The Actin Filament Content of Hair Cells of the Bird Cochlea Is Nearly Constant Even Though the Length, Width, and Number of Stereocilia Vary Depending on the Hair Cell Location

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Abstract. By direct counts off scanning electron micrographs, we determined the number of stereocilia per hair cell of the chicken cochlea as a function of the position of the hair cell on the cochlea. Micrographs of thin cross sections of stereociliary bundles located at known positions on the cochlea were enlarged and the total number of actin filaments per stereocilium was counted and recorded. By comparing the counts of filament number with measurements of actin filament bundle width of the same stereocilium, we were able to relate actin filament bundle width to filament number with an error margin (r^2) of 16%. Combining this data with data already published or in the process of publication from our laboratory on the length and width of stereocilia, we were able to calculate the total length of actin filaments present in stereociliary bundles of hair cells located at a variety of positions on the cochlea. We found that stereociliary bundles of hair cells contain 80,000–98,000 μm of actin filament, i.e., the concentration of actin is constant in all hair cells with a range of values that is less than our error in measurement and/or biological variation, the greatest variation being in relating the diameters of the stereocilia to filament number. We also calculated the membrane surface needed to cover the stereocilia of hair cells located throughout the cochlea. The values (172–192 μm^2) are also constant. The implications of our observation that the total amount of actin is constant even though the length, width, and number of stereocilia per hair cell vary are discussed.

THE hair cell bundles within cochleae of vertebrates vary in length in a systematic manner, with long bundles at the distal end, where the organism detects low frequencies, and short bundles at the proximal end, where the organism detects high frequencies (1–4, 6, 9, 14). The number of stereocilia per bundle also systematically changes, at least in the chick, but the pattern is the inverse of that of length, with small numbers of stereocilia at the distal end and large numbers at the proximal end (9). Because these two variables increase in opposite directions, we considered the possibility that the total mass of stereocilia per bundle might be a constant, or to express this in terms of molecules, the total amount of actin present within each hair cell bundle might be a constant in all cells of the cochlea. If this is true, then what we have already learned about the morphogenesis of the stereocilia and hair cell bundles (8, 10, 11) and their relation to actin filaments must be integrated with cell controls that limit the pool size of actin monomers.

A number of additional facts make this a horrendous problem to prove or disprove. First, the cochlea is not one dimensional, but has width such that one encounters ∼45 hair cells in a transverse walk across the width of the cochlea (9).

On such a transverse walk, the length of the longest stereocilia gradually increases as one approaches the superior edge (13); at the same time the width progressively decreases (12). How much change in width and length is a function of the position of the hair cell not only across the cochlea but along it. In short, hair cells lie on a two-dimensional grid. Second, each hair cell bundle contains stereocilia arranged into rows of increasing height, the so-called staircase (11). Third, the widths of the stereocilia in a particular bundle are not constant, but change in a prescribed way, with the tallest stereocilia thinner than shorter stereocilia within a single bundle (12).

One would predict at this point that comparing the actin content in hair cells at different positions on the cochlea would be a hopeless task. This would be true if it were not for the fact that all these variables such as length, width, and number of stereocilia are determined biologically with precision (7, 9, 11, 13); e.g., with an error margin of 5–10% from ear to ear. To us, the ear is the most precisely regulated actin machine so far described.

The purpose of this communication is to present our counts on the number of stereocilia per bundle as a function

of the position of the hair cell in two axes. We will then, by direct counts of actin filament number per stereocilium and from measurements of the widths of the filament bundles, determine the number of actin filaments per bundle. Combining these data with already published information from our laboratory (9, 11, 13), on the length of stereocilia as a function of location of the hair cell on the cochlea in two axes, we are then in a position to calculate the total content of actin in hair cell bundles located at different positions along the cochlea. We will then explore the implications of our findings and how our observations relate to what we have already learned about the morphogenesis of the stereocilia and hair cell bundles (8, 10, 11).

Materials and Methods

Male chicks 7–14 d old of the White Leghorn variety were obtained from Truslow Farms (Chestertown, MD). At this stage the skull is still largely cartilaginous, which facilitates dissection.

Scanning Microscopy

The techniques used in dissection, fixation, dehydration, removal of the tectorial membrane, critical point drying, sputter coating, and mounting are covered in Tilney and Saunders (9) and Tilney et al. (13). Of interest to this report is that the best fixation protocol that we found was to fix the cochleae by immersion in 1% OsO_4 in 0.1 M phosphate buffer at pH 6.2 for 1 h. After dehydration, critical point drying, and sputter coating, the specimen was examined in an AMR 1,000 scanning electron microscope operated at 20 kV using a lanthanum hexabromide filament. To count the number of stereocilia per bundle, the viewer must look directly down on the bundle. Along the superior edge of the cochlea, this does not present a problem, as the bundle extends straight up from the surface. However, the stereociliary bundles along the inferior edge lie over the apical surface of the hair cell with the tallest row of stereocilia covering the other rows; this makes counting difficult. We presume that this behavior is caused by shrinkage of the tectorial membrane during fixation, because it is attached only to the superior surface. To count the number of stereocilia in bundles at the inferior edge, we took advantage of ripples in the surface of the cochlea or small tears that occurred during preparation of the tissue. In these regions, the stereociliary bundles were upright or at least not entirely flattened so that accurate counts could be obtained. It was necessary to constantly readjust the tilt of the cochlea so we could see the bundles end on. Those in which the stereocilia splayed out were not included as, because of superposition artefacts, accurate counts could not be made.

We photographed cells located at six points along both the superior and inferior edges of the cochlea. In most cases, at least six cells were photographed at each position. These photographs were taken at a magnification of 12,000–16,000 and the negatives were enlarged photographically three times. From these prints, we could easily count all the stereocilia present in the bundles. Those that had stereocilia obscured by others were not counted.

Transmission EM

Because our ultimate purpose is to know the number of actin filaments present within the stereocilia in hair cell bundles located at prescribed regions of the cochlea, it was necessary to modify our protocol for dissection and fixation because, as already mentioned, of the problems of shrinkage of the tectorial membrane. To obtain perfect cross sections of the stereociliary bundles at defined positions on the cochlea is very difficult, even under ideal conditions when the bundles are fixed in their upright stance, because the cochlea is cupped. If fixation causes them to lie over the surface of the hair cells, then to get perfect cross sections of the bundles becomes a Herculean task. What we did to obviate fixation-induced flattening of the bundles was to remove the tectorial membrane from the cochlea before fixation. Our procedure was as follows. We cut away the tissues that surround the cochlear duct. Then, with fine forceps, we removed the tegmentum vasculosum, thereby exposing the sensory epithelium covered only by the tectorial membrane. During this procedure the preparation was immersed in Hanks' solution. The preparation was then immersed in papain (P3125; Sigma Chemi-

cal Co., St. Louis, MO) in fresh Hanks' solution. 40 μl of papain (supplied at 16–40 U/mg at a concentration of 1 mg/ml) was added to 10 cm^3 of Hanks' and digestion allowed to proceed for 30 min at room temperature. The cochlea was then transferred to fresh Hanks' solution at 4°C and examined using the darkfield mode on the dissecting microscope. The tectorial membrane can be visualized in this mode and can be grabbed with fine forceps and stripped off the sensory epithelium.

