Characterization of Some Invertebrate Tropomyosins*

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ABSTRACT: Physicochemical studies have been carried out on tropomyosins from crayfish (two species), oyster, abalone, and blowfly. Equilibrium sedimentation in high ionic strength buffers indicated that all these tropomyosins exist as self-association systems. The meniscus depletion method showed that the minimum molecular weights (65,000–86,000) were in the same range as values reported for vertebrate tropomyosins. Because of the strong self-association the analysis of the equilibrium sedimentation data did not give an unequivocal answer to the monomer molecular weight. In 7 M guanidine hydrochloride abalone tropomyosin gave a subunit size of 33,500 ± 2000 indistinguishable from the values found for vertebrate tropomyosins; oyster and blowfly tropomyosins gave slightly lower values. Crayfish tropomyosins were heterogeneous in denaturing solvents and a homogeneous subunit was not obtained even after gel filtration. However electron microscope observations on the periodicity of tactoids suggest that the molecular lengths of all invertebrate and vertebrate tropomyosins are identical.

The evidence presented suggests a common subunit molecular weight for all tropomyosins. It is postulated that the heterogeneity of crayfish tropomyosin in denaturing solvents is a result of contaminating proteases. Amino acid analyses are reported for crayfish, oyster, and abalone tropomyosins, and oyster and abalone paramyosins. Crayfish tropomyosin contains neither SH groups nor SS bonds, whereas the two molluscan tropomyosins appear to have their polypeptide chains linked by a disulfide bond.

There is convincing evidence that tropomyosins from all types of vertebrate muscles (skeletal, cardiac, and smooth) possess a common subunit molecular weight (McCubbin *et al.*, 1967; Woods, 1968, 1969b). The almost completely α-helical rod-shaped molecules are composed of two noncovalently linked polypeptide chains of mol wt 34,000 ± 2000. Although there have been some comparative chemical studies on invertebrate tropomyosins (Tsao *et al.*, 1956; Jen and Tsao, 1957; Kominz *et al.*, 1958; Bailey and Ruegg, 1960) their subunit structure has not been investigated. Woods (1968) reported that the molecular weight of crayfish tropomyosin in a denaturing solvent (8 M urea) was lower than that found for vertebrates. The solutions in 8 M urea were heterogeneous and high-speed equilibrium sedimentation showed the presence of species of molecular weight less than 10,000. In this paper the nature of the heterogeneity of crayfish tropomyosin in dissociating solvents is studied further. Since crayfish tropomyosin is salted out over a different range of ammonium sulfate concentration to that for vertebrate tropomyosins particular attention was paid to the fractionation procedure in order to obtain preparations of maximum α-helix content. The physicochemical properties and subunit structures of tropomyosins from the adductor muscle of oyster, the foot muscle of abalone, and blowfly are also reported.

Experimental Section

Isolation of Tropomyosins. The invertebrate species used in this investigation were: seawater crayfish, *Jasus lalandei;* freshwater crayfish, *Cherax destructor* (commonly called yabbie); oyster, *Crassostrea commercialis;* abalone, *Notohaliotis ruber* (black lip abalone or red ear shell); blowfly, *Calliphora stygia.* The seawater crayfish and oysters were obtained live from a local seafood supply; the freshwater crayfish were collected from a water hole; the abalone were caught in southern Tasmania. The blowflies were kept in the deep freeze until 200 g were collected. Alcohol–ether-dried fibers were prepared as in Bailey's method (1948) which was followed with slight modifications (Woods, 1967). The acid precipitation step was always carried out in the presence of 1 M KCl. This facilitated handling of the tropomyosins all of which were extremely viscous after dialysis against water to remove ammonium sulfate. In addition, the presence of 1 M KCl gives a better separation of tropomyosin from troponin (Ebashi *et al.*, 1968). The ammonium sulfate precipitation ranges varied for different tropomyosins. The two crayfish tropomyosins precipitated in the range 30–50% (w/v) saturation. Subsequently it was found that this fraction consisted of two fractions which precipitated approximately at 30–40 and 40–50% saturation, respectively. These fractions are discussed further under Results. Following Kominz *et al.* (1962) whole blowflies were homogenized with three volumes of 50% ethanol in a Waring Blendor in the first stage of the preparation of the alcohol–ether-dried fibers. Blowfly tropomyosin precipitated between 45 and 70% saturation of $(NH_4)_2SO_4$, the same range as for vertebrate tropomyosins.

