SOME PHYSICO-CHEMICAL PROPERTIES OF *PINNA NOBILIS* TROPOMYOSIN

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In recent communications BAILEY[1,2,3] has reported the isolation and properties of a water-insoluble tropomyosin isolated from the adductor muscles of the bivalve *Pinna nobilis*, a member of the Pseudolamellibranch class. Preliminary viscosity and double refraction of flow studies (BAILEY[1]) suggest that this globulin tropomyosin is extremely asymmetric. The present paper deals with the determination of the molecular weight of the protein by two independent methods: sedimentation–diffusion and light scattering. On the basis of the observed angular dissymmetry of light scattering, estimates of the length of the molecule have been made, assuming various possible molecular shapes. In addition, the following constants were evaluated: sedimentation constant, diffusion constant, refractive index increment, partial specific volume and frictional ratio.

MATERIALS AND METHODS

Protein samples

These were kindly supplied by Dr. K. BAILEY who prepared them from the whole adductors of *Pinna nobilis* by the ethanol method (BAILEY[1,3]). Material prepared in this way was shown to be electrophoretically homogeneous at pH 6.9; furthermore, as an analytical criterion of purity, it contained less than 0.05% tryptophan. All of the measurements reported herein were carried out on tropomyosin preparations dissolved in KCl-phosphate solutions of either of two compositions: 0.5 *M* KCl, 0.035 *M* Na_2HPO_4, 0.015 *M* KH_2PO_4, pH 7 or 1 *M* KCl, 0.035 *M* Na_2HPO_4, 0.015 *M* KH_2PO_4, pH 7. Protein concentrations were determined by the micro-Kjeldahl method, taking 18.2% as the N content of the protein (BAILEY[3]). Alternatively, concentrations were sometimes determined interferometrically by measuring the refractive index difference, Δn, between solution and solvent. For this purpose an experimental value of 0.1880 was used for the specific refractive index increment, dn/dc, of the protein (see below).

Partial specific volume ($\bar{v}$)

Partial specific volume determinations were made at 20 ± 0.05° C with a pycnometer of 9.24 ml capacity. Partial specific volumes were calculated from the equation (KRAEMER[4]):

$$1 - \bar{v}\varrho = \frac{1 - w_2}{m}\frac{dw_2}{dm} \qquad (1)$$

where m is the mass of solution, w_2 is the weight fraction of the protein (0.01 times the concentration by weight) and ϱ is the density of the solution.

Sedimentation

The sedimentation measurements were carried out in a model E Spinco ultracentrifuge at 59,780 r.p.m. (260,000 × g) at several protein concentrations. The sedimentation constant at each concentration point was calculated from the slope of a plot of the log of the distance of the boundary from the axis of rotation against time. An allowance of 0.025 cm was made for the stretching

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of the rotor at 59,780 r.p.m. (TAYLOR[5]). The temperature rise of the rotor was recorded throughout each run by means of a thermistor located in the base of the rotor. All the sedimentation constants were corrected to the viscosity and density of water at 20° C as described by SVEDBERG AND PEDERSEN[6].

Diffusion

Diffusion experiments were carried out in a moving-boundary electrophoresis apparatus constructed in the Department of Colloid Science by Mr. E. G. Richards under the supervision of Dr. P. Johnson. A standard Tiselius electrophoresis cell was used as a diffusion cell. It was filled so that initially the entire right limb above the bottom section and the left limb of the top section contained solvent, while the left limb of the centre section and the bottom section contained protein solution. The starting boundary was formed by moving the top and bottom sections into alignment with the fixed centre section. A fine stainless steel capillary tube was then lowered into the cell to the level of the optical axis, and the boundary was sharpened by the technique of capillary aspiration (at a rate of 36 ml/h) in accordance with the procedure of KAHN AND POLSON[7]. The measurements were made at 25 ± 0.01° C. In order to ensure the preservation of the protein at this temperature a drop of thymol was added to both solution and solvent during the dialysis process prior to diffusion. The concentration gradient of the diffusing protein was recorded photographically over a 72 h period using the Schlieren scanning technique. Diffusion constants were computed by the maximum-height area method (NEURATH[8]) and by the method of second moments (LAMM[9]). The viscosity of the medium was determined experimentally. All of the diffusion constants were corrected to the viscosity of water at 20° C, as described by GOSTING[10].

