Comparative Physicochemical Studies on Vertebrate Tropomyosins*

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ABSTRACT: Physicochemical studies have been carried out on tropomyosins isolated by Bailey's method from the skeletal and heart muscles of the sheep and ox, and from the muscle of fowl gizzard. Equilibrium sedimentation at high ionic strength showed all the tropomyosins to be heterogeneous and the molecular weight concentration plots suggest a system of macromolecules which dissociates on dilution. However the results do not conform to the requirements of a rapidly polymerizing system in that the molecular weights as a function of concentration in the centrifuge cell for different initial concentrations do not overlap to form a continuous smooth curve. The possibility of heterogeneity arising from a slow self-association or the presence of contaminating proteins is discussed. The ultracentrifuge and viscosity results indicate that tropomyosins from the various sources exist in different states of aggregation. High-speed sedimentation equilibrium experiments at high ionic strength indicate that the minimum molecular weight for tropomyosins from all sources is close to the value of 68,000 previously found for rabbit tropomyosin. Molecular weight determinations in 8 M urea gave values about one-half of the minimum found in salt solution. Within the limits of the experimental methods of molecular weight determinations all the tropomyosins consist of subunits similar in mass and identical with the recent figure of 33,500 ± 2,000 reported for rabbit tropomyosin. The homogeneity of the subunits was examined by gel filtration, chromatography on DEAE-cellulose, and gel electrophoresis. Optical rotatory dispersion measurements and ultraviolet absorption data are reported. Quantitative amino acid analyses showed small differences between the tropomyosins, that for fowl gizzard tropomyosin showing the same trends as reported by other investigators for smooth muscle tropomyosins. Proline was absent from fowl gizzard tropomyosin and from a column-purified preparation of rabbit skeletal tropomyosin. The variable proline figures in other tropomyosins may arise from preparation impurities.

Since the isolation of tropomyosin by Bailey (1948) there have been several comparative chemical and physicochemical studies on tropomyosins from various sources (Tsao *et al.*, 1955; Jen and Tsao, 1957; Kominz *et al.*, 1957; Katz and Converse, 1964; McCubbin *et al.*, 1967; Carsten, 1968). Tsao *et al.* (1955) found marked differences in the physicochemical properties of tropomyosins from skeletal, cardiac, and smooth muscles and between vertebrate and invertebrate species. Chemical differences have been shown to exist between skeletal and smooth muscle tropomyosins (Kominz *et al*, 1957; Carsten, 1968). On the other hand, the studies of McCubbin *et al.* (1967) suggest that skeletal and cardiac tropomyosins are identical in terms of molecular size, shape, secondary and tertiary structures, and substructure.

In a preliminary report (Woods, 1968) differences were noted in the apparent molecular weights at finite protein concentrations between skeletal and cardiac tropomyosins when examined by equilibrium sedimentation in high ionic strength buffer at pH 7. The minimum molecular weights determined by the high-speed equilibrium method (Yphantis, 1964) however were all close to the value found for rabbit skeletal tropomyosin (Woods, 1967). Equilibrium sedimentation and osmotic pressure studies on rabbit tropomyosin led to the conclusion that tropomyosin monomers[1] and higher molecular weight species existed in chemical equilibrium (Woods, 1967). In the present paper the heterogeneity at high ionic strength of tropomyosins prepared from skeletal, cardiac, and smooth muscles of vertebrate species is further examined by low- and high-speed equilibrium sedimentation. Intrinsic viscosities, optical rotatory dispersion parameters, and amino acid analyses are also given. In addition equilibrium sedimentation and gel filtration studies in 8 M urea are reported in order to determine the size and homogeneity of the subunits.

Experimental Procedure

Isolation of Tropomyosins. The tropomyosins were isolated by the method of Bailey (1948) with the precautions mentioned in a previous publication (Woods, 1967). The hearts and leg muscles from heifers aged 8–12 months and sheep aged 15 months were used for the preparation of the bovine and ovine tropomyosins. The meat and hearts were obtained from animals freshly slaughtered by the Division of Animal Health, CSIRO. Fresh gizzards from adult domestic fowls were obtained from a commercial poulterer. The muscles from ten gizzards were pooled to prepare the gizzard tropomyosin; all the other preparations were from single animals. Gizzard tropomyosin was noticeably more viscous than the others and in the absence of salt was difficult to handle. The isoelectric precipitation step was therefore always carried out in the presence of 0.5 M KCl and this procedure was adopted in the preparation of all the tropomyosins. To obtain preparations of maximum α-helical content the isoelectric

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[1] The two-chain α-helical structure postulated for tropomyosin is designated monomer. The polypeptide chains of the monomer obtained by further dissociation in urea or guanidine hydrochloride are called subunits (Woods, 1967).

precipitation and ammonium sulfate fractionation were carried out at least three times. The ratio of the 277:260 nm extinction was used as a criterion of the presence of nucleic acid contamination. If the ratio was under 1.8 the solution was chromatographed on a DEAE-cellulose column although the results presented in Table V suggest that this procedure may not be completely effective in removing nucleic acids.

Methods. The procedures used for equilibrium centrifugation, gel filtration, and chromatography on DEAE-cellulose have been given in a previous publication (Woods, 1967). 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 Yphantis (1964). All ultracentrifuge experiments were performed at 20°. Velocity sedimentation was carried out at 59,780 and 67,770 rpm. The apparent partial specific volumes used in all calculations were 0.739 ml/g in high ionic strength buffers and 0.728 ml/g for tropomyosin in 8 M urea (Kay, 1960).

