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Are Cytoplasmic Microtubules Heteropolymers?

(chick embryo brain/subunits/amino-acid composition)

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ABSTRACT **Colchicine-binding protein, considered to be microtubule protein, was purified from chick embryo brain by column chromatography in one step on DEAE-Sephadex. The active colchicine-binding unit is a dimer, MW 115,000 ± 5000, which is composed of two nonidentical monomeric units. The two subunits are separable by urea-acrylamide gel electrophoresis after they have been reduced and acetylated. Sodium dodecyl sulfate–acrylamide gel electrophoresis indicates that the subunits both have molecular weights of 55,000 ± 2000. The amino-acid compositions of the two subunits showed statistically significant differences in six amino-acid residues. These results indicate that colchicine-sensitive cytoplasmic microtubules are heteropolymers.**

Extensive research during the past few years has indicated that colchicine, a potent antimitotic agent, interacts with the subunits of "labile" microtubules and prevents their normal assembly (1–14). Because of the high affinity of colchicine for microtubule proteins, it has been possible to isolate these proteins from a number of sources by the use of radioactive colchicine. The most extensively studied protein, obtained from adult mammalian brain (13), is a dimer composed of subunits of MW 55,000–60,000, with an amino-acid composition similar to that of the flagellar microtubules of sea-urchin sperm tails (15, 16). The subunits of the colchicine-binding dimer have been assumed to be identical (13, 15), and identity with "stable" flagellar microtubules has been strongly suggested (1, 13). However, Stephens has presented evidence (17) that the microtubules comprising the flagellar outer fibers are composed of at least two subunit components (Tubulin A and Tubulin B), which he identifies with subfibers A and B. A recent report by Witman (18) indicates that perhaps more than two components are present, and their distribution between fibers A and B is more complex. These data raise two questions: are the subunits of the colchicine-binding dimer identical, as has been previously indicated, and if so, do they correspond to one of the stable flagellar tubule subunits?

In this report, we describe the purification of a colchicine-binding microtubule protein from chick embryo brain. The protein is a dimer consisting of two electrophoretically separable subunits of MW 55,000. Our results indicate that the two monomeric polypeptide chains are distinctly different, and that cytoplasmic microtubules, rather than being polymers containing identical polypeptides, are *heteropolymers*, composed of two different protein chains.

Abbreviation: MW, molecular weight.

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MATERIALS AND METHODS

The purification procedure was modified from that of Weisenberg *et al.* (13). Freshly dissected brains from 14- to 19-day-old chick embryos were homogenized for 30 sec in phosphate–glutamate buffer [20 mM sodium phosphate–100 mM sodium glutamate (pH 6.8)] with a motor-driven Teflon–glass homogenizer. The homogenate was centrifuged at 100,000 × *g* for 60 min, and the supernatant (which usually contained approximately 100 mg of total protein in 6–8 ml) was applied to a 2.5 × 6 cm column of DEAE-Sephadex, which had been previously equilibrated with 100 mM NaCl–20 mM sodium phosphate (pH 6.8). The supernatant was allowed to run into the column, and was followed by an additional 10–20 ml of the equilibrating buffer. The column was next eluted with 45–50 ml of 400 mM NaCl–20 mM sodium phosphate (pH 6.8), which eluted approximately 80–85% of the total supernatant protein from the column. The colchicine-binding (microtubule) protein was eluted with a linear 400–800 mM NaCl gradient [20 mM sodium phosphate (pH 6.8)], with the peak of the protein appearing at approximately 520 mM NaCl. The flow rate of the column was maintained at 40 ml/hr. All steps were performed at 0–4°C. Sodium chloride concentrations were determined with an Aminco-Cotlove chloride titrator (American Instrument Co). The purification procedure was standardized with bound radioactive colchicine, as described in the legend of Fig. 1. Radioactive colchicine was not present in subsequent experiments, except in the determination of the molecular weight of the colchicine–protein complex.

The colchicine-binding activity of the purified protein was determined according to methods described by Wilson (19) and Wilson *et al.* (20). The preparation of [*acetyl*-^{3}H]colchicine (170 Ci/mol) was described previously (21). Protein was determined by absorption at 280 nm or by the method of Lowry *et al.* (22). When necessary, protein solutions were concentrated with an Amicon ultrafiltration device.

