

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

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ABSTRACT A comparison of hair cells from different parts of the cochlea reveals the same organization of actin filaments; the elements that vary are the length and number of the filaments. Thin sections of stereocilia reveal that the actin filaments are hexagonally packed, and from diffraction patterns of these sections we found that the actin filaments are aligned such that the crossover points of adjacent actin filaments are in register. As a result, the cross-bridges that connect adjacent actin filaments are easily seen in longitudinal sections. The cross-bridges appear as regularly spaced bands that are perpendicular to the axis of the stereocilium. Particularly interesting is that, unlike what one might predict, when a stereocilium is bent or displaced, as might occur during stimulation by sound, the actin filaments are not compressed or stretched but slide past one another so that the bridges become tilted relative to the long axis of the actin filament bundle. In the images of bent bundles, the bands of cross-bridges are then tilted off perpendicular to the stereocilium axis. When the stereocilium is bent at its base, all cross-bridges in the stereocilium are affected. Thus, resistance to bending or displacement must be property of the *number* of bridges present, which in turn is a function of the number of actin filaments present and their respective lengths. Since hair cells in different parts of the cochlea have stereocilia of different, yet predictable lengths and widths, this means that the force needed to displace the stereocilia of hair cells located at different regions of the cochlea will *not* be the same. This suggests that fine tuning of the hair cells must be a built-in property of the stereocilia. Perhaps its physiological vulnerability may result from changes of stereociliary structure.

In the preceding paper we (21) demonstrated that each hair cell of the cochlea is able to specify with remarkable precision the length, width, number, and location of the stereocilia that emanate from its apical surface. Within each stereocilium is an organized bundle of actin filaments (8, 20) held together by macromolecular cross-bridges (5, 6, 17, 20). This bundle runs from the tip of the stereocilium to its base where some of the filaments extend into the apical cytoplasm of the cell as a rootlet. The rootlet filaments in turn are cross-bridged to a

second population of filaments that run perpendicular to the rootlet filaments (11, 20). If the membrane limiting the stereocilium is removed by detergent extraction leaving the core filament bundle, this bundle remains stiff and rigid (9), indicating that the actin filament bundle provides the shape and rigidity of each stereocilium. Furthermore, in organisms exposed to high intensity sound the stereocilia often appear floppy or bent (12, 13). One of the most obvious lesions in these noise-damaged hair cells, at least in the lizard, is a loss in

integrity of the actin bundles at the base of the stereocilia (22), again indicating that the actin filaments provide the rigidity and shape of each stereocilium.

From the observations presented above it seems reasonable that the length of a stereocilium would be regulated by the actin filaments within it, more specifically, by the length of the actin filaments. One would also expect that the width of each stereocilium would be regulated by the number of actin filaments within it, since the membrane forms a tight sleeve around the filament bundle (see reference 20). Lastly, the distribution of stereocilia on the hair cell should be determined by the distribution of actin filament bundles. Thus, if we want to understand how an individual cell controls the length, width, number, and distribution of its stereocilia, we must ask how the cell controls the length, number, and distribution of the actin filaments within each stereocilium.

Over the years much energy has been expended in our laboratories and in those of others in trying to understand how a cell determines the location of its actin filaments, the polarity of the actin filaments, and how many filaments are assembled (5, 20). It seems to us that the hair cell is a particularly interesting object in this regard. A study of the differentiation of the hair cell should provide clues as to how a cell controls these parameters, because the final products, the stereocilia, have well-defined lengths and widths. Thus, in an embryo of a certain age a measurement of the lengths of the stereocilia should tell us whether the actin filaments have assembled to their maximum length or not, or a measurement of the widths of the stereocilia should tell us whether all the actin filaments in the stereocilia are established or more have yet to be started (10). In essence we can assay what the cell is doing by examining, in a scanning microscope, the length and width of its stereocilia and then confirm what has been done by examination of thin sections. It is equally important to determine when the actin filaments become cross-bridged together into the precise paracrystalline array seen in adults. In microvilli, for example, polymerization of actin filaments is complete before cross-bridging occurs (3). For this we must examine the thin sections in detail.

Before presenting our adventures with the chick embryonic cochlea (a subsequent report), we must first describe how the actin filaments are organized in the stereocilia and in the cuticular plates of chicks, since these observations are essential for subsequent reports. What we will show in this report is that the packing of the actin filaments in the bird cochlea is different from that which we described in lizard cochleae, although the same bonding rule is used (see references 6 and 20). More specifically, although the actin filaments are packed with paracrystalline order in both systems, in birds the actin filaments lie on a hexagonal lattice (this report), while those in lizards display a liquidlike order (6). This feature actually makes the analysis of how the actin filaments become packed in the embryo and what regulates what in these embryos more amenable to investigation. But more exciting is that this type of packing allows us to find out what happens to the actin filaments when a stereocilium is displaced, such as might occur during sound stimulation. What we found was that the filaments appear to be able to slide past one another slightly, thereby maintaining the straightness of the stereocilium.

MATERIALS AND METHODS

Preparation of the Cochlea and Fixation for Electron Microscopy: Chicks 1- to 2-wk of age were sacrificed by decapitation and the lower jaw and soft tissues covering the nasopharynx were dissected away. Further

dissection from this ventral orientation involved the scraping away of the cartilage, which exposed the cochlea. Initially, we attempted to fix the cochlea in situ briefly before removing the bony labyrinth as was carried out by Tanaka and Smith (19). Although this method gives adequate fixation of the hair cells as a whole, for our studies the preservation of the actin filaments in the stereocilia and in the cuticular plate was definitely inadequate. We found that the best way to preserve these actin-containing regions was to delay fixation until we had removed the cochlea completely from its home in the bony labyrinth and then to fix it by immersion in 1% glutaraldehyde (Electron Microscope Sciences, Fort Washington, PA) in 0.1 M phosphate buffer at pH 7.0 for 30 min, followed by a brief rinse in 0.1 M phosphate buffer. The isolated cochleae were then fixed in 1% OsO₄ in 0.1 M phosphate buffer at pH 6.2 for 30 min at 0°C. An alternate method involved fixation of the cochleae, again by immersion in a solution containing 1% OsO₄ and 1% glutaraldehyde in 0.1 M phosphate buffer at pH 6.2 for 30 min at 0°C. The latter fixative was prepared immediately before immersion of the cochleae. After fixation the cochleae were washed three times in cold water and stained en bloc with 0.5% uranyl acetate for 3 h to overnight, then dehydrated rapidly in acetone and embedded in Epon 812.

Detergent Extraction: The isolated cochleae were immersed in a solution containing 1% Triton X-100 (Sigma Chemical Co., St. Louis, MO), 30 mM Tris, and 3 mM MgCl₂ at pH 7.5 for 5 min at 0°C with frequent agitation, then fixed in 1% glutaraldehyde in 30 mM Tris and 3 mM MgCl₂ at pH 7.5 for 30 min at 0°C. After washing, the detergent-extracted cochleae were postfixed in 1% OsO₄ in 0.1 M phosphate buffer at pH 6.2 for 30 min at 0°C, then rinsed in water, stained en bloc, dehydrated, and embedded as outlined above.

Electron Microscope Procedures: The blocks were mounted appropriately and trimmed. 1- μ m sections were cut and stained with 1% toluidine blue and examined in the light microscope to ensure that the stereocilia were oriented advantageously, and then thin sections were cut with a diamond knife on a Sorvall Porter-Blum Ultramicrotome II (Sorvall-Dupont, Newtown, CT), picked up on uncoated grids, and stained with uranyl acetate and lead citrate. The thickness of the sections proved to be critical. The thinnest sections, e.g., grey to transparent, were not the best in which to see the arrangement of bridges in the stereocilia; rather, sections whose color was silver to very pale gold proved to be optimal.

Diffraction Patterns and Filtered Images: Diffraction patterns were generated on the computer. In brief, the original negatives were scanned on a 50- μ m raster using an Optronics P1000 (Optronics International, Chelmsford, MA). Optical densities from the scan were used to compute a diffraction pattern according to the procedures presented in DeRosier and Moore (4). The diffraction pattern is displayed on a grey-scale graphics terminal (Grinnell model GMR-27; Grinnell Systems Corp., Santa Clara, CA). Filtered images were calculated on the computer (2) and the result displayed on the graphics terminal. Photographs were made directly from the video monitor using a 35-mm camera.

Computer-generated Model: The computer-generated model in Fig. 12 was produced at the Brandeis University's Feldberg Computer Center on a PDP-10 using PLUTO, a graphics package developed by Sam Motherwell, Cambridge University, Cambridge, England. A Tektronix model 4001 (Tektronix, Beaverton, OR) with a hard-copy attachment (model 4631) was used to record the plots.

RESULTS

We have cut sections through the cochlea at the proximal, central, and distal regions. Although the length, number, width, and distribution of the stereocilia are different in different parts of the cochlea as indicated in the preceding paper (21), the basic morphology of the hair cells is similar. For this report we have confined our attention to that region of the cochlea adjacent to the oval window or at a position \sim 2 mm from the distal end in 1-wk-old chicks. We chose this region because the oval window provides a convenient "landmark" in embryos, enabling us, when we trim the block, for example, of a 10-d-old embryo, to readily compare the structure of the stereocilia at this region in a thin section with their counterparts in thin sections of the chick hatchling cochlea. Obviously, for scanning microscopy this poses no problem since one can examine the entire cochlea at one time, but for thin sections we must know where we are on the cochlea before cutting sections. This is especially important because the cochlea differentiates from the proximal towards the distal end (16).