Optical rotatory dispersion measurements were made with a Perkin-Elmer spectropolarimeter, Model 141, at wavelengths of 365, 405, 436, 546, and 578 nm. Values of b_0 from the Moffitt equation were determined graphically as described by Urnes and Doty (1961). Linear plots were obtained with a value of 212 nm for the parameter, λ_0. The Jasco spectropolarimeter UV/5 was used for measurements at lower wavelengths.

Intrinsic viscosities were measured at 25° with Ostwald viscometers which had flow times for water of 200 sec.

For amino acid analyses the samples were hydrolyzed *in vacuo* with 6 N HCl at 110° for 24 hr. The amino acid content of the hydrolysate was estimated by the technique of Spackman *et al.* (1958) by means of a Beckman-Spinco 120B automatic amino acid analyzer. Special attention was paid to the determination of SH groups and proline. The SH groups were estimated as cysteic acid on performic acid oxidized protein or as CM-cysteine on the reduced and alkylated protein. Some of the hydrolysates were also run at five times the normal loading to obtain more accurate estimates of the CM-cysteine (or cysteic acid) and proline contents. Since there was good agreement between the cysteic acid and CM-cysteine values on the one preparation, only the latter are quoted in the results.

The *S*-carboxymethyl derivatives of tropomyosin and of the proteins used for calibrating the Sephadex G-200 column were prepared as described previously (Woods, 1967).

Results

Tropomyosins at Neutral pH and High Ionic Strength. All the preparations were examined by sedimentation velocity in 1.1 M NaCl–0.025 M sodium phosphate buffer (pH 7.0). The two ovine skeletal tropomyosins and one of the bovine skeletal preparations showed a small peak moving faster than the main peak and appeared to be more heterogeneous than their cardiac counterparts. The bovine skeletal and cardiac tropomyosins from a second animal appeared visually to be similar as regards boundary spreading on velocity sedimentation showing only a single peak. The smooth muscle tropomyosin from fowl gizzard gave a single peak in the ultracentrifuge at all concentrations.

The $s_{20,w}$ values were measured from the velocity of the maximum ordinate at six to eight concentrations in the range 0.1–0.9 g/100 ml and the concentration dependences are given by the following relations on least-squares analysis (c in g/100 ml); ovine skeletal (preparations 1 and 2), $1/s_{20,w} = 0.360 + 0.131c$; bovine skeletal (preparation 2), $1/s_{20,w} = 0.370 + 0.129c$; bovine cardiac (preparations 1 and 2), $1/s_{20,w} = 0.389 + 0.114c$; bovine cardiac (preparation 3), $1/s_{20,w} = 0.378 + 0.085c$; fowl gizzard, $1/s_{20,w} = 0.352 + 0.152c$; rabbit skeletal (Woods, 1967), $1/s_{20,w} = 0.372 + 0.130c$. The standard errors of the intercept and slope of the preceding equations are all less than 0.010 and 0.015, respectively. The two preparations from sheep hearts were only run at one concentration. Their $s_{20,w}$ values were 2.48 S at 0.41 g/100 ml and 2.44 S at 0.53 g/100 ml which are slightly higher than the ovine skeletal tropomyosins at the same concentration. Preparations varied as evidenced by the significantly different concentration dependences of the bovine cardiac tropomyosins. This variability between preparations is characteristic of previous findings for rabbit tropomyosin (Woods, 1967) where there was considerable scatter in the $1/s_{20,w}$ *vs.* c plots when a large number of preparations were included in the one graph. There was also a corresponding variability in the intrinsic viscosity, $[\eta]$, and its concentration dependence as shown by values for $[\eta]$ of 0.40 and 0.44 dl per g found for two preparations of rabbit tropomyosin (Table I).

Table I presents the $s^0_{20,w}$ values, intrinsic viscosities, and the constants, k', which are derived from the Huggins (1942) equation $\eta_{sp}/c = [\eta] + k'[\eta]^2c$. The specific viscosity–concentration plots were linear up to 1 g/100 ml except that for fowl gizzard tropomyosin which deviated from linearity above 0.5 g/100 ml. The values of these parameters found by other investigators at neutral pH and high ionic strength are also included for comparison as well as some results for acid solutions where homogeneity of the preparations was assumed.

Low-speed equilibrium sedimentation results previously reported for bovine and ovine tropomyosins (Woods, 1968) were carried out by the midpoint method of Yphantis (1960). These were repeated on different protein preparations with longer columns and slightly higher speeds and the molecular weights were calculated over the whole column by the more exact procedures of Richards *et al.* (1968). Figure 1 presents the molecular weight as a function of concentration for bovine skeletal, bovine cardiac, and fowl gizzard tropomyosins. At the higher concentrations thermodynamic nonideality is present characterized by an increase in M_w(app) as the solution is diluted. The curve passes through a maximum for the bovine tropomyosins. Fowl gizzard tropomyosin shows only the nonideal behavior although the leveling off of the molecular weight at the lowest concentration may point to a decrease at even lower concentrations. The behavior is similar to that expected for a nonideal self-associating system. With the exception of the highest concentration for bovine skeletal tropomyosin, the plots of log J (J = fringe number) as a function of r^2 (r = distance from centre of rotation) showed curvature indicative of heterogeneity.

