BIOCHEMICAL STUDIES OF THE EXCITABLE MEMBRANE OF *PARAMECIUM TETRAURELIA*

III. Proteins of Cilia and Ciliary Membranes

ANDRÉ ADOUTTE, RAJEEV RAMANATHAN, ROBERT M. LEWIS, ROLAND R. DUTE, KIT-YIN LING, CHING KUNG, and DAVID L. NELSON

From the Laboratory of Molecular Biology and the Departments of Biochemistry and Genetics, University of Wisconsin-Madison, Madison, Wisconsin 53706. Dr. Adoutte's present address is the Centre de Génétique Moléculaire du Centre National de la Recherche Scientifique, 91190 Gif-sur-Yvette, France. Dr. Dute's present address is the Department of Biology, St. Ambrose College, Davenport, Iowa 52803. Dr. Ling's present address is the Department of Physiology, University of North Carolina, Chapel Hill, North Carolina 27514.

ABSTRACT

As a first step in the biochemical analysis of membrane excitation in wild-type *Paramecium* and its behavioral mutants we have defined the protein composition of the ciliary membrane of wild-type cells. The techniques for the isolation of cilia and ciliary membrane vesicles were refined. Membranes of high purity and integrity were obtained without the use of detergents. The fractions were characterized by electron microscopy, and the proteins of whole cilia, axonemes, and ciliary membrane vesicles were resolved by SDS polyacrylamide gel electrophoresis and isoelectric focusing in one and two dimensions. Protein patterns and EM appearance of the fractions were highly reproducible. Over 200 polypeptides were present in isolated cilia, most of which were recovered in the axonemal fraction. Trichocysts, which were sometimes present as a minor contaminant in ciliary preparations, were composed of a very distinct set of over 30 polypeptides of mol wt 11,000-19,000. Membrane vesicles contained up to 70 polypeptides of mol wt 15,000-250,000. The major vesicle species were a high molecular weight protein (the "immobilization antigen") and a group of acidic proteins with mol wt ~40,000. These and several other membrane proteins were specifically decreased or totally absent in the axoneme fraction. Tubulin, the major axonemal species, occurred only in trace amounts in isolated vesicles; the same was true for *Tetrahymena* ciliary membranes prepared by the methods described in this paper. A protein of mol wt 31,000, pI 6.8, was virtually absent in vesicles prepared from cells in exponential growth phase, but became prominent early in stationary phase in good correlation with cellular mating reactivity. This detailed characterization will provide the basis for comparison of the ciliary proteins of wild-type and behavioral mutants and for analysis of topography and function of membrane proteins. It will also be useful in future studies of trichocysts and mating reactions.

There is much current interest in cell motility
involving actin and myosin or tubulin and dynein.
Motility must be regulated with respect to external
stimuli to be useful to the cell or the organism.
There has been relatively little study of the control
of motility at the biochemical level. The surface
membranes of many cells clearly transduce various
stimuli to motile responses. Such membranes must
consist of various binding sites, receptors, ion
channels, and ion pumps to account for their
functions. The components, topology, and ultra-
structure of such membranes therefore warrant
investigation. Although the mechanism of motility
of the axoneme (the ubiquitous 9 + 2 microtubular
assembly and its accessory structures) has been
extensively analyzed, the study of the membrane
that controls the axonemal mobility has been
scanty. This paper is a systematic study of the
Paramecium ciliary membrane, whose controlling
functions have been physiologically demonstrated.

Paramecium is an exceptionally favorable or-
ganism for studies of membrane excitability and
of ciliary regulation; it is a unicellular eucaryote
amenable to electrophysiological, genetic, and bio-
chemical study (13, 29, 30, 36, 40, 45). The motile
cilia of Paramecium are enclosed within an excit-
able membrane that regulates the direction of the
ciliary power stroke and, thereby, the path of this
free-swimming protozoan. Mechanical, chemical,
or electric stimuli cause depolarization of the sur-
face membrane, allowing the influx of Ca^{++}
through voltage-sensitive Ca^{++} channels in this
membrane (21, 22). The increased internal Ca^{++}
concentration caused by this influx produces a
change in the power stroke and hence in the
direction of swimming. Deciliated cells are viable
but electrically inexcitable; they apparently lack
Ca^{++} channels. As such cells regenerate cilia, they
regain electric excitability (19, 47). Thus, the cili-
ary membrane is functionally specialized; it is the
exclusive locus of voltage-sensitive Ca^{++} channels
and perhaps of some other receptors, channels,
and pumps that play roles in sensory transduction
and membrane excitation. Although there has
been extensive electrophysiological study of the
Paramecium membrane, the present work is the
first detailed analysis of its protein components.

Several procedures are known that specifically
release cilia from ciliated protozoans while leaving
the cell otherwise intact (26, 72). Studies of the
phospholipid composition of the ciliary membrane
of Paramecium (4, 34) have provided evidence that
this membrane is chemically differentiated. Elec-
tron microscopy has also revealed organized arrays
of particles at the base of the cilia (20). Hansma
and Kung (30) resolved the major proteins of
Paramecium cilia and ciliary membranes by acryl-
amide gel electrophoresis. They found that the
ciliary membrane fraction contained 12-15 pep-
tides of mol wt 25,000-150,000, and one (the major
membrane protein) of mol wt ~250,000. This ma-
jor membrane protein is immunologically related
to the "immobilization antigen" (30).

Because of the importance of obtaining ciliary
membranes uncontaminated by axonemal proteins
or other subcellular components, a scheme has
been developed for the isolation, without the use
of detergents, of ciliary membranes and demem-
branated axonemes. The protein composition of
cilia, axonemes, and ciliary membranes was ex-
amined using more effective techniques for resolv-
ing membrane proteins than were available to
Hansma and Kung. We now show that a set of
~70 polypeptides are in the ciliary membrane
fraction, and that another set of at least 125 poly-
peptides are found in purified axonemes. For sev-
eral major proteins of each fraction, no overlap in
the electrophoretic patterns occurred. These tech-
niques will be used in the biochemical comparison
of wild-type cells with behavioral mutants, and in
studies of ciliary membrane function.

MATERIALS AND METHODS

Stocks and Cultures

Isolation of Subcellular Fractions

The fractionation scheme is summarized in Fig. 1. Cells were harvested by centrifugation at room temperature at 200 g_{max} for 1–2 min in an HNS centrifuge (Damon/IEC Div., Damon Corp., Needham Heights, Mass.) with pear-shaped oil testing centrifuge vessels and washed in 4°C Dryl's solution (1 mM NaH_2PO_4, 1 mM Na_2HPO_4, 2 mM Na_3 citrate, 1.5 mM $CaCl_2$, pH 6.8) (18) three times. With fragile strains, cell washes were often carried out in room temperature Dryl's solution. All subsequent steps in the fractionation scheme were carried out at 4°C. Upon being transferred to cold Dryl's solution, the cells extruded trichocysts, which were separated from the cells by centrifugation at 200 g_{max}; they formed a distinct fluffy layer on top of the cell pellet. When we wished to purify trichocysts, this fluffy layer was resuspended, washed several times by centrifugation in Dryl's solution, and then centrifuged at 850 g_{max} for 2 min to sediment any contaminating cell bodies. Trichocysts were recovered from the supernatant fluid by centrifugation at 18,000 g_{max} for 20 min. Further purification of trichocysts was carried out on the sucrose gradient described below.

Cells were deciliated by a modification of the procedure used by Hansma and Kung (30). Washed cells were resuspended in a 1:1 mixture of Dryl's solution and STEN (0.5 M sucrose, 20 mM Tris-Cl, 2 mM EDTA, 6 mM NaCl, pH 7.5) for 10 min (at which time all cells were immobilized). The cilia were detached by adding concentrated $CaCl_2$ and KCl to a final concentration of 10 mM Ca^{++} and 30 mM K^+. The progress of deciliation was monitored by observing a drop of the suspension under the phase contrast microscope. Deciliation usually took between 5 and 10 min and was 80–90% complete (see Fig. 2). With this procedure, no blistering or lysis of cells was observed, a crucial condition for the purity and integrity of the ciliary preparation. The suspension was centrifuged at 850 g_{max} for 2 min in the HNS centrifuge to pellet cell bodies, and the supernatant was recentrifuged under the same conditions to completely eliminate remaining cell bodies. Cilia were recovered from the supernatant by centrifuging at 28,000 g_{max} for 20 min in a Sorvall RC2-B centrifuge (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.).

Ciliary membrane was removed by vortexing the ciliary pellet in freshly prepared 1 mM Tris, 0.1 mM EDTA, pH 8.3 (Tris-EDTA) for ~2 min. This suspension was centrifuged at 48,000

FIGURE 1 Preparation of ciliary fractions. A schematic diagram of the procedures used to prepare the fractions discussed in the text. Approximate protein yields at all points in the fractionation scheme are also indicated.

g_{max} for 30 min to recover all particulate material. The pellet obtained was suspended in 10 mM Tris, pH 8.0, to a concentration of ~5–10 mg protein ml^{-1}, and 0.2–0.3 ml was layered on a sucrose step gradient. The sucrose gradient consisted of 0.7 ml of 66% (wt/wt) sucrose overlaid with 1.7 ml each of 55 and 45% sucrose and 0.7 ml of 20% sucrose in 10 mM Tris, pH 8.0. The gradient was poured and left at 4°C for ~1 h before being used. The gradient was centrifuged in an SW 50.1 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 45,000 rpm for 1.5 h at 4°C to separate the different fractions. Ciliary membranes banded within the 45% sucrose layer, incompletely demembranated cilia banded within the 55% layer, and axonemes, trichocysts, and the few remaining bacteria banded at the interface of the 55 and 66% layers of sucrose. The bands were collected from the gradient, checked for purity by phase-contrast microscopy, and washed by centrifugation in 20–30 vol of 10 mM Tris, pH 8.0, before being analyzed by electron microscopy and polyacrylamide gel electrophoresis. In early experiments, pellet 3, Fig. 1, resuspended in 10 mM Tris, was layered on a 50% (wt/wt) sucrose cushion and spun at 35,000 rpm in an SW 50.1 rotor (30). In this case, membranes banded at the top of the cushion and a pellet, consisting of incompletely demembranated cilia, axonemes and trichocysts, was obtained.

Supernatant fractions were precipitated by the addition of 10 vol of cold acetone, allowed to stand for at least 1 h at 4°C, and centrifuged at 12,000 g_{max} for 30 min. The pellets were dried under a stream of nitrogen before analysis by gel electrophoresis.

Scanning Electron Microscopy

The paramecia were fixed while in 4 mM $CaCl_2$, 1 mM Tris-Cl, pH 7.2. An excess of the instantaneous fixation solution of Tamm (see reference 48) with final concentration of 2% OsO_4, 2% glutaraldehyde, 0.5 M Na-K PO_4, pH 7.2, was pipetted onto the swimming cells, and the suspension was left at room temperature for 10–15 min. The cells were washed in 20-mesh Nitex bags (Tetko Inc., Elmsford, N. Y.), dehydrated through an ethanol series, and critical point dried. Cells were then sprinkled on stubs covered with conductive glue, coated with carbon-platinum, and viewed on a JSM U3 scanning electron microscope.

Transmission Electron Microscopy

The samples were fixed for 1 h in 2% glutaraldehyde in 0.08 M s-collidine buffer (pH 7.2) at room temperature. After several buffer rinses the material was postfixed in 1% OsO_4 for 45 min. It was subsequently washed and stained en bloc with 0.5% aqueous uranyl acetate for 2 h and was dehydrated in an ethanol series followed by propylene oxide and embedment in Spurr's resin (62). Silver interference sections were taken on a Reichert Om U3 ultramicrotome (American Optical Co., Buffalo, N. Y.), stained with lead citrate, and photographed with a Philips 300 electron microscope.

