Comparison of Solubility Properties of α-Paramyosin, β-Paramyosin, and Acid-Extracted Paramyosin†

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ABSTRACT: The solubility properties of paramyosin in the zones of pH and ionic strength in which aggregation occurs were initially studied using preparations isolated by a method originally described by Bailey (Bailey, K. (1956), *Pubbl. Stn. Zool. Napoli 29,* 26). Other preparations yielding apparently different protein components have been described by Hodge (Hodge, A. J. (1952), *Proc. Natl. Acad. Sci. U.S.A. 38,* 850) using acid conditions, and Stafford and Yphantis (Stafford, W. F., and Yphantis, D. (1972), *Biochem. Biophys. Res. Commun. 49,* 848) have identified α-, β-, and γ-paramyosin using various times and temperatures of extraction with or without ethylenediaminetetraacetic acid. We have found that acid-extracted paramyosin is very similar if not identical to α-paramyosin, but that both acid and α forms differ considerably from β- and γ-paramyosin. β-Paramyosin precipitates abruptly from solution in a narrow zone of pH below neutrality, and increases in ionic strength shift the zone of precipitation toward lower pH values. In contrast, both acid and α-paramyosin show gradual aggregation with changing pH at lower ionic strength (<0.3) but sharp transitions similar to β-paramyosin at higher ionic strength (>0.3). Transitions were also found at lower pH (ca. 4.0) which were not mirror images of transitions at higher pH (ca. 7.0). Viscosity measurements show that acid extracted paramyosin is close in behavior to a native extract obtained by extraction in mild, nondenaturing media containing mixed antibiotics. Each of these extracts differed considerably from β-paramyosin. Mild, nonhydrolytic procedures employed by others to remove small, noncovalent bonded components or to separate protein complexes were not effective in converting α- to β-paramyosin. Comparison of extraction procedures strongly supports the suggestion of Stafford and Yphantis that β- and γ-paramyosin are hydrolytic products of α-paramyosin and that the proteases responsible may be of bacterial origin.

The solubility of paramyosin as a function of pH and ionic strength was studied by Johnson et al. (1959) some years ago using the optical density to measure protein remaining in solution after centrifugation at a *g* value sufficient to remove large aggregates. The concentration of protein in solution dropped sharply with pH in the region slightly below neutrality, and the precise point at which this occurred depended on the ionic strength. Higher ionic strengths shifted the transition zone to lower pH values, without changing the shape of the curves.

The protein used in the work of Johnson et al. (1959) was prepared from molluscan adductor muscles by the method of Bailey (1956) which involved denaturation of proteins other than paramyosin by addition of ethanol to a concentration of 70 vol %. We have examined the solubility of paramyosin prepared by the method of Hodge (1952), in which acetic acid and citrate buffered solutions are used to denature proteins other than paramyosin. The pH and ionic strength dependence of Hodge's preparation (acid paramyosin) was similar to that of the Bailey protein only at higher ionic strength (>0.4), and the transition zone along the pH scale was no longer sharp, especially at lower ionic strength (<0.3).

Different molecules are apparently extracted by each of these methods. This difference was clarified when Stafford and Yphantis (1972) published data showing that short extraction (<1 h) using the Bailey method but adding 10 mM EDTA to the extraction medium yielded a paramyosin preparation which migrates more slowly on sodium dodecyl sulfate–polyacrylamide gels. The difference in mobilities between this preparation and the original Bailey preparation indicated that the former, called α-paramyosin by Stafford and Yphantis, has an apparent molecular weight about 5% higher than the Bailey paramyosin (β-paramyosin, see below). They described a third component (γ-paramyosin) which migrated on gels ahead of the β component and appeared to be a proteolytic breakdown product of β-paramyosin.

We have examined the solubility of α-paramyosin and have compared it with the behavior of the Hodge (acid) preparation. Our results indicate that the acid-extracted protein is similar, if not identical, to α-paramyosin. Both differ from the original Bailey and Johnson et al. preparations which from our results may have contained a mixture of α-, β-, and γ-paramyosin, the exact content of the mixture depending on the duration of the extraction (see Stafford and Yphantis, 1972).

