Preliminary Biochemical Characterization of the Stereocilia and Cuticular Plate of Hair Cells of the Chick Cochlea

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Abstract. The sensory epithelium of the chick cochlea contains only two cell types, hair cells and supporting cells. We developed methods to rapidly dissect out the sensory epithelium and to prepare a detergent-extracted cytoskeleton. High salt treatment of the cytoskeleton leaves a "hair border", containing actin filament bundles of the stereocilia still attached to the cuticular plate. On SDS-PAGE stained with silver the intact epithelium is seen to contain a large number of bands, the most prominent of which are calbindin and actin. Detergent extraction solubilizes most of the proteins including calbindin. On immunoblots antibodies prepared against fimbrin from chicken intestinal epithelial cells cross react with the 57- and 65-kD bands present in the sensory epithelium and the cytoskeleton. It is probable that the 57-kD is a proteolytic fragment of the 65-kD protein. Preparations of stereocilia attached to the overlying tectorial membrane contain the 57- and 65-kD bands. A 400-kD band is present in the cuticular plate. By immunofluorescence, fimbrin is detected in stereocilia but not in the hair borders after salt extraction. The prominent 125 Å transverse striping pattern characteristic of the actin cross-bridges in a bundle is also absent in hair borders suggesting fimbrin as the component that gives rise to the transverse stripes. Because the actin filaments in the stereocilia of hair borders still remain as compact bundles, albeit very disordered, there must be an additional uncharacterized protein besides fimbrin that cross-links the actin filaments together.

MUCH structural information has been published in recent years on hair cells of the cochlea. Most of these studies have concentrated on the stereocilia and the underlying cuticular plate as this is the region of the cell crucial to sensory transduction. Attention has been directed to the structure (stereocilia and cuticular plate) because the cochlea is an amazingly specialized and stereotyped "actin machine" from which we might gain insights into how actin assembly and cross-bridging may be regulated in cells generally. To extend our knowledge of both sensory transduction and this actin machine, what is needed is a chemical characterization of these structures. Because of the extreme difficulty in obtaining enough material for a biochemical characterization, only two publications have appeared which have attempted to show on an SDS polyacrylamide gel the macromolecules present in the hair cell (11, 20). Both of these reports are of limited use as they involve the whole epithelium, not purified stereocilia. In contrast, a number of investigators have tried to identify the major components of the stereocilia and cuticular plate using immunofluorescent techniques or electron microscopy. The antibodies tested have been prepared against proteins in the brush border of intestinal epithelial cells; e.g., actin, myosin, tropomyosin, α actinin, fimbrin, villin, and spectrin.

There are three major difficulties with such a strategy. First, the stereocilia and cuticular plate may have major proteins in them that are specific to hair cells and not present in intestinal epithelial cells. Second, some investigators have reported the presence of a protein at certain locations in hair cells, yet others using a different antibody to the same protein, show that it appears to be absent. Two examples are: antifimbrin, localized to the stereocilia and cuticular plate by Slepecky and Chamberlin (22) and by Sobin and Flock (23), but only in the stereocilia by Drenckhahn et al. (11); and antimyosin, in one case localized in stereocilia and cuticular plates (16), yet in another report only in the cuticular plate, not the stereocilia (10). Third, the antibody methods do not really tell us if the brush border and hair cell proteins are identical or if they just share common domains.

An independent method to determine precisely what proteins are present in the stereocilia and in the cuticular plate and in what molar ratios to actin is needed. The most straight forward way to do this is to isolate the stereocilia and/or cuticular plate and biochemically determine what proteins are present in what relative concentrations. This is by no means a trivial task, as will be shown, because to obtain enough material to give adequate bands on a single lane of a silver stained SDS polyacrylamide gel (6-cm-long by 0.75-

mm-thick), the sensory epithelia of minimally five cochleae must be dissected free from all surrounding organs (the cochlea is, after all, a complex organ containing many cell types) and combined.

We will describe in great detail the methods used to obtain our results hoping to stimulate others to study this fantastic system as we believe the hair cell and particularly its differentiation in embryos will ultimately give us insights into how the actin cytoskeleton is formed.

Materials and Methods

The Cytoskeleton Preparation

Our initial cytoskeleton preparation was made by immersing the sensory epithelium in a solution containing 1% Triton X-100, 100 mM Pipes, 5 mM $MgSO_4$, and 5 mM EGTA at pH 6.8. More recently we modified this solution although the end result is exactly the same. We now use 1% Triton X-100, 0.3 mM $MgSO_4$ in 20 mM Pipes at pH 6.8. To either solution the proteolysis inhibitor, PMSF (Sigma Chemical Co., St. Louis, MO), at a concentration of 0.1 mg/ml was added just before immersion. A stock solution of 10 mg/ml PMSF in proponol was kept in the freezer until use. In either case the epithelium was left in the solution at 4°C for 5 min. The membranes were solubilized and the soluble proteins released. The residual insoluble cytoskeleton was concentrated by centrifugation (10,000 *g* for 3 min).

Isolation of the "Hair Borders"

Early on in this study we prepared hair borders by suspending the cytoskeleton preparation in 1 M NaCl, 3 mM $MgCl_2$, and 30 mM Tris at pH 8.0. Later we used 1 M NaCl, 0.3 mM $MgCl_2$ in 20 mM imadizole at pH 7.0. Both solutions contained PMSF and gave the same result. The cytoskeleton was left in the solution at 4°C for 10 min. The insoluble material, which contains the hair borders (the chromatin disappears, being broken down into histones and DNA, and the apical end of the supporting cells solubilizes, as do junctions, etc.), was now pelleted at 10,000 *g* for 1 min. This pellet and pellets of the sensory epithelium, the cytoskeleton preparation, and the tectorial membranes were either run immediately, after boiling, by SDS-PAGE or immediately frozen and maintained in the frozen state at −80°C until the samples were thawed and subjected to electrophoresis.

SDS-PAGE and Immunoblotting

The samples were analyzed by SDS-PAGE using minigels, 6 × 7 cm by 0.75 or 1.5 mm in thickness. 8 or 10% gels were poured using the buffer formulations of Laemmli (15) and then silver stained according to the method of either Sammons et al. (21) (Gelcode, Pierce Chemical Co., Rockford, IL) or Merrill et al. (19) to increase the sensitivity for detection of the proteins. In general we found that the Merrill stain and the 0.75-mm-thick gels proved to be most effective as the quantity of protein was extremely small. For each lane we knew the number of cochleae with which we started, a value we refer to as "ear equivalents" and accordingly our dilution into sample buffer was one ear equivalent into 5 μl of buffer. At least five ear equivalents were needed for a single lane.

For immunoblotting, after separation the proteins were transferred to nitrocellulose sheets (29) and antibodies against fimbrin, villin, myosin, or gelsolin (the latter three were kindly donated by Drs. Burgess [University of Miami] and Lazarides [California Institute of Technology, Pasadena]) were added. BSA was used as the blocker. The secondary antibody, alkaline phosphatase–coupled anti-rabbit IgG was purchased from Promega Biotec (Madison, WI).

Light Microscopy and Immunofluorescence

Dissections were carried out using an Olympus dissecting microscope with transmitted and darkfield illumination. In the early stages in the dissection an external light source is necessary. The sensory epithelium and various extractions of it were monitored with a Zeiss compound microscope equipped with phase contrast or Nomarski interference contrast using a 95× oil immersion objective lens.

For immunofluorescence studies the isolated sensory epithelium and cytoskeleton were fixed for 30 min with 3.7% paraformaldehyde in 50 mM phosphate buffer at pH 6.3, washed three times in phosphate buffer, and "blocked" with 20 mM Pipes, 0.1% Triton X-100, 1.5 mM $MgCl_2$, and 1% serum albumin at pH 6.8 for 30 min. The tissue was then incubated in affinity-purified antifimbrin (12) for 1.5 h, washed three times, and incubated in goat anti-rabbit IgG conjugated with rhodamine (Sigma Chemical Co.) for 30 min. To examine hair borders, the sensory epithelium, still connected to the tectorial membrane, was incubated in 1% Triton X-100 with 1 M NaCl and 20 mM Pipes at pH 6.8 for 10 min, which solubilizes the cytoplasmic components but leaves the hair borders weakly attached to the tectorial membrane. The DNA in the chromatin becomes uncoiled and wraps around the hair borders keeping them from floating away. The hair borders plus tectorial membrane were then pipetted gently into 1% Triton in 20 mM Pipes and 1 mM $MgCl_2$ at pH 6.8. After resting there for 5 min, a drop of hair border and tectorial membrane was deposited onto a polylysine coated slide and processed for immunofluorescence as described above.

Electron Microscopy

For scanning electron microscopy the isolated tectorial membrane was fixed in 1% OsO_4 in 0.1 M phosphate buffer at pH 6.3, dehydrated in acetone, critical point dried, and sputter coated. The specimens were attached to stubs and examined with an AMR 1000 scanning electron microscope.

