Geometry of the Myosin Dimer in High-Salt Media. I. Association Behavior of Rod Segments from Myosin†

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ABSTRACT: Evidence is presented from high-speed meniscus depletion sedimentation and concentration dependence of reduced viscosity studies demonstrating that light meromyosin (LMM) and headless myosin (rod) exist in a rapidly reversible equilibrium, probably of the monomer $\rightleftharpoons$ dimer mode at high ionic strength. The free energy of association of these particles is similar to that found previously for myosin indicating that the interaction sites responsible for association of the parent molecule reside primarily in the LMM segment. Intrinsic viscosities of the dimer species were derived from the concentration dependence of the reduced viscosity. The lengths of the monomer and dimer species have been determined from their respective intrinsic viscosities and molecular weights and the geometry of the parent myosin dimer has been deduced from the overlap lengths of LMM and rod dimers taken in conjunction. The possibility that this dimer is the basic building unit of the thick filament of muscle is discussed.

We have recently demonstrated (Godfrey and Harrington, 1970a,b) from differential velocity sedimentation and high-speed sedimentation equilibrium studies, that myosin molecules are in rapid, reversible equilibrium with a dimeric species at high ionic strength (0.5 M KCl–0.2 M PO_4^{2-}–0.01 M EDTA, pH 7.3). Similar conclusions have been reached by Herbert and Carlson (1971a) from spectral broadening and intensity measurements of laser light scattered from solutions of myosin under comparable ionic conditions. It has been known for several years (Jakus and Hall, 1947; Noda and Ebashi, 1960; Huxley, 1963; Zobel and Carlson, 1963; Josephs and Harrington, 1966; Kaminer and Bell, 1966a,b) that reduction of the salt concentration of such myosin systems by dilution or dialysis results in the formation of filamentous structures which are similar in a number of respects to the natural thick filaments of muscle. Thus it seems possible that the dimeric species present in the high-salt medium could be the initiating, and possibly the building unit used in assembly of the thick filaments. A major feature of these filaments is the existence of a smooth central region of about 0.15–0.20 μ in length with projections on either side extending out to the tapered ends. Huxley has proposed (1963) that the smooth midsection of the thick filament is constructed through antiparallel (tail-to-tail) packing of the myosin molecules so that the projecting heads, corresponding to the cross bridges in striated muscle, are oriented away from the smooth central zone. Elsewhere, in the cross-bridge region which constitutes the bulk of the filament mass, electron microscope and low-angle X-ray diffraction evidence (Huxley, 1963; Huxley and Brown, 1967) points to parallel (head-to-tail) packing of the monomeric myosin units. X-Ray diffraction studies of living muscle (Huxley and Brown, 1968) indicate large perturbations in the cross-bridge regions during active contraction suggesting that their arrangement in the thick filament structure is fundamental to the contractile mechanism.

The evidence that a dimer species exists in high salt leads to some interesting questions on the mechanism of assembly of the filament. If the dimer is composed of two myosin molecules in antiparallel configuration then one could suppose that if this dimer species is present at low ionic strength the bare central region of the filament could be constructed by lateral assembly of the dimer units. Further linear growth of the filament is presumably restricted to addition of monomeric units since addition of antiparallel dimers would lead to a cross-bridge array incompatible with the presently accepted distribution of cross bridges along the native thick filament surface (Huxley, 1963; Huxley and Brown, 1967).

If the dimer has parallel geometry then the bare central zone could be constructed from antiparallel association of the dimer species. Further growth of the filament could be achieved by addition of monomer or by addition of the same dimer species. Thus a knowledge of the geometry of the initiating dimer species would be expected to help clarify the self-assembly process of myosin molecules in formation of the thick filament of native muscle.

The solubility properties of light meromyosin (LMM) and "headless" myosin (rod) are very similar to those observed for myosin. Both particles show a remarkable tendency to associate in ordered arrays at low-salt concentrations. When the ionic strength of LMM is reduced to 0.1 or 0.2 M, spindle-shaped aggregates are formed of indefinite length and diameter and with a pronounced axial periodicity of about 430 Å (Szent-Gyorgyi, 1953; Philpot and Szent-Gyorgyi, 1954; Huxley, 1963), as seen by the negative staining technique in electron microscopy. Rod preparations of chicken myosin in which the globular segments of the myosin molecules have been removed from the rod by papain digestion, form both bipolar and polar ribbon-like aggregates when precipitated in the presence of divalent cations and potassium thiocyanate at low ionic strength (Cohen *et al.*, 1970; Harrison *et al.*, 1971). These studies indicate that the interaction sites responsible for association of myosin are likely to be located along the rod segment of the myosin molecule and very probably are within the LMM segment since subfragment II (the segment of the rod lying between LMM and the globular head) shows no apparent tendency to associate either with itself or with LMM at low ionic strength (Lowey *et al.*, 1969).

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From the information given above, it seems likely that both LMM and rod should exhibit association behavior in high-salt media similar to myosin. Moreover, since these particles are uniform rods of high axial asymmetry, the measured hydrodynamic properties should be extremely sensitive to the geometry of the associated species.

In the study to be presented below, we report high-speed sedimentation equilibrium and concentration dependence of viscosity experiments demonstrating that these particles are indeed in rapid, reversible chemical equilibrium with a dimeric species. The hydrodynamic behavior of the two associating systems has been utilized to deduce the geometry of the dimer species formed in high-salt media. In the following paper (Burke and Harrington, 1972) we present evidence on the geometry of the myosin dimer deduced from studies of the hydrodynamic behavior of macromodels. A preliminary account of our findings has already been published (Burke and Harrington, 1971).

