

Equimolar Heterodimers in Microtubules

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ABSTRACT Two equimolar β chains can be resolved from sea urchin sperm flagellar and scallop gill ciliary tubulins, and from certain brain tubulins as well, using the Triton X-100-acid-urea polyacrylamide gel system commonly used for histone analysis. The β chains are identified as such from their mobility on urea-free SDS PAGE, from amino acid composition, and from tryptic peptide distribution. Scallop β chains have almost identical amino acid profiles but they differ by one tryptic peptide. Optimal conditions for β chain resolution are very species-dependent, with some closely related species showing either maximal or no β chain separation. In addition, beef brain tubulin on Triton X-100-acid-urea electrophoresis and scallop gill ciliary tubulin upon isoelectric focusing in the presence of SDS show two approximately equimolar α chains. These data, indicating equimolar amounts of two potentially different tubulin heterodimers from a variety of microtubule types, support a model for microtubule structure wherein protofilaments consist of alternating heterodimers of two kinds, generating a 16-nm (2-dimer) axial repeat.

The heterodimer concept for tubulin structure has been supported by strong experimental evidence for over a decade. Bryan and Wilson (8) were primarily responsible for demonstrating that cytoplasmic microtubules contained two dissimilar polypeptide chains, α and β , in equal ratio, based upon amino acid composition differences in the electrophoretically resolved subunits. Ludueña and co-workers (22) later proved by chemical cross-linkage that these two chains existed as a heterodimer. Witman and colleagues (32) showed that subfractionation of *Chlamydomonas* flagellar outer doublets yielded equimolar electrophoretic species from all protofilaments except possibly those of the junctional wall, while Meza and co-workers (24) demonstrated equimolar subunits in sea urchin sperm tail outer doublets, regardless of the type of tubule or the portion of the tubule wall that was solubilized. Using primary sequence data, Ludueña and Woodward (21) demonstrated that α and β tubulin chains were evolutionarily related and that the respective α and β chains derived from vertebrate brain and echinoderm sperm flagella were highly conserved proteins, in spite of their different organellar and species origin. In contrast, Stephens (27) showed that the α and the β chains of sea urchin cytoplasmic, ciliary, and flagellar tubulins could be distinguished on an organellar basis, using amino acid composition and high-resolution peptide mapping. The homologous α and β chains were closely related among and within the organelles but differed, organelle-specifically, in local regions of the polypeptide chain, to an extent far greater than would be expected from simple posttranslational modification. However, the homologous chains were electrophoretically indistinguishable and

were consistently found in a 1:1 molar ratio. Thus the bulk of chemical evidence has supported the idea that the tubulins are a highly conserved class of heterodimeric protein, with a single dimer type most likely forming uniformly polymeric microtubules.

The structural situation may not be as simple as this basic chemical evidence would indicate. Early work by Witman and co-workers (32) with *Chlamydomonas* flagella revealed subclasses of both α and β tubulin upon isoelectric focusing, prompting them to suggest that multiple dimer types form a microtubule. More recently, Kobayashi and Mohri (13) found similar isoelectric heterogeneity in chromatographically resolved α and β tubulin chains from sea urchin sperm flagella. Perhaps most significantly, Bibring and colleagues (6) electrophoretically resolved two equimolar α chains in both mitotic apparatus and ciliary tubulin from sea urchin. They proposed that two different heterodimer types ($\alpha_1\beta$ and $\alpha_2\beta$) formed copolymeric microtubules, with the two dimers arranged along the protofilaments of the tubule in alternating fashion. This model was particularly attractive because it could account for a 16-nm (2-dimer) repeat sometimes detected in optical diffraction patterns of negatively stained microtubules but not expected from a uniform, single heterodimer arrangement (2, 17).

Multiple isotypes of tubulin have been reported in brain by numerous authors but it was not clear whether the observed microheterogeneity was a consequence of mixed cell populations, developmental stage, or both. Feit and co-workers (11) first noted isoelectric heterogeneity of both α and β tubulin in

porcine brain and suggested multiple heterodimers. Lu and Elzinga (20) chromatographically resolved two distinct α chains from calf brain while Little (18) separated two β chains from porcine brain by similar methods. Marotta and colleagues (23) electrophoretically resolved rat brain tubulin into two α chains which, in turn, were separable into three isoelectric variants; a major and a minor β chain were also detected. Similar isoelectric variants were reported by Gozes and Littauer (12) and even further complexity was observed with brain maturation. In most of these cases, the isoelectric heterogeneity was attributed to posttranslational modification or stage-specific gene products. In none of these studies, however, was there a consistent 1:1 stoichiometry between either the α or the β tubulin isotypes and hence such data neither supported nor negated the possibility of a fixed ratio of specific heterodimers.

The difficulty in resolving two equimolar α chains (6), coupled with the notorious ill-behavior of α tubulin on various electrophoretic systems (7), and the further possibility of heterogeneity introduced through posttranslational modification or preparational artifact, has not left the proposal of two equimolar heterodimer types on very firm grounding. The data presented in this report offer independent evidence, derived mainly from a unique electrophoretic system, that there are two equimolar β chains in tubulin preparations from various sources. This separation is achieved through differential detergent binding and thus most likely reflects hydrophobic differences between two otherwise indistinguishable β chains. Preliminary accounts of these findings have been presented in abstract form (29, 30).

