Actin Filaments, Stereocilia, and Hair Cells of the Bird Cochlea I. Length, Number, Width, and Distribution of Stereocilia of Each Hair Cell Are Related to the Position of the Hair Cell on the Cochlea

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ABSTRACT Located on the sensory epithelium of the sickle-shaped cochlea of a 7- to 10-d-old chick are ~5,000 hair cells. When the apical surface of these cell is examined by scanning microscopy, we find that the length, number, width, and distribution of the stereocilia on each hair cell are predetermined. Thus, a hair cell located at the distal end of the cochlea has 50 stereocilia, the longest of which are 5.5 μm in length and 0.12 μm in width, while those at the proximal end number 300 and are maximally 1.5 μm in length and 0.2 μm in width. In fact, if we travel along the cochlea from its distal to proximal end, we see that the stereocilia on successive hair cells gradually increase in number and width, yet decrease in length. Also, if we look transversely across the cochlea where adjacent hair cells have the same length and number of stereocilia (they are the same distance from the distal end of the cochlea), we find that the stereocilia of successive hair cells become thinner and that the apical surface area of the hair cell proper, not including the stereocilia, decreases from a maximum of 80 μm^2 to 15 μm^2. Thus, if we are told the length of the longest stereocilium on a hair cell and the width of that stereocilium, we can pinpoint the position of that hair cell on the cochlea in two axes. Likewise, if we are told the number of stereocilia and the apical surface of a hair cell, we can pinpoint the location of that cell in two axes.

The distribution of the stereocilia on the apical surface of the cell is also precisely determined. More specifically, the stereocilia are hexagonally packed and this hexagonal lattice is precisely positioned relative to the kinocilium. Because of the precision with which individual hair cells regulate the length, width, number, and distribution of their cell extensions, we have a magnificent object with which to ask questions about how actin filaments that are present within the cell are regulated. Equally interesting is that the gradient in stereociliary length, number, width, and distribution may play an important role in frequency discrimination in the cochlea. This conclusion is amplified by the information presented in the accompanying paper (Tilney, L. G., E. H. Egelman, D. J. DeRosier, and J. C. Saunders, 1983, *J. Cell Biol.*, 96:822–834) on the packing of actin filaments in this stereocilia.

Actin is the most commonly encountered protein in nonmuscle cells, where it can account for up to 15% of the total protein of the cell. It is now well established that this protein is not only involved with motile events such as pseudopodial movements, cytokinesis, clot retraction, and extension of the acrosomal process but also is the major component of the cytoskeleton, providing a frame that determines cell shape and symmetry. Because of the obvious importance of actin, cell biologists have been trying to determine the rules governing its assembly and disassembly. More specifically, they have asked these questions: What controls the correct positioning of actin filaments in cells? How is the polarity of assembled filaments regulated?

THE JOURNAL OF CELL BIOLOGY · VOLUME 96 MARCH 1983 807-821

How is their number determined? What might limit the length of the assembled filaments?

Two prominent examples come to mind: skeletal muscle cells and the hair cells of the ear. In both, not only is the distribution of the actin filaments controlled but, more surprisingly, the lengths are precisely regulated. For example, in skeletal muscle cells the lengths of the actin filaments in each sarcomere are fixed and in hair cells there is a population of sensory microvilli (stereocilia) within which are actin filaments with precisely regulated lengths. From these two examples, then, we recognize that at least some cells have the capability of defining the lengths of actin filaments very precisely, more precisely, in fact, than is predicted by kinetic studies on isolated solutions of actin. Perhaps all cells have this ability, but under most circumstances we have not recognized it.

We have become particularly curious about how the length of an actin filament bundle might be regulated (4). The stereocilium seemed to us to be an especially convenient biological object because, by measuring its length, e.g., by light microscopy or by scanning microscopy, we immediately know the length of the actin filament bundle within it (22). Before trying to determine how an embryonic hair cell controls the lengths of its actin filaments, we carefully examined the stereocilia of neonatal chicks to provide a basis for comparison with differentiating embryos. The precision with which the number and dimensions of the stereocilia are specified makes this an even better system to study than we had initially thought, but it is equally exciting that our studies have focused our attention on features of hair cells, e.g., length, width, and number of stereocilia, which may be important in frequency discrimination during sensory transduction. Most investigators have concentrated on the physical properties of the basilar membrane, an extracellular layer on which the hair cells sit, since it was thought that this extracellular membrane is the major determinant of the "Place principle" of frequency coding in the cochlea (3). More specifically, they followed up von Bekesy's (1) classic and elegant studies, which showed that different frequencies stimulated different parts of the basilar membrane but neglected to examine the sensory transducers themselves. The notion that the mechanical properties of the stereocilia may be important to frequency coding finds some support from indirect evidence in the lizard cochlea (25). Additional evidence for this contention is included in the accompanying paper (23).

MATERIALS AND METHODS

Dissection: 7- to 10-d-old chicks were sacrificed with an overdose of urethane. After decapitation, the lower jaw and the soft tissues covering the nasopharynx were dissected away. Further dissection from this ventral orientation involved scraping away the cartilage and exposing the bilateral cochleae.

For scanning microscopy, the skull, which is still cartilagenous at this age, was cut along the midline. The temporal bone, containing each cochlea, was then dissected away from the rest of the skull. Soft tissue around the oval and round windows was removed, and the cartilage over the scala tympani was dissected away. The ear was fixed by immersion. After fixation (see below), the ears were dehydrated in acetone up to 75% and were stored in acetone at 4°C until further dissection. Under a dissection microscope, the overlying cartilage and bone were completely removed to expose the sickle-shaped cochlea. The tegmentum vasculosum was then removed and the tectorial membrane was lifted free with fine forceps, thereby revealing the hair cell field lying beneath. The removal of the tectorial membrane was difficult, but with practice minimal damage was inflicted on the sensory epithelium. At all stages the cochleae remained immersed in acetone.

Fixation: The cochleae were fixed by immersion in 1% OsO_4 in 0.1 M phosphate buffer at pH 6.0 for 45 min at 4°C. Initially, we tried fixing the cochleae in a variety of ways. For example, we used glutaraldehyde with and without perfusion, and followed with osmication. We also tried a solution containing both glutaraldehyde and OsO_4. Many of the fixation methods, although adequate for fine structure study, caused shrinkage of the tectorial membrane, which in turn forced the stereocilia to lie over on the surface of the hair cells and were, thus, not good for scanning microscopy. Following the report of Tanaka and Smith (20), we found that fixation in OsO_4 without glutaraldehyde pretreatment or fixation in a solution containing both glutaraldehyde and OsO_4 appeared to produce the best results for examination by scanning microscopy.

Preparation for Scanning Microscopy: After fixation and dehydration in acetone, where the final dissection was carried out, the cochleae were critical point dried in CO_2 in a Sorvall apparatus (Sorvall-Dupont, Newtown, CT), sputter coated with gold palladium with a Polaron sputter coater (Polaron Instruments, Inc., Lexington, PA), and viewed with an AMR 1000A scanning electron microscope (SEM).

Measurements of Surface Area: Cochleae were oriented in the SEM so that the viewer was looking directly down on the surface of a local patch of hair cells. Photomicrographs were taken and enlarged photographically. The distance from the distal end of the cochlea to the "patch" being observed was also measured off the photographs. Since the surface of the cell is somewhat irregular in shape, the most accurate way to measure the surface area is to cut out the surface of a hair cell from the micrograph and weigh it. The weight is then compared with the weight of a piece of photographic paper of known surface area. Important to this procedure is the fact that the weight of photographic paper is remarkably constant, varying <3% from sheet to sheet. Measurements of surface area were obtained at a number of positions along the epithelium.

Lengths of the Tallest Stereocilia: We initially measured the lengths of the tallest stereocilia directly from the TV monitor, using its calibration display and a pair of calipers. These measurements were compared with measurements from photographs, and no systematic error was found. As the study progressed, all measurements were made from photographic records.

Number of Stereocilia per Cell: We found that the stereocilia splay out somewhat during preparation and thus accurate counts of the number of stereocilia per hair cell were difficult to obtain. More accurate determinations could be made by shaving off the stereocilia and counting the scars that were left on the apical surface of the cell. We removed the stereocilia by brushing the surface of the sensory epithelium, after the tectorial membrane was removed, with a small piece of filter paper. During this procedure the cochleae remained covered with acetone. This technique worked well at the distal and middle regions of the cochlea where the stereocilia are long, but at the proximal end they were difficult to remove. Fortunately, at this end, splaying is not a problem since the stereocilia are short, allowing us to make accurate counts directly off scanning electron micrographs.

Widths of the Stereocilia: Photomicrographs of stereocilia from hair cells at varying positions along the sensory epithelium were evaluated for width. Micrographs were taken at × 16,000 and enlarged to give an effective magnification of at least × 40,000 so that accurate measurements could be made. We measured the widths of the longest stereocilia only near the distal tips because that portion of the stereocilium is closest to its insertion into the tectorial membrane and because measurements near the apical surface of the cell are confounded by the taper of the stereocilium.

RESULTS

Histology of the Cochlea

This brief section is included to orient the reader who is unfamiliar with the basic morphology of the bird cochlea. More detailed descriptions can be found in the classic two-volume study of the vertebrate ear by Retzius (14) and in a monograph by Held (7). Several excellent papers have appeared in more modern times, amplifying these earlier observations (2, 8, 9, 19, 21). New information will be presented in the following sections.

The sensory epithelium of the cochlea of a bird is sickle-shaped (Fig. 1). The superior or convex side of the epithelium rests on a fibrocartilagenous plate overlying the cochlear nerve ganglion cells, while the inferior or concave side of the sensory epithelium lies on the basilar membrane without any underlying support or cartilage (see Fig. 2). Covering this epithelium is a fibrous extracellular membrane, the tectorial membrane (Fig. 2), which is attached near the superior edge; it makes contact with the longest stereocilia.

