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Major Membrane Protein Differences in Cilia and Flagella: Evidence for a Membrane-Associated Tubulin†

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ABSTRACT: The membrane of both sperm flagella and gill cilia of the scallop *Aequipecten irradians* may be selectively solubilized in 1% Triton X-100, 30 mM tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl), pH 8, and 3 mM $MgCl_2$, leaving the axoneme totally intact. This membrane fraction represents about 20% of the total protein of the respective organelle. Analysis of the flagellar membrane by sodium dodecyl sulfate ($NaDodSO_4$)-polyacrylamide gel electrophoresis revealed one principal protein component, periodic acid-Schiff (PAS) positive and migrating with an apparent molecular weight of 250 000. The remaining minor proteins, none of them PAS positive, accounted for less than one-third of the total flagellar membrane fraction. Analysis of the ciliary membrane also revealed one major protein component, weakly PAS positive and migrating with an apparent molecular weight of 55 000. The remaining minor proteins represented about one-third of the total ciliary membrane fraction; two components with molecular weights of 100 000 and 43 000 predominated. The latter could be substantially reduced by purification of the cilia on a sucrose density gradient and was assumed to be actin, derived by vesiculation of the brush border during deciliation. The principal ciliary membrane protein, that of 55 000 daltons, was resolved into two equimolar components on $NaDodSO_4$-Tris-glycine-polyacrylamide gels, comigrating with the α and β chains of outer fiber tubulin. S-carboxymethylation caused increased splitting of the two components and concomitant migration with similarly treated ciliary tubulin. Preparative gel electrophoresis yielded separate components whose cyanogen bromide cleavage products were virtually identical in size distribution with those obtained from outer fiber α and β chains; tryptic peptides corresponded almost exactly to those of authentic tubulin subunits but certain positional differences indicated possible side chain modification. At 25 °C both whole cilia and its solubilized membrane fraction bound colchicine while whole flagella and the 9 + 2 axoneme from either organelle did not. Thus certain molluscan flagellar membranes primarily contain a 250 000-dalton glycoprotein but ciliary membranes have a modified tubulin as the major protein component. At an electron microscopic level, flagellar membranes have a distinct trilamellar "unit membrane" structure while ciliary membranes appear thinner and considerably less distinct, perhaps reflecting the protein compositional differences in the membranes of these otherwise morphologically identical organelles.

Cilia and flagella differ principally in their length, mode of beat and location. Cilia are relatively short (25 μm or less), bending at the base for the "power" stroke and propagating this bend helically up the shaft during the "recovery" stroke, while flagella are usually much longer (often by a factor of 2 or 3) and continuously propagate near-planar, sinusoidal waves toward the tip. In higher organisms, cilia are found covering the surface of several kinds of epithelial cells, often occurring in discrete patterns or assemblages, while flagella occur singly and are typically found only in sperm; they thus have their origin in two quite different cell lines. At an ultrastructural level, however, the two organelles are virtually indistinguishable.

Two kinds of evidence point to chemical differences in the 9+2 axonemes of cilia and flagella. Immunologically, antibodies prepared against sea urchin sperm outer doublet microtubules quantitatively cross-react differently with doublets prepared from blastula cilia (Fulton et al., 1971), implying subtle antigenic differences in their respective microtubules or microtubule-associated proteins (cf. Linck, 1976). Biochemically, fractionation studies performed on the axonemes of molluscan gill cilia and sperm flagella show that the B-subfiber and central pair tubules differ markedly in solubility in the two organelles and that the mode of association of the dynein ATPase is likewise characteristically different (Linck, 1973b). Similar solubility differences have been found in sea urchin blastula cilia and sperm flagella (Stephens, 1976b). These results suggest that at least some structural proteins are different, either the tubulin dimers making up the respective homologous microtubules, the linkage and spoke proteins holding them together, or both.

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Furthermore, the membranes of cilia and flagella differ in their relative solubility properties. Concentrations of Triton X-100 near the critical micelle concentration (≧0.015%) rapidly disrupt flagellar membranes (cf. Gibbons and Gibbons, 1972; Stephens, 1970), while substantially higher concentrations (≧0.5%), longer time periods, or multiple extractions are needed to disperse and remove ciliary membranes efficiently (cf. Stephens and Linck, 1969; Linck, 1973a). These solubility differences could simply reflect compositional differences in the constituent lipids; to date no comparative lipid analysis of these two membranes has been carried out. However, some previous observations have indicated that the membrane fractions from the two organelles in certain species differ markedly in their protein composition (Stephens, 1974, 1975a). This current report attempts to document and quantitate these differences more fully, presenting evidence that tubulin is the major protein component of ciliary but not flagellar membranes.

Experimental Procedures

Sperm Flagella, Gill Cilia, Axonemes, and Membrane Fractions. Sperm tails were isolated from exudates of minced testes of the bay scallop *Aequipecten irradians* by homogenization of the sperm suspension for 2 s in a Sorvall Omnimixer, followed by sedimentation of sperm heads at 1000*g* for 5 min and free tails at 10 000*g* (Stephens, 1970; Linck, 1973a). One additional differential centrifugation from cold sea water containing 10^{-4} M EDTA[1] was performed to assure purity. The sperm flagella were then suspended in 40 volumes of 10 mM Tris-HCl, pH 8, and sedimented at 10 000*g* for 10 min in order to reduce the salt concentration prior to extraction of the membrane.

Cilia were isolated from thoroughly washed gills of *Aequipecten irradians* by treatment with hypertonic sea water (containing 32 g of additional NaCl per L) for 15 min at 10 °C, followed by sedimentation of cell debris at 1000*g* for 5 min and cilia at 10 000*g* for 10 min (Stephens and Linck, 1969; Linck, 1973a). The cilia were purified further by either of two methods. Differential sedimentation from cold sea water at 10 000*g* for 10 min, repeated twice, freed the cilia of small vesicles, derived from the tissue during deciliation, and the variably contaminating mucus and silt, characteristic of gill tissue. Alternatively, the cilia were suspended in a minimal volume of sea water (<1 mL), layered atop a 40-mL, 5–30% linear sucrose gradient (made up in 30 mM Tris-HCl, pH 8, 3 mM $MgCl_2$, and 0.1 mM EDTA), and spun at 5000*g* for 15 min at 20 °C in a Sorvall HS-4 swinging bucket rotor. The cilia sedimented as a narrow band to a point about three-quarters of the way down the gradient, while small vesicles remained at the top and the silt and mucus sedimented completely. With either method, the purified cilia were suspended in 40 volumes of 10 mM Tris-HCl, pH 8, and sedimented at 10 000*g* for 10 min to reduce the salt or sucrose concentration prior to extraction of the membrane.

Pellets of either flagella or cilia were thoroughly suspended in 10 volumes of a solution consisting of 1% Triton X-100, 30 mM Tris-HCl (pH 8), 3 mM $MgCl_2$, and 0.1 mM EDTA, extracted at 0–4 °C for 30 min, and the suspension was sedimented at 25 000*g* for 15 min. The supernatant of solubilized membrane protein was carefully removed and the pellet of 9+2 axonemes was resuspended up to the original volume with fresh Triton extraction solution, resulting in a stoichiometric ratio of membrane and axoneme fractions. Occasionally, this procedure was repeated twice more in order to obtain three successive extracts. When it was desirable to reduce both the volume and the Triton content of the fractions for gel electrophoresis, they were mixed with 10 volumes of cold acetone. The precipitated protein was recovered by sedimentation at 10 000*g* for 5 min, and the resulting pellets were freed of acetone under reduced pressure.

In an effort to obtain an intact membrane fraction without the use of detergents, isopycnic centrifugation on sucrose or CsCl gradients was employed. To a 5-mL linear 35–60% sucrose gradient (made up in 10 mM Tris-HCl, pH 8), 0.3 mL of whole cilia at 3 mg/mL was applied and centrifugation was carried out at 4 °C for 12 h in a Beckman SW 50L rotor at 40 000 rpm (Witman et al., 1972). Alternatively, a solution of 2.8 M CsCl in 10 mM Tris-HCl, pH 8, was used to suspend whole cilia to a final concentration of 0.5 mg/mL and the suspension was spun under the above conditions for 24 h (Snell, 1976). In the former case the cilia banded near the bottom of the gradient, while in the latter case they banded nearly at the top; in neither case was there evidence for a separate membrane band.

$NaDodSO_4$–Polyacrylamide Gel Systems, Staining and Quantitation. Samples were analyzed on $NaDodSO_4$–5% polyacrylamide gels using three different buffer systems. The 0.1 M phosphate (pH 6.9) system of Shapiro et al. (1967) was used early in the study to monitor results. The 0.04 M Tris–acetate (pH 7.4) system of Fairbanks et al. (1971) was employed with extracts high in Triton and in cases where carbohydrate was to be determined. The 0.025 M Tris–glycine (pH 8.3) system of Bryan (Bryan, 1974; Stephens, 1975b) was used when it was desirable to resolve α and β subunits of tubulin but, because of competitive detergent binding, its use was limited to fractions low in Triton X-100. In all cases, a sample buffer concentrate was prepared at two or ten times the specified concentration and was mixed with the membrane and axoneme fractions in either 1:1 or 1:9 ratios. Samples were heated to 100 °C for 2 min, cooled, and quantitatively applied to gels with microliter pipets.

