Self-Association in the Myosin System at High Ionic Strength. I. Sensitivity of the Interaction to pH and Ionic Environment*

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ABSTRACT: The differential velocity sedimentation technique has been employed to examine the self-interaction of monomeric myosin at high ionic strength in conjunction with velocity sedimentation and intermediate-speed (LaBar type) sedimentation equilibrium experiments. A large and reproducible change in the differential sedimentation rate of myosin in 0.5 M KCl was observed when solutions differing only in pH were compared. This effect was amplified by lowering the salt concentration to 0.35 M or by increasing the protein concentration.

A decrease in sedimentation coefficient as well as a decrease in molecular weight of this system was observed in the presence of phosphate. Results are interpreted as indicating the presence of a rapidly reversible monomer–dimer equilibrium which is pH and ionic strength dependent under the conditions employed.

Studies of the association of myosin molecules to form higher molecular weight filaments at low ionic strength have been reported by several laboratories in recent years (Jakus and Hall, 1947; Noda and Ebashi, 1960; Zobel and Carlson, 1963; Huxley, 1963; Kaminer and Bell, 1966a,b; Josephs and Harrington, 1966, 1967). Over the pH range 6.2–7.3 three heterogeneous weight classes of filaments are seen in velocity sedimentation experiments, but in the pH range 8–8.5 (μ = 0.15) a single, well-defined polymer species is observed ($s^0_{20,w}$ = 150 S) which has been shown to be in rapidly reversible equilibrium with monomeric myosin (Josephs and Harrington 1966, 1968). The position of the monomer $\rightleftharpoons$ polymer equilibrium is sensitive to salt concentration, pH, and the hydrostatic pressure. In view of the demonstrably large affinity for self-association under these conditions, it may be questioned whether this interaction is completely damped out even at the high salt concentrations (~0.5 M KCl) where myosin has been assumed to exist as an essentially monodisperse, monomeric species.

In the present study we have utilized the sensitive differential sedimentation velocity technique of Richards and Schachman (1959) to explore this question. Taken in conjunction with companion velocity sedimentation and sedimentation equilibrium experiments, our results indicate a significant amount of monomer–monomer interaction even at high salt concentration and suggest the presence of a heretofore undetected monomer $\rightleftharpoons$ dimer equilibrium which is sensitive to pH and salt concentration.

Materials and Methods

Chemicals. Glass-distilled water was used throughout; inorganic salts and reagents were reagent grade. Bis-tris (bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane) was synthesized by the method of Lewis (1966). This buffer has a pK of about 6.8 in the cold and was used whenever the presence of phosphate was undesirable.

Preparation of Myosin. Rabbit skeletal myosin was extracted and isolated immediately from back and thigh muscle at 4° by the method of Kielley and Bradley (1956) as modified by Kielley and Harrington (1960) with the following changes. (1) Before the first $(NH_4)_2SO_4$ fractionation, the pH of the myosin solution was raised to 6.75; this modification significantly increased the final yield. (2) The centrifugation step at 0.25 M KCl was omitted. (3) EDTA (0.01 M) was added to the 0.5 M KCl used in all solubilization steps. Myosin prepared by the above procedure is virtually free of lipids, actomyosin, and 5′-adenylic deaminase, three contaminants sometimes found in myosin preparations (Holtzer, 1956; Brahms and Brezner, 1961; Kielley and Bradley, 1956). Assays for phospholipids, fatty acids, and cholestrol, each sensitive to less than 2 μg of lipid/mg of myosin were negative (Albrink, 1959; Huang *et al.*, 1961; Van Handel and Zilversmit, 1957). Preparations analyzed for the presence of 5′-adenylic deaminase by the procedure followed by Richards *et al.* (1967) exhibited an average of four Kalckar units of activity, or about 0.1 μg of active enzyme/mg of myosin. The myosin preparations were found to be free of actomyosin by the test of Rice *et al.* (1963).

After centrifugation at 25,000 rpm for 1 hr to remove any particulate material, a number of fresh myosin preparations (1.5–2.5% dialyzed against 0.5 M KCl–0.01 M EDTA, pH 7) were examined in the ultracentrifuge. In most cases solution and dialysate were run in double-sector cells to enhance detection of small schlieren peaks. Almost all were free of both slower and faster moving material. This finding is in contrast to the report of Dreizen *et al.* (1967) that $(NH_4)_2SO_4$ fractionated myosin solutions invariably contain aggregated myosin easily seen in the schlieren optical system. Heavy aggregate peaks did appear in these solutions after a period of several days as they normally do in all myosin preparations maintained in 0.5 M KCl.

Analytical Ultracentrifuge Techniques. The differential velocity sedimentation method of Richards and Schachman

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(1959) was used to detect small differences in s for myosin in dissimilar solvent environments. In this procedure, the two sectors of an interference cell are filled equally with the two test solutions. The Rayleigh fringe pattern which reflects the difference in concentration between the two sector solutions records a difference concentration peak caused by the unequal radial positions of the sedimenting boundaries. Typical difference peaks are shown in Figure 1. The area under the peak continuously changes in response to the differing sedimenting rates of the two boundaries, and it is careful measurements of these areas at different measured radial positions that are the raw data of this technique.

