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City University of New York PH.D. 1986

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A STUDY OF AN UNUSUAL CYTOSKELETON IN TWO SPECIES OF THE PHYTOFLAGELLATES OCHROMONAS

BY

CAROLE ODDOUX

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1986

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

A STUDY OF AN UNUSUAL CYTOSKELETON IN TWO SPECIES OF THE PHYTOFLAGELLATES OCHROMONAS

by

Carole Oddoux

Advisors: Professor Sheldon Aaronson and Professor Robert Bittman

Relatively little work has been done on the cytoskeletal structure of lower eukaryotic or plant systems. While vertebrate systems may be easier to study because their tissue cells are specialized for a limited set of activities and cytoskeletal requirements, it is the lower eukaryotes that must engage in the total spectrum of activities, and therefore require multifunctional cytoskeletons. The complex and often unexpectedly different cytoskeletons of lower eukaryotes frequently makes these systems particularly difficult to study. Other complicating factors include the presence of active proteases, phenolases and pigments that may destroy proteins or interfere with standard biochemical techniques.

In this thesis, the cytoskeletons of two species of Ochromonas were studied. The adverse effects of proteolysis, phenolase activity and pigments on protein isolation and polyacrylamide gel electrophoresis were combatted by the use of the proteolytic inhibitors benzamidine,

1,10-orthophenanthroline and PMSF, and the use of the alkaline buffer system of Piccioni et al. [(1982) "Methods in Chloroplast Molecular Biology", Elsevier Biomedical Press, N. Y. p 985.] for electrophoresis.

Possible evidence for the presence of small amounts of actin was provided by positive anti-actin immunofluorescence. In addition, a protein comigrating with muscle actin eluted from a DEAE-cellulose column at a KCl concentration of 0.1 M - 0.35 M. A DNAase I inhibition activity also eluted in these fractions. Negative NBD-phallicidin results could indicate that the actin is present in non-filamentous form or in amounts below the detection limit of this assay. SEM and TEM of cold Triton X-100-extracted cells indicated the presence of a complex mesh-like cytoskeleton consisting of filaments of various sizes which are not accounted for by the tubulin or actin that could possibly be present. SDS-polyacrylamide gel electrophoresis revealed the presence of four major skeletal proteins of about 95.5 (or 111), 68, 40, and 30 kD. S1 labeling of Triton X-100-extracted cells was inconclusive because of the complex pattern created by the dense meshworks of these cytoskeletons.

Taken together, the results indicate that if actin is present, it is only present as a minor component of the cytoskeleton, possibly, in non-filamentous form. The complex cytoskeleton consists of four major proteins besides actin and tubulin. These proteins could possibly be intermediate filament-related or entirely new cytoskeletal proteins.

ACKNOWLEDGEMENTS

This thesis is dedicated to my mother who not only supported my entering the field of science even in the face of seemingly insurmountable odds but also taugnt me the meaning of courage and love. Her willingness to ride with me the roller coaster that is the life of a graduate student, has made this thesis possible.

The accomplishment of any difficult task requires not only perseverence but also cooperation from many sources. It has been my very good fortune to find so many generous and helpful people both at Queens College and at other institutions throughout the city. I would like to thank the members of my advisory committee for their extraordinary cooperation under very difficult and unusual circumstances, which made this thesis possible; My mentor Dr. Sheldon Aaronson for his continued confidence in my abilities even when I became doubtful and for giving me the opportunity to try new things and to fight for what I believe in; My second mentor Dr. Robert Bittman for his time and patience with a project over which none of us had any control; Dr. Thomas Haines for his imagination and excitement which reminded me of what science is all about when I sometimes lost sight of it and, for his generous support on so many levels; Dr. Saul Puszkin for taking the time to teach me immunofluorescence and for showing me that there are different points of view; Dr. John Condeelis whose insightful questions and careful attention to my work sent me looking in the right places for the answers I needed, for teaching me the critical analysis so important in scientific work and, for suggesting the NBD-phallicidin experiment and generously providing the NBD-phallicidin.

I especially thank Dr. Eugene Morris, ever generous with his knowlege, time and, support, for teaching me the fine arts of electron microscopy and photography and, for reminding me of the importance of one's sense of humor.

My thanks to Dr. Mildred Gordon for opening her laboratory at City College to me; To Dr. John Lee for making his magnificent collection of microscopes available to me for my immunofluorescence work; To Drs. Seymour Hutner and Margaret Clarke for providing cultures of O. malhamensis and D. discoudeum, respectively. My thanks to Dr. Elizabeth Boylan for many valuable discussions and for generously providing, along with Ms. Tova Rothschild and Ms. Mary Ellen Calatri, all of the rat tissue needed for these experiments.

I am grateful to the members of the Queens College Faculty of both the Chemistry and Biology Departments for their advice, assistance and inspiration throughout both my undergraduate and graduate careers, and for helping me to develop my own teaching abilities. My thanks to the Queens College staff for helping to keep things running smoothly and making the dark days brighter, to Mr. Robert Wurman for his friendship and help in resurrecting dead equipment and to Mr. Thomas Hayden III for his kind support and inspiration.

Finally, I would like to thank my fellow graduate students for their friendship, support and assistance. My special thanks to Ms. Helen Sabesvari and Ms. Mary Ellen Calatri for making it possible for me to meet the deadline.

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I INTRODUCTION

The literature in the field of cytoskeletal research is quite extensive; although much work has been done in this field, much more remains to learned. Most of the research to date has concerned itself with the cytoskeletal structure of vertebrate cells. Relatively little work has been done on lower eukaryotic or plant systems, yet in many ways these may be the most interesting systems. While vertebrate systems may be easier to study because their tissue cells are specialized for a limited set of activities and cytoskeletal requirements, it is the lower eukaryotes that must engage in the total spectrum of survival activities, and therefore require multifunctional cytoskeletons. It is becoming increasingly clear that lower eukaryotes have developed a variety of cytoskeletal systems to answer their varied needs. The generalizations we can make about vertebrate systems must be used with caution in nonvertebrate systems, as they may not apply in many cases.

This introduction is intended to describe the various functions of the cytoskeleton, the major cytoskeletal components known, the methods used to study them, and the available information on the particular lower eukaryotic system studied in this thesis, i.e. two species of Ochromonas. This thesis should provide a starting point for understanding the unusual cytoskeleton of these organisms and the experimental approaches required for their study.

A. Cytoskeletal Functions

The importance of cellular architecture in almost every area of cell behavior is beginning to emerge. Traditionally the structural elements of the cell, commonly referred to as the cytoskeleton, have been implicated in the maintenance of cell shape, intracellular arrangement and movement of organelles and chromosomes, cellular motility, plasma membrane activities such as phagocytosis, pinocytosis and exocytosis, and the formation and stabilization of cellular junctions and receptors. In recent years, the cytoskeleton has been shown to play a role in the mitogenic response of cells to growth factors [Gospodarowicz et al. (1978)] and in cellular differentiation [Hay (1981); Bissell et al. (1982); Benya and Shaffer (1982) and Chang et al. (1983)]. Furthermore, a change in cytoskeletal organization usually accompanies transformation. Imposition of cytoskeletal organizational change on a normal cell does not lead to a stablely transformed cell [Rifkin et al. (1979)] but it can dramatically change the ability of transformed cells to metastasize [Raz and Ben-Ze'ev (1983)]. In this context it is interesting to note that the Gardner-Rasheed feline sarcoma virus contains part of the gamma-actin gene in tandem with an oncogene [Naharro et al. (1983)].

Although our understanding of the structure of the cytoskeleton, the cellular activities in which it is engaged, and the biochemical mechanisms behind its activities is by no means complete, the knowledge that we do have has become a useful tool for learning other things about the cell. For example, since some of the components of the cytoskeleton are well conserved, differences in these molecules can be used to trace

the evolution of organisms [Little (1985); Adoutte et al. (1985); Heath (1980)]. Also, the distribution of some cytoskeletal proteins and their isoforms in vertebrates is tissue specific [Vandekerckhove and Weber (1978a); Vandekerckhove and Weber (1978b); Cooper et al. (1985)]; this has prompted pathologists to use the cytoskeletal protein distribution of cells to ascertain the tissue of origin of neoplastic cells [Cooper et al. (1985)]. An understanding of the different types of cytoskeletons may eventually lead to chemical or immunological weapons against specific cell types.

Clearly, a great deal of valuable information can be gained about many important cellular processes, by the study of the cytoskeleton and the wealth of literature in the field is a testament to its popularity as a research topic.

B. Major Cytoskeletal Components

1. Microtubules

Microtubules are straight, tube-like structures approximately 24 nm in diameter with a hollow 15 nm core. The 5 nm walls of microtubules generally consist of 13 protofilaments arranged so that the monomeric subunits of the protofilaments form a three-start right-handed helix [Gaskin and Shelanski (1976)]. While this general definition is usually applicable, variations do exist. Microtubules with diameters of 18-34 nm have been reported [Slautterback (1963)]. Dense elements have been reported in the

lumens of microtubules in neurons [Wuerker and Kirkpatrick (1972); Hama (1966); Peters and Vaughn (1967)], blood cells [Behnke (1967)], and flagella [Phillips (1966); Kiefer (1970); Shay (1972)]. Cross-bridges linking tubules have been observed in the mitotic apparatus [Krishan and Buck (1965); Roth et al. (1966); Lu (1967); Hepler and Jackson (1968); Wilson (1969); Hepler et al. (1970)], neuronal cells [Palay et al. (1968); Smith et al. (1970)], cilia and flagella [Gibbons (1963); Chasey (1969)], cytopharengeal baskets [Tucker (1968); Tucker (1972)], and axopods [Tilney and Byers (1969); Harris (1970); Roth et al. (1970)]. At least some of these cross-bridges may consist of the mechanochemical protein, dynein, which exhibits a microtubule-stimulated ATPase activity and may be responsible for the sliding of microtubules, (Table 1) [Gibbons (1963); Gibbons and Rowe (1965); Summers and Gibbons (1973)]. Dynein binds to microtubules in a polar orientation, and microtubules can be decorated with purified dynein to display their intrinsic polarity [Shelanski et al. (1971)]. Other possibilities for the "arms" associated with microtubules are the high molecular weight (380-270 kD) microtubule-associated proteins termed HMW-MAPS which have been shown to form microtubules with "arms" when tubulin is polymerized in their presence [Dentler et al. (1975); Murphy and Borisy (1975)].

Microtubule protofilaments are linear polymers of tubulin subunits. The tubulin subunit is composed of one α- and one β-polypeptide which are similar in molecular weight (approximately 55 kD) but differ in amino acid composition [Feit et al. (1971);

Bryan and Wilson (1971); Lee et al. (1973)]. Isoelectric focusing has shown that tubulin is an acidic protein (pI = 5.2-5.4) [Olmsted et al. (1971)] with a number of isoforms whose distribution may be organelle specific [Adoutte et al.(1985)]. Both α- and β-tubulin bind GTP or GDP. For polymerization to occur, tubulin subunits must associate to form a nucleus from which there is bidirectional assembly with associated hydrolysis of bound GTP [Timasheff and Grisham (1980)]. Microtubules are polar and one end assembles quickly, while the other end assembles slowly. As a consequence, the two ends of the microtubule have different critical concentrations for polymerization and permit treadmilling of subunits through the microtubule when the free subunit concentration is between the critical concentrations of the two ends [Wegner (1976)]. Microtubule polymerization is affected by the concentration of divalent cations, pressure and temperature; it is inhibited by Ca^{+2}, EDTA, high pressure [Zimmerman and Marsland (1964)], and cold [Timasheff and Grishan (1980)]. Both the HMW-MAPS and another group of microtubule-associated proteins called tau (55-70 kD) associate with the sides of microtubules and enhance polymerization, (Table I). Microtubules may also be stabilized by phosphorylation as tubulin can be phosphorylated by a cAMP-stimulated protein kinase [Soifer et al. (1972); Goodman et al. (1970); DiBella et al. (1971)] and can itself act as a kinase [Soifer et al. (1972)].

Microtubules have been associated with diverse functions based upon the general occurrence of microtubules and the response of cells to microtubule-disrupting agents. They include:

chromosome movements during cell division [Zimmerman and Marsland (1964)], intracellular transport of material as observed in the axons of neurons [Kreutzberg (1969); Fernandez et al. (1971)], the saltatory movement of particles in cultured cells [Freed and Lebowitz (1970)], of melanin granules in melanocytes [Malawista (1971)], endosomes in macrophages [Bhisey and Freed (1971)] and ribosomes in telotropic ovaries [Stebbings (1971)]. Microtubules have been implicated in the maintenance of cell shape in erythrocytes [Behnke (1970)], nerve ganglia [Daniels (1972); Yamada et al. (1971)], protozoan cells [Bouck and Brown (1973)], and lens epithelium [Piatogirsky et al. (1972)] to name only a few cell systems. Microtubules have also been shown to play a role in the development of cell shape as demonstrated by experiments with microtubule-destabilizing agents in Arbacia embryos [Tilney and Byers (1969)], developing lens tissue [Piatogirsky et al. (1972)], neurons [Daniels (1972); Hier et al. (1972); Seeds et al. (1970)] and developing germinative plant cells [Sanger and Jackson (1971)]. The consistent presence of microtubules in cilia and flagella relates to their role in cell motility [Satir (1976)]. Cytoplasmic microtubules have also been implicated in the cellular motility of tissue culture cells [Goldman and Follet (1970); Bhisey and Freed (1971); Goldman (1971)]. How microtubules perform these many functions is, in many cases, unkown.

2. Microfilaments

Microfilaments are involved in many cellular functions such as ameboid motility, cytokinesis, capping, exocytosis, phagocytosis, microvillar extension, membrane organization, and transmembrane signalling [Miranda et al. (1974); Pollard and Weihing (1974); Wang et al. (1975); Korn (1980)]. Microfilaments are composed of subunits of the roughly 42 kD globbular protein actin (G-actin), helically arranged in a double-stranded filament (F-actin) with a diameter of 4-7 nm. The filament is a one start left-handed helix with a genetic pitch of 59Å. Microfilaments have definite polarity which is revealed by the binding of the myosin subfagments, heavy meromyosin (HMM) or subfragment 1 (S1), which results in an arrowhead pattern along the filament, delineating the barbed (fast assembly) and the pointed (slow assembly) ends [Korn (1982)]. G-Actin binds a single divalent cation (Mg^{+2} or Ca^{+2}) and a single ATP molecule. During polymerization, each actin-bound ATP is hydrolyzed resulting in a tightly bound ADP and the release of inorganic phosphate [Uyemura and Spudich (1980)].

The monomeric form of actin (G-actin) is favored at low ionic strength but above 25 mM KCl or 0.2 mM $MgCl_2$ G-actin will polymerize to yield F-actin. Under conditions that favor polymerization, F-actin will only form if the concentration of G-actin present is greater than the critical concentration (less than 0.1 mg/ml) [Gordon et al. (1977)]. Thus F-actin is always in equilibrium with G-actin in an amount equal to the critical concentration.

A large number of actin-binding proteins have been described besides the ATPase-stimulating interaction between actin and myosin, which is responsible for contraction. Their various modes of interaction, which may or may not be Ca^{+2} dependent, include the following:

1. Binding to the monomer; suppresses nucleation and may result in a slower rate of filament elongation.

2. Binding to the pointed or slow assembly end; increases nucleation and affects elongation rate as a function of actin concentration, resulting in a larger number of shorter filaments.

3. Binding to the barbed or fast assembly end; promotes nucleation but inhibits elongation resulting in fewer, shorter filaments.

4. Binding to the side of the actin filament; results in stabilization or destabilization of the filament.

5. Binding to two filaments; results in cross-linking of filaments and the formation of gels.

Table 2 provides a sampling of the diverse microfilament-binding proteins that have been described.

Actin is an evolutionarily well conserved protein and actins from different sources are usually quite similar. Significant differences have, however, been observed (Tables 4, 5, and 6). Actin exists as isoforms which were discovered by isoelectric focusing. Four isoelectric point classes of actins have been described: α, β, γ, and δ in decreasing order of acidity [Adelstein et al. (1979)]. Skeletal and cardiac muscle actins are of the α-class while smooth muscle consists of γ-actin [Rubenstein

and Spudich (1977)]. Most vertebrate non-muscle cells contain mainly β-actin and some γ-actins. Most lower eukaryotes have almost exclusively one type of actin (95%). Acanthamoeba, for example, consists of δ-actin [Gordon et al. (1977)]. Naegleria, an ameboid cell, which has the ability to convert to a flagellate in a nutrient-free aqueous environment, is unique in that its actin exists in at least three isoforms [Lai (1984)]. Another difference between actins from different sources is the molecular weight which may vary between 42 and 48 kD (Tables 4, 5, 6).

Actin from most sources contains the rare amino acid 3-methylhistidine which has frequently been used as a marker for actin [Stebbings and Hams (1979)]; Naegleria actin lacks this posttranslationally added residue [Sussman et al.(1984)]. Naegleria actin behaves like other actins in its ability to stimulate myosin ATPase and polymerize, indicating that 3-methylhistidine is not required for these activities.

Most actins have been shown to bind to DNAase I and inhibit its activity. While the reason for this is unknown, DNAase I inhibition has been used as an assay for actin [Blikstad (1978)] but it is not foolproof. Naegleria actin has a very low affinity for DNAase I [Sussman et al. (1984)] and an actin-binding protein has been found in Physarum that binds actin [Maruta et al. (1984)]. An Entamoeba histolytica protein has been shown to be actin by anti-actin immunochemistry, rhodamine-conjugated phalloidin, activation of myosin ATPase and, S1 decoration but it does not inhibit DNAase I [Gadasi (1982)].

HMM or S1 decoration of actin filaments has been regarded as a specific indication of actin yet a number of organisms (Tables 5 and 6) have been shown to have filaments that do not label with HMM or S1. Whether some of these may represent forms of actin that are unresponsive to HMM or S1, or are different kinds of filaments, is not yet clear.

The discovery of unusual actins opens up the possibility of their use to associate aspects of molecular structure with specific functions, extending the information that may be gained through genetic modification, for which few systems are suited.

Another important difference in cellular actin exists between muscle and nonmuscle actins. Nonmuscle cells have a high concentration of G-actin, 20-300 times higher than the critical concentration. This indicates the presence of an intricate regulatory mechanism for actin polymerization, necessitated by the more dynamic needs of nonmuscle cells, as the actins from muscle and non-muscle cells perform in the same way in vitro [Uyemura et al. (1978)].

3. Intermediate Filaments

Electron microscopy has identified a major filamentous system in the cytoplasm of eukaryotic cells, with a characteristic diameter of 10 nm. As their size is between that of actin filaments (7 nm) and microtubules (25 nm), they are called intermediate filaments (IF) [Ishikawa et al. (1968)]. Subsequent

biochemical and immunochemical experiments established IFs as a distinct fibrous system consisting of five major subunit classes subdivided according to the tissues in which they were first discovered (Table 3). Today we know that some of the IF subunits can coexist in some tissues, e.g. vimentin and desmin in muscle cells [Gard et al. (1979); Osborn et al. (1980)], glial filaments and vimentin in glial cells [Bennett et al. (1981); Yen and Fields (1981)], and keratin and vimentin in HeLa cells [Osborn et al. (1980)].