The white and yellow parts of the oyster adductor muscle, and the foot muscle of the abalone were used. Tropomyosin was isolated from the supernatant after the crystallization of paramyosin from the KCl extract of the alcohol–ether-dried fiber. Variable yields of protein of sometimes low α-helix content were obtained if the usual acid precipitation step and $(NH_4)_2SO_4$ fractionation were applied, therefore the procedure of Bailey and Ruegg (1960) was followed. The supernatant after precipitation of paramyosin was adjusted to pH 5.9 with acetate buffer (1 M sodium acetate–acetic acid, pH 5.4), centrifuged, the precipitate discarded, and the supernatant adjusted to pH 5 with 0.1 M acetic acid. After centrifuging, the precipitate was redissolved in 0.1 M KCl, adjusted to pH 7.5 with $NaHCO_3$ and any undissolved protein was removed. Cold ethanol was added to 20% (v/v) and any turbidity was removed by centrifugation. Ethanol was added to 40%, the gel centrifuged, redissolved in 0.1 M KCl, and the solution was then fractionated with neutral $(NH_4)_2SO_4$. Both the oyster

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and abalone tropomyosins precipitated between 30 and 45% saturation $(NH_4)_2SO_4$. Further purification was effected by following the usual acid precipitation and $(NH_4)_2SO_4$ fractionation.

Methods. The methods used for equilibrium centrifugation, gel filtration, gel electrophoresis, DEAE-cellulose chromatography, amino acid analysis, and optical rotatory dispersion have been given in previous publications (Woods, 1967, 1969a,b). Low-speed equilibrium sedimentation was carried out by the methods of Richards *et al.* (1968) and Yphantis (1960). High-speed equilibrium was carried out according to the procedures of Yphantis (1964). All ultracentrifuge experiments were carried out at 20°. Velocity sedimentation was carried at 59,780 and 67,770 rpm. Protein concentrations were generally determined from refractive index difference measurements made with a Brice-Phoenix differential refractometer and converted into concentrations by the refractive index increments given by Kay (1960).

The apparent specific volumes, ϕ', used in all calculations were those determined by Kay (1960). He found the following values for rabbit tropomyosin: 0.739 ml/g in high ionic strength buffers and 0.728 ml/g in 8 M urea. Kay's determinations were made on dialyzed solutions and are therefore the correct quantities to be used in the equations for the calculation of the anhydrous molecular weight in a multicomponent system such as 8 M urea (Casassa and Eisenberg, 1961). Since the values of ϕ' only refer to the solvent in which they were measured the use of the value determined in 8 M urea is questionable for experiments carried out in concentrated guanidine hydrochloride solutions. The apparent quantity ϕ' is defined by $(\partial\rho/\partial C_2)_\mu{}^0 = 1 - \phi'\rho^0$, where $(\partial\rho/\partial C_2)_\mu{}^0$ is the density increment at constant chemical potential, μ, of diffusible solutes and ρ^0 the density of the solvent. The dependence of ϕ' on ρ^0 and preferential interactions is given by eq 2 and 3 in the paper of Reisler and Eisenberg (1969) who have recently shown that lack of precise knowledge of partial volumes in guanidine hydrochloride may lead to erroneous molecular weights. There are no published data on the apparent volumes of tropomyosin in concentrated guanidine hydrochloride solutions and we did not have sufficient material to carry out density measurements. The value of ϕ' for paramyosin in 7.85 M guanidine hydrochloride was recently determined (Woods, 1969a) and found to be 0.010 ml/g less than the value in high ionic strength buffers. We may expect tropomyosin to behave similarly. As a control we have determined the molecular weight of rabbit tropomyosin in 7 M guanidine hydrochloride by the low-speed equilibrium method. Employing a value of 0.728 ml/g for ϕ' gave 33,300 for the molecular weight at zero concentration compared with a value of 34,400 obtained from a similar experiment in 8 M urea (Woods, 1967). The results suggest that values obtained in 7 M guanidine hydrochloride utilizing $\phi' = 0.728$ ml/g may be 3–4% low. On the other hand, invertebrate tropomyosins have a slightly different amino acid composition. The specific volumes calculated from summation of the specific volumes of the individual amino acids are approximately 0.008 ml/g lower than the corresponding volumes for vertebrates. The use of a value of ϕ' determined for a vertebrate tropomyosin in calculating the molecular weight of an invertebrate tropomyosin would therefore have the effect of making the values of M higher than they should be since the use of too high a value of ϕ' leads to a value of $1 - \phi'\rho$ which is too low. This effect would therefore partially compensate for the slightly different values of M determined in 8 M urea and 7 M guanidine hydrochloride. It was therefore concluded that the use of 0.728 ml/g for ϕ' in 7 M guanidine hydrochloride does not involve any serious error.

