THE CYTOSTOMAL APPARATUS OF *TETRAMITUS ROSTRATUS*

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By

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ABSTRACT

Microtubular systems are an integral and essential element of eukaryotic cells. Many of these systems display linkages between the microtubules. The aim of this study was to investigate one such system, the cytostomal apparatus of Tetramitus rostratus. It was hoped that some insight could be gained into the nature of inter-microtubule linkages as well as investigating conditions for possible isolation via differential solubilization.

The cytostomal apparatus consists of two sheaves of microtubules that originate at the basal bodies and extend through two-thirds of the length of the cell, apparently supporting the gullet. These microtubules are extremely stable: they are cold stable, calcium stable and griseofulvin/colchicine stable. They are highly cross-linked by fine filaments. These "linkers" have an average length of 107 nm. Linker width varies from the mid-portion (6 nm) to the ends (12 nm). In this and other respects they are similar to the highly contested microtrabecular lattice. The linkers were observed in positively stained samples and in thin-sections. Glutaraldehyde was found to have a very destructive effect on the linkers. A combination of paraformaldehyde and glutaraldehyde (2.25%/0.5%) was found not to have this effect over times that are normally used for

primary fixation.

Another component of the cytostomal apparatus is the system of longitudinal filaments that is especially well-revealed with high salt (0.25M NaCl or KCl) extraction. Since this extraction solubilizes the microtubules, it may allow the longitudinal filaments to be isolated. These filaments may be related to the tektin filaments found in flagellar microtubules. In addition, a novel set of cross-fibres is found to originate at the juncture of the two sheaves of microtubules and fan out across two-thirds of the width of the apparatus. These fibres were found to be especially stable in urea which may, again, allow for their isolation and characterization.

The linkers have been investigated for motility. Linker length was measured after treatment with ATP (which successfully reactivated the flagellar dynein) and after treatment with ATP followed by treatment with calcium (which efficiently halted all reactivated movement of the flagella). The length of the ATP treated linkers was very close (5 nm) to the control linkers and their morphology was indistinguishable. The length of the calcium treated linkers was considerable shorter (20 nm) but the morphology suggested that this was artifactual rather than due to a physiological cause.

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INTRODUCTION

Filaments pervade the eukaryotic cell. There are microtubules, intermediate filaments, microfilaments, 2 nm filaments, possibly a microtrabecular lattice and very likely filaments that have not yet been identified. These filaments provide an elaborate scaffolding which is surrounded by the less rigid elements of the cell. While the filament system of individual cells does perform some of the same functions as the metazoan skeleton, the term "cytoskeleton" can be misleading. Unlike the metazoan skeleton, the cytoskeleton is a dynamic entity that is intimately involved in movement both within the cell and of the cell.

The amoebo-flagellate Tetramitus rostratus is a good example of a cell with an extensive filament system. Even if only the microtubule-based systems are considered, the flagellate stage has four such systems or microtubule classes: flagellar, spindle and cytoskeletal microtubules as well as a more unusual class - the cytostomal microtubules. The flagellar and spindle microtubules are considered to be motile while the cytoskeletal microtubules are generally believed to be structural. The disposition of the cytostomal microtubules has not yet been determined although it has been suggested that they may be involved in peristaltic

contractions (Dingle and Larson, 1985). There are about sixty roughly parallel microtubules that support the cell gullet or cytostome. The most striking aspect of these microtubules is that they are extensively cross-linked by fine, wavy elements. These "linkers" are the most likely candidate for providing the motive force in any proposed cytostomal movement. It should be noted that the linkers share characteristics with a highly controversial filament system - the microtrabecular lattice. Investigation of the linkers is the primary objective of the present work. More introductory information on this system will be given following the presentation of some background information that is necessary to fully appreciate it.

1.1 The Microtrabecular Lattice: Fact or Artifact?

It has been proposed that the microtrabecular lattice (MTL) is the ultimate glue in the gemish of filaments - compartmentalizing the cytoplasm, anchoring organelles, joining together the other filament types (Wolosewick and Porter, 1976). Wolosewick and Porter introduced the term "microtrabecular lattice" since this system was reminiscent of the trabecular structure of spongy bone. The MTL has, however, been the center of some controversy. Is it real or is it artifact? While the evidence is inconclusive, it is an attractive concept. Polymers of the "trabeculin" (Satir, 1984) class of subunits weave an intricate lattice that

extends throughout the cytoplasm organizing, supporting, connecting. The trabeculin filaments are small but their precise size seems to depend on the method of preparation. Measurements after removal of embedding medium and critical point drying yield 11 nm in midregions (Walch et al, 1986). High voltage transmission electron microscopy (TEM) following critical point drying yields 2-3 nm in midregions but 10 nm at ends whereas quick freezing yields a constant diameter of 15 nm (Wolosewick and Porter, 1979). The filaments have a characteristic anastomosing appearance - they branch and fuse with each other. They are seen consistently by Wolosewick and Porter in spite of efforts to vary preparative procedures and hence rout out artifacts (Wolosewick and Porter, 1976).

Even so, the very existence of the MTL has been questioned. Heuser and Kirschner (1980), in examining rapidly frozen, freeze-dried specimens that had been rotary replicated with platinum report the absence of microtrabeculae. They found a complex cytoskeletal network that they determined was composed primarily of microtubules, intermediate filaments and microfilaments (actin filaments). They did not find microtrabeculae and they suggest that the MTL found previously was actually a distortion of these known cytoskeletal filaments produced by aldehyde fixation and dehydration. Supporters of the MTL argue that in this case, the MTL was solubilized in the extraction prior to freezing.

Perhaps the strongest criticism of the MTL comes from Hans Ris (1985). In order to investigate the possibility that the MTL is an artifact, Ris subjected a variety of model fiber systems to variations in critical point drying. He found that if traces of either ethanol or water were present in the CO_2, normally discrete filaments (actin, collagen, microtubules etc.) became distorted and fused, appearing as a network of anastomosing, tapering filaments that is characteristic of the MTL. Ris thus concludes that the MTL is a surface tension artifact produced during critical point drying. It should be noted that critical point drying has been used consistently in visualization of the MTL.

Bridgman and Reese (1984) present a third alternative. Although they did not observe the MTL per se, they emphasize numerous interconnections between filaments while maintaining that the individual filaments are discrete. The diameter of these filaments is 4-6 nm - about the right size for microtrabeculae. The only difference is that anastamosing of filaments was not observed.

Although the evidence may appear to favour one view or another, it is, at the present time, inconclusive. The MTL may exist as depicted by Wolosewick and Porter (1976, 1979) or it may represent a distorted view of known elements of the cytoskeleton. Reality may, in fact, lie between these two poles. We have learned enough only to conclude that in

any system where an MTL-like filament is observed, the consideration of artifacts is crucial.

1.2 Microtubules and Associated Proteins

Many of the linkages found within cells involve microtubules. There are linkages between microtubules and organelles, microtubules and other filament types, and microtubules and microtubules. Such linkages are generally believed to provide support and organization and/or provide the motive force for some sort of movement, and thus can be considered to be part of the MTL. Since they figure prominently in this discussion, a general consideration of microtubules and associated proteins is appropriate.

Microtubules: Tubulin and Protofilaments

Microtubules are composed primarily of the protein tubulin. At the TEM level, a microtubule is revealed as an apparently hollow cylinder with a rim of protofilaments that appear globular in cross-sections. These protofilaments are most clearly identifiable in preparations treated with tannic acid. Although the majority of microtubules contain 13 protofilaments, microtubules of some cell types characteristically display a different number *e.g.*, 15 in crayfish sperm and 12 in crayfish and lobster nerve cords (Burton *et al*, 1975).

Each protofilament is a multimer of tubulin heterodimers. There are two species of tubulin, α and β,

that can be considered to be charge isomers (Bryan et al, 1971). α and β tubulin form a tightly-associated dimer that, along with various accessory proteins, assembles into extended multimers that comprise the protofilaments.

Microtubule Associated Proteins

Prominent amongst the accessory components in tubulin assembly are "MAPs" - microtubule-associated proteins. MAPs are proteins that copurify with tubulin (usually through three cycles of depolymerization/repolymerization as per Shelanski et al, 1973). Well-characterized MAPs include the high molecular weight (HMW) MAPs, MAP-1 and MAP-2, which have a molecular weight greater than 250 kd and tau proteins which have a molecular weight range of 55 - 62 kd (Cleveland et al, 1977a).

Both tau and HMW MAPs are associated with tubulin in vivo as shown by immunofluorescence (Sherline and Schiavone, 1977). They are known to affect the assembly properties of tubulin, which has important ramifications for intracellular control of microtubule formation. Tubulin, with MAPs removed, normally will not undergo assembly (Cleveland et al, 1977a, Sloboda et al, 1976) unless relatively high concentrations of Mg^{2+} and glycerol or 10% dimethylsulfoxide (DMSO) are present (Burton and Himes, 1978). Both tau (Cleveland et al, 1977a) and HMW MAPs (Kim et al, 1978) have been found to promote tubulin assembly in a stoichiometric

(as opposed to catalytic) fashion. There is one tau for every ten α or β tubulin chains and one MAP-2 for each nine tubulin dimers. It has been suggested (Cleveland et al, 1977b) that tau promotes assembly by effectively increasing the local tubulin concentration.

It is also possible that MAPs are involved in regulating microtubule stability under various conditions. There is evidence that suggests that cold-stable microtubules have this property conferred upon them by a Ca2+-dissociable, low molecular weight substance (Webb and Wilson, 1980). There is also evidence that suggests that Ca2+-lability is modulated by a microtubule associated protein (Schliwa et al, 1981).

Appearance of HMW MAPs under the electron microscope seems to be preparation dependent. They are seen as filamentous projections on microtubules in thin sections (Dentler et al, 1975; Kim et al, 1979; Murphy and Borisy, 1975; Vallee and Borisy, 1977; Zingsheim et al, 1977). On the other hand, they appear to be globular in negatively stained preparations (Amos, 1977; Zingsheim et al, 1977). Until recently, the reason for this has been unclear. However, Langford (1983) has shown that MAPs can appear as filamentous projections in negatively stained preparations, as well as thin sections as long as the MAPs were adsorbed to the grid surface prior to contact with uranyl acetate. If

allowed to react with the uranyl acetate before adsorption, the globular configuration was observed. Langford concludes that the extended configuration is the normal one. He notes that glutaraldehyde also causes this coiling into globular structures. Thus, MAPs can be sensitive to stains and fixatives.

MAPs: Non-artifactual elements of the MTL

As previously stated, microtubules are involved in many types of linkages. Where the linking protein(s) can be identified, it is often found to be either MAP-2 or dynein (the ATPase responsible for ciliary and flagellar beating). These linkages should be considered to be part of the MTL. Although the existence of the MTL may be questioned, wherever MAP-2 or dynein has been identified, there is little doubt that these elements are real. Both MAP-2 and dynein have been isolated and subsequently identified using electrophoresis. The identification of specific bands as MAP-2, for example, is considerably strengthened by the observation that assembly of tubulin in the presence of these purified MAPs yields microtubules with projections whereas assembly without MAPs yields smooth microtubules (Murphy and Borisy, 1975). Thus, we are confident of the electrophoretic properties of MAP-2 and this serves to help in identifying MAP-2 candidates. In addition, specific antibodies have been made to MAP-2. Any cellular element that is labelled by a

MAP-2 antibody is probably a closely related protein. Similarly persuasive evidence for the positive identification of dynein exists. It should be noted that while we can positively identify both MAP-2 and dynein, elucidation of their roles is much more difficult.

1.3 HMW MAPs and Filament Linkages

HMW MAPs have been implicated in many linkages. There is some evidence that suggests that there is an interaction between microtubules and actin filaments that requires MAPs. This has been shown by viscometry measurements of [(microtubles + MAPs) + (actin)] compared with [(microtubules - MAPs) + (actin)] (Griffith and Pollard, 1978; Pollard *et al*, 1984). The high viscosity when MAPs are present indicates that an interaction is occurring. MAPs have also been found to induce assembly of actin into bundles (Sattilaro *et al*, 1981). This may indicate that MAPs can cross-link actin filaments and, in fact, weakens the argument for a MAP-mediated microtubule-actin interaction. The increase in viscosity when MAPs are present may actually be due to actin bundling. The evidence is circumstantial at best and more investigation is definitely needed.

HMW MAPs may serve as links between microtubules and intermediate filaments. High molecular weight polypeptides that copurify with intermediate filaments are found to resemble HMW MAPs (Pytela and Wicke, 1980). Material that is

recognized by anti-MAP-2 antibodies is found to associate with both microtubules and intermediate filaments (Bloom and Vallee, 1983). As with actin microtubule associations, viscometry measurements indicate that there is an association between microtubules and neurofilaments (Runge et al, 1981). Although most of the evidence for actual linkages is circumstantial, some cross-links that measure 4-6 nm in diameter and 20-30 nm in length have been visualized between neurofilaments and microtubules (Hirokawa, 1982).

MAP-2 may also be involved in microtubule-microtubule interactions. Such a role has been proposed in connection with the highly ordered, parallel microtubules found in the dendritic cytoskeleton (Vallee and Bloom, 1984) The idea is supported by the fact that immunofluorescence studies show that MAP-2 is present in the marginal band of nucleated erythrocytes - a bundle of microtubules that lies just below the plasma membrane in non-mammalian vertebrates (Sloboda and Dickerson, 1980). Once more, the evidence is very circumstantial but warrants further investigation.

1.4 Dynein: The "Force Protein"

MAP-2 has been the subject of much research but another microtubule-associated protein has been studied perhaps even more intensely. Dynein, named from the Greek ("dyne-in" = "force-protein"; Gibbons and Rowe, 1965), is an

ATPase that is found associated with microtubules, primarily in cilia and flagella. Wherever dynein is found, there is movement.

Microtubules were first associated with movement in cilia and flagella. These organelles are composed of a circular array of nine doublet microtubules surrounding two central microtubules - the classic "9 + 2" organization of the axoneme. There are many cross-connections between microtubules in the axoneme. There are links between doublet microtubules and the central microtubules. There are nexin links between doublets. Most importantly however, a pair of dynein arms reaches from each A subfibril to the B subfibril of the adjacent doublet.

A sliding filament mechanism for ciliary and flagellar beating was first suggested by Afzelius (1959) and in more detail, with supporting evidence, by Satir (1968). Microtubule sliding was elegantly demonstrated in reactivated axonemes where telescoping of filaments was observed (Gibbons, 1971). The involvement of the dynein arms in force production was shown by Gibbons and Gibbons (1973) in experiments where the outer arms were selectively removed and a decrease in beat frequency was observed. Dynein was known to be an ATPase (Gibbons, 1966) but these experiments implicated it in microtubule sliding.

The physical appearance of dynein has been especially well-revealed in quick frozen deep-etch images. Goodenough and Heuser show that the dynein molecule consists of an elliptical head, two spherical feet, a slender stalk and an interdynein linker (Goodenough and Heuser, 1984, 1982). The feet contact the A microtubule, the stalk contacts the B microtubule with the head in between. Addition of ATP transforms the almost perpendicular stalk-B microtubule connection to one with a distinct basal tilt. This is a weaker connection and it is proposed that it allows microtubule sliding. Similar results have been reported by Tsukita et al (1983).

Dynein and Dynein-like ATPases in Other Systems

Dynein has been firmly established as a microtubule-associated protein that cyclically cross-links adjacent doublets in the axoneme thus providing the motive force for microtubule sliding. As a result of this established role, often when movement involving microtubules is being studied, a search for dynein is initiated.

The method of force generation for chromosome movement during mitosis is a highly contested issue. There are two principal theories that both involve microtubules. One theory suggests that treadmilling is responsible for the movement. The spindle fibres are shortened via preferential depolymerization at one end (Inoue and Ritter, 1975). The

other theory is a sliding filament model much like that described above. Cross-bridges between spindle microtubules can be visualized using TEM (Hepler et al, 1970). Vanadate is an ATPase inhibitor that affects dynein (and the oubain-sensitive Na+/K+ ATPase but not myosin or the Ca2+ ATPase of the sarcoplasmic reticulum) and is found to inhibit separation of chromosomes (Cande and Wolniak, 1978). Together these observations support the involvement of dynein in chromosome movement. However, mitosis is a complex event and thus support for one theory does not automatically exclude the other.

Dynein has also been suggested to be involved in other microtubule-based motility. The axostyle is an organelle, made up primarily of microtubules, that is found in some protozoa especially termite flagellates such as Saccinobaculus. The axostyle is fairly well established as a locomotory organelle (Grimstone and Cleveland, 1965). Several highly organized rows of singlet microtubules extend through the cell often forming a tight spiral. These microtubules are linked by cross-bridges both between and within the rows. An ATPase is found to be located within the inter-row cross-bridges (Bloodgood, 1975). There is evidence that this ATPase is "dynein-like": its size is comparable; it has the same molecular weight; its reactivation characteristics are similar (Mooseker and Tilney, 1973).

Quick freeze, deep-etch analysis suggests that it is the intra-row bridges that are dynein-like both in morphology and sensitivity (Heuser, 1986). These bridges, like dynein, are stable to low salt concentrations but are extracted by high salt. The orientation of the bridges is found to be affected by ATP. In isolated axostyles, frozen in the absence of ATP, i.e., non-motile or "rigor" axostyles, an upward tilt of the bridges is more common. In situ and in ATP-reactivated axostyles, a downward tilt is more common. It was also found that the bridges tend to have a downward tilt in bent axostyles as opposed to an upward tilt in straight axostyles. It would appear that ATP affects the morphology of the intra-row bridges.

Dynein-like ATPases have been suggested in other systems. Stentor is capable of contracting 20-25% of its extended length in milliseconds. Two filament systems lie immediately below the cell surface: the myonemes and the km fibres. The myonemes are believed to be responsible for the contraction while the km fibres are thought to be involved in the subsequent cell extension. The km fibers are composed of parallel, overlapping microtubule ribbons. Cross-bridges have been found and microtubule sliding inferred from micrographs at various stages (Huang and Pitelka, 1973). Although there is no direct evidence for it, a dynein-like ATPase has been hypothesized. Dynein has also been suggested

to be involved in the saltatory movement of pigment granules in the scales of the squirrel fish Holocentrus ascensionis since vanadate and EHNA (erythro-9-3-(2-hydroxy-nonyl) adenine) block this movement (Beckerle and Porter, 1982).

1.5 ATPases Distinct from Dynein

Kinesin

Recently, it has been shown that dynein is not the only ATPase associated with microtubules. Previously all ATPases were dynein or "dynein-like". However, identification of proteins involved in axonal transport reveal ATPases that are distinct from dynein. Axonal transport of organelles has long been associated with microtubules. Movement occurs simultaneously in both directions suggesting that two transport mechanisms may be involved. Cross-bridges have been shown between both anterogradely and retrogradely moving organelles and their substrate microtubules (Miller and Lasek, 1985). An ATPase, distinct from dynein, has been shown to be involved in this transport (Vale et al, 1985). It has been named kinesin from the Greek "kinein" ("to move") and differs from dynein both by molecular weight and enzymatic behaviour. Kinesin has a molecular weight of 110-120 kd with less abundant polypeptides of 65, 70 and 80 kd. This is distinct from the much heavier dynein whose ATPase subunit alone has a molecular weight greater than 300 kd with the entire complex

weighing more than 1 x 10^6 daltons. The two proteins are enzymatically different as well. AMP-PNP (adenylyl imidodiphosphate), a non-hydrolyzable ATP analogue, decreases the affinity of dynein for microtubules but increases the affinity of kinesin for microtubules. Concentrations of n-ethyl maleimide that inhibit dynein leave kinesin largely unaffected. Kinesin has been localized in the mitotic spindle of sea urchin eggs (Scholey et al, 1985) and there is speculation that it may be involved in the force production for chromosome movement.

A MAP-2-like Protein

Another ATP-binding protein has been identified in axoplasmic vesicle preparations (Gilbert and Sloboda, 1986). This protein is recognized by antibodies to MAP-2 and is distinct from both kinesin and dynein. It is also, however, slightly different from MAP-2. Electrophoretic migration characteristics suggest that this protein has a weight between MAP-1 and MAP-2. It has been found that this 292 kd protein cannot be phosphorylated under conditions that phosphorylate most MAP-2's. It should be noted that while a cAMP-dependent protein kinase (responsible for phosphorylating MAP-2) is characteristically associated with MAP-2 (Vallee and Bloom, 1984), there is no evidence to suggest that MAP-2 itself has kinase activity. Thus, this MAP-2-like protein is distinct from MAP-2 per se not only by

its molecular weight but also by the fact that it quite likely is an ATPase, a force generating protein.

1.6 Other Microtubule Linkages

There are other systems where microtubule linkages are either not believed to be involved in movement or where the evidence is somewhat less convincing. For example, the cytopharyngeal basket in *Nassula* is a very complex organelle composed primarily of microtubules but also of many accessory proteins. A central palisade of microtubules exists that exhibits intermicrotubule linkages. It has been postulated that these linkages are involved in the movements of the basket that are associated with feeding (Tucker, 1978). There is circumstantial evidence that suggests that the eversion and re-invagination of the cell surface at the top of the basket is due to the antagonistic forces of microtubule sliding and contraction of a ring of microfilaments.

In *Giardia*, a gut-living flagellate, there is a complex ventral cytoskeleton known as the ventral disk or striated disk. This disk is believed to provide support for the organism while it attaches to its host via a suction force that originates elsewhere (Holberton and Ward, 1981). A major proportion of the ventral disk is made up of "microribbons" which, in turn, are made up mostly of tubulin (Holberton and Ward, 1981, Holberton, 1981). These

microribbons are linked by a regular array of cross-bridges that presumably help provide support. These bridges have been isolated and found to have a weight of 30 kd (Crossley & Holberton, 1983). "Giardin" antibodies bind to the microribbons (Crossley et al, 1986). No active role has been suggested for the ventral disk since addition of ATP did not result in any movement although axonemes did resume bending.

1.7 MAPs and the MTL

Table 1 lists the dimensions of most of the microtubule associated proteins that have been discussed. Both their lengths (10-180 nm) and their widths (0.2-20.5 nm) tend to be quite variable. Most of these widths (with the exception of dynein) fall quite readily into the proposed width of microtrabeculae. Length of microtrabeculae is, by definition, variable. These linking elements do share characteristics with microtrabeculae other than dimensions merely by the fact that they connect things.

