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Purification and assembly *in vitro* of tubulin from *Trypanosoma brucei brucei*

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Trypanosome tubulin was purified to near homogeneity by chromatography on DEAE-Sephadex, Amicon filtration and assembly–disassembly *in vitro*. Polymerization of the tubulin *in vitro* yielded long, structurally normal, microtubules and some sheet structures on addition of GTP and incubation at 37 °C, in either the presence or the absence of Mg^{2+}. Tubulin assembly was disrupted by glycerol and a selection of microtubule-reactive drugs. Immunological analysis of the purified tubulin revealed tyrosinated and acetylated α-tubulin, in addition to defining the migration characteristics of the α- and β-tubulin on one-dimensional SDS/polyacrylamide gels. This is the first isolation of trypanosome tubulin with the ability to form structurally normal microtubules independent of the addition of taxol or nucleating microtubule fragments. The development of the purification procedure thus provides an important step for subsequent study of microtubule-associated protein–tubulin and plasma-membrane–microtubule cytoskeleton interactions of trypanosomes, and increases the potential for development of tubulin-based anti-trypanosome drugs.

INTRODUCTION

The parasitic protozoan *Trypanosoma brucei brucei* is a member of the African trypanosome group which includes among its members the causative agent of sleeping sickness, a catastrophic disease of humans (Ormerod, 1979). Development of effective drugs to combat the disease and prevention by immunoprophylaxis have led recently to a renewed consideration of the organism's cytoskeleton as a drug-sensitive target.

The microtubule-based trypanosomatic cytoskeleton is tightly organized, in part, into two highly ordered subcellular structures, namely the subpellicular microtubules and the flagella axoneme (Russell *et al.*, 1984; Gull *et al.*, 1986; Schneider *et al.*, 1987; Sherwin *et al.*, 1987; Seebeck *et al.*, 1988*a*; Gallo & Precigout, 1988; Sherwin & Gull, 1989; Woods *et al.*, 1989). The subpellicular microtubules consist of a specific cortical array of cross-linked microtubules which are connected to the cytoplasmic membrane (Angelopoulos, 1970; Schneider *et al.*, 1987; Sherwin & Gull, 1989; Woods *et al.*, 1989). The cross-linkers are visible in the electron microscope (Vickerman, 1985; Sherwin & Gull, 1989), and putative microtubule–microtubule and microtubule–membrane cross-linking proteins have been isolated from the trypanosomes (Bramblett *et al.*, 1987; Seebeck *et al.*, 1988*b*; Schneider *et al.*, 1988*a*,*b*). The flagellum attachment zone, that region where the flagellum and cell body meet, is a specialized region of the membrane skeleton which has been partially characterized through the use of monoclonal antibodies (Gallo *et al.*, 1988; Woods *et al.*, 1989). The flagellum has a typical 9+2 microtubule axoneme in association with the paraflagellar rod (Cachon *et al.*, 1988), a structure partially composed of a doublet of proteins with molecular masses of 68000 and 72 000 Da (Russell *et al.*, 1983; Gallo & Schrevel, 1985; Saborio *et al.*, 1989) and also containing a protein doublet antigenically related to spectrin (Schneider *et al.*, 1988*c*; Alcina *et al.*, 1988; Woods *et al.*, 1989). Maintenance of cell division, shape and motility are dependent on this plasma-membrane–microtubule cytoskeleton interaction and, as such, it is potentially a very important site for the action of anti-trypanosome drugs.