TABLE I: Optical Properties of Invertebrate Tropomyosins.[a]

	$E^{1\%}_{1\,cm}$ (277 nm)	E_{277}/E_{260}	b_0 (deg)	a_0 (deg)	m'_{232} (deg)
Crayfish[b]	2.5 (2.2–3.1)	1.9 (1.7–2.2)	−665 (±20)	+40 (±20)	−16,100 (±700)
Blowfly	2.2	1.1	−655	+25	
Oyster	3.2	2.2	−640	+15	
Abalone	3.0	1.9	−660	−10	−16,400

[a] Solvent 1.1 M NaCl–0.025 M sodium phosphate (pH 7.0). [b] These figures are for the fraction precipitating between 30 and 40% saturation with $(NH_4)_2SO_4$. There was considerable variation between preparations and the figures quoted are the mean values for nine preparations from the tail muscle of *J. lalandei;* the range of values found is given in parentheses.

Results

Optical Properties. The optical properties of the invertebrate tropomyosins are given in Table I. The crayfish tropomyosin results are for the fraction precipitating between 30 and 40% saturation with $(NH_4)_2SO_4$. In the preparative procedure, conditions were sought which gave maximum α-helical content of the fractions and the highest ratio for E_{277}/E_{260}. Generally crayfish tropomyosin fractions precipitating in the range 30–40% saturation were more helical and gave higher values of E_{277}/E_{260} than fractions precipitating at either higher or lower $(NH_4)_2SO_4$ concentrations. Two preparations however were separated by precipitation with $(NH_4)_2SO_4$ in the ranges 30–40 and 40–50% saturation to give two fractions of almost identical properties as regards optical rotation and ultraviolet absorption. Although fractions of uniformly high α-helix content could be prepared the ultraviolet absorption properties were not reproducible. Molecular weight studies were carried out on the 30–40% fraction except where stated otherwise. The optical properties of the molluscan tropomyosin preparations on the other hand were far more reproducible.

Molecular Weights at High Ionic Strength and Neutral pH. On velocity sedimentation in 1.1 M NaCl–0.025 M sodium phosphate buffer at pH 7 all the tropomyosins gave a single sharp peak at high protein concentrations. However, at concentrations below 0.5 g/100 ml, the peaks were asymmetric with spreading toward the trailing edge. Figure 1 presents the results of $s_{20,w}$ as a function of protein concentration for crayfish tropomyosin and includes runs on five preparations. The value of $s^0_{20,w}$ was 3.01 S. The sedimentation coefficients of the 40–50% $(NH_4)_2SO_4$ fractions of crayfish tropomyosin were more variable and for several preparations gave $s^0_{20,w}$ values in excess of 3.5 S. The variation of $s_{20,w}$ with concentration for abalone tropomyosin is shown in Figure 2. The value of $s^0_{20,w}$ was 3.08 S which is close to the value found for crayfish tropomyosin.

Apparent weight-average molecular weights, M_w (app), as a function of concentration were determined by low-speed equilibrium sedimentation and the results are given in Figure 3 for tropomyosins from three invertebrates. The molecular weights represent values calculated over the whole column by

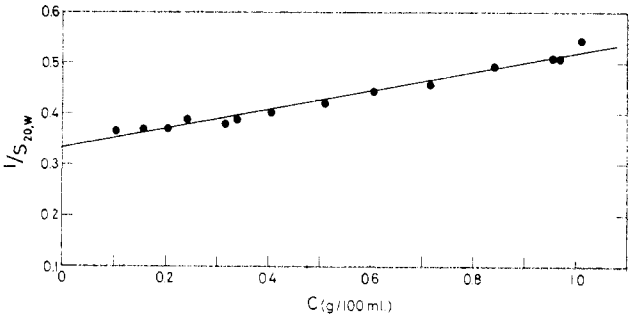

FIGURE 1: Sedimentation velocity as a function of protein concentration (g/100 ml) for crayfish tropomyosin in 1.1 M NaCl–0.025 M sodium phosphate (pH 7). A least-squares fit to the data gives $1/s_{20,w} = 0.332 + 0.188C$.

the procedures of Richards *et al.* (1968). M_w (app) first increases and then decreases as the concentration is decreased. The form of these plots can be described by a combination of thermodynamic nonideality and dissociation on dilution. As discussed in a previous paper (Woods, 1969b) the criterion of Adams and Fujita (1963) was applied to see whether the system is one in which there is a rapid dissociation–association. For crayfish tropomyosin, the molecular weight averages, $M(r)$, for a series of radial distances, r, were calculated for each initial concentration of Figure 3. The results are similar to those obtained for vertebrate tropomyosins (Woods, 1969b) in that the points from different initial concentrations and speeds do not merge to form a continuous smooth curve as expected for an associating system in rapid equilibrium. As found previously no obvious interaction with the FC 43 layering oil could be detected.

