

# Interactions between Actin Filaments and between Actin Filaments and Membranes in Quick-Frozen and Deeply Etched Hair Cells of the Chick Ear

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**ABSTRACT** Replicas of the apical surface of hair cells of the inner ear (vestibular organ) were examined after quick freezing and rotary shadowing. With this technique we illustrate two previously undescribed ways in which the actin filaments in the stereocilia and in the cuticular plate are attached to the plasma membrane. First, in each stereocilium there are threadlike connectors running from the actin filament bundle to the limiting membrane. Second, many of the actin filaments in the cuticular plate are connected to the apical cell membrane by tiny branched connecting units like a "crow's foot." Where these "feet" contact the membrane there is a small swelling. These branched "feet" extend mainly from the ends of the actin filaments but some connect the lateral surfaces of the actin filaments as well. Actin filaments in the cuticular plate are also connected to each other by finer filaments, 3 nm in thickness and  $74 \pm 14$  nm in length. Interestingly, these 3-nm filaments (which measure 4 nm in replicas) connect actin filaments not only of the same polarity but of opposite polarities as documented by examining replicas of the cuticular plate which had been decorated with subfragment 1 (S1) of myosin. At the apicolateral margins of the cell we find two populations of actin filaments, one just beneath the tight junction as a network, the other at the level of the zonula adherens as a ring. The latter which is quite substantial is composed of actin filaments that run parallel to each other; adjacent filaments often show opposite polarities, as evidenced by S1 decoration. The filaments making up this ring are connected together by the 3-nm connectors. Because of the polarity of the filaments this ring may be a "contractile" ring; the implications of this is discussed.

Although actin is now recognized as the most commonly encountered protein in eucaryotic cells, information on its interactions with other components of the cells is less forthcoming. This is in part due to the enormous number of protein molecules with which it interacts. For example, in a recent review Schliwa (26) listed 15 proteins which interact with actin *in vitro*. This list did not include the muscle proteins, i.e., myosin, tropomyosin, troponin, and  $\alpha$ -actinin, the red blood cell proteins such as the spectrins and Band 4.1 (see reference 2), invertebrate sperm proteins of which there are at least six that bind to actin *in vitro* (see reference 30), the brush border or microvillar proteins such as fimbrin (3), axon proteins such as fodrin (18), nor the glycolytic enzymes which bind to actin with binding constants as high as that of myosin (22). Thus, there are at least 30–40 different protein molecules that bind to actin *in vitro*.

What we would like to know is: Do these molecules interact with actin naturally (*in vivo*) and, if so, what do they do? In some cases, in particular in skeletal muscle, we have a pretty good idea of how at least some of these proteins interact with actin, but in other cells it is not clear what role, if any, these proteins play *in vivo*. Studies *in vitro* can, of course, unearth clues as to their function in the cell, although this can lead to results which have no meaning *in vivo*. In fact, the history of the field, beginning with the phenomenon of superprecipitation (29), is littered with interesting experiments and observations which do not help us to understand how a cell assumes a specific shape, changes its shape, moves, or allows substances to move into or out of its interior.

Rather than to isolate a protein from a cell or part of a cell and attempt to determine how it functions *in vivo* by studying its antics *in vitro*, we have started by simply observing a cell or

part of the cell and in this way try to define what it actually uses as equipment. One way to do this is to fix a cell, dehydrate it, embed it in plastic, and examine it in thin sections. The difficulty with this technique is that the medium used for embedding the specimen obscures structures whose densities match that of the plastic (32). A method with which to obviate this problem (used primarily in the laboratory of Keith Porter, University of Colorado, Boulder, CO) is to fix, dehydrate, and critical-point dry a cell, and then examine it in the dried state. Although this method is effective, it would be even better to be able to examine nonfixed, undehydrated tissue, particularly since it is now clear that actin, unless protected by tropomyosin, myosin, or its subunits, or phalloidin, is easily fragmented by  $\text{OsO}_4$  (19). Furthermore, dehydration causes substantial changes in the organization of actin in smooth muscle (28) and it has recently been shown that glutaraldehyde itself causes actin to break down by forming intrafilament crossbridges (17). A way to examine the tissue at high resolution without the obvious artifacts caused by fixation, dehydration, or embedding is by rapidly freezing the cell or tissue and examining a replica after it has been fractured and deeply etched. Such a technique has the additional advantage of enabling us to examine the actin filament organization in three dimensions so that we can establish what structures are attached to an actin filament and where they are found (7, 12, 13). Unfortunately, examination of intact cells with any one of the above techniques has potential artifacts included. These artifacts result because soluble proteins and salts present within an intact cell will either precipitate on existing structures or possibly form artifactual structures during fixation, dehydration, embedding, or deep etching. The only way to completely avoid these artifacts is to work with isolated parts of the cell after washing in low tonicity media and then to examine them with pre- or postfixation, dehydration, or embedding (see reference 12). The fast-freezing and deep-etching technique is ideal under these circumstances (7, 12, 13). Even so, it is important to examine intact cells as well, bearing in mind that artifactual structures may be present.

There are, of course, a great many actin-containing cells that should be examined by this technique. But what is most useful at this point is to examine a cell, or part of a cell, in which the actin filament organization is extensive, yet predictable, so that we know exactly where to look and what to expect. Equally important is for us to select a cell type in which the region to be examined lies at or very near the free surface. This is critical because excellent freezing without ice crystal formation can only be obtained 10–15  $\mu\text{m}$  from the surface that contacts the cooled copper block. We chose the apical surface of the mechanoreceptor or hair cell of the vertebrate ear since this system fits both criteria (see references 4, 9, 10, 14, 31).

Every hair cell has projecting from its surface large, specialized microvilli (stereocilia); within each stereocilium in an extensively cross-linked bundle of actin filaments (31). At the point of contact between the stereocilium and the apical portion of the cell there is an enormous network of actin filaments, the so-called cuticular plate. The central filaments in the stereocilia are believed to enter into the cuticular plate as a rootlet; they are, in turn, cross-linked to the actin filaments there.

The acoustico-vestibular system is ideal for the freezing technique because the stereocilia on the hair cells of the saccular macula and/or utricular macula extend from a large, nearly flat surface to make contact with the otolith crystals. After removal of these crystals, the flat surface can be pushed against the cooled copper block so that the apical surface of

the hair cell will lie within the first 5  $\mu\text{m}$  of the cooled copper block, a region in which the best freezing will occur. We used chicks as experimental animals because until 3 wk of age the skull is cartilaginous which greatly facilitates the rapid excision of these organs.

We, therefore, examined the apical surface of the hair cell or the acoustico-vestibular system by the fast freezing-deep etching technique. Our studies, besides giving us a fascinating three-dimensional view of these cell types, have revealed a number of interesting structural features of the interaction of actin filaments with themselves and with membranes which have not been observed before. These observations not only help us to determine what types of molecules to look for from the vast number already described but also give a structural basis which, by tightly coupling the cytoskeleton to the membrane and to itself, provides a framework so that the mechanical displacement of part of the cell is transferred accurately and directly to the membrane for electrical potential changes.

## MATERIALS AND METHODS

Two- to three-week-old white leghorn chicks were decapitated and the saccular macula and utricular macula were rapidly dissected out. These maculae were then placed in potassium buffer (80 mM KCl, 5 mM  $\text{MgCl}_2$ , 3 mM EGTA, 30 mM HEPES at pH 7.4) on ice and the otolith was removed by fine forceps or by flushing the buffer through a syringe with a #27 gauge needle. Some maculae were quick frozen directly in potassium buffers; others were fixed with 1% glutaraldehyde in potassium buffer for 1 h on ice, washed with distilled water, and quick frozen. Another group of maculae was processed as outlined below.

### Detergent Extraction

Maculae were incubated in 1% Triton X-100 in potassium buffer for 10 min on ice. Some of the maculae were fixed with 1% formaldehyde in 80 mM KCl, 5 mM  $\text{MgCl}_2$ , 3 mM EGTA, 30 mM HEPES at pH 7.2 on ice for 1 h and quick frozen; others were washed after detergent extraction with potassium buffer and processed as indicated below.

### S1 Decoration

Demembrated and washed maculae were incubated in a solution containing potassium buffer and 1 mg/ml of subfragment 1 of myosin (S1) for 20 min or for 1 h at room temperature. The S1 was kindly supplied by Dr. A. Weber of the University of Pennsylvania. After washing, the maculae were fixed with 1% glutaraldehyde containing 0.2% tannic acid (1) in potassium buffer for 30 min, then rinsed in a solution containing 100 mM KCl and 5 mM  $\text{MgCl}_2$  and then stained with 1% uranyl acetate dissolved in 100 mM KCl and 5 mM  $\text{MgCl}_2$  for 1 h at 4°C. Some of the maculae were then quick frozen; others were postfixed in 1%  $\text{OsO}_4$  in 0.1 M phosphate buffer at pH 6.2 (31) before uranyl acetate staining, then washed three times in water to remove the phosphate ions, and incubated in 0.5% uranyl acetate for 3 h at 0°C. The specimens were then dehydrated in acetone and embedded in Epon. Thin sections were cut on a Sorvall Porter-Blum ultramicrotome MTI (DuPont Instruments, Newtown, CT) with a diamond knife, stained in uranyl acetate and lead citrate, and examined with a JEOL 100 CX electron microscope.

### Quick Freezing

Some maculae were removed from the organism, incubated briefly in potassium buffer, then quick-frozen without fixation. These we call fresh samples.