The relative change in $s(\Delta s/s_{av})$ is obtained from a function derived from the difference between two statements of the conservation of mass for two systems at sedimentation velocity with differing sedimentation coefficients and can be written as (Richards and Schachman, 1959):

$$(1/c_0 x_m^2) \int_{x_m}^{x_p} \Delta c x \mathrm{d}x = (\Delta s/s)[(x_p^2/x_{av}^2) \ln (x_{av}/x_m)] \qquad (1)$$

The integral represents the difference in mass between the two solutions, each in a sector-shaped volume, from the meniscus, x_m, to a point in the solution plateau region, x_p; it can be accurately approximated by the area under the concentration difference peaks multiplied by x_{av}, the average radial position of the two boundaries (taken as the peak maximum). The concentration of both solutions at zero time is c_0. Differences in loading concentrations can be accommodated by modification of eq 1, but in the present work, $c_{01} = c_{02}$ in all experiments. $\Delta s/s$ is then determined from the slope of a plot of the left-hand expression against the bracketed expression on the right evaluated at several different radial positions of the concentration-difference peak.

The Rayleigh interference optical system was aligned following the procedure outlined in the manual supplied with the instrument. All runs were made in 12-mm cells with double-sector aluminum-filled epon centerpieces, the interior surfaces of which were coated with Krylon clear acrylic spray (Borden Chemical Co.). The limbs were filled nearly to capacity with identical volumes of protein solution using a 0.5-ml Hamilton precision syringe. The myosin concentrations in the two cell sectors in each experiment were identical; this was assured by gravimetric dilution from a stock myosin solution with appropriate buffers differing either in added metabolites or in pH (see Results section). With myosin, it was found that measurable data (*i.e.*, resolvable peaks over 75% of the column length) could be obtained from runs in which the cell loading concentration ranged from 2 to 7 mg per ml, and the rotor speed was maintained at 30,000 rpm or less. At higher speeds, the gradients of the boundary became too steep to be recorded by the interference optical system. The narrow-slit symmetrical mask supplied by the manufacturer was used to produce a wider interference envelope making it possible to resolve larger concentration-difference peaks. Interference patterns were recorded on Kodak spectroscopic, type II-G, plates at regular intervals; sometimes longer than normal exposure times (up to 40 sec) were required with the narrow-slit mask in place. Difference peak areas were measured by tracing the peak images projected on the viewing screen of a Nikon Shadow Graph Model 6 microcomparator equipped with a 50-X projecting lens; the tracings were then measured with a planimeter. Base lines could be accurately estimated without separate blank runs. Six to eight peaks, spaced evenly from meniscus to cell bottom, were normally measured along with their average radial positions. Cell-loading concentrations were based upon 280-mμ absorbancies (see below) which were converted into centimeters of fringe displacement assuming $\mathrm{d}J/\mathrm{d}c = 4.133$ fringes/mg per ml of myosin concentration in 0.5 M KCl (Woods *et al.*, 1963). A number of cell blank runs were made with identical solutions in both sectors as checks on the bilateral symmetry of the double-sector centerpieces. The apparent differences in sedimentation rates observed in the two channels were never greater than 0.25% in each case and well within the experimental error.

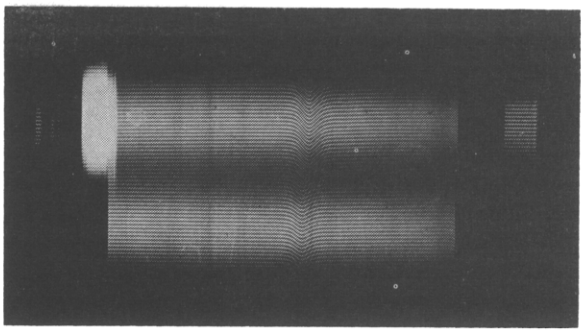

FIGURE 1: Concentration difference peaks from two differential sedimentation velocity experiments with myosin solutions at different pH values. Myosin (450 μl of 2.9 mg/ml) in 0.5 M KCl–0.01 M EDTA–0.025 M Tris–0.025 M bis-tris, in each limb of two double-sector cells. Regular cell: sector 1, pH 6.3; sector 2, pH 7.1; 0.6° down wedge cell: sector 1, pH 6.3; sector 2, pH 7.9; 30,000 rpm; 6°. Exposure taken 520 min after reaching speed.

Sedimentation velocity experiments were carried out in the conventional manner employing 12-mm cells with either single-sector Kel-F or double-sector aluminum-filled epon centerpieces.

Sedimentation equilibrium experiments at intermediate speeds followed a modification of the procedure outlined by LaBar (1965). Protein solutions were always exhaustively dialyzed against solvent and centrifuged at 25,000 rpm for 1 hr or more to remove particulate matter prior to their use in these experiments. The two sectors were filled to column heights of 1.6–2.1 mm with solution and dialysate. Experiments were carried out at 6° at rotor speeds of 5200–6000 rpm. Runs generally began with brief periods of overspeeding; sedimentation equilibrium was considered attained when fringe patterns were stable over periods of 8 hr or more. Rotor speeds were then raised to 18,000 rpm, or higher, as quickly as possible and the downward movement of the Rayleigh interference fringes in the meniscus region recorded at 2-min intervals for a period of 10–20 min on Kodak Spectroscopic type II-G plates. Photographs after progressively longer periods were taken until depletion was complete. (The redistribution of solvent components which occurs when the rotor speed is increased for meniscus depletion is negligible and does not affect the fringe-drop measurements. This is true even for those experiments in which the menisci levels in the two cell channels were not exactly matched.) Blank corrections were measured from patterns obtained from separate runs with dialysate in both sectors.