While the five classes of IFs can be distinguished both biochemically and immunologically, indicating that they exhibit extensive regions of amino acid sequence divergence, they do share some unusual properties. They are extremely stable and can withstand exposure to low ionic strength buffers, high salt solutions (3 M KCl), non-ionic detergents, and a pH range of at least 2.5-8.5 without loss of morphological integrity. They may often be dissolved in alkaline salt solutions, urea in concentrations in excess of 4 M and in guanidine hydrochloride in concentrations greater than 2 M, as well as in detergents such as SDS and sarkosyl [Shelanski et al. (1971); Davison and Winslow (1974); Yen et al. (1976)]. Some, as in the case of the neurofibrillary tangles found in the brain cells of Alzheimer's disease patients, are so highly cross-linked, that they completely resist solubilization [Selkoe et al. (1982)].

Even when the subunits have been purified under denaturing conditions of low pH, high concentrations of urea, guandinium hydrochloride or SDS they will reassemble into filaments whose

structure and morphology is indistinguishable, by electron microscopy or X-ray diffraction, from native IFs. IFs do not appear to require other proteins, nucleoside triphosphates or divalent cations for polymerization [Steinert et al. (1976)]. The in vitro conditions required for their disassembly are so extreme that they are not likely to occur in vivo [Geisler and Weber (1981a)]. Despite such stability the organization of IFs is dynamic and actively controlled by cells. The ability of cells to change the organization of their IFs is evident in epithelial cell lines. In these cell lines, prekeratin filaments disappear at the onset of mitosis by unravelling into protofilament-like structures which then condense into cytoplasmic spheres that are apparent throughout mitosis [Horwitz et al. (1981); Franke et al. (1982)]. The cytoplasmic spheres move into the daughter cells, where they subsequently reform a new filamentous network. Disassociation of desmin filaments also occurs in chicken gizzard cells [Geiger and Singer (1980)]. Furthermore, interference with protein synthesis with cycloheximide or with toxins that inhibit protein synthesis (e.g. diphtheria toxin or endotoxin A) reversibly disaggregates vimentin filaments in some cells [Sharpe et al. (1981)]. The observation that proteolysis by calcium-activated proteases associated with vimentin [Nelson and Traub (1981)], neurofilaments [Day (1980)], and desmin [Azanza et al. (1979)] rapidly dissassembles IFs has lead to speculation that degradation may be the normal cellular route for disassembly of IFs.

Different classes of IFs also show similarities in their assembly characteristics. Desmin, vimentin and glial fibrillary

acidic protein each assemble as a homopolymer of a single peptide. Neurofilaments purify as three component polypeptides (Table 3) but under appropriate conditions the 68 kD protein will reassemble into a homopolymer in the absence of the 145 kD and 220 kD proteins. The resulting neurofilaments are smooth-walled rather than fuzzy as the native filaments are. This suggests that the 220 kD and the 145 kD proteins may be IF binding proteins [Geisler and Weber (1981b)]. The keratins comprise a highly complex family of polypeptides. It has not been possible to assemble, in vitro, an IF out of a single cytokeratin, although various combinations of two or more can assemble in vitro. This limited reassembly potential of the cytokeratins has made it possible to demonstrate the co-assembly of cytokeratin with other IF proteins. By choosing conditions under which neither IF protein alone would form filaments, it has been possible to demonstrate the assembly of mixtures of heterologous IF proteins [Steinert et al. (1981)], indicating considerable homology between different IF classes. The isolation of a monoclonal antibody that crossreacts with all classes of intermediate filaments further supported the notion that all the classes of IF proteins are ultrastructurally related [Pruss et al. (1981)]. Amino acid sequence data have shown that the IF proteins are related both between different species for a given class and between the different classes [Geisler and Weger (1981)].

These results suggest that all IF classes contain regions with similar sequences that define common α-helical domains arranged in a coiled-coil conformation and are responsible for the

assembly of morphologically similar IFs [Renner et al. (1981); Lazarides (1981)]. The α-helical domains are flanked by non-α-helical domains that vary in size, configuration and amino acid sequence in the different classes and are responisble for the differences observed [Lazarides (1981)]. The existence of structurally homologous areas on all IF classes permits copolymerization of different subunits. By varying the expression of any one filament subunit in relation to other subunits, a cell can potentially achieve a wide possibility of polymers with subtle structural differences, tailored to the differentiated state of the cell [Lazarides (1981)].

What is the function of IFs? Based upon their characteristic three-dimensional distribution in the cytoplasm as revealed by indirect immunofluorescence and electron microscopy, and by their high insolubility under physiological conditions, IFs have been presumed to be constituents of the cytoskeleton and as such play a structural role in vertebrate cells as mechanical integrators of cellular space [Lazarides (1980); Osborn et al. (1982)]. Clearly they do interact in some way with microtubules as their distribution pattern is altered when microtubules are depolymerized by colcemid treatment (see section C3) [Lazarides (1979); Blose and Chacko (1975)] and the finding that MAP2 binds to neuronal IFs causing an increase in viscosity in mixtures of microtubules and neurofilaments [Bloom and Vallee (1983)] supports this. Furthermore the attachment of bundles of cytokeratin filaments (tonofilaments) in epithelial tissue [Geiger et al. (1983); Cowen and Garrod (1983)], and desmin filaments in cardiac myocytes

[Thornell and Ericksson (1981); Franke et al. (1982); Kartenbeck et al. (1983)] to desmosomes is well documented. This provides a strong structural framework for these tissues

More recent studies show that early embryonic cells have no significant amounts of IF proteins [Jackson et al. (1980); Brulet et al. (1980)] while other studies indicate that IF proteins are present but in nonfilamentous form [Lehtonen et al. (1983)]. Microinjection of antibodies specifically directed against IF proteins (see section C3) leads to the collapse of the IF meshworks without affecting cellular activities previously proposed to be mediated by cytoplasmically extended IFs [Gawlitta et al. (1981); Klymkowsky (1981); Lin and Fermisco (1981); Klymkowsky (1982); Klymkowsky et al. (1983)]. Thus the extension of IF networks in the cytoplasm of vertebrate cells may not necessarily be a prerequisite for proper cell functioning.

In light of the recent data indicating that IFs are DNA-binding proteins [Carnevali and Filetici (1981); Leibovitch et al. (1981); Wortzman and Baker (1981); Bjursell et al. (1979); McGhee and Felsenfeld (1980); Cartwright et al. (1982)], Traub et al. (1985 a,b)] have proposed that IF proteins have a nuclear function, perhaps gene regulation, and that cytoplasmic IFs may serve as a storage mechanism to maintain a low cytoplasmic concentration of free nucleic acid-binding IF proteins. IF proteins are then released as needed by Ca^{+2}-activated proteases [Nelson and Traub (1981a); Nelson and Traub (1982); Nelson and Traub (1983)]. This hypothesis may be supported by the finding that IF proteins are phosphorylated, which may prevent the binding interaction

between IF-associated proteins and IF core proteins [Julian and Mushymski (1982); Williams and Jones (1982)] and that the level of IF protein phosphorylation increases during mitosis [Celis et al. (1985)]. The idea that IF proteins may have some sort of nuclear function is further supported by the finding that the major structural component of the nuclear envelope of mammalian cells, the nuclear lamina, is composed of three proteins called lamins A, B, and C. These lamin proteins show strong homology with IF proteins [McKeon et al. (1986)] and increased phosphorylation of the lamin proteins occurs before nuclear envelope disintegration in prophase [Gerace and Blobel (1980); Miake-Lye and Kirschner (1985)].

A possible nuclear role for IF proteins has profound implications for the distribution of IF proteins. The fact that the general organization of the nucleus and the process of mitosis itself are so well conserved in eukaryotes probably indicates that IF-like proteins are present in all eukaryotic cells. In fact, they have been found in a few protozoa, plants and fungi as well as vertebrates and invertebrates (Table 7).

4. Microtrabeculae

The fourth and newest class of cytomatrix structures is referred to as the microtrabecular lattice. The microtrabecular lattice is an irregular mesh of fine fibers of varying length whose diameter ranges from less than 2 nm to more than 10 nm. All of the other fibrous elements in the cell, as well as its

organelles, are thought to be embedded in the microtrabeculae, which would act as the cell's ground substance.

The existence of the microtrabecular lattice was implied by high voltage electron micrographs [Wolosewick and Porter (1977); Buckley and Porter (1975); Byers and Porter (1977)]. As the microtrabeculae are not easily visible in embedded samples, the microtrabecular lattice has become the subject of much debate. Opponents of the theory have called them artifacts caused by condensation of the protein-rich cytosol during fixation or dehydration. In an effort to address this issue, Wolosewick and Porter (1979) prepared cultured cells by various fixation and dehydration methods, both embedded and nonembedded. In all instances they found evidence for the microtrabecular lattice although varying degrees of preservation were obtained. Most notable are the partial disassembly of the lattice by OsO_4 which has also been reported for actin filaments and microtubules [Pollard et al. (1976); Boyles et al. (1985)]. In addition, embedding samples rendered the microtrabecular lattice much less visible. This indicates that while the methods for preparing samples for electron microscopy have progressed considerably in recent years, we must still exercise caution in interpreting electron micrographs.

What protein or proteins would be likely candidates for this fourth cytoskeletal system? It is not likely that a single protein, which would make up a system as extensive as the microtrabecular lattice would have escaped notice. Rather, it has been proposed [Fulton (1984)] that the various cytoskeletal binding

proteins which cross-link various filament types might constitute the microtrabeculae. A striking new candidate for microtrabecular proteins is spasmin, a protein which undergoes conformational changes in the presence of calcium. As detailed in the next section, spasmin was originally described in protozoa [Amos (1975)] and has subsequently been antigenically detected near the centriole of mammalian cells [Fulton (1984)]. Thus some of the cytoskeletal oddities thought to occur only in isolated cases in protozoa, may have far reaching implications for our understanding of mammalian cytoskeletal systems.

5. Other Filament Systems

The various mechanisms of nonmuscle motility are generally believed to have their basis in the filament systems already discussed. Furthermore, the movements accomplished by the active cytostructures are ATP-dependent and consist of a sliding interaction between a filamentous component and an ATPase activity-containing component or result from treadmilling of the filament componenets. Increasing evidence for other motility systems is coming to light through the study of protozoa (Table 8).

One alternative motility system occurs in vorticellid ciliates [Amos (1975); Routledge (1978); Yamada and Asai (1982)] and consists of a bundle of 2-3 nm microfilaments termed the spasmoneme. The spasmoneme is closely associated with a network of membranous saccules analogous to the sarcoplasmic reticulum of

muscle cells. The spasmoneme contracts much faster than the fastest of striated muscles and requires Ca^{+2} but not ATP for contraction. These filaments are smaller than actin filaments, do not decorate with HMM and actually contract rather than sliding past another structure. Electrophoresis has shown them to consist of two major components of 18 and 20 kD molecular weight, referred to as spasmin (or spastin) A and B, respectively. Recently antibodies to these proteins have been shown to cross-react with striated rootlets of flagellated cells and the structures near the centriole of mammalian cells [Fulton (1984)] suggesting that spasmin or a related protein may be far from a curiosity occurring in a few limited systems.

Other 2-3 nm filaments have been shown to contract somewhat more slowly than spasmin but also in a calcium-dependent manner. These filaments are not directly associated with the endoplasmic reticulum. During contraction, these filaments actually coil into tubular structures which may or may not show periodicity. While these organisms are generally difficult to work with because they are not easily preserved with fixatives, many other instances of unusual filaments are coming to light. Too little has been done at this point to speculate about their mode of action or relationship to any of the proteins already discussed.

It is becoming clear that these unusual systems should not be overlooked as they may provide valuable insights into the cytoskeletons of mammalian cells or provide valuable tools for the protection of mammalian cells against eukaryotic parasites.

C. Methods Used for the Study of the Cytoskeleton

The most logical approach to the study of something as complex as the cytoskeleton, is to sequentially separate each component and to characterize its morphology, genetics, function and biochemical properties and then to reassemble the various characterized components in order to obtain a complete picture. In practice, this is not exactly what happens since it is often what presents itself that one studies, rather than a systematic stepwise analysis of each component. We must always be aware of the shortcomings of our techniques and of our understanding of any new system. Some of the methods and tools used in the study of the cytoskeleton include the following:

1. The Cell System

One valuable tool for the study of the cytoskeleton is the specific cell system employed. All cells are specialized for a particular set of functions and lifestyles. The multitude of different cell types available for study allows one to select a system that is specialized for a particular set of functions which in turn allows for the isolation of some cytoskeletal functions from the rest of the many possible functions. In addition, contrasting different cytoskeletons can provide valuable insight into the regulation, control and mode of action of these different systems. As helpful as specialized systems are, one must always

remember that a particular system is one isolated case; conclusions about other systems must, of necessity, be made with caution.

2. Microscopy

The most fundamental approach to the study of the cytoskeleton has been to "look" at it by means of various microscopic methods. The electron microscope gave scientists their first clear look at the complex fibrous network that makes up the cytoskeleton [Roth and Daniels (1962); Manton and Clark (1952); Fawcett and Porter (1954)] and indeed the names given to the various components (microtubules, microfilaments, thick filaments, intermediate filaments and microtrabeculae) come from their appearance in the electron microscope. Important information about the general organization of filamentous components has been obtained by this method, such as the arrangement of microtubules in the mitotic apparatus [Roth and Daniels (1962)], the 9 + 2 arrangement of microtubules in flagella and cilia [Manton and Clark (1952); Fawcett and Porter (1954)], the bundles of actin filaments in stress fibers [Buckly and Porter (1967)], and microvilli [Mooseker and Tilney (1975)], etc.

A significant contribution to our knowledge of the substructures of these fibrous elements has also come from electron microscopic studies. The arrangement of the 13 protofilaments in the microtubule [Ledbetter and Porter (1964)] is a good example.

Although, today the more elegant technique of X-ray diffraction is often used to obtain a three-dimensional picture of molecules, this method has largely confirmed the results obtained from electron microscopic studies [Mandelkow et al. (1977)]. The electron microscope has also proven useful in elucidating interactions between various filament types, between filaments and organelles and between filaments and membranes.

Microscopy has even become an important analytical tool. Purified proteins can be polymerized and examined under the electron microscope to determine the filament class to which they belong. Methods also exist for specific staining or labeling of cytoskeletal constituents. They may involve the specific binding and decoration pattern of cytoskeletal proteins to particular cytoskeletal fibers such as the characteristic arrow-head complexes formed by the myosin fragments, heavy meromyosin (HMM) and subfragment 1 (S1), when they bind to actin filaments [Pollard et al. (1970)] or dyenin when it binds to microtubules [Shelanski et al. (1971)]. These patterns are quite specific and usually readily observable by electon microscopy. Other methods require the attachment of a fluorescent, enzymatic, or electron dense marker to the specific binding protein or drug. Typical fluorescent markers include fluorescein, rhodamine, and 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) [Bullock and Petrusz (1982); Barak et al. (1980)]. Enzyme markers have been used for both light and electron microscopy. The most popular enzyme label has been horseradish peroxidase but alkaline phosphatase and glucose oxidase have also been used [Sternberger (1979)].

Electron dense markers such as ferritin [Webster et al. (1978)] or colloidal gold are also available for electron microscopy.

Besides the previously mentioned HMM (or S1) and dyenin interactions, other specifically binding molecules have been used successfully to label cytoskeletal components. They include the specific, although little understood interaction of the enzyme DNAase I with actin [Blikstad et al. (1978)] and the specific interaction of phallacidin with microfilaments [Barak et al. (1980)]. By far the most useful and generally applicable specific binding molecule is the antibody, since antibodies can be made at will for almost all purified proteins. They can be polyclonal, consisting of a population of different antibody molecules which as a group are specific to many different domains of the target molecule, or they may be monoclonal, consisting of a single antibody molecule specific to one particular domain of the target molecule.

Still another visual method for cytoskeletal analysis involves the observation of live cells microinjected with labeled antibodies, cytoskeletal subunits or drugs. Since fluorescence signals can be markedly enhanced by computer technology, small amounts of fluorescent probes and low levels of stimulating ultraviolet light may be used, thereby minimizing toxic effects. Such techniques provide a method for analysis of the dynamic interactions of cytoskeletal subunits with their respective structures [Fulton (1984)] and antibodies can be used to sequester a specific cytoskeletal component, thereby effectively removing it from the cytoskeleton [Gawlitta et al. (1981)].

As useful as these microscopic techniques are, they are not without their pitfalls and proper precautions must be taken when using them. For example, electron microscopy and all of the other microscopic methods employing dead cells require that the cells be preserved by fixation. Although great strides have been made in this technology, problems still exist and it is known that some fixatives alter the structure of cytoskeletal proteins. A most notable example is the effect that OsO_4 and dehydration agents have on actin filaments [Boyles et al. (1985)]. Fixatives also alter the tertiary structure of proteins sometimes making them unrecognizable to a given probe. Furthermore, fixatives preserve structure mainly by crosslinking the molecules of the cell together in situ. This means that the normal fluidity of the cell can be destroyed and probes, especially large ones such as antibodies, may be unable to diffuse to their intended targets. The cross-linking ability of fixatives is not specific and can often lead to a general stickiness of the cellular constituents which in turn can result in nonspecific sticking of the probe. To avoid some of these problems, frozen sections for binding of the probe can be used; however, freezing too can have effects on the cell, and this is a difficult technique to employ. Also after the probe has been introduced, the cell must still be fixed.

Electron microscopy requires extremely thin sections for adequate resolution and this necessitates the embedding of biological material in resin. The resin may itself have electron scattering properties that can obscure certain cytoskeletal components such as the microtrabecular lattice [Wolosewick and

Porter (1979)]. Whole mounts are therefore attempted in cells that are well spread and consequently relatively thin, or cells that have had their cytoplasmic contents extracted by a detergent such as Triton X-100 [Edds (1977)]. Thicker cells can be broken apart by the cleaving critical point dryed cells on grids by means of adhesive tape (dry cleaving) [Mesland et al. (1981); Traas (1984)]. The advent of high voltage electron microscopy has also aided the evaluation of thicker specimens [Wolosewick and Porter (1979)].

The probes themselves present still another problem. For example, HMM is believed to cause polymerization of actin into filaments [Oosawa and Kasai (1981)] and DNAase I causes depolymerization of actin filaments thereby possibly altering the appearance of the cytoskeleton [Blikstad et al. (1978)]. The lack of reaction with a probe must also be interpreted with caution. For example, there are documented instances where some actins have been shown to be actin by other methods but do not bind DNAase I [Gadasi (1982)]. In another instance, filaments the size of intermediate filaments were observed, which did not label with HMM [Buckley et al. (1978)]. Treatment of these filaments with proteases resulted in their being decorated when exposed to HMM, indicating that the structure consisted of an actin core filament covered with another protein.

The techniques used to open holes in the membrane such as detergent extraction or glycerination, of necessity, also open holes in organellar membranes, lysosomes in particular, which could release potentially damaging enzymes such as proteases or

cause leaching of soluble cellular components that may have a regulatory effect on the insoluble portion of the cytoskeleton.

Probes that carry fluorescent tags pose a problem when used in cells that have pigments that are fluorescent, such as chlorophyll, carotenoids, etc. This can sometimes be overcome by selecting a tag whose excitation wavelength does not excite the endogenous pigments, by solvent extraction, by subtraction of the endogenous fluorescence using filters, or by selection of appropriate exposures during photography.

