# Substructure of Paramyosin. Correlation of Helix Stability, Trypsin Digestion Kinetics, and Amino Acid Composition†

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ABSTRACT: The substructure of the clam muscle protein paramyosin was examined. Analysis of the kinetics of tryptic proteolysis suggests that two reaction classes are present in paramyosin. Measurements of viscosity and mass during the digestion reaction show that the fast reaction class is likely a clustered set of susceptible peptide bonds. A light meromyosin particle, "light paramyosin," was isolated from the proteolytic reaction products at the end of the fast reaction. Paramyosin was found to have a multiphasic "melt" curve ($[m']_{233\,nm}$ *vs.* temperature), including two cooperative transitions with $T_m$ values of 44 and 64°. The structure melting at 44° (amounting to about one-third of the paramyosin mass) was shown to be the region preferentially cleaved to low molecular weight peptides during the preparation of light paramyosin. These data identify the trypsin-sensitive region as the helical region of low thermal stability. Helix stabilizing parameters were estimated according to several current theories from the amino acid composition of the two regions and shown to give a good correlation with the observed thermal stabilities. The possible significance of the segment of low thermal stability in the catch mechanism of molluscan muscle is considered.

Paramyosin is the fibrous protein found in large amounts in the adductor muscles of bivalve molluscs. These muscles exhibit the catch state, a steady-state phase of contraction in which tension is maintained for long periods of time following stimulation. The characteristic features of the catch state and the various theories that have been proposed to explain the phenomenon have been discussed by Szent-Gyorgyi *et al.* (1971). As a result of their recent electron microscope investigations on the structure of the thick filaments in these muscles, Szent-Gyorgyi *et al.* (1971) have concluded that these structures are assembled with a core of paramyosin molecules. Myosin molecules are believed to be oriented in bipolar array along the surface of the filament. They postulate that a phase change in the paramyosin core alters the myosin–actin interaction leading to the catch state. This proposal, like the earlier one of Johnson *et al.* (1959), suggests that changes in the packing arrangement of the paramyosin molecules within the filament core may be fundamental to the catch state.

A number of investigations support the idea that regions of differing stability occur along the length of the paramyosin rod. Noelken and Holtzer (1964) and Riddiford (1966) showed that the two-chain, $\alpha$-helical, conformational pattern of paramyosin melts not as a single cooperative unit, but rather in discrete steps upon increasing the concentration of the denaturant, guanidine-HCl. Kay and Smillie (1964) and Bailey and Milstein (1964) found that trypsin senses two well-defined classes of peptide bonds in paramyosin. It seemed worthwhile to examine the substructure of paramyosin in more detail with the hope of relating some structural feature of the molecule with the specialized contractile activity of these muscles.

In the work to be described below we compare the substructure of paramyosin as it is reflected in the kinetics of tryptic proteolysis and in thermal denaturation experiments. Our findings indicate that the two classes of peptide bonds detected in the proteolysis kinetics can be correlated with two distinct structural regions of the molecule. Each of these regions melts cooperatively with midpoints of the transition, $T_m$, about 20° apart. The amino acid composition of the peptides released from the segment of low thermal stability indicates that this region contains a higher percentage of $\alpha$-helix destabilizing residues. The thermal stability ($T_m$ = 44°) and size of this segment (~570 residues) and its sensitivity to proteolytic digestion are strongly reminiscent of the properties of the trypsin-sensitive hinge region of myosin (Burke *et al.*, 1973). It seems possible that this segment of the paramyosin molecule could be the site of a physiologically important structural transition relevant to the development of tension in the catch state.

## Materials

Trypsin (recrystallized twice) and soybean trypsin inhibitor (recrystallized three times) were obtained from Worthington. The trypsin was treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone according to the procedure of Schoellmann and Shaw (1963) to remove chymotryptic activity. Trypsin was stored in 0.001 M HCl in the freezer. Water was distilled in an all-glass still. Medium and large clams (*Mercenaria mercenaria*) were obtained live from a local distributor.

## Methods

*Paramyosin Preparation.* Paramyosin was prepared from the clam, *Mercenaria mercenaria*, by the method described in detail by Riddiford and Scheraga (1962). Paramyosin prepared by this method exhibits one major band on electrophoresis in sodium dodecyl sulfate gels (100 $\mu$g of protein/gel) and sediments as a single peak in velocity centrifugation studies. The electrophoresis procedures were those of Weber and Osborn (1969) except that the gels were composed of 2% acrylamide and 0.5% agarose. All of our paramyosin preparations showed

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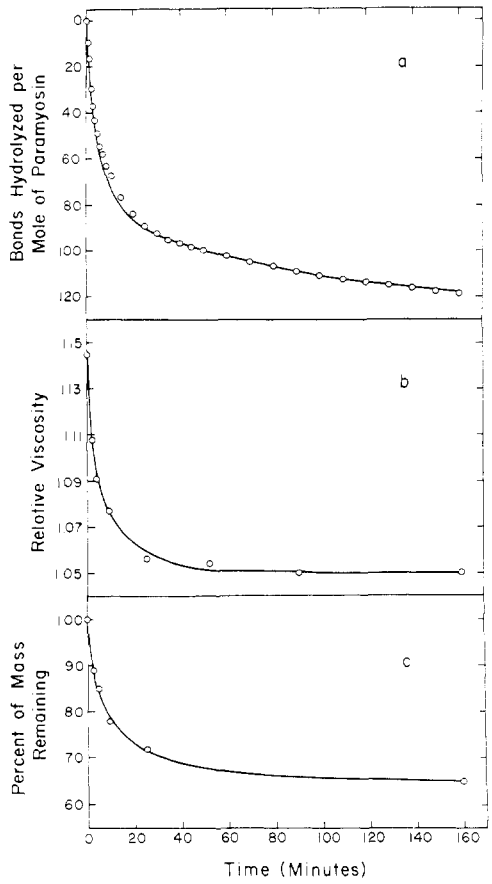


FIGURE 1: Kinetics of trypsin digestion at 25°. (a) Plot of bonds hydrolyzed *vs.* time of digestion. The smooth curve shown represents the equation: bonds hydrolyzed = $128 - 81e^{-k_1 t} - 47e^{-k_2 t}$, where $k_1 = 0.175$ min$^{-1}$ and $k_2 = 0.0103$ min$^{-1}$. In this equation 81 and 47 are the total fast bonds and total slow bonds, respectively. The circled points are the data from the pH-Stat experiment. (b) Plot of relative viscosity *vs.* time. Viscosity measurements were made as described on diluted aliquots from a trypsin digestion experiment. (c) Plot of percentage of mass remaining *vs.* time of digestion. Mass was obtained from the area of schlieren photographs.

a 260/280 nm absorbance ratio less than 0.45 and a 320/277 nm ratio less than 0.03. In some of the experiments reported we employed solutions of paramyosin prepared from lyophilized material which had been stored in a desiccator at 4°. The lyophilized paramyosin was dissolved in distilled water at ice bath temperatures and dialyzed against 0.6 M KCl–0.01 M $PO_4^{2-}$–0.01 M EDTA, pH 7. The resulting solution was centrifuged at 45,000*g* for 90 min to remove aggregate.

