Relationship of Structure to Function in Myosin. I. Subunit Dissociation in Concentrated Salt Solutions*

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ABSTRACT: Rabbit skeletal myosin is shown from sedimentation velocity and sedimentation equilibrium experiments to be dissociated by concentrated salt solutions, pH 7, 4°, into a heavy-chain core and a light component (12% of the total protein) that comprises approximately 2.7 light chains of an average molecular weight of 20,600. In order of decreasing effectiveness in dissociating light component from myosin, the cations may be listed as Ca^{2+}, Mg^{2+}, Li^{+}, NH_4^{+}, Na^{+}, and K^{+}; and the anions may be listed as *p*-toluenesulfonate, trichloracetate, SCN^{-}, I^{-}, Br^{-}, Cl^{-}, and SO_4^{2-}. Salts acting *via* a predominant cationic effect (Ca^{2+}, Mg^{2+}, and Li^{+}) also lead to reversible changes in the sedimentation properties of the heavy-chain core at salt concentrations above the transitions for light-chain dissociation. There is almost complete reassociation of light chains with heavy-chain core on redialysis against 0.4 M KCl, provided that salt denaturation was brief and thiol groups were protected. The light chains undergo aggregation during prolonged salt treatment, especially in the absence of thiol protection; and the purified heavy-chain core aggregates to at least dimer level in 0.4 M KCl, despite brief salt treatment and thiol protection. On cellulose acetate electrophoresis, the light component yields four major bands and one or two minor bands. Comparable electrophoretic heterogeneity is obtained after dissociation of light component from myosin by 4 M LiCl, 1 M KSCN, and 2 M guanidine, and at pH 11; however, prolonged salt or alkaline denaturation of myosin, especially in the absence of thiol protection, leads to blurring and on occasion merging of the different electrophoretic bands. Short treatment of myosin in 4.7 M NH_4Cl results in the dissociation of 3–4% light component.

This fraction contains protein that migrates in the fast electrophoretic band and also contains myokinase in trace proportion.

It was shown early that rabbit skeletal myosin yields light and heavy components on treatment in urea and guanidine solutions and at alkaline pH (Tsao, 1953; Kominz *et al.*, 1959; Szent-Györgyi, 1960; Wetlaufer and Edsall, 1960). Although the apparent absence of light component on dissociation of myosin in 5 M guanidine (Kielley and Harrington, 1960; Small *et al.*, 1961; Young *et al.*, 1962; Brahms and Kay, 1963) led to the belief that the low molecular weight component derived from peptide fragments or contaminant proteins (Perry, 1961), more precise analysis of sedimentation velocity, sedimentation equilibrium, and diffusion data demonstrated dissociation of myosin by 5 M guanidine into light and heavy components that are poorly resolved by hydrodynamic methods (Dreizen *et al.*, 1966, 1967). Detailed ultracentrifugal experiments in this laboratory indicated that myosin (molecular weight 468,000 ± 10,000) is dissociated at pH 11 into a heavy-chain core of 420,000 weight and a light component that comprises 12% of myosin and contains 2.7 (±0.3) light polypeptide chains of average weight 20,200; the myosin core is dissociated by 5 M guanidine into two heavy polypeptide chains, each of 212,000 weight (Gershman *et al.*, 1966, 1969; Dreizen *et al.*, 1967). The light component dissociated from carboxymethylated myosin at pH 11 yields three or four bands on cellulose acetate electrophoresis (Gershman *et al.*, 1966). Locker and Hagyard (1967a,b) have independently described electrophoretic heterogeneity and comparable stoichiometry (15%) and molecular weight (17,000–20,000) for the light component dissociated from myosin by acetylation; however, Frederiksen and Holtzer (1968), while confirming the 12% stoichiometry for light alkali component, reported a molecular weight about 30,000 for the light chains.

Some of these findings might be related at least in part to alkaline hydrolysis of myosin, and it is significant in this respect that myosin is also dissociated into light and heavy components in concentrated salt solutions, pH 7, 4° (Gershman *et al.*, 1968). This paper describes ultracentrifugal data on salt dissociation and reassociation of myosin, and electrophoretic experiments on light component dissociated from myosin in concentrated salt solutions. Preliminary accounts have been reported (Gershman and Dreizen, 1969a,b).

Methods

Rabbit skeletal myosin was prepared by procedures described previously (Szent-Györgyi, 1951; Dreizen *et al.*, 1966), and stored in 0.4 M KCl–0.005 M $NaHCO_3$ (pH 7) until use. The preparations were kept at 4° throughout.

Experiments on salt-treated myosin involved addition of

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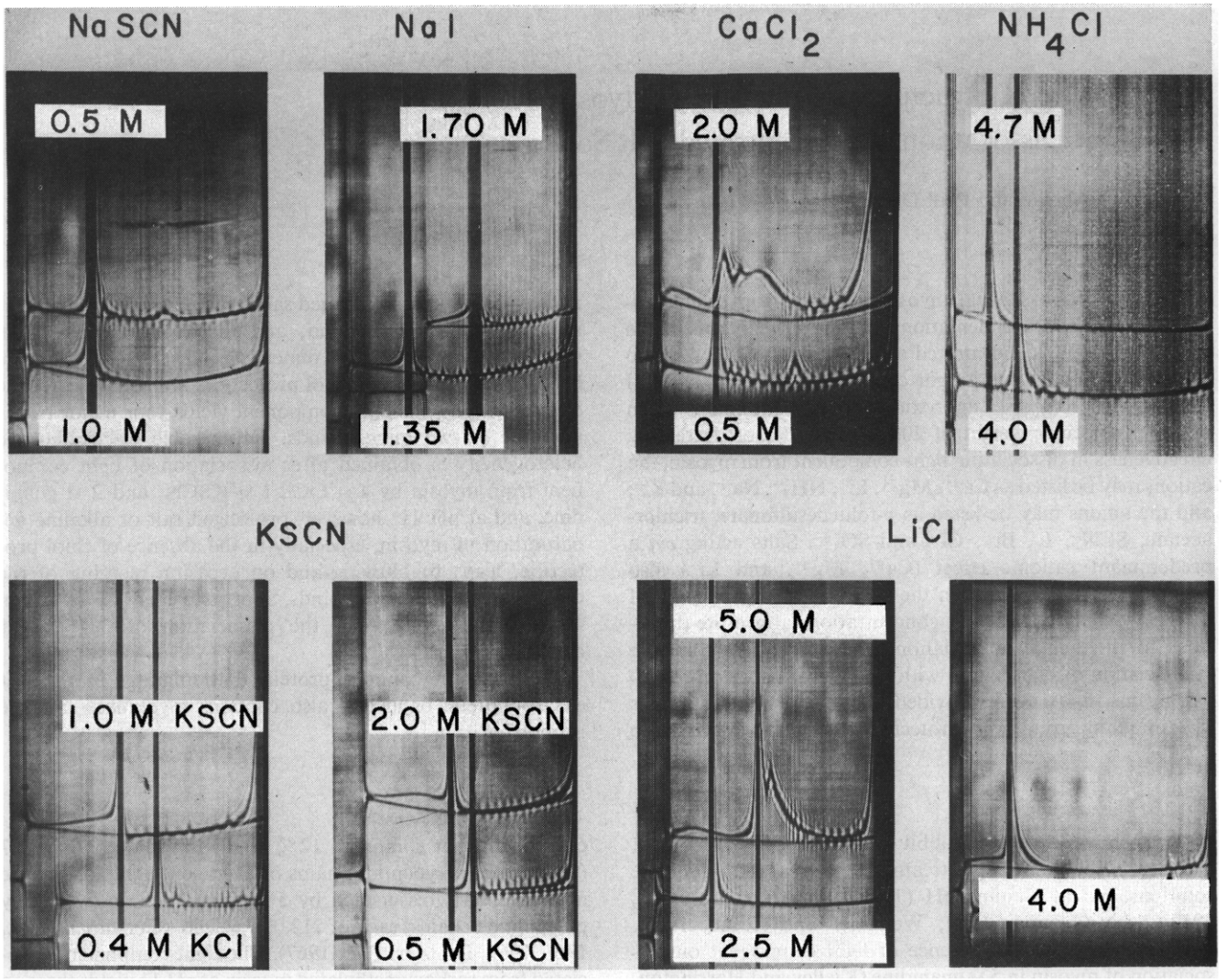

FIGURE 1: Sedimentation velocity of myosin at concentrations indicated, pH 7, 4°, 52,640 rpm, phase-plate angle 70° except as noted. NaSCN solutions: 12 and 1.1 mg per ml in each cell, 168 min. NaI solutions: 18 and 1.6 mg per ml in each cell, phase-plate angle 80°, 290 min. $CaCl_2$ solutions: 18 and 1.6 mg per ml in each cell, 212 min. NH_4Cl solutions: 9.3 mg/ml, 48 min. KSCN solutions: 19 mg/ml, 300 min. LiCl solutions: (left) 19 mg/ml, 280 min; (right) 12 mg/ml, phase-plate angle 75°, 152 min. Note that in some experiments double-sector cells contain protein in both compartments.