MATERIALS AND METHODS

Protein Preparations

Outer doublet and central pair tubulin fractions were prepared from sperm tails of the sea urchins *Tripeustes gratilla* (Hawaii) and *Strongylocentrotus droebachiensis* (Massachusetts) by short-term dialysis of demembrated axonemes as described previously (27). Outer doublet A- and B-subfiber tubulin fractions were obtained from gill cilia of the bay scallop *Aequipecten irradians* by long-term dialysis (16, 31). Beef, chick, and skate brain tubulins were prepared and purified by *in vitro* polymerization using standard procedures (15, 26).

Polyacrylamide Gel Electrophoresis

TRITON X-100-ACID-UREA: The system of Zweidler (33) was used, varying urea from 0 to 8 M, Triton X-100 (or Nonidet P-40) from 0 to 1%, and the total acrylamide concentration from 5 to 10% (1% bis) in horizontal slab gradients (0.15 \times 10 cm) for subunit resolution survey purposes. Parallel sample comparisons were then made on slabs or tubes of fixed, maximally resolving formulation. Early in this study, after a thorough pre-run to remove persulfate (which could cause gross protein oxidation), the gels were scavenged by running 0.5 M mercaptoethylamine to prevent oxidation by free radicals. Confirmatory runs were later done with the more stable methylthiopropylamine (1%), at the suggestion of Dr. Alfred Zweidler (Institute for Cancer Research, Philadelphia, PA). Salt-free protein samples were routinely dissolved in 8 M urea and 1% mercaptoethanol. Occasionally, Triton X-100 and acetic acid were added to render the samples uniform with respect to the running gel. Some samples were prepared with 5% thiodiglycol to control for possible methionine oxidation before electrophoresis.

SODIUM DODECYL SULFATE: For subunit identification, second-dimension analysis used the method of Laemmli (14), the first dimension being equilibrated in sample buffer according to O'Farrell (25). Maximal resolution of α and β tubulin subspecies was obtained only with SDS containing ~30% tetradecyl sulfate (4, 31).

PREPARATIVE ELECTROPHORESIS: Tubulin subunits from *Aequipecten irradians* cilia were excised from Zweidler system tube gels (1 \times 13 cm) containing 0.25% Triton X-100 and 6 M urea. To locate the proteins, the gels were briefly surface stained in 10% trichloroacetic acid saturated with Coomassie Blue G-250. The proteins were electrophoretically eluted from gel slices into dialysis bags, using 5% acetic acid, 8 M urea, and 1% mercaptoethanol as an electrolyte. The samples were then either analyzed directly on the same gel system or carboxamidomethylated under alkaline conditions (31) and dialyzed exhaustively

against distilled water for amino acid analysis and peptide mapping. Protein was determined by the method of Lowry and co-workers (19).

STAINING AND DENSITOMETRY: Gels were stained overnight with gentle agitation in at least 20 vol of stain in accord with the recommendations of Fairbanks and colleagues (10). Coomassie Blue R-250 was used at the prescribed concentrations while Fast Green was substituted at twice the amount. Gels stained with Coomassie Blue were scanned with white light, and those stained with Fast Green with 650 nm light, in an Ortec 5310 densitometer. Integrated optical density was linear with protein concentration for both stains when peak optical densities fell below 0.5 OD.

Analytical Procedures

Amino acid analysis of resolved, reduced and carboxamidomethylated subunits was performed on a Dionex D-500 automated, single column analyzer (AAA Laboratory, Mercer Island, WA), using 24-, 48-, and 96-hr hydrolyzates. Serine and threonine were extrapolated to zero hydrolysis time, whereas other values were averaged.

Tryptic peptide maps, using nanomole amounts of reduced and carboxamidomethylated subunits, were obtained by the high resolution fluorescent thin-layer methods published previously (28). Two independently prepared samples of each subunit type were generally analyzed in duplicate.

Isoelectric focusing was performed by a combination of established procedures (1, 25). Tubulin samples were made 1% in SDS, 2.5 mM in Tris-glycinate (pH 8.3), and 2% in mercaptoethanol, then heated for 10 min at 85°C. Urea and Nonidet P-40 (NP-40) were added to final concentrations of 9 M and 10%, respectively, and the sample was applied immediately to 0.3 \times 10 cm 4T/5C acrylamide gel cylinders containing 9 M urea, 2% NP-40, 1.6% pH 4/6 ampholyte, and 0.4% pH 3/10 ampholyte (Bio-Rad Laboratories, Richmond, CA). Focusing was for at least 8,000 V-h. Gels were stained by the Fairbanks procedure (10) after removal of ampholytes with 10% trichloroacetic acid (10 vol, 1 h) followed by 25% isopropanol/10% acetic acid (3 \times 10 vol, 3 h each).

RESULTS

Resolution of 9 + 2 Tubulins with Triton X-100-acid-urea Electrophoresis

The 5% acetic acid-6 M urea-6 mM Triton X-100 system of Zweidler (33), as formulated for routine histone separations, resolves *Tripeustes* serum flagellar and *Aequipecten* gill ciliary tubulin fractions into consistent, unique patterns (Fig. 1). In all cases, three major bands are obtained, typically in an approximate 2:1:1 ratio from slowest migrating (band 1) to fastest (band 3). The nature of this pattern does not vary with the age of the sample and is not influenced by the continued presence of thiodiglycol (an inhibitor of methionine oxidation) during preparation. With ciliary B-tubulin, the subunit ratio drops to 2:1:<1 when, for reasons of uniformity, the sample contains 5% acetic acid (Fig. 1, lane B, and Fig. 3, below).

