MICROTUBULE-MEMBRANE INTERACTIONS IN CILIA

II. Photochemical Cross-linking of Bridge Structures and the Identification of a Membrane-associated Dynein-like ATPase

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ABSTRACT

Photochemical cross-linking of both *Tetrahymena* and *Aequipecten* ciliary membrane proteins with the lipophilic reagent 4,4'-dithiobisphenylazide links together a high molecular weight dynein-like ATPase, membrane tubulin, and at least two other proteins. Electron microscopy of detergent-extracted cilia reveals that the cross-linked complex remains attached to the outer-doublet microtubules by a microtubule-membrane bridge. Cleavage of the reagent's disulfide bond releases the bridge-membrane complex and the dynein-like membrane-associated ATPase. Electron microscopy was used to ensure that the dynein-like protein did not result from the solubilization of the dynein arms attached to the outer-doublet microtubules. The dynein-like protein has been isolated using sucrose gradients and is similar to axonemal dynein with respect to its sedimentation characteristics, nucleotide specificity, and divalent cation requirements. Photochemical cross-linking of ciliary membrane proteins in vivo results initially in the modification of ciliary beat and, eventually, in the cessation of ciliary movement. These results suggest that a dynein-like ATPase comprises the bridge which links the ciliary membrane to the outer-doublet microtubules and that this bridge is involved in the modulation of normal ciliary movement.

KEY WORDS cilia · flagella · microtubules · dynein · tubulin · cross-linking

Although a great deal is known about the structure, function, and biochemistry of the 9 + 2 axoneme of cilia and flagella, little attention has been paid to the membrane that envelops these organelles. Since isolated and demembranated ciliary and flagellar axonemes can be reactivated to beat with apparently normal waveform, it has been thought that the membranes serve primarily as either regulators or barriers to ion flow (21, 40).

Morphological studies of the distal tips of cilia and flagella have, however, revealed the presence of the central microtubule cap, which links the central-pair microtubules to the membrane (11, 13, 47) and distal filaments, which link the tips of the outer-doublet microtubules to the membrane (11). Moreover, the lateral aspects of outer-doublet microtubules also appear to be associated with ciliary and flagellar membranes by bridges (1, 4, 7, 46, 49), and cytochemical studies have suggested that an ATPase activity is associated with the

bridges (10, 32, 39). Intramembrane particles that may coincide with the bridges have also been reported (49).

Although the bridges have been observed, their structure is not well understood. Short stretches of periodic bridges can, occasionally, be observed in longitudinal section of cilia but, more frequently, the bridges are not observed and the membrane appears somewhat wavy, with the appearance of a billowing sleeve around the axoneme (9, 49). The full extent of the outer-doublet microtubule-membrane association may, however, be more extensive than the present morphological data indicate, since the amount of membrane billowing can be reduced by varying fixation conditions. A concomitant increase in the number of bridges, however, has not been observed.

The primary ATPase in cilia and flagella is dynein, a high molecular weight protein which comprises the paired arms attached to the A-tubule of each outer doublet and which is responsible for force generation between adjacent outer-doublet microtubules. Since radial spokes are thought to interact cyclically with the central-pair microtubules during ciliary and flagellar movement (56) and since cytochemical studies have suggested the presence of an ATPase in the region of the spoke heads and the central sheath (3, 8, 10, 39), it is possible that another ATPase may exist in this region. To date, however, biochemical studies have not supported this hypothesis (18). Gibbons (22) first reported that ciliary membranes of *Tetrahymena pyriformis* contain substantial ATPase activity and, more recently, similar observations have been made with *Chlamydomonas* flagella (18, 57). Although the membrane-associated ATPase in *Chlamydomonas* may be calcium-specific (57), the role of the membrane ATPase was not understood. Cytochemical studies have indicated that this ATPase may be associated with the outer-doublet microtubule-membrane bridges which link the membrane to the axoneme (10, 32, 39). Since a great number of morphological studies have revealed similar bridges between cytoplasmic microtubules and membrane vesicles (2, 6, 19, 20, 29, 31, 35, 42, 44, 50, 52), the characterization of microtubule-membrane bridges in cilia may more clearly illustrate the roles of bridges in a variety of cytoplasmic systems.

The results reported here demonstrate that ciliary membranes are physically connected to the outer-doublet microtubules by bridges and that a high molecular weight protein with properties sim-

ilar to those of dynein, the ATPase responsible for ciliary and flagellar motility, is associated with these membranes. The biochemical nature and possible function of the bridges connecting the outer-doublet microtubules with the membranes are examined using the lipophilic, cleavable, bifunctional reagent, 4,4'-dithiobisphenylazide, which, through the photogeneration of a bifunctional nitrene, can cross-link proteins that are integral to or that insert into the lipid bilayer. A preliminary account of this work was presented at the 1978 Biophysical Society meeting (16) and to the American Society for Cell Biology (15).

MATERIALS AND METHODS

Cilia Isolation

T. pyriformis (strain BIV) were grown to late log phase (3–6 $\times 10^5$ cells/ml) in 2% proteose peptone at 25°C in Erlenmeyer flasks on a shaker. The cilia were isolated by either of two methods: dibucaine treatment described by Thompson et al. (55) or the calcium-ethanol-sucrose method of Gibbons (22), with no significant modification except for reducing volumes to liter-quantities in the later case.

The bay scallop *Aequipecten irradians* was maintained in running seawater at ~10°C. Cilia were isolated from excised gills by brief treatment with hypertonic seawater and enriched by differential centrifugation from seawater, as described in detail previously (54). To avoid osmotic effects, the cilia were not washed at low ionic strength or gradient-purified.

Photochemical Cross-linking

Cilia were suspended to a protein concentration of ~5 mg/ml. *Tetrahymena* cilia were suspended in "wash buffer" (0.25 M sucrose, 30 mM Tris-HCl, pH 7.6, 25 mM NaCl, 5 mM MgCl₂, and 0.5 mM EDTA), and *Aequipecten* cilia were resuspended in filtered seawater buffered with 10 mM Tris-HCl, pH 8.0. Under a red safelight, a 0.01 M solution of 4,4'-dithiobisphenylazide (Pierce Chemical Company, Rockford, Ill.) was prepared in absolute ethanol. The solution was added with stirring to the cilia suspension to produce a final concentration of 10^{-4} M (ethanol = 1%) and the mixture was incubated in the dark for 10 min. Based on total membrane protein present (~20% of the ciliary protein), these parameters are essentially the same as those used by Mikkelsen and Wallach (37) for erythrocyte ghost preparations.

The suspensions were placed in small (10 × 75 cm) Pyrex test tubes and irradiated with an unfiltered SL 3660 UV lamp (Ultraviolet Products, San Gabriel, Calif.) at a distance of 3 cm. A curved, aluminum foil reflector was placed ~2 cm behind the sample tube. Tests performed with reagent blanks in ethanol indicated that, under these conditions of illumination, ~90% of the reagent was exhausted within 30 min, based on the optical density decrease in the solution monitored at 262 nm. Routinely, *Tetrahymena* and *Aequipecten* cilia were irradiated for 30–45 min at an ambient temperature of 20° or 5°C, respectively, corresponding to 5°C below their normal growth temperature. Radiational heating caused a 4°–5°C rise by the end of the irradiation period. A fine glass rod was used to stir the sample intermittently. All of these operations were performed under subdued room

light, shown not to activate control reagent blanks to any meas-
urable extent. Parallel samples that contained cilia and the
reagent but that were not irradiated served as experimental
controls.

Analysis of Products

At the end of the irradiation period, the samples were cooled
to 0°C and the cilia were sedimented at 15,000 g for 10 min in a
swinging bucket rotor. The cilia were resuspended in 5-10 vol of
0.25-1.0% Nonidet P-40 (NP-40; Particle Data Laboratories,
Ltd., Elmhurst, Ill.) in wash buffer (*Tetrahymena*) or 30 M Tris-
HCl, pH 8.0, 3 mM MgCl₂ (*Aequipecten*) and were incubated for
15-30 min at 0°C to solubilize the membrane. The resulting
axonemes were recovered by centrifugation as described above
and were resuspended in a volume of detergent equal to that
removed. Generally, these operations were carried out in 3-ml
polypropylene conical centrifuge tubes (No. BB 411-1; BoLab
Incorporated, Derry, N. H.) while transfers and resuspensions
were performed with 250-μl automatic pipettes equipped with
polyethylene tips. The use of such non-wetting, small-scale de-
vices ensured minimal loss and allowed accurate stoichiometric
sample handling and dilution.

For sodium dodecyl sulfate-polyacylamide gel electrophoresis
(SDS-PAGE), the method of Fairbanks et al. (17) was used
because of its insensitivity to detergents. The resuspended axo-
nemes and solubilized membrane fractions were mixed with $\frac{1}{3}$
vol of fourfold concentrated sample buffer without reducing
agent and immediately brought to 100°C for 2 min. Using glass
microliter pipettes, the samples were accurately loaded onto 0.5
× 10-cm 5% polyacrylamide gels and electrophoresed at 10 V/
cm. Staining was performed as described by Fairbanks et al. (17).

For the analysis of high molecular weight cross-linked prod-
ucts that accumulate at the gel-buffer interface, the top 1-2 mm
was carefully sliced from several replicate gels, incubated in 1
vol of SDS-sample buffer containing reducing agent (5% mer-
captoethanol) for 30 min, then heated to 100°C for 5 min. The
cleaved products were analyzed by (a) placing the gel slice
directly atop a fresh SDS-polyacrylamide gel and electrophores-
ing the now-reduced proteins from it, (b) extracting the slices
overnight and electrophoresing an aliquot of the extract, or (c)
stacking the slices into a glass tube and electrophoresing the
protein into a dialysis bag. Use of the first method tended to
enrich for higher molecular weight proteins which were not as
readily lost from the gel slice during incubation, while the second
method enriched for lower molecular weight proteins for the
converse reason. The third technique, although much more la-
borious, resulted in maximal recovery of all components. Such
electrophoretically-eluted samples were concentrated at high
voltage to a visible pool, removed from the dialysis bag with a
microsyringe, and precipitated with 10 vol of cold acetone before
re-electrophoresis in a small volume of sample buffer (53). At
this point, the unreduced axoneme and membrane samples were
reduced with 5% mercaptoethanol and run in parallel with the
eluted product.

Sucrose Gradient and ATPase Analysis

Ciliary membrane fractions were prepared as described above
while dynein was prepared from axonemes, either by low ionic
strength dialysis (22) or by high salt extraction (30). 1-ml samples
were loaded on 12-ml, 10-40% linear sucrose gradients containing
10 mM NaCl and 10 mM Tris-HCl, pH 8.0, and were centrifuged
for 12-16 h at 100,000 g (35,000 rpm in a Beckman SW-40 rotor
[Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.]; 2°C).

Typically, gradients containing membrane extract and ciliary
dynein were run in parallel. Protein was determined in gradient
fractions by the method of Lowry et al. (34), ATPase by the
Pollard and Korn (45) modification of the Martin and Doty (36)
technique, and protein composition by SDS-PAGE (17).

Enzyme assays were carried out at 20°C in a medium con-
taining 0.1 M KCl, 50 mM Tris-HCl, pH 8.0, 5 mM divalent
cation (magnesium or calcium), and 1 mM EDTA was used
when the divalent cation was omitted. Inhibition by vanadate
(28) was assayed at 2 μM, using a 10 mM stock solution of
NaVO₃ prepared in 10 mM Tris-HCl, pH 8.0.