Central to gaining a biological appreciation of the membrane–microtubule complex and its usefulness as a target for drug therapy is a thorough characterization of the composite tubulin, something only feasible if the tubulin is available in a purified assembly-competent state. For example, it is possible to test the drug sensitivity of purified tubulin *in vitro*, permitting a more rational choice of drugs for analysis *in vivo* and easier interpretation of results obtained upon exposure of trypanosomes to drugs. Purified tubulin may be added to trypanosome cell-free extracts, leading, owing to the increased tubulin concentration, to microtubule formation under conditions in which it normally does not occur. Recovery of the microtubules and adherent non-tubulin proteins (Vallee, 1982; Bramblett *et al.*, 1987; Seebeck *et al.*, 1988*b*; Schneider *et al.*, 1988*a*; Campbell *et al.*, 1989) constitutes a method of procurement of trypanosome microtubule-associated proteins ('MAP'), some of which may be involved in organization of subpellicular microtubules. Finally, homologous trypanosome tubulin is required if the membrane–cytoskeleton complex is to be faithfully reconstructed *in vitro* (Dolan *et al.*, 1986), a process which will undoubtedly lead to a greater understanding of the control of trypanosome cell shape and division.

Although immunological analysis of trypanosome tubulin is progressing rapidly (Gallo & Anderton, 1983; Gull *et al.*, 1986; Schneider *et al.*, 1987; Sherwin *et al.*, 1987; Gallo & Precigout, 1988; Chang & Flavin, 1988; Sasse & Gull, 1988; Woods *et al.*, 1989), attempts to

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isolate trypanosome tubulin and to assemble it *in vitro* into structurally normal microtubules have been only partially successful (Stieger *et al.*, 1984; Russell *et al.*, 1984; Dolan *et al.*, 1986; Bramblett *et al.*, 1987), thus prompting the work described herein. We now report the purification of assembly-competent tubulin from *Trypanosoma brucei brucei* to near homogeneity by a fast and simple procedure. The tubulin has been characterized by immunological methods. Assembly under different conditions, including in the presence of microtubule-reactive drugs, has been performed. The results clearly open the way for characterization of trypanosome tubulin *in vitro* and its interaction with associated proteins and drugs.

EXPERIMENTAL

Incubation of trypanosomes

Procyclic forms of *Trypanosoma brucei brucei* (stock 427) were grown at 27 °C in tissue-culture flasks containing SDM-79 medium (Brun & Schonenberger, 1979) supplemented with 10% (v/v) foetal-calf serum and 7.5 μg of haemin/ml.

Tubulin purification

Preparation of cell-free supernatant. Trypanosomes, grown to $(2–4)\times10^7$ cells/ml, were harvested from 2 litres of medium by centrifugation at 1400 ***g*** for 5 min at 4 °C and washed once with 100 ml of PEME (100 mM-Pipes/2 mM-EGTA/1 mM-$MgSO_4$/0.1 mM-EDTA) supplemented with 4 M-glycerol, 0.1 mM-GTP and 50 μg of leupeptin/ml at pH 6.9. The washed cells were resuspended in 6 ml of the supplemented PEME, passed through a French press (Apex type 3010-4) at setting 70 and sonicated for 4 × 30 s, with a 2 min incubation in an ice/water bath between each sonication, at the highest setting of an MSE 150 W MK2 ultrasonic disintegrator. The sonicated cells were incubated on ice for 30 min, centrifuged at 40000***g*** for 30 min at 4 °C, and the supernatant was recovered and re-centrifuged under the same conditions for 20 min. The resulting supernatant was either used immediately or frozen at −80 °C.

DEAE-Sephadex chromatography. The cell-free supernatant was applied at a flow rate of 25 ml/h to a 28 ml DEAE-Sephadex column packed in a 30 ml disposable syringe and equilibrated with PEME containing 0.2 M-KCl, 0.1 mM-GTP and 12.5 μg of leupeptin/ml. The column was washed with the equilibration buffer after application of the sample, and adhering protein was eluted with PEME containing 0.6 M-KCl, 0.1 mM-GTP and 12.5 μg of leupeptin/ml. Peak fractions were pooled and either used immediately or frozen at −80 °C.

