the 185,000 monomer on the SDS gel (note that the other tail piece 2b cannot be observed on the 5% gel). The next cuts are at the 18,000 MW light chain creating a nick in the head region and at a region further along the tail. This is evidenced by the appearance of the 165,000 MW fragment which comes from the 3a) product dissociated in SDS and by the appearance of the 125,000 and 60,000 MW fragments resulting from the 2a') product dissociating in SDS. The further appearance of the 105,000 MW fragment and the increase in the intensity of the 60,000 MW fragment indicate that the 3a) product shown in Fig. 8 is having its 18,000 MW light chain cleaved, thereby nicking its head region and producing product 3a') which when dissociated in SDS would give the above mentioned bands. This evidence indicates that the Dictyostelium discoideum myosin is first cleaved by TPCK-trypsin at a point near the end of

the tail region. Once this heavy chain cut has been made, the light chain can be cut by the trypsin whether it is associated with the product from the first heavy chain cut or the product of the second heavy chain cut. This requirement for a first heavy chain cut is supported by the absence of a band running at 150,000 MW on the SDS gel (this would represent a heavy chain monomer with nicked head region from the original intact 210,000 MW heavy chain). Note that the solubility/insolubility of the 16,000 (uncleaved light chain), 17,000 (cleaved light chain) and 60,000 MW SDS bands (nicked head region) is due to the association of these fragments with the larger 2a') and 3a') cleavage products during the dialysis and separation steps and that these fragments have independent characteristics only under non-native conditions.

Interpreting the data from the electronmicrographs in terms of the proposed model is done under the assumption that the methods for preparing the molecule for microscopy were as close to native conditions as possible. In light of this, the micrograph of the pellet fraction showing a two-headed myosin molecule with a shorter tail agrees with the model. Although one might not expect to see a nick in the head region or a 1000 MW piece clipped off a light chain, one would expect to notice a 50,000 MW shorter tail region (it is assumed that the tail region which is proposed to run at 25,000 MW on SDS gels would have a MW of 50,000 if it were in its dimer form). Using the muscle unit of 78.6 MW/Å for single rod fragment and multiplying this by the 282 Å difference between the intact molecule and the pellet molecule (see pages18 and 19 in Results for

further details) one gets a MW of about 22,000 for the tail length missing in terms of monomer tail weight. This agrees well with the proposed 25,000 MW soluble SDS band as that missing tail piece.

Analysis of the supernatant micrograph becomes more difficult since one is dealing in the model with two cleavage products: the short tail region (2b) and the shorter double-headed molecule (3a'). The heterogeneous nature of the supernatant micrograph is in agreement with the proposal that more than one kind of cleavage product is present in that fraction. However, the model would predict that the micrograph would show a heterogeneity of 66% tail segments of 282 Å and 34% double-headed short-tailed molecules. This assumes that if 100 molecules are exposed to TPCK-trypsin under the SDS conditions that all 100 would have the tail region cleaved off but only about 50 would have the second heavy chain cut producing the soluble double-headed short-tailed fragment. Thus, one would have a ratio of cut off tail to short-tailed soluble molecule of 2:1. Instead, the supernatant micrograph in Fig. 5 shows a heterogeneity of 60% single headed myosin and 40% assorted fragments of a shorter double-headed molecule (see p. 19 of results for primary data). Several explanations are possible here. First, the interpretation that the supernatant fraction was 60% single heads may be wrong since a 282 Å end section of tail may not appear linear by itself but may curl into a ball so that what appeared to be single heads was actually pieces of tail that were curling up. The other explana-

tion is that when the second heavy chain cut was made, the cleavage product 3a') was so short that though the molecule could hold together under the native conditions of the non-dissociating gel system and so ran as one band, it could not hold together under the manipulations for microscopy and that the micrograph of the supernatant fraction represents dissociated fragments of the two postulated soluble cleavage products. More micrographs and varying conditions of sample preparation may help to clarify this apparent conflict.

The proposed model for TPCK-trypsin cleavage of D.d. myosin is consistent with the ATPase assays of the different fractions. The general conclusions from the assays is that while the trypsin cleaved myosin molecule keeps its capacity to cleave ATP under high salt conditions and in the presence of Ca^{2+}, the cleavage somehow destroys the molecule's ability to use actin to accelerate the hydrolysis of ATP. The actin-activated ATPase of myosin requires a site for actin association and sites for binding and splitting ATP. The intact Ca^{2+} high salt ATPase of the myosin fragments argues for an intact ATP binding and splitting site, and the decoration experiment (see p.19) argues that the actin binding site is intact. Why then is the actin-activated ATPase activity so low? The proposed model for TPCK-trypsin cleavage allows for several explanations for the observed lack of actin-activated ATPase in either the pellet fraction, supernatant fraction, or cleavage before dialysis fraction. It seems possible that the mechanism by which the Dictyostelium discoideum myosin ATPase is activated by actin may be different that that for muscle myosin. Perhaps,

cleaving off the tail piece destroys the actin-activated ATPase, in which case D.d. myosin is unlike muscle myosin in that it requires its tail region for intact activity. More probably, it is the light chain cleavage or nick in the head that is responsible for the loss of the D.d. actin-activated ATPase activity. The light chain of the D.d. myosin molecule may be a necessary part of the molecule's enzyme action. On the other hand, since it is postulated that a nick in one of the globular heads occurs in both cleavage products 2a') and 3a'), the loss of actin-activation of the myosin ATPase may be a result of this nicking in the head region.

While this model agrees reasonably well with the data obtained, it is put forth only as one way of interpreting the observations and is not meant to exclude other possible models.

B.) Comparison to Muscle Myosin

Although the above model is based on many assumptions and is not a definitive account of the events occurring when D.d. myosin is exposed to TPCK-trypsin, there are some conclusions that can be definitely drawn from the data obtained on Dictyostelium discoideum myosin and its relationship to muscle myosin.

With regard to general intact structure, it appears that, like other non-muscle myosins, Dictyostelium discoideum has a general shape and size similar to skeletal muscle mysoin, as judged by electron microscopy. The intact Dictyostelium myosin also demonstrates the capacity to have its ATPase activated by actin like muscle myosin.

Trypsin cleavage of Dictyostelium myosin indicates differences relative to muscle myosin. From the number of cleavage products and the rate of cleavage of Dictyostelium versus muscle myosin, it seems probable that the amino acid sequence and, perhaps, the conformation of Dictyostelium myosin is different such that it is more susceptible to trypsin cleavage than muscle myosin. Further, the solubility of the 165,000 molecular weight fragment of Dictyostelium myosin relative to the soluble 130,000 MW HMM fragment of muscle myosin might indicate that the non-muscle myosin has a shorter region of aggregation in its tail and therefore a longer region that would be free to move when the Dictyostelium myosin is in a thick filament state. While the evidence points to localization of the actin binding site and the Ca^{2+} high salt ATPase in the globular region, the poor actin-activated ATPase is disappointing. The assays were carried out within ten hours after cleavage, and myosin which was taken through the same experimental steps (using a trypsin-inhibitor complex in place of incubation with trypsin) had intact activity. Thus, the enzyme does not simply denature under these conditions. The more probable conclusion, as discussed above, is that cleavage of the non-muscle myosin removes or inactivates some part of the myosin that is required for the actin-activated ATPase observed in the intact molecule but not required for binding or splitting ATP under high salt, Ca^{2+} buffer conditions. The approximate 100 $\mathring{A}$ longer tail of the D.d. myosin versus muscle myosin may be relevant to such a functional difference.

