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Studies on the Proteins of Fish Skeletal Muscle

5. MOLECULAR WEIGHT AND SHAPE OF COD FIBRILLAR PROTEINS†

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It was shown in a previous paper in this series (Connell, 1958) that unaggregated myosin is present in extracts of cod skeletal muscle, and that attempts to isolate the monomeric protein from the extracts by employing the usual method of precipitating by dilution failed because it aggregated spontaneously. It has now been confirmed that the

tendency of cod myosin to aggregate is much greater than that of the rabbit protein. Measurements of the molecular weight and shape of unaggregated cod myosin have been undertaken therefore to decide whether some feature of these parameters could explain the unusual behaviour of the fish protein.

Although many attempts to prepare unaggregated cod myosin from extracts containing it have

† Part 4: Connell (1958).

been made in this Laboratory, none has proved successful. However, it has been found possible to prepare monodisperse cod myosin by separating it in the preparative ultracentrifuge after the dissociation of actomyosin with adenosine triphosphate (ATP) or sodium pyrophosphate at 0°. The method stems from Weber's (1956) work on the ultracentrifugal separation of rabbit myosin and Hamoir's (1951) finding that treatment of carp actomyosin with ATP gives rise in the ultracentrifuge to a single component whose sedimentation rate corresponds to that of rabbit myosin.

Although it is possible to determine accurately the sedimentation coefficient of monodisperse cod myosin, the instability of the protein renders the determination of diffusion coefficients less accurate, and it was therefore important to obtain a value for the molecular weight independent of the diffusion coefficient. Archibald's (1947) procedure of measuring the pre-equilibrium conditions obtaining in an ultracentrifuged solution as developed for proteins by Klainer & Kegeles (1955) and Ginsburg, Appel & Schachman (1956) offers an ideally rapid method of molecular-weight determination, and it has been therefore applied to cod myosin.

The molecular weight of an impure preparation of cod G-actin appeared to be near that of rabbit G-actin dimer (Connell, 1954), but it was decided to reinvestigate this by purifying the cod protein according to Mommaerts' (1952) procedure and examining its sedimentation behaviour.

Natural rabbit actomyosin, that is the actomyosin prepared by extracting fresh rabbit muscle, appears to be polydisperse (Weber & Portzehl, 1952a) and there seems little justification for attempting to obtain an accurate value for the molecular weight of this complex. On the other hand, Hamoir (1951, 1955a, b) speaks of carp actomyosin as consisting of one component. Also, the importance which natural actomyosin has assumed as a model substance in experiments designed to study the effects of technological processes on meat and fish demands that more information about this complex be obtained. In the present investigation, the order of the molecular weight of cod actomyosin has been estimated from turbidity measurements at different wavelengths.

EXPERIMENTAL

Materials

Buffers. A buffer of pH 7.5 and ionic strength 0.05 of the following composition was used: 3.38 mM-KH₂PO₄-15.5 mM-Na₂HPO₄. Potassium chloride or iodide was added to this buffer to bring the total ionic strength up to the values quoted below. Sodium thiosulphate (6 mM) was added to the buffers containing KI, to guard against the liberation of free iodine.

Myosin. All preparative operations were conducted near 0°. A mixture of cod myosin and its aggregates was prepared from skeletal muscle excised from 'live' cod (*Gadus callarias* L.) which had been killed only a few minutes previously. The muscle was coarsely minced and extracted with 3 vol. of slowly stirred KCl-phosphate buffer ($I=0.3$) for 10 min. The mixture was centrifuged at 3000 *g* for 15 min. and the supernatant solution diluted by the slow addition during stirring of 6 vol. of water. The precipitate generally showed a 'sheen' on stirring. Precipitation was allowed to proceed for 1 hr. although longer periods sometimes appear to be necessary, and the precipitate was collected by centrifuging at 3000 *g* for 15 min. The pad of myosin was dissolved by the addition of an equal volume of KCl-phosphate buffer ($I=1.0$) and the protein was re-precipitated by the addition of 10 vol. of water as above. After dissolving the centrifuged precipitate the solution was either examined immediately, or dialysed against KCl-phosphate buffer ($I=0.5$).

Monodisperse myosin was prepared from cod actomyosin prepared as described below. The actomyosin was prepared either from muscle excised immediately after the death of the fish or from muscle which had been frozen *pre-rigor*. Apart from the smaller yield of actomyosin obtained from muscle frozen *pre-rigor* no difference in either the actomyosin or the myosin obtained therefrom has ever been detected between these two types of muscle. The final solution of actomyosin used for the preparation of myosin was dialysed against KCl-phosphate buffer ($I=0.5$) for no longer than about 20 hr., because some evidence has been obtained that small quantities of myosin which contaminate the actomyosin aggregate during this period and appear in the final myosin supernatant solution. In addition the actomyosin itself aggregates and the yield falls. To the dialysed actomyosin solution was added either 5 mM-ATP + 5 mM-MgCl₂ or mM-sodium pyrophosphate + mM-MgCl₂. The reagents were all neutralized and their volume was sufficiently small to affect the concentration of buffer only negligibly. The treated actomyosin was immediately centrifuged in a cooled preparative 'A' rotor of a Spinco ultracentrifuge at an average of 102 000 *g* for 2½ hr. The centrifuge tubes then contained a water-clear supernatant solution, an almost transparent and gel-like pellet and a small white opaque precipitate at the tip. The top 40–50% layers of the supernatant myosin solution were immediately removed. Tubes containing untreated cod actomyosin centrifuged in the same way contained a much looser and more opaque pellet and a more opaque supernatant solution whose opacity decreased towards the meniscus.