The molecular weight of the purified microtubule protein–colchicine complex, obtained in the experiment described in Fig. 1, was determined by the method of Andrews (23) on a 2.5 × 65 cm column of Sephadex G-100. The following protein standards were used: chymotrypsinogen A, MW 25,000; ovalbumin, MW 45,000; serum albumin, MW 68,000; gamma-globulins (human), MW 160,000. Purified protein isolated in the absence of colchicine was reduced and carboxymethylated

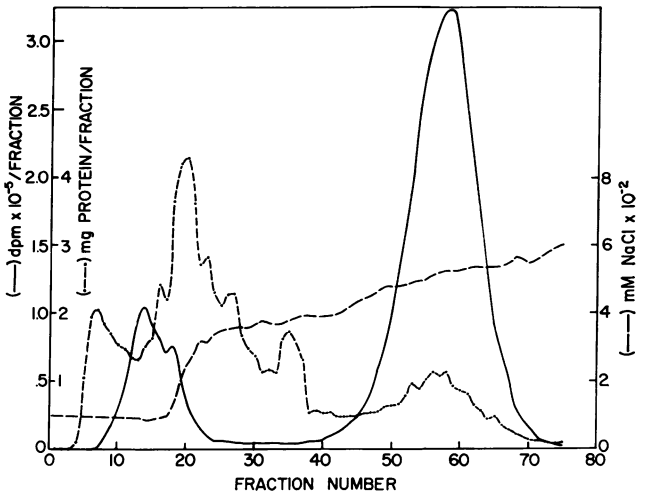

FIG. 1. Purification of microtubule protein from chick-embryo brain. A 100,000 × *g* supernatant brain extract, 6.5 ml, containing 99.5 mg of total protein, was incubated with 2.5 μM [*acetyl*-^{3}H]colchicine for 2 hr at 37°C; then 6.0 ml of this mixture, containing 91.8 mg of protein, was applied to the column. 2-ml fractions were collected. The NaCl concentration was changed from 100 to 400 mM at fraction 8, and the 400–800 mM NaCl gradient was begun at fraction 28 (mixing-chamber volume, 125 ml). The labeled colchicine that appeared in the peak between fractions 40 and 70 was bound to microtubule protein, as determined by methods described elsewhere (19, 20). The free colchicine peak appeared between fractions 10 and 20. *Solid line*, radioactivity; *closed circles*, protein; *dashed line*, NaCl concentration.

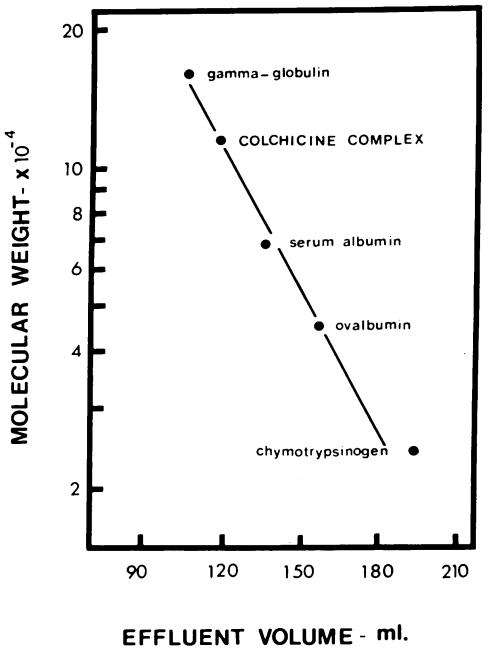

FIG. 2. Molecular-weight determination of purified protein-colchicine complex. Fractions from several areas of the peak of radioactive protein–colchicine complex (Fig. 1) were pooled and filtered through a calibrated Sephadex G-100 column. The labeled complex appeared as a single sharp peak at an effluent volume corresponding to a molecular weight of approximately 115,000. The recovery of labeled colchicine still bound to protein was 70%.

(24) and subjected to urea–acrylamide gel electrophoresis (25) or sodium dodecyl sulfate–acrylamide gel electrophoresis (26).

Molecular weights of the reduced and carboxymethylated subunits were determined by coelectrophoresis with reduced and carboxymethylated standards (bovine serum albumin, actin, and β-lactoglobulin) and by comparison with these and other standards (DNase I and II). Gels were stained in 0.025% Coomassie Brilliant Blue prepared in 50% aqueous methanol–10% acetic acid, and destained in 5% methanol–7.5% acetic acid. Gels were scanned with a Joyce–Loebl densitometer.