C.) Further Questions and Experiments

The results of these experiments help to identify and formulate questions that should be asked about the non-muscle Dictyostelium discoideum myosin and actin interaction and how this produces movements such as pseudopod formation.

One obvious question is: can a soluble non-muscle fragment be made that has intact actin activated ATPase? Use of other enzymes (such as chymotrypsin and papain as well as variations in the cleavage conditions such as ionic strength and divalent cation concentrations) may result in the production of a non-muscle HMM or HMM S-1 analog. If, however, smaller soluble fragments of D.d. myosin are produced and none have intact actin-activated ATPase, then the basic assumptions about non-muscle myosin's similarity to muscle myosin must be reviewed.

Another, perhaps more interesting, question raised in these experiments is can the actin-activated ATPase for the trypsin cleavage products be restored by appropriate recombinations? One possibility is that the 18,000 MW light chain is required for intact activity and that its cleavage to the 17,000 MW piece destroys this. One might try light chain recombination experiments to determine which or in what combination the light chains are required to give the non-muscle myosin its actin-activated ATPase activity. The final proof would be to take one of the cleavage products with destroyed light chain, add back proper light chain to it, and monitor the return of the actin-activated activity.

Another possibility is that the cleaved off tail portion is required for intact actin-activated ATPase activity. One method to determine if this is a probable explanation is to co-monitor the appearance of the 25,000 MW fragment on 12% gels and the loss of actin-activated activity of the myosin molecule as it is being cleaved. If the tail portion were required for intact activity, then its appearance should correlate with a loss of actin-activated activity. Proof that this tail piece, covalently linked, is required for intact activity would be difficult to obtain since reattachment of the piece to the myosin is not currently feasible. One might try to purify the first cleavage product proposed by the model (2a) which has only a cut in the heavy chain and no cut in the light chain yet. If it had already lost its actin-activated ATPase activity and no other structural difference compared to the intact molecule were detected, it would serve as strong evidence indicating that the cleaved off piece is required for intact activity. If this seems to be the case, one might try to set up a relationship between this piece as a primitive light chain comparable to the 25,000 MW third light chain found in muscle myosin. This could be done by checking to see if the tail fragment has any amino acid sequence in common with one of the other Dictyostelium light chains.

Answers to these questions could lead to a better understanding of how non-muscle myosin is composed, how it interacts with non-muscle actin and how valid the assumptions have been as to the analogy between muscle and non-muscle contractile systems.

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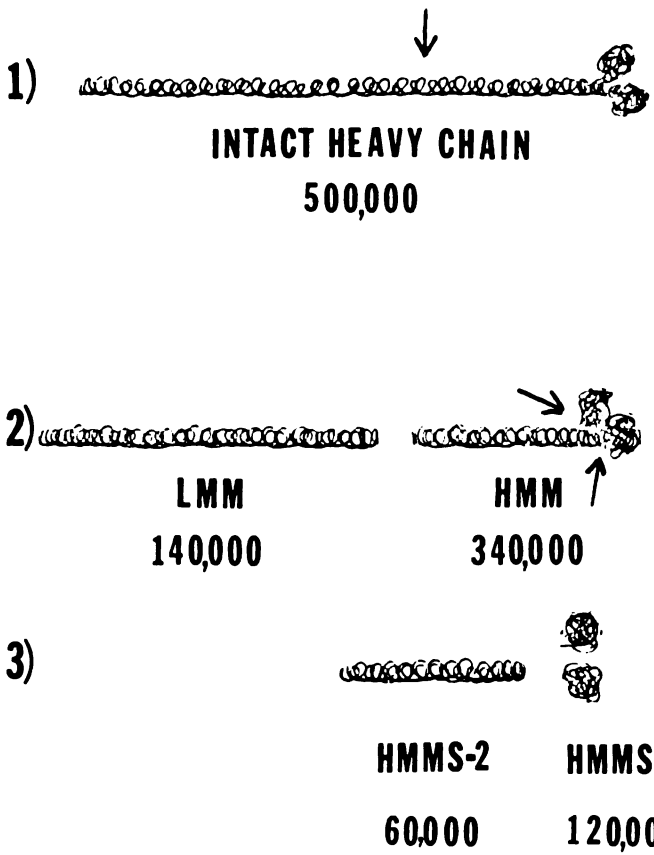
Fig. 1. Results of Trypsin & Papain Cleavage on Muscle Myosin

Schematic representation of trypsin and papain cleavage of rabbit skeletal muscle myosin. Light chains are not shown. Arrows indicate sites of cleavage of the molecule in its native state. Note that trypsin attacks the molecule first at a region in the tail portion and later cleaves at a less sensitive region near the heads producing the same HMMS-1 as papain. Common names for the resulting native cleavage products are listed below their illustrations along with the molecular weights determined by the Archibald methods of sedimentation velocity. The diagram is drawn to a scale of 0.5 mm = 100 Å. Weights of the denatured fragments as determined by SDS gels are as follows: intact heavy chain, 200,000; light meromyosin (LMM), 70,000; heavy meromyosin (HMM), 130,000; rod, 110,000; heavy meromyosin subfragment - 1 (HMMS-1), 90,000; heavy meromyosin subfragment-2 (HMMS-2), 30,000. Lengths of native fragments as determined by electron micrographs are as follows: intact heavy chain, 1500 Å; rod, 1400 Å; HMMS-1, 100 Å; HMMS-2, 500 Å. Length values are from Subunits in Biological Systems, Part A, ed. Timasheff, S. & Fasman, G. (1971) Dekker, Inc. N.Y. "Myosin: Molecule and Filament" by Susan Lowey, p. 201. SDS MW values were taken from Weeds, A. & Pope, B., J. Mol. Biol. (1977) 111, 129-157.

TRYPSIN



PAPAIN

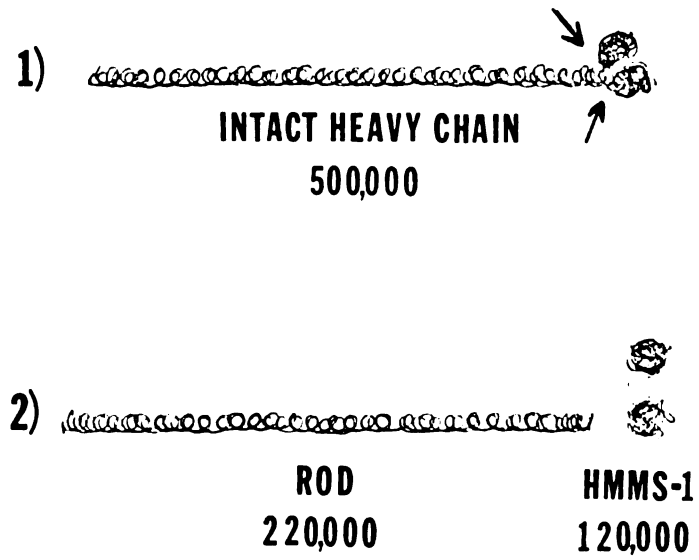


FIG. 1

Fig. 2. Gel Scans of Tryptic Time Course Cleavage of D.d. Myosin

5% acrylamide tube gels were scanned. Details of procedure are given in Materials and Methods. The time course pattern reads from top to bottom. Note that though some products appear to go through a heterogeneous stage, they do appear as homogeneous peaks at some point and are treated as such. The molecular weights given across the bottom are approximate weights determined from Rf analysis of the tube gels with internal standards noted in Materials and Methods. The weights given in the figure are for those Dictyostelium products and intact heavy chain determined to be the most prominent.

