In Vitro Assembly of Tubulin from Nonneural Cells (Ehrlich Ascites Tumor Cells)†

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ABSTRACT: Microtubules from nonneural cells (Ehrlich ascites tumor cells) could be assembled in vitro by two cycles of polymerization and depolymerization. The formation of microtubules is favored by 4 M glycerol. The tubules are morphologically identical with neurotubules and are temperature sensitive. In contrast to cycled tubulin from brain, neither high molecular weight (HMW) components nor 36/30 or 20S species (rings) have been observed. Gel permeation chromatography of the depolymerized material yielded a fraction with a $s^0_{20,w}$ value of >200 and another fraction of 6 S. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the protein in the gel fraction with the higher S value showed that it consists almost entirely of tubulin, the rest being distributed in 20–25 minor bands. The other fraction is composed of tubulin and a larger amount of nontubulin proteins. On electron microscopic examination, the first fraction contains large aggregates consisting of dense clusters of filaments. These aggregates had no tendency to form microtubules, whereas the 6S fraction could be easily polymerized into microtubules. The results demonstrate that HMW proteins are not essential for the in vitro formation of microtubules from ascites tumor cells and that rings are neither intermediates nor required nucleation centers in tubule assembly.

The study of cytoplasmic microtubules has been greatly facilitated by the development of conditions for the in vitro assembly of tubulin from brain (Weisenberg, 1972; Borisy and Olmsted, 1972; Shelanski et al., 1973) which has allowed further analysis of tubulin. Several groups have reported that aggregates of tubulin called rings are formed in partially purified tubulin from mammalian brain (Olmsted et al., 1974; Kirschner et al., 1974; Weingarten et al., 1974; Erickson, 1974, 1975; Rebhun et al., 1975; Doenges et al., 1976). Some investigators have suggested that these rings are intermediates in the assembly process and have proposed mechanisms by which ring structures and tubulin subunits are involved in tubule formation (Kirschner et al., 1974; Erickson, 1974; Olmsted et al., 1974). These conclusions are based on observations of the assembly sequence initiated by addition of GTP at 37 °C (Kirschner et al., 1975). Observations on the assembly sequence initiated by mixing phosphocellulose purified tubulin with assembly promoting factors suggest, however, that rings are not required for either the nucleation or elongation reactions (Bryan, 1976). Microtubule-associated proteins and especially high-molecular-weight (HMW) ones have been observed by several authors in cycled neurotubulin preparations (Borisy et al., 1974, 1975; Cande et al., 1974; Dentler et al., 1974; Erickson, 1974; Gaskin et al., 1974; Murphy and Borisy, 1975). It has been stated that tubulin requires these HMW proteins (Murphy and Borisy, 1975; Keates and Hall, 1975; Sloboda et al., 1976) and/or other protein factors (Weingarten et al., 1974; Bryan et al., 1975) for self assembly into microtubules.

Attempts to isolate tubulin from nonneural cells using these techniques developed for brain tissue have previously been unsuccessful (Bryan, 1975; Rebhun et al., 1975; Bryan et al., 1975; Kane, 1975, 1976). Recently, however, in vitro assembly of microtubules from a homogeneous cell line (rat glial cell clone C_6) has been described by Wiche and Cole (1976), from 3T3 and SV-3T3 cells by Fuller et al. (1975), and from a variety of other cells by Nagle and Bryan (1976). We report here the first successful in vitro polymerization of tubulin from nonneural Ehrlich ascites tumour (EAT) cells (Lettré et al., 1972) and present evidence that cycled tubulin from these cells lacks both HMW components and ring-like structures.

