

Evidence for a Tubulin-containing Lipid-Protein Structural Complex in Ciliary Membranes

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ABSTRACT The proteins and lipids of the scallop gill ciliary membrane may be reassociated through several cycles of detergent solubilization, detergent removal, and freeze-thaw, without significant change in overall protein composition. Membrane proteins and lipids reassociate to form vesicles of uniform, discrete density classes under a variety of reassociation conditions involving detergent removal and concentration. Freed of the solubilizing detergent during equilibrium centrifugation, a protein-lipid complex equilibrates to a position on a sucrose density gradient characteristic of the original membrane density. When axonemal tubulin is solubilized by dialysis, mixed with 2:1 lecithin/cholesterol dissolved in Nonidet P-40, freed of detergent, and reconstituted by freeze-thaw, vesicles of a density essentially equal to pure lipid result. If the lipid fraction is derived through chloroform-methanol extraction of natural ciliary membranes, a moderate increase in density occurs upon reconstitution, but the protein is adsorbed and most is removed by a simple low ionic strength wash, in contrast to vesicles reconstituted from membrane proteins where even high salt extraction causes no loss of protein. The proteins of the ciliary membrane dissolve with constant composition, regardless of the type, concentration, or efficiency of detergent. Analytical ultracentrifugation demonstrates that monodisperse mixed micelles form at high detergent concentrations, but that membranes are dispersed to large sedimentable aggregates by Nonidet P-40 even at several times the critical micelle concentration, which suggests reasons for the efficacy of certain detergent for the production of ATP-reactivable cell models. In extracts freed of detergent, structured polydisperse particles, but not membrane vesicles, are seen in negative staining; vesicles form upon concentration of the extract. Membrane tubulin is not in a form that will freely undergo electrophoresis, even in the presence of detergent above the critical micelle concentration. All chromatographic attempts to separate membrane tubulin from other membrane proteins have failed; lipid and protein are excluded together by gel filtration in the presence of high concentrations of detergent. These observations support the idea that a relatively stable lipid-protein complex exists in the ciliary membrane and that in this complex membrane tubulin is tightly associated with lipids and with a number of other proteins.

Tubulin is found as the major protein component of molluscan ciliary but not flagellar membranes, constituting at least two-thirds of the total detergent-solubilized ciliary membrane-matrix fraction (19). The tubulin may be labeled selectively with vectorial probes (20) and can be cross-linked to itself and to other major membrane proteins with a lipophilic, bifunctional reagent (6), which implies that it is an integral membrane protein. The tubulin of the membrane differs from that of the 9+2 axoneme in its relative degree of hydrophobicity, as judged by amino acid composition and differential detergent binding (21). The detergent-solubilized tubulin of the ciliary membrane-matrix fraction can be reassociated with

natural lipids by detergent removal followed by a simple freeze-thaw cycle (22). This tubulin and numerous other proteins remain associated with the reconstituted membrane after rigorous salt treatment, which suggests that these proteins are all integral. When membrane reconstitution is performed in the presence of high amounts of brain tubulin, the brain tubulin does not exchange with the ciliary membrane tubulin. Taken together, these data suggest that tubulin and various other ciliary membrane proteins may be closely associated with one another and with lipids, forming a discrete complex (22). The purpose of this report is to present further evidence for this concept through multiple successive membrane re-

constitutions (recycling), reconstitution by detergent removal followed by concentration, analysis of the mechanism of membrane solubilization, gradient centrifugation of solubilized membrane constituents in the absence of detergent, and electrophoresis or column chromatography of these components in the presence of detergent.

MATERIALS AND METHODS

Ciliary Proteins and Membrane Extract: Cilia were isolated from excised gills of the bay scallop *Aequipecten irradians* by high salt release and differential centrifugation, as previously described (22). The cilia were extracted with 10 vol of either (a) 3 mM MgSO₄, 30 mM Tris-HCl (pH 8), or (b) 0.5 mM MgSO₄, 1 mM EGTA, 1 mM guanosine triphosphate, 100 mM morpholino-ethane-sulfonic acid (pH 6.4), each containing 0.5 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol, with concentrations of various detergents as determined by the experimental protocol. The suspensions were held on ice for a minimum of 10 min, and then centrifuged at 45,000 g for 15 min, yielding pellets of 9+2 axonemes and supernatants of detergent-solubilized membrane and matrix components. Nonidet P-40 (NP-40)¹ (Particle Data, Inc., Elmhurst, IL) was removed by gentle agitation with 1/3 vol of SM-2 Bio-Beads (Bio-Rad Laboratories, Richmond, CA) for 2 h at 4°C (11), while octyl glucoside (OG) (Calbiochem-Behring Corp., San Diego, CA) was removed by exhaustive dialysis against 10 mM Tris-HCl (pH 8), 3 mM MgSO₄, and 0.1 mM dithiothreitol for 36–48 h at 4°C (10). Axonemal tubulin (from B-tubules plus central pair) was solubilized by exhaustive dialysis of the membrane-free axonemes against a low ionic strength buffer containing 0.1 mM EDTA at 4°C for 36–48 h (15). Skate brain tubulin was prepared by *in vitro* polymerization as previously described (22).

Reconstitution of Ciliary Membranes: The detergent-free extracts were frozen in liquid nitrogen and then thawed at room temperature, which resulted in membrane formation (22). When required, the frozen extracts were stored at –80°C. Alternatively, the extracts were concentrated 8–10-fold at 4°C in dialysis bags by external application of either Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, NJ) or Aquicid-II (Calbiochem-Behring Corp.).

Liposome-Tubulin Reconstitution: Soybean lecithin and cholesterol in a 2:1 weight ratio were dissolved in chloroform-methanol (2:1 vol/vol) or, alternatively, natural lipids were extracted from isolated cilia with chloroform-methanol. In either case, a solution containing ~1 mg of lipid was evaporated under N₂, dissolved in 2 ml of a solution containing 0.25% NP-40 and 1–2 mg of solubilized axonemal tubulin, briefly bath-sonicated (30 s), and centrifuged at 45,000 g for 15 min. The mixed micelles were freed of detergent with Bio-Beads as described above, then frozen and thawed.

Isopycnic Centrifugation: Linear 20–60% (wt/wt) sucrose gradients were formed by layering equal volumes of sucrose solutions in 5% increments, and then allowing them to stand for 24 h at 4°C. The gradients were buffered with 10 mM Tris-HCl (pH 8). Ultracentrifugation was carried out for 8–24 h (for reconstituted vesicles) or 24–48 h (for solubilized vesicles) in a Beckman SW 40 rotor (Beckman Instruments, Inc., Palo Alto, CA) at 4°C. Equilibrated gradients were photographed by intense overhead illumination against a dark background. For analysis, gradients were pumped from the bottom, using a long, blunt hypodermic needle inserted carefully through the gradient. The density calibration for the gradients was established by marking the position of each sucrose increment during initial layering and then taking the mid-point of the increment to be the final equilibrium density.

