Fractionation of *Tetrahymena* Ciliary Membranes with Triton X-114 and the Identification of a Ciliary Membrane ATPase

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Abstract. Cilia were isolated from *Tetrahymena thermophila*, extracted with Triton X-114, and the detergent-soluble membrane + matrix proteins separated into Triton X-114 aqueous and detergent phases. The aqueous phase polypeptides include a high molecular mass polypeptide previously identified as a membrane dynein, detergent-soluble α and β tubulins, and numerous polypeptides distinct from those found in axonemes. Integral membrane proteins partition into the detergent phase and include two major polypeptides of 58 and 50 kD, a 49-kD polypeptide, and 5 polypeptides in relatively minor amounts. The major detergent phase polypeptides are PAS-positive and are phosphorylated in vivo. A membrane-associated ATPase, distinct from the dynein-like protein, partitions into the Triton X-114 detergent phase and contains nearly 20% of the total ciliary ATPase activity. The ATPase requires Mg^{++} or Ca^{++} and is not inhibited by ouabain or vanadate. This procedure provides a gentle and rapid technique to separate integral membrane proteins from those that may be peripherally associated with the matrix or membrane.

CILIARY and flagellar membranes are associated with a number of metabolic and structural processes, including olfaction (Chen et al., 1986), Ca^{++} ion regulation (Travis and Nelson, 1986), regulation of ciliary motility (Gustin and Nelson, 1987; Dentler et al., 1980), microtubule assembly (Dentler, 1980*a*), and gliding (Bloodgood, 1977). Each of these properties require membrane proteins to be properly oriented and localized along the ciliary and flagellar shaft. Little is known about the structure or organization of ciliary membrane proteins but Stephens et al. (1987) proposed that a detergent-resistant membrane skeleton, composed partly of "membrane tubulin", is associated with the cytoplasmic face of scallop gill ciliary membranes and that this skeleton may provide structural rigidity to the membrane as well as attachment sites for the microtubule-membrane bridge proteins (Dentler et al., 1980).

Ciliary membranes and detergent-soluble "membrane + matrix" fractions are composed of 25–50 (or more) different polypeptides, as resolved on one-dimensional SDS-PAGE (Chen et al., 1986; Dentler, 1980*b*; Adoutte et al., 1980). To understand the functions of these proteins, it is necessary to identify the locations of these proteins in the membrane and identify proteins that may be associated with the membrane skeleton, if such a skeleton exists in all cilia. In this study, *Tetrahymena* ciliary membranes were fractionated with Triton X-114 to separate and identify integral membrane proteins (Bordier, 1981; Chen et al., 1986; Stephens, 1985*a*; Pryde and Phillips, 1986) and to determine if the major detergent-soluble proteins, previously identified as tubulins (Dentler, 1980*b*), are integral membrane proteins. The results show that the major *Tetrahymena thermophila* membrane proteins migrate at 50 and 58 kD, partition into the Triton X-114 detergent phase and are glycosylated and phosphorylated in vivo. The detergent-soluble *Tetrahymena* ciliary tubulin partitions into the aqueous phase and is unlikely to be an integral membrane protein.

Since ciliary and flagellar membrane ATPases have been described in a number of organisms (see Dentler, 1981; Travis and Nelson, 1986), the Triton X-114 aqueous and detergent phase fractions were examined for ATPase activity. The results show nearly 20% of the total ciliary ATPase activity is released by Triton X-114, most of which partitions into the detergent phase. The membrane ATPase is insensitive to vanadate and ouabain and requires divalent cations. The function of the ATPase is unknown but it could be a membrane pump or be related to the movements of ciliary surface proteins (Williams et al., 1985; Bloodgood, 1987).

Materials and Methods

Isolation of Cilia and Triton X-114 Fractions

Cilia were isolated from *Tetrahymena thermophila*, strain B-255 (from E. Orias, University of California, Santa Barbara) using dibucaine as previously described (Suprenant and Dentler, 1988). Cells were grown in 2% proteose peptone (Difco Laboratories Inc., Detroit, MI), 0.1 mM $FeCl_3$, and 0.0025% penicillin-streptomycin in 2.8-liter fernbach flasks at room temperature on an orbital shaking table. For most experiments, 3 liters of cells grown to late log phase, harvested and deciliated in 300 ml of proteose peptone with 300 mg of dibucaine. In some experiments, cells were washed three times in HNMK (50 mM Hepes, pH 6.9, 36 mM NaCl, 1 mM KCl,

0.1 mM $MgSO_4$)[1] and deciliated in HNMK and 1 mg/ml dibucaine with results identical to those obtained when cells were deciliated in protease peptone. Cells were routinely examined by phase microscopy after deciliation to insure that the cells were intact.

Cilia were washed twice in PEMKS (50 mM Pipes, pH 7.1 with KOH, 3 mM $MgSO_4$, 0.1 mM EGTA, 10 mM potassium acetate, 1 mM EDTA, 250 mM sucrose) or HEEMS (50 mM Hepes, pH 7.1, 3 mM magnesium acetate, 1 mM EGTA, 1 mM EDTA, 250 mM sucrose) as described in the text. DTT (0.1 mM) and 0.4 mM leupeptin were added to the final washed cilia and was included in all subsequent steps. PMSF (29 mM) was prepared in propanol and was added in a 1:1,000 dilution to each solution immediately before use. Washed cilia were suspended in 5–10 ml of PEMKS or HEEMS and were made 1% in ice-cold Triton X-114. The suspension was incubated on ice for 20 min and was mixed every 2 min with a pasteur pipette. Axonemes were then pelleted by centrifugation at 27,000 *g* for 20 min (4°C) in a rotor (model JA-20; Beckman Instruments Inc., Palo Alto, CA). The supernatant was carefully removed, placed in a conical glass centrifuge tube, and then warmed to 30°C in a water bath for 5 min to induce cloud formation. The sample was centrifuged for 10 min at 1,800 g_{max} at room temperature (model Centra 7; International Equipment Co., Needham Heights, MA). The clear aqueous phase was removed from the cloudy detergent phase and each fraction was placed on ice. For most studies, the aqueous phase was extracted two or three times by cooling, adding Triton X-114 to 1% (final concentration), incubating 2–3 min on ice, warming to 30°C, and centrifuging as described above. Greater than three extractions of the aqueous phase did not result in any significant quantity of protein partitioning to the Triton X-114 detergent phase.

For SDS-PAGE or ATPase assays, the Triton X-114 detergent phase sample was diluted with PEMKS to a volume equal to that of the aqueous phase and the diluted sample was placed on ice until all detergent micelles disappeared. Removal of detergent with Bio Beads SM-2 (Bio-Rad Laboratories, Cambridge, MA) was attempted but passage of 5 ml of the diluted detergent phase through three separate 3-ml columns of Bio Beads failed to remove all of the detergent, as judged from the formation of detergent micelles in the eluate at room temperature.