For transmission microscopy the sensory epithelium or extracts of it were fixed by immersion for 30–45 min in 1% OsO_4, 1% glutaraldehyde (from an 8% stock purchased from Electron Microscope Services, Fort Washington, PA) in 0.1 M phosphate buffer at pH 6.3 at 4°C. The fixative was made up immediately before use. The fixed material was washed three times in water at 4°C and then enbloc stained with 0.5% uranyl acetate 3 h to overnight at 4°C. The specimens were dehydrated in acetone and embedded in Epon. Thin sections were cut with a diamond knife and examined with a Philips 200 electron microscope after staining with uranyl acetate and lead citrate.

Results

Removal of the Cochlear Duct

It is most important that young chicks be used. Chicks 7–14 d of age are optimum because the cochlea is mature, yet the skull is still mainly cartilaginous, allowing the removal of the cochlea with fine forceps. Older animals have bony skulls making the removal of the cochlea increasingly difficult. Chicks are sacrificed by decapitation. The skin and underlying muscles are cut away with a sharp pair of scissors thereby exposing the tympanic membrane (ear drum). The connective tissue immediately posterior to the tympanic membrane is grasped by a blunt pair of forceps and pulled anteriorly, removing both the connective tissue and the tympanic membrane. In most cases the stapes comes away with the tympanic membrane. If not, it is removed with a finer forceps. At this point both the oval and round windows are visible and the bony-cartilaginous bar separating these windows is removed. Fine forceps are run along the inside of the cartilaginous wall of the cochlea which splits open the blue cartilage covering the medial part of the duct. This cartilage is then folded back revealing the white otoconia at the lagena or distal end of the cochlea. The proximal end of the basilar papilla is now grasped firmly with forceps and with a shaking motion the entire basilar papilla is removed and placed into cold Hanks medium buffered to pH 7.4 with Hepes. This method of removing the basilar papilla takes <1 min and is all carried out under a dissecting microscope.

Isolation of the Sensory Epithelium

The isolated cochlea in the petri plate containing the Hanks medium is now viewed with transmitted light (Fig. 1 *a*). The cochlea is held by one pair of fine forceps and with a second pair the tegmentum vasculosum is removed and the otoconia

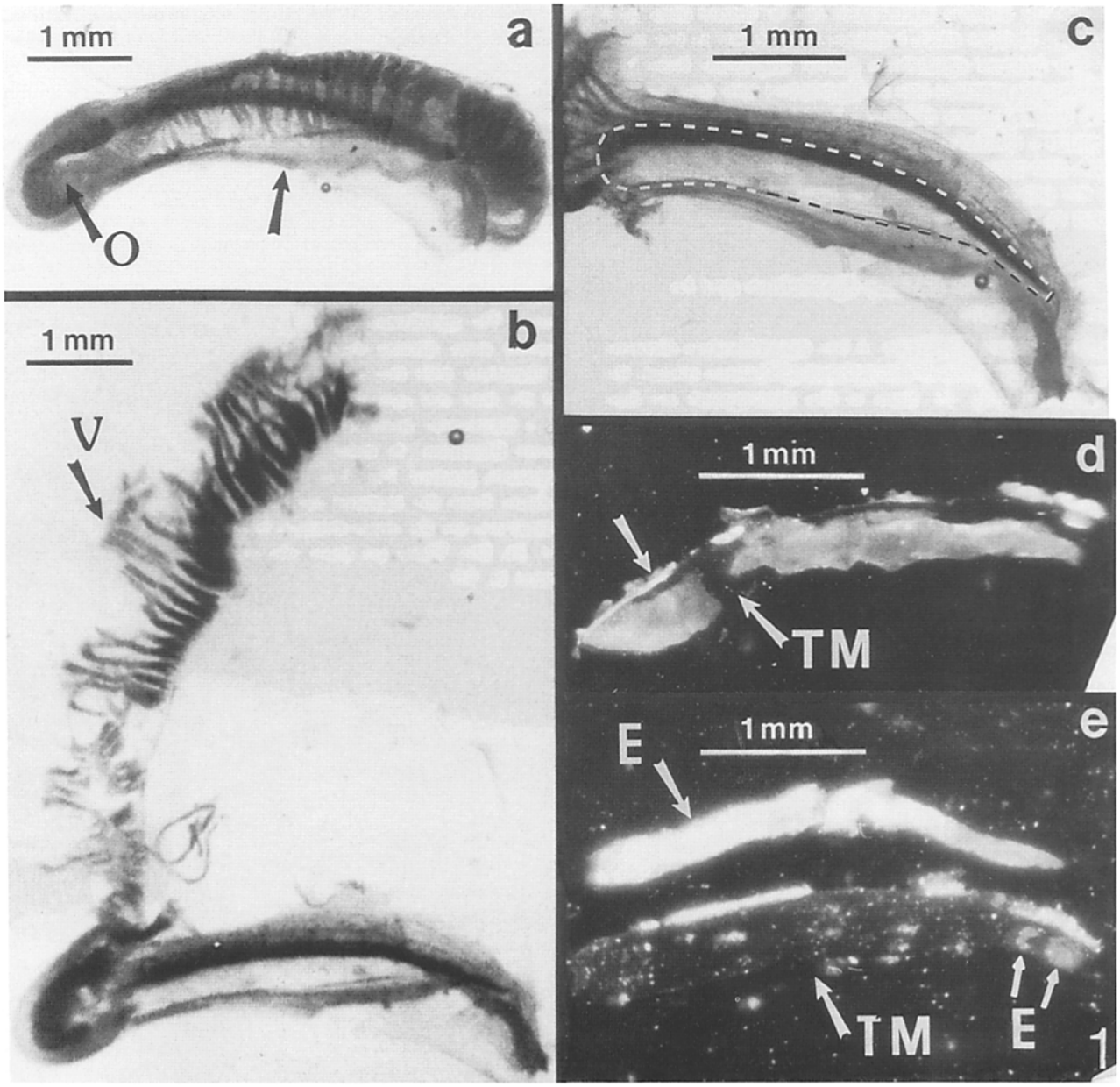


Figure 1. Intermediate stages in the isolation of the sensory epithelium. (*a*) Entire cochlea of the chicken taken through a dissecting microscope. The octoconia (*O*) are present at the distal end. The arrow indicates a spur of tissue that can be grasped with fine forceps before removal of the tegmentum vasculosum. (*b*) The tegmentum vasculosum (*V*) has been pulled away from most of the rest of the cochlea. The viewer is now looking directly down on the sensory epithelium. (*c*) The tegmentum vasculosum has been removed as have the otoconia. The dotted line indicates the position of the sensory epithelium. The epithelium can be seen if the cochlea is tilted somewhat. (*d*) The sensory epithelium with its associated tectorial membrane is dissected away from the rest of the cochlea and examined in the darkfield mode of the dissecting microscope. A break in the sensory epithelium allows us to see the tectorial membrane (*TM*) which is nearly transparent. The arrows point to the homogene cells that remain attached to the tectorial membrane. (*e*) The sensory epithelium (*E*) is gently pried away from the tectorial membrane (*TM*) with needles. The tectorial membrane still has two tiny fragments of sensory epithelium (*E*) attached to it (*arrows*). The bright cells at the superior margin are homogene cells. Other bright spots are dirt and otoconia.

scraped out (Fig. 1 *b* and *c*). Papain (Type III with an activity of 16–40 U/mg (P3125) obtained from Sigma Chemical Co.) is added to the cochlea in fresh Hanks solution. 25 μl of papain is added to 10 cc of Hanks and digestion is allowed to proceed for 30 min at room temperature. The cochlea is then transferred to fresh Hanks solution at 4°C containing 0.5 mM para-chloroaminobenzoate (PCMB) to inactivate the papain and the sensory epithelium is teased away from the cochlea proper (Fig. 1 *c*). Using the darkfield mode on the dissecting microscope the tectorial membrane is visible (Fig. 1 *d*) and can be grasped with fine forceps and the sensory epithelium stripped off (Fig. 1 *e*). Alternatively the sensory epithelium together with the overlying tectorial membrane can be dissected off the basilar membrane using 27 gauge (injection) needles. These needles turn out to be ideal as they taper and the exact point has a burr presumably formed during manufacture. Using these same needles the tectorial membrane can be separated from the sensory epithelium.

Sometimes the sensory epithelium can be teased away as a single piece. In most cases it comes away in two to five fragments. The isolated epithelium and/or fragments from 5 to 10 cochleae are pipetted into an Eppendorf tube and concentrated by centrifugation (10,000 *g* for 15 s). This we refer to as the sensory epithelium. It consists of only two cell types, hair cells and supporting cells. As a control, tectorial membranes are pipetted into a second Eppendorf tube and concentrated by centrifugation.

Fine Structure of the Sensory Epithelium and Its Extracted Fractions

Untreated Sensory Epithelia. A low magnification electron micrograph of a portion of the isolated epithelium reveals the basic structure (Fig. 2). Seen in this micrograph are hair cells separated by supporting cells. Since the epithelium strips away from underlying tissues at the level of the basal lamina, there are only two cell types present. The supporting cells in the isolated sensory epithelium tend to lose cytoplasmic density as if the plasma membrane were no longer completely intact. In contrast, the hair cells look identical to those of an untreated cochlea (14, 25, 27). Since the hair cell cytoplasm does not extend all the way to the basal lamina, yet that of the supporting cells does, it is probable that the plasma membrane of the supporting cells is compromised at its basal surface during removal of the sensory epithelium from the basal lamina.

