Subunit Structure of Oyster Paramyosin

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Paramyosin from the oyster *Crassostrea commercialis* was studied by equilibrium sedimentation. In non-denaturing solvents the minimum molecular weight is 208 000. Dissociation into subunits requires complete disruption of the α -helix. This occurs at pH 7 in guanidine hydrochloride solutions of concentration greater than 7 M in the presence of a disulphide-bond-reducing agent. Solutions of the protein in concentrated guanidine hydrochloride are polydisperse and contain species of low molecular weight (approx. 25 000) comprising approx. 5% to 10% of the protein. The molecular weight of the main component is estimated to be 97 000 and the paramyosin molecule contains two of these subunits. From the present observations no decision can be made as to whether or not the small component (or components) represents part of the paramyosin molecule. Preferential binding of guanidine hydrochloride to the extent of 0.13 g./g. of protein was shown in solutions of paramyosin in 7.85 M-guanidine hydrochloride.

It has recently been shown that the highly α -helical muscle proteins tropomyosin (Bailey, 1948) and paramyosin (Bailey, 1956) are dissociated to subunits in denaturing solvents such as concentrated urea or GuHCl* (Woods, 1965, 1967; Olander, Emerson & Holtzer, 1967; McCubbin & Kay, 1968). The results are in accord with the concept that each protein is comprised of two polypeptide chains. To establish the stoichiometry of the subunit structure the heterogeneity should be examined in both mild and dissociating solvents. This has only been attempted in a few cases. In non-denaturing solvents there is a rather wide variation in the published molecular weights for both tropomyosin and paramyosin from different species (summarized by Seifter & Gallop, 1966; Milstein, 1967). The almost twofold difference between the molecular weights of the paramyosins from *Pinna nobilis* (Kay, 1958) and *Venus mercenaria* (Lowey, Kucera & Holtzer, 1963) is beyond the range of the experimental uncertainties and has not so far been satisfactorily explained. An examination of the molecular properties of paramyosin from a larger number of species should eventually lead to an understanding of the significance of the variations in the molecular weights of this protein.

The present paper gives an account of molecular-weight studies by the sedimentation-equilibrium method of the paramyosin from the oyster *Crassostrea commercialis*, which occurs in large quantities

off the east coast of Australia. Measurements are reported in non-denaturing and in strongly denaturing solvents to determine the subunit structure. Paramyosins from the oyster and *Pinna nobilis* were first isolated by Bailey (1956, 1957), but in subsequent papers (Kay, 1958; Kay & Bailey, 1959) only the molecular properties of *Pinna nobilis* paramyosin were reported.

MATERIALS AND METHODS

Preparation of paramyosin. Fresh oysters (*Crassostrea commercialis*) were obtained from a local supply. They were opened and the adductor muscle was excised. Paramyosin was extracted from the whole muscle (both white and yellow parts combined) by the ethanol method of Bailey (1956). The only modification was to include EDTA in all the aqueous solutions used in the isolation procedure to minimize the possibility of heavy-metal ion catalysis of the oxidation of thiol groups. Glass-distilled water was used for the preparation of all solutions and the isolation and purification were carried out at 5°. The protein was recrystallized three times by successive dialyses of the solution in 1 M-KCl against decreasing concentrations of KCl to a final concentration of 0.15 M. After the third crystallization the protein solution in 1 M-KCl was dialysed against increasing concentrations of $(\text{NH}_4)_2\text{SO}_4$, pH 8.5, to 30% (w/v) saturation. After centrifugation the precipitate was dissolved in a buffer solution consisting of 1.1 M-NaCl-25 mM-sodium phosphate adjusted to pH 7.0 and dialysed against the same buffer to free the solution from $(\text{NH}_4)_2\text{SO}_4$. During the preparative procedure it was found that if paramyosin crystals were centrifuged at a high speed they compacted to a tough translucent gel, which would not

* Abbreviation: GuHCl, guanidine hydrochloride.

subsequently dissolve. Preparative centrifugation of paramyosin was therefore carried out in a Servall machine at 2500g for 20 min. to separate precipitates, but at higher speeds (35 000g) when the supernatant was required. The final product was clarified in the Spinco model L preparative ultracentrifuge at 50 000 rev./min. (150 000g) for 20 min.