Fixation

The sensory epithelium was then fixed by immersion for 30–45 min in 1% OsO_4, 1% glutaraldehyde (from an 8% stock purchased from Electron Microscope Services, Fort Washingtin, PA) in 0.1 M phosphate buffer at pH 6.3 at 4°C. The fixative was made up immediately before use. After fixation, the cochleae were washed three times in cold water and en bloc stained with 0.5% uranyl acetate for 3 h to overnight, dehydrated rapidly, and embedded in Epon 812.

The blocks were mounted and trimmed. 1-μm sections were cut and stained with 1% toluidine blue and examined in the light microscope to ensure that the stereocilia were oriented advantageously. Thin sections were then cut with a diamond knife on a Porter-Blum ultramicrotome II (Sorvall-Dupont, Newtown, CT), picked up on grids, and stained with uranyl acetate and lead citrate. They were examined with a Philips 200 or 410 electron microscope.

If the sections of the stereocilia were within 20° of a perfect cross section, the tilting stage and rotation holder of the Philips 410 allowed us to obtain images in which we could count the total number of filaments in each stereocilium. If the sections were >20° off a perfect cross section, then tilting resulted in plastic too thick to obtain ideal images of individual filaments. For counting filament numbers, we enlarged the micrographs of the stereocilia to a magnification of 400,000 or 500,000. Under a lit magnifier, we then counted the filaments, marking them with a grease pencil as we counted. What made this procedure so difficult is that in cutting the cross sections one must know the location of the stereociliary bundles on the cochlea in two axes. Furthermore, to obtain the requisite cross sections, the block has to be repeatedly tilted and rotated.

Results

Because the length, width, and number of stereocilia per hair cell vary depending upon the location of the hair cell on the cochlea in two axes, it is essential at the outset to establish the terminology that we will use. The chicken cochlea is sickle shaped (Fig. 1) with a broad, distal end tuned to low frequencies and a thinner, proximal end tuned to high frequencies. The tallest stereocilia on hair cells located along the superior edge are approximately the same diameter, whereas those on the inferior edge increase in width as one approaches the proximal end.

Number of Stereocilia

We examined the stereociliary bundles of hair cells located on the superior and inferior edges of the cochlea at 12 positions along the cochlea. Representative bundles are illustrated in Fig. 2. Our counts of the total number of stereocilia per hair cell are presented in Fig. 3. Both by inspection and even better from our counts, it is obvious that except at the distal end of the cochlea, there are invariably more stereocilia per bundle at the superior margin than on the inferior margin at comparable distances from the proximal end of the cochlea. Furthermore, the disparity in numbers on these two margins decreases toward the distal end. This by itself would insure that the slopes of the graphs for stereocilia number vs. location on the cochlea for the superior and inferior margins would not be parallel. Thus, whereas the graph of number vs. position at the superior edge is nearly a straight line, a comparable graph of numbers vs. position at the inferior edge is a curve (Fig. 3).
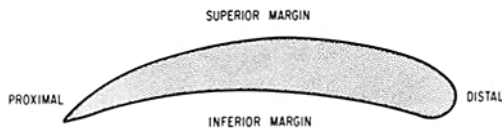
Figure 1. Drawing of the chicken cochlea to illustrate the terminology we will use.

Number of Steps in the Staircase

Micrographs of the stereociliary bundles looking from the superior edge of the cochlea toward its inferior edge reveal that the bundle is composed of stereocilia of increasing length with short ones in the front of the bundle and longer ones in the back. We call this arrangement of stereocilia the staircase, although it is difficult to distinguish individual rows or steps of the staircase because we do not find individuals all of the same length on a step. However, if we move directly up the staircase from its front to its back following a 1,0 lattice plane from the base of the bundle to its apex (9, 12), a plane we will henceforth refer to as the 1,00 lattice plane to distinguish it from the other 1,0 lattice planes situated at 60° to this 1,00 lattice plane, each successive stereocilium encountered is systematically longer than the one that precedes it. We can count the number of individuals that we encounter on this 1,00 lattice plane. The absolute number is somewhat noisy, but by counting a number of these 1,00 lattice planes, an average number for a particular bundle can be estimated (see arrow in Fig. 4). The main reason for the "noise" is that if the stereocilia are bent laterally somewhat during preparation it is difficult to see accurately the 1,0 lattice line. We counted steps in stereociliary bundles located on both the superior and inferior margins (Fig. 5). Whereas the average number of individuals encountered in going up the 1,00 lattice plane of the staircase on the inferior edge was roughly the same, on the superior edge the number increased as one approached the proximal end of the cochlea. Thus, the dramatic difference in the number of stereocilia on the superior and inferior margins at the same distance from the distal end of the cochlea documented in the last section is accommodated, at least in part, by increasing the number of staircase steps in the bundles located on the superior margin. Furthermore, the number of individuals per step is greater on those bundles located on the superior margin than on the inferior margin, at least at the proximal end of the cochlea and the overall widths of the stereocilia are thinner on the superior edge.

In short, what is apparently happening is that both the number of rows and the number of individuals per row are increasing on the superior edge relative to comparable positions on the inferior edge of the cochlea.