Figure 2

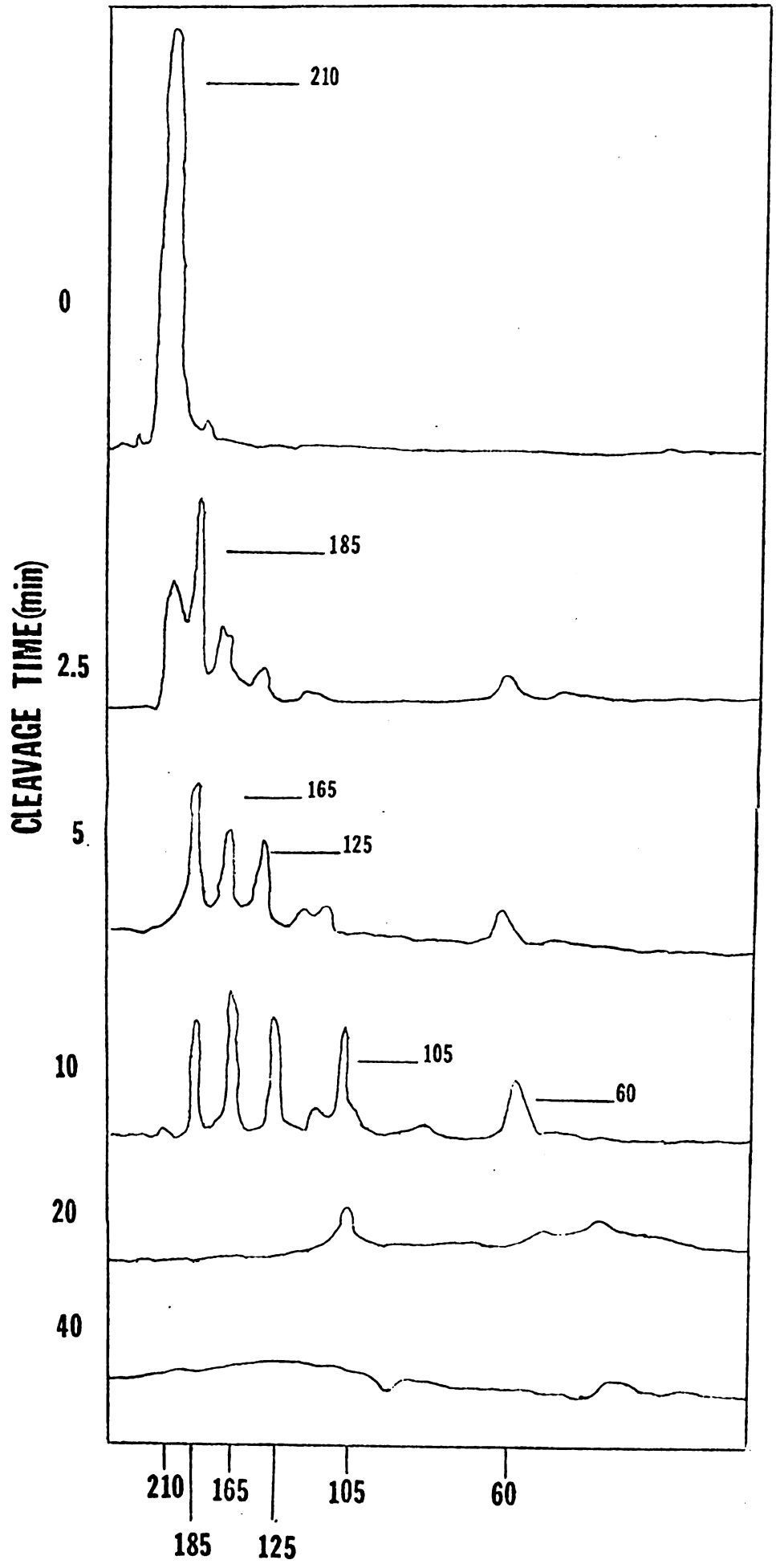

210
185
165
125
105
60
0
2.5
5
10
20
40
CLEAVAGE TIME(min)
210
185
165
125
105
60

Fig. 3. Native Gels of Tryptic Time Course Cleavage of D.d. Myosin

The picture of native gels run in a continuous fashion shows 3% acrylamide tube gels with the time of cleavage noted at the bottom of each gel. Bends in gels are due to their extreme flexibility that results from the very low percentage of acrylamide. Note that at 5 minutes the intact myosin is cleaved to one other product, that all intact myosin is gone by 10 minutes, and that a new product results from the first cleavage product after 20 minutes of incubation. Gels were run for twelve hours at 4^{o} C and were stained with Coomassie blue. Concentration of trypsin used for this experiment was one-half of the normal concentration. For further details, see Materials and Methods.

