INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI®

SC

Analysis of Microtubule Assembly *in vitro*

I. Role of Microtubule Organizing Centers (MTOCs) in Controlling Assembly of Microtubules

II. Roles of the Initiation Proteins Composing MTOCs and Associated with Brain Microtubules in Tubulin Assembly

A Dissertation

Presented to the School of Graduate Studies

of

The University of Ottawa

in partial fulfillment of requirements for a

Doctorate of Philosophy

in Biology

by

Mark E. Stearns

1978

© M.E. Stearns, Ottawa, Canada, 1978

UMI Number: DC52568

INFORMATION TO USERS

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleed-through, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

UMI®

UMI Microform DC52568
Copyright 2007 by ProQuest LLC
All rights reserved. This microform edition is protected against unauthorized copying under Title 17, United States Code.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ACKNOWLEDGEMENTS

A thesis is seldom the work of only one person, and I would like to take this opportunity to thank several people who have been of invaluable assistance in the preparation of this thesis.

Many of the experiments were carried out in collaboration with honors students Mr. Robert Marchand, Mr. Yvan Bourbonnais, and Mr. Robert Allore, and with graduate students Mr. Joe Connolly, Mr. Mervin Sabey, and Mr. Bruce White in this laboratory. Their help in experiments is gratefully acknowledged. I would also like to thank Dr. Andrew Massalski, Mr. George-Ben Tchavtchavadze, and Mr. Jacques Helie for technical assistance and for preparation of figures used in this text; Dr. Ed Yeung and Mr. Kem Rogers for reading of the text; and Mrs. Angela Yeung for typing the manuscript.

My supervisor, Dr. David L. Brown has had a tremendous influence on the progress of this work, both as a source of ideas and as a stimulating critic of the experiments and the results. As his "ever humble" graduate student, I thank him for his interest and guidance freely given during

the course of this work.

I dedicate this thesis to my wife Linda Jade for her contributions made both directly and indirectly to the completion of this degree.

Finally, I thank Dave and the people in our lab for the many good times I have had while a student here.

TABLE OF CONTENTS

ABSTRACT

In the alga Polytomella basal body rootlets function as microtubule-organizing centers (MTOCs) controlling the positioning, initiation and patterning of cytoskeletal microtubules (Brown and Stearns, 1978).

Procedures are described for the isolation and purification of intact basal body rootlet complexes. Quantitative electron microscopic and viscometry studies show that rootlet MTOCs are functionally intact and competent to initiate assembly of microtubules from both crude and phosphocellulose-purified brain tubulin preparations. Thin sections confirm that the microtubules are initiated from amorphous sites directly on rootlets in patterns closely resembling patterns of microtubules seen in situ. Biochemical analysis of the composition of rootlet-MTOCs shows that MTOCs consist of trypsin-sensitive protein(s) which can be extracted for purification by brief dialysis against a low ionic strength Tris-EDTA solution. Electron microscopic studies of treated complexes confirm that the complexes remain intact but incompetent to promote microtubule assembly. PAGE gels of the high speed supernatants of extracted complexes indicate a number of rootlet-associated proteins (RAPs) are removed. When RAPs are incubated at 37°C with phosphocellulose-purified tubulin

in 0.1M PIPES, 1mM $MgCl_2$, EGTA, pH 6.4 made 2mM in turbidiometry studies show microtubules are assembled.

Procedures are described for purification of the tubulin-initiating proteins of RAPs preparations and of microtubule-associated proteins (MAPs) obtained from temperature-cycled beef brain. Tubulin-initiating proteins (or TIPs) in RAPs preparations and microtubule-initiating protein ("the-MAP") in MAPs preparations are selected for by using centrifugation to pellet early assembly products (protofilaments) formed after brief incubation of fractions with tubulin. Phosphocellulose chromatography is used to further purify microtubule-initiating proteins in the protofilament pellets. PAGE gels of protein preparations show a single 330,000 mol. wt. protein ("the-MAP") in cycled brain tubulin (S-3) and 4 closely spaced 190-200,000 mol. wt. proteins in RAPs extracts (TIPs) function to promote assembly of protofilaments. The role of "the-MAP" and TIPs in promoting phosphocellulose-purified tubulin assembly is examined and compared using optical techniques (negative staining, turbidometry and viscometry). Negative staining studies show during initiation that "the-MAP" and TIPs stimulate formation of protofilaments and protofilament sheets as assembly intermediates which then form a complete microtubule wall. Intact rootlet-MTOCs are shown to initiate assembly of tubulin by the same

mechanism. Turbidometry studies demonstrate that "The-MAP" and TIPs stimulate increases in initiation kinetics and suggest that "the-MAP" may also stimulate the elongation of microtubules. Viscometry studies show that "the-MAP" will stimulate microtubule elongation on rootlet-MTOCs whereas TIPs do not, but instead initiate new microtubules. Experiments using S^{35} labeled TIPs confirm that TIPs specifically function in initiation which is consistent with their composing MTOCs.

Finally, electron microscopic studies of the structural properties of microtubules initiated by "the-MAP" and TIPs show the tubules have smooth surfaces. These microtubules become coated with arms and closely resemble S-3 microtubules if they are briefly incubated with crude MAPs, indicating that separate MAPs function to form arms on tubules. The microtubules are cold-sensitive and MAPs or "the-MAP" microtubules disassemble to form rings whereas TIPs microtubules disassemble to protofilaments. Analytical ultracentrifugation studies confirm these results. The main conclusion drawn from these studies is that microtubule-initiating proteins (TIPs) associate with MTOCs to control initiation of protofilaments during assembly of microtubules <u>in vivo</u>.

RESUME

Chez l'algue Polytomella, les radicules du corps basal fonctionnent comme centres d'organisation des microtubules (MTOCs) contrôlant la position, l'initiation et le patron de microtubules cytosquelettiques (Brown et Stearns, 1978)

Des procédures sont décrites pour l'isolation et la purification des complexes intacts du corps basal radiculaire. Des études viscométriques et quantitatives au microscope électronique ont démontré que les MTOCs-radiculaires sont intacts du point de vue fonctionnel et compétents pour initier l'assemblage des microtubules à partir de préparations de tubuline cerveaux à l'état pur ou purifié à la phosphocellulose. Des sections microscopiques ont confirmé que les microtubules sont initiées de sites amorphes directement sur les radicules, dans un patron ressemblant aux vus in situ. Des analyses biochimiques de la composition des MTOCs radiculaires démontrent que les MTOCs consistent de protéines sensibles à la trypsine, qui peuvent être extraites pour la purification par une courte dialyse avec une solution Tris-EDTA de basse teneur ionique. Des études au microscope électronique de complexes traités confirment que ceux-ci restent intacts mais incompétents à amener l'assemblage des microtubules. Des gels de polyocrylamide des supernatants extraits à haute vitesse des complexes indiquent qu'un certain nombre de protéines associées aux radicules (RAPs) sont enlevées. Quand ces RAPs sont incubées à 37^{o}C avec de la

tubuline purifiée au phosphocellul e dans 0.1M PIPES, 1mM $MgCl_2$, EGTA, amMGTP à un pH 6.4, des études turbidiométriques ont démontré que les microtubules sont assemblées.

Des procédures sont décrites pour la purification des protéines initiatrices de la tubuline des préparations RAPs et des protéines associées aux microtubules, MAPs obtenues du cerveau de boeuf au cours du cycle de polymérisation à 37°C. Les protéines initiant la tubuline (TIPs) dans les préparations de RAPs et la protéine initiant les microtubules ("la MAP") dans les préparations MAPs, sont sélectionnées pour centrifuger en boulette les produits d'assemblage primaires formés après une brève incubation des fractions avec de la tubuline. La chromatographique phosphocellulaire est utilisée pour purifier davantage les protéines initiatrices dans les boulettres de protofilaments. Des gels de polyocrylamide des préparations protéiques démontrent qu'une seule protéine de 330,000 daltons ("la MAP") dans la tubuline de cerveau (S-3) et quatre 190-200,000 daltons espacés étroitement dans les extraits RAPs (TIPs) fonctionnent à promouvoir l'assemblage des protofilaments. Le rôle de "la-MAP" et des TIPs pour promouvoir l'assemblage de la tubuline purifiée à la phosphocellulose est examiné et comparé en utilisant des techniques optiques (coloration négative, turbidiométrie, viscométrie). Des études à la coloration négative démontrent que durant l'initiation "la MAP" et les TIPs stimulent la formation des protofilaments et des feuilles de protofilaments comme intermédiaires de montage qui forment alors

des microtubules complets. Les MTOCs radiculaires intacts sont révélés comme étant les initiateurs dans l'assemblage de la tubuline par le même mécanisme. Des études de turbidiométrie démontrent que "la MAP" et les TIPs stimulent la cinétique d'initiation et suggèrent que "la MAP" peut aussi stimuler l'allongement des microtubules. Des études de viscométrie démontrent que "la MAP" stimulera l'allongement des microtubules sur les MTOCs radiculaires tans que les TIPs initieront de nouvelles microtubules. Des expériences utilisant des TIPs marqués au S^{35} confirment que les TIPs fonctionnent spécifiquement à l'initiation, ce qui est en accord avec leur MTOCs.

Finalement, des études au microscope électronique des propriétés structurales des microtubules initiées par "la MAP" et les TIPs démontrent que les tubules ont une surface lisse. Ces microtubules deviennent couvertes de brase (et ressemblent de près aux microtubules S-3) si elles sont incubées brièvement avec des MAPs à l'état pur, indiquant qu'une MAP différente fonctionne pour former les bras sur les tubules. Les microtubules sont sensibles au froid et les microtubules MAPs ou "la MAP" se défont pour former des anneaux, tandis que les microtubules TIPs se défont en protofilaments. Des études analytiques de l'ultracentrifugation confirment ces résultats. La conclusion principale qui peut être tirée de ces études est que les protéines initiant les microtubules (TIPs) s'associent avec les MTOCs (<u>in vivo</u>) qui contrôlent l'initiation des protofilaments durant l'assemblage des microtubules.

SOURCE OF CHEMICALS

Triton - X-100 - Fisher

lead citrate - Fisher

bovine serum albumin standards - Sigma Chemical Co., St. Louis, Mo.

coomassie brilliant blue 250-R - Polysciences Inc., Warrington, Penn.

protein standards:

cytochrome C, catalase - General biochemicals, Chagrin Fall, Ohio

egg albumin, chymotrypsinogen A - Sigma Chemical Co., St. Louis, Mo.

bovine serum albumin - Calbiochem, La Jolla, Calif.

GTP - Sigma

$NaSO_4$-S^{-35} - New England Nuclear

Aquasol - Fisher

PIPES - Sigma

PC - Whatman Ltd. - Fisher Chemicals Ltd.

SDS - Fisher

TRIS - Fisher

Glycerol - Fisher

DMSO - Fisher

EDTA - Fisher

EGTA - Fisher

Phosphate - Fisher

LIST OF ABBREVIATIONS

Chemicals

PC - Phosphocellulose

PIPES - (piperazine-N-N'-bis [2-ethane sulfonic acid])

SDS - sodium laurylsulfate

Tris - 2-amino-2-hydroxymethyl-1, 3-propanediol

GTP - guanosine triphosphate

DMSO - dimethyl sulfoxide

EGTA - [ethylene (oxyethylene-nitrilo)] tetraacetic acid

EDTA - disodium ethylenediamine-tetraacetate

Terminology

MAPs - microtubule accessory proteins

RAPs - rootlet-associated proteins

HMW - high molecular weight

MTOCs - microtubule-organizing centers

MAB - microtubule assembly buffer (see Materials and Methods)

TIPs - microtubule-initiating proteins

GDMP - 50% glycerol (v/v); 10% DMSO; 5mM $MgCl_2$, 5mM sodium phosphate pH 6.9

A_{350} - absorbance at wavelength of 350 nanometers

S_{w20} - sedimentation coefficient relative to water at 20°C

Stains

PTA - phosphotungstic acid

UA - uranylacetate

EXPLANATION OF TERMINOLOGY

Microtubule-Associated Proteins: Depending on the buffer conditions used, different amounts of several non-tubulin proteins have been shown to coassemble with tubulin to promote its assembly. Different groups studying tubulin assembly have employed different terminology to refer to these accessory proteins. Borisy's group find that at least two proteins with molecular weights of 271,000 and 286,000 coassemble with tubulin which they term high molecular weight (or HMW) proteins (see Murphy and Borisy, 1975). Rosenbaum's group find similar proteins having slightly higher molecular weights of 300,000 to 350,000 daltons and they have termed these proteins microtubule-associated proteins (or MAPs) (see Sloboda et al., 1976a). Kirschner's group have found that low molecular weight proteins of < 70,000 represent the major non-tubulin species and they have termed these proteins tau (see Weingarten et al., 1975).

In studies reported in this thesis, I found in contrast to the above studies that there are many non-tubulin proteins of a wide range of high and low molecular weights in temperature-cycled brain tubulin preparations. Unless otherwise specified, the term MAPs has been used to refer to high and low molecular weight non-tubulin proteins present in

temperature-cycled preparations. One of the MAPs of a molecular weight of 330,000 has been purified from MAPs and I have arbitrarily referred to this protein as "the-MAP".

MTOC-Associated Proteins: Proteins obtained in crude Tris-EDTA extracts of isolated basal body rootlet complexes have been called rootlet-associated proteins (RAPs) since in functional assays the extraction of complexes was found to prevent assembly of tubulin on the rootlet MTOCs. In microtubule assembly studies using purified tubulin protein, four high molecular weight proteins (190,000 to 220,000 present in crude RAPs preparations were found to function in initiating microtubule assembly. These proteins have been purified from RAPs and in this thesis are termed tubulin-initiating proteins (TIPs) since they have been found to function specifically in initiation of microtubules and in doing so they appear to act like intact MTOCs and associate at one end of the forming microtubule.

INTRODUCTION

FOREWORD

The microtubule is a basic cellular element found in at least one stage in the life cycle of eukaryotic cells. In the cell , microtubules are often organized to form diverse types of organelles which can carry out various cellular functions. These functions range from cell motility and intracellular transport to the formation and maintenance of cell shape.

In attempts to understand the structural and functional roles of microtubules and microtubule organelles, one useful approach has been to examine their structural properties. Electron microscopic observations have shown that a strong correlation exists between the structural and the functional characteristics of microtubules composing axonemes, spindles, and the cytoplasmic organelles of cells (Porter, 1966). The question that comes to mind is whether

these structural and functional properties of microtubules are controlled by a precise cellular mechanism.

Inoué (1964) discovered that microtubules exist in a dynamic equilibrium with their subunits. This indicated that factors affecting the equilibrium, or in other words the assembly-disassembly, of microtubules would also directly regulate the numbers and lengths of microtubules in microtubule organelles. The same factors may therefore indirectly control the functional capacity of microtubule structures.

One way we have gained insights into how cells might modulate microtubule structure and function is by studying conditions affecting tubulin assembly. In these studies, progress has largely been made through examining the reassembly of microtubules following disassembly induced by anti-microtubule agents such as pressure, low temperature and colchicine (Bouck and Brown, 1976). An alternate approach is through in vitro experiments that have followed Weisenberg's (1972) finding that microtubules could be assembled in vitro. The objective of these experiments has been to answer some of the questions raised by in vivo investigations into the conditions and the fundamental mechanism controlling assembly in living cells.

Perhaps one of the most significant observations made in *in vivo* studies has been that microtubule-organizing centers (MTOCs) serve to control initiation and patterning during assembly of functional microtubule organelles (Porter, 1966; Bouck and Brown, 1976). The role of these centers must be taken into account in any attempt to resolve the mechanism by which microtubule assembly occurs. Resulting *in vivo* studies have for the most part been limited to morphological observations. However, by taking advantage of Weisenberg's basic discovery, it should be possible to develop an *in vitro* assembly system which resembles that seen *in vivo*. Using *in vitro* conditions, one can then investigate the role of MTOCs in assembly and maintenance of potential microtubule organelles.

The ambition of this thesis has been to develop an *in vitro* assembly system to study this problem in terms of

(a) the roles of MTOCs and

(b) the roles of protein components of the MTOCs and microtubules that are involved in assembly.

DISCOVERY

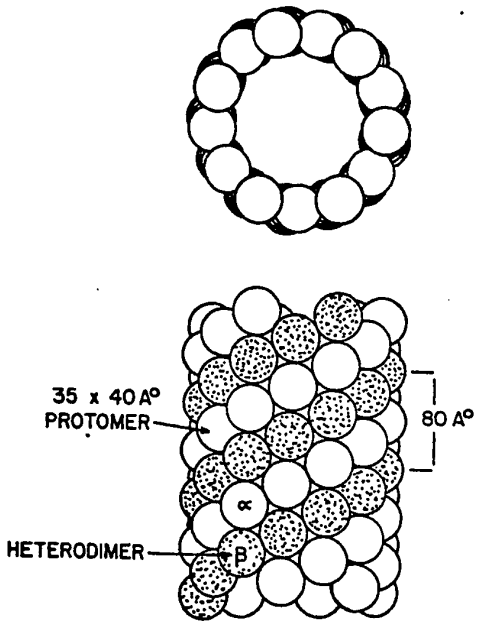
Interest in microtubules has originated largely from microscopic studies showing that microtubules occur in all eukaryotic cells as functionally important elements. Microtubules were first discovered as a prominent cellular component with the advent of electron microscopy in the 1950s. Manton and Clarke (1952) and later Fawcett and Porter (1954) reported microtubules are the main component forming the 9 + 2 structure in cilia. Several scattered reports had indicated that similar tubular elements are also present in the cytoplasm of cells (Grasse, 1956; Beams et al., 1959; Roth, 1959; Roth and Daniels, 1962; and Harris, 1962). With the discovery and use of glutaraldehyde as a fixative (Sabatini et al., 1963) microtubules were consistently found as cytoplasmic components in plant and animal cells (Ledbetter and Porter, 1963; Slautterback, 1963). Microtubules have since been found in all eukaryotic cells at one or more stages in their life cycle.

STRUCTURE AND BIOCHEMISTRY

Tubular structures having an outer diameter of about 23-27 nm and of varying length have generally been called microtubules. When fixed in glutaraldehyde, microtubules appear in cross sections as an electron-dense ring

surrounded by a 10 nm diameter halo. The ring consists of 13-15 protofilaments forming a 4-5 nm thick wall (Tilney et al., 1973). Optical diffraction studies have indicated that the protofilaments are composed of protein subunits exhibiting a 4-5 nm packing diameter and arranged in parallel arrays that give the globular subunits in adjacent protofilaments a half-staggered appearance (Grimstone and Klug, 1966; Cohen et al., 1971,1975). Using computer diffraction patterns of electron micrographs and filtered images from optical diffraction studies, Amos and Klug (1974) and Linck and Amos (1974) found that microtubules have a 4-5 nm lattice and exhibit an 8 nm repeat. They have interpreted this to mean that 8 nm dimers are the basic repeating subunit of the protofilaments and of microtubules. An artist's conception of a complete microtubule is seen in figure 1 showing the protofilaments and dimers in half staggered arrays.

Biochemical studies have shown that the principal component of microtubules is a protein termed tubulin (Mohri, 1969). Tubulin from different sources exhibits an interspecies homogeneity in molecular weight, sedimentation coefficient, amino acid composition, amino acid sequence, and immunological cross reactivity (see reviews by Stephens and Edds, 1976; and Snyder and McIntosh, 1976). On a gross biochemical level, tubulin can be separated into classes of monomers,



1

α tubulin and β tubulin, that associate as heterodimers with a molecular weight of 110,000 to 120,000 and a sedimentation coefficient of 6S. It turns out that α and β tubulin are two electrophoretically separable proteins of identical molecular weights of 55,000 but different amino acid composition (Lee et al., 1973; Bryan, 1974). The dimer is the monomeric form of tubulin which assembles microtubules given the correct physiological conditions by hydrophobic bonding of longitudinal and lateral binding sites. It is assumed in the model in fig. 1 that dimers exist as heterodimers of slightly different α and β tubulin subunits and that each protofilament contains equal amounts of α and β tubulin (Witman et al., 1972).

The biochemical studies have shown that tubulin exhibits a number of characteristic properties. Tubulin binds to alkaloids such as colchicine, vinblastine, and podophyllotoxin that inhibit its assembly, and to molecules such as magnesium, guanosine triphosphate and other nucleotides which promote its assembly. The drug colchicine binds specifically on a mole-to-mole basis with tubulin dimers and induces disassembly of microtubules (Borisy and Taylor, 1967; Weisenberg et al., 1968). Consequently, the binding of colchicine has been used as a basic criterion for identification of tubulin protein and for studies

relating microtubule structure to function. GTP binds to tubulin at a ratio of 2 moles to 1 mole of dimer (Stephens et al., 1967; Yanagisawa et al., 1968) and Mg^{++} binds at a ratio of 1 to 1 on a molar basis with the dimer. Both Mg^{++} and GTP are thought to stimulate assembly of tubulin by promoting strong structural changes in dimers to stabilize them for assembly. The dimer has a tightly bound non-exchangeable GDP which is transphosphorylated by GTP or ATP bound to an exchangeable site to promote assembly (Olmsted and Borisy, 1973). The biochemical data has provided helpful information about the factors involved in assembly in vivo but most importantly has permitted the development of conditions for in vitro assembly of tubulin.

DISTRIBUTION AND FUNCTION

Microtubules are found in a wide variety of plant and animal cells forming many diverse types of microtubule organelles. They are seen as single tubules dispersed in the cytoplasm of cells, and as the central element forming structures such as cytoplasmic organelles, spindle fibres, axostyles, axonemes, centrioles, and basal bodies. The functional roles of these microtubule organelles can be classified into three broad categories: cell motility, intracellular transport, and the establishment and maintenance of cell shape. In each of these categories, the

microtubules take on a pattern suited to the particular function.

Cell Motility: Microtubules in cilia, flagella and axostyles are arranged in a highly structured pattern to form an axoneme. Microtubules in axonemes can generate a bending motion by sliding past one another to provide the motive force (Satir, 1965; Brokaw, 1972; Warner, 1972; McIntosh, 1973; Bloodgood, 1975). The energy source for microtubule mediated motility is generated by an ATPase-dependent energy-transducing mechanism. In cilia, flagella, and axostyles the energy for motility depends on an ATPase of dynein arms coating the microtubules (Gibbons, 1965; Anderson et al., 1968; Gibbons and Gibbons, 1972; Burton, 1973; Mooseker and Tilney, 1973; Bloodgood, 1975). It is not known at this time if cytoplasmic microtubules involved in transport also have a similar ATPase-dependent energy-transducing mechanism.

Cellular Transport: Cytoplasmic microtubules are organized in many cell types to provide the structural framework for the movement of cellular components. A classical example is the spindle microtubules which are oriented and disposed in such a fashion as to provide a structural framework for the movement of chromosomes (de Harven and Bernhard, 1956; Roth and Daniels, 1962; Harris, 1962.)

Experimental disruption of spindle microtubules

with agents such as colchicine (Inoué, 1952a), cold (Inoué, 1952b, 1964; and Inoué and Sato, 1967), pressure (Zimmerman and Marshland, 1964) and UV irradiation (Inoué, 1964) has been shown to stop chromosome movement. Studies of this nature suggest that microtubules are indeed important elements for guiding cell division.

Another example is nerve cells, where microtubules constitute a prominent component of slender axon extensions (Palay, 1956; Elfvin, 1961; Gonatas and Robbins, 1964; Smith et al., 1975). Microtubule-disrupting agents will block protein transport in these cells (Kreutzberg, 1969; Sjöstrand et al., 1970; Fernandez et al., 1971) suggesting that microtubules provide the directional guidance and the motive force for axonal transport.

Finally, a striking example which clearly shows microtubules have a role in transport is in the melanophore cell where microtubules are seen extending from the center of the cell to provide a framework for movement of melanin granules during pigment contraction and dispersion (Bikle, Tilney, and Porter, 1966; Porter, 1966; Murphy, 1975; Byers and Porter, 1977). Pigment transport is immediately inhibited by anti-microtubule agents (low temperature and colchicine), showing that microtubules directly control their movement.

Cell Shape: In many developing organisms, localized changes in cell shape often occur as a product of intracellular changes which involve the assembly of cytoplasmic microtubule organelles. For example, during induction in the lens placode the cell assembles large numbers of microtubules in parallel near the cell cortex to enable formation of an elongating cell region (Byers and Porter, 1964; Porter, 1966). Similarly, cell elongation in spermatids of developing spermatozoa depends on assembly of microtubules in parallel arrays. In the primary mesenchyme cells in the gastrula of sea urchins, microtubules are prominent elements essential for formation of extended pseudopodia in these cells (Gibbins, Tilney and Porter, 1966).

Protozoans offer examples of cells which exhibit long, slender extensions that are formed and maintained by microtubules. In the heliozoan Tokophrya (Rudzinska, 1965), extensions termed axopodia are formed by microtubules organized in arrays which form central and peripheral chann els for the transport of particles. In axopodia of the heliozoan Actinosphaerium, microtubules are arranged in two coiled interlocking arrays to form the axial filament (Kitching, 1964; Tilney and Porter, 1965). If axopodia are exposed to low temperatures (Tilney and Porter, 1965), high pressure (Tilney, Hiramoto and Marshland, 1966) and colchicine (Tilney, 1968a) the microtubules rapidly dis-

assemble concomitant with a disappearance of axopodia. Upon reassembly of normal filaments normal functional axopodia are reformed.

In general, what the morphological studies have shown is that cells organize microtubules to form specific structures designed to carry out specific cell functions. It is also evident from the above discussion that the assembly and maintenance of these microtubules in their proper patterns is essential for the organelles to properly function.

MICROTUBULE ASSEMBLY in vivo

The fact that microtubule assembly is a highly controlled cellular event has been clearly demonstrated in in vivo studies. One system which has received considerable attention in these studies is the spindle, a highly transient microtubule system. Inoué (1952a, b) showed with polarization optics that low temperature or colchicine would reversibly abolish birefringence of the mitotic spindle. In addition, he showed that spindles also lose birefringence under normal conditions during chromosome movement. He consequently hypothesized that the microtubules of spindle fibres are not static structures, but, indeed, are in a constant dynamic state of assembly-disassembly.

The Dynamic Equilibrium Theory: Inoué (1964) and Inoué and Sato (1967) expanded on the initial studies. Birefringence,

they observed, is reversibly diminished in spindle fibres with cold, hydrostatic pressure and anti-mitotic drugs. With the removal of treatment, microtubules reassemble and spindle birefringence is regained. This recovery is not a result of new protein synthesis since the presence of actinomycin D and puromycin does not affect results. Their conclusion was that microtubules exist in a dynamic equilibrium with a pool of subunits, or tubulin. They further suggested that small changes in the intracellular environment are sufficient to shift this equilibrium from monomer to polymer or vice versa (see fig. 2).

There have been a number of studies showing that agents like heavy water will stabilize the spindle (Gross and Spindel, 1960; Marshland and Zimmerman, 1965) and in effect actually increase the numbers of microtubules in the spindle (Wunderlich and Weirich, 1972; Burgess and Northcote, 1969). This fact alone would suggest a pool of subunits is available for assembly. Stephens (1973) has experimentally tested the dynamic equilibrium hypothesis using sea urchin eggs in active metaphase. Eggs grown at $4^{o}C$ exhibited spindles with few microtubules (and a large subunit pool) in comparison to eggs at $8^{o}C$ which had more microtubules (and a smaller pool). The data shows a dynamic equilibrium does exist as a function of cellular environment. Some of the factors experimentally shown to affect this balance are included in fig. 2.

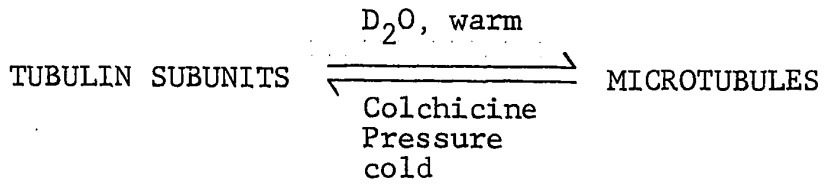


Fig. 2. Dynamic equilibrium between tubulin protein and microtubules.

This fundamental property of microtubules in spindles may also apply to the microtubules in other organelles. Anti-microtubule agents in fig. 2 have been shown to cause disassembly of microtubules in a variety of organelles. Tilney and others (1966; 1967; 1968a; 1971) have shown microtubules in axopodia of Actinosphaerium or Echinosphaerium will reversibly disassembly and reassemble with kinetics similar to microtubules in the mitotic apparatus. In developing sea urchin embryos the equilibrium of cytoplasmic microtubules to subunits is sensitive to anti-mitotic agents (Tilney and Gibbins, 1968, 1969). Similarly, in fully developed cell systems like in the alga Ochromonas (Bouck and Brown, 1973; Brown and Bouck, 1973, 1974) or in the alga Polytomella (Brown and Stearns, 1978) cytoskeletal microtubules have been shown to exhibit a sensitivity to anti-mitotic agents and appear to also exist in equilibrium with tubulin subunits. Protein synthesis is not essential for

reassembly of microtubules since cycloheximide does not inhibit the reaction (Bouck and Brown, 1973). The existence of a pool of tubulin subunits in equilibrium with microtubules has been neatly demonstrated in studies with sea urchin eggs and embryos using pulse labeling techniques in conjunction with gel electrophoresis (Raff et al., 1971, 1972). Raff and others showed that a pool of tubulin subunits is present from egg to gastrula and that there is a constant turnover of these subunits. Similarly, a turnover of tubulin has been found to occur during stages of the cell cycle (Robbins and Shelanski, 1969) and during differentiation of neuroblastoma cells (Morgan and Seeds, 1975). It is evident from these studies that one way in which the cell could control equilibrium in microtubule assembly is to control the balance of polymer to subunits.

In contrast to cytoplasmic microtubules, microtubules of cilia and flagella axonemes are insensitive to cold, colchicine or pressure treatments, although their initial formation or regeneration can be inhibited by colchicine and pressure (Young et al., 1972; Rosenbaum and Carlson, 1969; Rosenbaum et al., 1969). Despite this difference between the microtubules, Rosenbaum et al. (1969) have discovered an important similarity. They proved the existence of a tubulin pool for microtubules of axonemes: they found that after amputating the flagella of Chlamydomonas, protein

synthesis is not required for regeneration of at least 50% of the normal flagellum. More recently, Brown and Bouck (1973) showed in Ochromonas that when flagella are amputated they regenerate again in cycloheximide but not in colchicine.

Thus, the overall conclusion is that like microtubules in the spindle, cytoplasmic and flagella microtubules also exist in a dynamic equilibrium of monomer to polymer.

One difficult and obvious problem which arises from these studies is how does the cell regulate assembly and/or disassembly of microtubule organelles?

Control of Microtubule Distribution: Having established that microtubules are arranged in regular arrays in the cell, our attention can now be focused on understanding how this microtubule patterning is controlled during cell development.

As implied earlier, control of the spatial and temporal distribution of microtubules in most cell systems resides with intracellular structures which appear amorphous and electron dense in nature in electron microscopy studies (Porter, 1966; Bouck and Brown, 1976). These sites have been referred to as foci (Porter, 1966), orienting centers (Inoué and Sato, 1967), nucleating centers (Tilney, 1968b) and microtubule-organizing centers, MTOCs (Pickett-Heaps, 1969). The term MTOCs have been used in this thesis to describe such sites since it is the most descriptive of the terms used.

Microtubules are usually free at one end and embedded at the other end in MTOCs located on the surface of centrioles (Pickett-Heaps, 1971), on centriolar basal body complexes (Gonatas and Robbins, 1964), on the nuclear envelope (Robinow and Marak, 1966; Freed and Lebowitz, 1970; Jessen and Moc, 1972), and on kinetochores (Brinkley and Nicklas, 1968). In the protozoans, cytoplasmic structures such as centroplasts (Tilney, 1971), templates (Tucker, 1977), rhizoplasts and kinetobeaks (Brown and Bouck, 1974) and rootlets (Brown et al., 1976; Brown and Stearns, 1978) function as MTOCs.

It is largely from the work with protozoans that an understanding has developed of the various roles MTOCs serve in controlling spatial distribution of microtubules. The parameters of control of MTOCs include regulating positioning, initiation and patterning of microtubule arrays. Positioning, as would be expected, is controlled by virtue of the MTOC's own position in the cell (see review by Bouck and Brown, 1976). As for MTOCs initiating assembly, this has been demonstrated by electron microscopic studies. For example, it has been shown in cells with a closed spindle that MTOCs in the form of amorphous plaques on the nuclear membrane serve to initiate assembly of spindle microtubules (Moens and Rapport, 1971). In another example, Tucker (1971a, b) has discovered that during reassembly of microtubules after

cold shock treatment in the ciliate Nassula, a template or MTOC serves to control both initiation and patterning of the microtubules. He found that as microtubules extend from the MTOC the pattern is stabilized by intermicrotubule linkers (Ockleford and Tucker, 1973; Tucker, 1977). The alga Ochromonas presents a comparable example. Here, cell shape depends on two separate MTOCs, a rhizoplast and a kinetobeak which are positioned in the cell to initiate assembly of cytoskeletal microtubules in precise arrays that in turn determine and maintain cell shape (Bouck and Brown, 1973; Brown and Bouck, 1973). Bouck and Brown showed that disassembly of the cytoskeletal microtubules (with colchicine and hydrostatic pressure) disrupts cell shape, and that during cell recovery from treatments, the MTOCs initiate assembly of microtubules in the correct pattern to re-establish the original cell shape (Brown and Bouck, 1974). Similarly, in the cell wall-less alga Polytomella, the maintenance of cell shape depends on flagella rootlets which serve as MTOCs to control assembly and patterning of cytoskeletal microtubules. During recovery of these cells from treatments with anti-microtubule agents (colchicine, and pressure) or during cell excystment, rootlet-MTOCs control the positioning, initiation, directionality, and patterning of the cytoskeletal microtubules (Brown and Stearns, 1978).

Tilney (1971) has suggested as an alternate hypothesis that cells might control microtubule assembly via the linkers found bridging microtubules. He found in the protozoan Actinosphaerium that after cold shock disruption, the microtubules are initiated by MTOCs but only in random patterns. He observed that coincident with the attachment of linkers on the sides of microtubules, the tubules are arranged in their proper order indicating that the linkers, and not MTOCs, control patterning. In examining if linkers are important for patterned assembly of microtubules in the alga Polytomella, Brown and Stearns (1978) found that when pressure is used to displace rootlet-MTOCs away from membrane-attached linkers, the MTOCs still retain control over initiation and patterning of microtubules. It would therefore appear that in most cases linkers function secondarily to maintain tubules in a pre-established pattern. However, the possibility should not be ruled out that in cells having less predominant MTOCs, linkers may play a more important role in controlling the assembly of microtubules.

Temporal Control of Assembly: In addition to spatial control MTOCs also exercise temporal control over assembly of microtubules. For example, partially isolated mitotic centers of activated clam eggs will promote assembly of clam egg or brain tubulin protein. Tubulin from either fertilized or unfertilized eggs is assembled providing the MTOCs are

obtained from fertilized eggs. This means that at the activation step a modification of the MTOC and not the tubulin is essential for assembly to occur (Weisenberg, 1973; Weisenberg and Rosenfeld, 1975). Providing another example of temporal control, Brown and Bouck (1974) have shown that during shape regeneration in Ochromonas the kinetobeak MTOC initiates assembly of microtubules to first establish anterior asymmetry of the cell before the rhizoplast MTOC initiates assembly of microtubules that form the tail. It generally appears, then, that MTOCs regulate assembly of tubulin essentially to control the distribution and timing of appearance of microtubules in the cell. The mechanism by which MTOCs can control initiation and assembly of microtubules is not understood, nor is it known if MTOCs function in conjunction with other cellular components in order to control formation of microtubules.

The temporal control of microtubule assembly could depend on factors such as the gradual modification of microtubule proteins or on subtle changes in the cellular environment itself. In studies of protein kinase-dependent phosphorylation during the cell cycle in Hela cells, an increase in phosphate content in tubulin was observed from interphase to mitosis (Piras and Piras, 1974), indicating that a modification of tubulin may be necessary for assembly.

Yet, given this data, one now questions whether the

control of assembly is simply a function of MTOCs and microtubule proteins. In other words, can modification of MTOCs or tubulin alone control assembly, or do other cellular constituents have a role?

Several studies have indicated that subtle changes essential for assembly may depend on ionic conditions and cyclic nucleotide levels in the cell. A role for Ca^{++} in assembly has been suggested by work showing addition of Ca^{++} to nerve tissue disrupts microtubules and simultaneously increases amounts of monomer with a binding affinity to colchicine (Gillespie, 1971). Similarly, spindle birefringence is diminished by dinitrophenol or methylxanthines which cause increases in Ca^{++} levels. Changes in cAMP levels may also indirectly control tubulin assembly. For example, cAMP has been shown to promote axonal elongation (Roisen et al., 1972a, b) and dibutryl cAMP has been found to promote increases in cell length and in the numbers of microtubules per unit cell volume in Chinese hampster ovary cells (Porter et al., 1974). Cyclic-AMP is postulated to exert its effect via a cAMP-dependent protein phosphorylation or possibly as an allosteric activator of an enzyme (Murray and Froscio, 1971; Magun, 1973). There is some evidence which indicates that cGMP and cAMP may work in concert to control assembly of tubulin (Kram and Tomkins, 1973). The generally accepted concept is that cells control microtubule assembly by

controlling subtle changes in the balance of Ca^{++} and of cyclic nucleotide levels (Gillespie, 1971; Kirkland and Burton, 1972; Mazia et al., 1972; Rasmussen et al., 1975).

