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The Intrinsic Viscosity of Myosin and the Interpretation of its Hydrodynamic Properties

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Early work suggested a particle weight of about 800 000 for the myosin molecule, with an axial ratio (J) of 100:1 (Portzehl, 1950), but more recently it has appeared that some of the data employed were in error. For the sedimentation coefficient, a detailed account has been given of the probable cause of this error in terms of molecular-transformation reactions occurring during the experiments (Johnson & Rowe, 1960). There may well have been similar errors in much recent as well as early diffusion work; also in viscosity, light-scattering and osmotic-pressure experiments. There has as yet, however, been no general agreement upon revised estimates of the size and shape of the myosin molecule.

For the complete definition of an ellipsoid of revolution hydrodynamically equivalent to the myosin molecule, three independent (usually kinetic) parameters must be known, together with the partial specific volume ($\bar{v}$). The method of Scheraga & Mandelkern (1953) then provides a

convenient means for the calculation of the axial ratio and the effective volume/g. (V) of this ellipsoid. Let us take the extrapolated sedimentation and diffusion coefficients, $[S_{20}^0]$ and $[D_{20}^0]$, and the intrinsic viscosity, $[\eta]$, as the three kinetic parameters. $[S_{20}^0]$ has been accurately determined under conditions where myosin is known to be stable (Holtzer & Lowey, 1956, 1959; Johnson & Rowe, 1960), as also has $[D_{20}^0]$ (Parrish & Mommaerts, 1954), although the value given by Parrish & Mommaerts should be confirmed by an independent and equally thorough study. But the values given in the literature for $[\eta]$ show a surprising degree of scatter, the following being among those reported (dl./g.): 2.2 (Mommaerts, 1945, and Weber, 1950*a, b*); 1.8–2.0 (Weber & Portzehl, 1951; Portzehl, Schramm & Weber, 1951); 2.3, 2.2 and 2.4 (Holtzer & Lowey, 1956). Since the completion of this work Holtzer & Lowey (1959) have reported a value of 2.17 at 25°, and a rather higher value (2.24) at 0.7°. In view of the occur-

rence of a fair degree of scatter, Holtzer & Lowey do not consider this difference to be significant.

In the work here reported the difficulties in the accurate viscometry of myosin solution have been to a large extent overcome by the use of a modified viscometer and a carefully defined technique. An accurate value for $[\eta]$ is reported, which is used in calculating the dimensions of the equivalent ellipsoid of revolution.

EXPERIMENTAL

Materials

Water. All water used was thrice-distilled, as previously described (Johnson & Rowe, 1960).

Reagents. All reagents used were of A.R. quality.

Buffer solution. All experiments were performed in a stock buffer solution, pH 6.7 and 1.0-6.0, having the following composition (g./l.): KCl, 41.0; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 4.0; KH_2PO_4 , 2.2.

Myosin. Myosin was prepared from rabbit skeletal muscle as previously described (Johnson & Rowe, 1960). The purified protein was stored in stock buffer solution at 2-4° in sealed, sterile flasks.

Methods

Viscosity determinations. All viscosity determinations were performed with capillary viscometers reproducibly located on rigid, detachable mounts in a lagged water thermostat equipped with cooling coils. The temperature could be maintained to within $\pm 0.02^\circ$ over the range 5-25°.

Simple Ostwald and Ubbelohde viscometers, maintained at 20°, were used originally, but were found to give very poorly reproducible results with myosin solutions. In the light of experience gained of the general properties of myosin, the following peculiar difficulties can be listed:

(i) Myosin undergoes transformation reactions at room temperatures, and these are accompanied by viscosity changes (Lowey & Holtzer, 1959*a*; P. Johnson & A. J. Rowe, in preparation).

(ii) 'Threads' of denatured protein readily form in the bulk of the solution, and obstruct flow through the capillary.

(iii) A slight amount of surface foam is very easily formed during manipulation of solutions. Accurate measurements require the observation of a clean, well-defined meniscus.

(iv) Myosin has a tendency to precipitate on glass surfaces (Rupp & Mommaerts, 1957). Even if the bulk concentration is little affected, a film of protein may line the capillary, both narrowing the bore and also tending to entrap air bubbles during re-charging of the viscometer.