One-dimensional SDS Gels

A number of discontinuous SDS polyacrylamide gel systems based on Laemmli's (37) original formulation were tested. The best resolution of proteins over a wide molecular weight range was achieved by using 1.5-mm-thick polyacrylamide gradient slab gels (2). "Short" gels (16 × 10 cm) containing a linear 7.5–15% acrylamide gradient and a 3% stacking gel (49) were routinely used; all gels shown here are of this type. Occasionally, "long" gels (16 × 30 cm) containing a linear 6–20% acrylamide gradient were also used. Samples were dissolved in 2% SDS,
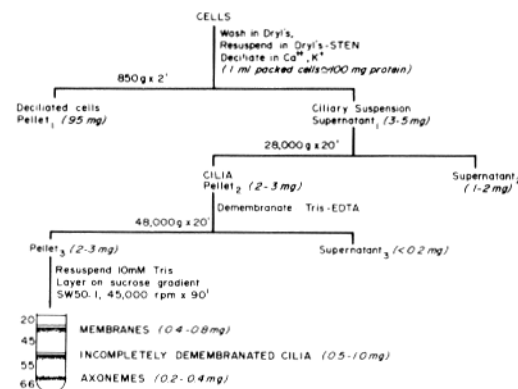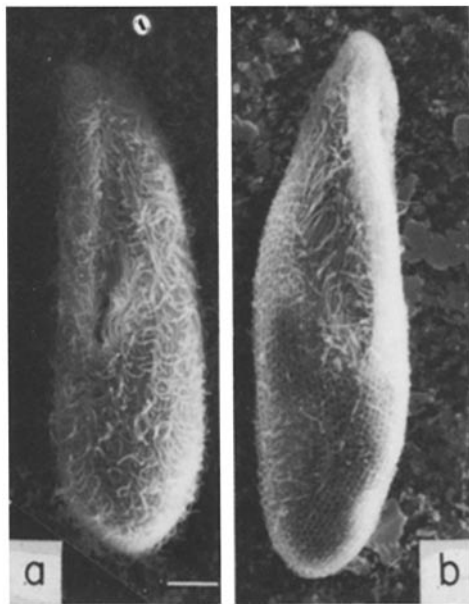
FIGURE 2 Scanning electron micrographs of normal (a) and deciliated (b) paramecia. Note that the oral cilia have not been detached in b. Bar, 10 μm. (Photographs kindly provided by Charlotte Omoto.)

heated at 100°C for 5 min, then stored at −20°C. After determination of the protein concentration (41), aliquots were added to an equal volume of two times sample buffer (20% glycerol, 10% 2-mercaptoethanol, 2% SDS, 125 mM Tris, pH 6.8) (37), heated again, and loaded on the gels. Loads ranged from 25 to 150 μg protein in volumes of 10–80 μl. Gels were run at 20 mA constant current with tap water cooling. Dansylated cytochrome c was used as a fluorescent indicator of the level of migration reached by low molecular weight proteins (33). The run was stopped when this marker was ~1 cm from the bottom of the gel. Gels were stained overnight in 0.1% Coomassie brilliant blue dissolved in ethanol:H₂O:acetic acid (9:9:2), destained in 7.5% acetic acid, and photographed before drying. Molecular weight standards (all from Sigma Chemical Co., St. Louis, Mo., except where mentioned) were myosin heavy (mol wt 220,000) and light (17,000) chains (kindly provided by Dr. M. Greaser, University of Wisconsin-Madison), conalbumin (86,000), bovine serum albumin (68,000), catalase (60,000), actin (44,000) (provided by Dr. Greaser), ovalbumin (43,000), DNAase I (34,000), α-chymotrypsinogen A (26,000), and myoglobin (17,000). Molecular weights of sample proteins were extrapolated from a plot of the R_f vs. log molecular weight of the standards (71), which is essentially linear in this type of gradient gel except in the very high molecular weight range.

One-dimensional Isoelectric Focusing (IEF) Gels:

IEF was carried out in 3% polyacrylamide slab gels as described by Ames and Nikaido (3) with some modifications.

The gel mixture, slightly modified from O'Farrell (46), contained 3% acrylamide (2.84%, wt/vol, acrylamide and 0.16%, wt/ vol, bis-acrylamide), 2% wt/vol Biolyte 3/10, 10 M urea, 4% (wt/ vol) NP-40, 0.00035% (wt/vol), riboflavin 5'-P, and 0.025% (vol/ vol) N,N,N,N-tetramethylenediamine. (TEMED).

Protein samples (100–200 μg) solubilized in 2% SDS were diluted with 80–120 μl of a solution containing 10 M urea, 2% (wt/vol) Bio-Lyte 3/10, 4% (wt/vol) Nonidet P-40, 5% (vol/vol) 2-mercaptoethanol, and 10% (wt/vol) sucrose. After being loaded in the sample well, each sample was overlayed with 20 μl of a solution of 2.5 M urea, 0.5% Bio-Lyte 3/10, 1% NP-40, and 1.25% mercaptoethanol.

The cathode and anode solutions were 0.4% monoethanolamine and 0.1 M H₃PO₄, respectively. IEF was started at 100 V. The voltage was then increased by increments during the following 1.5 h to 300 V, and held at that value for the next 9.5–10 h. The run was concluded at 400 V for 1 h or at 800 V for 30 min. The apparatus was connected to a water cooling system during the run.

To determine the pH gradient of the gels, a 1-cm strip was cut from one side of a gel along the axis of IEF after the run. The strip was cut into 0.5-cm pieces, which were soaked in 1 ml of distilled water for 24 h. The samples were then degassed and their pH was measured with a microelectrode.

Fixing, staining, and destaining of the gels were done by the methods of Efstratiades and Kafatos (23).

Two-dimensional Gels

The first-dimension IEF gels were prepared according to the methods described by O'Farrell (46), with the following exceptions: (a) Samples were suspended in 2% (wt/vol) SDS and heated for 5 min at 100°C. A few minutes before loading, the samples were adjusted with an IEF solution so that the final composition was 9 M urea, 0.4% (wt/vol) SDS, 4% (wt/vol) NP-40, 5% (vol/vol) mercaptoethanol, and 2% (wt/vol) ampholytes (0.4% LKB 3.5/10, 0.8% LKB 5/7, 0.8% LKB 7/9). (b) The final gel composition was 3% acrylamide (2.84% (wt/vol) acrylamide, 0.16% (wt/vol) bis-acrylamide), 9.5 M urea, 4% (wt/vol) NP-40, and 2% (wt/vol) ampholytes (0.4% LKB 3.5/10, 0.8% LKB 5/7, 0.8% LKB 7/9), 1 mg/ml arginine, and 1 mg/ml lysine. The final concentration of ammonium persulfate was 0.033% (wt/vol). No TEMED was used. (c) Degassed 0.4% (vol/vol) monoethanolamine was used as the cathode solution. (d) The pre-electrophoresis step was omitted. (e) The 11-cm gels were run for 10 h at a constant 800 V.

The pH gradient of the IEF gels was determined as described above. After a 1-h equilibration the first-dimension gels were run on SDS 7.5–15% polyacrylamide gradient slab gels prepared according to the methods described above for one-dimension SDS gels.

Chemicals

Ampholytes used in one-dimensional IEF gels were Bio-Lyte 3/10 from Bio-Rad Laboratories (Richmond, Calif.), and those used in two-dimensional electrophoresis were Ampholines 3.5/ 10, 5/7 and 7/9 from LKB Instruments, Inc. (Rockville, Md.). SDS was obtained from BDH Biochemicals, Ltd. (Poole, England), acrylamide and N,N'-methylene-bis-acrylamide (Bis) from Bio-Rad Laboratories, and Nonidet P-40 from Bethesda Research Laboratories (Rockville, Md.). Sarkosyl was obtained from Ciba-Geigy Corp. (Ardsley, N. Y.), Triton X-100 was the scintillation grade from Research Products International Corp. (Elk Grove Village, Ill.), and urea was the ultra pure grade from Schwarz/Mann Div., Becton, Dickinson & Co. (Orangeburg, N. Y.).

RESULTS

Deciliation

Phase-contrast light microscopy, transmission, and scanning electron microscopy all showed that the procedure for deciliation was successful; few somatic cilia remained attached to the body (Fig. 2). Cilia in the oral apparatus were not removed by this procedure. Deciliated cells were otherwise intact.

The cilia broke in the transition zone, the region within which the central tubules terminate and in which the necklaces and plaques (arrays of intramembrane particles) are located (20). Satir et al. (57) have presented evidence that the cilia of *Tetrahymena* break in this region, and Blum (10) has reviewed the data on diverse organisms indicating that the transition zone of the cilium is the preferred breaking point. Deciliation partitioned the plaques with the detached cilia (Fig. 3 d and e) and necklace with the cell body (Fig. 3f). Membrane fusion apparently occurred, as the break-points were covered by membranes.

Thin-section transmission electron microscopy showed that pellet 2 (Fig. 1) consists of cilia (Fig. 3 a). Like the cilia in intact paramecia, they consist of complete axonemes enclosed in unit membranes (Fig. 3 b). However, in some cases, the membrane ballooned out and no longer enwrapped the axoneme tightly. Occasionally, empty vesicles and bare axonemes were seen. If trichocysts were not eliminated during cell washes, they were also seen in this pellet as well as a few bacteria. (Trichocysts are membrane-bounded, expansile organelles that lie in regular arrays just beneath the *Paramecium* surface [31].)

Fractionation of Cilia on Sucrose Gradients

The cilia, when vortexed for short periods (≤ 2 min) in low ionic strength medium, yielded a suspension containing ciliary membranes, incompletely demembranated cilia (IDC), and axonemes free of membrane (pellet 3, Fig. 1). Although a brief period of vortexing was essential to the separation of vesicles from IDC, we found that vortexing the ciliary suspension for extended periods of time (up to 30 min) did not increase membrane yield or break down the IDC; this membrane-enclosed unit seemed to be structurally stable under the low ionic strength conditions employed. Optimum separation of these components into

three broad bands was achieved in a continuous 10–66% (wt/wt) sucrose gradient in 10 mM Tris, pH 8.0, run for 2 h at 45,000 rpm in an SW 50.1 rotor. The buoyant densities of these fractions were determined by centrifugation to equilibrium (17 h) in such a gradient. The three bands and their buoyant densities corresponded to: (a) ciliary membranes (1.15–1.2 g/ml); (b) IDC (1.25 g/ml); (c) axonemes (1.28 g/ml) and trichocysts (1.29 g/ml). On these continuous gradients the ciliary membranes often separated as two to three discrete bands, with buoyant densities <1.21 g/ml. Protein patterns of these membrane subfractions on SDS polyacrylamide gel electrophoresis (PAGE) were identical except for one striking difference: a protein of mol wt 19,000 was selectively enriched for in the lighter (less dense) membrane fraction (data not shown). About 30–40% of the protein and 60–70% of the phospholipids of whole cilia are recovered in the ciliary membrane fraction (Fig. 4). This proportion of membrane protein may represent an overestimate (see Discussion). For routine separations of these fractions we chose to use the more convenient step gradients described in Materials and Methods. This resolved the mixture into three sharp and well-separated bands (Fig. 4) after centrifugation for 1.5 h at 45,000 rpm in an SW 50.1 rotor.

Phase-contrast microscopy showed that the band within the 45% sucrose layer consisted of ciliary membrane vesicles of heterogeneous size and refractility. Electron micrographs showed that this fraction consisted of pure membrane vesicles. In thin sections, these vesicles were up to 0.7 μm in diameter (Fig. 5 a and b). They were occasionally multilamellar, and the laminae were not tightly stacked in most cases. An amorphous fuzzy layer was occasionally seen on the vesicles. This material may be related to the fuzzy coat observed in the surface of cilia in intact *Paramecia*. Besides this fuzzy material, no other recognizable structure was seen in the membranes.

The band of intermediate density, found within the 55% sucrose layer, consisted of incompletely demembranated cilia. Electron micrographs of this fraction showed axonemes coiled within the membrane (Fig. 5 c). There were also some axonemes in this fraction.

Occasionally, we also obtained a minor band of IDC above the main band. The structures in this fraction, unlike the IDC, contain only a small fragment of axoneme enclosed in a membrane. In agreement with the cytological observation, SDS

PAGE showed that this minor fraction contained mainly ciliary membrane proteins and the major axonemal proteins (data not shown).

The band at the interface of the 55 and 66% sucrose layers consisted mostly of axonemes free of the ciliary membrane (Fig. 5 d). Trichocysts and bacteria, if not completely removed before decilia-

tion, were also found in this fraction of the step gradient. Attempts to separate the axonemes from the trichocysts by the introduction of a 60% sucrose layer were unsuccessful.