When such one-dimensional Zweidler gels are analyzed by two-dimensional techniques, the subunit identity becomes immediately apparent, at least as defined by mobility in SDS (Fig. 2). The major single band migrates as an α chain while the two nearly equal minor bands migrate as β chains, the fastest on the Triton X-100-acid-urea system (band 3) corresponding to the slowest on the alkaline, discontinuous SDS system (β_1). Band 3 is sometimes diminished in amount, relative to the other subunits. This is evidently due to aggregation in the first dimension since β tubulin may be detected in the second dimension as a spot below the origin (Fig. 2, arrow). If the first-dimension gel is insufficiently equilibrated in the second-dimension stacking buffer (i.e. if the pH is too low), both β chains are diminished or absent.

Optimal conditions for the resolution of tubulin subunits may be determined by running the protein as a single loading across the top of a slab gel containing a horizontal gradient in which the parameter in question is varied (33). The results of such an analysis of scallop ciliary B-tubulin may be seen in Fig. 3, where all four variables in the system are investigated.

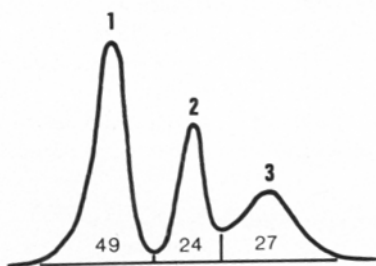
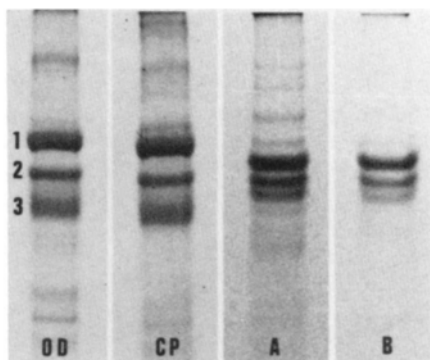


FIGURE 1 Tubulin fractions from outer doublet (OD) and central pair (CP) of *Tripneustes* sperm tails and the A- and B-subfiber fractions from *Aequipecten* cilia run on the Triton X-100-acid-urea system of Zweidler (33) under conditions typically used for histone resolution (6 mM Triton/6 M urea). The fractions yield three bands, generally two of approximately equal staining intensity and one of double intensity. Coomassie Blue staining after 1,200 V-h of electrophoresis on a 10T/1C gel. The densitometer scan is of *Tripneustes* outer doublet tubulin. The figures under the curves represent the percentage of total integrated optical density for the three bands.

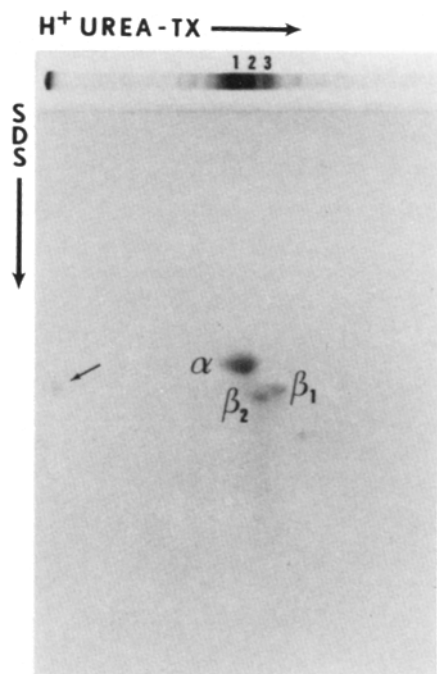


FIGURE 2 Identification of the Triton-acid-urea bands from *Aequipecten* B-tubulin by two-dimensional electrophoresis. Coomassie Blue staining, 1,400 V-h of electrophoresis on a 10T/1C gel containing 4 mM Triton and 6 M urea. Second-dimension electrophoresis on the Laemmli (14) system reveals that the fastest moving chain on the Triton-acid-urea system (band 3) corresponds to the slower moving β chain in SDS (β_1). Band 3, when depleted in the first

Maximal separation of band 2 from band 3 is obtained at 6 M urea, whereas band 1 is separated from band 2 maximally between 2 M and 4 M urea. Since band 3 merges with band 2 at and below ~ 4 M urea, the 6 M condition may be considered optimal for separation of the three subunits. Using similar arguments, the concentration of NP-40 is optimal between 0.25% and 0.4% (~ 4 –6 mM). A low degree of cross-linkage (at

