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Physarum Actin. Observations on Its Presence, Stability, and Assembly in Plasmodial Extracts and Development of an Improved Purification Procedure†

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ABSTRACT: Actin is readily extracted from plasmodia of *Physarum polycephalum* by low ionic strength solutions which do not solubilize the plasmodial myosin. The actin in such extracts exists predominantly as a monomer which slowly denatures, apparently via removal of bound nucleotide, and is subsequently proteolyzed. However, the native monomeric actin can be induced to assemble into polymeric arrays under appropriate solvent conditions. Actin assembly is dependent on the addition of ATP and is a function of KCl and $CaCl_2$ concentrations. These observations have allowed the development of an improved actin purification scheme which is simple, rapid, and efficient, yielding ~60 mg of protein from 100 g of plasmodium. The actin thus obtained is pure, stable, and comparable to that obtained by previously described procedures. Furthermore, the observations suggest that actin polymers may be metastably assembled in vivo and raise the possibility that actin assembly, and plasmodial movements, could be regulated via alterations in intracellular concentrations of nucleotide and/or divalent cation.

Since the demonstration, in the late 1960's, that plasmodia of the myxomycete slime mold *Physarum polycephalum* contain actin and myosin (Hatano and Oosawa, 1966; Adelman and Taylor, 1969a,b; Hatano and Ohnuma, 1970) such proteins have been isolated from a wide variety of other nonmuscle sources (Pollard and Weihing, 1974; Goldman et al., 1976). These nonmuscle actins and myosins closely resemble their muscle counterparts, the degree of evolutionary conservation being particularly striking in the case of the actins. Thus, for example, *Physarum* plasmodial actin is like its muscle homologue in terms of sedimentation and diffusion coefficient, molecular weight, nucleotide binding, filament formation, and interaction with heterologous as well as homologous myosin (Hatano and Oosawa, 1966; Adelman and Taylor, 1969a,b; Hatano, 1973). Moreover, the two proteins show substantial similarity in amino acid composition (Hatano and Oosawa, 1966) and at least partial sequence homology (Jockusch et al., 1974).

In contrast to the similarity of their constituent actins and myosins, nonmuscle systems show considerable diversity, both among themselves and in comparison with muscle, in the details of their movements. It is of obvious interest to determine the extent to which this phenomenological diversity reflects subtle differences between the constituent macromolecules and to this end it is necessary to characterize at least a few nonmuscle actins and myosins in detail. For such studies *Physarum* plasmodia remain a particularly suitable system, for reasons which dictated their earlier use; they manifest extremely vigorous protoplasmic streaming (Kamiya, 1959) and can be easily cultured in large quantities. Furthermore, the various stages of the *P. polycephalum* life cycle display a broad range of interesting motility phenomena and the availability of genetic approaches to this system makes it a logical choice for systematic studies of motility-related macromolecules (Jacobson et al., 1976).

This paper, the first of a new series on motility-related proteins from *Physarum,* describes observations on the actin present in crude plasmodial extracts which may have bearing on our understanding of the in vivo organization of plasmodial actin and which have allowed the development of an improved protocol for its purification. These results also form the basis for a refined plasmodial myosin isolation procedure and for studies of factors which interact with, and modulate the assembly and/or interaction of, the actin and myosin (manuscripts in preparation). Some aspects of these studies have appeared in preliminary form (Adelman, 1974a,b; Jacobson et al., 1976).

Materials and Methods

Culturing and Harvesting of Plasmodia. The *P. polycephalum* cultures used for these experiments were all derived from a single sample of M_3c VIII spherules supplied by Dr. Joyce Mohberg (McArdle Laboratory, University of Wisconsin). Spherule and microplasmodial stocks (used primarily for restarting plasmodial cultures) were maintained according to Daniel and Baldwin (1964). Plasmodia were cultured on oatmeal flakes and harvested after migration over agar surfaces (Adelman and Taylor, 1969a). As in the earlier work, freshly harvested plasmodia were used for all experiments; however, plasmodia were not washed with water prior to weighing and homogenization.

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Preparation of Extracts. Plasmodia were homogenized in 1–4 volumes (mL/g) of appropriate buffer using 8–10 passes of a Teflon pestle, motor-driven tissue grinder (A. H. Thomas Co., size C, pestle rotating at 500–1000 rpm) followed, occasionally, by 1–3 passes in a tight-fitting hand-operated tissue grinder (A. H. Thomas Co.) of the TenBroeck type (1931). Homogenates were left, with occasional stirring, for 15–60 min at 3 °C and then centrifuged to remove particulates, usually for ~30 min at 150 000–200 000g_{max}. The supernatant fluid was decanted through a layer of nytex cloth (No. 130; Tobler, Ernst, and Traber, Inc., New York, N.Y.) to remove fatty scum. All subsequent operations, unless otherwise noted, were carried out at 0–3 °C.

Two types of alternative extracts were occasionally used for comparative purposes. Plasmodial *exudates* ("Cell sap"; Adelman and Taylor, 1969a) were produced by centrifuging intact plasmodia [20 min-39K-SW41 (250 000); i.e. for 20 min at 39 000 rpm in a Beckman rotor SW41 under which conditions the maximum g force is ~250 000g] and removing the clear yellow fluid expressed. *Microplasmodial extracts* were prepared by centrifuging microplasmodia (2–5 min at ~500–1000g_{max}), washing them once in TM,[1] recentrifuging, and processing the resulting pellets as for plasmodia.

Protein Measurement. Samples were either dialyzed vs., or suspended in, the appropriate buffer or were acid precipitated and washed (Adelman et al., 1973) prior to analysis by the method of Lowry et al. (1951).

ATPase Assays. After dialysis, usually vs. TE,[1] samples were analyzed according to Adelman and Taylor (1969a) except that the high KCl, calcium-ATPase medium (which is fairly specific for myosin) contained 500 mM KCl, 5 mM $CaCl_2$, 1 mM ATP, 50 mM Tris, pH 8.0.

Electrophoresis. Samples were analyzed by electrophoresis in polyacrylamide slab gels using the discontinuous sodium dodecyl sulfate buffer system of Neville (1971) superimposed on an exponential acrylamide gradient gel. Details of the protocol including sample preparation, staining (Fairbanks et al., 1971) and destaining, photography, and densitometry of the gels will be presented elsewhere. For molecular weight estimations a mixture of standard proteins (S) was run in the left- and right-hand channels of each gel; the left-hand axis indicates the subunit molecular weights ($\times 10^{-5}$) of the standards, which included rat muscle myosin (2.00), β-galactosidase (1.30), bovine plasma albumin (0.68), catalase (0.60), ovalbumin (0.43), α-chymotrypsinogen A (0.26), ribonuclease A (0.14), cytochrome c (0.12), and insulin (a poorly resolved doublet plotted as ~0.03).

To the right of all gel photos are arrows indicating the top of the gel (upper reservoir-stacking gel interface) (T), the stacking gel–running gel interface (I), the bottom of the gel (B), and additional arrows as discussed in the text.