In some regions of the cochlea there is a bulging outwards of the hair cell plasma membrane immediately adjacent to the supporting cells, an area of cytoplasm not underlain by cuticular plate. We presume that this is due either to the fact that the tectorial membrane is mechanically pulled away from the sensory epithelium thus putting shear on the apical surface of the hair cells or to the lack of O_2 in our solutions. The membrane is intact in this region so that if it were pulled away during dissection, it resealed before fixation. Consistent with this observation is the fact that the density of the hair cell cytoplasm is unaltered. The inset in Fig. 2 is a transverse section of a stereocilium. The dots are the actin filaments. In most cases these filaments appear hexagonally packed. Fig. 3 is a longitudinal section of a stereocilium from the isolated epithelium. The 125 Å transverse striations are the cross-bridges between individual filaments (4, 26, 28). These are more obviously seen if the page is rotated by 90°.

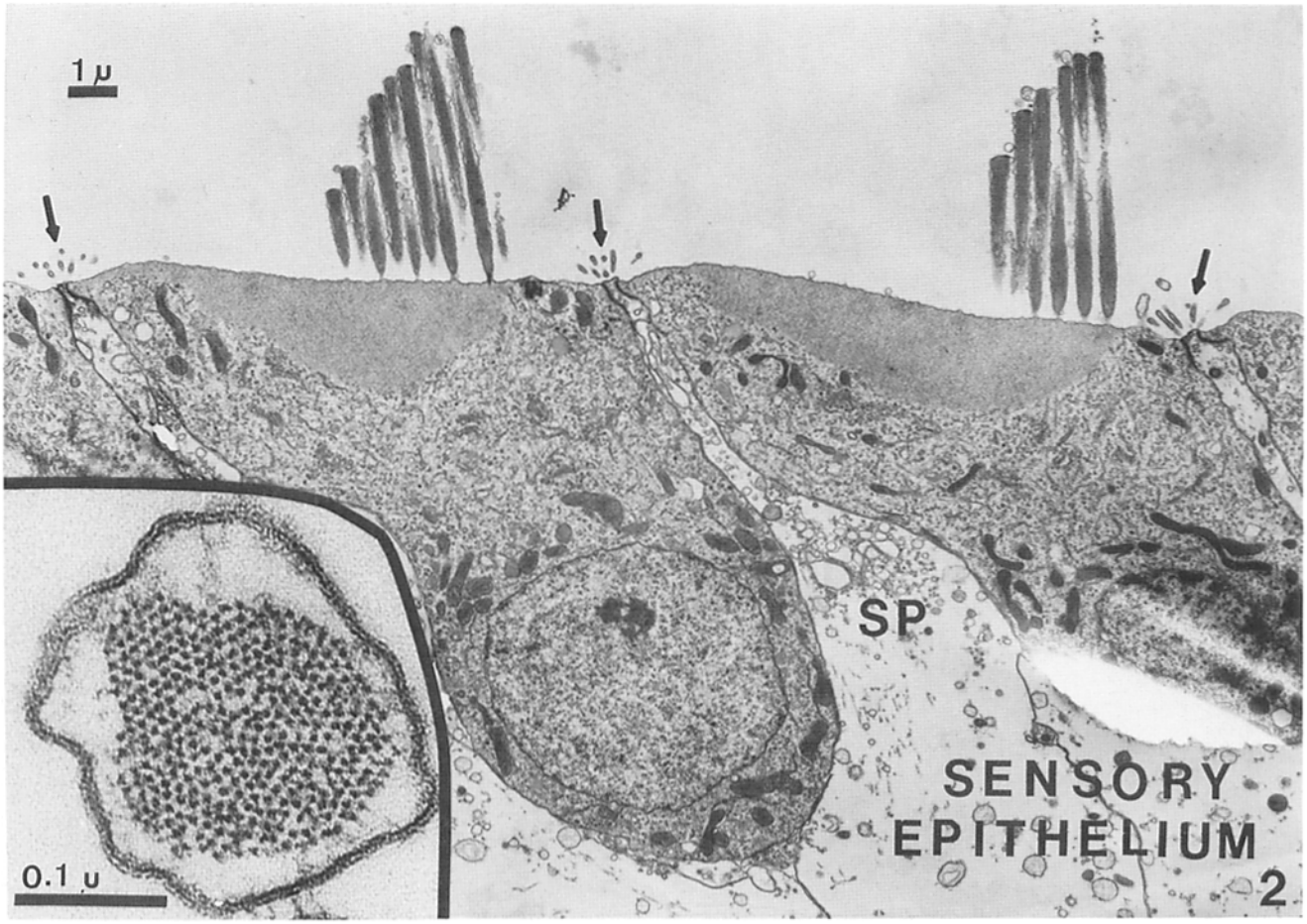


Figure 2. Thin section through a portion of the isolated sensory epithelium. The apical end of two hair cells with their stereocilia and cuticular plates are present in this micrograph. Between the hair cells are supporting cells (*SP*) whose cytoplasm appears "washed out". The arrows indicate microvilli on the apical end of supporting cells. In the intact cochlea the tectorial membrane would be connected to these microvilli and the stereocilia would insert into it. (*Inset*) Transverse section through a stereocilium. The dots are the actin filaments cut in transverse section. In general the actin filaments are hexagonally packed although there are regions where the packing is imprecise.

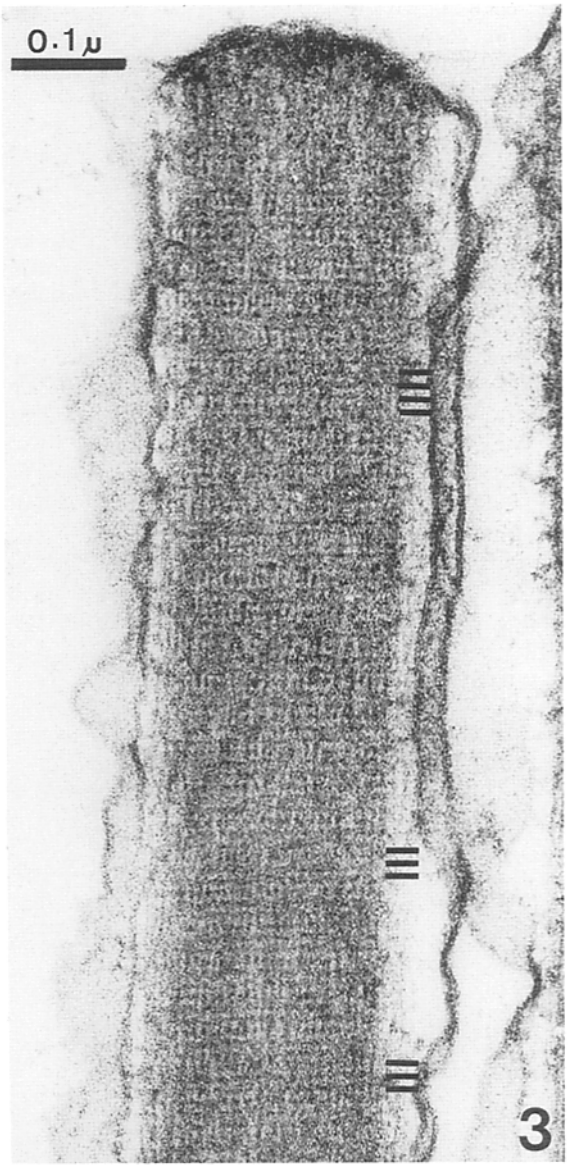


Figure 3. Longitudinal section through a stereocilium from the isolated sensory epithelium. Fine, transverse, periodic stripes are visible which are even more dramatically seen if the stereocilium is looked at on its side (rotate the journal 90°). These are the bridges between actin filaments. Some of the bridges are indicated by the lines.

The Cytoskeleton. The sensory epithelium is extracted with Triton X-100 to make a cytoskeletal preparation. By light microscopy the epithelium appears less dense, but otherwise intact, until the preparation is "vortexed", homogenized, or pipetted vigorously. As observed in thin sections, detergent extraction induces the removal of most of the cytoplasmic density and it dissolves the membranes and membrane-bound organelles such as mitochondria, ER, granules, and vacuoles (Fig. 4). The chromatin remains intact, although the nuclear envelope becomes solubilized. Polysomes and ribosomes disappear from their normal cellular locations. They tend to stick to the actin filaments making up the cores of the stereocilia. Although the plasma membrane that limits the stereocilia and apical surface of the cuticular plate is almost completely solubilized, at the cell junctions the plasma membrane remains. Fragments of the plasma membrane are often found between what was once the lateral margins of hair cells and supporting cells.

Of interest to this report is the cytoskeleton. The actin filaments and their arrangement in the stereocilia and cuticular plate appear essentially unaltered by detergent extraction. The transverse stripes across the filaments in the stereocilia at 125-Å intervals indicate that the cross-bridges between the filaments remain (Fig. 4) and the actin filaments maintain their basic hexagonal packing. Likewise the cuticular plate has the same substructure as in unextracted cells (DeRosier, D. J., and L. G. Tilney, manuscript submitted for publication). Bundles of microtubules remain in what is left of the supporting cells. Intermediate filaments are found around the chromatin, but are completely absent from the cuticular plate. These tend to be sparse and easy to miss in thin sections. We presume that, although they are a very minor fraction of the cytoskeleton and easy to "miss" unless the micrographs are carefully examined, they hold the chromatin in the cell ghosts in the same location as the nucleus in unextracted cells. In hair cells the nuclei reside near the apical surface, while in supporting cells they are more basal (Fig. 4).