Other maculae were fixed with glutaraldehyde. Another group of maculae was incubated in potassium buffer then Triton-extracted. Some of the Triton-extracted maculae were fixed; others were decorated in S1 after detergent extraction, but before fixation. If decoration was attempted, the maculae were then washed in buffer and fixed. All the fixed maculae were rinsed briefly in distilled water containing 15% methanol before freezing.

The maculae were positioned on a thin slice of lung on the specimen holder such that the apical surface of the sample faced the pure copper block cooled by liquid helium (see reference 8). Towards the end of this study some of the fixed samples were cut into sections ~400  $\mu\text{m}$  in thickness by a Sorvall Tissue Chopper (DuPont Instruments). The sections were then rinsed in distilled water containing 15% methanol and placed on the lung such that the cut surface faced the cooled

copper block. From these samples we could obtain a longitudinal view of the hair cells.

### Freeze Fracturing and Electron Microscopy

The frozen samples were freeze fractured at  $-196^{\circ}\text{C}$  at  $2-5 \times 10^{-7}$  Torr in a Balzers 400 freeze etch machine (Balzers, Hudson, NH), etched for 5 min at  $-95^{\circ}\text{C}$ , recooled to  $-120^{\circ}\text{C}$ , and replicated by rotary shadowing with platinum and carbon. After shadowing, the tissue was dissolved with Purex or chromesulfuric acid. The replicas were then cleaned by immersing them in three changes of distilled water, and picked up on Formvar-carbon coated 75-mesh grids. The replicas were examined in a JEOL 100 CX or 200 CX electron microscope at 100 kV and were photographed in  $\pm 10^{\circ}$  tilt for stereomicroscopy.

### Scanning Electron Microscopy

For scanning microscopy the maculae were prepared as described above and fixed in 1% glutaraldehyde and 2% formaldehyde in 0.1 M cacodylate buffer at pH 7.4 for 2 h. They were dehydrated through a graded series of acetone and washed in absolute isoamylacetate and critical point dried. After sputter coating with gold, they were examined with a Hitachi HSF-2 scanning electron microscope.

## RESULTS

### Anatomy of the Chick Gravity Receptor Organ

The utricular macula and saccular macula are disc-shaped organs (Fig. 1a) composed of sensory hair cells and supporting cells. On the apical surface of each hair cell is one kinocilium and 80–100 specialized microvilli (stereocilia) (Fig. 1b). The otolith sits on top of these stereocilia and exerts mechanical force on the stereocilia when the posture of the animal is changed. There are two types of hair cells, type I and type II. The former assume a flasklike form with the neck portion constricted; from 2 to 10 hair cells are encapsulated by a huge afferent nerve chalice (9). In contrast to type I, type II hair cells are cylindrical in outline and receive button-shaped syn-

apses (9). Aside from the innervation and overall cell shape, the morphologies of the apical region of each cell type are nearly identical.

### Stereocilia

**MATERIAL ATTACHED TO THE SURFACE OF THE MEMBRANE:** Because mechano-electrical transduction occurs either at the surface of the stereocilium or at the apical surface membrane at the base of the stereocilium, it is of interest to know if there are specialized clusters of intramembrane particles on this membrane. A number of reports using classical freeze-fracture techniques have appeared which show that intramembrane particles are sparse and randomly distributed on the plasma membrane limiting the apical cell surface and the surface of the stereocilium. We have confirmed this observation on both fixed and fresh tissues. What we have found that is new, or at least not previously appreciated, is that in unfixed maculae the stereocilia are connected together by an extensive extracellular net of anastomosing fibrils, each  $\sim 10$  nm in diameter (Fig. 2a and b), which is very much like the glycocalyx on the intestinal microvilli (12). We presume that it is this net of material that tends to hold the stereocilia together so that they move as a unit. We also find this net in fixed preparations which were washed with distilled water and methanol before freezing (Fig. 2b). However, this net is not observed clearly by the usual thin-section technique or by scanning electron microscopy, so it must, at least in part, be eliminated by fixation, dehydration and/or critical point drying for the conventional methods. Likewise, this net is not visible in conventional freeze-fracture replicas because the cryoprotectants used prevent deep etching.

When we observed unfixed samples after deep etching, we were initially concerned that the salt could condense and form

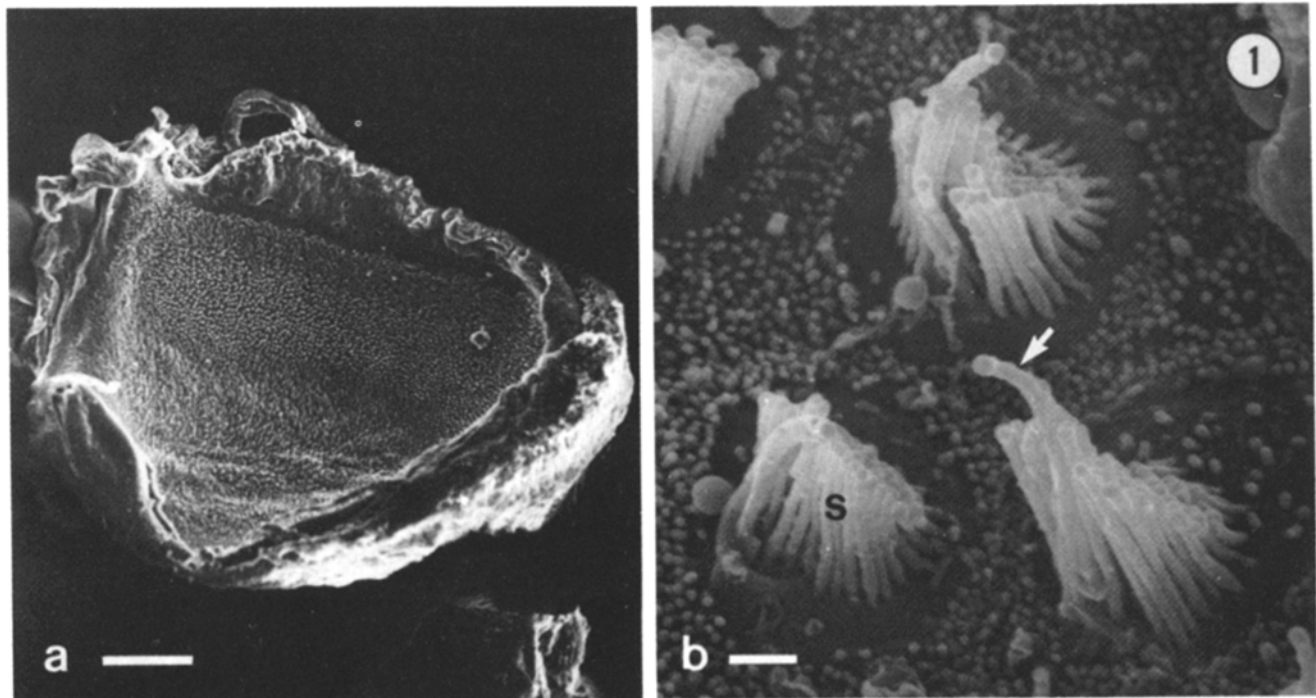


FIGURE 1 (a) Low magnification scanning electron micrograph of the saccular macula. The otolith has been removed from this disc-shaped organ revealing the tufts of stereocilia (white dots) projecting up towards the viewer. Bar,  $100 \mu\text{m}$ .  $\times 120$ . (b) Higher magnification of the macula illustrated in a showing the apical surface of three hair cells. Located on each hair cell is a tuft of stereocilia (S) and a single kinocilium (arrow). Surrounding these cell extensions is a smooth portion of the plasma membrane beneath which is the cuticular plate. Short microvilli extend from supporting cells that surround each hair cell. Bar,  $0.1 \mu\text{m}$ .  $\times 9,000$ .