Nothing remained on top of the gradient, but occasionally a small amount (<5% of the total protein) of material which appeared to be aggre-
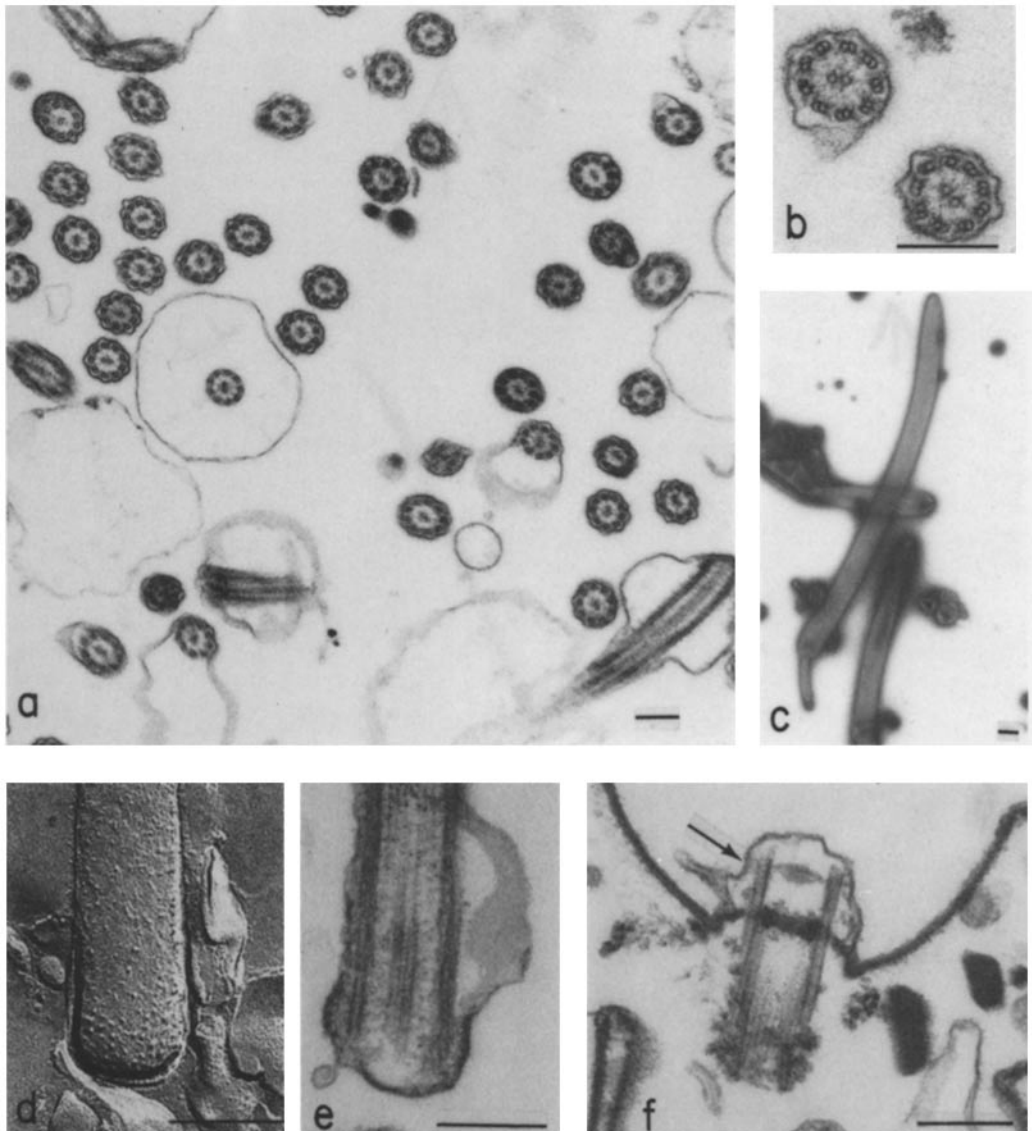

FIGURE 3 Isolated cilia. (a) Thin-section transmission electron micrograph of this pellet; (b) cross-section of cilia in a at a higher magnification; (c) negatively-stained whole cilia; (d) freeze-fracture replica of an isolated cilium showing particles at the base similar to the plaque particles (courtesy B. J. Byrne); (e) thin-section through the base of an isolated cilium showing the plaque complex; (f) thin-section electron micrograph of a deciliated paramecium showing that the detachment of the cilium occurs above the axosomal plate (arrow). Bar, 0.25 μm.

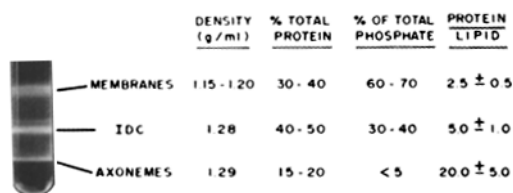| DENSITY (g/ml)	% TOTAL PROTEIN	% OF TOTAL PHOSPHATE	PROTEIN/LIPID
MEMBRANES 1.15-1.20	30-40	60-70	2.5 ±0.5
IDC 1.28	40-50	30-40	5.0 ±1.0
AXONEMES 1.29	15-20	<5	200 ±50

FIGURE 4 Composition of sucrose gradient fractions. Protein/lipid ratios are microgram protein/nanomole phosphate. Phosphate was determined by the method of Chen et al. (16).

gated axonemes sedimented through the 66% sucrose. SDS PAGE showed that the major proteins of this pellet were tubulins and dyneins. Up to 2.0 mg of protein could be reproducibly separated in the 5-ml gradient under the conditions employed. Fig. 1 also shows yield estimates at the various stages of the fractionation scheme.

While we used the sucrose step gradient described for routine preparations of the ciliary subfractions, the purest membranes, as judged by microscope observation and SDS gel analysis of the fraction, were obtained on the continuous sucrose gradients (see Discussion). Recentrifugation of ciliary membranes again on sucrose step gradients did not significantly improve the purity of the preparation.

Pattern of Cilia, Axonemes, and Membranes

ONE-DIMENSIONAL SDS GELS: Whole cilia and the various gradient fractions were first systematically compared, side by side, on one-dimensional SDS polyacrylamide slab gels. The patterns obtained in 100 gels from 75 independent experiments were highly reproducible. A representative example of the fractions from a step gradient is shown in Fig. 6. Several points emerged from this analysis:

(a) Whole cilia display a complex pattern of polypeptides; over 60 well-resolved bands were invariably present. In gels with the best resolution, up to 80 bands could be seen, and this is known from two-dimensional gels to be an underestimate of the total number of polypeptides (see below).

These polypeptides are present in very disproportionate amounts; three regions contribute over 80% of total ciliary proteins. These are, in order of decreasing intensity: (1) the two tubulin bands (identified on the basis of their mol wt, 53,000 and 55,000, the fact that they are also the major axo-

nemal component, and their isoelectric point (9), as described below); (2) a broad band at ~250,000 mol wt that corresponds to the surface immobilization antigen (discussed below); (3) a broad area of mol wt over 300,000, composed of at least six bands. By analogy with what is known from cilia and flagella of other species (27, 38, 52, 70) and also on the basis of the intensity of the bands and their total recovery in the axonemal fraction, several of these species are probably dynein subunits.

In addition to these main bands there is a large number of bands of medium intensity and characteristic groupings such as the triplet at mol wt 100,000-105,000, the bands of mol wt 42,000-44,000, and the set of four bands of similar intensity with mol wt between 19,000 and 42,000. All of these bands are useful landmarks when comparing slots on the same or different gels.

(b) Axonemes displayed an overall complexity similar to that of cilia in one-dimensional gels, and most of their bands were identical with those of cilia. The two most significant differences between cilia and axonemes were: (1) the relative increase in intensity of the tubulins and dyneins in axonemes; and (2) the total absence or very marked decrease of a set of bands in axonemes. The most striking example was the band of mol wt 250,000 (immobilization antigen) in cilia, which was totally absent from pure axonemes. Additional major bands belonging to this category were of mol wt 42,000-44,000; 31,000; 65,000; 87,000; and 19,000.

(c) Membrane vesicles and axonemes represented ~40 and 60% of the total ciliary protein, respectively. When protein from vesicles, axonemes, and cilia was loaded in the proportion: 150 μg of membrane protein, 125 μg of axonemal protein, and 125 μg of cilia, vesicles displayed a pattern which was both very distinct from and much simpler than that of axonemes and cilia (Fig. 6). The prominent features of the vesicle pattern were: (1) the mol wt 250,000 protein (totally absent from axonemes), which represented ~75% of total membrane proteins, and which was shown (30) to be immunologically related to the immobilization antigen, a major surface protein of Paramecium (24, 55); (2) the very small amounts of tubulins and dyneins, the major axonemal components; (3) a set of bands of mol wt 93,000; 87,000; 65,000; 63,000; 42,000-44,000; 31,000; and 19,000, which also correspond to bands that were decreased or absent in axonemes. We found a reproducible variation in the amount of one of the
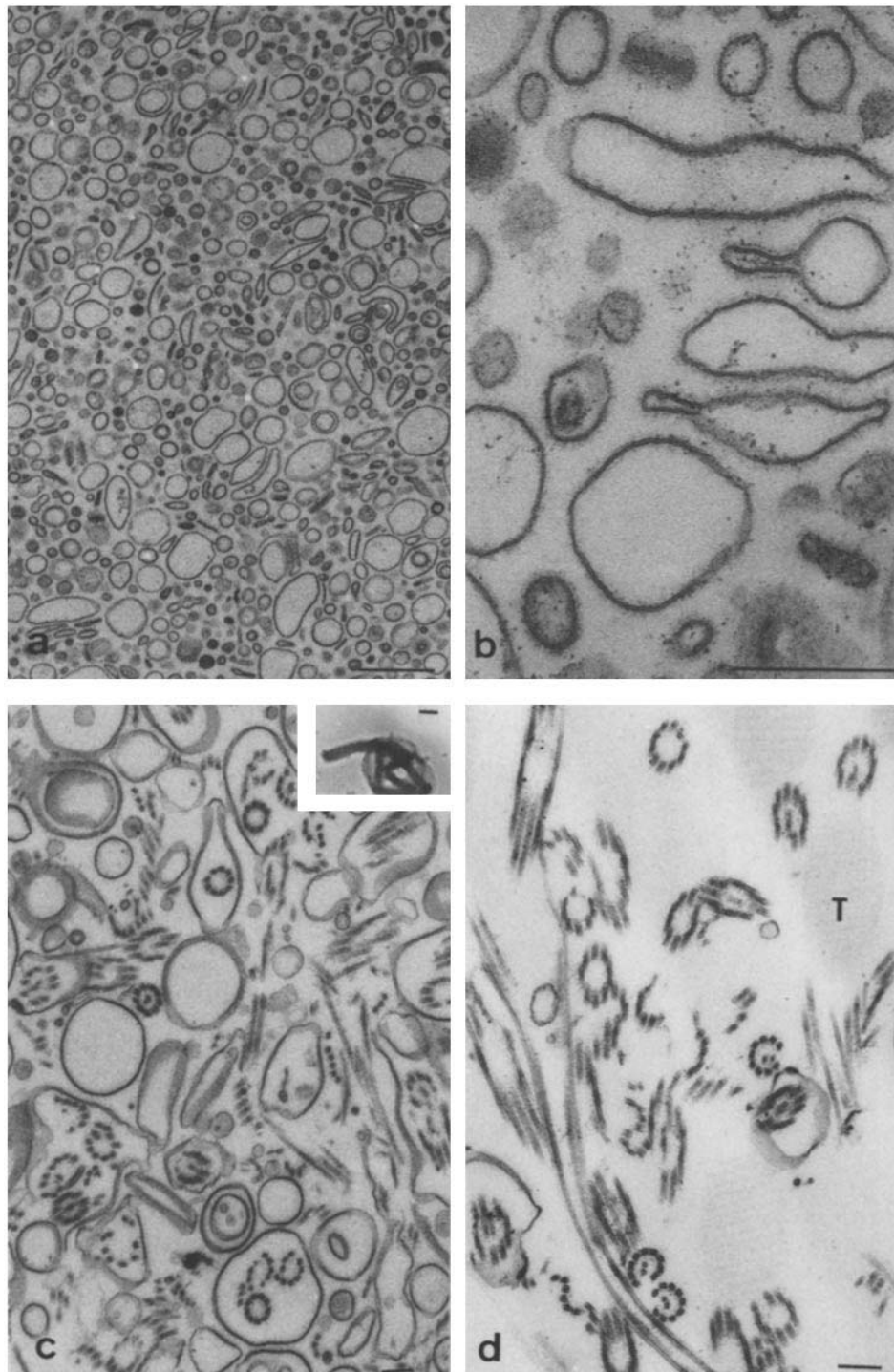
FIGURE 5 Thin-section electron micrographs from the three gradient fractions. (a) Membrane vesicles; (b) same at a higher magnification; (c) IDC; *inset* is one such cilium, negatively stained, showing fractured axoneme enclosed by the membrane; (d) axonemes; note that this fraction also contains some trichocysts (T). Some axonemal breakdown is seen in both c and d. Bar, 0.25 μm.

mol wt
(x10⁻³)

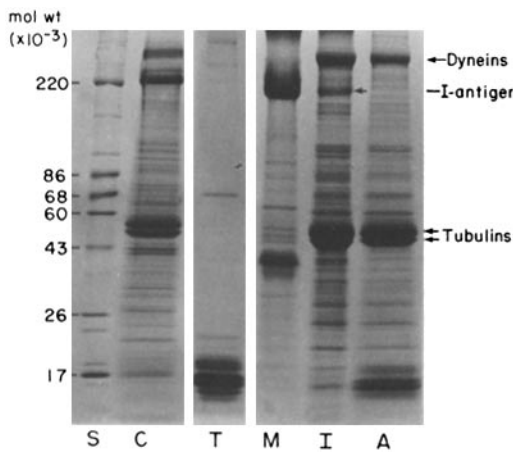

←Dyneins
─I-antigen

±Tubulins

S C   T M I A

FIGURE 6 One-dimensional SDS gel of ciliary fractions. (S) Molecular weight standards; (C) whole cilia, 125 μg protein; (T) trichocysts, 40 μg; (M) ciliary membranes, 150 μg; (I) IDC, 125 μg; (A) axonemes, 125 μg.

membrane proteins (mol wt 31,000) as a function of the growth stage of the cells (Fig. 11). Although this protein was barely detectable in cells at mid-logarithmic phase, it was a major component in cells at early stationary phase, and subsequently decreased in late stationary phase. The rest of the protein pattern remained similar throughout the three stages. The band was present in cells of both mating types (VII and VIII). As expected from earlier studies (43, 59, 60), mating reactivity assayed with testers appeared in early stationary phase (data not shown).