Width of the Stereocilia and Number of Actin Filaments Present within Them

Tilney and Saunders (9) reported on the width of the stereocilia of hair cells located at a series of positions along both the superior and inferior margins of the cochlea. These measurements were made off scanning EM micrographs taken of back views of the staircase because in this way the entire stereocilium could be analyzed, not just the tips, which are all that is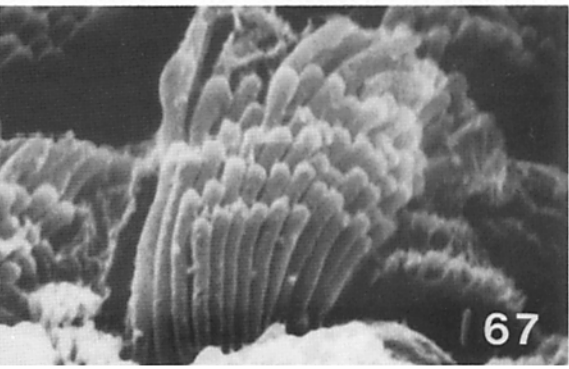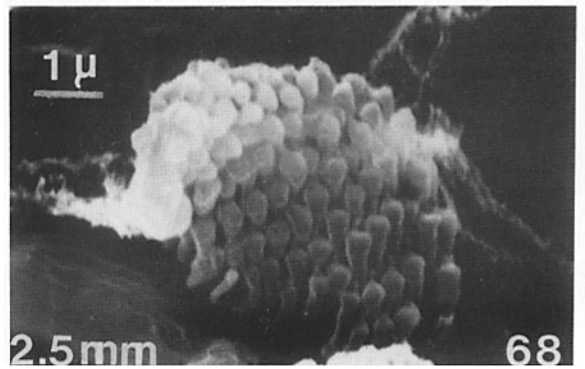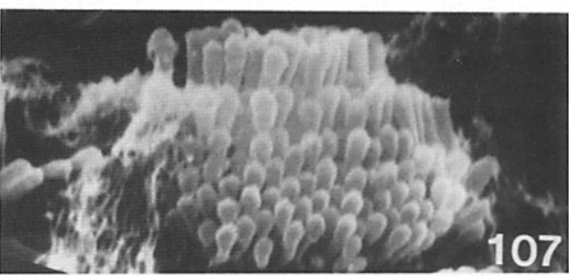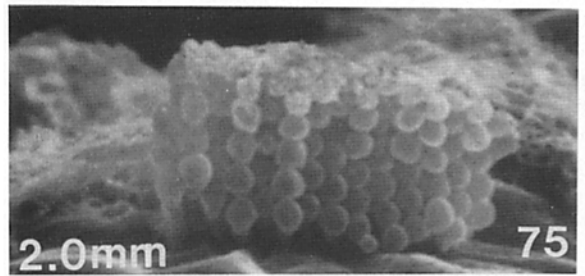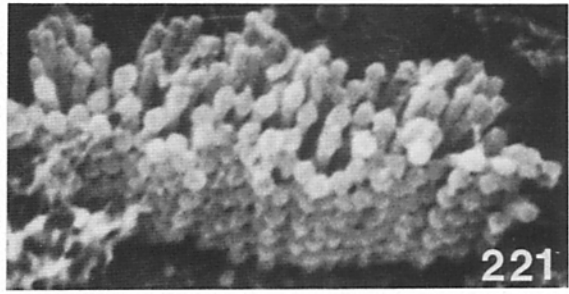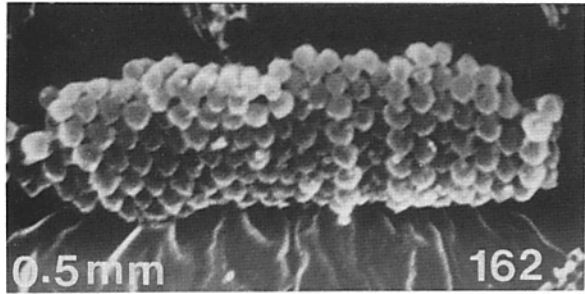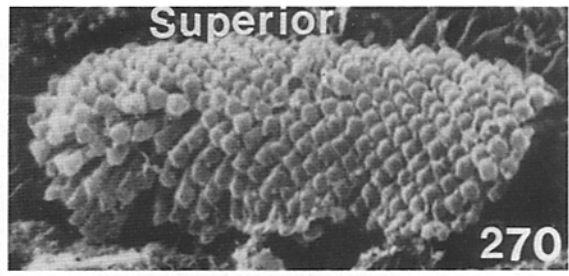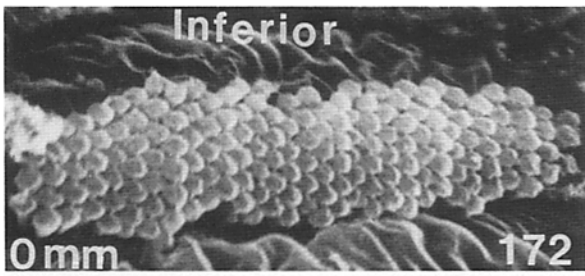 visible in frontal views. Measurements of widths are not valid at the tips because the plasma membrane tends to bulge out there, giving false readings. Unfortunately, views of the back side of the bundle only give us information on the tallest stereocilia in the bundle.

To get data on the width of stereocilia located in rows other than the tallest and to determine the number of actin filaments present in individual stereocilia, we cut thin sections through the stereociliary bundles at a variety of locations on the cochlea. Because our purpose here is to determine the total amount of actin in each stereociliary bundle, accurate measurements of the width of the stereocilia are crucial, as a small error in measurement of width produces a rather large error in filament number because the diameter of the stereocilium is approximately proportional to the square of the number of actin filaments present in that stereocilium.

There are problems measuring the width of stereocilia in thin sections. First, the membrane surrounding the stereocilium often bulges or blebs irregularly away from the filament bundle (see Figs. 6, 7, and 8). We presume that this is the result of inadequate fixation and/or dehydration and embedding and is therefore artefactual. Second, since the stereocilia taper at their bases, where they contact the apical surface of the hair cell, a transverse section through a stereocilium must be cut well away from the tapered region and accordingly near the stereociliary tips. This is less of a problem at the distal end of the cochlea where the stereocilia are long, but is hard to manage at the proximal end where the stereocilia are short. Third, we have to know where a particular bundle whose cross sections we are photographing is located on the cochlea in two axes. This can be accomplished, albeit with a serious trial to one's patience and sanity, at the distal end of the cochlea where the cochlea is broad and the stereocilia long, but at the proximal end where the cochlea is narrow and the stereocilia short, this is a horrendous problem. To make matters worse, the cochlea is severely cupped in the proximal region.

These problems notwithstanding, we were able to obtain a number of beautiful cross sections of the bundles at the distal end and a few at the proximal end that were sufficiently good to be able to count all the filaments in individual stereocilia. From our counts we are able to visualize directly the biological variation between adjacent cells and between stereocilia in the same row within a single cell (Figs. 6, 7, and 8). In all but a few cases, the maximum difference in actin filament number between stereocilia that lie in the same row is 15% within a single cell and 20% between adjacent cells.