Antibodies have their own set of problems. Polyclonal antibodies prepared from pooled sera may contain many other antibodies [Bullock and Petrusz (1982)] and affinity purified material should therefore be used. Monoclonal antibodies are specific for a particular protein domain. Narrow domains may be present in many proteins and therefore give rise to nonspecific positives or may be domains that are not conserved in different species thereby limiting the crossreactivity of the antibody. Some antibodies recognize a particular three dimensional conformation and consequently will not bind if the target protein has been denatured in some way such as by fixation or by detergents [Sternberger (1979)]. Antibodies are also frequently prone to nonspecific reactions mainly due to attractions to the Fc portion of the antibody molecule. The best defense against these problems with the use of antibodies is the incorporation of good positive and negative controls. Excellent negative controls include the use of preimmune serum from the animal in which the antibody was prepared, obtained before the animal was injected

with the antigen or, to precipitate the antibody by binding it to the antigen, thereby leaving behind all of the other components of the serum that could possibly generate a nonspecific reaction.

3. Drugs

Cytoskeletal drugs exist that specifically bind to tubulin or actin. They have two basic modes of action. They will either bind to the monomer and poison further assembly of the polymer or they will bind to the polymer and stabilize it.

The first cytoskeletal drugs discovered were the antimitotic drugs, of which colchine is the oldest. Nocodazole, oncodazole and colcemid all bind to the tubulin dimer at the same site that colchicine binds [Dustin (1978)]. The drug-dimer complex then binds to the fast assembly end of the microtubule preventing further assembly from that end. Since the slow assembly end of the microtubule has a much higher critical concentration for assembly, microtubule formation will cease which will lead to a net depolymerization of microtubules that are in dynamic equilibrium. Colchicine exhibits nonspecific binding to cell membranes sticking mainly to the nucleoside transporter. This nonspecificity can be subtracted from the specific reaction by the use of the control drug, lumicolchicine, whose binding to the tubulin dimer is inactivated by light while still maintaining the same pattern of non-specific binding. Nocodazole is more specific than colchicine and its results are more easily reversed since it

dissociates more rapidly than colchicine. Vinblastine and vincristine bind to the tubulin dimer at a site different from that of colchicine and they lead to aggregation and crystal formation which in turn reduces the concentration of free tubulin monomer available for assembly [Fulton (1985)]. All of the drugs mentioned so far prevent the assembly of microtubules and as such will exhibit highly specific effects on different classes of microtubules [Fulton (1984)]. Flagellar microtubules are unaffected once assembled, mitotic microtubules are prevented from forming, and cytoplasmic microtubules take one to several hours to disappear [Fulton (1984)]. The differential response of the different classes of microtubules to the drugs provides information about the dynamic equilibrium of each class as do the delayed effects such as bundling or formation of foci, or effects on other filament systems.

In contrast to the above-mentioned drugs, taxol stabilizes the microtubule by binding to it stoichiometrically and abolishing the lag phase during polymerization [Horwitz et al. (1982)]. This property has led to its use as a stabilizing agent for microtubules during extractions.

Microfilaments are affected by drugs whose mode of action corresponds to that of the antimitotic agents. The cytochalasins are a group of drugs that bind reversibly to actin monomers. The actin-cytochalasin complex in turn binds to the fast assembly end of the microfilament preventing further assembly from that end [Flanagan and Lin (1980)]. Cytochalasin B has the drawback of inhibiting glucose transport at the cell membrane, however,

dihydrocytochalasin B and cytochalasin D do not affect glucose transport [Atlas and Lin (1978)]; cytochalasin D has the higher affinity for actin. Phalloidin and phallacidin stabilize microfilaments by binding stoichiometrically to polymerized actin and lowering the critical concentration reversibly [Fulton (1984)]. This specificity for F-actin has proven to be a valuable tool for labeling microfilaments and NBD-phallacidin is commonly used for this purpose.

No small metabolites that directly affect intermediate filaments are known. At present, the only means of disassembling intermediate filaments in vivo is by microinjection of anti-intermediate filament antibodies [Fulton (1984)]. Useful information can sometimes be obtained from studies using drugs which primarily affect other molecules, but such data are often difficult to interpret (see section B3).

Also valuable for the study of the cytoskeleton are a number of drugs whose primary sites of action are known and which have marked secondary effects on the cytoskeleton and its organization. For example, drugs such as dinitrophenol which inhibit energy metabolism will affect all energy-dependent activities of the cytoskeleton. Several cytoskeletal proteins such as spectrin are calmodulin-binding proteins and are therefore affected by anti-calmodulin or calcium modulating agents [Fulton (1984)]. The effects of antimitotic drugs and drugs affecting protein synthesis, on IFs has already been mentioned. The use of drugs with secondary effects on the cytoskeleton aids in the elucidation of cytoskeletal protein interactions and the mechanisms that control various cytoskeletal activities.

4. Detergent Extraction

For the cytoskeleton, separation is usually accomplished by extraction with a nonionic detergent, such as Triton X-100, which results in the solubilization of the membrane and washing out of all of the soluble cytoplasmic components [Brown et al. (1974); Lenk et al. (1977); Schliwa and Van Blerkom (1981)]. One is then left with a cytoskeletal preparation that can be used for electron microscopy, biochemical analysis (such as by electrophoresis), purification and isolation of a cytoskeletal component [Fey (1984)], or reaction to particular conditions or drug treatments. Several important points must be kept in mind when using these preparations. First, anything that is attached to the cytoskeleton is not likely to be extracted. Such attached noncytoskeletal components include the nuclear matrix [Fey et al. (1973)], polyribosomes [Lenck et al. (1977); Fulton et al. (1980); Cervera et al. (1981)], and the plasma membrane [Ben-Ze'ev (1979)]. The inability to remove these noncytoskeletal elements produces a more complex structure than might be expected but on the other hand is useful for analyzing such cytoskeletal attachments and for purifying the attachment proteins. Secondly, the extraction of the soluble cellular constituents may mean the removal of regulating factors or filament subunits which play an important role in the dynamic interactions of the cytoskeleton. As Triton-extracted cells may react differently than whole cells, it is important to compare results with in vivo systems.

A further complication regarding the use of such extracted cells is the effect of the extracting buffer on the cytoskeleton. A good example is the effect that Ca^{+2} has on the calmodulin-binding portions of the cytoskeleton. Extraction in a Ca^{+2}-containing or conversely an EGTA-containing buffer may therefore yield very different products. The ionic strength of the buffer will have an effect on the solubility and therefore extractability of certain components. The temperature also plays an important role since cytoplasmic microtubules will depolymerize in the cold. Consequently, one must be careful to check the conditions used before comparing data.

5. Purification and Characterization

The cornerstone of biochemical research has always been the purification and characterization of the molecular constituents of the given system and cytoskeletal research is no different. A complete picture of any process requires an understanding of both its structural and the biochemical aspects. Not only is purification and characterization a valuable method for proving the identity of an isolated molecule, it also provides a basis for comparing subtle differences between the newly isolated molecule and other closely related species which in turn makes it possible, along with *in vitro* studies, to learn about the mechanism by which the isolated molecule carries out its biochemical activities.

Purification schemes make use of the individual characteristics of the molecule under study and anything that sets it apart from the others can be used. The main structural proteins of the cytoskeleton are distinguished from the rest of the cellular constituents by their ability to polymerize. The conditions, mainly variations in ionic strength, required for the polymerization of the various filament types are different so that conditions can be chosen where cycles of polymerization and depolymerization can frequently yield an almost pure preparation, contaminated only with proteins that specifically bind the protein of interest. The particular conditions required for the polymerization of a given filament type are often come upon accidentally, when a change in viscosity is observed. Once the conditions are known, they may be applied to a different system when searching for that protein. This has been the approach to the purification of actin and myosin from muscle or vimentin from lens [Geisler and Weber (1981b)], etc. Such an approach only works well for a system that has large amounts of the protein sought so that the critical concentration for polymerization can be achieved. In addition, a polymerization-depolymerization purification scheme requires incubations of crude extracts, centrifugations and then dialysis to exchange the buffer, all long processes which are not suited to a system containing active proteases that cannot be adequately inhibited by antiproteolytic agents. Many lower eukaryotes have small amounts of some of the cytoskeletal components and have large amounts of active, difficult to control proteases, making a polymerization-depolymerization purification

scheme useless in these systems [Pollard and Ito (1970)]. Furthermore, the cytoskeletal proteins may have some biochemical differences (such as a different critical concentration for polymerization) or are regulated by other proteins which would prevent them from behaving as expected during purification.

Another purification method makes use of the specific binding affinity of the desired molecule for a molecule with which interaction will result in precipitation of the complex. This is the basis for the purification of actin by suprapreciptation with myosin or immunoprecipitation where the interaction is between the desired protein and an antibody directed against it. Both of these methods require that the two protein components be present in a particular ratio or precipitation will not occur, and they both require incubations which may be damaging if proteases are rampant.

Another important factor in selecting a purification scheme is the fact that nonmuscle systems have complex regulatory mechanisms which may lead to subpopulations of molecules. Thus a purification scheme using polymerization-depolymerization cycles may enrich for only certain populations of molecules and not yield a representative sample [Uyemura (1980)].

When the system one wishes to study is not amenable to the above-mentioned purification schemes, more traditional broad-based procedures must be used. These usually include some form of column chromatography, the most popular of which are ion exchange, gel filtration, and affinity chromatography.

After purification the proteins must be characterized to be sure of their identity and to learn more about them. Aside from the typical determinations of molecular weight, amino acid composition and isoelectric point, cytoskeletal proteins are usually characterized in terms of their ability to form filaments. Changes in viscosity are used to determine under what conditions (ionic strength, presence of Ca^{+2}, Mg^{+2}, nucleotides, other proteins or peptides, temperature, and pH) they may form filaments. Electron microscopy provides a method for determining the size and shape of the filament formed in vitro. Whether or not the given protein binds to other cytoskeletal proteins can be answered by using cytoskeletal proteins as probes on electroblots or looking for effects such as superprecipitation, geling and, solubilization when the purified protein is mixed with other cytoskeletal proteins in vitro [Aamodt and Williams (1984)]. Such information is invaluable in ascertaining the activity of a newly isolated unknown protein.

Although purification and characterization of an unknown protein are, in many ways, the most important part of any biochemical analysis, many systems have problems that may prevent the researcher from achieving these goals. The one must therefore make inroads with other methods until enough information is obtained to solve the problems of purification.

All of the methods detailed above, along with the techniques of molecular biology, provide significant information about a particular aspect of the cytoskeleton. In order to obtain a complete picture, all

of the methods must be used. In practice, however, such complete data collection is not possible. Instead, systems are chosen that are specialized in certain ways and therefore facilitate the implementation of certain techniques for their study. As a result all of the techniques mentioned are being used to address the questions of cytoskeletal function, structure and regulation, but in many different systems. Scientists must therefore extrapolate to other systems in order to obtain a complete picture. Such extrapolation is dangerous, particularly in light of the increasing evidence that different types of cytoskeletons exist.

D. OCHROMONAS

Ochromonas danica and Ochromonas malhamensis (Poterioochromonas malhamensis) are classified as chrysomonads. They are naked phytoflagellates and as such possess both plant (phototrophic) and animal (phagotrophic) characteristics [Aaronson and Baker (1959)], in fact they may survive by either means of nutrition, exclusive of the other. Ochromonas is characterized by two anterior flagella which are different in length, orientation and external appendages called mastigonemes [Bouck (1971)]. The cells have a distinctive teardrop (pyriform) shape yet lack either a cell wall or pellicle. In the case of Ochromonas danica the shape is maintained by two sets of microtubules - one associated with the anterior region of the cell next to the flagellar insertion point called the kinetosome, and one associated with the rhizoplast, tail and spindle [Bouck and Brown (1973)]. In the case of

Ochromonas malhamensis, only one microtubule organizing center exists, that of the kinetosome. O. malhamensis lacks a rhizoplast and the microtubules of the kinetosome are extended to shape the tail [Schnepf et al. (1977)]. Actively motile O. malhamensis usually do not have a very pronounced tail but will form one as a means of attachment to a substratum. The tail region of Ochromonas is of special interest as it seems to have the capacity for controlled bending and is highly labile when exposed to unfavorable conditions [Bouck and Brown (1973)]. Fine filaments have been observed in the tail of O. danica [Bouck and Brown (1973)] as well as elsewhere in the cell [Kahan et al. (1978); Aaronson and Behrens (1974); Aaronson and Behrens (1973)].

These phytoflagellates may be grown in the dark and cannibalism, which involves engulfment of a complete Ochromonas by another, is not uncommon in older cultures indicating that these cells can undergo considerable distortion when necessary. In fact, these cells have a very active surface capable of phagocytosis and pinocytosis [Aaronson and Baker (1959)], formation of large [Aaronson et al. (1971a)] and small projections (microvilli) [Kahan et al. (1978)], and excretion of vesicles, membranes, macromolecules and small molecules by exocytosis [Aaronson et al. (1971a,b)]. Futhermore, these activities can be manipulated by changing the growth conditions. For example, the cells can be induced to pinocytose actively by growth in the dark on simple soluble nutrient media. They can be induced to phagocytose actively by growth in the dark on bacteria. They can also be induced to perform these activities as photosynthetic cells so that the effects due to the presence of chloroplasts can be ascertained. Ochromonas can be induced to pinocytose in the light by growing photoheterotrophically

with the obligate requirement for biotin satisfied only by pinocytosis of soluble biotin. They may even be induced to phagocytose while photosynthesizing by photophagotrophic growth using phagotrophy of bacteria to satisfy the obligate requirement for biotin. Even exocytosis can be controlled. O. danica excretes acid phosphatase into its medium and this excretion can be stopped by cycloheximide or stimulated by chloramphenicol base [Aaronson and Patni (1978)], or glucose-1-phosphate, glucose-6-phosphate or phosphoglycolic acid [Patni and Aaronson (1977)]. The ability to manipulate these biological processes may provide the means to study the biomolecules involved in these processes.

Ochromonas have very unusual plasma membranes containing chlorosulfolipids [Chen et al. (1976)], which enable them to exist at a pH of 5. In fact resuspension of these cells in pH 7 buffer results in lysis of the cells (personal observation). The plasma membrane of O. danica can be isolated in quantity and has been partially characterized [Patni (1974); Billmire (1974)].

E. EXPERIMENTAL OBJECTIVES

In light of the active cell surface of Ochromonas, the ease with which cell surface activities can be controlled and the existence of a method for the purification of large amounts of plasma membrane, the following experiments were proposed:

1. Purify O. danica actin.
2. Characterize O. danica actin and compare it to mammalian actin.

3. Purify quantities of O. danica plasma membrane and search for membrane-actin interactions by sequential extractions of membranes, exposure of membranes to purified actin and, analysis of membranes for the presence of actin-binding proteins.

As O. danica is evolutionarily far-removed from mammals, it was hoped that some significant differences in O. danica actin might be discovered, as has been the case for some other lower eukaryotic systems (see section B2). Such unusual actins are useful in determining which features of a molecule are required for particular activities. In addition, actin-membrane interactions are understood in relatively few systems and the mechanisms by which actin can control the membrane activities with which it is believed to be involved are still largely unknown [Carraway and Corothers Carray (1985); Geiger (1983); Wehing (1979)].

Initial attempts to purify actin from both acetone powders and whole cells, by cycles of polymerization-depolymerization were fruitless, largely due to proteolysis. Subsequent introduction of proteolytic inhibitors and the use of column chromatography yielded somewhat improved results. Monitoring of samples by electrophoresis was difficult due to smearing caused by the presence of pigments, phenolases and proteinases.

While efforts were undertaken to improve the above procedures, microscopy experiments were performed to determine whether or not actin was actually present. The results of such experiments indicated the presence of only small amounts of actin.

Thus the focus of this thesis has shifted from the purification and characterization of actin to the development of techniques by which the unusual cytoskeleton of these organisms could be studied. In the course of this work a cytoskeleton has been revealed that performs the activities normally associated with actin but consists largely of other fibrous elements, thereby opening up a whole new set of significant questions.

II MATERIALS AND METHODS

A. Organisms and Growth Conditions

Ochromonas danica, strain L933/2 Pringsheim, was maintained in a chemically defined medium (heterotrophic medium) [Aaronson and Baker (1959)] in a 10 ml volume in black-taped screw-cap test tubes in a refrigerated incubator at 25°C, in the dark. Experimental cultures were grown in heterotrophic medium under the same conditions in 500 ml volumes in one liter screw-cap Erlenmeyer flasks. Cultures were incubated 4-5 days for log phase cells.

Ochromonas malhamensis, strain 933-1 Pringsheim (kindly supplied by Dr. Seymour Hutner, Haskins Laboratories - Pace University, NY), was maintained in HL5 medium [Cocucci and Sussman (1970)] under the same conditions as O. danica.

Dictyostelium discoideum, strain Ax-3 (kindly supplied by Dr. Margaret Clarke, Albert Einstein College of Medicine, NY), was maintained in HL5 medium in a 20 ml volume in 50 ml screw-cap Erlenmeyer flasks in a refrigerated shaker incubator at 25°C. Experimental cultures were grown in HL5 medium under the same conditions in 500 ml volumes in one liter screw-cap Erlenmeyer flasks. Cultures were incubated 2-3 days for log phase cells. Cells were also stored by inducing amebas to fruit on non-nutrient agar (0.01 M potassium phosphate buffer, pH 6.4, 2% agar). Two milliliters of log phase culture were applied to the surface of the non-nutrient agar in a petri dish and incubated at 25°C for 2 days. After fruiting, the plates were

stored at 4°C for as long as several months and cells were scraped off the plate with a sterile loop when needed and placed into HL5 medium.

B. Protein Purifications

Actin was purified from ACI brown rat hind leg muscle (kindly supplied by Ms. Tova Rothschild and Dr. Elizabeth Boylan, Queens College of The City University of New York). Rats were sacrificed by decapitation or etherization and kept at 0°C while removing muscle. Muscle was frozen until use. Acetone powder was prepared by the method of Margossian and Lowey (1982) and stored at -20°C until use. Actin was prepared from acetone powder by the method of Pardee and Spudich (1982). F-actin was stored at -20°C either as a pellet or in 50% glycerol.

S1 was prepared from glycerinated ACI brown rat myofibrils by the method of Cooke (1972) and was stored at 4°C for use within one week or at -20°C in 50% glycerol for long term storage.

Vimentin was prepared from ACI brown rat or rabbit (Pel Freeze) lenses as per Geisler and Weber (1981). The procedure was taken only as far as the ethanol precipitation after extraction in urea. The crude vimentin pellet was then used directly for electrophoresis.

Desmin was prepared from chicken gizzard (Pel Freeze) by the method of Huatt et al. (1980).

C. Immunofluorescence

Cells were pelleted by spinning for 5 minutes at 5,000 rpm in a Sorvall RC-2B refrigerated centrifuge in an SS-34 rotor. Cells were resuspended in 1/10 of the original volume of fresh sterile medium. Concentrated cells were then incubated for 30 minutes on coverslips coated with 0.1% poly-L-lysine (Sigma) by the method of Mazia et al. (1975). Unattached cells were then rinsed off in phosphate buffered saline (PBS - 0.17 M NaCl, 3.0 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.02% Thimerosal) fixed for 10 minutes in 3.7% formaldehyde in PBS and washed five times in PBS (2 minutes each). The coverslips were then immersed in -20°C acetone for 10 min and allowed to air dry. The cells were incubated for 15 minutes at 37°C in 50% nonimmune goat serum (Pel Freeze), to block nonspecific binding sites. After washing once in PBS the cells were incubated for 30 minutes at 37°C in anti-actin antibody diluted 1:10 in PBS (Miles) and then washed 5 times in PBS. Finally the cells were incubated for 30 minutes in fluorescein conjugated goat anti-rabbit serum diluted 1:150 (Pel Freeze) in PBS, washed five times in PBS and mounted in 50% glycerol.