Amicon concentration. The 0.6 M-KCl fraction from DEAE-Sephadex was concentrated to 2–3 ml in an Amicon ultrafiltration assembly equipped with a Diaflow PM10 membrane of nominal exclusion 10000 Da. The solution was stirred gently with a magnetic stirrer, and concentration was performed at 30 lb/in^2 of N_2 (~ 200 kPa). After ultrafiltration, the preparation was further concentrated by overnight dialysis at 4 °C with stirring against 125 ml of PEME containing 8 M-glycerol, 0.1 mM-GTP and 25 μg of leupeptin/ml. The final volume of the solution was 0.7 ml.

Assembly/disassembly of tubulin. The dialysed solution was removed from the dialysis bag, and centrifuged, with transfer of the supernatant to a fresh tube for the second spin, at 40000 ***g*** for 30 min and 20 min at 4 °C. The second centrifugation was required to remove particulate material unavoidably obtained from the loosely packed primary pellet upon transfer of the supernatant. Tubulin in the supernatant was assembled in the presence of 10 mM-Mg^{2+} and 1.8 mM-GTP for 30 min at 37 °C and the microtubules were collected by centrifugation at 40000 ***g*** for 30 min at 20 °C. Since the microtubules packed poorly, only about 70% of the supernatant was removed, 50 μl of PEME containing 1.8 mM-GTP was added to the loose pellet, and the preparation was re-centrifuged at 40000 ***g*** at 20 °C, after incubation at 37 °C for 15 min. The supernatant was removed and the surface of the pellet rinsed gently with 100 μl of PEME containing 0.1 mM-GTP at 37 °C. The pellet was resuspended in 200 μl of PEME containing 0.1 mM-GTP and incubated on ice for 30 min with occasional gentle vortex-mixing. The suspension was then centrifuged at 40000 ***g*** for 30 min at 4 °C and the resulting supernatant containing the purified tubulin was recovered. The tubulin was stored at −70 °C.

SDS/polyacrylamide-gel electrophoresis

Samples were electrophoresed on 10% polyacrylamide gels by the method of Laemmli (1970), with SDS from Sigma (Clayton *et al.*, 1980). The proteins were stained with Coomassie Blue R250, or they were transferred to nitrocellulose.

Immunoblotting

After one-dimensional gel electrophoresis, proteins were transferred to nitrocellulose membranes (Schleicher and Schuell) essentially as described by Towbin *et al.* (1979) and probed with primary and secondary antibodies (Dakopatts) as described by Birkett *et al.* (1985). To ensure that efficient transfer of proteins had occurred, blots were stained with Ponceau S (Sigma) and gels were stained with Coomassie Blue R250 after blotting. The antibodies DM1A and DM1B, which recognize α- and β-tubulin respectively, were obtained from Dr. S. Blose and are described in Blose *et al.* (1984). 6-11B-1, specific for acetylated α-tubulin, was from Dr. G. Piperno and is described in Piperno & Fuller (1985). YL1/2, which reacts with tyrosinated tubulin and is described in Kilmartin *et al.* (1982), was obtained from Dr. J. Kilmartin. The β-tubulin-specific antibody, KMX, is described in Birkett *et al.* (1985). Before use, DM1A, DM1B, YL1/2 and KMX were diluted 1:500, and 6-11B-1 was diluted 1:20, with 10 mM-Tris/HCl (pH 7.4) containing 140 mM-NaCl and 0.1% (v/v) Tween 20 (TBS-Tween).

Tubulin assembly assay

Purified trypanosome tubulin in PEME was incubated with 1.8 mM-GTP at 37 °C for 30 min at a final concentration of 2 mg/ml. Where indicated, glycerol was added to 4 M and Mg^{2+} to 10 mM. Microtubule-reactive drugs used in assembly assays were prepared as stock solutions in PEME (colcemid and vinblastine), dimethyl sulphoxide diluted to 20% with PEME (maytansine and taxol) or ethanol diluted to 50% with PEME (nocodazole). The concentrations of the stock solutions were such that a 1:10 (v/v) dilution in the assembly

