Primary Structural Differences among Tubulin Subunits from Flagella, Cilia, and the Cytoplasm†

R. E. Stephens

ABSTRACT: Tubulin α and β chains were obtained from sea urchin sperm flagella A-tubules, B-subfibers, and central pair; from the A-tubule and B-subfiber enriched fractions of embryonic cilia; and from vinblastine-tubulin precipitates of unfertilized eggs. They were compared by both amino acid analysis and high-resolution, fluorescent thin-layer tryptic peptide mapping. Amino acid composition data indicate that all homologous chains examined are different, within the limits of this technique. Upon peptide mapping, homologous chains from either flagellar or ciliary A-tubules and B-subfibers differ subtly in terms of peptide presence, position, amount, or relative fluorescence, indicating subfiber specificity within the same organelle. Substoichiometric amounts of some peptides support recent observations that each outer-doublet member may contain two types of α chain and two types of β chain, with the possibility that one of each may be shared between doublet members. In addition to these minor differences between tubulin subunits of specific subfibers, the homologous subunits from the two organelles show marked peptide positional differences, allowing the outer-doublet tubulins of flagella to be readily distinguished from those of cilia. Corresponding chains of flagellar central pair and vinblastine-tubulin likewise contain certain characteristic peptides which permit them to be distinguished both from each other and from those of ciliary or flagellar outer doublets. The α chains of vinblastine-tubulin, flagellar central pair, and ciliary outer doublets all contain a small, hydrophilic, cathodic peptide that is not found in flagellar outer-doublet α chains. Conversely, the α and β chains of outer-doublet tubulins from both cilia and flagella contain another prominent, more cathodic peptide that is absent in the corresponding chains of flagellar central pair or vinblastine-tubulin. This major difference proves that the vinblastine-precipitated tubulin of the unfertilized egg is truly cytoplasmic in origin and fate, containing no significant amount of tubulin destined for later cilia formation. The overall similarity in peptide maps of homologous subunits, coupled with the limited number of organelle-specific peptides, would indicate that the tubulins are a highly conserved class of protein, having only local regions of variability to provide functional specificity.

The tubulins are a class of heterodimeric proteins which serve as building blocks for microtubules of varied function. Regardless of source—cytoplasmic, ciliary, or flagellar—they are composed of two dissimilar but related polypeptide chains (α and β) with molecular weights of 55 000, have characteristic amino acid compositions, contain 2 mol of bound guanine nucleotide, and form stoichiometric complexes with certain "antimitotic" drugs, in particular, colchicine and vinblastine. Ever since four microtubule classes were defined by Behnke and Forer (1967) on the basis of relative stability (cytoplasmic, ciliary or flagellar central pair, B-subfiber, and A-tubule), there has been some question as to whether a common tubulin pool can be used to assemble a mitotic apparatus and later give rise to embryonic cilia or even whether the individual "9 + 2" microtubules of cilia are composed of identical tubulin dimers.

The evidence for distinct tubulins in cilia or flagella comes from a variety of systems and approaches. Tubulin fractions obtained from sea urchin sperm flagella outer-doublet B-subfibers and A-tubules were found to differ markedly in solubility properties and amino acid composition but showed subtle differences in tryptic peptide maps (Stephens, 1970). Later work on this same system verified the amino acid composition and peptide map differences for the electrophoretically resolved tubulin α and β chains from the respective subfibers, implying at least two distinct types of dimer, one for each subfiber (cf. Stephens, 1975a, 1976a). In this regard, Witman and co-workers (1972) demonstrated that *Chlamydomonas* flagella outer-doublet tubulin could be separated into five bands on isoelectric focusing, again suggesting several kinds of dimer in doublet microtubules or portions thereof. Protein synthetic studies of ciliogenesis and regeneration in sea urchin embryos have shown that the tubulin of the A-tubule can differ in specific activity by nearly a factor of two from that of the B-subfiber, indicating separate pools of subfiber-specific dimers (Stephens, 1976a, 1977a). Also, optical reconstruction of electron micrographs has demonstrated that the B-subfiber and A-tubule differ in their dimer surface lattices, with that of the former being a 3-start, left-handed helix while that of the latter is a 5-start, right-handed helix (Amos and Klug, 1974), thus providing circumstantial evidence for differences in bonding properties of the respective tubulins in the outer-doublet members. Finally, preliminary tryptic mapping studies have shown marked positional differences in two major peptides from outer-doublet and central-pair tubulins, allowing these two kinds of tubulin to be clearly distinguished (Stephens, 1976a).

In terms of differences among tubulins of different organelles, Olmsted and colleagues (1971) showed that the α chain of neuroblastoma (cytoplasmic) tubulin could be resolved electrophoretically from that of *Chlamydomonas* flagella outer doublets, suggesting α-chain nonidentity, albeit in different species. Using a single species, Bibring and co-workers (1976) were able to resolve both sea urchin cytoplasmic and ciliary, but not flagellar, α chains into two nonidentical pairs of electrophoretic variants. This would indicate differences in at least the α chains of cytoplasmic, ciliary, and flagellar tubulins and suggests further that there may be two kinds of dimer in certain microtubules. Quantitative differences in the cross-reactivity of sea urchin mitotic apparatus, ciliary, and flagellar tubulins

† From the Marine Biological Laboratory, Woods Hole, Massachusetts 02543. *Received February 2, 1978.* This work was supported by United States Public Health Service Grants GM 20,644, GM 21,040, and GM 70,164 from the National Institute of General Medical Sciences.

0006-2960/78/0417-2882$01.00/0

to an antibody against sperm flagella axonemes have been reported (Fulton et al., 1971), implying that there are both common and unique antigenic determinants in the tubulins from these different organellar sources. Similarly, an antibody prepared against *Naegleria* outer-doublet tubulin was found not to cross-react with cytoplasmic tubulin (Kowit and Fulton, 1974). Isotope dilution experiments later showed that at least 70% of the flagellar tubulin was synthesized de novo during ameboflagellate transformation, indicating that the majority of tubulin destined for flagella was not drawn from a preexisting cytoplasmic pool (Fulton and Kowit, 1975).

Tubulin α and β chains derived from both chick brain and sea urchin sperm outer doublets have been partially sequenced, revealing an unusual degree of conservatism. Ludueña and Woodward (1973) determined the first 25 residues at the NH_2 terminus of each chain. They found that the α and β subunits were quite distinct proteins but clearly related to one another in that 11 positions were identical and 9 of those that differed could be accounted for by single base-pair mutations. The initial α-chain sequences of tubulins in these distant species were identical, while the β chains differed at only one residue. Nothing concrete can be said about the remaining 95% of the molecule from this limited sequence data. However, the fact that homologous α or β chains from tubulins from a variety of sources cannot be separated easily from one another by common electrophoretic, isoelectric, or chromatographic means would suggest that these molecules have maintained constant mass, charge, and sequence relationships throughout the evolution of both species and organelles.

None of these approaches have singly and unequivocally demonstrated primary structural differences among cytoplasmic, ciliary, and flagellar tubulins in the same organism. This report attempts to accomplish such an end through a comparison of homologous α and β chains derived from ciliary and flagellar A-tubules and B-subfibers, flagellar central pair, and prefertilization cytoplasmic tubulin of the sea urchin, using a sensitive, high-resolution, fluorescent peptide-mapping procedure (Stephens, 1978). Preliminary accounts of some of this work have appeared in symposia and abstracts (Stephens, 1975a, 1976a,b, 1977b).

