

# Cytoskeletal–Membrane Interactions: A Stable Interaction between Cell Surface Glycoconjugates and Doublet Microtubules of the Photoreceptor Connecting Cilium

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**Abstract.** The ciliary base is marked by a transition zone in which Y-shaped cross-linkers extend from doublet microtubules to the plasma membrane. Our goal was to investigate the hypothesis that the cross-linkers form a stable interaction between membrane or cell surface components and the underlying microtubule cytoskeleton. We have combined Triton X-100 extraction with lectin cytochemistry in the photoreceptor sensory cilium to investigate the relationship between cell surface glycoconjugates and the underlying cytoskeleton, and to identify the cell surface components involved.

Wheat germ agglutinin (WGA) binds heavily to the cell surface in the region of the Y-shaped cross-linkers of the neonatal rat photoreceptor cilium. WGA binding is not removed by prior digestion with neuraminidase and succinyl-WGA also binds the proximal cilium, suggesting a predominance of *N*-acetylglucosamine containing glycoconjugates. Extraction of the photoreceptor plasma membrane with Triton X-100 removes the lipid bilayer, leaving the Y-shaped cross-

linkers associated with the axoneme. WGA-binding sites are found at the distal ends of the crosslinkers after Triton X-100 extraction, indicating that the microtubule–membrane cross-linkers retain both a transmembrane and a cell surface component after removal of the lipid bilayer. To identify glycoconjugate components of the cross-linkers we used a subcellular fraction enriched in axonemes from adult bovine retinas. Isolated, detergent-extracted bovine axonemes show WGA binding at the distal ends of the cross-linkers similar to that seen in the neonatal rat. Proteins of the axoneme fraction were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose. WGA labeling of the nitrocellulose transblots reveals three glycoconjugates, all of molecular mass greater than 400 kD. The major WGA-binding glycoconjugate has an apparent molecular mass of ~600 kD and is insensitive to prior digestion with neuraminidase. This glycoconjugate may correspond to the dominant WGA-binding component seen in cytochemical experiments.

THE ciliary plasma membrane represents a distinct plasma membrane domain differing functionally, biochemically, and electrophysiologically from that of the remainder of the cell (3, 10, 14, 24, 32, 38, 50). The base of the cilium delineates the junction between restricted domains and is marked by the structural specialization of both the membrane and the underlying axoneme. Freeze fracture of the plasma membrane reveals rows of intramembranous particles encircling the base of the cilium, the ciliary necklaces (19, 22), strikingly distinct from the particle poor membrane over the remainder of the cilium. Underlying this membrane specialization is the axoneme transition zone, a 9 + 0 arrangement of microtubule doublets connecting the basal body to the 9 + 2 motile axoneme. Y-shaped cross-linkers project from each microtubule doublet at the junction of the A and B subfibers to the adjacent plasma membrane (22). The role of these connections and the ciliary necklaces

is not known. However, one hypothesis is that the connections function in the generation or maintenance of distinct membrane domains.

To study the interactions between membrane or cell surface components and the underlying microtubule cytoskeleton we have taken advantage of the unique features of the photoreceptor sensory cilium. Vertebrate photoreceptors have a nonmotile cilium, the connecting cilium, which represents an extended form of the transition zone seen in motile cilia (47). As in motile cilia, this region of the photoreceptor cilium marks the junction between two distinct membrane domains. Other notable common features include the 9 + 0 arrangement of microtubules arising from the basal body, the Y-shaped microtubule–membrane cross-linkers (hereafter referred to as cross-linkers), and the rows of necklace particles seen by freeze fractures (7, 33, 47). In addition, the cell surface of the connecting cilium bears a distinct array

of glycoconjugates as detected by concanavalin A (Con A)<sup>1</sup> and wheat germ agglutinin (WGA) binding (6, 25, 26). The localization of glycoconjugates overlying the cross-linkers has led to the suggestion that membrane components of the cilium are anchored to axonemal microtubules by the cross-linkers.

We have taken advantage of the Triton insolubility of axoneme-membrane cross-linkers (2, 18, 23), and the rich array of glycoconjugates on the ciliary surface, to study the distribution of surface glycoconjugates of developing rat photoreceptors before and after detergent extraction. We report here that, in most parts of the photoreceptor, Triton extraction removes the cell surface glycoconjugates along with the phospholipid bilayer. In contrast, cell surface glycoconjugates of the proximal cilium remain attached via cross-linkers to the axoneme, despite the near complete removal of the phospholipid bilayer. Using detergent-extracted bovine axonemes we have identified three high molecular mass glycoconjugates, all greater than 400 kD which remain associated with the cross-linkers after detergent extraction. Thus, we have identified a group of high molecular mass glycoconjugates as cell surface components of a transmembrane assemblage which is stably anchored to the ciliary cytoskeleton. Parts of this work have been presented previously in abstract form (6, 27).

## Materials and Methods

### Preparation of Neonatal Rat Retinas

Fisher strain albino rats were obtained at day 6 of postnatal development. Animals were killed in room light by cervical dislocation and eyes were surgically removed. After removal of the cornea, iris, and lens, retinas were dissected into 0.01 M phosphate buffer containing 0.14 M NaCl at pH 7.2 (PBS) at room temperature (22–23°C).

### Detergent Treatment and Tannic Acid Staining

To examine the structure of axoneme-membrane cross-linkers directly, some retinas were fixed in a mixture of saponin, tannic acid, and glutaraldehyde (34). To extract membranes before fixation, retinas were incubated in PBS containing 0.5% Triton X-100 for periods ranging from 30 to 120 s. In preliminary experiments brief (30–60 s) exposure of freshly dissected retinas to Triton-X 100 was insufficient to demembranate photoreceptors completely. In contrast, treatments exceeding 120 s so thoroughly disrupted the retina as to make subsequent analysis difficult. Thus, a 120-s extraction period was used. Extracted retinas were washed in PBS and fixed in 1.25% glutaraldehyde in 0.065 M phosphate buffer (pH 7.4) with or without 1% tannic acid. Tannic acid was included for structural studies but was omitted in all experiments that involved lectin staining. Tissue was subsequently washed in 0.065 M phosphate buffer, postfixated in 1% OsO<sub>4</sub> for 1 h, dehydrated in ethanol and propylene oxide, and embedded in a mixture of Embed 812 and Araldite.

### Lectin Staining of Neonatal Rat Retinas

Glutaraldehyde-fixed retinas were washed in PBS followed by PBS containing 1 mM glycine (7) before incubation in PBS containing lectin-ferritin conjugates for 90 min. Ferritin conjugates of WGA or succinyl-WGA (E-Y Laboratories, San Mateo, CA) were used at concentrations of 0.25 or 0.5 mg/ml. In each lectin-binding experiment a specificity control involving coincubation with the hapten sugar, *N,N'*-diacetylchitobiose, was included. Some retinas were treated for 3 h at 37°C with 1 U neuraminidase (Sigma Chemical Co., St. Louis, MO) in 0.05 M citrate-phosphate buffer, pH 5.5, with 0.3 mg/ml BSA. After lectin labeling, tissue was washed through several changes of PBS and then osmicated and processed for embedment as described above.

1. *Abbreviations used in this paper:* Con A, Concanavalin A; WGA, wheat germ agglutinin.

## Axoneme Preparation from Frozen Bovine Retinas

Frozen, dark-adapted, bovine retinas were obtained from George A. Hormel & Co. (Boston, MN) and fractionated essentially by the method of Fleischman et al. (17, 18). Preparation of outer segments was carried out at 4°C using buffer A (buffer A contains 10 mM Pipes, 5 mM MgCl<sub>2</sub>, adjusted to pH 7.0 with 0.1 mM phenylmethylsulfonyl fluoride (PMSF) added immediately before use). Retinas were thawed and rod outer segments were broken off by gentle vortexing in buffer A with 50% (wt/wt) sucrose and filtered through two layers of cheesecloth. The rod outer segment preparation was then centrifuged for 1 h at 13,000 g and crude rod outer segments were collected from the top. Crude rod outer segments were injected at the bottom of a 25–45% (wt/wt) sucrose gradient in buffer A and centrifuged for 2 h at 13,000 g. Purified rod outer segments were collected from near the middle of the gradient taking care to leave behind the contaminating material below. All steps up to this point were carried out under red safe lights.

Before detergent extraction, rod outer segments were concentrated. Rod outer segments were diluted with buffer A containing 25% (wt/wt) sucrose and placed over a cushion of buffer A with 50% (wt/wt) sucrose followed by centrifugation for 1 h at 13,000 g. Rod outer segments were collected at the 50% interface and detergent extracted by adding an equal volume of buffer B (10 mM Pipes, 5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 0.1 mM PMSF, 2% Triton X-100, pH 7.0) and incubated at least 1 h. Axonemes were then separated from detergent-soluble material by centrifugation over a 45–65% (wt/wt) sucrose linear gradient in buffer B for 3 h at 13,000 g. 600- $\mu$ l fractions from this final gradient were collected from the bottom of the gradient using a peristaltic pump.

### Protein Composition of Fractions from Axoneme Preparation

Fractions generated in the final 45–65% sucrose gradient were analyzed for protein composition using SDS-PAGE according to the method of Laemmli (31). Equal volumes of each fraction were loaded onto the gel. After electrophoretic separation, proteins were visualized by silver staining (36) and immediately photographed. In preliminary experiments a 6–10% gradient acrylamide gel was used. By direct staining of gels with WGA it was determined that the glycoconjugates in the axoneme fractions were of extremely high molecular mass. Therefore, in order to increase resolution, subsequent experiments were done using a 3–6% gradient. All data presented in this paper are from 3–6% gels.