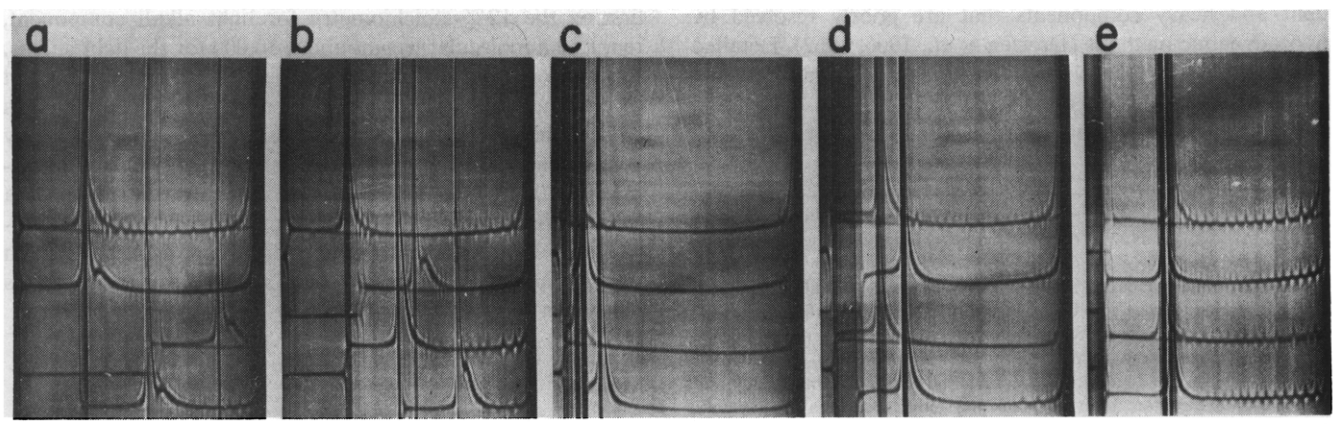

FIGURE 2: Sedimentation velocity of LiCl-treated myosin, pH 7, 4°, phase-plate angle 70°. (a) Myosin stored in 3 M LiCl–0.001 M EDTA for 24, 1, 0, and 4 hr (from top down); at 48,000 rpm for 328 min, 7 mg/ml. (b) Samples used in part a were dialyzed against 0.4 M KCl–0.001 M EDTA; 7 mg/ml; at 52,000 rpm for 112 min. From top down, 24-, 0-, 4-, and 1-hr storage in 3 M LiCl. (c) Myosin stored in 4 M LiCl–0.001 M EDTA for 24, 1, 0, and 4 hr (from top down); at 52,000 rpm for 64 min, 8 mg/ml. (d) Same as part c, at 216 min. (e) Samples used in parts c and d were dialyzed against 0.4 M KCl–0.001 M EDTA; 8 mg/ml; at 52,000 rpm for 120 min.

TABLE I: Molar Salt Concentration for Dissociation of Light Component from Myosin at Neutral pH and 4°.[a]

	Li^+	NH_4^+	Na^+	K^+
Thiocyanate	0.5	0.6	0.6	0.6
Iodide	0.7	1.2	1.4	1.8
Bromide	2.0	3.0	3.0[b]	2.7[b]
Chloride	3.5	4.7[b]	*c*	*c*
Sulfate	*c*	*c*	*c*	*c*

[a] Transition for light-chain dissociation also occurs in 1.6 M $CaCl_2$, 1.6 M $MgCl_2$, 0.3 M sodium *p*-toluenesulfonate, and 0.3 M sodium trichloroacetate. [b] Partial dissociation of light component. Experiments were not done at higher salt concentrations due to high solvent density (NaBr and KBr) or salt saturation (NH_4Cl). [c] No evident dissociation of light component at salt concentrations up to saturation.

concentrated salt solution to myosin or dialysis of myosin against the appropriate salt solution at pH 7 and 4°. The use of dithiothreitol (Calbiochem) or EDTA in these solutions is explicitly described. Analytical grade reagents were used throughout, and Ultra Pure $(NH_4)_2SO_4$ reagent was purchased from Mann Research Laboratories. Experiments with iodide salts were on freshly prepared solutions.

Light-Chain Purification. The light component was isolated from native myosin or from the precipitate fraction derived from NH_4Cl–$(NH_4)_2SO_4$ fractionation of native myosin by one of the following methods: (1) Alkali dilution, based on a method described earlier (Gershman *et al.*, 1966), in which myosin in 0.4 M KCl was titrated with Na_2CO_3–NaOH to pH 11.0, promptly diluted tenfold with water, titrated to pH 7 with 1 M KH_2PO_4, and centrifuged at 10,000 rpm for 10 min. The supernatant was concentrated to about 5 mg/ml of protein by dialysis against Sephadex G-200 or polyvinylpyrrolidone (A. H. Thomas Co.), and dialyzed against 0.4 M KCl (pH 7). Dithiothreitol was present throughout. (2) LiCl–citrate salting out, in which equivalent volumes of 8 M LiCl–0.002 M dithiothreitol and myosin in 0.4 M KCl were mixed, promptly salted out with one to two volumes of saturated potassium citrate–4 M LiCl (pH 7), and centrifuged at 10,000 rpm for 10 min. The supernatant was treated as in part 1, and on occasion dialyzed against water to precipitate any residual heavy-chain and light-chain aggregates. (Variations are described in text.) (3) KSCN–citrate salting out, in which equivalent volumes of 2 M (or 4 M) KSCN–0.002 M dithiothreitol and myosin in 0.4 M KCl were mixed, salted out with one volume of 1.5 M potassium citrate–1 M (or 2 M) KSCN (pH 7), and centrifuged at 10,000 rpm for 10 min. Supernatant was treated as in 1. (4) Guanidine–citrate salting out, in which myosin was dissociated in 2 M guanidine–0.002 M dithiothreitol (Gershman *et al.*, 1969), salted out at 0.7 M potassium citrate, and treated as in part 3. (5) NH_4Cl–$(NH_4)_2SO_4$ salting out, in which equivalent volumes of myosin and 5 M NH_4Cl–0.003 M dithiothreitol were mixed, with solid NH_4Cl added to 4.7 M; the solution was salted out with one volume of saturated $(NH_4)_2SO_4$–4.7 M NH_4Cl, and centrifuged at 10,000 rpm for 10 min. Supernatant was treated as in part 1, and precipitate was dissolved and dialyzed against 0.4 M KCl–0.001 M dithiothreitol.