Detergent-resistant membrane vesicles were purified by suspending the axoneme + membrane pellet after one extraction of cilia with Triton X-114 in cold PEMKS. The suspension was layered over a sucrose step gradient with 1.5 ml of 40% sucrose–PEMK (PEMKS without the sucrose; wt/wt), 0.5 ml 30% sucrose–PEMK (wt/wt), and 1.2 ml 20% sucrose–PEMKD (wt/wt), and the gradient was centrifuged for 120 min, 4°C, at 50,000 rpm in a rotor (model SW-65; Beckman Instruments Inc.). Membrane vesicles were identified as a thin white band at the interface between the 30 and 40% sucrose solutions. A faint white band was also seen at the interface between the PEMKS and the 20% sucrose but this fraction was not recovered. Axonemes were pelleted. The white band at the 30–40% interface was removed, diluted with PEMK, split into at least two aliquots, depending on the quantity of material in each experiment, and was centrifuged at 20,000 rpm, 4°C, for 30 min in a rotor (model JA-20; Beckman Instruments Inc.). One pellet was fixed for electron microscopy and remaining pellets were frozen at −80°C for subsequent electrophoretic analysis.

In vivo ^{32}P-labeling and Autoradiography

Cells were grown to ~10^5 cells/ml in 2% proteose peptone, harvested by centrifugation, and washed into 1.3 liter of sterile HNMK supplemented with 1 mM glucose, 0.0025% penicillin-streptomycin. Carrier-free ^{32}P (as phosphate) was added (1 mCi) and cells were incubated on a shaker at room temperature for 17 h. Cells were harvested by centrifugation, suspended in 2% proteose peptone, and cilia were amputated and isolated as described above. Cilia were demembranated with NP-40 or with Triton X-114, and the various membrane and matrix fractions were separated as described above. Samples were loaded and run on 1.5-mm-thick 4–16% polyacrylamide slab gels (see below). Gels were fixed following the method of Fairbanks et al. (1971). The inclusion or omission of Coomassie Blue from the gels had no effect on the labeling patterns observed, although both stained and unstained gels were autoradiographed. After fixation, gels were dried onto filter paper using a gel drier (model 443; Bio-Rad Laboratories). Dried gels were autoradiographed with pre-flashed X-O Mat film (Eastman Kodak Co., Rochester, NY) and a Cronex Lightning Plus intensifying screen (DuPont Co., Wilmington, DE). Typically, films were exposed at −80°C for 7 d and developed for 6 min in D-19.

Sucrose Gradient Separation of Dynein and Detergent-released Polypeptides

Triton X-114 aqueous and detergent phase ciliary fractions were prepared in HEEMS as described above. Axonemal dynein was prepared by suspending the axonemes in HEEM and then adding NaCl to 0.6 M. The suspensions were incubated on ice for 30 min and the axonemes were pelleted for 25 min at 48,000 *g*, 4°C. The best resolution of ATPase peaks was obtained if the NaCl supernatant was dialyzed against 200 vol of cold HEEM for 3 h followed by centrifugation at 48,000 *g* for 15 min at 4°C to remove precipitated material. 2-ml samples were layered over a 5–20% (wt/wt) linear sucrose gradient in HEEM and were centrifuged for 12 h at 32,000 rpm in a rotor (model SW-41; Beckman Instruments Inc.) at 4°C. Dynein, aqueous phase, and detergent phase samples were run in parallel. Fractions (18 drops [~0.5 ml]) were collected from the bottom of each centrifuge tube. The volume of each fraction varied somewhat depending on the concentration of the detergent. For initial experiments, the protein in each fraction was detected by reading the absorbance at 280 nm but this was discontinued because the Triton X-114 generally condensed at room temperature and interfered with the absorbance measurements.

For ATPase assays of gradient fractions, 15 μl of each dynein fraction, 25 μl of each aqueous phase fraction, and 10 μl of each detergent phase fraction was added to 1 ml (total volume) of 50 mM Hepes, pH 7.1, 3 mM magnesium acetate, 0.5 mM EGTA, 0.1 mM DTT, and 0.4 mM leupeptin. Assays were started by adding 2 mM ATP and were carried out at room temperature for 30 min. For SDS-PAGE, 100 μl aliquots of each sample were loaded on a 20-well separation gel.

ATPase and Protein Assays

ATPase activities were carried out using the method of Atkinson et al. (1973). The assay is sensitive and reproducible but is sensitive to lubrol impurities. Consistent results were obtained with Surfact-Amps PX (a 10% lubrol solution obtained from Pierce Chemical Co., Rockford, IL). 1-ml assays were run at room temperature in 50 mM Hepes, pH 7.1 to which the appropriate concentrations of magnesium acetate or $CaCl_2$ were added and each reaction was started by the addition of the appropriate nucleotide. For most assays, the reaction mixture contained 2 mM ATP and 2 mM magnesium acetate. Sodium ortho vanadate was made as a concentrated stock in 0.1 M NaOH. Most assays were run for 20 min and controls were run to insure that neither the buffer components nor the Triton X-114 had any effect on the assay procedure. Protein concentrations were determined using the method of Bradford (1976) or the BCA assay by Pierce (Pierce Chemical Co.).

Electrophoresis

SDS-PAGE was carried out with a modification of the discontinuous system of Laemmli (1970). Separation gels were composed of a continuous gradient of 4–16% acrylamide and were adjusted to pH 8.8 or 8.3 with HCl immediately before pouring the gels. Gels were run at constant current in a slab gel apparatus (model SE-600; Hoefer Scientific Instruments, San Francisco, CA) at 10°C and were fixed and stained using Coomassie Blue (Fairbanks et al., 1971) or Silver (Pratt et al., 1984). Gels were photographed with Technical Pan film (Eastman Kodak Co.) developed in HC-110. Molecular mass standards included myosin (205 kD), β-galactosidase (116 kD), phosphorylase B (97 kD), BSA (66 kD), egg albumin (49 kD), and carbonic anhydrase (29 kD). PAS staining was carried out by a modification of the method of Fairbanks et al. (1971).

Proteins were blotted from unfixed gels onto nitrocellulose using the procedure of King et al. (1986). Nitrocellulose sheets were cut into strips, incubated with tubulin antibodies for 1 h at room temperature, and the tubulin antibodies were visualized with peroxidase goat anti-mouse IgG (model No. 62-6520; Zymed Laboratories, San Francisco, CA). Monoclonal tubulin antibodies that bind to *Tetrahymena* α tubulin were obtained from ICN ImmunoBiologicals (Lisle, IL; Clone No. DM-1A) and to *Tetrahymena* β tubulin was identified with Tu9B obtained from Dr. L. I. Binder, University of Alabama.

Gels were scanned (model No. CS-930 scanner; Shimadzu Scientific In-

1. *Abbreviations used in this paper*: HEEMS, 50 mM Hepes, pH 7.1, 3 mM magnesium acetate, 1 mM EGTA, 1 mM EDTA, 250 mM sucrose; HNMK, 50 mM Hepes, pH 6.9, 36 mM NaCl, 1 mM KCl, 0.1 mM $MgSO_4$; PEMKS, 50 mM Pipes, pH 7.1 with KOH, 3 mM $MgSO_4$, 0.1 mM EGTA, 10 mM potassium acetate, 1 mM EDTA, 250 mM sucrose.

struments Inc., Columbia, MD) and peaks were cut and weighed to determine the relative quantities of proteins in each sample. Gels were scanned at 650 nm for Coomassie Blue and at 560 nm for PAS stain. Nitrocellulose blots were scanned at 650 nm using the reflection mode.