*Protein Concentration Determination.* The paramyosin concentration was determined by extinction using $\epsilon^{1\%}_{277\text{ nm}}$ of 3.05 as reported by Riddiford and Scheraga (1962). The concentration of paramyosin in 0.6 M KCl–0.01 M $PO_4$–0.01 M EDTA, pH 7, and in 0.2 M KCl, pH 2, was also determined using a synthetic boundary cell and Rayleigh interference optics (assuming 40 fringes are equivalent to a 1% solution). The concentration determined by extinction agreed with the refractive index value (±3%). The concentration of light paramyosin was estimated from a synthetic boundary experiment, again assuming a 1% solution equivalent to 40 fringes. For all experiments stock solutions were diluted gravimetrically.

*Tryptic Digestion Procedures.* The pH-Stat kinetic studies (25°) were carried out essentially as described by Mihalyi and Harrington (1959). A paramyosin to trypsin weight ratio of 120 was employed except as specifically noted. Under these conditions the initial velocity was first order with respect to both trypsin and paramyosin concentration. We employed paramyosin concentrations in the range 4–8 mg/ml in volumes of 6–10 ml and the pH of the unbuffered 0.6 M KCl solutions was maintained at 8.6 with 0.1 N NaOH. Data from the pH-Stat experiments were analyzed by the graphical method of Leonis (see Mihalyi and Harrington, 1959). This method can be used to obtain the kinetic constants for a process composed of two parallel first-order reactions if the rate constants are sufficiently different. The log rate *vs.* time plot used in this analysis is very sensitive to noise in the data. Although the raw data points were smoothed with a five-point sliding cubic smoothing formula (Savitzky and Golay, 1964), the kin tic parameters of the slower first-order processes were found to be variable and sensitive to small drift corrections. The faster first-order kinetic parameters were found to be very reproducible and, in spite of the variability in the slow reaction parameters, the data were always closely fit by assuming that two processes of markedly different rate constants were occurring simultaneously. A representative analysis is presented in Figure 1a.

To obtain samples at intermediate times of digestion for physical studies the proteolysis of 12 ml of 3.6 mg/ml paramyosin solution was followed in the pH-Stat and at the indicated times (Figure 1b and 1c) 1.0 ml of sample was withdrawn and added to 0.1 ml of cold soybean trypsin inhibitor (2× the trypsin concentration). The zero-time value was prepared by adding trypsin and soybean trypsin inhibitor, previously mixed, to a paramyosin aliquot. The resulting solutions were diluted with buffer before they were examined in the viscometer.

The light paramyosin fragment was prepared for subsequent physical studies by incubating 107 mg of paramyosin with 0.5 mg of trypsin in the pH-Stat until the recording unit indicated the hydrolysis of 72 peptide bonds per molecule. Soybean trypsin inhibitor (1.0 mg) was added to stop the reaction followed by 3 vol of ethanol and the resulting suspension was stirred overnight at 4°. This mixture was centrifuged and the supernate retained for isolation of released peptides. The precipitate was collected and dissolved in 0.6 M KCl–0.01 M $PO_4^{2-}$–0.1 M EDTA, pH 7, and the solute again precipitated by addition of 3 vol of ethanol. The precipitate was again dissolved in buffer and the resulting solution brought to 40% saturation in ammonium sulfate. The precipitated light paramyosin was collected by centrifugation. This preparation sedimented as a single peak in velocity centrifugation. To demonstrate purity and to show that few peptide bonds were hydrolyzed, an 86-bond light paramyosin (100 μg of protein/gel) was examined with the sodium dodecyl sulfate gel procedure employing 2% acrylamide and 0.5% agarose. A single band with a mobility corresponding to a molecular weight of 70,000 g/mol was observed. No other dye binding material was observed in these experiments.

The alcohol supernate (containing the released peptides) was dried with a rotary evaporator at 30° and the residue dissolved in distilled water. This alcohol supernate was contaminated by some trypsin and soybean trypsin inhibitor (≤4%). Peptides were separated from contaminating paramyosin and other large proteolytic fragments on a Sephadex G-150 column (46 × 2 cm) equilibrated and eluted at room temperature with 0.5 M ammonium bicarbonate, pH 7.6. Two completely resolved peaks were observed in the elution profile, a small peak at the column void volume followed by

a more diffuse larger peak. The fractions in this latter peak, containing the peptides, were pooled and dried by rotary evaporation at 45° to remove the ammonium bicarbonate. This material was dissolved in distilled water and prepared for amino acid analysis. The small amount of residual KCl (~0.015 M) in these preparations did not affect the resolution of the amino acids on the analyzer columns.

Paramyosin (31 mg) was digested with trypsin (0.15 mg) at 25° in the pH-Stat until 59 bonds/molecule had been hydrolyzed. The reaction was then stopped with soybean trypsin inhibitor (0.3 mg). One-half of this mixture was chromatographed on a Sephadex G-150 column (46 × 2 cm) equilibrated and eluted at room temperature with 0.5 M ammonium bicarbonate at 25°. The elution was monitored by recording the optical density profile at 230 nm.

*Ultracentrifugation.* The Spinco Model E analytical ultracentrifuge was used to follow the progress of the trypsin digestion experiment. Samples obtained at various times during proteolysis were sedimented in double sector cells at 48,000 rpm and the area of the single schlieren peak observed was determined by weighing enlarged photographic tracings. Corrections were made for radial dilution.

