

CHICK BRAIN ACTIN AND MYOSIN

Isolation and Characterization

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ABSTRACT

Brain actin extracted from an acetone powder of chick brains was purified by a cycle of polymerization-depolymerization followed by molecular sieve chromatography. The brain actin had a subunit molecular weight of 42,000 daltons as determined by co-electrophoresis with muscle actin. It underwent salt-dependent g to f transformation to form double helical actin filaments which could be "decorated" by muscle myosin subfragment 1. A critical concentration for polymerization of 1.3 μM was determined by measuring either the change in viscosity or absorbance at 232 nm. Brain actin was also capable of stimulating the ATPase activity of muscle myosin. Brain myosin was isolated from whole chick brain by a procedure involving high salt extraction, ammonium sulfate fractionation and molecular sieve chromatography. The purified myosin was composed of a 200,000-dalton heavy chain and three lower molecular weight light chains. In 0.6 M KCl the brain myosin had ATPase activity which was inhibited by Mg^{++} , stimulated by Ca^{++} , and maximally activated by EDTA. When dialyzed against 0.1 M KCl, the brain myosin self-assembled into short bipolar filaments. The bipolar filaments associated with each other to form long concatamers, and this association was enhanced by high concentrations of Mg^{++} ion. The brain myosin did not interact with chicken skeletal muscle myosin to form hybrid filaments. Furthermore, antibody recognition studies demonstrated that myosins from chicken brain, skeletal muscle, and smooth muscle were unique.

KEY WORDS microfilaments · myosin ·
brain · antibody

Since the pioneering work of Hatano and Oosawa in 1966 (29), the contractile proteins actin and myosin have been isolated from a variety of nonmuscle sources including amoeba, blood platelets, leucocytes, fibroblasts, sperm, and even plants (for review, see references 14, 59). Actins from muscle and nonmuscle sources have shown remarkable similarities: both have similar molecular weights and amino acid compositions, form polymers in the presence of salt, decorate with

HMM, and activate myosin Mg-ATPase activity (38, 59). Likewise, nonmuscle or cytoplasmic myosins have important functional properties which resemble those of muscle myosin, including the ability to form bipolar filaments, to bind to actin, and ATPase activity which can be stimulated by K^+ -EDTA (59).

One cell type having several different motile functions is the nerve cell. During embryogenesis, nerve cell processes move over long distances to form synaptic contacts, and, once connections are established, materials synthesized in the cell body are transported the length of the neuron by a

process known as axoplasmic transport (16, 28, 41). It is quite likely that both actin and myosin are involved in these and other (35) neuronal functions.

In 1968, Puszkin, Berl, and co-workers (5, 61, 62) began investigations of actin and myosin in mammalian brain. Using techniques established for the purification of muscle proteins, they succeeded in isolating a protein complex which resembled muscle actomyosin. Cell fractionation techniques were subsequently used to demonstrate that a large amount of the actomyosin was associated with the nerve ending or synaptosomal fraction (6, 63). Peptide mapping techniques have been used by other researchers to demonstrate the presence of both actin (22) and tropomyosin (21) in cultured chicken dorsal root ganglion nerve cells.

The present studies were designed to characterize brain actin and myosin of higher purity than heretofore available. This paper deals with the isolation and characterization of these proteins, while the accompanying work describes the localization of actin and myosin in nerve cells. Some of the results described here have been published in abstract form (38, 39, 40).

MATERIALS AND METHODS

Protein Purification

ACETONE POWDER: Whole brains from 1-d-old chicks were minced, rinsed with 0.15 M NaCl, 10 mM imidazole, pH 7.0, and extracted with 10 vol (vol/wt) of acetone at room temperature for 10 min. After filtration on a Büchner funnel, the residue was re-extracted three times with acetone. The final acetone powder was air-dried overnight and then stored at -18°C .

BRAIN ACTIN ISOLATION: Brain actin was isolated from the acetone powder by a modification of the 0.6 M KCl method of Spudich and Watt (70). Modifications included a shorter extraction time (10 min at 0°C) and polymerization in 100 mM KCl and 1 mM MgCl_2 . In addition, the final g-actin was chromatographed on a 2.6×60 cm Sephadex G-200 column equilibrated in Buffer A (2 mM Tris-Cl buffer, pH 8.0, 0.2 mM ATP, 0.5 mM mercaptoethanol, 0.2 mM CaCl_2), with a 20-cm pressure head.

BRAIN MYOSIN ISOLATION: Brain myosin was prepared by the KI method described by Pollard et al. (57). Modifications included homogenization in extraction buffer (size C Thomas glass homogenizer [Thomas Tool & Die Inc., Sun Valley, Calif.] with motor-driven Teflon pestle) and altered limits for ammonium sulfate fractionation (1.2–2.0 M fraction). Briefly, this method involves preparation of an actomyosin fraction by low ionic strength precipitation and ammonium sulfate frac-

tionation. Myosin is then separated from actin and other smaller proteins by chromatography on a Biogel A-15m column (Bio-Rad Laboratories, Richmond, Calif.) in the presence of KI.

MUSCLE PROTEINS: Skeletal muscle myosins were prepared from adult chicken breast muscle or rabbit back muscle by the method of Kielley and Harrington (37). The residue remaining after extraction of myosin was used to prepare an acetone powder (76) from which actin was extracted by the method of Spudich and Watt (70). Smooth muscle myosin was isolated from chicken gizzard by the method of Barany et al. (3). Myosin subfragment S_1 was prepared from rabbit muscle myosin by the method of Margossian and Lowey (48).

Actin Polymerization

VISCOMETRY: Polymerization of actin was monitored viscometrically with a no. 100 Cannon-Manning semi-micro viscometer (Cannon Instrument Co., State College, P. A.) having an outflow time for Buffer A of 66.3 seconds at 21°C . Brain g-actin was polymerized by addition of KCl to 75 mM and MgCl_2 to 2 mM.

ABSORBANCE AT 232 NM: The rate and extent of actin polymerization was also assayed at 21°C by monitoring the increase in absorbance at 232 nm. This method has been used by Spudich and Cooke (72) to study the polymerization of *Dictyostelium* actin and is based on the original observations of Higashi and Oosawa (31) that actin polymerization is accompanied by a significant change in absorbance at 232 nm. To initiate polymerization, 1-ml of g-actin in Buffer A was rapidly added to a 1.3-ml quartz cuvette (1-cm path length) which contained 0.025 ml of 3 M KCl and 80 mM MgCl_2 . This resulted in a final salt concentration for polymerization of 75 mM KCl, 2 mM MgCl_2 . The reaction was monitored with a Gilford recording spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio). No correction was made for absorbance as a result of turbidity, since turbidity contributed $<20\%$ to the A_{232} (unpublished observations and reference 15).

ATPase

Myosin ATPase reactions were carried out at 37°C in 0.6 M KCl, 10 mM imidazole, pH 7.0, with either 2 mM Mg^{++} , 1 mM Ca^{++} , or 2 mM ethylenediamine tetraacetic acid (EDTA) and a substrate concentration of 2 mM ATP. Actin activation assays were performed in 15 mM KCl, 5 mM imidazole, pH 7.0, 1 mM MgCl_2 , and 1 mM ATP. Reactions were initiated by the addition of myosin, and were linear for the times used. Inorganic phosphate was determined colorimetrically by the method of Martin and Doty (49) as modified by Pollard and Korn (56).