Gels were routinely stained for protein with 0.25% Coomassie blue in 50% methanol–10% acetic acid for 2–4 h at 45 °C (Weber and Osborn, 1969). For quantitation, 0.25% Fast Green (Gorovsky et al., 1970) was used in the same solvent and at the same temperature but, in this case, the stain was replaced with fresh stain after 2 h and staining was continud an additional 2–3 h. In either staining procedure, at least 10 volumes of stain were used in order to sufficiently dilute the $NaDodSO_4$ from the gels, thus assuring maximal staining. Gels were diffusion-destained with 5% methanol–7.5% acetic acid at 45 °C and stored in 7.5% acetic acid.

Carbohydrate was detected by the periodic acid–Schiff (PAS) method of Fairbanks et al. (1971), followed exactly as published. When both carbohydrate and protein staining were desired, two parallel sets of gels were run, both were fixed in 2-propanol–acetic acid as called for in the PAS procedure, and then one set was stained for protein as above while the other was stained for carbohydrate.

Gels were scanned with a Joyce-Loebl MK-IIIC double-beam recording microdensitometer. Fast Green stained gels were scanned with a 650-nm filter, while 550 nm was used for PAS-stained gels; Coomassie blue stained gels were scanned with white light. The system was calibrated using gels on which were run known amounts of bovine serum albumin (up to 25

[1] Abbreviations used: PAS, periodic acid–Schiff reagent; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; PPO, 2,5-diphenyloxazole.

μg) and which were stained according to the above procedures; areas under the protein peaks were determined by planimetry. Typically, samples were run at several different loadings to guarantee that they would fall within the calibration curve. In addition, total protein in the sample was determined by the Lowry method (Lowry et al., 1951), standardized with bovine serum albumin.

Tubulin Subunit Separation and Comparative Peptide Mapping. A-tubule and B-subfiber fractions of ciliary axonemes were prepared by exhaustive dialysis against 1.0 mM Tris-HCl, pH 8, and 0.1 mM EDTA for 48 h (Linck, 1973b), while a ciliary membrane fraction was prepared by Triton X-100 extraction as outlined above. All three fractions were precipitated at pH 4.5 and the resulting pellets taken up in 5 M guanidine hydrochloride, 0.1 M mercaptoethanol, and 30 mM Tris-HCl, pH 8. Reduction was conducted under a nitrogen atmosphere for at least 1 h at 25 °C, after which a 10% molar excess of 1.1 M iodoacetic acid in 1.0 M Na_2CO_3 was added (final pH was 9) and the samples were allowed to stand for 30 min at 25 °C under a nitrogen atmosphere in the dark. The reaction was quenched with a twofold molar excess of mercaptoethanol and the samples were dialyzed first against two changes of 100 volumes of cold 0.01 M sodium phosphate, pH 7, and then against 100 volumes of preparative electrophoresis buffer (2.5 mM Tris–glycine, pH 8.3, and 0.1% sodium dodecyl sulfate) at room temperature for at least 6 h.

The respective α and β subunits of the A-tubule, B-subfiber, and membrane tubulin fractions were obtained in milligram quantity by fluorescent preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Stephens, 1975b). Subunits intended for cyanogen bromide cleavage were used directly, while those intended for tryptic digestion were precipitated with 9 volumes of cold acetone, washed once with cold acetone, and dialyzed exhaustively against 0.1 M NH_4HCO_3 at 4 °C.

For cyanogen bromide cleavage, 1 mg of pure subunit in 0.2 mL of elution buffer (0.1% sodium dodecyl sulfate and 10 mM NH_4HCO_3) was mixed with 0.8 mL of 90% formic acid, a 100-fold excess of CNBr (vs. methionine content) was added, and the mixture was incubated at 25 °C for 24 h. The sample was evaporated to dryness under a stream of nitrogen and taken up in 0.25 mL of electrophoresis sample buffer, and the resulting CNBr peptides were analyzed by size distribution on $NaDodSO_4$–12.5% polyacrylamide gels, having a 10:1 acrylamide–methylenebisacrylamide ratio, containing 8 M urea, and utilizing a Tris–phosphate buffer system (Swank and Munkres, 1971; Stephens, 1975c). Staining was with 0.25% Coomassie blue in 50% methanol–10% acetic acid at room temperature, with agitation for 6–8 h, followed by diffusion-destaining with 7% acetic acid, also at room temperature.

For tryptic peptide mapping (Ritschard, 1964), 1 mg of pure subunit in 0.1 M NH_4HCO_3 was incubated with DCC-trypsin (Calbiochem) at an enzyme-to-protein ratio of 1:100 for 24 h at 25 °C. The sample was evaporated to dryness with a stream of nitrogen and taken up in 0.1 mL of 0.1 M NH_4HCO_3. Aliquots of 50–75 μg were spotted on 20 × 20 cm silica gel G thin-layer chromatographic plates (Analtech) and subjected to ascending chromatography with chloroform–methanol–ammonia, 2:2:1, at 25 °C in a DeSaga multiplate chamber and then air-dried overnight. Electrophoresis in a perpendicular direction was carried out on a Shandon single-plate thin-layer electrophoresis apparatus at 1000 V for 45 min, using a pH 3.5 solvent system of water–acetic acid–pyridine, 489:10:1. The platen was cooled to 8–10 °C with circulating, refrigerated water. The plates were oven-dried at 110 °C for 1 h and cooled to room temperature. The peptides were visualized by spraying with 0.025% fluorescamine, stabilized with 10% triethylamine in methylene chloride (Felix and Jimenez, 1974), and photographed under 360-nm ultraviolet illumination for direct comparison (Stephens, 1976a).

Colchicine Binding Assay. Samples of whole cilia or flagella, axonemes, and membrane fractions at concentrations of 0.4–0.5 mg/mL were incubated with 125 μM [^{3}H]colchicine (5 μCi/μmol) in 0.1 M sodium glutamate and 10 mM sodium phosphate, pH 6.8, at 25 and 37 °C. Generally, 1.0 mM GTP and 0.1 mM dithiothreitol were present during the incubation period. After appropriate incubation times, aliquots containing 100–300 μg of protein were removed and assayed by absorption to stacks of 4 Whatman DE-41 DEAE-cellulose disks held in 15-mL Millipore filter units, essentially according to the method of Borisy (1972). The disks were thoroughly washed five times with 10-mL aliquots of glutamate–phosphate buffer, aspirated to near-dryness for 1 min, and then placed in 10 mL of PPO–Triton X-100–toluene scintillation fluid (New England Nuclear Formula 950A). After overnight leaching, the samples were counted in a Beckman LS-350 liquid scintillation counter. A known amount of [^{3}H]colchicine was applied to a set of damp disks and counted as a standard. Using the amount of protein applied and the resulting counts retained, the amount of colchicine bound per given weight of protein was calculated for each experimental point.

Analytical Ultracentrifugation. Ciliary membranes were dissolved in 0.1% Nonidet P-40, 30 mM Tris-HCl, pH 8, and 3 mM $MgCl_2$, and dialyzed against 100 volumes of this solvent overnight at 4 °C. When necessary, the solution was concentrated by ultrafiltration prior to dialysis. The material was run at 25 °C in a Spinco Model E analytical ultracentrifuge, equipped with schlieren optics, at a speed of 42 040 rpm. A double sector cell was used in order to detect any detergent micelles in the solvent. Sedimentation constants were corrected to standard conditions, using the density and viscosity of the Nonidet solution, but the values were not extrapolated to infinite dilution since the material was heterogeneous and also showed little observable concentration dependence.

Electron Microscopy. Cilia, flagella, and their detergent-extracted axonemes in 10 mM Tris-HCl, pH 8, were sedimented at 15 000*g* for 15 min in a swinging bucket rotor, and the resulting thin, hemispherical pellets were fixed for 30–60 min at 0 °C with 3% glutaraldehyde in 0.1 M sodium phosphate, pH 7, rinsed for three periods of 20 min with cold phosphate buffer, and then post-fixed with 1% OsO_4 in phosphate buffer for an additional hour at 0 °C. The samples were dehydrated at room temperature with a graded series of ethanol solutions for 30-min periods, rinsed twice with anhydrous ethanol, twice with propylene oxide, and embedded in Araldite 506 by standard procedures. Sections of 600–700 Å were cut with a Du Pont diamond knife, stained with uranyl acetate and lead citrate, and observed and photographed with an RCA 3G or a Philips 300 electron microscope.