A Comparison of Tall vs. Short Hair Cells

As mentioned by earlier investigators (10, 18, 19) and in the first paper in this series (21), tall and short hair cells are distinguished by their position on the basilar membrane, by the length or height of the cell, by their type of innervation, and by their surface area. In a single thin section, only some of these features can be seen. It is more convenient to cut a section transversely across the cochlea and identify short and tall hair cells by their positions. In this brief comparison we will touch upon only those details that have not been described before or

upon features of these two types of cells that will be needed for discussion either here or in a subsequent publication. For greater detail on these two types of cells, see Tanaka and Smith (19), Takasaka and Smith (18), and Hirokawa (10).

The apical surface of a tall and short hair cell can be compared by examination of Figs. 1 and 2. As expected, both cells have stereocilia arranged in rows of ascending height and both are separated from adjacent hair cells by a supporting cell on whose apical surface is a tuft of short microvilli. It is obvious from the micrographs that in tall hair cells (Fig. 1) the stereocilia extend from the center of the apical end of the cell,

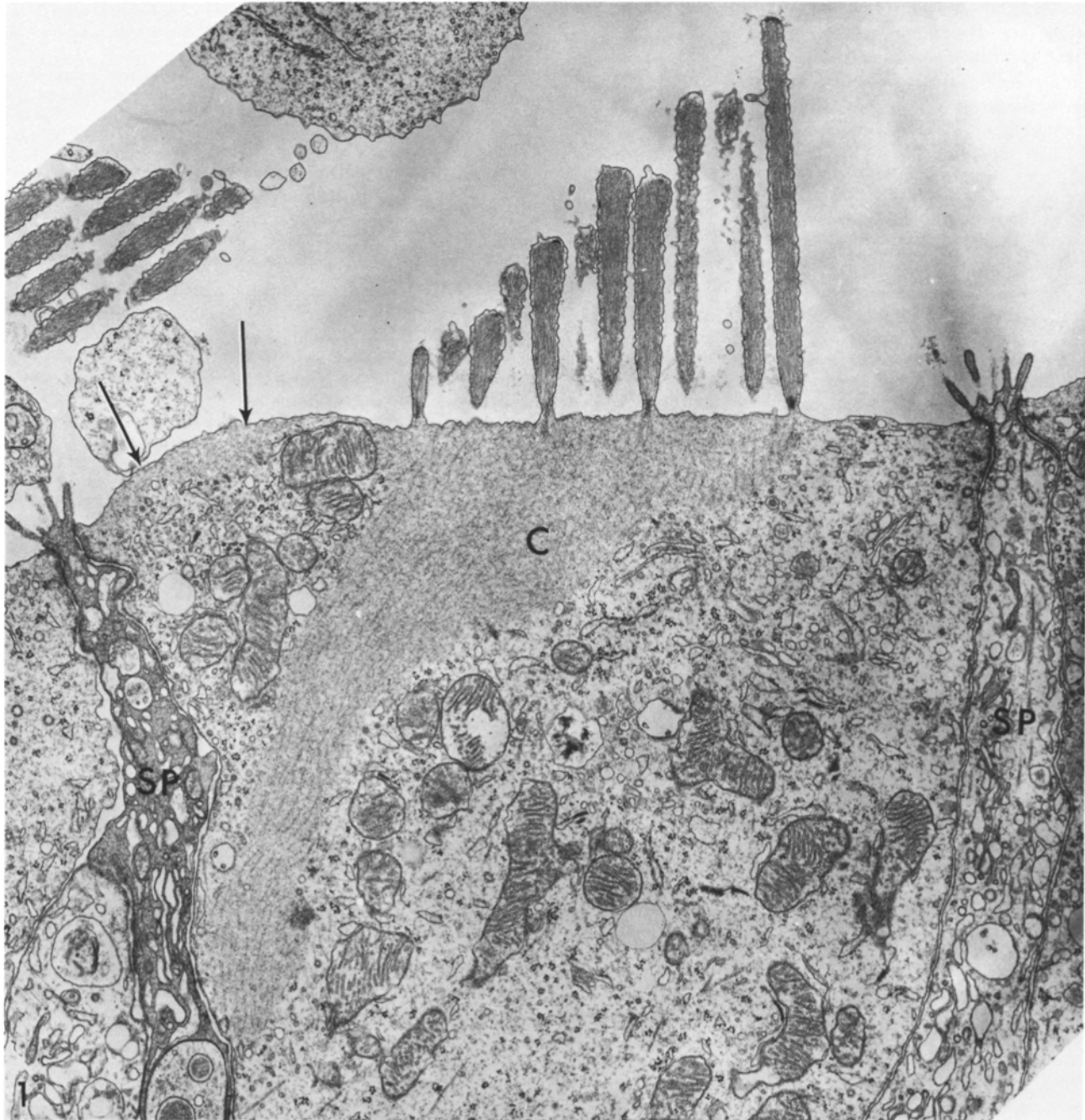


FIGURE 1 Longitudinal section of the apical end of a tall hair cell. Separating this cell from adjacent hair cells are supporting cells (SP) on whose apical margins are short microvilli. Beneath the stereocilia one sees the cuticular plate (C), which extends obliquely towards the lateral margin of the cell. Within it are fine, longitudinally oriented striations, which are the rootlet filaments. The arrows indicate some cuticular plate material that extends from the side of the cuticular plate on which we find the shortest stereocilium to the lateral margin of the cell. It is of interest that no such material extends from the side of the cuticular plate on which the tallest stereocilia are attached. $\times 18,600$.

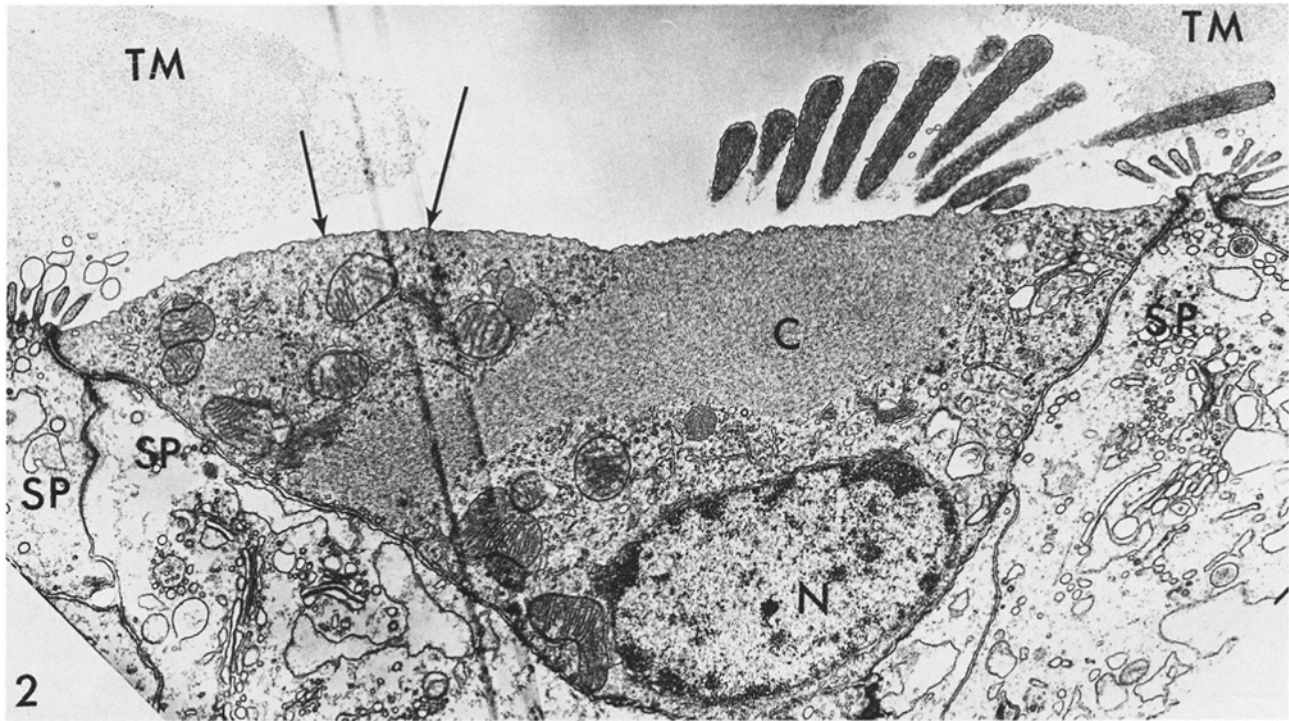


FIGURE 2 Longitudinal section of the apical end of a short hair cell. Separating this cell from adjacent hair cells are supporting cells (SP) on whose apical margins are short microvilli. Beneath the stereocilia is the cuticular plate (C), which extends towards the lateral margin of the cell. The arrows indicate some cuticular plate material that extends from the side of the cuticular plate on which we find the shortest stereocilia to the lateral margin of the cell. No such material is present beneath the apical membrane that connects the cuticular plate on whose surface are the tallest stereocilia with the lateral margins of the cell. The nucleus (N) of this short hair cell and portions of the tectorial membrane (TM) are indicated. $\times 12,000$.

whereas in short hair cells they are eccentrically placed (Fig. 2). In both, they tend to be situated directly above the nucleus. This means that in short hair cells the nucleus is also displaced toward one margin of the cell. Interestingly, it is always the margin nearest the inferior surface of the cochlea.