Protein Determination

Protein concentration was determined by the modified method of Lowry et al. (46) described by Schacterle and

Pollack (66). A standard bovine serum albumin solution (Pentex Biochemical, Kankakee, Ill.) was used for calibration.

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Fairbanks et al. (19) with 5% disk or slab gels. Protein samples were heated to 100°C for 2–3 min before being applied to the gels. Electrophoresis was carried out for 3–4 h (6 mA/disk gel or 80 mA/140 × 110 × 2 mm slab gel).

Immunology

Antibodies to purified chicken brain, gizzard, and breast myosins were raised in young New Zealand rabbits. 1–2 mg of protein in 0.6 M KCl were homogenized in an equal volume of Freund's complete adjuvant and injected into the rear foot pads and leg muscles. The same amount of protein in incomplete adjuvant was injected 21 d later, and the rabbits were bled 7 d after the second injection.

Ouchterlony double-diffusion reactions were performed in 1.0% agarose in 0.3 M KCl, 10 mM imidazole, pH 7.0. The reaction was carried out in a moist chamber at room temperature for 48 h. Gels were washed overnight in buffer, briefly washed in H₂O, and photographed with indirect illumination.

Electron Microscopy

NEGATIVE STAINING: A drop of protein solution was placed on a carbon-over-Formvar-coated 300-mesh copper grid and was rinsed after 30 s with several drops of buffer. After rinsing with two drops of 0.02% cytochrome *c* in 1% amyl alcohol (50), the specimen was negatively stained with 1% aqueous uranyl acetate and examined in a Philips 201 electron microscope operated at 60 KV.

PARACRYSTALS: Actin paracrystals were formed by dialyzing f-actin against buffer containing 5 mM Tris, 0.1 mM CaCl₂, 0.25 mM ATP, and 50 mM MgCl₂, pH 7.6 (71). Myosin paracrystals were induced by dialysis against 0.1 M KCl, 10 mM imidazole, pH 7.0, 25 mM MgCl₂ (58).

S₁ DECORATION OF ACTIN: A drop of f-actin was placed on a grid and rinsed after 30 s with several drops of 0.1 M KCl, 10 mM imidazole, pH 7.0. A drop of S₁ solution (0.3 mg/ml in 0.1 M KCl, 10 mM imidazole, pH 7.0) was added to the grid and allowed to react for 45 s at room temperature. After washing with 0.1 M KCl, the grid was negatively stained as described above.

RESULTS

Purification of Brain Actin

The low ionic strength Buffer A extracted several proteins from the acetone powder of chick

brain, with the major protein migrating at 42,000 daltons on SDS-PAGE. Addition of KCl and MgCl₂ to the low ionic strength extract caused the assembly of some of the brain protein which could then be collected by centrifugation. Like muscle actin, the sedimented protein could be depolymerized by dialysis against Buffer A. The composition of this depolymerized protein after clarification by centrifugation is shown in Fig. 1*b*. The 42,000-dalton species comprised 53% of the protein at this state of purification, representing <10% of the total amount of 42,000-dalton protein originally extracted from the acetone powder.

Electron microscopic analysis of this preparation revealed many amorphous aggregates (Fig. 2*a*). Upon the addition of KCl and Mg⁺⁺, bundles of 5–6-nm filaments were formed (Fig. 2*b*). In addition to the filament bundles, small spool-like particles were seen in the background (see also Fig. 3*b*, insert). To obtain a purer preparation, the above material was fractionated by Sephadex G-200 chromatography. Two protein peaks were obtained (Fig. 1*a*): the first peak coincided with the void volume and contained several proteins ranging in molecular weight from 12,000 to 96,000 daltons (Fig. 1*c*); the second peak contained the 42,000-dalton protein which comprised ~90% of this fraction (Fig. 1*d*).

Electron microscopy of Peak I revealed aggregated material (Fig. 3*a*), similar to that shown in Fig. 2*a*. However, after addition of salt to Peak I (0.1 M KCl, 1 mM MgCl₂), large numbers of discrete spool-like structures could be observed (Fig. 3*b*). These structures appeared as hollow cylinders measuring 11 × 15 nm. Viewed end-on, they formed a ring which seemed to be composed of globular subunits. These rings had an o.d. of 14 nm and a central lumen 5 nm in diameter.

Peak II, which contained the purified 42,000-dalton protein, had no obvious structure when examined in the electron microscope. After addition of KCl and Mg⁺⁺, large numbers of single filaments similar to muscle actin, as well as bundles of filaments, were seen (Fig. 3*c*). The further characterization of this 42,000-dalton protein as brain actin is described below. In all of these studies, the highly purified actin (Peak II material) was used.

Characterization of Brain Actin

MOLECULAR WEIGHT OF BRAIN ACTIN: Purified brain actin co-electrophoresed

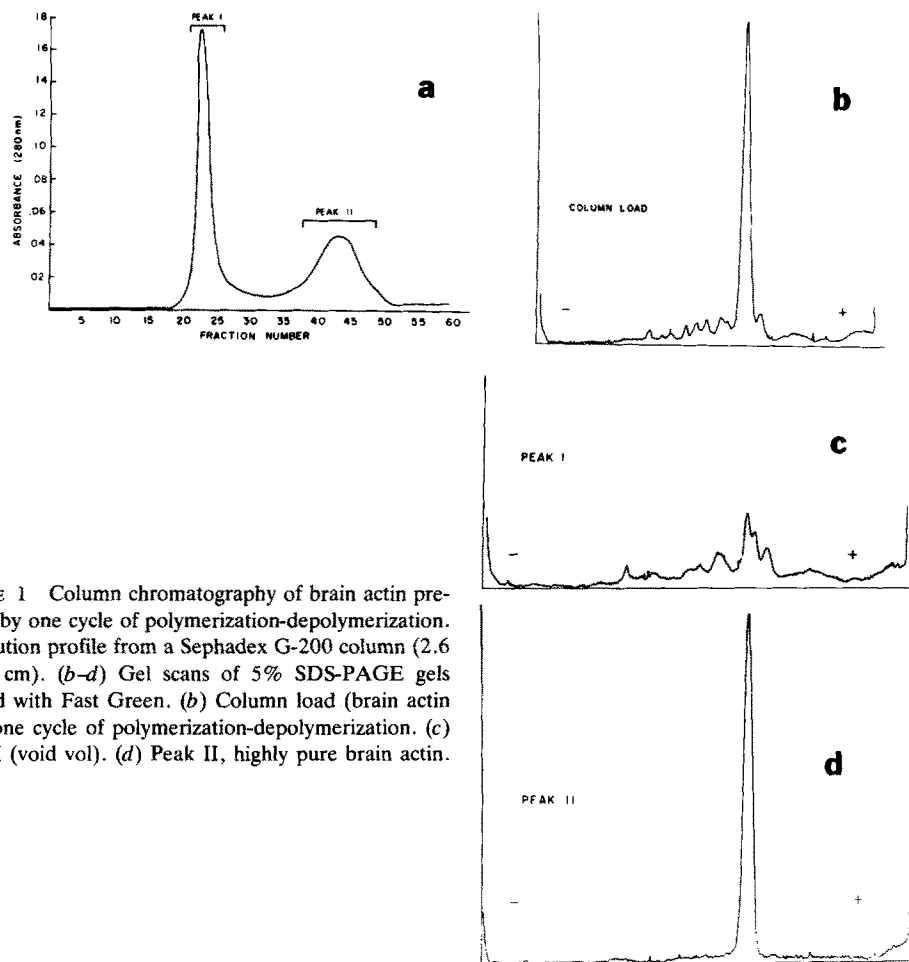


FIGURE 1 Column chromatography of brain actin prepared by one cycle of polymerization-depolymerization. (a) Elution profile from a Sephadex G-200 column (2.6 × 60 cm). (b-d) Gel scans of 5% SDS-PAGE gels stained with Fast Green. (b) Column load (brain actin after one cycle of polymerization-depolymerization). (c) Peak I (void vol). (d) Peak II, highly pure brain actin.

with breast actin at 42,000 daltons on 5% SDS polyacrylamide gels.

