Biochem. J. (1967) **103**, 634

Isolation of *Aulacomya* Paramyosin

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(*Received* 30 *September* 1966)

Tropomyosin A or paramyosin has been isolated from the adductor muscle of *Aulacomya magellanica*. It has in common with other tropomyosins A the method used for extracting it from adductor muscle, its solubility, facility of crystallization, ammonium sulphate range of precipitation, amino acid composition and behaviour when digested with trypsin. As a particular feature it exhibits an unusual low viscosity for this type of tropomyosin. Its molecular weight, determined by the Archibald approach-to-sedimentation-equilibrium method, is 258000 ± 16000.

Since its isolation (Bailey, 1946) from the fibrils of rabbit skeletal and cardiac muscle, tropomyosin was found in striated and smooth muscle (Sheng & Tsao, 1954; Tsao, Tan & Peng, 1955; Sheng, Tsao & Peng, 1956; Jen & Tsao, 1957) of a number of different species, both vertebrate and invertebrate. It is the only component of muscle that has been subjected to any determined comparative study. This has been facilitated by the fact that it is a protein of peculiar properties which is relatively easy to crystallize. There are two types of tropomyosin, distinguished mainly by their solubilities: paramyosin or tropomyosin A, which is insoluble in water and precipitated at 20–35% saturation of ammonium sulphate; and tropomyosin B, which is soluble in water at neutral pH and precipitated at above 40% saturation of ammonium sulphate. The latter has been isolated from the striated and smooth muscle of vertebrates and invertebrates, whereas paramyosin has been obtained only from invertebrate smooth muscle, where it is present in large amounts. It may be responsible for the ability of the tonic muscle of molluscs to sustain tension for long periods without extra consumption of energy (Rüegg, 1958, 1961; Johnson, Kahn & Szent-Györgyi, 1959).

The present paper reports the isolation of paramyosin in crystalline form from *Aulacomya magellanica* specimens collected in Puerto Deseado (Argentina), and some of its properties, which permit it to be compared with tropomyosins extracted from several molluscs of different origins.

MATERIALS AND METHODS

Ethanol-dried powder from adductor muscle of Aulacomya magellanica. An ethanol-dried powder was obtained, by a modification of the method of Bailey (1956) for the whole adductor muscle of *Pinna nobilis*. Living specimens kept at 0–4° in sea water were obtained from the Centro de Investigaciones de Biología Marina (Puerto Deseado, Argentina). The adductor muscles were excised and frozen until a reasonable amount of tissue had been collected. The ethanol method was then applied but with only 2–3 vol. of cold water. After being dried in ether, the powder was spread out to dry at room temperature. This powder can be kept in the refrigerator for a year or more. The protein was prepared by starting from the ethanol-dried powder and following Bailey's (1956) procedure. Samples were crystallized three times.

Total nitrogen. This was determined by a modification (Chibnall, Rees & Williams, 1943) of the Kjeldahl method.

α-Amino nitrogen. This was determined with ninhydrin according to modifications described by Chibnall, Mangan & Rees (1958).

N-*Terminal groups.* These determinations were carried out by Sanger's (1945) method. The protein content of the DNP-protein was assumed to be 70% (Porter & Sanger, 1948; Bailey, 1951), allowance being made for water and DNP content. Correction factors for losses in the hydrolysis and chromatography (20% and 10% respectively) were applied. The DNP-amino acids were run on buffered paper in the phthalate–2-methylbutanol-2-ol system of Blackburn & Lowther (1951). The chromatography standards of DNP-amino acids used were prepared as described by Sanger (1945).

C-*Terminal groups.* These were determined by using carboxypeptidase A, a three-times-recrystallized water suspension from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.). Locker's (1954) procedure was followed. The protein was dissolved in M-$(NH_4)_2CO_3$ and the enzyme/protein ratio used was 1:50 (w/w). Salt was eliminated from the samples by drying them in a desiccator two or three times. Then they were run by descending chromatography in butanol–acetic acid–water (4:1:5, by vol.). Controls of the protein and the enzyme were run simultaneously. The paper was quantitatively developed by dipping it in a 0·5% ninhydrin solution in acetone and leaving it in the dark for more than 12 hr. The paper was then dipped in a solution containing Cu^{2+} ions (Kawerau & Wieland, 1951) and left to dry for 30 min. The spots were cut out of the paper and eluted in 1 ml. of methanol in the

dark with occasional stirring for not less than 4hr. Colour was read in a Coleman spectrophotometer at 530mμ, calibration curves being obtained with standard solutions of amino acids.