At the mid distal end the tallest two to three rows of stereocilia are rather thin containing on average 225–240 filaments depending on which cell is examined (see Figs. 6 and 7). The shorter three to four rows of stereocilia contain on the average 450–500 (see Figs. 6 and 7). At the proximal inferior edge of the cochlea, we have cross sections through the tallest two to three rows of stereocilia; the shorter rows, because they are only a fraction of a micrometer long, are not in the plane of the section (Fig. 8). (To go closer to the cell surface is risky, as we might be in the tapered region.) The tallest row has on average 420 filaments and the shorter rows included in that section have on average 760. We obviously need more information at the proximal end. The difficulty in getting more cross sections here and at the same time knowing exactly where we are, albeit on the superior or inferior side of the cochlea, cannot be overestimated; we thus changed
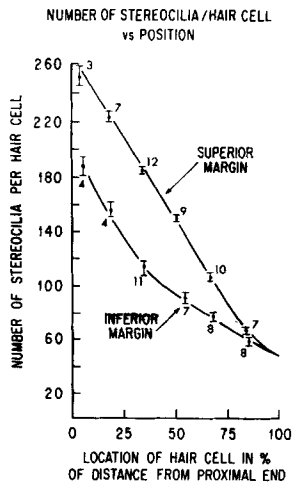
Inferior

Superior

0 mm. 172

270

0.5 mm 162

221

1.0 mm 100

190

1.5 mm 98

151

2.0 mm 75

107

1 µ

2.5 mm 68

67

NUMBER OF STEREOCILIA/HAIR CELL
vs POSITION
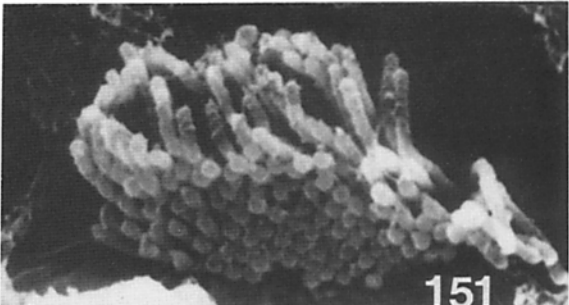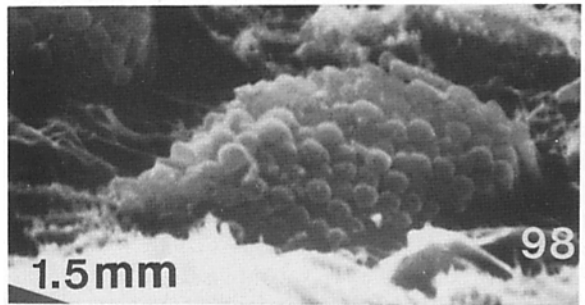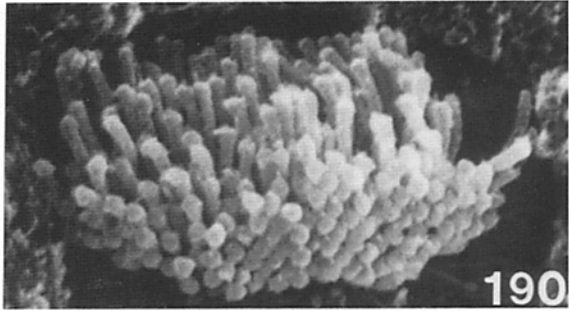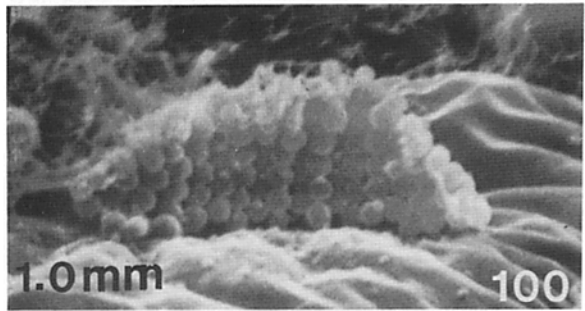
Figure 3. Plot of the number of stereocilia per hair cell relative to the position of the hair cell on the cochlea. Cells located at the extreme proximal end are at 0% on this graph with cells at the extreme distal end at 100%. The bars indicate one SD and the number above or below it indicate the number of hair cells measured.

Figure 4. Drawing of a section through a stereociliary bundle. The tallest rows (*top*) have stereocilia whose diameters are thinner than the shorter rows. The arrow indicates the 1,00 lattice plane.

our approach and cut thin sections through the width of the cochlea perpendicular to the long axis.

Every hair cell encountered was photographed and a montage was constructed. We examined the stereocilia of every hair cell in these sections and measured the width of the filament bundle (not the diameter of the stereocilium, as the membrane blebs out irregularly, as already mentioned). In oblique sections, the bundle is oval in shape and its thinner diameter is an accurate measurement of the width of the bundle. In longitudinal sections, because the filament bundle is cylindrical, the best measurement of the width of the bundle is the thickest part of the bundle. From these sections and the fact that the staircases of all hair cells point towards the superior edge (13), we can easily tell if we are examining the tallest or shortest rows of stereocilia on an individual hair cell and it is possible to get rather accurate measurements of the filament bundle widths. We also measured the widths of the same filament bundles from which we had counted the number of filaments (e.g., Figs. 6, 7, 8, and others not included here). From our counts of the number of filaments and measurements of the diameters of the same bundles, we can calculate our measurement error by performing a regression analysis with the number of filaments plotted against the square of the diameter of the filaments because the diameter is a function of the filament number (Fig. 9) and then calculating the r^2 from the correlation cofficient. We obtained a value of 0.84 or an error of relating filament number by direct counting to bundle diameter of 16%. This value takes into account differential shrinkage of the bundle during fixation, errors in counting (which are negligible), errors in measuring the diameter of the bundle, and errors in measuring the true diameter of the bundle, as sometimes it is not
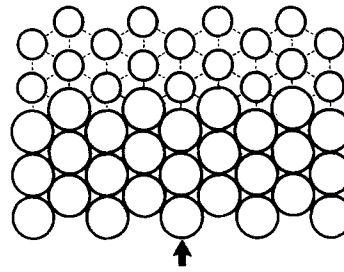
perfectly cylindrical. We presume that the reason that the line does not go through the origin is that there is some biological slip at either very small or very large diameters, or both.

Combining our information on filament bundle diameter obtained by transects cut across the cochlea (perpendicular to its axis), detailed information reported in Tilney et al. (11) in Fig. 4, and from the data in Fig. 9, we have in Fig. 10 expressed the filament number per stereocilium relative to the position of the hair cell on the cochlea. The bars here indicate our errors encountered in relating filament number to bundle diameter (Fig. 9) or 16% on either side of the central point. The smaller number in each case is the number of actin filaments in the tallest row of stereocilia, the larger number that in the shorter rows.