Electron Microscopy

Small aliquots of cilia or axoneme samples were removed
after irradiation and/or detergent extraction and centrifuged at
10,000 g for 10 min in 3-ml conical tubes. The resulting pellet
was fixed for 1 h with 2% glutaraldehyde in 0.1 M Na-phosphate
buffer, pH 7.0, washed with buffer, postfixed for 30 min with 1%
osmium tetroxide in 0.1 M Na-phosphate buffer, washed with
distilled water, stained en bloc for 1 h with 1% aqueous uranyl
acetate, and dehydrated with ethanol and propylene oxide. The
pellets were cut into quarters and embedded in Epon-Araldite.
Thin sections were cut with a diamond knife, stained with
methanolic uranyl acetate, and examined with a Philips 300
electron microscope. Whole cells were fixed, stained, and embed-
ded as described elsewhere (9).

For quantitation, thin sections of axoneme pellets were as-
sayed for patches of membrane attached to outer-doublet micro-
tubules or for other axonemal structures. In each case, quantita-
tion was carried out "blind," with samples being identified only
after quantitation was completed. To standardize the procedure,
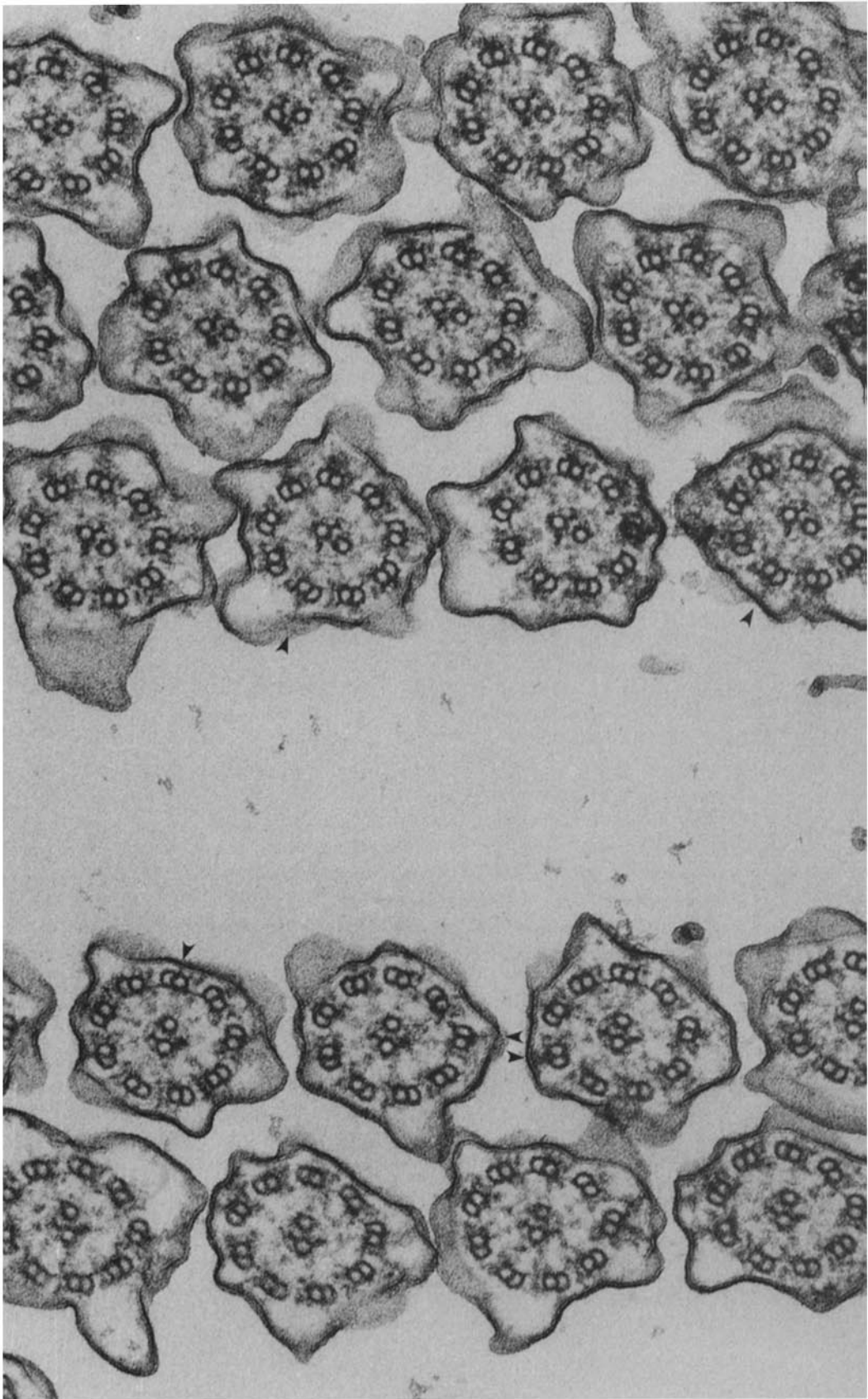only clear cross-sections of axonemes were analyzed.

Irradiation Effects In Vivo

Excised *Aequipecten* gill filaments or late log phase cultures of
Tetrahymena were incubated with 4,4'-dithiobisphenylazide at a
final concentration of 10⁻⁴ M for at least 5 min. *Tetrahymena*
were subsequently concentrated tenfold by gentle centrifugation.
Samples were observed at room temperature with a Zeiss phase-
contrast microscope equipped with a Zeiss Model IV-F1 epi-
fluorescence condenser. The irradiated area was limited by means
of an adjustable field diaphragm in the epi-illuminator. Irradia-
tion, initiated and terminated by means of an opaque field stop,
was performed at 334.2/365 nm (Hg lines) using a "UV excita-
tion" filter module and a red suppression filter. Still photographs
were obtained with an Olympus PM-10-A automated camera
(Olympus Corporation of America, New Hyde Park, N. Y.) at
timed intervals. Continuous filming was performed with a Sage
501 microcinematographic unit (Sage Instruments Div., Orion
Research Inc., Cambridge, Mass.), typically at 18 frames/s.

RESULTS

Outer-doublet Microtubule-Membrane
Bridges in Tetrahymena

Bridges between the outer-doublet microtubules
and the surrounding membranes could be clearly
visualized in cross-sections of Tetrahymena cilia
(Fig. 1). The bridges were filamentous extensions
of the outer-doublet microtubules and, in cross-

section, could be clearly distinguished both from the radial spokes, which extended centripetally from the outer doublets, and from the dynein arms, which extended from the lateral surface of one A-microtubule of an outer-doublet microtubule toward the B-microtubule of an adjacent outer-doublet microtubule. Bridges between outer-doublet microtubules and membranes appeared to attach either to the A-microtubule of each outer doublet or to the point at which the A- and B-microtubules of each outer-doublet join. The precise location of this attachment appeared somewhat variable and could not, therefore, be unambiguously distinguished.

The length and thickness of each outer-doublet microtubule-membrane bridge was somewhat variable and depended on the distance between each outer-doublet microtubule and the adjacent membrane. The number of bridges observed in different cross-sections of *Tetrahymena* cilia was also variable. Although two or more outer-doublet microtubules in any single ciliary cross-section were often observed to be attached to the membrane by bridges, the attachment of all nine outer-doublets to the adjacent ciliary membrane was rarely observed in any single cross-section of a *Tetrahymena* cilium. As shown in Fig. 1, the ciliary membrane frequently appeared to balloon outward from the axoneme and was only held close to the outer-doublet microtubules when the outer-doublet microtubule-membrane bridges were observed. Microtubule-membrane bridges were not uniformly spaced along a single outer-doublet microtubule but appeared at somewhat irregular intervals. When the bridges were observed, however, they often were grouped in clusters which contained four or more bridges. The periodicity of bridges within these clusters measured approximately 220 nm (Fig. 2, arrows) and was not clearly distinguishable from the periodicity of the dynein arms. Although a variety of fixation procedures have been tested, no conditions have been found, to date, which reveal bridges along the whole length of an outer doublet. It remains unclear, therefore, whether there were bridges in vivo which were not preserved or stained or whether all bridge sites are not filled along the outer doublets. It is important to note that even in patches of membrane which have been linked to axonemes by cross-linking reagents, the bridges were not always visible.

In addition to the bridges observed between the outer-doublet microtubules and the ciliary membrane, bridges were also observed to attach an electron-dense accessory fiber both to outer-doublet microtubules and to the ciliary membrane in several specialized cilia and flagella (41, 49). In both intact (*inset*, Fig. 2) and detergent-extracted cilia (Fig. 3), the accessory fiber was attached to the outer-doublet microtubules at a site which appeared to correspond to the site of the outer-doublet microtubule-membrane bridge. The outer-doublet microtubules may, therefore, have a bridge site which can be attached to a variety of different structures, including membranes and certain specialized accessory fibers.

Isolation and Detergent Extraction of Tetrahymena Cilia

Although the outer-doublet microtubule-membrane bridges in cilia fixed *in situ* appeared to link the membrane to the outer-doublet microtubules, it was not clear whether this attachment represented a real association in vivo or whether this was an artifact of the preparation procedure for electron microscopy. To more clearly determine whether a physical bridge attachment existed in vivo, cilia were isolated and kept in a high-ionic-strength buffer in which the cilia remained straight (as assayed by phase microscopy). It was expected that conditions which preserved the appearance of straight cilia would also serve to stabilize the bridges, since cilia isolated under other conditions, in which the cilia appeared as beads (47, 58), resulted in a curling of the axoneme within the membrane, and, presumably, dissociation of the microtubule-membrane bridges. The isolated, straight *Tetrahymena* cilia were then extracted by the addition of Nonidet P-40 to the medium, and the cilia were incubated for a few minutes at 4°C, and then were subsequently pelleted and examined by thin-section electron microscopy. Although the majority of the ciliary membranes were dissolved by the detergent treatment, some patches

FIGURE 1   Cross-section of *Tetrahymena* cilia fixed *in situ*. Bridges (arrows) connect outer-doublet microtubules to the ciliary membranes. The ciliary membrane generally balloons outward from the axonemes when bridges are not observed linking the membrane to the outer-doublet microtubules. × 100,000.

of membrane were more resistant to the detergent treatment and remained attached to the outer-doublet microtubules (Fig. 3). In many, but not all cases, the bridges were observed to link the membranes to the outer-doublet microtubules. These results clearly demonstrated that the bridges must physically bind the membrane to the outer-doublet microtubules.

Ultrastructural Analysis of Cross-linking

To more clearly characterize the nature of the bridges which link the membrane to the outer doublets, the lipophilic cross-linking reagent 4,4'-dithiobisphenylazide was employed. The reagent will partition into the membrane bilayer in the dark, and, upon irradiation with light, highly reactive nitrenes are generated which then cross-link to a variety of C-H bonds (see reference 43 for a discussion of nitrene chemistry). The irradiated suspension becomes increasingly pale yellow as the nitrene is formed and subsequently reacts; the yellow color co-sediments with the cilia. Moreover, the yellow color co-sediments with the axonemes even after most of the ciliary membrane has been solubilized with Nonidet P-40. The bulk of the photochemically cross-linked products, therefore, either remains attached to the axoneme or co-sediments with it.