### Preparation of Axoneme Fractions for Electron Microscopy

Fractions potentially containing axonemes were identified by an enrichment in tubulin. The migration position of tubulin in polyacrylamide gels was determined by staining a nitrocellulose transblot with a monoclonal antibody directed against tubulin (see below). Fractions enriched in tubulin were pooled, diluted with buffer B, and centrifuged 1 h at 13,000 g. The resulting pellet was resuspended in buffer C (10 mM Pipes, 5 mM MgCl<sub>2</sub>, 5 mM EGTA, pH 7.0) with 0.5% glutaraldehyde and allowed to fix for 30 min on ice. Glutaraldehyde was then added to a final 2.0% and incubation was continued for an additional 3 h. The material was then pelleted, osmicated, and processed as above for electron microscopy.

The staining of axoneme fractions with WGA-ferritin before embedding was accomplished by first fixing axoneme fractions for 30 min with 0.5% glutaraldehyde, followed by an additional 30 min in 2.0% glutaraldehyde. The material was then washed by repeated centrifugation and resuspension first with buffer C, followed by PBS, PBS with 1 mM glycine, and finally again with PBS. The fractions were then incubated for 90 min with WGA-ferritin (0.5 mg/ml) in PBS, washed with PBS as above, pelleted, and fixed for an additional 30 min in 2.5% glutaraldehyde. The pellet was then osmicated and processed for electron microscopy as above.

Axoneme fractions were also embedded in L R White resin (Poly-sciences, Inc., Warrington, PA) for direct labeling of thin sections with WGA. Axoneme fractions were pelleted, resuspended in buffer C with 0.5% glutaraldehyde, and incubated for 30 min on ice. Additional glutaraldehyde was added to 2.0% and incubation continued for 1 h. Buffer C containing glycine to a final concentration of 1 mM was added for a further 20-min incubation. The material was then centrifuged and the pellet washed with buffer C. Samples were dehydrated to 70% ethanol and fixed en bloc for 1 h with 2% uranyl acetate in 70% ethanol (15). Dehydration was continued to 100% ethanol followed by embedment in L R White resin. Plastic was polymerized for 1 d in a vacuum oven. Thin sections of L R White-embedded material were mounted on grids for labeling with WGA. Grids were

preincubated 10 min in PBS with 1% BSA and then labeled with biotinylated WGA (5 µg/ml) in the presence or absence of 0.1 M *N,N'*-diacetylchitobiose in PBS-1% BSA for 60 min at room temperature. Grids were washed with PBS, and WGA-binding sites detected with streptavidin-5-nm gold.

### Quantitation of WGA-binding Sites

To quantitate the distribution of WGA-binding sites in the axoneme-enriched fraction, the percentage of the volume occupied by axonemes was first determined. This was done using a point intersect counting method (55) on random micrographs of the axoneme pellet, and resulted in an estimation of 4% of the pellet volume being occupied by axonemes. Background was defined as gold particles sticking to L R White in the absence of tissue. Background staining was then determined by counting the number of gold particles associated with the plastic in micrographs taken of areas away from the pellet (two particles per µm<sup>2</sup>). In an area of the pellet, equivalent in size to that used for counting background staining, each gold particle was recorded as either being associated with a cross-linker, or not associated with a cross-linker. In that the axonemes account for 4% of the pellet, 4% of the number of background gold particles was subtracted from the number of gold particles associated with the cross-linkers. Likewise, because 96% of the pellet is nonaxonemal, 96% of the number of background gold particles was subtracted from the number of gold particles not associated with cross-linkers. Thus background was subtracted from each group according to the percentage of the pellet that it occupied. The corrected counts could still be considered a slight underestimate for the cross-linker-associated gold particles because background counts were based on the area of whole axonemes, and gold particles were counted as cross-linker-associated, not whole axoneme.

### Electrophoretic Blots

Proteins were electrophoretically transferred (53) from 3–6% polyacrylamide gels to nitrocellulose under two sets of conditions. To transfer low molecular mass proteins a buffer of 25 mM Tris, 192 mM glycine, 25% methanol (41) was used, blotting for 90 min at 400 mA. For the transfer of high molecular mass proteins a buffer containing 49.6 mM Tris, 384 mM glycine, 0.1% SDS, 20% methanol (29) was used and blotting was for 14–16 h at 30 V. Efficiency of transfer was visually determined by staining nitrocellulose blots for total protein using Aurodye (Janssen Life Sciences Products, Piscataway, NJ).

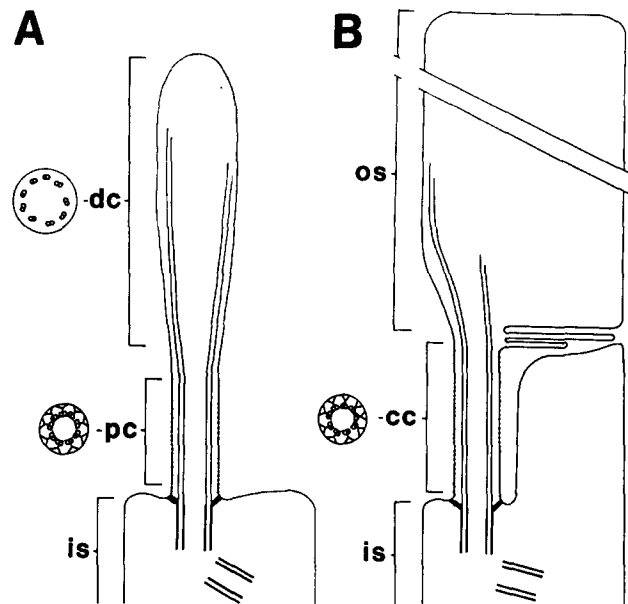
For staining with antibodies or lectins, nitrocellulose blots were blocked with 3% BSA in PBS for either 60 min at 60°C or overnight at 4°C, and washed five times at 5 min each with PBS-0.1% Tween-20. For all lectin experiments a modified PBS containing 0.137 M NaCl, 3 mM KCl, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 0.7 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub> was used. Blots were then incubated for 60 min at room temperature in the presence of primary antibody, 5 µg/ml WGA-biotin, or 5 µg/ml Con A-biotin (E-Y Laboratories) in PBS with 1% BSA and washed as above. Control experiments included a 30-min preincubation of the biotinylated lectin with the appropriate hapten sugar, *N,N'*-diacetylchitobiose or  $\alpha$ -methylmannopyranoside at 0.1 M. For blots immunostained with either a monoclonal antibody to tubulin B-5-1-2 (gift from Dr. Gianni Piperno, reference 42) or anti-opsin antiserum (produced by Dr. Brian Matsumoto in this laboratory) a biotinylated secondary antibody was applied in PBS-1% BSA and allowed to incubate for 60 min at room temperature. Blots were then washed as above. Finally, all blots were incubated with streptavidin-biotinylated horseradish peroxidase complexes (Amersham Corp., Arlington Heights, IL) in PBS-1% BSA for 20 min, washed as above, and visualized with diaminobenzidine and H<sub>2</sub>O<sub>2</sub>. After color development, blots were washed with water, dried, and photographed immediately.

Blots treated with neuraminidase before WGA staining were first blocked with BSA, then incubated for 18 h in 1 U neuraminidase as above for neonatal rat retinas. Each neuraminidase experiment included samples of fetuin and ovalbumin as positive and negative controls, respectively, for the action of neuraminidase. Fetuin or ovalbumin were applied to nitrocellulose using a slot-blot manifold and the nitrocellulose was included in the same incubation chamber with the electrophoretic blots of axoneme proteins.

## Results

### WGA Binding to the Neonatal Rat Cilium

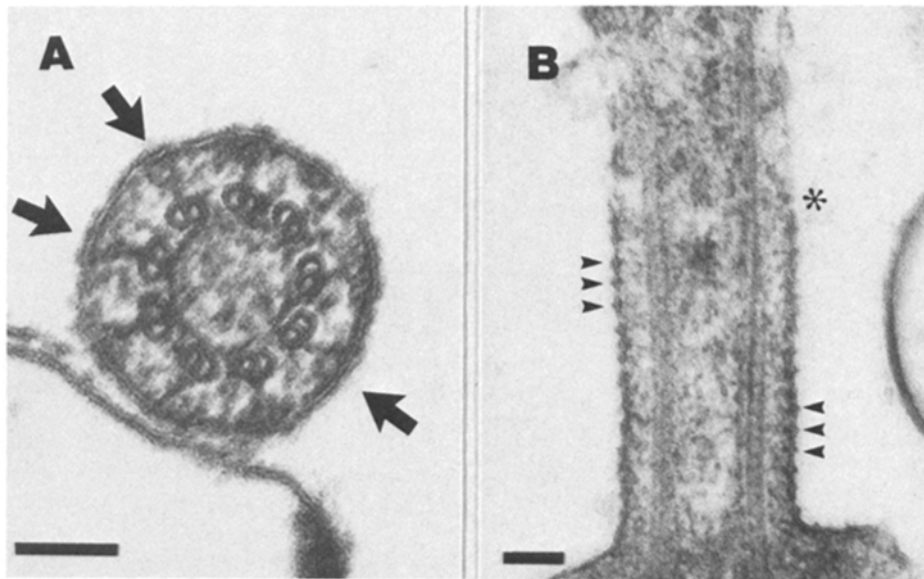
During development of neonatal rat photoreceptors, the cilium exhibits distinct proximal and distal regions before the



**Figure 1.** Diagram illustrating the organization of the connecting cilium and outer segment of neonatal and adult photoreceptors (A) Neonatal photoreceptor apical inner segment (is) and developing outer segment. The microtubules of the proximal cilium (pc) bear Y-shaped cross-linkers and are more highly ordered than those of the distal cilium (dc). The plasmalemma of the proximal cilium has a beaded appearance not seen in the distal cilium. (B) The connecting cilium (cc) of the adult rod photoreceptor is structurally similar to the proximal cilium of the neonate, including the beaded appearance of the plasmalemma. The rod outer segment (os) develops from the distal cilium.

formation of an organized outer segment (Fig. 1; see also Besharse et al. [7]). The proximal region possesses ciliary necklaces and surface lectin-binding sites comparable to those seen in the connecting cilium of adult cells. Axoneme-membrane cross-linkers are a prominent feature of the proximal region. In transverse section, cross-linkers are Y-shaped. Each cross-linker extends from the junction between A and B subfibers of a microtubule doublet to the overlying plasmalemma where its arms exhibit a broad zone of contact (Fig. 2 A). The longitudinal arrangement and structure of cross-linkers is, however, difficult to determine. In favorable longitudinal sections, the plasma membrane lipid bilayer of the proximal cilium is interrupted by beaded structures repeating at 32 nm which are lacking in the distal cilium (Fig. 2 B). The distal cilium plasma membrane is further distinguished by a high concentration of the integral membrane protein opsin, which is characteristic of the mature photoreceptor outer segment (7, 37). The axonemal microtubules of the distal cilium are less ordered in their arrangement and lack the Y-shaped cross-linkers seen in the proximal cilium.