A careful study of the fine structure of the stereocilia, which will be documented in a subsequent section, reveals no differences in the organization of the actin filaments within each cell type even though there are more actin filaments in the stereocilia of short hair cells than in tall hair cells at the same distance from the distal end of the cochlea. This was expected since the width of the stereocilia as revealed by scanning microscopy (see reference 21) is greater for short hair cells than for tall hair cells. This is an important point because a measurement of the width of a stereocilium in a scanning micrograph gives one a direct measurement of the number of actin filaments in it.

Beneath the stereocilia is a complex network of actin filaments called the cuticular plate (see reference 11). In both tall and short hair cells the bulk of the plate lies directly beneath the stereocilia, but as it descends towards the nucleus it begins to sweep laterally so that ultimately its most basal end makes contact with one side of the cell (Figs. 1 and 2). It is of interest that the tallest stereocilia are always situated on the margin of the cuticular plate that is farthest from the connection that the plate makes with the lateral margin of the cell (Figs. 1 and 2). Likewise, in both tall and short hair cells, at the margin of the cuticular plate nearest the shortest stereocilia, we see a thin layer of filamentous material extending all the way to the supporting cell (see arrows in Figs. 1 and 2). This material is associated with the apical plasma membrane. On the margin of the cuticular plate adjacent to the tallest stereocilia, this

amorphous layer is either absent altogether or much reduced in comparison with the margin adjacent to the shortest stereocilia. In poorly fixed cells or cells from animals exposed to intense noise (unpublished observation), the margin adjacent to the tallest stereocilia often explodes or blebs outwards, while the margin adjacent to the shortest stereocilia is unaffected. This indicates that the amorphous material stabilizes the apical surface of the cell.

Organization of the Actin Filaments in the Cuticular Plate

To better visualize the organization of the actin filaments and their interconnections within this exceedingly complex region of the cell, we extracted the cells first with the detergent, Triton X-100, and cut longitudinal sections through the cuticular plate. The detergent extraction allowed us to recognize longitudinally running rootlet filaments, which extend from the base of the stereocilia through the actin filaments making up the cuticular plate proper (Fig. 3). Extending from these rootlet filaments, which curve toward the lateral margin of the cell, are thin connecting filaments, each $\sim 30 \text{ \AA}$ in diameter. These connections often appear periodic. Although the chemical nature of these connectives is still not known, they are clearly not actin since they do not decorate with S1 of myosin and they are too slender to be myosin (see references 11 and 20).

Transverse sections through the cuticular plate reveal that the rootlet filaments (Fig. 4, circles) extend into the cuticular plate in bundles. We also see actin filaments running perpendicular to the rootlet filaments or parallel to the apical surface of the cell. It is of particular interest that the rootlet filaments

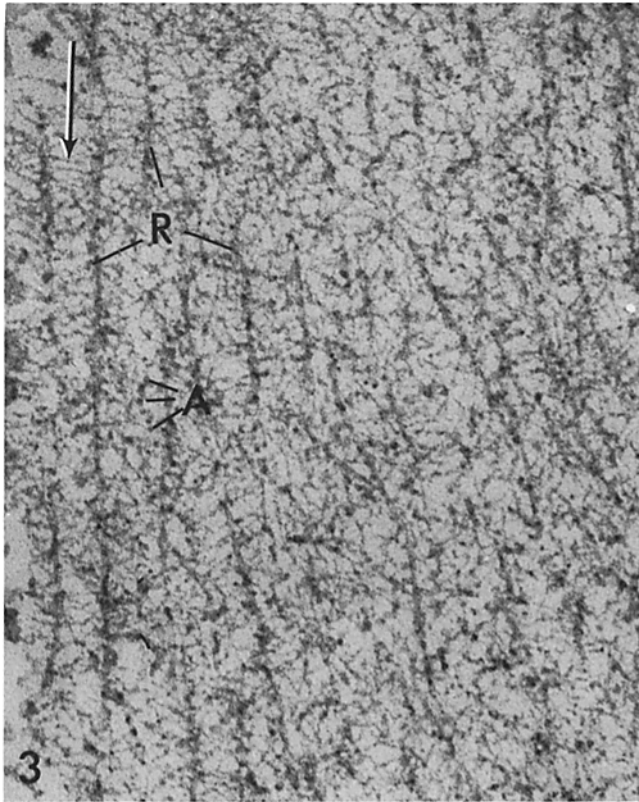


FIGURE 3 Longitudinal section through a portion of the cuticular plate of a detergent-extracted hair cell. Connecting adjacent rootlet filaments (*R*) are fine 30-Å connectors (arrow). The dots indicated by *A* are actin filaments cut in cross section. These parallel the apical surface of the cell. $\times 111,000$.

are easily distinguishable from other actin filaments in the cuticular plate and from the 30-Å filaments because they are ~ 80 Å thick, as compared with the actin filaments, which are 50 Å thick, and with the connecting filaments, which are 30 Å thick.

Connection between the Stereocilium Proper and the Rootlet

Near the base of the stereocilia, where contact is made with the apical surface of the cells, the stereocilia taper dramatically. The actin filaments in the center of the stereocilium, as in the lizard (20), extend all the way to the base, while the actin filaments at the periphery end on the tapered membrane of the stereocilia. We see no morphological specialization of that ending. At the base of the stereocilium the filaments become enmeshed in a plug of electron-dense material that is not removed by detergent extraction (Fig. 4). Rootlet filaments extend from this dense material into the cuticular plate (Fig. 5). Unfortunately, this dense material obscures the relationship between the rootlet filaments and the filaments that extend into the dense material from the stereocilium proper. Thus, we are not sure whether the rootlet filaments are continuous with the small number of filaments that extend from the center of the stereocilium into this dense material or whether there are two populations of filaments that extend into the dense material, one from the cuticular plate and the other from the stereocilium proper. What is clear, however, is that the size, shape, and organization of the dense material, as well as the number of actin filaments that insert into this material (either

from the stereocilium or from the cuticular plate), are the same regardless of whether the stereocilium is from a tall or short hair cell or whether the cell is located at the distal or proximal end of the cochlea.

Organization of Actin Filaments in the Stereocilia

Within each stereocilium is an organized bundle of actin filaments that lie parallel to one another (Fig. 6*a*). At the tip of the stereocilium they are attached to the membrane by some electron-dense material. The actin filament bundle that resides in each stereocilium is also attached to the plasma membrane limiting it by some fine connections. These connections are difficult to preserve in fixed material, but we are able to detect them in detergent-extracted stereocilia (Fig. 9*b*). Here they appear as periodic bumps extending from the surface.

In transverse sections the actin filaments in the stereocilia are hexagonally packed, although sometimes this packing is imperfect (Fig. 6*b*). How much of this imperfection is due to improper fixation and how much exists naturally *in vivo* is difficult to predict, but our best preserved material, as judged by the organization of the filaments in longitudinal sections (see below) and the lack of blebbing of the membranes, shows the highest degree of hexagonal symmetry. Detergent extraction does not affect this type of packing. The lines included in Fig. 6*b* should aid the reader in appreciating the hexagonality.

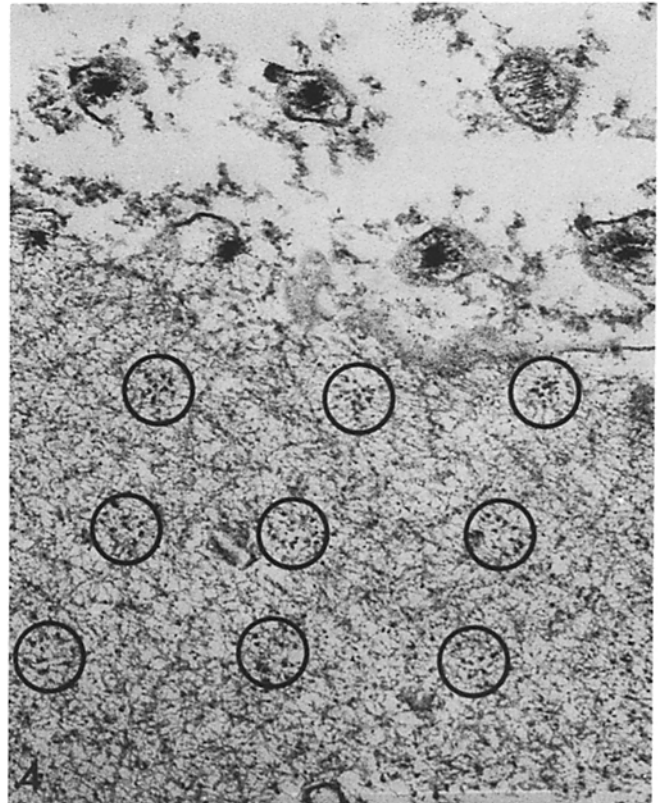


FIGURE 4 Thin section cut parallel to the apical surface of a detergent-extracted hair cell. In the upper portion of the micrograph are sections through the bases of several stereocilia. In the lower part of the micrograph the plane of section cuts across the apical end of the cuticular plate. The circles around the dense dots indicate the bundles of rootlet filaments cut in transverse section. Between these bundles are numerous filamentous elements which primarily lie parallel to the apical surface of the cell. $\times 78,000$.