CRITICAL CONCENTRATION OF ACTIN POLYMERIZATION: The g to f transformation of purified brain actin was demonstrated quantitatively by the change in absorbance at 232 nm. Addition of salt (75 mM KCl, 2 mM MgCl₂) to the globular actin caused a change in A₂₃₂, indicating the formation of actin polymer (Fig. 4). When monitored viscometrically, a rapid rise in specific viscosity was also observed.

The critical concentration for brain actin polymerization was 0.06 mg/ml, higher than the value of 0.025 mg/ml determined for muscle actin (Fig. 5a). To insure that the changes measured by A₂₃₂ represented polymer formation, the critical concentration for brain actin polymerization was also determined by viscometry (Fig. 5b). The value obtained by this method was 0.05 mg/ml.

DECORATION OF ACTIN FILAMENTS WITH S₁: Brain actin filaments interacted with muscle myosin subfragment S₁ to form "arrowhead" structures with a characteristic repeat of 35 nm (Fig. 6). The arrowheads could be removed by washing the grid in buffer containing 1 mM ATP.

BRAIN ACTIN MG PARACRYSTALS: Purified brain actin assembled into paracrystals when dialyzed against buffer containing 50 mM MgCl₂. Paracrystals obtained from chicken brain and breast actin exhibited the characteristic 35-nm banding pattern resulting from alignment of the helical cross-over points of the individual actin filaments (Fig. 7).

ACTIN ACTIVATION OF MYOSIN MG-ATPASE: Actin isolated from the chicken brain activated the Mg-ATPase of chicken breast muscle myosin, as illustrated in Fig. 8A. With increasing

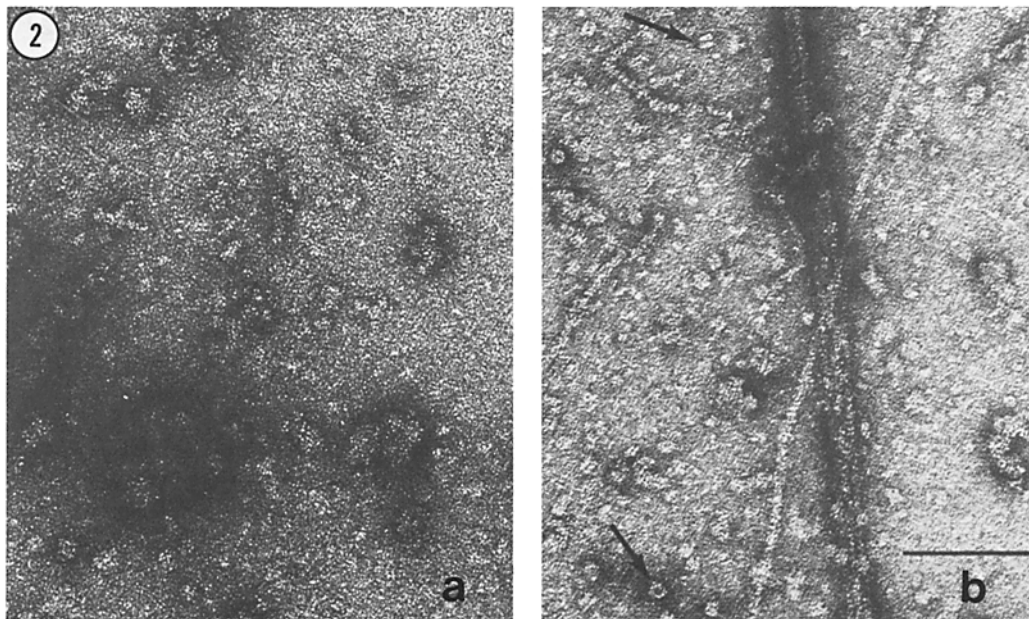


FIGURE 2 Negative stain of brain actin purified by one cycle of polymerization-depolymerization (G-200 column load). (a) Protein in Buffer A appeared amorphous. (b) Protein after addition of 75 mM KCl, 2 mM MgCl₂. Note actin filaments and spool-like structures (arrows). Bar, 0.1 μ m. \times 175,000.

amounts of brain actin, there was a linear increase in ATPase activity of the skeletal muscle myosin. In the homologous system using breast actin and breast myosin, a much greater activation was achieved (Fig. 8B).

Purification of Brain Myosin

The crude extract obtained from 70 day-old chick brains had a low K⁺-EDTA ATPase specific activity (Table I) and contained many proteins (Fig. 9A). Crude actomyosin was separated from the extract by lowering the pH and ionic strength (Fig. 9B). This step, as well as the subsequent ammonium sulfate fractionation, enriched for brain myosin as indicated by the increased K⁺-EDTA ATPase activity and decreased Mg⁺⁺-stimulated ATPase activity (Table I).

The 1.2–2.0 M ammonium sulfate fraction, which contained substantial amounts of actin and myosin (Fig. 9C), was fractionated by Biogel A 15m chromatography. The brain myosin, which eluted shortly after the void volume, was identified as a peak of K⁺-EDTA ATPase activity. This peak was concentrated by pressure dialysis to yield 3 mg of purified brain myosin (0.07% of the total brain protein) which had a K⁺-EDTA ATPase specific activity of 0.37 mol PO₄/mg myosin/min.

In a typical preparation, the myosin was >80% pure, as determined by densitometry of SDS-PAGE gels.

Characterization of Brain Myosin

SUBUNIT COMPOSITION: SDS-PAGE of the purified brain myosin revealed a 200,000 mol wt species which co-electrophoresed with the heavy chain of skeletal muscle myosin (Fig. 9). In addition to the heavy chain, the brain myosin contained three light chains. The molecular weights of these polypeptides, estimated from their mobilities relative to marker proteins of known molecular weight, were 16,000, 18,000, and 21,000 daltons. The 16,000- and 21,000-dalton light chains were consistently seen in each preparation, while the presence of the 18,000-dalton chain was somewhat variable. On a heavily loaded gel, however, the 18,000-dalton chain could always be detected (Fig. 9E).

