THE DISTRIBUTION OF MICROTUBULES IN POLYTOMELLA

by

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This thesis is submitted in partial fulfillment of the requirements for a Master of Science Degree at the University of Ottawa.

January 15, 1974.

ACKNOWLEDGEMENTS

The cooperation and assistance of the following associates in the preparation of this thesis is gratefully acknowledged.

Supervision and Guidance.

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Leo Simone, Dr.
New York State University,
Potsdam Campus, U.S.A.

Consultation.

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Canada Department of the Environment

Use of Scanning Electron Microscope

Dr. G.H. Haggis,
Canada Department of Agriculture,
Research Division,
Dominion Experimental Farm

Photographic Advice and Use of Facilities

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ABSTRACT

The distribution of microtubule systems in the quadriflagellate unicell, Polytomella, is detailed. The motile vegetative cell has a single, anterior cruciform papilla which contains a dense microfibrillar network but no microtubules or other organelles. Four equal flagella insert at approximate right angles to each other into depressions at the base of the papilla. The four basal bodies are embedded in an amorphous electron-dense material, and opposite basal bodies are connected by striated fibers. Two structurally distinct rootlets, one consisting of closely appressed microtubules (microtubular rootlet), the other of fine fibers with alternating electron-dense and electron-transparent regions (striated rootlet), insert between each pair of adjacent basal bodies. Cytoplasmic microtubules terminate on all eight rootlets, diverge, and extend just beneath the plasma membrane to the cell posterior. Studies correlating microtubule reappearance and shape regeneration in cells exposed to hydrostatic pressure indicate that a) these rootlets function as nucleating sites for microtubule assembly, and b) the cytoplasmic microtubules associated with these sites function in development and maintenance of cell shape. Synchronous division in Polytomella can be induced using

temperature shocks. Formation of the mitotic spindle is intranuclear, and nuclear division precedes replication of the flagellar and cytoplasmic microtubule systems.

Résumé

La distribution des systèmes de microtubules dans l'unicellulaire quadriflagellé, Polytomella, est détaillée. La cellule végétative mobile a une unique papille cruciforme antérieure laquelle contient un réseau fibrillaire dense mais sans microtubules ou autres organelles. Quatre flagelles égales s'insèrent dans les dépressions à la base de la papille en formant un angle droit entre chacun d'eux. Les quatre corps basaux sont entourés d'une substance amorphe, dense aux électrons. Des fibres striées relient les corps basaux opposés. La cellule contient deux "rootlets" structurellement différents, le premier constitué de microtubules étroitement juxtaposées ("rootlet" microtubulaire). Le deuxième de fibres fines avec des régions alternativement denses et transparentes ("rootlet" strié), s'insèrent entre chaque paire de corps basaux adjacents. Les microtubules cytoplasmiques se terminent sur les huit "rootlets", divergent, puis s'étendent directement sous la membrane cellulaire jusqu'à l'extrémité postérieure de la cellule. Les études mettant en corrélation la réapparition des microtubules et la régénération de la forme des cellules exposées à une pression hydrostatique indiquent que: a) ces "rootlets" fonctionnent comme "nucleating sites" lors de l'assemblage de microtubules, et b) les microtubules cytoplasmiques associés à

ces sites, jouent un rôle dans le développement et le maintien de la forme de la cellule. La division synchronisée chez Polytomella peut être induite par l'utilisation de chocs thermiques. La formation du fuseau mitotique est intranucléaire, et la division nucléaire précède la réplication des systèmes de microtubules flagellaires et cytoplasmiques.

The occurrence of microtubules was first reported in animal cells by Slautterback (1963) and in plant cells by Ledbetter and Porter (1963). The widespread distribution of microtubules was recognized with the advent of improved procedures for fixation of both plant and animal cells (Sabatini, Bensch and Barrnett, 1963). The fixative glutaraldehyde, followed by post-fixation in osmium tetroxide, made the preservation of these tubules possible.

Microtubules are slender, unbranched structures which measure between 200 Å and 250 Å in diameter. In cross-section these tubules appear as a dense cylindrical wall around a less dense centre. Seen in cross-section, the dense wall is believed to be made up of 11 to 13 closely associated, globular subunits, each measuring approximately 40 Å in diameter (Ringo, 1967a, Newcomb, 1969).

Because of the widespread occurrence of microtubules, a variety of speculations was made as to their possible role or roles in cells.

Microtubules are involved in movement of cilia and flagella. These organelles were known to consist of minute fibrils. The advent of the electron microscope made the detailed study of the cilia and flagella possible. Using negatively stained preparations, Grigg and Hodge (1949) reported 11 fibrils in sperm tails. Manton and Clarke (1950) were the first to recognize the structural uniformity of plant flagella. Manton (1952) concluded, from her many observations, that flagella and cilia consisted of 9 outer fibrils and 2 structurally different central fibrils. Much work has been done on the ultrastructure, the mechanisms of movement and the biochemistry of these motile organelles, and review articles are available (Gibbons, 1968, Joyon and Mignot, 1969, Warner, 1972).

Microtubules also function in chromosome movement in the mitotic spindle apparatus. Inoué and Sato (1967) hypothesized that the contraction and the elongation of the spindle microtubules and filaments were responsible for the regular mitotic movements of chromosomes. They contend that the spindle fibers can elongate by condensation and polymerization of the spindle protein molecules thereby providing forces to push chromosomes; conversely, depolymerization of spindle fibers accounts for their shortening and contraction.

Microtubules may also function in the distribution and translocation of particles within the cell. They may also define cytoplasmic streaming channels, thus compartmentalizing cytoplasmic materials (Porter, 1966, Newcomb, 1969, Tilney, 1971a).

In many animal and protistan cells, the microtubules are believed to play a cytoskeletal role. These tubules are oriented and distributed in the cytoplasm of cells in sufficient numbers to account for the development and maintenance of cell asymmetries (Porter, 1966, Tilney, 1968a). Within the axopodia of Actinosphaerium nucleofilum is an axoneme which is composed of two interlocking coils of microtubules (Tilney and Porter, 1965). If the microtubules in the axopodia are kept in a depolymerized form using hydrostatic pressure (Tilney, Hiramoto and Marsland, 1966), low temperature (Tilney and Porter, 1967) or colchicine (Tilney, 1968b), the axopodia are unable to reform; regrowth of the cell extensions begins when these agents are removed and microtubules are present in the redeveloping axopodia. Recently, Bouck and Brown (1973) described the entire complement of microtubules in Ochromonas and using microtubule depolymerizing agents, such as colchicine and hydrostatic pressure, demonstrated an obligatory role for cytoplasmic microtubules in establishing and maintaining cell shape (Brown and Bouck, 1973).

Vinblastine has been shown to cause the breakdown of microtubules into filaments. The formation of large cytoplasmic crystals, apparently composed of microtubule protein subunits and bound vinblastine, is also observed (Marantz et al., 1969, Olmsted et al., 1970, Bryan, 1971, Behnke and Forer, 1972). Microtubules are responsible for elongation of cells of the neural anlage which occurs during the neurulation in Xenopus. Embryos treated with vinblastine sulfate lose their elongate shape due to the disruption of microtubules (Karfunkel, 1971).

Microtubules are believed to play a role in the formation and elongation of the filopodia of the secondary mesenchyme cells in Arbacia. Development is arrested in embryos treated with colchicine or hydrostatic pressure (Tilney and Gibbins, 1968). Since these agents cause disassembly of cytoplasmic microtubules, it appears that the microtubules are related to the production of cell shape in this multicellular embryo.

In plant cells which possess rigid, supportive cell walls, microtubules, if they are involved in the deposition of the wall microfibrils, may function in controlling cell shape. It has been assumed that microfibrillar orientation at the time of deposition is under microtubular control. This is supported by observations correlating the parallel orientation of the microtubules and the newly deposited microfibrils in growing primary walls, and in the developing thickening of secondary walls (Newcomb, 1969).

Microtubules have been shown to function in the development and maintenance of cell shape. Questions arise regarding the regulation of flagellar and cytoplasmic microtubule appearance and their distribution in cells since the factors which determine the orientation and assembly of microtubules are important for they ultimately control cell differentiation. There are sites within cells on which microtubules terminate. The microtubules, which are free at one end, appear to make contact with identifiable sites such as the surface of centrioles (Pickett-Heaps, 1971), parts of the nuclear envelope (Robinow and Marak, 1966), kinetochores (Brinkley and Nicklas, 1968), and rhizoplasts (Bouck and Brown, 1973). These sites have been called foci (Porter, 1966), orienting centres (Inoué and Sato, 1967), nucleating centres (Tilney, 1968a), microtubule-organizing centres (Pickett-Heaps, 1969), and nucleating sites (Tilney and Goddard, 1970). These nucleating sites, with which the tubules are associated, usually appear as diffuse, amorphous, electron-opaque material. Bouck and Brown (1973) have identified different presumptive nucleating sites in <u>Ochromonas</u> which are all kinetosome related. Loss of cell shape in response to a variety of microtubule depolymerizing agents is believed to proceed toward the nucleating sites. Experiments, using depolymerizing agents, strongly suggest that the temporal and spatial control of microtubule assembly, in regenerating cells, is related to these recognizable nucleating sites (Brown and Bouck, 1973). The isolation and biochemical characterization of the nucleating material will help determine its exact role in control of microtubule assembly.

Tilney and Byers (1969) suggest that the axonemal pattern in Actinosphaerium is controlled by the substructure of the tubules and the links or bridges which connect them. This accordingly would achieve the most stable configuration of the tubules by selecting the energetically most favourable bonding pattern which is assumed to be the axoneme. In Raphidiophrys, the orientation of microtubules into the axonemal pattern is suggested to result from the formation of bridges between adjacent tubules at some distance from the nucleating site (Tilney, 1971b). On the other hand, Tucker (1970) postulates that the microtubule pattern in the developing cytopharyngeal basket in Nassula is defined at the points of tubule initiation (i.e. nucleating sites) and that the cross-connections function in stabilizing the existing microtubule distribution. Heat shocks inhibit the initiation of rod tubules in Nassula. Temperature shocks cause abnormal arrangement of these initiation sites but longitudinal growth of existing tubules continues. Hepler and Jackson (1969) found that the antimitotic herbicide, isopropyl-N-phenyl carbamate (IPC) causes reorientation of spindle microtubules in dividing Haemanthus so that a multipolar spindle apparatus is formed. They postulate that IPC may be affecting the organization of the spindle microtubules. By combining

IPC and hydrostatic pressure, it is possible to obtain spherical populations of Ochromonas in which all the microtubules are depolymerized or converted to 'macro-tubules'. When the pressure is released and the IPC washed out, the population synchronously regenerates its cell shape. The tubules first appear associated with the presumptive nucleating sites and gradually extend to the cell's posterior (Brown and Bouck, 1973).

The structural component of microtubules, isolated from diverse sources, such as flagella and neurotubules, is a protein called tubulin. Although the tubulins have been shown to be similar in sedimentation coefficients, and molecular weights (Shelanski and Taylor, 1968, Weisenberg, Borisy and Taylor, 1968), microtubules from different sources respond differently to various physical and chemical treatments (Behnke and Forer, 1967). Tilney and Gibbins (1968c) showed that certain antimitotic agents such as colchicine and high hydrostatic pressure had differential effects on the cytoplasmic and ciliary microtubules of sea urchin gastrulae. Brown and Bouck (1973) reported a differential sensitivity in Ochromonas, between two sets of cytoplasmic microtubules as well as between the cytoplasmic and flagellar microtubules. These different degrees of stability between the various populations of microtubules may be partially accounted for by variations in tubulin composition (Fine, 1971, Olmsted et al., 1971, Feit et al., 1971).

The development of the flagellar apparatus involves the synthesis of microtubule protein and its assembly into the characteristic "9 + 2" axonemal configuration. Naegleria can be induced to transform from the amoeba stage (having no flagella) into flagellates (generally having 2 flagella) thus providing a system for the study of flagellar development (Dingle and Chandler, 1966). The basal bodies appear to arise de novo during the transformation of Naegleria from amoeba to flagellate. The axonemal pattern is apparently ordered under the influence of the basal body pattern. Flagellar development has also been studied in a number of systems in which the flagellar apparatus duplicated during cell division: Peranema trichophorum (Tamm, 1967), Oedogonium cardiacum (Hoffman, 1966, Pickett-Heaps, 1971), (Chlamydomonas reinhardi (Johnson and Porter, 1968). In most cases where flagella or cilia disappear in morphogenesis, the loss is by shortening (i.e. 'retraction', 'withdrawal', 'degeneration') (Tamm, 1967, Johnson and Porter, 1968).

Amputation-induced flagellar regeneration presents a useful tool for the study of microtubule synthesis and assembly in the external flagellum. Analysis of regeneration kinetics reveals the complex nature of this process which is generally characterized by a lag phase (no apparent flagellar elongation) and an elongation phase (lengthening of the flagellar shaft until the original length is regained) (Dubnau, 1961, Rosenbaum and Child, 1967, Tamm, 1967, Rosenbaum et al., 1969, Rosenbaum and Carlson, 1969).