As outlined in the above examples of temporal, spatial and ionic controls of tubulin assembly, in vivo studies provide some insights into the way formation of microtubule organelles is manipulated in cell development. However, to determine the precise nature of the mechanism of controlling assembly, and ultimately the structure and function of microtubules, studies have been conducted in vitro.

MICROTUBULE ASSEMBLY in vitro

The development of in vitro assembly studies can be attributed to two important discoveries, the first being that brain tissue contains 10% tubulin protein (Borisy and Taylor, 1967) and secondly that high speed extracts of cold solubilized brain tubulin would assemble normal microtubules when warmed to 37°C with small amounts of GTP and Mg^{++} if Ca^{++} was removed (Weisenberg, 1972). Procedures for purifying assembly-competent tubulin to more than 80% homogeneity were developed using cycles of temperature induced assembly-disassembly of microtubules (Borisy et al., 1974; Shelanski et al., 1973). Recently, similar methods have been used to cycle tubulin extracted from tissue culture cells (Nagle et al., 1977). Thus, an easy access to large quantities of

functionally competent tubulin has stimulated a burgeoning number of *in vitro* studies of tubulin assembly.

The objectives of these studies have been basically two fold: to analyze conditions influencing assembly and to determine the mechanism governing tubulin assembly. Considerable progress has been made in the *in vitro* work partly because it is possible to quantitatively monitor assembly using optical techniques (turbidometry, flow birefringence, viscometry, and electron microscopy).

The main conditions found to regulate tubulin assembly *in vitro* have been summarized in Table I with references to sources of the work. The Table clearly shows that the assembly of tubulin *in vitro* occurs within physiologically significant ranges for ionic strength, pH, temperature, nucleotides, Mg^{++}, and Ca^{++}. Under these conditions, tubulin assembly resembles formation of microtubules *in vivo* in that (a) it occurs at low levels of tubulin (0.2 mg/ml); (b) the microtubules exist in a dynamic equilibrium with subunits at a critical equilibrium concentration of 0.2 mg/ml; (c) the tubulin is sensitive to anti-microtubule drugs and physical conditions (pressure, temperature) known to inhibit assembly *in vivo*; (d) the tubulin exhibits a requirement for high heat capacities involving positive enthalpies and entropies. This latter property suggests that a release of water is the basic mechanism driving assembly *in vivo* and

TABLE I. CONDITIONS GOVERNING TUBULIN ASSEMBLY in vitro

FACTORS	ASSEMBLY	DISASSEMBLY	REFERENCES
Temperature	>15°C; <40°C optimal 37°C	<10°C; >40°C	Borisy et al., 1974 Olmsted et al., 1974
pH	5.8 - 7.6 optimal 6.0 - 6.9	<5.8; >7.6	Olmsted et al., 1974 Gaskin et al., 1974; 1975 Lee et al., 1974
Nucleotides	1-3 mM GTP (and other nucleotides)		Kuriyama and Sakai, 1974 Gaskin et al., 1974; 1975
Buffers	Ionic strength 0.08-0.01 Good Buffers 1 mM EGTA, Mg^{++}	>150 mM Na^{+} >1 mM Ca^{++} >150 mM K^{+}	Olmsted et al., 1974 Gaskin et al., 1974; 1975 Haga et al., 1974
Critical Concentration	Crude Extract = 0.8 mg/ml Temperature Cycled = 0.2 mg/ml Pure Tubulin = 0.2 mg/ml		Olmsted et al., 1974 Borisy et al., 1974 Gaskin et al., 1974
Energy of Activation and Assembly	+ve enthalpies +ve entropies	Reverse conditions	Gaskin et al., 1974; 1975 Lee et al., 1974 Haga et al., 1974
Accessory Proteins	High molecular weight (HMW or MAPs) > 250,000 daltons Tau 52-62,000 daltons Protein Kinase 280,000 daltons		Borisy et al., 1975 Sloboda et al., 1976a,b Weingarten et al., 1974; 1975 Penningroth et al., 1976 Shigekawa and Olsen, 1975
Exogenous Agents	Glycerol, D_2O, Sucrose, DMSO, > 10 mM Mg^{++}, Polycations, Zn^{++}, DTT		Stephens and Edds, 1976 Himes, 1977 Herzog and Weber, 1977
Other Inhibitors	Colchicine, Podophyllotoxin, Vinblastine		Wilson et al., 1975 Wilson and Bryan, 1974 Salmon, 1975a; 1975b

in vitro (Anfinsen, 1973). This suggestion is supported by studies showing that water-seeking compounds like D_2O will stimulate tubulin assembly under *in vivo* and *in vitro* conditions.

The Mechanism of Tubulin Assembly: With this development of *in vitro* conditions for assembly of normal microtubules under near physiological conditions, it has been possible to examine the mechanism of assembly. The result has been a growing number of studies in the past four years attempting to resolve this mechanism in terms of the proteins involved at the molecular level.

One of the first findings was that temperature-cycled brain tubulin contains about 15% non-tubulin protein which coassembles with tubulin. Apparently depending on the laboratory and on the temperature cycling techniques used, the accessory proteins were found to be either of a high molecular weight class of proteins (Murphy and Borisy, 1975; Sloboda *et al.*, 1976) or of a low molecular weight class (Weingarten *et al.*, 1975). When tubulin was purified from these accessory proteins, the tubulin would not self-initiate microtubules, but it has been demonstrated that either class of these accessory proteins could function to promote the initiation of microtubule assembly (Murphy and Borisy, 1975; Weingarten *et al.*, 1974). In addition to promoting initiation, the two classes of accessory proteins

have each been found to also function in promoting assembly during the elongation phase of microtubule formation (Sloboda et al., 1976a; Witman et al., 1976). Recently, the high molecular weight proteins have been shown to function in a third capacity in stabilizing the assembled microtubules (Murphy et al., 1977). Whether the low molecular weight proteins also function in this capacity has not been tested.

One of the important questions with respect to in vivo studies is how the accessory proteins actually promote tubulin assembly? In response to this question, the mechanism of assembly has been demonstrated using negative staining electron microscopy. The studies show that both high and low molecular weight accessory proteins induce formation of 42 to 49 nm rings or discs. Rings then serve as initiating sites somehow "nucleating" the assembly of tubulin protein (Borisy et al., 1974; Kirschner et al., 1975a, b; Sloboda et al., 1976b). It turns out that rings consist of tubulin and accessory proteins in a 23:1 molar ratio and under assembly conditions the rings appear to unwind and form protofilaments which aggregate to make a microtubule wall (Erickson, 1974, 1975; Kirschner et al., 1975a, b; Sloboda et al., 1976b). The stabilized microtubule helix seeds assembly of tubulin dimers and during elongation accessory proteins associate with tubulin to

promote elongation directly (Sloboda et al., 1976b; Witman et al., 1976), or bind to tubules to stabilize them against disassembly, thereby indirectly promoting elongation (Murphy et al., 1977). At the molecular level, the accessory proteins, or MAPs (see Terminology), are thought to actually shift the equilibrium to promote assembly by somehow causing the longitudinal and lateral binding of tubulin to form microtubules.

A major problem with these studies is whether rings are involved in tubulin assembly in vivo. Rings are not seen in situ, admittedly, it would be difficult to find such small structures, and because rings do not appear to form any part of MTOCs that normally initiate assembly of microtubules. Recently, studies with tubulin protein obtained from cell cultures and from brains of poikilotherms (Nagle et al., 1977; Langford, 1977; Stearns et al., 1978) have shown protofilaments but not rings form as assembly intermediates. Rings can be induced in these preparations using MAPs obtained from brains of homeotherms (Nagle et al., 1977; Stearns et al., 1978), suggesting that rings are peculiar to these proteins and not essential as "nucleating" sites for assembly to occur.

Another area of contention has been whether the low molecular weight MAPs represent a distinct class of proteins or whether they are breakdown products of high molecular

weight MAPs. Kirkpatrick (1970) originally showed that microtubules isolated intact contain tubulin and several high molecular weight MAPs. However, temperature cycling studies have shown that low molecular weight MAPs are also associated with purified microtubules. Sloboda et al. (1976a) have shown that aging of high molecular weight MAPs will generate peptides of the low molecular weight species which can then function to stimulate tubulin assembly, and which cross-react immunologically with antibodies to high molecular weight MAPs. In contrast to Sloboda, Connolly et al. (1978) have demonstrated that antibodies made to the high and low molecular weight proteins are immunospecific, and that they do not cross-react. Recently, Cleveland et al. (1977a) have demonstrated that the MAPs of high and low molecular weights are indeed composed of distinct peptides. Furthermore, they show that although the different MAPs will simultaneously co-assemble with tubulin, the active component which promotes tubulin assembly are a low molecular weight MAPs termed tau. Murphy et al. (1977), on the other hand, have shown that in preparations of temperature-cycled tubulin containing both types of MAPs, the high molecular weight MAPs are the active component promoting tubulin assembly. The MAPs exhibit no apparent cooperativity (Cleveland et al., 1977a) and the problem as to which of the MAPs normally controls tubulin assembly remains unresolved.

A strong criticism of the above studies is that MAPs associate non-specifically with tubulin *in vitro* and may not be involved in assembly *in vivo*. In support of this argument, other MAPs of molecular weights 49K, 82K and 170K daltons have recently been implicated in promoting tubulin assembly in tissue culture and brain tubulin extracts (Nagle *et al.*, 1977; Schmitt *et al.*, 1977; Seeds and Maccioni, 1978). Furthermore, a variety of exogenous agents and positively charged proteins have been shown to substitute for MAPs to non-specifically promote tubulin assembly (see Table I). In favor of MAPs having some function in formation of microtubules *in vivo* is evidence showing that (a) both the high and the low molecular weight MAPs will co-purify with tubulin in stoichiometrically constant amounts through many cycles of assembly-disassembly (Borisy *et al.*, 1974; Sloboda *et al.*, 1976a, b; Penningroth *et al.*, 1976); and (b) antibodies made to one of the high molecular weight MAPs and to a low molecular weight MAPs (or tau) will specifically react with microtubules *in situ* (Connolly *et al.*, 1977, 1978; Sherline and Schiavone, 1977).

The assembly of microtubules is a highly organized event *in vivo*, in many cases controlled by MTOCs. In the random assembly of microtubules *in vitro*, the proteins or the mechanism involved may not be representative of

assembly in living cells. One approach would be to isolate MTOCs for studies of assembly onto these centers. But attempts to isolate MTOCs have met with very little success, and limited progress has been made in understanding how microtubules assemble on MTOCs. Studies with isolated axonemes, basal bodies, or neurotubules used to "seed" tubulin assembly have shown that tubulin assembles on heterologous "seeds" and does not require accessory proteins during the elongation phase of assembly (Allen and Borisy, 1974; Burns and Starling, 1974; Olmsted et al., 1974; Snell et al., 1974; Binder et al., 1975; Kuriyama and Miki, 1975; Murphy et al., 1977). A single report by Witman et al. (1976) maintains that small amounts of low molecular weight MAPs (tau) are required for elongation but this report has been refuted by Murphy et al. (1977). In assembly experiments using partially isolated mitotic centers, spindle poles and kinetochores have been shown to serve as assembly sites for partially purified brain tubulin containing varying amounts of MAPs (Snyder and McIntosh, 1975; McGill and Brinkley, 1975; Telzer et al., 1975; Gould and Borisy, 1976). To test if mitotic centers and not factors like inter-microtubule linkers or MAPs in the tubulin preparations used are responsible for assembly, procedures must first be developed for isolating mitotic centers. A distinct disadvantage of using mitotic centers

for such studies is that they are unstable and assembly from the centers appears unpatterned and random so that the effect of the MTOCs on assembly would be difficult to ascertain under even the best conditions.

To answer many of the questions concerning the process of tubulin assembly, it is necessary to first isolate stable MTOCs which promote assembly of microtubules in patterned arrays. Some of the problems which could be examined would be: testing the role of MTOCs in microtubule assembly; determining the mechanism of tubulin assembly in terms of proteins involved and intermediates formed in initiation of microtubules; testing buffer conditions for assembly; testing sensitivities of these microtubules to factors known to affect their stability *in vivo*. The main objective would of course be to simulate the assembly event *in vivo* in order to use information obtained *in vitro* to understand the properties of microtubules in cells.

In the alga *Polytomella*, rootlet-MTOCs represent highly structured examples of MTOCs and cytoskeletal microtubules emanating from these sites take on an easily identified pattern to maintain the elongated shape of cells (see figs. 3 and 4 in this thesis and figs. 2, 11c, 16, and 18 in Brown *et al.*, 1976). Isolating MTOCs from a microtubule assembly system of this nature would therefore provide for an ideal *in vitro* assembly system. In preli-

minary studies it was discovered that rootlet-MTOCs could be isolated from Polytomella in large numbers as an intact and very stable basal body rootlet complex like that seen in fig. 4. The isolated rootlet-MTOCs were found to be functionally intact and to exhibit a capacity to promote rapid assembly of exogenous preparations of crude brain tubulin (Stearns et al., 1976). Based on these preliminary studies it was possible to begin a careful analysis of tubulin assembly using a reconstituted system closely resembling that seen in vivo.

One objective of the work has been to determine if MTOCs actually stimulate formation of microtubules and to test the role of MTOCs in controlling initiation and patterning of microtubules. As part of this work, the biochemical composition of rootlet-MTOCs has been investigated to show if specific proteins are associated with MTOCs to initiate tubulin assembly. The compositional studies led to the development of procedures for extracting and purifying microtubule-initiating proteins found to be associated with MTOCs. In attempts to analyze the process of tubulin assembly at the molecular level, the objective of the second part of this thesis has been to identify and purify the microtubule-initiating proteins in extracts of MTOCs and in MAPs preparations. The capacity of these purified initiating proteins to stimulate tubulin assembly has been

compared (a) by kinetic analysis and (b) in morphological studies of assembly intermediates initiated. In the morphological studies, intermediates assembled by intact MTOCs have also been examined to determine how assembly might occur *in vivo*.

In this thesis I will report results showing:

1. Procedures for isolating intact basal body rootlet complexes from *Polytomella*;

2. The role of rootlet-MTOCs in controlling assembly of crude and purified brain tubulin protein;

3. The biochemical composition of rootlet-MTOCs;

4. Procedures for the identification and purification of microtubule-initiating proteins in crude extracts of rootlet-MTOCs and in crude preparations of brain microtubule accessory proteins;

5. The capacity of the purified initiating proteins of MTOCs and of brain microtubules to promote tubulin assembly.

Fig. 3 Median longitudinal section through the cell showing the distribution of cell organelles and general cell shape of the flagellate Polytomella.

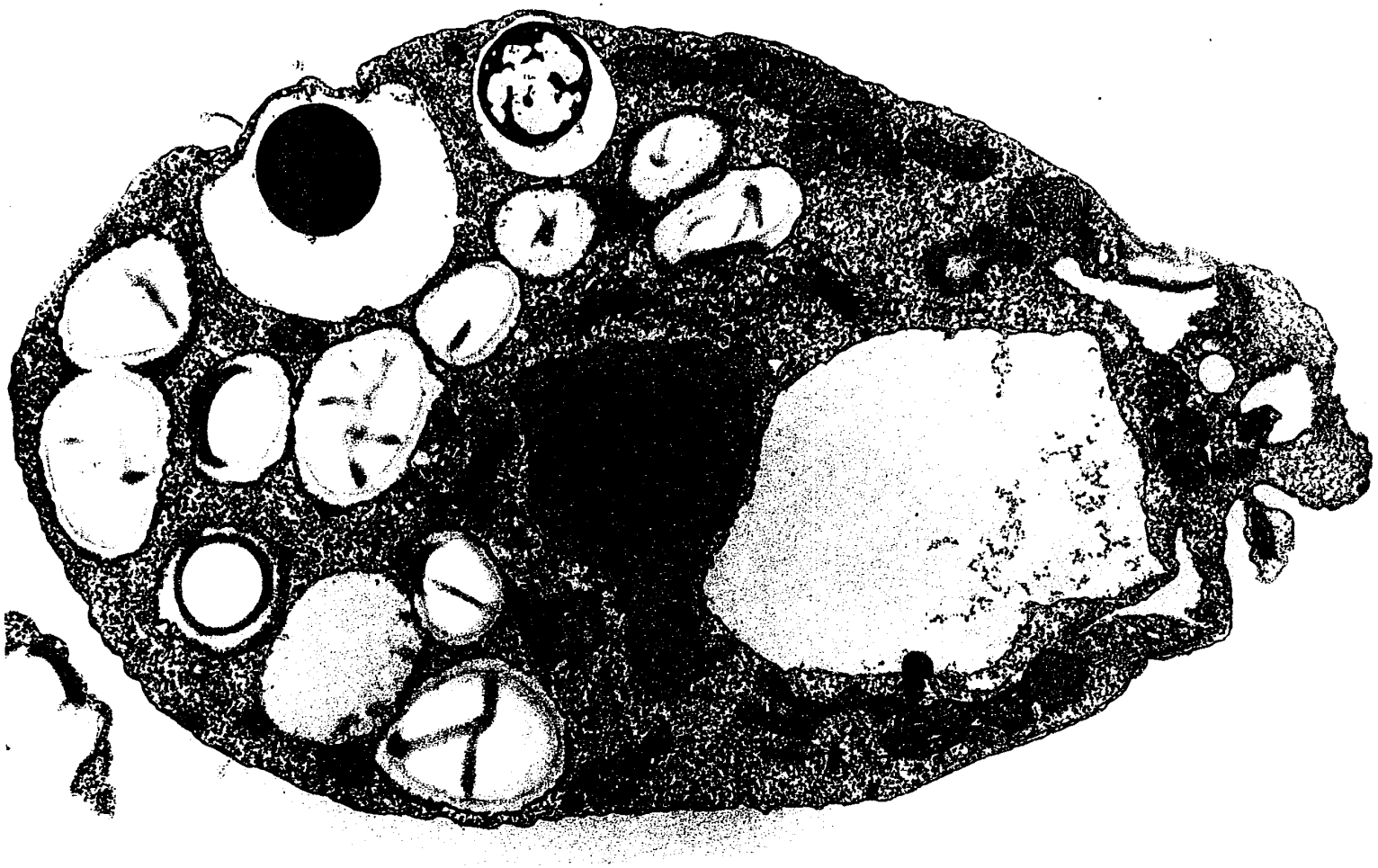
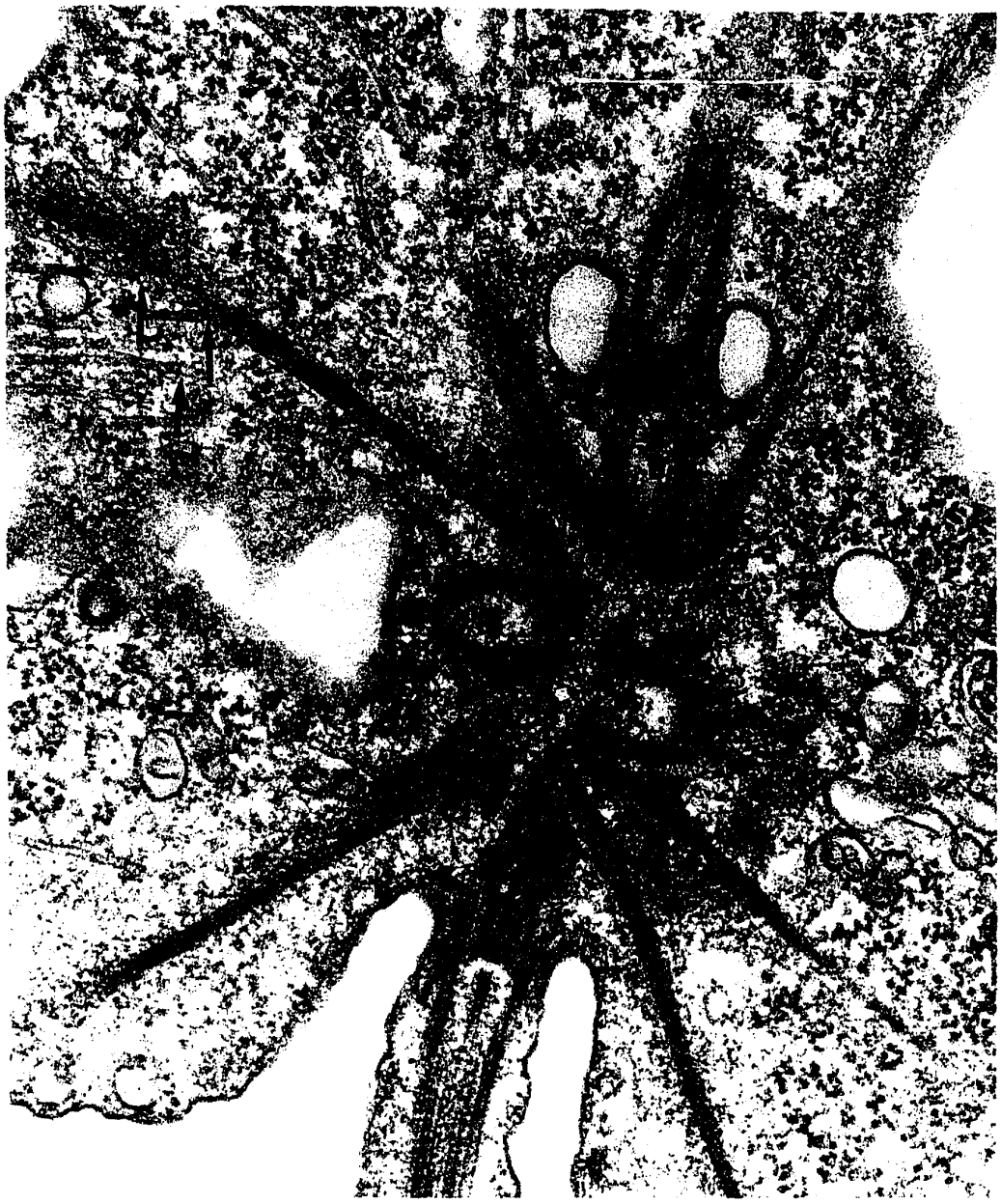


Fig. 4 Cross section through the anterior papilla region of the cell showing the four basal bodies (B) and attached rootlets (R). Cytoplasmic microtubules terminate (arrows) on amorphous material or sites associated with rootlets.



MATERIALS AND METHODS

CELL CULTURES

Cultures of Polytomella agilis Aragoa were grown in 4-liter erlenmeyer flasks in a complex medium containing 0.1% tryptone, 0.2% yeast extract, and 0.2% sodium acetate. The cultures were grown at 25°C in a dark constant-temperature incubator (Sheeler et al., 1968). Cultures were inoculated from log phase cultures giving a population density after inoculation of approximately 10^4 cells/ml. Mean generation time is 4.7 hours (Brown et al., 1976), and cultures were used in middle exponential growth phase approximately 40 hours after inoculation at a cell density of 2-5 x 10^6 cells/ml. Cells in 2-liter culture volumes were harvested at 23°C by centrifugation in a Sorval RC2-B centrifuge with a GSA head in 290 ml polycarbonate bottles at 4,000 rpm (or 2600 g) for 5 min. Cells were washed by resuspension in fresh growth medium and centrifugation in a IEC Clinical Centrifuge in 50 ml conical tubes at number 6 setting for 2 min.

ISOLATION OF BASAL BODY ROOTLET COMPLEXES

The procedures for isolating the basal bodies and attached rootlets as intact complexes is outlined in fig. 5a.

Five liters of cells at a density of 2-5 x 10^6 cells/ml were collected by centrifugation at 3000g for 5 min, washed

twice in fresh growth medium, and deflagellated by vortex agitation in a fluted tube (Rosenbaum and Child, 1969). The cell bodies were separated from the flagella by centrifugation (3000g, 5 min) in two washes of fresh medium and were then resuspended in 20 ml of a microtubule-stabilizing medium (Filner and Behnke, 1973; Forer and Zimmerman, 1974) containing 50% (vol/vol) glycerol, 10% (vol/vol) dimethyl sulfoxide, 5 mM $MgCl_2$, 5 mM NaH_2PO_4, at pH 6.9 (GDMP solution). The mixture was made 0.1% (vol/vol) Triton X-100 and the cells were broken by gentle vortical agitation in the fluted tube used for deflagellation. The released intact basal body-rootlet complexes could be detected by phase microscopy. The complexes were retained in the supernates following centrifugations at 3000g, 5 min and 4000g, 5 min, which pelleted large cell debris. The complexes were then pelleted at 12000g for 10 min, resuspended by vortex agitation in 5 ml of 5% sucrose containing 0.1% Triton X-100 and centrifuged at 3000g, 5 min to remove remaining debris. The supernate containing the complexes was centrifuged at 8000g, 10 min and the resultant pellet of complexes was resuspended in 0.7 ml of 5% sucrose and layered on a 3 ml gradient of equal volumes of 25%, 40%, 60% sucrose. Following centrifugation at 3000g, for 60 min, the complexes banded at the 40/60 interface. The band was pipetted from the gradient, diluted to 5% sucrose with distilled water, and centrifuged at 8000g,

10 min to pellet the complexes. The pellet was resuspended in a microtubule assembly buffer (MAB) containing 0.1 M piperazine-N, N-bis (2-ethane sulfonic acid), 1mM ethylene bis-(oxyethylene-nitrilo) tetraacetic, 1mM $MgCl_2$, 2.0mM guanosine triphosphate, at pH 6.4. A sorvall SS-34 rotor was used for all preparative centrifugation steps and a Beckman, SW-56 rotor was used for sucrose gradient centrifugation.

The presence of the complexes could be monitored by light microscopy at each stage of the isolation procedure (Fig. 5b). The number of complexes per ml was determined using a hemocytometer (Spencer Bright-Line Improved Neubauer) and diluted to a constant number of 2×10^6/ml in MAB for the microtubule assembly experiments. Light micrographs of the complexes were obtained using Zeiss phase-contrast optics and a Zeiss Ukatron UN 60 electronic flash.

TREATMENTS OF ISOLATED COMPLEXES

Trypsin digestion: Purified intact complexes were suspended in 5% sucrose containing 10 ug/ml trypsin and incubated at 37°C. After 7 min, excess soybean trypsin inhibitor (100 ug/ml) was added to stop the reaction. In control experiments excess inhibitor and trypsin were added simultaneously.

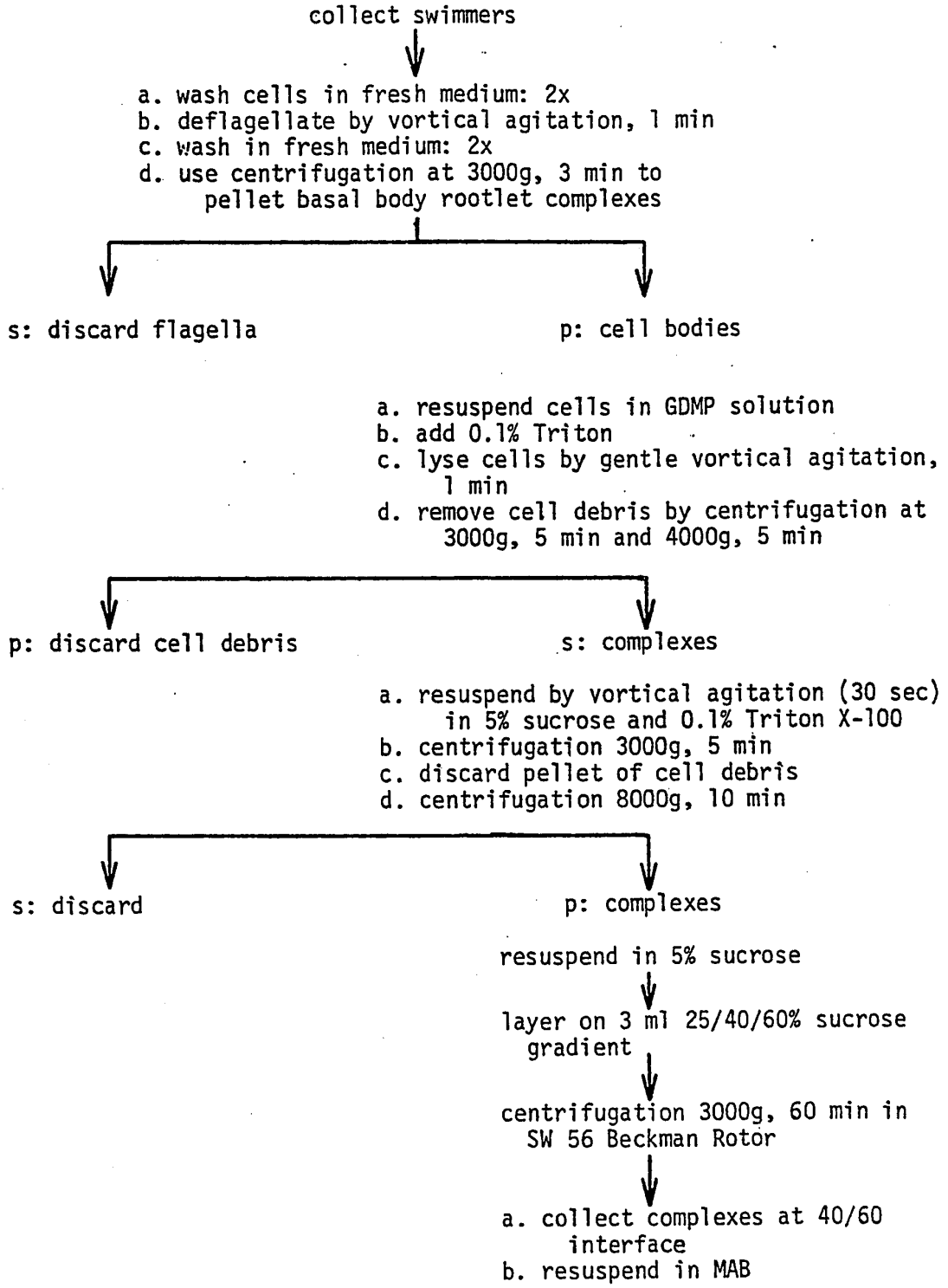


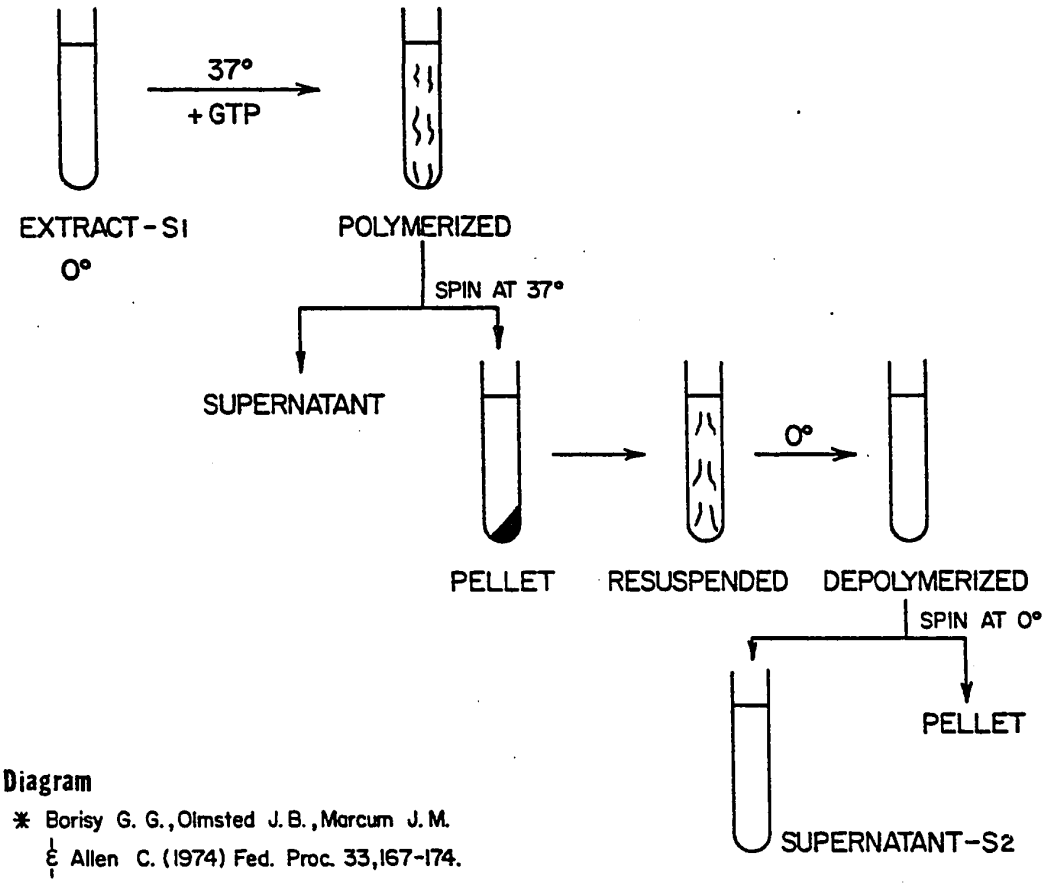
Fig. 5a. Procedure for the purification of basal body rootlet complexes from Polytomella

Extraction of rootlet-associated proteins (RAPs): Complexes in 5% sucrose were dialyzed at 4°C against 1mM Tris, 0.1mM Disodium Ethylenediamine-tetraacetate (EDTA), 0.01% vol/vol 2-mecaptoethanol, pH 8.0 (Bloodgood et al., 1976) for 20 min. The complexes were pelleted at 10,000g, 15 min and the supernate from this re-centrifuged at 100,000g for 30 min to obtain a high speed supernate containing rootlet associated proteins (RAPs).

PREPARATION OF MICROTUBULE PROTEIN

Brains of 16 to 21 day-old-chick embryos and beef brains were homogenized in a Waring blender in cold MAB and the homogenate centrifuged at 39,000g, 30 min to obtain a crude high speed supernate (S-1) containing microtubule proteins. To further purify brain tubulin, microtubule proteins in S-1 were subjected to two cycles of temperature-dependent assembly and disassembly by methods of Borisy et al., (1974). The procedure used is illustrated in the following diagram for one cycle and the second cycle is a repetition of these steps. To further purify tubulin, twice cycled S-3 samples were chromatographed on phosphocellulose following the methods of Weingarten et al., (1975). Accordingly, 1-2 ml of cycled microtubule proteins (S-3) at 8-10 mg/ml was applied to 1.5 x 8 cm columns containing 5 ml phosphocellulose (Whatman P-11) pre-equilibrated by

Diagram showing steps in purification of tubulin protein. Brain tissue is homogenized in a cold buffer (MAB) to extract tubulin protein and the homogenate centrifuged at high speed to obtain an S-1 extract containing tubulin protein (25% by weight). The tubulin is assembled at 37°C and microtubules pelleted by centrifugation then resuspended in fresh MAB at 0°C to disassemble the microtubules. Non-specific aggregates are removed by a cold centrifugation step and the supernatant containing tubulin protein (60% by weight), S-2, is retained for a second cycle to obtain an S-3 preparation of tubulin protein containing 75% tubulin and 25% microtubule-associated proteins (MAPs) that coassemble with tubulin (see Borisy and Olmsted, 1972; Borisy et al., 1974).



Diagram

* Borisy G. G., Olmsted J. B., Marcum J. M.
Allen C. (1974) Fed. Proc. 33,167-174.

washing with MAB. Unbound tubulin was eluted with MAB near the void volume and was immediately used for assembly experiments. The absorbed fraction, consisting of microtubule-associated proteins (MAPs) was eluted with MAB containing 550 mM NaCl and the salt removed by dialyzed against MAB.

Protein determinations were by the methods of Hartree, (1972) modified from Lowry et al., (1951). The percentage of tubulin in crude or cycled microtubule protein preparations was determined from densitometric scans of 10% SDS gels (Weber and Osborn, 1969) stained with Coomassie Brilliant Blue 250 R.

Purification of a microtubule initiating protein: "The-MAP" was purified from MAPs by incubating MAPs at about 0.3 mg/ml with tubulin at 2 mg/ml at 37°C briefly so that protofilament assembly intermediates formed. The mixture was then immediately centrifugated at 39,000 X g for 15 min to pellet the protofilaments. The pelleted protofilaments were cold solubilized in fresh MAB and the proteins composing protofilaments fractionated by chromatography on 1 ml of phosphocellulose in 1.5 x 1 cm columns at 4°C. Tubulin elutes in MAB near the void volume and the absorbed fraction containing "the-MAP" elutes in MAB made 550mM NaCl. Fractions containing "the-MAP" were concentrated with a Millipore immersible molecular separator vacuum filtration

apparatus or by dialysis against G-200 powder and used for assembly experiments.

To purify initiating proteins from RAPs preparations, the RAPs supernatant was dialyzed against MAB for 1 hr, then the microtubule-initiating proteins (TIPs) were selected for and purified by methods identical to those used to purify "the-MAP" from crude MAPs of brain microtubules (see section on Purification of "the-MAP"). The phosphocellulose-purified initiating proteins, "the-MAP" and TIPs were incubated with fresh tubulin for assembly experiments.