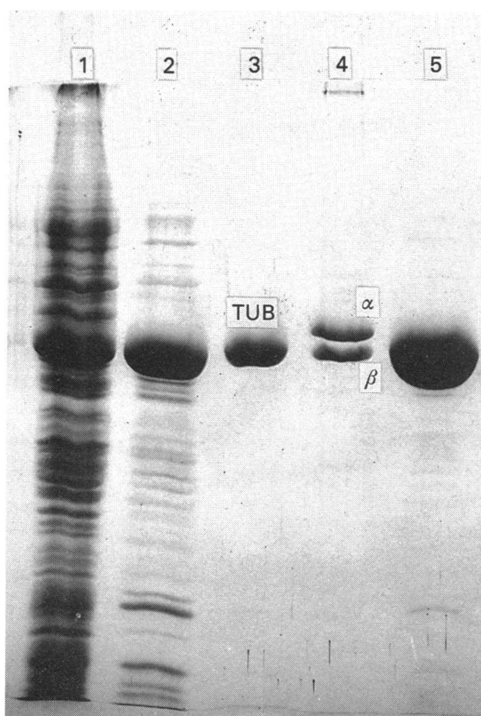

Fig. 1. SDS/polyacrylamide-gel electrophoresis of trypanosome tubulin during purification

Fractions from different stages of the trypanosome tubulin purification procedure were electrophoresed on one-dimensional SDS/polyacrylamide gels and stained with Coomassie Blue R250. The lanes contained: 1, cell-free supernatant (80 μg); 2, 0.6 M-KCl DEAE fraction after concentration against an Amicon Diaflow PM10 membrane and dialysis against PEME containing 8 M-glycerol (32 μg); 3, purified (cycled) trypanosome tubulin (10 μg); 4, sheep brain microtubule protein prepared by three cycles of assembly/disassembly (8 μg); 5, purified (cycled) trypanosome tubulin (40 μg). Abbreviation: TUB, tubulin.

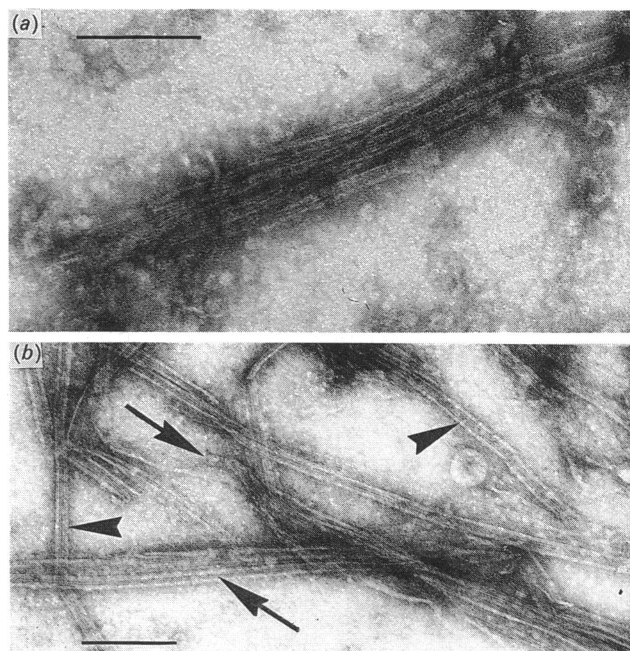

Fig. 2. Electron micrographs of negatively stained trypanosome microtubules assembled in fractions obtained during purification

(*a*) Tubulin was assembled in cell-free extracts of trypanosomes by addition of taxol to 20 μM and incubation at 37 °C. (*b*) Tubulin in 0.6 M-KCl DEAE fractions, after concentration against an Amicon Diaflow PM10 membrane and dialysis against PEME containing 8 M-glycerol, was assembled by addition of GTP to 1.8 mM and incubation at 37 °C. Samples were prepared for electron microscopy as described in the Experimental section. Arrowheads indicate structurally normal microtubules, and arrows indicate tubulin sheets. The bars represent 0.2 μm in (*a*) and (*b*).

mixture resulted in the final drug concentrations indicated in Table 2. Stock solutions were used immediately after preparation and then discarded. Controls, consisting of solvents in the absence of drugs, were tested.