Quantitation of Actin Sedimentation. Extracts were diluted to give (in a final volume of 10 mL) the protein concentrations and solvent conditions indicated in the appropriate figure legend and were incubated for 30 min at 3 °C in plastic centrifuge tubes prior to centrifugation for 4 h either at 39 K (42.1) or 41 K (60 Ti), the g_{max} in each case being ~168 000. These conditions were chosen to sediment completely all proteins of $s^0_{20,w} \geq 40$ S which should include fully polymerized *Physarum* actin (Adelman and Taylor, 1969b). Since small oligomers and even actin monomer would be at least partially sedimented under these conditions there is an inherent "background" of at least 5–10%. The amount of actin in the pellet was estimated by gel densitometry. Although replicate experiments and duplicate scans of a given sample agreed to within ±5%, the precision of the assay is less since it involves assumption of some baseline whose value depends on the extent of overlap from nearby bands. Judging from the values obtained by assigning either "minimal" or "maximal" baselines, quantitation of the actin in such samples as channels 1–16 of Figure 3 was accurate to ±10–20% while estimates of the actin in crude extracts had ~25–50% error limits.

Miscellaneous. Thin-layer chromatography on PEI-cellulose plates was carried out according to Randerath and Randerath (1967). The general procedures of Adelman and Taylor (1969a,b) were followed for column chromatography, viscometry, electron microscopy, centrifugation, etc. Rat muscle actin was prepared essentially according to Spudich and Watt (1971) while rat muscle myosin was prepared by a combination of conventional techniques involving low ionic strength precipitation and high-speed centrifugation to remove contaminating actin (M. R. Adelman, unpublished).

Enzyme grade disodium ATP from Sigma or ICN Nutritional Biochemicals was used for assays while Sigma grade II disodium ATP was used for preparative purposes. Trizma base, catalase, bovine plasma albumin, ovalbumin, α-chymotrypsinogen A, ribonuclease, cytochrome c, insulin, ADP, cAMP, and $PhMeSO_2F$ were purchased from Sigma; ADP, AMP, ITP, CTP, UTP, and GTP were from P-L Biochemicals; AMPPPNP was from ICN Nutritional Biochemicals; β-galactosidase was from Worthington; and AMPPCP was from Miles. All solutions were prepared in Ultra Pure water (Continental Water Conditioning Corporation) and were Millipore filtered (0.45 or 1.2 μm) prior to use.

Results

(a) *Actin in Plasmodial Extracts.* When *Physarum* plasmodia were homogenized in TM buffer containing various concentrations of KCl or sodium pyrophosphate (PP_i), the resulting extracts all contained similar amounts of protein, but their calcium-activated ATPase activity was an increasing function of the KCl or PP_i present in the homogenization medium (Figure 1). Comparable, but less-detailed, results presented earlier (Adelman and Taylor, 1969a) had led to the use of TM·PP_i to extract *Physarum* actomyosin. Examination of such plasmodial extracts by sodium dodecyl sulfate gel electrophoresis (Figure 2) revealed a large number of discrete polypeptide bands. In the high molecular weight region of such gels at least one band (mol wt ~225 000) could be seen which increased in intensity in parallel with the ATPase activity of the extracts; this is the major polypeptide of plasmodial myosin (Adelman, 1975; Jacobson et al., 1976). As was expected, the myosin-rich extracts also contained a prominent band at ~45 000 daltons which is the position to which purified muscle or plasmodial actins migrate. Contrary to expectation, however, Figure 2 reveals the stain intensity in the actin region of the gels was nearly independent of the extraction solvent, being essentially as great in TM extracts as in ones prepared with high KCl or PP_i. Although previous experiments had indicated

[1] Abbreviations used are: TM, 10 mM Tris-maleate, 5 mM HSEtOH, pH 7.0; TM·PP_i, TM plus 50 mM sodium pyrophosphate; TE, 10 mM Tris, 1 mM EDTA, 5 mM HSEtOH, pH 8.0; TAMP, 1 mM Tris, 1 mM ATP, 5 mM HSEtOH, 5 mM PP_i, pH 7.8; TAM, 1 mM Tris, 1 mM ATP, 5 mM HSEtOH, pH 7.8; EDTA, (ethylenedinitrilo)tetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; cAMP, cyclic adenosine 3′:5′-monophosphoric acid; GTP, guanosine 5′-triphosphate; ITP, inosine 5′-triphosphate; CTP, cytidine 5′-triphosphate; UTP, uridine 5′-triphosphate; AMPPNP, adenosine 5′-(β,γ-imino)triphosphate; AMPPCP, adenosine 5′-(β,γ-methylene)triphosphate; $PhMeSO_2F$, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; PP_i, inorganic pyrophosphate; HSEtOH, β-mercaptoethanol; BP, proteolytic breakdown fragment.

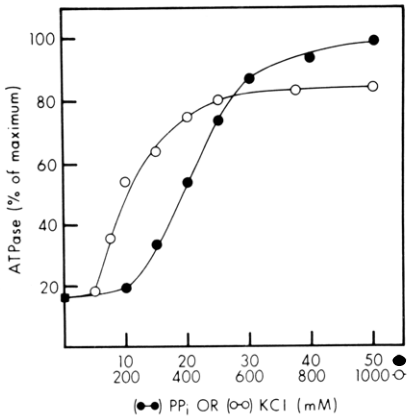

FIGURE 1: Calcium-activated ATPase of various plasmodial extracts. Plasmodia were homogenized (1 g/3 mL of solution) in TM buffer with KCl or PP_i added to give the indicated final concentrations. Homogenates were incubated for 60 min at 3 °C and centrifuged 20 min–39K–42.1 (168 000), and the supernates (extracts) were dialyzed vs. TE prior to protein and ATPase assay. The data presented here are from two experiments involving both KCl and PP_i extracts and two additional ones in which only PP_i extracts were prepared. The various extracts had protein contents which ranged from 3.25 to 5.50 mg/mL but within any one experiment the range was only ~10%, with TM extracts having slightly less protein than high KCl or PP_i extracts. Calcium ATPase data were computed as specific activity (μM P_i mg^{-1} min^{-1}) and then, to allow comparison between different experiments, were expressed as a percent of the maximum specific activity measured in the given experiment. The maximum specific activities ranged from 0.026 μM mg^{-1} min^{-1} to 0.039. The measured values ranged about the indicated means by no more than ±10% of the maximum and for most points the range was about ±5% of the maximum: (■) TM extracts; (○) TM plus various concentrations (mM) of KCl; (●) TM plus various concentrations (mM) of PP_i.

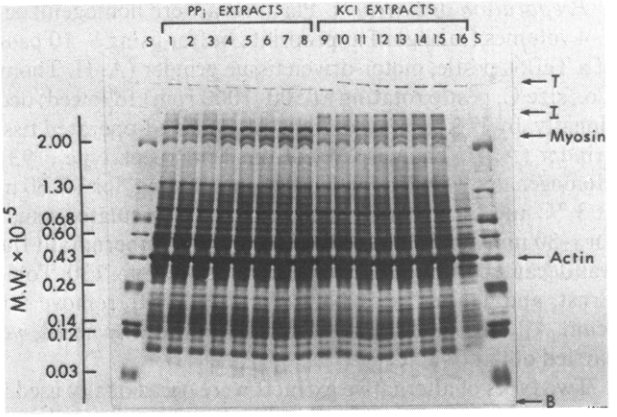

FIGURE 2: Sodium dodecyl sulfate gel analysis of various plasmodial extracts. The extracts were prepared exactly as for Figure 1 but were not dialyzed prior to analysis. Each channel received an identical aliquot (by volume) of an extract prepared with TM plus various levels of PP_i (channels 1–8) or KCl (channels 9–16). Analysis of the protein content by acid precipitation and washing (see Materials and Methods) indicated the applied aliquots contained ~37 to ~48 μg of protein with the PP_i extracts consistently closer to the upper value and the KCl ones closer to the lower values. The extraction buffers contained TM plus either PP_i or KCl at the millimolar levels indicated. Channels 1–8 (PP_i): (1) 0; (2) 10; (3) 15; (4) 20; (5) 25; (6) 30; (7) 40; (8) 50. Channels 9–16 (KCl): (9) 0; (10) 100; (11) 150; (12) 200; (13) 300; (14) 400; (15) 500; (16) 1000. The decreased stain intensity in channel 16 (cf. 9–15) indicates that only at 1000 mM KCl did the formation of a precipitate (presumably potassium dodecyl sulfate) complicate the gel analyses.