Reduced and carboxymethylated protein subunits were separated by urea–acrylamide gel electrophoresis (25) with 2.5 × 10 cm gels. The protein bands, made visible after rapid fixation with cold 10% perchloric acid, were cut out of the gels, macerated, neutralized by dialysis vs. 50 mM Tris·HCl (pH 8.0), and centrifuged to remove the acrylamide fragments. Solutions of the subunits were concentrated, dialyzed against distilled water, and dried by lyophilization. Aliquots of each subunit were resubmitted to gel electrophoresis as a final check for purity. The amino-acid compositions of the reduced and carboxymethylated subunits were determined on a Beckman Model 120 amino-acid analyzer.

RESULTS

A typical purification of microtubule protein from chick-embryo brain is illustrated in Fig. 1. The high concentration of colchicine-binding protein in this embryonic tissue made ammonium sulfate fractionation unnecessary, and permitted a single-step purification. In several experiments the percentage of microtubule protein in total protein (based on the amount of protein under the bound colchicine peak and the total protein in the 100,000 × *g* supernatant) varied from 10 to 20%. Some of this variability is due to the homogenization procedure, in which the release of microtubule protein into the soluble fraction is highly variable, and to the use of embryos in different stages of development.

The colchicine–protein complex was eluted from DEAE-Sephadex as a single peak (Fig 1); purification of the protein was approximately 5-fold. When the complex was chromatographed on Sephadex G-100 by the procedures of Andrews (23), it was eluted as a single sharp symmetrical peak with an apparent molecular weight of 115,000 ± 5000 (Fig. 2). A similar molecular weight has been obtained (19) for the colchicine complex in crude supernatant extracts.

The colchicine-binding properties of the purified protein have not yet been investigated in detail, but they seem on preliminary examination to be similar to those in crude extracts (19). The addition of GTP (1 mM) or several active vinca alkaloids (unpublished results) stabilizes the colchicine-binding activity of the protein, and binding activity is prevented or destroyed by podophyllotoxin and copper(II) (27). Approximately one colchicine molecule binds to two purified dimer units at a colchicine concentration of 2.4 μM; no attempt has been made to saturate the binding site. This value was obtained by determination of the initial binding activity of the protein by the time-decay assay procedure described previously (19). The half-life of the colchicine-binding activity in phosphate–glutamate buffer, pH 6.8, in the absence of any added stabilizing agents, is approximately the same as in crude extracts (4 hr at 37°C). The colchicine-binding activity of purified protein is considerably less stable at high salt con-

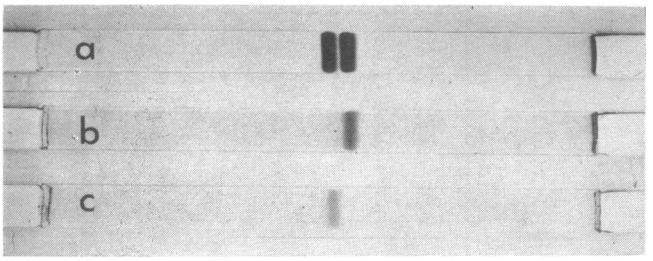

FIG. 3. Urea–acrylamide gel electrophoresis. *a*, Reduced and carboxymethylated subunits; *b*, purified upper component, α; and *c*, purified lower component, β. Conditions: 5% acrylamide–0.2% bisacrylamide gels in 8 M urea; running buffer, 5 mM Tris–glycine (pH 8.0). Stained with Coomassie blue.

centration (above 200 mM NaCl) than is the activity of the protein in crude supernatant brain extracts.

The proteins in the colchicine-binding fraction were concentrated by ultrafiltration, reduced and carboxymethylated, and subjected to electrophoresis in 8 M urea–acrylamide gels. Fig. 3*a* illustrates the usual pattern observed. Two closely-spaced major bands were always found; several variable slower-moving components and one faster-moving component were sometimes observed in different preparations. The densitometer scans of the gels revealed that the contaminating components never represented more than 1–2% of the total protein. The gel scans (Fig. 4) also showed that the ratio of stain in the bands in a number of experiments varied from 1.1 to 1.3 (upper/lower = α/β). This was not a function of the dye used, since similar results were obtained with Fast Green and Amido Black.

