Identification and characterization of microtubule proteins from myxamoebae of *Physarum polycephalum*

Anne ROOBOL, Christopher I. POGSON* and Keith GULL
Biological Laboratory, University of Kent, Canterbury CT2 7NJ, U.K.

(Received 29 January 1980)

Cell extracts of myxamoebae of *Physarum polycephalum* have been prepared in such a way that they do not inhibit assembly of brain microtubule protein *in vitro* even at high extract-protein concentration. Co-polymers of these extracts and brain tubulin have been purified to constant stoichiometry and amoebal components identified by radiolabelling. Amoebal tubulin has been identified as having an α -subunit, mol.wt. 54 000, which co-migrates with brain α -tubulin and a β -subunit, mol.wt. 50 000, which co-migrates with *Tetrahymena* ciliary β -tubulin. Non-tubulin amoebal proteins that co-purify with tubulin during co-polymer formation have been shown to be essential for microtubule formation in the absence of glycerol and appear to be rather more effective than brain microtubule-associated proteins in stimulating assembly. The mitotic inhibitor griseofulvin {7-chloro-2',4,6-trimethoxy-6'-methylspiro[benzofuran-2(3H),1'-cyclohex-2'-ene]-3,4'-dione}, which binds to brain microtubule-associated proteins and inhibits brain microtubule assembly *in vitro*, affected co-polymer microtubule protein in a similar way, but to a slightly greater extent.

Although cytoplasmic microtubules are a ubiquitous feature of eukaryotic cells, their constituent proteins have only been purified in large quantities from brain and, more recently, in much smaller amounts, from a few mammalian cell lines (Nagle *et al.*, 1977; Bulinski & Borisy, 1979; Doenges *et al.*, 1979). Very little is known about microtubule proteins in non-flagellated lower eukaryotes.

Purification of microtubule proteins by cycles of assembly and disassembly is the method of choice because this purifies not only the major component tubulin, an acidic protein consisting in brain of an α -subunit (mol.wt. 55 000) and a β -subunit (mol.wt. 53 000), but also other minor components, the microtubule-associated proteins, which are basic proteins covering a wide molecular-weight range and which stimulate microtubule formation *in vitro* (Murphy & Borisy, 1975). A major problem in purifying microtubule protein from non-neural cells by this method is the presence in cell extracts of factors that inhibit microtubule assembly *in vitro* (Bryan & Nagle, 1976). To define the nature of these inhibitory components in a lower eukaryote, we have

studied the assembly of brain tubulin in the presence of cell extracts of myxamoebae of the slime mould *Physarum polycephalum*. We have been able to completely relieve the inhibition of microtubule assembly by this cell extract and during the course of our work it became apparent that appreciable quantities of myxamoebal microtubule protein were co-polymerizing with brain tubulin through several cycles of assembly. In the present paper we identify the myxamoebal microtubule proteins by radiolabelling and show how the presence of these proteins modifies the assembly characteristics of brain tubulin.

Experimental

Myxamoebae of *P. polycephalum* strain CL d were obtained from Dr. J. Dee, University of Leicester, Leicester, U.K. and were routinely grown for 6–7 days in liquid culture or a semi-defined medium containing 1% Bactopeptone (Oxoid), 0.5% yeast extract (Oxoid), 0.5% glucose (McCullough & Dee, 1976). Since amoebae will not grow on a fully defined medium, they were radiolabelled indirectly as follows. *Escherichia coli* K12 were grown for 40 h in a sulphate-free defined medium containing 2.5 μ Ci of carrier-free $^{35}\text{SO}_4^{2-}$ /ml (The Radiochemical Centre, Amersham, Bucks., U.K.). The bacteria, which took up 80% of the radiolabel, were harvested by

Abbreviations used: Pipes, 1,4-piperazinediethanesulphonic acid; SDS, sodium dodecyl sulphate; HP, hot pellet; CS, cold supernatant.

