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Filamentous proteins of Myxococcus xanthus and Escherichia coli: Evidence for the presence of actin-like proteins in prokaryotes.

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FILAMENTOUS PROTEINS OF *MYXOCOCCUS XANTHUS* AND *ESCHERICHIA COLI* : EVIDENCE FOR THE PRESENCE OF ACTIN-LIKE PROTEINS IN PROKARYOTES

by

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ABSTRACT

Microfilaments with actin-like properties are demonstrated in extracts of Escherichia coli B/r. Attempts to extract an actin-like protein from the gliding myxobacter Myxococcus xanthus, by several methods, revealed similar filaments. The actin-like nature of the myxobacter filaments was not conclusively demonstrated. Other products of these extractions include large, fibrous complexes, and ring-shaped structures, which might be implicated in the gliding motility mechanism.

DEDICATION

To the countless billions, plus one.

ACKNOWLEDGEMENTS

The assistance and guidance of Dr. McCurdy is acknowledged, as is his enthusiasm, which initially inspired me to study microbiology. He has left me with impressions that I shall not soon forget.

Thanks are extended to Drs. Fackrell and Taylor for their advice and criticism offered during the course of this study and the writing of this thesis.

The gift of plate-grown M. xanthus offered by Bill Dobson is gratefully acknowledged.

The advice and friendship of Dr. Jim Ho, in addition to his example as a researcher, are fondly recalled and deeply appreciated.

Foremost appreciation is felt for my parents for their support throughout the course of my studies.

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LIST OF ABBREVIATIONS

ALP - actin-like protein

ATPase - adenosine triphosphatase

^{o}C - degrees celsius

cm. - centimetre

DEAE-cellulose - diethylaminoethyl cellulose

DTT - dithiothreitol

EDTA - ethylenediaminetetraacetic acid

g - force of gravity

gm. - gram

HMM - rabbit skeletal muscle heavy meromyosin

hr. - hour

HSEtOH - B-mercaptoethanol

kV - kilovolts

M - molar concentration

mA - milliampere

min. - minute

nm - nanometre

Pi - inorganic phosphate

PIPES - piperazine-N,N'-bis(2-ethanesulfonic acid)

s. - second

SDS-PAGE - sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Tris - tris(hydroxymethyl-aminomethane)

µg - microgram

INTRODUCTION

Cellular locomotion among microbes occurs by various means. The ciliates and flagellates rely upon a dynein-microtubule system for movement (Satir 1976), while amoeboid forms appear to move through the interactions of muscle-like contractile proteins (Hitchcock 1977, Korn 1978, Pollard and Weihing 1974). Bacterial movement has been attributed to the rotation of either the helical flagellum of eubacteria (Silverman and Simon 1974) or the 'axial filaments' of spirochetes (Bromely and Charon 1979, Canale-Parola 1978, Carleton et al 1979). However, a small yet diverse group, the gliding bacteria, undergo a form of movement that has yet to be mechanistically understood.

Gliding is a non-flagellar form of surface translocation in which the movement continuously and regularly follows the long axis of the cell (Henrichsen 1972). To date, no specific organelle has been associated with gliding, though intensive ultrastructural studies have been carried out. Freeze-fracture preparations of the gliding cyanophyte Oscillatoria princeps have revealed parallel arrays of helically wound, 5 to 8 nm filaments on the surface of the trichome (Halfen and Castenholz 1970). A similar study of the gliding myxobacter Myxococcus xanthus demonstrated periplasmic particles which are occasionally seen as a linear organization (Burchard and Brown 1973). Thin sections of M. xanthus have revealed intracellular microfilaments and periodic structures arranged longitudinally within the cells (Burchard 1977, Schmidt-Lorenz and Kuhlwein 1968). However, none of these uncharacterized, fibrillar structures have been associated with gliding motility.

The flexibility of some gliders, such as the myxobacteria (Henrichsen

1972) and particularly Flexibacter which actively flexes while moving (Dayrell-Hart 1979), suggests the involvement of a contractile process. The helical appearance of a class of motility mutant of Myxococcus xanthus, isolated by MacRae and McCurdy 1976), may indicate the presence of a contractile apparatus gone wrong.This mutant is particularly interesting in view of the discovery of a spiral-shaped, gliding, mycoplasma-like organism (Davis and Worely 1973), though the possibility of the shape of these cells being due to cell wall factors and not a contractile mechanism cannot be ignored (Carleton et al 1979, Mendelson 1976).

In many contractile processes the interaction of the proteins,actin and myosin, has been implicated as the mechanism of contraction. The presence of an actin-like protein, identified by the concomitant existence of a 45,000 dalton polypeptide with 6 nm diameter, HMM-decorating filaments, has served as the marker of an actomyosin system (Pollard and Weihing 1974). With the suggestion of an actin-like protein possibly existing in the bacterium Escherichia coli (Minkoff and Damadian 1976) it became expedient to search for such a protein in a gliding bacterium if the contractile mechanism of gliding was to be argued.

This investigation is initially involved with applying the definitive criteria for actin to the 'actin-like protein' of E. coli, as obtained by the Minkoff and Damadian (1976) procedure . Following that, the same procedure as well as other actin-isolating methods are applied to the gliding myxobacter Myxococcus xanthus , with the hope of establishing the presence of an actin-like protein in a gliding bacterium and thereby implicate an actomyosin system in the gliding motility mechanism.

MATERIALS AND METHODS

Microorganisms

All cultures were obtained from the University of Windsor culture collection.

Escherichia coli B/r, grown on T-Soy agar (Difco), was innoculated into 400 ml of KA medium (Minkoff and Damadian 1976) and shaken at 30°C. After 12 hr., the exponentially growing culture was innoculated into 8 litres of the same medium in a 12 litre carboy, aerated by a sparger which was attached to a filtered air supply, and incubated at 30°C. Growth was monitored with a Klett-Summerson colorimeter, (equipped with a No.57 green filter) after aseptically withdrawing samples. Cells in late logarithmic growth were harvested at 18 hr. by batch centrifugation at 10,400 g for 20 min., and washed twice with phosphate buffer (pH 7.0). Cells were stored as a pellet at -20°C.

Myxococcus xanthus strains M36 and M300 were cultivated in 30 ml. of either CT broth (Dworkin 1962) or SP broth (McCurdy 1963). After 24 hr. the culture was innoculated into 1 litre of medium in 2.8 litre flasks and shaken in the dark at 30°C. After 30 hr., the logarithmically growing cells were harvested by centrifugation and stored as mentioned above. Plate cultures of M36 were grown on SP agar (McCurdy 1963) at 30°C in the dark in sealed vegetable crispers. After 40 to 48 hr. the cells were washed with phosphate buffer (pH 7.0), collected by centrifugation and stored at -20°C.

Chemicals and Proteins

All chemicals used were reagent grade. Electrophoresis reagents were obtained from Bio Rad. Molecular weight markers, trypsin, soyabean trypsin inhibitor, DEAE-Cellulose and dipotassium adenosine triphosphate were obtained from Sigma Chemical Co. All solutions were prepared in double distilled water.

Electron Microscopy

Samples were applied dropwise onto formvar-coated, carbon-reinforced, 200 mesh copper grids, using a capillary pipette. After 1 min. the sample was withdrawn and a drop of filtered 1% Uranyl Acetate was applied for 30 s. A filter paper wedge was used to blot off the stain, and the grid was air dried .

To test for decoration by heavy meromyosin (HMM), after the initial application of the sample, a drop of HMM [350 µg/ml in: 10 mM Tris-Cl (pH 7.5), 1 mM ethylenediaminetetraacetate-tetrasodium (EDTA), 1 mM B-mercaptoethanol (HSEtOH), 0.1 M KCl] was applied to the grid for 1 min. The excess was removed and the grid was stained as above.

Specimens were examined in either a Hitachi HU-12 at 75 KV, or in a Phillips 201c at 60 KV, each equipped with a cold finger.

Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate (SDS-PAGE)

The gel system of Laemmli (1970) was used to fabricate 10% gels in either 0.3× 8.5 ×14.0 cm. slabs, or 10.0 ×3.0 cm. tubes. Separation gels were cast the preceding day and 3% stacking gels were cast prior to each run. Samples in the sample buffer of Laemmli (1970) were stacked at 3 mA per gel and separated at 10 mA per slab or 3 mA per tube gel in Bio Rad slab and tube electrophoresis units. When necessary, samples

were dialyzed overnight against sample buffer (less glycerol) to rid them of potassium and to avoid precipitation. The gels were stained at 30°C and destained using Bio Rad diffusion destainers, according to the method of Fairbanks et al (1971). Electrophoretic mobilities of protein bands in tube gels were calculated by the formula of Weber and Osborn (1969).

Extraction and Purification of Rabbit Muscle Contractile Proteins

A/ Muscle Pretreatment

A rabbit was killed by ether anaesthesia, rapidly skinned, eviscerated, and packed in ice water for 15 minutes. In the cold, the back and hind quarters muscles were cut off and minced in a meat grinder. The mince was then treated by the method of Feuer et al (1948), except that all steps were carried out in the cold, that the mince was homogenized in a Waring blender in the high salt buffer, and that the homogenate was centrifuged at 10,400xg for 20 minutes. The fibrous pellet was used for extracting actin, and the supernatant was used for extracting myosin after it was filtered through several layers of cheese cloth.

B/ Actin Extraction

After a washing with 0.01 M carbonate-bicarbonate buffer (Feuer et al 1948), the fibrous pellet was extracted twice in cold (-20°C) acetone. The fibrous mass was collected on filter paper in a Buchner funnel, by vacuum filtration. This fibre was vacuum-stored overnight over dessicant and paraffin, in the cold. A dessicator, at -20°C was used for long term storage.

Thirteen gm. of the dried fibre was shredded and suspended in 200 ml. of low ionic strength buffer (3mM imidazole, 0.1mM $CaCl_2$, 0.5mM ATP, 1mM B-HSEtOH, pH 7.5 with HCl. Gordon et al 1976) , stirred 30 minutes

in the cold , and spun at 10,400xg for 30 min. The cloudy supernatant was spun again at 100,000xg for 1.5 hr. This supernatant was applied to a DEAE-Cellulose column and eluted according to the method of Gordon et al (1976). Alternate fractions were scanned at 290 nm, and the KCl concentration in each was measured using a Phillips conductivity meter with a 1 cm^2 dip-type electrode, calibrated with KCl standards.

C/ Myosin Purification

Following the method of Lowey and Cohen (1962), the high salt supernatant from muscle pretreatment was dialyzed against 10 volumes of 5 mM HSEtOH overnight. The resulting precipitate was collected by centrifugation at 10,400xg for 30 min. The pellet was suspended in 10 mM phosphate buffer (pH 6.8), containing 0.5 M KCl, 5mM HSEtOH, and homogenized in a Teflon-glass homogenizer. This solution was made to 200 ml. in the same buffer, stirred for 1 hr., then spun at 30,000xg for 1 hr. The supernatant was then precipitated as before. the final pellet was resuspended in 5 mM HSEtOH.

Using the procedure of Tsao (1953), the 40-60 % ammonium sulfate precipitate was obtained. This suspension was spun at 10,400xg, the pellet collected and dissolved in 0.5 M KCl, 5 mM HSEtOH, 10 mM phosphate buffer (pH 6.8), then dialyzed against 400 volumes of 5 mM HSEtOH overnight. The resulting precipitate was collected again by centrifugation and dissolved in 1 M KCl, 20 mM PIPES-NaOH (pH 6.8), 10 mM HSEtOH. It was then spun at 30,000xg to clarify, and the final supernatant was mixed 1:1 with glycerol and stored at -20°C.

Heavy Meromyosin Preparation

The cold-stored myosin was dialyzed against the reaction buffer of Lowey and Cohen (1962), then digested according to their method. After stopping the reaction with 1% soyabean trypsin inhibitor, the mixture was dialyzed against 10 mM phosphate buffer (pH 7.0) overnight, then spun for 2 hr. at 48,000xg . The supernatant was dialyzed against 0.1 M KCl, 20 mM Tris-Cl (pH 7.5), 1 mM EDTA, 5 mM HSEtOH overnight, mixed 1:1 with glycerol, and stored at -20°C.

To test for the presence of contaminating actin, the preparation was analyzed by SDS-PAGE. Activity of the preparation was determined by its ability to form arrowhead complexes with rabbit muscle actin.

Escherichia coli ALP Extraction

An acetone powder was prepared from the fragmented cells of E. coli B/r and extracted according to the method of Minkoff and Damadian (1976) as outlined in Fig. 1.