WGA-ferritin binds all photoreceptor surfaces, but is particularly prominent over the proximal cilium (Fig. 3, A and B; see also Besharse et al. [7]). Because WGA binds sialic acid in addition to *N*-acetylglucosamine (9, 35), we have further characterized the WGA-binding sites using neuraminidase digestion and succinyl-WGA. Incubation of retinas with neuraminidase to remove sialic acid residues before lectin binding eliminates WGA binding to material in the extracel-



**Figure 2.** Electron micrographs illustrating the structure of the connecting cilium of intact neonatal rat photoreceptors. (A) Transverse section through the proximal cilium reveals Y-shaped cross-linkers (arrows) extending from each microtubule doublet to the overlying plasma membrane. (B) In well-oriented sections the lipid bilayer of the proximal cilium is interrupted by beaded structures projecting through the plasma membrane (arrowheads), which are lacking in the distal cilium (above the asterisk). Bars, 0.1  $\mu\text{m}$ .

lular space around photoreceptors (data not shown), but does not remove cell surface binding (Fig. 3 C). Succinyl-WGA, previously reported not to bind to sialic acid (35), also binds to all photoreceptor surfaces (Fig. 3 D). Binding of succinyl-WGA is, however, consistently less intense than that seen with WGA and appears in strings extending from the cell surface. It is not clear whether the differences in labeling density seen in Fig. 3 are due to variability within each group or represent actual differences between treatments. The addition of the hapten sugar *N,N'*-diacetylchitobiose blocks binding of WGA (Fig. 3 E). Although not quantitative, these observations suggest that, in the neonatal rat, the dominant WGA-binding sites on photoreceptor surfaces do not contain sialic acid.

#### WGA Binding to Triton-treated Photoreceptors

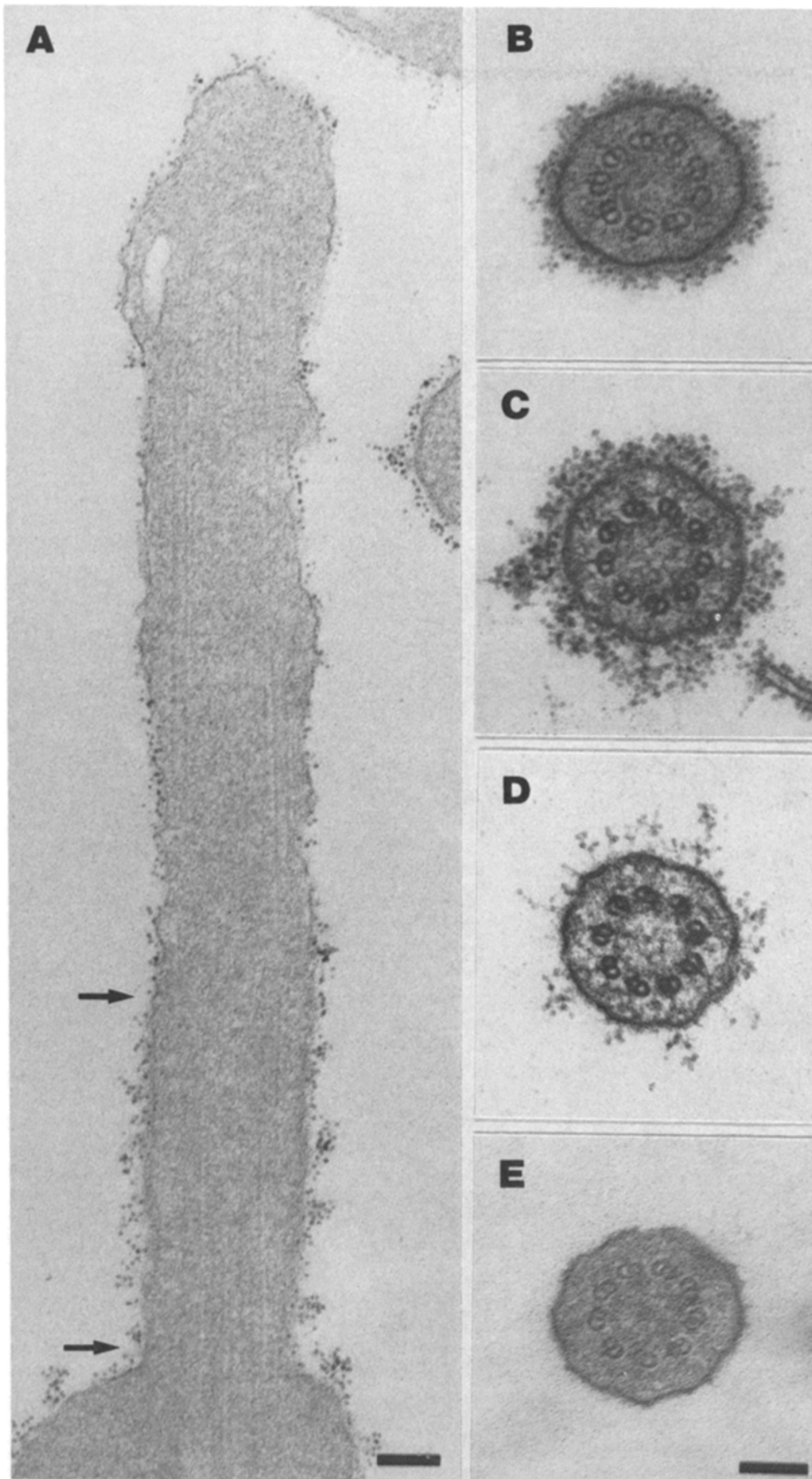
In previous studies designed to isolate axonemes from bovine outer segments, it was noted that axoneme-membrane cross-linkers resist detergent extraction (18). The extraction procedure for bovine outer segments has been adapted for use with isolated neonatal rat retinas. In retinas treated for 2 min with a buffer containing 0.5% Triton X-100, much of the phospholipid bilayer is removed leaving the cross-linkers associated with the axoneme (Fig. 4, arrows). Cross-linkers are most easily studied in transverse section (Fig. 4, A-D) where amorphous tufts are attached by a short stem to most of the doublets. Occasionally, remnants of the phospholipid bilayer are observed in some of the tufts (Fig. 4, arrowheads), but in most cases the entire bilayer appears to be extracted. In longitudinal sections of Triton-extracted photoreceptors, cross-linkers are seen only in the proximal region (Fig. 4 E) appearing as elongated, amorphous structures.

Over most photoreceptor plasmalemmal surfaces, Triton extraction removes the phospholipid bilayer along with virtually all WGA-binding sites (Fig. 5 A). However, at the proximal cilium WGA-binding sites are consistently associated with the amorphous tufts cross-linked to the axoneme (Fig. 5, A and B, arrows). In agreement with results from intact photoreceptors, preincubation of Triton-extracted retinas with neuraminidase does not remove WGA binding (Fig. 5