Quantitative amino acid analysis. This was performed in a Beckman Spinco model MS automatic amino acid analyser. Samples were hydrolysed with constant-boiling HCl in a sealed evacuated tube at 105° for 18hr.

All of the following physical determinations were carried out in 0·5M-KCl in 0·05M-phosphate (0·035M-Na_2HPO_4–0·015M-NaH_2PO_4) buffer, pH7·0.

Ultracentrifugation. Measurements were carried out in a Spinco model E analytical ultracentrifuge at 59780 rev./min. at several protein concentrations at 20°. The logarithm of the distance of the boundary from the axis of rotation was plotted against time. The slope of the straight line obtained was used in calculating the sedimentation coefficients at each concentration of protein. The sedimentation coefficients were corrected to the viscosity and density of water at 20° as described by Svedberg & Pedersen (1940). A determination of the molecular weight by the Archibald approach-to-sedimentation-equilibrium method was performed according to Elias (1960) at 8225 rev./min. for 20min. The standard 12mm. 4° synthetic-boundary cell was used with a drop of Dow-Corning 555 silicone in the bottom. The molecular weight was calculated from the meniscus by applying the formula:

$$\frac{1}{\bar{M}} = \frac{(1-\bar{v}\rho_{solv.})\omega^2 A x_m}{RTz_0}$$

where $\bar{M}$ is the average molecular weight, $\bar{v}$ the partial specific volume of the protein, $\rho_{solv.}$ the density of the solvent, ω the angular velocity, A the area measured in the first photograph, x_m distance of the meniscus from the axis of rotation, R the gas constant, T the absolute temperature and z_0 the limit $z_m(t\rightarrow 0)$, z_m being the height of the meniscus. Values for the bottom of the cell were not used to avoid silicone–protein interaction.

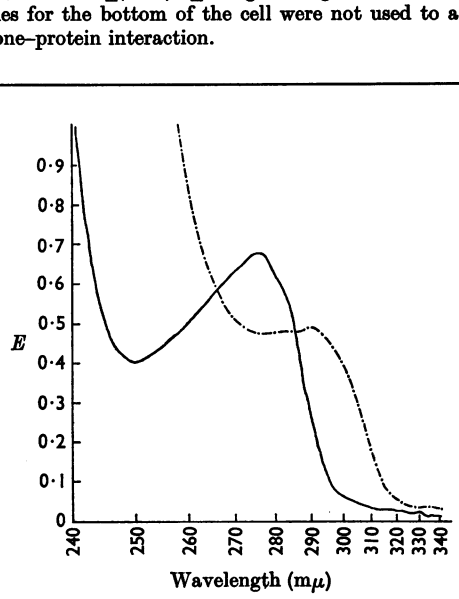

Fig. 1. Ultraviolet spectrum of *Aulacomya magellanica* paramyosin solutions in: –·–·–, 0·5M-KCl–0·1N-NaOH; ——, M-KCl, pH7·5.

Viscosity measurements. These measurements were made with an Ostwald-type viscometer having a flow time of 46sec. for the solvent used. The measurements were carried out at 25±0·1°.

Absorption spectrum. The spectrum of paramyosin was determined in a Beckman DK2A spectrophotometer. At neutral pH the spectrum showed a maximum at 276mμ and a minimum at 250–251mμ (Fig. 1). The E_{260}/E_{280} ratio was 0·885 for the three-times-crystallized preparation, indicating some contamination with nucleic acid. After a fourth crystallization the ratio decreased to 0·787, suggesting that contamination with 1–2% of nucleic acid might still be present. The extinction coefficient ($E^{1\%}_{1cm.}$) determined in a Beckman DU spectrophotometer was 3·4 at 280mμ in M-KCl at pH7·5 and 3·7 at 276mμ in the same solvent. These values were the same when the protein was crystallized three or four times.

RESULTS

Table 1 shows the yield of ethanol-dried powder obtained from fresh muscle and the yield of protein obtained in turn from the powder. The recovery for powder and paramyosin is slightly larger in *Aulacomya* than in *Pecten*. The protein yield is much larger still in *Pinna*. When the protein solution obtained by extracting the muscle powder in M-potassium chloride was dialysed against 0·1–0·2M-potassium chloride at neutral pH, crystals were obtained (Fig. 2). These fine needles, forming rosettes, are similar to those of tropomyosin A obtained by Bailey (1956).