Instead of measuring the fringe drop at the meniscus, as

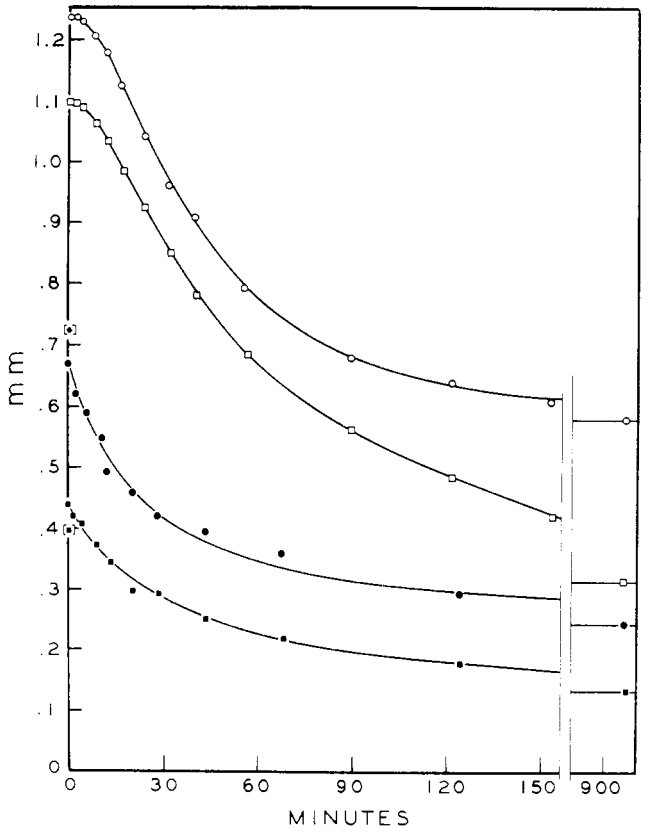

FIGURE 2. Two methods of measuring fringe depletion for LaBar intermediate-speed sedimentation equilibrium technique. (●, ■) Method outlined by LaBar (1965): bracketed symbols are zero time fringe-displacement values from low- (equilibrium) speed patterns. (○, □) Method outlined in text: data from four different intermediate-speed runs.

suggested by LaBar (1965), a point was chosen 100–300 μ centrifugal to it on the Rayleigh patterns. A plot of concentration against time at this point results in a sigmoidal-shaped curve which can be extrapolated with accuracy to zero time (see Figure 2). Thus, all measurements of the depletion are made from patterns taken at the higher speed rather than having to rely on the fringe position recorded at equilibrium (at the lower speed) as the zero time point on these plots. This procedure eliminates the error, substantial in most runs, due to window optical distortion occurring when the rotor speed is increased to its new value. The asymptotic-like function resulting from measurements at the meniscus, on the other hand, cannot be extrapolated back in time with accuracy because time zero is not known with sufficient precision. The inclusion of this modification transforms the LaBar method into the preferred sedimentation equilibrium technique for determining molecular weights down to very low concentrations when molecular weight values over a wide concentration range from one run are advantageous (*e.g.*, in the study of interacting systems).

Absorbancy, Specific Volume, and Refractive Index Increment Determinations. Extinction coefficients ($\epsilon_{280}^{1\%}$) were measured for myosin in 0.5 M KCl–0.01 M EDTA (pH 7.3), plus varying concentrations of potassium phosphate buffer by comparison of the extinctions for such solutions with that of an identical solution minus any phosphate, for which $\epsilon_{280}^{1\%}$ is known. A solution of myosin (25 ml) dialyzed against 0.5 M KCl–0.01 M EDTA (pH 7.3) was tared at 6° against the same solution on a two-pan torsion balance (±2 mg). To one of the solutions was added 25 ml of the dialysate and to the other 25 ml of a solution containing 0.5 M KCl–0.01 M EDTA, and twice the molarity in phosphate as was desired in the final solution. Duplicate gravimetric dilutions of these solutions were made to within an appropriate concentration range and their absorbancies (280–320 mμ) read on a Zeiss PMQ II spectrophotometer. $\epsilon_{280}^{1\%}$ values were calculated for myosin in these phosphate-containing solvent systems based on their extinctions relative to the companion solutions without phosphate assuming an $\epsilon_{280}^{1\%}$ of 5.50 $\%^{-1}$ cm^{-1} in the reference solvent (Gellert and Englander, 1963).[1]

TABLE I: $\epsilon_{280}^{1\%}$, dn/dc, and ϕ for Myosin in 0.5 M KCl–0.01 M EDTA (pH 7.3), Plus Various Concentrations of Phosphate, at 6°.

M PO_4^{2-}	ϕ' (ml/g)	dn/dc ($\Delta J/\%$)	$\epsilon_{280m\mu}^{1\%}$[a]
0	0.719	41.33[b]	5.50[c]
0.05	0.721		5.52
0.20	0.728	40.76	5.55
0.50	0.752		5.58

[a] At room temperature. [b] Value taken from Woods *et al.* (1963). [c] Value taken from data of Gellert and Englander (1963).[1]

The refractive index increment, dn/dc_{540}, for myosin in 0.5 M KCl–0.2 M phosphate–0.01 M EDTA (pH 7.3 at 6°) was measured in the usual way by layering dialysate over a known concentration of solution in a double-sector capillary-type synthetic boundary cell.