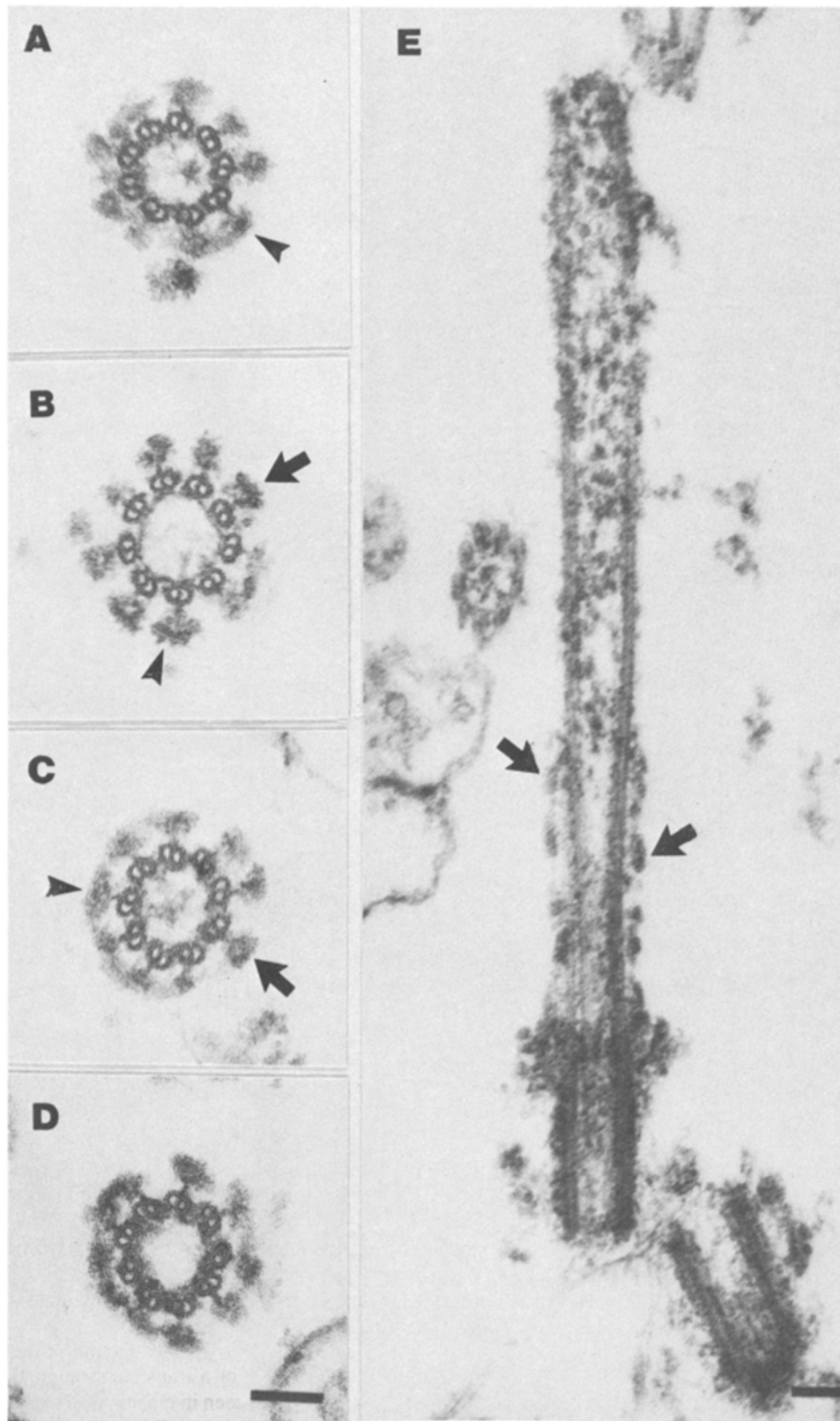
C). The addition of *N,N'*-diacetylchitobiose does block WGA binding (Fig. 5 D) indicating the interaction is specific. Thus, it can be concluded that WGA-binding sites, comparable to those seen at the cell surface of the proximal cilium, remain cross-linked to the axoneme after extraction with Triton.

#### Protein Composition of Subcellular Fractions Enriched in Axonemes

Although the neonatal rat preparation described above (Figs. 2-5) is ideal for electron microscopic analysis, it does not provide sufficient material for biochemical analysis. To identify glycoconjugate components of the cross-linkers, we used a subcellular fraction enriched in axonemes prepared from frozen bovine retinas (17). Axonemes were prepared from isolated bovine outer segments by extraction with Triton X-100 followed by sucrose density gradient centrifugation. Fractions from the sucrose gradient were collected beginning at the bottom of the gradient and protein composition of each fraction determined by SDS-PAGE. Fig. 6 shows a typical fractionation. Lane 1 represents fraction 1 (65% sucrose), and lane 16 represents fraction 16 which contains detergent-soluble material that did not enter the sucrose gradient. Fraction 16 is characterized by heavily stained bands centered at molecular weights of  $\sim 37, 58, 81, 104, 124,$  and  $142$  kD (Fig. 6, asterisks). These bands would be expected to represent opsin, the dominant 37,000-mol wt membrane protein of the rod outer segment, which is known to form multimers under the denaturing conditions used (16). Such bands are lacking in fractions 1-14 (Fig. 6). Using a polyclonal antiserum directed against frog opsin, we found that the heavy multimeric bands that did not enter the gradient (i.e., fraction 16), are opsin immunoreactive, whereas material entering the gradient is not opsin immunoreactive (data not shown). The migration position of tubulin was determined by immunoblot analysis that used a monoclonal antibody directed against tubulin (Fig. 6 B). Fractions potentially containing axonemes were then identified by an enrichment in tubulin. The migration position of tubulin in Fig. 6 A is indicated by the arrow. Fractions 3 through 7 are enriched



**Figure 3.** Electron micrographs illustrating the distribution of WGA binding sites on neonatal rat photoreceptors. (A) Ferritin-conjugated WGA binds the entire photoreceptor surface, although binding at the proximal cilium (arrows) is heavier and located a greater distance from the plasma membrane than WGA binding at the distal cilium. (B) Transverse section showing heavy WGA-ferritin binding around the proximal cilium. (C) Neuraminidase treatment before WGA labeling does not remove WGA binding to the proximal cilium. (D) Succinylated WGA binds the proximal cilium (though to a lesser degree than WGA) forming strings of ferritin particles extending from the plasmalemma. (E) The addition of *N,N'*-diacetylchitobiose blocks the binding of WGA. Bars, 0.1  $\mu$ m.

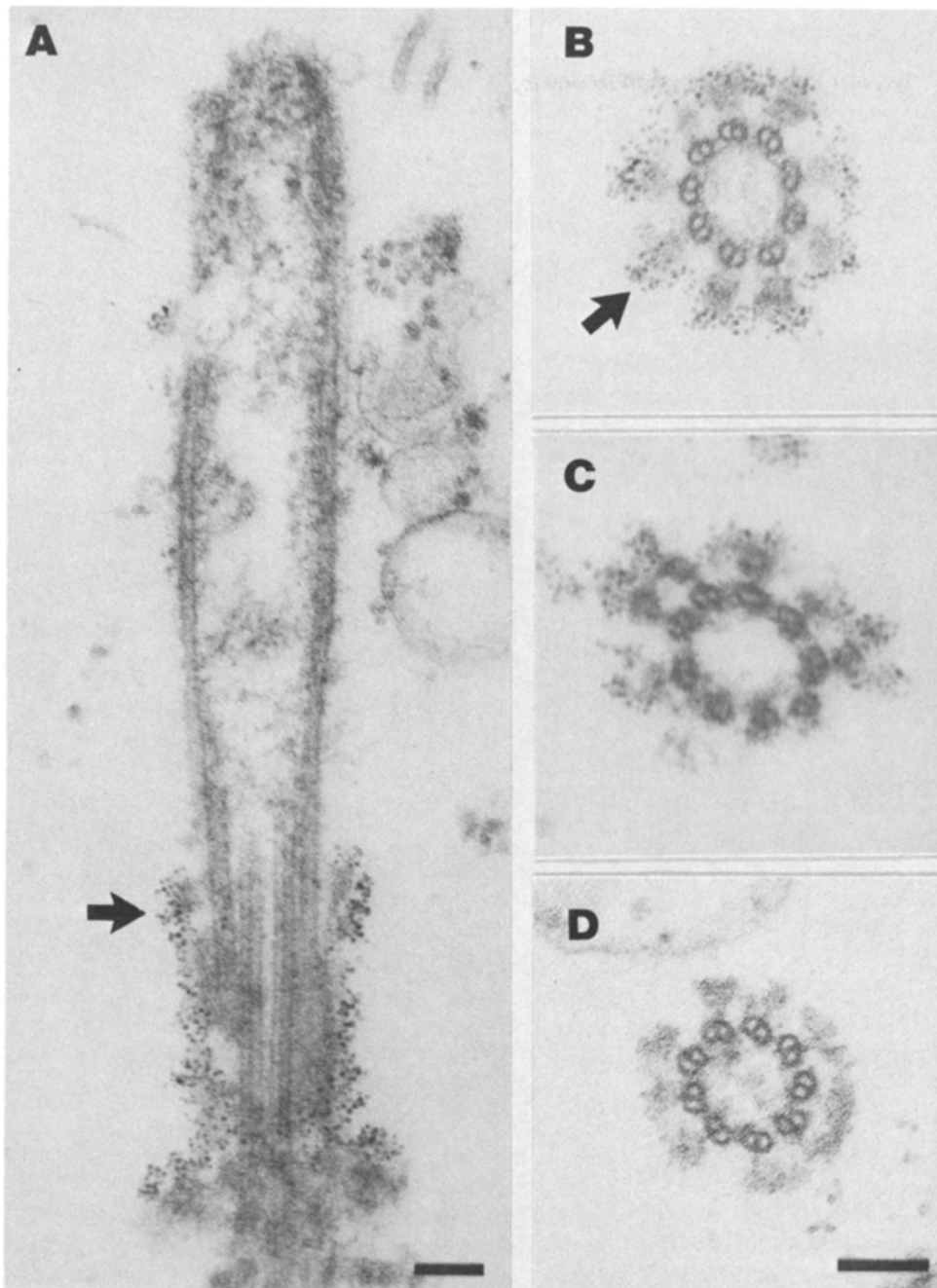


**Figure 4.** Electron micrographs illustrating photoreceptor cilia of Triton X-100-extracted neonatal rat retinas. Extraction of isolated 6-d-old rat retinas for 120 s with 0.5% Triton X-100 results in the complete removal of the distal cilium plasma membrane. The proximal cilium is characterized by the retention of amorphous tufts attached to axonemal microtubules by short stems (*arrows*). Although remnants of phospholipid bilayer are occasionally observed (*arrowheads*), most of the plasma membrane appears to be removed. (*A-D*) Transverse sections. (*E*) Longitudinal section. Bars, 0.1  $\mu\text{m}$ .

in tubulin and, therefore, potentially contain axonemes.