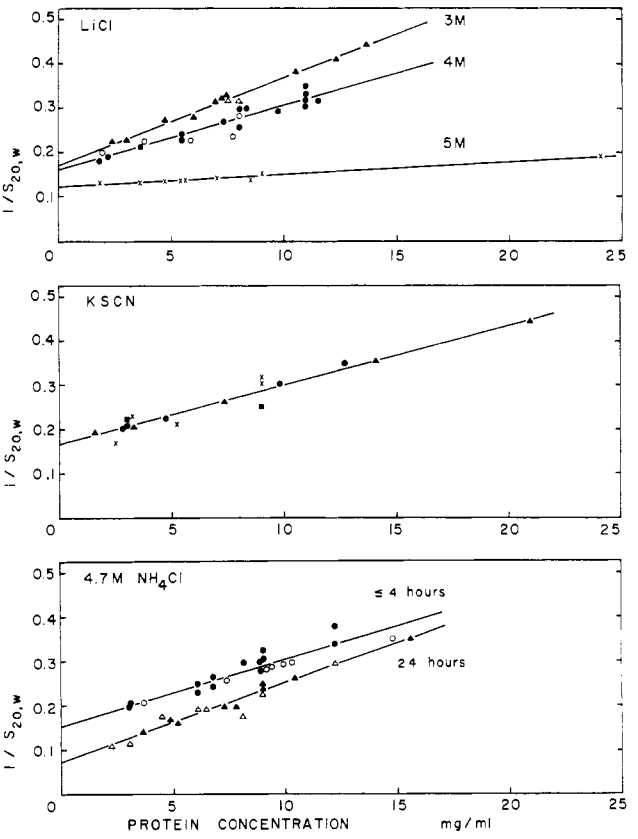

FIGURE 3: Reciprocal of sedimentation coefficient (S) for myosin or heavy-chain core plotted against initial concentration of myosin, pH 7, 4°. Upper: 3 M LiCl (▲); myosin stored in 3 M LiCl for 24 hr (△); 4 M LiCl (●); myosin stored in 4 M LiCl for 24 hr (○); 5 M LiCl (×). Middle: 0.5 M KSCN (●), 1 M KSCN (▲), 2 M KSCN (■), 4 M KSCN (×). Lower: myosin stored in 4.7 M NH_4Cl for less than 4 hr (●); same solutions dialyzed against 0.4 M KCl (○); myosin stored in 4.7 M NH_4Cl for 24 hr (▲); same solutions dialyzed against 0.4 M KCl (△). Lines indicate least-squares regression.

Analytical Ultracentrifugation. The experiments were done at 4° in a Beckman Model E ultracentrifuge equipped with temperature control unit and, for the later work, with electronic speed control. The proportions of light and heavy components were determined from sedimentation velocity and sedimentation equilibrium experiments, with total protein concentration from synthetic boundary experiments (at 3–6 mg/ml) or optical density measurements. In sedimentation velocity experiments (at 4–9 mg/ml), schlieren measurements were corrected for radial dilution and Johnston–Ogston effect (Trautman *et al.*, 1954; Gershman *et al.*, 1966). High-speed sedimentation equilibrium experiments (Yphantis, 1964) involved successive equilibria at two or more rotor speeds. For multicomponent analyses (Gershman *et al.*, 1966, 1969; Gershman, 1968), the molecular weight and concentration of light component were determined from interference patterns at the higher speed(s); and the molecular weight of the next heavy component was determined from interference patterns at the lower speed(s), correcting for the presence of known light component. Calculations were done with an Olivetti-Underwood 101 computer.

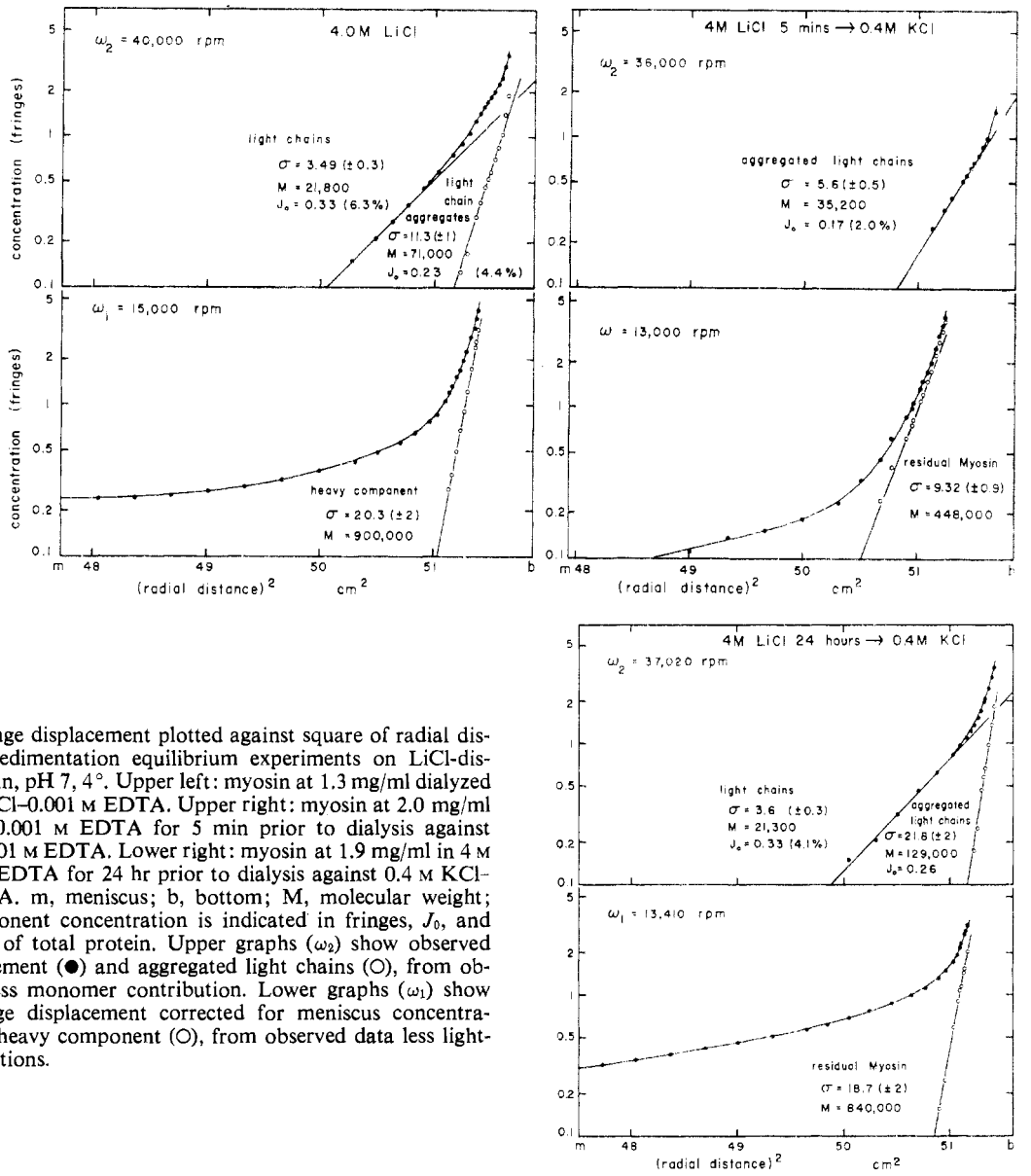

FIGURE 4: Fringe displacement plotted against square of radial distance, from sedimentation equilibrium experiments on LiCl-dissociated myosin, pH 7, 4°. Upper left: myosin at 1.3 mg/ml dialyzed against 4 M LiCl–0.001 M EDTA. Upper right: myosin at 2.0 mg/ml in 4 M LiCl–0.001 M EDTA for 5 min prior to dialysis against 0.4 M KCl–0.001 M EDTA. Lower right: myosin at 1.9 mg/ml in 4 M LiCl–0.001 M EDTA for 24 hr prior to dialysis against 0.4 M KCl–0.001 M EDTA. m, meniscus; b, bottom; M, molecular weight; average component concentration is indicated in fringes, J_0, and as percentage of total protein. Upper graphs (ω_2) show observed fringe displacement (●) and aggregated light chains (○), from observed data less monomer contribution. Lower graphs (ω_1) show observed fringe displacement corrected for meniscus concentration (●), and heavy component (○), from observed data less light-chain contributions.