Materials and Methods

All experiments were carried out at 5.0° unless otherwise stated. Glass distilled water was used throughout and the reagents were of analytical grade; trypsin and papain were purchased from Worthington and the former was further treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (Cyclo Chemical Corp., Los Angeles, Calif.) according to the procedure of Schoellmann and Shaw (1963) to remove any residual chymotryptic activity. Papain was insolubilized by complexing with cellulose as reported by Kominz *et al.* (1965). To ensure against contamination by trapped soluble papain, the cellulose–papain was placed in a small column and washed with papain diluent until no activity could be detected in the eluent. The insoluble enzyme conjugate was then dispersed in a glass homogenizer prior to determination of activity by the standard pH-Stat procedure.

Preparation of the Proteins. Rabbit skeletal myosin was prepared by the method of Kielley and Bradley (1956), employing modifications described by Godfrey and Harrington (1970a). For digestion of myosin with cellulose–papain to produce rod, the activity of the insoluble enzyme was adjusted such that at 25°, 0.25 ml of the suspension hydrolyses 0.02 to 0.04 μequiv of Bz-L-ArgOEt per minute in 6 ml of the standard substrate mixture. The rod was prepared by digesting myosin (0.7–0.8%) at pH 6.6 for 5 min at 25° with cellulose–papain employing a volume ratio of 6:100 (enzyme to protein). The reaction mixture was subsequently diluted with dialysate to facilitate removal of the cellulose–papain by filtration through a Millipore filter (1.2 μ) employing a plastic Swinnex adaptor and the filtrate was immediately dialyzed against low ionic strength buffer (0.01 M PO_4^{2-}, pH 6.8), which was changed several times over a period of up to 48 hr. The precipitate containing rod and residual myosin was separated by centrifugation at 20,000 rpm for 20 min, collected, and washed several times with fresh low ionic strength buffer. The above precipitate was subsequently redissolved with stirring in 0.5 M KCl, 0.01 M EDTA, pH 7.0. Denaturation of residual myosin was achieved by dropwise addition of three volumes of absolute alcohol over a period of approximately 30 min with constant stirring. The precipitate containing denatured, insoluble myosin, and precipitated rod was then dialyzed against 0.5 M KCl, 0.01 M EDTA, pH 7.3. The denatured, insoluble myosin was removed by centrifugation at 50,000 rpm for 60 min and the supernatant containing the soluble rod was collected. The rod solution was applied to a column (2.5 × 100 cm) of Sephadex G-200 previously equilibrated with 0.5 M KCl, 0.2 M PO_4^{2-}, 0.01 M EDTA, pH 7.3; this procedure effectively removed any residual low molecular weight, peptide material. Fractions containing the protein were pooled and concentrated by alcoholic precipitation and redissolved by dialysis into 0.5 M KCl, 0.2 M PO_4^{2-}, 0.01 M EDTA, pH 7.3. Tryptic LMM was prepared as described previously (Young *et al.*, 1964) but in addition it was purified using the same procedure as that employed for rod.

Myosin concentrations were determined spectrophotometrically in a Zeiss PMQ 11 or Gilford spectrophotometer employing $\epsilon_{280}^{1\%}$ 5.55 (Godfrey and Harrington, 1970a). In the case of the rod fragments, the number of fringes at 546 mμ corresponding to a 1% solution was determined by synthetic boundary experiments; the concentrations of these solutions (nitrogen-free buffers in this case) were determined by micro-Kjeldahl analyses employing nitrogen contents obtained by triplicate amino acid analyses on rod and LMM. The fringe number for a 1% solution of rod and LMM was found to be 39 and 40 fringes, respectively, and these values were routinely employed for concentration determinations on these particles. The problem of contamination of the rod and LMM preparations by low molecular weight impurities is worthy of mention. Since both LMM and rod are prepared by proteolytic digestion of myosin, it is likely that contamination by peptide material will occur. However, we feel that this type of contamination is effectively eliminated by the fractionation procedures employed to isolate rod and LMM. The main problem occurs with preparations of the rod. If proteolysis with papain is carried out for too long a period of time, degradation of the rod to LMM may occur, so that the rod preparation will contain contaminating amounts of the lower molecular weight LMM. As far as we know, there are no adequate procedures which will effectively fractionate these two components. In the present investigation only rod preparations with intrinsic viscosities of 2.50 dl/g or higher have been used; preparations with lower intrinsic viscosities are undoubtedly mixtures of LMM and rod and have not been studied (see Lowey *et al.*, 1969). In the case of LMM, we are dealing with the "final" product of proteolysis and since trypsin cleaves the myosin rod at the "hinge" region, the only low molecular weight impurity is the released peptide material which is removed by fractionation on G-200 Sephadex.

Molecular Weight Determinations. We employed the high-speed meniscus depletion method of Yphantis (1964) for molecular weight determinations. The Rayleigh interference optical system was used for all of the equilibrium sedimentation studies and fringe displacements were analyzed by the computer method of Roark and Yphantis (1969). The data were obtained by essentially identical experimental procedures as those described by Godfrey and Harrington (1970b). Apparent partial specific volumes (ϕ') of 0.728 and 0.710 for myosin and LMM (rod), respectively, were employed (Godfrey and Harrington, 1970a; Young *et al.*, 1964).