Adams (1964) has shown that for a self-associating system molecular weight averages measured over the whole cell do not represent the average molecular weight of the original sample. The molecular weight averages, $M(r)$, calculated at a given radial distance, r, must be used to obtain information on the association. For a system in both sedimentation and rapid chemical equilibrium, the values of $M(r)$ are a

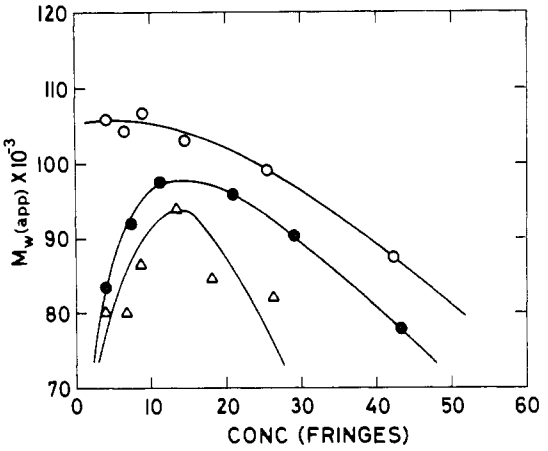

FIGURE 1: Low-speed equilibrium sedimentation of tropomyosin in 1.1 M NaCl–0.025 M sodium phosphate (pH 7) at 20°; weight-average molecular weight, M_w(app), plotted as a function of concentration expressed in interference fringe numbers. A 1% solution in a 12-mm cell at 546 nm corresponds to 41.3 fringes. (○) Fowl gizzard tropomyosin, (●) bovine skeletal tropomyosin, and (△) bovine cardiac tropomyosin.

function only of the concentration at that point (Adams and Fujita, 1963). Figure 2 presents a plot of $M_w(r)$ as a function of fringe number for each of the protein concentrations of Figure 1 for bovine skeletal tropomyosin. It is evident that the experimental points from different initial concentrations and speeds do not merge to form a continuous smooth curve as expected for an associating system. The results of some high-speed equilibrium experiments are also included in Figure 2 and these also do not overlap with the low-speed points. Similar results were obtained for rabbit skeletal, bovine cardiac, and fowl gizzard tropomyosins. Adams and Lewis (1968) recently reported that it was only possible to obtain overlap of the data from different experiments with β-lactoglobulin A if the FC 43 layering oil was omitted. They showed that the FC 43 fluorocarbon oil caused precipitation of the protein. In this laboratory paramyosin was shown to gel in the presence of FC 43 (Woods, 1969). However, no interaction of tropomyosin with FC 43 could be detected and the omission of layering oil did not give overlapping curves. In one experiment with the six-channel centerpiece the fringes were labeled by two methods: (a) by the conservation of mass in the cell (this was the method used routinely for the low-speed equilibrium results reported in this paper) and (b) by location of the hinge point by means of the white light fringe (Richards *et al.*, 1968). The values of the meniscus fringe number, J_m, calculated by the two methods agreed to within 0.15 fringe for the three solution–solvent pairs. Since the value of C_m in this experiment was approximately 14 fringes, all the material in the cell appears to be included in the molecular weight calculations and there appears to be no removal of protein at the solution–FC 43 interface. However this does not exclude the possibility that FC 43 may induce some aggregation. There was some variability in the molecular weight data between different preparations for the same tropomyosin. There were no obvious effects on the results of time and temperature of the initial dialysis as one might expect if slow equilibria were involved. Since tropomyosin has been shown to undergo end-to-end

TABLE 1: Comparison of Intrinsic Viscosities and Sedimentation Coefficients of Tropomyosins.[a]

Source of Tropomyosin	$[\eta]$	k'	$s^0_{20,w}$ (S)	References
Rabbit skeletal	0.18	3.15		Mueller (1966)
	0.21[b]		2.51	Carsten (1968)
	0.34	1.9	2.59	Holtzer *et al.* (1965)
	0.43[c]		2.53	Katz and Converse (1964)
	0.44	1.38	2.69	Woods (1967)
	0.40	2.19		and this study
	0.57			Tsao *et al.* (1951)
Rabbit heart	0.43[c]		2.53	Katz and Converse (1964)
Ox skeletal	0.48	3.09	2.70	This study
Ox heart	0.37	2.56	2.68	McCubbin *et al.* (1967)
	0.44	1.45	2.57 and 2.65	This study
Pig heart	0.45			Tsao *et al.* (1955)
Sheep skeletal	0.21[b]		2.51	Carsten (1968)
	0.43	4.79	2.78	This study
Sheep uterus	0.43[b]		2.82	Carsten (1968)
Human uterus	0.18[b]		2.48	Carsten (1968)
Duck gizzard	0.65			Tsao *et al.* (1955)
Fowl gizzard	0.47	4.48	2.84	This study

[a] Except where indicated measurements were made at neutral pH and high ionic strength (1.0–1.2). Since k' depends upon the solvent, it is only given for these solvent conditions. [b] 0.09 M KCl–0.01 M HCl. [c] 0.3 M KCl–0.01 M HCl.

association (Kay and Bailey, 1960) changes in polymerization with time may be reflected in viscosity measurements. Several preparations of tropomyosin, initially 1.0 g/100 ml, were diluted fivefold and the viscosity was followed with time. There was a small decrease (approximately 5%) in the viscosity number over 24 hr to a constant value within experimental error. The magnitude of this change was not sufficient to allow any definite conclusions to be reached.