ATPASE ACTIVITY: In Table II, the ATPase specific activities of chicken brain, breast, and gizzard myosins in 0.6 M KCl are compared. Skeletal myosin (17) as well as some cytoplasmic myosins (2, 13, 47, 59) exhibit increased Mg-ATPase activity at low ionic strength when actin is added (actin activation). The purified brain

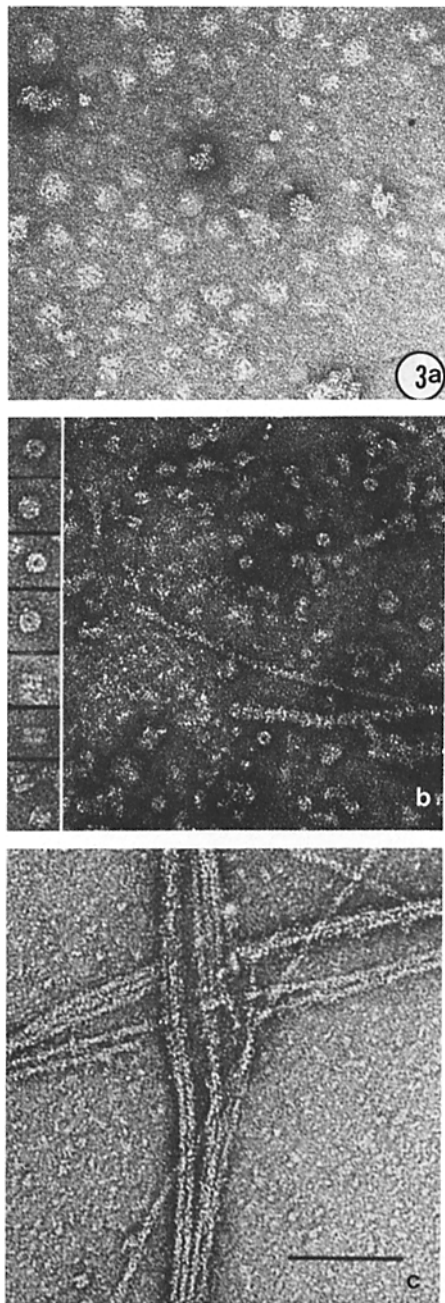


FIGURE 3 Sephadex G-200 fractionated proteins, negatively stained with uranyl acetate. (a) Peak I proteins in Buffer A appeared amorphous. (b) Peak I proteins after addition of 75 mM KCl, 2 mM $MgCl_2$. Note appearance of spool-shaped structures which occasionally formed long concatamers. (inset) Higher magnification of the spool-like structures seen in side and end-on views. (c) Peak II (actin) protein after addition of 75 mM KCl, 2

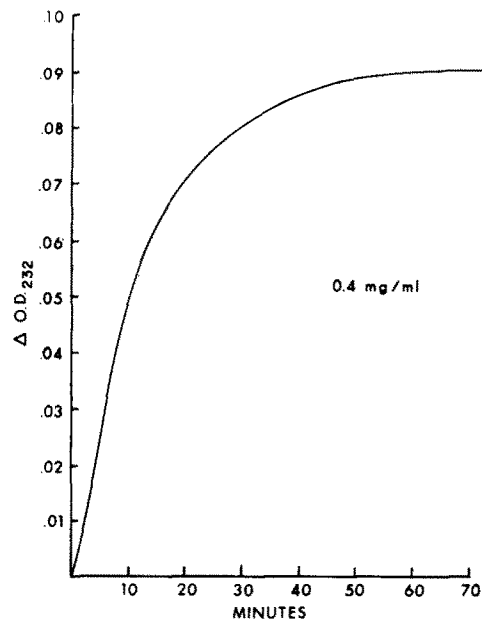


FIGURE 4 Salt-induced polymerization of brain actin as a function of time. 1 ml of brain actin (0.4 mg/ml) was polymerized by addition of KCl (75 mM) and $MgCl_2$ (2 mM). Change in A_{232} as a function of time.

myosin, however, showed no actin activation with either brain or breast actin.

FILAMENT FORMATION: In low ionic strength, brain myosin self-assembled to form bipolar filaments (Fig. 10a, inset). These bipolar filaments resulted from interactions between the "tail" portions of individual myosin molecules. The "head" regions of the molecules formed the fuzzy ends of individual bipolar filaments. The filaments averaged $0.3 \mu m$ in length and had a central bare zone of $0.15 \mu m$. There was a strong tendency for individual bipolar filaments to interact with each other. End-to-end associations of these bipolar filaments were frequently observed in the electron microscope (Fig. 10a), and lateral associations among bipolar filaments were occasionally seen (Fig. 10b). Addition of 25 mM $MgCl_2$ to the dialysis buffer induced the formation of brain myosin "paracrystals." Fig. 11a illustrates the periodic nature of these structures. At higher magnification, it could be seen that these myosin Mg-paracrystals were composed of many

mM $MgCl_2$ formed long 6-nm wide filaments which also associated to form bundles of filaments. Bar, $0.1 \mu m \times 152,000$.

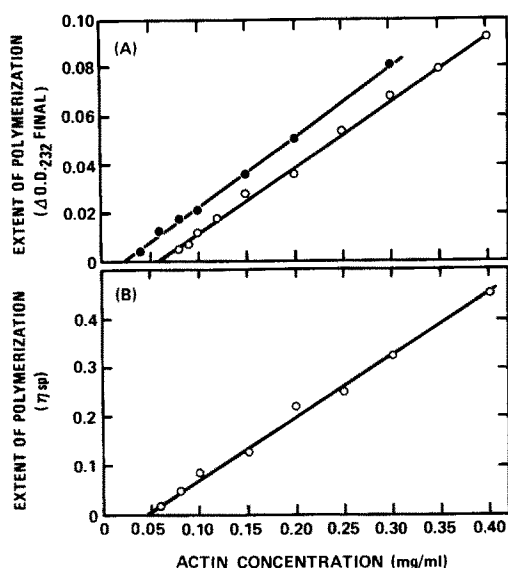


FIGURE 5 Degree of polymerization of actin as a function of actin concentration. (a) Purified chicken breast muscle g-actin (●) and column purified chicken brain g-actin (○) were polymerized by the addition of KCl (75 mM) and $MgCl_2$ (2 mM). The extent of polymerization represents the total change in A_{232} after completion of polymerization. Point of intersection with the abscissa indicates critical concentration for polymerization; brain actin = $1.4 \mu M$, breast actin = $0.6 \mu M$. (b) Polymerization of brain actin as measured by viscometry. Each point represents the total change in viscosity after completion of polymerization. Critical concentration for polymerization = $1.2 \mu M$. ($N_{sp} = N_{rel} - 1$).

individual bipolar aggregates arranged end-to-end and side-to-side (Fig. 11b).

FORMATION OF HYBRID BIPOLAR FILAMENTS: Since it was recently demonstrated that myosin molecules isolated from heterologous sources interact to form hybrid filaments (33, 60), similar studies were carried out to investigate the behavior of brain myosin. Purified chicken brain, breast, and gizzard myosins were dialyzed separately against 0.1 M KCl, 10 mM imidazole, pH 7.0. The average lengths of the filaments were $0.3 \mu m$ for both the brain and gizzard myosins, while breast myosin assembled into much larger filaments having an average length of $1.3 \mu m$. Dialysis of a 1:1 mixture of breast and gizzard myosins resulted in the formation of a single class of intermediate length filaments. These hybrid filaments were $0.35 \mu m$ long, slightly larger than pure gizzard myosin filaments ($0.30 \mu m$), but substantially smaller than the $1.3 \mu m$ breast

myosin filaments.

