ELECTRON MICROSCOPE OBSERVATIONS ON

MYOSIN FROM *PHYSARUM POLYCEPHALUM*

VIVIANNE T. NACHMIAS

From the Department of Biology, Haverford College, Haverford, Pennsylvania 19041

ABSTRACT

Myosin has been separated from *Physarum polycephalum* actomyosin in confirmation of the results of Hatano and Tazawa. In an intermediate step, myosin-enriched actomyosin has also been obtained. The mean yield of free myosin was 4.4 mg from 100 g of mold. It was obtained as water-clear solutions at $\mu = 0.055$ with calcium ATPase activity of up to 0.5 μM P_i/min per mg. Negatively stained preparations were examined by electron microscopy. *Physarum* myosin in 0.5 M KCl interacted with actin from rabbit skeletal muscle to form polarized arrowhead complexes similar to but less regular than those of natural actomyosin from muscle or myosin-enriched *Physarum* actomyosin. The *Physarum* myosin-enriched actomyosin at low ionic strength displayed evidence of head-to-tail and tail-to-tail aggregation attributable to the myosin component. Yet *Physarum* myosin alone did not produce detectable filaments at $\mu = 0.055$ at pH 7, 6.5, or 5.8, nor when dialyzed against 0.01 M ammonium acetate, nor when the dielectric constant of the medium was reduced. However, aggregation approaching the extent of 'thick filaments' up to 0.3 μ long was found in some preparations of myosin-enriched actomyosin put into solutions containing adenosine triphosphate. Myosin alone in such solutions did not form filaments. The results are compatible with the idea that head-to-tail aggregations are favored by actin-myosin interactions in *Physarum*, possibly due to alignment of the extended or tail portions of this myosin molecule.

INTRODUCTION

Previous work (Hatano and Oosawa, 1966; Hatano and Tazawa, 1968; Adelman and Taylor, 1969 a, 1969 b; Hatano and Ohnuma, 1970) has shown that both an actin and a myosin can be prepared and purified from *Physarum polycephalum*, the plasmodial slime mold which exhibits vigorous cytoplasmic streaming (Kamiya, 1959). Both proteins are, in a number of respects, similar to their counterparts prepared from vertebrate striated muscle but with certain intriguing differences. These proteins represent the first available material with which to study actin-myosin interactions in vitro from a system whose motility involves cytoplasmic streaming rather than muscle contraction. Hence, the ways in which the pro-

teins differ from their counterparts in muscle are especially interesting, as they may shed light on what is unique to the mechanochemical phenomenon of cytoplasmic streaming.

In this paper we are concerned with one property which distinguishes *Physarum* myosin from muscle myosin, namely, its solubility in salt solutions of low ionic strengths such as 0.1–0.03 M univalent salt. This solubility is in remarkable contrast to the solubility of *Physarum* actomyosin, which precipitates at low ionic strength. We have slightly modified the method of Hatano and Tazawa (1968) to prepare both myosin and myosin-enriched actomyosin from *Physarum*. We present some data on the purity and some enzy-

matic characteristics of the myosin prepared in this way, and then use the two fractions for structural study by electron microscopy of negatively stained preparations. First, we confirm the finding of Hatano and Ohnuma (1970) that the *Physarum* myosin prepared in this way is soluble at low ionic strengths, and we show that this is the case *despite* the existence of an extended "tail-like" portion similar to that of muscle myosin. Adelman and Taylor (1969 b) have also shown previously that highly purified *Physarum* myosin prepared by a different technique was soluble at low ionic strengths, but conclusive evidence for an extended tail portion was not present. Second, we find that, although cysteine was not detectable in *Physarum* myosin within the limits of the available methods by Hatano and Ohnuma (1970), preliminary enzymatic studies using sulfhydryl inhibiting compounds show that this myosin must almost certainly be a sulfhydryl enzyme, as indeed they suspected. Third, we confirm by electron microscopy previous findings by viscometry and enzymology (Hatano and Ohnuma, 1970; Adelman and Taylor, 1969 b) indicating that *Physarum* myosin interacts with rabbit striated muscle actin, and we show that, as in the converse case, the actomyosin complex displays polarity and takes the form of the arrowhead structures similar to those first discovered in vertebrate striated muscle actomyosin (Huxley, 1963). Fourth, we show by electron microscopy that *Physarum* myosin does not form filaments under a variety of conditions that might favor such aggregation. On the other hand, we find some evidence of short (0.3–0.4 μ long) filaments associated with actin in myosin-enriched actomyosin preparations treated with solutions containing adenosine triphosphate (ATP), magnesium salts, and sometimes EGTA.

The results therefore suggest that *Physarum* myosin is capable of forming short filaments but under physiological conditions only as a result of actin-myosin interactions. This is interesting in view of the recent finding (Kessler and Nachmias, 1970; Kessler, in preparation) of thick filaments in sectioned material from glycerinated *Physarum* undergoing spherule formation. It is possible that a different charge distribution along the tail regions of *Physarum* myosin requires that these regions be aligned much more precisely than is the case for muscle myosin, in order that interactions leading to filament formation take place. Possibly, interactions with actin, either binding

TABLE I

Flow Diagram for Myosin and Actin from Physarum polycephalum (Modified from Hatano and Tazawa, 1968)

Extract fresh mold in 0.35 M KCl, 12 mM EDTA, pH 8.2 for 2 hr. Centrifuge × 2. From supernatant,

(1) 1st low (0.08 M) KCl precipitate at pH 6.5 = crude actomyosin. Wash, redissolve in 0.6 M KCl.

(2) 2nd or 3rd low (0.06 M) KCl precipitate at pH 6.5 = actomyosin. Redissolve in 0.6 M KCl 0.06 M imidazole pH 7, and make 5 mM in ATP and 1 mM in MgCl₂ . Centrifuge 100,000 g for 3 hr.

(3) Supernatant (S₁) = dissociated myosin + "short" actin.

(4) Pellet (P₁) = crude actin pellet; resuspend and recentrifuge 60,000 g 30 min, dialyze supernatant and convert to G, then F-actin → Purified actin (P₂).

(5) S₁ is dialyzed vs. 0.05 M KCl 0.005 M imidazole (100–200 vols) × 2 → precipitate (P₃) = myosin-enriched actomyosin. → supernatant (S₂) = partially purified myosin.

of the myosin to actin filaments or part of the process of contraction itself, favor such alignment. Even so, only short filaments are formed. The hypothesis that thick filaments form as a result of contraction is considered.

MATERIALS AND METHODS

Culture of Physarum

The mold was grown by the previously described method (Nachmias et al., 1970) except that only plasmodia which had migrated to a layer of plastic wrap free of food material was collected. These plasmodia were rapidly migrating, relatively free of slime, and could be harvested with a minimum of damage before homogenization. The preparation is summarized in Table I.

One comment should be made here. At step 2, the actomyosin precipitate is redissolved at high ionic strength. It is then made 5 mM in ATP and 1 mM in MgCl₂ and is centrifuged at 100,000 g for 3 hr at 5°C. In a control sample monitored at 15°C in a viscometer, the viscosity remained near the lowest level for more than 3 hr. The resultant pellet (P₁, Table I) has a yellow lower layer and a translucent upper layer consisting mainly of actin-like filaments, which can be further processed to yield crude or purified actin. The supernatant (S₁), which appears water-clear at high ionic strength, contains

myosin plus 'short actin' (Hatano and Ohnuma, 1970; Nachmias and Ingram, 1970).