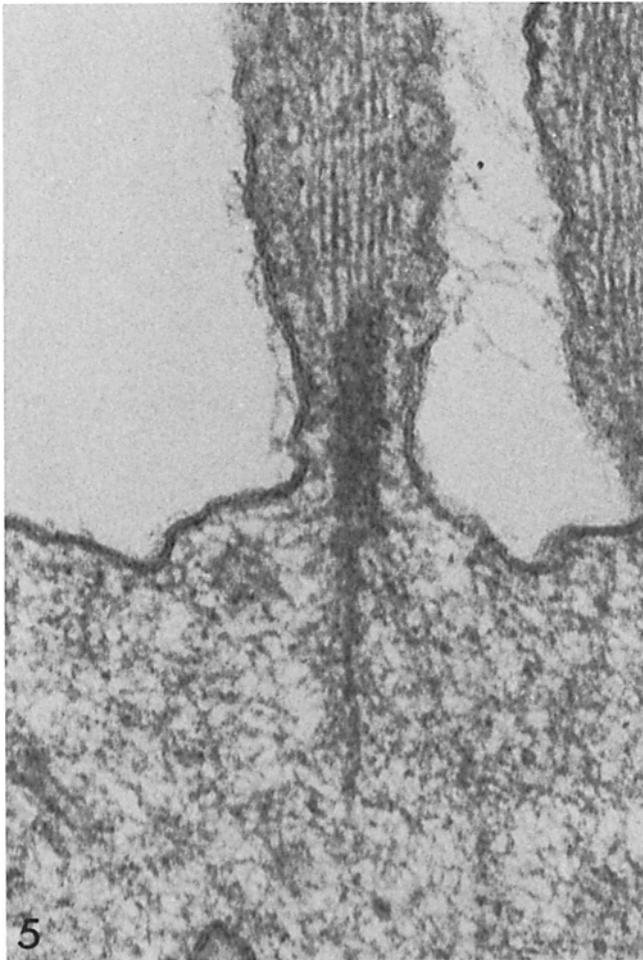


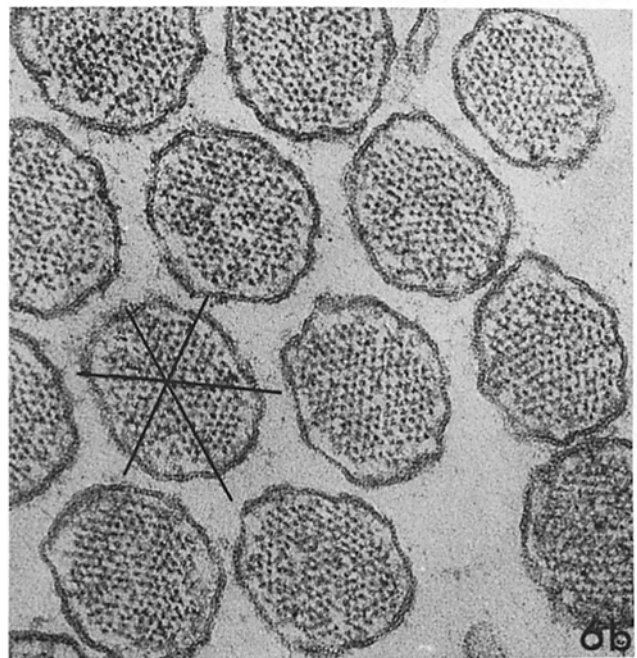
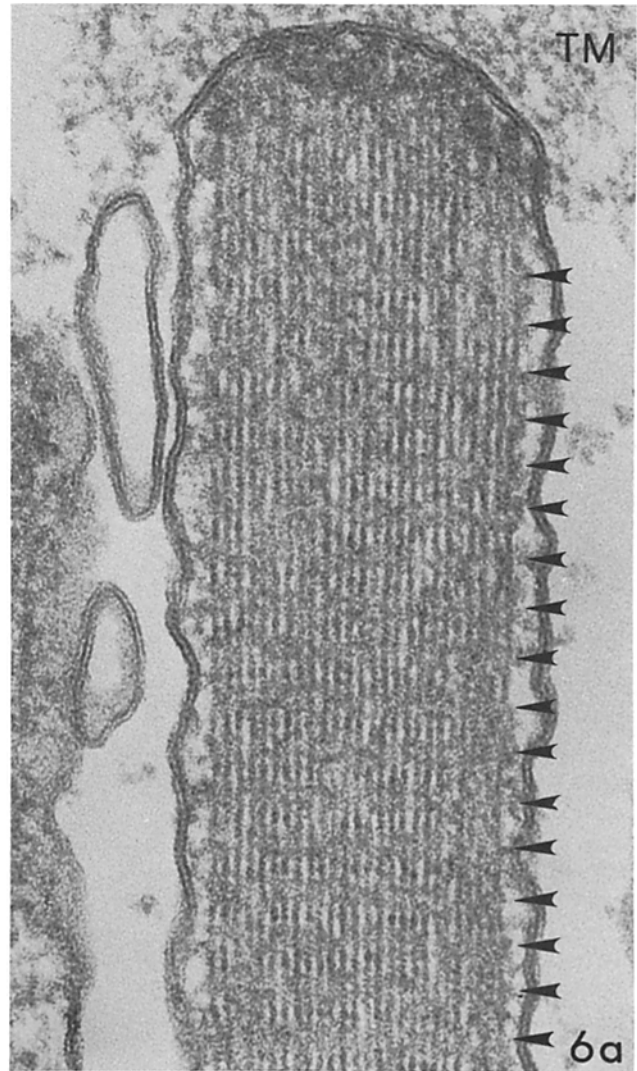
FIGURE 5 Longitudinal section cut through the base of a stereocilium. This section illustrates the central plug of dense material that is connected on its apical end to the actin filaments in the center of the stereocilium and on its basal end to the rootlet filaments. $\times 167,000$.

The filaments appear to be ordered along three sets of row lines that intersect at 60° with respect to each other.

A careful inspection of these cross sections reveals that adjacent filaments, seen as dots in the micrographs, are connected together by thin cross-connections. If the section is thin and somewhat oblique to the axis, we see a festooned pattern as indicated in Fig. 7, reminiscent of that already described for lizards (6, 20). This festooned pattern gives us information on the organization of the bridges (see reference 5).

When we examine longitudinal sections of the stereocilia at high magnification, we get images such as those depicted in Fig. 8a and d. A striking feature of the images is the set of transverse stripes arising from the cross-bridges. These are most easily seen in the filtered images shown in Fig. 8c and f. In Fig. 8a and c the stripes seem uniformly spaced every 125 Å (see arrows), whereas in Fig. 8d and f the stripes have an

FIGURE 6 (a) Longitudinal section through the tip of a stereocilium. The filament bundle terminates near the tip in some electron-dense material that is attached to the membrane. Also attached to the tip of this stereocilium is a portion of the tectorial membrane (TM). Close examination of the filament bundle reveals that there are periodic bands extending across the bundle (see arrows). $\times 240,000$. (b) Transverse section through a number of stereocilia. The filaments



within each stereocilium are arranged on a hexagonal lattice. This can most easily be appreciated by looking at the lines that mark the hexagonal lattice on which the filaments lie. $\times 127,000$.

