

HETEROGENEITY OF THE ALPHA SUBUNIT OF TUBULIN AND THE VARIABILITY OF TUBULIN WITHIN A SINGLE ORGANISM

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ABSTRACT

When tubulins obtained from particular microtubules of the sea urchin (ciliary doublet A tubules, flagellar doublet microtubules, and mitotic microtubules) are analyzed by electrophoresis in a polyacrylamide gel system containing sodium dodecyl sulfate and urea, heterogeneity of the alpha subunit, and differences between the tubulins are revealed. The alpha subunit of tubulin from mitotic apparatus and from A microtubules of ciliary doublets is resolved into two bands, while the alpha subunit of flagellar doublet tubulin gives a single band. The mitotic and ciliary tubulins differ in the mobilities of their two alpha species, or in the relative amounts present, or both. The existence of differences between the tubulins has been confirmed by a preliminary analysis of their cyanogen bromide peptides.

Tubulin, the constituent molecule of microtubules, is dimeric in saline solutions. It has a molecular weight of approximately 110,000, but dissociates under denaturing conditions into polypeptide chains of molecular weight near 55,000 (see, e.g., reference 8). Two nonidentical chains, commonly termed the α - and β -chains, have been resolved in polyacrylamide gel electrophoresis. Available evidence supports the interpretation that each tubulin molecule is a heterodimer containing both chains. The chains are usually found in equimolar amounts, regardless of the source of tubulin (7, 9, 15, 16, 30), and the few contrary reports (22, 39) may result from failure of quantitation in polyacrylamide gel electrophoresis (6). When undissociated tubulin is exposed to cross-linking reagents, the cross-linked heterodimer $\alpha\beta$ is formed preferentially (28). Based on this evidence, a single heterodimeric species of tubulin molecule would account for the polypeptide chains hereto-

fore resolved in polyacrylamide gel electrophoresis.

There is, however, evidence that tubulin is heterogeneous. Stephens (36), and more recently Safer (34), report differences in the tubulins making up the A and B tubules of flagellar doublets; these tubulins were at first identified, respectively, with the α - and β -chain (36), but this view is no longer held (15, 34, 39). Isoelectric focusing of tubulin from doublet microtubules (39), and also from cells which contain no doublets (15), may yield four or five bands under dissociating conditions. These observations suggest a greater heterogeneity in tubulin than has been resolved in polyacrylamide gel electrophoresis.

The question of tubulin heterogeneity includes a question with important functional implications: are tubulins from different microtubule systems within a single organism identical? Surprisingly little precise information exists on this point.

Stability differences between microtubules have been interpreted both as indicating (3) and as not indicating (38) differences in their constituent tubulins. Strong similarities between tubulins from all animal sources are well documented (e.g., 11, 27); tubulin appears to be a highly conserved molecule, and this fact can perhaps be taken to suggest that tubulins within a single organism are identical. However, available data, though sparse, have not supported this view. Both Fulton et al. (17) and we (5) have compared tubulins from different microtubule systems of the sea urchin by immunochemical means. In each case, cross-reaction with antibody was obtained, but clear *quantitative* differences in the reaction were also found. Safer's preliminary report (34) indicates that peptide differences exist between ciliary and flagellar tubulins in the lamellibranch molluscs.

We will here describe a polyacrylamide gel electrophoresis system which resolves the α -chain of sea urchin mitotic apparatus and ciliary doublet A microtubule tubulins into two species, which we will call α_1 and α_2 . When this level of resolution is applied to a comparison of tubulins from different microtubule systems of the sea urchin, differences are revealed. Both tubulin heterogeneity and differences in tubulins from different microtubules are thus demonstrable at the level of electrophoresis of the dissociated subunits in polyacrylamide gels.

MATERIALS AND METHODS

Preparation of Microtubules and Tubulins

The sea urchins used were *Strongylocentrotus purpuratus* from Pacific Bio-Marine Supply Co., Venice, California. Gametes and embryos were handled at 15°C unless specified otherwise. Mitotic apparatus was isolated at the first cleavage division by the method of Kane (23), as described previously (5). Sperm flagella were cut from sperm at 4°C in a blender, in isotonic citrate medium at pH 6.5 (4), and collected by centrifugation. Outer doublet microtubules were obtained from flagella by a modification (6) of the method of Stephens et al. (37). Cilia were obtained by the method of Auclair and Siegel (2) from embryos between hatching and gastrula stages. Eggs were fertilized, washed, and allowed to develop to hatched blastulae in Millipore-filtered artificial seawater containing penicillin (0.25 mg/ml) and streptomycin (0.25 mg/ml). Cilia were detached by suspending the embryos in seawater containing an additional 3% sodium chloride. After 1 min, the medium was restored to isotonicity by the addition of 50% seawater, with stirring. Embryos were collected by centrifugation,

resuspended in seawater with antibiotics, and used for further cycles of harvesting after regeneration of cilia. The supernate containing detached cilia was cooled over ice, then handled at 4°C. It was centrifuged for 6 min at 600 g to remove cell debris, then centrifuged for 30 min at 9,250 rpm in a Sorvall GSA rotor (DuPont Instruments, Sorvall Operations, Newtown, Conn.) to collect cilia. Cilia were then treated by a procedure identical to that used to obtain outer doublet microtubules from sperm flagella. Electron microscopy of the ciliary microtubule preparations, carried out after negative staining with 1% uranyl acetate, showed that the preparation consisted of singlet microtubules, some of which remained in axonemal groupings. Linck (25) observed a selective loss of the B tubule during isolation of doublet microtubules from gill cilia, but not sperm flagella, of *Aequipecten irradians*. From this result, and also because the A tubule is a complete microtubule and more stable than the B (3, 36), we infer that the preparation consists of A tubules. One tubule from each central pair may also be present (25).

Tubulin was obtained from mitotic apparatus and ciliary and flagellar microtubules by extraction with organic mercurial (5). In the case of mitotic apparatus, the extraction medium was 0.02 M *p*-chloromercuriphenylsulfonic acid (Sigma Chemical Co., St. Louis, Mo.) in 0.01 M phosphate buffer, pH 6.4, which was added 1:1 to packed mitotic apparatus in isolation medium. In the case of ciliary and flagellar microtubules, the extraction medium was 0.02 M *p*-chloromercuriphenylsulfonic acid in 0.01 M borate buffer, pH 9.0, which was added 4:1 to pellets of microtubules. Extraction was allowed to proceed for 1 h, and solubilized tubulin was recovered as described previously (5) as the supernate of high-speed centrifugation.