If we examine the surface of the sensory epithelium in more

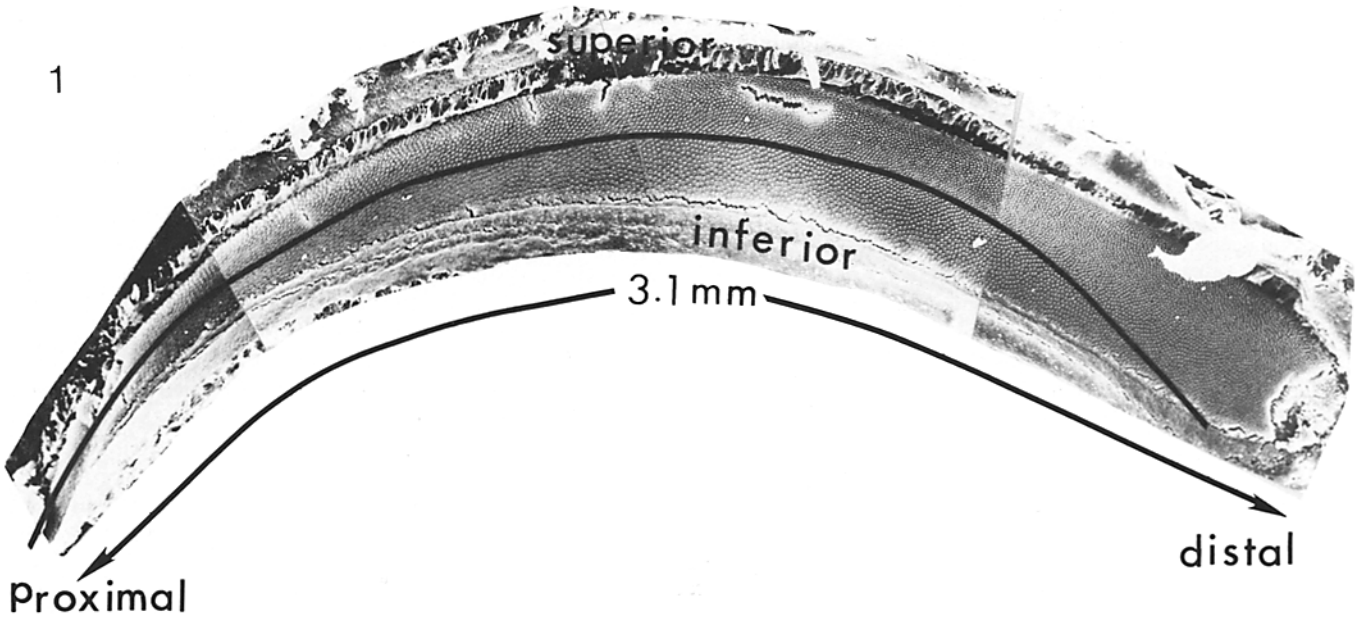


FIGURE 1 Low magnification scanning electron micrograph of the entire cochlea of a 7-d-old chick viewed from directly above. We have indicated the distal and proximal ends of the cochlea, which respond to low and high frequencies, respectively, and the superior and inferior margins. The solid line on the cochlea indicates the approximate distribution of tall and short hair cells with the short hair cells lying on that margin of the cochlea nearest its inferior edge, and the tall hair cells lying nearest the superior edge. The separation indicated here comes from the work of Tanaka and Smith (21). × 65.

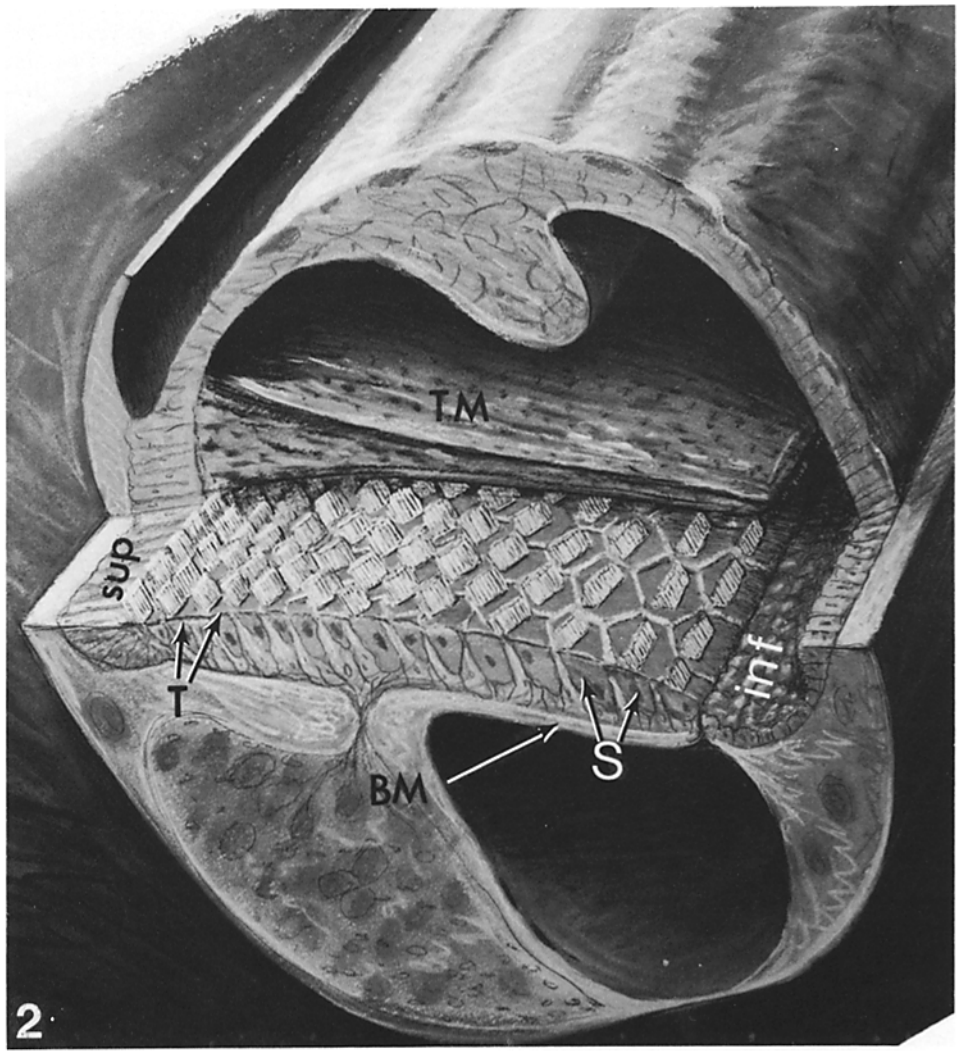


FIGURE 2 Drawing of a portion of the cochlea of a 7-d-old chick. This drawing was modified from one published by Tanaka and Smith (21). The sensory epithelium, composed of hair cells and supporting cells, rests on the basilar membrane (*BM*). The hair cells, located above the portion of the basilar membrane that is not overlain by tissue, are short hair cells (*S*). These are nearest the inferior margin (*inf*) of the cochlea. The tall hair cells (*T*) are located near the superior margin (*sup*). The tectorial membrane (*TM*), a portion of which was removed to allow us to look at the surface of the sensory epithelium, is situated just above the stereocilia with the longest stereocilia making contact with it. A careful examination of the tufts of stereocilia shows that those closest to the inferior and superior margins have their tufts parallel to these surfaces. Those in the center of the papilla are oriented at 45° to those at either margin.

detail, we find that it is composed of two types of cells, hair cells and supporting cells (Fig. 2). Adjacent hair cells, identified as such by their population of stereocilia sprouting from an otherwise smooth surface, are separated from one another by a ridge of microvilli. Thin sections reveal that all of these microvilli extend from the supporting cells that surround and thus separate each hair cell from its neighbors (see the accompanying paper [23]). When viewed from the surface, the hair cells are seen to be closely packed, although lacking precise order such as hexagonal packing (see Fig. 11).

The hair cells have been divided into two types based upon the overall length of the cells and whether or not they are located over the fibrocartilagenous plate. The tall hair cells are found over this plate, while the short hair cells are found primarily over the basilar membrane (Fig. 2). The approximate distribution of short and tall hair cells as was proposed by Tanaka and Smith (21) is diagrammatically depicted in Fig. 1. Besides the height and location of the cell, two other differences between short and tall hair cells are reported in the literature. First, the main innervation of tall hair cells is by afferent neurons, in contrast to the short hair cells, which are innervated largely by efferent neurons. Second, the apical surface of the short hair cell is large (see Figs. 2 and 4) while that of the tall hair cell is much smaller.

Each hair cell has polarity. This polarity is easily recognized because the stereocilia are organized into rows of increasing height in a "staircase-like" pattern (see Fig. 12). Behind the center of the tallest row may be a single cilium or kinocilium which, in many mature hair cells, is represented only by the basal body.

THE NUMBER OF HAIR CELLS PRESENT IN THE SENSORY EPITHELIUM RELATIVE TO THE DISTANCE FROM THE DISTAL END OF THIS EPITHELIUM: In scanning micrographs of the surface of the sensory epithelium (see Fig. 1), we can measure the width of the epithelium as a function of the distance from the distal end of the papilla (Fig. 3 *a*). We use the distal end as our point of reference because it is always clearly delineated, whereas the exact terminus of the proximal end is difficult to establish precisely, particularly since the sensory epithelium not only tapers but twists (it does not lie on a plane). Thus, if we are looking directly down on the wide or distal end, the proximal end lies almost perpendicular to our observation point. We measured the overall length of the papilla in a large number of preparations. In most of them, the distance from the distal to the proximal tip is 3.0–3.1 mm in length. Thus, if we were to measure the distance from the distal tip to a particular point, this distance would be the same in every chick at this stage in development. Occasionally, we found papillae which were shorter, the shortest being 2.6 mm.