1.8 The Cytostomal Apparatus of Tetramitus rostratus

Most of the linkages discussed can be placed in the MTL genre but none so much so as the linkages found in the cytostomal apparatus of the amoebo-flagellate Tetramitus rostratus. It is these linkages that will now be examined in detail.

TABLE 1

DIMENSIONS OF VARIOUS MICROTUBULE-ASSOCIATED LINKAGES

System	Width(nm)	Length(nm)	Source
MAP-2	-	165 - 180*	Voter & Erikson, 1982
	-	30 - 40	Zingsheim et al, 1977
HMW MAP	5.6	18.9	Murphy & Borisy, 1975
Dynein:			
perpendicular	20.5	27	Tsukita et al, 1983
tilted	16.7	32.7	
Mitotic Spindles	0.2-0.5	10 - 40	Hepler et al, 1970
Axostyle (within row bridges)	3	16	Mooseker & Tilney, 1973
Stentor (inter-ribbon)	5 - 6	18 - 20	Huang & Pitelka, 1973
Nassula	7	20	Tucker, 1978
Mitochondria-Microtubules	6.5-12.5	13	Smith et al, 1977
Neurofilament-Microtubules	4 - 6	20 - 50	Hirokawa, 1982
Axoplasmic Vesicles-Microtubules	8	16 - 18	Miller & Lasek, 1985

*65 - 80 nm of this length is attached to microtubules

Tetramitus rostratus is a virtually unknown amoebo-flagellate which is believed to be related to the somewhat better known Naegleria. Like Naegleria, Tetramitus has three stages - cyst, amoeba and flagellate. Unlike Naegleria, in Tetramitus the stable flagellate stage can both feed and divide. It is this stage with its four classes of microtubules (flagellar, cytoskeletal, spindle and cytostomal) that is most interesting in terms of filament systems. There are four anterior flagella that propel the streamlined conical-shaped flagellate. The distinctive cell shape is maintained by the prominent cytoskeletal microtubules.

An opening, encircled by an anterior collar extends posteriorly through two-thirds of the flagellate. This opening, generally considered to be the primary site of feeding, is referred to as the cytostome. The cytostome is supported by a palisade of about 60 microtubules that can be liberated intact from the remainder of the cell via either sonication (Balamuth et al, 1983) or detergent lysis (Dingle and Larson, 1985).

The isolated cytostomal apparatus consists of the microtubules as well as a basal body-rootlet complex including prominent striated rootlets (Dingle and Larson, 1981). The flagella can be left on or removed as desired. Perhaps the most striking aspect of the cytostomal

microtubules is the fact that they are highly cross-linked. The cross linking elements are aptly christened "linkers". The maximum linker length is 120 nm while the diameters vary from 5 nm - 20 nm (Dingle and Larson, 1985). This alone makes them MTL-like. In fact, the length of the linkers sets them apart from most of the microtubule-associated proteins previously discussed. In addition, the characteristically wavy appearance of the linkers corresponds well with the appearance of microtrabeculae.

Although there is no evidence for motility of these linkers, a thorough investigation has not yet been made. It is not implausible that the cytostomal apparatus participates actively in ingestion via a type of peristaltic contraction. From the brief review above, it is apparent that if there is movement of cytostomal microtubules, the linkers are the prime candidates for the generation of the motive force.

One of the objectives of the present work is to investigate the possible motility of the system. Another, more basic, objective is to investigate the nature of the linkers - this includes visualizing them with different methods to ascertain that they are not artifactual. The final objective is to investigate the sensitivities and stabilities of the system in order to facilitate future purification of the various elements via differential solubilization.

Chapter 2

MATERIALS AND METHODS

2.1 Culturing *Tetramitus rostratus*

The medium used to support the bacteria was "HYP" which consisted of 7.5 mM Hepes (N-2-Hydroxyethyl Piperazine-N'-2-ethanesulfonic acid), pH 7.3, 0.2% Difco yeast extract and 0.2% Difco bacto-peptone. This culture medium was autoclaved immediately following preparation and was subsequently opened only under sterile conditions.

The food organism used was *Enterobacter aerogenes*. Although *Tetramitus* would feed on many other types of bacteria, growth was most consistently successful with *E. aerogenes* and thus this bacterium was used throughout this work. Permanent stocks of bacteria were kept either as: a). streaks on agar plates, periodically subcultured, grown for 24 h at 33°C and then maintained at 4°C, or b). frozen in 20% Glycerol in Penassay Broth (PAB) [1.75% Difco antibiotic medium 3 in distilled water]. Working stocks were obtained by placing a loop of bacteria (from either a plate or a frozen culture) into 4 ml of PAB and incubating for 24 h at 33°C.

Tetramitus rostratus flagellates were maintained in liquid culture with the live bacterium *Enterobacter aerogenes*

as food. Cultures were maintained both stationary at room temperature and agitated at 33°C. Both agitation and surface area of the liquid culture are key factors in determining the rate of growth and final density.

Room Temperature Subculture

These stocks were maintained as a back-up, primarily in case of a 33°C culture "crash". Occasionally, Tetramitus cultures would fail to thrive despite concerted efforts to maintain optimal conditions. The cause of this phenomenon has not yet been determined although there seemed to be a seasonal component involved - cultures were more likely to crash in spring and fall than at other times. The room temperature stocks were more stable and were affected less by these variations in viability.

To a 250 ml Bellco flask were added 20 ml HYP, 0.2 ml working stock E. aerogenes and 2 ml Tetramitus rostratus from the previous culture. This results in a starting Tetramitus density of 3×10^5 cells/ml. The culture reached stationary phase by three to four days with a final density of 2.5×10^6 cells/ml.

Note: i). All bacterial transfers were made by way of sterile technique.

ii). Tetramitus densities were determined with a Coulter Counter, Model Zf. The sample was diluted in

0.5% NaCl and counted at threshold 10, 1/amplification 2 and 1/aperture current 2. These Coulter Counter settings were chosen so that only Tetramitus was counted - neither single nor clumped bacteria were included in these counts. Throughout this work, "cells/ml" has been used to denote Tetramitus density as determined using the Coulter Counter at these settings.

33°C Subculture

Cultures were grown in a number of different flask types. 250 ml Bellco flasks, 1 l Pyrex Erlenmyer flasks and 2.5 l Pyrex flasks were all used. All flasks were loosely covered throughout with foil caps or Bellco stainless steel caps. Up to 10% of the capacity of each flask was used. A small amount (approximately 1% of final volume) of E. aerogenes working stock was added to a volume of HYP. This was placed in a New Brunswick Gyratory shaker, model G-25 (approximately 60 rev/min) at 33°C for 2 h. At this point, sufficient T. rostratus were added to yield a starting density of 3×10^5 cells/ml.

The cells reached stationary phase by 36 to 48 h with a final density of 2.5 to 3.0×10^6 cells/ml.

2.2 Isolation of the Cytostomal Apparatus

Isolations were carried out with cells that were washed free of most of the bacteria and starved for 12 to 24

hours. This starvation period was necessary in order to yield a relatively clean lysate.

Washing Procedure

A stationary phase culture was transferred to several 50 ml conical-bottomed tubes. These were centrifuged for 3 min at 400xg (setting six) in a table top clinical centrifuge (model CL, International Equipment Co.). The centrifuge was allowed to come to a stop without braking. The supernatant was decanted and the pellet was gently resuspended in 7.5 mM Hepes, pH 7.3 (Hepes). This procedure was repeated twice more. After the third centrifugation, the volume was brought up to approximately one-half the original volume, placed in a clean flask and left at room temperature.

The Isolation

The isolation was carried out with cells that had been washed as above and starved for 12 to 24 h. 1.0 to 2.0 x 10^8 cells were used for each isolation.

Cytostomal apparati could be isolated with flagella intact or removed. If the cells were to be deflagellated, the procedure was as follows. The cells were pelleted as in the wash procedure, resuspended in 4 ml of Hepes, transferred to a 50 ml round-bottomed tube and vortexed at maximum speed for 40 s. This resulted in greater than 95% deflagellation.

The cells were then transferred back to a conical-bottomed tube and brought up to 20 ml with Hepes.

If the cytostomal apparati were to have their flagella left on, the last centrifugation and vortex were omitted and the isolation was started at this point. The next step was to separate Tetramitus from the remaining bacteria (and flagella, if deflagellated). This was accomplished via a sucrose gradient. A 12 ml 0 - 0.4 M continuous sucrose gradient in Hepes was made using a simple two-chamber gradient maker and a Gilson Minipuls II pump. The cells were spun for 3 min at 400xg as before and resuspended in 1 ml of Hepes. This was carefully layered on top of the sucrose gradient which was then spun for 1 min at 400 g and allowed to come to a rest without braking. The Tetramitus banded together approximately halfway down the tube with a band of single bacteria (and flagella) above the Tetramitus band and bacterial clumps below it. The band was removed with a Pasteur pipette and immediately brought up to 20 ml with Hepes.

The cells were then pelleted as before and this time were resuspended in Tris-glycerol [10% glycerol, 30 mM Tris (Tris(hydroxymethyl) aminomethane), pH 8.0] and spun once more. This final spin was followed by a resuspension in 1 ml Tris-glycerol. To this was added 100 ul of 10% TX-100 (Sigma Chemical Co., St. Louis, MO) - a final concentration of

0.91%. This mixture was vortexed at top speed for 20 seconds (or longer - up to 1 minute until complete lysis had occurred as determined by phase microscopy). Upon completion of lysis, $MgSO_4$ was added to 3 mM final concentration.

2.3 Ghosting *Tetramitus rostratus*

All cells to be ghosted were washed and run through a sucrose gradient as in the preparation for cytostomal apparatus isolation. Ghosting was carried out in 1.5 ml Eppendorf tubes. 1.5 ml of 2.0×10^6 cells/ml (a total of 3.0×10^6 cells) were placed in the Eppendorf tube and spun at 400xg in a clinical centrifuge for 2 min. The supernatant was discarded and the pellet was resuspended in iced TEM (30 mM Tris, 2 mM EGTA, 5 mM $MgSO_4$, pH 8.0). A volume of iced TEMT (30 mM Tris, 2 mM EGTA, 5 mM $MgSO_4$, 2% TX-100, pH 8.0) was added and the solution was agitated for one minute. The agitation consisted of 30 inversions.

The relative volumes of TEM and TEMT were adjusted depending on the final detergent concentration desired (i.e., extent of ghosting desired). The sum of the volumes was always 0.5 ml in order to maintain a constant geometry and density during agitation. For example, if 1.0% TX-100 ghosts were desired, 0.25 ml TEM and 0.25 ml TEMT would be used but if 0.1% TX-100 ghosts were desired, 0.475 ml TEM and 0.025 ml TEMT would be used.

2.4 Reactivation of Extracted Cells

Motility could be restored to the flagella of ghosted cells or isolated cytostomal apparati. There were many variables that affected the success of reactivation and these are considered in the Results. The basic procedure was as follows. Cells were ghosted as outlined above. Detergent concentration (and occasionally agitation) was varied to produce the desired extent of extraction. A volume of ghosted cells in iced TEMT (with varying TX-100 concentrations) was mixed with an equal volume of iced reactivation buffer and brought to room temperature. The reactivation buffer contained 30 mM Tris, pH 8.0, 3 mM $MgSO_4$ and varying concentrations of adenosine-triphosphate (ATP) (Catalogue # A-5394, Sigma Chemical Co., St. Louis, MO) and ethyleneglycol - bis - (B-amino-ethylether) N,N'-tetra-acetic acid (EGTA) (Sigma Chemical Co., St. Louis, MO). This resulted in final concentrations of 30 mM Tris, pH 8.0, 4 mM $MgSO_4$ and varying ATP and EGTA concentrations. In some cases KCl or NaCl was added prior to reactivation.

Flagellar reactivation would commence immediately as monitored by phase microscopy. Some beating would continue for up to 30 min without addition of further ATP although the percent reactivated would decrease during this time. All percent reactivations reported in the Results were determined

within the first five minutes when reactivation was at the highest level.

2.5 Exposure to Varying Chemical Conditions

Throughout this work, cytostomal apparati (and occasionally ghosted cells) were exposed to a wide range of chemical conditions. Samples to be exposed were washed free of the detergent by centrifugation in a clinical centrifuge (3 min at 400 g for ghosts; 7 min at 650xg (setting seven) for isolated apparati) and resuspended in 30 mM Tris, pH 8.0. A volume of the washed sample was then mixed with an equal volume of the necessary solution to yield the desired final concentrations. All exposures were carried out in this manner with the exception of pH variations which necessitated resuspension directly into the final solution.

2.6 Positive Staining for Electron Microscopy

Cytostomal apparati and ghosted cells were prepared for electron microscopy by sticking them down on formvar/carbon coated grids followed by staining with uranyl acetate.

Cytostomal apparati in detergent solution would not stick well to grids and thus were washed prior to attempting this procedure. The washing was accomplished by bringing the solution up to 10 ml with 30 mM Tris, 3 mM $MgSO_4$, pH 8.0 (Tris-Mg), transferring to a 15 ml conical bottomed tube and

spinning for 7 min at 650 g in a clinical centrifuge. The supernatant was decanted, the rim of the tube was wiped with a Kimwipe to remove all the supernatant and the pellet was resuspended in a small volume (approxiamtely 100 ul) of Tris-Mg. This same difficulty was encountered with ghosted cells in detergent but in this case was overcome with volume/drying changes during staining since centrifugation damaged the ghosts.

Ten to twenty microlitres of the sample was placed on a formvar/carbon coated grid sitting on Parafilm. The cytostomal apparati/ghosts were allowed to settle for about 3 min at which point the liquid was drawn from underneath the grid with damp filter paper. The grid was then floated on top of a small drop of 0.5% aqueous uranyl acetate for 2 min. The uranyl acetate staining was followed by a rinse in a drop of distilled water. The grids were allowed to dry on filter paper and were usually observed with phase microscopy prior to examination with the electron microscope. This examination allowed for a light microscope level evaluation of the condition of the apparati. It also allowed for an evaluation of the success of the staining procedure. As stated above, some difficulty was encountered in sticking ghosts to grids (this difficulty was occasionally encountered with isolated cytostomal apparati as well). A light microscope examination allowed this to be monitored.

2.7 Preparation for Thin Section Electron Microscopy

With the exception of the fixatives used, a standard fixation protocol was followed. The primary fixative used was either 2.0% glutaraldehyde in Tris-Mg or 2.25% paraformaldehyde/0.5% glutaraldehyde in Tris-Mg. The pH of the primary fixative was adjusted to 8.0 after all the components were mixed together. The secondary fixative was somewhat atypical, consisting of 50 mM cacodylate, pH 7.4, 0.5% OsO_4 and 0.8% $K_3Fe(CN)_6$ (Macdonald, 1984). No pH adjustment was made following the addition of OsO_4 and $K_3Fe(CN)_6$.

Equal volumes of the sample and double strength primary fixative were mixed together, left for 60 minutes and then pelleted. This was followed by three 5 minute rinses in 50 mM cacodylate, pH 7.4 (cacodylate). The pellet was resuspended in the secondary fixative, left for 60 min and then pelleted once more. There were three more 5 min cacodylate rinses followed by 10 min in each of an iced ethanol series (30, 50, 70, 85, 95%). The samples were then brought to room temperature and treated to two 15 min exposures to 100% ethanol followed by 10 min in ethanol:propylene oxide (1:1) and two 15 min exposures to 100% propylene oxide. Spurr's resin was introduced diluted 1:1 with propylene oxide for 60 min and then 2:1 with propylene oxide for the next 60 min. The samples were then

left in 100% Spurr's, with three or four changes, overnight and all day. The samples were then transferred to BEEM capsules and polymerized at 70°C for 16 to 24 h.

Silver and gold sections were cut using glass knives and an LKB ultratome III. Grids were floated on 70% methanol saturated with uranyl acetate for 15 to 20 min, rinsed with 50% methanol followed by water and immediately floated for 2 to 3 min on lead citrate (Reynolds, 1965) drops on Parafilm on a bed of NaOH pellets. This was followed by rinsing with water. The grids were examined in a Philip's 300 electron microscope at 60 KV using a 30 um objective aperture.

Chapter 3

THE CYTOSTOMAL APPARATUS RECONSTRUCTED: FACT OR ARTIFACT?

In *Tetramitus rostratus* the cytostome (cell gullet) extends through approximately two-thirds of the cell. It is at its widest at the anterior, tapers towards the bottom and curves very slightly throughout its length. This description is reconstructed from examination of living cells with phase contrast microscopy and by TEM of fixed, sectioned cells. As previously observed (Dingle and Larson, 1985), a series of microtubules lies immediately below the surface of the gullet, apparently supporting the cytostomal membrane. The image of these microtubules and associated elements (together termed the "cytostomal apparatus"), to be described in detail below, remains unchanged despite many variations in preparative techniques. A diagrammatic representation of the organization of the cytostomal apparatus within the cell is shown in Figure 1.

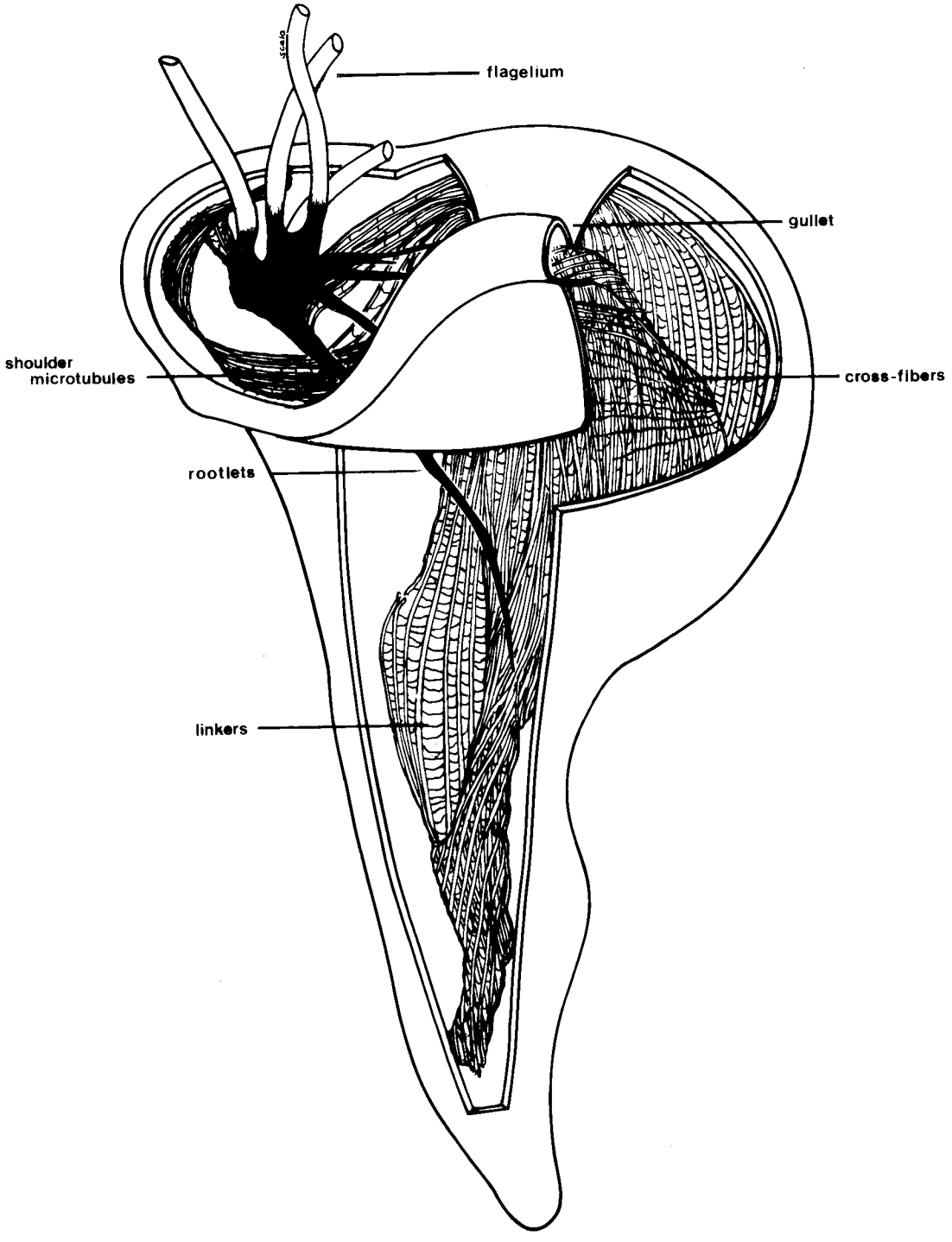
3.1 Preparative Techniques

There are three fundamental preparations that have been used and they can be examined using somewhat different techniques. A brief explanation of both the preparations and the techniques used to examine them is necessary in order to fully appreciate the results that follow.

Figure 1

Tetramitus Diagram

A diagrammatic representation of *Tetramitus rostratus* highlighting the organization of the cytostomal apparatus within the cell. Approximately 9,000X.


flagelium
gullet
shoulder
microtubules
cross-fibers
rootlets
linkers

The three basic preparations that have been used vary the treatment of the cell prior to fixation. The cell can either be essentially untreated, as is the case with intact cells, or extracted with detergents. The degree of extraction can be controlled through manipulation of both detergent and magnesium concentrations, as well as agitation. Light to moderate extraction results in cell "ghosts" from which all but the most stable elements are extracted. A more extreme extraction results in complete lysis yielding isolated cytostomal apparati. The procedure for each of these preparations has been outlined in the Materials and Methods section. These three key preparations - intact cells, ghosted cells and isolated cytostomal apparati - form the starting point for all other variations.

Each different preparation can be examined using different techniques. The cytostomal apparatus of intact cells is best revealed with thin section electron microscopy. While both ghosted cells and isolated cytostomal apparati can also be examined in this manner, "positive stain" electron microscopy has proved to be very informative (this preparative technique will be referred to as "positive staining"). The "positive staining" technique requires some explanation. The procedure, which is described in detail in Materials and Methods, is very similar to the more common technique of negative staining. Both techniques are carried

out with unembedded specimens that are placed directly on formvar/carbon-coated grids and both often employ uranyl acetate as a stain. The difference between the techniques is in the type of staining that results. In negative staining, the stain surrounds the specimen without actually binding to or penetrating the specimen itself. A negative stain can therefore be washed away. Positive staining, on the other hand, requires that the stain does bind to and/or penetrate the specimen. Thus, a positive stain is not easily rinsed away. The staining of the cytostomal apparatus, using the "negative stain" technique, is definitely an example of positive staining and the technique shall be referred to as such.