Viscosity Determination. An Ostwald-type viscometer of relatively long capillary, and average shear gradient of 158 sec^{-1} was used for viscosity measurements. The kinetic energy correction was found to be negligible. The viscometer stand was constructed in such a fashion that the viscometer was always situated in the same position in the bath. Temperature variation was monitored with a Beckmann thermometer and was found not to exceed ±0.02°. Protein stock solutions were centrifuged at 20,000 rpm for 60 min to remove particulate matter, and the supernatant was carefully withdrawn. All dilutions were carried out gravimetrically employing solvents

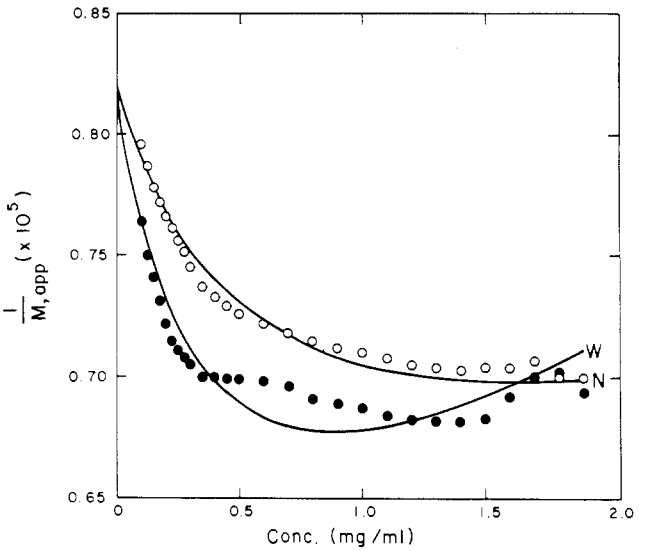

FIGURE 1: $1/M_{app}$ *vs.* concentration plot for LMM Fr. 1 in 0.5 M KCl, 0.2 M PO_4^{2-}, 0.01 M EDTA, pH 7.3, at 5°. Circles, filled and unfilled, represent weight and number averages, respectively, and are the average of 12 runs at 8000–20,000 rpm. The smooth curves represent computer simulated data for a monomer $\rightleftharpoons$ dimer reversible association with the parameters listed in Table I.

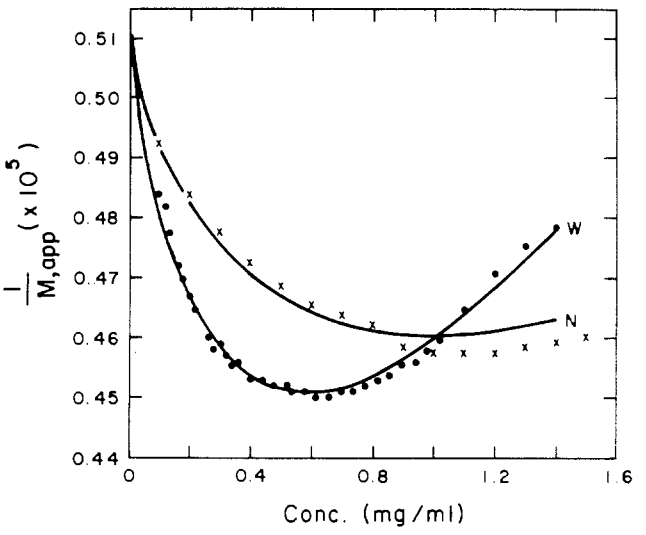

FIGURE 2: $1/M$ *vs.* concentration plot for myosin rod in 0.5 M KCl, 0.2 M PO_4^{2-}, 0.01 M EDTA, pH 7.3, at 5°. The circles and crosses represent weight and number averages, respectively (average for four runs at 14,000 and 17,000 rpm). The smooth curves represent computer simulated $1/M$ *vs.* concentration plots for a monomer $\rightleftharpoons$ dimer reversible association with parameters given in Table I.

freshly filtered through Millipore filters (0.8 μ). Viscosity measurements were made on 2.0-ml samples and were repeated four to five times on each solution.

The reduced viscosity is given by eq 1 where t and t_0, ρ and

$$\frac{\eta_{sp}}{c} = \frac{t - t_0}{ct_0} + \frac{1}{\rho_0}\left(\frac{\partial\rho}{\partial c}\right)_{\mu}^{0}\frac{t}{t_0} \qquad (1)$$

ρ_0 are the outflow times and density of solution and solvent, respectively. The second term on the right-hand side of equation (1) does not vanish in the limit $c \rightarrow 0$, but it is negligibly small in comparison to the large intrinsic viscosities with which we are dealing here.

Adsorption of protein to the capillary wall can be a serious source of error, particularly in the low concentration range, but in the present study this phenomenon does not appear to be contributing significantly to the measurement of η_{sp}/c since no anomalous upward curvature is detected in the Huggins plot for these proteins. Furthermore, plots of $t - t_0$ *vs.* concentration of protein were found invariably to extrapolate through zero at zero concentration (Reisler and Eisenberg, 1970). The possibility that the shear gradient within the capillary could produce non-Newtonian flow in solutions of these very asymmetric particles has been examined and on the basis of hydrodynamic analysis (Yang, 1961) this effect is expected to be negligible even for the most asymmetric rod dimer. The reduced viscosity *vs.* concentration profiles were analyzed by a least-squares treatment of the data. Normal equations were formed from eq 6, 7, and 8 (see Results section) and a solution obtained for the residual of each unknown using standard matrix methods (Deming, 1964). The new residual value was used to obtain estimates of the unknowns. Iteration was carried out until the residual was $\leq 0.01\%$ of the previous value of this unknown.

Results

Equilibrium Sedimentation Studies. Figures 1 and 2 present the results from twelve high-speed meniscus depletion experiments with LMM and four with "rod" in a solvent system consisting of 0.5 M KCl, 0.2 M PO_4^{2-}, 0.01 M EDTA, pH 7.3. Various cell loading concentrations (0.35–0.96 mg/ml, LMM; 0.5–1.2 mg/ml, rod) and rotor speeds (8000–20,000 rpm, LMM; 14,000–17,000 rpm, rod) were employed in these studies. The two sets of points plotted in each figure are the reciprocal of the apparent weight average ($1/M_{w,app}$) and number average ($1/M_{n,app}$) molecular weights at the different concentrations shown within the liquid column. Each point is the apparent reciprocal moment ($1/M_{w,app}$ or $1/M_{n,app}$) obtained by averaging the value estimated from the Roark–Yphantis computer program at that concentration over all ultracentrifuge runs. The presence of a minimum in the reciprocal moment–concentration profiles shown in Figures 1 and 2 and the apparent convergence of the reciprocal moments in each plot at a common infinite dilution value are a strong indication of nonideal associating systems. In general, such systems are expected to show a decrease in the reciprocal molecular weight with increasing concentration as the result of the formation of higher molecular weight species which is exactly counterbalanced at some concentration by the nonideality of the system which tends to reduce the apparent molecular weight.