Materials and Methods

Tubulin from EAT cells was prepared with slight modifications of the procedure of Shelanski et al. (1973) originally used for the isolation of tubulin from mammalian brain. Routinely, 40–60 g (wet weight) of EAT cells (G+ and Gϕ strain) was taken from tumor-bearing mice, washed twice by centrifugation in ice cold buffer containing 0.1 M Mes,[1] 0.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM EGTA, pH 6.5 (Mes buffer). For each 10 mL of packed, washed cells, 1 mL of additional Mes buffer was added and this thick suspension was homogenized in an Ultraturrax homogenizer and the homogenate was centrifuged at 100 000g for 60 min at 4 °C. The supernatant was made 1 mM in GTP and 4 M in glycerol, warmed to 37 °C for 45 min, and centrifuged at 100 000g for 60 min at 30 °C to obtain supernatant (H_1S) and pellet (H_1P) fractions. H_1P was resuspended in Mes buffer (10% volume of H_1S) by gentle homogenization in a Dounce homogenizer, chilled on ice for 30 min, and centrifuged at 100 000g for 60 min at 4 °C to obtain supernatant (C_1S) and pellet (C_1P). This supernatant, containing 10–15 mg of protein/mL, was made up to 4 M glycerol and stored at −20 °C. For induction of the second cycle of assembly, GTP was added to the C_1S and the solution was incubated at 37 °C and centrifuged at 30 °C as above, resulting in pellet (H_2P) and supernatant (H_2S). H_2P was resuspended in 4–5 mL of Mes buffer, homogenized,

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[1]Abbreviations used: Mes, 2-(N-morpholino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid, disodium salt; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid.

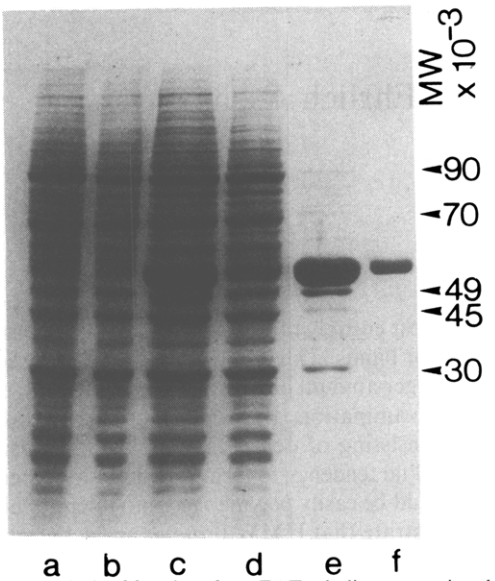

FIGURE 1: Analysis of fractions from EAT tubulin preparations by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. (a) Whole extract; (b) H_1S; (c) C_1S; (d) H_2S; (e) C_2S; (f) DEAE-cellulose purified porcine brain tubulin.

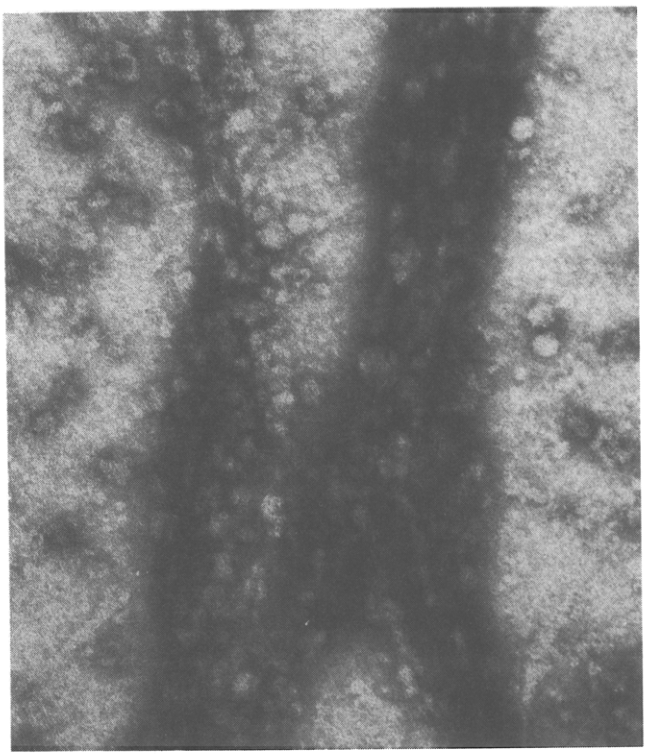

FIGURE 2: Electron micrograph of a cold solution of tubulin (C_1S) before GTP addition and warming up to 37 °C, showing large aggregates consisting of dense clusters of filaments. No rings could be observed (×120 000).

cooled at 0 °C, and clarified by centrifuging at 100 000*g* at 4 °C. Supernatant C_2S contained 1–1.5 mg of protein/mL.

Protein concentrations were determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

Polyacrylamide–sodium dodecyl sulfate gel electrophoresis was carried out on slab gels according to the method of Laemmli (1970).