To avoid the gradual aggregation of tubulin, mercurial extracts were dialyzed against freshly prepared 8 M urea overnight at room temperature, and reduced and carboxymethylated according to Crestfield et al. (10). The preparations were then dialyzed in the dark against freshly prepared 8 M urea. If required, the material was stored in 8 M urea at -20°C, followed by dialysis into fresh medium. Protein determinations were carried out by the method of Lowry et al. (26).

Repurification of Tubulin on Diethylaminoethyl Cellulose (DEAE-Cellulose)

Reduced and alkylated preparations of tubulin (1-5 mg) were applied in 8 M urea + ammonium chloride-ammonia buffer, 0.08 M in ammonium chloride, pH 9.3, to a 0.9 × 15 cm column of Whatman DE-52 preequilibrated with the same medium, and were eluted with increasing concentrations of the same buffer in 8 M urea. The buffer used was chosen to minimize deamination of lysine residues by cyanate released from urea (35); for the same reason, only freshly prepared urea solutions were

used. Tubulin elutes from the column as a single peak at approx. 0.24 M ammonium chloride. The peak fractions were pooled, and tubulin was precipitated by the addition of 7 vol of ethanol, recovered after several hours of incubation by centrifugation (no detectable protein remained in the supernate) and redissolved in 8 M urea.

Polyacrylamide Gel Electrophoresis

The following system, which contains SDS and urea, was used to resolve the α -chain of tubulin into two components. Monomers, riboflavin, and *N,N,N',N'*-tetramethyl-ethylenediamine (TEMED), were obtained from Eastman Kodak Co., Rochester, N. Y. The resolving gel contained 5% (wt/vol) acrylamide (electrophoresis grade), 0.165% *N,N'*-methylenebisacrylamide (6.3% acrylamide, 0.2% bisacrylamide were also used in some cases), 8 M urea (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y., ultrapure), 0.1% SDS, 0.07% ammonium persulfate, 0.029% (vol/vol) TEMED, and buffer containing, per 400 ml, 18.15 g Tris (primary standard, Fisher Scientific Co., Pittsburgh, Pa), and 19.8 ml standardized 1 N HCl (Fisher).

Spacer and sample gels, identical in composition except for the presence of sample, contained 2% (wt/vol) acrylamide, 0.5% bisacrylamide, 8 M urea, 0.1% sodium dodecyl sulfate (SDS), 0.0005% riboflavin, and buffer containing, per 400 ml, 2.498 g Tris (primary standard), and 19.8 ml of 1 N HCl (standardized). TEMED was omitted, as the small volumes required were difficult to measure accurately and it tended to raise the pH.

A note on the pH of the gels is in order. Our pH meter, equipped with a Fisher 13-639-3 glass electrode, consistently gives pH readings for Tris buffers in 8 M urea (at 24°C) that are 0.6 U higher than values calculated from $pK = 8.1$ for Tris. That is, the meter readings indicate an apparent $pK = 8.7$ for Tris under these conditions. This discrepancy in pK depends on the presence of urea, since in the absence of urea the meter readings are within 0.1 pH U of values calculated from $pK = 8.1$. The pH indicator dye bromothymol blue (Fisher), added as a 0.001% solution to pH 6.7 Tris buffer, fails to confirm the discrepancy: its absorption spectrum is almost identical whether or not the buffer contains 8 M urea. Since these results show that indirect measurements of pH are unreliable in this system, we have specified our buffers by their precise compositions rather than by their measured pH.

Electrode buffers contained 14.4% (wt/vol) glycine, 3% Tris, 0.1% SDS, and 10^{-5} % bromphenol blue. Electrophoresis was carried out in 0.5×14 cm gels at 0.6 mA per gel during stacking and 1.2 mA per gel during resolution. A run time of about $4 \frac{1}{2}$ h allowed migration of the bromphenol blue marker to near the bottom of the gels. Gels were fixed in 5% trichloroacetic acid (TCA), 5% sulfosalicylic acid, stained in 0.1% Coomassie blue in 45% methanol, 10% acetic acid, and destained in 5% methanol, 7.5% acetic acid. Gels were scanned at 565

nm in a GCA/McPherson model EU-701 B recording spectrophotometer equipped with a gel scanner (GCA/McPherson Instrument, Acton, Mass.); the areas under each peak, taken as the areas between verticals dropped from the nearest trough or horizontal point of the scan, were determined by cutting out and weighing the appropriate sector of the scan.

Mapping of Cyanogen Bromide Peptides by Isoelectric Focusing

Tubulin preparations in 8 M urea were diluted to 3.25 mg protein per ml, and 0.3 ml of 1 N HCl in 8 M urea was added per milliliter protein solution (measured final pH = 2.0; final protein concentration, 2.5 mg/ml). Cyanogen bromide, 50 mg per ml of sample, was added, and the mixture was allowed to react at 24°C in the dark for 24 h. A hydrolysis time of 48 h produced no change in the results. Samples were then lyophilized and stored.

Isoelectric focusing was carried out in 0.5×10 -cm polyacrylamide gels formulated according to Righetti and Drysdale (33), except that the gels were made 8 M in urea (29), which we found to be indispensable for reproducibility and good resolution in the peptide maps. Gels were polymerized at 15°C, using carefully temperature-equilibrated solutions; gel mixtures were deaerated for 90 s before adding initiators, and for 30 s more after their addition. The gel mixtures were centrifuged at low speed (130 g) during polymerization in a floor model International centrifuge (International Equipment Co., Needham Heights, Mass.). To achieve this, the gel mixtures were added to tubes, capped at the bottom, which had previously been mounted through a one-hole stopper in centrifuge tubes filled with water; the water prevented extrusion of the gel mixture from the bottom of the gel tube by balancing the pressure. The centrifugation step eliminates a tendency of the peptide bands to form wavy surfaces which complicates comparisons between peptide maps.