We were particularly concerned in these studies with the possibility that heterogeneity of the LMM and rod preparations would mask the presumed association reaction. Variations in rotor speed, giving a difference in centrifugal field of about 6-fold in the case of LMM and about 1.5-fold for rod, were employed to detect low molecular weight material which would tend to elevate the reciprocal moment values near infinite dilution with increasing rotor speed. In fact, the infinite dilution values obtained by extrapolation of reciprocal moment–concentration plots from individual runs were the same within ($\pm 3\%$) for the rod and ($\pm 7.5\%$) for LMM. The larger variation of the reciprocal moments in the LMM experiments is likely due to a broader length distribution of these particles. Moreover, the $1/M$ *vs.* concentration profiles showed no apparent trend either with changing speed or with differing cell loading concentrations thus satisfying a cardinal re-

TABLE I: Monomer ⇌ Dimer Reversible Association Parameters for LMM, Rod, and Myosin in 0.5 M KCl, 0.2 M PO_4^{2-}, 0.01 M EDTA, pH 7.3. High-Speed Sedimentation Equilibrium Data (5°).

Particle	M_1 (g/mole)	B_1 (mole dl/g²)	BM_1 (dl/g)	K_2 (dl/g)	K_2 (liter/mole)	ΔF (cal/mole)
Myosin[a]	458,000	6.60×10^{-6}	3.02	10	2.3×10^5	−6860
Rod	198,000	8.44×10^{-6}	1.67	8	0.8×10^5	−6300
LMM	122,000	8.5×10^{-6}	1.04	10	0.6×10^5	−6100

[a] Godfrey and Harrington, 1970b.

quirement of an associating system. There is some perturbation in the experimental points, especially in the case of LMM. This could arise from the presence of small amounts of a higher *n*-mer (*cf.* Godfrey and Harrington, 1970b) or it may result from cell window distortion since this deviation seemed to be more pronounced at the higher speeds.

Since detailed analysis of similar plots in the case of myosin demonstrated the existence of a monomer ⇌ dimer equilibrium in the same solvent system, the $1/M$ *vs.* concentration profiles of Figures 1 and 2 were analyzed assuming this mode of association. The monomeric molecular weight M_1 of each of these systems was established by smooth extrapolation of the data points to infinite dilution. This parameter was then used to determine K_2 (the equilibrium constant) and B (the second virial coefficient) which define the nonideal monomer–dimer self-association reaction according to eq 2 and 3.

$$1/M_{w,app} = 1/2M_1 + 1/[2M_1(4K_2c + 1)^{1/2}] + Bc \quad (2)$$

$$1/M_{n,app} = 1/2M_1 + 1/\{M_1[(4K_2c + 1)^{1/2} + 1]\} + (1/2)Bc \quad (3)$$

Equations 2 and 3 were solved for K_2 and B at a number of concentrations (over the range 0.1–1.6 mg/ml) and the results used to establish the best fit to the simulated reciprocal moment *vs.* concentration plot with the experimental data. Computer-calculated reciprocal apparent molecular weight moments using the parameters M_1, K_2, and B assigned to each system (see Table I) are shown as solid curves in Figures 1 and 2. The simulated $1/M$ *vs.* concentration profiles fit the experimental data within 2.5% over the concentration range 0.1–1.6 mg/ml.

Modes of association higher than monomer–dimer have not been tested in the present study. A detailed analysis of the myosin system for the presence of monomer–dimer–trimer (M–D–T) or monomer–dimer–trimer–tetramer (M–D–T–T) modes of association indicated that if trimer and tetramer species are involved in the association reaction, they exist in very low concentration. We believe the binding sites on myosin to be primarily, if not entirely, located on the LMM segment of the molecule for reasons given in the introduction. This conclusion is supported by the molar free energies of association of LMM and rod estimated from the monomer–dimer equilibrium constant (Table I) which are closely similar to that of myosin. Thus it seems likely that the association mode established for myosin in high ionic strength is mirrored in the association behavior of LMM and rod. This conclusion is consistent with the sedimentation behavior of these two proteolytic fragments in that they exhibit single hypersharp boundaries in velocity sedimentation experiments as expected from Gilbert (1959) theory for rapidly reversible monomer ⇌ dimer equilibria.

Viscosity Studies. The sedimentation equilibrium experiments demonstrating a monomer ⇌ dimer reversible equilibrium for LMM and rod suggest that viscosity–concentration studies should also reflect the association reaction. The intrinsic viscosity of asymmetric particles is quite sensitive to their axial ratio, a/b, varying approximately with $(a/b)^{1.8}$ (Tanford, 1961). In the case of a uniform rigid rod large variations are expected in the intrinsic viscosity of a dimer species depending on the degree of overlap. For example, the intrinsic viscosity of a dimer obtained by end-to-end association and *that* by complete side-to-side overlap should differ by a factor of about 12. Thus the concentration-dependence profile of the reduced viscosity, η_{sp}/c, should reflect the increasing (or decreasing) average asymmetry of the particles with increasing protein concentration. For nonassociating systems the concentration dependence of η_{sp}/c is generally well represented by a virial expansion in which terms of c^2 or higher are usually neglected at low concentrations