Length of the Stereocilia

There is no way to measure accurately the length of all the stereocilia in a bundle, because in frontal views of the staircase there is a superposition of stereocilia over each other so that one cannot see the tapered basal end of each stereocilium. We can, however, measure the length of the shortest row of stereocilia from frontal views and the tallest row from views of the backside of the staircase and the next tallest step by subtracting the next to last step in the staircase obtained from frontal views (11) from the entire length of the tallest row measured from rear views of the bundle (11, 13).

The last step in the staircase on the superior edge of the cochlea is considerably taller than any preceding steps, whereas on the inferior edge this is not the case (11). This difference accounts almost exclusively for the difference in height on the superior and inferior edges of the cochlea if one compares cells at comparable distances from the proximal end of the cochlea. Thus to arrive at a value for the length of the stereocilia in a single cell we separated the last step or tallest rows from the shorter rows. To obtain the average length of the shorter rows we averaged the length of the shortest row with that of the next tallest (Table I).

Figure 2. Montage illustrating the stereocilia of hair cells located at 12 positions on the cochlea. Six of these stereociliary bundles are located on the inferior edge (*left column*) and six along the superior edge (*right column*). The pair of bundles at the top of this figure are located at the extreme proximal end of the cochlea; this is indicated by the notation, 0 mm. As one moves down this montage, we have illustrated representative stereociliary bundles located at 0.5-mm intervals from the extreme proximal to the distal end. The number in the corner of each micrograph is the actual count of the total number of stereocilia in each bundle. These counts were made from enlargements of these pictures. Notice that as one goes from the proximal towards the distal end the total number of stereocilia per hair cell decreases. However, at each position there are more stereocilia in bundles located along the superior edge than along the inferior edge.
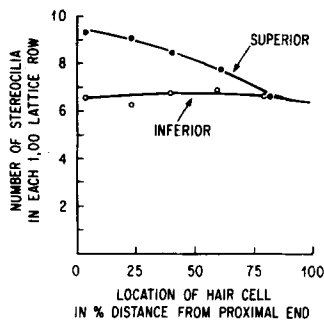
Figure 5. Graph depicting the average number of stereocilia encountered going up the 1,00 lattice plane (from the bottom to the top of the staircase) plotted as a function of the position of the hair cell on the cochlea. Data are included for cells located along both the superior and inferior margins.

presented in this figure are in micrometers of actin present within the stereocilia of each hair cell. How we arrived at these final values, e.g., the actual numbers used, is depicted in Table I.

## Calculations on the Amount of Membrane Surface Area Covering the Stereocilia

Using the information obtained in this report on the number of stereocilia and the total number of steps in the 1,00 lattice plane, the average length of the stereocilia, and the average diameter of the stereocilia (11), we also calculated the membrane surface area that covers the stereociliary bundle on hair cells located at six positions on the cochlea (Table II). To obtain these values we added to the diameters of the filament bundle the thickness of plasma membrane on either side of the bundle and approximate space seen in our electron micrographs between the filament bundle and the plasma membrane. In practice, this means adding 0.05 μm to each filament bundle diameter. We then obtained the surface area by treating the stereocilia as cylinders each with a single lid ($\pi dh + \pi r^2$), because the basal end is confluent with the plasma membrane limiting the apical surface of the hair cells.

Calculations on the Amount of Actin Per Stereociliary Bundle

Using the information presented here on the total number of stereocilia, the total number of steps in the 1,00 lattice plane, the length of the stereocilia from Tilney et al. (11), Fig. 4, and the number of rows in the final step (12), we can get a measurement of the total length of actin filaments in all the stereocilia of a single hair cell. This was done for six locations on the cochlea and plotted in Fig. 11. The values

Table I. Data Used to Calculate the Total Amount of F Actin Present in the Stereocilia of Hair Cells Located at Different Positions on the Cochlea

		Number of stereocilia	Average length	Average filament number	Actin per hair cell
			μm		*μm*
Superior distal	Tall	24	4.5	226	24,400
(2.5 mm)	Short	48	2.8	437	58,700
					83,100
Superior mid	Tall	40	2.8	201	22,500
(1.5 mm)	Short	100	1.4	376	57,900
					80,400
Superior proximal	Tall	56	1.8	220	22,200
(0.5 mm)	Short	168	0.9	437	66,100
					88,300
Inferior distal	Tall	30	4.0	226	27,100
(2.5 mm)	Short	60	2.1	437	55,100
					82,200
Inferior mid	Tall	20	2.5	445	22,300
(1.5 mm)	Short	80	1.3	716	74,500
					96,800
Inferior proximal	Tall	32	1.7	467	25,400
(0.5 mm)	Short	128	0.8	716	73,300
					98,700

Data on the total number of stereocilia in hair cells located at discrete points on the cochlea are taken from Fig. 3. The total number of 1,00 lattice rows is taken from Fig. 5 and the number of individual stereocilia that are tall or short is taken partly from direct counts; e.g., Fig. 2 and partly from information in Tilney et al. (12). The average length of stereocilia is taken from Fig. 4 (p. 360) in Tilney et al. (11). The average filament number is taken from Fig. 10.