Myxococcus xanthus Extractions

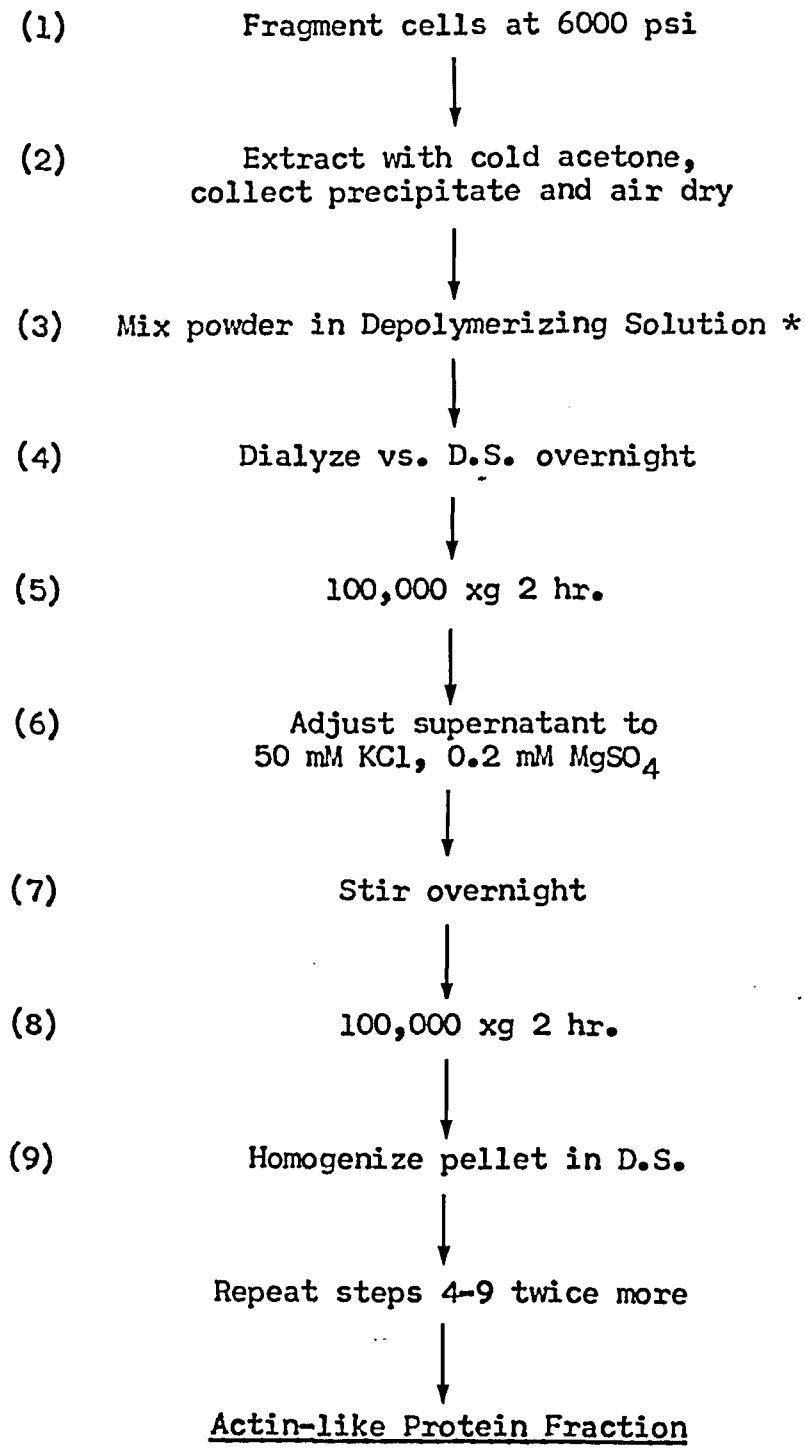
A/ Method of Minkoff and Damadian (1976)

One hundred gm. of strain M36, cultivated in SP broth, was subjected to this procedure. The final product was then treated with 0.6 M KCl as outlined by Stossel and Hartwig (1976) to investigate the possible existence of an 'actin-binding protein'.

B/ Method of Neimark (1977)

Ninety gm. of SP broth cultivated M36 was treated by the method as outlined in Fig. 2.

Figure 1 METHOD FOR EXTRACTING ACTIN-LIKE PROTEIN FROM ESCHERICHIA COLI (Minkoff and Damadian 1976)



* Depolymerizing Solution (D.S.): 0.2 mM ATP, 0.5 mM HSEtOH, 10 mM Tris-HCl (pH 7.6).

Figure 2 MYXOCOCCUS XANTHUS M36 EXTRACTION BY THE METHOD OF NEIMARK (1977)

(1) 90 gm. cells suspended in 200 ml. of :

0.6 M KCl, 15 mM Tris-Cl (pH 8.5), 2.5% n-butanol, 5 mM DTT.

Homogenize and stir 16 hr. at 4° C

(2) ↓ 30,000xg, 30 min.

(3) Supernatant (S1)
Dilute 7-fold with 10 mM acetate buffer (pH 6.3)

(4) ↓ -30,000xg, 30 min.

(5) Pellet (P2)
Dissolve in 8 mM Tris-Cl (pH 7.5), 0.6 M KCl, 3 mM DTT.

(6) ↓ 140,000xg 1 hr.

(7) Supernatant (S)
adjust to 5 mM Mg-ATP

(8) ↓ 140,000xg 1 hr.

Pellet (P4)
repeat steps 5 to 8

↙ Pellet (P5)

↘ Supernatant (S5)
Dialyze vs. 0.075 M KCl, 3 mM DTT., 10 mM Tris-acetate (pH 6.5)

↓ 10,400xg, 20 min.

Supernatant (S6)
Incubate at 4°C overnight

C/ Method of Gordon *et al* (1976)

Two attempts to extract actin-like protein from the two strains of *M. xanthus* were carried out, using this method.

(i) Eighty-five gm. of plate-grown M36 were dispersed in 200 ml. of low ionic strength buffer with a Teflon-glass homogenizer. The cell paste was passed through a French Pressure Cell at 10,000 psi, made to 250 ml. in the same buffer, and spun at 30,000xg for 30 min. The supernatant was spun again at 100,000xg for 1.5 hr., and the resulting supernatant was applied to the DEAE-Cellulose column and eluted in 25 ml. fractions at approximately 50 ml. per hr.

(ii) In a modified version of this procedure, an acetone powder was prepared from 180 gm. of fragmented M300 cells, grown in CT broth. The powder was extracted according to the method of Minkoff and Damadian (1976), and the resulting high speed supernatant was then applied to the DEAE-Cellulose column and eluted in 8 ml. fractions as before.

Myosin Adenosine Triphosphatase Assay

To test the activity of the myosin preparation, the procedure of Mabuchi (1976) was used. Actin activation of myosin ATPase was assayed using the procedure of Burke *et al* (1974). The release of inorganic phosphate was measured by a modified version (10-fold volume reduction) of the method of Martin and Doty (1949).

Protein Quantitation

Protein was measured by the dye-binding method of Bradford (1976). For samples with less than 10 ug protein per ml., the modification of Spector (1978) was employed.

E. coli Extraction

A protein fraction, which was obtained by polymerization cycles of the Minkoff and Damadian (1976) method, appeared in E.M. preparations as filament bundles, obscured filaments and as the occasional bare filament of 6 nm diameter (Figs. 3 abc). Attempts to decorate these filaments with HMM led to the appearance of 'arrowhead' complexes along short lengths of some of the filaments, with a periodicity of 39 nm (Figs. 3 de).

M. xanthus Extraction by the Method of Minkoff and Damadian (1976)

Subjecting the cells of M. xanthus M36 to this procedure produced a fraction similar to that obtained from E. coli. When the soluble extract was 'polymerized' by the addition of KCl and $MgCl_2$, filament bundles and the occasional bare filaments (Fig. 4) could be seen amid linear arrays of obscured filaments (Fig 5a) following overnight incubation at $4^{o}C$. This was a distinct change from the amorphous appearance of the soluble extract, prior to the addition of salts (Fig. 5b).

Since it has been reported that the existence of an 'actin-binding protein' may obscure the appearance of actin filaments (Stossel and Hartwig 1976), the myxobacter fraction was adjusted to 0.6 M KCl and spun at 100,000 g for 1 hr. The resulting supernatant was dialyzed to low ionic strength and low pH in 5mM Tris-maleate buffer (pH 6.9). Overnight, a precipitate formed which was was collected by centrifugation and redissolved in 0.6 m KCl, 10 mM Tris-Cl (pH 7.5). When examined by E.M., negatively stained specimens demonstrated a homogeneous preparation

Figure 3. Electron micrographs of E. coli ALP and rabbit muscle actin.

Fig. 3a. Filament bundle in E. coli ALP preparation.

Fig. 3b. Obscured filaments in E. coli ALP preparation.

Fig. 3c. Bare, 6 nm diameter filaments (F) in E. coli ALP preparation.

Fig. 3d,e. HMM decoration of E. coli filaments, demonstrating arrowhead complexes (indicated by markers).

Fig. 3f. HMM decoration of rabbit muscle actin (arrowhead complexes are indicated by markers).

In these and all other electron micrographs, unless otherwise noted, the bar represents 100 nm.

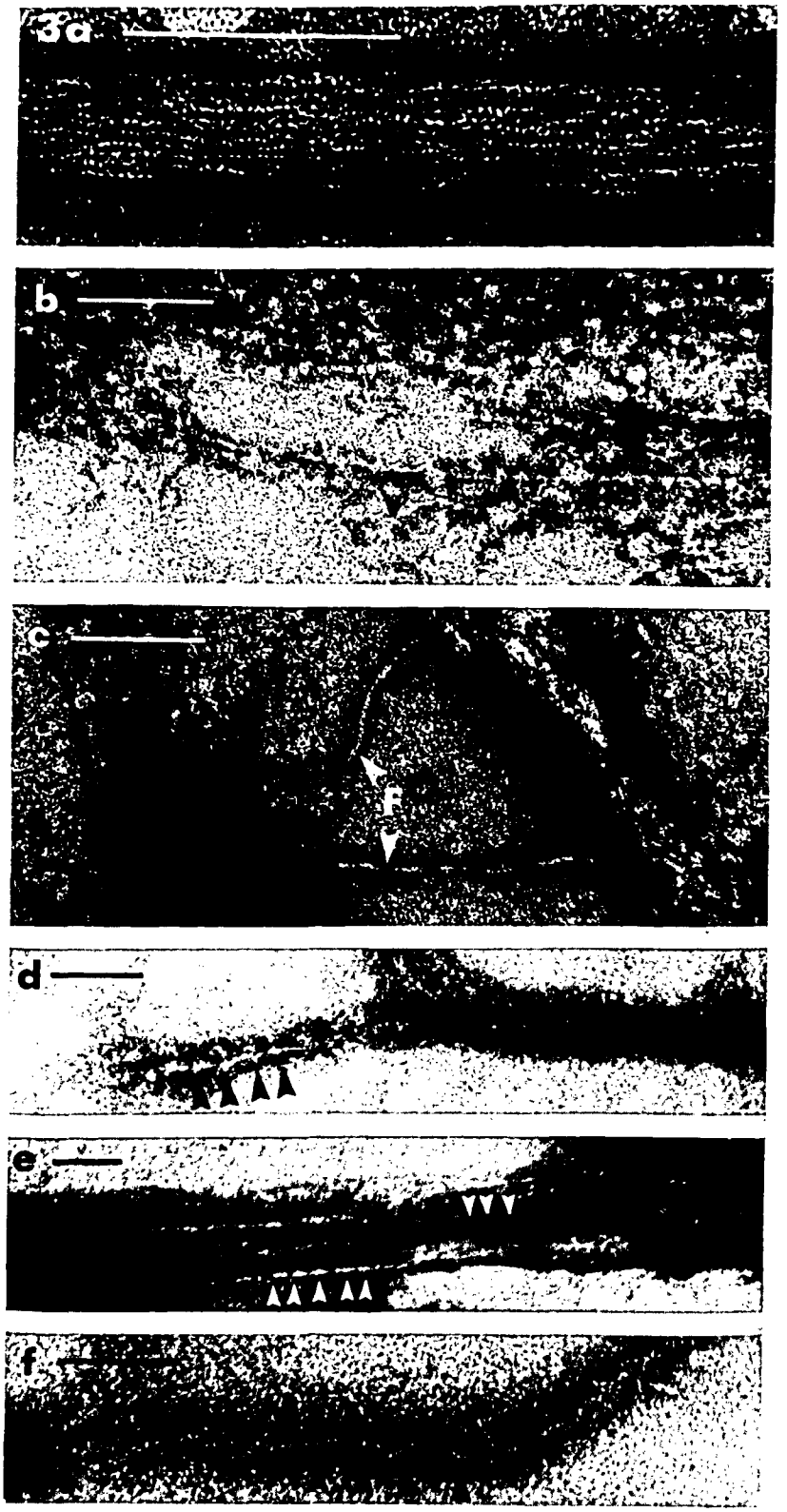


Figure 4. Electron micrographs of M. xanthus M36 protein extracted by the procedure of Minkoff and Damadian (1976).