Hair Borders. When we perfuse high salt into a preparation of detergent-extracted sensory epithelia while viewing the preparation with the light microscope, the nuclear mass is seen to gradually disappear due to the solubilization of the histones and the hair borders float away (Fig. 5 *a*). In thin sections of pellets of extracted epithelia treated with high salt the major components are the hair borders. The other material in these pellets cannot be identified. The cuticular plate is easy to recognize with bundles of stereocilia extending from it. When hair borders are examined at low magnification in thin sections (Fig. 5 *b*), the organization of the stereocilia appears unchanged and the actin filaments making up the stereociliary core are held tightly together and do not fray. Furthermore, the shape of the cuticular plate is unchanged. At higher resolution, however, the packing of the actin filaments in the stereociliary bundle is seen to be altered. As seen in longitudinal sections (Fig. 6 *a*) the actin filaments do not remain perfectly parallel such as seen in unextracted stereocilia or those extracted with Triton in low salt, but instead present a somewhat wavy profile (Fig. 6 *a*). More importantly the transverse striping which has been shown to be due to the cross-bridges is no longer visible (compare Figs. 2 and 4 with Fig. 6). In transverse sections the actin filaments are no longer hexagonally packed (Fig. 6 *b*), but lie in short rows which are frequently curved. These rows can be parallel to each other or can be oriented at varying degrees to other rows. Individual filaments are rarely found. The short rows of filaments must be connected together because the stereocilia cores do not fragment or fray, even after extraction in high salt for 3 h.

Although it might be possible that fixation has induced a change in the packing of the actin filaments, it seems unlikely because the actin filaments, notoriously difficult to preserve by conventional fixatives remain intact in the cuticular plate. Nevertheless, to see if improper fixation was a consideration, we fixed the "hair borders" in a variety of ways. First, we fixed a pellet of hair borders in the same way as we fix pellets of sensory epithelia or detergent extracted epithelia. Second, thinking that high salt might influence fixation, we washed the hair borders in low salt after high salt extraction, repel-

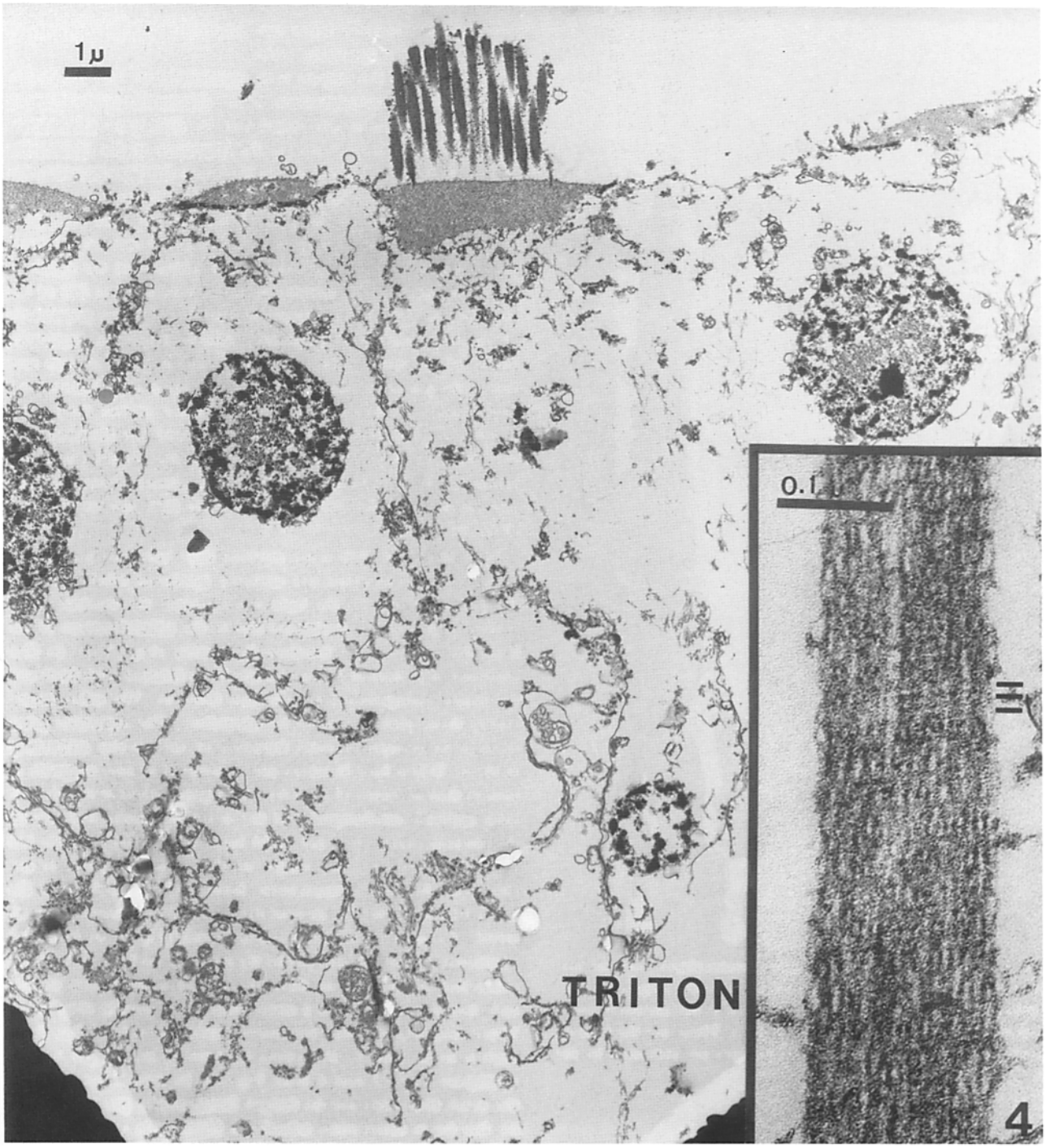


Figure 4. Thin section through a detergent-extracted sensory epithelium. The membranes surrounding the hair cells and supporting cells are solubilized as are the membraneous organelles such as the mitochondria, ER, and vacuoles. The nuclear envelope is also solubilized, but the chromatin stays intact. (*Inset*) The actin filament organization in the stereocilia and cuticular plate are not affected by the detergent extraction. Longitudinal sections of the actin filament bundle formerly in the stereocilia show the 125 Å periodic transverse striations present in the unextracted cell (see lines).

leted them and fixed them. As a control we took some low salt, detergent-extracted epithelia and left them in this medium for the same length of time as it took to extract, wash, and repellet the high salt preparation. Third, thinking that the osmium might be the problem because osmium tends to destroy actin filaments (18), we fixed a pellet of hair borders in glutaraldehyde alone, without osmication. Fourth, we changed the concentration of magnesium and used different buffers for the high salt incubation step before fixation. We used the same buffers and magnesium concentration on detergent-extracted epithelia in low salt as controls. All these protocols gave the same result indicating that the change in packing and apparent loss of cross-bridges observed is not induced by improper fixation.