Apparent specific volumes (ϕ') defined according to Casassa and Eisenberg (1964) were calculated from the densities of solution and dialysate determined at 6° in a 25-ml Leach pycnometer. Variations in these measurements led to an estimated precision for each ϕ' determination of about ±0.003 ml/g. Myosin concentrations were determined from 280- to 320-mμ absorbancies and the $\epsilon_{280}^{1\%}$ values previously determined; buoyancy corrections were made. Results of the absorbancy, refractive index increment, and specific volume determinations are presented in Table I, where it will be noted that the addition of 0.5 M phosphate results in a 4% increase in ϕ' over its value in KCl alone. This sensitivity to phosphate concentration was unexpected and demonstrates the need for caution in assuming the constancy of ϕ' for proteins in even mildly differing solvent systems. As far as we are aware, this is the first report of a dramatic response of ϕ' to phosphate concentration for any protein.

These results prompted us to select the 0.2 M, rather than

[1] It should be noted that Gellert and Englander (1963) report a value of 5.43 $\%^{-1}$ cm^{-1}. However one of six determinations averaged to obtain this figure stands more than two standard deviations from the mean. When it is discarded, the new mean is 5.50 $\%^{-1}$ cm^{-1}.

TABLE II: Selected Differential Sedimentation Velocity Results of Myosin in the Presence (or Absence) of Contraction Related Metabolites.[a]

Expt	Sector 1 (M)	Sector 2 (M)	% Δs
V-1	2.6×10^{-3} PO_4^{2-}		0[b]
V-2	3.2×10^{-4} EDTA	3.2×10^{-4} EDTA	0
	2.6×10^{-5} ATP	2.6×10^{-5} ATP	
	2.6×10^{-4} $CaCl_2$	1.7×10^{-4} $CaCl_2$	
VI-1	2.5×10^{-5} ATP	2.5×10^{-5} Adenosine	0
VI-2	3.0×10^{-4} EDTA		0
VI-3	3.0×10^{-4} EDTA	3.0×10^{-4} EDTA	0
	2.0×10^{-4} $CaCl_2$	1.6×10^{-4} $CaCl_2$	
XII-1	3.0×10^{-3} AMP	3.0×10^{-3} ATP	0.13
	3.0×10^{-3} $K_4P_2O_7$	1.8×10^{-3} $CaCl_2$	
		1.8×10^{-3} $MgCl_2$	
		1.8×10^{-3} EGTA	
XII-2	3.0×10^{-3} $K_4P_2O_7$	1.8×10^{-3} EGTA	0.14
XII-3	3.0×10^{-3} $K_4P_2O_7$	1.8×10^{-3} EGTA	−0.25
XIII-1	Identical with expt XII, except sectors were filled in the reverse sense.		−0.01
XIII-2	Identical with expt XII, except sectors were filled in the reverse sense.		1.10
XIII-3	Identical with expt XII, except sectors were filled in the reverse sense.		−0.14

[a] In all runs, myosin concentration was 0.5–0.6% in 0.5 M KCl–0.02–0.04 M bis-tris, pH 6.7–6.9. Temperature was 6°. [b] First five runs were estimated as 0 ± 0.5%. Sensitivity of difference peak size to Δs is large making such estimates possible.

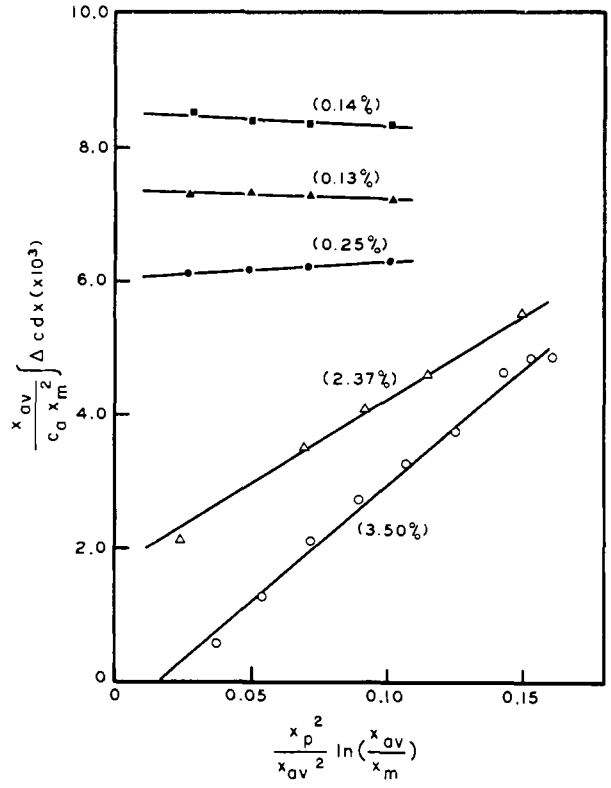

FIGURE 3: Examples of differential sedimentation velocity data plotted to yield $\Delta s/s$. Slope of function = $\Delta s/s$ (eq 1). Curves have been spaced arbitrarily on the ordinate for visualization; when plotted correctly the functions extrapolate to a value close to zero reflecting close matching of the menisci of the two sectors. Solid symbols from run XII (see Table II). (Δ) Myosin (2.9 mg/ml) in 0.5 M KCl at pH 7.9 and 6.4. (○) Myosin (5.0 mg/ml) in 0.35 M KCl at pH 7.8 and 6.4. All runs were at 6°, 28,000 rpm.

the 0.5 M PO_4^{2-} system, for the velocity sedimentation studies and the sedimentation equilibrium experiments described below and in the following paper (Godfrey and Harrington, 1970). For, as ϕ' and solvent density increase in value, the buoyancy factor ($1 - \phi'\rho$) approaches zero and molecular weights determined by sedimentation equilibrium become increasingly sensitive to errors in ϕ'. The buoyancy factor for the 0.2 M PO_4^{2-} system is 0.234, but only 0.182 for the 0.5 M PO_4^{2-} system. This difference results in a 35% greater sensitivity to error in ϕ' for the latter system.