Results

Polypeptide Composition of the Membrane + Matrix

Extraction of *Tetrahymena* cilia with PEMKS and NP-40 reveal the same major 55-kD polypeptides and high molecular mass dynein-like polypeptides as previously reported (Fig. 1, *NA* and *NS*) (Dentler, 1980*b*; Dentler et al., 1980). To further fractionate the detergent-soluble proteins and identify those likely to be integral membrane proteins, cilia were extracted with PEMKS and Triton X-114 at 4°C, axonemes were pelleted, and the supernatant warmed to 30°C to separate the hydrophobic detergent phase from the soluble aqueous phase. Analysis of the fractions with SDS-PAGE (Fig. 1, *TA*, *TS*, and *TP*) reveals that the major polypeptides released into NP-40 are resolved into at least four major polypeptides, two of which partition into the detergent phase while two to four polypeptides remain in the aqueous phase. Repeated extractions of the aqueous phase with Triton X-114 result in the sedimentation of progressively lower quantities of protein but, regardless of the number of extractions, each detergent phase sample contains the same number and ratio of polypeptides, as resolved by SDS-PAGE (Fig. 1, *TP*).

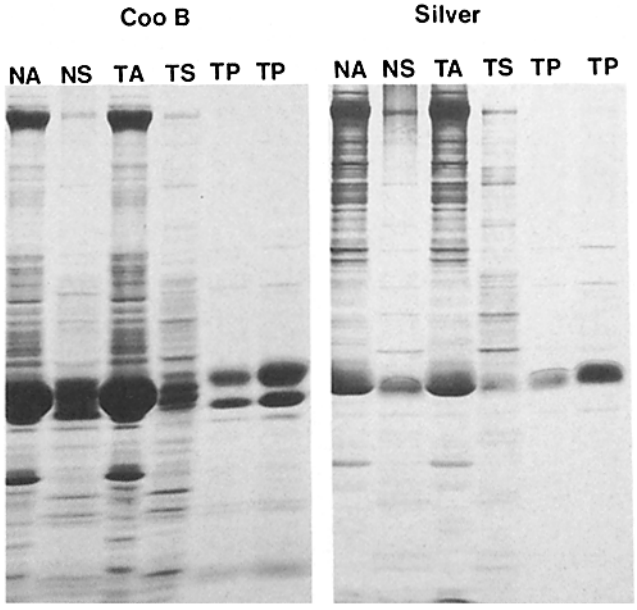

Figure 1. SDS-PAGE showing the composition of Coomassie Blue- (*Coo B*) and silver- (*Silver*) stained axoneme and membrane fractions of isolated *Tetrahymena* cilia. *NA* and *NS* are the axoneme and detergent-soluble fractions, respectively, obtained by 1% NP-40 extraction. *TA*, *TS*, and *TP* represent the Triton X-114 axoneme, aqueous phase, and detergent phase, respectively, obtained after extraction of cilia with 1% Triton X-114. Cilia were isolated and extracted in PEMKS and the pH of the separation gel was 8.6. These conditions released a high molecular mass polypeptide that comigrated with axonemal dynein (*NS* and *TS*) and the resolution of the major Triton X-114 detergent phase polypeptides into 58- and 50-kD species. The 58- but not the 50-kD polypeptide was stained with silver.

Identification of the major membrane polypeptides by SDS-PAGE depends on the pH of the separation gel and the staining method employed. As judged by Coomassie Blue staining, the major Triton X-114 detergent phase polypeptides migrate with apparent molecular masses of 50 and 58 kD in pH 8.6 separation gels. Only the 58-kD polypeptide stains with silver (Figs. 1 and 2). The remainder of the polypeptides that partition into the Triton X-114 detergent and aqueous phases stain equally well with Coomassie Blue and silver, with the exception of the high molecular mass membrane vesicle protein whose staining is slightly enhanced by silver staining (Fig. 2).

Densitometry of Coomassie Blue–stained gels reveal that the major 50- and 58-kD polypeptides comprise 25 and 65% of the Triton X-114 detergent phase protein, respectively (Table I). The remaining polypeptides, which comprise <10% of the detergent phase protein, include those of 126, 104, 69, 49, and 29 kD (see Fig. 7). The 50- and 58-kD polypeptides comprise ~3 and 7%, respectively, of the total ciliary protein. Nearly half of the total Triton X-114 solubilized protein remains in the aqueous phase. The major aqueous phase polypeptides migrate in a broad band at 50–60 kD (Figs. 1 and 2) and include bands that comigrate with α and β tubulins and bands that comigrate with the detergent phase polypeptides. Small quantities of the detergent phase 50- and 58-kD polypeptides remained in the aqueous phase after repeated extractions with Triton X-114 and the 69- and 29-kD polypeptides are found in both detergent and aqueous phases. A small quantity of detergent-resistant membrane vesicles are present in the axoneme fractions (data not shown). These vesicles were purified from the axoneme fractions on sucrose gradients and contain the major detergent phase polypeptides and the same minor bands present in the Triton X-114 detergent phase plus a high molecular mass polypeptide that migrated more slowly than the dynein heavy chains from axonemal or aqueous phase fractions (Fig. 2).

Tubulins were identified by immunoblots of detergent and aqueous phase polypeptides with α and β tubulin antibodies. To compare these results with those described in an earlier study, cilia were extracted with NP-40, which releases α and β tubulins (Figs. 3 and 4). Tubulins are present in the NP-40

Table I. Composition of Tetrahymena "Membrane + Matrix"

	Percent of fraction	Percent of total ciliary protein
TX-114 axonemes		77
Axonemal tubulin	41	32
Axonemal dynein (HMW)	8	6
TX-114 aqueous phase		
Total protein		11
Aqueous 55 kD + tubulin	28	3
TX-114 detergent phase		
Total protein		11
58 kD	65	7
50 kD	25	3

The polypeptide composition of the major ciliary axonemal and membrane proteins as determined from scans of Coomassie Blue–stained gels. Based on molar ratios, the 55- + 58-kD polypeptides are present in 2–6× greater quantities than ciliary dynein.

