THE ACTOMYOSIN-LIKE PROTEIN

OF

NAEGLERIA GRUBERI AMOEBA

AN INVESTIGATION INTO SOME PROPERTIES OF THE ACTOMYOSIN-LIKE PROTEIN OF *NAEGLERIA GRUBERI* AMOEBA

by

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SCOPE AND CONTENTS:

Amoeboid motion is thought to be due to the action of an actomyosin-like protein present in the cytoplasm of amoeba. A co-ordinated network of microfilaments of the actomyosin-like protein, 70 Å in diameter, may be the mechanical means of accomplishing amoeboid motion. The microfilaments formed of the actomyosin-like protein, may be capable of rapid association and dissociation in vivo.

In this thesis, the cytoplasm of Naegleria gruberi amoeba has been shown to possess a protein similar to actomyosin. Characterization of the ATPase activity, superprecipitating ability, electrophoretic behaviour and microfilament producing ability reveal that the actomyosin-like protein of Naegleria gruberi amoeba is quite similar to the analogous protein in Physarum polycephalum. Naegleria gruberi may be an ideal organism in which to study the interconversion of one form of a biologically active macromolecule to another. In different stages of the life cycle, amoeboid motion, flagellar beating and mitotic spindles are present. It is possible that the same contractile molecules in different forms may perform different functions.

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INTRODUCTION

Although the phenomenon of amoeboid motion has been recognized for well over a century, only within the last twenty years have efforts been made to understand its molecular basis. Concomitant studies of other intracellular phenomenon of a contractile nature, such as muscular contraction, saltatory particle movement, chromosome movement during mitosis, cytoplasmic streaming, and flagellar beating suggest that common mechanisms of operations may be in effect. It may be postulated that certain cell types are capable of utilizing one form of a contractile protein to perform one function, and in a different form this same contractile protein could perform a different function. One organism which may be capable of doing this is the soil amoeba Naegleria gruberi since, in various stages of its life cycle, it does exhibit amoeboid motion, chromosome separation, and flagellar beating.

Actomyosin-like proteins from many diverse cell types have been characterized, (see table 1). A growing body of evidence of non-muscular contractility at the cellular level indicates mechanisms similar to muscle contraction may be responsible for cell movement, both by a single cell or by a group of cells. The precise molecular organization of these actomyosin-like proteins is unknown, but they most probably are arranged into some sort of ordered array (2). In vitro studies of these molecules must ultimately be related to their state in vivo (3). Proteins similar to actin, myosin, and actomyosin are most probably involved in non-muscular motility.

TABLE I

Comparison of actomyosin-like proteins
adapted from (I)

SOURCE	EXTRACTION MEDIUM	EFFECT OF ATP ON EXTRACT	REPRECIPITATION	EFFECT OF ATP ON INSOLUBLE FORM	REFERENCE
Rabbit muscle actomyosin (myosin B)	0.6 M KCl pH 8-9	Fall of viscosity reversed as ATP is split.	As load-bearing fibers or a gel below 0.2 M KCl	Rapid contraction of fibers. Superprecipitation of gel.	Weber & Portzehl (1952) Advances Protein Chemistry 7: 161-252.
Amoeba proteus	0.6 M KCl on water insoluble residue.	ATP is split.	-	-	Simard-Duquesne & Couillard (1962) Experimental Cell Research 28: 85-91.
Physarum polycephalum (myxomyosin)	1.7 M KCl	Fall of viscosity reversed as ATP is split.	20-40% saturated ammonium sulfate.	-	T'so et al (1956) J. General physiology 39: 325-47.
Physarum polycephalum (Plasmodial myosin B)	0.6 M KCl pH 8.9	"	Below 0.1 M KCl	Slow superprecipitation.	Nakajima (1960) Protoplasma 52: 413-36.

SOURCE	EXTRACTION MEDIUM	EFFECT OF ATP ON EXTRACT	REPRECIPITATION	EFFECT OF ATP ON INSOLUBLE FORM	REFERENCE
Physarum polycephalum (Plasmodial myosin B)	0.4 M KCl 15 mM EDTA pH 8.2	Fall of viscosity reversed as ATP is split.	Below 0.1 M KCl	Strong and rapid superprecipitation.	Hatano & Tazawa (1968) Biochimica Biophysica Acta 154: 507-19.
Naegleria gruberi amoebae (Actomyosin-like protein)	0.4 M KCl 15 mM EDTA pH 8.2 + digitonin 1.0 mg/ml	ATP is split.	"	"	Lastovica (unpublished results)

Muscle fibers containing essentially only the contractile elements may be prepared by glycerol extraction. Such fibers which contain the actomyosin in an insoluble state, will contract similarly to isolated actomyosin, or to intact muscle fibers (4). Insoluble contractile models of non-muscular cells have likewise been prepared by glycerol extraction of tissue cells (5), sperm tails (6), amoebae (7), and myxomycete plasmodia (8).

It is universally accepted that the protein actomyosin plays a key role in the contractile process of striated muscle. Actomyosin, also called myosin B, is routinely extracted after 24 hours from a mince of striated muscle by the use of a solution of neutral buffered salt, the Weber-Edsall solution, (0.06 M KCl, 0.04 M $KHCO_3$, 0.01 M K_2CO_3). Haga, et al (9), have investigated this extraction procedure, and conclude that the pure myosin, also called myosin A, is initially extracted from the muscle, then combines with the polymer form of actin (F-actin), to form actomyosin, and this process is completed by 20 hours. Actomyosin, at pH 7.0, precipitates at or below an ionic strength of $\mu = 0.1$, while at an ionic strength of $\mu = 0.4$ or greater, actomyosin is completely soluble (10).

In contrast to precipitation, superprecipitation occurs at low ionic strength, ie., $\mu = 0.05 - 0.15$, at pH 7.0 in the presence of low concentrations of ATP and Mg^{+2}ion. When this process occurs in solution, the particles of actomyosin decrease in size, increase in density and rapidly settle. Superprecipitation has long been considered to be the in vitro analogue of contraction, and involves not only physical interaction of the actin with the myosin, but also a change in the enzymatic properties of the myosin ATPase, so that it is activated rather than inhibited by the Mg^{+2}ion. The addition of higher concentrations of ATP

at low ionic strengths dissolves, or "clears" the actomyosin suspension. Clearing has been reported to be due to the dissociation of actin and myosin, and has been considered to be the in vitro analogue of relaxation (11, 12). Like relaxation and contraction, clearing and superprecipitation has been considered to be an all or nothing phenomenon. However, Eisenberg and Moos (13), suggest that superprecipitation and clearing may not be strictly analogous to contraction and relaxation in vivo. Their simultaneous ATPase, viscosity, and turbidity measurements indicated that enzymatic activation of myosin could occur in the cleared state. This would imply that myosin and actin do physically interact in the cleared state.

Actomyosin from striated muscle possesses ATPase activity, the extent of which is dependent on the ionic strength (14), and is activated by the Ca^{+2} ion at both high (μ=0.5) and low (μ=0.1) ionic strengths. Mg^{+2} ion inhibits the ATPase activity at high ionic strength, but will stimulate the enzymatic activity at low ionic strength (15). It is well known that actomyosin ATPase activity may be inhibited by a number of compounds such as Salyrgan, the sodium salt of o-(3-hydroxymercuri-2-methoxypropyl) phenoxyacetic acid, and p-chloromercuribenzoic acid, (PCMB). The inhibition caused by PCMB may be prevented, in part, by cysteine. Both these inhibitors act on the sulfhydryl groups present on the enzyme.

Actomyosin, dissolved in 0.5-0.6 M KCl, at pH 7.0 has a high viscosity. If low concentrations of ATP are added to the actomyosin solution, the viscosity will drop rapidly (16). It is thought that this viscosity drop is due to the dissociation of the actomyosin into its

constituent actin and myosin, and as the ATP is dephosphorylated by the myosin, the viscosity will gradually increase to its original value, as the actin and myosin recombine to form actomyosin. This ATP-induced viscosity drop may be repeated several times on the same sample.

The dissociation of the actomyosin complex into its constituent actin and myosin is dependent not only on the ionic strength (17), but the ATP concentration as well. Weber (18) succeeded in preparing myosin and actin from actomyosin by interacting the actomyosin in 0.6 M KCl with 5mM ATP and 10^{-3} M Mg^{+2} ion, and centrifuging for 3 hours at 100,000 x g. "Interaction inhibitors" (19) may also be involved in the dissociation process. Marsh (20) was the first to discover that striated muscle contained an "interaction inhibitor", or relaxing factor. Elegant work by Ebashi and co-workers has resolved the mode of action of this inhibitor (21). They have found the protein tropomyosin in collaboration with the protein troponin sensitized myosin and actin interaction to Ca^{+2} ion, and concluded that the sensitivity of a contractile system to Ca^{+2} ion depended only on the Ca^{+2} binding ability of the protein troponin. Further work on non-muscle actomyosin-like proteins may reveal similar "interaction inhibitors."

Myosin has been studied in detail: it is an elongate molecule, about 1600 Å long by 20-40 Å wide (22), whose molecular weight is now generally accepted to be 500,000 (23). Actin has usually been obtained from an acetone dried powder of muscle by extraction with water (24). G-actin, the globular, monomeric form (M.W. 60,000) of this protein is obtained with this procedure. In 0.1 M KCl, with trace Mg^{+2} and Ca^{+2} ion, approximately 30-40 G-actin monomers polymerize into a long fibrous molecule

of F-actin (M.W. $2x10^6$), comprised of two chains of G-actin subunits twisted around one another in a helix. This elongate F-actin molecule is then associated with myosin to form the actomyosin complex.

Proteins similar to striated muscle actin, myosin, and actomyosin have been isolated from a number of non-muscle sources. Hatano and Oosawa (25) have recently isolated the actin-like protein from an acetone powder of the slime mold, Physarum polycephalum. These workers have studied the polymerization of this actin-like protein (26), and its interaction with rabbit muscle myosin (27). Using the procedure of Weber (18), Hatano and Tazawa (28) have succeeded in isolating the myosin-like protein from the actomyosin-like protein from Physarum.

Actomyosin-like proteins from amoeba and slime mold plasmodium have been isolated by a number of workers. Lowey, in 1952 (29), was the first to isolate an actomyosin-like protein from Physarum plasmodium by extraction with 1.2 M KCl and 0.1 K_2HPO_4. Ts'o, et al, obtained myxomyosin from Physarum by salting out with $(NH_4)_2 SO_4$ and centrifugation (30). Nakajima (31) in 1960, isolated plasmodial myosin B by methods similar to the extraction of actomyosin from rabbit striated muscle, using Weber-Edsall solution. However, these extraction procedures gave actomyosin-like preparations which were relatively impure. Hatano and Tazawa (28), used a final concentration of 15mM EDTA during extraction of their actomyosin-like protein (plasmodial myosin B). This procedure proved to be very effective in preventing contamination of the actomyosin-like protein with globular material. A clear and very pure preparation of plasmodial myosin B was obtained, without any indication of denaturation.

Thus, the contractile cytoplasm of Physarum polycephalum consists of two kinds of protein, an actin-like and a myosin-like protein. The

interaction of filaments of the actin-like and the myosin-like proteins in the presence of ATP and divalent cations presumably generates the motive force responsible for streaming in Physarum plasmodia by a mechanism in some way related to the homologous phenomena in muscle.

It is unknown exactly how structures such as microfilaments and microtubules found in streaming systems are related to physiological events of contraction in the cell. Microtubules are prominent in many types of cell: they may form the elements of the mitotic spindle, of centrioles, and the axonemes of flagella and cilia. Maintenance of cell shape is also thought to be a function of microtubules, and this provision of a rigid framework may aid in cytokinesis (32, 33). Although microtubules have been associated with contractility, ie., chromosome movement during mitosis, and flagellar beating, the most probable organizational element of amoeboid motion is the microfilament. However, suggestions have been made that microfilaments are merely subunits of microtubules (34, 35).

Similarities exist between muscle actin, Physarum plasmodial actin, and the microtubules of flagella, cilia, and of the mitotic apparatus. Hatano and Oosawa (25, 27), and Adelman, et al (34) have reported that the actin-like protein of Physarum was similar to muscle actin. The monomeric forms of both proteins (molecular weight about 60,000) exist as globular subunits at low ionic strength with one mole bound nucleotide per mole protein. Mazia (82) stated that the subunit isolated from sea urchin mitotic apparatus is similar to muscle G-actin with respect to a molecular weight of about 60,000, a sedimentation constant of about 4s, and the number of sulfhydryl groups bound to the monomer. An important difference is that the mitotic apparatus subunit contains six times as much bound nucleotide as G-actin. Recent work by Kieffer, et al (83) indicates the

smallest subunit of sea urchin egg mitotic apparatus is a 2.5s protein of 33 Å diameter and molecular weight of 34,000. Stephens (84) and Renauld (85), reported that the protein subunits of Tetrahymena cilia outer fibers, sea urchin flagella, and muscle actin have common characteristics. The three proteins possess a molecular weight of about 60,000, a sedimentation constant of 4s, similar amino acid composition, similar electrophoretic behaviour and bind a mole of nucleotide per mole protein. Tetrahymena and sea urchin subunits bind guanine nucleotides, while actin binds adenosine nucleotides.

Microfilaments are present in amoeboid cytoplasm after treatment of various kinds such as incubation with surface bound dye (36) or with an injection of a fixative (37). Microfilaments, of a diameter of about 70 Å have been seen in amoeba by electron microscopy (38,39). Wolfarth-Bottermann (60,61) reported that these fibrils form a coherent network in Physarum plasmodial cytoplasm. Two types of filaments have been seen by Nachmias (40) in the cytoplasm of the amoeba Chaos chaos; thin filaments 40-60 Å in diameter and thicker filaments 120-150 Å in diameter, which could have been aggregations of the thinner filaments. Nagai and Kamiya (41), have studied glycerol extracted Physarum plasmodia and have found the 70 Å diameter microfilaments present in the cytoplasm tend to aggregate on the addition of 5mM ATP, 5mM Mg^{+2} ion, and 30 mM KCl.

The electron microscope studies of Hatano and Tazawa (28) demonstrate that the plasmodial myosin B molecule is about 100 Å in diameter in both 0.05 M and 0.5 M KCl without ATP. These results compare favourably with those of Nagai and Kamiya on the fibrils present in the cytoplasm of glycerinated Physarum plasmodia (8). When ATP was added to dissociate the plasmodial myosin B into its constituent myosin-like and

actin-like components, electron micrographs revealed filaments very similar to plasmodial F-actin, about 75 Å in diameter. These workers suggest that the plasmodial myosin B molecule consists of a core of actin-like protein surrounded by globular units of myosin-like protein. This is a unique arrangement of the contractile elements, as in striated muscle the myosin and actin filaments alternate in the regions of overlap.