ASSEMBLY STUDIES

Viscometry: The effect of added intact complexes on the assembly of crude or purified tubulin preparations was assayed by viscometry. In comparative studies, the assembly occurring in two samples containing the same concentration of tubulin, to one of which was added 2×10^6 complexes/ml, were measured simultaneously using two Oswald viscometers (2 ml chamber volumes and buffer flow times of 155.6 sec). In a second set of experiments the initial rates of assembly and the viscosities attained at equilibrium were determined with a fixed concentration of purified tubulin and variable numbers of complexes or with a fixed number of complexes and variable tubulin concentration. In a third set of experi-

ments, a constant number of complexes (0.5 x 10^6/ml) were incubated with a constant amount of tubulin (2 mg/ml) and increasing amounts of "the-MAP" and TIPs. In all of these studies, samples at 4°C were added to viscometers, pre-equilibrated and maintained at 37°C in a temperature controlled water bath, and assembly was monitored continuously to equilibrium.

Turbidity: The assembly kinetics of purified tubulin incubated with the microtubule-initiating proteins, crude MAPs, "the-MAP", and TIPs was monitored at A_{350} using a Gilford Model 2400 equipped with a temperature controlled four chambered cuvette holder. Samples were checked by electron microscopy to ensure that changes in turbidity corresponded to microtubule assembly.

Electron microscopy: Negative staining was routinely used to detect the presence of microtubules in assembly studies. Samples were taken and fixed by the addition of an equal volume of 4% glutaraldehyde in MAB at 37°C. For negative staining a drop of the fixed sample was deposited on 400 mesh carbon coated grids, washed with drops of glutaraldehyde and 0.4% photo-flo, and stained with 1% phosphotungstic acid (PTA) or with 1% uranylacetate, 0.1% cytochrome C. In one set of experiments, to determine the extent of assembly onto intact complexes during early incubation periods (up to 5 min), a constant number of complexes

(2×10^6/ml) were incubated with varying amounts of crude chick brain tubulin. The numbers of microtubules assembled onto the basal body rootlets were counted from enlarged prints of 10 complexes for each tubulin concentration and time interval. In a second set of experiments, microtubule assembly intermediates initiated by "the-MAP" and TIPs were examined by fixing samples after brief incubation times for negative staining.

For thin sectioning the complexes or microtubules were pelleted and fixed at 20°C for 1 hr in 2% glutaraldehyde in 0.1 M sodium phosphate buffer at pH 7.2, and post-fixed at 4°C for 1 hr in 1% osmium tetroxide in the same buffer, then dehydrated in an acetone series of 10, 30, 50, 70, 90 and 100%. Samples were then infiltrated and embedded in Spurr's plastic (Spurr, 1969). Thin sections were cut using a diamond knife on a Porter Blum MT2-B ultramicrotome and sections stained with 2% uranylacetate (in 5% ethanol) and 1% lead citrate (Reynolds, 1963) for examination in an AEI-EM 6B electron microscope.

LABELING STUDIES - SULFATE ^{35}

Batch cultures of Polytomella agilis aragoa were grown in 1-liter quantities for 12 to 14 generations in media containing 0.125 mCi/ml $NaSO_4^{35}$ (New England Nuclear). Cells were harvested at log phase, complexes isolated and

rootlet-associated proteins extracted by methods described to obtain a high speed supernatant extract containing rootlet-associated proteins. The specific activity of the extract was calculated from a standard curve comparing radioactive counts to Lowry values and this value was used to determine amount of TIPs-S^{35} associating with tubulin in assembly experiments. For assembly studies phosphocellulose-purified beef tubulin was incubated at 37°C with TIPs-S^{35} and the mixture centrifuged to pellet microtubules formed. Microtubules were resuspended in warm MAB for negative staining studies or in 5 ml Aquafluor for scintillation counting on a Beckman LS-233 Beta Counter.

ANALYTICAL ULTRACENTRIFUGATION

Samples of freshly prepared 2 X cycled beef brain tubulin (S-3), tubulin (6S), and tubulin (6S) mixed with crude MAPs, "the-MAP' and TIPs were dialyzed 1 hr against excess MAB at 5°C prior to centrifugation. Varied concentrations of S-3, and 6S were subjected to centrifugation to determine the molecular weights of components in these preparations. In other experiments a set concentration of 6S at 8 mg/ml was mixed with varied amounts of either crude MAPs, "the-MAP", or TIPs from 0.2 to 0.5 mg/ml at 5°C and the different mixtures centrifuged to determine the molecular weights of components. To examine molecular weights of

microtubule disassembly products the above types of samples were incubated at 37°C and microtubules then disassembled at 5°C prior to analytical ultracentrifugation. A Beckman model E with a single sector cell and Schlieren optics was used for all the experiments. Rotor speed was 48,000 rpm at a constant temperature of 5°C and pictures were taken at 4 min intervals. $S_{20,W}$ values were obtained by plotting values at various concentrations of protein used and extrapolating the sedimentation coefficient to infinite dilution. Samples were examined by negative staining before and after centrifugation as another means of assaying the types of structures present in the samples.

GEL ELECTROPHORESIS

SDS gels: Samples were denatured and prepared for gel electrophoresis using methods of Weber and Osborn (1969). Samples were made 0.01M PO_4 (from 0.1M stock, pH 7.2), 3% SDS, 1% 2-mercaptoethanol and placed in a boiling water bath for three minutes. Samples were then allowed to sit 6 - 12 hours, and then dialyzed for 12 hours against one liter of 0.01M PO_4, 1% SDS, 10% glycerol, 1% 2-mercaptoethanol.

Gels were made 6 to 10% polyacrylamide containing 0.1% SDS and were pre-electrophoresed for three hours prior to protein application. The running buffer used was 0.1% SDS, 0.1M PO_4, pH 7.2. Proteins were loaded on gels with 0.001%

bromophenol blue tracking dye and separated by electrophoresis at 8 ma/gel for tube gels and 30 ma/gel for 0.75 mm dia. slab gels. Gels were run until dye neared the bottom of the gel (5 to 6 hours for disc gels and 10 to 12 hours for slab gels). Gels were removed with a continuous stream of running buffer from a syringe using a fine gauge needle, and standardly fixed overnight in 20% sulfosalycylic acid, then stained in 0.5% aqueous Coomassie Brilliant Blue 250-R and destained overnight in 7% acetic acid.

Disc gels were 5 mm in diameter and approximately 85 mm in length, slab gels were 40 mm wide, 100 mm long and 0.75 mm thick. A 20 well slot was usually used. To ensure detection of all proteins present in samples, a series of increasing amounts of protein from 20 ug to 150 ug of protein were routinely examined by electrophoresis for each sample.

To prepare a standard curve for the SDS gel system, protein standards used were myosin from rabbit skeletal muscle, cytochrome C, catalase, bovine serum albumin, egg albumin, and chymotrypsinogen A. 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, 1969).

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

Gels were scanned with a Vitatron LD 100 densitometer with a linear transport mechanism at 3 cms/min with a narrow slit aperature at 546 nm.

RESULTS

MICROTUBULE ORGANELLES OF POLYTOMELLA

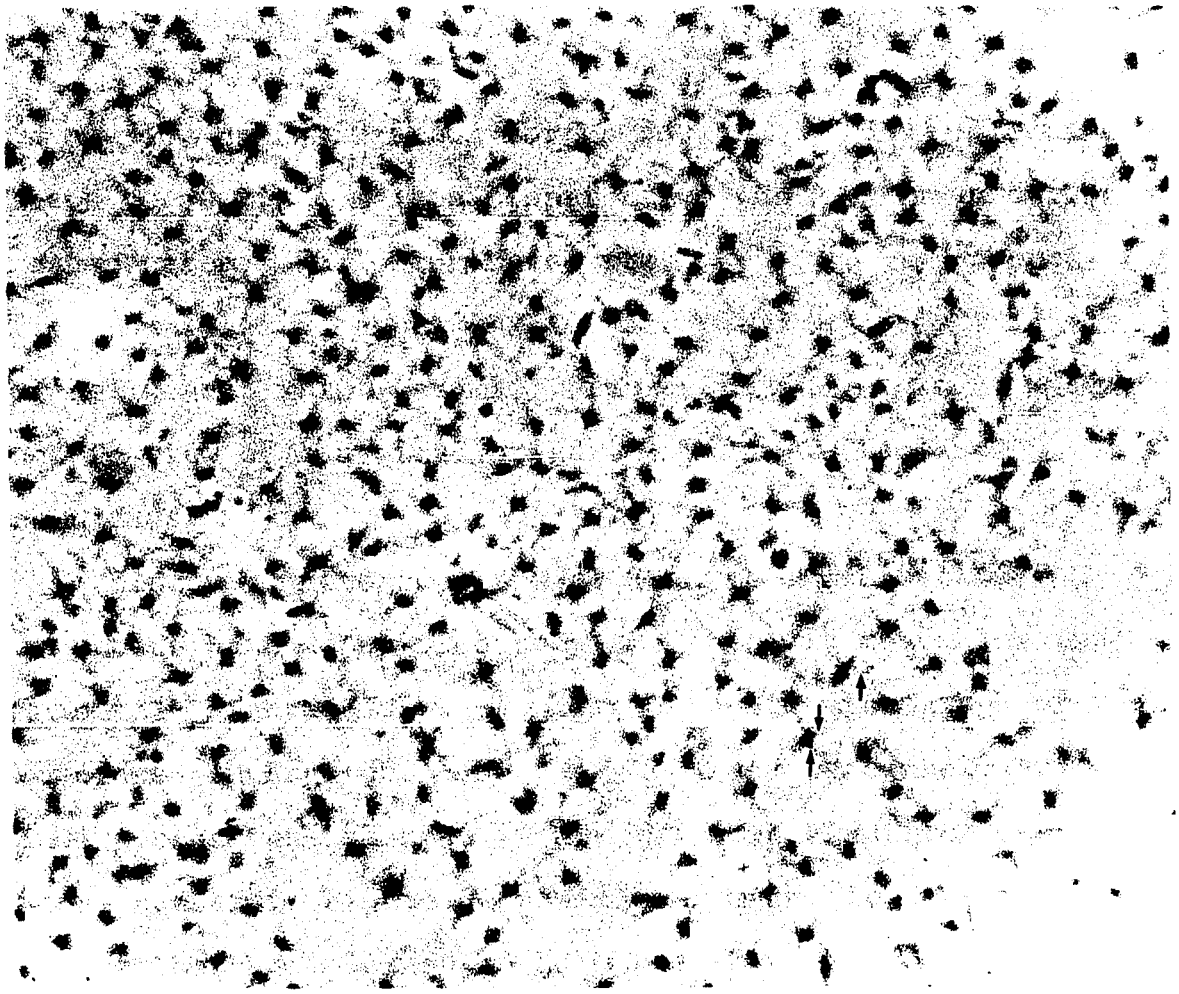
Polytomella agilis is a single-celled colorless green alga that exists in a free swimming vegetative form in exponentially growing cultures. The motile cells are 12 um long by 7 um wide and appear elongate with a single anterior projection called the papilla (see fig. 3 in the introduction). The organism is a quadriflagellate and the flagella emerge from depressions at the base of the anterior papilla. The external appearance of motile cells, the structural organization of the cells and their microtubule system have been described in detail by Brown et al. (1976). The distribution of the microtubules in Polytomella has been discussed in the introduction to familiarize the reader with the structural organization of basal body rootlet MTOCs in vivo. Essentially, the structure consists of four basal bodies linked together to serve as anchors for the flagella and rootlets. The rootlets are coated with an amorphous electron dense material and serve as rootlet-MTOCs for controlling assembly of cytoskeletal microtubules (see fig. 4 and Brown and Stearns, 1978).

ISOLATION OF BASAL BODY ROOTLET COMPLEXES

Procedures have been described in the Materials and

Methods section of this thesis for isolating intact basal body rootlet complexes from Polytomella. The key to purification of large quantities of MTOCs is to maintain the complexes as intact and stable structures which can be easily detected by light microscopy and monitored throughout the isolation procedure (fig. 5b). This is accomplished by the gentle lysis of deflagellated cells into a microtubule-stabilizing medium containing a high percentage of glycerol (GDMP). When the cell bodies are suspended in this solution they immediately shrink and appear crenulated. The addition of low concentrations of Triton X-100 (below 0.10%) causes little or no lysis by itself but the cells are quite fragile and are easily lysed by mechanical agitation. A gentle vortex treatment at this stage lyses virtually all the cells and releases the 4 basal bodies with 8 attached rootlets as intact complexes. If the flagella are not removed prior to the lysis step many of the complexes either remain attached to the cell membrane or are apparently disrupted so that yields of intact complexes are greatly reduced. The gentle lysis of the cells leaves much of the cell debris as large fragments which can subsequently be removed by low speed centrifugation leaving the complexes in the very dense GDMP solution. At this stage the complexes can be removed from GDMP at a higher centrifugation speed and are stable to subsequent purification steps (fig. 5a). It is important

Fig. 5b Flash photomicrograph of purified basal body rootlet complexes. The presence of the intact complexes can be monitored by phase microscopy throughout the isolation procedure. Each black dot (arrows) represents four attached basal bodies and the rootlets are seen as faint projections. x3,200.



to use low centrifugation forces for each of the pelleting steps to permit complete dispersal of the complexes during resuspension. Following these procedures yields of purified complexes at about 3×10^7 are obtained from 5 liters of cells at 5×10^6 per ml. Figure 5b shows a light microscopic view of a typical preparation of the isolated complexes.

Electron microscopy shows that the organization and structure of the purified complexes closely resembles that in situ. Figure 6 shows a negative stain micrograph of the complexes in fig. 5b. The basal bodies are densely stained and rootlets are seen as fibers (R) emanating from these dense centers. Figure 7 is a higher magnification view of one of these complexes and it shows that the dense centers in figure 6 consist of 4 basal bodies to which the rootlets are attached to form an intact basal body rootlet complex. Figure 8 shows the four basal bodies are still interconnected by fibers into 2 opposing pairs and the rootlets remain attached at the anterior ends of the basal body. The electron-dense material of the rootlet MTOCs is retained and in longitudinal section appears as a coating along the sides of the rootlets (arrows). The cytoskeletal microtubules, which terminate on this material in the intact cell, are no longer present.

Fig. 6 Negative stain (1% PTA) micrograph of isolated complexes. Rootlets (R) are seen to extend from the densely stained centers containing basal bodies (B).

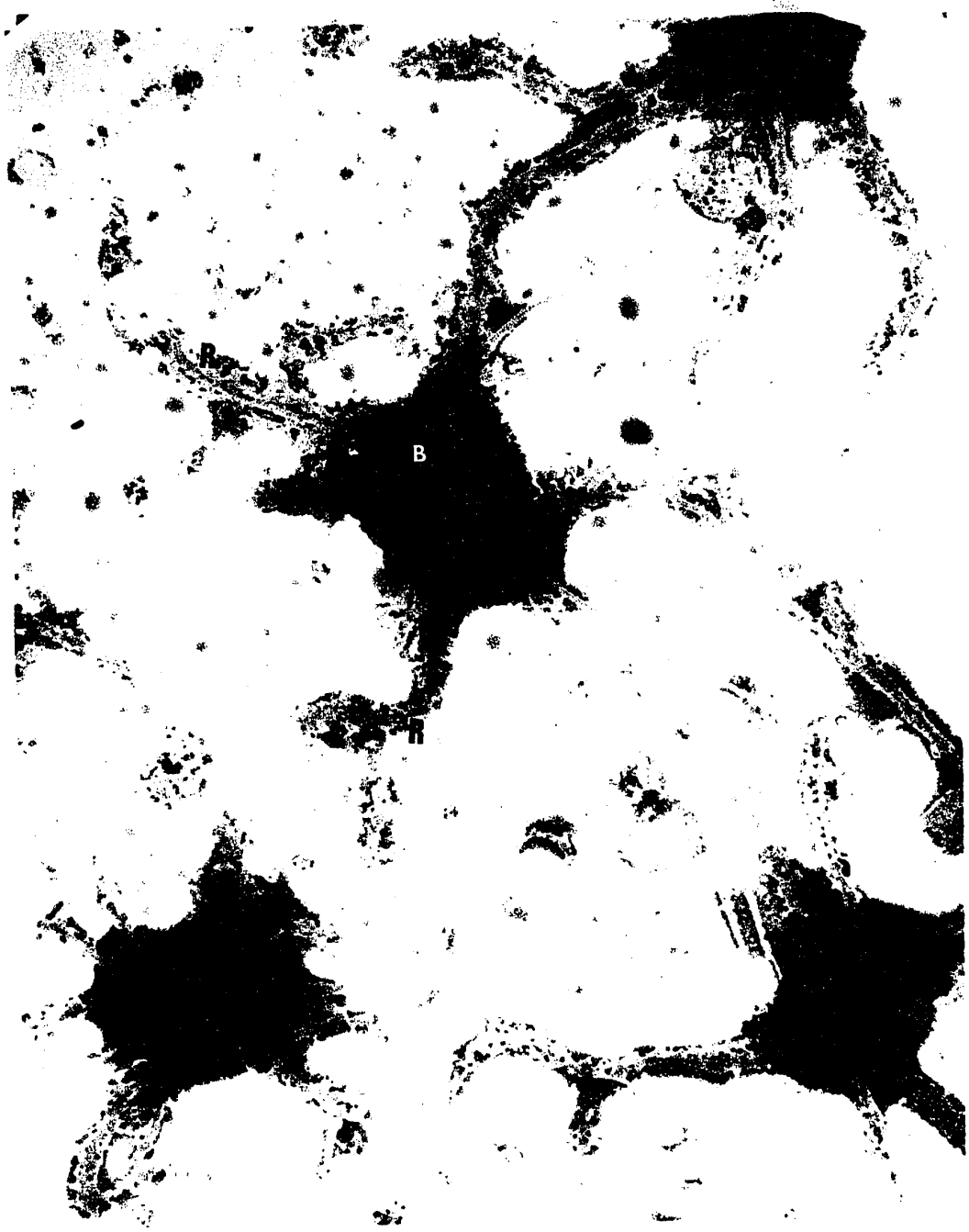

B

Fig. 7 A higher magnification view of a complex that has been dodged in printing to show the four basal bodies and rootlets.

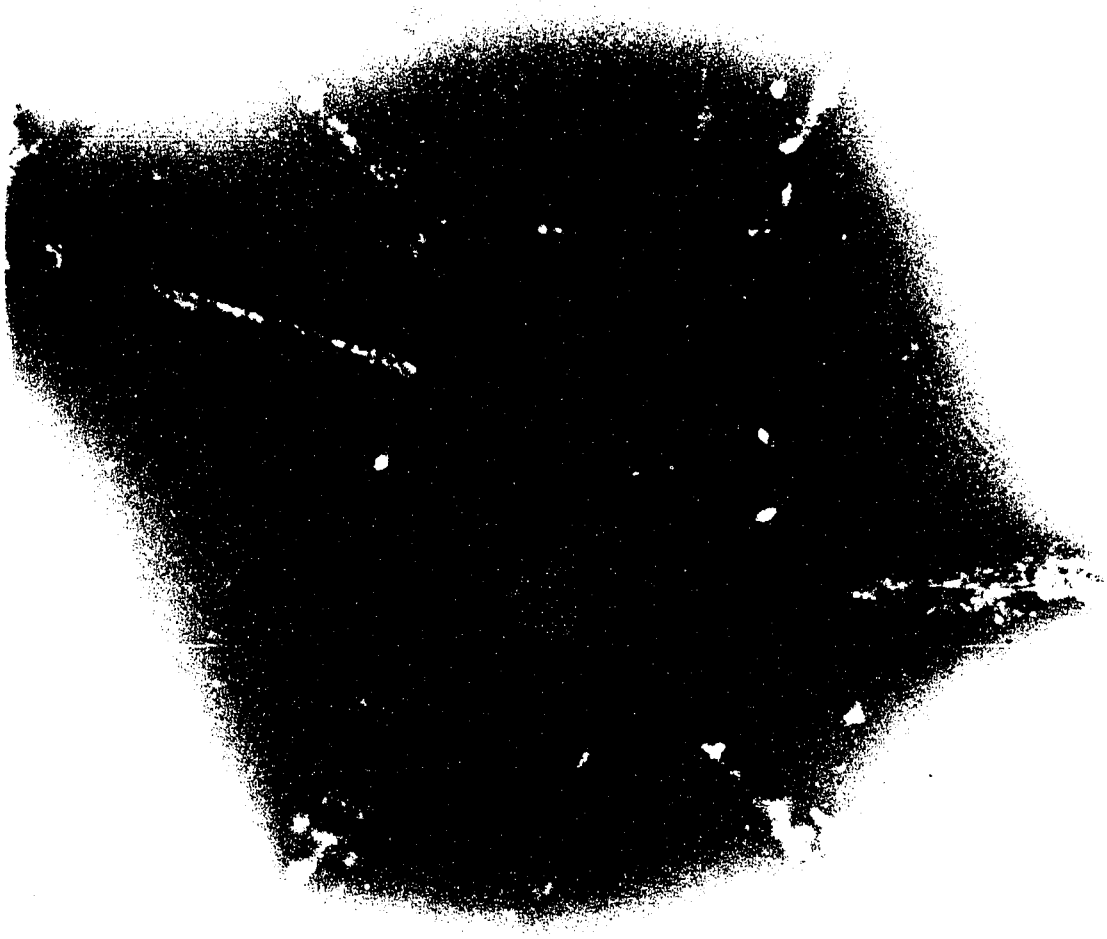


Fig. 8 Transverse section through an isolated complex. If compared with a similar *in situ* view, fig. 4, the complex appears intact. Four basal bodies and attached rootlets are present and rootlets have retained a coated appearance. (arrows) Cytoplasmic microtubules detach during the isolation steps. Some membrane is often associated with the distal end of the basal bodies.

ISOLATED MTOCs STIMULATE MICROTUBULE ASSEMBLY

To test if the isolated basal body rootlet complexes retain a capacity to initiate tubulin assembly *in vitro*, complexes were incubated with brain microtubule proteins. Figure 9 is a negative stain view of one complex after incubation with tubulin (1.25 mg/ml) for 5 min showing large numbers of microtubules associated with the complex. It is difficult to determine the attachment points of the microtubules. In thin-sections (figs. 10, 11) it is clear that the microtubules are assembled at specific sites, in a row on the electron-dense material coating the sides of the rootlets (arrows). The pattern of these *in vitro* assembled microtubules closely resembles the pattern of cytoskeletal microtubules in intact cells (see Brown and Stearns, 1978). The distal ends of the basal bodies are capped by membrane (fig. 8) and little or no assembly is detected at these sites (figs. 10, 11) except when long incubation times are used.

The extent of assembly onto complexes was determined by negative staining and viscometry. Figure 12a-c shows examples of complexes incubated for 1, 2 and 5 min periods at a constant tubulin concentration and figure 12 d-f shows examples taken after a 5 min incubation with increasing concentrations of tubulin protein. It is apparent that both the numbers and lengths of microtubules assembled are depen-

Fig. 9 A complex after incubation with brain tubulin extracts (S-1) at 37°C. Tubulin was at 1.25 mg/ml and the incubation time was for 5 min before the reaction was stopped with 4% gluteraldehyde and the complexes prepared for negative staining with 1% PTA.

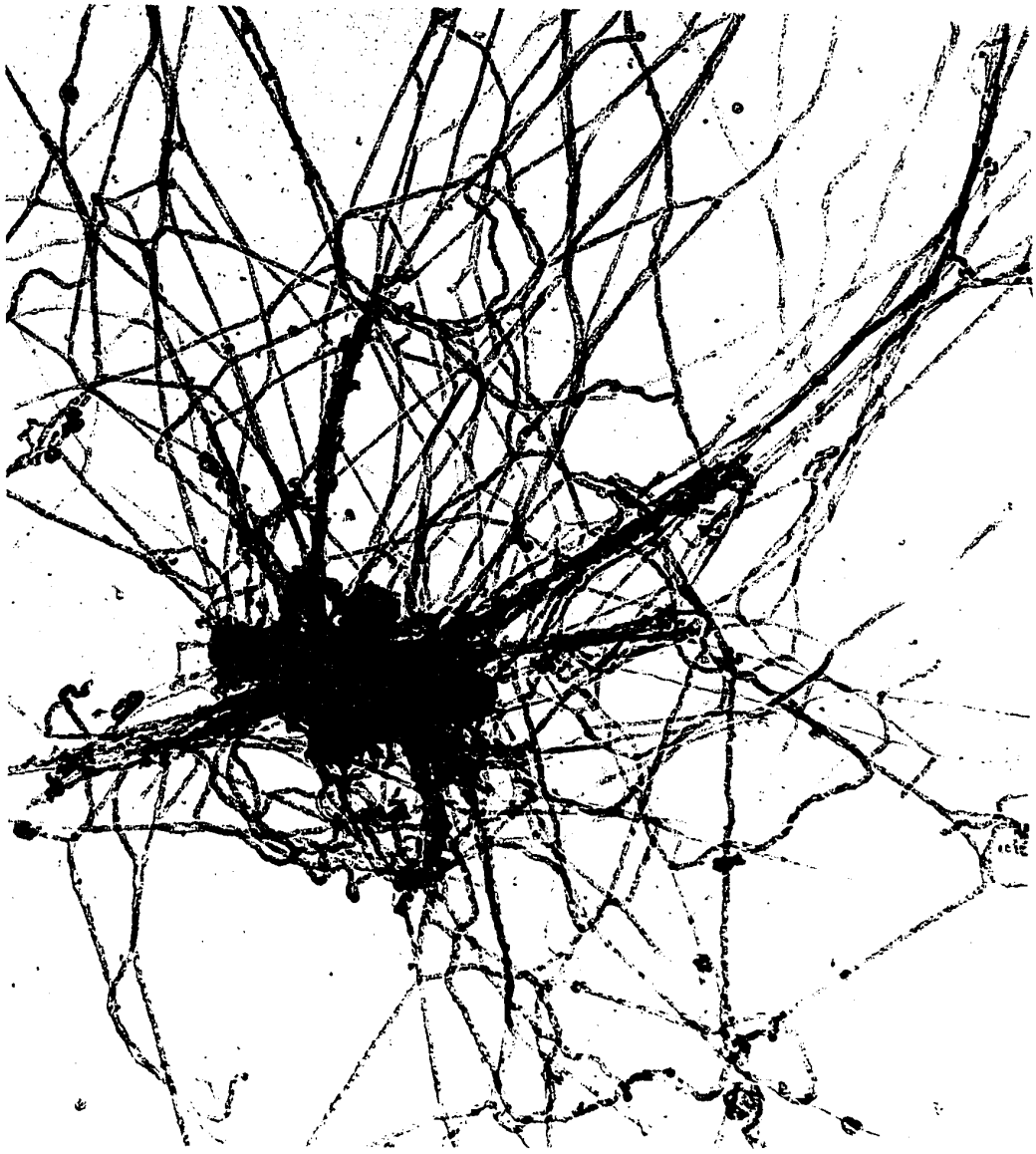


Fig. 10-11 Thin section views of complexes in fig. 9. Fig. 10 shows 3 basal bodies, attached rootlets and associated brain microtubules. Fig. 11 shows in a higher magnification view, part of 2 basal bodies and one rootlet to illustrate the tendancy of microtubules to assemble in parallel arrays at the same angle to the rootlet.


10

11

Fig. 12 Negative stain composite of brain microtubules assembled onto basal body rootlets. The reaction was stopped with 4% glutaraldehyde and complexes stained with 1% PTA. Fig. 12 a-c demonstrates at 1.25 mg/ml tubulin in (S-1) the extent of tubulin assembly onto complexes after incubation times of 1, 2 and 5 min. Fig. 12 d-f shows after a 5 min incubation period, the degree of assembly on complexes at 0.8, 1.10 and 2.54 mg/ml tubulin in S-1 extracts. In samples for fig. 12 a, b, d nearly all the microtubules are seen attached to complexes whereas in samples for fig. 12c, e, f, independently assembled microtubules were seen in the background on grids.

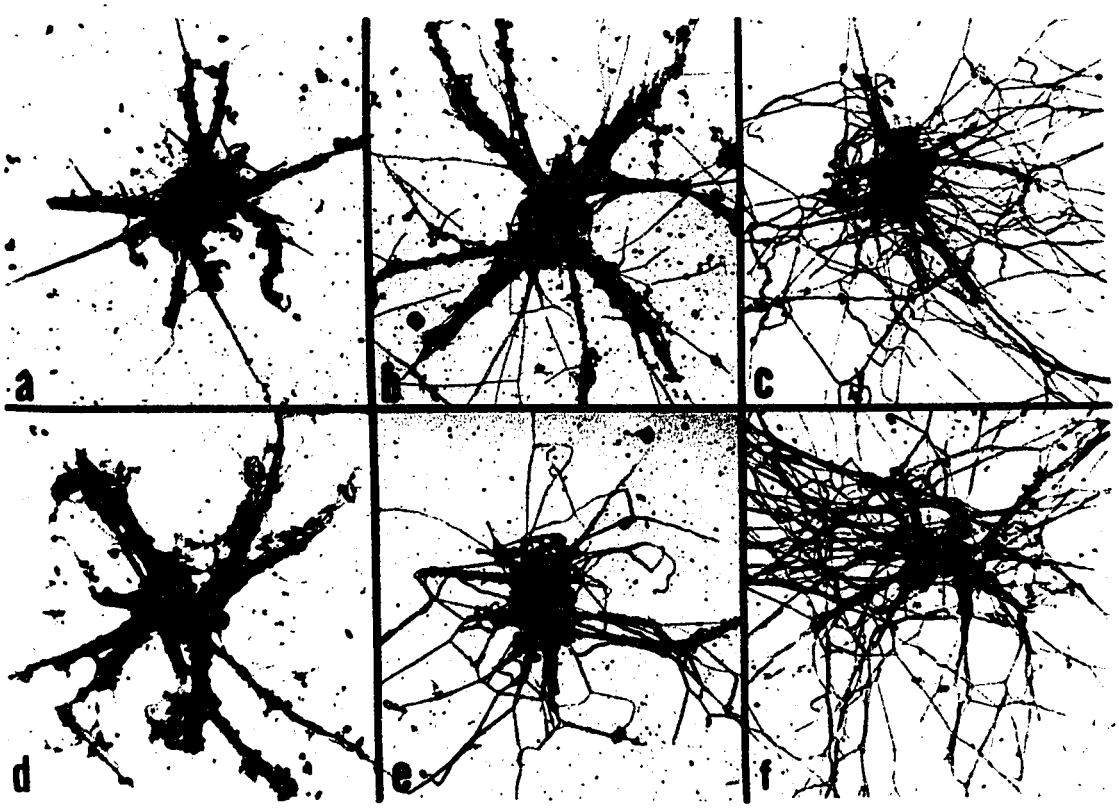

a
b
c
d
e
f

Fig. 13 An average of the numbers of microtubules per complex was obtained from enlarged micrographs of 10 complexes for different time points and for several tubulin protein concentrations tested at 0.8 (◉); 1.0 (◘); 1.84 (▲) and 2.54 (●) mg/ml. The graph quantitatively compares the extent of microtubule assembly onto complexes at 2 x 10^6 complexes/ml.

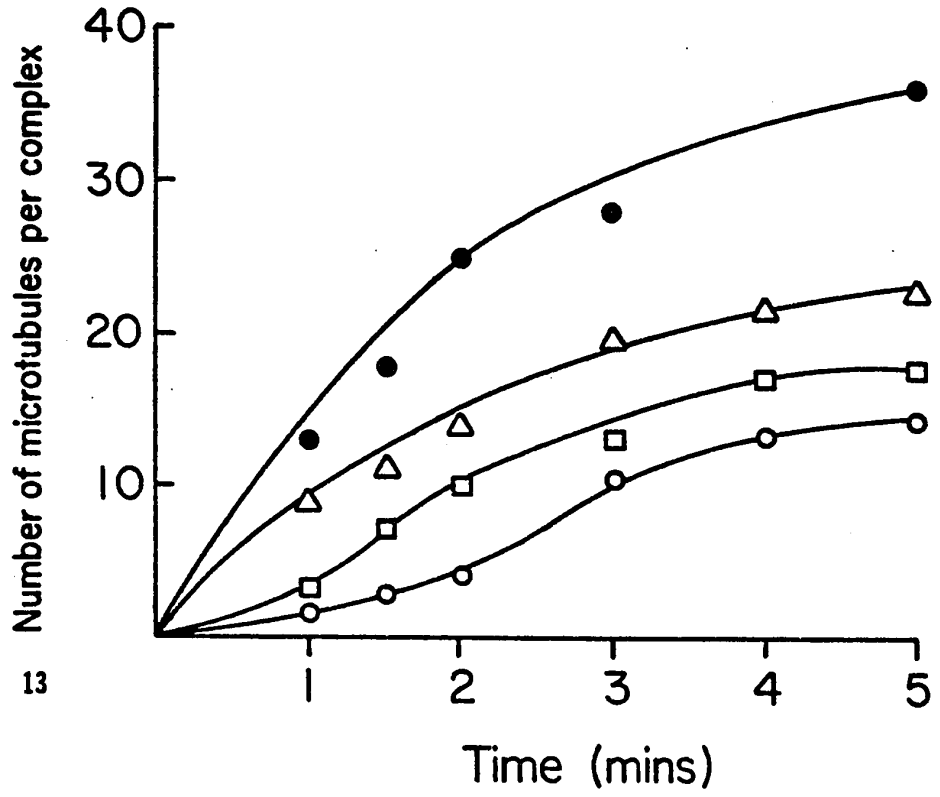

40
30
20
10
Number of microtubules per complex
1
2
3
4
5
Time (mins)

Fig. 14 Graph comparing the changes in specific viscosity (η_{sp}) to equilibrium of crude brain tubulin (S-1)(o) and crude brain tubulin (S-1) mixed with 2 x 10^6 complexes/ml (•) at 1.25 mg/ml tubulin protein. Curves are averaged from 10 separate experiments in which freshly prepared tubulin protein was used. Bars represent 2 standard deviations. In the absence of complexes, an extended initial lag phase of about (10 min) was observed before an increase in viscosity occurred. The addition of complexes to samples decreased the initial lag phase (to 5 min) and increased the final viscosity values attained. The results indicate complexes provide initiating sites which can immediately start microtubule assembly without the lag phase normally observed for brain tubulin.

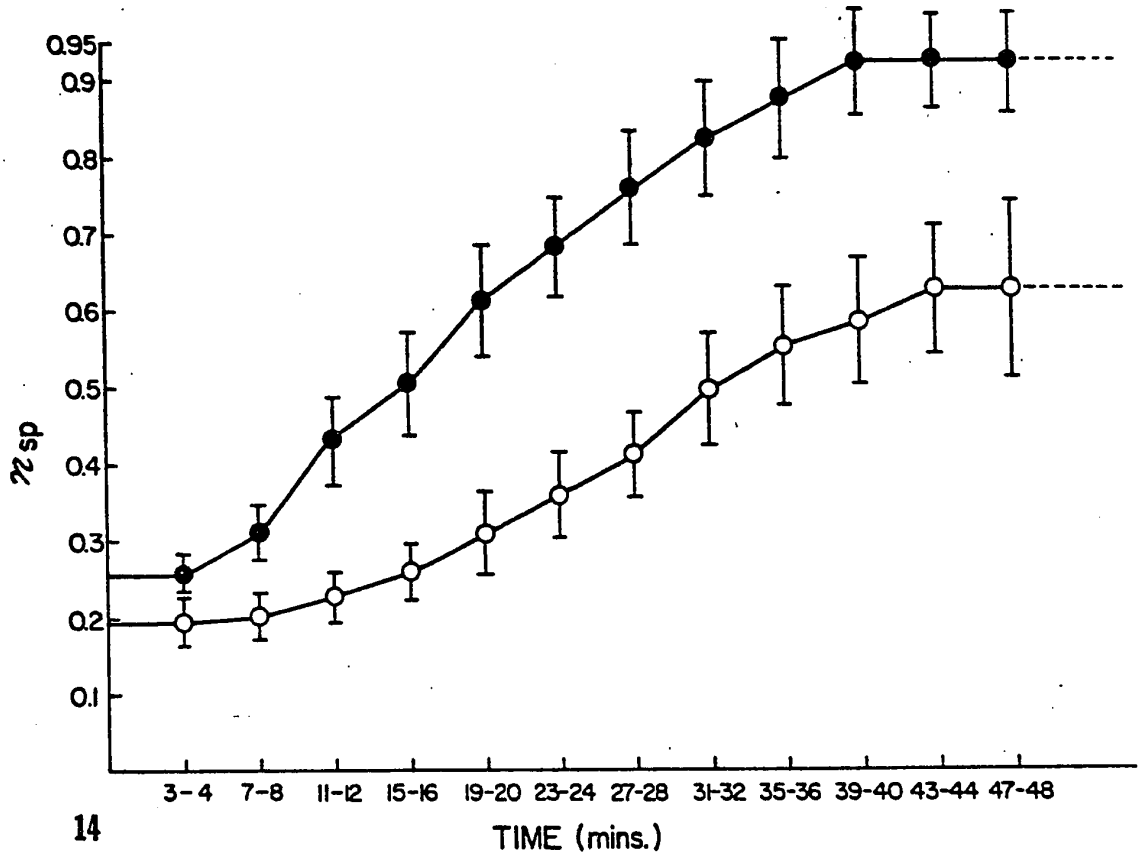

0.95
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
r^2_{sp}
3-4
7-8
11-12
15-16
19-20
23-24
27-28
31-32
35-36
39-40
43-44
47-48
TIME (mins.)