Results

As reported previously (Stephens, 1970; Stephens and Levine, 1970; Gibbons and Gibbons, 1972; Linck, 1973a), brief Triton X-100 treatment of flagella and cilia results in the selective solubilization of the membrane. This point is documented in Figure 1 which illustrates isolated scallop sperm tails (a), the resulting flagellar axonemes (b), isolated scallop gill cilia (c), and the resulting ciliary axonemes (d). In both cilia and flagella, membrane fusion readily occurs during centrifugation but the flagellar membrane is frequently removed as

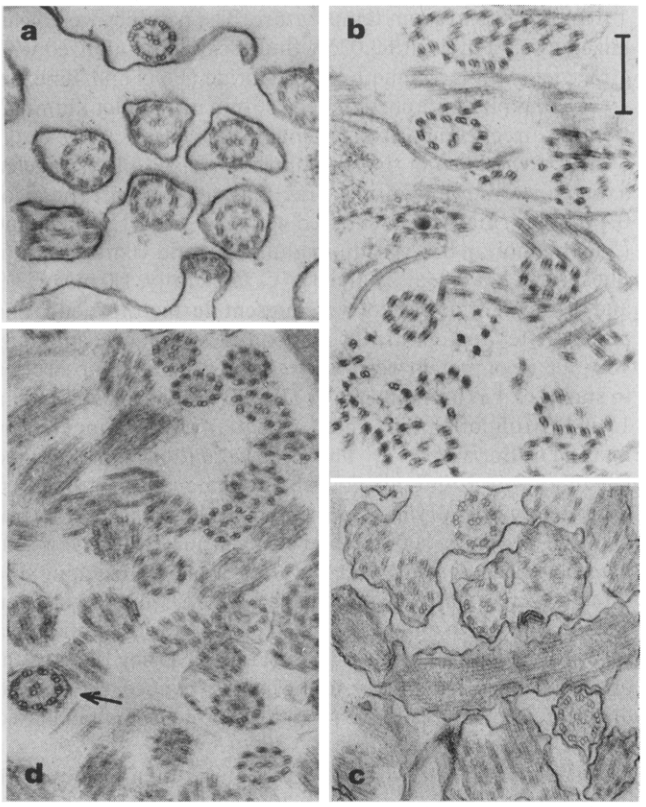

FIGURE 1: Isolated sperm flagella (a) and gill cilia (c) of *Aequipecten irradians* and the flagellar (b) and ciliary (d) axonemes which result from extraction with Triton X-100. The arrow in d indicates the basal region of the ciliary axoneme to which some (specialized?) membrane frequently remains attached. Scale: 0.25 μm.

an open sheet (cf. Witman et al., 1972) while the ciliary membrane remains adherent and reseals, sedimenting with the axonemes. Characteristically, flagellar axonemes easily disrupt during centrifugation into ribbons of nine or fewer outer doublets and a free central pair while ciliary axonemes remain as discrete 9+2 cylinders.

At higher magnification the flagellar membrane appears as a very distinct 80-Å trilamellar "unit membrane" (Figure 2a), while the ciliary membrane appears thinner and less distinct (Figure 2b). Although not noted previously, reference to the above citations will reveal that this difference is quite evident in already published work. The unique appearance of the ciliary membrane is not due to isolation with a hypertonic medium and subsequent washing in a hypotonic one, for fixation of gill cilia in situ gives the same general appearance of the membrane (Figure 2c). This apparent structural difference between flagellar and ciliary membranes in the same organism is currently being investigated with high-resolution thin-sectioning and freeze-fracture techniques; the results will appear at a later date.

In contrast to the flagellar membrane, solubilization of the ciliary membrane is not total, even after three successive detergent extractions. As much as 10% of the membrane (estimated by length of profiles in cross-section) remains detergent resistant. This residual, fragmented membrane is frequently found associated with the *base* of the cilium (evident from interdoublet and doublet-membrane connections, Figure 1d, arrow) and may be composed of different materials than that of the ciliary shaft proper. Since the membrane at the basal region of cilia is morphologically distinct, bearing intramembranous particles which form a characteristic "ciliary necklace" (cf. Gilula and Satir, 1972), this result is not too surprising.

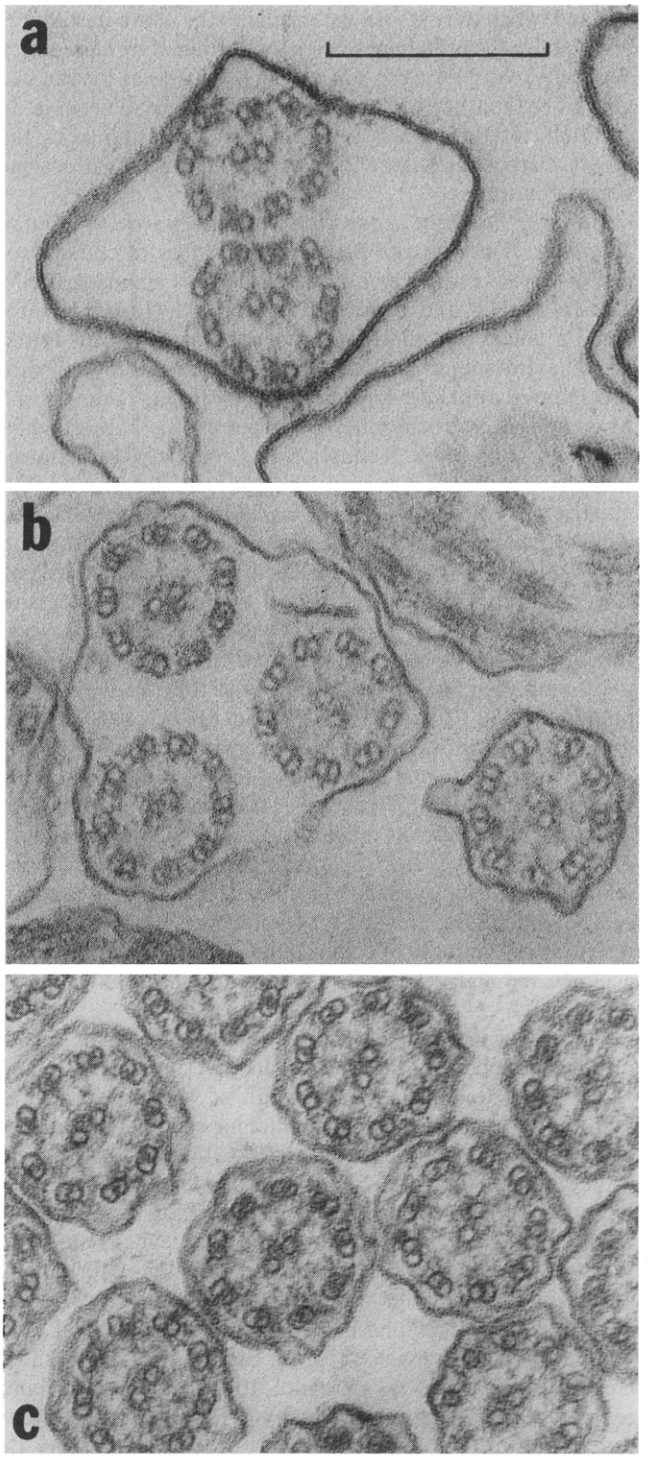

FIGURE 2: High magnification cross-sections of isolated sperm flagella (a) and gill cilia (b) illustrating the relatively thick and distinct trilamellar "unit membrane" of the flagellum in contrast to that of the cilium. A cross-section of gill cilia fixed in situ (c) shows that the comparatively less distinct ciliary membrane is not the result of the isolation procedure (micrograph by Dr. R. W. Linck). Scale: 0.25 μm.

Flagellar Membrane and Axoneme. When scallop sperm flagella were compared quantitatively with Triton-extracted axonemes by $NaDodSO_4$–polyacrylamide gel electrophoresis on a Tris–glycine system, they were found to be nearly identical except for the absence of a prominent high-molecular-weight band in the axoneme (Figure 3). No significant change could

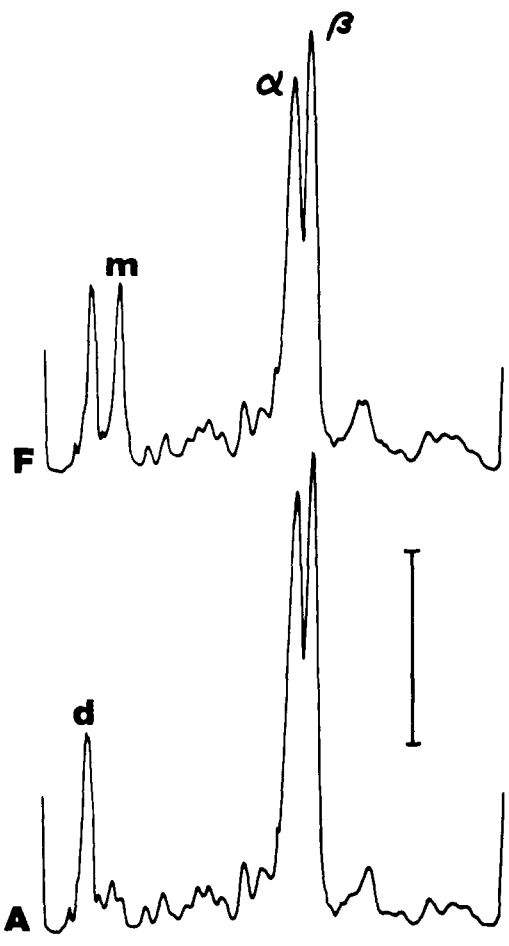

FIGURE 3: Sperm flagella (F) and the "9+2" axoneme (A) remaining after Triton X-100 extraction to remove the membrane are compared stoichiometrically on $NaDodSO_4$–Tris–glycine–polyacrylamide gels, stained quantitatively with Fast Green. One prominent band (m) of slightly greater mobility than dynein (d) is absent in the axoneme, while the tubulin subunits (α/β) show no significant change in amount. Loading: F (57 μg); A (47 μg). Scale: 0.5 OD unit at 650 nm.