Electrophoresis. Cellulose acetate electrophoresis was done with a Beckman Microzone system at room temperature, in 0.75 M sodium barbital (pH 8.6) at 225 V for 40–60 min. The protein was stained with coomassie brilliant blue (Mann), and cellulose acetate strips were stored in light paraffin oil to provide a transparent medium for photographs or densitometric recording. Use of coomassie blue permitted better definition of the minor electrophoretic components than was earlier obtained on use of ponceau S (Gershman *et al.*, 1966). Occasional artifacts from trapped air or diffraction by oil are easily recognized.

Other Methods. Protein concentration was determined from optical density at 2800 Å, using a Zeiss PMQ-II spectrophotometer, with correction for Rayleigh scattering from optical density at 3200 to 6000 Å. Values for extinction coefficient and partial specific volume were as noted (Gershman *et al.*, 1966, 1969).

Myokinase was assayed by the procedure of Richards *et al.* (1967), in which ATP that is formed from ADP in the presence of myokinase (Sigma) is determined by subsequent liberation of P_i by Ca-activated myosin ATPase. The myokinase reaction was carried out in 0.5 M KCl, 0.1 M Tris, 0.01 M $MgCl_2$, and 0.01 M dithiothreitol, at 30°; and aliquots were removed at set times from 0 to 24 hr.

Results

Dissociating Effect of Salt. Figure 1 demonstrates the dissociation of myosin into light and heavy components in concentrated salt solutions at pH 7 and 4°; schlieren patterns are shown from sedimentation velocity experiments at salt concentrations above and below the transition for light-chain dissociation from myosin. Table I indicates the molar salt concentration for dissociation of approximately half the

TABLE II: Sedimentation Equilibrium Experiments on LiCl-Treated Myosin.

	Rotor Speed (rpm)	Light Component				Heavy Component	
		Monomer		Aggregate			
		Mol Wt	%	Mol Wt	%	σ	Mol Wt $\times 10^{-5}$
4 M LiCl	40,000	21,800	6.3	71,000	4.4		
	15,000	21,800	6.3	71,000	4.4	20.3	9.0
4 M LiCl[a]	39,460	20,000	6.3	77,000	5.3		
	16,200	20,000	6.3	77,000	5.3	19.2	7.3
4 M LiCl[b]	39,490	20,100	7.7	57,000	4.8		
	16,200	20,100	7.7	57,000	4.8	14.3	6.0
4 M LiCl–0.4 M KCl[c]							
5 min[a]	36,000		0	35,200	2.0		
	13,000		0	35,200	2.0	9.3	4.5
1 hr[a]	36,000	20,400	3.3	150,000	1		
4 hr[a]	37,020	20,500	3.3	120,000	2		
24 hr[a]	37,020	21,300	4.1	130,000	3		
	13,410	21,300	4.1	130,000	3	18.7	8.4
Second cycle LiCl–citrate precipitate[b] in 0.4 M KCl, pH 7[b]	36,000		0		0		
	15,000		0		0	21.4	9.5
At pH 11.0[a]	36,000	22,000	1	80,000	1		
At pH 11.0[a]	37,020	20,800	2	92,000	1		

[a] Solutions contain 0.001 M EDTA. [b] Solutions contain 0.001 M dithiothreitol. [c] Native myosin was stored in 4 M LiCl (pH 7) for indicated time and then dialyzed against 0.4 M KCl (pH 7) over 48 hr.

total light component from myosin in different salt solutions at pH 7 and 4°. In order of decreasing effectiveness in dissociation of light component, the cations form the series: Ca^{2+}, Mg^{2+}, Li^{+}, NH_4^{+}, Na^{+}, and K^{+}; and the anions form the series: *p*-toluenesulfonate, trichloroacetate, thiocyanate, iodide, bromide, chloride, and sulfate. The myosin structure remains intact in NaCl, KCl, and sulfate solutions; a fraction of the light component is dissociated from myosin in 4.7 M NH_4Cl, 2.7 M KBr, and 3 M NaBr; and the entire light component is dissociated at sufficiently high molar concentrations in all other salt solutions tested. The results derive from sedimentation velocity experiments initiated promptly after addition of myosin to concentrated salt solutions, with comparable data in the presence or absence of 0.001 M EDTA or 0.001 M dithiothreitol. The transition concentration is unchanged following 24-hr treatment of myosin in strongly dissociating salts (KSCN and LiCl, for example), but is diminished slightly following 24-hr treatment in mildly dissociating salts (NH_4Cl, for example).

Salt treatment may also effect the sedimentation properties of the main myosin component. Thus, treatment of myosin in solutions of Li^{+}, Ca^{2+}, and Mg^{2+} salts at concentrations above the transition for light-chain dissociation is accompanied by immediate and extensive aggregation of the main component (Figure 1). In addition, light and heavy components both undergo irreversible aggregation during prolonged storage under dissociating conditions, especially in the absence of EDTA or dithiothreitol.

Detailed ultracentrifugal studies were conducted on myosin in LiCl, KSCN, and NH_4Cl solutions, representing strong "cationic effect," strong "anionic effect," and mild denaturation, respectively.

LiCl Solutions. In 3 M LiCl, schlieren patterns show only slight dissociation of light component (~2%) from myosin, and the proportion of trailing component is not increased during 24-hr storage in 3 M LiCl (Figure 2a). The main schlieren boundary has an $s^0_{20,w}$ value of 5.7 S in 3 M LiCl (Figure 3), but a leading edge indicates the occurrence of some aggregation (Figure 2a) that is not reversible on dialysis against 0.4 M KCl (Figure 2b).

In 4 M LiCl, the entire light component is dissociated from myosin within the period required for the formation of schlieren boundaries (Figure 2c,d). Measurements of schlieren area corrected for radial dilution and Johnston–Ogston effect indicate that the light component comprises 12.3 (±1)% of myosin. The heavy component has an $s^0_{20,w}$ value of 6.2 S, with somewhat less concentration dependence of sedimentation in 4 M LiCl than in 3 M LiCl (Figure 3). The sedimentation properties are unchanged during 24-hr treatment in 3 or 4 M LiCl.

In 5 M LiCl, the main component displays an increase in $s^0_{20,w}$ to 8.2 S and nearly complete loss of the concentration dependence of sedimentation (Figure 3). These effects are largely reversible on dialysis against 0.4 M KCl, provided that the duration of LiCl treatment was short and 0.001 M dithiothreitol was present throughout.

TABLE III: Sedimentation Equilibrium Experiments on NH_4Cl-Treated Myosin.

		Light Component					
		Monomer		Aggregate		Heavy Component	
	Rotor Speed (rpm)	Mol Wt	%	Mol Wt	%	σ	Mol Wt $\times 10^{-5}$
4.7 M NH_4Cl	40,000	19,000	4.1	98,000	2		
4.7 M NH_4Cl[a]	40,000	22,600	3.8	81,000	2	11.0	5.2 (±0.3)
	14,000	22,600	3.8	81,000	2		
4.7 M NH_4Cl to 0.4 M KCl[b]							
5 min[a]	39,460	20,300	2.2	125,000	<1		
	13,410	20,300	2.2	125,000	<1	10.7	4.78 (±0.2)
5 min[c]	40,000	19,100	2.0	150,000	1		
	13,000	19,100	2.0	150,000	1	9.6	4.56 (±0.2)
1 hr	40,000	20,100	2.8	85,000	2		
	13,000	20,100	2.8	85,000	2	9.7	4.50 (±0.2)
24 hr[c]	39,460	21,200	3.5	140,000	2		
	13,410	21,200	3.5	140,000	2	13.2	5.88 (±0.3)
24 hr	40,000	22,500	4.0	60,000	3		
	13,000	22,500	4.0	60,000	3	12.8	5.9 (±0.3)
NH_4Cl–$(NH_4)_2SO_4$ precipitate fraction in 0.4 M KCl, pH 7[c]	14,290 (44 hr)					12.0	4.62 (±0.2)
	14,290 (80 hr)					12.1	4.66 (±0.2)
At pH 11.0	44,000	20,100	5.6	108,000	3		
	36,000	20,700	7.5	121,000	2		
	13,000	(20,400)	(6.5)	(115,000)	(2.5)	8.8	4.26 (±0.2)
At pH 11.0	40,000	21,300	8.4		0		
At pH 11.0[c]	40,000	21,300	9.6		0		

[a] Solutions contain 0.001 M dithiothreitol. [b] Native myosin was treated in 4.7 M NH_4Cl at pH 7 for indicated times, and the dialyzed against 0.4 M KCl at pH 7, over 48 hr. [c] Solutions contain 0.001 M EDTA.