The problem of capillary adsorption is discussed below. The remaining difficulties have been overcome by the use of a modified viscometer and by working at $5 \pm 0.02^\circ$ with a rigidly defined technique.

The modified capillary viscometer used was similar to one described by Nawab, Goring & Mason (1957) and is illustrated in Fig. 1. Described as a 'non-foaming' viscometer, it is so designed that where a certain amount of surface foam appears during manipulation this can be spilled over into the upper reservoir A by suction at C, with the con-

necting tube F sealed off by the three-way tap E. The detailed technique of measuring and dilution was as described by Nawab *et al.* (1957). Briefly, the viscometer was charged from the lower reservoir B by suction at C, allowing solution to overspill into A until the lower meniscus reached the mark Z: the fall of the upper meniscus between the levels X and Y was then timed. The capillary was at all times kept filled with solution, and the lower bulb Q was used solely to prevent the meniscus from entering the capillary after each reading. Dilutions were made in the reservoir B, tap E being opened to drain the upper reservoir. The efficiency of the dilution procedure was checked by control experiments with coloured fluids. For experiments with variable shear-rate, a system of variable large-capacity reservoirs was connected at C, the resulting constant pressures being read from a water manometer by means of a cathetometer.

After normal cleaning (chromic acid; water; A.R. acetone) the viscometer was dried by sucking through it a stream of filtered air. Solutions were freed from dust and protein 'threads' by filtration under gravity through cotton-wool plugs. This method was found to be superior to the use of removable sintered-glass tips as filters on pipettes used in transfer, a technique which was also tried but abandoned as liable to produce foaming.

Values for flow-times were accepted only if the capillary was visibly free from obstruction and if several readings consistent to ± 0.15 sec. (< 0.2 g./100 ml.) or to ± 0.25 sec.

Fig. 1. 'Non-foaming' capillary viscometer used for myosin solutions. Capillary length, 6.3 cm.; internal diam. 0.05 cm. (approx.). Minimal charging volume, 10 ml. For description of parts see the Experimental section.

(higher concentrations) could be obtained. By these criteria results from second and subsequent dilutions were invariably rejected, and often those from first dilutions also. Latterly, dilution *in situ* has been abandoned, and the viscometer cleaned, dried, filled and equilibrated for each concentration. Acceptable results were then usually obtained.

The kinetic characteristics of the viscometer were determined by the method described by Merrington (1949). For a given viscometer

$$\eta = \rho[At - (B/t)], \quad (1)$$

where η is the dynamic viscosity of the fluid under investigation, ρ is the density of the fluid, t is the flow time and A and B are constants. The term B/t represents a correction for the kinetic energy of the fluid leaving the capillary.

Water and aqueous glycerol solutions, whose exact composition was found from density determinations, were employed as standards. Details of the density and viscosity of aqueous glycerol solutions were taken from the tables of Bosart & Snoddy (1927) and Sheely (1932). The results obtained are shown in Table 1. The quantity B is seen to be too small to be measured with any accuracy. For a typical experiment with a myosin solution, with t 250 sec., the correction term (B/t) amounts to 0.2% of (At) , taking B 0.9. Because of the smallness of this correction and the uncertainty of its exact value, it has not been applied to the data reported.

Measurements of protein concentration. Portions of solution were removed from the viscometer after each experiment, and the nitrogen content was estimated by the micro-Kjeldahl method (Pregl & Roth, 1945). A nitrogen content of 16.2% was assumed for myosin (Mommaerts & Parrish, 1951).

RESULTS

It was shown by Mommaerts (1945) that the relative viscosity of dilute myosin solutions shows little shear-rate dependence. This was confirmed in the present work by following flow-times at various pressure-heads and several concentrations (Fig. 2). The linearity of the plots demonstrates that the effect of varying shear was negligible under the conditions employed.

The collected data from four preparations are shown in Figs. 3 and 4. Above a protein concentration of 0.2 g./100 ml. both the relative viscosity ($\eta_{rel.}$) and the reduced specific viscosity ($\eta_{sp./c}$)

Fig. 2. Reciprocal flow-times of myosin solutions at 20° with various pressure heads. Concentrations: c_1 , 0.46; c_2 , 0.42; c_3 , 0.28; c_4 , 0.21 g./100 ml. Solutions in stock buffer solution.