Light scattering

Experimental. Light-scattering measurements at a wavelength of 546 mμ were made at 90° to the incident beam in a light-scattering apparatus described by GORING AND JOHNSON[11]. Measurements of dissymmetry, *i.e.* of the ratio z of scattered intensities at 60° and 120°, were carried out concurrently with the 90° measurements. Tropomyosin solutions were clarified by initial high speed centrifugation (20,000 × g) for 30 min followed by ultrafiltration through collodion membranes prepared as described by GORING AND JOHNSON[12]. All of the experiments were carried out at room temperature, 18 ± 2° C.

Theory. For a system of point scatterers the turbidity, τ, due to concentration fluctuations is related to the molecular weight, M, according to the following expression given by DEBYE[13]:

$$\frac{Hc}{\tau} = \frac{1}{M} + 2Bc \tag{2}$$

where c denotes the concentration, B is the interaction constant determined by the thermodynamic properties of the system and

$$H = \frac{32\pi^3 n_o^2 \left(\frac{dn}{dc}\right)^2}{3N\lambda_o^4} \tag{3}$$

The refractive index of the solution is given by n, that of the solvent by n_o; N is Avogadro's Number ($6.02 \cdot 10^{23}$) and λ_o is the wavelength of the light in vacuo.

Equation (2) is only valid for a system in which the solute particles are small in comparison with the wavelength of the light. When the particle size exceeds approximately one-twentieth the wavelength of light (about 300 A) interference will occur between light waves scattered from the same particle (*intra*particle interference) and this will result in a diminution of the scattered light (DOTY AND EDSALL[14]). In order to preserve the usefulness of equation (2) under such circumstances, it is necessary to apply a correction to the turbidity value as obtained by measuring the scattered light at 90°. The corrected turbidity, $\tau_{corr.}$, is given by the relation:

$$\tau_{corr.} = \tau' \cdot \frac{1}{P(90)} \tag{4}$$

where τ' is the turbidity value as obtained by measuring the scattered light at 90° in a calibrated light-scattering photometer and $P(90)$ is a particle-scattering factor accounting for the decrease of scattering due to *intra*particle interference and derivable from the dissymmetry ratio z (DOTY AND STEINER[15]). Equation (2) may now be rewritten to take into account the effect of *intra*particle interference as:

$$\frac{Hc}{\tau_{corr.}} = \frac{1}{M} + 2Bc \tag{5}$$

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Apart from its use in determining the turbidity correction factor, the dissymmetry ratio affords a measure of the size of the protein molecule in solution assuming that it can be represented by various possible molecular models. Graphs have been constructed relating the scattering dissymmetry to the characteristic particle dimension for spherical, coiled and rod-shaped particles (DOTY AND STEINER[15]; JOHNSON[16]).

EXPERIMENTAL RESULTS

Partial specific volume

Fig. 1 shows a plot of m, the mass of solution held by the pycnometer to w_2, the weight fraction of solute for *Pinna* tropomyosin in a buffer solution of pH 7 and ionic strength of 1.1 (1 M KCl, 0.035 M Na_2HPO_4 and 0.015 M KH_2PO_4). The slope of the line, dm/dw_2, was drawn according to the method of least squares and when used in equation (1) gave a value of 0.736 $\pm$ 0.005 for the partial specific volume. The $\bar{v}$ value of the protein was also determined at an ionic strength of 0.6 (0.5 M KCl, 0.035 M Na_2HPO_4 and 0.015 M KH_2PO_4) and was found to be 0.724 $\pm$ 0.005. Both of these values are in good agreement with a $\bar{v}$ value of 0.729 calculated for *Pinna* tropomyosin on the basis of its amino acid content and using the specific volumes of the amino acid residues as given by COHN AND EDSALL[17].