Naegleria gruberi (42, 43), is a small soil amoeba which can exist in three morphologically distinct states in its life cycle, as cyst, amoeba, or flagellated cell. The cytoplasm of Naegleria amoebae and Physarum plasmodia contain a contractile protein similar in many respects. By the use of the cyst-lysing procedure developed by Werth and Kahn (45), and the cytoplasmic contractile protein extraction procedure of Hatano and Tazawa (28), protein extracts of Naegleria cysts were tested for the ability to form microfilaments under the influence of low concentrations of the chelating agents, EDTA and EGTA. These cyst contractile protein extracts never produced microfilaments, while control amoebae subjected to the same extraction procedure always produced microfilaments. It is possible that the contractile protein present in the amoeba may be absent, or not biologically active in the cyst form. The excystment process of Naegleria has been well investigated (44). The reversible amoeba to cyst transition in Naegleria will allow studies to be undertaken of possible alternate forms of the actomyosin-like protein.

In the flagellated state, the cells assume a fusiform shape and the cytoplasm does not usually exhibit streaming, as do the amoeboid cells. It is possible that the contractile protein present in the amoeba may have been utilized for the production of the contractile elements of the flagella. Naegleria flagellates have never been observed to divide

and Naegleria amoeba about to divide exhibit reduced amoeboid motion. Since the mitotic cycle involves chromosome separation under the influence of the contractile elements of the mitotic spindle, it is suggested that the amoeboid contractile protein may be serving yet another function. Work by Fulton and Guerrini (46) dealing with the mitotic synchrony of Naegleria should provide quite valuable insights into this fundamental question of cell economy.

Forsheit and Hayashi (47), have reported that colchicine in concentrations sufficient to inhibit mitosis does not affect myosin or actomyosin ATPase activity. Adelman, et al (34) have found that ^{3}H-colchicine gave a high specific binding activity (cpm/mg protein) with sea urchin mitotic apparatus, while less than a hundreth the activity was obtained with Physarum polycephalum cells, myosin, F-actin or G-actin. If colchicine binds to Naegleria mitotic apparatus and not to the cytoplasmic actomyosin-like protein of Naegleria, then studies of possible interconversion may be undertaken.

The precise organization of the contractile proteins of the amoeba has still to be resolved, but it is conceivable that a network of labile contractile microfilaments could be present in the cytoplasm. Microfilament-like structures have been produced in vitro from contractile protein extracts of Naegleria amoeba, by the addition of 2.5 mM EDTA, EGTA or ATP. However, contractility in this system may not result from the organized array of interdigitating elements similar to the actin and myosin filaments in striated muscle.

It is conceivable that a cell can express different contractile functions, such as amoeboid motion, chromosome separation and flagellar beating, by utilizing the same contractile molecules in different ways.

Naegleria gruberi would appear to be the organism of choice for a detailed study of the above phenomena. In vitro studies of these contractile molecules may then provide valuable insight into the molecular basis of these different types of cell movement.

MATERIALS AND METHODS

Stocks

The experimental work was done with the amoebo-flagellate, Naegleria gruberi, strain NEG-4c. These cells were grown in association with bacterium, Aerobacter aerogenes. Both the NEG-4c strain of Naegleria, and stock cultures of Aerobacter were supplied by Dr. C. Fulton of Brandeis University. Cells for stock plates were cultured in 10 cm petri dishes of autoclaved NM media, according to the method of Fulton and Dingle (43). Stock cultures of NEG-4c were prepared by spreading 0.1 ml of an overnight Penassay broth culture of Aerobacter on an NM plate. The bacteria were spread with a glass spreader stored in 70% ethanol and flamed before use. A bacteriological loop of NEG-4c cysts was transferred to one point near the edge. Occasionally, "2x" cells would arise with double the cell volume, and four, or twice the usual number of flagella. Precautions were taken to maintain the strain originally supplied by periodically reisolating "1x" clones from stock plates. Stock culture of Aerobacter were maintained at 22 °C on agar slants of Difco Penassay broth (antibiotic media 3).

Media

Two nutrient agar media were used for maintenance and growth of Naegleria (43). NM medium was used for the maintenance of stock plates of Naegleria. This medium contained (in grams per liter of distilled water): Difco Bacto-peptone, 2.0; Dextrose, 2.0; K_2HPO_4, 1.5; KH_2PO_4, 1.0; and

Difco Bacto-agar, 20.0. PM medium was used for large batch growth of Naegleria amoebae. The composition of this medium is the same as NM, except that PM contains double the amount of Bacto-peptone. Naegleria cells were grown in association with the bacterium, Aerobacter aerogenes. Overnight 34 °C liquid cultures of Aerobacter were grown in Difco Penassay broth (antibiotic media 3).

Growth of amoeba

To obtain amoebae for extraction of the cytoplasmic contractile protein, a suspension of about $3x10^5$ cysts from an NEG-4c stock plate was spread on PM with 0.3 ml of Aerobacter in a 9"x14" Pyrex baking dish covered with a single layer of aluminium foil. Usually, nine baking dishes were plated at the same time, and incubated at 34°C.

The amoebae excyst and grow exponentially on the growing bacteria until, as growth approaches stationary phase, the amoebae clear the bacterial lawn. Routinely, the baking dishes were harvested when 70-80% cleared at about 34 hours. Approximately eight grams wet weight of cells were obtained from nine baking dishes.

Amoebae and cysts were counted using a Coulter electronic particle counter, Model F (Coulter Electronics, Hialeah, Florida). Cells were suspended or diluted in 0.4% NaCl prepared in distilled water. Appropriate dilutions were counted in the Coulter counter, with a 100μ aperture, a current aperture of 32, attenuation of 2 and a threshold setting of 20. Cells were vortexed briefly before counting to break up any clumps of cells or bacteria.

Isolation of contractile protein

The extraction procedure was based on a modification of the method of Hatano and Tazawa (28). At about 75% clearing of the bacterial lawn, one baking dish was flooded with 100 ml of 2mM Imidazole buffer, pH 6.8. The cells were suspended from the agar surface using a flamed, cooled glass spreader. The cell suspension was poured into the next baking dish, until four or five dishes had been washed in this way. This procedure was repeated with fresh buffer twice more. The pooled cell suspension was run through cheesecloth moistened with buffer to remove agar chips. The cells were centrifuged at low speed to pellet the amoebae, leaving the contaminating bacteria in the supernatant. Four centrifugations at 1600 x g for 1 minute, in 50 ml lusteroid tubes in an International Clinical centrifuge fitted with a swinging bucket head, were sufficient to remove all but a few of the contaminating bacteria.

The final combined pellet was resuspended to a known volume with 2mM Imidazole buffer, pH 6.8 and vortexed for 5 seconds. Samples were taken at this point for Coulter counter or Lowry protein determinations. This cell suspension was spun down once more in the I.E.C. centrifuge and the supernatant was carefully Pasteur pipetted off and discarded. The pellet was weighed, and its volume was determined by measuring the distilled water in another centrifuge tube, equal in volume to that of the pellet. One volume of 0 °C freshly prepared 0.8 M KCl, 30 mM EDTA (pH 8.2) containing 2 mg/ml digitonin, was added to the pellet, which was then vortexed 15-20 seconds and stored at 0-5°C. Digitonin was not stable in this solution for more than 12 hours.

After about 20 minutes, essentially all the amoebae had lysed

An equal volume of 0°C 0.4M KCl, 15mM EDTA (pH 8.2) solution was then added and the suspension was vortexed for 5 seconds. The pH of the resulting solution was adjusted with cold 1 N KOH to 8.2, and stirred in a cold room for 2 hours. Next, this slurry was centrifuged at 10,000 x g for 30 minutes in a refrigerated centrifuge (Sorvall, Model RC2B, SS-34 rotor) to pellet the cell debris. The supernatant was carefully Pasteur pipetted through a cheesecloth moistened with imidazole buffer, pH 8.2, into a chilled 50 ml graduated cylinder, the volume of the supernatant was measured, and 4 volumes of cold glass distilled water were added. The pH of the resulting solution was adjusted to 6.5 with cold dilute acetic acid, and the solution was stored at 0-5°C overnight to precipitate the contractile protein.

The resulting turbid solution was centrifuged in the RC2B refrigerated centrifuge, to pellet the precipitated protein. Centrifugation was carried out first in 50 ml tubes at 12,000 x g for 10 minutes, then finally, in a conical 11 ml tube at 7,000 x g for 10 minutes. The supernatant was carefully Pasteur pipetted off and discarded, then the pellet volume was determined. The pellet was made up to 0.5 M KCl, 0.018 M imidazole buffer, pH 6.8 with stock solutions of 3 M KCl, 0.175 M imidazole buffer, pH 6.8 and glass distilled water.

This solution was transfered to a small beaker and stirred about 1 hour in the cold room until the protein was evenly dispersed. Contaminating particulate matter was pelleted by centrifugation in a Spinco Model L-265B at 60,000 x g for 30 minutes. The supernatant which was clear and colourless, was carefully Pasteur pipetted through cheesecloth moistened with cold imidazole buffer pH 6.8 to remove contaminating lipid

material and stored at 0-5°C. The protein concentration of this solution was determined by the Lowry method (48).

Protein determination

A modification of the Lowry method (48) was employed, using Bovine serum albumin (B.S.A.) as a standard.

Reagents:

(a) Grams per liter of glass distilled water: Na_2CO_3, 20.0; Sodium potassium tartrate 0.2; NaOH, 4.0.

(b) 0.5% $CuSO_4.5H_2O$.

(c) 50 ml of reagent (a) and 1.0 ml of reagent (b), rapidly mixed just before use.

(d) Folin-Ciocalteau reagent diluted to half-strength with glass distilled water.

(e) Standard B.S.A.

Stock standard B.S.A. 1.0 mg/ml

Working standard B.S.A. 200 γ/ml

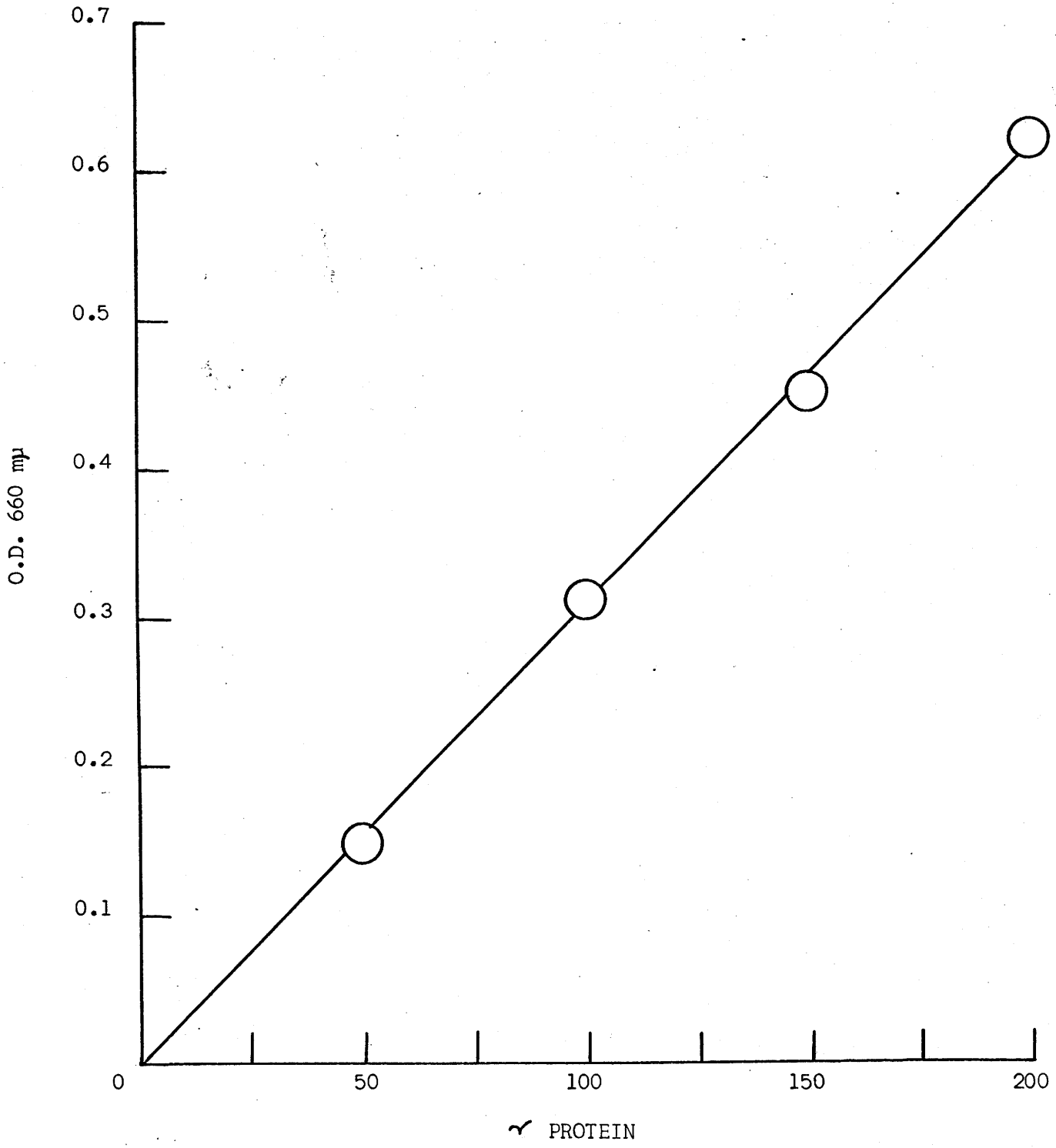
Procedure:

B.S.A. standards of 50, 100, 150 and 200 γ B.S.A. were prepared in 1.0 ml volumes of glass distilled water. To each tube, 3.0 ml of reagent (c) was added, then the tubes were vortexed 5 seconds each. The tubes were allowed to stand at room temperature for 15 minutes. Exactly 0.5 ml of reagent (d) was added to each tube, which was vortexed 5 seconds. The tubes stood at room temperature for 45 minutes, and were read at O.D. 660 mμ. The developed colour is stable for about 2 hours. With 100 γ of B.S.A. standard, an O.D.660 mμ . of about 0.3 was obtained.

Figure 1: LOWRY PROTEIN REFERENCE CURVE

This curve demonstrates the linearity of the Bovine serum albumin (B.S.A.) standard in the range 0 to 200✓protein. B.S.A. standards and unknown samples were determined at 660 mμ, in a 1.0 cm light path in a Bausch & Lomb Spectronic 20 spectrophotometer. The concentration of the samples was determined from the B.S.A. standard curve. A B.S.A. standard curve was run with each protein determination.