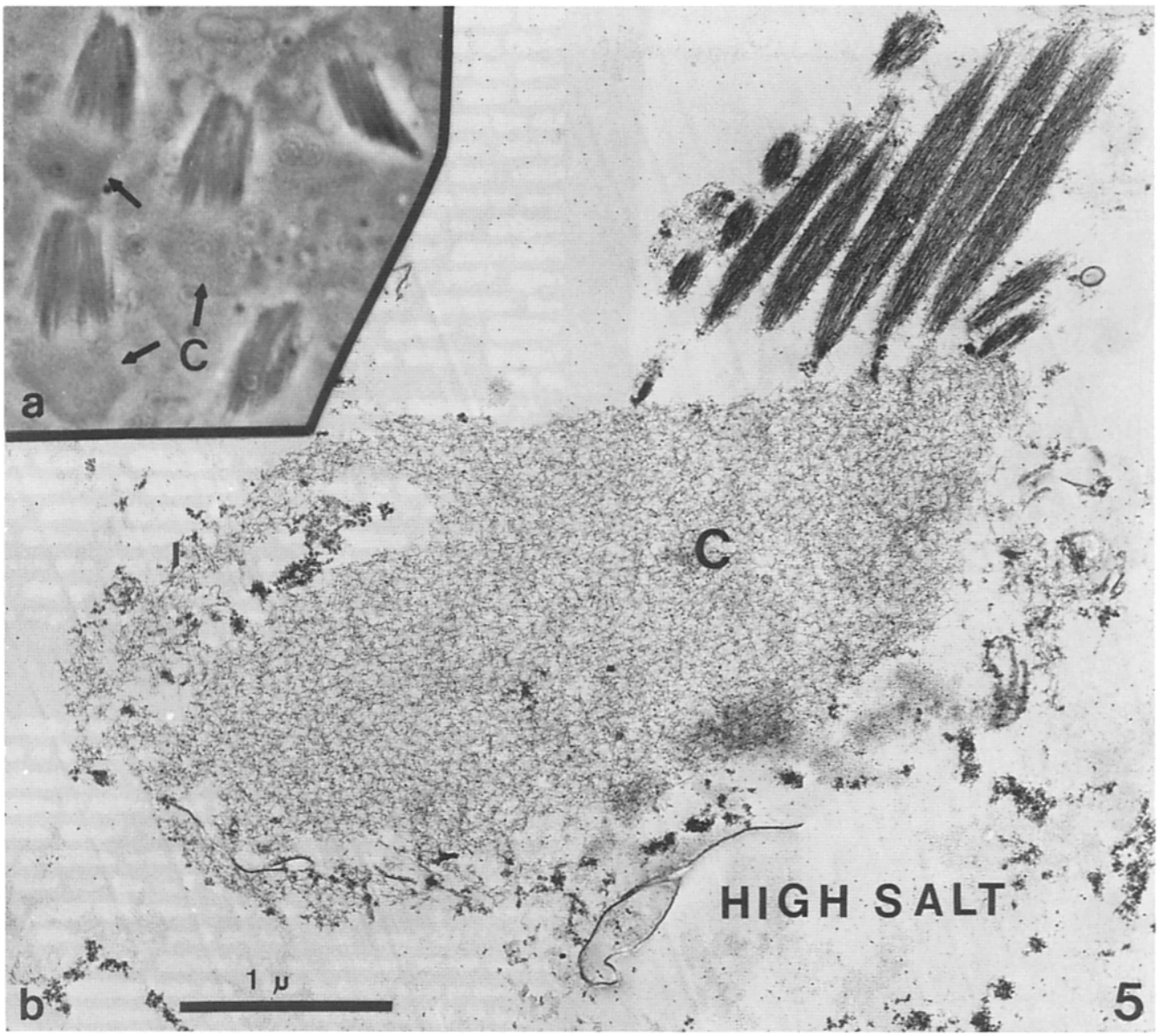


Figure 5. (*a*) Light micrograph (phase contrast) of a high salt, Triton-extracted sensory epithelium. Visible here are the "hair borders" of five hair cells. The core filament bundles of the stereocilia are still connected to their cuticular plates (*C*). The hair borders float freely in solution. All the other structures including the chromatin have been solubilized. (*b*) Low magnification, thin section through a hair border. Notice that except for few membrane remnants the rest of the cell including the junctional complexes are solubilized by the high salt. Remaining are the cuticular plate and its attached stereocilia.

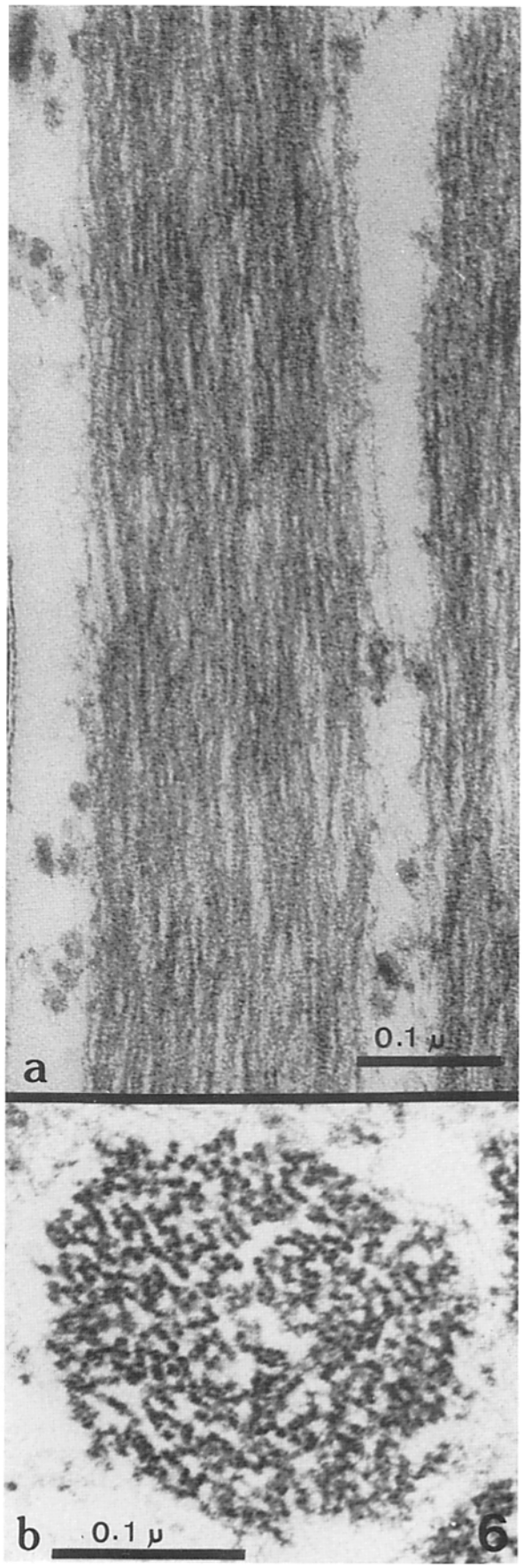
SDS-PAGE

Since the tectorial membrane is our greatest source of contamination, we will begin by describing its behavior on an SDS polyacrylamide gel (Fig. 7 *a*). The tectorial membrane consists of a number of bands, most prominent of which are two bands of 37 and 175 kD. There is a less prominent band at 30 kD. We find minor components at 65 and 43 kD which vary in intensity depending upon the preparation.

When we examine the unextracted sensory epithelium minus the tectorial membrane, we see a large number of bands, as expected (Fig. 7 *b*). The most prominent is a 23-kD protein which Oberholtzer et al. (20) have identified as calbindin, a calcium binding protein. As others have noticed, with some silver staining techniques extremely prominent bands such as these give a negative image. There is also a prominent band near the dye front which is presumably due to the histones. The second most prominent band has a molecular weight of 43,000 and corresponds to actin. There are a number of other bands. Most noticeable are those at 28, 33, 38, 50, 57, 65, 73, 87, and 95 kD.

On gels of sensory epithelia which were extracted with detergent in low salt we find a reduction in the number of bands as well as in the amount of certain bands indicating that they are not bound to the cytoskeleton or are very weakly bound (Fig. 7 *c*). In essence this method removes soluble proteins. The most prominent band removed is the calbindin band at 23 kD.

In contrast to the sensory epithelium and the low salt, detergent extracted epithelium, gels of the "hair borders", sensory epithelia that have been extracted with detergent and high salt (1 M NaCl), show a remarkable simplification in



band number (Fig. 7 *d*). Most prominent is the 43-kD band which presumably is actin. Below this the bands are very weak and in some gels absent altogether. Above the 43-kD band there are prominent intermediate bands at 57 and 65 kD and near the top of the gels, a series of bands whose molecular weights must be considerably larger than myosin heavy chain (200,000). Since in this part of the gel the proteins do not migrate proportional to the log of their molecular weights, we do not know the actual molecular weights of these bands, but estimate that the major one is in the 400,000 range. There is a pair of bands even higher than this whose real molecular weights are unknown, but must be in the 500,000 to million range (Fig. 7 *d*). Thus the hair border fraction consists of only three major bands, 43, 57, and 65 kD.

As mentioned in Materials and Methods, our most sensitive silver staining was the Merrill stain on 0.75-mm-thick gels. To have enough protein to adequately visualize the bands we needed for each lane a minimum of five sensory epithelia or "five ear equivalents" of starting material. We tried roughly to quantitate the relative amount of protein in the hair borders, a difficult and capricious task with silver staining techniques as the staining saturates. Using gels of different loadings our best estimates of the ratio of actin to the two other major bands (57 and 65 kD) was 6:1.

Immunoblots

Since our purpose in this communication is to establish methods with which to identify the proteins present in the stereocilia and cuticular plate unambiguously, we subjected the intact sensory epithelium and the hair borders to immunoblotting techniques using antibodies to chicken actin-binding proteins. We first applied antifimbrin antibodies to blots of the sensory epithelium (Fig. 8 *a*) and hair border fractions (Fig. 8 *b*) and found that in both preparations the 65 and 57 kD bands stain. Thus the three major proteins in our hair border fractions can be identified. The 43 kD protein must be actin based on its size and stoichiometry and the 57 and 65 kD components a fimbrin-like molecule.

Initially we thought that there must be two fimbrin-like proteins in the stereocilia as we ran dozens of gels of variously extracted sensory epithelia with and without the protease inhibitor, PMSF, and consistently obtained the same result, although we ran immunoblots on preparations of intestinal epithelia and did not find two bands. The reviewers of this manuscript kindly pointed out to us that the 57-kD protein is likely to be a proteolytic fragment of the 65-kD protein. They argued that if fimbrin is proteolysed in vitro with any of a variety of proteases including papain the 65 kD (68 kD by others) protein is broken down to a first major proteolytic fragment of 57 kD (17). Accordingly we went back

Figure 6. Longitudinal section through a portion of a detergent and high salt extracted stereocilium. Although the actin filaments form a compact bundle, they are not perfectly straight and the transverse stripes indicative of the cross-bridges are not seen. (*b*) Transverse section through a detergent and high salt extracted stereocilium. The actin filaments are not hexagonally packed, but instead appear in short rows.