Experimental Procedures

Protein Extractions. The acid extraction of paramyosin was performed by a modification of the method of Hodge (1952). Blended adductor muscles of *Mercenaria mercenaria* were extracted twice in 0.16 M potassium citrate solutions (pH 3.4) for 2 min and allowed to stand in 0.16 M potassium citrate and 0.1 M KCl overnight. The preparation was then centrifuged and the pellet blended in 0.4% acetic acid. This mixture was allowed to stand overnight, thus bringing the paramyosin back into solution and leaving behind other, denatured, contractile proteins. The suspension was centrifuged and the pellet discarded. Paramyosin was purified by dialysis against 0.1 M

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potassium phosphate (KP_i)[1] at pH 6.0, centrifuged, and resuspended in 0.1 M KP_i and 0.6 M KCl at pH 7.5. The dialysis, centrifugation, and resuspension steps were repeated two more times.

The protein thus obtained showed a ratio of optical density at 280 to 265 nm of 1.6 or higher, indicating little contamination. The corrected value of $E_{1cm}^{1\%}$ at 277 nm was found to be 3.20. This value was used subsequently to determine paramyosin concentrations in solubility studies.

Paramyosin (native) was also prepared by a method similar to that of Bailey (1956) and Ruegg (1961) in which differential solubilities were used to separate the proteins, but our procedure was modified to include a mixture of antibiotics. The first steps involve suspension of blendored and washed (0.1 M KCl) white adductor muscles of *Mercenaria mercenaria* in 0.6 M KCl at pH 7.5 for 24 h in the presence of a mixture of penicillin, streptomycin, and Mycelin. The protein complex actomyosin is then differentially removed by (a) slowly lowering the ionic strength to 0.15 and the pH to 6.3, (b) centrifuging to obtain a precipitate which is suspended in 0.6 M KCl at 7.8, and (c) finally slowly lowering the ionic strength at this pH to 0.25. Paramyosin is left in the supernatant and is purified by recrystallization as described in the preceding paragraph.

α-Paramyosin was prepared by the second method (ethanol precipitation) of Stafford and Yphantis (1972). Purified β-paramyosin was prepared by using this method without EDTA. "Ethanol" paramyosin was prepared by the method of Johnson et al. (1959) modified to include 0.5 mM EDTA in all solutions and overnight extraction in the 0.6 M KCl solution. This method yielded protein which could not be distinguished from the 1959 preparation. In all studies in which acid extracted, α- and β-paramyosins were compared, the purity of the preparations was checked on polyacrylamide gels using the methods of Shapiro et al. (1967) and Stafford and Yphantis (1972).

Measurement of Solubility Behavior. Solubility of paramyosin was measured in the transition zones where paramyosin comes out of solution as the pH or ionic strength is changed. This occurs on the pH scale at values between 2.0 to 3.8 and between 6.0 and 7.5. In the pH range between 3.8 and 6.0, little or no protein remains in solution, and above 7.5 and below 2.0, the protein is in all likelihood not totally in monomeric form, although paracrystals cannot be separated from such solutions by centrifugation (Delaney and Krause, 1976). We also found that the equilibrium between paracrystals and paramyosin in solution was sensitive to pressures generated in higher speed centrifugation (16 000*g* and above); thus removal of precipitates was done at lower speeds.

In attempts to measure the kinetics of paracrystal formation (to be reported elsewhere), we noted a residual optical density at 320 nm which we attributed to turbidity. In the studies described below we subtracted the value of OD at 320 from that at 277 nm to correct the concentration of paramyosin for this residual turbidity.

In comparing the solubility of paramyosin extracted by the acid method (hereafter called acid paramyosin) with that extracted by the method of Johnson et al. (1959), similar methods were employed. At low temperature (1 °C), protein solutions in the concentration range of 1.0 to 2.0 mg/mL and at the highest pH and ionic strength employed were dialyzed for 48 h against solutions of appropriate ionic strength and pH. Equilibrium was thus approached by prolonged dialysis. However, in studies at 25 °C, we employed a faster method which gave results similar if not identical to equilibrium dialysis but which reduced possible bacterial contamination. Paramyosin was brought into solution by exhaustive dialysis against distilled water at 1 °C, and the concentration was brought to 2.0 mg/mL. Aliquots (5 mL) of this solution were quickly mixed with 5-mL amounts of solutions at appropriate ionic strength and pH to give desired final values and shaken intermittently in a water bath at 25 °C for 2 h. All samples were centrifuged at the same temperature at speeds adequate for removal of the precipitate, and the concentration of protein remaining in the supernatant was determined spectrophotometrically, as outlined above.

