# THE ISOLATION AND PROPERTIES OF FISH MYOSIN

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SUMMARY

A method for the preparation of myosin from carp red muscle and from mullet white and red muscles is described. The mullet myosin obtained is only very partially centrifuged off at ionic strength 0.05 and pH 7.2 at 25,000 $\times$ *g*. It is necessary to lower the pH to 6.4 in order to complete the precipitation. Apart from this anomaly, the preparations isolated from carp and mullet muscles behave as rabbit myosin from the viscosimetric, electrophoretic and ultracentrifugal points of view. The rate of sedimentation at zero concentration is 6.03 $\pm$ 0.12 S, somewhat lower than the corresponding value for cod myosin and about identical with recent values found for rabbit myosin. Electrophoresis of fish myosin in borate buffer at pH 10.5 gives much more symmetrical gradients than in phosphate buffer of pH 7.2 suggesting that the former solution could be more favourable for the study of the monomeric form of myosin. The stability of the myofibrils of white and red fish muscles in presence of extractants of high ionic strength appeared to be very different. Their disruption is easy in the case of white muscle while the behaviour of red muscle is reminiscent of rabbit muscle. The submicroscopic organisation of the myofibrils in white and red fish muscles is obviously very different.

## INTRODUCTION

Myosin can be readily prepared from muscles of warm blooded vertebrates. Rabbit myosin has been extensively 'investigated' and found by a number of criteria[2–5] to be similar to rat and mouse myosin. However, the isolation of myosin from muscles of cold blooded vertebrates is much more difficult[6]. In this case, actomyosin is obtained when the methods devised for rabbit myosin are used. However, CONNELL[7] prepared cod myosin by extracting coarsely minced muscle for 5 min with a phosphate buffer of pH 6.5, containing 0.2 *M* KCl and 0.01 *M* $Na_4P_2O_7$ (total *I* 0.35). The myosin was precipitated by dialysis of the extract against water, washed with dilute phosphate buffer, redissolved and purified by reprecipitation under the same conditions. The viscometric and electrophoretic behaviour of this preparation did not differ from those of rabbit myosin, but it exhibited some polydispersity in the ultracentrifuge with a much higher and more concentration dependent rate of sedimentation. Furthermore,

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it had a low ATP sensitivity but this has been attributed recently to the interference of pyrophosphate[8]. One of the present authors has described another method of preparation[4,5]. Slices of frozen carp muscle, cut with a freezing microtome, were extracted for 10 min with a phosphate buffer of $I$ 1.0 and pH 5.5. Actomyosin was removed by dialysis to $I$ 0.25 and pH 7.2 and the myosin precipitated by dilution with water to $I$ 0.025. From some points of view (*e.g.* solubility, viscosity, electrophoretic mobility) this preparation behaved as rabbit myosin, but on ultracentrifugation it showed two closely sedimenting peaks ($S_{20,w}$ 4.1–4.8 and 4.5–5.0 S) (*cf.* Fig. 1b). As a single peak is obtained by ultracentrifugation of carp actomyosin in the presence of ATP[5,9] some alteration of the myosin molecule or of its state of aggregation must have occurred during the preparation. In the course of the present work further results have been published by CONNELL[8]. During ultracentrifugal analysis of codling extracts of various ionic strengths, he observed a sharp peak sedimenting with the rate varying from 4 to 8 S in extracts of $I$ 0.3 (0.05 $I$ phosphate, 0.25 $M$ KCl) and pH 6.5 or 7.5. This myosin was precipitated by reducing the ionic strength to approx. 0.05 and had the same electrophoretic mobility as rabbit myosin and practically zero ATP sensitivity. Its actin combining power was not very high and after precipitation two or three peaks in the range 4–8 $S$ appeared in the ultracentrifuge. CONNELL concluded that aggregation occurs during precipitation or even in extracts held overnight at 0°. During the course of the preparation of the present manuscript CONNELL[10] has described a new preparation of cod myosin (with similar properties) by ultracentrifugal separation after dissociation of cod actomyosin with ATP or pyrophosphate. A serious disadvantage of this method is that it is not possible to obtain myosin solutions more concentrated than 0.4 %. This is because the concentration of actomyosin must not exceed 0.7 % if contamination of the myosin preparation is to be avoided. (The latter is in accordance with similar observations made independently by one of us (M.B.S.) in which myosin is separated by ultracentrifugation after dissociation of actomyosin by ATP, pyrophosphate or at high ionic strength.)