$$\eta_{sp}/c = [\eta] + k'[\eta]^2c + \ldots \quad (4)$$

where k', the dimensionless Huggins constant, is about 0.4 to 0.5. Assuming that the specific viscosity of a monomer–dimer mixture will be an additive function of the specific viscosities of each species present, we may express the specific viscosity of the equilibrium system as

$$\eta_{sp_i} = \sum_{i=1}^{2} ([\eta]_i + k'[\eta]_i^2c_i)c_i \quad (5)$$

where c_i is the concentration of the ith species (g/dl). The measured reduced viscosity at a concentration c will be given by

$$(\eta_{sp}/c)_{mixture} = ([\eta]_m + k'[\eta]_m^2c_m)(c_m/c) + ([\eta]_d + k'[\eta]_d^2c_d)(c_d/c) \quad (6)$$

where c_m and c_d represent the concentration (g/dl) of monomer and dimer, respectively. The concentrations of monomer and dimer are governed by the relationships

$$K_2 = \frac{c_d}{(c_m)^2} \quad (7)$$

and

$$c = c_m + c_d \quad (8)$$

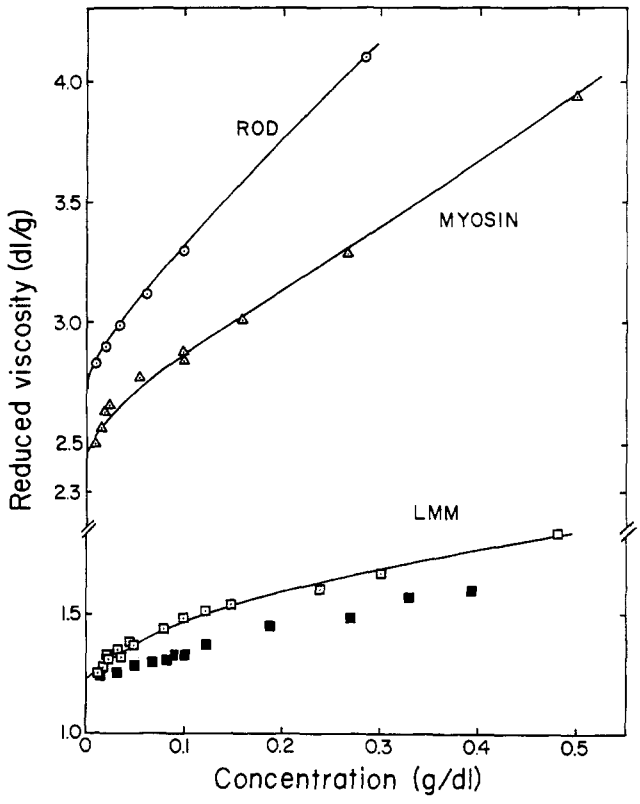

FIGURE 3: Reduced viscosity *vs.* concentration plots for myosin (△), LMM (□), and rod (○) in 0.5 M KCl, 0.20 M PO_4^{2-}, 0.01 M EDTA, pH 7.3, at 5°. ■ is for LMM in 0.5 M KCl, 0.5 M PO_4^{2-}, 0.01 M EDTA, pH 8.5. The latter has been normalized to $\phi' = 0.710$ to account for the ϕ' change occurring when LMM is placed in the solvent containing 0.5 M PO_4^{2-}. The smooth curves represent computer simulated η_{sp}/c *vs.* concentration plots for a monomer ⇌ dimer reversible association with parameters given in Table II.

At a concentration c the fractions of monomer and dimer present are

$$\frac{c_m}{c} = \frac{-1 + (1 + 4K_2c)^{1/2}}{2K_2c} \quad (9)$$

and

$$\frac{c_d}{c} = \frac{2K_2c + 1 - (1 + 4K_2c)^{1/2}}{2K_2c} \quad (10)$$

If the dimer has the same asymmetry as the monomer ($[\eta]_d = [\eta]_m$) eq 6 will simplify to give the following equation:

$$\frac{\eta_{sp}}{c} = [\eta]_m + k'[\eta]_m^2\left(\frac{2c_m^2 - 2c_mc + c^2}{c}\right) \quad (11)$$

If the asymmetry of dimer is greater than monomer, the η_{sp}/c *vs.* c plot will increase with slope greater than $k'[\eta]_m^2$ in the low concentration range and will approach a slope of $k'[\eta]_d^2$ at high concentrations. The initial slope ($c = 0$) will depend on the value of K_2 and will increase with increasing values of K_2. For the case where the asymmetry of dimer is less than monomer the η_{sp}/c *vs.* c profile will decrease with increasing concentration with initial slope less than $k'[\eta]_m^2$, pass through a minimum at a concentration depending on the value of K_2, and then increase with slope approaching $k'[\eta]_d^2$. The intrinsic viscosity of the dimer in such plots cannot be obtained by extrapolation of the limiting slope to infinite dilution, since the intrinsic viscosity (apparent) varies with concentration. Equation 6 can be solved for $[\eta]_d$ and K_2 using eq 9 and 10.