Synthetic boundary experiments on paramyosin and the light paramyosin were performed by the method described by Chervenka (1969). The fractional fringe was read on a series of interference photographs (from 4 to 10). A plot of fringe displacement *vs.* time (after reaching speed) was made and the zero-time value obtained by extrapolation.

Meniscus depletion sedimentation equilibrium experiments were performed on light paramyosin according to the ultracentrifugation procedures used by Godfrey and Harrington (1970). Loading concentrations of 0.4 mg/ml and 0.8 mg/ml in 0.6 M KCl–0.01 M $PO_4^{2-}$, pH 7, and speeds of 13,000 rpm and 15,000 were used. The data from the solution and solvent runs were analyzed with the computer program of Roark and Yphantis (1969). The partial specific volume of paramyosin ($\bar{V}$ = 0.730 ml/g) determined by Kay (1960) was used in the molecular weight calculations.

*Viscometry.* Viscosity measurements were made on 2.0 ml of solution in Ostwald-type viscometers with shearing stresses of 2.4 dyn/cm² (rate of shear ~150 $sec^{-1}$). No kinetic energy and shear dependence corrections were necessary. Viscosity measurements with paramyosin solutions require special precautions because irreversible aggregation is readily induced by mixing. This problem was minimized by centrifugation of stock solutions at 45,000*g* for 90 min to pellet aggregate. All pipetting was performed with wide bore pipets. Gravimetric dilutions of the stock solutions were mixed by very gentle inversions of the capped vessel; the solution was then allowed to mix completely by diffusion in the cold. The aggregation reaction was also minimized by carrying out measurements at low temperature. Aggregation and fiber formation were found to be reduced in the presence of 0.01 M EDTA or at low pH. The temperature was maintained near 5° with variation less than 0.02°. The viscosity of paramyosin solutions was found to decrease slightly (~5%) over the range from 5 to 25°. The outflow times for the solvents at 5° were about 140 sec in our viscometers.

Viscometry was used to follow the progress of the digestion reaction. Here the samples, obtained as described previously, were diluted one-fifth with 0.6 M KCl–0.01 M $PO_4^{2-}$–0.01 M EDTA, pH 7, for measurement at 5°.

*Polarimetry.* A Cary Model 60 spectropolarimeter with a jacketed 0.1 dm pathlength cell was employed. Optical rotatory dispersion experiments were performed on solutions 1.7–4 mg/ml. Rotations were measured over the range 600–320 nm and the parameter, $b_0$, was obtained from a linear least-squares fit of $[m']_\lambda^T(\lambda^2 - \lambda_0^2)$ *vs.* $1/(\lambda^2 - \lambda_0^2)$ (Moffitt, 1956). In all reported calculations a $\lambda_0$ value of 212 nm and a mean residue weight of 115 were used. The refractive index of the solvent was calculated at each wavelength and temperature (see Fasman, 1962).

Measurements of the Cotton trough rotations (300–220 nm) were made on dilute protein solutions (~0.08 mg/ml) in 0.6 M KCl–0.01 M $PO_4^{2-}$, pH 7. The rotation melt experiments were all carried out at 233 nm. The protein solution at 5° was placed into a jacketed cell and the temperature was increased in a stepwise manner. When the temperature was shifted, the $[m']_{233\text{ nm}}$ rapidly approached a new plateau; after 15–20 min only very slight changes in rotation were detectable. The $[m']_{233\text{ nm}}$ reached after incubation for 20 min at the indicated temperature was the value used in our calculations. The steps in temperature were not equally spaced and, in the transition regions, they were very close together. These melt studies therefore are not true equilibrium melts.

All rotations were corrected by subtracting the solvent rotation at the appropriate temperature and wavelength. The temperature reported was the average of the temperature entering and leaving the jacketed polarimeter cell.

*Calculation of Fraction Helix vs. Temperature Curves.* The thermal coefficient of a 100% $\alpha$-helical coiled coil was determined from an expanded plot of $[m']_{233\text{ nm}}$ *vs.* temperature in the 7–40° range for light paramyosin. The value obtained, 61°/°C, was used to calculate the rotation of a 100% helix at each temperature. The $[m']_{233\text{ nm}}$ value of the completely unfolded paramyosin and light paramyosin was taken to be −2100° at all temperatures. Riddiford (1966) found that paramyosin in 7 M guanidine-HCl exhibited a value for $[m']_{233\text{ nm}}$ of −2090° at 25° and 50°. Both the light paramyosin and paramyosin were assumed to be 100% helical at 5°. Thus the fraction helix, $f_H$, was calculated.

$$f_H = \frac{[m']_{233\text{ nm}}^{T,\text{obsd}} + 2100^\circ}{[m']_{233\text{ nm}}^{T,100\%\text{ helix}} + 2100^\circ}$$

*Amino Acid Analysis.* Paramyosin and light paramyosin were dialyzed against distilled water in preparation for analysis. The released peptides were prepared as described above. The paramyosin composition reported in the Results section is an average of four different analyses (two different paramyosin preparations hydrolyzed on different occasions and analyzed in duplicate). The light paramyosin and released peptide compositions were obtained by averaging duplicate analyses. Other light paramyosin and released peptide fractions were prepared and analyzed and found to be similar to the data reported here for the 72-bond preparation.

Samples in acid-washed ignition tubes were evaporated to dryness with a rotary evaporator. To these tubes, 10 $\mu$l of 0.1 M phenol and 0.5 ml of 6 N HCl were added and the tubes were constricted with an $O_2$ flame. The tubes were evacuated to below 20 $\mu$ and then sealed. Hydrolysis was performed in a boiling toluene bath (110°) for 24 hr. The hydrolysates were then analyzed for amino acid composition by the method of Spackman *et al.* (1958) on a Beckmann 120B amino acid analyzer. Since we are comparing 24-hr samples with a generally similar overall composition no corrections were made for the destruction of serine and threonine.