Electron microscopy revealed that, although the axonemal structures were morphologically unaffected by cross-linking, the cross-linking did increase the number of detergent-resistant patches of membrane that were linked to the outer-doublet microtubules (Fig. 4A, and Table I). Quantitation of the number of axonemes with membrane patches attached after detergent extraction of cilia (Table I) showed that cross-linked cilia retained two to seven times more membrane patches than did the controls that were not cross-linked. The most dramatic examples of cross-linked membranes attached to axonemes were seen in experiments in which the most extensive detergent extraction was used (Table I, exps. III and IV). The lipophilic cross-linker, therefore, stabilized the bridges which link the membranes to the outer-doublet microtubules.

The effect of 4,4'-dithiobisphenylazide is revers-

ible by reduction. Electron microscopy showed that reduction resulted in the disappearance of the membrane patches which were stabilized by the (oxidized) cross-linking reagent (Fig. 4B). Quantitation of these results are shown in Table I. Dithiothreitol at low concentrations was nearly ineffective in releasing patches from cross-linked cilia (exp. I) and only minimally effective at higher concentrations (exp. II). Cold (exp. III) or warm (exp. IV) extraction with relatively high concentrations of mercaptoethanol, however, were quite effective in releasing cross-linked membrane. In addition to this expected effect on cross-linked membranes, however, the few membrane patches which were still linked to control cilia were also released, possibly by the reduction of protein-protein disulfide bonds which may have formed by auto-oxidation. Auto-oxidation of proteins forming the membrane-microtubule bridge may explain why any membrane patches are retained by control cilia and why the percentage is so variable.

## Electrophoretic Analysis of Cross-linking

Since electron microscopy showed that the microtubule-membrane bridges were stabilized by cross-linking, electrophoretic analysis was carried out to determine which proteins may be involved in attaching the membrane to the outer doublets. Cross-linked and control axonemes (detergent-extracted cilia) and detergent-solubilized ciliary membranes were electrophoresed in the absence of reducing agents. Gels of cross-linked samples showed a pale yellow disk of material at their origins that did not enter the gel to any significant extent. The yellow disks were then removed and the proteins in these disks were reduced and re-electrophoresed (see Materials and Methods) to determine which ciliary proteins were cross-linked. Figs. 5 and 6 represent this basic protocol performed with cilia of Tetrahymena and Aequipecten, respectively.

Fig. 5 shows gels of axonemes from cilia which were incubated with 4,4'-dithiobisphenylazide for 10 min and then cross-linked by irradiation ($A[X]$) or held in the dark ($A[O]$) for 30 min before detergent treatment. Although cross-linking re-

FIGURE 2 Longitudinal section of Tetrahymena cilia fixed in situ. Outer-doublet microtubule-membrane bridges appear in groups along outer-doublet microtubules (arrows). × 88,000. (inset) Thin section of a Tetrahymena cilium with an accessory fiber. Accessory fibers are only found in certain oral apparatus cilia (see reference 38). × 55,000.
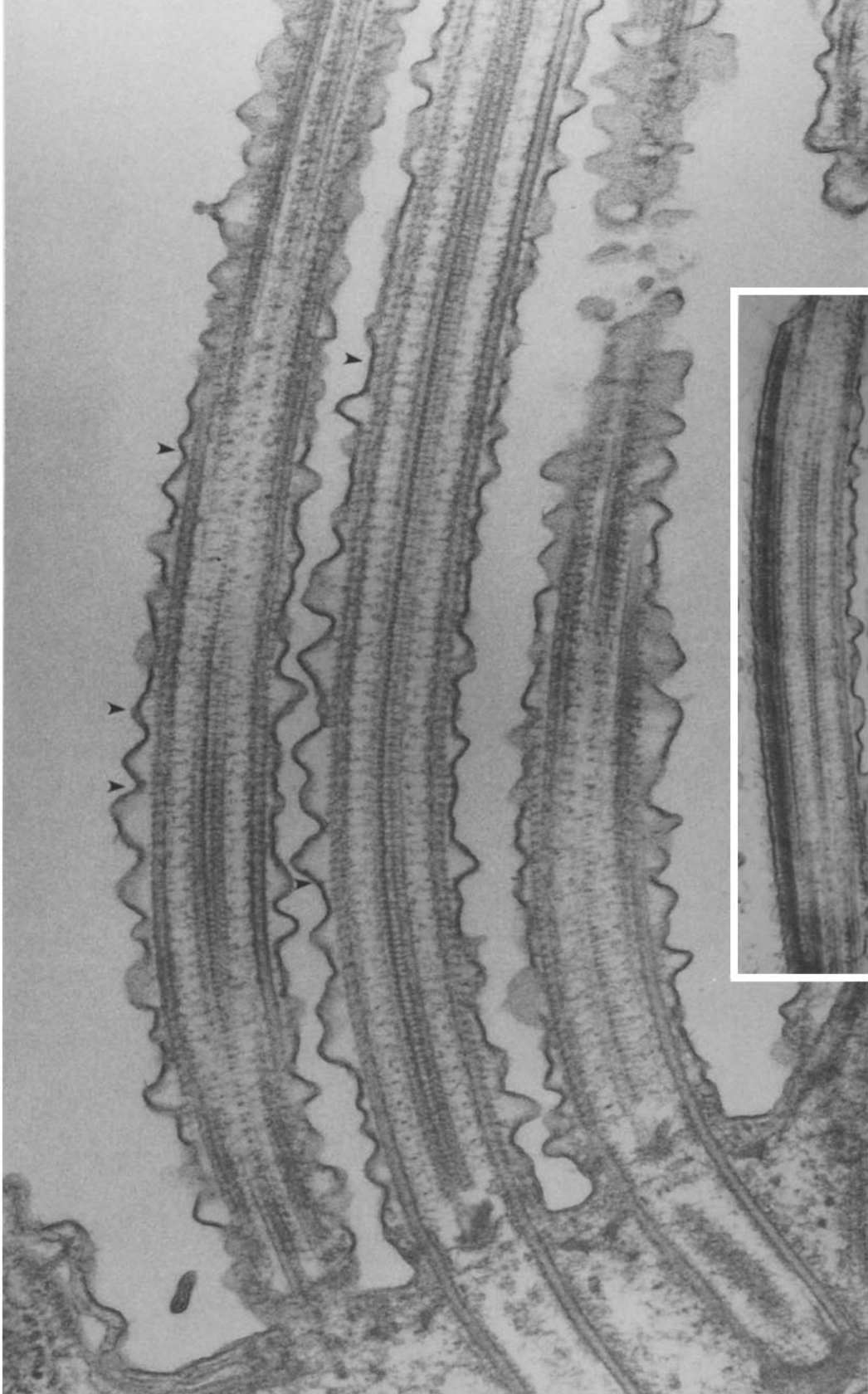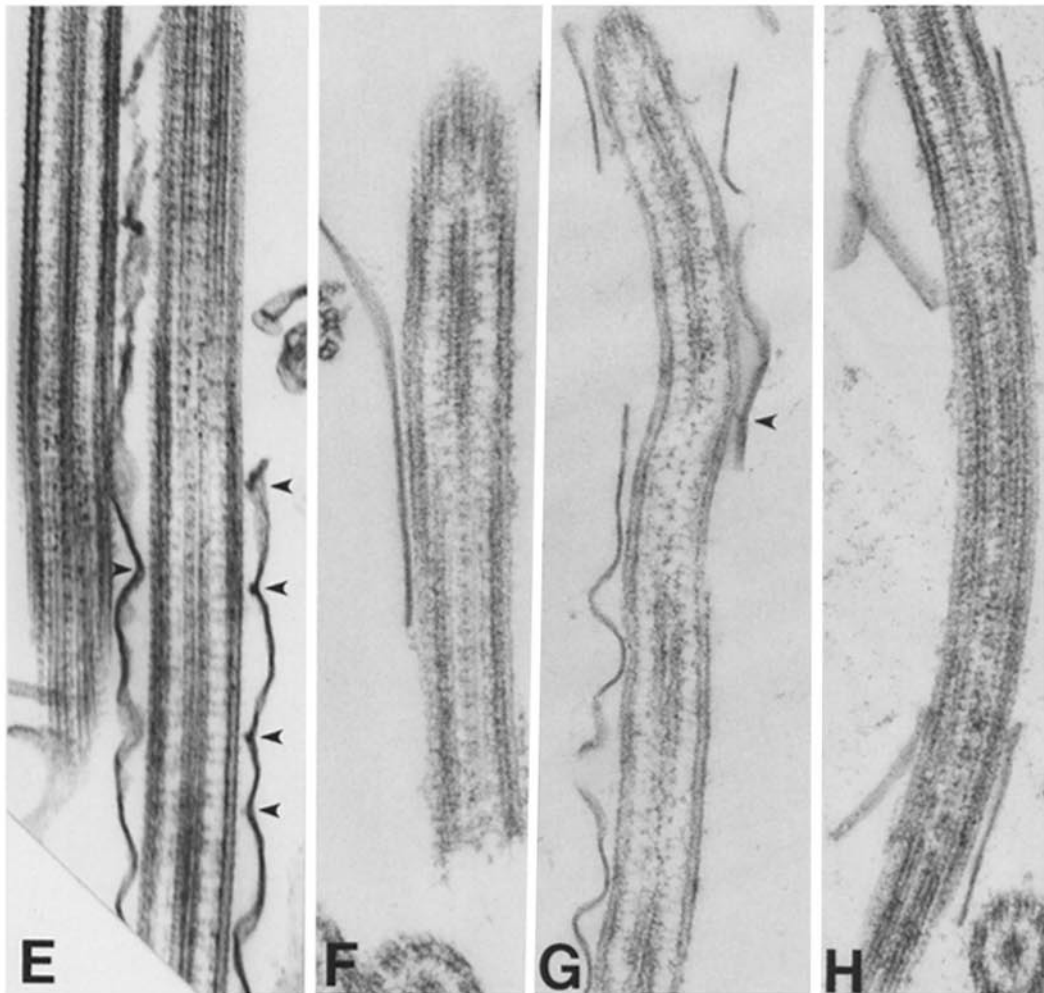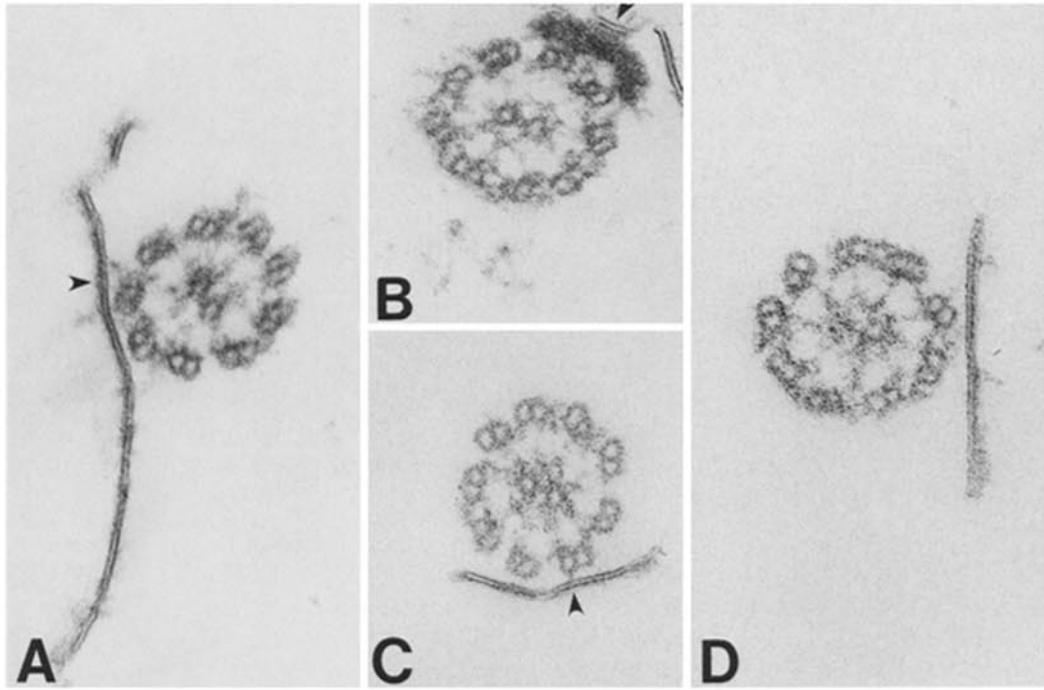
sulted in the accumulation of material in a disk at the gel origin (Fig. 5, A[X]), no new bands appeared nor were any former bands diminished (compare A[O]) with A[X]). The cross-linking did not, therefore, measurably affect any of the major axonemal proteins, indicating that the cross-linker did not penetrate into the axoneme proper to any significant extent during incubation. Reduction of the cross-linked axonemes with mercaptoethanol (A'[X]) resulted in the loss of most, but not all, of the cross-linked material at the gel origin. There were no significant differences in the composition of either reduced or nonreduced axonemes (A[O] vs. A' [O]), indicating that there was probably no significant amount of natural intermolecular disulfide bonding to the axoneme and, more significantly, that random disulfides were not introduced during sample preparation under oxidizing conditions. Moreover, when isolated and demembranated axonemes were incubated with the reagent and subsequently irradiated, no cross-linking of axonemal components occurred. The cross-linking reagent, therefore, only affected proteins in or closely associated with the ciliary membrane.