Several experiments were done in an attempt to alter the ratio of α/β; these included (*a*) altering the extent of inactivation of colchicine-binding activity by prior incubation for various periods of time before reduction and carboxymethylation; (*b*) subfractionation of the active colchicine-binding protein peak, in an attempt to show some separation of possible "homo-dimers"; and (*c*) variation in the amounts of protein applied to the gels. No significant perturbations in the ratio of α/β were observed. There was no correlation between extent of inactivation of colchicine-binding activity and ratio of the subunits. Similarly, no separation or preferential segregation of possible homodimers was observed on DEAE-Sephadex chromatography.

In addition, the subunits were applied to urea–acrylamide gels of increasing acrylamide concentrations (28). Plots of the

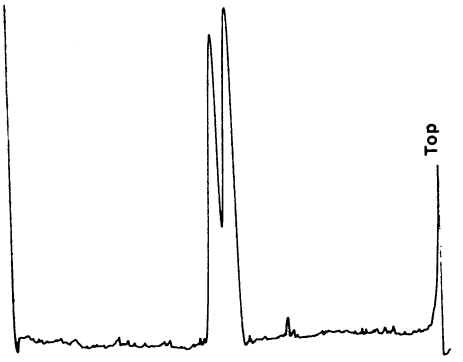

FIG. 4. Densitometer scan of gel stains in Fig. 3*a*. The ratio of α/β for this experiment was 1.25–1.30.

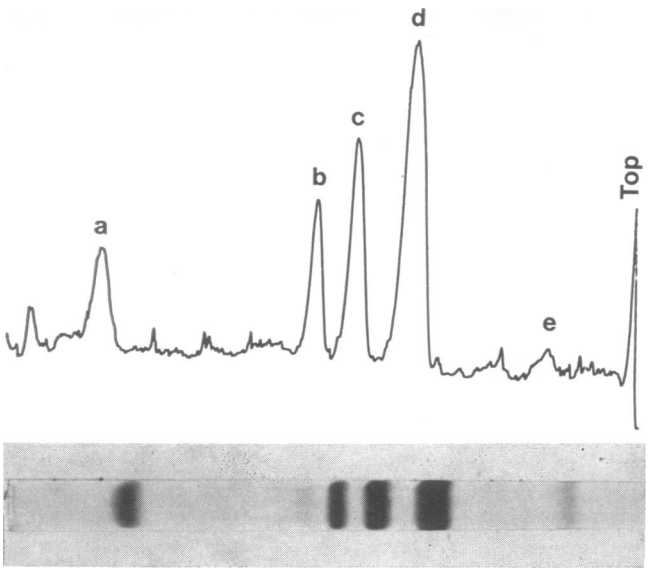

FIG. 5. Sodium dodecyl sulfate–acrylamide gel electrophoresis of reduced and carboxymethylated purified colchicine-binding proteins and reduced and carboxymethylated standards. *a*, β-lactoglobulin; *b*, actin; *c*, microtubule subunits; *d*, bovine serum albumin; *e*, bovine serum albumin dimer. 5% acrylamide–0.2% bisacrylamide gels, 0.1% sodium dodecyl sulfate in 0.1 M sodium phosphate buffer (pH 7.0).

logarithm of relative mobility against gel concentration gave parallel lines, which indicates that the subunits are charge isomers, not size isomers. This tends to rule out the possibility that the subunits were incompletely dissociated in 8 M urea and were perhaps in a monomer–dimer equilibrium. The fact that the separated subunits, electrophoresed again under similar conditions, each behaved as a single species rules out simpler electrophoresis artifacts.

The molecular weights of the two subunits were determined by sodium dodecyl sulfate–gel electrophoresis. Fig. 5 illustrates a typical gel and its corresponding densitometer scan. The microtubule proteins run as a single component (labeled *c*) with an apparent molecular weight of 55,000 ± 2000, as

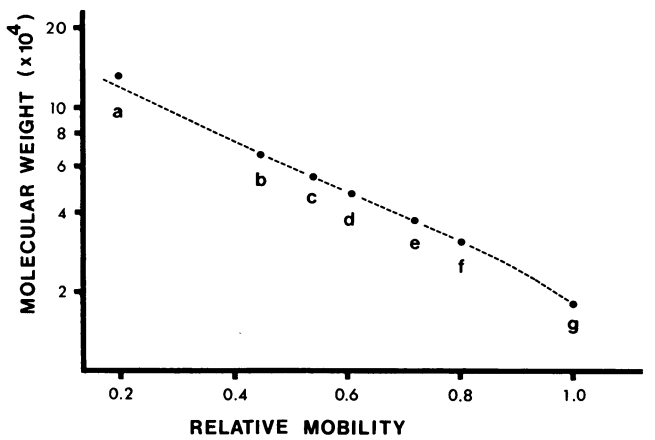

FIG. 6. Semilogarithmic plot of molecular weight against relative mobility on sodium dodecyl sulfate gels of reduced and carboxymethylated proteins. *a*, Bovine serum albumin dimer; *b*, bovine serum albumin; *c*, microtubule subunits; *d*, actin; *e*, DNase II; *f*, DNase I; and *g*, β-lactoglobulin. Conditions as for Fig. 5.