Table II presents the results of high-speed sedimentation equilibrium experiments. The molecular weights of the smallest species were determined graphically from the plots of log y (where y = fringe displacement in centimeters) as a function of r^2 giving most weight to fringe displacements in the range 0.5–2.0 fringes (Yphantis, 1964). Initial concentrations were mostly in the range of 1–2 mg/ml and several speeds were used to increase the sensitivity of detection of low molecular weight species (Yphantis, 1964). In the majority of cases the plots of log y with respect to r^2 were nonlinear indicating the presence of species of higher molecular weights. This is in agreement with the heterogeneity observed in the low-speed runs. In some experiments lower molecular weight

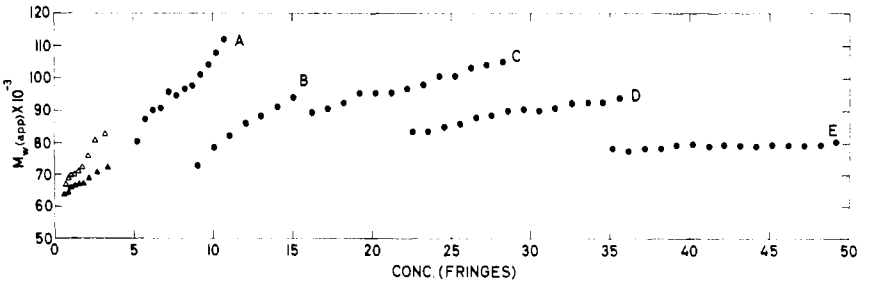

FIGURE 2: Equilibrium sedimentation of bovine skeletal tropomyosin in 1.1 M NaCl–0.025 M sodium phosphate (pH 7) at 20°. M_w(app)(r) plotted as a function of fringe number. (●) Low-speed equilibrium, speed 7928 rpm for the three highest concentrations and 9945 for the two lowest, column height approximately 1.3 mm. Initial concentrations in milligrams per milliliter were: A, 1.7; B, 2.6; C, 5.2; D, 6.9; and E, 10.4. (▲△) High-speed equilibrium results, speed 29,500 rpm, column height 3 mm; (▲) initial concentration 1.5 mg/ml; (△) initial concentration 0.15 mg/ml.

species were present, in particular, the tropomyosins from sheep, and these would tend to make the estimates for ovine tropomyosin somewhat low. The minimum molecular weights of skeletal and cardiac tropomyosins appear to be identical within the limitations of the high-speed method and they are within the range of minimum values found for rabbit skeletal tropomyosin (Woods, 1967). The values for fowl gizzard tropomyosin show a trend with speed and concentration. At higher speeds and initial concentrations the value approaches that for the other tropomyosins. Thus if fowl gizzard tropomyosin is also an associating system then the equilibrium must be in favor of higher molecular weight species. This is apparent from the low-speed results presented in Figure 1 where at a concentration of three fringes (0.07 g/100 ml) the molecular weight is 105,000 compared with a minimum value near 70,000 from the high-speed experiments (Table II).

TABLE II: Minimum Molecular Weights of 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
Sheep skeletal	1.5	59,500[b]	60,300[b]	55,600[b]
Sheep heart	1.5	60,000	61,800	
Ox skeletal (1)	2.1		63,400	57,400[b]
Ox skeletal (2)	1.5		65,200	62,300[b]
	0.5		66,800	
	0.15		68,600	
Ox heart (1)	1.9		67,800	
Ox heart (2)	1.5		66,500	65,100
Fowl gizzard	1.4	72,100	73,000	67,400
	0.5		77,700	
	0.15		80,000	

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

Tropomyosins in Acid Solution. On the basis of the results of Tsao *et al.* (1951) homogeneity of tropomyosin preparations is often assumed in acid solutions (for example, Carsten, 1968) although sedimentation equilibrium has shown that rabbit tropomyosin is heterogeneous under these conditions (Woods, 1967). Figure 3 presents the molecular weight as a function of fringe displacement from a high-speed equilibrium experiment on fowl gizzard tropomyosin in 0.09 M KCl–0.01 M HCl. Heterogeneity is clearly evident and the plots for the two speeds are not superimposable. The curves would extrapolate to a value between 60,000 and 70,000.

Tropomyosins in 8 M Urea. The weight-average molecular weights of the various tropomyosins in 8 M urea are given in Table III. Provided the SH groups are prevented from forming disulfide bridges by reaction with *N*-ethylmaleimide the chains can be dissociated without the necessity of a reducing agent, indicating for all the species the absence of disulfide bonds between the chains of the native molecule.

All the tropomyosins gave a single peak on gel filtration through Sephadex G-200 in 8 M urea with a sharp leading edge and some asymmetry on the trailing side. The calibrating proteins exhibited a sharper trailing edge as expected for a

TABLE III: Weight-Average Molecular Weights of Tropomyosins in 8 M Urea.[a]

Source of Tropomyosin	0.1 M Mercaptoethanol	0.02% *N*-Ethylmaleimide
Sheep skeletal	34,200	31,300
Sheep Heart	34,900	
Ox skeletal	33,700	33,300
Ox heart	32,800	36,000
Fowl gizzard	34,700	34,700

[a] Experimental conditions: 8 M urea–0.2 M NaCl–0.01 M Tris-HCl (pH 7.6) with either 0.1 M mercaptoethanol or 0.02% *N*-ethylmaleimide. Sedimentation equilibrium experiments were carried out by the midpoint method of Yphantis (1960) at four concentrations in the range 0.2–0.9 g/100 ml employing 1-mm columns and speeds of 14,290 and 16,200 rpm. The molecular weights were extrapolated to zero concentration from plots of $1/M_w$(app) with respect to c.