Table II. Shows absolute increases in specific viscosity (η_{sp}) for a range of tubulin levels in comparative experiments identical to fig. 14. Values represent total increases in viscosity are included for after 10 min and 40 min incubation periods within one standard deviation.

(+) tubulin incubated with complexes.

(-) tubulin incubated in the absence of complexes

Table II

Tubulin mg/ml	Increases in specific viscosity			
	10 min		40 min	
	+	−	+	−
0.8	0	0	0.35 ± 0.03	0.20 ± 0.02
1.0	0.10 ± 0.01	0	0.55 ± 0.04	0.31 ± 0.02
1.25	0.15 ± 0.05	0.03 ± 0.03	0.68 ± 0.06	0.40 ± 0.04
1.5	0.38 ± 0.04	0.14 ± 0.02	0.75 ± 0.06	0.52 ± 0.03
2.0	0.46 ± 0.05	0.20 ± 0.06	0.85 ± 0.05	0.66 ± 0.06

dent on the concentration of tubulin used and the length of incubation. Figure 13 shows the numbers of microtubules ending on rootlets over a five min period for four different tubulin concentrations tested (0.8 to 3.4 mg/ml). No assembly occurs when complexes are incubated with crude extracts of microtubule proteins containing less than 0.8 mg/ml tubulin protein. At 0.8 mg/ml tubulin the numbers of microtubules increases to a maximum of about 11 per complex after 5 min incubation. With the higher concentrations of tubulin used there is a corresponding increase in number of microtubules at each time interval. The tubulin in the crude brain extracts used in these experiments will independently self-assemble, but at these early time points the assembly occurs preferentially onto complexes.

The negative stain results indicate that the rootlet MTOCs promoted the assembly of tubulin in the crude extracts. This effect is also evident when the kinetics of self-assembly of crude tubulin and the kinetics of assembly in the presence of rootlets are compared viscometrically. Figure 14 shows in one example at a tubulin concentration of 1.25 mg/ml tubulin protein that there is a gradual self-assembly of the tubulin fraction alone reaching an equilibrium of polymer and dimers after about 40 min. Table II shows absolute increases in viscosity for increasing tubulin levels during initiation (at 10 min) and at equilibrium (at 40 min) for

tubulin protein alone and for tubulin incubated with complexes. With complexes present both the initial rate of tubulin assembly and the final equilibrium viscosity are increased. Complexes incubated alone or added to microtubules do not cause an increase in viscosity indicating assembly of microtubules on rootlet-MTOCs is what enhances viscosity values.

MTOCs INITIATE ASSEMBLY OF PURIFIED TUBULIN

The tubulin protein purified by phosphocellulose chromatography for use in these studies does not self-assemble in the MAB buffer at concentrations up to 6 mg/ml. For each tubulin preparation, samples were routinely tested by negative staining to ensure they did not self-assemble and samples were also examined for purity using gel electrophoresis (see fig. 20). When complexes are added to the purified tubulin, negative staining shows assembly onto the rootlet-MTOCs at a critical tubulin concentration of 0.2 mg/ml, which is near physiological levels.

Thin-sections (fig. 15) show that the microtubules are initiated in rows from material coating the sides of the rootlets. The pattern of microtubules assembled is the same as that described in the earlier experiments using crude microtubule proteins (figs. 10, 11).

Figures 16a and 16b show the kinetics of assembly of

Fig. 15 a, b. Thin sections of a rootlet attached to two basal bodies. Fig. 15a shows view *in situ* of the pattern of cytoskeletal microtubules. Fig. 15 b shows view *in vitro* of purified brain tubulin microtubules in a pattern closely resembling that seen *in situ*.

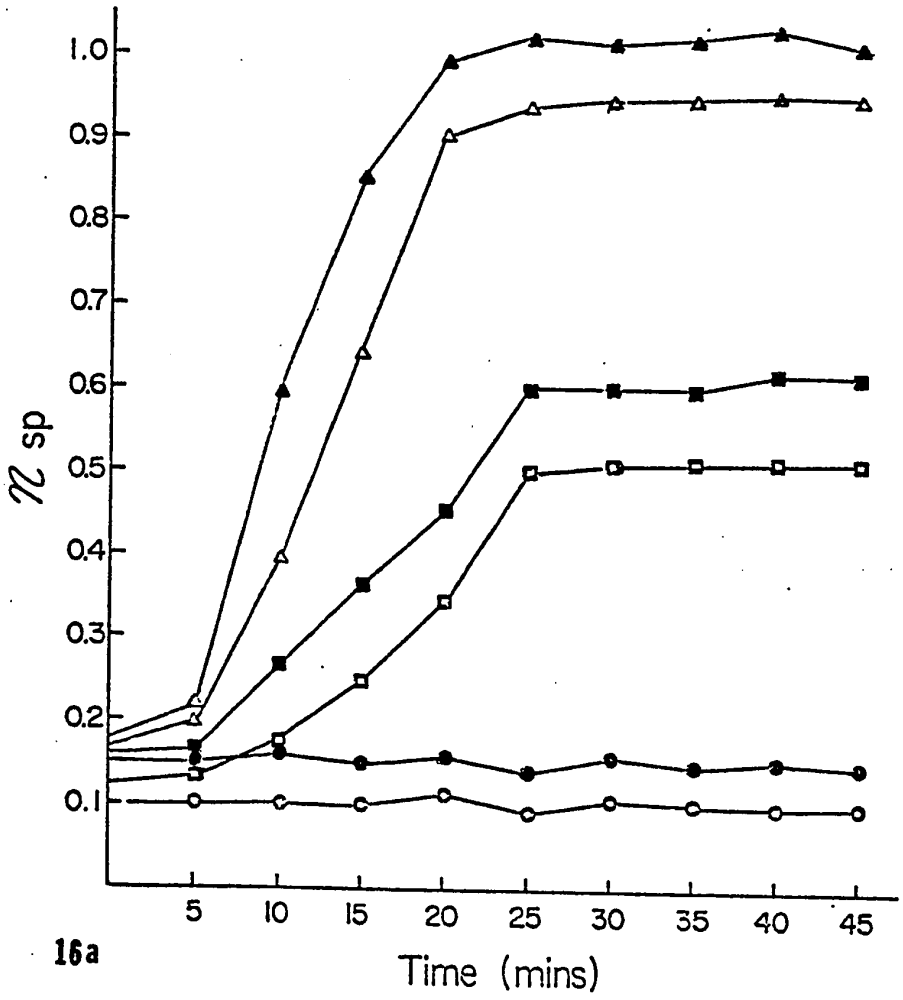

a
b

the purified tubulin initiated by rootlet-MTOCs. Incubation of the tubulin alone or of complexes alone at 37°C produce no change in viscosity. At a constant number of complexes (2×10^6/ml) increasing the concentration of tubulin increases the initial assembly rate and the viscosity values attained at equilibrium (fig. 16a). The reverse experiment (fig. 16b) using a fixed high concentration of tubulin (2.5 mg/ml) show that these parameters are also sensitive to the number of initiating sites available. These data also show that increasing the number of complexes above 2×10^6/ml (the number used in fig. 16a) does not further enhance assembly kinetics.

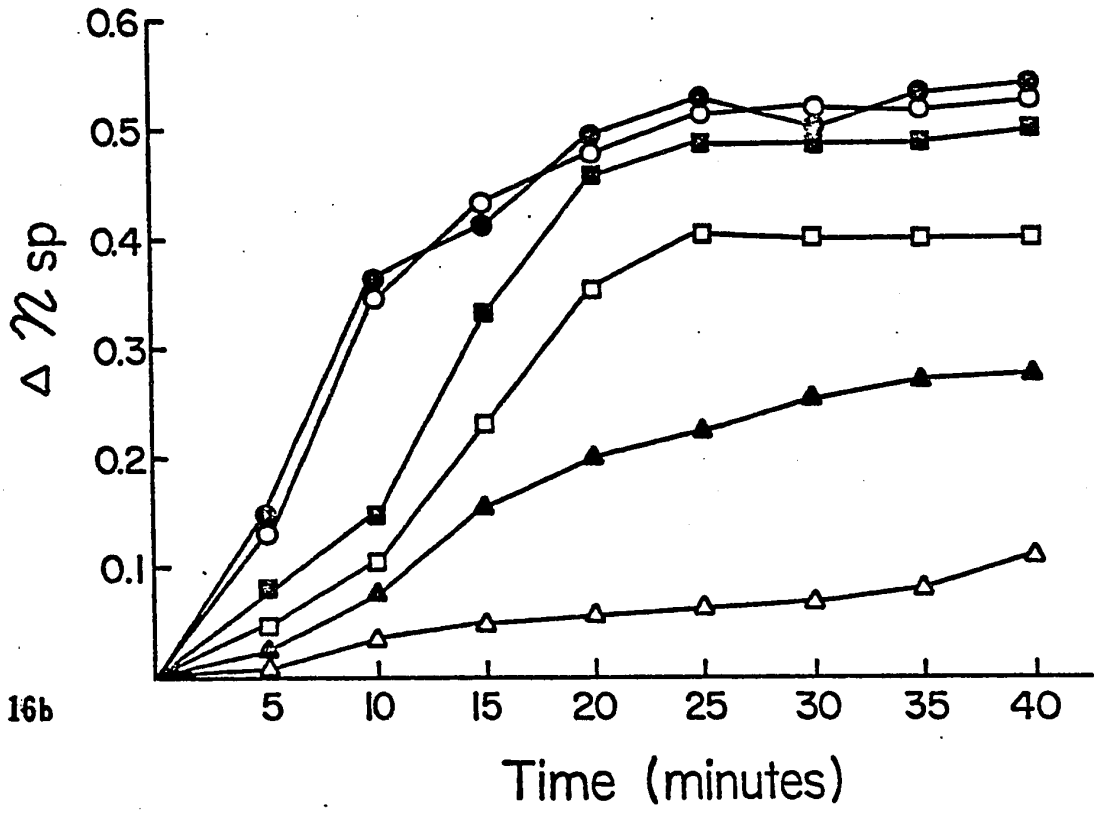
EXTRACTION OF MICROTUBULE-INITIATING PROTEINS

Brief trypsinization destroys the capacity of the rootlet-MTOCs to initiate the assembly of crude or purified tubulin indicating that the initiation sites are composed, at least in part, of proteins. The rootlet-associated proteins (RAPs) functioning in initiation can be removed from intact complexes by a brief extraction with a low ionic strength Tris-EDTA solution. The structure of the complexes appear unchanged by the brief extraction (fig. 17) but they will no longer promote assembly of microtubules. The extract contains trypsin-sensitive proteins which will initiate the assembly of purified tubulin. Figure 18 shows

Fig. 16 a, b. Increases in viscosity for the assembly of phosphocellulose purified tubulin onto complexes. 16a, complexes are at 2 x 10^6/ml and tubulin was at 0.5 (□), 1.0 (■), 2.0 (△), 3.0 mg/ml (▲). Controls show there is no viscosity change for complexes at 2 x 10^6/ml (●), or for tubulin at 3 mg/ml (○). The graph shows the initial rate and the final extent of assembly is dependent on initial tubulin-levels used. 16b, Total changes in viscosity at a constant amount of tubulin (2.5 mg/ml) and a decreasing number of complexes at 3 (●), 2 (○), 1 (■), 0.5 (□), 0.1 (▲), 0.05 x 10^6 (△) complexes/ml. Decreasing the number of complexes/ml decreases the extent of assembly.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
η_{sp}
5
10
15
20
25
30
35
40
45
Time (mins)

16a



16b

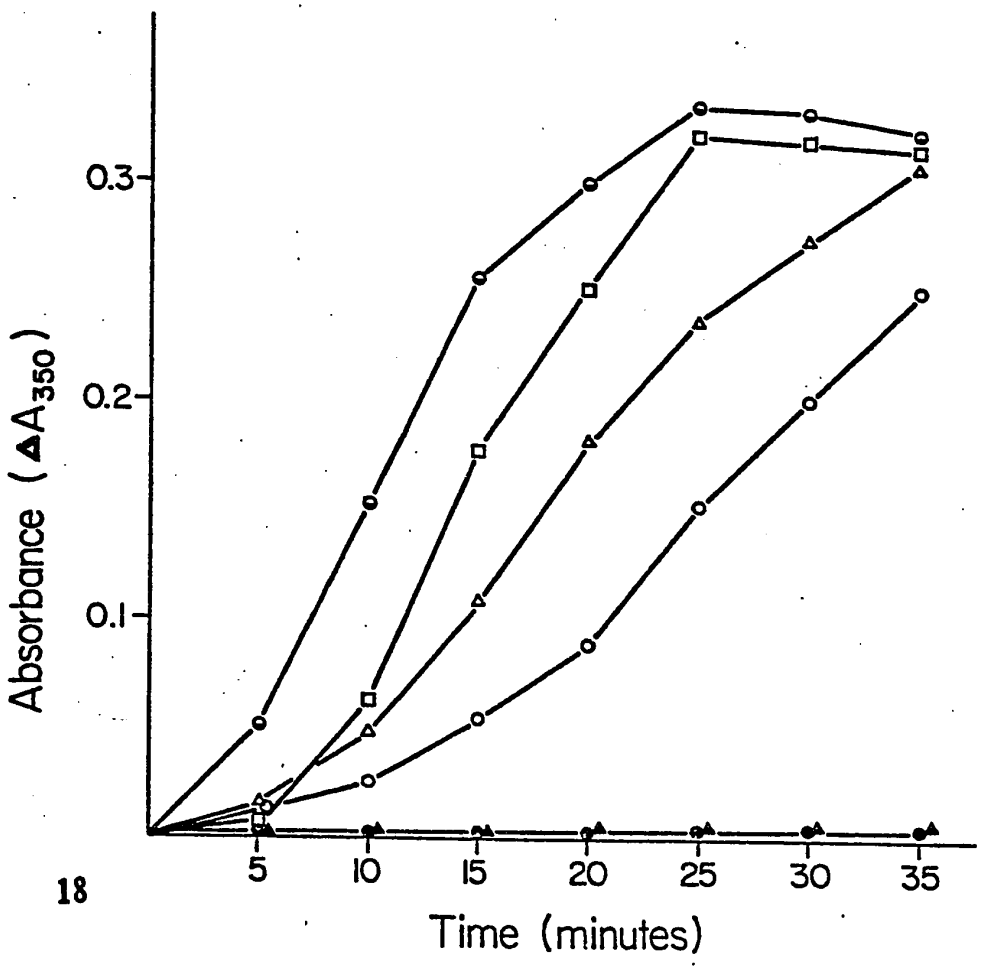
Fig. 17 Thin sections of a basal body rootlet complex previously extracted in Tris-EDTA at 4°C for 20 min. The basal bodies and rootlets appear completely intact. x100,000.

the kinetics of assembly, assayed by turbidity, for a constant concentration of purified tubulin (2.0 mg/ml) and for varying amounts of RAPs. These results indicate that increasing the concentration of RAPs increases the initial rate of assembly with little apparent effect on the equilibrium attained. This is consistent with evidence that the intact rootlet MTOCs function in initiation of assembly.

PURIFICATION OF MICROTUBULE INITIATING PROTEINS

High speed supernatants from low ionic strength extracts of intact rootlet MTOCs contain a number of rootlet-associated proteins, RAPs, fig. 19a, and these extracts promote assembly of phosphocellulose-purified brain tubulin (fig. 18). The microtubule-initiating proteins (TIPs) can be selected from extracts by briefly incubating RAPs with tubulin at 37°C and using centrifugation to pellet protofilament assembly intermediates, fig. 19b. Pellets can be cold solubilized and TIPs separated from tubulin using phosphocellulose chromatography, fig. 19c. TIPs incubated with fresh brain tubulin stimulate assembly of microtubules which gels show to contain tubulin and TIPs consisting of 4-high molecular weight proteins ranging from 190,000 to 220,000 daltons, fig. 19d. The purified TIPs, shown in figure 19c, have been used in assembly experiments reported in this thesis.

Fig. 18 Turbidity, A_{350}, changes at a constant amount of tubulin protein (2.0 mg/ml) and increasing amounts of crude rootlet extract for 0.10 (o), 0.20 (Δ), 0.30 (□), 0.40 (●) mg/ml. A decrease in the initial lag phase occurs with increasing amounts of extract but there is no apparent increase in the final absorbance at equilibrium. Tubulin protein (2.0 mg/ml) in the absence of crude rootlet extract (▲) and rootlet extract in the absence of tubulin (●) showed no change in absorbance at 37°C.


Absorbance (ΔA_{350})
0.3
0.2
0.1
5
10
15
20
25
30
35
Time (minutes)

2 X temperature-cycled beef tubulin (S-3) contains about 75% tubulin and a large number of microtubule-associated proteins (MAPs) which co-assemble with tubulin, fig. 20a. S-3 can be fractionated into purified tubulin (fig. 20c, d) and MAPs (fig. 20b) fractions using phosphocellulose chromatography. The microtubule-initiating component can be selected from MAPs and purified using the same procedures described for purifying TIPs. Figure 21 shows gels of MAPs (fig. 21a), tubulin (fig. 21b) and the proteins in protofilament pellets, fig. 21c. Protofilaments are shown to contain tubulin and a single MAP which has been purified using phosphocellulose chromatography and is shown to consist of a single 330,000 dalton protein, "the-MAP" (fig. 21d). When TIPs and "the-MAP" are incubated with fresh tubulin, they promote microtubule assembly. The capacity of these two types of initiating proteins to promote tubulin assembly was compared using several optical measurement techniques. The molecular weight of the tubulin monomers, of MAPs, "the-MAP", and TIPs was determined using 10% SDS PAGE-gels by comparison to a set of known standards. A standard plot is represented in fig. 22.

INTERMEDIATES IN INITIATION OF TUBULIN ASSEMBLY

Negative staining: Figure 23a-e shows "the-MAP" incubated with tubulin initiates assembly intermediates which are

Fig. 19 SDS-PAGE gels (a) under loaded 10% gel of rootlet-associated proteins (RAPs) extracted from intact rootlets; (b) 7% SDS gel of proteins composing protofilaments; (c) TIPs purified using phosphocellulose chromatography; (d) 7% SDS gel of microtubules pelleted from TIPs and tubulin mixtures.

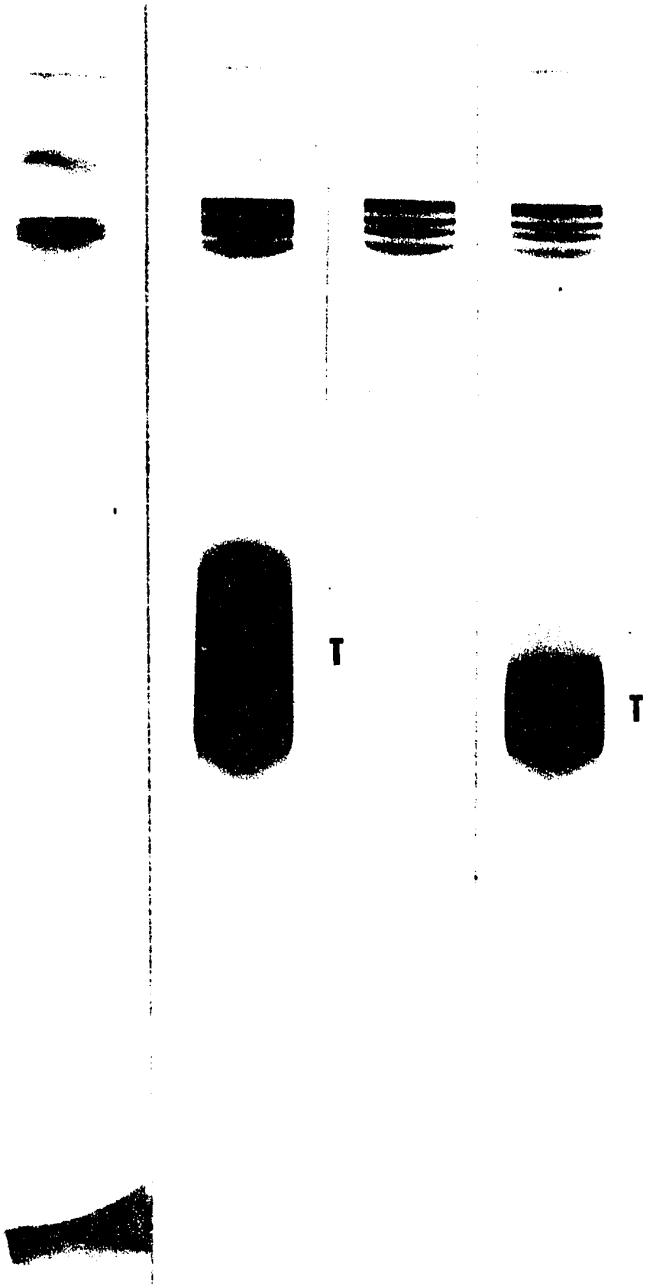

T
T

Fig. 20 10% SDS-PAGE gels of beef brain microtubule proteins. (a) 2X cycled S-3 at 100 ug; (b) phosphocellulose-fractionated microtubule associated proteins, MAPs, at 50 ug; (c,d) phosphocellulose-purified tubulin, (c) at 50 ug, (d) at 100 ug.

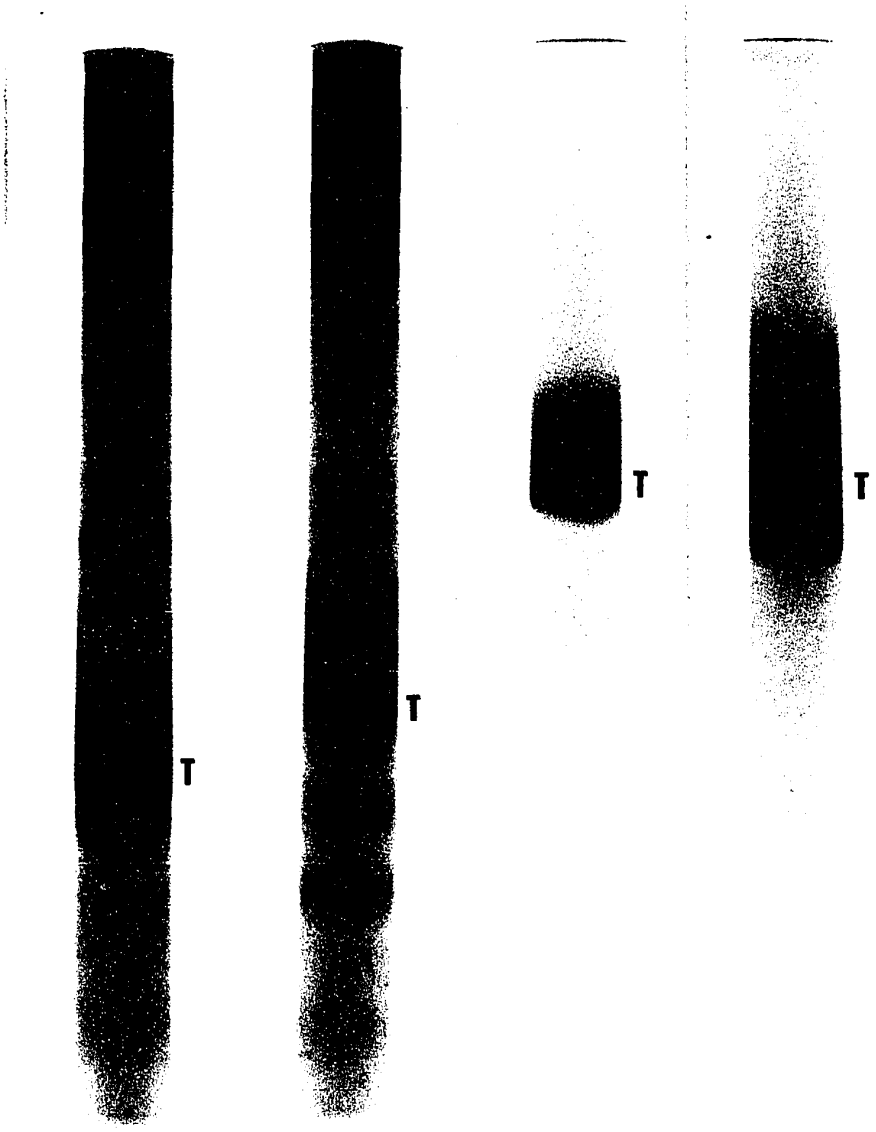

T
T
T
T

Fig. 21 7% SDS-PAGE gels (a) MAPs; (b) tubulin; (c) proteins composing protofilaments formed by briefly incubating MAPs with tubulin at 37°C; (d) "the-MAP" purified from disassembled protofilaments in (c).

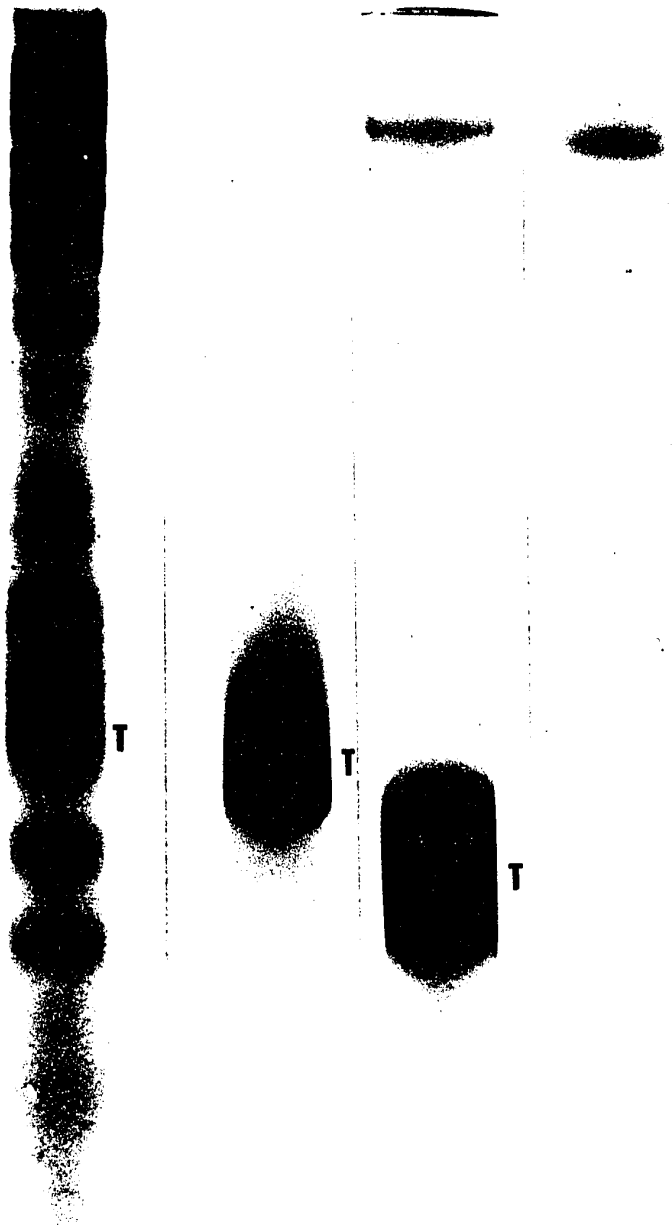
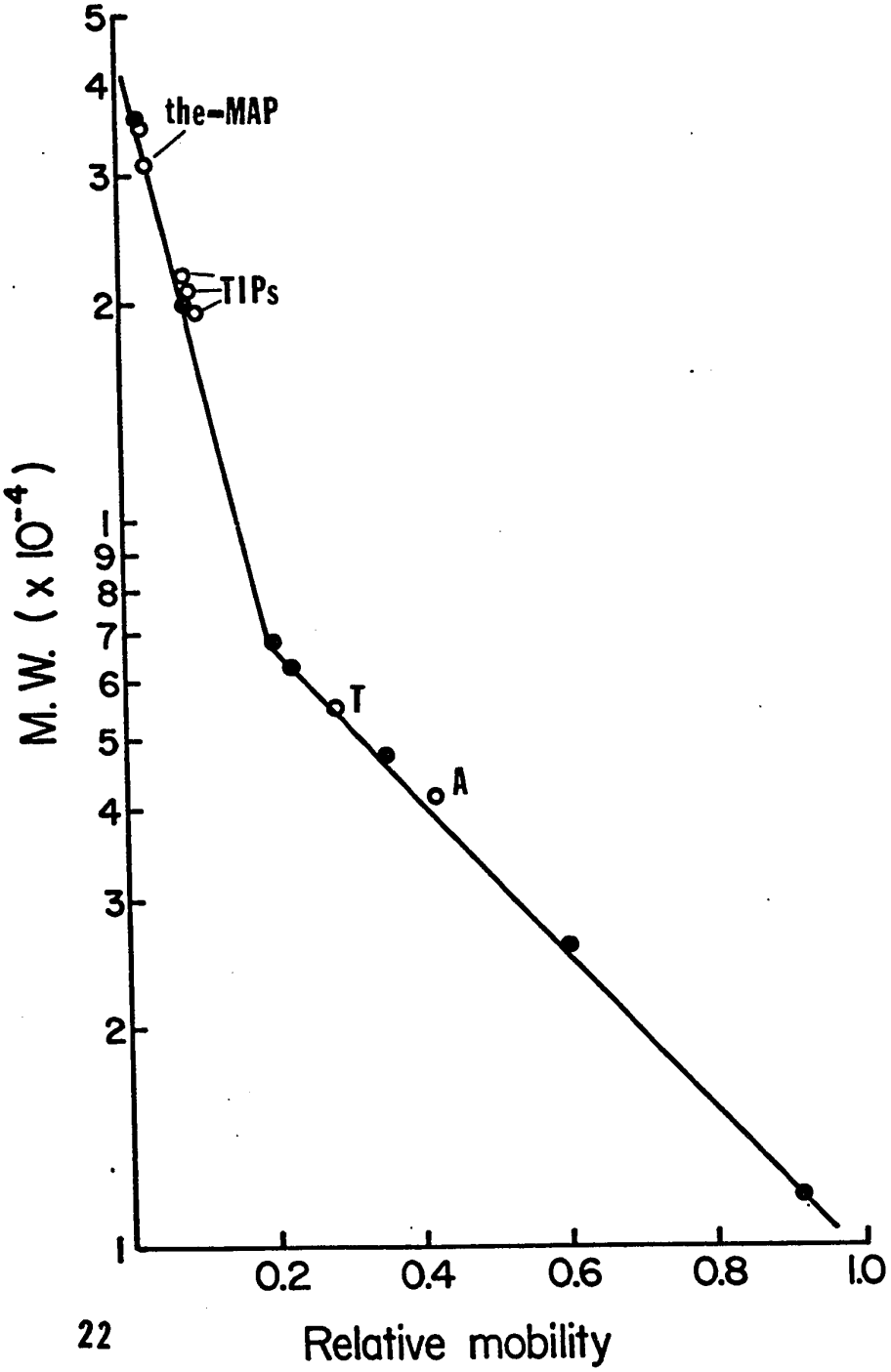

T
T
T

Fig. 22 Standard curve for molecular weight determinations by 10% SDS gel electrophoresis (Weber and Osborn, 1969). Standard proteins used are axonemal dynein (Bloodgood et al., 1976); bovine serum albumin, catalase, cytochrome C, egg albumin, chymotrypsinogen A, rabbit skeletal myosin. T (brain tubulin); A (brain actin).


5
4
3
2
1
9
8
7
6
5
4
3
2
1
M.W. (x 10⁻⁴)
the-MAP
TIPs
T
A
0.2
0.4
0.6
0.8
1.0
Relative mobility

similar to those which have been reported previously for temperature-cycled brain tubulin (Kirschner et al., 1975a; Sloboda et al., 1976b). When "the-MAP" and tubulin are separately incubated in MAB containing GTP at 4°C or at 37°C they do not form any recognizable structures. When these proteins are mixed together, at 4°C rings are formed (fig. 23a) and at 37°C these rings are seen to gradually disappear as microtubule assembly intermediates appeared. Figure 23b shows rings are still the prevalent structures after 10 to 20 sec at 37°C but that protofilaments begin to replace rings as the most abundant structure. Rings associated with the ends of microtubules may be coincidental or they may represent rings in the process of unfolding to protofilaments. Figure 23c shows that these protofilaments aggregate together by 30 to 45 sec, leading in turn to assembly of sheets of protofilaments at 90 to 120 sec (fig. 23d) and finally these sheets fold to make the complete microtubules by about 180 sec, fig. 23e.

Figure 24a-e shows that when incubated at 37°C similar assembly intermediates can be initiated by TIPs incubated with brain tubulin. In contrast to MAPs, at 4°C, no recognizable aggregates are seen (fig. 24a) and no rings appear unless some of "the-MAP" is added to the mixture, indicating that rings are structures induced by the brain accessory proteins. At 37°C, protofilaments are the first microtubule assembly intermediates to appear after 20 sec (fig. 24b) and these proto-

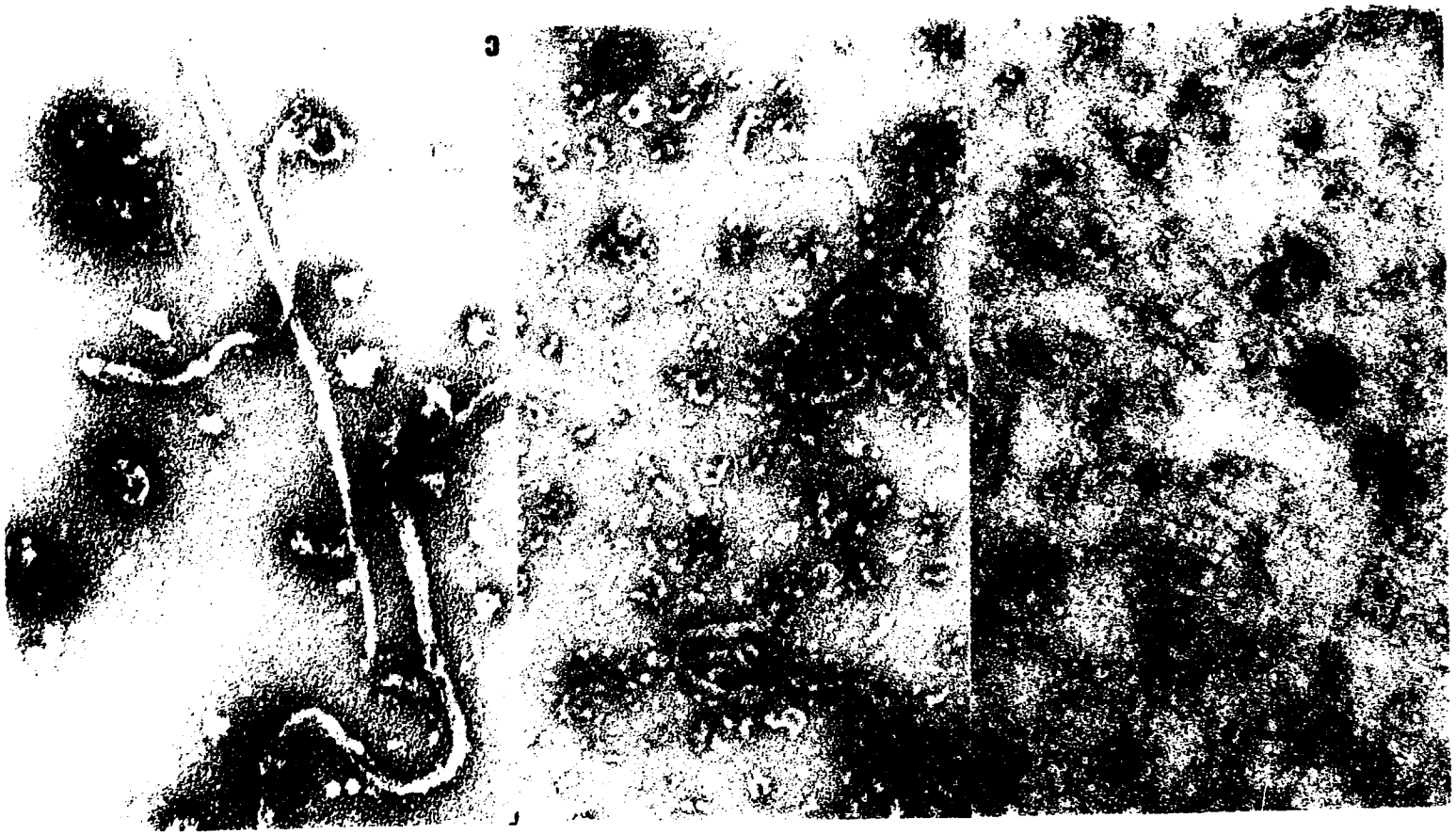
filaments form aggregates of protofilaments at 30 - 45 sec (fig. 24c) leading to assembly of sheets of protofilaments at about 90 to 120 sec (fig. 24d) and at 120 to 180 sec these sheets in turn fold to form microtubules, fig. 24e. The assembly intermediates initiated by TIPs closely resemble those described for "the-MAP" with the exception that rings were not seen.