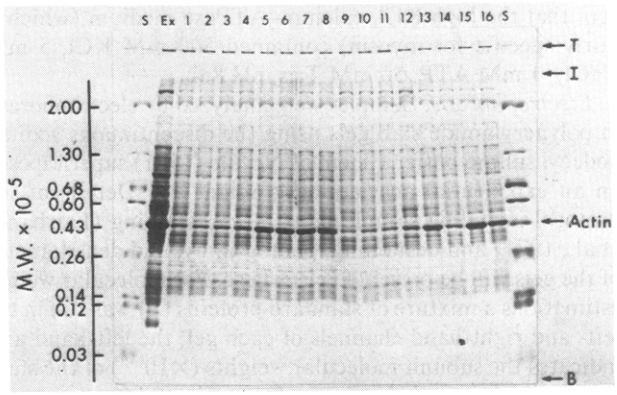

FIGURE 3: Sedimentation of actin under various conditions. A TM extract was prepared under standard conditions (Materials and Methods) and its protein content was measured to be ~7.15 mg/mL. The extract was then diluted to a final concentration of either 6.44 mg/mL (channels 1–8) or 0.715 mg/mL (channels 9–16) using appropriate stock solutions to give as final conditions (in addition to the protein and TM buffer) the following components: (channels 1 and 9) no addition; (2 and 10) 100 mM KCl, 1 mM $MgCl_2$; (3 and 11) 100 mM KCl, 1 mM $CaCl_2$; (4 and 12) 1 mM ATP; (5 and 13) 100 mM KCl, 1 mM ATP; (6 and 14) 100 mM KCl, 1 mM ATP, 1 mM $MgCl_2$; (7 and 15) 100 mM KCl, 1 mM ATP, 1 mM $CaCl_2$; (8 and 16) 100 mM KCl, 1 mM ATP, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$. Incubation and centrifugation were as described in Materials and Methods. After centrifugation the supernates were discarded and the pellets were resuspended in a volume of buffer equal to the volume of extract from which the pellet derived. The gel channels then received equal volumes of the untreated extract (e.g., ~57 μg) or of the pellets derived therefrom. Thus, if actin sedimentation is complete and all material at mol wt 45 000 is actin, the intensity of the actin band in a particular channel should equal that in the channel containing extract.

actin to be a major component of the plasmodia (ca. 2–4% of the protein; Adelman and Taylor, 1969b) there was of course no reason to assume that all of the material present in this region of the gels was actin.

Indeed, preliminary attempts to demonstrate that the 45 000-dalton material in TM extracts was actin were uniformly unsuccessful: this reflected not only an initial failure to understand the state of the actin but also the unsuitable nature of many traditional actin assays, such as viscometry, electron microscopy, and myosin–ATPase activation assays, for systems of this type (see Discussion). As shown by the gel profiles in Figure 3 little of the 45 000-dalton material was sedimented (Figure 3, channel 1) under conditions (Materials and Methods) chosen to sediment plasmodial F-actin, and the addition of KCl (100 mM) alone, or in combination with 1 mM $MgCl_2$ or 1 mM $CaCl_2$ (Figure 3, channels 2 or 3), did not alter this result. Column chromatography on Sephadex G-200 (Figure 4) under any of these solvent conditions revealed that most of the material in question emerged at $K_D \sim 0.5$ which is the position expected for a globular protein of mol wt ~45 000. While these results suggested that the 45 000-dalton material represented either denatured actin, or non-actin-like proteins, or a mixture of both, further studies revealed that the material could be made to sediment under appropriate solvent conditions and the following experiments clearly demonstrate that most of the 45 000-dalton protein in the crude extracts is actin.

Experiments such as those in Figures 3 and 4 indicated that the actin was present as an unusual, but nondenatured, monomer (the presence of small amounts of low *n*-mers was not excluded) which could be made to assemble into a sedimentable polymer, or aggregate (i.e., high *n*-mer), by adding ATP; they also suggested a role for KCl, $CaCl_2$, and $MgCl_2$ in modulating this assembly (Figure 3, channels 4–8). Furthermore, the assembly was, under some conditions, significantly affected by total protein concentration (Figure 3, compare channels 9–16 with 1–8). Detailed studies were then undertaken with two specific objectives: (1) to define optimal conditions for recovery of actin from TM extracts, thus allowing the further purification of the protein, and (2) to gain some

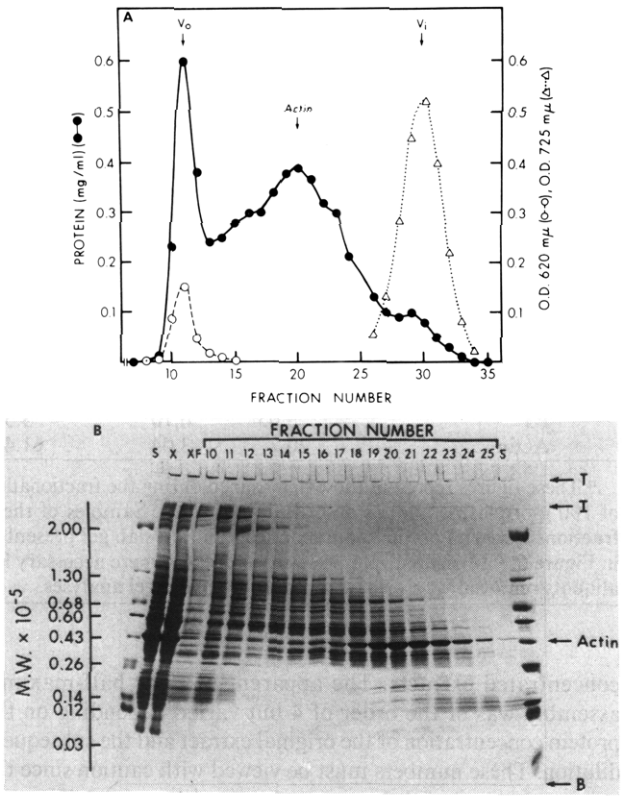

FIGURE 4: Extraction and chromatography of actin in KCl + $MgCl_2$. Freshly harvested plasmodium was homogenized (1 g/2 mL) in TM buffer containing 100 mM KCl and 1 mM $MgCl_2$ and was *immediately* centrifuged (~10 mL/tube) 20 min–39K–42.1 (168 000). The extract (X) was quickly filtered through an 8-μm Millipore filter to produce XF. As judged by protein (both X and XF contained ~7.70 mg/mL) and sodium dodecyl sulfate analyses (Figure 4B) this filtration removed little if any protein; it did, however, result in improved resolution during the subsequent chromatography and prevented the sample basket of the column from clogging with some gelatinous material (slime?). Immediately after filtration 3 mL of XF was applied to a Sephadex G-200 column (2.5 cm × 25.5 cm high) which was equilibrated in, and eluted (downward flow; ~25 mL/h) with, the extraction buffer. Fractions of ~5-mL volume were collected and analyzed for protein content (Figure 4A). Equal aliquots (60 μL) of selected fractions were then analyzed by sodium dodecyl sulfate gel electrophoresis (Figure 4B). (A) Elution profiles of protein (●-●), blue dextran (○- - -○), and inorganic phosphate (△- - -△) on the column. Blue dextran (detected by OD 620 nm) and inorganic phosphate (detected in the standard ATPase assay as OD 725 nm) were chromatographed together in a separate but identical run of the column to provide calibrations of the void volume (V_0) and the included volume (V_i). (B) Sodium dodecyl sulfate gel analyses of X and XF (~62 μg of protein each) and of selected fractions from the column elution. As judged by visual inspection the actin band was most intense in fraction number 20 and this is indicated by the labeled arrow in A.