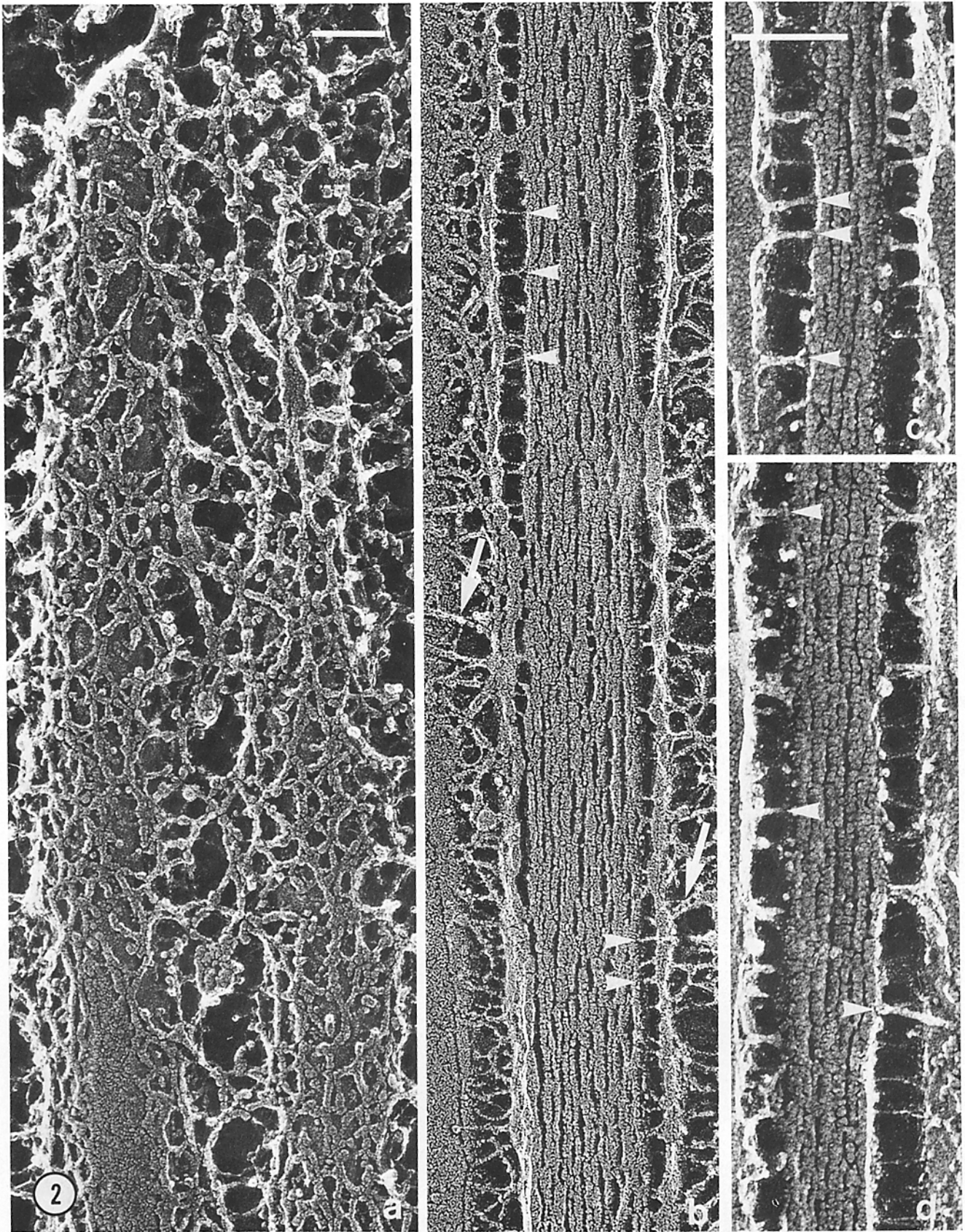


FIGURE 2 Micrographs of quick-frozen, deeply etched, and rotary shadowed stereocilia. (a) Unfixed sample. Connecting adjacent stereocilia is an anastomosing network of filamentous material. Bar,  $0.1 \mu\text{m}$ .  $\times 133,000$ . (b) Fracture through a stereocilium in a fixed material washed with distilled water just before freezing. An anastomosing network of glycocalyx (arrows) is connecting adjacent stereocilia. A bundle of actin filaments inside the stereocilia is connected to the membrane by lateral arms  $4 \text{ nm}$  in diameter (arrowheads). Bar,  $0.1 \mu\text{m}$ .  $\times 133,000$ . (c and d) Fracture through a stereocilium in an unfixed sample revealing a bundle of actin filaments connected to the membrane surrounding the stereocilium by lateral arms  $4 \text{ nm}$  in diameter (see arrowheads). In this unfixed preparation, the space between the core bundle and the membrane is wider than normal. Bar,  $0.1 \mu\text{m}$ .  $\times 210,000$ .



artificial structures, perhaps even giving us the appearance of a net. We demonstrated that this is not the case, in two ways. First, in fixed-distilled water-washed preparations we still see a net, thus it cannot be residual salt (Fig. 2*b*). Second, the salt-induced artificial structures described by Miller and Lassignol (20) are produced when a sample is freeze dried. There, the etching time is 1 h at  $-90^{\circ}\text{C}$  in contrast to our study in which we etched for only 5 min at  $-95^{\circ}\text{C}$ . In our preparations we have never encountered these artificial structures caused by condensation of salt, because a much shallower layer of ice is removed than in the case of freeze drying. This we can confirm by comparing a quick-frozen fixed tissue which contains the same amount of salt as a fresh tissue with a quick-frozen tissue which was washed extensively with distilled water after fixation, but before freezing. Thus, as Hirokawa and Heuser (12) and Hirokawa (11) mentioned earlier, salt did not cause any artificial structure in the extracellular space and in the cytoplasm by this degree of shallow etching.

**INTERACTION BETWEEN ACTIN FILAMENTS AND MEMBRANES IN STEREOCILIA:** As demonstrated by Flock and Cheung (4) for the stereocilia in the vestibular system and by Tilney et al. (31) in the cochlea, within each stereocilium is a tightly packed bundle of actin filaments. Until now, no one has shown clearly any morphological connections between this bundle of filaments and the membrane limiting the stereocilium except at the base of the stereocilium (31).

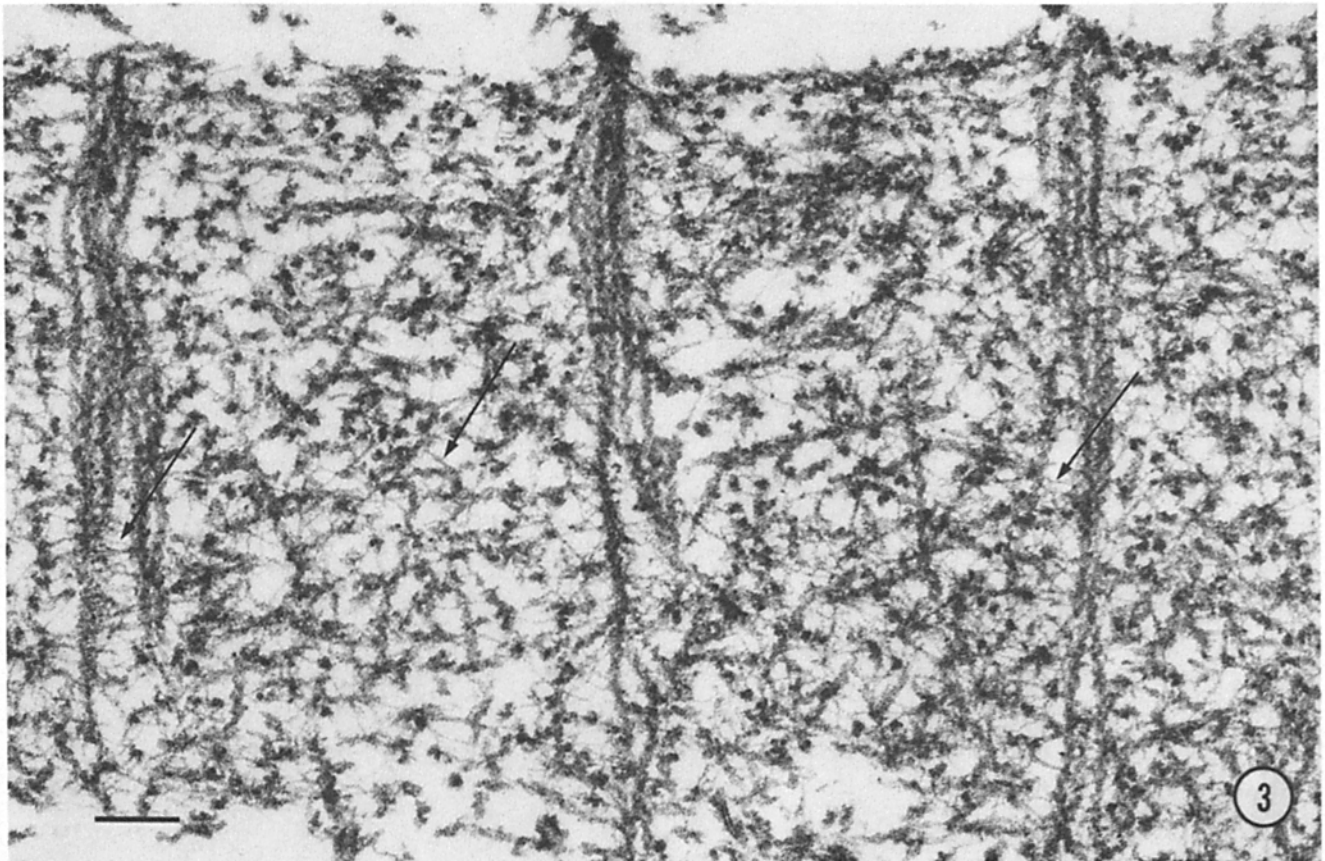
In our replicas of unfixed maculae we found that there are numerous lateral connections between the core filaments and the membrane (Fig. 2*c* and *d*). In the replicas, these connec-

tions are  $\sim 4$  nm in diameter and up to 30 nm in length. Since the distance from the filament bundle to the membrane is usually much narrower ( $\sim 10$  nm) (the greater distance seen here may be due to incubation in a potassium-rich fluid), these lateral connections must be elastic or at least able to elongate. A careful examination of these connections shows that they insert on the membrane at a single point (Fig. 2*c* and *d*). We found that these connections tend to be labile to fixation, but sometimes they could be observed in the fixed samples washed with distilled water before freezing (Fig. 2*b*). Unlike the situation in fresh samples in fixed material, the length of the connectors is much less; they average  $\sim 15$  nm in length.

At the base of the stereocilium where it makes contact with the apical surface of the hair cell we find a number of fine connections extending from the filament bundle to the plasma membrane. These connections resemble the spokes of a wheel where the rim of the wheel is the inside of the plasma membrane. At the center of the wheel these "spokes" connect to a material that is electron dense in transmission microscopy. This material is, in turn, attached to the actin filaments in this region (see reference 31).