When a solution of paramyosin crystals was examined in the ultracentrifuge a symmetrical peak was observed (Fig. 3). A plot of $S_{20,w}$ as a function of concentration gave a straight line, which on extrapolation to zero gave $S^0_{20,w}$ 3·13s (Fig. 4).

The molecular weight of the protein was determined by the Archibald approach-to-sedimentation-equilibrium method, as described in the Materials and Methods section, and the molecular weight of

Table 1. *Yield of ethanol-dried powder and paramyosin obtained from adductor muscle of different molluscs by using Bailey's* (1956) *method*

	Yield (%)	
	Ethanol-dried powder (% of wt. of fresh muscle)	Paramyosin (% of wt. of ethanol-dried powder)
Pinna nobilis	—	2·3
	—	2·5
Pecten maximus	17·7	0·5
	16·5	0·7
Aulacomya magellanica	21·6	0·7
	20·0	0·9

γ-globulin was determined as a control. The value of 160000 ± 10000 was obtained for γ-globulin, which coincides with 156000 given by Porter (1950) and by Hughes (1954). The value obtained for *Aulacomya* paramyosin was 258000 ± 16000.

Specific viscosity/concentration was plotted against concentration (Fig. 5) and an intrinsic viscosity value of 0·84 was obtained by extrapolating the straight line to zero concentration.

Alanine was the main *N*-terminal amino acid shown by the fluorodinitrobenzene method (Table 2). This method suggests that the molecular weight of paramyosin is 210000, which is lower than that obtained by the Archibald approach-to-sedimentation-equilibrium method, but not sufficiently so to suggest a dimeric structure. The finding of DNP-glutamic acid (1 residue/320000g.) in *Aulacomya* is reminiscent of Bailey's (1957) observation of one unidentified residue/480000g. for *Pinna nobilis* paramyosin, identified in the present work as glutamic acid. The origin of this *N*-terminal glutamic acid is not clear but that it is unlikely to be due to contaminant high-molecular-weight material is emphasized by the absence of proline in the amino acid analysis.

Digestion with carboxypeptidase A gave serine in the proportion of 1 mole of residue/430000g. of protein, much less than 1 mole of amino acid/mole

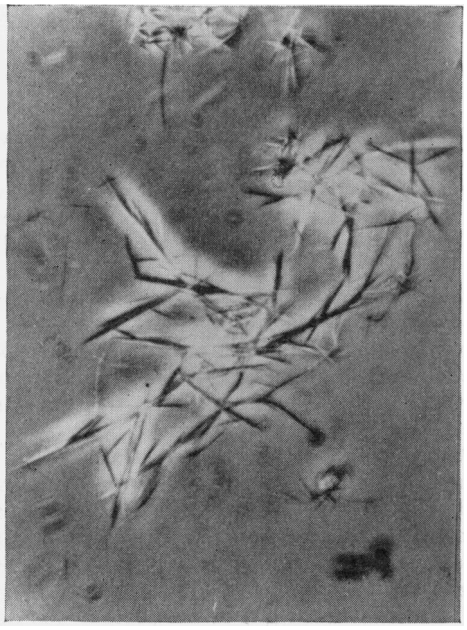

Fig. 2. Crystals of paramyosin from *Aulacomya magellanica* in 0·1 M-potassium chloride, pH 7, observed with a phase-contrast attachment. Magnification ×600.

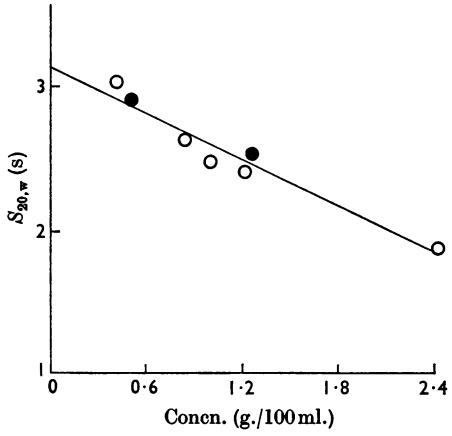

Fig. 4. Dependence of the sedimentation coefficients of *Aulacomya magellanica* paramyosin on concentration. The protein was dissolved in 0·5 M-KCl in 0·05 M-phosphate (0·035 M-Na_2HPO_4–0·015 M-NaH_2PO_4) buffer, pH 7·0. ○ and ● indicate different preparations.