Brain myosin, on the other hand, did not interact with skeletal muscle myosin. A 1:1 mixture of brain and breast myosins dialyzed against the 0.1 M KCl buffer gave rise to two distinct classes of filaments rather than hybrid filaments of intermediate length. The brain myosin formed characteristic $0.3 \mu m$ bipolar filaments, seen in Fig. 12 as large aggregates as a result of the strong head-to-head interactions of the individual filaments. In the background of the same figure, tapered $1.3 \mu m$ filaments of skeletal muscle myosin are visible. Under these conditions, then, the brain and breast myosins did not interact to form hybrid structures of altered length.

Antibodies to Myosin

Fig. 13A shows the strong reaction between the anti-brain myosin serum and the brain myosin antigen. There was no cross reaction with chicken smooth (gizzard) muscle myosin or with skeletal muscle myosin from either chicken or rabbit. The antibody to gizzard myosin only reacted with the gizzard myosin antigen; there was no cross reaction with the other chicken myosins or with the rabbit skeletal myosin (Fig. 13B). Similarly, anti-

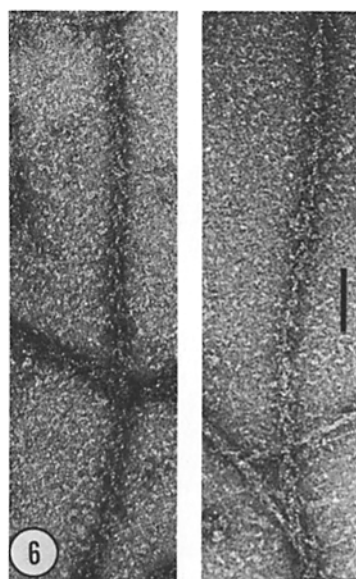


FIGURE 6 Brain actin (G-200 Peak II) decorated with S_1 . Brain actin polymerized in 0.1 M KCl, 1 mM $MgCl_2$ was reacted with rabbit muscle myosin S_1 to form characteristic "arrowhead" associations. Bar, $0.1 \mu m$. $\times 86,000$.

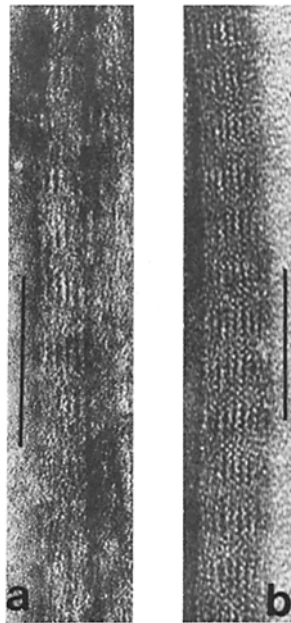


FIGURE 7 Actin paracrystals. g-actin (0.5 mg/ml) was dialyzed overnight against 5 mM Tris, 0.1 mM CaCl_2 , 0.25 mM ATP, and 50 mM MgCl_2 , pH 7.6, to induce the formation of paracrystals. Cross striations with a repeat distance of 35 nm are a result of the parallel alignment of individual actin filaments having their helical cross-over repeats in register. (a) Chicken brain actin. (b) Chicken breast actin. Bar, 0.1 μm . (a) $\times 225,000$. (b) $\times 200,000$.

breast myosin serum only interacted with breast myosin antigen (Fig. 13C).

DISCUSSION

Brain Actin

PURIFICATION

Actin was first isolated from nervous tissue in 1967 by Berl and co-workers. Although purity was not rigorously demonstrated, these workers were able to show a g to f transformation, stimulation of myosin Mg-ATPase, and the presence of 3-methyl histidine (62). Using peptide mapping techniques (7, 22), heavy meromyosin (HMM) labeling (12, 42, 43), and chemical isolation (38, 52), others have subsequently provided evidence for the presence of actin in nerve cells.

In the present study, brain actin was prepared by using procedures developed for muscle actin purification. The method involved extraction of a

chick brain acetone powder and proved to be rapid and simple. Although others have had limited success using this approach for nonmuscle actin purification (52, 71), it consistently gave satisfactory yields of pure actin. It was necessary to include molecular sieve chromatography in the purification scheme, because nonactin aggregates composed of several different proteins co-purified with the actin (Fig. 1b). These amorphous aggregates appeared to be converted to discrete ring-shaped structures by 0.1 M KCl, and individual particles often stacked to form linear aggregates (Figs. 2b and 3b). Such salt-dependent changes in the aggregation state probably explain why these ring-shaped structures co-purified with actin through cycles of polymerization and depolymerization. However, in low ionic strength buffer the 42,000-dalton actin monomer could be separated from the much larger nonactin aggregates by molecular sieve chromatography (Figs. 1 and 3).

Ring-shaped particles similar in size to those described here have been observed by others. For example, Harris obtained particles having the same dimensions from purified erythrocyte membranes (27). Shelton et al. (69) enriched for similar ring-shaped particles from homogenates of several different eukaryotic cells, and Stossel and Hartwig (75) suggested that macrophage actin binding protein formed structures like those described here. In addition, Taylor et al. (78) have seen similar "barrel-shaped" structures in motile extracts of *Amoeba proteus*. Although these particles are found in a variety of cell types, their function remains unknown.

CHARACTERIZATION OF BRAIN ACTIN

POLYMERIZATION OF BRAIN ACTIN: The critical concentration for polymerization of the purified brain actin, determined by two different methods, was between 0.05 and 0.06 mg/ml (Fig. 5). This value is similar to the critical concentration for polymerization of muscle actin (34), *Dicystostelium* actin (72), and *Acanthamoeba* actin (25).

Quite different results were obtained by Bray and Thomas (9). They found that chick brain actin remained monomeric under conditions that greatly favored polymerization of muscle actin. However, at concentrations >1 mg/ml, some of the brain actin did polymerize and also formed paracrystal-like structures. At concentrations as high as 2 mg/ml, these researchers found that only 20% of the actin was in f-form. In contrast, brain

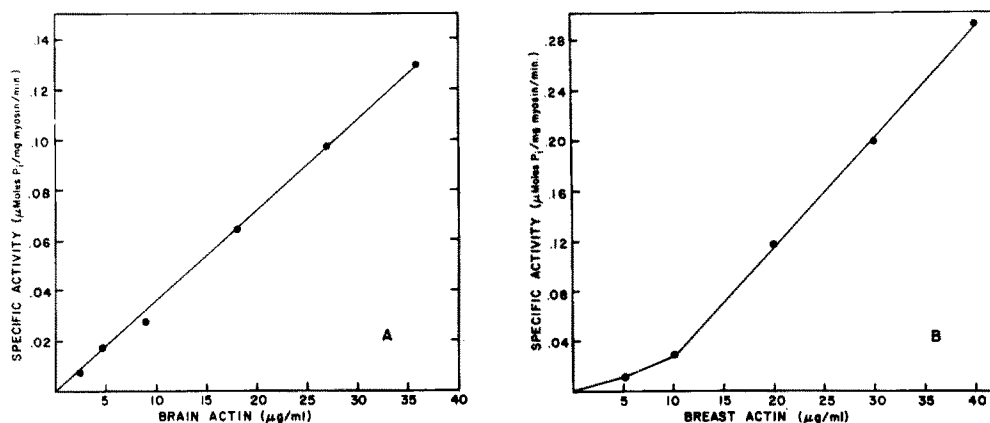


FIGURE 8 Activation of myosin Mg-ATPase as a function of actin concentration. 50 μg of chicken breast muscle myosin were incubated with different amounts of purified brain (a) or breast muscle (b) actin for 30 min at 37°C.