LOWRY PROTEIN REFERENCE CURVE



ATPase assay

Reagents:

Stock solutions of:

3M KCl, pH 6.8

Imidazole buffer, 0.175 M, pH 6.8

1.0 M $CaCl_2$

1.0 M $MgCl_2$

50 mM ATP, pH 6.8

Procedure:

Protein, KCl, $CaCl_2$, imidazole buffer, and glass distilled water were made up to a known volume at the desired concentration, and stirred at room temperature for 10 to 15 minutes. A substrate and an enzyme blank were taken before the addition of ATP to a final concentration of 1.0 mM. The time course at 22°C was initiated by the rapid addition of the ATP. Each sample removed from the reaction vessel was added to a separate acid-washed conical 11.0 ml centrifuge tube, in which 0.1 ml of 40% TCA and glass distilled water were present, such that the final volume was 1.1 ml. The tubes were vortexed briefly, and centrifuged in the I.E.C. clinical centrifuge at 1600 x g for 2 minutes to pellet the protein. Exactly 1.0 ml of the supernatant was carefully removed, and Pasteur pipetted into a clean acid washed test tube. The inorganic phosphate was determined by a modification of Sumner's method (49).

Inorganic phosphate (for ATPase assay)

A modification of Sumner's procedure (49) was employed, using monobasic sodium phosphate as a standard.

Reagents:

(a) 6.6% ammonium molybdate $(Mn_4)_6Mo_7O_{24}\cdot 4H_2O$.

(b) 7.5 N H_2SO_4.

(c) Ferrous sulfate solution. 0.5 gm $FeSO_4\cdot 7H_2O$ in 5.0 ml of glass distilled water with 0.1 ml of 7.5 N H_2SO_4.

(d) Standard $NaH_2PO_4\cdot H_2O$.

Stock standard 1.0 mM
Working standard 1.0 µmole/ml

Procedure:

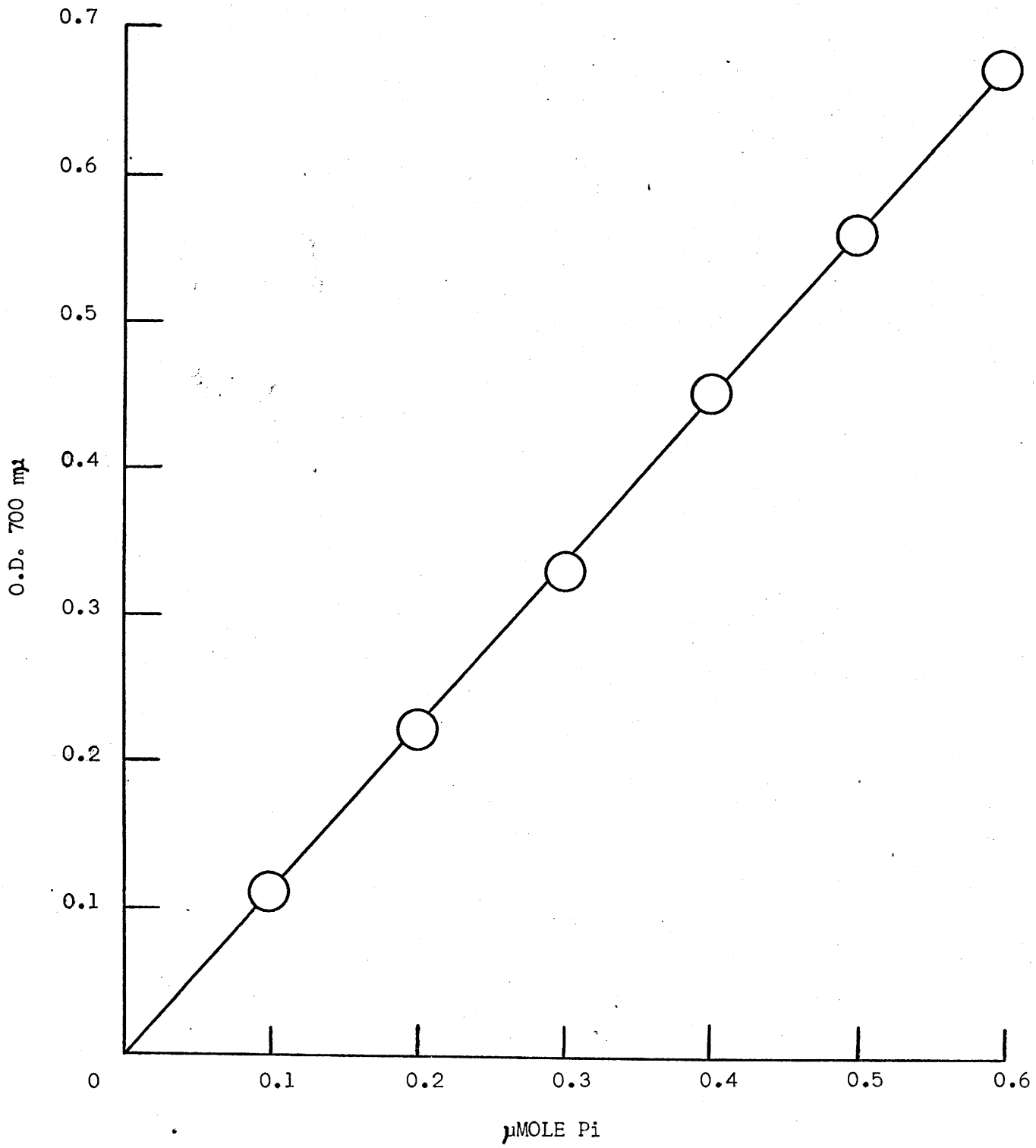
Standards of 0.15, 0.30, 0.45, 0.60 µmole Pi in a volume of 2.16 ml were prepared. The unknown samples were made up to a volume of 2.16 ml with glass distilled water. Exactly 0.3 ml of reagent (a) was added to each tube, which was then vortexed 5 seconds. Next, 0.3 ml of reagent (b) was added to each tube, which was then vortexed 5 seconds. Finally, 0.24 ml of reagent (c) was added to each tube which was vortexed 5 seconds. The tubes stood at room temperature for 5 minutes, and were read at O.D. 700 mµ. The developed colour is stable for about 30 minutes. With 0.3 µmole of phosphate standard, an O.D. 700 mµ of about 0.33 was obtained.

NOTE: Acid washed and thoroughly rinsed glassware was used to remove traces of contaminating phosphate. Reagent (c) is stable for 2 hours and was made up just before use. Reagents (a) and (b) are stable indefinitely.

Figure 2: INORGANIC PHOSPHATE REFERENCE CURVE

This curve demonstrates the linearity of the inorganic phosphate standard in the range 0 to 0.6 µmoles of inorganic phosphate. The inorganic phosphate standards and unknown samples were made up to a final volume of 3.0 ml. The optical density of the inorganic phosphate standards and samples was determined at 700 mµ, in a 1.0 cm light path in a Bausch & Lomb Spectronic 20 spectrophotometer. The concentration of the samples was determined from the inorganic phosphate standard curve. An inorganic phosphate curve was run with each phosphate determination.

Pi REFERENCE CURVE



Superprecipitation

A modification of the procedure of Bemis, et al (50) was used.

Reagents:

(a) 0.1 M $MgCl_2$

(b) 0.1 M $CaCl_2$

(c) 0.1 M EDTA

(d) 0.1 M EGTA

(e) 50.0 mM ATP

Equipment:

A Beckman recording spectrophotometer, Model DU, fitted with a Gilford Multiple Sample Recorder, Model 2000, was used with 1.0 ml cuvettes.

Procedure:

Protein, divalent cation, chelating agents and glass distilled water were added to the desired concentrations in a total volume of 0.98 ml in a 1.0 ml cuvette. A baseline was established at O.D. 550 mμ. The cuvette was removed, the superprecipitation reaction was initiated by the addition of ATP to a final concentration of 1.0 mM to the cuvette, which was rapidly mixed, and returned to the DU. Progress of the superprecipitation reaction was followed by the increase in O.D. at 550 mμ, at a chart speed of 4 and a maximum sensitivity of 1.0.

Viscosity

Professor S. Hatano of Nagoya University, kindly supplied specifications for the viscometers used in his studies of Physarum polycephalum plasmodial myosin B (28). These Ostwald-type viscometers had volumes of 0.6 ml and flow times for buffer at 22°C of about 30 seconds.

Disc electrophoresis

Disc electrophoresis at 0-5°C of Naegleria contractile protein extracts was accomplished by the method of Davis (51). A Canalco disc electrophoresis apparatus (Canal Industrial Corp., Rockville, Maryland) was used in conjunction with a Buchler D.C. power supply (Buchler Co., Fort Lee, N.J.). 50 ✓in 50 λ protein sample was mixed with 50 λ of saturated sucrose to increase the density and was carefully applied to the top of the polymerized polyacrylamide gel. The upper and lower reservoirs of the electrophoresis apparatus were carefully filled with 0.04 M Tris glycine buffer pH 8.3. 1 ml of 0.001% Bromphenol Blue in water was stirred into the upper buffer. Any air spaces in the tubes above the sample gels, were displaced with buffer by using a blunt glass rod. A hanging drop of buffer was then placed on the bottom of each gel tube, and the upper reservoir was then lowered so that the bottoms of the gel tubes were immersed in the lower reservoir buffer. The power supply was connected and the current set to 5 ma/gel tube.

Electrophoresis was carried out until the Bromphenol Blue tracking dye had migrated about 75% of the length of the gel tubes. This usually took one and a half hours. At the termination of electrophoresis the power supply was turned off, the gel tubes were removed from the apparatus, and the gels removed from the tubes by rimming under water with a fine needle. Staining was accomplished by the procedure of Chrambach, et al (52), using Coomassie Brilliant Blue R 250.

Materials

Sources for chemicals were as follows: Digitonin from Nutritional Biochemicals Co., Cleveland, Ohio; EGTA, EDTA, ATP, AMP, B.S.A., and Salygran from Sigma Chemical Co., St Louis, Mo.; Difco Bacto-agar, Difco antibiotic medium 3 from Difco Laboratories, Detroit, Mich.; Sepharose 4B from Pharmacia (Canada) Ltd., Montreal, P.Q.

All inorganic reagents were of analytical grade, and were obtained from Fisher Scientific Co., Fairlawn, New Jersey. All solutions were prepared using glass distilled water.

RESULTS

General properties

Using a modification of the extraction procedure of Hatano & Tazawa (28), a protein with contractile properties was obtained from the cytoplasm of Naegleria gruberi amoebae. This protein possesses properties characteristic of actomyosin-like proteins, ie., it is soluble in 0.5 M KCl and insoluble in 0.1 M KCl, superprecipitates at low ionic strength, and has an ATPase activity which is almost completely inhibited by Salyrgan. The protein forms microfilaments in the presence of chelating agents such as EDTA and EGTA.

This evidence indicates the existence of a contractile protein similar to actomyosin found in the cytoplasm of Naegleria amoebae. This protein comprises about 0.1% of the wet weight of the cells and about 1.3% of the total cellular protein.

Sepharose 4B elution

Sepharose 4B elution was employed as an indication of the relative purity of the contractile protein extract. The Sepharose 4B was made up in 0.5 M KCl, 10 mM Tris buffer, pH 7.6 in a 1.5 x 30 cm column. A 1.0 ml sample containing 2.8 mg of protein in 0.5 M KCl, 10 mM Tris buffer, pH 7.6 was mixed with 0.2 ml of saturated sucrose. This mixture was applied to the top of the Sepharose 4B column in a cold room, and the flow rate was adjusted to 1 drop every 25 seconds. Sixty tubes of 1.7 ml

volume each, were collected by an LKB Ultrorac fraction collector, type 7000. Each tube was assayed for protein by reading at O.D. 280 mμ and by the Lowry method (48).

Figure 3 illustrates the elution profile. Both methods of protein estimation gave good agreement, and indicated a single peak eluting just after the void volume. This single peak represents more than 80% of the protein applied, and indicates the minimum molecular weight of the contractile protein preparation is greater than 200,000. Tubes numbered 15-20, produced microfilaments upon the application of EDTA or EGTA to a final concentration of 2.5 mM.

An experiment was done to attempt dissociation of the actomyosin-like complex into its constituent actin-like and myosin-like proteins. The addition of 5 mM ATP and 1 mM Mg^{+2} ion to another elution caused the appearance of 5 protein peaks. Peak I eluted at the same position as the actomyosin-like peak in the prior experiment. Peaks II - IV, could represent aggregated or monomer myosin-like protein, and polymerized or monomeric actin-like protein (fig. 4).

Further modification of this method will make it possible to obtain pure actin-like and myosin-like protein by dissociation and Sepharose chromatography of the actomyosin-like protein in the presence of ATP and Mg^{+2} ion.

Figure 3: SEPHAROSE 4B ELUTION OF NAEGLERIA AMOEBA ACTOMYOSIN-LIKE PROTEIN

Conditions:

Sepharose 4B in 0.5 M KCl, 10 mM Tris buffer, pH 7.6 was made up in a 1.5 x 30 cm column. A 1.0 ml sample containing 2.8 mg of protein in 0.5 M KCl, 10 mM Tris buffer pH 7.6 was mixed with 0.2 ml saturated sucrose to increase sample density. This mixture was carefully applied to the column at 5°C, and the flow rate was adjusted to 1 drop every 25 seconds. Sixty tubes of 1.7 ml volume each, were collected on an LKB Ultrorac fraction collector, type 7000.