Electron microscopy

Samples for electron microscopy were fixed by diluting 1:1 in PEME containing 4% (v/v) glutaraldehyde, placed on Formvar-coated carbon-stabilized grids, negatively stained with aq. 1% uranyl acetate and examined in a Philips 410 electron microscope.

Protein determination

Protein concentration was determined by the Bio-Rad method as described by the supplier, with bovine serum albumin as the standard protein.

RESULTS

Electrophoresis of cell-free supernatants from homogenized trypanosomes revealed a multitude of bands after Coomassie Blue staining, with a major band in the tubulin region of the gel (Fig. 1, lane 1). The major band interacted with anti-tubulin antibodies after blotting to nitrocellulose (results not shown). We were unable to polymerize the tubulin in the cell-free extracts unless taxol, at 20 μM, was added, resulting in the assembly of short, ill-formed, microtubules (Fig. 2*a*). The taxol was kindly given by Dr. Matthew Suffness, Natural Products Branch, NCI, Bethesda, MD, U.S.A. Initial attempts to purify the tubulin by use of phosphocellulose P-11 chromatography and $(NH_4)_2SO_4$ precipitation proved unsuccessful, mainly owing to difficulty in resolubilizing the $(NH_4)_2SO_4$ precipitate. Chromatography on DEAE-Sephadex followed by concentration against an Amicon Diaflow PM10 membrane and dialysis in PEME containing 8 M-glycerol yielded a protein solution in which

Table 1. Purification of trypanosome tubulin

Fraction	Volume (ml)	Protein concn. (mg/ml)	Total protein (mg)	Protein recovery (%)
Cell-free supernatant	10	15	150	100
DEAE 0.6 M-KCl	33	0.16	5.3	3.5
Amicon	0.7	5.4	3.8	2.5
Cycled tubulin	0.2	4.7	0.9	0.6