Fractions enriched in tubulin were pelleted and prepared for electron microscopic examination. Pellets have a heterogeneous composition (Fig. 7) including an abundance of amorphous and filamentous material (Fig. 7, *asterisk*). In ad-

dition, the pellets are highly enriched in intact axonemes (Fig. 7, *arrows*) retaining basal bodies (Fig. 7, *open arrows*), and often bearing extended striated rootlets. By stereometric analysis, using a point intersect counting method (55), axonemes make up  $\sim 4\%$  of the pellet by volume. Detergent ex-

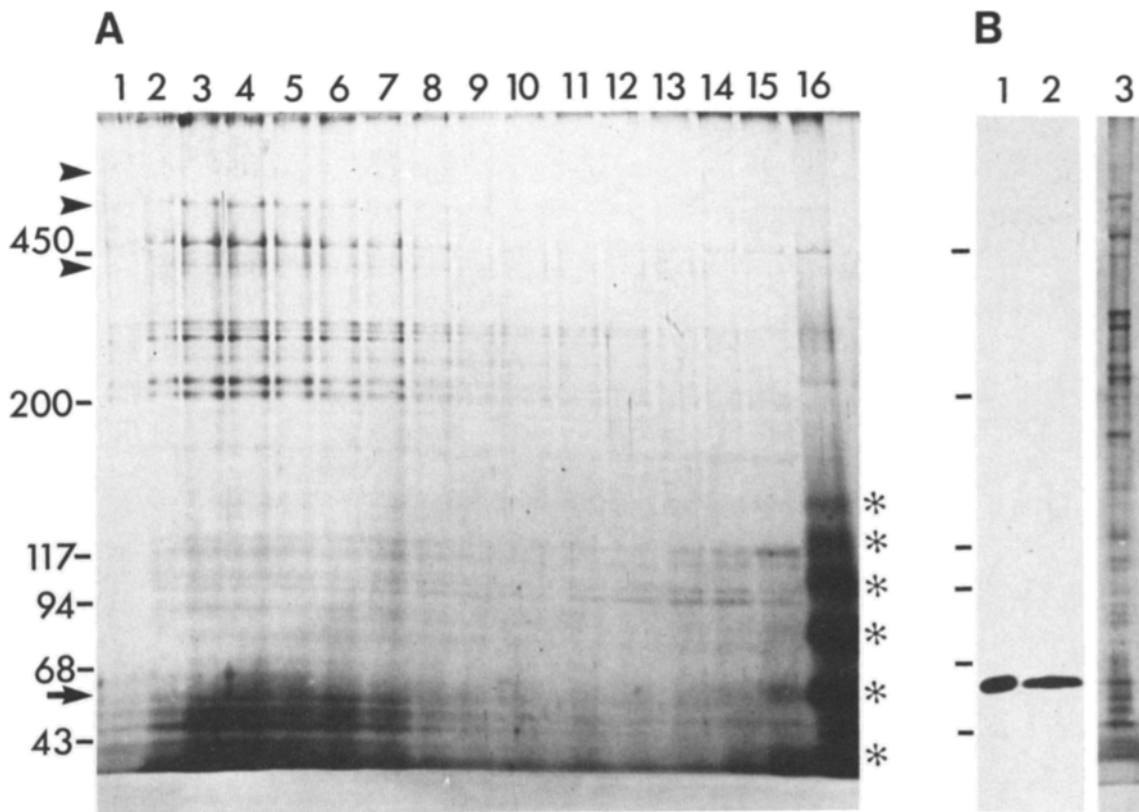


**Figure 5.** Electron micrographs illustrating WGA binding to photoreceptor cilia of detergent treated neonatal rat retinas. (A and B) Extracted retinas were labeled with ferritin-conjugated WGA. Ferritin particles decorate the distal ends of the tufts. (C) Neuraminidase treatment before WGA labeling does not remove WGA-ferritin binding from the crosslinkers. (D) Addition of *N,N'*-diacetylchitobiose blocks binding of WGA to the tufts. Bars, 0.1  $\mu\text{m}$

tracted bovine axonemes are morphologically similar to those of the neonatal rat described earlier (see Fig. 4); displaying the 9 + 0 structure of nonmotile cilia and lacking both central pair microtubules and dynein arms (Fig. 8 A). Although the membrane has been completely extracted, axonemes retain the microtubule-membrane cross-linkers extending from the microtubule doublets along much of the length of the axoneme (Fig. 8 B). Transition fibers (20) or alar sheets (1) emanating in a spiral pattern from the junction of axonemal doublet microtubules and basal body triplet microtubules also remain associated with the axoneme (Fig. 8, B and C, *open arrows*). Axonemes are routinely surrounded by a halo devoid of staining which can be seen in both transverse and longitudinal section (see Figs. 7 and 8).

#### **Lectin Labeling of Isolated Axonemes**

To determine if isolated bovine axonemes retain glycoconjugate components similar to those seen in the neonatal rat, fractions containing axonemes were labeled with WGA-ferritin, and prepared for electron microscopy. Axoneme-membrane cross-linkers label heavily with ferritin particles (Fig. 9), implying the presence of a transmembrane cross-linker component similar to that seen in the neonatal rat. Due to nonuniform penetration of the lectin and difficulties in washing out the lectin when using the preembedding labeling technique, we repeated the WGA labeling using the postembedding labeling technique. Thin sections of axoneme pellets embedded in LR White resin were mounted on grids and incubated with biotinylated-WGA followed by streptavidin-



**Figure 6.** Protein composition of fractions generated in axoneme purification. (A) Fractions were removed from the bottom of the 45–65% sucrose gradient, protein composition resolved by SDS-PAGE (3–6% gradient) and visualized by silver staining. Lanes 1–16 represent equal volumes of fractions 1–16, respectively. The arrow on the left indicates the migration position of tubulin which is enriched in fractions 3–7. The arrowheads on the left indicate the migration positions of the three high molecular mass WGA-binding glycoconjugates (the uppermost of these is barely discernable on this gel). Asterisks to the right mark opsin and its multimers. Numbers to the left indicate migration positions of molecular mass markers in kD. (B) Transblot showing tubulin immunoreactivity. (Lanes 1 and 2) Tubulin immunoreactivity using B-5-1-2 antibody which reacts with  $\alpha$ -tubulins. (Lane 1) Migration position of sea urchin sperm tubulin, the original antigen used for antibody production. (Lane 2) Tubulin-enriched fraction from bovine axoneme fractionation showing the antibody detects a single band with the same migration position as sea urchin sperm  $\alpha$ -tubulin. (Lane 3) Silver-stained gel of tubulin enriched fraction showing protein composition lane 2.

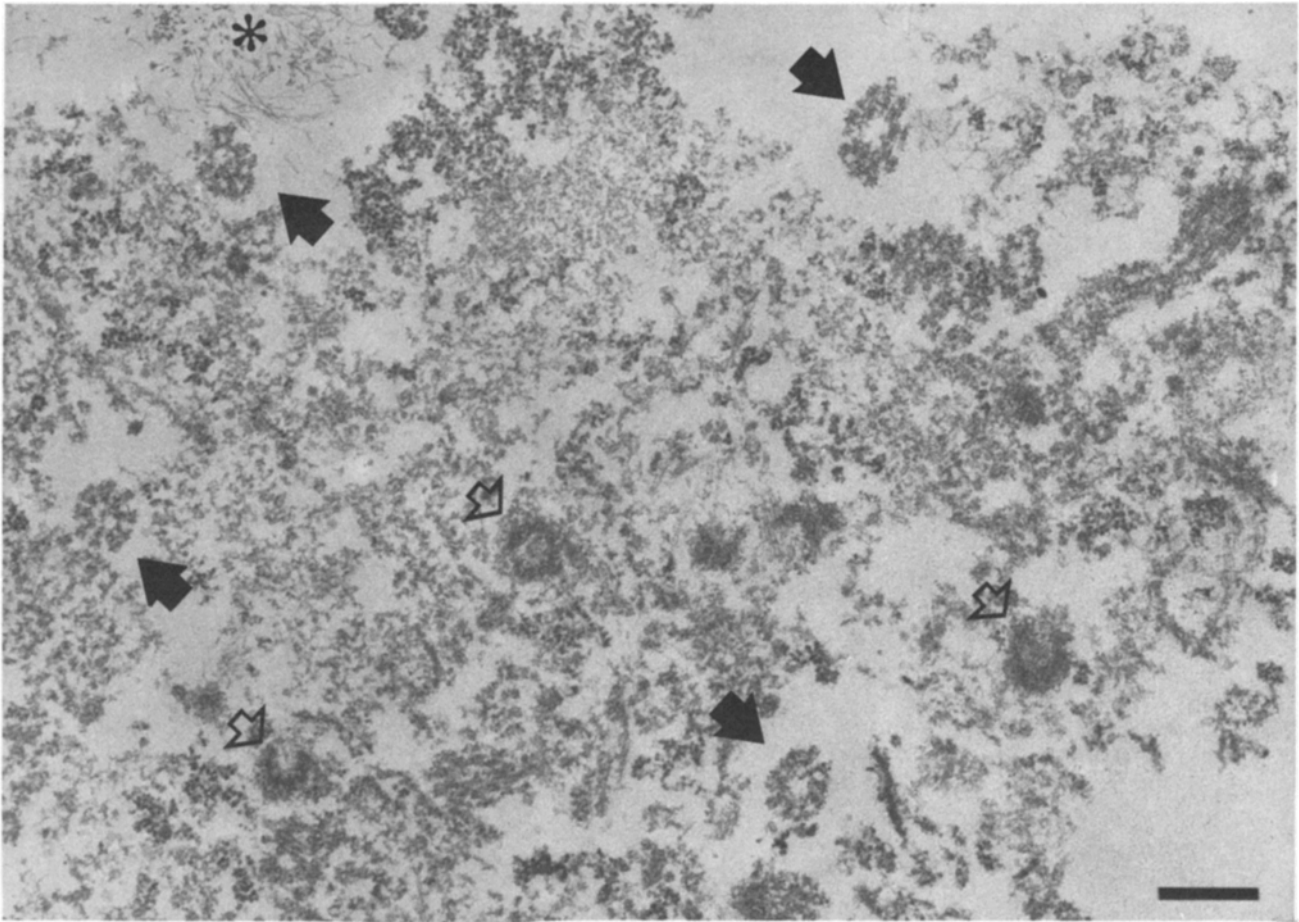
5-nm colloidal gold (Fig. 10, A–D). Gold particles are associated primarily with the distal ends of cross-linkers. In that only sites at the surface of the section are available to bind WGA, the degree of labeling is less than that obtained using preembedding labeling.