SEPHAROSE 4B ELUTION OF NAEGLERIA AMOEBA ACTOMYOSIN-LIKE PROTEIN

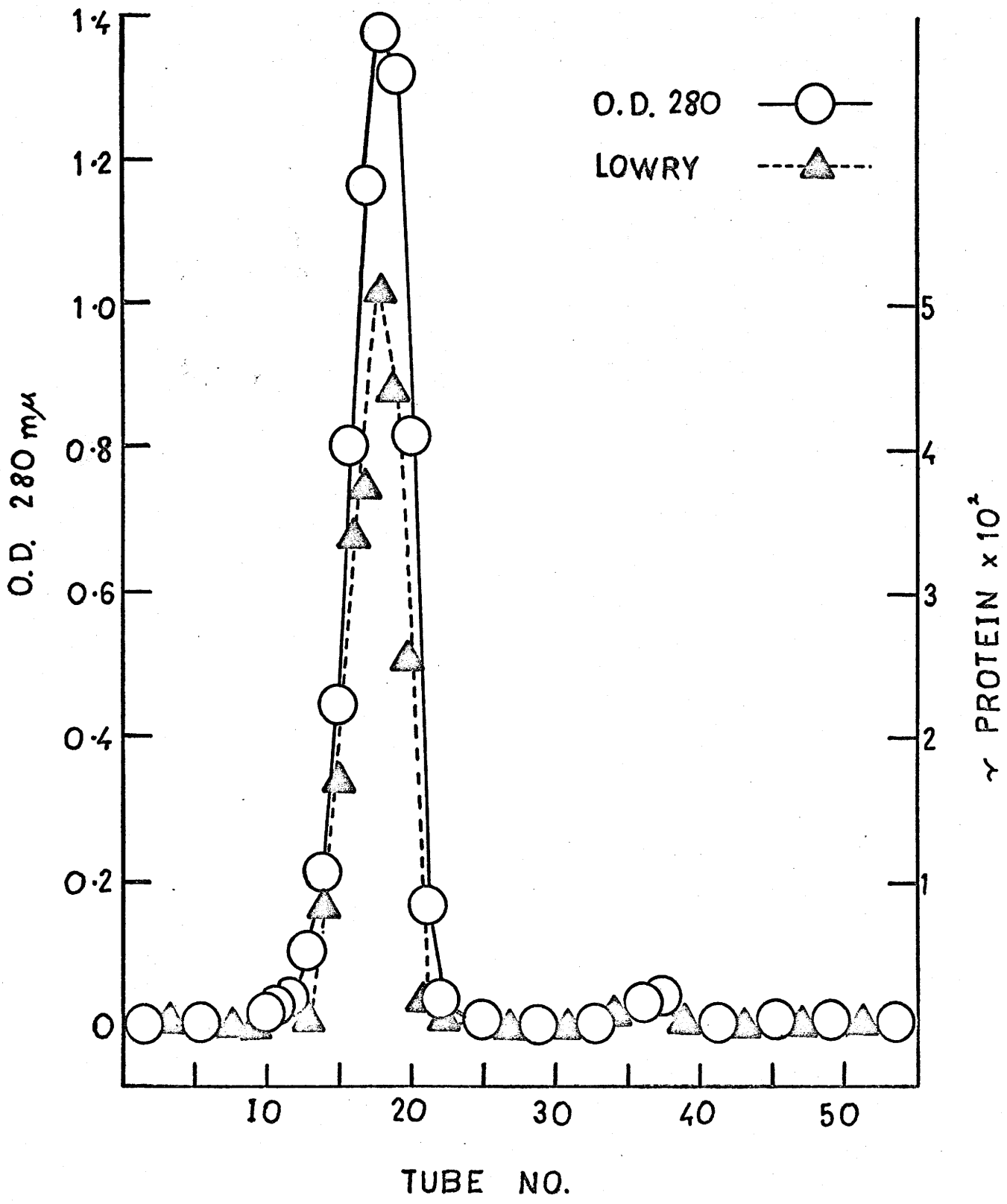


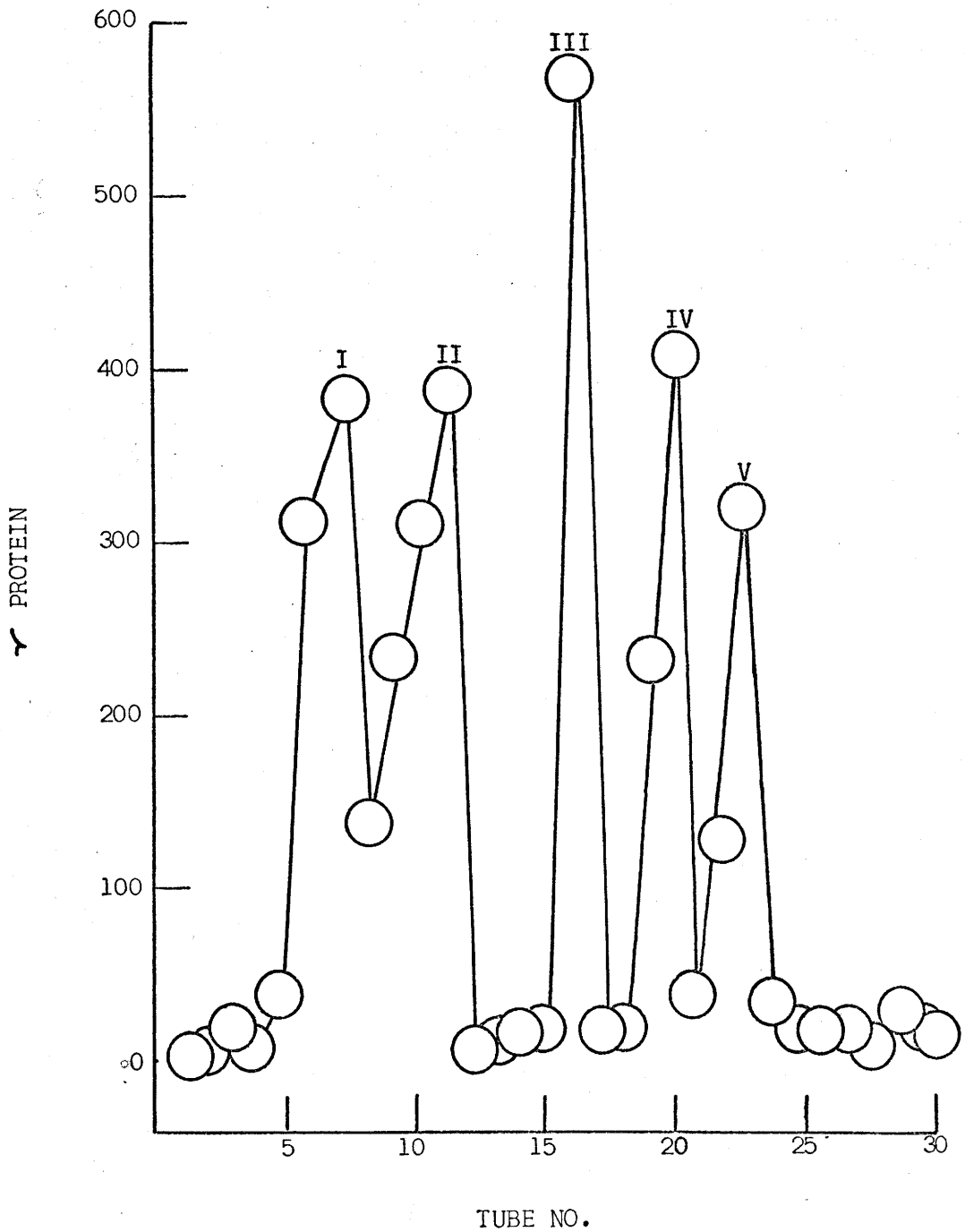
Figure 4: SEPHAROSE 4B ELUTION ON NAEGLERIA AMOEBA ACTOMYOSIN-LIKE PROTEIN WITH ATP AND Mg^{+2} ION.

Conditions:

Sepharose 4B in 0.5 M KCl, 10 mM Tris buffer, pH 7.6, 1 mM Mg^{+2} ion and 5 mM ATP was made up in a 1.5 x 30 cm column. A 1.0 ml sample containing 3.8 mg of protein in 0.5 M KCl, 10 mM Tris buffer pH 7.6 was mixed with 0.2 ml saturated sucrose to increase density and 0.3 ml of eluant. This mixture was carefully applied to the column at 5°C, and the flow rate was adjusted to 1 drop every 15 seconds. Thirty tubes of 2.6 ml volume each, were collected on an LKB Ultrorac fraction collector, type 7000.

SEPHAROSE 4B ELUTION ON NAEGLERIA AMOEBA

ACTOMYOSIN-LIKE PROTEIN WITH ATP AND Mg^{+2} ION.



ATPASE PROPERTIES

(a) General features

The cytoplasmic contractile protein from Naegleria amoebae demonstrates true ATPase activity (E.C. 3.6.1.3.), which splits off the terminal phosphate from ATP. This protein demonstrates a number of other enzymatic properties that are common to actomyosin contractile proteins. It splits 0.05-0.16 μmole P_i/min/mg protein which compares favourably with other non-muscle contractile systems (28, 30, 31). In 0.5 M KCl, the ATPase acticity is strongly enhanced by Ca^{+2}ion, with an optimum occuring at 2.5 x 10^{-2}M Ca^{+2}ion, while Mg^{+2}ion in the concentration range 10^{-5} - 10^{-1} M consistently inhibited the ATPase activity. The pH optimum of the ATPase in 0.5 M KCl was found to be 6.8. Sulfhydryl inhibitors such as p-chloromercuriphenylsulphonic acid inhibited ATPase activity, and this inhibition was partially prevented by cysteine. Salyrgan, a powerful inhibitor of actomyosin ATPase inhibited the Naegleria ATPase by over 80%.

(b) Substrate specificity

Figure 5 illustrates the hydrolysis of ATP, ADP, AMP and pyrophosphate by Naegleria actomyosin-like protein in 0.5 M KCl. The release of P_i is linear for the first 4 minutes, while a plateau is reached by 8 minutes. The Naegleria protein extract has the greatest activity toward ATP, while no measurable activity toward AMP or pyrophosphate was detectable. ADPase activity is present, possibly due to a contaminating myokinase (adenylate kinase). Myokinase reacts in the following manner:

$$2\ ADP \rightleftharpoons ATP + AMP$$

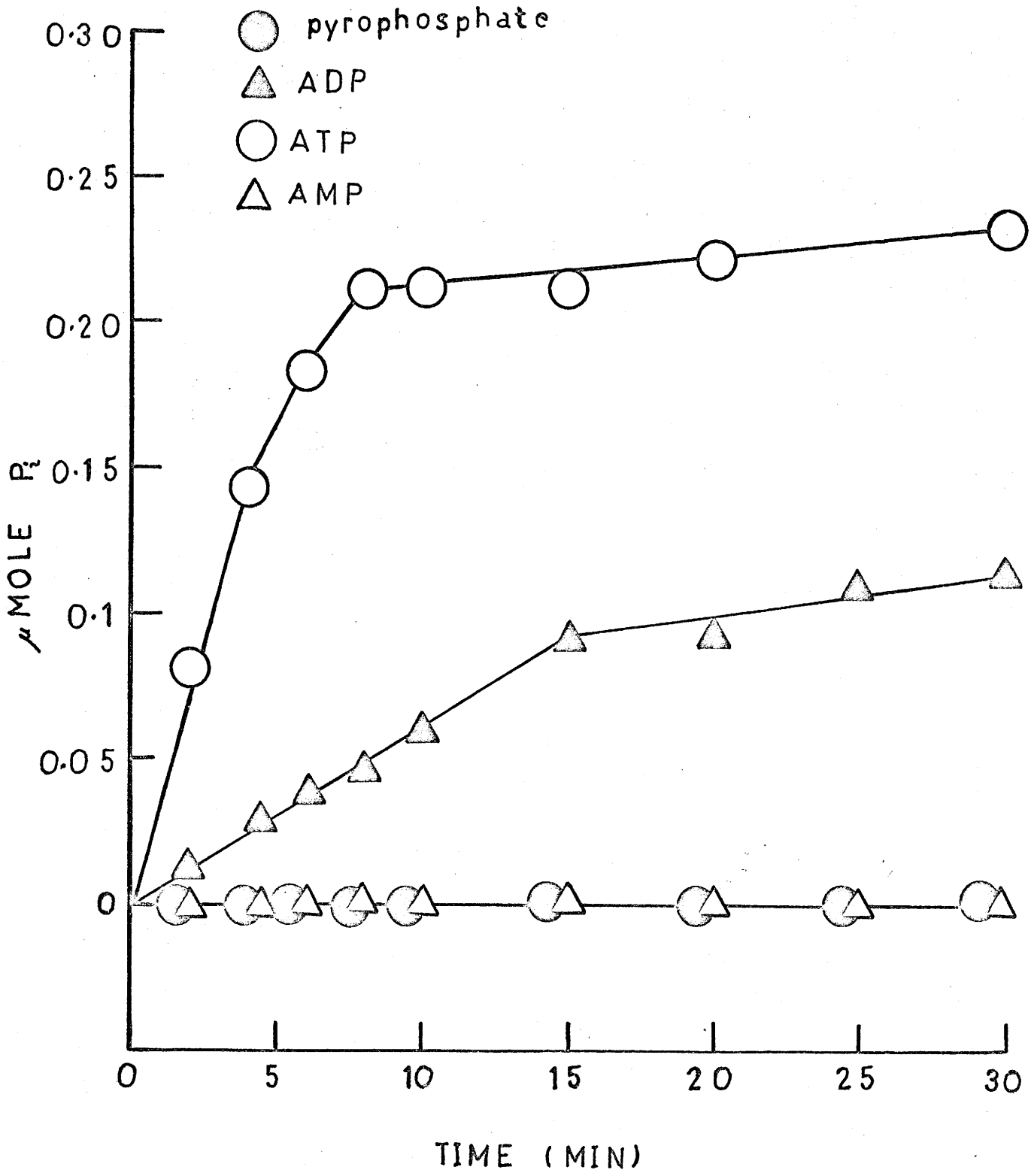
The ATP formed in this reaction could then be acted upon by the ATPase present to liberate inorganic phosphate.

Figure 5: HYDROLYSIS OF ATP, ADP, AMP AND PYROPHOSPHATE BY NAEGLERIA ACTOMYOSIN-LIKE PROTEIN

Conditions:

0.5 M KCl, 0.018 M imidazole buffer (pH 6.8), 2.5×10^{-2} M Ca^{+2}, 1 mM ATP,ADP, AMP or pyrophosphate, 0.47 mg protein, 23°C.

HYDROLYSIS OF ATP ADP, AMP, AND PYROPHOSPHATE BY NAEGLERIA ACTOMYOSIN-LIKE PROTEIN



(c) Specific activity (μmole P_i/mg protein/minute)

Specific activities were determined within the first 2 minutes of the initiation of the enzyme reaction by ATP. Twice precipitated Naegleria contractile protein had a specific activity of 0.160 which compares favourably with similar preparations from other organisms (Table 2). Precipitation of the protein was acconplished by lowering the ionic strength from 0.5 to 0.1 with glass distilled water.

TABLE 2: Specific activity of some non-muscle ATPases in 0.5 M KCl

System	Specific activity	Reference
Naegleria twice precipitated protein*	0.160	Lastovica, unpublished
Physarum plasmodial myosin B	0.270	Nakajima (31)
Physarum plasmodial myosin B	0.420	Hatano & Tazawa (28)
Physarum myxomyosin	0.004	T'so, et al (30)

*As protein loss was excessive, (about 40 - 50%), the second precipitation step recommended by Hatano & Tazawa (28) was not routinely performed. Lower specific activities of 0.05 -0.08 resulted with once precipitated protein preparations.