Experimental Procedures

Protein Preparations. Flagella were prepared from sperm of the sea urchin *Strongylocentrotus droebachiensis* by decapitation and differential centrifugation, essentially according to methods described earlier (Stephens, 1970). Axonemes were obtained by demembranating the flagella with 0.25% Nonidet P-40 (NP-40; Particle Data Laboratories) in 30 mM Tris-HCl (pH 8), 3 mM $MgCl_2$, 0.1 mM EDTA, and 0.1 mM dithiothreitol for 15 min, sedimenting them at 10 000*g* for 10 min, and then washing once with 10 mM Tris-HCl (pH 8). The axonemes were suspended at a concentration of about 5 mg/mL in 1 mM Tris-HCl (pH 8), 0.1 mM EDTA, and 0.01% mercaptoethanol and then dialyzed at 0–4 °C against 100 volumes of this solution, changed four times over a 36–40-h period. The suspension was made 10 mM in Tris-HCl (pH 8) and the axonemes were sedimented at 75 000*g* for 30 min. The supernatant, consisting of solubilized central-pair tubulin plus dynein, was retained on ice. The pellet of outer doublets was suspended to a concentration of about 3 mg/mL in 1 mM Tris-HCl (pH 8) and 0.1 mM dithiothreitol, heated rapidly to 40 °C, held there for 2 min to "melt" the B-subfiber (cf. Stephens, 1970), cooled rapidly to 0 °C, made 10 mM in Tris-HCl (pH 8), and sedimented at 150 000*g* for 30 min to recover the A-tubules as a pellet and the B-tubulin as the supernatant. The fractions were retained on ice for further processing.

Cilia were harvested from *S. droebachiensis* embryos at the midgastrula to late gastrula stage by suspending the embryos in 10 volumes of hypertonic sea water for 2 min at 0 °C to remove the cilia, followed by gentle centrifugation to sediment the denuded embryos. The cilia were recovered from the supernatant by centrifugation at 15 000*g* for 15 min in a Sorvall HB-4 swinging-bucket rotor, washed once with 10 mM Tris-HCl (pH 8) to lyse any contaminating cell debris and lower the salt concentration, and then demembranated by extraction with 20 volumes of 0.25% NP-40 in 30 mM Tris-HCl (pH 8), 3 mM $MgCl_2$, and 0.1 mM dithiothreitol for 15 min. The resulting axonemes were recovered by centrifugation at 15 000*g* for 15 min, suspended at a concentration of about 2 mg/mL in 1 mM Tris-HCl (pH 8), 0.1 mM EDTA, and 0.01% mercaptoethanol, and then dialyzed at 0–4 °C against 100 volumes of this solution, changed three times over a 30–36-h period. The material was then centrifuged at 75 000*g* for 30 min. The supernatant, consisting primarily of solubilized tubulin from the nine B-subfiber and one central-pair member plus the bulk of the dynein, and the pellet of A-singlets plus the remaining central-pair member were retained on ice for later steps. To provide sufficient cilia for this study, multiple batches of embryos were grown and repeatedly deciliated up to ten times each. For further details and documentation of this fractionation, see Stephens (1977a).

To further purify the A-tubule fractions, the final pellets from the above flagellar and ciliary fractionation schemes were suspended in 10 volumes of 0.5% Sarkosyl (NL-97; Geigy Industrial Chemicals) in dialysis buffer and extracted for 30–60 min at 0 °C to solubilize all but the junctional protofilament ribbon and associated proteins (Linck, 1976). The samples were spun at 150 000*g* for 30 min to sediment the ribbon fraction, and the resulting supernatant of flagellar or ciliary A-tubulin was retained on ice. These various procedures for flagellar and ciliary fractionation are summarized in a flow diagram (Figure 1).

Vinblastine crystals of cytoplasmic tubulin were prepared in accord with procedures devised by Bryan (1971). Freshly shed, unfertilized eggs of *S. droebachiensis* were suspended in 20 volumes of sea water containing 10^{-4} M vinblastine sulfate (Eli Lilly Co.) and 0.01% each of sulfadiazine, penicillin, and streptomycin and then incubated with gentle agitation at 8 °C for 36–48 h to induce maximal crystal growth (Figure 2a). The eggs were sedimented with a hand centrifuge, suspended in 10–20 volumes of 1 M urea buffered at pH 8 with 5 mM Tris-HCl, stirred gently for about 2 min, and again hand centrifuged. The eggs were quickly lysed in 3 volumes of a solution containing 1% NP-40, 10 mM Tris-HCl (pH 8), and 1 mM EDTA, releasing the vinblastine–tubulin crystals. These were recovered by centrifugation at 2000*g* for 15 min and washed twice with a solution of 0.1% NP-40 in 100 mM KCl, 10 mM Tris-HCl (pH 8), and 1 mM $MgCl_2$. The final pellet of purified crystals (Figure 2b) was suspended in 20 volumes of 10 mM Tris-HCl (pH 8) and retained on ice for further processing.

Subunit Separation. The solubilized flagellar A, B, and central-pair tubulin, ciliary A- and B-enriched fractions, and vinblastine-precipitated tubulin were each made 5 M in guanidine hydrochloride, 50 mM in Tris-HCl (pH 8), 1 mM in EDTA, and 0.1 M in mercaptoethanol. Reduction proceeded under nitrogen for 30–60 min at 20–25 °C, after which a 10% molar excess of 1.1 M sodium iodoacetate in 1 M Na_2CO_3 was added, the sample was flushed with nitrogen, the mixture was allowed to stand in the dark for 30 min at 25 °C, and then fi-

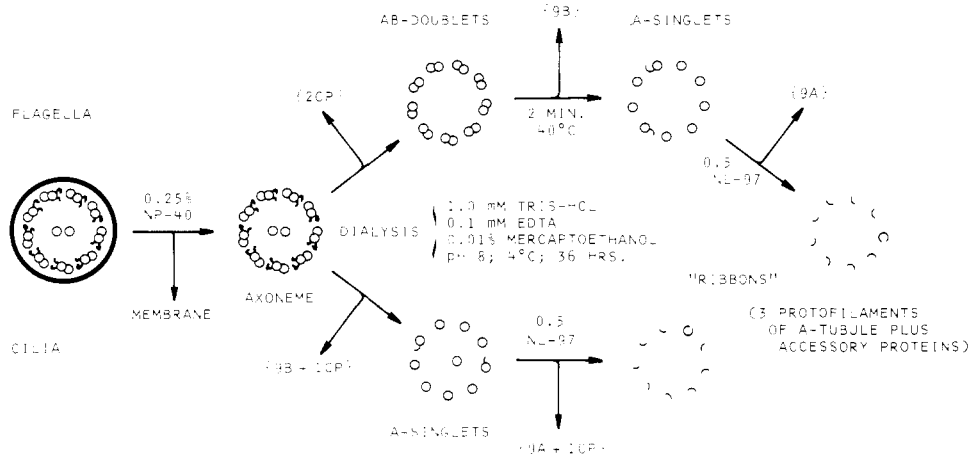

FIGURE 1: Flow diagram for the fractionation of flagella and cilia into specific subfiber tubulins. Brackets indicate the fractions analyzed in this study.

nally quenched with a twofold molar excess of mercaptoethanol. The samples were exhaustively dialyzed against cold 10 mM sodium phosphate (pH 7), and then a 10% aliquot was dansylated for preparative gel electrophoresis (Stephens, 1975b). At this stage, samples could be frozen indefinitely.

Pure α and β subunits of the respective tubulins were resolved by fluorescent preparative NaDodSO$_4$-polyacrylamide gel electrophoresis, removed from the gels by excision, and eluted, concentrated, and freed of excess NaDodSO$_4$ by electrophoretic elution-electrodialysis as described in detail previously (Stephens, 1975a,b). The samples were precipitated and further freed of NaDodSO$_4$ by the addition of 10 volumes of cold acetone, recovered by centrifugation, briefly air-dried, and then frozen until use. Prior to precipitation, aliquots were taken for analytical gel electrophoresis in order to estimate recovery and ascertain the degree of separation (Figure 3).