In contrast to the axoneme, the detergent-solubilized membrane fractions (M, here loaded at five times the stoichiometric ratio with respect to the corresponding axoneme gels), showed extensive dimerization of the 55,000-dalton component (shown by a reduction in the density of the 55,000-dalton band and a concomitant increase in a 110,000-dalton band), a marked decrease in a very high molecular weight band, and the disappearance of numerous minor bands (M[X] vs. M[O]). Upon reduction with mercaptoethanol (M' pair), both cross-linked and non-cross-linked samples appeared similar, although the amounts of a very high molecular weight protein, proteins with molecular weights of ~62,000, 55,000, and 48,000, and certain minor proteins of intermediate molecular weight were significantly diminished by cross-linking (M' [X] vs. [O]). These "lost" proteins were apparently retained by the axoneme and were not solubilized by the detergent.

Removal of the pale yellow disk of cross-linked

product from gels of unreduced axonemes, reduction, and re-electrophoresis resulted in the recovery of components that were lost from the membrane fraction. Gel B of Fig. 5 is a sample of the reduced product, eluted electrophoretically, and loaded stoichiometrically with respect to the membrane fraction. The major constituents missing from the cross-linked membrane (M' [X]) were almost fully recovered in gel B and, therefore, most likely corresponded to the protein complex associated with the outer-doublet microtubule-membrane bridge.

Gels of axonemes from Aequipecten cilia which were incubated with reagent for 10 min and then held in the dark or irradiated, respectively, for 45 min are shown in Fig. 6 (A[O] and A[X], respectively). No difference is seen between the control and irradiated sample. In Aequipecten cilia, the cross-linked material at the gel origin, which was so obvious in Tetrahymena cilia, is entrapped in mucus which accumulates at the gel-buffer interface, preventing its penetration into the gel. Thus mucus is responsible for most of the stained material seen at the tops of all gels, control or otherwise, and is particularly evident in the membrane fractions. Unlike the case of Tetrahymena, no ob-

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FIGURE 3 Thin sections of isolated Tetrahymena cilia that have been extracted in suspension with detergent and subsequently sedimented. Patches of ciliary membrane remain tightly bound to outer-doublet microtubules by the bridges (arrows). Free membranes, unassociated with microtubules, were rarely observed. In B, both an accessory fiber and a patch of membrane has remained linked to the axoneme throughout the extraction procedure. (A–D) × 100,000; (E) × 64,000; (F) × 55,000; (G) × 61,000; (H) × 54,000.
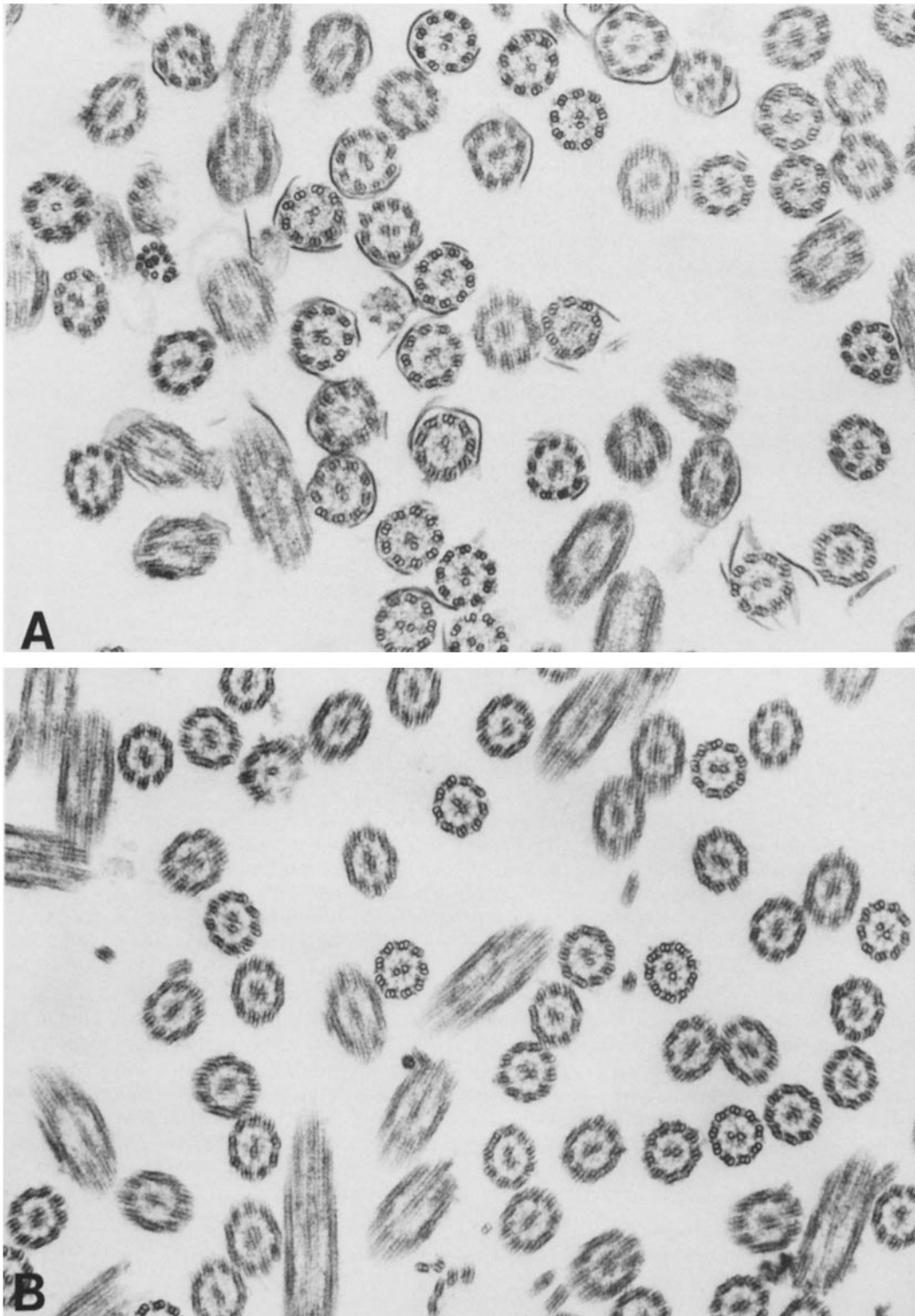
FIGURE 4   Cross-sections of *Tetrahymena* cilia fixed after cross-linking membrane proteins with 4,4'-dithiobisphenylazide and extracting the membrane with detergent (*A*) and after reduction of the cross-linking reagent with mercaptoethanol (*B*). Patches of membrane are cross-linked to outer-doublet microtubules upon irradiation. After reduction of the reagent's disulfide bond, all of the membrane patches are released from the outer-doublet microtubules.
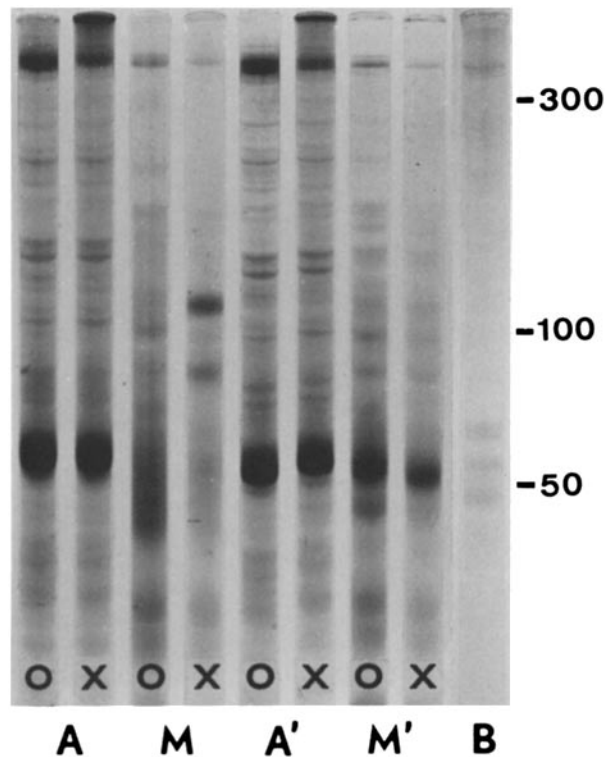
FIGURE 5 SDS-PAGE analysis of cross-linked products from *Tetrahymena* ciliary membranes. *A*, Axonemes, detergent-extracted, without (*O*) and with (*X*) prior UV cross-linking of membrane constituents. Highly cross-linked proteins accumulate at the gel-buffer interface, *A*(*X*). *M*, Membrane extract from cross-linked (*X*) and control (*O*) cilia, loaded at five times stoichiometric ratio with respect to the axoneme samples. The major band on the control gel disappears after cross-linking, being replaced with a major band of twice the molecular weight (110,000). The very high molecular weight band diminishes appreciably, *M*(*X*). *A'*, Axoneme samples, as in *A*, but reduced. Some cross-linked protein still remains atop the irradiated sample (*X*), causing the dynein band immediately below it to migrate anomalously. *M'*, Membrane samples, as in *M*, but reduced. The very high molecular weight band, the major (55,000-dalton) band, and bands immediately above and below it are significantly reduced (*X*) in comparison with the control (*O*). *B*, Bridge proteins, recovered from axonemes of cross-linked samples by removing, reducing, and extracting the highly cross-linked material from the gels. Loaded stoichiometrically with respect to the membrane samples, the proteins "lost" from the membrane are recovered.

vious dimerization of the 55,000-dalton protein occurs in the cross-linked membrane fraction (*M*[*X*] vs. *M*[*O*]). Rather, there is a decrease in proteins with molecular weights of 62,000, 55,000, and 48,000 plus the very high molecular weight band and a 100,000-dalton glycoprotein (54). Representative examples of axoneme gels from which the tops have been removed (pair *S*) demonstrate that the very high molecular weight band (dynein) in such gels was not removed with the disk and thereby included when the disk was reduced. Gel pairs *A'* and *M'* represent the reduced axoneme and membrane samples, respectively. The control

and irradiated axonemes are identical while the membrane fractions show the same basic differences as their unreduced counterparts. Extracts of reduced slices from the tops of gels of irradiated axoneme samples yield back the membrane proteins lost to the axoneme as a result of cross-linking (Fig. 6, gel *B*).