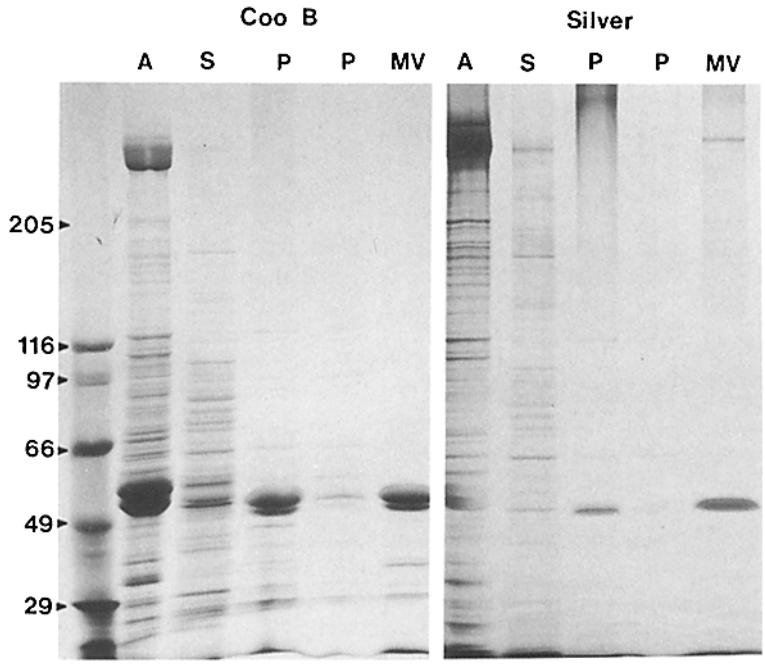

Figure 2. Comparison of the major Triton X-114 axonemal (*A*), aqueous phase (*S*), and detergent phase (*P*) polypeptides with those of purified membrane vesicles (*MV*). Cilia were extracted in PEMKS and separated at pH 8.3. Molecular mass markers are shown in the left lane and identical gels are stained with Coomassie Blue (*Coo B*) and silver (*Silver*). The membrane vesicles are enriched in a high molecular mass polypeptide that migrates slightly higher than does any of the axonemal dyneins or the membrane dynein that partitions into the Triton X-114 aqueous phase (compare *S* with *MV*). Other than the high molecular mass polypeptide, the composition of the membrane vesicles is identical to that of the Triton X-114 detergent phase.

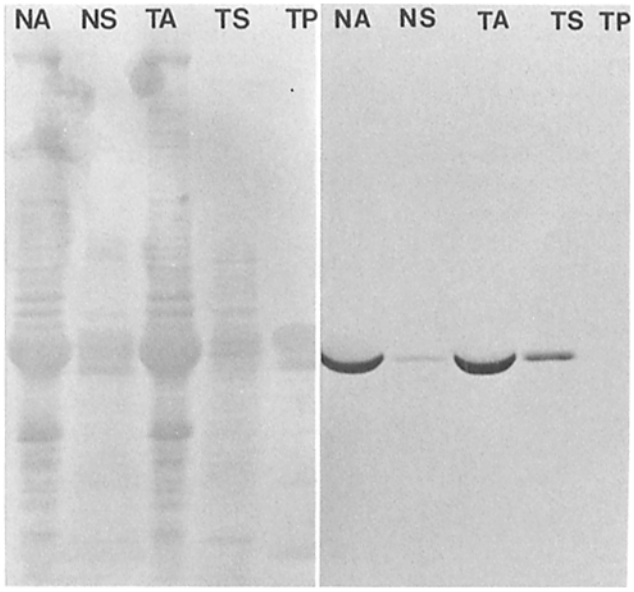

Figure 3. Identification of α tubulin in NP-40 (*NS*) and Triton X-114 (*TS* and *TP*) fractions of cilia. *NA* and *TA* are the axonemal polypeptides after extraction with NP-40 or Triton X-114, respectively. *NS* is the NP-40 detergent soluble fraction and *TS* and *TP* are the Triton X-114 aqueous and detergent phase polypeptides. Cilia were extracted in PEMKS and separated on a pH 8.3 gel. An Amido black-stained nitrocellulose blot is shown on the left and a corresponding immunoblot stained for alpha tubulin is on the right. *Tetrahymena* α tubulin migrates ahead of β tubulin (see Suprenant et al., 1985). Tubulin is released from the axonemes with each detergent and partitions into the Triton X-114 aqueous, but not detergent, phase. Since most of the 58-kD protein extracted with Triton X-114 partitions into the detergent phase, the proportion of tubulin to total protein in the aqueous phase is greater than that in the NP-40 supernatant. The major detergent-soluble proteins in *Tetrahymena* cilia are, therefore, not tubulins.

soluble extract and partition into the Triton X-114 aqueous phase. No tubulin partitioned into the detergent phase in four separate experiments. Therefore, the major membrane proteins found in *Tetrahymena* ciliary membranes are not tubulin and tubulin does not partition into the Triton X-114 detergent phase.

Identification of Glycosylated and Phosphorylated Membrane Polypeptides

Previous studies reported that ciliary membrane tubulin is glycosylated (Stephens, 1977; Dentler, 1980*b*), so samples of the Triton X-114 detergent and aqueous phase polypeptides were separated by SDS-PAGE and were stained using the PAS procedure. The results (Fig. 4) show that the major PAS-positive bands in the NP-40 extracts partition into the Triton X-114 detergent phase. At the highest sensitivity of the densitometer, the axonemes contained only a trace of PAS-positive material. Trace amounts of PAS-positive material were present in the Triton X-114 aqueous phase but it is unclear whether this is due to glycosylated tubulin or to incompletely separated detergent phase polypeptides. These results show that the detergent-phase polypeptides are glycosylated and the major 50- and 58-kD glycosylated ciliary membrane proteins are not tubulins.

Since tubulin is associated with the detergent-soluble membrane fraction and since phosphorylated tubulin can bind to lipid vesicles in vitro (Hargreaves et al., 1986), *Tetrahymena* cells were grown with ^{32}P and isolated cilia examined to determine if the detergent-solubilized tubulin is phosphorylated in vivo. The NP-40 soluble fractions contained two phosphorylated polypeptides of 50 and 58 kD (Fig. 5) as well as material at the leading edge of the gel, presumably phospholipids. Fractionation of the cilia with Triton X-114 reveals that most of the phosphorylated protein partitions

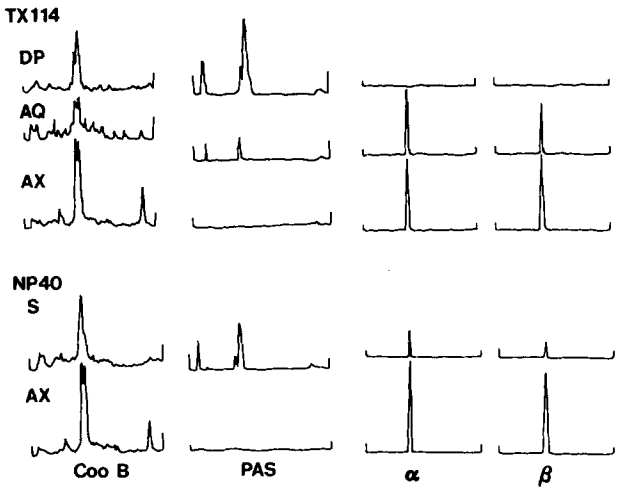

Figure 4. Scans of Coomassie Blue-stained gels (*Coo B*), identification of PAS-positive bands (*PAS*), and immunoblots stained with α (*α*) and β(*β*) tubulins. Cilia were extracted in PEMKS and polypeptides separated on pH 8.25 gels. Triton X-114 (*TXll4*) detergent phase (*DP*), aqueous phase (*AQ*), and axonemes (*AX*) are compared with NP-40 (*NP40*) supernatants (*S*) and axonemes (*AX*).The tops of each gel is toward the left. The major PAS-positive ciliary proteins migrate in the 50–58-kD region of detergent-soluble proteins but not in the axonemes. Most of the PAS-positive polypeptides found in the detergent extracts partition into the Triton X-114 detergent phase. In contrast, all of the α and β tubulin in the detergent extracts partitions into the Triton X-114 aqueous phase. Tubulins are not, therefore, the major PAS-positive ciliary proteins.

into the detergent phase and only a small amount remains in the aqueous phase (see Fig. 3). The labeled aqueous phase polypeptides comigrate with bands in the detergent phase fraction. Numerous phosphorylated axonemal polypeptides are present in cilia extracted with NP-40 and Triton X-114. The major phosphorylated band migrates at ~55 kD and may represent phosphorylated tubulin but is most likely due to the presence of membrane vesicles, which contain the phosphorylated detergent phase polypeptides.