So far twenty-nine preparations have been made in this way with substantially the same result; in most of them pyrophosphate was used because of the expense of ATP. If the concentration of actomyosin in the treated solution exceeded about 0.7% it contaminated the myosin presumably because the dissociation was incomplete. The concentration of the myosin solution was never more than about 0.4%, which has imposed restrictions on the range of observations possible with the protein since it must be examined immediately if it is to remain monodisperse.

The viscosity of myosin solutions obtained by treating cod actomyosin with ATP did not fall when they were treated with ATP (the test cannot be applied to solutions containing pyrophosphate), indicating the virtual absence of

actomyosin. Ultracentrifugal analyses of myosin solutions prepared by dissociating cod actomyosin almost always showed a single slowly diffusing component (Fig. 1a). Occasionally traces of much heavier or lighter components are discernible, presumably actomyosin and sarcoplasmic proteins unremoved from the original actomyosin respectively. The area (corrected for dilution in a sector-shaped cell) of the myosin peak in the ultracentrifuge picture falls by about 5–10% during a run; this may be due to some systematic error in the subtraction of the base line of the picture or to the presence in the myosin of small

amounts of material which have approximately the same sedimentation rate and which give rise to very rapidly spreading boundaries. No difference between the myosins prepared from actomyosin treated with either ATP or pyrophosphate has been detected (see Results).

Attempts to apply Szent-Györgyi & Szent-Györgyi's (1956) KI method of preparing myosin to fish yielded only traces of protein resembling myosin.

Actin. Acetone-dried powders were prepared by Bárány, Bárány & Guba's (1957) procedure from muscle removed from cod killed a few minutes before. The powder was extracted for 1 hr. at room temperature with 20 vol. of water, which was stirred occasionally by hand. The paste was filtered through a Büchner funnel and the filtrate, containing 0.3–0.5% of protein, was treated substantially according to Mommaerts' (1952) procedure. This procedure involves polymerizing the actin and separating it from light unpolymerized protein by high-speed centrifuging. Two polymerizations were carried out: about 75 and 90% of the actin polymerized and could be centrifuged in the first and second polymerizations respectively. The F-actin was depolymerized in 0.1 mM-ATP adjusted to pH 8–8.5 with NaHCO_3 . The final solution, containing 0.3–0.5% of protein, was dialysed against either 0.1 mM-ATP, pH 8–8.5, or KI-phosphate buffer ($I=0.66$) to continue the depolymerization, or against KCl-phosphate buffer ($I=0.3$ or 0.5) to repolymerize the actin. Ultracentrifugal analysis of the crude aqueous extract of powder showed the presence of two heterogeneous components having nearly the same rate of sedimentation, but the purified preparations showed only a single diffuse boundary (Fig. 2).

Actomyosin. Coarsely minced muscle either from cod killed only a few minutes beforehand or frozen *pre-rigor* was extracted for 18–24 hr. with 5–10 vol. of KCl-phosphate buffer ($I=0.5$), with slow mechanical stirring. The brei was centrifuged at 300 g for 30 min. and the cloudy, very viscous supernatant solution diluted by the addition, with stirring, of 10 vol. of water. After allowing to stand $\frac{1}{2}$ –1 hr. the precipitate was collected by centrifuging at 3000 g for 30 min. and redissolved by the addition of an equal volume of KCl-phosphate buffer ($I=1.0$). After one or two further precipitations in the same way, the final solution was dialysed against KCl-phosphate buffer ($I=0.5$). For the turbidity measurements only unfrozen muscle was used, and the final dialysis was for 2–3 days against several changes of 0.5 M-KCl.

Methods

Ultracentrifuging. The Spinco model E equipped with a Rotor-Temperature Indicator and Control unit was used. With very few exceptions (indicated below) the temperature of the runs has been kept between 0 and 5°, and the samples have been loaded into precooled rotors. For runs requiring a knowledge of the whole refractive-index gradient in the cell (e.g. Archibald, 1947 procedure), the bar of the schlieren set up was replaced by a wire of 0.0089 cm. diameter (43 s.w.g.), and measurements were taken at the mid-point of the photographed image of this wire. The photographic plates were measured with a two-way travelling microscope reading to 0.01 mm.

Sedimentation coefficients (S) were calculated as follows from photographs taken at intervals of 16 or 32 min.:

$$S = \frac{\Delta x}{\Delta t \bar{x} \omega^2} \quad \text{or} \quad \frac{\ln(x_2/x_1)}{\Delta t \omega^2},$$

Fig. 1. Sedimentation diagrams of cod myosin prepared by ultracentrifuging of dissociated cod actomyosin. KCl-phosphate buffer, pH 7.5 ($I=0.50$): (a) 6000 sec. after reaching full speed of 59 780 rev./min.; concentration 0.28 g./100 ml. (12 mm. cell); bar angle 70.0°; temperature 1.00°. (b) 4320 sec. after reaching full speed of 7247 rev./min.; concentration 0.40 g./100 ml. (18 mm. cell); wire angle 70.0°; temperature 4.60°. M denotes the position of the meniscus.