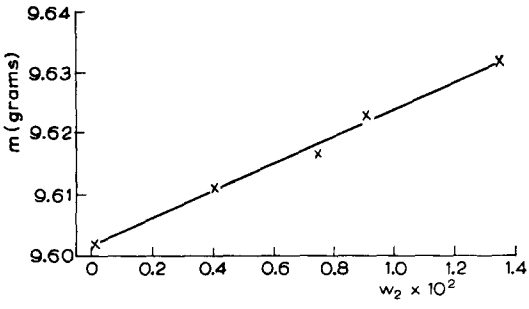

Fig. 1. Plot of m (mass of a given volume of solution of tropomyosin for which the solute weight fraction is w_2) against w_2.

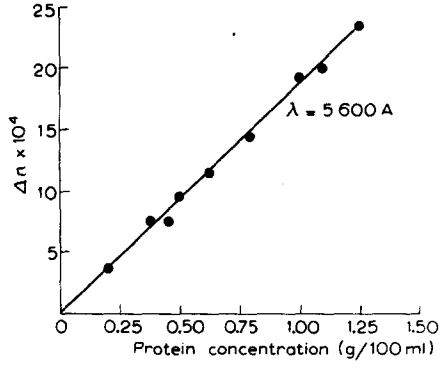

Fig. 2. Refractive index differences, Δn, of tropomyosin solutions in phosphate-buffered KCl (pH 7, ionic strength 1.1) measured between tropomyosin solutions of different concentrations and their respective solvents.

Specific refractive-index increment

Solutions of tropomyosin in phosphate-buffered KCl solution (pH 7, ionic strength 1.1) were prepared and were dialyzed against a large volume of solvent of the same composition. Measurements in the Zeiss interferometer, using white light (λ = 5600 A) to label the zero-order band, established the refractive index difference, Δn, between tropomyosin solutions and their respective diffusates. The protein concentrations were determined by the micro-Kjeldahl technique. The results of such measurements are given in Fig. 2 from which a value for dn/dc of 0.188 was determined. The Debye factor, H, in the light-scattering equation (see equations (2) and (3)) was evaluated for *Pinna* tropomyosin as $3.95 \cdot 10^{-6}$, taking the refractive increment of the protein as 0.188 at 5600 A and 20° C. In actual fact the light-scattering measurements were made at 5461 A (green light of the mercury arc); however GORING[18] has shown that

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the value of dn/dc at 5600 A differs by only 0.3% from that at 5461 A, making any correction to the latter wavelength insignificant.

Sedimentation

Sedimentation determinations were carried out on tropomyosin dissolved in phosphate-buffered KCl solutions at two levels of ionic strength, 0.6 and 1.1. The peaks in the ultracentrifuge were symmetrical and maintained a normal distribution with time suggesting that the protein had all the features of homogeneity. Fig. 3 shows a plot of the $s_{20,w}$ values as a function of concentration. The data at both ionic strengths can be represented by the single full line of Fig. 3 obtained by the method of least squares. The sedimentation constant extrapolated to zero concentration, (s^0_{20}), is 3.1. This value is in good agreement with that obtained by KOMINZ, SAAD AND LAKI[19] for tropomyosins isolated from a wide variety of sources.

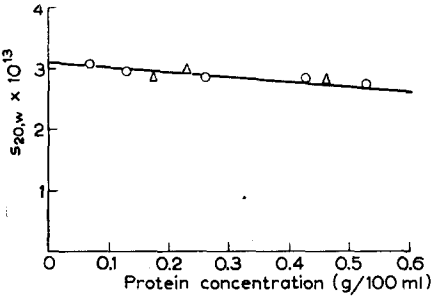

Fig. 3. Sedimentation constants of *Pinna nobilis* tropomyosin in phosphate-buffered KCl. The circles (○) represent solvent medium of 1 *M* KCl, 0.035 *M* Na_2HPO_4, 0.015 *M* KH_2PO_4, pH 7. The triangles (△) represent solvent medium of 0.5 *M* KCl, 0.035 *M* Na_2HPO_4, 0.015 *M* KH_2PO_4, pH 7.