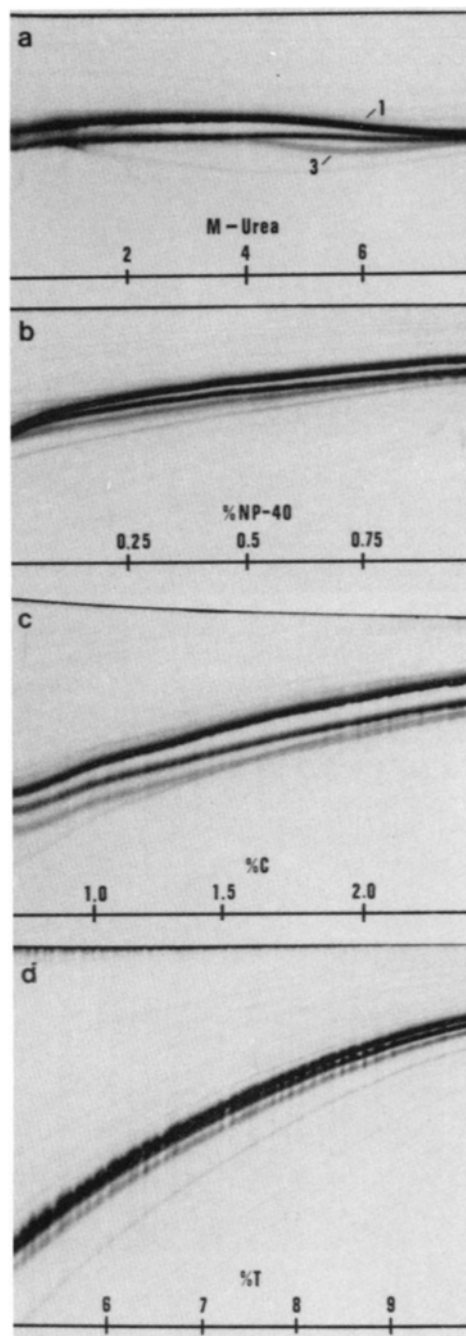


FIGURE 3 Optimization of conditions for β chain separation of *Aequipecten* ciliary B-tubulin on horizontal gradient gels of the Zweidler formulation. (a) 0–8 M urea, 0.25% nonidet P-40 (NP-40), 1,500 V-h on a 10T/1C gel. (b) 0–1.0% NP-40, 6 M urea, 1,500 V-h on a 10T/1C gel. (c) 0.63–2.5% Bis, 6 M urea, 0.25% NP-40, 2,500 V-h on a 10% gel. (d) 5–10% acrylamide, 6 M urea, 0.25% NP-40, 1,500 V-h on a 1C gel.

dimension by aggregation, appears in the second dimension as a spot below the origin (arrow).

or below 1% bis) and a high gel strength (9% or above) will likewise optimize separation. Essentially identical optima are obtained for *Tripneustes* sperm flagella tubulins, although (as in Fig. 1) the separations are somewhat greater for the same running time (data not shown).

Using the data from Fig. 3d, a Ferguson plot may be constructed to evaluate the electrophoretic properties of the subunits (Fig. 4). On a plot of the log of the mobility versus gel concentration, a linear function is generally obtained, the slope of which is a measure of molecular size while the y -intercept is a measure of the free electrophoretic mobility (9). Since the mobilities of bands 2 and 3 determine parallel lines, the Triton-tubulin mixed micelles that they represent are of the same relative size but must differ in relative charge. Since the mobility of band 1 does not parallel that of the other two bands, the band 1 mixed-micelle must differ from the others in size; it must also differ in free electrophoretic mobility since it does not extrapolate to the same point as either band 2 or 3 at zero acrylamide concentration. This analysis assumes proper theoretical behavior for Triton-protein micelles on the Zweidler system, a point which has never been rigorously demonstrated. The fact that the relationships here are strictly linear is at least encouraging.

Separation of Equimolar β Chains from Additional Sources

Is the 2:1:1 tubulin subunit ratio illustrated above characteristic only of morphologically complex 9 + 2 microtubules or can it be demonstrated for cytoplasmic microtubules as well? When skate (elasmobranch) brain tubulin is run under conditions found generally optimal for axonemal tubulin separation (6 mM Triton/6 M urea), only a simple 1:1 α/β pattern is seen. However, if Triton is held constant at 4 mM and the urea is varied from 0 to 8 M, a 2:1:1 subunit ratio is found but only at a comparatively high urea concentration and long running time (Fig. 5). Thus the general phenomenon discussed above

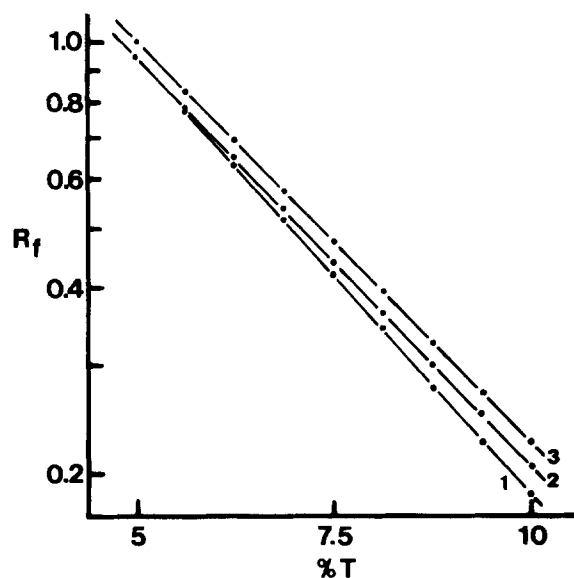


FIGURE 4 Ferguson plot of the mobilities of *Aequipecten* ciliary B-tubulin subunit-detergent micelles, derived from Fig. 3d. Bands 2 and 3 yield parallel lines (same size but different relative charge) while band 1 is neither parallel nor intersects either line at 0% (different size and free electrophoretic mobility). The mobility of band 3 was used as a relative reference point since there is no ion front reference due to the prolonged running time.

applies to cytoplasmic tubulins but with somewhat different optima.