The observation of one of us (G.H.) that myosin can also be extracted at pH 6.5 from fresh carp red muscle and the interest in this laboratory in the isolation of myosin as part of a programme on aggregation reactions has led us to reinvestigate the isolation and properties of fish myosin.

## EXPERIMENTAL

### *Materials and methods*

The earlier experimental work was carried out on specimens of a fresh water fish, mirror carp (*Cyprinus carpio* L) weighing not less than 1 kg, and was limited to red muscle of the lateral line. Later both white and red muscles from a common marine fish living in sub-tropical waters, the mullet (*Mugil cephalus* L), were separately investigated. In view of the small amount of red muscle present in both carp and mullet, four or five fish were generally used for a red muscle extract while one or two were sufficient for a white muscle extract.

The myosin preparations, obtained as described in the next section, were dialysed against a phosphate buffer of $I$ 0.35 and pH 7.1 (0.0077 $M$ $NaH_2PO_4$, 0.0308 $M$ $Na_2HPO_4$, 0.25 $M$ NaCl) and examined by several methods in this solvent.

The protein content was determined by the micro Kjeldahl method of MCKENZIE

AND WALLACE[11], assuming a total nitrogen content of 16.7 %[12]. The viscosimetric behaviour was studied in an Ostwald viscosimeter in a constant temperature bath at 1° (± 0.03°). The relative viscosity of the preparations was determined in the absence of ATP ($\eta_{rel}$) and in the presence of ATP ($\eta_{rel, ATP}$), in order to compare their relative viscosity with that of rabbit myosin[13] and to calculate the ATP sensitivity. The latter is defined by[14],

$$\text{ATP sensitivity} = \frac{\log\eta_{rel} - \log\eta_{rel, ATP}}{\log\eta_{rel, ATP}} \times 100$$

Ultracentrifugal measurements were carried out in a Spinco model E ultracentrifuge at 59,780 rev./min and room temperature. A Spinco phase plate was used in place of the Schlieren bar. The sedimentation coefficients were corrected to water at 20° assuming PARRISH AND MOMMAERTS'[15] value of 0.728 for the partial specific volume. Electrophoresis measurements were carried out in the cell described by DUBUISSON, DISTECHE AND DEBOT[16] and in the usual Tiselius cells. In the former, the Longsworth scanning method of recording and in the latter, the Philpot cylindrical lens method and the apparatus of MCKENZIE, ROSE AND SMITH[17], were used.

*Isolation of myosin*

Live fish were decapitated and skinned immediately. Both kinds of muscle were isolated carefully and brought to the cold room where they were minced without delay and the rest of the preparation carried out. Finely ground white muscle, unlike red muscle, formed a gelatinous mass when mixed with the extraction fluid and gently stirred. In order to reduce this extraction of actomyosin, coarsely minced tissue was used in the myosin extractions from white muscle. This was obtained with a 'Sunbeam' electric household mincer driven slowly. Red muscle was minced more finely, using a Baird and Tatlock (Type C18/001) tissue mincer.

The minced muscle was gently stirred for 10 min with two volumes of a phosphate buffer of pH 6.6 and $I$ 1.0 (0.25 $M$ $KH_2PO_4$, 0.25 $M$ $K_2HPO_4$) or 0.5 (0.125 $M$ $KH_2PO_4$, 0.125 $M$ $K_2HPO_4$) containing 1 mg/ml of disodium ATP (Sigma). Comparative experiments made on mullet white muscle suggest that the increase in ionic strength of the extraction fluid from 0.5 to 1.0 does not improve the yield of the preparation and $I$ 0.5 was used in later preparations. The role of the ATP will be discussed below.

The muscle debris was separated by centrifuging at 25,000 × $g$ for 10 min. A liquid layer of lipid material accumulated at the top of red muscle extracts and a much thinner one in the case of white muscle extracts. It was removed as completely as possible and the supernatant dialysed in Visking cellulose tubing for 18–25 h against three changes of 2.1 l of a phosphate buffer of pH 7.2 and $I$ 0.25, obtained by dilution of the $I$ 0.35 buffer. The use of an efficient rocking device for equilibration of the solution in order to remove completely the actomyosin present was necessary. Conductivity measurements provided a convenient check of equilibration. In the case of mullet extracts, the ionic strength of the buffer used in dialysis was lowered to 0.20 without notably lowering the yield of myosin.