### The Cuticular Plate

**INTERACTIONS BETWEEN ACTIN FILAMENTS:** If we strip away the membranes with detergent, decorate the actin filaments with S1, and then cut thin sections through the center of the cuticular plate parallel to the rootlet, we get the image depicted in Fig. 3. Between the rootlet filaments are many



**FIGURE 3** A thin section of a hair cell that had been detergent-extracted, then incubated in a solution containing S1. Longitudinal section through the cuticular plate showing three rootlet bundles. Surrounding these rootlets are numerous decorated filaments connected by thin, 3-nm cross-linking filaments (arrows). These cross-linking filaments connect rootlet filaments with each other and with actin filaments in the cuticular plate. The horizontal running actin filaments are cross-linked with each other, as well. Bar,  $0.1 \mu\text{m}$ .  $\times 113,000$ .

short, decorated filaments which are the actin filaments of the cuticular plate cut in transverse and oblique section. Thus, the rootlet filaments and the cuticular plate filaments lie perpendicular to each other; rarely do we see cuticular plate filaments which run parallel to the rootlet filaments. Also present in Fig. 3 are numerous 3-nm filaments which do not decorate and thus do not contain actin. These 3-nm filaments connect the adjacent actin filaments in the cuticular plate. Some of these 3-nm filaments seem to connect rootlet filaments with some of the filaments in the cuticular plate proper (see arrows in Fig. 3).

What we wanted to establish next was the nature of these cross-linking 3-nm filaments. (In the replicas these filaments are actually 4 nm.) For example, we would like to know whether the cross-linked actin filaments are of the same or opposite polarity. What are the lengths of the 3-nm filaments and do they branch? These questions can only be adequately answered by stereomicroscopy because of superposition artifacts. Furthermore, replicas are preferable because they give us a remarkable 3-dimensional image, many times better than that in plastic-embedded material since there is no drop in observability caused by the embedding medium. Accordingly, we decorated the actin filaments with S1, fast froze and deep etched the maculae, and obtained replicas. It should be remembered that in replicas the decorated actin filaments appear "ropelike" (7). Recently, it has been noticed in well-frozen samples, whether they are fresh or fixed, that one can recognize the polarity of the decorated actin filaments (Heuser and Cook, manuscript in preparation). This polarity is more evident in samples fixed in tannic acid and glutaraldehyde, because such fixatives further stabilize the filaments and thus tend to prevent fine distortions during freezing. A careful examination of the surface of the "ropes" shows that the gyres are somewhat angular which allows us to determine the polarity of the filaments (Fig. 4). Often it is easier to see this polarity by squinting along the filaments in the micrograph. In the insert of Fig. 4a we have included a micrograph of a filament at high magnification. The polarity of this filament is indicated by the arrow. The clue for the determination of its polarity comes from the asymmetry of each gyre which appears fatter on one side than on the other.

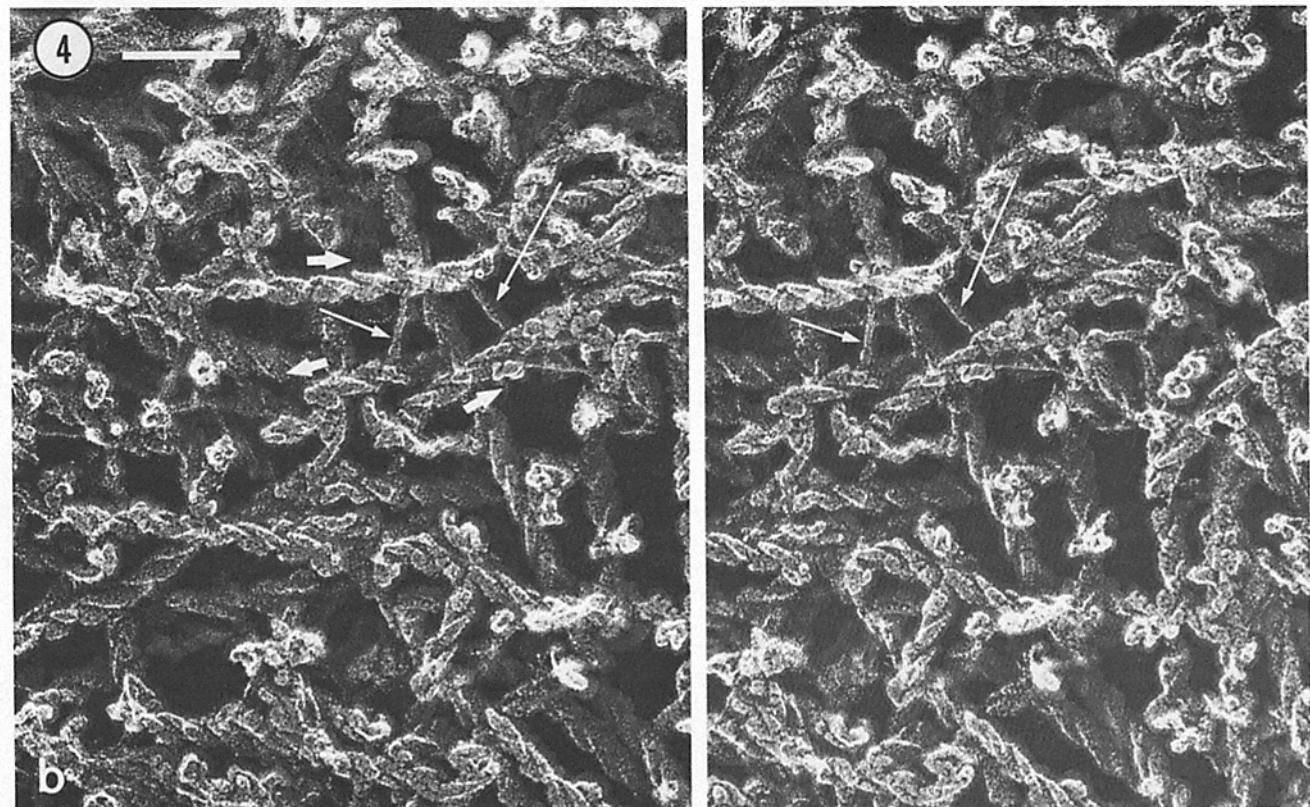
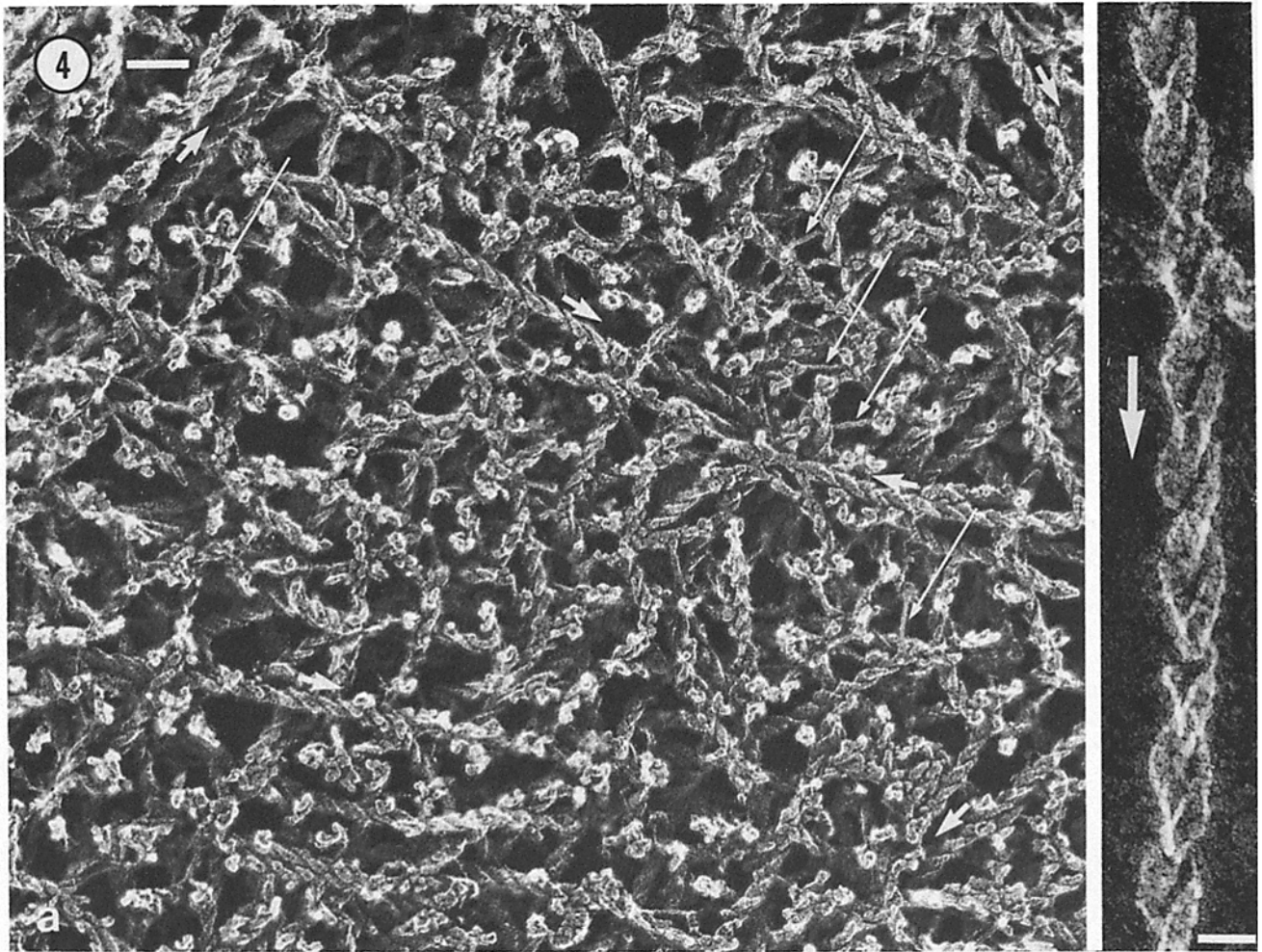
In Fig. 4 we present representative areas of the cuticular plate. The fine, long arrows depict the 3-nm cross-connections. What was unexpected is that the cross-connections connect actin filaments of the same as well as opposite polarity (Fig. 4b, thick arrows). We confirmed that the cross-connections were actually connecting adjacent filaments, not just passing beneath or above the decorated filaments, by examining stereo photographs (Fig. 4b). We measured 85 3-nm filaments and found that they measure  $74 \pm 14$  nm in length. Sometimes the 3-nm filaments branch, but most are unbranched.

