ISOLATION AND PURIFICATION OF MICROTUBULE PROTEIN FROM *POLYTOMELLA AGILIS*

by

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ABSTRACT

Polytomella agilis (Chlorophyta) exists as a free-swimming, quadriflagellate unicell in exponentially growing cultures. Electron microscopic observations indicate there is an extensive cytoplasmic microtubule system, and the characteristic 9+2 axonemal structure.

Isolation and purification procedures for flagellar axonemes using chemical or mechanical deflagellation are described. Electrophoretic analysis of purified axonemes (assayed by electron microscopy) in urea containing polyacrylamide gels reveals axonemal microtubules to be composed of two proteins, designated alpha tubulin and beta tubulin (after Bryan and Wilson, 1971). In SDS containing polyacrylamide gels, the two tubulins move as a single band, indicating identical molecular weights. The monomer molecular weight is determined by SDS gel electrophoresis (method of Weber and Osborn, 1969) to be 57,000.

Two methods are used for the purification of pig brain microtubule protein. 1) In vitro polymerization: pig brains are homogenized in 0.1M PIPES, 1mM EGTA, pH 6.9, and a high speed supernatant (25,000 *g*) is prepared. When this supernatant is made 2.5mMGTP and incubated at 37°C, microtubules form in vitro. These microtubules are cold labile, and by using cycles of polymerization (at 37°C) and depolymerization (at 0°C), microtubule protein is purified. 2) Column chromatography: microtubule protein

can also be purified by applying pig brain high speed supernatants to columns of Sephadex DEAE A-50, and eluting with a discontinuous salt gradient. Incubation of the sample with ^{3}H-colchicine prior to application to the column allows for localization of the tubulin by assaying ^{3}H-colchicine binding. Subsequent analysis of ^{3}H-colchicine binding fractions by gel electrophoresis reveals microtubule protein to be present with very little contaminating material.

Using both of these schemes as model systems, attempts are described to isolate cytoplasmic microtubule protein from deflagellated cells of Polytomella. High speed supernatants of Polytomella were fractionated on Sephadex DEAE A-50 columns following the procedure for purifying pig brain microtubule protein. Urea gel electrophoresis of fractions corresponding to peak colchicine binding reveals a number of acidic proteins, two of which are seen to co-migrate with purified flagellar microtubule protein. Following the in vitro polymerization system for pig brain, there appears to be a non-specific aggregation of protein in high speed extracts of Polytomella; no identifiable microtubules are seen. In contrast to the in vitro polymerization of pig brain microtubules, this aggregation is insensitive to colchicine, is not dependent on the presence of GTP, and is not reversible by low temperature. Aggregated protein may be pelleted at 25,000 g. When this pellet is analysed by polyacrylamide

gels containing urea, many bands are seen, and two of these bands co-migrate with purified flagellar microtubule protein; the supernatant does not contain co-migrating bands. Low levels of ^{3}H-colchicine binding can be detected in high speed supernatants of Polytomella, and there is increased ^{3}H-colchicine binding in the aggregated protein. These results provide some evidence that the protein bands which co-migrate with flagellar microtubule protein are cytoplasmic microtubule protein. To confirm this, it will be necessary to extract these co-migrating proteins for comparative amino acid analysis.

RESUME

Polytomella agilis, lorsqu'en croissance exponentielle, est un quadriflagellé mobile, unicellulaire. Les observations en microscopie électronique indiquent la présence d'un système étendu de microtubules cytoplasmiques, ainsi qu'une structure axonémale typique de 9+2.

Des méthodes d'isolation et de purification d'axonèmes flagellaires utilisant la déflagellation chimique ou mécanique sont décrites. L'analyse électrophorétique des axonèmes purifiés (verifié par la microscopie électronique) sur les gels urée-polyacrylamide démontre que les microtubules axonémales sont composées de deux protéines: la tubuline alpha et la tubuline beta (d'après Bryan and Wilson, 1971). Sur les gels SDS-polyacrylamide, les deux tubulines se déplacent en une seule bande, indiquant des poids moléculaires identiques. Le poids moléculaire du monomère, déterminé par électrophorèse sur gel SDS (méthode de Weber et Osborn, 1969), est de 57,000.

Deux méthodes sont utilisées pour la purification de protéines microtubulaires de cerveau de porc. 1) La polymérization in vitro: les cerveaux de porc sont homogénisés dans une solution de PIPES, 0.1M, EGTA, 1mM, pH 6.9, et un surnageant est obtenu après centrifugation à vitesse élevée (25,000 g). Après l'addition de GTP, 2.5mM, et une incubation a 37°C, des microtubules se forment in vitro.

Puisque ces microtubules sont sensibles au froid, l'utilisation de cycles de polymérisation à 37°C et de dépolymérisation à 0°C permet la purification de protéines microtubulaires. 2) La chromatographie sur colonne: en appliquant le surnageant obtenu après centrifugation à haute vitesse sur une colonne de Sephadex DEAE A-50, suivi d'élution avec gradient discontinu de sel, la protéine microtubulaire est purifiée. Incubation de l'échantillon avec la colchicine-H^3, suivi de fractionnement sur colonne, permet la localisation de tubuline par analyse de colchicine-H^3 attachée. L'analyse subséquente par électrophorèse sur gel des fractions démontrant l'attachment de colchicine-H^3, indique la présence de protéine microtubulaire, avec contamination négligeable.

En utilisant ces deux méthodes comme modèles, des essais ont été faits pour isoler la protéine de microtubules cytoplasmiques de cellules déflagellées de Polytomella. Les surnageants obtenus après centrifugation à haute vitesse de Polytomella ont été fractionnées sur colonne de Sephadex DEAE A-50, suivant la même méthode que pour la purification de protéine microtubulaire de cerveau de porc L'électrophorèse sur gels d'urée des fractions correspondant a l'attachement maximum de colchicine-H^3, indique la présence de plusieurs protéines acidiques, dont deux se déplacent conjointement avec la protéine microtubulaire flagéllaire purifié. Suivant la même méthode que celle pour la polymérisation in vitro de microtubules de cerveau

de porc, les résultats indiquent la présence d'une agrégation non-spécifique de protéines dans les surnageants de Polytomella, obtenus après centrifugation à haute vitesse. Aucune microtubule ne peut être identifiée. Cette agrégation de protéines est insensible à l'action de la colchicine, est irréversible à basse température, et ne dépend pas de la présence de GTP, contrairement à ce qui a été observé dans la polymérisation in vitro de microtubules de cerveau de porc. Cette agrégation est precipitée à 25,000 g. L'analyse de ce precipité sur gels urée-polyacrylamide démontre plusieurs bandes, dont deux se déplacent conjointement avec la protéin purifiée de microtubules flagéllaires. Le surnageant ne contient aucune de ces deux bandes. Des niveaux minimes d'attachement de colchicine-H^3 au surnageant obtenu par centrifugation à haute vitesse peuvent être détectés, ainsi qu'un niveau d'attachment plus élevé pour l'aggrégation de protéine. Ces résultats donnent des indications que les bandes qui se déplacent avec la protéine microtubulaire de flagèlles, sont composées de protéin microtubulaire cytoplasmique. Afin de confirmer cette hypothèse, il sera nécessaire d'extraire ces protéines et en faire une analyse comparative d'acides aminées.

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LIST OF ABBREVIATIONS

DEAE	diethylaminoethyl
EDTA	disodium ethylenediamine-tetraacetate
EGTA	[ethylene(oxyethylene-nitrilo)] tetra-acetic acid
GDP	guanosine diphosphate
GTP	guanosine triphosphate
IPC	isopropyl N-phenylcarbamate
PC buffer	67mM KPO_4, 100mM KCl, pH 6.8
PE buffer	0.1M PIPES, 1mM EGTA, pH 6.9
PG buffer	20mM sodium phosphate, 100mM sodium glutamate, pH 6.8
PIPES	(piperazine-N-N'-bis[2-ethane sulfonic acid])
SDS	sodium lauryl sulfate
TEEP	15mM Tris, 2.5mM EDTA, 11% ethanol, 30mM KCl, pH 7.8
Tris	2-amino-2-hydroxymethyl-1, 3-propanediol

INTRODUCTION

STRUCTURE AND DISTRIBUTION OF MICROTUBULES

Microtubules may be defined as a class of long tube-like or cylindrical organelles, approximately 220-260 A° in diameter. In cross-section, they are composed of an electron dense cylindrical wall surrounding a less dense core.

Tubule-like structures (now known to be microtubules) were reported several times during the 1950's (Grasse, 1956; Beams et al., 1959; Roth, 1959) but it was with the advent of gluteraldehyde fixation (Sabatini et al., 1963) that these structures were uniformly encountered in cells. Their occurence was reported almost simultaneously as 230-270 A° tubular elements in plant cells by Ledbetter and Porter (1963), and in Hydra by Slautterback (1963). They have been observed in all eukaryotic cells so far studied. They are not known to exist in prokaryotes.

The general structure of a microtubule is consistent with a protofilament model (Ringo, 1967), in which the wall of the tubule is composed of a number of protofilaments approximately 40-50 A° in diameter. Recently, Tilney et al. (1973) have shown this number of protofilaments to be thirteen in a wide range of phylogenetically different organisms. X-ray diffraction data from wet

gels of sea urchin flagellar outer fiber doublets (Cohen et al., 1971) reveals that subunits with a 40-50 A° packing diameter form protofilaments parallel to the axis of the microtubule. The globular subunits in adjacent protofilaments are half staggered (fig. 1).

In cilia and flagella, the axoneme is composed of microtubules in the typical 9+2 arrangement (Manton and Clarke, 1952; Gibbons and Grimstone, 1960; Porter, 1966). Centrioles and basal bodies are characterized by their distinctive arrangemnt of microtubules (Fulton, 1971a) and the fibrous component of the mitotic spindle is also composed of microtubules (Roth and Daniels, 1962; Nicklas, 1971). They are also universally distributed through the cytoplasm of plant and animal cells (for a detailed review see Newcomb, 1969; Olmsted and Borisy, 1973a).

FUNCTION

By the very fact of their diverse distribution, one would expect microtubules to be involved in a variety of cell functions.

Microtubules have been implicated, by association, in the movement of mitotic chromosomes. The anti-mitotic effects of the plant alkaloid, colchicine, were first described in 1889 (Pernice, cited in Eigsti et al., 1949). In 1952 (a), Inoué reported a system in which the birefringence of the mitotic spindle could be measured using polarizing microscopy. Upon the addition of colchicine,

the birefringence of the spindle was destroyed and chromosome movement stopped. This action was reversible upon removal of the colchicine. In light of later work (Taylor, 1965), it has been postulated that colchicine disrupts the action of the spindle by binding directly to microtubule protein, causing microtubules to depolymerize. Use of cold (Inoué, 1952b), pressure (Zimmerman and Marsland, 1964), or UV microbeam irradiation (Inoué, 1964) have given similar results with disruption of spindle microtubules and the blockage of mitosis.

Microtubules have also been shown to be associated with axonal transport, both on a morphological basis (Smith et al., 1974), and on the basis of disruption of activity by microtubule depolymerizing agents. Several workers (Kreutzberg, 1969; Fernandez et al., 1971; Sjöstrand et al., 1970) have shown that application of colchicine or the Vinca alkaloid, vinblastine, to nerves in vivo will block the transport of marker proteins down the nerve fibers.

In many animal and protistan cells, microtubules are believed to play a cytoskeletal role. In the axopodia of Echinosphaerium (Actinosphaerium) is an axoneme composed of two rows of microtubules spiralling around a central core (Tilney and Porter, 1965). When the microtubules are depolymerized using hydrostatic pressure (Tilney et al., 1966), low temperature (Tilney and Porter,

1967), or colchicine (Tilney, 1968a), the axopodia retract. When these agents are removed, the axopodia will regenerate and the original microtubular pattern is again seen.

Bouck and Brown (1973) have described the distribution of microtubules in the alga, Ochromonas danica. They subsequently demonstrated (Brown and Bouck, 1973), by the use of the microtubule depolymerizing agents colchicine and hydrostatic pressure, an obligatory role for cytoplasmic microtubules in the development and maintenance of a species-specific cell shape in Ochromonas.

Microtubules have also been implicated in cell motility (in flagella), and in the external movement of substances past cells (in cilia). In these systems microtubules are believed to slide past one another (Brokaw, 1972), causing the movement of the flagellum or cilium. The energy mechanism of this reaction is not known in detail, although it has been shown that the projections emanating from the A tubule of the axoneme are an ATPase known as dynein (Gibbons, 1965). It has also been shown that demembranated axonemes of sea urchin sperm can be reactivated (i.e. become motile) in vitro by the addition of exogenous ATP (Gibbons and Gibbons, 1972).

MICROTUBULE ASSEMBLY

1. In Vivo: By using the system he developed for measuring spindle birefringence, Inoué demonstrated spindle fibers in a living mitotic spindle (Inoué, 1953).

Such fibers could easily be seen associated with chromosomes in fixed material (Schrader, 1944). Inoué hypothesized that birefringence was due to the fibrous component of the spindle. Reversible loss of this birefringence could be demonstrated using colchicine (Inoué, 1952a) or cold (Inoué, 1952b). From these results he postulated that the spindle fibers were not static structures but existed in a dynamic state of flux, with the recognition that spindle fibers were bundles of microtubules. This postulate has been expanded into the dynamic equilibrium theory of microtubule assembly (Inoué, 1964). This theory states simply that there exists a pool of microtubule protein within a cell, and an equilibrium reaction exists between the polymerized state (microtubules) and the non-polymerized state (protein subunits). (For a re-examination of the dynamic equilibrium model, see Stephens, 1973.)

There are several agents which can shift the equilibrium of the mitotic spindle. Substitution of heavy water (D_2O) for normal water (H_2O) in high concentrations has been shown to "freeze" mitosis (Gross and Spindel, 1960; Marsland and Zimmerman, 1965) by overstabilizing the structure of the spindle. D_2O has also been shown to enhance the birefringence of the mitotic spindle by increasing its volume and protein content (Inoué et al., 1963; Sato et al., 1966). In the dynamic equilibrium model, structured water is believed to dissociate from the protein molecules upon polymerization. Heavy water

molecules are held together more tightly than ordinary water molecules (Sidgwick, 1950). D_2O would thus remove structured water from protein molecules and shift the equilibrium towards polymerization, with a resultant enhancement in birefringence (Inoué and Sato, 1967). Agents such as cold (Inoué, 1952a), colchicine (Inoue, 1952b), vinblastine (Malawista et al., 1968) or hydrostatic pressure (Zimmerman and Marsland, 1964) push the equilibrium towards the non-polymerized state. Upon removal of any of these agents, spindle birefringence returns. That this was not simply due to a de novo synthesis of protein was demonstrated by Inoué and Sato (1967) in studies which showed equivalent spindle birefringence recovery in the presence of Actinomycin D, or puromycin.

The dynamic equilibrium theory may also be applied to the assembly of microtubules in other systems. Tilney et al. (1966, 1967, 1968a) have demonstrated that the reversible assembly and disassembly of microtubules in the axoneme of Echinosphaerium is similar to that for the mitotic spindle. Brown and Bouck (1973) have shown that hydrostatic pressure can cause the disappearance of cytoplasmic microtubules in Ochromonas. De novo protein synthesis is not required for their regeneration as they wil reassemble in the presence of cycloheximide. Similarly Rosenbaum et al. (1969) have demonstrated the presence of a microtubule protein pool in Chlamydomonas

by amputating both flagella and assaying flagellar regeneration in the presence of cycloheximide or colchicine. Flagella will regenerate to about half their length in cycloheximide. However, in the presence of colchicine, all flagellar regeneration is blocked.

Is this equilibrium reaction a mediated one? That is, does the protein assemble and disassemble because of its inherent structure (Lauffer et al., 1958), or is there some center within the cell which regulates this process? The evidence for such centers is mostly ultrastructural, and it simply points to sites within cells on which microtubules terminate. The microtubules, which are free at one end, appear to make contact with identifiable cell 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 centers (Tilney, 1968b), microtubule organizing centers (Pickett-Heaps, 1969), and nucleating sites (Tilney and Goddard, 1970). Tubules are shown ending on these sites, which usually appear as amorphous, diffuse, electron dense material. Bouck and Brown (1973) have identified such sites in Ochromonas. By combining hydrostatic pressure and the herbicidal mitotic inhibitor isopropyl N-phenylcarbamate (IPC) it is possible to obtain spherical populations of cells in which all

microtubules are depolymerized or converted to macrotubules. When pressure is released, and the IPC washed out, the population synchronously regenerates its cell shape. The tubules first appear associated with these presumptive nucleating sites and gradually extend to the cell's posterior (Brown and Bouck, 1974).

2. In Vitro: Recently, an in vitro polymerization reaction for microtubules has been established using brain homogenates (Borisy and Olmsted, 1972b; Weisenberg, 1972) that shares many of the characteristics of in vivo assembly. Microtubules, morphologically identical to those seen in vivo, can be prepared in vitro when brain homogenates in Good buffers (Good et al., 1966) containing EGTA and GTP are incubated at 37°C. In vitro polymerization is temperature sensitive (Borisy et al., 1974); colchicine sensitive (Borisy et al., 1974); has a nucleotide requirement (Weisenberg, 1972); and is strongly dependent upon tubulin concentration in the homogenate (Olmsted and Borisy, 1973b). Microtubules formed in vitro are cold labile and colchicine labile. In brain preparations maintained at 0°C, no microtubules form, but ring-like structures (ca. 300 A° in diameter) are seen (Borisy and Olmsted, 1972b). These structures are not seen in vivo. When homogenates are raised to 37°C, microtubules appear with a concomitant disappearance of the ring-like structures. It has been postulated that these rings somehow serve as initiation sites for tubule formation in vitro.

Tubulin represents roughly 25% of the protein in a pig brain homogenate (Olmsted and Borisy, 1973b). This concentration is many fold greater than that seen in most organisms. An in vitro assembly reaction has, as yet, only been demonstrated in systems in which tubulin represents a significant amount of the protein present (Raff et al., 1974). It is not known therefore, how closely this in vitro reaction resembles an in vivo situation in any other system other than brain and this is presently under study by many workers.

CHEMISTRY

Microtubules from all sources are essentially morphologically identical (Porter, 1966). They are polymers of a protein which has been named tubulin (Mohri, 1968).

The first successful studies in which microtubules as identifiable structures were isolated and the biochemical properties of their constituent protein, tubulin, analysed used axonemes of cilia and flagella. In 1959, Child isolated ciliary axonemes from Tetrahymena. His results suggested that they were composed of a single homogenous protein. Renaud et al. (1968), however, also working with Tetrahymena, showed that outer doublet protein, after extensive reduction and alkylation, could be resolved into two very closely spaced bands on urea containing polyacrylamide gels. Outer doublet protein was shown to have a molecular weight of approximately 104,000

in aqueous solution, with a sedimentation coefficient of 6S. In guanidine hydrochloride, the molecular weight fell to 55,000 suggesting tubulin existed as a dimer. Several workers (Stephens, 1970; Everhart, 1971; Witman et al., 1972b) have confirmed that flagellar microtubules in cilia and flagella isolated from a variety of sources are composed of two discrete proteins. Approximate molecular weight determinations were also confirmed by ultracentrifugation (Shelanski and Taylor, 1968; Stephens, 1968) and by SDS polyacrylamide gel electrophoresis (Witman, 1972a).

An association has also been demonstrated between purified flagellar microtubules and bound nucleotides, usually GTP or GDP. Several authors have reported one mole of guanine nucleotide is bound per 60,000 g. protein (Stephens et al., 1967; Shelanski and Taylor, 1968; Yanagisawa et al., 1968). The direct significance of this bound nucleotide is not known although it has been postulated that transphosphorylation between guanine and adenine nucleotides might play a significant role in cell motility (Yanagisawa et al., 1968).