Fig. 4a. Filament bundles.

Fig. 4b. 6 nm diameter filament (F).

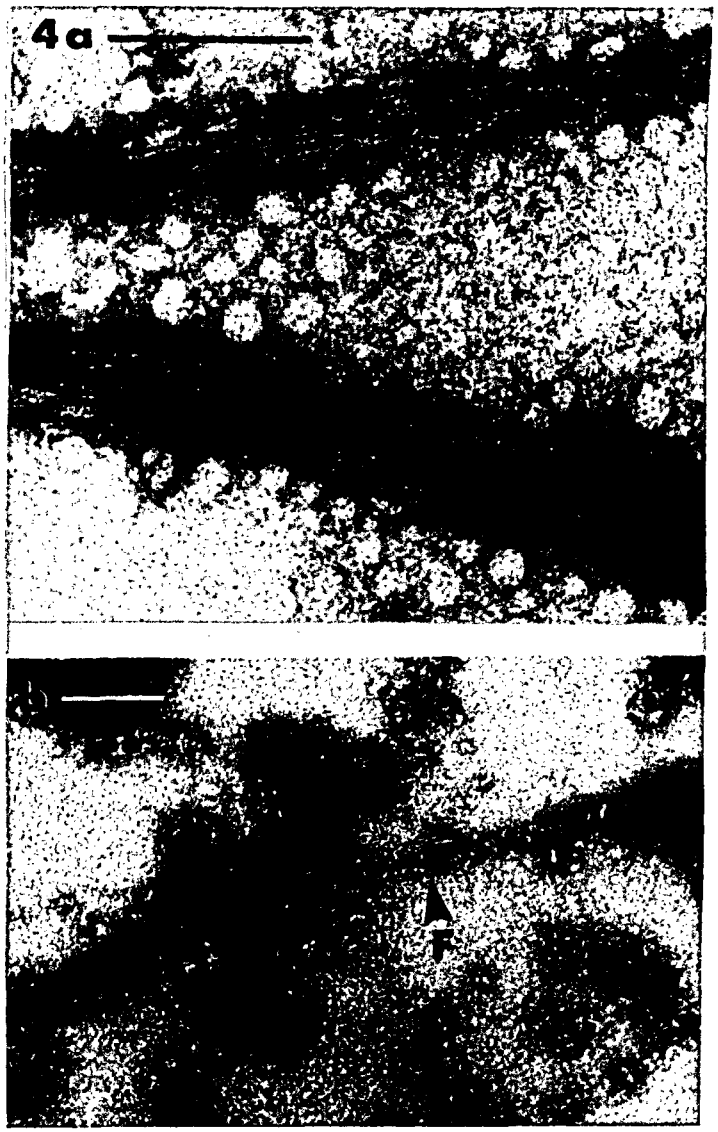
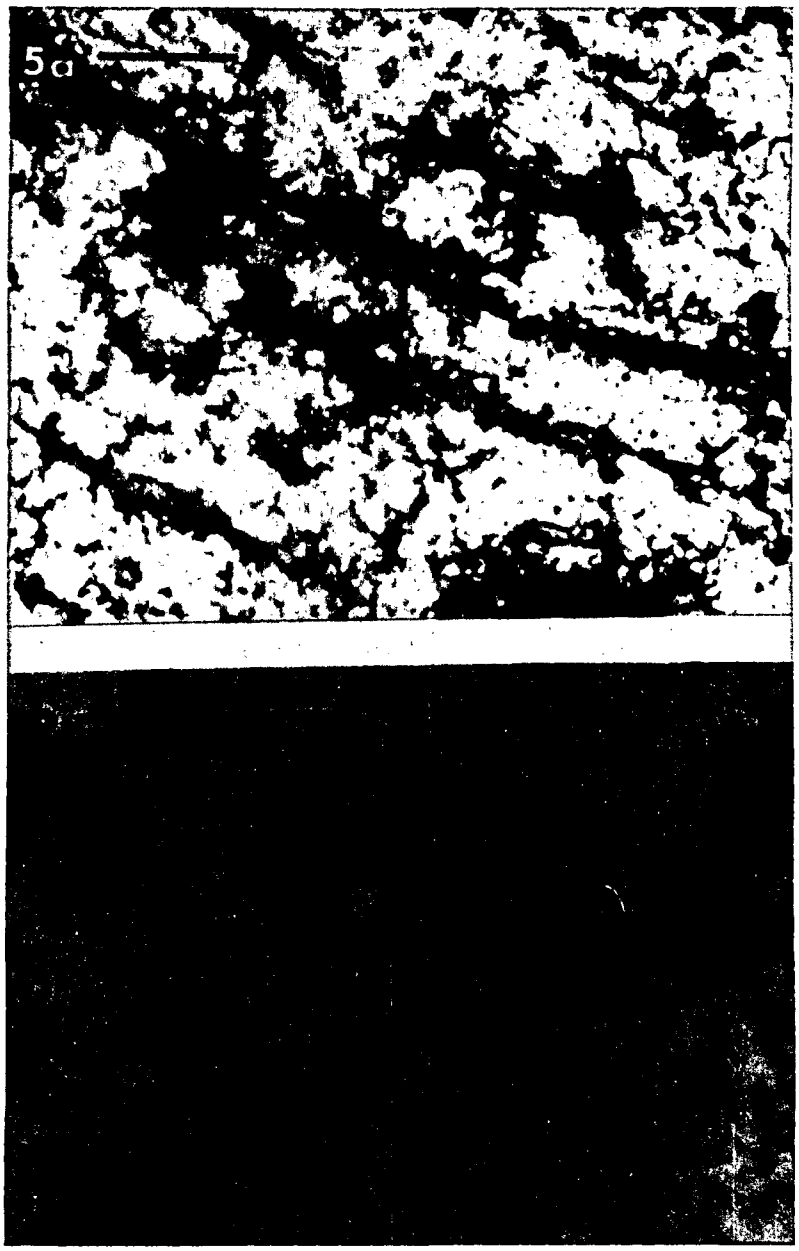


Figure 5. Electron micrographs of M. xanthus M36 protein extract, prepared by the method of Minkoff and Damadian (1976).

Fig. 5a. Polymerized extract showing linear arrays.

Fig. 5b. Soluble extract.

In these figures the bar represents 1 micron.



of 'tetrameric structures' (Fig. 6a). SDS-PAGE analysis of this fraction demonstrated the existence of but a single component, with a molecular weight of approximately 10,000 daltons (Fig. 6b).

The remaining 100,000xg pellet, when resuspended in polymerizing solution (Minkoff and Damadian 1976) and examined by E.M., showed no apparent change, and the filaments therein defied decoration with HMM (data not shown). Treatments with 1 % SDS, 8 m urea, or heating to 60°C for 30 min., did not disperse the filament bundles nor dissociate the obscuring material.

M. xanthus Extraction by the Method of Neimark (1977)

After 16 hr. of incubation in the extraction buffer, over 90 % of the cell suspension was converted to spheroplasts, as determined by phase contrast microscopy (data not shown). Upon clarification by centrifugation, the cell lysate appeared as a network of linear arrays when examined by E.M. Analysis of the lysate by SDS-PAGE demonstrated a polypeptide band of approximately 45,000 dalton (Fig. 7) comprising 6 % of the extracted protein (6 % of the total area of the gel profile scan, using a planimeter.)

Upon diluting the extract seven-fold and lowering the pH to 6.3 with acetate buffer, the resulting precipitate was collected by centrifugation and redissolved in high salt buffer. After spinning to clarify, the resulting supernatant was adjusted to 5 mM Mg-ATP and spun again at high speed. This pellet (P5), when resuspended in high salt buffer and examined by E.M., appeared to consist of amorphous clumps and linear arrays. The occasional bundle of well resolved filaments and single, 6 nm diameter filaments were seen in addition to lattice structures

Figure 6. Tetrameric protein of M. xanthus M36.

Fig. 6a. Electron micrograph of tetrameric proteins.

Fig. 6b. Photograph of SDS-PAGE tube gel showing a single, 10,000 dalton polypeptide band (arrow) in the preparation.

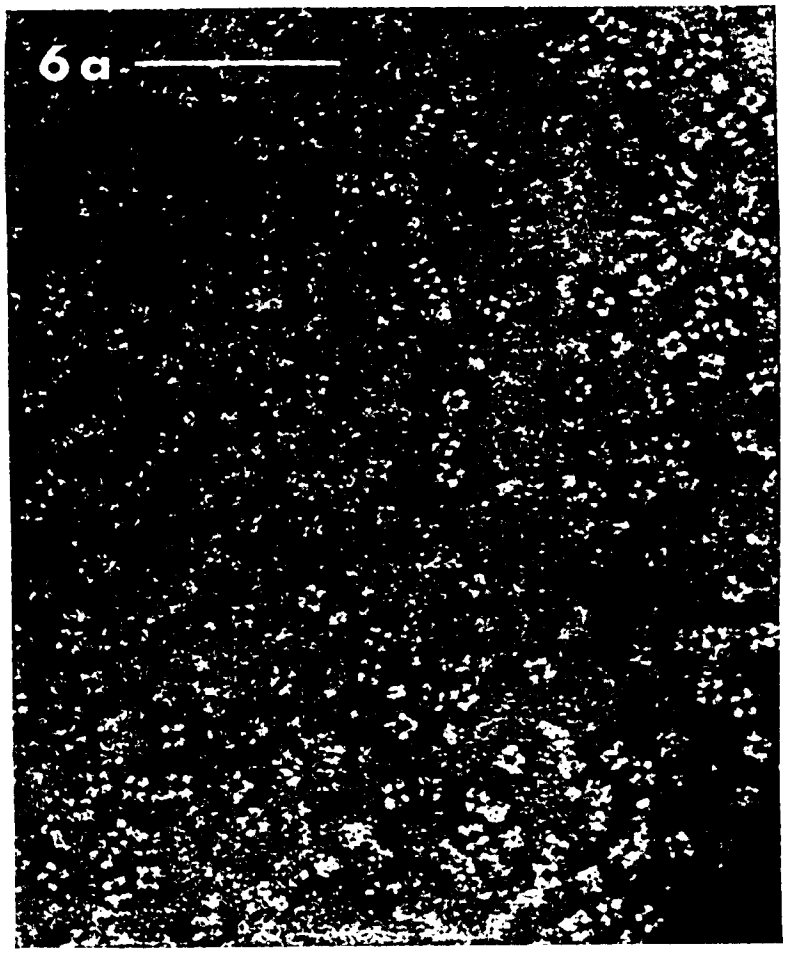
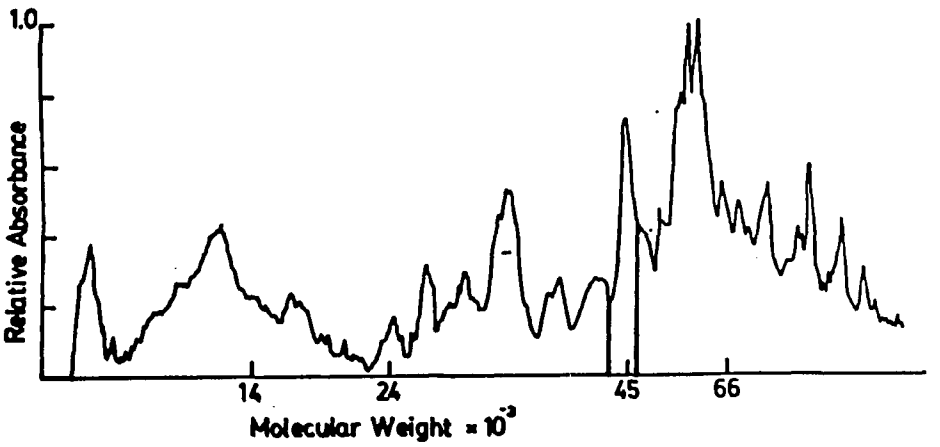


Figure 7. Densitometer scan of SDS-PAGE tube gel, showing the distribution of polypeptides extracted from M. xanthus M36 by the procedure of Neimark (1977). The relative absorbance is plotted against moecular weight in daltons. Molecular weight markers are; lysozyme (14,000 daltons), trypsinogen (24,000 daltons), ovalbumin (45,000 daltons), bovine serum albumin (68,000 daltons). The 45,000 dalton peak represents 6 % of the area of the profile.