* Present address: Department of Biochemistry, University of Manchester, Oxford Road, Manchester M13 9PL, U.K.

centrifugation and re-suspended in one-quarter of the original culture volume of a sulphate-free basal salts medium and autoclaved for 15 min at 69 kPa. Amoebae were grown in this medium for 6 days (six generations) during which they took up 26% of the radiolabel initially present. Cells were harvested by centrifugation at 500g for 1–2 min at 4°C, washed once in approx. 1 litre of cold 0.9% (w/v) NaCl, once in approx. 200 ml of cold 0.1 M-Pipes, 2 mM-EGTA, 1 mM-MgSO₄, 0.1 mM-EDTA, pH 6.9, and once in an equal volume of cold assembly buffer (0.1 M-Pipes, 2 mM-EGTA, 1 mM-MgSO₄, 0.1 mM-EDTA, 1 mM-GTP, 50 µg of leupeptin/ml, pH 6.9). Cells grown on autoclaved bacteria were washed in five changes of 0.9% NaCl before washing with the Pipes buffers, after which they were free of bacterial debris. The washed cell pellet was weighed, re-suspended in an equal volume of cold assembly buffer and sonicated by 5 × 5 s bursts at 9 µm in an MSE 150 W sonicator. Extract was prepared by centrifuging the sonicated material at 130 000g for 30 min at 4°C. The supernatant was removed with a syringe to avoid the lipid overlay. Before addition to the co-polymerization incubation, the extract was dialysed for 90 min at 4°C against 2 vol. of a solution containing 8 M-glycerol 0.1 M-Pipes, 2 mM-EGTA, 1 mM-MgSO₄, 0.1 mM-EDTA, 0.1 mM-GTP and 10 µg of leupeptin/ml (pH 6.9).

Microtubule protein was purified from fresh sheep brain by two cycles of assembly (Roobol *et al.*, 1976) and the pellet from the second polymerization (HP₂) was stored for up to 2 months in liquid N₂. Immediately before use HP₂ protein was re-suspended in the appropriate cold buffer, depolymerized at 0°C for 30 min and then the suspension was cleared by centrifugation at 130 000g for 30 min at 4°C. The supernatant is termed CS₂ protein.

Tubulin was prepared from CS₂ protein for co-polymerization with amoebal extracts in two ways. For dimethyl sulfoxide-treated tubulin, CS₂ protein was polymerized in 0.4 M-Pipes/1 mM-EGTA/0.5 mM-MgSO₄, 1 M-glycerol/10% dimethyl-sulphoxide/0.5 mM-GTP (pH 6.9) at 37°C for 20 min and harvested at 100 000g for 20 min at 26°C (Himes *et al.*, 1977). Under these conditions the polymer formed was free of high-molecular-weight microtubule-associated protein components. The pellet was resuspended in a small volume of assembly buffer, depolymerized at 0°C for 30 min, and then the suspension was cleared by centrifugation at 130 000g for 30 min at 4°C. The supernatant was then used in co-polymerization incubations.

Phosphocellulose-column-prepared tubulin (PC tubulin) was prepared by fractionating CS₂ protein on phosphocellulose (Whatman P11) equilibrated with 25 mM - Pipes/2 mM - EGTA/1 mM - MgSO₄/0.1 mM-EDTA/0.1 mM-GTP (pH 6.9) (PC column

buffer). Protein eluting in the void volume (PC tubulin) was collected and stored in liquid N₂ for 1–3 days. Brain microtubule-associated protein components were eluted with 0.8 M-KCl buffer and stored in liquid N₂. Before addition to co-polymerization incubation, PC tubulin was thawed and dialysed for 90 min at 4°C against 2 vol. of a solution containing 8 M-glycerol, 0.1 M-Pipes, 2 mM-EGTA, 1 mM-MgSO₄, 0.1 mM-EDTA, 0.1 mM-GTP, 10 µg of leupeptin/ml (pH 6.9).