TABLE I
Purification of Chicken Brain Myosin

Fraction	Total protein mg	K ⁺ -EDTA ATPase		Mg ⁺⁺ ATPase	
		Total activity μmol PO ₄ /min	Specific activity μmol PO ₄ /mg/min	Total activity μmol PO ₄ /min	Specific activity μmol PO ₄ /mg/min
Homogenate	4182.0	50.2	0.012	242.0	0.058
Actomyosin	2285.0	47.3	0.021	86.5	0.038
Ammonium sulfate (1.2- 2.0 M)	370.0	14.8	0.040	11.0	0.030
Purified myosin	3.0	1.1	0.370	0.0015	0.0005

Reactions were carried out in 1-ml volumes at 37°C for 30 min as described in Materials and Methods.

actin prepared as described in the present study would, under similar circumstances, form >95% f-actin. The reason for these differences is not clear. Perhaps the acetone powder procedure removes some moiety which inhibits polymerization. Also, because only a small per cent of the total brain actin is recovered by this method, it may be that a particular class of actin has been selected for.

ACTIN ACTIVATION: Purified brain actin was shown to stimulate muscle myosin Mg-ATPase (Fig. 8A). With increasing amounts of brain actin, the activity of the myosin ATPase increased linearly in a manner similar to that described for muscle actin (17). In the homologous system in which breast muscle actin was used to stimulate breast muscle myosin, greater activation of myosin Mg-ATPase was obtained (Fig. 8B). It should be noted that relatively low concentrations of actin

were used in these studies, and that the effects of saturating amounts of actin are not known. The reason for the differences between brain and breast actins is not clearly understood, although other researchers have reported lower levels of activation of muscle myosin ATPase by nonmuscle actins. For example, Spudich found that, at higher concentrations, muscle actin was more than twice as effective as *Dictyostelium* actin in stimulating muscle myosin HMM (71), and low levels of activation have been reported for *Acanthamoeba* and platelet actins (8, 80). Although brain actin in most respects resembles muscle actin, peptide mapping and sequence studies have demonstrated that differences in primary structure do exist (18, 26), and these differences are likely to be functionally important. For example, subtle changes in the concentrations of various ions or protein co-factors may be needed for brain actin to fully

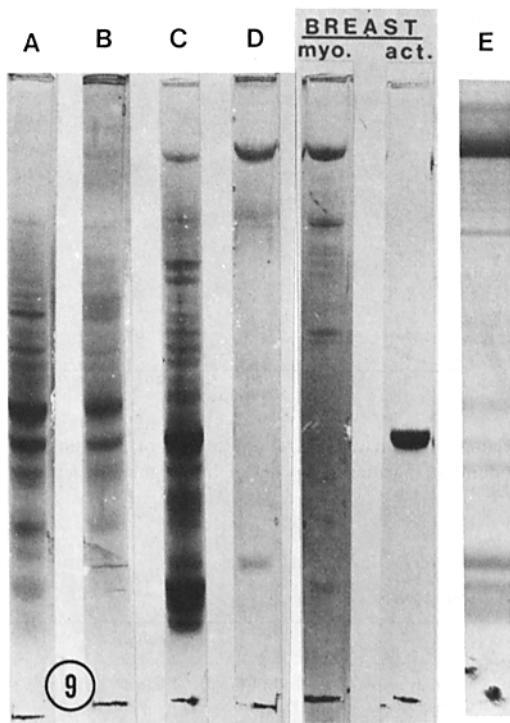


FIGURE 9 Brain myosin purification analyzed on 5% SDS-PAGE. (a) Extract supernate. (b) Low ionic strength precipitation of actomyosin. (c) 1.2-2.0 M ammonium sulfate fraction (column load). (d) Biogel A 15-m purified brain myosin. Standards of chicken breast muscle myosin and actin. (e) Portion of heavily loaded slab gel emphasizing the three light chains.

activate myosin, thus providing fine control over actomyosin interactions in rapidly changing cellular processes.

Brain Myosin

PURIFICATION AND SUBUNIT COMPOSITION

The pioneering investigations of myosin in nervous tissue were carried out by Berl et al. (6) who isolated myosin from whole brain, as well as from purified nerve endings (synaptosomes). This myosin was characterized by Ca^{++} -activated ATPase activity, ATP-sensitive binding to actin, and superprecipitation of actomyosin at low ionic strength. Incorporating the original observations of Szent-Györgyi (76), Berl used potassium iodide in his purification procedure to depolymerize actin, thus enhancing separation of myosin from

actin (6). This innovative approach was improved upon by both Pollard et al. (57) and Nachmias (54), and has since been used to prepare very pure myosin from a number of nonmuscle sources (2, 11, 54, 57, 73). These improved procedures were used to prepare the brain myosin described here. While this work was in progress (38, 39), Burridge and Bray also reported on a highly purified chick brain myosin (11).

SDS-PAGE analysis of the purified chick brain myosin showed it to be free of actin and composed of a 200,000-dalton heavy chain and three light chains. Smooth muscle myosin has two light chains (36), as do most cytoplasmic myosins (e.g., platelet [1], *Acanthamoeba* [59], *Dictyostelium* [13], and *Physarum* [54]). In contrast, striated muscle myosin has three light chains (44, 65, 79). In a detailed study of myosins isolated from a variety of chicken tissues, it was found that two light chains were present in oviduct, fibroblast, platelet, liver, and kidney (11). Myosin isolated from brain and sympathetic nerve, however, had three light chains, similar to those found in the present study. Variability in the amount of 18,000-dalton light chain suggests that this brain myosin subunit is weakly associated with the rest of the molecule and may be more sensitive to some step in the purification than are the other light chains. It is known, for example, that one of the light chains of scallop myosin can be removed with EDTA (77), and that the 17,000-dalton light chain of rabbit striated muscle myosin can be removed with mercurial reagents (23, 30, 45) or concentrated salt solutions (24).

ATPASE

The conditions under which purified muscle myosin hydrolyzes ATP are unique; at high ionic strength, Mg^{++} ion inhibits the activity (10) while Ca^{++} or EDTA stimulates ATPase activity (67, 68). Most cytoplasmic myosins have similar enzymatic properties (59). Purified chick brain myosin had ion requirements for ATPase activity which paralleled those of skeletal muscle myosin (Table

TABLE II
Myosin ATPase in 0.6 M KCl ($\mu\text{moles PO}_4/\text{mg}$
myosin/min)

Myosin	Mg^{++}	Ca^{++}	K^+ -EDTA
Brain	0.0005	0.185	0.370
Gizzard	0.075	0.326	0.488
Breast	0.041	0.213	1.665