Protein synthesis is required for total flagellar regeneration. The addition of cycloheximide immediately after flagellar amputation resulted in almost complete inhibition of flagellar elongation in Euglena (Rosenbaum and Child, 1967). Partial regeneration of flagella occurs in Chlamydomonas in concentrations of cycloheximide inhibiting to protein synthesis (Rosenbaum et al., 1969).

Colchicine and its derivative, Colcemid, also inhibit flagellar and ciliary regeneration. The elongation of ciliary buds in cultured fibroblasts is inhibited by Colcemid (Stubblefield and Brinkley, 1966). Colchicine inhibits ciliary elongation in cilia regenerating Tetrahymena (Rosenbaum and Carlson, 1969), and flagellar regeneration in Chlamydomonas (Rosenbaum et al., 1969) and in Ochromonas (Brown and Bouck, 1973). Inhibitory concentrations of colchicine were shown to have no effect on RNA (Rosenbaum and Carlson, 1969) or protein synthesis (Rosenbaum et al., 1969). Colchicine binds to microtubule subunit protein (Borisy and Taylor, 1967). The binding of colchicine to flagellar microtubule protein in Chlamydomonas (Rosenbaum et al., 1969) and to spindle microtubule protein in Pectinaria (Inoué and Sato, 1967) is completely reversible. In both these systems the microtubular protein is kept in the depolymerized state by colchicine and when the colchicine is washed out, microtubule reassembly occurs in the absence of new protein synthesis. New protein synthesis is required for flagellar regeneration in Ochromonas after colchicine is washed out (Brown and Bouck, 1973), suggesting that the flagellar microtubular subunit protein is irreversibly altered by exposure to colchicine in their system.

Microtubules are linked with two main functions in cells: movement of cells and cell parts (e.g. chromosomes) and production of cell form. The roles played by these tubules are important factors in the regulation of cell differentiation and in the control of motility systems. A single organism in which both function and control of the different populations of microtubules could be studied would aid the further characterization of tubules, bridges, and nucleating sites. Such an experimental system could be used to correlate the interactions between the factors involved in microtubule biogenesis.

Polytomella agilis presents an ideal system for the study of nucleating sites (terminology of Tilney and Goddard, 1970) and for their possible role in microtubule initiation. Two distinct sets of microtubules are associated with the interphase cell: the flagellar microtubules and the cytoplasmic microtubules which may contribute to the development and/or maintenance of cell shape. Microtubule distribution in *Polytomella* is described in Section I and possible nucleating sites for cytoplasmic microtubule assembly are located.

To determine that microtubules function in establishing and maintaining cell shape in *Polytomella*, the regeneration of cytoplasmic microtubules and the role of the presumptive nucleating sites in the reassembly process were studied using microtubule depolymerizing agents. This work will be described in Section II.

SECTION I

The colourless unicell Polytomella agilis Aragoa (Volvocales, Tetrablepharidaceae, Pringsheim, 1955) exists in a free swimming form and a cyst form. The transition to the encysted form has been studied ultrastructurally. There is an accumulation of starch bodies, mitochondria and ribosomes disappear and a thick 3-layered wall is developed (Gittleson, Alper and Conti, 1969). Moore et al., (1970) have reported that the developing cyst wall is fibrous and consists of at least 6 layers of different electron density external to the plasma membrane. It is difficult to distinguish between the cells destined to encyst and those destined to die (10% of the cells encyst); according to these authors, these facts account for the structural abnormalities observed. Soluble proteins from disrupted motile and encysted cells were found to differ in electrophoretic mobilities. Concentrations of constituent proteins vary between the motile cells and the cysts. There was greater amylolytic activity in the soluble proteins from the motile fraction than in those from the encysted fraction (Sheeler, Cantor and Moore, 1968, 1970).

Moore et al., (1970) have described the ultrastructure of P. agilis emphasizing the distribution and fine structure of the cell organelles in the trophic and encysted stages of the life cycle. The microtubules were briefly discussed and it was suggested that the tubules which are oriented longitudinally beneath the plasma membrane may play a structural or skeletal role in this quadriflagellate cell.

The present study describes the distribution of microtubules in _Polytomella agilis_. _Polytomella_ has extensive microtubular systems which are associated with presumptive nucleating sites.

METHODS AND MATERIALS

Polytomella agilis Aragoa (L 193) was originally obtained from the Culture Collection of Algae at Indiana University, Bloomington, Indiana. *P. agilis* was grown in batch cultures of 400 ml in 1000 ml Erlenmyer flasks in a complex medium containing 0.1% tryptone, 0.2% yeast extract and a 0.2% sodium acetate at pH 6.8 (Sheeler *et al.*, 1968). The cells were incubated at 25° C in the dark. In order to study the growth kinetics, 1 ml aliquots of cells in batch cultures were taken at various time intervals, fixed in 0.5% glutaraldehyde and counted in a haemacytometer.

LIGHT MICROSCOPY

Living cells were photographed with a Zeiss microscope equipped with Nomarski optics and a Zeiss 60 W.s. electronic flash. The cells were observed in growth medium plus Protoslo (Carolina Biological Supplies) which increases the viscosity of the medium and facilitates photographing swimming cells.

SCANNING ELECTRON MICROSCOPY

Cells were fixed in 0.5% glutaraldehyde in 0.05M phosphate buffer pH 7.4 (Gerhardt and Berger, 1971) and then resuspended in distilled water. The cells were freeze dried for 12 hours in a Speedivac Freeze-Drier.

They were then coated with a gold-platinum alloy and examined in a Cambridge Stereoscan electron microscope.

TRANSMISSION ELECTRON MICROSCOPY

P. agilis was fixed at room temperature (22° C.) for 1 hour in 0.5% glutaraldehyde buffered with 0.05M phosphate pH 7.4 and washed three times in phosphate buffer (22° C.). The cells were post-fixed for 1 hour in 1.0% osmium tetroxide (OsO_4) in phosphate buffer at 0° C, washed three times in phosphate buffer (0° C) and dehydrated in a graded acetone series. The cells were then infiltrated with Spurr's hard plastic (Spurr, 1969) and polymerized at 60° C for 18 hours. Sections cut on a Sorvall Porter-Blum MT-2B ultramicrotome were collected on formvar coated-copper grids. The sections were stained in a saturated uranyl-acetate solution (2% W/V) in 50% ethanol followed by lead citrate (Reynolds, 1963), and were viewed in an A.E.I. EM-6B electron microscope.

PARTIAL SYNCHRONIZATION OF POLYTOMELLA

In order to increase the number of dividing cells in the population, cells were subjected to temperature shocks (Cantor, Bartholomew and Thieman, 1966). A batch culture inoculated with 10^5 cells was placed at 25° C for 6 hours (one generation time), transferred to 9° C for 22 hours and then returned to 25°. One ml samples were taken at various intervals, fixed and counted in a haemacytometer. Upon return to 25° C, the cell population doubled in less than 2 hours. During this period, cells were fixed for electron microscopy as previously described.

OBSERVATIONS

CELL ORGANELLES

In the exponential phase of growth, virtually 100% of the cells are in the motile form (Fig. 1). This quadriflagellate cell is elongate (about 12 um long and 7 um wide) with a single anterior projection called the papilla. Scanning electron microscopy was undertaken in order to get a precise three-dimensional picture of Polytomella. Scanning electron micrographs show the four anterior flagella emerging in two opposite pairs from depressions at the base of the X-shaped papilla (Figs. 2-3).

A longitudinal section through the cell reveals the regular distribution of the majority of organelles (Fig. 4). This regularity makes it possible to recognize the orientation of any section. The central nucleus is bound by a nuclear envelope whose continuity is broken by nuclear pores; the pores are often plugged by granular electron opaque material (Moore et al., 1970). The nucleoplasm is homogeneous except for a large, well defined nucleolus which contains dense fibrillar areas and granular areas. Several Golgi bodies (dictyosomes) surround the nucleus. They consist of flattened, saucer shaped cisternae which are surrounded by vesicles. The mitochondria, located mainly at the anterior end of the cell, are large,

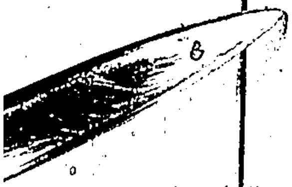

Fig. 1: Flash photograph of a swimming *Polytomella* x 4,000
Fig. 2: Scanning electron micrograph of *Polytomella* showing the ovoid shape, the anterior papilla (P) and the 4 flagella. x 4,000
Fig. 3: Head on view of *Polytomella* showing the 4 flagella emerging from depressions at the base of the papilla. x 9,000.

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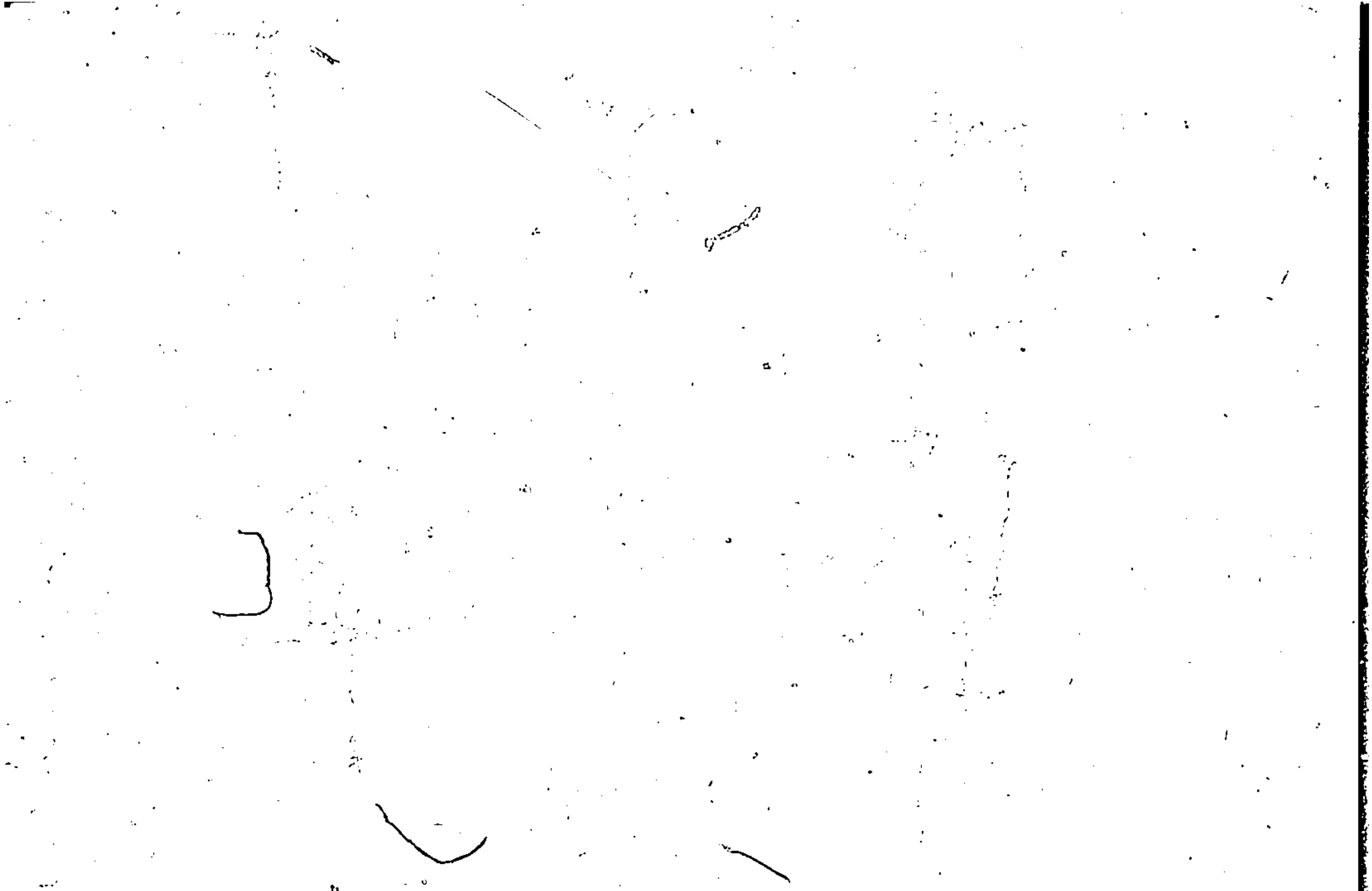

Fig. 4: Median longitudinal section of *Polytomella*. P, papilla, G, golgi body, L, leucoplast, S, starch, V, vacuole, M, mitochondria, N, nucleus. The arrow indicates the basal body region x 15,000.

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elongate, and often convoluted organelles in which the cristae are randomly distributed. The leucoplasts (or proplastids in the terminology of Moore et al., 1970) are generally found at the cell posterior. In exponentially growing cultures, these organelles have a granular matrix and in older cultures, the leucoplasts contain many starch grains.