Amino Acid Analysis. Each of the precipitated, purified subunit fractions was suspended at a concentration of 0.5–1.0 mg/mL in cold 10 mM sodium phosphate (pH 7) and dialyzed exhaustively against this buffer in order to eliminate residual glycine and acrylamide by-products introduced at the preparative electrophoresis step. The samples were evaporated to dryness with a stream of nitrogen and then hydrolyzed at 110 °C in 6 N HCl under vacuum for 24, 48, and 96 h. The hydrolysates were analyzed on a Durrum D-500 automated, single-column amino acid analyzer. Serine and threonine were extrapolated to zero hydrolysis time, whereas 96-h values were used for valine.

Peptide Mapping. The basic procedure for fluorescent thin-layer peptide mapping of nanomole quantities of protein is given in detail elsewhere (cf. Stephens, 1978) but outlined here briefly in regard to specific conditions. The purified subunit fractions were suspended in and exhaustively dialyzed against cold 0.1 M ammonium bicarbonate (pH 8) and then digested with DCC-treated trypsin (Calbiochem) for 24 h at 25 °C, using an enzyme/protein ratio of 1:100. The samples were evaporated to dryness with a stream of nitrogen, dissolved in 0.1 M ammonium bicarbonate at a concentration of approximately 0.1 nmol/μL, and frozen until use.

Each subunit (1–1.5 nmol) was applied in duplicate to 20 × 20 cm silica gel G plates (Analtech; 250-μm thickness) and subjected to ascending chromatography with either of two chromatographic solvent systems: chloroform–methanol–ammonium hydroxide (2:2:1, v/v) or 1-propanol–ammonium hydroxide (7:3, v/v). The plates were air-dried overnight. Electrophoresis was then performed with a pyridine–acetic acid–water buffer at either pH 3.5 (1:10:489, v/v) or 6.5 (100:3:897, v/v) on a water-cooled (8–10 °C) Shandon single-plate high-voltage electrophoresis unit at 1000 V for 45 or 35 min, respectively, for the two pH values. The plates were oven dried at 110 °C for at least 45 min.

The accumulated plates were sprayed with 0.025% fluorescamine (Hoffmann-LaRoche) in acetone. Those run at pH 3.5 were postsprayed with 5% triethylamine in acetone to stabilize the fluorescamine–peptide reaction products. The plates were photographed under 366-nm UV illumination (XX-15 lamps) on Kodak Plus-X 35-mm film, using UV-haze and medium-yellow (K2, No. 8 Wratten) barrier filters. Image reversal (to improve contrast) was achieved by copying the resulting negative and printing the copy negative on high-contrast paper (Stephens, 1978).

Maps were compared by direct superposition of negatives, by tracings of enlargements, and also by comigration of samples. Only peptides present on all replicate maps were considered significant. At least two totally independent preparations of each dimer type were analyzed. As has already been demonstrated (Stephens, 1978), the presence of varying amounts of residual NaDodSO$_4$ has no significant influence on either the tryptic digestion or the final peptide pattern.

Results

Amino Acid Composition of Specific Tubulin Subunits. With the reservation that amino acid composition is neither a very sensitive nor a particularly accurate method for protein comparison, the tubulin subunits derived from specific organellar sources nevertheless do show certain reproducible qualitative differences. Subunits from blastula ciliary microtubules were in short supply and so were not compared by this methodology. Sufficient material was obtained from sperm flagellar A, B, and central-pair microtubules and from prefertilization vinblastine-precipitated egg tubulin to allow duplicate analyses at three hydrolysis times.

The composition of the subunits derived from "stable" doublet A- and B-tubules of sea urchin sperm flagella is given in Table I. The data closely match that reported earlier for the same resolved subunits analyzed on a two-column instrument (Stephens, 1975a). When the corresponding α and β subunits are averaged, these data also agree in principle with composition differences found for the unresolved A- and B-tubulin dimers (Stephens, 1970). A significantly higher content of lysine and arginine is found in both subunits of the A-tubulin dimer than in the B-tubulin α and β chains. The α subunit of

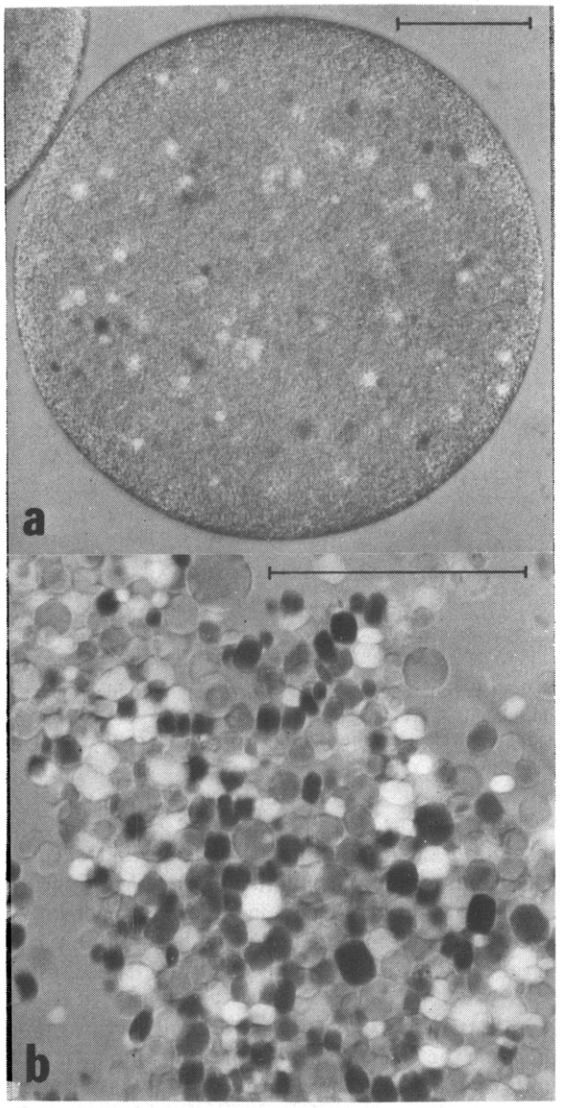

FIGURE 2: Eggs of *Strongylocentrotus droebachiensis* incubated with vinblastine sulfate to induce large quantities of vinblastine–tubulin crystals (a) which may then be isolated by detergent lysis (b). Polarized light microscopy. Scale marker = 50 μm.

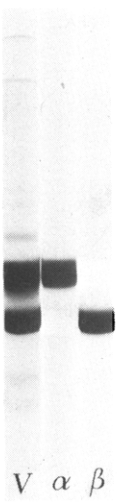

FIGURE 3: Analytical $NaDodSO_4$–polyacrylamide gel electrophoresis of reduced and alkylated vinblastine-precipitated cytoplasmic tubulin (V) and its subunits (α/β) separated by preparative electrophoresis.

TABLE I: Amino Acid Composition of Outer-Doublet Tubulin Chains.[a]

Residue	αA	αB	βA	βB
Ala	41.1	41.3	32.5	31.6
Arg	23.8*	21.2*	25.0*	22.6*
Asx	40.3*	44.3*	45.5*	51.2*
CMC	8.9	9.2	7.9	7.3
Glx	62.9*	65.8*	66.0*	68.6*
Gly	47.0	43.0	45.2	39.8
His	12.2	12.2	10.9	10.6
Ile	22.1	22.5	18.3	18.2
Leu	35.8*	34.0*	35.5	35.1
Lys	23.3*	20.5*	20.8*	17.5*
Met	8.3	8.6	15.1	15.2
Phe	18.4	18.9	19.4*	22.4*
Pro	22.7*	23.7*	23.3	22.7
Ser	28.1	27.7	30.8	30.8
Thr	29.2	30.5	31.1	32.7
Tyr	15.4	15.4	14.9	14.9
Val	30.1	30.6	27.3*	28.6*

[a] Expressed as mol/54 000 g; tryptophan, which averages 4 mol per chain in unresolved dimers, was not determined. Serine and threonine were extrapolated to zero hydrolysis time; 96-h value was used for valine. Asterisks denote those amino acids which differed in homologous chains by ±2 standard deviations or more; two independent samples were analyzed at three hydrolysis times each; CMC = carboxymethylcysteine.