7



(Fig. 8a). The 6 nm filaments can be seen to possess a period of 44 nm, approximating that of rabbit muscle actin filaments (Fig. 9). The lattice structures have a spacing of about 9 nm and appeared to be closely associated with fine filaments (Fig. 8b).

SDS-PAGE analysis of P5 revealed a 45,000 dalton polypeptide band. Reaction of P5 with HMM did not result in the formation of arrowhead complexes when examined by E.M. (data not shown). An assay of this preparation's ability to activate the ATPase of rabbit muscle myosin at low ionic strength showed an activity only slightly above that of myosin alone, under the same conditions (Table 1).

After the second Mg^{2+}-ATP precipitation, the resulting supernatant (S5) was dialyzed to low pH and low ionic strength in acetate buffer to precipitate any dissociated components. Following centrifugation of the precipitate, the resulting supernatant (S6) , demonstrated in E.M. preparations large linear aggregations of a complex nature (Fig. 10a), after standing overnight. Attempts to separate these aggregations from fine filaments (Fig. 10c), led to their enrichment in pellets (Fig. 10c). Close examination of these aggregations shows them to be composed of fine filaments (Fig. 10d) and globular components (Fig. 10e). Analysis of the enriched pellet, by SDS-PAGE, demonstrated three polypeptide bands of 10,000 and 80,000 daltons (Fig. 10f). A very faint 85,000 dalton band was seen as well.

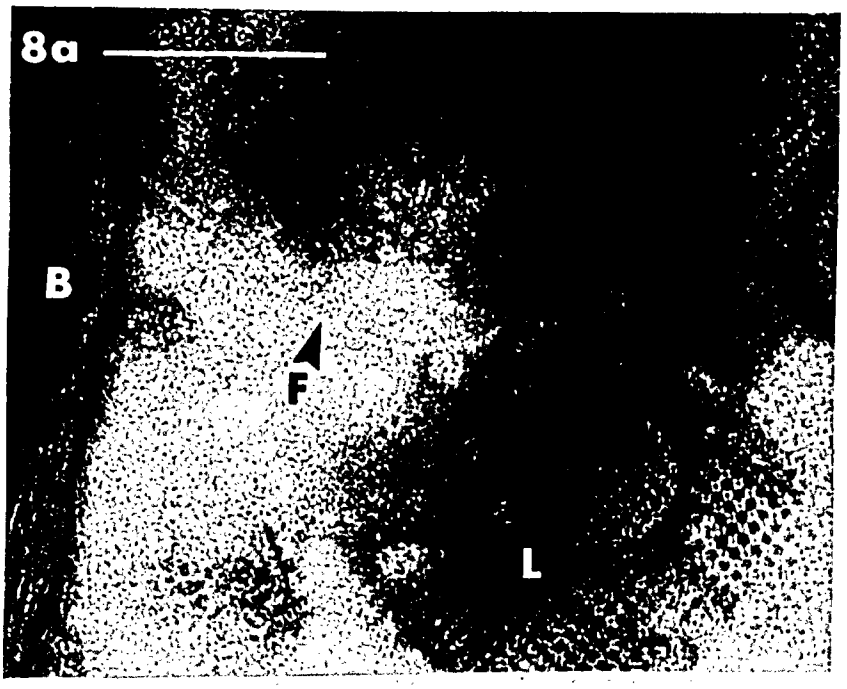
M. xanthus M36 Extraction by the Method of Gordon et al (1976)

A 200 ml. volume of cell lysate (12.5 mg. protein per ml.) was applied to the DEAE-Cellulose column. Fractions of the effluent were scanned at 290 nm and absorbances plotted against eluted volume (Fig. 11a)

Figure 8. Electron micrographs of P5, obtained by the method of Neimark.

Fig. 8a. Filament bundle (B), single 6 nm diameter filament (F), lattice structure (L).

Fig. 8b. Lattice structure with associated fine filaments (f).



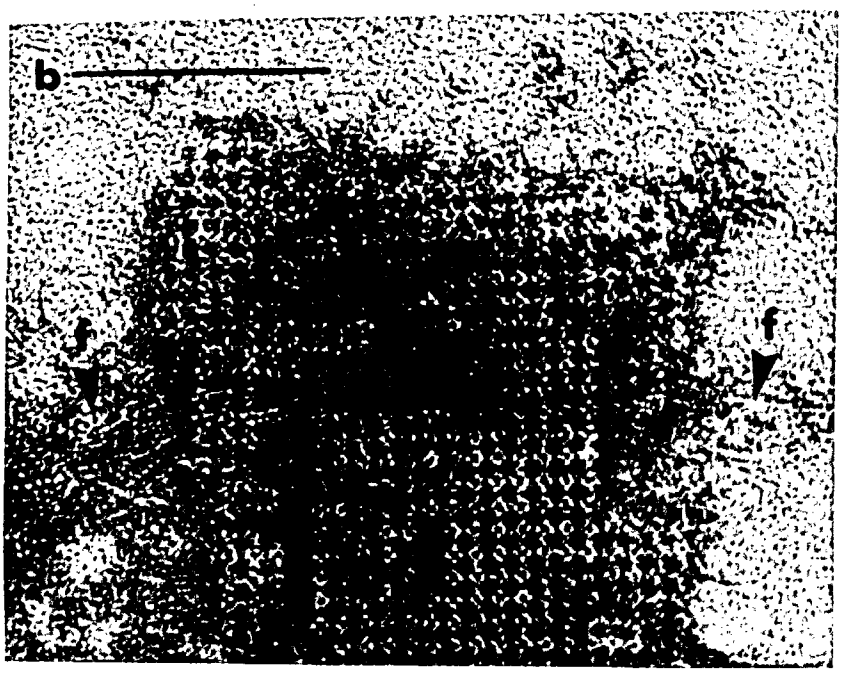
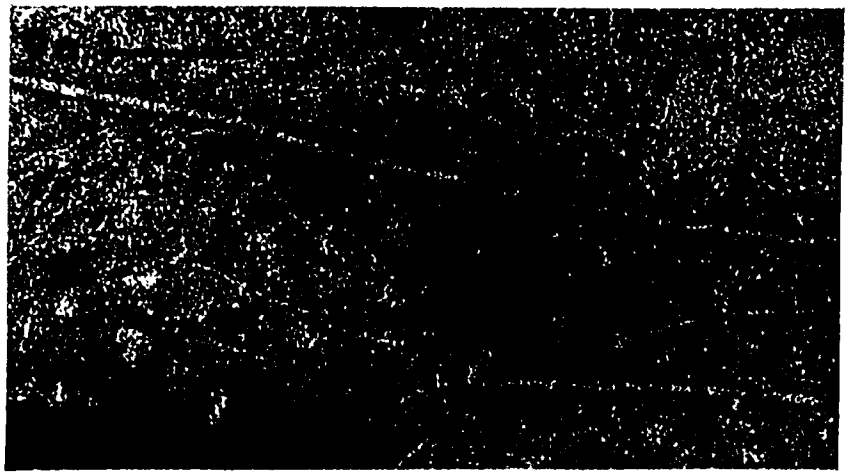


Figure 9. Electron micrographs comparing a 6 nm diameter filament of M. xanthus M36 (Fig. 9a) obtained through the Neimark procedure, with a rabbit muscle actin filament (Fig. 9b). The markers indicate the apparent crossover points of the helical filaments, spaced 44 nm (Fig. 9a) and 40 nm (Fig. 9b).



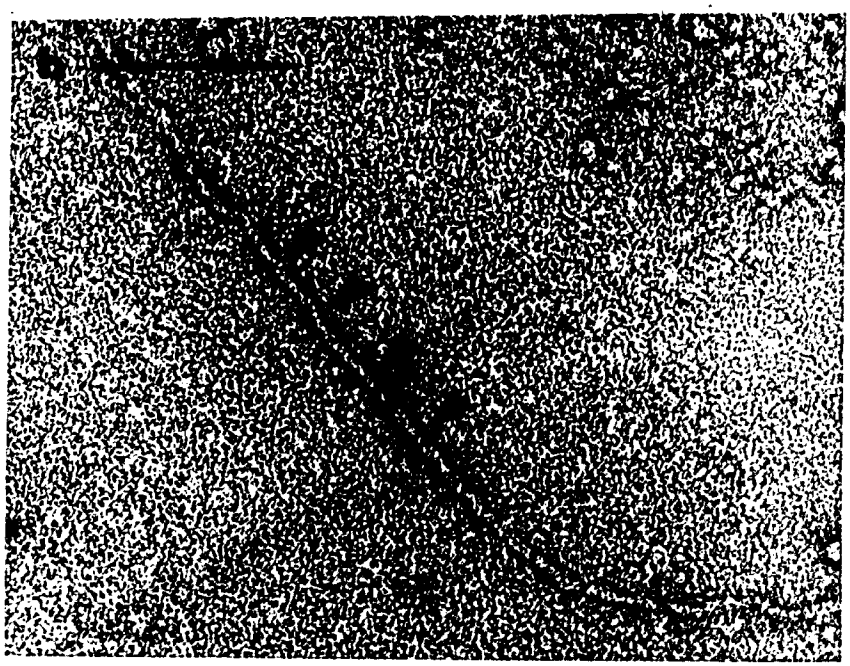


TABLE 1

ATPase ACTIVITIES OF VARIOUS PROTEIN EXTRACTS

SAMPLE	ADDITION*	SPECIFIC ACTIVITY (u mol Pi released / min. / mg. protein)	
		per mg. MYOSIN	per mg. Test Substance
1. MYOSIN (RABBIT)	5 mM $MgCl_2$	0.025	-
2. ACTIN + MYOSIN (RABBIT)	"	0.138	-
3. "	5 mM EDTA	0.000	-
4. M. xanthus ALP (Neimark Method)	5 mM $MgCl_2$	-	0.416
5. " + MYOSIN	"	0.030	-
6. "	5 mM EDTA	0.000	-
7. M. xanthus ALP (DEAE-Cellulose)	5 mM $MgCl_2$	-	0.220
8. " + MYOSIN	"	0.025	-
9. "	5 mM EDTA	0.000	-

*Reaction Buffer: 20 mM Tris-Cl, 20 mM Histidine-Cl, 50 mM KCl, pH 7.0.(Burke et al 1976)

Figure 10. Electron micrographs of linear aggregated complexes obtained by the the application of the Neimark procedure to M. xanthus M36 (Figs. 10a to 10e) and photograph of SDS-PAGE tube gel of the concentrated preparation (Fig. 10f). Fine filaments (f) are seen in association with the complexes. Three polypeptide band are seen in the SDS-PAGE gel; 10,000 daltons, 80,000 daltons, and a very faint 85,000 daltons band .