A comparison of *Tetrahymena* (*T*) and *Aequipecten* (*A*) cross-linked proteins, after reduction, is shown in Fig. 7. The samples were run at approximately equal protein concentration and were co-migrated with axoneme and myofibril protein standards for molecular weight calibration. The
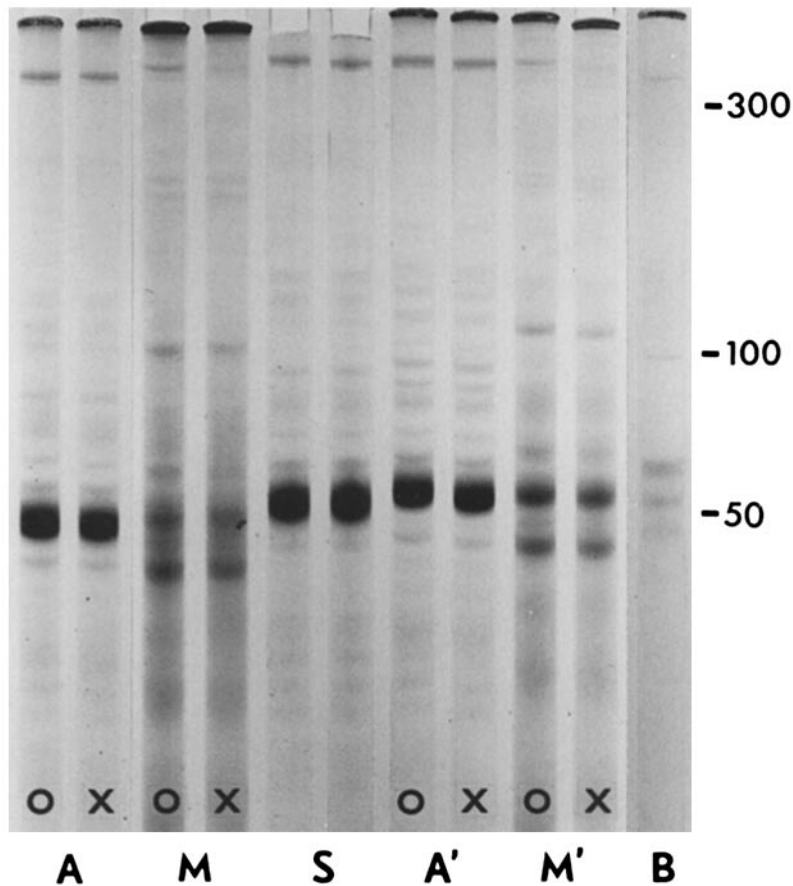
FIGURE 6 SDS-PAGE analysis of cross-linked products from *Aequipecten* ciliary membranes. *A*, Axonemes, detergent-extracted, without (*O*) or with (*X*) prior UV cross-linking of membrane constituents. Highly cross-linked proteins which accumulate at the gel-buffer interface are lost during staining-destaining due to entrapment in mucus. *M*, Membrane extract from cross-linked (*X*) and control (*O*) cilia, loaded at five times the stoichiometric ratio with respect to the axoneme samples. The major (tubulin) band, bands on either side of it, and the very high molecular weight band diminish substantially upon cross-linking, but the tubulin is not dimerized. *S*, Sliced gels of axonemes, illustrating that the dynein band was not accidently included when the gel tops were removed for reduction of cross-linked products. *A'*, Axoneme samples, as in *A*, but reduced. *M'*, Membrane samples, as in *M*, but reduced. The same proteins, recovered from axonemes of cross-linked samples by removing, reducing, and extracting the highly cross-linked material from gels. Loaded stoichiometrically with respect to the membrane samples, the proteins "lost" from the membrane are recovered.

very high molecular weight protein in both cases co-migrates with certain bands of ciliary dynein. The protein at ~62,000 daltons generally splits into two equimolar bands of 61,000 and 63,000 daltons. The 55,000-dalton band co-migrates with membrane tubulin of *Aequipecten* (54), splitting into α- and β-equivalents on Tris-glycine gels. The 55,000-dalton protein of *Tetrahymena* ciliary membranes has also been identified as a modified tubulin (12). The membrane fraction from both species shows varying amounts of a 48,000-dalton protein, often varying reciprocally with the major 55,000-dalton protein. In experiments where proteolysis was minimized, this protein was barely detectable and, therefore, it may be a cleavage product of the major 55,000-dalton protein of the membrane.

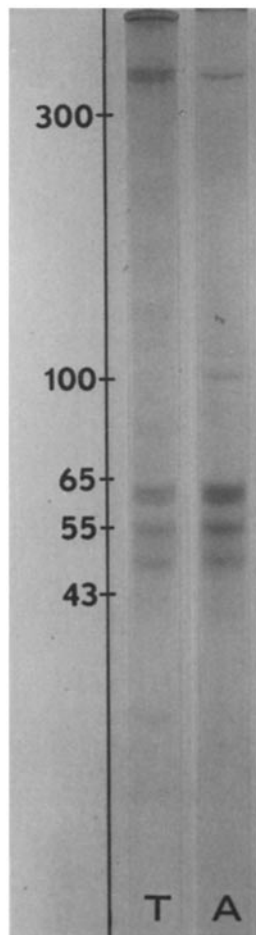That cilia from such widely separated species

FIGURE 7 Comparative SDS-PAGE analysis of outer-doublet microtubule-membrane bridge complex proteins. Proteins eluted from reduced gel slices of cross-linked product from axonemes of *Tetrahymena* (*T*) and *Aequipecten* (*A*) are compared at approximately equal total protein loading. Molecular weight scale in daltons × 10^{-3}.

should yield the same basic cross-linked protein profile is encouraging. The only reproducible differences seem to be in the relatively greater amount of the very high molecular weight protein found in *Tetrahymena* and in the cross-linking of a 100,000-dalton glycoprotein that occurs uniquely in *Aequipecten* ciliary membranes.

One other major species difference is noteworthy. The 55,000-dalton protein of *Tetrahymena* ciliary membranes is dimerized by the lipophilic cross-linker, often almost completely (Fig. 5, *M*[*X*] vs. *M*[*O*]). *Aequipecten* membrane tubulin may be cross-linked to much higher, polydisperse oligo-

mers since it is somewhat diminished after cross-linking (Fig. 4, *M*[*X*] vs. *M*[*O*]), but no obvious "new" bands of higher molecular weight are found on the gel. Cross-linked product accumulating at the gel-buffer interface of membrane samples yields tubulin upon reduction (Fig. 6, *B*), explaining the "reappearance" of a small amount of tubulin after reduction of the membrane fractions (Fig. 4, *M*'[*X*] vs. *M*'[*O*]). These differences in mode of cross-linking must reflect some difference in arrangement or mobility of tubulin within the lipid bilayer of the two membranes. A detailed report concerning the disposition of tubulin subunits within the ciliary membrane will appear at a later date. (Stephens and Dentler, manuscript in preparation).

Gradient Analysis of Ciliary Membrane

Electrophoretic analysis of detergent-solubilized ciliary proteins revealed the presence of high molecular weight proteins which co-migrated with axonemal dynein (Figs. 5 and 6); moreover, cross-linking experiments showed that these proteins were involved in the microtubule-membrane bridge complex (Fig. 7). It was important, therefore, to define the properties of these proteins and to compare them with those of axonemal dynein.

When either *Tetrahymena* or *Aequipecten* cilia were extracted with Nonidet P-40 under conditions where axonemal components should not be solubilized, 20–30% of the total Mg^{++}-ATPase activity was solubilized. This was based upon a comparison of the total of ATPase activity found in whole axonemes resuspended in detergent with the amount of ATPase in the detergent-solubilized membrane fraction. In *Aequipecten*, >75% of this activity was solubilized with one detergent wash, but in *Tetrahymena* the association was evidently greater, with one detergent wash removing $<\frac{2}{3}$ of the total. A second detergent wash was sufficient, in both cases, for membrane enzyme release since a third wash liberated little or no additional ATPase. At least $\frac{1}{4}$ of the Mg^{++}-ATPase in both types of cilia is, therefore, associated with the membrane fraction, with the important qualification that ATPase activity may be modified by detergent treatment, solubilization, or by uncoupling (27, 28). Regardless of absolute ratios, however, a surprising amount of ATPase is solubilized by detergent treatment.

Since the bulk of axonemal ATPase is localized in the dynein arms and since some ATPase may be associated with the radial spoke-central pair

microtubule complex (3, 8, 10), quantitative electron microscopy was carried out to ensure that the detergent-solubilized ATPase was not due to the solubilization of either the arms or the central pair-spoke complex. The data in Table II show that detergent extraction of *Tetrahymena* cilia had minimal effect on the solubilization of either the dynein arms or the central microtubules. Radial spokes were attached to all outer-doublet microtubules assayed. Identical results were obtained in over 14 experiments (a total of 3,412 arms and 886 central microtubules assayed), regardless of whether the axonemes assayed were washed once or several times during the 5- to 8-h experimental time period, as occurred with cross-linked and reduced axonemes. Only a small percentage of the detergent-solubilized membrane ATPase could have been derived from the solubilization of dynein arms or central microtubules, even if one assumes a substantial activation of the solubilized enzyme. Since the dynein arms in *Aequipecten* cilia are difficult to see even before extraction, quantitation was not attempted for this species.

To determine the nature of these membrane ATPases, Nonidet P-40 extracts of cilia were analyzed on sucrose gradients. Dialysis- or salt-solubilized axonemal dynein was used as a marker in parallel gradients. In detergent-solubilized membrane from *Tetrahymena* cilia, the bulk of the Mg^{++}-ATPase sediments as a 14S species, with some ATPase activity also occurring near the top and at the bottom of the gradient (Fig. 8). SDS-PAGE analysis reveals that the major ATPase peak coincides with the very high molecular weight protein shown above to undergo cross-linking with the other membrane components and which co-migrates with axonemal dynein on SDS-PAGE. Salt-extracted dynein from *Tetrahymena* axonemes sediments as two ATPase peaks, one at

14S and one at 30S, with both peaks coinciding with the expected high molecular weight bands, well-separated from the 6S central-pair tubulin and numerous minor bands of intermediate molecular weight (Fig. 8). The high activity of the 30S species compared with that of the 14S is due to the ionic conditions under which the extraction and assay were carried out (24). When *Aequipecten* ciliary membrane and dialysis-extracted dynein are similarly compared, the same basic results are obtained (Fig. 9). The majority of the membrane ATPase is found in one band, in this case also sedimenting coincident with axonemal dynein, known to be 14S in this species (33). On SDS-PAGE, the ATPase peak fractions yield very high-molecular weight polypeptide chains which co-migrate with axonemal dynein. The axonemal dynein sediments as a comparatively broad peak (possibly due to the presence of a 30S form), adequately separated from 6S central-pair tubulin and other minor structural components. Thus, the detergent-solubilized membrane fraction from both species contains a Mg^{++}-ATPase characterized by a sedimentation rate and minimal polypeptide chain weight quite similar to those of axonemal dynein.