Selective Extraction of an ATPase Activity in the Triton X-114 Detergent Phase

NP-40 extracts of *Tetrahymena* cilia separated on sucrose gradients were previously shown to contain two ATPase activities, one of which is a dynein-like protein that sediments at 14 S and contains polypeptides that comigrate with dynein and another ATPase is composed of polypeptides that migrate at ~55 kD (Dentler et al., 1980). Since most of the high molecular mass detergent-soluble polypeptides partition into the Triton X-114 aqueous phase and the major ~55-kD detergent-soluble polypeptides partition into the detergent phase, the aqueous and detergent phase fractions were assayed to determine if the detergent phase polypeptides are ATPases.

Both the detergent and aqueous phase fractions contain ATPase activity (Table II) and the total detergent-soluble protein contains nearly 20% of the total ciliary activity. To identify the polypeptides responsible for the ATPase activity, Triton X-114 aqueous and detergent phase ciliary fractions were separated on sucrose gradients and were compared with salt-extracted axonemal dynein. In some experiments, cilia were washed and extracted with Triton X-114 in PEMKS and proteins were separated on linear 10–40% sucrose gradients (not shown). The Triton X-114 aqueous phase contains the high molecular mass ATPase that cosediments with 14-S dynein as well as a second activity that remains near the top of the gradient. All of the ATPase activity in the Triton X-114 detergent phase remains near the top of the gradient and contains polypeptides identical to those found at the top of the aqueous phase gradients.

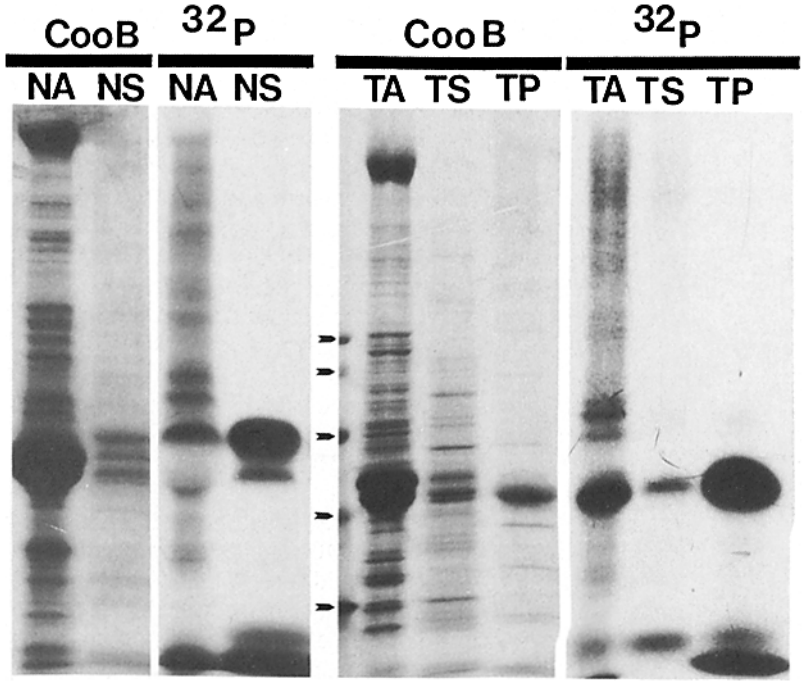

Figure 5. In vivo phosphorylation of ciliary polypeptides separated by NP-40 and Triton X-114. Cells were labeled with inorganic ^{32}P, cilia were isolated, extracted with 1% NP-40 in PEMKS, and polypeptides were separated by SDS-PAGE at pH 8.8 (left pair of gels). *NS*, NP-40 soluble protein; *NA*, axonemes. Triton X-114 solubilized proteins were warmed, separated into axoneme (*TA*), aqueous phase (*TS*), and detergent phase (*TP*) polypeptides on pH 8.4 gels (right pair of gels) which do not separate the 50- and 58-kD polypeptides. Coomassie Blue-stained gels (*Coo B*) and autoradiograms (^{32}P) are shown. The major phosphorylated polypeptides are found in the detergent-soluble extracts and comigrate with axonemal tubulins but partition into the Triton X-114 detergent phase, which does not contain tubulin. The labeled polypeptides that comigrate with tubulin in the axoneme fraction may be due to detergent-insoluble membrane vesicles (see text). Molecular mass markers on the pH 8.4 gels are (*top to bottom*) 116, 97, 66, 49, and 29 kD.

Table II. Ciliary Axoneme and Membrane Fraction ATPase Activities

Fraction	Specific activity	Total activity	Percent of total ciliary ATPase
	nM P_i min^{-1} mg^{-1}	*Sp Act × mg protein*	
Axonemes	1,800	46,800	81
Triton X-114 aqueous phase	519	908	2
Triton X-114 detergent phase	6,940	10,173	17

When isolated cilia are washed and demembranated in HEEMS, which is lower ionic strength than PEEMKS, little dynein is released. Separation of the ATPase activity on sucrose gradients reveals a single ATPase peak with virtually identical sedimentation rates in the Triton X-114 aqueous and detergent phases (Fig. 6, *D* and *F*). The dialyzed ciliary dynein is separated into 14- and 22-S peaks (Fig. 6 *B*). The polypeptide composition of the major ATPase peaks, from a 10–40% sucrose–HEEM gradient, is shown in Fig. 7. Neither of the dynein peaks contain bands that comigrate with the proteins released by Triton X-114 in HEEMS. Both the aqueous and detergent phases contain the major 50- and 58-kD polypeptides as well as minor polypeptides of 69 and 104 kD.

The polypeptide composition of the detergent and aqueous phase ATPases isolated in HEEM are similar, but not identical. To determine if the same ATPase is present in each fraction, the effects of inhibitors, divalent cations, and nucleotides were examined. Neither the aqueous nor the detergent phase ATPase activities are inhibited by 10 μM vanadate and up to 100 μM vanadate only slightly inhibits the activity. By contrast, sucrose gradient–purified 14- and 22-S dynein ATPase was almost completely inhibited by 10 μM vanadate. The effect of ouabain was examined to identify possible ciliary membrane Na^+/K^+ ATPases and the results of two experiments showed that up to 5 mM ouabain had no effect on the ATPase activity of the aqueous or detergent phase enzyme. The nucleotide specificities of the Triton X-114 aqueous and detergent phase ATPase activities are essentially identical (Table III). Each exhibit a preference for ATP but can also use GTP, CTP, and UTP. Each of the enzymes require divalent cations. The Ca^{++} and Mg^{++} activities are nearly identical at 1 mM (Table III) but higher concentrations of Ca^{++} show a slight, but reproducible, decline in the ATPase activity (Fig. 8).