#### INTERACTIONS BETWEEN ACTIN FILAMENTS AND

MEMBRANES IN THE CUTICULAR PLATE: With the exception of the stereocilium and kinocilium, the apical surface of the hair cell that overlies the cuticular plate is characteristically flat and smooth (Fig. 1b). At the same time, the horizontally arranged actin filaments making up the apical margin of the cuticular plate (see Fig. 3) come into proximity with the limiting plasma membrane. To determine what kind of association may exist here, one must procure replicas in which the fracture plane runs through the cytoplasm immediately adjacent to the plasma membrane and then, by etching down to the plasma membrane, any connections between the filaments and the membrane should become visible (see the drawing in Fig. 5). Such a fracture occurs infrequently because the free surface of the macula faces the cooled copper block and, therefore, etching will generally occur towards the cell center, not away from it. However, sometimes one is lucky and we managed to obtain some magnificent replicas, one of which is illustrated in Fig. 5. To understand the replica, we have included a drawing (Fig. 5) showing how the apical portion of the cell was fractured and etched. Note that, going from the top to the bottom, the fracture first passes through the cuticular plate, then passes near the plasma membrane, and finally fractures down the center of the plasma membrane, thereby exposing the *E* face of the membrane. This is easily appreciated if you imagine that the fracture plane runs through a part of cuticular plate instead of exclusively down the center of apical membrane. Of considerable interest is that we find a series of projections that extend from the actin filaments to make contact with the cytoplasmic surface of the plasma membrane (Figs. 5 and 6). These connectives generally extend from the ends of the actin filaments with only a few from the lateral surfaces (Figs. 5 and 6). The connectives are usually branched and the contact spot is not a point but consists of a cytoplasmic bump or lump. They appear as "crows" feet. This type of connection should be contrasted to the stereocilium or microvillus where the connection between the actin bundle and the membrane is always from the lateral surface of the filament and there is no bump on the membrane at the point of contact. In fresh cuticular plates, the substructure of the actin filaments and cross-connectives is not clear; instead, the filaments and cross-connectives appear covered with some material (perhaps soluble proteins) that give them a knobby appearance. After Triton extraction the network of actin filaments and cross-linkers appeared to be unchanged as in the fresh sample, although the surface details of the filaments is now discernible (see Fig. 5, insert). More specifically, the actin filaments appear with a fine striping characteristic of purified actin (see reference 7).

ORGANIZATION OF ACTIN FILAMENTS ADJACENT TO THE TIGHT AND INTERMEDIATE JUNCTIONS: There are two discrete populations of actin filaments located in the apical cell cytoplasm just lateral to the membrane specializations.

FIGURE 4 Quick-frozen, deeply etched, and rotary shadowed replica of hair cells that were detergent extracted and decorated with S1 for 10 min at room temperature, fixed with glutaraldehyde and tannic acid, washed with distilled water, and quick frozen. (a) The decorated actin filaments now appear as a double-stranded rope, although careful scrutiny of this rope reveals that the gyres are angular. Thus the polarity of the filaments can be seen. To aid the viewer we have included in the insert to the right, a decorated actin filament illustrated at high magnification. The polarity of this filament is indicated by the large thick arrow. *Insert*, Bar, 0.2  $\mu$ m.  $\times$  391,000. In a proper the thick arrows indicate the polarity of the filaments; the thin arrows indicate the 3-nm connections that link adjacent actin filaments. Bar, 0.1  $\mu$ m.  $\times$  86,000. (b) Stereopair to illustrate at higher magnification its connection of adjacent actin filaments by the 3-nm connectives. As in Fig. 6 a, the thick arrows indicate the polarity of the filaments the thin arrows the cross connections. A cross-linker between actin filaments of the same polarity is indicated by the long, thin arrow, and a second cross-linker between actin filaments of opposite polarity is indicated by the shorter, thin arrow. Bar, 0.1  $\mu$ m.  $\times$  162,000.





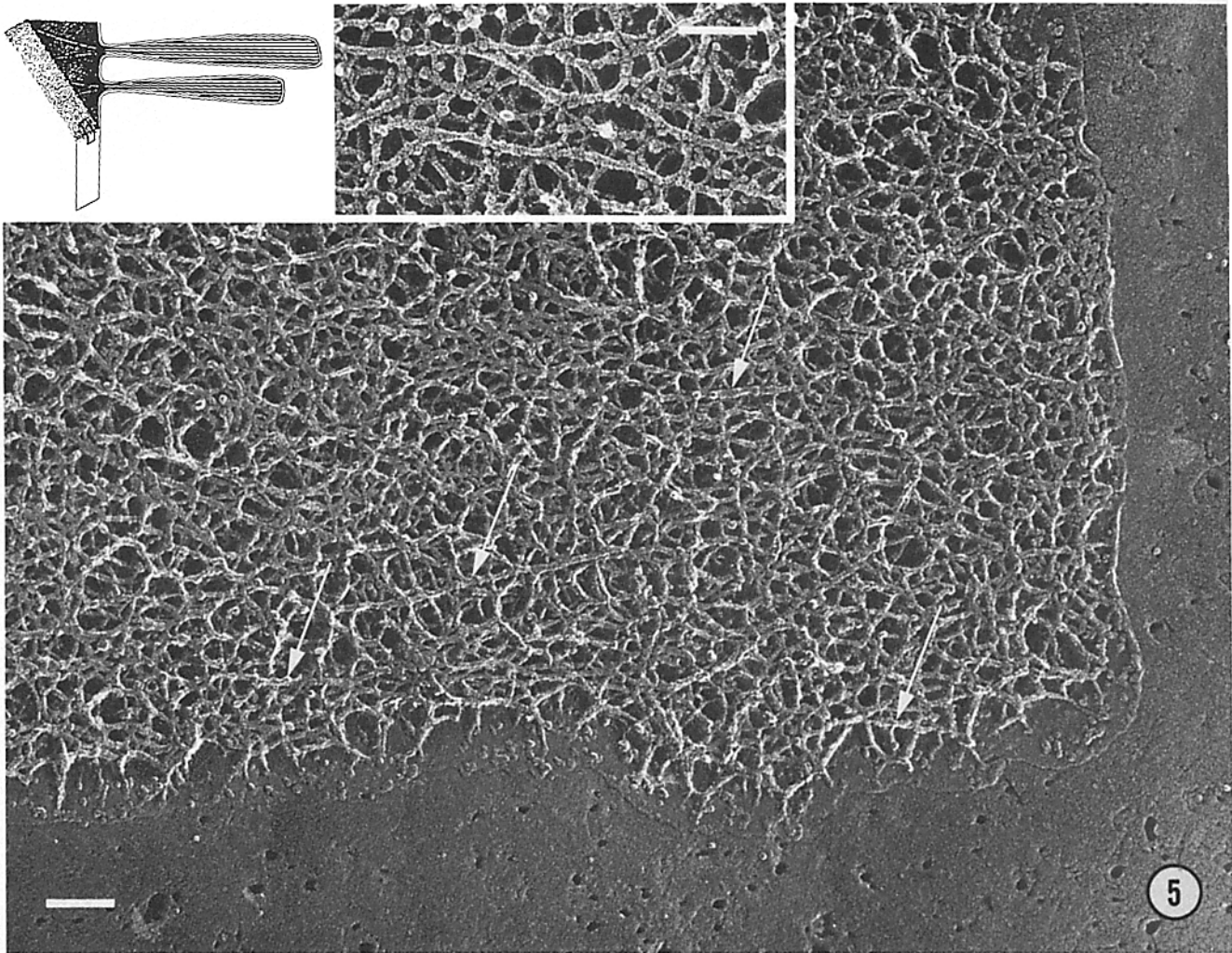


FIGURE 5 Quick-frozen, deeply etched, and rotary shadowed replica of an unfixed macula. The orientation of the fracture is illustrated in the inserted drawing. Starting from the upper left hand of this drawing, the fracture first passes through the cuticular plate, making the true cytoplasmic surface of the plasma membrane visible, then the fracture jumps into the plasma membrane, thereby exposing its *E* face. A comparable fracture is illustrated here. Of greatest interest is the connection of the filaments of the cuticular plate with the membrane; this is shown at high magnification in Fig. 6. The arrows point to actin filaments. Bar,  $0.1 \mu\text{m}$ .  $\times 96,000$ . (*Insert*) Cuticular plate, detergent-extracted, fixed and washed with distilled water before freezing, showing network of actin and 3-nm filaments. The filaments appear to be clearer than those in the fresh sample, yet the basic structure of the cuticular plate looks the same as in the fresh sample. Bar,  $0.1 \mu\text{m}$ .  $\times 112,000$ .

First, we find a meshwork of actin filaments which runs parallel to, but just inside, the tight junction (Fig. 7). Even after Triton extraction, these junctions with their network of intramembrane fibrils remain. Actin filaments are closely associated with the inside of the membrane, but precisely how they interact with the tight-junction intramembrane fibrils is a subject for further study. The second population is much more extensive and is located adjacent to the intermediate junction or zonula adherens. This population forms a ring or girdle around the apical end of the cell (Fig. 8). The actin filaments that comprise this ring run parallel to each other. After decoration with S1, the polarity of these filaments can be ascertained (Fig. 8). Of particular interest is that filaments of both polarities are present in this bundle (see arrows in Fig. 8*b, c*). Sometimes thin filaments were observed to link between decorated actin filaments (Fig. 8*b*). Undecorated filaments were also occasionally seen interposed between decorated filaments. An example of this can be seen to the right of the left arrow in Fig. 8*b*.