Microtubules in ciliary and flagellar axonemes are quite stable and remain intact under rather harsh isolation procedures. Therefore, purity of axonemal preparations can easily be assayed by electron microscopy. However, cytoplasmic microtubules are very labile, and initial attempts to purify microtubule protein by isolating intact

microtubules met with failure. This problem was circumvented by developing an assay system for microtubule protein using the anti-mitotic drug colchicine. Inoué (1952a) had shown colchicine blocks mitosis with resultant disruption of spindle fibers. Taylor (1965) demonstrated that this anti-mitotic effect of colchicine was probably due to the colchicine binding directly to the structural protein of the spindle. Borisy and Taylor (1967a) subsequently showed, using ^{3}H-colchicine, that the majority of bound ^{3}H-colchicine was in a noncovalent complex with a macromolecule. This complex was shown to have a sedimentation coefficient of 6S, and it was suggested that the protein was a subunit of microtubules. A similar colchicine binding complex was subsequently isolated from a variety of sources (Borisy and Taylor, 1967b; Shelanski and Taylor, 1967; Wilson and Friedkin, 1967) and found to have a molecular weight of 110,000 and a sedimentation coefficient of 6S.

Direct correlation was made between morphologically identifiable tubules and this 6S colchicine binding fraction using isolated spindles and sea urchin sperm tails. Using low ionic strength buffers, microtubules could be extracted from isolated spindles with a simultaneous appearance of a 6S protein peak in the extract. This correlated with the fact that greater than 80% of the ^{3}H-colchicine binding activity was contained in the extract (Borisy and Taylor, 1967b). Similar results were

obtained with microtubules from sea urchin sperm tails (Shelanski and Taylor, 1967).

Based on this work, isolation schemes for colchicine binding protein were developed for brain tissue (Weisenberg et al., 1968; Olmsted et al., 1970; Bryan and Wilson, 1971). This purified fraction had a dimer molecular weight of 110,000-120,000, could be dissociated into a 55,000-60,000 molecular weight monomer, and had similar amino acid composition and electrophoretic patterns as purified outer doublet protein. Electrophoretic patterns, identical to those obtained for this colchicine binding complex, have been obtained from intact microtubules isolated from brain (Kirkpatrick et al., 1970). Thus, this colchicine binding complex is generally assumed to be microtubule protein.

As with flagella and cilia, an association has been shown between this protein and bound nucleotides. Weisenberg et al. (1968) indicated 0.5-0.8 moles of guanine nucleotide bound per 120,000 g. protein isolated. However, studies using ^{3}H-GTP indicated two nucleotide binding sites: at one site, exchange of bound and free nucleotide occured with a half life of less than fifteen minutes; but the guanine nucleotide still bound to the protein after purification indicated a second site where little or no exchange occured. This tightly bound nucleotide appears significant in maintaining the native configuration of the protein (Weisenberg et al., 1968). It has

also been postulated that the binding and hydrolysis of guanine nucleotides may play a role in microtubule polymerization (Olmsted and Borisy, 1973a).

Morphological data (Cohen and Rebhun, 1970) indicates about 6-12% of the total protein in the spindle is microtubules. Numerous studies on isolated mitotic spindles implicated a variety of subunits as the possible precursor of spindle microtubules depending on the isolation method used (Dirksen, 1964; Kane, 1967; Miki-Nomura, 1968). However, selective solubilization procedures have allowed for the specific study of spindle microtubule protein. Using low ionic strength solutions, the appearance of a 6S colchicine binding fraction has been associated with the disappearance of microtubules from hexylene glycol stabilized spindles (Borisy and Taylor, 1967b). Bibring and Baxandall (1971) selectively solubilized microtubules from the isolated spindle apparatus using meralluride sodium. This protein extract possessed very similar properties to outer doublet tubulin, including molecular weight, amino acid composition, precipitability by vinblastine, and electrophoretic mobility.

All these results point to the hypothesis that microtubules are polymers of a 120,000 molecular weight protein dimer with a sedimentation coefficient of 6S. This may be resolved into a monomer of 55,000-60,000 molecular weight with a sedimentation coefficient of 3-4S. Microtubules are known to consist of at least two proteins of

approximately equal molecular weight which can be separated by gel electrophoresis. On the basis of their electrophoretic mobility, these two tubulins have been denoted tubulinα (faster moving species) and tubulinβ (slower moving species; Bryan and Wilson, 1971). It is not known whether the dimeric subunit of microtubules is a homodimer, or a heterodimer, although several models have been proposed for the distribution of tubulin in the microtubule (Witman et al., 1972c; Tilney et al., 1973). Fig. 1 represents a schematic model of a microtubule, showing the arrangement of protofilaments, and suggesting a distribution scheme for tubulin (from Tilney et al., 1973).

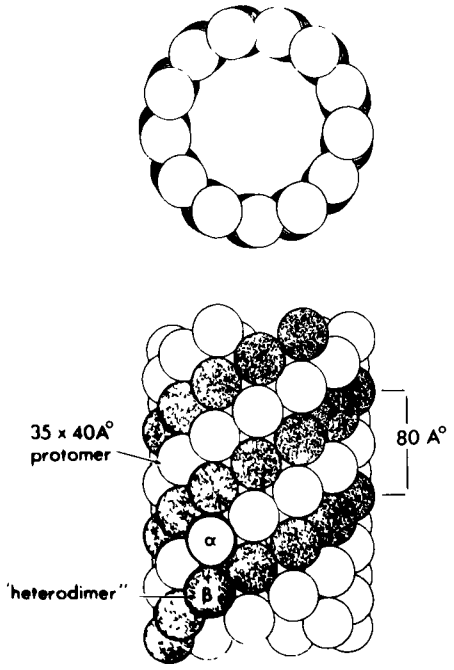


Fig. 1 The model (From Tilney et al., 1973) has 13 protofilaments composed of heterodimers. Each half of the dimer is assumed to be a 35 x 40 A^{o} globular subunit (either α- or β-tubulin).

Are corresponding tubulins from different sources identical? Isolated from very different sources, they possess similar electrophoretic mobilities, amino acid compositions and molecular weights (Luduena and Woodward, 1973). But different sets of microtubules have been shown to respond differently to the same treatments. In 1967, Behnke and Forer presented evidence for four classes of microtubules. Using crane fly spermatids and rat tracheal cilia, they tested their lability to cold, heat, colchicine, and pepsin treatment. On the basis of resultant morphological evidence of lability, they proposed four classes of microtubules: cytoplasmic microtubules, the central pair tubules, A tubules, and B tubules. Tilney and Gibbins (1968c) showed differential effects of colchicine or hydrostatic pressure on cytoplasmic and ciliary microtubules of sea urchin gastrulae. Brown and Bouck (1973) also showed differences between the two sets of cytoplasmic microtubules of Ochromonas, as well as its flagellar tubules. The question remains whether these differences of stability reflect direct differences in protein composition or are there in situ stabilizing forces, or other agents which account for them?

The answer to the previous question lies in being able to isolate and biochemically characterize different sets of microtubules from the same organism. At this time,

there is only one report of such an attempt. Safer (1973) describes a comparison of tubulin of gill cilia and sperm flagella from lamellibranch molluscs. Using cyanogen bromide cleavage, and subsequent SDS gel electrophoresis, he found subtle but consistent differences between corresponding tubulins. It is to this same question that we have directed ourselves, in part, in this project.

Polytomella agilis presents an ideal system for the study of microtubules and microtubule protein. It exists both as a free swimming, motile organism, and as a dormant cyst. During encystment (due to nutritional depletion of the medium), flagella are retracted, and cytoplasmic microtubules disappear. In fresh medium, cysts will germinate with a concomitant regeneration of the cytoplasmic and flagellar microtubular systems. The vegetative form, with which we are concerned in this report, is a quadriflagellate organism possessing an extensive system of cytoplasmic microtubules (Patenaude, 1974). It also possesses an intranuclear spindle and populations of cells can be partially synchronized using cold shocks.

Polytomella possesses three distinct sets of microtubules during its life cycle. It is the long range aim of this research to isolate each set of microtubules, and characterize and compare their microtubule protein. Since microtubules are lost during encystment, and subsequently regenerated when cysts germinate, it may be

possible to study the synthesis and subsequent assembly of microtubule protein. This is, of course, dependent upon developing a system for assaying microtubule protein.

This thesis will report on:

1. methods for the isolation and purification of flagellar microtubules from Polytomella
2. in vitro polymerization of brain microtubules and a subsequent purification of brain microtubule protein
3. application of this model system to Polytomella in an attempt to purify cytoplasmic microtubule protein.

MATERIALS AND METHODS

CULTURES

Cultures of Polytomella agilis Aragoa were grown in Erlenmeyer flasks or in 9 liter serum bottles (Pyrex B7750) in a complex medium containing 0.1% tryptone, 0.2% yeast extract, and 0.2% sodium acetate. All cultures were incubated at 25°C in the dark (Sheeler et al., 1968). Original cultures (L 193) were obtained from Indiana Algal Culture Collection (Starr, 1964). Further axenic cultures were a generous gift of Dr. Joseph Moore, California State University at Northridge. Cultures were inoculated from stationary phase cultures giving a population density after inoculation of approximately 10^4 cells/ml. Mean generation time is 4.7 hours, and cultures were used in late exponential phase (approximately 36 hours after inoculation) at a cell density of 2-5 x 10^6 cells/ml.

HARVESTING OF CELLS

Cells in 100-500 ml. cultures were harvested at room temperature by centrifugation in an IEC Clinical Centrifuge in 50 ml. conical tubes at number 7 setting for 2 minutes. Cells in 1000-4000 ml. cultures were harvested by centrifugation in a Sorvall RC2-B refrigerated centrifuge with GSA head in 290 ml. polycarbonate bottles at 4,000 rpm (2600 g) for 5 minutes. Flagella were detached and

isolated from sedimented cells by one of the following procedures:

FLAGELLAR DETACHMENT

1. Manual Deflagellation: Cells were harvested and resuspended in approximately 0.5% original culture volume in fresh media. 5 mls. was placed in a fluted test tube (20 x 150 mm.) and agitated on a Vortex mixer (Scientific Industries, Springfield, Mass.) at full speed for 90 seconds (Rosenbaum and Child, 1967). Flagellar detachment was greater than 99%.

2. TEEP Deflagellation: Flagella were also removed from cells by a modification of the procedure of Witman et al. (1972b) for isolating flagella from Chlamydomonas. Cells were harvested, washed once in 10 mM Tris, pH 7.8, and then resuspended in 1.5% original culture volume in 10 mM Tris. This cell suspension was cooled to 10°C, and then 4 to 5 volumes of ice cold (0-2°C) 15 mM Tris, 2.5 mM EDTA, 11% ethanol, 30 mM KCl, pH 7.8 (TEEP) was added, and this mixture was stirred on a magnetic stirrer. 1M $CaCl_2$ was added immediately sufficient to bring final calcium concentration to 15 mM. Cells were deflagellated within about 60 seconds. Deflagellation was greater than 95%.

FLAGELLAR ISOLATION

Flagella were separated from cell bodies by

centrifugation in an IEC Clinical Centrifuge at number 5 setting for 2 minutes, or in a Sorvall RC2-B with SS-34 head, at 4,000 rpm (1900 g) for 5 minutes. Supernatants, containing flagella, were centrifuged in either 15 ml. Corex tubes and the SS-34 head at 14,500 rpm (25,000 g) for 30 minutes, or 290 ml. polycarbonate bottles and the GSA head at 12,500 rpm (25,000 g) for 30 minutes. Pellets were resuspended in 5% sucrose, and given two low speed spins at 1500 rpm (SS-34 head) to pellet unremoved bodies and starch from broken cells. Flagella were then pelleted at 25,000 g, resuspended in 5% sucrose, placed over a discontinuous 5%/40% sucrose gradient in nitrocellulose tubes and centrifuged for 60 minutes at 2,000 rpm (400 g) in a Beckman L2-65B preparative ultracentrifuge. Flagella banded out at the interface and could be removed with a Pasteur pipette. The pellet contained starch granules and cell bodies.

REMOVAL OF FLAGELLAR MEMBRANES

Flagella were pelleted from 5% sucrose (25,000 g, 30 minutes) and resuspended in 1 mM Tris, pH 8.3. Six times this volume of 1 mM Tris, pH 8.3 containing 0.02% Non-Idet P-40 (Shell Chemicals, London, England) was added and this suspension was kept on ice for 5 minutes. Demembranated flagella (axonemes) were pelleted as per whole flagella. The pellet obtained was resuspended in 5% sucrose and layered over a 5%/40% discontinuous sucrose

gradient and centrifuged at 12,500 rpm (16,000 g) for 60 minutes. Axonemes were pelleted through the 40% sucrose but detached membranes remained as a whitish band at the interface.

MICROSCOPY

Deflagellated cells and isolated flagella were examined with a Zeiss Universal microscope equipped with Nomarski optics. Pictures were taken with electronic flash at 60 W.s. on Ilford Pan F and developed in Diafine.

For electron microscopy whole cells were pelleted and fixed in 0.5% gluteraldehyde in 0.05M PO_4 buffer, pH 7.4 for 1½ hours, postfixed in 1% OsO_4 in the same buffer, and dehydrated in 10, 30, 50, 70, 90 and 100% acetone. Samples were then infiltrated and embedded in Spurr's plastic (Spurr, 1969). Flagella and axonemes were pelleted hard in 15 ml. Corex tubes at 18,000 rpm for one hour. Pellets were fixed with 4% gluteraldehyde, in 0.1M PO_4 buffer, pH 7.4, postfixed in 1% OsO_4 in the same buffer, dehydrated, infiltrated and embedded as above. Sections were cut with a diamond knife on a Porter-Blum MT2-B ultramicrotome, picked up on uncoated 200 mesh copper grids, stained with uranyl acetate (2% in 50% ethanol) and lead citrate (Reynolds, 1963) and examined in an AEI-EM6B electron microscope.

For observations by negative staining a drop of the sample to be examined was placed on a formvar and carbon

coated 200 mesh grid. After 60 seconds, the drop was removed with filter paper, and a drop of aqueous 1% uranyl acetate was placed on the grid for 60 seconds and then drawn off with filter paper. Grids were allowed to dry and examined as above.

DETERMINATION OF PROTEIN CONCENTRATION

Protein concentrations in all samples were determined by the method of Lowry et al. (1951), with bovine serum albumin standards (Sigma Chemical Co., St. Louis, Mo.).

ELECTROPHORESIS

1. Urea Gels: Protein samples were prepared for analysis on urea containing polyacrylamide gels by the method of Renaud et al. (1968). Reduction: samples were made 0.12M 2-Mercaptoethanol, 8M urea, and dialysed for 12-24 hours against a large excess of 8M urea, 0.12M 2-Mercaptoethanol, 35mM Tris, 0.1% EDTA, pH 8.8. Alkylation: one part 1.1M sodium iodoacetate, 8M urea was added to nine parts reduced protein and placed in the dark for one hour. The sample was then dialysed in the dark for 4-6 hours against 30mM Tris, 8M urea, pH 7.8.

Proteins were separated by electrophoresis at 2-3 ma/gel in 7.5% polyacrylamide gels (Davis, 1964; Ornstein, 1964) containing 8M urea. Gels were 5 mm. in diameter and approximately 85 mm. in length. Running buffer was 0.028M glycine, 0.005M Tris, pH 8.3, and was pre-cooled

before electrophoresis. Bromphenol blue (aqueous 0.001%) was used as a tracking dye and the gels were run until the dye almost reached the end of the gel. Gels were removed with a continuous stream of water from a Pasteur pipette, and standardly fixed overnight in 20% sulfosalycylic acid, then stained in aqueous 0.5% Coomasie brilliant blue R (Polysciences Inc., Warrington, Penn.).

2. SDS Gels: Proteins were also analysed on SDS containing gels essentially according to the method of Weber and Osborn (1969). Samples were made 0.01M PO_4 (0.1M stock, pH 7.2), 3% SDS, 1% 2-Mercaptoethanol and placed in a boiling water bath for three minutes. Samples were allowed to sit for 6-12 hours, and then dialysed for 12 hours against a large excess of 0.01M PO_4, 1% SDS, 1% 2-Mercaptoethanol.

Gels were 10% polyacrylamide containing 0.1% SDS and were pre-electrophoresed for three hours prior to protein application. Running buffer was 0.1% SDS, 0.1M PO_4, pH 7.2. Proteins were loaded in glycerol containing bromphenol blue and run at 8 ma/gel. Gels were removed with a syringe and fine guage needle and fixed and stained as described.

To prepare a standard curve for the SDS gel system proteins used were cytochrome C, catalase (General Biochemicals, Chagrin Falls, Ohio) egg albumin, chymotrypsinogen A (Sigma) and bovine serum albumin (Calbiochem, La Jolla, Calif.). Proteins were dissolved in 5% sucrose and then

prepared as above. A standard curve was prepared by plotting values for relative mobility (R_m) against molecular weight (Weber and Osborn, 1967). R_m is defined as:

$$R_m = \frac{\text{distance of protein migration}}{\text{length of gel after destaining}} \times \frac{\text{length of gel before staining}}{\text{distance of dye migration}}$$

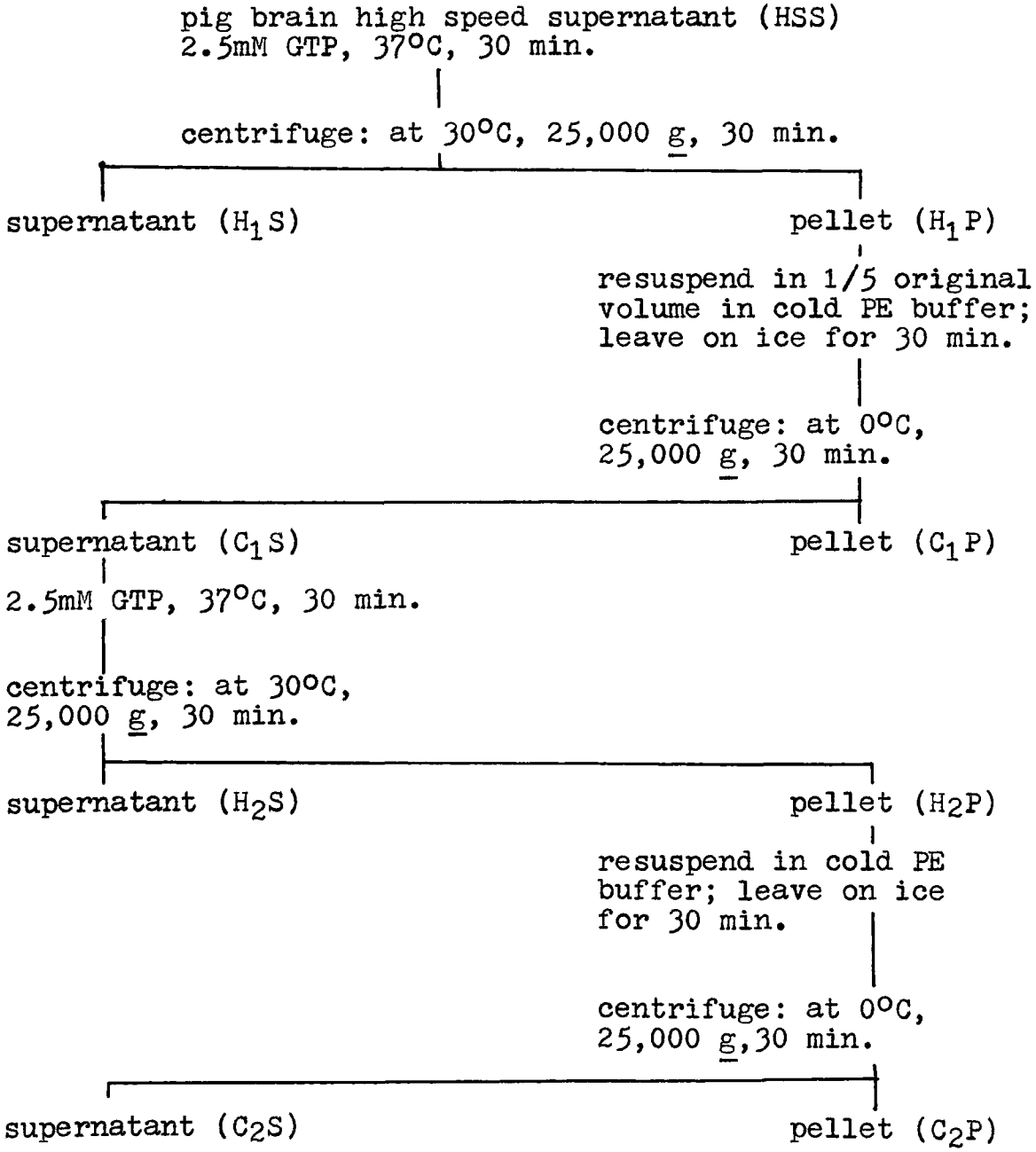
3. Gel Scans: Gels were scanned with a Vitatron LD 100 densitometer (O.H. Johns Scientific Ltd., Montreal, P.Q.) with a linear transport mechanism at 3 cms./min. with a narrow slit aperture at 546 nm.