Gels heavily loaded with membrane vesicles prepared by the continuous gradient procedure (100–200 μg of protein) displayed a more complex pattern comprising ~35 bands in addition to the major ones (Fig. 7), but showed only trace amounts of tubulins and dyneins and none of the other major axonemal proteins.

Membrane vesicles obtained from the 50% sucrose cushion (see Materials and Methods) yielded a pattern identical with that of Fig. 6. However, occasional contamination by incompletely demembranated cilia led to increased amounts of tubulin and of other axonemal proteins and prevented the unambiguous identification of minor membrane proteins.

(d) IDC displayed a pattern similar to that of whole cilia except that all the membrane bands were decreased in intensity relative to the axonemal ones (Fig. 6).

(e) The electrophoretic pattern of purified tri-

chocysts was characterized to ascertain their contribution to the ciliary pattern. Trichocysts displayed a typical and easily identifiable pattern (Fig. 6), made up of a cluster of bands concentrated around mol wt 17,000. Eight to ten bands were visible in one-dimensional gels in this area in addition to two minor bands at mol wt 23,000 and 70,000. Two bands were particularly abundant in the 17,000 cluster and occurred in an area where there were few ciliary proteins. Trichocyst contamination of cilia was therefore easily detected on gels. Furthermore, trichocyst proteins were completely absent from the vesicle fraction: whenever

←I-ANTIGEN

◀

←mol wt
 42-44,000
←mol wt
 31,000

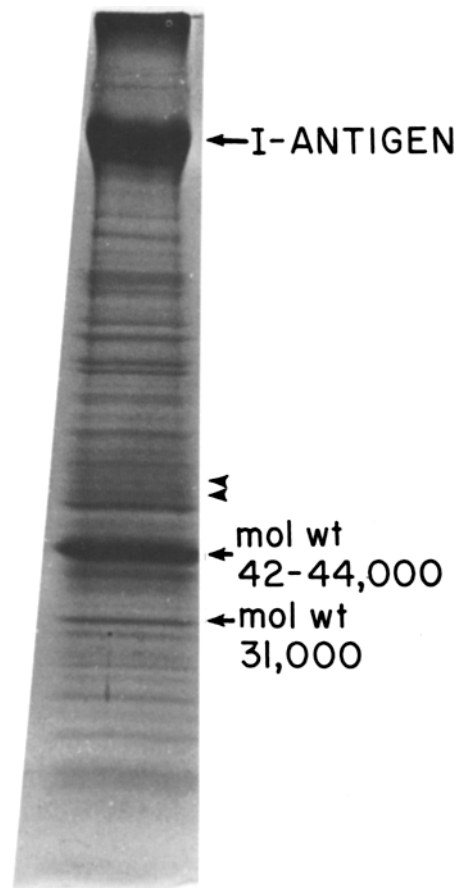FIGURE 7 SDS gel of ciliary membrane proteins. Highly purified ciliary membranes, obtained from a 10–66% continuous sucrose gradient were solubilized in 2% SDS, and 200 μg protein was electrophoresed. Arrows point to several major proteins, and arrowheads indicate the very low amount of tubulin in these membranes. The set of proteins at mol wt 42,000–44,000 is not well resolved in such heavily loaded gels.

trichocysts contaminated the initial ciliary preparation, they co-purified with axonemes in the step gradient.

In summary, the procedures used have enabled us to separate cilia into two pure and complementary fractions: the axonemes and membranes, made up of distinct polypeptides which, when added together, yielded a pattern similar to that of whole cilia. The demembranation by these procedures was incomplete and therefore yielded a third fraction, the IDC.

MEMBRANE PROTEINS RETAINED IN IDC: A comparison of the protein patterns of IDC and membrane vesicles (Fig. 6) showed that the major membrane proteins were present in the IDC, and appeared in the same relative ratio to each other as in the pure membrane fraction. However, it was important to ascertain whether there was any specific subset of membrane proteins retained in the incompletely demembranated cilia or whether the membranes retained were a random fraction of the total membrane. This was done in two ways: (a) demembranation of cilia was carried out in the presence of detergents (0.1% [wt/vol] Triton X-100 or 0.1% Sarkosyl); and (b) incompletely demembranated cilia, purified by the sucrose gradient procedure described, were retreated with detergents and/or Tris-EDTA.

The effects of Triton and Sarkosyl on these fractions were marked but different. As observed by phase-contrast microscopy, both detergents led to a disruption of the structure of the incompletely demembranated cilia, although Triton seemed more effective in this process. In the case of Triton-treated fractions, although the axonemes appeared intact, the membranes were barely visible, as though they had disintegrated. Conversely, while the Sarkosyl treatment resulted in intact membrane vesicles, there seemed to be some axonemal disintegration.

Separation of the detergent-treated fractions on sucrose density gradients led to results that were in agreement with our observations at the microscope; the band of incompletely demembranated cilia was markedly decreased or absent in the gradients, the Triton membrane band was smaller (in amount) than the Sarkosyl membrane band or the Tris-EDTA membrane band. Furthermore, the Triton membranes banded in the sucrose gradient at a higher (less dense) position than Tris-EDTA membranes, and their protein/lipid ratio was relatively lower (0.5 and 2.5 µg protein/nmol phosphate, respectively). Aggregation artifacts and

consequent cross-contamination of fractions also seemed to be a detergent-related problem.

We also compared the protein patterns of these fractions on SDS gels. As shown in Fig. 8, Sarkosyl membranes had a protein pattern very similar to that of Tris-EDTA membranes except that the Sarkosyl membranes contained slightly more tubulin. Supernatant$_3$ (Fig. 1) from Sarkosyl-treated, incompletely demembranated cilia had an SDS gel pattern nearly identical with that of supernate$_3$
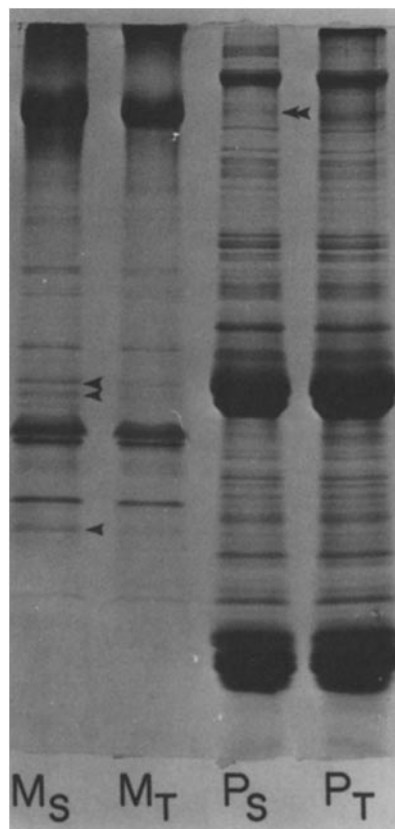
FIGURE 8 Comparison of membranes prepared with and without detergent. Cilia, demembranated using Tris-EDTA with or without Sarkosyl, were centrifuged on a 50% sucrose cushion as described in the text. SDS polyacrylamide gel of (M_S) ciliary membranes obtained in the presence of 0.1% Sarkosyl, 125 µg protein; (M_T) ciliary membranes obtained in Tris-EDTA, 125 µg; (P_S) pellet from Sarkosyl-treated cilia containing axonemes and trichocysts, 150 µg; (P_T) pellet obtained from Tris-EDTA-treated cilia containing axonemes, trichocysts, and some membrane, 150 µg. Single arrowhead points to proteins enriched in Sarkosyl-treated membranes. Double arrowheads indicate the total removal of I antigen in Sarkosyl-treated axonemes.

from the Tris-EDTA treatment, except for an increased amount of tubulins (data not shown). Triton membranes, while containing several membrane proteins, also contained greater amounts of tubulins and dyneins. The soluble fraction obtained after Triton treatment of cilia or incompletely demembranated cilia (supernatant$_3$ in Fig. 1) also contained tubulins and dyneins, pointing to the possibility of disruption of axonemal structure.

ONE-DIMENSIONAL IEF GELS: We have also used one-dimensional IEF in polyacrylamide slab gels to resolve the proteins of whole cilia and of each of the ciliary fractions recovered from sucrose gradients.

Isolated ciliary vesicles gave a pattern of protein bands that was clearly different from those of axonemes, incompletely demembranated cilia, or whole cilia (Fig. 9). Although the pH gradient in gels was not perfectly reproducible, the individual proteins in each ciliary fraction focused with characteristic and reproducible pIs. As expected from the SDS gels, the IEF gels of ciliary vesicles were dominated by a single protein, the immobilization antigen. This protein, which co-migrated with purified antigen kindly provided by Dr. H. Hansma, sometimes streaked rather than banding sharply (Fig. 9), but in about half of our gels, it focused in the region of pH 6.8–7.0.

To see the other proteins it was necessary to load the IEF gels heavily; typically, 200 μg of protein was applied, and between 25 and 30 distinct protein bands were visible by Coomassie staining. By comparing this pattern of vesicle proteins with that of axonemes and whole cilia, we were able to identify unambiguously at least 17 proteins unique to the ciliary membrane. In addition to the immobilization antigen, membranes contained: (a) two light bands (pI 7.0–7.1) frequently masked by the immobilization antigen but visible when it smeared; (b) a group of four bands (pI 6.5–7.0), two more intense than the others; (c) a light sharp band, with pI 6.0; (d) a pair of light bands (pI 5.6–5.8); (e) a group of four bands (pI 4.8–5.0), the least acidic of which was usually the least dense; (f) a group of three bands (pI 3.8–4.1), which in better-focused gels could be resolved into three minor and four major bands. This region of the gel frequently focuses less well than the rest, possibly because of SDS which accumulates in this region. In our experience, glycoproteins such as the immobilization antigen and the cluster near mol wt 42,000–44,000 focus less sharply than other
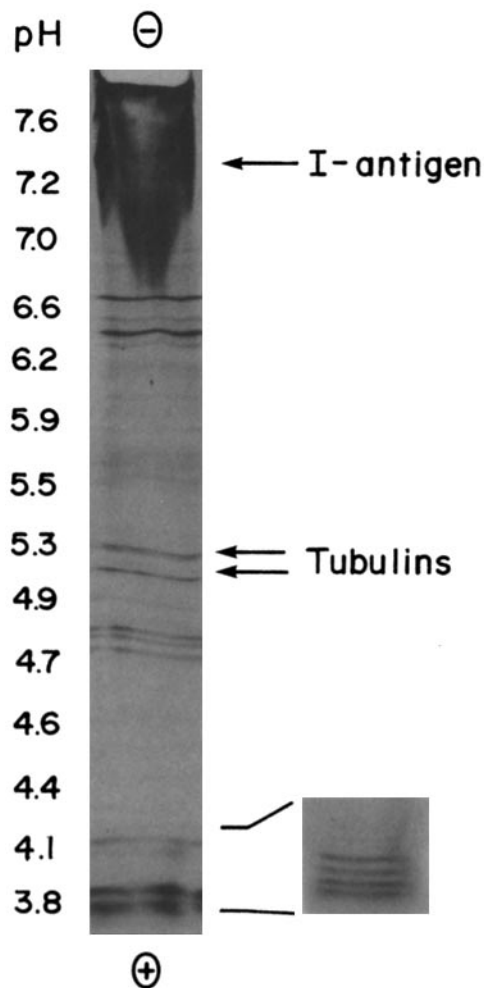

FIGURE 9 IEF gel of ciliary membrane vesicles. 200 μg protein of ciliary membrane vesicles was focused from cathode to anode. The pH gradient of the gel is plotted along the left side. Improved resolution of the acidic proteins was obtained by using less SDS to dissolve the sample (see inset).

ciliary proteins or tubulins, and thus appear to be less prominent in IEF gels than in SDS gels of the same sample. Also, glycoproteins such as the immobilization antigen appear in general to stain less well with Coomassie Blue than do nonglycosylated proteins.