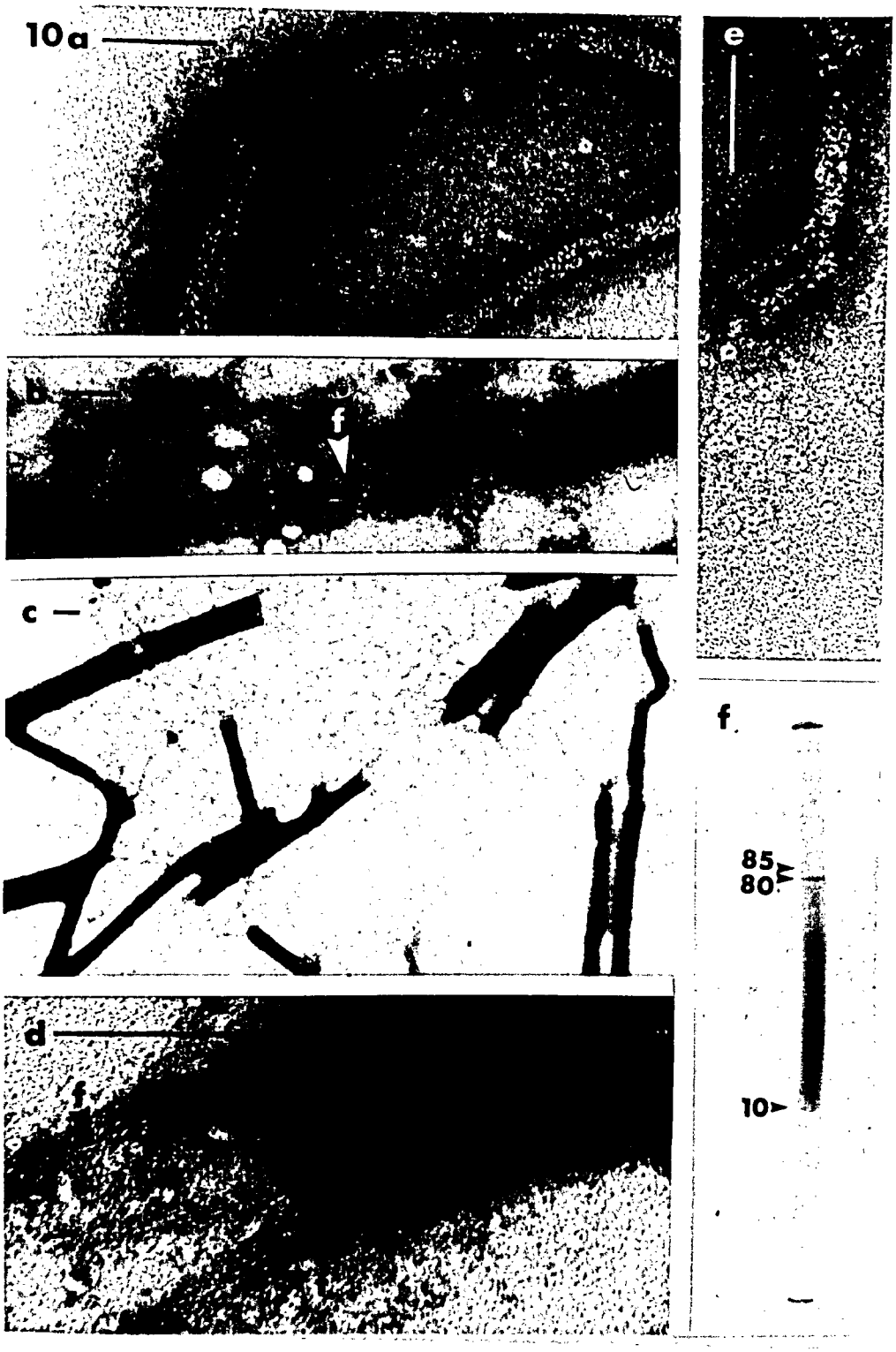
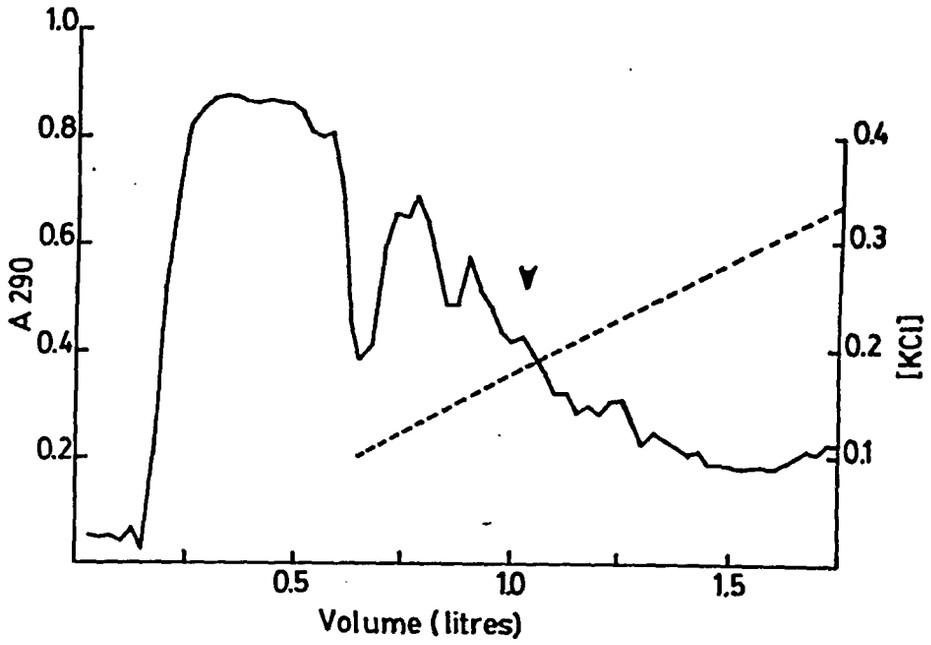
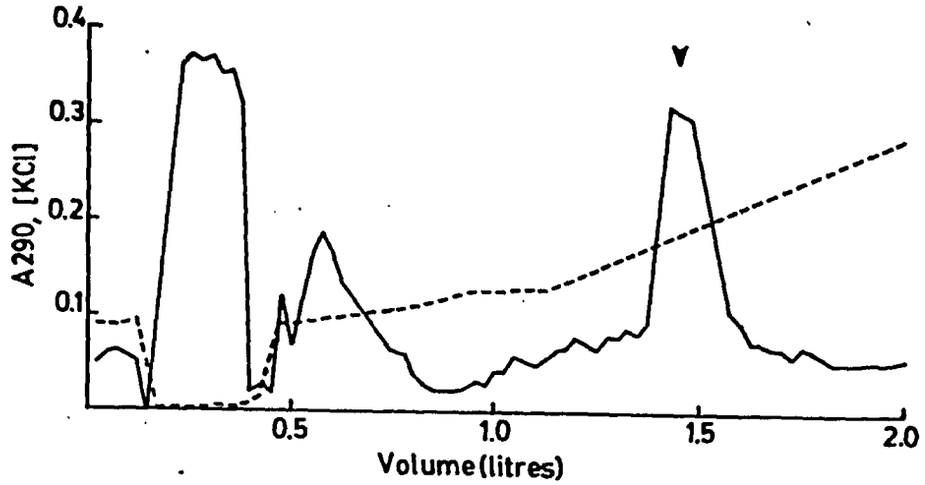


Figure 11. DEAE-Cellulose ion-exchange chromatography of M. xanthus M36 whole cell extract (Fig. 11a) and of extracted rabbit muscle fibre (Fig. 11b). The absorbance at 290 nm (solid lines) and the KCl concentration (dotted lines) of alternate fractions is plotted against the eluted volume. The arrows indicate the location in the salt gradient where actin is expected to elute (0.18 M KCl).

11a



b



Upon superimposing the salt gradient, a poorly resolved peak is observed in the region where rabbit muscle actin (Fig. 11b) and Acanthamoeba actin (Gordon et al 1976) are eluted.

To assay the presence of an actin-like protein, 2 mM $MgCl_2$ was added to 1 ml. samples of the fractions in the suspect peak. They were then incubated at 20° C for 1 hr. Upon E.M. examination, fractions 38 through 43 appeared to be similarily composed of linear arrays of obscured material (Fig. 12a). This material bears a resemblance to rabbit actomyosin under the same conditions (Fig. 12b). The occasional bundle of 3 nm diameter filaments was also seen in some of these fractions (Fig.12c). SDS-PAGE analysis revealed a 40,000 dalton component in these fractions (Fig. 13).

Fractions 38 through 43 were pooled, adjusted to 2 mM $MgCl_2$ and spun at 100,000 g for 3.5 hr. at 20°C. The pellets were homogenized in depolymerizing buffer (Gordon et al 1976) and exhaustively dialyzed against the same, then spun at 100,000 g for 3 hr. When examined by E.M., this supernatant appeared as amorphous masses (data not shown). Upon adding KCl and $MgCl_2$ to 0.1 and 2 mM respectively, and after 1 hr. at 24°C, 3 nm diameter filaments could be seen in the masses (data not shown).

Extraction of M. xanthus M300 Acetone Powder by the method of Gordon et al

A 200 ml. volume of extract from the acetone powder of M 300 (6.9 mg of protein per ml.) was applied to the DEAE-Cellulose column. The plot of 290 nm absorbance versus eluted volume is presented in Fig. 14a. A non-dialyzable, vividly orange pigment appeared to interfere with absorbance readings and may have led to the poorly resolved profile.

E.M. examination of $MgCl_2$-treated samples showed the widespread occurrence of linear arrays of material. The presence of a 45,000 dalton

Figure 12. Electron micrographs of fibrous components of M. xanthus M36 DEAE-Cellulose chromatography fraction 41 (Figs. 12a,c), and of rabbit muscle actomyosin (Fig. 12b)

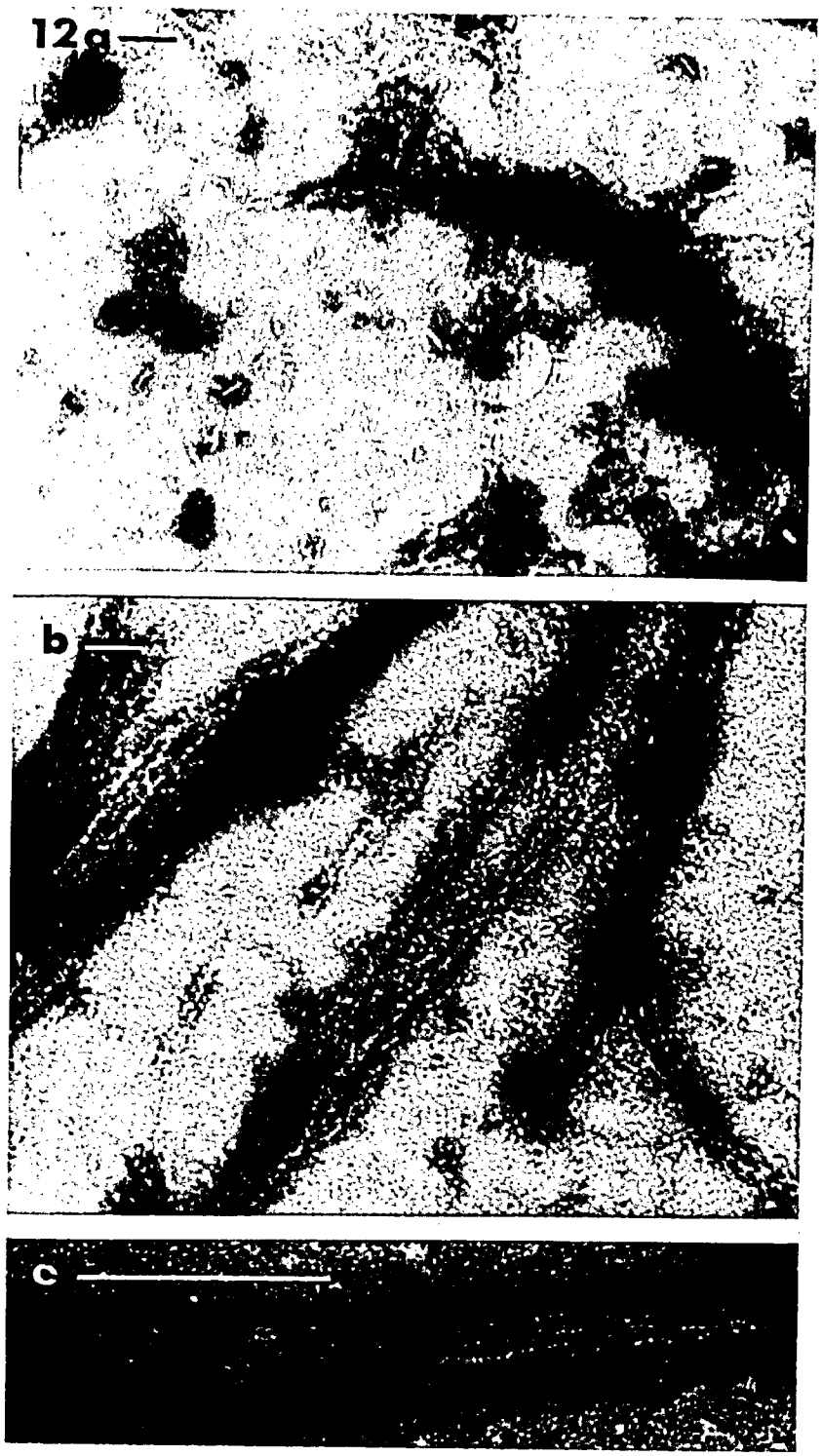


Figure 13. Photograph of SDS-PAGE tube gels of fractions eluted from DEAE-Cellulose chromatography of M. xanthus M36 whole cell extract. 1) 100,000xg supernatant of extract, 2) Fraction 13, 3) Fr. 29, 4) Fr. 32, 5) Fr. 35, 6) Fr. 36, 7) Fr. 39, 8) Fr. 41, 9) Pool of fractions 36 to 43, 10) Final preparation, 11) Rabbit muscle actin, 12) Molecular weight markers: (from top to bottom) bovine serum albumin (68,000 daltons), ovalbumin (45,000 daltons), pepsin (35,000 daltons), trypsinogen (24,000 daltons), lysozyme (14,000 daltons). The arrow marks the approximate region of 45,000 daltons.