Focusing was done in an ordinary polyacrylamide gel electrophoresis apparatus (Model 3-1071, Buchler Instruments, Inc., Fort Lee, New Jersey), which was air-cooled at 4°C. Samples of tubulin peptides previously lyophilized from 8 M urea, reconstituted with water to their volume before lyophilization, were made 0.02 N in sodium hydroxide immediately before use by the addition of sodium hydroxide in 8 M urea, placed on the gels, and run from cathode to anode. Samples of 0.2 ml, containing 0.1–0.2 mg peptides, were used; they were mixed with two drops of glycerol before placing on the gels. The cathode buffer was 0.02 N sodium hydroxide or 0.02 N sodium hydroxide in 8 M urea; the anode buffer was 8 M urea titrated with 17.9 ml of 85% phosphoric acid per liter (measured final pH, 2.5 at 6°C). All urea solutions were freshly made. Electrophoresis was done at 1 mA per gel until the voltage reached 400 V; focusing was then allowed to proceed at 400 V for 8 h (33) and 800 V for 1 h more (29).

Gels were fixed with gentle agitation in four changes of 5% TCA, 60 ml per gel, over a period of 48 h. This step removes the ampholytes. Staining was done to equilibrium, in two changes of 0.002% Coomassie blue in 15% TCA, 60 ml per gel, over a period of 24 h. The stain was freshly made, by dilution in 15% TCA of a 2% stock solution of Coomassie blue in methanol. Destaining was not required. The gels were stored in 0.0002% Coomassie blue in 7% acetic acid.

NH₂-Terminal Amino Acid Determination

NH₂-terminal amino acid determination was carried out on DEAE-purified tubulin according to Gray (18). Dansyl amino acids were identified by chromatography on polyamide layers.

RESULTS

Heterogeneity of Mitotic α -Tubulin

We have previously described a method of obtaining tubulin from the microtubules of sea urchin mitotic apparatus by extraction of the isolated apparatus with organic mercurial (5). Tubulin obtained in this way was reduced and alkylated (the mercurial is presumably removed in this step), and analyzed by electrophoresis in the SDS-urea gel system described (see Materials and Methods). Electrophoresis in this system resolves the α -band of mitotic tubulin into two bands (Fig. 1 *a*); we call the slower migrating band the α_1 band and the faster migrating one the α_2 band.

Repurification of the tubulin preparation by salt gradient elution from DEAE-cellulose in the presence of urea (Fig. 2) produces no change in the electrophoretic pattern. As a further check on the purity of the tubulin, an end-group analysis was performed. The only detectable NH₂-terminal amino acid is methionine, as is the case with tubulin from sea urchin sperm flagellar doublet microtubules (27, confirmed by us in this work) and chick and mammalian brain (13, 24, 27).

In our earliest results, resolution of α_1 - and α_2 -bands in polyacrylamide gels containing SDS and urea occurred only occasionally; it has become reproducible with modification and standardization of the technique, and appears to depend sensitively on the details of the gel system used. Resolution of α_1 - and α_2 -bands does *not* occur in a comparable gel system (6) containing only urea (Fig. 1 *b*), or in the SDS-containing system of Yang and Criddle (40), which was used by Lu-

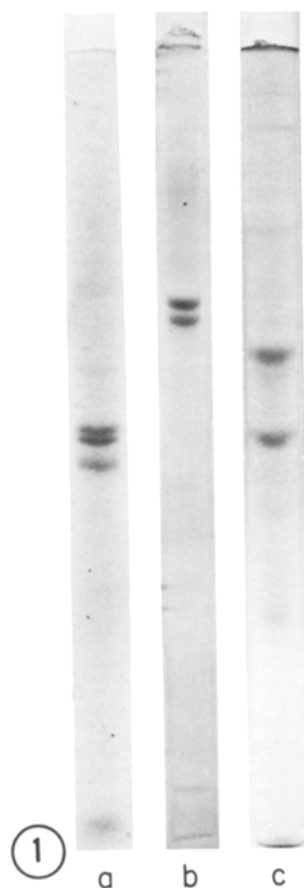


FIGURE 1 Electrophoresis of a single preparation of tubulin from sea urchin mitotic apparatus in three different polyacrylamide gel systems. (*a*) SDS-urea system, (*b*) urea system, (*c*) SDS-system (for details of gel system, see text). In the SDS-urea system, α -tubulin is resolved into two bands. The tubulin preparation used was an unrepurified mercurial extract, and the gels reflect the degree of purity of such a preparation. The loading for (*a*) was 10 μ g protein containing an estimated 5–7 μ g of tubulin. The loadings for (*b*) and (*c*) were 20 μ g and 10 μ g, respectively.

duena and Woodward (27) to give an excellent separation of α - and β -chains (Fig. 1 *c*).

Densitometric quantitation of SDS-urea gels stained with Coomassie blue shows that at loadings of DEAE-purified tubulin above 15 μ g the *combined* material in the α_1 - and α_2 -bands is approximately equal in amount to that in the β -band. In other words, the characteristic ratio of α - to β -tubulin is obtained, provided both α_1 - and α_2 -bands are taken to represent α -tubulin. At considerably lower loadings (Figs. 1 *a* and 4) the

combined α -species are present in considerable apparent excess over β -tubulin, but this also is characteristic of α - and β -tubulin, as we have previously shown (6). On gel systems which do not resolve α_1 - and α_2 -tubulin, α - and β -tubulin appear at sufficiently high loadings to be present in nearly equal amounts (Table I).