Results

Differential Velocity Sedimentation. The sensitivity of the differential velocity sedimentation method was investigated by a series of experiments in which only very small changes in s could be expected (see below), but which would also provide information of potential significance. These experiments were designed to detect a change in the sedimentation rate of the myosin molecule in the presence of metabolites known to be involved in the contractile process (*e.g.*, ATP, ADP, PO_4^{2-}, Ca^{2+}, and Mg^{2+}). Any alteration in the secondary or tertiary structure of myosin induced by these low molecular weight substances might well be accompanied by a change in the effective axial ratio which would in turn affect the sedimentation rate.

The rod section of myosin has a reported diameter of about 20 Å and the length of the molecule is about 1500 Å. Ignoring the larger diameter of the head section, the molecule can be approximated by a prolate ellipsoid with an axial ratio of about 75. If the average diameter of the molecule is assumed to remain essentially constant during some conformational change, the change in axial ratio will be proportional to the relative change in length. From the Perrin and Svedberg equations we estimate that the differential sedimentation velocity method could conceivably detect an alteration of about ±2% in the length of the myosin molecule assuming a ±1% change in s to be the limit of sensitivity of the technique.

Results derived from experiments testing a wide variety of environmental conditions for myosin are summarized in Table II. The second and third columns contain components of solutions introduced into the two limbs of a double-sector cell; the fourth gives the calculated per cent of Δs. The sign is positive when myosin in cell sector 1 exhibited the higher sedimentation rate. In these studies Ca^{2+} concentrations were sometimes controlled by $CaCl_2$–EDTA (or EGTA) buffer systems and EDTA was added to the control limbs in some runs to eliminate trace amounts of free divalent cation. AMP and pyrophosphate ion were introduced

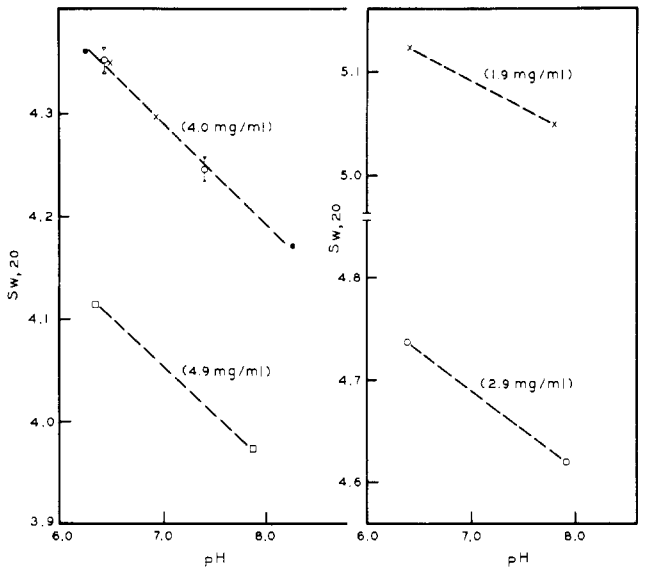

FIGURE 4: $s_{20,w}$ for myosin at various concentrations as a function of pH. Each pair of symbols are pH values for solutions in one differential sedimentation velocity experiment. Vertical bars (upper left) are estimated probable error for one run. All experiments at 6°, 24,000–30,000 rpm; solvent was 0.5 M KCl–0.01 M EDTA–0.025 M bis-tris–0.025 M Tris, at indicated pH values.

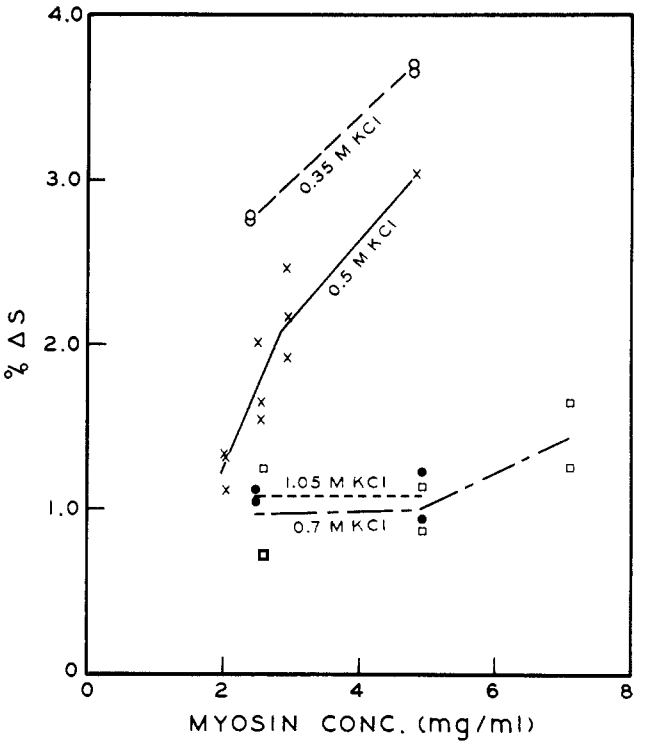

FIGURE 5: Per cent Δs of myosin at pH 6.4 and 7.8 as a function of myosin and KCl concentration. Each symbol is a result from one differential sedimentation velocity experiment. All runs were conducted at 6°, 24,000–32,000 rpm; additional solvent ingredients: 0.01 M EDTA–0.025 M bis-tris–0.025 M Tris. In all cases, the myosin at pH 6.4 exhibited the larger sedimentation rate.

into the control limb to maintain approximate salt and solution density equality. Bis-tris buffer was used to regulate the pH at 6.7–6.9 because phosphate, itself a metabolite, might possibly have had an inhibitory effect on metabolite–myosin interactions. Typical plots of data from these studies, calculated from eq 1, are seen in Figure 3 (solid symbols).