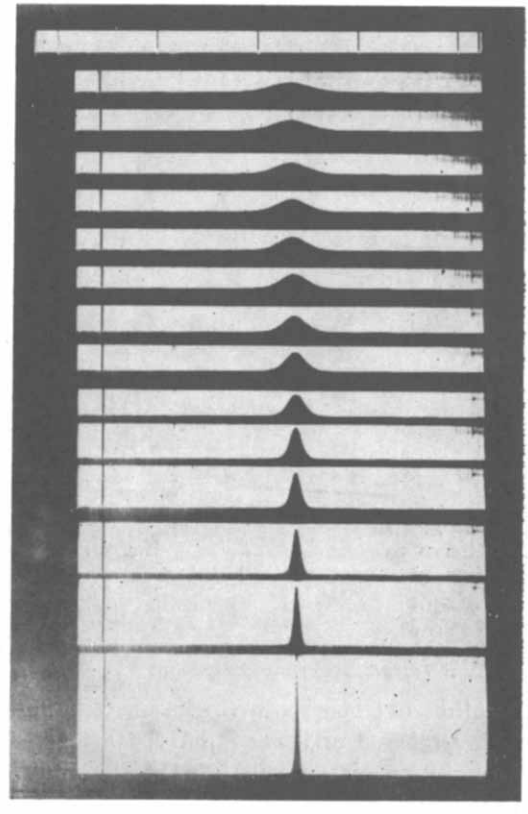

Fig. 4. A series of photographs obtained in a diffusion run on *Pinna nobilis* tropomyosin in phosphate-buffered KCl (pH 7, ionic strength 1.1) at 25° C. The protein concentration is 0.55% and the bar angle is 30°. The bottom picture represents the diffusion run at 48 min after sharpening the initial boundary. The other pictures, going from bottom to top, were taken at 154, 270, 379, 495, 1092, 1462, 1992, 2546, 2905, 3357, 3997, 4353, and 5441 min respectively after sharpening the initial boundary.

Diffusion

Diffusion measurements were performed on *Pinna* tropomyosin at pH 7, an ionic strength of 1.1 (phosphate-buffered KCl) and a temperature of 25° C. A series of photographs of a typical diffusion run are shown in Fig. 4, which serve to illustrate the excellent initial sharpness and symmetry of the peak. Fig. 5 shows the results of the second-moment method of calculating the diffusion constant. The ordinate

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represents the time in seconds and the abcissa is the square of the standard deviation, 6^2. The diffusion constant is obtained by taking half the slope of the line of Fig. 5

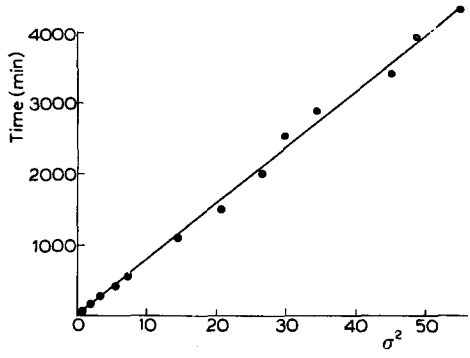

Fig. 5. A plot of time versus the square of the standard deviation, σ^2, for a typical diffusion experiment with *Pinna* tropomyosin in phosphate buffered KCl (pH 7, ionic strength 1.1).

and multiplying this quantity by the appropriate optical factors. The diffusion constant corresponding to the conditions of Fig. 5 is $2.47 \cdot 10^{-7}$ cm^2/sec. For water at 20° as a solvent D becomes $2.21 \cdot 10^{-7}$ cm^2/sec. Diffusion constants were also calculated according to the method of maximum height, area. The results of both methods of calculation are listed in Table I.