Figure 6. (*Center*) Low-magnification micrograph of a thin section through a bundle of stereocilia that extends from a hair cell located ∿2.5 mm from the proximal end of the cochlea and midway between the inferior and superior margins. The single kinocilium (*k*) identifies the tallest row of stereocilia. The shortest row of stereocilia is situated at the base of this plate. (*Top*) High magnification images of three stereocilia outlined on the center panel. (*Bottom*) On this tracing of the center panel we have placed our actual counts of the filament number in the high-magnification images of the stereocilia in the thin section illustrated in the center panel.
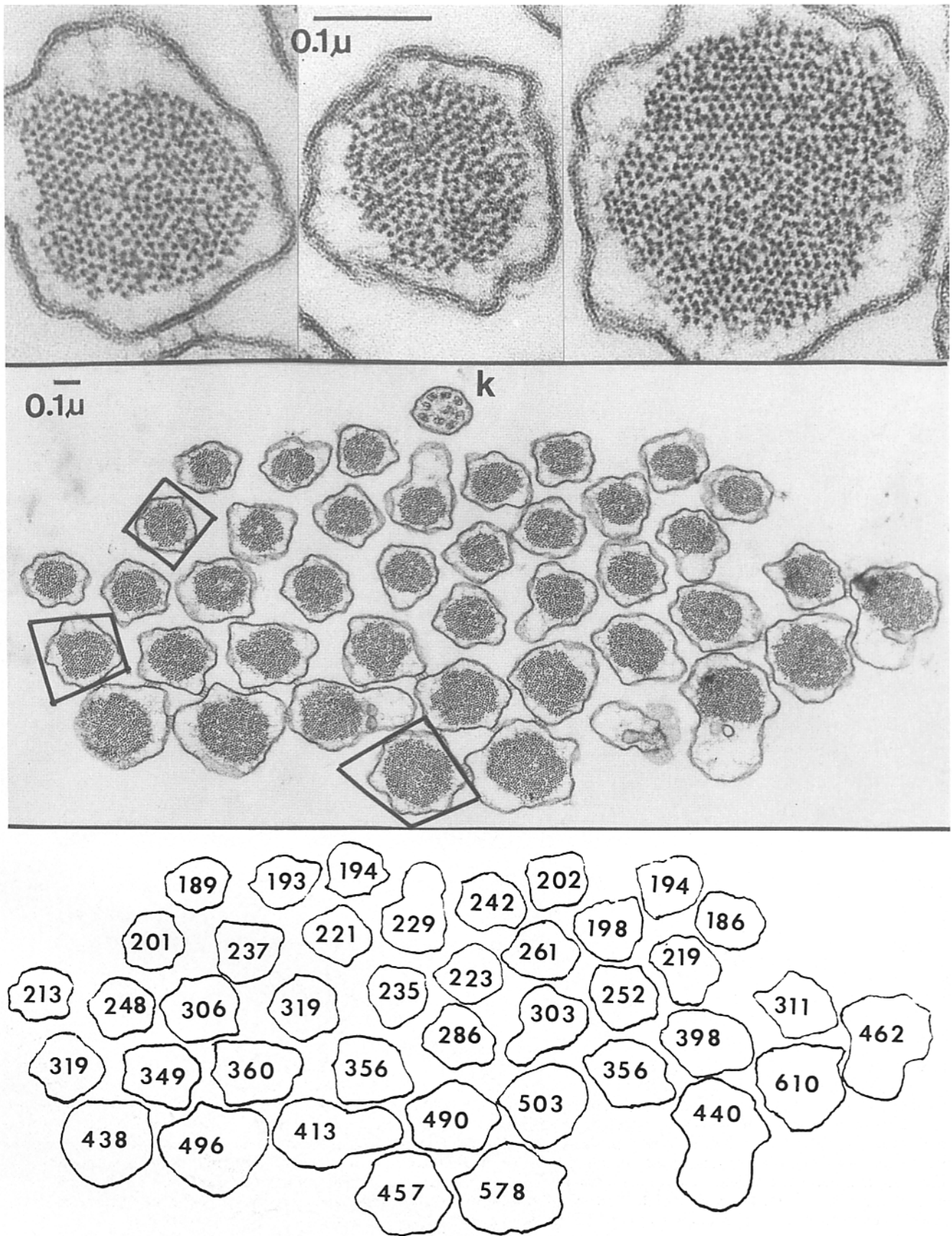
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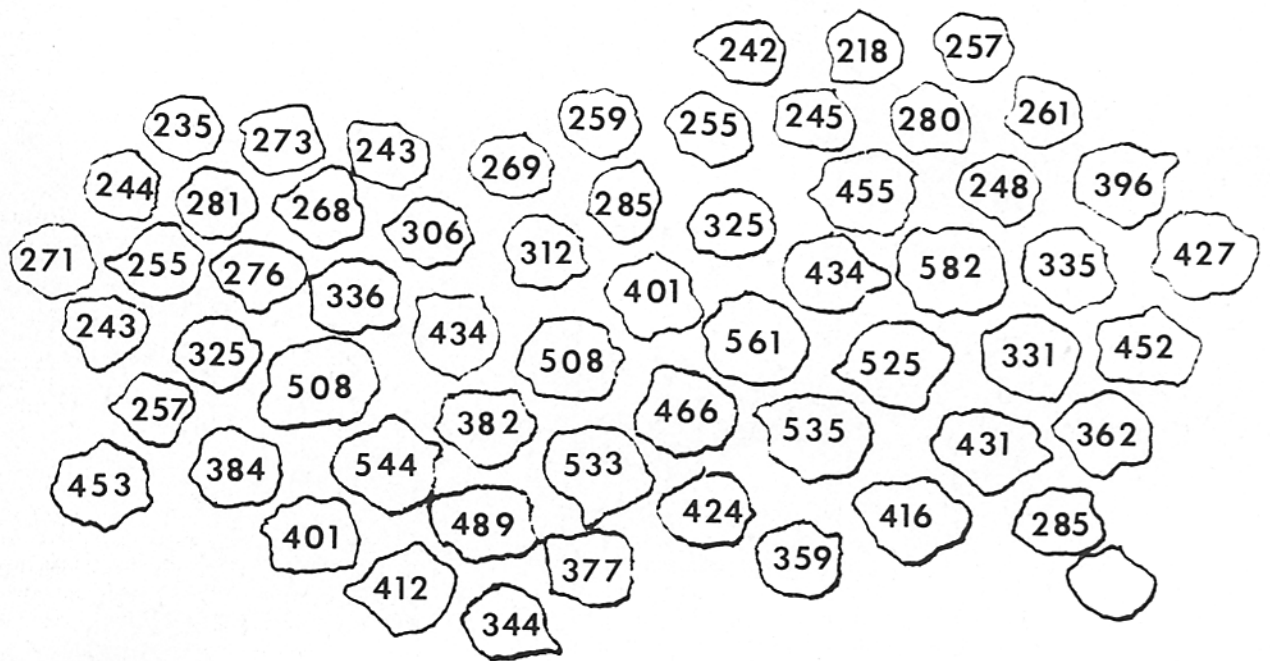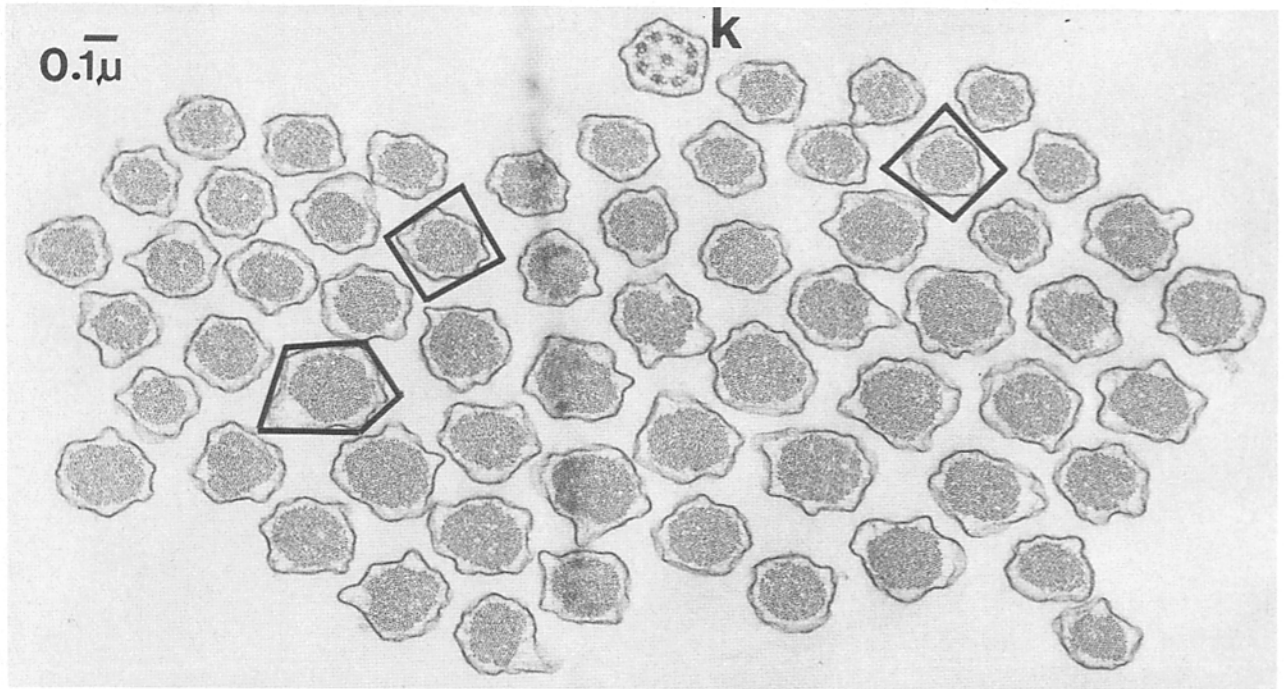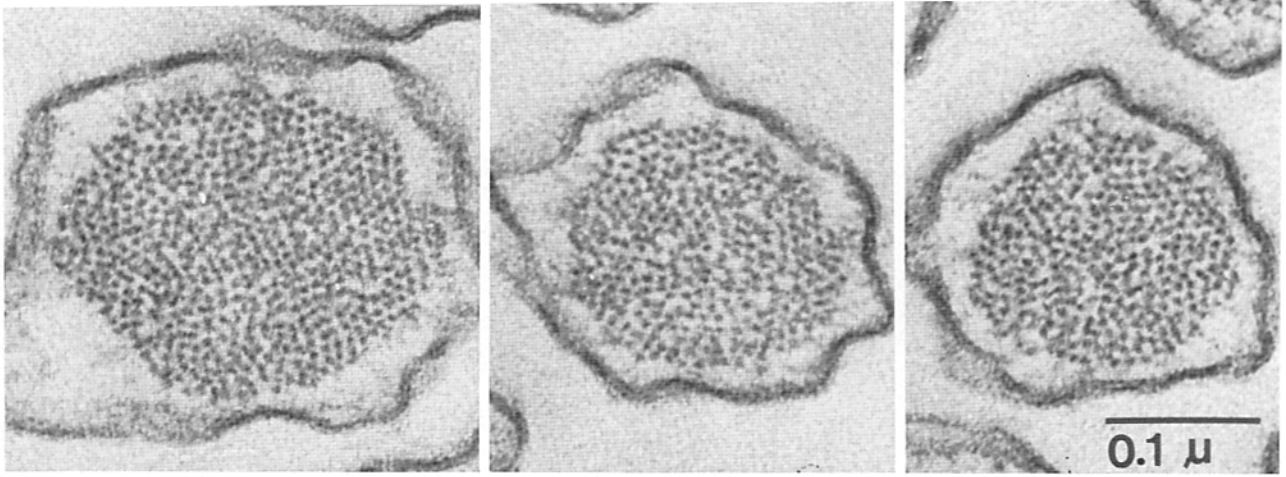
Figure 7. (*Center*) Low-magnification micrograph of a thin section through a bundle of stereocilia from a hair cell located nearby that illustrated in Fig. 6. Again the kinocilium (*k*) identifies the tallest row of stereocilia. (*Top*) High magnification images of three stereocilia outlined on the center panel. (*Bottom*) On this tracing of the center panel we have placed our actual counts of filament number in the high-magnification images of the stereocilia in the thin section illustrated in the center panel.
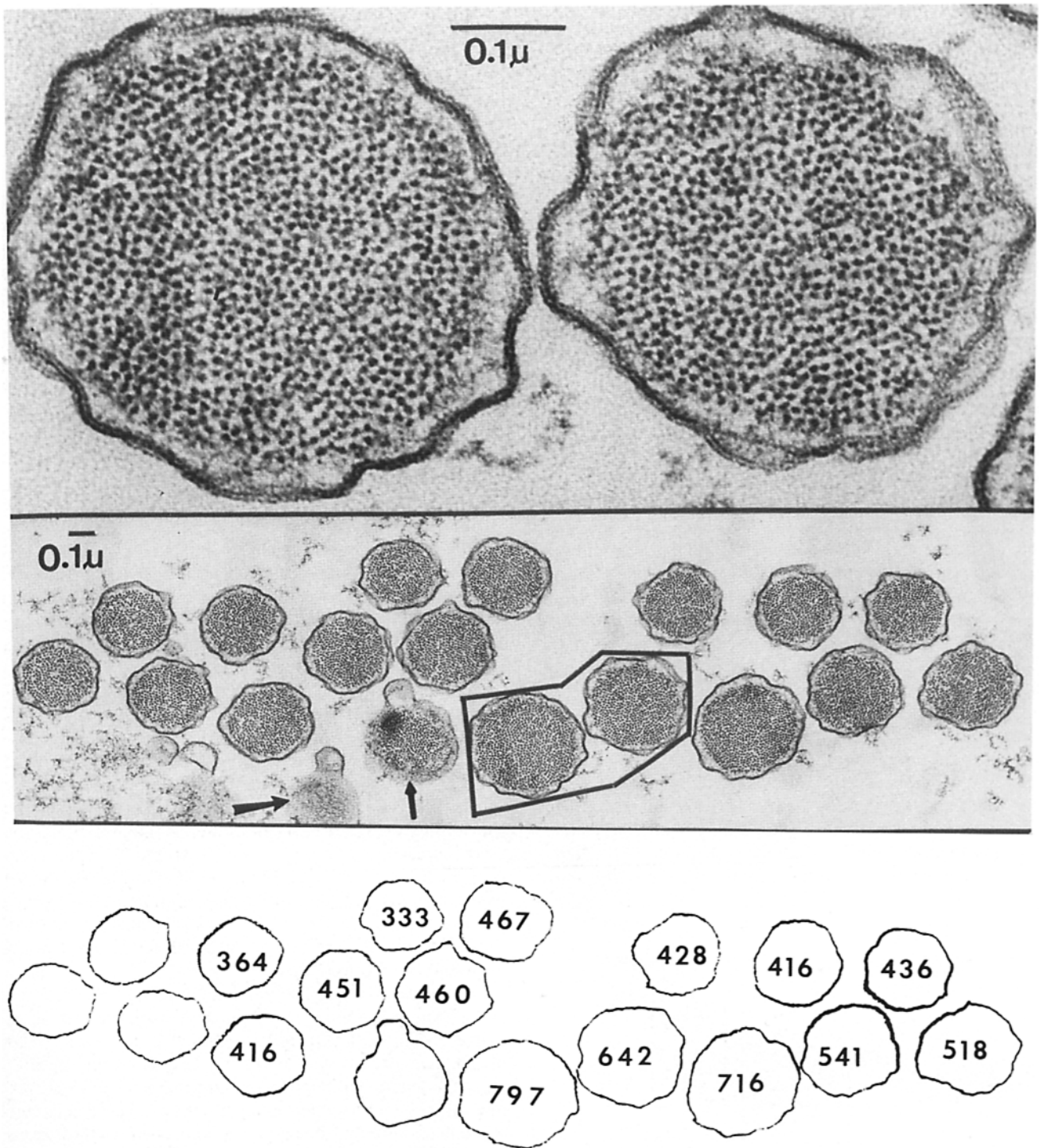