Protein concentrations. In dilute aqueous buffers the concentrations were either determined refractometrically after dialysis and converted into concentrations by the refractive-index increment (0.188 ml./g. at 546 nm.) reported by Kay (1960) or were determined by the micro-Kjeldahl method taking 18.2% as the N content of the protein (Bailey, 1957). In concentrated GuHCl solutions the concentration was calculated from the extinction at 276 nm.

Absorption spectrum. The spectrum of paramyosin was measured in 1.1 M-NaCl-25 mM-sodium phosphate buffer, pH 7.0, with a Beckman DB spectrophotometer. The spectrum was similar to that for *Aulacomya* paramyosin (Milstein, 1967) with a maximum at 276-277 nm. and a minimum at 250-251 nm. The $(\text{NH}_4)_2\text{SO}_4$ fractionation step decreased the E_{260}/E_{276} ratio from 0.65 to 0.55. $E_{1\text{cm}}^{1\%}$ at 276 nm. was 2.86 after correction for scattering by subtraction of the 340 nm. reading. These values are somewhat lower than those given by Milstein (1967), suggesting possibly less contamination with nucleic acid.

Optical rotatory dispersion. This was measured with a Perkin-Elmer model 141 spectropolarimeter in the wavelength range 365-578 nm. The b_0 values were calculated from the Moffitt equation as described by Urnes & Doty (1961). Linear plots were obtained with a value of 212 nm. for the dispersion parameter λ_0 . The mean residue weight was taken as 115. The b_0 values in 1.1 M-NaCl-25 mM-sodium phosphate buffer, pH 7.0, for the six preparations used in this study were between -650° and -690° .

Sedimentation velocity. Measurements were carried out at 20° in a Spinco model E analytical ultracentrifuge equipped with both schlieren and interference optics. In 1.1 M-NaCl-25 mM-sodium phosphate buffer, pH 7.0, the protein sedimented as a single peak with no evidence of other components. At protein concentrations of 4.6 and 1.0 mg./ml. the $S_{20,w}$ values were 2.40 and 3.00 s respectively. The latter value was within 3% of the values obtained by Milstein (1967) and Riddiford & Scheraga (1962) at this concentration.

Sedimentation equilibrium. Both low-speed and high-speed sedimentation-equilibrium techniques were used. The mid-point method of Yphantis (1960) utilizing the eight-channel centrepiece was used for some experiments; in others the six-channel centrepiece (Yphantis, 1964) or a Kel F-coated aluminium double-sector centrepiece was employed. Conventional low-speed equilibrium was carried out with Rayleigh interference optics (Richards & Schachman, 1959). The procedure followed and equations required for the calculation of molecular weights are given in previous papers (Woods, Himmelfarb & Harrington, 1963; Harrop & Woods, 1964). The procedures and methods of calculation for the high-speed equilibrium experiments were carried out as described by Yphantis (1964). Special care was taken to avoid evaporation and a resultant mismatch between the solution and solvent channels when handling proteins in concentrated urea and GuHCl solutions. The possible errors arising from the centrifugation of concentrated urea solutions were considered in an earlier paper (Woods, 1967). It was found

that when 8 M-urea was centrifuged in a double-sector cell at 44 700 rev./min. the urea distribution in the two sectors cancelled provided the column heights differed by less than 10%. The cells were therefore loaded in the high-speed equilibrium experiments so that solution and solvent columns were almost identical.

Protein solutions for sedimentation equilibrium were dialysed with gentle rocking for at least 24 hr. in 200 ml. tubes filled almost to capacity. Before use the dialysis tubing was washed in hot 1% NaHCO_3 and boiled in distilled water.