Rabbit Muscle Purified Actin

Actin preparations were generous gifts of Dr. S. Matačić. The acetone-dried powder of rabbit leg muscle was prepared by the method of A. Szent-Györgyi (1951). The dried powder was stored at $-15°C$. G-actin was extracted from the acetone-dried powder at 4°C with 5×10^{-4} M ATP and 2×10^{-4} M CaCl$_2$ at pH 7.5 (modified Rees-Young, 1967) for 20 min. The muscle residue was removed by centrifugation at 10,000 g for 15 min. The supernatant containing G-actin was centrifuged at 100,000g for 30 min. G-actin was converted to F-actin by the addition of 0.1 M KCl, 0.01 M Tris, pH 8.3, 5 mM MgCl$_2$, and 2 mM ATP. After 1 hr at room temperature, F-actin was collected by centrifugation for 2 hr at 100,000 g. The pellet was gently homogenized in the presence of the ATP-CaCl$_2$ solvent. Actin was dialyzed for 18 hr at 4°C against the ATP CaCl$_2$ solvent. After dialysis G-actin was transformed to F-actin again, and the same procedure described above was repeated two more times.

Rabbit Muscle Actin from Actomyosin

These preparations were made from natural actomyosin made by the method of Szent-Györgyi (1951). The actomyosin in 0.5 M KCl was made 5 mM in ATP and magnesium chloride and centrifuged for 2 hr. The pellet was washed and resuspended in 0.5 M KCl.

Heavy Meromyosin (HMM)

This was made by the method of Szent-Györgyi (1953), but using a 10-min incubation of myosin at low ionic strength with trypsin. The supernatant HMM was used within 3 days.

Spectrophotometry and Viscometry

Matched quartz cuvettes were used in a Zeiss PMQ II. When necessary, correction was made for light scattering by the linear extrapolation of optical densities in the range 350-310 nm. The viscometry checks were made in a 15°C water bath, using a Cannon semi-micro viscometer with an outflow time for water of 58 sec.

Enzymatic Activities

ATPase activity was estimated using a semi-micro version of the exact assay conditions of Adelman and Taylor (1969 b). The buffer was 0.05 M Tris, pH 8, the calcium ion concentration 10 mM, the KCl concentration 0.5 M, the incubation time 15 min at

room temperature (23° C to 26°C) or in a water bath at 26°C, and the total volume 0.4 ml. When ethylenediaminetetraacetate (EDTA) was used, it replaced CaCl$_2$. Inhibitors were added to the enzyme either during a preincubation stage or directly to the assay medium before addition of substrate. Activities were, in general, proportional to enzyme concentration. The inorganic phosphate released was estimated by the method of Taussky and Schorr (1952) and optical densities were read at 700 nm in a Gilford model 300 micro-sample spectrophotometer (Gilford Instrument Company, Oberlin, Ohio). Blanks were run with each assay point and standard curves were run with each assay. Protein concentrations were estimated by the technique of Lowry et al. (1951), using bovine serum albumin as a standard. Since calibration against direct measure of nitrogen has not been made, they must be regarded as approximate. For myosin solutions dialyzed essentially free of nucleotide, optical density at 278 nm was also used with the value $E_{1cm}^{1\%}$ 5.2 (Adelman and Taylor, 1969 b). The agreement with the Lowry technique was reasonably close, but as expected, the optical density usually gave a slightly higher estimate.

Reagents

All chemicals were reagent grade. Water was deionized with a mixed bed resin. ATP[1] was purchased as the disodium salt from Sigma Chemical Co., St. Louis, Mo., and was made up at 50 mM as samples neutralized to pH 6.9 and stored frozen until used. Samples were used within 1-2 months. N-ethyl maleimide (NEM) was a product of Nutritional Biochemicals Corporation, Cleveland, Ohio, and the iodoacetamide was obtained from the Aldrich Chemical Co., Inc., Milwaukee, Wis. Iodoacetamide (IA) was used within 2 days of preparation; the solution was colorless. Beta-mercaptoethanol (BME) was stored at 5°C in a dark bottle and was diluted directly before use. Dithiothreitol or dithioerythritol was made up directly before use. The D$_2$O was a product of International Chemical & Nuclear Corporation, Burbank, Calif.

Electron Microscopy

Negative staining was routinely carried out by the method previously described (Huxley, 1963; Nach-

---

[1] *The following abbreviations are used in this paper:* ATP, adenosine triphosphate; BME, beta-mercaptoethanol; Ca-ATPase, calcium-activated adenosine triphosphatase; EDTA, ethylenediaminetetraacetate; EGTA, ethylene glycol bis (β-aminoethyl ether) N,N'-tetraacetic acid; IA, iodoacetamide; NEM, N-ethyl maleimide.

Table II

Table II

Typical Yields of Fractions from Physarum Preparation 8–137 g Mold

Stage	Volume	Concentration	Yield	Yield mold
	(ml)	(mg/ml)	(mg)	mg/100 g
(1) Actinomyosin	15.2	2.5	38	27.8
(2) S_1 (Myosin + short actin)	15.2	1.0	15.2	11.1
(3) S_2 (Myosin)	12.0	0.6*	7.2	5.2
		0.43‡	5.2	3.8
(4) P_1 (crude actin)	9.0	1.5	13.5	9.8
(5) P_2 (Myosin-enriched actomyosin)	2.1	3.0	6.3	4.6

* Optical density.

‡ Lowry.

Table III

Yields and Properties of Partially Purified Physarum Myosin

Preparation No.	Physarum	Yield myosin (OD 278)	Lowry	Yield myosin	OD ratio, 278/260	Ca-ATPase
	g	mg	mg	mg/100 g		$\mu M P_i/min/mg$
1	126	10.6		8.4	1.54	
2	131	3.6		2.9	1.57	
					1.45*	0.47*
3	123	6.5		5.2	1.68‡	0.51‡
4	298	7.0		2.3	1.32	
5		4.2				
7	116	3.0	2.5	2.2–2.8		
8	137	7.2	5.2	3.8–5.2	1.54	
11	50		2.4	4.8		1.38§

Mean yield partially purified myosin: 4.4 mg/100 g mold.

* 0.5 M KCl.

‡ 0.05 M KCl.

§ Beta-mercaptoethanol (1 mM) in the preparation.

mias et al., 1970) and carbon-coated plain or holey formvar or collodion-filmed grids were examined in a Hitachi HU-11A microscope equipped with a 50 μ thin gold foil objective aperture at 50 kv and with beam currents of 15–20 μamps. Original magnifications were 20,000 or 40,000. The microscope was calibrated with a replica grating (E. F. Fullam, Inc., Schenectady, N. Y.). The reaction of actin or thin filaments with *Physarum* myosin or HMM was carried out directly on filmed grids at 5°C.