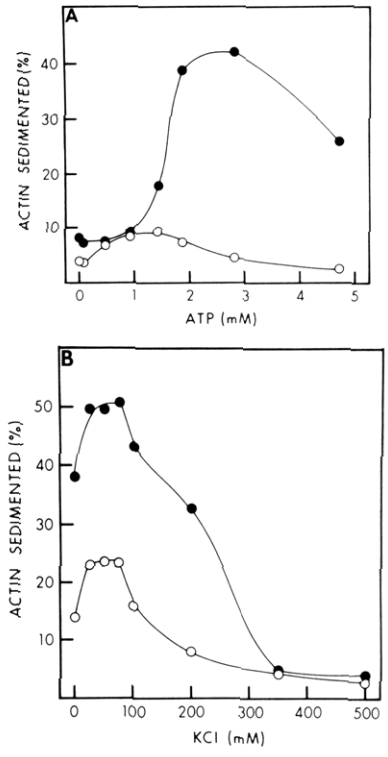

FIGURE 5: (A) Sedimentation of actin as a function of ATP concentration. Preparation of the extract, general conditions of incubation, centrifugation, and gel analyses were as described in Materials and Methods. A series of aliquots of the extract was run, at rather low sample loads, in order to facilitate a reasonably accurate estimate of the total actin present but as explained in Materials and Methods such estimates are probably subject to significant error and thus the percent scale on the ordinate may be in error; however, this is a constant error for all samples and may therefore be regarded as a scaling factor. The general shape, relative heights, etc., of such curves were very reproducible. All samples were incubated in TM with the indicated final concentrations of ATP (but *no* other additions) at final protein concentrations of either 4.95 mg/mL (●) or 0.55 mg/mL (○). (B) Sedimentation of actin as a function of KCl concentration. All manipulations and analyses were as described in A. All samples were incubated with the indicated concentrations of KCl and either 2 mM ATP, 4.16 mg of protein/mL (●) or 1 mM ATP, 0.57 mg of protein/mL (○). The apparent optima in this experiment were ca. 50–75 mM KCl but slightly higher values (100 mM) have also been observed.

insight as to the mechanism(s) controlling actin assembly in vitro and, ultimately, in vivo. Assembly studies were carried out over a range of protein concentrations since those at fairly high concentration (undiluted extracts) would be most useful in designing a practical isolation scheme while ones involving more diluted extracts might minimize any nonspecific protein–protein interactions and would allow at least modest control over ionic composition of the medium with minimal contribution due to plasmodial contents. Several experiments showed that under optimal conditions (~100 mM KCl, 1–2 mM ATP) actin assembly was nearly constant over the total protein concentration range from ~7.5 to ~1 mg/mL. Further experiments were therefore conducted either with minimally diluted extracts (initial concentration = 5–7 mg/mL) or with tenfold diluted extracts; in the latter case assembly was usually low even under optimal solvent conditions.

Figure 5A indicates the dependence of actin assembly on the concentration of added ATP. In undiluted extracts polymerization was optimal when [ATP] exceeded 2 mM whereas at lower protein concentration assembly appeared optimal at ~1 mM ATP. ATPase measurements suggested extensive hydrolysis of the added nucleotide under these experimental conditions but since interpretation of measurements of P_i liberation in such crude mixtures was ambiguous the fate of the added ATP was followed using thin-layer chromatography. The results of these experiments will be presented in detail elsewhere but, with relevance to the present studies, it should be commented that added ATP is rapidly broken down by a calcium-dependent, PP_i-inhibited enzyme(s) directly to AMP and that the AMP is further degraded in a series of reactions. Under conditions relevant to the present studies no more than a small percentage of the added ATP survives the first few minutes of incubation in the crude extract. Thus, the "true" optimum concentration of ATP is likely to be significantly lower than 1 mM. In diluted extracts assembly fell off as the [ATP] was raised above 2 mM, and 5 mM ATP reproducibly yielded no more actin assembly than was achieved without added ATP; inhibition by high ATP was also demonstrable at higher protein concentrations. As shown by the data in Figure

TABLE I: Specificity of ATP in Inducing Actin Sedimentation.[a]

Addition[b]	Substitution[c] (% of control)	Competition[d] (% of control)
1. None	13.5	100
2. ATP	100	97.5
3. ITP	86.3	102.5
4. GTP	15.2	72.5
5. CTP	14.8	35.5
6. UTP	14.4	16.8
7. AMPNP	96.3	69
8. AMPPCP	21.1	64
9. ADP	14.6	13
10. AMP	28.1	101
11. cAMP	20.4	82.5
12. PP_i	11.1	90
13. $PP_i \times 10$	10.4	64
14. EDTA	6.7	36.5
15. EGTA	9.6	47.5

[a] Actin sedimentation was assessed as described under Materials and Methods. [b] Compounds were added (lines 2–15) to a final concentration of 1 mM except in line 13 where PP_i was present at 10 mM. [c] All tubes contained TM extract at a final protein concentration of 0.69 mg/mL and in addition contained 0.10 M KCl, TM, and 10^{-4} M $CaCl_2$. In the presence of 1 mM ATP (line 2) sedimentation of actin was ~64% of that in the extract. This value was taken as the control, set equal to 100%, and all other values are expressed as a percentage of the actin sedimented in line 2. [d] All tubes contained TM extract at a final protein concentration of 0.71 mg/mL and in addition contained 0.10 M KCl, TM, 10^{-4} M $CaCl_2$, and 0.5 mM ATP. Thus, line 1 represents the sedimentation of actin in the presence of 0.5 mM ATP. Under this condition sedimentation was ~80% of the total in the extract and for purposes of comparison line 1 was set equal to 100 (control) and all other values are expressed relative to line 1. It should be emphasized that in line 2 the tube contained 0.5 mM ATP + 1.0 mM ATP (total = 1.5 mM ATP) while in lines 3–15 the basal 0.5 mM ATP was supplemented with the other compounds indicated.

TABLE II: Purification of Plasmodial Actin.[a]

Fraction	Vol[b] (mL)	Protein content (mg/mL)	Total protein (mg)
Homogenate	395	10.40	4108
Extract	346	6.00	2076
P1	172	0.75	129
S1	412	2.95	1215
P2	17.2	4.96	85.3
Crude Actin	33.3	10.20	340
Void	137	0.79	108
Intermediate	103	0.73	75.2
Column Actin	207	0.74	153.2
S2	203	0.34	69.0
P3	5.05	0.70	3.5
Actin	20.2	3.04	61.4

[a] These figures represent those determined during the fractionation of 100 g of plasmodium as described in the text. Samples of these fractions were run on the sodium dodecyl sulfate slab gel presented in Figure 6. [b] Measured volumes were corrected where necessary for aliquots removed, at a prior step, for protein and gel analyses.