Carp actomyosin was easily centrifuged off at 25,000 × $g$ for 15 min. In the mullet preparation some slowly sedimenting material occurred even at $I$ 0.20 and a centrifugation of 1 h at 80,000 × $g$ in a preparative ultracentrifuge (Spinco Model L)

was necessary. The clear supernatant was diluted to $I$ 0.05 in order to precipitate the myosin. A fraction (designated fraction I) was isolated by centrifuging at 25,000 $\times$ $g$ for 10 min (carp extract) or 30 min (mullet extract). In the case of mullet extracts a second fraction (designated fraction II) was also investigated. It was precipitated by acidification of the supernatant of fraction I with dilute acetic acid to pH 6.4 (see also RESULTS). Fractions I and II were separately washed twice with 0.03 $M$ KCl, dissolved and dialysed against the phosphate buffer of $I$ 0.35 and pH 7.2.

## RESULTS

### *Myosin prepared from carp red muscle*

The extractions were made at $I$ 1.0, both in the presence and absence of ATP. Fraction $I$ corresponded to approx. 0.5 % of the fresh muscle weight. Its relative viscosity did not differ from that of rabbit myosin and was unchanged on addition of ATP, suggesting that it was pure myosin. However, electrophoresis and ultracentrifugation of preparations from extracts made in the absence of ATP, revealed some heterogeneity. A small amount of slow moving material could be observed on electrophoresis. Two main peaks similar to those observed in extracts at pH 5.5[4,5] and a small amount of slowly sedimenting material were observed in the ultracentrifuge. The slow electrophoretic peak was only present in very small amount in preparations from extracts made in the presence of ATP (see Fig. 1a). In the ultracentrifuge the slow sedimenting component was not shown in the latter preparations but two fast moving peaks were present in addition to the main myosin peak (Fig. 1b). The variation in the amount of these fast components from one preparation to another suggested that they correspond to various myosin aggregates.

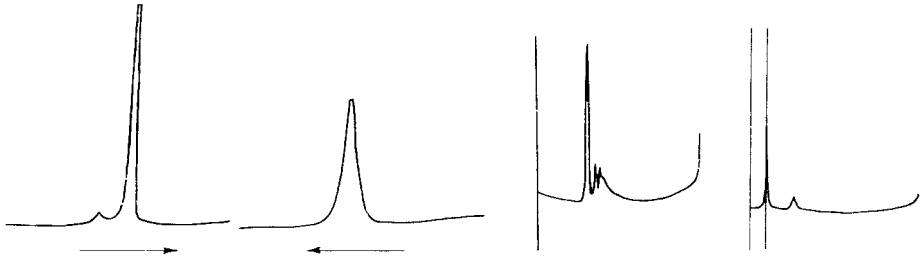


Fig. 1. a. Electrophoresis patterns of 0.5 % carp myosin in $I$ 0.35 phosphate buffer pH 7.1, after 1054 min. Voltage gradient 11.84 V/cm. Ascending limb on left. b. Ultracentrifuge pattern of 0.5 % carp myosin in $I$ 0.35 phosphate buffer pH 7.1, after 59 min at 59,780 rev./min. Phase plate angle 45°.

Fig. 2. Ultracentrifuge pattern of 0.87 % mullet myosin in pH 7.1 $I$ 0.35 buffer, after 24 min at 59, 780 rev./min, angle 75°, showing actomyosin (fast) peak.

### *Myosin prepared from white and red mullet muscle*

In extracts made without addition of ATP the amount of actomyosin appeared fairly high; an appreciable precipitate forming on dialysis at $I$ 0.25 and pH 7.2. The myosin obtained by dilution of the supernatant contained some actomyosin, having an ATP sensitivity of about 20 %. This contamination with actomyosin was also observed in the ultracentrifuge. As can be seen from Fig. 2, a small actomyosin peak

has separated quickly from the much slower and very sharp myosin peak. On the other hand the myosin aggregates shown in Fig. 1b were not observed in these preparations.