PIG BRAIN HOMOGENATES

Pigs' heads were obtained within sixty minutes of slaughter. Brains were removed, cortical tissue was dissected clean of white matter, minced, and washed twice with either cold 0.1M PIPES, 1mM EGTA, pH 6.9 (PE buffer; Borisy and Olmsted, 1972b) or cold 20mM sodium phosphate, 100mM sodium glutamate, pH 6.8 (PG buffer; Bryan and Wilson, 1971). One part of minced brain tissue (gram wet weight) plus one volume of buffer was then homogenized on ice with a motor driven teflon pestle in a glass homogenizer tube. Homogenates were centrifuged at 5,000 g for 10 minutes to remove large fragments and unbroken cells. The resultant supernatant was centrifuged at 25,000 g for one hour. This supernatant is designated as pig brain high speed supernatant.

PURIFICATION OF PIG BRAIN MICROTUBULE PROTEIN BY CYCLES OF POLYMERIZATION AND DEPOLYMERIZATION

Pig brain high speed supernatants in PE buffer were made 2.5mM GTP (General Biochemicals) and incubated at 37°C. The appearance of microtubules was monitored by negative staining. Tubulin was purified by two cycles of polymerization and depolymerization (Borisy et al., 1974). This procedure is outlined in the following diagram.



PURIFICATION OF PIG BRAIN MICROTUBULE PROTEIN BY COLUMN CHROMATOGRAPHY

Pig brain microtubule protein could also be purified in a one-step procedure on DEAE-Sephadex (Bryan and Wilson, 1971). High speed supernatants in PG buffer were applied directly to 2.4 x 7.0 cm. columns of DEAE Sephadex A-50 which had been pre-equilibrated with 100mM NaCl, 20mM $NaPO_4$, pH 6.8. The sample was allowed to run into the column and then eluted succesively with 15 mls. of 100mM NaCl, 20mM $NaPO_4$, pH 6.8, 50 mls. of 400mM NaCl, 20mM $NaPO_4$, pH 6.8, and an excess of 800mM NaCl, 20mM $NaPO_4$, pH 6.8. Columns were eluted at a flow rate of approximately 15 drops per minute and 40 drop fractions (2.4 mls.) were collected on an LKB fraction collector. Sample preparation and column chromatography were carried out at 4^oC.

^{3}H-COLCHICINE BINDING TO PIG BRAIN MICROTUBULE PROTEIN

^{3}H-colchicine was obtained from New England Nuclear in benzene-ethanol at a concentration of 2×10^{-4}M, and a specific activity of 5.0 curies/mM. A stock solution of colchicine was prepared by taking one part ^{3}H-colchicine plus ten parts PG buffer. The benzene-ethanol was removed under a stream of N_2, reducing the ^{3}H-colchicine concentration to 2×10^{-5}M. One part stock colchicine solution plus seven parts pig high speed supernatant in PG buffer were mixed giving a final colchicine concentration of 2.5×10^{-6}M (Bryan and Wilson, 1971). This sample was incubated at 37^oC

for two hours, placed on ice for 5 minutes and then applied directly to a DEAE Sephadex A-50 column and eluted as above.

^{3}H-colchicine binding was assayed by taking 0.1 ml. aliquots from each fraction into 10 mls. Aquasol (New England Nuclear) and counting in a Beckman liquid scintillation counter. Protein profiles were monitored at 280nm in a Gilford spectrophotometer (Model 2400) with digital absorbance. For quantitative protein determinations, measurements were done by the method of Lowry, and samples were reduced and alkylated for urea gels.

POLYTOMELLA HOMOGENATES

Cells grown in batch cultures were harvested and deflagellated by the procedures described. Cell bodies were washed once in fresh media, and once in cold buffer, either PE buffer, PG buffer, or 67mM KPO_4, 100mM KCl, pH 6.8 (PC buffer; Borisy et al., 1972c). Bodies were then resuspended in approximately 0.1% original culture volume, and a homogenate was prepared by either sonicating with a Branson sonicator (Branson Instruments, Danbury, Conn.) with pre-cooled microtip, at number 4 setting for 30 seconds, or by breaking the cell bodies in a French pressure cell (American Instrument Co., Silver Spring, Md.) at 16,000 psi. Homogenates were centrifuged at 12,000 g for 15 minutes, and the resulting supernatant was centrifuged at 25,000 g for 60 minutes. This supernatant is designated as Polytomella high speed supernatant.

AGGREGATION OF POLYTOMELLA MICROTUBULE PROTEIN

Polytomella high speed supernatants in PE or PC buffer were made 2.5mM GTP, and incubated at 37°C. Samples were examined by negatively-stained grids. Microtubule protein was monitored by centrifuging the sample, after four hours at 37°C, at 25,000 g (30 min. at 30°C) and preparing pellet and supernatant fractions for analysis by polyacrylamide gel electrophoresis. In all cases the presence of microtubule protein was monitored using both purified flagellar microtubule protein and pig brain microtubule protein as standards.

PURIFICATION OF POLYTOMELLA MICROTUBULE PROTEIN BY COLUMN CHROMATOGRAPHY

Polytomella microtubule protein could be partially purified in a one-step procedure on DEAE Sephadex. High speed supernatants in PG buffer were applied to DEAE Sephadex A-50 columns, allowed to run in, and eluted as described.

^{3}H-COLCHICINE BINDING TO POLYTOMELLA MICROTUBULE PROTEIN

1. Columns: One part stock ^{3}H-colchicine was mixed with seven parts Polytomella high speed supernatant, incubated at 37°C for two hours, placed on ice for 5 minutes and then applied to a DEAE A-50 column and eluted as above. Binding and protein profiles were also obtained in a similar fashion.

2. Disc Method: The method used is that of Borisy (1972a) developed for assaying colchicine binding in pig brain. Filter stacks of Whatman DE81 Chromedia paper were prepared by wetting four filters at a time, and letting them dry overnight at 60°C. Five mls. of either PC or PG buffer were added to a small Millipore Filter apparatus. A sample aliquot (usually 0.2 ml.) was then added and five more mls. of buffer were added. Suction was then applied and the filter stack was washed with four 10 ml. changes of buffer, allowed to run dry, blotted, and placed in a scintillation vial with 10 mls. of Aquasol. After 24 hours, the samples were counted.

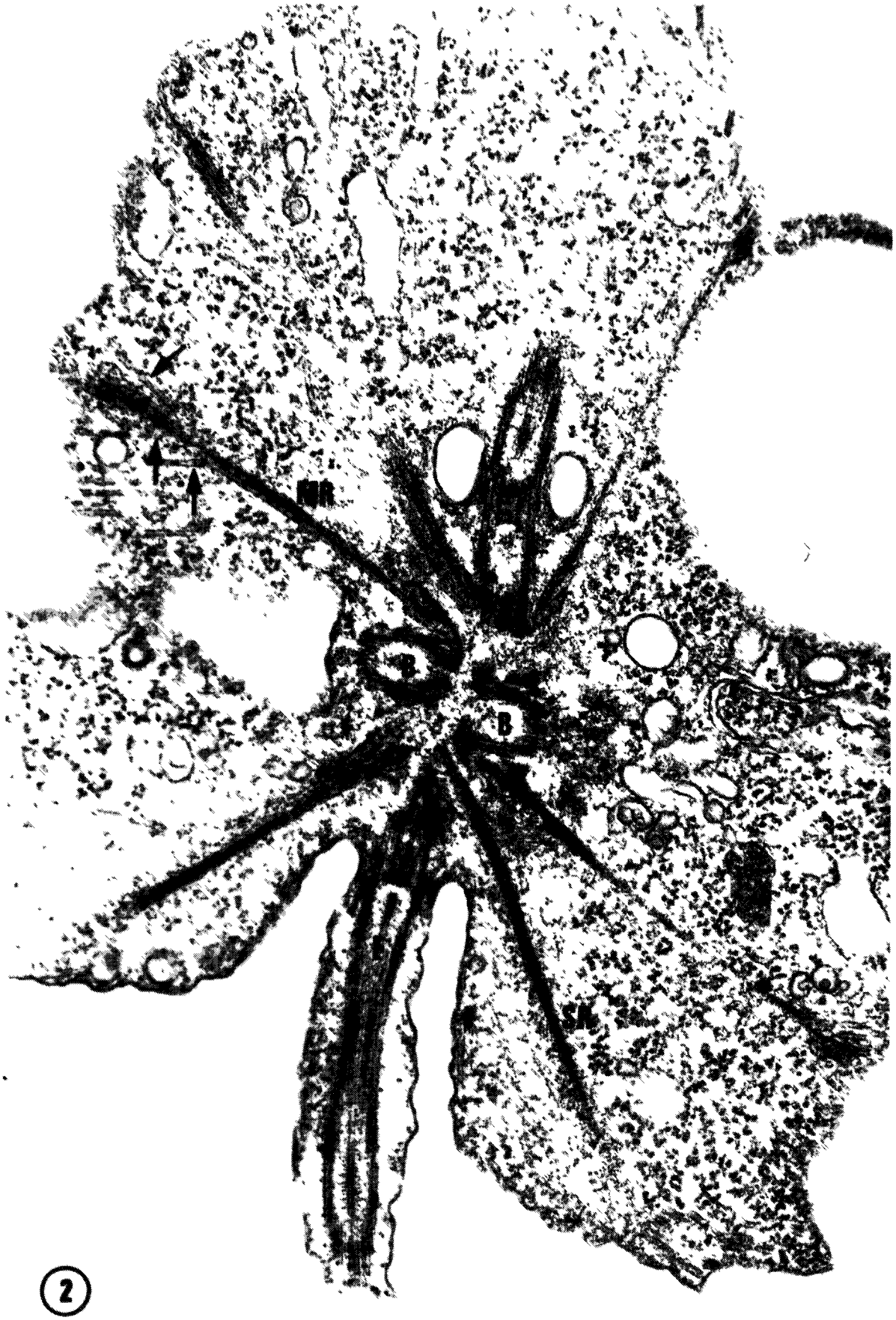
RESULTS

OBSERVATIONS ON WHOLE CELLS

Polytomella agilis exists in both a free swimming vegetative form and as a non-motile cyst. In exponentially growing cultures, virtually all cells are of the free swimming type. These cells are elongate, being about 12μ in length, and about 7μ in width. The cell is a quadriflagellate and the flagella emerge from depressions at the base of an anterior papilla. The distribution of microtubules in *Polytomella* has been described in detail elsewhere (Patenaude, 1974), and is presented briefly here to emphasize the extent of the microtubular systems.

Fig. 2 is a cross section just below the papillar region of the cell. The four basal bodies are organized into opposite pairs, termed an A pair and a B pair. Between adjacent basal bodies, there are two structurally different rootlet fibers. One of these consists of a striated fiber lying over two tubules and is designated as the striated rootlet. The other rootlet consists of four tubules in a 3 over 1 pattern and is called the microtubular rootlet. Large numbers of cytoplasmic microtubules appear to terminate on both of these rootlets (fig. 2). These microtubules extend to the cell's posterior forming the cytoplasmic microtubule network, shown diagramatically in fig. 4.

Fig. 2 Cross section just below the papillar region of the cell, showing the arrangement of basal bodies (B) and associated striated (SR) and microtubular (MR) rootlets. Cytoplasmic microtubules terminate (arrows) on these rootlet fibers. F, flagellum. x 49,500.



(2)

Fig. 3a Longitudinal section through the anterior papilla (P) of the cell, showing the flagellum (F) in longitudinal section, and its attachment to the basal body (B). x 63,000.

Fig. 3b Cross section of a flagellum, still attached to a cell, showing 9 outer doublets surrounding the 2 central pair tubules. Each doublet consists of an A tubule with dynein arms (projecting clockwise in this photograph) and an incomplete B tubule, sharing a common wall, or partition, with the A tubule. x 132,000.

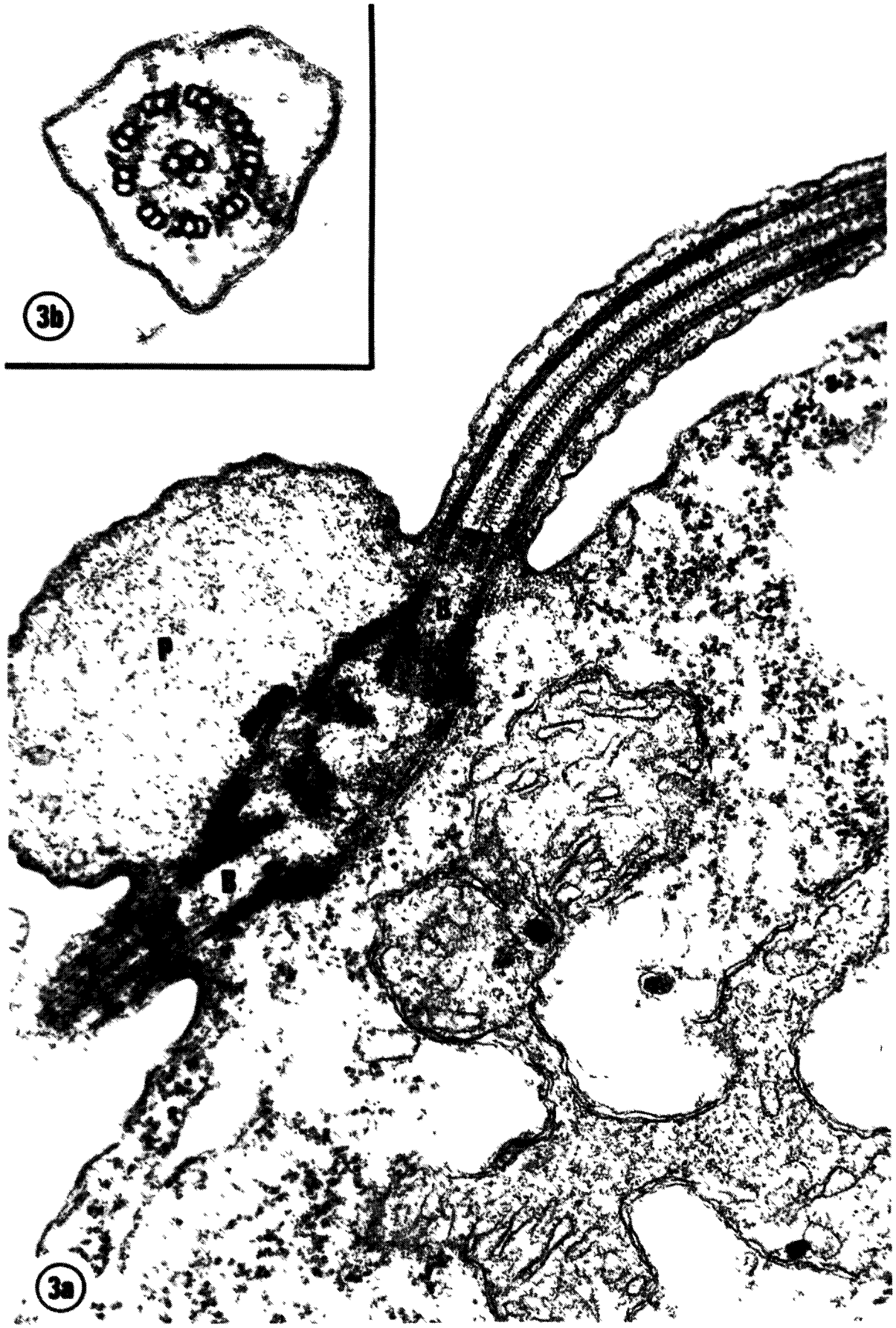

3b
3a

Fig. 4 Diagrammatic representation of the cytoplasmic microtubule system in Polytomella. The flagella, and their basal bodies, are organized into opposite pairs (A + B). Between each pair of adjacent basal bodies is a striated rootlet and a microtubular rootlet.

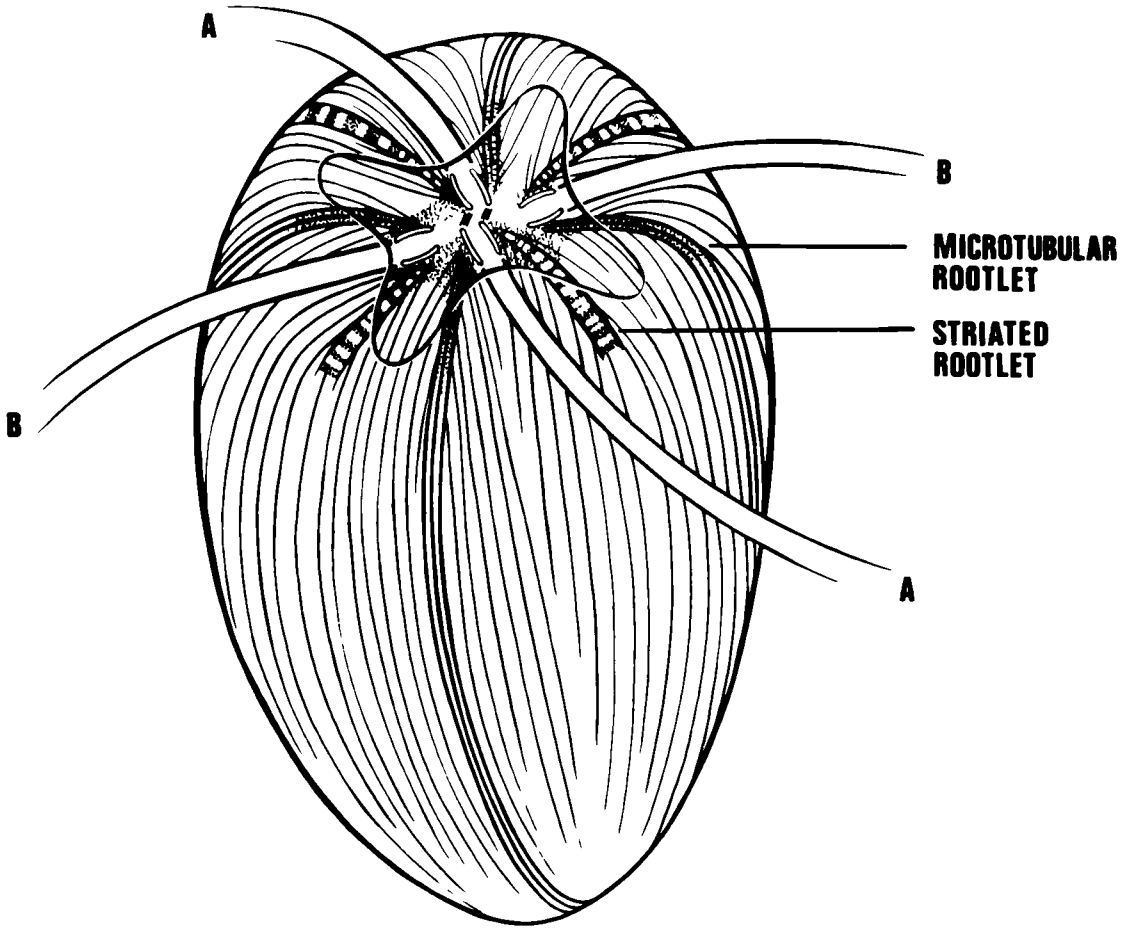

A
B
MICROTUBULAR ROOTLET
STRIATED ROOTLET
B
A

Fig. 3a is a longitudinal section through the anterior end of the cell, showing insertion of the flagella below the papillar region. The transition region between the flagellar axoneme and the basal body is evident. The inset (fig. 3b) is a cross section of a flagellum still attached to a cell, showing the characteristic 9+2 arrangement of tubules (i.e. 9 outer doublets surrounding 2 central pair tubules). Each doublet consists of a complete A tubule with dynein arms (projecting in a clockwise direction in this photograph), and an incomplete B tubule, sharing a common wall, or partition, with the A tubule.