Co-polymer incubations were initiated by mixing brain tubulin and dialysed extract from 40–60 g wet wt. of cells or 0.6 g of ³⁵S-labelled cells and adding the following components: leupeptin, 40 µg/ml; GTP, 0.9 mM; deoxyribonuclease (Sigma type 1), 10 µg/ml; ribonuclease (Sigma type 1-A), 20 µg/ml; ATP-acetate phosphotransferase (Sigma), 0.4 µg/ml; acetyl phosphate, 15 mM. After mixing the sample was incubated at 37°C for 45 min and aggregated material was harvested by centrifugation at 180 000g for 45 min at 26°C. The pellet, termed HP₁ was re-suspended in 2–3 ml of cold assembly buffer, depolymerized and the suspension was cleared by centrifugation. To the supernatant, termed CS₁, was added an equal volume of assembly buffer containing 8 M-glycerol and the mixture again polymerized at 37°C and centrifuged to give a pellet termed HP₂. This pellet was taken through one further cycle of cold depolymerization (CS₂) and warm assembly in the presence of 4 M-glycerol to yield a pellet termed HP₃, which was stored in liquid N₂ for up to 1 month. Immediately before use the HP₃ pellet was re-suspended in 1–2 ml of assembly buffer, depolymerized and the suspension was cleared by centrifugation.

Tetrahymena ciliary outer fibre protein was obtained by the method of Maekawa & Sakai (1978).

The kinetics of microtubule assembly at 37°C were monitored by turbidimetry (Gaskin *et al.*, 1974) at 400 nm in a Gilford recording spectrophotometer. The binding of the anti-mitotic drug griseofulvin {7-chloro-2',4,6-trimethoxy-6'-methylspiro-[benzofuran-2(3H),1'-cyclohex-2'-ene]-3,4-dione} to microtubule protein was measured by the method of Roobol *et al.* (1977a). Samples for electron microscopy were prepared by negative staining (Borisy *et al.*, 1974). SDS/polyacrylamide-gel electrophoresis was performed in 1-mm-thick slab gels of 7.5% acrylamide by the method of Laemmli (1970). Gels were stained for protein with Coomassie Brilliant Blue, dried and, where required, exposed to X-ray film (Kodirex) for 14–28 days. Molecular-weight markers for electrophoresis were: *E. coli* β-galactosidase (135 000), bovine serum albumin (68 000), muscle pyruvate kinase (57 000), bovine liver glutamate dehydrogenase (50 000), horse liver alcohol dehydrogenase (41 000) and soya-bean trypsin

inhibitor (21 500). Protein concentration was determined by the method of Lowry *et al.* (1951). GTP was assayed by the method of Grassl (1974).

All chemicals were AnalaR grade. Biochemicals were purchased from the Sigma Chemical Co. Leupeptin was from the Peptide Institute, Osaka, Japan. Randomly tritiated griseofulvin was prepared at The Radiochemical Centre, Amersham, Bucks., U.K., and purified as described previously (Roobol *et al.*, 1977a). Radioactivity was measured, after mixing samples with PCS scintillation 'cocktail' (Hopkin and Williams, Chadwell Heath, Essex, U.K.), in a Beckman liquid-scintillation counter.

Results and discussion

Conditions for copolymerization

Preliminary experiments, in which assembly of brain microtubules in the presence of amoebal extracts was studied, showed that the inhibition of assembly by the extract was due to proteolytic activity, GTPase activity and the presence of ribonuclease- and deoxyribonuclease-sensitive material. A range of proteolysis inhibitors were tested, including phenylmethanesulphonyl fluoride, 7-amino-1-chloro-3-L-tosylamidoheptan-2-one (Tos-Lys-CH₂Cl; 'TLCK'), 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one ('TPCK'), *p*-aminobenzamidine, Trasylol and several of the oligopeptide inhibitors such as leupeptin, antipain and pepstatin (Aoyagi & Umezawa, 1975). Of these, by far the most effective was leupeptin. At a concentration of >10 µg/ml leupeptin abolished proteolytic attack by the extract on endogenous protein, exogenous proteins such as casein and on brain high-molecular-weight microtubule-associated proteins, which are particularly sensitive to proteolysis (Vallee & Borisy, 1978), and partially relieved

inhibition by the extract of brain microtubule assembly. Also effective to a lesser extent were Tos-Lys-CH₂Cl, *p*-aminobenzamidine and antipain, although high concentrations (>100 µM) of Tos-Lys-CH₂Cl inhibited brain microtubule assembly. Phenylmethanesulphonyl fluoride, which is widely used to prevent proteolysis in cell extracts, was completely ineffective for amoebal extracts.

