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DUPLICATION OF THE FLAGELLAR APPARATUS,
CELL DIVISION AND MORPHOGENESIS IN THE
ALGA POLYTOMELLA

William Alexander Aitchison.

University of Ottawa
Faculty of Science and Engineering
Department of Biology

A thesis submitted in conformity with the requirements for a
doctoral degree at the University of Ottawa

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LIST OF ABBREVIATIONS

- MTOC: Microtubule Organizing Center
- MAPs: Microtubule Associated Proteins
- I.P.: Intraperitoneal
- HAT: Hypoxanthine-Aminopterin-Thymidine
- FITC: Fluorescein Isothiocyanate
- IgG: Immunoglobulin class G
- IgM: Immunoglobulin class M
- 1D-SDS-PAGE: One Dimensional-Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
- EM: Electron Microscopy
- PBS: Phosphate Buffered Saline
- PEG: Polyethylene Glycol
- DMEM: Dulbecco's Modified Minimal Essential Medium
- Tris: Tris[hydroxymethyl]aminomethane
- TEEP: 15mM Tris, 2.5mM EDTA, 30mM KCl, 11% absolute ethanol
- EDTA: Ethylenediaminetetraacetic Acid
- GDMP: 50% (vol/vol) glycerol, 10% (vol/vol) dimethyl sulfoxide, 5mM $MgCl_2$, 5mM NaH_2PO_4

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ABSTRACT

Microtubules in the quadriflagellate green alga Polytomella agilis are organized into five distinct populations: axonemes, basal bodies, rootlets, cytoskeleton and spindle. The axonemes, basal bodies and rootlets, together with the striated and non-striated interconnecting fibers, constitute the flagellar-basal apparatus. The flagellar rootlets have been shown to serve as MTOCs for the extensive cytoskeletal microtubule array (Stearns and Brown, 1979; 1981). These studies also strongly support a morphogenetic role for the flagellar apparatus. This study has extended these observations. The distribution and organization of all microtubule populations in the cell during mitosis and cytokinesis have been examined using electron microscopy, immunofluorescence, and immunogold labelling. The duplication and segregation of the flagellar apparatus and associated MTOCs during cell division and cell morphogenesis have also been examined.

Immunofluorescence microscopy, using monoclonal antibodies generated against Polytomella axonemal tubulin and components of the flagellar apparatus, proved to be a rapid and reproducible technique by which large numbers of cells could be examined. The monoclonal antitubulin recognizes all five microtubule populations in the cell. During mitosis, the elongate cell morphology is maintained by the persistent cytoskeletal microtubule array. In fact, all microtubule populations remain intact throughout mitosis. The flagellar apparatus maintains its interphase organization through late anaphase. The basal bodies replicate prior to mitosis and the new basal bodies remain associated with the parental basal bodies in the anterior of the cell

and are not involved in the organization of the spindle. The parental basal bodies remain flagellated and the cells are motile throughout mitosis. A closed, acentric spindle forms and elongates perpendicular to the long axis of the cell. Microtubules of the spindle appear to be initiated from a nuclear envelope associated MTOC and these microtubules are mainly distributed around the periphery of the nucleus.

Regressive staining was used to aid in the identification of chromosomes. These results, together with the distribution of the DNA specific stain Hoechst 33258, suggest that the electron dense bodies seen by electron microscopy in the nucleus throughout mitosis are chromosomes. Kinetochores, identified by their tri-laminar structure or by microtubules inserting in them, were not observed. It is hypothesized that the chromosomes are separated during spindle elongation through either lateral association with microtubules or by attachment to the nuclear envelope.

As the spindle elongates, the poles approach the plasma membrane on opposite sides of the cell. During mitosis, four additional elongating flagella appear. At cytokinesis, the eight flagella separate into two groups, each with two long and two shorter flagella. Concomitant with this separation, the parental flagellar apparatus separates and appears to be distributed equally to the daughter cells.

During separation, the parental basal bodies rotate and reorient, prior to the assembly of new flagellar rootlets. Absolute orientation of the basal bodies is achieved prior to new rootlet assembly and this is necessary for the development of proper cell morphology. This implies that the rootlet organizing center is firmly associated with

each basal body. Soon after assembly, each new rootlet is quickly capable of initiating the assembly of microtubules which contribute to the cytoskeletal array and, therefore, the morphology of the daughter cells.

RESUME

Les microtubules (Mts) dans l'algue Verte quadriflagellée Polytomella agilis, sont organisées en cinq populations distinctes: les Mts des axonèmes, des corps basaux, des "rootlets", du cytoquelette et du fuseau. Les axonèmes, les corps basaux et les "rootlets", avec les fibres interreliantes striées et non striées constituent l'appareil flagello-basal. Stearns et Brown (1979; 1981) ont démontré que les "rootlets" flagellaires servent de MTOCs (centre organisateur des microtubules). Ces études ont aussi suggéré un rôle morphogénique pour l'appareil flagellaire. Dans la présente étude nous avons poursuivi ces recherches. Nous avons étudié la distribution et l'organisation de toutes les populations de Mts dans la cellule pendant la mitose et la cytokinèse par microscopie électronique et marquage immulogique avec l'or colloïdal ainsi que par microscopie à immunofluorescence. La duplication et la ségrégation de l'appareil flagellaire et des MTOCs qui lui sont associés, durant la division cellulaire, ainsi que la morphogénèse des cellules ont aussi été étudiées.

La microscopie par immunofluorescence, en utilisant des anticorps monoclonaux préparés contre la tubuline axonémale et les composantes de l'appareil flagellaire de Polytomella s'est avérée une technique rapide et reproductible avec laquelle un grand nombre de cellules a pu être examiné. L'anti-tubuline monoclonal utilisé au cours de cette étude reconnaît les cinq populations de Mts dans la cellule. Pendant la mitose, la structure allongée de la cellule est maintenue par la persistance des Mts du cytosquelette. Toutes les populations de Mts demeurent intactes pendant la mitose. L'appareil flagellaire

maintient une organisation identique à celle de l'interphase jusqu'à l'anaphase tardive. Les corps basaux se dupliquent avant la mitose et les nouveaux corps basaux demeurent associés aux corps basaux parentaux dans la portion antérieure de la cellule. Ils ne sont pas impliqués dans l'organisation du fuseau. Les corps basaux parentaux demeurent flagellés et la cellule demeure motile pendant la mitose. Un fuseau acentrique, fermé, se forme et s'allonge perpendiculairement à l'axe long de la cellule. Les Mts du fuseau semblent être initiées à partir d'un MTOC associé à la membrane nucléaire. Ces Mts sont principalement distribuées autour de la périphérie du noyau.

La coloration régressive à l'EDTA a été utilisée pour permettre l'identification des chromosomes. Les résultats obtenus avec cette coloration ainsi que ceux obtenus après coloration avec le Hoechst 33258, suggèrent que les corps denses observés par microscopie électronique dans les noyaux au cours de la mitose sont les chromosomes. Les kinétochores normalement identifiés par leur structure trilaminaire et par la présence de Mts qui s'y insèrent n'ont pas été observés. Il est proposé que les chromosomes sont séparés pendant l'élongation du fuseau par leur association latérale avec les Mts ou par leur attachement à la membrane nucléaire.

Comme le fuseau s'allonge, les pôles se rapprochent de la membrane plasmique sur les cotés opposés de la cellule. Pendant la mitose, quatre nouveaux flagelles apparaissent. Pendant la cytokinèse les huit flagelles se séparent en deux groupes chacun avec deux flagelles longs et deux flagelles courts. En même temps qu'a lieu cette séparation les appareils flagellaires parentaux se séparent et semblent se distribuer également dans les cellules filles.

Pendant la séparation, les corps basaux parentaux tournent, et se réorientent avant l'assemblage des nouvelles "rootlets" flagellaires. L'orientation finale des corps basaux est atteinte avant l'assemblage des nouvelles "rootlets", ce qui est nécessaire pour le développement morphologique normal. Ceci implique que le centre organisateur des rootlets est fermement associé avec chaque corps basal. Rapidement après son assemblage chaque nouvelle "rootlet" est capable d'initier l'assemblage des Mts qui contribuent à l'arrangement du cytosquelette et par conséquent ils contribuent à la morphologie de la cellule.

INTRODUCTION

Microtubules are major cytoskeletal elements found in all eukaryotic cells (Dustin, 1984). They are composed of a protein, tubulin, ($M_R=110,000$) which is a dimer of two 55 Kd subunits. Two classes of subunits, alpha and beta, can be identified and studies suggest that the tubulin dimer is composed of a heterologous pair of these subunits.

Microtubules are formed by the polymerization of alpha- and beta-tubulin, as well as associated proteins, MAPs. Negative staining of microtubules demonstrates parallel, longitudinal, beaded protofilaments. The number of protofilaments, with a few exceptions, is thirteen. The protofilaments result from the longitudinal assembly of subunits. Each subunit exhibits a 4-5nm packing diameter and is arranged in parallel arrays that give the globular subunits in adjacent protofilaments a half-staggered appearance. In diverse conditions, microtubules tend to splay into protofilaments, indicating that the lateral bonding is weaker than the longitudinal links. The evidence indicates that the structural unit is the tubulin dimer. Although the precise location of the alpha and beta tubulins is not known, most data indicate an alternating pattern, both longitudinally and laterally.

The sequence of events that links dimers to form a microtubule is still not clearly established; it probably varies with particular conditions, both in vitro and in

vivo. Much of what is known about microtubule assembly is the result of Weisenberg's demonstration (1972) of the self-assembly of microtubules in vitro. He found that purified brain tubulin, in the absence of calcium, and in the presence of GTP and magnesium, would self-assemble into long tubular structures resembling microtubules seen in cells. The goal of the in vitro studies was to determine the mechanism of assembly in terms of the role of each component present. Microtubules are associated with other proteins which become incorporated into the microtubule structure. As a general class, these proteins are known as "microtubule associated proteins" (MAPs; Sloboda et al., 1975).

Microtubules mediate a number of crucial processes including cell division, cell motility, intracellular movement and organization and, as part of the cytoskeleton, the formation and maintenance of cell shape (Buckley and Porter, 1967, 1975; Dustin, 1984). They may be found as an elaborate system of rapidly assembled and disassembled singlet microtubules as seen in the mitotic apparatus, or as part of highly complex organelles such as the basal body (centriole). The diversity of structures, and therefore functions, in which microtubules play a role may arise from differences in the component tubulins. These differences may be a result of the differential expression of numerous tubulin genes or of post-translational modifications of the tubulin monomers. This diversity could also be determined by the presence of other components, such as MAPs or by

information contained within the various microtubule organizing centers (MTOCs). The discovery that the flagellar microtubules in the amoeboflagellate Naegleria gruberi were composed of tubulins distinct from microtubules found elsewhere in the cell (Kowit and Fulton, 1974) provided one of the first substantial indications that an organism's microtubules are not all identical. This led to the "multi-tubulin hypothesis" (Fulton and Simpson, 1976) which raised the possibility that cells generally possess several types of tubulin monomers. Numerous investigators have since shown this to be true in many cell types (Chlamydomonas: Brunke, Collis and Weeks, 1982; Polytomella: McKeithan and Rosenbaum, 1981; McKeithan et al., 1983; Trypanosome Crithidia: Gallo and Anderton, 1983; Russell and Gull, 1984; Tetrahymena: Suprenant et al., 1985). In these studies it was observed that the alpha-tubulin incorporated into the flagella and cilia is different than the alpha-tubulin of the cytoskeleton. It has since been shown that the tubulin is post-translationally modified by acetylation of the amino group of lysine (L'Hernault and Rosenbaum, 1985; Greer et al., 1985) and that this modification is dependent on flagellar assembly.

Other post-translational modifications of tubulin include the phosphorylation of tubulin (Eipper, 1974; Sandoval and Cuatrecasas, 1976) and the tyrosination of alpha-tubulin (Barra et al., 1973a). Synthesis of alpha-tubulin actually occurs with tyrosine at its C-terminus

(Valenzuela et al., 1981). This tyrosine is removed in vivo by a specific carboxypeptidase (Kumar and Flavin, 1981) exposing the penultimate glutamic acid residue, and this non-tyrosinated tubulin is the substrate for addition of tyrosine by another cellular enzyme, tubulin tyrosine ligase (Barra et al., 1974). The distribution of these tubulins has been examined by immunofluorescence (Gundersen, Kalnoski and Bulinski, 1984). Tyr-tubulin was found throughout the interphase network of microtubules and in the metaphase spindle whereas Glu-tubulin was present in a small subset of interphase microtubules and was absent from astral microtubules. It was however in the spindle although the authors were unable to determine whether any particular spindle fiber contained one or both species of tubulin. Recent evidence (Gundersen and Bulinski, 1986) indicates that Glu-tubulin is predominant in centrioles and primary cilia of PtK1 cells and the axonemes and basal bodies from human and sea urchin sperm. Thus, Glu-tubulin may be an indicator of stable assemblies of microtubules as opposed to labile microtubule assemblies where Tyr-tubulin is predominant.

Post-translational modifications are not the only method of producing specific populations of tubulins. In all eukaryotes examined, except for yeast, multiple genes for alpha- and beta-tubulin exist and more than one alpha- and one beta- can be expressed (Havercroft and Cleveland, 1984). Lewis, Lee and Cowan (1985) have shown that in mouse, there

exist five tubulin isotypes (two alpha and three beta) and that, although both alpha- and beta-tubulin are expressed in a roughly coordinate fashion in all tissues studied, the beta-tubulin expression is developmentally regulated.

The role of MAPs in generating subclasses of microtubules has also been investigated. MAPs are proteins that co-purify in constant stoichiometry with brain derived microtubules through in vitro assembly cycles. These include two high molecular weight families, MAP-1 and MAP-2, and a group of four 55-68 Kd proteins designated tau proteins. Recently, Job, Pabion and Margolis (1985) have shown that MAPs have properties that can account for the formation of different stability classes of microtubules both in vivo and in vitro.

The control and regulation of microtubule assembly during morphogenesis and during the cell cycle has been attributed to the MTOC (Pickett-Heaps, 1969). The existence of MTOCs was first postulated by Porter (1966) and this term is now applied to organelles of variable morphology, composition, distribution and organization. They are all related however, by the fact that they all possess the common function of nucleating microtubule assembly. Other important functions that have been attributed to MTOCs include when and where microtubules are to be assembled as well as their orientation, number and final length. Thus, the information required to define the precise arrangement of the microtubule may reside in the MTOC. How the MTOC

carries out these functions and what factors are involved are not known. Clearly an understanding of how MTOCs function in the regulation of microtubule assembly (temporal and spatial) requires a more detailed knowledge of MTOC composition and organization.

MICROTUBULE ORGANIZING CENTERS

(A) MORPHOLOGY

The centrosome, a pair of centrioles surrounded by osmiophilic pericentriolar material, is responsible for MTOC activity in a broad range of eukaryotes. But the centrosome is not the description of every MTOC. MTOCs exhibit a high degree of structural diversity. In the myxamoebae of Physarum polycephalum, the nucleating structure is a small, electron dense area at the proximal end of the centrioles, between the centrioles and the nucleus (Roobol, Havercroft and Gull, 1982; Clayton and Gull, 1982). Stearns and Brown (1981) have shown that the cytoskeletal microtubules of the alga Polytomella originate from a complex of nucleation sites distributed in a defined pattern on the flagellar rootlets (organizing centers). The nucleation sites appear as an amorphous granularity distributed along the sides of the rootlets.

The nucleating ability of the tri-laminar kinetochore has also been observed. This ability to assemble

microtubules has been shown in vitro, however, recent studies (Mitchison and Kirschner, 1984a) on microtubule assembly appear to cast doubt on the functional significance of this in vivo.

One of the most highly organized MTOCs yet described, and one that lacks the amorphous granularity commonly associated with MTOCs, is the polar ring of Eimeria sporozoites (Russell and Burns, 1984). The polar ring has a rigid and highly defined structure with a diameter of 350-400 nm. The subpellicular microtubules terminate at the ring but appear to have a lateral attachment to the ring rather than being inserted into it. It appears to control the number, spacing, directionality and orientation of the subpellicular microtubules.

The basal body, capable of nucleating the assembly of cilia and flagella, is one of the most structurally complex MTOCs known. It is a highly evolutionarily conserved organelle containing nine outer triplet microtubules linked together and to a central hub by, as yet, unknown proteins.

(B) COMPOSITION

The ability of MTOCs with very different structures, from phylogenetically distant organisms, to initiate the assembly of non-specific tubulin purified from brain tissue may indicate that common components and/or assembly mechanisms exist. The protein composition of pericentriolar

clouds is not known, its determination hampered by the inability to isolate it free from other contaminating material.

Using electrophoretic techniques, Anderson and Floyd (1980) examined chicken oviduct basal bodies. They showed that tubulin was the most prominent protein, representing approximately 20% of the total protein. Indirect immunofluorescence has been used to demonstrate the presence of calmodulin (Maihle et al., 1981) in the basal bodies of Paramecium. Turksen, Aubin and Kalnins (1982) described a protein, recognized by the immunoglobulins in some normal rabbit sera, of approximate molecular weight 50,000 daltons in both basal bodies isolated from Tetrahymena pyriformis and in extracts of chicken tracheal epithelial cells.

Nucleic acids have long been considered to be an integral component of basal bodies. Enzymatic digestion studies have shown the presence of RNA, but not DNA (Dippell, 1976; Heidemann, Sander and Kirschner, 1977). Basal bodies from Chlamydomonas or Tetrahymena, when injected into eggs of Xenopus laevis, induce aster formation (Heidemann et al., 1977). This ability was not affected if the basal bodies were treated with DNase I, alkaline phosphatase, wheat germ lipase, or lysozyme prior to injection. Treatment with RNase or trypsin however, eliminated this capacity.

The determination of kinetochore composition has been aided by the discovery that sera from patients having the

CREST variant of scleroderma stain the centromere region of mitotic chromosomes (Fritzler and Kinsella, 1980; Tan et al., 1980). Cox and Olmstead (1984) have demonstrated that four major antigens (34 Kd, 23 Kd, 20 Kd, and 14 Kd) are localized at the kinetochore, and although the structure of the kinetochore varies widely among organisms, the 14 Kd protein appears to be conserved in a number of evolutionarily distant species.

(C) FUNCTION

It is difficult to imagine how an amorphous, electron-dense, unstructured MTOC such as the pericentriolar cloud could provide the necessary information for the spatial control of microtubule assembly. One theory suggests that in the centrosome, the pericentriolar cloud is composed of numerous initiating sites, each one initiating growth of a microtubule. The arrangement of the initiating sites in the centrosome and their orientation could then determine the morphology of the structures built by the microtubules.

Clearly, since MTOCs differ morphologically and biochemically, it seems reasonable to assume that MTOCs also differ in the amount and/or quality of spatial information that they provide. There appears to be two basic ways in which microtubule patterns are established during the assembly of highly ordered microtubule arrays. The final microtubule array may be established as a result of intermicrotubule links. For example, Tucker (1977) has shown

that in the heliozoan Echinosphaerium nucleofilum, microtubules in the axopodial axonemes are packed and linked in a complex double-spiral array. After cold treatment to disassemble the microtubules, reassembly is initiated randomly from irregularly shaped clumps of dense material. Each clump acts as an MTOC for the assembly of one axoneme but they appear to do very little in the way of organizing the double-spiral array. This pattern is apparently generated after the microtubule nucleation by a self-linkage mechanism.

The alternative is that pattern is established as a result of a specific association between the microtubule nucleating elements and cytoplasmic structures. These structures, acting as templates, define the microtubule pattern as their assembly begins. This organized association between the nucleating sites and cytoplasmic structures therefore defines the microtubules positioning, direction, orientation and ultimately, the final pattern. Tucker (1977, 1982) examined the cytopharyngeal microtubules in the ciliate Nassula. Each cytopharyngeal microtubule bundle grows out from a basal body-associated MTOC, which is a flat, three-layered plate. Observations of the assembly of microtubules from the lower surface of the plate indicate that the nucleating elements are anchored to the plate and are regularly positioned thereby specifying microtubule position and orientation with considerable precision.

In the alga Ochromonas (Bouck and Brown, 1973), the

structured rootlet-MTOCs are constructed such that the distribution of microtubule initiation sites dictates the pattern of the microtubule array. After treatments to remove cytoplasmic microtubules and alter the cell's shape, reassembly of microtubules is initiated from two MTOCs (kinetobeam fibre and rhizoplast) in the original pattern during shape regeneration. These structures could, therefore, exert control over the position, orientation and pattern of microtubule distribution.

In the alga Polytomella (Brown, Massalski and Patenaude, 1976; Stearns and Brown 1981), the initiating sites are distributed along the sides of the flagellar rootlets, where they appear to initiate precisely ordered arrangements of cytoplasmic microtubules. This capacity to initiate a patterned array of microtubules is retained by the isolated rootlet MTOCs. In vitro these MTOCs are preferential sites for the assembly of brain microtubule proteins and will initiate the assembly of purified brain tubulin. The microtubules are initiated from the sites on the rootlets in a pattern closely resembling that of the cytoskeletal microtubules in vivo.

In most organisms, replication of the MTOC occurs in late interphase, shortly before spindle formation. Segregation of the MTOC begins at the onset of mitosis (Heath, 1978). But is the interphase MTOC, also responsible for the initiation and organization of the spindle?

(D) SPINDLE ORGANIZING CENTER

Microtubules form either the mitotic spindle or are used for movement and/or development of asymmetric cell shape when the cell differentiates. Generally, an organism does not possess all of the possible microtubule structures at the same time. Microtubules therefore, are used at different stages of the cell cycle for different and mutually exclusive purposes (Lloyd and Barlow, 1982). Generally, when cells divide, the cytoplasmic microtubules depolymerize. The tubulin protein from this structure is then used in the formation of the mitotic spindle. Transient, yet highly ordered structures, such as a mitotic spindle require some mechanism to effect its correct organization. This function is generally attributed to the cytoplasmic MTOC (centrosome), which duplicates just prior to the onset of mitosis. Therefore some MTOCs play a dual role; development of cell morphology and assembly and organization of the mitotic spindle.