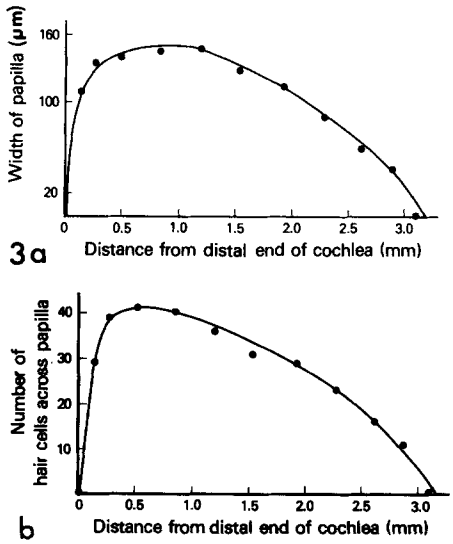
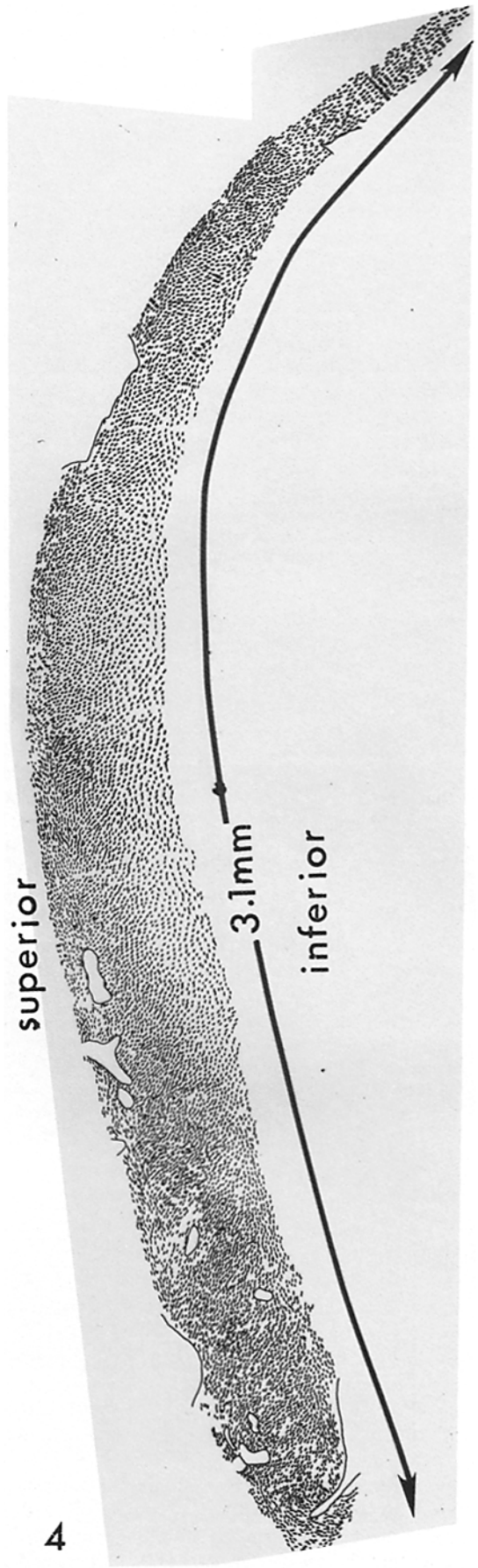


FIGURE 3 (*a*) Graph illustrating the width of the papilla (cochlea) in plotted as a function of the distance from the distal end of the cochlea. (*b*) Graph indicating the number of hair cells encountered as one crosses the papilla, plotted as a function of the distance from the distal end of the cochlea.

Measurements were also made of the number of hair cells that were present at a given location on the sensory epithelium. This was carried out by drawing across the micrograph of the sensory epithelium a line perpendicular to the long axis of the papilla at that point; the number of hair cells on that line was counted. Our data are expressed in Fig. 3 *b*. Of particular interest is that the graph for the width of the epithelium and the graph for the number of hair cells at a given position on the epithelium are very similar. Superficially, this indicates that the average diameter of the hair cell at one end of the papilla is similar to that at any other location.

THE ORIENTATION OR POLARITY OF A HAIR CELL RELATIVE TO ITS POSITION ON THE COCHLEA: The longest stereocilia on the short hair cells are always found nearest the inferior surface of the cochlea (Fig. 2). As we move across the cochlea to its superior edge in order to examine the tall hair cells, again we find that the staircase faces the superior surface (Fig. 2). Between these two surfaces, however, the orientation of the staircase differs in a predictable way from that just described. We have indicated this pattern in Fig. 4 by denoting the orientation of each hair cell with a short line. Notice that the hair cells in the center of the cochlea are oriented at a 45° angle relative to those at the margins. This feature is particularly apparent at the wider or distal three-quarters of the cochlea. This change in polarity is not abrupt but gradual. Thus, if we were to look across the cochlea, we would first see a short hair cell whose stereocilia are oriented towards the inferior surface and, as we progressed across the cochlea, we would see that with each successive hair cell the orientation would shift until the cell in the center is oriented at 45° to the inferior margin and then, as we progressed farther, we would see that it would shift back again (see Figs. 2 and 4).

THE SURFACE AREA OF THE APICAL SURFACE OF THE HAIR CELL EXPRESSED AS A FUNCTION OF ITS POSITION ON THE COCHLEA: We measured the surface areas of tall, short, and intermediate (those that lie between obvious tall and short hair cells) hair cells at varying positions along the length of the cochlea, as outlined in Materials and Methods. These measurements are expressed in Fig. 5. The short hair cells have the greatest surface area and the tall hair cells the least; thus, as we go across the cochlea, the inferior edge has cells with large surface areas, the superior edge those with small surface areas. What was unexpected is that the tall hair cells have approximately the same surface areas at any position on the cochlea, while the short hair cells have minimal surface areas at the ends but enormous areas in the center of the cochlea. It is difficult to relate these measurements directly with the data in Fig. 3 *b* on the number of hair cells at each position along the cochlea because we would be comparing measurements of surface areas expressed as a square function (μm^2), with numbers of hair cells, a linear term. Nevertheless, from Figs. 4 and 5, we know that the greatest surface area of short and inter-



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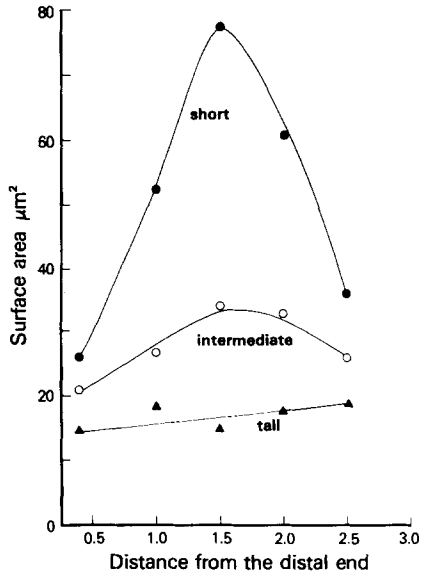

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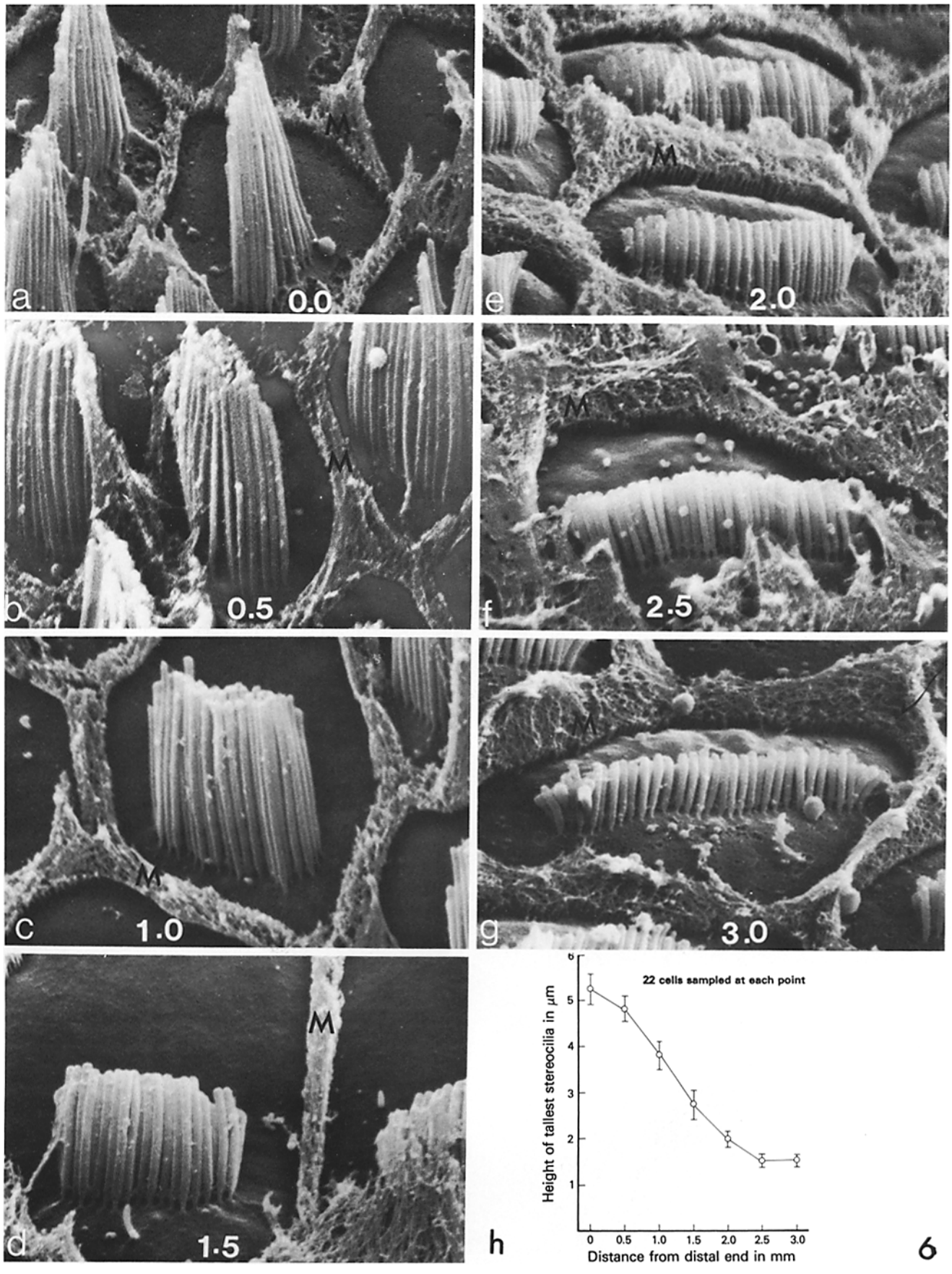
FIGURE 5 The apical surface area of short, intermediate, and tall hair cells plotted as a function of their positions from the distal end of the cochlea. Whereas the surface area of tall hair cells remains constant regardless of their locations, there is a dramatic increase in the surface areas of short hair cells ~1.5 mm from the distal end. At all points, however, short hair cells have larger surface areas than tall hair cells.

mediate hair cells is at ~1.5 mm from the distal end of the cochlea; yet from Fig. 3 both the number of hair cells and the width of the cochlea begin to fall off at this position. Thus, to account for the data presented in Figs. 3–5, at 1.5 mm from the distal end there must be fewer short hair cells and more tall hair cells than at other regions of the cochlea. This statement is true only if the area occupied by the supporting cells remains constant, a statement that qualitatively seems correct. We have not quantitated this, however.