In the case of mammalian brain, however, there is either minimal or no resolution of β subunits, depending upon source. With chick brain tubulin, there is a barely perceptible β separation, optimal under the same conditions as for axonemal tubulin but requiring threefold longer running times (data not shown). In contrast, beef brain tubulin shows no β separation under any conditions tried thus far. Rather, two nearly equally staining α chains are resolved, in a 1:1:2 ratio with β tubulin (Fig. 6a and b). The difference in the degree of β chain separation between *Tripneustes* sperm tail and *Aequipecten* ciliary tubulins was already noted above (Fig. 1). That this is neither simply a difference between sea urchins and molluscs nor between flagella and cilia is illustrated by the fact that flagellar tubulin from another sea urchin, *Strongylocentrotus*, is resolved only into α and β chains in a 1:1 ratio in 6 mM Triton and 6 M urea (Fig. 6c). The ability of the Zweidler system to resolve equimolar β chains (or α chains in one case)

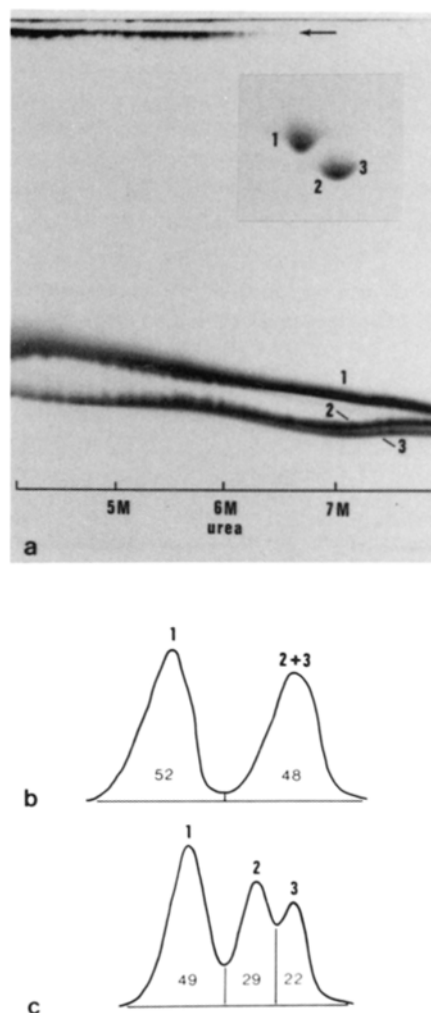


FIGURE 5 Resolution of skate brain tubulin on the Zweidler system, 4 M to 8 M urea region, at 4 mM NP-40. (a) At urea concentrations >6.2 M, three bands are obtained, identified as an α and two β 's (inset). Below 6 M urea, considerable aggregation occurs at the top of the gel (arrow) and the β tubulin is depleted. Fast Green staining, 3,500 V-h. (b) Densitometer trace through the 6.2 M urea region. The α (band 1) and β (bands 2 + 3) subunits are present in a 1:1 ratio. (c) Densitometer trace through the 7.7 M urea region. Three bands are present in an approximate 2:1:1 ratio, $\alpha:\beta:\beta$.

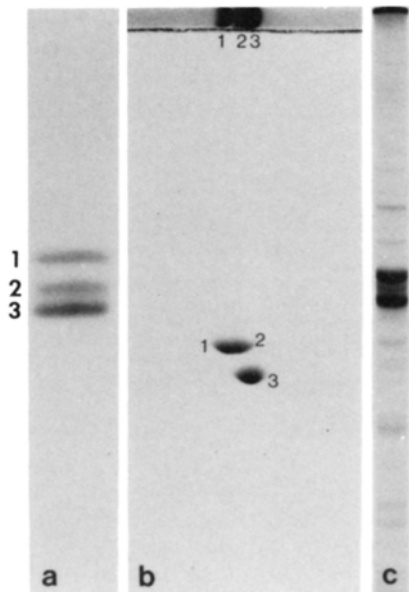


FIGURE 6 Resolution of beef brain and *Strongylocentrotus* outer doublet tubulins on the Triton-acid-urea system. (a) At 7 M urea, 4 mM Triton X-100, beef brain tubulin is resolved into a pattern that appears inverted with respect to that of skate brain, requiring extensive electrophoresis (4,800 V-h) for adequate separation. (b) When analyzed by two-dimensional methods, the two equally staining bands (1 and 2) are identified as α chains while band 3 migrates as a single β band. (c) Sperm tail outer doublet tubulin from *Strongylocentrotus* migrates as a closely spaced, equally staining α/β pair, regardless of conditions. The electrophoresis illustrated is on a 6 M urea, 6 mM Triton X-100, 10T/1C gel, run for twice the time as Fig. 1.

is highly species-dependent, rather than tissue- or organelle-dependent.