FLAGELLAR DETACHMENT AND ISOLATION

1. Mechanical Deflagellation: Mechanical deflagellation represents a very rapid and effective method for removing flagella from Polytomella. Deflagellation was always greater than 99%; however, some cell breakage was also seen. Axonemes from flagella prepared in this manner could be purified by repeated separations on discontinuous sucrose gradients, thus providing an effective microtubule protein standard. However, this method resulted in the loss of significant numbers of flagella.

2. Chemical Deflagellation: To obtain very pure preparations of flagella for subsequent electrophoretic analysis, TEEP deflagellation was used. When cells were treated with TEEP, most flagella fell off quickly. Some cells seem to be paralysed, while still retaining one or

two flagella; however, these usually fell off with stirring. Deflagellation was greater than 95%, and in contrast to mechanical deflagellation, there was very little cell breakage. Fig. 5a shows a typical field of TEEP-deflagellated cells containing cell bodies and detached flagella. Flagella could be purified as described, and these seemed straight and intact when viewed with Nomarski optics (fig. 5b). Flagella are stable in TEEP for several hours after deflagellation.

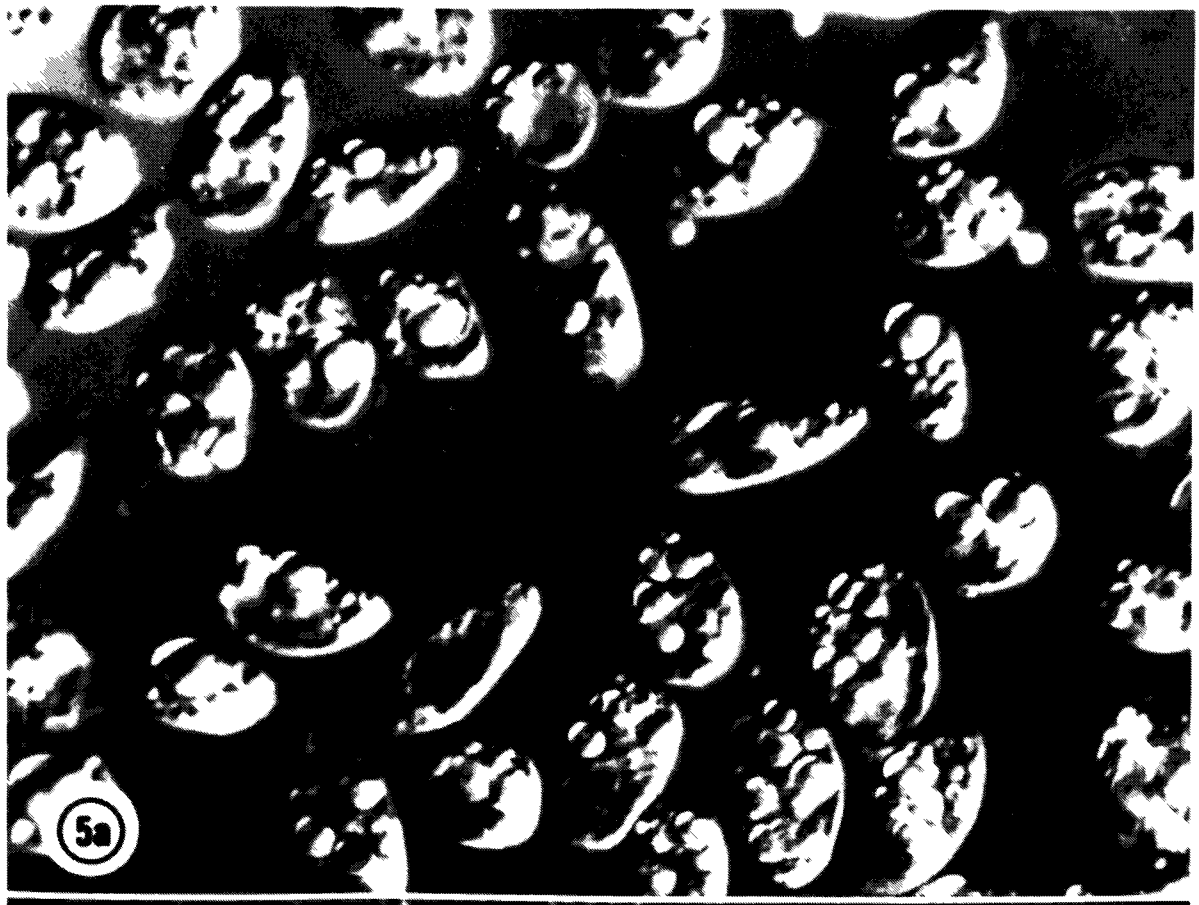
Fig. 6a is a thin section through a pellet of purified flagella. Flagellar axonemes and membranes are present with little contaminating material. In this particular field, the flagellar membrane is ruptured in most cases, and one of the central pair tubules missing. This does not appear to result from rupture of the membrane, since sections of flagella in which the membrane is intact also show a loss of one or both of the central pair tubules (fig. 6b). In flagella prepared by the TEEP method, there is a shrinkage of the matrix in which flagellar microtubules are embedded in comparison to that seen in sections of flagella from whole cells (fig. 3b).

ISOLATION OF AXONEMES

When flagella, resuspended in 1mM Tris, were mixed with Tris containing 0.02% Non-Idet P-40, there was an immediate decrease in the viscosity and turbidity of the solution. This treatment removed flagellar membranes,

Fig. 5a Nomarski micrograph of TEEP deflagellated cells showing cell bodies and detached flagella. x 3,000.

Fig. 5b Nomarski micrograph of purified flagella prepared by the TEEP deflagellation method. x 5,000.


5a

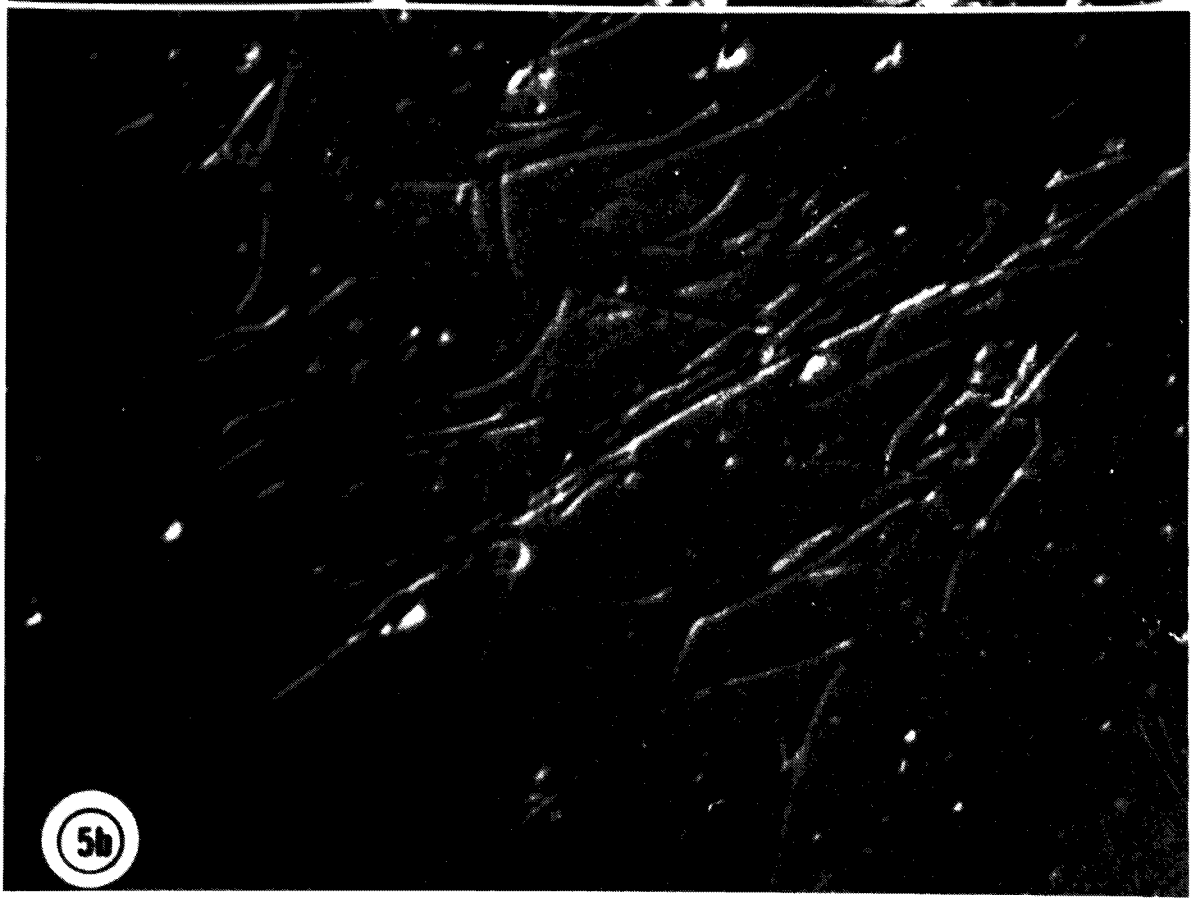

5b

Fig. 6a Thin section through a pellet of purified flagella. Note that the flagellar membrane is present, although it is ruptured in most cases, and one or both of the central pair tubules are lost. Flagella were prepared by the TEEP deflagellation method. x 51,000.

Fig. 6b High magnification cross section of an isolated flagellum, showing an intact flagellar membrane and loss of the central pair tubules. x 204,000.

6a
6b

leaving intact and partially intact axonemes. These axonemes could be pelleted through 40% sucrose, leaving the membranes as a whitish band at the 5%/40% interface. When the pelleted axonemes are examined by negative staining, intact and partially intact axonemes, with little contamination, are observed (fig. 7). If this pellet is fixed and sectioned, a small amount of contaminating membrane is seen. However, most of the pellet consists of intact axonemes, consisting of 9 outer doublets and one or both of the central pair tubules (figs. 8a, 8b).

ELECTROPHORETIC ANALYSIS OF AXONEMES

1. Urea Gels: Purified axonemes were prepared for electrophoresis on 7.5% polyacrylamide gels containing 8M urea. Electrophoretic analysis in this system revealed two closely spaced bands (fig. 9a) of approximately equal staining intensity. Very little other contaminating protein is seen on the gel. The slower moving of these two proteins has been designated α-tubulin, the faster moving β-tubulin (after the nomenclature of Bryan and Wilson, 1971). A densitometric tracing of this gel (fig. 9a) shows that these two bands account for greater than 90% of the protein present on the gel. No differences in electrophoretic patterns were seen between flagellar microtubule protein prepared by mechanical or chemical deflagellation.

Fig. 7 Electron micrograph of negatively stained axonemes after removal of flagellar membranes with Non-Idet P-40. x 27,000.


1

Fig. 8a Thin section through a pellet of purified axonemes after removal of flagellar membranes with Non-Idet P-40. In most cases, 9 outer doublets and one or both of the central pair tubules are seen. x 51,000.

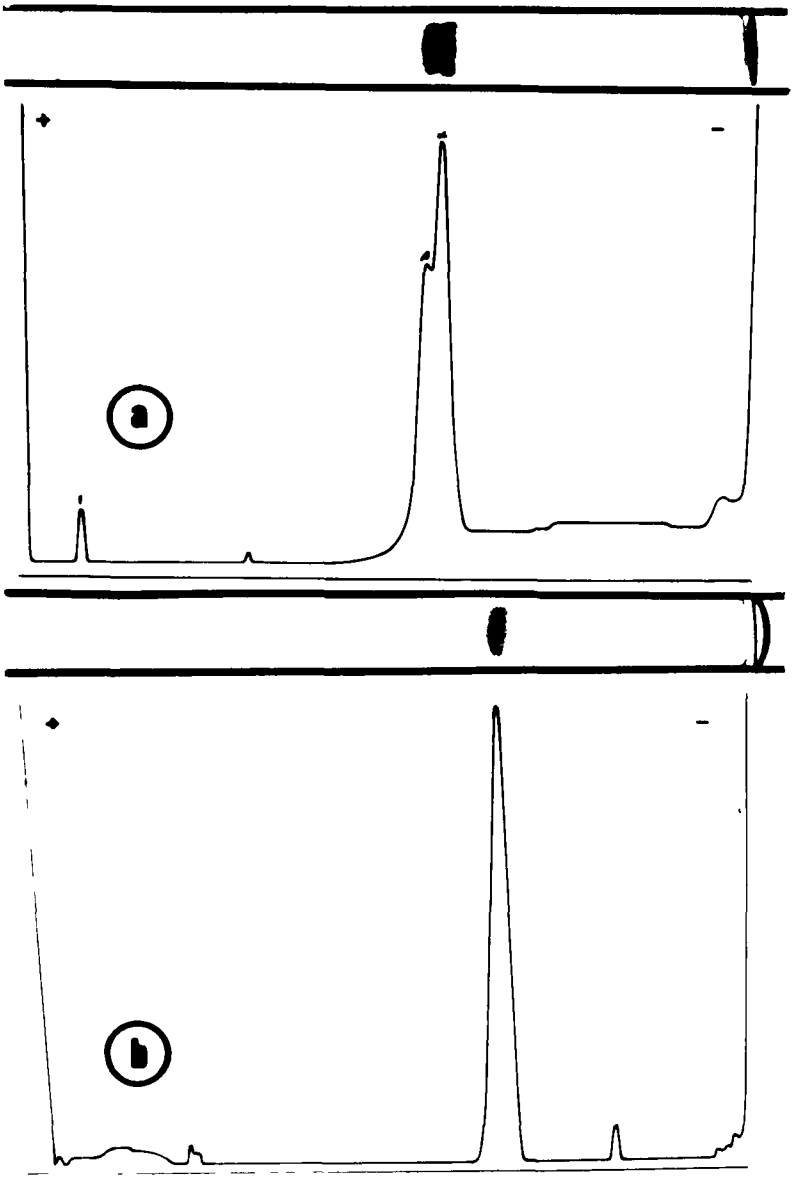
Fig. 8b High magnification cross section of an isolated axoneme, showing nine outer doublets and one of the central pair tubules. x 204,000.

8a
8b

Fig. 9a Gel and gel tracing of purified axonemal microtubules*. The two main bands seen are α- and β-tubulin. Gel is urea-containing 7.5% polyacrylamide.

Fig. 9b Gel and gel tracing of purified axonemal microtubules. In the SDS system, α- and β-tubulin run as a single band. Gel is SDS-containing 10% polyacrylamide.

*In this and all subsequent gel tracings, the top of the gel is denoted by a - sign, the bottom of the gel by a + sign; f indicates dye front; α and β indicate α- and β-tubulins.


a
b

2. SDS Gels: Purified axonemes were also prepared for electrophoresis on 10% polyacrylamide gels containing 0.1% SDS. Electrophoretic analysis in this system revealed most of the protein migrating as a single densely staining band. Few other contaminants are seen on the gel (Fig. 9b). A densitometric tracing of this gel reveals one major protein peak accounting for greater than 90% of the protein present on the gel.

The molecular weight of the tubulin monomer in this system was determined by comparison to a set of known standards. A standard plot is represented in fig. 10. When one places the R_m value for flagellar tubulin on this slope, it gives a monomer molecular weight of 57,000.

IN VITRO ASSEMBLY OF PIG BRAIN MICROTUBULES

If a pig brain high speed supernatant in PE buffer is maintained at 0^oC, or if no GTP is added, microtubules do not form. However, upon addition of 2.5mM GTP and incubation at 37^oC, microtubules appear within minutes. By thirty minutes incubation time, the process seem complete and masses of assembled microtubules are seen (fig. 11a). These tubules appear to be of normal morphology and when measured are 250-260 A^o in diameter (fig. 11b).

Microtubules formed in vitro are cold labile and this property may be used to obtain pig brain microtubule protein in a pure form by cycles of polymerization and depolymerization. This procedure is outlined in the following diagram.

Fig. 10 Standard curve for molecular weight determinations by SDS gel electrophoresis (Weber and Osborn, 1969). Standard proteins are (from top of slope): bovine serum albumin, catalase, ovalbumin, chymotrypsinogen A, cytochrome C. Relative mobilities for purified pig brain microtubule protein, and purified flagellar microtubule protein from Polytomella are indicated.