PAGE: The discontinuous ionic system of Laemmli (14) was used throughout for SDS gels. Linear 5–15% acrylamide gradients were cast in 1.5-mm × 10-cm slabs. For “native” gels, the same ionic system was used, except that OG was substituted for SDS and the gels were cast in 5-mm × 10-cm tubes, each containing a different detergent concentration. Gels were stained with Coomassie Blue R-250 by the method of Fairbanks et al. (8). Quantitation was by densitometry. Some slab gels were silver stained by the method of Wray et al. (28).

Light Scattering: The turbidity of suspensions of reconstituted membranes in the appropriate buffer was observed and recorded at 530 nm with a Beckman DB spectrophotometer (Beckman Instruments, Inc.) using buffer as a blank. Aliquots of concentrated detergent were added slowly with a microsyringe, employing gentle agitation. Readings were allowed to stabilize for at least 1 min before the next aliquot was added. Measurements were carried out at

20–22°C using several dilutions of the vesicle stock, typically beginning with 0.4–0.5 mg/ml protein (0.5–0.6 mg/ml lipid). The cuvette path length was 1 cm.

Analytical Ultracentrifugation: Observations were carried out at 20°C with a Beckman Model E ultracentrifuge (Beckman Instruments, Inc.), using schlieren optics with 12-mm double sector wedge and standard cells in an AN-D rotor. High and low molecular weight species were recorded by initiating a run at 20,410 rpm, and then increasing the speed to 42,040 rpm. Photographs were taken on Kodak (Eastman Kodak Co., Rochester, NY) metallographic plates.

Electron Microscopy: Membrane pellets were fixed for a minimum of 2 h at 0°C with 2.5% glutaraldehyde in 0.1 M sodium phosphate (pH 7), washed twice with this buffer, and then postfixed at room temperature with ferrocyanide-osmium (12). The material was stained en bloc for at least 3 h with 1% uranyl acetate, dehydrated with ethanol, and embedded in Epon-Araldite (17). Silver sections were cut with a diamond knife and then observed and photographed with a Zeiss EM-10C electron microscope. Negative staining was carried out on glow-discharged, carbon-coated Formvar films, using 1% uranyl acetate as a stain, after thorough buffer rinsing of the adsorbed material to prevent precipitation artifacts. Due to focusing artifacts arising from the granular nature of the membranous material, the edges of holes in the grid film were used to focus upon, then the adjacent material under study was moved to the image center and photographed.

RESULTS

Ciliary Membrane Reconstitution and Recycling

When cilia from the scallop *Aequipecten irradians* are suspended in 0.25–0.5% NP-40 in 3 mM MgCl₂, 30 mM Tris (pH 8) buffer, extracted on ice for 10 min, then spun down at 35,000 g, they yield a membrane-matrix supernatant and a pellet of 9+2 axonemes. The detergent may be removed effectively from the supernatant with SM-2 Bio-Beads and the membrane subsequently reconstituted by a simple freeze-thaw cycle. Nearly all of the supernatant proteins reassociate with the vesicles; calmodulin is the major one that does not (22).

Can such membranes be “recycled” and do their constituent proteins remain constant with successive cycles? The following experiments demonstrate that reconstituted ciliary membrane vesicles may be collected by centrifugation and then recycled several times by redissolving them in NP-40, spinning down any insoluble protein, removing the detergent again with Bio-Beads, then refreezing and rethawing the supernatant.

SDS PAGE analysis of the reconstitution is illustrated in Fig. 1. There is a successive loss to protein with each cycle of membrane reconstitution. The bulk of lost protein appears to be the result of denaturation, particularly evident as insoluble material in the second cycle (p₂). In spite of multiple reconstitutions, the overall protein composition of the resultant vesicles does not change appreciably. The lack of significant protein in any supernatants after the first (which contains the soluble ciliary matrix components) would indicate that if a critical concentration for membrane reconstitution exists at all, it must be very low.

Linear 20–60% sucrose gradient analysis of the recycled vesicles (Fig. 2) indicates that the loss of protein through recycling results in an expected proportional decrease in vesicle density. Gradients 1 through 4 contain pellet fractions P₁ through P₄. As previously reported (22), the first cycle gives a vesicle fraction banding at ~37% sucrose (protein content 45%) but after three more cycles, the vesicles band at 30–31% sucrose with approximately two-thirds of their original protein content. At this stage, the yield is <10%, based upon initial extract protein content.

¹ Abbreviations used in this paper: cmc, critical micelle concentration; NP-40, Nonidet P-40; OG, octyl glucoside.

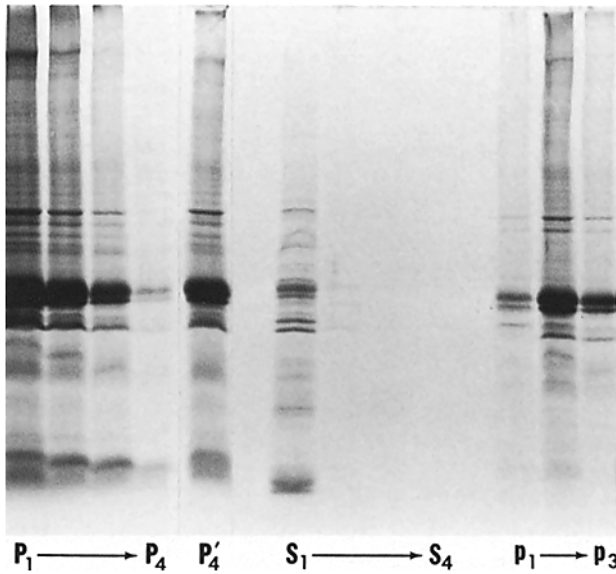


FIGURE 1 SDS PAGE gradient analysis of multiple membrane reconstitutions. The reconstituted vesicles (P_i) are recycled by redissolving them in NP-40, spinning down insoluble material (p_i), removing detergent again with Bio-Beads, refreezing, rethawing, and reconstituting (S_2 and P_2), etc. The first through fourth pellets (p_1 - p_4) are loaded stoichiometrically; the fourth pellet is then loaded at $8 \times$ concentration (P'_4). The supernatants (S_1 - S_4) and the denatured, NP-40-insoluble material (p_1 - p_3) are also shown loaded stoichiometrically. Coomassie Blue staining was used.