FIG. 3

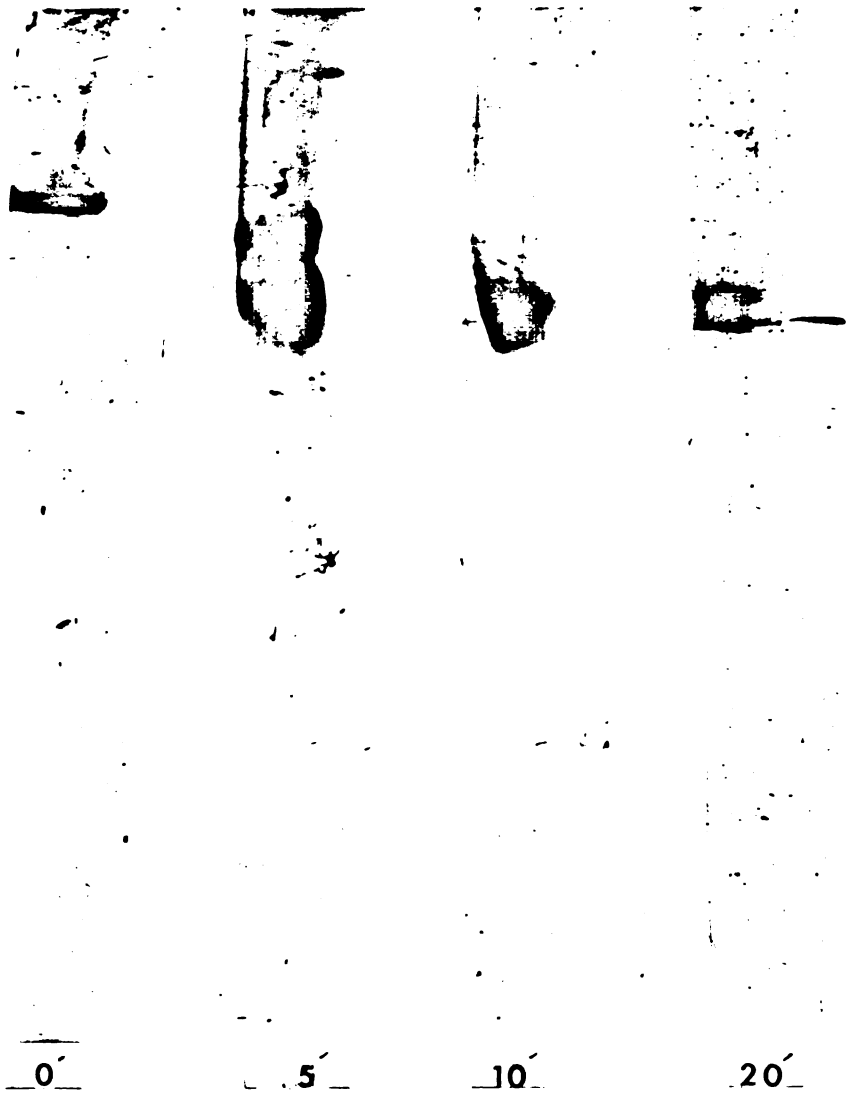


Table 1. Product Distribution After Dialysis of Trypsin-Cleaved D.d. Myosin

The table shows the distribution of D.d. myosin cleavage products obtained when D.d. myosin is incubated with TPCK-trypsin for 10 min, dialyzed against low salt, and spun at low speed. The supernatant is that material that is soluble in low salt, while the pellet is that which is insoluble in low salt. Percentage distribution was determined by quantitative analysis of gel scans of 5% SDS gels in the case of 210,000-60,000 MW products and of 12% SDS gels in the case of 60,000-16,000 MW products. The final percentage distribution for the 60,000 MW product was the average of the values obtained from the 5% and 12% gel scans. Such distribution analysis was based on the total protein recovered after dialysis and resuspension. It should be noted that some protein was lost between the cleavage step and the resuspension step, presumably during dialysis. Analysis of gel scans before and after separation gave the following net recoveries: 185,000 - 53%; 165,000 - 69%; 125,000 - 42%; 105,000 - 57%; 60,000 - 77%; 25,000 - 87%; 17,000 - 66%; and 16,000 - 70%. The control experiment of dialyzing intact D.d. myosin against low salt, spinning, and resuspending resulted in 95% of the intact myosin pelleting. Further details are covered in Materials and Methods. The column labeled "Solubility" refers to the fragments apparent solubility in low salt: S = soluble, I = insoluble, S/I = is both soluble and insoluble.

MW from SDS Gel	% in Supernatant	% in Pellet	Solubility
210,000(intact)	-	-	I
185,000	25	75	I
165,000	88	12	S
125,000	28	72	I
105,000	89	11	S
60,000	64	36	S/I
25,000	99	1	S
17,000	60	40	S/I
16,000	65	35	S/I

Fig. 4. SDS Gel of Light Chains of D.d. Myosin Cleaved by TPCK-Trypsin

This 12% acrylamide gel was run in order to observe the state of the D.d. myosin light chains which could not be observed on the 5% gel. M = intact rabbit skeletal muscle myosin; D = intact Dictyostelium discoideum myosin; DO = D.d. myosin exposed to trypsin/inhibitor complex as a control; DOP = Fraction of DO that pelleted when dialyzed against low salt buffer (supernatant fraction had too little protein in it to be visible on the gel); D 7.5 = D.d. myosin cleaved for 7.5 minutes with TPCK-trypsin; D 7.5S = Fraction of D 7.5 that remained soluble when dialyzed against low salt buffer; D 7.5P = Fraction of D 7.5 that pelleted when dialyzed against low salt buffer.

Arrows in the right margin point to the position of skeletal muscle myosin 25,000, 18,000 and 16,000 MW light chains. Note that DO shows the top heavy intact heavy chain band and the same light pattern as D standard and that, while DOP includes the intact heavy chain and light chains, it also shows an intermediate band running with the 25,000 MW muscle light chain. This band is presumably a result of heavy chain cleavage and may be due to activation of trypsin during the dialysis step.

Sample D 7.5 (cleaved D.d. myosin) shows multiple top bands probably analogous to bands seen in the 5% gel of D 7.5. The lower running dense band is probably the 60,000 MW piece. Note that the 25,000 MW intermediate band seen in DOP is present in even greater amount in D 7.5. Also note that the 18,000 MW light chain of D 7.5

appears to be cleaved to about 17,000 MW while the 16,000 MW light chain remains intact. Sample D 7.5S runs as D 7.5 although the intermediate band running at about 25,000 MW is enriched. Sample D 7.5P runs as D 7.5 except that no intermediate 25,000 MW band is present. For further discussion, see Results and Discussion.

Note also that the DOP sample from this experiment did not exhibit the intact actin-activated ATPase that it did in other experiments, although the Ca^{2+} high salt ATPase activity was intact as expected. Perhaps the presence of the intermediate 25,000 MW band correlates with this loss of actin-activated ATPase noted in all experimental samples.

FIG. 4

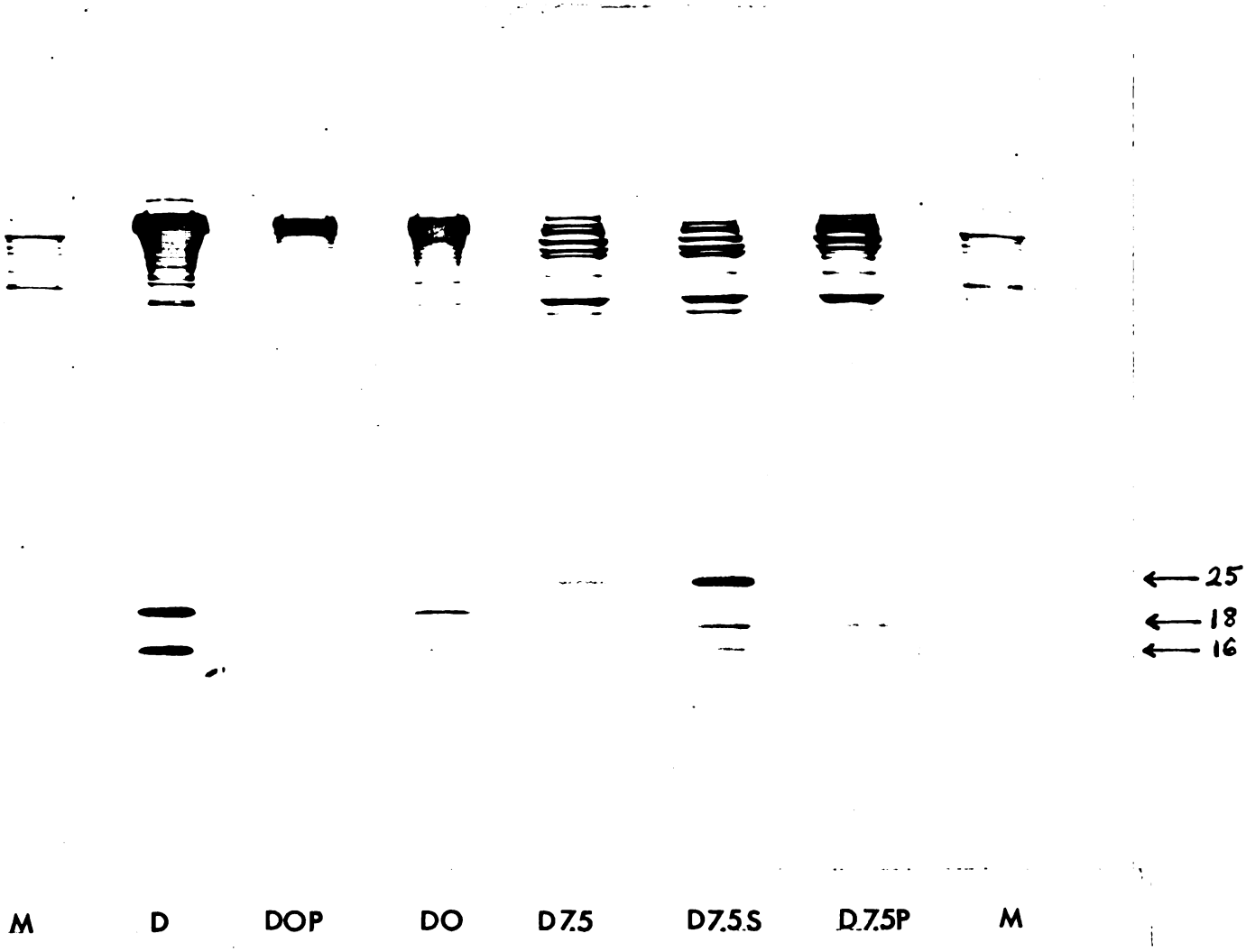


Fig. 5. Electron Micrographs of D.d. Myosin: Intact, Cleaved Pellet and Supernatant