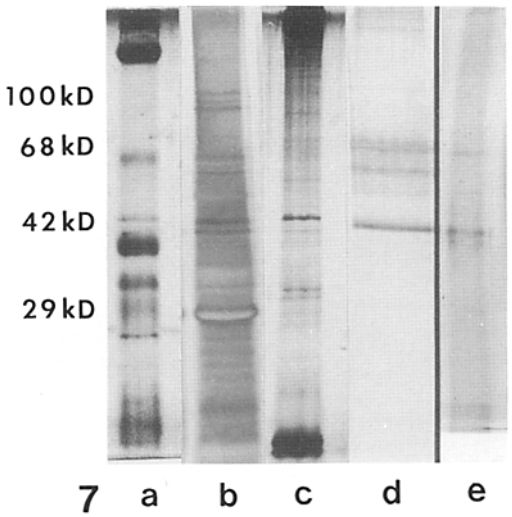


Figure 7. SDS-PAGE of fractions of the cochlea stained with silver. On the left are the molecular masses of representative bands. Lanes *a–e* are 10% gels. (Lane *a*) Tectorial membrane, five ear equivalents (Merrill); (lane *b*) sensory epithelium, six ear equivalents (Sammons); (lane *c*) Triton-extracted sensory epithelium, six ear equivalents (Merrill); (lane *d*) hair borders (high salt extract), six ear equivalents (Sammons); (lane *e*) Triton-extracted sensory epithelium with the addition of leupeptin, benzadine, and PMSF, boiled immediately in SDS, and run on an 8% gel (Merrill).

and ran more gels using a battery of protease inhibitors including PMSF, leupeptin, and benzamidine and found that the amount of the 57-kD protein varies from preparation to preparation and in one preparation was absent altogether (Fig. 7 *e*). Thus we now believe that the hair borders contain only two major proteins, actin and fimbrin. Because the unextracted sensory epithelium contains a 57- and 65-kD band on our immunoblots (Fig. 8 *a*), it is probable that proteolysis occurs because of the addition of papain which seems to be active even after boiling in SDS (Bretscher, A., unpublished data). Thus "chewing" of the proteins would occur in the gel mix and during electrophoresis.

We also made immunoblots using antivillin, antimyosin, and antigelsolin, antibodies which had been prepared against villin and myosin isolated from the brush borders of intestinal epithelial cells and against gelsolin from the chicken gizzard. None of these antibodies reacted with any of the proteins on the hair borders even though they reacted strongly to brush border and gizzard proteins, respectively, not surprisingly as there are no bands from that preparation at their respective molecular weights. Immunoblots using the intact sensory epithelium, however, showed a strong reaction with the antimyosin antibodies; this is reasonable as this protein has been shown by immunofluorescence to be present in hair cells (10, 11, 16).

Probable Location of the Proteins Seen on Our Gels

Since we have only three major bands in our preparation of hair borders, our next project was to try to determine which proteins were located in the stereocilia and which in the cuticular plate. We tried many procedures. For example, we attempted homogenization of the hair borders to shear off the stereocilia, extractions with a battery of detergents such as sarkosyl or digitonin, incubation using a variety of ionic solutions, and so on. All met with only marginal degrees of success. What proved most enlightening was to look carefully at the tectorial membrane as this extracellular layer, when run on a gel, contains a band of the same electrophoretic mobility as actin (Fig. 7) as well as the two bands that cross react with antibodies to fimbrin (Fig. 9 *a*). All the other bands present in Fig. 9 *a* (with perhaps the exception of the 110-kD) appear to be components of the tectorial membrane. Interestingly no 400-kD protein or proteins of even larger size are present (Fig. 9 *a*). The parallel control is the high salt extracted epithelium (Fig. 9 *b*).

We now know why actin and fimbrin are present in the tec-

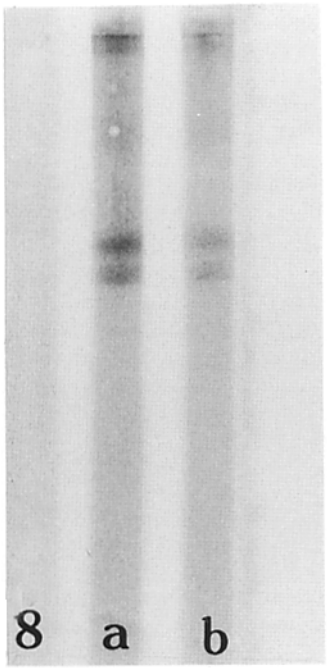


Figure 8. Immunoblot using anti-fimbrin antibody. (*a*) Unextracted sensory epithelium (five ear equivalents; (*b*) high salt extract (hair borders) (five ear equivalents).

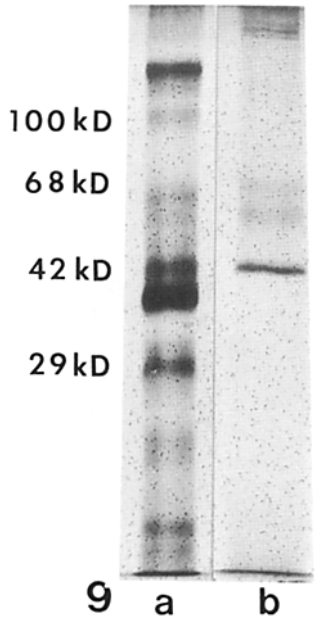


Figure 9. SDS-PAGE. (*a*) Tectorial membrane (five ear equivalents). (*b*) Hair border (high salt). Note that bands at the same electrophoretic mobility of actin and two fimbrin-like proteins are present in both lanes in addition to the bands known to be present in the tectorial membrane. The high molecular mass band present in lane *b* is not present in lane *a* and a band at 110 kD is present in the tectorial membrane fraction that is absent in hair borders. Stained by the Merrill technique and enhanced by recycling.

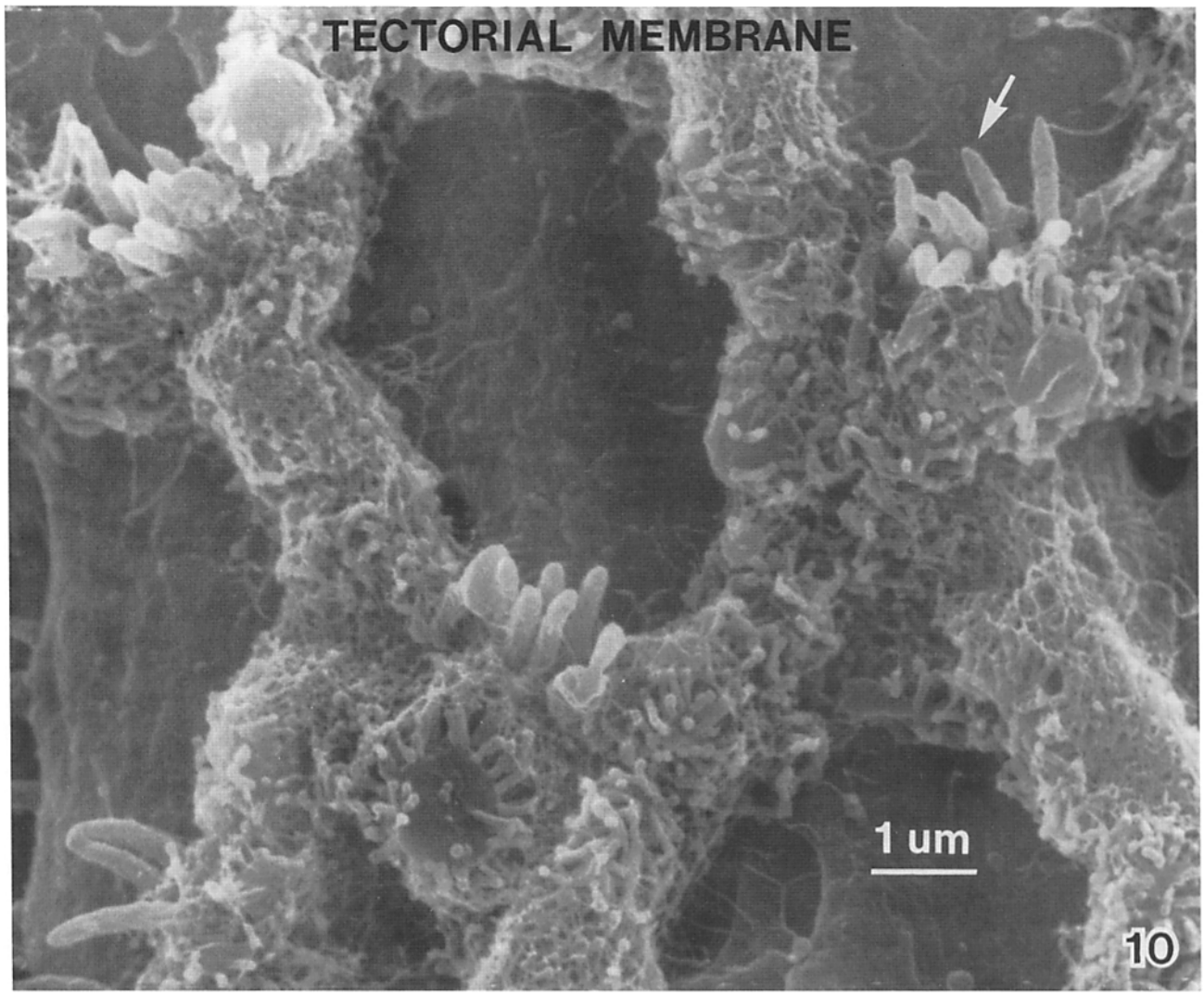


Figure 10. Scanning electron micrograph of the underside of the tectorial membrane. The round holes lie over the apical end of the hair cells, the fibrous material lies over the supporting cells located between the hair cells. Still attached to the fibrous part of the tectorial membrane are stereocilia. These stereocilia correspond to some of the longest stereocilia in each bundle. Examination of the free ends of these stereocilia shows that they are pointed (see *arrow*). This is because the stereocilia are broken off the apical cell surface at their tapered bases (see Fig. 2).