Partial-specific-volume measurements. A 25 ml. pycnometer was used for the determination of solution and solvent densities at 20° . The pycnometer was calibrated with distilled water. The apparent specific volume, ϕ' (defined in the Results section), in GuHCl was determined from the densities of the dialysed protein solution and the diffusate. After the measurement of ϕ' the GuHCl solution of the protein was dialysed against successive changes of 1 M-KCl and then precipitated by dialysis to 0.15 M-KCl. The protein was repeatedly washed with distilled water followed by centrifugation. The precipitate was freeze-dried and further dried over P_2O_5 . The apparent partial specific volume, $\bar{v}$, in GuHCl was determined from the density of solutions prepared by dissolving directly in GuHCl. In all the specific-volume determinations the protein concentration was determined from the extinction at 276 nm.

Reagents. GuHCl was prepared from analytical-grade guanidine carbonate and recrystallized from water. Urea was passed through a deionizing column before use to remove heavy-metal ions and cyanate. Mercaptoethanol was distilled under reduced pressure. All other chemicals were of analytical-grade quality.

RESULTS

Molecular weight in salt solutions. All sedimentation-equilibrium experiments in salt solutions were carried out in a buffer of 1.1 M-sodium chloride-25 mM-sodium phosphate, pH 7.0. Low-speed equilibrium experiments at 6995 rev./min. indicated heterogeneity and an accumulation of protein material at the base of the columns. It was found that the layering fluid (FC43 fluorocarbon oil) caused gelation of paramyosin when they were shaken together. Experiments were performed without the use of a liquid false bottom, but these also were not very satisfactory. The molecular weights along the cell were constant at about 230 000, then showed a sharp increase at the base of the cell. A similar behaviour was also observed in acid solutions at pH 2.

Since meaningful molecular weights could not be obtained by low-speed equilibrium methods the meniscus-depletion (high-speed) method of Yphantis (1964) was therefore used to determine the molecular weight of the smallest species present. This value will be independent of any aggregation due to interaction with FC43 and of any adsorption on the cell walls. A range of speeds (15 220-27 690

rev./min.) and protein concentrations was used to increase the sensitivity. A speed of 15220 rev./min. corresponds to a value of 5 cm.^{-2} for σ where $\sigma = \omega^2 M(1 - \bar{v}\rho)/RT$ and the symbols have the usual meaning; a speed of 27690 rev./min. corre-

sponds to a value of approx. 17 cm.^{-2} for σ . Plots of the logarithm of the fringe displacement, y , against r^2 (r =distance from centre of rotation) were linear up to about 3 fringes, then showed a suggestion of upward curvature indicative of the presence of higher-molecular-weight species (Fig. 1). With the ultracentrifuge used 1 Rayleigh interference fringe corresponds to a displacement of $284\text{ }\mu\text{m}$. Graphical estimates of the smallest component present were made by visually fitting the initial slopes from 0.5 to 2 fringes displacement. The results for four preparations are presented in Table 1. All values are within 7% of the mean 208000 and there is no significant dependence on either speed or initial protein concentration. Thus it seems reasonable to conclude that lower-molecular-weight species are not present in any great amount and the minimum values refer to monomeric paramyosin. The partial specific volume used in these molecular-weight calculations was 0.730 ml./g. reported by Kay (1960) and Lowey *et al.* (1963) for paramyosin at this ionic strength and pH.

Apparent specific volume in denaturing solvents. The difficulty of interpreting molecular-weight measurements in multicomponent systems such as protein-water-salt-urea has long been recognized (for review with relevant references see Casassa & Eisenberg, 1964). The anhydrous molecular weight of a macromolecule in a three-component system may be determined if quantitative knowledge is available of the preferential binding of one of the solvent components. This approach was followed by Kielley & Harrington (1960) in the determination of the molecular weight of myosin in 5 M-GuHCl .

Casassa & Eisenberg (1964) have shown that, by defining the solvent component as that solution which is in osmotic equilibrium with the protein solution across a semipermeable membrane, the molecular weight of the macromolecular component can be determined explicitly without evaluating

Fig. 1. High-speed equilibrium sedimentation of oyster paramyosin in 1.1 M-NaCl - $25\text{ mM-sodium phosphate buffer}$, pH 7, at 20° . The initial protein concentration was 1.5 mg./ml. The column length was 3 mm. $\log y$ was plotted as a function of r^2 , where y =fringe displacement in cm. and r =distance from centre of rotation in cm. Centrifugation in the Spinco model E centrifuge was at: ●, $15,220\text{ rev./min.}$; ▲, $27,690\text{ rev./min.}$

Table 1. *Minimum molecular weights of paramyosin determined by high-speed equilibrium sedimentation*

Experimental conditions were as follows: 1.1 M-NaCl - $25\text{ mM-sodium phosphate buffer}$, pH 7.0; 20° ; column height 3 mm.