The above pictures are heavy metal shadowed replicas of D.d. myosin. Details of methods are in Materials and Methods. A) Intact D.d. myosin, 65,000X. 25 molecules were measured to give an average value for the tail length of 1590 $\mathring{A}$ ± 14%. B) Picture of material that remains in the pellet after 7.5 minutes incubation with trypsin and dialysis against low salt buffer. The myosin molecule tail appears to be shorter and 20 molecules were measured to give an average tail length value of 1308 $\mathring{A}$ ± 10% 65,000X. C) Material that remained in the supernatant after cleavage with trypsin. 75,000X. Analysis of 6 such micrographs gave the following distribution of myosin particles; single heads, 60%; other pieces, 40%. Other pieces were broken down to: double heads, 15%; tails, 9%; and single head-tail particles, 16%.

FIG. 5

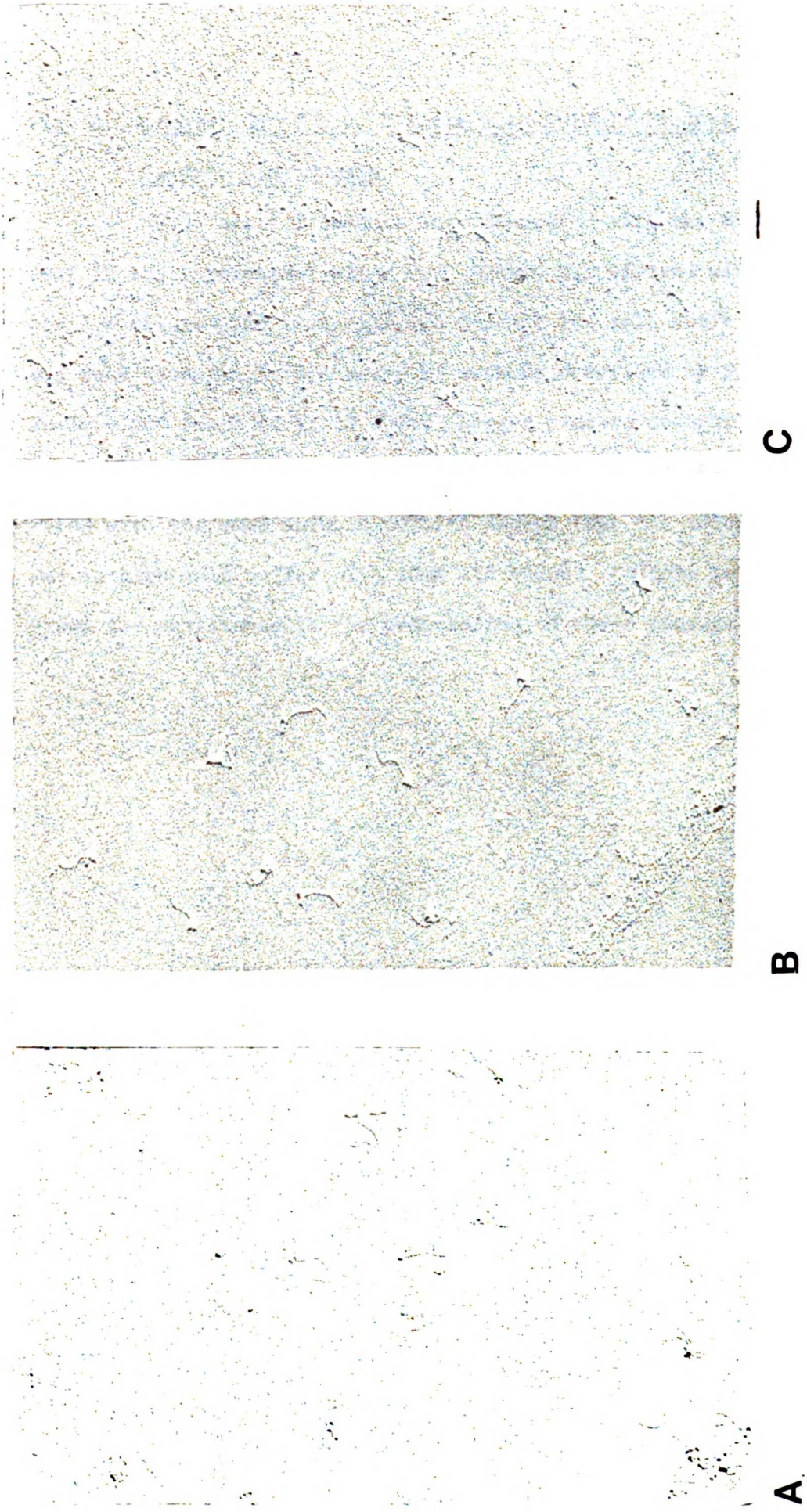


Fig. 6. Decoration of D.d. F-Actin with Soluble Product from Trypsin Cleaved D.d. Myosin

D.d. actin filaments were decorated with the material left in the supernatant after D.d. myosin was cleaved with trypsin for 7.5 minutes and then dialyzed against low salt buffer. Decoration was done essentially according to methods described by Spudich, Huxley, and Finch (27); 48,000X. Larger picture decoration with the soluble fragment in high salt buffer. Inset is D.d. F-actin decorated with the same soluble portion except that the soluble sample was in high salt buffer with 10mM ATP added. (Thanks to Dr. Susan Brown for assistance in the preparation of these micrographs.)