TABLE I

DIFFUSION CONSTANTS OF *Pinna nobilis* TROPOMYOSIN

Prot. concn. (g/100 ml)	*Method of calculation*	*Diffusion constant $\cdot 10^7$ (cm^2/sec)*	
		D_{25}	$D_{20,w}$
0.21	Maximum height, area	2.77	2.48
	Second moments	2.45	2.20
0.55	Maximum height, area	2.60	2.33
	Second moments	2.47	2.21

The ratio of the diffusion constant calculated by second moments to that calculated from measurements of maximum height and area is generally considered to be a measure of homogeneity, a homogeneous system resulting in a ratio of unity. In the present case ratios of 0.90 and 0.93 are obtained. It is however difficult at the present time to state whether this departure from unity is a true indication of heterogeneity in our system for the following reasons. First, a slight overestimation of the height of the diffusion curves of the order of 5–7% would account for the entire deviation from unity. This is most certainly a conceivable source of error with the sharper peaks, for here the tops are much more diffuse than their sides or the base line when viewed through a travelling microscope. Second, with our system consistently higher values for the diffusion constant (of the order of 7%) have been obtained by the maximum height, area method over the second-moment method for a good preparation of bovine serum albumin. The second-moment method gave a value of

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$6.66 \cdot 10^{-7}$ cm²/sec for D_{20}, in excellent agreement with that reported by BALDWIN *et al.*[20]. It has therefore been suggested[21] that with our system values of the diffusion constant obtained by the maximum height, area method may be subject to some systematic error which has not yet been properly located. This matter is currently under investigation in Dr. P. JOHNSON's laboratory. For this reason it is felt that the values of D obtained by the method of second moments are more reliable and they have been used exclusively in the calculation of molecular weights from sedimentation-diffusion data in this investigation. Furthermore, the method of second moments yields a weight average of D which is more directly comparable with light-scattering results which give a weight average molecular weight; on the other hand the method of maximum height, area yields a weight average of $1/\sqrt{D}$ which is not directly comparable with light-scattering measurements. Since the D values calculated by second moments showed no variation with concentration, they were averaged at the two concentrations to yield an average $D_{20,w}$ of $2.21 \cdot 10^{-7}$ cm²/sec*.

Light scattering

An example of measurements of the dissymmetry of scattering, z, at various protein concentrations is given in Fig. 6 for tropomyosin in phosphate-buffered KCl (pH 7, ionic strength 1.1). The dissymmetry values show no perceptible dependence on concentration and for this reason have been averaged at the different concentrations. The average z value is 1.23 as measured with green light of the mercury arc. This value can be used to calculate the molecular length, which has been done by means of the graphs constructed by JOHNSON[16]. The effective wavelength of the green light in the medium employed is 4080 A, and from the measured dissymmetry of 1.23, particle lengths of 1400 A for a rod-shaped entity and 957 A for a random monodispersed coil are calculated.

Because of the appreciable angular dissymmetry of scattering shown by tropomyosin solutions it was necessary to correct the turbidity values, as obtained by measuring the scattered light at 90° in our calibrated light-scattering photometer,

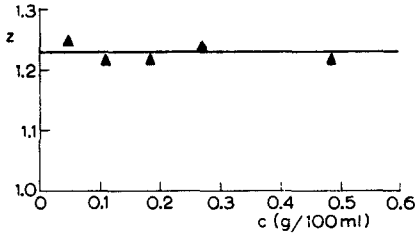

Fig. 6. Light-scattering dissymmetry, z, as a function of concentration for *Pinna* tropomyosin in phosphate-buffered KCl (pH 7, ionic strength 1.1).

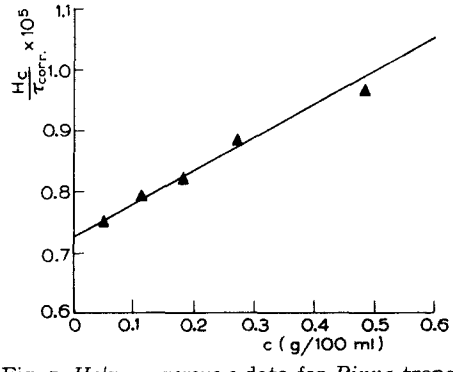

Fig. 7. $Hc/\tau_{corr.}$ *versus* c data for *Pinna* tropomyosin in phosphate-buffered KCl (pH 7, ionic strength 1.1).