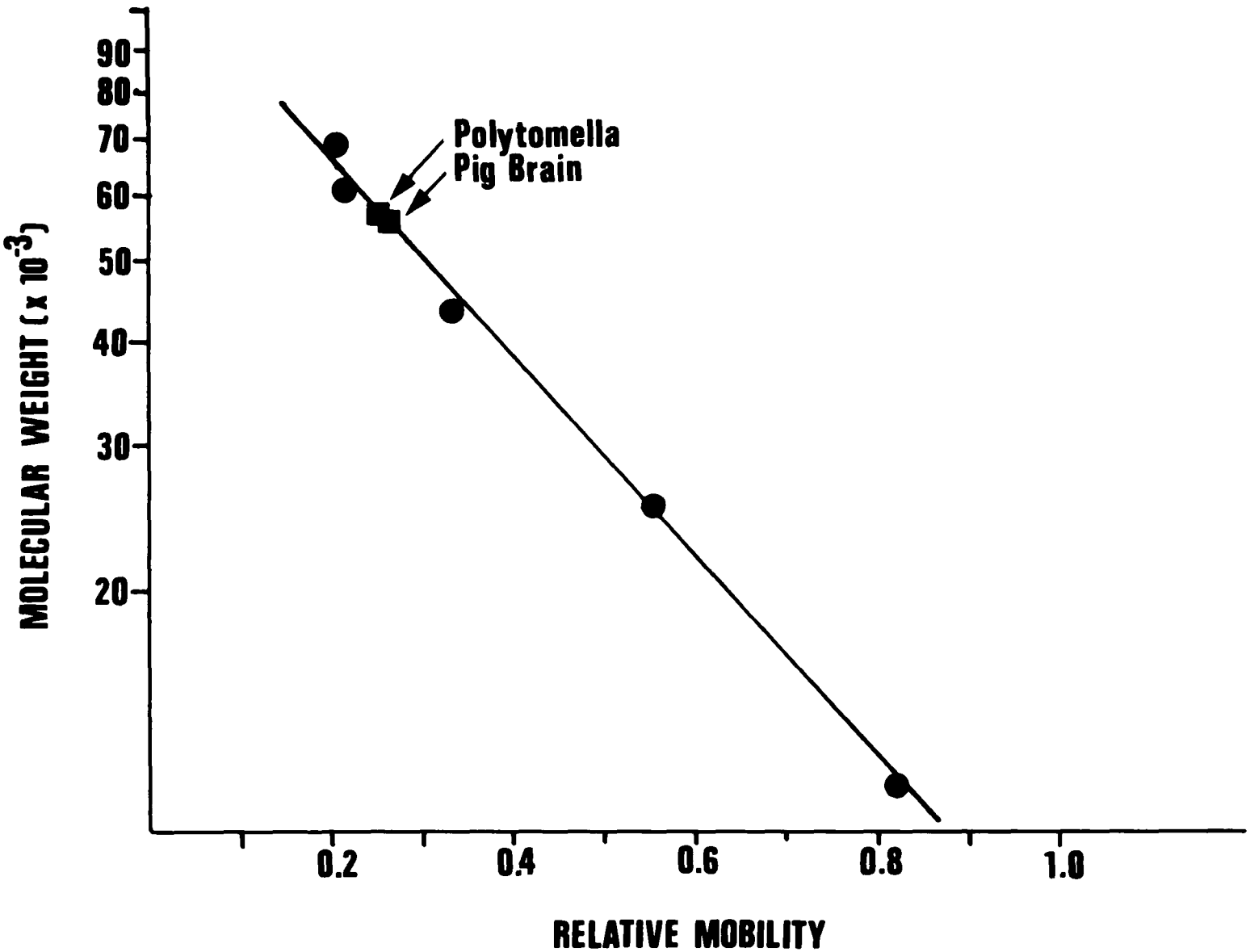
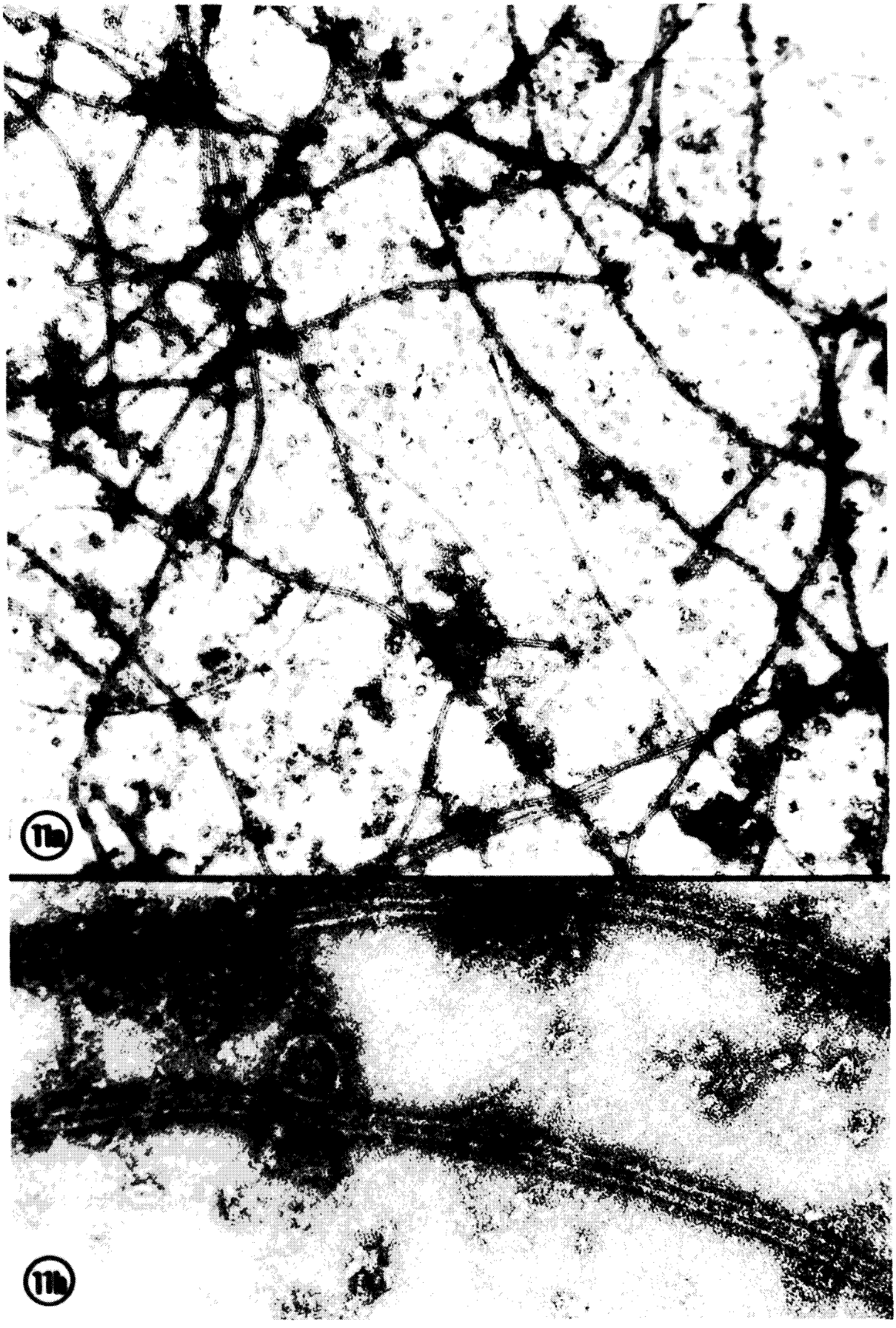
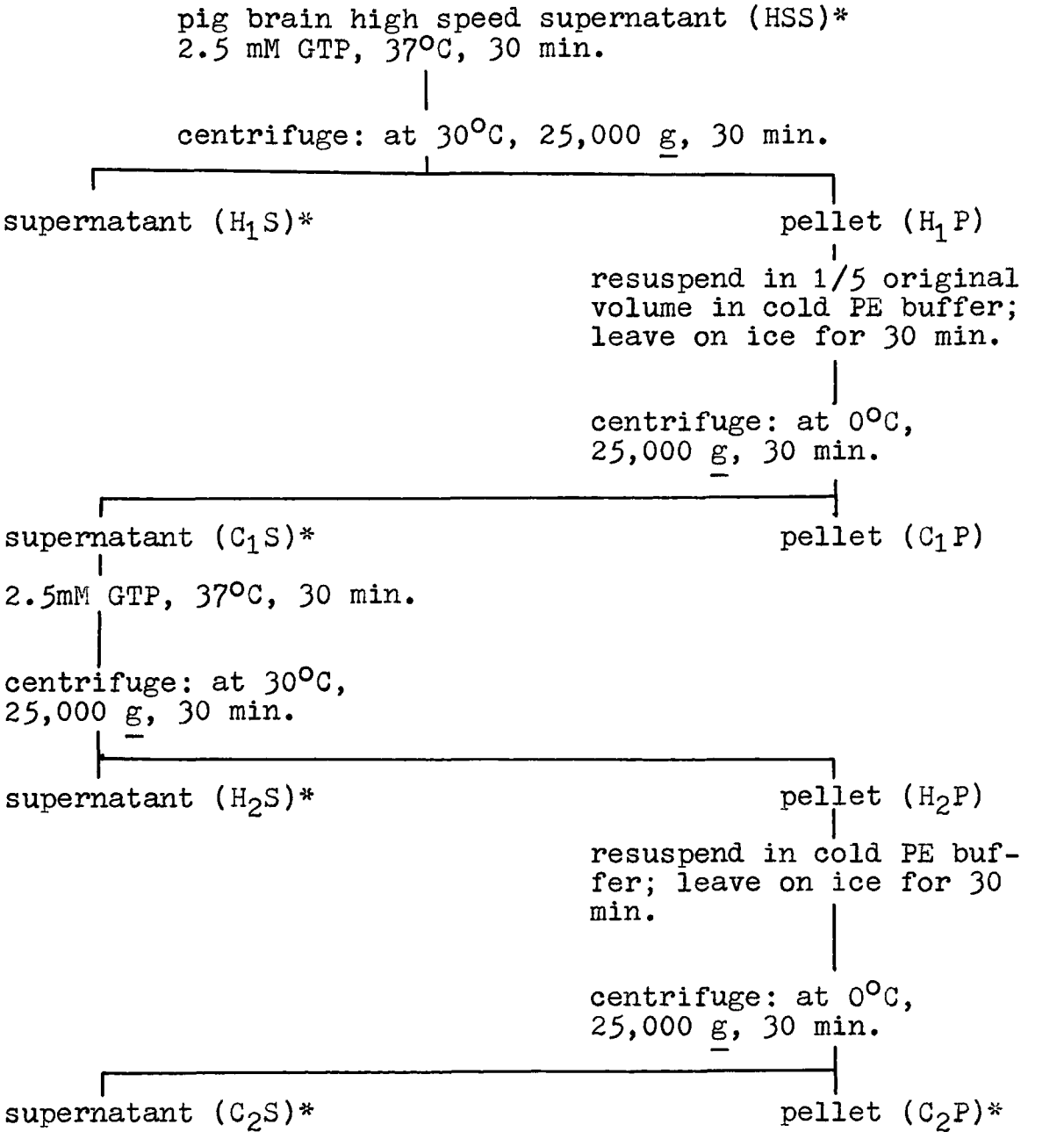

MOLECULAR WEIGHT (x 10^{-3})
90
80
70
60
50
40
30
20
Polytomella
Pig Brain
0.2
0.4
0.6
0.8
1.0
RELATIVE MOBILITY

Fig. 11a Field of negatively stained pig brain microtubules prepared by the in vitro polymerization method. x 25,500.

Fig. 11b Higher magnification view of repolymerized pig brain microtubules. x 80,000.


11a
11b



*Samples are prepared for analysis of SDS containing polyacrylamide gels.

It is important to note that in preparing microtubules in this fashion, and in purification by cyclic polymerization and depolymerization, the tubulin concentration in the solution must be maintained at greater than 1 mg./ml. (Olmsted and Borisy, 1973b). Below this concentration, very few microtubules will form in vitro.

Figs. 12 and 13 represent an electrophoretic analysis of samples prepared from this procedure. Figs. 12a and 12b show gels and gel tracings of pig high speed supernatant (HSS), and the supernatant obtained after first repolymerization (H_1S). In both fractions, although many other bands are seen, tubulin represents the major protein on the gel. The first hot pellet obtained (H_1P) was resuspended on ice, causing assembled microtubules to depolymerize. Microtubular aggregates and other insoluble material were removed by centrifugation in the cold (giving fraction C_1P which was discarded). The supernatant (C_1S) shows one major peak corresponding to microtubule protein (fig. 12c). C_1S was then made 2.5mM GTP and incubated at 37°C. After 30 minutes, polymerized material was pelleted. The supernatant (H_2S) contains unpolymerized tubulin and a number of other contaminating proteins (fig. 13a). The pellet, which contained cold-labile microtubules, was then cold solubilized and centrifuged at 0°C to give fractions C_2P and C_2S (figs. 13b, 13c). C_2S, as seen in fig. 13c, consists of 85-90% tubulin, with one major band of high molecular weight. This high molecular weight material consistently co-purifies with microtubule protein in this procedure.

The densely staining band obtained from this purification procedure for pig brain microtubules (fig. 13c) approximately co-migrates in SDS gels with the single band obtained with flagellar microtubule protein from Polytomella.

Fig. 12a-c (a) Gel and gel tracing of pig brain high speed supernatant (HSS). (b) Gel and gel tracing of pig brain supernatant after first repolymerization (H_1S). (c) Gel and gel tracing of first repolymerized pellet, cold solubilized (C_1S). In all cases, tubulin is the most prominent band on the gel. All gels are SDS-containing 10% polyacrylamide.

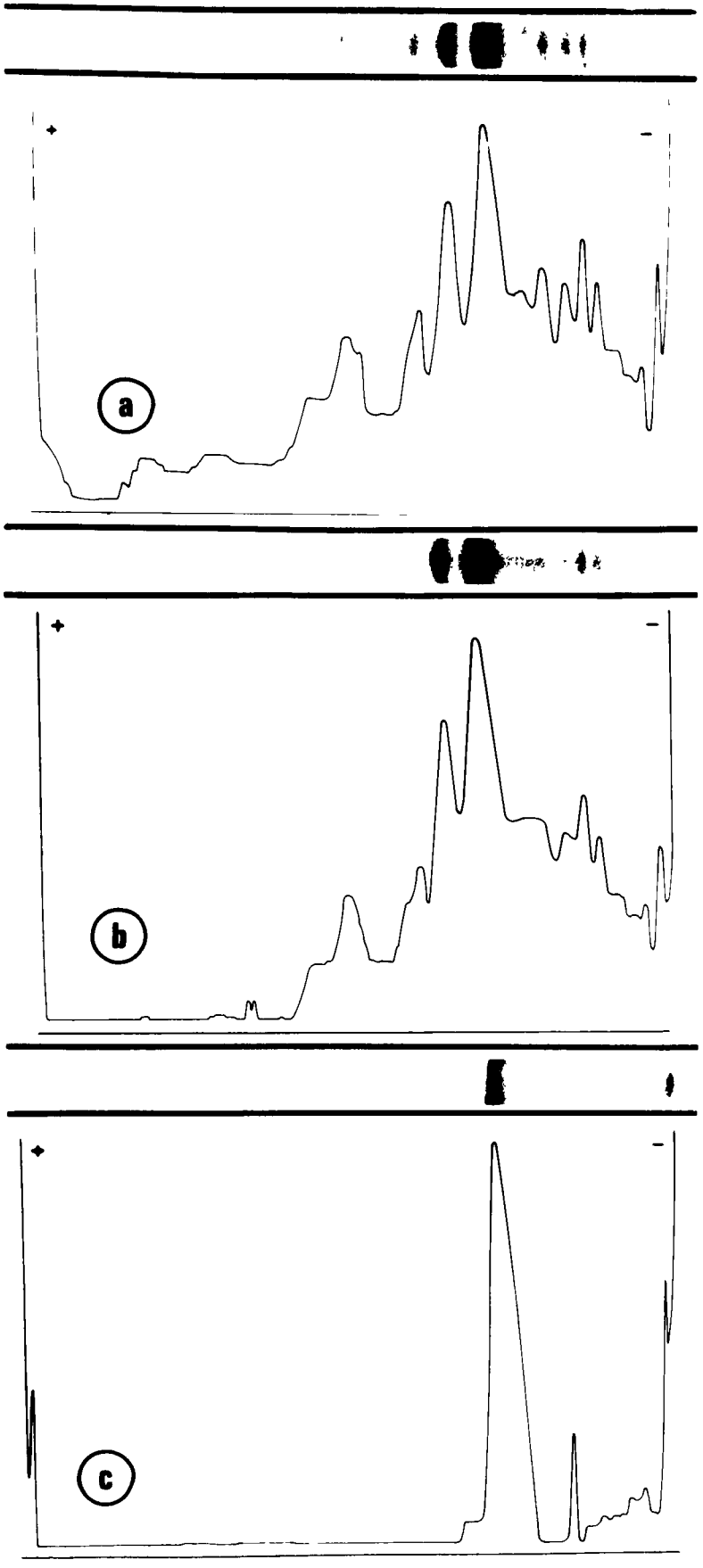
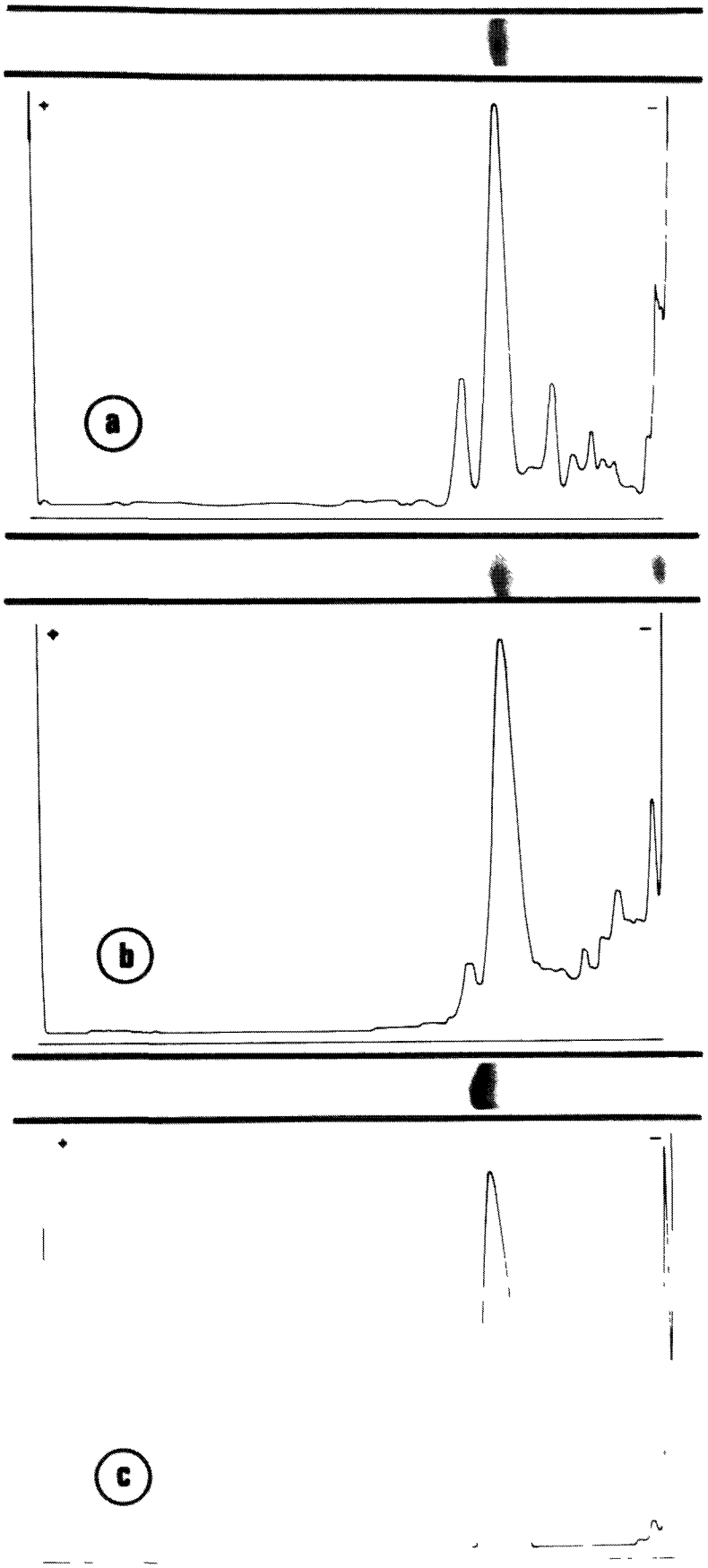

a
b
c

Fig. 13a-c (a) Gel and gel tracing of supernatant after second repolymerization (H_2S). (b) Gel and gel tracing of final pellet fraction obtained after pellet H_2P is cold solubilized and centrifuged at 0°C (C_2P). (c) Gel and gel tracing of supernatant after pellet H_2P is cold solubilized (C_2S). In fraction C_2S, tubulin represents 85-90% of the protein on the gel, and the only contaminant seen is a high molecular weight protein which co-purifies with tubulin. All gels are SDS-containing 10% polyacrylamide.


a
b
c

From the standard curve, a monomer molecular weight of 55,000 was assigned to pig brain microtubule protein (fig. 10).