Fig. 2. Sedimentation diagram of ultracentrifugally purified cod G-actin in 0.1 mM-ATP, pH 8.5, 8220 sec. after reaching full speed of 59 780 rev./min.; concentration 0.34 g./100 ml. (12 mm. cell); wire angle 70.0°; temperature 1.15°. M denotes the position of the meniscus.

where Δx is the distance moved by the maximum ordinate of the boundary from radial distances of x_1 to x_2 in a time interval of Δt , $\bar{x}$ is the mean radial distance and ω the angular velocity. No tendency of the values of S calculated as above either to increase or decrease during a run could be detected and therefore they were averaged for a single run. The average values of S were corrected to a liquid of the density and viscosity of water at 20° (Svedberg & Pedersen, 1940). The values at 20° of the partial specific volume used in this correction were 0.730 and 0.74 for cod myosin (see below) and actin respectively. The latter value is assumed and it is also assumed that the partial specific volume of myosin falls by 0.0005 per degree (Svedberg & Pedersen, 1940, Appendix II; Noda, Kubo & Lardy, 1954).

Archibald procedure. The procedure was essentially that of Klainer & Kegeles (1955) and Ginsburg *et al.* (1956) and makes use of the relationship:

$$M = \frac{RT}{(1 - \bar{v}\rho)} \frac{(\partial c/\partial x)_{x_0}}{\omega^2 c_{x_0}},$$

where M is the molecular weight of the solute, R is the gas constant, T is the absolute temperature, $\bar{v}$ is the partial specific volume of the solute, ρ is the density of the solution, $(\partial c/\partial x)_{x_0}$ is the value of the concentration gradient at either end of the centrifuged column of solution, x_0 is the radial distance of either end of the column, ω is the angular velocity and c_{x_0} is the concentration of solute at either end of the column.

In order to improve the definition of the image of the diagonal wire in these runs, the cell path-length was increased to 18 mm., which enabled larger angles of the wire (to the slit image at the wire) to be used. With this arrangement values of $(\partial n/\partial x)_{x_0}$, the refractive-index gradient at the top meniscus of the cell, of 0.2–0.6 cm. at the photographic plate were obtained for myosin of the greatest concentration available (about 0.4%). The total concentration of myosin was determined (in arbitrary units on the photographic plate) in a 12 mm. synthetic boundary cell and the values were multiplied by 1.5 in order to correct to the 18 mm. cell.

The photographic plates were aligned so that the middle of the image of the top meniscus furnished one co-ordinate and measurements of $\partial n/\partial x$ taken at 0.1 mm. intervals from this co-ordinate. At the speeds used (9230 and 7247 rev./min. in the analytical B rotor), the photographic image of the meniscus was itself about 0.1–0.2 mm. broad, so that uncertainty about its exact position might make up to 5% error in the determination of M (Cheng, 1957). Apart from the very early stages of a run (where it was not possible to make measurements) the refractive-index-gradient curve produced by myosin showed (Fig. 1b) at a distance of about 0.4–0.5 mm. from the meniscus a definite inflexion which is not apparent in the curve produced by low-molecular-weight proteins (e.g. bovine serum albumin) under the same conditions. Therefore the method of straight-line extrapolation used by Ginsburg *et al.* (1956) was not adopted, and instead the experimental points were plotted up to a distance of 0.2 mm. towards the position of the top meniscus. A smooth curve was drawn freehand through these points and continued up to the meniscus in order to obtain values of $\partial n/\partial x$ at the meniscus and at a point 0.1 mm. from it (Fig. 3). This procedure will introduce further errors into the determination of M , though they will probably at most be 5%.

It has been found possible to utilize only the gradient at the top meniscus in the calculation of M , since the bottom of the cell is not registered on the photographic plate and neither carbon tetrachloride nor dense silicone fluids could be used to form a registerable bottom meniscus, because they appeared to aggregate or precipitate cod myosin (but not bovine serum albumin). With the conditions described above, the average molecular weight of bovine serum albumin (The Armour Laboratories, Chicago, Ill., U.S.A.) was found to be 74 500, which is 6–7% higher than the generally accepted figure for this protein.

Diffusion. A Tiselius apparatus equipped with a diagonal-edge schlieren system was used at a constant temperature of 0.5°. The boundaries were sharpened by Kahn & Polson's (1947) method. Because of the rapid aggregation of cod myosin, readings were taken only during the first 24 hr.; after this time progressively lower values for the diffusion coefficient were obtained. The rate of diffusion was so slow and the concentration of protein so low that considerable difficulties were experienced in obtaining boundaries which remained undistorted during the first 24 hr. Less than half the experiments proved successful and it is therefore difficult to be really confident of the accuracy of the remainder. The diffusion coefficient was calculated by the maximum-ordinate-area method.