determined from a plot of log of molecular weight vs. mobility (Fig. 6). The molecular weight has also been determined on 10% acrylamide gels with the same results. The molecular weight of the subunits is just half that of the active dimer. The finding of a single protein band on the sodium dodecyl sulfate gels indicates that the subunits differ in charge, but not in size.

The microtubule subunits were separated on preparative urea–acrylamide gels; Fig. 3, *b* and *c*, illustrates the subsequent electrophoresis to establish purity. The amino-acid compositions of each subunit (Table 1) result from several determinations on each subunit. Timed acid hydrolyses were performed to allow accurate estimation of serine and threonine contents. Statistically significant differences were found in His, CM-Cys, Ser, Ala, Met, and Ile. Of these amino acids, only CM-Cys would impart a significant charge difference at the pH used for electrophoresis (pH 8.0). Carbamidomethylation of the subunits, which blocks the sulfhydryl residues with neutral groups, lowers the mobility, but two components are still detectable. This indicates that the carboxymethyl derivatives are not the only residues contributing to the charge separation.

The stoichiometry of the subunits was examined by carboxymethylation of the cysteine residues of the polypeptide chains with [^{3}H]iodoacetic acid. The amount of tritium in each polypeptide was determined after separation of the chains by urea–acrylamide gel electrophoresis. A ratio of α/β of 1.21 ± 0.10 was obtained (data from 10 gels). This ratio is in good agreement with the expected theoretical ratio of 1.25 (α/β), which is based upon the amino-acid composition of the two chains, and assumes a 1:1 stoichiometry of the subunits.

TABLE 1. *Amino-acid compositions of reduced and carboxymethylated microtubule subunits*

	Mol %		Residues/55,000	
	α	β	α	β
Lys	3.9 (±0.2)	3.8 (±0.3)	16 (±0.8)	16 (±1.0)
His	2.6 (±0.1)	2.2 (±0.1)	11 (±0.6)	9 (±0.4)
Arg	4.7 (±0.3)	4.7 (±0.2)	20 (±1.4)	20 (±0.8)
CM-Cys	2.4 (±0.2)	1.9 (±0.1)	10 (±0.8)	8 (±0.4)
Asp	10.3 (±0.4)*	11.1 (±0.8)*	43 (±1.5)	46 (±3.5)
Thr	6.8 (±0.2)	6.6 (±0.2)	28 (±0.7)	28 (±0.7)
Ser	6.2 (±0.1)	7.3 (±0.2)	26 (±0.4)	31 (±0.8)
Glu	13.5 (±0.4)	12.7 (±2.2)	56 (±1.7)	53 (±9.0)
Pro	4.7 (±0.5)	4.7 (±0.6)	20 (±2.0)	20 (±2.5)
Gly	8.9 (±0.3)	9.1 (±0.3)*	37 (±1.4)	38 (±1.1)
Ala	7.6 (±0.3)	6.9 (±0.2)	32 (±1.1)	29 (±1.0)
Val	7.9 (±0.3)	7.4 (±0.2)	33 (±1.5)	31 (±0.8)
Met	1.6 (±0.2)	2.5 (±0.3)*	7 (±0.8)	10 (±1.2)
Ile	5.3 (±0.1)	4.4 (±0.1)	22 (±0.6)	18 (±0.6)
Leu	7.2 (±0.3)	7.5 (±0.2)	30 (±1.3)	31 (±0.9)
Tyr	3.6 (±0.2)	3.5 (±0.3)	15 (±0.8)	15 (±1.4)
Phe	4.5 (±0.3)	4.8 (±0.1)	19 (±1.0)	20 (±0.5)

* 4 determinations.