If intact rootlet-MTOCs are substituted for TIPs and incubated with tubulin at 4°C, no recognizable aggregates such as rings are formed. When incubated at 37°C, figs. 25, 26a show that at 20 sec protofilaments are the first intermediates formed. At 30 to 45 sec aggregates of these protofilaments form (fig. 26b) and at 60 to 90 sec sheets of protofilaments appear which then fold to make complete microtubules (fig. 26c, d). These intermediates closely resemble those promoted by the purified initiating proteins suggesting that assembly of tubulin may occur by similar mechanism *in vivo*.

KINETICS OF TUBULIN ASSEMBLY

Turbidiometry (A_{350}): Turbidity can measure increases in the total amount of polymer formed (Berne, 1974). In fig. 27 increasing amounts of S-3 beef tubulin have been incubated to equilibrium at 37°C. The graph shows that a linear increase in absorbance occurs up to 0.6 units and

Fig. 23 a - f. 1% uranyl acetate negative stain show microtubule intermediates initiated by "the-MAP" (0.3 mg/ml) and tubulin (2 mg/ml) incubated at (a) 4°C and at 37°C for (b) 20 sec; (c) 30 to 45 sec; (d) 60 to 90 sec; (e) 120 to 180 sec. The rings gradually disappear at 37°C as protofilaments (c), aggregates of a few protofilaments (d), and sheets of protofilaments (e), form as intermediates to microtubules (f). Magnifications: (a) x120,000; (b) x80,000; (c) x80,000; (d) x90,000; (e) x90,000.



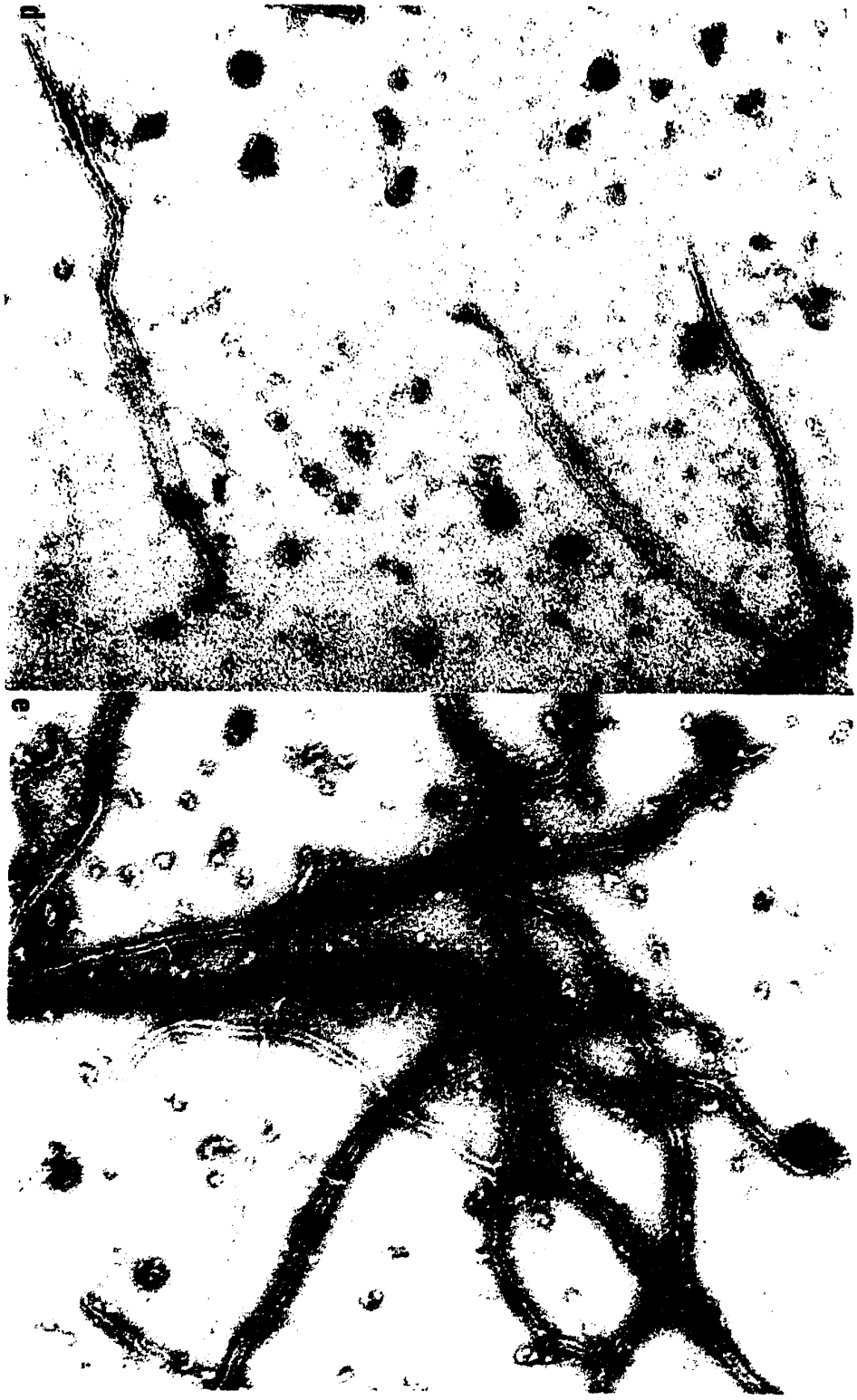
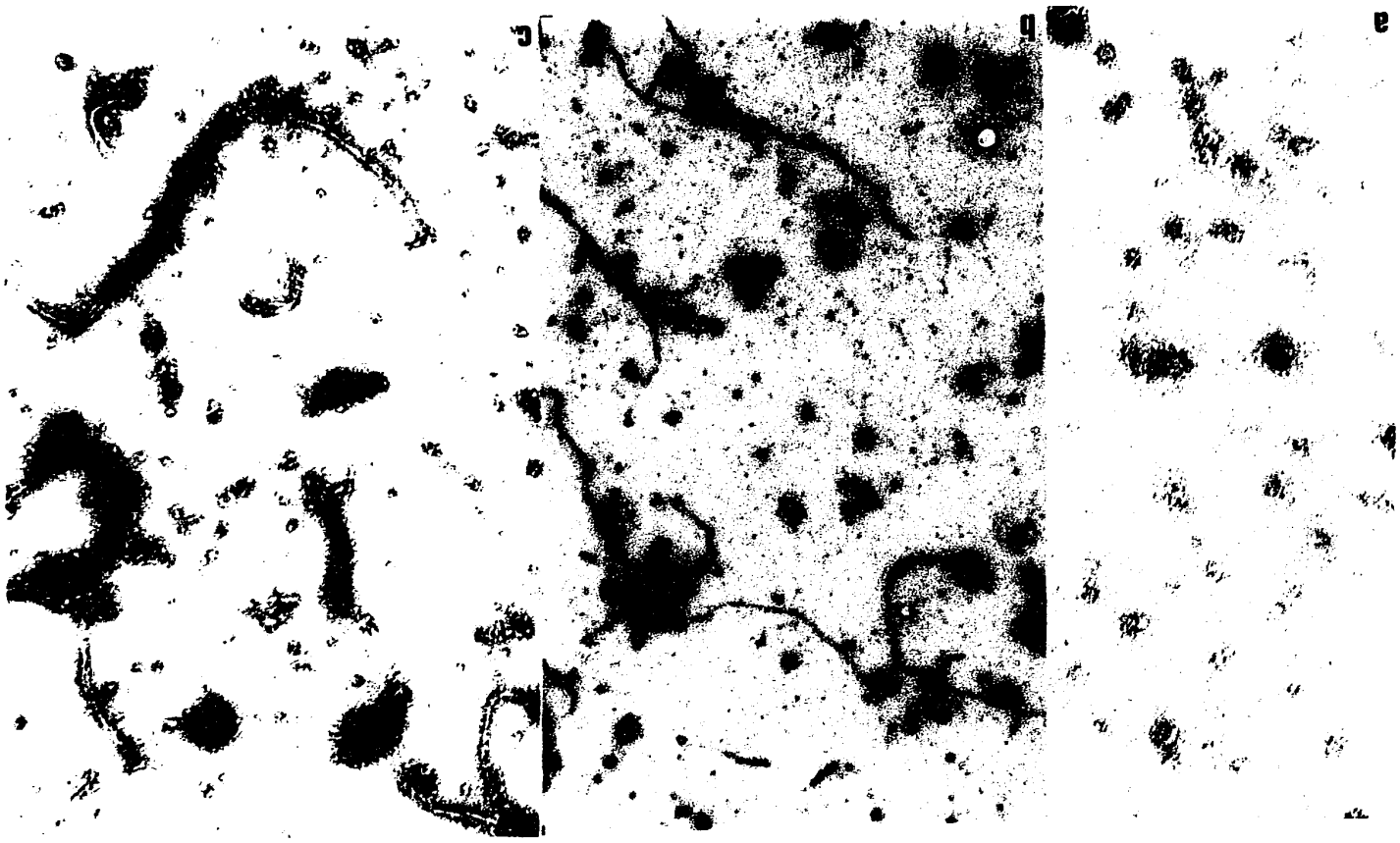

b
a

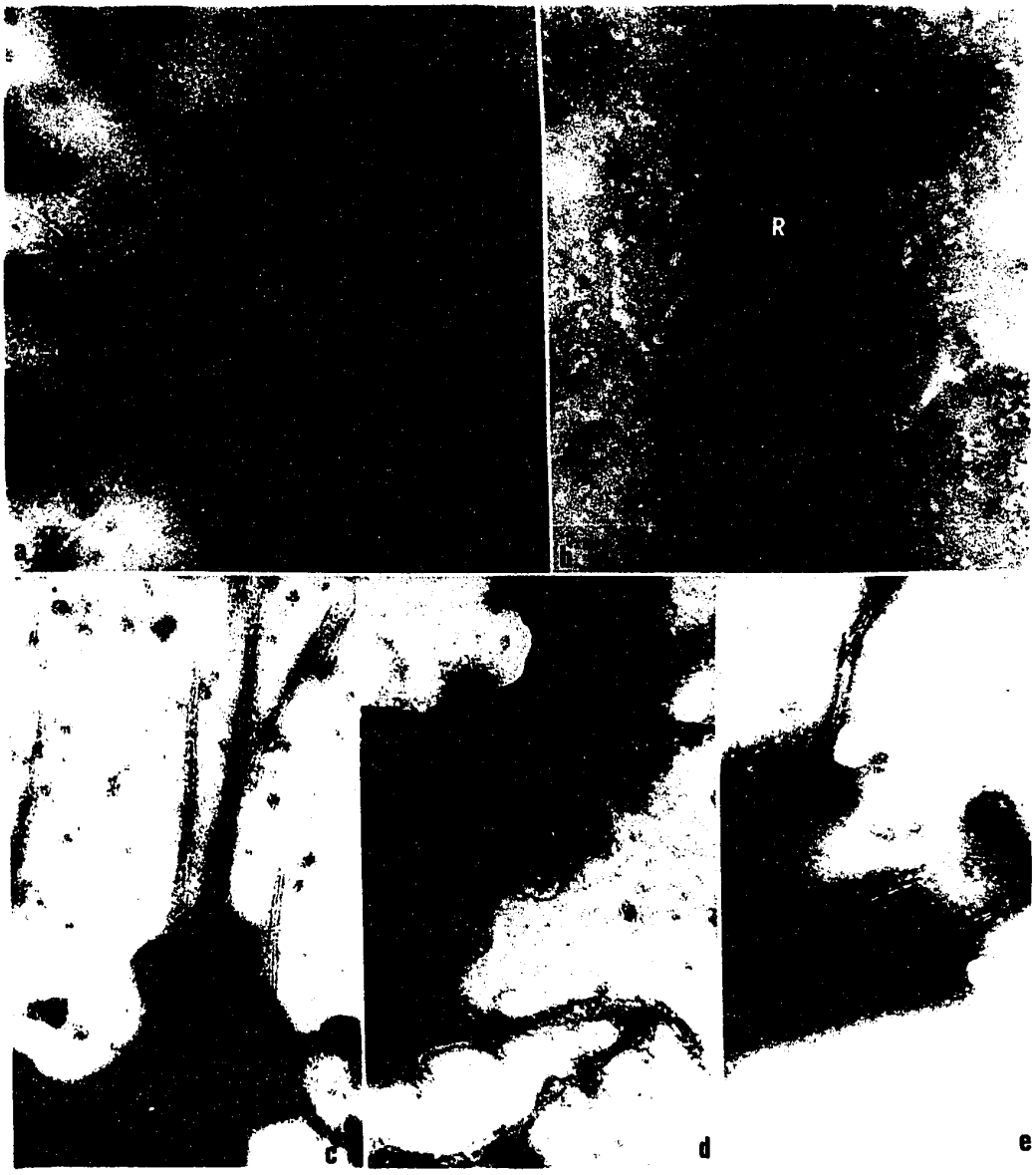
Fig. 24 a - e. Negative staining showing assembly intermediates formed by TIPs (0.3 mg/ml) and tubulin (2 mg/ml) incubated (a) at 4°C; and at 37°C for (b) 20 sec; (c) 30 to 45 sec; (d) 60 to 90 sec; and (e) 120 to 180 sec. With the exception of rings, the intermediates at each time interval are identical to those in fig. 23. Magnifications: (a) x50,000; (b) x50,000; (c) x25,000; (d) x90,000; (e) x90,000.


c
b
a

a
b

Fig. 25, 26. Intermediates of microtubules initiated by rootlet MTOCs and tubulin (2 mg/ml). Fig. 25 shows a complete basal body rootlet complex after incubation at 37°C for 15 sec. Protofilaments (arrows) are seen emanating from sides of rootlets (R). Magnifications: x20,000.
Fig. 26 a-d, a composite showing only the rootlets (R) of complexes after incubation at 37°C for (a) 15 sec; (b) 30 to 45 sec; (c) 60 to 120 sec; (d) 120 to 180 sec. The intermediates (arrows) are identical in morphology to those seen at similar time intervals in fig. 23, except no rings are seen. Magnifications: (a) x34,000; (b) x68,000; (c) x40,000; (d) x65,000; (e) x52,000.


R
25



26

above 0.6 the curve levels off. In studies reported in this thesis the turbidity values fall within the linear part of the curve. Figure 28 shows that at a constant amount of tubulin (2 mg/ml) increasing the amount of "the-MAP" from 0.05 to 0.55 mg/ml decreases the initial lag phase for assembly. Increases in turbidity values at equilibrium also occur. This increase may result from "the-MAP" promoting microtubule elongation to shift more tubulin dimers to the polymer form. Figure 29 summarizes the data in fig. 28 and shows that there is an exponential increase in turbidity values during the initiation phase (at 5 min) with increasing amounts of "the-MAP" used. The increases observed in turbidity at equilibrium level off at about 0.35 mg/ml of "the-MAP" and this value is assumed to be optimal for stimulating assembly to equilibrium for 2 mg/ml tubulin. In fig. 29, Lowry values of centrifugation pellets show 80-85% of the tubulin (2 mg/ml) is in polymer form at 0.35 mg/ml "the-MAP". Note that the absorbance values at equilibrium for purified tubulin (at 2 mg/ml) are almost half that reached by tubulin (at 2 mg/ml) in S-3. S-3 contains some actin which may also assemble to cause some increase in turbidity. An aggregation of microtubules with filaments may also account in part for the higher turbidity levels observed.

The capacity of TIPs to promote tubulin assembly has

Fig. 27 Relationship of total absorbance (A_{350}) at equilibrium to initial protein concentration. A linear increase in absorbance occurs to 0.6 mg/ml, and above this value a leveling off is observed. 2X cycled beef tubulin (6S) at increasing concentrations in MAB solution was incubated at 37°C for 30 min. The critical 6S concentration (Cc) is 0.2 mg/ml for assembly and tubulin values (mg/ml) represent total tubulin in polymer formed.

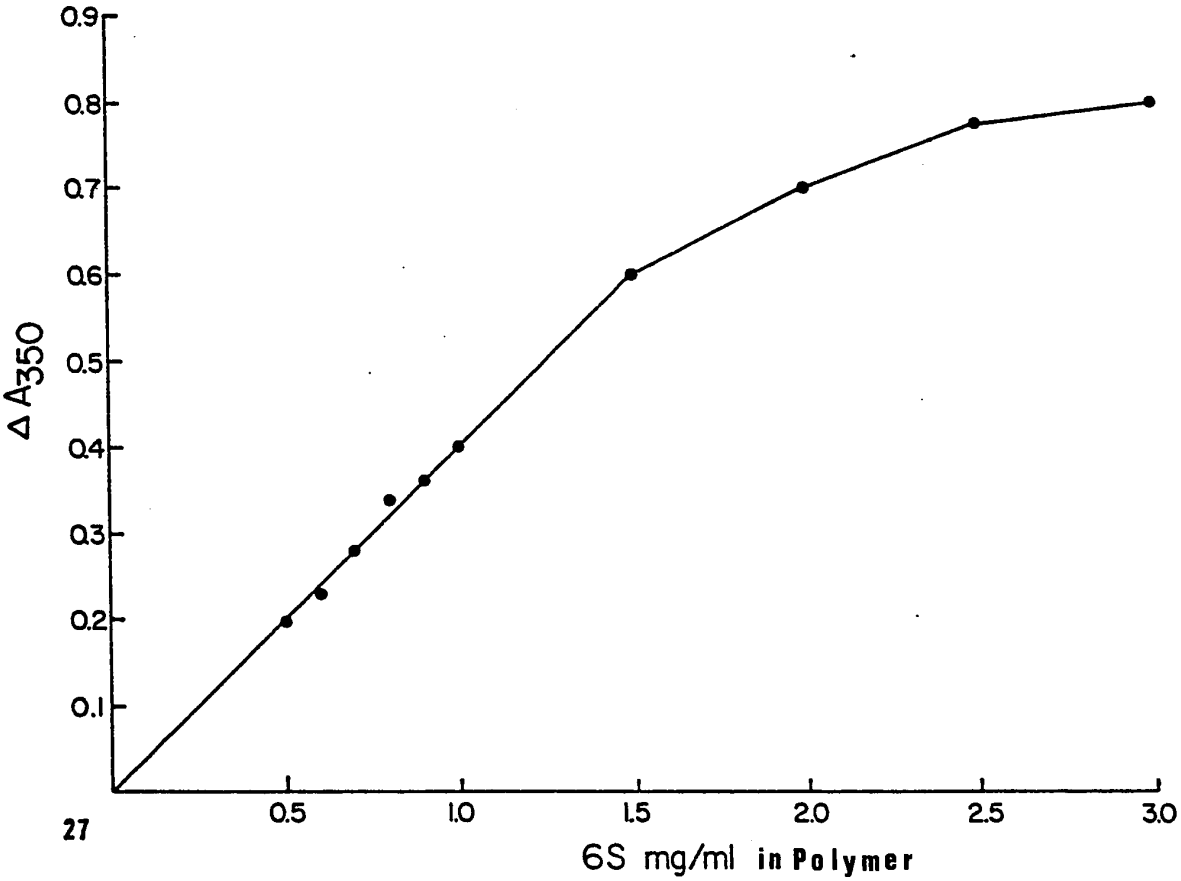

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
ΔA350
0.5
1.0
1.5
2.0
2.5
3.0
6S mg/ml in Polymer

Fig. 28 Stimulation of tubulin assembly (2 mg/ml) using increasing amounts of "the-MAP" at (A) 0.05 mg/ml; (B) 0.10 mg/ml; (C) 0.15 mg/ml; (D) 0.25 mg/ml; (E) 0.35 mg/ml; (F) 0.45 mg/ml; (G) 0.55 mg/ml. A decrease in the initial lag phase of assembly occurs with increasing amounts of "the-MAP" used and there is an apparent increase in the absorbance values at equilibrium (compare to fig. 29).

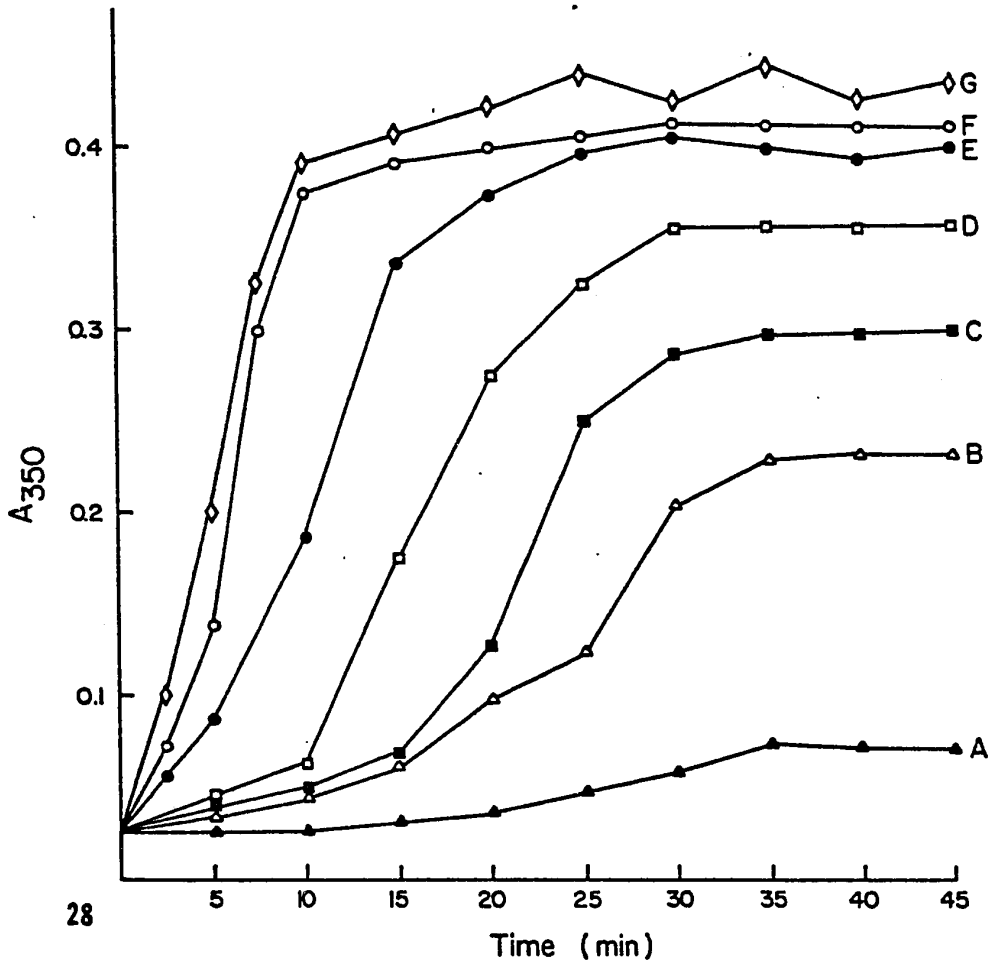
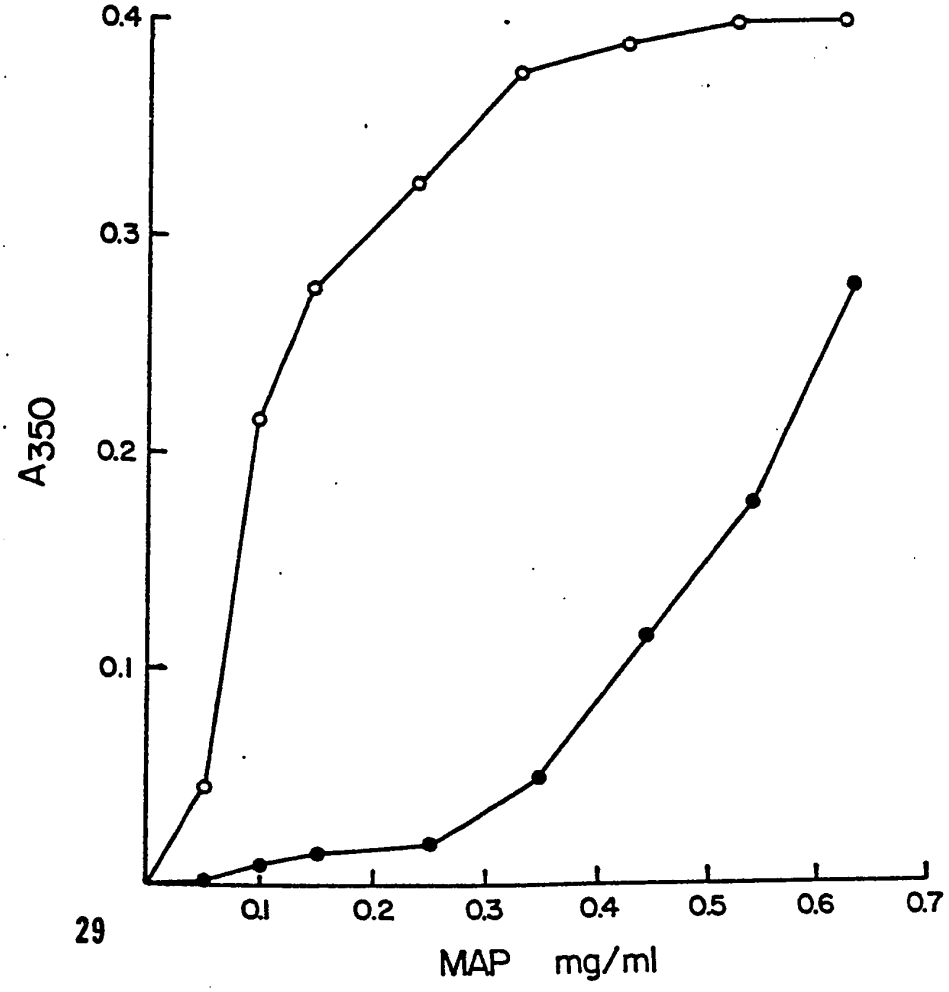

A350
0.4
0.3
0.2
0.1
5
10
15
20
25
30
35
40
45
Time (min)
G
F
E
D
C
B
A

Fig. 29 Replot of experiments in fig. 28 showing increases in the total amount of assembly (A_{350}) at 5 min (-●-●-) and at 30 min (-o-o-). An exponential increase occurs in initiation rates and 0.35 mg/ml "the-MAP" is optimum for maximum assembly of tubulin.


0.4
0.3
0.2
0.1
A350
0.1
0.2
0.3
0.4
0.5
0.6
0.7
MAP mg/ml

also been examined using turbidiometry. Figure 30 shows that using increasing amounts of TIPs at constant tubulin levels results in a decrease in the initial lag phase for assembly. We conclude that TIPs is active in the initiation of microtubules.

The significance of the fact that small amounts of TIPs (0.15 mg/ml) can promote assembly of tubulin to the same absorbance values observed for higher amounts of "the-MAP" (0.25 mg/ml) is not clear at this time. Figure 31 summarizes the data in fig. 30 and shows that the effect of increasing the amount of TIPs at a constant tubulin level is to stimulate an exponential increase in the initiation kinetics (measured after 5 min) and the extent of assembly at 10 min. The apparent optimal amount of TIPs for stimulating assembly of tubulin to a maximal equilibrium value is shown in fig. 31 to be approximately 0.35 mg/ml. This value which is similar to that observed for optimal assembly in the presence of "the-MAP".

Thus the optimal ratio of initiating protein to tubulin for both "the-MAP" and TIPs in microtubules at equilibrium is approximately 0.35 to 2 by weight (or 0.18 to 1.0) and the value represents about 16% initiation component to 84% tubulin. The microtubules formed by the initiating proteins are therefore of a similar relative composition when optimal amounts of these proteins are present.

To determine the effect of tubulin levels on assembly kinetics in the presence of excess initiating protein, changes in turbidity have been monitored in using constant amounts of "the-MAP" (fig. 32) and of TIPs (fig. 33) at 0.3 mg/ml and increasing tubulin levels up to 2 mg/ml.

Fig. 30 Stimulation of tubulin (2 mg/ml) assembly with increasing amounts of TIPs at (A) 0.10 mg/ml; (B) 0.15 mg/ml; (C) 0.25 mg/ml; (D) 0.35 mg/ml; (E) 0.45 mg/ml; (F) 0.55 mg/ml; (G) 0.65 mg/ml. A decrease in the initial lag phase of assembly occurs with increasing amounts of TIPs.

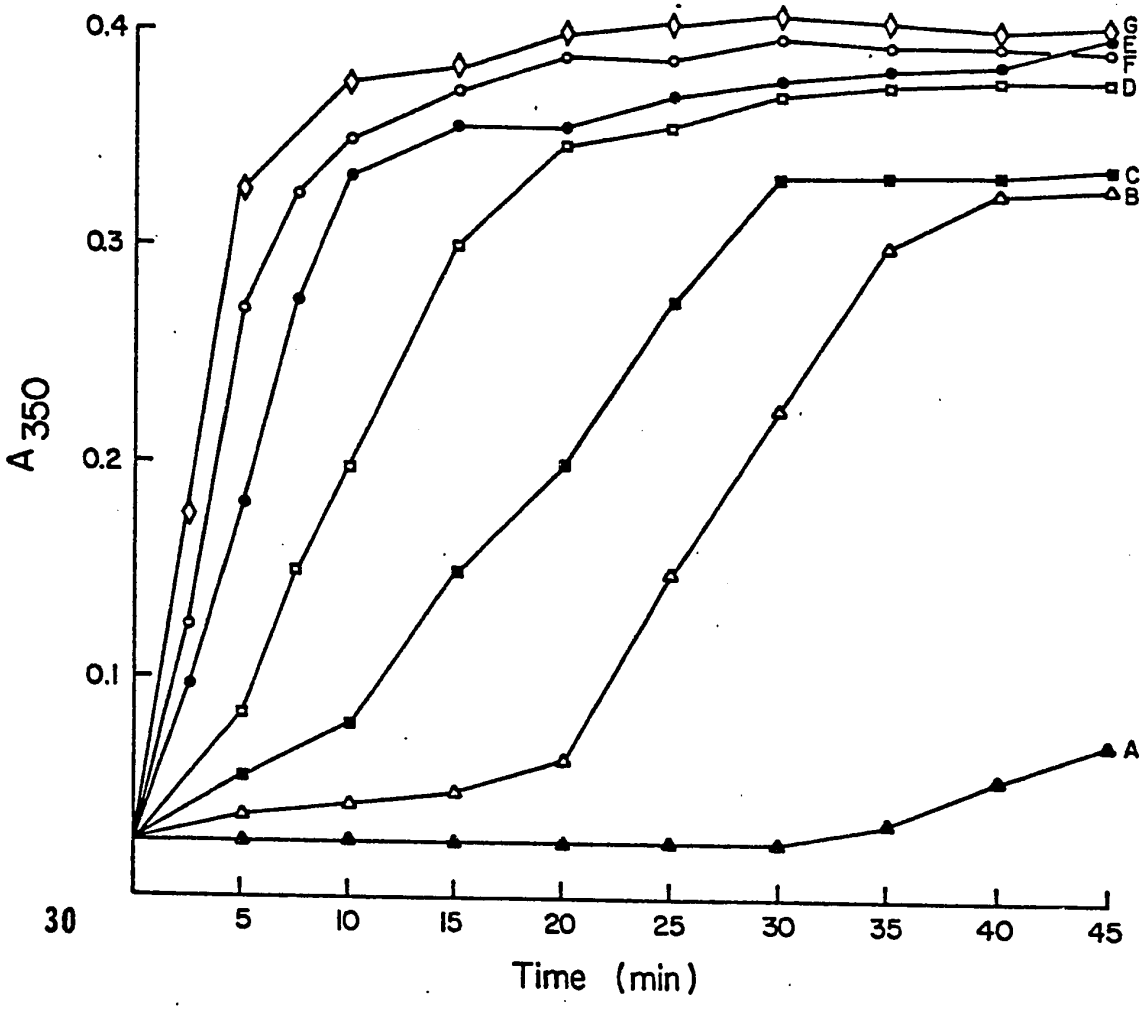
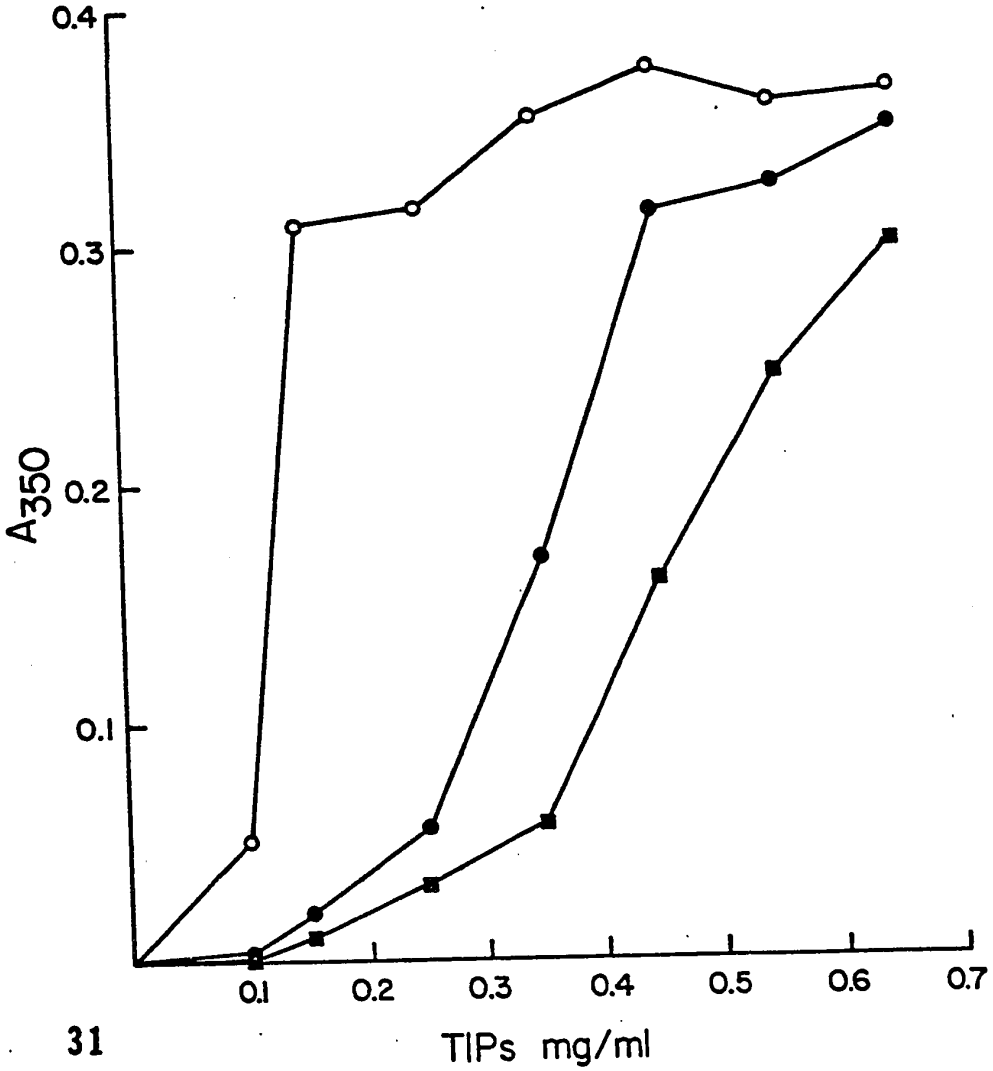
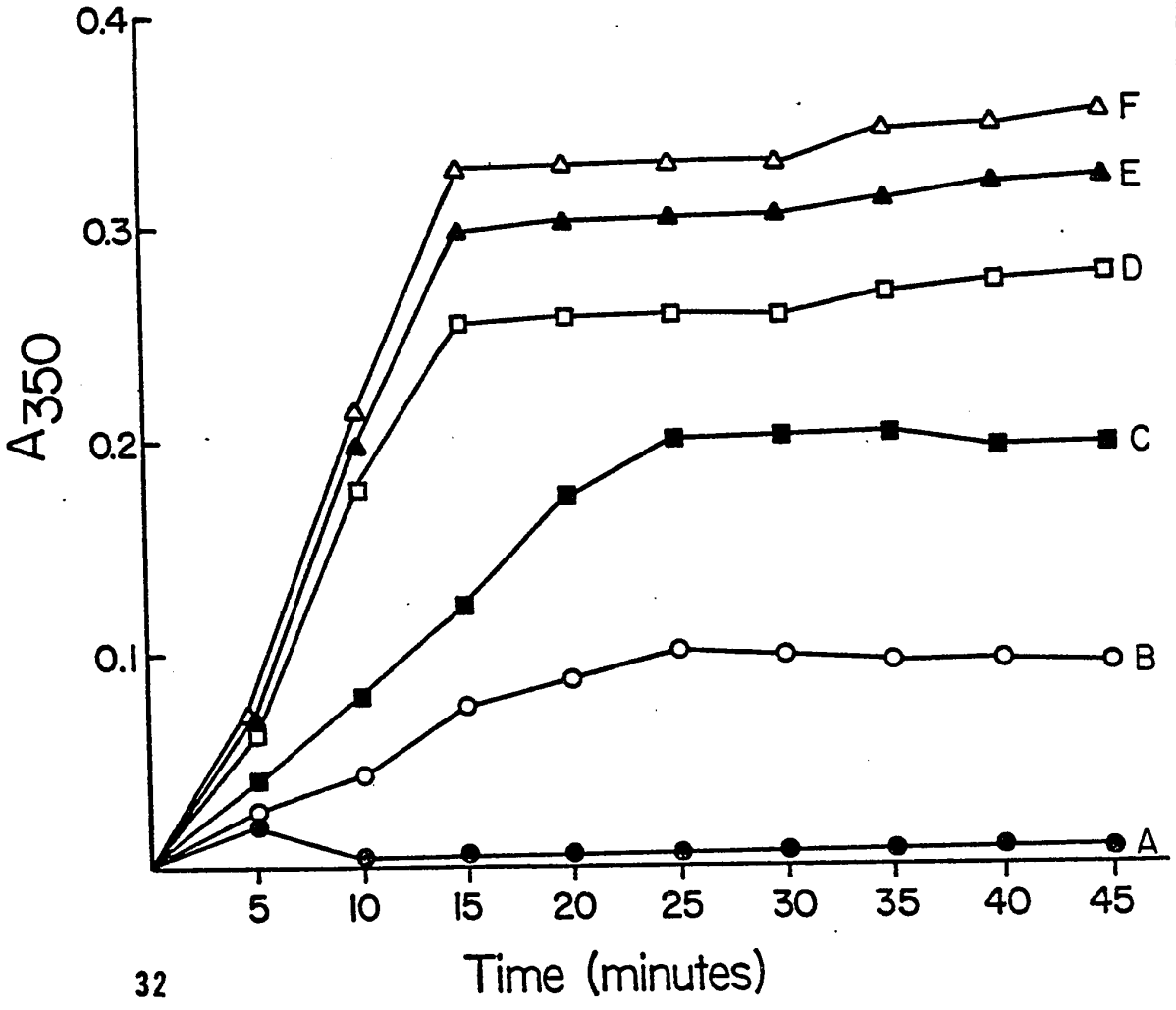

0.4
0.3
0.2
0.1
A 350
30
5
10
15
20
25
30
35
40
45
Time (min)
G
E
F
D
C
B
A

Fig. 31 Replot of experiments in fig. 30 shows increases in the total amount of assembly at 5 min (-□-□-), at 10 min (-o-o-) and at 30 min (-o-o-). An exponential increase is observed for initiation rates and 0.35 mg/ml TIPs is optimum for maximum assembly of tubulin.