To investigate the properties of these major membrane ATPases further, the peak fractions from sucrose gradients of membrane and dynein preparations from both species were analyzed in terms of ion and nucleotide specificities. The comparative data are given in Table III. The membrane ATPase from both species can be activated by either Mg^{++} or Ca^{++}, has no significant ATPase activity in K^{+}-EDTA, and shows a clear substrate preference for ATP over GTP, all properties considered to be general characteristics of dynein (25). There are quantitative differences between the membrane enzyme and axonemal dynein, how-

TABLE II

Occurrence of Axonemal Structures after Detergent and Salt Extraction of Tetrahymena Cilia

Exp.	No. of outer-doublet microtubules with:			Central microtubules	
	2 Arms	1 Arm	0 Arms	Present	Absent
Detergent-extracted	97 ± 2% (3319)	2 ± 1% (72)	1 ± 1% (21)	97 ± 2% (863)	2 ± 2% (23)
Salt-extracted	5 ± 1% (24)	3 ± 1% (18)	93 ± 6% (542)	63 ± 18% (44)	37 ± 18% (27)

Cross-sections of ciliary axonemes were quantitated for the presence of 0, 1, or 2 arms attached to the A-microtubules of each outer-doublet microtubule and the presence or absence of the central microtubules after extraction of the axonemes with 1% Nonidet P-40 or 1% Nonidet P-40 plus high salt (24). Axonemes with only one central microtubule were scored as having no central microtubules. The results are an average of 15 separate experiments. The numbers of axonemes or outer-doublet microtubules scored are in parentheses.
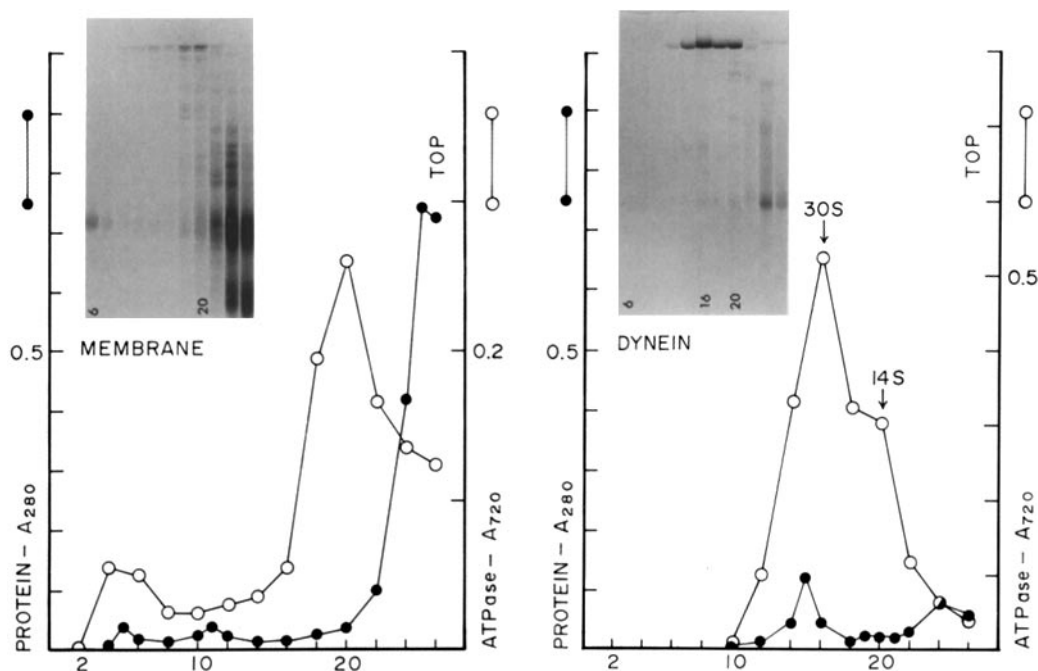
FIGURE 8 Sucrose gradient analysis of *Tetrahymena* ciliary membrane proteins and salt-extracted axonemal dynein. Fraction 20 contains a 14S ATPase in both cases. SDS-PAGE (*insets*) indicates a characteristic, high molecular weight band in this same fraction (fourth gel from right; even fractions were run). A 30S species is present in *Tetrahymena* dynein and dominates the gradient profile. 12-h gradient run.

ever. In comparison with dynein, the membrane ATPases consistently appear to be less discriminating toward GTP and show less inhibition by vanadate ion, an ion considered to be a potent inhibitor of dynein ATPase (28). The differences are minimal, however, and were only evident at very low concentrations of vanadate (5 μM or less). At higher concentrations (>10 μM), both dynein and membrane ATPases were totally inhibited. The differences may also be due to the association of the membrane ATPase with lipid or other membrane components that were not well separated on the gradient. The major ATPase from detergent-treated cilia, therefore, has the general characteristics of a dynein: nucleotide and divalent cation specificity plus native size and minimal polypeptide chain weight.

Although the ultrastructural data indicated that no significant amount of the enzyme was derived from solubilization of obvious structures within the axoneme, it was possible that the ATPase was contained in the ciliary matrix and was released upon solubilization of the membrane by detergent.

Cross-linking data showed, however, that the detergent-solubilized ATPase was associated with the complex formed by linking the membrane to the outer-doublet microtubules: when the total, detergent-released ATPase from control *Tetrahymena* cilia is compared with that from irradiated cilia, the cross-linking process decreased the released ATPase by a factor of two. This is about the same as the amount of very high molecular weight band lost from the membrane fraction as a result of cross-linking (cf. Fig. 5, $M[X]$ or $M'[X]$ vs. $M[O]$ or $M'[O]$). Gentle reduction of the axoneme samples containing the cross-linked membrane patches results in the release of nearly half of this retained ATPase (Table IV). This correlates well with the amount of membrane patch material released (Table I, exp. IV) and the reappearance of the very high molecular weight band on SDS-PAGE. Thus, there is a reasonable relationship between the relative amount of dynein-like membrane ATPase retained in photochemical cross-linking, the number of outer-doublet microtubule-membrane bridge patches stabilized, and the pro-
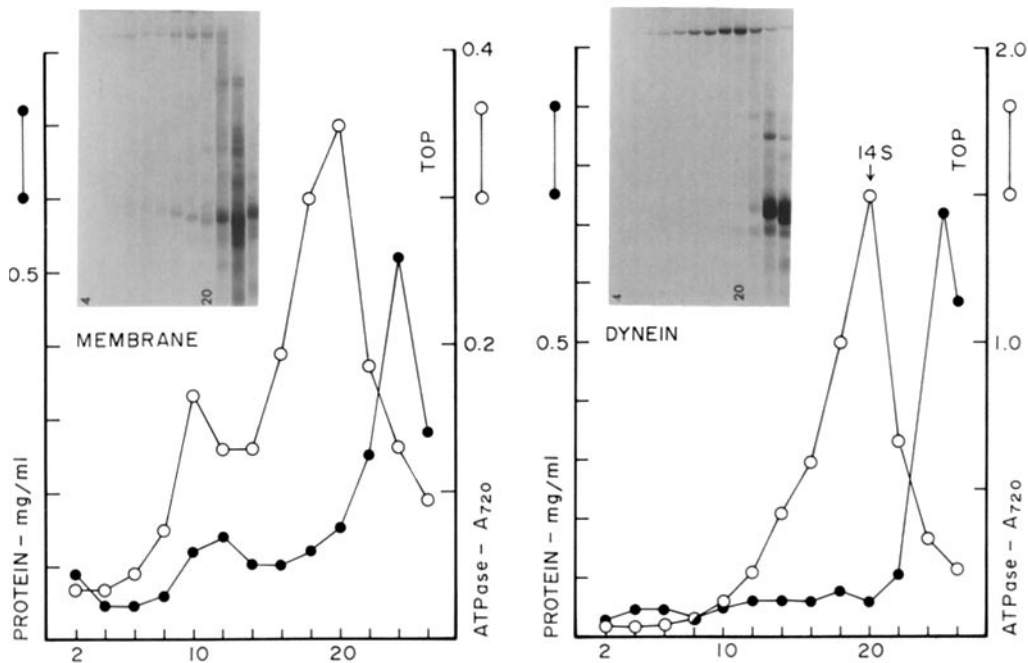
FIGURE 9 Sucrose gradient analysis of *Aequipecten* ciliary membrane proteins and dialysis-extracted axonemal dynein. Fraction 20 contains a 14S ATPase in both cases. SDS-PAGE (*insets*) indicates a characteristic, high molecular weight band in the same fraction (fourth gel from right; even fractions were run). No 30S dynein species has been reported in *Aequipecten*, but the skewed ATPase profile suggests that one may be present. 12-h gradient run.

TABLE III

Specific Activity of Ciliary ATPases (μM P_i/mg-min)

	Mg^{++} ATPase	Ca^{++} ATPase	K$^+$ EDTA ATPase	Mg^{++} GTPase	Mg^{++} ATPase + 2 μM V(V)
Aequipecten dynein, *14S*	1.50	0.52	0.00	0.009	0.94
Aequipecten membrane, *14S*	0.26	0.11	0.00	0.019	0.19
Tetrahymena dynein, *30S*	0.73	0.73	0.00	0.037	0.40
Tetrahymena membrane, *14S*	0.14	0.09	0.00	0.037	0.11

Average of two sets of independent experiments, under conditions detailed in Materials and Methods. Variance $\simeq$ ±10%. V(V) designates vanadate anion (23).

portion of very high molecular weight protein lost from membrane extracts and then recovered from the axoneme.

Functional Effects of Cross-linking

Incubation of excised *Aequipecten* gill tissue in 10^{-4} M 4,4′-dithiobisphenylazide at 10°C in the dark has no effect on the movement of cilia or the viability of the cells when compared with tissue receiving neither reagent nor carrier ethanol. In either case, cilia continue to beat normally over much of the surface of the gill for several days, terminating only upon gross tissue disintegration.

Irradiation of the ciliated gill epithelium by epi-illumination with long wavelength UV light has no effect on control tissue but will produce a dramatic arrest of ciliary movement in tissue incubated with the reagent. Cilia which move together as units, such as the laterofrontals, will stop after only a few seconds of irradiation, generally arrested in the power stroke (Fig. 10). The longer, less coordinated, frontal cilia (particularly those at the distal tip of the gill filament) require longer exposure (5–15) and are usually arrested in an extended position. In either case, lower concentrations of the reagent (10^{-5} to 5×10^{-5} M), reduced

TABLE IV

Membrane ATPase Release Data for Control and Cross-linked Cilia (Exp. IV from Table I)

	Control cilia	Cross-linked
	%	%
First detergent wash	61.5 ⎱ 72.7	34.3 ⎱ 49.5
Second detergent wash	11.2 ⎰	15.2 ⎰
Detergent plus [—SH]	27.2	50.5

Data expressed as percentage of Mg^{++} ATPase units released versus total units present in all washes. Detergent and mercaptoethanol had minimal effects on the enzymatic activity and their presence was corrected for with reagent blanks. The control and cross-linked cilia were aliquots from the same preparation and were treated in parallel.

incubation time, or shorter UV exposure will permit slow recovery of normal movement after cessation of irradiation, generally requiring ten's of seconds. This reversal may be due to either membrane turnover or a natural reduction and cleavage of the cross-linking reagent. Incubation with the reduced, monofunctional reagent (4-thiophenylazide) at 10^{-4} M followed by irradiation did not result in cessation of movement, indicating that the actual cross-linking, rather than simple nitrene-protein coupling, was responsible for ciliary arrest.