* Another diffusion run was carried out at concentration 0.48% and yielded a $D_{20,w}$ value of 1.95, when computed by the method of second moments. Because of a marked asymmetry of the peaks which suddenly developed during the last 24 h of the run, it is suspected that the sample had developed a bacterial contamination. For this reason it is not included in the average.

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by the particle-scattering factor, $P(90)$, in accordance with equation (4). From JOHNSON's graphs the factor for correcting the turbidity for rods and coils of the above specified lengths is 1.26. (Actually the value of $P(90)$ depends upon the particle shape as well as the dissymmetry ratio, z; however, at the levels of z obtained for tropomyosin, the dependence on shape is negligible.) The values of $Hc/\tau_{corr.}$, as obtained for *Pinna* tropomyosin in phosphate-buffered KCl (pH 7, ionic strength 1.1) are plotted as a function of c in Fig. 7, where the best straight line has been drawn by the method of least squares. The intercept of $Hc/\tau_{corr.}$ at zero concentration is the reciprocal of the weight average molecular weight, M, according to equation (5). The observed intercept value of $0.73 \cdot 10^{-5}$ yields a molecular weight of 137,000 $\pm$ 5000.

DISCUSSION

The molecular weight of *Pinna* globulin tropomyosin can now be calculated from the sedimentation–diffusion data using the usual Svedberg equation (SVEDBERG AND PEDERSEN[6]):

$$M = \frac{RT\,s}{D(1 - \bar{v}\varrho)} \tag{6}$$

where R is the gas constant, T the absolute temperature, ϱ the density of the solvent, and the other quantities are as previously defined. Using $s = 3.1 \cdot 10^{-13}$, $D = 2.21 \cdot 10^{-7}$ and $\bar{v} = 0.736$, a molecular weight of 131,000 is computed. This value is in good agreement with the value of 137,000 $\pm$ 5,000 found by light scattering.

The ratio of the frictional resistance of the hydrated molecule to that of the anhydrous equivalent sphere, f/f_0, may be calculated from the constants obtained in the above determinations. This ratio was calculated by two alternative methods using equations given by SVEDBERG AND PEDERSEN[6] which involve: (a) $s_{20,w}$ and $D_{20,w}$ and (b) $s_{20,w}$ and M_e, the molecular weight determined by a method other than sedimentation-velocity. Both of these methods of calculation yielded a value of 2.9 for the frictional ratio of the protein. This value, one of the highest ever recorded for a protein of comparable molecular weight is indicative of the pronounced asymmetry of the tropomyosin molecule, a fact already observed for rabbit tropomyosin (BAILEY *et al.*[22]).

Measurements of the angular dissymmetry of light scattering are compatible with the view that the tropomyosin molecule is either a rod of molecular length 1400 A or a random monodispersed coil of length 957 A. It is possible to make a choice between these two models using an approach which HOLTZER AND LOWEY[23] applied to the interpretation of similar data for myosin. They assumed that the rod-like model was made up of a rigid string of spherical beads of individual diameter d and of total length L. The theory of KIRKWOOD AND RISEMAN[24] and KIRKWOOD AND AUER[25] relate d and L of the above model with the experimentally observed intrinsic viscosity, (η), and sedimentation constant, (S). In the equations given, d is the only quantity not directly measured; hence the consistency of the rod model depends upon whether a single value of this quantity fits both the viscosity and sedimentation behaviour. KIRKWOOD AND AUER[25] have given the following viscosity relation:

$$(\eta) = \frac{24 \cdot \bar{v} \cdot J^2}{9000\, l_n J} \tag{7}$$

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where J is the axial ratio and $\bar{v}$ is the partial specific volume. Using a value of 0.736 for $\bar{v}$ and 2.40 for (η) (BAILEY[1]), a value of 72.1 is obtained for J. The light-scattering length for a rod model is 1400 A, and this fact together with J yields 19.4 A for the diameter.