To determine if cross-linkers are the major WGA-binding structure in the axoneme-enriched fraction, we calculated the percentage of total gold particles associated with the cross-linkers. Gold particles were counted as being either associated with, or not associated with, cross-linkers in a random collection of micrographs of the pellet ( $n = 58$ ). Only gold particles found directly on cross-linkers or within  $\sim 50$  nm were considered associated with cross-linkers. After correcting for background staining, we estimated that 85% of all gold particles ( $n = 3,222$ ) were associated with the cross-linkers. We regard this as a conservative estimate because axonemes are less readily identified in L R White-embedded material and only structures definitely identifiable as axonemes were considered when counting cross-linker-associated gold particles. The addition of *N,N'*-diacetylchitobiose blocks labeling of the cross-linkers (Fig. 10 E), indicating that WGA labeling is specific.

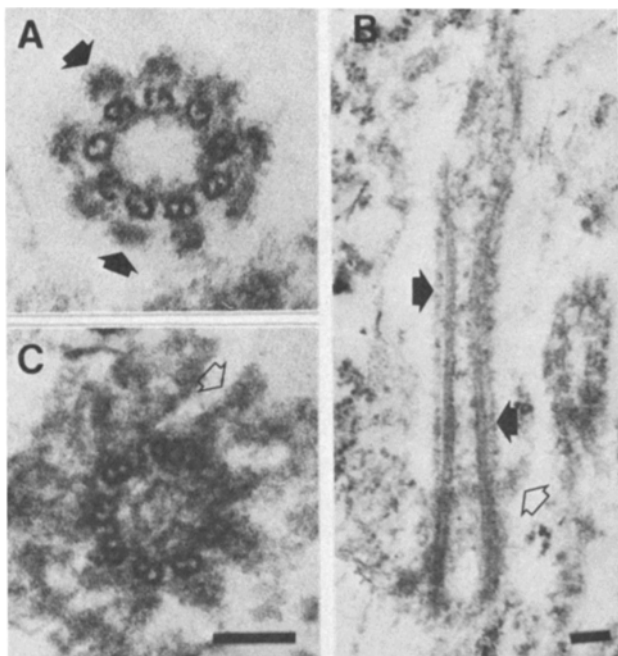
#### Identification of Axoneme-associated Glycoconjugates

Lectin staining of transblots has been used to identify which components of the axoneme fractions contain WGA-binding sites. In preliminary experiments proteins of the axoneme fractions were separated by SDS-PAGE using a 6–10% acrylamide gradient and the gel directly stained with WGA using the technique of Wood and Sarinana (56). These experiments revealed three WGA-stained bands near the top of the gel (data not shown). Subsequently, we used 3–6% gels and electrophoretic transfer to nitrocellulose for WGA staining to increase resolution and decrease the gradient of background staining seen with direct staining of gels. Using transfer conditions which can drive large proteins from the polyacrylamide gel, three major high molecular weight glycoconjugates are visualized (Fig. 11 A, lane 3 arrowheads); all three have apparent molecular masses above 400 kD. The dominant WGA-binding glycoconjugate has an approximate molecular mass of 600 kD. A faintly stained WGA-binding component of  $\sim 260$  kD is seen in the detergent-soluble fraction (Fig. 11 A, lane 4), but opsin, the dominant WGA-binding protein of rod outer segment, is not detected. Because opsin is a characteristic component of the soluble fraction, and be-





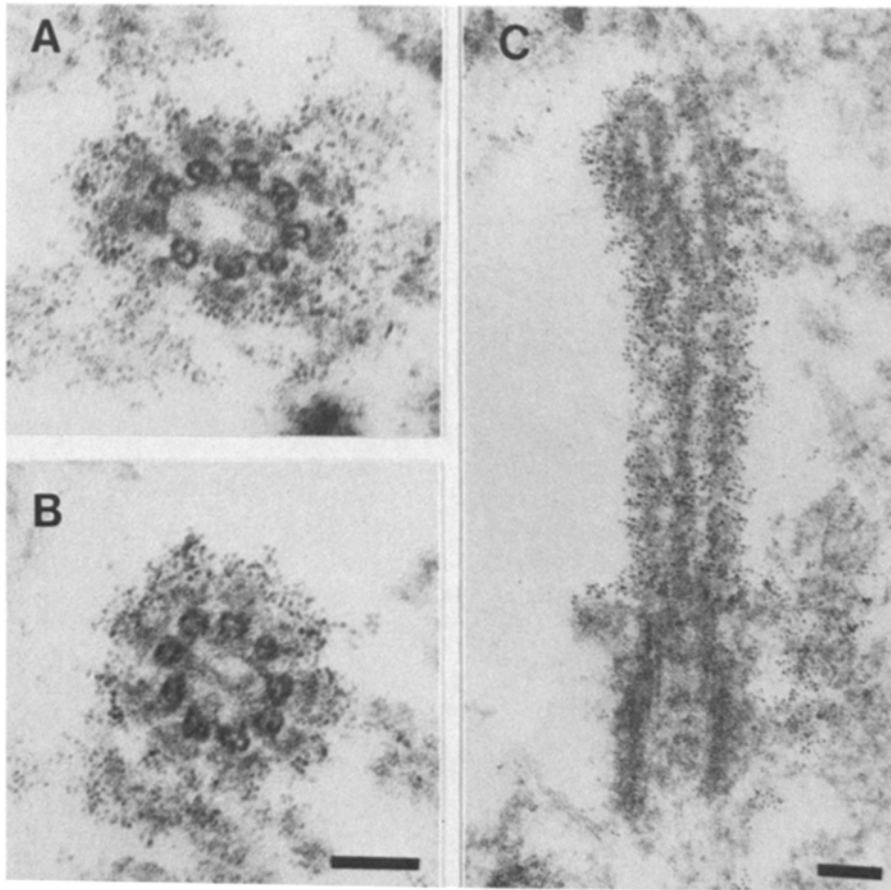
**Figure 7.** Low power electron micrograph illustrating the overall composition of material in fractions enriched in tubulin. The pellet contains numerous axonemes (*solid arrows*) and basal bodies (*open arrows*) as well as amorphous and filamentous material (\*). Note that axonemes are surrounded by a halo devoid of staining. Bar, 0.5  $\mu\text{m}$ .



**Figure 8.** Electron micrographs illustrating the structure of individual bovine axonemes. (A) Transverse section through an in-

dividual axoneme shows the 9+0 structure with all doublet microtubules bearing axoneme-membrane crosslinkers (*arrows*), and the complete removal of the lipid bilayer. (B) In longitudinal section the cross-linkers are distributed along much of the length of the axoneme (*solid arrows*), with transition fibers (*open arrow*) at the junction of the basal body and doublet microtubules. (C) Transverse section through the distal basal body where triplet microtubules change to doublet microtubules. Transition fibers, or alar sheets (*open arrows*), emanate spirally from the microtubules. Bars, 0.1  $\mu\text{m}$ .

cause bands corresponding to the expected position of opsin and its multimers are present in the silver stained gel (Fig. 11 A, lane 2), we assumed that low molecular mass proteins were not being retained by the nitrocellulose. Poor retention of low molecular mass proteins during transblotting could prevent us from detecting low molecular weight, WGA-binding components in the axoneme fraction. Therefore, we carried out electrophoretic transfers using conditions that retain opsin on the nitrocellulose. Using these conditions, large proteins are not as efficiently transferred. The amount of protein loaded to each lane was also increased to further enhance the likelihood of detecting minor WGA-binding components. Under these conditions, op-



**Figure 9.** Electron micrographs of axonemes labeled, before embedment, with WGA-ferritin. (A and B) Transverse sections with ferritin particles heavily labeling the distal ends of the cross-linkers. (C) Tangential, longitudinal section with heavy labeling along the length of the axoneme. Bars, 0.1  $\mu\text{m}$ .