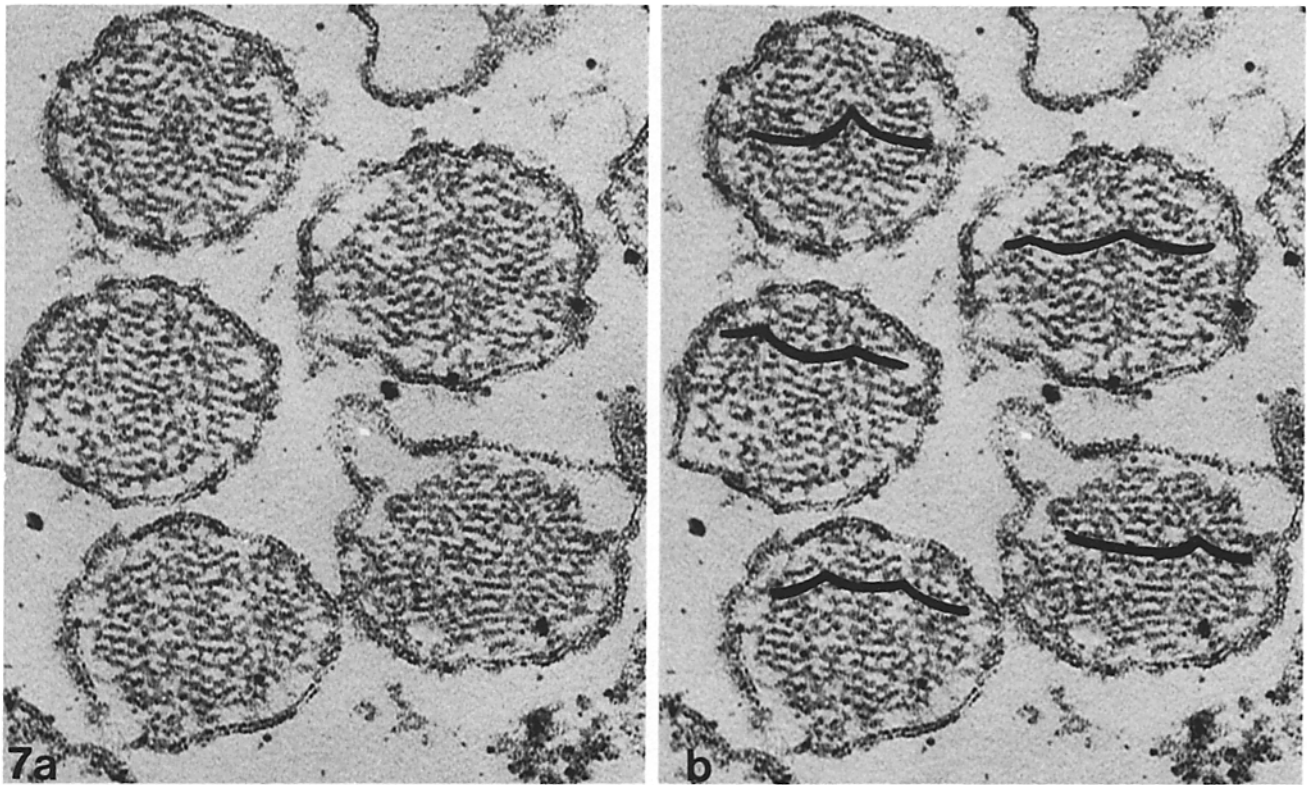


FIGURE 7 Thin transverse section through five stereocilia. Careful examination of this section reveals that the filaments are organized in a "festooned" pattern. This pattern is illustrated in *b*, in which we have drawn over the micrograph in *a*. This pattern appears because the section is very thin ($<300 \text{ \AA}$) and because the stereocilia are cut at an oblique angle to their long axes, rather than exactly normal to them. It is a property of the bridges that connect adjacent actin filaments in the bundle. $\times 160,000$.

apparently different pattern in which the stripes are alternately separated by 125 \AA and 250 \AA (see arrows). Another difference in the two images is that the vertically oriented filaments are clearly seen in Fig. 8*d* and are essentially absent in *a*.

Accompanying the images are their diffraction patterns seen in Fig. 8*b* and *e*. Both patterns show a meridional reflection at $1/125 \text{ \AA}^{-1}$ (i.e., the third layer line) and an off-meridional reflection on the $1/375 \text{ \AA}^{-1}$, or first layer line. The pattern in Fig. 8*e* shows the second layer line at $2/375 \text{ \AA}^{-1}$. The reflections on the first and second layer lines lie in a vertical row. This row line arises from interfilament interference and tells us that all the actin filaments are aligned with their crossover points in register.

Structural Changes Accompanying Bending of the Stereocilia

In some cases the striping on the actin bundle or the pattern produced by the bridges is perpendicular to the bundle axis; in other cases it does not extend perpendicularly across the bundle but is situated at an oblique angle to the bundle. In studying this phenomenon, we noted the following: if the stereocilia extend from the hair cell exactly perpendicular to the apical surface of the cell, then the bridges are found perpendicular to the axis of the stereocilium (Fig. 9). On the other hand, if the stereocilia extend at an oblique angle to the apical cell surface, then the bridges are found at an angle to the stereociliary axis (Fig. 10). The key to their behavior is that the bridges remain parallel to the apical surface of the hair cell when the stereocilium is displaced such as might occur when it is moved naturally by mechanical stimuli. This phenomenon can even

be seen in an individual stereocilium that somehow was bent, then fixed (Fig. 11*d*). The basal end of the stereocilium is not bent and the bridges are found running perpendicular to the axis. In the bent portion the bridges remain parallel to those below them, even though they are now running at an oblique angle to the new (now bent) axis.

DISCUSSION

Packing of the Actin Filaments in the Stereocilia of the Bird

Since each actin filament in the stereocilium is composed of monomers arranged in a double helical pattern, and since all the actin filaments are aligned such that the crossover points of their helices are in register, a fact established from the diffraction patterns (see Results), it stands to reason that bridges connecting adjacent actin filaments must bond at specific sites along the filament. This point can be best appreciated by a close examination of Fig. 12, which shows a model of several filaments sitting on the vertices of a hexagonal lattice. The filaments are positioned so that the lowest subunit of each filament is pointing in the same direction; thus, the crossover points are fixed in register. Bridges are inserted between the filaments at positions in which the actin subunits are pointing towards each other, as is observed in the electron micrographs. In Fig. 12*b*, filaments *A* and *B* are bound by a cross-bridge (here indicated by a rod) at subunit 1. Notice that as we travel up these two filaments the subunits no longer point towards each other until one-half turn of the helix has been completed. At this point, filaments *A* and *B* are again cross-bridged to each other. Filaments *A* and *C* can also be bound together but,

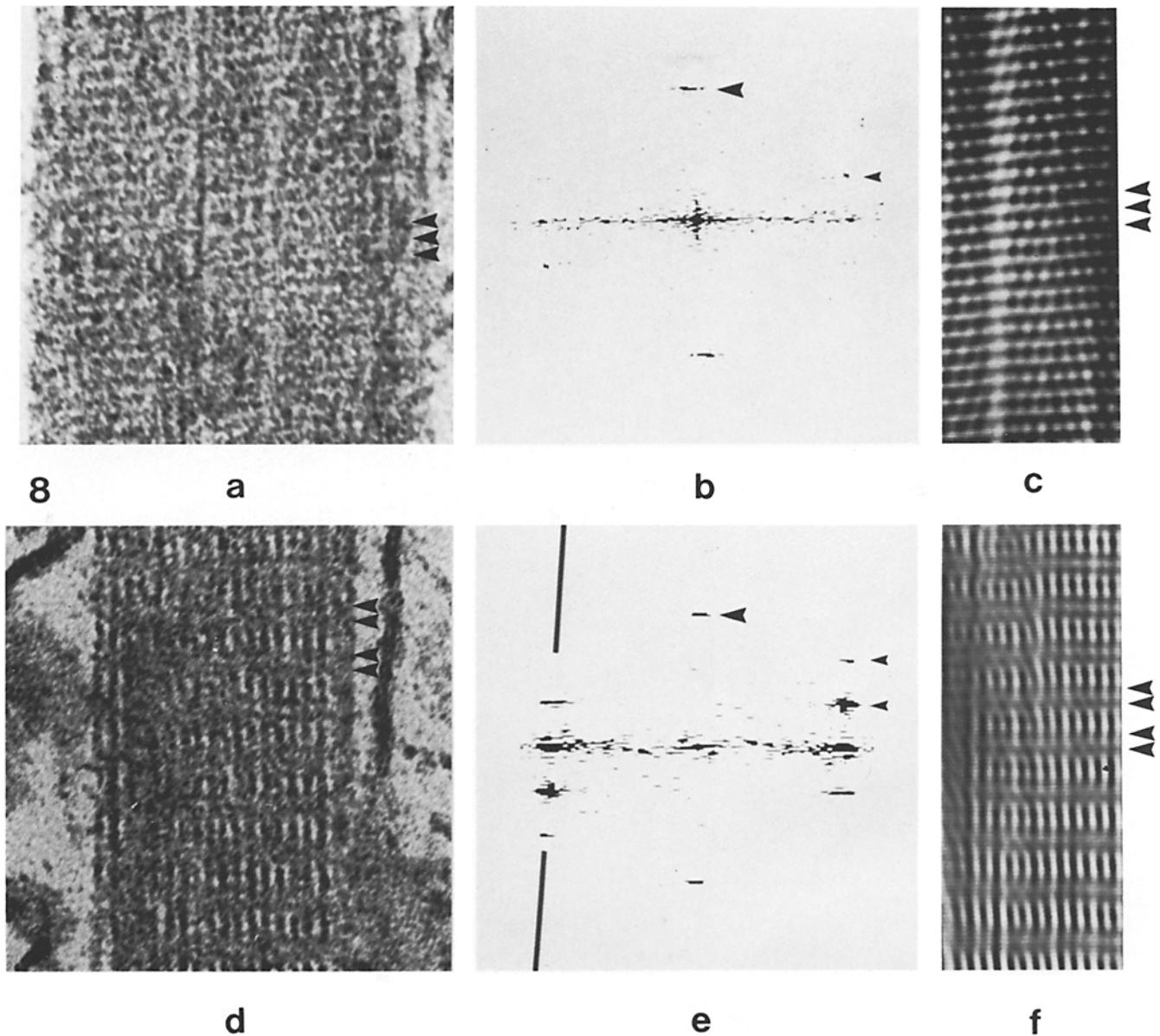


FIGURE 8 (a) Longitudinal section of a detergent-extracted stereocilium cut to illustrate a near 1,1 view. Prominent here are striations separated at 125 Å intervals (see arrows). Notice that the longitudinal striations corresponding to the filaments are not visible as they are in the 1,0 view (Fig. 7 d). $\times 310,000$. (b) Computed diffraction pattern of a. The main reflection seen here is the meridional reflection at $1/125 \text{ \AA}^{-1}$ (see larger arrow). Barely visible is a reflection on the first layer line at $1/375 \text{ \AA}^{-1}$ (see smaller arrow). (c) Filtered image of b. The arrows indicate the position of the bridges. (d) Longitudinal section of a detergent extracted stereocilium cut to illustrate the 1,0 view. The arrows depict the striations that are separated from each other by 125 and 250 Å. $\times 310,000$. (e) Computed diffraction pattern of d. Notice the reflections on the first and second layer lines at $1/375 \text{ \AA}^{-1}$ and $1/187 \text{ \AA}^{-1}$, respectively (see small arrows). These arise from the helical symmetry of actin. On the meridian is a strong reflection at $1/125 \text{ \AA}^{-1}$ (see larger arrow). It arises from the cross-bridges. One of the vertical row lines arising from the packing of filaments is indicated by the tilted vertical line. (f) Filtered image of d. The arrows indicate the position of the bridges.

because of the helical symmetry of the filament, bonding occurs at subunit 5, not 1. Filaments B and C bond at subunit 10 and so forth. Thus, the actin helix specifies the position of the cross-bridges.