TABLE IV: Molecular Weights of Tropomyosins in 8 M Urea from High-Speed Sedimentation Equilibrium and Gel Filtration.[a]

Source of Tropomyosin	Mol Wt from Sedimentation Equilibrium: Init Concn (mg/ml)	Minimum[b]	$\bar{M}_n$	$\bar{M}_w$	$\bar{M}_z$	Mol Wt from Elution Vol
Sheep skeletal	0.15	35,400	35,600	35,000	34,500	31,700
Ox skeletal (1)	0.15	35,700	35,000	33,600	32,500	32,600
Ox skeletal (2)	0.15	35,900	35,100	34,500	33,330	34,700
Ox heart (2)	0.14	33,100	32,600	32,300	31,000	34,800
	1.0	32,900				
Fowl gizzard	0.20	35,900	33,300	31,500	29,800	33,100

[a] The proteins were treated with mercaptoethanol in 8 M urea and reacted with sodium iodoacetate to prevent SH reactions. After dialysis to remove excess reagents the proteins were submitted to gel filtration on a calibrated column of Sephadex G-200 in 8 M urea–0.2 M NaCl–0.01 M Tris-HCl (pH 7.6). The peak tubes were examined in the ultracentrifuge in the same solvent at 44,770 rpm, 20°, and a column height of 3 mm. [b] This was estimated graphically and also from $M_n(r)$ and $M_w(r)$ extrapolated to zero fringe displacement.

nonideal solution (Winzor and Nichol, 1965) so the asymmetry observed with the tropomyosins is attributed to the presence of some lower molecular weight material. Estimates of the molecular weights were made from the elution volumes of the peak tubes and are shown in the last column of Table IV. The peak tubes were also examined by high-speed equilibrium sedimentation. The minimum molecular weights were estimated from fringe displacements above 100 μ and there was no evidence from smaller fringe displacements of lower molecular weight species. The results are presented in Table IV. In all cases the plots of log y as a function of r^2 showed curvature indicative of nonideality. As a consequence the average molecular weights, $M(r)$, decrease through the cell and $\bar{M}_n > \bar{M}_w > \bar{M}_z$. The values $\bar{M}_n$, $\bar{M}_w$, and $\bar{M}_z$ refer to an average concentration over the cell contents and will be higher at zero concentration. From the concentration dependence found in low-speed runs, $\bar{M}_w$ will increase by about 5% (there are approximately five fringes at the base for the experiments in Table IV), $\bar{M}_n$ by a lesser amount, and $\bar{M}_z$ by a larger amount to give near identical values for the three average molecular weights. The absence of smaller molecular weight species indicates a high degree of homogeneity, and the mean value of all centrifuge data is close to 35,000.

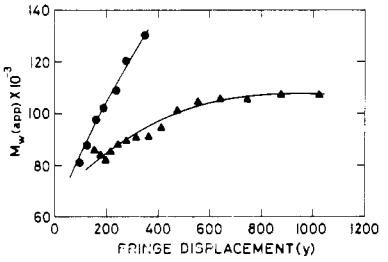

FIGURE 3: High-speed equilibrium sedimentation for fowl gizzard tropomyosin in 0.09 M KCl–0.01 M HCl at 20°, column height 3 mm, M_w(app)(r) plotted as a function of fringe displacement in microns (1 fringe = 284 μ). Initial concentration was 1.4 mg/ml. (●) 39,460 rpm and (▲) 27,690 rpm.

The CM derivatives of the tropomyosins were chromatographed on DEAE-cellulose in 8 M urea–0.01 M Tris-HCl (pH 7.6) at 20° and eluted with a linear gradient to 0.5 M KCl. More than 90% of the ultraviolet-absorbing material was eluted in one main asymmetrical peak at the same salt concentration as found for rabbit tropomyosin (Woods, 1967). When submitted to acrylamide gel electrophoresis in 8 M urea containing thioglycollic acid a single band was obtained in all cases (Woods, 1968). If the gels were overloaded some material moving faster than the main peak could be detected. Mixtures of the various tropomyosins

TABLE V: Optical Properties of Vertebrate Tropomyosins in 1.1 M NaCl–0.025 M Sodium Phosphate (pH 7.0).

Source of Tropomyosin	$E^{1\%}_{1\,cm}$	E_{277}/E_{260}	b_0 (deg)	a_0 (deg)	m_{232} (deg)
Sheep skeletal	3.28	2.3	−610	−18	−14,930
Ox skeletal (2)	3.10	2.2	−620	0	−15,000
Ox heart (2)	3.32	2.2	−650	+30	−15,800
Ox heart (3)	4.01	1.5	−630	+20	−15,400
Ox heart (3[a])	3.41	2.0	−630	0	−16,000
Fowl gizzard	2.18	2.0	−650	+20	−15,500
Rabbit skeletal (37)	3.76	1.6	−635	+15	−15,200
(37[b])	3.21	1.9	−600	−15	−15,400
(41)	3.20	2.2	−580	−15	−15,300
(42)	3.75	1.6	−615	+5	−15,800
(42[a])	3.43	1.9	−610	−5	−15,700
(43)	3.23	2.3	−660	+20	−16,500

[a] Remeasured after passage through DEAE-cellulose equilibrated with 0.5 M KCl in order to remove any nucleic acid contaminants. [b] The SCM derivative was submitted to gel filtration in 8 M urea on Sephadex G-200. Tubes from the main peak were pooled excluding the tails, dialyzed to remove the urea, precipitated at pH 4.5, dissolved in the NaCl–phosphate buffer, and equilibrated against the same buffer.

TABLE VI: Amino Acid Compositions of Tropomyosins (moles/10^5 g).

	Skeletal			Cardiac		Smooth Fowl (Gizzard)
	Rabbit[a]	Ox	Sheep	Ox	Sheep	
Lys	115	120	113	120	124	115
His	6	6	7	6	6	9
Arg	43	45	49	44	42	51
CM-Cys	2.8–4.6[b]	4.7	4.7	3.9	Not done	3
Asp	92	93	90	92	82	83
Thr	23	24	23	23	23	24
Ser	39	35	35	39	37	38
Glu	220	226	215	221	228	235
Pro	0–4.1[b]	3.7	5.0	1.4	4.3	0
Gly	13	15	15	10	13	14
Ala	113	108	108	113	103	108
Val	29	26	31	26	26	35
Met	20	18	21	15	18	19
Ile	33	31	32	32	31	27
Leu	98	90	97	99	109	94
Tyr	16	17	17	18	16	11
Phe	4	6	7	5	8	4

[a] Mean of analyses on four preparations. [b] Range of values found for seven preparations (see text).

also moved as a single band on gel electrophoresis. When the reducing agent was omitted from the gels slower moving bands appeared and these are attributed to higher molecular weight species produced by oxidation of SH groups to produce disulfide-bonded species.

