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Chemical Differences Distinguish Ciliary Membrane and Axonemal Tubulins†

R. E. Stephens

ABSTRACT: Tubulin was prepared by exhaustive dialysis solubilization from axonemal A and B subfibers and by detergent solubilization of the membrane of cilia from the scallop *Aequipecten irradians*. The respective α and β chains were isolated by preparative sodium dodecyl sulfate ($NaDodSO_4$)-polyacrylamide gel electrophoresis and then compared by amino acid analysis and high-resolution two-dimensional tryptic peptide mapping. Minor amino acid and peptide differences distinguished A- and B-subfiber-derived tubulin subunits from each other, but far more significant amino acid differences distinguished the membrane-derived subunits from those of the axoneme. Peptide mapping revealed that each membrane tubulin subunit contained two major peptides not coincident with those of the axoneme and vice versa, but many corresponding peptides differed markedly in relative intensity. The α and β subunits from these sources showed virtually identical isoelectric points. Certain $NaDodSO_4$-polyacrylamide gel systems, sensitive to differential detergent binding, allowed the membrane-derived subunits to be distinguished from those of the axoneme. Under nondenaturing conditions, the membrane-derived tubulin bound both anionic and cationic detergents more strongly than axonemal tubulin. These data indicate that ciliary membrane tubulin is a distinct molecule whose subunits have the same molecular weight and isoelectric point as those of axonemal tubulin but differs chiefly in terms of nonpolar, conservative substitutions. These chemical differences argue against the artifactual origin of the protein from breakdown of the axoneme.

In a comparison of membranes from molluscan gill cilia with those from sperm flagella, it was found that the latter were composed chiefly of a 250 000-dalton glycoprotein while the former contained a like amount of 55 000-dalton, mildly PAS-positive (periodic acid–Schiff base-positive)[1] protein. The 55 000-dalton ciliary membrane protein was identified as a tubulin on the basis of electrophoretic separation into two equimolar chains, size distribution of CNBr fragments, tryptic peptide mapping, and colchicine binding (Stephens, 1977a). In a study utilizing a variety of membrane preparation methods, Dentler (1980) showed that, regardless of the method, the ciliary membrane of *Tetrahymena* was also composed chiefly of a 55 000-dalton, PAS-positive protein whose combined equimolar subunits yielded tryptic peptides characteristic of a tubulin dimer. A preliminary vectorial labeling study indicated that molluscan ciliary membrane tubulin was an integral membrane protein (Stephens, 1977b) while a later study demonstrated cross-linkage of both the molluscan and the ciliate 55 000-dalton protein within the membrane bilayer by a lipophilic, bifunctional reagent (Dentler et al., 1980).

By no means is the claim for a 55 000-dalton, tubulin-like major ciliary membrane protein a universal one. Two sets of workers (Adoutte et al., 1980; Brugerolle et al., 1980) find only small amounts of such a protein in membrane vesicles prepared from *Paramecium* cilia. Flagellar membranes typically contain none (Witman et al., 1972; Stephens, 1977a), although there are several reports to the contrary (Otter, 1978; Adair & Goodenough, 1978). The novelty of tubulin—a classic cytoplasmic protein—in a ciliary membrane, the dominance of tubulin as a structural component of the 9 + 2 axoneme, and the near absence of tubulin in membranes of related organelles or organisms raise the obvious question of artifact.

Based upon simple quantitation, it was argued previously that breakdown of the axoneme during isolation was a very unlikely source for the tubulin found in detergent-solubulized membrane fractions (Stephens, 1977a; Dentler, 1980). Even if both labile central pair members dissolved totally, they could account for only one-third of the tubulin found in the membrane fraction. However, the amorphous ciliary matrix might contribute unpolymerized axonemal tubulin awaiting assembly. This protein would be subject to solubilization by detergent or to entrapment during vesicle preparation. Such a protein might be identifiable as arising from 9 + 2 microtubules through some specific chemical property.

This report presents further biochemical characterization of the molluscan 55 000-dalton ciliary membrane protein, concluding that it is a tubulin isotype differing significantly in its chemical properties from the tubulin of the axoneme. A preliminary account of this work was presented at the 24th Annual Meeting of the Biophysical Society, New Orleans, LA, June 1-5, 1980 (Stephens, 1980).

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; PAS, periodic acid–Schiff base; DCC, dicyclohexylcarbodiimide; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

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Experimental Procedures

Protein Preparation. Cilia were isolated from the gill of the bay scallop *Aequipecten irradians* by hypertonic shock, purified by differential centrifugation, and demembranated with 0.25% Nonidet P-40, 3 mM $MgCl_2$, and 30 mM Tris-HCl (pH 8), and the resultant 9 + 2 axonemes were sedimented at 25000*g* for 15 min. They were then fractionated into A tubules and solubilized B subfibers by long-term, low ionic strength dialysis as described in detail elsewhere (Linck, 1973; Stephens, 1977a). The A tubules were recovered by centrifugation at 25000*g* for 15 min. Nonidet P-40 was removed from the membrane extract by gentle agitation with one-third volume of Bio-Beads SM-2 (Holloway, 1973). The protein from both the membrane extract and the solubilized B subfibers was precipitated at 0 °C by adjusting the pH to 4.5 with 0.1 M acetic acid; the flocculent precipitate was recovered by low-speed centrifugation.

A tubules, B-subfiber protein, and detergent-solubilized membrane fractions were reduced and carboxymethylated with sodium iodoacetate in 5 M guanidine-HCl as described previously (Stephens, 1977a). Alternatively, they were carboxamidomethylated with iodoacetamide, taking care to maintain a pH of 8.0–8.5 during the course of the reaction. The protein was freed of excess guanidine-HCl and mercaptoethanol by exhaustive dialysis, a 10% aliquot was dansylated, and the total sample was subjected to fluorescent preparative NaDodSO$_4$–polyacrylamide gel electrophoresis (Stephens, 1975, 1977a). The resultant solutions of α- and β-tubulin were dialyzed exhaustively against either 0.1 M ammonium bicarbonate (pH 8) for tryptic digestion or 0.01 M sodium phosphate (pH 7) for acid hydrolysis. This procedure results in near-quantitative recovery of electrophoretically pure tubulin subunits, as documented previously for this same and related fractionations (Stephens, 1975, 1976, 1977a, 1978a).