Sedimentation equilibrium experiments were performed following dialysis of myosin against 4.0 M LiCl. A representative experiment (Figure 4, left) shows light chains, aggregated light chains (~71,000 average weight), and a heavy component of about 900,000 weight. In three such experiments (Table II), the light chains have an average monomer weight of 20,600, and the total light component (including aggregated material) comprises 11.6 (±1.5)% of myosin; the residual heavy-chain core is aggregated even in the presence of EDTA or dithiothreitol.

After dialysis of LiCl dissociated myosin against 0.4 M KCl, most of the light component reassociates with the heavy chain core. Thus, schlieren patterns (Figure 2e) show complete loss of the trailing component on dialysis against 0.4 M KCl following storage of myosin in 4 M LiCl for as long as 24 hr. Similarly, a sedimentation equilibrium experiment on myosin treated for 5 min in 4 M LiCl and then dialyzed against 0.4 M KCl shows residual dissociation of only 2% aggregated light chains; the reconstituted myosin has a molecular weight on the order of 450,000 (Figure 4, middle). However, prolonged LiCl treatment results in diminished reassociation of light chains with heavy-chain core (Table II), and on dialysis of myosin against 0.4 M KCl after 24-hr treatment in 4 M LiCl, as much as 7% light component remains dissociated, about half of which has aggregated to the 130,000 weight level; the residual myosin is also aggregated (Figure 4, right). The apparent difference in the extent of subunit reassociation as determined by sedimentation velocity and sedimentation equilibrium experiments is presumably related to the higher protein concentration (thereby enhancing subunit reassociation) in sedimentation velocity experiments, and to sedimentation of aggregated light chains as a broad boundary that merges with the schlieren boundaries for monomer light chains ($s^0_{20,w}$ = 2 S) and reconstituted myosin ($s_{20,w}$ < 4 S at protein concentrations above 8 mg/ml).

An attempt was made to characterize the purified heavy-chain core following dissociation of myosin in 4 M LiCl. Myosin was added to an equivalent volume of 8 M LiCl and promptly salted out in 60% saturated potassium citrate–4 M LiCl; the precipitate fraction was redissolved in 4 M LiCl, and again salted out with citrate. Second cycle precipitate was dialyzed against 0.4 M KCl (pH 7) or further dialyzed against 0.1 M Na_2CO_3–0.4 M KCl (pH 11). Dithiothreitol or EDTA was present throughout. Ultracentrifugal experiments (Table II) indicate that the precipitate fraction contains a residual 1–2% light component (21,000 weight) and another 1% aggregated light component, but the heavy-chain core has again aggregated to at least dimer level.

KSCN Solutions. Sedimentation velocity experiments indicate the dissociation of 12.5% light component on

treatment of myosin in 1 M KSCN. The heavy component is characterized by a single sharp boundary at KSCN concentrations up to 4 M, and $s^0_{20,w}$ value (5.9 S) and concentration dependence of sedimentation remain the same when KSCN concentration is increased from 0.5 to 4 M KSCN (Figure 3). A sedimentation equilibrium experiment on myosin dialyzed against 1 M KSCN indicates the presence of approximately 11% light component of average weight 23,600, and a heavy component of about 410,000 molecular weight.

NH_4Cl Solutions. Sedimentation velocity experiments initiated promptly after increase in salt concentration to 4.7 M NH_4Cl indicate the dissociation of approximately 4% light component from native myosin (Figure 1, 5), and the residual myosin has a sedimentation coefficient of 6.5 S (Figure 3). The extent of light-chain dissociation is similar in the presence or absence of 0.001 M EDTA or 0.001 M dithiothreitol. Prolonged NH_4Cl treatment is accompanied by increase in the proportion of dissociation light component to 6% (Figure 5) and aggregation of the residual myosin (Figure 3). Sedimentation equilibrium experiments on myosin dialyzed against 4.7 M NH_4Cl confirm the dissociation of 6% light component and show aggregation of the dissociated light component and the residual myosin (Table III).

Following brief treatment of myosin in 4.7 M NH_4Cl and dialysis against 0.4 M KCl, sedimentation velocity experiments indicate virtual absence of any trailing component, without significant change in the sedimentation properties of the main component (Figure 3). On sedimentation equilibrium (Table III), approximately 3% light component (in part aggregated) remains dissociated from the reconstituted myosin. The aggregation that occurs on prolonged NH_4Cl treatment is irreversible, as indicated by an $s^0_{20,w}$ value of 13 S (Figure 3) and additional leading peaks on sedimentation velocity, and a limiting molecular weight of 590,000 on sedimentation equilibrium (Table III). Similarly, the NH_4Cl-dissociated light component is only slightly reassociated (sedimentation velocity) or not reassociated (sedimentation equilibrium) with the residual myosin after 24-hr treatment in 4.7 M NH_4Cl.

In order to characterize the residual myosin after removal of the NH_4Cl-dissociated light component, myosin in 4.7 M NH_4Cl was salted out with an equivalent volume of saturated $(NH_4)_2SO_4$–4.7 M NH_4Cl, centrifuged at 10,000 rpm for 10 min; and the precipitate was dissolved in and dialyzed against 0.4 M KCl (pH 7). High-speed sedimentation equilibrium reveals that the residual myosin in the precipitate fraction has a monomer weight of 462,000 (±20,000) (Table III). After dialysis of this fraction against 0.4 M KCl–0.1 M Na_2CO_3 (pH 11.0), sedimentation velocity indicates the presence of 8–10% light component, and sedimentation equilibrium indicates a heavy component of 426,000 (±20,000) weight and a light component that comprises 9.2 (±1)% of the protein, with monomer weight 20,700 (Table III).

Electrophoresis of Light Chains of Myosin. Cellulose acetate electrophoresis was performed on light component isolated from myosin by LiCl–citrate fractionation or the alkali dilution method; all purification procedures were done simultaneously in order to exclude possible electrophoretic differences from genetic variations in myosin, denaturation of myosin, or the electrophoretic procedure itself. The experiments indicated four major electrophoretic components and one or two minor electrophoretic components for light chains