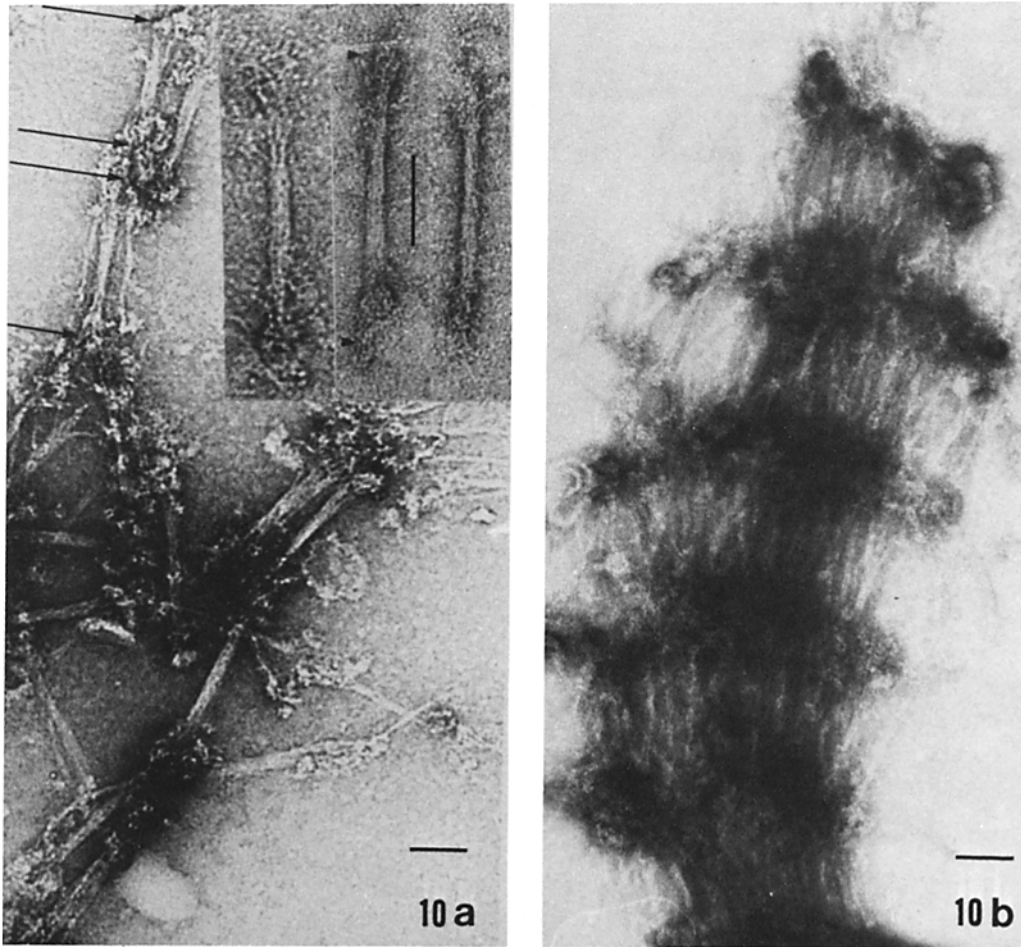


FIGURE 10 Electron micrographs of brain myosin aggregates negatively stained with uranyl acetate. Brain myosin (0.1 mg/ml) was dialyzed overnight at 4°C against 0.1 M KCl, 10 mM imidazole, pH 7.0. (a) Several individual bipolar filaments showing end-to-end interactions. (*inset*) Individual filaments measure 0.3 μm . Fuzzy regions at the ends contain the "head" portion of the myosin molecules, while the central bare regions are composed of the "tails." (b) Bipolar filaments associated both end-to-end and side-to-side. Bar, 0.1 μm . (a) $\times 78,000$. (a, *inset*) $\times 120,000$. (b) $\times 78,000$.

II) (see also references 38, 39), and Burridge and Bray have confirmed this finding (11).

Perhaps a more biologically relevant property of myosin is the ability of its ATPase activity to be stimulated by actin under physiological conditions (17). In the present studies, however, it was not possible to demonstrate such actin activation for purified myosin with either brain or breast actin. A possible explanation for this lack of stimulation is that a protein co-factor was removed during purification. Indeed, there is evidence from other nonmuscle systems that accessory proteins are required for actin activation of myosin ATPase

(55, 74). Another possibility is that this property of myosin was very labile (3, 54), and that KI treatment or some other manipulation during purification destroyed its ability to be stimulated by actin.

FILAMENT FORMATION

In muscle, myosin is located in the thick filaments of the myofibril, in the form of bipolar filaments (32), and similar bipolar filaments can be obtained by dialyzing purified muscle myosin against buffers containing <0.2 M salt. In some nonmuscle cells, thick filaments, which are be-

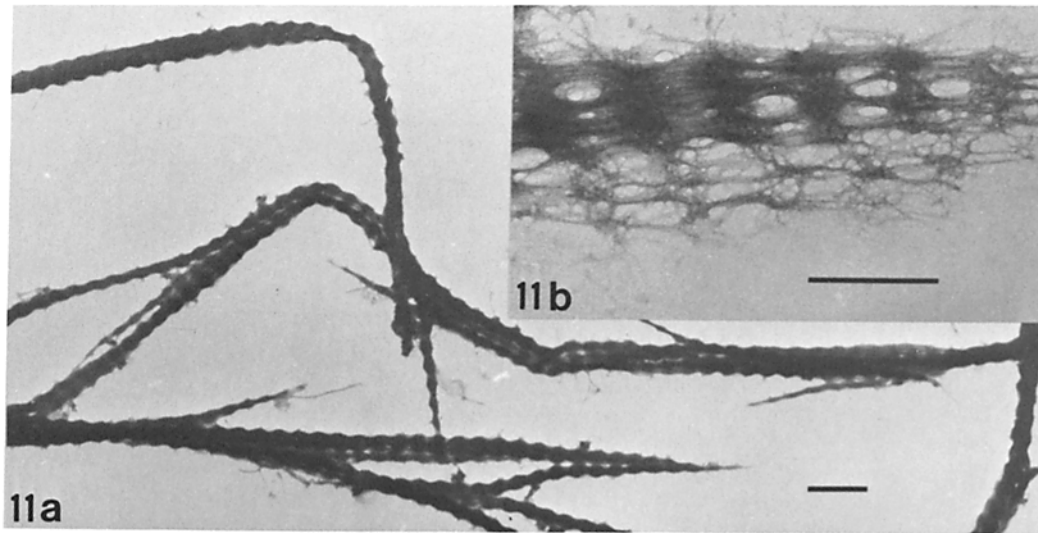


FIGURE 11 Electron micrographs of myosin Mg-paracrystals negatively stained with uranyl acetate. Purified chicken brain myosin (0.14 mg/ml) was dialyzed overnight against 0.1 M KCl, 25 mM MgCl₂, 10 mM imidazole buffer, pH 7.0 (a) Long paracrystals of brain myosin with varicosities produced by myosin heads. Bar, 1.0 μ m. (b) Higher magnification revealing the end-to-end and side-to-side interactions of individual filaments. Bar, 0.5 μ m. (a) \times 8,000. (b) \times 34,400.