Gel permeation chromatography of microtubule protein solutions was performed by applying 4 mL of fraction C_1S (10–15 mg of protein/mL) in Mes buffer to a 50 cm × 1.5 cm column containing Bio-Gel A-15m, 100–200 mesh (Bio-Rad). Elution was with Mes buffer at 4 °C.

Sedimentation velocity experiments were done using a preparative Beckman ultracentrifuge Model L 75-B, equipped with a schlieren optics accessory. $s^0_{20,w}$ values were determined for samples sedimented in 12-mm single sector cells by extrapolation of the sedimentation coefficients to infinite dilution. All experiments were done with an analytical An-D rotor at a rotor speed of 50 000 rpm.

Samples for electron microscopy were prepared by negative staining. One drop from each sample was applied to a carbon-coated Formvar film cast on a copper grid for 30 s and stained by rinsing with 15 drops of 1% aqueous uranyl acetate. Excess stain was removed with filter paper and the grid was air-dried.

Results

Purification of Tubulin from EAT Cells. Isolation of tubulin from EAT cells by repeated cycles of polymerization–depolymerization requires that protein concentrations in the cytosol be kept sufficiently high (>20 mg/mL). Otherwise, microtubule assembly does not occur. Addition of glycerol (4 M) to the assembly buffer is also necessary. Under these conditions tubulin assembly was spontaneous and reversible. Fractions of the two cycling steps were characterized by electrophoretic analysis on polyacrylamide–sodium dodecyl sulfate slab gels, using highly purified porcine brain tubulin as marker.

Figure 1a shows that tubulin is not a major component in EAT cell extracts. In contrast there is a much higher concentration of tubulin in brain cytosol extracts (not shown). A successive cycle of assembly and disassembly resulted in the enrichment of tubulin in the C_1S supernatant (Figure 1c). It is apparent that tubulin is the main component in this fraction; however, a large number of other nontubulin proteins are still present. A second cycle of assembly–disassembly dramatically improved the purity of the tubulin preparation (Figure 1e), although some higher and lower molecular weight bands could still be observed. Most of the contaminants in C_1S remain in the supernatant (H_2S) after the second cycle and do not assemble with microtubules (Figure 1d).

No HMW proteins are present in these gels, indicating that in vitro assembly of tubulin from EAT cells does not require these components.

Characterization of Polymerization Products by Electron Microscopy. To characterize the morphological structure of polymerized material and to delineate the pathway of tubule assembly, we made electron microscopic examinations. No ring-like structures were observed in samples (C_1S) prior to addition of GTP and warming up to 37 °C. However, a few large aggregates consisting of dense clusters of filaments resembling denatured protein were always observed (Figure 2). About 1 min following addition of GTP, glycerol, and transfer to 37 °C, short protofilaments and sheets of protofilaments are observed but no rings could be seen (Figure 3a). EAT tubulin samples mixed with a small amount of neurotubulin (C_2S) showed a number of rings indicating that these structures could be observed in control preparations. Within 2 min, longer protofilaments or sheets are observed (Figure 3b). By 3 min the electron micrographs contained varying amounts of long sheets and some well-formed microtubules (Figure 3c). After 10 min of incubation at 37 °C, the electron micrographs showed intact microtubules almost exclusively (Figure 3d).