MITOSIS

The process of mitosis ensures the equal distribution of nuclear material between the daughter cells. In animal cells, mitosis is characterized by chromosome condensation and nuclear envelope breakdown at prophase, equatorial arrangement of chromosomes and their attachment to spindle fibers at metaphase, separation and poleward movement of

chromosomes at anaphase and finally chromosome decondensation and reformation of the nuclear envelope at telophase.

The most prominent structural elements of the spindle apparatus are microtubules. While the mechanism(s) by which the microtubules carry out their function (e.g. generating force for the movement of chromosomes) is not known, it is clear that the microtubules undergo dynamic changes in their arrangement and distribution. At least four types of microtubules can be distinguished in most spindles; those that run the length of the spindle from pole-to-pole (continuous), those that connect the pole and the kinetochores of the chromosomes (chromosomal or kinetochore), those that do not connect with a kinetochore and do not extend pole-to-pole (non-kinetochore), and those which surround the centrosome and lie in the cytoplasm (astral).

The spindle MTOC, like the interphase MTOC, exhibits a wide range of structural variation in different organisms. As well as being known as MTOCs, they are also known as nucleus associated organelle (NAO; Girbardt, 1977), spindle pole body (SPB; Aist and Williams, 1972), spindle plaque (Robinow and Marak, 1966), and nucleus associated body (NAB; Roos, 1975). The one ubiquitous characteristic of these MTOCs appears to be that they are composed of variously arranged amorphous, electron-dense material.

In cells with centrioles the interphase microtubules,

and in mitosis the spindle microtubules, radiate from the centriole associated pericentriolar cloud. This is also true for many cells which have a flagellate stage in their life cycle (Heath, 1977). As stated above, replication of the centrioles, or basal bodies, occurs prior to mitosis, so that at each spindle pole there is usually a pair of centrioles. But many organisms, even those which do possess a flagellate stage in their life cycle, do not form centric spindles, i.e., centrioles or basal bodies are not positioned at the spindle poles. Nevertheless, these organisms are able to form fully functional spindles. It has long been known that centrioles are absent entirely from higher plants, yet spindle organization is still accomplished. In many protozoans (Naegleria gruberi; Dingle and Fulton, 1966; Tetramitus rostratus; Fulton, 1970), centrioles are absent during part of the life cycle. During this cycle, fully functional spindles continue to form and the cells can proceed through mitosis. Many of the fungi (Heath, 1977) and algae (Coss, 1974; Floyd, 1978) studied do not have truly centric spindles; spindle organization being accomplished in various ways. In some flagellate algae, the basal bodies dissociate from their flagella and migrate to occupy positions between the spindle pole and the metaphase plate, a position referred to as lateral (Mattox and Stewart, 1974). Many organisms proceed through mitosis by organizing a spindle within the intact nuclear envelope. If polar fenestrae are present, centrioles (basal bodies) may

still be involved in spindle formation, however, in a completely closed spindle, spindle organization is accomplished without any centriolar (basal body) involvement. Because microtubules do not traverse the nuclear envelope, it is expected that all organisms in which the nuclear envelope remains intact during mitosis will eventually be shown to possess intranuclear MTOCs of some type (Heath, 1977). These MTOCs generally appear as electron-dense, osmiophilic plaques which lie adjacent to, or within the nuclear envelope, at the spindle pole. These organisms may well contain two separate MTOCs; one for the assembly of the interphase microtubule array and one for the assembly of the spindle.

ROLE OF MICROTUBULES AND THE MTOC IN CELL MORPHOGENESIS

In addition to their functions in mitosis, microtubules also play a large role in the development and maintenance of cell morphology. During the establishment of the interphase cell morphology following cell division (Brinkley et al., 1975) or during the recovery of cells from anti-microtubule drug treatments (Osborn and Weber, 1976a; 1976b), microtubules are initiated from specific sites which correspond to the centrosome (Brinkley et al., 1981). Solomon (1980) has also shown that neuroblastoma cells recovering from nocodazole treatments redevelop their detailed morphology seen prior to the treatment.

A cytoskeletal function for microtubules has been

proposed and confirmed experimentally in a variety of cell types (Tilney, 1966; Goldman, 1971). For example, amoebae of Naegleria gruberi can differentiate rapidly into swimming flagellated cells. During this transformation, the amoebae round up into spherical cells, following which the cells are rapidly converted into flagellate shaped cells with an asymmetric oval shape. Concomitant with this shape transformation, an extensive array of microtubules is assembled and are seen to follow the cell contour, suggesting a microtubular role in the development of the cell morphology (Walsh, 1984).

The clearest demonstration of microtubules performing dual roles in development and maintenance of cell shape occurs in the algae. In the alga Ochromonas (Bouck and Brown, 1973; Brown and Bouck, 1973), the normal asymmetric form appears to be directly dependent on the continued presence of cytoplasmic microtubules. Treatment of cells with microtubule depolymerizing agents (colchicine, hydrostatic pressure) causes microtubule disassembly and a loss of cell shape. Cell shape is not regenerated unless microtubules are permitted to reassemble. In regenerating cells, the reassembly of microtubules correlates precisely with formation of the characteristic cell shape.

Similar results have been obtained from studies of the cell wall-less alga Polytomella (Brown et al., 1976; Stearns and Brown, 1981). The cytoskeletal microtubules occur in a row under the plasma membrane and terminate in a

characteristic pattern on rootlets attached to the flagellar basal bodies. During recovery of cells from treatments which disassemble the existing microtubules, the cytoskeletal microtubules are observed to initially reassemble from the rootlet MTOCs in the pattern seen prior to treatment. A loss of cell shape occurs with the loss of microtubules and the regeneration of the elongate cell shape is dependent on, and occurs concurrent with, the reassembly of the cytoplasmic microtubules. Thus, in some motile algae, the flagellar rootlets play an important role in the development of cell morphology. Flagellar rootlets are structures common to all motile algal cells. They may be present as a striated fibrous rootlet, a microtubular rootlet, or a combination of both (compound). They are attached to the flagellar basal bodies and, together with striated and/or non-striated fibers which interconnect the basal bodies, comprise the flagellar-basal apparatus.

FLAGELLAR APPARATUS AS AN MTOC

In a very broad sense, the flagellar apparatus has been a highly conserved structure. It has been found not only in all motile algal cells, but also in fungi and protozoa as well as in the cilia (ciliary basal apparatus) associated with the epithelial surfaces of the respiratory and reproductive systems in invertebrates and vertebrates. Despite the overall conservative nature of the flagellar apparatus, there is extensive structural diversity. This

diversity exists with respect to numbers of flagella, flagellar appendages, basal body and transition region ultrastructure, fibrous structures interconnecting basal bodies and flagellar rootlet systems (Melkonian, 1982). In order to understand the significance of the variability, and why such a conserved structure has evolved in so many ways, it is necessary to examine their morphology and their functions.

The structural organization of the flagellar apparatus has been well described for many organisms (Ringo, 1967; Melkonian, 1975; Brown et al., 1976; Moestrup, 1982; O'Kelly and Floyd, 1985). Our present knowledge regarding the structural organization of the flagellar apparatus comes from electron microscopy of thin sections and some serial reconstructions. These structural studies have not led to a satisfactory understanding of its function. Knowledge regarding flagellar apparatus function has been hampered by the fact that little is known about its biochemical composition. This has resulted from the inability to isolate the apparatus free from other cell constituents.

The function(s) of the flagellar apparatus has been difficult to assess. Most of the theories regarding the role of the flagellar apparatus (and its components) are based solely on morphology and only recently have some functions of flagellar rootlets (Salisbury and Floyd, 1978; Stearns and Brown, 1981), basal bodies and interconnecting striated fibers (Wright, Chojnacki, and Jarvik, 1983; Hoops et al.)

1984; Adams, Wright and Jarvik, 1985) been evaluated. These structures have been suggested to function in control of flagellar motion and anchoring of flagella. Evidence has also been presented that the flagellar apparatus plays a significant role in cellular morphogenesis. Hoops and Witman (1985) have suggested that because of the timing of formation of the flagellar apparatus and its positioning in the apex of the cell, these structures could have important functions in the development and maintenance of cell morphology. To fully understand how the flagellar apparatus functions though, it is important to attempt to define the apparatus in terms of the biochemical properties of its individual components.

(A) BASAL BODIES

The basal body is a complex specialized structure within the flagellar apparatus. The structure of the basal body has been highly conserved and even in phylogenetically distant organisms there is little variability. The structural core of the organelle is made up of nine sets of three microtubules. These triplets are linked together by means of lateral projections and to the central ring through radial projections (Cappuccinelli, 1980). Both pattern and direction of microtubule assembly in the axoneme appear to result, at least in part, from the distal polymerization onto an existing pattern of tubules in the basal body (Fulton, 1970). The two most accepted proposals for basal

body function are: (1) as the initiation site for flagellum/cilium formation (Fawcett, 1970), and (2) as a cytoplasmic anchoring device that holds the flagellum/cilium in the cell (Fawcett, 1961). These theories are based on the observations that all organisms studied so far form their flagellum from the distal end of a basal body and that the basal body contains accessory structures (e.g. interconnecting fibers and rootlets) that appear to be adapted for anchoring the basal body within the cell.

Although the basal body seems essential for flagellar formation, it is apparently not required for flagellar movement because the flagella of certain mammalian and insect sperm are motile in the absence of basal bodies (Phillips, 1970, 1974; Wooley and Fawcett, 1973). Also, isolated, demembranated flagella lacking basal bodies can be reactivated (Allen and Borisy, 1974; Bessen, Fay and Witman, 1980). These reactivated flagella beat with normal waveforms and even change their waveforms in response to calcium, the normal physiological effector. Similar conclusions have been reached from studies of flagellar activity during cell division. In dividing cells of Polytoma (Gaffal, 1977; Gaffal and Schneider, 1980) basal bodies separate from the flagella, yet the flagella remain functional. A similar situation exists in the green alga Chlorogonium (Hoops and Witman, 1985). In dividing cells, the basal bodies detach from the parental flagella, which remain motile in the absence of associated basal bodies, rootlets and striated

fibers. In fact, there is no difference in the behavior of the flagella of interphase and dividing cells. These results indicate that not only are basal bodies not required for flagellar movement but that all elements directly involved in the control of flagellar movement must be located in the flagellar shaft distal to the basal body.

ATPase activity has been demonstrated associated with the basal bodies (Anderson, 1977). This ATPase activity is distinguishable from that found in flagella and suggests that this organelle has an energy-requiring function and thus argues for a more active rather than passive role in the cell.

(B) STRIATED AND NON-STRIATED INTERCONNECTING FIBERS

Among the components of the flagellar apparatus that show great structural diversity are the striated and non-striated fibers which act as interbasal body connectors. Their ubiquitous presence suggests that these fibers play an important role in flagellar function. Possible functions that have been proposed for the striated fibers include an active role in the initiation or coordination of flagellar movement (Hyams and Borisy, 1975; Brown et al., 1976; White and Brown, 1981), a passive role in anchoring the cilia and flagella to resist the forces resulting from flagellar beating (Pitelka, 1974), and a role in the development or maintenance of the proper positioning of the basal bodies (Pitelka, 1974; Holley, 1982). There has been, however, no

experimental evidence for any of these functions. Recently, Chlamydomonas mutants which lack normal striated and microtubular rootlets (Hoops, Wright, Jarvik and Witman, 1984) have been used to evaluate the functions of these structures. Their results suggest that the striated fibers are not required for the initiation or control of flagellar motion, but are important in determining the rotational orientation of the basal bodies. Similar results were seen in the comparison of dividing and non-dividing cells in the alga Chlorogonium (Hoops and Witman, 1985), where the flagella continue to beat normally in the absence of the basal bodies and striated fibers. In a careful examination of isolated axonemes from Chlamydomonas, Hoops and Witman (1983) have shown that there are consistent morphological differences between several outer doublets resulting in an inherent structural polarity. The striated fibers were always associated with specific basal body triplets suggesting a role in developing and/or maintaining the correct rotational orientation. A consequence of improper basal body positioning and orientation has been shown to be a defect in the partitioning of cellular contents at cytokinesis.

In intact cells, it has been observed that the striated connecting fibers change length and shape during the flagellar beat cycle (Melkonian, 1978; Melkonian and Preisig, 1983). In Chlamydomonas, the large distal striated fiber has been shown to be calcium sensitive, resulting in a

narrowing of the angle between the two basal bodies, perhaps by contraction of the striated fiber (Hyams and Borisy, 1978). A similar observation has been made in the cell wall-less, biflagellate alga Dunaliella bioculata (Marano, Santa-Maria and Krishnawamy, 1985). The periodicity of the striation pattern in the striated fiber connecting the two basal bodies was quite variable, as was the angle between the basal bodies, suggesting a relationship between the spacing of the striations and the angle of the two basal bodies. Although no active contractile role has been demonstrated, ATPase activity has been cytochemically observed in the striated fibers of Polytomella agilis (White and Brown, 1981).

The presence of non-striated fibers is not as widespread as that of striated fibers (Melkonian, 1979). The most likely function would be in the support and maintenance of basal body position and orientation.

(C) FLAGELLAR ROOTLETS

Flagellar rootlets are microtubular or fibrous structures associated with basal bodies and which terminate somewhere in the cytoplasm. In most algal classes, both microtubular and fibrous rootlets occur in the flagellar apparatus (Melkonian, 1983). The function of the rootlets is believed to be in the absorption of stress generated by the movement of the flagella (Ringo, 1967; Satir and Ojakian, 1979; Stearns and Brown, 1981), but as with other structures

in the flagellar apparatus, these are assumed functions based solely on morphology, in the absence of clear experimental evidence.

Fibrous rootlets are of two main types; microtubular rootlet associated fibers (system I), and fibrous rootlets composed of a bundle of filaments (system II) (Melkonian, 1980). The system II fibers appear to be calcium-regulated. Salisbury and Floyd (1978) demonstrated contraction in the quadriflagellate green alga Platymonas. The system II rootlets from the alga Tetraselmis striata have also been examined (Salisbury, Baron, Surek and Melkonian, 1984). The results indicated that the rootlets were simple ion-sensitive, contractile organelles composed predominantly of a 20,000 dalton calcium-binding phosphoprotein, and that this protein is largely responsible for the motile behavior of the organelle. Antiserum raised against this protein has been shown to cross react with flagellar rootlets in other green algae and with the centrosome of mammalian cells (Salisbury, 1986) demonstrating the conserved nature of this protein.

Evidence for the contractility of system I fibers is still lacking, however, ATPase activity along the system I fibers of Polytomella (White and Brown, 1981) has been demonstrated, indicating that this rootlet may have a more active function than was previously assumed.

Goodenough and Weiss (1978) have examined the rootlet system in gametes of Chlamydomonas reinhardtii. The striated

fiber overlying the two-stranded rootlet makes direct contact with the mating type structure suggesting that the association may have a morphogenetic function or the fiber may function in signal transmission during mating structure activation.

Knowledge concerning the biochemical composition of fibrous roots has been greatly limited by the difficulty of thorough purification. The results available suggest that fibrous roots are heterogenous and probably use different proteins to perform their function (Stephens, 1975; Dingle and Larson, 1981; Melkonian, 1983; Marano et al., 1985)

Microtubular flagellar rootlets occur in all motile algae and contain specific numbers of microtubules per rootlet (for review see Moestrup, 1982). They have been thought to function in maintaining the constant and regular disposition of most cell organelles in flagellated algal cells (e.g. mitochondrion, eyespot, mating structure, nucleus) (Moestrup, 1982). In addition, they have been shown to play a significant role in cell morphology by serving as MTOCs for the assembly of cytoskeletal microtubules (Stearns and Brown, 1979, 1981).

Much of what we know about the functions of the flagellar apparatus have come from the examination of mutants. Cells which lack, or are defective, in any of the flagellar apparatus structures are either capable or incapable of performing certain functions. Careful examination of these cells allows one to eliminate, as

possible functions, those functions that the defective cell is still able to perform. This method has drawbacks as it is difficult to attribute a particular function to a specific flagellar apparatus component. The role of the flagellar apparatus in the development of cell morphology however, is a function that is directly attributable to the flagellar apparatus. Nowhere is this more clearly shown than in the green alga Polytomella agilis.

POLYTOMELLA AS A MODEL SYSTEM

Polytomella agilis is a colorless, cell wall-less, quadriflagellate, unicellular alga belonging to the Chlorophyceae, Volvocales (Pringsheim, 1955). It exhibits many of the structures that are common to the flagellar apparatus of all motile algal cells. Brown et al., (1976) have described in detail the morphology of the flagellar apparatus. The basal bodies are organized into two pairs with each pair having distinctive interconnecting fibers. The "A" pair is connected distally by a large, striated fiber and proximally by two smaller striated fibers. The "B" pair is connected by a single, non-striated fiber at the proximal end of the basal bodies. The four basal bodies are held together by connections to rootlets which terminate between adjacent "A" and "B" basal bodies.

The flagellar rootlets are of two structural types; one consists of four microtubules in a 3 over 1 pattern (microtubular rootlet), and the other consists of a striated

fiber overlying two microtubules (compound). One rootlet of each type terminates between adjacent basal bodies, for a total of eight, and extends toward the cell posterior passing just beneath the plasma membrane. So tight is this association, the basal bodies and eight flagellar rootlets can be isolated as an intact unit (Stearns, Connolly and Brown, 1976).

A highly patterned array of cytoplasmic microtubules occur in a row under the plasma membrane and terminate in a characteristic pattern on the rootlets. All eight flagellar rootlets serve as attachment sites for this array of microtubules (Brown et al., 1976). A cytoskeletal role for the cytoplasmic microtubules was shown (Stearns and Brown, 1981) by observing cells before and after treatment to disassemble the microtubules. The cells become spherical when the cytoplasmic microtubules are disassembled and regeneration of the elongate cell shape is dependent on, and occurs concomitant with, the reassembly of the cytoplasmic microtubules. These microtubules were seen to reassemble in the same pattern seen prior to treatment. The rootlets do not simply serve as attachment sites for microtubules but, in fact, appear to promote microtubule polymerization under conditions where little or no free microtubule assembly occurs. This capacity to initiate microtubule assembly is retained in vitro by the isolated basal body rootlet complexes (Stearns and Brown, 1979, 1981).

In the intact cell, the microtubules are assembled

from the sides of the microtubular component of each rootlet and only on a limited length of the rootlet in the anterior portion of the cell. Also associated with this area of the rootlets is an amorphous, electron-dense material, the nature and composition of which, remains obscure. It has been suggested that the amorphous material may include assembly sites that are prepatterned on the rootlets.

Having firmly established that the flagellar rootlets are the MTOCs for the interphase cytoskeletal microtubules and that these microtubules are important in the development and maintenance of cell morphology, I have set out to do the following:

(1) To examine the distribution and organization of all microtubule populations in the cell (axonemes, basal bodies, rootlets, cytoskeleton and spindle) during mitosis and cytokinesis. This has been accomplished by electron microscopy of thin and serial thin sections and by indirect immunofluorescence (on large numbers of cells) using a monoclonal antitubulin I have generated against Polytomella tubulin.

(2) To examine the duplication and segregation of the flagellar apparatus during mitosis and cytokinesis and its effect on cell morphogenesis in the daughter cells. To do this, I have produced monoclonal antibodies to components of the flagellar apparatus and used them in indirect immunofluorescence assays.

(3) To examine, by electron microscopy, basal body

replication and their segregation to the daughter cells at cytokinesis.

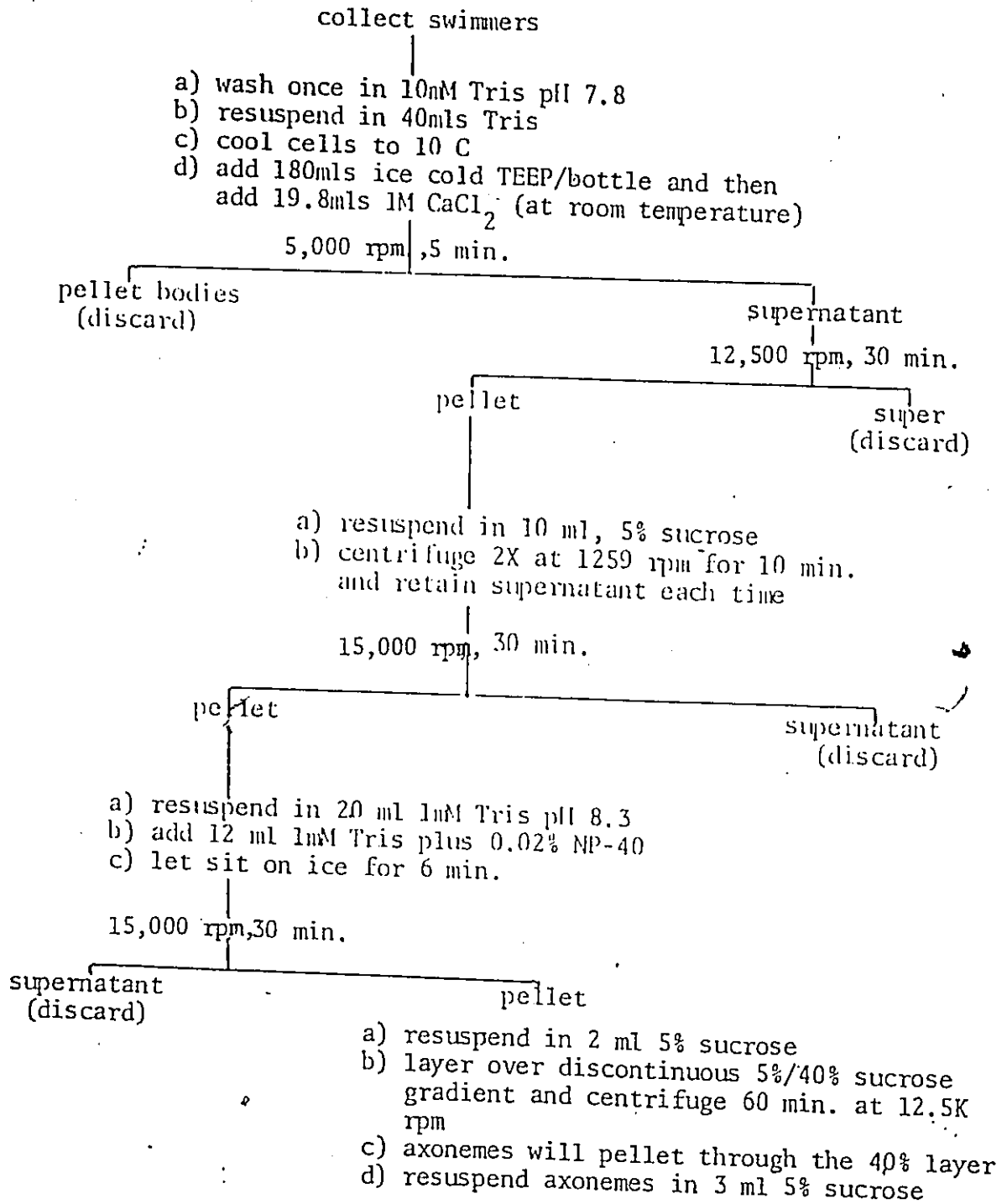
MATERIALS AND METHODS

Batch cultures of Polytomella agilis Aragoa were grown as previously described (Brown, Massalski and Patenaude, 1976). Cells were grown in 4-liter erlenmeyer flasks in growth medium containing 0.1% tryptone, 0.2% yeast extract and 0.2% sodium acetate.