B-tubulin has a higher proline content and the β subunit contains more phenylalanine then the A-tubule-derived counterparts. Significant differences are also seen in leucine and valine, with the first being higher in the A-tubulin α chain, while the second is higher in the B-tubulin β chain. Differences in aspartic and glutamic acids (or their corresponding amides) are difficult to quantitate precisely, but these computer-calculated analyses consistently show the subunits of B-tubulin to be higher in these amino acids than those of A-tubulin, a conclusion not totally in agreement with previous values determined by more subjective graphical methods (Stephens, 1970, 1975a). Differences in glycine content were also not corroborated by these analyses, and the large fluctuations sometimes seen in this amino acid could result from residual glycine introduced during preparative electrophoresis.

The amino acid composition of subunits from the comparatively "labile" singlet microtubules of the flagellar central pair and the egg cytoplasm is given in Table II. In terms of basic amino acids, the α chain of central-pair tubulin has two more residues each of arginine and lysine than its cytoplasmic counterpart. Conversely, the cytoplasmic β chain contains one more residue each of these amino acids than does that from central pair, although these differences are barely significant. In comparison with their outer-doublet counterparts, the α and β chains from either central-pair tubulin or vinblastine–tubulin are lower in either arginine, lysine, or both. Although the homologous α and β chains of central pair and cytoplasmic tubulin do not differ appreciably from each other in alanine content, the singlet-derived α chains are slightly lower in alanine (39 vs. 41 residues) than their doublet-derived counterparts, while the opposite is true for the corresponding β chains (35 vs. 32 residues). Both chains of vinblastine–tubulin, but particularly the α chain, have a notably higher content of phenylalanine and tryosine than those of the central pair or either of the outer-doublet tubulins, a fact that may reflect

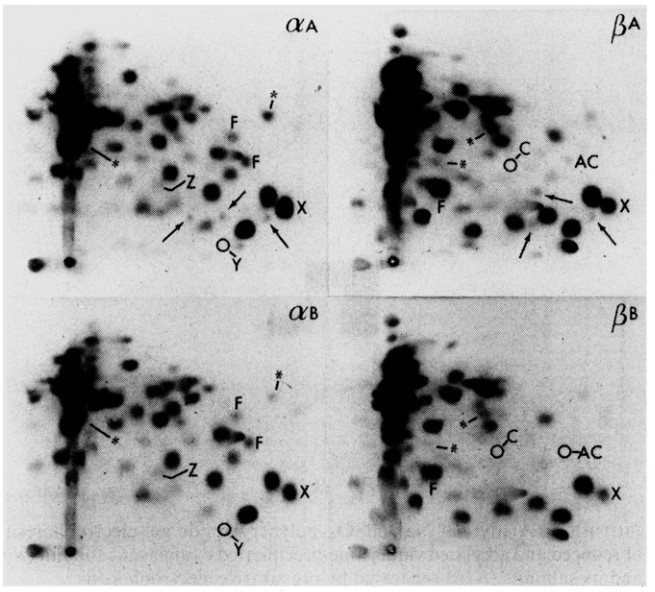

FIGURE 4: Tryptic peptide maps of flagellar A-tubule and B-subfiber tubulin subunits (αA/βA and αB/βB, respectively). Chromatography with chloroform–methanol–ammonium hydroxide (ascending) and electrophoresis at pH 3.5 (cathode to the right). See text for explanation of symbols.

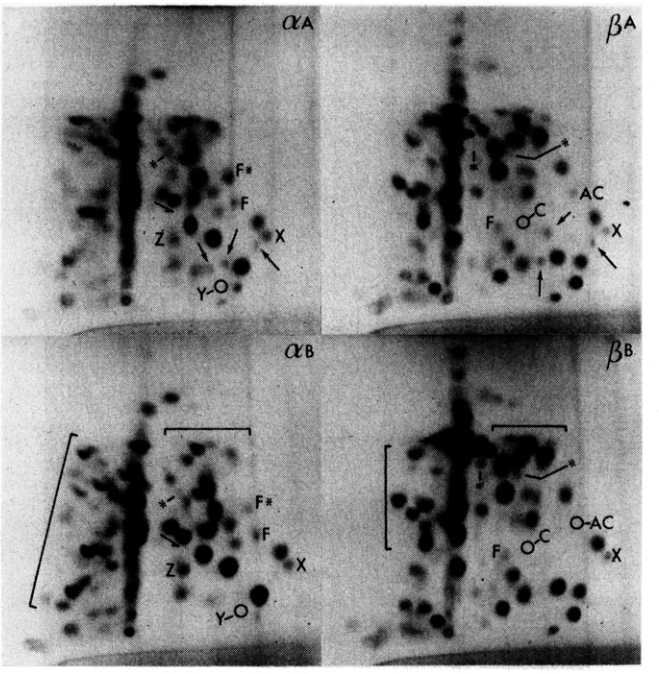

FIGURE 5: Tryptic peptide maps of flagellar A-tubule and B-subfiber tubulin subunits. Chromatography with 1-propanol-ammonium hydroxide (ascending) and electrophoresis at pH 6.5 (cathode on the right). See text for explanation of symbols.

TABLE II: Amino Acid Composition of Singlet Tubulin Chains.

Residue	αC	αV	βC	βV
Ala	39.0	39.8	34.5	35.7
Arg	21.4*	19.9*	19.7*	20.9*
Asx	46.1	46.4	48.3	51.3
CMC	9.2*	10.6*	7.6	7.3
Glx	63.2*	59.5*	66.7	64.8
Gly	40.6*	36.7*	44.4*	36.3*
His	12.3	12.6	10.2	10.0
Ile	25.5*	24.0*	18.4	17.9
Leu	36.8*	35.4*	33.9*	36.1*
Lys	21.7*	19.6*	16.2*	17.2*
Met	9.8	10.3	15.9	16.2
Phe	18.1*	21.3*	21.4*	24.5*
Pro	24.6*	22.6*	23.5*	22.7*
Ser	26.2	23.9	33.6*	29.1*
Thr	29.2	31.6	31.6	31.3
Tyr	14.3*	20.3*	14.6*	17.4*
Val	31.6*	35.1*	29.1*	30.8*

[a] Expressed as mol/54 000 g; tryptophan, which averages 4 mol per chain in unresolved dimers, was not determined. Serine and threonine were extrapolated to zero hydrolysis time; 96-h value was used for valine. Asterisks denote those amino acids which differed in homologous chains by ±2 standard deviations or more; two independent samples were analyzed at three hydrolysis times each; CMC, carboxymethylcysteine.

posttranslational modification of the cytoplasmic dimer via a tyrosine ligase (cf. Raybin and Flavin, 1977). Both the α and β subunits of central-pair tubulin have a higher proline content than their cytoplasmic equivalents, differing by 2 and 1 residues, respectively, but the difference for the β chain, though consistent, is marginal. Other major differences are seen in glutamic acid and glycine, while smaller differences are detectable in isoleucine, leucine, and valine between the α chains and in leucine, serine, and valine between the β chains of these singlet-derived dimers. Although the cysteine content of the cytoplasmic β chain does not differ from that of any of its flagellar counterparts, the number of cysteines in the α chain appears to be somewhat higher, in basic agreement with values reported for sea urchin egg and avian brain tubulin α chains by Bryan (1972) and Bryan and Wilson (1971), respectively.

Overall, there appears to be more similarity in amino acid composition between central pair and vinblastine-tubulin subunits than between the equivalent subunits of central pair and outer doublets, even though all of the latter are derived from the same organelle. The major differences occur in the basic amino acids, arginine and lysine, in the nonpolar amino acids, alanine, leucine, isoleucine, phenylalanine, and valine, and to some extent in proline. On this basis, one would expect tryptic cleavage to produce a certain number of characteristic peptides for each type of subunit, with some peptides differing in relative hydrophobicity.