Two types of membrane-bound vacuoles are evident in P. agilis. The four contractile vacuoles, found beneath the papilla, are similar to those described in other green algae (Hobbs, 1971). Other large vacuoles which are found throughout the cell contain osmophilic granules, the nature of which is unknown. Ribosomes are present throughout the cytoplasm, associated with the endoplasmic reticulum, and with the nuclear membrane. There is no evidence for a cell wall or pellicle anywhere around the plasma membrane.

Polytomella agilis contains three distinct sets of microtubules: the flagellar microtubules, the cytoplasmic microtubules and nuclear microtubules.

FLAGELLAR MICROTUBULES

A flagellum can be divided into four parts for purposes of discussion: the flagellar tip, the flagellum proper, the transitional region, and the basal body which anchors the flagellum in the cell. Fig. 5a shows a longitudinal section through the flagellum proper, the transitional zone and the basal body. The transitional zone is located between the flagellum proper which is external to the cell and the basal body which is embedded in amorphous electron-opaque material located beneath the papilla.

In the flagellar tip (Fig. 5b), the nine peripheral doublets are gradually lost and only the central pair of microtubules extend to the very tip. The matrix material is less evident.

In the flagellum itself (Fig. 5c), the characteristic 9+2 axoneme pattern is embedded in matrix. There are no hairlike projections or mastigonemes attached to the flagellar membrane anywhere along its length.

The transitional region can be subdivided into three parts. At the distal end only the nine outer doublets are observed (Fig. 5d); the central pair does not extend into this region and very little matrix material is present in the central region. The peripheral tubules appear to be connected one to another by a filament which attaches an A tubule of one doublet to a B tubule of an adjoining

Fig. 5a: Longitudinal section through a flagellum and basal body. F, flagellum, BB, basal body x 49,000.
Fig. 5b: Cross-section through the flagellar tip x 56,500.
Fig. 5c: Cross-section through the flagellum proper x 56,500.
Fig. 5d to g: Cross-section progressing down through the transitional region. 5d, e, f x 56,500. 56 x 52,000.
Fig. 5h: Cross-section through the basal body x 56,500,

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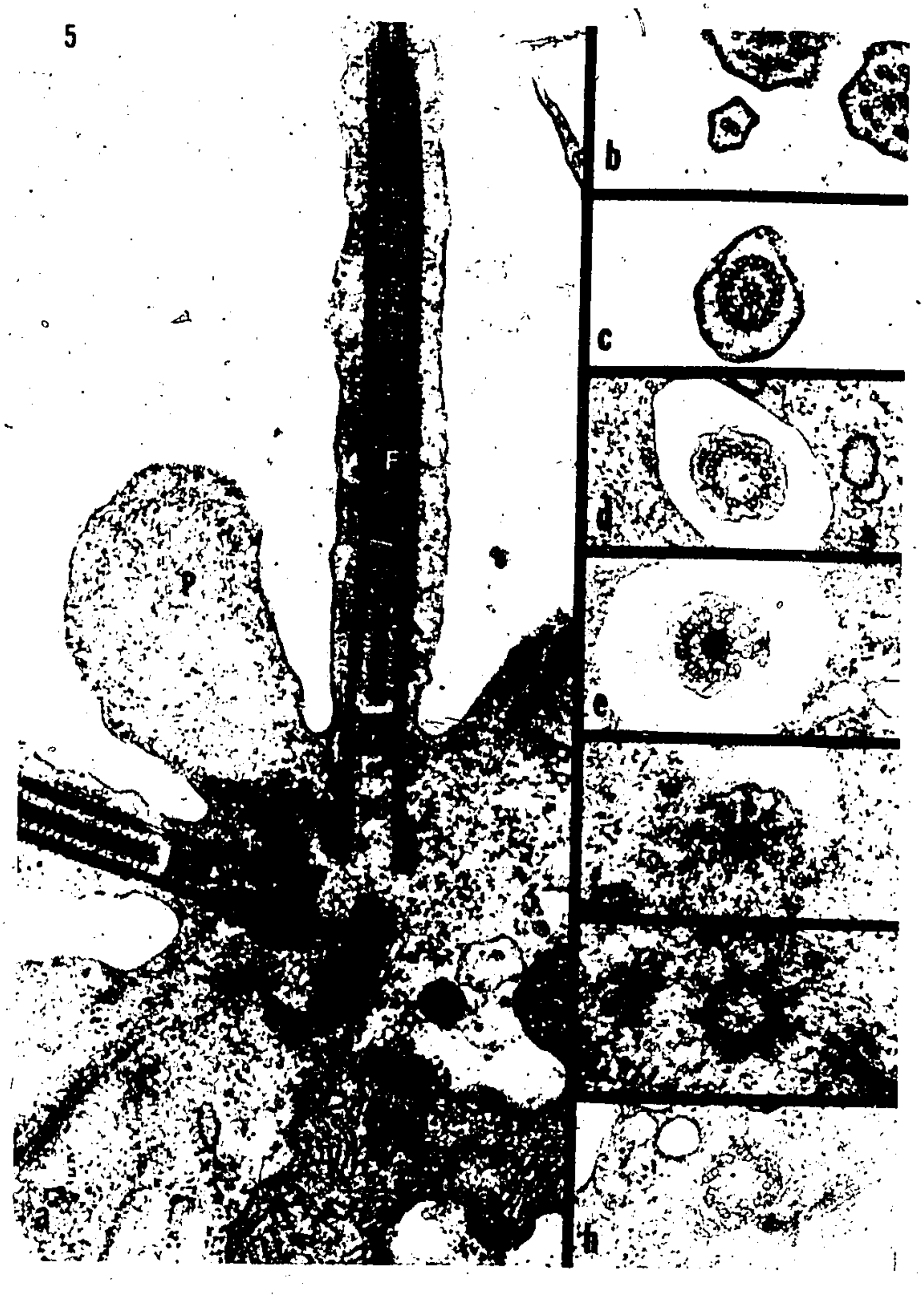
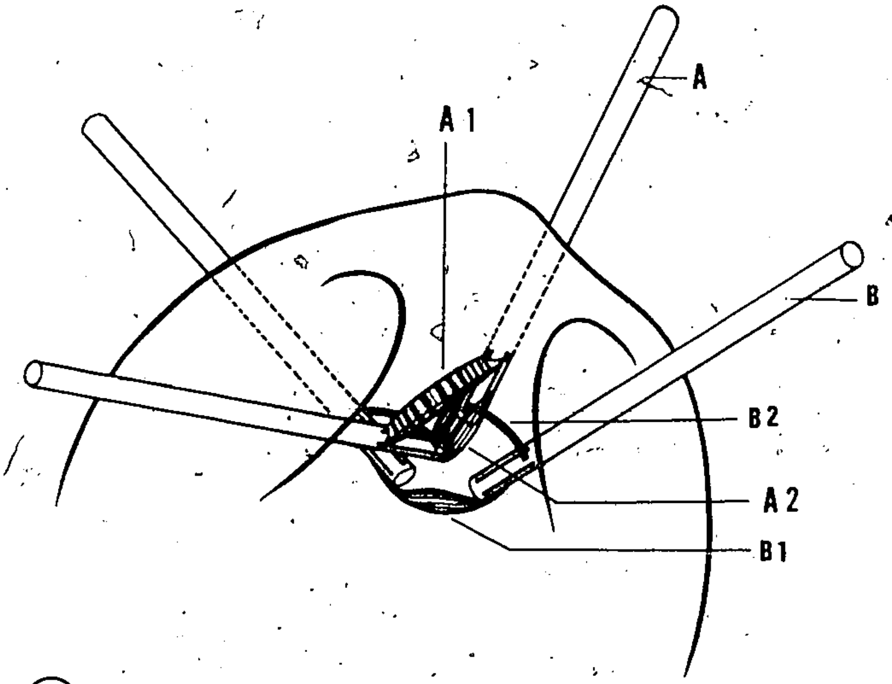


doublet. The middle area of the transitional zone (Fig. 5e) is characterized by a stellate structure (Lang, 1963) also called a star-body (Olson and Kochert, 1970). Each of the nine outer doublets is connected by filaments forming V-shaped connections which seem to extend from a central dense core. This central core also called an axial filament (Olson and Kochert, 1970) disappears at a lower level leaving only the nine pointed star-structure within the ring formed by the outer doublets (Fig. 5f). In longitudinal view, the stellate structure appears as an electron-opaque cylinder internal to the peripheral doublets. The midregion of the transitional zone penetrates into the cell proper. At the proximal end of the transitional region only the nine peripheral doublets are observed (Fig. 5g) in the same arrangement as that seen at the distal end of the transitional zone. The most distal part and the proximal part of the transitional region can be distinguished in that the latter is surrounded by the cytoplasm. The connections which exist between the nine doublets in the proximal region are difficult to distinguish since this structure is surrounded by amorphous, electron-opaque material.

The peripheral fibers of the basal body stain very densely as seen in a longitudinal view (Fig. 5a). A cross section through this region (Fig. 5h) reveals nine

Fig. 11: Schematic diagram of *Polytomella agilis* showing the basal body arrangement. A, A flagellum, B, B flagellum, A1, striated fiber connecting A pair basal bodies at their distal ends, A2, proximal fiber connecting A pair of basal bodies at their proximal ends, B1, large fiber connecting B pair of basal bodies at their proximal ends, B2, short fiber connecting B basal body to amorphous material at base of A basal body.

triplets with no apparent structure in the lumen. A filament connects an A tubule and a C tubule of adjacent triplets.



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CYTOPLASMIC MICROTUBULES

A series of cross sections progressing down through the papilla and into the basal body region demonstrates the orientation of the basal bodies as well as the distribution of the cytoplasmic microtubules. The X-shaped papilla (Fig.6) lacking organelles, contains a network of microfilaments of about 50 Å in diameter (Fig. 7).

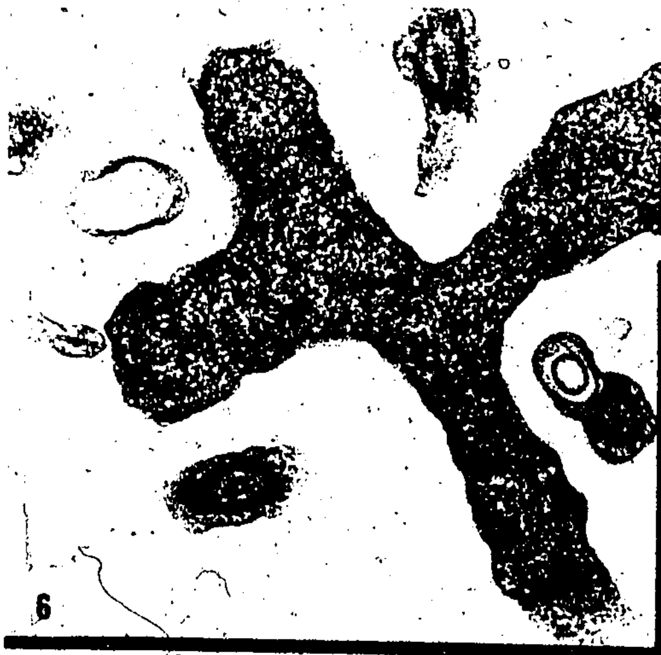
A diagrammatic representation of the basal body complex shows the relationship between the basal bodies and their associated fibers (Fig. 11). In a section just posterior to the papilla adjacent basal bodies are observed to lie at right angles to each other but slightly offset from center (Fig. 8). One pair of basal bodies, called the A pair, is more anterior than the other pair, the B-pair. The A pair which are connected at their bases by two proximal fibers (A_2 fibers) are 100 Å to 300 Å apart (Fig. 8). The B pair which is deeper set in the cell, are 300 Å to 4000 Å apart (Fig. 8). The A pair of basal bodies is connected at their distal portions by a short striated fiber (A_1 fiber, Fig. 9) similar to that described in Chlamydomonas (Ringo, 1967). The B pair of basal bodies is attached at their proximal ends by a large fiber (B 1 fiber) which appears to consist of a bundle of fibrillar material (Fig. 10). At their distal end a short fiber (B_2 fiber) connects the B basal bodies to amorphous, electron opaque material at the base of the A pair of basal bodies (Fig. 10).

Rootlets extend out from the basal body complex. Between adjacent basal bodies there are two structurally different rootlets (Fig. 12) which are coated with and terminate in the amorphous, electron-opaque material in which the entire basal body complex is embedded. One rootlet which has a crossbanded appearance is called a striated rootlet (Fig. 13). The pattern of crossbanding in this rootlet is quite simple. There are two faint (electron-lucent) lines which are separated by a fine dense (electron-opaque) line; groups of these faint lines are separated by a dense line about 250 Å wide. A similar rootlet that has a slightly different crossbanding has been described in <u>Stigeoclonium</u> (Manton, 1964a). The other rootlet, called the microtubular rootlet, consists of closely juxtaposed microtubules (Figs. 14 and 15). This rootlet, in which there is no crossbanding, consists of four or five microtubules arranged in two layers of a 3 over 1 or a 4 over 1 configuration (Fig. 16). Seen in a cross-section, the striated rootlet appears as a bundle of dense material (Fig. 16).