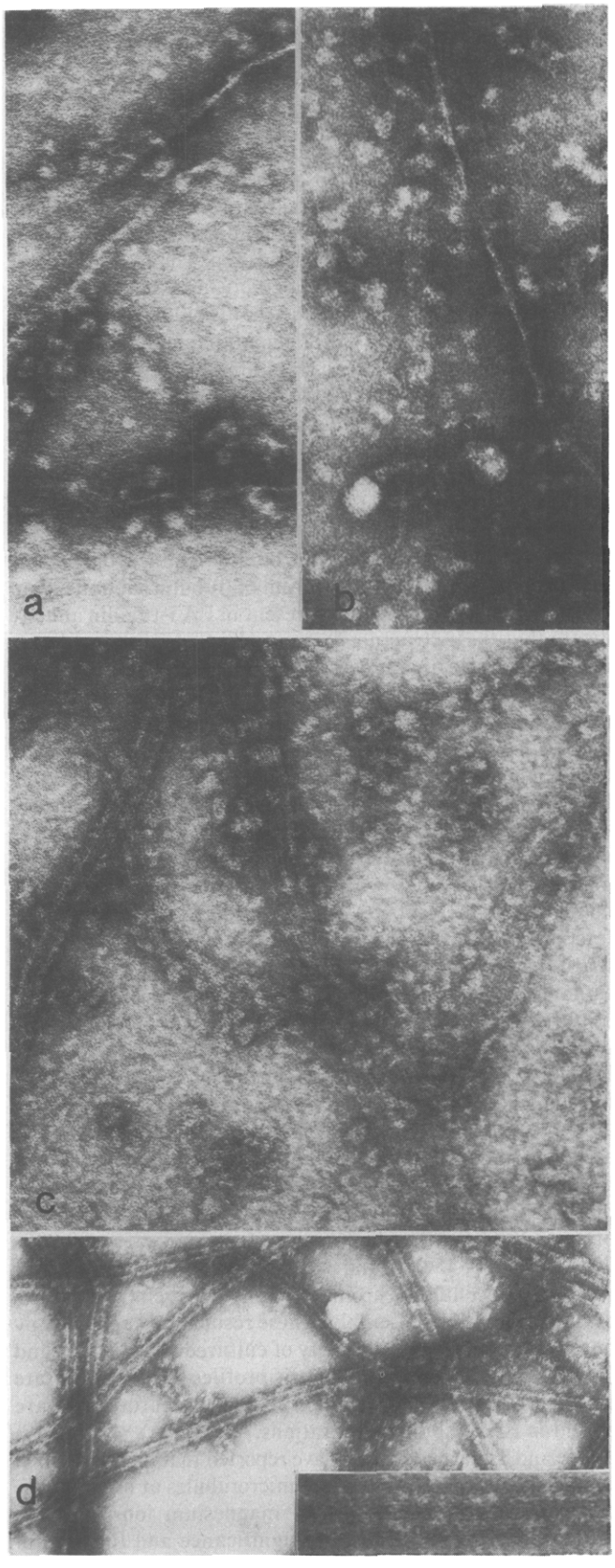

FIGURE 3: Pathway of microtubule assembly. (a) One minute following addition of GTP to C_1S and warming to 37 °C. The first detectable structures are short protofilaments and sheets of protofilaments (×120 000). (b) Two minutes after transfer to 37 °C. Longer protofilaments or sheets of protofilaments are observed (×120 000). (c) After 3 min at 37 °C, long sheets and some well-formed microtubules are detectable (×120 000). (d) Ten minutes following incubation at 37 °C. Intact microtubules are found almost solely (×60 000; inset, ×160 000).