## DISCUSSION

With the technique of quick freezing and deep etching, we have discovered a number of structural features on the organization of actin filaments, specifically on interactions with themselves and with membranes, which have not been seen before. Many of these features, although clearly important in mechanical transduction, are probably general features of all epithelial cells. More specifically, we have shown: (*a*) that actin filaments are attached to membranes not only along their lateral surfaces but at the tips by fine, branching wisps; (*b*) that adjacent actin filaments in the cuticular plate are cross-linked by 3-nm filaments which, surprisingly, connect filaments of both the same and opposite polarities; (*c*) that adjacent to the zonula adherens is a bundle of actin filaments which forms a loop or ring around the apical surface of the cell; the polarities of the actin filaments in this loop are mixed such that they could act as a contractile ring, thereby stiffening the apical



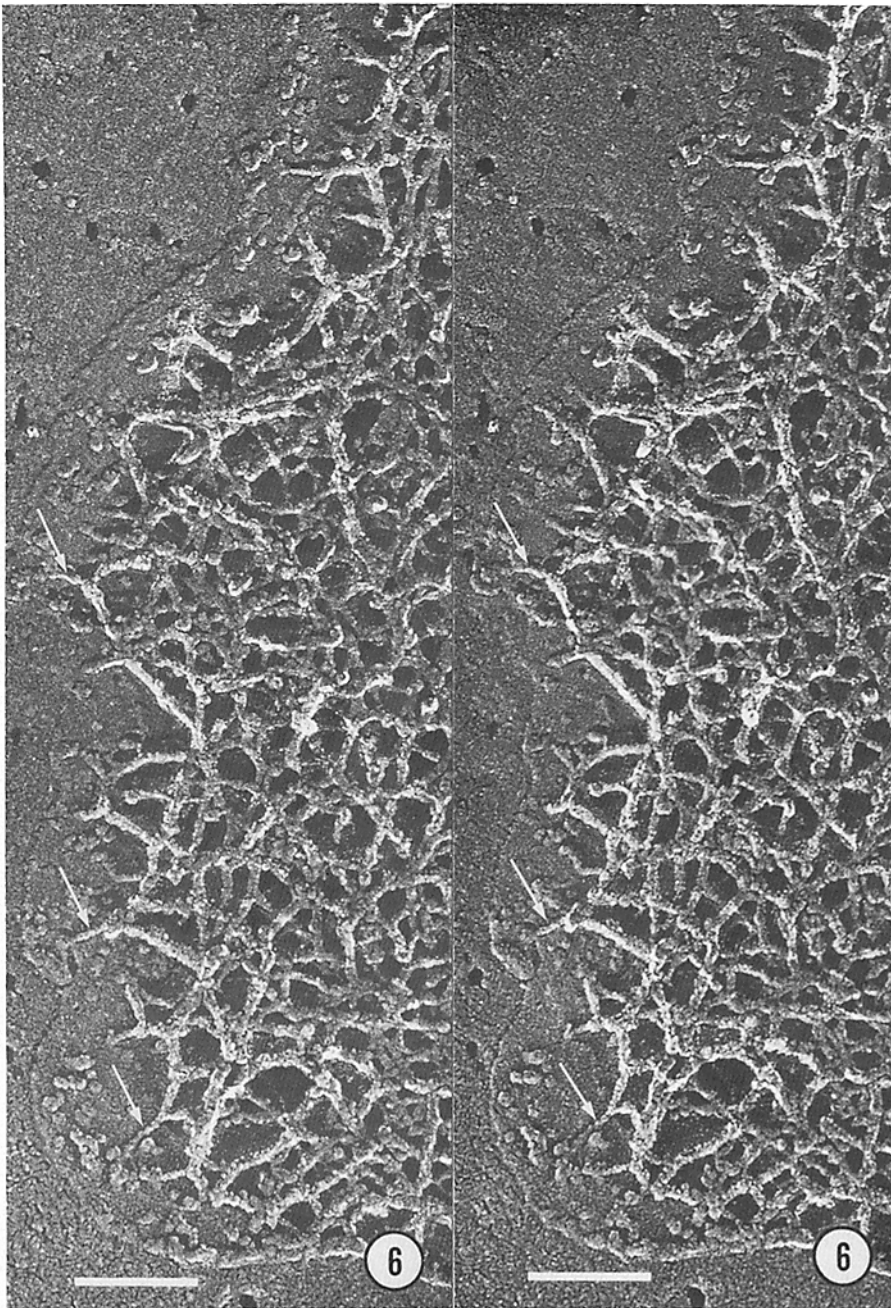


FIGURE 6 Stereopair of a high-magnification image of a portion of Fig. 5. The arrows indicate some of the numerous wisps that connect the ends of the actin filaments to the membrane. Bar,  $0.1 \mu\text{m}$ .  $\times 162,000$ .