The Km's for the Triton X-114 aqueous and detergent phase ATPases are 100 and 93 μM, respectively (Fig. 9). Cilia were also isolated and extracted with HEEM–NP-40 and the Km of the detergent-released ATPase was 90 μM (Fig. 9). Therefore, the same ATPase activity is present in cilia extracted with HEEM and either NP-40 or Triton X-114. These data indicate that the same ATPases are present in both the aqueous and detergent phase fractions isolated from HEEMS–Triton X-114 extracted cilia. They have virtually identical requirements for Ca^{++} and Mg^{++}, similar Km's, both are insensitive to vanadate and ouabain, and the peaks of ATPase activity eluted from sucrose gradients contain the same major polypeptides.

Discussion

Triton X-114 is a useful tool with which to solubilize membranes and separate hydrophobic integral membrane proteins from those that are more water soluble (Bordier, 1981). Previous studies of ciliary membrane proteins solubilized with Triton X-114 found relatively few polypeptides partitioning into the detergent phase. The detergent phase fraction from scallop ciliary membranes contains a small amount of a 20-kD protein (Stephens, 1985*a*) and the detergent phase of frog olfactory cilia contains only one 95-kD protein (Chen et al., 1986). When applied to *Tetrahymena* ciliary membranes, the majority of detergent-soluble polypeptides remain in the aqueous phase while only a few polypeptides, that comprise nearly 50% of the total detergent-soluble protein, are integral membrane proteins. One advantage to this method is that it separates detergent-soluble tubulin from two major integral membrane proteins that comigrate with tubulin in one-dimensional SDS-PAGE (Dentler, 1980*b*). With the separation of these polypeptides, the major phosphorylated and glycosylated membrane polypeptides can now be identified and at least one previously unidentified ciliary membrane ATPase is now partially purified. The major detergent phase polypeptides are exposed to the cell surface since they are PAS-positive. Furthermore, these polypeptides are labeled in living cells with a biotinylated probe that binds cell surface but not axonemal proteins (Dentler, W. L., and N. Franano, manuscript in preparation). The observation that the major membrane polypeptides are phosphorylated may reveal a role of phosphorylation/dephosphorylation in the membrane protein function or may reveal their association with phospholipids. The role of phosphorylation remains to be determined.

The initial impetus for this study was to characterize membrane-associated tubulin in *Tetrahymena* and to determine if phosphorylation may regulate its association with the membrane, as suggested by Hargreaves et al. (1986). Tubulins are found in detergent-soluble extracts of scallop gill cilia and *Tetrahymena* cilia (Stephens, 1977, 1983, 1985*b*, 1986; Dentler, 1980*b*) and proteins that comigrate with tubulins are present in *Tetrahymena* ciliary membrane vesicles (Dentler, 1980*b*). The major 55-kD detergent-soluble polypeptides are phosphorylated in vivo, which is consistent with tubulin phosphorylation being involved in its association with the membrane. However, Triton X-114 fractionation of the detergent-soluble polypeptides revealed that the major 50- and 58-kD polypeptides partition into the detergent phase and these polypeptides, not tubulin, that is phosphorylated in vivo.

Is tubulin associated with *Tetrahymena* ciliary membranes? The results reported here are consistent with those reported by Stephens (1985*a*), in that the detergent-soluble tubulin partitions into the Triton X-114 aqueous, but not detergent phase. These results indicate that tubulin is unlikely to be an integral membrane protein but they do not prove that it is not associated with the membrane since some chromaffin granule membrane proteins partition into the aqueous phase (Pryde and Philips, 1986). It is unlikely that the tubulin is freely soluble in the intact cilia. Examination of thin sections of isolated cilia fixed before detergent extraction revealed that virtually all ciliary membranes were broken open and, although attached to the axonemes, they were not sufficiently intact to prevent the release of soluble tubulin or