THE LENGTHS OF THE LONGEST STEREOCILIA ON A HAIR CELL RELATIVE TO THE POSITION OF THE HAIR CELL ON THE PAPILLA: To accurately measure the lengths of the longest stereocilia, we found it necessary to orient the cochlea in the SEM so that we are looking at the "back side" of the staircase (Fig. 6*a–g*). In other words, we must orient the cochlea so that we are looking from the inferior towards the superior edge of the cochlea (see Fig. 1). The types of images we are measuring are illustrated in Fig. 6*a–g*. On the micrographs, we have indicated the approximate location of the hair cell in millimeters from the distal end of the cochlea. In Fig. 6*h* we have plotted the length of the longest stereocilium as a function of position. For each point in this graph we measured at least 20 separate cells. The lengths of the tallest stereocilia

FIGURE 4 Scanning micrographs of an entire cochlea similar to that illustrated in Fig. 1 were enlarged (9 ft in length) and overlain with tracing paper. The orientation of the stereociliary bundles on each hair cell was indicated in the tracing paper by a short line. Note that the bundles at the superior and inferior margins of the papilla lie parallel to these margins, yet those in the center lie at an oblique angle (near 45°) to the margins. Also note that, in the central two-thirds of the cochlea, stereociliary bundles attached to short hair cells located near the inferior margin (arrow) are separated at considerable distances from each other.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M
d
1.5
M
h
22 cells sampled at each point
Height of tallest stereocilia in μm
0
1
2
3
4
5
0
0.5
1.0
1.5
2.0
2.5
3.0
Distance from distal end in mm

6

vary from 5.3 μm at the most distal end to 1.5 μm at the proximal end of the cochlea. Both by inspection of the micrographs (Fig. 6 *a–g*) and from the graph (Fig. 6 *h*), it is clear that there is an orderly decrease in the heights of the longest stereocilia from the distal to the proximal end of the cochlea. To be sure that this pattern is not a fluke from the first cochlea we studied in detail, we examined six additional cochleae; the results appear in Fig. 7. What is remarkable is that at a given location on the cochlea the maximum lengths of the stereocilia are accurately programmed with little variation from animal to animal. This statement is true whether we are examining stereocilia from a tall or short hair cell at the same distance from the distal end of the cochlea.

THE NUMBER OF STEREOCILIA PER HAIR CELL AS A FUNCTION OF POSITION OF THE HAIR CELL ON THE PAPILLA: The number of stereocilia per cell can be estimated in thin sections cut across the bundle of stereocilia or by counting the stereocilia in a scanning electron micrograph. Both of these methods have built-in errors. For example, if the thin section is not cut near the cell surface, it is possible to exclude the shortest row or rows of stereocilia. Likewise, when one is counting the stereocilia in a scanning electron micrograph, superposition of stereocilia is often a problem. This can be partly rectified by carefully orienting the sensory epithelium so that one is looking straight down on the stereociliary bundle. Often, however, the bundle splays apart so that even though the orientation is perfect some stereocilia obscure others. This is particularly true of hair cells with longer stereocilia such as those towards the distal end. To obviate these problems we "shaved" off the stereocilia and thus could obtain an accurate count of the number of stereocilia per cell by counting the scars that remained on the apical surface of the hair cell. Although this technique worked excellently at the distal end of the cochlea, the stereocilia proved very difficult to remove at the proximal end. However, at the proximal end, splaying of the stereocilia was less pronounced because of their shortness and they could be counted directly. The types of images we see are illustrated in Fig. 8. The images illustrated here are taken at ~0.5-mm intervals from the distal end of the cochlea and are oriented such that the kinocilium, if present, would be located at the top of each micrograph. Particularly well shown in this figure are the short tufts of stereocilia from the supporting cells that separate adjacent hair cells.

In Fig. 9 we present our data on the number of stereocilia per hair cell as a function of the distance from the distal end of the papilla. Each point on this graph represents counts we made on 6–60 adjacent hair cells. Information from seven different cochleae is included on this figure. The number of stereocilia per hair cell ranges from 50 at the distal end to 300 at the proximal end.

To determine how precisely adjacent hair cells control the

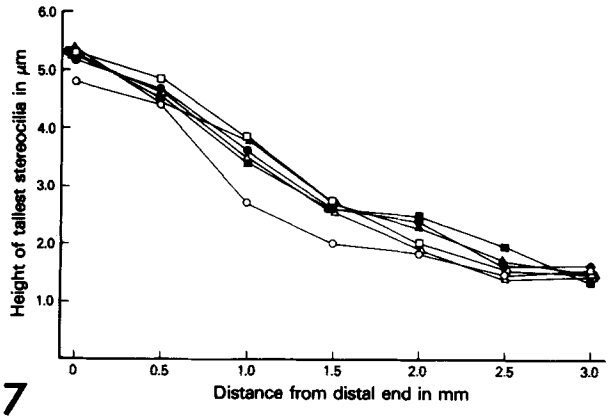

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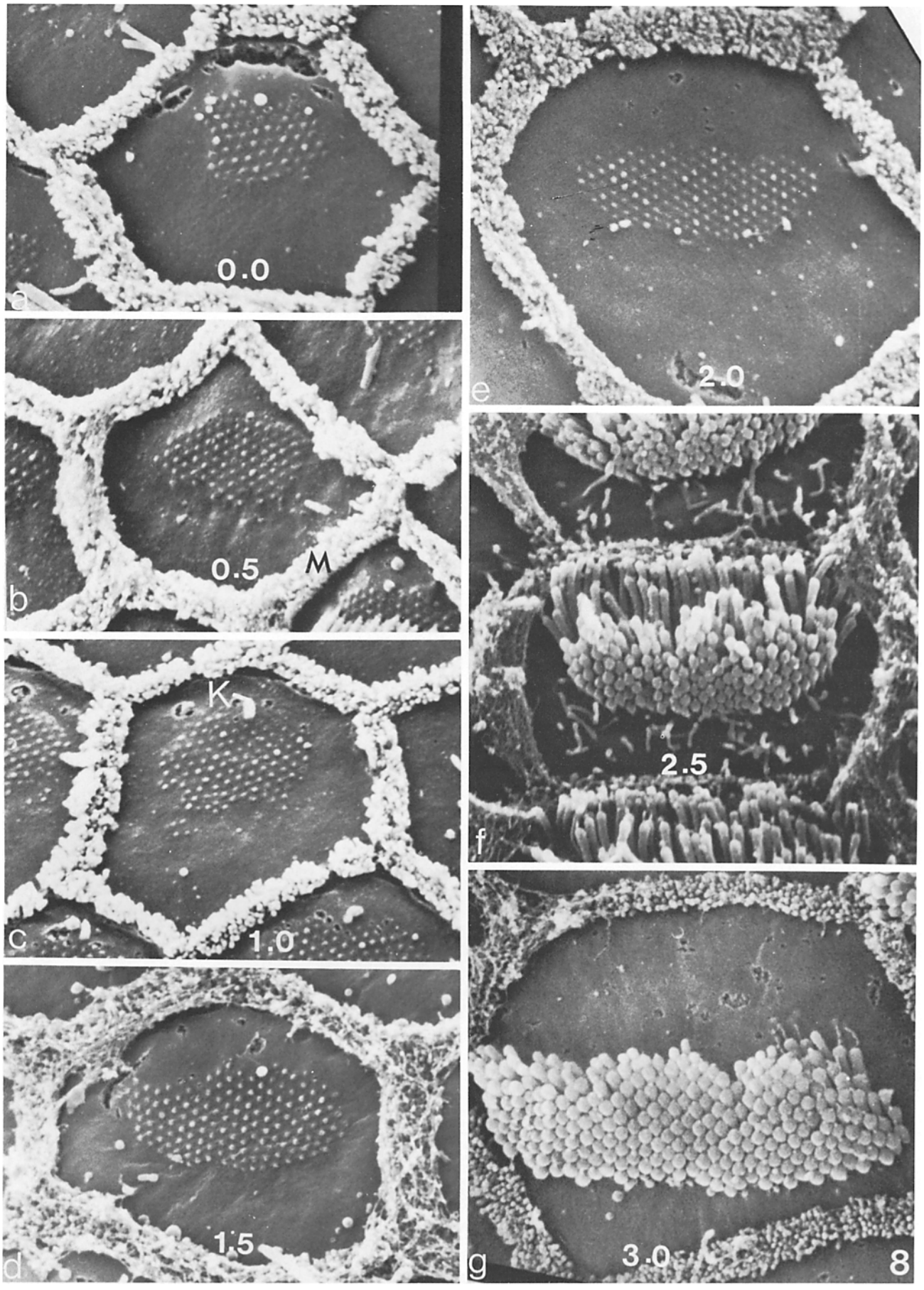
FIGURE 7 Graph depicting the height of the tallest stereocilia plotted as a function of the distance from the distal end of the cochlea from information taken from six different cochleae. For each point, 22 cells were sampled. Notice that there is a systematic decrease in the heights of the tallest stereocilia as one travels from the distal towards the proximal end of the cochlea. Ears no. 4 (●), 8 (△), 12 (▲), 14 (■), 15 (○), and 17 (□).