It was apparent from Table II that no dramatic, reproducible change in sedimentation coefficient was detected under the conditions imposed. These negative results are consistent with those of earlier workers who were unable to detect an alteration in secondary structure under similar environmental conditions by either optical rotatory dispersion (Gratzer and Lowey, 1969) or tritium exchange measurements (Segal and Harrington, 1967). Differential velocity experiments of Gratzer and Lowey utilizing the schlieren optical system also failed to detect changes in s for myosin greater than $\pm 5\%$ in the presence of ATP.

The Effect of pH. A large and reproducible change in sedimentation rate is found when solutions differing only in pH are compared. Plots of data from these studies are shown in Figure 3 (open symbols). Results from a number of runs at four different myosin concentrations are displayed in Figure 4. These results appear as absolute s values based upon the finding that at pH 6.8 the sedimentation coefficient of myosin in 0.5 M KCl fits the two-term sedimentation–concentration equation (eq 2). It can be seen from the differences in sedimentation rate of 0.4% myosin at pH 6.3 and at various higher pH values that the effect is apparently linear with pH. It is also sensitive to the myosin concentration; the relative response of s to pH changes decreases by a factor of about 2.5 when the myosin concentration is reduced from 4.9 to 1.9 mg per ml. In addition, decreasing the ionic strength of the myosin solution enhances the effect. Both of these latter relationships are shown in Figure 5. The four sets of points are from runs at different KCl molarities with each point representing one experiment. In all runs, solutions at pH 6.4 were compared with those at pH 7.8. Because absolute s values were not determined in KCl concentrations other than 0.5 M the ordinate is per cent of Δs.

In seeking an explanation for the pH–ionic strength effect, various alternatives have been examined. One phenomenon frequently found responsible for modifying sedimentation rates, the primary charge effect, can be rejected on theoretical grounds. The very high ionic strength of the solutions and the relatively small difference in net charge on the myosin molecule (Mihalyi, 1951) at pH 6.4 and 7.8 allow, at most, a change in s of about 0.01% (Pedersen, 1958). Another possible explanation, that a significant secondary salt effect is present, seems unlikely also because of the very low and similar molecular weights of the major counter ions, K^+ and Cl^-. The strong concentration dependence of the effect does not favor a pH-induced change in the axial ratio as the principle cause, either, since such a change would be reflected in a change in the intrinsic sedimentation coefficient values as well. However a pH-dependent association of myosin to one or more larger species is consistent with the data. The suppressing effect of both low myosin concentration and high ionic strength on the differential sedimentation rate suggests the presence of a reaction leading to associated species in which electrostatic interactions play a major role (Harrington and Josephs, 1968). Moreover, the near Gaussian shapes of the concentration difference peaks and the lack of secondary peaks (see Figure

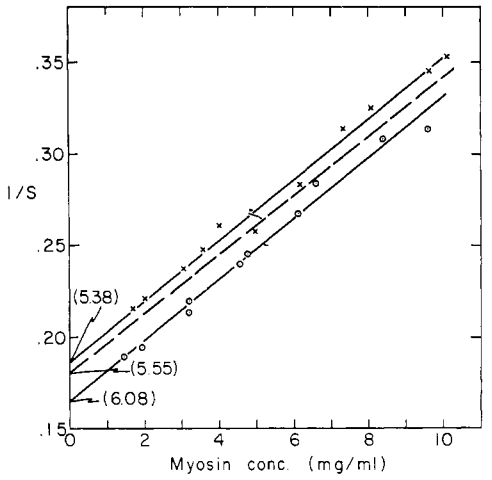

FIGURE 6: $1/s_{20,w}$ *vs.* concentration for myosin in the presence and absence of 0.2 M PO_4^{2-}. (×) Myosin in 0.5 M KCl–0.2 M PO_4^{2-}–0.01 M EDTA, pH 7.3; (○) myosin in 0.5 M KCl–0.01 M EDTA, pH 7; (---) 0.2 M PO_4^{2-} solvent curve normalized for buoyancy differences between the two solvent systems. All experiments at 6°, 52,000 rpm.