Fig. 3. Reduced specific viscosity of myosin in stock buffer solution as a function of concentration. $\square$, Myosin VI; $\circ$, myosin VII; $\triangle$, myosin VIII at 5°; $\bullet$, myosin IX at 25°, extrapolated to zero time of incubation.

Fig. 4. Reduced specific-viscosity data for myosin at concentrations below 0.3 g./100 ml. Symbols are defined in Fig. 3.

Table 1. Values for the characteristic constants A and B

Values are for the 'non-foaming' capillary viscometer, determined by the use of aqueous glycerol solutions and water as standards.

Concn. of glycerol soln. used (%)	A	B
5.45	0.00719 (± 0.00011)	0.4 (± 0.6)
12.05	0.00724 (± 0.00007)	1.4 (± 0.6)

cease to be linear with respect to concentration, as noticed by Szent-Gyorgyi (1951). Below this concentration, the relation between η_{sp}/c and c is linear to a good approximation. The line drawn was determined by the method of least squares for the data from myosin VII and myosin VIII (Fig. 4). From the intercept, a value for the intrinsic viscosity $[\eta] = 2.35 \pm 0.01$ dl./g. has been obtained. Further uncertainty arises from doubts about the nitrogen content of the protein. Thus Dr K. Bailey (personal communication) considers the value 16.7% to be more correct than that (16.2%) assumed here, and use of the higher figure would yield an intrinsic viscosity of 2.42. The result given here falls within the range of values obtained by previous workers (also subject to uncertainty in the nitrogen content), and the problem of reproducibility appears to have been successfully overcome.

Two results obtained with myosin IX at 25° are included in Figs. 3 and 4. As is shown by P. Johnson & A. J. Rowe (in preparation), the viscosity of myosin solutions increases with time at this temperature, and these results have been obtained by extrapolation of values of η_{sp}/c to zero time of incubation. The concordance with results at 5° provides further evidence that the hydrodynamic behaviour of native myosin is consistent over this temperature range (cf. Johnson & Rowe, 1960).

DISCUSSION

Is capillary adsorption occurring? The possibility of adsorption of protein on the walls of the capillary has not been specifically ruled out in the above-described experiments. Moreover, the fact that values for η_{sp}/c as determined at low concentration lie above the line which might be extrapolated from higher concentration (> 0.4 g./100 ml.) might suggest such an adsorption. However, two sets of considerations make it very unlikely that $[\eta]$ is in error for this reason.

First, Öhrn and others have described the effect of capillary adsorption theoretically, and observed it empirically in solutions of synthetic polymers in organic solvents (Öhrn, 1955, 1956; Tuijnman & Hermanns, 1957; Takeda & Endo, 1956; Rutherford & Pepper, 1959; Huque, Fishman & Goring, 1959). Their interpretation has, however, been disputed by Boyer & Streeter (1955). Experimentally, a steep rise in the reduced specific viscosity as the concentration falls has been generally found, followed by a 'fall-over' and steep drop as the concentration approaches zero. This differs markedly from the behaviour of myosin solutions.

Secondly, for adsorption to occur it would have to be: (i) Complete within equilibration time. At 5° the viscosity of myosin solutions does not change

with standing in the viscometer. (ii) Completely reproducible between different chargings of the cleaned viscometer, different preparations and different temperatures (5° and 25°). (iii) Preferential on the capillary walls, rather than on the bulbs and reservoirs of the viscometer.

Postulate (i) is quite reasonable: (ii) and (iii) are most unlikely to be true. Even allowing for immobilized solvent contributing to the adsorbed layer, it may be calculated that the total amount of protein present at the lowest concentration (about 1 mg.) is quite insufficient to provide adequate generalized adsorption. Further, at low protein concentrations (< 0.2 g./100 ml.), adsorption if appreciable should be directly observable as a significant fall in the solution concentration. Only small decreases have been observed experimentally. Moreover, after storing myosin solution in a cleaned viscometer, and then carefully draining, we have been unable to demonstrate anything more than a very small ($< 0.5\%$) rise in flow-times for either protein solution or solvent over that obtained with a clean viscometer, unless gross visible obstruction of the capillary had occurred. Thus positive evidence for capillary adsorption is lacking, and indirect evidence strongly suggests that it was not an important factor in these experiments.