Prep. no.	Initial concn. (mg./ml.)	Graphical estimate of lowest mol.wt. present		
		15220 rev./min.	17980 rev./min.	27690 rev./min.
1	1.5	200000	—	222000
2	1.4	199000	—	202000
3	1.2	208000	—	197000
4	1.5	—	211000	—
	0.4	—	221000	—
	0.1	—	214000	—
Mean of all determinations			208000	

Table 2. *Apparent partial specific volumes of paramyosin in 7.85 M-GuHCl (20.0°)*

10^3c (g./ml.)	ϕ' (ml./g.)	10^3c (g./ml.)	$\bar{v}$ (ml./g.)
15.6	0.721	8.6	0.729
12.9	0.721	6.9	0.734
11.6	0.719		
10.7	0.722		
9.9	0.718		
8.5	0.718		
Mean	0.720		0.731

the preferential interactions with solvent components. The application of this treatment to myosin in GuHCl solutions has been discussed by Woods *et al.* (1963). The apparent specific volume obtained from a comparison of solution and diffusate densities, i.e. at fixed chemical potential of all the diffusible components, is designated ϕ' and is given by:

$$\phi' = \frac{1}{\rho_s} \left(1 - \frac{\rho - \rho_s}{c} \right) \quad (1)$$

where ρ and ρ_s are the densities of solution and solvent respectively and c is the concentration of anhydrous protein in g./ml. The insertion of ϕ' into equilibrium-molecular-weight equations leads to the molecular weight of the anhydrous protein species.

Table 2 shows the values of ϕ' and the partial volume, $\bar{v}$, for paramyosin in 7.85 M-GuHCl. Because of the shortage of protein, $\bar{v}$ was only measured at two concentrations, but the difference between ϕ' and $\bar{v}$ is significant. They are related by the following equation (Casassa & Eisenberg, 1961):

$$\phi' = \bar{v} - \xi \left(\frac{1}{\rho_s} - \bar{v}_g \right) \quad (2)$$

where ξ is the mass of guanidine bound preferentially/g. of protein and $\bar{v}_g$ is the partial specific volume of GuHCl. For values of $\rho_s = 1.186$ (7.85 M-GuHCl) and $\bar{v}_g = 0.760$ ml./g. (Kielley & Harrington, 1960), eqn. (2) gives preferential binding of GuHCl to the extent of 0.13 g./g. This result is very similar to the results with lobster myosin in 5 M-GuHCl (Woods *et al.* 1963), where the same method gave 0.14 g./g. of protein. Recent work (Hade & Tanford, 1967; Noelken & Timasheff, 1967) also indicates that denatured globular proteins in concentrated GuHCl preferentially bind GuHCl to a small extent.

The value of 0.720 for ϕ' was used for all molecular-weight determinations in GuHCl. Eqn. (2) does, however, indicate that if the preferential binding remains constant then ϕ' will be somewhat lower at lower concentrations of GuHCl. This is not important in this work, since the main interest

is in concentrations of GuHCl greater than 7 M where complete unfolding of the protein occurs.