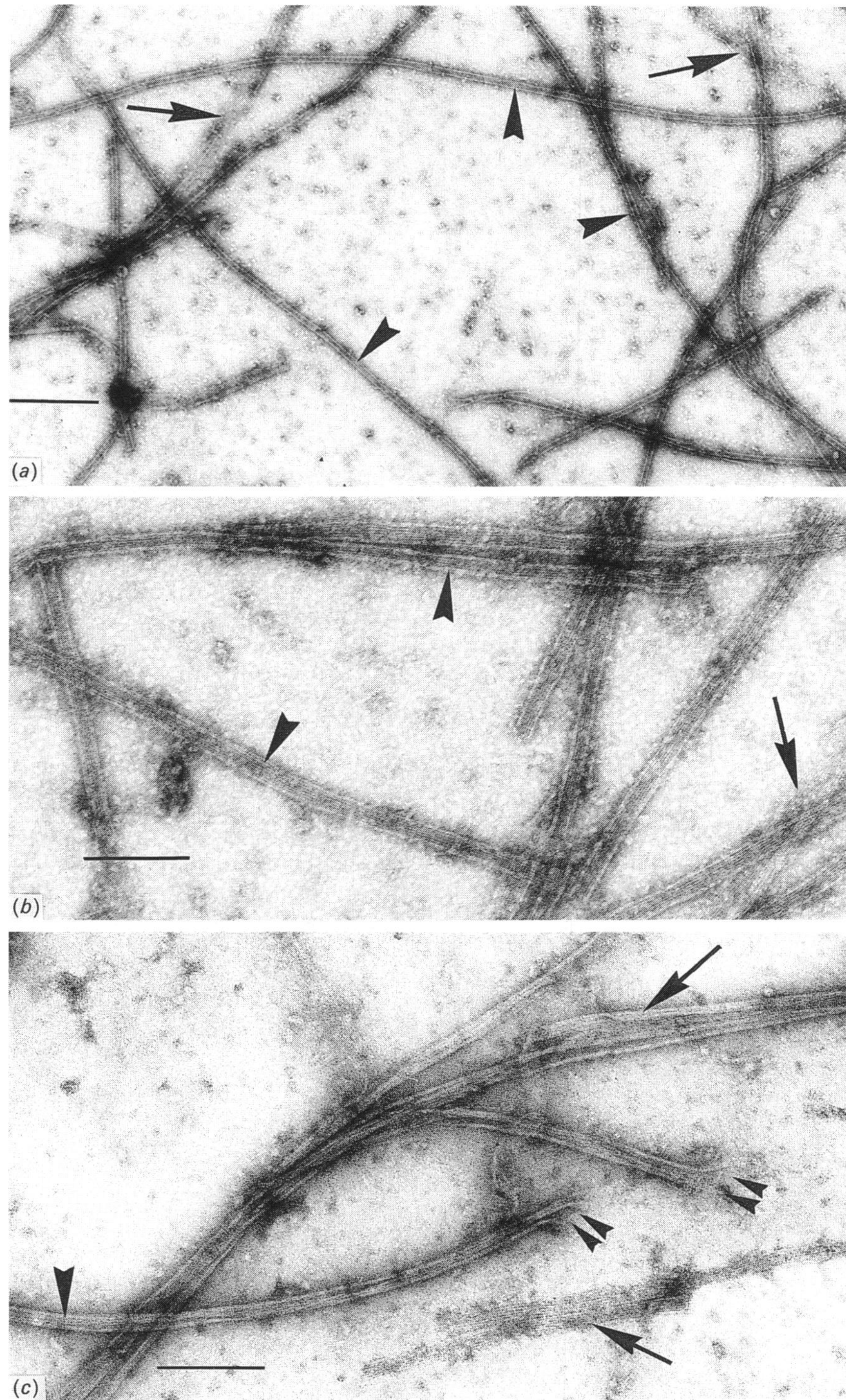

Fig. 3. Electron micrographs of negatively stained trypanosome microtubules assembled from purified tubulin

(*a*) and (*b*) Purified trypanosome tubulin, at 2 mg/ml, was assembled at 37 °C upon addition of GTP to 1.8 mM and Mg^{2+} to 10 mM; (*c*) assembly as for (*a*) and (*b*), but glycerol at a final concentration of 4 M was present. Samples were prepared for electron microscopy as described in the Experimental section. Arrowheads indicate structurally normal microtubules, and arrows indicate tubulin sheets. Note the frayed microtubule ends in (*c*), indicated by double arrowheads. The bar represents 0.5 μm in (*a*) and 0.2 μm in (*b*) and (*c*).

the tubulin assembled into long, often poorly formed, microtubules on addition of GTP (Fig. 2*b*). Addition of Mg^{2+} at 10 mM had little apparent effect on microtubule ultrastructure at this stage of purification (results not shown). On SDS/polyacrylamide gels, the Amicon-concentrated 0.6 M-KCl fraction from DEAE-Sephadex exhibited a major band of tubulin and several contaminating polypeptides (Fig. 1, lane 2). Electrophoresis of the material obtained after assembly/disassembly of the Amicon-concentrated DEAE fraction revealed a single band when 10–20 μg of protein was applied to the gel (Fig. 1, lane 3). At very high loadings (40 μg), a few

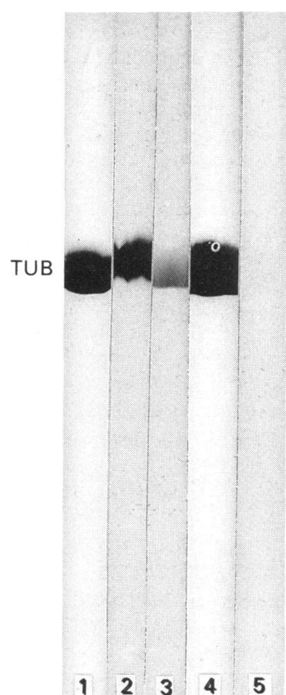

Fig. 4. Western blot of purified trypanosome tubulin probed with anti-tubulin monoclonal antibodies