5B, actin assembly required, in addition to ATP, the presence of KCl. Observations on diluted extracts suggested assembly was maximal at 50–100 mM KCl and fell off as the salt concentration was raised. Even in undiluted extracts 500 mM KCl inhibited actin assembly.

Table I is representative of a series of experiments which examined the specificity of ATP in causing actin assembly. While these data derive from diluted extracts to which KCl and $CaCl_2$ had been added, similar results have been obtained with undiluted extracts (no KCl or $CaCl_2$ added) and with diluted extracts to which KCl but no $CaCl_2$ was added. Only ITP and the ATP analogue AMPPNP could substitute effectively for ATP. Pyrophosphate was without significant effect and the chelators EDTA and EGTA depressed sedimentation below the "no addition" value (cf. lines 14 and 15 with line 1). Of the nucleotides tested for their ability to compete with ATP (at a 2:1 ratio and under conditions where the excess ATP itself did not depress assembly), ADP and UTP showed strong inhibition, while all others had more modest effects. At very high levels PP_i was capable of causing some inhibition, perhaps due to its chelating ability, since both EDTA and EGTA were inhibitory.

Because the inhibition by EGTA was reversible with added calcium while that by EDTA was not reversed with magnesium (data not shown) and in view of the obvious augmentation in actin sedimentation observed with added calcium in, e.g., Figure 3 (cf. channel 15 with channel 13), several experiments with calcium buffer systems were carried out. These studies (not shown) revealed a strong calcium requirement in diluted extracts but no demonstrable one (using 3 mM EGTA) in concentrated extracts. The apparent pCa for half-maximal assembly was of the order of 4 but varied depending on the protein concentration of the original extract and the subsequent dilution. These numbers must be viewed with caution since the calcium content of the extracts was not determined. The conclusion that calcium was required for stable assembly of the actin in crude extracts was supported by a series of experiments analogous to that presented in Figure 4: only with extraction and subsequent chromatography in the presence of KCl, ATP, *and* calcium was it possible to demonstrate the elution from G-200 of most of the actin as a polymer.

The inhibitory effect of $MgCl_2$ suggested by Figure 3 (cf. channel 14 with channel 13) was reproducible but only demonstrable with moderately diluted extracts analyzed at 3 °C. In general, under suboptimal conditions of ATP, KCl, and/or Me^{2+}, actin assembly was slightly enhanced by incubation at 20 °C (cf. 3 °C) and was a decreasing function of pH over the range 6.0 to 8.0 (data not shown).

Two sets of observations were made on the stability of the actin monomer: these concern its denaturation and proteolytic breakdown and will be described in detail elsewhere. However, they are mentioned briefly here because of their relevance to the design of the purification scheme (Results, section b). When the actin in TM extracts was assembled by the addition of KCl and ATP it remained stable for many hours and, even after overnight storage at 3 °C, most of the actin was sedimentable. In contrast, if the TM extract was stored with the actin in the monomeric state (even with sufficient PP_i and sucrose to totally inhibit the proteolysis discussed below), the ability of the actin to assemble upon subsequent addition of KCl and ATP was gradually lost. The half-life of this effect was about 4–6 h, thus placing constraints on the purification scheme. When TM extracts were stored at 3 °C, the actin slowly disappeared, apparently due to the action of endogenous protease(s). Numerous commercially available inhibitors failed to block this breakdown, with the exception of $PhMeSO_2F$ which had a marginal effect. However, PP_i (5–10 mM) and/or sucrose (0.50 M) were fairly effective in preventing actin degradation and use is made of the former in the purification scheme. In view of the proteolysis, adequate controls were carried out to ensure that the failure of the actin in a TM extract to sediment in a given situation was not simply due to augmented degradation.

(b) *Purification of Plasmodial Actin.* The observations presented in Results, section a, concerning the extraction of actin by TM buffer, the conditions required for actin assembly, and factors related to its stability have allowed the development of an improved procedure for the purification of plasmodial actin. The protein content of each fraction during a representative preparation is given in Table II and sodium dodecyl sulfate gel profiles of the various fractions are shown in Figure 6.

(1) The freshly harvested plasmodia were homogenized in 3 volumes (mL/g) of TM containing 5 mM PP_i and 0.5 mM $PhMeSO_2F$. The inclusion of PP_i at this step and during column chromatography (step 7 below) not only improved the yield of actin but also reduced the contamination of the final product with the proteolysis fragment BP (see below). The inclusion of $PhMeSO_2F$ was essentially precautionary. Inclusion of sucrose to provide maximal protection against proteolysis was found impractical since the elevated viscosity necessitated longer centrifugation and thus actin losses due to denaturation were increased. The protein content of the homogenates thus produced varied from ~3 to 4% of the wet weight of plasmodium. Crude quantitation of the actin in the homogenate by gel electrophoresis suggested that the actin represented ~5–10% of the total plasmodial protein.

(2) The homogenate was immediately centrifuged 30 min–40K–45Ti (186 000) to remove particulates. The sedimented residue was used for the preparation of myosin (Adelman, 1975; Jacobson et al., 1976; detailed manuscript in preparation) or was discarded. The supernatant, after filtration through nytex cloth to remove fatty scum, constituted the crude extract, which contained ~50% of the plasmodial protein and most of the actin. Electrophoretic analyses suggested that ca. 15% of the protein in the extract was actin.

(3) The crude extract was immediately recentrifuged 60 min–40K–45Ti (186 000). The resulting pellets (P1), although quite large, were fairly clear, and contained rather little protein (Table II) and almost no actin (Figure 6, channel 3). The removal of this material (possibly glycogen and/or polysaccharide slime) considerably facilitated subsequent manipulations and had it not been for the gradual denaturation of the actin which occurred during such "preprocessing" it might have been desirable to prolong this centrifugation.

(4) The supernatant from step 3 was diluted by ca. 10–20% to give as final conditions 100 mM KCl and 2 mM ATP. After 30 min of incubation at 3 °C the solution was centrifuged 5 h–44K–45Ti (230 000) to yield supernatant (S1) and pellet fractions. Analysis of S1 revealed it to have ~60% of the protein in the crude extract (Table II) but at most 10–20% of the original actin (Figure 6, channel 4).

(5) The slightly turbid yellow pellets from step 4 were resuspended in 0.10 vol (relative to the initial extract) of TAMP containing 0.5 mM $PhMeSO_2F$ and were dialyzed vigorously vs. 1 L of TAMP for 4–5 h. While more prolonged dialysis would have led to more complete depolymerization of the actin [and therefore better resolution during the ensuing chromatographic separation, (step 7 below)] this was found to be offset by lower yields of actin and greater content of BP (see below) if dialysis was prolonged.

(6) The dialyzed material from step 5 was centrifuged 60 min–45K–60Ti (204 000) to yield pellet (P2) and supernatant (crude actin) fractions. P2 contained very little protein (Table II) and almost no actin (Figure 6, channel 5) but its removal was essential to preventing clogging of the G-200 column used in the following step.