Molecular properties in 8 M-urea. The molecular weight of paramyosin in 8 M-urea-0.1 M-mercapto-ethanol (buffer: 0.2 M-sodium chloride-10 mM-tris-hydrochloric acid, pH 7.6) was determined at four concentrations by the mid-point method of Yphantis (1960) at a speed of 9341 rev./min. The infinite-dilution value was 211 000. The solution showed heterogeneity when examined by the high-speed method of Yphantis (1964). This is shown in Fig. 2(b) for an initial protein concentration of 1.5 mg./ml. and a speed of 33 460 rev./min. Fig. 2(a) gives the blank-corrected fringe displacements in the initial region up to approx. 50 μ m. net displacement. Inspection of this graph shows that the base line in this experiment can be chosen to better than $\pm 5 \mu$ m., which was the degree of accuracy attained in the experiments of Yphantis (1964). Thus the deviation from linearity at low fringe displacements in Fig. 2(b) is well beyond the experimental error of the choice of the zero concentration level and is an indication of the presence of species of low molecular weight (approx. 25 000) together with a species of much higher molecular weight. This was confirmed in many subsequent experiments. At lower speeds, where there was a shorter flat region near the meniscus, it was found advisable to measure at closer intervals of the radial co-ordinate than for the experiment shown in Fig. 2. Heterogeneity in 8 M-urea was further indicated in an experiment at a much lower initial concentration (0.15 mg./ml.) and speed (17 980 rev./min.), which gave values for the number-, weight- and z-average molecular weights (M_n , M_w and M_z) of 161 000, 180 000 and 183 000 respectively for a displacement at the base of 4 fringes. The close proximity of M_w and M_z is an indication of the absence of very aggregated species. The possibility of an equilibrium was investigated by calculating the molecular weights at points in the centrifuge cell by the procedures of Yphantis (1964) and plotting these as a function of fringe displacement. For an equilibrium system the molecular weight should be a function only of the concentration at

Fig. 2. High-speed equilibrium sedimentation of oyster paramyosin in 8M-urea-0.2M-NaCl-10 mM-tris-HCl, pH 7.6, containing 0.1M-mercaptoethanol at 20°. The initial protein concentration was 1.5mg./ml. The column length was 3mm. and centrifugation in the Spinco model E centrifuge was at 33460 rev./min. (a) Fringe displacements, corrected for blank, plotted as a function of the comparator x-coordinate for determination of the zero concentration level (broken line). The bars correspond to $\pm 5 \mu\text{m}$. fringe displacement. (b) $\log y$ plotted as a function of r^2 . Error bars are as for (a).

that point (Adams & Fujita, 1963). The plots of molecular weight as a function of fringe displacement for paramyosin under various conditions of speed and initial concentration did not overlap and it was therefore concluded that this was not an associating system.

Since there is a clear separation of the low-molecular-weight and high-molecular-weight species at 33460 rev./min. (Fig. 2) the molecular weight of the main species was determined by subtraction of the displacements due to the smaller

Fig. 3. Molecular weight of oyster paramyosin as a function of GuHCl concentration. ●, 0.1M-Mercaptoethanol added; ▲, 0.02% *N*-ethylmaleimide added.

molecular weight from the whole curve. This procedure, referred to by Yphantis (1964) as a 'quick and dirty' method, yielded 207000 for the molecular weight of the main species. For equilibrium at a lower speed (17980 rev./min.) the same procedure yielded 199000 for the molecular weight.

It is clear that complete dissociation into component polypeptide chains does not occur in 8M-urea-0.1M-mercaptoethanol at pH 7.6. Similar results were also obtained at pH 9.5. Optical-rotatory-dispersion measurements on oyster paramyosin in 8.6M-urea at pH 7 gave a value for b_0 of -240° , which indicates that considerable α -helical structure still remained. Cohen & Szent-Gyorgyi (1957) also found that urea did not abolish completely the α -helix of clam paramyosin.

Conditions necessary for dissociation into subunits in GuHCl. The molecular weight as a function of GuHCl concentration in the presence of 0.1M-mercaptoethanol at pH 7.3 is presented in Fig. 3. The M_w values were extrapolated to zero concentration from short-column equilibrium experiments at four protein concentrations carried out in the eight-channel cell of Yphantis (1960) at speeds in the range 8766-12590 rev./min. The molecular weight shows a rapid decrease between 5.5 and 7.0M, where it reached a minimum at approx. 90000. At low concentrations of GuHCl (less than 5.5M) the molecular weights are greater than the

values in 1M-salt or 8M-urea. Dissociation into subunits is incomplete in 6M-GuHCl, a concentration that is generally considered sufficient to break all non-covalent interactions within and between polypeptide chains (Tanford *et al.* 1967). Optical-rotation values indicated that the polypeptide chains were not completely unfolded at GuHCl concentrations below 7M. The b_0 values at various concentrations of GuHCl were: 4.8M, -220° ; 6.1M, -120° ; >7 M, 0° . The results are similar to those published for clam paramyosin (Noelken & Holtzer, 1964), where it was also found that 7M-GuHCl was required to destroy the α -helical structure completely. At pH 8.6 a slightly lower concentration of GuHCl (approx. 6.5M) is sufficient for dissociation into subunits.