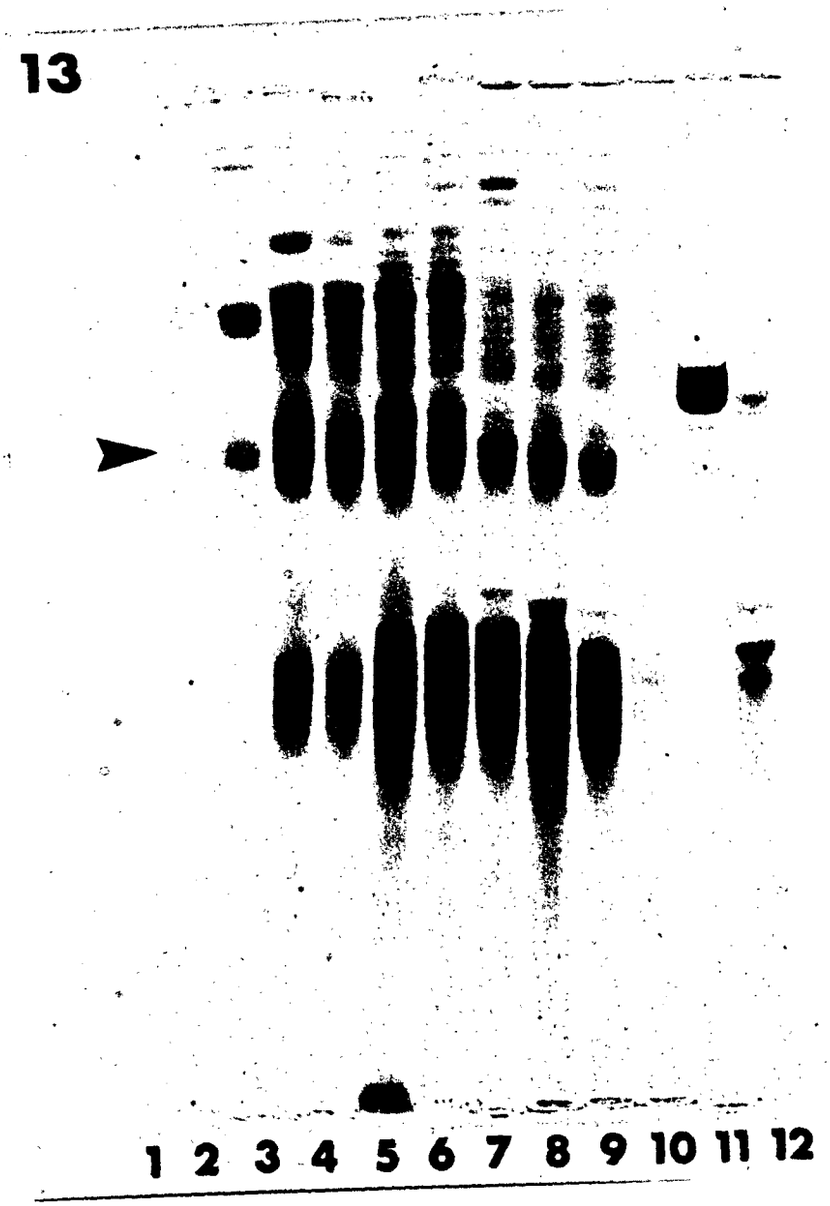
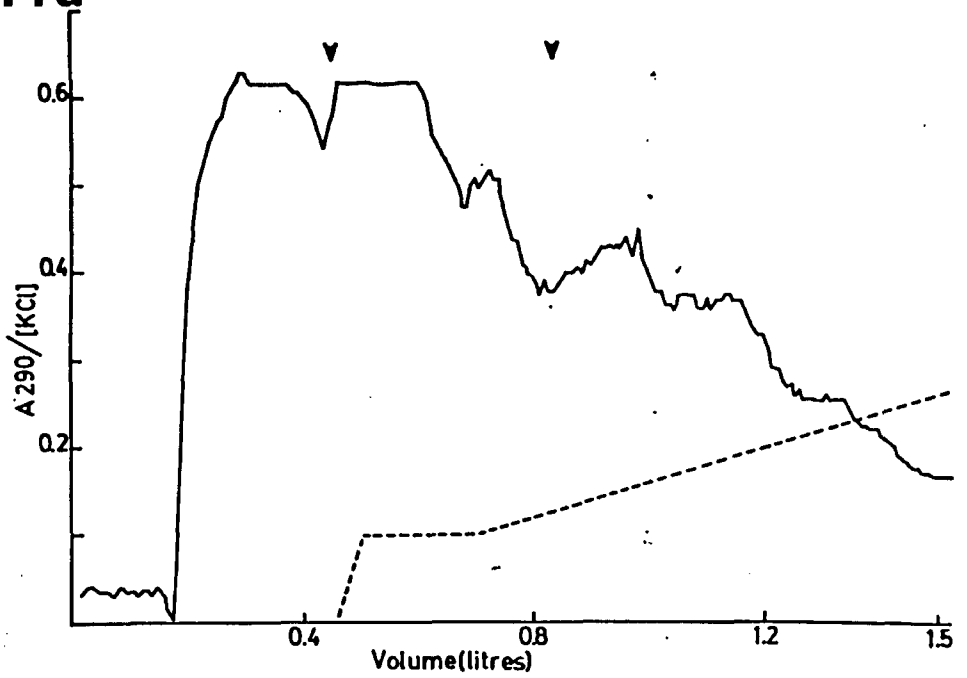


Figure 14. DEAE-Cellulose ion-exchange chromatography of an extract of M. xanthus M300 acetone powder.

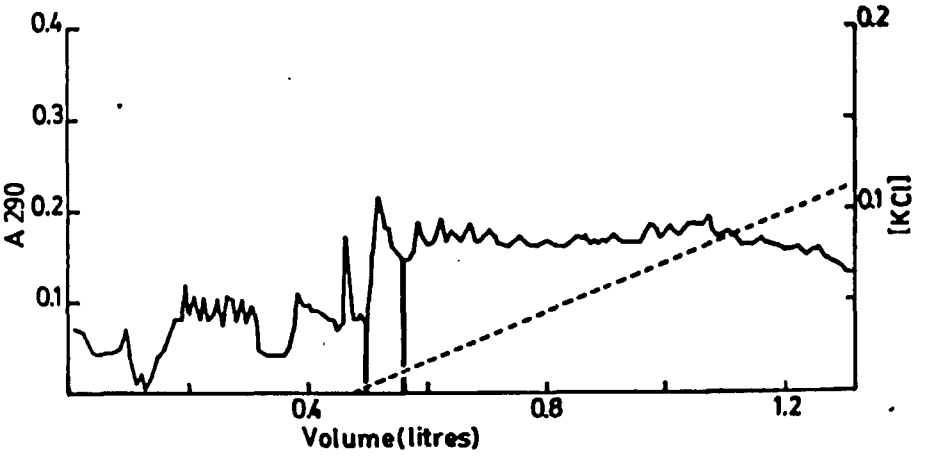
Fig. 14a. Elution profile of extracted acetone powder. Arrows mark the bounds of the fractions demonstrating linear arrays in E.M. specimens, and 45,000 dalton polypeptide. These fractions were pooled and rechromatographed.

Fig. 14b. Elution profile of the rechromatographed, pooled fractions. Vertical bars mark the peak containing fractions which demonstrate linear arrays and 3 nm diameter filaments.

14 a



b

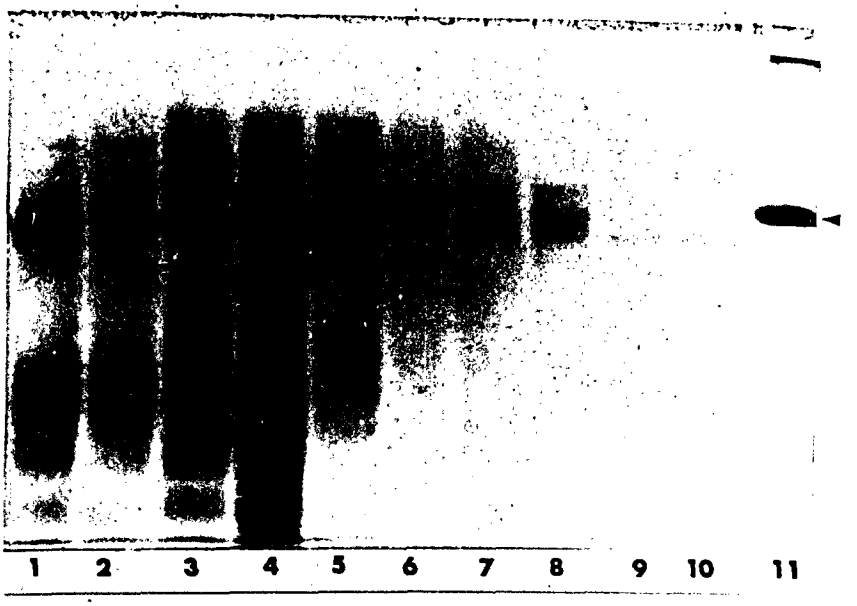


polypeptide was equally widespread (Fig. 15), though the bulk of this material appeared in fractions 59 through 109 concomittent with the linear arrays. These fractions were pooled, adjusted to 2 mM $MgCl_2$ and spun at high speed with the hope of concentrating any actin-like component. After homogenizing the pellet and exhaustively dialyzing in depolymerizing buffer, a high speed supernatant of this preparation was again applied to a DEAE-Cellulose column (0.5 cm x 20 cm). This time, the sample was eluted with a more gently increasing salt gradient (Fig. 14b). An A_{290} plot of the effluent (Fig. 14b) revealed several peaks, with only one corresponding to the occurence of linear arrays containing 3 nm filaments and few, short, 6 nm filaments (Fig. 16), when adjusted to 0.1 M KCl and 2 mM $MgCl_2$. When these pooled fractions were assayed for an ability to activate myosin Mg-ATPase, only a very low level was observed, as was a low intrinsic ATPase activity (Table 1). Reaction of this preparation with HMM failed to result in the appearance of any arrowhead complexes in E.M. preparations.

Also seen in this preparation were ' ring-shaped structures' of about 15 nm diameter, in the form of linear aggregations and in association with 3 nm filaments (Fig 17).

Figure 15. Photographs of SDS-PAGE slab gels of selected fractions of DEAE-Cellulose chromatography of M. xanthus M300 acetone powder extract.
Fig. 15a. 1) Fraction 36, 2) Fr. 38, 3) Fr. 59, 4) Fr. 65, 5) Fr. 76, 6) Fr. 87, 7) Fr. 92, 8) Fr. 101, 9) Fr. 109, 10) Fr. 117, 11) actin (arrow) and myosin.
Fig. 15b. 1) Fr. 125, 2) Fr. 133, 3) Fr. 142, 4) Fr. 149, 5) Fr. 157, 6) Fr. 176, 7) pool of fractions 134 to 148, 8) 186,000xg supernatant of extracted acetone powder, 9) 100,000xg supernatant of extracted acetone powder, 10) 186,000xg pellet of extracted acetone powder, 11) actin (arrow) and myosin.