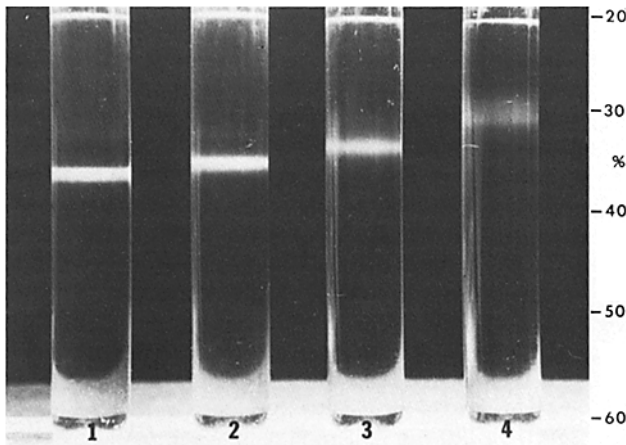


FIGURE 2 Linear 20-60% sucrose gradient analysis of recycled vesicles: gradients 1 through 4 contain pellet fractions P_1 through P_4 . Centrifugation was for 8 h at 150,000 g at 4°C .

Reconstitution by Concentration

It was suggested previously that ciliary membrane reconstitution by freeze-thaw is simply a consequence of protein and lipids being concentrated during freezing (22). But will simple concentration produce the same density of vesicle as freeze-thaw? When the NP-40-solubilized, detergent-free ciliary membrane supernatant is concentrated 10-fold, using Sephadex or Aquicide, vesicles and membrane fragments reform (Fig. 3*a*), but their morphology is somewhat less uniform than previously reported. When the initial membrane extraction is carried out in microtubule polymerization buffer at pH 6.4 and the extract is freed of detergent with Bio-Beads, relatively uniform, small, closed vesicles form upon 10-fold

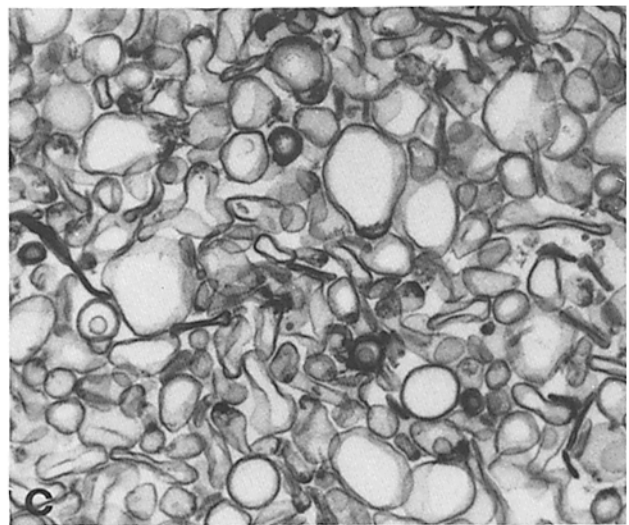
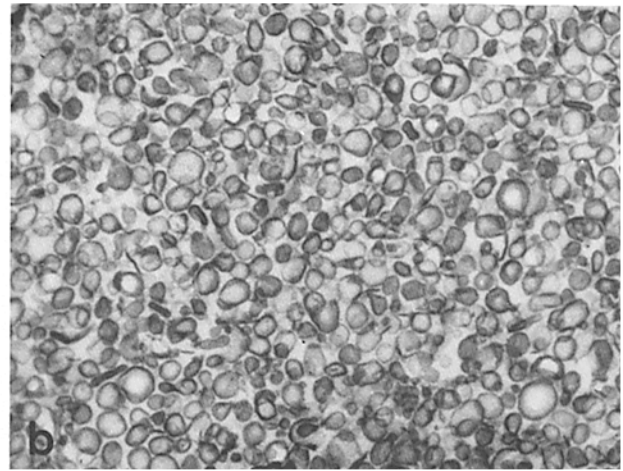
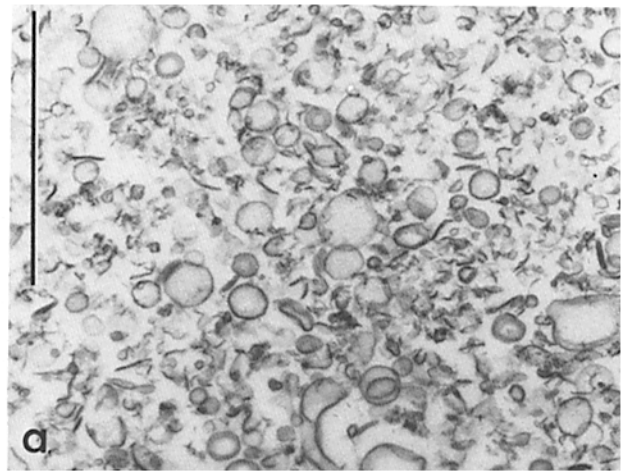


FIGURE 3 Electron microscopy of reconstitution by concentration or detergent dialysis. (a) Detergent-free ciliary membrane supernatant, derived through NP-40 solubilization at pH 8, freed of detergent with Bio-Beads, then concentrated 10-fold. (b) Extraction performed in microtubule polymerization buffer (0.1 M morpholinoethane-sulfonic acid, 1 mM guanosine triphosphate, 0.5 mM Mg^{++} , 1 mM EGTA [pH 6.4]), freed of detergent with Bio-Beads, then concentrated 10-fold. (c) Extraction performed with OG at 50 mM, detergent removed by dialysis, then concentrated 10-fold. Bar, $1 \mu\text{m}$. $\times 36,500$.

concentration (Fig. 3*b*), in this case more uniform than those produced by freeze-thaw. When the initial extraction is performed using 50 mM OG in the above-mentioned Mg-Tris buffer at pH 8 and the detergent is removed by exhaustive dialysis, large closed vesicles of variable size form upon 10-fold concentration (Fig. 3*c*). Vesicles of identical morphology to these are obtained if the extract is first concentrated and then dialyzed to remove detergent (not shown), but the density distribution is remarkably different (see below). Under all of the conditions employed, only unilamellar vesicles form, with no evidence for multilamellar vesicles, the presence of which would be indicative of inadequate initial solubilization (27).

Equilibrium analysis on 20–60% linear sucrose gradients reveals that the reconstitution conditions critically determine the final density distribution of the vesicles. When reconstituted from OG, dialyzed, and then concentrated, most vesicles band at 34% sucrose but the remainder of the population extends uniformly throughout the gradient above the major band (Fig. 4*a*). When reconstituted from an NP-40 extract at pH 8 by detergent removal followed by concentration, the vesicles band at 37% sucrose with a minor band at 31% (Fig. 4*b*), whereas reconstitution at pH 6.4 in microtubule polymerization buffer results in vesicles that band at 34% sucrose (Fig. 4*c*). These results for NP-40 solubilization/concentration are virtually identical to those obtained previously by freeze-thaw under the same two buffer conditions (22). In contrast, when the extraction is performed with OG, first concentrated 10-fold and then dialyzed, vesicles of comparatively low density form, mainly banding at 26% sucrose but extending down to 31% (where bands also occur in the NP-40 samples [Fig. 4, *b* and *c*]). Under the latter condition, the concentration of OG must approach 500 mM (15%) before dialysis. Consequently, considerable delipidation must occur, resulting in lipid-rich vesicles and, presumably, delipidated proteins which should sediment much deeper into the gradient.