TWO-DIMENSIONAL GELS: Coomassie Brilliant Blue staining of the two-dimensional gels of cilia revealed up to 250 polypeptides in the mol wt range of 9,000 to over 300,000 and the pI range of 4.5–8.5 (Fig. 10a). The most prominent spots were: (a) the two tubulin subunits (mol wt ~55,000; pI
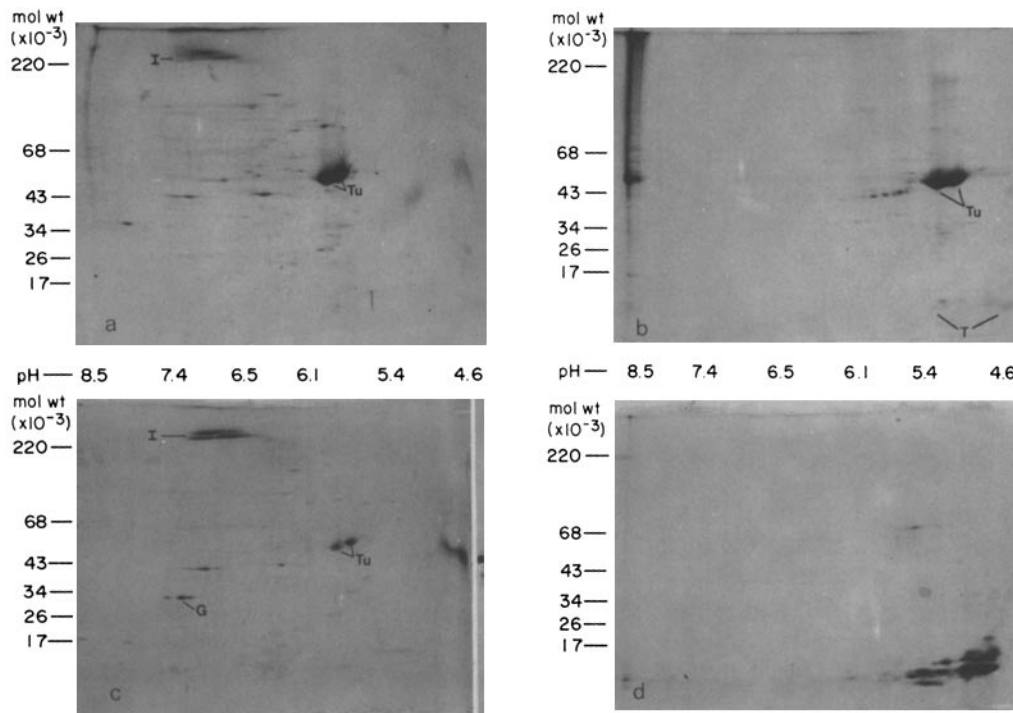
FIGURE 10 Two-dimensional gels. Gels were run and stained with Coomassie Blue as described in Materials and Methods. (a) Whole cilia. (b) Axonemes. The cluster of spots (T) in the extreme lower right section of the gel is caused by contaminating trichocysts. (c) Membranes. The inset shows improved resolution of the acidic arc (see Results). (d) Trichocysts. (Tu) Tubulin. (I) Immobilization antigen. (G) mol wt 31,000 protein.

5.4–5.5); (b) the immobilization antigen (mol wt ~250,000; pI 6.4–6.8); and (c) a large arched band (mol wt 34,000–46,000; pI ≤ 4.5).

The distortion of proteins into a large arc at the acidic end of the gel appeared to be an SDS artifact. If a relatively large volume of SDS was used to dissolve the sample, the SDS collected at the acidic end of the IEF gel and caused it to swell. This caused a distortion of the polypeptides focusing at that portion of the gel. If a relatively small amount of SDS was applied with the sample to the IEF gel, the distortion by SDS was reduced, and the large arc was resolved into six spots of similar molecular weight and pI (Fig. 10c).

At least 125 polypeptides were visible in gels of axonemes (Fig. 10b). Two-dimensional gels of cilia and axonemes were superimposable over large regions, but they revealed several striking differences between the two fractions. The axoneme fraction was relatively enriched for tubulin. Axonemes were also highly enriched for three major and eight minor polypeptides of ~50,000

mol wt and with pI's of 5.6–6.3. Several other polypeptides were enriched in the axonemal fractions, compared to the cilia fraction, and were missing from the membrane fraction. In contrast, the immobilization antigen and the cluster of proteins at pI ≤4.5, mol wt 34,000–46,000, were visible only in trace amounts. Several other polypeptides which appeared as major and minor spots on the gels of cilia were missing from the gels of axonemes. Spots corresponding to dyneins were not seen on two-dimensional gels; this is probably caused by lack of focusing in the first dimension.

Over 70 polypeptides were resolved on gels of the membrane vesicle fraction (Fig. 10c). The most prominent spots on the gel were: the immobilization antigen; the cluster of six spots of mol wt 34,000–46,000, pI ≤4.5; a spot at mol wt 31,000, pI 6.8 that varies in intensity with the stage of growth; a spot at mol wt 43,000, pI 5.9; a spot at mol wt 41,000, pI 6.3; and a set of three spots at mol wt 100,000, pI 5.8–5.9. The tubulin subunits also appeared as major spots in the gel, but in

greatly reduced amounts when compared to the relative intensity of the tubulin spots in the gels of cilia. (The possible reason for this observation has been discussed above.) The prominent cluster of spots at mol wt 50,000, pI 5.6–6.3, from the axoneme fraction was not visible in the gel of the membrane vesicle fraction.

Trichocysts (Fig. 10 d) consisted of a group of up to 40 polypeptides in the mol wt range of 11,000–19,000 and the pI range of ≤4.5–6.2. Minor spots were also visible at mol wt 68,000, pI 5.7 and 5.9; mol wt 61,000, pI 5.8 and 5.9; mol wt 41,000, pI 6.1; mol wt 45,000, pI 5.0; mol wt 31,000, pI 4.8; mol wt 26,000, pI 4.8 and 4.9; mol wt 23,000, pI 5.2; and mol wt 20,000, pI 5.1. Some of these minor spots may represent nonreduced multimers of the major trichocyst polypeptides.

Absence of Bacterial Contamination

Most of the experiments described in this paper were done on cells grown in bacterized Cerophyl medium. Light and electron microscope observation of washed *Paramecium* showed only very small numbers of bacteria, and several lines of evidence show that bacteria did not contribute to the protein patterns described above. Ciliary fractions (Fig. 1) from *Paramecium* cultured axenically (see Materials and Methods) gave protein patterns identical to the pattern obtained using bacterized cultures (cf. Figs. 6 and 12). When proteins of *Enterobacter aerogenes* and of cilia were compared on SDS gels, none of the major bacterial proteins was found in ciliary fractions. Cerophyl cultures treated with penicillin before harvesting and fractionation to remove most of the bacteria showed the same complement of ciliary proteins as untreated cultures (data not shown).

Absence of Proteolysis

The polypeptide patterns of the various fractions, obtained using gel electrophoresis, were very reproducible. Other lines of evidence show that proteolytic artifacts did not contribute to these patterns: (a) Protein patterns of fractions obtained in the presence of a proteolytic inhibitor such as phenylmethylsulfonyl fluoride or two sulfhydryl blockers (*N*-ethylmaleimide and iodoacetic acid) were identical to those obtained in the absence of the inhibitor. (b) Incubation of ciliary fractions with ^{125}I-labeled bovine serum albumin resulted in no degradation of the labeled substrate (data not shown). (c) Occasionally, cell lysis occurred at

deciliation, releasing cell proteases into the ciliary supernatant (supernatant$_1$, in Fig. 1). The polypeptide pattern obtained in such cases was totally different from the normal pattern and reflected nonspecific proteolysis of ciliary proteins. (d) The addition of exogenous proteases to cells or ciliary fractions resulted in very different protein patterns on SDS gels (data not shown).

Ciliary Proteins of Tetrahymena

Ciliary fractions of *Tetrahymena* were prepared as in Fig. 1, and the proteins in these fractions were analyzed by one-dimensional SDS gels. The major proteins of the ciliary membranes had mol wt of 64,000 and 20,000, and there were about 25 other minor proteins. The pattern of membrane proteins in *Tetrahymena* was quite different from that in *Paramecium*, but *Tetrahymena* vesicles, like those of *Paramecium*, contained only trace amounts of tubulin and high molecular weight proteins (300,000 daltons) (Fig. 13).

DISCUSSION

Many behavioral, electrophysiological, and ultrastructural studies of *Paramecium* have been done and a collection of mutants abnormal in their electrophysiological characteristics has been isolated (36, 45). We anticipate intensive biochemical and cell-biological investigation of the *Paramecium* membrane. The main objective of the present work is to provide, using current technology, a detailed analysis of the ciliary membrane proteins. Modifications were made in the methods of isolation of this membrane fraction, based mainly on previous work carried out with *Tetrahymena* (26), *Chlamydomons* (72), and *Paramecium* (30). The fractions obtained were subjected to microscope and improved electrophoretic analysis. We have now obtained a membrane vesicle preparation of high purity and integrity in moderately good yield and have established its electrophoretic pattern in one-dimensional SDS and IEF gels, as well as in two-dimensional gels. In all, ~70 proteins characteristic of the ciliary membrane were identified. During the course of this work the electrophoretic patterns of whole cilia, axonemes, and trichocysts were also established. Our evaluation of the methods used and the significance of the findings are given below.

Assessment of the Methods Used

PURITY OF THE MEMBRANE FRACTION:

One advantage of studying ciliary membranes is that cilia can be specifically and efficiently detached from the cell, providing a very useful preliminary fractionation, in which all intracellular membranes are eliminated. The disadvantage of the system, however, is that the cilia contain a very complex internal structure, the axoneme (the "9 + 2" microtubular framework of the cilium with all its accessory structures). The procedures to be used must therefore first provide a pure and abundant ciliary fraction, then allow the recovery of a membrane fraction uncontaminated with axonemal material.

Of the various deciliation procedures used, all provided ciliary fractions displaying very similar patterns on one-dimensional SDS gels, but the STEN Ca^{++}-shock procedure described was found to be the most reproducible, rapid and efficient, as well as the one least damaging to the cells. This appears to be caused by the omission of ethanol from Gibbons' (26) STEEP (sucrose-Tris-ethanol-EDTA-phosphate) procedure and the addition of a high concentration of sucrose for cell stabilization. Occasionally cell breakage occurred, but only with cells in late stationary phase or fragile mutant strains. In these instances it was readily detected by phase-contrast microscopy and the experiments were not pursued further, as breakage was invariably accompanied by proteolysis. The only contaminants of ciliary preparations are bacteria and trichocysts. Bacteria are largely eliminated by the successive cell washes. Four lines of evidence described in Results show that they do not contribute to the electrophoretic patterns. Contamination by trichocysts is substantially reduced by the cold shock procedure before deciliation. Some additional trichocyst discharge may, however, still occur at the time of deciliation. Furthermore, the cold shock procedure must sometimes be avoided when one is dealing with fragile strains. In these cases a substantial number of trichocysts are present in the ciliary pellet. However, the electrophoretic pattern of trichocysts (a cluster of proteins of mol wt ~17,000) is clearly distinct from that of cilia (Fig. 6), and trichocysts (together with bacteria) band at a position distinct from that of membranes in sucrose gradients (Fig. 1).

The point at which cilia break off from the cell body has been precisely determined and, in agreement with studies on Tetrahymena (57), was found to lie between the "plaques" and the "necklace" (Fig. 3 d-f), two arrays of organized intramembrane particles located at the ciliary base. That the

plaques are indeed recovered in the ciliary fraction is a point to be stressed as they correspond to a highly organized membrane region with connections to the axoneme (20) and may play an important role in ciliary motility or its regulation. The procedure we used to deciliate cells does not remove the oral cilia, which make up <10% of the total cilia (Fig. 2), and it is conceivable that the pattern of proteins in those cilia differs from that of the somatic cilia, which we have studied.