Purification of Flagellar Axonemes

Flagellar axonemes were purified by a modification of the procedure of Witman et al., (1972), as described in Brown and Rogers (1978) and outlined in Fig 1. Fifteen liters of cells at 2.5×10^6 cells/ml were collected in 6 large polycarbonate bottles (290 ml volume), washed once in 10 mM Tris, pH 7.8 and then resuspended in a total of 240 ml (40 ml/bottle) 10mM Tris pH 7.8. The cells were cooled to 10°C in an ice bath. To each bottle, 180 ml of ice cold TEEP was added (15mM Tris, 2.5mM EDTA, 30mM KCL, 11% absolute ethanol, pH 7.8) followed by 19.8 ml of CaCl_2 (CaCl_2 kept at room temp). The mixture was stirred for 60 sec and the cell bodies were separated from the flagella by centrifugation at 5,000 rpm for 5 min (polycarbonate bottles were centrifuged in Beckman RC2-B centrifuge equipped with a GSA rotor). The supernatant containing the flagella was centrifuged at 12,500 rpm for 30 min and the pellet resuspended in 10 ml of

PURIFICATION OF FLAGELLAR AXONEMES



5% sucrose. The flagella were further purified from cell debris by centrifuging the supernatant twice at 1,250 rpm for 10 min (Beckman RC2-B centrifuge and SS-34 rotor). The flagella were then collected by centrifugation at 15,000 rpm for 30 min, resuspended in 2.0 ml of Tris (1 mM, pH 8.3), to which 12 ml of 1mM Tris plus 0.05% Non-Idet was added. The flagella were placed on ice for 6 min, pelleted by centrifugation at 15,000 rpm for 30 min and then resuspended in 2.0 ml of 5% sucrose. This was layered over a discontinuous 5%/40% sucrose gradient and centrifuged at 12,500 rpm for 60 min. The axonemes, which pellet through the 40% sucrose layer, were resuspended in 5% sucrose and frozen at -20°C .

Isolation of Basal Apparatus Complexes

The procedure for basal apparatus isolation is outlined in Fig 2. Fifteen liters of cells at a density of 2.5×10^6 cells/ml were collected by centrifugation at 3,500 rpm for 5 min, washed twice in fresh growth medium and deflagellated by vortex agitation in a fluted tube. The cell bodies were separated from the flagella by centrifugation (3,500 rpm, 5 min) in two washes of fresh medium and were then resuspended in 10.0 ml of a microtubule stabilizing medium (Filner and Behnke, 1973; Forer and Zimmerman, 1974) containing 50% (vol/vol) glycerol, 10% (vol/vol) dimethyl sulfoxide, 5mM MgCl_2 , 5mM NaH_2PO_4 , at pH 6.9 (GDMP). The solution was made 0.1% Triton X-100 and the cells were then lysed by gentle

- wash cells in fresh medium: 2x
- deflagellate by vortical agitation, 1 min
- wash in fresh medium: 2x
- centrifuge at 3,500 rpm, 3 min to pellet cells

p: cell bodies

- resuspend cells in GDMF solution
- add 0.1% Triton X-100
- lyse cells by gentle vortical agitation, 1 min
- centrifuge to remove cell debris
3,500 rpm, 5 min

s: complexes

- centrifuge 2x (8,000 rpm, 5 min and 8,000 rpm, 10 min)
- resuspend pellets in 5% sucrose and 0.1% Triton X-100
- centrifuge 3,500, 5 min
- discard pellet of cell debris
- dilute super 1:1 with 5% sucrose and centrifuge 3,500 rpm for 10 min

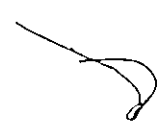
s: complexes

- centrifuge 2x (4,000 rpm, 10 min and 6,000 rpm, 10 min)
- resuspend pellets in 5% sucrose

vortex agitation in the same fluted tube used for deflagellation. The intact basal apparatus complexes were separated from cell debris by centrifugation at 3,500 rpm for 5 min. The supernatant, containing the basal apparatus complexes, was centrifuged twice (8,000 rpm, 5 min and 8,000 rpm, 10 min), the pellets were resuspended by vortex agitation in 5 ml of 5% sucrose containing 0.1% Triton X-100 and again centrifuged at 3,500 rpm for 5 min to remove remaining debris. The supernatant was diluted 1:1 with 5% sucrose and the basal apparatus complexes were further separated from cell debris by centrifugation at 3,500 for 10 min. The complexes were collected by centrifuging the supernatant twice (4,000 rpm, 10 min and 6,000 rpm, 10 min). The resulting pellets were resuspended in 5% sucrose and frozen at -20°C .

Production of Monoclonal Antibodies

Flagellar axonemes or isolated basal apparatus were used as immunogens in the production of monoclonal antibodies. Female Balb/c-BYJ mice (Charles Rivers) were injected intraperitoneally (I.P.) with 100ug of protein every ten days for a total of 300ug of protein. Mice were tail bled after the third I.P. injection and the serum was checked for antibody production by indirect immunofluorescence using Polytomella (as described below for screening of hybrids). Mice showing low, or a lack of



antibody production were not used for fusion. Ten days following the last I.P. injection and four days before fusion, the mice were injected intravenously with another 100ug of protein. Fusion was carried out according to the protocol of Kennett (1980). Spleens, from mice showing antibody production, were removed and splenocytes isolated by perfusion with culture medium (DMEM-10% fetal calf serum) (Flow Laboratories) by injection with a 26-gauge needle at several sites, thereby forcing medium into the spleen. Red blood cells were lysed by addition of 0.17M ammonium chloride (Fisher Scientific) following which the cells were counted and their viability checked by trypan blue exclusion. The cells were mixed with 10^7 cells of the myeloma cell line SP2/0. (Shulman, Wilde and Kohler, 1978). Cells were washed free of serum and 0.2 ml of 30% PEG (Koch-Light) in medium without serum was added. After 8 min the cells were pelleted and the 30% PEG diluted by addition of 5 ml of medium (no serum), followed by 5 ml of medium (20% serum). Cells were again pelleted and resuspended in 30 ml of hybridoma (HY) medium (see Appendix 1). The 30 ml of cells were then evenly distributed into 6, 96 well microtiter plates (Linbro Scientific). Aminopterin (Sigma) was added the following day, and again 6-7 days later, to make hypoxanthine-aminopterin-thymidine (HAT) selective medium (Littlefield, 1964). Clones appeared macroscopically within two weeks.

Screening of Hybrids

Hybrids were screened by an indirect immunofluorescence assay (Chaly et al., 1984) using Polytomella cells. Coverslips (35x50 mm) were coated with 0.1% poly-L-lysine (Sigma) and air-dried. Polytomella cells were deflagellated (Brown and Rogers, 1978), washed twice in fresh medium, and layered over the coverslips. The cells were allowed to settle for 10 min after which they were fixed either with 95% ethanol for 15 min on ice, or with absolute methanol at -20°C for 8 min and air-dried. The coverslips were then placed upon templates consisting of 24 evenly spaced spots. Culture supernatants (2.5ul) from hybrids showing growth were placed over each spot and the coverslip was incubated for 30 min at room temperature. The coverslips were then rinsed in phosphate buffered saline (PBS), pH 7.0, and the entire coverslip was then flooded with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG; gamma-chain specific (Cappel-Cooper Biomedical) for 30 min. Following another wash in PBS, the coverslips were mounted in 50% glycerol-PBS with 0.1% p-phenylenediamine to retard fluorescence bleaching. Areas where the culture supernatant had been applied were then outlined with a marking pen. The areas of the coverslip between each supernatant application provided a control exposed only to the secondary antibody. Hybrids producing immunoglobulin specific for the immunogen were cloned three times in sloppy agar. Antibody was produced as ascites fluid in Balb/c-BYJ mice.

Immunofluorescence

For microscopic observation of cell division, cultures were synchronized by the method of Cantor and Klotz (1971). Cells, at a density of 1×10^5 cells /ml, were incubated at 25°C for 6 hours, following which they were placed at 9°C for 20 hours. Sixty minutes prior to harvesting, the cells were reincubated at 25°C . For immunofluorescent staining, cells (100ml) were harvested, deflagellated, washed twice in fresh medium and then processed as described above. Prior to mounting, coverslips were stained for 2 min with the DNA specific fluorochrome Hoechst 33258 (American Hoechst Corp.) (Hilwig and Gropp, 1972). Samples were observed in a Zeiss Universal photomicroscope equipped for epifluorescent illumination and photographed on Ilford XPI-400 b/w film and printed on Ilfospeed #5 paper.

Immunoblotting

Immunoblotting was performed using Polytomella axoneme, whole cell or basal apparatus proteins, and phosphocellulose purified (Stearns and Brown, 1981) bovine brain tubulin. Proteins were separated in 10% SDS polyacrylamide gels according to Brasch (1982) and then transferred electrophoretically to nitrocellulose (BioRad Laboratories) following the method of Burnette (1981). Total transferred proteins were visualized by staining with amido black.

Molecular weights were estimated by comparison with a molecular weight standards kit (BioRad Laboratories). Immunostaining used horseradish peroxidase-conjugated goat anti-mouse IgG (Cappel Laboratories Inc.) and diaminobenzidine (Polysciences Inc.).

Electron Microscopy

Synchronized cells were prepared for serial thin sectioning as previously described (Brown et al., 1976). Briefly, cells were fixed at 20°C for one hour in 2% glutaraldehyde in 0.05M sodium phosphate buffer, pH 7.2, and post-fixed at 4°C for 1½ hours in 1% osmium tetroxide in the same buffer. Cells were then dehydrated in a graded acetone series of 10%, 30%, 50%, 70%, 90% and 100%. After incubation in propylene oxide, cells were infiltrated and embedded in Epon. Thin sections were cut using a diamond knife on a Porter-Blum MT2-B ultramicrotome and were collected on formvar-coated slot grids. Sections were stained with 2% uranyl acetate (in 50% ethanol) and 1% lead citrate (Reynolds, 1963) for examination in a Philips 201 electron microscope.

For immunogold labelling, synchronized cells were fixed in suspension in methanol at -20°C for 8 min. After rinsing in PBS, the cells were stained with the antitubulin 5A6 for one hour at room temperature, rinsed in PBS, and incubated with protein A:gold (5nm particle size: Janssen Life

Sciences Products) for one hour at room temperature. After rinsing in PBS, the cells were fixed with glutaraldehyde followed by osmium tetroxide and prepared for electron microscopy as described above.

Regressive staining (Bernhard, 1969) was carried out on thin sections mounted on formvar-coated grids in order to differentiate between chromosomes and dispersed nucleolus in the mitotic nucleus. The times of incubation with uranyl acetate, EDTA, and lead citrate varied from section to section. It was important that the EDTA (0.2M aqueous) be aged at least four days before use.

RESULTS

The organization of the microtubular systems in Polytomella has previously been examined (Brown et al., 1976). The four basal bodies of the flagella are organized into two pairs, each with distinctive interconnecting fibers and orientation in the cell (Fig 3). Eight rootlets extend from the basal bodies and serve as attachment sites for cytoskeletal microtubules (Fig 4). The basal body rootlets are of two structural types; microtubular rootlets, consisting of four microtubules in a 3 over 1 pattern and compound rootlets, consisting of a striated fiber overlying two microtubules (Fig 5). One rootlet of each type terminates on each of the four basal bodies. In the anterior region, near the basal bodies, the microtubular component of each rootlet is coated with a densely staining material. It is in this region only, that the rootlets serve as attachment sites for the cytoskeletal microtubules (Fig 5).

Monoclonal Antitubulin 5A6

Polytomella axonemes were used as immunogen to produce a monoclonal antitubulin (designated 5A6). This antibody has been characterized by microscopic methods and by immunoblotting. By immunofluorescence, it appears to detect all of the microtubule-containing structures in Polytomella:

FIGURE 3: Schematic diagram of the arrangement of the four basal bodies and their interconnecting fibers. A, basal bodies of the "A" pair of flagella; B, basal bodies of the "B" pair of flagella; D, distal striated fiber of the "A" pair; P, proximal striated fiber of the "A" pair; Pn, proximal, non-striated fiber of the "B" pair (from Brown et al., 1976).

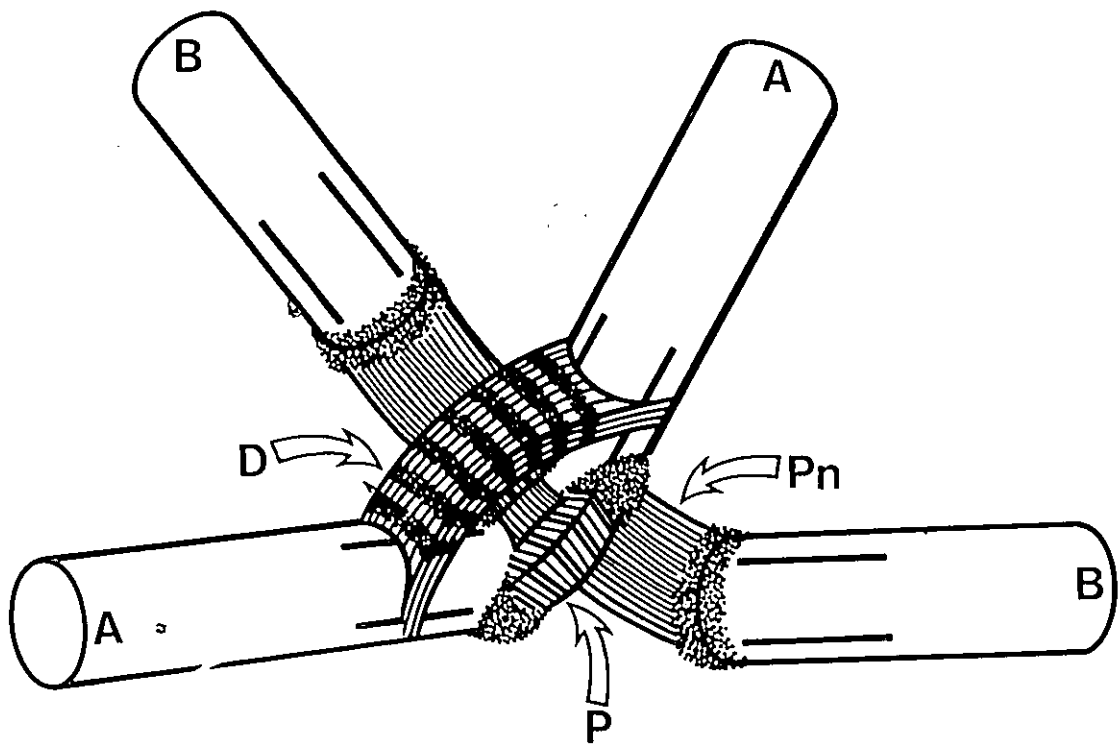


Figure 4: Schematic diagram showing the flagellar basal apparatus organization and the overall distribution of cytoskeletal microtubules in Polytomella. The four flagella are organized into two pairs (A and B pairs). The rootlets, both compound (CR) and microtubular (MR), extend from the basal bodies to the cell posterior. The anterior region of each of the eight rootlets serves as attachment sites for the cytoskeletal microtubule array (from Stearns and Brown, 1981).

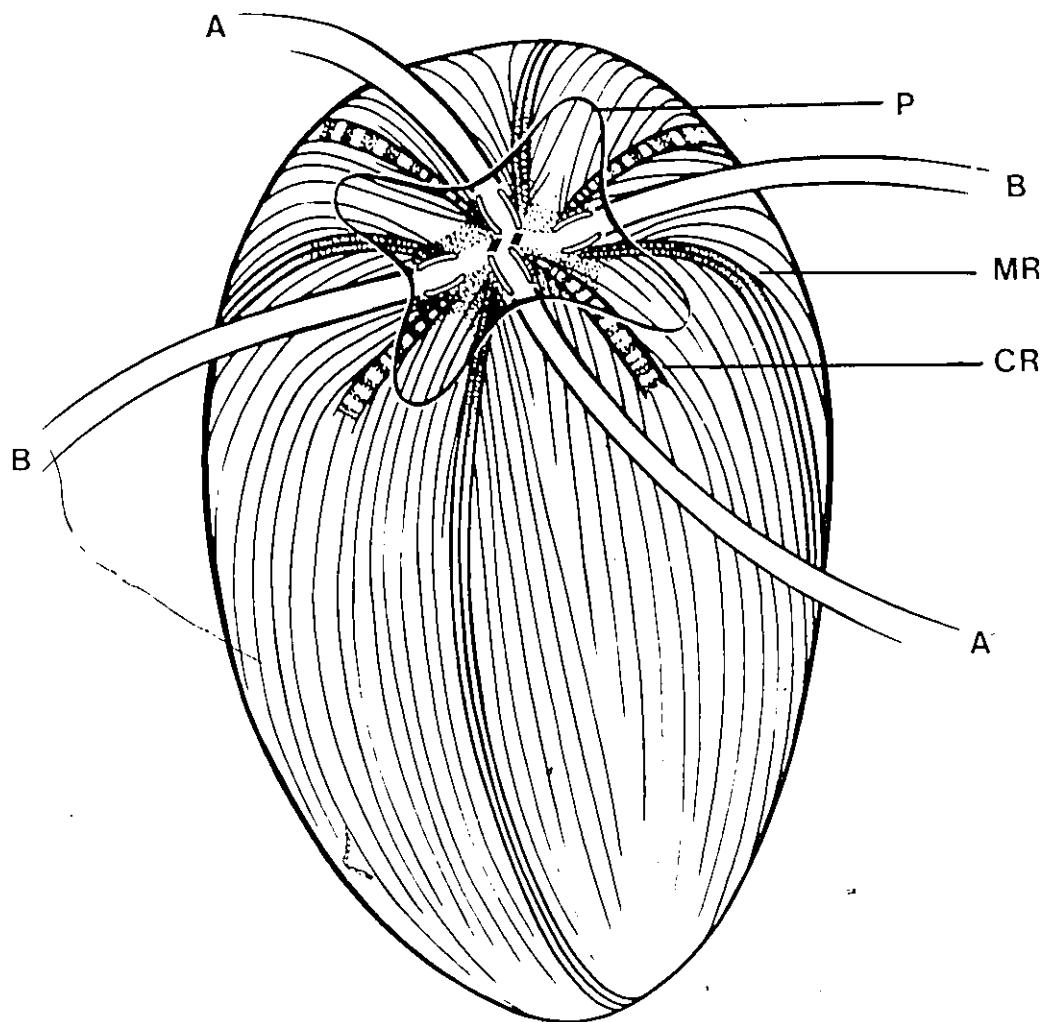
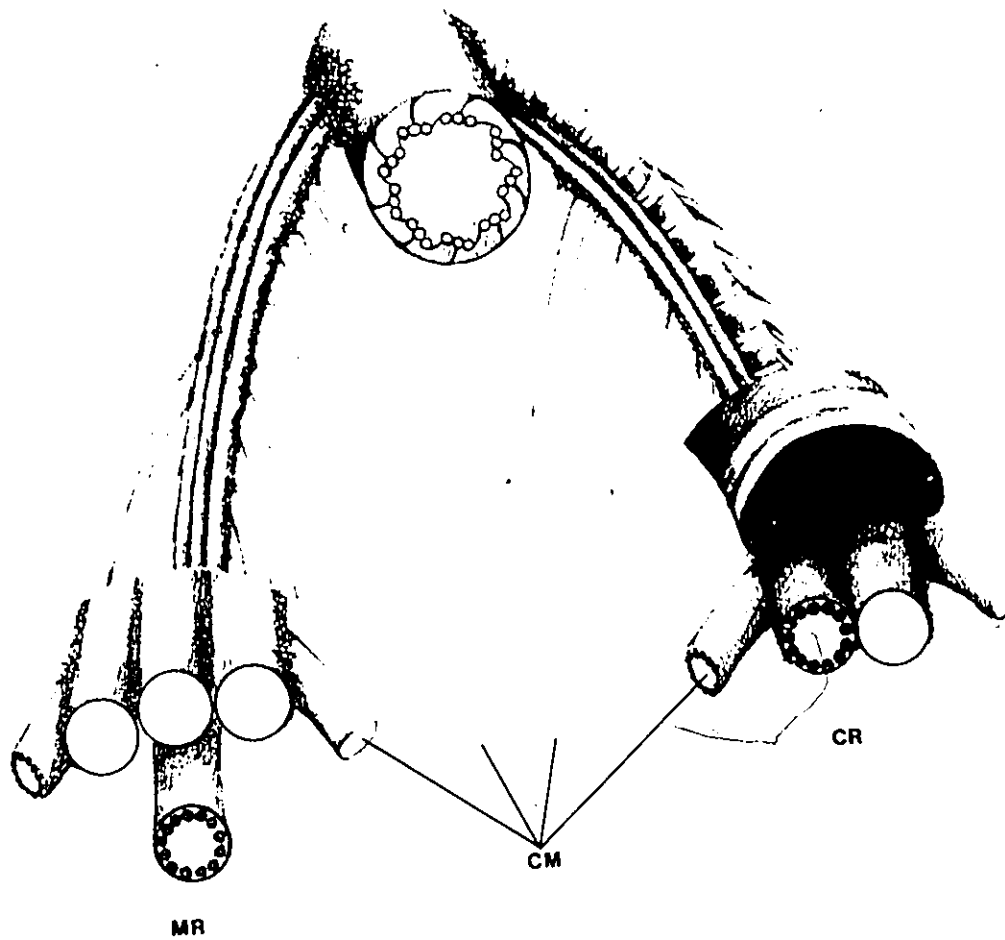


FIGURE 5: Diagrammatic representation of the two types of rootlets attached to each basal body. MR, microtubular rootlet; CR, compound rootlet. Cytoskeletal microtubules (CM) terminate in a regular pattern on both sides of the rootlets (from Stearns and Brown, 1981).



the four basal bodies and the microtubular portion of the rootlets extending from them (Fig 6a), the cytoskeletal microtubules (Fig 6b), the spindle (Fig 7b) and the flagellar axonemes. This antibody is detected by FITC-conjugated goat anti-mouse IgG, but not by FITC-conjugated goat anti-mouse IgM. Immunogold labelling and electron microscopy confirm the identity of all of these structures (Fig 6c and 7c).