Sedimentation of NP-40-Solubilized Membrane Components in a Detergent-free Gradient

If solubilized membrane components are gently freed of detergent, can a lipid-protein complex of a density characteristic of the original membrane be isolated from a sucrose gradient? When reconstituted membranes are solubilized with NP-40 concentrations between 2 and 17 times the critical micelle concentration (cmc), centrifuged, and the supernatant is then applied to 20–60% linear sucrose gradients and sedimented to equilibrium, visible vesicle bands at ~31% sucrose result in all cases, with some less visible material at 39–40% sucrose in samples solubilized at the lower detergent concentrations (Fig. 5, *a–d*). The latter sucrose density is slightly greater than the point to which intact vesicles reconstituted at pH 8 normally equilibrate. Identical results were obtained with 50 mM OG solubilization (data not shown).

The gradients were separated into convenient fractions and concentrated SDS sample buffer was added directly to the high sucrose-containing samples. These fractions were directly subjected to SDS PAGE analysis. The lowest and highest NP-40 conditions are illustrated in Fig 5, *e* and *f*, respectively. The protein distributions on the gradients are nearly indistinguishable, in spite of how much detergent was used in the initial solubilization. In all cases, the solubilized material,

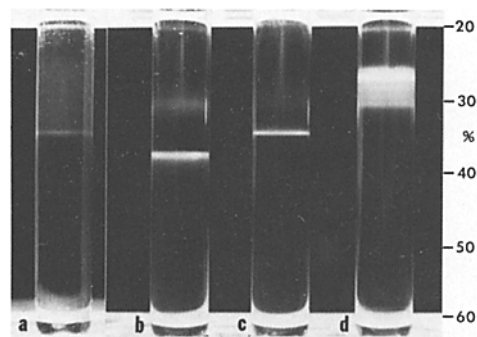


FIGURE 4 Analysis of concentration- or dialysis-reconstitution with 20–60% linear sucrose gradients. Centrifugation was for 12 h at 150,000 g at 4°C. (a) Reconstitution from OG, dialyzed, and then concentrated. (b) Reconstitution from an NP-40 extract, pH 8 Tris, freed of detergent with Bio-Beads and then concentrated 10-fold. (c) Reconstitution from an NP-40 extract, pH 6.4 morpholinoethane-sulfonic acid, freed of detergent with Bio-Beads and then concentrated 10-fold. (d) Extraction performed with OG, concentrated 10-fold, and then dialyzed.

after losing detergent by centrifugation through the detergent-free sucrose, equilibrated at or near the density to which reconstituted membranes equilibrate (fractions 4–5), although vesicles large enough to scatter light were only obvious at 31% sucrose. With the exception of a 40,000 mol-wt band in fraction 2, the relative protein composition of the fractions is fairly constant. The horizontal distortion of the lane positions results from the direct use of samples having very high sucrose concentrations. The paucity of lower molecular weight bands is a consequence of the lowered sensitivity of silver staining in the higher acrylamide concentrations of the gel gradient after destaining background.

Vesicle Reconstitution Using Pure Lipids in the Presence of Axonemal Tubulin

Since brain tubulin can be incorporated into liposomes (2) and since the possibility exists that the membrane tubulin and associated proteins found in reconstituted vesicles might somehow arise artifactually through the incorporation of axonemal tubulin and other proteins into reconstituted vesicles, dialysis-solubilized B-subfiber and central pair tubulins (plus various associated minor proteins) were added to detergent-solubilized pure lipids and the reconstitution performed.

Two kinds of vesicle reconstitution were attempted. First, a mixture of lecithin and cholesterol approximating the composition of ciliary membrane lipid (22) was detergent-solubilized in the presence of soluble axonemal proteins in a 1:1 weight ratio (approximately that of the natural membrane) and reconstituted by detergent adsorption and freeze-thaw. Upon sucrose gradient analysis, there is no difference in buoyant density between pure lipid vesicles and the major population of vesicles formed in the presence of axonemal tubulins (Fig. 6*a* vs. Fig. 6*b*), but a minor population of vesicles band at ~25% sucrose, which indicates some minimal protein association (Fig. 6*b*). Secondly, a natural mixture of lipids was extracted from whole cilia and used in the same type of reconstitution with twice the ratio of protein to lipid. Here, most of the vesicles formed in the presence of protein band at 28% sucrose, indicating considerable protein association (Fig. 6*c* vs. 6*d*). However, these vesicles lose most of their associated protein when subjected to a simple low ionic

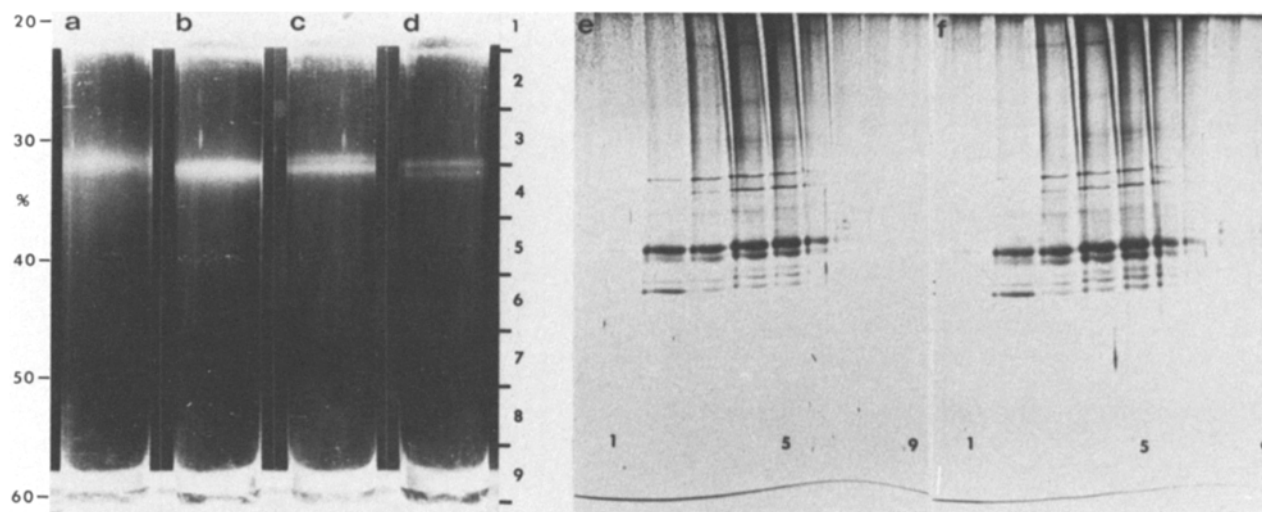


FIGURE 5 Sedimentation of NP-40 solubilized membrane components on sucrose gradients. Reconstituted membranes solubilized with NP-40 concentrations of 0.032, 0.063, 0.125, and 0.250%, applied to 20–60% linear sucrose gradients a–d, respectively, and sedimented to equilibrium (24 h). For SDS PAGE analysis, gradients were separated into nine fractions, concentrated SDS sample buffer was added, the fractions were electrophoresed on 5–15% gradient gels and then silver stained. Gel e is from gradient a (2 × cmc); gel f is from gradient d (16 × cmc). Variation in lane width and front migration is a consequence of the increasing concentration of sucrose in successive samples while the apparent inequality of tubulin bands is a consequence of differential staining.