The results of molecular weights determined by the meniscus depletion method are given in Table II. Initial protein concentrations were generally in the range 1–2 mg/ml in order to detect the presence of low molecular weight species. The molecular weight of the smallest species present was determined graphically from the plot of log y (where y = fringe displacement) as a function of r^2, giving most weight to fringe displacements in the range 0.5–2.0 fringes as suggested by Yphantis (1964). For all three tropomyosins at high fringe displacements the plots were nonlinear with upward curvature indicating the presence of higher molecular weight species. The behavior is in accord with the low-speed results (Figure 3) which show that the system is an associating one.

The mean value of the minimum molecular weight for crayfish tropomyosin at the two lower speeds from Table II

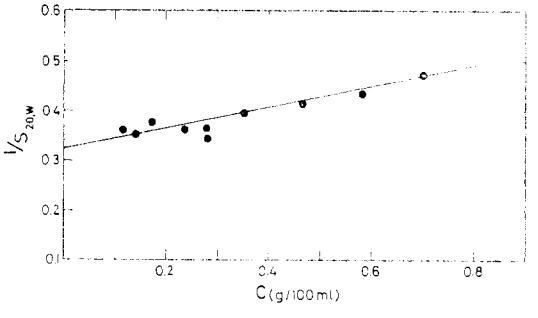

FIGURE 2: Sedimentation velocity as a function of protein concentration for abalone tropomyosin in 1.1 M NaCl–0.025 M sodium phosphate (pH 7). A least-squares fit to the data gives $1/s_{20,w} = 0.325 + 0.189C$.

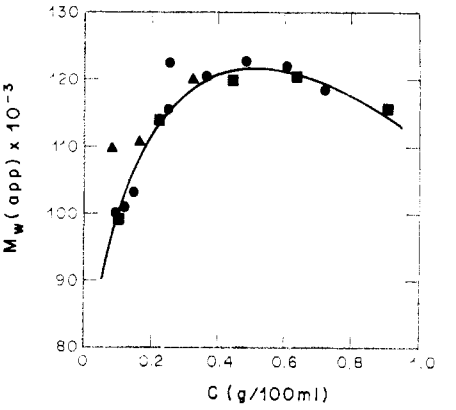

FIGURE 3: Low-speed equilibrium sedimentation of tropomyosin in 1.1 M NaCl–0.025 M sodium phosphate at pH 7, 20°, column height 1.3 mm, 8766 rpm. Cell-average molecular weight, M_w (app), as a function of concentration, $(C_m + C_b)/2$. (■) Crayfish, (●) abalone, and (▲) oyster.

is 70,700 and at the highest speed 67,500. In two preparations (5 and 6) which are not listed in Table II considerable amounts of low molecular weight species were apparent (mol wt 10,000–16,000) and the log y *vs.* r^2 plots showed continuous curvature. From runs at 39,460 and 44,770 rpm it was possible to use the subtractive procedure of Yphantis (1964) to obtain an estimate of the higher molecular weight species present. This gave values ranging from 60,000 to 70,000 for the main species. The molecular weight of the crayfish tropomyosin molecule would appear to be close to 70,000. Because of the pronounced tendency to aggregate and in some instances the presence of lower molecular weight species this figure must be accepted with some reservation.

There is a wide spread in the values of the minimum molecular weight for oyster tropomyosin similar to that observed for crayfish tropomyosin (Table II). These variations are outside the experimental error of the method and in many cases could also be directly attributed to the presence of some low molecular weight material. However, the abalone tropomyosin did not show the presence of low molecular weight species and values of M_w between 85,000 and 87,000 were obtained irrespective of initial concentration or speed. In an associating system the monomer molecular weight will only be obtained from an equilibrium sedimentation experiment if dissociation to monomer is nearly complete in the concentration range in which the measurements are made. In the graphical method used in this paper to analyze the meniscus depletion experiments we have estimated the molecular weight from the slopes of the log y *vs.* r^2 plots in the range 0.5–2.0 fringes. Extrapolation of the low-speed sedimentation equilibrium curves (Figure 3) for both abalone and crayfish tropomyosine would indicate considerable association at a concentration of 2 fringes. Neither the mode of aggregation, *i.e.*, whether monomer–dimer, monomer–trimer, etc., nor the thermodynamic nonideality term can be deduced from the conventional method of approach. This would require a more detailed analysis similar to that employed by Godfrey and Harrington (1970) to the myosin system. Thus the results do not allow us to give a value for the monomeric molecular weight of the molluscan tropomyosins.