We have also determined the ratio of α_1 - to α_2 -tubulin; in mitotic tubulin, the α_1 - and α_2 -species appear to the eye to be present in exactly equal

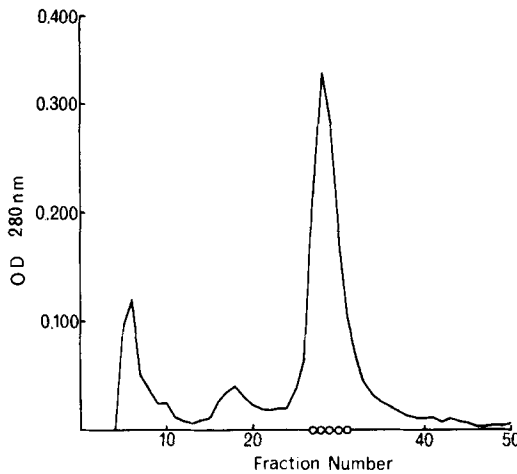


FIGURE 2 Repurification of a mercurial extract of tubulin by salt gradient elution from DEAE-cellulose in the presence of urea, using increasing concentrations of an ammonium chloride-ammonia buffer for elution. Open circles indicate the fractions pooled for the repurified preparation. Mercurial extracts of flagellar doublet microtubules (shown) and mitotic apparatus give essentially similar elution profiles. Polyacrylamide gel electrophoresis of the repurified tubulin preparations, at loadings of up to 40 μ g, shows no detectable impurities.

amount; densitometry confirms this impression within the accuracy to be expected in view of the close spacing of the tubulin bands (Table I). In contrast to α - and β -tubulins, the apparent equality of α_1 - and α_2 -tubulins is not restricted to high loadings of tubulin, but persists at all loadings tested.

To further analyze the relationship between tubulin chains, a Ferguson plot according to Hendrick and Smith (20) was constructed (Fig. 3). Both α_1 - and α_2 -tubulins give straight lines on this plot. Each species therefore behaves, in gels of different pore size, like an entity with a definite size and electrophoretic mobility. The lines are closely parallel to each other and to the line given by β -tubulin. This is taken to show that the migrating species differ in charge rather than size (20), but it should be borne in mind that the migrating species in this case are mixed micelles of protein and SDS, and that neither gels containing urea alone nor gels containing SDS alone resolve α_1 - and α_2 -bands (see Discussion).

Comparison of Tubulins

Tubulins from widely disparate sources have heretofore been reported to give identical patterns in polyacrylamide gels. The one reported exception has been in the case of the β -chains of tubulins from neuroblastoma cells and from *Chlamydomonas* flagella (31). We have applied the level of resolution afforded by the SDS-urea system to a comparison of tubulins from three species of microtubule of the sea urchin: mitotic microtubules, flagellar doublet microtubules from sperm, and the A microtubule of the ciliary doublets from the hatched embryo. Each tubulin was ob-

TABLE I
Relative Amounts of Tubulin Subunits in Mitotic Tubulin, Determined by Densitometry after Coomassie Blue Staining

Gel system	Amount of α_1 (%)	Amount of α_2 (%)	Amount of α (or of $\alpha_1 + \alpha_2$) (%)	Amount of β (%)
SDS	—	—	52.7	47.3
Urea	—	—	46.8	53.2
SDS-urea	30.1	24.8	54.9	45.1

The values listed are averages for 3–5 gels. Amounts are expressed as percentage of total tubulin present. SDS-urea gels were loaded with 15–40 μ g of DEAE-purified tubulin. For details of the gel systems, see text. SDS and urea gels were loaded with 15–25 μ g of mercurial extract.

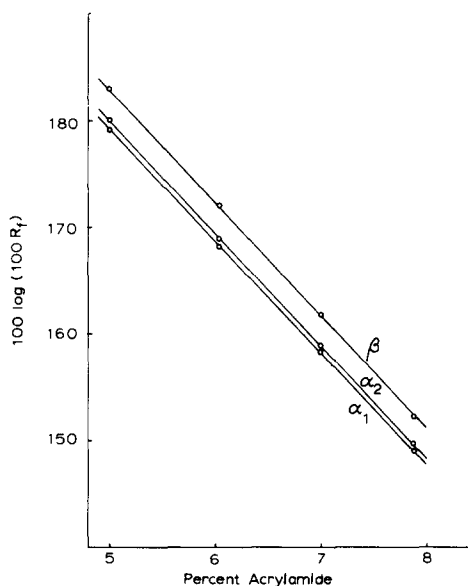


FIGURE 3 Ferguson plot for α_1 -, α_2 -, and β -tubulins from mitotic apparatus (SDS-urea system). R_f denotes the relative mobility of a component with respect to a bromphenol blue marker. The 5% acrylamide system indicated on the abscissa is the system described in Materials and Methods. The higher percentage systems were obtained by increasing the acrylamide concentration to the indicated percentages, increasing the bisacrylamide concentration proportionately, and leaving the system otherwise unaltered.

tained by extraction with mercurial, and reduced and carboxymethylated before electrophoresis. The electrophoretic patterns obtained from these tubulins are different (Fig. 4). In tubulin from sperm flagellar doublet microtubules, α_1 - and α_2 -bands are not resolved. In mixture gels of flagellar doublet and mitotic tubulins, α -tubulin of flagellar doublets migrates with, or close to, α_2 -tubulin from mitotic apparatus. The absence of resolvable α -tubulins is not characteristic of doublet microtubule systems, however, since α_1 - and α_2 -tubulins are resolved in tubulin from the A tubule of ciliary doublets. The absence of the B tubule from the ciliary preparation cannot of course account for the presence of an *extra* band in ciliary tubulin. A microtubule from each central pair may be present in these preparations (25), but we doubt that it could account for the total amount of either of the α -subunits which is present; in particular, it could not account for the presence of the α_1 -subunit, which is clearly different in mobility from flagellar α -tubulin, and is, if anything,

present in excess over the α_2 -subunit. The electrophoretic pattern for the ciliary tubulin is moreover clearly different from that for mitotic tubulin. The exact basis of the difference is difficult to ascertain, because of the low degree of separation of ciliary α -bands. Judging strictly from appearance, the ciliary α -species are closer in mobility than are the α -species of mitotic tubulin, and the ciliary α_1 -species is present in greater amount than the α_2 -species. However, each of these differences alone could cause the appearance of the other. An excess of α_1 -chain would place the α_2 -peak on a steeply rising slope, thereby shifting it toward the α_1 -peak. On the other hand, since the peaks are asymmetrical, with an extended trailing edge, an apparent excess of α_1 -species could result from the location of the α_1 -peak on the trailing edge of a closely spaced α_2 -peak. In Fig. 5, asymmetrical triangles are used as models for the peaks, and a profile similar to that of the α bands of the ciliary tubulin is constructed by the addition of equal peaks. It is hoped that a computer analysis of the scans will help to determine the exact difference between the mitotic and ciliary tubulins.