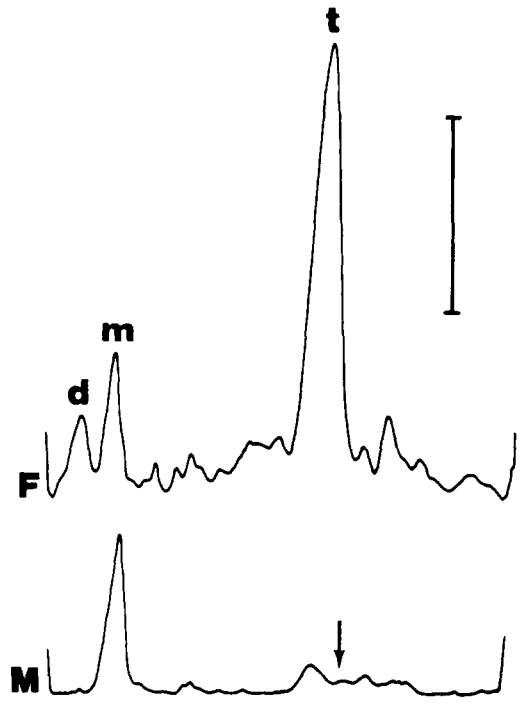

FIGURE 4: Flagella (F) and the Triton-soluble membrane fraction (M) are compared on $NaDodSO_4$–sodium phosphate–polyacrylamide gels, stained with Fast Green. The membrane contains one dominant protein (m), while tubulin (t) appears to be nearly absent in the membrane extract (arrow). Loading: F (50 μg); M (9 μg). Scale: 0.5 OD unit at 650 nm.

be detected in the tubulin content. When flagella were Triton-extracted, from 17 to 20% of the total protein was solubilized, based both on Lowry protein determination and on planimetry of the gel scans. When flagella and membrane extract were compared directly (using a phosphate or Tris–acetate buffer system to minimize detergent interference), the conclusions were basically the same. The membrane fraction was dominated by a single, high-molecular-weight protein and there was little, if any, protein migrating coincident with tubulin (Figure 4). Analyzed in this manner, the membrane fraction represented 18–21% of the total flagellar protein. Of the total protein in the membrane fraction, the high-molecular-weight material typically represented at least 67% and, when highly purified flagella were rapidly extracted, the figure could exceed 90%.

The molecular weight of the major flagellar membrane protein was estimated relative to bovine serum albumin (68 000) and its dimer and to scallop muscle paramyosin (106 000), myosin heavy chain (200 000) and its dimer, using 3% polyacrylamide gels (Linck, 1973b) and the three buffer systems discussed in Experimental Procedures. These standards behaved linearly on a plot of log molecular weight vs. mobility, regardless of buffer composition. For comparison, human red blood cell spectrin (Fairbanks et al., 1971) was run with the flagellar membrane protein. On the Tris–glycine system, the one most sensitive to anomalous $NaDodSO_4$ binding (Bryan, 1974), the two spectrin bands showed a maximal separation, migrating with a MW_{app} of 230 000 and 265 000. The flagellar membrane protein migrated between them with an apparent molecular weight of 243 000. On the sodium phosphate system, the spectrins migrated with MW_{app}'s of 215 000 and 235 000, while the membrane protein remained almost unchanged at 248 000 in comparison with the Tris–glycine system. Finally, on the Tris–acetate system the spectrins had apparent molecular weights of 225 000 and 245 000 with the flagellar membrane protein migrating with a MW_{app} of 265 000. Under no conditions did the membrane protein comigrate with either component of spectrin. All three proteins behaved anomalously on these gel systems if one considers the standards to be absolute.

Ciliary Membrane and Axoneme. When scallop gill cilia were compared electrophoretically with Triton-extracted axonemes, no obvious differences were seen except for a significant decrease in the tubulin band of the axoneme, to the extent of 11–13%. In contrast to sperm flagella, no high-molecular-weight membrane protein band could be detected in gill cilia. When the ciliary axonemes were compared directly with the membrane extract, the major protein of this extract was found to be of comparatively low molecular weight, comigrating with the tubulin of the axoneme (Figure 5). As in flagella, the membrane fraction of cilia represents 17–19% of the total protein, again based both on densitometry and direct protein determination. The major band, in turn, represents 55–65% of the total membrane fraction protein, depending upon the relative purity of the cilia preparation. When run on $NaDodSO_4$–Tris–glycine gels, the major ciliary membrane protein split into two equally staining bands which comigrated with the α and β subunits of tubulin from the axoneme (Figure 6).

Successive extractions of ciliary axonemes yielded 15–20%

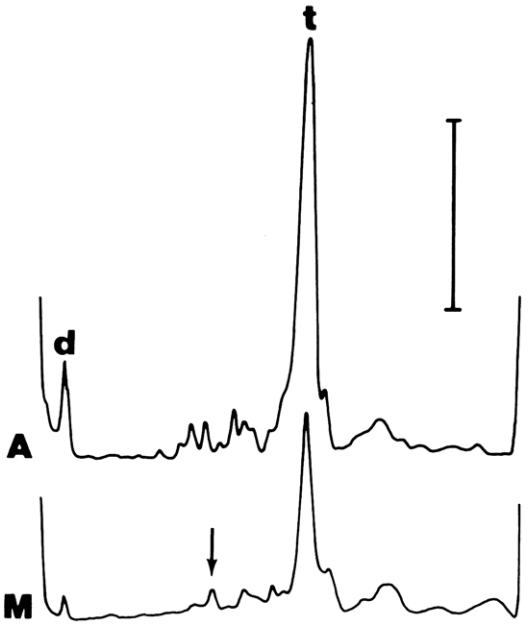

FIGURE 5: Gill ciliary axonemes (A) are compared with the Triton-soluble membrane fraction (M) on $NaDodSO_4$–Tris–acetate–polyacrylamide gels, stained quantitatively with Fast Green. No trace of a 250 000-dalton protein is present in the membrane fraction; instead the major protein migrates with tubulin (t). The arrow indicates a 100 000-dalton protein also consistently present. Loading: A (24 μg); M (13 μg, 2.5× stoichiometric ratio). Scale: 0.5 OD unit at 650 nm.

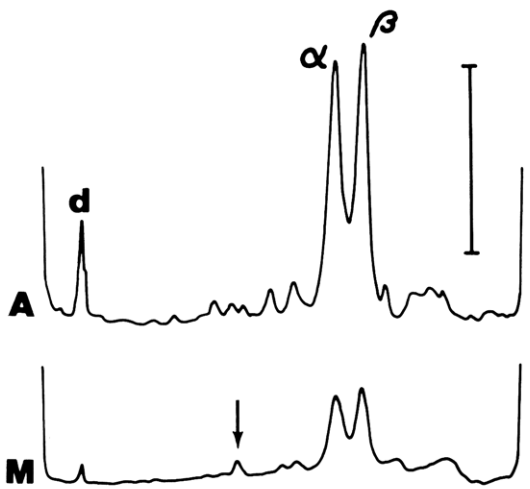

FIGURE 6: Ciliary axonemes (A) are compared with the Triton-soluble membrane fraction (M) on $NaDodSO_4$–Tris–glycine–polyacrylamide gels, stained with Fast Green. The major membrane protein splits into two equally staining bands, comigrating with the tubulin subunits (α/β). The arrow indicates the 100 000-dalton protein. Loading: A (21 μg); M (11 μg, 2.5× stoichiometric ratio). Scale: 0.5 OD unit at 650 nm.

additional protein, with virtually all of this appearing in the second extract. The amount was roughly proportional to the ratio of pellet volume to supernatant in the first extraction, perhaps reflecting the removal of residual, interstitial extract during the second extraction. A third extraction, even extended to a period of several hours, removed little or no (<1%) additional protein. The same phenomenon was true for the flagellar extraction; in either case, doubling the initial extraction volume roughly halved the amount of membrane protein obtained in the second extraction.