A large number of microtubules appear to terminate on both the striated and the microtubular rootlets (Figs. 14 and 15). These microtubules, which form the cytoplasmic microtubular system, diverge and extend just beneath the plasma membrane to the cell posterior. The microtubules are distributed in a single row around the cell periphery (Fig. 17). At four points around the cell, there are groups of three or four closely apposed microtubules (Fig. 18) that are probably extensions of the microtubular rootlets which have a similar structure in the basal body region. Microtubules are quite evenly spaced between these apposed microtubules. Details of how these tubules end in the posterior region of the cell are not known.

A schematic drawing of an idealized Polytomella cell shows the arrangement of the striated and microtubular rootlets between the basal bodies and the orientation of the cytoplasmic microtubules which terminate on the rootlets (Fig. 19).

Fig. 6: Cross section through the X-shaped papilla showing portions of the flagella in the depressions x 37,500.
Fig. 7: An oblique section through the anterior end of the cell showing a portion of the papilla x 25,000.
Fig. 8: Cross section through the basal body region showing the A pair of basal bodies (A) and the B pair (B). Two proximal fibers (A_2) connecting the basal bodies are observed x 40,000.



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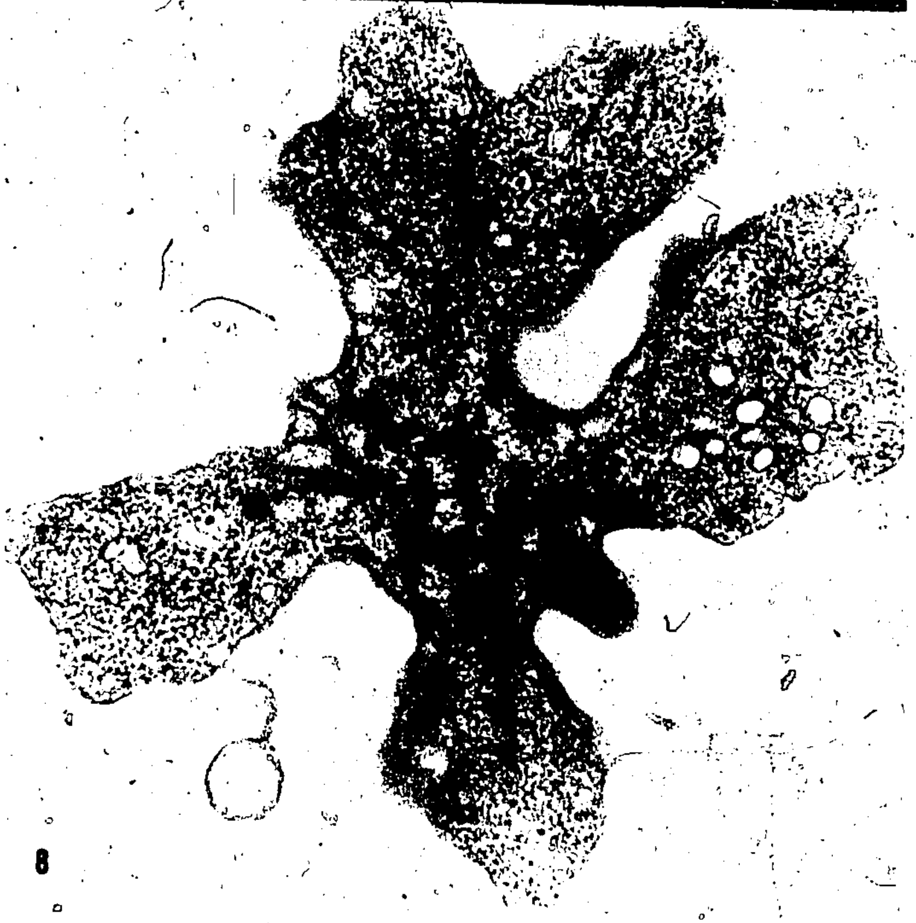

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Fig.9: Longitudinal section through the A pair of basal bodies. A1, striated fiber connecting the distal portions of the A basal bodies, A2, proximal fibers connecting the proximal ends of the A basal bodies, B1, a large fiber seen here in cross section which connects the proximal ends of the B basal bodies x 55,000.
Fig. 10: Longitudinal section through the B pair of basal bodies. B1, a large fiber connecting the proximal ends of the B basal bodies, B2, distal fibers which connect the B basal bodies to amorphous material at the base of the A basal bodies, A1, striated fiber seen in cross section which connects the A pair of basal bodies x 34,000. (P, papilla, F, flagellum).

9

10

Fig. 12: Cross section just below the papilla in the flagellar region. A, A basal body, B, B basal body, F, flagellum, MR, microtubular rootlet, SR, striated rootlet, Mt, cytoplasmic microtubules, V, vacuole x 42,500.

12

Fig. 13: Cross section through the basal body region showing the striated rootlet (SR) between adjacent basal bodies. A, A basal body, B, B basal body x 71,250.

Fig. 14: Oblique section through the anterior end of the cell showing the microtubular rootlet (MR). x 75,000

Fig. 15: Oblique section through the anterior end of the cell showing the microtubular rootlet (MR) and the cytoplasmic microtubules (Mt) terminating on this rootlet x 71,250.

Fig. 16: Longitudinal section through the cell showing the microtubular rootlet (MR) and the striated rootlet (SR) in cross section. Mt, cytoplasmic microtubules x. 170,000 (F, flagella).


13


14


16

Fig. 17: Cross section through the central nucleus. L, leucoplast, S, starch, M, mitochondria, G, golgi body, NP, nuclear pore, Nu, nucleolus, V, vacuole .x 25,000

Fig. 18: It is a higher magnification of the inset in Fig. 17. Note the group of three juxtaposed microtubules (arrow) and the single microtubules (Mt). x 50,000.

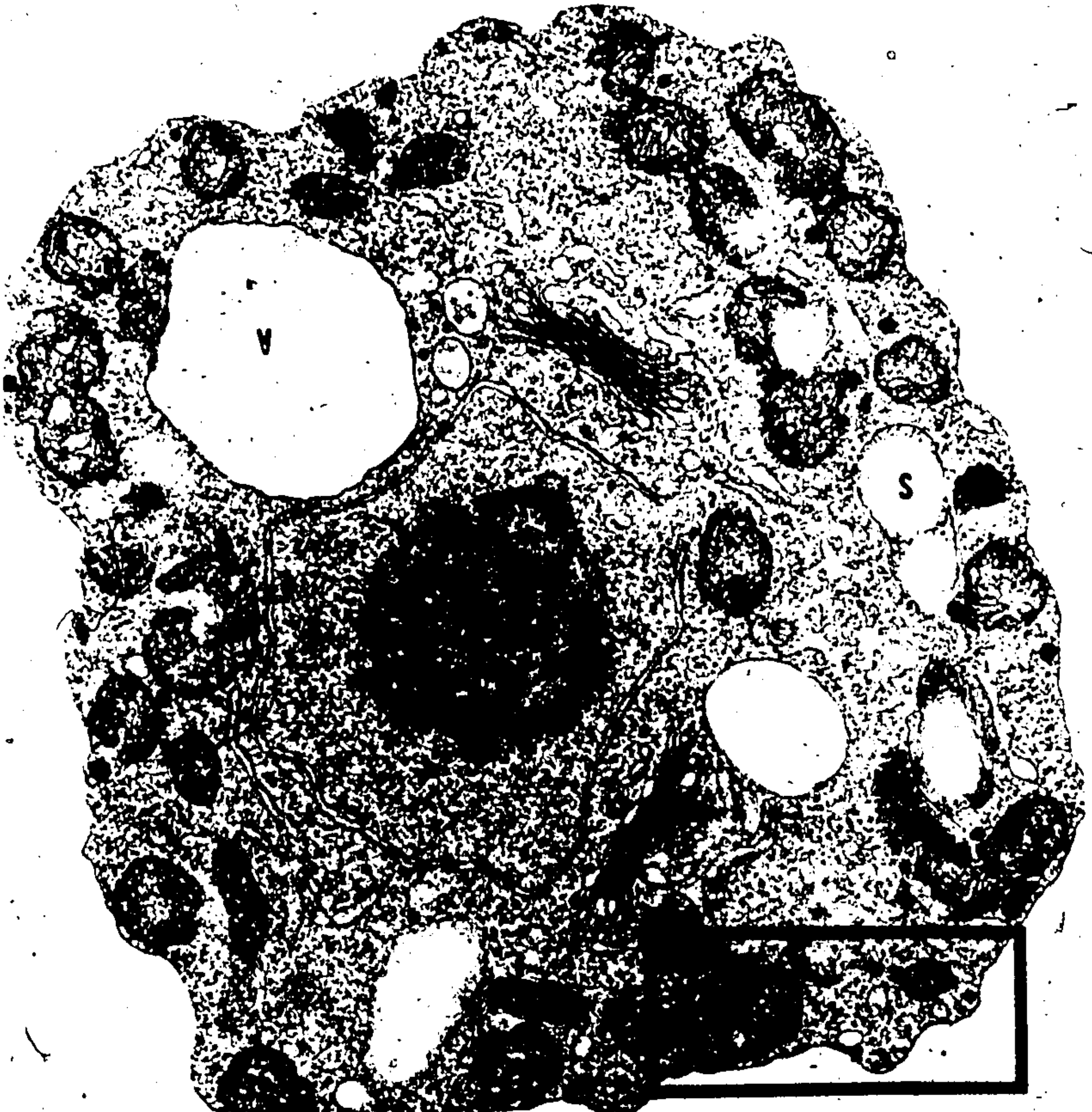

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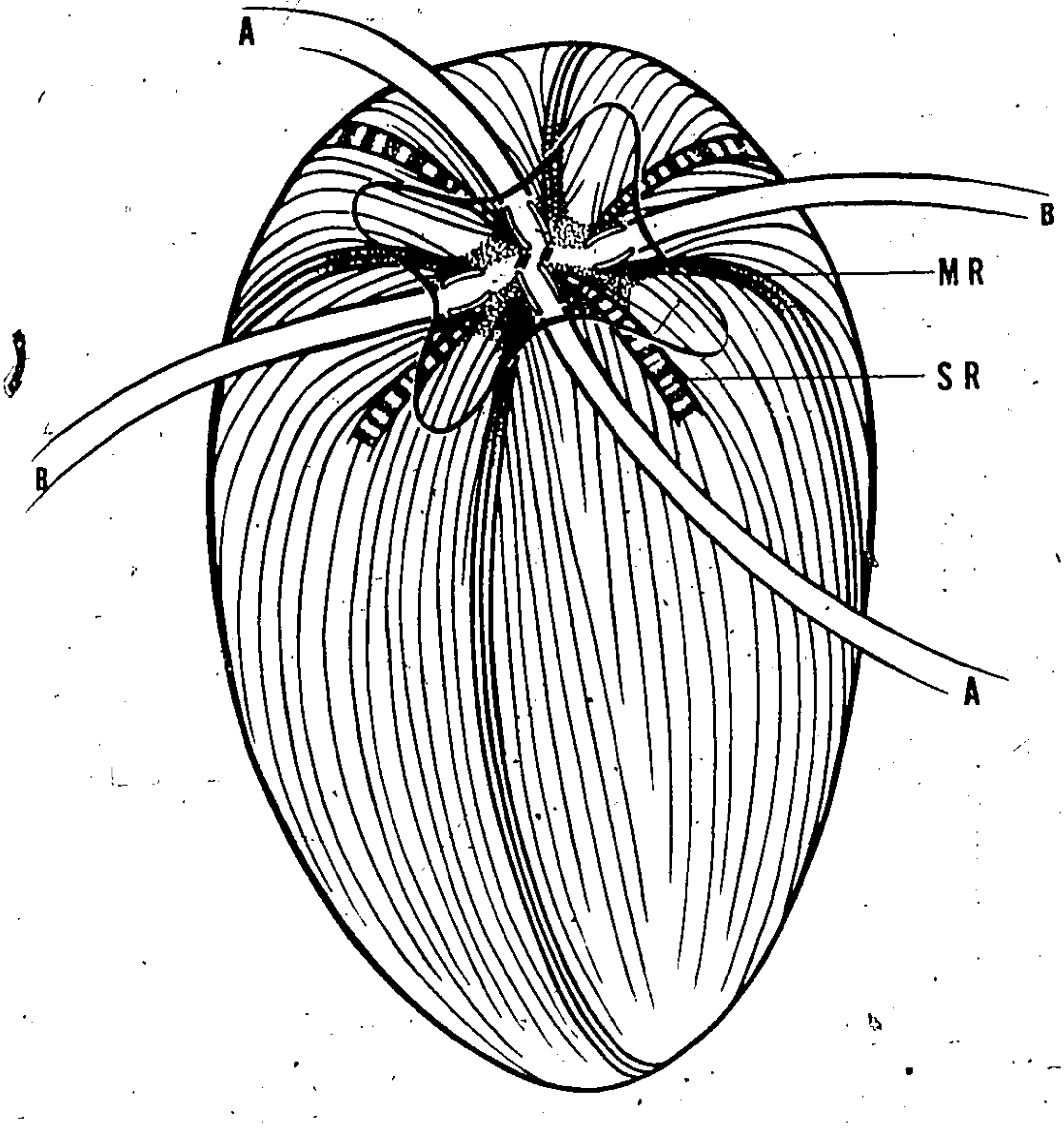
Fig. 19: Diagrammatic representation of the basal body and rootlet arrangement and their relationship to cytoplasmic microtubules. The relative sizes of the basal body complex, rootlets and tubules are not to scale. A, A flagella, B, B flagella, SR, striated rootlet, MR, microtubular rootlet.