The following controls were used: 1. The entire staining sequence was used but anti-actin antibody was eliminated. 2. Anti-actin antibody was precipitated from the serum by reacting for 30 minutes with purified rat actin filaments resuspended in PBS, and centrifuging in a Beckman L5 ultracentrifuge for 3 hours at 36,000 rpm in an SW-36 rotor. 3. Dictyostelium discoideum was stained as a positive control as this organism has large amounts of actin.

Cells were examined with a Zeiss microscope equipped with epifluorescence. They were photographed with Kodak Tri-X film using Kodak Wratten filters #58 and 99 to filter out the gray background flourescence of the cells. All photos were taken and printed under identical conditions.

D. NBD-Phallacidin Staining

NBD-phallacidin from Molecular Probes Inc. was the generous gift of Dr. John Condeelis (Albert Einstein College of Medicine). NBD-phallacidin staining was performed by the method of Detmers et al. (1985) with the following exceptions: 1. Cells were not treated with autolysin as neither Ochromonas nor Dictyostelium have cell walls. 2. Cells were attached to coverslips immediately after fixation and were treated with 1% bovine serum albumin in PDF buffer (40 mM $NaPO_4$, pH 6.4, 20 mM KCl, 0.7 mM $CaCl_2$, 2 mM $MgSO_4$) followed by two rinses with PDF.

The controls used were: 1. Negative control - Entire procedure with the exclusion of the NBD-phallacidin. 2. Positive control - Entire procedure performed on Dictyostelium.

E. Preparation of Cytoskeletons

Cells were pelleted by spinning for 5 minutes at 5,000 rpm in a Sorvall RC-2B refrigerated centrifuge in a GSA rotor. Cells were then washed by resuspension in cold (4°C) heterotrophic medium in the case of O. danica or pH 6 buffer (10 mM potassium phosphate buffer) in the case

of O. malhamensis and D. discoideum and repelleted. Cytoskeletons were prepared by extracting in cold TSB (2% Triton X-100, 40 mM KCl, 10 mM imidazole-chloride, pH 7.0, 10 mM EGTA, 2 mM $MgCl_2$, 2 mM NaN_3, 2 mM phenylmethylsulfonylfluoride (PMSF), 2 mM benzamidine) on ice for 30 minutes and pelleting at 11,000 rpm for 30 minutes. The cytoskeletons were then washed by resuspension in TSB and repelleted. The cytoskeletons were finally washed twice in cold LSW (40 mM KCl, 10 mM PIPES buffer, pH 6.8, 1 mM EGTA, 2 mM $MgCl_2$, 0.65% NaN_3, 2 mM PMSF, 2 mM benzamidine).

F. Electron Microscopy

1. Negative Staining of Cytoskeletons

Cytoskeletons of O. danica, O. malhamensis, and D. discoideum were each attached to formvar carbon-coated 300 mesh copper grids by the method of Mazia et al. (1975). Occasionally the whole cells were attached to the grids and the extractions were performed by incubation of the grids in a drop of the appropriate buffer in a petri dish kept on ice and blotting with filter paper to remove each solution. The grids were then stained with 0.5% uranyl acetate, blotted and allowed to air dry. Grids were examined with a Phillips 300 electron microscope.

2. S1 Staining of Cytoskeletons

Cells of all three organisms, and purified rat F-actin were each attached to grids in the same manner as for negative staining above. Grids with adhering cytoskeletons or actin filaments were then rinsed 3 times in standard salt solution (6 mM potassium phosphate buffer, pH 6.0, 5 mM $MgCl_2$, 0.1 M KCl). Grids were then incubated for 30 minutes with a 2 mg/ml S1 solution. As controls, duplicate grids were incubated with standard salts solution or alternatively with S1 and 5 mM ATP. The grids were then washed once with bacitracin solution (0.1 mg/ml) or with bacitracin-ATP solution (0.1 mg/ml bacitracin and 5 mM ATP) for ATP controls. All grids were then stained with uranyl acetate as for negative staining above.

3. Embedding of Cytoskeletons

Pellets of cytoskeletons from all three organisms were each fixed overnight in 2% glutaraldehyde and 0.2% tannic acid and then washed three times (10 minutes each) in PBS. Pellets were then fixed overnight in 2% OsO_4 in PBS and subsequently washed once (10 minutes) with PBS. Samples were dehydrated by passing through 25%, 50%, 75% ethanol (14 minutes each) and then through 95%, 95%, absolute, absolute, absolute (20 minutes each) and finally embedded in Spurr's medium (medium hardness). Sectioned blocks were then examined as for negative staining.

4. Glycerination and S1 Staining of Whole Cells

Cells of O. danica and O. malhamensis were glycerinated and reacted with S1 according to the method of Pollard et al. (1970). Control samples were incubated with S1-ATP (5 mM ATP) or glycerination buffer (0.1 M KCl, 0.005 M $MgCl_2$, 0.006 M sodium phosphate, 5% glycerol). Samples were then fixed, embedded sectioned and examined as described for embedded cytoskeletons.

5. Scanning Electron Microscopy of Cytoskeletons

Cells of O. danica, O. malhamensis, and D. discoideum were attached to glass coverslips as above and were detergent-extracted as described for cells on grids. After the coverslips were washed in LSW Buffer, they were incubated in 3% glutaraldehyde in PBS for 45 minutes and washed three times in PBS. The coverslips were then incubated for 45 minutes in 1% OsO_4 and washed three times in distilled water. Samples were dehydrated in 50% and 75% acetone (overnight) and then rinsed three times in 100% acetone (10 minutes each). Samples were critical point dried, gold coated and examined on a JOEL-JSM-2 or a Cambridge-S4 scanning electron microscope.

G. Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed on whole cells and Triton-extracted cells of all three organisms by the method of Laemmli (1970). Results were not satisfactory so several variations in sample preparation were tried. They include the following:

1. Solubilization of cytoskeletons in a range of NaCl concentrations up to 1 M.

2. Solubilization of cytoskeletons in Laemmli sample buffer whose pH was gradually lowered by addition of HCl until SDS precipitated.

3. Solubilization in 4 M urea.

4. Use of the alkaline Piccioni (1982) sample buffer designed for plants with the following composition: 60 mM Na_2CO_3, 60 mM DTT, 2% SDS, 12% sucrose and 0.04% bromphenol blue. Samples could also be prepared by resuspending the sample in three parts Piccioni Solution A (0.1 M Na_2CO_3, 0.1 M DTT) for storage and then addition of two parts Piccioni Solution B (5% SDS, 30% sucrose, 0.01% bromphenol blue) immediately before use.

5. Extraction of samples with ethyl ether, 2:1 chloroform:methanol or acetone.

6. Sonication of samples in sample buffer.

7. Treatment of samples with DNAase and RNAase as per Hari (1981).

8. Use of antiproteolytic agents: PMSF (2 mM), benzamidine (2 mM), 1,10-orthophenanthroline (0.1%) and N-ethylmaleimide (0.0054%)

during the collection and extraction of cells.

9. Use of the antiphenolase agents: sodium tetraborate (0.2 M), sodium ascorbate (0.25 M), sodium bisulfite (0.02M) and sodium diethyldithiocarbamate (0.02 M) during the collection and extraction of cells.

Procedures 5-8 were used in connection with procedure 4. Samples were aliquoted and stored at -20°C until use. Immediately before use they were boiled for 2 minutes and centrifuged at 11,000 rpm for 20 minutes at room temperature. Ten percent polyacrylamide or 10-20% gradient polyacrylamide gels were run in a Bio Rad Protean Cell and were then stained in Coomassie Blue [Diezel et al. (1972)] or Fast Green [Bertolini et al. (1976)]. Gels were scanned using a Hoeffer Model CS300 Scanning Densitomer, photographed with Kodak Technical pan film using a Kodak #25 Wratten filter, and developed with D-19 developer.

H. Purification of O. danica Actin

Purification of O. danica actin was attempted by the method of Sikora and Marzluf (1982), (Figure 1). Column samples were monitored for actin using the DNAase I-methyl green assay of Sikora and Marzluf (1982) or the DNAase I-hyperchromicity assay of Blikstad (1978). Protein concentration was determined using the Bradford method (1976) and KCl concentration was followed using conductivity measurements. Column samples were perpared for electrophoresis by desalting on a Sephadex G-25 column (Pharmacia PD-10) and adding an equal volume of 2X-concentrated Piccioni sample buffer. Alternatively samples were

desalted and concentrated simultaneously using Amicon's Centricon protein concentration cone and resuspending in Piccioni's Sample Solution A for storage.

I. Electroblotting and Immunoprobing

SDS-polyacrylamide gels were blotted onto nitrocellulose (Bio Rad) using the method of Towbin et al. (1979). Blots were transferred from overnight to 2 days at 40 mV and then for 3 hours at 60 mV using a Bio Rad Transblot apparatus. The blots were then air-dried and stained with amido black [Towbin et al. (1979)]. After photography, blots were destained overnight in Tween-20 in 0.02 M Tris, pH 7.5, 3% NaCl, and 0.02% Thimerosal (TTBS) at 50°C. Blots were then washed in TBS (0.02 M Tris, pH 7.5, 3% NaCl) for thirty minutes and then the background was blocked using 2% goat serum in TBS. Blots were incubated with primary serum 1:100-1:5000 for 30 minutes at 25°C. The Vectastain Avidin-Biotin System (Vector Labs) was used to visualize the location of the primary antibody. The horseradish peroxidase, glucose oxidase, and alkaline phosphatase systems were used. All solutions were made at recommended concentrations in TTBS-2% goat serum except the avidin-biotin complex which was diluted in TTBS and blots were washed 6 times (15 minutes each) in TTBS between each solution used. Substrates consisted of 4-chloro-1-napthol for horseradish peroxidase, Vectastain substrate kits I-III for glucose oxidase and alkaline phosphatase. The problem of nonspecific activity was addressed in several ways:

1. The primary antibody was diluted to a range of concentrations from 1:100-1:5,000.

2. Blots were blocked with avidin and biotin prior to probing (Vector Labs Kit).

3. Blots were blocked with 0.01% poly-L-lysine in TTBS and 2% goat serum.

4. Blots were blocked with 0.1 M maltose, 0.1 M N-acetylglucosamine in TTBS and 2% goat serum.

5. Avidin-biotin complex was prepared in 0.1 M sodium carbonate buffer, pH 9.5.

6. Avidin-biotin complex was prepared in 5% skim milk powder in TTBS.

7. Avidin-biotin complex was incubated with D. discoideum cytoskeletons for 30 minutes at room temperature and then centrifuged for 30 minutes at 11,000 rpm before use.

After completion of the substrate reaction, blots were rinsed in distilled water, air dried and stored in the dark.

III RESULTS

A. Immunofluorescence

Immunofluorescence microscopy was performed in the hope of finding evidence for the presence of actin in these species of Ochromonas and, if found, to localize it within the cell. The polyclonal antibody used was pooled unadsorbed rabbit serum prepared from rabbits injected with chicken muscle actin purified to homogeneity (Miles). This antibody was known to react specifically with stress fibers in cultured chicken, mouse and human fibroblasts and with muscle actin but there was no prior evidence to indicate that it was crossreactive with the actin of lower eukaryotes. The problem of autofluorescence due to the photosynthetic pigments of the chloroplast, was overcome by extracting most of the photosynthetic pigments with acetone and then filtering the residual gray fluorescence with Kodak Wratten filters #58 and 99.

Dictyostelium. discoideum was used as a positive control since it is a lower eukaryote which contains large amounts of actin. While the antibody was not known to cross react with D. discoideum actin, such a test provided valuable information about the degree of crossreactivity of the antibody. A negative result with Dictyostelium would have necessitated the use of skeletal muscle sections as positive controls and might indicate that this antibody is generally not crossreactive with lower eukaryotic actins. The staining of D. discoideum with this antibody (Figure 2) resulted in intense, diffuse staining that was not localized in any particular area of the cell, as is typical for unspread, suspended cells. The staining was almost completely

eliminated when anti-actin serum was preabsorbed with muscle actin filaments. Elimination of the antiactin in the staining protocol also prevented staining of D. discoideum. These results indicate that the antiserum does crossreact with the nonmuscle actin of at least some lower eukaryotic species and can, therefore, be used as a positive control for comparison with the Ochromonas species.

Both O. danica and O. malhamensis showed diffuse staining of the cytoplasm but not of the central vacuole (Figures 3 and 4). The intensity of staining was darker for O. malhamensis but still did not approach that of D. discoideum. The flagella did not stain and both controls for each organism were negative. Thus actin does appear to be present in both of the species of Ochromonas studied although in much smaller amounts than that present in Dictyostelium. Of the two Ochromonas species studied, O. malhamensis probably contains more actin because of the greater intensity of staining under identical conditions of antibody concentration, washing, and photography. In addition the actin is not localized in any particular area of the cell (i.e. it is present wherever there is cytoplasm) under the conditions used. It still remains to be seen whether or not the actin becomes localized if the cell becomes engaged in cellular functions requiring an active cell surface such as phagocytosis, exocytosis or mating. A sudden localization of actin filaments occurring in response to mating has been observed in Chlamydomonas [Detmers et al. (1985)].

The very low background fluorescence of the controls indicates that the use of acetone extraction and Kodak Wratten filters removes almost all of the autofluorescence of these photosynthetic cells. Any nonspecific binding of the serum is apparently adequately controlled by

preabsorption of goat serum prior to incubation with the antiserum. The significant positive signal indicates that much, if not all of the actin, originally present in the cells remains after the extraction procedure. This procedure therefore makes possible the use of immunofluorescence in a traditionally difficult photosynthetic system.

B. NBD-Phallacidin Staining

Microfilaments are seldom observed in published electron micrographs of Ochromonas and when they are found, they are usually in the tail region. The specific binding of NBD-phallacidin was used to determine whether any of the actin observed by immunofluorescence was filamentous and to determine its location in the cell should any F-actin prove to be present.

Dictyostelium. discoideum was used as a positive control and produced the diffuse staining pattern typical of unspread cells. This staining was absent if NBD-phallicidin was omitted from the protocol. Neither species of Ochromonas showed any staining at all with NBD-phallicidin indicating that the actin in these cells is present in nonfilamentous form or that so little F-actin is present that it is below the detection limit of this assay. The likelihood that the cell's F-actin would, for some reason, be inaccessible to the probe is small because the G-actin of the cell was accessible to the larger antibody probe used for immunofluorescence under similar staining conditions. The possibility that some of the actin may be in filamentous form but coated with some other protein or in some way protected from access to

the probe cannot be ruled out. Protease treatment of fixed, acetone-extracted cells could make protein coated F-actin accessible to NBD-phallicidin if any were present.

C. Electron Microscopy

1. Negative Staining of Cytoskeletons

In order to obtain a close-up view of the cytoskeleton of Ochromonas, negatively stained preparations of cold Triton X-100-extracted cells were observed by transmission electron microscopy (TEM). The largely actin cytoskeleton of D. discoideum was used for comparison.

Negative staining of cytoskeletonss of D. discoideum showed an organized cytoskeleton consisting of a network of actin cables (Figure 5). In some cases the edges of the cytoskeleton were teased apart from the main cytoskeleton, revealing fine filaments about 7 nm in size. Ochromonas danica and O. malhamensis (Figures 6 and 7) show a more intricate cytoskeleton which appears to be a closely spaced network of different types of filaments. The basic shape of the cell is maintained and there are spaces where the vacuoles would be located. Although cold treatment would be expected to depolymerize tubulin, flagella stripped of their membranes and some microtubules are quite apparent but the microtubules do not make up the entire cytoskeletal network. In

addition, mastigonemes (flagellar projections used to increase the surface area of the flagellum), are common. Filaments teased from their networks are either long and straight or highly branched and they appear to range in size from 3 - 30 nm. Some of the larger filaments may be complex cables. Thus the cytoskeletons of these chrysomonads are more comples than that of Dictyostelium. In view of the complexity of filament types, the cytoskeletons of these cells may be expected to contain more than one major protein component.

2. S1 Staining of Cytoskeletons

The specific binding of S1 to F-actin was used in an effort to determine whether or not any of the filaments observed by negative staining were actually F-actin. The arrowhead pattern characteristic of F-actin binding of S1 was observed for purified rat muscle actin filaments and was abolished by ATP. The cytoskeletons of D. discoideum, O. danica and O. malhamensis produce a very complex pattern that could not be resolved into arrowheads and so the results are questionable. The extra handling of the samples required for this process evidently breaks off most of the nicely teased out portions of the cytoskeleton, as few, if any, of these filaments are seen in the above preparations as are seen in plain negatively stained preparations. The thick three-dimensional matrix of the cytoskeletons must be broken apart in some manner in order to expose single filaments so that the arrowhead pattern can be distinguished. The procedure called dry

cleaving, which uses adhesive tape to pull apart critical point dryed cells on electron microscopy grids [Traas (1984); Mesland (1981)], could prove useful in this regard. An alternative approach would be to use a probe such as colloidal gold conjugated to an antibody to produce a pattern that is easier to distinguish than arrowheads would be.

3. Embedding of Cytoskeletons

In an effort to clarify the complex pattern of filaments observed by negative staining, cytoskeletons were embedded and sectioned. It was hoped that this might expose single filaments that could more easily be measured and S1 labeled.

The overall shape of the cells were maintained in the embedded cytoskeletons, as were the spaces indicating the position of the vacuoles, just as they were in the negatively stained preparations. Some filamentous structures were evident in the crysomonads' cytoskeletons but they appeared fuzzy and poorly preserved. Their location and length indicates that they might be flagellar microtubules. No structure or filaments were resolvable within the dense cellular matrix which itself was very fuzzy and appeared poorly preserved (Figure 8).

Problems with the preservation of cytoskeletal elements have been previously observed and have been attributed to OsO_4 fixation and dehydration [Boyles and Bainton (1979); Maupin-Szamier and Pollard, (1976); Pollard and Maupin (1982); Small and Langanger

(1981)]. Attempts have been made to protect cytoskeletal elements from the deliterious effects of fixation and dehydration by coating with tannic acid. Tannic acid results in a thickening of filaments which may hide details of interest [Seagull and Heath (1979)] and is often unreliable. More recently the use of amines in the initial aldehyde fixative has been shown to be useful for the preservation of actin filaments [Boyles et al. (1985)]. Such a fixative might also preserve other cytoskeletal elements which would enable the successful embedding of cytoskleletons of the type attempted here. The ability to embed cytoskeletons with adequate preservation would also open the door to labeling studies with S1 and immunoprobes.

4. Glycerination and S1 Staining of Whole Cells

It was hoped that the identification and localization of actin filaments in Ochromonas could be accomplished with the widely accepted method of S1 labeling of glycerinated cells. However, attempts to S1 label microfilaments in glycerinated preparations of whole cells proved unsuccessful. Extremely poor preservation of the chrysomonads was obtained and the the cells were largely fragmented. No filaments or microtubules were evident at all.