FIG. 6

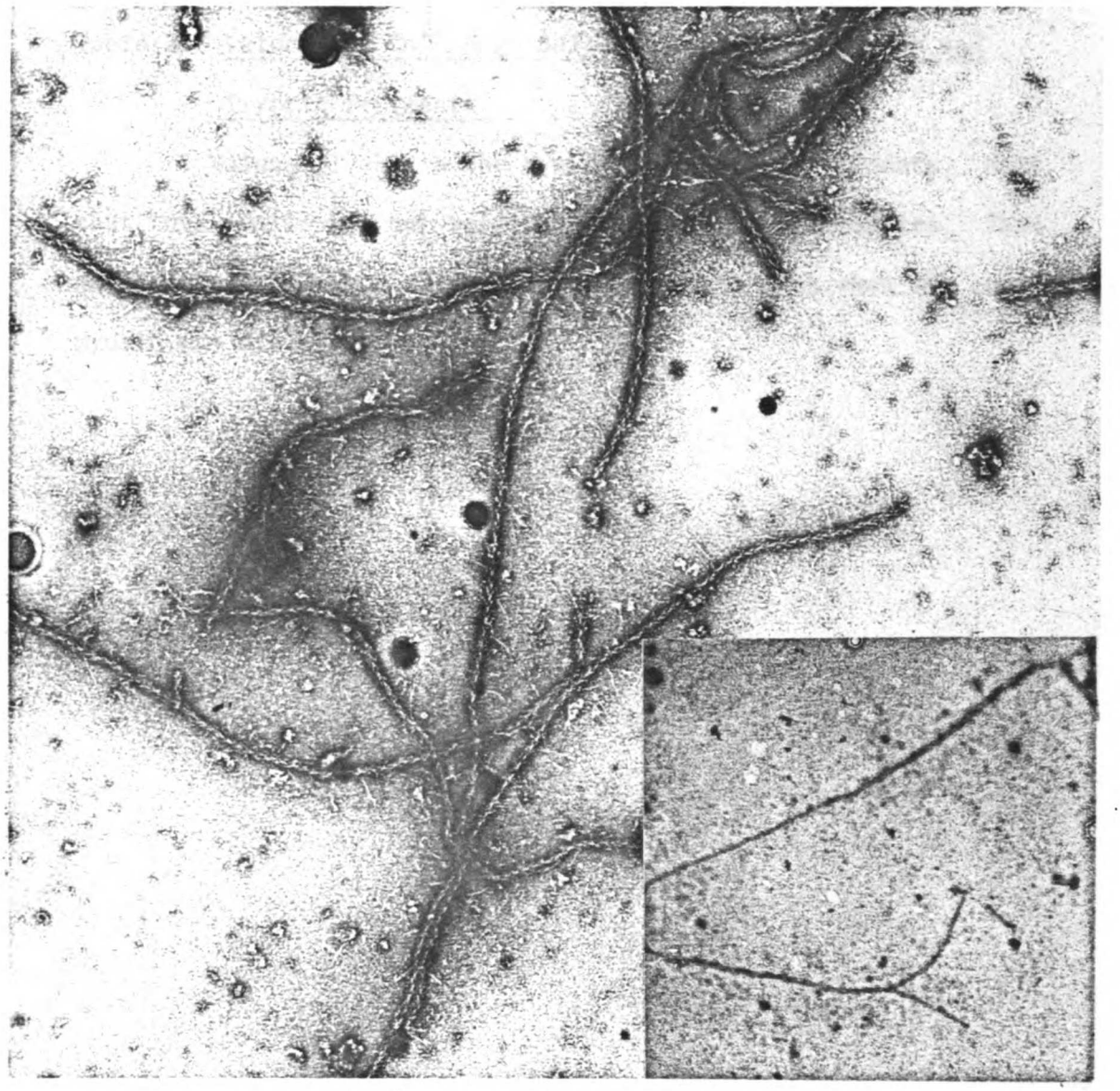


Table 2. Values for Ca^{2+} High Salt ATPase of D.d. Myosin and Tryptic Fragments

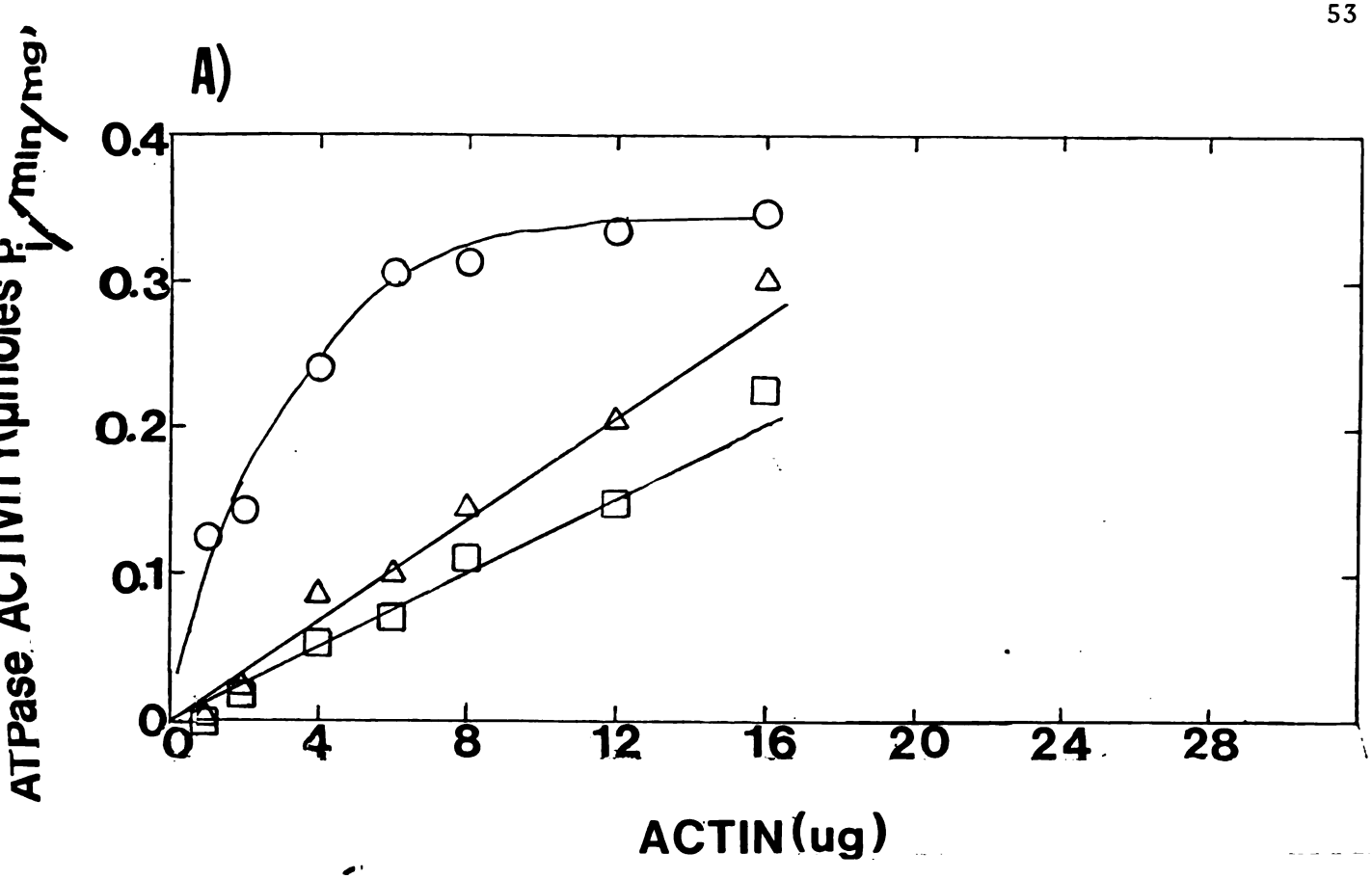
Values given are the average of three experiments. Note that total units are not retrieved for the control samples. This may be explained by a constant 20-35% protein loss when the control myosin was dialyzed and spun.

Sample	TOTAL UNITS (μ moles P_i/min)	ATPase SPECIFIC ACTIVITY (μ moles P_i/min/mg protein)
DO	.17	.36
DOS	--	--
DOP	.09	.31
D 7.5	.22	.35
D 7.5S	.10	.36
D 7.5P	.12	.31

Fig. 7. Actin Activated ATPase Versus Increasing Actin Concentration

Graph A shows the results of the control experiment done with intact muscle myosin (O). HMM (Δ), and HMM S-1 (□). Note that while the intact myosin exhibits saturation kinetics, the graphs for HMM and HMM S-1 do not in the range of actin tested. Graph B shows the results of intact D.d. myosin (O) and the material obtained from the supernatant (Δ) after differential solubility separation of cleaved D.d. myosin. Note that the intact D.d. myosin exhibits the same kind of saturation kinetics as the intact muscle myosin, but that the supernatant fraction is not as readily activated as the muscle HMM or HMM S-1 in graph A. Although data shown here is for muscle myosin with muscle actin and for D.d. myosin with D.d. actin, all reciprocal experiments were done and similar results were obtained regardless of which actin was used to activate the myosin. All reactions were done in 0.1 ml of reaction mixture with 5 μg of myosin sample. Specific activity is in mg of myosin sample. Further details are in Materials and Methods.