31

Fig. 32 Stimulation of tubulin assembly at a constant amount of "the-MAP" (0.3 mg/ml) for increasing amounts of tubulin protein at (A) 0.2 mg/ml; (B) 0.5 mg/ml; (C) 0.8 mg/ml; (D) 1.0 mg/ml; (E) 1.5 mg/ml; (F) 2.0 mg/ml.



32

Fig. 33 Stimulation of tubulin assembly at a constant amount of TIPs (0.3 mg/ml) for increasing amounts of tubulin protein at (A) 0.2 mg/ml; (B) 0.5 mg/ml; (C) 0.8 mg/ml; (D) 1.0 mg/ml; (E) 1.5 mg/ml; (F) 2.0 mg/ml.

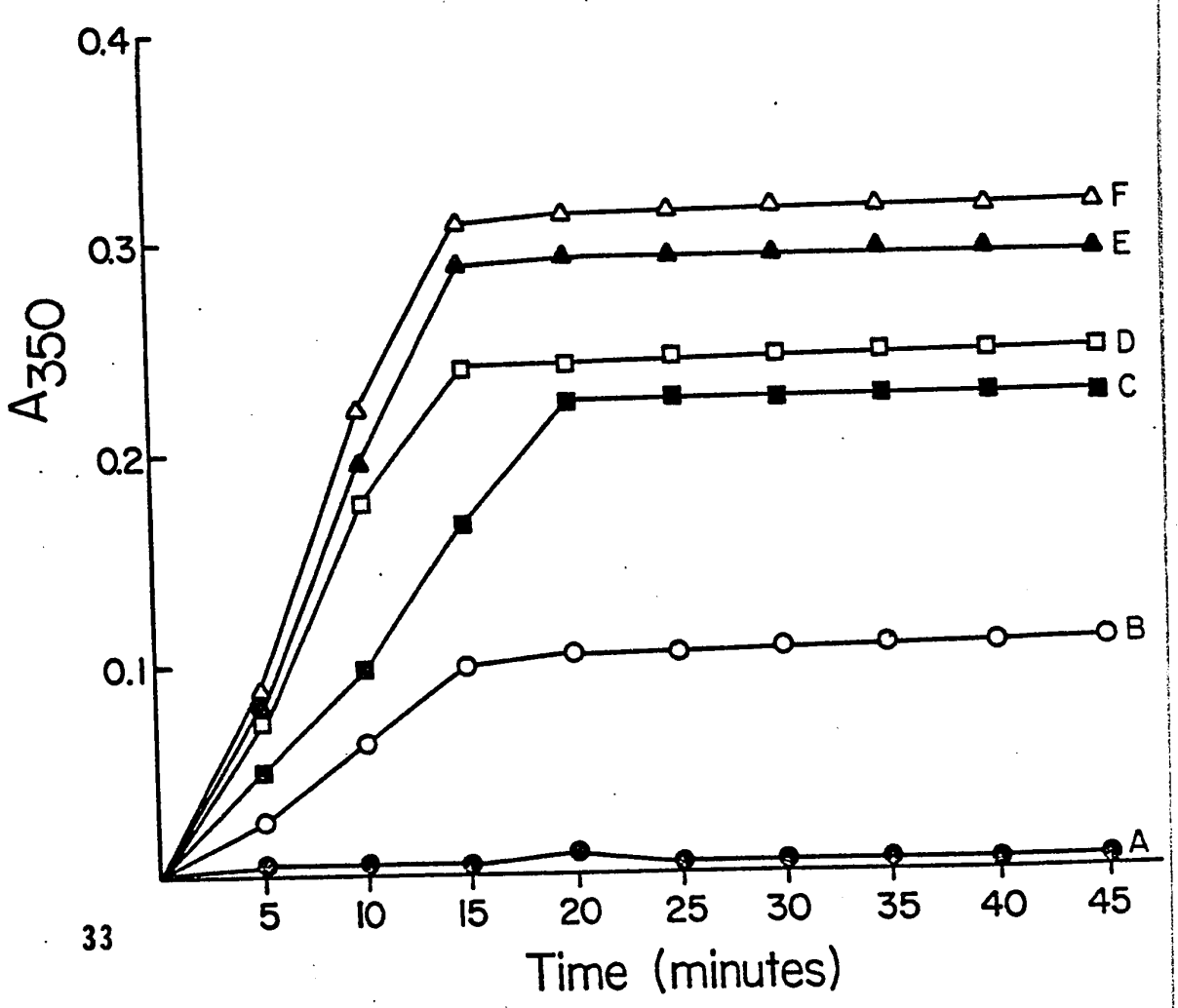
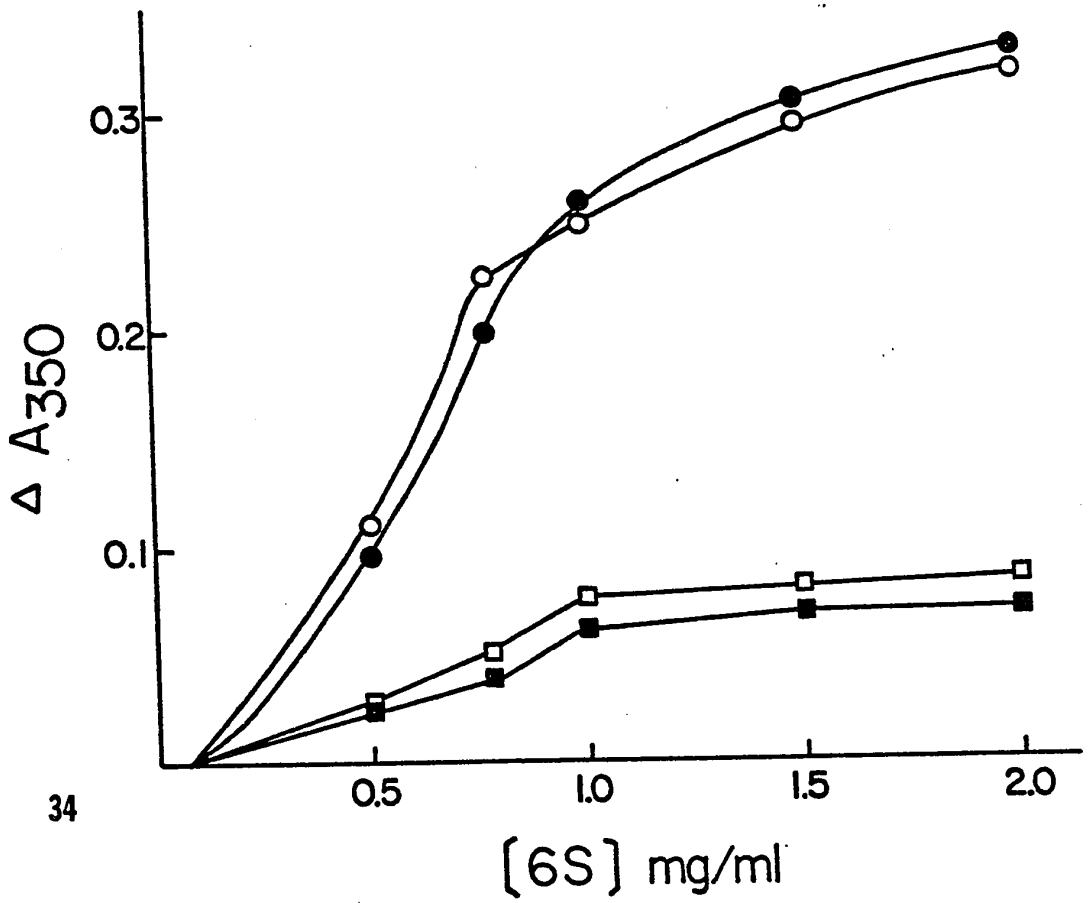

0.4
0.3
0.2
0.1
A350
F
E
D
C
B
A
5
10
15
20
25
30
35
40
45
Time (minutes)

Fig. 34 Summary of results in fig. 32, 33 showing the total increases in absorbance after incubation at 37°C for 5 min for "the-MAP" (-■-■-), for TIPs (-□-□-)and for 30 min for "the-MAP" (-●-●-), for TIPs (-o-o-). Increases in the tubulin concentration to 1.0 mg/ml rapidly increases initiation rates and final equilibrium levels. Above 1.0 mg/ml no increase in initiation rates occurs and increases in equilibrium levels tend to level off.


0.3
0.2
0.1
Δ A350
0.5
1.0
1.5
2.0
[6S] mg/ml

For "the-MAP" and for TIPs, the initiation rates and the equilibrium values increase with an increase in the amount of tubulin used. The result indicates that assembly kinetics are also dependent on the availability of tubulin protein. Figure 34 summarizes the results in figs. 32, 33 showing initiation rates, at 5 min, and equilibrium values reached at 30 min. The greatest increase in initiation rates and in equilibrium values occurred for tubulin levels up to 1 mg/ml. Above 1 mg/ml tubulin no significant increase in the initiation rates occurs, probably because the amount of initiating protein becomes limiting at this point. Coincidently, the increases in apparent equilibrium values also level off but it is not clear if this is of any significance.

Viscometry: In the turbidiometry studies, the effect of "the-MAP" and TIPs on initiation of microtubules has been measured but it is not clear from the data whether these proteins (particularly "the-MAP") also can promote tubulin assembly during microtubule elongation. It is necessary to first devise an experimental system which separates these two events. In earlier viscometry work in fig. 16b it was shown that at 2 mg/ml tubulin, reducing the number of rootlet-MTOCs to 0.5×10^6 results in a reduction in the initiation rates as well as the extent of assembly after 30 min. This provides an ideal system by

which to test if "the-MAP" and TIPs can function to promote microtubule elongation. To samples containing a constant number of intact rootlet-MTOCs (0.5×10^6/ml) and tubulin at a constant level of 2 mg/ml, increasing amounts of "the-MAP" (fig. 35) or TIPs (fig. 36) have been added and the kinetics of tubulin assembly compared to equilibrium at 30 min. Figure 35 shows that addition of the "the-MAP" does not have a significant effect on initiation rates unless increased to as high as 0.3 mg/ml. In comparison, fig. 36 shows that addition of even small amounts of TIPs does stimulate the initiation rates of assembly by 5 min. The direct effect of TIPs on initiation is found to indirectly enhance assembly rates during the elongation phase (e.g. by 10 min) presumably as a result of TIPs starting more microtubules or sites which promote more rapid assembly of tubulin (see Johnson and Borisy, 1977). Figures 35 and 36 show addition of "the-MAP" and TIPs both have an overall effect of increasing the final extent of tubulin assembly at equilibrium in rootlet-MTOC, tubulin mixtures. The results show "the-MAP" functions to promote microtubule elongation, while TIPs specifically serves only to initiate microtubules. Negative staining studies of samples in figs. 35 and 36 have confirmed the viscometry results and shows that addition of "the-MAP" does not result in initiation of independent microtubules by 10 min.

Fig. 35 Stimulation of tubulin (2.0 mg/ml) assembly in the presence of rootlet MTOCs at 0.5 x 10^6/ml by increasing amounts of "the-MAP" at (A) 0 mg/ml; (B) 0.1 mg/ml; (C) 0.15 mg/ml; (D) 0.20 mg/ml; (E) 0.3 mg/ml. "The-MAP" stimulates increases in equilibrium levels apparently by promoting elongation of microtubules (compare to fig. 36).

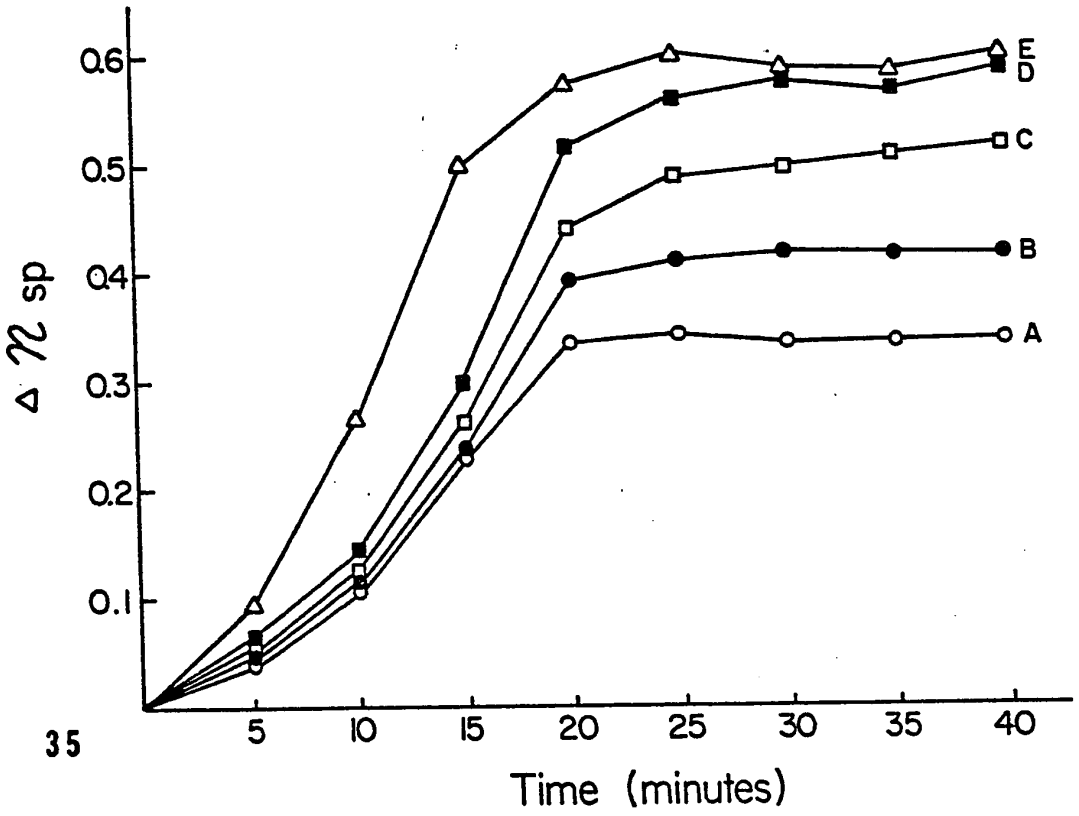
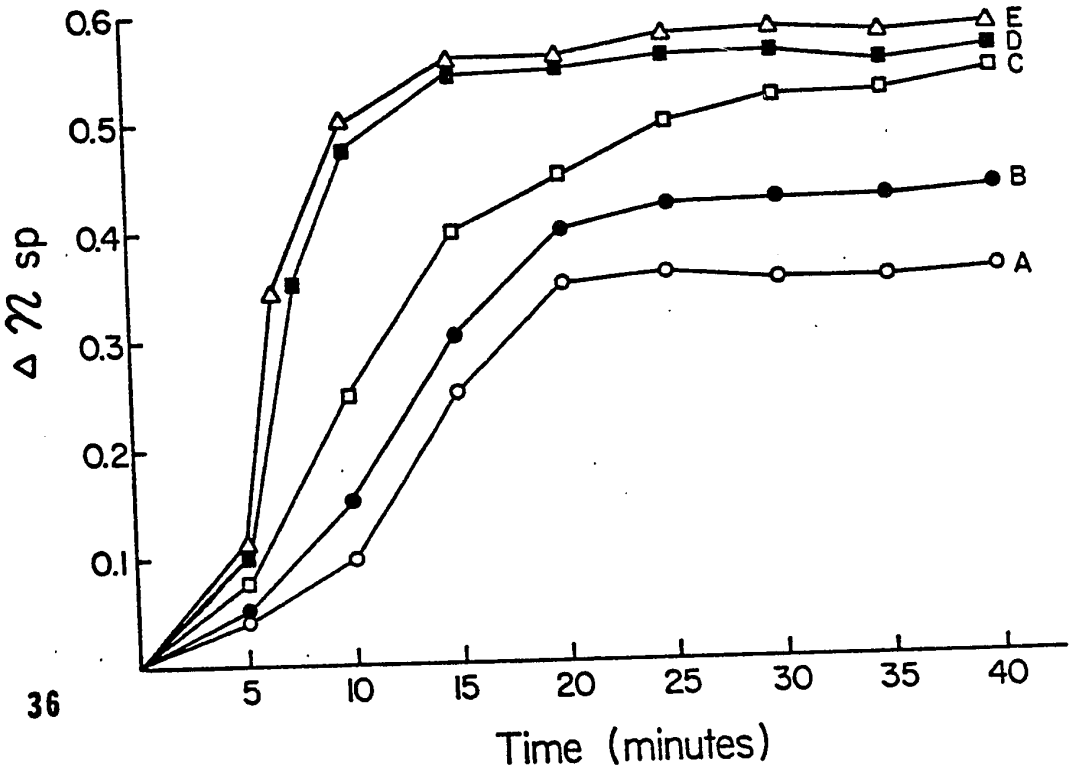

0.6
0.5
0.4
0.3
0.2
0.1
Δ η sp
5
10
15
20
25
30
35
40
Time (minutes)
E
D
C
B
A

Fig. 36 Stimulation of tubulin (2.0 mg/ml) assembly in the presence of rootlet MTOCs at 0.5 x 10^6/ml by increasing amounts of TIPs at (A) 0 mg/ml; (B) 0.1 mg/ml; (C) 0.15 mg/ml; (D) 0.20 mg/ml; (E) 0.3 mg/ml. TIPs stimulates initiation rates to increase the specific viscosity at equilibrium (compare to fig. 34).


0.6
0.5
0.4
0.3
0.2
0.1
Δ η sp
5
10
15
20
25
30
35
40
Time (minutes)
E
D
C
B
A

However, addition of TIPs does result in initiation of large numbers of microtubules by 5 min which were not attached to the rootlet-MTOCs.

LABELING STUDIES: TIPs-S^{35}

TIPs labelled with ^{35}S provide a more sensitive technique for determining the effect of low concentrations of TIPs on tubulin assembly. Figure 37 is a standard curve used to determine the specific activity for TIPs-S^{35}, at 4×10^5 cpm/mg protein, and this value was used to calculate the amounts of TIPs associated with microtubules in assembly experiments in figs. 38, 39. Figure 38 shows that in experiments where high tubulin levels are employed (2 mg/ml) all of the TIPs-S^{35} incubated with the tubulin is found to incorporate in the microtubules during the initiation phase of tubulin assembly. In experiments using very low amounts of TIPs-S^{35} (fig. 38), the initiation event is slowed to 10 min by which time all the TIPs-S^{35} is used. Figure 39 shows that when different amounts of TIPs-S^{35} are incubated with varying amounts of tubulin protein (0.5, 1.0 and 2.0 mg/ml), the amount of TIPs-S^{35} incorporated into the microtubules after a 10 min incubation period depends on the initial amount of TIPs added. The amount of TIPs-S^{35} incorporated in the microtubules does not vary over a wide range of tubulin concentrations tested (fig. 39). The

Fig. 37 Linear relationship of protein levels determined by Hartree method to cpm. A specific activity for TIPs-S^{35} of 4×10^5 cpm/mg protein was used in fig. 37, 38 to determine TIPs associating with tubulin in assembly of microtubules.

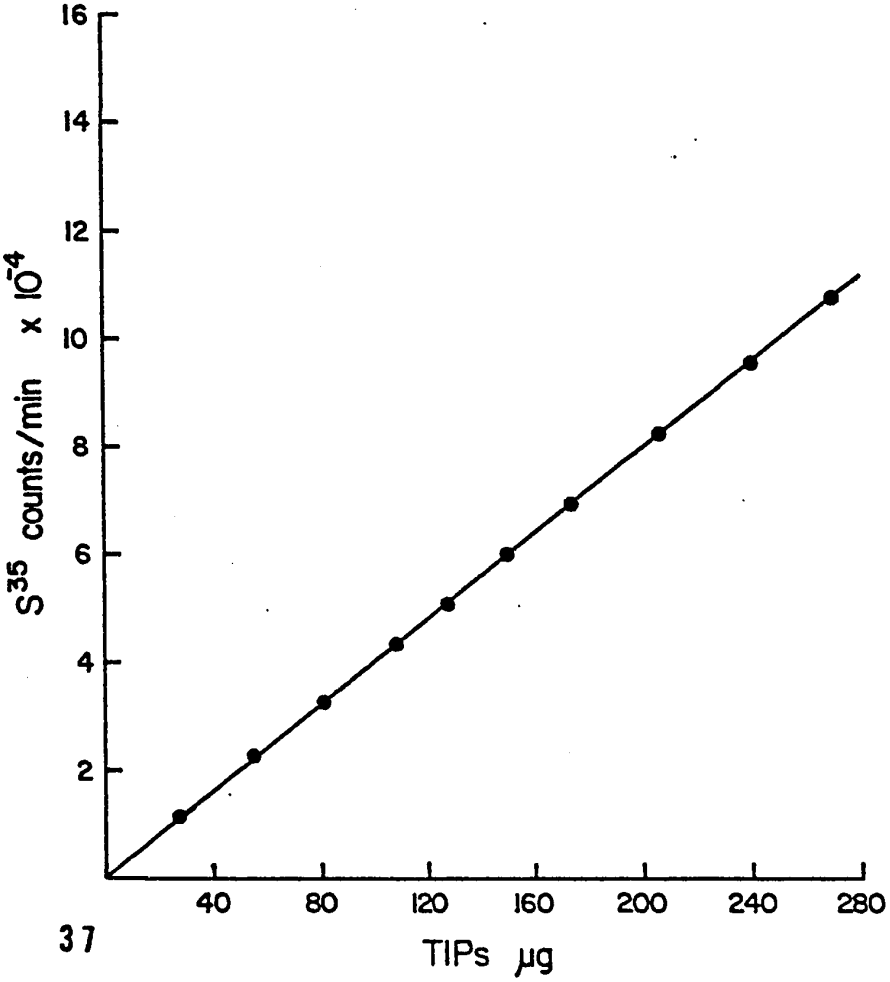

16
14
12
10
8
6
4
2
S^{35} counts/min x 10^{-4}
40
80
120
160
200
240
280
TIPs µg

Fig. 38 Amounts of TIPs-S^{35} incorporated in microtubules at 37°C at a constant tubulin level (2 mg/ml) and increasing amounts of TIPs-S^{35} at (A) 0.07 mg/ml; (B) 0.13 mg/ml; (C) 0.26 mg/ml. 0.5 ml aliquots were taken from samples incubated 5, 10 and 30 min and pellets dissolved in scintillation fluid for counting.

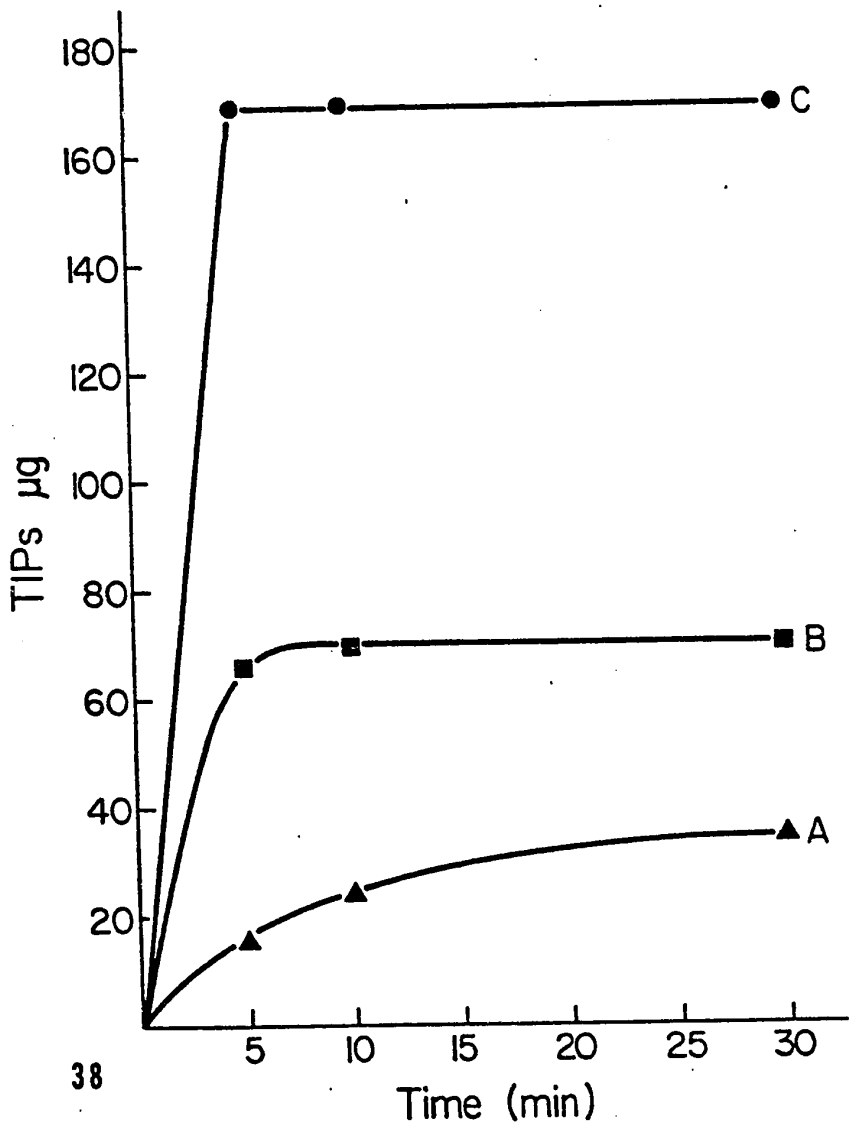
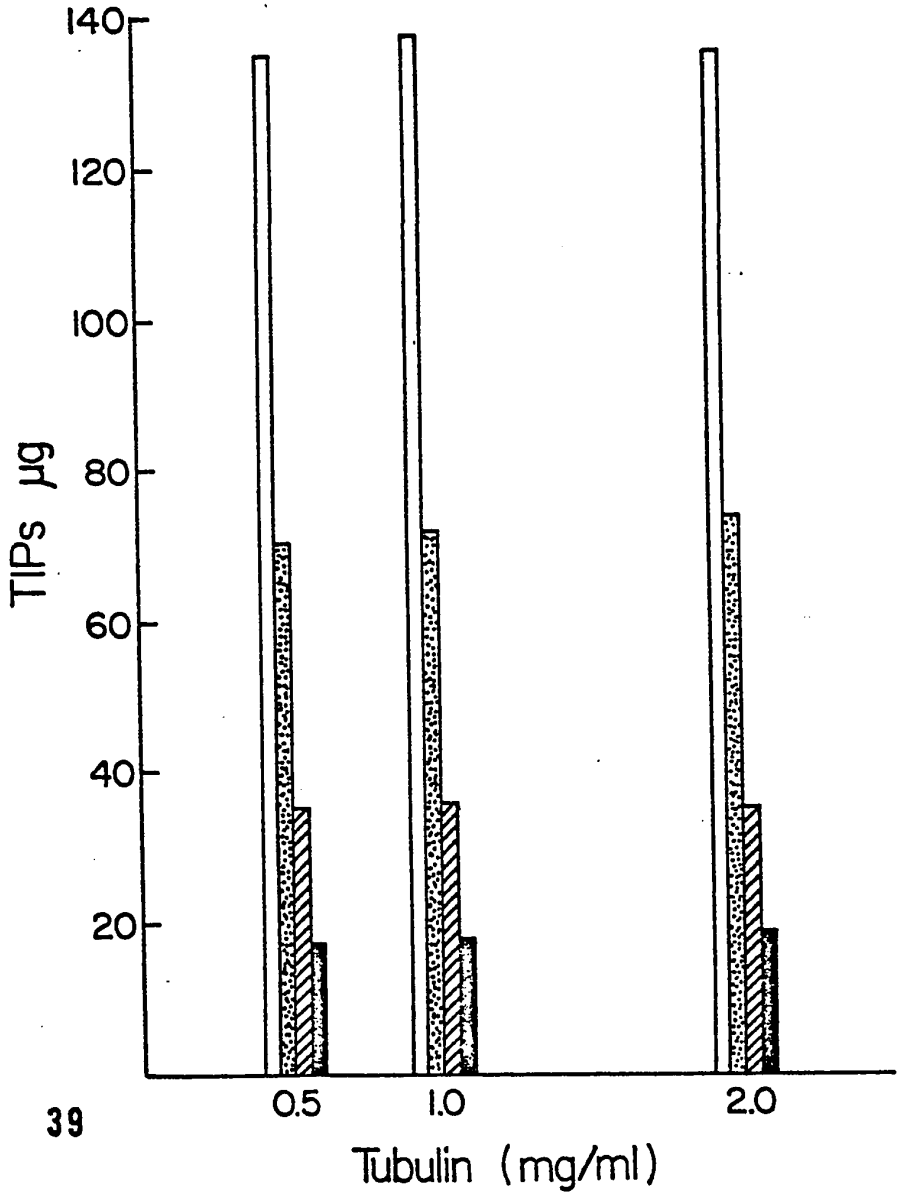

180
160
140
120
100
80
60
40
20
TIPs µg
C
B
A
5
10
15
20
25
30
Time (min)

Fig. 39 Amounts of TIPs-S^{35} incorporated in microtubules at 37°C at varying tubulin levels and increasing amounts of TIPs-S^{35} at (a) 0.26 mg/ml, ▯ ; (b) 0.13 mg/ml, ▯ ; (c) 0.07 mg/ml, ▨ ; (d) 0.04 mg/ml, ■ . Samples were incubated for 10 min at 37°C and 0.5 ml aliquots centrifuged and pellets counted.


140
120
100
80
60
40
20
TIPs µg
0.5
1.0
2.0
Tubulin (mg/ml)

results from labeling studies conclusively demonstrate that TIPs is involved specifically in the initiation on microtubules which is consistent with the kinetic data and with TIPs composing MTOCs.

STRUCTURAL ANALYSIS OF ASSEMBLY PRODUCTS

Thin sectioning: Temperature-cycled brain microtubules have been shown previously by others to take on a coated appearance having arms along the microtubule surface (Dentler et al., 1975; Murphy and Borisy, 1975; Sloboda et al., 1976a, b). Figure 40a, b shows that in longitudinal and cross sectional views of the microtubules formed by tubulin at 2 mg/ml incubated with 0.3 mg/ml "the-MAP" (fig. 40a) and with 0.3 mg/ml TIPs, fig. 40b. These microtubules have smooth surfaces and tend to be clumped side-by-side in centrifugation pellets, fig. 40b. These smooth microtubule (fig. 41a) are found to take on a coated appearance, fig. 41b, c, after they have been briefly incubated for 3 min with crude MAPs (at 0.2 mg/ml) prior to centrifugation to pellet them. Figure 41b, c shows the microtubules now have arms coating their surfaces which apparently prevent their clumping. These microtubules closely resemble S-3 microtubules. The sections show that one or more of the MAPs protein other than "the-MAP" contributes to formation of the arms.

Fig. 40 a, b. (a) "The-MAP"-initiated microtubules; (b) TIPs-initiated microtubules. The microtubules are smooth surfaced and often clumped side-by-side. Magnifications: (a) x90,000; (b) x120,000.

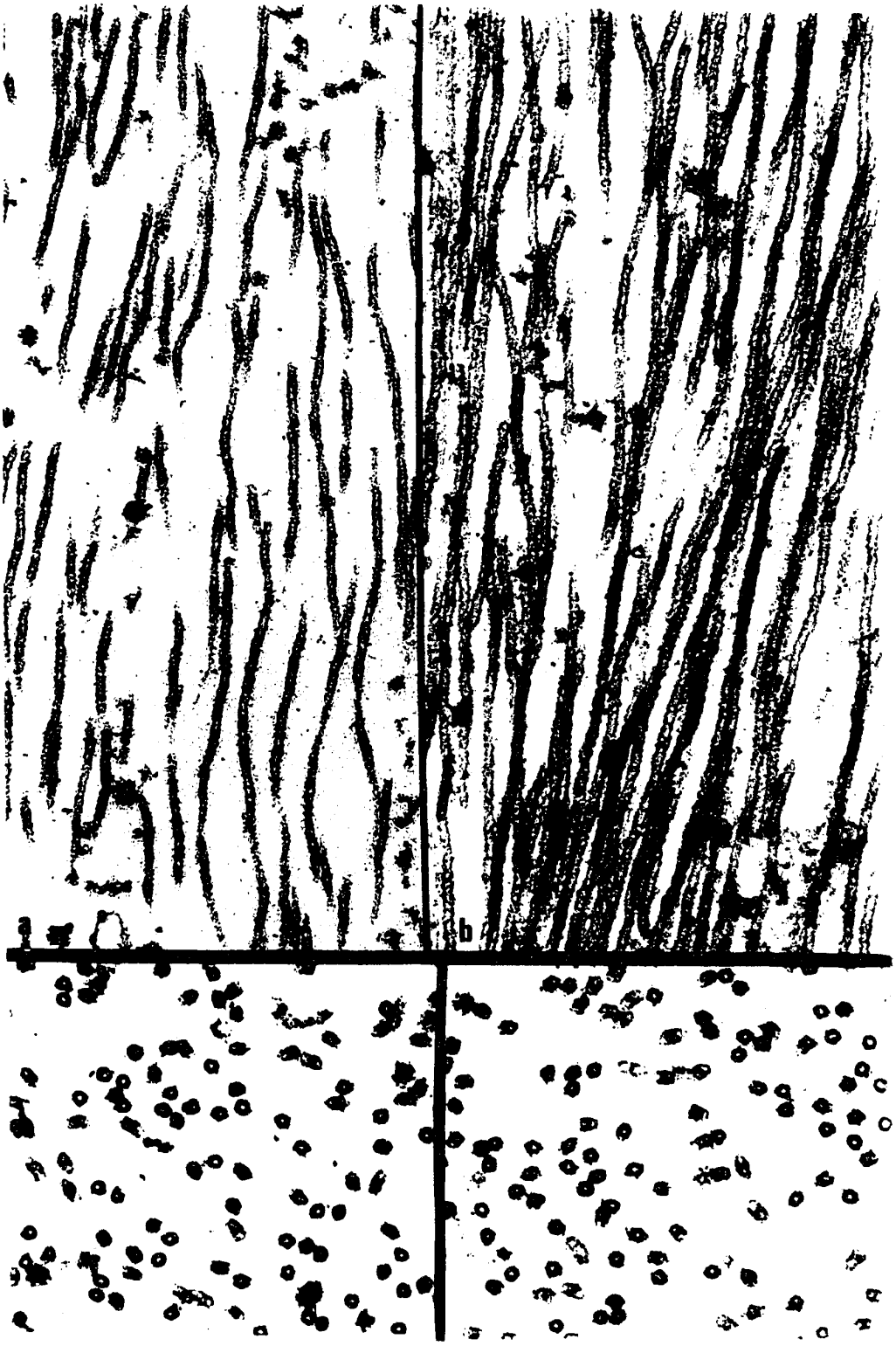

b

Fig. 41 a - c. (a) TIPs-initiated microtubules; (b) TIPs microtubules briefly incubated with MAPs before pelleting; (c) higher magnification view of (b). Smooth microtubules in (a) are coated with arms in (b), (c) and they no longer clump side-by-side. Magnifications: (a) x100,000; (b) x100,000; (c) x180,000.