To avoid some of the problems observed in the permeabilization of algal cells by glycerination Detmers et al. (1983) have used saponin for this purpose. Such a procedure could prove useful for Ochromonas but would probably need to be modified to deal with the

active proteases and the unusual plasma membrane of these organisms. Studies must first be undertaken to determine which combination of antiproteolytic agents will completely control the active proteases of these organisms. In addition, if Dr. Haines' theory (City College - personal communication) that the plasma membranes of the chrysomonads release protons when perturbed, which may in turn activate proteases, is correct, it will be necessary to develop a buffer system that is sufficiently alkaline to titrate the released protons to neutrality. Another important factor would be the effect of the fixative employed upon the cell's cytoskeletal elements which have already been mentioned above in connection with the embedding of cytoskeletons. A successful procedure would need to address all of these issues.

5. Scanning Electron Microscopy

The cytoskeletons of both species of Ochromonas, in comparison to those of Dictyostelium, were examined by means of scanning electron microscopy (SEM). This was done in order to determine the three-dimensional organization of the cytoskeleton and to verify the results obtained by TEM.

SEM of Dictyostelium cytoskeletons (Figure 9) revealed a lacey network similar to that observed by TEM. The overall shape of the cell was maintained although it was frequently flattened or collapsed. Microfilaments or microtubules were not resolved but patches of membrane were clearly evident adhering to the cytoskeletons.

The cytoskeletons of both species of Ochromonas consist of a spongy cellular matrix (Figure 9) that is much denser than that of Dictyostelium. Like the cytoskeleton of D. discoideum, that of the chrysomonads maintains the overall shape of the cell, is easisly collapsed under the conditions employed, and has patches of plasma membrane on the cell surface. These results are consistant with those obtained by TEM. Furthermore, these results explain the difficulties encountered in attempting to S1 label negatively stained preparations because such a dense meshwork in an unspread cell would be expected to yield a complex pattern that would preclude the resolution of arrowheads.

D. Electrophoresis

SDS-polyacrylamide gel electrophoresis has emerged as a powerful technique for the localization and quantitation of proteins of a given molecular weight in complex biochemical mixtures but good results are seldom obtained with photosynthetic plant tissue. Attempts to utilize this technique to scan the chrysomonad cells for a protein comigrating with muscle actin and to obtain information about the number, relative amounts and, molecular weights of the proteins making up the cytoskeletons of these cells were unsuccessful at first.

Initial attempts at electrophoresis yielded extremely smeared bands for both O. danica and O. malhamensis although D. discoideum yielded acceptable results. In addition, the cytoskeletons of the chrysomonads were highly insoluble and resisted solubilizaton in salt, low pH, and

4 M urea. Sonication to break up pellets was of no benefit either. Attempts to extract the yellowish pigments in the cytoskeletal preparations of the chrysomonads, which might interfere with the solubilization and migration of proteins, resulted in such severe denaturation of the proteins that they became even less soluble. A major improvement in the resolution of the gels was obtained by using the alkaline buffer system of Piccioni et al. (1982), although the electrophoresis pattern deteriorated upon storage of the samples. Treatment with DNAase and RNAase to remove large nucleic acid molecules that could interfere with migration was of no benefit. The introduction of the antiproteolytic agents PMSF, benzamidine and 1,10-orthophenanthroline (Figures 10 and 11) enhanced resolution significantly This indicates that at least part of the problem was due to the generation of a large number of protein fragments of different molecular weights, which complicated the gel pattern. However, deterioration upon storage in complete Piccioni buffer at -20°C was still evident.

After some time it became evident that the best resolution obtainable was produced when the antiproteolytic agents mentioned were used during the initial isolation of the sample and when the sample was subsequently resusupended and stored in Piccioni Sample Buffer A. In addition the samples had to be run as soon as possible after thawing and addition of Piccioni Sample Buffer B. Evidently the samples deterioriated the fastest in the presence of SDS and did so even at -20°C. Deterioration was slowed considerably if SDS was not added before storage.

The phenolases present in plant tissue have been implicated as a causative factor of band smearing in electrophoresis [Kelly and Adams

(1977)] and so a number of antiphenolase agents, including sodium tetraborate, sodium bisulfite, sodium ascorbate and sodium diethyldithiocarbamate were tried in the hopes of improving the banding pattern. No major enhancement of banding was observed with any of these agents.

Electrophoresis of both whole cells and Triton-extracted cells of D. discoideum showed an extremely prominent band at 42 kD which comigrated with purified rat muscle actin. In fact the densitometer tracing indicates that actin is the major protein of the cell (over 10%) (Figure 12). The cytoskeletons of D. discoideum yielded complex electrophoretic patterns but certain prominant bands appear to be enhanced as compared to the whole cell. These include the 95.5, 68, 40 and 30 kD bands.

The cytoskeletons of O. danica also yielded a complex electrophoretic pattern (Figure 13). The 42 kD band comigrating with rat muscle actin was a minor component of the cell and did not appear enhanced in the cytoskeleton. The 111, 66, 40, and 30 kD proteins, on the other hand were enhanced by Triton extraction as was a low molecular weight protein triplet. The densitometer trace of the whole cell indicated that tubulin is the major protein of these cells.

The results for O. malhamensis were similar to those of O. danica with tubulin as the major protein and the 42 kD protein was a minor cellular component that was not enriched by Triton extraction. The proteins enriched by Triton extraction consisted of the 95.5, 68, 41, 30 kD proteins and the low molecular weight protein triplet (14, 15 and 17 kD) (Figure 14). Ochromonas malhamensis differs from O. danica in that its high molecular weight major cytoskeletal protein is somewhat smaller

than that of O. danica (95.5 kD compared to 111 kD) and the amount of the 42 kD protein present is greater.

Thus all three organisms have a set of four non-actin and non-tubulin proteins of similar size, that are enriched when the cells are extracted in cold Triton X-100. How similar in structure and function these proteins actually are cannot be gauged at this time and nothing is known about their role in the cell. They may be traditional cytoskeletal components such as intermediate filament proteins, or they may instead be part of the nuclear matrix or even something new. Certainly the complex electrophoretic pattern of the cytoskeletons of all of these organisms indicates that many cellular components are not extracted. The scanning electron micrographs indicate that patches of plasma membrane are still present after extraction. One may also expect polyribosomes to resist extraction [Lenk et al. (1977); Fulton et al. (1980); Cervera et al. (1981)]. Determinations of the identity and function of the major proteins must therefore await purification, characterization and localization with specific immunoprobes.

The major difference between the cytoskeletons of Dictyostelium and Ochromonas is that the major protein of Dictyostelium is actin while that of Ochromonas is tubulin. Note that the flagellar tubulin of Ochromonas is not cold sensitive so that it is not extracted by treatment with cold Triton X-100 and tubulin is still the major protein present in the cytoskeleton after cold extraction. While cold extraction is more efficient for Dictyostelium tubulin, electrophoresis of the cytoskeletons indicates that a considerable amount of tubulin is left behind.

Another significant difference between the electrophoretic pattern of Dictyostelium and Ochromonas is the presence of the intense low molecular weight triplet. It is very likely that these low molecular weight proteins are proteolytic fragments of higher molecular weight cytoskeletal components, as they do not appear to be present in appreciable amounts in whole cells. Although the proteolytic inhibitors PMSF, benzamidine and 1,10-orthophenanthroline were successfully used to enhance banding, indicating that serine and metalloenzyme proteases are present, the continued deterioration of the samples stored in SDS and the broadening of the tubulin peak in the cytoskeletal preparations indicates that not all of the proteases present were controlled by the proteolytic inhibitors used. In order to completely rule out proteolysis, complex sample preparation methods such as those suggested by Weber et al. (1973) utilizing guanidine hydrochloride or performic acid to completely denature all proteins, may need to be used. Further studies must also be undertaken to elucidate the nature of the proteases involved so that reagents may be found to control them during the Triton X-100 extraction procedure as the major uncontrolled proteolytic effect seems to be occurring at this stage.

E. Purification of O. danica Actin

Attempts to prepare O. danica actin by the standard method utilizing cycles of polymerization and depolymerization were unsuccessful, probably due to the presence of active proteases and only small amounts of actin which could have different polymerizaton

characteristics or regulators than muscle actin. The more traditional biochemical method of Sikora and Marzaluf (1982) utilizing ion-exchange and affinity chromatography, was therefore selected.

DEAE-cellulose chromatography resulted in the elution of a 42 kD protein over a KCl concentration range of 0.1 - 0.35 M which is typical for actin (samples 33-43) (Figure 15). Electrophoresis of the DEAE-cellulose chromatograpy samples indicated enrichment of a 42 kD protein in this range (Figure 16). DNAase I inhibition occurred at three points in the elution profile: towards the end of sample loading, at samples 32-40 and at samples 45-53 where essentially no protein eluted. This last peak is probably due to the elution of DNA.

Thus a 42 kD protein comigrating with muscle actin, as shown by the actin-column sample mixing studies (Figure 16b) elutes from a DEAE-cellulose column at a KCl concentration consistent with muscle actin. Furthermore, DNAase I inhibition, as assayed by both the methyl green method of Sikora and Marzaluf (1982) and, the hyperchromicity method of Blikstad et al. (1978), follow the elution pattern of this 42 kD protein. Other factors such as proteases could possibly result in DNAase I inhibition and would require a protease that has charge characteristics similar to those of actin in order to elute as one would explect actin to elute. Even if the DNAase I inhibition observed was due to protein binding rather than to proteolysis it could be caused by a protein other than actin. In Physarum, at least four functionally distinct 42 kD actin binding proteins have been observed that closely resemble the structure of actin but are themselves incapable of polymerization [Maruta et al. (1984)]. These proteins regulate actin polymerization and one of them shows a strong affinity for DNAase I.

Proof that the 42 kD protein of Ochromonas is actually actin must therfore await purification and characterization of this protein.

Attempts to further purify the actin by affinity chromatography on DNAase I-sepharose were not successful and no protein was found to elute from the column indicating excessive dilution, proteolysis, phenolase activity, or a defective DNAase I column. Future studies will require:

1. Characterization of the DNAase I column with purified muscle actin.
2. The incorporation of more proteolytic inhibitors as the proteases of these organisms become better understood.
3. The incorporation of antiphenolase agents.
4. The introduction of more efficient means of protein concentration.

F. Electroblotting and Immunoprobing

Electroblotting and immunoprobing are invaluable techniques for judging the structural relatedness of different proteins. While techniques employing antibodies can have many problems as indicated in the introduction, it was hoped that electroblots could provide insight into the identity of the 42 kD Ochromonas protein, particularly in light of the positive results obtained by immunofluorescence. It was also hoped that the Pruss antibody (1981), which reacts with the intermediate filament core common to all intermediate filament proteins, could be used to determine whether or not any of the major cytoskeletal proteins are intermediate filament-related.

Attempts to immunoprobe electroblots have been unsuccessful due to the binding of the avidin-biotin complex to a doublet band around 60 kD for all three organisms. None of the methods used in an attempt to block this binding were effective, indicating that this 60 kD protein was not an avidin- or biotin-binding protein. Nor was it related to endogenous activity of the visualization enzyme as different enzymes all yielded the same results. The other blocking measures employed were designed to interfere with lectin binding of the glycoprotein avidin or with nonspecific charge interactions. Further studies will have to be undertaken to determine whether or not this nonspecific reaction interferes with the specific antibody binding desired. If it does not, then this effect could be ignored. Another approach to this problem could be to treat the blot with the avidin-biotin complex of one enzyme and then to immunoprobe using the avidin-biotin complex of another enzyme for visualization. Still another approach would be to use an entirely different visualization system such as I^{125}-protein A and autoradiography.

IV DISCUSSION

The chrysomonad phytoflagellates, O. danica and O. malhamensis have a complex asymmetrical shape and an extremely active cell surface. The role of microtubules in the maintenance of cellular shape in these organisms has been studied [Bouck and Brown (1973); Schnepf et al. (1977)] and is fairly well understood. The methods used by these phytoflagellates to maintain such an active cell surface are not understood and in fact are poorly understood in most protozoa. The large number of activities these cells must engage in requires that they have a complex cytoskeleton that is responsive to external stimuli.

Cell surface activities have traditionally been associated with actin and so it is logical to expect to find some form of actin (which could possibly be modified) in these phytoflagellates. Evidence supporting the theory that actin is present in the chrysomonads studied has been obtained in the form of positive anti-actin immunofluorescence data and the elution of a 42 kD protein from a DEAE-cellulose column at a KCl concentration consistant with actin. In addition, a DNAase I inhibition activity elutes in the same fractions in which this 42 kD protein elutes.

Actin has been difficult to demonstrate in a number of protozoa for several reasons. One reason is that actin is often a minor component of protozoal cells and is often highly regulated so that it may not be polymerized at all times [Detmers et al. (1985)]. Alternatively the actin may be modified in some way and therefore have slightly different properties. Still another barrier to the demonstration of actin is the presence of active proteases in protozoa which will degrade proteins

before one has the opportunity to analyze them. Analysis is made still more difficult by the presence of photosynthetic pigments and phenolases in the photsynthetic species. Almost all of these barriers to the demonstration of actin in cells apply to the two species of Ochromonas studied in this thesis.

The densitometer tracings of the SDS polyacrylamide gels of these organisms clearly indicates that tubulin is the major protein in these cells and the 42 kD actin candidate is a very minor component. Furthermore, it is not enriched in the Triton-extracted cells, indicating that most of it is probably unpolymerized. This appears to be verified by the negative results of the NBD-phallacidin staining. While such negative NBD-phallacidin staining results could also be explained by the presence of actin filaments coated with another protein [Buckley et al. (1978)] and therefore inaccessible to the stain, one would expect those filaments to be even more inaccessible to a large antibody molecule. Yet in the case of the chrysomonads the immunofluorescence staining was positive although weaker than for D. discoidium, whose major protein is actin, suggesting that these two phytoflagellates have much less actin and what little there is exists largely in unpolymerized form. If any actin filaments are present in the chrysomonads, they must be very few in number. While the evidence presented suggests the presence of actin in the two species of Ochromonas studied it is by no means adequate proof. Methods will have to be developed which will allow purification and characterization of the 42 kD protein, in order to be sure of the presence of actin in these species of Ochromonas.

The Ochromonas species studied have very active proteases which were the major barrier to purification of actin. Proteases are a

complex, varied group of enzymes whose functions stretch far beyond digestion; it has been demonstrated that limited proteolysis has a key role in the regulation of a wide range of cellular processes [Holzer and Heinrich (1980)]. The calcium-activated protease found in association with intermediate filaments may be an example of such a regulatory role for a protease. As the proteolytic activity of Ochromonas is partially inhibited by PMSF, benzamidine and 1,10-orthophenanthroline, some of the proteases of Ochromonas must be metalloenzymes and some must be of the serine type [North (1982)]. Ochromonas also evidently contains proteases that are not inhibited by the above inhibitors but are stimulated by SDS. Another interesting property of Ochromonas which may play a role in the activity of their proteases is the highly acidic membrane they possess which drastically lowers the pH of the surrounding medium when perturbed (Dr. Thomas Haines - City College of CUNY - personal communication). Many proteases are activated by low pH [North (1982)] so this effect may be important for controlling the activity of proteases. It is interesting to note that both chrysomonads and D. discoideum show enrichment of four similar non-actin, non-tubulin proteins upon Triton X-100 extraction. The low molecular weight triplet appears only in the two species of Ochromonas and is greatly enriched in the Triton X-100 extracted cells. Whether or not they are significant unique components of the cytoskeleton or are proteolysis products remains to be determined.

A survey of the literature shows relatively few electrophoretic experiments performed on photosynthetic cells. This is largely due to the adverse effects photsynthetic pigments have on electrophoretic mobility as well as the general presence of active proteases and

phenolases. The difficulty encountered in performing electrophoresis experiments on the two species of Ochromonas studied is, in part, due to these factors. Another problem in doing electrophoresis on these organisms is the extreme insolubility of at least some of the cytoskeletal proteins. Such insolubility in nonalkaline buffers is typical for IFs and may indicate the presence of IF-like proteins in the cytoskeletons of these two species of Ochromonas. The electron microscopic data clearly indicate the presence of a complex mesh-like cytoskeleton consisting of filaments of different sizes. The known distribution of tubulin and the small amount of actin demonstrated cannot account for the structure and the size of some of the filaments seen and the highly branched nature of some of them is not inconsistant with the presence of IFs. It is, however, becoming increasingly clear that protozoa have unusual cytoskeletal proteins and the possibility that the complex cytoskeleton of Ochromonas has such unknown proteins cannot be ruled out. It is also clear from the pieces of plasma membrane observed in the scanning electron and the complex electrophoresis pattern that non-cytoskeletal elements are attached to the cytoskeletons of these cells. Study of such attached cellular components may eventually lead to an understanding of how the cytoskeleton may mediate cellular processes.

In conclusion, the major contribution of this thesis has been to develop methods by which the cytoskeleton of these difficult to handle species of phytoflagellate may be analyzed electrophoretically. Evidence suggesting the presence of actin in these phytoflagellates has been presented but because of the problems encountered in working with these systems, absolute proof of the presence of actin in these

phytoflagellates has not been possible. The methods developed in this thesis are a starting point for learning how to manipulate phytoflagellates. Much work must still be done before this system can be worked with as easily as the more popular ameboid systems. The most important problem requiring solution is the control of the proteases. The proteases of these organisms affect the use of detergent extraction, electrophoresis, protein purification, and electron microscopy for the study of their cytosleletons. Other methods that need to be developed are procedures for the adequate fixation and display of cytoskeletal elements for electron microscopy. Fine-tuning of the actin purification procedure is also necessary so that the 42 kD protein can be purified, characterized and its identity proven. The development of such procedures would also facilitate the purification of the other major cytoskeletal proteins.

Another important accomplishment of this thesis is the demonstration of the complex, fibrous nature of the cytoskeletons of these species of Ochromonas. The results of the chromatography, immunoflourescence and the electrophoretic studies suggest that the 42 kD actin candidate protein is only a minor component of the cytoskeleton. Although proteolytic digestion of most of the 42 kD protein cannot be entirely ruled out, it is unlikely because the sharper tubulin peak observed in the electrophoresis of whole cells indicates that proteolysis is much reduced in such preparations, yet the 42 kD protein is not a major cellular constituent. The possibility still exists that the weaker immunofluorescence signal observed in the chrysomonads compared to the slime mold could result from a lower antibody affinity for the chrysomonad actin rather than from a lower

concentration of actin. Such an effect would be inconsistent with the electrophoresis and column chromatography data, however, while the cytoskeletons of these organisms have been shown to be complex and fibrous in nature, actin is only a minor component and is probably mainly present in unpolymerized form. This opens the question of what the cytoskeleton _is_ made of. The most likely candidate, consistent with the data obtained so far, would be an intermediate filament-like protein. There appear to be different ways of using cytoskeletal proteins; moreover IF proteins have been found in _Tetrahymena_ [Numata et al. (1982)]. The possibility that entirely new cytoskeletal proteins may be discovered cannot be ruled out.