FIG. 7


A)
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0.2
0.1
0
0
4
8
12
16
20
24
28
ACTIN(ug)

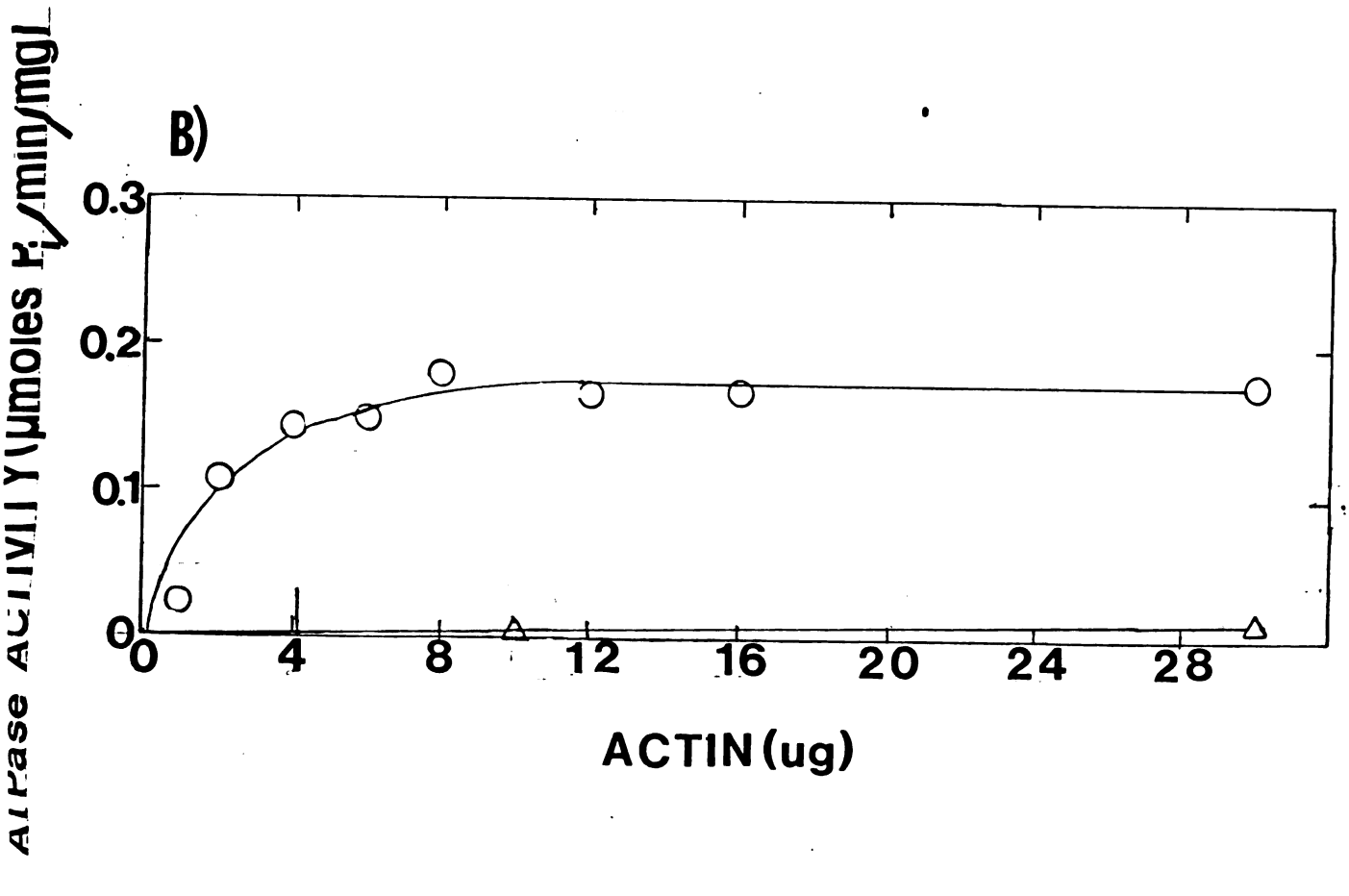

B)
ATPase ACTIVITY(μmoles Pi/min/mg)
0.3
0.2
0.1
0
0
4
8
12
16
20
24
28
ACTIN(ug)

Fig. 8. Model for TPCK-Trypsin Cleavage of D.d. Myosin

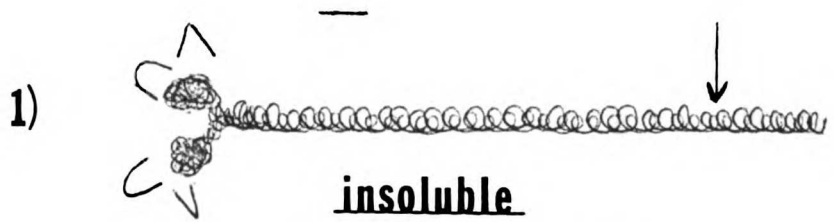
The proposed scheme for how trypsin cleaves D.d. myosin is shown above. The heavy chain is drawn to a scale of 1 mm = 100 Å. The length of the intact D.d. myosin is about 1700 Å (1600 Å for the measured tail region - See Fig. 5 - and an approximate 100 Å for the globular head region).

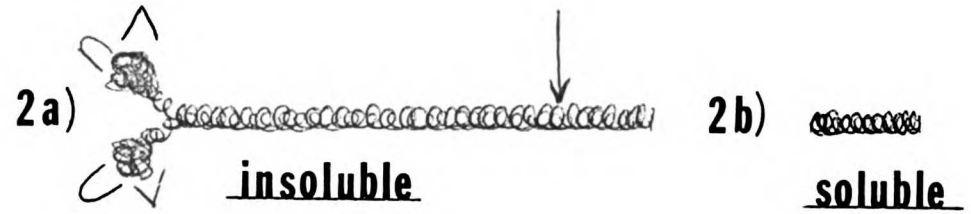
The first part of Fig. 8 illustrates the cleavage sites in the heavy chain. Note that the light chains are included though not drawn to scale and are /\ = 16,000 MW and /\ = 18,000 MW light chain. The first cleavage in the intact molecule occurs at the end of the tail region liberating an approximately 1400 Å double-headed myosin (2a) and a now soluble piece of tail about 300 Å long (2b). Product 2a) retains the insoluble properties of the parent molecule, 1). The next heavy chain cut is further along the tail and produces an even shorter double-headed molecule about 1150 Å in length (3a) and a degraded piece of tail (3b). Product 3a) is now soluble in low salt.

The light chain cleavage site shown in Part II of Fig. 8 shows the 18,000 MW light chain (∩) cleaved in 2a) and 3a) to a piece about 17,000 MW (⌐) in 2a') and 3a'). The solid arrow points to the cleavage site in 2a) and 3a) while the dotted line, extending from the arrow, marks the nick made in the globular region which is shown as an extended head region in 2a') and 3a') with dotted-line arrows. The reason only one globular head is nicked per molecule may be due to kinetics or differences in the heads.