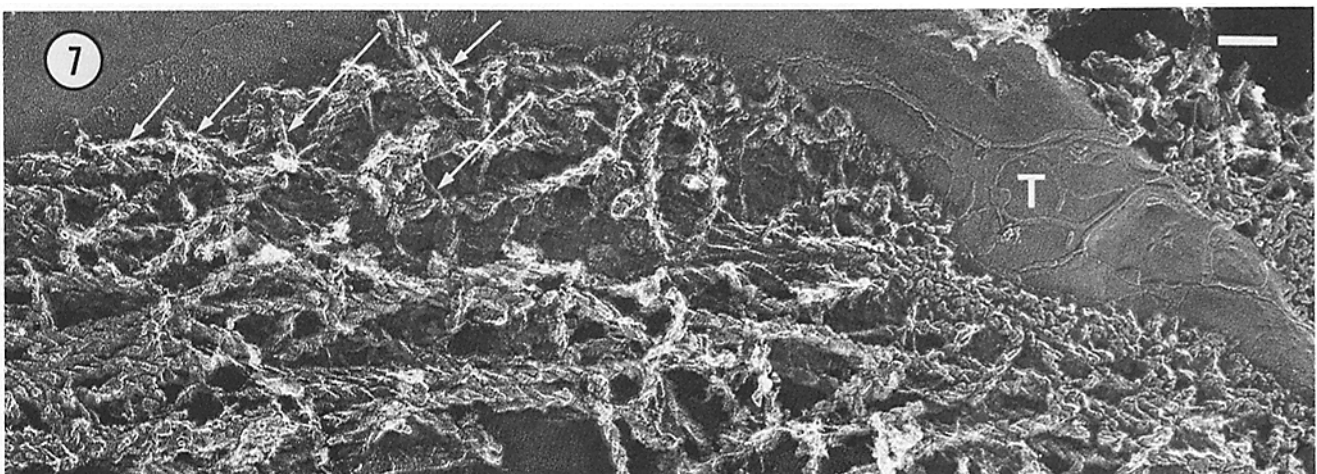
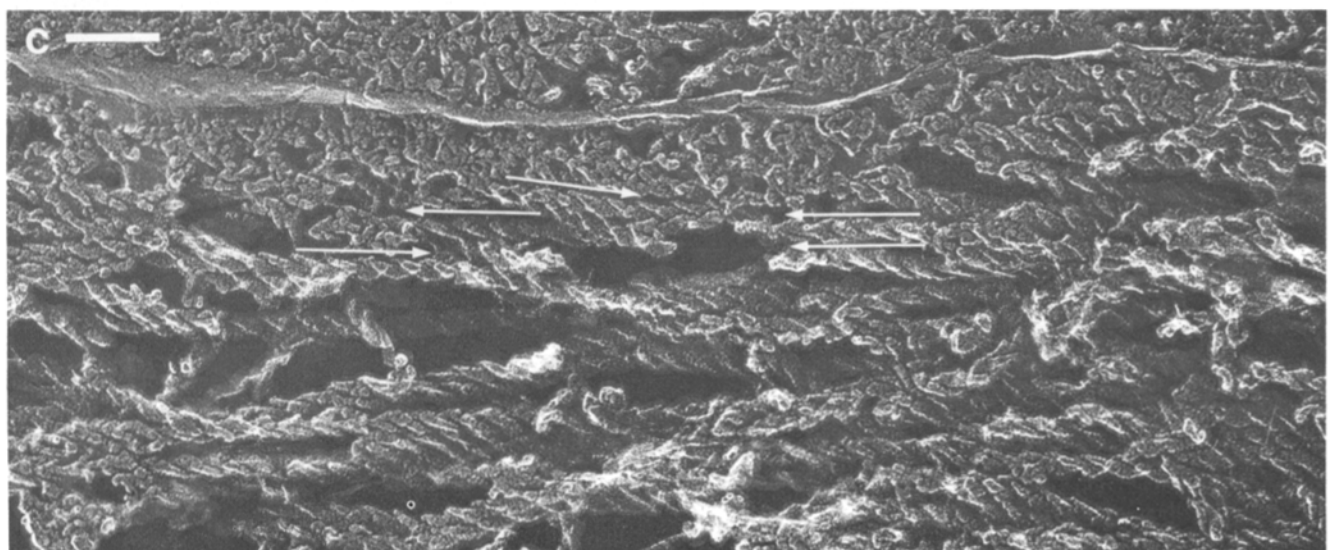
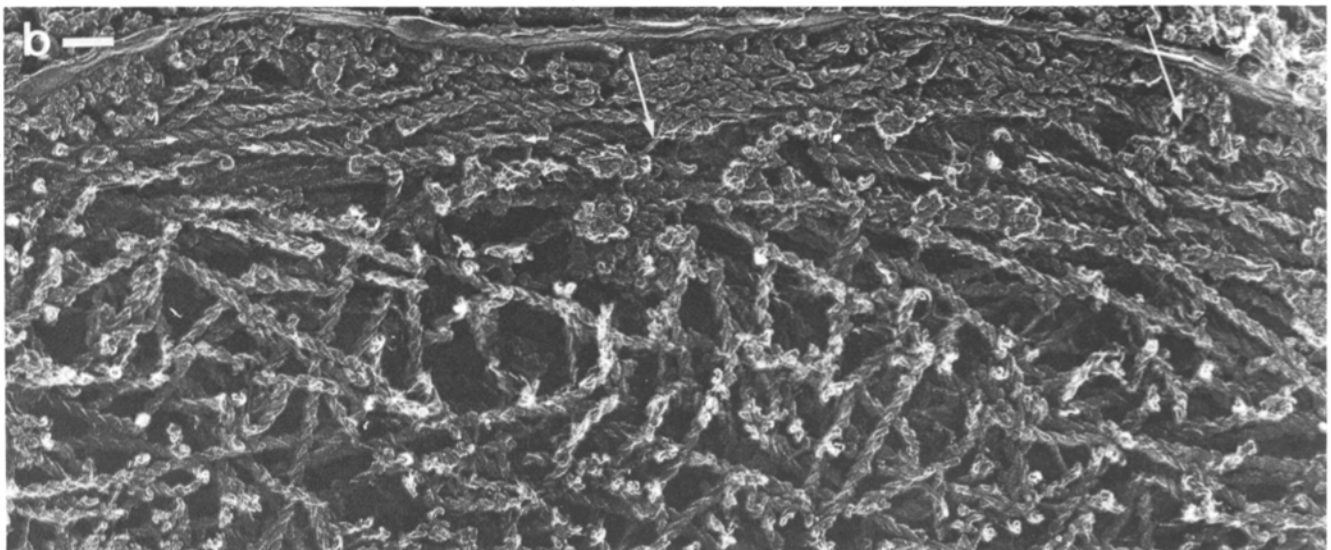
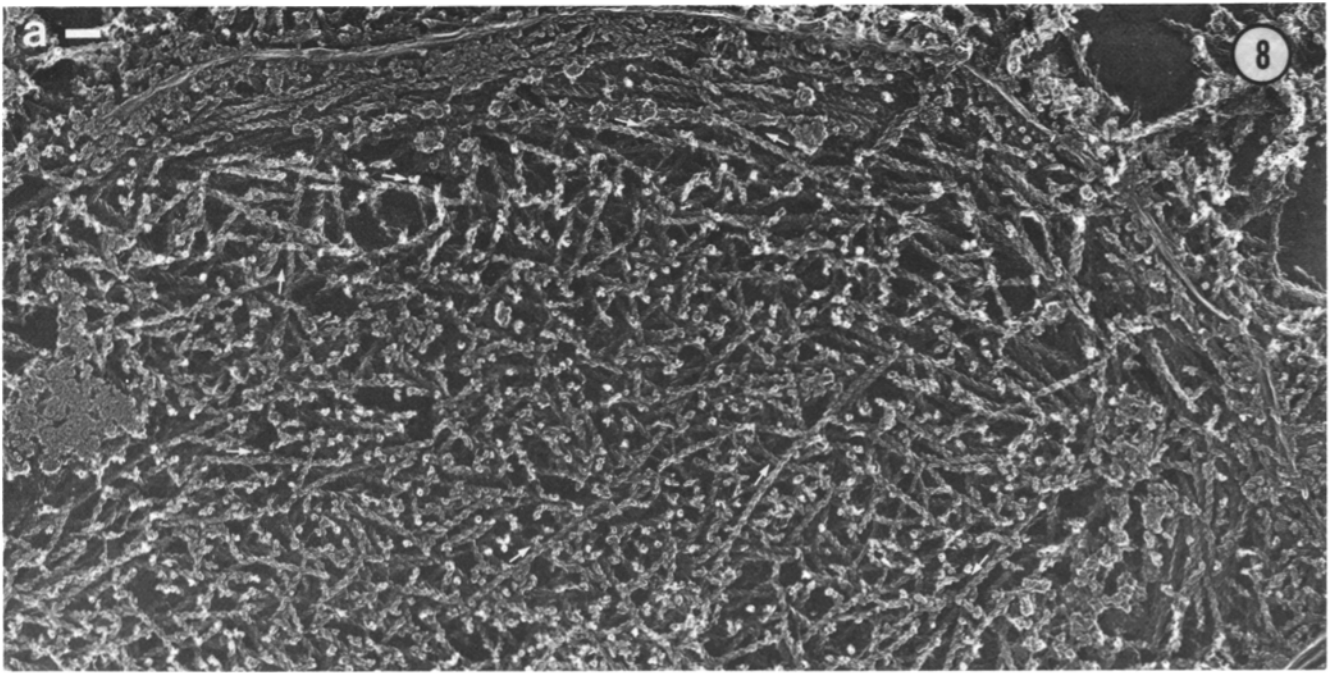


FIGURE 7 Replica of the actin filament network inside the tight junction in a quick-frozen, deeply etched, and rotary shadowed hair cell that had been detergent-extracted and decorated with S1. Often, the tight junction (*T*), although it is distorted, remains after Triton extraction. Adjacent to this junction is a network of S1-decorated filaments. The actin filaments (short arrows) are connected together by undecorated connectives (long arrows). Bar,  $0.1 \mu\text{m}$ .  $\times 78,000$ .



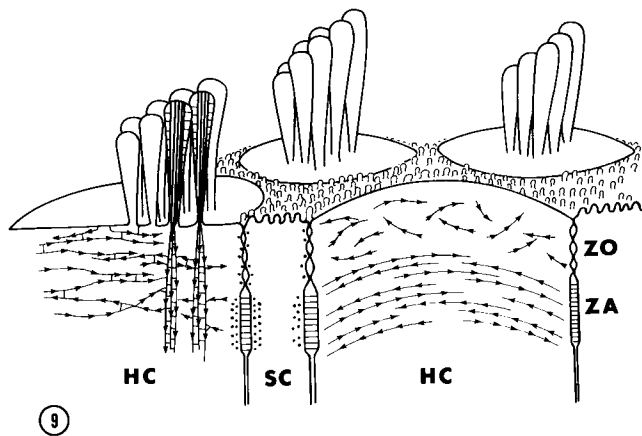


FIGURE 9 A schematic drawing which shows the organization of actin filaments in the stereocilia, cuticular plate, and inside the tight junction (ZO) and intermediate junction (ZA). Arrows indicate the polarity of actin filaments. HC, hair cell. SC, supporting cell. Lateral arms in the stereocilia, cross-linking filaments in the cuticular plates, and short filaments between actin and plasma membrane are drawn as thin short connectives. A detailed explanation is included in the text.

surface of the cell; and (d) that there are a few actin filaments that parallel the string of membrane particles making up the tight junction. These findings are summarized in Fig. 9. We will discuss these four findings in more detail below.

#### Actin Filament-Membrane Interactions

From the work of Hudspeth and Jacob (14) it is clear that mechanical displacement of the stereocilia induces, in a manner yet to be defined, a change in the conductance of ions across the limiting membrane; this ion flux in turn elicits a generator potential in the hair cells. In our study we have shown that the cytoskeleton of the hair cell, which includes the actin filament bundle in the stereocilium proper, as well as some of the actin filaments in the cuticular plate, is connected to the limiting membrane. Thus, by tightly coupling the plasma membrane to the cytoskeleton, small mechanical changes are transferred to the limiting membrane so that ion movements can be initiated. More specifically, we have shown that three types of connections exist between actin filaments and the limiting membrane.

First, we found lateral connections between the actin filament bundle in the stereocilia and the limiting membrane (Fig. 9). These connections are reminiscent of the connections between the actin filament bundle and the membrane in intestinal microvilli as described by Mooseker and Tilney (21) and by Mukherjee and Staehelin (23). However, unlike the situation in intestinal microvilli, these connections have not previously been described in stereocilia even though epithelial cells and

hair cells were fixed by the same investigators (us) in the same way and from the same species. Nevertheless, it was not difficult to find these connections in samples quick-frozen and deep-etched, indicating that the cross connections in the stereocilia may be labile to fixation and dehydration for thin-section method, in contrast to those in intestinal epithelial cells where they can be found in cells fixed in a variety of media. This difference in lability towards fixation and/or dehydration may indicate that these connections are chemically distinct cross-bridges.

Second, we demonstrated connections between the actin filaments in the cuticular plate and the apical surface membrane (Fig. 9). In fact, this is the first demonstration of how actin filaments which are not in a bundle contact the plasma membrane at their ends. What we see here are small, often branching, wisps or fingers connecting the ends of the filaments to the membrane. The main reason why these connections have not been seen before, we believe, is that in a given thin section these connections would be difficult to recognize and we could not be sure that they are real because they would have little density above the plastic background. By examining replicas after freeze etching, we see broad surfaces on which actin filaments are attached to the membrane. These fingerlike connections occur at the tips of the actin filaments; at the plasma membrane, they associate with fine lumps or swellings. These lumps on the cytoplasmic surface of the plasma membrane do not occur on the membrane of the stereocilium or on the membrane at the base of the stereocilium (where the spokelike attachment inserts), indicating that this bridge must be different in chemical nature from other actin-membrane attachments. With a study combining immunocytochemistry with freeze etching, as we did for myosin in the brush border (see reference 13), we should ultimately be able to clarify the nature of these cross-links and to identify which of the known proteins corresponds to the cross bridge and which to the "lump", etc.