RESULTS

Comments on the Preparation

Tables II and III summarize typical results with a number of preparations made in the same fashion. The only variations were in the inclusion of a wash step of the first low-ionic strength pre-

cipitate, use of sulfhydryl protecting agents, and speed of preparation. Systematic study of these variables and their effect on yields was not made, but we found that BME added to the second precipitate during dissolution increased the enzymatic activity of the myosin prepared from the actomyosin. We also found that by omitting the wash step (see step 2, Table I) and performing the entire extraction procedure in 12 hr instead of 24 hr, we increased the final yield by a factor of 2 to 3, but obtained a less pure preparation. The mean yield of actomyosin, when prepared according to the details in Material and Methods, was close to 30 mg from 100 g of original wet weight of mold. The actomyosin was a white, slightly turbid solution in 0.5 M KCl, 0.05 M imidazole.
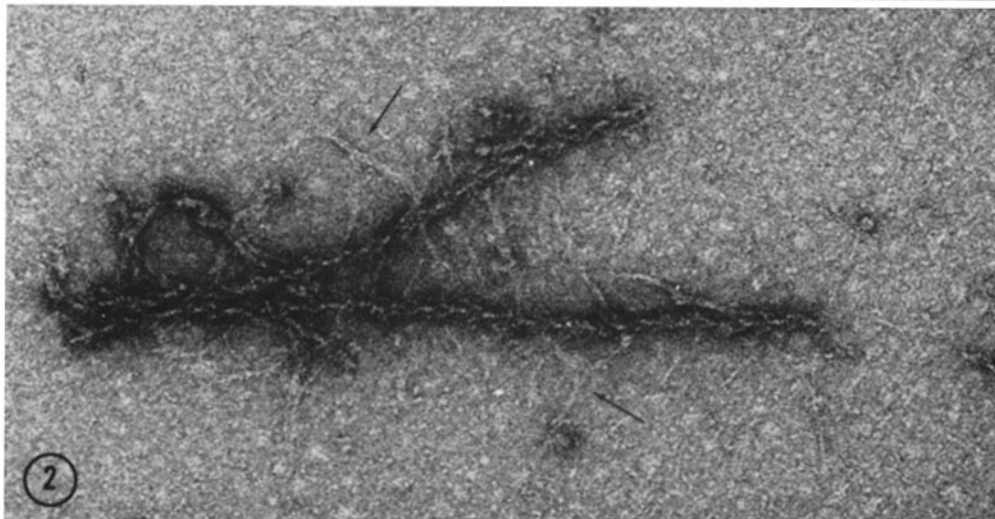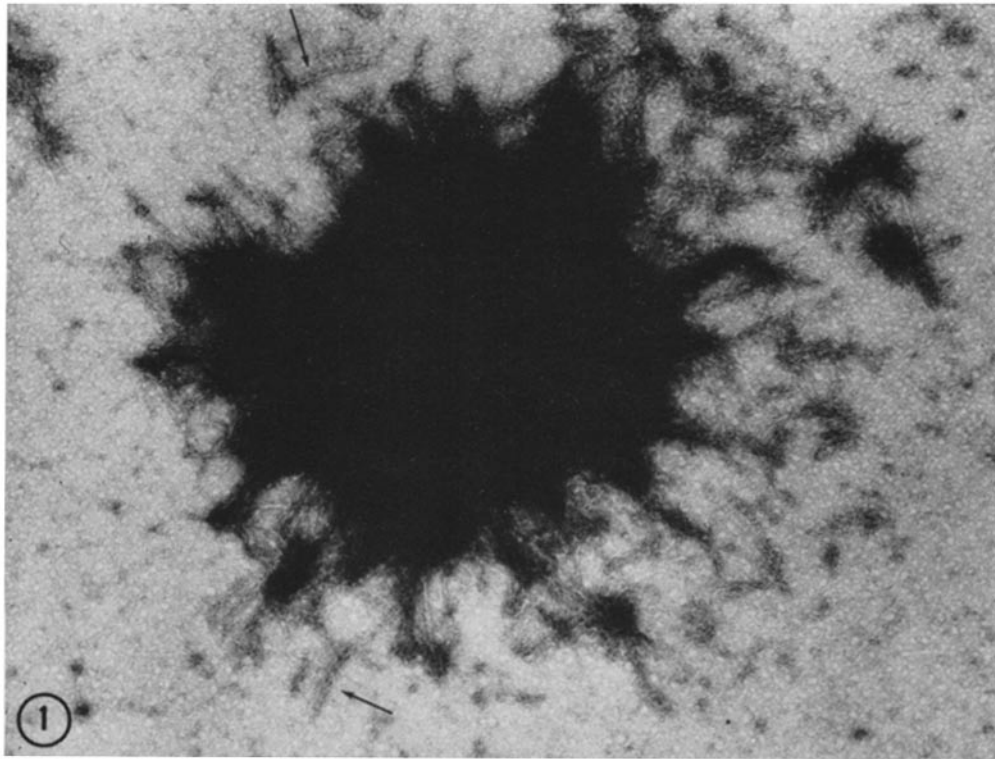
FIGURE 1 Massive clump several micra wide from the myosin-enriched actomyosin fraction. Note decorated actin at periphery (arrows). × 25,000.

FIGURE 2 A fragment from another myosin-enriched actomyosin fraction, at higher magnification. Note the regular appearance of the arrowhead structures (compare Figs. 6 a and 6 b) and the lateral extensions which often come together (arrows) from a single filament. × 120,000.
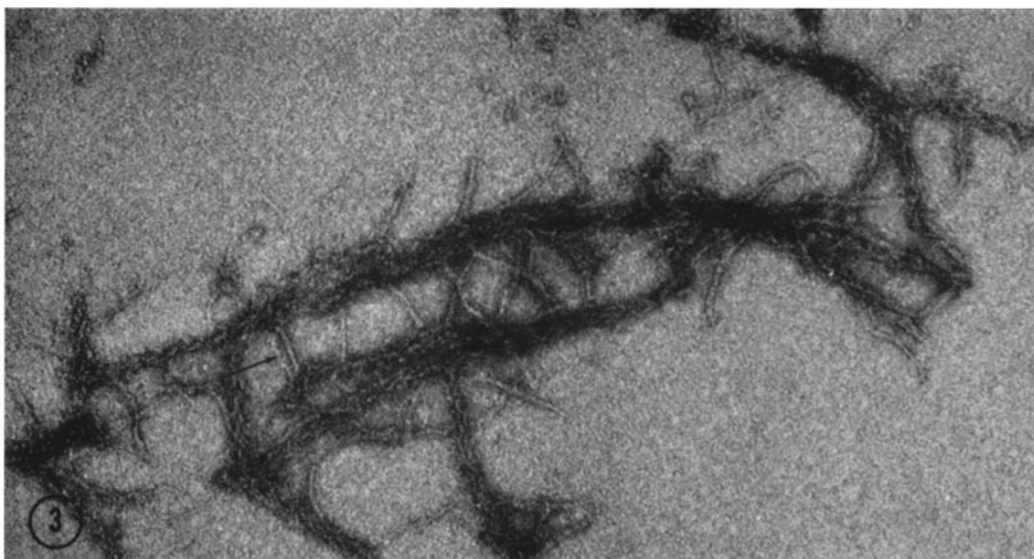
FIGURE 3   Another configuration seen in myosin-enriched *Physarum* actomyosin. Note the ladder-like appearance of the aggregate with extensions (arrow) joining from two different filaments. × 88,000.