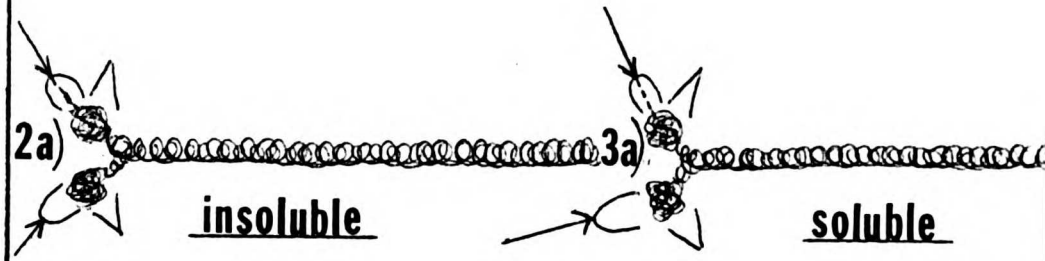
The characteristic solubility of each product is noted underneath their illustration. The three bands seen on native gels are also noted: 1) is the intact top band; 2a') is the first new band; 3a') is the second faster running band.

I) HEAVY CHAIN CUTS





II) LIGHT CHAIN CUT (nicked head region)



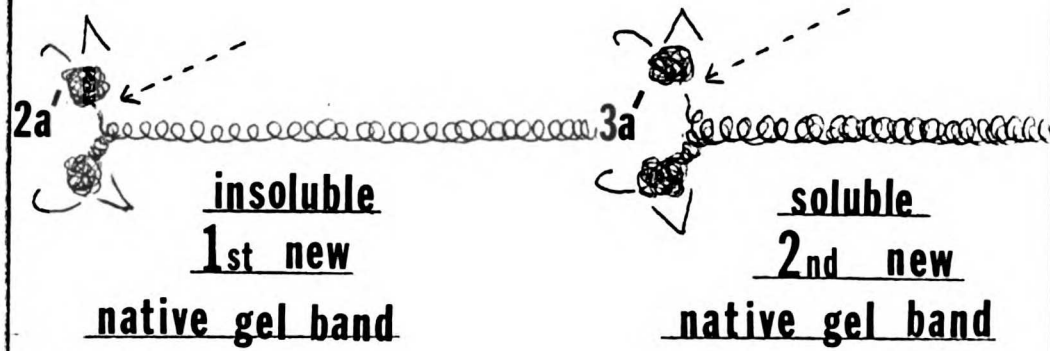


FIG. 8

Fig. 9. SDS Analysis of Cleavage Model

This scheme illustrates what happens when the proposed cleavage products 2a'), 2b'), and 3a') from Fig. 8 are separated by differential solubility and then run in the presence of SDS on a dissociating, discontinuous gel system. The dotted-line arrows indicate the nicks in the head regions of product 2a') and 3a').

The pelleted, insoluble, 2_a') product dissociates to give 5 fragments: head-short tail fragment (185,000); short tail fragment missing nicked head (125,000); nicked head region (60,000); cleaved light chain (17.000); and intact light chain (16,000).

The lower half of Fig. 9 shows how the soluble products, that remain in the in the supernatant after low salt solubility separation (products 2b) and 3a')), dissociate in the presence of SDS. Product 2b) simply dissociates from an approximately 280 Å long dimer tail piece to two monomers of about 25,000 MW each (only one is shown). Soluble product 3a') dissociates in a manner similar to insoluble product 2a'). The heavy chain and light chains dissociate in SDS to give: head-shorter tail fragment (165,000); shorter tail fragment missing nicked head (105,000); nicked head region (60,000); cleaved light chain (17,000); intact light chain (16,000).

SDS ANALYSIS OF MODEL

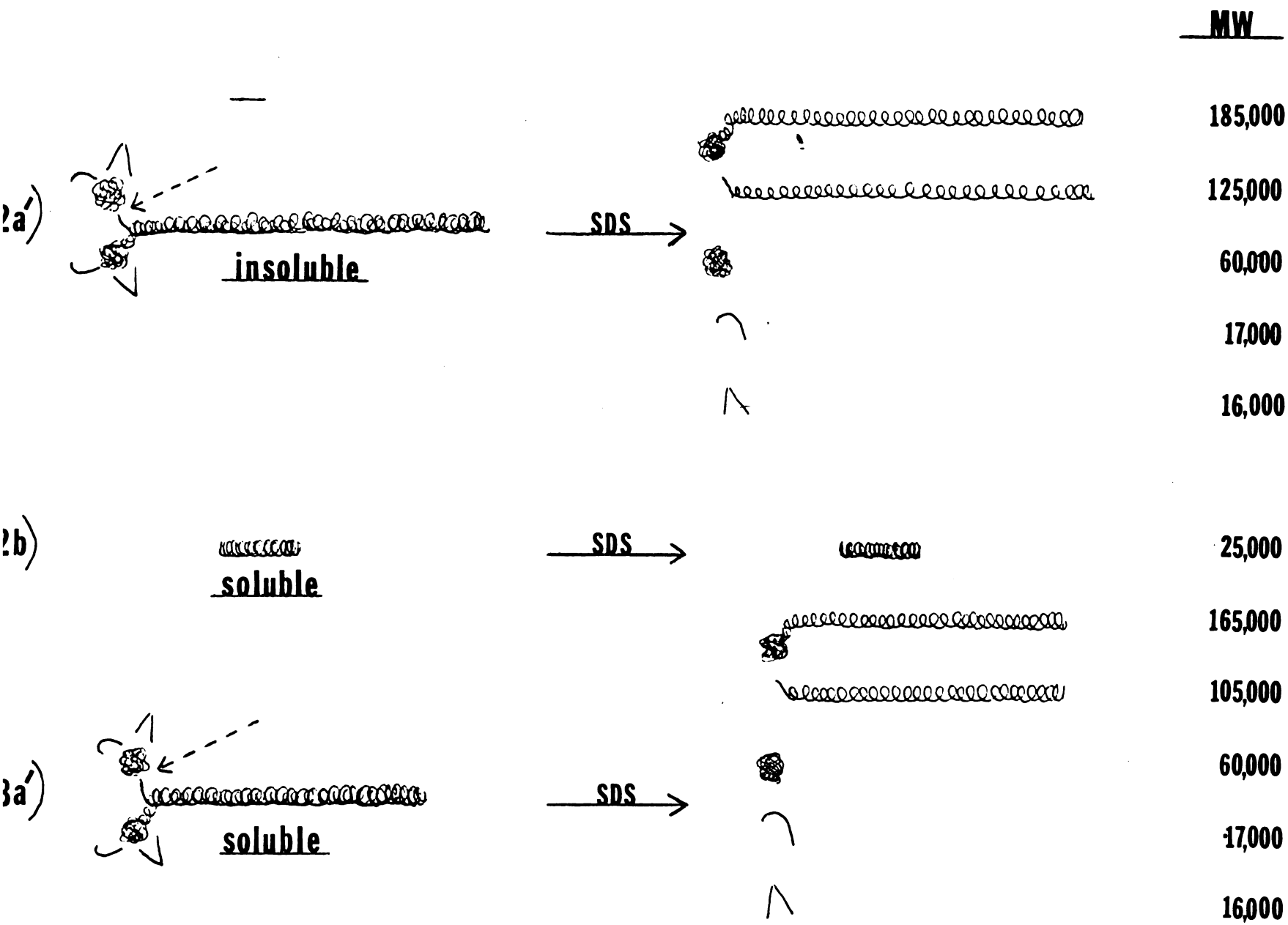


FIG. 9