The difference obtained in the electrophoretic patterns of the ciliary and flagellar tubulins is particularly significant because the procedures used to obtain these tubulins are almost identical. Only the steps at which cilia are detached from embryos, and sperm tail from sperm, differ in that the cutting media are different, and different factors may be released into the medium by embryos and by sperm. We have done an experiment to test the possibility that the electrophoretic difference between the ciliary and flagellar tubulins might arise at this step. In this experiment, the cutting media were made identical, and sperm were exposed to supernatant factors which might have been released by embryos during the detachment of cilia. Cilia were amputated from blastulae (see Materials and Methods) by exposure to hypertonic medium, followed by restoration of the medium to isotonicity. Embryos were centrifuged out of the suspension at 15°C, and cilia collected by centrifugation at ice temperature. Previously undiluted sperm were then resuspended in the supernate from the ciliary procedure. The sperm tails were detached from heads by agitation with a vortex mixer, and the suspension was further diluted with ciliary supernate to a concentration of sperm flagella approximately equal (by microscope examination) to the prior concentration of cilia. The suspension was handled so that the time

of exposure of sperm flagella to ciliary supernate at 15°C and at 0°C was approximately equal to the previous times for cilia. Thereafter, sperm flagella and cilia were handled exactly in parallel, to obtain microtubules and tubulins. The results obtained in this experiment were unaltered: α_1 - and α_2 -bands were resolved in the ciliary tubulin, but not in the flagellar tubulin.

To further confirm the existence of differences between tubulins from mitotic apparatus and flagellar doublets, the DEAE-repurified tubulins were subjected to cyanogen bromide cleavage, and the resulting peptides were compared by isoelectric focusing in polyacrylamide gels containing urea (Fig. 6). Most of the peptides from the two sources are indistinguishable, but the patterns reproducibly include major peptide bands specific to each tubulin. A single sample of tubulin from the ciliary A microtubule has also been compared with mitotic apparatus and flagellar doublet tubulins. Due to the difficulty of obtaining this tubulin in high yield, it was not repurified after mercurial extrac-

tion, and was placed on the gels at insufficient loading, but one of the most heavily staining peptides of the ciliary pattern had no counterpart in either mitotic or flagellar doublet tubulin.

DISCUSSION

Is the α_1 - or α_2 -Chain an Impurity?

Both α_1 - and α_2 -chains behave like tubulin in the following ways: both are present in isolated mitotic apparatus and ciliary A microtubules; both are extracted by organic mercurial, which extracts tubulin selectively (5); both elute from DEAE-cellulose in the tubulin peak; and both have methionine end-groups. Moreover, the two α -chains apparently co-migrate in all gel systems used except the SDS-urea system, as shown by the absence in these gel systems of additional bands of strengths comparable to the tubulin bands, and by the presence of α -tubulin in an amount (relative to β -tubulin) equal to the *sum* of α_1 and α_2 . By the same argument, if α_1 or α_2 as revealed by the

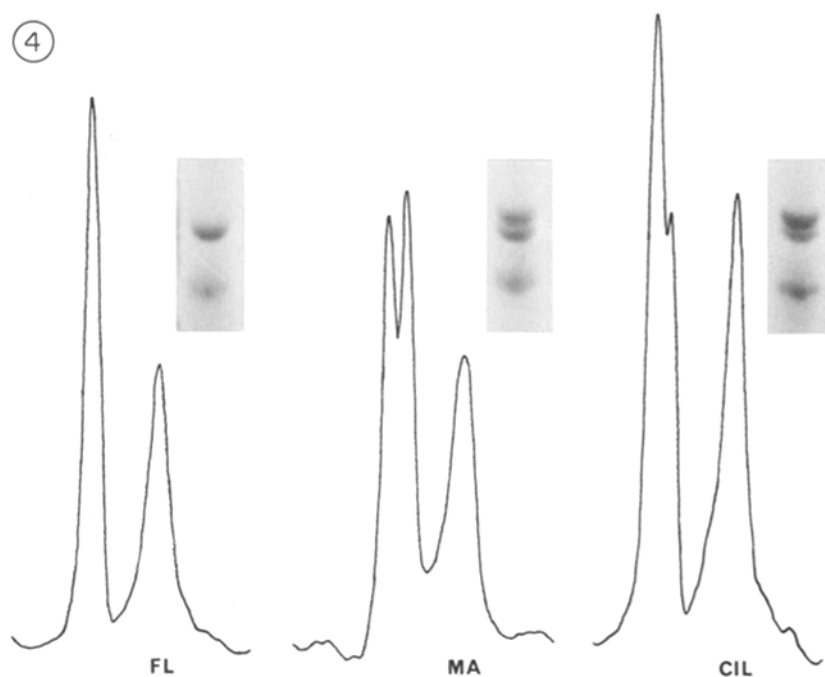


FIGURE 4 Comparison of tubulins from three different microtubule systems of the sea urchin by polyacrylamide gel electrophoresis in the SDS-urea system. *FL*: tubulin from doublet microtubules of sperm flagella. *MA*: tubulin from mitotic apparatus. *CIL*: tubulin from the A microtubule of ciliary doublets. All gels were loaded with 8 μ g of mercurial extract protein. Densitometer scans of the tubulin bands are shown, as well as photographs of the tubulin region of the gels. All three patterns are clearly different. It is not self-evident whether the ciliary and mitotic apparatus patterns differ in the mobilities of the α -subunits, the relative amounts present, or both (see text).

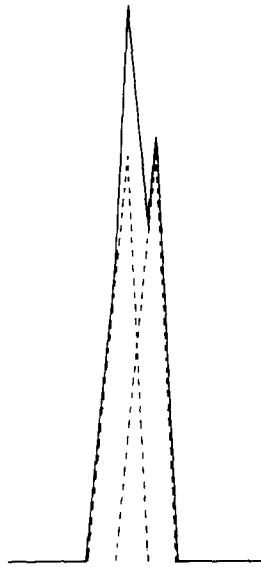


FIGURE 5 Illustrates how the α -band pattern of tubulin from the ciliary A microtubule could arise from two closely spaced α -species present in equal amount, in view of the fact that the bands are asymmetrical, with an extended trailing edge. Asymmetrical triangles are used as models of peaks of a densitometer scan. The solid line, which resembles the scan of the ciliary α -peaks, is the sum of the two dotted lines.