3.2 The Isolated Cytostomal Apparatus

Positive staining reveals much about the cytostomal apparatus. The cytostomal microtubules can be liberated from the cell by a vigorous 20 second vortex in 0.9% TX-100 and 10% glycerol. The microtubules, along with several other components remain together as a unit which is referred to as the cytostomal apparatus (Fig. 2). The backbone of this unit is a microtubule array that consists of two sheaves, both originating at the basal bodies and extending in opposite directions. These microtubules are apparently supporting the prominent anterior "collar" that is visible with both phase contrast microscopy and scanning electron microscopy (SEM)

Figure 2

Isolated Cytostomal Apparatus

This cytostomal apparatus was liberated from the intact cell by a vigorous 20s vortex in 0.91% TX-100, 10% glycerol, 30mM Tris pH 8.0 as outlined in Materials and Methods. The preparation was washed with Tris-Mg and a sample was positively stained for 2 min with 0.5% aqueous uranyl acetate. 9,000X.

Bar represents 1 um.

(Dingle and Larson, 1985). The two sheaves meet at the lower edge of the cytostomal opening and continue posteriorly to support the cytostomal membrane. Each sheaf, at its widest point, consists of about 25 microtubules and tapers to less than half that number towards the posterior. The microtubules have a typical outer diameter of about 27 nm which is well within the range of "normal" microtubules.

Other components of the cytostomal apparatus are the four anterior flagella and associated basal bodies. The flagella can be removed fairly easily but the basal bodies are tightly associated with the rest of the cytostomal apparatus. There are a number of striated rootlets (two long and approximately six short), some of which characteristically run in parallel with a microtubule for some distance with no visible space between them suggesting that there may be some connections between the two. There are also about 20 cross-fibres that run approximately perpendicular to the microtubules, extending for one-half to two-thirds of the width of one sheaf and originating just below the area where the two sheaves meet.

The most striking aspect of the cytostomal apparatus is the presence of the "linkers" that join adjacent microtubules (Fig. 3). Measurements of various characteristics of the linker-microtubule array have been summarized in Table 2. The linkers are numerous and semi-

Figure 3

High Magnification Linker Array

A high magnification view of the microtubules and linkers from the anterior single sheaf portion of the isolated cytostomal apparatus. Positively stained with 0.5% aqueous uranyl acetate. 110,000X.

Bar represents 0.1 um.

TABLE 2

DESCRIPTIVE STATISTICS OF THE ISOLATED CYTOSTOMAL APPARATUS*

	MEAN	STD DEV	N**	MIN	MAX	RANGE
LINKER LENGTH	107.2	15	600	60.3	153.9	93.6
MICROTUBULE SPACING	85.7	17	79	50.8	131.7	80.9
LINKER WIDTH						
OVERALL	9.3	5.0	368	0.8	28.3	27.5
MID	6.6	2.7	184	0.8	23.0	22.2
EDGES	12.0	5.3	184	0.8	28.3	27.5
LINKER SPACING	37.0	19.7	200	3.8	130.1	126.3

* All measurements are in nm.

** Six separate isolated cytostomal apparati were used with measurements divided equally between them. The measurements were made consecutively (when possible) from the anterior to the posterior of the apparatus with the exception of the microtubule spacing meaurements which were made consecutively across the width of the cytostomal apparatus.

regularly spaced. The linker array initially appears regular, but close examination reveals considerable irregularity. The average spacing between successive linkers is 37 nm, but the range of values is very great, from 4 nm to 130 nm. In fact, Figure 4 shows the distribution of the spacings and indicates that both extremely long and extremely short spacings are quite rare.

The linkers are not linear, but have a somewhat wispy, wavy appearance. They do vary considerably in length (60 - 150 nm), but have an average length of about 107 nm and this is fairly constant between individual cytostomal apparati. It should be noted that both extremely long and extremely short linkers tended to be grouped together. The clusters of extreme values were usually located in the same area of the cytostomal apparatus, frequently at the edge. Microtubule spacing and linker length are compared in Figure 5. Whereas the average linker length is 107 nm, the average intermicrotubule distance is only 85 nm, about 20% shorter. As might be expected, the linkers are generally not straight but rather have a characteristic wavy appearance.

The width of the linkers was difficult to measure both due to their fine nature and because a lot of variation existed even within one linker. Overall, the average width was 9.3 nm with values ranging from 0.8 to 28.3 nm. However, closer analysis revealed a pattern to this variation. The

Figure 4

Linker Spacing is Variable

Frequency distribution of linker spacing. Spacing between successive linkers was measured in six separate isolated cytostomal apparati (see Table 2). Measurements were made on projections of negatives using the MOP-3 digitizing unit.

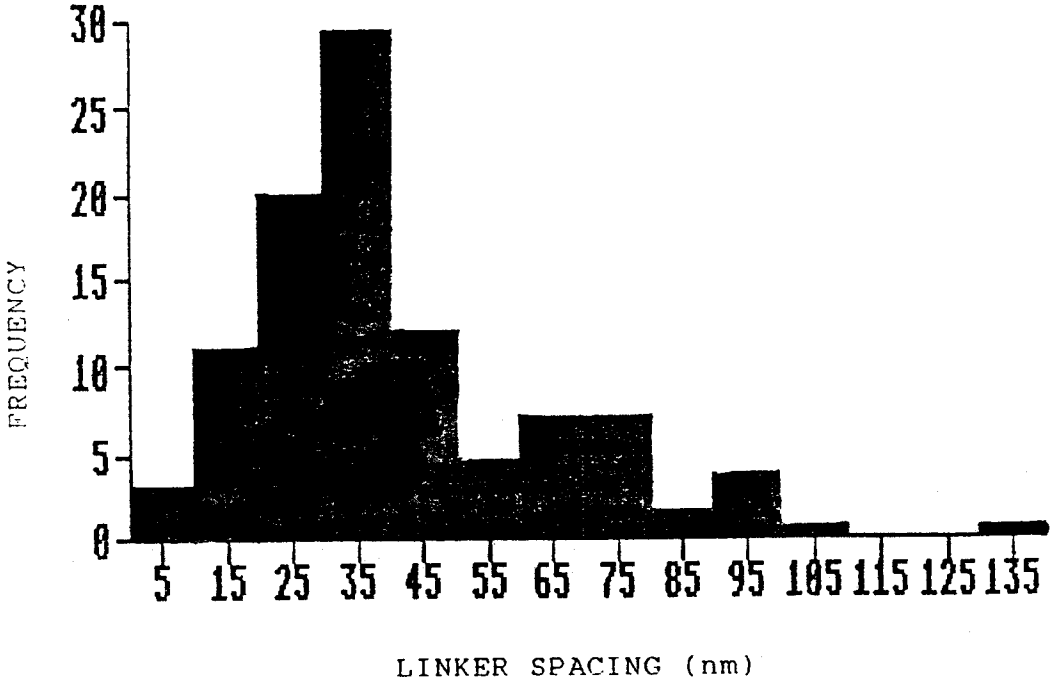
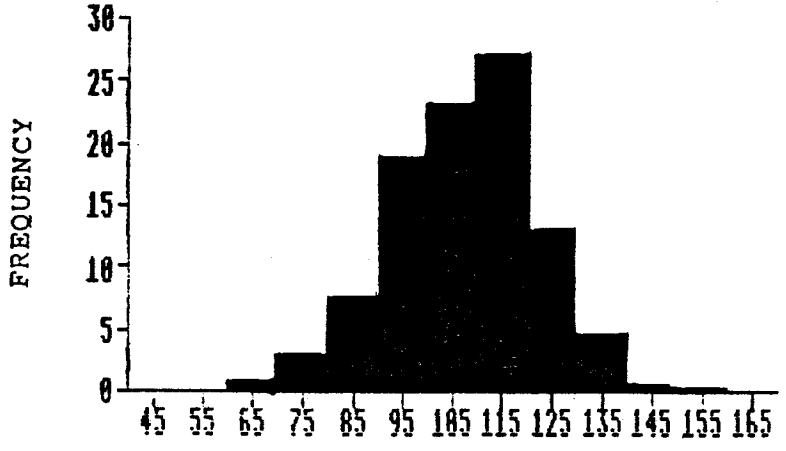

30
25
20
15
10
5
0
FREQUENCY
5 15 25 35 45 55 65 75 85 95 105 115 125 135
LINKER SPACING (nm)

Figure 5

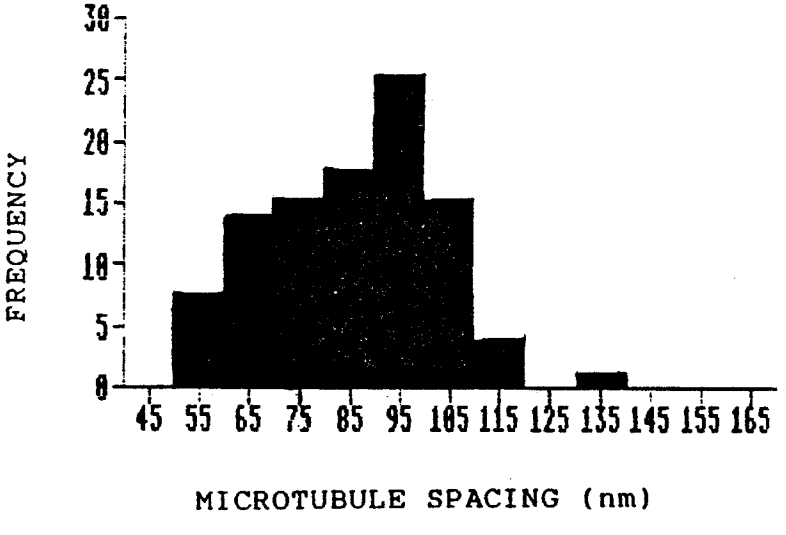
Linker Length (A) is Greater than Microtubule Spacing (B)

Frequency distribution of linker length and microtubule spacing. Linker length (A) and spacing between microtubules (B) were each measured in six separate isolated cytostomal apparati (see Table 2).

A LINKER LENGTH DISTRIBUTION



B MICROTUBULE SPACING DISTRIBUTION



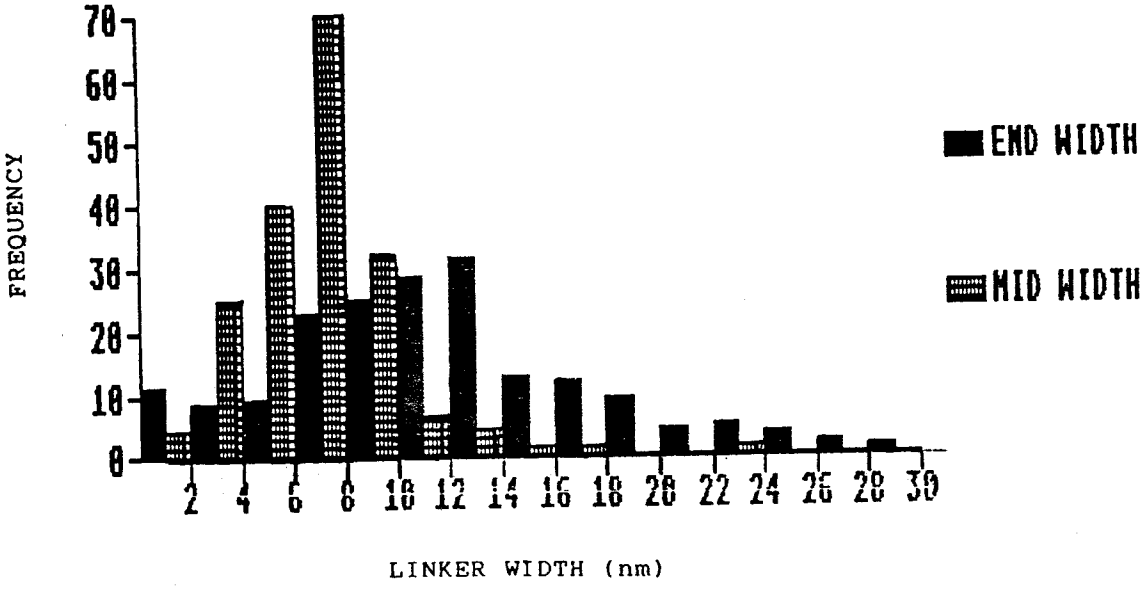
mid-regions had an average width of 6.6 nm. The ends of the linkers, particularly at the junction with the microtubule, tended to be both thicker and more variable - they average 12.0 nm with a standard deviation that is twice that of the mid-widths. The difference between mid and end-widths can be seen very clearly in Figure 6.

The cytostomal apparatus isolation is quite simple and the linkers are invariably present. However, certain variables must be controlled in order to achieve optimal results. Results have been considered to be optimal if the preparations are clean, relatively uniform with few apparent gaps in the linker lattice and if they exhibit microtubules that correspond well to appearances in the literature. Among these variables are pH, buffer, detergent type, cell density and length of starvation. In order to obtain relatively clean preparations, the use of starved cells was crucial. Even though the cells are washed free of most of the bacteria, some bacteria do remain in vacuoles in the cell. Starvation allows digestion to occur and thus yields a much cleaner lysate. It also renders the cell much more susceptible to detergent lysis - non-starved cells are much more resistant to detergents than are cells starved for 12 - 24 hours. Another important factor is cell density during lysis. Greater than 2×10^8 cells/ml yielded messy, damaged microtubules and linkers. The specific detergent used also affected the condition of the linker arrays. A number of

Figure 6

Linker Width Varies from Mid to End Points

Frequency distribution of linker width. Measurements were made in six separate isolated cytostomal apparati (see Table 2). The width was measured at the mid-point of the linker as it extends between the microtubules and at the end of the linker just before it attaches to the microtubule. No attempt was made to discriminate between the two ends of the linker.


FREQUENCY
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
LINKER WIDTH (nm)
END WIDTH
MID WIDTH

detergents were used and their effectiveness evaluated. Nonidet-P-40 (NP-40) sufficient to yield adequate lysis gave somewhat damaged arrays while Triton-X-100 was much more gentle with respect to the linkers. CHAPS (3-[(3-Cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate), used at the same concentration as TX-100 in order to yield adequate lysis, had the same effect as NP-40. Sarkosyl at this concentration was catastrophic - total solubilization - and thus no further attempts with lower concentrations were made. Both Brij 58 and Tween 20 were much more gentle than TX-100 - so much so that adequate lysis could not be obtained, even with a 10% detergent concentration. Of the detergents used, TX-100 was clearly the most useful in terms of attaining both adequate lysis and good linkers.

Both pH and buffer had an effect on the linkers. The optimal pH was found to be 8.0 and the best buffer was Tris. Tris was superior to Hepes when used at pH 8.0. Little difference was found between Hepes and Pipes. An increase in pH to 9.0 had a strikingly negative effect with both the linkers and microtubules "fading away". Lower pHs (7.3, 6.9) also had a negative effect on the linkers, although the microtubules were undamaged. The linker array became uneven at lower pHs - as if some linkers were missing. There was also an increase in "debris" - perhaps broken linkers - attached to the microtubules at lower pHs.

The optimal conditions for the isolation of cytostomal apparati were found to be the use of 0.9% TX-100 in Tris at pH 8.0. In spite of the differences found between the various preparations, even sub-optimal conditions yield linker arrays that exhibit most of the typical characteristics described above.

3.3 Tetramitus Ghosts

The cytostomal apparatus had the same appearance in ghosted cells as it did when isolated from the cell. Positively stained images revealed a gently curved set of cytostomal microtubules within a cage of cytoskeletal microtubules (Fig. 7). Linkers were readily apparent in the anterior, single sheaf region. The cytostomal microtubules can be thought of as tracing out a mirror image or backwards elongated "S". This gentle, almost S-shaped curve was also apparent in isolated cytostomal apparati. In this orientation, it was found that the nucleus was invariably located on the inside of the upper curve - in fact some bridges between the nucleus and cytostomal microtubules have been visualized with TEM. The cytostomal microtubules were seen to extend through two-thirds of the length of the cell.

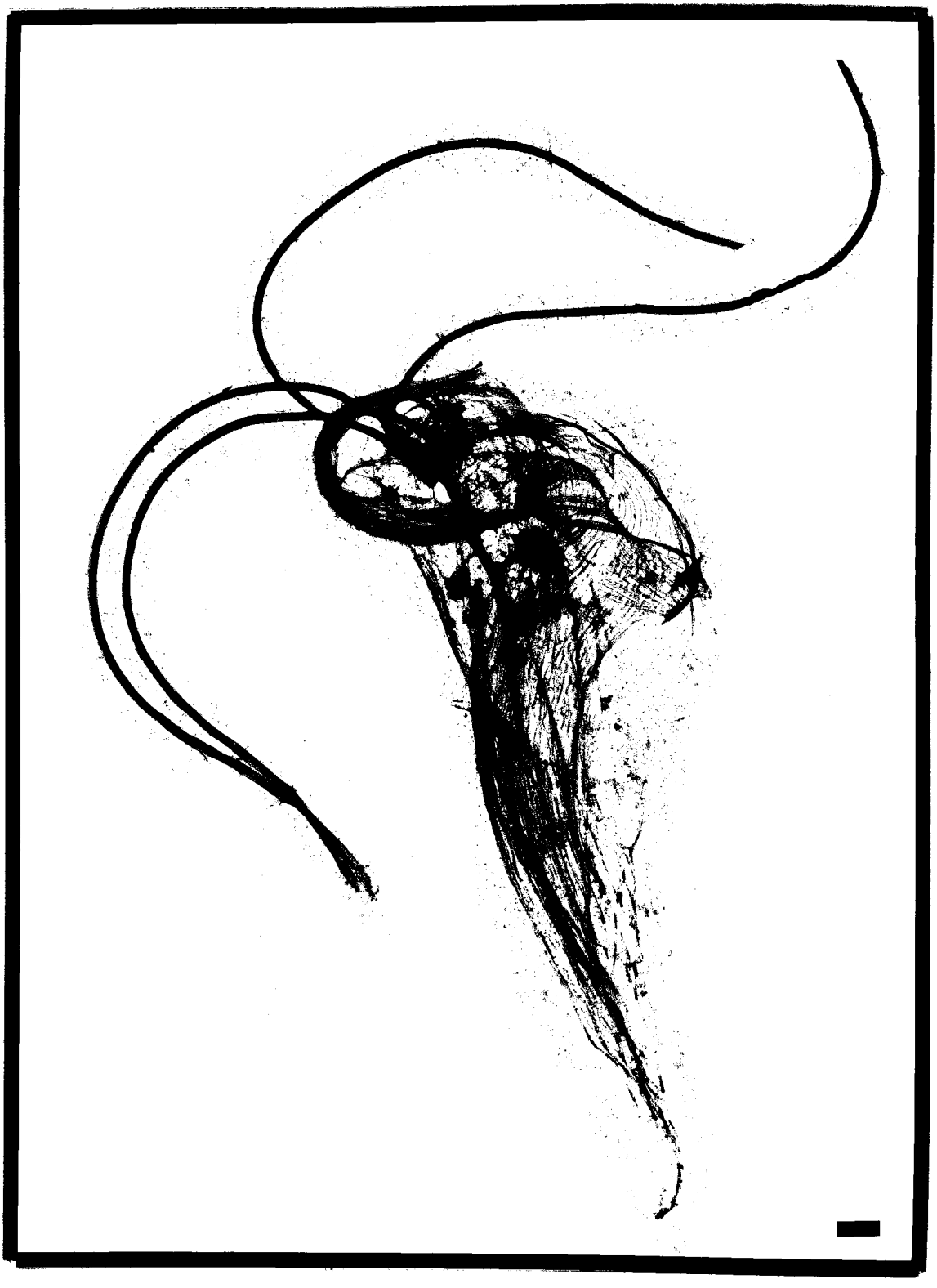
Tetramitus can be ghosted to varying degrees depending on the concentration of detergent used and the

Figure 7

Positively stained ghosted cell.

This cell was ghosted using a final concentration of 0.1% TX-100 in 30 mM Tris, pH 8.0, 2 mM EGTA, 5 mM $MgSO_4$. Positively stained with 0.5% aqueous uranyl acetate. The suggestion of a halo of material surrounding the specimen has been caused by the removal of the background in the rest of the micrograph. 7,200X.

Bar represents 1 um.



extent of agitation. Regardless of the extent of ghosting, using from 0.1% to 2.0% TX-100, the same overall view of the cytostomal apparatus emerges. Up to 2.0% TX-100 can be used without absolute cell lysis (*c.f.*, 0.9% TX-100, which is used to obtain isolated cytostomal apparati) because 5 mM $MgSO_4$ is present to help maintain cell shape *i.e.*, the cytoskeletal microtubules, and this in some way increases resistance to lysis.

Thin section electron microscopy shows that the ghosts are thoroughly extracted (Fig. 8). Most of the cytoplasm has been removed leaving primarily the microtubules, nucleus and a few large vacuoles. The amount of cytoplasm present does vary with the extent of ghosting but, in all cases, very little remains when compared to intact cells. Careful observation reveals linkers in these thin sections (Fig. 9a). Due to the nature of thin sections, arrays like those seen with positive staining cannot be expected. The plane of section is crucial and difficult to control. Thus, both the regularity of the array and the abundance of the linkers are not apparent in thin sections. Nevertheless, linkers can be found in those areas where the plane of section is fortuitous. The linkers are more difficult to discern in very lightly ghosted cells (0.01% TX-100) since more cytoplasm is obscuring them. Careful examination of micrographs (Fig. 9b) shows that linkers are

Figure 8

Thin-Sectioned Ghosted Cell

This cell was ghosted using a final concentration of 0.1% TX-100. The sample was prepared for thin-sectioning as outlined in Materials and Methods using 2.25% paraformaldehyde/0.5% glutaraldehyde as the primary fixative. N - nucleus; Bb - basal bodies; PM - plasma membrane; mt - cytostomal microtubules. 30,000X.

Bar represents 1 um.