Optical Properties. Table V presents the ultraviolet absorption and optical rotatory dispersion parameters of the tropomyosins discussed in this paper. The variability in extinction coefficients at 277 nm and the 277:260-nm extinction is attributed to nucleic acid contamination which can be reduced by passage through DEAE-cellulose. The values of $E_{1\,cm}^{1\%}$ are in reasonable agreement with values quoted for rabbit skeletal tropomyosin (Holtzer *et al.*, 1965; Woods, 1967) and bovine cardiac tropomyosin (McCubbin *et al.*, 1967).

Ooi (1967) however quotes a significantly lower value of 2.5 for $E_{1\,cm}^{1\%}$ for rabbit skeletal tropomyosin and Hartshorne and Mueller (1969) a value of 2.9 for tropomyosin prepared by a modified procedure. The extinction coefficient of fowl gizzard tropomyosin is significantly less than that of the others and this is a reflection of the lower tyrosine content of this protein (Table VI).

The optical rotatory dispersion data indicate that the proteins are almost completely α helical as deduced from the b_0 value and the magnitude of the trough of the Cotton effect at 232 nm. The values of m'_{232} range from $-15{,}000$ to $-16{,}500°$ and are slightly higher than McCubbin and Kay's (1969) values for bovine cardiac tropomyosin. The results suggest that a 100% α helix would have a value near $-16{,}000°$. The peak of the Cotton effect at 198 nm was measured in 0.6 M sodium fluoride at pH 7 for fowl gizzard tropomyosin and gave $m'_{198} = +68{,}000°$. As seen for one of the rabbit tropomyosin preparations (37) the conformation changes induced by 8 M urea could be reversed to give optical rotatory properties indistinguishable from the native protein.

Amino Acid Composition. Table VI gives the amino acid compositions of the tropomyosins studied in this paper. For comparative purposes all the results have been converted into the basis of 870 residues/10^5 g, that is, to a mean residue weight of 115. The results for rabbit skeletal tropomyosin are in good agreement with published figures (Kominz *et al.*, 1957; Katz and Converse, 1964; Carsten, 1968) and those for sheep skeletal tropomyosin are similar to those of Carsten (1968) when all comparisons are made on the basis of 870 residues/10^5 g. There are small differences between the skeletal and cardiac tropomyosins from the one species and all the skeletal and cardiac tropomyosins are very similar. The amino acid composition of fowl gizzard tropomyosin shows similar trends to those reported for other smooth muscle tropomyosins (Carsten, 1968; Kominz *et al.*, 1957) which were found to contain more arginine, histidine, glutamic acid, and valine and less aspartic acid, lysine, isoleucine, and tyrosine than skeletal tropomyosins. However because of the variability between the three skeletal tropomyosins (Table VI) a comparison with the amino acid composition of fowl skeletal tropomyosin would be preferable to confirm these differences.

The range of values found for CM-cysteine for seven preparations of rabbit tropomyosin was 2.8–4.6 moles/10^5 g of protein with a mean figure of 3.8 moles/10^5 g. This figure is lower than some recent estimates of the SH content of rabbit tropomyosin where 5–7 groups/10^5 g were indicated (Drabikowski and Nowak, 1965; Mueller, 1966). The mean value of the proline content of rabbit skeletal tropomyosin was 3.3 moles/10^5 g and the range 2.6–4.1 moles/10^5 g. Proline could not be detected in one preparation of rabbit tropomyosin which had been further purified by fractionation on DEAE-cellulose in 8 M urea and by passage through Sephadex G-200 in 8 M urea. The CM-cysteine and proline contents of the cardiac and skeletal tropomyosins from the

sheep and ox showed a similar variability. Bovine cardiac tropomyosin showed a lower proline value (1.4 moles/10^5 g), and none could be detected in fowl gizzard tropomyosin. The glycine content of the bovine cardiac tropomyosin seems significantly lower than for the others and agrees with a similar finding by Kominz *et al.* (1957).

Discussion

The results of this investigation indicate that vertebrate tropomyosins from all types of muscle and from different species are heterogeneous when studied by equilibrium sedimentation both at high ionic strength, pH 7, and also at acid pH values at lower ionic strengths. The results agree with those previously found in this laboratory for rabbit skeletal tropomyosin (Woods, 1967). The tropomyosin solutions show indications of dissociation on dilution but do not fulfill the criterion of a rapid association–dissociation equilibrium. The apparent molecular weight at a point in the centrifuge cell is not a unique function of concentration as required for a system in sedimentation and rapid chemical equilibrium (Adams and Fujita, 1963). This is shown by the results in Figure 2 where it is seen that the plots of $M_w(r)$ as a function of concentration for different operating speeds and initial concentrations do not form a continuous curve. The reason for this was not due to interaction with FC43 as found in some other systems (Adams and Lewis, 1968). At the speeds used in the low-speed sedimentation equilibrium experiments pressure effects are not expected to be important. A similar situation has been reported recently by Nichol (1968) for chymotrypsinogen A. One explanation is that the association is slow compared with the time of sedimentation equilibrium. However no definite evidence was obtained from either sedimentation or viscosity measurements of a slow equilibrium. Since the ultracentrifuge experiments do indicate dissociation on dilution (Figure 1), a more likely explanation for the observations of Figure 2 is that some species are present which do not take part in the association. These could be disulfide-bonded species since it has been shown that the SH groups of tropomyosin are readily oxidized (Drabikowski and Nowak, 1965). In some preparations of rabbit skeletal tropomyosin acrylamide gel electrophoresis indicated the presence of bands corresponding to disulfide-bonded species (Woods, 1967). However crayfish tropomyosin is devoid of SH groups and behaves similarly to the vertebrate tropomyosins (E. F. Woods, unpublished results).