SDS-urea system were considered an impurity, then the other species would be present in only half of the amount required for equimolarity with β -tubulin. Moreover, the apparent equimolarity of α_1 - and α_2 -tubulins themselves in tubulin from mitotic apparatus (and possibly also from ciliary A microtubules, see Fig. 5) is inconsistent with expectation if either one of them is an impurity. We conclude that both α_1 - and α_2 -species are components of tubulin.

Are the α_1 - and α_2 -Chains Artifacts?

Several considerations rule out the possibility that the cleavage of the α -tubulin band is a purely electrophoretic artifact specific to the SDS-urea system. There are two internal controls: the β -band is not cleaved, and the α -band of flagellar doublets is not cleaved. Moreover, a Fergusson plot shows that the α_1 - and α_2 -species behave as entities having definite sizes and electrophoretic mobilities.

A chemical modification of α -tubulin occurring during preparation would not in general be expected to give reproducible and equimolar

amounts of α_1 - and α_2 -tubulins in the case of mitotic apparatus tubulin. A proteolytic cleavage of the α -chain, if carried to completion, would yield two equimolar species from a single α -chain, but both products would not have a molecular weight similar to that of the original α -chain; both would not therefore migrate at or near the α -position in gel systems which discriminate primarily by molecular weight. An irreversible bimolecular reaction among α -chains, $2\alpha \rightarrow \alpha_1 + \alpha_2$, would also, if carried to completion, produce two species of

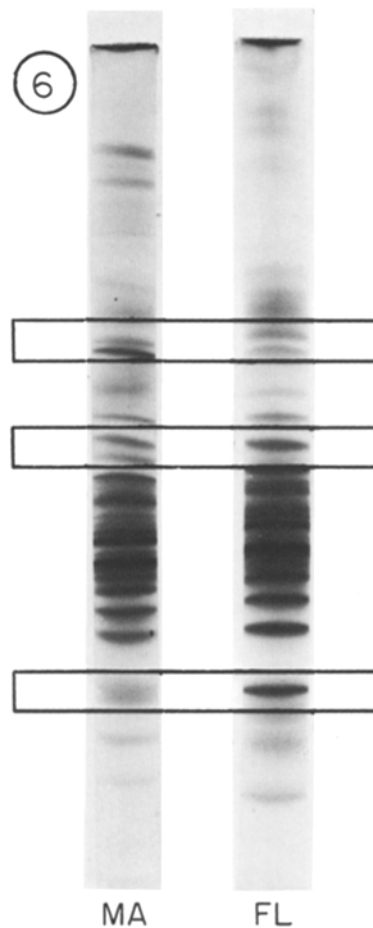


FIGURE 6 Comparison of the cyanogen bromide peptides of DEAE-repurified tubulins from sea urchin mitotic apparatus (*MA*) and doublet microtubules of sperm flagella (*FL*). The peptides were analyzed by isoelectric focusing in polyacrylamide gels containing 8 M urea. Most of the peptides of the two tubulins are indistinguishable by this method, but differences are also evident. The most conspicuous of these have been emphasized by brackets.

α -chain in equimolar amounts. This reaction is a spontaneous reaction of the α -chain, requiring no added reagents. It might as well occur *in vivo* as during our experimental procedure. Conceivably, such a reaction might take place during the preparative steps rather than *in vivo*, and so produce resolvable α_1 - and α_2 -chains from mitotic apparatus and ciliary tubulins, but not flagellar tubulin. However, this suggestion has little weight of probability; it is simpler to assume that α_1 - and α_2 -tubulins are real.

Are Tubulins from Different Microtubule Systems Different?

Unless α_1 - and α_2 -tubulin are artifacts, we have shown that both mitotic tubulin of sea urchins and tubulin from A tubules of ciliary doublets differ from flagellar doublet tubulin. It is further likely that mitotic tubulin and tubulin from the A tubule of ciliary doublets are basically different, differing in the mobilities of their α -species as their electrophoretic patterns suggest, not merely in the relative amounts of the two species present. Further analysis will be required to confirm this point.

Even if α_1 - and α_2 -tubulins were considered to arise during our preparative steps, the fact that they arise in mitotic and ciliary doublet A tubulins but not in flagellar doublet tubulin would show intrinsic differences in the tubulins, unless this could be attributed to differences in preparation. The methods of preparation used for the ciliary and flagellar tubulins are, however, very similar, and an experiment in which the procedures were made virtually identical, and in which sperm flagella were exposed during cutting to supernatant factors that may have been released from embryos during the detachment of cilia, produced no alteration of the results. Moreover, the differences in tubulins have been confirmed by a preliminary analysis of their cyanogen bromide peptides. The results indicate that each tubulin has specific peptides, so that each tubulin differs by the presence of molecular regions not present in the others. Apparently, differences exist in the tubulins of different microtubule systems.

Factors Affecting the Resolution of α -Chains

Although we have made no systematic study of factors affecting the resolution of α -chains, the use of gels containing both SDS and urea appears to be critical. The Ferguson plot for this system (Fig.

3) suggests that the micelles formed with SDS by the α -chains differ more in charge than in size. If a substantial amount of this charge difference were intrinsic to the polypeptide chains, one would expect the chains to have a mobility difference in gels containing only urea. Therefore the charge difference may reflect primarily a difference in bound SDS. Since the bands are not resolved in systems containing SDS only, there does not appear to be a *major* difference in the tendency of the chains to bind SDS, but any existing difference might be magnified in the presence of urea, which would be expected to promote the release of SDS from the chains. With little SDS bound, SDS would account for a higher percentage of total charge than of total mass, and a difference in binding might appear primarily as a difference in charge.

Other factors which clearly improve the resolution of α -bands are the use of low percentages of acrylamide and bisacrylamide in the resolving gel, and the use of low loadings of tubulin, preferably below 10 μ g of mercurial extract protein (Figs. 1 *a* and 4). Certain other aspects of our procedure might also play a part in the resolution of α -bands. These are: (*a*) the use of tubulins obtained from particular microtubule systems rather than whole-cell tubulin, which could conceivably be highly heterogeneous in subunit composition; (*b*) the use of both sample and spacer gels, which are sometimes omitted at the risk of convective disturbance of the pattern; (*c*) the use of gel buffers which are slightly more alkaline than those of the usual Ornstein-Davis system (12, 32); (*d*) the presence of SDS in the electrode buffers as well as the gels; (*e*) the use of lower than usual percentages of acrylamide and bisacrylamide in the stacking gels; and (*f*) the omission of TEMED from the stacking gels, which allows a more precise control of pH.