⑲

SPINDLE MICROTUBULES

Partial synchronization of batch cultures of Polytomella was obtained by subjecting them to temperature shocks (Fig. 20). After return to 25° C, the cell population doubles in less than 2 hours. During this time interval many cells are observed in division.

The sequence of cell division as seen at the light microscope level is as follows (Fig. 21 a-g):

1) nuclear division appears to occur prior to basal body and flagellar replication
2) when nuclear division is complete, the flagellar apparatus replicates (Fig. 21a)
3) two papilla are evident as the 2 sets of flagella gradually move apart (Figs. 21b - 21e)
4) when they are at opposite ends of the cell (Fig. 21f) cytokinesis occurs (Fig. 21g).

The spindle microtubules are present only during nuclear division. Nuclear division is marked by the presence of an intact nuclear envelope (Fig. 22). The microtubules which form the spindle apparatus appear to be formed independently of the flagellar and cytoplasmic microtubules (i.e. spindle microtubules are not associated with the basal body complex or with the rootlets). Spindle microtubules traverse the nucleoplasm and are oriented towards the poles where there is no involvement of basal bodies or centrioles (Fig. 23) but numerous nuclear pores, fenestrae, are observed in this region (Fig. 22). In the nucleoplasm, the prominent interphase nucleolus has dispersed. The spindle microtubules are directed towards the chromosomes on which no specialized kinetochore regions have been recognized.

Fig. 20: Growth kinetics of a control culture of *Polytomella* and kinetics of a culture subjected to temperature shock.

⑳

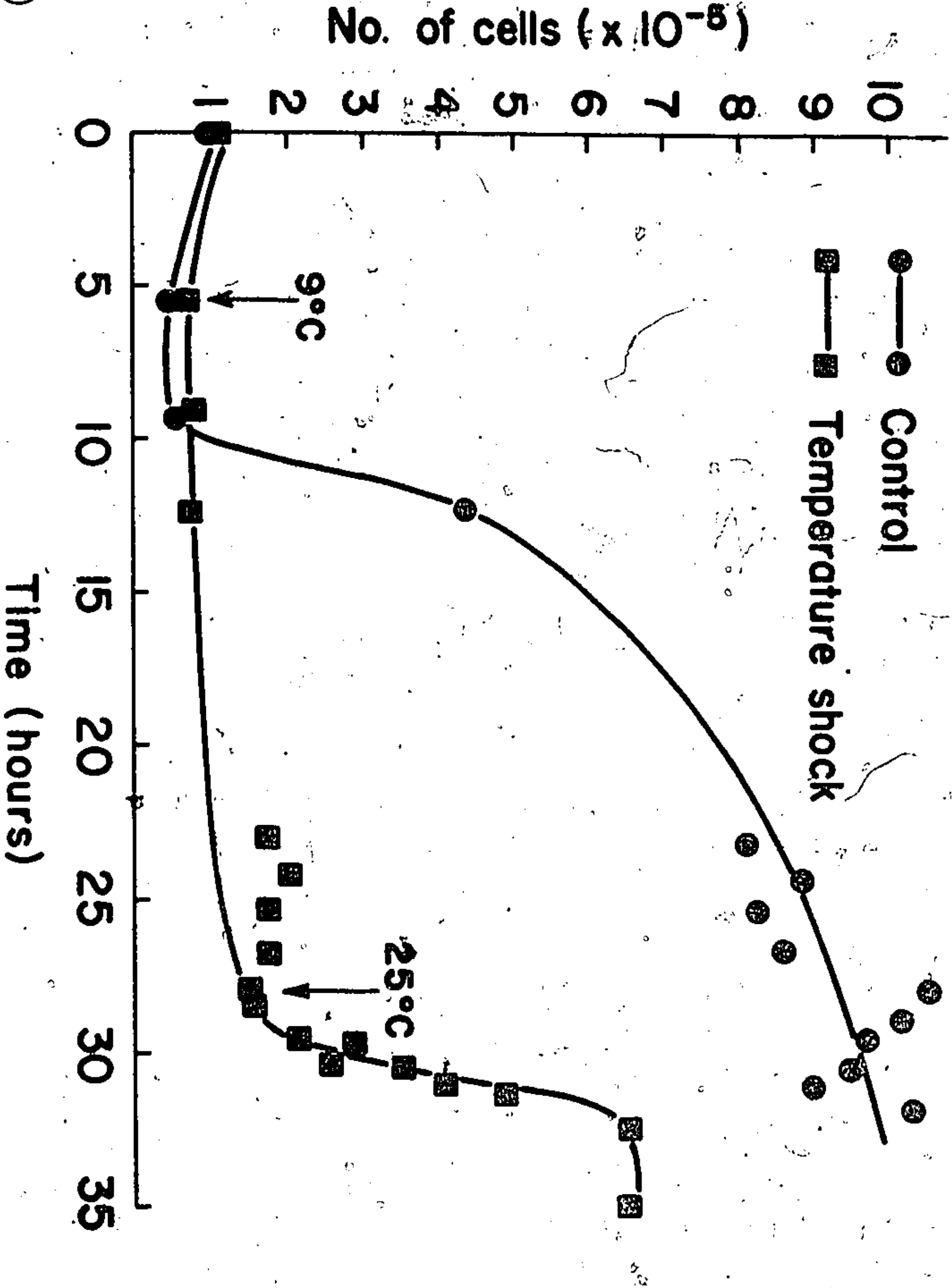

No. of cells (x 10⁻⁸)
1
2
3
4
5
6
7
8
9
10
Control
Temperature shock
9°C
25°C
0
5
10
15
20
25
30
35
Time (hours)

Fig. 21: Division of Polytomella agilis as seen by light microscopy. x 3,500

Fig. 21a: Two nuclei are present (arrows) and the flagellar apparatus has replicated.

Fig. 21b to e: The papilla becomes evident and the two sets of flagella gradually move apart.

Fig. 21f: The two sets of flagella are at opposite ends of the cell.

Fig. 21g: Cytokinesis occurs.

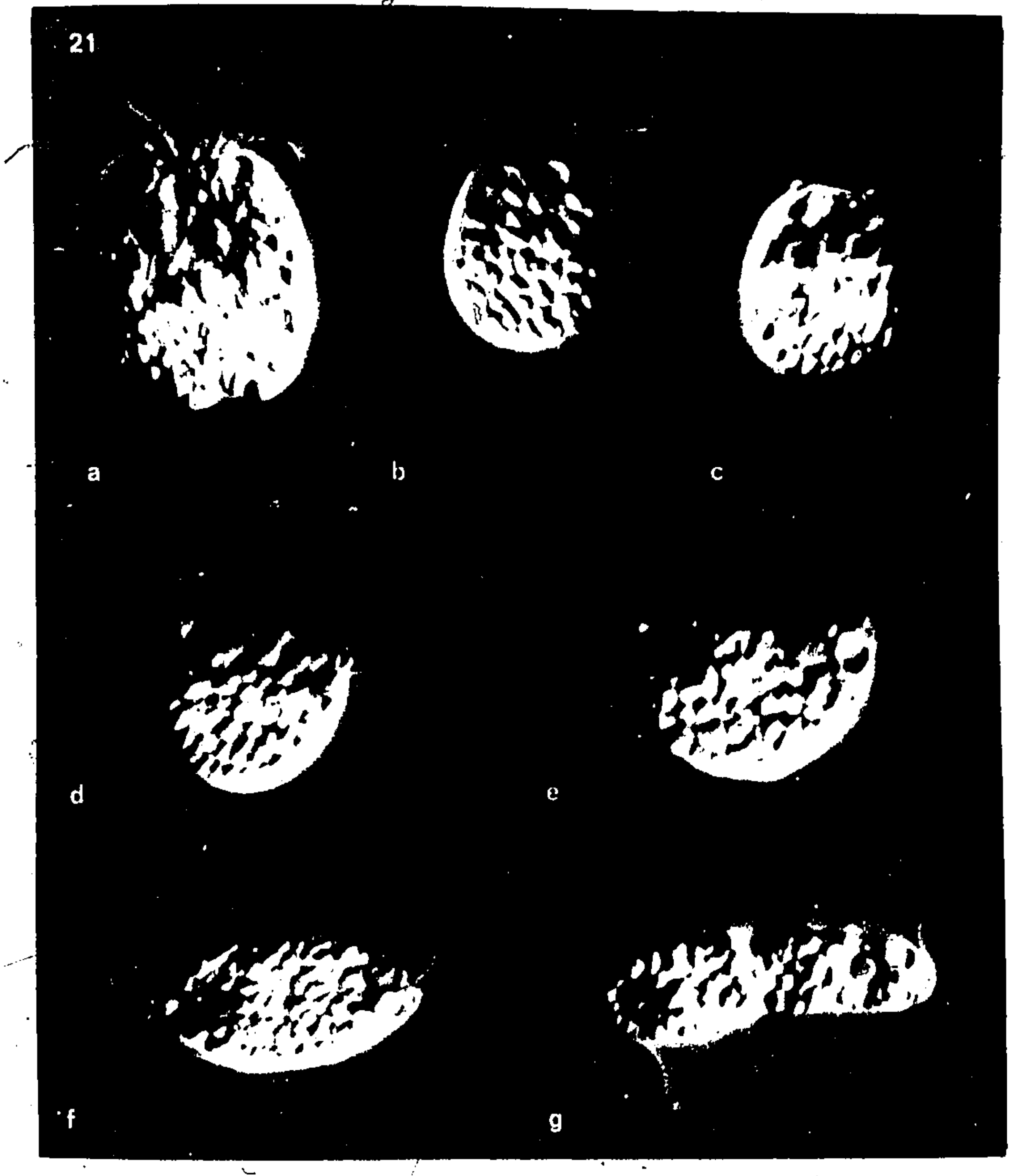

21
a
b
c
d
e
f
g

Fig. 22: Longitudinal section of a dividing nucleus. Fenestrae are observed at one of the poles (arrows) S Mt, spindle microtubules, x 32,500

22

Fig. 23: Portion of a dividing nucleus showing one pole. S Mt, spindle microtubules, G. golgi body. x 106,000.


G

SECTION 2

Section 2 deals with the distribution of cytoplasmic microtubules in <u>Polytomella agilis</u> as they relate to the development and/or maintenance of cell shape.

The physical and chemical agents used in this study include hydrostatic pressure, cycloheximide, colchicine and isopropyl-N-phenyl carbamate.

MATERIALS AND METHODS

Cells were harvested in the log phase of growth (5-7 x 10^5 cells/ml) for the following experiments.

Chemical Treatments

The effects of various drugs were tested on *Polytomella*: colchicine and cycloheximide (Sigma Chemical Co., St. Louis, Mo.), isopropyl-N-phenyl carbamate (IPC) (PPG Industries, Inc., Chemical Division, Pittsburgh, Pa.), and vinblastine sulfate (Eli Lilly & Co. (Canada) Ltd., Toronto, Ont.). Cells harvested by low speed centrifugation were resuspended in a treatment medium and incubated at 25° C. Drugs used in all experiments were dissolved in fresh sterile media just prior to use.

Deflagellation

Cells harvested by low speed centrifugation were resuspended in a fluted glass tube (Rosenbaum and Child, 1967) in 2 mls of a treatment medium. Flagella were mechanically amputated from populations of cells by agitation with a Vortex mixer at top speed for 90 seconds. Following flagellar amputation, the cells were gently concentrated with a clinical centrifuge and resuspended in treatment media or control media and incubated at 25° C. Samples taken at various time intervals during these regeneration experiments, were fixed in Lugol's iodine (6 g KI and 4 g I_2/100 ml distilled H_2O) and observed under a Zeiss Photomicroscope equipped with Nomarski optics.

Hydrostatic Pressure

The hydrostatic pressure apparatus used for the pressure treatments is that described by Brown and Bouck (1973). Cells harvested by low speed centrifugation were resuspended in 7.5 or 8.0 x 10^{-4} M IPC and then subjected to 6000 or 6500 psi for 30 minutes. Upon release of pressure, the cells were concentrated by gentle centrifugation, resuspended in different treatment media and incubated at 25° C. Cell shape was monitored by observing cells under a Zeiss microscope equipped with Nomarski optics. At various time intervals, cells were fixed for electron microscopy as previously described (Section 1).