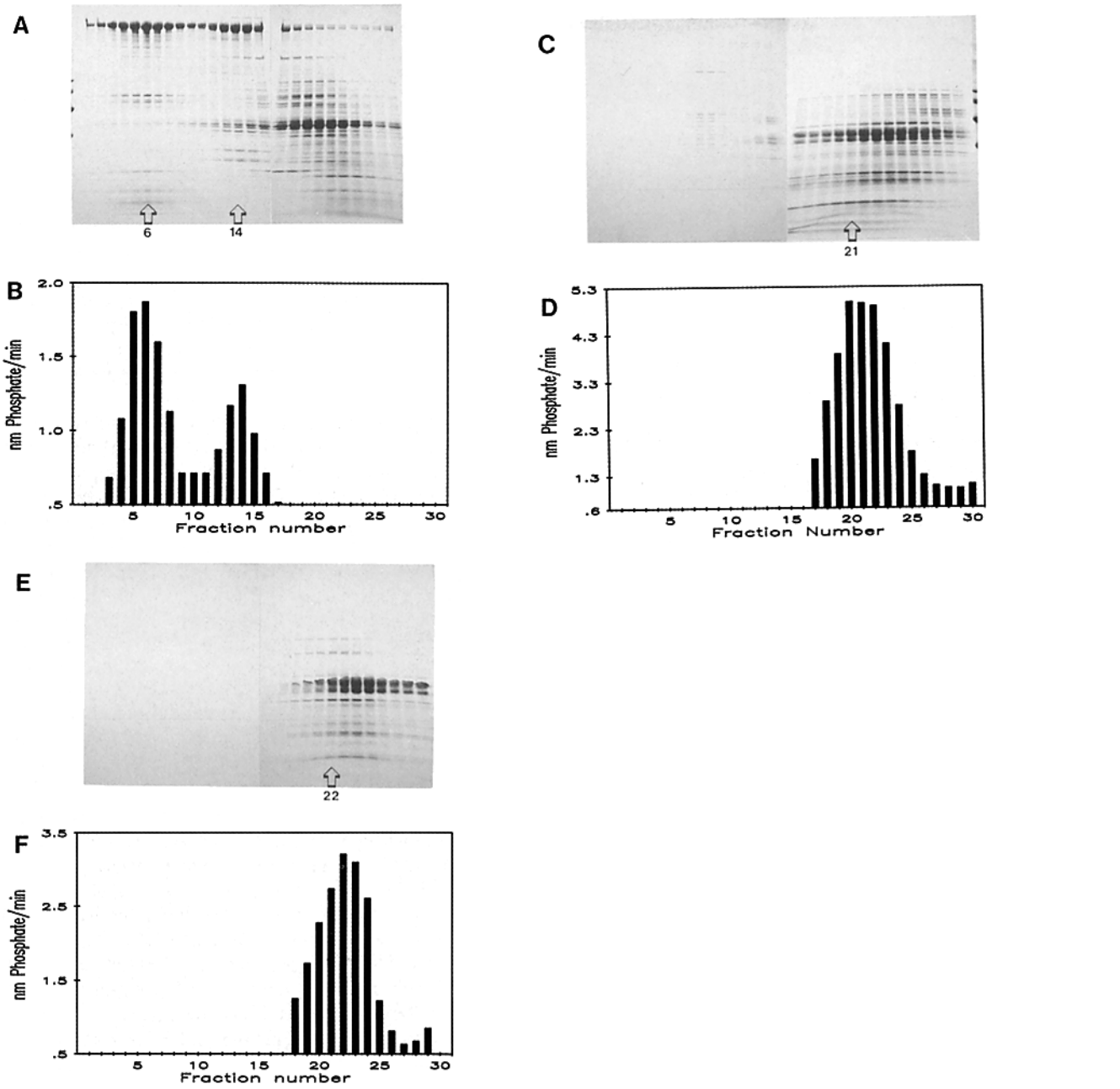

Figure 6. Analysis of ciliary fractions isolated in HEEMS plus Triton X-114. Fraction No. 1 was at the bottom of each gradient. Salt-extracted and dialyzed dynein is shown in *A* and *B*. Triton X-114 aqueous (*C* and *D*) and detergent (*E* and *F*) phases are shown in *E* and *F*. ATPase activity is expressed in nmols P_i released/min. Each fraction of the dynein (25 μl/assay), aqueous phase (25 μl/assay), and detergent phase (10 μl/assay) was assayed for ATPase activity and 100 μl aliquots of each fraction was assayed by SDS-PAGE. The 22- (fraction 6) and 14-S (fraction 14) dynein ATPases were clearly resolved (*A* and *B*). Since extraction in HEEMS does not release the membrane dynein seen in Fig. 1, the peaks of ATPase activity in both the aqueous and detergent phases sediment near the top of the gradient and contain the same polypeptides. Comparison of the polypeptides found in the peaks of ATPase activity is shown in Fig. 7.

any other soluble ciliary protein (data not shown). Despite the presence of disrupted membranes and an exposed matrix, the tubulins (and other aqueous phase polypeptides) were only released from the axonemes by the addition of detergent. Some of the soluble tubulin could be due to microtubule breakdown, since examination of thin section axonemes after detergent-extraction showed a small amount of microtubule breakdown and a portion of the soluble tubulin was assembled into microtubules in vitro upon addition of taxol (data not shown). However, the amount of released tubulin is greater than that expected from the examination of thin section axonemes after detergent extraction (see Stephens, 1986). These results do not, therefore, rule out the possibility that tubulin is associated with *Tetrahymena* ciliary membranes but they do show that, contrary to a previous report (Dentler, 1980*b*), the detergent-soluble tubulins are not the

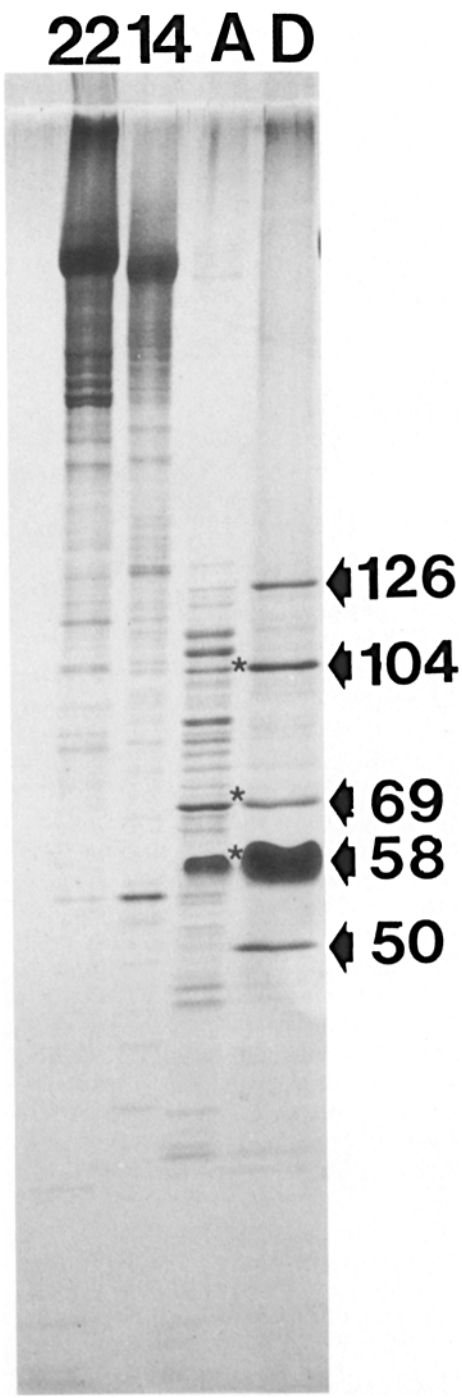

Figure 7. Comparison of the polypeptide composition of the ATPase peaks in Fig. 2. The 22- and 14-S dyneins each contain major heavy chains but have different light and intermediate chains. The Triton X-114 aqueous and detergent phase polypeptides contain numerous bands with identical mobility (*), including the major 50- and 58-kD polypeptides and 69- and 104-kD polypeptides. The 50- and 58-kD polypeptides are not resolved on this pH 8.3 separation gel.

major components of the detergent-soluble fractions, they are not glycosylated, and they are unlikely to be integral membrane proteins. The major membrane polypeptides migrate at 50 and 58 kD, partition into the Triton X-114 detergent phase, and are PAS-positive. These results are consistent with other studies of *Paramecium* and *Tetrahymena* membranes showing that tubulins are not the major membrane proteins, if they are membrane protein at all (Adoutte et al., 1980; Brugerolle et al., 1980; Merkle et al., 1981; Schultz et al., 1983; Stephens, 1986).

Table III. Nucleotide and Ion Specificities of Ciliary ATPases

Fraction	ATP/GTP	ATP/CTP	ATP/UTP	Mg^{2+}/Ca^{+2}
Axonemes	1.7	0.6	1.0	1.0 ± 0.3
Triton X-114 aqueous phase	2.1	1.5	1.3	1.2 ± 0.1
Triton X-114 detergent phase	2.6	1.3	1.0	1.2 ± 0.1

Comparison of the specific Mg^{++}-ATPase activities and divalent cation requirements for ciliary ATPases. Three different preparations of each fraction were tested for nucleotide specificity in the presence of 2 mM magnesium acetate. Mg^{++}/Ca^{++} stimulation of ATPase activity was determined for 12 axoneme preparations and 16 Triton X-114 aqueous and detergent phase fractions.

The presence of a substantial amount of ciliary and flagellar membrane-associated ATPase activity has been described in other biochemical (Gibbons, 1965; Travis and Nelson, 1986) and cytochemical studies (see Dentler, 1981) but neither the identity nor the function of the membrane-associated ATPases are understood. There are at least two detergent-soluble *Tetrahymena* ciliary membrane ATPases, one of which has been identified as a high molecular mass dynein-like protein forming a bridge linking the doublet microtubules to proteins in the ciliary membrane (Dentler et al., 1980) and partitions into the Triton X-114 aqueous phase. The release of the dynein-like protein from the axonemes depends on the buffer composition, since it could be released in PEMKS but little of the high molecular mass ATPase was released in HEEMS. Based on its solubility, the high molecular mass ATPase is probably most tightly associated with the axonemes. Since it does not partition into the detergent phase it is unlikely to be an integral membrane protein.