Separation and Analysis of Scallop β Chains

To prepare pure β tubulin chains for further analysis, the B-tubulin fraction from *Aequipecten* cilia was subjected to electrophoresis on preparative scale gels of a 4 mM Triton/6 M urea formulation, visualized by light staining, and electrophoretically eluted from excised bands. A typical preparative gel is illustrated in Fig. 7, accompanied by analytical gels of the three subunits analyzed directly after elution (Fig. 7, 1-3) and bands 2 and 3 after carboxamidomethylation (Fig. 7, 2' and 3'). It should be obvious that the excised bands run with the same relative migration rates as before, i.e. one band is not generated artifactually from another. Furthermore, the mobilities of bands 2 and 3 are not influenced by cysteine derivatization, except for the generation of a very minor species of band 2 which migrates slightly faster than band 3. Similar results are obtained by derivatization before preparative electrophoresis, further minimizing the possibility that cysteine oxidation could account for the β chain mobility differences. Direct protein determination of the freshly eluted subunits yields a weight ratio of 2:1.1 (± 0.1):0.8 (± 0.2) for bands 1, 2, and 3, respectively, in basic agreement with the ratios obtained by densitometry.

Amino acid analysis of bands 2 and 3 further supports their identification as β chains. There are no statistically significant differences between the average composition of these chains and the composition of the unresolved β tubulin from *Aequipecten* B-subfiber reported previously (31). There are minor

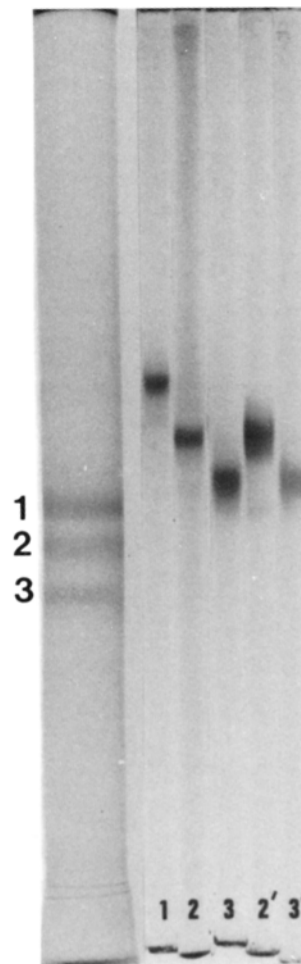


FIGURE 7 Preparative electrophoresis of *Aequipecten* ciliary B-tubulin subunits on the Zweidler system; 6 M urea, 4 mM Triton X-100, 10T/1C gel, 1.0 \times 12.5 cm, run for 3,000 V-h. Samples of the eluted bands before (1, 2, and 3) and after (2' and 3') carboxamidomethylation were monitored on 0.25 \times 10 cm gels of the same formulation, run for 1,800 V-h. The preparative gel was briefly surface-stained before excising the three bands.

differences between the two resolved β chains, although their significance as true primary structural variation may be marginal. Deviations are seen in serine and valine (band 2 is lower in serine and higher in valine by about 2 residues each) but these residues normally present difficulties in precise analysis; both appeared higher in the resolved chains than in the earlier analysis. A difference of 2-3 residues is seen in glycine, band 3 being higher than band 2. Here the analysis of the unresolved chains shows a value midway between those found for the separated chains and hence this difference is probably real. Although not statistically significant, band 3 appears to have one more arginine residue than band 2. If this difference is real, tryptic peptide maps of the two chains should reveal an additional peptide in band 3.

Fluorescent thin-layer peptide mapping of bands 2 and 3 shows patterns typical of β tubulin (31), whether electrophoresed at pH 3.5 after a relatively nonpolar chromatographic solvent or at pH 6.5 after a fairly polar solvent (Fig. 8). As predicted from the amino acid composition, band 3 yields one additional major peptide in comparison with band 2 (arrows). The peptide is comparatively neutral and hydrophobic. All other peptides, major and minor, are absolutely coincident and of the same relative intensity in the two β chain types. If other primary structural variations do exist, they must be too highly conserved to be revealed by this method.