Origin and Function of α_1 - and α_2 -Tubulins

Our findings indicate the presence of at least two dimeric species of tubulin molecule, $\alpha_1\beta$ and $\alpha_2\beta$, in some microtubules. Since there is as yet no information concerning the origin and function of these molecules, little more can be done at this time than to list possibilities. Since mitotic microtubules are considered to be in dynamic equilibrium with unpolymerized tubulin (21), we suppose that both species of tubulin are present in the soluble tubulin pool, and are already present at the time of microtubule assembly. We do not know

whether the molecules are present in the pool in equimolar amount, or whether their apparent equimolarity in mitotic microtubules is determined during assembly. In the latter case (and possibly even in the former) the microtubule would presumably consist of an alternating arrangement of the two molecules. It should be noted that tubulin molecules can be arranged in this way on what is generally agreed (1, 14, 19) to be the microtubule lattice (Fig. 7). The required disposition of dimers on the lattice is identical to that described by Amos and Klug for the A tubule of flagellar doublets (1). The alternating arrangement gives an axial periodicity of 160 Å, a spacing which has been detected in doublet microtubules (1, 19). In terms of this model, a possible function of tubulin heterogeneity is to confer on the microtubule an intrinsic periodicity longer than the dimer spacing, which could play a part in the organization of periodic structures associated with microtubules.

Assuming that both tubulin molecules are already present in soluble tubulin before the assembly of microtubules, it is still not known whether they are translated from distinct transcripts, reflecting the activity of different genes, or whether they are produced by posttranslational modification of a single precursor. At the chemical level, we do not know whether the α_1 - and α_2 -chains represent truly distinct amino acid sequences, or whether the differences between them are restricted to terminal or side chain modifications imposed on a single basic sequence. In either case, the point at which the presence of the two tubulin molecules is determined could well be a control point for tubulin function.

Origin and Function of the System-Specificity of Tubulin

The system-specificity of tubulin indicated by our data extends to stable microtubules, which are not necessarily in dynamic equilibrium with a soluble pool. Therefore it cannot be assumed that system-specificity arises before microtubule assembly. It may be caused by localized modification of polymerized microtubules, in which case it could function only in determining the higher order systems to be constructed from microtubules, and in microtubule function. If, however, specific tubulins exist before polymerization, then the specific tubulins for different microtubule systems might coexist in a cell at one time; in this case, tubulin would be selected during polymerization from a

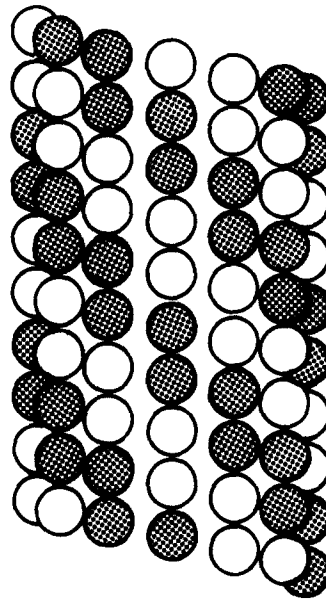


FIGURE 7 A model in which two different tubulin dimers are arranged in alternating fashion on the microtubule lattice. Circles represent monomers. Axially oriented shaded pairs of circles represent one tubulin dimer; unshaded pairs represent the other. Rows of like dimers run diagonally between the protofilaments and rows of the shallower three-start helix. For a thirteen-protofilament microtubule, this helix of like molecules forms a 16-start helix of monomers, or 8-start helix of dimers. The axial period of this helix accommodates four each of the two dimeric tubulin molecules.

The upper member of each shaded pair might be regarded as an α_1 -chain, and the upper member of each unshaded pair as an α_2 -chain. The lower members would then be β -chains. The α_1 -chains would occupy equivalent lattice positions, as would the α_2 -chains. However, β -chains would occupy two nonequivalent positions, corresponding to the lower members of shaded and unshaded pairs. These positions could be filled by nonidentical, but as yet unresolved, β -chains, or by identical β -chains whose combining properties have been influenced by their prior association with α_1 - or α_2 -chains.

heterogeneous pool. One function of tubulin specificity would then necessarily be to determine the specificity of microtubule polymerization. This might be done either via a specific polymerization site, or via a site participating in a specific activation of tubulin for assembly.

As is the case with α_1 - and α_2 -tubulins, we do not know whether system-specific tubulins arise by translation of different transcripts or by posttranslational modification of a single precursor; these

possibilities would differ in their implications for the control of microtubule function.