By immunoblotting, the monoclonal detects Polytomella axonemal tubulin (Fig 8) and a co-migrating band in preparations of total cell and isolated basal apparatus protein. It also detects purified bovine brain tubulin as well as very clean detection of tubulin on immunoblots of total cell protein from mouse and bovine lymphocytes and 3T3 fibroblasts, but detects no antigen in preparations from higher plant cells. When bovine brain tubulin is electrophoresed under conditions which allow for separation of the alpha- and beta- subunits, the monoclonal detects only the alpha- subunit (Fig 9).

NUCLEAR DIVISION IN POLYTOMELLA

The major features of nuclear division have previously been examined (Dubois, 1982). The sequence of events can be summarized as follows; disappearance or dispersal of the large interphase nucleolus and the appearance of a uniformly, darkly stained nucleoplasm, formation of a

FIGURE 6: Detection of various microtubule containing structures of Polytomella with monoclonal antitubulin 5A6. Basal body, rootlet (a) and cytoskeletal (b) microtubules are detected (X2,000). Electron micrograph (c) shows immunogold labelling of the basal body (B), the cytoskeletal microtubules (single arrow), the microtubular portion of one rootlet (double arrow) and the axoneme (Ax) (X26,000).

7



FIGURE 7: Surface and mid-plane views of the same cell are shown with both cytoskeletal (a) and spindle (b) microtubules stained (X2,000). Electron micrograph (c) shows immunogold labelling of spindle microtubules (X13,000). Arrow shows point of attachment for spindle microtubules.

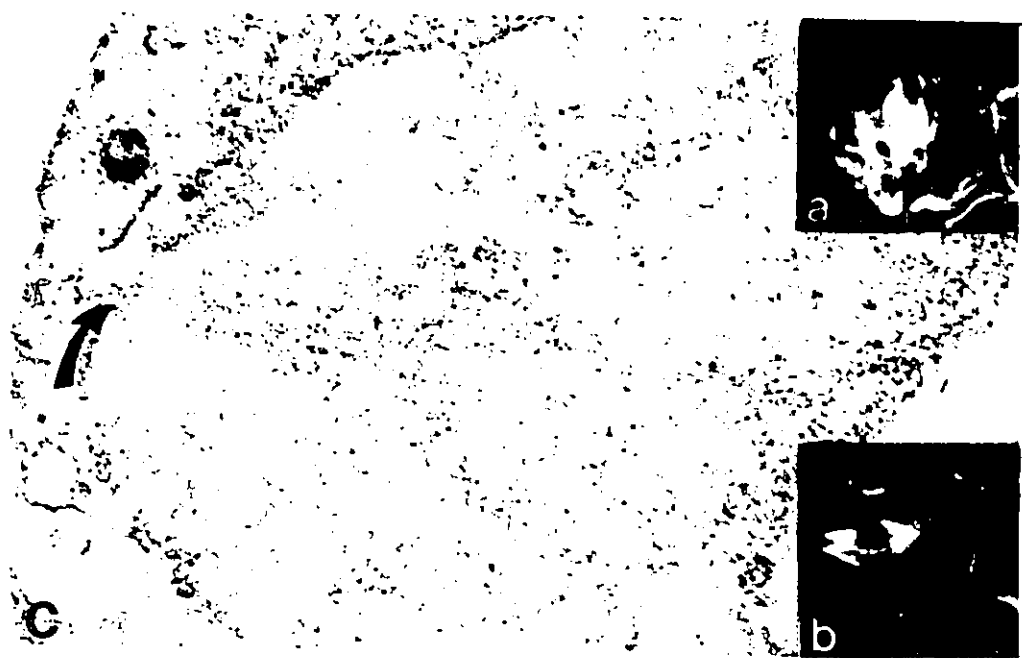


FIGURE 8: Immunoblotting of antitubulin monoclonal 5A6. Lanes A and B show total protein transfer of isolated basal apparatus and whole cell respectively. Lane C shows transfer of molecular weight standards (top to bottom; phosphorylase B, bovine serum albumin, ovalbumin and soybean trypsin inhibitor). Lanes D-J show immunoblots of whole cell (D), isolated basal apparatus (E), purified axonemes (F), bovine lymphocyte whole cell extract (G), murine lymphocyte whole cell extract (H), 3T3 whole cell extract (I) and whole cell extract from the plant *Vicia hajastana*(J). (See appendix II for sample preparation).

92.5 K

66.2 K

45 K

31 K



A

B

C

D

E

F

G

H

I

J

FIGURE 9: Immunoblot of antitubulin 5A6. Lanes A and B show Coomassie blue staining of molecular weight standards and 3X cycled bovine brain tubulin respectively. Lane C shows electrophoretic transfer of bovine brain tubulin and Lane D illustrates that the antitubulin 5A6 recognizes the alpha-tubulin subunit.

92.5 K



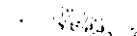
66.2 K



45 K



31 K



A

B

C

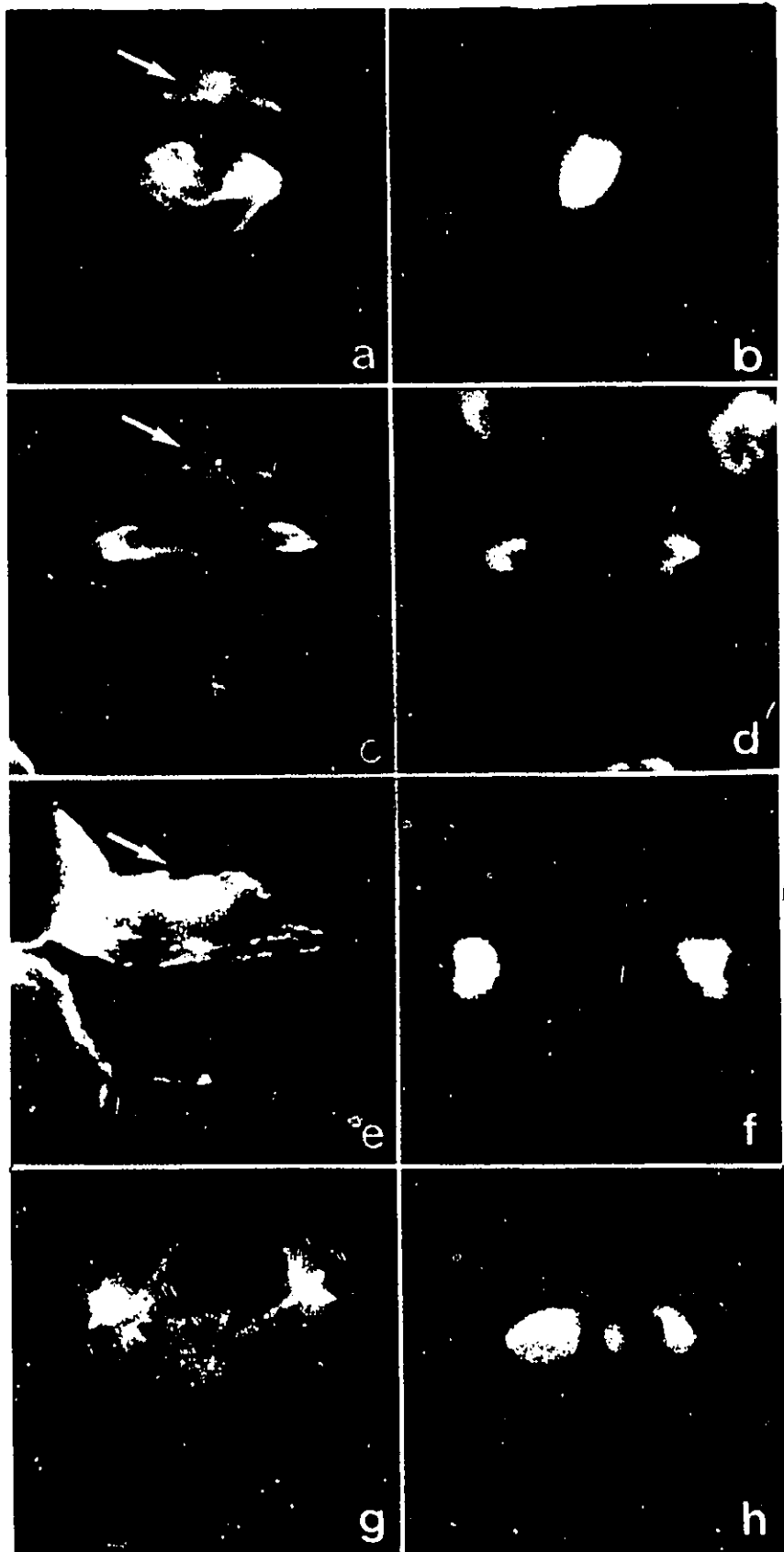
D

spindle-shaped nucleus with the poles oriented perpendicular to the long axis of the cell, loss of spindle shape late in mitosis as nucleus becomes more cylindrical and finally nuclear division to form two daughter nuclei which lie close to each other in the center of the cell.

Immunofluorescence

The distribution and organization of the microtubule populations were examined in large numbers of dividing cells, with the corresponding chromosome/nuclear staining using Hoechst 33258. Interphase cells exhibit an extensive cytoskeletal microtubule array extending from the anterior most regions of the flagellar rootlets and extending the length of the cell (see Fig 6b). Hoechst staining of nuclei at interphase shows diffuse staining of the nucleoplasm and a distinct absence of staining of the nucleolus (Fig 10h shows nuclei that have reassumed their interphase appearance). Prophase and prometaphase microtubule organization have not been observed. Microtubules are first detected in the nucleus at a stage resembling metaphase (Fig 10a). Here the spindle has a "typical" metaphase shape and contains microtubules which appear to extend from pole-to-pole as well as from the poles to the equator. The Hoechst/nuclear staining shows a dense staining mass situated between the two spindle poles (Fig 10b). In anaphase and telophase (Fig 10c and 10e), the nucleus elongates perpendicular to the long axis of the cell. There

FIGURE 10: Immunofluorescent staining of spindle and cytoskeletal microtubules with antitubulin 5A6, with the corresponding chromosome/nuclear staining using Hoechst 33258. Metaphase spindle(a) with pole-to-equator microtubules and dense Hoechst staining at the equatorial plate(b). Anaphase(c) microtubules appear to be predominantly pole-to-pole. Chromosome masses are well separated(d). Late anaphase/telophase(e) shows elongated spindle with spindle poles touching the plasma membrane on opposite sides of the cell. Chromosome masses still located at the poles(f). Early stage in cytokinesis(g) with cytoplasmic microtubules extending from each basal apparatus. Chromatin (Hoechst staining) is located in the center of the cell and each nucleus has reassumed its interphase appearance(h) (X2,000). (Arrows in a,c,e indicate anterior end of cell).

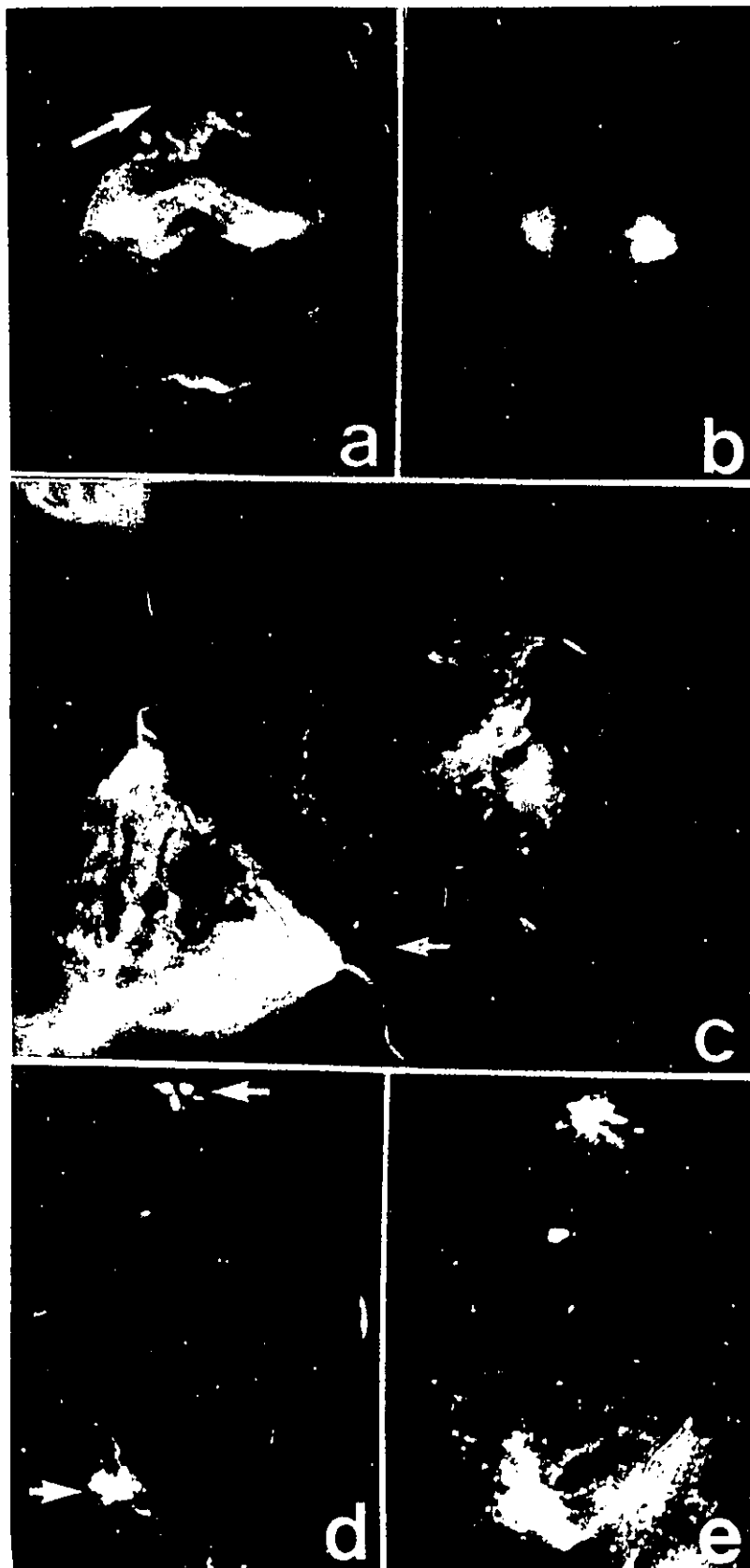


appear to be fewer microtubules than at metaphase, and those that are present appear to extend from pole-to-pole. The chromatin masses are now separated and each mass is associated with one of the spindle poles (Fig 10d and 10f). Following mitosis, the spindle collapses, and the two daughter nuclei lie next to each other in the center of the cell. By Hoechst staining, each nucleus has already taken on an interphase appearance. Cytokinesis proceeds in an anterior to posterior direction. The duplicated basal bodies now separate (Fig 10g) and the cytoskeletal microtubule array is assembled from each new basal apparatus. The nuclei separate concomitant with the separation of the flagellar apparatus. The cells have remained flagellated (Fig 11a,b) and motile throughout mitosis and at cytokinesis, four additional elongating flagella appear (Fig 11c). The eight flagella segregate into two groups, each with two long and two shorter flagella, which separate towards opposite poles of the cell. At a late stage of cytokinesis, the basal apparatus of each daughter cell are separated by 180° (Fig 11d) and the interphase microtubule array has been assembled (Fig 11e).

Electron Microscopy

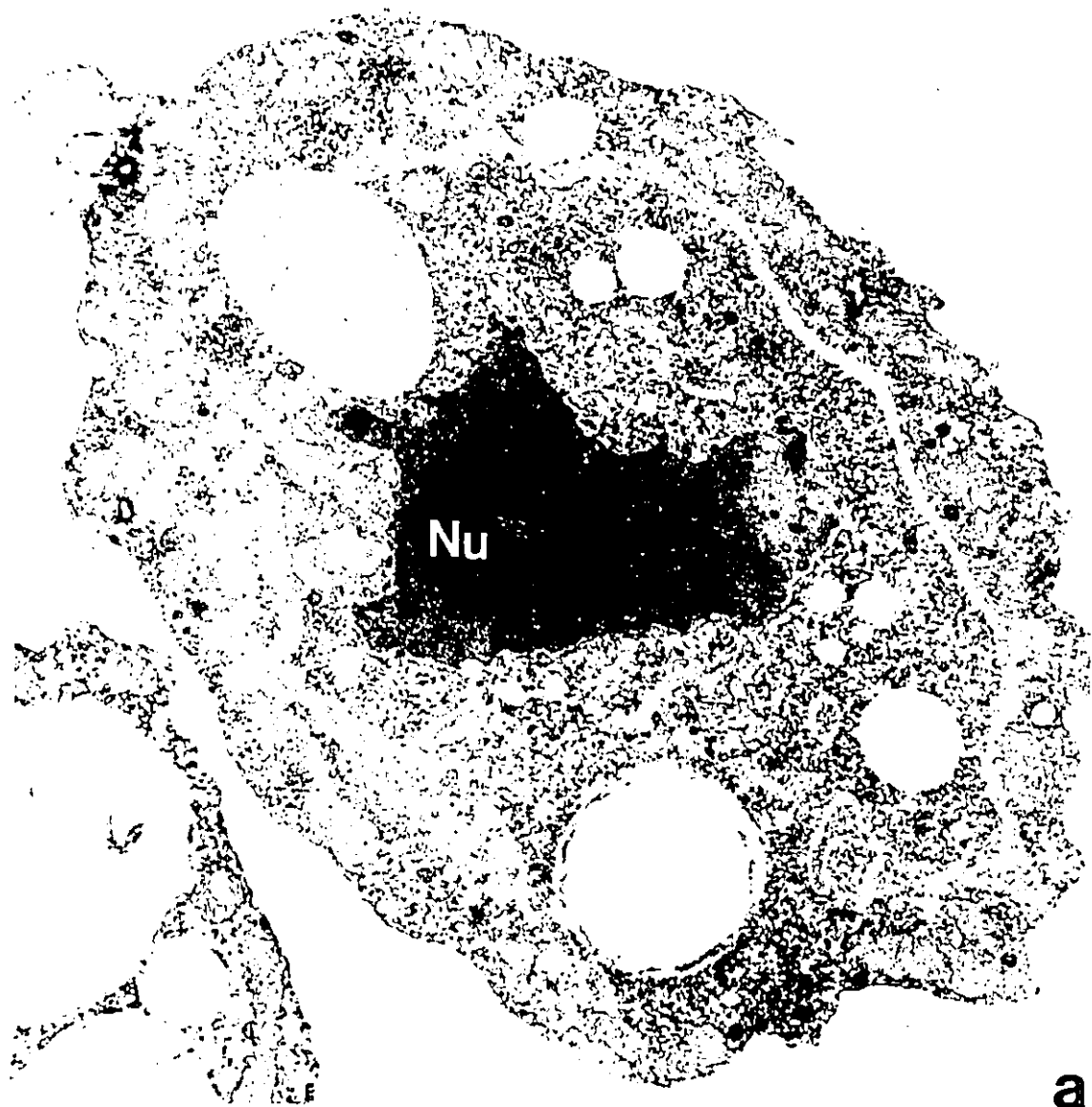
The non-mitotic nuclei are irregular in shape and have a prominent, central nucleolus (Fig 12). In interphase, the large nucleolus is very densely stained with respect to the

FIGURE 11: Mitotic cells remain flagellated throughout mitosis. Fig(a) shows anaphase cell stained with 5A6 with the corresponding Hoechst staining(b). Note flagella in (a) are still attached to cell and are located at the anterior of the cell. Early in cytokinesis(c) new flagella begin to appear and the flagella segregate into two groups, each with two long and two shorter flagella (arrows). Late in cytokinesis, the basal apparatus (arrows in d) are located at opposite ends of the cell and cytoskeletal microtubules can be seen extending from both(e) (X2,000) (d and e are same cell).



7:

FIGURE 12: Median longitudinal section of interphase cell shows irregular shaped nucleus(N) and large central, dense nucleolus(Nu) (X13,000). 



a

remainder of the nucleoplasm. In prophase, the nucleolus appears to disperse, and the nucleoplasm is more densely stained. Elongation of the nucleus is correlated with the appearance of a band of four to five microtubules, which extend from one side of the nucleus to the other (Fig 13). Very early in mitosis, microtubules are seen extending into the nucleoplasm, from a site on the intact nuclear membrane (Fig 14). Cytoskeletal microtubules are visible lying next to the plasma membrane.

The nucleus continues to elongate to form a spindle shaped structure with an intact nuclear envelope. Numerous microtubules are now seen extending from the spindle pole, which is lying close to the plasma membrane (Fig 15), particularly near the nuclear envelope. Fig 16 is a reconstruction from serial longitudinal sections (12 sections) through the pole of an elongating metaphase nucleus. Clear acetate film was placed over each section and the microtubules were tracked. Large numbers of microtubules are present, with the majority of them extending from the pole and running along the nuclear envelope. The spindle shaped nucleus continues to elongate and the poles become more tapered and approach very close to the plasma membrane on opposite sides of the cell (Fig 17). Serial sectioning of the nuclei in this stage reveal the absence of polar fenestrae. The microtubules appear to terminate on the nuclear envelope. No other polar structure has been observed. Centrioles (basal bodies) were never observed near

FIGURE 13: Early stage in mitosis. In cross-section(a), a small bundle of microtubules is seen lying next to the nucleolus (X60,000). Adjacent longitudinal serial sections (b,c) shows that these microtubules extend from one side of the nucleus to the other (arrows) (X20,000).