From table 2, it can be seen that the specific activity of Naegleria is much greater than T'so's myxomyosin prepatation, and compares favourably with the results reported by Hatano & Tazawa, and Nakajima for plasmodial myosin B.

(d) Effect of Ca^{+2} and Mg^{+2} ions on the ATPase activity

It is well known that Ca^{+2}ion has an activating, and Mg^{+2}ion, a depressing effect on the ATPase activity of striated muscle actomyosin in 0.5 M KCl. The actomyosin-like protein of Physarum has been reported (28, 30, 31) to respond in a similar manner to Ca^{+2}ion and Mg^{+2}ion. Figure 6 illustrates the ATPase activity in the presence of Ca^{+2}ion or Mg^{+2}ion. At a concentration of 2.5×10^{-2} M, Ca^{+2}ion stimulated, while Mg^{+2}ion at a similar concentration, inhibited the ATPase activity of the Naegleria protein.

The effect of different concentrations of Ca^{+2}ion or Mg^{+2}ion was compared with ATPase activity with no Ca^{+2}ion or Mg^{+2}ion present. Figure 7 illustrates this relative activity, ie., the specific activity with ATP and Ca^{+2}ion or Mg^{+2}ion present, divided by the specific activity with ATP alone. In the concentration range of 10^{-5} M to 10^{-1} M, the Ca^{+2}ion always stimulated the ATPase activity. Maximal stimulation was observed at a concentration of 2.5×10^{-2} M Ca^{+2}ion, the relative activity being about 6 times greater than in the absence of Ca^{+2}ion. In the concentration range of 10^{-5} M to 10^{-1} M, Mg^{+2}ion was always found to inhibit the ATPase activity, this inhibition appeared to be linear, increasing with Mg^{+2}ion concentration. Thus, Naegleria actomyosin-like protein is similar to actomyosin and Physarum myosin B with respect to Ca^{+2}ion stimulation and Mg^{+2}ion inhibition of the ATPase activity in 0.5 M KCl.

Figure 6: EFFECT OF Ca^{+2} AND Mg^{+2} ON THE TIME COURSE HYDROLYSIS OF ATP BY NAEGLERIA ACTOMYOSIN-LIKE PROTEIN

Conditions:

0.5 M KCl, 0.018 M imidazole buffer (pH 6.8), 2.5×10^{-2} M Ca^{+2} or Mg^{+2}, 10^{-3} M ATP, 0.442 mg protein, 22°C.

EFFECT OF Ca^{+2} AND Mg^{+2} ON THE TIME COURSE HYDROLYSIS OF ATP BY NAEGLERIA ACTOMYOSIN-LIKE PROTEIN

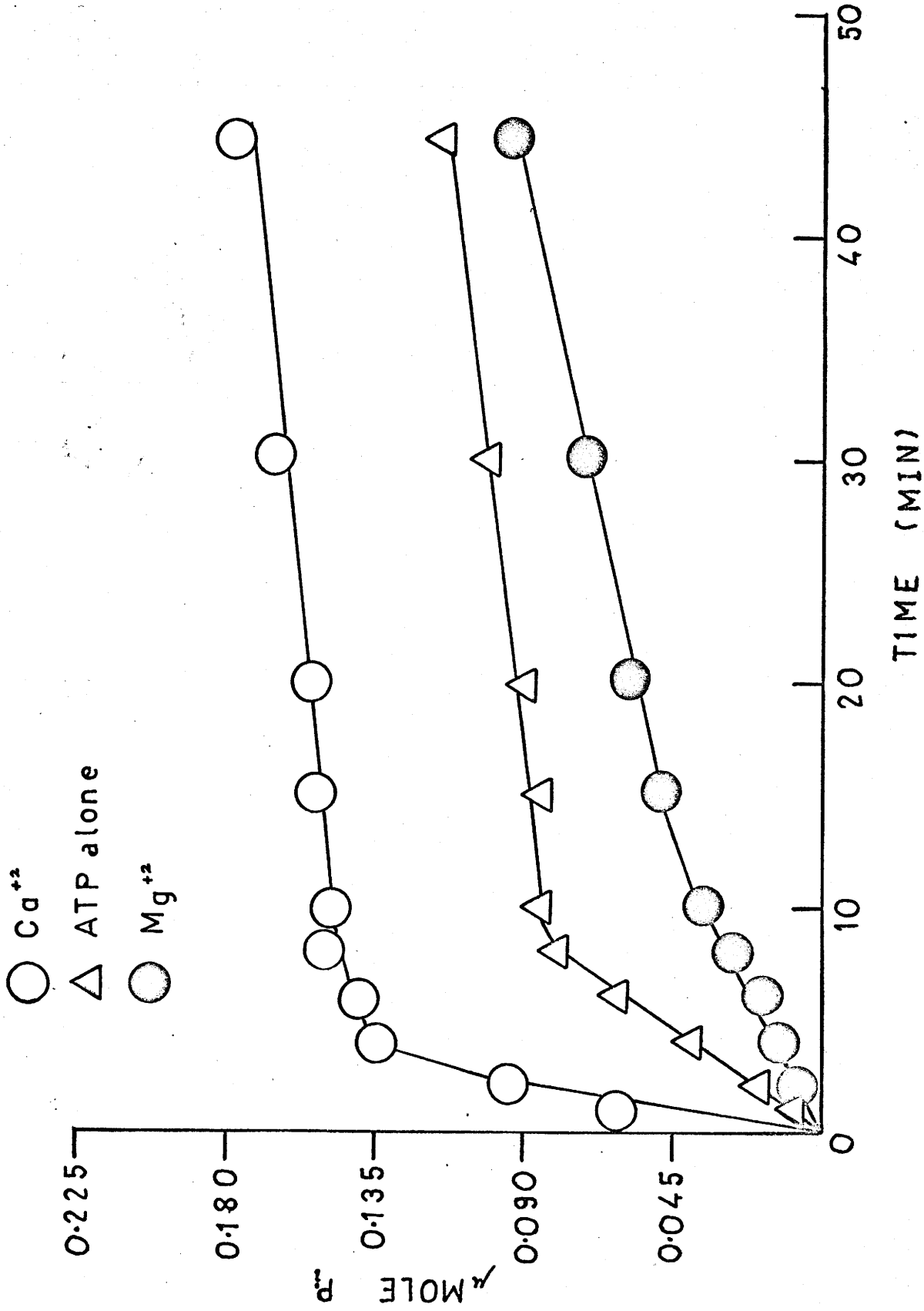


Figure 7: EFFECT OF Ca^{+2} AND Mg^{+2} ION ON THE HYDROLYSIS OF ATP BY AMOEBA ACTOMYOSIN-LIKE PROTEIN

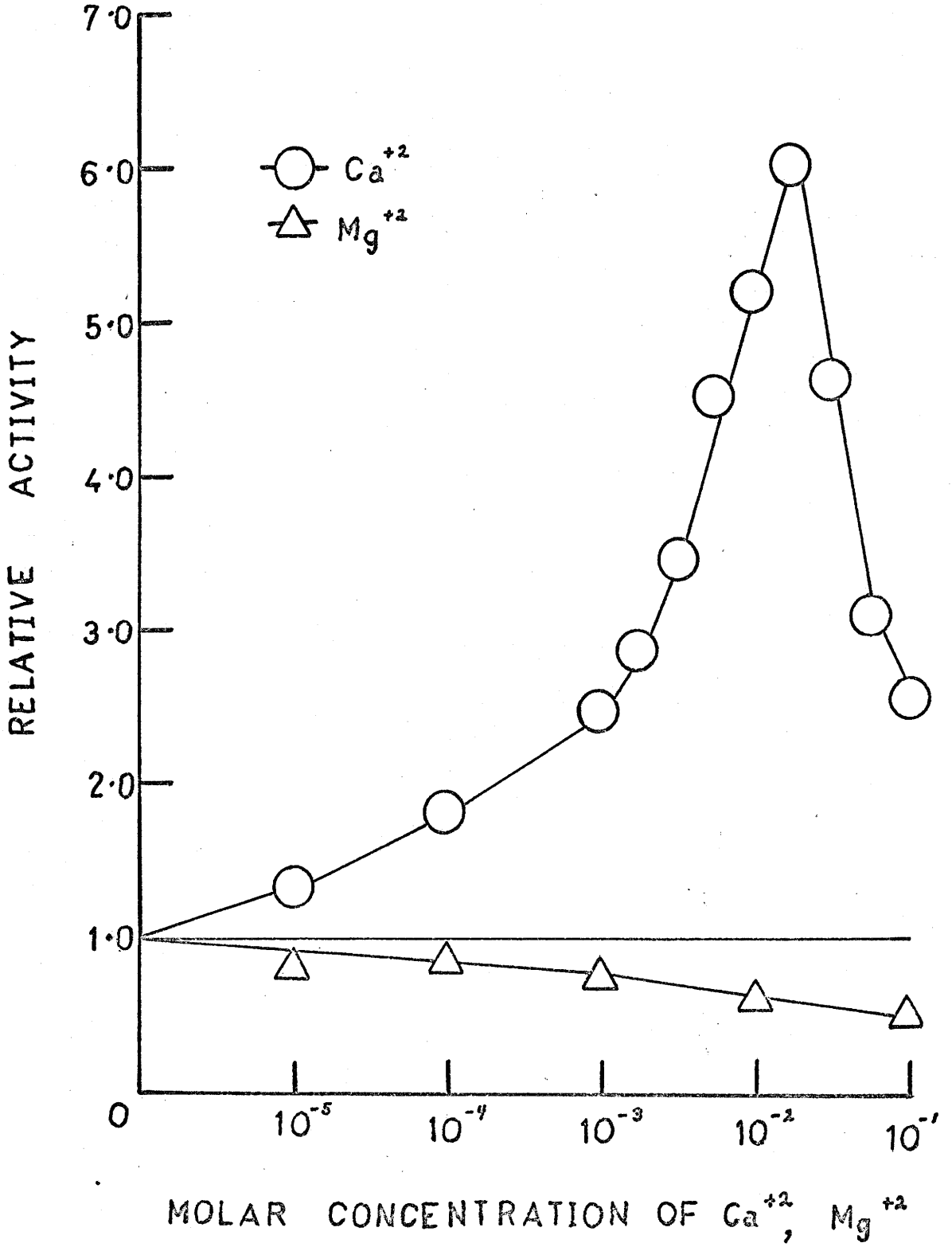
Conditions:

0.5 M KCl, 0.018 M imidazole buffer (pH 6.8), Ca^{+2} or Mg^{+2} in the concentration range 10^{-5} to 10^{-1} M, 10^{-3} M ATP, 0.44 mg protein, 22°C.

The relative activity is defined as:

$$\frac{\text{specific activity + ATP + ion}}{\text{specific activity + ATP}}$$

EFFECT OF Ca^{+2} AND Mg^{+2} ION ON THE HYDROLYSIS OF ATP BY AMOEBA ACTOMYOSIN-LIKE PROTEIN



(e) pH optimum

The pH optimum of the ATPase activity of the actomyosin-like protein was determined in the range 4.7-8.4. The ATPase activity in the pH range 6.1-7.0 was determined in increments of 0.1 pH unit. Figure 8 illustrates these results. A distinct optimum is observed at pH 6.8, while enzymatic activity decreased at pH values below 6.6. A "false" or alkaline optimum is seen in the pH range 7.0-8.4. This alkaline optimum has no distinct value, but rather rises with increasing hydroxyl ion concentration, until at a sufficiently high hydroxyl ion concentration, the enzyme is destroyed.

(f) Effect of P.C.M.P.S., a sulfhydryl inhibitor

It is generally believed that the sulfhydryl groups of actomyosin are involved in the ATPase activity of the enzyme. The precise function of the sulfhydryl groups is not known, but they are initially connected with the binding sites for ATP hydrolysis, and blocking the sulfhydryl groups with inhibitors such as P.C.M.B. (p-chloromercuribenzoate) will curtail enzymatic activity. Young (70) gives evidence for two classes of sulfhydryl groups in myosin, and both classes of sulfhydryl groups must be blocked before enzyme inhibition occurs. Hotta (76) proposed that in myosin the active site consists of two classes of sulfhydryl groups and histidine. The activity of P.C.M.B. inhibited ATPase may be partially restored by excess cysteine.

10^{-4}M p-chloromercuriphenylsulfonic acid (P.C.M.P.S.), a more soluble form of P.C.M.B., was incubated with the Naegleria protein for 5 minutes at 22°C. Another sample was treated similarly, then reacted with

5×10^{-4} M cysteine for 30 minutes. A control tube with no P.C.M.P.S. or cysteine was also prepared. The enzyme reaction was initiated by addition of ATP to a final concentration of 1.0 mM. After suitable incubation, the reaction was stopped, and the P_i liberated was determined by Sumner's method (49). It was found that 10^{-4} M P.C.M.P.S. inhibited the ATPase reaction by 66%, while addition of 5×10^{-4} M cysteine partially reversed this inhibition, so that the ATPase was inhibited by only 27%. This experiment indicates that the ATPase of the Naegleria protein, like actomyosin, is capable of being inhibited by a specific sulfhydryl blocking agent P.C.M.P.S., and this inhibition may be partially removed by cysteine.

(g) Effect of Salyrgan

Salyrgan, or the sodium salt of O-[(3-hydroxymercuri-2-methoxypropyl) carbamyl] phenoxyacetic acid is a powerful inhibitor of actomyosin ATPase activity. Salyrgan, like P.C.M.B., binds specifically to the sulfhydryl groups involved in ATP hydrolysis. Salyrgan inhibition may be partially removed by treatment with β-mercaptoethanol. Figure 9 illustrates that 10^{-2} M salyrgan inhibits Naegleria actomyosin-like protein ATPase by over 80%.