Amino acid incorporation

A labelling experiment was carried out to determine the effect of isopropyl-N-phenyl carbamate (7.5 x 10^{-4} M IPC) and cycloheximide (20 ug/ml CHI) on amino acid incorporation into trichloroacetic acid (TCA) precipitable protein. This was determined using the filter paper disc method (Mans and Novelli, 1961).

Cells were collected by low speed centrifugation and resuspended in sterile media. Uniformly labelled L-amino acid-C^{14} mix (New England Nuclear Corp., Boston, Mass.) was used, the total activity being 0.025 mCi per

5-ml reaction mixture. The 5-ml samples of cells were incubated at 25° C in 25-ml Erlenmeyer flasks. 100 ul samples were taken at various intervals with an Oxford pipette, spotted on 2.3 cm filter paper discs and stored in cold 10% TCA. The extraction procedure used to process the filter discs for liquid scintillation counting was that of Rosenbaum <u>et al</u>., (1969). The scintillation fluid contained Triton-X-100 and toluene with 0.4% PPO and 0.01% POPOP (1V:2V) (Patterson and Greene, 1965). The samples were counted in a Beckman LS-233 scintillation counter.

Sterile conditions were maintained throughout the experiment as determined by periodic light microscope examinations of samples.

RESULTS

Effects of some chemical and physical agents on log phase *Polytomella*.

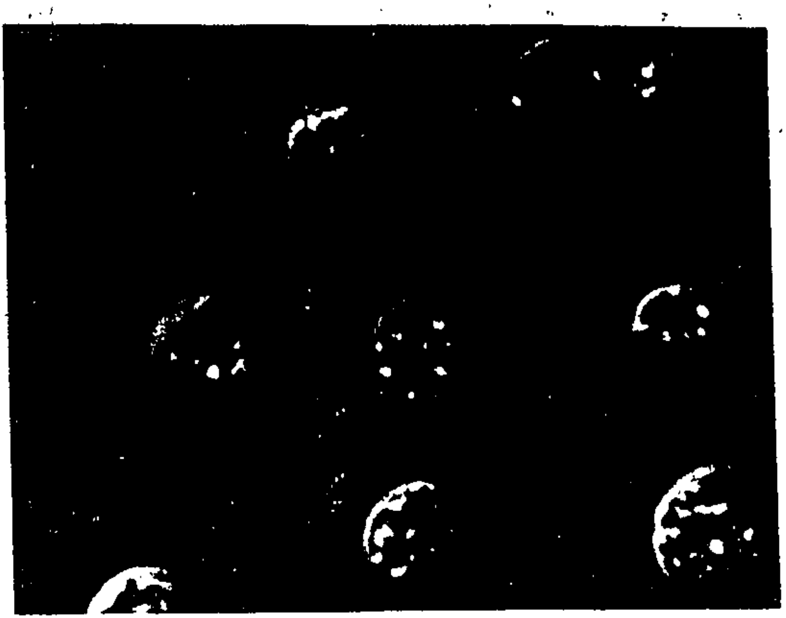
The effects of various physical and chemical agents were tested on log phase cells (5-7 x 10^5 cells/ml) of *Polytomella*. Colchicine (1%), cycloheximide (20 ug/ml), cold and IPC (7.5 or 8.0 x 10^{-4}M) have no apparent effect on cell shape after 3 hours as determined by both light and electron microscopy. Both the flagellar and cytoplasmic microtubules are stable in the presence of these depolymerizing agents.

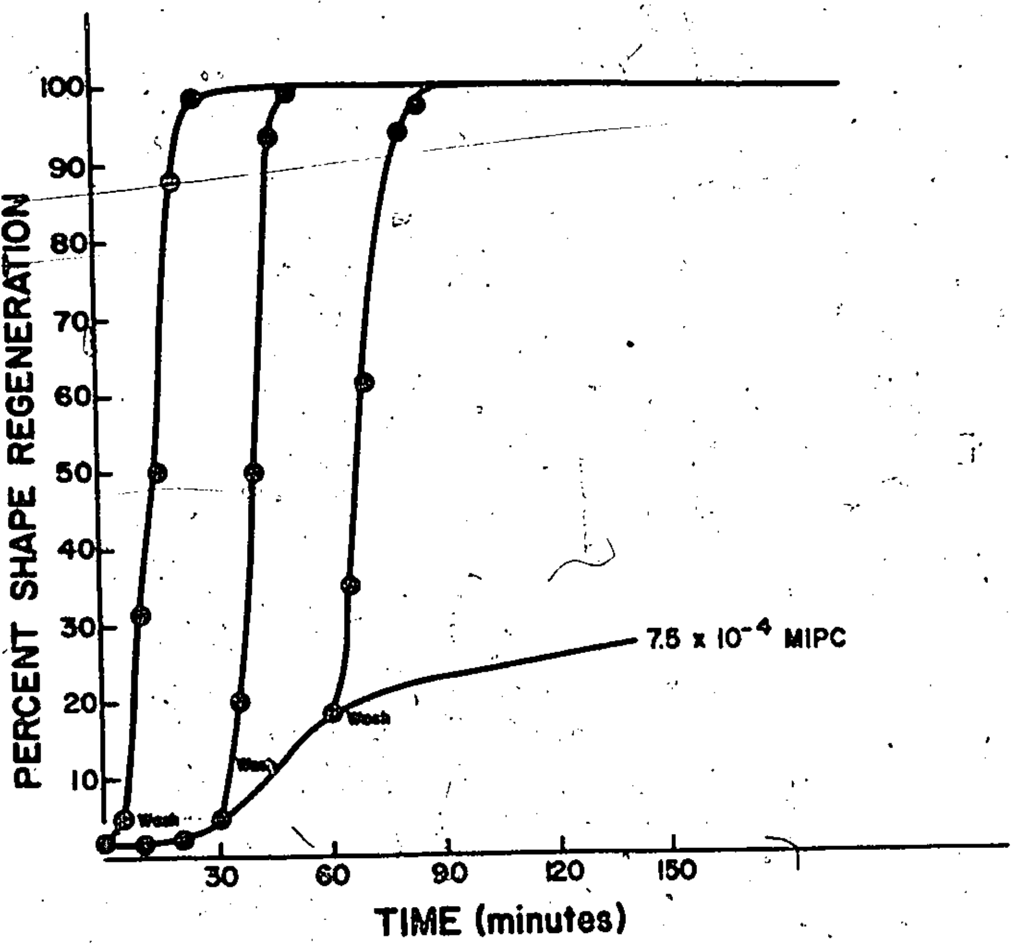
Hydrostatic pressure (6500 - 7000 psi) causes loss of cell shape in *Polytomella*. Cells pressurized at 7000 psi for 30 minutes are spherical and when the pressure is released, the cells begin to regenerate their shape immediately. The entire cell population regains its original cell shape within 15 minutes.

In cells pressurized at 7000 psi, the papillar region can be identified at the electron microscope level although it has lost its characteristic X-shape (Figs. 25 - 27). The basal body complex is disorganized and flagellar axonemes are observed within the cells. Many cytoplasmic microtubules are still seen in the majority of cells (Figs. 26 and 27), but they are not in their normal positions.

Fig. 24a: Population of rounded cells produced after IPC-pressure treatment (6500 psi and 7.5 x 10^{-4}M IPC for 30 minutes) x 1,500

Fig. 24b: Graph of cell shape recovery after IPC-pressure treatment (6500 psi and 7.5 x 10^{-4}M IPC for 30 minutes).




PERCENT SHAPE REGENERATION
100
90
80
70
60
50
40
30
20
10
Wash
Wash
Wash
7.5 x 10⁻⁴ MIPC
30
60
90
120
150
TIME (minutes)

24 b

Figs. 25 to 27: Sections through cells pressurized at 7000 psi for 30 minutes. (O time).

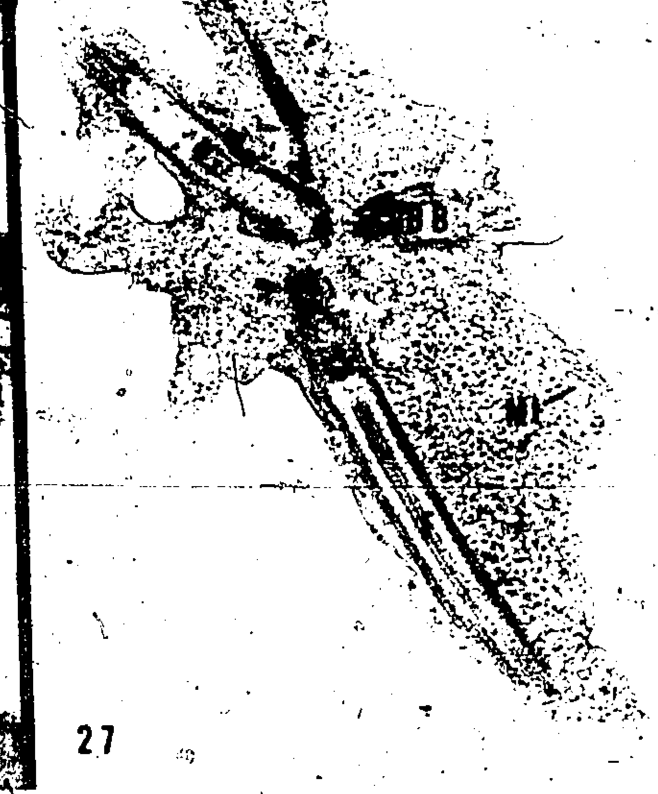
Fig. 25: Longitudinal Section. Note the disorganization of the papillar region (arrow). F, flagella, M, mitochondria, S, starch. x 18,750.

Fig. 26: Longitudinal section. BB, basal body, F, flagella, Mt, cytoplasmic microtubules . x 30,000.

Fig. 27: Cross section through the basal body region. BB, basal body, F, flagella, Mt, cytoplasmic microtubules. x 42,500

25

26



27

Effect of isopropyl-N-phenyl carbamate (IPC) plus hydrostatic pressure

Cells pressurized at 7000 psi regenerate their cell shape very rapidly when the pressure is released. By combining IPC (7.5 x 10^{-4} M) with relatively low hydrostatic pressure (6000 psi for 30 minutes), populations of spherical cells are produced (Fig. 24a) in which the majority of the cells remain rounded. The effect of IPC on cell shape is completely reversible within 15 minutes when the IPC is washed out. Partial recovery of cell shape is observed in cells which remain in IPC (7.5 x 10^{-4} M) for 2 hours after IPC-pressure treatment (Fig. 24b).

In rounded cells fixed for electron microscopy immediately after IPC-hydrostatic pressure treatment, the papillar region is not evident and the flagellar basal bodies and their associated rootlets are disorganized (Figs. 28 and 29). The rootlets remain associated with a basal body and they are apparently not altered by the IPC-pressure treatment. Both the striated rootlets (Figs. 28 and 31) and the microtubular rootlets (Figs. 30 and 31) can be distinguished. Some of the fibers which normally connect the basal bodies are observed to be disoriented (Fiber B1, Fig. 31 and Fiber A1, Fig. 32). The flagellar axonemes are seen in the cytoplasm (Figs. 31 and 32) accounting for the observation that many of the cells appear

Figs. 28 to 32: Sections through the basal body region of IPC-pressure treated cells at 0 time (6500 psi and 7.5×10^{-4}M IPC for 30 minutes.

Fig. 28: x 28,000

Fig. 29: x 57,500

Fig. 30: Cross section of the microtubular rootlet (MR) x 60,000.

Fig. 31: x 44,000

Fig. 32: x 37,500

F, flagella, SR, striated rootlet, BB, basal bodies, Bl, large fiber connecting the B basal bodies, Mt, cytoplasmic microtubules, R, rootlets.

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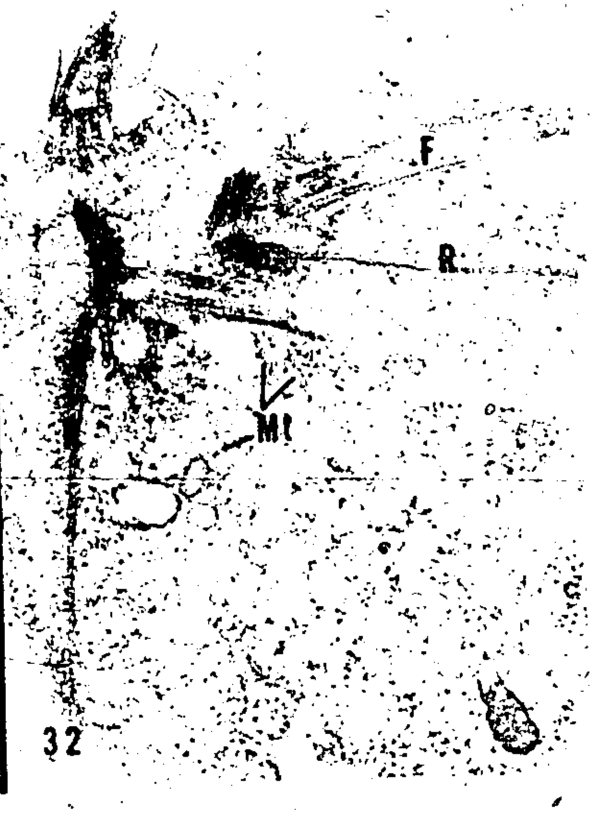
29

30

31



32

Figs. 33 to 35: Sections through cells in 7.5 x 10^{-4}M IPC after IPC-pressure treatment (6500 psi and 7.5 x 10^{-4}M IPC for 30 minutes). (2 hrs. after pressure releas

Fig. 33: Cross section through the nucleus. N, nucleus, M, mitochondria, S, starch. Note the flagellar axonemes in the cytoplasm (arrows) x 14,000.