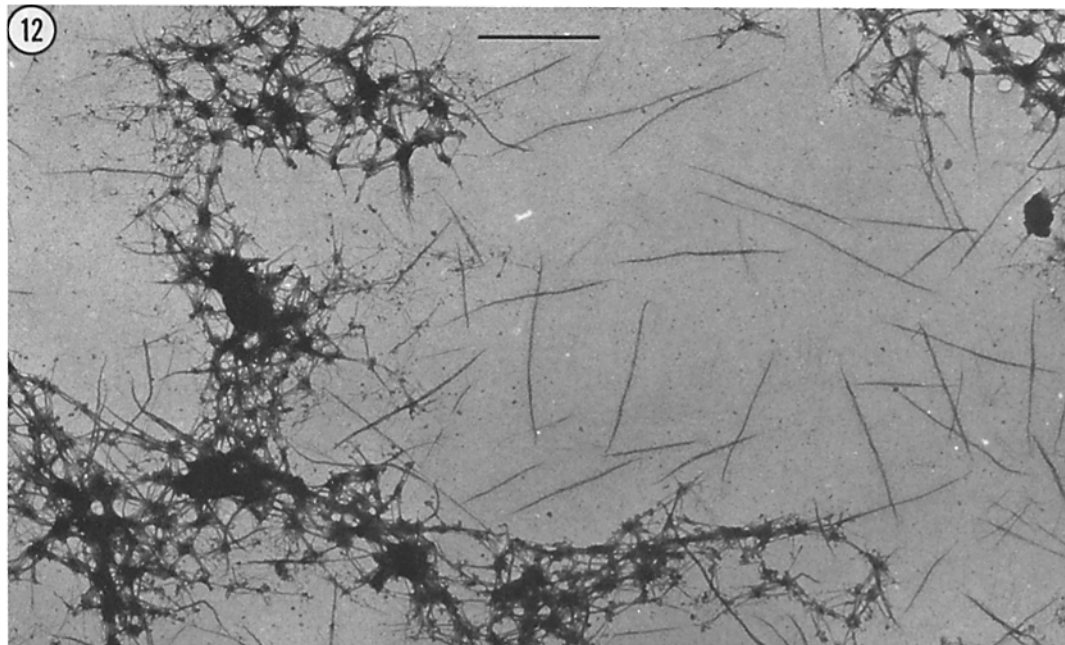


FIGURE 12 Electron micrographs of negatively stained myosin bipolar filaments. 1:1 mixture of chicken breast muscle and brain myosins (final concentration of each, 0.24 mg/ml). Small brain myosin bipolar filaments formed a network as a result of end-to-end interactions. Tapered breast myosin filaments assumed their characteristic length of 1.3 μ m. Bar, 1.0 μ m. \times 16,000.

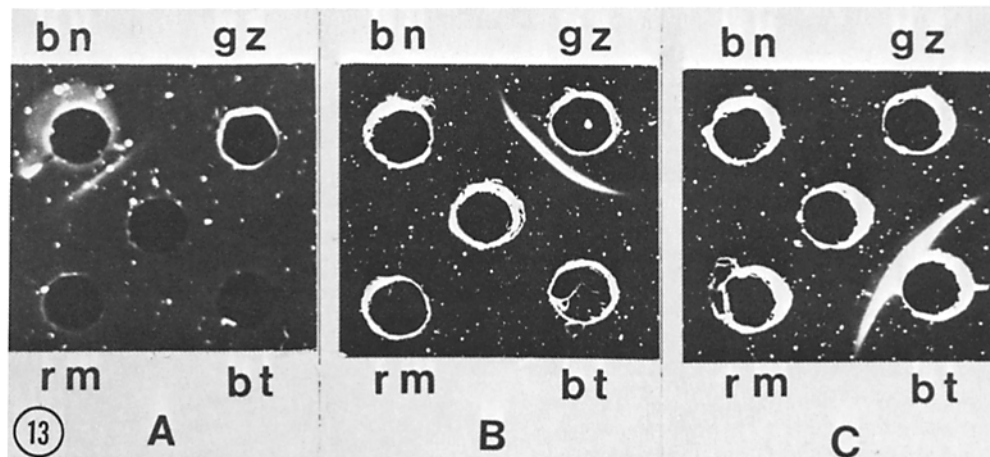


FIGURE 13 Ouchterlony double-diffusion analysis of myosin antibodies. Antiserum in center wells, myosins (0.5 mg/ml) in outer wells: *bn*, chicken brain myosin; *gz*, chicken gizzard myosin; *bt*, chicken breast muscle myosin; and *rm*, rabbit muscle myosin. Reactions are with: (a) antibody to brain myosin; (b) antibody to gizzard myosin; and (c) antibody to breast myosin.

lieved to be composed of myosin, also have been seen by electron microscopy (4, 51, 53, 78). Furthermore, most purified cytoplasmic myosins are capable of self-assembling (11, 13, 38, 59).

Individual molecules of purified brain myosin self-assembled in a typical tail-to-tail manner to form bipolar filaments (Fig. 10). The myosin interactions were enhanced by Mg^{++} ion, resulting in the formation of long paracrystalline arrays which had nodular periodicities (Fig. 11). This Mg^{++} effect was first reported for platelet myosin (58) and then for brain myosin (38). Burridge and Bray (11) were able to induce similar arrays by prolonged dialysis of brain myosin against 0.1 M 2-(*N*-morpholino)ethane sulfonic acid (MES) buffer. Although current models for the role of myosin in nonmuscle motility have incorporated bipolar filaments (59, 71), the physiological significance of the larger aggregates is not clear.

COMPARISON OF BRAIN MYOSIN WITH MUSCLE MYOSIN

Myosins isolated from three different chicken tissues (brain, gizzard, and breast muscle) were compared by a variety of methods. Although molecular weight determinations and Stokes radii measurements (data not shown) showed myosin from breast and brain to be essentially identical, brain myosin assembled into short bipolar filaments while breast myosin formed much larger filaments. In addition, brain myosin could form

head-to-head associations, while similar interactions were never seen with breast myosin. Therefore, even though the individual molecules had similar overall dimensions, molecular interactions during self-assembly differed markedly. In mixing experiments, these two myosins did not co-assemble but rather gave rise to two distinct classes of filaments (Fig. 12). This was in contrast to mixing experiments using breast and gizzard myosins, where hybrid filaments of intermediate length were obtained. The findings that gizzard and breast myosins co-assembled was in agreement with results reported by Kaminer et al. (33). These observations suggest important differences in the molecular interactions for bipolar filament formation between brain and breast muscle myosins.

The fact that brain myosin did not interact with muscle myosin differed from results reported for another cytoplasmic myosin. Pollard (60) showed that human platelet myosin was capable of interacting with human skeletal myosin to form intermediate-sized hybrid bipolar filaments. Although no obvious interactions between brain and breast myosin were seen in the present study, a small amount of co-assembly that did not alter the final length of individual filaments would not have been detected. Also, differences in purity and the possible presence of factors which modified co-assembly might explain the observed differences.

Antibody recognition studies were carried out

to further investigate the relationships between the different myosins. As shown in Fig. 13, the chicken cytoplasmic, smooth muscle, and skeletal muscle myosins (from brain, gizzard, and breast, respectively) appeared to be antigenically distinct. The results for the chick muscle myosins agreed with the findings of others (20, 64).

These antibody results, together with differences in filament formation and in ATPase specific activities (Table II), suggest that myosins from the three sources are different gene products. Additional support for this contention comes from the work of Barany et al. (3) in which it was demonstrated that chicken breast and gizzard myosins have different amino acid compositions. Moreover, using chemical cleavage of myosin heavy chain, Burridge and Bray (11) have recently shown major polypeptide differences among breast, gizzard, and brain myosins.

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