15a



b

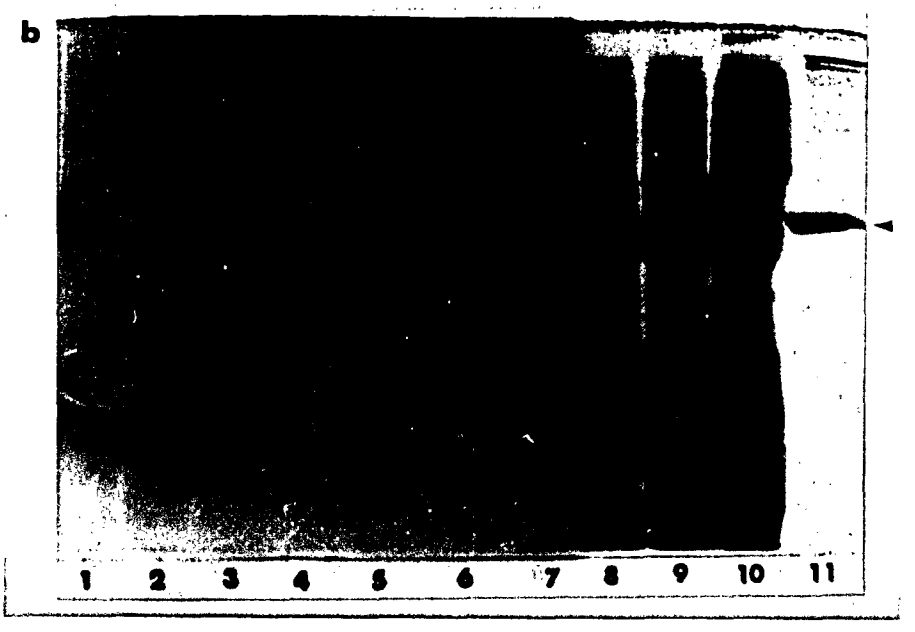
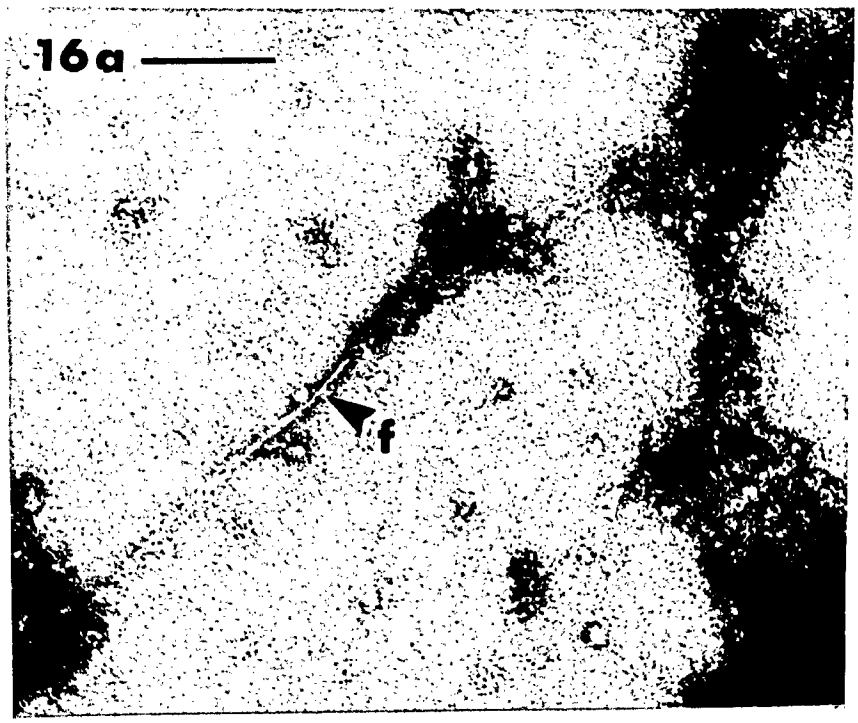


Figure 16. Electron micrographs of M. xanthus M300 3 nm (f) and 6 nm (F) filaments seen in eluted fractions of rechromatographed, pooled fractions (see peak marked by vertical bars in Fig. 14b.).



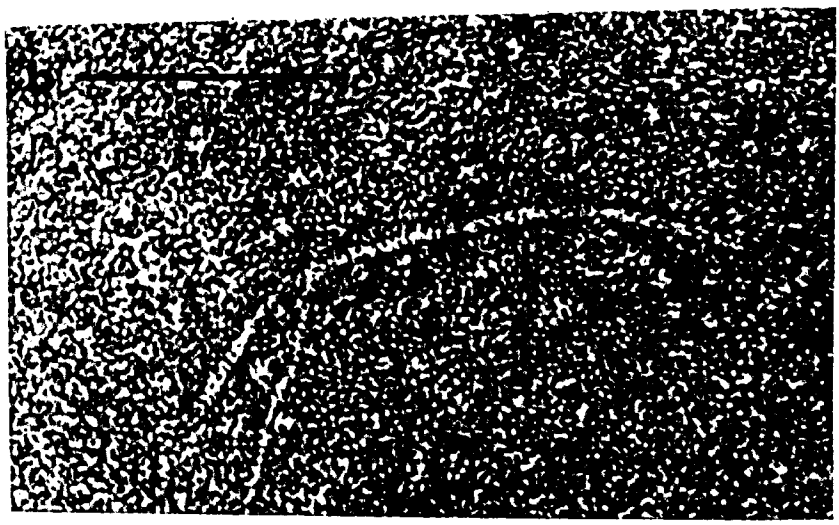
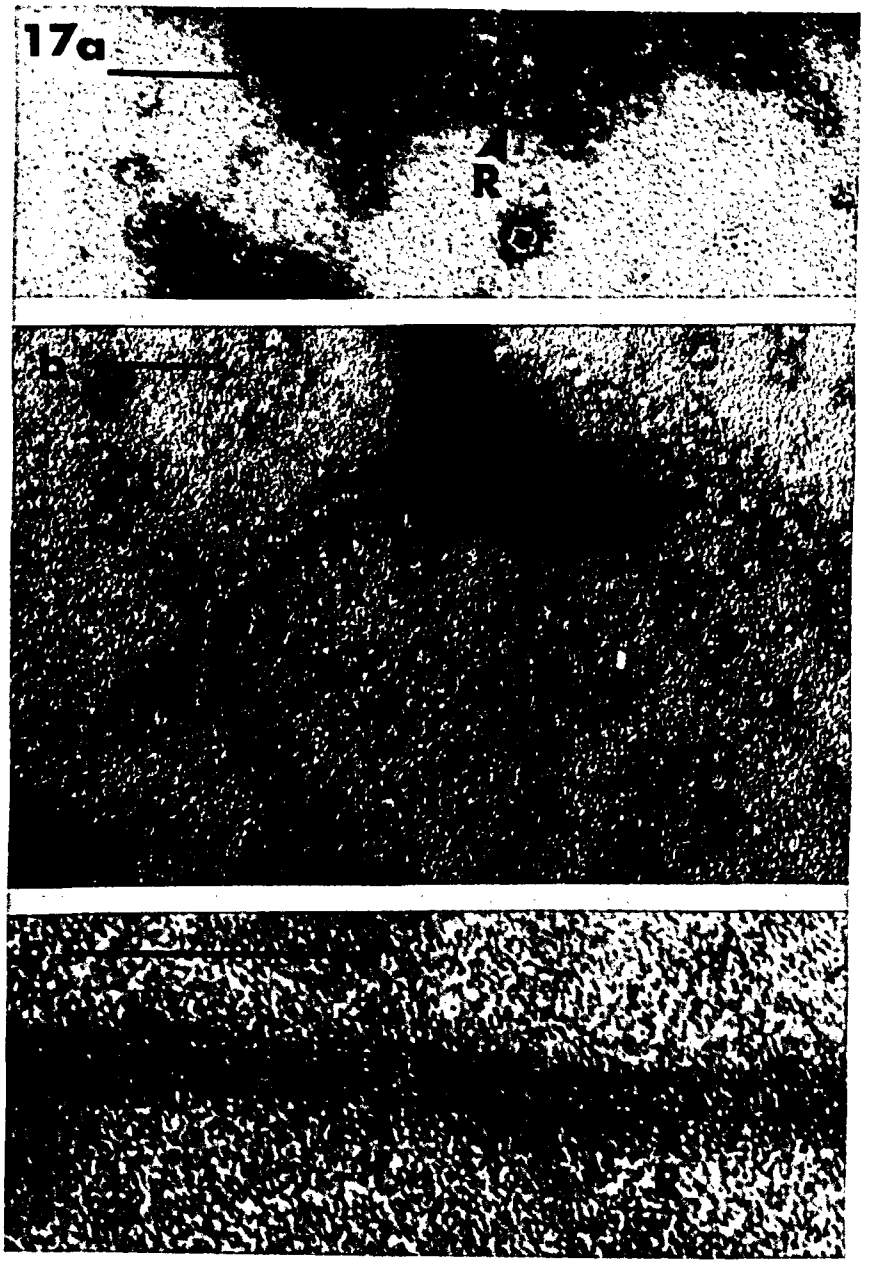


Figure 17. Electron micrographs of ring-shaped structures (R) of M. xanthus M300, seen in eluted fractions of rechromatographed, pooled fractions (see peak marked by vertical bars in Fig. 14b.). In Fig. 17c 3 nm diameter filaments (f) are seen in association with ring-shaped structures (R).



DISCUSSION

The results demonstrating 6 nm diameter filaments in E coli which decorate with heavy meromyosin confirm the existence of an actin-like protein in this prokaryote. Minkoff and Damadian (1976) had attempted to attribute actin-like properties to a protein of E.coli simply by the cosedimentation of a reversibly aggregating, 45,000 dalton protein with rabbit muscle myosin; the specificity of the interaction with myosin was not clearly shown. Subsequently a specific myosin interaction was demonstrated by Nakamura and Watanabe (1978) with a similar protein of E. coli which was shown to activate the ATPase of rabbit muscle myosin. These workers also demonstrated the existence of a myosin-like protein as well in E. coli.

The filamentous nature of the E. coli ALP was not demonstrated by either of these studies, particularly not by the reversible 'aggregation' described by Minkoff and Damadian. This 'aggregation' could have been a non-specific association of proteins, mediated by the addition of salts, which would then pellet by ultracentrifugation. The results presented in this thesis clearly show the filamentous nature of the E. coli ALP by the demonstration of filament bundles and 6 nm diameter filaments (Fig.3).

Previously it has been suggested that E. coli ALP is, in fact, the protein synthesis Elongation Factor Tu (Ef-Tu) of E. coli (Beck et al 1978, Rosenbusch et al 1976). Ef-Tu was shown to undergo polymerization under the conditions favouring E. coli ALP 'aggregation', and that mainly large bundles of filaments, as well as single filaments would form (Beck et al 1978). However, attempts by these workers to decorate the Ef-Tu filaments with HMM failed to produce arrowhead complexes. Also, myosin ATPase failed to be activated; it was, in fact, inhibited by Ef-Tu. Subsequent optical

diffraction analysis and immunochemical studies of Ef-Tu have shown the two proteins to be distinct (Wurtz *et al* 1978). The HMM decoration of the *E. coli* filaments, shown in this thesis (Fig. 3), confirms their actin-like nature, distinguishing them from Ef-Tu further.

The function of *E. coli* ALP has yet to be clearly demonstrated. Minkoff and Damadian (1976) have presented evidence suggesting the involvement of ALP in the maintenance of cell 'tonus', by regulation of sodium and potassium transport across the cytoplasmic membrane. The evidence supporting this theory derives from the occurrence of mutant strains which possess an ALP requiring a significantly higher concentration of potassium for its 'aggregation'. It was presumed that the ALP filaments (not then demonstrated as such) would serve as a 'cytoskeleton', allowing a contractile process to sieve out larger sodium ions and favour the retention of smaller potassium ions.

While the 'cytotonus theory' remains to be conclusively proven, one role for ALP that can be dismissed is that of motility, for motility in *E. coli* has been ascribed to flagellar rotation and not to any contractile process (Silverman and Simon 1974). The general association of actin and myosin with contractile-motile phenomena among eukaryotes would seem to favour the possibility that an actomyosin may yet be involved in some form of bacterial motility. A contractile mechanism to explain gliding motility in bacteria has been repeatedly promoted (Doetsch and Hageage 1968, Henrichsen 1972), and in view of the recent discovery of an ALP in *Mycoplasma* (Neimark 1977), which displays a form of gliding , it is still a tantalyzing hypothesis.

The application of the Minkoff and Damadian procedure to the gliding myxobacter, *Myxococcus xanthus*, failed to provide conclusive evidence for

the existence of an ALP in this organism. While 6 nm filaments could be resolved in E.M. specimens (Figs. 4,9) they would not decorate with HMM. The obscured and bundled appearance of these filaments, as well as their apparent inability to interact with HMM, suggest the presence of some other factor(s) with possible regulatory function.

Nakamura and Watanabe (1978) succeeded in purifying E. coli ALP to two components, with a 56,000 dalton polypeptide appearing to bind tenaciously with the ALP. It was suggested that this polypeptide may be responsible for the poor interaction of ALP with rabbit muscle myosin, as evidenced in the low level of myosin ATPase activation. This might also be the case with an ALP of M. xanthus.

Stossel and Hartwig (1976) demonstrated an 'actin-binding protein' which appears to regulate both the polymerization of actin and the acto-myosin interaction in macrophages. Crude extracts of macrophages were shown to contain arrays of obscured actin filaments. Treatments with 0.6 M KCl released the 'binding protein', a ring-shaped aggregate, which was purified by precipitation at low pH and low ionic strength. A similar treatment of the M. xanthus extract produced a purified fraction of 'tetrameric' proteins, having a subunit molecular weight of 10,000 daltons (Fig. 6). The actual nature of this protein is unknown, though its relative abundance in the cell (7mg. protein per 22 gm. of acetone powder, or 170 gm. of wet cells) suggests significance. Unlike the macrophage actin filaments, the M. xanthus filaments were not dissociated from the obscuring material by these high salt treatments.

Clearly a more sophisicated procedure, other than differential solubilization, is called for if bacterial ALP is to be purified. Even the high temperature, ammonium sulfate, and urea treatments of E. coli ALP by Nakamura and Watanabe (1978) did not lead to purity; treatments

with high temperature, urea and SDS also failed to dissociate the material obscuring the filaments of M. xanthus.