One minor but consistent component of the ciliary membrane extract was a protein migrating at about 100 000 daltons

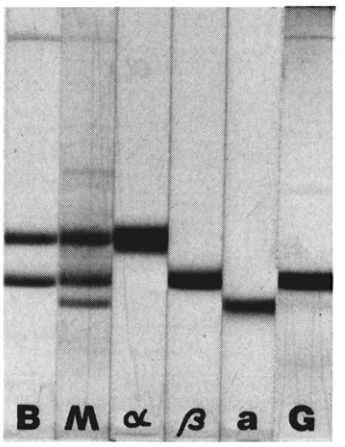

FIGURE 7: After S-carboxymethylation, the major ciliary membrane protein (M) shows an increased separation of its two subunits, comigrating with the similarly treated subunits of B-tubulin (B), when run on $NaDodSO_4$–Tris–glycine–polyacrylamide gels. Preparative gel electrophoresis separates the membrane subunits from each other (α/β) and from an actin-like protein (a) which contaminates crude ciliary membrane preparations. Gradient purification of cilia results in a membrane fraction (G) nearly devoid of the 43 000-dalton component, here run on an $NaDodSO_4$–Tris–acetate gel system. These gels were stained with Coomassie blue to accentuate minor bands.

(Figures 5 and 6, arrows), *not* corresponding in molecular weight to a tubulin dimer. In addition, a component migrating slightly slower than dynein occurred near the top of the gel, but was somewhat variable in amount. Upon further purification of the cilia, both of these components were enhanced while all other minor bands decreased in relative amount. In terms of total percentages, no significant amount of material was found migrating with the dye front or remaining at the gel interface.

Thus Triton-solubilized flagellar and ciliary membrane fractions were each found to represent about one-fifth of the total organelle protein and each were dominated by one major component. In both cases this major species accounted for about two-thirds of the total membrane protein. However, the major flagellar membrane component migrated as one band with an average molecular weight of 250 000 while the ciliary counterpart comigrated with tubulin, both on gels where tubulin migrated as a single band and where it was resolved into two subunits. The major flagellar membrane component was never detected in the ciliary membrane fraction nor was any significant amount of a protein comigrating with tubulin found in the flagellar membrane fraction.

Comparison of the Ciliary Membrane Protein with Tubulin. Reduction and alkylation of tubulin markedly enhances the separation between α and β subunits on low ionic strength $NaDodSO_4$ gel systems, apparently because additional charged groups (carboxymethylcysteine) increase the anomalous $NaDodSO_4$ binding of the α subunit (Bryan, 1974). When the presumptive ciliary membrane tubulin was reduced and alkylated, its subunits comigrated with those of B-subfiber tubulin treated in a like manner (Figure 7, B vs. M). Resolution of membrane subunits and separation by preparative gel electrophoresis was easily achieved (Figure 7, α vs. β). The membrane sample illustrated was from unpurified cilia, chosen because of the relative abundance of a 43 000-dalton protein often found associated with the membrane fraction in such preparations; preparative electrophoresis resulted in a clean separation of this protein from the presumptive membrane α and β chains (Figure 7, a). For comparison, a membrane

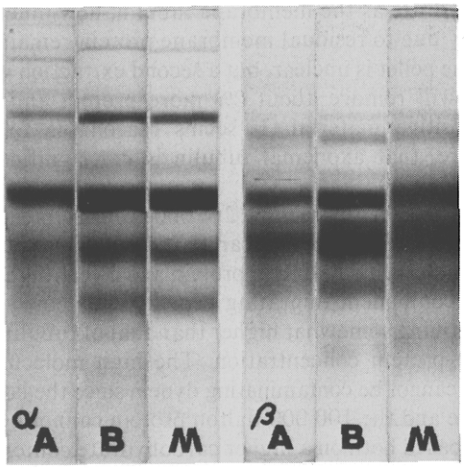

FIGURE 8: Cyanogen bromide digestion of the resolved α and β chains from ciliary A-tubule (A), B-subfiber (B), and membrane (M) tubulins and analysis on highly cross-linked $NaDodSO_4$-polyacrylamide gels reveals that the presumptive membrane tubulin subunits are cleaved into CNBr peptides with a size distribution virtually identical with those of authentic tubulin (Coomassie blue staining).

fraction derived from gradient purified cilia, run on a Tris–acetate gel system (Figure 7, G), is included to illustrate the near-absence of the 43 000-dalton protein in such preparations.

Cyanogen bromide cleavage of the resolved presumptive α and β chains from the ciliary membrane, analyzed on the Swank and Munkres (1971) gel system, resulted in a distribution of peptides not discernibly different from those obtained through cleavage of authentic tubulin subunits derived from A-tubule or B-subfiber tubulins (Figure 8, α_A or α_B vs. α_M; β_A or β_B vs. β_M). Subtle differences in minor bands could sometimes be seen but these were certainly no greater than those detected between homologous subunits of the authentic tubulins. Thus the subunits of the ciliary membrane protein must have a primary structure wherein methionine positions are identical with those of corresponding α and β chains of the subfiber tubulins.

The 43 000-dalton protein was cleaved by CNBr into a class of peptides migrating closely together and corresponding in no obvious way to those from either membrane protein subunit. It thus appears unlikely that it was derived from either subunit by limited proteolytic cleavage during the isolation procedure. The peptides correspond in general size class and distribution to those expected to arise from cleavage of actin, based on the known sequence of rabbit actin, but considering the possible species differences and the lack of adequate resolution, this can hardly be regarded as conclusive. The near-absence of the 43 000-dalton protein in membrane fractions derived from gradient-purified cilia minimizes further consideration of its identity or importance.

Tryptic peptide mapping provides additional proof that the major ciliary membrane protein is a tubulin dimer. Figure 9 illustrates composite peptide maps of homologous α and β chains from A-tubule and B-subfiber-derived axonemal tubulins and the corresponding α and β chains from the ciliary membrane fraction. As reported previously (Stephens, 1976a,b), the α chains from the two subfiber tubulins produce nearly identical peptide maps, differing at best by the relative intensity of certain peptide spots but not in the presence or position of any obvious peptides. The β chains differ, however, with that derived from the A-tubule having several additional spots not found in the B-tubulin counterpart, a finding in accord with amino acid compositional differences in the "homologous" outer fiber subunits.

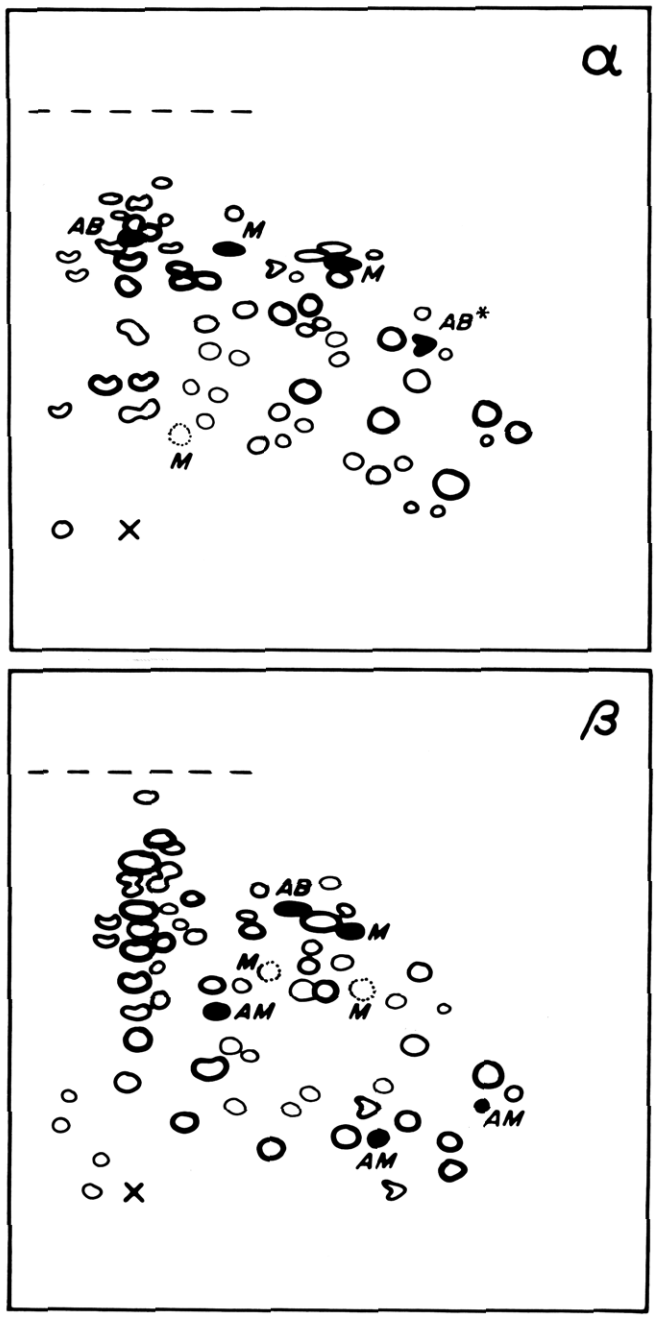

FIGURE 9: Comparative tryptic peptide mapping of α and β subunits of A-tubule, B-subfiber, and membrane-derived tubulins on thin-layer silica gel G plates shows a very high degree of homology. Peptides held in common are indicated by open circles, the line-thickness of which indicates relative intensity. Peptides unique to a particular subunit are filled and marked with letters indicating their source as A-tubulin (A), B-tubulin (B), or membrane-derived tubulin (M); e.g., "AB" designates a peptide not found in the membrane-derived tubulin but present in A- and B-tubulins. The asterisk (*) in the α-chain map designates a peptide that appears to be shifted to the right in the membrane-derived α chain. Chromatographic direction was vertical; electrophoresis with cathode on the right. Composites are from 4–6 maps of each subunit type; fluorescamine visualization.