Molecular Weights in Denaturing Solvents. Difficulties were encountered in the determination of the subunit size of crayfish tropomyosin in 8 M urea. The values found for M_w, determined by low-speed equilibrium sedimentation, were variable

TABLE II: Minimum Molecular Weights of Invertebrate Tropomyosins by High-Speed Equilibrium Sedimentation.[a]

Source of Tropomyosin	Initial Concn (mg/ml)	Graphical Estimate of Smallest Mol Wt Present: 27,690 rpm	29,500 rpm	39,460 rpm
Crayfish (1)	1.9		75,500	69,100
(2)	1.6		65,800	
(3)	1.4	68,700		67,500
(4)	1.2		66,200	58,500[b]
(7)	1.4	71,200		75,900
	0.12		80,600	
(8)	1.3	67,200[b]		66,700[b]
Oyster (1)	1.1		66,100	
(2)	1.5		84,400	
(3)	1.3	65,200		64,700
	0.2	73,700		
Abalone	1.0	86,800		85,200
	0.12	86,900		

[a] Experimental conditions: 1.1 M NaCl–0.025 M sodium phosphate, pH 7.0, 20°, column height, 3 mm. [b] Some species of lower molecular weight were evident from the log y *vs.* r^2 plots at small fringe displacements (<100 μ).

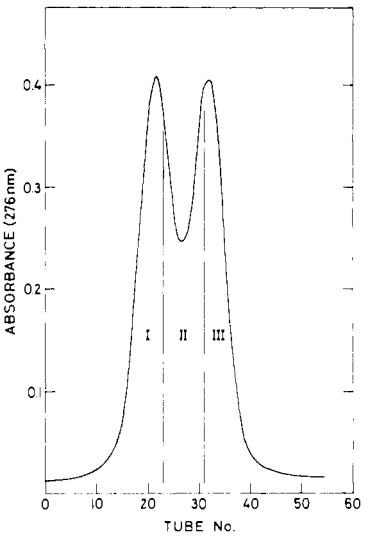

FIGURE 4: Gel filtration of crayfish tropomyosin (preparation 7) on a Sephadex G-200 column (2.0 × 40 cm) equilibrated with 8 M urea–0.2 M NaCl–0.01 M Tris-HCl (pH 7.6); 200 mg of protein in 12 ml of solvent was loaded; flow rate 14 ml/hr, sample size 4.7 ml.

and ranged from 17,000 to 32,800 for the nine preparations examined. Even for a single preparation molecular weights were variable and on one preparation values of M_w ranging from 17,000 to 25,500 were found for separate samples dialyzed against 8 M urea. On acrylamide gel electrophoresis a similar nonreproducibility was encountered. Sometimes a single band was observed moving identically with rabbit tropomyosin (see Woods, 1968) but in other runs material moved ahead of the main band. High-speed equilibrium experiments indicated heterogeneity with species of molecular weight less than 10,000.

Gel filtration on Sephadex G-200 in 8 M urea was employed to further fractionate the subunits. The 30–40% $(NH_4)_2SO_4$ fraction always gave a peak with marked asymmetry toward the trailing edge. Although the elution volume of the peak tube corresponded to a molecular weight between 30,000 and 39,000, sedimentation equilibrium measurements showed that the trailing edge contained molecular weight species in the range 5000–10,000. In two preparations two major peaks were obtained as shown in Figure 4 the elution volumes of which correspond to molecular weights of 35,000 and 5000, respectively. Attempts to obtain a homogeneous subunit by repeated passage through Sephadex G-200 were not successful. When fraction I (Figure 4) was dialyzed to remove the urea about 90% of the original α-helix content could be recovered, but fraction III could not be refolded to the α-helical conformation. There were differences in the amino acid compositions, particularly the tyrosine and phenylalanine contents which showed an almost 3-fold increase through fractions I–III. This was reflected in the values of $E^{1\%}_{1\,cm}$ (277 nm) which were 1.7, 2.2, and 4.6 for fractions I, II, and III, respectively. Thus on a mass basis the low molecular weight peak represents less than one-third of the total. All of the preceding results on crayfish tropomyosin were for the species *J. lalandei*. Tropomyosin from the crayfish, *C. destructor*, behaved similarly and the results are not therefore reported.