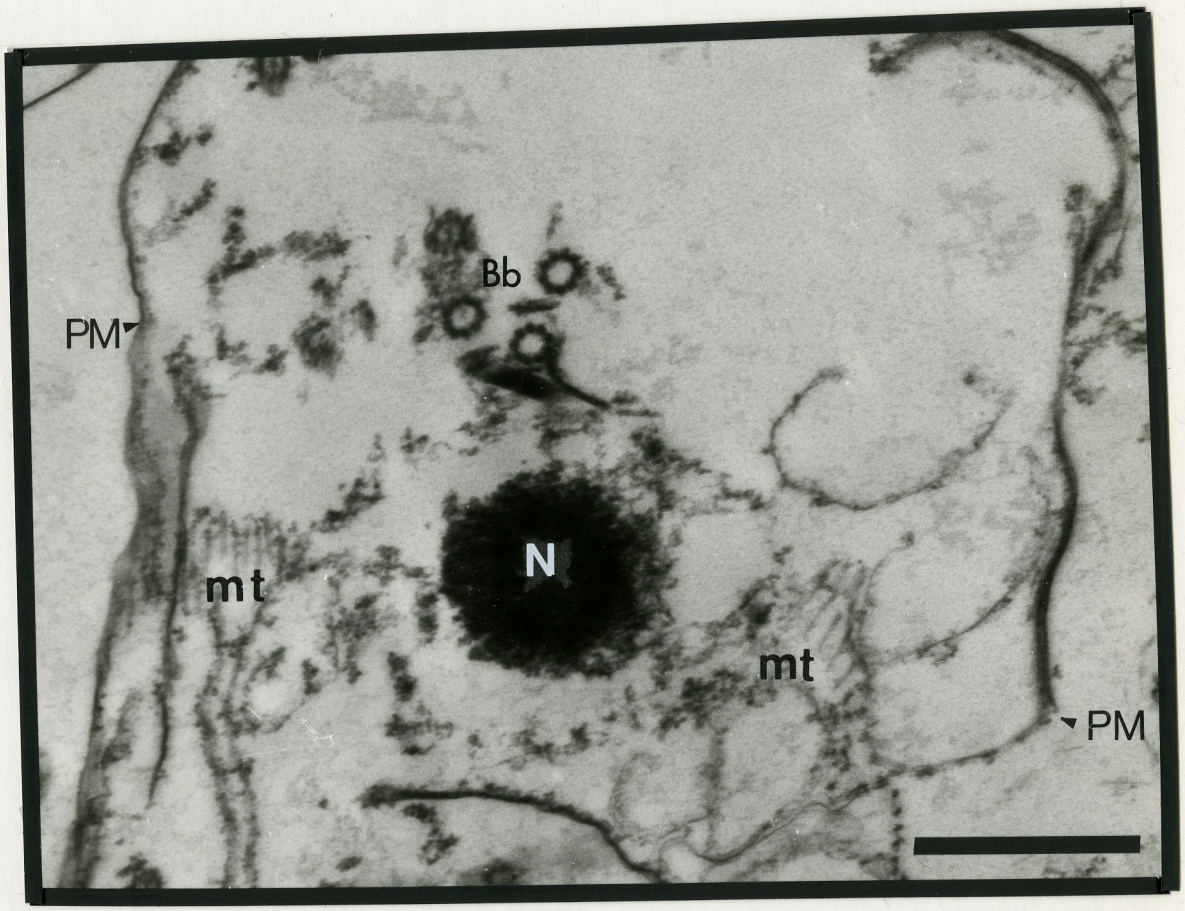

Bb
PM
N
mt
mt
PM

Figure 9

Linkers in thin sections.

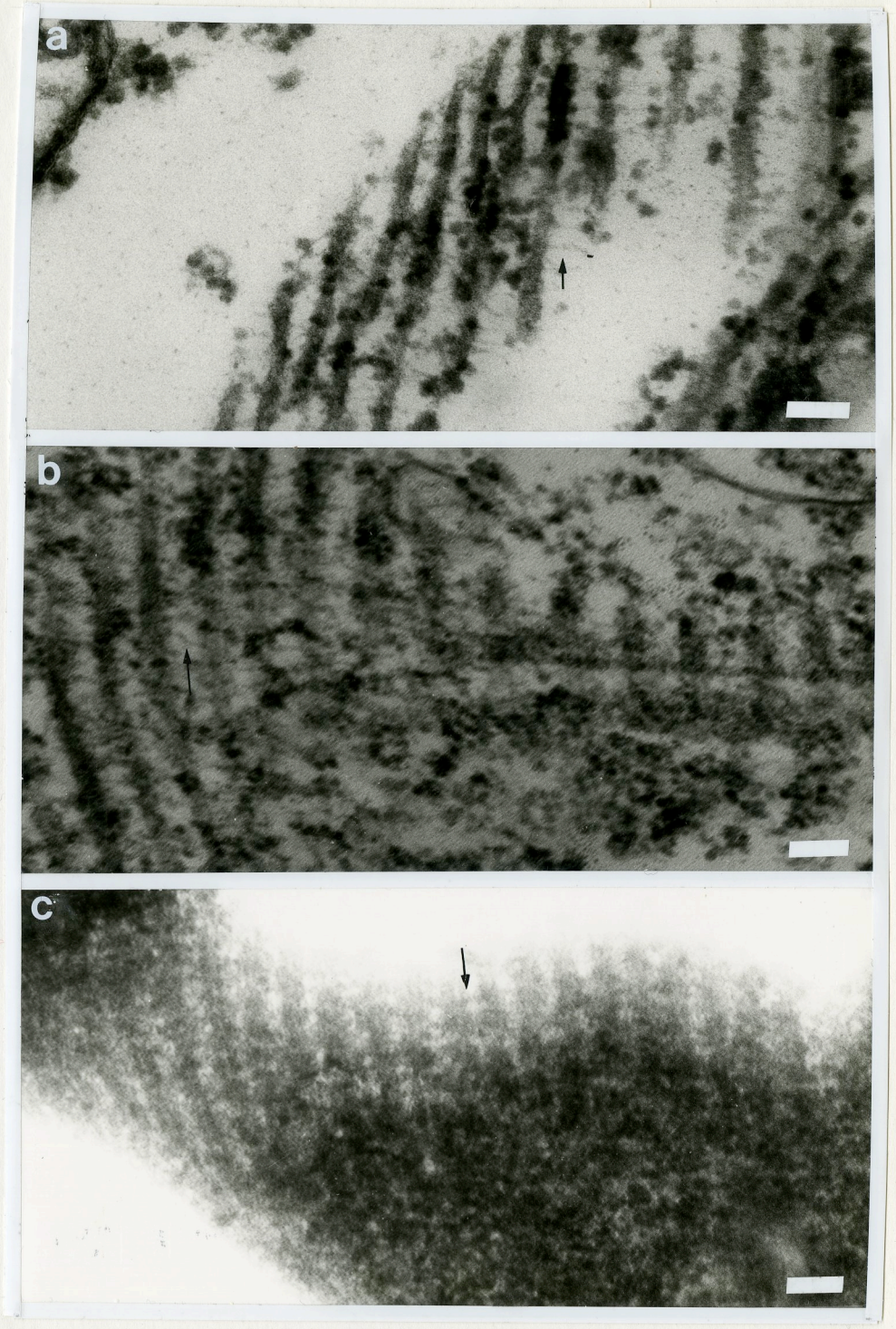
Each of these samples was prepared using 2.25% paraformaldehyde/0.5% glutaraldehyde as the primary fixative. Linkers are clearly visible (arrow) in 0.1% TX-100 ghosts (a). They are more difficult to discern but still apparent (arrow) in 0.01% TX-100 ghosts. In unghosted cells (c), it is very difficult to positively identify linkers although some hints of linkers are present (arrow).

a). 90,000X.

b). 90,000X.

c). 80,000X.

Each bar represents 0.1 um.


a
b
c

present in these 0.01% TX-100 ghosts. A through-focal series would show this even more clearly.

The same difficulty presents itself more forcefully when intact cells are observed. In thin sections, microtubules can be seen to line the cytostome. The microtubules are regularly spaced as in isolated cytostomal apparati and ghosted cells and maintain the same orientation. However, in this case connections between the microtubules are almost impossible to identify positively (Fig. 9c). Still, hints of linkers are present suggesting that they may simply be masked by the cytoplasm.

While it is possible that artifacts may be introduced in the act of removing the cytoplasm to facilitate observation, the essential image of the cytostomal apparatus remains unchanged throughout these different preparations. Each preparation adds more information but does not present any evidence that conflicts with observations from the other preparations. Thin sections of intact cells show most clearly that the gullet <u>is</u> lined with microtubules; ghosted cells are the most informative with respect to the organization/orientation of the cytostomal apparatus (and cytostome) within the cell; isolated cytostomal apparati are the most useful in terms of characterizing the linkers.

Chapter 4

CYTOSTOMAL MICROTUBULES ARE REMARKABLY STABLE

Microtubules are often classified and characterized according to their stability. Some microtubules, such as those in the spindle, are quite labile while others are very stable like those in cilia and flagella. Still other microtubules e.g., cytoskeletal, have stabilities that lie between these two extremes. As reported in the Introduction, microtubule stability is often thought to be mediated through the HMW-MAPs or other associated proteins. The cytostomal microtubules have been found to be extremely stable. While the basis for this stability has not been investigated, the effects of several agents/conditions that normally cause disassembly of cytoskeletal microtubules has been documented below.

Due to the lack of an enclosing membrane, isolated cytostomal apparati are excellent material for these experiments. The open nature of the isolated apparatus completely eliminates the permeability concerns inherent in some other systems. In addition, the cytostomal microtubules of the isolated apparatus can be examined at the EM level with a minimum of manipulation. Therefore, most of these experiments have been carried out with isolated cytostomal apparati, although ghosted cells were also used on occasion.

4.1 Cold Temperatures

Low temperatures (0 - 4°C) have traditionally been used to solubilize microtubules (Shelanski et al, 1973). However, not all microtubules can be solubilized in this manner. The cytostomal microtubules clearly belong to the cold-stable class. Not only can they be left on ice throughout isolation and experimentation, they can also be stored for three to four days at 4°C. The morphology of the microtubules suggests that they are perfectly normal and undamaged: they have a typical diameter (27nm) and have lengths measured in micrometers. Since the major effect of low temperatures on cytoplasmic microtubules is complete disassembly, usually within five minutes (Roth, 1967), the cytostomal microtubules are cold-stable.

The majority of the other cytoskeletal microtubules do not remain following the cytostomal apparatus isolation. This is true even when complete lysis is not attained. When lysis is incomplete, the result is not the well-defined ghost previously observed but simply a membrane-enclosed cytostomal apparatus. The difference between the two, of course, is the presence of the cytoskeletal microtubules. Occasionally, a few cytoskeletal microtubules are found but these are rare. It would seem that during the ghosting procedure most of the cytoskeletal microtubules are maintained at 0 - 4 °C by the 5 mM $MgSO_4$ that is present in the ghosting solution.

Although both the cytostomal and cytoskeletal microtubules can be considered to be cold-stable, the cytostomal microtubules are much more stable than the cytoskeletal microtubules.

4.2 Colchicine and Griseofulvin

The effects of colchicine and griseofulvin, classic microtubule "depolymerizing" agents, on the cytostomal apparatus have been investigated. Concentrations ranging from 10^{-6} M to 10^{-2} M, found to be effective in the literature (Dustin, 1978), were tested. Tetramitus is relatively unaffected by high (10 mM) colchicine or griseofulvin concentrations. A lengthy exposure to 10 mM griseofulvin or colchicine ($>$ 24 hours) of isolated cytostomal apparati left many microtubules intact (Fig. 10a). At the light microscope level, the apparati appeared similar to the controls but a decrease in density was observed. This may indicate that some of the apparati were being partially or completely disassembled.

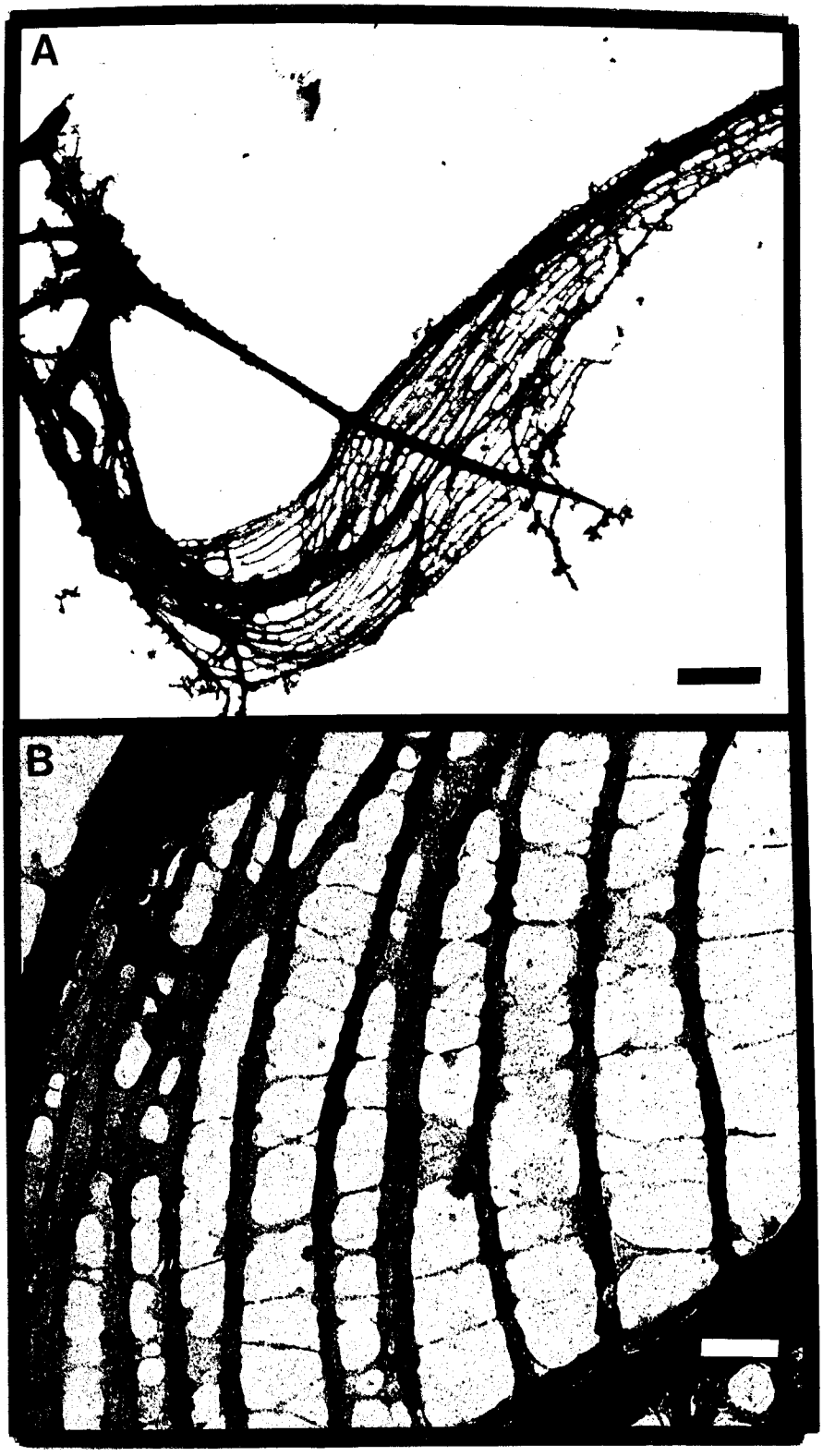
A much shorter period of exposure - 30 minutes - to 1 mM griseofulvin or colchicine had very little effect on the microtubules of the cytostomal apparatus (Fig. 10b). The number of microtubules was virtually unaffected and their length was only slightly diminished. Linkers could still be visualized although they were fewer in number, straighter and

FIGURE 10

Cytostomal Apparatus is Griseofulvin Stable

A). Positively stained isolated cytostomal apparati exposed to 10 mM griseofulvin for 24 h at room temperature. The isolated apparati were washed with Tris-Mg, resuspended in 30 mM Tris and then mixed 1:1 with 20 mM griseofulvin in 30 mM Tris, pH 8.0. 12,000X.
Bar represents 1 um.

B). Positively stained isolated cytostomal apparati exposed to 1 mM griseofulvin for 30 min. The apparati were prepared as in a) and mixed 1:1 with 2 mM griseofulvin in 30 mM Tris, pH 8.0. 110,000X.
Bar represents 0.1 um.


A
B

thicker than normal. Treatment with 5 x 10^{-5} M griseofulvin or colchicine had virtually no effect on the microtubules or linkers. When ghosted cells were treated with 5 x 10^{-5} M griseofulvin/colchicine and subsequently examined by fluorescence microscopy, a normal number of both cytostomal and cytoskeletal microtubules were observed.

4.3 Calcium

Calcium is also known to have a profound effect on microtubules. Microtubules lacking MAPs are rapidly disassembled by micromolar concentrations of calcium (Schliwa et al, 1981). Microtubules with MAPs are almost completed disassembled in 1 minute by 1 mM $CaCl_2$. The cytostomal microtubules were found to be calcium-stable. Treatment of isolated cytostomal apparati with even 50 mM $CaCl_2$ for 30 minutes did not eliminate the microtubules (Fig. 11a) although a decrease in density was once again observed with the light microscope. Certainly, there was some damage to the microtubules but they were definitely still present. The linkers appeared somewhat "stretched" and some of them may have been missing. Nevertheless, the presence of the linkers was still readily apparent. The microtubules did have a slightly uneven appearance, looking as if small debris had become stuck to them. The nature of this "debris" was not determined. Treatment with 10mM $CaCl_2$ yielded slightly less damage with the linkers exhibiting a more normal morphology.

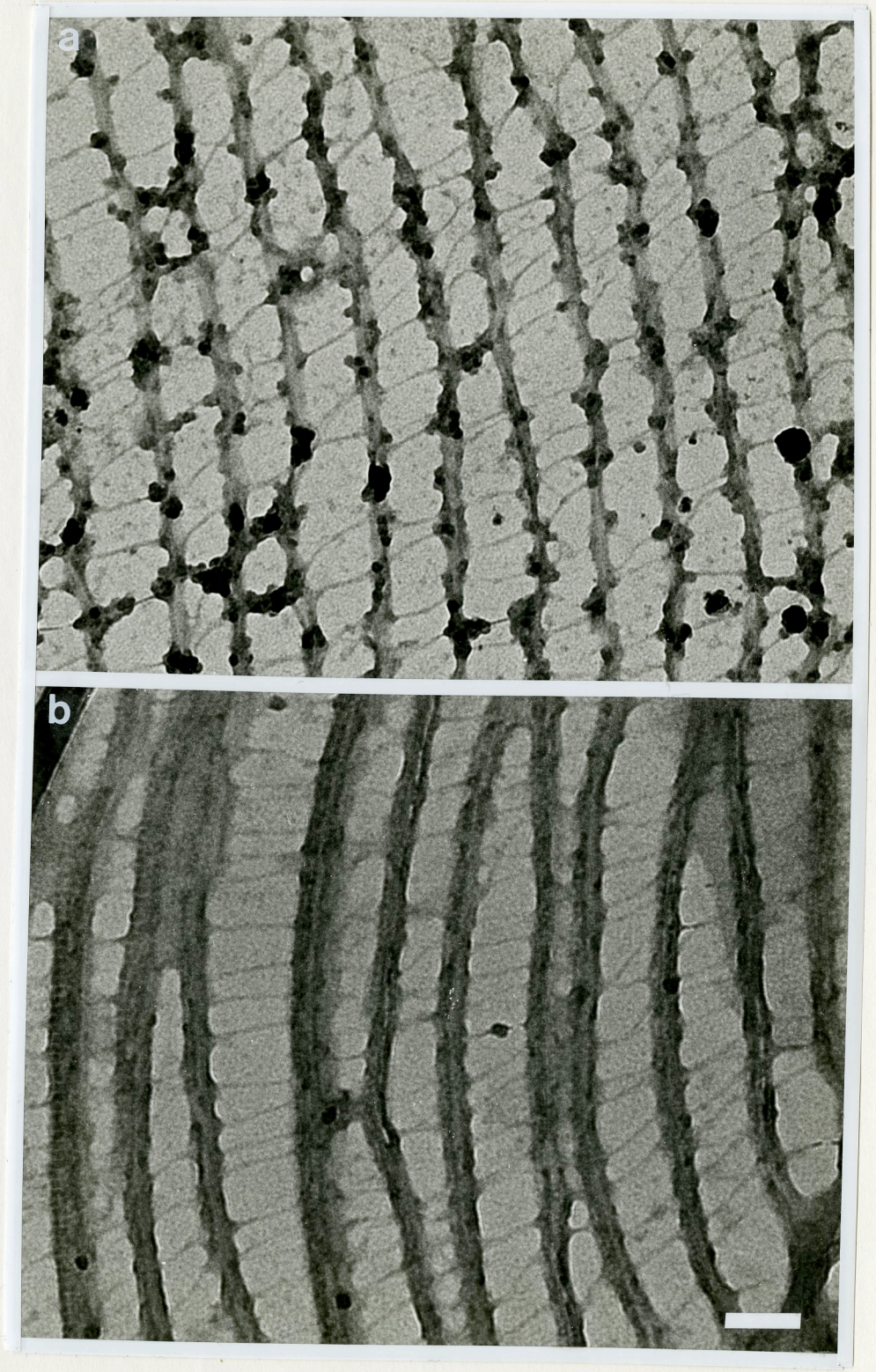
Figure 11

Cytostomal Apparatus Unaffected by Calcium Treatment

a). Positively stained isolated cytostomal apparatus exposed to 50 mM calcium for 30 min. 110,000X.

b). Positively stained isolated cytostomal apparatus exposed to 1 mM calcium for 30 min. 110,000X.

Bar represents 0.1 um.


a
b

Treatment with more physiological concentrations of 1 mM $CaCl_2$ (Fig. 11b) resulted in microtubules and linkers that were similar to the positively-stained array shown in Fig. 3. When these results are compared with the fact that 1 mM $CaCl_2$ disassembles many microtubule types in one minute, it is apparent that the cytostomal microtubules should be considered to be calcium-stable.