However, it should be borne in mind that generalized adsorption of protein on the viscometer walls could cause a drop in bulk protein concentration even though flow-times were but little affected. Thus during filling and charging of the viscometer, a solution (10 ml.) is exposed to at least 100 cm.^2 of glass surface. A uniformly adsorbed film $10^3 \text{ \AA}$ thick would deplete the protein content by 1.3 mg., although the flow time for a capillary of diameter 0.05 cm. would be increased by only 0.16%. Thus the protein concentration should be measured after equilibration in the viscometer, the only remaining source of possible error then being adsorption on the pipette during the transfer to the Kjeldahl-digestion flask.

Comparison with other values for $[\eta]$. Weber's low value for $[\eta]$ (Portzehl *et al.* 1951) may be ascribed to the fact that it was obtained from results at concentrations greater than 0.2 g./100 ml. The present value falls within the range of other values quoted to within the various experimental errors: however, it appears to be just significantly higher than the most recent value given by Holtzer & Lowey (1959) (2.17 ± 0.1 dl./g. at 25°). Probably differences in techniques of concentration measurement account for this discrepancy. Holtzer & Lowey obtained c values by dilution from stock solution: as is described above, adsorption on the walls of the dilution vessels and of the viscometer could result in these c values

being erroneously high, and hence in low values for η_{sp}/c . These latter values might well decrease with increasing temperature, as described by Holtzer & Lowey, since surface denaturation and adsorption would be expected to increase with increasing temperature. For these reasons, and also because the Ostwald-Fenske viscometers used by Holtzer & Lowey are less well suited to the system and give a greater scatter of results than the 'non-foaming' viscometer, we consider that the present value for $[\eta]$ is to be preferred.

Calculation of the size and shape of the equivalent ellipsoid. It was shown by Scheraga & Mandelkern (1953) that the axial ratio of the equivalent ellipsoid may be calculated from a single function, β , defined by

$$\beta = N \cdot \eta \left(\frac{[S][\eta]}{(1-\bar{v}\rho)} \right)^{\frac{1}{2}} \left(\frac{[D]}{RT} \right)^{\frac{1}{2}}, \quad (2)$$

where η is the viscosity of the solvent, ρ is the density of the solution and $[S]$, $[\eta]$, $[D]$ and $\bar{v}$ are respectively the extrapolated sedimentation coefficient, reduced specific viscosity, diffusion coefficient and partial specific volume of the solute, under a given set of conditions. For myosin, the above-mentioned quantities (with the possible exception of $[D]$) have recently been measured with accuracy at low temperatures where myosin is stable. The solvents used were closely similar in composition, and it has been shown that small variations in ionic strength, composition and pH do not affect the sedimentation or light-scattering behaviour of myosin solutions (Parrish & Mommaerts, 1954; Holtzer & Lowey, 1959; Johnson & Rowe, 1960, and present work). The intrinsic viscosity of the native protein does not appear to vary with temperature over the range 5–25°, and it has already been shown that this is also true for the sedimentation coefficient over the range 3–19° (Holtzer & Lowey, 1959; Johnson & Rowe, 1960). Thus we can be reasonably confident that all the data refer to identical or at least equivalent experimental conditions. Substituting $[S]_{20}^0 = 6.43$ s, $[\eta] = 2.35$ dl./g., $\bar{v}_{20} = 0.725$, $[D]_{20}^0 = 1.05 \times 10^{-7}$ c.g.s.u. and values for η_{20}^0 and ρ_{20}^0 , a value $\beta = 2.83 \pm 0.06$ is yielded. An axial ratio $J = 34.5 \pm 5$ is obtained by interpolation from Scheraga & Mandelkern's tables of the function: this corresponds to an effective volume $V = 2.52$ ml./g. (by application of the equation of Simha, 1940). A possible error of $\pm 2\%$ has been assumed for the diffusion coefficient: other errors in the evaluation of β are relatively small.