Peptide Mapping. The respective α- and β-tubulin chains from the ciliary A-tubule, B-subfiber, and detergent-solubilized membrane fractions were digested for 24 h at 25 °C with DCC- or TPCK-treated trypsin at a 1:100 enzyme:protein ratio. The peptides (0.5–1.1 nmol; 25–60 μg) were spotted on silica gel G or GHL (Analtech) thin-layer plates and subjected to ascending chromatography with either chloroform–methanol–ammonium hydroxide (2:2:1 v/v) or 1-propanol–ammonium hydroxide (7:3 v/v), followed by perpendicular electrophoresis with pyridine–acetic acid–water at either pH 3.5 (1:10:489 v/v) or pH 6.5 (100:3:897 v/v). The resolved peptides were visualized at 366 nm with fluorescamine, stabilized and enhanced with triethylamine, and then photographed through appropriate barrier filters. The resultant negatives were copied, and the internegatives were printed on high-contrast paper. Details of this mapping procedure appear in detail elsewhere (Stephens, 1978a,b).

Amino Acid Analysis. Samples of A-tubule, B-subfiber, and membrane-derived α- and β-tubulin were evaporated to dryness under vacuum and then hydrolyzed in 6 N HCl at 110 °C for 24, 48, or 96 h. Two different samples, of carboxymethylated and of carboxamidomethylated subunits, were analyzed at each of these three hydrolysis times on a Durrum D-500 automated, single-column amino acid analyzer (AAA Laboratory, Mercer Island, WA). Serine and threonine were extrapolated to zero hydrolysis time. With the exception of the latter amino acids, two independent sets of three values were thus obtained for each subunit type from the three protein sources in question. The values for each subunit type were averaged, and the means for each source were compared by Student's *t* test.

Isoelectric Focusing. The two-dimensional method of O'Farrell (1975) was used to determine relative purity and subunit identity in the tubulin fractions. The isoelectric dimension was performed essentially as described, the only variations being in the sample treatment, which was occasionally by the method of Ames & Nikaido (1976), and in the use of a pH 4–6 major ampholyte (Bio-Rad Laboratories: Bio-Lyte 4/6). The second dimension was carried out on a uniform 7.5T/2.6C polyacrylamide slab. Isoelectric point determination was performed on a 0.15 cm thick × 10 cm long slab gel of the O'Farrell (1975) formulation but using a pH 4–6 major ampholyte. Empty lanes, adjacent to the sample in question, were excised, cut into 1-cm segments and extracted with 1 mL of 0.1 M NaCl for 6 h, and the pH was determined by conventional means.

Comparative NaDodSO$_4$–Polyacrylamide Gel Electrophoresis. Two closely related gel systems were utilized on replicate samples of tubulin fractions. A simple, continuous 0.1% NaDodSO$_4$ and 25 mM Tris–glycine (pH 8.3) buffer system was used for both preparative electrophoresis and sample monitoring; the gel composition was 5.0T/2.6C (Bryan, 1974; Stephens, 1975). The standard, discontinuous system of Laemmli (1970) was employed with 5-mm i.d. × 10 cm long gel cylinders and 1.5 mm thick × 10 cm long gel slabs; the gel composition was 7.5T/2.6C. The running buffer in both of these systems was identical. The samples were applied to both systems in 1% NaDodSO$_4$, 1% mercaptoethanol, 10% sucrose, and 2.5 mM Tris–glycine (pH 8.3). The gels were run at 10 V/cm, constant voltage. Staining was by the method of Fairbanks and co-workers (Fairbanks et al., 1971).

Charge-Shift Electrophoresis. The amphiphilic vs. hydrophilic nature of the dialysis-solubilized B-subfiber tubulin and the detergent-solubilized membrane tubulin was compared under nondenaturing conditions by high-voltage agarose electrophoresis in the presence of Triton X-100 and either sodium deoxycholate or cetyltrimethylammonium bromide (Helenius & Simons, 1977). A Shandon flatbed electrophoresis unit was employed, the platen being cooled to 4 °C by water circulation. Bovine serum albumin served as a hydrophilic mobility standard. Proteins (10–20 μg) were applied to 1.5 × 5 mm wells in the center of a 6 × 7 cm 1% agarose slab of 2.0–2.5-mm thickness. Buffer compositions, electrophoresis conditions, and staining procedures were exactly as described by Helenius & Simons (1977).

Results

Comparative Tryptic Peptide Mapping. Tryptic peptides of carboxymethylated α- and β-tubulin from the A-tubule, B-subfiber, and detergent-solubilized membrane fractions of gill cilia were subjected to chromatography in chloroform–methanol–ammonium hydroxide and electrophoresis at pH 3.5. Examples of the resultant fluorescamine-visualized peptide maps are illustrated in Figure 1. As shown previously with composite tracings (Stephens, 1976, 1977a), the α chains derived from the A tubule and B subfiber are virtually identical while the β chains differ in the presence of substoichiometric amounts of three peptides found only in the A-tubule-derived chain (Figure 1, β-B, circles). One peptide, not noted previously, is found in the α chain of the B subfiber but not in that of the A tubule (Figure 1, α-A, circle; α-B, asterisk).

The membrane-derived subunits map very much like tubulins, having over 90% coincidence of peptides. The membrane α and β chains each contain a pair of prominent peptides not found in the axonemal counterparts (Figure 1, α-M/β-M, arrows). Likewise, there are three peptides in the α chain and four in the β chain that are prominent in the axonemal subunits

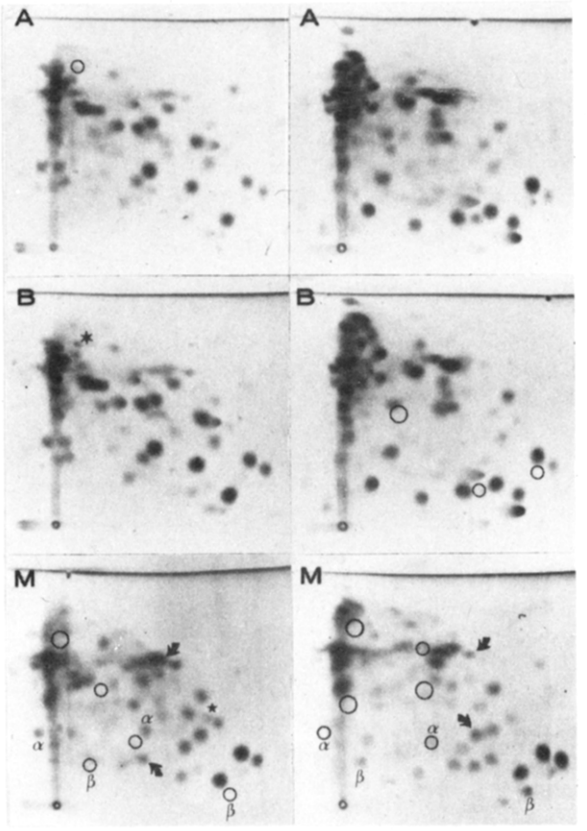

FIGURE 1: Tryptic peptide maps of carboxymethylated A-tubule (A), B-subfiber (B), and membrane-derived (M) α- and β-tubulins (left and right panels, respectively). Chromatography in chloroform–methanol–ammonium hydroxide; electrophoresis at pH 3.5. α chains (left panel): Peptide in B (asterisk) is absent in A (circle). β chains (right panel): Three peptides in A are absent in B (circles). Both chains: Two peptides are present in M (arrows) which are absent in A and B; other peptides (unmarked circles in M) are characteristic of A and B. Greek-lettered spots and circles designate α- or β-chain-specific peptides, indicating little cross-contamination. Star in M, shifted peptide.