number of stereocilia, we measured 60 adjacent cells on one small part of the cochlea and plotted the number of stereocilia on a histogram (Fig. 10). (The error in counting is negligible. By mistake, we ended up counting the scars on a number of hair cells several times and the differences in the counts were either none or 1, but seldom more.) Except for seven individuals, the mean number was 95 ± 5. Thus, the biological accuracy of these cells in determining the number of stereocilia is ~5%. These variations seem to be less than that suggested by a Poisson distribution. On this histogram we found two individuals whose counts were much farther apart than the others, namely 77 and 110. Of interest is that these cells were neighbors; if we average their combined stereocilia, we obtain a mean value for the 2 cells of 94. It is tempting to interpret this result in mechanistic terms, e.g., during the last division unequal numbers of "stereociliary determinants" were given to the two daughter cells. Two other regions were sampled in which we counted at least 30 adjacent hair cells per region. One had a mean of 50 ± 5, the other a mean of 150 ± 5. This indicates that the hair cell is able to regulate the number of stereocilia by ± 5. However, at the proximal end where there are up to 300 stereocilia per cell, the variation is somewhat more than this. The sample size here was, unfortunately, much smaller (12 cells) due to our failure in removing the stereocilia from the cells. Thus, to get accurate counts, we had to search for cells in which all the stereocilia were perfectly upright.

An examination of Fig. 11, one of the micrographs from which we concluded that the accuracy in determining the

FIGURE 6 (*a–g*) Scanning electron micrographs of representative bundles of stereocilia that extend from the apical surface of hair cells located at discrete positions on the cochlea. The view illustrated here is the view of the stereocilia we would see when looking from the inferior margin of the cochlea towards its superior margin. These positions were determined by measuring the distance from the distal end of the cochlea to the hair cell in question. We have indicated this distance in millimeters on the micrographs (white letters). Cochleae are ~3.1 mm in length in 7-d neonatal chicks, and the micrographs included here are taken at 0.5-mm intervals. All of the micrographs are shown at the same magnification. The body of the hair cell is easy to determine because the apical surface is smooth. Adjacent hair cells are separated by a ridge of microvilli (*M*) that extend from the surface of supporting cells that separate adjacent hair cells. × 8,400. (*h*) Graph of the height of the tallest stereocilia on a hair cell plotted as a function of the distance from the distal end of the cochlea. This graph was derived from a single cochlea. Each point on the graph represents the data from at least 22 adjacent hair cells. Most of the micrographs illustrated in *a–g* were from this cochlea.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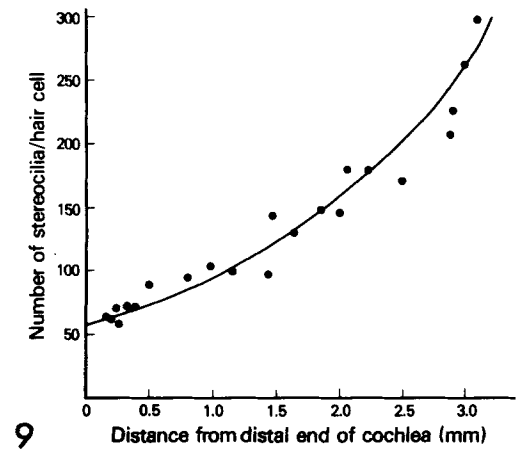

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FIGURE 9 Graph depicting the number of stereocilia per hair cell as a function of the distance from the distal end of the cochlea. The information plotted here comes from seven cochleae.

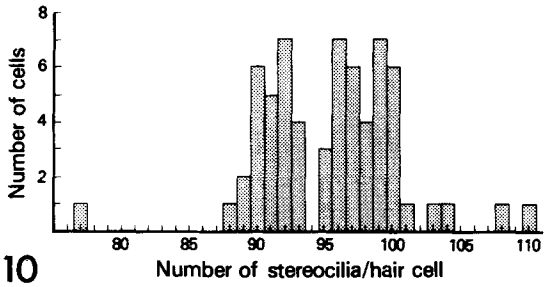

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FIGURE 10 Bar graph illustrating the precision with which adjacent hair cells control the number of stereocilia which appear on their surfaces. As seen in this figure, most of the hair cells at this location on the cochlea have 95 ± 5 stereocilia per cell. We do not know whether the bimodal distribution depicted here is real or not.

number of stereocilia is 5%, shows little variation in number of stereocilia in adjacent cells. From these data, one might expect that the points plotted in Fig. 9 would fall closer to the curve. It should be remembered, however, that the data for this curve were accumulated from seven different cochleae. Since variations in the overall length of the cochlea occur in 7-d neonatal chicks (as already mentioned, most cochleae measure ~3.1 mm in length, yet cochleae of 2.6–3.2 mm in length have been found), this will influence the exact positioning of the points on Fig. 11 by affecting the *x*-axis. Since there can be some variation here due to the total lengths of the cochleae, we expect that the number of stereocilia from hair cells of different organisms would show more variation than within a single organism.

The data in Fig. 10 produce what looks like a curve of two peaks, one around 91, the other around 97. We have no way of knowing whether this is a sampling error or whether this is a real finding.

Organization of Stereocilia on the Surface of a Hair Cell

If we look straight down on the surface of a hair cell, we see the tips of the stereocilia (Fig. 8*g*). Close examination reveals that they are hexagonally packed, an observation that can be confirmed by looking at the apical surface of hair cells whose stereocilia have been "shaved" off. In the shaved preparations there can be no possibility of a rearrangement of the stereocilia during processing for scanning electron microscopy (Figs. 8*a–e* and 11).

Now let us examine the bundle of stereocilia on hair cells as we might see them by looking from the superior towards the inferior edge of the cochlea (see Fig. 1). In this view we have the opportunity to examine the "staircase" pattern created because the stereocilia on one side of the bundle are short, those on the opposite side long. More particularly, we were anxious to examine how precise the "staircase" is. Counter to our expectations, we found that the stereocilia are not organized into rows of increasing height, a true staircase. Rather, there was a gradual increase in the heights of the stereocilia from the shortest in the front of the bundle to the tallest at the back, but they were not arranged in rows of uniform height (Fig. 12). This is true of all cells whether they are located in the proximal or distal part of the cochlea.

What we would like to know is how the hexagonal lattice of stereocilia is oriented in relation to the shortest and longest stereocilia. It is well known that each unit in a hexagonal bundle lies such that three lines oriented at 120° to each other can be drawn through that unit. These lines connect the unit's six nearest neighbors and represent the 1,0 view of the bundle (see reference 4). This can be easily appreciated by looking at the shaved preparations (Figs. 8*a–e* and 11). From the position of the single kinocilium or, if absent, the basal body of the kinocilium (see Figs. 8*c* and 11), we have a marker for the orientation or polarity of the bundle because this structure always resides immediately behind the center of the tallest stereocilia. In all the >1,000 shaved hair cells examined, the bundle is invariably oriented in the same way. We have drawn lines on a bundle of stereocilia illustrated in Fig. 11 which represent the three 1,0 views. If we look straight back from the shortest to the tallest stereocilia, we find that we are looking along a 1,0 lattice line. The lines of stereocilia forming the staircase are oriented at 90° to this 1,0 direction. They cannot lie along 1,0 lattice lines because these lines are all oriented at 120° to each other, not 90°. Rather, the staircase rows lie along 1,1 lattice lines. In the 1,1 view, adjacent stereocilia are sepa-

FIGURE 8 Scanning electron micrograph of the apical surfaces of hair cells located at 0.5-mm intervals from the distal end of the cochlea. These distances are indicated on the micrographs. In *a–e* the stereocilia have been "shaved" off, leaving scars. This allows us to accurately count the number of stereocilia per cell. In *f* and *g*, the stereocilia have not been removed because they are very short and, therefore, extremely difficult to "shave" off. In all seven micrographs the hair cell depicted is oriented in the same way. If the kinocilium (*K*) is present, or if the stub is present, it will be located nearest the top of the page. Surrounding each hair cell is a tuft of microvilli (*M*) that extends up from the supporting cells that lie immediately beneath. By inspection of these micrographs it is clear that the number of stereocilia per cell is lowest at the distal end and that there is a systematic increase in number as one travels towards the proximal end. For this figure we chose representative cells, not just tall hair cells. Thus, the increase in surface area in *e–g* is not meant to represent a systematic increase in area. × 12,500.