Comparative Tryptic Peptide Mapping. Nanomole quantities of peptides from electrophoretically resolved α and β chains of sperm flagellar A, B, and central-pair tubulins, ciliary A and B fractions, and vinblastine-precipitated egg tubulin were compared at both pH 3.5 and 6.5, using different solvents for the chromatographic step to optimize the separation of peptides in both dimensions. Representative maps for all of these comparisons are presented here for direct inspection. Since all possible combinations could not be run at the same time, the relative degree of separation or the absolute migration distance in one or both dimensions will vary between sets. Consequently, peptides are most easily identified in terms of their general pattern, much as stars in a constellation are identified by their relative position within the group. The number of major peptides observed was generally 10–15% in excess of the number predicted from the amino acid composition of the respective subunit, indicating either a certain degree of incomplete digestion or else the presence of several very similar polypeptide chains. Since the patterns were consistent and reproducible for various preparations or digestions

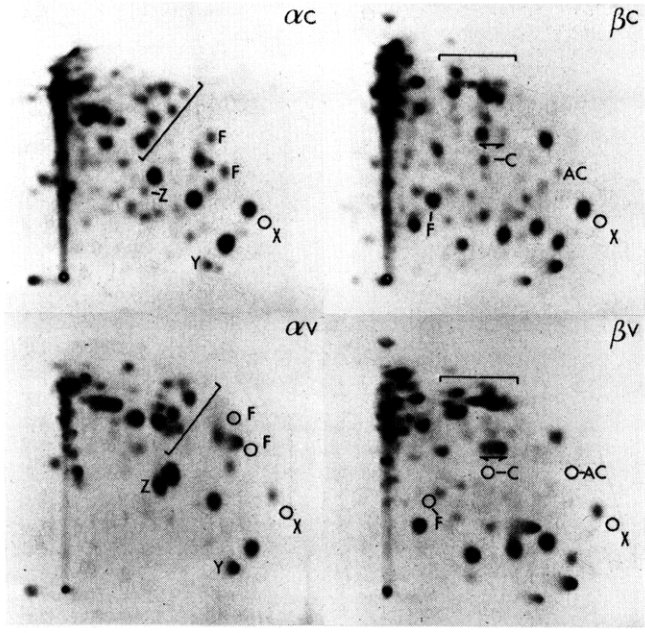

FIGURE 6: Peptide maps of flagellar central pair and vinblastine-tubulin subunits (αC/βC and αV/βV, respectively), at pH 3.5. Conditions as in Figure 4.

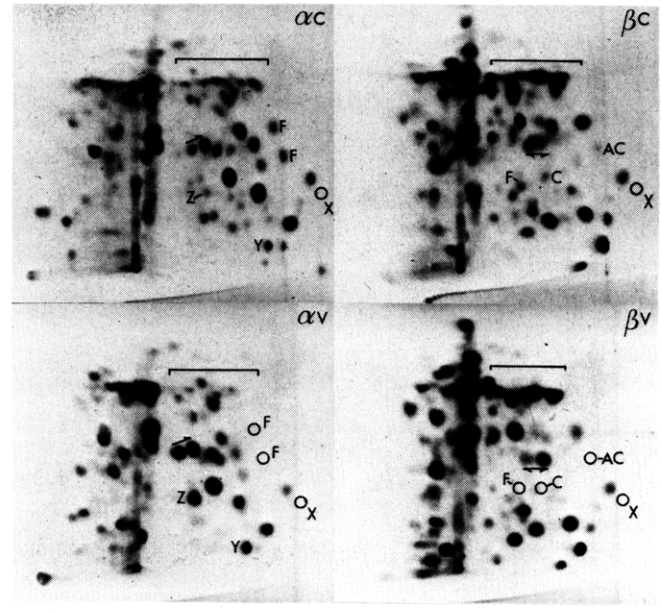

FIGURE 7: Peptide maps of flagellar central pair and vinblastine-tubulin subunits at pH 6.5. Conditions as in Figure 5.

of the same material, the presence of such peptides should not influence the final interpretation for purposes of comparison by subunit type.

A comparison of tryptic peptides from the α and β chains of tubulins derived from the A-tubule and B-subfiber of flagellar outer doublets is presented in Figures 4 and 5, where electrophoresis was carried out at pH 3.5 and 6.5, respectively. Regardless of the conditions of chromatography or electrophoresis, over 90% of the peptides in homologous chains are coincident. In fact, even the α and β chains appear to have at least one-third of their peptides in common, perhaps reflecting the postulated common ancestral origin of the two tubulin subunits (Ludueña and Woodward, 1973). As noted previously (Stephens, 1976a), three "extra" peptides are found in each of the subunits obtained from the A-tubulin dimer (Figures 4 and 5, arrows). These sets of three peptides appear in the same relative order under both sets of pH conditions in the respective subunits. When estimated by relative fluorescence, these peptides occur in roughly equimolar amounts relative to one another but in about half-molar amounts relative to most major peptides on the maps. This may indicate the presence of two distinct kinds of α and β chains in the A-tubule (i.e., mixed dimer types) but only one of each kind of chain in the B-subfiber. Certain other spots on the maps of homologous subunits differ in relative fluorescence intensity (Figures 4 and 5, asterisks), but direct comigration of the samples will not render these peptides separable with either chromatographic system or at either pH. Since different peptides may vary in the relative fluorescence of their fluorescamine reaction product, these could be distinctly different peptides with very conservative substitutions. Fluorescence differences may also reflect relative amount, and certain short sequences may be represented more than once in one chain but not in its homologue. Alternatively, since the separation of the A and B fractions is not absolute (some protein is solubilized from the tips of the A-tubules as the B-subfiber is melted, while some B-subfiber material remains attached to the A-tubule), certain peptides characteristic of one subfiber type may have arisen from contaminating tubulin of the other subfiber. The unique "minor" peptides in the lower right of the maps and the variable-intensity peptides above the origin and near the center of the maps appear to correspond to the characteristic A-tubulin peptides which were reported earlier to distinguish the unresolved chains of the A-tubule and B-subfiber-derived tubulin dimers (Stephens, 1970).

When digests of the α and β chains from central-pair and vinblastine-precipitated tubulin are compared at either pH 3.5 (Figure 6) or 6.5 (Figure 7), the homologous subunits are easily distinguished from each other and from those of the outer doublets. The most obvious difference is the total *absence* of a prominent peptide found in both α and β chains from the outer-doublet tubulins, designated as peptide X on Figures 4–7. Almost equally obvious is the *presence* of a unique peptide in the α chain of both central-pair and vinblastine tubulin, designated as peptide Y in these same figures. This peptide occurs in about twice the amount in the cytoplasmic α chain as in the central-pair counterpart; this may be judged from the marked fluorescence intensity difference in peptide Y in both Figures 6 and 7. Similarly, a less cathodic, more hydrophobic peptide is prominent in the α chain of cytoplasmic tubulin but somewhat less discernible in any of the flagellar tubulin counterparts. This peptide has been designated as Z in Figures 4–7. Conversely, a peptide is found in the β chain of central-pair tubulin that is not seen in any of the other β chains (Figures 4–7, marked C) and at least two other peptides in flagellar α chains and one in flagellar β chains are not readily detected in the respective cytoplasmic homologue (Figures 4–7, marked F). Also, many spots vary in intensity or relative position on maps of the homologous subunits of central-pair and cytoplasmic tubulin (Figures 6–7, brackets, double half-arrows). One obvious peptide in the α chain is shifted in the outer-doublet tubulin subunits in comparison with those of singlet tubulins (Figures 5 and 7, single half-arrow). Differences also occur in some of the faint, minor spots appearing primarily in the central-pair α chain. Though some of these may be real, many vary with the preparation and thus may be spurious background arising either from incomplete cleavage or from limited proteolysis during preparation. Even without consideration of any of these peptides, it should be evident from the five major peptide differences (X, Y, Z, C, and F) that the α