Figure 3 presents reduced viscosity–concentration plots of LMM, rod and myosin in 0.5 M KCl, 0.2 M PO_4^{2-}, 0.01 M EDTA, pH 7.3 buffer at 5°. It will be seen that the viscosity increases with increasing concentration as expected for an associating system in which the associated species has a higher asymmetry than monomer. Thus these experiments lend strong support to the sedimentation equilibrium studies indicating that the three species are in rapid reversible equilibrium under these ionic conditions. It should be noted that earlier studies (Young *et al.*, 1964; Lowey *et al.*, 1969) of the viscosity–concentration dependence of these particles failed to detect downward curvature since measurements were generally made at concentrations above 0.5 mg/ml. However, the very high asymmetry of the particles under study allows accurate measurement of the reduced viscosity at concentrations well below this value. At concentrations of 0.5 mg/ml the difference in outflow time of solution (LMM) and solvent was about 9 sec in the viscometer employed in this study. Further support for the presence of an associating system is provided by viscosity measurements of LMM in high phosphate (lower curve of Figure 3). It has been shown in earlier work that increasing amounts of phosphate ion shifts the monomer–dimer ratio toward monomer (Godfrey and Harrington, 1970a). The reduced viscosity *vs.* concentration profile in a solvent system consisting of 0.5 M KCl, 0.5 M PO_4^{2-}, 0.01 M EDTA, pH 8.5, showed only slight upward curvature with increasing concentration consistent with a repression of the equilibrium constant for dimerization in this solvent system. Herbert and Carlson (1971a) have recently reported a decrease in the monomer ⇌ dimer equilibrium constant of myosin from 10.6 dl/g in 0.2 M PO_4^{2-} to 1.30 dl/g in 0.5 M PO_4^{2-}.

The experimental data shown in Figure 3 have been fitted by a least-squares analysis computer program (see "Materials and Methods") in which η_{sp}/c, c, k' and $[\eta]_m$ have been used to estimate $[\eta]_d$ and K_2 over the concentration range of the measurements. A constant value of the Huggins constant, $k' = 0.45$, was assumed in these calculations although variations in the constant between 0.4 and 0.5 were found to give a negligibly small error in the estimation of $[\eta]_d$ and K_2. The intrinsic viscosity of monomer, $[\eta]_m$, was obtained by extrapolation of the measured η_{sp}/c data to infinite dilution. Derived values of $[\eta]_d$ and K_2 which best fit the experimental points are given in Table II and the simulated monomer–dimer curves generated from them are shown in Figure 3. The same viscosity behavior was also observed for myosin chromatographed by the procedure of Richards *et al.* (1967) yielding $[\eta]_d = 3.30$ dl/g. In the case of myosin, a value of 10 dl/g was assigned to K_2, since this value was found previously in the high-speed sedimentation experiments using an identical solvent (see also Herbert and Carlson, 1971a). The equilibrium constants (liters/mole) of association derived from the viscosity data are within the same range as those derived from the high-speed sedimentation equilibrium experiments, but neither set of data can be considered to yield an equilibrium constant with the same level of confidence as that of myosin which was established on the basis of a much more detailed investigation. Nevertheless, the molar free energies of dimerization derived from the two independent studies are consistent in assigning most if not all of the binding sites for association of myosin in high salt to the LMM segment.

TABLE II: Monomer ⇌ Dimer Reversible Association Parameters for LMM, Rod, and Myosin in 0.5 M KCl, 0.2 M PO_4^{2-}, 0.01 M EDTA, pH 7.3. Viscosity Data (5°).

Particle	$[\eta]_m$ (dl/g)	$[\eta]_d$ (dl/g)	K_2 (dl/g)	K_2 (liter/mole)	ΔF (cal/mole)
Myosin	2.45	3.11	10[a]	2.3×10^5[a]	−6860[a]
Rod	2.65	3.44	27	2.8×10^5	−6960
LMM	1.23	1.90	5.5	0.33×10^5	−5800

[a] Godfrey and Harrington, 1970b.

Discussion

The high-speed meniscus depletion and viscosity experiments presented in this paper provide evidence for a rapid, reversible association of LMM and rod in high salt. A monomer ⇌ dimer equilibrium appears to be the most likely mode of association, since both the reciprocal molecular weight *vs.* concentration profile at sedimentation equilibrium and reduced viscosity *vs.* concentration plots give a good fit for this mode of association to the experimental data. In the case of LMM, the viscosity behavior expected for a monomer ⇌ dimer equilibrium is observed out to a protein concentration of about 1% (for rod to about 0.3%; higher levels of concentration were not measured because of the inordinately long ouflow times encountered). The formation of higher order aggregates in significant amount would be expected to produce large deviations in the experimental and simulated monomer–dimer curves, particularly at the higher concentration levels, unless the higher *n*-mer was identical in asymmetry with that of dimer.

The asymmetry of the LMM and rod dimers deduced from the viscosity studies provides information on the geometry of the myosin dimer found in high salt. Consider the two general types of myosin dimers, antiparallel and parallel, shown in Figure 4, in which the rod segment lying between LMM and the head (subfragment II) is cross hatched for identification. For simplicity, we assume the LMM and rod segments of the myosin molecule to have lengths of 860 and 1290 Å, respectively, and to be displaced in the various dimer geometries by a period of 430 Å. A study of the models reveals that the *overlap distance* for the LMM and rod segments of each myosin dimer, when taken together, establishes the geometry of the parent dimer species. For example, consider the antiparallel structure (a) in which the LMM segments are overlapped by 430 Å and the rod segments are overlapped by 1290 Å. This length of overlap for the LMM segments is also true for the lower antiparallel structure (c) and the central, parallel structure (e). The *rod* segment overlap of 1290 Å is compatible with models a and d. However, the overlap lengths of 430 Å for LMM and 1290 Å for rod taken together uniquely define structure a. Similar considerations apply to other degrees of overlap. Looking at the system of dimers in another way, it will be seen that *the length* of the LMM dimer, when taken in conjunction with *the length* of the rod dimer, establishes the geometry of the parent species.