*Calculation of Helix Potential.* The helix-forming power statistic of Robson and Pain (1971) was used to calculate the

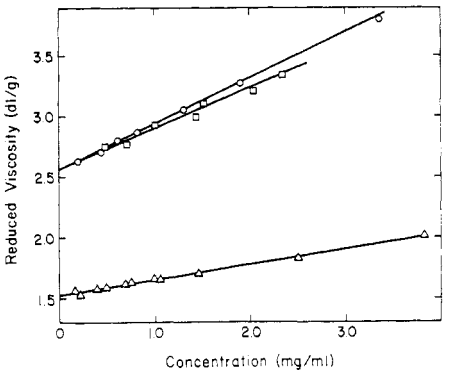


FIGURE 2: Reduced viscosity *vs.* concentration for paramyosin and light paramyosin at 5°. (○) Paramyosin in 0.2 M KCl, pH 2; (□) paramyosin in 0.6 M KCl–0.01 M $PO_4$–0.01 M EDTA, pH 7.0; (▽) light paramyosin in 0.6 M KCl–0.01 M $PO_4^{2-}$, pH 7.0.

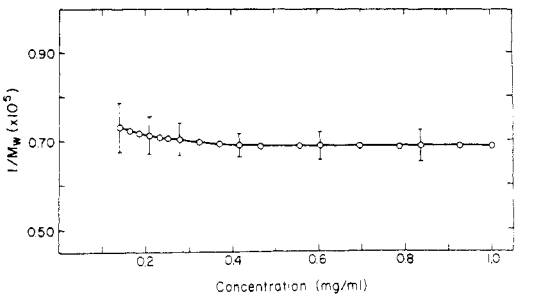


FIGURE 3. Reciprocal weight average molecular weight *vs.* concentration for light paramyosin. Each circled point is the average of three point averages for runs at different loading concentrations and rotor speeds (0.4 mg/ml at 13,000 rpm, 0.8 mg/ml at 13,000 rpm and 15,000 rpm). The vertical bars are 90% confidence limits for means calculated. The solvent was 0.6 M KCl–0.01 M $PO_4^{2-}$, pH 7.0.

helix-forming tendency of proteins, proteolytic fragments, and peptide mixtures from the amino acid composition. Each residue percentage is multiplied by the helix-forming power assignment and these are summed to obtain the helix potential in nats/100 residues. A positive, linear correlation was observed for the range 33–100% helix (the nats/100 residues increases from 3.5 to 5.0) when this parameter was calculated for 11 proteins of known $\alpha$-helix content.[1]

## Results

*Kinetics of Trypsin Digestion.* Figure 1a shows the time dependence of peptide bond hydrolysis of paramyosin at 25° in the presence of trypsin (weight ratio 120:1). The tryptic digestion of paramyosin appears to be biphasic with a very rapid phase of digestion during the first 20 min of the reaction followed by a much slower phase which persists for over 2 hr. Analysis of the pH-Stat data according to the procedure described in the Methods section indicates that the overall reaction can be closely described by a sum of two independent first-order reactions with widely separated rate constants. Simulated reaction curves were constructed from the derived kinetic parameters and a typical profile is shown in Figure 1a as the smooth curve which gives a reasonable fit to the data (circled points). Although the rate measurements showed some variability from one day to the next, the data always appeared to be biphasic and the fast reaction rate constant was always more than an order of magnitude greater than the slow reaction rate constant.

This kinetic pattern could indicate that paramyosin has a distinct, localized region where susceptible bonds are clustered or that the labile peptide bonds are spaced along the length of the molecule. That the fast reaction class probably represents a clustered set of susceptible bonds is suggested by the results shown in Figures 1b and 1c. To obtain these data, paramyosin was digested with trypsin in a pH-Stat and at various times samples were removed and mixed immediately with soybean trypsin inhibitor. Aliquots of these samples were examined in the ultracentrifuge and in a viscometer as described in the Methods section. The relative viscosity (Figure 1b) falls rapidly during the early stages of the digestion reaction and levels off at a time corresponding to completion of the fast reaction process followed in the pH-Stat. It seems clear that the rod-shaped paramyosin molecule is undergoing a marked decrease in asymmetry in the early phase of the reaction consistent with transverse cleavage of the particle.

Figure 1c shows that the size of the particle also decreases in the early stages of the reaction. Velocity sedimentation patterns of the reacting mixture obtained at various times of digestion show only a single sedimenting boundary whose area decreases rapidly during the first 30 min of digestion and then becomes invariant with respect to time thereafter.

The fast reaction appears to digest one-third of the molecule to small fragments. Approximately two-thirds of the mass of paramyosin is resistant to fragmentation, as shown by Figure 1c, in spite of the fact that many bonds are hydrolyzed. When the digestion mixture was chromatographed on a G-150 column at a stage corresponding to 59 bonds cleaved no discrete intermediate sized fragment was observed. Two peaks were seen in the elution profile with paramyosin and light paramyosin eluting at the void volume position and the released peptides at a position just in front of the KCl peak. If an intermediate sized particle is formed by trypsin hydrolysis, its existence must be transitory.

It is clear that the physical properties of the paramyosin molecule are markedly altered by proteolysis and that the changes in these properties occur during the fast reaction. The results taken together suggest that trypsin hydrolyzes an interior, trypsin-sensitive belt. Digestion from one or both ends of the molecule would not be expected to produce the rapid decrease in viscosity. Moreover, cleavage of labile peptide bonds spaced at random along the length of the molecule would not yield the observed *plateau* in viscosity and mass seen in Figures 1b and 1c.

*Isolation and Properties of a Light Meromyosin-Like Particle from Paramyosin.* The results summarized in Figure 1 indicate that at the completion of the fast reaction a high molecular weight particle remains which is relatively resistant to further fragmentation. It will be noted that this behavior is also observed when myosin is digested with trypsin to form the stable light meromyosin fragment. In the case of paramyosin, the large difference between the fast and slow rate constants ($k_f/k_s \cong 20$) suggests that the light paramyosin particle could be formed with very few internal proteolytic clips, and the sodium dodecyl sulfate gel pattern of light paramyosin, which exhibits a single migrating band of mol wt $\sim$ 70,000, supports this conclusion (see Methods).

It was of interest to compare the physical properties of this fragment with that of light meromyosin and for this purpose a particle was isolated after 72 peptide bonds had been hydrolyzed (see Methods). Results from some of the physical

[1] Unpublished results.