The origin of the low molecular weight material may be due to the action of tissue proteases which act during the early stages of the isolation or which survived the ethanol treatment. After limited proteolysis the molecule may still exist as an α-helical rod, similar to the situation in light meromyosin which has been prepared by enzymic digestion of myosin. In 8 M urea the molecule breaks down into a heterogeneous mixture. Since rabbit tropomyosin has been shown to be readily attacked by trypsin (Ooi, 1967), DFP, which inhibits tryptic- and chymotryptic-like activity, was included in the initial wash liquors in one preparation. This preparation was no different to the others. In an examination of tropomyosins from lower animals Pan *et al.* (1964) found breakdown on storage which they attributed to the presence of tenaciously held proteolytic impurities. They found that their tropomyosin preparations were inactive toward synthetic substrates for trypsin, chymotrypsin, pepsin, and allied cathepsins but they were able to hydrolyze hemoglobin. Several other cases of proteolytic damage to tissue proteins during preparative procedures have been recorded. A recent example is from the work of Nordwig and Hayduk (1969) where they found that subunits of liver fluke collagen were labile and could not be characterized by disc electrophoresis and this was attributed to contamination by proteases.

The molluscan tropomyosins showed different behavior to crayfish tropomyosin in 8 M urea. In addition to a main component of molecular weight in the region of 30,000–35,000 some aggregate was present, therefore measurements were made in 7 M guanidine hydrochloride–0.1 M mercaptoethanol. The results for abalone tropomyosin are shown in Figure 5 from which a value of 34,500 is obtained for the subunit molecular weight. The meniscus depletion method gave a value of 32,500. The log y *vs.* r^2 plots from the high-speed runs showed curvature typical of thermodynamic nonideality. A high degree of mass homogeneity existed in this solvent since the values of M_n and M_w extrapolated to zero fringe displacement were identical. Oyster tropomyosin gave somewhat lower values which were 30,400 from low-speed equilibrium, and 31,000 by

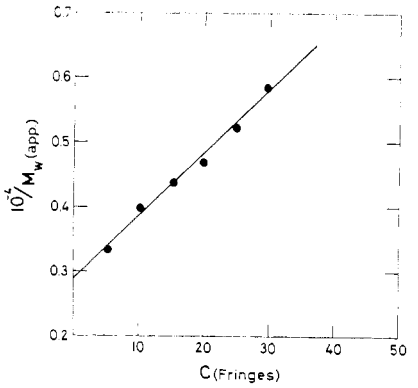

FIGURE 5: Cell average, $1/M_w$(app), for abalone tropomyosin in 7 M guanidine hydrochloride–0.1 M mercaptoethanol as a function of concentration (interference fringe numbers); column height 1.3 mm; 22,000 rpm, 20°.

the meniscus depletion method. No lower molecular weight species were detected but the concentration dependence of M_w (app) was not as great as for abalone tropomyosin and this probably indicates a slight heterogeneity.

The molluscan tropomyosins were not dissociated into subunits unless a disulfide bond reducing agent was present. When guanidine hydrochloride was employed with the addition of *N*-ethylmaleimide to prevent oxidation of SH groups, dissociation to subunits did not occur. These observations indicate that the polypeptide chains are probably joined by a disulfide bond. This may be present *in vivo* but it is possible that the SH group or groups of the individual chains are oxidized to SS during the isolation procedure.

Blowfly Tropomyosin. Only a small quantity of blowfly tropomyosin was available for physicochemical examination. The optical properties are given in Table I. The low value of 1.06 for the ratio E_{277}/E_{260} indicates some nucleic acid contamination. Molecular weights in 1.1 M NaCl–0.025 M sodium phosphate (pH 7) were measured by the meniscus depletion method. The minimum molecular weight for the main component was estimated to be in the range 65,000–75,000 and as with the other tropomyosins, dimers of this molecular weight were also present in solution. The only published figure for the molecular weight of an insect tropomyosin is that of Kominz *et al.* (1962) who found by low-speed equilibrium sedimentation, values of 65,600 for adult blowfly (*Phormia regina*) and 84,400 for the larval tropomyosin. They attributed the higher figure as due to greater polymerizability of the larval protein.

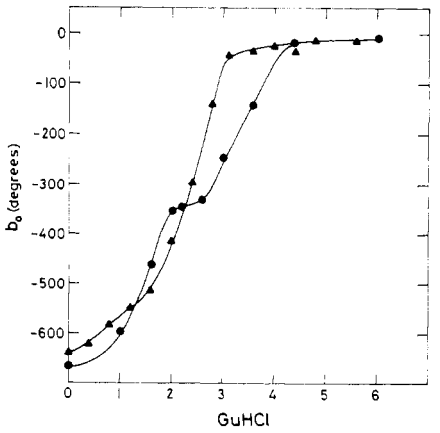

FIGURE 6: Denaturation of rabbit skeletal (▲) and abalone (●) tropomyosins by guanidine hydrochloride containing 0.05 M mercaptoethanol.

TABLE III: Amino Acid Compositions of Tropomyosins from Crayfish, Oyster, and Abalone and Paramyosins from Oyster and Crayfish (moles/10^5 g).