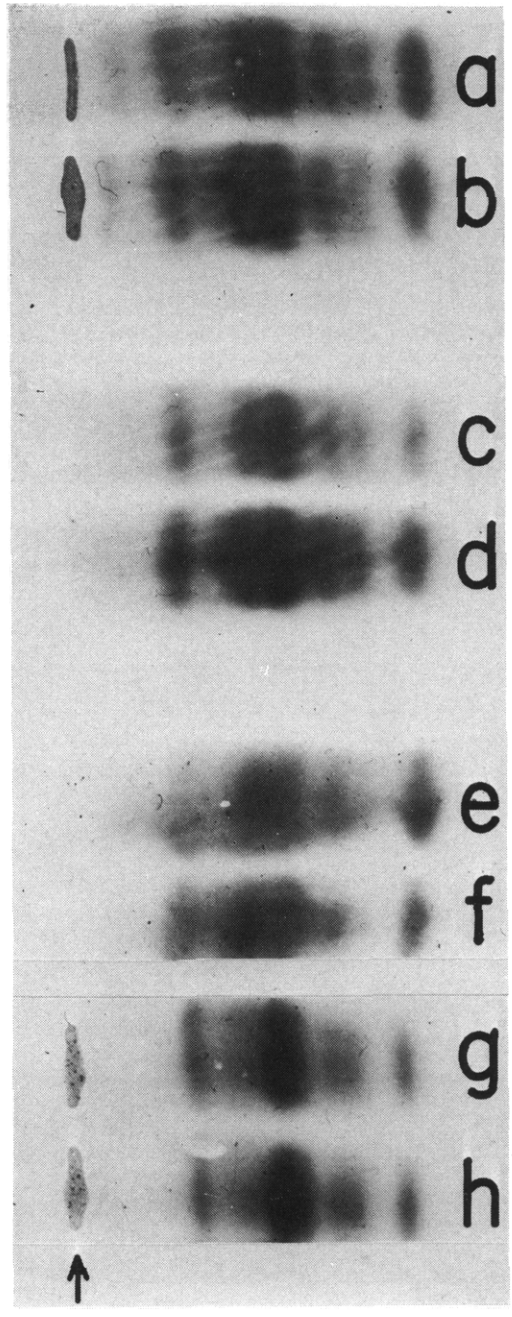

FIGURE 5: Cellulose acetate electrophoresis of myosin light chains. (a, b) 4 M LiCl–citrate salting out; (c, d) pH 11 dilution; (e, f) water-soluble fraction from 4 M LiCl–citrate salting out; (g, h) same as parts a and b, stored 1 day. Anode to right in this and other experiments. Arrow indicates origin.

dissociated from myosin at pH 11 and in 4 M LiCl. Representative electrophoretic patterns from one of the experiments (Figure 5) show the overall similarity in mobility and relative intensity for light chains isolated by the two methods. The LiCl-dissociated light chains also contain a small amount of trailing material and some residual protein at the origin; these components derive from aggregated protein that may be precipitated and removed on further dialysis against water. Following 1-day storage of the LiCl-dissociated light chains in 0.4 M KCl–0.001 M dithiothreitol, there is some blurring

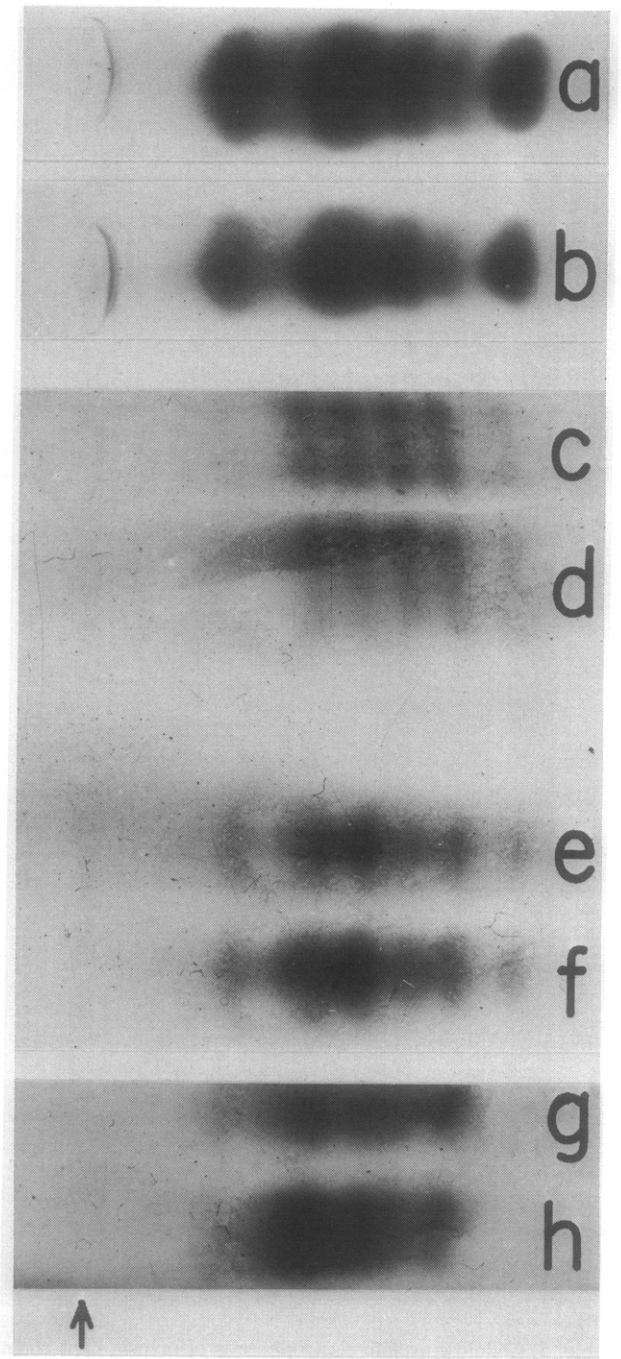

FIGURE 6: Cellulose acetate electrophoresis of myosin light chains. (a, b) 24 hr 2 M guanidine–citrate salting out; (c, d) 1 M KSCN–citrate salting out; (e, f) 2 M KSCN–citrate salting out; (g, h) 24 hr 2 M KSCN–citrate salting out.

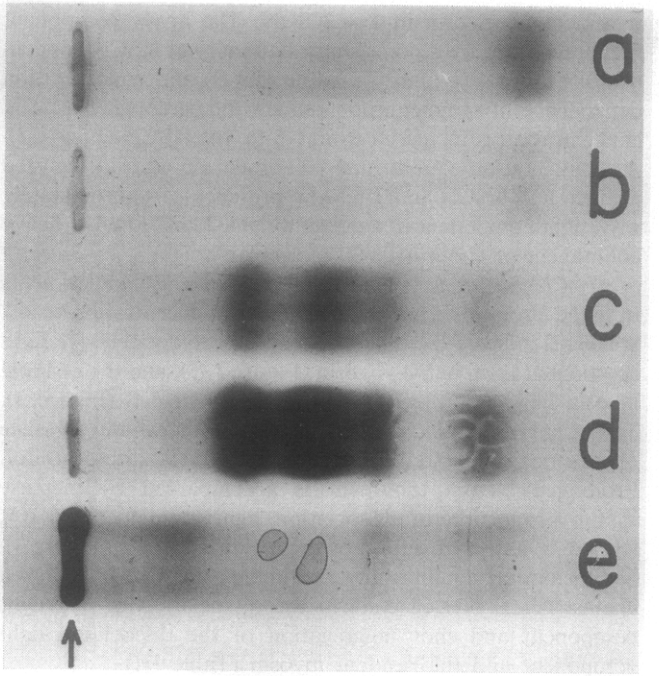

FIGURE 7: Selective dissociation of light component from myosin by 5 min NH_4Cl–50% saturated $(NH_4)_2SO_4$ salting out: (a, b) Supernatant fraction; (c, d) light alkali component from precipitate fraction; (e) residual dissociated light component after 5-min treatment of myosin in 4 M LiCl and dialysis against water.

of the discrete band pattern without formation of additional electrophoretic components (Figure 5g,h).

In order to determine the effect of irreversible denaturation on electrophoretic patterns, the same preparation of myosin was stored for 1 week in 0.4 M KCl–0.005 M $NaHCO_3$–0.001 M dithiothreitol (pH 7) and then treated in 4 M LiCl for 5 min to 72 hr prior to salting out in 60% saturated potassium citrate. On cellulose acetate electrophoresis the supernatant fraction from 5 min LiCl treatment showed loss of the discrete band pattern found 1 week earlier, and the light component isolated by alkali dilution from the 5-min LiCl–citrate precipitate fraction migrated as a slow diffuse band extending to the origin; sedimentation equilibrium of this fraction indicated aggregated light component with molecular weight on the order of 110,000. On prolonged LiCl treatment, there was progressively less fast electrophoretic component in the LiCl–citrate supernatant fraction and concomitant increase in the proportion of aggregated protein in the LiCl–citrate precipitate fraction. Similar experiments on light chains isolated after prolonged alkaline treatment of aged myosin preparations showed transformation of the discrete band pattern into a diffuse poorly defined distribution, without formation of additional discrete bands.