TABLE I: Physical Properties of Paramyosin and the Light Paramyosin.

| | Light Paramyosin[a] | Paramyosin |
|---|---|---|
| Mol wt (g/mol) | 145,000 | 210,000[b] |
| Intrinsic viscosity (dl/g) at 5° | 1.52 | 2.54[c] |
| Huggins constant, $k'$ | 0.55 | 0.48 |
| Moffitt parameter, $b_0$ | −626 | −682 (5.5°)[c]<br>−680 (23°)[c]<br>−673 (6°)[d] |
| Reduced mean residue rotation, $[m']_{233\ nm}$ | −16,000° | −16,000° (5°)[a] |

[a] Measured in 0.6 M KCl–0.01 M $PO_4^{2-}$, pH 7, 5°. [b] Woods, 1969. [c] Measured in 0.6 M KCl–0.01 M $PO_4^{2-}$–0.01 M EDTA, pH 7. [d] Solvent was 0.2 M KCl, pH 2.

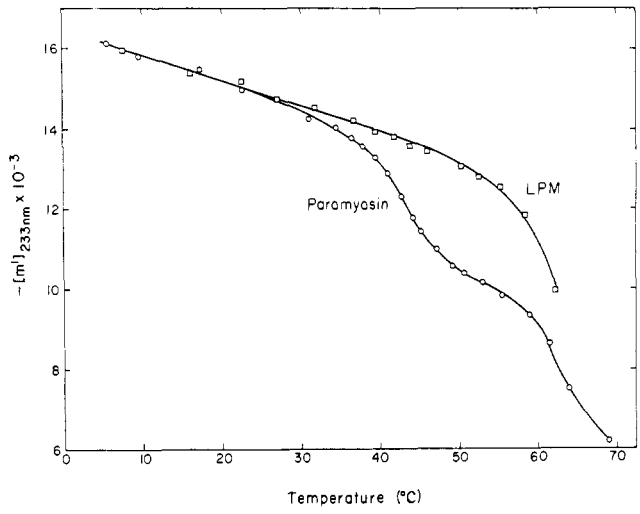


FIGURE 4: Reduced mean residue rotation *vs.* temperature for paramyosin (○) and light paramyosin (□). Solvent was 0.6 M KCl–0.01 M $PO_4$, pH 7.

studies on paramyosin and the proteolytic fragment (light paramyosin) are summarized in Figures 2 and 3 and Table I.

Viscosity measurements on light paramyosin and on paramyosin are shown in Figure 2. The relatively high intrinsic viscosity measured for light paramyosin of 1.52 dl/g indicates that this proteolytic fragment has retained the rodlike character of paramyosin but with a significant reduction in length. It is of interest that the reduced viscosity *vs.* concentration plots of paramyosin and light paramyosin at pH 7 in high salt (0.6 M KCl) are linear over the concentration range 0.2–4.0 mg/ml. Under similar ionic conditions both the rod segment of myosin and light meromyosin exhibit upward curvature with increasing protein concentration, consistent with a rapidly reversible monomer–dimer association reaction (Harrington and Burke, 1972). The finding that the viscosities of paramyosin at pH 2 and 7 are virtually identical also differs from the results with the myosin rod (Burke *et al.*, 1973).

Meniscus depletion sedimentation equilibrium experiments on light paramyosin demonstrate that the isolated particle is relatively homogeneous with respect to size. A weight-average molecular weight of 145,000 g/mol was obtained by extrapolating from the linear part of the $1/M_w$ *vs.* concentration curve (Figure 3). Each point plotted is obtained by averaging the value at the concentration over three sets of experimental conditions (loading concentration and rotor speed). At loading concentrations of 0.4 and 0.8 mg/ml and speeds of 13,000 and 15,000 no significant variation in the reciprocal molecular weight *vs.* concentration plots was detected *above* 0.3 mg/ml of protein concentration in the solution column. At higher speeds and lower cell loading concentrations, upward curvature became more pronounced in the low concentration region of the $1/M_w$ *vs.* concentration curve. This suggests that the upturn at very low concentrations (<0.3 mg/ml) results from particle heterogeneity.

A summary of other physical studies on the light paramyosin is presented in Table II. The $b_0$ of −626 and $[m']^{7.5}_{233\ nm}$ of −16,000° indicate that the particle, like paramyosin, is virtually 100% α helical. The assembled data of Table I demonstrate that light paramyosin is a rigid rod comprising about two-thirds of the mass of the original paramyosin molecule. Although the molecular weight and the intrinsic viscosity of light paramyosin are somewhat higher than the corresponding values for myosin light meromyosin, it seems clear that the fragment formed at the end of the fast reaction phase of tryptic digestion of paramyosin is a particle very similar in physical properties to that of light meromyosin.

*Rotation Melt Experiments.* The biphasic kinetic pattern observed during tryptic digestion of paramyosin suggests that the enzyme may be sensing regions of differing helical stability along the length of the rod. Indeed it has been known for some time that the paramyosin structure does not melt with a single, cooperative, helix–coil transition under denaturing conditions. Riddiford (1966) and Olander (1971) have measured the reduced mean residue rotation (and the Moffitt parameter, $b_0$) *vs.* guanidine-HCl concentration demonstrating the presence of discrete steps over the range in concentration of the denaturent where the native molecule unfolds.

When the optical rotation at 233 nm of a dilute solution of paramyosin (~0.08 mg/ml in 0.6 M KCl–0.01 M $PO_4^{2-}$, pH 7) is measured over the temperature range 5–70°, the melt profile shown in Figure 4 is obtained. Earlier workers (Riddiford and Scheraga, 1962) have noted the tendency of paramyosin solutions to aggregate on heating. However, measurements at the Cotton trough (233 nm) allow the use of protein solutions sufficiently dilute to obtain reliable data up to ~70° before the development of significant turbidity.

The thermal denaturation of paramyosin shows three stages. In the low-temperature range from 5 to 25°, the rotation increases slowly with increasing temperature. Since the change in $[m']_{233\ nm}$ is not accompanied by any change in the Moffitt parameter, $b_0$ (Table I), in this temperature range, we believe that this rotation change reflects some heat-induced changes in the optical rotatory power of the coiled coil without significant change in the α-helix content. It is important to note in this regard that the initial $d[m']_{233\ nm}/dT$ for both light paramyosin and paramyosin is 61°/°C.