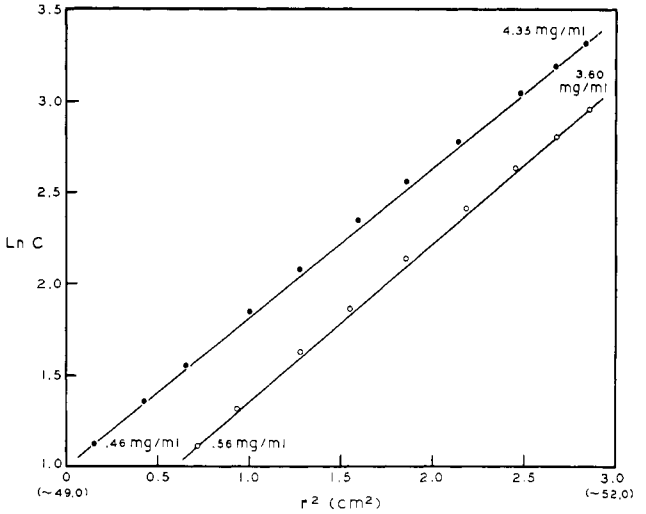

FIGURE 7: In *c vs.* r^2 of data from intermediate-speed runs in the presence and absence of 0.2 M PO_4^{2}. (●) (low phosphate) Myosin (1.56 mg/ml) in 0.5 M KCl–0.02 M PO_4^{2-}–0.01 M EDTA, pH 7.0; 5200 rpm; 6°; (○) (high phosphate) 1.68 mg/ml of myosin in 0.5 M KCl–0.2 M PO_4^{2-}–0.01 M EDTA, pH 7.3; 6000 rpm; 6°. Abscissa in generalized units to accommodate both curves.; concentrations at menisci and bottom of columns are indicated.

1) as well as the single hypersharp boundary observed in velocity sedimentation experiments indicate that dimer in rapidly reversible equilibrium with monomer is a likely candidate for the higher associated species. Only simple self-associating systems such as monomer–dimer or monomer–dimer–trimer sediment as single, nonbimodal gradient peaks according to the well-documented Gilbert (1959) theory of masstransport (see also Nichol *et al.*, 1964).

Sedimentation Velocity Experiments. One way to conclusively demonstrate reversible association in the high-salt myosin system is to somehow suppress the association so that hydrodynamic and molecular weight measurements will be more characteristic of the monomer species. Preliminary sedimentation velocity experiments in this laboratory suggested a gross lowering of the sedimentation rate of myosin in the presence of 0.5 M phosphate. This finding, and the report some years ago by Holtzer and Lowey (1959) that the aggregation rate in myosin solutions was suppressed in the presence of phosphate ion, prompted us to investigate the properties of myosin in 0.5 M KCl–0.01 M EDTA, plus varying concentrations of *o*-phosphate.

In both 0.5 M KCl and in the 0.5 M KCl–PO_4^{2-} solvent systems freshly prepared myosin sedimented as a single sharp boundary devoid of faster or slower moving shoulders. After about 5 days at 6° myosin solutions without phosphate began to show the faster moving boundaries associated with aged myosin preparations (Holtzer and Lowey, 1959; Johnson and Rowe, 1960). But in the presence of phosphate, the formation of heavier species is retarded for long periods of time. A 2.1% myosin solution dialyzed for 8 months in the cold against 0.5 M KCl–0.01 M EDTA–0.5 M PO_4^{2-} (pH 7.3) still sedimented as a single boundary; no very fast peaks preceded it. A 3-week-old sample in the 0.2 M phosphate system showed similar behavior. Subsequent DEAE-Sephadex A-50 chromatography (see paper II of this series) of the 8-month-old solution did reveal a shoulder on the front side of the myosin-containing peak suggesting the presence of some higher molecular weight aggregate. Nevertheless, these results confirm conclusively the capacity of phosphate to suppress the formation of very heavy species.

The effect of 0.2 M phosphate on the sedimentation rate of myosin in freshly prepared solutions can be seen in Figure 6. Myosin solutions over the concentration range of about 1.5 to 10 mg/ml in 0.5 M KCl–0.01 M EDTA (pH 7.3), with and without 0.2 M PO_4^{2-} added, were sedimented at 6° and 52,000 rpm. Plots of $1/s_{20,w}$ *vs.* concentration gave two nearly parallel linear functions which extrapolated to values differing by about 12%. Thus for each:

$$1/s_{20,w} = 0.165 + 0.0169c \text{ (mg/ml) in KCl alone} \quad (2)$$

$$1/s_{20,w} = 0.186 + 0.0167c \text{ (mg/ml) in KCl plus } PO_4^{2-} \quad (3)$$

At infinite dilution $s_{20,w}$ in the KCl–EDTA solvent agrees very well with published values for myosin in 0.5 M KCl (Trayer and Perry, 1966; Chung *et al.*, 1967; Gershman *et al.*,[2] 1969). When the data for myosin in the presence of phosphate is corrected for the difference in buoyancy in the two solvents (see Table I) the dashed line seen in Figure 6 is obtained, reducing the difference between the two intrinsic s values to about 9%. Other factors may contribute to this difference in s values, but a shift in the monomer:dimer ratio (toward monomer in the presence of PO_4^{2-}) could easily account for it. In this case, the normalized $s_{20,w}$ values would be expected to extrapolate to the same point on the ordinate after some upward curvature near zero concentration. However, results from the sedimentation equilibrium studies presented in the following paper indicate, that as a result of the very large virial coefficient and association constant, sedimentation coefficients at concentrations much lower than 1.5 mg/ml are un-

[2] Solvent used by Gershman *et al.* (1969) was 0.4 M KCl–0.05 M PO_4^{2-}.