Where direct comparisons of the solubilities of the various types of paramyosin were made, identical procedures were employed throughout. A solution at the highest ionic strength and pH employed was brought to concentrations of paramyosin of 1–2 mg/mL by prolonged dialysis in the presence of antibiotics or sodium azide with subsequent removal by centrifugation of any precipitate remaining. This solution was then dialyzed against solutions of appropriate ionic strength and pH with several intervening changes of external solutions. The dialysate was then centrifuged at the same temperature and the concentration of protein remaining in solution determined. Phosphate buffers were used throughout. We found that accurate solubility measurements are difficult to obtain in ranges of concentration above 2.0 mg/mL. Such solutions are unstable, and the slightest change in a parameter such as pH or temperature, or even shaking the solution, leads to aggregation. This is perhaps due to the strong tendency of paramyosin to form *n*-mers in concentrated solutions.

Viscosity measurements were made on acid, ethanol, and native paramyosin solutions at low ionic strength using a no. 100 Ostwald-Cannon-Fenske viscometer with 0.63-mm bore in a thermostated water bath at 20 ± 0.02 °C. Flow times were measured to the nearest hundreth of a second with a spring-driven stopwatch. Flow time of the buffer without protein was 70 s. At higher protein concentrations, the flow times often decreased by a second or more in subsequent runs; however, the error due to this effect was very small.

Results

Figure 1 shows the dependence of the solubility of acid paramyosin on pH in the transition ranges from pH 6.0 to 8.0 at 25 °C and at various ionic strengths. The techniques employed were similar enough to those employed by Johnson et al. (1959) to permit direct comparison of the acid preparation with that of the preparation (ethanol) published by them. The most striking difference lies in the sharpness of the transition along the pH scale at ionic strengths below 0.35. "Ethanol" paramyosin passes into solution in a narrow range of pH at all of the ionic strength values studied, whereas acid paramyosin shows a gradual transition, which was more marked as the ionic strength is lowered below 0.35. At ionic strength below 0.15, acid paramyosin becomes virtually insoluble, whereas ethanol paramyosin is still soluble above pH 7.0 at ionic strength as low as 0.05. Figure 3A gives a direct comparison of the pH dependence of solubility of the two preparations at two different ionic strengths.

The pH dependence of solubility of acid paramyosin at 1 °C is seen in Figure 2; comparison of this figure with the profile shown in Figure 1 indicates that lower temperatures decrease solubility, especially at lower ionic strength. Similar temperature dependence was observed for α-paramyosin for which a plot of the ionic dependence of solubility (pH 7.0) at 20 and 4 °C is shown in Figure 3B. It can be seen that the lower tem-

[1] Abbreviations used: P_i, inorganic phosphate; EDTA, ethylenediaminetetraacetic acid.

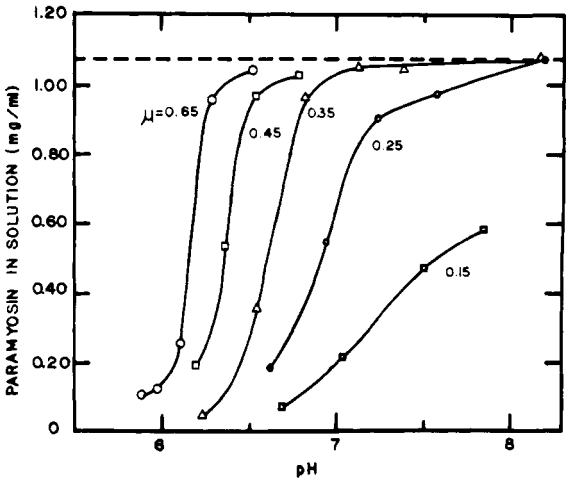

FIGURE 1: Solubility behavior of acid-extracted paramyosin as a function of final solution pH at various ionic strength values shown. Method: quick mixing of protein in low ionic strength medium with solutions necessary to give final pH and ionic strength. Dotted line shows total protein concentration of solution before centrifugation. Points indicate protein remaining in solution after centrifugation. Temperature was 25 °C.