The diffusion coefficient has been also computed from one velocity ultracentrifuge run with Baldwin's (1957) treatment of Fujita's (1956) equation. In this the height-area ratio of the peaks in successive photographs is corrected for the sharpening effect produced on the diffusion gradient of molecules whose sedimentation is concentra-

Fig. 3. Method of extrapolating concentration-gradient curves in the Archibald procedure. $\partial n/\partial x$ is the value of the concentration gradient (actually refractive-index gradient) measured in cm. at the photographic plate. ○, Experimental points; ●, extrapolated points.

tion-dependent, and this ratio squared, $[(A_0/H) \cdot F(\xi_m)]^2$, is plotted against $(e^\tau - 1) [1 + (1 - \lambda)^{\frac{1}{2}}]^2$, where A_0 is the area of the peak corrected for dilution during sedimentation in a sector-shaped cell, H is the height of the peak, $F(\xi_m)$ is a function incorporating the concentration-dependence of sedimentation, and τ and λ are terms incorporating the elapsed time from the start of the experiment. For homogeneous solutes this plot is linear and the slope is $2D/\omega^2 S_0$, where D is the diffusion coefficient of the solute at the temperature and solute concentration of the experiment, ω is the angular velocity and S_0 is the sedimentation coefficient of the solute at infinite dilution and at the temperature of the experiment. In the present experiment the sedimentation coefficient at infinite dilution and the concentration-dependence of sedimentation found for cod myosin (see below) have been used in the calculation.

Viscosity. An Ostwald viscometer (B.S. 188:1957, no. 1) held at 0.5° has been used. The efflux time with buffer was 449 sec. and the rate of shear calculated by Kroepelin's (1929) formula varied between 454 and 272 sec^{-1} for the buffer and most concentrated protein solution respectively. The kinetic-energy correction was negligible.

Turbidity measurements. A Unicam SP. 500 spectrophotometer was used with matched 4 cm. cells. Dialysed cod-actomyosin solutions in 0.5M-KCl, pH 6.3, were centrifuged at 20 000 g until the turbidity was constant, and samples of the supernatant solution were diluted with 0.5M-KCl, quickly warmed to room temperature and their optical densities determined at selected wavelengths between 361.1 and 670.8 $m\mu$. The mean temperature of the solutions at the beginning and end of the readings was used in the computation of refractive index. Protein concentrations ranged from 0.05 to 0.3%. The refractive index of the solvent was calculated from the values given for water in the International Critical Tables, vol. VII (taking account of wavelength and temperature), and the average specific-refractive-index increment of 0.5M-KCl was determined with a dipping refractometer (0.00460). The refractive-index increment of cod actomyosin was assumed to be the same as that of rabbit actomyosin (Harkness & Wassermann, 1954). M was calculated according to Harkness & Wassermann (1954) from the reciprocal intercept of plots of $(Hc/\tau)_{c \rightarrow 0}$ against $(n_0/\lambda)^2$, where τ/c is the reduced turbidity and H is a factor incorporating the wavelength (λ), the refractive-index increment of actomyosin and the refractive index of the solvent (n_0).

Optical rotation. An instrument reading to 0.01° was used. Observations were made at a temperature of $23.5 \pm 0.5^\circ$ in 4 dm. tubes with sodium light.

Partial specific volume. Determinations were made in 10 ml. density bottles at 20.0° . The myosin solutions (concentration about 0.35%) were dialysed beforehand at 0° for 4 days against several changes of KCl-phosphate buffer ($I=0.5$).

Protein concentration. Usually micro-Kjeldahl determinations of nitrogen were used; sometimes they were supplemented by gravimetric determinations of the protein insoluble in 10% trichloroacetic acid. The nitrogen content of dry, ash-free cod myosin was found to be 16.4%; those of actin and actomyosin were assumed to be both 16.0%.

RESULTS

Sedimentation of cod myosin. Within the temperature range of $0-7^\circ$ the values of the corrected sedimentation coefficient (Fig. 4) are consistent, and show no systematic variation with temperature, type of muscle (frozen or fresh) or type of dissociating reagent (ATP or pyrophosphate). Each point represents one distinct preparation. Application of the method of least squares leads to the following values ($S = S_0 - kc$): $S_0 = 6.43 \times 10^{-13}$ (standard error 0.08×10^{-13}), $k = 3.18 \times 10^{-13}$ (standard deviation 0.39×10^{-13}), correlation coefficient 0.89. c is the initial protein concentration in g./100 ml. A single determination of the sedimentation coefficient of rabbit myosin prepared by diluting an extract of rabbit muscle gave a value in KCl-phosphate buffer ($I=0.5$) (Fig. 4) which did not differ significantly from that of cod myosin at the same concentration.

Two runs with monodisperse cod myosin at temperatures of 10.8° and 13.4° and protein concentrations of 0.32 and 0.51% gave respectively values of 5.60 and 5.31×10^{-13} for the sedimentation coefficient. These values are significantly different from the values at the same concentration expected from Fig. 4, the first barely so. A run at 21.8° with cod myosin which was monodisperse at 5° showed several small peaks, the sedimentation coefficient of the slowest being 6.60. Presumably aggregation occurred at this relatively high temperature during the period of approximately 30 min. before starting the acceleration of the rotor. The concentration of the protein corresponding to the slowest peak could not be measured accurately but was probably about 0.05 g./100 ml. These results at high temperatures indicate that the corrected sedimentation coefficient of cod myosin is temperature-dependent, as appears to be the case with

Fig. 4. Concentration-dependence of sedimentation coefficient, O, of cod myosin; ●, of rabbit myosin.

rabbit myosin (Laki & Carrol, 1955; Parrish & Mommaerts, 1954).

Diffusion of cod myosin. The diffusion coefficient of cod myosin was found to vary very little with concentration between 0.19 and 0.40 g./100 ml., the mean value of six determinations being 1.10×10^{-7} cm.² sec.⁻¹, standard error 0.05×10^{-7} . The shape of the diffusion curve of the final photograph of one run (Fig. 5) deviated from ideality in showing considerable sharpening but no skewing.