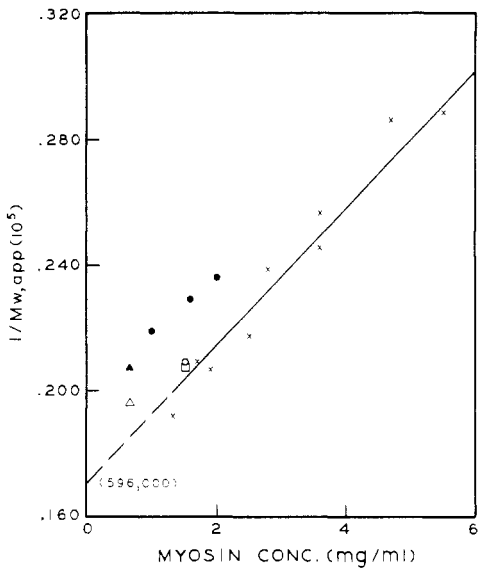

FIGURE 8: Comparison of cell-average $1/M_{w,app}$ values for myosin in high salt concentration from low-, intermediate-, and high-speed sedimentation equilibrium experiments in the presence and absence of 0.2 M PO_4^{2-}. (×) and (—) Short-column low-speed runs (also some Archibald runs) in 0.5 M KCl; 5°, 4059–5609 rpm (Woods *et al.*, 1963); (○) Average of two intermediate speed runs in 0.7 M KCl–0.02 M PO_4^{2-}–0.01 M EDTA, pH 7.2; 6°; 5200 rpm. (□) Average of two intermediate-speed runs in 0.35 M KCl–0.02 M PO_4^{2-}–0.01 M EDTA, pH 7.1; 6°; 5200 rpm; (●) three intermediate-speed runs in 0.5 M KCl–0.2 M PO_4^{2-}, 0.01 M EDTA, pH 7.3; 6°; 6000 rpm; (△) average of 21 high-speed runs in 0.6 M KCl–0.025 M histidine, pH 6.8; 4°; 8766 rpm; ln *c vs.* r^2 plot analysis (Chung *et al.*, 1967); (▲) average of 21 high-speed runs in 0.5 M KCl–0.2 M PO_4^{2-}, 0.01 M EDTA, pH 7.3; 6°; 9,000–12,000 rpm; ln *c vs.* r^2 plot analysis (Godfrey and Harrington, 1970). See paper II of this series for comment on the last two values.

doubtedly required to record this phenomenon in either solvent system.

Sedimentation Equilibrium Experiments. A number of sedimentation equilibrium runs were made at intermediate rotor speeds (about 6000 rpm for myosin) employing the modification of the LaBar procedure outlined earlier. Figure 7 shows typical ln *c vs.* r^2 plots of myosin in both high and low phosphate solvent systems. The upper (solid circle) data were obtained from an experiment with myosin dialyzed against 0.5 M KCl–0.02 M PO_4^{2-}–0.01 M EDTA (pH 7). The ln *c vs.* r^2 plot exhibits pronounced monotonic downward curvature across the entire liquid column characteristic of a nonideal, nonassociating species. Similar behavior was observed with myosin in 0.35 M KCl or 0.70 M KCl. The least-squares-fitted slopes from four such plots yielded cell-average reciprocal apparent weight-average molecular weights presented in Figure 8. The line is taken from Woods *et al.* (1963) and represents the best linear fit to values from Archibald and short-column low-speed equilibrium experiments with rabbit skeletal myosin in 0.5 M KCl.

Reciprocal cell-average values from LaBar experiments with myosin dialyzed against 0.5 M KCl–0.01 M EDTA–0.2 M PO_4^{2-} (pH 7.3) (high phosphate) are also shown in Figure 8. The ln *c vs.* r^2 plots (the intermediate loading concentration run is presented in Figure 7) exhibit the same marked monotonic downward curvature as the plot in the absence of high phosphate.

These results suggest that the apparent molecular weight for myosin is not measurably affected by variations in KCl concentration from 0.35 to 0.7 M, in the presence of low phosphate.[3] Addition of high concentrations of PO_4^{2-}, however, shifts the apparent weight-average moments to significantly lower values. This effect parallels the velocity sedimentation results presented earlier in the two solvent systems. The lack of an inflection point within the downward curving ln *c vs.* r^2 plots from both series of runs clearly indicates a continuous decrease in the apparent molecular weight with increased concentration. Thus it would appear that no upturn in the $1/M_{app}$ *vs.* concentration function characteristic of nonideal systems with pronounced self-association was present. This interpretation was later verified by computer analysis of a number of these runs using the method described in the following paper. On the basis of these results alone, myosin either does not associate, or, if it does, the characteristic behavior of the molecular weight moments for such systems must be located in the concentration range below that which can be effectively analyzed by the LaBar approach (*i.e.*, below *ca.* 0.4 mg/ml). This was true for myosin in both high and low phosphate solvent systems.

Conclusion

The differential sedimentation velocity experiments provide evidence for the presence of a pH-sensitive, reversible, monomer–*n*-mer reaction for myosin in high salt concentration (0.35 M KCl). Moreover, the apparent decrease in sedimentation coefficient and weight-average molecular weight in phosphate indicates that this anion acts to displace the equilibrium in favor of monomer. Since myosin sediments as a single, sharpened boundary in sedimentation velocity experiments, a rapidly reversible monomer–dimer equilibrium appears to be the most likely mode of association. The major disagreement with these conclusions comes from the linear behavior of $1/M_w$ *vs.* concentration plots derived from the sedimentation equilibrium experiments. A nonideal system in rapid chemical equilibrium would be expected to exhibit upward curvature as a result of the counterbalancing effects of association and the expression of nonideality. As we will demonstrate in paper II of this series, the very large virial coefficient of myosin effectively overshadows the monomer–dimer equilibrium at all concentrations above about 0.5 mg/ml and consequently the association cannot be detected in low- and intermediate-speed equilibrium studies.

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[3] The differential velocity experiments (Figure 5) suggest indirectly that the monomer–dimer equilibrium may be sensitive to KCl concentration as well as pH but the effect may be too small (<3%) for easy detection by sedimentation equilibrium measurements of $M_{w,app}$.

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