As all the data were obtained at a high ionic strength, the possible effect of selective solvation on the term $(1-\bar{v}\rho)$ must be considered (Schachman & Lauffer, 1950; Brand & Johnson, 1958). Assuming (as an outside estimate) that 0.5 ml. of water is

selectively associated with 1.0 g. of dry protein, then the term $(1-\bar{v}\rho)$ must be replaced by $1-\bar{v}\rho+0.5(\rho_{\text{water}}-\rho)$, and β becomes 2.88. This increase in β is within the cumulative experimental uncertainty, and the effect of selective solvation on β is not further considered.

The effective volume of the equivalent ellipsoid is large, suggesting that a considerable amount of solvent may be associated with the molecule. Correspondingly, the axial ratio is lower than that which would be calculated assuming zero solvation for the molecule from $[\eta]$ and $\bar{v}$ ($J = 70$), or from $[D]_{20}^0$, $[S]_{20}^0$ and $\bar{v}$, which yield a value $J = 86$. These latter values, which involve the assumption of $V = \bar{v}$, are in good agreement with those derived by Holtzer & Lowey (1959), using the same assumption, from the equations of Riseman & Kirkwood (1950) and Kirkwood & Auer (1951), which treat the molecule as a rigid string of spherical beads.

An independent estimate of the size and shape of the hydrodynamic particle is clearly desirable. The theory derived by Fessler & Ogston (1951) and Ogston (1953) for the dependence of S_{20}^0 upon concentration offers the possibility of obtaining such an

estimate from the parameters $d\left(\frac{1}{S}\right)/dc$, $[S]_{20}^0$, $[\eta]$

and $\bar{v}$. It has been found that the variation of S_{20}^0 with concentration for myosin can be well described in terms of this theory; allowance being made for the fact that as the concentration approaches zero, one of the constant terms (k) in Fessler & Ogston's equations itself varies with concentration, an effect to be expected from earlier work by Emersleben (1925) and Sullivan (1941) but not incorporated in Fessler & Ogston's treatment. A value for $d(1/S)/dc = 0.0956$ has been measured, mean sedimenting concentrations being used as suggested by Kegeles & Gutter (1951). Assuming values $k = 3$, $\zeta = 0.67$ for the constant terms, values of $M = 543\,000$, $J = 32.7$, $V = 2.71$ have been obtained as described by Ogston (1953). The selection of values for k and ζ is to a considerable extent arbitrary. For sedimenting protein systems Ogston (1953) used the value $k = 1.7$ with initial protein concentrations in calculating values of $d(1/S)/dc$. If his results are recalculated by using estimated mean concentrations during sedimentation they are no longer decisive in favouring a value lower than $k = 3$. The latter value is supported also by calculation for model systems (Emersleben, 1925). With initial concentrations in sedimentation and $k = 1.7$, $\zeta = 0.67$ (Ogston, 1953), then we obtain $J = 26.4$, $M = 565\,000$. The agreement with the previous results is very satisfactory, but it should be emphasized that the Fessler-Ogston treatment is to a considerable extent an empirical one, and too much significance should not therefore be attached to the extent of this agreement.

Molecular weight of myosin. With the data given above, the sedimentation-diffusion method yields a value $M = 540\,000 \pm 15\,000$. This agrees reasonably well with the most careful estimates by the light-scattering method (Rupp & Mommaerts, 1957; Holtzer & Lowey, 1956, 1959). However, recent estimates by the 'Archibald' technique have yielded values as low as 420 000 (Mommaerts & Aldrich, 1958; Hippel, Schachman, Appel & Morales, 1958; Lowey & Holtzer, 1959*b*), and this lower value has been quoted as the best available estimate for M in several reviews of the field (e.g. Morales, 1960; Huxley, 1960).