ANALYTICAL ULTRACENTRIFUGATION

Negative staining studies have shown "the-MAP" stimulates tubulin to form rings at 4°C. TIPs, however, at 4°C does not promote formation of rings or of any other recognizable structures. Analytical ultracentrifugation studies have confirmed these observations. Figure 42a shows purified tubulin exhibits a single 6S peak. Freshly mixed samples containing "the-MAP" and tubulin at 5°C exhibit an additional 33S peak (fig. 42b) but samples containing TIPs and tubulin exhibit only a 6S peak as in fig. 42a. To check if rings are formed as microtubule disassembly products, the microtubules were disassembled at 5°C for 30 min and subjected to centrifugation. Figure 42c shows a 33S peak is present in samples containing "the-MAP". Figure 42d shows the 6S peak has a broad leading edge in samples containing TIPs. Negative staining studies show rings present in "the-MAP" preparations and residual protofilaments in samples containing TIPs. The protofilaments tend to disappear after longer periods at 5°C. Addition of "the-MAP" to TIPs preparations preserves these protofilaments in a stable form as rings.

Sedimentation Analysis: Examination of phosphocellulose purified tubulin, or purified tubulin incubated with "the-MAP" in the analytical ultracentrifuge shows peaks with extrapolated sedimentation coefficients ($S^{o}_{20,W}$) of 5.9S for tubulin and 33 ± 1S for rings at 5°C in MAB (fig. 43).

Fig. 42 a - d. Peaks after 24 min at 48,000 rpm (5°C) photographed at 65° for (a) tubulin, 8 mg/ml; (b) tubulin, 6 mg/ml plus MAPs, 0.4 mg/ml; (c) cold disassembled proteins of "the-MAP" (0.4 mg/ml) and tubulin (8 mg/ml); (d) cold disassembled proteins of TIPs (0.4 mg/ml) and tubulin (8 mg/ml).

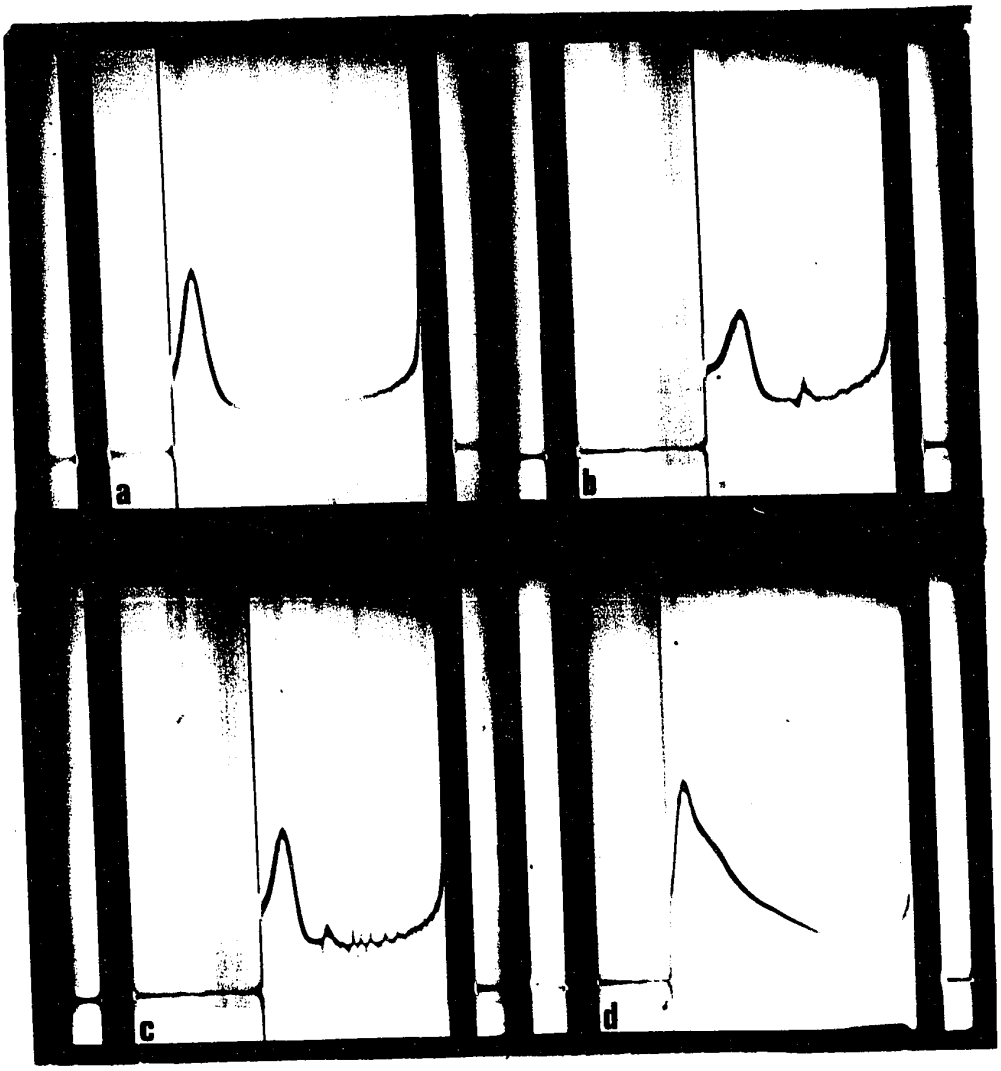
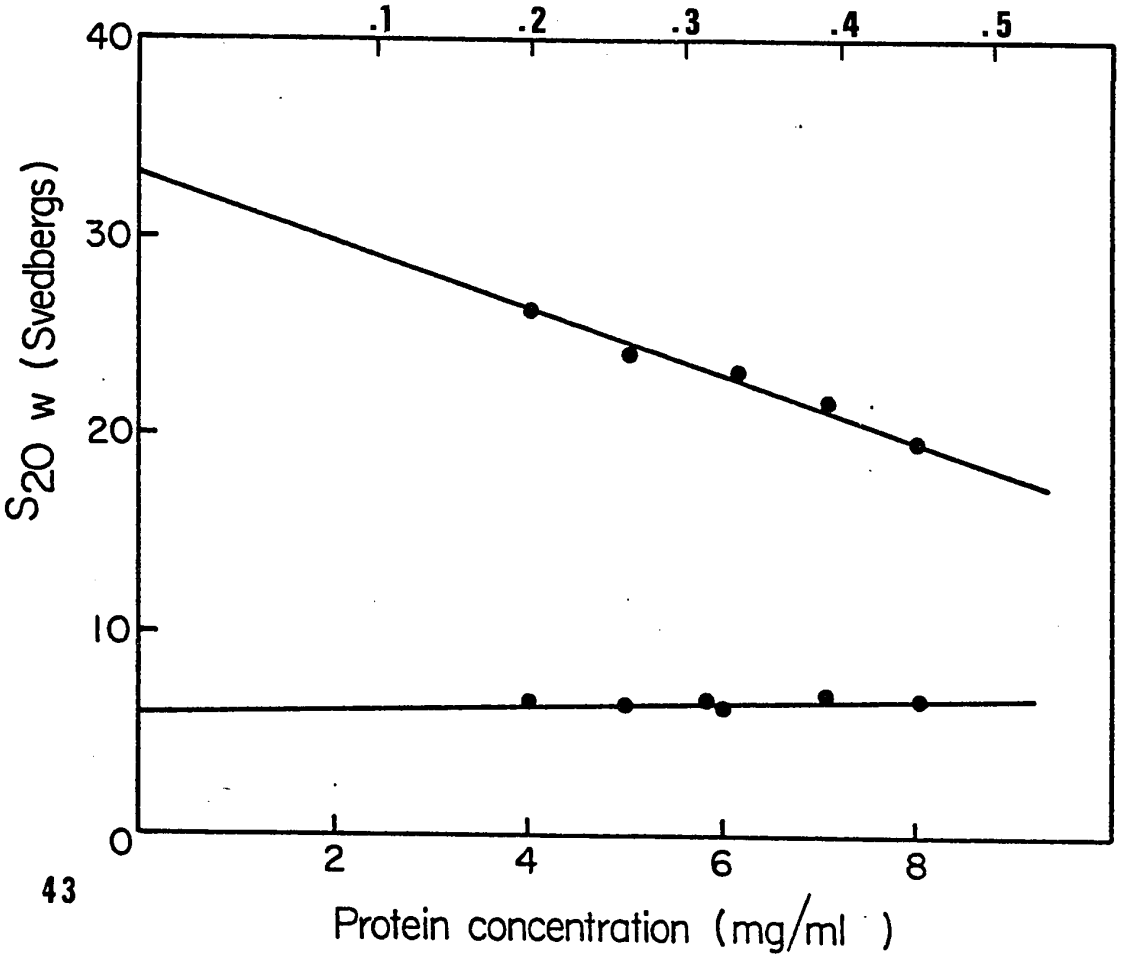

a
b
c
d

Fig. 43 Sedimentation coefficients, S_{w20} for rings in 6S plus "the-MAP" mixtures (33 ± 1S and for phosphocellulose-purified tubulin (5.9S). Sedimentation values have been determined by extrapolation to infinite dilution.

Top Scale: Concentrations of "the-MAP" using 6S at 8 mg/ml.

Bottom Scale: Concentrations of purified 6S.

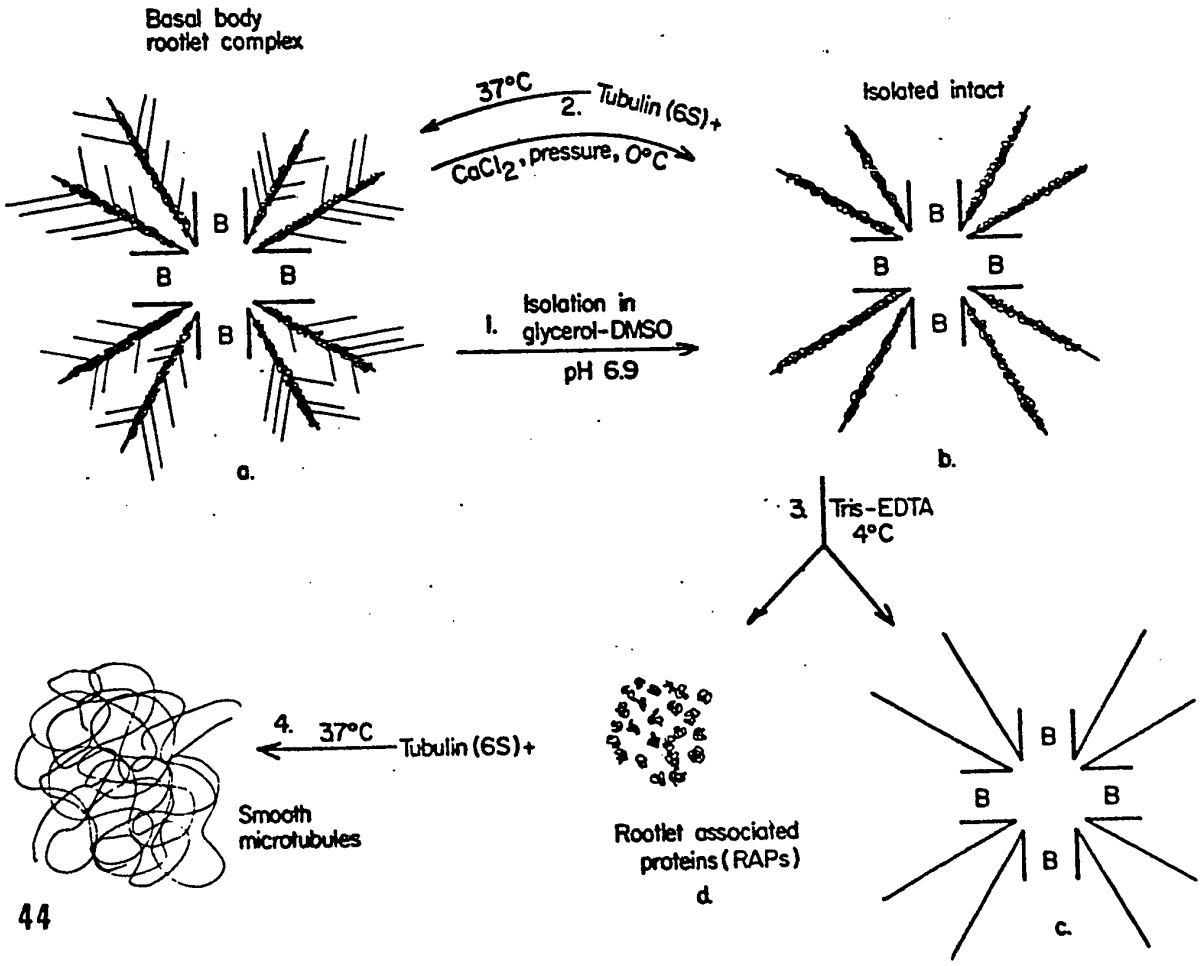

.1
.2
.3
.4
.5
40
30
20
10
0
2
4
6
8
$S_{20\,w}$ (Svedbergs)
Protein concentration (mg/ml)

DISCUSSION

The main results that this thesis reports on the properties of rootlet-MTOCs are summarized in figure 44. We have isolated and purified rootlet-MTOCs which function *in vivo* in the assembly of a patterned array of cytoskeletal microtubules. The isolated MTOCs do not retain these microtubules but otherwise appear structurally intact. They are functional *in vitro* in re-initiating the original pattern of microtubules utilizing brain tubulin. Most importantly, the proteins necessary for this initiation can be extracted and will initiate purified tubulin assembly.

In vivo studies of some organisms have shown that the development of highly patterned arrays of microtubules appears to be primarily controlled at the site of assembly by MTOCs (Porter, 1966; Tucker, 1977; Bouck and Brown, 1976). In others, linkers formed between microtubules appear to be the controlling elements in establishing the pattern (Tilney, 1971). More recently, it has been postulated that linkers as well as MTOCs may function in microtubule initiation (Bardele, 1977). In *in situ* studies of *Polytomella* (Brown and Stearns, 1978) we showed that the pattern of cytoskeletal microtubules in *Polytomella* is established at the point of initiation by MTOCs and we concluded that linkers are not involved at least in initial patterning. The *in vitro*

Fig. 44 Model diagram summarizing *in vitro* assembly studies using basal body rootlet MTOCs.


Basal body
rootlet complex
37°C
2. Tubulin (6S)+
CaCl2, pressure, 0°C
Isolated intact
B
B
B
B
B
B
1. Isolation in
glycerol-DMSO
pH 6.9
a.
b.
3. Tris-EDTA
4°C
4. 37°C
Tubulin (6S)+
Smooth
microtubules
Rootlet associated
proteins (RAPs)
d.
c.

results showing that patterned assembly occurs on isolated MTOCs confirm this conclusion (compare figs. 10, 11; figs. 15a, b).

Several groups have indicated by electron microscopy that several apparently unstructured MTOCs either partially isolated or in cell lysates will serve as assembly sites for brain microtubule proteins (Borisy et al., 1975; Gould and Borisy, 1976; McGill and Brinkley, 1975; Snyder and McIntosh, 1975; Telzer et al., 1975). Following high speed centrifugation at 230,000g, 90 min., microtubule proteins used in these studies slowly self-assemble and the MTOCs have been shown to stimulate this assembly (Borisy et al., 1975; Gould and Borisy, 1977; Snyder and McIntosh, 1975). Similarly, isolated rootlet-MTOCs have been shown by negative staining to stimulate rapid assembly of high speed supernatants of brain tubulin (see also Stearns et al., 1976). These MTOCs are highly structured and have been extensively purified in the form of a basal body rootlet complex in numbers sufficient for use in quantitative studies that measure the capacity of an MTOC to promote tubulin assembly. Viscometric results reported in this paper show that this presence of rootlet-MTOCs has a significant effect on stimulating initiation of microtubule assembly (fig. 14; Table II). Negative staining observations have confirmed this result and have shown that in the presence of complexes, microtubules will reassemble

specifically on rootlets and the number and length of these tubules quickly increases with longer incubation times (figs. 12a-f; 13). Some independent self-assembly of microtubule proteins does occur, but the majority of the microtubules form on the rootlets during the early incubation periods, indicating that MTOCs promote assembly. By using an easily identified MTOC this study shows that MTOCs can actively stimulate tubulin assembly, and also that they do not simply serve as sites for the attachment of self-assembled microtubules. One concludes from these results that MTOCs serve as assembly sites for initiating microtubules. Thin sectioning of the complexes which have been incubated with tubulin show conclusively that the microtubules are assembled in rows directly on recognizable sites on rootlets.

The independent assembly of brain tubulin described in the above experiments occurs as a result of the presence of non-tubulin accessory proteins which can promote tubulin assembly (Murphy and Borisy, 1975; Keates and Hall, 1975; Sloboda et al., 1976a, b; Weingarten et al., 1975). To eliminate the possibility that these proteins are required for MTOCs to initiate microtubules, I have temperature-cycled microtubule proteins to enrich for tubulin and used phosphocellulose chromatography to prepare purified tubulin free of accessory proteins. Purity was judged on the basis of two criteria for each batch of tubulin prepared: a) electro-

phoretic purity for a range of 10 to 100 ug of protein loaded on gels, and b) inability to self-initiate at concentrations of 6 mg/ml in MAB at 37°C. Electron microscopy studies showed that in the presence of intact rootlet-MTOCs there is no independent assembly of tubulin; this means that preparations of rootlet-complexes do not contain initiating material which has dissociated from the rootlets. Tubulin is assembled on the rootlet-MTOCs at critical concentrations of 0.2 to 0.3 mg/ml tubulin, values well within possible physiological levels. Viscometric results have supported the structural evidence showing that tubulin self-assembles on MTOCs (figs. 16a, b). When the tubulin level is kept constant and the number of rootlet-MTOCs gradually increased from 0.05×10^6 to 3×10^6, a corresponding increase in the kinetics of initiation of microtubules is observed to occur (fig. 16a). The result demonstrates that MTOCs are actively promoting tubulin assembly by providing sites to initiate assembly. It is noteworthy that below a critical number of complexes of approximately 1×10^6/ml, a decrease in the equilibrium values occurs, showing that in assembly experiments in earlier studies (figs. 14, 16, Table II) assembly was not limited by MTOCs at a level of 2×10^6/ml.

The problem of whether accessory proteins (MAPs) are essential for tubulin assembly on "microtubules seeds" has been examined by others, but the results have so far been

contradictory. Witman et al. (1976) have reported that phosphocellulose-purified tubulin will not assemble onto the ends of axonemal seeds unless small amounts of low molecular weight MAPs (tau) are added to mixtures. Murphy et al. (1977), on the other hand, have found that DEAE-sephadex-purified tubulin does not require any accessory protein to assemble on axonemal seeds. Results from the present studies with rootlet-MTOCs confirm Murphy's findings and show that phosphocellulose-purified tubulin does not require accessory proteins to assemble on MTOCs or on ends of microtubules initiated by MTOCs.

The tubulin assembly promoted by the rootlet-MTOCs does not appear to be due to the presence of remnants of cytoskeletal microtubules. None were seen in the negative staining or in thin-sectioning studies of the isolated complexes. In addition, treatment of isolated complexes with several agents (2mM $CaCl_2$, low temperature, and hydrostatic pressure) known to depolymerize microtubules had no effect on their ability to initiate microtubules. If the purified brain tubulin microtubules assembled onto rootlets are depolymerized by these treatments, the stable rootlets-MTOCs will again initiate assembly (fig. 44), indicating that the initiation function resides with the amorphous MTOC structures.

Rootlet-MTOCs are ideal for composition studies since they have easily identified sites and they can be isolated in

large quantities. In a set of experiments for this thesis, trypsin treatments indicate that the microtubule-initiating component of these MTOCs consists of proteins exposed on the surface of the rootlets. In another set of experiments, a low ionic strength Tris-EDTA solution was used to extract rootlet-associated proteins from rootlets. Bloodgood and Rosenbaum (1976) have reported that extensive dialysis of isolated flagella axonemes in this solution for 48 hours will extract a high molecular weight protein which initiates assembly of tubulin protein. In the present study similar procedures, as outlined in fig. 5a, were used for isolating complexes. However, during isolation the flagella were removed from Polytomella to eliminate the possibility of axonemes contaminating preparations of complexes. Also unlike the axonemal extraction experiments, rootlet complexes in this work were only briefly dialyzed (20 min) against Tris-EDTA, and this amount of time was found to be sufficient to remove rootlet-associated proteins (RAPs) from rootlet-MTOCs. Functional assays showed that the extracted MTOCs would not initiate microtubules and so confirmed that the initiating proteins had been removed. Like intact MTOCs, the RAPs in extracts are trypsin-sensitive and will initiate tubulin assembly. Turbidity, A_{350}, studies reveal in experiments to test the ability of RAPs to promote assembly, that at a constant tubulin level increasing the amounts of RAPs results

in a decrease in the initial lag phase for assembly. This finding indicates that RAPs contain proteins which function in initiation (fig. 44).

In general, two conclusions can be made from these in vitro assembly studies with isolated rootlet-MTOCs. First, the primary function of MTOCs is to control initiation and to establish the patterning of microtubules. Second, MTOCs consist of microtubule-initiating proteins which function to stimulate tubulin assembly. These results are consistent with and confirm in vivo observations that MTOCs control formation of microtubules (Porter, 1966; Bouck and Brown, 1976; Brown and Stearns, 1978).

In a second part to this thesis, these structural-functional studies with MTOCs were extended to examine the process by which microtubules are initiated at the molecular level. For these studies, procedures were first developed for purifying the initiating proteins (TIPs) present in RAPs extracts. By using purified TIPs, it is then possible to measure the effect that these proteins have on tubulin assembly. The importance in taking this precaution is that contaminating proteins are removed and this ensures that the results reflect the contribution that measurable quantities of TIPs have on tubulin assembly.

The methods employed for the purification of TIPs depend on the fact that a slow initiation stage is associated with

tubulin assembly. At the protein concentrations used, initiation of protofilaments took 20 sec and the first microtubules do not appear until 180 sec. Because of the time difference between these two events, centrifugation could be used to pellet out protofilaments after briefly incubating samples to start assembly (i.e. 10 sec). The maximum total incubation period for samples is about 100 sec, which includes the time it takes for the rotor to reach maximum centrifugation speed. At high centrifugal force (i.e. 39,000g) assembly apparently stops since negative staining shows the main structures that are removed from mixtures are protofilaments. Using this approach for purifying TIPs has two advantages: it permits positive identification of the initiating proteins (TIPs), and most importantly, it enables purification of these proteins for assembly studies.

SDS gels show that the pelleted protofilaments consist of tubulin and four high molecular weight proteins (190,000 to 220,000 daltons). To purify TIPs, these protofilaments were cold solubilized and fractionated using phosphocellulose chromatography. Using the above procedures it was possible to obtain approximately 600 ug of biologically active TIPs from about 6×10^7 basal body rootlet complexes which can be obtained from 10 liters of cells. Yields of this magnitude are sufficient for investigation of the role of these proteins in tubulin assembly.

Studies of the process of tubulin assembly *in vitro* have already been carried out by others who have used MAPs of brain microtubules to promote assembly (see Introduction). In brief, these studies have shown that chromatographically purified tubulin will not self-initiate, but requires a number of proteins termed MAPs which normally co-assemble with temperature-cycled tubulin in stoichiometrically constant amounts to promote the assembly of tubulin. Using these MAPs, most parameters of tubulin assembly have been examined and most of the available techniques for monitoring tubulin assembly have been standardized in these studies. These MAPs have been shown to have at least two functions: to initiate formation of a stable microtubule helix and to encourage the elongation of these fragments.

In interpreting results from similar work with a new factor such as TIPs, it makes sense to compare the data to similar results obtained using MAPs. One of the problems with comparing results to published data is that most of these studies have been with MAPs preparations containing several proteins which appear to have different functions depending on the group (i.e. compare Sloboda *et al.*, 1976b to Murphy *et al.*, 1977) and it is difficult to draw meaningful comparisons in attempting to determine the effect of TIPs on assembly. It is imperative therefore to first purify one or more MAPs before attempting to analyze their functions

in assembly. Since the main function of TIPs is to serve as an initiating factor, it is logical to purify a similar initiating factor from MAPs and then compare the roles of these two factors in tubulin assembly.

Thus, procedures that were employed for purifying TIPs from RAPs were used to purify the initiating component of MAPs from temperature-cycled beef brain. The crude MAPs containing high and low molecular weight MAPs, and from these a single 330,000 daltons protein ("the-MAP") was found to initiate tubulin assembly and during centrifugation it pelleted as a component of protofilaments.[1] After purification of "the-MAP" by phosphocellulose chromatography, the ability of "the-MAP" and TIPs to stimulate assembly of phosphocellulose-purified tubulin was compared using negative staining, turbidiometry and viscometry. The results from these studies have been dealt with here in the discussion in two categories, structural studies and kinetic studies.

---

[1]This result implies low molecular weight MAPs (tau) does not function to initiate tubulin assembly. However, the conditions used may be the major factor for this inactivity and further studies comparing the abilities of different MAPs to promote tubulin assembly under different conditions are required before firm conclusions are drawn. See Introduction for discussion of the controversies over which of the MAPs is active in promoting tubulin assembly.

STRUCTURAL STUDIES - INTERMEDIATES IN TUBULIN ASSEMBLY

In structural studies, the assembly of microtubules from temperature-cycled brain tubulin or purified tubulin reconstituted with either high or low molecular weight MAPs has been shown by negative staining to involve rings, protofilaments and protofilament sheets as intermediates (Erickson, 1974; Kirschner et al., 1975a; Sloboda et al., 1976b). The rings are proposed to serve as "nucleating centers" which initiate the assembly of tubulin protein (Borisy et al., 1974; Sloboda et al., 1976b). Recently, however, several reports have indicated that rings are not uniformly present in tubulin extracts from different sources. Furthermore, these studies show rings are not necessary for assembly of tubulin to occur in extracts prepared from cultured cells (Nagle et al., 1977) or from fish brains (Langford, 1977; Stearns et al., 1978). It was found that the addition of MAPs obtained from brains of beef or pig would induce formation of rings, indicating that rings are peculiar to the MAPs obtained from homeotherms (see Nagle et al., 1977; Stearns et al., 1978). The present study shows that when beef brain tubulin is incubated with MAPs or "the-MAP" at $4^{o}C$, tubulin will form rings but the same tubulin does not form rings if instead it is incubated with TIPs. In spite of this difference in these two tubulin mixtures, negative stain studies show that at $37^{o}C$ purified TIPs and "the-MAP" both initiate

tubulin to form identical types of intermediates at 37°C in the assembly of tubulin. That is, tubulin is seen to assemble protofilaments and protofilament sheets which in turn fold to form a complete microtubule wall. These results would strongly suggest that protofilaments are an important assembly intermediate, but that rings are not essential as either "nucleating centers" or as intermediates, as was suggested by the work of Borisy et al. (1974) and Sloboda et al. (1976a, b).

In the studies with MAPs, rings are seen as low-temperature aggregation products, which suggests that they may appear only after cold disassembly of microtubules in tubulin mixtures containing TIPs. To test this possibility, analytical ultracentrifugation (fig. 42) and negative staining techniques were utilized. Fig. 42 shows that rings (33S) form during cold disassembly of "the-MAP" microtubules, but that following cold disassembly of TIPs microtubules only protofilaments remain. Also, no rings are present, indicating again that rings are a product peculiar to MAPs. When MAPs or "the-MAP" are added to the protofilament preparations, rings (33S) form within 30 sec at 4°C. This confirms the fact that MAPs induce rings. In other studies in which these MAP-induced rings are incubated at 37°C, the rings disappear concomitant with the appearance of protofilaments and the assembly of tubulin. It is likely then that rings are aggregates of

coiled protofilaments which either rapidly dissociate at 37°C, or uncoil to form protofilaments which are then incorporated into microtubules. The latter hypothesis is supported by negative stain studies by Erickson (1975) which show rings uncoiling into protofilaments in the process of tubulin assembly.

Next, to test if intact rootlet-MTOCs initiate tubulin assembly by the same mechanism as TIPs, steps in the assembly of purified tubulin on MTOCs were examined. The present study demonstrates that MTOCs initiate assembly via protofilaments and protofilament sheets as the principal intermediates, and so suggests that this mechanism may be utilized *in vivo*. In addition, rings were not present in the initial mixtures nor do they appear after cold disassembly of the microtubules, therefore indicating that rings are not a part of an MTOC mediated assembly system.

It should be noted that when high tubulin concentrations are used, or when the tubulin is pre-warmed before mixing with MAPs, TIPs or MTOCs, no intermediates are observed and complete microtubules appear almost instantaneously. This observation has also been made previously in work by Nagle *et al.* (1977) utilizing pre-warmed tubulin. This result suggests that an alternate mechanism for tubulin assembly may exist where tubulin may simply add on MTOCs or aggregate around MAPs and TIPs to start assembly by making a ring(s)

of dimers on which the tubulin continues to add so that the complete microtubule grows out from this nucleus. If this mechanism is correct, it would fit in with the ring "nucleating center" hypothesis of Borisy et al. (1974). However, I suggest as a very likely alternative that under the conditions used in the above experiments the reaction was simply too fast to detect any intermediates that occur. Further studies are necessary to satisfactorily resolve this question.

KINETIC STUDIES

Turbidometry, which is sensitive to amounts of polymer (Berne, 1974), has been used to demonstrate that preparations of high molecular weight MAPs (Sloboda et al., 1976a, b) and low molecular weight MAPs (Penningroth et al., 1976) function to promote both initiation and elongation kinetics during tubulin assembly. Their results have shown that increasing the amounts of either of these MAPs at constant tubulin levels causes decreases in the lag period for initiation and also enhances elongation rates and the final extent of tubulin assembly. Turbidometry studies reported in this thesis show that both "the-MAP" and TIPs serve to promote initiation of microtubules, a finding that agrees with the negative staining observations showing these proteins are active in initiation. However, the data also shows that "the-MAP" (like crude MAPs used in the work of Sloboda) stimulates an increase in the elongation rates, as is

indicated by changes in the slopes of the family of curves in Fig. 28. In addition, this shows that increases in amounts of "the-MAP" enhances the final extent of tubulin assembly. TIPs by comparison, although appearing to stimulate increases in elongation rates, does not affect the extent of assembly at equilibrium at least over a similar range of protein concentrations tested. These facts hint strongly that "the-MAP" has an additional role to initiation in promoting both the elongation and/or the final extent of microtubule assembly at equilibrium.

The problem with this interpretation, and for that matter the conclusions drawn by Sloboda et al. (1976a) from similar data, is that the influence that "the-MAP" has on initiation cannot be measured separately from its effect on elongation kinetics by the experimental technique used. The importance of this point is emphasized by experimental data shown in figs. 32, 33. These figures reveal that in studies where TIPs and "the-MAP" are kept at constant levels and the amount of tubulin increased, the effect of increasing tubulin levels on initiation kinetics cannot be separated from its concomitant effect on increasing rates of microtubule elongation. Again this conclusion is based on simultaneous changes in the slopes of the family of curves shown in figs. 32, 33. Thus initiation kinetics and elongation rates are closely linked and the effects of "the-MAP" on each of these cannot be separately measured in the above type of experimental system.

In an alternate approach using microtubule "seeds" to serve as assembly sites (in order to eliminate the initiation step), Murphy et al. (1977) have discovered that at constant tubulin levels changes in the MAPs concentration does not affect the rate of microtubule elongation. In other experiments they found that microtubule samples having more MAPs are more stable and do not disassemble as readily at lowered temperatures as the microtubules in preparations containing fewer MAPs. Based on these two types of experiments they concluded that the main function of MAPs is to stabilize microtubules against disassembly and thus to indirectly increase the extent of tubulin assembly at equilibrium. This would indicate that the apparent effect MAPs and "the-MAP" has on elongation in the turbidometry studies reported by Sloboda and myself, actually results from MAPs and "the-MAP" working to stimulate initiation and to stabilize microtubules against disassembly, an effect which gives the mistaken impression that MAPs are directly stimulating elongation of microtubules.

To further examine this problem, I have taken advantage of the observation made in viscometry studies in Fig. 16b that, at a constant tubulin level, reducing the number of rootlet-MTOCs (which here serve as the equivalent of the "seeds" used by Murphy et al. (1977) causes a reduction in both initiation rates and apparent rates of microtubule elongation. Fig. 35 shows that under conditions utilizing reduced numbers of complexes (curve o in fig. 16b) and at a constant tubulin level, increases in amounts of "the-MAP"

have the effect of stimulating assembly during the elongation stage of microtubule assembly (i.e. during the 5-30 min interval). In addition the final extent of assembly at equilibrium is enhanced. Negative staining studies show that "the-MAP" does not initiate independent assembly after 5 min and does so only to a limited extent after 10 min incubation. Taken together, the data suggest in contrast to Murphy et al (1977) that "the-MAP" functions to both promote microtubule elongation as well as to shift equilibrium in favor of microtubules. Whether the effect of "the-MAP" on elongation is causal to its effect on the extent of microtubule assembly is not clear from the present data but is certainly a very likely possibility.

What about TIPs - could these proteins taken from MTOCs also promote microtubule elongation? The turbidity data in Fig. 30 suggests this may be the case. In similar studies to the above viscometry studies with "the-MAP", I found that, in contrast to "the-MAP", if equivalent amounts of TIPs are substituted for "the-MAP" they had a detectable effect on stimulating tubulin assembly during the first 5 min of incubation (compare fig. 36 and Fig. 35). Negative staining studies of samples, showed that microtubules assembled independent of the MTOCs. These microtubules are observed as early as 1 min in samples with greater that 0.1 mg/ml TIPs, and in 5 min incubation intervals, large numbers of independent tubules are seen. In control samples containing 0 mg/ml TIP, all the microtubules are associated with MTOCs

and no independently assembled microtubules are seen. Taken in conjunction with the negative staining data for "the-MAP", the results for TIPs suggest that they function only in initiation of microtubules and that they do not have a role in promoting microtubule elongation. However, increases in the amount of TIPs used in these turbidometry studies did have an effect on the slope and the extent of assembly at equilibrium. Since the electron microscopy studies show TIPs functions specifically in initiation, this result is taken to mean that the effect of TIPs is on initiation rates and that the apparent effect on the elongation kinetics of tubulin assembly occurs as an indirect result of increased initiation rates.

To ensure that this is not an erroneous conclusion, the question of whether TIPs function specifically in initiation was re-examined using labeled TIPs-S^{35} obtained by growing cells in $NaSO_4^{35}$. Time course studies using centrifugation to pellet the microtubules show that, independently of the tubulin levels used, all the TIPs-S^{35} added to the samples associates with the tubulin in microtubules within a 5 min incubation interval. The same result is obtained for a wide range of TIPs-S^{35} concentrations tested. Also, TIPs is seen to initiate microtubules at low levels (0.04 mg/ml) which is in keeping with the possibility that TIPs may be localized in small amounts on rootlets to initiate microtubules.

Turbidometry studies indicate that in the mixtures of

0.26 mg/ml TIPs and 1 mg/ml tubulin, more than enough TIPs is available for initiation of maximum assembly, thus the excess TIPs in these mixtures could be used in elongation if TIPs has a role in this step. However, the labeling studies show all the TIPs is used in initiation under these conditions. It would appear that when excess TIPs is available, it must aggregate at the tips of microtubules. I conclude that TIPs function specifically in initiation of microtubules and this finding is consistent with the fact that TIPs originate from rootlet-MTOCs. Currently, studies are underway in this lab to examine if TIPs form any recognizable structures in association with the tips of microtubules.

As a matter of interest, the microtubules from the above kinetic studies were prepared for thin-sectioning. Temperature-cycled microtubules have previously been shown in thin-sections to be coated with arms (Dentler et al., 1975; also observed by the author). TIPs and "the-MAP" microtubules are shown in the present study to have smooth surfaces and to be clumped side-by-side in pellets (see figs. 40a, b). However, if they are incubated with crude MAPs briefly before pelleting, the smooth tubules take on a coated appearance so that they no longer appear clumped (figs. 41b, c). This means that one or more of the MAPs other than "the-MAP" must form the arms on microtubules. This result supports an earlier conjecture made in this discussion that different MAPs may have different functions in promoting tubulin

assembly. Further kinetic studies comparing "the-MAP" and MAPs should resolve whether MAPs contains proteins with distinct function from "the-MAP". An obvious possibility suggested by work of Murphy et al. (1977) is that MAPs forming arms have the responsibility of stabilizing microtubules.

SUMMARY

If we are to fully understand control of microtubule assembly in living cells, it is essential to first examine the important components involved in this assembly and to understand the steps by which microtubules are assembled. In general, the goal of the *in vitro* studies has been to analyze the process of assembly in terms of the role of MTOCs and in terms of protein components found to function in this event in purified microtubule preparations.