Yields, Purity, and Properties of the Partially Purified Myosin

The mean yield of partially purified myosin was 4.4 mg from 100 g of mold. This myosin appeared as a water-clear solution at either 0.5 or 0.05 M KCl. Nevertheless, a preliminary trial in the ultracentrifuge[2] suggested that some more rapidly sedimenting material occurred in the myosin at low ionic strength. Although a systematic study of the specific activity was not made here, observations on *Physarum* actomyosin yielded values of 0.1–0.2 μM P_i/min per mg, compared with values of 0.5 μM P_i/min per mg for partially purified myosin. Thus, a twofold to fivefold purification was achieved. The yield of the myosin is clearly reduced by the myosin bound up in the myosin-enriched fraction, and at present this is a drawback in obtaining maximum yields of myosin by this procedure. The myosin has OD ratios 278/260 nm of 1.3–1.6 (Table III) and Ca-ATPase activities of 0.4–0.5 μg P_i/min per mg without sulfhydryl protection. Small differences in the 278/260 ratios may be due to the presence of G-actin or traces of residual ATP. After storage for a few days, the activity dropped to about half

---

[2] Courtesy of Dr. Joel Rosenbloom, University of Pennsylvania.

this figure and occasionally it was lower than this originally. We found that BME significantly increased the rate of ATP hydrolysis by free myosin while IA or NEM inhibited the activity. Both EDTA and $MgCl_2$ inhibited the ATPase when they replaced calcium in the assay.

Preliminary results showed that the partially purified myosin, as detected by ATPase activity, ran with the void volume on Agarose 6 B and gave only three heavy bands on 5% SDS polyacrylamide gels, one strong band being close to the position of muscle myosin. However, a number of lighter bands were also present.

Electron Microscope Observations

ACTOMYOSIN: As previously described (Nachmias et al., 1970; Nachmias and Ingram, 1970), *Physarum* actomyosin was observed to consist largely of actin-like filaments with occasional material adhering to the filaments; some of the adherent material displayed polar attachments.

MYOSIN-ENRICHED ACTOMYOSIN: The number of such polar attachments was greatly increased in the 'myosin-enriched actomyosin' fraction which precipitates upon dialysis of the S-1 fraction (see also Nachmias and Ingram, 1970). Fig. 1 shows a massive clump composing part of such a precipitate. These clumps of material,
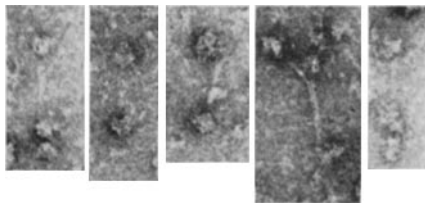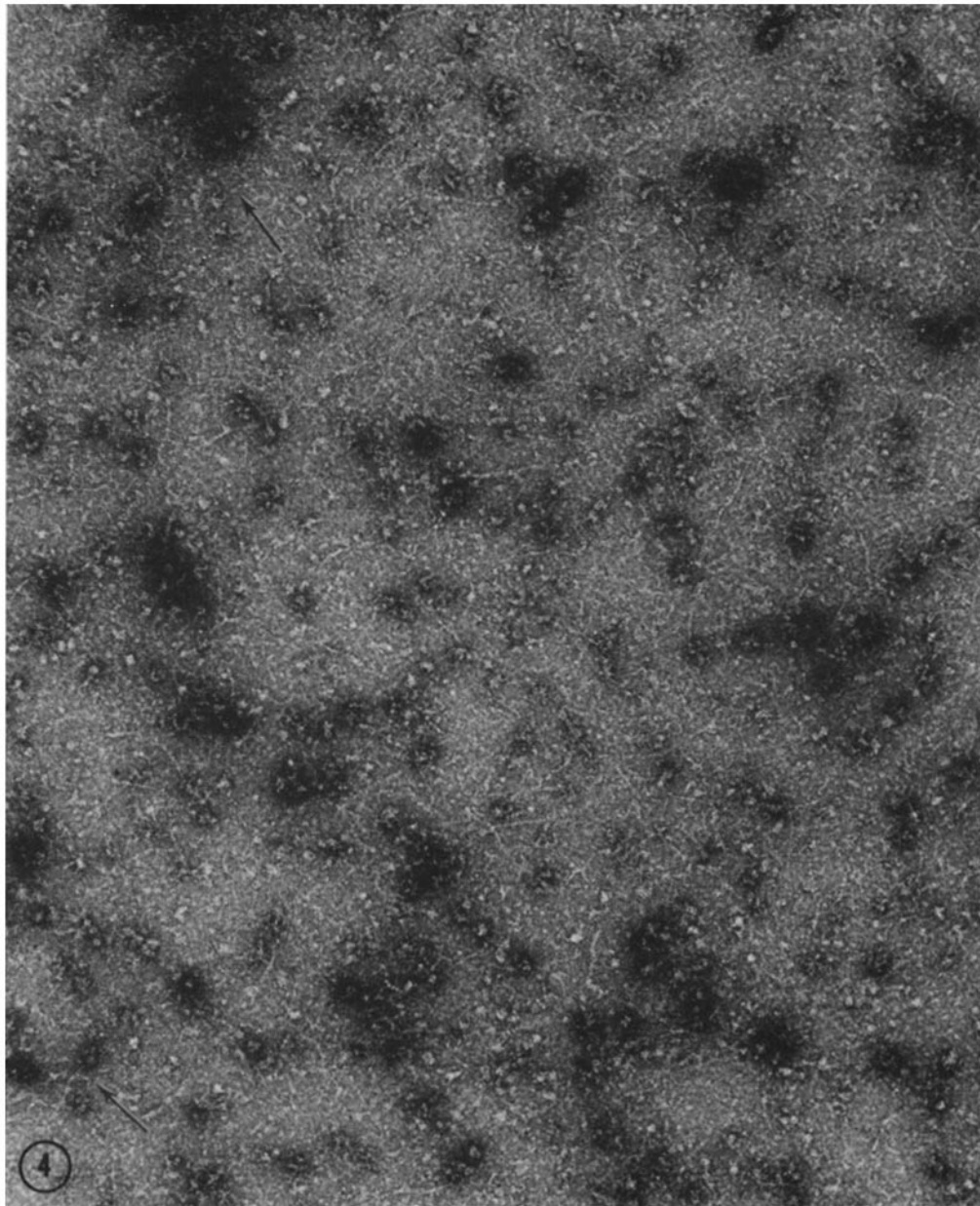
FIGURE 4 Myosin supernatant fraction (S₂, Table I) in 0.05 M KCl, plus 15% isopropanol. Virtually no actin filaments are seen (compare Figs. 8 and 9. Arrows point to thin extensions joined to globules. Insert, examples of dimer-type configurations. × 66,000.

FIGURE 5 *Physarum* myosin supernatant fraction (S$_2$, Table I) concentrated to ∼1 mg/ml and reacted with purified rabbit F-actin. Arrow indicates well-formed arrowhead structure. × 100,000.

several micra in diameter, showed clear-cut arrowhead structures on their periphery (arrows, Fig. 1). When the precipitate was dispersed or incompletely centrifuged, we found material which displayed a more open structure. Sometimes the fully decorated appearance of the filaments was more obvious, as in Fig. 2; at other times, the ladder-like structures shown in Fig. 3 predominated. In both cases, the long extensions from the arrowhead complex are seen to interact with one another; in Fig. 2 many are arranged with interactions from arrowheads on the same filament (head-to-tail), while in Fig. 3 it appears that extensions from two different actin filaments, and hence with heads pointing in opposite directions (tail-to-tail), nevertheless interact so as to connect the two or more actin strands.

ACTIN AND MYOSIN: The P$_3$ pellet was found to consist of actin-like filaments, while the myosin

remaining in the S-2 fraction appeared as globular material whether examined directly or after addition of isopropanol (Fig. 4).