4.4 EDTA/EGTA

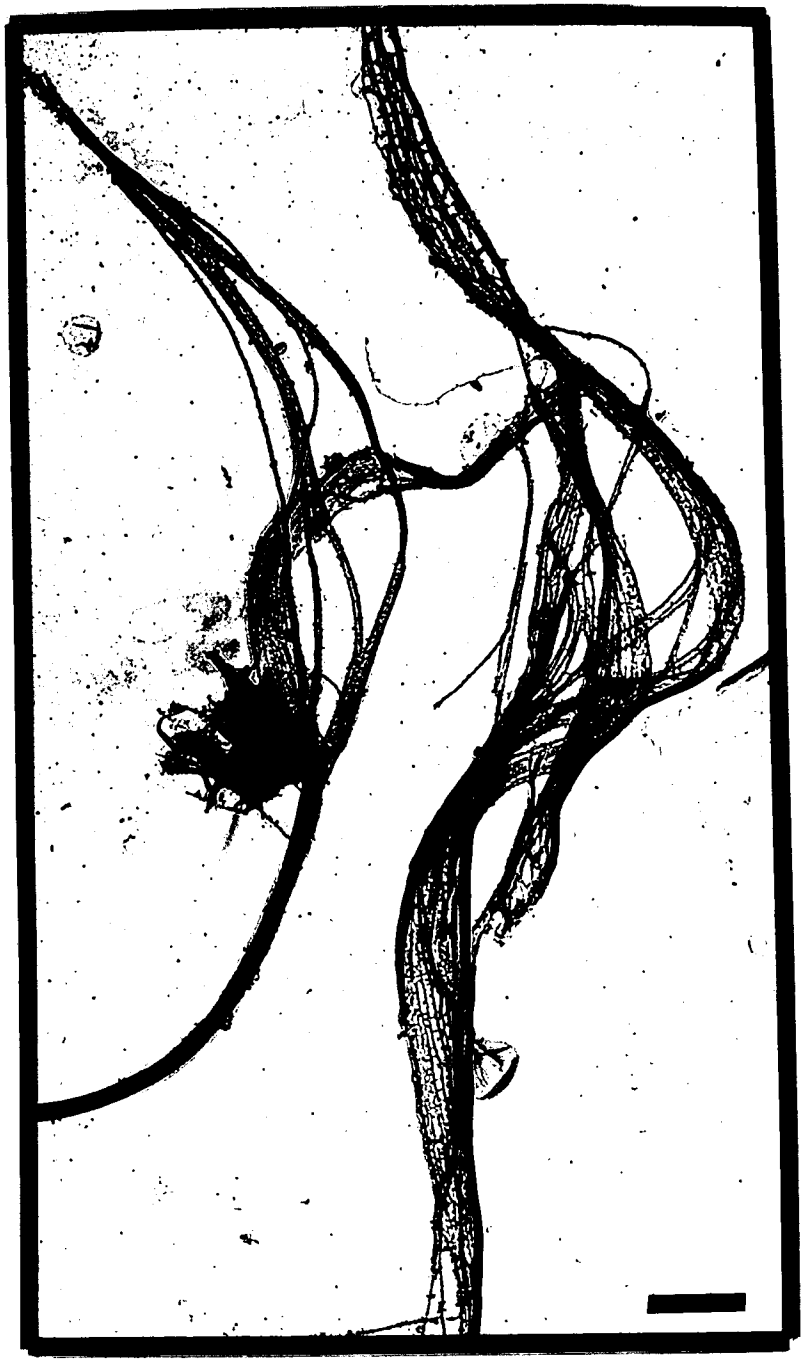
Since the divalent cations magnesium and calcium are important in microtubule stability, the chelating agents EGTA and EDTA would be expected to have an effect on microtubules. Treatment with 1 mM EDTA or EGTA for short periods of time (60 minutes) has very little effect on isolated cytostomal apparati. The microtubule-linker arrays are control-like in appearance. However, exposure for an extended period of time ($>$ 24 hours) has a very definite effect. The unit that has been referred to as the cytostomal apparatus is no longer a unit. With the light microscope, the apparati appear smaller, fainter and sometimes without basal bodies. This impression is confirmed with the electron microscope where it is seen that the two sheaves of microtubules have become separated from each other and, in fact, often from the basal bodies as well (Fig. 12). Some basal bodies were seen attached to just one or two microtubules. While the isolated cytostomal apparatus has, in effect, been torn apart, it is

Figure 12

EDTA Leaves Microtubules Intact

These isolated cytostomal apparati were exposed to 1 mM EDTA for 24 h at room temperature. Positively stained with 0.5% aqueous uranyl acetate. 13,000X.

Bar represents 1 um.



not clear that the microtubules have been disassembled. Many, many microtubules were present on the grid. Some were single sheafs (or lattices), others were in groups of two or three and still more were singlet microtubules. Thus, it was not certain that the microtubules were being disassembled - they may have all been present but were simply freed from the tight association that holds them together in the cytostomal apparatus. Wherever microtubules were in close proximity some linkers were present. The linkers were stretched taut and in places were broken or missing. Whenever the microtubules were separated from each other, even if they were attached to the same basal body, the linkers were no longer apparent.

Treatment with EGTA was less damaging than treatment with EDTA. The effects were similar but less pronounced as if the same events were occurring but more slowly. When treated with EGTA for an extended period of time, the groups each contained more microtubules i.e., there were fewer singlet microtubules and more single sheafs. Still, as with EDTA, it was difficult to determine if the microtubules were being disassembled or merely disbanded.

Chapter 5

OTHER FACTORS AFFECTING CYTOSTOMAL APPARATUS STABILITY

Cytostomal microtubules and their associated linkers do display some sensitivities. While they are resistant to many microtubule disassembling agents, the linkers turn out to be considerably more fragile when exposed to many common chemicals or treatments. This array is not as firmly held together as the previous chapter may have implied.

5.1 Sonication

The most catastrophic treatment was found to be sonication. A minimum level of sonication - ten seconds at the minimum setting - results in almost complete disassembly. Light microscope examination suggested that only the basal bodies remained and electron microscope examination confirmed that the basal body/rootlet complex was essentially intact. However, virtually no microtubules were observed.

5.2 Gradient Materials

During the course of the work on isolated cytostomal apparati, attempts were made to purify the preparation. Some of these experiments were attempts at selective solubilization. Others were attempts at a physical separation. Many density gradients were attempted and several gradient materials were employed. Unfortunately, it

was found that a number of the gradient materials had deleterious effects on the linker array.

Sucrose was found to be tremendously damaging to the isolated cytostomal apparatus. The entire array appeared shrivelled and torn. Therefore, sucrose was not pursued as a gradient material. Two iodinated gradient materials - Metrizamide and Nycodenz - were investigated with equally negative results. Treatment of isolated cytostomal apparati with either Metrizamide or Nycodenz, at the concentrations that would be necessary for a gradient, resulted in microtubule-linker arrays that were barely recognizable with both the linkers and microtubules badly damaged.

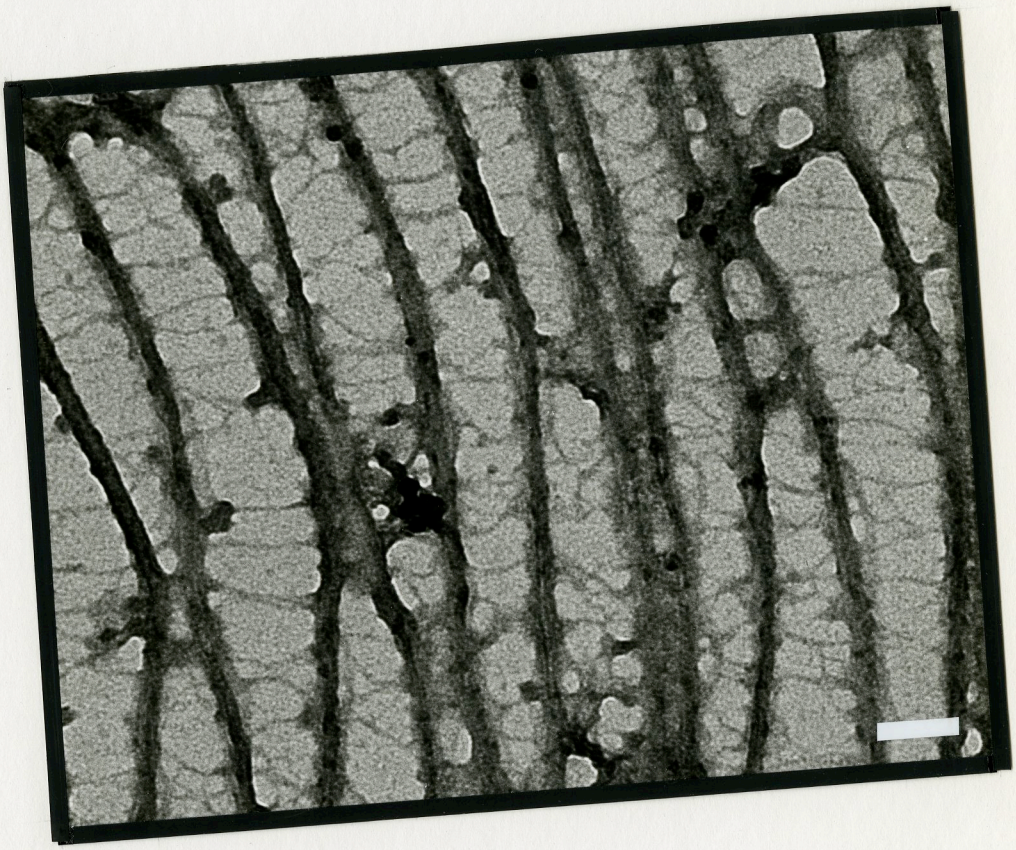
Due to the deleterious effects of sucrose, Metrizamide and Nycodenz, extensive attempts at gradient purification were not made with these materials. Ficoll, however, was investigated thoroughly. Treatment of isolated apparati with Ficoll resulted in linkers that were somewhat sub-optimal. While the microtubules were unharmed, the linkers were sometimes broken or missing in places (Fig. 13). Even so, the array had many of the typical characteristics and therefore gradients were attempted. A partial separation could be achieved with 5 - 15% Ficoll in 30 mM Tris, 3 mM $MgSO_4$ at pH 8.0. Up to 2 x 10^8 isolated cytostomal apparati in a maximum volume of 1 ml were layered on top of a 12 ml

Figure 13

Gradient Material Ficoll Damages the Cytostomal Apparatus

This linker array is from an isolated cytostomal apparatus exposed to Ficoll. The preparation was layered on a 5 - 15% continuous Ficoll (in 30 mM Tris, pH 8.0) gradient and centrifuged for 1 min at 300g in a clinical centrifuge. Positively stained with 0.5% aquoeous uranyl acetate. 110,000X.

Bar represents 0.1 um.



gradient which was then spun for 5 minutes at 300 g in a clinical centrifuge.

Since the purification with Ficoll was only partial and the linkers were sub-optimal, another gradient material was investigated. Glycerol had no deleterious effects on the linker-microtubule array - in fact it has a stabilizing effect. A superior separation was achieved with a glycerol step-gradient. A gradient of 30/60/80% glycerol in 30 mM Tris, 3 mM $MgSO_4$, pH 8.0 was overlaid with 1 to 2 x 10^8 cytostomal apparati in solution (as above) and spun for 20 min at 9150 g in a Sorval Superspeed RC-2B centrifuge with the HB-4 rotor. Membrane that escaped solubilization during lysis was completely banded at the 30/60% interface. Small debris banded here as well as at the upper 30% interface. While not all the cytostomal apparati banded together, many were present at the 60/80% interface. These were predominantly singlet apparati. Doublets, triplets and clumps of apparati were found progressively through the 80% glycerol layer. The recovered cytostomal apparati had microtubule-linker arrays that were control-like in appearance.

5.3 Low and high pH treatments

The optimal pH for the isolated cytostomal apparatus was found to be 8.0. The effect of slight variations in pH was reported earlier. To recapitulate, dropping the pH by

about one unit or less (8.0 to 7.3/6.9) resulted in some broken and/or missing linkers while the microtubules were apparently unaffected. The effect of larger changes in pH is documented below.

Decreasing the pH was found to damage the linkers much more than the microtubules. At pH 4.0, the morphology of the microtubules appeared virtually normal, but linker morphology was drastically altered (Fig. 14a). The linker array described in Chapter 3 was practically non-existent. In its place were broken, snapped, missing and faded linkers. Spherical blobs were attached to the microtubules, often where a missing linker could be hypothesized. Treatment with pHs between the optimal pH 8.0 and the severely damaging pH 4.0 resulted in linker arrays with intermediate characteristics progressively increasing the number of linkers affected with decreasing pH. Cytostomal apparati treated with pH 7.3, 6.9, 6.5, 6.0, 5.5, 5.0 and 4.5 were all examined. However, addition of 35 um Taxol to the solutions was found to stabilize the linkers to low pH (Fig. 14b).

Treatment with pHs above optimal damaged both the linkers and the microtubules. This time, the damage followed a completely different pattern. Exposure to pH 9.0 and above resulted in apparati that had a faded appearance (Fig. 15). This was noticed at the light microscope level and the

Figure 14

Low pH Damage (a) Can be Prevented with Taxol (b)

a). This cytostomal apparatus was isolated and washed with Tris-Mg as described previously. It was then resuspended in 30 mM Tris, pH 4.0 and allowed to sit at room temperature for 2.5 h. Positively stained with 0.5% aqueous uranyl acetate. 110,000X.

b). This cytostomal apparatus was treated as in a) but 35 um Taxol was present in the resuspending solution. Exposure is for 1 h. Positively stained with aqueous uranyl acetate. 110,000X.

Bar represents 0.1 um.

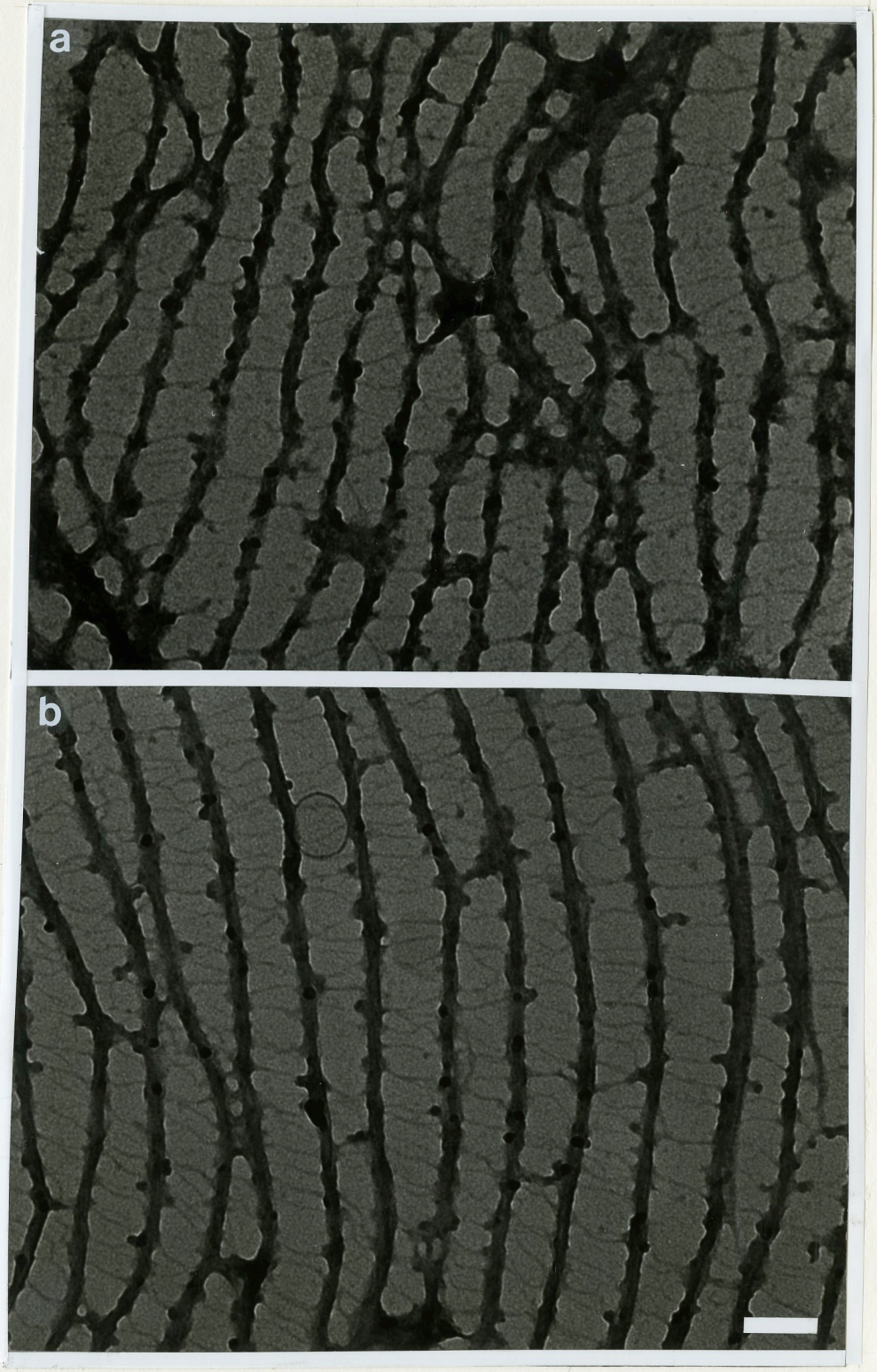
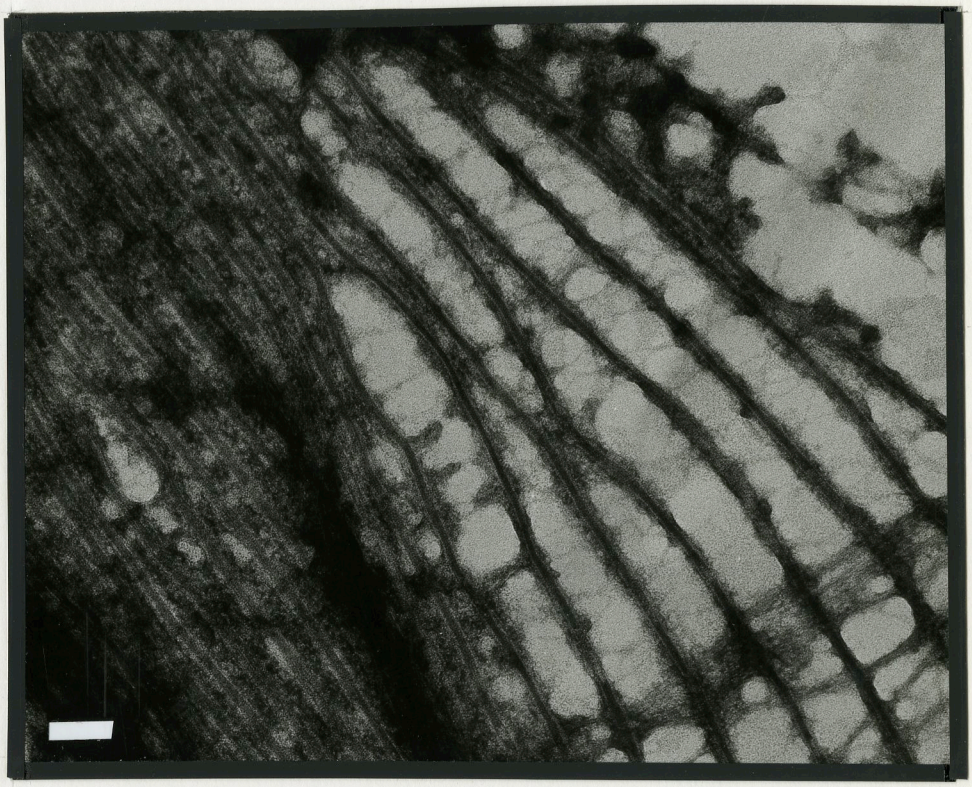

a
b

Figure 15

High pH Treatment Collapses the Linker Array

This apparatus was isolated and pelleted as previously described. It was then resuspended in 30 mM Tris, pH 11.0. This exposure is for 2.5 h. Positively stained with 0.5% aqueous uranyl acetate. 80,000X. Bar represents 0.1 um.



cause became apparent when the preparation was examined with the electron microscope. There appears to be a general solubilization of both linkers and microtubules that increases with duration of exposure. The linkers are not snapped or broken, nor do "blobs" decorate the microtubules. The extent of the solubilization also depends on the pH - the higher the pH, the more rapid the solubilization. The same basic pattern was followed for pHs 9.0, 10.0, 11.0 and 12.0. Addition of taxol did not completely stabilize the apparati to high pHs, for the most part, it merely delayed the previously observed effects. The microtubules and linkers seemed to be equally stabilized.

5.4 Urea

Relatively low concentrations (0.15 M) of the protein denaturing agent urea have been found to disassemble microtubules while allowing for reformation after its removal (Shigenaka et al, 1971). A different study, using 0.8 M urea, and a different system, found no effect on assembled microtubules (Roth, 1967). There is system variability with respect to susceptibility to urea. In this case, the cytostomal microtubules were found to be quite susceptible. While not completely disassembled by exposure to 0.15 M urea for 60 minutes, the cytostomal microtubules were badly damaged and fewer in number. The linkers were gravely affected - there were considerably fewer of them and they are

more difficult to discern. There was a loss of density at the light microscope level and the apparati took on a ragged appearance. Urea concentrations of 0.8M were also used and while many of the microtubules were intact after 30 minutes, they were very badly damaged by 60 minutes (Fig. 16). Thus, although the microtubules and linkers were not completely destroyed by urea treatment, the damage was extensive and the cytostomal microtubules should be considered to be urea-sensitive.

5.5 Salt

Exposures to high salt concentrations (0.25M NaCl/KCl for 30 minutes) badly affects the microtubules - they are broken and are very uneven (Fig. 17). At the light microscope level, the preparation actually looks cleaner while the cytostomal apparati look somewhat fainter. The linkers are still readily apparent and appear almost normal. Treatment of this type reveals a previously unobserved longitudinal element that has been separated from the microtubules as seen in Figure 17. The thickness of the longitudinal elements appears to be approximately the same as that of the linkers at their midpoint i.e, approximately 7 nm. These longitudinal elements run the full length of the microtubules.

Lower salt concentrations still have a deleterious effect on the microtubules. Treatment with 100 mM NaCl or KCl for 10 minutes yields poor microtubules although the

Figure 16

Urea Treatment Leaves Cross-Fibres Intact

This positively stained isolated apparatus (a) was exposed to 0.8 M urea in 30 mM Tris, pH 8.0 for 60 min. 13,000X. Bar represents 1 um.

The cross-fibres are left essentially intact (b). 30,000X. Bar represents 0.5 um.