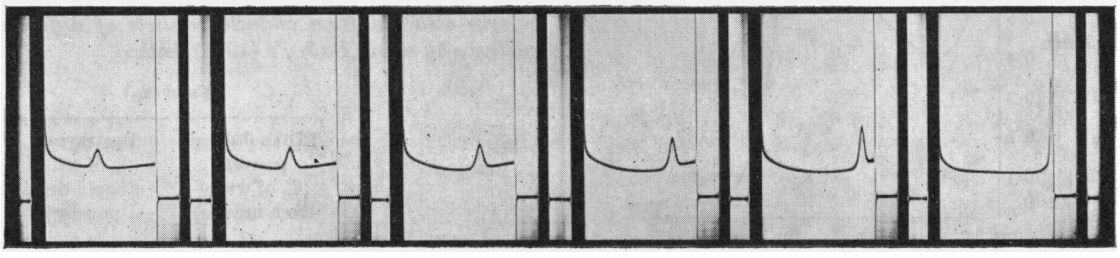

Fig. 3. Ultracentrifuge patterns of *Aulacomya magellanica* paramyosin. Photographs were taken at 6, 38, 70, 102, 134 and 166 min. (right to left) after reaching top speed (56100 rev./min.). The concentration was 0·4g./100ml. The run was carried out in 0·5 M-KCl in 0·05 M-phosphate (0·035 M-Na_2HPO_4–0·015 M-NaH_2PO_4) buffer, pH 7·0, at 20°.

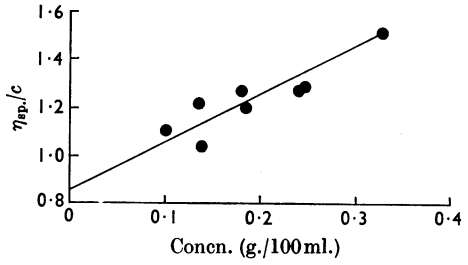

Fig. 5. Specific viscosity/concentration of *Aulacomya magellanica* paramyosin solutions as a function of concentration. Experiments were carried out in 0·5M-KCl in 0·05M-phosphate (0·035M-Na_2HPO_4–0·015M-NaH_2PO_4) buffer, pH7·0, at 25°.

Table 2. *N-Terminal residues in* Aulacomya magellanica *and* Pinna nobilis *paramyosin determined by the fluorodinitrobenzene method*

	Amount of protein containing 1 mole of DNP-residue (g.)		
	DNP-Ala	DNP-Glu	DNP-Gly or DNP-Ser
Aulacomya	194000	283000	370000
Aulacomya	222000	346000	—
Pinna	—	451000	—

of protein, accepting 260000 as a maximum value for the molecular weight of paramyosin. Digestion also produced traces of threonine, alanine and methionine or valine (1 mole of residue/$1·3 \times 10^6$g. of protein).

The amino acid composition is shown in Table 3. Like the amino acid pattern of other tropomyosins, this one is characterized by the absence of proline, and the presence of traces of tryptophan and cystine, small amounts of glycine, tyrosine and phenylalanine and a very large amount of dicarboxylic acids (in particular, glutamic acid) and of basic amino acids. Its lysine/arginine ratio resembles that of paramyosins from other sources (Table 4).

A suspension of *Aulacomya* paramyosin in 0·23M-borate buffer, pH8·2, in a concentration of 2·7mg./ml. was treated with trypsin (1:30, w/w) at 30° for 24hr. The reaction was stopped by the addition of an equal volume of 3% (w/v) trichloroacetic acid and α-amino nitrogen was then determined in the supernatant by the ninhydrin method. After 24hr. the α-amino nitrogen in the trichloroacetic acid-soluble fraction was 5·09% of the total nitrogen. The extent of the reaction was therefore slightly less for this protein than for *Pinna* or

Table 3. *Amino acid analysis of* Aulacomya magellanica *paramyosin*

	Composition (moles of residue/10^5g. of protein)
Lys	70·0
His	7·6
NH_3	(124·0)
Arg	89·6
Asp	119·0
Thr	37·8
Ser	55·4
Glu	222·0
Pro	0·0
Gly	14·4
Ala	80·3
CyS	4·6
Val	22·4
Met	14·9
Ile	24·6
Leu	93·5
Tyr	12·5 (11·7)*
Phe	5·3
Trp	3·5*
Total	877·4

* Calculated by the method of Beaven & Holiday (1952) from the data of Fig. 1.