Isoelectric Focusing in the Presence of Detergent

Previous studies of *Aequipecten* axonemal and membrane-

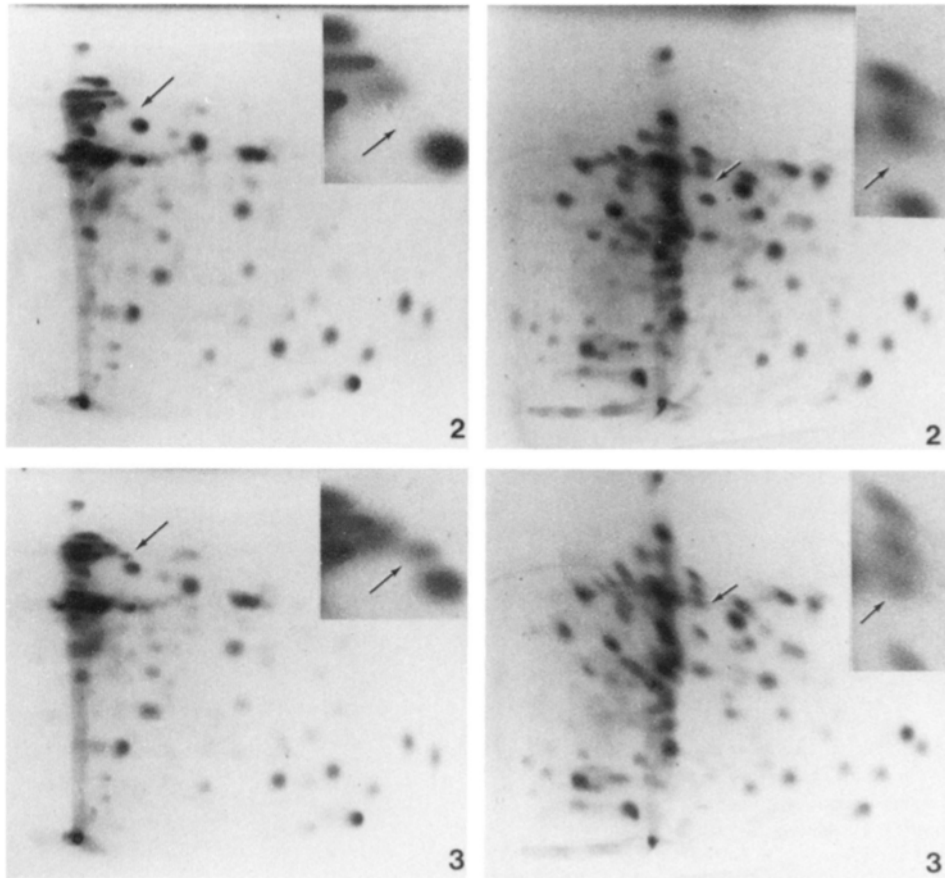


FIGURE 8 Fluorescent thin-layer tryptic peptide analysis of β chains (bands 2 and 3) from *Aequipecten* ciliary B-tubulin. Chromatography with chloroform-methanol-ammonium hydroxide and electrophoresis with pyridinium acetate, pH 3.5 (left pair) and chromatography with *n*-propanol-ammonium hydroxide, followed by electrophoresis with pyridinium acetate, pH 6.5 (right pair). One "extra" peptide, small, neutral, and quite nonpolar, is evident in the maps of band 3 (2 versus 3 and insets; position denoted by arrows), but the maps are otherwise identical.

associated tubulins by conventional isoelectric focusing (25) revealed single major α and β chains (31). However, when SDS is present initially in the sample (1), each major band splits into two bands of approximately equal staining intensity (Fig. 9). The α and β chain pairs are separated by 0.03 pH units while the α pair differs from the β pair by ~ 0.14 pH units (measured trough-to-trough), essentially the same degree of separation noted previously for the α/β split but with different absolute values for the isoelectric points (31). This pattern is established within the first 2,500 V-h of focusing and does not change appreciably thereafter. Although SDS is said to dissociate from proteins early in electrofocusing, accumulating as a narrow band at the anodic end of the gel (1), the simplest explanation for the phenomenon described here is that the two α and β chain subsets are rendered separable due to differing amounts of tightly bound detergent, characteristic of each chain type. Consistent with this conjecture is the observation that the α and β pairs become unequal when substantially higher voltage gradients are used (>100 V/cm versus 40 V/cm).

DISCUSSION

Under appropriate conditions, a Triton X-100-acid-urea polyacrylamide gel system resolves two equimolar β chains from sea urchin sperm flagellar and scallop gill ciliary tubulins and from skate and chick brain tubulins as well. The degree of β -splitting depends upon the specific source of the tubulin, somewhat like the α/β split on conventional SDS systems, where the phenomenon is well known to be species dependent. The β separation is even more species dependent since closely related organisms can show either maximal or no separation

(e.g. *Tripneustes* vs. *Strongylocentrotus* sperm tail tubulins). The separation is clearly a difficult one to achieve. However, the fact that 1:1 β -splitting can be shown in tubulins of various organelles and species would suggest that the phenomenon has some structural significance.

On the Zweidler Triton-acid-urea system, under the proper conditions, the $\alpha:\beta:\beta$ ratio is consistently 2:1:1 in both 9 + 2 and cytoplasmic tubulins. Deviation from this ratio occurs when the urea concentration in the sample is below ~ 6 M or when the sample is acidified. The ratio falls to 2:1: <1 due to the aggregation of the faster moving β chain, band 3. In second-dimension analysis on a discontinuous SDS system, the more slowly migrating β chain (equivalent to band 3 in the Zweidler system) is sometimes reduced in relative amount when the sample is more acidic than prescribed. In both systems, the problem is more noticeable at low protein concentrations. The fact that the band 3 β chain is subject to aggregation may explain the "failure to quantitate" β tubulin in a 1:1 ratio with α tubulin at low protein loadings on a discontinuous, urea-containing system, as reported by Bibring and Baxandall (5). Such cases may reflect depletion of the less-soluble β chain as a consequence of its aggregation before or during stacking. Under extreme conditions, band 3 may be totally absent, giving an $\alpha:\beta$ (band 1:band 2) ratio of 2.0:0.9–1.0 from a sample which had an $\alpha:\beta$ ratio of 1:1 by conventional SDS gel electrophoresis. This is particularly a problem with *Aequipecten*, less noticeable with skate brain, and not apparent with *Tripneustes*.