As the solution is heated further the protein undergoes two well-defined cooperative transitions, indicating that the paramyosin molecule has regions of different thermal stability. The melt data were used to calculate fraction helix at each temperature as described under Methods. From this plot (Figure 5), it can be seen that approximately 30% of the

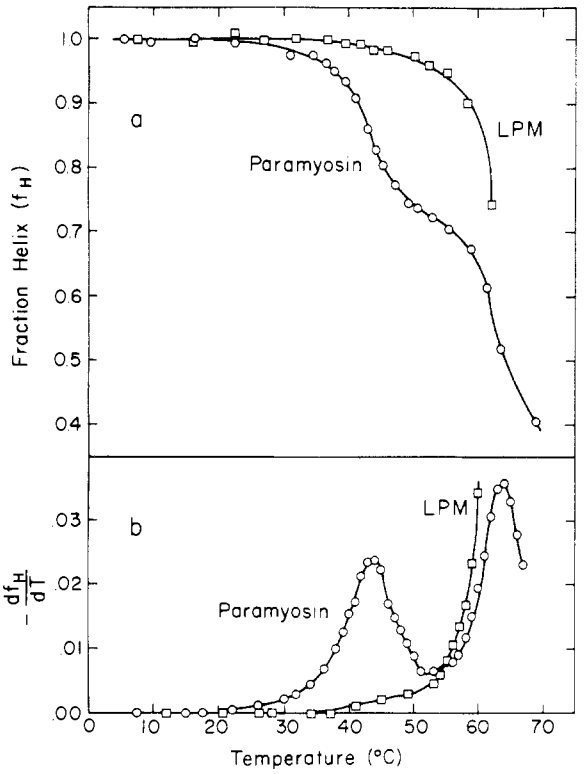


FIGURE 5: Thermal denaturation of paramyosin and light paramyosin: (a) fraction helix *vs.* temperature for paramyosin (○) and light paramyosin (□); calculated as described under methods from the data in Figure 4; (b) derivative plot of above.

helical structure is lost upon heating to 55°. A derivative plot was obtained numerically with a quadratic-fitted first derivative formula (Savitzky and Golay, 1964) using equally spaced points read from the smoothed fraction helix *vs.* temperature curve. The presence of two cooperatively melting regions in paramyosin is clearly demonstrated in this derivative plot (Figure 5) with midpoints of the transitions ($T_m$) of 44 and 64° obtained from the peak maxima.

If the fast reaction observed in the tryptic digestion kinetics of paramyosin has its origin in proteolysis of a less stable helical region, then the melt profile of light paramyosin isolated at the completion of the fast reaction should reflect the loss of this section of the structure.

That this is indeed the case can be seen from the melting profiles and the derivative plot of light paramyosin presented in Figures 4 and 5. The first cooperative transition of paramyosin ($T_m$ = 44°) is now absent from the rotation *vs.* temperature profile and the fraction helix *vs.* temperature plot. At temperatures above 62° light paramyosin aggregated as shown by the onset of turbidity so that the melting profile could not be taken to completion. Figures 4 and 5 demonstrate that the tryptic fast reaction results from proteolysis of the region of low thermal stability in the paramyosin structure.

*Amino Acid Composition of the Released Peptides.* Since the physical studies described above indicate that paramyosin has discrete helical regions of differing thermal stability, we thought that these differences might be reflected in the amino acid composition of the peptides released during the fast reaction phase. Table II shows the amino acid composition of paramyosin, light paramyosin, and the released peptides. The composition of native paramyosin shown here agrees

TABLE II: Amino Acid Composition of Paramyosin, Light Paramyosin, and Released Peptides.[a]

| Amino Acid | Residues/100 Residues | | |
|---|---|---|---|
| | Paramyosin | Light Paramyosin | Released Peptides |
| Lysine | 7.4 | 6.9 | 8.3 |
| Histidine | 0.6 | 0.4 | 1.6 |
| Arginine | 10.1 | 8.7 | 11.5 |
| Aspartic acid | 13.1 | 14.5 | 10.5 |
| Threonine | 4.2 | 4.7 | 3.3 |
| Serine | 5.0 | 4.3 | 6.9 |
| Glutamic acid | 21.1 | 20.6 | 20.5 |
| Glycine | 2.2 | 1.8 | 3.5 |
| Alanine | 12.2 | 14.1 | 9.7 |
| Valine | 4.3 | 4.1 | 4.7 |
| Methionine | 1.5 | 1.0 | 2.7 |
| Isoleucine | 3.4 | 3.3 | 4.1 |
| Leucine | 12.1 | 13.5 | 9.1 |
| Tyrosine | 2.1 | 1.7 | 2.9 |
| Phenylalanine | 0.8 | 0.5 | 0.9 |

[a] Proline and half-cystine were not detected.

quite well with that reported by Riddiford (1966). Self-consistency of the amino acid analysis data for the light paramyosin and the released peptides can be shown by adding two-thirds of the percentage for light paramyosin to one-third of the peptide percentage. This total gives good agreement with the composition presented for paramyosin.

Table III presents the amino acid analysis results in a form that readily permits a comparison of light paramyosin with the released peptides by focusing on those amino acids which differ, and which are predicted to have a significant effect on relative helical stability. It can be seen that the digested region contains a significantly lower amount of leucine and alanine and a higher amount of glycine and serine. In column 3 (Table III) the helix-forming or helix-breaking tendency of these residues is given (Ptitsyn, 1969, and Kotelchuck and Scheraga, 1968) and in column 4 a quantitative measure of the $\alpha$-helix forming power of the differing residues is presented according to the formulation of Robson and Pain (1971). Inspection of Table III reveals that leucine and alanine both have a strong tendency to form an $\alpha$-helical conformation and a reduction in the density of these residues would be expected to destabilize the $\alpha$ helix. Glycine, which has helix-breaking potential, is present in significantly higher amounts in the trypsin-sensitive region. Serine has the greatest tendency to destabilize the $\alpha$ helix of all the residues. Thus, the amino acid composition of this region shows a pattern consistent with its thermal stability as judged from the optical rotation *vs.* temperature results. We conclude that the trypsin-sensitive region of the paramyosin molecule has an amino acid composition which favors a reduction in stability of the $\alpha$ helix.