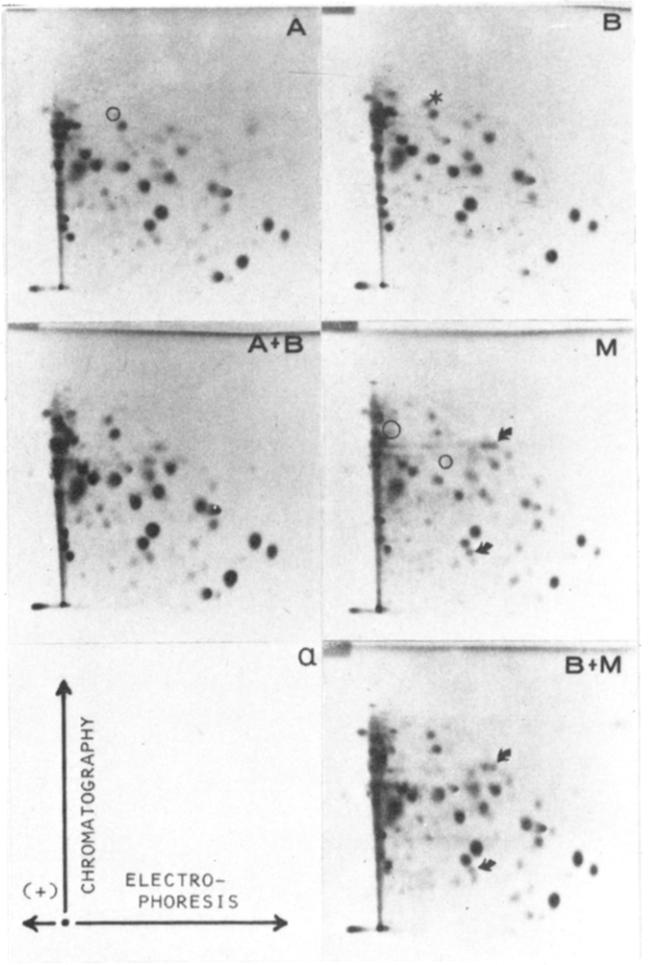

FIGURE 2: Tryptic peptide maps of carboxamidomethylated α-tubulin from the A-tubule (A), B-subfiber (B), and membrane (M) fractions. Chromatography in chloroform–methanol–ammonium hydroxide; electrophoresis at pH 3.5. Peptide in B (asterisk) is absent in A (circle). Two peptides are present in M (arrows) which are absent in A and B, while two peptides are absent in M (circles) which are present in A and B.

but absent or reduced in intensity in the membrane counterpart (Figure 1, α-M/β-M, unmarked circles). A prominent comet-shaped peptide (Figure 1, α-M, star) is diminished in intensity and shifted in position in the membrane α chain. These differences between membrane-derived and axoneme-derived chains confirm and extend those reported previously, performed with less material and presented as tracings (Stephens, 1977a).

By use of a more controlled derivatizing procedure, comparable samples were carboxamidomethylated and subjected to preparative electrophoresis. Having quite different charge parameters than when carboxymethylated, it was determined that the tubulin subunits migrate with considerably less separation, apparently a result of differential detergent binding. Differences in migration of the two derivatives result in relative positional differences of 10.5 mm for the α chains and 3.5 mm for the β chains on a 15-cm preparative gel run 25% longer than the ion-front migration time. Consequently, if peptide map differences were due to comigratory contaminants, one might not expect the same contaminating proteins in both the carboxymethyl and carboxamidomethyl derivatives. Peptide maps of the carboxamidomethylated α and β chains, performed at both pH 3.5 and 6.5, are illustrated in the following four figures.

In terms of α chains, the single peptide distinguishing the B-subfiber-derived subunit noted above is obvious at both pH 3.5 and 6.5 (Figures 2-A and 3-A, circles; Figures 2-B and 3-B, asterisks). Its minimal presence in the A-tubule-derived subunit is likely due to incomplete separation of subfibers during fractionation.

The presence of two prominent, unique peptides in the membrane-derived α chain is also obvious (Figures 2-M and 3-M, arrows), as is the absence of two prominent peptides characteristic of axonemal α-tubulin (Figures 2-M and 3-M, circles). Comigration of samples confirms these identifications (Figures 2-B/M and 3-B/M vs. B + M). From the relative positions on the respective maps, these characteristic peptides are the same ones identified in the carboxymethyl derivatives.

In the case of the β chains, three substoichiometric peptides are found characteristically in the A-tubule-derived subunit (Figures 4-B and 5-B, circles), as noted above for the carboxymethyl derivative. In addition, there is one prominent peptide found in the B-subfiber β chain not found in the A-tubule counterpart (Figures 4-A and 5-A; circles; Figures 4-B and 5-B, asterisks). This peptide was apparently not resolvable in the carboxymethyl derivative.

The membrane-derived β chain contains two prominent peptides not found in axonemal β chains and is missing two peptides characteristic of axonemal chains (Figures 4-M and 5-M, arrows, circles). Mixed-sample comigration confirms the identification (Figures 4-B/M and 5-B/M versus B + M). As in the case of the α chain, the characteristic membrane