(7) The crude actin fraction (nytex filtered to remove any traces of floating lipid scum) was then subjected to gel chromatography on Sephadex G-200 in TAMP. Assay of collected fractions (Figure 7A) revealed a major protein peak at the void volume and another at $K_D \sim 0.5$; sodium dodecyl sulfate gel analyses (Figure 7B) showed that actin eluted primarily, but not exclusively, in the second peak. For routine analyses fractions corresponding to the voided peak and the intermediate zone (see legend to Figure 7) were pooled. Both the void and intermediate pools (Table II; Figure 6, channels 8 and 9) contained significant amounts of actin, possibly because the brief dialysis (step 5) was insufficient for complete depolymerization.

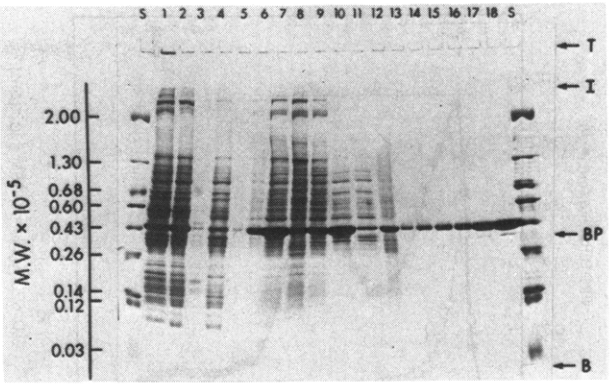

FIGURE 6: Sodium dodecyl sulfate gel analysis of various fractions obtained during purification of *Physarum* plasmodial actin. The samples analyzed here are the same as those for which protein contents are given in Table II. The samples were as follows: (channel 1) 83.2 μg (protein) of homogenate; (2) 48 μg of extract; (3) 3.0 μg of P1; (4) 28.3 μg of S1; (5) 2.0 μg of P2; (6) 8.2 μg of crude actin; (7) 40.8 μg of crude actin; (8) 31.6 μg of void; (9) 29.2 μg of intermediate; (10) 29.6 μg of column actin; (11) 13.6 μg of S2; (12) 17.5 μg of P3. Channels 13–18 contain purified actin at loads of 1.0, 2.0, 3.0, 4.0, 10.0, and 20.0 μg, respectively. The arrow labeled BP indicates the minor contaminant in the actin (see text). It should be noted that the samples in channels 1–6 all derive from the same amount of plasmodium and thus allow, by visual inspection, an estimate of the partitioning of the actin in the early stages of the fractionation. Similarly, channel 11 contains the same amount of fluid (S2) as the material (column actin, channel 10) from which it was derived which demonstrates visually the efficiency of sedimentation of actin vis-a-vis other proteins.

(8) Fractions corresponding to the included peak were pooled as "column actin" (Figure 6, channel 10) and were diluted by ~10% with a concentrated buffer solution to give as final conditions 100 mM KCl, 0.1–1.0 mM $CaCl_2$, pH 6.2–6.5, TM. After brief incubation at 37 °C to warm the mixture it was further incubated for 30 min at room temperature. The inclusion of $CaCl_2$, the use of a moderately low pH, and incubation at or slightly above room temperature were found to be crucial to achieving efficient recovery of actin from the fairly dilute solution (Table II; protein concentration usually ca. 0.6–0.8 mg/mL) present at this stage. Furthermore, it was essential that the crude actin be dissolved as indicated in step 5 so that the input to the G-200 column be at the appropriate concentration (usually 7–10 mg/mL).

(9) The repolymerized column actin was then centrifuged 2.5 h–45K–60Ti (204 000) at 20 °C, yielding supernatant (S2) and pellet fractions. Analyses of S2 (Table II; Figure 6, channel 11) revealed that it contained ~50% of the protein but, under favorable circumstances, only a small percentage (10–20%) of the actin originally present in the column actin.

(10) The pellets from the preceding step were resuspended in the desired volume (usually 10–20 mL) of TAM and dialyzed for 18–24 h vs. 1 L of this buffer. Brief centrifugation [15 to 30 min–39K–42.1 (168 000)] removed a small amount of aggregated material, P3, Table II; Figure 6, channel 12)

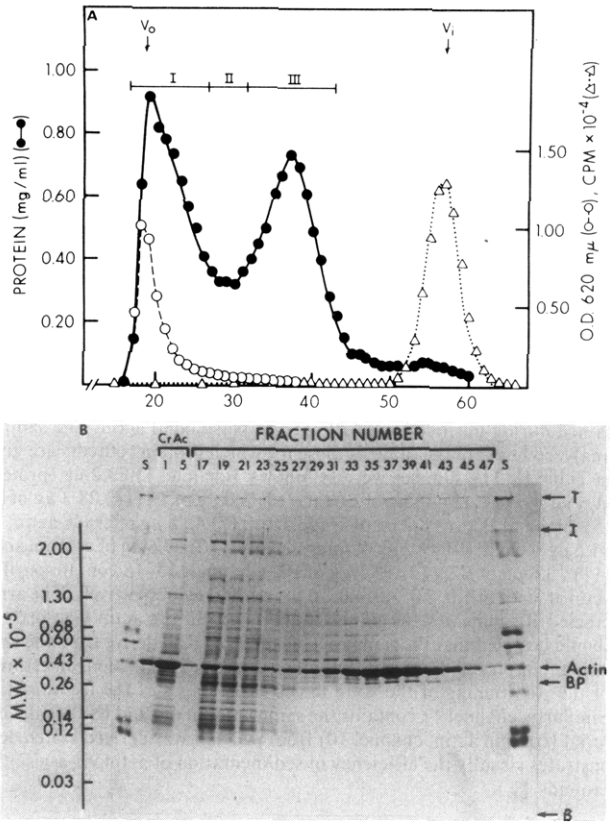

FIGURE 7: Chromatography of crude actin on Sephadex G-200. Crude actin was prepared as described in the text. The sample (254 mg of protein in 36.8 mL of TAMP) was applied to a Sephadex G-200 column (5 cm diameter × 45 cm high) equilibrated in, and eluted (upward flow) with, TAMP at a flow rate of ~49 mL/h. Fractions (~16.2 mL) were collected and analyzed for protein content. The bars labeled *I*, *II*, and *III* indicate the fractions pooled to give the void, intermediate, and column actin fractions, respectively, in this experiment which (possibly because of the somewhat low concentration of the crude actin) yielded only slightly more than 50 mg of pure actin. (A) Distribution of protein (●—●), blue dextran (○- - -○), and γ-^{32}P-labeled ATP (△···△) in the fractions eluted from the column. Blue dextran (detected by OD 620 nm) and labeled ATP (detected by liquid scintillation counting) were chromatographed together in a separate but otherwise identical run in order to provide calibrations of V_0 and V_i. (B) Sodium dodecyl sulfate gel electrophoresis of the input crude actin (Cr Ac) and of various fractions eluted from the G-200 column. Channels labeled 1 and 5 contain 5.5 and 27.6 μg of crude actin, respectively. The other channels contain 20-μL aliquots of the indicated column fractions. The arrow labeled BP indicates the proteolytic fragment which coelutes with the actin.

which contained some actin and a number of contaminants.

The supernatant from the above spin constituted the purified actin preparation; Table II indicates a recovery of ~60 mg of protein from 100 g of plasmodium at this stage which was the most common yield. Occasionally slightly higher yields (usually with elevated trace impurities, see below) were obtained; more frequently slightly lower yields (40–50 mg/100 g) were encountered; this was usually the case when the column actin was somewhat more dilute than specified above.