PHYSARUM MYOSIN PLUS RABBIT ACTIN: When this myosin was dialyzed against buffered 0.5 M KCl and interacted with purified rabbit muscle actin on support grids, arrowhead structures were again formed. Figs. 5 and 6 show examples of somewhat incompletely decorated and moderately decorated purified rabbit muscle actin interacted with *Physarum* myosin supernatant. When more complete decoration was obtained, the structure no longer had a clear-cut arrowhead appearance; this may be partly due to the presence of the long extensions which in some areas appeared to adhere to the filaments, thus obscuring the regularity of the arrowheads. For comparison, the regular appearance of rabbit thin filaments from natural actomyosin reacted with crude heavy
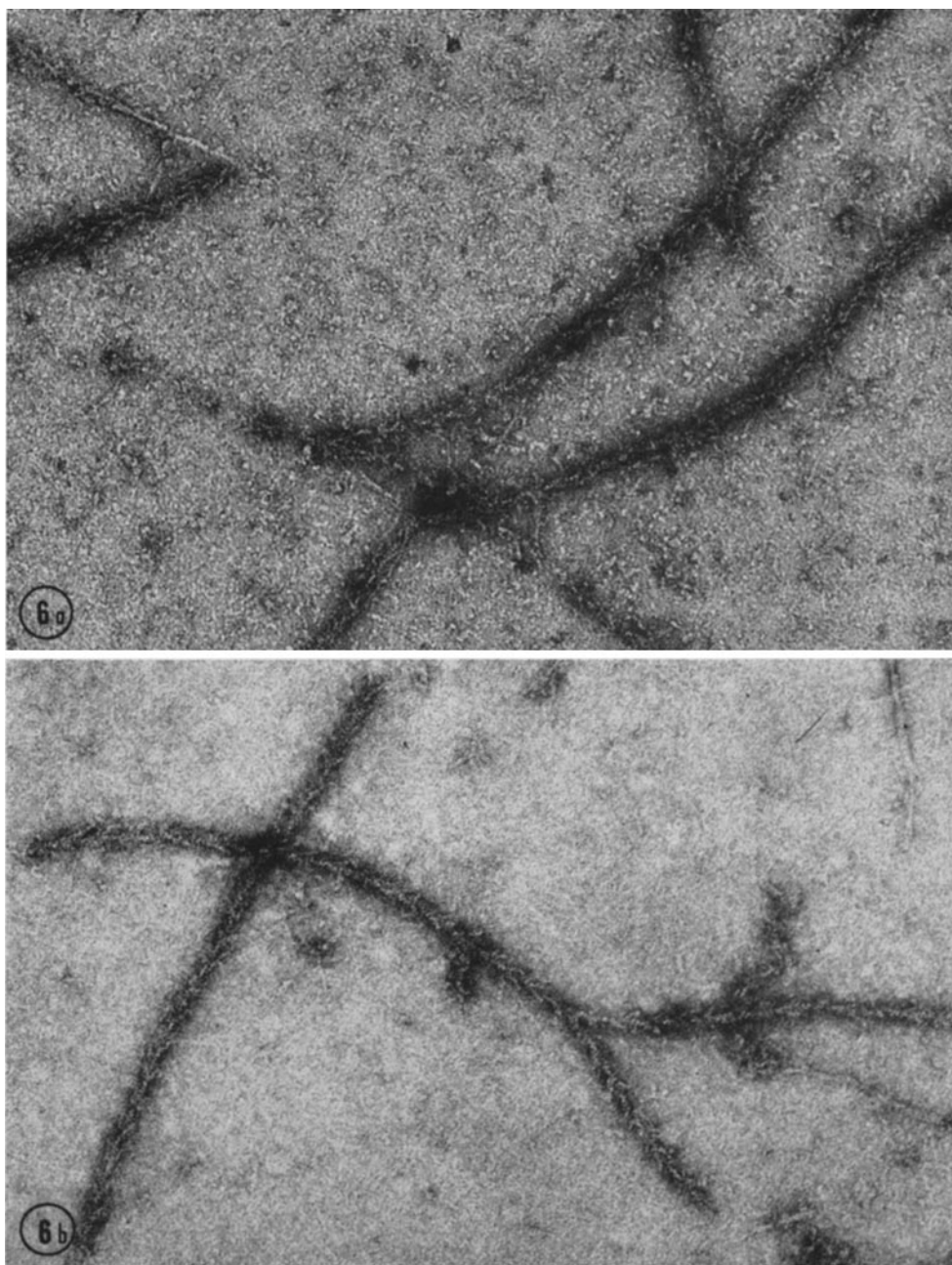
FIGURE 6 *a* *Physarum* myosin supernatant fraction (S$_2$, Table I) reacted, as in Fig. 5, with purified rabbit F-actin. Here, however, a large excess of myosin was used. Note irregular appearance of arrowheads compared to Figs. 2 and 6 b. Compare with sparser decoration in Fig. 5. × 88,000.

FIGURE 6 *b* Heavy meromyosin from rabbit striated muscle reacted with thin filaments prepared from rabbit actomyosin. × 122,000.

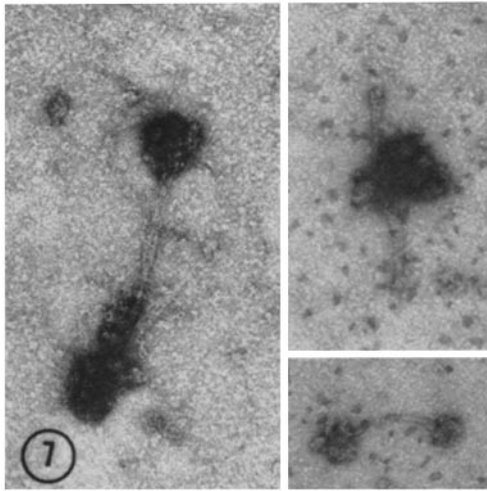FIGURE 7 Examples of different types of aggregations seen in *Physarum* myosin (S₂) dialyzed or diluted with D₂O at pH 8.5. × 100,000.

meromyosin from rabbit myosin is shown (Fig. 6 *b*). See also Fig. 2.

MYOSIN AGGREGATES: A series of experiments was carried out to see whether the *Physarum* myosin might form aggregated structures similar to the thick filaments of striated muscle myosin, which forms filaments readily when the ionic strength is lowered to 0.1 and the pH is 6.5 (Huxley, 1963). Preliminary trials with muscle myosin showed that indeed filaments were readily formed under these conditions. Because the sliding filament model of muscle contraction has been so successful in explaining various aspects of contraction in striated vertebrate muscle, and because it has often been invoked without basic alterations for other systems, we thought that it was important to try a variety of conditions that might induce filament formation from *Physarum* myosin. We used conditions which might enhance either ionic bonding, such as lowering the dielectric constant of the medium, or hydrogen bonding, or conditions which empirically have been found by previous workers to result in long filaments when used with striated muscle myosin. The following conditions were therefore tried with the *Physarum* myosin supernatant (S₂): (*a*) At an ionic strength of 0.055, the pH was altered over the range 6.5–5.8; (*b*) The myosin was diluted with isopropanol or ethylacetate; (*c*) The myosin was diluted with or dialyzed against 45% D₂O solutions at neutral or alkaline (pH 8.5) conditions; (*d*) The myosin was dialyzed

against 0.01 M ammonium acetate (pH 7); (*e*) The myosin was treated with a 'relaxing solution' containing 5–10 mM ATP and 5–8 mM MgCl₂ and 1–3 mM EGTA (Hinssen, 1970). In addition, *Physarum* actomyosin or myosin-enriched actomyosin was treated with such relaxing solutions.