Demembranation of the ciliary fraction was effected by a number of methods. The one described in this paper (Fig. 1), inspired by Gibbons' "dialysis" method (26), involves a brief vortexing in a low ionic strength buffer. This method has several advantages: it is mild and rapid, and releases membranes in the form of vesicles which, when further separated on sucrose gradients, are very pure. Except for specific experiments discussed below, no further treatment either with detergents or with KI as a microtubule-dissolving agent was used. Similar methods were used with Chlamydomonas (72) and Ochromonas (15). The exact process by which the membranes are detached from the axonemes is not known. The disadvantage of the method is that demembranation is not complete; a high proportion of axonemes coil and remain enclosed in membranes. These IDC can be efficiently separated from the membrane vesicles in sucrose gradients (Figs. 1 and 4). There is therefore some loss of membrane material into this fraction. As discussed below, preliminary data suggest that it is not a specific membrane fraction that is lost with incompletely demembranated cilia.

The criteria for purity of the membrane vesicles are both microscopic and electrophoretic. Observation of the bands from sucrose gradients by phase and electron microscopy shows that the vesicle fraction contains very few visible axonemal structures (Fig. 5a). Because the density of axonemes, trichocysts, and bacteria is much higher than that of the membrane vesicles, they separate completely from membranes under strong centrifugal force. The only common contaminant of membrane preparations is a small and variable amount of membrane vesicles enclosing a small part of an axoneme. These are best eliminated from the membrane band by separation in continuous sucrose gradients. For routine purposes, however, this small contamination is tolerable, as discussed below. These microscope observations closely match the electrophoretic analysis of the gradient fractions. The systematic comparison of

whole cilia, axonemes, and membrane preparations by the three electrophoretic techniques used (Figs. 6, 7, 9, and 10) clearly shows that the membrane vesicles are composed of a subset of ciliary proteins that are specifically absent in axonemes. Furthermore, membrane vesicle preparations contain only small amounts of tubulins, although tubulins represent ~50% of total ciliary proteins and are extremely prominent in the axoneme fraction. Several other major bands present in axonemes are absent from membrane patterns. Several lines of evidence show that these proteins specifically removed from cilia indeed correspond to the membrane proteins. First, they are recovered in a membrane vesicle fraction in which 60–70% of the ciliary lipids are also recovered. Secondly, they contain an excellent internal marker, the immobilization antigen, which is known to be located at the cell surface (24, 55). This protein is totally absent from axonemes but it accounts for ~75% of the protein of membrane vesicles. Thirdly, it is in this fraction that the major iodinated proteins are recovered after radioiodination of external proteins with lactoperoxidase (R. Ramanathan and D. L. Nelson, manuscript in preparation). In summary, the clear segregation of the ciliary proteins into two distinct fractions, vesicles and axonemes, which show no overlap in protein patterns, provides very good evidence for the purity of the vesicle fraction.

Axonemal contamination is minimal, and contamination by cytoplasmic, bacterial, or trichocyst protein has been ruled out, but the other possible source of nonmembrane proteins in the membrane vesicles is the ciliary matrix. Although we have not studied this possibility systematically, the electrophoretic analysis of the various supernatants produced at each step of the procedure starting from deciliation (Fig. 1) suggests that membrane vesicles are not contaminated by soluble matrix proteins. The ciliary supernatant contains 1–2% of total cell protein (J. J. Rauh and D. L. Nelson, manuscript in preparation). It displays many bands which are distinct from those of the cilia, and has been shown to contain a very active Ca^{++}-ATPase (L. M. Riddle, J. J. Rauh, A. E. Levin, and D. L. Nelson, manuscript in preparation). In contrast, the supernatant obtained after demembranation contains very little protein or ATPase activity and its electrophoretic pattern is basically that of the membrane vesicles. It is possible, therefore, that most of the diffusible matrix proteins are lost at the time of deciliation.

IS THE VESICLE FRACTION REPRESENTATIVE OF THE WHOLE CILIARY MEMBRANE? Three questions can be raised concerning the extent to which the membrane protein patterns described reflect the actual in vivo composition of the ciliary membrane. (a) Are certain membrane proteins lost during membrane preparation? (b) Is there any artifactual modification of these proteins during extraction or analysis? (c) Is our vesicle preparation typical of the whole ciliary membrane? Several lines of evidence indicate that the fraction analyzed is intact and unaltered. First, as described in Results, there is good evidence ruling out proteolytic damage in the preparation. Secondly, the pattern of the main externally iodinated surface proteins is the same whether whole cells or isolated membrane vesicles are analyzed, indicating retention of these proteins throughout purification (R. Ramanathan and D. L. Nelson, manuscript in preparation). Finally, the supernatant after demembranation (supernatant₃, Fig. 1), the fraction that is most likely to contain any detached membrane components, contains little protein. The electrophoretic pattern of this fraction is identical to that of membranes, suggesting that it only contains small unpelleted membrane vesicles.

However, the procedure for ciliary membrane purification outlined in this paper involves some loss of membrane material into the IDC fraction. This loss decreases the final yield of membranes and also raises the question as to whether some selective loss is taking place, that is, whether there is a specific subset of membrane proteins that remain associated with the axonemes. The plaques, for instance, may be retained because of their links with the axoneme.

For several reasons we think it likely that the membranes remaining in the IDC fraction are similar to those of the purified membrane fraction. (a) Purified IDC when reextracted with Tris-EDTA yield membrane vesicles that give a gel pattern identical to that of the initial vesicle preparation (Fig. 8). (b) The main membrane bands (immobilization antigen, 42,000 mol wt cluster, 31,000 mol wt), that can be easily identified in IDC, appear to be in the same ratio with respect to each other as they are in purified membranes. (c) The efficiency of "demembranation" was sometimes lower than usual. However, the membranes recovered did not differ significantly from those of typical experiments. It is therefore likely that the membranes released from the cilia are a

random and representative sample. As described in Results, attempts at more extensive release of membranes by use of detergents can lead to apparently artifactual results, and will be discussed below. On the whole, then, the loss of membranes in the IDC fraction was considered acceptable in view of the purity and integrity of the membrane fraction obtained.

YIELD: The problem of yield is particularly serious with *Paramecium*. Paramecia do not grow to very high densities (when compared to *Tetrahymena*, for instance). As shown in Fig. 1, 0.5–1 ml of "packed cells" per liter are obtained in the medium used in this study, corresponding to 50–100 mg of protein. This yields 1–3 mg of total ciliary proteins, of which 30–40% represents membrane proteins (Fig. 4). These figures represent maximal yields, but in practice, the recovery at each step is less; for example, recoveries from the gradient were ~60%. The fact that the membrane gradient band represented 30–40% of the total protein recovered from the gradient (as determined by the method of Lowry et al. (41) in numerous experiments) was surprising because it was expected, on the basis of the ultrastructure of a cilium, that the great majority of proteins would be concentrated in the axoneme. The ciliary membrane shows scattered particles when observed by freeze-etching (except for the plaque area). Additional membrane is also present in the IDC, raising the actual proportion of membrane protein even more. In addition, it was invariably noted that higher amounts of membrane proteins had to be loaded on the gels to yield patterns of intensity similar to those of axonemes, suggesting that the actual protein concentration of the membrane sample might be overestimated. However, determination of protein concentration by an independent method (25), as well as measurement of the amount of radioactivity present in the various gradient bands after growth of the cells in ^{35}S medium, yielded similar values (data not shown). It remains unclear therefore whether the membranes truly represent 30–50% of the total ciliary proteins or whether some factors lead systematically to an overestimation of the protein concentration of this sample. Among these, two may be cited: preferential recovery of membrane vesicles in the gradient bands (as opposed to axonemes, for instance) and introduction of a bias in the three methods used to determine protein concentration because of the preponderance of one protein type in the sample (the immobilization antigen). For instance, the immobilization antigen is known to be very rich in cysteine, therefore probably distorting the ^{35}S-based measurement.

Cultures of ~5–10 liter must be grown to obtain ~1 mg of ciliary membrane proteins. These amounts are sufficient, however, for most analytical purposes using gel electrophoresis and Coomassie staining of protein bands. We are presently attempting to increase the sensitivity of detection by labeling the proteins with ^{35}S and autoradiographing the gels. For preparative purposes various attempts by us and others are being made to increase cell yields, especially by using synthetic medium.

Pattern of Membrane Proteins

The purest membrane preparations obtained by centrifugation in continuous sucrose density gradients contain only trace amounts of tubulin and therefore their electrophoretic pattern reflects the degree of membrane complexity. Such preparations have yielded up to 52 separate bands in one-dimensional SDS gels, two of which are faint tubulin bands; two others are faint dynein bands. As there probably are some overlapping bands, these numbers must be considered as a lower estimate. In fact, two-dimensional gels suggest that up to 70 components may be present in these membranes. This represents a degree of complexity previously undetected in ciliary and flagellar membranes, and is comparable to that of the membranes of *Escherichia coli* and of cultured mammalian cells (3). Of the 70 components, 25–30 characteristic ones are routinely identified in one-dimensional SDS gels (Fig. 7) and ~20 in one-dimensional IEF gels (Fig. 9). These polypeptides are present in very disproportionate amounts. As already shown by Hansma and Kung (30), ~75% of the membrane protein is represented by a band of mol wt 250,000, which is related to the surface antigen of *Paramecium*. Two bands of antigen with slightly different migration properties in SDS and IEF gels were often observed, and this appeared to be related to the temperature and conditions of cell growth, as already noted by Hansma and Kung. The antigen was often smeared in IEF slab as well as tube gels, covering a broad pH region centered at pH 7. In some cases, it yielded a sharp band at the same pH. This is quite different from the acid isoelectric points (3.9–4.2) determined by Steers (63) for purified antigen molecules of different serotypes. Purified antigen isolated according to the method used by Steers

(54) focused in the same area as the one present in membrane preparations (data not shown). The reason for this discrepancy is not known. Most of the proteins in our IEF gels appear to have reached equilibrium. The antigen, which is a very large molecule and has to traverse the whole gel to reach the acidic region, might still not be focused under the conditions used. In work to be reported elsewhere we show that this abundant high molecular weight protein constitutes the "fuzz" that covers the whole surface of Paramecium, including the cilia. A major protein of high molecular weight was also found in the flagellar membranes of Chlamydomonas (8, 58, 72) and of Euglena (12).

Next in abundance to the antigen is a cluster of four proteins of mol wt ~40,000. These proteins have the interesting property of being similar not only in molecular weight but also in isoelectric point, yielding a set of closely spaced bands at the acidic end of IEF gels. They may therefore belong to a set of related proteins. Because of the similarity in molecular weight between this protein cluster and actin and the frequent association between actin and cell membranes, it is possible that one of the bands in this cluster may be actin. A small amount of actin has recently been reported to be present in Chlamydomonas flagellar axonemes (51). However, when co-electrophoresed with rabbit actin in one-dimensional SDS gels, the major proteins of the cluster band below it, and furthermore the isoelectric point of these proteins is much more acidic than that of actins from various sources. No major band co-electrophoresing with rabbit muscle myosin heavy chain was found in our membrane preparation.

Another protein band of interest is the one of mol wt 31,000. This is the only band showing reproducible variation in amount, depending on the growth stage at which the cells are harvested: it is barely detectable in full logarithmic phase of growth, becomes prominent when the cells are in early stationary phase, then decreases in "starved" cells (Fig. 11). These variations show a strong parallel to those of cellular mating reactivity, which is known to be totally absent in exponentially growing cells ("well fed"), to be induced by mild starvation, then to decrease and eventually to disappear when starvation is prolonged. Mating of Paramecium cells of complementary mating type is also known to involve interactions between the cilia of the two mates (43), and in fact ciliary membrane vesicles capable of agglutinating cells of the opposite mating type have been recently
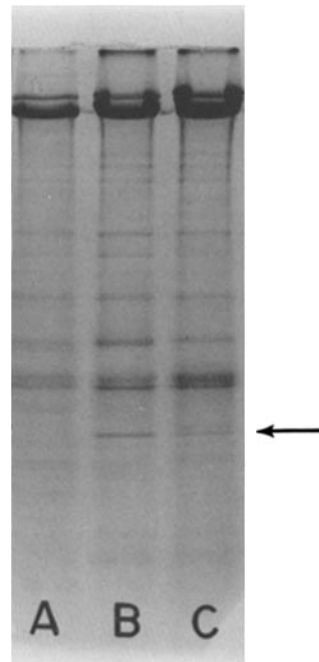

FIGURE 11   Growth stage variation of a membrane protein: SDS polyacrylamide gel showing the pattern of ciliary membrane proteins prepared from cells in (A) mid-logarithmic phase; (B) early stationary phase; and (C) late stationary phase. Arrow indicates the variation in protein of mol wt 31,000. Approximately 100 μg protein was loaded in each lane.

obtained in Paramecium caudatum, demonstrating that the mating substances are located on the ciliary membranes (35). It may therefore be that this protein is in some way involved in the mating reaction, although the possibility of completely different roles, related to the modifications occurring in cell physiology during these transitory periods, cannot be excluded. The available mutants affecting mating reactivity (see reference 60) may enable us to test the presumptive functions of this protein.