Nucleic acids are a common contaminant of tropomyosins and their presence may affect the polymerization behavior. Hamoir (1951) has shown that nucleotropomyosins are more heterogeneous than tropomyosins. One of the bovine preparations showed evidence of some nucleic acid contamination (3 in Table V) and this did in fact show a slightly higher $s^0_{20,w}$ value than the other two. On the other hand, preparations which appeared essentially free of nucleic acid showed heterogeneity on low-speed equilibrium sedimentation and the point-average molecular weights from different experiments did not superimpose (see Figure 2). It is difficult to prepare tropomyosin completely free from other proteins and the salt-dependent aggregation complicates the detection of contaminants. Investigations on rabbit skeletal tropomyosin in aqueous buffers and in 8 M urea (Woods, 1967) indicated the presence of a small proportion of proteins other than the main component in tropomyosins prepared by Bailey's (1948) procedure. The presence of another protein not greatly contributing to the heterogeneity itself but promoting the aggregation of tropomyosin could explain the molecular weight results at finite concentration. Ebashi and Kodama (1965) have shown that troponin promotes the aggregation of tropomyosin. Troponin can be detected by its ability, when in combination with tropomyosin of regulating the interaction of myosin and actin (Ebashi, 1963; Ebashi and Ebashi, 1964). The tropomyosins studied in this paper have not been tested for this activity, but Carsten (1968) prepared tropomyosins by the Bailey (1948) method and found them to be free of troponin. Yasui *et al.* (1968) consider that even the best tropomyosin preparations contain traces of troponin. They showed that troponin moved faster than tropomyosin on disc gel electrophoresis. I find traces of such faster moving bands in many tropomyosin preparations and they may be more readily seen if the gels are overloaded (Woods, 1967). Hartshorne and Mueller (1969) imply that all high molecular weight species which are seen in classical Bailey-type tropomyosin preparations at high ionic strength are manifestations of the tropomyosin–troponin complex. This is a possible explanation of the low-speed equilibrium observations. The troponin content of the fowl gizzard tropomyosin must however be extremely small because of the absence of proline. Troponin has been reported to contain about 30 moles of proline/10^5 g (Hartshorne and Mueller, 1968; Ebashi *et al.*, 1968) although the actual proline contents of tropomyosins are uncertain. Fowl gizzard tropomyosin gives the highest molecular weights (Figure 1) and the low-speed sedimentation equilibrium data show the same noncoincidence of molecular weight–concentration plots from runs at different initial concentrations and speeds.

The high-speed equilibrium results (Table II) show that the smallest species present at high ionic strength is between 60,000 and 70,000. A more accurate figure is difficult to obtain since quite small quantities of low molecular weight species could have a marked influence on this estimate and account for the variability of the values given in Table II.

The results of this investigation can be readily reconciled with those of Tsao *et al.* (1955) who reported 89,000 for the molecular weight of porcine cardiac tropomyosin and 153,000 for duck gizzard tropomyosin. Their determinations were made by osmotic pressure measurements in the concentration range 1–5 g/100 ml and their plots of π/c (where π = osmotic pressure) as a function of c were linear with a positive second virial coefficient. It is clear from Figure 1 that low-speed sedimentation equilibrium data extrapolated from high concentrations would give differing molecular weights for the various tropomyosins whereas the high-speed results (Table II) suggest a common minimum molecular weight. In other comparative studies (Katz and Converse, 1964; Carsten, 1968) absolute methods of molecular weight determination have not been employed but the recent measurements of McCubbin *et al.* (1967) and McCubbin and Kay (1969) on bovine cardiac tropomyosin offer a better comparison. They concluded that their preparations were monomeric since the intrinsic viscosity reached a minimum value in solutions above an ionic strength of 0.6. Below this salt concentration they showed that bovine cardiac tropomyosin is heterogeneous and consists of a monomer in equilibrium with its aggregates. At higher ionic strengths there was no tendency to polymerize.

In spite of the disagreement about the aggregation of bovine cardiac tropomyosin at high ionic strength the value of the monomer molecular weights reported here are in reasonable agreement with the estimates of McCubbin *et al.* (1967). The only difference in procedure in the two laboratories is the presence of dithiothreitol during the isolation of tropomyosin by McCubbin *et al.* (1967). The results reported in Table III indicate that –S–S– bonds are not present to any great extent in the tropomyosins studied in this laboratory since dissociation to subunits occurs without the necessity of a reducing agent.