Figure 8. (*Center*) Low-magnification micrograph of a thin section through a portion of a bundle of stereocilia located on the inferior margin of the cochlea ∼0.5 mm from the proximal end. Because the stereocilia are short, the largest in the bundle being only 2.0 μm long and the shortest 0.2 μm, and because they taper near their attachments to the cell surface in bundles located near the proximal end, only sections such as this near the tips of the stereocilia give us satisfactory values for stereociliary diameters. This also means that only the tallest rows are included in the section. Arrows indicate a grazing view of the tips of two stereocilia. Of course, these grazing views will be only of the shorter rows. (*Top*) Higher magnification images of two stereocilia outlined on the center panel. (*Bottom*) Tracing of the center panel. The number in each stereocilium indicates our actual counts of the number of actin filaments in that stereocilium. The three stereocilia on the left are blank as the angle of tilt of the stereocilia did not allow us to count the number accurately. The blank stereocilium in the center (with the blebbed membrane) was not counted because the section only grazes the surface of this stereocilium.
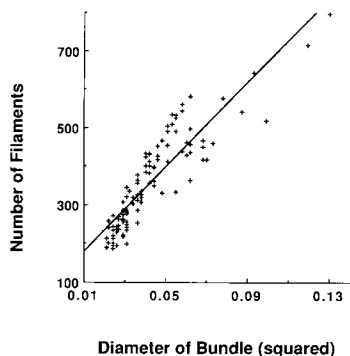
Figure 9. From micrographs such as those illustrated in the center panels of Figs. 6–8, only magnified many fold, we measured the diameter of the filament bundle in each stereocilium. On this graph we plotted the square of the diameter of the bundle relative to our actual counts of the number of actin filaments in the bundle. The line indicates a regression analysis.