Fig. 34: Longitudinal section through the basal body region. BB, basal body, F, flagellum, M, mitochondria x 22,000.

Fig. 35: Oblique section through basal body region. F, flagellum, BB, basal body, Mt, cytoplasmic microtubules x 22,000.

33

34

35

deflagellated when viewed under the light microscope. Cytoplasmic microtubules are absent (Fig. 28) or are rarely seen in these treated cells (Fig. 32).

Cells which remain in IPC (7.5×10^{-4}M) for 2 hours after IPC-pressure treatment appear very similar to cells fixed immediately after the treatment. The cells do not regain their shape (Fig. 33). The basal body region is still disorganized (Fig. 34) and flagellar axonemes are still observed in the cells (Figs. 33 and 35). The cells do not regain a normal complement of cytoplasmic microtubules although a few microtubules are observed in some cells (Fig. 35).

By 5 minutes recovery after IPC-pressure treatment (i.e. when cells are in control media) the basal bodies and rootlets are partly reorganized below the developing papilla (Fig. 36). Flagellar axonemes are still observed in the cells (Figs. 36 and 38). By 15 minutes recovery, the pairs of basal bodies can be recognized. In Fig. 37, the A pair of basal bodies is seen and one of the B basal bodies is observed. Cytoplasmic microtubules reassemble and appear to extend from the rootlets to the cell posterior concurrent with the redevelopment of cell shape (Figs. 38 and 39).

Cells pressurized at 6000 psi for 30 minutes in 8.0×10^{-4}M IPC do not regenerate cell shape after 3 hours

Fig. 36 and 37. Recovery series after IPC-pressure treatment.

Fig. 36: Cross section through the basal body region of a cell after 5 minutes recovery. F, flagellum x 37,500

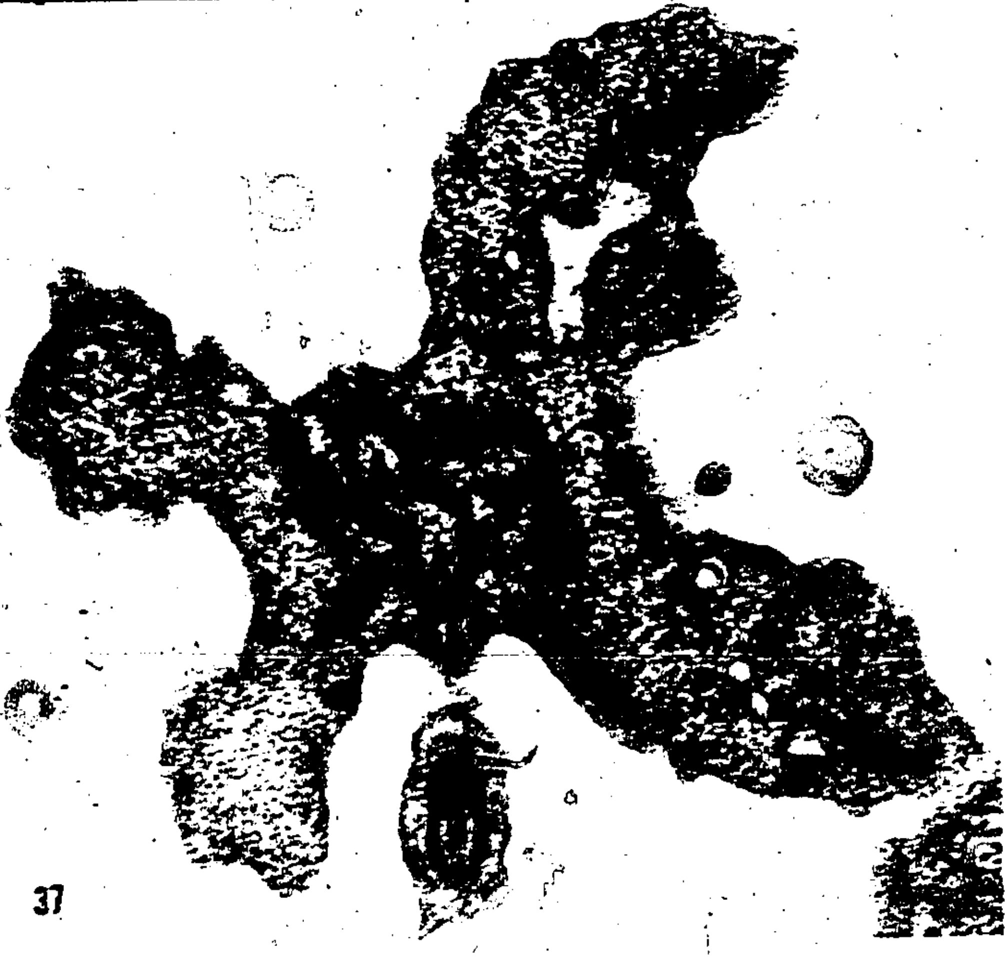
Fig. 37: Cross section through the basal body region of a cell after 15 minutes recovery. A, A basal bodies, F, flagellum, Mt, microtubules x 37,500.

Fig. 36 and 37. Recovery series after IPC-pressure treatment.

Fig. 36: Cross section through the basal body region of a cell after 5 minutes recovery. F, flagellum x 37,500

Fig. 37: Cross section through the basal body region of a cell after 15 minutes recovery. A, A basal bodies, F, flagellum, Mt, microtubules x 37,500.

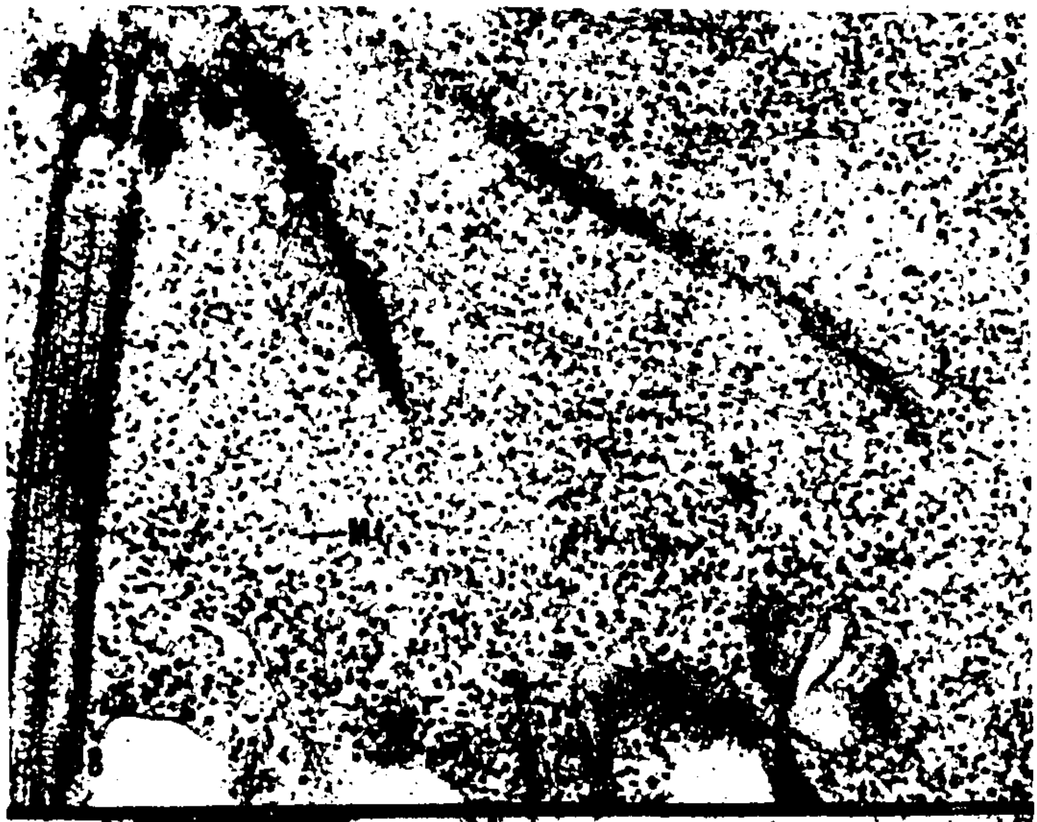
36



37

Fig. 38: Oblique section through the anterior end of a cell after 5 minutes recovery in control media after IPC-pressure treatment. F, flagellum, MR, microtubular rootlet, SR, striated rootlet, Mt, cytoplasmic microtubules x 52,500.

Fig. 39: Glancing section through the anterior end of a cell after 15 minutes recovery in control media after IPC-pressure treatment. F, flagellum, MR, microtubular rootlet, Mt, cytoplasmic microtubules x 60,000



39

in the same concentration of IPC. There is, however, some cell breakage when cells are pressurized in concentrations of IPC above 7.5 x 10^{-4}M. Cytoplasmic microtubules are absent in spherical cells fixed for electron microscopy. Complete recovery of cell shape is observed in cells resuspended in control media.

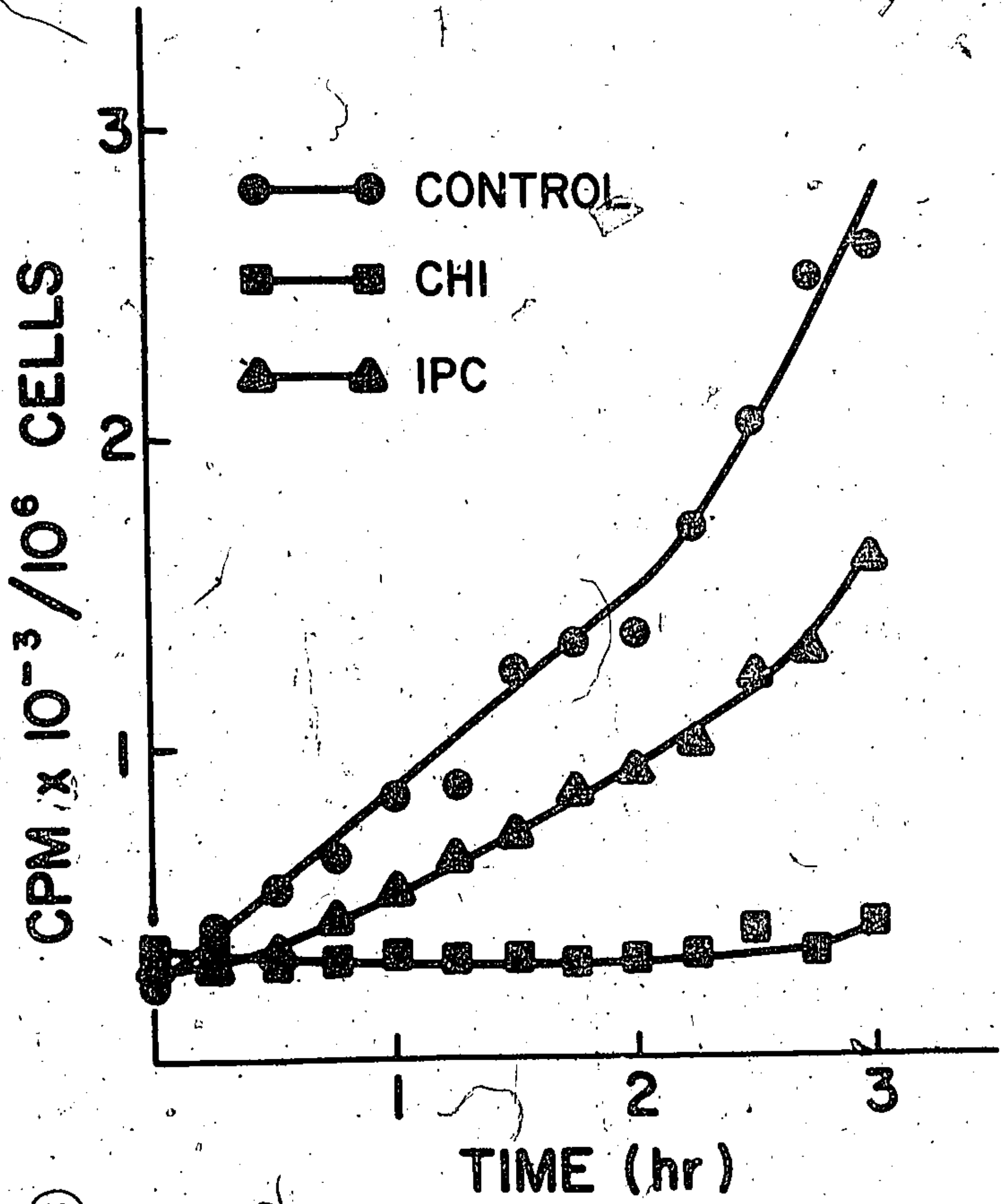
When cells treated with IPC-hydrostatic pressure are returned to control media or cycloheximide containing media (20 ugm/ml), complete shape regeneration is observed; cells returned to colchicine containing media (1%) only partially recover their shape.