Our results may be summarized as follows: (*a*) Myosin alone: Significant aggregations were found only in the D₂O experiment where dialysis was against pH 8.5. Examples of the aggregates found under these conditions are shown in Fig. 7. It can be seen that both head-to-head and tail-to-tail types of aggregations are seen. In fact, the head-to-head type of aggregation, with whisker-like extensions jutting out from a central mass, was, if anything, more frequent than the occurrence of filament-like structures, which nevertheless were also seen frequently. (*b*) Actomyosins: When *Physarum* actomyosin was treated with the relaxing solutions, we first noted a 'clearing response', that is, by comparison with the control sample, the experimental sample appeared to go into solution. However, on standing (usually 12–18 hr), 'super-precipitation' followed, i.e., the experimental sample contained dense precipitate at the bottom of the tube, while the control sample still contained a flocculent, lightly settled precipitate. When we examined the actomyosin early after treatment, we found only clear actin-like filaments, often in laterally aggregated bundles (Figs. 8 and 9). After longer standing, we obtained some material which did not resemble actin but, on the other hand, did not really resemble short thick filaments, in that no globular heads were visible at the extremities. Myosin-enriched actomyosin treated in this way showed in some preparations more clear-cut filament-like structures (Fig. 10). These measure up to 0.3 μ long. However, we did not find clear-cut filaments, *free of actin*, in either type of experiment.

DISCUSSION

Yields and Purity

ACTOMYOSIN: Our yields of actomyosin were consistently only a third of those reported by Hatano and Tazawa (1968) and obtained regularly by them (Hatano, personal communication). The reasons for this are not clear. However, if we omitted a repeat centrifugation of the original extract and a wash step on the first precipitate, and finished the entire preparation in 12 hr rather than 30 hr, we did obtain higher yields, up to 90 mg per
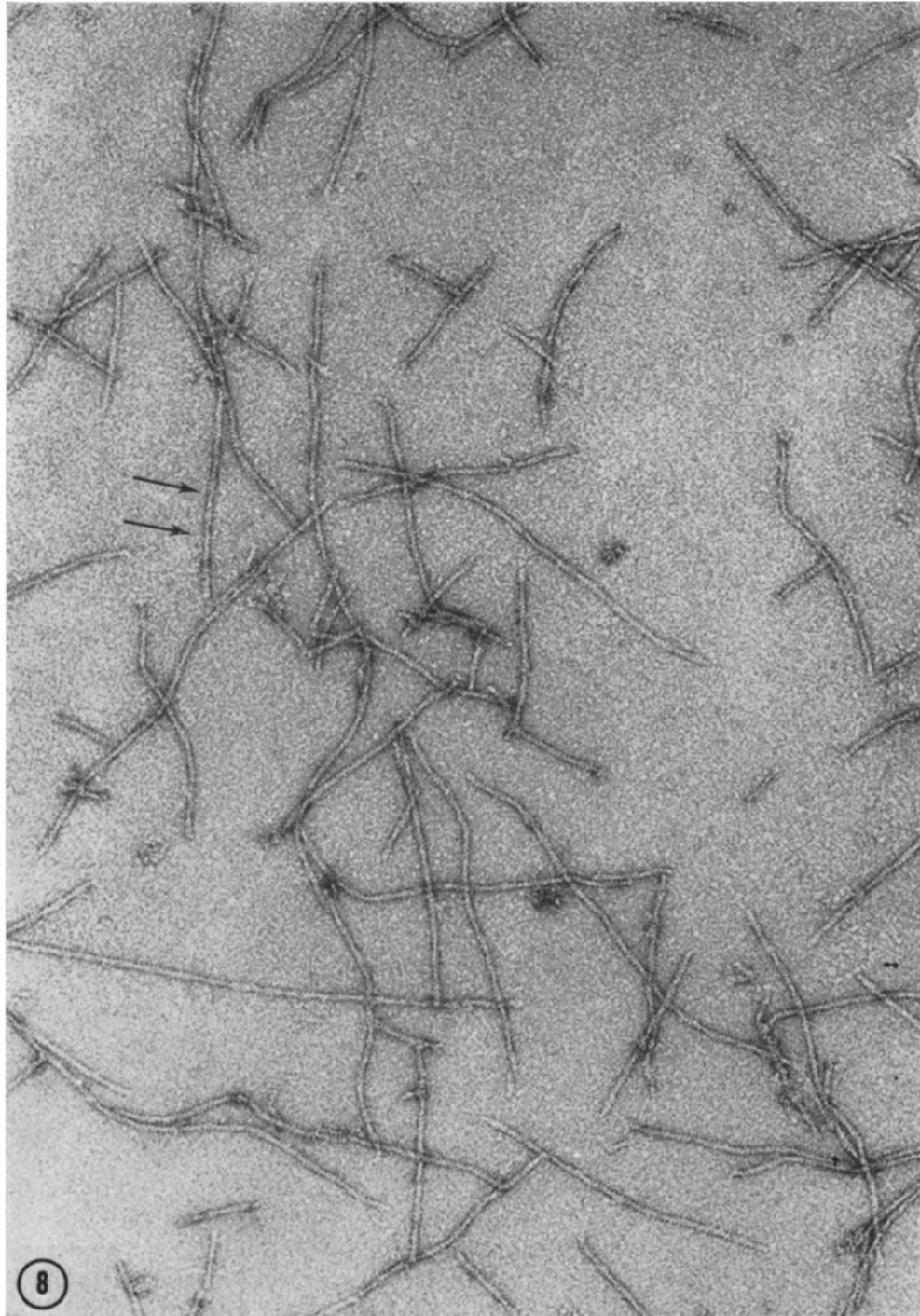
FIGURE 8 *Physarum* actomyosin treated with relaxing solution (see text). Actin-like filaments are usually seen, as here. Arrows point to crossover points on the actin. × 110,000.
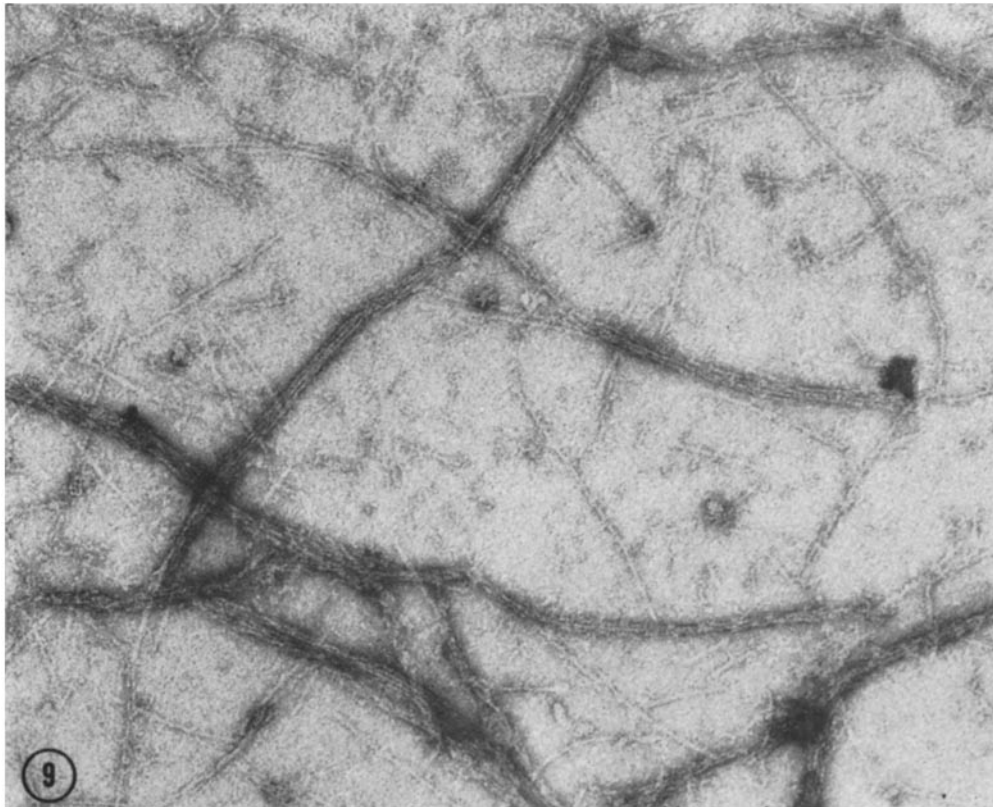
FIGURE 9   Same type of preparation as Fig. 8, but to show lateral aggregation of filaments which was often seen. × 44,000.