Fairly high concentrations (0.5–2 mM) of GTP are required for microtubule assembly *in vitro* and we found the amoebal extract contained a fairly active GTPase (approx 0.3 µmol of GTP hydrolysed/mg of extract protein per h). High concentrations (>5 mM) of GTP and ATP actually inhibited microtubule assembly, but 15 mM-acetyl phosphate together with acetate kinase (EC 2.7.2.1) maintained GTP at a concentration that supported microtubule formation in the presence of extract. It has been shown that microtubule assembly is inhibited by polyanions (Bryan *et al.*, 1975), and we have found that treatment of the amoebal extract with ribonuclease and deoxyribonuclease partially alleviated inhibition of brain microtubule assembly by the extract.

Amoebal extracts prepared in the presence of leupeptin, treated with ribonuclease and deoxyribonuclease and supplied with the GTP-generating system, did not inhibit brain tubulin assembly even at high extract-protein concentrations and in fact the protein recoveries of co-polymerized material were higher than those of PC tubulin or dimethyl sulphoxide-treated tubulin alone (Table 1). The proportion of amoebal protein in the co-polymer CS₂ (Table 2) was not sufficient to account for these enhanced recoveries, which are probably due to stimulation of tubulin assembly by factors in the extract. Since at the extract concentrations used in the co-polymerization incubations (40–50 mg of protein/ml) the extract alone did not form microtubules on warming,

Table 1. Preparation of co-polymers from myxamoebal cell extracts and brain tubulin

Purification step	Total (mg of protein)				Total (c.p.m.)	
	Dimethyl sulphoxide-treated tubulin alone	Dimethyl sulphoxide-treated tubulin + extract	PC tubulin alone	PC tubulin + extract	PC tubulin + ³⁵ S-labelled extract	PC tubulin + ³⁵ S-labelled extract
Tubulin added (final concentration)	34.8 (1.1 mg/ml)	33.0 (1.1 mg/ml)	40.0 (1.1 mg/ml)	38.0 (1.3 mg/ml)	34.0 (9.7 mg/ml)	2.5 × 10 ⁸ extract
HP ₁	24.1	125.2	13.0	87.7	—	—
CS ₁	18.2	58.6	—	56.0	16.2	9.3 × 10 ⁶
HP ₂	16.0	22.8	9.2	24.6	—	—
CS ₂	15.0	19.2	—	16.6	8.8	2.1 × 10 ⁶
HP ₃	9.4	15.0	7.1	13.4	—	—
CS ₃	8.5	13.8	6.4	12.1	7.2	1.6 × 10 ⁶
% recovery of tubulin or % recovery of counts	24%	42%	16%	32%	21%	0.64%

Table 2. *Composition of co-polymer CS₃*

The percentage composition was estimated by SDS/polyacrylamide-gel electrophoresis followed by staining with Coomassie Brilliant Blue. The stained bands were scanned at 650 nm in a Gilford model 250 spectrophotometer equipped with linear transport and the peak areas were determined by integration. The molecular-weight values were determined by mobility of bands separated by SDS/polyacrylamide-gel electrophoresis and detected by autoradiography relative to the mobility of protein standards (see the text) in the system used (Laemmli, 1970).

Component protein	Protein (% of total)	Molecular weight
Brain tubulin + A ₁	91.9	54000
A ₂	6.8	50000
A ₃	Not detectable	46500
A ₄	1.3	43000

Fig. 1. *SDS/polyacrylamide-gel electrophoresis of co-polymer protein*

Protein was precipitated from column fractions by addition of an equal volume of cold acetone and the precipitate was allowed to form overnight at -20°C . The resulting precipitate was dissolved in 50–1000 μl of Laemmli sample buffer depending on its size. (a) Co-polymer CS₂, protein stain; (b) co-polymer CS₂, autoradiograph; (c) *Tetrahymena* ciliary tubulin; (d) co-polymer CS₃, protein stain; (e) co-polymer CS₃, autoradiograph; (f) phosphocellulose void peak, protein stain; (g) and (h), phosphocellulose void peak, autoradiograph; (i) DEAE tubulin peak (Fig. 3), protein stain; (j)–(l), phosphocellulose salt-eluted peak, autoradiograph; (m) brain microtubule-associated protein, protein stain.

the microtubules formed with brain tubulin present must be true co-polymers.