A helix potential parameter was calculated for the total amino acid compositions of paramyosin, light paramyosin, and the released peptides (Table IV). The trypsin-sensitive region (*i.e.*, the released peptides) exhibited significantly lower helix potential (4.25) than light paramyosin (4.97) and the native paramyosin (4.76). The differences in these poten-

TABLE III: Differences in the Distribution of Certain Amino Acids in the Released Peptide Region[a] and the Light Paramyosin.

| Residue | Diff between No. Calcd and No. Obsd[b] | Assignmt for Residue by Theor[c] and Statist[d] Approach | Helix-forming Power of Residue[e] | Probable Effect on Helix Stability in Released Peptide Region |
|---|---|---|---|---|
| Leucine | −25 | Helix forming | +0.11 | Destabilize |
| Alanine | −25 | Helix forming | +0.09 | Destabilize |
| Glycine | +10 | Helix breaking[f] | −0.05 | Destabilize |
| Serine | +15 | Helix breaking | −0.07 | Destabilize |

[a] 565 residues are estimated to be in this region. This number was calculated from the estimated mass (65,000 g/mol, obtained by subtracting the mass of light paramyosin (145,000 g/mol) from the mass of paramyosin (210,000 g/mol). [b] The calculated number is the number of residues expected if this region had the same composition as light paramyosin. Difference = (% composition in light paramyosin) (565 residues) − (% composition in released peptides) (565 residues). [c] Kotelchuck and Scheraga, 1968. [d] Ptitsyn, 1969. [e] Values are in nats (Robson and Pain, 1971). The helix-forming power of the residues was found to vary from −0.07 nats to +0.12 nats. Negative values indicate helix-disrupting tendencies. [f] This residue was helix indifferent according to energy calculations (Kotelchuck and Scheraga, 1968).

tials, which reflect *all* the residue differences, also correlate with the observed thermal stabilities.

In view of this finding it was of interest to compare the helix potential parameter of peptides released during the fast reaction phase of tryptic proteolysis of myosin. Segal *et al.* (1967) have isolated peptides released at various stages of the fast reaction phase and determined their amino acid composition. Using this information the helix potential was estimated at various stages of the fast reaction and this parameter *vs.* the number of bonds digested is plotted in Figure 6. In the initial stages of trypsin digestion, the released peptides show a strikingly low helix potential, suggesting that these peptides are from nonhelical and/or less stable $\alpha$-helical regions of the myosin molecule. The helix-stabilizing potential then increases rapidly and begins to plateau as the fast reaction phase is completed.

## Discussion

Optical rotatory dispersion measurements of paramyosin indicate that the protein is fully $\alpha$ helical and the invariance of the Moffitt parameter at 5 and 23° ($b_0$ = −682 and −680, respectively) shows that the conformational state of the polypeptide chains is not significantly perturbed over this temperature range. Thus, the temperature coefficient of optical rotation, $d[m']/dT$ = 61°/°C, in the region below the lower transition temperature (44°) appears to be a fundamental property of this two-chain $\alpha$ rope rather than an unfolding of the helical structure. A similar temperature coefficient (66°/°C) was found for the poly-L-glutamic acid $\alpha$ helix by Warashina and Ikegami (1972). We find virtually the same temperature coefficient of the light paramyosin particle over the temperature range 5–50°. This result, coupled with the high value of the Moffitt parameter ($b_0$ = −626) and the rotation at the Cotton trough ($[m']_{233}$ = −16,000°), indicates that the substructure of this proteolytic fragment retains the $\alpha$-helical conformation of the parent protein up to temperatures approaching the single cooperative transition temperature ($T_m \approx 64°$) observed for light paramyosin. It seems clear from the information given above that the presence of a trypsin-sensitive region cannot be directly related to an unfolded polypeptide segment embedded within the predominantly $\alpha$-helical rod of paramyosin. Such a segment would have to occupy approximately one-third the mass of the paramyosin molecule, contrary to the optical rotatory dispersion data. Yet the enzyme must be sensing significant differences in the secondary structure, since the rate of peptide bond cleavage in the trypsin-sensitive segment is well over an order of magnitude faster than that in the light paramyosin segment of the rod. Moreover, the presence of the two reaction

TABLE IV: Helix Potential of Paramyosin, Light Paramyosin, and the Released Peptides.

| | Helix Potential in nats/100 residues[a] |
|---|---|
| Paramyosin | 4.76 |
| Light Paramyosin | 4.97 |
| Released Peptides | 4.25 |

[a] Calculated using the amino acid compositions given in Table II as described in Methods.

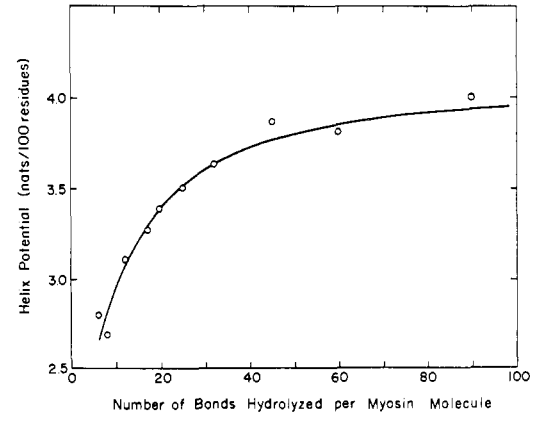


FIGURE 6: Helix potential of the peptides released from myosin during the course of tryptic proteolysis. The amino acid analysis results used for these calculations were obtained from Segal *et al.* (1967). Proline and cysteine were not included.

classes cannot be ascribed simply to differences in the enzyme structure specificity since subtilisin and chymotrypsin exhibit a similar biphasic kinetic pattern (Kay and Smillie, 1964).

If the trypsin digestion studies of Figure 1 are correlated with the melting curves of paramyosin and light paramyosin of Figures 4 and 5, it seems likely that the enzymatic digestion behavior has its origin in the relatively low thermal stability of the trypsin-sensitive segment. We interpret the data as indicating that this segment is a less tightly folded helix, *i.e.*, it spends more time populating different conformations than the light paramyosin segment of the paramyosin molecule. This breathing reaction at 25° is sufficient to open a set of peptide bonds for rapid trypsin hydrolysis. We might assume for simplicity that peptide cleavage requires (1) that a polypeptide segment must be in a specific conformation for proteolysis to occur and (2) that the resistance to proteolysis of any other conformation will be a function of the stability of that conformation. For example, a protease might require that the substrate peptide backbone within the enzyme cleft be in a $\beta$ conformation. Then a helical or other systematic structure which is very stable, *i.e.*, one which spends a small part of its time exploring alternative conformations, would be expected to be relatively resistant to proteolysis. This line of argument is similar to that suggested by Segal and Harrington (1967) for the proteolytic sensitive belt of myosin, on the basis of tritium exchange studies. They observed that a class of peptide hydrogen atoms (495 hydrogen atoms based on a molecular weight of 458,000), thought to arise from this segment of the myosin rod, exchanged much more readily with solvent than other regions of the light meromyosin structure. The comparatively high accessibility of this class of peptide hydrogen atoms is consistent with the observed high accessibility of the peptide bonds of this region to proteolysis.