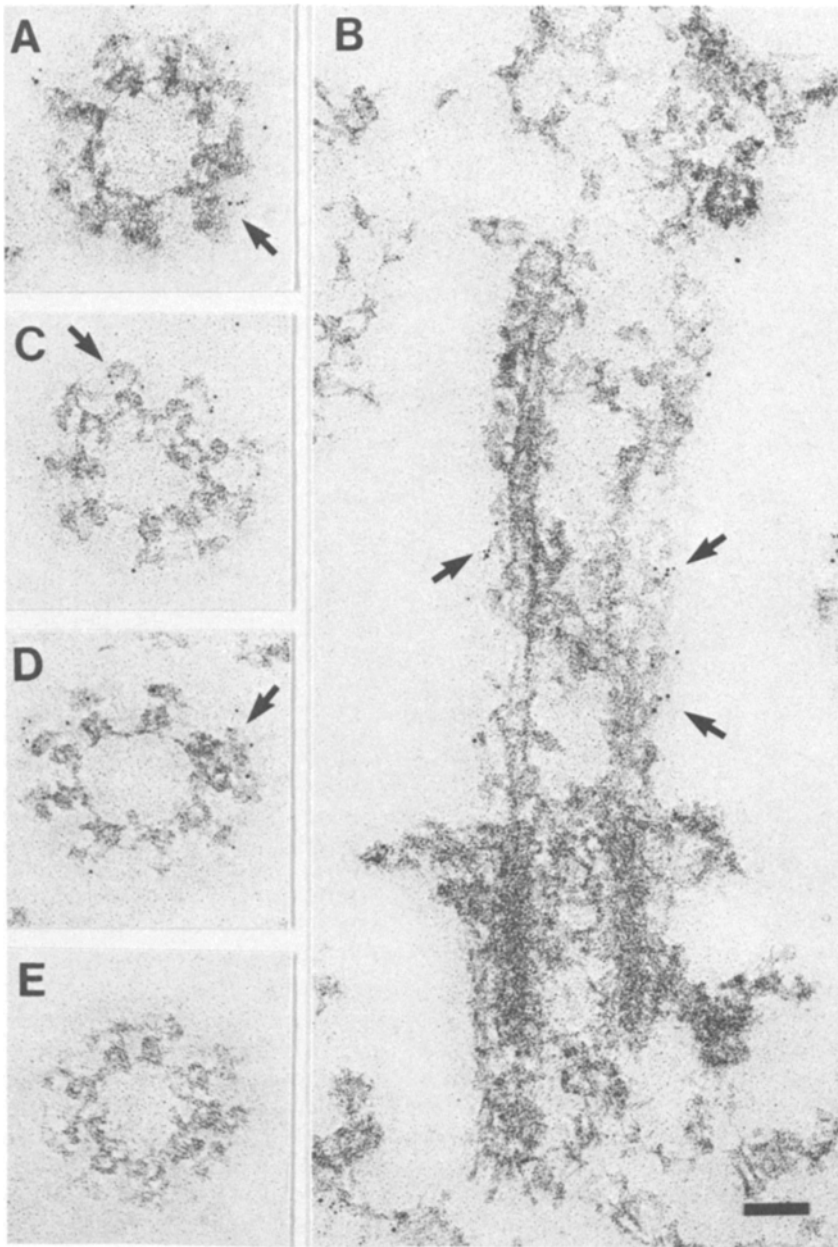
sin and its multimers are easily identified in the detergent-soluble fraction as major WGA-binding proteins (Fig. 11 B, lane 4). In addition, 260- and 220-kD WGA-binding proteins are seen in the detergent-soluble fraction. The same major high molecular mass glycoconjugate seen in lane 3 (Fig. 11 A) is the dominant staining component of the axoneme fraction (Fig. 11 B, lane 3). Because of decreased transfer, the highest molecular mass minor component is, however, lacking. In addition, several lower molecular mass bands are faintly stained. These are considered to be minor components because they do not appear on directly stained gels and are faintly visible even though their transfer to nitrocellulose greatly exceeds that of the high molecular mass components. Because the opsin monomer (37 kD) was not efficiently retained using the electrophoretic transfer conditions illustrated in Fig. 11 B, these data could not rule out the existence of a WGA-binding component smaller than  $\sim 40$  kD in the axoneme fraction. This is unlikely, however, because the direct staining of SDS polyacrylamide gels revealed only the three high molecular mass glycoconjugates described above (data not shown).

Inspection of the protein composition of all fractions from the axoneme isolation reveals the three high molecular mass glycoconjugates (Fig. 6, *arrowheads*) to be enriched in the same fractions enriched in tubulin. This is consistent with the idea that the high molecular mass glycoconjugates are components of the axonemal cross-linkers which either span the bilayer or are associated at the cell surface.

Further characterization of the high molecular mass glycoconjugates using neuraminidase digestion or staining with Con A yield results consistent with surface-staining studies from the neonatal rat retina. Transblots of axoneme fractions subjected to digestion by neuraminidase followed by WGA staining reveal that, whereas the two minor WGA-binding components are neuraminidase sensitive, the 600 kD major WGA-binding glycoconjugate is not (Fig. 12 A). Although not localized electron microscopically on detergent-extracted photoreceptors, Con A is known to bind the connecting cilium of neonatal rat photoreceptors with a similar distribution (7, 25). The 600 kD WGA-binding glycoconjugate is the sole Con A-binding component in the axoneme fraction (Fig. 12 C).

### Discussion

We have identified cell surface glycoconjugates which display a stable interaction with the underlying cytoskeleton of the connecting cilium. These may serve in maintaining the polarized distribution of membrane components by preventing random diffusion between domains. Alternatively, they may serve to facilitate transport of membrane components across the connecting cilium. Morphologic and functional similarities between the photoreceptor connecting cilium and the transition zone of motile cilia lead to the proposal that the cross-linker structure performs a similar role in both systems.

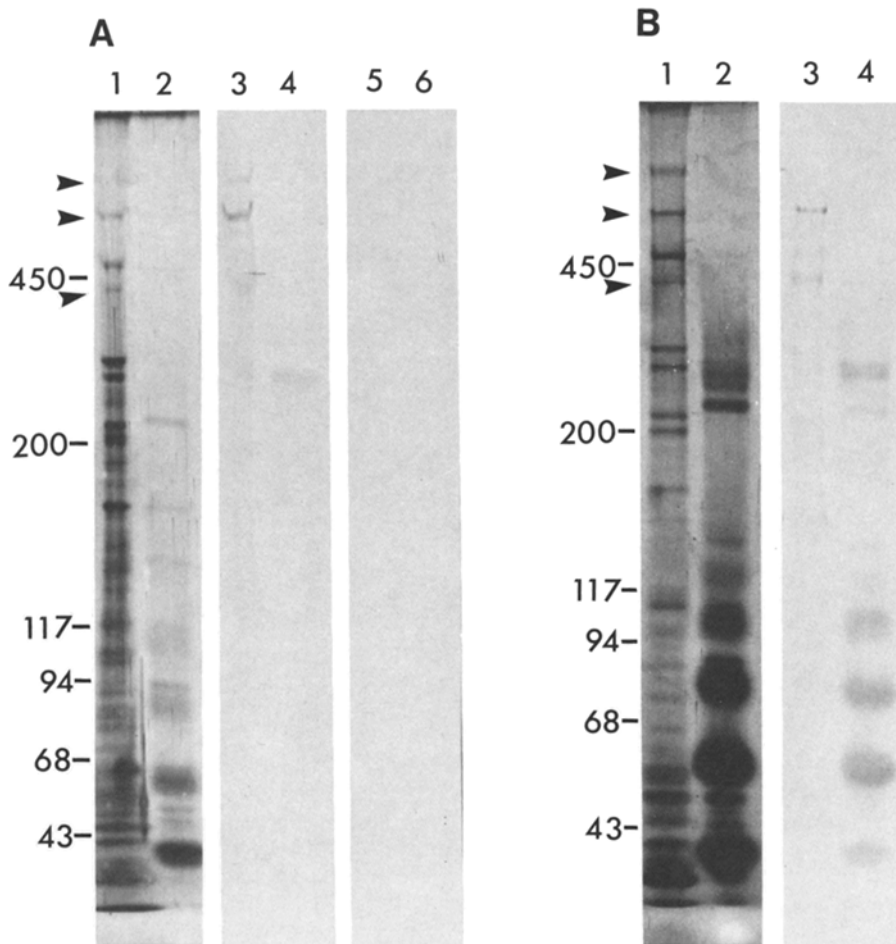


**Figure 10.** Electron micrographs of axonemes in L R White resin labeled, post-embedment, with WGA-colloidal gold. (*A-D*) 5-nm gold particles are seen decorating the distal ends of the cross-linkers (*arrows*). (*E*) The addition of *N,N'*-diacetylchitobiose blocks labeling with WGA. Bar, 0.1  $\mu\text{m}$ .

The vertebrate retinal photoreceptor is a highly polarized cell providing an opportunity to study the mechanisms of both the generation and the maintenance of membrane domains. Throughout its lifetime the photoreceptor must continually generate and maintain a polarized distribution of opsin, the visual apoprotein. The protein synthesis and processing machinery necessary for opsin production are housed in the inner segment. After cotranslational insertion into membrane, and passage through the Golgi apparatus, opsin is packaged into vesicles and transported to the periciliary region. Fracture-label studies have confirmed the presence of immunoreactive opsin in vesicles near the apical region of the inner segment (12). Vesicles are thought to fuse with the plasma membrane (8, 12, 39, 40) and opsin is then transported to the outer segment. Upon reaching the base of the outer segment, opsin is incorporated into newly forming disks (58). The presence of opsin-containing vesicles at the

base of the cilium, in addition to the cilium being the only persistent connection between the inner and outer segments, both suggest that the cilium is the route of transport between the inner and outer segments. There is, however, no direct evidence for this model.

The distribution of opsin immunoreactivity suggests that the connecting cilium may, in fact, not be the route of transport. Opsin immunoreactivity, while high on the outer segment, is virtually absent from the ciliary plasma membrane (7, 37, 40). Rapid transport of opsin across the cilium could still explain the low opsin immunoreactivity along the connecting cilium in static images; however, alternative models must be considered (see Besharse [5] for review). The distribution of opsin immunoreactivity also implies a role for the connecting cilium in the maintenance of photoreceptor polarity. Opsin in the outer segment is highly mobile within the plane of the membrane (43) possibly requiring a mechanism



**Figure 11.** WGA-stained transblots of axoneme and detergent-soluble fractions. (A) Proteins transferred to nitrocellulose under conditions which efficiently transfer large proteins. Lanes 1 and 2 are silver-stained section of gel representing axoneme (1) and detergent-soluble (2) fractions. Lanes 3-6 are blots incubated with WGA-biotin in the absence (3 and 4), or presence (5 and 6), of *N,N'*-diacetylchitobiose and visualized by streptavidin-horseradish peroxidase complex followed by diaminobenzidine. Odd-numbered lanes are axoneme fractions and even-numbered lanes are detergent-soluble material. Arrowheads indicate the three major WGA-binding glycoconjugates. (B) Proteins transferred under conditions which retain low molecular weight proteins. Lanes are as for A. Numbers to the left indicate migration positions of molecular mass markers in thousands.

to prevent diffusion back to the inner segment. Thus, the cell must both efficiently deliver opsin to the outer segment, and prevent its diffusion back to the inner segment, though the mechanisms for these two processes need not be different.