Table 1 - MICROTUBULE-ASSOCIATED PROTEINS

PROTEIN	SUBUNIT MW	FUNCTION	SOURCE
Tubulin	55,000 53,000	Forms backbone of microtubule	Widespread, enriched in brain
MAP_1	340,000	A HMW-MAP, it may be similar to ankyrin	Brain
MAP_2	270,000	A HMW-MAP, forms lateral arm projections on microtubules and promotes assembly, interacts with neurofilaments	Brain
Tau	55,000- 62,000	Multiple polypeptides, promote nucleation and elongation	Brain
Dynein	400,000 85,000 small	ATPase, sliding of microtubules	Cilia, flagella

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TABLE 2 - MICROFILAMENT-ASSOCIATED PROTEINS

PROTEIN	SUBUNIT	SOURCE	FUNCTION	REF
Actin	43,000	Muscle and nonmuscle cells	Major component of microfilaments	1,2
Myosin	200,000	Muscle and nonmuscle cells	Contraction, ATPase activity	1,2
	18,000-20,000			
	15,000-17,000			
MONOMER-BINDING PROTEINS				
Profilin	16,000	Spleen, brain, thymus	Forms complex with G-actin, Calcium dependent	3-5
Fragmin	43,000	Physarum	Forms complex with G-actin, Calcium dependent	6,7
DNAase I		Extracellular	Function unknown	8
BARBED (FAST ASSEMBLY) END-BINDING PROTEINS				
Gelsolin	91,000	Macrophage	Calcium dependent	9-11
Villin	95,000	Microvilli	Calcium dependent	12-17
Capping protein	90,000	Platelets	Calcium dependent	8
Capping protein	31,000	Acanthamoeba	Calcium independent	18
	29,000			
Capping protein	65,000	Platelets	Calcium independent	19
POINTED (SLOW ASSEMBLY) END-BINDING PROTEINS				
Brevin	90,000	Serum	Shortens filaments without increasing free monomer concentration, calcium insensitive	19,20
Acumentin		Macrophages	Calcium insensitive	21
β-actinin	37,000	Muscle		22
	34,000			
SIDE BINDING PROTEINS				
Tropomyosin	42,000	Smooth and skeletal muscle	Calcium insensitive, stabilizes F-actin and regulates contraction	1,2,23
	37,000			
Severin		Dictyostelium	Calcium sensitive, destabilizes F-actin	8
Troponin	18,000	Skeletal	TnC - Calcium-binding subunit	24
	20,856	muscle	TnI - Troponin-inhibiting subunit	
	37,000-45,000		TnT - Tropomyosin-binding subunit Regulates contraction	
CROSS-LINKING PROTEINS				
α-actinin	100,000	Muscle and nonmuscle cells	May be calcium dependent depending on source	25-30
Spectrin I	240,000	Erythrocytes	Calcium dependent	31-34
Spectrin II	220,000	Other systems	Closely related to spectrin I	35
TW260/240	240,000	Intestinal brush border	Closely related to fodrin	36
Fodrin	240,000	Neural tissue	closely related to TW260/240	37
Vinculin	130,000	Smooth Muscle, cultured cells	Linkage of stress fibers to membrane	38-43
Filamin	250,000	Smooth muscle, macrophage		44-49
Caldesmon	150,000	Smooth muscle	Calmodulin dependent	50
Actin binding	120,000	Dictyostelium		51,52
Actinogelin	115,000	Ehrlich ascites		53
Fimbrin	68,000	Microvilli	Calcium dependent	54-56
Fascin	58,000	Sea urchin egg		57
Gelactin I-IV	23,000 38,000	Acanthamoeba		58

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TABLE 3. INTERMEDIATE FILAMENT-ASSOCIATED PROTEIN

PROTEIN	SUBUNIT	COMMENTS	SOURCE	REF
Keratin	40,000	Enormous tissue diversity, several different polypeptide per cell	Epithelium (keratinizing and non-keratinizing)	1-4
Vimentin	58,000	Different molecular weights reported 52,000; 54,000; 57,000	Mesenchymal cells	1-4
Desmin	55,000		Skeletal, smooth, and cardiac muscle	1-4
Neurofilament Protein	68,000 160,000 210,000	Component of filament core Coassembles with the 68,000 peptide Peripherally bound to neurofilaments	Most neural cells	1-4
Glial fibrillary acidic protein	51,000		Astrocytes	1-4
Synemin	230,000	Associates with desmin in muscle. Highly susceptible to proteolysis. Associates with vimentin in avian erythrocytes	Embryonic and adult chicken muscle Avian erythrocytes	5 6
Paranemin	280,000	Highly susceptible to proteolysis. Associates with vimentin and desmin	Embryonic avian muscle	2-7
IF associated	210,000	May function in muscle differentiation	Rat skeletal muscle	8-9
IF associated	95,000	May function in muscle differentiation	Rat skeletal muscle	8-9
Plectin	300,000	Associates with vimentin 1:20; bundles vimentin filaments in vitro	Glioma cells	10

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Table 4. Evidence for actin in fungi[a]

	Anti-actin immuno-chemistry	Rhodamine-conjugated phalloidin or similar method	Comigration with skeletal muscle actin	KCl solubility	% of total cell or cytoskeletal protein	Evidence for actin gene(s)	DNAse I inhibition	Activation of Mg^{2+} myosin ATPase	3-1 or NBD decoration	Apparent molecular weight (Kd)	Ref
Ascomycetes											
Neurospora crassa	+(2)		+(2)	+(2)	1-2% of total		+(2)		+(1)	42	1,2
Saccharomyces cerevisiae			+(2)	+(2)	(2)	+(3)	+(3)	+(17)	+(3)	42-43(3,17)	2,3,17
Schizosaccharomyces pombe		+(16)									
Basidiomycetes											16
Ustilago sphaeroli		+(16)									
Myxomycetes											
Physarella dictyoides	+(14,15)	+(15)	+(12,14)		8-25% of total						
Physarella mucoroides					(5)	+(11)		+(12)	+(6)	43,46-48(6,14)	5,6,11,12,14,15,16
Physarum polycephalum	+(5)				6% of total(18)						
Plasmodiophorella curvatum						+(7)				42-45(8,9,13)	5,7,8,9 [a]in nucleus 10,13,16
									+(10)		

[a]Myxomycetes may be more closely related to amoeboid protozoa, also called Myxozoans

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Table 5 Evidence for actin in algae

	Anti-actin immunochemistry	Rhodamine-conjugated phalloidin or similar method	Comigration with skeletal muscle actin	EGl solubility	% of total cell or cytoskeletal protein	Evidence for actin gene(s)	DNAse I inhibition	Activation of Mg^{2+} myosin ATPase	S-1 or HMM decoration	Apparent molecular weight (Kd)	Ref	
Chlorophyta												
Acetabularia mediterranea	+(1)										1	
Chara corallina									+(7)		7	
Chlamydomonas sp.*			+(12)				+(12)			A2(12)	12	*only in flagella
Coleochaete scutata									+(5)		5	
Dunaliella viridis	+(6)					+(6)				A2(6)	6	
Ernodesmis verticillata									+(1)		1	
Hydrodictyon sp.									+(4,5)		4,5	
Vitella sp.	+(3)								+(3)		3	actin not isolated
Bacillariophyta												
Amphora coffeaeformis										?43*	10	*could not show actin by variety of techniques
Navicula cuspidata		+(2)									2	
Carrophyta												
Carrageenus canina*	+		+								This lab	minor protein in cytoskeleton
Cyclotella cryptica*	+		+								This lab	
Synura petersenii*									+(8)		8	attached to scale forming vesicle
S. echinulata									+(8)		8	

* These species are also classified among the phytoflagellates in the Protozoa

Pyrrophyta												
Crassodinium glaucum									-(9)		9	
Exuviaella cordata									-(9)		9	
Gymnodinium sanguineum									-(9)		9	
Peridinium westii									-(9)		9	
Prorocentrum micans									-(9)		9	
Euglenophyta										43(12)	12	
Distigma proteus											11	
Euglena sp.	+(12)	+(11)	+(12)									

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Table 6 Evidence for actin in Protozoa

	Anti-actin immuno-chemistry	Rhodamine-conjugated phalloidin or similar method	Comigration with skeletal muscle actin	KCl solubility	% of total cell or cytoskeletal protein	Evidence for actin gene(s)	DNAse I inhibition	Activation of Mg^{2+} myosin ATPase	S-1 or HMM decoration	Apparent molecular weight (Kd)	Ref
Mastigophora											
Giardia lamblia			±(1)							84[a]	1 [a]very little actin
G. duodenalis	+(2)										2
G. muris	+(2)										2
Herpetomonas samuelpessoai	+(4)									42(4)	4
Naegleria gruberi -(23)[a]	+(24)					+(25)	±(23)	+(23)	+(23)	43(23)	3,23 [a]Don't crosss react with 24,25 actin from *Acanthamoeba*, *Dictyostelium* & *Physarum*
Trypanosoma cruzi	+(4)										
Sarcodina											
Acanthamoeba castellani	+22			+(6)	20-30%(7)	+(5)	+(6)	+(22)	+(4)	+42(6)	4,5,6,22
Amoeba proteus	+ (?)										
Chaos carolinensis									+(8)		7,8
Entamoeba histolytica	+(10)	+(9)		+(9a)	15-20% of total cell(11)		-(9a)	+(9a,b)	+(9)	45(9a) 48(9b)	9,10
Ciliata											
Dileptus gellini									+(11)		11
Isotricha prostoma									-(12)		12
Paramecium tetraurelia	+(14)								+(13)		13,14 Mainly phagocytic
Pseudomicrothorax dubius	+(15)										15 organelles (minor)
Spirostomum terse								±(5)			5
Tetrahymena harveyana									+(16)		16
T. pigmentosa						+(5)					5
T. pyriformis			-[a](17,18)								17,18 [a]no actin
T. thermophila											
Sporozoa											
Gregarina garnhami	-(21)		-(20,21)								20,21
G. blaberae			-(20)								20
G. rhynchobia			-(20)								20
Sarcocystis muris										43[a](19)	19 [a]very little of
Sarcocystis tenella			-(20)								20 this protein

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Table 7. INTERMEDIATE FILAMENTS IN LOWER EUKARYOTES

PROTEIN	ORGANISM	FILAMENT DIAMETER	PI	SUBUNIT MW	COMMENTS	REFERENCES
	Amoeba proteus	10 nm			Stable at pH 5.0-9.0. May be linked to microtubules	1
FFP	Tetrahymena pyriformis	14 nm	6.7	38,000	Tetramer of 140,000 MW. Coprecipitates with myosin	2
	Candida					3
Vimentin (like)	Dictyostelium discodium	9 nm		59,000 62,000		4,5
Tektin	Sea-urchin sperm	2-3 nm		47,000 55,000	Limited protolysis shows homology with desmin Solubility similar to IFs. Interacts with tubulin	6,7
	Gregarina	10-12 nm		52,000		8

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Table 8. NON-ACTIN MICROFILAMENTS IN LOWER EUKARYOTES

PROTEIN	ORGANISM	FILAMENT DIAMETER	SUBUNIT MW	PI	COMMENTS	REFERENCES
ENDOPLASMIC RETICULUM-ASSOCIATED MICROFILAMENTS						
Spasmin A (Spastin A)	Vorticellid ciliates: Vorticella, Carchesium Zoothamnium	2-3 nm	18,000 20,000	4.7-4.8	Actually appear to contract No sliding of filaments Spasmin B = Ca^{++} binding Extremely fast contraction	1-3
Spasmin B (Spastin B)			22,000(minor) 16,000 17,000			
MYONEMAL BUNDLES FORMING MICROFILAMENTS						
	Perichous ciliates: Stentor, Spirostomin	4 nm relaxed 10 nm contracted			Entire filament thicken (by coiling) during contraction calcium dependent	4-8
	Axopods: Sticholonche zanclea	2-3 nm			Calcium dependent coils during contraction, attached to microtubules	9
	Dinoflagellates: Leptodiscinae Kofoidirinae Noctilicinae	3 nm 7.5 nm			Contracted filaments show periodicity, calcium dependent	10 11,12
	Basal bodies of flagellates					
	Actinopods: Acantharia				Calcium dependent	13
	Ciliate: Isotricha prostoma	6 nm	58,000 63,000		Soluble in KI	14
	Ciliate: Isotricha porstoma	4-5 nm	11,000 23,000		KCl insoluble Periodic beaded structure	15
	Sporozoa: Gregarines	10 nm	752,000			16
	Flagellates:					
Giardian	Giardia lamblia	2-3 nm	30,100 (Several components)	5.8-6.2	Insoluble in high salt (pH 6.5) Interacts with tubulin	17,18
	Amoeba proteus	3 nm				19
	Euglena gracilis	5-8 nm			Electrophoresis shows no 43-45 kD band	20

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Figure 1. ACTIN ISOLATION PROCEDURE AS PER SIKORA AND MARZLUF (1982)

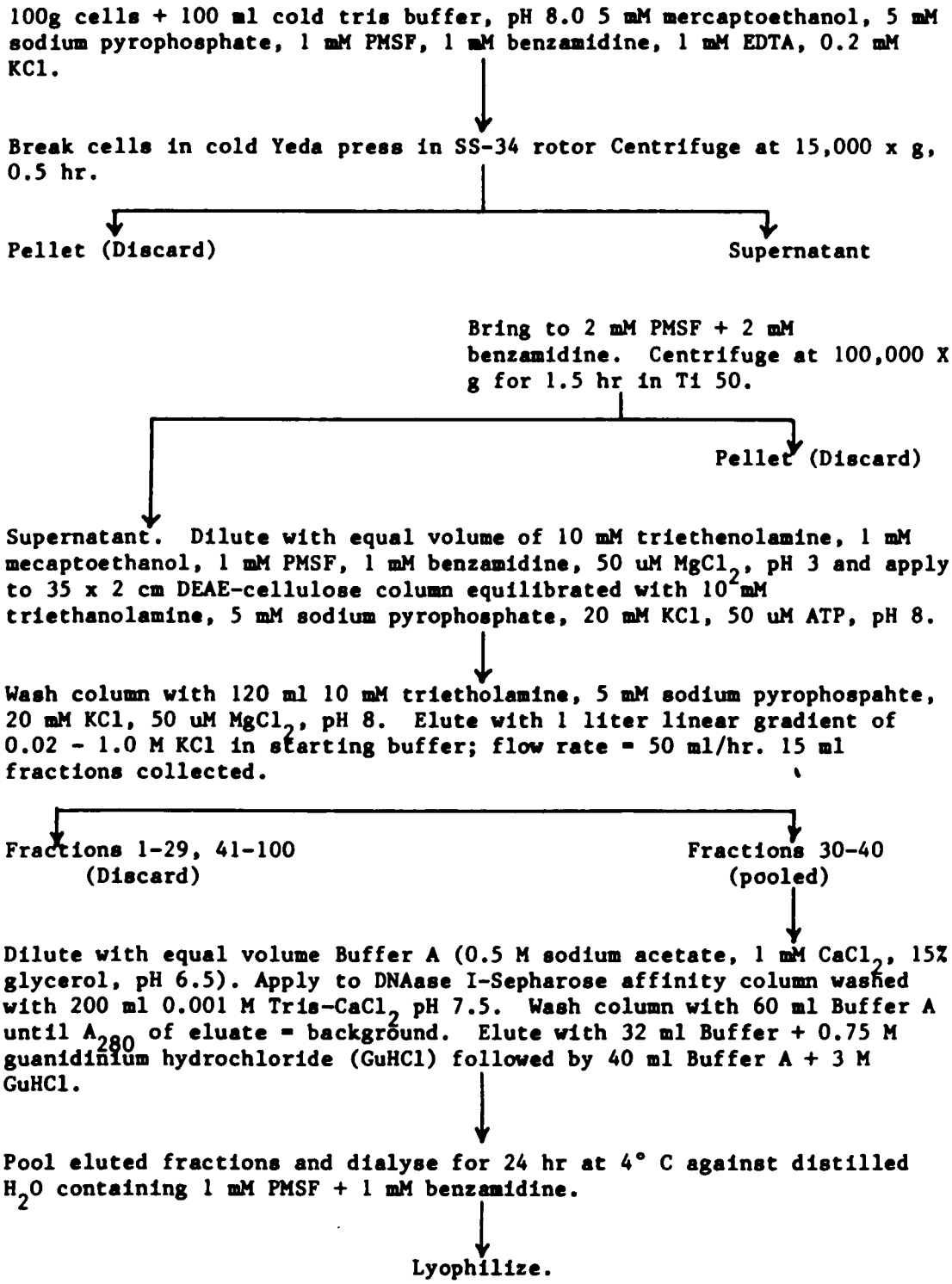


Figure 2. Immunofluorescence of D. discoideum (Magnification 1000X)

A. Phase contrast micrograph of cells treated with rabbit anti-actin serum and visualized with fluorescein-conjugated goat-anti-rabbit antibody.

B. Fluorescence micrograph of cells in A.

C. Control. Phase contrast micrograph of cells treated with rabbit anti-actin from which the anti-actin antibody was precipitated using muscle actin filaments. Cells were vusualized as in A.

D. Fluorescence micrograph of cells in C.

Figure 3. Immunofluorescence of O. danica (Magnification 1000X)

A. Phase contrast micrograph of cells treated with rabbit anti-actin serum and visualized with fluorescein-conjugated goat-anti-rabbit antibody.

B. Fluorescence micrograph of cells in A.

C. Control. Phase contrast micrograph of cells treated with rabbit anti-actin from which the anti-actin antibody was precipitated using muscle actin filaments. Cells were vusualized as in A.

D. Fluorescence micrograph of cells in C.

Figure 4. Immunofluorescence of O. malhamensis (Magnification 1000X)

A. Phase contrast micrograph of cells treated with rabbit anti-actin serum and visualized with fluorescein-conjugated goat-anti-rabbit antibody.

B. Fluorescence micrograph of cells in A.

C. Control. Phase contrast micrograph of cells treated with rabbit anti-actin from which the anti-actin antibody was precipitated using muscle actin filaments. Cells were vusualized as in A.