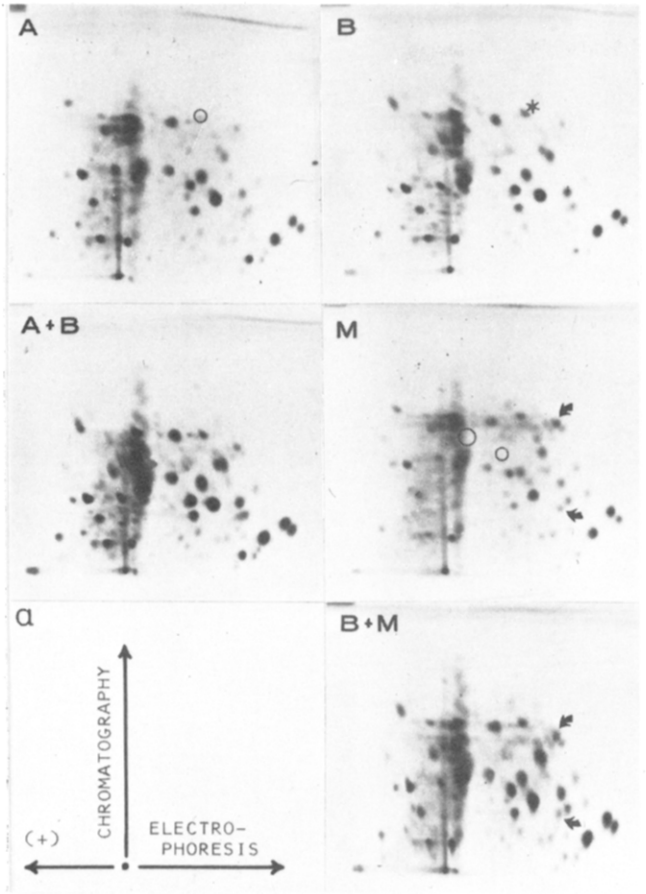

FIGURE 3: Tryptic peptide maps of carboxamidomethylated α-tubulins. Chromatography in 1-propanol–ammonium hydroxide; electrophoresis at pH 6.5. Same samples and designations as in Figure 2.

β-chain peptides appear to be the same as those detected in carboxymethylated samples. Particularly evident in all of the β-chain comparisons, but true of the α-chain comparisons to some extent, are marked differences in the relative fluorescence of positionally equivalent peptides. This is evident for both carboxymethyl and carboxamidomethyl derivatives and is accentuated at pH 6.5.

In contrast to digests of carboxymethylated membrane subunits, those which are carboxamidomethylated show numerous "ghost" spots, varying with the preparation. This is especially obvious in the β chain and appears to be a consequence of incomplete digestion. In general, more material remains at the origin in the carboxamidomethylated samples.

Thus, regardless of derivative, preparation, or mapping conditions, the putative α and β chains from the detergent-solubilized membrane fraction of cilia map much like authentic, axonemal tubulins, having greater than 90% coincidence of peptides. However, under all of these various conditions, both the α and β chains of membrane tubulin each contain two major, characteristic peptides not found in axonemal tubulins, while the latter likewise contain two unique peptides not found in the membrane-derived subunits. Moreover, many of the spots which are coincident differ in their relative degree of fluorescence.

Amino Acid Composition. The α and β chains of the A-tubule, B-subfiber, and detergent-solubilized membrane tubulins from both carboxymethylated and carboxamidomethylated samples were analyzed after 24, 48, and 96 h of acid hydrolysis. No significant composition differences were noted between the two types of derivative. Where major differences occurred between homologous subunits (see below), the means for the two derivative types generally differed by no more than ±5% or 1 standard deviation (SD). Consequently, the data from the two types of derivative were grouped together and further analyzed statistically by Student's *t* test. The comparative amino acid composition of the α- and β-tubulin chains is presented in Tables I and II, respectively.

In the case of α-tubulin, highly significant differences ($P < 0.001$) are seen between the A-tubule- and B-subfiber-derived proteins in arginine and glycine. Smaller differences ($P < 0.05$) occur in leucine, lysine, and phenylalanine. Such differences are consistent with the subfiber-specific tubulin differences reported previously (Stephens, 1976), using samples carboxymethylated in 8 M urea. If *both* axonemal α chains

Table I: Amino Acid Composition of α-Tubulin[a]

amino acid	A	B	M
Ala	38.1 ± 1.9	36.4 ± 2.1	34.0 ± 0.7*
Arg	*24.6 ± 1.2*	21.7 ± 0.4	20.6 ± 1.6
Asp	46.3 ± 0.4	44.7 ± 0.8	*49.4 ± 0.8*
S-CMC[b]	10.8 ± 0.3	11.5 ± 0.6	*8.0 ± 0.6*
Glu	57.8 ± 3.5	55.9 ± 2.0	50.6 ± 5.1
Gly	41.2 ± 2.1	*47.1 ± 1.1*	42.5 ± 0.8
His	12.2 ± 0.7	12.9 ± 0.9	*9.8 ± 0.7*
Ile	23.2 ± 0.5	23.4 ± 0.4	*24.5 ± 0.5*
Leu	37.4 ± 1.1*	35.3 ± 0.7	36.4 ± 1.5
Lys	23.5 ± 1.5*	21.8 ± 1.2	*28.4 ± 2.7*
Met	10.2 ± 0.8	10.0 ± 0.3	11.5 ± 0.4*
Phe	19.6 ± 0.6*	21.0 ± 0.8	22.0 ± 1.5
Pro	19.1 ± 1.3	20.0 ± 2.3	21.8 ± 2.1
Ser	25.9 ± 1.3	25.3 ± 1.3	*30.4 ± 1.5*
Thr	30.1 ± 1.5	30.5 ± 1.5	30.1 ± 1.5
Tyr	18.0 ± 0.3	19.1 ± 1.0	17.0 ± 1.2*
Val	31.5 ± 1.1	33.0 ± 1.1	32.7 ± 1.1

[a] Tubulin derived from ciliary A tubules (A), B subfibers (B), and detergent-solubilized membrane (M). Average of 24-, 48-, and 96-h hydrolysis values; Ser and Thr extrapolated to zero hydrolysis time. Values are expressed as moles per 54 000 g ± 1 SD (±5% for Ser and Thr). Italicized numbers represent values which differ significantly at $P < 0.001$ (or >15% for Ser). Asterisks represent differences where $P < 0.05$. Differences in M were scored only when deviating from both A and B. [b] *S*-(Carboxymethyl)cysteine.

Table II: Amino Acid Composition of β-Tubulin[a]

amino acid	A	B	M
Ala	30.4 ± 1.6	28.3 ± 1.3	33.0 ± 2.1
Arg	*25.9 ± 0.3*	23.3 ± 1.0	21.9 ± 1.0
Asp	49.4 ± 1.4	48.9 ± 1.5	47.9 ± 1.0
S-CMC[b]	11.6 ± 1.7*	9.0 ± 0.3	8.0 ± 1.7
Glu	61.3 ± 0.8	59.5 ± 2.1	50.5 ± 5.8*
Gly	38.3 ± 0.8	*47.3 ± 0.5*	42.1 ± 1.3*
His	10.4 ± 0.5	10.8 ± 0.8	*8.4 ± 0.8*
Ile	18.0 ± 0.5	16.9 ± 0.5	*24.8 ± 0.5*
Leu	37.7 ± 0.7	36.6 ± 0.6	38.6 ± 1.8
Lys	*22.1 ± 1.2*	18.8 ± 0.6	*29.2 ± 3.0*
Met	14.4 ± 1.4	15.5 ± 1.3	13.3 ± 1.9
Phe	21.8 ± 0.7	*24.0 ± 0.6*	21.5 ± 2.4
Pro	21.2 ± 1.3*	19.3 ± 1.5	19.8 ± 1.9
Ser	30.5 ± 1.5	30.4 ± 1.5	33.1 ± 1.7
Thr	31.8 ± 1.6	33.2 ± 1.7	30.3 ± 1.5
Tyr	16.2 ± 0.3	*17.4 ± 0.5*	15.1 ± 1.2*
Val	28.6 ± 1.3*	30.5 ± 0.9	32.2 ± 1.2*