	Crayfish	Oyster		Abalone	
	Tropomyosin	Tropomyosin	Paramyosin	Tropomyosin	Paramyosin
Lys	87	86	60	88	60
His	3	4	11	1	7
Amide NH_3	(105)	(111)	(129)	(88)	(127)
Arg	64	58	85	61	91
CMCys (or $CySO_3H$)	<1[a,b]	2[a]	5[a]	3[b]	6[b]
Asp	112	104	111	105	110
Thr	29	40	39	44	35
Ser	37	40	46	33	45
Glu	218	239	185	229	200
Pro	<1	2	1	<1	<1
Gly	14	7	17	13	19
Ala	105	98	106	100	98
Val	36	27	31	29	39
Met	24	15	13	20	12
Ile	16	33	29	31	34
Leu	103	91	108	95	102
Tyr	12	15	14	15	10
Phe	10	7	9	6	7

[a] Determined as carboxymethylcysteine after reduction and reaction with sodium iodoacetate. [b] Determined as cysteic acid by the method of Moore (1963).

Investigations of the subunit size were carried out in 8 M guanidine hydrochloride–0.1 M mercaptoethanol. Low-speed equilibrium sedimentation gave a value of 29,000 for M_w. The meniscus depletion method indicated a main component of 34,700 together with some low molecular weight species. The subunit size could not be determined with great precision but was within the limits 32,000 ± 4000.

Denaturation. The values of the Moffitt parameter, b_0, as a function of guanidine hydrochloride concentration are shown in Figure 6 for abalone tropomyosin and rabbit skeletal tropomyosin. The curve for abalone tropomyosin shows two distinct stages whereas rabbit tropomyosin exhibits a smooth transition curve. Although there is little difference in the guanidine hydrochloride concentration at which the tropomyosins are half-unfolded as judged from the changes in b_0, rabbit tropomyosin unfolds more readily beyond this point. Similar results were also obtained when the parameter a_0 was plotted.

A comparison of the temperature transitions at pH 3 of rabbit skeletal and abalone tropomyosins was also made. The heat denaturation of rabbit tropomyosin at this pH has been shown to take place in distinct stages (Woods, 1968). The temperature transition curve for abalone tropomyosin showed similar features and the temperature at which the molecule was half-unfolded was within 1° of that for rabbit tropomyo-

in. The behavior was different to that found for crayfish ropomyosin (Woods, 1968) which gave a relatively smooth ransition with a transition temperature about 7° lower than he corresponding value for rabbit tropomyosin.

Amino Acid Analysis. Table III gives the amino acid analyes of crayfish (mean of four preparations), oyster, and abalone ropomyosins. Proline and half-cystine (determined as CM-Cys or $CySO_3H$) were detected in trace amounts only in crayish tropomyosin and this suggests that they do not occur in his protein. There is half-cystine/34,000 in both oyster and balone tropomyosins. Evidence given earlier suggests that his occurs as cystine. The crayfish tropomyosin is close in mino acid composition to that of the lobster (Kominz *et al.*, 958). The ratio of Lys/Arg is 1.37 which agrees with the ndings of Bailey and Ruegg (1960) and Kominz *et al.* (1962) hat invertebrate tropomyosins have a lower value for this atio. The ratios of Lys/Arg for the oyster and abalone tropoyosins are 1.48 and 1.44, respectively, and lie between the xtreme values (1.3 and 1.9) for the two types of molluscs iven by Kominz *et al.* (1962). Thus classification of molluscs nto two types on the basis of this ratio may be more artificial han real; a range of values may well exist. Since the parayosins from oyster and abalone were isolated on the way hrough the tropomyosin preparations their amino acid ompositions are also recorded in Table III. The analyses re in line with published results for other species which are haracterized by lower Lys/Arg and Glu/Asp ratios than or the tropomyosins.

Discussion

The broad salting-out range encountered with crayfish ropomyosin has also been observed by Maruyama (1959) with ropomyosin from a different species of crayfish. He found wo fractions which precipitated between 35 and 40% saturaion of $(NH_4)_2SO_4$ and between 40 and 60%. In a later paper, Maruyama *et al.* (1968) reported that the 22.5 to 37.5% fracon was found to be the most effective in restoring the calcium ensitivity of desensitized crayfish actomyosin. Hence this raction must contain a considerable amount of troponin. We nd that the fraction with the highest α-helix content precipiates between 30 and 40% saturation with $(NH_4)_2SO_4$, the 2–30% fraction is less helical and the 40–50% fraction generlly less helical but not always so. Hartshorne and Mueller 1969) have discussed in detail the variables involved in prearing pure tropomyosin and troponin from vertebrate nuscle. The crayfish, oyster, and abalone tropomyosins precitate between 30 and 45% saturation with $(NH_4)_2SO_4$ and it s this fraction which is discarded in the preparation of vertebrate tropomyosins. However, the solubility in water and ilute salt solutions, the amino acid composition and almost 00% α-helix content are sufficient to establish these proteins s tropomyosins. The corresponding paramyosins from oyster nd abalone differ from the tropomyosins in that they are recipitated at 0.2 ionic strength in neutral solutions, are alted out at lower $(NH_4)_2SO_4$ concentrations and possess a ys/Arg ratio in the range 0.6–0.8.