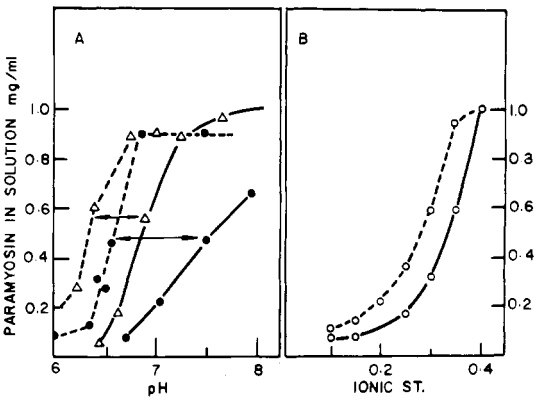

FIGURE 3: (A) Comparison of solubility behavior of acid extracted paramyosin with ethanol paramyosin. Dotted lines show points replotted from data published by Johnson et al. (1959); solid lines show points replotted from Figure 1. For both plots, points indicated as △ are taken at ionic strength of 0.35; (●) data taken at 0.15 ionic strength. Arrows show shift of pH–solubility curves between ethanol and acid paramyosin at each ionic strength. (B) Ionic strength dependence at two temperatures of α-paramyosin prepared by method 2 of Stafford and Yphantis (1972). (O- - -O) at 20 °C; (O—O) 4 °C.

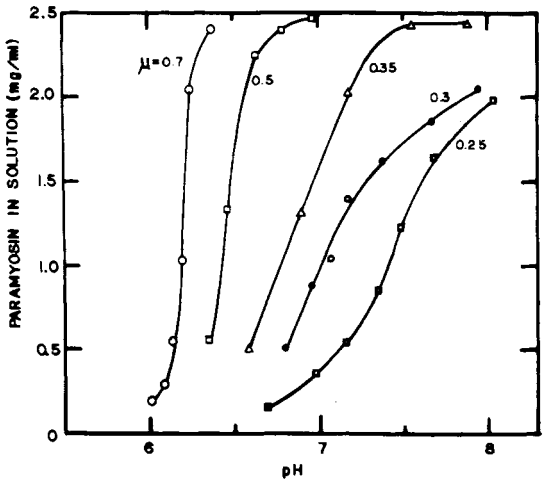

FIGURE 2: Solubility behavior of acid paramyosin at 1 °C as a function of final solution pH at various ionic strength values. Method: equilibrium dialysis with solutions at various ionic strength and pH. Initial protein concentration was 2.5 mg/mL. Points indicate protein remaining in solution after centrifugation.

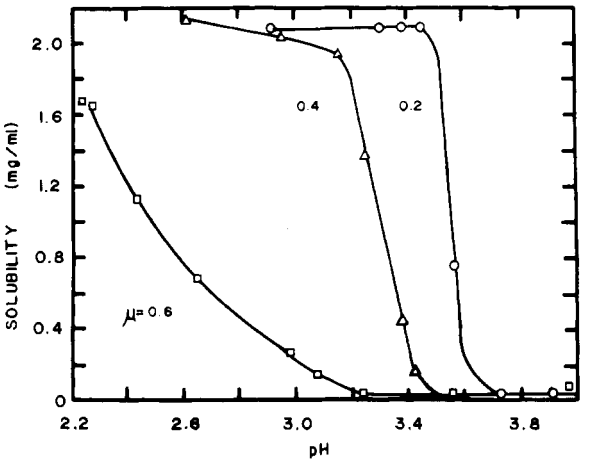

FIGURE 4: Solubility behavior of acid (α) paramyosin below pH 4.0 and at various ionic strength values shown. Temperature was 1 °C. Method: equilibrium dialysis; initial protein concentration 2.09 mg/mL for μ = 0.2 and 0.6 and 2.16 mg/mL for μ = 0.4.

perature brings both acid and α-paramyosin out of solution at a much higher pH or ionic strength.

We have also investigated the solubility transition zone around pH 3.0 for both acid and ethanol paramyosin. As shown in Figure 4, acid paramyosin at 1 °C passes quickly into solution at pH below 4.0 and the transition now becomes broader at *higher* ionic strengths rather than at lower ionic strength, as seen in the higher pH zone (Figure 2). The pH dependence below 4.0 is far from a mirror image of changes near neutrality, and thus the processes involved in paracrystal formation are probably entirely different in the two ranges. Ethanol paramyosin behaves similarly in the lower pH range but again with sharper transitions on the pH scale at all ionic strengths. Hodge (1952) employed the effects of ionic strength at lower pH in his studies of preparatory methods.

In view of the great differences between acid and ethanol paramyosin, it seemed profitable to examine differences between these proteins and α-paramyosin prepared by the second method of Stafford and Yphantis (1972). It was also obvious from examination of curves like those in Figures 1 and 2 that the major transitions occurred in the range of ionic strength between 0.15 and 0.35. Since this range probably includes the physiological range of internal ionic strength of muscle cells, it seemed reasonable to use the ionic strength as the independent variable instead of pH. The significance of this is seen in the plot of the solubility of α-paramyosin in range of ionic strength between 0.1 and 0.4 at three different pH values, as shown in Figure 5B. Note the relative sharpness of the transition at pH 7.0 in the range of ionic strength between 0.2 and 0.3. Note also that small changes in pH in the range of 6.5 to 7.0 produce large changes in solubility at a given ionic strength.