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REFERENCES

1. AMOS, L. A., and A. KLUG. 1974. Arrangement of subunits in flagellar microtubules. *J. Cell Sci.* **14**:523-537.
2. AUCLAIR, W., and B. W. SIEGEL. 1966. Cilia regeneration in the sea urchin embryo: evidence for a pool of ciliary proteins. *Science (Wash. D. C.)*. **154**:913-915.
3. BEHNKE, O., and A. FORER. 1967. Evidence for four classes of microtubules in individual cells. *J. Cell Sci.* **2**:169-192.
4. BIBRING, T., and J. BAXANDALL. 1969. Immunological studies of 22S protein from isolated mitotic apparatus. *J. Cell Biol.* **41**:577-590.
5. BIBRING, T., and J. BAXANDALL. 1971. Selective extraction of isolated mitotic apparatus. Evidence that typical microtubule protein is extracted by organic mercurial. *J. Cell Biol.* **48**:324-339.
6. BIBRING, T., and J. BAXANDALL. 1974. Tubulins 1 and 2. Failure of quantitation in polyacrylamide gel electrophoresis may influence their identification. *Exp. Cell Res.* **86**:120-126.
7. BRYAN, J. 1972. Vinblastine and microtubules. II. Characterization of two protein subunits from the isolated crystals. *J. Mol. Biol.* **66**:157-168.
8. BRYAN, J. 1974. Biochemical properties of microtubules. *Fed. Proc.* **33**:152-157.
9. BRYAN, J., and L. WILSON. 1971. Are cytoplasmic microtubules heteropolymers? *Proc. Natl. Acad. Sci. U. S. A.* **68**:1762-1766.
10. CRESTFIELD, A. M., S. MOORE, and W. H. STEIN. 1963. The preparation and enzymatic hydrolysis of reduced and S-carboxymethylated proteins. *J. Biol. Chem.* **238**:622-627.
11. DALES, S. 1972. Concerning the universality of a microtubule antigen in animal cells. *J. Cell Biol.* **52**:748-754.
12. DAVIS, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N. Y. Acad. Sci.* **121**:404-427.
13. EIPPER, B. A. 1974. Properties of rat brain tubulin. *J. Biol. Chem.* **249**:1407-1416.
14. ERICKSON, H. P. 1974. Microtubule surface lattice and subunit structure and observations on reassembly. *J. Cell Biol.* **60**:153-167.
15. FEIT, H., L. SLUSAREK, and M. L. SHELANSKI. 1971. Heterogeneity of tubulin subunits. *Proc. Natl. Acad. Sci. U. S. A.* **68**:2028-2031.
16. FINE, R. E. 1971. Heterogeneity of tubulin. *Nat. (New Biol.)*. **233**:283-284.
17. FULTON, C., R. E. KANE, and R. E. STEPHENS. Serological similarity of flagellar and mitotic microtubules. *J. Cell Biol.* **50**:762-773.
18. GRAY, W. R. 1972. End-group analysis using dansyl chloride. In *Methods in Enzymology*. C. H. W. Hirs and S. N. Timasheff, editors. Academic Press, Inc., New York. **25B**:121-138.
19. GRIMSTONE, A. V., and A. KLUG. 1966. Observations on the substructure of flagellar fibres. *J. Cell Sci.* **1**:351-362.
20. HENDRICK, J. L., and A. J. SMITH. 1968. Size and charge isomer separation and estimation of molecular weights of proteins by disc gel electrophoresis. *Arch. Biochem. Biophys.* **126**:155-164.
21. INOUÉ, S., and H. SATO. 1967. Cell motility by labile association of molecules. The nature of mitotic spindle fibers and their role in chromosome movement. *J. Gen. Physiol.* **50**:259-288.
22. JACOBS, M., and A. MCVITTIE. 1970. Identification of the flagellar proteins of *Chlamydomonas reinhardtii*. *Exp. Cell Res.* **63**:53-61.
23. KANE, R. E. 1965. The mitotic apparatus. Physical-chemical factors controlling stability. *J. Cell Biol.* **25**(1, Pt. 2):137-144. (Suppl.).
24. LEE, J. C., R. P. FRIGNON, and S. N. TIMASHEFF. 1973. The chemical characterization of calf brain microtubule protein subunits. *J. Biol. Chem.* **248**:7253-7262.
25. LINCK, R. W. 1973. Chemical and structural differences between cilia and flagella from the Lamellibranch mollusc, *Aequipecten irradians*. *J. Cell Sci.* **12**:951-981.
26. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
27. LUDUENA, R. F., and D. O. WOODWARD. 1973. Isolation and partial characterization of α - and β -tubulin from outer doublets of sea-urchin sperm and microtubules of chick-embryo brain. *Proc. Natl. Acad. Sci. U. S. A.* **70**:3594-3598.
28. LUDUENA, R., L. WILSON, and E. M. SHOOTER. 1974. Cross-linking of tubulin: evidence for the heterodimer model. *J. Cell Biol.* **63** (2, Pt. 2):202 a. (Abstr.).
29. MERZ, D. C., R. A. GOOD, and G. W. LITMAN. 1972. Segregation of membrane components using isoelectric focusing in polyacrylamide gels. *Biochem. Biophys. Res. Commun.* **49**:84-91.
30. MEZA, I., B. HUANG, and J. BRYAN. 1972. Chemical heterogeneity of protofilaments forming the outer doublets from sea urchin flagella. *Exp. Cell Res.* **74**:535-540.
31. OLMSTED, J. B., G. B. WITMAN, K. CARLSON, and

- J. L. ROSENBAUM. 1971. Comparison of the microtubule proteins of neuroblastoma cells, brain, and *Chlamydomonas* flagella. *Proc. Natl. Acad. Sci. U. S. A.* **68**:2273-2277.
32. ORNSTEIN, L. 1964. Disc electrophoresis-I. Background and Theory. *Ann. N. Y. Acad. Sci.* **121**:321-349.
33. RIGHETTI, G. P., and J. W. DRYSDALE. 1971. Isoelectric focusing in polyacrylamide gels. *Biochim. Biophys. Acta.* **236**:17-28.
34. SAFER, D. 1973. Comparison of ciliary and flagellar microtubule subunits. *J. Cell. Biol.* **59**:299 a. (Abstr.).
35. STARK, G. R., W. H. STEIN, and S. MOORE. 1960. Reactions of the cyanate present in aqueous urea with amino acids and proteins. *J. Biol. Chem.* **235**:3177-3181.
36. STEPHENS, R. E. 1970. Thermal fractionation of outer doublet microtubules into A- and B-subfiber components: A- and B-tubulin. *J. Mol. Biol.* **47**:353-363.
37. STEPHENS, R. E., F. L. RENAUD, and I. R. GIBBONS. 1967. Guanine nucleotide associated with the protein of the outer fibers of flagella and cilia. *Science (Wash. D. C.)*. **156**:1606-1608.
38. TILNEY, L. G., and J. R. GIBBINS. 1968. Differential effects of antimitotic agents on the stability and behavior of cytoplasmic and ciliary microtubules. *Protoplasma.* **65**:167-179.
39. WITMAN, G. B., K. CARLSON, and J. L. ROSENBAUM. 1972. *Chlamydomonas* flagella. II. The distribution of tubulins 1 and 2 in the outer doublet microtubules. *J. Cell Biol.* **54**:540-555.
40. YANG, S., and R. S. CRIDDLE. 1970. *In vitro* biosynthesis of membrane proteins in isolated mitochondria from *Saccharomyces carlsbergensis*. *Biochemistry.* **9**:3063-3072.