D. Fluorescence micrograph of cells in C.

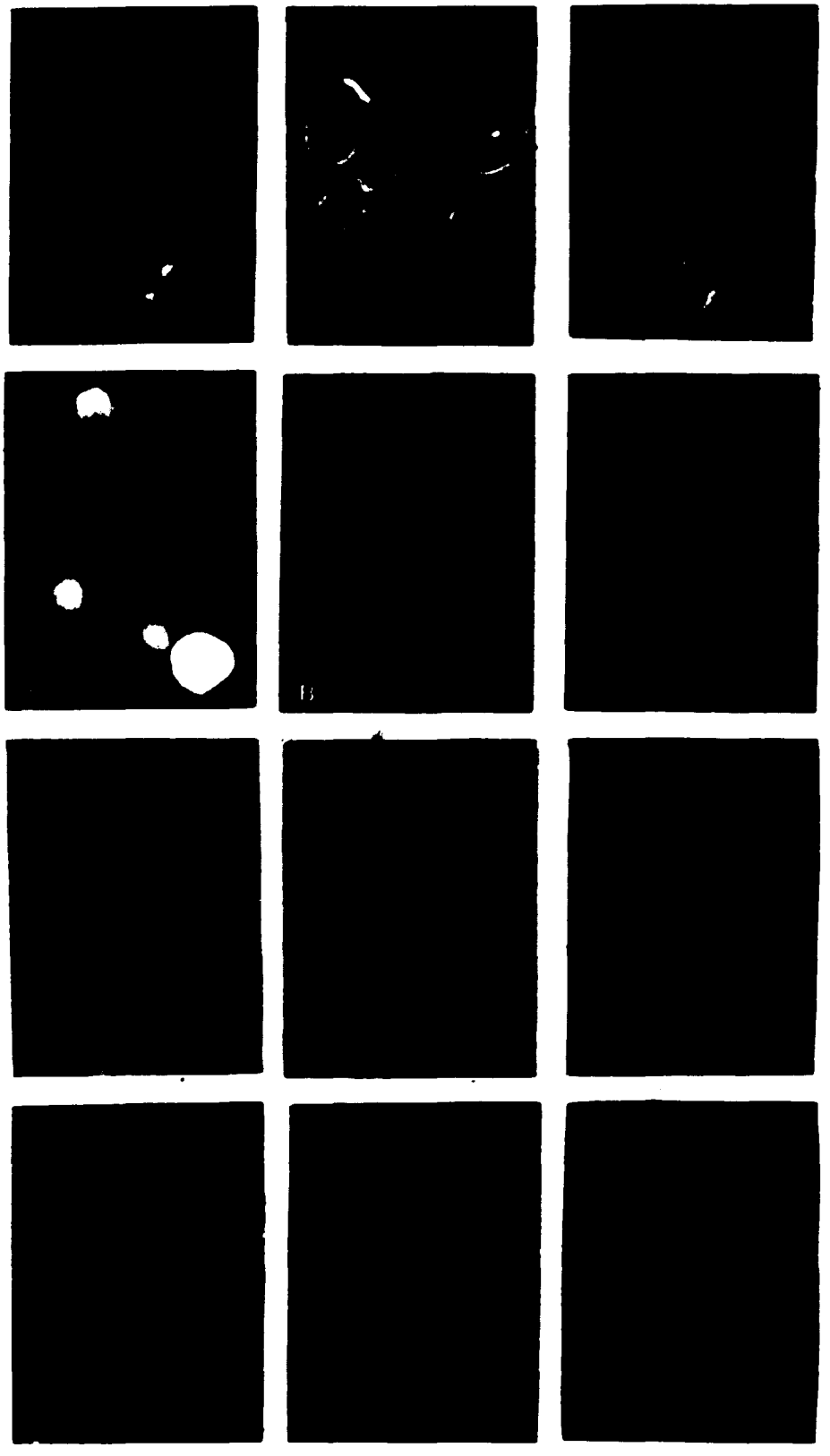


Figure 5. Transmission Electron Microscopy of Negatively-Stained *D. discoideum* Cytoskeletons

A. Magnification - 25,000X.

B. Higher magnification of A. Magnification - 100,000X.

C. Magnification - 50,000X. Note complex meshwork that is exposed when cytoskeletons are broken apart.

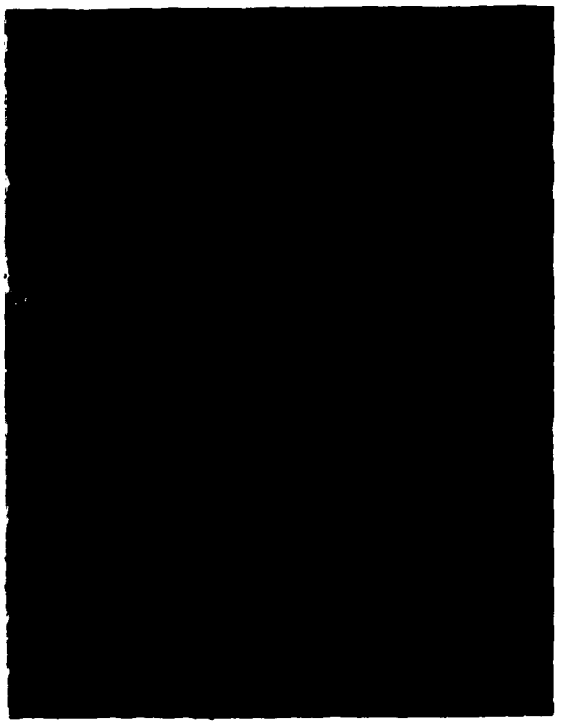


Figure 6. Transmission Electron Microscopy of Negatively-Stained *O. danica* Cytoskeletons

A. Magnification - 10,000X. Note that flagellar microtubules are cold stable.

B. Higher magnification of A. Magnification - 25,000X.

C. Magnification - 50,000X. Note the microfilaments (MF) and the fragment of flagellar axoneme with exposed microtubules (MT).

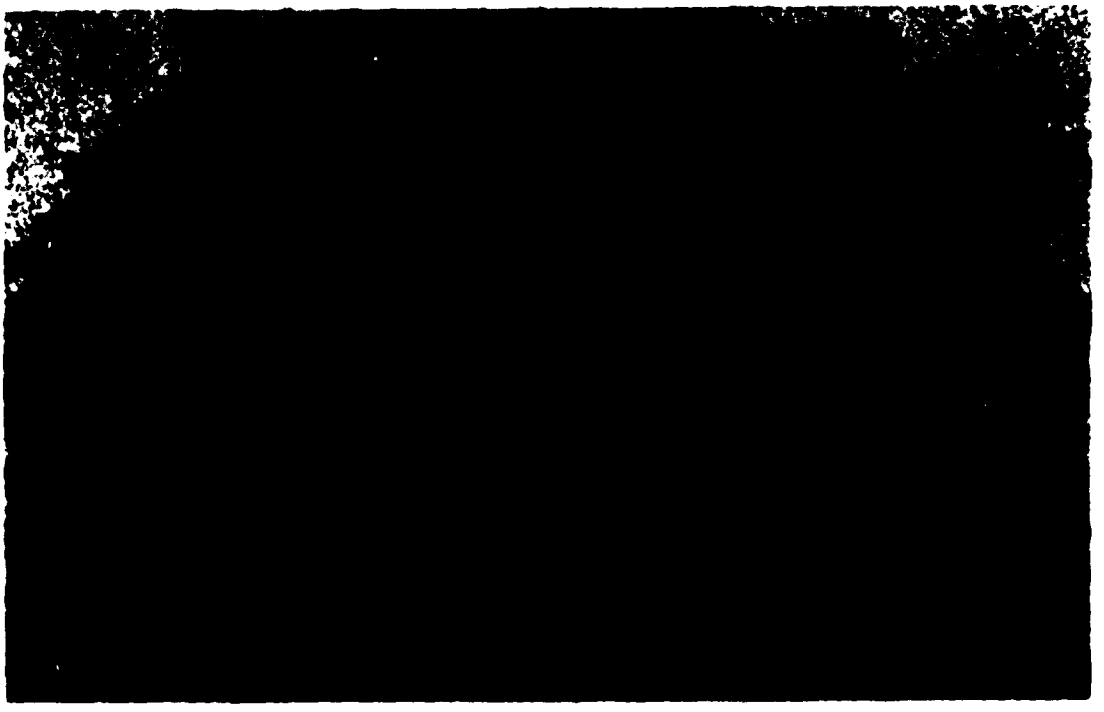
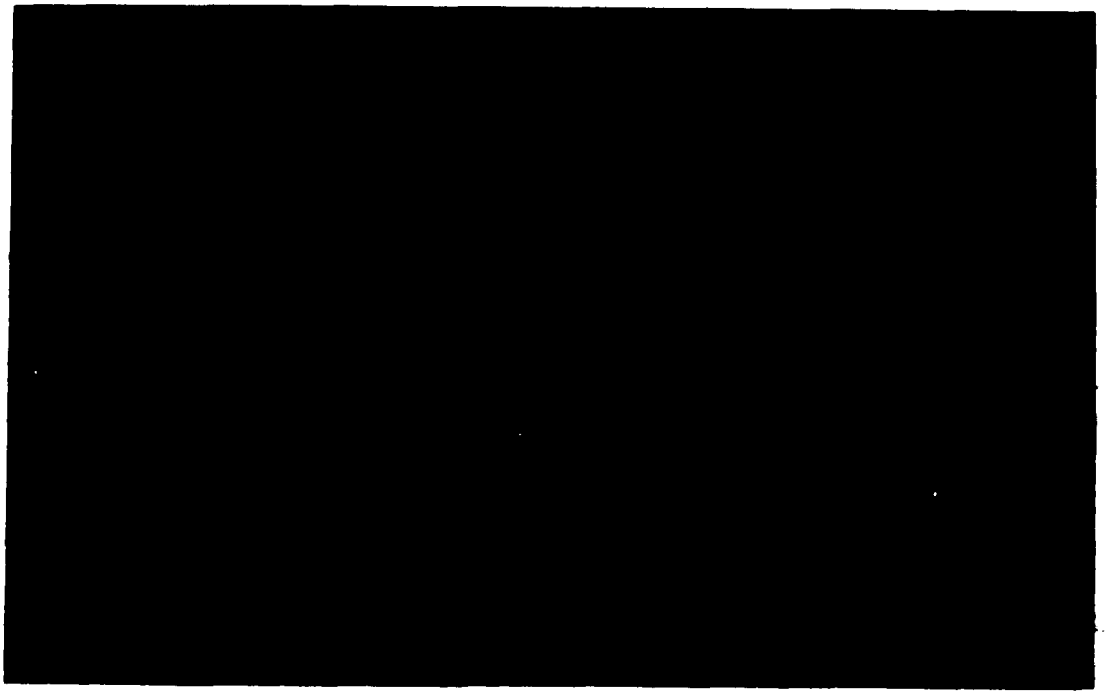


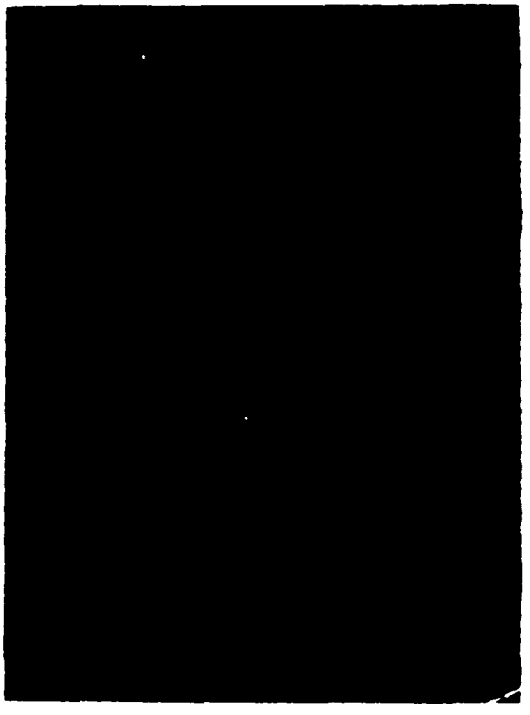
Figure 7. Transmission Electron Microscopy of Negatively-Stained *O. malhamensis* Cytoskeletons

A. Magnification - 25,000X. Note the presence of microfilaments that appear to run the length of the organism.

B. Higher magnification of A. Magnification - 100,000X.

C. Magnification - 100,000X. Note the complex meshwork that is exposed when cytoskeletons are broken apart.





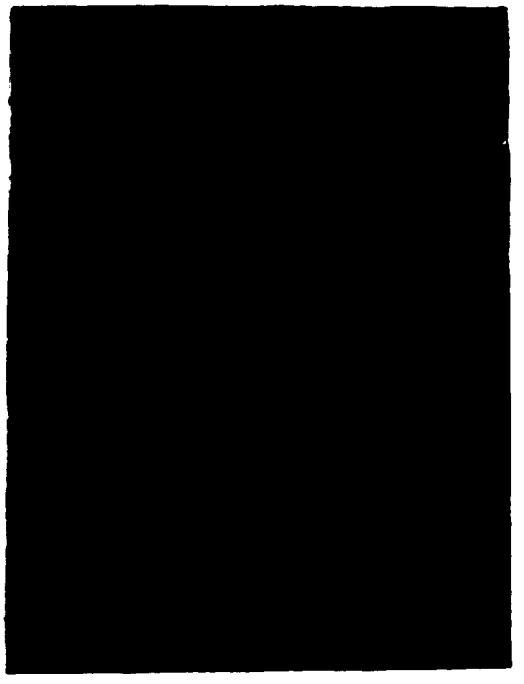


Figure 8. Transmission Electron Microscopy of Embedded O. danica Cytoskeletons

A. Magnification - 100,000X.

B. Magnification - 25,000X. Note location and size of filament-like structures indicating that they may be partially destroyed microtubules from the flagellum axonemes.

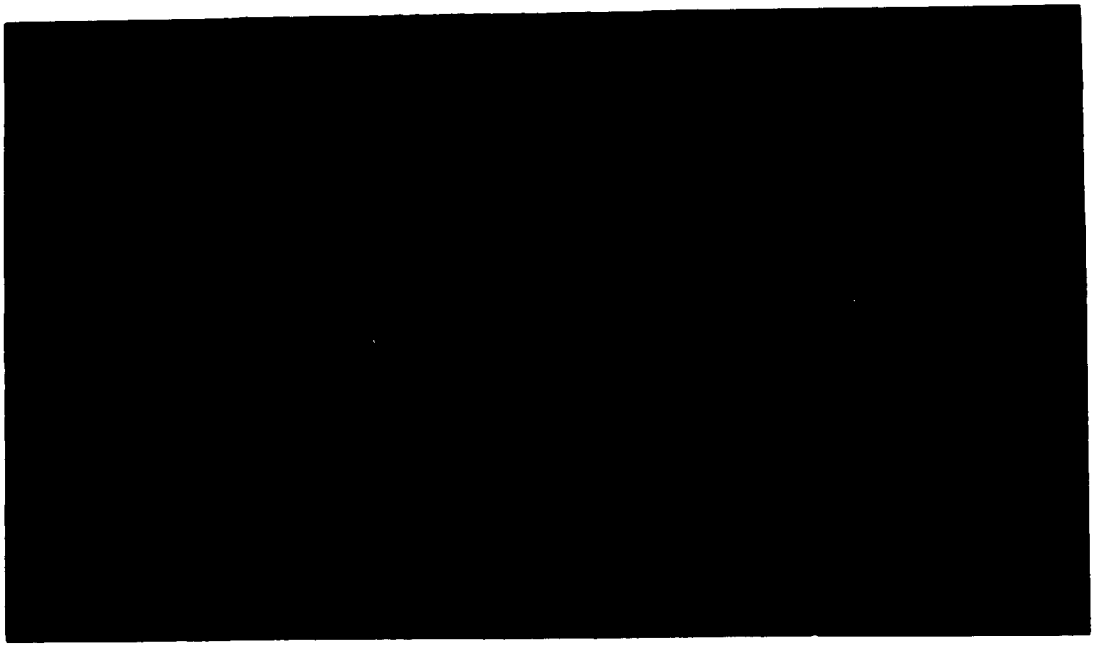


Figure 9. Scanning Electron Microscopy of Cytoskeletons

A. D. discoideum

B. O. danica

C. O. malhamensis

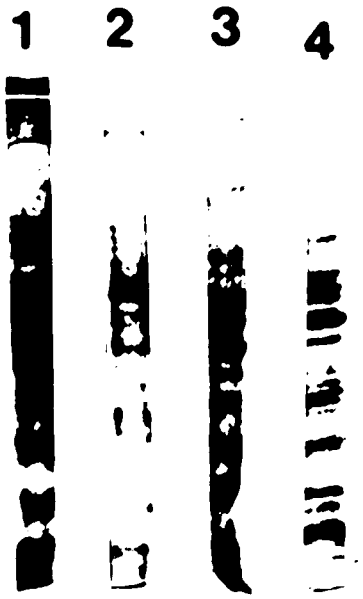


Figure 10. Effect of Proteolytic Inhibition on SDS-PAGE Electrophoresis

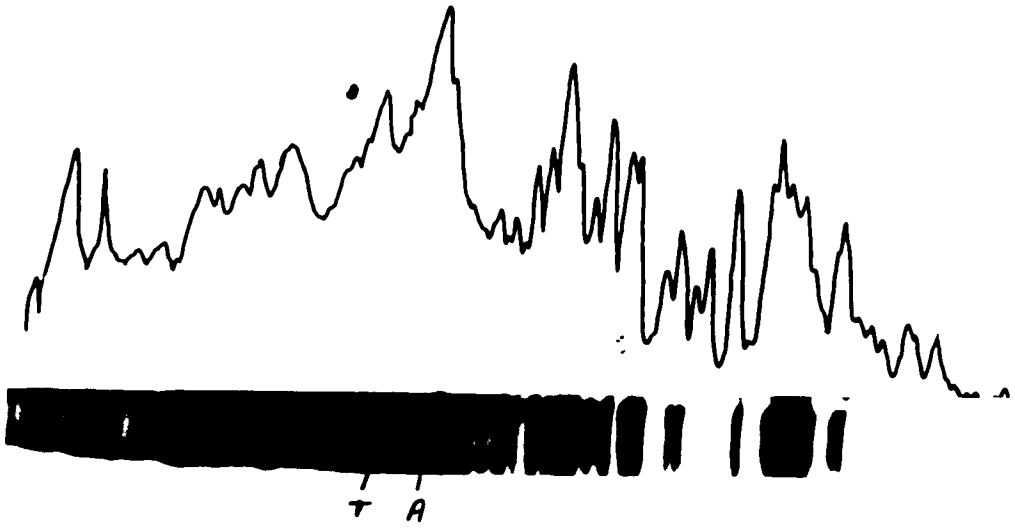
1. O. danica cell homogenate.
2. Same as 1 but with 2 mM PMSF and benzamidine. Note reduced smearing and apparent increase in molecular weight of major bands.
3. D. discoideum cell homogenate.
4. D. discoideum cell homogenate treated as in 2, with similar results.

Figure 11. SDS-Electrphoresis in Piccioni Buffer and Antiproteolytic Agents

Samples prepared with 2 mM PMSF, 2 mM benzamidine and 1% 1,10-orthophenanthroline.

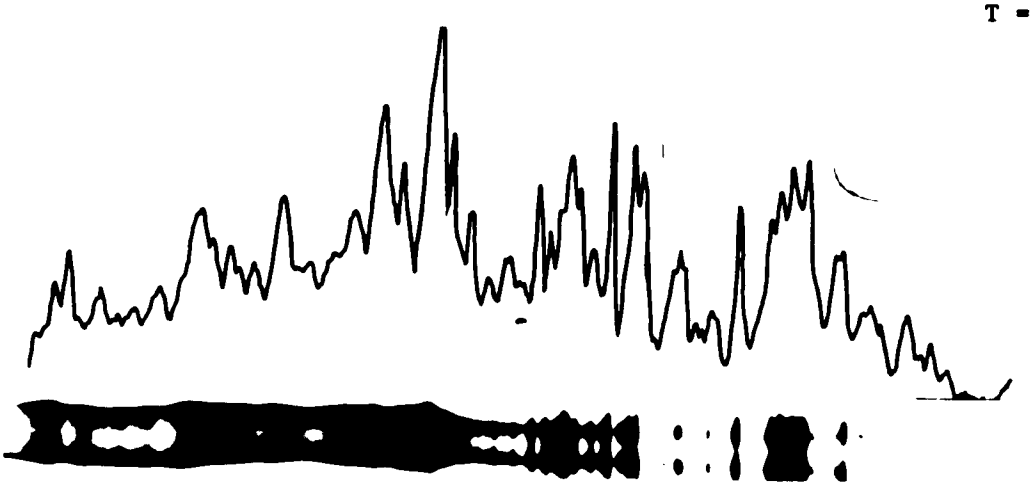
1. High molecular weight standard.
2. D. discoideum cells.
3. D. discoideum cytoskeletons.
4. O. malhamensis cells.
5. O. malhamensis cytoskeletons.
6. Actin.
7. O. danica cells.
8. O. danica cytoskeletons.
9. Desmin.
10. Desmin.
11. O. danica cells.
12. O. danica cells.
13. Low molecular weight standard.