Addition of ATP to the extraction fluid increased the proportion of myosin in the extracts. The actomyosin extracted could be more easily removed by dialysis at $I$ 0.20 and pH 7.2. Dilution of the supernatant from $I$ 0.20 and pH 7.2 to $I$ 0.05 produced a notable increase in turbidity. By centrifugation at 25,000 $\times g$ for 30 min, the turbidity of white muscle extracts was greatly reduced but not completely removed, while water clear solutions were obtained from red muscle. The amount of myosin (fraction I) isolated under these conditions was approx. 0.3 and 0.1 % of the fresh muscle weight for white and red muscle respectively.

It was somewhat surprising that the major part of the myosin extracted did not sediment under these conditions. However, on lowering the pH of the supernatant below 6.7 further precipitation of myosin occurred. The final pH for the precipitation was varied over the range 6.1 to 6.7 and it was found that pH 6.3 to 6.4 was most suitable. Although this fraction (fraction II) was isolated at a lower pH than fraction I, it redissolved more easily in the presence of salt and gave a clearer solution (possibly due to the presence of more lipid in fraction I). Furthermore, higher yields were obtained, namely approx. 1.1 and 0.2 % for white and red muscle respectively.

Both fractions I and II from white and red muscle were examined viscometrically. The ATP sensitivities of these preparations were zero and their viscosities were similar to that of rabbit myosin.

Some ten preparations of fraction I, and fraction II were examined in the ultracentrifuge. All of these were studied in $I$ 0.35 phosphate buffer of pH 7.1 at a concentration of approx. 0.9 % and some of them at several concentrations over the range 0.1–1.0 %. Phase plate angles of 40, 45 and 50° were used in order to detect more easily small amounts of any aggregated material which might be present.

In the more concentrated solution (approx. 0.9 %) all of these preparations showed a major sharp peak sedimenting at a rate characteristic of myosin. Most of them also showed a second small peak (2–3 % of the total protein) of more highly aggregated material sedimenting at a faster rate. Three of the preparations showed a small amount of a third peak of aggregated material (approx. 1 % of the total protein). The range of S values of these aggregates at a total protein concentration of 0.9 % was 4–6 S. Typical sedimentation patterns are shown in Fig. 3.

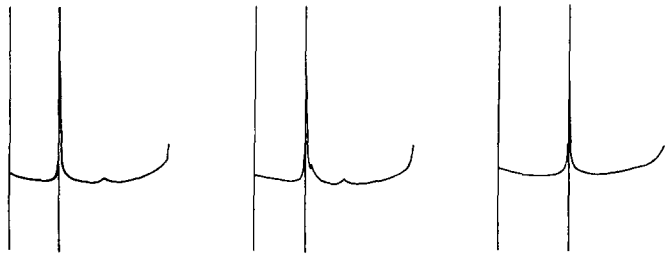


Fig. 3. Ultracentrifuge patterns of 0.9 % mullet myosin in pH 7.1, $I$ 0.35 buffer. Angle 50°, speed 59,780 rev./min. a. Fraction I (preparation $K_w$), after 78 min. b. Fraction I (preparation $L_w$), after 70 min. c. Fraction II, after 110 min.

In some of the preparations at 0.5 % concentration no aggregated material was detectable, but in others it was observed. Below 0.5 % concentration no aggregated material was detectable. ATP sensitivities of all these preparations were zero.

Values of sedimentation coefficient for the myosin peak at various protein concentrations for a number of preparations are shown in Fig. 4. Fractions I and II of white and red muscle preparations were similar from the point of view of rate of sedimentation and purity. All values of $S_{20}$ for 0.6 % and below were used to calculate the regression line:

$$S = S_0 - kC$$

where

$$S_0 = 6.03 \pm 0.12 \text{ and } k = 2.99 \pm 0.27$$

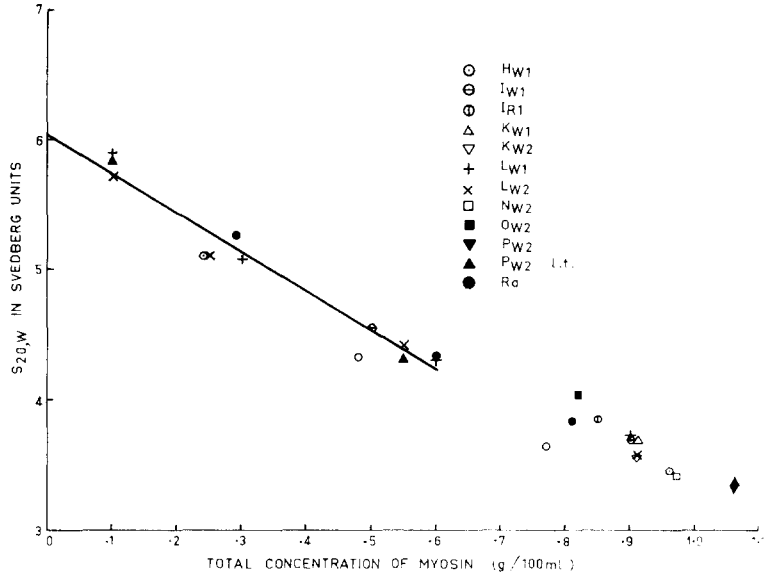


Fig. 4. Sedimentation coefficient data for various preparations of mullet myosin at different concentrations. The letters H, I etc. are laboratory identifications for different lots of fish from which the preparations were made. The subscripts R and W, 1 and 2 indicate red and white muscle preparations and fractions I and II respectively. The subscript l.t. indicates the run was done at low temperature. The three points $R_a$ are for a preparation of rabbit myosin made in this laboratory for comparison. The regression line is drawn for values of 0.6 % and below.

The $S_0$ value of 6.03 ± 0.12 S may be compared with values of 6.43 ± 0.08 S obtained recently by CONNELL[10].

Electrophoresis measurements were made in $I$ 0.35 phosphate buffer of pH 7.1 and the following typical behaviour was observed. Both ascending and descending boundaries showed a single peak. After approx. 15 h a very small amount of aggregated material showed up on the ascending side as a very small second peak moving more slowly than the main peak (Fig. 5a). Sometimes this peak was just perceptible on the descending side. After approx. 24 h this showed as two very small peaks on the ascending side (Fig. 5b). It will be noted that the descending pattern is slightly asymmetrical and that the ascending peak shows an hypersharp front with an asymmetric tail. This type of pattern is observed with myosin and actomyosin[3] and is similar to

that observed for associating dissociating systems (for the caseins by McKENZIE AND WAKE[20], for the lactoglobulins by OGSTON AND TILLEY[21], OGSTON AND TOMBS[22] and McKENZIE AND SMITH[23]). In TSAO borate buffer of pH 10.5 (0.104 *M* $H_3BO_3$, 0.1 *M* NaOH) where there should be a much reduced tendency to aggregation, the myosin pattern is much more symmetric (Fig. 5c). A single peak of normal shape was found both for the ascending and descending boundaries. This pattern is more normal than that observed by TSAO[24] for rabbit myosin at the intermediate pH of 9.0. The electrophoretic behaviour observed at pH 7.1 is not due to true heterogeneity of the preparation.

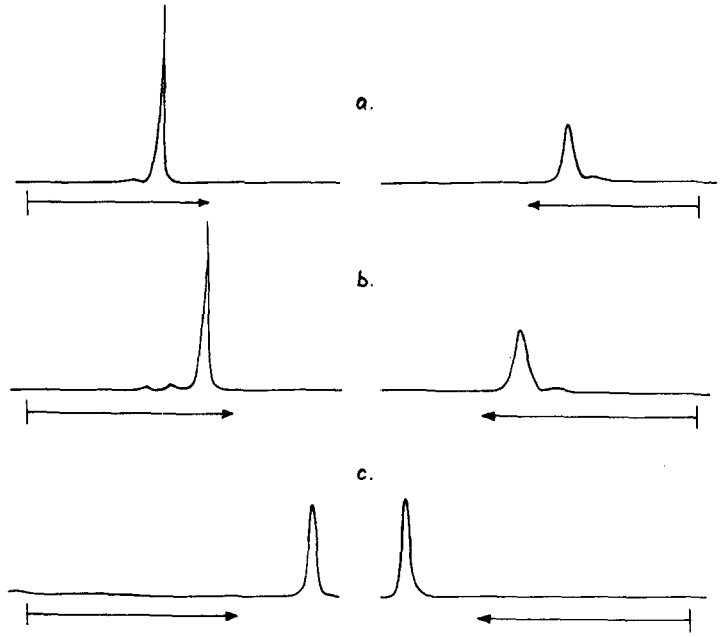


Fig. 5. Electrophoretic patterns of 0.6 % mullet myosin (Fraction II). a. In *I* 0.35 phosphate buffer pH 7.1, voltage gradient 1.6 V/cm, after 1025 min, angle 50°. b. The same, after 1404 min, angle 40°. c. In TSAO borate buffer pH 10.5, voltage gradient 2.3 V/cm, after 945 min, angle 50°.