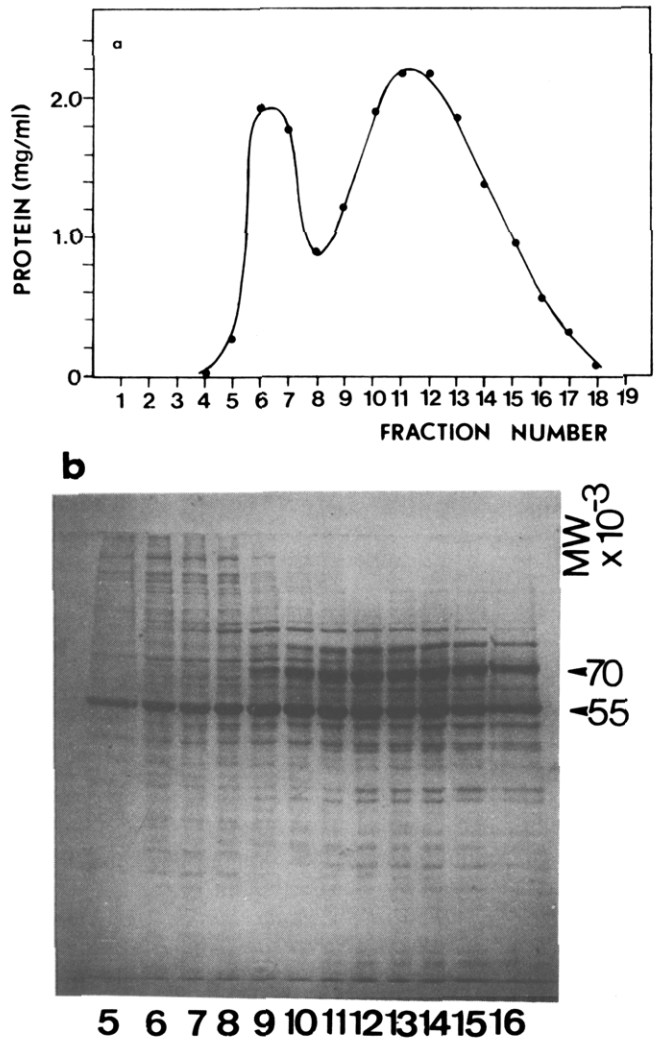

FIGURE 4: Agarose column fractionation of cold-depolymerized microtubules as described in Materials and Methods (a). Analysis of each fraction by sodium dodecyl sulfate–polyacrylamide gel electrophoresis is shown in b.

The diameter of these filaments was 25 ± 3 nm, similar to the dimensions of microtubules from brain tissue. This assembly sequence is similar to that outlined by Bryan (1976). No microtubules could be seen after cooling to low temperature (0 °C) or following addition of calcium ions (not shown).

Fractionation of Depolymerized Microtubules. Electron microscopic examinations of depolymerized EAT tubulin suggested that no ring-like structures are involved in cell tubulin polymerization. Thus it was necessary to describe more fully what components were present in depolymerized microtubules. Fractionation of tubulin solutions (C_1S) on agarose columns at 4 °C revealed two peaks. The first peak, eluting in the excluded volume of the column, contained about 20% of the applied protein. The other peak, which contained about 70% of the protein, appeared in the included volume (Figure 4a). Similar elution profiles have been described for the separation of rings and tubulin subunits from mammalian brain by Kirschner et al. (1974) and Erickson (1974). Electron microscopic examination of material from the excluded fraction contained neither rings nor spirals. The only morphologically identifiable material was large aggregates similar to those found in unfractionated tubulin preparations (Figure 2) which are presumably denatured protein.

Samples from the two fractions were further analyzed by

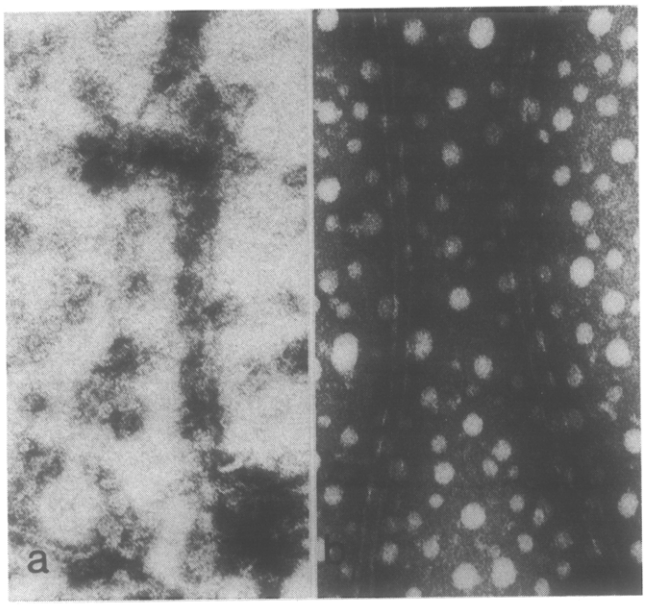

FIGURE 5: Electron micrographs of tubulin preparations separated by chromatography on agarose A-15. (a) Leading fraction incubated 30 min at 37 °C with 1 mM GTP and 4 M glycerol, showing irregular formed aggregates but no microtubules (×120 000). (b) Trailing fraction (6S subunit) after identical incubation; long intact microtubules (×120 000).

analytical ultracentrifugation. The excluded fraction contained a >200S species but no 30 or 36S components, while the included fraction appeared to be 6S tubulin.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis of the >200S and 6S Proteins. Analysis of the agarose column fractions by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 4b) showed that the leading peak consists almost entirely of tubulin, the rest being distributed in 20–25 minor bands. The trailing fractions contain a lot of higher and lower molecular weight bands in addition to tubulin. However, no HMW bands have been observed. The gels of the trailing fractions show besides tubulin a second major component of approximately 70 000 molecular weight. It is not yet clear if this protein is similar to the τ factor, a protein fraction reported by Weingarten et al. (1975) and Witman et al. (1976) to be necessary for tubulin assembly in brain tissue.