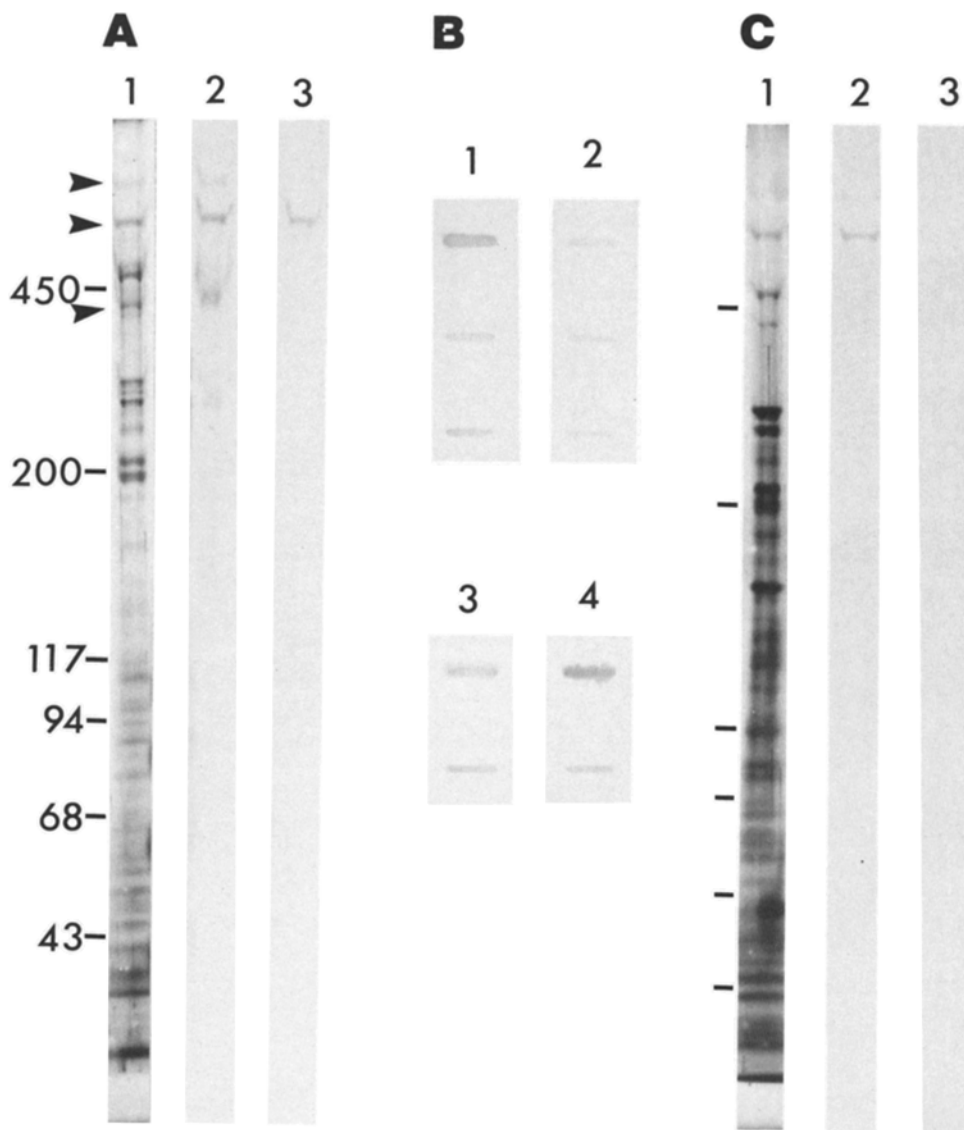
The structure of the connecting cilium is consistent with a role in maintaining distinct membrane domains. Y-shaped cross-linkers seen in transverse section run the entire length of the connecting cilium, reaching from each microtubule doublet to the overlying plasma membrane (7, 47). The distribution of cross-linkers along the longitudinal axis is difficult to determine, although the membrane takes on a beaded appearance with a 32-nm repeat in the neonatal rat, similar to that of the ciliary necklaces (7). Given the expanse of membrane contact made by the cross-linkers, as well as the number of necklace rows extending the length of the connecting cilium, it is reasonable to propose that the cross-linkers perform a barrier function.

The surface of the photoreceptor connecting cilium is rich with glycoconjugates (7, 25, 26). To determine the relationship between surface glycoconjugates and the underlying cross-linkers, we combined detergent extraction with lectin cytochemistry. Detergent extraction of the plasmalemma, while efficiently removing the lipid bilayer, does not remove the cross-linker structures which remain stably associated with axonemal microtubules (18). Extraction of neonatal rat retinas with Triton X-100 followed by WGA-ferritin shows labeling at the distal ends of the cross-linkers. Similar results

are obtained using isolated bovine axonemes. Thus, the cross-linkers reach not only to the plasma membrane, but have an integral membrane portion which extends to the extracellular space. Membrane-microtubule interactions have been described in other systems; these deal primarily with microtubule-associated ATPases (13, 21, 44, 49, 54) and the association of microtubules with secretory granules (51). However, these associations have shown neither the extreme stability (resistance to detergent extraction) nor the trans-membrane connections seen in the connecting cilium.

To further define the structure of the cross-linkers, we sought to identify individual protein components of the crosslinkers. Subcellular fractions enriched in bovine axonemes were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose for staining with WGA. Three high molecular mass glycoconjugates were revealed, all of molecular weight greater than 400 kD. The major WGA-binding protein has tentatively been assigned a molecular mass of 600 kD based on extrapolation from sea urchin sperm dynein heavy chains at 450 kD. However, since the relationship between migration and molecular mass is not necessarily linear in this range, the molecular mass assignment must be considered tentative (4, 28).

The interactions between cross-linkers and other components of the connecting cilium appear more extensive than previously described. Subcellular fractions enriched in axonemes reveal an additional feature of the cross-linkers when



**Figure 12.** Transblots showing Con A binding, and effects of neuraminidase on WGA binding, to axoneme fractions. (A) Effect of neuraminidase treatment on WGA binding to transblots. Lane 1 is a silver-stained gel. Transblots of axoneme fractions were treated with (lane 3) or without (lane 2) neuraminidase before staining with WGA. Neuraminidase digestion does not remove staining of the 600 kD major WGA-binding glycoconjugate, but the two minor WGA-binding components are both neuraminidase sensitive. The WGA staining along the left edge of lane 2 on the bottom half of the blot is opsin from the neighboring lane on the transblot. Arrowheads indicate the three major WGA-binding glycoconjugates. (B) Controls for neuraminidase activity. Fetuin (1 and 2) and ovalbumin (3 and 4) were applied to nitrocellulose using a slot-blot manifold and included in the same incubations with the axoneme transblots illustrated in A (1 and 3 controls, 2 and 4, neuraminidase-treated). WGA staining of fetuin is known to be neuraminidase-sensitive whereas ovalbumin is not sensitive to neuraminidase digestion. Protein amounts on slot blots were (from top to bottom) 100, 10, and 1  $\mu$ g of fetuin (1 and 2), 10 and 1  $\mu$ g of ovalbumin (3 and 4). WGA staining of fetuin, but not ovalbumin is sensitive to neuraminidase digestion as predicted. (C) Con A staining of axoneme fraction transblots. The 600 kD glycoconjugate stains specifically with Con A (lane 2) as revealed by blocking with  $\alpha$ -methylmannopyranoside (lane 3). Lane 1 is a silver-stained gel. Molecular mass markers (in kD) are the same for A and C.

pellets are viewed by conventional thin section electron microscopy. In pellets, axonemes are routinely surrounded by an unstained area extending beyond the cross-linkers to what would be, in vivo, extracellular matrix space. This suggests that the cross-linkers possess not only a transmembrane component but also interact with an extensive extracellular component, and might actually serve to connect the axoneme to the extracellular matrix.

The exact nature of the 600 kD glycoconjugate, as well as the relationship between extracellular matrix components and the WGA-binding glycoconjugates, is presently unclear. One possibility is that the WGA-binding component is an integral membrane proteoglycan (11, 30, 46). Such an interac-

tion between an integral membrane proteoglycan and the cytoskeleton has been suggested in mammary epithelial cells and cultured fibroblasts where a membrane intercalated heparan sulfate proteoglycan may link extracellular matrix components to intracellular actin filaments (45, 57). WGA has been shown to bind glycosaminoglycans (52). The unusually large size of the WGA-binding component, as well as electron microscopic images of intact photoreceptors where strings of succinyl-WGA-ferritin are seen extending great distances from the plasmalemma, could be consistent with the presence of a WGA-binding glycosaminoglycan. This might explain why the intramembraneous particles of photoreceptor ciliary necklaces partition with the E face in freeze-fracture

images (7, 33, 47). An extensive extracellular oligosaccharide, or an extracellular matrix connection, would not be readily pulled through the outer leaflet as would be necessary to partition with the P face.

The numerous morphologic similarities between the connecting cilium and the motile cilium transition zone have been described (47). In addition, they share common lectin-binding characteristics. The transition zone plasmalemma of quail oviduct cilia also bears both WGA- and Con A-binding sites (48). In addition, the WGA-binding sites are not sensitive to neuraminidase treatment, and are not removed by chloroform methanol extraction after fixation (48).

The increasing list of features shared by the photoreceptor connecting cilium and transition zone of motile cilia further the idea that they are homologous structures, and suggests that the cross-linkers serve a similar role in both systems. That this region is placed at the junction between distinct membrane domains implies that the cross-linker may perform a barrier or selective transport function. Through the identification of individual components of the cross-linkers we will be better able to define their actual function.

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