Pecten paramyosin (Bailey & Milstein, 1964). After 24hr. of tryptic digestion, material is left that is precipitated with 3% trichloroacetic acid. This supports the idea that this tropomyosin is of the insoluble type (tropomyosin A or paramyosin), since those of the soluble type or type B are completely attacked by trypsin and after 2hr. of digestion no trichloroacetic acid-insoluble material is left (Milstein, 1966).

DISCUSSION

Several features permit the characterization of the tropomyosin isolated from *Aulacomya magellanica* as paramyosin or tropomyosin A type of protein, i.e. the method used for its extraction from adductor muscle, solubility properties, facility of crystallization, range of precipitation with ammonium sulphate, amino acid composition and behaviour when digested with trypsin (Table 4). In all these properties it is very similar to the paramyosin obtained from other molluscs. The value of its intrinsic viscosity is, however, very low compared with that of *Pinna nobilis* paramyosin (Kay & Bailey, 1959) and not very different from rabbit (Tsao, Bailey & Adair, 1951) or *Pecten* tropomyosin B (Rüegg, 1959). The intrinsic viscosity can be used to calculate the viscosity increment (Mehl, Oncley & Simha, 1940), and with

Table 4. *Analytical and physicochemical properties of some tropomyosins*

	Tropomyosin B			Tropomyosin A			
Animal.....	Rabbit	*Pecten maximus*	Ox	*Pinna nobilis*	*Venus mercenaria*	*Pecten maximus*	*Aulacomya magellanica*
Muscle	Skeletal	Striated	Uterus	Adductor	Adductor	Adductor	Adductor
Precipitation range in $(NH_4)_2SO_4$ (% saturation)	41–70%[a]	30–60%[e]	40–70%[g]	23–29%[i]	28–40%[h]	35–40%[e]	21–34%
Crystalline form	Plates[a]	Plates[e]	Fine needles[g]	Needles[i]	Needles	Needles[e]	Needles
Glutamic acid residues (moles/10^5g. of protein)	211[b]	198[e]	213[g]	183[j]	169[h]	158[e]	222
Glutamic acid/aspartic acid ratio	2·4	1·8	2·6	1·6	1·5	1·3	1·9
Lysine/arginine ratio	2·6	1·9	1·9	0·8	0·7	1·0	0·8
Mol.wt.	53000–65000[c]	100000[f]	59000[g]	135000[k]	228000–618000[m] 220000[n]		258000
Intrinsic viscosity (dl./g.)	0·5[c]	0·5		2·4[i]	1·9		0·8
Molecular shape (a/b)	29[c]			71	64		38·5
Acid precipitate (insoluble protein core) after 24hr. of digestion with trypsin	None[d]	None[d]		Core left[i]		Core left[d]	Core left
Solubility in neutralized water	Soluble[a]	Soluble[e]	Soluble[g]	Almost insoluble[i]	Almost insoluble	Almost insoluble[e]	Almost insoluble

[a]Bailey (1948).
[b]Kominz, Hough, Symonds & Laki (1954).
[c]Tsao *et al.* (1951).
[d]Milstein (1966).
[e]Bailey & Rüegg (1960).
[f]Rüegg (1959).
[g]Kominz, Saad, Gladner & Laki (1957).
[h]Kominz, Saad & Laki (1957).
[i]Bailey (1956).
[j]Bailey (1957).
[k]Kay (1958).
[l]Milstein & Bailey (1961).
[m]Riddiford & Scheraga (1962).
[n]Lowey *et al.* (1963).

this value the axial ratio can be calculated. The viscosity increment is given by:

$$\nu = \frac{[\eta]100}{\bar{v}}$$

deduced from Simha's equation, where $[\eta]$ is the intrinsic viscosity and $\bar{v}$ the partial specific volume. The value for $\bar{v}$ given by Kay (1958) for *Pinna nobilis* paramyosin is used since the partial specific volume does not vary much from protein to protein (Pedersen, 1945; Kay, 1960). The axial ratio thus obtained for *Aulacomya* paramyosin is much lower (see Table 4) than that of *Pinna* and *Venus* paramyosin, suggesting that the molecule is less asymmetric or more hydrated than that of *Pinna* paramyosin.