In Figure 5A, a comparison is made between the solubility behavior at pH 7 of α-, β-, acid, and ethanol paramyosin. Note that the difference between α- and acid paramyosin is not significant, whereas β-paramyosin is soluble over the range of ionic strength in which α- and acid paramyosin solubility is rapidly changing. Ethanol paramyosin is soluble over virtually the entire range. Acid paramyosin also migrates on polyacrylamide gels at mobilities similar to α-paramyosin, but, as

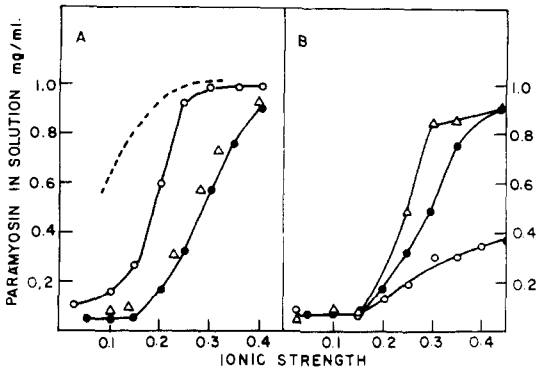

FIGURE 5: (A) Comparison of solubility behavior as a function of ionic strength of α-paramyosin (Stafford and Yphantis, 1972, method 2) (●—●); acid paramyosin (△—△); β-paramyosin (Stafford and Yphantis, method 2, but with no EDTA present) (○—○); ethanol paramyosin (mixture of β- and γ-paramyosin obtained by method of Johnson et al. (1959)) (- - - -); method for all measurements, equilibrium dialysis. Temperature was 20 °C. (B) Ionic strength dependence of α paramyosin at pH 7.5 (△), 7.0 (●), and 6.5 (○). Temperature was 20 °C.

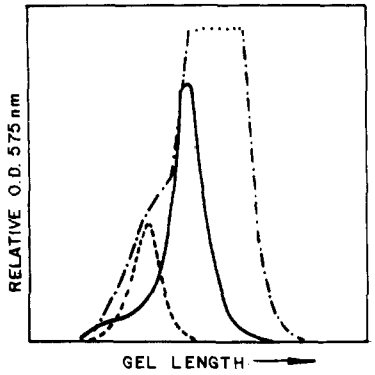

FIGURE 6: Optical density scan at OD_{575nm} of Coomassie blue stained polyacrylamide gels showing relative positions of α (- - -), β (—), and ethanol (·····) paramyosin peaks. Standard protein markers used. Gels were loaded proportionally to relative yield of each proteins, which were, in mg/g of tissue: (α) 5.1; (β) 7.05; and (ethanol) 18.5. Major peak for ethanol preparation was close to the γ peak published by Stafford and Yphantis (1972).

found by Stafford and Yphantis and confirmed in our work, β-paramyosin consistently migrates faster than α. On this basis we would conclude that acid and α-paramyosin are essentially the same, whereas β paramyosin is very different. Figure 6 gives a sodium dodecyl sulfate gel scan of the three preparations plotted so as to show relative yields of protein during extraction. Note also in Figure 6 that prolonged extraction (ethanol paramyosin) leads to the appearance of a faster component which is no doubt identical to the γ form of Stafford and Yphantis even though 0.5 mM EDTA is included in the extraction. In fact, prolonged extraction yields predominantly γ-paramyosin.

We found in earlier unpublished work that ethanol paramyosin was soluble at very low ionic strengths; both acid and α-paramyosin behave similarly. Both are soluble in the range of ionic strengths below 0.01 at room temperature, and, as mentioned in Experimental Procedures, acid paramyosin can be brought into solution by exhaustive dialysis against distilled water. This has permitted both the electric birefringence behavior of the various paramyosins (Delaney and Krause, 1976) and the viscosity of paramyosin solutions at low ionic strength to be studied.

Figure 7 shows a plot of the viscosity as a function of concentration of solutions of paramyosin in 0.01 M KP_i at pH 7.5.