Figure 8: pH OPTIMUM OF NAEGLERIA ACTOMYOSIN-LIKE PROTEIN ATPase

Conditions:

0.5 M KCl, 0.018 M imidazole buffer (pH 4.7 - 8.4), 2.5×10^{-2} M Ca^{+2}, 10^{-3} M ATP, 0.45 mg protein, 23°C.

The P_i of each sample was determined exactly 2 minutes after the addition of ATP.

pH OPTIMUM OF NAEGLERIA ACTOMYOSIN-LIKE PROTEIN ATPase

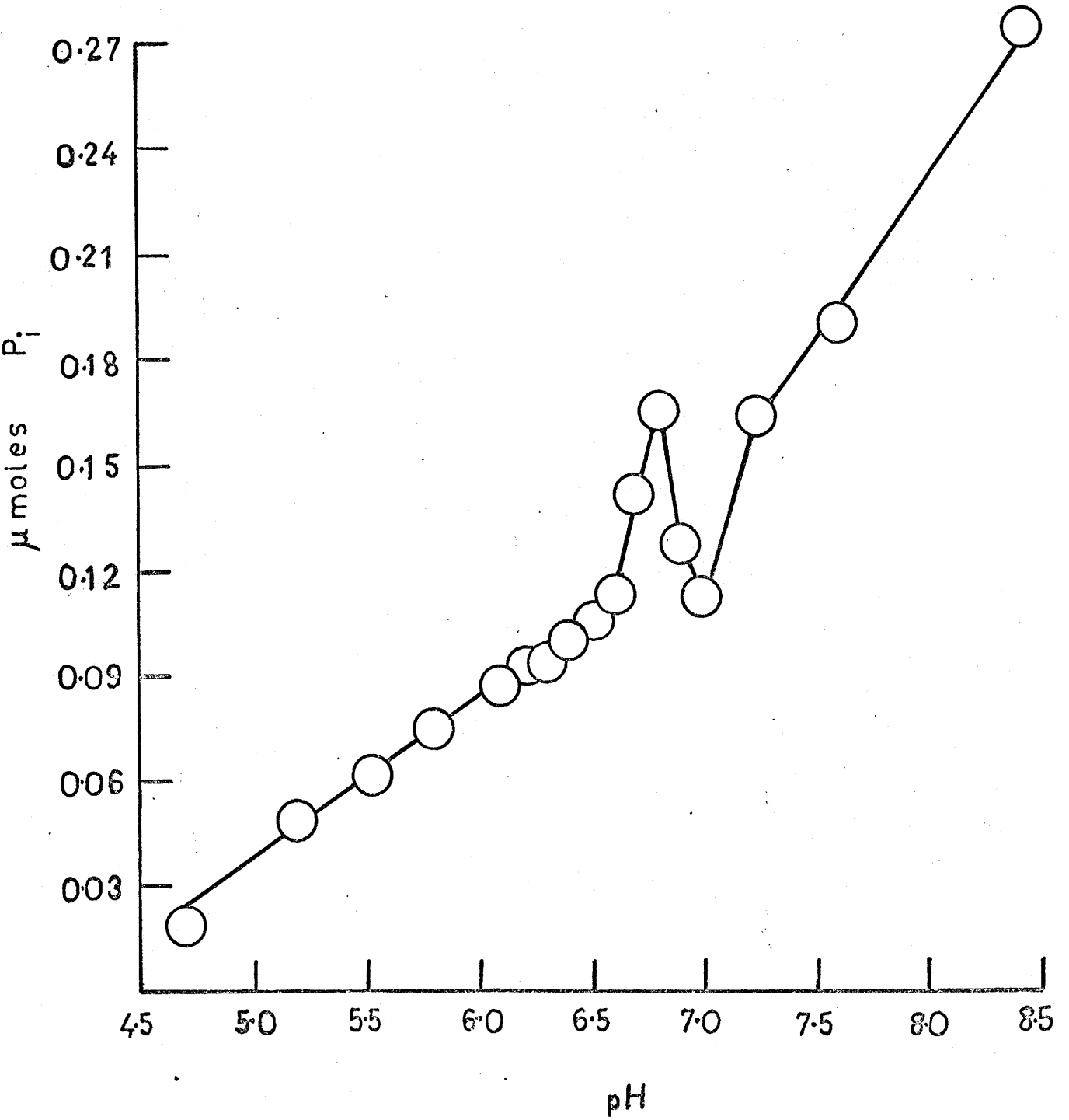
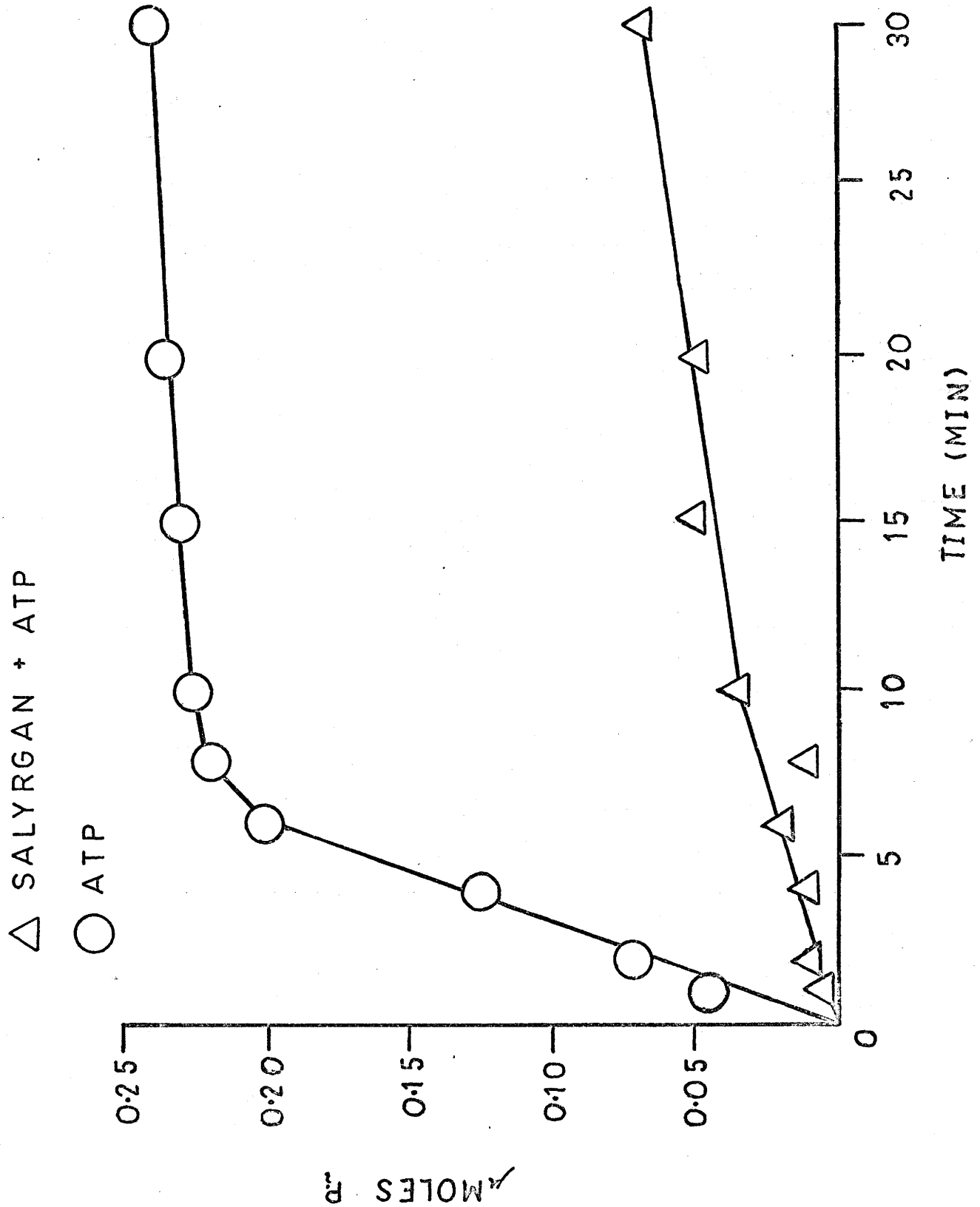


Figure 9: SALYRGAN INHIBITION OF NAEGLERIA ACTOMYOSIN-LIKE PROTEIN ATPase

Conditions:

0.5 M KCl, 0.018 M imidazole buffer (pH 6.8), 2.5×10^{-3} M Ca^{+2}, 10^{-3} M ATP, 0.430 mg protein, 10^{-2} M salyrgan, 22°C.

SALYRGAN INHIBITION OF NAEGLERIA ACTOMYOSIN-LIKE PROTEIN ATPase



Superprecipitation

The importance of superprecipitation as an *in vitro* analogue of contraction has been emphasized by Szent-Györgyi, who considers this phenomenon as a characteristic feature of actomyosin-like protein (10). Non-muscle actomyosin-like proteins with the ability to superprecipitate have been demonstrated in cat and rat brain by Pushkin, *et al* (56) and in *Physarum polycephalum* by Nakajima (31), and Hatano & Tazawa (28).

Superprecipitation of actomyosin is activated by Mg^{+2} ion and inhibited by Ca^{+2} ion at an ionic strength of μ=0.1 (11). Divalent cations are essential for superprecipitation, as chelating agents such as EDTA or EGTA, completely inhibit superprecipitation. Under the proper conditions of pH and ionic strength, superprecipitation may be preceded by clearing, which has been taken to be the *in vitro* analogue of muscular relaxation (12) Hatano & Tazawa (28), have reported that clearing occurs within several seconds after the addition of ATP to *Physarum polycephalum* plasmodial myosin B extracts.

The actomyosin-like protein of *Naegleria* amoebae was tested for superprecipitating ability by a modification of the procedure of Bemis, *et al* (50). Reaction mixtures were made up to 1.0 ml volumes, and a base line of absorption at 550 mμ was established with the Beckman D.U. spectrophotometer fitted with a Gilford 2000 chart recorder. The superprecipitation reaction was initiated by removing the cuvette, rapidly adding and mixing ATP to a final concentration of 10^{-3} M and returning the cuvette to the spectrophotometer. This resulted in a delay of 10-15 seconds prior to recording. The progress of superprecipitation was followed as a rise in O.D. 550 mμ after the return of the cuvette to the spectrophotometer.

Figures 10 and 11, representing the same experimental sample, illustrate the superprecipitation phenomena in Naegleria actomyosin-like protein. Figure 10 illustrates that 10^{-3} M Mg^{+2} stimulates, while 10^{-3} M Ca^{+2} inhibits the superprecipitation reaction. 10^{-3} M ADP would not induce superprecipitation of the Naegleria extract. Figure 11 illustrates that divalent cations are essential for superprecipitation to occur, as both 5 mM EDTA or EGTA completely inhibits superprecipitation. If excess Mg^{+2} ion is added with 5 mM EDTA, the inhibition is partially removed. With respect to superprecipitation, the actomyosin-like protein of Naegleria behaves like actomyosin (11, 12) and the actomyosin-like protein of Physarum (28).

Microfilaments

In vitro production of microfilaments from motile cytoplasm extracts by the action of low concentrations of chelating agents such as EDTA or EGTA has been demonstrated by Wohlforth-Bottermann for Physarum (61), and by Morgan, et al for Amoeba proteus (64). Divalent cations may be essential to the integrity of the actomyosin-like protein molecules in solution, and binding of these ions by chelating agents results in the formation of microfilaments.

A simple procedure for the production of microfilaments in vitro from the cytoplasm of Naegleria was devised. A single drop of the contractile protein solution was placed on a clean, dry slide and next to this drop, an equal sized drop of EDTA or EGTA was placed. Since ATP has limited chelating ability, it was also an effective agent for the production of microfilaments. A clean, dry, coverslip was carefully lowered so

Figure 10: SUPERPRECIPITATION OF NAEGLERIA ACTOMYOSIN-LIKE PROTEIN

Conditions:

0.1 M KCl, 0.018 M imidazole buffer (pH 6.8), 10^{-3} M Ca^{+2} or Mg^{+2}, 10^{-3} M ATP or ADP, 0.1 mg protein, 23°C.

SUPERPRECIPITATION OF NAEGLERIA ACTOMYOSIN-LIKE PROTEIN

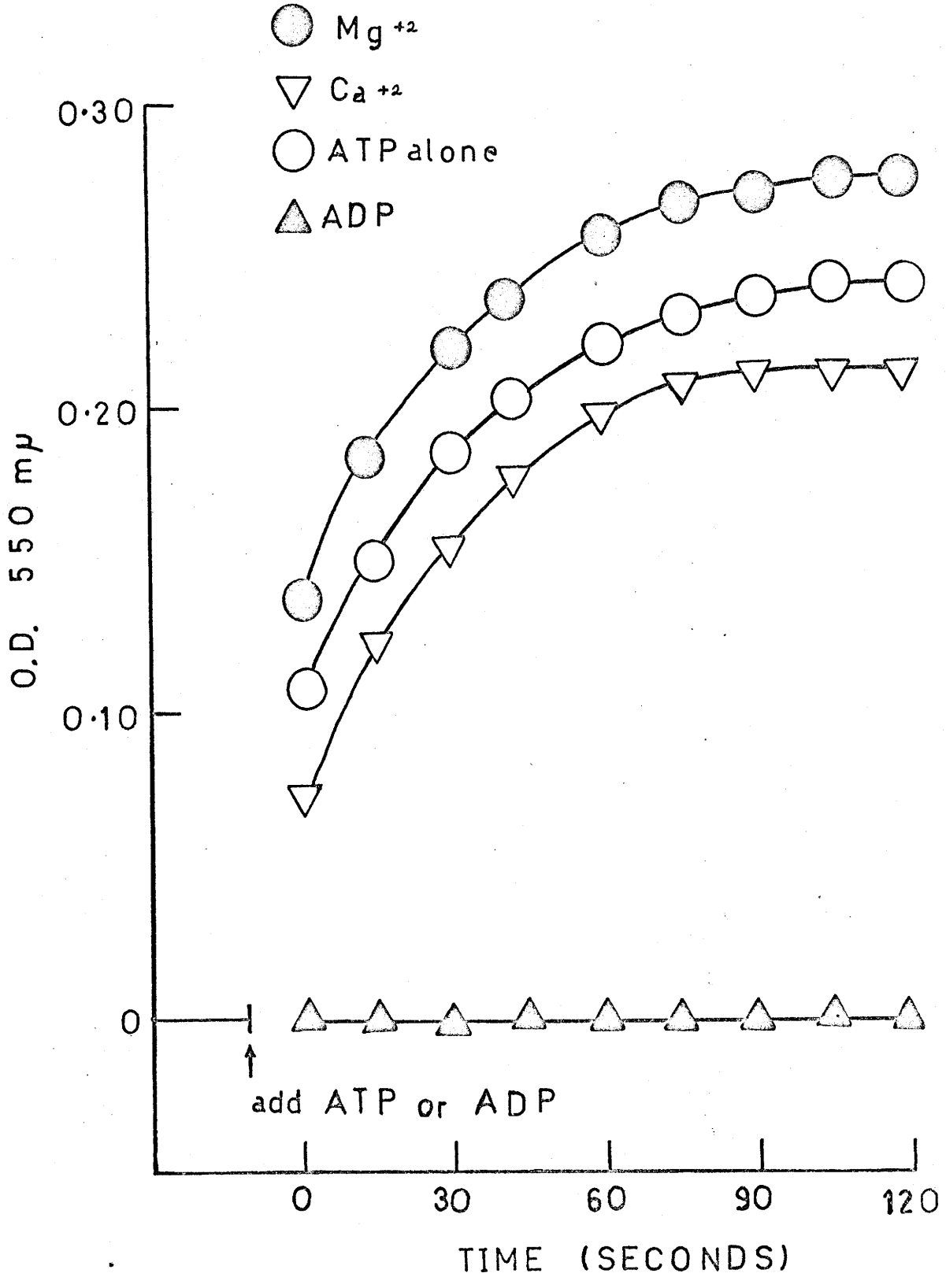
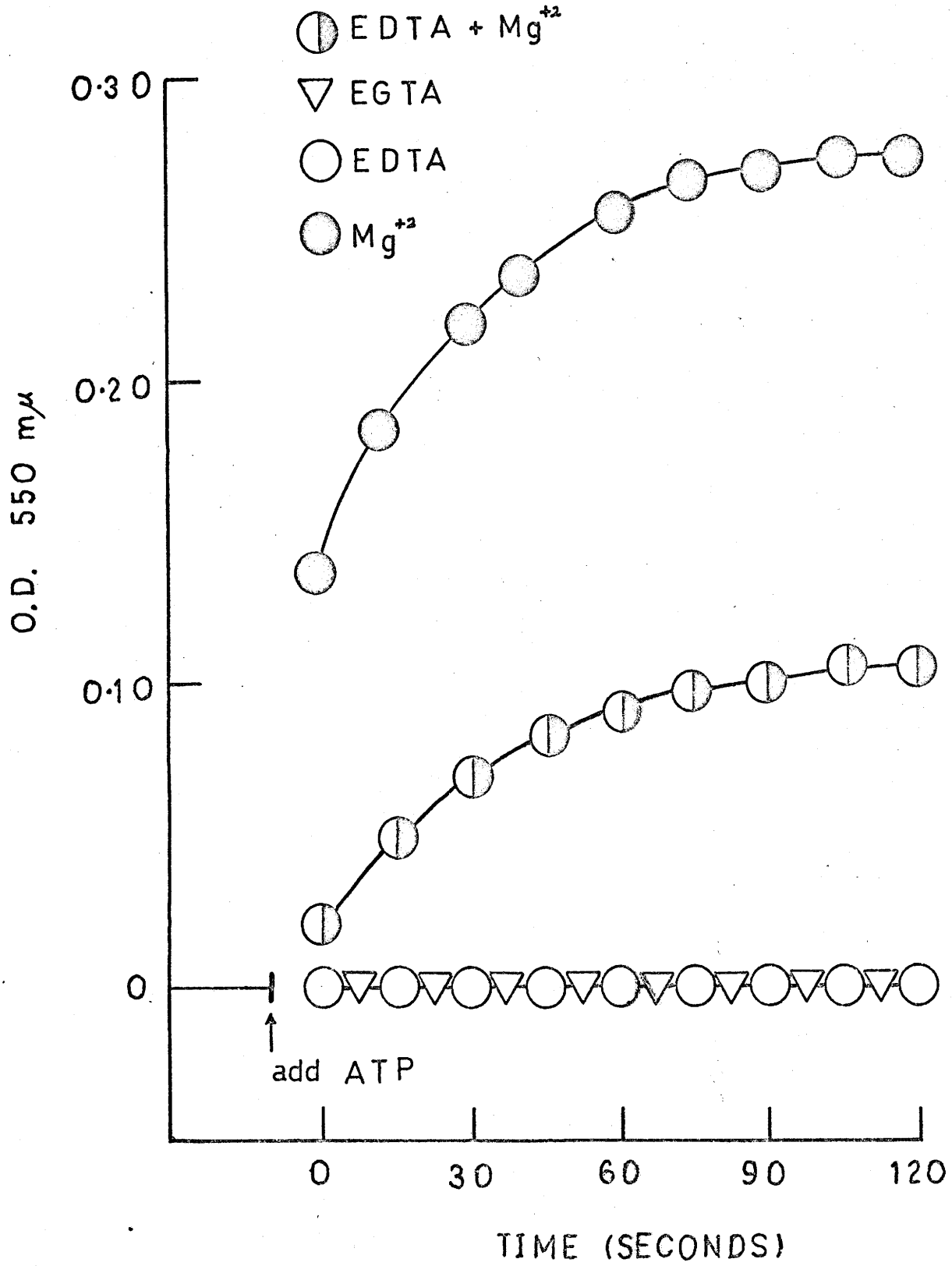


Figure 11: SUPERPRECIPITATION OF NAEGLERIA ACTOMYOSIN-LIKE PROTEIN

Conditions:

0.1 M KCl, 0.018 M imidazole buffer (pH 6.8), 10^{-3} M Mg^{+2}, 10^{-3} M ATP, 5 x 10^{-3} M EDTA or EGTA, 0.1 mg protein, 23°C.

SUPERPRECIPITATION OF NAEGLERIA ACTOMYOSIN-LIKE PROTEIN



the two drops merged, and this combined drop was viewed under phase contrast microscopy.

In the concentration range studied of 2.5 to 50.0 mM (final concentration), both EDTA and EGTA produced microfilaments (fig. 12). The higher concentrations of chelating agent tended to produce rather compact granular masses of microfilaments. Under ideal conditions, if the cover slip was carefully applied, a fine network of microfilaments of about 0.25 μ in diameter by up to 100 μ long, was observed, and measured by ocular micrometer. Occasionally, the cover slip was incorrectly applied and the microfilament mesh would "roll up" on the slide. A final concentration of 2.5 mM ATP also produced somewhat granular microfilaments.

These experiments were done with the amoeboid form of Naegleria gruberi. The cyst form of Naegleria was tested for microfilament formation, but no microfilaments could be produced. Amoebae extracted by the same procedures as used for the cysts invariably produced microfilaments. Naegleria flagellated cells were capable of producing microfilaments. These microfilaments may have been produced by contaminating amoebae, or those cells that possessed flagella and still demonstrated amoeboid motion. It should be possible to make quantitative estimations of amounts of microfilament producing protein of cyst, amoeba and flagellated cells. Excess EDTA or EGTA added to a known amount of protein from cyst, amoeba or flagellate should precipitate all the microfilament forming protein which may be then centrifuged. Then the precipitates and supernatants could be assayed for protein.

This ability of the extracts to form microfilaments may be useful in further Sepharose 4B experiments, since this ability may be a means

of identifying the actomyosin-like protein from a number of elution peaks on the same sample eluted through Sepharose 4B.

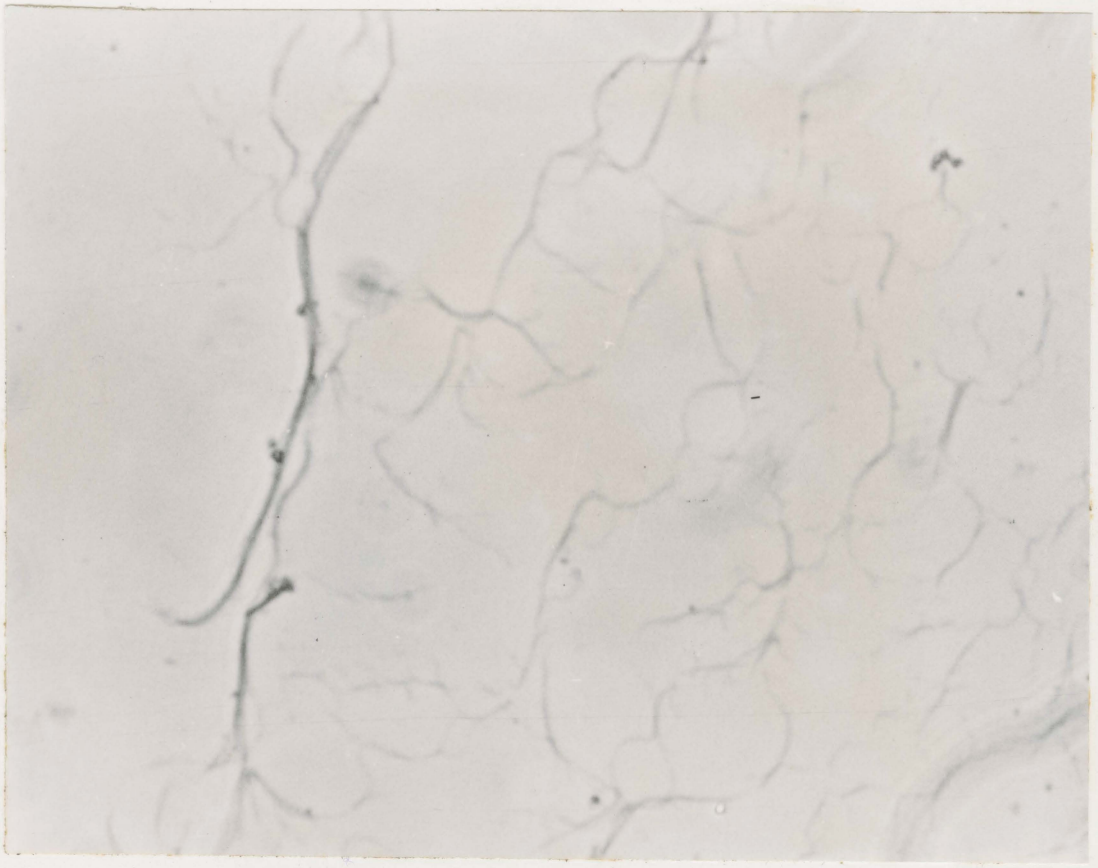


Figure 12: THE IN VITRO PRODUCTION OF MICROFILAMENTS FROM NAEGLERIA ACTOMYOSIN-LIKE PROTEIN.

Conditions:

One drop of the Naegleria extract was examined under phase contrast microscopy. No microfilaments could be seen. An equal sized drop of 5 mM EDTA was placed next to the Naegleria extract and allowed to merge. Numerous microfilaments were then seen. The above figure illustrates these microfilaments, and on the left, the large, dense microfilament appears to be composed of finer elements. X 1440

Viscosity

Actomyosin and non-muscular actomyosin-like proteins such as Physarum plasmodial myosin B (28-31) have repeatedly demonstrated a marked viscosity drop when ATP was added to the protein solution in 0.5 M KCl. This drop in viscosity is thought to be due to dissociation of the actomyosin into its component actin and myosin. As the ATP present is dephosphorylated by myosin, the actin and myosin reassociate, causing the viscosity to increase. This ATP-induced viscosity drop may be demonstrated repeatedly on the same sample.

Following the viscosity procedure of Hatano and Tazawa (28), and using Ostwald-type viscometers of 0.6 ml capacity and flow times for water of about 30 seconds, attempts were made to observe an ATP-induced viscosity drop in Naegleria extracts. The experiments were unsuccessful, as initial viscosities (before ATP was added) were too low to measure. Modification of Hatano and Tazawa's extraction procedure (28) for Naegleria involved the use of digitonin for cell lysis, and an overnight reprecipitation of the contractile protein. The longer reprecipitation time employed for Naegleria may have allowed the F-actin-like material to depolymerize into monomers, which would account for the low initial viscosities observed.

Further modification of the extraction procedure, possibly by addition of Mg^{+2} ion to promote polymerization of the actin-like monomers may be effective in obtaining a high viscosity extract. When this is accomplished, another technique will be available for the in vitro study of Naegleria actomyosin-like protein.

Disc electrophoresis

Disc electrophoresis of Naegleria actomyosin-like extracts was accomplished by the method of Davis (51), and the staining procedure of Chrambach, et al (52). Most of the protein ran as a single component, but occasionally several fainter bands were present. Denatured protein was always seen at the top of the gel surface (fig. 13).

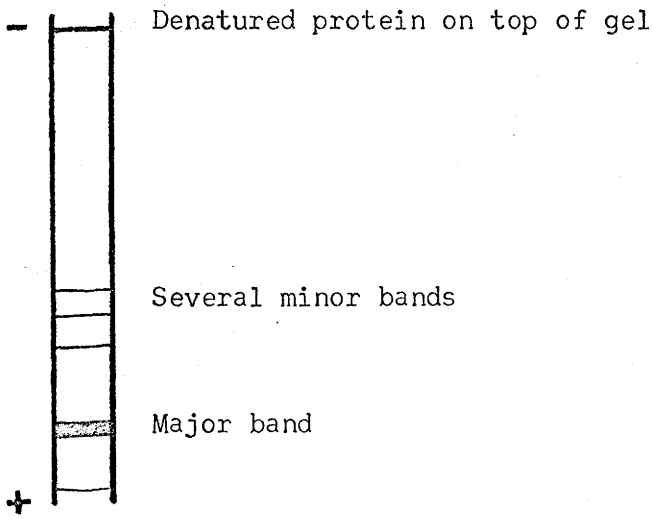


Figure 13: DISC ELECTROPHORESIS OF NAEGLERIA ACTOMYOSIN-LIKE PROTEIN.

Conditions:

50 ✓ protein applied to top of gel. Electrophoresis carried out at 4° C, 200V, 5 ma/tube for 75 minutes.

DISCUSSION

A growing body of evidence indicates that the slime mold Physarum polycephalum (28-31) and the protozoan Amoeba proteus (7) contain a cytoplasmic contractile protein similar in many respects to actomyosin. Actomyosin-like proteins present in the cytoplasm of over fifty diverse cell types have been reported [see Table 1 and the excellent review article by Jahn and Bovee (65)]. These non-muscle actomyosins are chemically quite similar to actomyosin, as "hybrids" may be produced in vitro. For example, the plasmodial actin-like protein of Physarum will combine with rabbit muscle myosin to produce a biologically active actomyosin-like protein possessing ATPase activity, superprecipitating ability and undergoing an ATP-induced viscosity drop similar to that of muscle actomyosin (25)

The amoeboid form of Naegleria gruberi has been found to contain a cytoplasmic contractile protein similar in many respects to the actomyosin-like protein found in Physarum polycephalum or Amoeba proteus. Comparison of these contractile proteins from Naegleria, Physarum and Amoeba reveal similar behaviour with respect to solubility in various concentrations of salts, substrate specificity, specific activity, pH optimum, effect of divalent cations, and the effect of sulfhydryl inhibitors on the ATPase activity. The superprecipitating ability and the in vitro production of microfilaments was similar in these three proteins.