Since a fourth cycle of assembly/disassembly did not alter the proportion of protein bands shown in Table 2, except for band A₄, all the results described below were obtained with CS₃ protein.

Identification of the amoebal components in the co-polymer

Autoradiography of ³⁵S-labelled CS₂ and CS₃ proteins separated by SDS/polyacrylamide-gel electrophoresis revealed four bands, A₁–A₄ (Fig. 1). The molecular-weight estimation for these components is given in Table 2. Band A₄ is very likely actin and did not co-purify with tubulin in the third and fourth assembly cycles. The distortion of band A₁ indicates co-migration with brain α - or β -tubulin, which were not resolved in this gel system. Brain α - and β -subunits did separate when 8 M-urea was

present during electrophoresis and in this system band A₁ migrated between the brain α - and β -subunits, as did the α -subunit of *Tetrahymena* ciliary tubulin. Band A₂ co-migrated with the β -subunit of *Tetrahymena* ciliary tubulin with or without 8 M-urea present.

In the hope of establishing which of the radiolabelled bands were amoebal tubulin, the CS₃ protein was applied to a column (1 cm $\times$ 6 cm) of phosphocellulose equilibrated with PC column buffer and eluted with this buffer at a flow rate 10.8 ml/h. Of the recovered radiolabel 98.4% was eluted in the void volume and this contained proteins A₁, A₂ and A₃ (Fig. 1). After washing the column with 5 vol. of equilibration buffer, the remaining 1.6% of radiolabel was recovered by eluting the column with column buffer containing 0.8 M-KCl. The salt-eluted peak contained many radiolabelled proteins (Fig. 1), the major ones being A₂ and A₃. The non-tubulin

proteins derived from co-polymer did not contain prominent high-molecular-weight proteins, which are so typical of brain microtubule-associated proteins (Kirschner, 1978). Protein A_1 was much decreased in this fraction and this leads us to the conclusion that this acidic protein is the α -subunit of amoebal tubulin. Although on the autoradiogram band A_2 appeared to have similar intensity in both the void and salt-eluted fractions, the protein staining of this band showed much greater intensity in the void fraction than in the salt-eluted fraction, in which it was barely detectable. This observation suggests that the A_2 band is in fact two proteins, one moderately radiolabelled, which behaves like a β -subunit of tubulin, and a second heavily labelled, which behaves like a microtubule-associated protein. Band A_3 could only be detected by autoradiography and it appeared in both void and salt-eluted fractions of the phosphocellulose column. The void-fraction protein alone did not form microtubules on warming (see below), so it is unlikely that this is a microtubule-associated protein component. If band A_3 contains a protein at all, it must be present in very small quantities (although very heavily radio-labelled) and does not fulfil any known role in microtubule formation.

Fig. 2. Effect of protein concentration on extent of microtubule formation

CS_3 samples of proteins in assembly buffer were diluted in the same buffer, assembly was initiated by warming to $37^\circ C$ and the change in absorbance at 400 nm was monitored in a Gilford model 250 recording spectrophotometer. Symbols: ●, brain microtubule protein; □, PC tubulin; △, dimethyl sulphoxide-treated tubulin; ■, PC tubulin co-polymer; ▲, dimethyl sulphoxide-treated tubulin co-polymer.

Assembly characteristics of the co-polymer

Co-polymer CS_3 protein assembled to form microtubules on warming in glycerol-free assembly buffer. The microtubules formed in this way were cold-labile and depolymerized by the addition of 5 mM- $CaCl_2$. Co-polymer formed from both dimethyl sulphoxide-treated tubulin and PC-tubulin showed a marked decrease in the critical concentration for microtubule assembly in the absence of glycerol when compared with control material prepared from dimethyl sulphoxide-treated tubulin or PC tubulin alone (Fig. 2). We also observed that a given concentration of co-polymer protein gave a greater increase in turbidity than did a similar concentration of brain microtubule protein. These data indicate that amoebal microtubule-associated protein components that stimulate microtubule formation have been co-purified with the brain tubulin. The proportion of non-tubulin components in co-polymer preparations as judged by autoradiography (1.6%), is, however, much lower than in brain microtubule preparations (8–12%).