A final point requiring discussion is the question of membrane-associated tubulin. Two reports, one by Stephens (66) using scallop gill cilia, and the other by Dentler (17) using Tetrahymena cilia, conclude that tubulin constitutes the major ciliary membrane protein (up to 50% of total membrane protein), in addition to dynein in the case of Tetrahymena. In Paramecium, in over 100 independent membrane preparations carried out according to the method described in this paper, we have only observed substantial amounts of tubulin
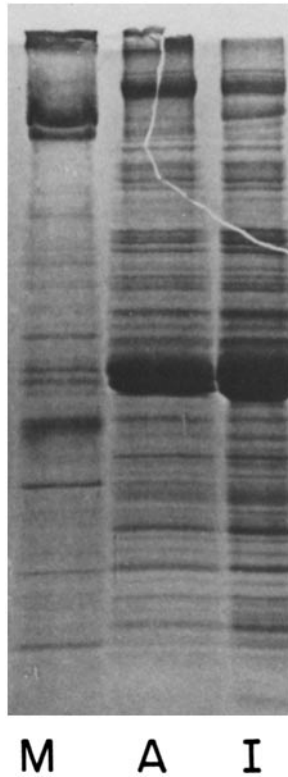
M A I

FIGURE 12   One-dimensional SDS gel of ciliary fractions from axenically grown cells. (*M*) ciliary membranes, 150 μg protein; (*A*) axonemes, 125 μg; (*I*) IDC, 125 μg.

(i.e., >5 or 10% of total membrane proteins) when the membrane vesicles were significantly contaminated with axonemal structures, as seen by phase-contrast microscopy. Furthermore, as pointed out above, in membrane vesicles purified on continuous gradients of sucrose, tubulin represents <1% of the protein.

Because of this major discrepancy, we prepared ciliary membranes of *Tetrahymena pyriformis*, strain NT-1, with the methods described in this paper, and we also prepared *Paramecium* ciliary membranes in the presence of 0.1% Triton (a detergent used both by Stephens and, in some experiments, by Dentler). The *Tetrahymena* vesicles have only trace amounts of tubulin and no dynein (Fig. 13), whereas *Paramecium* "Triton vesicles," although somewhat enriched in membrane proteins, display very heavy tubulin bands (data not shown). The divergent results therefore appear to be caused not by species differences but by differences in the methods used.

Several lines of evidence argue in favor of the idea that our methods provide a representative profile of the ciliary membrane proteins. As discussed above, there is good evidence that we are indeed dealing with a pure membrane fraction. Were tubulin an abundant membrane component, as suggested by Stephens and Dentler, it might be absent from our membrane preparation if we were dealing with a nonrepresentative fraction of the ciliary membrane. However, two findings make
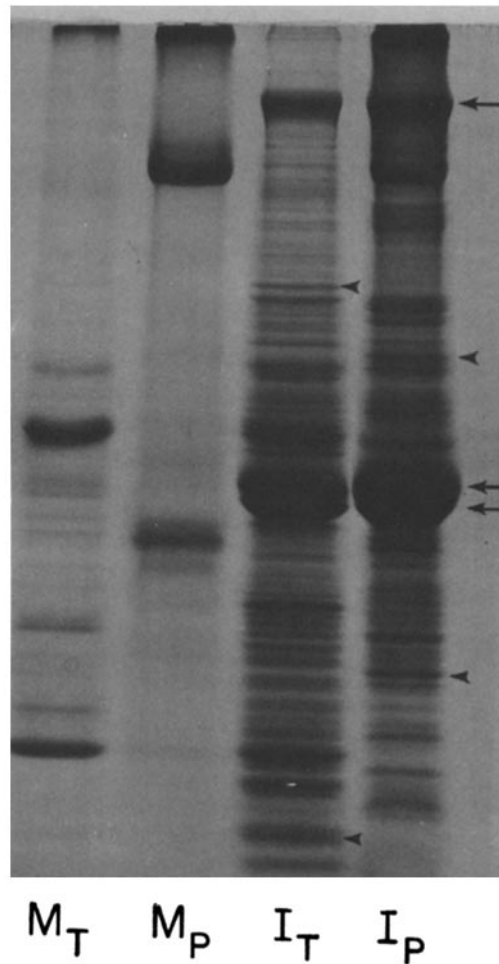

M$_T$ M$_P$ I$_T$ I$_P$

FIGURE 13   Comparison of *Tetrahymena* and *Paramecium* ciliary fractions. SDS polyacrylamide gel of (*M$_T$*) ciliary membranes from *Tetrahymena*, 100 μg protein; (*M$_P$*) ciliary membranes from *Paramecium*, 100 μg; (*I$_T$*) IDC from *Tetrahymena*, 125 μg; (*I$_P$*) IDC from *Paramecium*, 125 μg. The similarity in the tubulins and dyneins of the two species is pointed out (arrows), as are also a few prominent differences in the patterns of the IDC (arrowheads).

this seem unlikely: (a) the membrane fraction recovered accounts for 60–70% of total membrane phospholipid and (b) the membrane associated with IDC, when reextracted with Tris-EDTA, displays a pattern similar to that of the initial preparation. We think that the origin of the discrepancy lies in the way demembranation is achieved.

As described in Results, the use of Triton to solubilize the membrane apparently leads to artifactual results; although axonemes appear well preserved, membrane vesicles can hardly be seen, as if the membranes were disrupted. Similar observations were made by other authors (66, 72). In fact, the fraction analyzed by Stephens was Triton-solubilized material (after pelleting of axonemes) which he equated with the membrane constituents. The amount of membrane removed after Triton extraction is also lower, and the Triton vesicles have a lower buoyant density. Such Triton vesicles might randomly entrap solubilized ciliary proteins, the most abundant of which is tubulin, in addition to membrane proteins, yielding the observed pattern. These vesicles would appear to be very pure in the electron microscope.

Observations in several other systems support our interpretation. Ciliary membrane vesicles of *Paramecium caudatum* prepared by a method very different from ours, not involving detergents, display a similar complexity and contain relatively small amounts of tubulin (35). Little tubulin was also found in *Chlamydomonas* flagellar membranes extracted in the absence of detergents (8, 58, 72). In contrast, in two other studies on *Tetrahymena* ciliary membranes involving Triton solubilization of the membrane, a heavy band in the molecular weight range of tubulin was reported (14, 67).

In summary, because it is possible, in the absence of detergents, to obtain an abundant ciliary membrane fraction containing very little tubulin, this protein cannot be considered to be a major ciliary membrane constituent in *Paramecium*. It is not known, however, whether the small amount of tubulin still found in the membrane vesicles of *Paramecium* represents a very low level of contamination by axonemal or matrix proteins or whether this is a true membrane component. Such a low level of contamination would not be surprising in view of the considerable amount of tubulin present in whole cilia. However, the recent report in *Chlamydomonas* (1) of a small amount of tubulin displaying apparently specific association with the flagellar membrane leaves open the possibility of a small amount of intrinsic membrane tubulin in *Paramecium*.

Pattern of Axonemal Proteins

Axonemes were not the fraction of main interest in this analysis. However, they were characterized in detail during the course of the systematic comparison of the various gradient fractions. It should be noted that the axonemal fraction analyzed may have suffered some damage in the course of purification which involves a low ionic strength treatment in the presence of EDTA, a condition known to cause the beginning of axoneme breakdown (39, 65). Electron microscope observations indeed show that many axonemes lacked at least one of the two central tubules, and additional breakdown was often visible. In agreement with other authors, we found that solubilization of membrane proteins with 0.1% Triton yielded well-preserved axonemes (26, 66, 72), whereas 0.1% Sarkosyl, an ionic detergent, which releases the membrane in the form of vesicles, usually caused extensive axoneme solubilization (39, 72).

In spite of the possible loss of a few components from the axonemal fraction, the pattern of the remaining structure is highly complex, being made up of ~125 protein spots in high resolution two-dimensional gels. (Fig. 10b). A similar complexity had already been clearly shown in one other study involving *Chlamydomonas* axonemes using similar electrophoretic methods (32, 50). Comparison of the one-dimensional SDS gel pattern of *Paramecium* axonemes with those of the ciliary or flagellar axonemes available in the literature indicates only two clear areas of similarities: those of tubulins and dyneins. This is best illustrated by co-electrophoresing on the same slab gel the samples to be compared, as we did for *Paramecium* and *Tetrahymena* (Fig. 13). Between these two related protozoa, the tubulin and dynein areas appear to be exactly superposable, whereas considerable variation occurs between the other axonemal bands. That tubulins are highly conserved proteins is well established (42), as is the fact that dyneins of several species are proteins of very high molecular weight (27, 38, 52, 70). Extensive divergence between the other axonemal bands of *Paramecium* and *Tetrahymena* on SDS gels is more surprising, considering the similarities in the ultrastructure and function of the cilia in these two organisms. Similar situations have, however, already been found in the comparison of the structural proteins from the pellicles of various *Tetrahymena* species

(69), as well as for soluble enzymes (11). Together with additional evidence, this has led to Nanney's suggestion that "the molecular distances between the species of *Tetrahymena* are very large, in contrast to the morphologic distance among the same species" (44) and therefore that considerable fluctuation in molecular structure has occurred within a functionally constrained architectural framework. This important concept can obviously be extended to account for the observed divergences between the axonemal proteins of *Paramecium* and *Tetrahymena*.

Pattern of Trichocyst Proteins

Trichocysts have been extensively analyzed both cytologically (see reference 31) and genetically (53, 56) and are presently being used as model systems to analyze exocytosis and mechanisms of organelle positioning within cells (5, 7). Therefore, the electrophoretic pattern of purified trichocysts, which was established mainly to ascertain the extent to which they may contaminate the ciliary fractions under analysis, can now be exploited for the biochemical study of the organelle per se. A limitation in our analysis must, however, be stressed: the fraction that has been characterized corresponds to extruded trichocysts, that is, organelles containing the trichocyst body and tip but devoid of the membrane that normally surrounds the trichocyst in its intracellular form and that presumably plays an important role in the positioning of the organelle beneath the cell membrane. In a previous study (64), trichocysts were shown to yield two bands of mol wt 17,000 and 36,000 on SDS gels and only a single unit at 17,000 after reduction by 2-mercaptoethanol. The present work confirms and extends these results by showing that trichocysts, upon reduction and electrophoresis in our one-dimensional SDS gel system, yield a set of closely spaced bands ~17,000 mol wt (Fig. 6). The demonstration of much higher complexity in that area than previously observed is clearly caused by improvements in gel resolution. A few additional minor bands of higher molecular weight are also consistently observed. Complexity of trichocyst proteins is more strikingly illustrated in IEF slab gels and in two-dimensional gels (Fig. 10). Because the isoelectric points of the proteins in the 17,000 cluster are very distinct, they spread over 2 pH units in IEF slab gels and yield up to 40 spots in two-dimensional gels. This complexity is not too surprising in view of the elaborate ultrastructure of the trichocyst tip with its set of distinct

overlapping sheaths (6). The electrophoretic pattern is dominated by a few major bands of similar isoelectric points. These may correspond to the proteins making up the trichocyst body.

In conclusion, this paper has set forth the methods for obtaining subfractions of *Paramecium* cilia, particularly the membranes, and has provided a detailed electrophoretic characterization of these fractions. These methods are now being used as the basis for a variety of projects in progress. The present work emphasizes optimal resolution of membrane proteins because of the likely possibility that the ion channels responsible for cell excitability, in which we are ultimately interested, are quantitatively minor constituents of the membrane (22). Systematic comparison of membrane proteins from wild-type and behavioral mutants has now indicated that minor differences can be demonstrated by the techniques used. Other studies of membrane subfractionation and of the topology and function of the membrane proteins are also underway.

REFERENCES

1. ADAIR, W. S., and U. W. GOODENOUGH. 1978. Identification of a membrane tubulin in *Chlamydomonas* flagella. *J. Cell Biol.* 78(2, Pt. 2): 54 a (Abstr.).
2. AMES, G. F.-L. 1974. Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs: membrane, soluble and periplasmic fractions. *J. Biol. Chem.* 249:634–644.
3. AMES, G. F.-L., and K. NIKAIDO. 1976. Two-dimensional gel electrophoresis of membrane proteins. *Biochemistry.* 15:616–623.
4. ANDREWS, D., and D. L. NELSON. 1979. Biochemical studies of the

excitable membrane of *Paramecium tetraurelia*. II. Phospholipids of ciliary and other membranes. *Biochim. Biophys. Acta.* **550**:174-187.