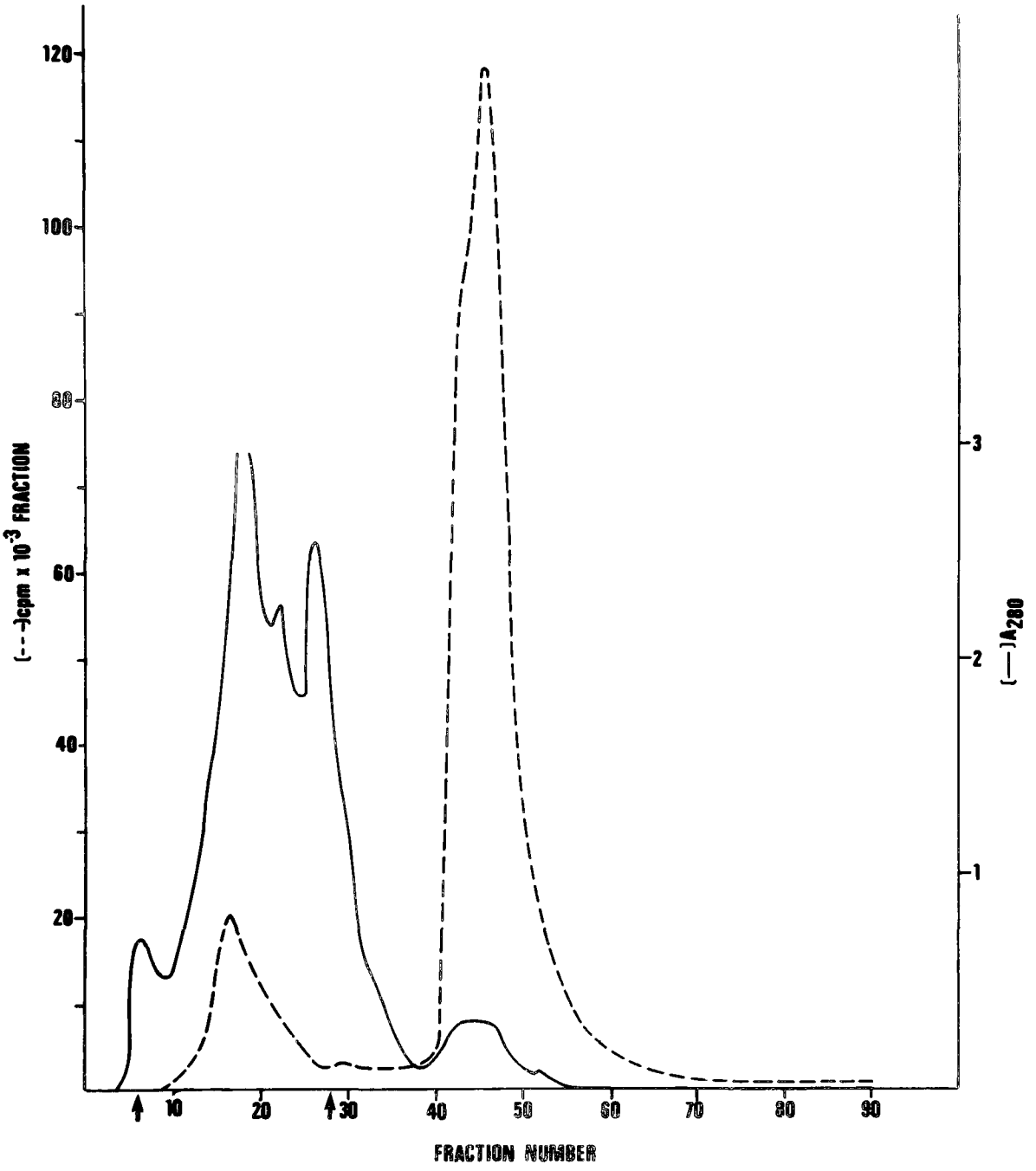
In urea containing polyacrylamide gels, pig brain tubulin approximately co-migrates with flagellar microtubule protein; however, only one band is seen (fig. 15a). If gels are run for twice the normal length of time, the resultant band becomes much more diffuse, but two bands could not be resolved. Varying the amount of protein applied to the gel did not affect this result.

PURIFICATION OF PIG BRAIN MICROTUBULE PROTEIN BY COLUMN CHROMATOGRAPHY

Pig brain microtubule protein can also be purified in a one step column chromatography procedure on Sephadex DEAE A-50. Pig high speed supernatants in PG buffer, incubated with 2.5×10^{-6}M ^{3}H-colchicine, were cooled on ice, applied to columns of DEAE A-50 and eluted at 4°C. This purification for pig brain tubulin is represented in fig. 14.

After addition of 800mM NaCl, colchicine binding protein elutes from the column as a single peak. The bound colchicine peak is approximately six times greater than the unbound peak, and this peak coincides with a broad protein peak. Fraction 46 of this protein peak (maximum value for bound colchicine), and pooled fractions 41-50 (the entire protein peak), were reduced and

Fig. 14 Purification of microtubule protein from pig brain on 2.4 x 7.0 cm. Sephadex DEAE A-50 column. A 25,000 g supernatant was incubated with 2.5 x 10^{-6}M colchicine for 2 hours at 37°C. 4 mls. of this mixture was cooled to 0°C, applied to the column, and 40 drop fractions (2.4 mls.) were collected at a flow rate of 15 drops/min. Arrows indicate approximate points at which eluting salt concentration was changed. Protein was eluted successively with 15 mls. of 100mM NaCl, 50 mls. of 400mM NaCl, and an excess of 800mM NaCl. All elution buffers were 20mM $NaPO_4$, pH 6.8. Dashed line, radioactivity; solid line, absorbance at 280 nm.


120
100
80
60
40
20
(- - -)cpm x 10^-3 FRACTION
3
2
1
(—)A280
10
20
30
40
50
60
70
80
90
FRACTION NUMBER

alkylated for analysis of urea gels. The results are seen in figs. 15c and 15b, respectively.

When either of these fractions is analysed on urea containing polyacrylamide gels, they are seen to contain one major band, with very little contaminating protein. This protein (possessing significant colchicine binding activity) co-migrates with pig brain tubulin obtained in the previous procedure and with flagellar microtubule protein, although once again only one band is seen. Note that the high molecular weight species purifying with tubulin in the cyclic polymerization procedure (fig. 13c) is not seen when tubulin is purified on DEAE Sephadex columns (fig. 15c).

AGGREGATION OF POLYTOMELLA MICROTUBULE PROTEIN

In all cases where aggregation of microtubule protein was examined, cell bodies from manually deflagellated cells were used in order to avoid interfering effects of ethanol. When a Polytomella high speed supernatant (HSS) in PE or PC buffer is maintained at 0°C, no aggregation occurs and the solution remains clear. When this fraction is analysed on urea containing polyacrylamide gels, many bands are seen (fig. 16a). In contrast to a pig high speed supernatant, where tubulin represents the major band on the gel, no bands could be identified as tubulin on the basis of co-migration with flagellar microtubule protein. Upon incubation at 37°C, the solution quickly becomes

Fig. 15a Gel and gel tracing of fraction C_2S obtained from pig brain repolymerization procedure. Gel is urea-containing 7.5% polyacrylamide.

Fig. 15b Gel and gel tracing of pooled fractions 41-50 (see fig. 14) obtained from fractionation of pig brain high speed supernatant on Sephadex DEAE A-50. Gel is urea-containing 7.5% polyacrylamide.

Fig. 15c Gel and gel tracing of fraction 46 (maximum colchicine binding value) obtained from fractionation of pig brain high speed supernatant on Sephadex DEAE A-50. Gel is urea-containing 7.5% polyacylamide.

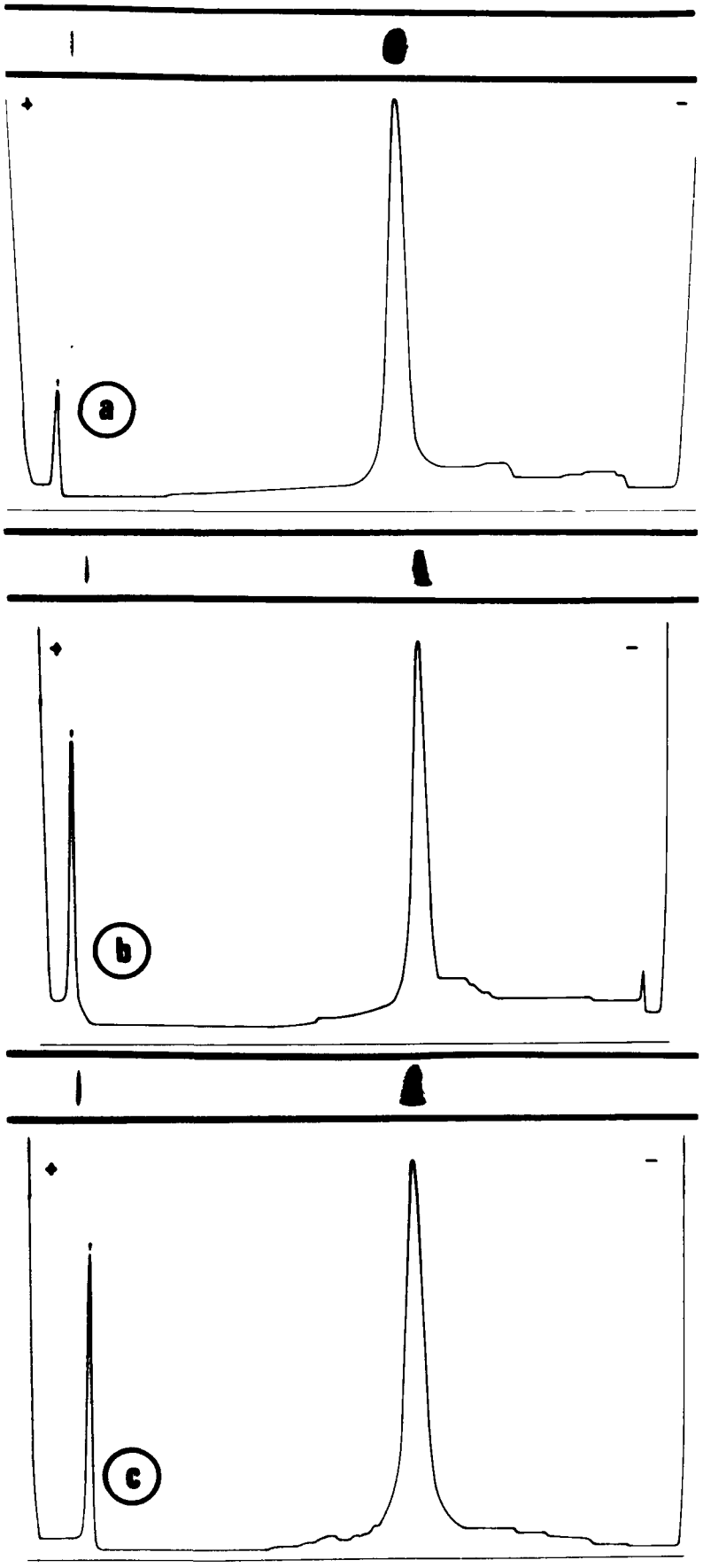
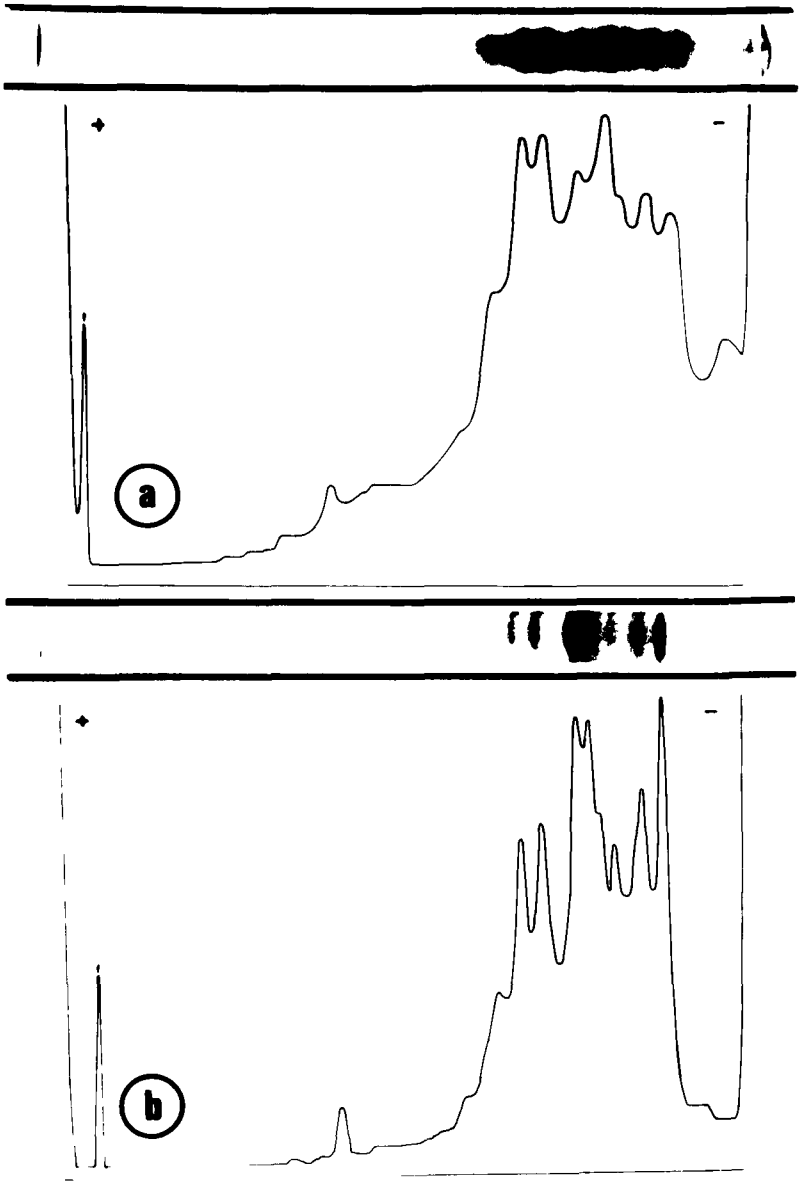

a
b
c

Fig. 16a-b (a) Gel and gel tracing of Polytomella high speed supernatant (HSS). (b) Gel and gel tracing of supernatant fraction H_1S obtained after an in vitro polymerization procedure with Polytomella high speed supernatant. In both gels, major bands are not seen in the area where purified flagellar microtubule protein runs (compare with fig. 17a). Gels are urea-containing 7.5% polyacrylamide.


a
b

turbid and by four hours is opaque in appearance. If this change is monitored with negatively stained grids, no microtubules are seen at any time. After four hours, this sample is centrifuged at 30°C to give a pellet fraction (H_1P) and supernatant fraction (H_1S). When fraction H_1S is analysed on urea containing gels (fig. 16b), several bands are seen, but these are all at the top of the gel, and no bands are seen in the area where flagellar microtubule protein would run. If fraction H_1P is examined (fig. 17a)*, two bands are seen which co-migrate with purified flagellar microtubule protein. If this pellet fraction H_1P is examined on SDS containing gels, a major band is seen which co-migrates with flagellar microtubule protein (fig. 17b).

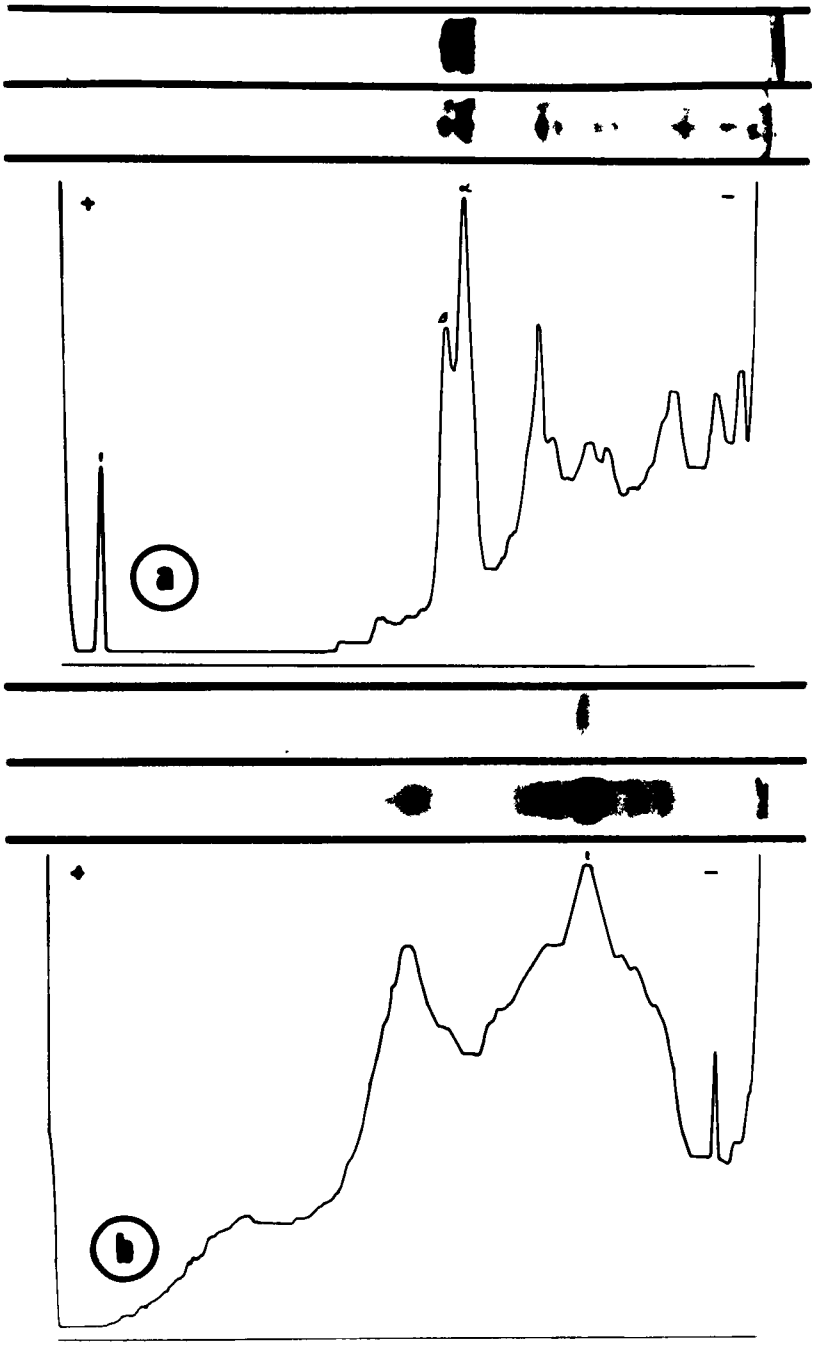
Though no morphologically distinguishable microtubules are seen, there seems to be an aggregation of microtubule protein by this procedure. The next step was to determine if this aggregation of protein was sensitive to the same agents as the in vitro assembly of pig brain microtubules.

1. Cold Lability: After four hours, Polytomella high speed supernatants were placed on ice for 30 minutes and then centrifuged at 0°C. Alternatively, pellet fraction H_1P was resuspended (or resuspended with homogenization)

*Although there is no definitive evidence that these bands are tubulin, they have been labelled α and β on the basis of co-migration with purified flagellar microtubule protein.

Fig. 17a Gel and gel tracing of pellet fraction H_1P obtained after in vitro polymerization with Polytomella high speed supernatant. Two major bands are present, which co-migrate with α- and β-tubulins from purified axonemes, as seen in the upper gel. Gel is urea-containing 7.5% polyacrylamide.

Fig. 17b Gel and gel tracing of pellet fraction H_1P. Many more proteins are seen on the gel, but a major band is observed which co-migrates with purified flagellar microtubule protein, as seen in the upper gel. Gel is SDS-containing 10% polyacrylamide.


a
b

in either cold PE or PC buffer, left on ice for 60 minutes, and then centrifuged at 0°C. The resultant pellet and supernatant fractions were examined on urea containing polyacrylamide gels.

2. Nucleotide Dependency: GTP was added to Polytomella high speed supernatants at concentrations of 0, 1.0, and 2.5mM. After four hours incubation at 37°C, pellet fractions (H_1P) and supernatant fractions (H_1S) were analysed on urea containing gels.

3. Colchicine Sensitivity: Polytomella high speed supernatants were made 100μM in colchicine (a concentration which inhibits formation of microtubules in vitro in pig brain homogenates; Borisy et al., 1974). After four hours incubation at 37°C, fractions H_1S and H_1P were analysed on urea containing gels.