[a] Tubulin derived from ciliary A tubules (A), B subfibers (B), and detergent-solubilized membrane (M). Average of 24-, 48-, and 96-h hydrolysis values; Ser and Thr extrapolated to zero hydrolysis time. Values are expressed as moles per 54 000 g ± 1 SD (±5% for Ser and Thr). Italicized numbers represent values which differ significantly at $P < 0.001$ (or >15% for Ser). Asterisks represent differences where $P < 0.05$. Differences in M were scored only when deviating from both A and B. [b] *S*-(Carboxymethyl)cysteine.

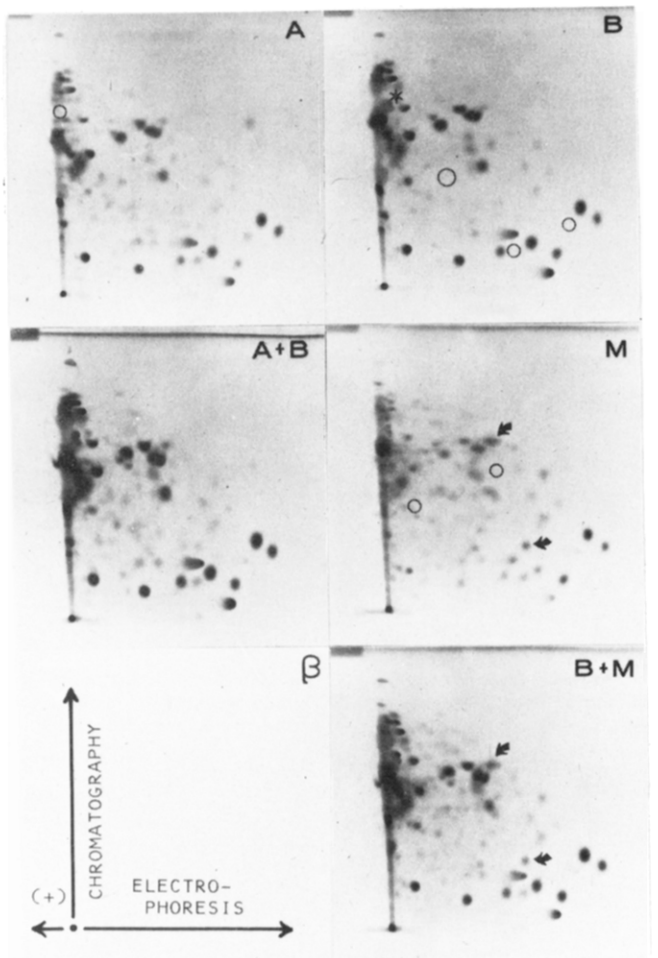

FIGURE 4: Tryptic peptide maps of carboxamidomethylated β-tubulin from the A-tubule (A), B-subfiber (B), and membrane (M) fractions. Chromatography in chloroform–methanol–ammonium hydroxide; electrophoresis at pH 3.5. Peptide in B (asterisk) is absent in A (circle). Three peptides are present in A which are absent in B (circles). Two peptides are present in M (arrows) which are absent in A and B, while two peptides are absent in M (circles) which are present in A and B.

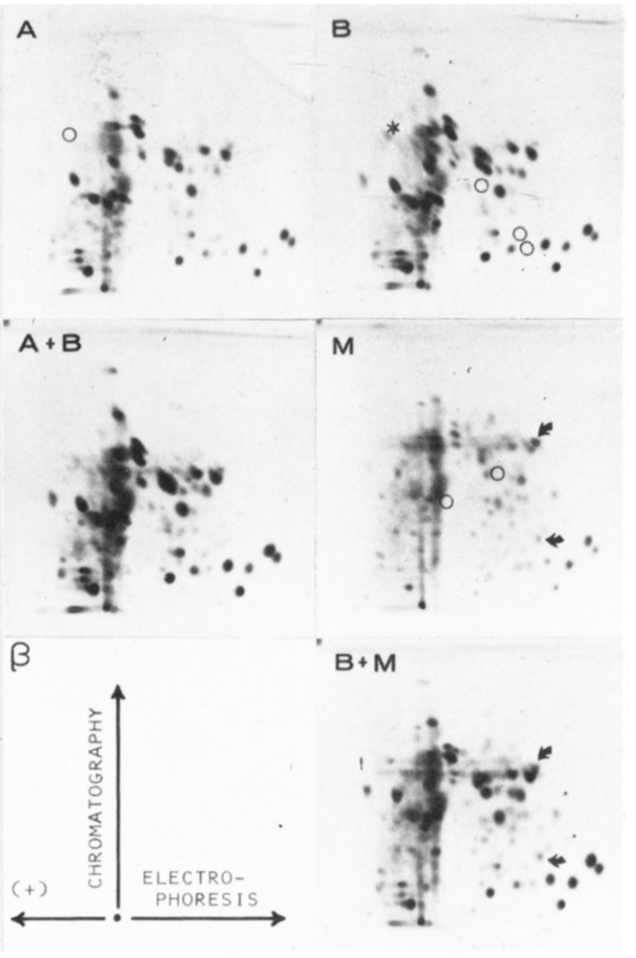

FIGURE 5: Tryptic peptide maps of carboxamidomethylated β-tubulins. Chromatography in 1-propanol–ammonium hydroxide; electrophoresis at pH 6.5. Same sample and designations as in Figure 4.

are compared with the membrane-derived chain, however, there are highly significant differences in six amino acids (Asx, Cys, His, Ile, Lys, and Ser) and lesser differences in three more (Ala, Met, and Tyr).

The β-tubulin chains also show several significant differences between the axonemal counterparts. Highly significant differences occur in arginine, glycine, and lysine while lesser differences appear in cysteine, proline, and valine. Again these differences are consistent with a previous analysis (Stephens, 1976). In comparison with *both* axonemal β chains, the membrane-derived counterpart shows highly significant differences in three amino acids (His, Ile, and Lys) and less significant differences in four other residues (Glx, Gly, Tyr, and Val). The most striking and highly reproducible difference is in isoleucine, where there are 25 residues in the membrane β chain but only 17–18 in the outer doublet chains.