The $s^0_{20,w}$ values and molecular weights in high ionic strength uffer are higher for invertebrate tropomyosins than those for ertebrates under comparable conditions (Woods, 1969b) ndicating greater polymerizability. Similar conclusions were eached by Maruyama (1959) from flow birefringence studies n crayfish tropomyosin. Differences in polymerization beavior of tropomyosins from different sources have been bserved in several laboratories (Tsao *et al.*, 1956; Kominz *et al.*, 1962; Woods, 1969b). Variable molecular weights were obtained when apparent molecular weights were extrapolated to zero concentration depending on the concentration range in which measurements were made (Tsao *et al.*, 1956; Kominz *et al.*, 1962). The present paper and previous results on vertebrate tropomyosins (Woods, 1967, 1969b) indicate that at high ionic strength all tropomyosins are self-associating systems. Variable molecular weights found for tropomyosins from different sources are readily explained on recognition of this fact. Further research is necessary to determine the mode of aggregation and the equilibrium constant for the association of each tropomyosin. Because of the strong tendency to associate reliable values for the monomeric molecular weight of invertebrate tropomyosins were not obtained. The value of 86,000 (Table II) obtained by the meniscus depletion method for abalone tropomyosin represents an upper limit.

The subunit size of abalone tropomyosin falls within the range of values found for the vertebrate tropomyosins, *viz.*, 33,500 ± 2000. Oyster and blowfly tropomyosins gave values for the subunit molecular weight about 5–10% lower but in both of these tropomyosins there was some evidence of lower molecular weight species. Although the tropomyosins from the two species of crayfish behaved normally in salt solutions, in denaturing solvents they dissociated into nonstoichiometric amounts of fragments. The possibility that the molecules may have suffered proteolysis has been discussed earlier. Thus although the molecular weights suggest a subunit size for the invertebrate tropomyosins very similar to that found for vertebrates, the results do not give an unequivocal answer.

There is no evidence in this work and the preceding papers on the subunit structure of tropomyosins from various sources (Woods, 1967, 1968, 1969b; McCubbin *et al.*, 1967) to suggest nonidentity of the chains of tropomyosin. The disc electrophoretic patterns of Yasui *et al.* (1968) also support the concept of closely similar chains. The gel electrophoresis experiments of Bodwell (1967) which indicate two dissimilar subunits were performed on protein that had been in dilute alkali for 12 hr. As pointed out by Bodwell in a footnote to his paper, the subunits described could conceivably be two major fragments which have arisen by alkali cleavage.

Under suitable conditions tropomyosins crystallize in various polymorphic forms (Caspar *et al.*, 1969). The tactoidal form is characteristic of all tropomyosins and Millward and Woods (1970) have shown that tactoids from crayfish and oyster tropomyosins show the same axial periodicity as those formed from vertebrate tropomyosins. We have also shown that tactoids from abalone tropomyosin have this characteristic 400-Å periodicity. If this period defines a minimum length for the molecule, then the minimum molecular weight of a fully α-helical polypeptide chain of this length would be 31,000. If the overlap on aggregation is the same for all the tropomyosins then it seems reasonable to conclude that the subunits of both vertebrate and invertebrate tropomyosins have the same mass. The banding patterns of the tactoids from invertebrate tropomyosins are different from those observed for vertebrate skeletal and cardiac tropomyosins and this can be attributed to differences in chemical composition and thus to differing reactions to the electron stain.

Invertebrate tropomyosins differ from vertebrate tropomyosins in the following properties: amino acid composition, in particular the Lys/Arg ratio; salting-out range with $(NH_4)_2SO_4$; degree of polymerizability; banding patterns of the tactoids in the electron microscope; the course of denaturation with guanidine hydrochloride; difficulty of dissociation into subunits. Molluscan tropomyosins have one disulfide bond

between the polypeptide chains, crayfish tropomyosin has neither SH nor SS bonds, whereas vertebrate tropomyosins all have at least one SH group per polypeptide chain but no SS bonds. The absence of SH groups in crayfish tropomyosin would suggest that SH groups of tropomyosin cannot be implicated in the relaxing mechanism of muscle in agreement with the work of Yasui *et al.* (1968).

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