Third, we described a population of cross bridges at the point of contact between the stereocilium and the apical cell surface. The cross bridges appear as spokes radiating out from the filament bundle in the stereocilium. In thin sections, the filament bundle in this region is covered by some electron dense material. Spokes, similar to what we describe here in our freeze-etched preparations, were described by Tilney et al. (31) in the stereocilia of the alligator lizard cochlea, but in that preparation, in order to see them, the cochlea had to be treated with the detergent, Triton X-100. This detergent in turn solubilized the lipid bilayer so that it was difficult to determine whether or not the spokes indeed contacted the plasma membrane. Our results here, using the freeze-etch technique, establish that connection conclusively.

It is worthwhile to reiterate that the connections we have been describing do not appear to be artifacts caused by precip-

FIGURE 8 In this replica a macula was quick frozen after detergent extraction and decorated in S1 for 1 h, then deeply etched and rotary shadowed. The hair cell seen here was cross fractured at the level of the intermediate junction so that any rootlet filaments would be cross fractured. (a) At this low magnification we can see a portion of the lateral membrane at the intermediate junction. Just inside this membrane is a prominent bundle of decorated actin filaments which encircle the cell as a ring. This bundle seems to be distinct from the actin filaments in the cuticular plate. The polarity of the decorated actin filaments is indicated by the arrows. Bar, 0.1  $\mu\text{m}$ .  $\times 44,000$ . (b) A higher magnification of the actin filament bundle inside the intermediate junction. As in a, the small arrows indicate the polarity of the actin filaments. Note that some are of opposite polarities. The long arrows indicate undecorated connecting elements between actin filaments. Bar, 0.1  $\mu\text{m}$ .  $\times 67,000$ . (c) Yet higher magnification of the decorated actin filament bundle just inside the intermediate junction. The polarity of the filaments is clearly visible (see arrows). Some of the decorated actin filaments have the same polarity, others have the opposite. Bar, 0.1  $\mu\text{m}$ .  $\times 123,000$ .

itation of salts during the etching process particularly in light of a recent report by Miller and Lassignal (20) who pointed out that freeze-drying of fresh samples can cause the appearance of artifactual networks of various sorts. Our conclusion is based on the following observations. First, to carry out freeze-drying the etching time must be minimally 10 times longer than that used in this study. Second, a comparison of replicas of fixed (the fixative contains the same amount of salt as fresh samples) and quick-frozen material with samples that are fixed, yet washed extensively with distilled water before freezing, revealed no "new" filamentous structures (see Hirokawa and Heuser [12] and Hirokawa [11] for more details). Third, careful examination of the extracellular space between cells or the cytoplasmic surface of the membrane along the lateral surface of the cell at the nonjunctional area in unfixed samples reveals no fine filaments like those we see connecting the actin filaments to the membrane, etc. Thus the limited etching done in this study (relative to the enormous amount of etching needed to freeze-dry a sample) appears not to induce any gross artifacts. We should also be cautious about possible artifacts induced by condensation (during etching) of the soluble proteins on formed elements in fresh samples. This issue is extensively examined in a separate paper (11). Previously, Hirokawa and Heuser (12) noted in the intestinal brush border that a granular material (presumably soluble proteins) obscured the clear identification of cytoskeletal elements. After examining many types of cells, we know that the amount of this granular material or the concentration of soluble proteins could be different in different kinds of cells as well as in distinct parts of cells. For example, in intact intestinal epithelial cells (either unfixed or fixed) not only are the connectives (see reference 11) between adjacent actin bundles in the rootlet obscured by a granular material but the individuality of the filaments making up the rootlet cannot be seen (12). In contrast, when the axopodia of unfixed, intact, protozoa such as *Echinospaerium* are examined (Heuser and Tilney, unpublished observations) the substructure of the microtubules and its cross bridges are readily seen. An intermediate situation occurs in axons where the cytoskeletal elements are coated with a granular material, probably caused by the condensation of soluble components on them (11), but this granular material does not obscure these elements as often happens in replicas of other unfixed cells (11).

Because we can clearly see filaments in the cuticular plate of unfixed maculae, we conclude that the concentration of soluble components in this region of the cell is relatively low since we can recognize actin filaments and many of the fine, cross-linking filaments. Thus, this was a fortuitous choice of material for examining intact cells. Even so, the surface of the actin is obscured so that we cannot clearly identify the banding pattern seen in purified actin filaments (7) indicating the presence of material on these filaments.

A comparison of unfixed cuticular plates with detergent-extracted, and in other experiments, with detergent-extracted and S1-decorated cuticular plates allows us to begin to understand what this actin gel looks like. Furthermore, because of this comparison we know that when we look at the attachments of actin filaments to the membranes, we are looking at real structures, not at structures produced by the condensation of soluble components.

### *Interaction between Actin Filaments*

Tilney et al. (31) demonstrated that the rootlet actin filaments

are cross-linked with each other and with the actin filaments in the cuticular plate by 3-nm filaments. They also showed that the actin filaments in the cuticular plate are connected to each other by 3-nm filaments. Our study confirmed and extended this earlier report by demonstrating that these cross-connections adjoin actin filaments of both the same and opposite polarities! This result is strikingly different from the in vitro observations of Hartwig et al. (6) on actin-filament "actin binding protein" (220,000-dalton protein) interactions, in which actin filaments with fixed polarities are cross-connected. Likewise, the cross bridging we see here is not the same as that speculated by Schliwa and Van Blerkom (27) from critical point dried specimens of tissue culture cells. The fact that filaments of the same and opposite polarities are both connected by these fine filaments indicates that these cross bridges must be able to rotate upon themselves. The result, of course, is that the 3-nm filaments will hold the actin filaments in a gel.

### *Actin Filament Bundle at the Apico-Lateral Margin of the Cell: Is it a Contractile Ring*

At the lateral margins of the cell we described a ring of actin filaments; the polarity of these filaments is mixed such that if myosin were present, sliding of the filaments past one another could occur. If the filaments in turn were attached to the plasma membrane in this region, constriction of the apex of the cell would occur. The presence of filaments at the apico-lateral surface of epithelial cells has been reported by Flock et al. (5) for hair cells and by Rodewald et al. (25) and Hull and Staehelin (15) for the intestinal epithelial cell. Although Flock et al. (5) and Rodewald et al. (25) demonstrated that these filaments are actin by decoration with S1, both of these investigators did not understand the orientation of the filaments as forming a ring just inside and parallel to the lateral plasma membrane at the intermediate junction. This is not surprising, for if we examine thin sections of a loop of filaments, there is only one way that one can identify these filaments as forming a loop—that is to cut sections across the short axis of the cell at the apical end or to cut a section perpendicular to the orientation of the stereocilium or microvillus. In longitudinal sections the looplike nature of this bundle would not be appreciated. The technique of deep etching, which we used in this study, allows us to easily recognize the three-dimensional character of this loop because we can etch down to  $\sim 0.5 \mu\text{m}$ , yet still resolve what the filaments are doing. Thus the circularity of this bundle becomes obvious in fractures through it.

In our replicas, the ring appears to have autonomy, i.e. most of the filaments that comprise it run parallel to the lateral plasma membrane as a bundle. Thus, if motion is generated by this ring, it would constrict the apical end of the cell in a manner similar to what is thought to occur during cytokinesis. It is interesting, parenthetically, that we still do not know the polarity of the filaments in the contractile ring in dividing cells and thus, how the force for cytokinesis would be generated. Our observation on the hair cells, then, is the first detailed morphological report of a potential contractile ring.

This ring of filaments surrounding the apical margins of the cell is likely to be found in epithelial cells in general. For example, we (unpublished observations) found similar rings at similar positions in intestinal epithelial cells which may play a role in maintaining the stiffness of the apical cell surface. Moreover, this actin ring contracts after incubation with ATP (unpublished observation). The same kind of contractile cir-



cumferential actin bundle has been reported in pigmented epithelial cells (24). The function of this ring in hair cells may be to squeeze the surface of the cell and thus to push the stereocilia upwards. This would tend to increase the sensitivity of the stereocilia and may be an important function in the mammalian cochlea because the outer hair cell could lift up the tectorial membrane from the inner hair cells. This may explain the efferent input to these cells. Alternatively, this ring may function in some way in explaining the sounds that come from the ear. Kemp (16) was the first to record these sounds by placing a microphone in the ear canal just anterior to the ear drum. He demonstrated that, after stimulation of the ear with sound, an echo which has a different frequency from that of the initial sound can be recorded from the ear canal. This suggests that the cochlea itself can produce a vibration. Even more interesting is that when a microphone is placed in the ear canal of an individual complaining of tinnitus or "ringing in the ear," this microphone picks up a vibration or sound at the time of the "ringing." Perhaps it is this actin ring which, by contracting mechanically, distorts the stereocilia which in turn leads to this vibration.

We wish to express our gratitude to John Heuser for the use of his laboratory, for encouragement, and at the same time for tolerance in putting up with our chaos, not to mention being patient when the chicks inadvertently grew into roosters and hens. We also wish to thank Annemarie Weber for her kind and repeated gifts of S1, and Molly Tilney for her great help in preparing the manuscript.

N. Hirokawa was supported by the George Meany MDA post doctoral fellowship and by grants to John Heuser from the U.S. Public Health Service and the Muscular Dystrophy Association of America. L. G. Tilney, on leave from the University of Pennsylvania, was supported by National Institutes of Health grant #HD14474.

Received for publication 28 December 1981, and in revised form 4 June 1982.

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