torial membrane fraction. What happens is that the stereocilia that are attached to the tectorial membrane often break off from the sensory epithelium and are carried off with the tectorial membrane when it is stripped away (Fig. 10). Thus when gels are run of the tectorial membrane, we see stereociliary proteins as well as tectorial membrane proteins, but no cuticular plate proteins. When the underside of the tectorial membrane is examined by scanning electron microscopy, it is seen to resemble a honeycomb (Fig. 10), as pointed out initially by Takasaka and Smith (24). The microvilli of the supporting cells make contact with the edges of the cells of the honeycomb, while the stereocilia insert into the pores (Figs. 2 and 10). When we look at the under surface of a tectorial membrane that was teased away from the sensory epithelium, we find, in most cases, a few stereocilia still attached (Fig. 10). These stereocilia have broken off from the hair cells at their tapered bases (Fig. 10, *arrow*).

The 110-kD band deserves further mention as in some tectorial membrane preparations it is either not present or present in very reduced amounts. In short it could be a proteolytic artefact derived from the 175-kD tectorial membrane band, or it could be a component of the stereocilia. In summary then, the stereocilia are composed of actin and fimbrin and possibly a 110-kD protein, but lack the 400-kD proteins, which, by elimination, must be cuticular plate proteins. The cuticular plate, then, appears to have actin (12, 26, 27) and the 400-kD proteins. By immunofluorescence (see below) we now know that fimbrin is not present in the cuticular plate.

Immunofluorescence Studies with Antifimbrin

As pointed out by the reviewers of this manuscript, we are faced with a paradox. Since high salt solubilizes fimbrin in vitro (1) and at the same time induces the loss of the trans-

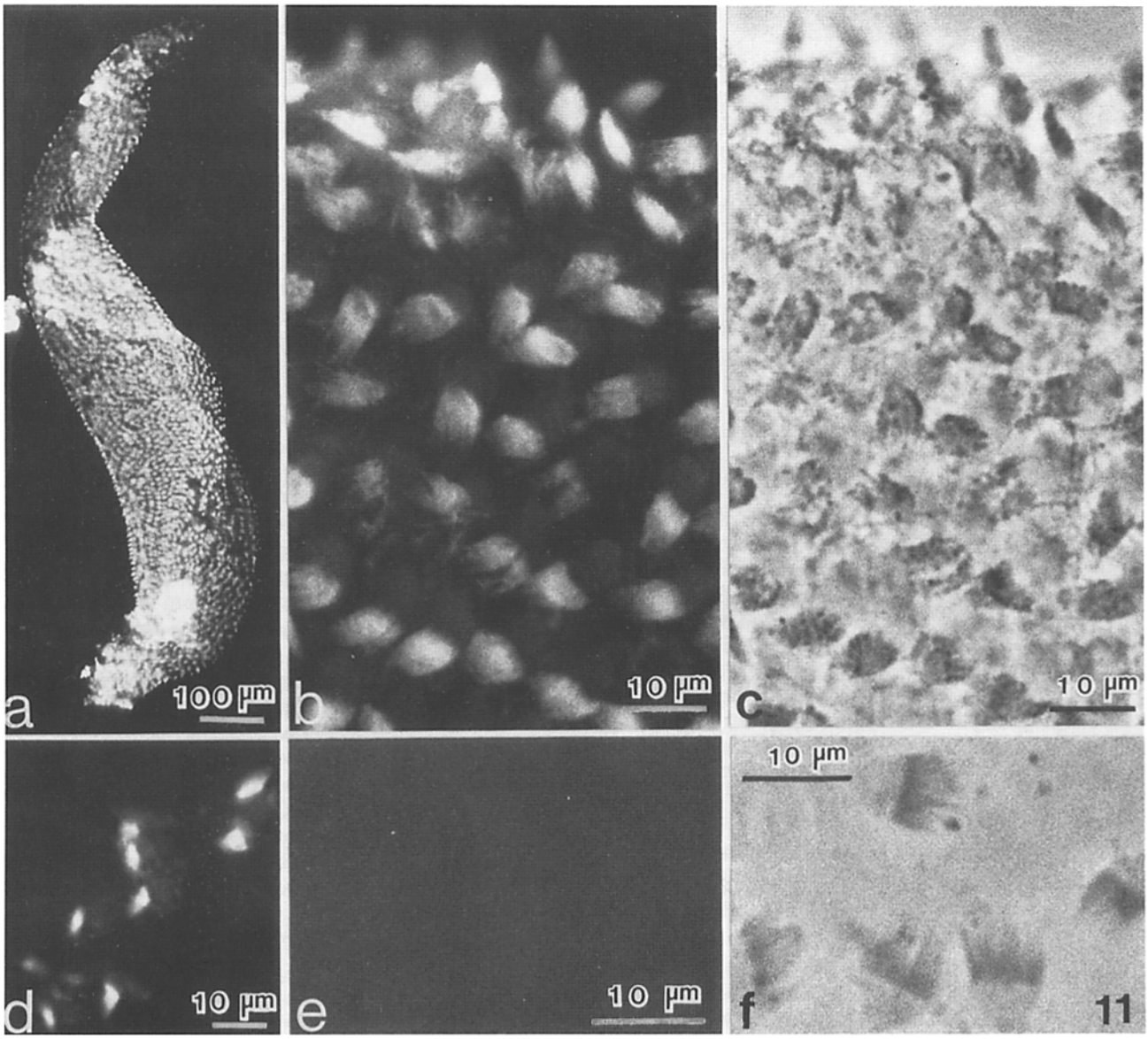


Figure 11. (*a*) Whole mount of the sensory epithelium stained with an antifimbrin antibody. Each small spot is the stereociliary bundle of a single hair cell. (*b*) A portion of the whole mount at higher magnification, again stained with antifimbrin. The individual stereociliary bundles stain intensely, but the cuticular plate is unstained. (*c*) Phase contrast image of the whole mount region in *b*. (*d*) The sensory epithelium extracted with Triton in low salt to make a cytoskeletal preparation. This is then stained with antifimbrin. Note that the stereociliary bundles stain intensely. (*e* and *f*) The sensory epithelium is extracted with Triton in high salt, a procedure that gives rise to hair borders, depicted in *f* with phase contrast microscopy. The hair borders are then incubated in antifimbrin. No staining can be detected (*e*).

verse striping pattern on the stereocilia (hair borders), a pattern that is due to the cross-bridges (4, 5), why do gels and immunoblots of the hair borders show the presence of fimbrin? To determine if fimbrin really is present in the stereocilia of hair borders, we compared by immunofluorescence the staining of stereocilia in the isolated sensory epithelium (Fig. 11, *a* and *b*), the cytoskeletal preparation (Fig. 11 *d*), and the hair borders (Fig. 11 *e*). The stereocilia in the sensory epithelium and the cytoskeletal preparations stain beautifully with the antifimbrin antibody (Fig. 11, *b* and *d*), in contrast to the hair borders that are completely unstained (Fig. 11 *e*). From these results we can conclude that the transverse stripes in the intact stereocilia are, in fact, due to fimbrin cross-bridges. Since the actin filaments in the high salt extracted stereocilia are still connected together and do not splay apart even after incubation in high salt for 3 h, it seems likely that there is a second species of cross-bridge whose identity remains unknown. Examination of the hair border preparation by fluorescence microscopy after staining with antifimbrin antibody reveals that the DNA often contains intense spots of fluorescence as if the fimbrin extracted from the hair borders precipated and stuck to the DNA. This may explain why fimbrin is present on gels of the hair borders, but not physically present in the hair borders themselves.

Discussion

We have described how one can isolate and begin to identify by SDS gels, immunoblots, and immunofluorescent techniques proteins in the apical surface of hair cells of the cochlea. It is our hope that our isolation methods and/or modifications of them will interest others more talented than the senior authors in biochemical procedures in characterizing the proteins in this fascinating cell type. Using current silver staining techniques it is possible to catalog the proteins present in the sensory epithelium, the cytoskeleton, and in the high salt extracted hair borders. We have in a preliminary way started to do just that. We find that in the hair borders there are a small number of major bands on an SDS polyacrylamide gel. The three major bands can be identified as actin, the major component of both the stereocilia and cuticular plate, and two proteins (65 and 57 kD; the latter we believe is a proteolytic fragment of the 65-kD), which cross react with antibodies prepared against fimbrin, an actin-binding protein first described in brush borders of intestinal epithelial cells (2). All three major bands are present in the stereocilia. Evidence is presented that one of the remaining bands, ~400 kD protein, appears to be a component of the cuticular plate.