The results of applying Fujita's (1956) equation to one sedimentation experiment at a myosin concentration of 0.33 g./100 ml. are shown in Figs. 6 and 7. The plot of $[(A_0/H) \cdot F(\xi_m)]^2$ against $(e^x - 1) [1 + (1 - \lambda)^{\frac{1}{2}}]^2$

shows an upward curvature indicating that the preparation is heterogeneous. The magnitude of the correction for the sharpening effect with this protein is shown by the plot of $[A_0/H \cdot 1.128]^2$ against $(e^x - 1) [1 + (1 - \lambda)^{\frac{1}{2}}]^2$, 1.128 being the value $(2/\sqrt{\pi})$ assumed by $F(\xi_m)$ when the sedimentation of the solute is not concentration-dependent. Apparent diffusion coefficients, D^* , (Fig. 7), have been calculated from the data of Fig. 6 assuming that the curves pass through the origin. The value of the diffusion coefficient at zero time, after correction to 20°, is about 1.2×10^{-7} cm.² sec.⁻¹.

Partial specific volume of cod myosin. A value of 0.735 at 20° and a mean concentration of 0.34 g./100 ml. was found with Kraemer's equation 115 (Svedberg & Pedersen, 1940). The value at infinite dilution is probably slightly less than this, which brings it into close agreement with Parrish & Mommaerts' (1954) value of 0.728 for rabbit myosin at 26°. A value of 0.730 at 20° has been used in the calculations of sedimentation coefficient and molecular weight.

Fig. 5. Normalized diffusion curve of cod myosin. O, Experimental points of the diffusion curve in normalized co-ordinates. The full line is the normal curve; 94 140 sec. after the start of the experiment; concentration 0.40 g./100 ml.

Molecular-weight and frictional ratio of cod myosin from sedimentation and diffusion results. Insertion of the values for sedimentation coefficient and diffusion coefficient, determined from a stationary boundary, in the Svedberg equation gives a value of 530 000 for the molecular weight with a standard deviation of about 30 000. The frictional coefficient is 3.62. From Perrin's (1936) equation for prolate

Fig. 6. Height-area plots of the concentration-gradient curve of cod myosin in an ultracentrifuge run at 42 040 rev./min. O, Plot corrected, and ●, uncorrected, for concentration-dependence of sedimentation. Initial concentration 0.33 g./100 ml.

Fig. 7. Apparent diffusion coefficients of cod myosin, D^* , calculated from the points of Fig. 6. O, Corrected, and ●, uncorrected, for concentration-dependence of sedimentation

Table 1. *Molecular weight of a cod-myosin preparation calculated from successive exposures of an Archibald experiment*

Temperature 1.40°; concentration 0.44 g./100 ml.; 9230 rev./min.

Time after reaching full speed (min.)	33	65	97	129	151
Molecular weight/10 ⁵	5.5	5.4	6.4	5.9	5.4

Fig. 8. Concentration-dependence of sedimentation of cod-myosin aggregates. O, Dimer; ●, trimer. Full line is taken from Fig. 4 and refers to myosin.

ellipsoids of revolution, the latter corresponds to axial ratios of about 70 and 60 assuming 10 and 30 % of hydration respectively.

Molecular weight of cod myosin from Archibald procedure. The mean of four determinations at temperatures of 1.4–6.4° and concentrations of 0.23–0.44 g./100 ml. was 590 000, standard deviation 50 000. The molecular weight calculated from successive exposures (Table 1) showed no trend with time, indicating that the preparations were essentially monodisperse.

Intrinsic viscosity of cod myosin. Values of η_{sp}/c were plotted against c for four preparations, and extrapolated to infinite dilution to give an intrinsic viscosity of 1.80, standard deviation 0.05. η_{sp} and c are the specific viscosity and concentration in g./100 ml. respectively. This value corresponds to axial ratios of 58 and 50 for prolate ellipsoids of revolution with 10 and 30 % of hydration respectively (Simha, 1940).

Optical rotation of cod myosin. One preparation had $[\alpha]_D^{25} = -29^\circ$ in KCl-phosphate buffer ($I = 0.5$, protein concentration 0.32 g./100 ml.) and -104° in KCl-phosphate buffer ($I = 0.5$) made up to 8M-urea (protein concentration 0.17 g./100 ml.) respectively. These results remained constant for 24 hr., the solutions being kept at 0°.

Aggregation of cod myosin. The process of aggregation appears to be a much more rapid one than with rabbit myosin. Amounts of oligomers estimated at about 5–10 % of the total protein content are present in cod-myosin solutions kept at 0° for 18–20 hr., whereas solutions of rabbit myosin can be kept unchanged for several days under the same

conditions. Approximate sedimentation coefficients of the two main peaks which sediment more rapidly than monodisperse myosin are shown in Fig. 8. At infinite dilution the values are about $9.0\text{--}9.5 \times 10^{-13}$ and $10.5\text{--}11.0 \times 10^{-13}$, which suggests that they are respectively the dimer and trimer of a side-to-side myosin-aggregation process. For very elongated molecules, the sedimentation coefficients of the side-to-side dimer and trimer are respectively about $\sqrt{2}$ and $\sqrt{3}$ times that of the monomer (Weber & Portzehl, 1952b); for myosin this would give values of about 9.1×10^{-13} and 11.0×10^{-13} for the dimer and trimer respectively. The type of aggregation therefore appears to resemble that undergone by rabbit myosin.