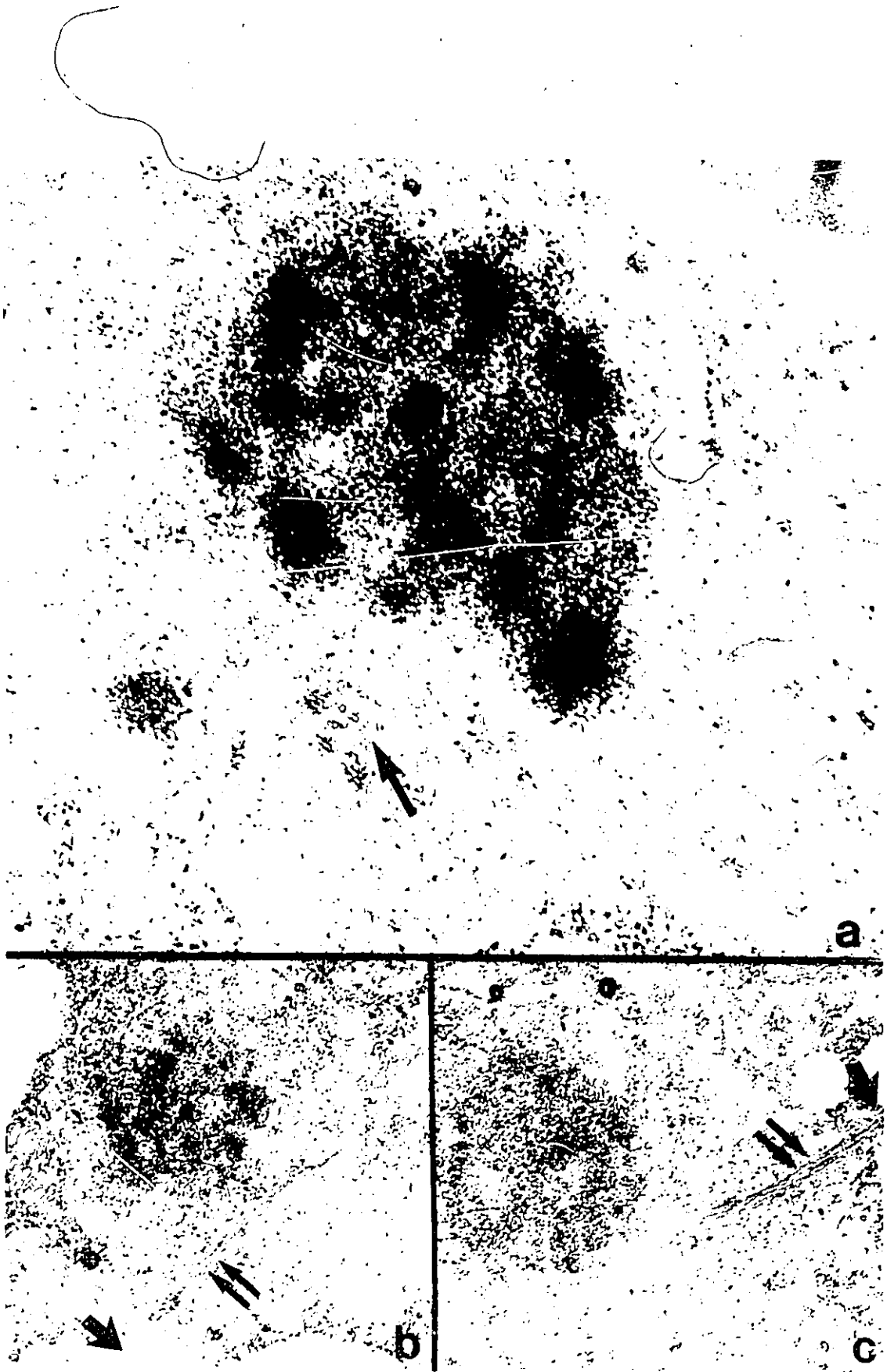




FIGURE 14: As nucleus begins to elongate, microtubules are seen extending from polar regions. The nucleolus has dispersed and cytoskeletal microtubules are present (arrow) (X22,500).

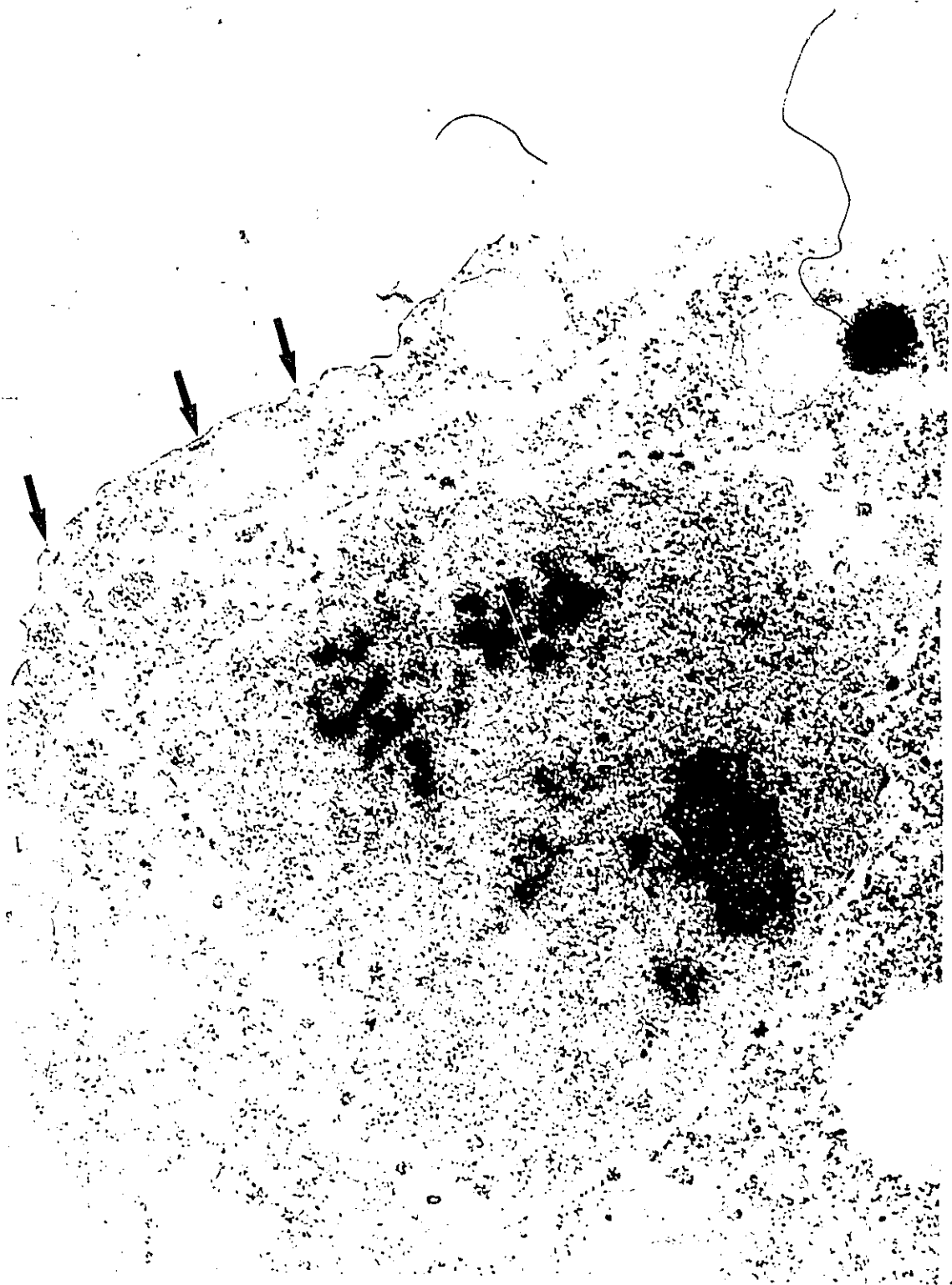


FIGURE 15: Glancing section of spindle pole lying next to the plasma membrane. Large numbers of microtubules are clearly visible (X30,000).



8

FIGURE 16: Reconstruction of serial longitudinal sections through a mitotic nucleus (metaphase/anaphase) showing organization and arrangement of microtubules. Microtubules are seen extending from the spindle pole (S.P.) and running along the nuclear envelope (N.E., dashed line) (X30,000).

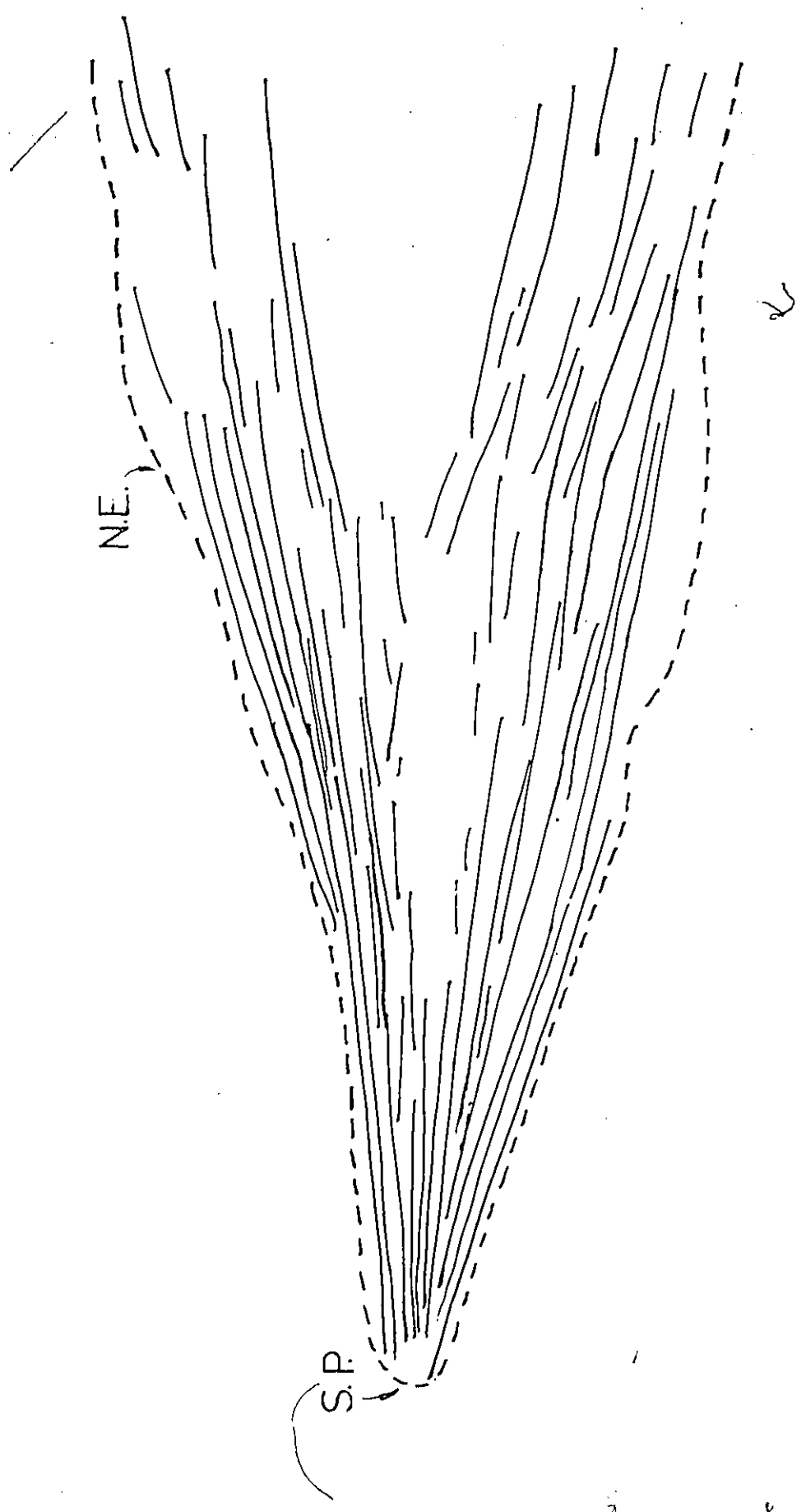
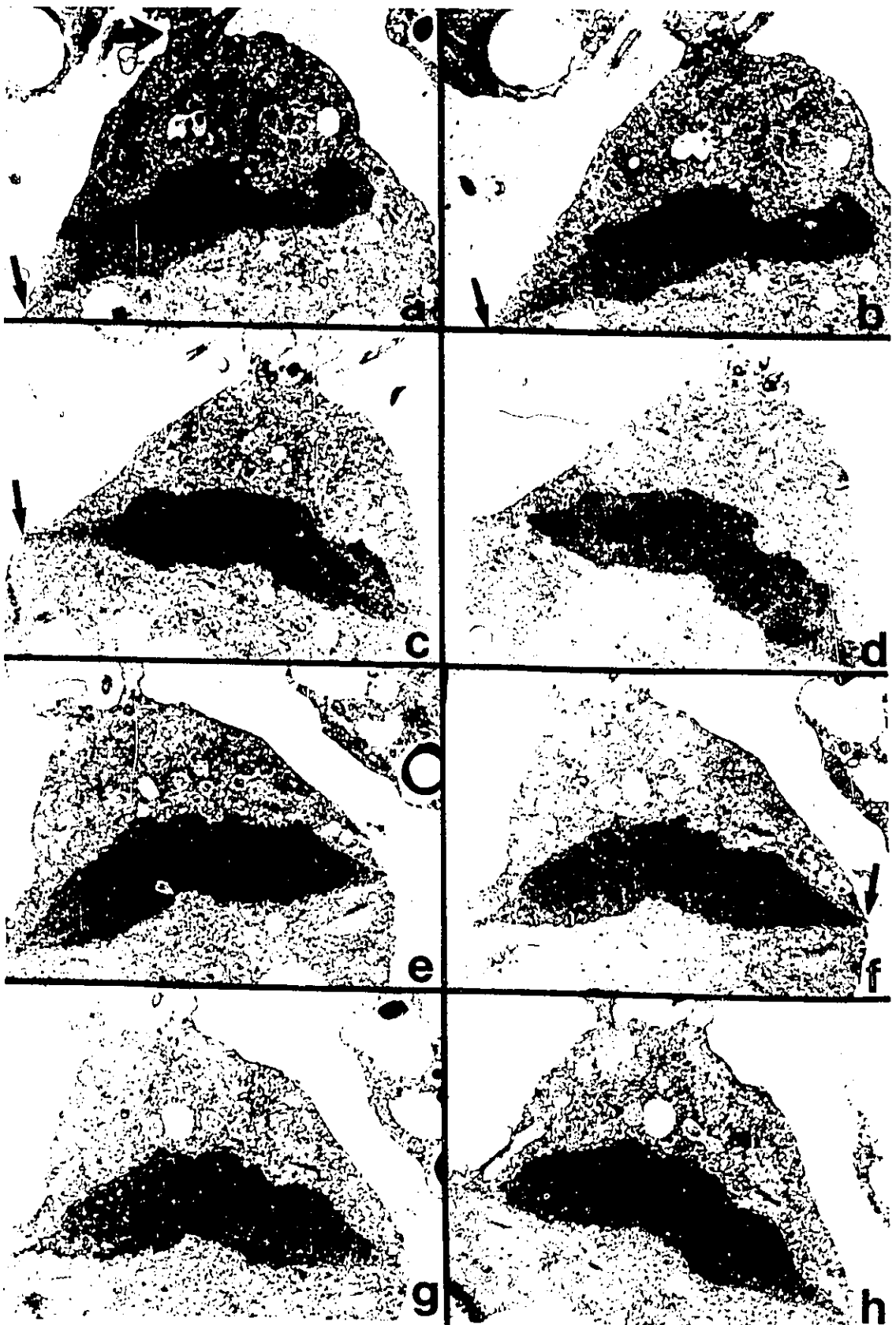


FIGURE 17: Non-consecutive serial longitudinal sections through a cell in anaphase. The nucleus has elongated perpendicular to the long axis of the cell and the poles are touching the plasma membrane on opposite sides of the cell (arrows in a,b,c and f). Note also that the basal apparatus is still intact in the anterior of the cell (large arrow in a,b) (X4,500).



the spindle poles. At a later stage of mitosis (Fig 17), basal bodies can be seen in the anterior papilla region of the cell, far removed from either spindle pole.

As mitosis proceeds, the spindle poles become less tapered and the overall shape of the nucleus is cylindrical. The chromatin is clearly separated into two masses. Following this, the nucleus constricts in the central region and the nucleus takes on a dumb-bell shaped appearance (Fig 18). The poles are no longer near the plasma membrane. Microtubules are still present in the region of constriction, between the two chromosome masses (Fig 19a,b). After nuclear division, the two daughter nuclei formed lie very close to each other in the anterior of the cell (see Fig 10g). The daughter nuclei remain close together and reassume the appearance of the interphase nucleus with a well defined nucleolus surrounded by a less densely stained nucleoplasm. The nuclei then separate concomitant with the separation of the flagellar apparatus.

Regressive Staining

Regressive staining, using the method of Bernhard (1969), was performed on mitotic cells to assist in the identification of chromosomes. Chromosome identification was a problem since no kinetochores (and microtubule attachment sites) were observed. This method allows preferential staining of certain structures, among them

FIGURE 18: At a later stage of mitosis, the nucleus becomes less tapered and begins to constrict. The chromosome masses are clearly separated at this stage (X22,500).

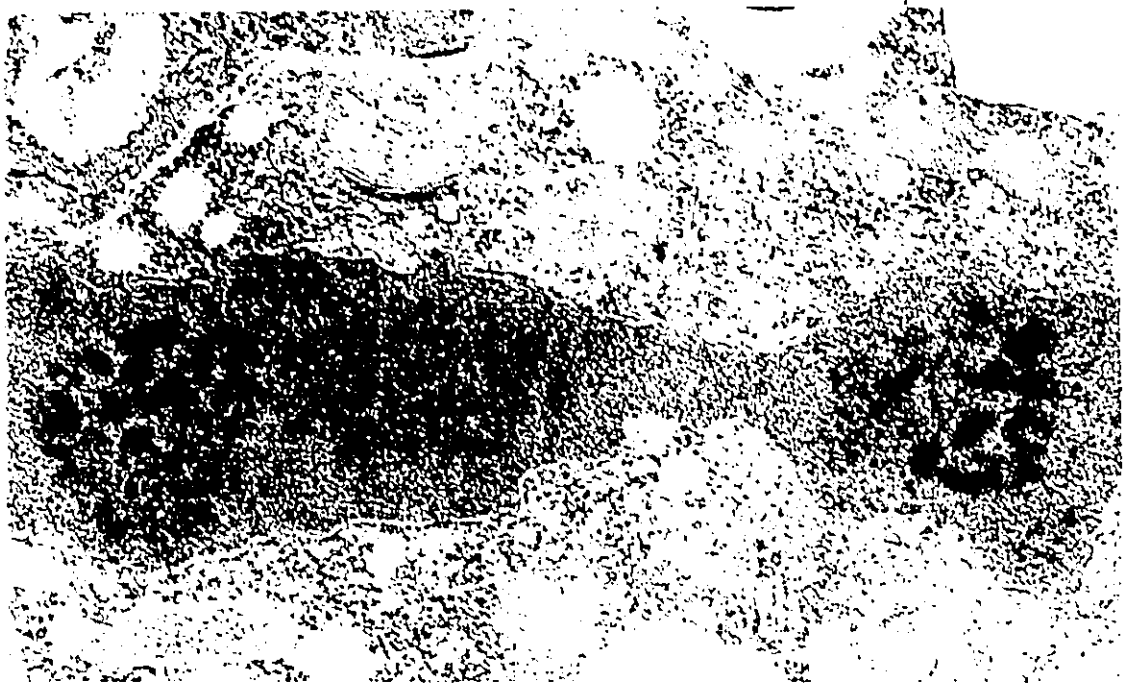


FIGURE 19: Fig(a) and (b) show nuclei in very late mitosis. Microtubules can still be seen the region of constriction (arrow in a) (X30,000).

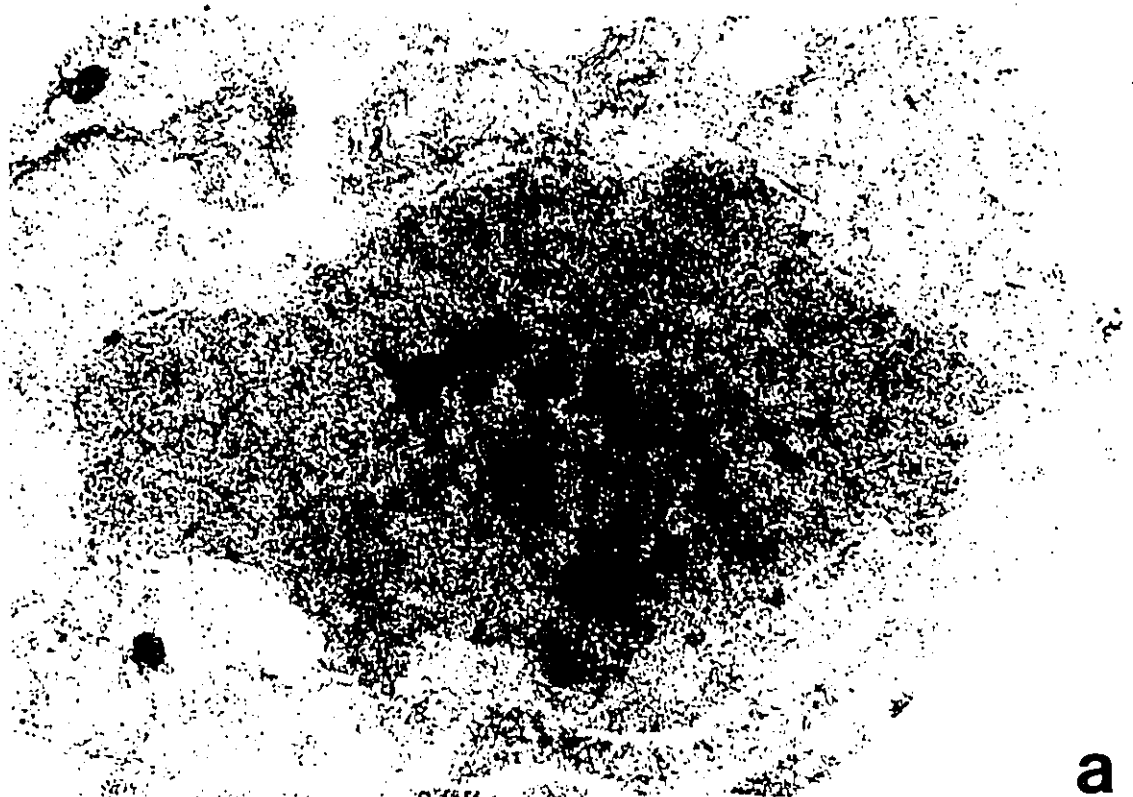


ribonucleoproteins, and is based on the chelating ability of EDTA. Fig 20a shows a mitotic cell in the early stages of nuclear elongation after staining with uranyl acetate and lead citrate. Densely staining bodies in the nucleus are clearly visible. Fig 20b is the adjacent section of the same cell incubated in 0.02M EDTA after briefly staining with uranyl acetate. The uranyl acetate enhances the contrast of cell structures known to contain both RNA and DNA. The action of the chelating agent EDTA is not to extract DNA, but to remove the uranium linked with chromatin without producing the disappearance of the stain bound to the ribonucleoprotein structures. By briefly staining in lead citrate afterwards, the contrast of the uranium staining areas which remained, was slightly enhanced. This suggests that the densely staining bodies at this and all later stages of mitosis, are chromosomes.