The light component dissociated from myosin on treatment in 2 M guanidine and 1 or 2 M KSCN also yield five or six electrophoretic bands (Figure 6), and prolonged storage in KSCN solutions results in blurring of the band pattern. Although the relative intensities of the different electrophoretic bands vary somewhat from experiment to experiment, the patterns show comparable electrophoretic mobilities following LiCl, KSCN, guanidine, and alkaline dissociation of myosin. As a crude marker, bovine serum albumin migrates faster than the light chains of myosin (Figure 8c).

Electrophoresis was performed on the light components in supernatant and precipitate fractions after treatment of myosin in 4.7 M NH_4Cl and salting out at 50% saturated $(NH_4)_2SO_4$. The supernatant fraction after 5 min NH_4Cl treatment is composed predominantly of protein that migrates in the fast electrophoretic band, whereas the precipitate fraction yields all other electrophoretic bands and some fast component (Figure 7a–d). The same electrophoretic findings

TABLE IV: Fractionation of Myokinase Activity of Native Myosin.

	Myosin		Myosin + 3 moles of Myokinase
	nmoles of P_i/min per mg	nmoles of P_i/min per mole	nmoles of P_i/min per mg
Native protein	0.96	0.45	45
NH_4Cl–$(NH_4)_2SO_4$ fractions			
Precipitate	0.059	0.026	7
Supernatant	26	0.52	200

were obtained repeatedly in each of six different preparations of myosin. Following 4-hr NH_4Cl treatment prior to 50% $(NH_4)_2SO_4$ salting out, the supernatant fraction contains additional protein from one of the middle electrophoretic bands, and the precipitate fraction shows merging of the discrete band pattern (Figure 8e,f); these findings are consistent with ultracentrifugal evidence of progressive light-chain dissociation and irreversible aggregation during prolonged NH_4Cl treatment of myosin (Table III).

There is further evidence for preferential dissociation and reassociation of the light chains of native myosin. About half of the total light component is dissociated from myosin in 3.5 M LiCl, and this fraction contains the fast electrophoretic component and additional protein from one or two slower electrophoretic bands (Figure 8a). Essentially similar findings are obtained for the supernatant fraction after treatment of myosin in 4.7 M NH_4Cl and salting out at 50–100% saturated $(NH_4)_2SO_4$. Also, a small fraction of light component remains dissociated from myosin following treatment in 4 M LiCl and dialysis against 0.4 M KCl (Table II); this fraction yields aggregated material, some fast-migrating protein, and proportionately little protein migrating in the intermediate zone (Figure 7e).

Localization of Myokinase Activity of Myosin. The myosin preparations displayed 0.96 nmole of P_i/min per mg of myokinase activity (Table IV). Samples of myosin were treated in 4.7 M NH_4Cl–0.01 M dithiothreitol for 5 min and salted out at 50% saturated $(NH_4)_2SO_4$; supernatant and precipitate fractions were dialyzed against 0.5 M KCl–0.1 M Tris–0.001 M dithiothreitol (pH 7.5). The supernatant fraction retained 26 nmoles of P_i/min per mg of myokinase activity, approximately the entire myokinase activity of native myosin on a molar basis, while the precipitate fraction retained only a small proportion of the original myokinase activity (Table IV).

Myokinase (Sigma) was added to native myosin in 3:1 molar ratio, and this mixture exhibited myokinase activity approximately 50-fold greater than native myosin (Table IV). On NH_4Cl–$(NH_4)_2SO_4$ fractionation of the myokinase–myosin mixture, most of the myokinase activity was recovered in the supernatant fraction, which exhibited nearly 10-fold greater activity than the supernatant fraction isolated from native myosin alone.

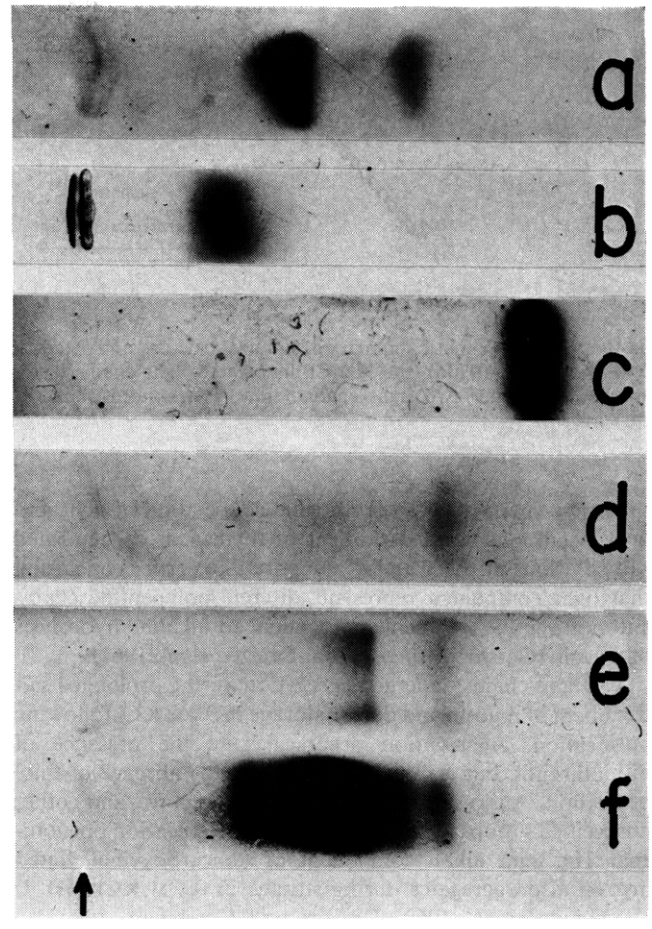

FIGURE 8: Cellulose acetate electrophoresis. (a) Supernatant from 3.5 M LiCl–citrate salting out of myosin; (b) rabbit skeletal myokinase (Sigma); (c) bovine serum albumin (Armour); (d) supernatant from 5 min NH_4Cl–50% saturated $(NH_4)_2SO_4$ salting out of myosin; (e, f) supernatant and precipitate, respectively, from 4 hr NH_4Cl–50% saturated $(NH_4)_2SO_4$ salting out of myosin.

On cellulose acetate electrophoresis (Figure 8b), rabbit skeletal myokinase migrates more slowly than the fast electrophoretic component that comprises the predominant part of the NH_4Cl–$(NH_4)_2SO_4$ supernatant fraction.

Discussion

Approximately 12% light component of average molecular weight about 20,600 is dissociated from the heavy-chain core on treatment of myosin in 4 M LiCl and 1 M KSCN, pH 7, 4°. The stoichiometry indicates 2.7 light chains on the average per myosin molecule (468,000 weight), and is in agreement with the previous data on the dissociation of myosin at pH 11 and in 2 M guanidine (Gershman *et al.*, 1966, 1969) (Figure 9). Dialysis of salt-dissociated myosin against 0.4 M KCl is accompanied by almost complete reassociation of light chains with the heavy-chain core, provided that salt treatment was short and thiol groups were protected throughout. For example, following 5-min treatment of myosin in 4 M LiCl, only 2% of aggregated light chains remain dissociated from the renatured myosin, and electrophoretic experiments confirm that the major middle bands have reassociated with

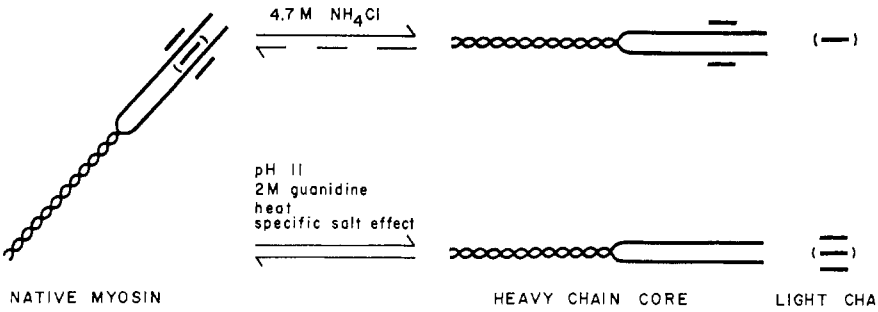

FIGURE 9: Diagrammatic summary of subunit structure and interactions of rabbit skeletal myosin. As shown in the following paper (Dreizen and Gershman, 1970b), the NH_4Cl-dissociated light chain (fast electrophoretic band) is not essential for Ca-ATPase or ADP binding, whereas the other two light chains (slow electrophoretic bands) appear to be essential for the Ca-ATPase and the ADP binding of myosin.

the heavy-chain core. The reversible dissociation of light and heavy components on treatment of myosin in concentrated salt solutions at pH 7 and 4° supports the earlier conclusion that light component represents discrete polypeptide chains and not peptide fragments consequent to alkaline hydrolysis of myosin (Gershman *et al.*, 1966; Dreizen *et al.*, 1967).