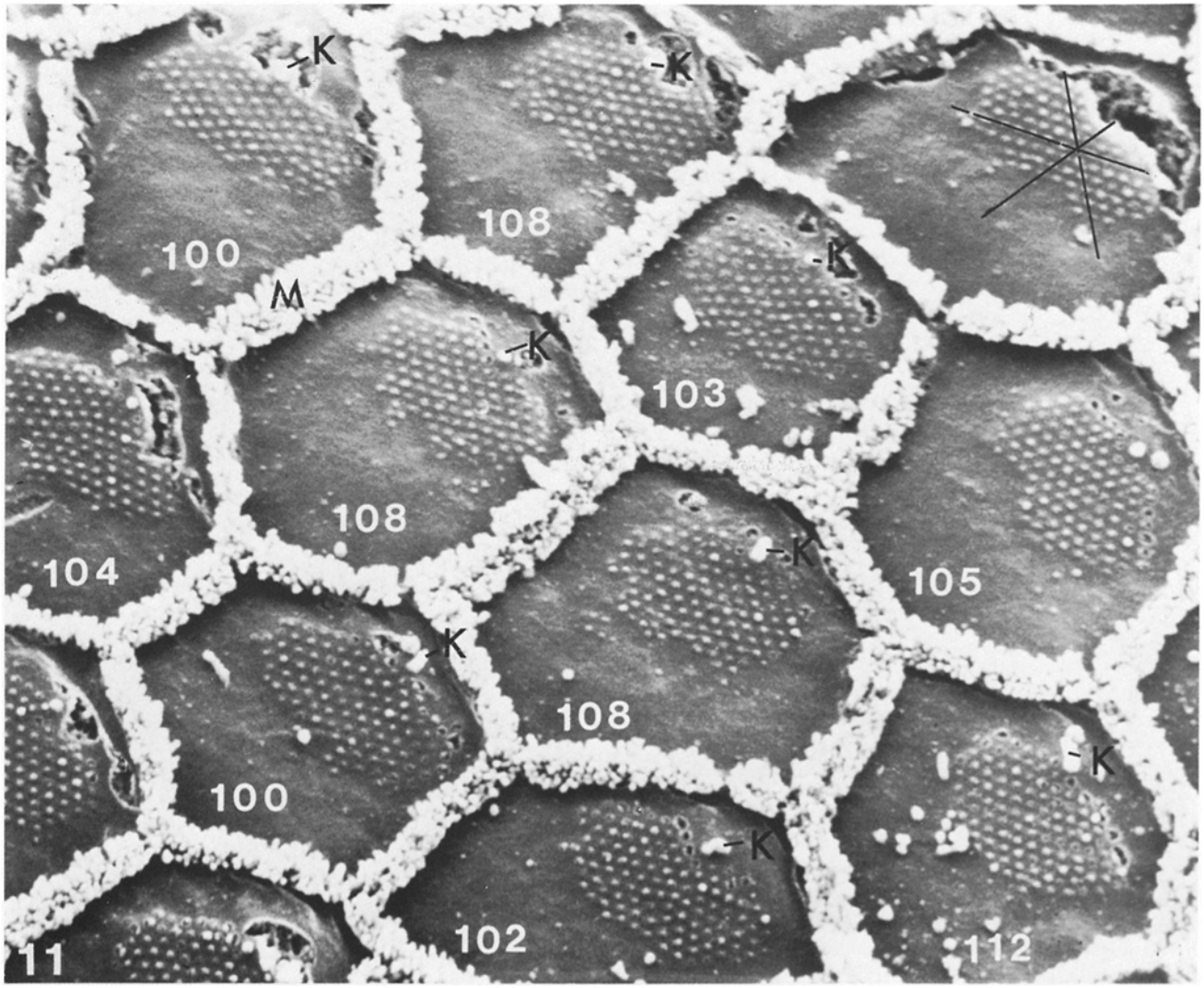

FIGURE 11 Scanning electron micrograph of a small portion of the cochlea from which the stereocilia have been shaved off. The kinocilium (*K*), if present, or a stub of it, if present, is located at approximately 2 o'clock. We have included on this figure our counts of the number of stereocilia per cell. Notice that even though several cells have exactly the same number of stereocilia per cell, the exact arrangement of stereocilia in the bundle (beyond the facts that they are hexagonally packed and the lattice planes are always precisely oriented relative to the kinocilium) is somewhat variable. In one of the hair cells, we have drawn over the scars of stereocilia to indicate the 1, 0 lattice plane. × 12,000.

rated from each other by a larger space than in the 1,0 views because it is not nearest neighbors that are adjacent but rather two sides of the hexagon. Thus, the simple row lines (i.e., 1,0 lines) along which the stereocilia touch do not form the steps of the staircase. The steps instead are the 1,1 lines (an abstracted view of this is illustrated in Fig. 13).

To count the number of steps, we must count the number of 1,1 lines encountered in going from the shortest to the tallest stereocilia. For the hair cells illustrated in Figs. 8 and 11, we encountered 15–19 steps.

Organization of Stereocilia on the Apical Surface of a Hair Cell Relative to the Position of the Hair Cell on the Cochlea

As noted earlier, there is a sixfold increase in the number of stereocilia per hair cell from the distal to the proximal end of the cochlea. Yet the number of stereocilia that we encounter in traveling on a 1,0 row from the shortest stereocilia towards the longest in a bundle is quite constant, usually being seven or eight (see Fig. 8), or, to put it another way, the width of the bundle of stereocilia is quite constant. This means that at the distal end of the cochlea the bundle should be square, yet at the proximal end it should be greatly elongated or rectangular in order to accommodate all the stereocilia that must be present. This also means that at the distal end of the cochlea there are only a few tall stereocilia, whereas at the proximal end there are many tall stereocilia (see Fig. 8). This may be important because movement of the tallest stereocilia in any bundle may play more of a role in sensory transduction than displacement of the shorter stereocilia.

There is an additional observation on the organization of stereocilia on each hair cell that must be taken into account. Unlike the situation in lizards (4) and in mammals (16), where only the tallest stereocilia in any bundle differ in lengths depending upon their location on the cochlea, in birds all the

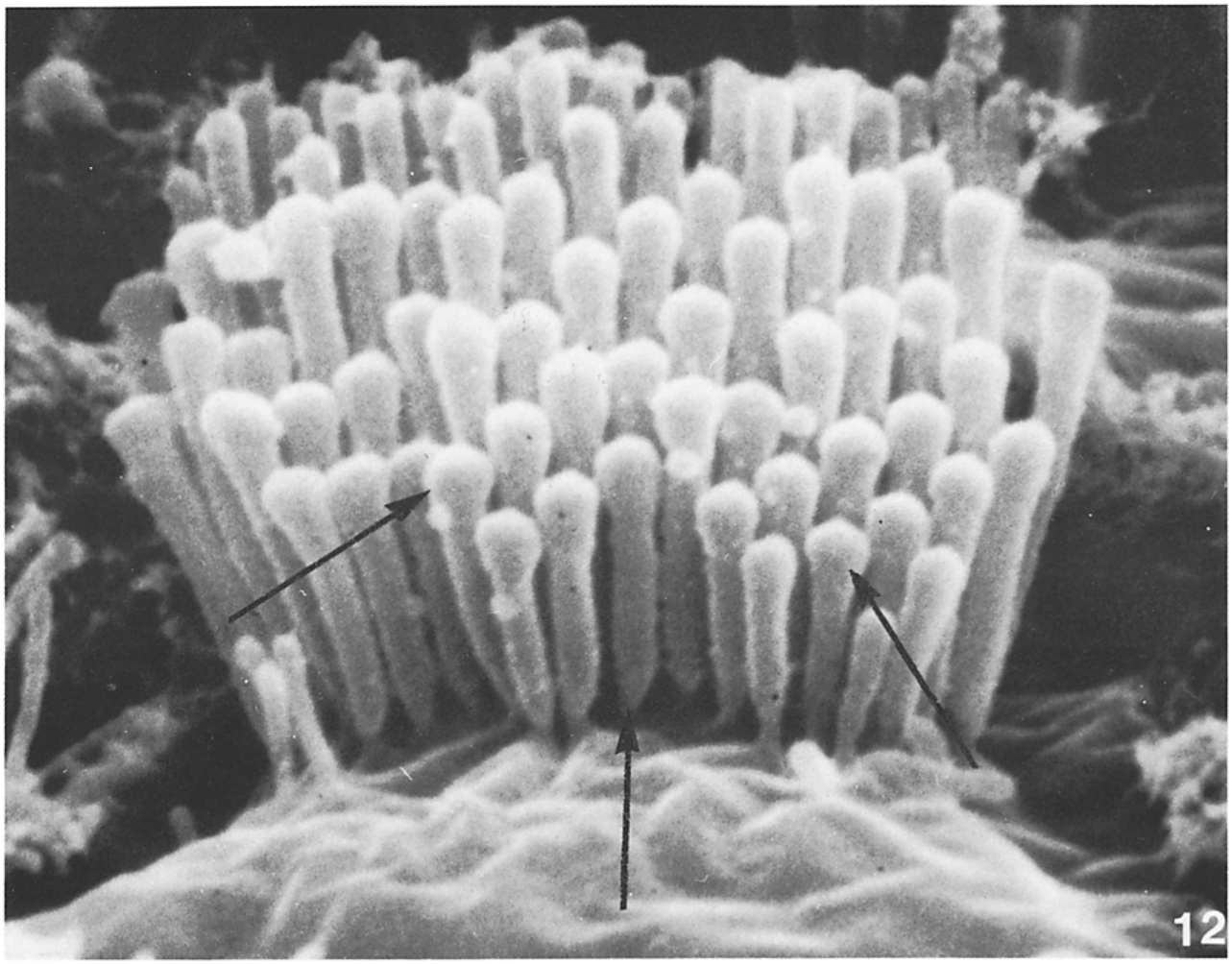

FIGURE 12 Scanning electron micrograph of the stereociliary bundle that extends from the apical surface of a single hair cell. The view was taken by looking at the hair cell from the superior margin of the cochlea towards the inferior margin. Of interest is that, although there is a progressive increase in height of the stereocilia in going from the front of the bundle towards the back, the lengths of the stereocilia are not precisely controlled. The arrows indicate the 1, 0 planes. Notice that if there were rows of stereocilia of increasing height, these rows would not lie on a 1, 0 plane but rather on the 1, 1 lattice plane. × 31,360.

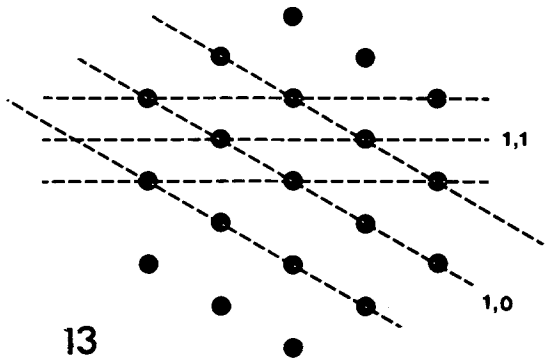

FIGURE 13 Drawing illustrating the 1,0 and 1,1 lattice planes on a series of hexagonally packed dots. Note that in the 1,0 view nearest neighbors are connected, while in the 1,1 view nearest neighbors are not connected. Since the stereocilia are oriented on a hexagonal lattice, it is interesting to determine how this lattice is related to the position of the kinocilium or to know how the stereocilia are displaced. In this diagram the kinocilium would be situated at the top of the bundle of dots. Thus, if there is a true staircase of stereocilia, it would be a 1,1 staircase.

stereocilia in the bundle vary in their lengths. Thus, at the distal end of the cochlea not only are the tallest stereocilia longer than the tallest on cells at the proximal end, but, as we move along the 1,0 row from the tallest to the shortest, stereocilia of intermediate height on the distal end are much taller than their respective members on hair cells at the proximal end of the cochlea. To look at this another way, if one looks at the bundle of stereocilia from one of its sides, the profile is different in hair cells in different parts of the cochlea.