The aggregation does not appear to be dependent upon the presence in the molecule of intact sulphhydryl groups (at least those that are freely reactive), because samples of monodisperse cod myosin which were treated with *N*-ethylmaleimide until they no longer gave a nitroprusside reaction aggregated as quickly as untreated samples.

Aggregation is promoted by diluting solutions of cod myosin until the protein precipitates. This effect, first noted with extracts (Connell, 1958), also occurs with solutions of monodisperse cod myosin. It is not prevented by precipitating the protein into and redissolving it in solutions containing either 0.01M-sodium cysteinate or 0.01M-ethylenediaminetetra-acetic acid at pH 7. Ultracentrifugal analysis of a mixture of cod myosin and its aggregates to which 3M-urea had been added immediately before showed a main component sedimenting at a rate much slower than that of myosin, and two or three smaller components sedimenting at rates similar to those of myosin and its aggregates at similar concentration. Apparently urea at this concentration, in contrast with its effect on aggregated fibrinogen (Caspary & Kekwick, 1957), disaggregates cod myosin into fragments smaller than the monomer. Urea at concentrations of 6–7M is known to dissociate rabbit-myosin monomer but at a relatively slower rate (Snellman & Erdős, 1948; Tsao, 1953).

It was shown that cod myosin is definitely more sensitive than rabbit myosin to precipitation by extracting simultaneously, and with the same batch of solution, muscle removed from recently killed cod and rabbit and working up the extracts (see Experimental section) exactly in parallel. The cod preparation showed in the ultracentrifuge the

presence of much dimer and trimer, but the rabbit myosin remained perfectly homogeneous throughout the run even though it was examined 3 hr. after the cod myosin. Various authors have noted a tendency of rabbit myosin to aggregate on repeated precipitation (Snellman & Erdős, 1948; Portzehl, Schramm & Weber, 1950).

Sedimentation coefficient, molecular weight and frictional ratio of cod G-actin. The sedimentation coefficients of a limited number of purified cod G-actin preparations in a number of solvents are given in Fig. 9. The protein remaining in the supernatant solution of runs containing KCl ($I=0.3$) is presumably inactive actin, i.e. actin no longer able to polymerize; these preparations were obtained by prolonged dialysis against KCl ($I=0.3$). The values of the sedimentation coefficient determined in water are susceptible to serious errors due to charge effects but do not appear to be very much out of line with the values obtained in the presence of salt (cf. Mommaerts, 1952). The extrapolated sedimentation coefficient is about 3.3×10^{-13} , which taken together with the diffusion coefficient of cod G-actin (Connell, 1954), $2.3 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$, gives a molecular weight of about 130 000 and frictional ratio of 2.7. Assuming 30% of hydration, the latter value corresponds to an axial ratio of 25 for a prolate ellipsoid of revolution.

Molecular weight of cod actomyosin. Turbidity measurements on three preparations gave figures in the region of 6, 6 and 8×10^7 for the molecular weight of cod actomyosin.

DISCUSSION

There are so many divergencies in the data for myosins prepared by different laboratories that it is useful to summarize the results if comparison

Fig. 9. Concentration-dependence of sedimentation of ultracentrifugally purified cod G-actin. O, 0.1 mM-ATP, pH 8.5; □, KCl-phosphate, pH 7.5 ($I=0.3$); Δ, KI-phosphate, pH 7.5 ($I=0.4$); ●, KI-phosphate, pH 7.5 ($I=0.66$).

between cod and other animal myosins is to be made (Table 2).

The group of sedimentation coefficients above 7×10^{-13} were all obtained with either oil- or air-turbine ultracentrifuges, and the remaining, significantly lower group, all with Spinco machines; it is likely that part of the discrepancy between the two groups is traceable to differences in rotor-temperature calibration (Edsall, 1953). The scatter in the values obtained with Spinco machines is perhaps no greater than that for some other proteins when they are examined in different laboratories, but it also seems that the sedimentation behaviour of myosin is complicated by two different effects: aggregation and the effect of temperature on the frictional coefficient of the myosin monomer. Most authors describe myosin as a simple, sharp peak in the ultracentrifuge and it is not likely therefore that the variations in sedimentation coefficients are the result of a side-to-side type of aggregation (Holtzer, 1956; Holtzer & Lowey, 1956), since the oligomers formed are easily separable from the monomer by virtue of their much higher sedimentation coefficients ($> 9 \times 10^{-13}$). End-to-end aggregation would yield oligomers with sedimentation coefficients only slightly different from that of the monomer, and it is possible that some variability in sedimentation behaviour as well as the heterogeneity of cod myosin observed in the present work may be ascribed to this phenomenon. It is a reasonably well authenticated fact that the sedimentation coefficient of monodisperse myosin, even after correction in the usual way, shows a positive temperature-dependence (Blum & Morales, 1953; Parrish & Mommaerts, 1954; Laki & Carrol, 1955). This could be explained either by an end-to-end aggregation process whose equilibrium position, like that of side-to-side aggregation, is temperature-dependent, or by a variation of the frictional coefficient of the molecule with temperature. In either case it is possible to obtain a correct value of the average molecular weight from the sedimentation results by combining the data, as in the present work, with results obtained from diffusion experiments made at the same temperature.