The light chains undergo aggregation during prolonged salt treatment of myosin and during storage in 0.4 M KCl following purification. Aggregation occurs despite the presence of dithiothreitol, but is clearly enhanced in the absence of thiol protection, suggesting that sulfhydryl groups and other unspecified groups are implicated in the aggregation phenomena. The light alkali component of noncarboxymethylated myosin also aggregates during storage in 0.4 M KCl (pH 7) (Gershman *et al.*, 1969; Dreizen and Gershman, 1970a,b), and we have suggested that aggregation of light chains might possibly account for the molecular weight values of 29,000 to 33,600 that were determined by Frederiksen and Holtzer (1968) from viscosity and light-scattering measurements on the purified light alkali component.

Following LiCl–citrate fractionation of myosin, the purified heavy-chain core aggregates to at least dimer level, despite brief LiCl exposure and the presence of dithiothreitol. Comparable aggregation was found after alkaline and guanidine purification of the heavy-chain core, with or without prior carboxymethylation or the presence of dithiothreitol (Gershman *et al.*, 1966, 1969; Dreizen *et al.*, 1967); the present data are in accord with the interpretation that removal of light chains may uncover sites on the heavy-chain core than enter into aggregation phenomena, *via* disulfide bridges and/or other covalent bonds (Gershman *et al.*, 1969).

The basis of salt denaturation of proteins has been controversial (Gordon and Jenks, 1963; von Hippel and Wong, 1965), and more than one phenomenon would appear to be involved in the effect of salt on myosin. Salts acting *via* a predominant cationic effect lead to change in the sedimentation properties of the heavy-chain core at salt concentrations above the transition for light-chain dissociation. Thus, there is virtual loss of the concentration dependence of sedimentation and increase in the $s^0_{20,w}$ value of the heavy-chain core on increase from 4 to 5 M LiCl. The changes are largely reversible on dialysis of LiCl-treated myosin against 0.4 M KCl–0.001 M dithiothreitol, and presumably reflect a marked structural change of the heavy-chain core during LiCl treatment. Comparable changes (not studied in as great detail) were found on treatment of myosin with other Li^+ salts (Table I), $CaCl_2$, and $MgCl_2$. In contrast, those salts acting *via* a predominant anionic effect (KSCN, for example) do not significantly affect the sedimentation properties of the heavy-chain core, even at ionic strength well above the transition for light-chain dissociation. The anionic series for light-chain dissociation (Table I) is essentially identical with the series for preferential anion binding by bovine serum albumin (Scatchard and Black, 1949) and myosin (Ghosh and Mihályi, 1952). One might suppose that preferential anion binding to lysyl or other basic residues may alter electrostatic charges on light and heavy chains in the region of their contact zone and thereby disrupt subunit interactions without drastic change in the heavy-chain core, at least, as manifest by sedimentation properties. This kind of effect would be comparable in a way with the effect of alkali on myosin, in which case proton ionization from lysyl or other basic residues at pH 10.5 leads to light-chain dissociation without significant change in the sedimentation properties of the heavy-chain core (Gershman *et al.*, 1966, 1969).

Light-Chain Heterogeneity. In previous studies, the light component that was isolated from carboxymethylated myosin after dialysis to pH 11 yielded three or four bands on cellulose acetate electrophoresis, whereas the light component from identically treated but noncarboxymethylated myosin yielded two poorly defined electrophoretic bands (Gershman *et al.*, 1966). Locker and Hagyard (1967a) reported electrophoretic heterogeneity of light component isolated from acetylated myosin and light component acetylated following isolation from myosin, and Oppenheimer *et al.* (1967) have described extensive heterogeneity following succinylation of myosin. Nevertheless, it remained uncertain whether the electrophoretic heterogeneity of carboxymethylated (or otherwise modified) light component represented structural differences in the light chains or merely charges differences in the number of chemically modified groups; and, indeed, Weeds (1967) has reported that the light chains show similarity in peptide maps and identical thiol sequences, suggesting their derivation from a single precursor. The present experiments are thus significant in demonstrating that light component dissociated from unmodified myosin at pH 11, in 4 M LiCl, in 1 or 2 M KSCN, and in 2 M guanidine is in all instances electrophoretically heterogeneous on cellulose acetate, with four major bands and one or two minor bands of comparable mobility. Although the mobility of proteins on cellulose acetate is not subject

to precise analysis, electrophoretic experiments on human oxyhemoglobins A and S under identical conditions would suggest that the light-chain bands differ by increments of one or possibly two charges per light chain.

It is unlikely that the major light-chain bands are artifacts of denaturation. A discrete band pattern is obtained reproducibly on use of fresh myosin preparations, rapid alkaline or salt treatment, thiol protection, and initiation of electrophoresis immediately after light-chain purification. On the other hand, there is merging of the band pattern and increase in the trailing material on use of myosin preparations older than 1 week, prolonged alkaline or salt treatment, or lengthy storage of purified light chains, especially without thiol protection. The minor variations in mobility and relative intensity of the different electrophoretic components appear to have a complex origin, including effects from aggregation, light-chain heterogeneity in different skeletal muscles (Locker and Hagyard, 1967c), and selective dissociation of light chains under borderline dissociating conditions.

A part of the light component is dissociated from native myosin in 4.7 M NH_4Cl. Short NH_4Cl treatment of native myosin leads to irreversible dissociation of about 3% light component, that has a molecular weight of approximately 20,700 (±1,000) on sedimentation equilibrium and thus comprises 0.7 (±0.2) mole of light chain/mole of myosin. The light component dissociated on short NH_4Cl treatment is composed predominantly of protein migrating as a fast electrophoretic band, but prolonged NH_4Cl treatment (or treatment in 3.5 M LiCl) also leads to dissociation of protein from one or more of the middle electrophoretic bands.

The present data confirm that rabbit skeletal myosin exhibits trace myokinase activity (Richards *et al.*, 1967). Although rabbit skeletal myokinase has 21,000 molecular weight (Noda and Kuby, 1957), this protein does not comprise a major fraction of the myosin light component, since myokinase and the light chains differ with respect to C-terminal group (Olson and Kuby, 1964; Kominz *et al.*, 1959; Gershman *et al.*, 1966), amino acid analysis (Mahowald *et al.*, 1962; Kominz *et al.*, 1959; Locker and Hagyard, 1967b), and sulfhydryl peptides (Weeds, 1967). The trace myokinase activity of myosin preparations is recovered in the supernatant fraction from NH_4Cl–$(NH_4)_2SO_4$ salting out. Myokinase migrates more slowly on cellulose acetate than the light chains of myosin, a finding consistent with the appreciably higher ratio of acidic to basic residues in the myosin light component (Kominz *et al.*, 1959; Locker and Hagyard, 1967b) than in myokinase (Mahowald *et al.*, 1962). It thus appears that myokinase activity derives from a trace contaminant in myosin that is fractionated on NH_4Cl–$(NH_4)_2SO_4$ salting out, and is not to be identified with the fast electrophoretic band that comprises most of the NH_4Cl-dissociated light component.

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