In the case of the membrane-derived tubulin subunits, they correspond almost exactly in their tryptic peptide distribution to their axonemal counterparts. One prominent peptide is "missing" in each of the membrane chains (Figure 9, AB), with two additional peptides appearing in the α chain and one in the

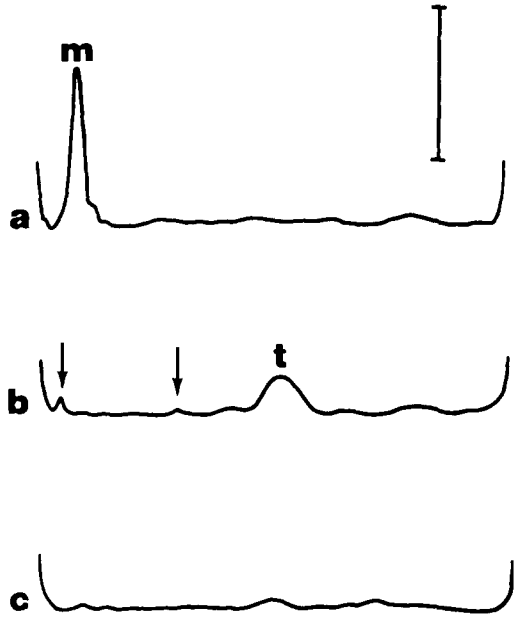

FIGURE 10: Staining with the periodic acid–Schiff reagent shows that the major flagellar membrane protein (a) is a glycoprotein, and also that the ciliary membrane tubulin (b) is weakly PAS positive, in contrast to the ciliary axoneme (c) which shows little significant reaction. Arrows indicate the 100 000-dalton and very high molecular weight proteins, always present in ciliary membrane extracts and also PAS positive. Loading: (a) 9 μg; (b) 26 μg; (c) 60 μg. Scale: 0.1 OD unit at 550 nm.

β (Figure 9, M). One peptide (Figure 9, AB*) appears to be shifted in position in the membrane α chain, sometimes comigrating with the spot to the immediate right. The membrane β chain contains the three peptides unique to the A-tubulin β chain (Figure 9, AM). Both membrane subunits show additional "ghost" spots and some of the major peptides differ markedly in relative fluorescence intensity in comparison with their apparent subfiber homologues.

Since nearly all tryptic peptides found in authentic tubulin have their counterpart in the subunits of the ciliary membrane protein, the membrane protein can be considered a tubulin. However, the fact that there are clear positional differences would indicate that the membrane subunits are not strictly identical with those of the axoneme. This may simply reflect some posttranslational side chain modification, resulting in an increased relative electrophoretic migration of the "missing" axonemal peptides to produce the "new" spot or spots in the membrane tubulin fingerprints. The results are certainly consistent with this hypothesis.

Carbohydrate Staining. Reaction with periodic acid–Schiff reagent indicates that the major flagellar and ciliary membrane proteins are both PAS positive while ciliary axoneme tubulin is not to any significant extent (Figure 10). The upper trace is the same flagellar membrane sample as in Figure 4, containing 9 μg of total protein or about 6 μg of the 250 000-dalton protein. The center trace is the same ciliary membrane sample as in Figure 6 but at twice the loading, thus containing about 17 μg of membrane tubulin. The lower trace is the same axoneme sample as in Figure 5 but at 2.5 times the loading, thus containing about 40 μg of tubulin. From the ratio of applied ciliary to flagellar membrane proteins (3:1) vs. the ratio of PAS-staining intensity (1:2), it can be estimated that membrane tubulin contains, at a maximum, only about one-sixth as much carbohydrate as its high molecular weight flagellar membrane counterpart. Based on the degree of PAS staining, the axoneme tubulin could contain only about 15% as much carbohydrate as the membrane tubulin; how much of this is actually due to residual membrane protein remaining in the axoneme pellet is unclear, but a second extraction with Triton X-100 will remove about 2% more protein and nearly all PAS-positive material. It seems reasonable to conclude, therefore, that axonemal tubulin is not significantly PAS positive.

In the flagellar extract the 250 000-dalton protein is the only component with significant carbohydrate content but in ciliary fraction the 100 000-dalton protein and a very high molecular weight component migrating near dynein show a degree of PAS staining somewhat higher than that of tubulin, based on relative protein concentration. The high molecular weight protein cannot be contaminating dynein since the latter is PAS negative and the 100 000-dalton protein cannot be a tubulin dimer, based both on a higher carbohydrate content and on a different migration rate.

Interestingly, if the samples were concentrated by acetone precipitation or carried through the reduction and alkylation procedure, the major flagellar membrane protein and the minor ciliary components remained unchanged in their degree of PAS reactivity, but the ciliary membrane tubulin was much diminished. For example, the parallel counterparts of the subunits illustrated in Figure 7 (α and β) had barely detectable PAS staining at loadings of 25 μg. Whatever was responsible for the majority of the PAS-positive reactivity in the membrane tubulin was evidently not tightly bound since it was removed during acetone treatment or by unfolding the polypeptide chain during reduction and carboxymethylation. The association is evidently sufficiently strong, however, to withstand $NaDodSO_4$ electrophoresis. We have not yet identified this component.

Colchicine Binding. At 25 °C, neither whole flagella nor their axonemes had any significant colchicine binding activity after 60-min incubation. However, at 37 °C both had measurable activity in the range of 0.005–0.01 mol of colchicine bound per 100 000 g of total protein after a 30-min incubation and this activity either increased slightly with time or remained relatively constant, up to 90 min. This marginal colchicine binding was most likely due to the slow solubilization of axonemal microtubules at elevated temperatures, yielding a 6S tubulin able to bind the drug, a fact demonstrated earlier by Wilson and Meza (1973). Having values consistently lower than those of flagellar axonemes, ciliary axonemes also possessed a slight but measurable binding activity at 37 °C but none at 25 °C.

Whole cilia, however, bound colchicine at 25 °C to the extent of 0.01–0.02 mol per 100 000 g of total protein, measured at 30 min, with this activity remaining essentially constant over a 90-min assay period. Nearly all of this activity appeared to reside in the membrane fraction. At 60 min, the Triton X-100 extract had a maximal binding of 0.05–0.14 mol of colchicine per 100 000 g of total protein, or 0.08–0.23 mol per mol of tubulin in the sample. Values were roughly half these at 30 or 90 min. Activity decayed rapidly and erratically at 37 °C and even upon standing on ice the extract lost half of its activity in about 6 h. No significant difference in colchicine binding activity was demonstrable between membrane tubulin solubilized with 0.25% Nonidet P-40 or with 0.5% Triton X-100, measured at the same final detergent concentration. Nonidet P-40 was tested because of a report that Triton X-100 inhibited colchicine binding of brain tubulin while Nonidet P-40 did not (Bhattacharyya and Wolff, 1975); this same report noted that the continued presence of detergent during incubation reduced the extent of colchicine binding. Partial inhibition by Triton X-100 may explain why colchicine binding in the ciliary

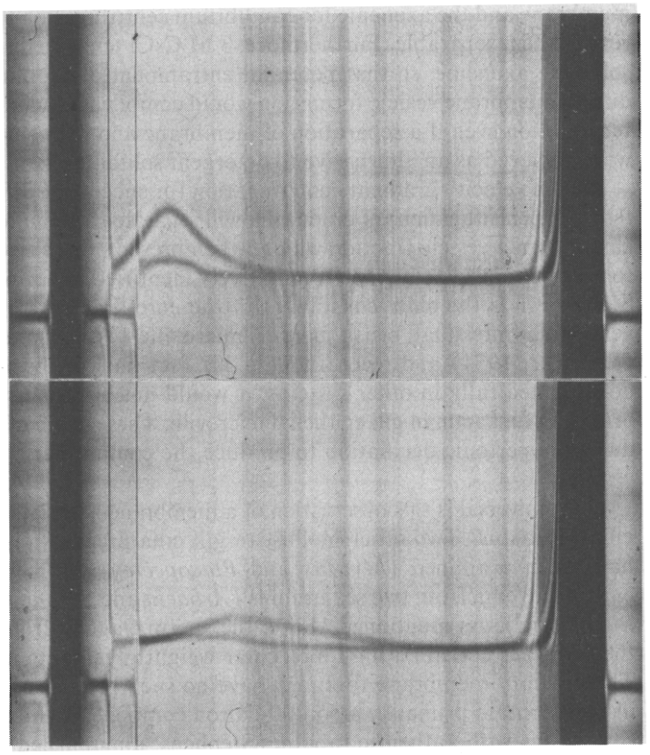

FIGURE 11: Analytical ultracentrifugation of the ciliary membrane dissolved in 0.1% Nonidet P-40. The upper frame is 64 min after reaching a speed of 42 040 while the lower is 160 min later. Temperature: 25 °C. Bar angle: 70°.

membrane fraction never approached the theoretical value of 1 mol of colchicine per 110 000 g of protein. Because of variability, lability, and limited material, the colchicine binding properties of the membrane tubulin were not characterized further.