Parrish & Mommaerts (1954) suggest that the corrected sedimentation coefficient of rabbit myosin may be dependent upon the angular velocity of the rotor. In the present work two determinations at 42 040 rev./min. of the sedimentation coefficient of cod myosin did not differ significantly from the bulk of the results, which were obtained at 59 780 rev./min.

In view of the variability of S_0 for myosin it is hardly surprising that large differences are found for the concentration-dependence of sedimentation. That for cod myosin is, however, not out of line

with the results for rabbit myosin. The low value of k obtained for carp myosin (Hamoir, 1951, 1955*a, b*) is probably the result of using the concentration of actomyosin from which the myosin was derived by dissociation rather than the concentration of myosin itself.

The diffusion coefficient of cod myosin agrees well with latest estimates for rabbit myosin. The absence of any concentration-dependence is surprising for such an asymmetric molecule as cod myosin, and it is suspected that the results at concentrations below 0.2 g./100 ml. are too low; a similar suggestion has been made for rabbit myosin (Laki & Carrol, 1955). On the other hand, the extremely large dependence on concentration of the diffusion coefficient of rabbit myosin found by Portzehl *et al.* (1950) is difficult to reconcile with the fact that the concentration-dependence of diffusion should be less than that of sedimentation (Singer, 1947).

The molecular weight of cod myosin both from sedimentation and diffusion and from the Archibald procedure are in good agreement with the latest

results on rabbit myosin with sedimentation-diffusion (Laki & Carrol, 1955) and light-scattering (Rupp & Mommaerts, 1957; Holtzer, 1956). The same is true of the axial ratio of cod myosin calculated from the frictional coefficient, though the axial ratio calculated from the intrinsic viscosity is slightly lower. Low values of the axial ratio calculated from viscosity data in contrast with those calculated from sedimentation and diffusion data are also obtained for some other very asymmetric proteins, e.g. tropomyosin and fibrinogen. (Since this paper was prepared two groups of workers have obtained a molecular weight of about 420 000 for rabbit myosin with the Archibald procedure.)

It is not possible to attribute the marked instability of cod myosin to some peculiarity of its molecular weight or general shape. The structural similarity between the cod and rabbit proteins appears to extend to the configuration of the secondary structure of their molecules, since the optical rotation of cod myosin is almost the same as that given for rabbit at the same wavelength

Table 2. *Collected data on the skeletal-muscle myosin of various animals*

Animal	$10^{13} \times S_{20, w}^*$	$10^{13} \times k^\dagger$	$10^7 \times D_{20, w}^*$	$10^{-5} \times M^\ddagger$	$[\eta]^\S$ (100 ml. g. ⁻¹)	Reference
Rabbit	—	—	—	—	2.5	1, 2
Rabbit	7.2 (20)	3.3	0.5 (15)	15 (SD)	—	3
Rabbit	7.1 (20)	3.3	0.87–0.90 (20)	8.29–8.88 (SD) 8.4 (O)	2.2	4
Rabbit	7.1 (20)	5.2	—	—	—	5
Rabbit, rat, mouse	6.2 (7)	5.0	—	—	2.0	6
Rabbit	6.66–6.85 (23.6)	5.21	1.05 (1.1)	—	—	7
Rabbit	6.15 (4)	2.6	1.0 (1.0)	5.0¶ (SD)	—	8
Rabbit	6.38–6.43 (25)	—	—	5.3 (LS)	2.2–2.4	9
Carp	6.55 (20)	1.6	—	—	—	10
Frog	6.6 (20)	—	—	—	2.0 (rabbit)	11
Rabbit	—	—	—	—	1.7	12
Rabbit	—	—	—	6.5** (LS)	—	13
Cod	6.45 (0.5)	3.18	1.10 (0.5)	5.3 (SD) 5.9 (A)	1.80 —	14

* Sedimentation and diffusion coefficients at infinite dilution; figures in parentheses are temp. of measurement.

† Values in the equation: $S = S_0 - kc$ (c in g./100 ml.). Where k is not stated explicitly in the reference it has been estimated from the published figures.

‡ Calc. from sedimentation and diffusion (SD), osmometry (O), light-scattering (LS) and Archibald procedure (A).

§ Intrinsic viscosity, values from references 4, 11 and 12, are viscosity number (Portzehl, Schramm & Weber, 1950) $\times 10$, which is only an approximate conversion.

|| If $V = 0.73$ is used, these values are nearer 7.7–8.0.

¶ Value given in reference 8; correct value appears to be 5.5.

** Provisional estimate; correct value may be lower.

References to Table 2

- Guba & Straub (1943).
- Mommaerts (1945).
- Snellman & Erdös (1948).
- Portzehl *et al.* (1950).
- Johnson & Landolt (1951).
- Miller, Golder, Eitelman & Miller (1952)
- Parrish & Mommaerts (1954).
- Laki & Carrol (1955).
- Holtzer & Lowey (1956).
- Hamoir (1951).
- Hamoir & Reuter (1956).
- Kerekjato (1952).
- Rupp & Mommaerts (1957).
- Present work.

(Cohen & Szent-Györgyi, 1957). The explanation for the readiness with which cod myosin aggregates probably lies rather in some idiosyncrasy of molecular topography or reactivity of surface groups. Certain soya-bean-protein fractions aggregate in a similar way to cod myosin when their solutions are diluted, and the interaction of sulphhydryl groups has been shown to be involved in the process (Briggs & Wolf, 1957). What evidence there is suggests that the aggregation of cod myosin is not of this kind; it has been also noted (J. J. Connell, unpublished work) that the sulphhydryl content of cod myosin appears to be not very different from that of rabbit myosin.