The results collected in Table I from various investigations on tropomyosins show variability in the values of $[\eta]$, k', and $s^0_{20,w}$. The concentration dependence of $s_{20,w}$ values shows a similar variability. Although differences in the $s_{20,w}$ values are not large, they probably represent considerable differences in molecular weight since the sedimentation coefficients of rodlike macromolecules are not very sensitive to molecular weight. The investigators whose results are quoted in Table I considered that the physical constants refer to "monomeric" tropomyosin with the exception of the sheep uterus tropomyosin (Carsten, 1968) which was more aggregated. The range of values found for $[\eta]$ for rabbit skeletal tropomyosin (0.18–0.57 dl/g) is rather wide for a homogeneous protein. The accepted molecular model for rabbit skeletal tropomyosin is two α helices side by side and is based on the X-ray diffraction pattern (Cohen and Holmes, 1963), the almost 100% α-helical content (Cohen and Szent-Gyorgi, 1957), the light-scattering and hydrodynamic properties (Holtzer *et al.*, 1965), electron microscopy (Rowe, 1964), and subunit molecular weight of 33,500 ± 2,000 (Woods, 1967). The length of such a molecule would be 400–500 Å and its diameter 18–20 Å with an axial ratio, p, of about 24:1. Kirkwood and Auer (1951) give the following relation between $[\eta]$ and p, for rodlike macromolecules; $[\eta] = 24p^2/9{,}000\rho \ln p$, where ρ is the density of the particle. For $p = 24$, $\rho = 1.35$ ($\bar{v} = 0.739$), and assuming no hydration, $[\eta]$ should be 0.36 dl/g. Hydration would decrease ρ and increase $[\eta]$. Thus values of $[\eta]$ in the vicinity of 0.2 dl/g (Table I) are incompatible with the model generally accepted for the tropomyosin molecule. The low values may result from side-to-side association and although Mueller (1966) considered this to occur in neutral solvents together with end-to-end association he regarded the intercept (0.18 dl/g) of the reduced viscosity–concentration plots as characteristic of monomeric tropomyosin. The values of the Huggins constant, k', are high and variable (Table I). This again points to varying degrees of aggregation and is supported by actual molecular weight measurements (Figures 1 and 2). Holtzer *et al.* (1965) and McCubbin *et al.* (1967) reject this explanation because they found no anomalous concentration dependence in molecular weight measurements.

The results of studies of tropomyosins in 8 M urea by equilibrium sedimentation, gel filtration, and gel electrophoresis indicate a common subunit size. The polypeptide chains are not linked by disulfide bonds. The range of values found for the subunit size of rabbit tropomyosin was 33,500 ± 2,000 (Woods, 1967) and the results reported in Tables III and IV fall within this range. The mean value of 35,000 from Table IV is slightly higher than the value found for rabbit tropomyosin but within the experimental uncertainty in the determinations. Thus it seems reasonable to conclude that the polypeptide chains of all vertebrate tropomyosins are identical in mass. The minimum molecular weight in salt solutions should be double the subunit size on the basis of the two chain model, that is 67,000 ± 4,000. The experimental results (Table II) are in accord with this figure.

The amino acid composition suggests a close chemical similarity in the skeletal and cardiac tropomyosins. The differences between the amino acid compositions of the two tropomyosins from each species are somewhat greater than found by Katz and Converse (1964) who found rabbit skeletal and cardiac tropomyosins to be almost identical. Small chemical differences could easily account for the differences observed in the polymerizability. Jen and Tsao (1957) suggest a parallelism between the arginine content and polymerizability when different tropomyosins are compared. While such correlations are difficult to establish with certainty, the higher degree of polymerizability of fowl gizzard tropomyosin does not conflict with Jen and Tsao's (1957) observations.

Although steps were taken to obtain more accurate CM-cysteine and proline figures, the results are variable when different preparations from the same species are examined. Thus on the basis of the proline figures it is not possible to decide whether the two polypeptide chains are dissimilar. The absence of proline in some preparations suggests the possibility that all the proline may arise from troponin in the preparations. This may account for the variability in the SH content found in this and other investigations since troponin has, in addition to a higher proline content (Hartshorne and Mueller, 1968), a significantly greater number of SH groups (Yasui *et al.*, 1968).

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Use of N^{im}-Dinitrophenylhistidine in the Solid-Phase Synthesis of the Tricosapeptides 124–146 of Human Hemoglobin β Chain*

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ABSTRACT: The tricosapeptide H-Pro-Pro-Val-Gln-Ala-Ala-Tyr-Gln-Lys-Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-Ala-His-Lys-Tyr-His-OH, corresponding to residues 124–146 of the β chain of human hemoglobin, was synthesized by the solid-phase method.

The imidazole rings of the two histidine residues were protected by the dinitrophenyl group and were deprotected by the thiolysis method of Shaltiel. Crystalline N^{α}-*t*-butyloxycarbonyl-N^{im}-2,4-dinitrophenyl-L-histidine was synthesized and shown to couple rapidly and efficiently by the dicyclohexylcarbodiimide procedure. The dinitrophenyl group was stable during the subsequent synthetic steps and in the cleavage step, but could be removed cleanly at the end under mild conditions by treatment with mercaptoethanol. This promises to be a very effective way to handle histidine during solid-phase peptide synthesis.

The C terminus of the β chain of human hemoglobin plays an important part in the determination of the biological activity of this protein. Thus, after the removal of His-146 and Tyr-145 by carboxypeptidase A hemoglobin showed a marked change in its affinity for oxygen and a considerable reduction of the Bohr effect (Rossi-Fanelli *et al.*, 1964). In order to study some of the chemical and physical properties associated with the carboxyl end of the chain we have undertaken the synthesis of the β-chain 124–146 tricosapeptide of human hemoglobin, H-Pro-Pro-Val-Gln-Ala-Ala-Tyr-Gln-Lys-Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-Ala-His-Lys-Tyr-His-OH (Braunitzer *et al.*, 1961).

One of the important problems associated with the synthesis of histidine-containing peptides has been the selection of a suitable protecting group for the imidazole nitrogen of the amino acid. The benzyl group has been used most often for histidine but it does not mask the basicity of the imidazole ring and, in addition, it can be difficult to remove. In cases where catalytic hydrogenolysis is slow or impossible it has been necessary to use sodium in liquid ammonia to remove the benzyl group, and to accept the low yields and side reac-

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