Although the axial ratio of a rigid rod is difficult to determine with a high degree of accuracy from viscosity measurements, its length can be deduced with reasonable confidence, providing that the molecular weight is known. We will demonstrate in the following paper (Burke and Harrington, 1972) that dimeric structures formed through side-by-side association of rigid rods can be treated hydrodynamically as equivalent cylinders of length and volume equal to that of the dimer. From the definition of intrinsic viscosity, the length of prolate ellipsoids of large axial ratio is given by the equation (Yang, 1961)

$$L_{ellipsoid} = 2a = 6.82 \times 10^{-8} ([\eta]M)^{1/3}(p_e^2/\nu_e)^{1/3} \quad (12)$$

where p_e is the axial ratio of the ellipsoid and ν_e the viscosity increment. We assume for large p that $L_{ellipsoid} \cong L_{cylinder}$. The apparent lengths of monomer and dimer structures of LMM and rod deduced from the intrinsic viscosities and molecular weights presented in Tables I and II are summarized in Table III. Apparent lengths of the two monomeric particles estimated in this way are in reasonable agreement with the average length assigned to LMM and rod by Lowey *et al.* (1969) from length *vs.* number histograms derived from electron micrographs (730 Å LMM and 1370 Å rod). More recent electron microscopic data in conjunction with sodium dodecyl sulfate gel electrophoresis (Harrison *et al.*, 1971) indicate lengths of 900 and 1440 Å for LMM and rod, respectively. The length of the LMM dimer, 1320–1380 Å, depending on the assumed molecular weight of monomer, gives an overlap very close to one-half (430–450 Å) of the monomer particle length. Similarly, the length of the rod dimer, 1910–2015 Å,

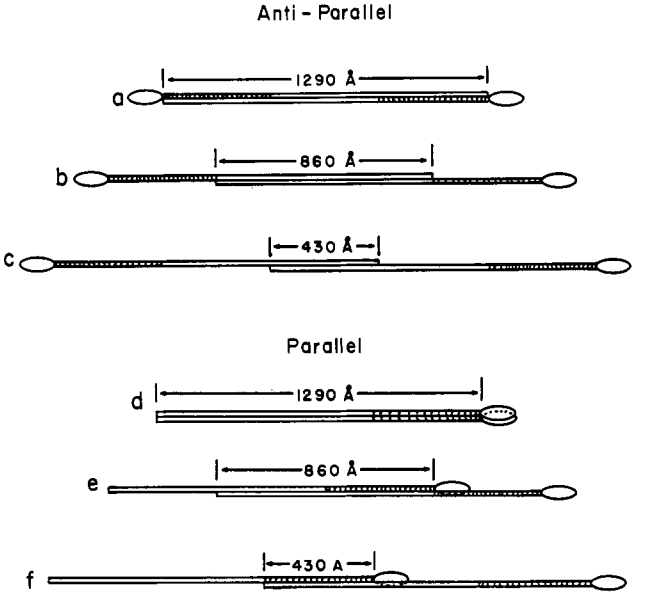

FIGURE 4: Schematic representation of various geometries for the myosin dimer. The rod secton of the myosin molecule is subdivided into its two components subfragment II (cross-hatched) and LMM (unmarked) for convenience.

TABLE III: Lengths of Monomeric and Dimeric LMM and Rod Based on Eq 12.

Particle	$[\eta]$ (dl/g)	Length[a] (Å)	Length[b] (Å)
LMM (monomer)	1.23	915	875
LMM (dimer)	1.90	1380	1320
Rod (monomer)	2.65	1440	1380
Rod (dimer)	3.42	2015	1910

[a] Mol wt of LMM and rod: 1.40×10^5 g/mole; 2.30×10^5 g/mole, respectively (Lowey *et al.*, 1969). [b] Mol wt of LMM and rod: 1.22×10^5 g/mole; 1.98×10^5 g/mole, respectively.

is consistent with an overlap of about 60% (850–865 Å) of its monomer length. It will be seen that the overlap observed for the LMM dimer is compatible with models a, c, or e of Figure 4 and the rod overlap with models b or e. The only model which fits both sets of experimental data is e, a parallel structure in which one myosin molecule is displaced 430–530 Å with respect to its neighbor. We should note that the intrinsic viscosity expected for a fully overlapped rod dimer is 1.43 dl/g, a value approximately half that estimated for $[\eta]_d$ of rod (Burke and Harrington, 1972). Moreover, the η_{sp}/c *vs.* concentration curve expected for a fully overlapped rod species should exhibit significant downward curvature at low concentrations.

In the treatment given above, three major assumptions have been made: (1) the association of rod and LMM in the free state corresponds to the dimerization occurring when they are incorporated into the myosin structure, (2) in the high-salt solvent system the myosin dimer is constrained to adopt one specific type of dimer geometry, and (3) the LMM and rod segments behave hydrodynamically in solution as rigid rods both in the monomer and dimer states. The first assumption is supported by the molar free energies of association of LMM which appear to be, within the experimental error in determining equilibrium constants, the same as that for myosin, indicating that the binding sites responsible for association must reside primarily, if not entirely, in the LMM segment of the molecule. Moreover, Lowey and her collaborators (1969) have demonstrated that subfragment II of HMM has no apparent tendency to associate over a wide range of ionic strengths near neutral pH. It seems unlikely, therefore, that the subfragment II segment of the myosin molecule is contributing significantly in the association process. The second assumption is based on the likelihood that a specific dimer species is employed in the assembly of the thick filament. Successful assembly of the growing aggregate from an ensemble of myosin dimers constructed of differing geometry would require a complicated and improbable sequential addition of the dimer species according to their geometries. Dilution by dialysis from the high-salt solvent system employed in the present study results in the formation of filament structures with close topological similarity to that of the native thick filament. If a multiplicity of dimer species were present we would expect a variety of polymorphic structures to form. Indeed, Cohen *et al.* (1970) and Harrison *et al.* (1971) have observed the formation of ribbon-like aggregates from dilute-salt solvent systems of rod preparations in the presence of potassium thiocyanate and divalent cations. They suggest that the initiating species for these systems may be an antiparallel dimer. Their studies would indicate that the rod preparations of myosin are similar to tropocollagen systems in that the specific ionic constituents of the medium may have a profound effect on the mode of organization of the aggregates. A striking feature of the ordered aggregates of myosin rod preparations under these ionic conditions (potassium thiocyanate and divalent cation) is their apparent tendency to grow indefinitely by lateral accretion of rods suggesting that if such structures form from an antiparallel rod dimer, as proposed, this geometry may favor side-by-side organization rather than the linear type of growth characteristic of thick filament formation. In any event, if systematic aggregation proceeds through addition of a dimer species we would expect the building unit to have a unique geometry.