The order of the molecular weight of cod actomyosin determined by turbidity is about one-half or one-third of the values obtained for rabbit actomyosin with the same method (Harkness & Wassermann, 1954), but very similar to those for rabbit actomyosin from light-scattering measurements (Blum & Morales, 1953) or for carp actomyosin from sedimentation and viscosity measurements (Hamoir, 1955*a*).

The value of the sedimentation coefficient of cod G-actin and the resulting value for molecular weight, when combined with the diffusion coefficients, substantiate the earlier observations (Connell, 1954) that this protein closely resembles rabbit G-actin and in particular agree with the conclusion from osmotic-pressure measurements that it exists mainly in the form of the dimer of molecular weight about 150 000. The value for the axial ratio of cod G-actin estimated from sedimentation and diffusion also agrees with Tsao's (1953) viscometric value for the rabbit-protein dimer, though in view of the approximate nature of the data this agreement is perhaps fortuitous.

SUMMARY

1. Solutions of unaggregated cod myosin have been prepared by ultracentrifugal separation after dissociation of cod actomyosin with adenosine triphosphate or pyrophosphate.

2. The sedimentation and diffusion coefficients of cod myosin at infinite dilution have been measured and the molecular weight and shape calculated from these data with the measured partial specific volume.

3. Investigation of the diffusion behaviour both in stationary boundaries and in boundaries in the ultracentrifuge indicate that the preparations of cod myosin are heterogeneous.

4. The molecular weight of cod myosin has been also determined by the Archibald procedure and the axial ratio assessed from the intrinsic viscosity.

5. Some aspects of the aggregation of cod myosin have been examined.

6. Cod actin has been purified in the ultracentrifuge and its sedimentation coefficient determined.

7. The order of magnitude of the molecular weight of cod actomyosin has been determined from turbidity measurements.

8. The size and shape of the cod fibrillar proteins are very similar to those of the rabbit proteins. The unusual properties of the fish proteins cannot be explained therefore on this basis.

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The Changes in the Total Vitamin B₆ and the Pyridoxal Phosphate Content of Cells of *Bacillus sphaericus* during Growth and Sporulation: their Possible Relationships with $\alpha\epsilon$ -Diaminopimelic Acid Metabolism

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The present study was prompted by the observation that vegetative cells of *Bacillus sphaericus* contained no $\alpha\epsilon$ -diaminopimelic acid (DAP), whereas sporulating cells and spores contained 1–2% (w/w) of DAP (Powell & Strange, 1957). A connexion between DAP metabolism and sporulation was already suspected: from structural considerations and the analogous conversion of lysine into piperidine-2-carboxylic acid (Rothstein & Miller, 1953; Lowy, 1953) it seemed possible that DAP was the precursor of pyridine-2,6-dicarboxylic acid (dipicolinic acid), a substance synthesized in large amounts by sporulating cells (Powell, 1953; Powell & Strange, 1956). In other *Bacillus* species there was, however, no connexion between sporulation and the appearance of DAP as a cell constituent: vegetative cells, sporulating cells and spores all contained approximately equal amounts of DAP. *B. sphaericus* was also exceptional among *Bacillus* species (Antia, Hoare & Work, 1957) in possessing an active DAP decarboxylase system requiring pyridoxal phosphate as a cofactor (Powell & Strange, 1957). A comparison of the DAP decarboxylase activities of vegetative cells, sporulating cells and spores showed that although the decarboxylase activity of *B. sphaericus* cultures decreased at sporulation, the activity of strongly sporulating cells was still 50% of that of vegetative cells. There was therefore no sudden disappearance of the decarboxylase at sporulation to account for the appearance of DAP; nor was its activity in

disrupted cells inhibited by dipicolinic acid (Powell & Strange, 1957). There remained the further possibilities that (a) other unidentified substances synthesized during sporulation inhibited the decarboxylase or (b) that sporulation was associated with a disturbance of total vitamin B₆ or, more particularly, of pyridoxal phosphate synthesis. The present report is chiefly concerned with the latter possibilities and also includes some data on DAP decarboxylase and vitamin B₆ synthesis during spore germination and development.

EXPERIMENTAL

Materials

Organisms. Most of the work was done with *B. sphaericus*, National Collection of Type Cultures (N.C.T.C.) 9602, since this strain usually sporulated strongly and gave 'clean' spore suspensions, i.e. suspensions containing very little vegetative debris, after growth for 24 hr. in the test medium. A few observations were made on a laboratory strain of *Bacillus cereus* which was used in a previous study (Powell & Strange, 1956). Some growth tests were made with *B. sphaericus* N.C.T.C. 7582 var. *fusiformis*, an asporogenous variant of *B. sphaericus* N.C.T.C. 7582 (Powell & Hunter, 1955) and a laboratory strain of *B. sphaericus* var. *rotans*.

Chemicals. Pyridoxal hydrochloride and pyridoxine hydrochloride were supplied by L. Light and Co., pyridoxal phosphate by S. A. F. Hofmann-La Roche and Co., deoxypyridoxine hydrochloride by Nutritional Biochemical Corp. and isonicotinylhydrazine by Ward, Blenkinsop and Co.