Incubation of *Tetrahymena* with reagent in the dark likewise has no obvious effect on motility or viability. Dividing cells may be seen many hours after introduction of the reagent, at least in the 10^{-4} M range employed here.

Observing the effect of irradiation on the motility of these ciliates poses certain tactical problems since the cells move quite actively. At a cell density of 5×10^5/ml (late log phase under our growth conditions), no one cell remains long enough within the UV-illuminated area to be affected noticeably. When the cells are concentrated tenfold by centrifugation, however, their density is sufficient so that partially-immobilized cells tend to be retained within the UV field by their inability to reverse when encountering a neighbor. Fig. 11 illustrates this effect. Cells rapidly move randomly across the field (Fig. 11 a) but when the center of the field is illuminated (Fig. 11 b), those cells remaining within the beam for 2–5 s are partially immobilized (Fig. 11 c) due to the arrest of their somatic cilia in the power stroke. The longer oral cilia continue to beat at least 10–20 s longer, causing the cells to spiral within the field and become entangled with the neighboring cells (Fig.

11 d), accumulating as a large, writhing mass within the irradiated area. Further irradiation of the now-entrapped cells causes eventual cessation of the oral cilia, generally in the extended position, as was also the case with the frontal cilia in gill tissue. Unlike the situation with the gill, however, many cilia are lost during the irradiation-immobilization process, so the light-induced arrest of *Tetrahymena* cilia cannot be considered reversible.

Irradiation of *Tetrahymena* without reagent has no effect on movement except that the cells appear to avoid the area of epi-illumination rather than accumulate within it. After ~90 min, incubated *Tetrahymena* become totally unresponsive to UV irradiation, presumably due to some internalization of the reagent through membrane turnover. The cells are maximally responsive to light-induced ciliary arrest within the first 30 min after introduction of the reagent. This is in contrast to *Aequipecten* where a 10-min incubation is usually required to get any reproducible effect and where responsiveness is retained for many hours.

DISCUSSION

Filamentous bridges have been observed between microtubules and membranes in both cilia and flagella (1, 7, 49) as well as in a variety of nerve axons and secretory cells (2, 6, 19, 20, 29, 31, 35, 42, 44, 50–52). Although these associations have been proposed to link membranes to the microtubules and, in some cases, to provide a basis for microtubule-associated movements, their nature is not at all well understood. Cilia and flagella provide excellent systems in which to study microtubule-membrane interactions since they can be easily isolated with the bridges intact. Data reported here show that physical linkage, via the bridges, must exist in cilia fixed *in situ* since the membrane associated with *Tetrahymena* outer-doublet microtubules is frequently observed to balloon outward from the axoneme except where bridges are observed, in which case the membrane is more closely apposed to the outer doublets. Additional evidence that the bridges bind the membrane to the outer doublets is the observation that patches of membrane remain attached to the microtubules even after the cilia have been isolated, extracted with detergent, and extensively washed by centrifugation.

In cross-sections of cilia, the bridges clearly link the membrane to the outer-doublet microtubules, although in any given cross-section all nine of the outer doublets are rarely observed to be bridged
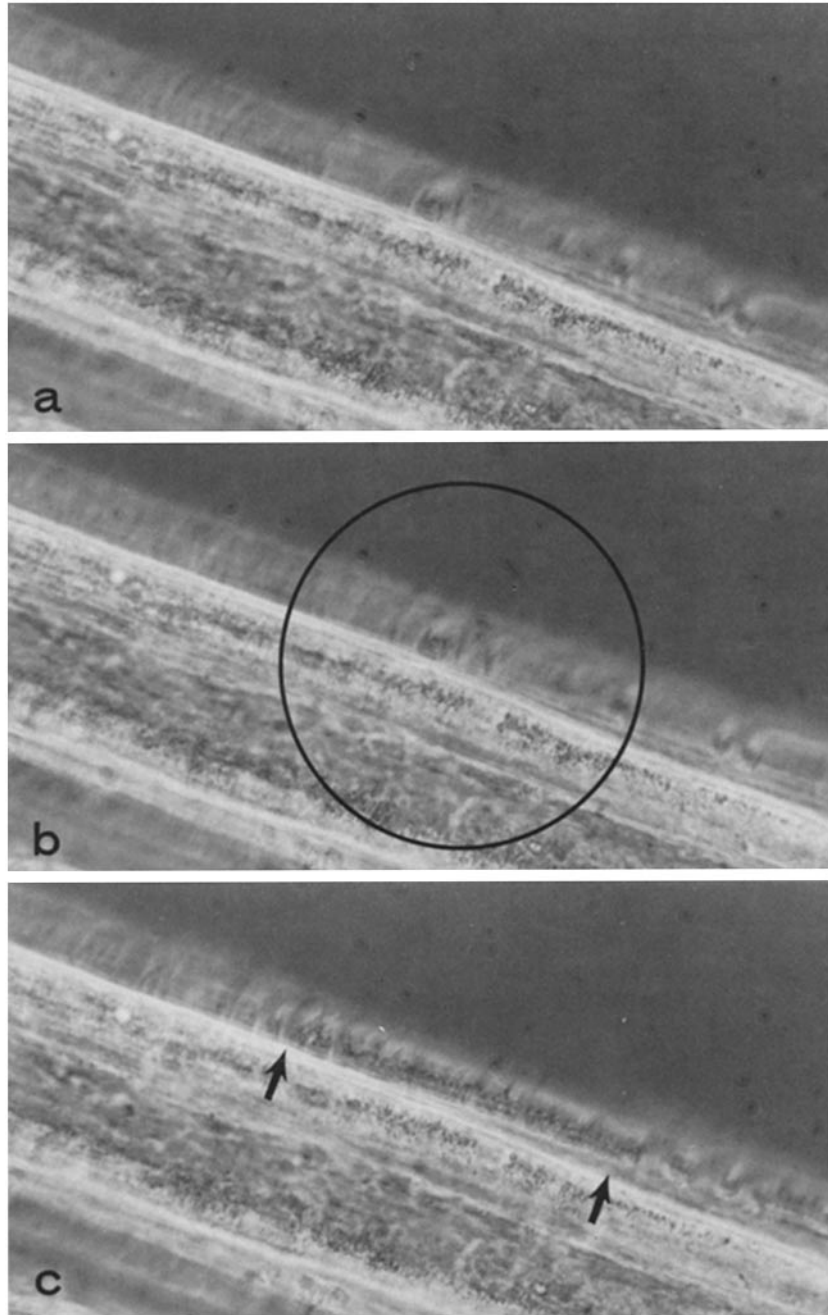
FIGURE 10 In vivo photochemical cross-linking of ciliary membrane proteins in *Aequipecten* gill filaments. Cilia beat actively in the presence of 10^{-4} M 4,4'-dithiobisphenylazide (*a*). A beam of long wavelength UV light from an epi-illumination system (*b*) causes local cessation of ciliary movement within 5 s (*c*). The cilia are arrested in the power stroke, seen between the arrows in *c*. Phase-contrast microscopy. × 400.
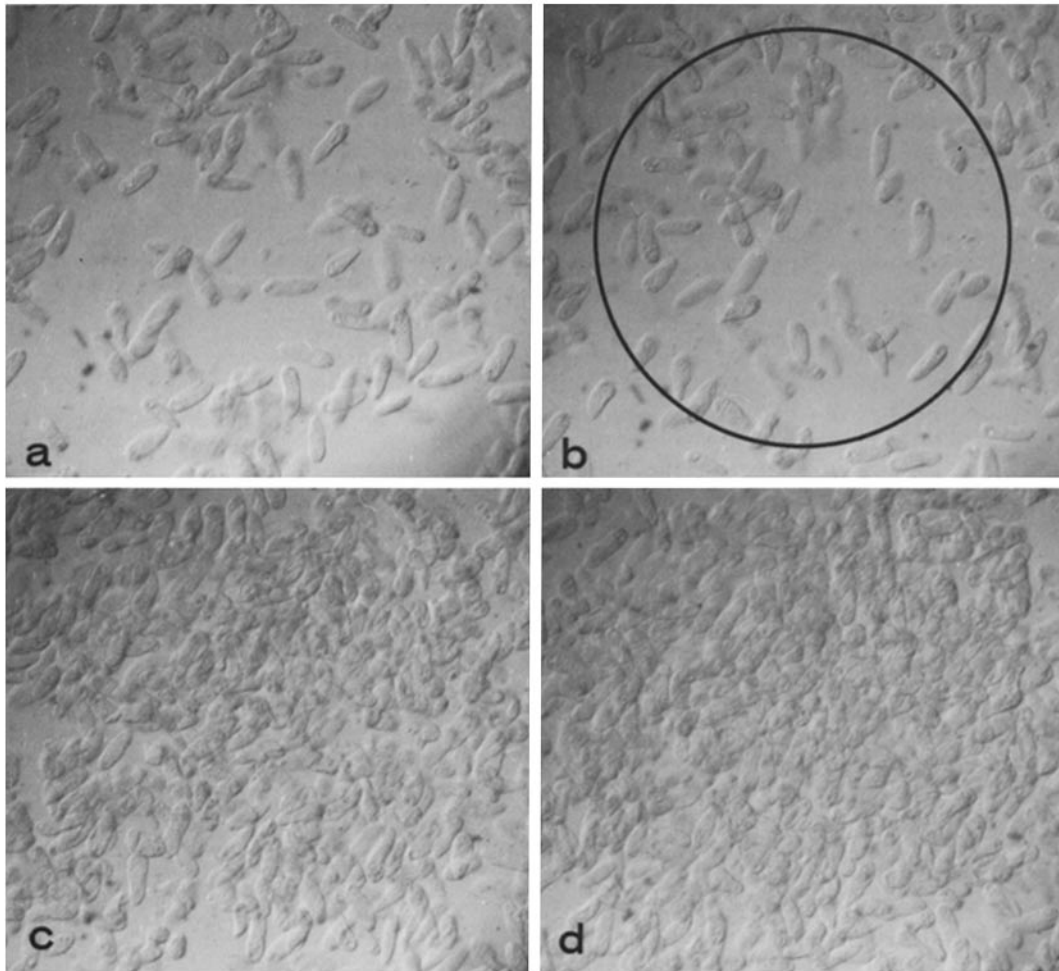
FIGURE 11   In vivo photochemical cross-linking of ciliary membrane proteins in *Tetrahymena*. The ciliates swim randomly across the field in the presence of 10^{-4} M 4,4'-dithiobisphenylazide (*a*). After 2–5 s, a beam of UV light from an epi-illumination system (*b*) causes ciliary arrest in cells within the beam (*c*). Further irradiation results in massive cell accumulation in the irradiated area (*d*) only 1 min later because the cells spiral and cannot reverse. Oblique, bright-field illumination. × 160.

to the membrane. In longitudinal sections of cilia, however, the bridges are rarely observed to bridge more than very short patches of membrane to the outer doublets. The periodicity of bridges in these patches appears to vary somewhat, between the 16- and 22-nm, with the 22-nm spacing being that most commonly observed. Unfortunately, this is also the longitudinal spacing of the dynein arms. Since both arms and bridges would be present within the thickness of the thin sections, the unambiguous resolution of outer-doublet microtubule-membrane bridges is not, at present,

achieved. In cross-sections, however, the microtubule-membrane bridges are clearly observed to be distinct from the dynein arms.