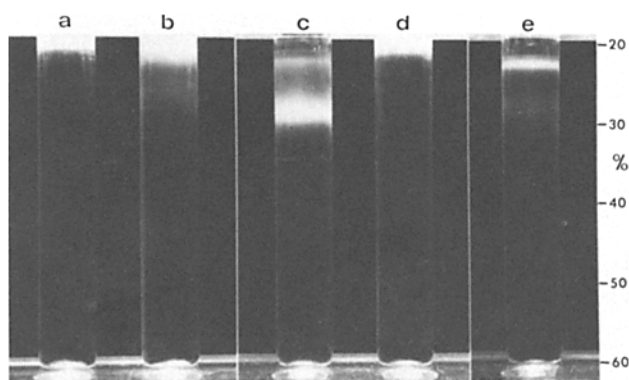


FIGURE 6 Vesicle reconstitution with pure lipids and axonemal tubulins. (a) 2:1 lecithin/cholesterol pure liposomes; (b) the same but made in the presence of excess axonemal proteins; (c) liposomes made from natural ciliary membrane lipids and axonemal proteins; (d) natural ciliary membrane liposomes without added protein; (e) vesicles recovered from c by dilution in 10 mM Tris, centrifugation, and re-analysis.

strength Tris wash, banding just into the 20% sucrose region (Fig. 5e), in contrast to vesicles made directly (or recycled) from ciliary membrane extracts, where the same Tris wash, followed by extensive extraction with 1 M iodide or thiocyanate, leaves the buoyant density unchanged (22).

Thus, using axonemal tubulin and solubilized lipids, no appreciable material bands at 37% sucrose, the equilibration density characteristic of membranes formed by direct reconstitution from the natural membrane extract. Any axonemal protein that does “reconstitute” with natural membrane lipids is mainly lost upon washing.

Detergent Solubilization of Reconstituted Vesicles and Ciliary Membranes

The mechanism of detergent action on ciliary membrane

vesicles could give some insight into the nature of the membrane’s constituents. Do detergents solubilize membranes in a monophasic manner and do certain protein constituents extract selectively?

When reconstituted vesicles, prepared by freeze-thaw at either pH 8 or pH 6.4, are resuspended in Mg-Tris buffer and the turbidity is monitored, solubilization can be estimated by the decrease in optical density resulting from the addition of small aliquots of detergent (Fig. 7). Using either NP-40 or OG, the suspensions sharply decrease in turbidity at a detergent concentration corresponding to the respective published cmc’s (0.015% for NP-40; 25 mM for OG). Except at considerably higher vesicle concentrations than used here, the point of 50% optical density decrease is independent of vesicle dilution or initial concentration of the added detergent aliquot. Illustrated because of its common use to permeabilize cells selectively, Brij-58 does not produce a sharp decrease in vesicle turbidity and its ability to solubilize lipids depends critically upon vesicle concentration (Fig. 7c; note log scale), a probable consequence of its very low cmc (0.009%; see Discussion).

When intact cilia are suspended in corresponding series of dilutions of OG or NP-40, only OG maximally solubilizes the membrane near its cmc, as judged by maximum protein in the supernatant after centrifugation and analysis by gradient SDS PAGE (Fig. 8). In NP-40, maximal solubilization occurs at >10× cmc, between 0.125 and 0.25% (Fig. 8a, lanes 6–7). No more protein is solubilized at the cmc (lane 3 = 0.016%) than is freed by vesiculation of the membrane in the low ionic strength buffer alone (lane 1). In OG, maximal solubilization takes place just above the cmc (Fig. 8b, lanes 4 and 5, 30–40 mM OG). Regardless of detergent concentration, the same ratios of the various membrane and matrix proteins are solubilized, indicating no selective protein removal. Virtually identical results were also obtained with these two detergents using reconstituted membranes and sedimentation at 50,000 g (data not shown).

Analytical Ultracentrifugation of NP-40 and OG Solubilization of Reconstituted Vesicles

The apparent discrepancy in the degree of "solubilization" of ciliary membranes, judged from turbidity of vesicles versus centrifugation of cilia or reconstituted membranes, may be rationalized on the basis of the sedimentation properties of the resultant detergent-lipid-protein micelles. The action of the two detergents, NP-40 and OG, is strikingly different.

In the case of NP-40 solubilization, large aggregates sediment quickly before the rotor reaches speed when the detergent concentration is 0.1% (Fig. 9a), whereas at 0.25% NP-40 (maximal solubilization, Fig. 8a), a broad band sediments at about 6S, twice the speed of the detergent micelles (slower "peak" in this double-sector cell). Only at 1% NP-40 is a reasonably uniform 4S peak observed, riding atop a 3S detergent peak (Fig. 9b). With OG solubilization, at 25 mM (cmc)

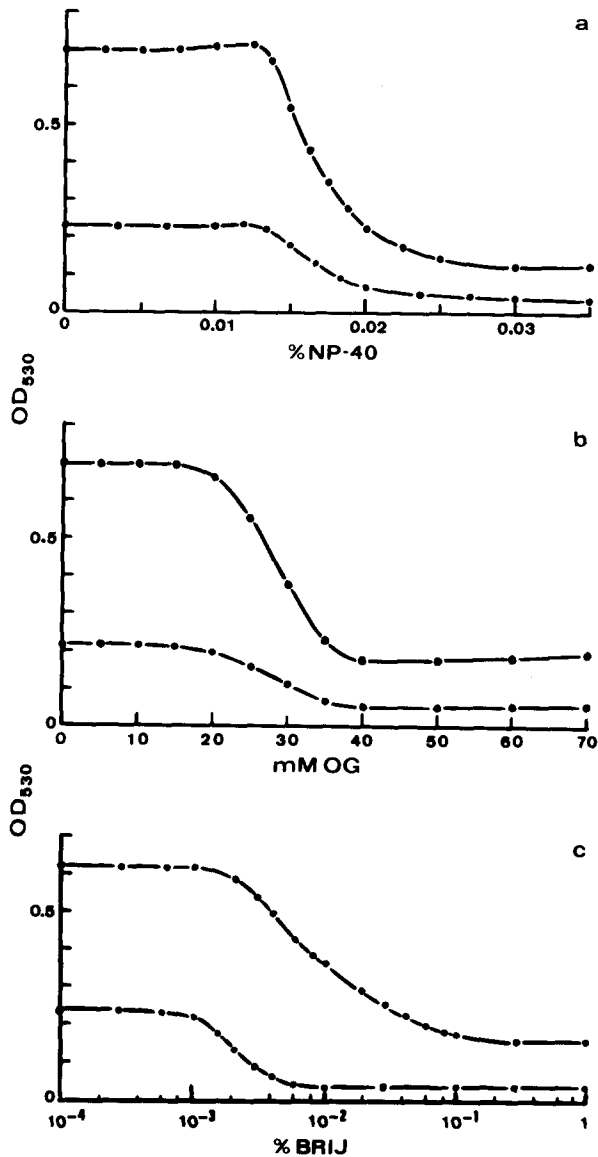


FIGURE 7 Detergent solubilization of reconstituted vesicles. Reconstituted vesicles were resuspended in buffer, then monitored at 530 nm during the addition of small aliquots of detergent. (a) NP-40 (cmc = 0.0155% or 0.24 mM); (b) OG (cmc = 25 mM or 0.7%); (c) Brij-58 (cmc = 0.009% or 0.08 mM). The lower curve in each case is a 1:2 dilution of the initial preparation.