Obviously what is needed in the future are better procedures to inhibit proteolysis, a more complete analysis of minor components, and information on the location of these proteins. Such studies can be approached both biochemically using SDS gels and immunoblot analyses and with immunological location techniques at the light and electron microscope level.

The Change in Packing of the Actin Filaments in the Stereocilia of the Hair Bundles during Extraction

As shown in earlier publications (4, 5) because of the helical symmetry of actin, cross-bridges cannot form at any random position along the filaments. Their location is dictated by the topography of the actin filament such that cross-bridges can form only when the subunits on adjacent filaments face each other (4, 5). If cross-bridging is maximized, which it is in a hexagonally packed bundle, cross-bridges will be found every 125 Å, which in turn will cause the periodic 125 Å transverse striping seen in the electron micrographs of stereocilia (Fig. 3 and Fig. 4, *inset*). If the filaments do not lie on a hexagonal lattice and are instead oriented as in a liquid crystal, these stripes will not be present even though the degree of cross-bridging is only a little less than in the hexagonal bundle (4, 8). Of interest here is that in our thin sections of the hair borders the actin filaments are no longer hexagonally packed and accordingly no stripes are present, but the filaments are still connected together in short, curved rows, even in preparations extracted for 3 h in high salt. Furthermore examination of these hair borders by immunofluorescent techniques using affinity-purified antibodies against fimbrin shows that fimbrin no longer stains the stereocilia (Fig. 11). From these two sets of observations it seems inescapable that fimbrin is the cross-bridge that accounts for the 125-Å stripes seen in our thin sections, a conclusion that had been suspected, but not proved by earlier studies. The unresolved question is what is the chemical identity of the cross-bridge that remains in the hair borders. It is unlikely that it is fimbrin as high salt readily extracts fimbrin from bundles (1) and immunofluorescence techniques show it to be absent (Fig. 11 *e*). It is more likely a yet unidentified cross-bridge. Consistent with this is the observation that in microvilli of intestinal epithelial cells there are two actin cross-bridging proteins, fimbrin, and villin (3, 9, 13), suggesting that to get an ordered bundle two cross-bridging proteins must be present. (In stereocilia the second is clearly not villin; see Results). Why two species of cross-bridges are necessary requires more investigation.

The puzzling observation is why on gels and immunoblots of the hair border fractions do the fimbrin bands remain although fimbrin is no longer bound to the actin filaments. As mentioned in Results, this is possibly due to the fimbrin sticking to the uncoiled DNA in the hair border preparation.

The Cuticular Plate

In contrast to the stereocilia, the morphology of the cuticular plate in "hair borders" fixed 10 min after incubation in high salt seems identical to that seen in untreated cells, but after 3 h there have been changes. This will be the subject of a further communication as it will help us analyze the structure of the cuticular plate. From our gels of the isolated tectorial membranes we conclude that the 400-kD protein must be a component of the cuticular plate. It is possible that this polypeptide is involved in linking the actin filaments together. Such a notion would be consistent with the amount of the 400-kD protein in the hair borders, since on a molar basis the components of the cuticular plate are small relative to those in the stereocilia proper (the amount of actin in the cuticular plate is only 6–10% of that present in the stereocilia) and the observation of DeRosier and Tilney (manuscript submitted for publication) that each actin filament in the cuticular plate is covered by whiskers ~500 Å in length and 30 Å in diameter, dimensions consistent with a molecule of 400 kD. Other proteins, again in small amounts, may also be critical here.

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References

1. Bretscher, A., 1981. Fimbrin is a cytoskeletal protein that cross links F-actin in vitro. *Proc. Natl. Acad. Sci. USA.* 78:6849–6853.
2. Bretscher, A., and K. Weber. 1980. Fimbrin, a new microfilament associated protein in microvilli and other cell surface structures. *J. Cell Biol.* 86:353–340.
3. Coluccio, L. M., and A. Bretscher. 1989. Reassociation of microvillar core proteins: making a microvillar core in vitro. *J. Cell Biol.* 108:495–502.
4. DeRosier, D. J., and L. G. Tilney. 1982. How actin filaments pack into bundles. *Cold Spring Harbor Symp. Quant. Biol.* 81:525–540.
5. DeRosier, D. J., and L. G. Tilney. 1984. The form and function of actin a product of its unique design. *Cell Muscle Motil.* 5:139–169.
6. Deleted in proof.
7. Deleted in proof.

8. DeRosier, D. J., L. G. Tilney, and E. Egelman. 1980. Actin in the inner ear: the remarkable structure of the stereocilia. *Nature (Lond.)*. 287: 291–296.
9. Drenckhahn, D., and R. Dermietzel. 1988. Organization of the actin filament cytoskeleton in the intestinal brush border: a quantitative and qualitative immunoelectron microscope study. *J. Cell Biol.* 107:1037–1048.
10. Drenckhahn, D., L. Kellner, H. Mannherz, U. Gröschel-Stewart, J. Kendrick-Jones, and J. Scholey. 1982. Absence of myosin-like immunoreactivity in stereocilia of cochlear hair cells. *Nature (Lond.)*. 300:531–532.
11. Drenckhahn, D., T. Schäfer, and M. Prinz. 1985. Actin, myosin, and associated proteins in the vertebrate auditory and vestibular organs. Immunocytochemical and biochemical studies. *In* Auditory Biochemistry. D. Drescher, editor. Charles C. Thomas Publisher, Springfield, IL. 312–335.
12. Flock, A., A. Bretscher, and K. Weber. 1983. Immunohistochemical localization of several cytoskeletal proteins in inner ear sensory and supporting cells. *Hear. Res.* 6:75–89.
13. Glenny, J. R., P. Kaulfus, P. Matsudaira, and K. Weber. 1981. F-actin binding and bundling properties of fimbrin, a major cytoskeletal protein of microvillus core filaments. *J. Biol. Chem.* 256:9283.
14. Hirokawa, N. 1978. The ultrastructure of the basilar papilla of the chick. *J. Comp. Neurol.* 181:361–374.
15. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680–685.
16. MacCartney, J. C., S. D. Comis, and O. Pickles. 1980. Is myosin in the cochlea a basis for active motility? *Nature (Lond.)*. 288:491–492.
17. Matsumura, S. Y., and F. Matsumura. 1985. Purification and characterization of an F-actin bundling 55-kilodalton protein from HeLa cells. *J. Biol. Chem.* 260:5087–5097.
18. Maupin-Szamier, P., and T. D. Pollard. 1978. Actin filament destruction by osmium tetroxide. *J. Cell Biol.* 77:837–852.
19. Merrill, C. R., D. Goldman, and M. L. VanKeuren. 1982. Simplified silver protein detection and image enhancement methods in polyacrylamide gels. *Electrophoresis.* 3:17–23.
20. Oberholtzer, J. C., M. E. Schneider, M. C. Summers, J. C. Saunders, and F. M. Matschinsky. 1986. Developmental appearance of a major basilar papilla-specific protein in the chick. *Hear. Res.* 23:161–168.
21. Sammons, W., L. D. Adams, and E. E. Nishizawa. 1981. Ultrasensitive silver-based staining of polypeptides in polyacrylamide gels. *Electrophoresis.* 2:135–141.
22. Slepecky, N., and S. C. Chamberlain. 1985. Immunoelectron microscopic and immunofluorescent localization of cytoskeletal and muscle-like contractile proteins of inner ear sensory hair cells. *Hear. Res.* 20:245–260.
23. Sobin, A., and A. Flock. 1983. Immunohistochemical identification and localization of actin and fimbrin in vestibular hair cells in the normal guinea pig and in a strain of the waltzing guinea pig. *Acta Oto-larynogol.* 96:407–412.
24. Takasaka, T., and C. A. Smith. 1971. The structure and innervation of the pigeon's basilar papilla. *J. Ultrastruct. Res.* 35:20–65.
25. Tanaka, K., and C. A. Smith. 1978. Structure of the chicken's inner ear: SEM and TEM study. *Am J. Anat.* 153:251–271.
26. Tilney, L. G., and D. J. DeRosier. 1986. Actin filaments, stereocilia, and hair cells of the bird cochlea IV. How the actin filaments become organized in developing stereocilia and in the cuticular plate. *Dev. Biol.* 116:119–129.
27. Tilney, L. G., and J. C. Saunders. 1983. Actin filaments, stereocilia, and hair cells of the bird cochlea I. The length, number, width and distribution of stereocilia of each hair cell are related to the position of the hair cell on the cochlea. *J. Cell Biol.* 96:807–821.
28. Tilney, L. G., E. H. Egelman, D. J. DeRosier, and J. C. Saunders. 1983. Actin filaments, stereocilia, and hair cells of the bird cochlea II. Packing of actin filaments in the stereocilia and in the cuticular plate and what happens to the organization when the stereocilia are bent. *J. Cell Biol.* 96:822–834.
29. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.