Fig. 3. Resolution of co-polymer CS_3 on A-50 DEAE-Sephadex

CS_3 protein (9.18 mg) in a solution containing 0.1 M-Pipes, 2 mM-EGTA, 1 mM- $MgSO_4$, 0.1 mM-EDTA, 0.1 mM-GTP, 5 μg of leupeptin/ml, 0.25 M-KCl, pH 6.9, was applied to a column (2 cm $\times$ 4 cm) of A-50 DEAE-Sephadex equilibrated with the same buffer at $4^\circ C$. The column was developed with a linear 0.25–0.65 M-KCl gradient in the same buffer with a flow rate of 11.5 ml/h and 10 min fractions were collected. As soon as possible portions of fractions were dialysed against 100 vol. of a solution containing 0.1 M-Pipes, 2 mM-EGTA, 1 mM- $MgSO_4$, 0.1 mM-EDTA, 0.1 mM-GTP, 2 μg of leupeptin/ml, pH 6.9, for 1 h at $4^\circ C$, and then assayed for ability to form microtubules alone and when mixed with brain tubulin at a final concentration of 2.4 mg/ml by turbidimetry. Symbols: ●, mg of protein/fraction; ▲, change in absorbance at 400 nm in assembly assay; ----, KCl concentration (M).

Table 3. *The effect of ion-exchange chromatography on phosphocellulose and DEAE-Sephadex on assembly of co-polymer CS₃ protein (10–15 mg) in phosphocellulose column buffer was applied to a column (1 cm × 7 cm) of phosphocellulose equilibrated and eluted with phosphocellulose column buffer at a flow rate 10.8 ml/h at 4°C. After elution of the void peak the column was washed with 5 column vol. of equilibration buffer, then developed with 0.8 M-KCl column buffer. The details of the DEAE-Sephadex column are given in the legend to Fig. 3. The tubulin-containing fractions, 500 µl of each salt-eluted fraction and 500 µl of brain microtubule-associated protein were immediately dialysed against 100 vol. of a solution containing 0.1 M-Pipes, 2 mM-EGTA, 1 mM-MgSO₄, 0.1 mM-EDTA, 0.1 mM-GTP, 2 µg of leupeptin/ml, pH 6.9, for 1 h at 4°C. Immediately after dialysis protein solutions were made 1 mM with respect to GTP and assayed for microtubule formation at 370 nm by turbidimetry. Control CS₃ protein was exposed to the same buffers, for the same amount of time, as fractionated samples.*

Assembly condition	Change in absorbance at 400 nm
Phosphocellulose chromatography	
1. Co-polymer control (3.7 mg/ml)	1.015
2. Void peak (3.7 mg/ml)	0.000
3. Void peak (3.7 mg/ml) + salt-eluted peak*	0.030
4. Void peak (3.7 mg/ml) + brain microtubule-associated protein (0.36 mg/ml)	0.341
DEAE-Sephadex chromatography	
1. Co-polymer control (2.4 mg/ml)	0.750
2. Tubulin peak (1.5 mg/ml)	0.000
3. Brain tubulin (2.4 mg/ml) + peak fraction from Fig. 3 (0.14 mg/ml)	0.078
4. Brain tubulin (2.4 mg/ml) + brain microtubule-associated protein (0.31 mg/ml)	0.245

* Protein concentration was too low to detect.

The stimulatory activity in co-polymer CS₃ could be separated from tubulin by ion-exchange chromatography on phosphocellulose (Table 3) or DEAE-Sephadex (Fig. 3, Table 3). We have, however, been unable to establish conditions under which reconstitution of dialysed non-tubulin co-polymer components with the tubulin-containing fraction resulted in any marked restoration of assembly capacity. At best approx. 10% of the initial assembly capacity could be restored in such experiments (Table 3). A variety of conditions have been tested without success. Addition of brain microtubule-associated protein components to the co-polymer tubulin fractions readily induced microtubule formation and gave rise to an absorbance change consistent with a similar concentration of brain microtubule protein rather than of co-polymer protein (Table 3).