As indicated by the serial loading of the plasmodial actin shown in Figure 6 (channels 13–18) the protein is apparently quite pure. Except at very high load levels only one band, mol wt ~45 000, was detected. The only significant contaminant, comprising ~1–2% of the Coomassie blue stained material, was a polypeptide of mol wt ~34 000–38 000, and several lines of evidence suggest this to be a proteolytic breakdown fragment (BP) of the actin itself (see Discussion). Thus, for example, close examination of Figures 6 and 7B reveals more of BP in the column actin than was present in the input crude actin.

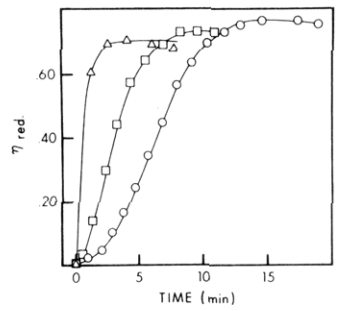

FIGURE 8: Repolymerization of purified actin as monitored by viscometry. After the G-actin in TAM was allowed to equilibrate to the ambient temperature (20 °C) and the zero baseline was established, the experiments were begun (at "zero" time) by dilution of the sample (10% by volume) to give, as final conditions, 1.1 mg of protein/mL, 10 mM Tris-maleate, pH 7.0, and 100 mM KCl (○), 100 mM KCl plus 1 mM $MgCl_2$ (△), or 100 mM KCl plus 1 mM $CaCl_2$ (□).

Furthermore, the contamination with BP was significantly greater with earlier preparations made without the use of PP_i and $PhMeSO_2F$ (Jacobson et al., 1976) and was found to increase upon storage of actin preparations of slightly lower purity than those described here (data not shown). In view of the similarity in apparent molecular weight between BP and the subunit of many tropomyosins it is perhaps worth noting that the elution of BP in the low molecular weight region of the G-200 column (Figure 7B) rules out its possible identification as tropomyosin since the latter would [as noted previously (Jacobson et al., 1976)] be voided from this column. With extremely high load levels (not shown) it was possible to demonstrate the presence of several extremely minor (<0.1%) contaminants in the actin; these could be eliminated by a repeated cycle of polymerization and sedimentation (which also reduced the level of BP) and such additional manipulations may be advisable for the detailed physicochemical studies of the actin currently in progress.

In TAM buffer plasmodial actin preparations exhibited low solution viscosity which rose sharply upon the addition of KCl (Figure 8); the formation of typical F-actin filaments was also confirmed by electron microscopy (not shown). As with previous preparations of plasmodial actin (Adelman and Taylor, 1969b) polymerization in the presence of KCl + $MgCl_2$ (Figure 8) proceeded more rapidly and plateaued at a slightly lower value of η_{red}. The response to KCl plus $CaCl_2$ (Figure 8) was intermediate. The actin preparations described here were quite stable: the viscosity rise upon addition of KCl ± $MgCl_2$ was virtually unaffected by 24-h storage (in TAM) at room temperature or by prolonged storage (1–2 weeks) at 0 °C. However, attempts to recover activity after lyophilization have met with limited success.

Discussion

The finding that *Physarum* plasmodial actin was readily extracted in low ionic strength buffers which did not solubilize the myosin was somewhat surprising since previous studies (Adelman and Taylor, 1969a) had indicated that the actin (detected as a rapidly migrating boundary in sedimentation velocity experiments), like the myosin (monitored by calcium-ATPase assays), was only extracted with moderately high KCl or PP_i. It seemed of interest to further examine the state of the actin in TM extracts not only in order to purify the protein but also because such studies represent a possible means of linking, on the one hand, the characterization of completely purified proteins and, on the other, phenomenological investigations of whole plasmodia (Kamiya, 1959) or of disrupted plasmodial "models" (Hatano, 1970).

Although many of the results presented here may prove to be unique to the plasmodial system it seems probable that at least some of them have more general implications. Thus, for example, other workers have reported detecting nonpolymerizable actins and have speculated as to possible regulatory mechanisms (see, e.g., Hinnsen, 1972; Tilney, 1975; Bray and Thomas, 1976). The effects of KCl and ATP described here may be related to the findings that sea urchin egg actin is polymerized by KCl and ATP (Kane, 1975, 1976) and that filament formation in "motile" amoebae extracts is dependent on ATP (Thompson and Wolpert, 1963; Pollard and Ito, 1970). It is also interesting to note, in the present context, that much of the actin of smooth muscle is readily extracted by low ionic strength buffers (Sobieszek and Bremel, 1975).

In view of the possible general implications of these results it seems important to stress the logic of the approach taken and the critical nature of the assays chosen. Because the objectives were to gain some understanding of the state of the actin in TM extracts and to develop an efficient purification procedure, certain of the techniques often applied in actin studies were of little value here. Attempts to activate the ATPase of rat muscle or *Physarum* myosin with TM extracts have consistently failed (unpublished), which is perhaps not surprising in view of the numerous impurities present. Also, it has thus far proven impossible to demonstrate either by viscometry or negative stain electron microscopy (unpublished) the presence of fibrous actin polymers in TM extracts under conditions (fairly high protein concentration, KCl and ATP present) where detection should have been possible and where assembly into polymeric arrays was demonstrable by the sedimentation assay. While the negative viscometric and electron microscopic data are subject to trivial explanations, they may indicate that the actin assembly occurring in the crude extracts is not of the conventional G $\rightarrow$ F type but rather the formation of either "amorphous" aggregates or of fairly short fiber fragments. This serves to emphasize the value of the sedimentation assay applied here, in conjunction with column chromatography and gel electrophoresis, to monitor (albeit in a crude fashion) the extent of actin polymerization (or aggregation) as a function of experimental manipulations without introducing a specific bias as to the type of polymer (aggregate) being generated.

The experiments presented in Results, section a, namely the failure of the protein to sediment unless certain solvent conditions are satisfied, as well as its elution position on Sephadex G-200, strongly suggest that the actin exists in TM extracts in a monomeric form; in particular the elution of the actin from Sephadex G-200 at $K_D \simeq 0.5$ rules out the existence of small oligomers and makes it unlikely that the actin is tightly bound to any other protein unless this is one of rather low molecular weight. This is also important for understanding why the actin (although not denatured) remains unpolymerized under conditions which do polymerize purified *Physarum* or muscle actin, since it would appear to rule out mechanisms such as blockage via tight association with other proteins, as may well occur in some nonmuscle systems (Tilney, 1975), and as has been demonstrated in the case of the interaction of muscle actin with deoxyribonuclease I (Mannherz et al., 1975; Hitchcock et al., 1976). Because the actin in TM extracts to which KCl and $MgCl_2$ have been added is degraded by the endogenous protease(s) as rapidly as that in the TM extract alone (data not shown) it does not seem necessary to suggest that an "F-actin–monomer" (Rich and Estes, 1976) exists, whose polymerization is blocked at some later stage in the sequence.