## DISCUSSION

It has generally been considered that the myosin of cold blooded vertebrates does not differ from that of warm blooded vertebrates[3–6]. This opinion has been based mainly on the behaviour of the myosin peak observed during the ultracentrifugation of carp[9] or frog actomyosin[6] in the presence of ATP. The preparations obtained so far from frog[6], carp[4,5], or cod[7] differ from rabbit myosin. Even with cod myosin prepared by his improved methods CONNELL[10], found that it rapidly underwent side by side aggregation and he was unable to prevent this change.

Somewhat different results have been obtained in the present study of fish myosin. The tendency towards aggregation appears to vary from one species to another. Carp myosin behaves fairly similarly to cod myosin but mullet myosin appears about as stable as rabbit myosin. It can be kept for several days in the cold or be precipitated at low ionic strength without change in the ultracentrifugal pattern.

On the other hand a very partial precipitation of fish myosin at $I$ 0.05 and pH 7.2 is observed suggesting an apparently greater solubility of this myosin. However, the two fractions isolated do not differ appreciably in viscosimetric, ultracentrifugal and electrophoretic behaviour from rabbit myosin. Preliminary results by one of us (G. H.), on fraction II indicate that prolonged centrifugation at 100,000 $\times g$ will bring down most of this "soluble" fraction at pH 7.1. In any case, fish myosin appears to form at low ionic strength and neutral pH an interesting colloidal system worthy of further investigation. The difference in electrophoretic patterns at pH 7.1 and 10.5 is also of interest. It seems unlikely that this is due to irreversible changes. Electrotitration[26,27] has shown that rabbit and cat myosin are stable in this pH range. A much more likely explanation is to be found in the effect of pH on the tendency to association. Myosin may not exist in the monomeric form at moderate concentration and neutral pH. Whether one or more peaks will be observed for an associating dissociating system on electrophoresis or ultracentrifugation will depend on a number of factors, some of which have been discussed by GILBERT[25]. (CONNELL[10] assumes that his monodisperse preparation of cod myosin examined in the ultracentrifuge with the limited concentration range possible (below 0.4 %) is monomeric. This may not be so.) The effect of pH, temperature and amides on myosin is being further studied in our laboratory.

The striking differences of extractibility observed in fish white and red muscles should also be noted. In the case of white muscle, the swelling of the muscle brei is much more pronounced, the amount of myosin extracted higher; the easier disruption of the myofibrils by salts favours also the extraction of actomyosin. Mullet red muscle does not swell notably even after a finer comminutation, and dissolves only slowly and partially under the conditions used. Its behaviour is reminiscent of rabbit muscle. It was well known that the sarcoplasms of white and red fish muscle have a very different protein composition[4,5]. The present results suggest that the submicroscopic organization of the myofibrils is also very different.

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# LIGHT SCATTERING IN SOLUTIONS OF NATIVE AND GUANIDINATED RABBIT TROPOMYOSIN

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## SUMMARY

1. The fundamental molecular weight of rabbit tropomyosin has been established by light scattering as 54,050 ± 1000 at pH 2 and 52,600 ± 1000 at pH 12.

2. Light scattering measurements have been carried out in the same solvent media as the earlier osmotic pressure work. The aggregation of tropomyosin particles as the ionic strength is diminished is clearly brought out by this technique, the molecular weight increasing from 52,900 at I = 1.1 to 101,000 at I = 0.1. These values are about 20 % lower than the figures deduced from osmotic pressure and are probably more reliable in view of the lower protein concentrations used.

3. Light scattering measurements have been extended to the ionic strength range below 0.1 where aggregation is most pronounced. The data suggest that up to the hexamer stage the polymerization process is end-to-end.

4. The preparation and characterization of a guanidinated derivative of rabbit tropomyosin by reaction with S-methylisothiourea are described.

## INTRODUCTION

Previous investigations have shown that the particle weight of rabbit tropomyosin, owing to its reversible polymerization and depolymerization, is a sensitive function

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