One of the initial objectives of this research was to purify basal body rootlet complexes from *Polytomella*. Procedures were developed for isolating intact complexes using a microtubule-stabilizing media used for isolating intact mitotic apparatus. An ability to detect complexes at the light microscope level facilitated purification of large numbers of complexes for assembly studies. A subsequent objective of this research was to examine the extent of control purified MTOCs could exert on tubulin assembly. These studies demonstrated that the MTOCs are prepatterned on rootlets to control initiation and patterning of microtubules. It was clear from this work that the MTOCs promote formation of an initial microtubule fragment on which tubulin could self-assemble during elongation. This self-assembly appears to be stimulated by accessory proteins of

brain microtubules which may promote assembly or stabilize microtubules against disassembly. As a second part to this work, I have purified the microtubule-initiating proteins from rootlet-MTOCs, i.e. TIPs, and compared their capacity to promote tubulin assembly to a purified accessory protein, "the-MAP", of brain microtubules. It turns out that TIPs function specifically in initiation of microtubules. The evidence suggests that TIPs may aggregate with tubulin to form a presumptive initiating site for tubulin assembly. "The-MAP" apparently differs from TIPs in associating along the microtubule in stoichiometric amounts to promote tubulin assembly.

Ideally, for the stoichiometry studies one should employ an homologous assembly system. To this end, I have recently developed methods for purifying cytoplasmic tubulin of Polytomella. The tubulin assembles on isolated rootlet-MTOCs or is stimulated to assemble by TIPs. Several low molecular weight non-tubulin accessory proteins have been chromatographically separated from tubulin extracts of Polytomella and have been found to also stimulate tubulin assembly. It should now be possible to grow cells in label and obtain labeled TIPs, tubulin and accessory proteins for studies examining the minimal and maximal stoichiometries of tubulin to TIPs and/or accessory proteins during assembly. By this approach, it should be possible to gain a more

complete understanding of how the cell might control microtubule assembly.

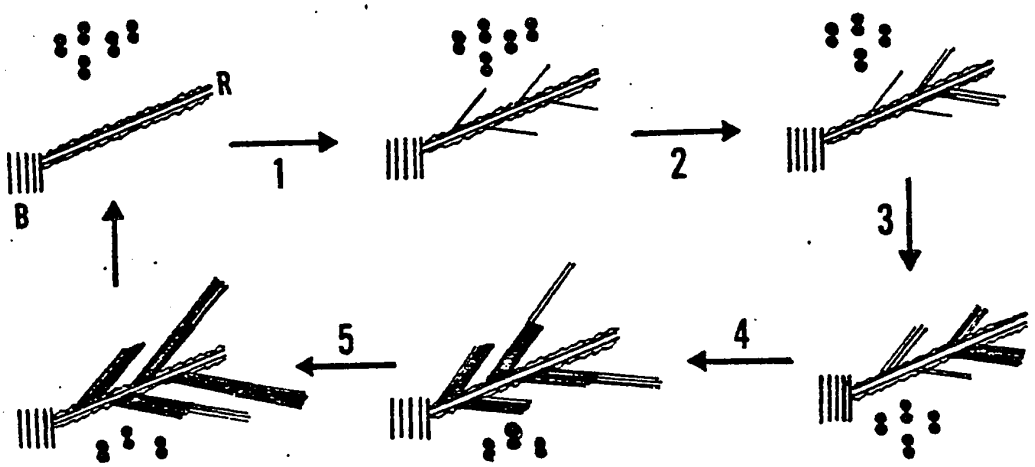
CONTROL OF ASSEMBLY in vivo

Assembly of tubulin on rootlet-MTOCs in vitro has provided a plausible model system for understanding the process of assembly in living cells. Fig. 45 diagramatically represents the basic steps that are involved in this event. The model shows that assembly involves at least two important steps, initiation and elongation. The initiation step is subject to control by MTOCs while the elongation step is only regulated by the availability of tubulin protein. Our studies with "the-MAPs" of brain microtubules suggest that non-tubulin accessory proteins may be modulating assembly during elongation. That there may be a third step in assembly in which accessory proteins control disassembly of microtubules has been suggested by work of Murphy et al. (1977) on MAPs.

It is therefore reasonable to postulate that, in cells, appropriately placed MTOCs could regulate the initiation and spatial distribution of microtubules. Accessory proteins and tubulin protein may control the rate and extent of this assembly. This interpetation is consistent with in vivo observations that rootlet-MTOCs control microtubule assembly in Polytomella (Brown and Stearns, 1978). The ability of these factors to control tubulin assembly would of course

be subject to changes in other cellular factors such as cation levels (mostly divalent), nucleotide levels (i.e. GTP), and perhaps on the covalent modification of microtubule proteins (such as by tyrosylation, phosphorylation, and alkylation).

Fig. 45 Model diagram from negative staining studies (figs. 25, 26) showing the steps by which rootlet-MTOCs might initiate tubulin protein assembly *in vivo*. The rootlet-MTOCs marked (R) are shown attached to a basal body (B). In assembly, tubulin dimers (●) associate with MTOCs to make protofilaments (straight lines) which associate longitudinally until enough are present to fold and form a stable microtubule helix. Tubulin dimers would assemble onto ends of these tubules. The tubules may exist in dynamic equilibrium with subunits (Inoué 1964) and disassemble into subunits in response to changes in the cellular environment.



45

REFERENCES

ALLEN, C., and G.G. BORISY. 1974. Structural polarity and directional growth of microtubules of Chlamydomonas flagella. J. Mol. Biol. 90: 381.

AMOS, L., and A. KLUG. 1974. Arrangement of subunits in flagella microtubules. J. Cell Sci. 14: 523.

ANDERSON, W.A., P. PERSONNE, and J. ANDRE. 1968. Chemical compartmentalization in Helix spermatozoa. J. Microscop. Paris 7: 367.

ANFINSEN, C.B. 1973. Principles that govern the folding of protein chains. Science 181: 223.

BARDELE, C.F. 1977. Comparative study of axopodial microtubule patterns and possible mechanisms of pattern control in the centrohelian heliozoa Acanthocystis, Raphidiophrys and Heterophrys. J. Cell Sci. 25: 205.

BEAMS, H.W., T.N. TAHMISIAN, R.L. DEVINE, and E. ANDERSON. 1959. Studies on the fine structure of a gregarine parasitic in the gut of the grasshopper, melanoplus differentialis. J. Protozool. 6: 136.

BERNE, B.J. 1974. Interpretation of the light scattering from long rods. J. Mol. Biol. 89: 755.

BIKLE, D., L.G. TILNEY, and K.R. PORTER. 1966. Microtubules and pigment migration in the melanophores of Fundulus heteroclitus L. Protoplasma 61: 322.

BINDER, L., W. DENTLER, and J.L. ROSENBAUM. 1975. Assembly of chick brain tubulin onto flagellar microtubules from Chlamydomonas and sea urchin. Proc. Natl. Acad. Sci. U.S.A. 72: 1122.

BLOODGOOD, R.A., and J.L. ROSENBAUM. 1976. Initiation of brain tubulin assembly by a high molecular weight flagellar protein factor. J. Cell Biol. 71: 322.

BLOODGOOD, R.A. 1975. Biochemical analysis of axostyle motility. Cytobios. 14: 101.

BORISY, G.G., and E.W. TAYLOR. 1967. The mechanism of action of colchicine. Binding of colchicine-^{3}H to cellular protein. J. Cell Biol. 34: 525.

BORISY, G.G., and J.B. OLMSTED. 1972. Nucleated assembly of microtubules in porcine brain extracts. Science 177: 1196.

BORISY, G.G., J.B. OLMSTED, J.M. MARCUM and C. ALLEN. 1974. Microtubule assembly in vitro. Fed. Proc. 33: 167.

BORISY, G.G., J.M. MARCUM, J.B. OLMSTED, D.B. MURPHY, and K.A. JOHNSON. 1975. Purification of tubulin and associated high molecular weight proteins from porcine brain and characterization of microtubule assembly in vitro. Ann. N.Y. Acad. Sci. 253: 107.

BOUCK, G.B., and D.L. BROWN. 1973. Microtubule biogenesis and cell shape in Ochromonas. I. The distribution of cytoplasmic and mitotic microtubules. J. Cell Biol. 56: 340.

BOUCK, G.B., and D.L. BROWN. 1976. Self-assembly in development. Ann. Rev. Plant Physiol. 27: 71.

BRINKLEY, B.R., and R.B. NICKLAS. 1968. Ultrastructure of the meiotic spindle of grasshopper spermatocytes after chromosome micromanipulation. J. Cell Biol. 39: 16a.

BROKAW, C.J. 1972. Flagellar movement: A sliding filament model. Science 178: 455.

BROWN, D.L., and G.B. BOUCK. 1973. Microtubule biogenesis and cell shape in Ochromonas. II. The role of nucleating sites in shape development. J. Cell Biol. 56: 360.

BROWN, D.L., and G.B. BOUCK. 1974. Microtubule biogenesis and cell shape in Ochromonas. III. Effects of the herbicidal mitotic inhibitor isopropyl N-phenylcarbamate on shape and flagellum regeneration. J. Cell Biol. 61: 514.

BROWN, D.L., A. MASSALSKI, and R. PATENAUDE. 1976. Organization of the flagellar apparatus and associated cytoplasmic microtubules in the quadriflagellate alga Polytomella agilis. J. Cell Biol. 69: 106.

BROWN, D.L., and M.E. STEARNS. 1978. Microtubule organizing centers in the flagellate Polytomella. I. Development and function in vivo. J. Cell Biol. - submitted for publication.

BRYAN, J. 1974. Biochemical properties of microtubules. Fed. Proc. 33: 152.

BURGESS, J., and D.H. NORTHCOTE. 1969. Action of colchicine and heavy water on the polymerization of microtubules in wheat root meristem. J. Cell Sci. 5: 433.

BURNS, R.G., and D. STARLING. 1974. The in vitro assembly of tubulins from sea-urchin eggs and rat brain: use of heterologous seeds. J. Cell Sci. 14: 411.

BURTON, P.R. 1973. Some structural and cytochemical observations on the axial filament complex of lung-fluke spermatozoa. J. Morphol. 140: 185.

BYERS, B., and K.R. PORTER. 1964. Oriented microtubules in elongating cells of the developing lens rudiment after induction. Proc. Natl. Acad. Sci. 52: 1091.

BYERS, H.R., and K.R. PORTER. 1977. Transformation in the structure of the cytoplasmic ground substance in Erythrophores during pigment aggregation and dispersion. I. A study using whole-cell preparations in stereo high voltage electron microscopy. J. Cell Biol. 75: 541.

CLEVELAND, D.W., S.-Y. HWO, and M.W. KIRSCHNER. 1977a. Purification of tau, a microtubule associated protein which induces assembly of microtubules from purified tubulin. J. Mol. Biol. 116: 207.

CLEVELAND, D.W., S.-Y. HWO, and M.W. KIRSCHNER. 1977b. The physical and chemical properties of purified tau factor and the role of tau in microtubule assembly. J. Mol. Biol. 116: 227.

COHEN, C., S.C. HARRISON, and R.E. STEPHENS. 1971. X-ray diffraction from microtubules. J. Mol. Biol. 59: 375.

COHEN, C., D. DeROSIER, S.C. HARRISON, R.E. STEPHENS, and J. THOMAS. 1975. X-ray patterns from microtubules. Ann. N.Y. Acad. Sci. 253: 53.

CONNOLLY, J.A., V.I. KALNINS, D.W. CLEVELAND, and M.W. KIRSCHNER. 1977. Immunofluorescent staining of cytoplasmic and spindle microtubules in mouse fibroblasts with antibody to tau protein. Proc. Natl. Acad. Sci. U.S.A. 74: 2437.

CONNOLLY, J.A., V.I. KALNINS, D.W. CLEVELAND, and M.W. KIRSCHNER. 1978. Intracellular localization of the high molecular weight microtubule accessory protein by indirect immunofluorescence. J. Cell Biol. 76: 781.

De HARVEN, E., and W. BERNHARD. 1956. Etude au microscope electronique de l'ultrastructure du centriole chezles vertebrates. Z. Zellforsch. mikrosk 45: 378.

DENTLER, W.L, S. GRANETT, G.B. WITMAN, and J.L. ROSENBAUM. 1975. Directionality of brain microtubule assembly in vitro. Proc. Natl. Sci. 71: 1710. .

ECKHART, B.S., and J.A. SNYDER. 1978. Combined immunofluorescence and high-voltage electron microscopy of cultured mammalian cells, using an antibody that binds to glutaraldehyde-treated tubulin. Proc. Natl. Acad. Sci. U.S.A. 75: 334.

ELFVIN, L.-G. 1961. Electron-microscopic investigation of filament structures in unmyelinated fibers of cat splenic nerve. J. Ultrastruct. Res. 5:51.

ERICKSON, H.P. 1974. Assembly of microtubules from preformed ring-shaped protofilaments and 6S tubulin. J. Supramol. Struc. 2: 393.

ERICKSON, H.P. 1975. The structure and assembly of microtubules. Annals of the New York Acad. Sci. 253: 60.

FAWCETT, D.W., and K.R. PORTER. 1954. A study of the fine structure of ciliated Epithelia. J. Morphol. 94: 221.

FERNANDEZ, H.L., P.R. BURTON, and F.E. SAMSON. 1971. Axoplasmic transport in the crayfish nerve cord. The role of fibrillar constituents of neurons. J. Cell Biol. 51: 176.

FILNER, P., and O. BEHNKE. 1973. Stabilization and isolation of brain microtubules with glycerol and dimethylsulfoxide (DMSO). J. Cell Biol. 59: 99a.

FORER, A., and A.M. ZIMMERMAN. 1974. Characteristics of sea-urchin mitotic apparatus isolated using a dimethylsulfoxide/glycerol medium. J. Cell Sci. 16: 481.

FREED, J.J., and M.M. LEBOWITZ. 1970. The association of a class of saltatory movements with microtubules in cultured cells. J. Cell Biol. 45: 334.

GASKIN, F., C.R. CANTOR, and M.L. SHELANSKI. 1974. Turbidiometric studies of the in vitro assembly and disassembly of porcine neurotubules. J. Mol. Biol. 89: 737.

GASKIN, F., C.R. CANTOR, and M.L. SHELANSKI. 1975. Biochemical studies on the in vitro assembly and disassembly of microtubules. Annals of New York Acad. Sci. 253: 133.

GIBBINS, J.R., L.G. TILNEY, and K.R. PORTER. 1966. Microtubules in primary mesenchyme cells of sea urchin embryos. Anat. Rec. 154: 347a.

GIBBONS, I.R. 1965. Chemical dissection of cilia. Arch. Biol. 76: 317.

GIBBONS, B.H., and I.R. GIBBONS. 1972. Flagellar movement and adenosine triphosphatase activity in sea urchin sperm extracted with Triton X-100. J. Cell Biol. 54: 75.

GILLESPIE, E. 1971. Colchicine binding in tissue slices: decrease by calcium and biphasic effect of adenosine 3'5'-monophosphate. J. Cell Biol. 50: 544.

GONATAS, N.K., and E. ROBBINS. 1964. The homology of spindle tubules and neuro-tubules in the chick embryo retina. Protoplasma 59: 377.

GOULD, R.R., and G.G. BORISY. 1976. The pericentriolar material in chinese hamster ovary cells nucleates microtubule formation. J. Cell Biol. 73: 601.

GRASSE, P.-P. 1956. L'ultrastructure de Pyrsonympha vertens (Zooflagellata pyrsonymphina): les flagelles et leur coaptation avec le corps, l'axostyle contractile, le paroxostyle, le cytoplasme. Arch. Biol. 67: 595.

GRIMSTONE, A.V., and A. KLUG. 1966. Observations on the substructure of flagellar fibres. J. Cell Sci. 1: 351.

GROSS, P.R., and W. SPINDEL. 1960. Mitotic arrest by deuterium oxide. Science 131: 37.

HAGA, T., T. ABE, and M. KUROKAWA. 1974. Polymerization and depolymerization of microtubules in vitro as studied by flow birefringence. FEBBS Letters 39: 291.

HARRIS, P. 1962. Some structural and functional aspects of the mitotic apparatus in sea urchin embryos. J. Cell Biol. 14: 475.

HARTREE, E.F. 1972. Determination of protein. A modified Lowry method for linear photometric response. Anal. Biochem. 48: 422.

HERZOG, W., and K. WEBER. 1977. In vitro assembly of pure tubulin into microtubules in the absence of microtubule-associated proteins and glycerol. Proc. Natl. Acad. Sci. U.S.A. 74: 1860.

HIMES, R.H., P.R. BURTON, and J.W. GAITO. 1977. Dimethyl sulfoxide-induced self-assembly of tubulin lacking associated proteins. J. Biol. Chem. 252: 6222.

INOUÉ, S. 1952a. The effect of colchicine on the microscopic and submicroscopic structure of the mitotic spindle. Expt. Cell Res. Suppl. 2: 305.

INOUÉ, S. 1952b. Effects of temperature on the birefringence of the mitotic spindle. Biol. Bull. 103: 416.

INOUÉ, S. 1964. In: Primitive Motile Systems in Cell Biology. R.D. Allen and N. Kamiya, eds. p.549. Academic Press, New York.

INOUÉ, S., and H. SATO. 1967. Cell motility by labile association of molecules. The nature of mitotic spindle fibers and their role in chromosome movement. J. Gen. Phys. 50: 259.

INOUÉ, S., H. SATO, and R.W. TUCKER. 1963. Heavy water enhancement of mitotic spindle birefringence. Biol. Bull. 125: 380.

JESSEN, H., and H. MOE. 1972. The fine structure of macrophages in the enamel organ with special reference to the microtubular system. Z. Zellforsch. 126: 466.

JOHNSON, K.A., and G.G. BORISY. 1977. Kinetic analysis of microtubule self-assembly in vitro. J. Mol. Biol. 117: 1.

KEATES, R.A.B., and R.H. HALL. 1975. Tubulin requires an accessory protein for self assembly into microtubules. Nature 257: 418.

KIRKLAND, W.L., and P.R. BURTON. 1972. Cyclic adenosine monophosphate-mediated stabilization of mouse neuroblastoma cell neurite microtubules exposed to low temperatures. Nature New Biol. 240: 205.

KIRKPATRICK, J.B., L. HYAMS, V.L. THOMAS, and P.M. HOWLEY. 1970. Purification of intact microtubules from brain. J. Cell Biol. 47: 384.

KIRSCHNER, M.W., L.S. HONIG, and R.C. WILLIAMS. 1975a. Quantitative electron microscopy of microtubule assembly in vitro. J. Mol. Biol. 99: 263.

KIRSCHNER, M.W., M. SUTER, M. WEINGARTEN, and D. LITTMAN. 1975b. The role of rings in the assembly of microtubules in vitro. Ann. N.Y. Acad. Sci. 253: 90.

KITCHING, J.A. 1964. The axopods of the sun animalcule Actinophrys sol. In: Primitive Motile Systems in Cell Biology, edited by R.D. Allen and N. Kamiya. New York: Academic, p. 445.

KRAM, R., and G.M. TOMKINS. 1973. Pleiotypic control by cyclic AMP: interaction with cyclic GMP and possible role of microtubules. Proc. Natl. Acad. Sci. 70: 1659.

KREUTZBERG, G.W. 1969. Neuronal dynamics and axonal flow. IV. Blackage of intra-axonal enzyme transport by colchicine. Proc. Nat. Acad. Sci. U.S.A. 62: 722.

KURIYAMA, R., and H. SAKAI. 1974. Viscometric demonstration of tubulin polymerization. J. Biochem. 75: 463.

LANE, N.J., and J.E. TREHERNE. 1970. Lanthanum staining of the neurotubules in axons from cockroach ganglia. J. Cell Sci. 7: 217.

LANGFORD, G.M. 1977. In vitro assembly of dogfish brain tubulin and the induction of coiled ribbon polymers by calcium. Exper. Cell Res. 111: 139.

LEDBETTER, M.C., and K.R. PORTER. 1963. A "microtubule" in plant cell fine structure. J. Cell Biol. 19: 239.

LEE, J.C., R.P. FRIGON, and S.N. TIMASHEFF. 1973. The chemical characterization of calf brain microtubule protein subunits. J. Biol. Chem. 248: 7253.

LEE, Y.C., F.E. SAMSON, L.L. HOUSTON, and R.H. HIMES. 1974. The in vitro polymerization of tubulin from beef brain. J. Neurobiol. 5: 317.

LINCK, R.W., and L.A. AMOS. 1974. Flagellar doublet microtubules: selective solubilization of minor component proteins. J. Cell Biol. 63: 194a.

LOWRY, O.H., N.J. ROSEBROUGH, A.L. FARR, and R.J. RANDALL. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193: 265.

MAGUN, B. 1973. Two actions of cyclic AMP on melanome movement in frog skin. J. Cell Biol. 57: 845.

MANTON, I., and B. CLARKE. 1952. An electron microscope study of the spermatozoid of sphagnum. J. Expt. Bot. 3: 265.

MARSLAND, D., and A.M. ZIMMERMAN. 1965. Structural stabilization of the mitotic apparatus by heavy water in the cleavage eggs of Arbacia punctulata. Increased resistance to pressure-induced disorganization. Expt. Cell Res. 38: 306.

MAZIA, D., C. PETZELT, and R.D. WILLIAMS. 1972. A co-activated ATPase in the miotic apparatus of the sea urchin egg (isolated by a new method). Exper. Cell Res. 70: 325.

McGILL, M., and B.R. BRINKLEY. 1975. Human chromosomes and centrioles as nucleating sites for the in vitro assembly of microtubules from bovine brain tubulin. J. Cell Biol. 67: 189.

McINTOSH, J.R. 1973. The axostyle of saccino-baculus II. J. Cell Biol. 56: 324.

MOENS, P.B., and E. RAPPORT. 1971. Spindles, spindle plaques, and meiosis in the yeast Saccharomyces cerevisiae Hansen. J. Cell Biol. 50: 344.

MOHRI, H. 1969. Amino acid composition of "tubulin" constituting microtubules of sperm flagella. Nature 217: 1053.

MOOSEKER, M.S., and L.G. TILNEY. 1973. Isolation and reactivation of the axostyle. Evidence for a dynein-like adenosine triphosphatase in the axostyle. J. Cell Biol. 56: 13.

MORGAN, J.L., and N.W. SEEDS. 1975. Tubulin constancy during morphological differentiation of mouse neuroblastoma cells. J. Cell Biol. 67: 136.

MURPHY, D.B. 1975. The mechanism of microtubule dependent movement of pigment granules in teleost chromatophores. Annals of N.Y. Acad. Sci. 253: 692.

MURPHY, D.B., and G.G. BORISY. 1975. Association of high molecular weight proteins with microtubules and their role in microtubule assembly in vitro. Proc. Nat. Acad. Sci. U.S.A. 72: 2096.

MURPHY, D.B., K.A. JOHNSON, and G.G. BORISY. 1977. Role of tubulin-associated proteins in microtubule nucleation and elongation. J. Mol. Biol. 117: 33.

MURRAY, A.W., and M. FROSCIO. 1971. Cyclic adenosine 3':5'-monophosphate and microtubule function: specific interaction of the phosphorylated protein subunits with a soluble brain component. Biochem. Biophys. Acta Commun. 44: 1089.

NAGLE, B.W., K.H. DOENGES, and J. BRYAN. 1977. Assembly of tubulin from cultured cells and comparison with the neurotubulin model. Cell 12: 573.

OCKLEFORD, C.D., and J.B. TUCKER. 1973. Growth, breakdown, repair and rapid contraction of microtubule axopodia in the heliozoan, Actinophrys sol. J. Ultrastruct. Res. 44: 369.

OLMSTED, J.B., and G.G. BORISY. 1973 . Microtubules. Ann. Rev. Biochem. 42: 507.

OLMSTED, J.B., J.M. MARCUM, K.A. JOHNSON, C. ALLEN, and G.G. BORISY. 1974. Microtubule assembly: some possible regulatory mechanisms. J. Supramol. Struct. 2: 429.

PALAY, S.L. 1956. Synapses in the central nervous system. J. Biophys. Biochem. Cytol. Suppl. 2: 193.

PENNINGROTH, S.M., D.W. CLEVELAND, and M.W. KIRSCHNER. 1976. In vitro studies of the regulation of microtubule assembly. In: Cell Motility. Cold Spring Harbor Conferences on Cell Proliferation, editors R. Goldman, T. Pollard and J. Rosenbaum. p.1233.

PICKETT-HEAPS, J.D. 1969. The evolution of the mitotic apparatus: an attempt at comparative ultrastructural cytology in dividing plant cells. Cytobios 1: 257.

PICKETT-HEAPS, J.D. 1971. The autonomy of the centriole: fact or fallacy? Cytobios. 3: 205.

PIRAS, M.M., and R. PIRAS. 1974. Phosphorylation of vinblastine-isolated microtubules from chick-embryonic muscles. European J. Biochem. 47: 443.

PORTER, K.R. 1966. Cytoplasmic microtubules and their functions. In: Principles of Biomolecular Organization, editors G.E.W. Wolstenholme and M. O'Connor. p. 308.

PORTER, K.R., T.T. PUCK, A.W. HSIE, and D. KELLEY. 1974. An electron microscopic study of the effects of dibutyrl cyclic AMP on chinese hamster ovary cells. Cell 2: 145.

RAFF, R.A., G. GREENHOUSE, K.W. GROSS, and P.R. GROSS. 1971. Synthesis and storage of microtubule proteins by sea urchin embryos. J. Cell Biol. 50: 516.

RAFF, R.A., H.V. COLOT, S.E. SELVIG, and P.R. GROSS. 1972. Oogenetic origin of messenger RNA for embryonic synthesis of microtubule proteins. Nature 235: 211.

RASMUSSEN, H., and D.B.P. GOODMAN. 1975. Calcium and cAMP as interrelated intracellular messengers. Ann. N.Y. Acad. Sci. 253: 789.

REYNOLDS, E.S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17: 208.

ROBBINS, E., and M.L. SHELANSKI. 1969. Synthesis of a colchicine-binding protein during the HeLa life cycle. J. Cell Biol. 43: 371.

ROBINOW, C.F., and J. MARAK. 1966. A fiber apparatus in the nucleus of the yeast cell. J. Cell Biol. 29: 129.

ROISEN, F.J., R.A. MURPHY, M.E. PICHICHERO, and W.G. BRADEN. 1972a. Cyclic adenosine monophosphate stimulation of axonal elongation. Science 175: 73.

ROISEN, F.J., R.A. MURPHY, and W.G. BRADEN. 1972b. Dibutryl cyclic adenosine monophosphate stimulation of colcemid-inhibited axonal elongation. Science 177: 809.

ROSENBAUM, J.L., and K. CARLSON. 1969. Cilia regeneration in Tetrahymena and its inhibition by colchicine. J. Cell Biol. 40: 415.

ROSENBAUM, J.L., and F.M. CHILD. 1967. Flagellar regeneration in protozoan flagellates. J. Cell Biol. 34: 345.

ROSENBAUM, J.L., J.E. MOULDER, and D.L. RINGO. 1969. Flagellar elongation and shortening in Chlamydomonas. The use of cycloheximide and colchicine to study the synthesis and assembly of flagellar proteins. J. Cell Biol. 41: 600.

ROTH, L.E. 1959. An electron microscope study of the cytology of the protozoan Paranema trichophorum. J. Protozool. 6: 107.

ROTH, L.E., and E.W. DANIELS. 1962. Electron microscopic studies of mitosis in amebae. II. The giant ameba Pelomyxa carolinensis. J. Cell Biol. 12: 57.

RUDZINSKA, M.A. 1965. The fine structure and function of the tentacle in Tokophrya infusionum. J. Cell Biol. 35: 459.

SATIR, P. 1965. Studies on cilia. II. Examination of the distal region of the ciliary shaft and the role of filaments in motility. J. Cell Biol. 26: 805.

SABATINI, D.D., K. BENSCH, and R.J. BARNETT. 1963. Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. J. Cell Biol. 17: 19.

SALMON, E.D. 1975a. Pressure-induced depolymerization of spindle microtubules. I. Changes in birefringence and spindle length. J. Cell Biol. 65: 603.

SALMON, E.D. 1975b. Spindle microtubules: thermodynamics of in vivo assembly and role in chromosome movement. Ann. N.Y. Acad. Sci. 253: 603.

SCHMITT, H., R. JOSEPHS, and E. REISLER. 1977. A search for in vivo factors in regulation of microtubule assembly. Nature 265: 653.

SEEDS, N.W., and R.B. MACCIONI. 1978. Proteins from morphologically differentiated neuroblastoma cells promote tubulin polymerization. J. Cell Biol. 76: 547.

SHEELER, P., M. CANTOR, and J. MOORE. 1968. Growth characteristic of Polytomella agilis in batch cultures. Life Sci. 7: 289.

SHELANSKI, M.L., F. GASKIN, and G.R. CANTOR. 1973. Microtubule assembly in the absence of added nucleotides. Proc. Nat. Sci. 70: 765.

SHERLINE, P., and K. SCHIAVONE. 1977. Immunofluorescence localization of proteins of high molecular weight along intracellular microtubules. Science 198: 1038.

SHIGEKAWA, B.L., and R.W. OLSEN. 1975. Resolution of cyclic AMP stimulated protein kinase from polymerized-purified brain microtubules. Biochem. Biophys. Res. Commun. 63: 455.

SJÖSTRAND, J., M. FRIZELL, and P.O. HASSELGREN. 1970. Effects of colchicine on axonal transport in peripheral nerves. J. Neurochem. 17: 1563.

SLAUTTERBACK, D.B. 1963. Cytoplasmic microtubules. I. Hydra. J. Cell Biol. 18: 367.

SPURR, A.R. 1969. A low-viscosity epoxy-resin embedding. medium for electron microscopy. J. Ultrastruc. Res. 26: 31.

SLOBODA, R.D., W.L. DENTLER, and J.L. ROSENBAUM. 1976a. Microtubule-associated proteins and the stimulation of tubulin assembly in vitro. Biochem. 15: 4497.

SLOBODA, R.D., W.L. DENTLER, R.A. BLOODGOOD, B.R. TELZER, S. GRANETT, and J.L. ROSENBAUM. 1976b. Microtubule-associated proteins (MAPs) and the assembly of microtubules in vitro. In: Cell Motility. Cold Spring Harbor Conferences on Cell Proliferation, editors R. Goldman, T. Pollard and J. Rosenbaum. p. 1171.

SMITH, D.S., U. JÄRLFORS, and B.F. CAMERON. 1975. Morphological evidence for the participation of microtubules in axonal transport. Ann. N.Y. Acad. Sci. 253: 472.

SNELL, W.J., W.L. DENTLER, L.T. HAIMO, L.I. BINDER, and J. L. ROSENBAUM. 1974. Assembly of chick brain tubulin onto isolated basal bodies of Chlamydomonas reinhardii. Science (Wash. D.C.) 185: 357.

SNYDER, J.A., and J.R. McINTOSH. 1975. Initiation and growth of microtubules from mitotic centers in lysed mammalian cells. J. Cell Biol. 67: 744.

STEARNS, M.E., J.A. CONNOLLY, and D.L. BROWN. 1976. Cytoplasmic microtubule organizing centers isolated from Polytomella agilis. Science 191: 188.

STEARNS, M.E., R. ALLORE, and D.L. BROWN. 1978. Comparison of the microtubule initiating proteins in beef and catfish brain tubulin preparations. Exper. Cell Res. - in preparation.

STEARNS, M.E., and D.L. BROWN. 1978. Microtubule organizing centers in the flagellate Polytomella. II. Isolation and function in vitro. J. Cell Biol. - submitted for publication.

STEPHENS, R.E. 1973. A thermodynamic analysis of mitotic spindle equilibrium at active metaphase. J. Cell Biol. 57: 133.

STEPHENS, R.E., F.L. RENAUD, and I.R. GIBBONS. 1967. Guanine nucleotide associated with the protein of the outer fibers of flagella and cilia. Science 156: 1606.

STEPHENS, R.E., and K.T. EDDS. 1976. Microtubules: structure, chemistry, and function. Physiological Reviews 56: 709.

TELZER, B.R., M.J. MOSES, and J.L. ROSENBAUM. 1975. Assembly of microtubules onto kinetochores of isolated mitotic chromosomes of Hela cells. Proc. Natl. Acad. Sci. 72: 4023.

TILNEY, L.G., and K.R. PORTER. 1965. Studies on the microtubules in heliozoa. I. Fine structure of Actinosphaerium with particular reference to axial rod structure. Protoplasma 60: 317.

TILNEY, L.G., and K.R. PORTER. 1967. Studies on the microtubules in heliozoa. II. The effect of low temperatures on these structures in the formation and maintenance of the axopodia. J. Cell Biol. 34: 327.

TILNEY, L.G. 1968a. Ordering of subcellular units: the assembly of microtubules and their role in the development of cell form. Develop. Biol. Suppl. 2: 63.

TILNEY, L.G. 1968b. Studies on the microtubules in heliozoa. IV. The effect of colchicine on the formation and maintenance of the axopodia and the redevelopment of pattern in Actinosphaerium nucleofilum (Barrett). J. Cell Sci. 3: 549.

TILNEY, L.G., and J.R. GIBBINS. 1968. Differential effects of antimitotic agents on the stability and behavior of cytoplasmic and ciliary microtubules. Protoplasma 65: 167.

TILNEY, L.G., Y. HIRAMOTO, and D. MARSLAND. 1966. Studies on microtubules in Heliozoa. III. A pressure analysis of the role of these structures in the formation and maintenance of the axopodia of Actinosphaerium nucleofilum (Barrett). J. Cell Biol. 29: 77.

TILNEY, L.G., and J.R. GIBBINS. 1969. Microtubules and filaments in the filopodia of the secondary mesenchyme cells of Arabacia punctulata and Echinarachnius parma. J. Cell Sci. 5: 195.

TILNEY, L.G. 1971. How microtubule patterns are generated. The relative importance of nucleation and bridging of microtubules in the formation of the axoneme of Rhaphidiophrys. J. Cell Biol. 51: 837.

TILNEY, L.G., J. BRYAN, D.J. BUSH, K. FUJIWARA, M.S. MOOSEKER, D.B. MURPHY, and D.H. SNYDER. 1973. Microtubules: evidence for 13 protofilaments. J. Cell Biol. 59: 267.

TUCKER, J.B. 1971a. Spatial discrimination in the cytoplasm during microtubule morphogenesis. Nature 232: 387.

TUCKER, J.B. 1971b. Microtubules and a contractile ring of microfilaments associated with a cleavage furrow. J. Cell Sci. 8: 557.

TUCKER, J.B. 1977. Shape and pattern specification during microtubule bundle assembly. Nature 266: 22.

WARNER, F.D. 1972. Macromolecular organization of eukaryotic cilia and flagella. In: Advances in Cell and Molecular Biology, edited by E.J. DuPraw. New York: Academic, vol. II, p. 193.

WEBER, K., and M. OSBORN. 1969. The reliability of molecular weight determinations by dodecyl-sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244: 4406.

WEINGARTEN, M.D., A.H. LOCKWOOD, S.-Y. HWO, and M.W. KIRSCHNER. 1975. A protein factor essential for microtubule assembly. Proc. Natl. Acad. Sci. U.S.A. 72: 1858.

WEINGARTEN, M.D., M.M. SUTER, D.R. LITTMAN, and M.W. KIRSCHNER. 1974. Properties of the depolymerization products of microtubules from mammalian brain. Biochemistry 13: 5529.

WEISENBERG, R.C., G.G. BORISY, and E.W. TAYLOR. 1968. The colchicine binding protein of mammalian brain and its relation to microtubules. Biochemistry 7: 4466.

WEISENBERG, R.C. 1972. Microtubule formation *in vitro* in solutions containing low calcium concentrations. Science *177*: 1104.

WEISENBERG, R.C. 1973. Regulation of tubulin organization during meiosis. Am. Zool. *13*: 981.

WEISENBERG, R.C., and A.C. ROSENFELD. 1975. *In vitro* polymerization of microtubules into asters and spindles in homogenates of surf clam eggs. J. Cell Bil. *64*: 146.

WILSON, L. 1975. Microtubules as drug receptors: pharmacological properties of microtubule protein. Ann. N.Y. Acad. Sci. *253*: 213.

WILSON, L., and J. BRYAN. 1974. Biochemical and pharmacological properties of microtubules. In: *Advances in Cell and Molecular Biology*, edited by E.J. DuPraw. New York: Academic, vol. III, p. 21.

WITMAN, G.B., K. CARLSON, and J.L. ROSENBAUM. 1972c. Chlamydomonas flagella. II. The distribution of tubulins 1 and 2 in the outer doublet microtubules. J. Cell Biol. *54*: 540.

WITMAN, G.B., D.W. CLEVELAND, M.D. WEINGARTEN, and M.W. KIRSCHNER. 1976. Tubulin requires tau for growth onto microtubule initiating sites. Proc. Natl. Acad. Sci. U.S.A. *73*: 4070.

WUNDERLICH, F., and E. WEIRICH. 1972. Antimitotic agents and macronuclear division of ciliates: V. cytokinesis of tetra-hymena synchronized by deuteriumoxide. Exptl. Cell Res. *72*: 568.

YANAGISAWA, T., S. HASEGAWA, and H. MOHRI. 1968. The bound nucleotides of the isolated microtubules of sea-urchin sperm flagella and their possible role in flagellar movement. Exptl. Cell Res. *52*: 86.

YOUNG, P.G., D. YOUNG, and A.M. ZIMMERMAN. 1972. Action of hydrostatic pressure on cilia. Biol. Bull. *143*: 256.

ZIMMERMAN, A.M., and D. MARSLAND. 1964. Cell division: effects of pressure on the mitotic mechanisms of marine eggs (*Arabacia punctulata*). Exptl. Cell Res. *35*: 293.