Analytical Ultracentrifugation of the Detergent-Solubilized Tubulin. Late in this study it was discovered that Nonidet P-40 was superior to Triton X-100 for ciliary membrane solubilization, functioning quite effectively at concentrations fivefold lower. These nonionic detergents have nearly identical compositions (polyoxyethylene glycol *p-tert*-octylphenol) and are often used interchangeably. Why they differ in effectiveness is not at all clear, but a twofold difference in the solubilization of total membrane-bound protein in brain has been reported (Bhattacharyya and Wolff, 1975), in this case with Triton X-100 being more effective than Nonidet P-40.

The use of Nonidet P-40 at low concentrations permitted a hydrodynamic analysis of the tubulin employed in the colchicine binding assays, with minimal interference from sedimentation of detergent micelles. Figure 11 illustrates the ultracentrifugation of ciliary membrane tubulin at about 5 mg/mL in 0.1% Nonidet P-40, 30 mM Tris-HCl, pH 8, and 3 mM $MgCl_2$, compared with the detergent–salt solution alone. A small peak, due to detergent micelles, was evident in the solvent system but it sedimented at roughly half the rate as did the major protein component (Figure 11, upper frame). Upon extended centrifugation, the protein peak broadened considerably (Figure 11, lower frame), suggestive of a polydisperse population of sedimenting species. The $s_{20,w}$ for the major portion of the boundary was 3.8. The expected value for a tubulin dimer is 6 S (cf. Bryan, 1974), while that for the monomer is 2.8 S (cf. Stephens and Linck, 1969). The fact that an intermediate value was obtained would suggest that the boundary is due to protein–detergent complexes. Continuous observation during acceleration did not reveal any rapidly sedimenting material, indicating that the major detergent-solubilized ciliary membrane protein is a relatively small species, not inconsistent in size with that of a tubulin dimer. It seems unlikely that any higher aggregates of tubulin are present in the detergent-solubilized ciliary membrane fraction.

Discussion

The major protein component of the scallop sperm flagellar membrane is a 250 000-dalton glycoprotein. It makes up at least two-thirds of the total Triton X-100 extractable protein of the membrane fraction, with this fraction itself representing about one-fifth of the total flagellar protein. This protein is probably not related to red blood cell spectrin since spectrin electrophoretically migrates as a doublet, neither chain of which comigrates with the flagellar protein, and moreover spectrin is not PAS positive. The presence of carbohydrate makes the observed molecular weight of the flagellar glycoprotein suspect since glycoproteins may migrate anomalously on $NaDodSO_4$–polyacrylamide gel systems (Segrest et al., 1971). The Triton X-100 extract of flagella contain little or no tubulin, in spite of the well-known lability of the central pair microtubules, a potential source of extraneous tubulin. Its near-absence in the extract would also indicate that little or no free tubulin exists in the flagellar matrix.

Proteins of the same size class as the 250 000-dalton species have been observed in flagellar membranes of other organisms as well. Witman and co-workers (1972) found a protein with a molecular weight in excess of 200 000 as the major constituent of *Chlamydomonas* membranes; in this case a trace of tubulin and several other minor proteins were detectable. In *Ochromonas,* Chen and Haines (1976) detected five lower molecular weight protein components, the largest of which had a molecular weight corresponding to tubulin, and noted that the counterpart to the *Chlamydomonas* major protein was quite large, not migrating appreciably on their gel system. In echinoderm (sea cucumber) flagella, a high molecular weight protein was the dominant feature of a Triton X-100 extract of whole sperm (Tilney et al., 1973). Finally, Triton extracts of sperm flagella from two species of sea urchin (*Arbacia* and *Strongylocentrotus*) and two bivalve molluscs (*Mytilus* and *Placopecten*) have a protein complement not significantly different from that in Figure 4 (Stephens, unpublished). Although a phylogenetic survey has obviously not been attempted, it is encouraging to find very similar protein compositions in flagellar membranes from lower plants and higher invertebrates.

Scallop gill cilia or their Triton-soluble membrane fraction lack any evidence of a 250 000-dalton glycoprotein. Instead, a weakly PAS-positive 55 000-dalton component is found in an amount approximately equal to that of the major flagellar component. This protein has been identified as a tubulin on the basis of its resolution into two subunits comigrating with tubulin α and β chains, the coincidence of methionine positions in these subunits with those in authentic tubulin chains, the near-identity of tryptic peptides of these same subunits with those from axonemal tubulin, and a labile but measurable ability of the protein to bind colchicine.

That this "membrane tubulin" is not artifactual can be argued from several directions. If it were axonemal in source, arising from a time-dependent breakdown of microtubules, successive extractions should have significant amounts of tubulin present. A second extraction has less than 20% of the amount of protein as the first, with much of this being poten-

tially interstitial. A third extraction, even when carried out for several hours, contains essentially no tubulin. Long-term, low ionic strength dialysis is necessary to promote axoneme breakdown, resulting in dissolution of one central pair member and all 9 B-subfibers (Linck, 1973b). Extracted under identical conditions, the far more labile flagella showed little or no extraction of tubulin from the central pair, serving in a sense as a control for the parallel extraction of cilia. Furthermore, tryptic peptides of membrane and axonemal tubulin chains are not strictly identical. Finally, the tubulin in the membrane fraction is PAS positive while that from the axoneme is not. The ciliary matrix cannot be ruled out as a possible source of tubulin appearing in the Triton-soluble fraction but this would lead to the conclusion that there is no protein in the ciliary membrane in any amount comparable to that found in the flagellar membrane, a conclusion that would be nevertheless of some interest.

The presence of tubulin in the ciliary matrix would not be totally unexpected since, during growth or renewal, soluble tubulin from the cytoplasm must somehow be conveyed to the tip of the elongating organelle and the extraaxonemal space would be its most likely location. The absence of tubulin in the flagellar matrix might also be expected since mature sperm would not be adding to or turning over axonemal proteins. The fact that the tubulin in question is PAS-positive, while tubulin in the axoneme is not, may simply reflect the requisite removal of carbohydrate from a soluble matrix tubulin prior to its incorporation into axonemal microtubules. The reality of tubulin as an authentic membrane constituent still remains the simplest hypothesis, however, particularly in light of the relative difficulty with which detergent solubilizes ciliary membranes, their physical tenacity, and the unique appearance of these membranes in the electron microscope, all properties which might not be expected of a protein-free lipid bilayer.

The fact that the PAS-positive nature of the Triton-soluble tubulin is reduced dramatically by acetone treatment or carboxymethylation might indicate that a noncovalently bound moiety is responsible for the bulk of the carbohydrate associated with the tubulin. Bryan and co-workers (Hammond and Bryan, 1972; Wilson and Bryan, 1974) report a third, or "γ", subunit of the very low molecular weight, possibly a glycolipid or lipoprotein associated somehow with the colchicine binding site of the tubulin dimer. It may be that such molecules exist in conjunction with tubulin in order to render it lipophilic and aid in its incorporation into or onto a lipid bilayer.

Alternatively, sugar moieties may be bound to the protein through O-glycosidic linkages to serine or threonine, as has been suggested for synaptic membrane tubulin by Shelanski and colleagues (1972). Such linkages would be susceptible to β elimination at the somewhat alkaline pH used during either the protein precipitation or carboxymethylation. A variable fraction of the sugars might thus be lost, explaining the variable but reduced PAS reactivity of the membrane tubulin after further purification.

The inability to "float" ciliary membranes free from axonemes either on sucrose gradients of low ionic strength or on cesium chloride gradients of extreme ionic strength would indicate a fairly firm association between the membrane and the axoneme–matrix complex. This is in striking contrast to flagellar membranes where simple shearing will strip them from the axoneme, permitting easy gradient separation (e.g., Witman et al., 1972). Since membrane fusion occurs readily in centrifuged whole cilia (Figure 1c), the ciliary membrane itself must be quite fluid, resealing easily. Alternatively, there may not be a sufficient density difference between the ciliary membrane and the axoneme for equilibrium centrifugation to render them separable. Furthermore, 3 M CsCl tends to dissolve the axoneme, so that potential entrapment of tubulin during membrane vesicle formation would complicate the interpretation even if a separation of membrane and axoneme were achieved as an alternative to detergent solubilization.