A method which appeared to have promise was that which utilizes the property of actin binding tightly to anion exchange resin at low ionic strength (Gordon et al 1976). This method had proven most effective in purifying Acanthamoeba actin to homogeneity, and in high yield. When applied to acetone-dried muscle fibre, the method produced a major peak of actin in the expected region of the KCl gradient (Fig. 11a); A nearly homogeneous preparation of actin was demonstrated by SDS-PAGE (Fig. 13, gel 11), and beaded filaments therein were readily decorated with HMM to form arrowhead complexes (Fig. 3f).

The application of this procedure to the whole cell extract of M. xanthus resulted in a poorly resolved profile (Fig. 11a) perhaps due to an orange pigment interfering with ultra violet absorbance measurements. The fractions comprising a small 'blip' in the region where actin was expected to elute, proved to contain a 45,000 dalton component (Fig.13), and demonstrated linear arrays of obscured material in E.M. preparations when incubated with 2 mM $MgCl_2$. Together, these observations were suggestive of the presence of an ALP; but subsequent polymerization cycles, to which the pooled fractions were subjected, failed to yield filaments that could be decorated with HMM.

Even though the DEAE-Cellulose procedure had been applied to a whole cell extract of Acanthamoeba , it appeared that the whole cell extract of M. xanthus might contain components which could not be separated from an ALP by this method. The elution profile resulting from chromatography of the extracted M300 acetone powder (Fig. 14) proved to be even less satisfactory than that of M36 whole cells. Not only had a non-dialyzable,

vividly orange pigment interfered with absorbance measurements, but the suspect ALP (judged by the presence of a 45,000 dalton component in fractions demonstrating linear arrays in E.M. preparations) was distributed throughout the effluent (Figs. 14a, 15). Perhaps M300 differs from M36 or possibly the acetone treatment may have released membrane-associated components which interact with an ALP, affecting its elution. Nakamura and Watanabe (1978), in their study of E. coli , have shown that the bulk of the ALP is in the membrane fraction, possibly bound by a membrane-associated protein.

The most promising method used in this study of M. xanthus is that of Neimark (1977), which employed gentle lysis of cells under conditions which promote the extraction of actomyosin. This procedure led to a fraction which not only contained 6 nm filaments, concommittant with a 45,000 dalton polypeptide,but it also possessed the ability to slightly activate rabbit muscle myosin ATPase (Table 1). However, the filaments in this preparation did not appear to decorate with HMM, and whether this is related to the poor myosin ATPase activation is not known. This procedure also led to the appearance of large, aggregated structures (Fig. 10) which, while interesting in themselves, are even moreso in view of the discovery of similar structures in thin sections of M. xanthus (Burchard 1977, Schmidt-Lorenz and Kuhlwein 1968). It has been speculated that these structures might be involved in motility, but this has not been substantiated.

An actin-like protein was not readily extracted by the conventional procedures used. These procedures involved actin-specific polymerization-depolymerization reactions in crude extracts which may contain other interacting components. The work of Nakamura and Watanabe (1978) demonstrates

the avidity with which such a component will bind to ALP. Therefore another approach to the problem of prokaryote ALP purification and characterization is needed.

One approach that may lead to ALP purification could involve electrophoretic procedures. The relationship of the mass and charge for each protein is essentially unique. A preparative, native gel electophoresis, such as the system of Furlong et al (1973), coupled with an actin assay such as that of Gordon et al (1976) may serve to purify and identify an actin-like protein from any system. The more sophisticated procedure of preparative isoelectric focussing may be a more advantageous technique, for the isoelectric points of several actins are known (Gordon et al 1977) and an ALP or even several isomers might be isolated from a complex mixture by this means. By the reliance upon the intrinsic properties of a protein and not upon inter-protein interactions, this approach may prove effective in isolating an ALP from M. xanthus or any other gliding bacterium,and lead to its characterization.

But whether the gliding motility mechanism is of a contractile nature is now doubtful in view of recent evidence. Pate and Cheng (1979) have presented evidence that would suggest the involvement of rotary surface assemblies in the motility of some gliders. This finding is consistent with the motility mechanisms of other prokaryotes which rely on rotary elements (Silverman and Simon 1974, Bromely and Charon 1979). The ring-shaped structures observed in extracts of M. xanthus (Fig. 17) are similar in appearance to those seen in Flexibacter and Cytophaga (Pate and Cheng 1979) and may indicate the presence of a similar rotary 'motor' in myxobacteria.

The suggestive evidence for an actin-like protein in M. xanthus and

the demonstration of actin-like and myosin-like proteins in E. coli pose questions about their functions in prokaryotes. Bearing in mind the associations of contractile proteins with diverse functions in eukaryotes, the course of investigation into this realm of bacterial physiology is well laid out.

SUMMARY

The filamentous nature of an actin-like protein of Escherichia coli, and its ability to form arrowhead complexes with heavy meromyosin is demonstrated. To test the contractile theory for the mechanism of bacterial gliding motility several procedures, designed to extract actin-like protein, were applied to the gliding myxobacter Myxococcus xanthus. Filaments resembling those of E. coli were demonstrated, but they did not appear to decorate with heavy meromyosin. Associated proteins appeared to complex with the filaments, possibly interfering with purification and heavy meromyosin decoration. Other procedures which do not rely upon actin-specific interactions may be required for the purification of bacterial actin-like protein. In some extracts, large fibrous complexes and ring-shaped structures were observed. These structures may have implications in the gliding motility mechanism.

APPENDIX

CURRENT MICROBIOLOGY, Vol. 1 (1978), pp. 201–202

Current Microbiology
An International Journal

Heavy Meromyosin Decoration of Filaments from *Escherichia coli*

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Abstract. A fibrous protein complex extracted from *Escherichia coli* B/r by the method of Minkoff and Damadian [2] demonstrates arrowhead complexes when reacted with heavy meromyosin.

Recent studies have demonstrated the presence of a protein with actin-like properties in prokaryotes. Minkoff and Damadian [2] have shown that a reversibly aggregating protein complex of *Escherichia coli* mimics the polymerization cycle of actin. It also binds myosin, as judged by co-sedimentation and co-electrophoresis. This was confirmed recently by Nakamura and Watanabe [3], who also have demonstrated activation of the adenosine triphosphatase (ATPase) of rabbit skeletal muscle myosin and of a myosin-like protein of *E. coli*, though activity appears to have been quite low. Neimark [4], however, has shown the formation of arrowhead complexes between rabbit skeletal muscle heavy meromyosin (HMM) and 5- to 6-nm filaments in *Mycoplasma pneumoniae*.

Presently, knowledge of the interaction of the fibrous protein of *E. coli* with myosin is at best in a confused state. Interaction with myosin and the formation of arrowhead complexes are essential criteria in the identification of actin, and must be met if authentic actin is to be considered as having been demonstrated in *E. coli*.

We have used the procedure of Minkoff and Damadian [2] to prepare a protein extract of *E. coli* B/r that contains bundles of well resolved filaments (Fig. 1) as well as clumps of more dispersed, though obscured, filaments (Fig. 2) with an occasional bare filament of 6-nm diameter visible (Fig. 3). Attempts to decorate this preparation with HMM have led to the appearance of arrowhead complexes along short lengths of the filaments (Figs. 4 and 5). The 39-nm period of the complexes corresponds well with that observed in a wide range of eukaryotes [5] and in *Mycoplasma* [4]. We have also used this same method and obtained similar filaments from the gliding myxobacter, *Myxococcus xanthus*, but could not decorate them with HMM.

The limited appearance of arrowhead complexes and the obscured nature of the filaments, combined with evidence of the complex nature [1, 2,3] and poor myosin ATPase activating property

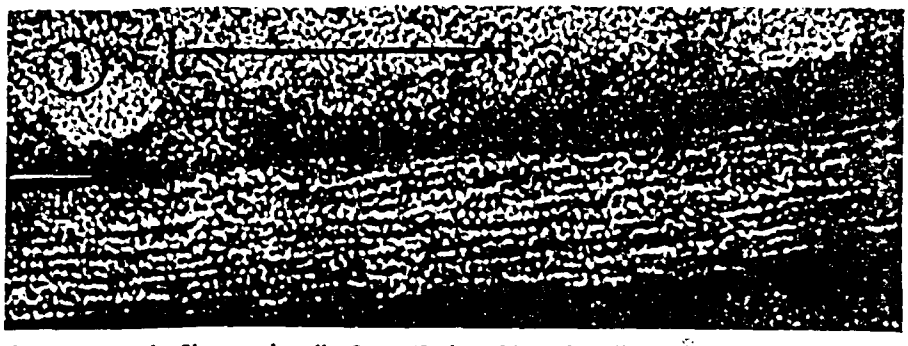

Fig. 1. Protein filament bundle from *Escherichia coli*. All specimens were applied as a drop with a capillary pipette onto carbon reinforced, formvar coated copper grids for 60 s. After removal of excess sample, a drop of 1% uranyl acetate (pH 3.8) was applied, then blotted with a filter paper wedge and air dried. Specimens were examined in a Hitachi Hu-12 at 75 kV or in a Phillips 201c at 60 kV, each equipped with cold finger. The bar represents 100 nm in each figure.

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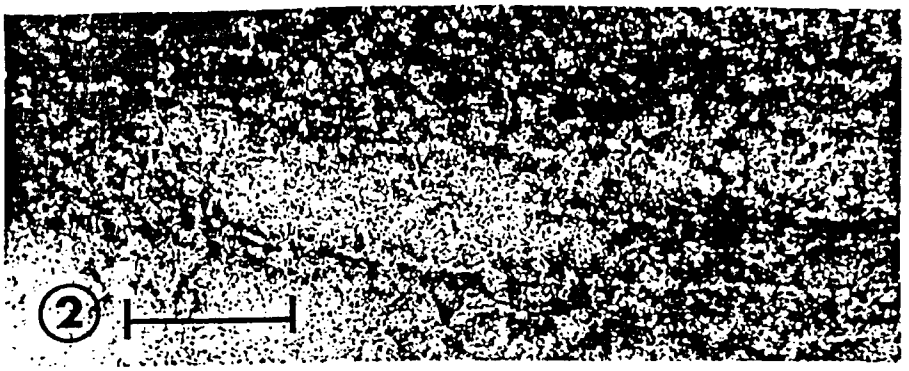

Fig. 2. Clumps of obscured filaments from *Escherichia coli*.

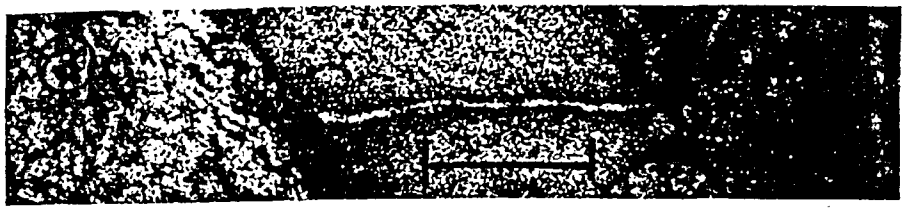

Fig. 3. Bare, 6-nm-diameter filament amid clumps of obscured filaments.

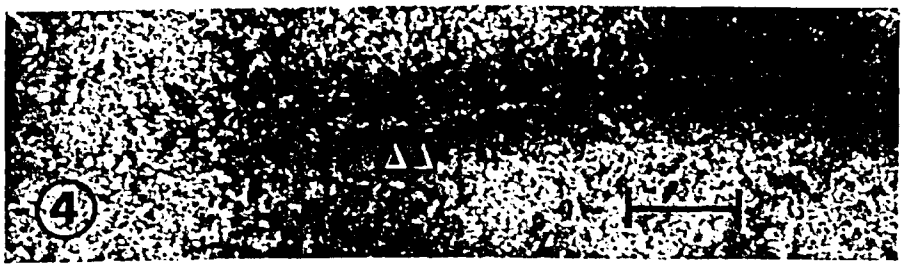

Fig. 4. Rabbit skeletal muscle heavy meromyosin (HMM) decoration of *Escherichia coli* filaments. After application of sample, a drop of HMM (350 μg/ml) in 10 mM Tris-HCl (pH 7.5), 1 mM disodium EDTA, 1 mM β-mercaptoethanol, and 0.1 M KCl was applied for 1 min and removed, then stained. Markers indicate arrowhead complexes.

Fig. 5. HMM decoration (see Fig. 4 legend for method).

[1,3] of this actin-like fraction, strongly suggest the close association of other protein(s), with possible regulatory properties (see also Nakamura and Watanabe [3]).

ACKNOWLEDGMENTS

We thank Arthur Forer for the generous gift of rabbit skeletal muscle heavy meromyosin and John Schuurman for his technical assistance.

This work was supported by National Research Council of Canada grant A1022

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