Purified trypanosome tubulin (12.5 μg) was electrophoresed on an SDS/10%-polyacrylamide gel, blotted to nitrocellulose and probed with the following antibodies: 1, DM1A, general anti-(α-tubulin); 2, 6-11B-1, anti-(acetylated α-tubulin); 3, YL1/2, anti-(tyrosinated α-tubulin); 4, DM1B, general anti-(β-tubulin); 5, blot of tubulin reacted only with secondary antibody to mouse immunoglobulins. A control blot with secondary antibody to rat immunoglobulins gave the same result as in lane 5, and is not shown. KMX, a general anti-(β-tubulin) monoclonal antibody, also reacted with the trypanosome tubulin (result not shown). Abbreviation: TUB, tubulin.

very lightly staining bands, all of lower molecular mass than tubulin, were visible (Fig. 1, lane 5). Whether these bands are contaminating proteins or breakdown products of the tubulin is uncertain. Protein yields during purification are summarized in Table 1.

The purified tubulin assembled readily at a final concentration of 2 mg/ml when GTP was added, yielding long, well-formed, microtubules of normal morphology, with ribbons or open-sheet polymers sometimes observed. Addition of Mg^{2+} had little effect on assembly of tubulin or on microtubule ultrastructure, but glycerol, in either the presence or the absence of Mg^{2+}, caused increased formation of open sheets and ribbons (Figs. 3*a*–3*c*). Addition of taxol to assembly mixtures containing purified tubulin yielded poorly formed microtubules of varying length, which were somewhat reminiscent of the polymers found upon taxol-induced assembly in cell-free supernatants (results not shown).

On one-dimensional SDS/polyacrylamide gels, the α- and β-tubulins were either not resolved (Fig. 1, lane 3) or resolved very poorly (results not shown), whereas on the same gels sheep brain tubulin separated clearly into two subunits (Fig. 1, lane 4). General monoclonal antibodies to α- and β-tubulin, as well as those that specifically recognize the acetylated and tyrosinated forms of α-

Table 2. Effect of tubulin-specific drugs on assembly of purified trypanosome tubulin

Effect on assembly of tubulin: +, assembly; —, no assembly.

Drug	Concn. (μM)	Effect on assembly
None	–	+
Taxol	20	+*
Colcemid	27	+†
Nocodazole	17	+‡
Maytansine	20	–
Vinblastine	40	–

* Taxol induced formation of structurally abnormal microtubules in cell-free supernatants and in tubulin preparations at different stages of purification.

† The microtubules were poorly formed and the amount of assembly appeared to be decreased when colcemid was present.

‡ The microtubules tended to form ribbons, but this also occurred in the presence of 5% ethanol, the final concentration of the nocodazole solvent within assembly reactions.

tubulin, all reacted well with purified trypanosome tubulin on Western blots (Fig. 4).

A variety of tubulin-reactive drugs were tested for their effects on the assembly of purified trypanosome tubulin (Table 2). Since only a small amount of tubulin was available, it was used in assembly assays initially judged by electron microscopy. Colcemid and nocodazole did not prevent tubulin assembly, but, as determined by negative staining of drug-exposed samples, colcemid induced formation of morphologically abnormal microtubules. Structural abnormalities apparent in microtubules when nocodazole was added also occurred upon addition of 5% ethanol, the final concentration of the drug solvent in assembly reactions. Trypanosome tubulin did not assemble in the presence of maytansine and vinblastine.