Effect of IPC and Cycloheximide on amino acid incorporation

IPC alone has no apparent effect on cell shape or microtubule integrity in *Polytomella* but shape regeneration is blocked when cells remain in IPC containing media after IPC-pressure treatment. IPC at a concentration of 7.5 x 10^{-4}M causes a partial inhibition of amino acid incorporation into TCA precipitable protein (Fig. 40).

Cycloheximide, which partially blocks flagellar regeneration, does not affect shape regeneration after IPC-pressure treatment. It has no apparent effect on cell shape or microtubules after 3 hours in non-regenerating cells. Cycloheximide (20 ugm/ml) completely blocks amino acid incorporation into TCA precipitable protein (Fig. 40).

Fig. 40: Incorporation of amino acid into control, IPC and cycloheximide (CHI) treated cells.



(40)

Regeneration of deflagellated cells

When flagella are mechanically amputated, they regenerate in control medium. Flagellar regeneration is partially blocked by cycloheximide (20 ugm/ml) and is completely inhibited by colchicine (1%) and IPC (7.5 x 10^{-4}M). These results were obtained by viewing fields of cells in the light microscope.

Discussion

Three sets of microtubules have been described for Polytomella agilis; the flagellar microtubules, the cytoplasmic microtubules and the nuclear microtubules.

Polytomella has four equal flagella which are structurally similar to those found in other green algae such as Polytoma (Lang, 1963), Stigeoclonium (Manton, 1964a) and Chlamydomonas (Ringo, 1967). A flagellum has the characteristic 9 + 2 axonemal pattern in the flagellar shaft. The central pair terminate proximally just above the cylinder in the transitional region and the nine peripheral doublets extend through the transitional region into the basal body at which point they become triplets (Fig. 5).

Cross sections through the cylinder of the transitional region show the nine doublets linked by fine fibrils in the stellate pattern characteristic of the flagella of green algae (Lang, 1963, Manton, 1964b, Ringo, 1967, and Hobbs, 1971).

The arrangement of the basal bodies in Polytomella is apparently unique among quadri-flagellate green algae which have been described ultrastructurally. In Polytomella, the A1 striated distal fiber and the A2 proximal fibers link the A basal bodies; this arrangement is similar to that found in the biflagellate Chlamydomonas

(Ringo, 1967). The B1 proximal fiber links the B basal bodies. The A and B basal bodies are joined by the B2 distal fibers. In the quadriflagellate genus Carteria, the distal fibers link adjacent basal bodies in some species while in others the distal fibers are attached to long electron-dense fibers which extend between and to one side of opposite basal bodies (Lembi, 1973). Fibers joining basal bodies have been reported in a number of biflagellate algae; Polytoma which is closely related to Polytomella (Lang, 1963), Chlamydomonas (Ringo, 1967a), Tetraspora (Lembi and Walne, 1971), and Eudorina (Hobbs, 1971). The basal bodies of multiflagellate zoospores and spermatozoids of Oedogonium are linked by fibers at their proximal and distal ends forming a ring of flagellar bases (Hoffman and Manton, 1963; Hoffman, 1970).

Ringo (1967a) has postulated that the striated fibers linking the basal bodies may function in coordination of flagellar movement in Chlamydomonas. The fibers interconnecting the basal bodies in Polytomella may also function in coordination as well as anchoring and supporting the flagellar complex. It has been reported (Gittleson and Jahn, 1968) that the two flagella on each side of the anterior end in P. agilis beat synchronously in a latero-posterior cilia type beat similar to the breaststroke action observed in Polytoma and in Chlamydomonas and that two of the flagella beat further back along the cell than the other pair. My observations would indicate that the B pair which is deeper set in the cell probably beats further back along the cell than the A pair. A more detailed study of the pattern of flagellar movement in Polytomella is needed to offer insight into the problem of how flagellar ultrastructure is related to flagellar activity.

Striated fibrils, roots and rootlets are terms used to describe similar structures most often found in association with the basal bodies of motile unicells. Three types of rootlets have been described: 1) a striated rootlet such as that found in Stigeoclonium zoospores (Manton, 1964): 2) closely associated bands of microtubules such as described in Stigeoclonium (Manton, 1964 a) and 3) a compound rootlet consisting of a striated fibril and juxtaposed microtubules as in Oedogonium (Hoffman, 1970).

Two of these types of rootlets are present in Polytomella, four microtubular rootlets and four striated rootlets. Both types of rootlets are embedded in and coated with the amorphous electron opaque material associated with the flagellar complex. The electron opaque material may function as the nucleating site for cytoplasmic microtubule assembly in Polytomella. The juxtaposed microtubules which comprise the microtubular rootlets extend to the cell posterior but are no longer coated with amorphous material. The striated rootlets which are coated with amorphous material for their entire length extend out from the basal body region to the cell periphery. The rhizoplast of the amoeba-flagellate Naegleria is a periodically banded structure similar to the striated rootlets in Polytomella. Simpson and Dingle (1971) have reported a variable periodicity in the *in situ* rhizoplast of Naegleria. Once the rhizoplast is isolated, the variability is greatly reduced. They suggest that the variable periodicities may relate to the possible contractile or elastic function of these organelles. The striated rootlets do not apparently have a contractile or elastic function in Polytomella. The striated rootlets as well as the microtubular ones function as nucleating sites for cytoplasmic microtubules which diverge and extend beneath the plasma membrane to the cell posterior. Polytomella has an extensive system of cytoplasmic microtubules whose distribution closely parallels the external shape of the cell.

The mitotic spindle in Polytomella is enclosed throughout division by the nuclear envelope. A "closed" spindle has been observed in yeast cells (Robinow and Marak, 1966), in plasmodia of Physarum (Aldrich, 1969), in a red alga Membranoptera (McDonald, 1972), in

Chlamydomonas (Johnson and Porter, 1968) as well as in some other algae. In Polytomella there is no apparent ultrastructural organization at the spindle poles although the nuclear membrane appears more porous in this region during the nuclear division (Fig. 23). Fenestrae, large pores in the nuclear envelope at the spindle poles, have been reported in Ulva (Lovlie and Braten, 1970), in Membranoptera (McDonald, 1972), and in Chlamydomonas (Johnson and Porter, 1968). There is no apparent involvement of basal bodies or centrioles at the poles in Polytomella. The flagellar complex apparently remains intact during the nuclear division. The spindle microtubules which form the spindle apparatus appear to be formed independently of the flagellar and cytoplasmic microtubules. The nucleating site for spindle microtubules may not be preserved when fixed for electron microscopy or it may be diffuse and therefore not distinguishable in sections. The chromosomes do not contain localized regions which might be referred to as centromeres. How the new nuclear membranes are formed and how cytokinesis occurs have so far not been established.

The cytoplasmic microtubules may be controlled by specific sites called nucleating sites (Tilney, 1968, Brown and Bouck, 1973) which initiate microtubule polymerization and orientation. The flagellar basal bodies and the rootlets on which the cytoplasmic microtubules terminate are embedded in amorphous electron opaque material which is believed to function as the nucleating site for these microtubular systems in Polytomella.

The cytoplasmic microtubules function in the development and maintenance of cell shape in Polytomella. When cells are pressurized at 7000 psi, populations of spherical cells are obtained in which the basal body complex is disrupted and cytoplasmic microtubules are still present. When the pressure is released, the cells immediately regenerate their cell shape and the basal body complex is reorganized.

By combining IPC and low hydrostatic pressure, populations of rounded cells are produced in which the loss of cell shape can be correlated with the disappearance of the cytoplasmic microtubules. When the pressure is released and the IPC washed out, cytoplasmic microtubule reassembly occurs concomitant with the regeneration of the characteristic ovoid cell shape. The cytoplasmic microtubules extend from the microtubular and striated rootlets towards the cell posterior. Shape regeneration can be blocked at any time by the antimitotic agent colchicine or by IPC. These results indicate that microtubules function in establishing and maintaining cell shape.

It is interesting to note that the striated-rootlets and the microtubular rootlets are apparently not affected by the IPC-pressure treatment. They are also often observed still associated with a basal body (Fig. 31) although the basal body complex is disrupted. This disruption is not due to IPC as it is seen after pressure treatment only. The fibers which connect the basal bodies are still present and, as the rootlets, are still associated at one end with a basal body.

After IPC-pressure treatment (7.5 x 10^{-4}M IPC at 6000 psi for 30 minutes), some cytoplasmic microtubules are still present. By increasing the concentration of IPC to 8.0 x 10^{-4}M, all the cytoplasmic microtubules are depolymerized but there is some cell breakage. It is possible that by combining lower IPC concentrations with longer exposure to hydrostatic pressure or higher IPC concentrations with lower pressure, populations of spherical cells containing no cytoplasmic microtubules may be obtained.

Microtubule depolymerizing agents such as colchicine, IPC and cold have no apparent effect on log phase cells after 3 hours. Both the flagellar and cytoplasmic microtubules are stable in the presence of these drugs.

Colchicine causes a loss of cell shape in *Ochromonas* (Brown and Bouck, 1973) and in *Arbacia* (Tilney and

Gibbins, 1969) due to the breakdown of cytoplasmic microtubules. It also causes a breakdown of the axoneme in the axopodium of Actinosphaerium (Tilney, 1968). It is interesting to note that colchicine has no effect on cell shape in log phase populations of Polytomella as would normally be expected. Colchicine apparently does bind to Polytomella microtubule protein since it inhibits flagellar regeneration and regeneration of cell shape in IPC-pressure treated cells. One possible explanation is that the intact microtubule systems of log phase cells are relatively stable in the presence of colchicine but once the tubules exist as subunits the colchicine can bind to them thus preventing assembly of the microtubules.

Cell shape in log phase cells is not affected by IPC after 5 hours or by cycloheximide after 3 hours. IPC causes a partial inhibition of amino acid incorporation into TCA precipitable protein and cycloheximide causes total inhibition. These results suggest that Polytomella has a large pool of microtubular protein and/or that there is a slow turnover of the protein.

Regeneration of cell shape occurs in the presence of cycloheximide after IPC-pressure treatment once the pressure is released and the IPC washed out. This suggests that the cells can reutilize the depolymerized microtubule protein subunits in the absence of new protein synthesis in order to rebuild their cytoplasmic microtubule system.

Both regeneration of amputated flagella and cell shape are blocked by IPC. IPC is likely affecting some macromolecule involved in the repolymerization process of flagellar and/or cytoplasmic microtubules (i.e. their nucleating site) or it could be binding reversibly to the microtubule protein. Hepler and Jackson (1969) reported that IPC causes reorientation of spindle microtubules within a dividing plant cell so that a multipolar spindle apparatus is formed. They postulate that IPC may bind to specific cellular proteins or macromolecules involved in the organization of spindle microtubules during division. IPC may therefore be affecting the nucleating sites of the spindle microtubules.

When flagella are amputated, flagellar regeneration is partially blocked by cycloheximide. Flagellar regeneration in Polytomella is partially dependent on new protein synthesis. Rosenbaum *et al*. (1969) suggest that Chlamydomonas cells have a limiting amount of some protein, a flagellar precursor, which is necessary for total regeneration; Polytomella may present a similar situation.

Flagellar microtubules are not depolymerized by the IPC-pressure treatment as are the cytoplasmic tubules. This observation suggests that the flagellar microtubules are more stable than the cytoplasmic ones. The differential sensitivity of the cytoplasmic and the flagellar microtubules to chemical and physical agents may be accounted for by variations in their tubulin composition (Olmsted et al., 1971).

Polytomella agilis which has no cell wall possesses a cytoplasmic microtubular system which parallels the cell's shape. The cytoplasmic microtubules which terminate on the microtubular and striated rootlets, diverge and extend just beneath the plasma membrane to the cell posterior. Studies correlating microtubule reappearance and shape regeneration in cells exposed to physical and chemical agents (hydrostatic pressure, hydrostatic pressure plus IPC) indicate:

1) the rootlets function as nucleating sites for cytoplasmic microtubule assembly

2) the cytoplasmic microtubules associated with these nucleating sites function in development and maintenance of cell shape.

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