The sedimentation equation given by KIRKWOOD AND RISEMAN[24] (assuming Stoke's law for the spherical rod segments) is:

$$(S) = (1 - \bar{v}\varrho_0)\, d^2 l_n \left(\frac{6\, M\, \bar{v}}{N\, \pi\, d^3}\right) \Big/ (18\, \bar{v}\, \eta_0) \tag{8}$$

where ϱ_0 and η_0 are the solvent density and viscosity respectively, N is Avogadro's Number and the other quantities are as previously defined. Substituting $3.1 \cdot 10^{-13}$ for (S), 1.039 for ϱ_0, 0.0098 for η_0, 0.736 for $\bar{v}$ and 137,000 for M into the above equation and solving for d yields a value of 17.5 A. This value is in good agreement, within the limits of error of all the experimentally measured quantities, with the value of d determined from the viscosity data, suggesting the adequacy of the rod model.

On the other hand the improbability of the random coil model can be shown by invoking the FLORY-FOX relation[26] for (η) viz:

$$(\eta) = \Phi \frac{R^3}{M} \tag{9}$$

where Φ is a universal constant, $2.1 \cdot 10^{21}$, R is the length in the case of a monodispersed coil and M is the molecular weight. Substituting 137,000 for M and 2.4 for (η) into the above equation yields a value of 538 A for R. This value is decidedly too low when compared with the value of 957 A obtained by light-scattering for a random monodispersed coil model.

It may also be of interest to compare the dimensions suggested by the rod model for *Pinna* tropomyosin (length 1400 A, average diameter 18.5 A) with those to be expected if one assumed that the molecule were a fully extended α-helix. Since the average amino acid residue weight is 115 (BAILEY[3]), the number of amino acid residues in a molecule of average molecular weight 134,000 is 1165. The α-helix of PAULING AND COREY[27] has a translation of 1.47 A/residue. Therefore the construction of the 1165 residues of the molecule into an α-helix would lead to a length of 1713 A. The average diameter is calculated to be 15.4 A, a value based on the average side chain extension of several proteins and the diameter of the α-helix (WAUGH[28]). The experimentally observed dimensions of the molecule are thus not compatible with the view that it is a fully extended α-helix. Whatever the precise configuration, the possibility of a double chain for the molecule is excluded.

It may be significant that the light-scattering length is equal to twice the period of 725 A found in intact fibrils of paramyosin (HALL *et al.*[29]; BEAR[30]) and also equal to the 1400 A repeating period of HODGE's reconstituted paramyosin, to which he gave the name paramyosin long-spacing fibrils (HODGE[31]).

ACKNOWLEDGEMENTS

I wish to thank Dr. KENNETH BAILEY, F.R.S. for valuable advice concerning the handling of *Pinna nobilis* tropomyosin and for many stimulating discussions; Dr.

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P. JOHNSON of the Department of Colloid Science for making available to me the facilities of his laboratory and for helpful advice; Mr. E. G. RICHARDS for an enjoyable collaboration on the diffusion work and Mr. N. BUTTRESS for carefully performing the ultracentrifuge runs.

SUMMARY

The molecular weight of the water-insoluble tropomyosin isolated from *Pinna nobilis* is 137,000 ± 5000 by the technique of light scattering and 131,000 by sedimentation–diffusion. The sedimentation constant extrapolated to zero concentration, s^0_{20}, is $3.1 \cdot 10^{-13}$ and the diffusion constant, $D_{20,w}$, $2.21 \cdot 10^{-7}$ cm²/sec. The frictional ratio is found to be 2.9, one of the highest recorded for a protein of comparable molecular weight. The partial specific volume is 0.736 ± 0.005 in good agreement with the value of 0.729 calculated for *Pinna* tropomyosin on the basis of its amino acid content. The observed angular dissymmetry of light scattering is compatible with the view that the molecule is either a rod-shaped entity of length 1400 A or a random monodispersed coil of length 957 A. The greater probability of the rod-shaped model over that of the random coil is discussed in the light of the theories of KIRKWOOD AND RISEMAN, KIRKWOOD AND AUER and of FLORY AND FOX. It may be significant that the length is equal to the period found in reconstituted paramyosin fibrils of invertebrate smooth muscle.

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Received September 12th, 1957