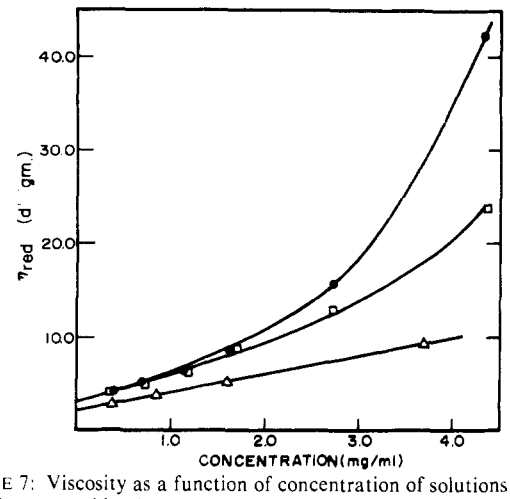

FIGURE 7: Viscosity as a function of concentration of solutions of paramyosin prepared by three methods. (△—△) Ethanol paramyosin (prolonged extraction by method of Johnson et al. (1959)); (□—□) native paramyosin (method of Ruegg (1961) with antibiotics added); (●—●) acid paramyosin (method of Hodge (1952)). Protein dissolved in 0.01 M KPO_4, pH 7.5.

Acid paramyosin shows a higher viscosity at all concentrations studied and also shows a marked upward curvature in concentration dependence. Ethanol paramyosin, on the other hand, shows a linear dependence on concentration. We also found that the viscosity of native paramyosin, prepared by mild, nondenaturing procedures, exhibits a concentration dependence similar to that of the acid preparation, as is seen in Figure 7, suggesting that the two may be identical molecules.

In early investigations on the differences between these forms, the upward curvature in the concentration dependence of acid paramyosin suggested that acid paramyosin may be similar to native tropomyosin and may contain components similar to troponin. In view of this possibility, we employed the methods which were used by Ebashi and Kodama (1965) to remove additional proteins from native tropomyosin. It should be recalled that, on the basis of amino acid composition, paramyosin has in the past been classified as a type of tropomyosin (A, or insoluble tropomyosin) by several authors (see Kominz et al., 1957). We attempted to remove possible bound proteins or smaller molecules by bringing acid paramyosin into solution in the following media: (a) 0.02 M Na_2CO_3 at pH 12.5; (b) 0.1 M citric acid at pH 1.8; (c) 2 M KCl at neutral pH for several hours; or (d) denaturing in 6 M guanidine hydrochloride with subsequent renaturation in phosphate buffer. The viscosities of these solutions when brought by dialysis back to 0.01 M KP_i at pH 7.5 exhibited no difference compared with untreated preparations. Ethanol paramyosin was also exposed to 0.4% acetic acid (conditions for acid extraction) for 3 days and acid paramyosin to 70% ethanol solutions for 18 h, and no change in the viscosity behavior of either preparation resulted. Thus the difference in solubility behavior of acid and ethanol paramyosin does not reside in alterations in the molecule which are sensitive to the extremes of pH or ionic strength, to denaturation in guanidine hydrochloride, or to denaturing agents (ethanol or acid) used during preparation. This lends strength to the suggestion of Stafford and Yphantis (1972) that the difference is instead due to hydrolytic removal of a small piece of the α-paramyosin molecule during extraction.

Discussion

In summary, ethanol extraction of paramyosin from the adductor muscles of *Mercenaria mercenaria*, using the original

method of Bailey (1956) and Johnson et al. (1959), yields a preparation which contains β-paramyosin or, in prolonged extractions, a mixture of β- and γ-paramyosin. If short extraction is employed in the presence of EDTA, predominantly α-paramyosin is obtained with little or no β- or γ-paramyosin (Stafford and Yphantis, 1972). Extraction by the acid method of Hodge (1952) yields a preparation which seems to be identical to the α form of Stafford and Yphantis (1972); solubility measurements, the concentration dependence of viscosity at low ionic strength, and gel electrophoresis behavior suggest that the acid preparation can in fact be designated as α-paramyosin. Furthermore, if paramyosin is extracted by the method of Ruegg (1961), in which denaturing agents are not used but in which mixtures of antibiotic agents are included, a preparation (native) is obtained which is similar to α-paramyosin (Figure 7).

The latter result is at first sight rather puzzling, in that the initial steps in the Ruegg method are similar if not identical to the initial steps in the method of Johnson et al. (1959). However, antibiotics were not employed in earlier ethanol methods. This would suggest that β- and γ-paramyosin may well be degradation products of α-paramyosin similar to the products observed in preparations of fibrinogen by Cohen and Tooney (1974). They showed that contamination by strains of *Pseudomonas aeruginosa* resulted in protease action which considerably modified the solubility and aggregation properties of the original fibrinogen.