This discrepancy is not easily explained. Selective solvation to the extent described earlier reduces the sedimentation-diffusion value for M to 514 000, but then the 'Archibald' values should be corrected in exactly the same ratio. It does not seem likely that the values for $[S]_0$, $[D]_0$ and $\bar{v}$ now used could be in cumulative error to the extent of 29 %, as is required by acceptance of the lowest 'Archibald' values. In fact these various quantities now seem to be known to a higher degree of precision, over a range of conditions, than for the great majority of 'well-defined' proteins. The chief remaining source of uncertainty lies in the value for $[D]_0$ obtained by Parrish & Mommaerts, which has not been independently confirmed; but values obtained by other workers have all been lower rather than higher (Weber, 1950*a*; Laki & Carroll, 1955).

Using an alternative expression for the β coefficient (Scheraga & Mandelkern, 1953)

$$\beta = \frac{N[S]\eta[\eta]^{\dagger}}{M^{\frac{1}{2}}(1-\bar{v}\rho)}, \quad (3)$$

and the lowest 'Archibald' value for M , an equivalent ellipsoid may be constructed independently of a value for the diffusion coefficient. The results yielded are: $\beta = 3.34$, $J = 143$, $V = 0.2$.

A recent comprehensive study in this Laboratory of published hydrodynamic data for proteins has shown that there is probably no well-established case where the effective volume is substantially smaller than the partial specific volume (E. G. Richards, personal communication). The J value obtained is also greatly in excess of the maximum permissible value calculated directly from the Simha equation assuming zero hydration. Thus the weight of evidence suggests that the low 'Archibald' values for M are in error, but the reason for this is not clear. Errors in determination of the meniscus position as described by Trautman (1958) as well as the inherent large uncertainty of the method are probably involved.

Conclusions. If the anomalous results yielded by the 'Archibald' method can be explained, then the behaviour of the myosin molecule in solution now

seems to be as well defined as current hydrodynamic theory will permit. In addition to a comprehensive diffusion study, the chief additional data which might usefully be obtained are on the flow-birefringence at low temperatures, the experiments of Joly, Schapira & Dreyfus (1955) being difficult to interpret since these were obtained at room temperatures.

If, as is usually thought, the myosin molecule is indeed a rigid, elongated structure, then a prolate ellipsoid of revolution should provide a fair model, and we may tentatively interpret our equivalent ellipsoid in physical terms. Its most striking feature is its large effective volume. This cannot be explained by normal estimates of solvation, and might be accounted for either in terms of a 'swept' or 'excluded' volume caused by rotation of an asymmetrical particle, or else in terms of a volume of solvent included 'within' the molecule. Since extensive solvent immobilization within the tertiary structure is unlikely, this latter possibility would correspond to a structure such as a hollow cylinder, the solvent within the cylinder being postulated as contributing to the hydrodynamic volume. Taking 1620 Å as the molecular length (Holtzer & Lowey, 1959), the axial ratio as 34.5 (for high axial ratios, prolate ellipsoids and cylinders of revolution are indistinguishable) and $M = 540\,000$, $\bar{v} = 0.725$, a value $V = 3.2$ is yielded for such a hollow cylinder, in reasonable agreement with that found for the equivalent ellipsoid. Electron-microscope studies are likely to offer the most direct further evidence on this problem, and an investigation by this technique is under way. Valentine (1960) has shown that the seed protein, legumin, appears as a hollow cylinder in electron micrographs.

SUMMARY

1. The problems involved in the viscometry of myosin solutions have been investigated, and many of the difficulties overcome by the use of a modified form of capillary viscometer. A value for the intrinsic viscosity of myosin,

$$[\eta] = 2.35 \pm 0.03 \text{ dl./g.},$$

is reported. With this value, together with

$$[S]_0 = 6.43 \text{ s}, [D]_0 = 1.05 \times 10^{-7} \text{ c.g.s.u.}, \\ \bar{v} = 0.725,$$

a β factor of 2.83 is calculated. The hydrodynamically equivalent ellipsoid possesses an axial ratio $J = 34.5$ and effective volume $V = 2.52 \text{ ml./g.}$ An independent treatment utilizing the sedimentation-concentration dependence is shown to give consistent results.

2. The values for the molecular weight obtained by the 'Archibald' method are shown to be inconsistent with the present data, and it is suggested that the Archibald values may be too low.

3. The properties of the equivalent ellipsoid are discussed in terms of possible molecular models, and the dimensions of one model, a hollow cylinder of revolution, are calculated.

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