very rapidly (185S) and slowly (22S) sedimenting species appear (Fig. 9c). These may correspond, respectively, to partially delipidated lipid-protein aggregates and to small protein-

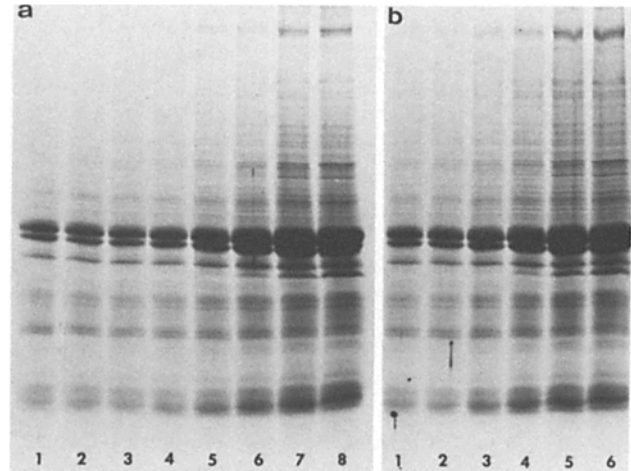


FIGURE 8 Detergent solubilization of ciliary membranes. Cilia were suspended in appropriate dilutions of NP-40 and OG detergents, extracted for 10 min, and centrifuged 35,000 g. The supernatants were analyzed by SDS PAGE. (a) NP-40: lane 1 is buffer alone; lane 8, 0.5% NP-40; lane 7, 0.25%; lane 6, 0.125%, etc. (b) OG: Lane 1 is buffer alone; others increase in 10-mM increments of OG to 50 mM (lane 6).

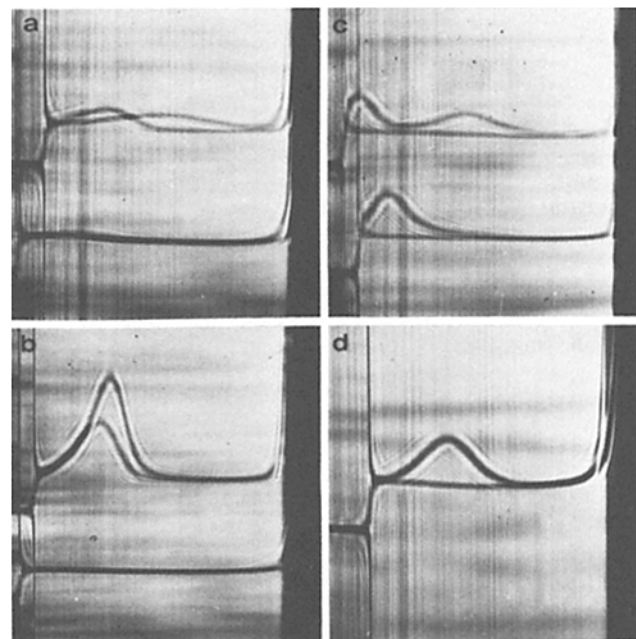


FIGURE 9 Analytical ultracentrifugation of NP-40 and OG solubilization of reconstituted vesicles. (a) NP-40 solubilization (160 min, 42,040 rpm). Lower pattern, 0.1% NP-40 (6.5 × cmc, Fig. 7); upper pattern, 0.25% NP-40 (maximal solubilization, Fig. 8a). Approximate sedimentation rate, 6S (vs. 3S of pure detergent micelles in the solvent sector). (b) NP-40 solubilization (184 min, 42,040 rpm). Upper pattern, 1% NP-40 (65 × cmc). Approximate sedimentation rate, 4S. Lower pattern, membranes without added detergent. (c) OG solubilization (6 min, 42,040 rpm). Upper pattern, 25 mM OG (cmc, Figs. 7b and 8b). Approximate sedimentation rates, 22S and 185S. Lower pattern, 10 mM OG (<cmc). Approximate sedimentation rate, 47S. (d) OG solubilization (176 min, 42,040): 35 mM OG (>cmc; maximum solubilization, Fig. 8b). Approximate sedimentation rate, 4S.

lipid-detergent micelles. In 10 mM OG (<cmc), particles of intermediate sedimentation rate appear, possibly corresponding to larger protein-lipid-detergent mixed micelles. However, in 35 mM OG (>cmc), a single, relatively homogeneous particle sediments at 4S and no aggregated material can be detected at lower speeds (Fig. 9d).

Thus the clearing of vesicle suspensions at the cmc of NP-40 is due to dispersal of the highly scattering vesicles into considerably smaller but still highly sedimentable micelles rather than the result of true solubilization. In contrast, OG completely disperses the membrane just above its cmc to particles of quite low sedimentation rate.

Electron Microscopy of the Detergent-free Extract

When the detergent is removed from the NP-40-solubilized ciliary membrane, little protein is sedimentable at 100,000 *g* (22). Analytical ultracentrifugation reveals only very heterogeneous material of relatively low sedimentation rate (small protein-lipid micelles?). Freeze-thaw, further concentration, warming, or simply standing for long periods on ice will give rise to membrane vesicles; only the first two do so efficiently. What does the detergent-free material look like at the ultrastructural level?

Negative staining of this initially "soluble" material reveals heterogeneous "particles," with a minimal size of ~15–25 nm (Fig. 10a). On close inspection, these appear to be aggregates containing 5–6 nm globules. When this material is allowed to partially evaporate on the grid, or when more concentrated material is used, incipient membrane vesicles appear, evidently arising through association of the aggregates (Fig. 10b). It could be argued that some detergent still remains associated with the protein-lipid micelles, preventing vesicle formation, but concentration (or freeze-thaw) will promote 100% vesicle formation from such a preparation. In addition, further treatment with Bio-Beads has no effect upon the appearance of the preparation; whatever little detergent that may still be associated with the protein-lipid micelles is either unaffected by further adsorption attempts or has no influence on the ultimate formation of vesicles.