We have stated already that in any local part of the cochlea the number of stereocilia per cell is remarkably constant and the lattice is consistently hexagonal. One wonders, therefore, how much variation exists in the organization of the stereocilia at the margins of the bundle. Does the bundle at any local region, for example, exist as a rectangular crystal, or does it vary somewhat in shape? Close examination of Fig. 11 shows that although the numbers of stereocilia per cell are remarkably constant the overall shape of the bundle varies from cell to cell. Thus, the cell seems to regulate the numbers of stereocilia per cell and the lattice very accurately, the boundaries of the bundle are not precisely specified.

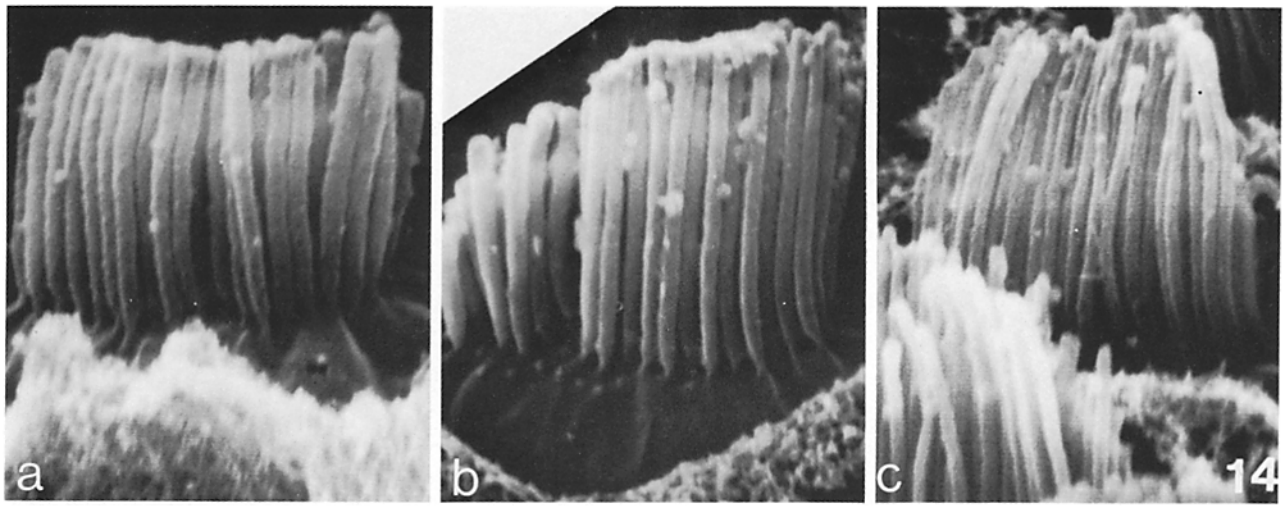

FIGURE 14 In this figure we have illustrated the stereocilia of three hair cells located at the same distance from the distal end of the cochlea. The view of the stereociliary bundle seen here is that looking at the cochlea from its inferior to its superior surface. In *a* we have included a short hair cell. In *b* an intermediate hair cell is depicted, and in *c* a tall hair cell. A comparison of the stereocilia of these three cells shows that tall hair cells have thin stereocilia, while those on short hair cells are thick. × 15,000.

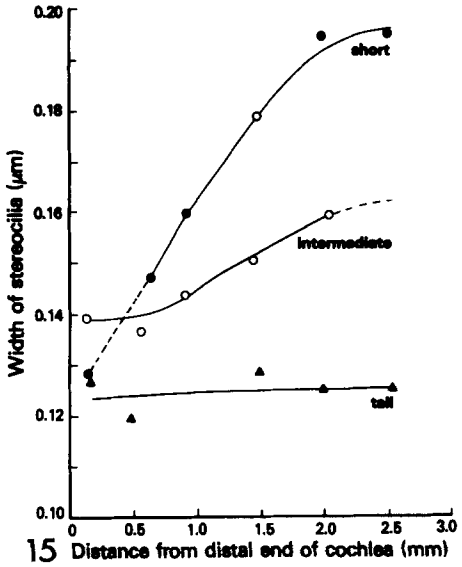

FIGURE 15 Graph depicting the width of the stereocilia on short, intermediate, and tall hair cells as a function of their positions from the distal end of the cochlea. Note that stereocilia of short hair cells are always thicker than those of tall hair cells except at the most distal end of the cochlea. Also, the stereocilia of tall hair cells have remarkably constant dimensions throughout the length of the cochlea, yet the stereocilia of short hair cells get progressively thicker as one travels towards the proximal end of the cochlea.

Widths of the Tallest Stereocilia as a Function of Their Position on the Cochlea

Cursory examination of the widths of the longest stereocilia of tall and short hair cells located at the same distance from the distal end of the cochlea reveals that they are dramatically different, with the stereocilia on the short hair cells consistently wider than those on tall hair cells. Representative examples are included in Fig. 14. Besides differences between tall and short hair cells, measurements of the diameters of stereocilia from short hair cells located at the distal, mid, and proximal regions of the cochlea reveal that the diameters are not constant (Fig. 15); instead, the stereocilia of short hair cells located near the proximal end of the cochlea are fat, nearly 0.20 μm in diameter, while those at the distal end of the cochlea are much narrower, <0.13 μm in diameter (Fig. 15). The diameters of stereocilia of tall hair cells, on the other hand, remain remarkably constant irrespective of whether the cell is found near the distal or the proximal end of the cochlea.

DISCUSSION

In this paper we demonstrated that the length, width, and distribution of stereocilia of each hair cell are dependent upon the position that the cell occupies on the sensory epithelium. Thus, at the distal end of the cochlea the stereocilia are longer, fewer in number, and generally narrower in width, while at the proximal end they are short, wide, and plentiful. Also, as one looks across the cochlea from its inferior to its superior margin, the widths of the stereocilia vary in a predictable fashion, being wide at the inferior margin and narrower at the superior margin. The polarity of every hair cell, as judged by the position of the stereociliary bundle, also varies in a predictable way depending upon the location of that cell on the cochlea, as does the surface area of the cell. Thus, if we are told the height and width of the tallest stereocilia, or the number and width of stereocilia on a certain hair cell, we can pinpoint the position of that cell on the cochlea in *two axes*. This position can be confirmed if we are told the polarity of the cell and the surface area of the cell. Thus, the cochlea exercises exquisite control over its sensory transducers, a fact that has not been appreciated before. Undoubtedly, this patterning is important in the process of sensory transduction, as will be mentioned below and in the accompanying paper (23). However, this degree of organization makes this system a model one for the cell biologist who is interested in studying how a cell regulates the length, width, number, and distribution of its cell extensions, because these features are governed with precision. Also, since the actin filaments within the stereocilia apparently control these parameters, the cell biologist is in a position to gain insights on how the length, number, and distribution of actin filaments are controlled, a situation which in a less stereotyped cell type would be extremely difficult or impossible.

Local Variability among Hair Cells

To evaluate the systematic variation in stereociliary length, width, number, and orientation, it is important to assess how precisely these variables are determined. This will also be important for future embryological studies in which we will try to determine how actin controls these variables. This chapter, then, provides a baseline for such studies.

VARIATIONS IN THE LATTICE: In all the thousands of cells so far examined the stereocilia are arranged on a hexagonal lattice. One wonders how this lattice is positioned in respect to the polarity of the cell which is determined more accurately by the position of the kinocilium. As mentioned in Results, the best way to examine this is to look at hair cells whose stereocilia and kinocilium have been "shaved" off, leaving scars. We found that in all cases the hexagonal lattice is oriented precisely relative to the kinocilium and the staircase. What was unexpected is that the ascending *rows* of stereocilia forming the staircase lie along 1,1 lattice lines and at 30° relative to the more obvious rows of stereocilia, i.e., lying along the 1,0 lines. The kinocilium sits just behind the center of the last 1,1 line which contains the tallest stereocilia (see Fig. 13).

VARIATIONS IN THE LENGTHS OF THE STEREOCILIA RELATIVE TO THE LATTICE: Even though within an individual hair cell the heights of the stereocilia progressively increase, careful viewing of the bundle reveals that the exact lengths of the stereocilia are not rigidly fixed. Thus, if one follows one of the 1,0 lattice planes, i.e., going from the shortest stereocilium to the longest, each successive stereocilium, while generally longer than the preceding one, is not longer by a predetermined amount. The amount of increase, instead, is variable. A comparison of adjacent rows in the 1,0 plane (going from the shortest to the tallest stereocilia) shows that the incremental increase is not related from row to row. This same type of variation occurs in lizard cochlea (unpublished observations) and in the vestibular systems of many organisms. In contrast to the above pattern, in the cochlea of mammals the inner hair cells have stereocilia organized into only three rows. To our knowledge, it is not yet known how the lattice is organized, e.g., whether these rows lie on the 1,0 plane or not.

The fact that every hair cell, irrespective of its type (acoustic, vestibular, or lateral line), has a bundle of stereocilia roughly organized relative to their lengths indicates that this feature is probably important functionally. However, since a precise control over these lengths is not seen, we must conclude that a precise accounting of these lengths is not critical for transduction.