There are certain features of the methods we have used to prepare α-paramyosin which suggest that it is obtained under conditions where bacterial growth is retarded or inhibited. These are (a) short term (1 h) extraction near 0 °C in the presence of fairly high (10 mM) concentrations of EDTA, (b) immediate suspension of the muscle brei in a citrate buffer at pH 3.4 and (c) the presence of antibiotics such as streptomycin, penicillin, and mycelin. Bacterial contaminants may well be carried over from the animal into the initial stages of extraction and may produce a protease during growth which specifically attacks a portion of the paramyosin molecule. When growth is inhibited in the presence of EDTA, in acid conditions or in the presence of antibiotics, proteolytic action would be minimal.

Failure of our attempts to convert α- to β-paramyosin, or vice versa, by mild, nonhydrolytic means would lend strength to the original suggestion of Stafford and Yphantis that paramyosin is attacked by at least two enzymes. These may well be bacterial exoproteases. Colwell and her collaborators (Colwell and Liston, 1960) have shown that shellfish, such as the oyster, carry within their body fluid a large population of bacterial forms which tend to be psychrophilic and halophilic, and which promote high proteolytic rather than saccharolytic activity. These may be typical of the marine habitat of the animal and thus may be carried over into the initial stages of extraction procedures in which bacterial flora are not eliminated. Since marine habitats are similar, the precise geographical origin of the animals (European, Asiatic, or North American waters) used in preparation of paramyosin may not influence the final results. For instance, the earliest studies by Bailey were done in the region of the Bay of Naples and yielded preparations of paramyosin apparently identical to those derived by us from North American shellfish.

Preliminary results in our laboratory indicate that a considerable number of bacteria are carried over into the initial stages of preparation of paramyosin. Growth tends to be retarded for the first hour or so of extraction in 0.6 M KCl at 1 °C under conditions where EDTA and/or antibiotics are present, but considerable growth appears to occur after the first hour even under these conditions. EDTA is known to retard bacterial growth; thus, this may explain the findings of Stafford and Yphantis (1972).

The solubility data suggest that conversion of α- to β-paramyosin increases the solubility at neutral pH and ionic strength of 0.2 to 0.4. Further degradation, producing a mixture of β- and γ-paramyosin, increases the solubility further (ethanol preparation shown in Figure 5A). In making comparisons with earlier solubility results of Johnson et al. (1959), it is safe to assume that their preparation consisted of either pure β- or a mixture of mainly β- and γ-paramyosin.

The major differences in solubility behavior are the following. Conversion to the β and/or β and γ forms sharpens the pH dependence and converts the ionic strength dependence to a simple shift of the solubility transition zone along the pH scale. Figure 3A shows a plot of the pH dependence of α paramyosin at 25 °C along with a replot of the curves for ionic strengths 0.15 and 0.25 as published by Johnson et al. (1959). The solubility transition zones of α-paramyosin are nearer neutral pH and the ionic strength now becomes a major factor, so much so that we have found it profitable to plot the solubility results for α-paramyosin on the ionic strength scale at constant pH.

There is considerable evidence linking the mechanical properties of catch muscles, such as the anterior byssus retractor muscle of *Mytilus edulis* (ABRM) to the solubility properties of paramyosin (Ruegg and Weber, 1963; Johnson, 1962). In closely examining the pH dependence of stress strain behavior of the glycerinated ABRM, we noted that sharp transitions in stretch resistance were not the rule; increases in stretch resistance began at pH values considerably higher than the pH at which β-paramyosin precipitates at comparable ionic strength (Hartt and Johnson, unpublished results). The results are much more correlated with the pH and ionic strength dependence of α-paramyosin solubility. Since this is no doubt the form of paramyosin present in the muscle, future studies of the possible role of paramyosin in functional states of the muscle should be based on the properties of the α form.