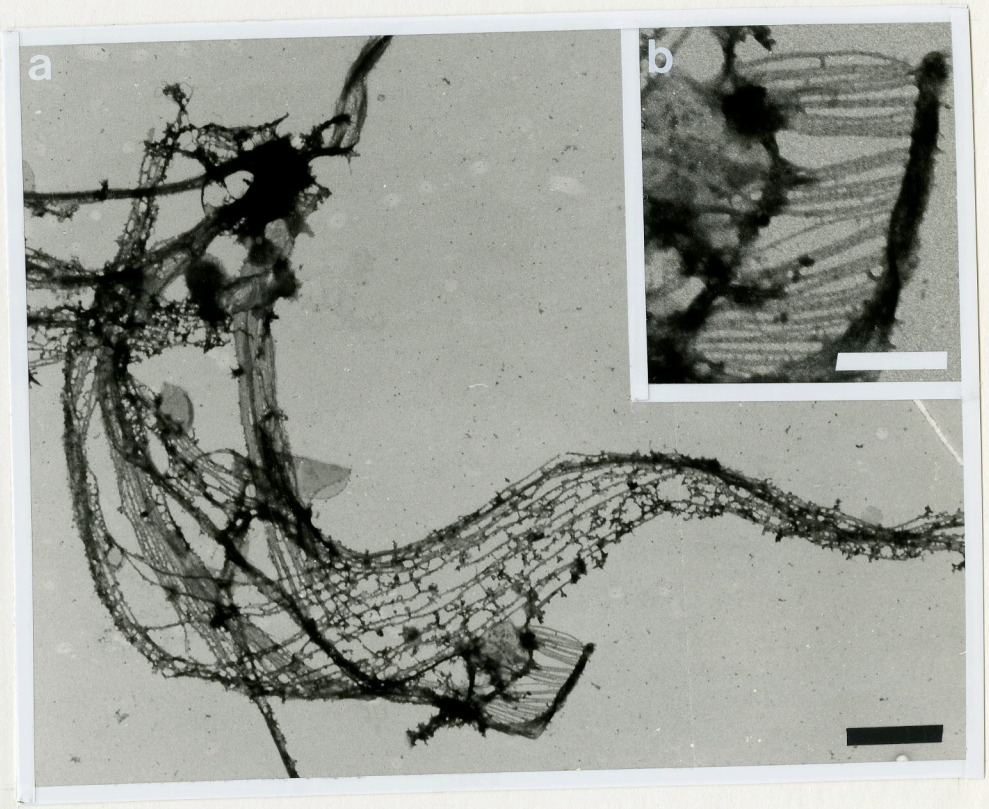
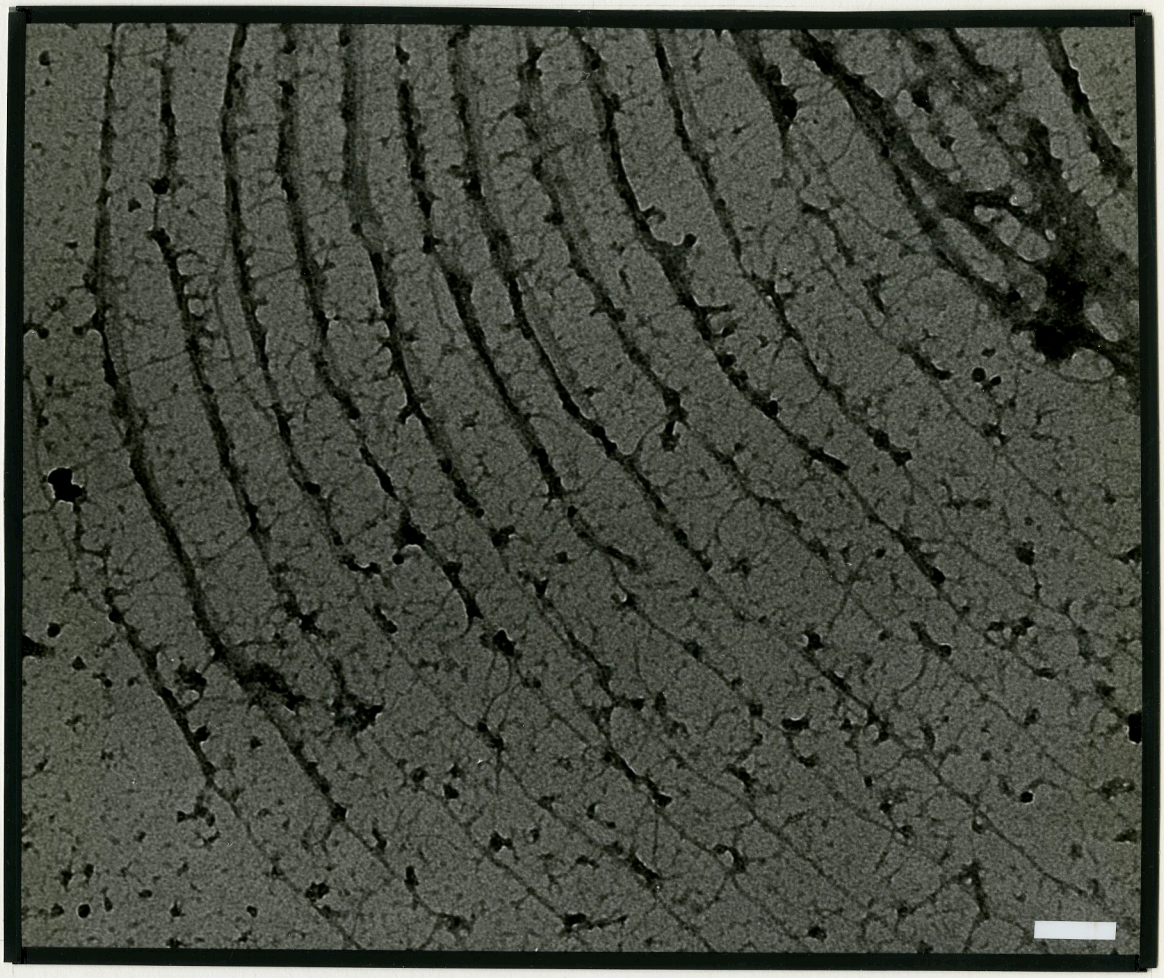

a
b

Figure 17

High Salt Extraction Reveals Longitudinal Elements

A positively stained isolated cytostomal apparatus treated with 0.25 M NaCl for 30 min. 110,000X. Bar represents 0.1 um.



linkers still look quite normal. A drop in salt concentration to 50 mM decreases the rate of damage such that apparati treated for 10 minutes have an appearance similar to control arrays. All samples treated with salt show the previously unobserved longitudinal elements. Both the cytostomal microtubules and linkers could be stabilized to 100 mM NaCl or KCl by addition of 50 μM Taxol.

Chapter 6

LINKER MORPHOLOGY CAN BE DRASTICALLY ALTERED BY FIXATIVES

The effect of various fixatives on the cytostomal apparatus was investigated. The fixatives used included osmium tetroxide, osmium ferricyanide and the aldehydes glutaraldehyde and paraformaldehyde. The aldehydes were used both separately and in combination with each other. Most exposures were for 60 minutes since this is the duration usually required in a thin sectioning fixation protocol. All results reported below are from experiments carried out at room temperature although similar experiments performed on ice yielded similar results.

6.1 1% Osmium tetroxide

Treatment with 1% OsO_4 for 30 minutes preserves the linkers quite well (Fig. 18a). They do appear to be a little less wavy than the control linkers and some of them have been broken. Spherical debris was present on both the microtubules and the linkers. The microtubules are very well preserved. Continued exposure for 60 minutes results in microtubules and linkers that had a faded appearance.

6.2 Osmium ferricyanide

Osmium ferricyanide was used at 0.5% OsO_4 and 0.8% $K_3Fe(CN)_6$ (MacDonald, 1984). This combination preserves the

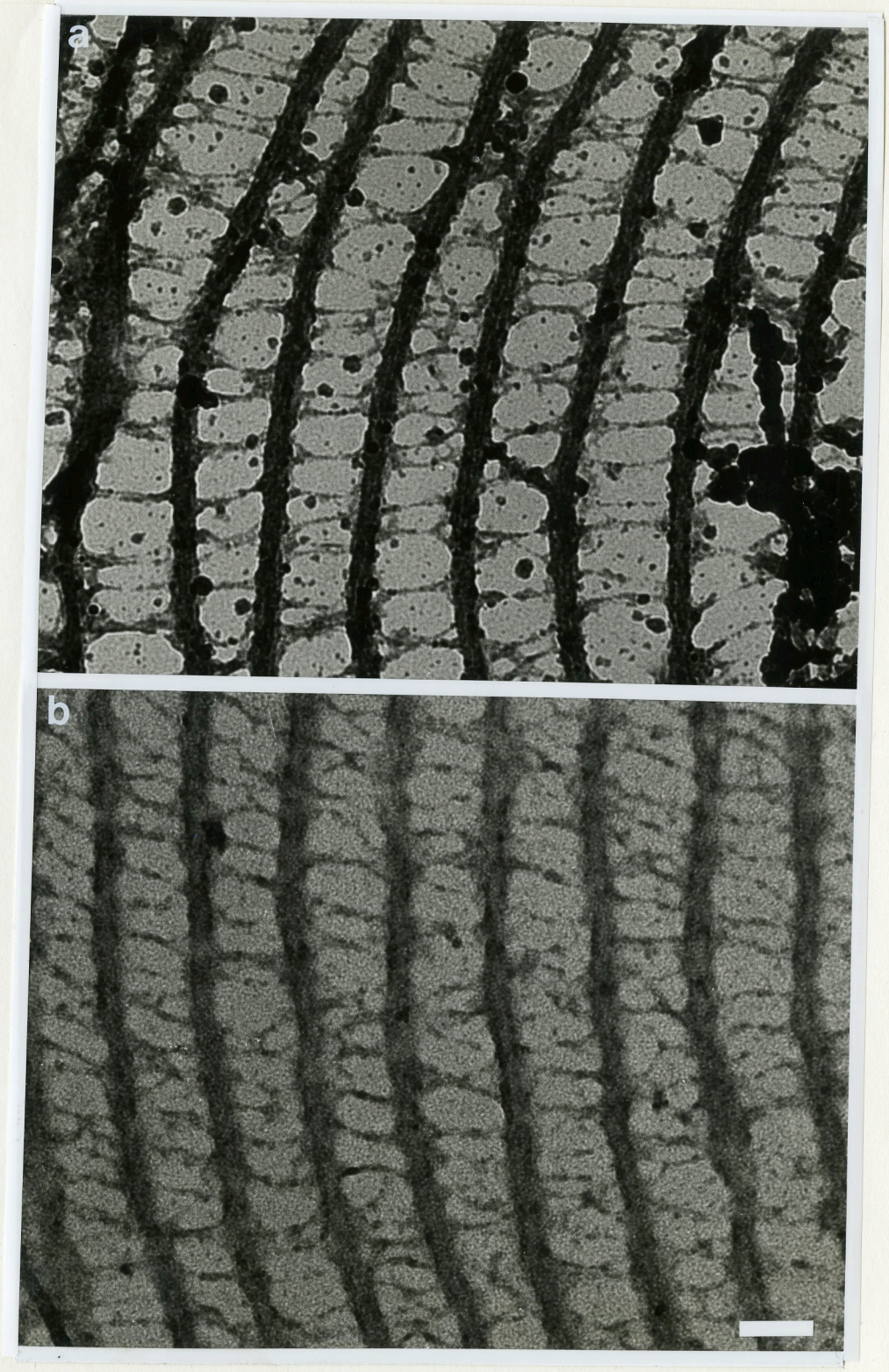
Figure 18

Osmium Damages Array but Leaves Linkers Intact

a). A positively stained, isolated cytostomal apparatus treated for 30 min with 1% OsO_4 in 30 mM Tris, pH 8.0. 110,000X.

b). A positively stained, isolated cytostomal apparatus treated with 30 min with 0.5% OsO_4, 0.8% $K_3Fe(CN)_6$ in 30 mM Tris, pH 8.0. 110,000X.

Bar represents 0.1 um.


a
b

linkers and microtubules extremely well. While the method used did tend to result in some precipitation, the linkers are most definitely present after both 30 and 60 minutes. In fact, they appear to be somewhat thicker than normal as if they have been coated with something (Fig. 18b). The microtubules also have this appearance. It was noted that the linkers tended to be straighter than usual, having lost some of their waviness. Thus, while both the linkers and microtubules appeared to have been preserved, they are now coated and seem to have lost some of the typical characteristics.

6.3 Glutaraldehyde

This most generally used primary fixative has a deleterious effect on the linkers at the commonly used concentration of 2%. Treatment for 5 minutes seems to have little effect, but by 15 and 30 minutes the appearance and distribution of the linkers has changed drastically (Fig. 19a and b). The linker distribution is no longer uniform (or "semi-regular") - it is haphazard as if some units have been randomly removed. The linkers that are present no longer exhibit the fine, wavy appearance that characterized the control linkers. The glutaraldehyde-treated linkers are much straighter and, in fact, often appear to be "stretched" almost to the breaking point. The microtubules, while apparently undamaged, are decorated with considerable amounts

Figure 19

Aldehyde Effects on Linker Morphology.

All micrographs are of positively stained, isolated cytostomal apparati treated with aldehydes at room temperature.

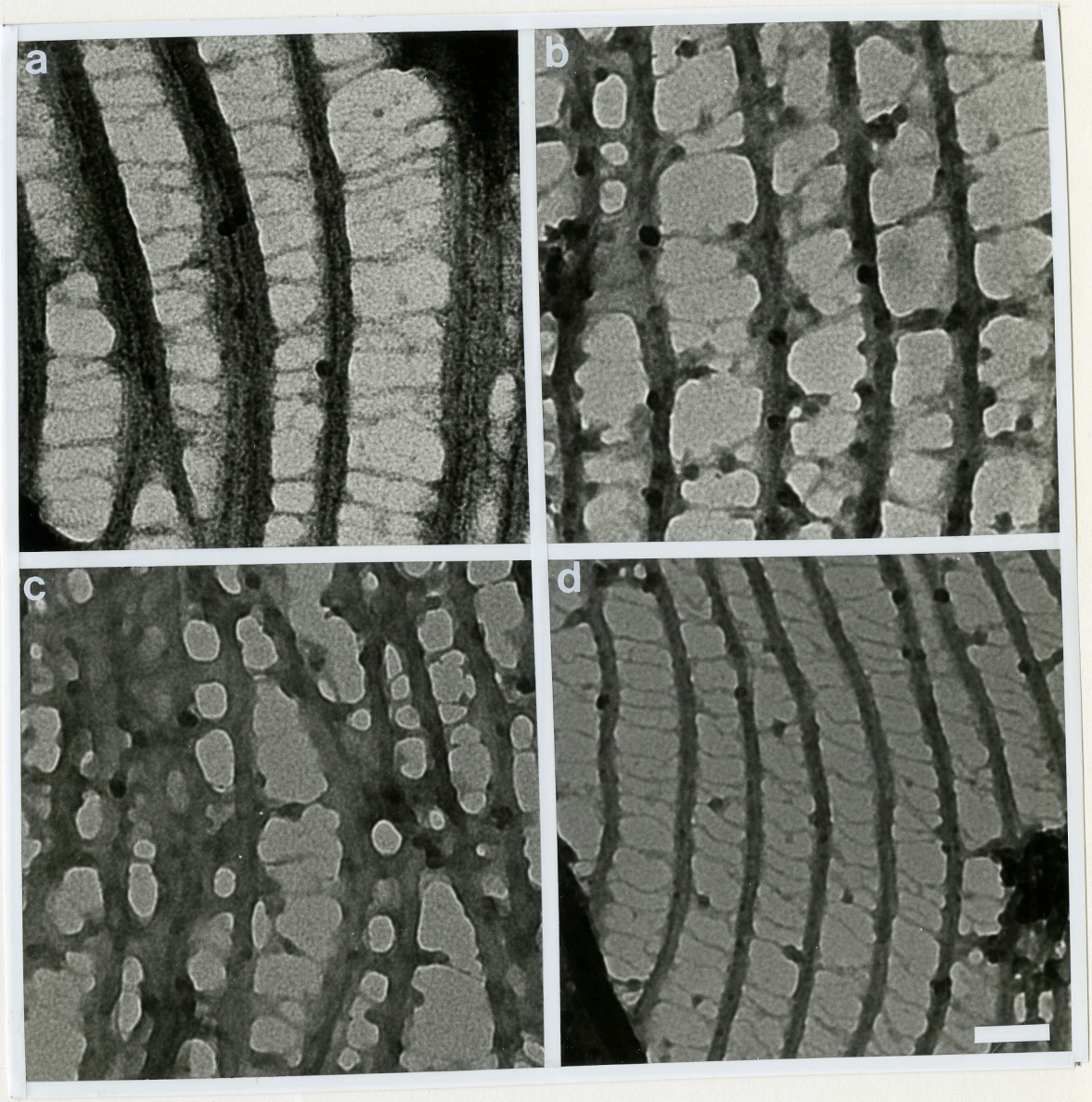
a). 5 min exposure to 2% glutaraldehyde in 30 mM Tris, pH 8.0. 110,000X.

b). 30 min exposure to 2% glutaraldehyde in 30 mM Tris, pH 8.0. 110,000X.

c). 60 min exposure to 2% glutaraldehyde in 30 mM Tris, pH 8.0. 110,000X.

d). 30 min exposure to 2.25% paraformaldehyde/0.5% glutaraldehyde in 30 mM Tris, pH 8.0. 110,000X.

Bar represents 0.1 um.


a
b
c
d

of material. Most of this takes the form of small blobs that are seen occasionally on control microtubules. These blobs are relatively uniform in size - approximately 22 nm in diameter. Almost everywhere they are present, a missing linker could be hypothesized. Continued exposure exaggerates these effects. Treatment for 60 minutes (Fig. 19c) results in a badly damaged array with very few linkers and with the microtubules littered with debris. A lower concentration of glutaraldehyde - 0.5% - results in the same pattern as 2.0%. The damage is not as extensive as with 2.0% although, almost paradoxically, it becomes evident more quickly with missing linkers being noted after only 5 minutes of exposure. A 60 minute exposure shows the now familiar pattern of missing linkers and the appearance of blobs on the microtubules. In this case, however, it is not as extensive as it was with 2.0% glutaraldehyde.

All these effects were observed using positive staining of isolated cytostomal apparati. The light microscope appearance is deceiving, especially in the early stages. The apparati look control-like except towards 60 minutes where they begin to look a little uneven. In addition, thin sections of ghosted cells treated with 2.0% glutaraldehyde were examined. In all cases (1.0%, 0.1% and 0.01% TX-100), no linkers were observed (Fig. 20c and d). The microtubules could be clearly visualized and some debris

Figure 20

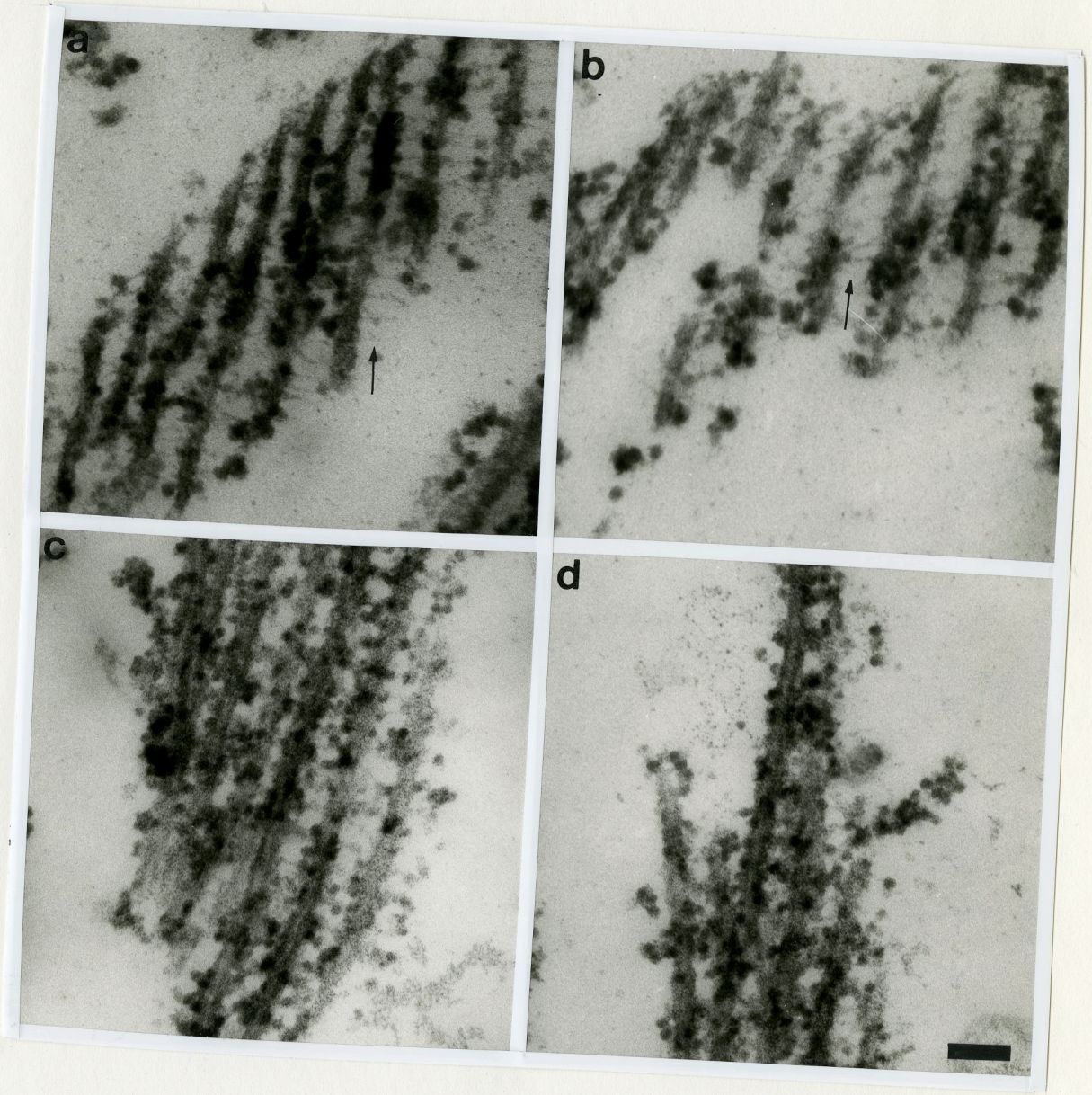
Linkers in thin sections.