The second ATPase is composed of lower molecular mass polypeptides and partitions into the Triton X-114 detergent

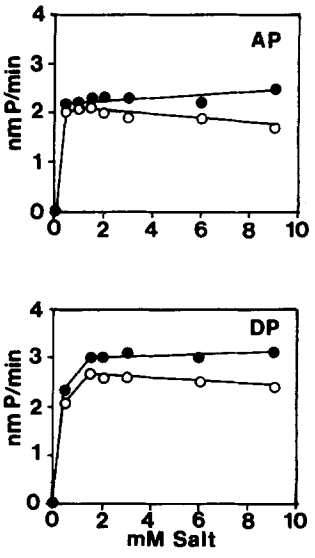

Figure 8. Effects of Mg^{++} (●) and Ca^{++} (○) on the ATPase activity found in the Triton X-114 aqueous phase (*AP*) and detergent phase (*DP*) fractions. Both enzymes required divalent cation and each used Ca^{++} or Mg^{++} with equal affinity at low concentrations of salt. At higher concentrations there was a slight, but reproducible, increase in the Mg^{++}-ATPase activity and a decrease in the Ca^{++}-ATPase activity.

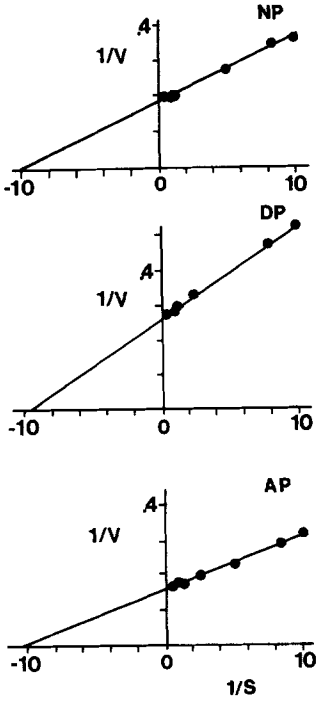

Figure 9. Lineweaver-Burke plots comparing the Km of the Mg^{++}-ATPase activity released from cilia with HEEMS and NP-40 (*NP*) with that found in the HEEMS-aqueous (*AP*) and detergent (*DP*) phases. Each of the preparations had essentially equal Km's, which strongly suggests that identical enzymes are found in each preparation.

phase, which strongly suggests that it is an integral membrane protein. Preliminary identification of the polypeptides comprising the ATPase can be made by comparing the polypeptide composition of the peaks of ATPase activity in the detergent and aqueous phases in HEEMS-extracted cilia, because the ATPases in each phase had essentially identical Km's, Ca^{++} and Mg^{++} stimulation, sensitivity to vanadate and ouabain, and sedimentation rate in sucrose gradients. In 10 separate experiments, the only major polypeptides that were common to both preparations were the 50- and 58-kD bands and a relatively minor 69-kD band. Attempts to separate the ATPase activity from Triton X-114 or from the major ciliary polypeptides contained in the Triton X-114 detergent phase were not successful. The major ciliary membrane ATPase may, therefore, be part of a complex containing the other detergent phase polypeptides (also see Dentler et al., 1980). Identification of the ATPase will depend on further purification or photoaffinity labeling.

Are these ATPases found in all cilia and flagella? The "membrane dynein" has only been identified in detergent extracts of scallop gill and *Tetrahymena* cilia (Dentler et al., 1980) so this report merely confirms its presence and purification from *Tetrahymena*. The detergent phase ATPase may be present in *Tetrahymena* but not in scallop gill cilia, since a comparison of the NP-40 soluble protein separated on sucrose gradients revealed a peak of ATPase that cosedimented with ~55-kD polypeptides in NP-40 extracts of *Tetrahymena* but not in scallop gill cilia (Dentler et al., 1980). If the detergent phase ATPase is present in *Tetrahymena* but not in scallop ciliary membranes, then the function of the ATPase may reflect different activities that occur along the ciliary surface of these two organisms. Since the extraction procedures described in this report are simple and can be carried out on small quantities of cilia, confirmation of similar ATPase activities in other cilia and flagella should easily be obtained.

The function of the detergent phase ATPase is unknown. One obvious role of a membrane-associated ATPase is to regulate ion flux across the ciliary membrane. Ca^{++}-stimulated ATPase activities have been reported in membrane or detergent extracts of *Tetrahymena* cilia (Satir, 1976), *Paramecium* cilia (Doughty, 1978; Brugerolle et al., 1980; Travis and Nelson, 1986), and *Chlamydomonas* flagella and the ATPase described here requires divalent cation. However, Mg^{++} and Ca^{++} show equal stimulation of the ATPase and high concentrations of Ca^{++} slightly, but reproducibly, decrease activity, which are properties that are not expected of a Ca^{++} pump. Neither is it a Na^+/K^+ pump, based on the lack of ouabain and vanadate inhibition, nor a dynein or kinesin-like ATPase, since it is not inhibited by vanadate (Cohn et al., 1987). The effects of inhibitors or Ca^{++}/Mg^{++} stimulation must be cautiously interpreted, however, since the enzyme was isolated in detergent and a related nonionic detergent, Triton X-100, significantly increases axonemal dynein ATPase (Gibbons and Fronk, 1979). Triton X-114 may either activate or alter the substrate specificity of the ATPase reported here. Attempts to remove the detergent from the enzymes were not successful.

The most prominent proteins exposed to the external face of *Tetrahymena* ciliary membranes include a family of species-specific immobilization antigens that migrate between 36 and 70 kD (Doerder and Berkowitz, 1986; Williams et al., 1985). The expression of specific antigens is species specific and depends on the temperature at which the cells are grown (Doerder and Berkowitz, 1986; Williams et al., 1985). These proteins are particulary interesting as models for the regulation of membrane protein synthesis and the transport of membrane-associated proteins to and along the cell surface. Their functions are, however, unknown, since the immobilization property is due to cross-linking with antisera, a situation that is unlikely to occur during the normal life of *Tetrahymena* or *Paramecium*. Based on their mobility in SDS-PAGE and their position in the ciliary membrane, the *Tetrahymena Ser H* immobilization antigen is similar to the major polypeptides found in the Triton X-114 detergent phase. However, the detergent phase polypeptide is PAS-positive while the *Ser H* immobilization antigens are not (Williams et al., 1985; Doerder and Berkowitz, 1986), although the lack of PAS staining of *Ser H* could be due to the low sensitivity of the PAS reaction. The major 52-kD immobilization antigen is exposed to the cell surface and can be iodinated (Williams et al., 1980) and preliminary results show that the major 50- and 58-kD detergent phase polypeptides are biotinylated by a cell-surface probe (Dentler, W. L., and N. Franano, manuscript in preparation). Clearly, there are similarities between the detergent phase polypeptides and the immobilization antigens but it is not known if they are identical. If they are identical, this fractionation procedure provides a more rapid and gentle method to isolate the antigens than has previously been used (Williams et al., 1985).

The Triton X-114 detergent phase polypeptides are major ciliary components: they comprise nearly 7% of the total ciliary protein by weight and are present in two to five times the quantity of the (axonemal) dynein heavy chains, based on molar ratios. It is likely that they serve important functions in the structure or function of ciliary membranes, including cell recognition and the movements of material within or on the surface of the ciliary membrane. The pres-

ence of an ATPase associated with these major proteins indicates that they may be important for either ion transport or cell-surface motility. If similar proteins are found in other ciliary membranes, the fractionation methods described here should be useful to isolate them for further analysis.

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