The value of the molecular weight obtained in the present investigation (258000 ± 16000) is much higher than the one obtained by Kay (1958) (131000–137000) for *Pinna* paramyosin or by Rüegg (1959) (100000) for *Pinna* tropomyosin B. These authors used the sedimentation-diffusion method, which may be more reliable than the Archibald method. Riddiford & Scheraga (1962), using the Archibald approach-to-sedimentation-equilibrium method, found values for the molecular weight of *Venus mercenaria* paramyosin varying from 228000 to 618000 according to the ionic strength and pH used. The first value was obtained for conditions similar to those used in this work and is not so different from 258000, the value obtained for *Aulacomya* paramyosin. The wide range of molecular weights obtained by these authors suggest that aggregation occurred in their preparation, and in fact they pointed out that their values are integral multiples of the sedimentation-diffusion value of 131000 found by Kay (1958) for *Pinna nobilis* paramyosin. The value obtained in the present work is also an integral multiple of that value, and therefore aggregation may also occur in *Aulacomya magellanica* paramyosin. The sedimentation coefficients for all the tropomyosins, including those of *Venus* and *Aulacomya*, are the same. The behaviour with regard to the approach-to-sedimentation-equilibrium is similar in the last two. Further, both contain 0·5 mole of cystine/mole and one cannot exclude the possibility of dimers joined by disulphide bridges. That is the case for rabbit tropomyosin, in which the molecular weight of the sub-units seems to be about 35000 (Mueller, 1966; Woods, 1966). Therefore unit molecular weights of 130000 are possible for *Aulacomya magellanica* paramyosin. However, the value (258000) of the molecular weight of *Aulacomya* paramyosin is very close to the one reported for *Venus* paramyosin by Lowey, Kucera & Holtzer (1963), who dismissed the possibility of substantial aggregation on the grounds of their viscosity and sedimentation measurements. The evidence for the presence of alanine as *N*-terminal in the proportion of one mole per mole of protein, assuming a molecular weight of 210000, is also against the idea of aggregation.

Calculating the number of amino acid residues in a molecule of molecular weight 235000 (which is the average of the value obtained from *N*-terminal and the Archibald approach-to-sedimentation-equilibrium molecular weight determinations), and using the translation value of 1·47 Å per residue (Pauling & Corey, 1953), the total length of the helix in one molecule can be worked out on the basis that the content of α-helix is 90% (Cohen & Szent-Györgyi, 1957). The length thus obtained is 3030 Å. Dividing this value by the reported molecule length in paramyosins of other species (Kay, 1958; Lowey *et al.* 1963) the answer 2·2 α-helical chains per molecule is obtained. This value agrees very well with a double-chain α-helical type of molecule, which is the model already suggested by several authors. Thus Riddiford & Sheraga (1962), working with *Venus mercenaria* paramyosin, suggested that their preparation is a lateral aggregate that may involve some type of supercoiled α-helices as postulated by Bear & Selby (1956); Lowey *et al.* (1963) referred to the *Venus* paramyosin molecule as made up of two adjacent α-helical chains; Cohen & Holmes (1963) indicated that X-ray-diffraction patterns of intact anterior byssus retractor muscle of *Mytilus edulis* give evidence of a coiled coil α-helical structure. On the other hand, the value of the diameter calculated from the axial ratio and molecular length is far larger than the average value obtained by Lowey *et al.* (1963). This may indicate that the molecule is highly hydrated or less asymmetric.

It seems therefore that, in spite of their identical sedimentation coefficients, gross differences (amino acid composition, solubility properties, trypsin digestion) between the two tropomyosins A and B are found in all the species studied so far. The viscosity (and the calculated axial ratio, which may be related to the asymmetry or hydration of the molecule) does not seem to be a constant characteristic of tropomyosin A.

We have to bear in mind that the species listed in Table 4 (*Pinna nobilis*, *Venus mercenaria* and *Aulacomya magellanica*) belong to different orders of the true lamellibranch division and therefore their differences may well be great (Bailey, Milstein, Kay & Smillie, 1964). *Pecten* and *Pinna*, on the other hand, belong to the same order, Pseudo-Lamellibranchia, and have extremely similar properties. *Aulacomya* and *Mytilus* are different genera of the same family, Mytilidae, and this also might be a good reason for expecting similarities between them.

Most of the experimental work described in this paper was done in the Division de Biologia Molecular, Instituto Nacional de Microbiologia, Buenos Aires, Argentina. I am grateful to Dr I. Pirosky for his unfailing support, to Dr O. Kühnemann for making possible the supply of the molluscs and to the Consejo Nacional de Investigaciones Cientificas y Tecnicas (Argentina) for a grant.

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