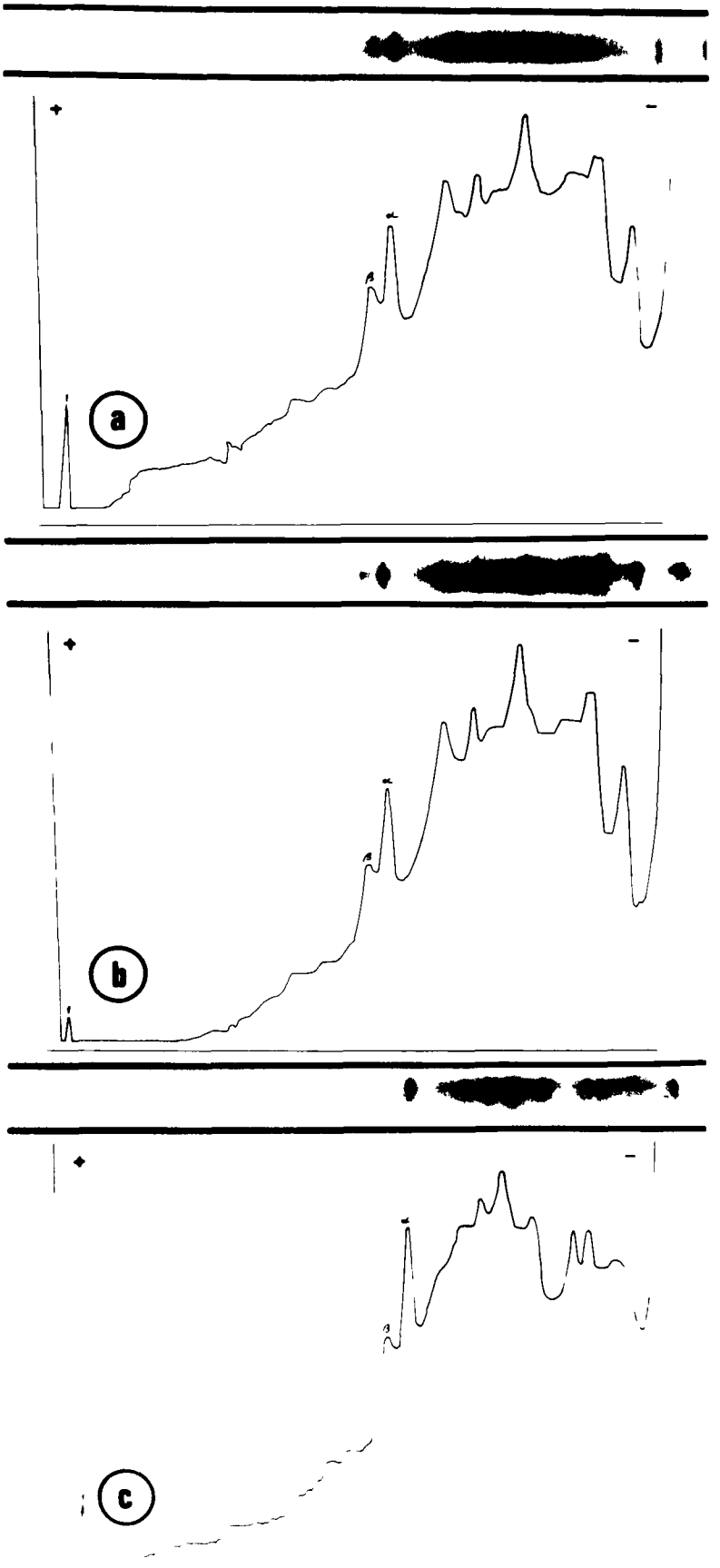
In each sample examined, pellet fraction H_1P was seen to contain our presumptive cytoplasmic microtubule protein (figs. 18a, 18b, 18c, respectively).

These results indicate there is a non-specific aggregation of Polytomella cytoplasmic microtubule protein, utilizing a pig brain in vitro repolymerization system.

REMOVAL OF POLYTOMELLA MICROTUBULE PROTEIN FROM PELLET FRACTION H_1P

Pellet fraction H_1P was subjected to three treatments (known to solubilize microtubule protein in other systems) in an attempt to specifically solubilize microtubule protein.

Fig. 18a-c (a) Gel and gel tracing of final pellet fraction after pellet fraction H_1P is cold extracted. (b) Gel and gel tracing of pellet H_1P from an in vitro polymerization with Polytomella in which no exogenous GTP was added. (c) Gel and gel tracing of pellet H_1P from an in vitro polymerization with Polytomella in the presence of 100 M colchicine. In each case, proteins which co-migrate with purified flagellar microtubule protein are labelled α and β. Gels are urea-containing 7.5% polyacrylamide.


a
b
c

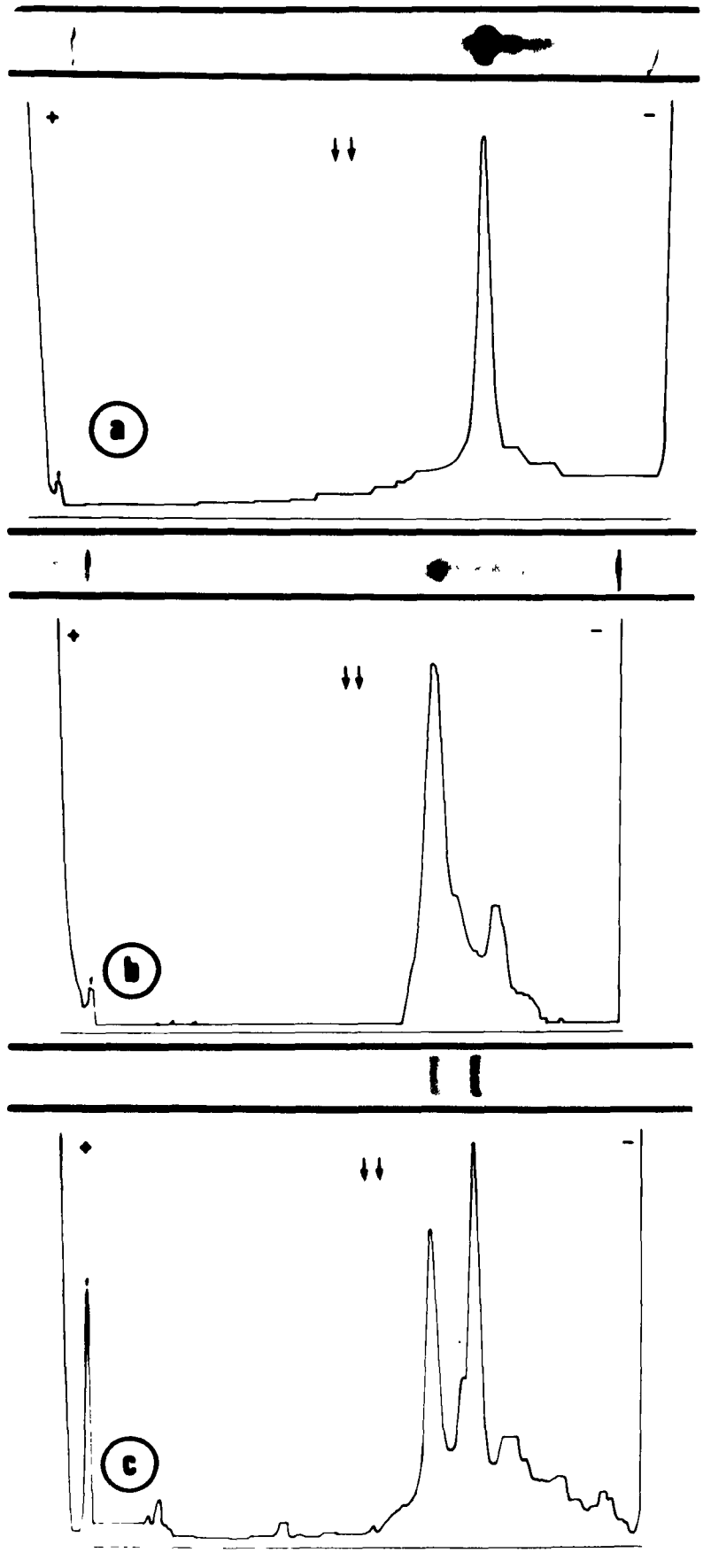
1. Sucrose-Tris-EDTA: Pellet H_1P was homogenized in cold 0.24M sucrose, 10mM Tris, 1mM EDTA, pH 7.0 (known to solubilize microtubule protein from Neuroblastoma; Olmsted et al., 1970), placed on ice for 60 minutes, and then centrifuged. Pellet and supernatant fractions were analysed on urea containing gels.

2. Sodium Phosphate-Sodium Glutamate: Pellet H_1P was homogenized in 20mM sodium phosphate, 100mM sodium glutamate, pH 6.8, and heated in a water bath at 50°C for seven minutes (will solubilize flagellar outer doublet microtubules; Wilson et al., 1972). The sample was centrifuged and pellet and supernatant fractions were analysed on urea containing gels.

3. Tris-Mercaptoethanol: Pellet H_1P was homogenized in 1mM 2-Mercaptoethanol, 10mM Tris, pH 7.5, and dialysed for 18 hours at 4°C against 1mM 2-Mercaptoethanol, 10mM Tris, 0.1mM EDTA, pH 7.5 (will solubilize spindle microtubules from Arbacia punctulata; Fulton et al., 1971b).

When supernatant fractions from each procedure were analysed, in no case were any bands seen on the gel where flagellar microtubule protein runs (Figs. 19a, 19b, 19c, respectively). When final pellet fractions were analysed after extraction, the profile observed was very similar to that obtained for fraction H_1P, and in all cases still contained our presumptive cytoplasmic microtubule protein.

Fig. 19a-c Gels and gel tracings of supernatants after pellet H_1P is subjected to various extraction treatments. (a) Sucrose-Tris-EDTA. (b) Sodium phosphate/sodium glutamate. (c) Tris-Mercaptoethanol. In all cases, arrows indicate where α- and β-tubulins run on corresponding standard gels. Gels are urea-containing 7.5% polyacrylamide.


a
b
c

PURIFICATION OF POLYTOMELLA MICROTUBULE PROTEIN BY COLUMN CHROMATOGRAPHY

A crude purification of Polytomella cytoplasmic microtubule protein could be achieved in a one step column chromatography procedure on Sephadex DEAE A-50. Polytomella high speed supernatants in PG buffer were applied to a column of DEAE A-50 and eluted at 4°C. The protein profile of this purification is represented in fig. 20.

After addition of 800mM NaCl, a broad peak is seen in the elution profile (fig. 20). Fraction 49 (binding peak) and pooled fractions 41-50 (containing entire peak) were reduced and alkylated for urea gels. Results are shown in figs. 21a and 21b.

There are a number of highly acidic proteins which run ahead of microtubule protein on the gel, and very little behind it. These bands represent a small fraction of the protein on the gel, but do co-migrate with flagellar microtubule protein.

^{3}H-COLCHICINE BINDING TO POLYTOMELLA MICROTUBULE PROTEIN

In an attempt to demonstrate that colchicine binding protein (microtubule protein) was contained in fraction H_1P, several colchicine binding assays were tried.

1. Columns: Samples of Polytomella HSS were incubated with 2.5×10^{-6}M colchicine, and then applied to a DEAE A-50 column and eluted as described. The binding profile from this elution is represented in fig. 20. There is a

Fig. 20 Purification of microtubule protein from Polytomella on 2.4 x 7.0 cm. Sephadex DEAE A-50 column. A 25,000 g supernatant was incubated with 2.5 x 10^{-6}M colchicine for 2 hours at 37°C. 4 mls of this mixture was colled to 0°C, applied to the column, and 40 drop fractions (2.4 mls.) were collected at a flow rate of 15 drops/min. Arrows indicate approximate points at which eluting salt concentration was changed. Protein was eluted successively with 15 mls. of 100mM NaCl, 50 mls. of 400mM NaCl, and an excess of 800mM NaCl. All elution buffers were 20mM $NaPO_4$, pH 6.8. Dashed line, radioactivity; solid line, absorbance at 280 nm.

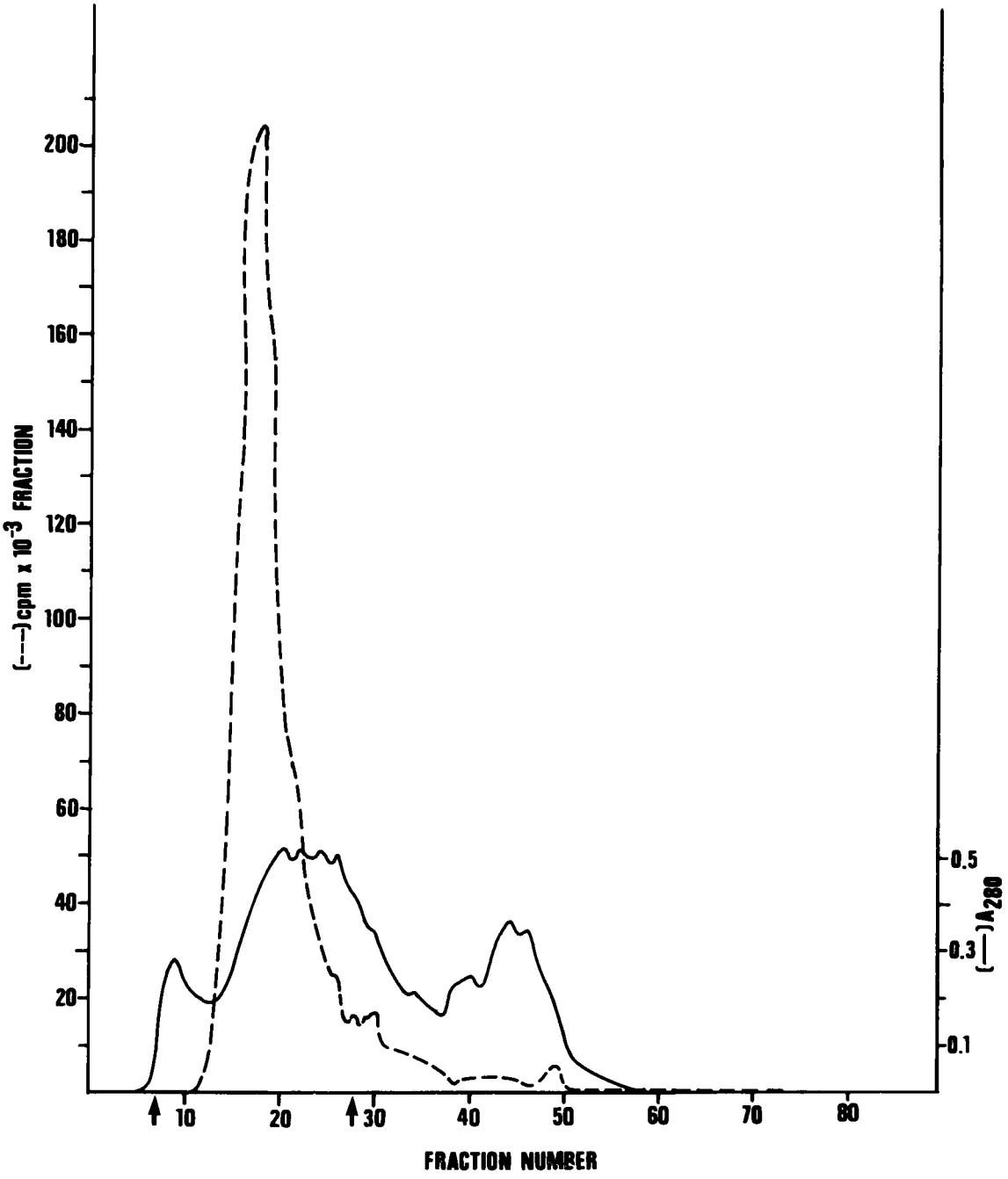
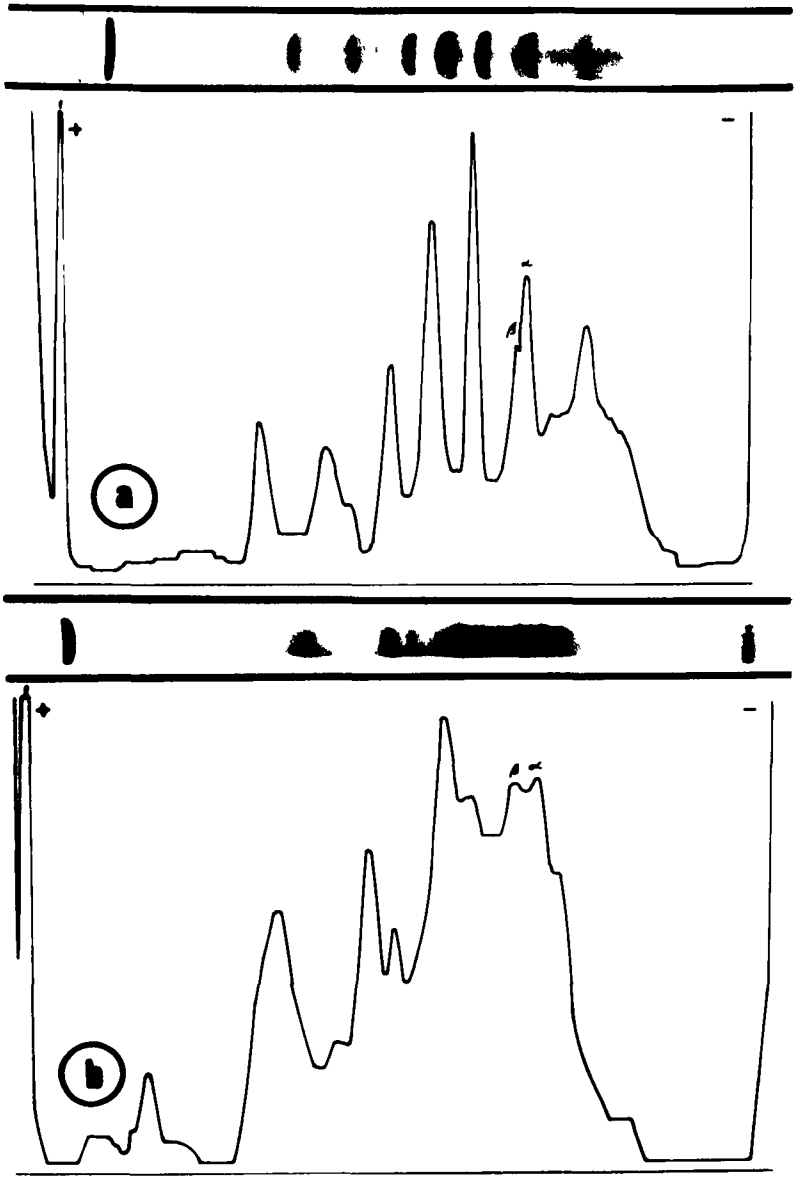

(---)cpm x 10^-3 FRACTION
200
180
160
140
120
100
80
60
40
20
0.5
0.3
0.1
(—)A280
10
20
30
40
50
60
70
80
FRACTION NUMBER

Fig. 21a Gel and gel tracing of fractions 41-50 (see fig. 20) obtained from fractionation of Polytomella high speed supernatant on Sephadex DEAE A-50. Gel is urea-containing 7.5% polyacrylamide.

Fig. 21b Gel and gel tracing of fraction 49, obtained from fractionation of Polytomella high speed supernatant on Sephadex DEAE A-50. This procedure has selectively solubilized the more acidic proteins, including 2 which co-migrate with purified flagellar microtubule protein. Gel is urea-containing 7.5% polyacrylamide.


a
b

small peak of bound colchicine that elutes under the broad protein peak containing microtubule protein. The ratio of unbound to bound colchicine in this preparation is on the order of 40:1.

Similarly, Polytomella high speed supernatants could be incubated with ^{3}H-colchicine and applied to pre-equilibrated columns of Sephadex G-100. When the elution profile was examined, a bound colchicine peak was seen to co-migrate with the broad protein peak coming off the column. This reached a maximum binding value of 565 cpm. An unbound colchicine peak, with a maximum binding value of over 400,000 cpm eluted at the bed volume of the column.

2. Discs: Homogenate fractions HSS, H_1P, and H_1S (in PG or PC buffer) were assayed in duplicate by the filter disc assay method. This was done both on a single time-point basis (Table I), and at three time points post incubation in order to obtain a zero-time binding value (Fig. 22). By taking binding values at three time points, it should be possible to extrapolate back to zero time and obtain a binding efficiency there. In order to do this a straight line should be seen for a slope. This is not the case (Fig. 22). It does seem to be significant, however, that with both methods used, and in all experiments run, fraction H_1P had the highest value for colchicine binding.