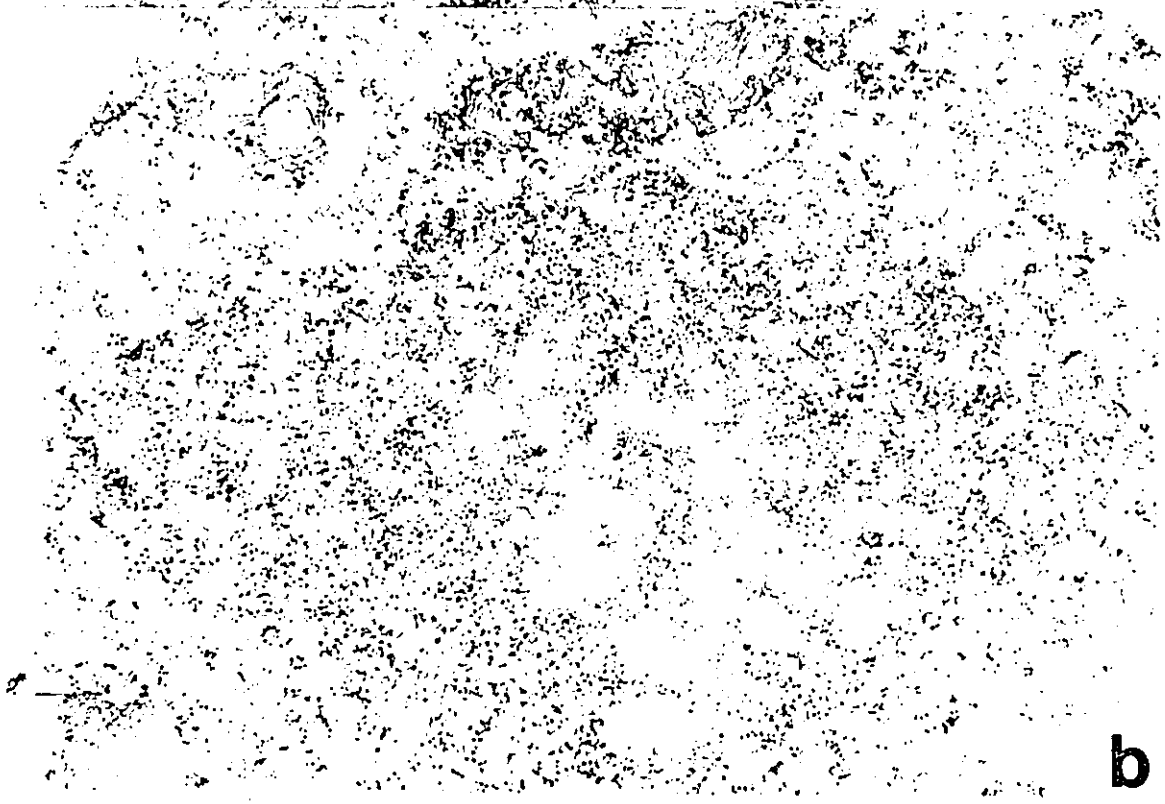
CYTOKINESIS: Light Microscopy

The first indication of cytokinesis in living cells is the duplication of the flagellar apparatus. These cells can be identified in the population because they do not exhibit the normal, rapid, forward motion and tend to swim in circles. In order to better understand the sequence of events during cytokinesis, monoclonal antibodies were generated against the flagellar apparatus. The separation and segregation of the flagellar apparatus was examined

FIGURE 20: Adjacent sections through mitotic (early metaphase) nucleus illustrating the appearance of the nucleus after conventional EM staining (lead citrate and uranyl acetate) (a) and after regressive staining with 0.02M EDTA (b). The densely stained masses in (a) have lost their stain (b) suggesting that they are chromosomes (X13,300).



a



b

using these antibodies and immunofluorescence microscopy. Two of the monoclonal antibodies appear to recognize four of the eight rootlets. The monoclonal designated 3D10 stains four rootlets in a cruciate pattern in interphase cells. This cruciate staining pattern remains unchanged in mitotic cells until late anaphase. The staining extends for approximately 2.5um along each rootlet from the anterior papilla region(Fig 21 a,b). In late anaphase or early telophase, the four rootlet structures separate (Fig 21c,d). Following mitosis ,as determined by the Hoechst staining and the position of the nuclei, the rootlet structures reassociate in two equal parts, lying parallel to each other (Fig 21e,f). As separation proceeds, new rootlet structures are seen forming perpendicular to the parental rootlets, re-establishing the original interphase cruciate pattern (Fig 21g,h).

The second monoclonal, designated 3H10, also stains in a cruciate pattern, apparently recognizing four of the eight rootlet structures (Fig 22a,b). This antibody is distributed along each rootlet and extends approximately 1.5um from the anterior papilla region. The cruciate staining pattern is maintained through anaphase. In telophase, the rootlet structures separate and reassociate into two equal pairs (Fig 22c,d). New rootlet structures are seen forming perpendicular to the parental rootlets.

Attempts to identify the antigens recognized by these antibodies were unsuccessful. In immunoblots of 1D SDS-PAGE

FIGURE 21: Immunofluorescent staining of four rootlet structures with antirootlet monoclonal 3D10 in dividing cells with the corresponding nuclear/chromosome staining with Hoechst 33258. The cruciate, interphase staining pattern is maintained throughout mitosis until late anaphase (a,b). At telophase (c,d), the rootlet structures begin to separate. Early in cytokinesis (e,f), they are segregated equally to the daughter cells. As cytokinesis proceeds, new rootlet structures are detected (arrows in g). The nuclei in (f and h) have reassumed their interphase morphology (X2,000).

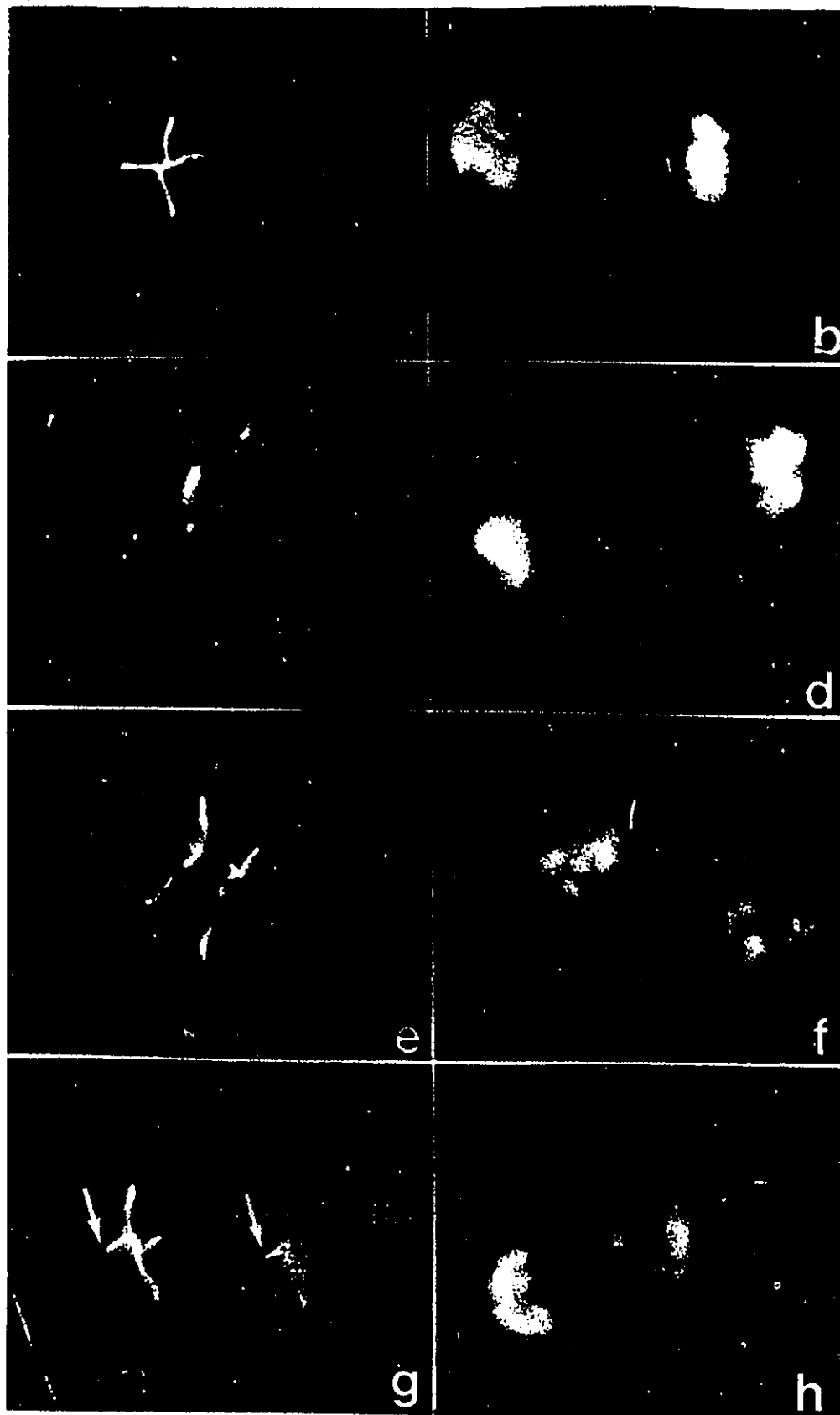
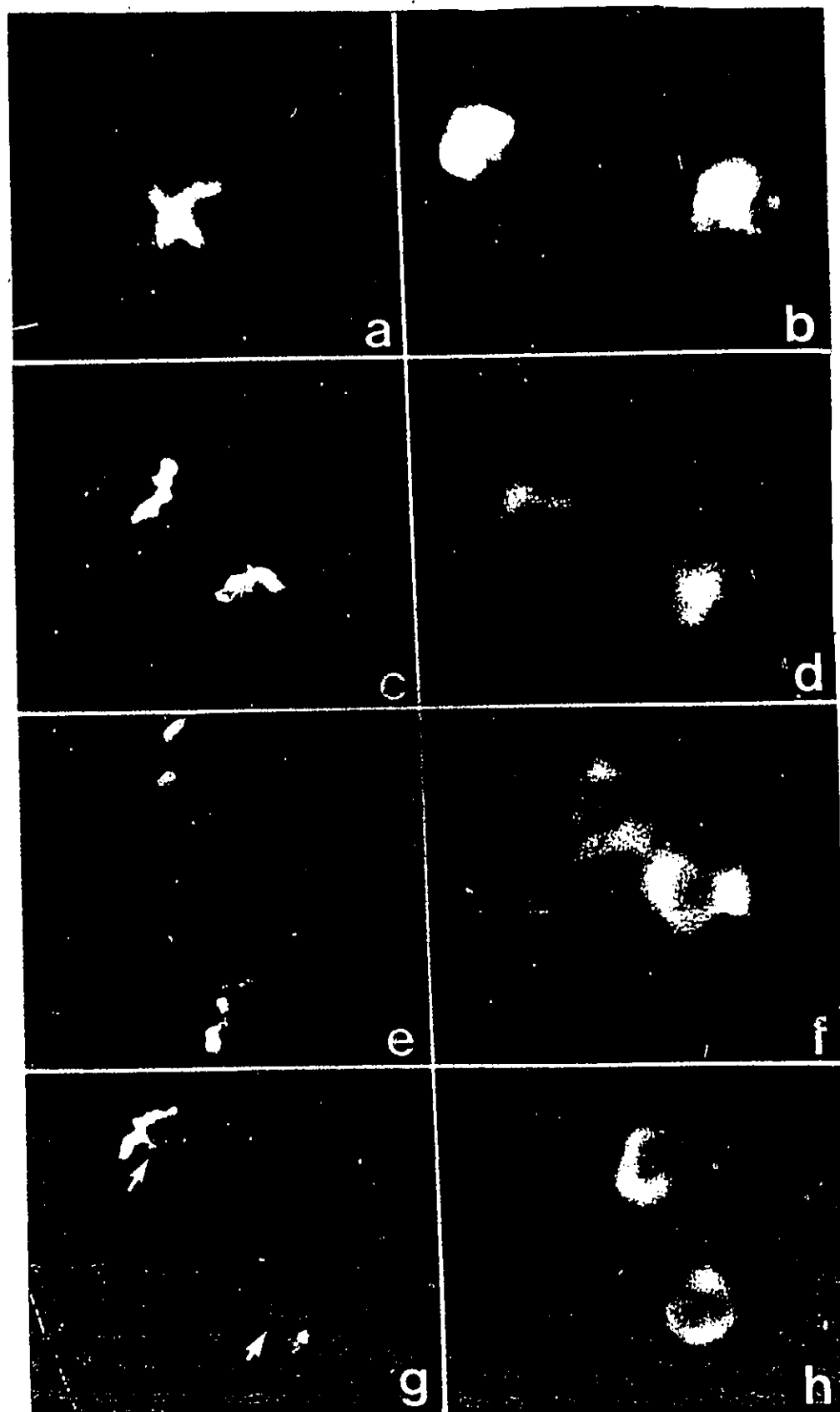


FIGURE 22: Immunofluorescent staining of four rootlet structures with the antirootlet monoclonal 3H10 with the corresponding nuclear/chromosome staining using Hoechst 33258. The cruciate interphase staining pattern is maintained until late mitosis (anaphase, a,b). Early in cytokinesis, the rootlet structures separate and segregate equally to the daughter cells (c,d). As cytokinesis proceeds (e,f), the rootlets have associated in two pairs. In late cytokinesis, two new rootlet structures per basal apparatus are detected (arrows in f and h) (X2,000).



of Polytomella whole cell or basal apparatus extracts, the antigens were not detected by either antibody. It is possible that the antigens recognized by the antibodies were sensitive to SDS denaturation. Attempts to immunoprecipitate the antigens were also unsuccessful. In double labelling experiments with both antibodies, labelling of all eight rootlets was never observed. This result, together with the overall immunofluorescent distribution of each antibody, suggests to us that the antibodies are recognizing different antigens on the same rootlet. Attempts to recognize the structures detected by the antibodies at the EM level were also unsuccessful due probably to poor penetration of the protein A:gold into the complex rootlet structure.

Electron Microscopy

As stated earlier, the post-mitotic cell shows two daughter nuclei lying close together in the central region of the cell. At this stage, eight basal bodies are present in the anterior region of the cell. The four new basal bodies are however, assembled very early in mitosis or even before the onset of mitosis. Serial longitudinal sections through the anterior region of a cell in prophase clearly shows the presence of the four parental basal bodies and the four newly formed basal bodies (Fig 23a-1). Serial cross sections through the same region shows the position of the newly formed basal bodies with respect to the parental ones

(Fig 24a,b). Formation of each new basal body appears to be initiated from the proximal region of a parental basal body. Even though the basal bodies have replicated at this early stage, the cell has only the eight parental flagellar rootlets, and the parental basal bodies are still in pairs, joined by their interconnecting fibers.

The onset of cytokinesis is marked by the separation of the flagellar apparatus. Fig 25a shows a cell in cytokinesis with the cleavage furrow extending from the anterior to the posterior. Higher magnification of the cleavage furrow shows that very few microtubules are present (Fig 25b). Fig 26(a-f) and Fig 27(a-f) are serial longitudinal sections through each new, anterior papilla region from a cell in cytokinesis, at a stage similar to that seen in Fig 25. A full complement of basal bodies is seen in one papilla region (Fig 26) however they are not yet in their normal interphase positions. The "B" pair of basal bodies are lying at an angle of approximately 180° to each other rather than the normal 150° . Also, they are not yet joined by the large, proximal, non-striated interconnecting fiber. The other papilla region (Fig 27) shows only two of the four basal bodies but also shows the association of both the compound and microtubular rootlets with the basal body and the cytoskeletal microtubules extending from them. The results here suggest that early in cytokinesis, after the flagellar apparatus has been segregated, the basal bodies and associated structures are still undergoing reorientation.

This process occurs without the basal bodies linked into pairs. Rejoining of the basal bodies into pairs, by the interconnecting fibers, does not occur until the basal bodies are properly aligned.

FIGURE 23: Non-consecutive serial longitudinal sections through basal apparatus of a cell in prophase. The entire basal apparatus is situated in the anterior of the cell far removed from the nucleus. All eight basal bodies (4 parental and 4 newly replicated) are present (parental, 2, 4, 5, 7 and newly replicated, 1, 3, 6, 8) in the anterior of the cell (X15,000).



FIGURE 24: Adjacent cross sections through basal apparatus shows the position of the newly replicated basal bodies (b) with respect to the parental basal bodies (a). The parental basal bodies exhibit counter clockwise rotation and the newly replicated basal bodies appear to be initiated from regions closely associated with the proximal regions of the parental basal bodies (X45,000).



a



b

FIGURE 25: Nuclear division is completed and the daughter nuclei(N) are at opposite sides of the cell. The cleavage furrow is being initiated from the anterior end (a). Fig b shows an enlargement of the cleavage furrow. Few microtubules are present. Those that are (arrow), probably are cytoskeletal microtubules assembled from each new rootlet(X30,000).