When all the actin filaments in the bundle have their cross-over points in register, or, to put it another way, when subunit 1 on each filament points in the same direction, the bridges that will form will line up to create a striated appearance. Neighboring filaments would then be cross-bridged once per half-turn of the two-start helix or every 375 Å, and, since there are three sets of neighbors in a hexagonally packed bundle,

there will be three striations per half-turn at $\sim 125\text{-\AA}$ intervals, as can be seen in Fig. 12 c.

We asked the computer to draw this bundle as it would appear when examined in the electron microscope lying on a grid. To do this, we simply remove the perspective so that filament D lies directly behind filament A. This view, known as the 1,1 view, is shown in Fig. 12 c. Another way of seeing what we have done is to mentally flip the bundle depicted from the top in Fig. 12 a on its back. As in Fig. 12 c, filament A will lie directly over filament D. In this view we see that there are three cross-bridges visible per half-turn of the helix, corre-

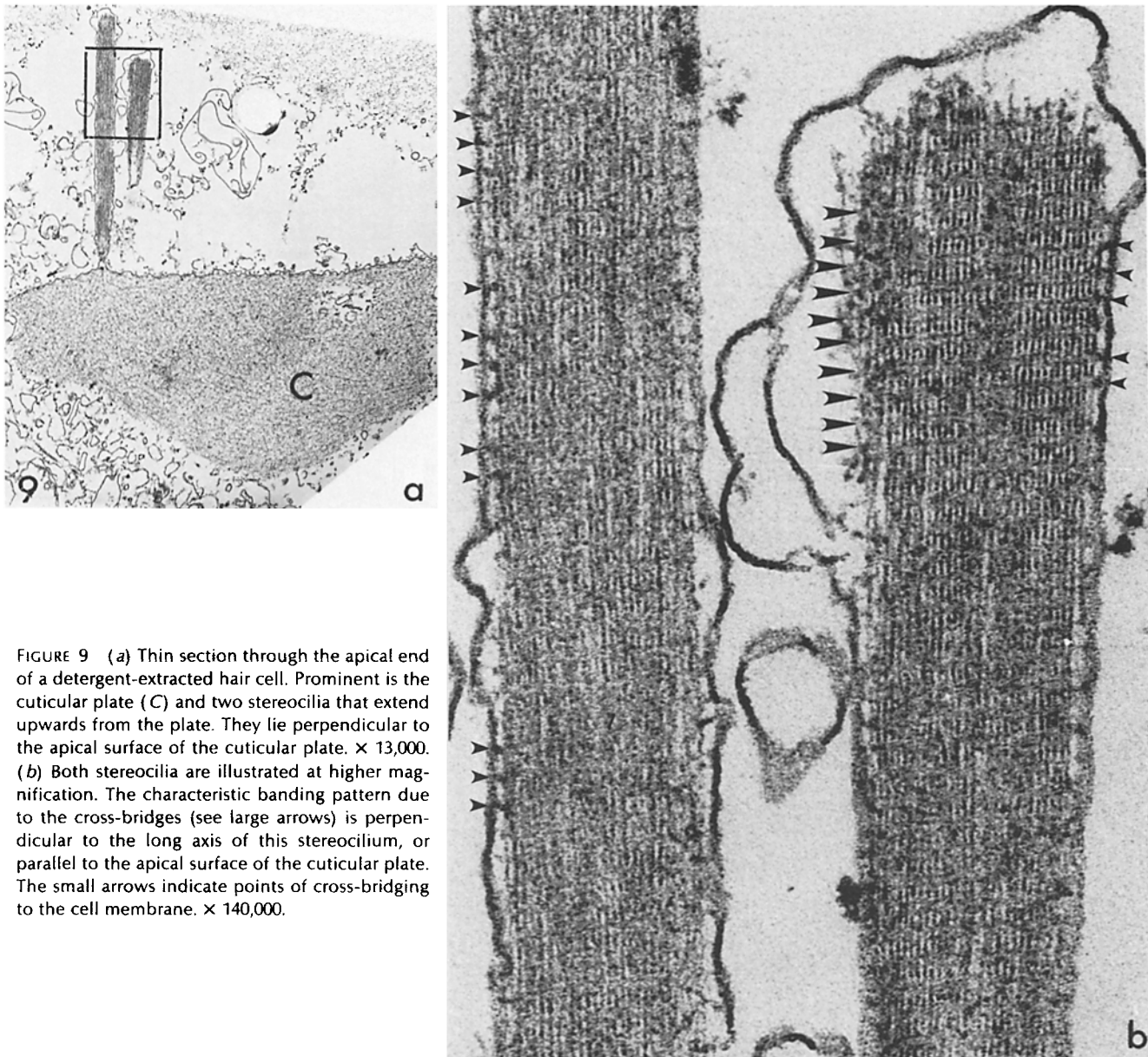


FIGURE 9 (a) Thin section through the apical end of a detergent-extracted hair cell. Prominent is the cuticular plate (C) and two stereocilia that extend upwards from the plate. They lie perpendicular to the apical surface of the cuticular plate. $\times 13,000$. (b) Both stereocilia are illustrated at higher magnification. The characteristic banding pattern due to the cross-bridges (see large arrows) is perpendicular to the long axis of this stereocilium, or parallel to the apical surface of the cuticular plate. The small arrows indicate points of cross-bridging to the cell membrane. $\times 140,000$.

sponding to the 125-Å striations visible on the real bundle (Figs. 8a and c).

What is superficially confusing is that in some longitudinal sections of the stereocilium we see a striping pattern consisting of two stripes separated by 125 Å, then a space of 250 Å, then two stripes, etc. (Figs. 8d and f). This is, in fact, what should be seen for a hexagonally packed bundle when viewed in the 1,0 rather than the 1,1 direction. The easiest way to explain this is for the reader to look down the row of filaments B, C, and D. This perspective view is shown in Fig. 12e. In this view the cross-bridges between A and C are hidden by filament A. These hidden cross-bridges are indicated on filaments A, B, and D by the filled circles. As mentioned for the 1,1 view, we can see how this image depicted in Fig. 12e arose by mentally flipping the bundle depicted in Fig. 12d on its back. Thus, we see two cross-bridges separated by 125 Å, then a gap of 250 Å, and two cross-bridges, etc. Fig. 12f shows how a hexagonally packed bundle of actin filaments would appear when looked at along the 1,0 direction (i.e., with the perspective removed).

The diffraction patterns shown in Fig. 8b and e are those expected for a bundle of actin filaments whose crossover points

are in register (6). It is very similar to the pattern obtained for the stereocilia of the lizard cochlea (20) except for the strong meridional reflection (at $1/125 \text{ \AA}^{-1}$) indicated in Figs. 8b and e. This reflection is missing in the pattern from lizard stereocilia. It arises as the result of a combination of the presence of the cross-bridges and the hexagonal arrangement of the actin filaments. Although the lizard stereocilia have cross-bridges, the filaments are not hexagonally arranged, so the ($1/125 \text{ \AA}^{-1}$) meridional reflection is not present (5), nor in the micrographs do we see striations at 125-Å intervals in the 1,0 and 1,1 views.

What Happens When a Stereocilium Is Bent?

The transverse striations in the micrographs of chick stereocilia make it possible for us to interpret what happens when the stereocilia are displaced such as presumably occurs during sound stimulation. In addition, we can obtain information on what happens when a stereocilium is bent not at its base but in the middle.