100 g of mold. However, this type of preparation contained visible evidence of yellow pigment which was only lost after a third precipitation, so that this does not constitute a complete explanation. Our actomyosin solutions were never water-clear as reported by Hatano and Tazawa (1968) but were consistently white, slightly turbid solutions.

MYOSIN: Our mean yield of "free myosin" was about half that found by Adelman and Taylor (1969 a, 1969 b) by a method in which myosin is separated from actin in the initial extraction at low ionic strength. However, an unknown fraction of myosin is precipitated with the actin fragments (Table I, step 5, page 3) in our procedure.

A significant drawback of the present procedure for preparing *Physarum* myosin is the 50% loss in the myosin-enriched fraction. Although this fraction is useful for certain studies, it would be valuable to be able to obtain a more complete separation. On the other hand, the myosin that is easily obtained in the S_2 fraction is moderately pure and

suitable for further purification. The OD 278/260 nm ratios of 1.3–1.6 suggest little nucleotide contamination, and the Ca-ATPase levels of 0.5–1 μM MP_1/min per mg are close to the values obtained by Adelman and Taylor (1969 b) for column purified myosin. The myosin we obtained is fairly stable; after concentration in sucrose, it lost about 50% activity in 5–6 days.

Hatano and Ohnuma (1970) could not detect sulfhydryl residues in *Physarum* myosin within the limits of the spectrophotometric method of Boyer used by them. Yet they found that parachloromercuribenzoate inhibited the myosin Ca-ATPase. We also found that the myosin displayed the properties of a sulfhydryl enzyme. The details will be presented elsewhere. Both NEM and IA inhibited the Ca-ATPase activity, and the effect of the NEM was prevented by early postincubation with BME. On the other hand, we found, as has been reported (Adelman and Taylor, 1969 b; Hatano and Ohnuma, 1970), that *Physarum* myosin is Mg in-

FIGURE 10   Myosin-enriched actomyosin treated with relaxing solutions (see text) for 18–24 hr. Some-times thick filaments are seen, as here, about 0.3–0.4 μ long, but connections to actin filaments still appear present. × 120,000.

hibited, like skeletal muscle myosin, but also EDTA inhibited, unlike skeletal muscle myosin. The significance of these similarities and differences is not clear, but may be related to certain crucial sulfhydryl groups. In conclusion, it may be said that *Physarum* myosin possesses the properties of an enzyme, some of whose sulfhydryl groups are essential for the Ca-ATPase activity.

Electron Microscopy

MYOSIN: In our experiments on filament formation by myosin alone, we used myosin at about 0.1–0.5 mg/ml. This is lower than the concentrations usually used when studying filament formation by striated muscle myosin, but it is close to the estimated myosin concentration in the slime mold in vivo (Hatano and Ohnuma, 1970). The conditions we tried were designed to enhance ionic or hydrogen bonding by the myosin. Both Kaminer and Bell (1966) and Sanger (in press) reported that, at constant ionic strength, lowering the pH below 7 to pH 6.5 results in increased length of filaments. However, varying the pH at an ionic strength of 0.055 did not result in detectable filament formation from *Physarum* myosin. Sanger also found that lowering the dielectric constant of the medium with propanol increased the S value of the aggregates formed from skeletal muscle myosin. Although the use of 5–30% isopropanol or 2% ethyl acetate did result in some suggestive evidence of dimer formation (Fig. 4), we did not find any evidence of filaments in the *Physarum* myosin preparations tested in this way.

Inoué and Sato had reported (1967) that 45% D_2O had a striking effect in increasing the birefringence of the mitotic spindle and had suggested that this is due to stabilization of the microtubule structures comprising the spindles. Hence, we also tried dialyzing our myosin preparations against 45% D_2O or diluting them to 45% D_2O. In one experiment, after dialysis at 8.5 we did obtain some filament-like structures (as in Fig. 7) but also obtained other types of aggregation. At neutral pH we did not obtain such filament structures.

ACTOMYOSIN: We do not believe that our results with *Physarum* actomyosin fully confirm the report of Hinssen (1970) on thick filaments from *Physarum* actomyosin aged in relaxing solutions. Although we obtained some aggregations that are very much like myosin tail aggregations (Fig. 10, and cf., Ikemoto et al., 1968), we did not find clear-cut thick filaments with globular head re-

gions. Our results appear much more as though further aggregation of myosin tail regions had occurred, while the head regions of the myosin remained or came to be in contact with actin.

PHYSARUM MYOSIN AND RABBIT ACTIN: We used the interaction of *Physarum* myosin with purified rabbit F-actin as one test for the presence of active myosin in our supernatant solutions. It is interesting that although we could uniformly get "decoration" of actin (Figs. 5 and 6), despite a number of trials, this decoration was never very regular in appearance. Compare Figs. 6 *a* and 6 *b*. At present, we do not know whether this irregularity is due to impurities in our myosin preparations, to some interference by the myosin tail regions, or whether it reflects a less good fit between the myosin and actin from these different organisms. In a previous study (Nachmias et al., 1970), the decoration of *Physarum* actin by muscle HMM-S1 seemed to be more regular in the highly purified preparation of *Physarum* actin, so that we cannot rule out such effects here.