Although a variety of fixation conditions have been tested in *Tetrahymena*, as well as other protozoans, and scallop gills, and *Ctenophore* cilia, it has not yet been possible to find bridges which link the entire length of the outer-doublet microtubules to the surrounding membrane. Moreover, under even fixation conditions in which the membrane is tightly apposed to the axoneme, the bridges cannot be resolved as highly periodic

structures. The frequency with which the bridges exist in vivo, therefore, remains unclear and new methods to preserve or stain the bridges must be developed to clarify their structure and arrangement in the axoneme.

We have previously reported that detergent washes of isolated cilia (12, 54) and isolated ciliary membrane vesicles (12) contain principally a 55,000-dalton tubulin-like protein that may be glycosylated, high molecular weight proteins that co-migrate with axonemal dyneins, and at least two other proteins with molecular weights of ~45,000–50,000 and 60,000 (12). Results reported here demonstrate that the lipophilic photoactivatable cross-linking reagent 4,4'-dithiobisphenylazide links the high molecular weight protein to a 55,000-dalton protein as well as to other proteins of 45,000–50,000 and 60,000 daltons. Electron microscopy reveals that the cross-linking binds patches of the ciliary membrane to the outer-doublet microtubules; cleavage of the reagent's disulfide bond releases the membrane patches from the outer-doublet microtubules.

The high molecular weight membrane protein has both sedimentation and ATPase characteristics very similar to those of axonemal dynein. Other data (12) demonstrate that the membrane-associated high molecular weight protein is composed of three polypeptides that co-migrate, on SDS-PAGE, with the lower molecular weight polypeptides of axonemal dynein. Quantitative electron microscopy demonstrates that this ATPase probably does not result from a loss of the dynein arms from the outer-doublet microtubules; the dynein-like ATPase is, therefore, probably associated with the high molecular weight membrane-associated protein. Cross-linking binds the ATPase activity to the axonemes, and this ATPase is released upon reduction of the cross-linking reagent.

Photochemical cross-linking of beating cilia in vivo results initially in modifying the ciliary beat pattern and, eventually, in the total cessation of movement. These results suggest that either free movement of the outer-doublet microtubule-membrane bridges in the lipid bilayer is essential for normal ciliary motility or that proteins within the membrane must be free to interact cyclically with the outer-doublet microtubules. A third but very unlikely possibility is that the cross-linked membrane becomes so rigid that the axoneme cannot move within it. Of particular importance is that virtually identical results were obtained with the cilia of a protozoan (Tetrahymena) and a mollusc (Aequipecten). The light-induced arrest described here resembles the calcium-arrest seen in mussel gills treated with ionophore (48), raising the possibility that the light effect is merely due to direct action of the photochemically-generated nitrene on some calcium channel or pump protein. Arguing against this is that the reduced, monofunctional form of the reagent is totally without effect, thus supporting the conclusion that photochemical cross-linkage of integral and/or peripheral membrane proteins is the primary cause of ciliary arrest.

We believe that these data indicate that the high molecular weight, dynein-like ATPase comprises the bridges which link ciliary membranes to the outer-doublet microtubules. Supporting this model is cytochemical evidence for an ATPase which exists between outer-doublet microtubules and ciliary membranes (10, 32, 39) and the observation that the size of the high molecular weight protein, based on its sedimentation properties and its molecular weight, is consistent with the size of the bridge observed in electron micrographs, i.e., approximately that of a dynein arm. Moreover, the membrane ATPase appears to be a dynein, and dynein typically interacts with either tubulin or microtubules. Since tubulin, or proteins with properties similar to those of tubulin, is a major ciliary membrane protein (12, 54), and since tubulin also forms the outer-doublet microtubules, the dynein-like protein could be logically placed between the microtubules and the membrane, perhaps functioning in a manner similar to that of a dynein arm bridging an A-microtubule to an adjacent B-microtubule in the axoneme.

Although these data suggest that the dynein-like membrane ATPase comprises the outer-doublet microtubule-membrane bridge, they clearly do not demonstrate the manner by which the bridge is linked to the microtubules and to the membrane. Several lines of evidence suggest that the dynein-like protein is most firmly attached to the membrane and that it is only weakly associated with the outer-doublet microtubule. When membranes of Aequipecten cilia are osmotically swollen, very low detergent concentrations will solubilize virtually all of the membrane and the membrane ATPase. Cross-linking does not stabilize the outer-doublet microtubule-membrane bridges in these swollen cilia, suggesting that the bridges have been physically pulled away from the outer-doublet microtubules. Furthermore, the dynein-like pro-

tein is present in isolated ciliary membrane vesicles and it can be released by detergent washes that solubilize the ciliary membrane.

On the other hand, a single detergent wash of *Tetrahymena* cilia will solubilize most of the ciliary membrane but will release only about half of the membrane ATPase; most of the remainder is released with a second wash. These data suggest that the dynein-like ATPase may be more firmly associated with the outer-doublet microtubules than with the membrane, although one might then argue that the small amount of membrane that was not solubilized by the first detergent wash was held to the outer-doublet microtubules by the remaining bridges. An alternate possibility is that the dynein-like ATPase is a bifunctional molecule which can reversibly bind to either the outer-doublet microtubules or the membrane. Diagrams illustrating these possible arrangements of the dynein-like ATPase are shown in Fig. 12. With the outer-doublet microtubules, as proposed in Fig. 12 C, the cross-linking reagent would only have to link one bridge to a membrane protein. Since

other proteins within the membrane would also be cross-linked to one another, the single bridge should be sufficient to link a whole complex of molecules to the microtubule. The same arguement can be applied if the cross-bridges bind in a bifunctional manner, as in Fig. 12 B. Finally, one cannot rule out the model in which the bridges are only weakly linked to the outer-doublet microtubule (Fig. 12 A); the cross-linking reagent may penetrate the bridge and bind it to the outer-doublet microtubule. If the ciliary membrane is as tightly apposed to the outer-doublet microtubules as fixation without osmium tetroxide suggests (Dentler, unpublished data), this becomes a more reasonable possibility.

It is important to understand the location of the proteins that 4,4'-dithiobisphenylazide is capable of cross-linking. Mikkelsen and Wallach (37) reported that spectrin was the principal cross-linked protein at pH 7-8. While spectrin may have hydrophobic portions that dip into the membrane lipids, spectrin is generally considered to lie subjacent to the membrane; the cross-linking reagent,
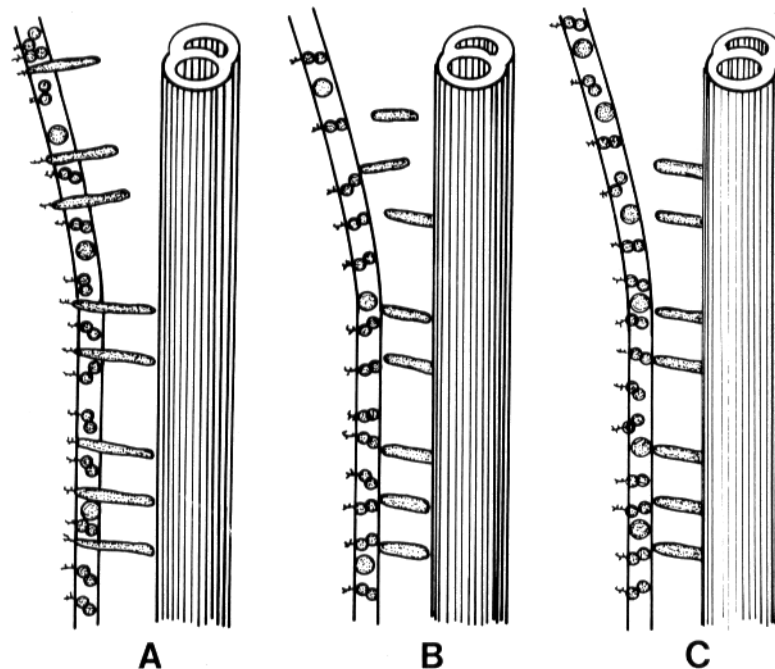

A   **B**   **C**

Figure 12   Three models for a mechanism of membrane attachment to outer-doublet microtubules via a dynein-like bridge. In *A*, the bridge protein is embedded in the ciliary membrane and is free to interact with the outer-doublet microtubules. In *C*, the bridge is attached to the outer-doublet microtubules, similar to the dynein arms, and interacts with proteins in the ciliary membrane. In *B*, the bridge is a bifunctional protein which can interact at either end with the outer-doublet microtubules and with the proteins in the ciliary membrane. See Discussion for data supporting these various models.

therefore, probably remained within the membrane (anchored by its rings) but the activated nitrenes were likely exposed at the cytoplasmic surface of the membrane. The results of Bayley and Knowles (5) demonstrated that nitrenes probably do not remain in the lipid bilayer and therefore do not cross-link chains of fatty acids. It is likely therefore that the nitrenes link either to proteins that lie immediately subjacent to the membrane surface (as would be expected for a protein that binds a membrane to a microtubule) or to intrinsic membrane proteins that have a small portion of their structure lying at the membrane surface; these proteins would provide an excellent site for the binding of a microtubule-associated protein responsible for linking membranes to microtubules.

Although the data presented here are insufficient to distinguish among the models presented in Fig. 12, it does appear that some longitudinal translocation of the bridges between the membrane and the outer-doublet microtubules is involved in ciliary motility. Whether the bridges are actually active in the motile process, functioning to modulate beat pattern, or whether they serve as passive restraints to prevent membrane shear remains to be determined. The presence of ATPase implies an energy-requiring step which could involve an active sliding of the membrane past the outer-doublet microtubules, utilizing the bridges as arms. Alternatively, an ATP-dependent make-break cycle may be needed to allow a membrane tethered by rigor linkages to be repositioned during active ciliary movement. It may be of interest that isolated, demembranated, reactivated *Tetrahymena* cilia are reported to beat in a spiral rather than the usual monophasic, planar manner characteristic of the cilium in vivo (23). Also, isolated, demembranated, reactivated molluscan gill cilia beat in a sinusoidal pattern, somewhat like sperm flagella (Stephens, unpublished observations). One could speculate that the ciliary membrane, modulating motility through the outer-doublet microtubule-membrane bridges, is responsible for the difference in the form of beat observed in cilia as opposed to flagella. Many cilia, and certain kinds of flagella, are capable of changing their form of beat and can undergo reversal. Parallel microtubule/dynein/microtubule and microtubule/dynein/membrane systems may function in concert to accomplish this.

The presence of a dynein-like membrane protein which may extend between the outer-doublet microtubule and the membrane is particularly interesting in light of a recent report of the movement of polystyrene beads along the surface of *Chlamydomonas* flagella (7). It is possible that these surface movements may be due to the direct attachment of beads to the dynein-like membrane protein, as would occur in Fig. 12A. Alternatively, the beads may attach to other proteins which in turn interact with the dynein-like protein, as would occur in Figs. 12B or C. Finally, it is interesting that the high molecular weight MAP proteins which comprise the projections attached to in vitro assembled neurotubules (14, 38) have also been implicated in the attachment of microtubules to membranes (50).

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