The low thermal stability of the trypsin-sensitive segment of paramyosin appears to have its origin in the higher level of helix-disrupting residues in this region. When the composition of peptides released during the fast reaction phase of tryptic proteolysis is compared with that of light paramyosin, which is a two-chain $\alpha$ rope of high thermal stability, a significant increase in the percentage of helix-destabilizing residues is seen. As shown in Table III, the composition differences can be examined by any of three different approaches with the same result, *i.e.*, the released peptide region should form a less stable $\alpha$ helix.

It is of considerable interest that an analysis of the helix-forming tendency of peptides released primarily from the trypsin-sensitive segment of myosin shows this region also to be of low $\alpha$-helical stability. The results on paramyosin have been obtained from analysis of the composition of all peptides released during the fast reaction phase. Segal *et al.* (1967) have isolated peptides released at various stages of the fast tryptic reaction of myosin, thus allowing estimation of the helix-forming tendency as a function of the number of peptide bonds cleaved (Figure 6).

The length of the *Mercenaria* paramyosin molecule has been established with high precision from banding patterns of aggregates precipitated in the presence of divalent cations. Cohen *et al.* (1971) report a molecular length of 1257 ± 35 Å for the native molecule. They interpret the polarity of the banding pattern as indicating that the molecular unit is polar and suggest that the two chains of the structure have very similar sequences and run in the same direction. Our results are consistent with this interpretation. The trypsin-sensitive segment occurs at one end of the molecule and both chains in this region appear to be equally susceptible to enzymatic attack. In the present study we have demonstrated that this segment, amounting to close to one-third of the mass of paramyosin, undergoes a cooperative helix–coil transition at a temperature (44°) approximately 20° lower than the light paramyosin region. The length of this segment, ~420 Å (*i.e.*, one-third of 1260), and its melt temperature correspond closely to the length and melt temperature of the trypsin-sensitive segment of myosin ($L$ = 450 ± 50 Å and $T_m$ = 44°) (see Burke *et al.*, 1973). Moreover, the length of the light meromyosin rod segment of vertebrate skeletal myosin which is used to construct the thick filament core is 860 Å (Pepe, 1967). We estimate the length of the light paramyosin segment of the paramyosin molecule to be ~840 Å. Although these striking similarities in structural features could be fortuitous, there is a strong hint that they are fundamental to the contractile properties of the two molecules.

The conclusion of Szent-Gyorgyi *et al.* (1971) that the paramyosin and myosin of molluscan smooth muscle are in mutual association to form a thick filament consisting of a paramyosin core surrounded by a layer of myosin led them to suggest a dynamic role for paramyosin in the contractile mechanism. They propose that the specific phenomenon of catch is generated as a result of a "phase" change or structural rearrangement of the paramyosin molecules in the filament core which is coupled to cross-bridge movement. In phasic, or normal contractile, activity the rate of cross-bridge detachment would be unaffected by the myosin–paramyosin interaction, whereas in catch a structural rearrangement in the molecular packing would alter the cross-bridge interaction.

The "phase" change envisioned by Szent-Gyorgyi *et al.* (1971) involves a large number of paramyosin molecules. As they noted, however, the X-ray diffraction observations of Millman and Elliott (1965) show no change in the spacing or intensity of the 145-Å reflection in the resting and catch states of these molluscan muscles. Thus, any change involving appreciable structural rearrangements in the paramyosin packing scheme within the filament core would seem to be excluded.

Our finding of a segment of low thermal stability in the paramyosin rod leads us to suspect that a cooperative phase change in individual molecules may occur within this region during catch. The bonding arrangement of paramyosin in the thick filament core, in which there is a systematic arrangement of gaps between molecules and no end-to-end bonding (Cohen *et al.*, 1971), could allow a conformational change within individual molecules without affecting the specific interactions between the overlap regions of neighboring molecules. Thus, the basic 145-Å spacing would be preserved.

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## Added in Proof

Following submission of this paper a report has recently appeared (Cowgill, 1972) demonstrating, in agreement with the present findings, that one-third of the paramyosin molecule is released by tryptic proteolysis and this segment exhibits a lower thermal stability than the remaining segment.

It appears from the work cited above that the most stable region of paramyosin is the N-terminal segment.

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# Studies on the "Hinge" Region of Myosin†

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ABSTRACT: The structural stability of the myosin rod and its smaller segments light meromyosin and subfragment II (isolated by papain digestion of myosin) has been examined to obtain more insight into the nature of the trypsin-sensitive region of the myosin rod. Although optical rotatory dispersion studies show that these rod segments are highly helical at ambient or lower temperatures, the kinetics of their proteolysis by trypsin suggest the presence of regions differing in accessibility to the protease. This conclusion is supported by thermal denaturation studies which demonstrate that the loss in helical structure of these rod segments occurs in cooperative phases. Tryptic digestion of myosin to the completion of the fast reaction results in a light meromyosin which exhibits only a single phase melt, indicating that the more heat labile phase may be correlated with the trypsin-sensitive region. Evidence is presented to show that the rod exhibits a substantial amount of flexibility under conditions where no discernible loss in helix content occurs. The possible significance of the trypsin labile helical region of the myosin rod to the contractile process is discussed.

A variety of proteolytic enzymes, including trypsin, chymotrypsin, and subtilisin, rapidly cleave the myosin molecule into two relatively homogeneous, high molecular weight fragments, light meromyosin and heavy meromyosin. Hydrodynamic and electron microscope studies have demonstrated that cleavage occurs predominantly within the helical rod segment in a region 700–1000 Å from the tail end of the molecule. More recently, Kominz *et al.* (1965) and Lowey *et al.* (1969) have shown that papain, which also cleaves the molecule in the rod region, has a slightly greater preference for attack at a second site near the globular (subfragment I) segments. The reason for the increased susceptibility of the polypeptide chains to proteolytic fission in the rod segment is unclear at the moment. Segal *et al.* (1967) observed a rapid release of proline-containing peptides in the early stages of

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