5. AUFDERHEIDE, K. J. 1978. The effective sites of some mutations affecting exocytosis in *Paramecium tetraurelia. Mol. Gen. Genet.* **165**:199-205.

6. BANNISTER, L. H. 1972. The structure of trichocysts in *Paramecium caudatum. J. Cell Sci.* **11**:899-929.

7. BEISSON, J., M. LEFORT-TRAN, M. POUPHILE, M. ROSSIGNOL, and B. SATIR. 1976. Genetic analysis of membrane differentiation in *Paramecium*: freeze-fracture study of the trichocyst cycle in wild-type and mutant strains. *J. Cell Biol.* **69**:126-143.

8. BERGMAN, K., U. W. GOODENOUGH, D. A. GOODENOUGH, J. JAWITZ, and H. MARTIN. 1975. Gametic differentiation in *Chlamydomonas reinhardii*. II. Flagellar membranes and the agglutination reaction. *J. Cell Biol.* **67**:606-622.

9. BERKOWITZ, S. A. 1977. Separation and characterization of microtubule proteins from calf brain. *Biochemistry.* **16**:5610-5617.

10. BLUM, J. J. 1971. Existence of a breaking point in cilia and flagella. *J. Theor. Biol.* **33**:257-263.

11. BORDEN, D., D. L. NANNEY, and G. S. WHITT. 1977. Isozymic analysis of the evolutionary relationships of *Tetrahymena* syngens. *Evolution.* **31**:91-102.

12. BOUCK, G. A., A. ROGALSKI, and A. VALAITIS. 1978. Surface organization and composition of *Euglena*: II. Flagellar mastigonemes. *J. Cell Biol.* **77**:805-826.

13. BROWNING, J. L., and D. L. NELSON. 1976. Biochemical studies of the excitable membrane of *Paramecium aurelia*. I. $^{45}Ca^{2+}$ fluxes across resting and excited membrane. *Biochim. Biophys. Acta.* **448**:338-351.

14. BUTZEL, H. M., and A. DECAPRIO. 1978. Ciliary membrane proteins of *Tetrahymena thermophila. J. Protozool.* **25**:267-269.

15. CHEN, L. L., and T. H. HAINES. 1976. The flagellar membrane of *Ochromonas danica. J. Biol. Chem.* **251**:1828-1834.

16. CHEN, P. S., JR., T. Y. TORIBARA, and H. WARNER. 1956. Microdetermination of phosphorus. *Anal. Chem.* **28**:1756-8.

17. DENTLER, W. L. 1978. Isolation and characterization of *Tetrahymena* ciliary membranes. *J. Cell Biol.* **79**(2, Pt. 2):284*a* (Abstr.).

18. DRYL, S. 1959. Effect of adaptation to environment on chemotaxis of *Paramecium caudatum. Acta. Biol. Exp.* (Warsaw). **19**:83-93.

19. DUNLAP, K. 1977. Localization of calcium channels in *Paramecium caudatum. J. Physiol.* **271**:119-133.

20. DUTE, R., and C. KUNG. 1978. Ultrastructure of the proximal region of somatic cilia in *Paramecium tetraurelia. J. Cell Biol.* **78**:451-464.

21. ECKERT, R. 1972. Bioelectric control of cilia. *Science* (*Wash. D. C.*). **176**:473-481.

22. ECKERT, R., and P. BREHM. 1979. Ionic mechanisms of excitation in *Paramecium. Annu. Rev. Biophys. Bioeng.* **8**:353-383.

23. EFSTRATIADES, A., and F. C. KAFATOS. 1970. The chorion of insects: techniques and perspectives. *Methods Mol. Biol.* **8**:1-124.

24. FINGER, I. 1974. Surface antigens of *Paramecium aurelia*. *In Paramecium*—A Current Survey. W. J. Van Wagtendonk, editor. Elsevier Scientific Publishing Co., New York. 131-164.

25. FLORES, R. 1978. A rapid and reproducible assay for quantitative estimation of proteins using Bromophenol blue. *Anal. Biochem.* **88**:605-611.

26. GIBBONS, I. R. 1965. Chemical dissection of cilia. *Arch. Biol.* **76**:317-352.

27. GIBBONS, I. R., E. FRONK, B. H. GIBBONS, and K. OGAWA. 1976. Multiple forms of dynein in sea urchin sperm flagella. *In* Cell Motility. R. Goldman, T. Pollard, and J. Rosenbaum, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 915-932.

28. HANSMA, H. G. 1974. Biochemical studies on the behavioral mutants of *Paramecium aurelia*: ion fluxes and ciliary membrane proteins. Ph.D. disssertation. University of California, Santa Barbara, Calif.

29. HANSMA, H. G. 1979. Sodium uptake and membrane excitation in *Paramecium. J. Cell Biol.* **81**:374-381.

30. HANSMA, H. G., and C. KUNG. 1975. Studies of the cell surface of *Paramecium*. Ciliary membrane proteins and immobilization antigens. *Biochem. J.* **152**:523-528.

31. HAUSMANN, K. 1978. Extrusive organelles in protists. *Int. Rev. Cytol.* **52**:197-275.

32. HUANG, B., G. PIPERNO, and D. J. L. LUCK. 1979. Paralyzed flagella mutants of *Chlamydomonas reinhardii* defective for axonemal doublet microtubule arms. *J. Biol. Chem.* **254**:3091-3099.

33. INOUYE, M. 1971. Internal standards for molecular weight determinations of proteins by polyacrylamide gel electrophoresis. Applications to envelope proteins of *Escherichia coli. J. Biol. Chem.* **246**:4834-4838.

34. KANESHIRO, E. S., L. S. BEISCHEL, S. J. MERKEL, and D. E. RHOADS. 1979. The fatty acid composition of *Paramecium aurelia* cells and cilia: changes with culture age. *J. Protozool.* **26**:147-158.

35. KITAMURA, A., and K. HIWATASHI. 1976. Mating-reactive membrane vesicles from cilia of *Paramecium caudatum. J. Cell Biol.* **69**:736-740.

36. KUNG, C., S.-Y. CHANG, Y. SATOW, J. VAN HOUTEN, and H. HANSMA. 1975. Genetic dissection of behavior in *Paramecium. Science* (*Wash. D.*

C.) **188**:898-904.

37. LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (*Lond.*). **227**:680-685.

38. LINCK, R. W. 1973. Chemical and structural differences between cilia and flagella from the lamelli-branch mollusc, *Aequipecten irradians. J. Cell Sci.* **12**:951-981.

39. LINCK, R. W. 1976. Flagellar doublet microtubules: fractionation of minor components and α-tubulin from specific regions of the A-tubule. *J. Cell Sci.* **20**:405-439.

40. LING, K. Y., and C. KUNG. 1979. Ba^{++} influx measures the duration of membrane excitation in *Paramecium. J. Exp. Biol.* **63**. In press.

41. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.

42. LUDUENA, R. F., and D. O. WOODWARD. 1973. Isolation and partial characterization of α- and β-tubulin from outer doublets of sea-urchin sperm and microtubules of chick-embryo brain. *Proc. Natl. Acad. Sci. U. S. A.* **70**:3594-3598.

43. MIYAKE, A. 1974. Cell interaction in conjugation of ciliates. *Curr. Top. Microbiol. Immunol.* **64**:49-77.

44. NANNEY, D. L. 1977. Molecules and morphologies: the perpetuation of pattern in ciliated protozoa. *J. Protozool.* **24**:27-35.

45. NELSON, D. L., and C. KUNG. 1978. Behavior of *Paramecium*: chemical, physiological and genetic studies. *In* Taxis and Behavior. G. L. Hazelbauer, editor. Chapman and Hall, London. 77-100.

46. O'FARRELL, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**:4007-4021.

47. OGURA, A., and K. TAKAHASHI. 1976. Artificial deciliation causes loss of calcium-dependent responses in *Paramecium. Nature* (*Lond.*). **264**:170-172.

48. OMOTO, C. K., and C. KUNG. 1979. The pair of central tubules rotates during ciliary beat in *Paramecium. Nature* (*Lond.*). **279**:532-534.

49. ORNSTEIN, L. 1964. Disc electrophoresis. I. Background and theory. *Ann. N. Y. Acad. Sci.* **121**:321-349.

50. PIPERNO, G., B. HUANG, and D. J. L. LUCK. 1977. Two-dimensional analysis of flagellar proteins from wild-type and paralyzed mutants of *Chlamydomonas reinhardii. Proc. Natl. Acad. Sci. U. S. A.* **74**:1600-1604.

51. PIPERNO, G., and D. J. L. LUCK. 1979a. An actin-like protein is a component of axonemes from *Chlamydomonas* flagella. *J. Biol. Chem.* **254**:2187-2190.

52. PIPERNO, G., and D. J. L. LUCK. 1979b. Axonemal adenosine triphosphatases from flagella of *Chlamydomonas reinhardii*. Purification of two dyneins. *J. Biol. Chem.* **254**:3084-3090.

53. POLLACK, S. 1974. Mutations affecting the trichocysts in *Paramecium aurelia.* I. Morphology and description of the mutants. *J. Protozool.* **21**:352-362.

54. PREER, JR., J. R. 1959. Studies on the immobilization antigens of *Paramecium.* III. Properties. *J. Immunol.* **83**:385-391.

55. PREER, JR., J. R. 1969. Genetics of protozoa. *In* Research in Protozoology. T. Chen, editor. Pergamon Press, Oxford. **3**:129-278.

56. RUIZ, F., A. ADOUTTE, M. ROSSIGNOL, and J. BEISSON. 1976. Genetic analysis of morphogenetic processes in *Paramecium.* I. A mutant affecting trichocyst formation and nuclear division. *Genet. Res.* **27**:109-122.

57. SATIR, B., W. S. SALE, and P. SATIR. 1976. Membrane renewal after dibucaine deciliation of *Tetrahymena*. Freeze-fracture technique, cilia, membrane structure. *Exp. Cell Res.* **97**:83-91.

58. SNELL, W. J. 1976. Mating in *Chlamydomonas*: a system for the study of specific cell adhesion. I. Ultrastructural and electrophoretic analysis of flagellar surface components involved in adhesion. *J. Cell Biol.* **68**:48-69.

59. SONNEBORN, T. M. 1970. Methods in *Paramecium* research. *In* Methods in Cell Physiology. D. M. Prescott, editor. Academic Press, Inc., New York. **4**:241-339.

60. SONNEBORN, T. M. 1974. *Paramecium aurelia. In* Handbook of Genetics. R. C. King, editor. Plenum Press, New York. 469-594.

61. SONNEBORN, T. M. 1975. The *Paramecium aurelia* complex of fourteen sibling species. *Trans. Am. Microsc. Soc.* **94**:155-178.

62. SPURR, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* **26**:31-43.

63. STEERS, E. 1961. Electrophoretic analysis of immobilization antigens of *Paramecium aurelia. Science* (*Wash. D. C.*). **1**:2010-2011.

64. STEERS, E., J. BEISSON, and V. T. MARCHESI. 1969. A structural protein extracted from the trichocyst of *Paramecium aurelia. Exp. Cell Res.* **57**:392-396.

65. STEPHENS, R. E. 1970. Thermal fractionation of outer fibre doublet microtubule into A and B subfiber components: A and B tubulin. *J. Mol. Biol.* **47**:353-363.

66. STEPHENS, R. E. 1977. Major membrane protein differences in cilia and flagella: evidence for a membrane-associated tubulin. *Biochemistry.* **16**:

2047–2058.

67. Subbaiah, P. V., and G. A. Thompson, Jr. 1974. Studies of membrane formation in *Tetrahymena pyriformis.* The biosynthesis of proteins and their assembly into membranes of growing cells. *J. Biol. Chem.* **249:** 1302–1310.

68. van Wagtendonk, W. J. 1974. Nutrition of *Paramecium.* In *Paramecium—A* Current Survey. W. J. Van Wagtendonk, editor. Elsevier Scientific Publishing Co., New York. 339–376.

69. Vaudaux, P. E., N. E. Williams, J. Frankel, and C. Vaudaux. 1977. Inter-strain variability of structural proteins in *Tetrahymena. J. Proto-*

zool. **24:**453–458.

70. Warner, F. D., D. R. Mitchell, and C. R. Perkins. 1977. Structural conformation of the ciliary ATPase dynein. *J. Mol. Biol.* **114:**367–384.

71. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determination by dodecyl sulfate polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244:**4406–4412.

72. Witman, G. B., K. Carlson, J. Berliner, and J. L. Rosenbaum. 1972. *Chlamydomonas* flagella. I. Isolation and electrophoretic analysis of microtubules, matrix, membranes and mastigonemes. *J. Cell Biol.* **54:** 507–539.