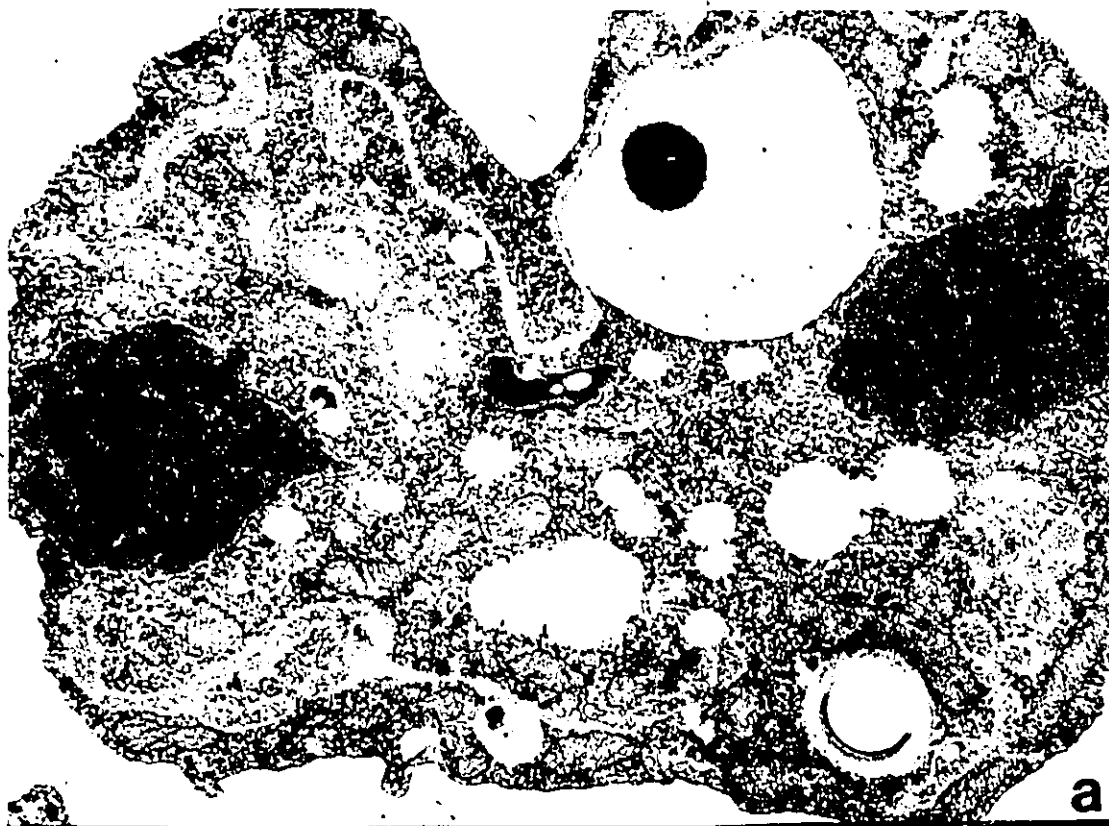
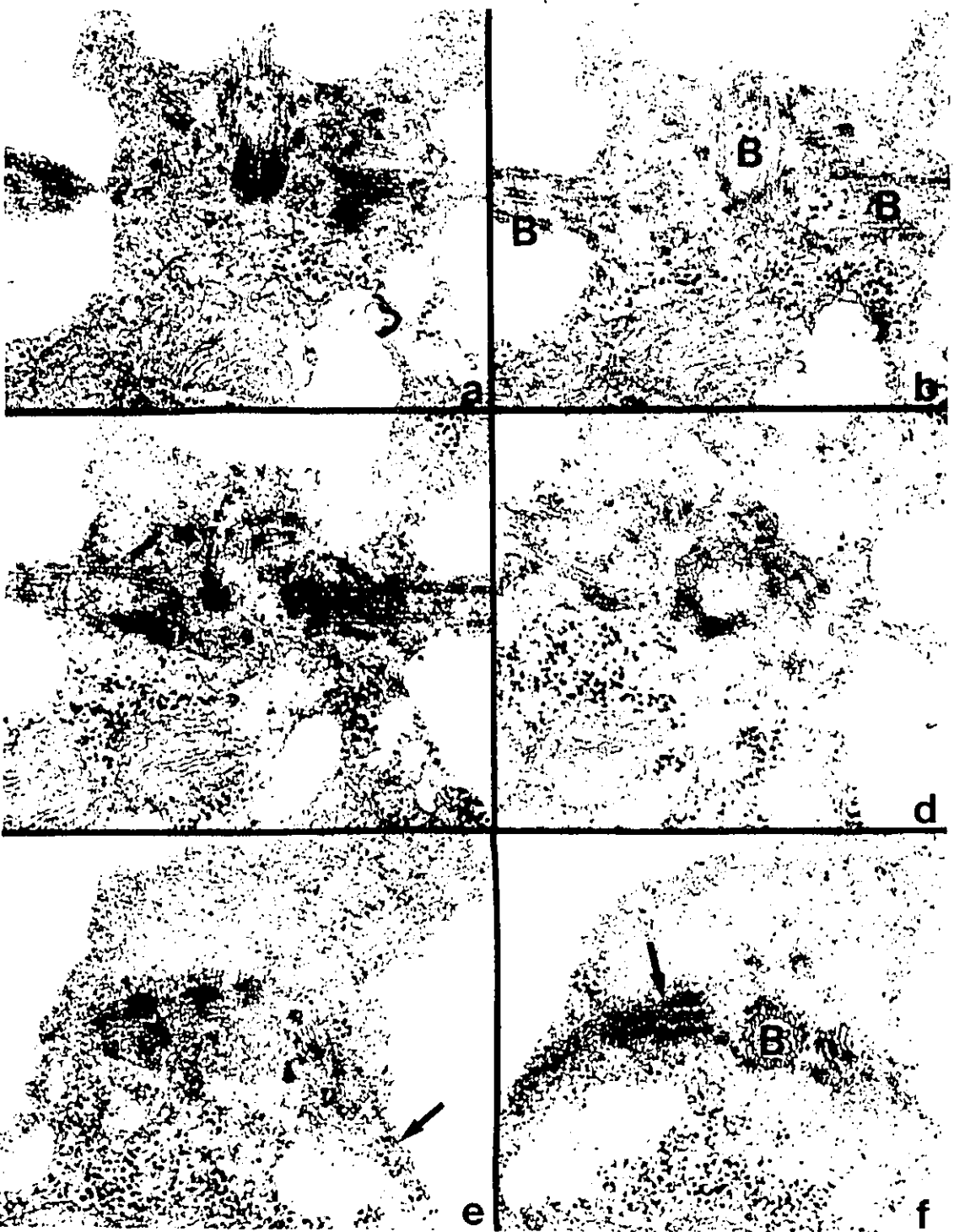
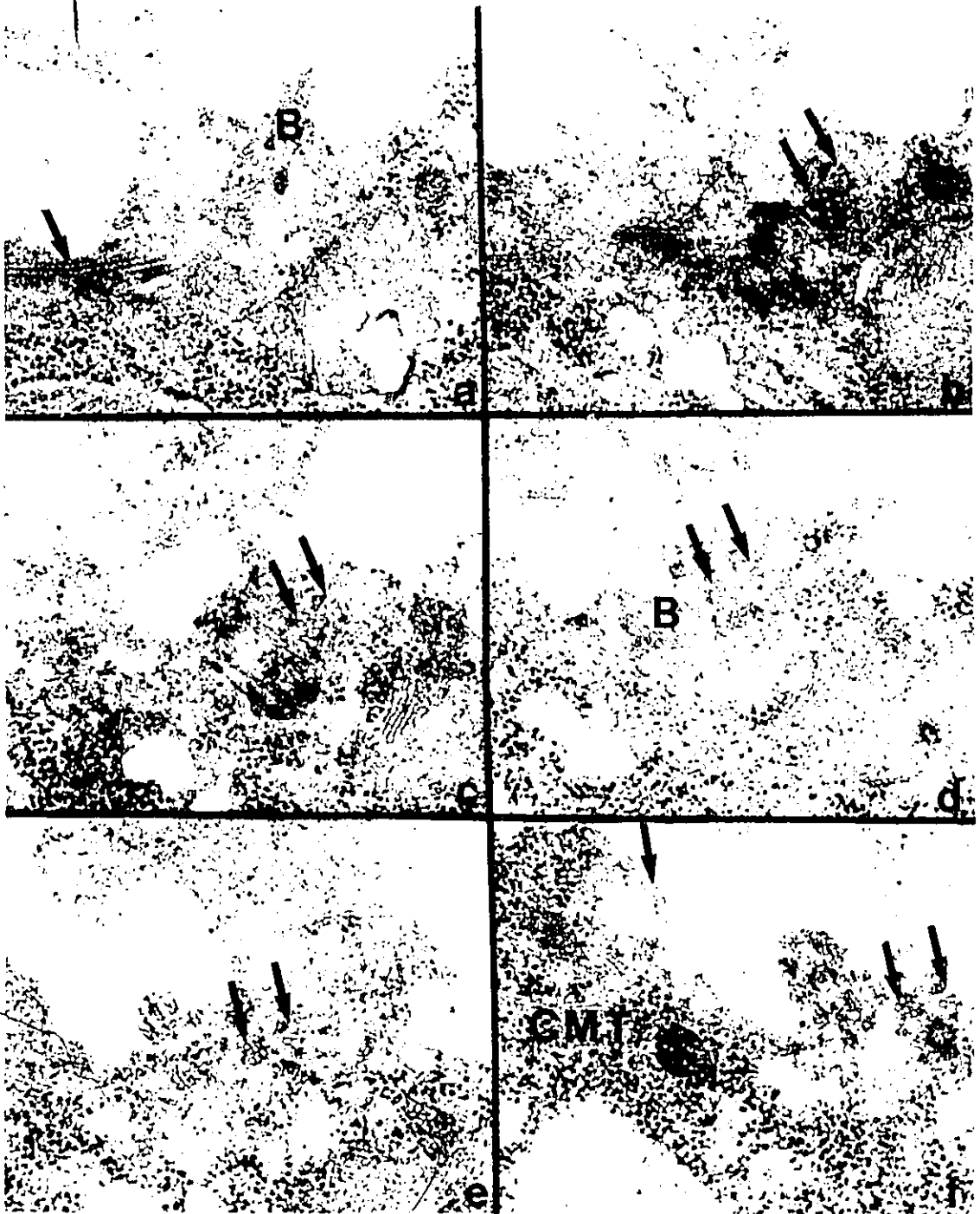


FIGURE 26 and 27: Serial longitudinal sections through the two basal apparatus of a cell in a stage similar to that seen in Fig 25. Fig 26 shows all four basal bodies (B) beginning to reform the interphase pattern. Arrows in (e) and (f) indicate flagellar rootlets. Fig 27 shows rootlet structures (compound and microtubular, arrows) with cytoskeletal microtubules assembled from the side of one rootlet (f, CMT) (X50,000).





Basal Body Segregation

How does a dividing cell equally distribute eight basal bodies to the daughter cells and then reorient them into their normal interphase organization? This appears to be accomplished by "breaking" the interconnecting fibers joining the parental basal bodies into pairs and allowing them to move individually (Fig 28a). Although not joined to each other, the basal bodies still retain their attachment with the flagellar rootlets (Fig 28b). The parental basal bodies also retain their attachment to a portion of their interconnecting fibers. One of the parental "A" basal bodies, separate from its partner, is shown with portions of the two proximal striated fibers still attached (arrows in Fig 28b). Serial cross sections through the papilla region indicate that once the interconnecting fibers are disrupted, the parental basal bodies begin to reorient (Fig 29a-d). Fig 29a shows two parental basal bodies (designated A and B). It is evident that the A basal body is no longer in its normal position and that it has begun to rotate in the direction of the B basal body. The proximal fibers joining the A pair of basal bodies are disrupted (Fig 29c,d) allowing this rotation to occur.

This model of basal apparatus segregation is summarized schematically in Fig 30. The parental basal bodies are represented by solid circles and the newly replicated basal bodies by broken circles. As separation begins the

FIGURE 28: Segregation of the basal bodies first involves disruption of the interconnecting fibers, freeing the basal bodies to move individually. Fig 28a shows a basal body with a microtubular rootlet (3 over 1) still closely associated. Fig 28b shows two parental basal bodies with associated rootlets still attached. The parental basal bodies also retain portions of the interconnecting fibers (28b; arrows indicate portions of the proximal striated fibers), (X40,000).

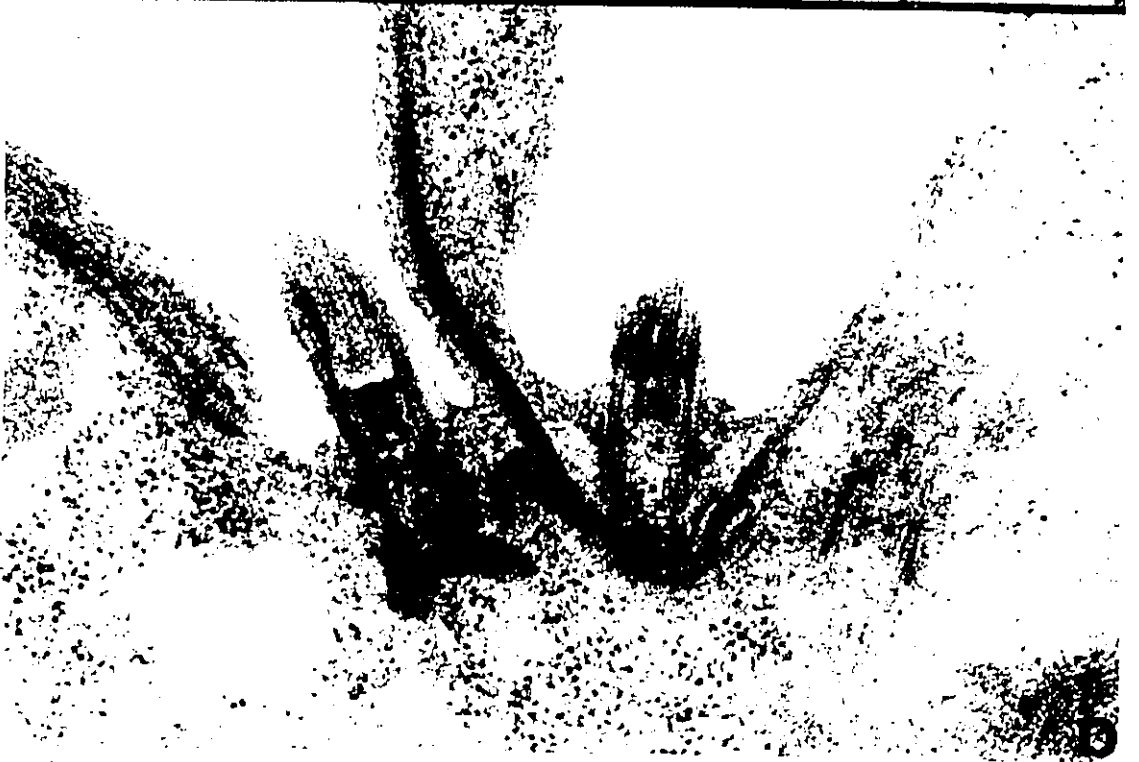
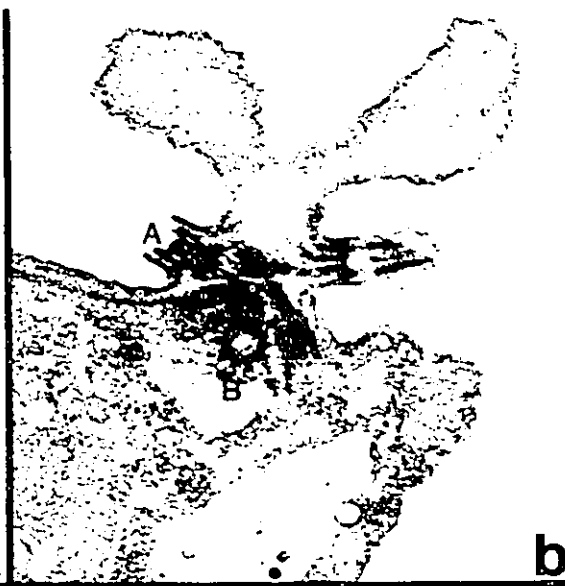




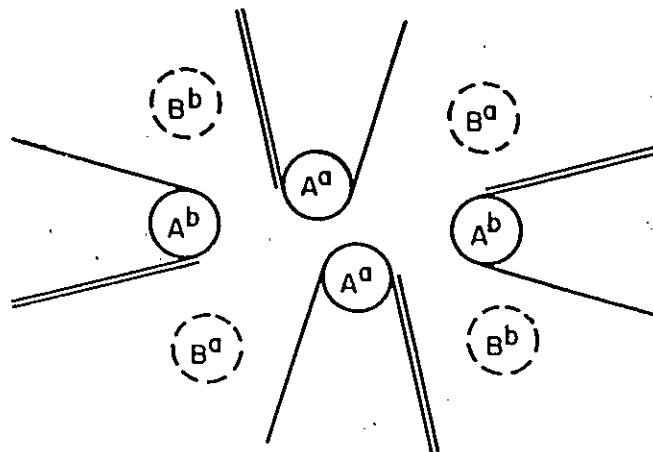
FIGURE 29: Serial sections through basal apparatus illustrating early stages of basal body movement and rotation. One of the parental "A" basal bodies (A) has begun to rotate towards the "B" parental basal body (B). This is clearly evident in 29d. (X30,000).



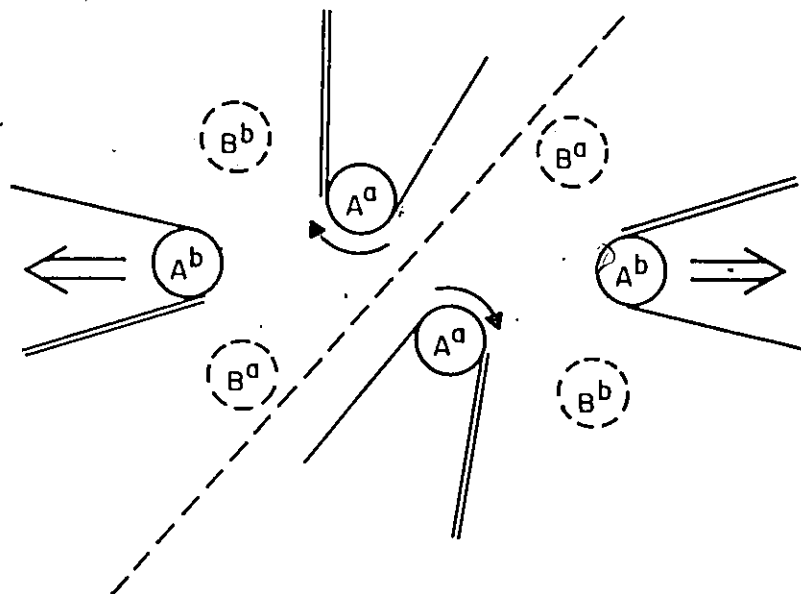
interconnecting fibers are disrupted and the parental "A" basal bodies start their rotation. The flagellar rootlets remain closely attached to the sides of each basal body. Rotation in this manner results in one parental "A" basal body and one parental "B" basal body comprising the new "A" pair in each of the daughter cells. The newly replicated basal bodies now become the "B" pair. The 90^0 rotation of the parental "A" pair also maintains the counter clockwise orientation of the basal bodies in the daughter cell. Once rotation is complete, the interconnecting fibers can reform and re-establish the normal interphase organization. New flagellar rootlets would then be assembled from the sides of each new basal body.

FIGURE 30: Schematic diagram showing arrangement of parental basal bodies (solid circles) and the new basal bodies (dotted circles). The single line attached to the basal bodies denotes compound rootlet and the double line denotes microtubular rootlet. For each basal body, the superscript designates the parental origin and the capital letter the fate of that basal body in the daughter cell. The parental basal bodies are arranged in two pairs ($A^a A^a$ and $A^b A^b$) and exhibit a counter-clockwise absolute orientation.

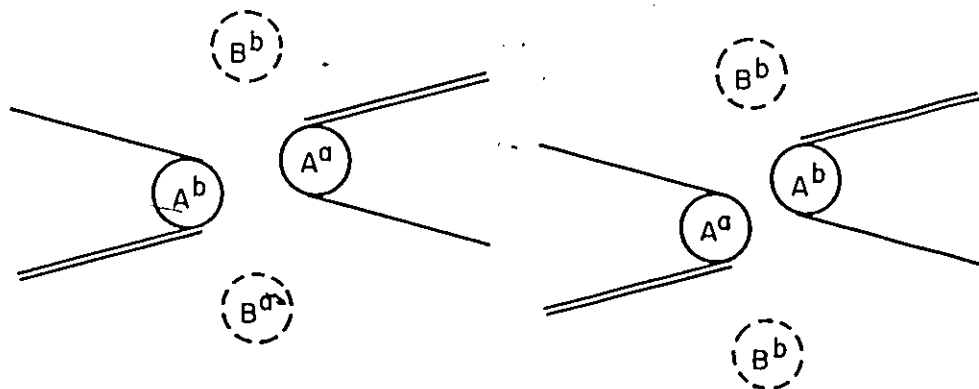
(a)



(b)



(c)



DISCUSSION

The spatial and temporal control of microtubule assembly, as well as microtubule orientation, number and final length are all roles which have been attributed to the MTOC. The MTOC therefore contains much of the information necessary to specify the assembly of a particular microtubular structure.

The assembly, and final pattern, of a microtubule-containing structure by the MTOC can be accomplished by various methods. The final pattern can be generated as a result of intermicrotubule links (Tucker, 1977) established after microtubule nucleation by the MTOC. Alternatively, the final pattern can be established as microtubule nucleation proceeds. One way of accomplishing this is to have the initiation sites patterned onto existing cytoplasmic structures which, in turn, specify the final microtubule pattern. In this way, the microtubule pattern is specified by the distribution of initiation sites upon the cytoplasmic structure but the orientation and direction of the microtubule assembly is determined by the position of the structure in the cell.

The alga Polytomella can be regarded as having two independent MTOCs. One MTOC functions in the assembly of the intranuclear spindle. The second MTOC is the entire flagellar apparatus, containing numerous initiating sites. There are initiation sites, residing on the rootlets, for

the cytoskeletal array, as well as initiation sites for the four axonemes, the basal bodies. There must also be initiation sites for the flagellar rootlets and the basal bodies.

Because of its position in the anterior of the cell and the time of formation, the flagellar apparatus is considered to have a major role in the development of cell morphology (Hoops and Witman, 1985). Stearns and Brown (1979, 1981) have already shown that the development and maintenance of cell morphology in Polytomella are due to the cytoskeletal array, assembled from initiation sites on the flagellar rootlets. The results presented in this thesis extend these observations and strongly suggest that the development of cell morphology is directly dependent upon the absolute orientation of the basal bodies. Although accurate segregation of the parental and newly formed basal bodies is essential, it is not sufficient to ensure the proper development of cell morphology. This only occurs when the proper absolute orientation of the basal bodies, in each daughter cell, is achieved. Once achieved, the assembly of new flagellar rootlets, from initiation sites firmly associated with the basal bodies, can begin. These new rootlets, whose orientation and direction are dictated by the position of the basal bodies, can then initiate new cytoskeletal microtubules. These new microtubules contribute to the cell morphology of the daughter cell.

Microtubules in Polytomella are organized into five

distinct populations; axonemes, basal bodies, rootlets, cytoskeleton and spindle. The distribution and organization of all five microtubule populations have been examined in dividing and non-dividing cells. Also, the duplication and segregation of the flagellar apparatus and the MTOCs associated with it have been examined. This is particularly important when considering a possible morphogenetic role for the flagellar apparatus.

As an approach to analyzing microtubule assembly and the duplication of the MTOCs associated with the flagellar apparatus in the flagellate Polytomella, monoclonal antibodies to Polytomella axonemal tubulin and to components of the flagellar apparatus were produced.

Monoclonal Antitubulin 5A6

Characterization of the monoclonal antitubulin used in this study has been performed by microscopic methods and by immunoblotting (Aitchison and Brown, 1986). It is clear from the data that this antibody recognizes the alpha subunit of tubulin and the epitope recognized is present in all microtubule populations in the cell. This epitope is not altered in any detectable way by the post-translational modification alpha tubulin undergoes during axonemal assembly. McKeithan and Rosenbaum (1981) observed that the alpha tubulin present in the cell body of Polytomella differed from the alpha tubulin present in the axoneme.

Recently, evidence from a number of researchers has shown that this modification is due to acetylation of the cell body alpha tubulin upon assembly into the axonemal structure (L'Hernault and Rosenbaum, 1985; Piperno and Fuller, 1985).

The epitope recognized by 5A6 is also present (by immunofluorescence and/or immunoblotting) in other protozoans, such as dinoflagellates (Brown et al., 1986), as well as in murine and bovine lymphocytes and murine 3T3 fibroblasts grown in culture. However, this antibody does not detect tubulin in higher plants, either by immunofluorescence (Seagull, personal communication) or by immunoblotting. Comparative peptide mapping studies on the alpha and beta subunits of tubulins from taxonomically distant species have revealed dramatic structural differences between the alpha subunits from plants and animals (Little et al., 1981; Morejohn and Fosket, 1982). Antibodies directed against plant (rose) alpha tubulin (Morejohn, Bureau, Tocchi and Fosket, 1984) show high cross-reactivity with algal tubulin (Chlamydomonas axonemes) indicative of their close phylogenetic distance. With increasing phylogenetic distance however, cross-reactivity decreased until a total lack of cross-reactivity with mammalian alpha subunits was observed.