VARIATIONS IN THE POLARITIES OF ADJACENT HAIR CELLS: Discounting the systematic variation in the orientation of the hair cell bundle documented in Fig. 4, careful examination of adjacent hair cells reveals that there is a 10–15° variation in the polarity of adjacent cells. This point is best appreciated when the stereocilia have been removed, leaving "scars," in which case we can very precisely compare the polarity of adjacent hair cells (Fig. 11).

VARIATIONS IN THE LENGTHS OF THE STEREOCILIA OF ADJACENT HAIR CELLS: Since there is so much variation in stereociliary length within a hair cell, we could only effectively compare the lengths of the longest stereocilia in adjacent hair cells. Here the variation from cell to cell is 10% (see Fig. 6h) and in some regions as high as 15%. Some of this variation could be related to measurement errors. For example, a careful scrutiny of Fig. 6*d* shows that the tallest stereocilia vary somewhat in length, so that if one measures the stereocilia on the right of the micrograph (where they are shorter), this parameter would affect the overall measurement. Also, sometimes and for unaccountable reasons, the tallest stereocilia become sheared off, possibly during the removal of the tectorial membrane, leaving scars which are visible only at high resolution. In looking over some of the figures we found instances in which, by mistake, we included these cells in our counts. Thus, we estimate that there is about a 10% variation in the lengths of the longest stereocilia of adjacent cells.

VARIATIONS IN THE NUMBER OF STEREOCILIA IN ADJACENT CELLS: Except at the most proximal end of the cochlea where there are large numbers of stereocilia per cell, we found that in adjacent cells, at least in the regions in which the sample size was large enough (30–60 individuals), there was a 10% variation in the number of stereocilia from cell to cell. Occasionally there was an exception that may ultimately be important in understanding how the cells regulate stereociliary length. For example, in the part of the cochlea where there are 95 ± 5 stereocilia per cell we found two touching cells, one of which had 77, the other 110, stereocilia. The mean of these is 94. Perhaps, during the mitosis that precedes hair cell differentiation, something interesting happened.

VARIATIONS IN HOW THE STEREOCILIA ARE ARRANGED ON THE HEXAGONAL LATTICE: HOW CONSTANT IS THE WIDTH OF THE BUNDLE?: As the number of stereocilia increases from the distal end of the cochlea towards the proximal end, we find that the bundle elongates, keeping the width of the bundle remarkably constant. What we would like to discuss in this subheading is how consistent is the width of the bundle or the dimensions of the bundles in adjacent hair cells where the numbers of stereocilia are the same. It turns out that there is a large variation here. For example, in Fig. 11 there are two hair cells in which the numbers of stereocilia are identical, but the edges are very different. The edges of the bundles are not like a crystal with six faces (even though all the stereocilia are hexagonally packed) but instead are jagged. The pattern of the edges, then, is very different from cell to cell even though adjacent cells manage to grow the same number of stereocilia per cell and to arrange them on a hexagonal lattice that itself is precisely positioned.

Although the width of the bundle is not precisely controlled, nor are the lengths of all the stereocilia within the bundle proper, e.g. no true staircase, the fact that the cells with more stereocilia make an elongated bundle rather than a larger, square bundle indicates that a staircase is important to function.

VARIATIONS IN THE WIDTHS OF THE LONGEST STEREOCILIA IN ADJACENT HAIR CELLS: Within an individual hair cell and between adjacent hair cells the widths of the stereocilia are remarkably constant. This can be seen primarily in sections cut transversely across bundles of stereocilia (see the accompanying paper [23]). In our scanning micrographs, measurements as accurate as those from our transverse sections examined by transmission microscopy are just not possible, but, again, within the errors of our measurements, variations in width are minimal.

VARIATIONS IN THE SURFACE AREAS OF ADJACENT HAIR CELLS: As depicted in Fig. 11, there is clearly some variation in the surface areas of adjacent hair cells. We have calculated that this variation is ~10–20% depending on whether one is considering local variability between tall or short hair cells. We should also mention that, although adjacent hair cells are separated by supporting cells, each hair cell is surrounded by five to seven hair cells. Thus, the number of "sides" of each

hair cell is somewhat variable. Nevertheless, hair cells are closely packed, although not hexagonally packed.

Systematic Variations in the Length, Number, Width, and Distribution of Stereocilia May Be Related to Frequency Discrimination

As mentioned in the introduction, different parts of the cochlea respond to different frequencies. Thus, in the bird ear the distal end of the cochlea "hears" low frequencies and the proximal end high frequencies; or, to put it into the current jargon in the literature, the cochlea has tonotopic organization. In short, the cochlea presents a high frequency map of the sound that impinges on it. Von Bekesy (1) was the first to show that this tonotopic organization of the cochlea is determined primarily by the properties of an extracellular membrane, the basilar membrane, on which the hair cells sit. Many investigators followed his innovative studies and confirmed them with more modern techniques (see reference 3 for references). Furthermore, individual hair cells in the cochlea have recently been penetrated with microelectrodes and it is clear that these cells are "tuned" to particular, yet predictable (based on their location), frequencies (15, 24, 26).

Lizard cochleae are particularly interesting in this regard. The lizard cochlea is tonotopically organized, yet, unlike the situation in higher vertebrates, the basilar membrane motion itself does not reflect the tonotopic organization (13, 25, 26). This means that individual cells are somehow "tuned" to the appropriate frequency (13); their tuning cannot be due to their location on an extracellular membrane since all positions oscillate at all frequencies. A logical place to look to explain the special tuning properties of the individual cells is the stereocilia, since these are the "detectors" of the oscillations. Although as yet no careful comparison of the stereocilia from hair cells in different parts of the cochlea has been carried out for a lizard cochlea, we do know that the lengths of the tallest stereocilia are systematically different in hair cells from different regions of the cochlea (4, 12, 24, 26).

Similar systematic variations in the lengths of the longest stereocilia in cells located at different parts of the cochlea have been found in mammals (11, 16) and in birds (this report). Since both birds and mammals are thought to discriminate frequencies based upon properties of the basilar membrane, systematic differences in the lengths of the stereocilia may amplify the capability of the organism for discriminating fine distinctions in frequency. In fact, it is possible that properties of the stereocilia such as length, width, number, etc., may be a major factor in frequency resolution. This concept should be explored further since most current interpretations of frequency resolution in the cochlea rely only on measurements of gross mechanical movements of the basilar membrane (10).

If all our observations are taken together, it is difficult to ignore the overwhelming fact that hair cells that are differently tuned have stereocilia of different, yet defined, lengths, widths, numbers, polarities, and surface areas. These variables are predictable and exist not only within an individual cochlea but are the same in every bird we have looked at. Some of these variables clearly will affect stereociliary stiffness, and this in itself may influence tuning. Other variables may be related to the sensitivity of this remarkable organ, and still others may be related to how the efferent input from the brain to the hair cells may modify the output.

The Stereociliary Bundle Is a Model for Understanding What Regulates the Length, Number, and Distribution of Actin Filaments

From the studies of Flock and Cheung (6) and Tilney et al. (22), it is now well established that within each stereocilium is an organized array of actin filaments which are cross-bridged to one another. In stereocilia which have been detergent extracted and decorated with S1, the component actin filaments splay apart (22). Some of these filaments can be followed in an individual section for at least a micron. This implies that some of the filaments, at least in the center of the bundle, extend the length of the stereocilium and most likely determine the overall height of the stereocilium (22). We also know that the widths of the stereocilia are accurately regulated. From earlier studies on the lizard cochlea (22) and from studies on the bird cochlea (see the accompanying paper [23]), we recognize that by measuring the width of each stereocilium we are, in fact, providing an accurate measurement of the number of actin filaments, since the membrane is a tight-fitting sleeve. And, finally, we know that the number of stereocilia per cell is accurately regulated. This tells us how many actin bundles per cell there are. Thus, an individual cell must somehow know how long to make the filaments, how many filaments to glue together into a bundle, and how many bundles to make. To top all this, there are systematic differences in these variables in hair cells at different parts of the cochlea.

Obviously, what we would ultimately like to know is how a hair cell, given a cytoplasmic pool of actin monomers, controls the assembly of these monomers so that stereocilia of precise dimensions appear on its surface. This is not a simple problem because each hair cell grows stereocilia of different, yet accurately prescribed, lengths so that each cell must assemble actin filaments of different lengths at different positions in its cytoplasm. Besides this awe-inspiring problem, each cell also "knows" and, therefore, regulates the number of actin filaments per bundle (in order to control the width of each stereocilium), and, since each hair is different in a predictable way, this number must be different in cells in different parts of the cochlea. The one thing that is apparent is that what we know about the antics of purified actin in vitro, i.e., critical concentration for assembly, "preferred end" for addition of monomers, treadmilling, etc., is not sufficient for an understanding of this problem. To put it another way, if we were to inject actin into a small container the size of a cell at any concentration and at any rate the experimenter desired, we would not get bundles of actin of different, yet prescribed, lengths and widths. Thus, it cannot be the concentration of actin alone that gives this pattern. Instead, there must be a multitude of controlling factors, i.e., nucleating factors, capping factors (both ends), cross-bridging factors, inhibiting and stimulating factors, etc., that control these phenomena. It sounds like an almost hopeless task to try to unravel how the hair cell controls the lengths, numbers, and distribution of its actin filaments. However, from this study we already know how precisely many of these variables are controlled and which are local variables from cell to cell and which are systematic and "controlled" variables. Because this cell type controls certain features so precisely and because by just looking at the top of the cell we have an excellent idea of what the cell is doing with its actin, by studying the genesis of this pattern in embryos we have some hope of gradually working out what a cell actually does. This will be the topic of a subsequent paper in this series.

We would particularly like to acknowledge the invaluable assistance of David DeRosier and Ed Egelman of Brandeis University who carefully read over the manuscript and showed us how to improve the text. They were especially helpful in assisting us to express in as simple a way as possible the 1,0 and 1,1 lattice planes and provided us with a drawing (Fig. 13) to illustrate the subject. We would also like to thank Doug Wray for maintaining the SEM and Emma Jean Battles for her help in fixing the cochlea, dissecting these free from the skull, and removing their tectorial membranes.

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