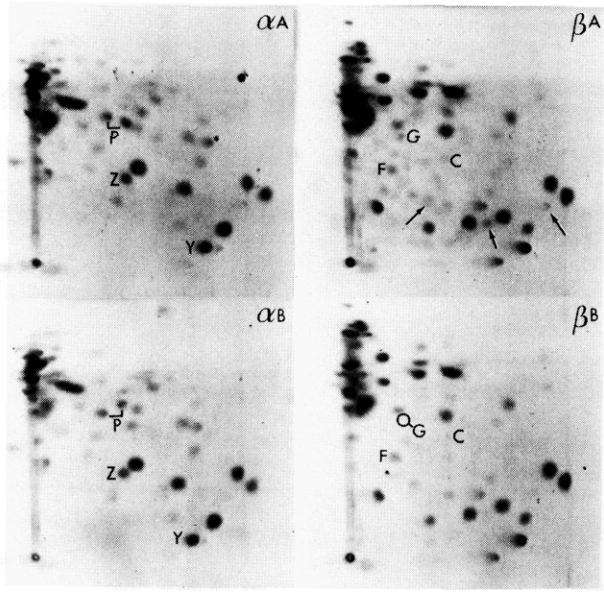

FIGURE 8: Peptide maps of ciliary A-tubule and B-subfiber tubulin subunits (αA/βA and αB/βB, respectively) at pH 3.5. See Figure 4 for conditions.

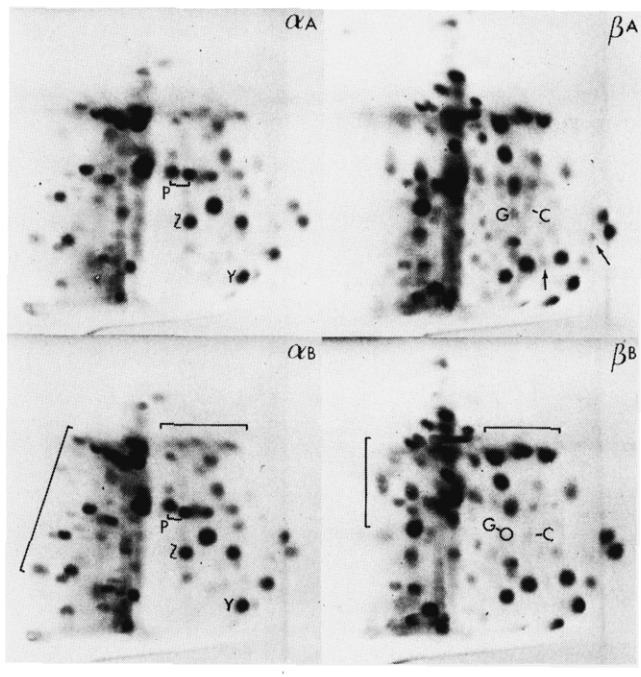

FIGURE 9: Peptide maps of ciliary A-tubule and B-subfiber tubulin subunits at pH 6.5. See Figure 5 for conditions.

and β chains of outer-doublet, central-pair, and cytoplasmic tubulins are distinct at a primary structural level. Interestingly, cytoplasmic and central-pair tubulins have certain prominent features in common, namely, the presence of peptide Y in the α chains and the absence of peptide X in both chains.

Peptide maps of A- and B-tubulin-enriched fractions from embryonic cilia run at pH 3.5 and 6.5 are illustrated in Figures 8 and 9, respectively. The interpretation here is slightly complicated by the fact that one member of the central pair is carried along with each tubulin fraction (13 out of 103 total protofilaments). This can explain *some* but clearly not *all* of peptide Y in the α subunit of both A- and B-tubulin. It may account fully for the trace of peptide C in the β subunits, assuming that ciliary and flagellar central pair have the same distinguishing peptides. Peptide C probably can be discounted when ciliary peptides are compared with flagellar ones, but the dominance of peptide Y appears to be a significant feature of ciliary outer-doublet α chains. Unlike the situation with flagellar α chains, that from the ciliary A-tubule cannot be distinguished unequivocally from that of the B-subfiber fraction by the presence of half-equivalent amounts of small, cathodic peptides. This is also the case for the α chains of the A- and B-tubulin fractions from scallop gill cilia, where the two homologous chains are indistinguishable (Stephens, 1976a, 1977c). However, as in both sea urchin flagella (above) and in scallop gill cilia, the β subunits from the two fractions *are* distinguishable by the presence of two small, cathodic peptides in the A-tubule-derived chain (Figures 8–9, arrows). Quite evident in the pH 3.5 maps but not as obvious at pH 6.5 is a positional difference (Figures 8–9, marked P), also detected in comigration, which *does* allow the A-tubule α chain to be distinguished from that of the B-subfiber. This is the only difference that has been detected; the maps are otherwise perfectly coincident. Furthermore, at neither pH do any of the α-chain peptides differ significantly in relative fluorescence between homologous chains.

It should be evident from comparison of Figures 4 and 8 (pH 3.5) or Figures 5 and 9 (pH 6.5) that neither the α nor the β subunit of either the A-tubule or B-subfiber-derived tubulin from embryonic cilia is equivalent to its sperm flagellar counterpart. The dominance of peptide Y in the ciliary α-chain maps is the most immediate distinguishing characteristic (Figures 4–5 vs. 8–9, marked Y). Positional differences in groups of both anodic and cathodic peptides are particularly obvious at pH 6.5 (Figure 9 vs. 5, brackets). At pH 3.5, peptide Z is barely discernible in flagellar subunits but is prominent in both of the ciliary α chains (Figure 8, marked Z), in spite of the lower sample loading. One "F-peptide", so prominent in the flagellar subunits, is substantially less obvious in the ciliary β chain (Figure 8, marked F). As is also the case with scallop gill cilia (Stephens, 1976a, 1977c), a peptide of intermediate chromatographic and electrophoretic mobility is present in the β chain derived from the A-tubule but is absent in that from the B-subfiber (Figures 8–9, marked G). This difference in subfiber-specific tubulin chains of cilia is not detectable in the sperm flagellar counterparts (Figures 4–5).

The maps presented here are representative of over 200 that have been run to date. The preparations used for Figures 4 and 5 were totally independent, produced a year apart. The same preparation was used for Figures 8 and 9. The former is comparatively underloaded and the image is understabilized, while the latter is optimally loaded and requires no stabilization. These represent examples of the worst and the best results typically obtained with this technique. In spite of such differences in sample origin or mapping procedures, the conclusions concerning the major common and unique peptides are basically the same. For convenience in comparison, these differences are summarized in Table III.

Discussion

Regardless of source, certain characteristics of the amino acid composition readily allow one to distinguish an α- from a β-tubulin chain. The α chains typically contain 12–13 histidines, 8–10 methionines, 8–10 cysteines, and 39–41 alanines, while the β chains contain 10–11 histidines, 15–16 methionines, 7–8 cysteines, and 32–36 alanines. Less significant differences occur among other amino acids, but, nevertheless, the α and β chains of tubulin have quite distinctive profiles (cf. Bryan and Wilson, 1971; Bryan, 1972; Ludueña and Woodward,

TABLE III: Characteristic Tryptic Peptides of Tubulin Chains.[a]

Peptide	αA_f	αA_c	αB_f	αB_c	αC	αV	βA_f	βA_c	βB_f	βB_c	βC	βV
X	++++	+++	+++	+++	0	0	+++	++++	++	++++	0	0
Y	0	+++	0	+++	++	+++	−	−	−	−	−	−
Z	+	+++	+	+++	+	++++	−	−	−	−	−	−
F	++	+	++	+	++	0	+++	+	++	+	+++	0
C	−	−	−	−	−	−	0	+*	0	+*	++	0
G	−	−	−	−	−	−	0	+	0	0	0	0

[a] Chain nomenclature: A, B, C, and V designate chains derived from A-tubules, B-subfibers, central-pair, and vinblastine-precipitated tubulin, respectively. The subscripts designate the source, flagella (f) or cilia (c). Relative intensities are indicated from 0 (absent) to ++++ (very intense). Asterisk denotes peptides which were probably derived from ciliary central pair. These evaluations were based primarily on maps run at pH 3.5, since these generally accentuate fluorescence differences.