Nondenaturing Electrophoresis in OG

Is tubulin or any other protein of the reconstituted, detergent-solubilized ciliary membrane separable by electrophoresis in the presence of the solubilizing detergent?

Using the discontinuous buffer system of Laemmli (14) but without SDS, reconstituted membranes may be dispersed with and electrophoresed in varying amounts of OG, through the cmc, on 5% polyacrylamide gels (Fig. 11a). In parallel, similar concentrations of skate brain tubulin or axonemal B-tubulin serve as controls. In direct contrast to soluble tubulins, which run as oligomers below the cmc of OG (25 mM) but aggregate somewhat above it, little membrane vesicle proteins run below the cmc. Above the cmc, a sharp protein band is seen, but two-dimensional gel electrophoresis of a sample resolved in 30 mM OG (Fig. 11b) reveals that this "band" contains only minor membrane proteins with a variety of molecular weights. Virtually all of the membrane tubulin and most other proteins remain at the top of the first-dimensional gel. In spite of the production of a 4S particle by OG above the cmc, this particle is sufficiently large such that it will not enter the polyacrylamide gel, even though brain tubulin oligomers with molec-

ular weights in excess of 1,000,000 migrate readily on this gel system (cf. 13).

Chromatography of Ciliary Membrane Proteins

Since it is well known that tubulin can be separated from associated proteins by salt elution on DEAE-Sephadex or Sephacryl, attempts were made to resolve the proteins of the detergent solubilized, reconstituted membranes. A variety of salt and pH conditions were tested, including the continued presence of 1% NP-40 (> 20 × mol ratio to lipids present in the sample). All failed to yield significant protein separations.

Nearly all of the membrane proteins remained bound to the column, eluting as a group at >1 M NaCl and trailing badly. Occasionally, a barely detectable amount of tubulin was found to elute at 0.5 M salt but this was only in cases where the presence of small amounts of central pair tubulin in the initial preparation could not be discounted.

Bio-Gel 5m was used in an attempt to estimate the size of this apparent complex of tubulin and associated proteins. In the presence of 0.25% NP-40, all of the proteins were excluded from the column, indicating a molecular weight for the protein-lipid-detergent complex of >5,000,000. Under extreme conditions (1–2% NP-40 and 10 mM dithiothreitol, [pH 8.3]), less than one-third of the tubulin was retained by the column. It eluted at points corresponding to molecular weights of about 250,000 and 125,000, indicating that very

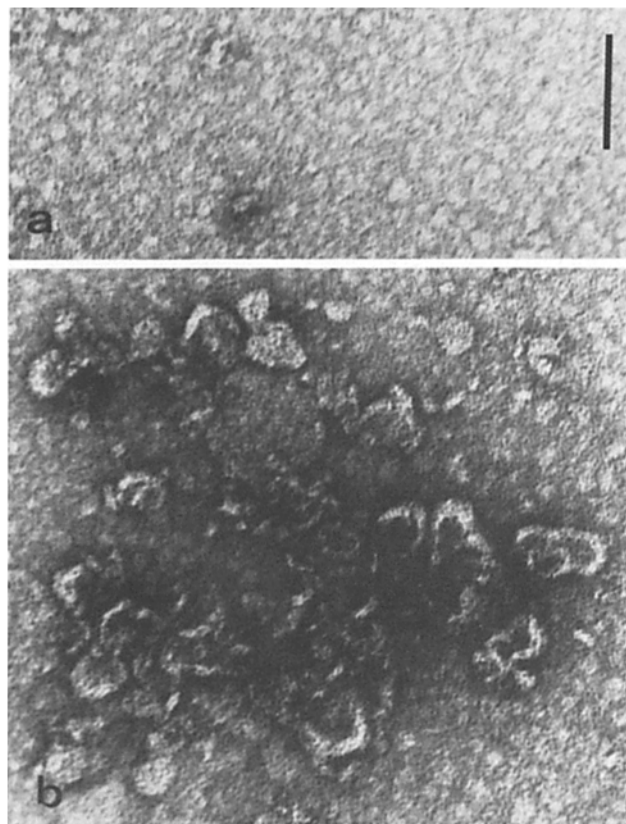


FIGURE 10 Electron microscopy of the detergent-free ciliary membrane-matrix supernatant before reconstitution. (a) Heterogeneous "particles," with a minimum size of ~15–24 nm are usually visible; upon close inspection, these appear to contain 5–6 nm globules. (b) If the preparation is allowed to evaporate somewhat, incipient vesicle formation is observed at the edges of the grid. Bar, 100 nm. × 150,000.

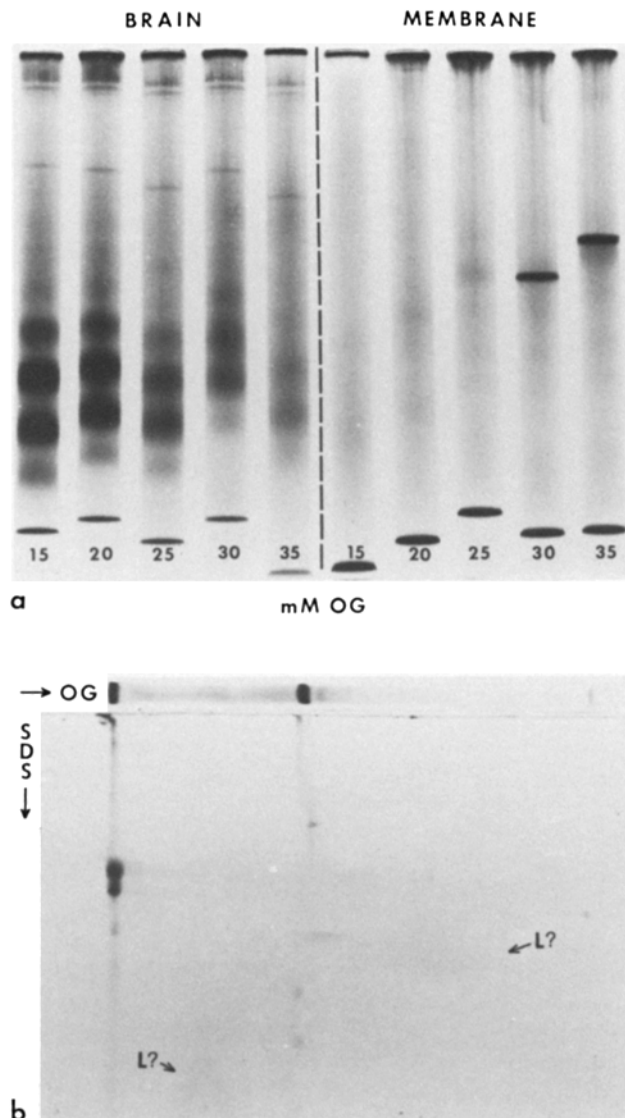


FIGURE 11 Electrophoresis in the presence of increasing concentrations of OG. (a) Reconstituted membranes (top right) and brain tubulin (top left) electrophoresed on 5% gels containing varying amounts of OG. (b) Two-dimensional gel electrophoresis of reconstituted ciliary membranes solubilized at 30 mM OG. Regions marked L? were particularly obvious during destaining and probably contain lipid or lipid-detergent micelles.

limited amounts of tubulin dimer and tetramer can be freed under delipidating conditions.