Figure 12. SDS-Electrophoresis of D. discoideum



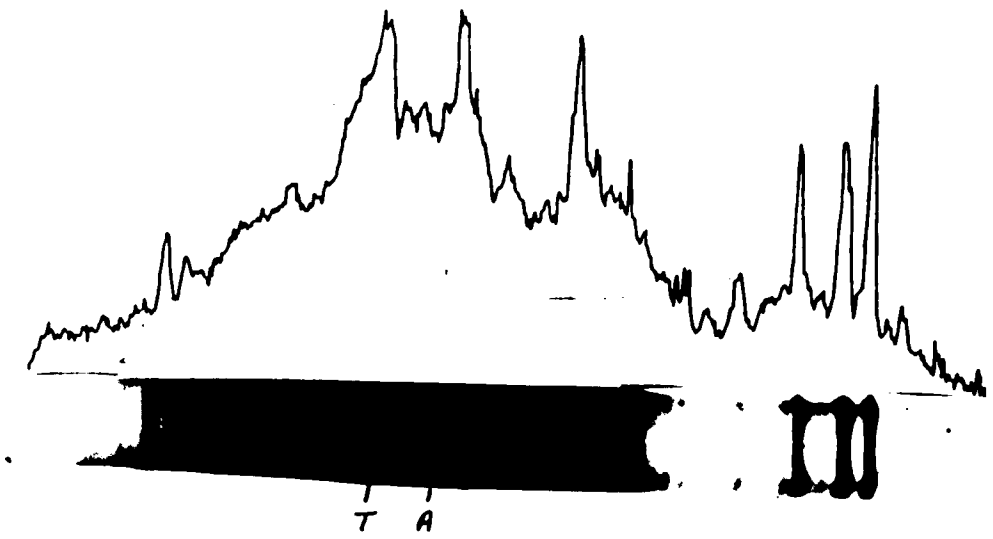
CYTOSKELETONS

A = Actin
T = Tubulin



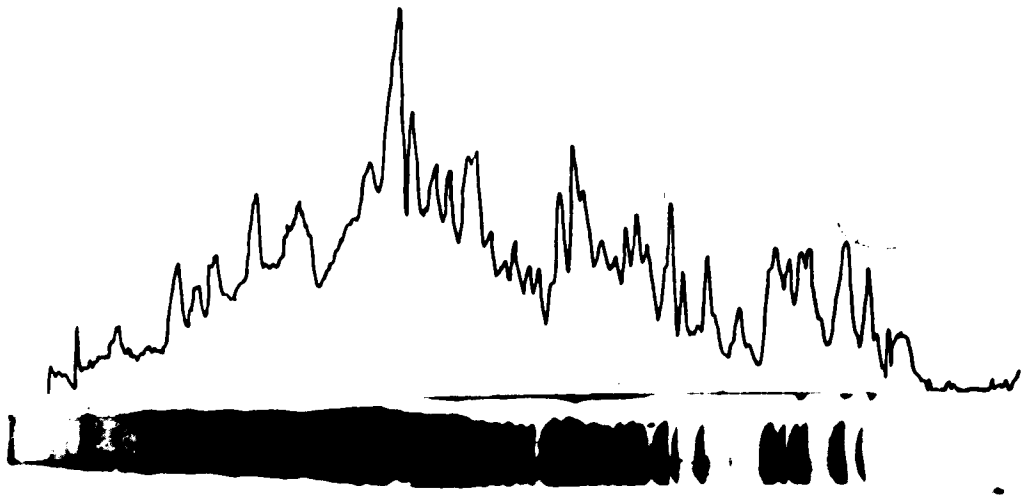
WHOLE CELLS

Figure 13. SDS-Electrophoresis of O. danica



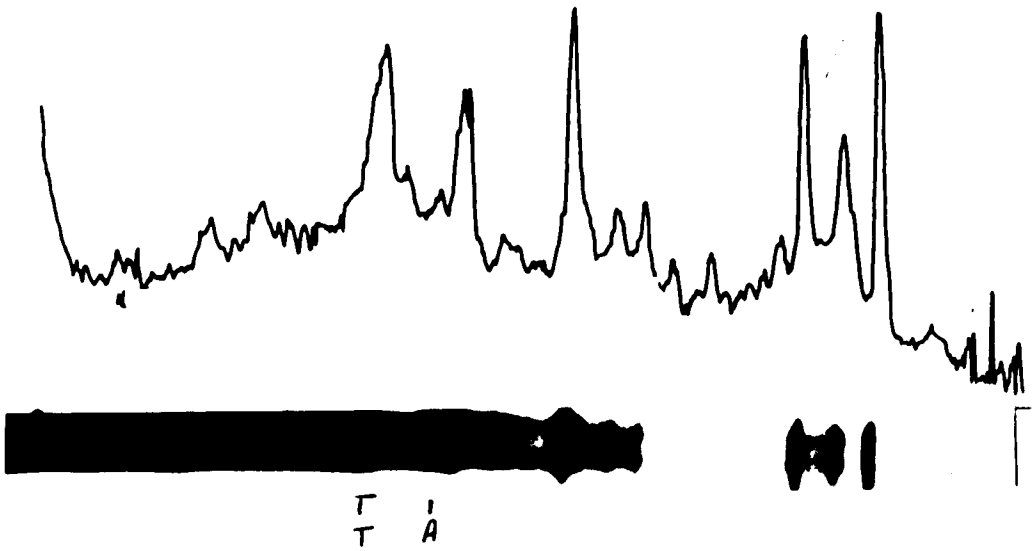
CYTOSKELETONS

A = Actin
T = Tubulin



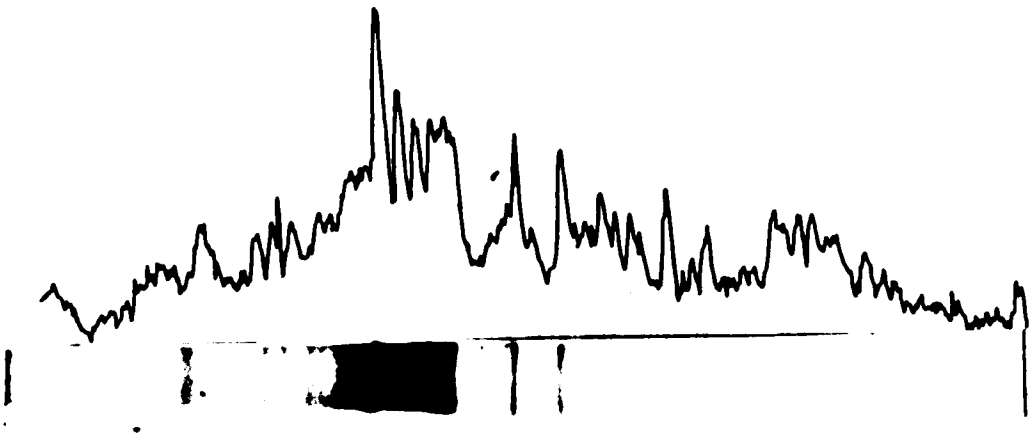
WHOLE CELLS

Figure 14. SDS-Electrophoresis of O. malhamensis



CYTOSKELETONS

A = Actin
T = Tubulin



WHOLE CELLS

Figure 15 - DEAE Cellulose Chromatography

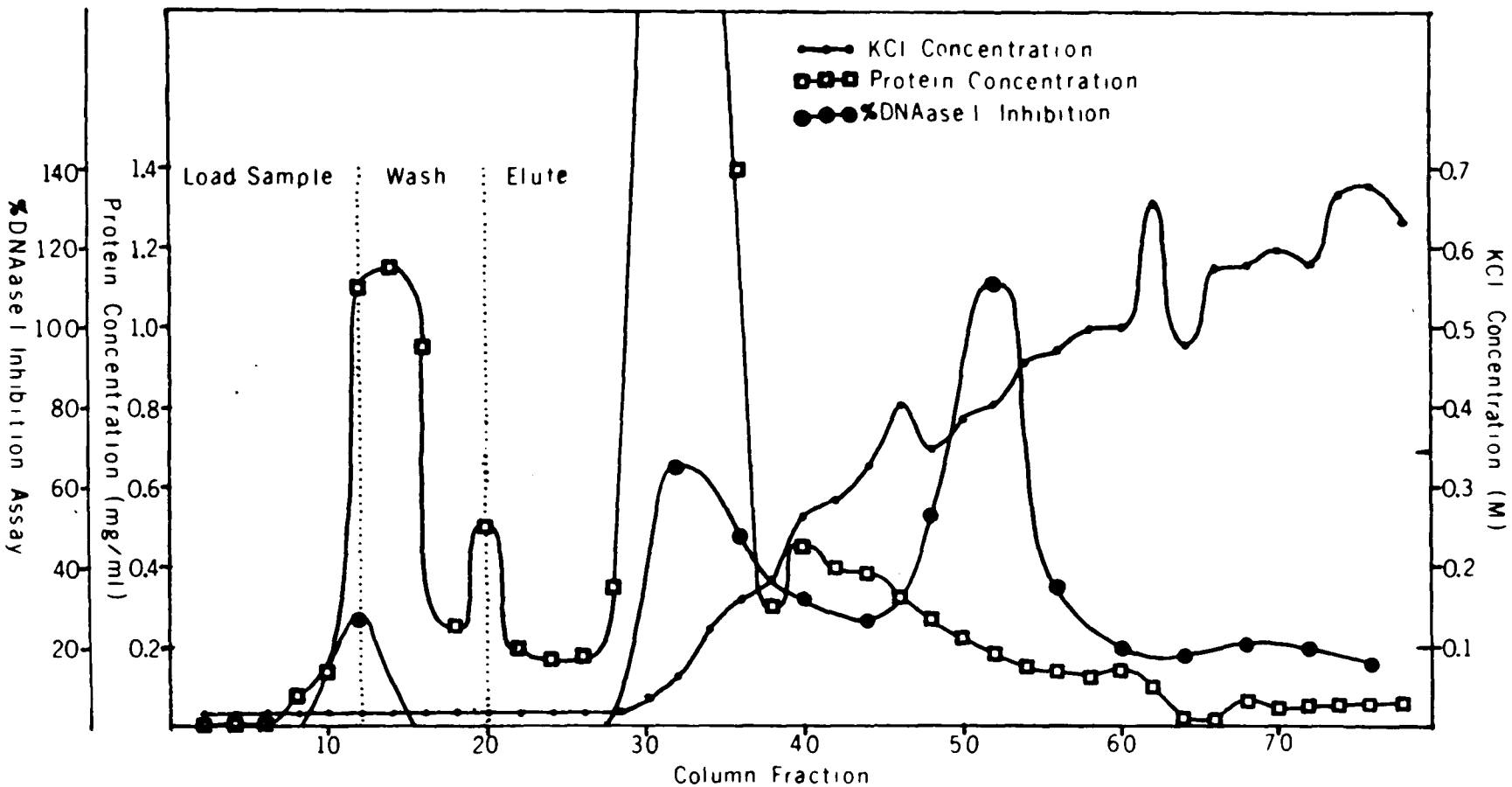


Figure 16. SDS-Electrophoresis of DEAE-Cellulose Column Samples

Symbols: A = Actin

M = Myosin

H = Cell Homogenate

Number = Column sample number

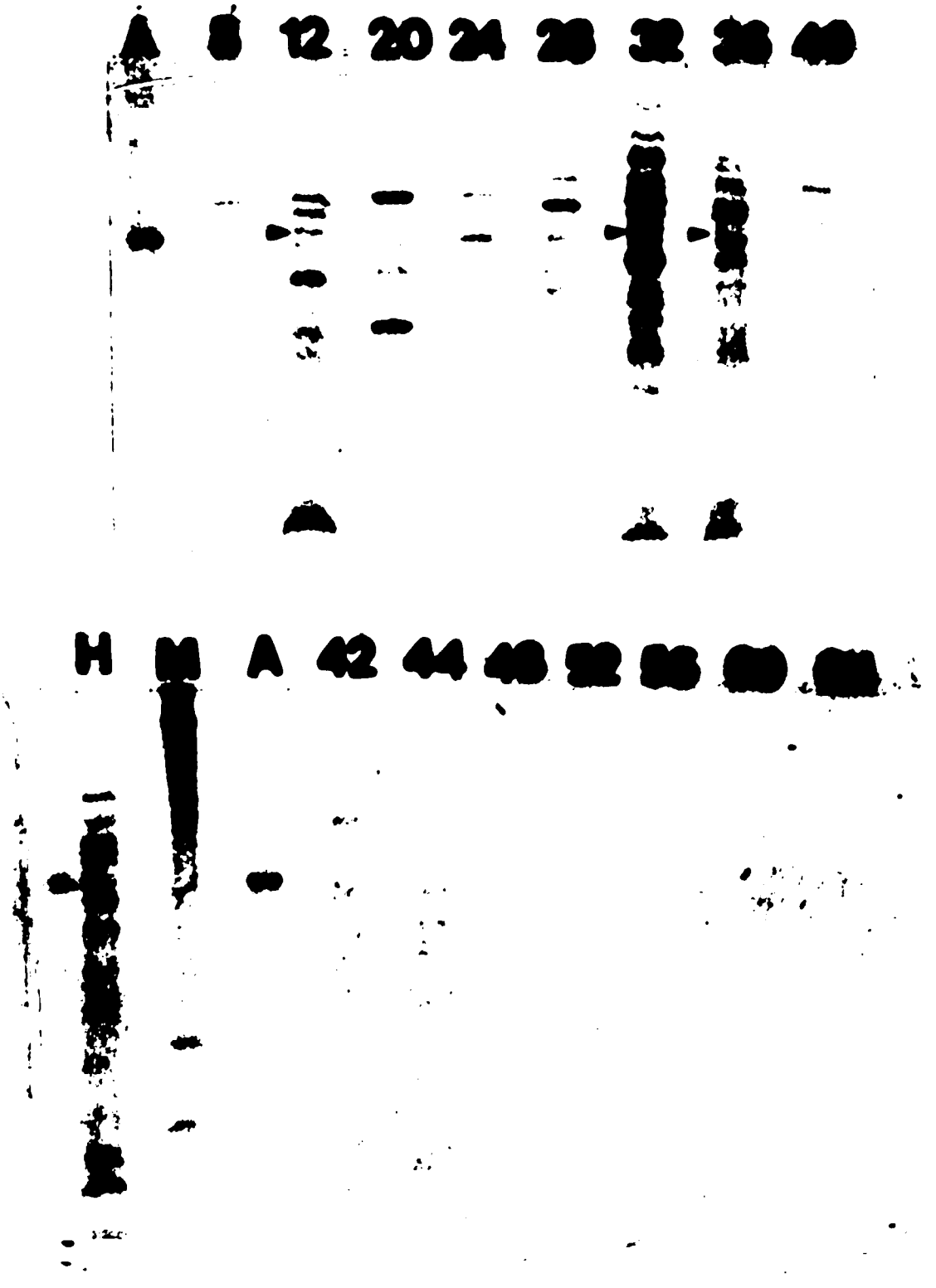
Number A = Column sample number with added muscle actin

Arrow = Indicates position of bands comigrating with muscle actin

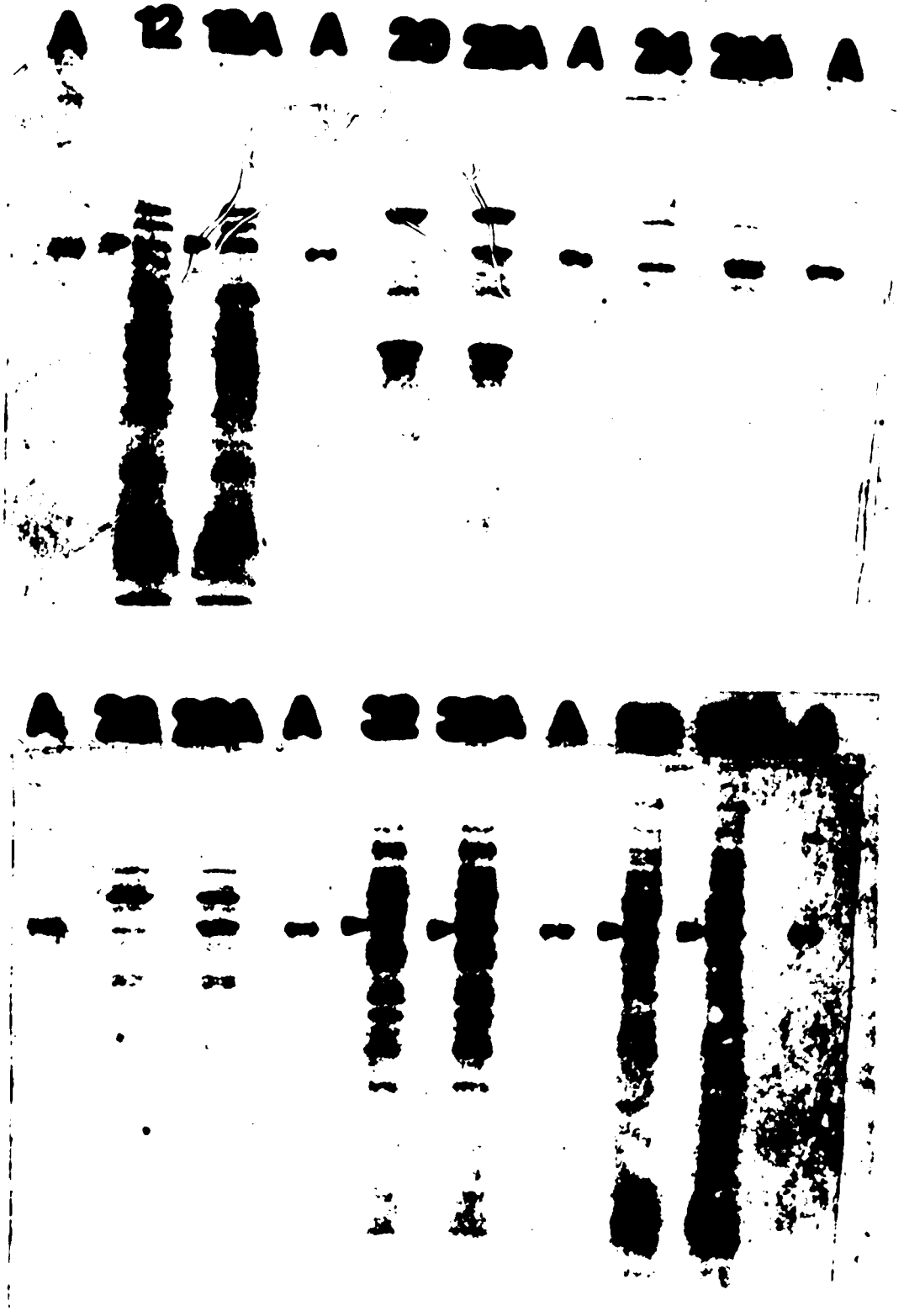
A. DEAE-cellulose column samples. Note that samples 12, 32 and 36 appear to have bands comigrating with muscle actin.

B. Verification of comigration of protein bands with muscle actin. Rat muscle actin was added to DEAE-cellulose column samples to verify that protein bands presumed to comigrate with muscle actin actually do comigrate.

16A



16B



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