The findings that the addition of optimal levels of ATP to TM extracts can bring about actin assembly and that the added ATP is rapidly degraded [presumably by the ATP–pyrophosphohydrolase of Kawamura and Nagano (1975)] may be central to understanding the state of the actin in such extracts in terms of our current information about muscle actin (see, e.g., Oosawa and Kasai, 1971; Cooke, 1975). The hypothesis can be advanced that the freshly prepared extracts contain monomeric actin which is essentially devoid of bound ATP and/or is in competition with other nucleotide binding factors for the traces of ATP available; whether the nucleotide binding site is completely unoccupied is not clear but the actin monomer is here presumed to be in an altered conformation which hinders polymerization. If ATP is added, polymerizability is restored. In the absence of ATP the monomer is slowly further altered such that functional restoration of bound nucleotide is no longer possible. Finally the irreversibly altered monomer becomes a substrate for the endogenous protease(s). The model elaborated here for the state of the actin monomer in TM extracts accounts for much of the specificity data of Table I. Thus, nucleotides which bind tightly to and/or substitute for ATP in the polymerization of muscle actin (ITP, AMPPNP; Oosawa and Kasai, 1971; Cooke, 1975) substitute in the present crude extract system; conversely, GTP and CTP which bind weakly to muscle actin (Oosawa and Kasai, 1971) do not promote actin assembly in TM extracts. On the other hand, until the thin-layer chromatographic analyses mentioned under Results, section a, are complete and other data are available, this simple model cannot unequivocably explain, e.g., the proteolysis inhibition by PP_i or some of the competition data in Table I.

Nevertheless, given the above picture of the state of the actin in TM extracts it was possible to develop a simplified actin purification protocol which is rapid (2 days) and like the previously described one (Adelman and Taylor, 1969b) does not involve the use of acetone powders. Yields of ~60 mg of protein per 100 g of plasmodium are obtained (significantly greater than those previously achieved; Hatano and Oosawa, 1966; Adelman and Taylor, 1969b) and studies still in progress suggest slightly higher yields may ultimately be achieved. While the total plasmodial actin content remains uncertain, the analyses reported here suggest a value of ~260 mg per 100 g of plasmodium (~7.5% of an assumed total of 3.5 g of protein/100 g wet weight) which falls in the mid-range of previously reported estimates (Adelman and Taylor, 1969b; Jacobson et al., 1976; Kessler et al., 1976). Because the method described here is applicable to microplasmodia, from which it is possible to obtain uniformly labeled actin of a high specific activity (manuscript in preparation), more accurate measurements of the actin content of plasmodia are now in progress using isotope dilution techniques. Since, in any case, the recovery of actin by this procedure is fairly high and losses of actin occur in small amounts at various stages in the purification (rather than a major loss at any one point) it seems unlikely that only one subspecies of actin has been selected, a consideration which is important if the possibility is to be evaluated in later studies that there are multiple "isoactins" corresponding to multiple functions. Sodium dodecyl sulfate gel analyses of the actin preparation described here reveal it to be essentially pure, containing at most a few percent of a minor polypeptide, BP, which is a breakdown product of the actin itself, probably similar to the fragment generated from muscle and platelet actins by the degradative action of thrombin and other proteases (Muszbek and Laki, 1974; Muszbek et al., 1975, 1976; Jacobson and Rosenbusch, 1976). The presence of even trace amounts of BP may be of some significance, however, since actin preparations with elevated levels of BP have been found to show a larger difference in the viscosity plateau values $\pm$ $MgCl_2$ than is demonstrated in

Figure 8 (unpublished) and since the low viscosity "magnesium-polymer" of Hatano and co-workers (Hatano et al., 1967; Hatano, 1973) probably reflects the interaction of actin with one or more contaminating factors (Jacobson et al., 1976). When stored in the G state in TAM buffer the actin described here is quite stable. Upon addition of KCl ± divalent cations the monomer polymerizes rapidly to F-actin as judged by viscometry and electron microscopy. Furthermore, these preparations are able to interact with and activate the ATPase of purified *Physarum* myosin (Jacobson et al., 1976). A more detailed characterization of this plasmodial actin will be presented elsewhere.

Finally, some comments can be advanced as to the possible implications of these findings vis-a-vis the in vivo assembly state of plasmodial actin. Since morphological studies (for a review see Komnick et al., 1973) suggest that plasmodial actin filaments are not tightly linked into a highly ordered structure analogous to the Z-line of striated muscle, the ease and rapidity with which the actin is extracted in a variety of buffer solutions (including ones containing KCl, ATP, and $CaCl_2$) are not surprising. Although plasmodial extracts clearly contain protease(s), neither exudates nor microplasmodial extracts degrade the actin in them and the latter appear to be nearly devoid of significant proteolytic activity (manuscript in preparation); it therefore seems unlikely, as well as unnecessary to hypothesize, that the ease of actin extraction is a result of the sort of proteolytic activity involved in the preparation of natural F-actins from striated muscle (Hama et al., 1965; Suzuki et al., 1973). However, the extensive release of the actin and its existence primarily as a monomer in TM extracts could be a consequence of the ATP-pyrophosphohydrolase activity discussed above. The physiological significance of this enzyme remains obscure: it is present in exudates as well as in microplasmodial and plasmodial extracts (unpublished) and may be a soluble cytoplasmic enzyme, but its "normal" activity is questionable in view of its high calcium requirement (Kawamura and Nagano, 1975) and the likelihood that most of the 20–40 mM Ca^{2+} content of plasmodia (Nagai et al., 1975a) is sequestered in mitochondria and/or various vacuoles (Ettienne, 1972; Braatz and Komnick, 1973). Disruption of the plasmodium might be expected to result in release of sequestered calcium, activation of the pyrophosphohydrolase, and the production of homogenates and/or extracts in which the actin is depolymerized but subject to metastable assembly, i.e. in which the monomer ⇆ polymer equilibrium is affected by very slight changes in, e.g., ATP and/or calcium concentration. Clearly it is tempting to draw an analogy between such in vitro metastability and recent electron microscopic studies (Nagai et al., 1975b; Wohlfarth-Botterman and Fleischer, 1976) which suggest a cyclic assembly-disassembly of actin filaments during the oscillatory contractions and shuttle streaming of plasmodial strands. Since the intracellular concentration of ATP has been estimated at ca. 0.2–0.4 mM (Hatano and Takeuchi, 1960; Chin and Bernstein, 1968; Fink, 1975) small localized changes in ATP concentration could affect the extent of actin assembly. There is some evidence for cyclic fluctuations in the free calcium of the plasmodium (Braatz, 1975; Ridgeway and Durham, 1976) and one could envision a number of types of calcium-mediated assembly control including direct effects on the actin or indirect effects involving, for example, the ATP-pyrophosphohydrolase or some low molecular weight calcium binding proteins analogous to those found in numerous muscle and nonmuscle systems (Kretsinger, 1976). The possibility of either direct or indirect calcium regulation of actin assembly is, in light of the scanty evidence for a muscle-like calcium control system in *Physarum* (Jacobson et al., 1976), particularly intriguing; such regulation would constitute a basis for calcium control of motility because assembled actin arrays are presumably required for the transmission of force and/or because F- but *not* G-actin activates the ATPase of myosin (Offer et al., 1972; Chantler and Gratzer, 1976).

Obviously such models are rather speculative and quite different from those upon which our current understanding of muscle systems is based. However, there is no compelling reason to expect diverse nonmuscle systems, even those having actin and myosin, to effect motility precisely as is done in various muscles. The tentative proposals advanced here make numerous specific predictions which are testable using the purified proteins and the various homogenates and crude extracts described above and such tests are currently in progress.

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