DISCUSSION

Theoretical Considerations

Eight independent observations support the idea that a relatively stable lipid-protein complex exists in the ciliary membrane of *Aequipecten* and that in this complex membrane tubulin is tightly associated with a number of other proteins. (a) The proteins of the membrane reassociate through several cycles without significant change in the overall protein composition. (b) Membrane proteins and lipids reassociate to form vesicles of uniform, discrete density classes under a variety of reassociation conditions. (c) Freed of solubilizing detergent during equilibrium centrifugation, a protein-lipid complex

equilibrates to a position on the gradient characteristic of the original membrane. (d) The ciliary membrane proteins dissolve with constant composition, regardless of the type, concentration, or efficiency of the detergent. (e) Monodisperse mixed micelles form at comparatively high detergent concentration, with no evidence for a 6S free tubulin or a multitude of micelle types at lower detergent levels (f) In extracts freed of detergent, quasi-structured "particles" are seen in negative staining, with no evidence for two separate phases of soluble protein and lipid vesicles. (g) Membrane tubulin is not in a form that will freely undergo electrophoresis, even in the presence of detergent above the critical micelle concentration. (h) All ion-chromatographic attempts to separate membrane tubulin from other membrane proteins to any practical extent have failed. Furthermore, the ciliary membrane proteins and lipids are excluded together from gel filtration columns in the presence of high concentrations of detergent, indicating strong association of membrane proteins and lipid.

What might be the function of such a complex? Perhaps it is a package of axonemal precursors, conveyed to the growing tip of the axoneme by membrane flow. This at least would be consistent with the large number of proteins present and their lack of correspondence in molecular weight to those of the axoneme. Outer doublets and central pair microtubules are attached to the membrane at the tip of the cilium through very specialized structures (7). These tip structures may be part of a system which functions to receive and process membrane-associated axonemal protein precursors (i.e., the proteins of the postulated lipid-protein complex), then direct the products to their appropriate assembly sites. Sardet and Tilney (18) have proposed a related type of mechanism for the origin of the sperm acrosome and its membrane. In this case, the unpolymerized actin of the periacrosomal region appears to be complexed with lipids; when the lipids are released, the actin polymerizes to form the acrosomal filament while the membrane of the acrosomal process is thought to be generated from released lipid.

Membrane tubulin and associated proteins could be involved more directly in membrane attachment or motility. A dynein-like, membrane-associated ATPase has been isolated from ciliary membranes and shown by cross-linking studies to be associated with membrane tubulin (6). This protein may interact with both membrane and axonemal tubulin, tethering the membrane to the axoneme in a passive but reversible manner, or it could function actively by propelling membrane domains along the axoneme, a concept consistent with the observed rapid movement of attached beads along certain flagellar membranes (1).

Finally, membrane tubulin may serve some enzymatic or sensory function. Deanin et al. (5) recently showed that when brain tubulin is inserted into reconstituted brain membranes, the inherent adenylate cyclase activity of the membrane is greatly enhanced. The mechanosensitive cilia of *Mytilus* gill (homologous to the scallop gill lateral cilia described here), also contain prominent membrane proteins that co-migrate with tubulin (23). Motility in *Mytilus* gill cilia is initiated by serotonin, evidently activating adenylate cyclase; cAMP in turn mediates the specific phosphorylation of three low molecular weight axonemal proteins (24). Chen and Lancet (4) have noted that frog olfactory cilia contain very high amounts of membrane-associated tubulin, in striking contrast to respiratory cilia, which contain little or none, suggesting some sensory role.

Practical Considerations

Several practical consequences are implicit in the findings presented here. Both NP-40 and OG can effectively solubilize ciliary membranes, but the latter does so at its cmc while much larger concentrations (at least $10 \times$ cmc) of the former are required. Considering the present cost of these two detergents, however, NP-40 is a clear choice for bulk preparation of cilia in spite of the higher concentrations required. Also, for reconstitution, NP-40 can be removed quickly by adsorption but OG removal takes a minimum of 18–24 h of dialysis, even then yielding only polydisperse vesicles upon concentration.

The ultimate reason for these differences in solubilizing ability may be simply rationalized through arguments made by Mimms et al. (16). Under the solubilizing conditions used in this present study, NP-40 at its cmc generally represents less than a 1:1 mole ratio of detergent to lipids (with Brij 58 being substantially lower), but OG at its cmc represents at least a fivefold molar excess of detergent over lipid. Mimms et al. (16) have shown that a 5–10-fold excess of detergent over lipid is required for optimal lipid solubilization. Thus, in spite of the fact that both NP-40 and OG disrupt or disperse ciliary membranes (as judged by turbidity) precisely at their respective cmc's, the latter is also able to solubilize much more efficiently just above its cmc because of an already high mole ratio of detergent to lipid. This argument is quite consistent with both the solubility and the ultracentrifugation studies presented above. In the case of Brij 58, with an unusually low cmc, solubilization takes on the nature of a titration due to the relatively high amount of lipid present when the detergent is at its cmc.

Both NP-40 and Triton X-100 have been used widely for producing ATP-reactivated "cell models," although this use has always been somewhat empirical. The efficacy of these detergents now can be easily understood in terms of their inability to efficiently solubilize membranes. The use of Triton X-100 in producing cell models (cf. 9) was originally suggested because I observed that ciliary membranes required several extractions at fairly high detergent concentration for thorough removal of the membrane; therefore, lower concentrations of Triton, if properly chosen, should simply permeabilize the membrane to small molecules such as ATP. With molluscan cilia, this is, in fact, the case. Tsuchiya (26) has used Triton X-100 just below the cmc to produce robust cell models of mussel gill cilia. Under these extraction conditions, proteins as small as calmodulin are retained by the ciliary membrane (25). This approach has proven to be quite valuable for the production of cell models that retain their differential calcium sensitivity and, presumably, the proteins responsible for this sensitivity (23). It should be obvious from Fig. 7a that membrane permeabilization versus dispersal depends critically upon the use of NP-40 (or Triton X-100) at or just below the cmc. Tsuchiya's extraction conditions correspond to the slight rise in optical density at about 0.012% NP-40, this being the point where detergent micelles should begin to form and fuse with the membrane but still below the point where any physical disruption would occur. Interest-

ingly, the data for Brij-58 (Fig. 7c), showing gradual membrane disruption over a concentration range an order of magnitude above the cmc, would indicate that this detergent might be useful for creating a very wide range of permeabilities, a fact consistent with its use in cell model studies wherein fairly large molecules have been introduced into cells (cf. 3).

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