It is clear from these data that the membrane-derived subunits have the overall composition characteristic of α- and β-tubulin [cf. Stephens (1978a)], but there are several highly significant differences in both chain types which set them apart from their axonemal counterparts. These differences are most marked in certain hydrophobic amino acids, particularly evident in the β chain.

Isoelectric Focusing. For the identification of components, unequivocally, for the purpose of isoelectric point determination, two-dimensional gels of the O'Farrell (1975) variety were run on dialysis-solubilized B-subfiber fractions and detergent-solubilized membrane fractions. The results are illustrated in Figure 6.

The bulk of the protein in the detergent-solubilized membrane fraction corresponds in position to the α and β chains of tubulin, as judged from the position of authentic tubulin chains from the B-tubulin fraction (Figure 6-M vs. 6-B). Few detectable proteins migrate in the $NaDodSO_4$ dimension aligned with either the α or the β chains of either tubulin type, indicating that the proteins judged by $NaDodSO_4$ mobility alone to be tubulin chains do consist principally of tubulins. A prominent 43 000-dalton protein with an isoelectric point comparable to that of an actin [cf. Garrels & Gibson (1976)] is a variable feature of both fractions (Figure 6-M and 6-B, asterisks). As concluded previously (Stephens, 1977a), this protein is most likely derived from contaminating microvilli.

For accurate determination of the isoelectric point, samples of the detergent-solubilized membrane fraction were run in parallel with dialysis-solubilized B tubulin on slab gels, using the O'Farrell (1975) first-dimension formulation, leaving some adjacent lanes vacant for direct determination of the pH gradient. The results are illustrated in Figure 6, right panel.

The detergent-solubilized membrane fraction consists mainly of two protein regions coincident with the α and β chains of B-tubulin, having isoelectric points of 5.29 ● 0.02 (SD) and 5.17 ± 0.02 (SD), respectively. Three other components, always found but somewhat variable in amount, have isoelectric points of 5.85, 5.43, and 5.15 (Figure 6a, b, and c,

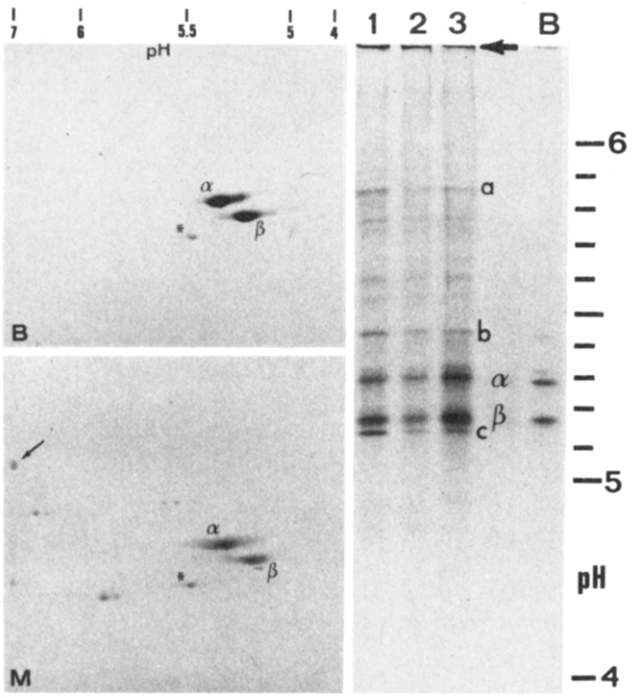

FIGURE 6: Two-dimensional gel analysis of B-tubulin (B) and membrane-derived (M) tubulin (left section) and isoelectric focusing of three different membrane extracts (1–3) in comparison with B-tubulin (right section). The two-dimension gels are dominated by α- and β-tubulin, and both contain a trace of a protein with the same isoelectric point and molecular weight as those of actin (asterisks). A 100 000-dalton membrane glycoprotein component barely migrates in the isoelectric dimension (M, arrow). The isoelectric points of the membrane-derived tubulin chains are not measurably different from those of B-tubulin (right section); the arrow indicates the 100 000-dalton membrane glycoprotein; a, the 40 000-dalton membrane protein; b, contaminating actin; and c, a probable β-chain cleavage product.

respectively). The first corresponds to a 40 000-dalton component which is probably a true membrane protein [cf. Dentler et al. (1980)], the second is the 43 000-dalton β- or γ-actin, and the third is most likely a β-chain cleavage product. Barely migrating into the first-dimension gel, but obvious in the second dimension, is a component with a pI > 6.5 and a molecular weight of 100 000, corresponding to a major glycoprotein of the ciliary membrane [cf. Stephens (1977a)]. It is denoted by arrows in Figure 6.

Variations in the isoelectric focusing system warrant some comment here. The use of a pH 4–6 major ampholyte in place of the more conventional pH 5–7 range keeps the unusually acidic tubulins near mid-range. When the sample is focused from the low-pH end of the gradient, the tubulin aggregates badly, forming trailing bands. Aggregation also occurs during sample preparation and may be minimized by adjusting the ampholyte–urea–Nonidet sample buffer to pH 9 before dissolving the sample and then immediately applying the sample to the basic end of the prefocused gel. The inclusion of $NaDodSO_4$ with the sample (Ames & Nikaido, 1976) greatly aids in solubilization, but the membrane-derived α-tubulin tends to streak badly even when the sample is previously subjected to alkaline conditions prior to focusing. In spite of pH adjustment and the inclusion of $NaDodSO_4$ to increase solubility, the membrane tubulin precipitates irreversibly upon freeze–thawing of the sample, necessitating the use of only fresh samples. Although quite adequate for reproducibly resolving axonemal tubulins, ampholyte brands other than Bio-Rad 4/6 yield variable results with the membrane-derived protein, generally in the form of multiple bands and increased aggregation.