0.1% TX-100 ghosts were prepared for thin-section TEM as described in Materials and Methods.

a) and b). The primary fixative used was 2.25% paraformaldehyde/0.5% glutaraldehyde. Linkers are readily apparent (arrows). 90,000X.

c) and d). The primary fixative used was 2% glutaraldhyde. 90,000X.

Each bar represents 0.1 um.


a
b
c
d

was seen surrounding them. While other fixatives (see below and Fig. 20) do allow identification of linkers in sectioned ghosts, none could be found in glutaraldehyde-fixed specimens.

6.4 Paraformaldehyde

This alternative to glutaraldeyde was investigated at 0.5% and 2.25% concentrations. Treatment for 1 minute with 0.5% resulted in damage that was as general as it was severe. In fact, all that was visible at the EM level was an amorphous mass. This was identified as a cytostomal apparatus for several reasons: the material was grouped in the approximate shape of a cytostomal apparatus; the debris was not found as a general background; nothing else resembling cytostomal apparati could be found although light microscope observations indicated that something was present. By 10 minutes, nothing even remotely resembling a cytostomal apparatus could be seen even at the light microscope level.

Treatment with 2.25% paraformaldehyde was less destructive although the cytostomal apparati did not emerge unscathed. The pattern was somewhat different from that with glutaraldehyde. By 15 minutes of exposure, the linkers were disappearing and becoming less prominent but there was no concomitant increase in blobs decorating the microtubules. However, with continued treatment (30 to 60 minutes), the

blobs once more become apparent, accompanied by an increased "loss" of linkers.

6.5 2.25% Paraformaldehyde and 0.5% Glutaraldehyde

This mixture of primary fixatives yields better results than either fixative alone. Exposure for 5 to 15 minutes resulted in microtubule-linker arrays that were indistinguishable from controls. Even a 30 minute treatment yields cytostomal apparati that are relatively undamaged (Fig. 18d) especially when compared with those treated with either fixative alone. Continued exposure for 30 minutes revealed that some damage was occurring. While, as in the controls, few blobs were present, the linkers now had a different appearance. They were quite straight and appeared to be stretched almost to the breaking point. These linkers had the same appearance as do the linkers that remained following glutaraldehyde treatment. It seemed likely that further exposure to the paraformaldehyde/glutaraldehyde mix would cause a loss of linkers and the decoration of microtubules.

When this mixture of fixatives was used to fix ghosts that were subsequently thin sectioned, linkers could be visualized (Fig. 20a and b). The visibility of the linkers was dependent on the degree of extraction of the ghost - the less cytoplasm that was present, the more clearly the linkers could be seen. This effect can be seen in Fig. 9a, b and c.

Chapter 7

Reactivated Tetramitus

7.1 Reactivated Flagella

Motility is often a characteristic of microtubule-based systems and thus it is possible that the cytostomal apparatus undergoes some form of movement. If this is so, the linkers are the prime candidates for the production of the active force. In order to determine if movement is occurring, the linkers and microtubules must be examined under conditions that are most likely to induce such movement.

The flagella of ghosted cells can be induced to beat upon addition of ATP. Although the basic procedure is outlined in Materials and Methods, the relative success of reactivation depended on many variables. These effects are documented below. Not all the parameters are known - differences in reactivation success were sometimes noted in spite of seemingly identical protocols. In fact, occasionally the phenomenon would not occur at all. Usually though, reactivation was quite successful with more than 70% of the ghosts having reactivated flagella. Under the right conditions (see below), this percentage could be increased to greater than 90%.

The "percentage of cells reactivated" or "percent reactivated" reported in this section refers to the percentage of ghosted cells (or, occasionally, isolated cytostomal apparati) with one or more flagella undergoing movement. The reactivation rarely included all four flagella. The majority of the cells - 65% - would have just one beating flagellum. About half as many cells (32.4%) would have two beating flagella and very few (3.0%) would have three of the four flagella beating. These proportions hold true for trials with a good percent reactivation (greater than 70%). In less successful trials, especially with fewer than 50% reactivated, the vast majority (generally above 80%) of the reactivated cells had only one beating flagellum. While it is possible that one specific flagellum was most likely to be reactivated, no attempt was made to determine if this was so and if so, to identify the flagellum.

While it was not quantified, the beat frequency was also found to vary with the success of reactivation. In some cases, the beat frequency was slow enough to clearly see a wave being propagated along the flagellum. At other times, the flagellum itself could only be visualized as a blur due to the rapidity of the beating. Under optimal conditions, the beating was strong enough to propel the ghost through the medium, although at a lower speed than live, swimming cells.

The extent of reactivation was controlled, both positively and negatively, by the presence and concentration of a number of substances in the ghosting and reactivation solutions (Fig. 21 and Table 3). Increasing the ATP concentration from 2 mM to 4 mM could cause the number of cells reactivated to rise from 70% to 90%. Further increases in ATP concentrations were considerably less dramatic giving an increase of only a few percent. Likewise, increasing the EGTA concentration from 1 mM to 2 mM increased the reactivation percent from 65 to 86%. A further increase to 4 mM resulted in an additional 7% enhancement to 93%.

The most effective of all the substances with positive effects was KCl. NaCl had a similar effect although not quite as pronounced. Addition of 12.5 mM KCl would usually cause an increase of 20% (70% to 90%) in percent reactivation. It was also found that addition of KCl to very poorly reactivating samples would bring them to a normal level (70%) of reactivation. Higher levels of KCl (up to 50mM) would raise the percent reactivation to greater than 90% and the beat frequency to such a level that swimming cells were not uncommon.

Reactivation occurred poorly in media that contained dithiothreitol (DTT). DTT is used in some reactivation protocols (Goodenough, 1983) but was found to impede reactivation in Tetramitus flagella. With control percent

Figure 21

Factors Affecting Reactivation Success

Reactivation success depended on the concentration of various chemicals in the reactivation medium. These effects are shown relative to the standard level of reactivation (see Table 3). Each bar represents the average of three trials detailed in Table 3.

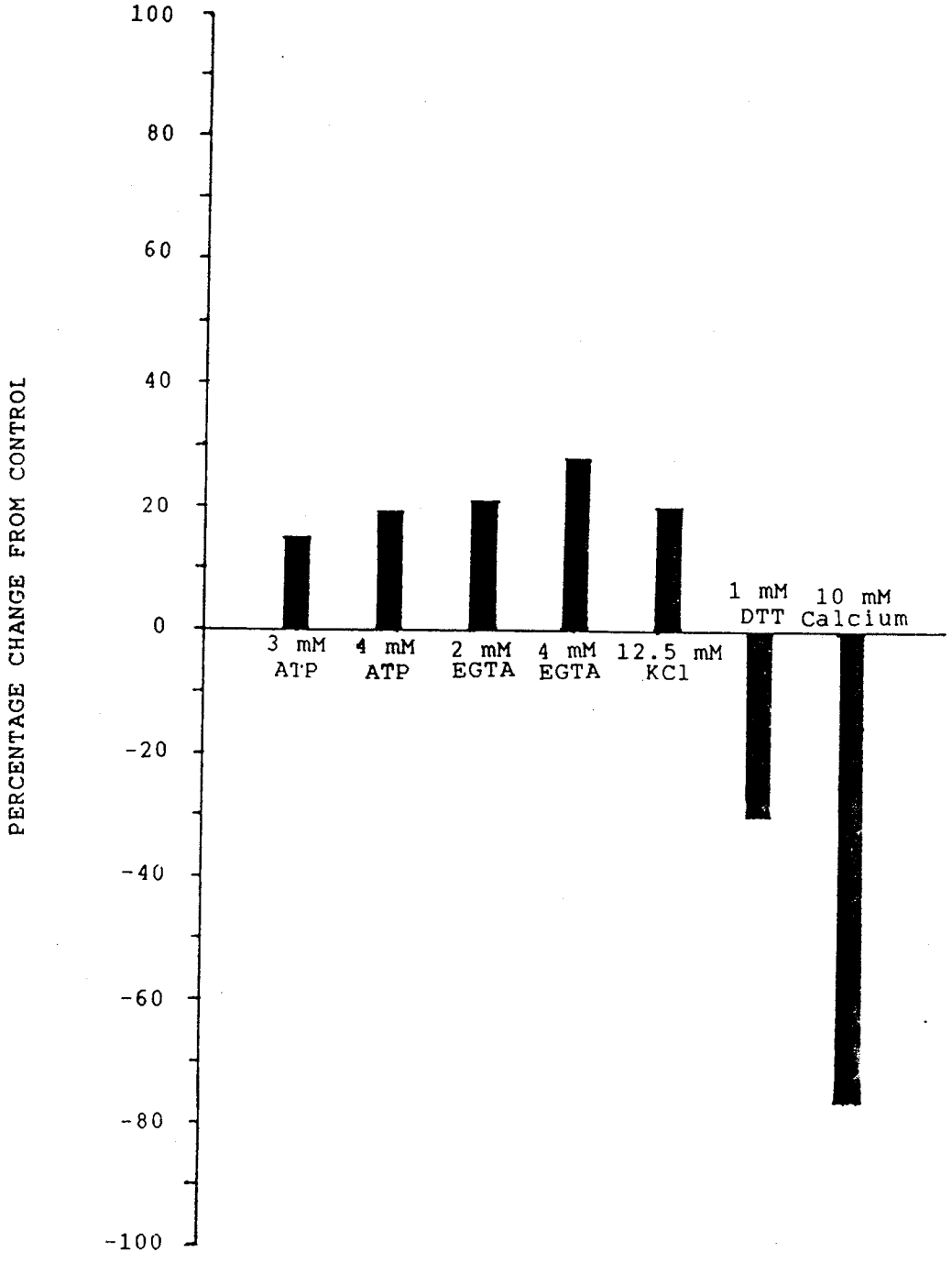

PERCENTAGE CHANGE FROM CONTROL
100
80
60
40
20
0
-20
-40
-60
-80
-100
3 mM ATP
4 mM ATP
2 mM EGTA
4 mM EGTA
12.5 mM KCl
1 mM DTT
10 mM Calcium

TABLE 3

CHEMICAL	CONCENTRATION	% REACTIVATED Trial I	Trial II	Trial III
ATP	2 mM (standard)*	75	68	71
	3 mM	90	83	87
	4 mM	87	94	92
EGTA	1 mM (standard)	58	72	63
	2 mM	87	86	87
	4 mM	92	94	95
KCl	0 mM (standard)	66	71	67
	12.5 mM	87	90	88
DTT	0 mM (standard)	65	64	67
	1 mM	35	37	34
Calcium	0 mM (standard)	76	73	75
Calcium	1 mM		0 - 20 **	
Calcium	10 mM	0	0	0

* The standard reactivation solution was 30 mM Tris, pH 8.0, 4 mM $MgSO_4$, 2 mM ATP and 1 mM EGTA. KCl, DTT and $CaCl_2$ were not normally included. Reactivation under standard conditions usually varied from 65% to 75%.

** Over many trials this reactivation percent was found to vary between 0 and 20%.

reactivation at 65%, parallel trials with 1 mM DTT resulted in reactivation percentages of only 34% to 37%. This effect has also been noted with ciliated epithelia (A.N. Legault, personal communication).

Centrifugation of the ghosts lowered their ability to be reactivated. Both 0.1% and 1.0% TX-100 ghosts were used. An increase in the extent of ghosting also lowered the percent reactivated regardless of whether the increased extraction was due to higher detergent concentrations or more vigorous agitation. This effect could be overcome by increasing either the ATP or KCl concentration. Duration of exposure to detergent prior to reactivation affected reactivation success - a 15 minute time lag between ghosting and reactivation resulted in an approximately 50% decrease in percent reactivation.

The most effective means of stopping beating cells is by addition of calcium (see Fig. 21 and Table 3). The concentration required to stop all the flagella from beating varied from preparation to preparation. 10mM calcium was always sufficient to stop 100% of the cells immediately. 1mM calcium was not always 100% effective but the result was still striking. Usually with 1 mM calcium, only a few percent (sometimes none) of the cells still had beating flagella but very occasionally this number could be as high as 20% (one-quarter the initial reactivation percentage).

7.2 Reactivated Linkers?

Linkers of reactivated ghosts/isolated cytostomal apparati could be examined. The most extracted of the well-reactivated preparations contained a mixture of thoroughly ghosted cells and isolated cytostomal apparati. The isolated cytostomal apparati had flagella that were as successfully reactivated as those of the ghosted cells. Linkers of both could be examined because the membrane around the shoulder of the cytostomal apparatus was susceptible to tearing thereby revealing the linkers underneath.

Linkers from control, reactivated and calcium-stopped samples were examined. These samples were never as clean as the isolated cytostomal apparatus preparation. This was due, in part, to the fact that handling (i.e., centrifugation) decreased reactivation success and thus was minimized. It was also due to the presence of ATP in the reactivation medium which apparently enhanced the "stickiness" of the preparation - most of the debris in solution stuck to the grid rather than being washed off as would normally occur. The presence of calcium in the stopped samples induced precipitation during the staining procedure. Nevertheless, linkers could be clearly seen in the shoulder area of both ghosts and cytostomal apparati and measurements were made.

Samples for measurement were chosen carefully. All reactivated samples chosen had a high level of reactivated

flagella greater than 90%. Calcium-stopped samples were equally successful - reactivation which was initially 90% or greater was decreased to 3% or less by 1 mM calcium. Percent reactivation initially at 90% or greater brought to 3% or less with 1 mM calcium. All these samples were immediately allowed to settle on grids and were then positively stained.

The parameter chosen for in-depth analysis was linker length. Some basic descriptive statistics are shown in Table 4. It is immediately apparent that these linker length means are considerably shorter than those determined for isolated cytostomal apparati. While this will be taken up in detail in the Discussion, at this point it should be noted that there is considerable variability in the data - the maximum lengths measured are about 135 nm and the minimum lengths are around 30 nm.

The linker lengths of the ATP-treated and control samples have similar distribution profiles (Fig. 22). The data span the same range with both maximum and minimum values being similar. While a two-sample t-test indicates that there is a trend towards statistical significance in the difference between the two means (see Table 5), this less than 5 nm difference (control - 78.7 nm; ATP-treated - 83.6 nm) is of questionable significance biologically.

The distribution profile of the linker lengths in the calcium-stopped sample is quite different from both the

TABLE 4

DESCRIPTIVE STATISTICS OF LINKER LENGTHS*

SAMPLE	MEAN	STD DEV	N**	MIN	MAX	RANGE
CONTROL	78.7	20.3	152	35.8	130.9	95.1
ATP-TREATED	83.6	21.4	98	27.2	145.2	118.0
CALCIUM-TREATED	63.6	15.4	98	34.2	110.1	75.9

* All measurements are in nm.

** 152 measured in 3 separate control cytostomes; 98 measured in 3 separate ATP-treated cytostomes; 98 measured in 5 separate calcium-treated cytostomes.

TABLE 5
TWO-SAMPLE T-TESTS

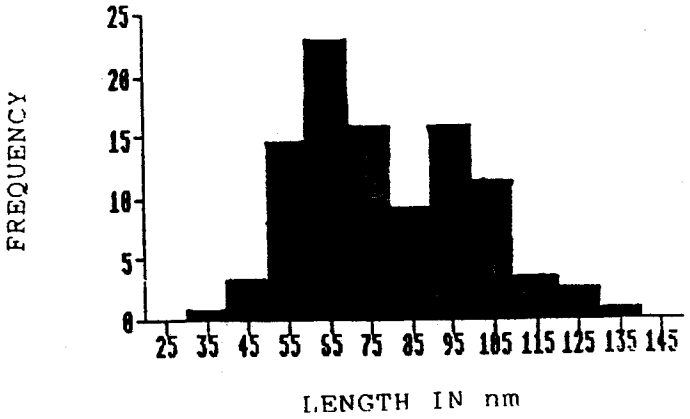
SAMPLES COMPARED	DIFFERENCE BETWEEN MEANS		P-VALUE	CONCLUSION
CONTROL - ATP-TREATED	4.9 nm	1.82	$0.05 < p < 0.1$	trend towards significance
CONTROL - CALCIUM-TREATED	15.1 nm	6.29	$p < 0.001$	very highly significant
ATP-CALCIUM-TREATED	20.0 nm	7.51	$p < 0.001$	very highly significant

Figure 22

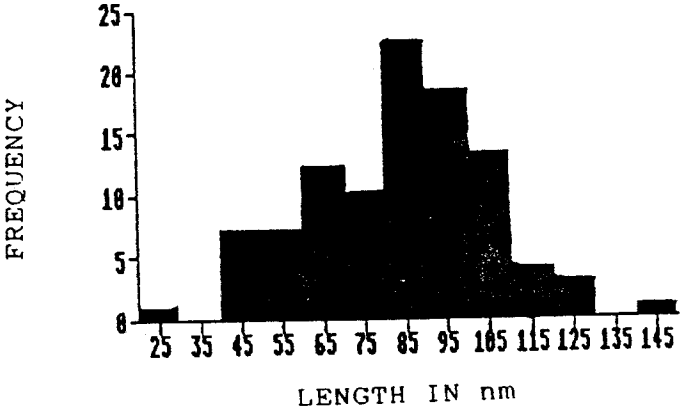
Variations in Control (A), ATP (B) and Calcium-Treated (C) Linker Lengths

Frequency Distribution of control, ATP and calcium treated linker lengths. Each bar represents the mean value of the data shown in Table 3.

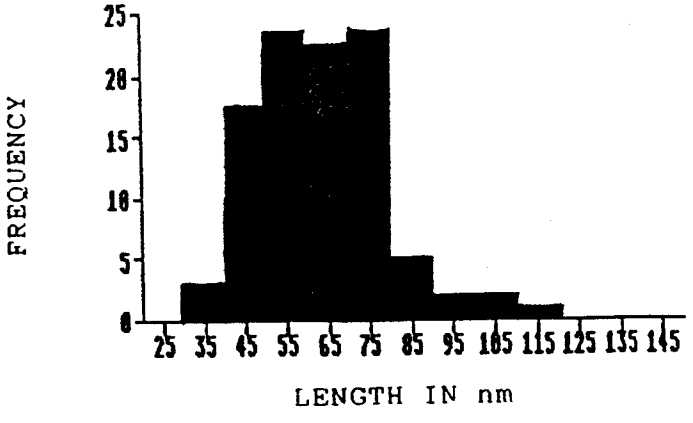
A CONTROL LINKER LENGTH



B ATP-TREATED LINKER LENGTH



C CALCIUM-STOPPED LINKER LENGTH



control and the ATP-treated linkers. The distribution has been shifted to the left with the shorter linker lengths predominating. While the minimum value of 34.2 nm is approximately the same as in the ATP-treated and control samples, the maximum value is somewhat lower (110 nm compared with 131 and 145 nm). The mean value for the calcium-treated linkers is 63.6 nm, fully 20 nm less than the ATP-treated sample. The difference between mean linker-length of the calcium-treated sample and both the control and ATP-treated samples is highly statistically significant (p 0.001 with a two-sample t-test as shown in Table 5). The biological significance of this will be considered in the Discussion.

The appearance of the linkers varies with the different treatments (Fig. 23). The control and ATP-treated linkers have an appearance similar to the "standard" described in detail in Chapter 3. Although the mean is lower, the fine, wavy appearance persists. The morphology of the calcium-treated linkers is somewhat different. They are noticeably both shorter in length and straighter than the control and ATP-treated linkers. In addition, it is much more common to find linkers that are literally broken and visibly disconnected from the adjacent microtubule. These broken linkers are not always broken in the same place - sometimes they are broken in the middle, sometimes towards the end - casting doubts on whether this is a "natural" occurrence.

Figure 23

Morphology of Reactivated Linkers

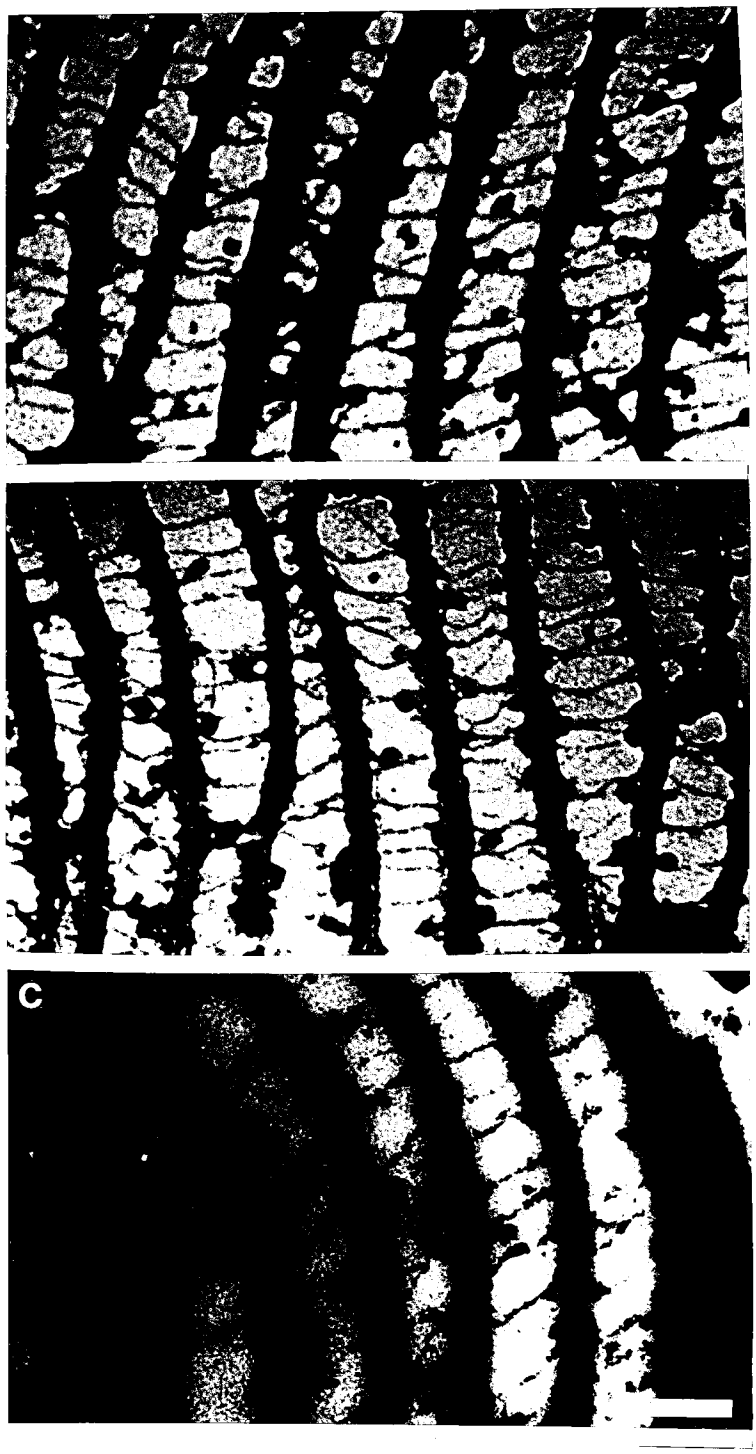
High magnification views of the anterior single sheaf region of 0.1% TX-100 ghosts. Positively stained with uranyl acetate.

a). Control. 110,000X.

b). Exposed to 2 mM ATP. 110,000X.

c). Exposed to 2 mM ATP followed by 1 mM Calcium. 110,000X.