Simple velocity gradient centrifugation (or successive differential centrifugation) of crude cilia will separate them quite easily from a vesicle fraction which, when present, gives rise to a 43 000-dalton component tentatively identified as actin. Since actin is the chief constituent of the core filaments of vertebrate intestinal brush border microvilli (Tilney and Mooseker, 1971) and, even though this fact has not been documented fully in other systems, it would not be too surprising to find actin in gill epithelial microvilli. These pinch off during hypertonic deciliation to produce the contaminating vesicles.

How universal is this observation of a membrane-associated ciliary tubulin? Two other molluscan gill cilia preparations have been examined (*Mytilus* and *Placopecten*), as have embryonic cilia from two sea urchins (*Arbacia* and *Strongylocentrotus*). As mentioned above, the sperm flagella from these species contain a high molecular weight glycoprotein. These ciliary membrane fractions have no such protein but instead contain primarily a 55 000-dalton component which comigrates with authentic tubulin (Stephens, unpublished). On the other hand, pure membranes from *Tetrahymena* cilia are reported to contain no detectable tubulin (Baugh et al., 1976). Other systems simply have not yet been analyzed.

The most thoroughly documented case for tubulin as a membrane constituent is in the synaptosomal fraction prepared from brain homogenates. Three groups of workers (Feit et al., 1971; Blitz and Fine, 1974; Kornguth and Sunderland, 1975) identified tubulin in crude synaptosome preparations on the basis of electrophoresis and peptide mapping, and another group (Cotman et al., 1974; Banker et al., 1974) found that isolated postsynaptic densities contained a major protein with a molecular weight of 53 000. Walters and Matus (1975) identified this protein as tubulin by tryptic peptide mapping and localized it in the postsynaptic junctional lattice by immunohistochemistry at the electron microscopic level. Like the ciliary membrane tubulin reported here, tryptic peptides of these synaptosomal tubulins were almost, but not quite, identical with their soluble brain counterpart (Feit et al., 1971; Blitz and Fine, 1974; Kornguth and Sunderland, 1975; Walters and Matus, 1975), suggesting some sort of possible modification. Regardless of interpretation, tubulin or tubulin-like molecules appear to exist at least as part of an extrinsic membrane element in the postsynaptic density.

Somewhat less direct evidence, based on colchicine binding, suggests that tubulin may be an integral component of the synaptosome plasma membrane as well (Feit and Barondes, 1971; Lagnado and Lyons, 1972). This membrane fraction typically shows a prominent 52 000-dalton protein component, regardless of the specific preparative methods (Banker et al., 1972; Karlsson et al., 1973; Morgan et al., 1973). However, all of these studies are open to the criticism that some or all of the observed tubulin may have come from axoplasmic sources or that the colchicine-binding activity may reside solely in any adhering postsynaptic densities.

A stronger argument for the reality of tubulin as an integral part of the synaptic plasma membrane is the fact that such tubulin is PAS positive while the soluble, axonal tubulin which might contaminate the preparation is not (Feit and Shelanski, 1975), as has been argued here for the ciliary membrane

counterpart. Feit and co-workers (1971) also demonstrated slow transport of tubulin to nerve endings and speculated that the observed incorporation of hexosamines into tubulin at nerve endings might be preparatory to its incorporation into membranes or to its secretion or degradation. Such a side-chain modification could explain the slight differences in tryptic peptide fingerprints seen when presumptive membrane tubulins are compared with cytoplasmic or axonemal counterparts.

Kadota and co-workers (1976) discovered that the presynaptic matrix material, a flocculent protein aggregate derived from fractionated synaptosomes and sometimes found associated with synaptic vesicles, could be induced to form helices and rings by interaction with vinblastine sulfate, producing crystalline forms not unlike those which form when the drug interacts with tubulin. This finding would suggest that tubulin can exist in a polymorphic, particulate form not directly a part of but associated with membranes, just as it exists as a more-ordered component of the postsynaptic density.

Bhattacharyya and Wolff (1975) have shown that both brain and thyroid tissue contain a membrane-associated colchicine-binding activity which shows a considerably greater thermal stability and a higher temperature optimum for binding than does soluble tubulin. When this membrane-bound activity was released through Nonidet P-40 solubilization, it adopted the binding properties of a typical free tubulin and, in addition, $NaDodSO_4$–polyacrylamide gel electrophoresis revealed a prominent band comigrating with authentic tubulin. The marked differences in properties of bound vs. free tubulin as a result of the membrane environment would argue strongly in favor of the validity of a membrane-bound tubulin.

Stadler and Franke (1974) characterized the colchicine-binding properties of liver membranes, using both microsomal and nuclear membrane fractions. They noted appreciably better binding in the latter, found the binding in both fractions to be usually heat stable, and showed that the binding was complex and dose dependent, a fact consistent with the presence of both high and low affinity binding sites. A lesser but significant binding of lumicolchicine indicated a certain degree of nonspecificity. These workers concluded that, even if all of the observed binding was due to a membrane-associated tubulin, only a minute amount of the total nuclear membrane protein could be tubulin.

Using isolated nuclear membranes from lily microsporocytes, Hotta and Shepard (1973) detected a membrane-bound colchicine-binding protein which could be solubilized with Nonidet P-40. It differed from its cytoplasmic counterpart (tubulin) in that it appeared to have a slightly lower molecular weight (100 000 vs. 110 000) and sedimentation coefficient (5.3 S vs. 5.6 S) and was far less sensitive to precipitation by vinblastine. Treatment of meiotic nuclear membranes with colchicine resulted in the loss of a DNA-binding protein which normally appears during meiotic prophase and is thought to be involved in chiasma formation. It and the colchicine-binding protein are not the same entity and the data would thus imply that tubulin-like molecules associated with the nuclear envelope are somehow involved in the attachment of DNA-binding proteins to the membrane, facilitating chromatin-membrane association.

Tubulin as an integral or associated part of membranes may be related to the observed increase in fluidity of plasma membranes after colchicine treatment, an effect usually ascribed to a disruption of submembranous microtubules which are thought to interact with the cell surface and minimize fluidity (e.g., Edelman et al., 1973; Berlin et al., 1974). Conversely, Wunderlich and co-workers (1973) found the opposite effect of the drug on the temperature-induced mobility of membrane-intercalating particles in *Tetrahymena*, namely an impairment of fluidity induced by colchicine. In either case, interaction of the drug may be directly with a membrane-bound tubulin, in some manner perturbing normal hydrophobic interactions involved in membrane stability or flow.

Evidence for a direct interaction between plasma membranes and microtubules comes from fluorescent energy transfer studies done with fluorescein-labeled polymorphonuclear leukocyte membranes and rhodamine-labeled brain tubulin (Becker et al., 1975). At 0 °C, where no in vitro tubulin polymerization could take place, no energy transfer could be shown, but polymerization of the tubulin by raising the temperature to 37 °C resulted in increasing resonance energy transfer between the membrane- and tubulin-bound chromophores, paralleling the increase in microtubule polymerization. The observed high efficiency of energy transfer indicated a considerable degree of interaction, suggesting that some membrane constituent may serve as a nucleation site for microtubule polymerization.

What functional role membrane tubulin might play in either ciliary movement or in membrane physiology in general is not at all obvious. Considering the varied organic molecules that tubulin can bind (e.g., colchicine, vinblastine, podophyllotoxin, GTP), tubulin could conceivably have some sort of chemoreceptive role, binding the relevant molecule, changing conformation, and thus influencing membrane fluidity, conductance, or transport. As a contractile protein, able to interact with the ATPase dynein and produce motion, tubulin existing as part of a membrane could provide enzymatic interaction sites whereby the membrane might be propelled (as a vesicle) or translocated (as a section of fluid membrane) by means of a cytoplasmic, dynein-based contractile system. The opposite may also apply, however, with a membrane-bound tubulin being conveyed passively via membrane flow to an advancing, microtubule-containing structure, such as a developing neuron or an elongating cilium. On the other hand, tubulin may serve only an inert structural role as an extrinsic membrane element, providing a rigidly associated, hydrophobic framework for ciliary and plasma membranes or the postsynaptic junctional lattice. In this potential dual role as both the building block of cytoplasmic microtubules and as an intrinsic or extrinsic component of membranes, tubulin must interact with specific lipids, lipoproteins, or glycolipids to promote its incorporation into or association with membranes.

Why such a significant difference in protein composition should exist in ciliary and flagellar membranes is also not clear. The sperm is a short-lived, self-contained, and self-controlled cell whose flagellum must propagate a continuous, unidirectional wave and whose membrane must simply prevent the diffusion of ATP and other metabolites. The ciliated epithelial cell has an electrically excitable membrane and the cell is coupled to others; the cilia are under ionic control with respect to direction and frequency of beat. The difference in protein composition of the organelle membrane may somehow reflect the functional differences in the respective cell type.

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