Physarum plasmodial myosin B extracts exhibit a reversible viscosity drop on the addition of ATP (28-31). Attempts to duplicate this

phenomena with Naegleria extracts were unsuccessful. Possibly, the extraction procedure used, a modification of Hatano and Tazawa's procedure (28), in which Naegleria actomyosin-like protein was allowed to precipitate out in a Mg^{+2} ion free, low salt (0.1 M KCl) solution favoured depolymerization of the polymer form of the actin-like protein present. This may be the case as low viscosities of Naegleria extracts, before the addition of ATP, were observed. The addition of Mg^{+2} ion to the reprecipitation media of Naegleria would favour polymerization of the actin-like monomers, and coupled with a shorter extraction time should result in a more viscous solution. The demonstration of an ATP-induced viscosity drop should then be possible.

Naegleria actomyosin-like protein, which comprises 1.3% of the total protein and 0.1% of the wet weight of the amoebae, is soluble in 0.5 M KCl and insoluble in 0.1 M KCl. The Physarum protein is contained in approximately the same amounts in the cell, about 0.1% of the wet weight of the plasmodia, and 1.2% of the total plasmodial protein (28). Hatano and Tazawa (28) reprecipitate their plasmodial myosin B extracts twice. Routinely, one precipitation of the Naegleria extract was done by reducing the ionic strength from 0.5 to 0.1 with glass distilled water, yielding about 10 mg of protein. A second precipitation yielded only 6 mg.

The Naegleria protein possess ATPase activity (E.C. 3.6.1.3.), i.e., it is capable of releasing P_i by splitting the terminal phosphate from ATP. This enzyme has no measurable effect toward AMP or pyrophosphate, but does possess ADPase activity which may be due to contaminating myokinase activity. That is, myokinase could act on two molecules of ADP to form one molecule of ATP and one molecule of AMP. The ATP

formed could then be acted upon by the ATPase present. The specific activity of Naegleria ATPase twice reprecipitated was about twice as high as that of once reprecipitated Naegleria extract. Twice reprecipitated extracts of Naegleria ATPase had a specific activity of 0.160. The specific activity of Physarum has been reported to vary from 0.040 to 0.420 (see Table 2).

Physarum plasmodial myosin B in vitro, has been reported to possess similar ATPase activity, and unresponsiveness toward AMP and pyrophosphate (28, 30, 31,). Nakajima (31), reported a myokinase contaminant of his Physarum preparations. Tso', et al (30) experienced contamination of their Physarum myxomyosin preparations, as only 75% of the protein moved in a single band in the electrophoresis apparatus and ultracentrifuge. Treatment with RNAase indicated these Physarum extracts contained about 10% RNA, some of which was reversibly bound to the myxomyosin.

The Sepharose 4B elution profile of Naegleria actomyosin-like protein (fig. 3) indicates a single peak, which may mean either a single protein or several proteins of similar elution properties. Addition of 5 mM ATP and 1 mM Mg^{+2} ion to the eluate produced an elution profile with several peaks (fig.4) One of the bands eluted at the same position as the protein component in fig.3, along with several bands eluting after this component which could represent the actin-like and myosin-like proteins. The use of Sepharose 4B chromatography and ATP and Mg^{+2} ion will make possible the study of the actin-like and myosin-like proteins from Naegleria.

Once reprecipitated actomyosin-like extracts of Naegleria appear to be homogeneous by the Sepharose 4B data (fig.3). The single peak

eluted in fig. 3 could represent a single protein, or several proteins of very similar elution properties. The disc electrophoresis data (fig. 13) indicates that while most of the protein migrates as a single band, several minor bands may be due to contaminating protein. The once reprecipitated extracts of Naegleria routinely extracted were undoubtedly contaminated, as twice reprecipitated Naegleria extracts had a higher ATPase specific activity. Since the Naegleria extracts possess ADPase activity, a likely contaminant would be myokinase. Reprecipitating the Naegleria actomyosin-like protein extracts two or three times should produce a homogeneous preparation.

The Naegleria ATPase exhibited a bimodal pH optimum curve (fig.8), with a distinct pH optimum in 0.5 M KCl at 6.8, a drop in activity about 7.0, and a rise to 8.4 in the alkaline or "false" pH range. Mommaerts, et al (53, 54) have reported that actomyosin possesses a "false" pH optimum. With actomyosin, the true optimum will lose its activity on aging, while the "false" or alkaline optimum will not. Bimodal pH optimum curves for ATPase activity, similar to that obtained with the Naegleria ATPase, have been reported for Physarum by Nakajima (31), and for striated muscle actomyosin by Mommaerts and Green (54). Pitts and Mast (66) have reported a similar bimodal pH optimum curve by varying external pH and observing the locomotory rates of Amoeba proteus. This is not to imply that external pH may directly influence the contractile process of amoeboid motion, as undoubtedly other metabolic processes are involved, but as Pautard (67, 68) suggests, local pH changes and relative protein concentrations may be important to amoeboid motion.

The ATPase activity of Naegleria protein is stimulated by Ca^{+2} ion

and inhibited by Mg^{+2}ion. In the concentration range of 10^{-1} to 10^{-5} M, Ca^{+2} always stimulated the ATPase activity. Maximal stimulation with Ca^{+2} was obtained with 2.5×10^{-2} M Ca^{+2}, where ATPase activity was stimulated about 6 fold greater than the activity in the absence of Ca^{+2}ions. While Mg^{+2}ion in the concentration range of 10^{-1} to 10^{-5} M, always inhibited the ATPase activity in the Naegleria protein, therefore inhibition of the ATPase is directly proportional to the concentration of the Mg^{+2}ion.

Nakajima (31) reported that Ca^{+2}ion stimulated Physarum plasmodial myosin B ATPase, with the maximal stimulation occuring at $2\text{-}4 \times 10^{-2}$ M Ca^{+2}ion, the activation was 6.6 fold greater than in the absence of Ca^{+2}ion. Hatano and Tazawa (28) reported with their preparation of Physarum plasmodial myosin B, Ca^{+2}ion stimulation occured with a 7.4 fold increase at the optimal Ca^{+2}ion concentration of 2×10^{-3} M Ca^{+2}. The Physarum ATPase (31) similar to Naegleria ATPase was inhibited by Mg^{+2}ion concentration from 10^{-1} M to 10^{-5} M.

Streaming cytoplasm is sensitive to sulfhydryl blocking agents such as P.C.M.B. Kamiya (69) observed that addition of 10^{-3} M P.C.M.B. caused cessation of protoplasmic streaming in Nitella within a few minutes, and addition of 10^{-2} M cysteine restored protoplasmic streaming. It is known that sulfhydryl groups are intimately associated with the site of hydrolysis of ATP by the myosin molecule. Young (70) suggests that that with striated muscle myosin, each functional subunit of the myosin has two types of sulfhydryl groups, selective blocking of the first type will elevate the Ca^{+2} activated ATPase and blocking of the second type will completely inhibit Ca^{+2} activated ATPase activity.

Hotta (76) has suggested that the active site of myosin ATPase

activity includes both types of sulfhydryl groups. A similar situation may exist with non-muscle myosin. The ATPase activity of Physarum plasmodial myosin B has been reported to be inhibited by P.C.M.B. (31), and this inhibition may be partially removed by excess cysteine. This is the situation with the Naegleria ATPase. Salyrgan, a heavy metal compound, binds to sulfhydryl groups, and has been used extensively in studies of actomyosin and myosin enzyme reactions. The salyrgan inhibition obtained with Naegleria ATPase is comparable with striated muscle actomyosin inhibition.

Superprecipitation, the in vitro analogue of contraction, has been considered to be a property of actomyosin-like proteins. In 0.1 M KCl, 1 mM ATP and Mg^{+2} ion, striated muscle actomyosin will superprecipitate (11-13), similar results have been reported for Physarum plasmodial myosin B (28, 31). The superprecipitation of actomyosin-like protein may be due to the formation of bonds between actin and myosin. These bonds may break and reform, drawing the actin and myosin molecules closer together. Takahashi and Yasui (77, 78) present electron microscopic evidence that this occurs for actomyosin. Moos, et al, (79) have suggested that a conformational change of the actin filaments may occur in superprecipitation, based on the exchangeability of the actin-bound nucleotide.

Prior to superprecipitation, clearing or the in vitro analogue of relaxation may occur under appropriate conditions. Hatano and Tazawa (28) have demonstrated this clear phase of Physarum plasmodial myosin B, but they report that as with actomyosin, clearing is difficult to detect because it occurs within seconds of ATP addition. These workers report the Physarum plasmodial myosin B exhibits strong superprecipitation, which was increased by Mg^{+2} ion and inhibited by Ca^{+2} ion. Physarum

plasmodial B preparation never superprecipitated in the presence of EDTA or EGTA, but when sufficient Mg^{+2} ion was added to EDTA, superprecipitation could occur.

The protein extracts of Naegleria showed strong and rapid superprecipitation in 0.1 M KCl which was stimulated by 1 mM Mg^{+2} ion, and inhibited by 1 mM Ca^{+2} ion. Divalent cations are absolutely essential for superprecipitation to occur, as 5 mM EDTA or EGTA will completely inhibit the superprecipitation of Naegleria protein (fig.11). Excess Mg^{+2} ion added with 5 mM EDTA will partially overcome this inhibition (fig.11).

Microfilaments, which may represent the mechanical means of accomplishing amoeboid motion, have been produced in vitro with extracts of the Naegleria actomyosin-like protein with the addition of chelating agents such as EGTA or EDTA, at a final concentration of 2.5 mM. In the light microscope, these microfilaments were about 0.25 μ in diameter, and may have been made up of smaller elements. 2.5 mM ATP also produced microfilaments, although these were somewhat granular.

Microfilaments have been seen in vivo in Physarum polycephalum (60-63) and Amoeba proteus (40). Microfilaments may be produced in vitro in both by the action of chelating agents such as EGTA, EDTA (61,64), or ATP (1). Wohlfarth-Bottermann (60) believes that these microfilaments possessing ATPase activity (61) and having a diameter of 60-80 Å, which is the same diameter as myxomyosin (30) and plasmodial myosin B (28) from Physarum, are the mechanical means of accomplishing amoeboid motion in Physarum. Hatano and Tazawa (28) are of the same opinion, although they state that their 75 Å diameter estimate of plasmodial myosin B was the same order of magnitude as plasmodial F-actin formed from the polymeriz-

ation of plasmodial G-actin. They conclude that the microfilament network found in Physarum cytoplasm consists of 75 Å diameter filaments of plasmodial F-actin. For a Myosin-like protein, present in the cytoplasm as dimer or trimer molecules associates with the plasmodial F-actin strands. Electron microscopic evidence indicates that these strands become about 100 Å in diameter (28). After contraction has been accomplished the globular myosin-like units dissociate themselves from the actin-like strands. In striated muscle, Hayashi (71) has recently suggested that fibrous actin cannot interact, that is, contract or superprecipitate with fibrous myosin, only some dispersed state of myosin, and vice versa.

The actomyosin-like extracts of Naegleria, Amoeba and Physarum, have been reported to possess the ability to produce microfilaments in vitro. It is reasonable to assume that if a coherent network of microfilaments is responsible for amoeboid motion, then these molecular associations must be labile, capable of being formed and dissociated quite rapidly and repeatedly in the cytoplasm. Hatano, et al (26) have found that Physarum polymerized plasmodial actin can exist in the fibrous form, or an alternate globular polymer, 100-600 Å in diameter, termed the "Mg polymer". No in vitro evidence of this "Mg polymer" has been demonstrated, but it is tempting to speculate that this polymer may enable rapid association and dissociation of the actin-like network in Physarum to occur.

Inoué and Sato (74) present evidence for the labile nature of the mitotic spindle subunits and conclude a pool of spindle subunits exist in the cell. Pools of the labile, contractile molecules of the mitotic apparatus have been reported in the sea urchin by Kane and Stephens (72, 73).

The major component of the mitotic apparatus is a protein of molecular weight of 880,000 with a sedimentation coefficient of 22 s. A protein similar to this 22 s component was extracted from unfertilized sea urchin eggs, and on the basis of sedimentation coefficients, optical rotatory dispersion, amino acid analysis, and electron microscopic studies, this protein was the same as the 22 s component from the mitotic apparatus. Apparently, the major protein component of the mitotic apparatus arises from a preformed pool comprising some 8% of the total cellular protein. Stephens (73) observed that the 22 s protein will reorganize and form filamentous aggregates of 40-60 Å diameter at pH 4.5 in 0.1 M ammonium acetate with 1 mM Mg^{+2} ion. These fibrils will aggregate laterally, and can be dissociated into 22 s subunits by raising the ionic strength or the pH.

Stephens (73) suggests that the 22 s particle may be an inactive form of the mitotic apparatus protein which could form microfilaments similar in diameter to those found in the cytoplasm of various cells (28,40). These 40-60 Å diameter microfilaments could form the microtubular elements of the mitotic apparatus.

It may be that the actomyosin-like protein in the cytoplasm of Naegleria amoeba can also form part of the mitotic apparatus of Naegleria. Colchicine, while inhibiting mitotic spindle formation, does not inhibit ATPase activity of actomyosin or myosin (47). If colchicine binds preferentially to either Naegleria actomyosin-like cytoplasmic protein or Naegleria mitotic apparatus protein then studies on the presumed conversion between the two proteins may be undertaken. Yanagisawa, et al (75) have recently found that the microtubules of sea urchin spermatozoa flagella contained bound GTP. One GTP molecule was bound to one 40 Å diameter

subunit of the microtubule. In this system guanosine may substitute for adenosine in the conversion of the monomer to the polymer form of the actin-like protein (tubulin). Shelanski and Taylor (76) have reported that the subunit of the microtubule of sea urchin flagella has a GTP binding site.

Using muscle F-actin in which the usual bound ADP had been replaced by CDP or IDP, Estes and Moos (86) found these substitutions did not affect the rate of exchange of the bound nucleotide with free ATP. The authors conclude that the specific nucleotide bound can affect the stability of F-actin. The nucleotide bound to the actin-like protein of Naegleria might be a control mechanism for various physiological states of the actin-like protein. Adenosine nucleotides may be bound to this protein in the amoeba, while other nucleotides, such as guanine may be bound to the flagellar actin-like component. Hayashi (81) has recently prepared actomyosin free of bound nucleotide, and still retaining biological activity. Possibly this technique could be applied to transformation of amoeba to flagellate actin in Naegleria.

In conclusion, this present study has shown that the amoeboid form of Naegleria gruberi contains an actomyosin-like protein which may be utilized for amoeboid motion. Naegleria may utilize this contractile protein to perform other contractile functions such as chromosome separation during mitosis, or flagellar beating. Naegleria gruberi could provide an ideal organism for a detailed study of the interconversion of one biologically active form of a macromolecule to an entirely different, biologically active form in different physiological states of the same organism.

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