Bar represents 0.1 um.


C

Chapter 8

DISCUSSION

The cytostome of Tetramitus rostratus is a deep ventral invagination that extends through two-thirds of the cell. It is supported by two sheaves of microtubules that both originate at the basal bodies. Each sheaf of approximately 60 microtubules is highly cross-linked by fine filaments. The microtubules and their associated elements have been termed the "cytostomal apparatus". It has been revealed to be a complex and integral part of the organism. During this study, many questions have arisen regarding both the ultrastructure and function of this microtubule-based organelle.

Fact or Artifact?

The cytostomal apparatus, as reconstructed from the various preparations, is described in detail in Chapter 3. The first major concern is whether this reconstruction is accurate. It is possible that one or more of the described features is, in reality, an artifact. This concern must be treated seriously since the very nature of electron microscopy requires that specimens be manipulated in ways that may introduce artifacts.

There are several general criteria that are used in evaluating preservation. These include preservation of

membrane continuity, a lack of empty spaces and similarity to the light microscope appearance of the living specimen (Glauert, 1975). A widely used criterion is that of beauty. In the words of D.W. Fawcett, 1964: "an image which is sharp, coherent, orderly, fine-textured and generally aesthetically pleasing is more likely to be true than one which is coarse, disorderly and indistinct". Another, perhaps more rigorous, method of routing out artifacts is to examine the same specimen type with several different preparatory methods. If the specimen looks the same despite preparative variations, then the image is likely to be a reasonably accurate representation of reality.

These methods of identifying artifacts can be used to evaluate the image of the cytostomal apparatus. All the different methods used in this work give the same basic appearance. There is little doubt regarding the reality of the microtubular organization. The same organization is found in isolated cytostomal apparati, ghosted cells (examined with positive staining and thin sections) and intact cells. Our interpretation of the basic organization of the cytostomal apparatus is almost certainly correct.

The presence and organization of the linkers must be questioned more closely. Although the linkers are readily apparent in isolated cytostomal apparati, they are much more

elusive in other preparations - especially thin sections. The argument has been made that this is due to surrounding cytoplasm obscuring the delicate linkers from view. Although this is a distinct possibility, it is also possibile that the linkers are artificially created during the isolation procedure. The detergent is the agent most likely to introduce artifacts. In fact, there is cause for some concern since, in this work, linkers have only been visualized in material that has been treated with detergents. The "linkers" in the micrographs could also be interpreted to be the remains of a sheet of material that has been incompletely extracted. This argument is supported by the occasional appearance of sheet-like material covering the microtubules. Careful observation, however, reveals that the linkers are visible through this sheet. In addition, other workers have observed linkers in specimens that were lysed by sonication rather than detergent (Balamuth et al, 1983). Thus, if linkers are artificially created by detergent extraction, they are also artificially created by sonication. While there are many cases of broken, torn and messy linker arrays, the "beauty principle" suggests that the "semi-regular" array, described in detail in Chapter 3, shown in Figure 3 and previously discussed by Dingle and Larson (1985) is the best depiction of reality. The fact that these arrays also have the best preserved microtubules supports this view.

Fixatives Change the Morphology

All fixation creates artifacts. The key is to both recognize and minimize these artifacts. Different fixatives preserve different cellular components best e.g., glutaraldehyde usually fixes proteins well and osmium fixes lipids well. However, there is always some loss of material and some reorganization of that which is preserved.

In this work, it has been found that certain fixatives either do not preserve the linkers or radically change their appearance. Both glutaraldehyde and paraformaldehyde used alone had this effect. With glutaraldehyde, the linkers "disappeared" with the concomitant appearance of "blobs" decorating the microtubules. Paraformaldehyde had the same basic effect although it occurred more slowly, allowing the "stretching" of the linkers to be more apparent as an intermediate stage. The blobs are identified as retracted linkers since they are not observed in control arrays and since wherever they are present, a linker is "missing". In addition, although these blobs are ribosome size (approximately 22 nm in diameter), they are not affected by RNAse (data not shown). Langford, 1986 noted that glutaraldehyde caused MAPs to assume an extended configuration rather than the normal globular form. In this work, the opposite effect is observed. Aldehydes apparently cause the normally extended linkers to retract

into a globular configuration. It was noted that lowering the glutaraldehyde concentration lessened the damage while lowering the paraformaldehyde concentration greatly increased the damage.

A mixture of glutaraldehyde and paraformaldehyde (0.5%/2.25%) does not have this effect over the short periods of time required for fixation. Even so, by an hour of exposure some stretching of the linkers has become apparent suggesting that the same events may be occurring but at a slower rate. This slower rate allows this mixture of fixatives to be used to fix specimens for EM without unduly damaging the linkers. The success of this fixative combination in this system is not explained by the standard explanation of the success of the glutaraldehyde /paraformaldehyde mixture. Traditionally, it is suggested that the paraformaldehyde (with the active ingredient of formaldehyde) penetrates more quickly and temporarily fixes elements that are subsequently more permanently fixed by glutaraldehyde (Glauert, 1975). This scenario does not explain the effect of this mixture on the cytostomal apparatus. The linkers do not require stabilization - without fixation their morphology is unchanged over time. Isolated cytostomal apparati, kept at $4^{o}C$ in Tris-Mg, are stable for up to three or four days. This morphological stability can be extended for up to a week if the apparati

are kept in a Tris-glycerol-sucrose-DMSO solution. In fact, treatment with paraformaldehyde decreases the linker stability. Even the glutaraldehyde-paraformaldehyde combination decreases the stability, albeit more slowly than either aldehyde alone. It seems reasonable to suggest that there may be an interaction between glutaraldhyde and paraformaldehyde that in some way minimizes the destabilization of the linkers.

All previous observations of Tetramitus rostratus linkers have been made only in preparations that did not utilize glutaraldehyde. Linkers have been seen in specimens preserved with osmium alone and with platinum shadowing (Outka & Kluss, 1967, Balamuth et al, 1983 respectively). Osmium has been found to preserve the linkers better than either aldehyde alone although exposures beyond 30 minutes resulted in a gradual, general solubilization. Osmium ferricyanide seemed to be gentler than osmium alone, apparently leaving the linkers intact but imparting a "coated" appearance to them. The relative effects of osmium and osmium ferricyanide are not unexpected since it has been found (MacDonald, 1984) that when osmium tetroxide reacts with ferricyanide it becomes reduced and is both less destructive to proteins and more reactive.

It is possible that filaments analogous to the linkers exist in other microtubule systems. The widespread use of glutaraldehyde as a primary fixative would destroy

these linker-like filaments prior to observation and thus prevent their detection. These proposed linkers in other systems could be revealed by employing a preparative technique that does not utilize glutaraldehyde or paraformaldehyde alone.

Stability of the Cytostomal Apparatus

Since microtubules are often classified and characterized according to their stabilities, cytostomal microtubule stability has been carefully investigated. These microtubules have been found to be cold stable, calcium stable and stable to concentrations of colchicine and griseofulvin that normally causes disassembly of cytoskeletal microtubules

The basis for this stability is uncertain. Usually, this type of stability is attributed to proteins associated with the tubulin rather than being an intrinsic property of the tubulin itself. It has been shown that cold-stability can be conferred upon microtubules by a calcium-dissociable, low molecular weight protein (Webb and Wilson, 1980). These microtubules, however, become soluble in the presence of 2 mM $CaCl_2$. Some microtubules, e.g. flagellar, are both cold-stable and stable to millimolar calcium. Although the basis for this dual stability is not clear, the cytostomal microtubules definitely fall into this category.

MAPs are believed to play a role in calcium stability. Tubulin alone is disassembled by micromolar calcium while millimolar concentrations are necessary to disassemble tubulin with MAPs. The addition of calmodulin restores the calcium sensitivity (Bender and Rebhun, 1986). It is believed that this is accomplished via the activation of a calmodulin-dependent kinase that phosphorylates MAP-2. Cold-stable microtubules are particularly sensitive to calmodulin probably due to the calmodulin-dependent kinase which has been found to be specifically associated with these microtubules (Vallano et al, 1986).

The basis of colchicine/griseofulvin resistance is even more uncertain. Colchicine (and probably griseofulvin) is believed to cause disassembly via an inhibition of net assembly at the addition or "A" end of the microtubule (Wilson and Farrell, 1986). This inhibition is believed to be caused by a kinetic capping mechanism at the "A" end and would have little or no affect on the "D" (net disassembly) end. Thus, these drugs ultimately result in the disassembly of treadmilling microtubules. Microtubules that are relatively unaffected by colchicine/griseofulvin (such as the cytostomal microtubules) are either resistant to the assembly-blocking mechanism or have a very low turnover rate. It is possible that resistant microtubules have these properties conferred upon them by microtubule-associated proteins.

The protein composition of the cytostomal microtubules is not known, therefore any attempt to explain their extraordinary stability will be conjecture. However, it seems likely that the stability has its basis in associated proteins. The presence of the HMW MAPs is probable and may help explain the calcium stability up to the millimolar range. The stability at and above this level is not explained by the known properties of MAPs and thus may involve other associated elements. Most of the described features of the isolated cytostomal apparatus are not affected by these various "disassembly" treatments. In particular, the linkers retain their prominence. It is possible that the linkers are not only themselves stable to these treatments but are also involved in the microtubule stability.

Taxol is well-known for its ability to stabilize microtubules and to promote their assembly. In this work it was found that taxol not only stabilized the microtubules to low salt concentrations and to variations in pH (from pH 4 to 11), it also stabilized the linkers. Thus, taxol not only affected the tubulin-tubulin bonds but also affected the tubulin-linker bonds. This is not the case with HMW MAPs. In fact, one common method of purifying HMW Maps (Vallee, 1982) relies on this differential stabilization. Microtubules with MAPs are treated with taxol and

subsequently exposed to high salt concentrations. The stabilized tubulin remains intact but the MAPs are solubilized. An essential difference between the HMW MAPs and the Tetramitus linkers is indicated by the fact that the cytostomal microtubules and linkers are stabilized equally by taxol.

Novel Features

This investigation of the cytostomal apparatus of Tetramitus rostratus has revealed several novel features. The investigations into differential solubility may allow future purification and characterization of some of these elements. Aside from the linkers, which will be discussed in depth below, there are two other features that merit consideration.

The "cross-fibres" that are described in Chapter 3 have never before been identified in Tetramitus rostratus. They can be discerned in previous literature micrographs (Dingle and Larson, 1985), but were not given any consideration. These cross-fibres are a unique feature dissimilar to all literature descriptions of microtubule-associated elements that we have seen. Although many complex cytoskeletal arrays with structural elements exist [e.g., the cytopharyngeal basket in Nassula (Tucker, 1968) and the disc cytoskeleton in Giardia (Holberton and Ward, 1981)] nothing resembling the cross-fibres has ever been observed. The

isolated cytostomal apparatus allows the cross-fibres to be observed more clearly than in any previous preparation. They are smaller in diameter than both the microtubules and the small flagellar rootlets. The cross-fibres appear to be relatively smooth - no striations have been observed. The fibres radiate from the junction of the two sheaves of microtubules. They are closest together at that point and progressively fan out as they extend across two-thirds of the width of the cytostomal apparatus. This organization suggests that the cross-fibres may play a role in holding the sheaves together. The cross-fibres are much more stable to urea than the remainder of the cytostomal apparatus. An extended urea extraction may permit their isolation and characterization.

Treatment of the isolated cytostomal microtubules with potassium or sodium chloride reveals a longitudinal element that runs parallel to the microtubules for their entire length. A 30 minute treatment with 0.25 M salt results in extensive solubilization of the microtubules while leaving the longitudinal elements essentially intact. The width of these elements is approximately the same as that of the linkers at their mid point i.e., 6 nm or less. These longitudinal elements are similar to the tektin filaments identified in flagellar microtubules (Amos et al, 1986). The tektin filaments have a diameter of 2 to 3 nm and a

description that matches the longitudinal elements. Tektin filaments are normally masked by tubulin but are highly insoluble and are purified with sarkosyl-urea extractions. In this work, the longitudinal elements were not only observed routinely with exposure to salt, but were also occasionally observed during the course of the urea treatments. The tektins consist of several polypeptides with weights ranging from 45 to 70 kd e.g. 47, 51, 55 for Strongylocentrotus purpuratus (Amos et al, 1986) although the specific weights can differ from species to species (Linck & Langevin, 1982). When purified isolated cytostomal apparati are run on SDS-Polyacrylamide gel electrophoresis (data not shown) several strong bands are evident in this range. It is possible that these bands could represent tektin filament proteins.

In order to positively identify the longitudinal elements as tektin filaments, either the sarkosyl-urea solubilization or an extended salt solubilization should be performed and the isolate electrophoresed. Decoration of longitudinal elements with tektin filament antibodies would also unambiguously identify them as a member of the tektin family.

The linkers are unusual for their genre because of both their length and their variation. Most connections between microtubules are much shorter than the 105 nm average for linkers. The only linkage that is longer is MAP-2 which

measures 165 to 180 nm when the length attached to the microtubule is included (Voter & Erikson, 1982). When measured in a more conventional manner, MAP-2 has a length of 30 to 40 nm and this represents the upper length of the microtubule linkages. Linkages in the mitotic spindle are 10 to 40 nm (Hepler et al, 1970); the interribbon linkages in Stentor are 18 to 20 nm (Huang & Pitelka, 1973); dynein has a length of 27 to 32 nm (Tsukita et al, 1983). In addition, most of these other connections are regularly spaced. The Tetramitus linkers are "semi-regularly" spaced - there is an overall appearance of order but considerable variation exists in the individual spacings.

The length variation of the linkers is also unusual. It should be noted that both extremely long and extremely short linkers tended to be clustered together, frequently at the edge of the array. While it is possible that different areas of the cytostomal apparatus may exhibit different characteristic linker lengths, it is also quite possible that these particular areas are subject to stress during the staining procedure. This stress may affect the way in which the edges dry onto the grid. Thus, the outer areas may collapse or spread out resulting in extremely short or long linkers respectively.

The other unusual aspect ot the linkers is their width variation. The widths range from a minimum of 0.2 nm to

a maximum of 23 nm. The mid width, however, is much more constant and averages 6.6 nm. This is very reminiscent of the pattern with microtrabeculae whose mid-width averages 2 to 3 nm and end-widths averages 10 to 15 nm (Wolosewick & Porter, 1979). Whether the linker width variation is real or artifactual is uncertain. Since the larger readings are invariably at the junction of the linker and microtubules, it is possible that this large width is, in fact, due to uneven staining with the uranyl acetate being accumulated at the junction point.

Are the Linkers Microtrabeculae?

Are the linkers microtrabeculae? The two filament types do share many features. As mentioned above, the variable width found to be characteristic of the linkers is also characteristic of microtrabeculae. In both cases, it is possible that the variation is due to the preparation and/or staining (positive staining for the linkers, critical point drying for the microtrabeculae) although these methods are quite distinct from each other.

Variable length is also an inherent feature of microtrabeculae since they are assumed to be multimers of the hypothetical unit trabeculin. Most segments of the MTL would be longer than the majority of connecting elements between microtubules, or filaments extending from microtubules such as the HMW MAPs. These variable features have been

considered to be peculiar to the microtrabeculae genre but are shared with the Tetramitus linkers.

The elusiveness of the linkers in thin sections is also shared with the MTL. The MTL has only been visualized using high voltage TEM. It is possible that this technique would reveal linkers in thin section more readily, even with intact cells. Of course, this particular characteristic is a direct result of the delicate nature of both filament classes (linker and microtrabeculae) and as such is not exclusively diagnostic of microtrabeculae. Still, the resemblances are striking and it seems reasonable to suggest that the linkers may be a part of the MTL. The linkers are observed without critical point drying and this fact may indicate that some of the features observed for the MTL are not strictly artifacts of critical point drying as has been suggested (Ris, 1980). However, the characteristic anastamosing feature of the MTL is absent in the linkers and thus represents either a real difference between the linkers and the MTL or a genuine artifact of critical point drying.

Are the Linkers Motile?

It has been suggested (Dingle and Larson, 1985) that the cytostomal apparatus may play an active role in ingestion and that the linkers could provide the motive force for this proposed movement. No evidence to support this hypothesis

has yet been found, but it has been investigated further here.

The obvious approach to the problem is to attempt to initiate movement and then to compare non-motile and motile apparati. Unfortunately, we lack knowledge of the proposed "linker ATPase" (it has been assumed that the motility would be powered by an ATPase). Thus, we have taken the activation of the flagellar motility system (and therefore the dynein ATPase) to be an indicator of the conditions necessary for the activation of the "linker ATPase". This assumption could be erroneous - the "linker ATPase" could require different conditions for its activation. If this is so, a failure to observe linker motility could well be due to the failure to provide the necessary conditions rather than the non-existence of said motility.

It does seem likely that conditions appropriate to flagellar motility would be appropriate for linker motility. Flagellar motility is considerably easier to monitor and thus extracted cells have been examined for flagellar reactivation under a variety of conditions. The basic protocol for flagellar reactivation was modified from Goodenough, 1983. Maximization of flagellar reactivation has been documented in Chapter 7.

Both ghosts and isolated cytostomal apparati could be reactivated. The linkers in the shoulder region of both can

be examined. Linker length was the parameter thought most likely to change in response to reactivation. It was measured in control samples, ATP-treated samples and calcium-stopped samples. Any proposed movement involving the linkers would be a cyclical event and it was hoped that the calcium treatment would allow all the linkers to be examined at the same point in the cycle.

Analysis of linker length in the three groups does reveal some differences. An immediate observation is that the "control" linker length differs considerably from the "control" length reported in for isolated cytostomal apparati (79 vs. 107 nm). This difference in linker length may be due to the differences in sample preparation. Highly extracted ghosts were chosen for measurements. In spite of the extent of ghosting, this was still a more "physiological" preparation than isolated cytostomal apparati. In fact, in these ghosts, the cytostomal apparati were observed to have a more three-dimensional appearance. In addition, the chemical conditions of the two preparations differ - the ghosting cell solution contained 2 mM EGTA and a slightly higher $MgSO_4$ level (5 instead of 3 mM). These differences may account for the observed difference in linker length measurements. It is probable that the lower value for the linker length (85nm) is closer to the *in vivo* length.

The linker lengths for the ghosted control and ATP-treated samples are quite similar (78.7 and 83.6 nm

respectively). There is a small difference between the control and ATP samples that translates to a trend towards statistical significance. This difference is probably not biologically significant, especially since the two samples are morphologically indistinguishable.

There is a statistically highly significant difference between the calcium-treated sample and both the control and ATP-treated samples. The average linker length in the calcium-treated sample is 63.6 nm - fully 20 nm less than the ATP-treated sample. The magnitude of this difference is such that it could represent a "contracted" linker. The question that remains is whether this difference is due to a naturally occurring event that has been captured for observation by the calcium treatment or whether it represents an artificial effect of the experimental treatments. Although it is tempting to conclude that these linkers are indeed contracting, careful observation at the EM level suggests that these linkers have, instead, undergone significant damage that subsequently changes their overall length. The linkers are often found to be broken but not in the consistent manner that would be expected if this was part of a cycle of attachment/detachment.

These experiments have failed to reveal linker motility. Although this may simply be a result of failing to supply the right ingredients, no evidence supporting the

hypothesis of linker motility has ever been observed. Therefore, the alternative hypothesis that the linkers play a predominantly structural role in the cytostomal apparatus must be given serious consideration.

SUMMARY

1. The cytostomal apparatus of Tetramitus rostratus has been found to be a complex and integral part of the organism. Its ultrastructure has been described in detail, having been reconstructed from positive staining of isolated cytostomal apparati and ghosted cells as well as from thin sections of ghosted cells. Attention has been focussed on the linkers. The linkers are extremely long for microtubule linkages - they have an average length of 107 nm. The width of the linkers varies from the mid to the end point (6 vs 12 nm). These features, along with their general appearance, suggests that they may belong to a genre of filaments referred to as the microtrabecular lattice.

2. The cytostomal microtubules and associated linkers have been found to be remarkable stable. The microtubules are cold stable, calcium stable and colchicine/griseofulvin stable.

3. Aldehyde fixation has been found to drastically affect linker morphology. Both glutaraldehyde and paraformaldehyde used alone were very destructive at both low and high concentrations. The best fixative for the linkers was found to be a combination of paraformaldehyde and glutaraldehyde

(2.25%/0.5%) was found to yield adequate fixation. The destructive effect of glutaraldehyde may explain the paucity of linker-like filaments in the literature.

4. A system of longitudinal filaments that runs in parallel with the microtubules was observed. These filaments are very similar to the tektin filaments found in flagellar microtubules. These filaments are unique for non-flagellar microtubules - they have not before been observed in cytoskeletal microtubules. They are especially well-revealed with high salt extraction and this may ultimately allow for their isolation and characterization.

5. Approximately twenty cross-fibres were found to originate at the juncture of the two sheaves of microtubules and fan out across the width of the cytostomal apparatus. These fibres are unlike any that have been seen in the literature. They are especially stable in urea and an extended urea extraction may allow for their isolation and characterization.

5. The linkers were investigated for motility. Linker length in control and ATP-treated samples was very similar - the means differ by 5 nm. Linker length in calcium treated samples was considerably shorter - 20 nm - but the morphology suggests that this difference is artifactual rather than the result of a physiological event.

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