It is not clear how the two β subunits are related at a primary structural level, beyond the fact that one additional tryptic peptide arises from band 3. This is consistent with an arginine difference seen in the amino acid composition and with the Ferguson plot analysis indicating that bands 2 and 3 represent

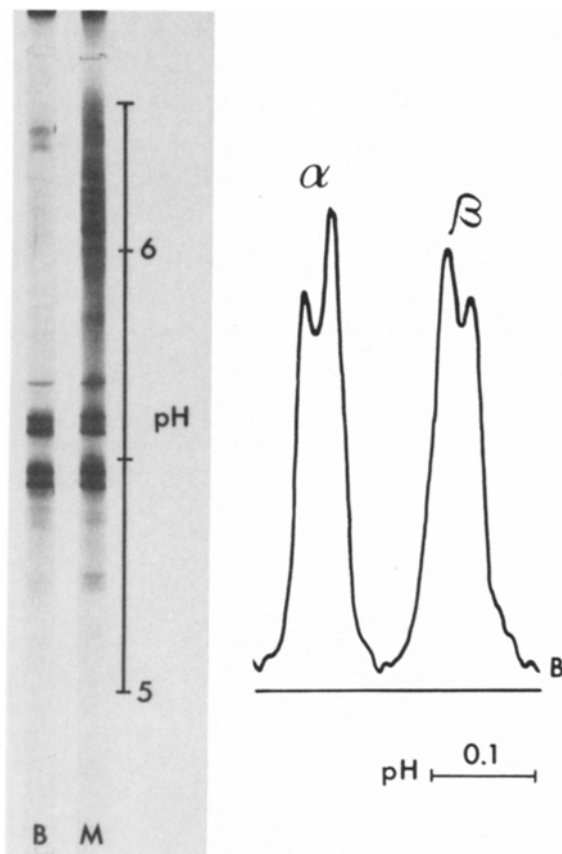


FIGURE 9 Tubulin from *Aequipecten* ciliary B-subfiber (B) and membrane fraction (M) electrofocused initially in the presence of SDS. Densitometry reveals that the α and β regions are each split into a pair of nearly equally staining bands, the β pair being the more acidic. Fast Green stain, 8,800 V-h.

mixed micelles of essentially the same size but of different relative charge. In contrast, in 6 M urea, without detergent, all three bands merge (Fig. 3b, origin), implying very similar mass-charge parameters for the unfolded polypeptide chains at acid pH. The charge difference must be very subtle, not being apparent in isoelectric focusing without ionic detergent, and must be emphasized as a result of mixed micelle formation in the Zweidler system. If band 3 represented a simple arginine substitution within the sequence of band 2, there should be two small tryptic peptides unique to band 3 and one larger peptide unique to band 2. This is not the case, implying an insertion or terminal addition of a short sequence unique to band 3.

Preliminary analyses of the unresolved β chains from both scallop cilia and skate brain tubulin revealed only amino-terminal methionine, so it is unlikely that band 2 could arise from band 3 by proteolysis of a small, arginine containing peptide from the NH_2 -terminal end. Carboxy-terminal tyrosylation (or detyrosylation) is an unlikely posttranslational β chain modification since this is reported only to occur in the α chain of brain tubulin. Selective deamidation during preparation or electrophoresis offers another artifactual possibility but would not be consistent with the tryptic mapping data. That one β chain is not the oxidation product of the other is proven by reelectrophoresis of both unmodified and carboxamidomethylated subunits from preparative electrophoresis. Furthermore, it seems highly unlikely that proteolysis, post-translational modification, deamidation, or oxidation would modify sea urchin flagellar, scallop ciliary, and skate brain

tubulin β chains (and beef brain tubulin α chains) all to exactly the same extent (50%). It is even less likely that β splitting is an artifact of the gel system since β tubulin from related sources does not split when run simultaneously with those that do.

The means by which the Zweidler Triton X-100-acid-urea gel system separates closely related polypeptides depends upon the competitive interaction of urea and detergent with the protein, yielding protein-detergent mixed micelles of characteristic size and charge. The method can be sensitive to single, uncharged amino acid differences and hence potentially able to detect conservative, nonpolar substitutions. The differences between β chains could be extremely subtle and sequence analysis may be required to provide a final answer.

The two equimolar tubulin subunits separated here by means of the Zweidler system from flagellar and ciliary tubulins are unquestionably β chains, based upon the independent criteria of SDS PAGE mobility, amino acid composition, and tryptic peptide distribution. This appears in contrast to the α chain heterogeneity reported by Bibring and co-workers (6). It may be that there are, in fact, both equimolar α and equimolar β chains in microtubules from various sources, as Figs. 6a and 9 would suggest. Alternatively, these workers may have observed β chain heterogeneity since they later report that the β chain may trail the α chain (i.e. reverse position) on urea-containing SDS PAGE (3). Regardless of interpretation, the independent demonstration of two equimolar β chains should rekindle interest in the type of alternating heterodimer model for microtubule structure that Bibring and collaborators (6) originally presented.

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Note Added in Proof: The two α -chains originally identified by Bibring and co-workers (6) have been confirmed as true α -chains through amino acid analysis and, furthermore, only batches of SDS which produce the correct migration order for α - and β -tubulin on SDS-urea gels are able to resolve two α -chains (Dr. Thomas Bibring, Vanderbilt University, personal communication). Thus these workers correctly identified the chain types.

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