The fact that 5A6 does not detect higher plant tubulin makes this result rather interesting. The epitope recognized by 5A6 appears to be conserved in organisms which produce, or have the ability to produce, flagellated and/or ciliated

cells sometime in their life cycle. It is possible that this epitope plays an important role in the ability of the alpha tubulin to be incorporated into the axonemal structure. It would be interesting to see if this epitope was conserved in lower plants, such as mosses and ferns, which do produce flagellated cells during their life cycle.

The distribution and organization of the five microtubule populations in dividing and non-dividing cells was examined by immunofluorescence. There is an extensive interphase microtubule array extending from the anterior most regions of each flagellar rootlet to the cell posterior. This microtubule population does not disassemble at mitosis, but remains intact. In fact, all microtubule populations (axonemes, basal bodies, rootlets and cytoskeleton) remain intact throughout mitosis. This implies that the spindle must be assembled from a pool of tubulin, independent of all other microtubule populations.

Replication of the basal bodies usually occurs very early in prophase, or prior to the onset of mitosis. In Polytomella, like some other green algae (Chlamydomonas), the new basal bodies appear to be closely associated with the proximal end of the mature ones. Observations in Chlamydomonas have shown that new basal bodies are firmly attached to the old ones by amorphous material, suggesting that this material serves as a nucleus for the initial stages of assembly (Cavalier-Smith, 1974). Gould (1975) noted that the probasal bodies in Chlamydomonas appeared as

- thin "annuli", consisting of nine rudimentary triplet microtubules firmly attached proximally to the mature basal bodies. Elongation of the basal bodies occurs just prior to cell division.

In Polytomella, new axonemes appear late in mitosis. New rootlets are detected soon after the onset of cytokinesis, followed closely by the assembly of new cytoskeletal microtubules from the sides of the new rootlets.

Cell Division

Throughout mitosis, the elongate morphology of the cell is maintained by the persistent cytoskeletal microtubule array. The cells remain flagellated and motile throughout mitosis. A closed spindle forms perpendicular to the long axis of the cell. During mitosis, the newly formed basal bodies mature and four additional elongating flagella appear. Following mitosis, the eight flagella segregate into two groups, each with two long and two shorter flagella. Concomitant with this separation, the rootlets of the parental basal apparatus separate and new rootlets are detected. The interphase cytoskeletal microtubule array assembles from each basal apparatus and the morphology of the two daughter cells is progressively established during cytokinesis.

Mitosis

Although many similarities exist between mitosis in Polytomella and mitosis in some closely related green algae (Chlamydomonas, Asteromonas, Dunaliella), there are some significant differences.

Polytomella organizes a spindle within a completely intact nuclear envelope. Generally, in organisms which have intranuclear spindles (e.g. Fungi), the spindle MTOC appears as an electron-dense aggregate lying next to the nuclear membrane. In Polytomella, even in serial sections through a spindle pole, a structure resembling this (or any other MTOC) has not been observed. In the two Chlamydomonas species studied, the spindles are organized from MTOCs located in the cytoplasm. The microtubules pass through polar fenestrae into the nucleus. In these cases, the flagellar basal bodies dissociate from their flagella, either by abscission or regression, and migrate to lateral positions to act as the spindle MTOCs (Johnson and Porter, 1968; Treimer and Brown, 1974; Cavalier-Smith, 1974).

The naked flagellates Asteromonas (Floyd, 1978) and Dunaliella (Marano, 1976) remain motile with the flagella attached to the original basal bodies. The entire basal body/flagellum complex then migrates to positions where it subsequently assembles the spindle. In these organisms, the absence of a cell wall permits the flagella to remain attached to the basal bodies and motile while migration of

the basal bodies toward the spindle pole is occurring. This is, of course, not possible in walled organisms and the basal bodies and the flagella must detach.

Does the migration of the basal bodies to the spindle pole have any functional significance beyond being a method of ensuring that each daughter cell receives its full complement of basal bodies? Marano, Santa-Marie and Fries (1984) found that mitosis in Dunaliella could be inhibited by the drug diazepam. Basal body duplication and separation were similarly inhibited, inducing an accumulation of abnormal mitoses with unipolar spindles. This suggests that the basal bodies are important in the bipolarization and elongation of the spindle. It appears then, at least in Dunaliella, the basal bodies function as a centrosome during mitosis.

The duplication of interphase MTOCs (and basal bodies) and migration to accomodate a new function in organizing the mitotic spindle has been observed in many cell types from fungi to animal cells. Within the algae, Ochromonas displays this phenomenon clearly. At interphase, two rootlet MTOCs (kinetobeam fiber and rhizoplast) initiate separate sets of cytoplasmic microtubules (Brown and Bouck, 1973, 1974). In mitosis, rhizoplasts are found at each spindle pole where they serve as MTOCs for the spindle apparatus. Following mitosis, the rhizoplast reassumes its function as an MTOC for the cytoplasmic microtubules. This alternation of function of the interphase MTOC does not occur in

Polytomella. The flagellar apparatus remains intact in the anterior of the cell until late mitosis; a second MTOC being responsible for spindle formation. The intact flagellar apparatus appears to be functioning in two ways; first, in maintaining cell polarity and secondly, in maintaining cell morphology.

Spindle Morphology

Microtubule arrangement and distribution during mitosis has been examined by immunofluorescence and by electron microscopy. Early in mitosis, even before nucleolus dispersal, a bundle of microtubules can be seen in the nucleus extending from one side to the other, possibly functioning in the initial stages of nuclear elongation. As the spindle begins to elongate, more microtubules are seen extending from both poles, particularly near the nuclear envelope. The number of microtubules appears to peak during organization of the metaphase spindle. Although difficult to determine by EM, by immunofluorescence it appears as a typical metaphase spindle with equatorial arrangement of chromosomes. During anaphase, the spindle elongates further, the poles become tapered and lie next to the plasma membrane on opposite sides of the cell. All of the microtubules still present at this stage, fewer than metaphase, now appear to extend pole-to-pole and the chromosome masses are separated and lie at opposite ends of the spindle. The spindle then

becomes less tapered and very few microtubules remain. As the nucleus constricts, some microtubules are still observed in the region of constriction. At telophase, the spindle collapses and the divided nuclei approach one another in the center of the cell.

It has already been stated that at least four types of microtubules exist in the typical mitotic spindle (astral, pole-to-pole, kinetochore and non-kinetochore). Polytomella contains no astral microtubules. The majority of microtubules present in late mitosis (anaphase, telophase) appear to be pole-to-pole and/or non-kinetochore microtubules. The presence of kinetochore microtubules has been difficult to assess due to the fact that kinetochores, identified by their trilaminar appearance or by the insertion of microtubules into the chromosome, have not been observed. This difficulty is further compounded by the inability to identify individual chromosomes in the mitotic nucleus.

In Polytomella, when viewed by EM, the interphase nucleus contains a large, central, densely staining nucleolus. This correlates well with what is seen by fluorescence microscopy using the DNA specific stain Hoechst 33258. During interphase, there is diffuse staining throughout the nucleus, with a distinct absence of stain in the area of the nucleus corresponding to the position of the nucleolus.

The large nucleolus disperses during mitosis. By EM,

the nucleoplasm is more densely stained than in interphase and the nucleus also contains numerous densely stained bodies. These densely staining bodies appear to be distributed throughout the nucleus early in mitosis and they are segregated to opposite poles during anaphase. Results obtained using the technique of regressive staining, in which EDTA is used to selectively destain DNA-containing structures while RNA-containing structures remain stained, suggest that the densely staining bodies seen in mitotic nuclei are chromosomes.

These results again correlate well with the fluorescent observations. The diffuse interphase staining disappears during mitosis. The chromatin condenses to the center of the nucleus and appears to be equally segregated to opposite poles during anaphase. As the nucleus reassumes its interphase appearance during cytokinesis, the chromatin decondenses and the nucleus again appears diffusely stained, except for the nucleolus.

Spindle microtubules have not been observed in association with these dense bodies. Serial sections through mitotic nuclei show that the microtubules extend from the polar regions and lie close to the nuclear envelope, with few microtubules seen penetrating the center of the nucleus.

Spindle Function

How do microtubules carry out their proposed functions during mitosis, i.e., spindle elongation and chromosome transport? Obviously, the actual function of the microtubules cannot be determined simply by examining their distribution and organization. However, a mitotic mechanism can be inferred by comparing the spindle morphology of Polytomella to spindles of other lower eukaryotes (fungi, diatoms, dinoflagellates) where spindle function has been investigated. Research in this area has led to the conclusion that there is more than one mechanism for chromosome transport (Pawletz, 1983). There is, in fact, evidence to support a variety of mechanisms, the mechanism differing with different spindle microtubule organization.

The fungi are a diverse group and exhibit extreme diversity in spindle morphology, however, the force generating mechanism is thought to be common. This involves an interaction between cytoplasmic microtubules, the nuclear envelope or spindle MTOC, and the cytoplasm (Aist and Williams, 1972; Heath and Heath, 1976; Aist and Berns, 1981; King, Hyams and Luba, 1982). Laser beam irradiation of late anaphase spindles of Fusarium resulted in a dramatic increase in the rate of separation of daughter nuclei. This, together with the large increase in astral microtubules, indicates that the interzone microtubules may function in the regulation of separation in anaphase, while the astral

microtubules, which exhibit close interactions with the plasma membrane, exert extranuclear forces pulling on the spindle (Aist and Berns, 1981).

Ultrastructural studies of nuclear division in the dinoflagellates have provided evidence suggesting that chromosome distribution occurs passively, through chromosome/nuclear envelope associations. In the dinoflagellate Syndinium (Ris and Kubai, 1974), two distinct types of microtubules exist; one bundle running pole-to-pole (nucleated by centrioles) within a channel and others which radiate from the poles. These latter microtubules attach directly to kinetochores which protrude through pore-like openings in the nuclear envelope. Although there is microtubule/chromosome interaction, these microtubules do not shorten in conjunction with the poleward progress of the chromosomes. This implies that the actual chromosome movement is generated by elongation of the pole-to-pole microtubules and chromosomes are being passively dragged along with the separating poles to which they are anchored by microtubules.

Diatom spindles are characterized by having a remarkable degree of organization (Pickett-Heaps and Tippit, 1978). The spindle initially forms outside the nucleus. Microtubule elongation proceeds until at prometaphase, the nuclear envelope breaks down and the spindle enters the nucleus. The metaphase spindle is characterized by two overlapping half spindles. Microtubules, not part of the

central spindle, radiate out and penetrate the chromatin (small, single-layer kinetochores have been observed). In Diatoma (McDonald et al., 1977), spindle elongation occurs by microtubule elongation in addition to microtubule sliding in the region of overlap. Spindle elongation in other diatoms (Hantzschia, Nitzschia: Tippitt, Pickett-Heaps and Leslie, 1980) occurs simply by microtubule sliding generated between half spindles in the region of overlap.

Based on a comparison with these observations, we can speculate on how the spindle in Polytomella functions. There are no astral microtubules and there appear to be no kinetochore microtubules. The possibility however, that there are lateral connections between the chromosomes and microtubules cannot be overlooked. Large numbers of microtubules are seen extending from the poles, running along the nuclear envelope. The spindle, from metaphase to telophase, is composed of either pole-to-pole microtubules or non-kinetochore microtubules which extend almost the entire length of the spindle. It would be necessary to track the microtubules through serial cross-sections from one pole to the other to make this distinction.

The spindle also undergoes extensive elongation, almost doubling. The elongation could be a result of microtubule elongation, microtubule sliding or a combination of both. If microtubule sliding was a major factor in elongation, one would expect to see, by immunofluorescence, greater intensity of stain in the region of overlap. This is not the

case as the intensity of stain is the same along the entire length of the microtubule bundle from one pole to the other suggesting very little sliding. The polar regions show a very high intensity of staining due to the large numbers of microtubules focusing at the poles. This arrangement of microtubules implies that spindle elongation results mainly from microtubule elongation. In this way the chromosomes, either through lateral attachment to microtubules or attachment to the nuclear envelope, are then passively separated.

Cytokinesis

Cytokinesis in Polytomella does not begin until after nuclear division is complete. The cleavage furrow is initiated from the anterior end of the cell without the involvement of a specialized microtubular structure. The existence of a complex cytokinetic apparatus consisting of microtubules, a phycoplast, is a common feature in the green algae (Pickett-Heaps, 1975). These microtubules are always oriented in the plane of cytokinesis (in contrast to the phragmoplast system of higher plants where the microtubules are oriented perpendicular to the plane of cytokinesis). The microtubules present in the region of the cleavage furrow in Polytomella are likely cytoskeletal microtubules assembled from the newly forming flagellar rootlets. These microtubules, as well as contributing to the establishment

of the cell morphology in the daughter cells, are acting ,by definition, as a phycoplast in that they are ensuring proper separation of cell constituents. They are not however, part of a specialized microtubular structure assembled specifically for this purpose.

The flagellar rootlets, as shown by the distribution of the antigens recognized by antibodies 3H10 and 3D10, do not separate until late mitosis. They are equally distributed to the daughter cells, following which, new rootlet structures are detected. The exact stage of basal body/flagellar rootlet separation is not known, nor is how they are distributed to the daughter cells. Observations of dividing cells show the parental flagella remain attached to the basal bodies during mitosis, and at the start of cytokinesis, four additional elongating flagella appear. The eight flagella segregate into two groups, each with two long and two shorter flagella, which separate towards opposite poles of the cell. It seems probable that each daughter cell receives two parental and newly formed basal bodies. Although it has yet to be shown conclusively, the two basal bodies of Chlamydomonas appear to be segregated semi-conservatively, with each daughter cell receiving one parental and one newly formed basal body (Adams, Wright and Jarvik, 1985 and references therein). The question that remains though, is how accurate segregation of the basal bodies occurs. In many cells, the positioning of the basal bodies at the spindle poles ensures equal distribution to

the daughter cells however, this is not observed in Polytomella. Recently, a nucleus-basal body connector has been observed in Chlamydomonas reinhardtii (Wright, Salisbury and Jarvik, 1985). The authors speculate that this connector may function to couple the basal body to the mitotic cycle. Alternatively, it might serve to properly localize basal bodies with respect to the nucleus ensuring equal distribution at cytokinesis. This suggestion was supported by the results obtained with a Chlamydomonas mutant (vfl-2) which contains a variable number of basal bodies per cell. These cells exhibit a defect in the nucleoflagellar connector inviting speculation that the variable basal body number phenotype is a result of the connector failing to localize the basal bodies precisely enough to guarantee equal segregation at cytokinesis.

Whatever the mechanism(s) involved, accurate segregation of the flagellar apparatus is imperative if the cell morphology of the daughter cells is to be properly established. Previous observations (Stearns and Brown, 1981) showed that the flagellar rootlets functioned in the assembly of the cytoskeletal microtubule array which is directly responsible for the development of cell morphology. Therefore absolute orientation of the flagellar rootlets is required. I suggest that the rootlet organizing center is located within, or firmly associated with, the basal body. Once proper basal body orientation has been achieved, new rootlet growth is initiated and proper cell morphology is established.

Model For Segregation of Flagellar Apparatus

Flagellar apparatus components show very specific positional relationships and, in some cases, may even be associated with specific basal body triplets (Hoops and Witman, 1983). Proper flagellar apparatus assembly therefore depends upon the conservation of the absolute orientation of the basal bodies during replication and segregation. Basal body absolute orientation is, in turn, dependent upon a series of reorganization events involving a rotation of the parental basal bodies during separation.

In Polytomella, the parental cell contains four basal bodies with a counter clockwise absolute orientation (O'Kelly and Floyd, 1984). These basal bodies are arranged in two pairs; an "A" pair and a "B" pair. Each basal body replicates in late interphase or early prophase. The position of the newly replicated basal bodies with respect to the parental ones is shown schematically in Fig 30a. Based on electron microscopic observations and observations of the distribution of the rootlet antigens recognized by 3D10 and 3H10 during mitosis and cytokinesis, it is proposed that segregation of the flagellar apparatus occurs in the following manner. Throughout most of mitosis, the organization of the flagellar apparatus remains unchanged from that seen in the interphase cells. In late mitosis, the striated and non-striated fibers interconnecting the basal bodies are disrupted, freeing the basal bodies to move

individually, rather than in pairs. Each basal body continues to retain its association with the rootlets. As separation proceeds, the parental "A" basal bodies rotate 90^0 , and occupy new positions facing the parental "B" basal bodies (Fig 30b). The associated rootlets also rotate at the same time. This rotation allows the counter clockwise absolute orientation to be conserved and initially results in the daughter flagellar apparatus reorienting at a 90^0 angle with respect to the parent (Fig 30c). The new "A" pair of basal bodies in one of the daughter cells then, is composed of one of the parental "A" pair (A1) plus one of the parental "B" pair (B1). The "B" pair of basal bodies in the daughter is then composed of the newly replicated basal bodies A2/ and B1/.

New rootlet assembly is not initiated until after parental basal body rotation and reorientation is complete. This suggests that the rootlet's position in the cell is determined by the position of the basal body. Once assembled, each new rootlet is quickly capable of assembling new microtubules which contribute to the establishment of the morphology of the daughter cell.

Re-establishment of the normal interphase organization also requires the reformation of the striated and non-striated interconnecting fibers. It is very difficult to determine whether the interconnecting fibers function to facilitate basal body orientation, or whether they reform after the basal body orientation has been achieved. Previous

studies, using Chlamydomonas, have shown that the striated fibers and microtubular rootlets are important in the development or long-term maintenance of proper basal body positioning and orientation (Hoops and Witman, 1983).

Because the cells used were deficient in both striated fibers and rootlets, the study could not distinguish which of these components was responsible. In the present study, the appearance of new rootlet assembly after the parental basal bodies have reoriented suggests that the rootlets are not involved in the positioning of the basal bodies. Whether or not the basal bodies are positioned and oriented before, during or after reformation of the interconnecting fibers is not known, nor is the exact mechanism of fiber reformation fully understood. Parental "A" basal bodies in the process of segregation often have remnants of the original interconnecting fibers still attached to them. The possibility exists then, that each parental "A" basal body retains portions of its interconnecting fibers and is able to assemble more fiber in order to associate with the appropriate parental "B" basal body during cytokinesis. Remnants of the large non-striated fiber interconnecting the "B" pair of basal bodies have never been observed. If this hypothesis is true, the net result is that all of the information necessary for morphogenesis of the flagellar apparatus is contained within the basal bodies themselves.

This is the first comprehensive study of flagellar apparatus duplication and segregation in the flagellated

green algae. Studies on biflagellate algae, especially Chlamydomonas, have suggested that the basal bodies segregate semi-conservatively. This is also true for the few available studies of quadriflagellate algae. The green alga Pyramimonas has four basal bodies with a counter clockwise absolute orientation. The basal bodies replicate prior to mitosis and the eight basal bodies begin to separate at preprophase into two groups each possessing two new and two old basal bodies (Pearson and Norris, 1975). These authors however, do not give any indication as to which parental basal bodies segregate to each daughter cell nor how this segregation occurs.

Similar observations have been made in Polytomella, with two long (parental) and two shorter (newly replicated) flagella seen segregating to each daughter cell at cytokinesis. But in Polytomella, the four parental basal bodies are not equivalent. The basal bodies of the "A" pair are situated more anterior in the cell than the "B" pair and are inserted at an angle of 110^0 - 120^0 to one another. They are linked distally by a large striated fiber and proximally by two smaller striated fibers. The "B" pair of basal bodies are situated slightly deeper in the cell at an angle of 150^0 to one another and are linked proximally by a single large non-striated fiber. The results show that these connections are disrupted during mitosis, with each basal body migrating as a single unit. The parental basal bodies are then distributed equally in a truly semi-conservative fashion (one "A" and one "B" basal body) to the daughter cells.

SIGNIFICANCE

This thesis represents a significant contribution to our knowledge of the structures and dynamics of the flagellar apparatus of Polytomella. The production of monoclonal antibodies has allowed the characterization of the microtubule populations and aspects of the rootlet system. The detailed ultrastructural investigation has clarified our general understanding of the duplication of the flagellar apparatus of this organism.

The relevance of these observations lie not only in the extension of knowledge in this specific area, but, more importantly in the advance in understanding of the eukaryotic centrosome in general. The flagellar apparatus of Polytomella represents a model system of choice for the understanding of the centrosome. The model describing basal body separation and segregation is particularly relevant here. It appears that the "B" set of basal bodies can be considered immature in terms of their accessory fibers and that these basal bodies gain a level of structural maturation as they proceed into their second cell cycle.

The success of the monoclonal antibody technique has shown that this technique can be used to dissect the centrosome biochemically. Given that one can isolate the basal apparatus of Polytomella intact in a microtubule assembly competent form, we are also provided with a unique

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in vitro system where specific functions of these antigens
can be observed.

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APPENDIX 1- HY CULTURE MEDIUM FOR HYBRIDOMAS

HY medium used for the culture of mouse hybridomas was prepared according to the method of Kennett (1980) and consisted of the following:

- Dulbecco's modified Eagle's medium with high glucose
- 10% NCTC 109 medium
- 20% heat-inactivated fetal calf serum (FCS)
- 0.15mg/ml oxaloacetate
- 0.05mg/ml pyruvate
- 0.2U/ml insulin
- 100U/ml penicillin
- 100ug/ml streptomycin

For HAT selection of hybridoma cell lines (Littlefield, 1964), the following ingredients were added to the HY medium:

- 0.1mM hypoxanthine
- 0.4uM aminopterin
- 16uM thymidine

After 6 days, aminopterin was omitted from the HY medium.

APPENDIX II- PREPARATION OF WHOLE CELL EXTRACTS

Whole cell extracts were prepared from:

- (a) Polytmella agilis
 - (b) Murine and bovine lymphocytes
 - (c) 3T3 mouse fibroblast cells in culture
 - (d) Vicia hajastana
- (1) Cells were resuspended in 1.0 ml of Tris buffer (10 mM, pH 7.8).
 - (2) Cells were then immersed in boiling water for 7-8 minutes.
 - (3) 10X volume of cold acetone was yhen added.
 - (4) cells were homogenized in a glass to glass homogenizer.
 - (5) The precipitate was pelleted by centrifugation at 2,000xg for 5 minutes.
 - (6) The pellet was washed 2X with cold acetone.
 - (7) The final pellet was allowed to air-dry and was finally resuspended in SDS sample buffer at an appropriate protein concentration (usually 1 mg/ml).