Let us start by considering two modes of bending of a pair of filaments. In Fig. 11b we depict a bundle of three cross-

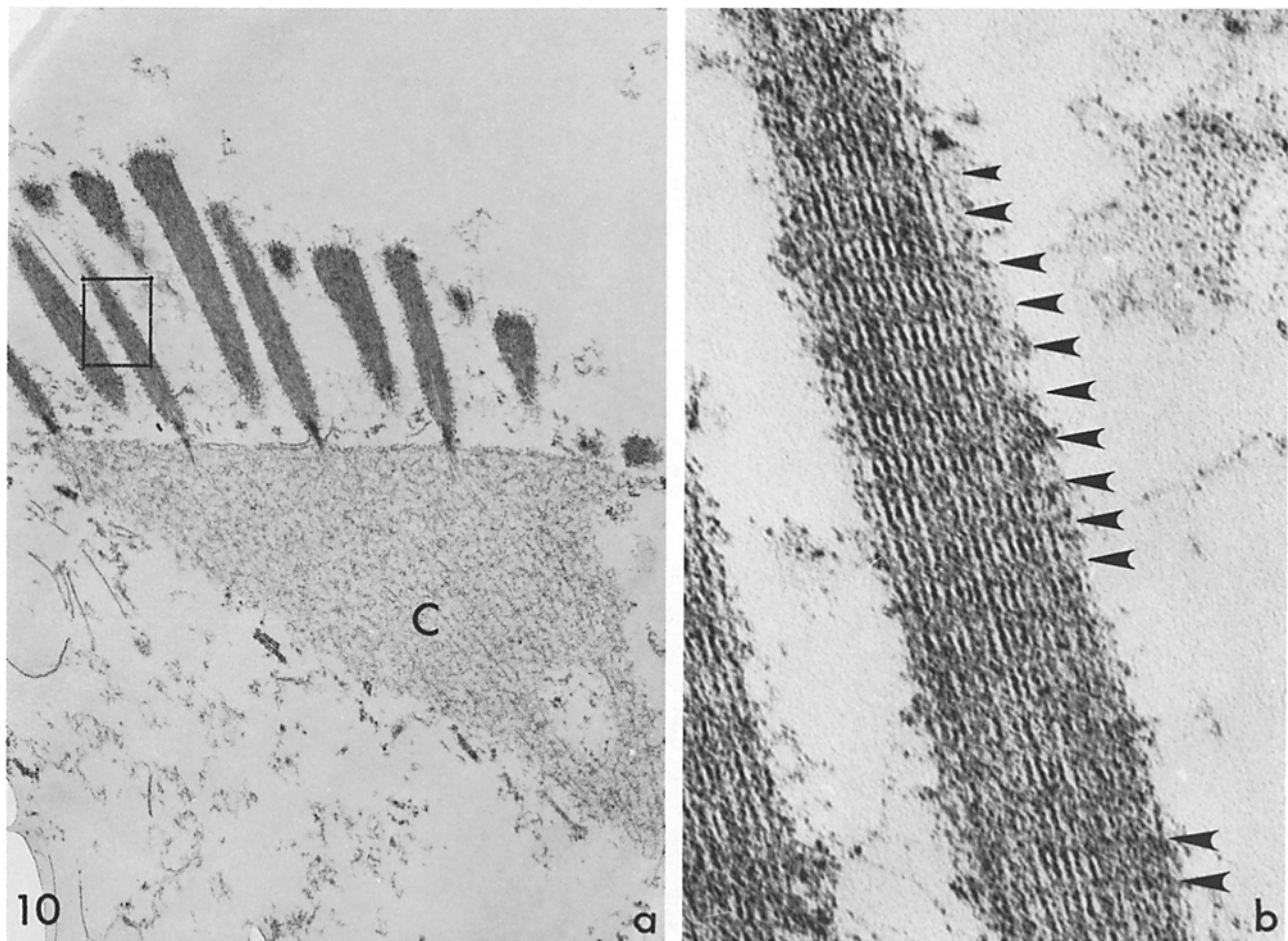


FIGURE 10 (a) Thin section through the apical end of a detergent-extracted hair cell. Prominent are the cuticular plate (C) and the stereocilia. In this micrograph the stereocilia lie at an oblique angle to the apical surface of the cuticular plate. The square indicates a portion of a stereocilium, which is shown at higher magnification in *b*. $\times 23,000$. (b) Of interest in this micrograph is that the banding pattern due to the cross-bridges remains parallel to the apical surface of the cuticular plate. It is no longer perpendicular to the long axis of the stereocilium. $\times 210,000$.

bridged filaments that are bent; the lefthand filament is stretched and the righthand one is compressed to achieve the bend. In such a situation the rows of cross-bridges remain perpendicular to the bundle axis and thus are no longer all parallel to each other. In Fig. 11*c* we illustrate another possibility, namely one in which the filaments are neither stretched nor compressed. Here the cross-bridges must bend in order to maintain bonding. In this situation the rows of cross-bridges do not remain perpendicular to the bundle axis but instead all remain parallel to each other.

When we examined the micrographs of a bent stereocilium such as in Fig. 11*d*, we found that the cross-bridges maintain themselves parallel to each other as suggested in Fig. 11*c*. Thus, the actin filaments do *not* stretch or compress but rather slip relative to each other so that the cross-bridges will appear tilted, since they remain attached to the same subunits. In this regard, there is evidence that the outer domain of the actin subunit can tilt by 10° , an amount similar to the tilt required when stereocilia are displaced (Egelman, E. H., and D. J. De Rosier, unpublished results). Thus the tilting of cross-bridges may in fact reflect the capacity of the actin subunit itself to tilt.

Now we can ask what happens when a stereocilium is displaced so that it bends at its base rather than its middle. Looking at the micrographs of stereocilia bent at their bases such as illustrated in Figs. 9 and 10, we clearly see that the cross-bridges remain parallel to the apical surface. What are

the implications of this observation? It means that bending of the stereocilium at its base not only affects the 20 filaments at the point of bending but affects *all* the filaments in the bundle.

Before we consider how this behavior affects the response of the stereocilia during sound stimulation, let us ask whether this effect is observed in other vertebrates. The lizard stereocilia are more difficult to analyze because they lack the 125-Å periodicity. Nevertheless, from the diffraction patterns of the stereocilia of the lizard cochlea, we have determined that the filaments slide past one another rather than being stretched or compressed. Our conclusion is based on the following observations. If the cross-bridges remain perpendicular to the bundle axis as a result of the stretching and compression of the filaments, then the row lines connecting strong intensities on the layer lines will always be exactly perpendicular to the layer lines. On the other hand, if there is a slippage of filaments past one another so that the filaments are not stretched or compressed, then the row lines will shift off the perpendicular by the amount of the bend. In optical diffraction patterns of the lizard stereocilia, row lines are frequently shifted off the perpendicular (see reference 20). Thus, we think they behave exactly like the stereocilia in birds.

It is possible that our observations on the behavior of the bridges when a stereocilium is bent are an artifact of the preparative procedure for electron microscopy. For example, perhaps in the native state, the actin filaments are stretched