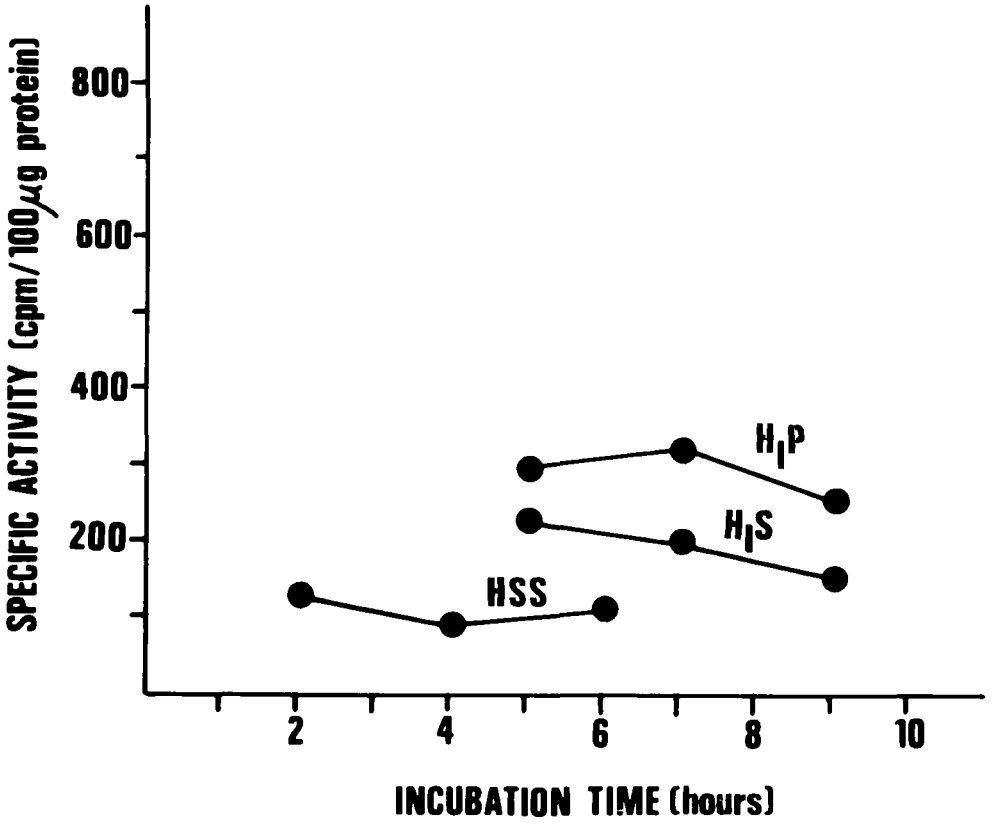
Table I Table I represents ^{3}H-colchicine binding in homogenate fractions of Polytomella. Each fraction was first obtained and subsequently incubated with $2.5 \times 10^{-6}M$ colchicine at $37^{o}C$ for 2 hours. Colchicine binding protein was assayed by the filter disc assay method in duplicate (see text for details).

Fig. 22 ^{3}H-colchicine binding values were obtained at three time points. High speed supernatant (HSS) from Polytomella was incubated with $2.5 \times 10^{-6}M$ colchicine, incubated at $37^{o}C$, and sampled at 2, 4, and 6 hours. Portions of this high speed supernatant were centrifuged to give H_1S and H_1P samples at 5, 7, and 9 hours. All colchicine binding assays were done in duplicate by the filter disc assay method.

TABLE I

^{3}H-COLCHICINE BINDING
IN HOMOGENATE FRACTIONS OF
POLYTOMELLA

FRACTION	SPECIFIC ACTIVITY (cpm/100μg protein)
HSS	1,589
H_1S	3,304
H_1P	10,557



DISCUSSION

One of the major questions raised in the Introduction to this thesis was whether the differential lability of microtubules reflected direct differences in protein composition or was due to other factors which promote the stability of certain microtubules, or certain parts of microtubules.

Roth et al. (1970) hypothesized that the differential lability of microtubules or segments of microtubules may be accounted for by stabilizing structural elements linking subunits of one microtubule to other microtubules or cellular structures. They subsequently showed that the microtubule arrangement in the heliozoan Echinosphaerium nucleofilum could be disrupted by the use of nickel or copper ions (Roth and Shigenaka, 1970), or dilute urea treatments (Shigenaka et al., 1971). On the basis of electron microscopic observations, they state that high lability of microtubules is correlated with reduced numbers of linkers between the microtubules.

There is additional ultrastructural evidence for this hypothesis. Hopkins (1970) showed that in axonemes from Chlamydomonas flagella, the more stable of the central pair tubules had two rows of longitudinal projections, while the more labile tubule had only one. Similarly, the radial spokes which are directed into the center of the axoneme from the outer doublets, are attached to the

A tubule, which is more stable than the B tubule.

The differential sensitivity of sets of microtubules has also been hypothesized to be due to dissimilarities in associations or connections between tubules. Tilney and Gibbins (1968c) showed differential sensitivities of axonemal and cytoplasmic microtubules to hydrostatic pressure, calcium free sea water, and D_2O.

A conflicting interpretation, based on similar results was presented by Behnke and Forer (1967; see Introduction). They proposed four classes of microtubules: A tubules, B tubules, the central pair tubules, and cytoplasmic tubules, differing in stability to a variety of treatments. They postulated these differences were due to different constituent elements (i.e. microtubule proteins) composing the microtubules. Kiefer (1970) has studied a naturally occurring degradation pattern in sperm axonemes in a sterile Drosophila mutant. He observed that central pair, A and B tubules were degraded in a specific sequence, and suggested that this was best explained by compositional differences among various flagellar microtubules.

This suggestion is supported by Witman's results (1972a). Using iso-electric focusing on urea-containing gels, he could resolve outer doublet microtubule protein of Chlamydomonas into five major bands. It is possible that each of these proteins forms a unique part of the

axonemal microtubules, and it is the differences in these proteins themselves which account for observed differences in tubule stability.

ANALYSIS OF FLAGELLAR MICROTUBULES

Methods are reported for the isolation and purification of flagella from Polytomella. Flagella can be isolated intact, and are essentially identical to flagella on cells, with two exceptions: 1) a shrinkage of the embedding matrix material; and 2) a loss of one or both of the central pair tubules of the flagellum. Isolated flagella had a much smaller cross-sectional diameter than flagella on cells, and in most cases the flagellar membrane remained intact. As noted above, the central pair tubules have been shown to be more sensitive than other tubules of the flagellum. This sensitivity may account for loss of the central pair tubule(s) during the lengthy isolation and fixation period required for Polytomella.

A method is also reported for the removal of the flagellar membrane using the non-ionic detergent Non-Idet P-40, and a subsequent separation into axonemal and membrane components. The axonemes are essentially intact, but once again there may be loss of the central pair tubules. Solubilization or partial solubilization of the B tubules is sometimes seen, leaving intact A tubules, or A tubules with a partial B tubule attached. The A tubules are the most resistent to detergent treatment.

Witman (1972a) has observed a similar pattern of microtubule sensitivity to detergents in flagella isolated from Chlamydomonas. He found that, with increasing Sarkosyl (another non-ionic detergent) concentrations, axonemal microtubules solubilized in the following order: one of the two central tubules; the other central tubule and the outer wall of the B tubule; the remaining portion of the B tubule; and then the A tubule with the exception of the three "partition" protofilaments.

Electrophoretic analysis of flagellar microtubules of Polytomella in urea-containing polyacrylamide gels reveals two closely spaced bands, similar to the pattern seen for flagellar microtubule protein from a variety of organisms. When the gel is scanned these two bands account for almost all of the protein present on the gel. Scans also reveal a consistent alpha to beta tubulin ratio of 1.1-1.2 to 1. When gels stained with fast green (a dye reported to give quantitative protein staining; Gorovsky et al., 1970), similar ratios were obtained.

To explain this ratio, several arguments must be examined. It may simply be explained on the basis of there being more alpha than beta tubulin in outer doublets. However, this is at variance with a heterodimer model, a model in which there is an equi-distributional pattern of alpha and beta tubulins throughout the microtubule (ref. fig. 1). Witman et al.(1972c) have presented a homodimer model for flagellar outer doublet microtubules (fig. 23).

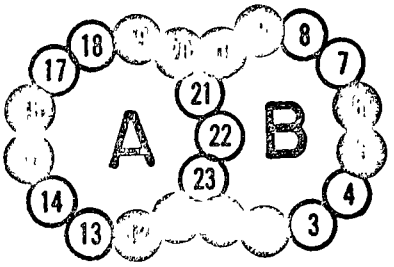


Fig. 23 A homodimer model for the distribution of α-tubulin (white subunits) and β-tubulin (black subunits) in outer doublets (from Witman et al., 1972c).

In solubilizing outer doublets they were able to obtain homogeneous preparations of the three partition protofilaments and subsequent electrophoretic analysis showed them to be composed of only alpha tubulin. In my preparations, the partial loss of central pair and B tubules would produce an enrichment of the partitions, producing an increased alpha to beta tubulin ratio, as predicted by the homodimer model.

However, the homodimer-heterodimer conflict has not been resolved. Meza et al.(1971) isolated the partition protofilaments from sea urchin flagellar doublets and showed them to be composed of alpha and beta tubulins in equal proportions. This is taken as strong evidence for a heterodimer model.

When flagellar microtubules were analysed on SDS-containing polyacrylamide gels, only one band was observed. Such a gel system separates proteins, for the most part, on the basis of their molecular weights. This would then indicate that alpha and beta tubulin are of the same molecular weight, but have different charges, and so different amino acid compositions. By SDS gel electrophoresis, a molecular weight of 57,000 was assigned to this tubulin monomer. This result is in agreement with published results of 55,000-60,000 (Renaud et al., 1968; Shelanski and Taylor, 1968; Weisenberg et al., 1968; Bryan and Wilson, 1971) for the tubulin monomer as determined both by SDS gel electrophoresis and ultracentrifugation studies.

In 1972 (a), Witman reported different molecular weights for alpha and beta tubulin. He found molecular weights of 56,000 and 53,000 respectively. The two tubulins, after reduction and alkylation, were separated in a urea-SDS system electrophoresed at alkaline pH. I have obtained similar separation of alpha and beta tubulins from Polytomella flagella in the same gel system. However, in the SDS gel system of Weber and Osborn (1969) for molecular weight determinations, run at neutral pH, the two tubulins move as a single band, indicating they are of the same molecular weight. Further to this point, Bryan (1972) reported that, if reduced and alkylated microtubule protein

is applied to urea-containing gels of increasing acrylamide concentration, a Ferguson plot, that is relative mobility versus gel concentration, gives parallel lines, indicating monomers are separating principally on the basis of charge.

AFFINITY CHROMATOGRAPHY

Tubulin is a very acidic protein and could be purified from pig brain high speed supernatants by fractionation on Sephadex DEAE A-50. The tubulin adheres tightly to the column and elutes only after application of high salt concentration buffer, at which time the vast majority of other proteins have been eluted (see fig. 14). ^{3}H-colchicine binding provides an effective marker for the localization of tubulin in this procedure. Fractions containing colchicine binding activity were examined by gel electrophoresis. Only one major band was seen on urea-containing polyacrylamide gels, and this band co-migrates with tubulin prepared by the in vitro polymerization procedure.

In the original paper describing this method (Weisenberg et al., 1968), colchicine binding protein was also shown to migrate as a single band on urea gels. However, in more recent studies (Bryan and Wilson, 1971; Luduena and Woodward, 1973), brain microtubule protein has been resolved into two tubulins. That my result was not an artifact of the preparation procedure was tested by preparing axonemes from Polytomella, at the same time, in the same solutions, and electrophoresing on the same set

of gels as the brain tubulin from the DEAE column. Neither very dilute protein concentrations, nor extended running time caused a separation of brain tubulin, whereas the flagellar microtubule protein separated into two bands.

Olmsted et al. (1971) reported microtubule protein isolated from either neuroblastoma or Chlamydomonas flagella could be resolved into two tubulin bands on urea-containing acrylamide gels. One of the flagellar tubulins co-migrated with one of the neuroblastoma tubulins, whereas the other tubulins differed in electrophoretic mobility, suggesting different tubulins were present. Failure to obtain separation of brain tubulin in the same gel system which clearly separates flagellar tubulins from Polytomella, may also indicate different tubulin proteins.

In an attempt to purify Polytomella cytoplasmic microtubule protein, high speed supernatants were fractionated on columns of Sephadex DEAE A-50, following the procedure used to purify pig brain tubulin. After addition of buffer containing 800mM NaCl, a broad protein peak elutes from the column. When this peak is analysed on urea gels, a number of acidic proteins are observed. Two of these co-migrate with purified flagellar microtubule protein. Although these gels were not stained for quantitative protein determinations, I would estimate these co-migrating bands to account for less than 10% of the protein on the gel.

Bound colchicine values from this experiment reached a peak value of 5800 cpm. Although this value represents only about 1/20 of bound counts obtained with pig brain supernatants, this does appear to represent significant ^{3}H-colchicine binding. The protein concentration of the bound ^{3}H-colchicine peak was about the same in both experiments (see figs. 14, 20). In the pig brain experiment, the bound peak reached a maximum value of 120,000 cpm and on polyacrylamide gels, tubulin represents a minimum of 90% of the protein present. From the gels of the bound peak from Polytomella high speed supernatants, it was estimated that the presumptive tubulin bands represent a maximum of 10% of the protein present on the gel. If we assume that the efficiency of ^{3}H-colchicine binding is identical in both systems, from these values one would predict a maximum bound ^{3}H-colchicine value of approximately 13,000 cpm. If the presumptive tubulin represents only 5% of the protein, the predicted bound value would be 6500 cpm. The observed value of 5800 cpm approaches this lower limit.

It should be remembered that this colchicine binding assay was performed under optimal conditions for brain tubulin (37°C; tubulin concentrations of at least 1 mg./ml.). It seems likely that for organisms such as Polytomella, growing at lower temperatures (ca. 25°C), the optimal conditions for ^{3}H-colchicine binding would be different.

It should be possible to obtain fractions enriched in tubulin from Polytomella high speed supernatants fractionated on a DEAE A-50 column, and eluted with a careful linear salt gradient. From these fractions, optimal ^{3}H-colchicine binding conditions for Polytomella tubulin could be determined.

As far as I know, significant colchicine binding has not been demonstrated in any plant system. It is unlikely that this low level of bound colchicine means that plant microtubules do not have binding sites for colchicine, as in vivo studies show plant microtubules are depolymerized by low concentrations of colchicine.

IN VITRO ASSEMBLY

A system has been described (using the procedure of Borisy and Olmsted, 1972b) for the in vitro assembly of microtubules from high speed supernatants of pig brain homogenates. Microtubules formed in vitro are identical in size and morphology to Polytomella singlet microtubules (obtained from axonemes by a prolonged treatment with 0.6M KCl, pH 8.3). This system provides a rapid purification procedure for pig brain microtubule protein. By cycles of polymerization and depolymerization, a very pure preparation of tubulin could be obtained. On SDS gels, one main tubulin band is seen, along with an associated high molecular weight protein, running right at the top of the gel. The nature of this high molecular weight species

is unknown. It co-purifies with tubulin in repeated cycles of polymerization (Borisy et al., 1974), but it is not clear whether it is part of the microtubules or an unrelated reversibly associating protein. When fraction C_2S is analysed on urea-containing polyacrylamide gels, only one band is seen. This band co-migrates with tubulin purified by fractionation of pig high speed supernatant on Sephadex DEAE A-50.

In the Introduction to this thesis, the possibility was raised that functionally different microtubules consist of different microtubule proteins. The obvious approach is to develop methods to isolate different sets of tubules from the same cell, for comparative biochemical analysis. Fractionation of high speed supernatants of Polytomella on Sephadex DEAE A-50 did not give sufficient yields of tubulin for comparative studies. A second course of action would be to isolate intact cytoplasmic microtubules using agents which stabilize these microtubules, such as hexylene glycol (Kane, 1965) or DMSO-glycerol (Filner and Behnke, 1973). Several attempts were made to do this with Polytomella, however these have met with limited success to date.

A third approach, following the in vitro polymerization scheme for pig brain, initially gave promising results, in that protein aggregating at 37°C did include easily identifiable proteins which co-migrated with flagellar microtubule protein. Even if a procedure for

in vitro polymerization of cytoplasmic microtubules from Polytomella could not be established, this seemed to be a reasonable method for obtaining significant amounts of Polytomella cytoplasmic microtubule protein.

Polytomella high speed supernatants contain a low concentration of tubulin as no bands could be identified as tubulin on the basis of co-migration with flagellar microtubule protein. When high speed supernatants were incubated at 37°C, they became turbid almost immediately. However, at no time during the course of this incubation were any microtubules seen (as monitored by negatively stained grids). Material aggregating at 37°C was pelleted and the pellet (H_1P) and supernatant (H_1S) were analysed on urea-containing polyacrylamide gels. H_1S was seen to contain a large number of bands towards the top of the gel, but none near the region where flagellar microtubule protein runs. H_1P also contains several proteins, two of which co-migrate with flagellar microtubule protein and are presumed to be cytoplasmic tubulin. If flagellar microtubule protein and H_1P protein are run on the same gel, there is an intensification of these tubulin bands.

Using pig brain in vitro polymerization as a model, the aggregation of microtubule protein in Polytomella high speed supernatants was assessed in terms of its sensitivity to agents which block in vitro assembly of pig brain microtubules. In the presence of cold, this aggregation was not reversible; and neither the presence

of colchicine, nor the absence of GTP prevented this aggregation. This appears then to be a non-specific aggregation of protein, stimulated by heat. Attempts were made to specifically solubilize tubulin from this pellet, using procedures known to solubilize tubulin from other sources. However, when pellet fractions were examined after extraction, all were seen to still contain the tubulin bands.

It is interesting to note here that initial experiments on in vitro polymerization of brain tubulin also produced an aggregation, with no identifiable microtubules seen. This aggregation could be prevented by cold or by colchicine, but was not reversible by either cold or colchicine. However, this aggregated protein consisted largely of tubulin (Borisy et al., 1972c). It was subsequently determined that the presence of a calcium chelator, and the use of the organic Good buffers, were essential for the formation of microtubules in vitro. We have not been able to establish conditions for assembly of Polytomella microtubules in vitro, however, the limiting factor is most probably a low concentration of tubulin in high speed supernatants.

Significant ^{3}H-colchicine binding was demonstrated (as discussed earlier) in fractions containing our presumptive tubulin eluted from a Sephadex DEAE A-50 column. Fraction H_1P could not be assayed for colchicine binding on DEAE A-50 columns since it did not resuspend well

and stayed at the top of the column, so a filter disc assay method was used. This method did not produce quantitative results, and incongruities were observed such as increased ^{3}H-colchicine binding in H_1S compared to HSS. However, in all experiments assayed, pellet H_1P was seen to contain the highest level of bound colchicine. This is taken as further evidence that microtubule protein is contained in pellet fraction H_1P.

The initial objective of this research was to develop methods to isolate tubulin from different sets of microtubules within Polytomella. Flagellar microtubule protein was easily purified by adapting methods used by others. The purification of cytoplasmic tubulin proved extremely difficult, and attempts to develop an in vitro polymerization procedure were unsuccessful. Only in systems in which tubulin is natively present in high concentrations has in vitro polymerization been reported. Tubulin represents a small fraction of the total protein present in a Polytomella high speed supernatant. It seems more reasonable now to obtain a preliminary purification of Polytomella tubulin on Sephadex DEAE A-50 using a careful linear salt gradient, and subsequently test in vitro polymerization. However, this posed serious technical problems. The experiments reported in this thesis were run with 10-20 liters of cells per run. Fractions had to be greatly concentrated, and

tubulin still represented only a small portion of the total protein. At the time, we were not equipped to run experiments with much more than 20 liters of cells.

The protein bands contained in pellet H_1P which are presumed to be cytoplasmic microtubule protein must be demonstrated to be tubulin by methods other than co-migration with purified tubulin. Work is now under way in this laboratory to run large amounts of fraction H_1P on a preparative gel system, and to extract these proteins in amounts sufficient for amino acid analysis.

Another possibility which seems more fruitful is the use of immunological techniques. Preliminary work has begun in conjunction with Dr. V.I. Kalnins of the University of Toronto. He has obtained antibodies to brain tubulins which have been eluted from SDS gels. Even after such drastic treatment, it seems that the protein still retains its antigenic properties. We are currently providing Dr. Kalnins with samples of purified whole axonemes, as well as tubulin eluted from gels. Antibodies prepared against these tubulins could be used: 1) to identify whether our presumed cytoplasmic microtubule protein is indeed tubulin; 2) to demonstrate differences between corresponding tubulins from axonemes and cytoplasmic microtubules; and 3) as a sensitive and quantitative assay method for detecting microtubule protein at various stages during the cell cycle of Polytomella.

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