The third assumption in the hydrodynamic treatment, that of particle rigidity, is supported by the high value of the exponent, α, in the Mark–Houwink eq ($[\eta] = KM^{\alpha}$) when the viscosity of two-stranded polypeptide rods of various lengths are examined. A plot of log $[\eta]$ *vs.* log $[M]$ for rod particles of uniform diameter including LMM, rod, paramyosin LMM (Halsey and Harrington, unpublished) and subfragment II is linear with slope, $\alpha = 1.6$. Values of α between 1.5 and 2.0 indicate a high degree of particle rigidity (Benoit *et al.*, 1967). The lengths of LMM and rod derived from the hydrodynamic measurements assuming them to be rigid rods are also in reasonable agreement with the lengths of these particles assuming them to be coiled coils of α-helices. The expected lengths based on the molecular weights of Table III are 780–910 Å (LMM) and 1300–1500 Å (rod). On the basis of these considerations, and assuming a length of 1600 Å (1450 Å for the rod (Cohen *et al.*, 1970) and 150 Å for the head) for the myosin monomer, we expect the parallel dimer to have a length of 2030–2130 Å. Herbert and Carlson (1971a,b) estimate the length of the myosin dimer to be 2120 ± 260 Å based on diffusion measurements of the monomer $\rightleftharpoons$ dimer equilibrium by spectral broadening of laser light scattering. Moreover, following the demonstration of parallel dimer geometry established in the present study, they have recently calculated the Z-average radius of gyration of the myosin dimer as a function of concentration for both parallel and antiparallel structures. These calculations also favor a structure of parallel geometry with an overlap distance of about 840 Å, employing a monomeric length of 1481 ± 130 Å.

Synthetic myosin filaments prepared by lowering the ionic strength of myosin solutions near neutral pH have a variability in length from 0.25 to 0.3 μ up to about 1.5 μ, but throughout this range of lengths the bare zone of 0.15–0.20 μ is always situated near the center of the structure, never at one end (Huxley, 1963). The filaments formed near pH 8.3 have a very sharp size distribution (mean length = 0.63 μ) and again the smooth region, devoid of "cross bridges," is situated near the center of the filament (Josephs and Harrington, 1966; Kaminer and Bell, 1966a,b). These observations are consistent with the hypothesis that the bare central zone is formed in the early stages of the assembly process by reversing the polarity of the myosin molecules making up this segment of the filament (Huxley, 1963). The parallel dimer which we believe to exist in high salt seems to us a very likely candidate for the initiating species in low-salt media. It has been shown in earlier work that the monomer $\rightleftharpoons$ dimer equilibrium is displaced toward dimer as the salt concentration is reduced (over the range 1.0–0.35 M) (Godfrey and Harrington, 1970a; Herbert and Carlson, 1971a). Thus it seems reasonable to assume that the parallel dimer geometry is maintained but the

equilibrium constant for dimer formation is increased as the ionic strength is decreased into the range where filament formation begins. If this dimer species is present in high concentrations under ionic conditions favoring filament formation, then it seems likely that the bare central zone is formed through antiparallel (tail-to-tail) association of the parallel dimer units. We assume growth of the filament to occur through head-to-tail addition of the same dimer units. A detailed investigation of the geometry of the myosin dimer at low-salt concentration is required to establish this proposal. Preliminary high-speed, sedimentation equilibrium experiments in this laboratory show that the myosin dimer is strongly favored[1] ($K_2 \cong 24$ dl/g) at low ionic strengths approaching that required for filament formation (0.20 M KCl, 0.005 M Veronal, pH 8.3). In light of these findings the earlier analysis of the myosin–polymer reversible association (Josephs and Harrington, 1968) based on a rapidly reversible monomer $\rightleftharpoons$ polymer equilibrium must now be reassessed on the basis of a monomer $\rightleftharpoons$ dimer $\rightleftharpoons$ polymer equilibrium system. The slower sedimenting single peak observed in velocity sedimentation studies of these systems very likely corresponds to a monomer $\rightleftharpoons$ dimer equilibrium boundary.

The dimer geometry deduced in the present study appears to be compatible with the repeat period of 430 Å for the helical array of cross bridges determined by Huxley and Brown (1967). A study of the model of the thick filament proposed by Pepe (1967) indicates that the entire structure can be constructed from a parallel type dimer in which the head of one molecule is displaced about 430 Å with respect to its neighbor. This parallel dimer can be used to construct a single row of cross bridges according to the Pepe model. If the same binding sites are maintained for such a set of dimers on both sides of the M-line region, and if the tails of myosin dimers abut in this region, the heads of a row on one side of the filament will be projecting *outward* away from the surface of the filament while those on the opposite side of the filament will project *inward* toward the center of the filament. To bring all cross bridges out to the surface in the model requires that the heads be rotated on one side of the M-line. It is intriguing that the restrictions of the model suggest that a conformational change in the flexible "hinge" segment of the myosin molecule may occur during the packing process. While this should not be interpreted as compelling support for the Pepe model, it nevertheless leads to an interesting speculation, namely that the presumed conformational change occurring within the hinge region is allowed at low ionic strength and prohibited at high ionic strength.

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[1] J. S. Barton and W. F. Harrington, unpublished.

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