IMPLICATIONS: Both Hatano and Ohnuma (1970), and Adelman and Taylor (1969 b) reported that free *Physarum* myosin was soluble at low ionic strengths (0.05–0.03), and yet in the ultracentrifuge both groups found evidence of an increase in the S value from near 6 in 0.5 M KCl to 9–11 in 0.03–0.05 M KCl. It is clear from the electron microscopy of *Physarum* actomyosin, presented here, that myosin "tails" exist, are quite long (near 1400 A), and are often seen to align at low ionic strength (Nachmias and Ingram, 1970, and Figs. 2 and 3). It is plausible, then, to assume that tail-to-tail interaction may take place in free myosin and be the cause of the modest increase in S values of free myosin at low ionic strengths. We have not been able, by negative staining, to obtain conclusive evidence for such tail-to-tail interaction in free myosin, although some examples suggest their presence (Fig. 4). On the other hand, our results are compatible with the further assumption that head-to-tail aggregations do not occur, under physiological conditions, in free myosin and so filaments are not formed. However, a greater degree of aggregation is actually found in actomyosin and myosin-enriched actomyosin treated with solutions containing ATP and Mg salt after superprecipitation has occurred. It may be, therefore, that alignment of the tail regions of the myosin occurs during interaction with actin (possibly as part of contraction), so

that a greater degree of thick filament formation can occur (see Fig. 10). One can show that with a 50% overlap as few as six monomers 1200 A long could construct a filament 0.4 μ long, while tail-to-tail interactions alone would only result in a structure about 0.2 μ long.

Some other studies in the literature on ameboid systems suggest that thick filaments may be formed as part of the process of contraction. Whether or not these are composed of myosin has not been shown. Holberton and Preston (1970) found thick filaments after ATP activation of glycerinated *Amoeba discoides* cells; Wolpert et al. (1964) found thick filaments after ATP treatment of *Amoeba proteus* cytoplasmic extract; Pollard and Ito (1970) concluded that thick filaments were present in cytoplasmic extracts before the start of contraction, but did find extensive numbers after streaming and contraction of the amoeba extracts was complete. We previously reported (Nachmias, 1964) thick filaments in *Chaos chaos* after treatments which induced pinocytosis. Schafer-Daneel (1967) found that thin and thick filaments appeared during glycerination of *A. proteus*, and that some further condensation occurred after an ATP-induced contraction. In conclusion, it can be said that there is some evidence that contraction may cause some changes in the arrangement of filaments in ameboid systems, but it is uncertain whether the appearance of thick filaments precedes contraction or results from contraction.

Until recently, thick filaments had not been seen in *Physarum*. Kessler (Kessler and Nachmias, 1970, and Kessler, in preparation) has found thick filaments, very much like those reported for various species of amebae, in glycerinated *Physarum* undergoing spherule formation. Whether or not such glycerination causes contraction is not certain; it may be that other aspects of actin-myosin interaction are sufficient to cause thick filament formation, and more work must be done on this. At present, crucial evidence is lacking for the assumption that the thick filaments found in sectioned amebae or *Physarum* are indeed myosin filaments, although such a conclusion seems likely.

The point to be emphasized here is that more extensive aggregation of *Physarum* myosin appears to take place when it is present under near physiological conditions together with actin, and some type of orienting effect of the actin seems to occur. We propose, then, the hypothesis that *Physarum* myosin interacts with actin as tail-to-tail oligomers

and that thick filaments result from a drawing together of the myosin oligomers as contraction proceeds, resulting in head-to-tail interactions. We have no clear proof for this at present. In support of this proposal, however, it must be emphasized that in an actomyosin contraction one does not require a structure as elaborate as a thick filament necessarily. Two actin filaments may be drawn together along the axial direction by, minimally, a tail-to-tail dimer of myosin, as long as the forces holding the dimer together are sufficient to withstand the tension exerted, and the actin filaments have appropriate (opposite) polarities. Such a model requires anchor points for the actin filaments so that tension may be exerted.

In conclusion, several predictions can be made from the hypothesis proposed here. First, at low ionic strength, *Physarum* myosin should form a tail-to-tail dimer or oligomer. Second, it should be possible to show anchor points for actin filaments on the cell membrane or on other structures and with a distinctive polarity in relation to such structures. Third, during contraction, actin filaments attached to such relatively fixed points should be moved relative to one another. Some of these predictions can be tested.

ADDENDUM

While this manuscript was in preparation we found that *Physarum* myosin further purified by gel filtration would form aggregates visible by electron microscopy when dialysed against 5 or 10 mM calcium salts. Such aggregates viewed by negative staining were both tail-to-tail and head-to-tail formations. In a few cases, completely formed, short filaments up to 0.45 μ long with a smooth central region and tufted ends were found. Therefore, it can now be said that *Physarum* myosin alone can form filaments. Preliminary work suggests that the extra purification is not necessary. However, it is still the case that actin-myosin interactions result in a greater degree of myosin head-to-tail interaction under *physiological* conditions than occur with myosin alone. Obviously,

the relative importance of the two conditions in vivo remains to be determined.

BIBLIOGRAPHY

ADELMAN, M. R., and E. TAYLOR. 1969 a. *Biochemistry.* 8:4964.

ADELMAN, M. R., and E. TAYLOR. 1969 b. *Biochemistry.* 8:4976.

HATANO, S., and J. OHNUMA. 1970. *Biochim. Biophys. Acta.* 205:110.

HATANO, S., and F. OOSAWA. 1966. *Biochim. Biophys. Acta.* 127:488.

HATANO, S., and M. TAZAWA. 1968. *Biochim. Biophys. Acta.* 154:507.

HINSSEN, H. 1970. *Cytobiologie.* 2:326.

HOLBERTON, D. V., and T. M. PRESTON. 1970. *Exp. Cell Res.* 62:473.

HUXLEY, H. E. 1963. *J. Mol. Biol.* 7:281.

INOUÉ, S., and H. SATO. 1967. *J. Gen. Physiol.* 50:259.

IKEMOTO, N., A. KITAGAWA, A. NAKAMURA, and D. GERGELY. 1968. *J. Cell Biol.* 39:620.

KAMINER, B., and A. BELL. 1966. *J. Mol. Biol.* 20:39.

KAMIYA, N. 1959. *Protoplasmatologia.* 8:1.

KESSLER, D., and V. T. NACHMIAS. 1970. *J. Cell Biol.* 47 (2, Pt. 2):105 a. (Abstr.)

LOWEY, S., H. SLAYTER, A. G. WEEDS, and H. BAKER. 1970. *J. Mol. Biol.* 42:1.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. T. RANDALL. 1951. *J. Biol. Chem.* 193:265.

NACHMIAS, V. T. 1964. *J. Cell Biol.* 23:183.

NACHMIAS, V. T., H. E. HUXLEY, and D. KESSLER. 1970. *J. Mol. Biol.* 50:83.

NACHMIAS, V. T., and W. C. INGRAM. 1970. *Science (Washington).* 170:743.

POLLARD, T. D., and S. ITO. 1970. *J. Cell Biol.* 46:267.

REES, M. K., and M. YOUNG. 1967. *J. Biol. Chem.* 242:4449.

SANGER, J. 1972. *Cytobiologie.* 4:450.

SCHAFER-DANEEL, S. 1967. *Z. Zellforsch. Mikrosk. Anat.* 78:441.

SOBER, H. A., and R. A. HARTE, editors. 1970. CRC Handbook of Biochemistry. The Chemical Rubber Co., Cleveland, Ohio. 2nd edition.

SZENT-GYÖRGYI, A. 1951. Chemistry of Muscular Contraction. Academic Press Inc., New York. 2nd edition.

SZENT-GYÖRGYI, A. G. 1953. *Arch. Biochem. Biophys.* 42:305.

TAUSSKY, H. M., and E. SCHORR. 1952. *J. Biol. Chem.* 202:675.

WOLPERT, L., C. M. THOMPSON, and C. H. O'NEILL. 1964. *In* Primitive Motile Systems in Cell Biology. R. D. Allen and N. Kamiya, editors. Academic Press Inc., New York.