Competence of Depolymerization Products for Tubule Assembly. The absence of rings in the electron microscopic examination of C_1S fractions brings into question the proposed role of rings in the mechanism of tubulin polymerization. In order to check whether other preexisting nucleation centers in our in vitro system are required for assembly, cold-depolymerized microtubules from C_1S, fractionated on agarose columns, were tested for ability to repolymerize. Fractions of the leading peak from agarose fractionation of C_1S, at 2–4 mg protein/mL, supplemented with GTP and 4 M glycerol were incubated at 37 °C for 30 min. Samples were examined by electron microscopy and found to contain irregularly formed aggregates similar to those found in the initial C_1S but no microtubules (Figure 5a). Fractions of the trailing peak exhibited long intact microtubules when examined in the electron microscope after incubation also at 2–4 mg protein/mL (Figure 5b). These results show that microtubules from EAT cells can be polymerized in vitro from totally dissociated tubulin and that preexisting nucleation centers are not required.

Discussion

Microtubules can be polymerized in vitro from nonneural EAT cells by the glycerol procedure without the involvement of HMW proteins and rings. Similar findings were made by Langford (1976) for dogfish brain tubulin polymerized by a very similar procedure. Microtubule self-assembly is favored by high protein concentrations in the cytosol and by the presence of glycerol. High protein concentrations may increase the amount of assembly-competent tubulin to a concentration necessary for spontaneous polymerization. The role of glycerol in enhancing the yield in the assembly–disassembly process is not yet clear. It has been found that tubulin prepared with the aid of glycerol has glycerol bound to it and may differ in its polymerization properties from tubulin prepared without glycerol (Detrich et al., 1976). Scheele and Borisy (1976) have found that depolymerized microtubules prepared in the presence of glycerol by the method of Weingarten et al. (1974) contained 6S and 36S species, while material prepared without glycerol by the method of Borisy et al. (1975) yielded 6S and 30S components. Kirschner et al. (1974) have stated that disassembled microtubules can be reassembled only from double rings or spirals but not from 6S tubulin subunits. Our results show that no rings are present in EAT tubulin and indicate that these structures are not obligatory intermediates in the polymerization process of EAT cell tubulin.

The absence of ring structures was demonstrated by sedimentation analysis and by electron microscopy of the fractionated cold depolymerized products. In addition, the fractionated cold depolymerization products have been analyzed for the ability to assemble and by analytical sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The leading peak, which normally contains ring structures and will assemble microtubules when cycled neurotubulin is fractionated, will not produce microtubules when cycled EAT cell tubulin is fractionated. The gel profiles and electron microscopy indicate that tubulin and a number of other proteins are present presumably as denatured filamentous aggregates. The included 6S tubulin peak which normally produces very few microtubules, however, readily produces microtubules when cycled EAT cell tubulin is fractionated. The gel electrophoresis profiles indicate, using crude C_1S preparations, that tubulin and an approximately 70 000 molecular weight nontubulin protein are the principal components, although a number of other proteins are present. Most of them are lost on a second cycle; however, the C_2S still contains besides tubulin some minor proteins having molecular weights of approximately 90 000, 70 000, 49 000, 45 000, and 30 000. Preliminary results show that phosphocellulose chromatography does remove proteins essential for a third assembly. These results are supported by our previous work with a variety of cultured cells (Nagle and Bryan, 1976). The electrophoresis profiles also demonstrate that the HMW proteins present in cycled neurotubulin are absent in EAT tubulin preparations.

Lee and Timasheff (1975) have reported that highly purified tubulin can be reconstituted into microtubules at high tubulin concentrations, higher levels of magnesium ions, and the presence of glycerol. Thus, the significance and function of tubule-associated proteins remain to be elucidated.

The results presented here show that tubulin from nonneural cells can be polymerized in vitro into microtubules. In vitro formation of microtubules from these cells does not appear to require the presence of rings or HMW components as has been proposed for microtubules from mammalian brain. Microtubules can be reconstituted from fully dissociated EAT cell tubulin.

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