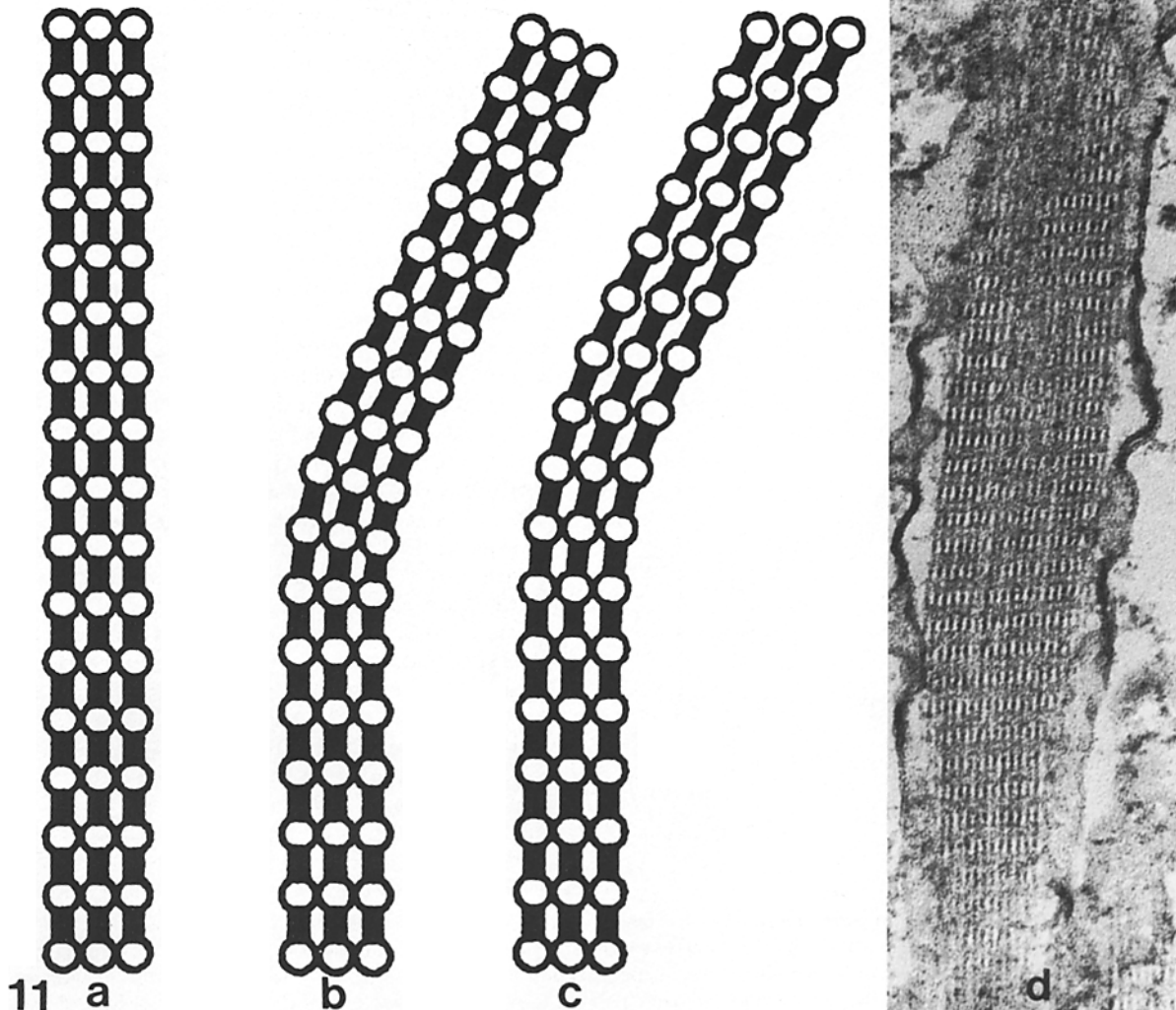


FIGURE 11 (a) Drawing depicting three filaments that lie parallel to each other. (b) Drawing illustrating what might happen if the filaments were bent. In this drawing the filament on the left is stretched, and that on the right compressed, so that the cross-bridges would remain exactly perpendicular to the filaments. (c) Drawing illustrating what might happen if the filaments were bent such that the filaments are neither stretched nor compressed. In this case the cross-bridges would no longer remain perpendicular to the filaments, but would remain parallel to each other. (d) Thin section through a bent stereocilium. Notice that the pattern of bridges fits c, but not b. Thus, the filaments must slide past one another during bending. $\times 147,000$.

and compressed and the cross-bridges unbent, whereas it is only after fixation that the opposite is seen. Observations of the actin bundles in the sperm of *Limulus* shed some light on this question. The sperm contains a coiled bundle of cross-bridged actin filaments. In the coil the bundle is not uniformly curved (i.e., circular) but is polygonal, consisting of sharpish bends (elbows) separated by straight regions (arms). In passing through an elbow the actin filaments are neither stretched nor compressed, but, as in the stereocilia, they slip relative to each other. This situation is found in negatively stained bundles, in thin sections of bundles, and in fast-frozen, etched bundles (5). Thus, for *Limulus*, the observations are not an artifact of preparation. However, the crosslinking proteins in *Limulus* and in the stereocilia are different, so the two cases are not strictly comparable. Nevertheless, what is common to both is that cross-linked actin filaments slip relative to each other rather than being stretched or compressed.

Resistance to Displacement Depends on the Length and Width of the Stereocilium

Now we can ask how the behavior of the cross-bridges affects

the response of the stereocilia during sound stimulation. The amount of movement of the stereocilium depends on a number of factors. It depends on the torque exerted on the stereocilium and the opposing of this force by the built-in rigidity of the bundle. There are two components of the bundle to consider during bending. One is the base of the stereocilium, which consists of 20 or so filaments engulfed in some dense material of unknown properties. This structure seems to be identical in every hair cell of the cochlea. Thus, it appears to contribute a constant term. The second component is the cross-bridges in the stereocilia. Strictly speaking, we cannot differentiate between a bending within the actin subunit, within the cross-bridges, or some combination of the two. For simplicity, however, we will treat the deformation as the bending of cross-bridges. Thus, the amount of resistance to displacement under a fixed torque will depend on the number of cross-bridges being bent.¹ Since we know the packing of the actin filaments

¹ By knowing the Young's modulus for actin, we can, in principle, set limits on the stiffness of the cross-bridges and, therefore, on the resistance of the stereocilium to bending. A complete analytical de-

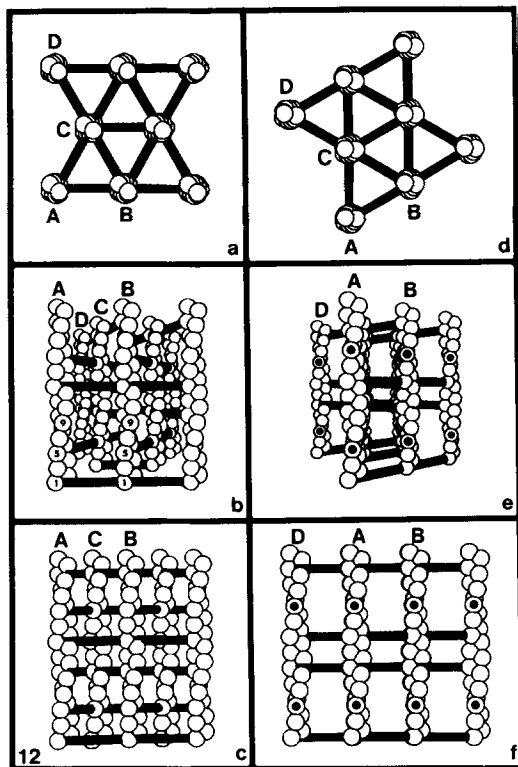


FIGURE 12 Computer-generated model of a cross-bridged bundle of actin filaments. (a) Top view. (b) 1,1 view with perspective. If one flips the bundle seen in *a* on its back, one sees this view. The cross-bridges are represented by the shaded lines. (c) 1,1 view without perspective. This image simulates what is seen in the electron microscope. A series of more or less evenly spaced transverse bands cross the filaments. (d) Top view. The same as in *a* but rotated 30°. (e) 1,0 view with perspective. If the bundle in *d* is now flipped, one generates this view instead of the 1,1 view. (f) 1,0 view without perspective. This image shows the characteristic two bands and a gap rather than three evenly spaced bands. It is easy to see that one band is missing because the cross-bridges are viewed end on and appear hidden behind filaments. The dots indicate the positions of hidden cross-bridges.

in the stereocilia, it is easy to calculate the number of cross-bridges per stereocilium. For each 375 Å of every filament there are three cross-bridges. Thus, the number of cross-bridges depends on both the length and number of filaments. For example, in the tall hair cells at the low frequency end of the cochlea the stereocilia are 5.5 μm in length and 0.12 μm in width (see the accompanying paper [21]). Each stereocilium, then, would contain 37,000 cross-bridges. In contrast, at the high frequency end of the cochlea each stereocilium is only 1.5 μm in length and 0.12 μm in diameter. It would contain 10,000 cross-bridges. Thus, the stereocilia from different ends of the cochlea would respond differently to an applied force.

In the preceding paper (21) we showed that in the bird the length and width of the stereocilia in fact vary systematically from the distal to the proximal end. These variations in turn must affect the mechanical properties and hence the tuning of the cochlea. This may explain some of the puzzling aspects of fine tuning.

For over 10 years, fine frequency discrimination of the

scription of this motion suffers from indeterminacy of a very high order, and therefore the problem must be solved numerically. This will be left for future work.

peripheral auditory system has been known to be physiologically vulnerable. Within 5 min after the onset of hypoxia there is a marked loss of fine tuning from single neuron recordings. Evans (7) stated: "It is difficult to conceive of the basilar membrane mechanics being so sensitive to metabolic changes." Hence, researchers proposed that the basilar membrane mechanics acted to give coarse tuning and that there existed a vulnerable second filter that provided the fine tuning.

Within the past several years, it has been shown that the basilar membrane motion is itself physiologically vulnerable, counter to Evans's (7) plausible comment. Rhode (15) stated that within 10 min after death there is a 10-fold decrease in amplitude of vibration. The question is, What part of this mechanical system is vulnerable? Given that some of the mechanical components of the cochlear partition, such as the tectorial and basilar membranes, are extracellular, it is unlikely that these are the site of the physiological vulnerability. What is more likely is that the hair cells coupling these to membranes are the vulnerable point. In particular, we think that the stereocilia themselves are responsible for fine frequency discrimination as well as the lability of basilar membrane motion, an idea suggested earlier (1, 24). Evidence for this has come from studies of acoustic trauma in which a loss of fine tuning is associated with a loss in rigidity of the stereocilia (12, 13). It is plausible that intracellular changes caused by hypoxia and metabolic poisoning could have a similar effect.

A more dramatic demonstration of the importance of the stereocilia to fine tuning comes from studies of the alligator lizard. In this organism the basilar membrane moves as a rigid structure for most sound frequencies and does not show the spatial variation associated with fine tuning. Recordings from hair cells that sit on the membrane, however, do show fine tuning. Since Mulroy (14) noted a gradation of stereocilia length along the papilla, Weiss et al. (23) suggested that fine tuning results solely from the mechanical properties (e.g., length and stiffness) among the stereocilia. A loss of fine tuning is seen in lizards subjected to acoustic trauma. This loss is accompanied by morphological changes in which the actin filaments in the stereocilia depolymerize where they are connected to the rootlet. In addition, cross-bridges between filaments disappear. Both effects result in a loss of the mechanical rigidity of the stereocilia (22).

Thus, the observations we are reporting here on the mode of stereocilia bending (and the relation between length and stiffness), combined with our previous report (22) on the effect of acoustic trauma on stereocilia rigidity, appear to shed some light on the mechanism of fine frequency selectivity and the source of its vulnerability.

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