Dissociation of paramyosin into the constituent polypeptide chains only occurs when a reducing agent is present. In the presence of 8M-GuHCl and *N*-ethylmaleimide, which reacts only with thiol groups, dissociation into subunits does not occur (Fig. 3). The polypeptide chains are apparently held together by one or more disulphide bonds. After treatment with mercaptoethanol in the presence of 8M-urea or 5M-GuHCl followed by alkylation with sodium iodoacetate, subsequent dissociation only occurs in 8M-GuHCl if mercaptoethanol is present. After reduction and alkylation in the presence of 8M-GuHCl subsequent dissociation only occurs in concentrations of GuHCl above 7M. Thus high concentrations of GuHCl (above 7M) are necessary both for the reduction of the disulphide bonds and for the subsequent dissociation into the constituent polypeptide chains. Determination of *S*-carboxymethylcysteine on the completely reduced and alkylated protein indicated $4.3 \text{ moles}/10^5 \text{ g}$. If all the cysteine residues occur in the disulphide form this means that there could be 4 such bonds in a molecule of molecular weight 200000.

Subunit molecular weight. The heterogeneity of the protein in GuHCl was examined by the high-speed equilibrium method at speeds in the range 29500–44770 rev./min. and some typical results are presented in Fig. 4. The observations indicate the presence of species of molecular weight about 20000–30000 and this might have been expected in view of results obtained in urea (Fig. 2). There was no indication that this low-molecular-weight component was in association equilibrium with higher species.

To characterize better the main component attempts were made to remove the small component by gel filtration on Sephadex G-200 in both 8M-urea and 5M-GuHCl. However, tubes from the leading edge and the peak when examined in 8M-GuHCl–0.1M-mercaptoethanol still showed the presence of low-molecular-weight material.

Fig. 4. Plot of $\log y$ as a function of r^2 for oyster paramyosin in 8M-GuHCl–0.1M-mercaptoethanol at 20° . The initial protein concentration was 1.0 mg./ml. The column length was 3 mm. Centrifugation in the Spinco model E centrifuge was at: ●, 31410 rev./min.; ▲, 44770 rev./min.

Chromatographic fractionation on DEAE-cellulose in 8M-urea was more successful. Approx. 80% of the material loaded was eluted in the main peak (Fig. 5). Although all fractions exhibited heterogeneity tubes from the main peak showed less of the low-molecular-weight components when examined by high-speed equilibrium in 8M-GuHCl–0.1M-mercaptoethanol.

There was considerable variability in the detection of low-molecular-weight components. In some experiments (Fig. 4) where the small component was clearly separated the fringe displacements due to this component were subtracted from the total displacement. Number- and weight-average molecular weights at points in the centrifuge cell, $M_n(r)$ and $M_w(r)$, were evaluated from the corrected observations and their reciprocals extrapolated to zero fringe displacement to eliminate concentration-dependence. Most weight was given to fringe displacements above $500 \mu\text{m}$. The corrected values of $M_n(r)$ and $M_w(r)$ showed typical thermodynamic non-ideality and extrapolated to identical values within experimental error. Where two speeds were employed in the one run the value of σ for the small component obtained at the higher speed was used to correct the results

at the lower speed. In some cases passage through DEAE-cellulose in 8M-urea (Table 3, Expt. 5) removed the small component and only thermodynamic non-ideality was apparent. In a replicate experiment (Table 3, Expt. 9) low-molecular-weight species could be detected after chromatography, but the equilibrium centrifugation was carried out at a higher initial protein concentration with increased sensitivity in the detection of small species. The form of the plots of $\log y$ versus r^2 is a function of the heterogeneity, non-ideality, centrifugal field and initial concentration. Thus runs over a range of speeds and initial concentrations are