1973). If one takes the amino acid composition differences seriously, however, one must also conclude that none of the homologous chains analyzed in this present study are identical by this criterion.

Again, regardless of source, one can always classify a subunit by its highly characteristic α or β peptide map. Such maps are remarkably similar overall, with most peptides having a corresponding counterpart, albeit sometimes with an altered mobility. Although shown here for the sea urchin, this is also true for the subunits of molluscan ciliary tubulin (Stephens, 1976a, 1977c). Thus, the general architecture of the tubulin α or β chain has been highly conserved with respect to species and organellar source, but a cursory inspection of the peptide maps will reveal a certain degree of "homology" even between the heterologous chains, both conclusions drawn originally by Ludueña and Woodward (1973) from primary sequence data.

Certain systematic differences in amino acid composition become evident when organellar source is taken into consideration. Both this and previously published data from a variety of sources (cf. discussion in Stephens, 1975a) would indicate a higher basic amino acid content for outer-doublet-derived tubulin chains than those from the central pair. The α chain of central pair, in turn, has a higher basic amino acid content than that from cytoplasmic tubulin. The β chain of cytoplasmic tubulin exceeds that of central pair in both lysine and arginine content, this being the exception to the generalization that tubulins derived from more stable tubules have more basic amino acids. These apparent differences are interesting in that no dramatic differences in isoelectric point have ever been reported for these tubulins, implying that the total charge for each subunit type has been highly conserved also. Microheterogeneity has been observed frequently for flagellar and cytoplasmic tubulins (e.g., Witman et al., 1972; Feit et al., 1977), but any correspondence between isoelectric variants and an organelle-specific tubulin has yet to be established.

Organelle-specific differences in the tubulins are readily revealed by rather dramatic additions, deletions, or shifts in the otherwise quite similar peptide maps of homologous subunits. Outer doublet tubulin subunits, whether from sperm flagella or embryonic cilia, contain a unique, highly basic peptide (X) that is absent from the corresponding subunits of either sperm flagella central pair or egg cytoplasmic tubulin. Homologous outer-fiber subunits from cilia are distinguished from those of flagella by numerous positional differences in hydrophobic peptides in both subunits and by the presence in ciliary α chains of less-basic, small peptide (Y) shared with cytoplasmic and sperm flagella central-pair α chains. In fact, in spite of the species difference, there appears to be more similarity between peptide maps of sea urchin embryonic and molluscan gill ciliary subunits than between homologous subunits of sea urchin sperm flagella and blastula cilia (cf. Stephens, 1976a, 1977c, for molluscan cilia subunit maps). Cytoplasmic and central-pair tubulin can be distinguished by the presence of several "9 + 2"-specific peptides (F) in both chains of the latter and by the abundance of an even less basic, moderately hydrophobic peptide (Z) in the α chain of the former. One can conclude from these comparisons that the homologous α and β chains from flagellar and ciliary outer doublets, flagellar central pair, and egg cytoplasmic tubulin all differ at a primary structural level. From the gross similarity, however, one must also conclude that any differences that are dispersed along the polypeptide chain must be a highly conserved nature (since most peptides either coincide or have near equivalents) and that any nonconserved differences which may lead to organelle-specific properties most likely occur in very local regions of the molecule (since unique sets of peptides are either present or absent). Like the immunoglobulins, the tubulins probably have constant and variable regions, with the latter lending them specificity with regard to assembly or bonding to accessory proteins.

The differences between homologous chains of the A-tubule and B-subfiber-derived tubulins are hardly dramatic. The α and β chains of flagella (but only the β chains of cilia) differ in that the A-tubule-derived chain contains several unique peptides in roughly half-equivalent amounts. Such peptides could arise from posttranslational modification of half of the chains or from the presence of two types of homologous chains in each dimer. That these differences are not artifactual is supported by the fact that the same unique peptides are detectable in the β chains of sea urchin flagella, sea urchin cilia, and scallop gill cilia tubulin. That they may arise from two kinds of homologous chains in each subfiber-specific tubulin dimer preparation is supported by recent findings of two electrophoretically resolved α chains in sea urchin ciliary A-tubules and mitotic tubulin (Bibring et al., 1976) and also in mammalian brain (Lu and Elzinga, 1977). Furthermore, Witman and co-workers (1972) reported that both α- and β-tubulin from *Chlamydomonas* flagella (then called tubulin 1 and 2, respectively) could be resolved further into at least two isoelectric variants, a fact that also holds true for electrophoretically pure α or β chains derived from the various sea urchin tubulin fractions discussed above (Stephens, in preparation). The presence of two kinds of α chain or two kinds of β chain would lead to a model in which microtubules are built of two alternating types of $\alpha\beta$ heterodimer (Bibring et al., 1971). If both chains had two homologues which could be combined to form the four possible dimer types, quite complex microtubule structural models could be constructed.

Nevertheless, a single primary structural difference in either an α or a β chain between the A-tubule and B-subfiber-derived dimers would indicate subfiber specificity, regardless of

whether there were also multiple chains of each type or one chain held in common between subfibers. The dominance of several unique peptides in the flagellar A-tubulin α and β chains, the positional differences seen in ciliary α chains, and the unique peptides in the ciliary A-tubulin β chain would all argue in favor of this point. The fact that the specific activity of both chains of tubulin in ciliary A-tubules is higher than that of the B-subfiber homologues after ciliary regeneration in the sea urchin embryo (Stephens, 1976a, 1977a) would indicate separate pools for the tubulins destined for the two members of the outer doublets and hence control by separate genes. Neither the peptide-mapping data nor this protein-synthesis study can eliminate the possibility that one dimer type might be shared between outer-doublet members while a unique, second type may be incorporated into one subfiber and not the other. This situation would certainly be consistent with the presence of half-equivalent amounts of some peptides or a 50% difference in specific activity between A-tubule and B-subfiber tubulins.

Other kinds of organelle-specific synthetic differences in sea urchin embryos would also point to distinct tubulin dimer types, independent of any biochemical data. Bibring and Baxandall (1977) demonstrated clearly that the tubulin incorporated into the mitotic apparatus at first division was drawn largely from a preexisting, prefertilization pool, for no more than 0.4% of the mitotic tubulin was synthesized between fertilization and (first) metaphase. Similarly, the bulk of the tubulin incorporated into blastula cilia was shown to be synthesized after fertilization at a rate which maximized to a near-constant, steady-state after first division (Stephens, 1972). Also, pulse–chase labeling before fertilization indicates that no more than 3% of the ciliary tubulin could be presynthesized (M. M. Pratt, in preparation). Taken together, these data imply that a large pool of prefertilization tubulin is destined for mitotic apparatus assembly but that little or no tubulin is synthesized before fertilization for later cilia formation. Quite independently, one can reach this same conclusion from the fact that the peptide maps of prefertilization, vinblastine-precipitated tubulin contain no detectable "9 + 2"-specific peptide (X) or the other, less obvious peptides characteristic of blastula ciliary tubulin subunits. Thus, prefertilization tubulin can be accurately described as cytoplasmic or mitotic in destiny, while the cilia-specific tubulins must be synthesized after fertilization.

The absence of the outer-doublet-specific peptide X and the presence of the cytoplasmic-specific peptide Y in the central-pair tubulin dimer was an unexpected result. The presence of flagella (or cilia) specific peptides (F) immediately distinguishes this flagellar protein from cytoplasmic tubulin, and one might conclude that the sequence associated with peptide X is involved somehow with the ability to form doublets or to bind doublet-specific proteins, since it is found only in tubulin chains derived from outer fibers. Conversely, peptide Y could reflect the property of forming singlets, but its marked presence in embryonic cilia negates this. The presence of peptide Y in cytoplasmic and ciliary tubulins might reflect the maternal origin of these proteins, but its presence in the sperm central-pair tubulin (formed when the diploid genome must be functional) argues against egg-specific proteins as such. The simplest explanation is that all of these tubulins represent separate gene products, made at a specific time for a specific function.