DISCUSSION

We describe here a rapid and simple process for purification of trypanosome tubulin which has the ability to assemble into structurally normal microtubules upon addition of GTP and warming. The method is based on other procedures used to purify non-neural tubulins (Russell *et al.*, 1984; MacRae & Ludueña, 1984), and depends for its success on obtaining a sufficient concentration of the tubulin to permit assembly *in vitro*. We have no indication of the presence of an inhibitor of tubulin assembly in our preparations. Indeed, addition of taxol to cell-free trypanosome extracts, which leads to a decrease in the concentration of tubulin required for assembly, is sufficient to allow tubulin polymerization. Concentration against an Amicon membrane followed by dialysis against PEME containing 8 M-glycerol were both necessary to achieve efficient assembly and to decrease losses owing to low concentrations of tubulin in the preparations. Precipitation of the tubulin with $(NH_4)_2SO_4$, used to concentrate tubulins from other sources (MacRae & Ludueña, 1984), was not useful in this case, since it was very difficult to resolubilize the pellet.

There have been other attempts to purify trypanosome tubulin, meeting with varying degrees of success. Stieger *et al.* (1984) used taxol to promote tubulin assembly in trypanosome cell-free supernatants, achieving abundant formation of smooth microtubules of variable length and some sheet structures. Since procedures for disassembly of taxol-stabilized microtubules were not available, the tubulin could not be recycled or further purified. In agreement with our findings, the trypanosome tubulin prepared by Stieger *et al.* (1984) did not co-migrate with mammalian neural tubulin, nor were the α- and β-tubulins well resolved.

Dolan *et al.* (1986) isolated pellicular tubulin from *Trypanosoma brucei* by Ca^{2+}-induced depolymerization of microtubules in preparations enriched for the membrane–cytoskeleton complex. The purified tubulin assembled upon addition of brain microtubule seeds (rings of tubulin), but, interestingly, the membrane–microtubule complex could not be reconstituted. It is possible, as proposed by Dolan *et al.* (1986), that the microtubule attachment sites migrate in the membrane when not connected to microtubules, and thus lose their ability to form the highly ordered membrane cytoskeleton. However, the assembly characteristics of the tubulin may be influencing the reconstruction, and it is now possible to test this idea. Our purified trypanosome tubulin is behaving as one would expect for a non-neural tubulin, that is, it assembles readily on addition of GTP and does not require MAP or other components to induce assembly at low tubulin concentrations (reviewed by Rafiee *et al.*, 1986). As such, the tubulin may be more able to participate in formation of the membrane cytoskeleton. It is necessary to remember, when comparing our results with those of Dolan *et al.* (1986), that they were working with pellicular tubulin, whereas we prepared tubulin from homogenates of the whole organism. Most evidence shows, however, that the various tubulin-containing compartments of the cell share similar tubulins, at least as far as post-translationally modified varieties are concerned (Gull *et al.*, 1986; Schneider *et al.*, 1987; Sherwin *et al.*, 1987; Sasse & Gull, 1988), and would probably have similar assembly characteristics.

The purified trypanosome tubulin can be assembled into structurally normal microtubules. Thus the formation of ribbon-like structures demonstrated by others (Russell *et al.*, 1984; Bramblett *et al.*, 1987) is probably not inherent in the structure of tubulin itself, as has been suggested for subpellicular tubulin from *Crithidia* (Bramblett *et al.*, 1987). Inclusion of glycerol in assembly reactions did tend to disrupt microtubule structure, although intact microtubules were also formed. Structural abnormalities were introduced into microtubules upon assembly in the presence of colcemid, and assembly was completely disrupted by maytansine and vinblastine. We are not aware of any other published results concerning the effect of microtubule-reactive drugs on purified trypanosome tubulin.

Reconstitution of the trypanosome membrane–cytoskeleton complex and complete analysis of trypanosome tubulin have been hindered by the lack of purified tubulin which has the ability to assemble into structurally normal microtubules. We demonstrate that this problem has now been overcome while providing initial characterization of purified trypanosome tubulin.

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