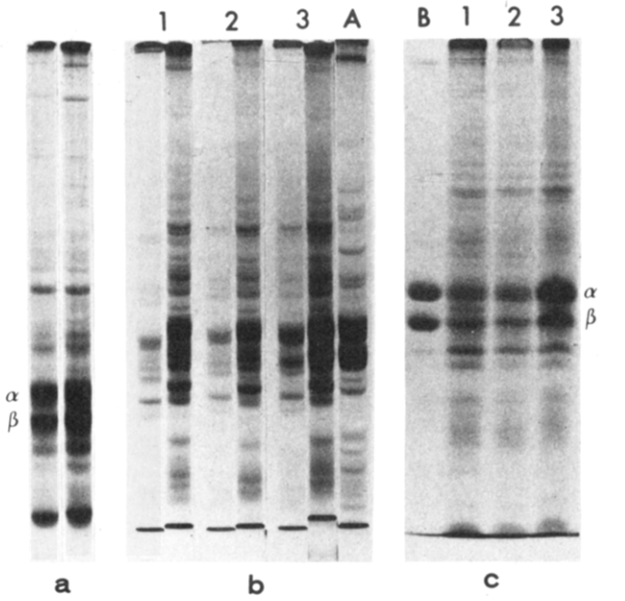

FIGURE 7: Comparative $NaDodSO_4$–polyacrylamide gel electrophoresis on three gel system configurations. In a, two different membrane preparations are run on a continuous ionic system, separating obvious α and β bands. In b, three different membrane extracts (1–3; same samples as in Figure 6) at two loadings each are run on a discontinuous ionic system in parallel with A tubules (A), using gel cylinders. The α- and β-chain regions of the membrane samples are complex. In c, the same samples (1–3) are run with B-tubulin on the same discontinuous ionic system, but using a gel slab. The α and β regions of all samples are well-defined.

Comparative $NaDodSO_4$–Polyacrylamide Gel Electrophoresis. The initial evidence that the 55 000-dalton protein of the ciliary membrane might be a tubulin was the fact that it split into two equally staining bands on a continuous $NaDodSO_4$–Tris–glycine system, separating farther when reduced and carboxymethylated, and comigrating with authentic tubulin subunits under both sets of conditions (Stephens, 1977a). Such a separation is illustrated in Figure 7a, where a reduced but underivatized total membrane extract was run. The excess Nonidet was removed with polystyrene beads to avoid detergent competition. A typical α/β slipt occurs, but there is no evidence for multibandedness, even upon extensive migration.

Dentler (1980) noted that the tubulin-like protein from *Tetrahymena* membranes was resolved into multiple bands when run on a discontinuous $NaDodSO_4$ gel system of the Laemmli (1970) formulation. This phenomenon is evident for *Aequipecten* ciliary membrane tubulin also, but only when the protein is run on a tube gel, not on a slab. Figure 7b,c compares three different samples of detergent-solubilized membrane fraction run in tubes and on a slab. These are the same samples run at the same time using the same stock acrylamide and buffers and subjected to the same voltage gradient. The first two samples were precipitated at the isoelectric point from solutions freed of Nonidet; the third was precipitated with trichloroacetic acid. Consequently, they should all be free of any excess detergent. On tubes, the α band shows signs of splitting into two equal bands which approximately comigrate with their axonemal counterpart (Figure 7b, 1, 2, or 3 vs. A). The β region consistently shows complex multiple banding, varying with the preparation (Figure 7b, 1 vs. 2 or 3). On a slab gel, however, these same samples exhibit well-behaved α and β bands, comigrating vs. authentic tubulin subunits (Figure 7c). When ultrapure $NaDodSO_4$ (C_{12} > 98%) is used, the multibandedness on the Laemmli tube gels is considerably diminished; samples which have been reduced and alkylated also show diminished multiplicity of bands (data not shown).

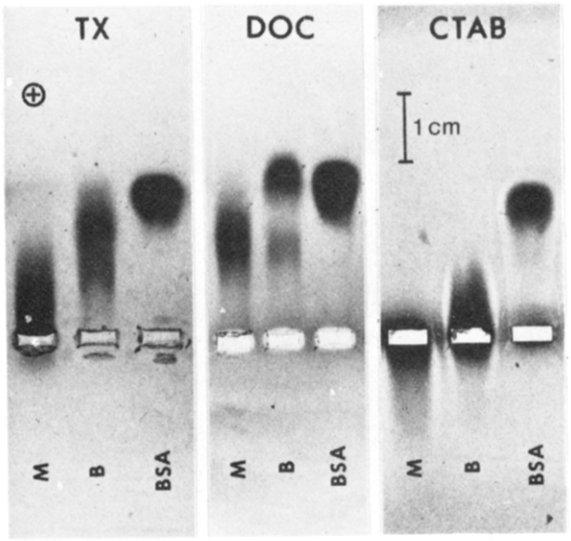

FIGURE 8: Charge-shift electrophoresis on 1% agarose. Membrane-derived tubulin (M), B-tubulin (B), and bovine serum albumin (BSA) are run in the presence of Triton X-100 (TX) alone, and with Triton X-100 and either sodium deoxycholate (DOC) or cetyltrimethylammonium bromide (CTAB). The two tubulin samples migrate differently under all conditions while BSA is relatively unaffected.

Charge-Shift Electrophoresis. The method of Helenius & Simons (1977) allows an estimation of the relative binding of an anionic and a cationic detergent to the tubulin derived from the dialysis-solubilized B subfiber of the axoneme with that from the detergent-solubilized membrane fraction. Typical results are illustrated in Figure 8.

With bovine serum albumin (BSA) as a mobility standard [since it is relatively unaffected by the presence of either deoxycholate (DOC) or cetyltrimethylammonium bromide (CTAB)], both axonemal B-tubulin and membrane tubulin show amphiphilic properties. In Triton X-100 (TX) alone, B-tubulin has an anionic mobility nearly equal to that of BSA, but the membrane-derived tubulin moves only about one-fourth as far. Both show considerable "trailing". In DOC, B-tubulin slightly exceeds BSA in anodic mobility while membrane tubulin migrates at about half the mobility of BSA. In the presence of CTAB, B-tubulin binds sufficient positively-charged detergent to decrease its mobility about 6-fold in comparison with that in TX alone, whereas BSA is only slightly retarded. In contrast, the membrane-derived counterpart binds sufficient CTAB to actually migrate slightly toward the cathode. Reciprocal treatment, i.e., parallel dialysis of membrane tubulin or long-term storage of B-tubulin with TX, does not affect these relationships. Thus, both tubulin types bind anionic and cationic detergents in amphiphilic fashion under nondenaturing conditions, in direct comparison with a hydrophilic control, but the membrane-derived tubulin binds substantially more.

Discussion

Using simple coincidence as a criterion, tryptic peptide maps of both carboxymethylated and carboxamidomethylated membrane α and β chains show near-identity with authentic tubulin chains derived from the axoneme. Just as there is not a strict identity between homologous chains from the A tubule and B subfiber [cf. Stephens (1976, 1977a, 1978a)], there are significant differences between membrane and axoneme homologues in the form of the reciprocal presence or absence of two sets of prominent peptides and also in the relative degree of fluorescence of many spots which are positionally equivalent. The latter may reflect differences in the relative amount of a particular sequence or in the quantum yield, a possible consequence of conservative sequence variation. On the basis of peptide map comparisons, there is far more homology between the putative membrane tubulin and tubulin from the axoneme than is seen among ciliary, flagellar, and cytoplasmic tubulins of the same organism [cf. Stephens (1978a)]. The membrane subunits are tubulins, but they are not the same tubulins as make up outer doublets.