Interaction of the co-polymer with the microtubule poison griseofulvin

Since our data indicated that the presence of amoebal microtubule-associated protein components stimulated assembly of brain tubulin to a different extent than did brain microtubule-associated protein components and we have shown that griseofulvin interacts with brain microtubule-associated protein rather than tubulin (Roobol *et al.*, 1977a), it was of interest to examine the interaction of this drug with co-polymer protein. Fig. 4 shows that microtubule formation from dimethyl sulphoxide-treated tubulin

co-polymer was more sensitive to inhibition by griseofulvin (IC₅₀ 40 µM; IC₅₀ is the inhibitor concentration required to produce 50% inhibition of microtubule assembly) than was a dimethyl sulphoxide-treated-tubulin control (IC₅₀ 115 µM) and brain microtubule protein (IC₅₀ 68 µM, Roobol *et al.*, 1976). When brain microtubule protein is mixed with griseofulvin at 4°C, a characteristic aggregate forms that gives rise to a high initial absorbance in assembly assays (Roobol *et al.*, 1977b; Weber *et al.*, 1976). There was no evidence of such aggregate formation from either co-polymer or tubulin alone. Fig. 5 shows that co-polymer protein bound considerably more griseofulvin than the tubulin control and slightly more than brain microtubule protein, which is consistent with the sensitivities of assembly towards the drug. It has been reported that *Physarum* amoebae (Mir & Wright, 1978) and mammalian cell lines (Grisham *et al.*, 1973; Weber *et al.*, 1976) are not especially sensitive towards griseofulvin *in vivo*, which is consistent with the similarity in IC₅₀ values and in drug-binding data for the co-polymer and brain microtubule protein.

In the absence of a method suitable for purifying microtubule protein from lower eukaryotes, isolation of these proteins by co-polymerization with brain tubulin depleted of its natural microtubule-associated protein components is a useful method for identifying probable microtubule proteins in these organisms. The technique has already been

Fig. 4. Effect of griseofulvin on microtubule assembly from tubulin CS₃ and co-polymer CS₃.

Stock solutions of griseofulvin in dimethylformamide were diluted in assembly buffer to the required concentration and to a final concentration of 2% (v/v) dimethylformamide. After addition of CS₃ protein at 0°C, microtubule formation at 37°C was monitored by turbidimetry. (a) Dimethyl sulphoxide-treated tubulin CS₃; (b) dimethyl sulphoxide-treated tubulin co-polymer CS₃. Curve 1, 2% (v/v) dimethylformamide; curve 2, 30 μM-griseofulvin; curve 3, 50 μM-griseofulvin; curve 4, 75 μM-griseofulvin; curve 5, 100 μM-griseofulvin.

successfully applied to yeast (Clayton *et al.*, 1979) and *Aspergillus nidulans* (Sheir-Neiss *et al.*, 1976). The co-polymer described in the present paper is unusual in that it has been purified to constant stoichiometry of components and, because care was taken to eliminate inhibitory components in the cell extract, it was obtained in sufficient quantity to permit a study of its assembly characteristics and its interaction with an anti-microtubule drug that interacts with microtubule-associated protein components. Our data have shown that some of the assembly characteristics of tubulin are modified by the type of microtubule-associated protein component present. Perhaps most importantly, the present study has provided a cell extract from a lower eukaryote that does not inhibit brain microtubule

Fig. 5. Binding of griseofulvin to co-polymer, brain microtubule protein and brain tubulin.

Griseofulvin binding was determined by the method of Roobol *et al.* (1977a). Symbols: ●, brain microtubule protein CS₂; (Δ), dimethyl sulphoxide-treated tubulin CS₃; (▲), dimethyl sulphoxide-treated tubulin co-polymer CS₃.

assembly at really high protein concentrations and that is therefore very promising material for further studies of assembly of lower-eukaryote microtubule protein *in vitro*.

We thank Mrs. Marianne Wilcox for excellent technical assistance, Professor H. Umezawa for gifts of antipain, pepstatin, chymostatin and phosphoramidon, Mr. Ray Newsam for assistance with electron microscopy and photographic work and the staff of Canterbury Abattoir for kindly supplying sheep brains. The investigation was funded by grants from the Medical Research Council, the Royal Society and the Wellcome Trust.

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