One could mount an argument for "prototubulin" chains which are cleaved at different points to produce organelle-specific tubulin(s) at the appropriate point in development. In the case of the sea urchin, the prefertilization tubulin appears to be a poor candidate for such a molecule, since it is the least complex tubulin investigated. One could also argue, however, that specific tubulins are built up through terminal additions to a cytoplasmic tubulin. The numerous positional differences between cytoplasmic and ciliary or flagellar outer-doublet tubulins would argue against this, as would all of the protein synthetic data discussed above. However, the lesser differences among certain tubulin subunits could be due, at least in part, to a specific cleavage, terminal addition, or amino acid side-chain modification. In terms of the last mechanism, Eipper (1972) has demonstrated that the α subunit of rat brain tubulin is phosphorylated, Piperno and Luck (1976) found β-chain phosphorylation in *Chlamydomonas* flagella, and Stephens (1975a) showed that both chains of sea urchin flagellar outer-doublet tubulins may be phosphorylated. No direct correlation has yet to be made between phosphorylation and either tubulin function or specificity. Tubulin derived from synaptosomes, but not that from the axoplasm, has been shown to be a glycoprotein (Feit and Shelanski, 1975), while ciliary membrane tubulin, but not that of the axoneme, is likewise a glycoprotein (Stephens, 1977c). Raybin and Flavin (1977) demonstrated that the brain tubulin α chain is tyrosylated via a tubulin–tyrosine ligase, but this modification does not appear to modulate in vitro tubulin assembly. Recently, Nath and Flavin (1977) showed that synaptosomal (membrane) tubulin is not tyrosylated (though it may be through addition of the ligase), while the cytoplasmic counterpart is. Glycosylation and tyrosylation thus appear to offer ways for specifying membrane-associated and cytoplasmic tubulins, respectively, from presumably the same molecule. A fourth posttranslational mechanism, methylation, is probably not involved in tubulin modification, since numerous studies have failed to demonstrate the presence of methylhistidine or methyllysine in either flagellar or brain tubulin. The presence of modified and unmodified versions of the same peptide may explain some of the "ghost" spots consistently seen in maps of certain tubulin chains, for example, in the α subunit of both cytoplasmic and central pair. Regardless of how they arose, the fact remains that there are differences in all of the tubulin subunits, raising the question of how a developing embryo sorts out such very similar molecules during specific microtubule formation.

Spatial and temporal controls of microtubule polymerization remain as major questions if one accepts the idea of several kinds of tubulin dimer in one cell. Temporal control of synthesis, such as has been shown to exist in the early embryo, clearly provides substantial preexisting cytoplasmic tubulin for mitotic apparatus assembly well before any appreciable ciliary tubulin has begun to be synthesized and even late in development the amount of ciliary tubulin is only a small fraction of the total tubulin present. Since cell division and cilia formation do not occur simultaneously but, rather, sequentially, one would only need to somehow inactivate the cytoplasmic tubulin and provide a functioning basal body for the (now activated?) ciliary tubulin to assemble upon. Considering the relative ease of heterologous polymerization in vitro, separating outer-doublet from central-pair tubulin during cilia formation appears to pose more of a problem, but recent work by Dentler and Rosenbaum (1977) provides a very interesting mechanism for spatial control. Outer doublets appear to grow from their free, distal end (the favored end in in vitro polymerization experiments), while the central pair is "capped off" at the distal end of the growing cilium and is evidently forced to grow from its proximal (nonfavored) end. Since both sets of tubules grow at essentially the same rate but in opposite directions, the outer doublets must recruit subunits from the distal tip of the cilium while the central pair probably incor-

porates tubulin immediately from the proximal, basal region. Subtle primary structural differences in the respective tubulins may provide the specific charge distribution or steric basis for favoring (disfavoring) incorporation of the proper tubulin at the proper, polar, physically distinct site, thus providing the required sorting mechanism.

The definition of a tubulin chain type by gross morphological fractionation and its characterization through peptide mapping have obvious limitations. To further clarify the nature of organelle-specific tubulins, two basic approaches must now be pursued. For a mixture of tubulins, such as that which might be obtained from a developing embryo or tissue, techniques must be devised which will enable one tubulin chain to be distinguished from its homologues through subtleties in either isoelectric point or relative hydrophobicity. The initial morphological identities, such as those defined here, should help to set the groundwork for this task. Once the functional identities of particular chains are established, the question of the nature of their specificity must be explored on a more refined primary structural level. Dissection of the postulated variable region(s) from the remainder of the molecule and determination of its comparative sequence should provide information on the kinds of substitutions responsible for the organelle specificity of particular tubulins.

References

Amos, L., and Klug, A. (1974), *J. Cell Sci. 14,* 523–549.

Behnke, O., and Forer, A. (1967), *J. Cell Sci. 2,* 169–192.

Bibring, T., and Baxandall, J. (1977), *Dev. Biol. 55,* 191–195.

Bibring, T., Baxandall, J., Denslow, S., and Walker, B. (1976), *J. Cell Biol. 69,* 301–312.

Bryan, J. (1971), *Exp. Cell Res. 66,* 129–136.

Bryan, J. (1972), *J. Mol. Biol. 66,* 157–168.

Bryan, J., and Wilson, L. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 1762–1766.

Dentler, W. L., and Rosenbaum, J. L. (1977), *J. Cell Biol. 74,* 747–759.

Eipper, B. A. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 2283–2287.

Feit, H., and Shelanski, M. L. (1975), *Biochem. Biophys. Res. Commun. 66,* 920–927.

Feit, H., Neudeck, U., and Baskin, F. (1977), *J. Neurochem. 28,* 697–706.

Fulton, C., and Kowit, J. D. (1975), *Ann. N.Y. Acad. Sci. 253,* 318–332.

Fulton, C., Kane, R. E., and Stephens, R. E. (1971), *J. Cell. Biol. 50,* 762–773.

Kowit, J. D., and Fulton, C. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 2877–2881.

Linck, R. W. (1976), *J. Cell Sci. 20,* 405–439.

Lu, R. C., and Elzinga, M. (1977), *Anal. Biochem. 77,* 243–250.

Ludueña, R. F., and Woodward, D. O. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3594–3598.

Nath, J., and Flavin, M. (1977), *J. Cell Biol. 75,* 280a.

Olmsted, J. B., Witman, G. B., Carlson, K., and Rosenbaum, J. L. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 2273–2277.

Piperno, G., and Luck, D. J. (1974), *J. Cell. Biol. 251,* 2161–2167.

Raybin, D., and Flavin, M. (1977), *J. Cell Biol. 73,* 492–504.

Stephens, R. E. (1970), *J. Mol. Biol. 47,* 353–363.

Stephens, R. E. (1972), *Biol. Bull. 142,* 489–504.

Stephens, R. E. (1975a), in Molecules and Cell Movement, Inoué, S., and Stephens, R. E., Ed., New York, N.Y., Raven Press, pp 181–206.

Stephens, R. E. (1975b), *Anal. Biochem. 65,* 369–379.

Stephens, R. E. (1976a), in Contractile Systems in Non-Muscle Tissues, Perry, S. V., Margreth, A., and Adelstein, R. S., Ed., Amsterdam, Elsevier/North-Holland Biomedical Press, pp 241–254.

Stephens, R. E. (1976b), *J. Cell. Biol. 70,* 95a.

Stephens, R. E. (1977a), *Dev. Biol. 61,* 311–329.

Stephens, R. E. (1977b), *Biol. Bull. 153,* 446–447.

Stephens, R. E. (1977c), *Biochemistry 16,* 2047–2058.

Stephens, R. E. (1978), *Anal. Biochem. 84,* 116–126.

Witman, G. B., Carlson, K., and Rosenbaum, J. L. (1972), *J. Cell Biol. 54,* 540–555.