The overall amino acid composition similarities demonstrate independently that the α and β chains of the membrane protein are clearly tubulin-like, but a number of important differences again do not support identity. It should be noted that many of the compositional differences seen here between the membrane and axonemal tubulin chains are really no greater than differences among bona fide tubulins from different organelles in the sea urchin [cf. Stephens (1978a)]. However, both the membrane α and β chains are uncharacteristically low in cysteine and histidine while the α chain is relative high and the β chain relatively low in methionine when compared with tubulins of the same or other species. The unusually high isoleucine content of the membrane β chain is more characteristic of an α chain. This fact and the methionine deviation could argue for cross-contamination of chain types, but peptide mapping, performed on these samples, rules out any significant cross-contamination since peptides characteristic of α chains are not seen in β-chain maps and vice versa (Figure 1-M, Greek letters and lettered circles).

The two principal components of the detergent-solubilized membrane fraction have isoelectric points indistinguishable from those of the α and β chains of B-tubulin from the axoneme. Two-dimensional gel analysis supports the validity of the one-step preparative electrophoresis for separation and isolation of the corresponding α and β chains of these tubulin fractions for peptide mapping and amino acid analysis, used here and previously (Stephens, 1976, 1977a). It is highly improbable that appreciable contamination with comigrating proteins could occur. It would be an unusual protein, indeed, to account simultaneous for the two equimolar peptide fragments found in both membrane chain types and for the marked deviation in isoleucine content seen only in the membrane β chain, especially from independently prepared carboxymethylated and carboxamidomethylated derivatives of different mobilities.

Isoelectric focusing fails under two related conditions. Samples introduced from the acidic end of the pH gradient either precipitate or trail badly; samples introduced from the alkaline end but kept at the pH of the ampholyte mixture (generally about pH 5.5) behave similarly. Both of these problems appear to be due to isoelectric precipitation, in spite of the continued presence of ~9 M urea. An extreme example of this situation results in a two-dimensional gel of a membrane tubulin fraction containing little or none of either tubulin chain but having most of the minor protein components. This difficulty may be remedied by making the protein sample alkaline and introducing it from the alkaline end of the gel. The B-subfiber-derived tubulin focuses well from $NaDodSO_4$-containing, neutral sample buffers, but the membrane-derived protein does not.

Unless the membrane tubulin sample is exposed to alkaline conditions of pH 9 or higher for at least 30 min, it often shows a multitude of isoelectric forms, ranging from about pH 6.0 to 5.2, migrating at the molecular weight of the tubulin subunits. When this occurs, the tubulin subunits are greatly diminished. The phenomenon varies with the preparation and the season. It was shown previously that membrane tubulin

was rendered PAS negative after reduction and alkylation at alkaline pH, implying cleavage at O-glycosidic linkages of carbohydrate moieties (Stephens, 1977a). The requirement for alkaline pretreatment to produce well-behaved membrane tubulin α and β chains is consistent with the hypothesis that hydrolysis of such bonds occurs during sample preparation, ultimately yielding a uniformly charged protein species.

The underivatized membrane tubulin can be resolved into components strictly comigratory with axonemal α- and β-tubulin chains only on certain gel system configurations. Reduced and alkylated samples behave well, however. One possible explanation, related to the isoelectric focusing problem discussed above, is that the protein subunits have a variable carbohydrate content. The resolution into variable multiple components may reflect the variable carbohydrate content through differential binding of $NaDodSO_4$ or higher chain length impurities, perhaps explaining why ultrapure $NaDodSO_4$ is far less effectual [cf. Matheka et al. (1977)]. Alternatively, there may be several chain types present, differing in primary structure and binding $NaDodSO_4$ (or other chain lengths) anomalously. A similar situation has been reported recently for tubulin subunits of chick oviduct cilia versus those of the basal bodies (Anderson & Floyd, 1980). The respective proteins have identical isoelectric points but can be clearly distinguished on certain urea-containing $NaDodSO_4$ gel systems. These differences are interpreted as due either to amino acid substitutions or to posttranslational modification. As to the geometry of the gel, multiple tubulins are simply better resolved where there is minimal sample height and maximal cross-sectional area for sample entry (i.e., minimization of the initial stacking zone thickness). In the comparison made in this study, the tube gel configuration has over twice the cross-sectional area as that of the slab.

On the basis of the differential detergent binding criteria set forth by Helenius & Simons (1977), both B-tubulin and membrane-derived tubulin behave as amphiphilic proteins, but they are cleary distinguishable. The comparatively higher binding of both anionic DOC and cationic CTAB by the membrane-derived tubulin implies that it is more amphilic in nature than its B-tubulin counterpart, perhaps reflecting the markedly higher isoleucine content of the former. The fact that B-tubulin is affected at all in this system probably reflects the fact that it has a certain degree of hydrophobic character, as evidenced by the association properties of the tubulins in general. Even hydrophilic proteins such as lysozyme and thyroglobulin show the charge-shift phenomenon to some extent (Helenius & Simons, 1977), evidently due to detergent binding to exposed hydrophobic pockets. The lower initial mobility of membrane tubulin in Triton X-100 alone is consistent with a comparatively more amphiphilic nature since it appears to form a larger (hence slower) detergent complex with Triton than B-tubulin. It is unlikely that the mobility difference seen between the two tubulins in Triton X-100 alone is due to a marked difference in protein particle size. Dialysis-solubilized B-tubulin is a typical 6S dimer, and the membrane-derived tubulin is also dimeric or even smaller (Stephens, 1977a). It is even less likely that the mobility difference between the two tubulins is due to a charge difference since their corresponding α and β chains have identical isoelectric points.

Taken together, these data show that membrane-derived tubulin is a tubulin isotype having chemical characteristics which clearly distinguish it from tubulin derived directly from the axoneme proper. Therefore, the tubulin obtained from detergent solubilization of the ciliary membrane cannot have arisen artifactually from the breakdown of the axoneme. However, the data presented here, standing alone, cannot eliminate the possibility that a nonaxonemal tubulin may exist within the amorphous ciliary matrix and is released during the extraction process.

The fact remains that the protein may be isolated from ciliary membrane vesicles (Dentler, 1980) and may be chemically cross-linked at or in the in situ membrane (Dentler et al., 1980). The net amino acid composition of the membrane-derived tubulin dimer shows it to be somewhat more hydrophobic than its axonemal counterparts, and it appears to interact more strongly with both anionic and cationic detergents. Such properties are consistent with the membrane-associated nature of the protein.

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