It is perhaps significant that the zone of the aggregation in α-paramyosin lies very near the pH and ionic strength values which might be found in the living muscle. Caldwell has found that the pH of the interior of the muscle lies slightly on the acid side of neutrality (pH 6.8–7.2, Caldwell, 1958). Although precise determination of the ionic strength within a muscle cell is difficult, if not impossible due to the size of some of the charge carriers and to intracellular ion binding, it probably lies in the range of 0.25 to 0.35. This is precisely the range in which transitions in aggregation are observed in α-paramyosin solutions, whereas β- and γ-paramyosins undergo such transitions at lower pH and ionic strength; the transition zone for β-paramyosin at $\mu = 0.25$ lies near pH 6.0 to 6.2, whereas that for α-paramyosin lies near pH 7.0 (Figure 3A). The conversion of α- to β-paramyosin thus represents an intriguing molecular problem, in that the removal of a small portion of the original molecule is sufficient to shift the zone of aggregation a considerable distance along the pH or ionic strength scale. When this molecular piece is present, molecular aggregation can be triggered by a small shift in pH and ionic strength, and perhaps also in the concentration of metabolites linked to the contractile process, under solute conditions close to those in which the catch contraction occurs in the living muscle.

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Solubility Properties of α-Reduced Paramyosin†

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ABSTRACT: It is now believed that the reduced form of α-paramyosin is that found in the living adductor muscles of molluscs. We have studied the solubility of a preparation of α-paramyosin obtained under reducing conditions. In contrast to the solubility profile of β-paramyosin, the α-preparation showed a rapid, almost linear decrease in solubility over the ionic strength range 0.35–0.25 at neutral pH. Solubility in this range was further decreased by the presence of physiologically small amounts of calcium ion. Lactate ion, which can accumulate during anaerobic glycolysis in molluscan muscles, also decreases the solubility at a level of 50 mM. In addition, the type of paracrystal formed by α-paramyosin differs greatly from those of β-paramyosin and paracrystal formed in the presence of lactate differs from those formed in buffer solutions. Reduced α-paramyosin is more sensitive to the above parameters than the preparations made without reducing agents. Moreover, the pH and ionic strength ranges in which greatest change in solubility behavior occurs are physiologic, as are the calcium and lactate ion levels effective in increasing intermolecular interactions. A model is proposed for α-paramyosin in which the extra 5% presumably removed in β preparations is a "sticky head" which protrudes from one end of the molecule and confers on it an increased tendency for interaction, particularly at physiological ionic strengths. Such molecules would be capable of promoting interactions between thick filaments which contain them, providing a means of accounting for the pH dependent stiffness observed in glycerinated preparations of molluscan catch muscles.

Paramyosin is a fibrous, α-helical protein found in many invertebrates which, in addition to purely structural functions, may also play a role in tonic (catch) contractions of such muscles as anterior byssus retractor muscle (ABRM) of *Mytilus edulis* and the white adductor of *Mercenaria mercenaria* (Johnson et al., 1959; Ruegg, 1961). In the past, this protein has been extracted with a high ionic strength buffer, followed by ethanol denaturation of actomyosin (Bailey, 1956). Another method of extraction is that of Hodge (1952) in which organic acids are used first to extract and then to denature proteins other than paramyosin. These methods yield preparations with different solubility, viscosity, and electrophoretic behavior. Until recently the reason for these differences was not known.

Stafford and Yphantis (1972) showed that paramyosin, extracted by the ethanol method, appears to be attacked by at least two proteolytic enzymes, active at high ionic strength and neutral pH. When EDTA[1] is added to the extracting solution in short (<1 hr) extractions, a new molecular species is isolated, with an apparent subunit molecular weight 5000 greater than that of protein extracted by the earlier ethanol procedure. We have shown in the preceding paper (Edwards et al., 1977) that this new protein, given the name α-paramyosin by Stafford and Yphantis (1972), has solubility characteristics very similar to the acid preparation of Hodge and also has the same apparent molecular weight.

It has been further shown that the four sulfhydryl groups of paramyosin exist in the reduced state in vivo (Stafford, 1973; Cowgill, 1974) and, for at least the adductor muscle of *Mercenaria mercenaria,* the reduced form of α-paramyosin seems to be the functional molecular species.

A relationship has been found between the solubility behavior of paramyosin and the mechanical behavior of catch muscles. The elastic modulus of the anterior byssus retractor muscle (ABRM) of *Mytilus edulis* is reduced drastically in pH ranges where α-paramyosin is soluble (Ruegg and Weber, 1963); however, the zone of pH in which the elastic modulus changes was not found to be nearly as sharp as the solubility changes of β-paramyosin (Johnson, 1962), and, in subsequent work, we found that there was considerable hysteresis in the pH dependence of the elastic modulus of the ABRM. In the search for better agreement between mechanical and molecular properties, solubility experiments previously done on acid, α-, and β-paramyosin were done on α-paramyosin prepared under

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[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; ABRM, anterior byssus retractor muscle.