Fig. 5. Chromatographic fractionation of oyster paramyosin on DEAE-cellulose in 8M-urea-10mM-tris-HCl, pH 7.6, at 20°. A 30mg. sample of protein was loaded in 7ml. of buffer. The column size was 2.3cm. $\times$ 13cm. The fraction size was approx. 3ml. Samples from fractions A and B were taken for molecular-weight determination (Table 3).

necessary to assess the various contributions to the form of the plots. Graphical estimation of the molecular weight of the main component by drawing the line of best slope from 0.5 to 2 fringes displacement generally gave low values (Table 3, column 5). These result from the extreme non-ideality decreasing the slope of the plots of $\log y$ versus r^2 at high fringe displacements and the presence of low-molecular-weight species acting to decrease the slope at small displacements. Frequently in these systems sigmoidally shaped plots resulted (Fig. 4).

The pitfalls in applying the meniscus-depletion method have been well presented by Yphantis (1964) and he emphasized that a system may appear homogeneous whereas in fact it is both heterogeneous and non-ideal. In another situation quoted by Yphantis (1964) the system may appear to be homogeneous when, for example, 10% of the molecules have one-quarter of the molecular weight of the main species. The estimation of the molecular weights of small species will in addition rely on accurate fitting of the base line, and this depends on reproducible cell blanks and for the experiments reported here exact cancelling of the distribution due to GuHCl. Thus estimates of the molecular weight of the small component that depend on fringe displacements less than 50 μ m. are consequently very variable (15 000-30 000). The lack of precision at low fringe displacements will also be reflected in estimations of the molecular weight of the main component when the subtractive procedure is applied. This is not as serious because most weight was placed on fringe displacements above 500 μ m.

Table 3 presents only a selection of the high-speed equilibrium results. In all, 15 estimates of

Table 3. *High-speed equilibrium sedimentation of oyster paramyosin in 8M-GuHCl*

All solutions contained 0.1M-mercaptoethanol and were adjusted to pH 7.3. The temperature was 20°, the column height 3mm. The graphical estimate was obtained from plots of $\log y$ versus r^2 by drawing the best line between 0.5 and 2 fringes displacement. The small component was estimated graphically from displacements less than 0.25 fringe. The main component represents values of $M_n(r)$ and $M_w(r)$ extrapolated to zero fringe displacement, giving most weight to displacements above 500 μ m. Where the small component was clearly separated these displacements were first subtracted from the total fringe displacement.

Expt. no.	Material	Initial concn. (mg./ml.)	Speed (rev./min.)	Molecular weight		
				Graphical estimate	Small component	Main component
1	Unfractionated	1.3	39 460	63 700	26 000	87 000
			29 500	66 200	Not separated	88 000
5	Fraction A (Fig. 5)	0.3	33 450	98 200	None	96 600
	Fraction B (Fig. 5)	0.6	33 450	94 400	None	95 400
9	Fraction A*	1.0	44 770	69 000	15 000	98 600
			31 410	73 700	36 000	87 800

* Peak tube from a replicate of the experiment shown in Fig. 5.

Fig. 6. Low-speed equilibrium sedimentation of oyster paramyosin in GuHCl-0.1M-mercaptoethanol at 20°. The column height was 1.1mm. Centrifugation in the Spinco model E centrifuge was at 17980 rev./min. $1/M_w(\text{app.})$ was plotted as a function of concentration (expressed in fringe numbers for a cell of path length 12mm). ●, Prep. 6 in 8.0M-GuHCl; ▲, pooled fractions (tubes 65-85, Fig. 5) concentrated and examined in 7.5M-GuHCl.

the size of the main component were made from ten ultracentrifuge runs and these varied from 86 000 to 102 000 with a mean value of 95 000. More accurate molecular weights for the entire sample were obtained from low-speed equilibrium sedimentation. These are presented in Fig. 6 and give a value for M_w of 92 000 for unfractionated paramyosin in GuHCl and 95 000 after chromatography on DEAE-cellulose. The agreement with the estimate from the high-speed experiments is probably fortuitous, since the fractionation does not completely remove low-molecular-weight species. The estimates of the main component do include some values less than M_w for the unfractionated sample (see e.g. Table 3, Expt. 1) and if these are excluded from the average of the high-speed results a value of 97 000 is obtained. It is difficult to place exact limits on this value. Because of the presence of low-molecular-weight species it must be greater than M_w (92 000), and since the results suggest that there are two polypeptide chains/molecule it must be less than one-half the value of 208 000 found in salt solutions. Thus reasonable limits would be ± 5000 .