Duplicate samples of each reduced and carboxymethylated subunit were hydrolyzed for 24, 48, and 72 hr by standard procedures. The percent destruction with time of serine and threonine was calculated, and extrapolation to zero-time hydrolysis allowed estimation of serine and threonine content. Values in parentheses represent the variation at the 95% confidence level as calculated by Student's *t* test (29) (data from 5 or 6 determinations on each chain).

DISCUSSION

The results demonstrate that colchicine-binding protein can be rapidly isolated and purified from chick embryonic brain, essentially in a single step. The active colchicine-binding protein isolated with this procedure is a dimeric unit of molecular weight, 115,000 (±5000), with an amino-acid composition similar to that found for other microtubule proteins. The subunits of the dimer differ both in amino-acid composition and in electrophoretic mobility. In addition, the reduced protein from chick embryo brain and from the colchicine-binding protein isolated from vinblastine-induced crystals (from *Strongylocentrotus purpuratus* eggs) are each separable into two components, which migrate together in acidic gel systems and under isoelectric focusing (to be published) and which seem to be similar in size (MW 55,000 in several sodium dodecyl sulfate–gel systems).

These findings suggest that the "labile" microtubules are composed of at least two subunits that are charge isomers. Which amino-acid residues contribute to the charge difference is not clear. Amino-acid compositions of the separate subunits revealed considerable similarities among the charged amino acids, except for the blocked cysteine residues. The possibility that the charge difference is due to some unknown prosthetic group, to the presence of an unequal number of glutamine and (or) asparagine residues, or the occurrence of phosphoryl or charged sugar residues cannot be ruled out.

There is evidence for heterogeneity of microtubule subunits from other sources, in particular from the "stable" microtubules (i.e., those insensitive to colchicine, low temperature, high pressure) in flagella and cilia. In Tetrahymena, Everhart (25) found that similar doublets could be extracted from acetone powders of whole cilia and ciliary outer fibers. Similarly, Stephens (17) has shown differences in the "fingerprints" and amino-acid compositions of thermally fractionated outer fibers of sea-urchin sperm tails. These differences have been interpreted in terms of a protein from subfiber A (Tubulin A) and a different protein from subfiber B (Tubulin B). Some preliminary work in this laboratory with sea urchins and a report (18) on *Chlamydomonas* ciliary proteins indicate, however, that the situation is more complex, since thermally fractionated preparations of Tubulin A and Tubulin B can be shown to contain at least two separable proteins.

The relationship of the multiple "stable" microtubule subunits to the "labile" microtubule subunits is unclear; a comparison (unpublished) by urea–gel electrophoresis between the "stable" and "labile" tubules (within a single species and between several different species) indicates that the subunits are electrophoretically indistinguishable. This suggests, first, that the chemical differences between "stable" and "labile" tubule subunits are probably slight, and second, that in evolutionary terms, there appears to be a marked similarity among the tubule proteins—in particular, there has been a strong conservation of both the size and charge properties of the subunits.

How are the subunits associated? Although the inability to fractionate the active dimer, and the finding of 1:1 stoichiometry, suggests that the dimer is a heterodimer ($\alpha\beta$), and consequently that tubules are heteropolymers, the possibility that there are two classes of homodimers ($\alpha\alpha$, $\beta\beta$) and consequently homotubules has not been rigorously excluded. A more thorough investigation (to be published) of several

other colchicine-binding proteins in another organism under different physiological conditions indicates, however, that the $\alpha:\beta$ ratio (as determined by dye binding) is invariant and identical with that found in chick brain. This reinforces the idea that the ratio of α/β is fixed. In addition, the heterodimer model provides a convenient rationale for the available data on nucleotide and colchicine binding. One subunit may have a higher affinity for nucleotide than the other, and only one of the subunits may possess a binding site for colchicine. Alternatively, both subunits could be required to form the colchicine-binding site. The heterodimer model, however, places certain restrictions on the arrangement of subunits in a microtubule. If we assume a protofilament arrangement of monomeric subunits (15) in a microtubule, it becomes apparent that a *homofilament* microtubule can be constructed only if the number of protofilaments is *even* (i.e., 12 or 14 in the usual model) while a *heterofilament* microtubule *always* results if an *odd* number (11 or 13) of protofilaments are assembled.

A final unanswered question concerns the function of the subunits. Their similarity precludes the possibility that they serve widely divergent functions, but the apparent invariance of the $\alpha:\beta$ ratio indicates that a simple isozyme relationship, in which subunits are interchangeable, is unlikely.

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