DISCUSSION

The molecular weight of 208 000 found for oyster paramyosin in non-denaturing solvents agrees well with the osmotic-pressure determinations by McCubbin & Kay (1968) and the Archibald data of Lowey *et al.* (1963) for *Venus mercenaria* para-

myosin, but is somewhat lower than the last-named workers' light-scattering value of 250 000. In view of the difficulties associated with the aggregation and the uncertainty of the correct experimental value of the refractive-index increment to be used in the light-scattering equation (Lowey *et al.* 1963) the ultracentrifuge value is to be preferred. Thus the paramyosins from *Venus mercenaria* and *Crassostrea commercialis* possess, within the experimental error of the determinations, a common monomeric molecular weight in non-denaturing solvents. The value found for *Aulacomya magellanica* paramyosin (Milstein, 1967) is about 25% higher, but the abnormally low intrinsic viscosity for this protein suggests that side-by-side aggregation may have occurred. Thus these three paramyosins appear to be identical in molecular weight and differ from the molecular weight of 135 000 found for *Pinna nobilis* (Kay, 1960).

Oyster paramyosin is only completely dissociated to subunits in the presence of a reducing agent and GuHCl concentrations above 7M. Dissociation is incomplete in urea at all concentrations. The dissociation to subunits closely follows the loss of α -helix as measured by optical rotatory dispersion.

The molecular weight of 97 000 for the main component in GuHCl is slightly less than one-half that found in mild solvents and is in agreement with the two-chain model for paramyosin. The results support the findings of Olander *et al.* (1967) and McCubbin & Kay (1968), but it should be noted that the conditions used by the latter workers, namely 8M-urea and 6M-GuHCl, were found in the present work to be insufficient for complete dissociation.

The homogeneity of paramyosin in GuHCl has not previously been investigated and the results reported here show the presence of a low-molecular-weight component or components of molecular weight about 25 000. These are considered to be non-covalently bound to the paramyosin molecule since they are only evident in denaturing solvents. A weight-average molecular weight of 92 000 in GuHCl is consistent with a main component of molecular weight 97 000 and the presence of 5-10% of species of molecular weight 25 000 or less. Exact quantitative determination is difficult, if not impossible, to establish and it is therefore not possible to decide whether or not the small components represent subunits of the paramyosin molecule. A similar situation exists with regard to small components in myosin (Dreizen, Hartshorne & Stracher, 1966; Locker & Hagyard, 1967; Gaetjens, Barany, Bailin, Oppenheimer & Barany, 1968).

There are some recent reports that concentrated GuHCl may cause main-chain degradation in proteins (Ansevin, 1968; Jaenicke & Knof, 1968).

Low values for the molecular weight of proteins in GuHCl have been reported to be a function of time and considered to be due to breakage of covalent bonds. This, of course, could explain the variability in the detection and apparent amounts of small components observed in paramyosin solutions in GuHCl. Since dialysis must be carried out for at least 24 hr. before an equilibrium experiment, which itself takes 24–48 hr., time effects are difficult to investigate. There is no evidence that neutral urea breaks covalent bonds in proteins, yet low-molecular-weight species are seen in this medium (Fig. 2). The limited number of molecular-weight results in 8M-urea, which does not completely dissociate paramyosin, suggest the presence of at least 5% of low-molecular-weight components, and one might expect more in 8M-GuHCl, which is a more effective dissociating agent. There is therefore justification in the conclusion that oyster paramyosin contains non-covalently bound low-molecular-weight components that do not arise from main-chain degradation when placed in GuHCl.

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