Figure 11. Combining the data included in Fig. 10 with data presented in Fig. 3 and in earlier publications from our laboratory we calculated the total length of actin filaments present in stereocilia located at six positions on the cochlea. These lengths in micrometers of actin in stereocilia in individual hair cells and their approximate positions are indicated on the cochlea. Details on the exact values used are presented in Table I.

Discussion

Two major new findings are presented in this report. First, we counted the total number of stereocilia present in hair cells located at 12 positions on the cochlea. These measurements show us that the number of stereocilia varies in a predictable way in two axes. Second, by actual counts of the number of actin filaments per stereocilium and the relationship of these counts to the diameter of the filament bundles in stereocilia we were able, using information published in other communications from our laboratory (11, 12), to obtain values on the total length of actin filaments present in the entire bundles of stereocilia of hair cells located at six positions on the cochlea. This forced us to look in detail at individual bundles. We found that the tallest or last step in the staircase has stereocilia that contain fewer actin filaments than the shorter or lower steps of the staircase. In essence, then, each hair cell bundle is more complex than was previously thought, a fact that will be crucial to subsequent studies on what regulates actin filament number and length. We found that each hair cell contains between 80,400 and 98,700 μm of actin filaments in its stereocilia. Since the cuticular plate, the only other source of actin filaments in the hair cell, contains only

6% of the total actin (DeRosier, D. J., and L. G. Tilney, unpublished observations) and the cuticular plates of hair cells located throughout the cochlea appear to be approximately the same density and volume, this means that every hair cell on the cochlea has the same amount of actin with a variance level of 10% which is within our error of measurement (16%).

The relationship between filament bundle diameter and filament number is our major error because the diameter is proportional to the square of the filament number. Errors in counting are miniscule and errors in measuring stereociliary length and number small. Thus our major error is relating filament bundle diameter to filament number. Furthermore, biological variation in these values is in the range of 5–10%. If we had a better way of measuring the number of actin filaments in each stereocilium, this variance would be less than what we mention here because on the inferior margin of the cochlea from the proximal end to about midway along it, the shorter rows of stereocilia are all wide and at the same time short. Since the stereocilia taper near their connections to the apical surface, the shortest rows of stereocilia contain less actin than one would suspect by measuring the width of the bundles near the tips, which in practice we have to do. Therefore these particular values are inflated more than shorter stereocilia elsewhere on the cochlea. This error would make our values even closer.

Our conclusion that the total amount of actin per hair cell is constant even though each hair cell is different from its neighbors in the length of its stereocilia, the width of its stereocilia, and the number of its stereocilia, and accordingly the length and number of actin filaments present, implies that somehow each hair cell must make a quantum amount of actin and during development must regulate its assembly into structures which vary in length, width, and number. Thus a particular hair cell can make a few long stereocilia or a lot of short stereocilia and by changing the width of the stereocilia in the bundle come out with the same amount of actin per bundle. To turn this statement around, which is what the cell is doing biologically, by regulating the length of the actin filaments, the number of actin filaments per bundle, and the number of bundles, the cells, by using the same amount of actin, vary the physical dimensions of the stereocilia in two axes, a situation that the ear presumably requires for frequency selectivity in hearing.

Equally interesting is that our calculations on the membrane surface that covers the stereocilia located at varying
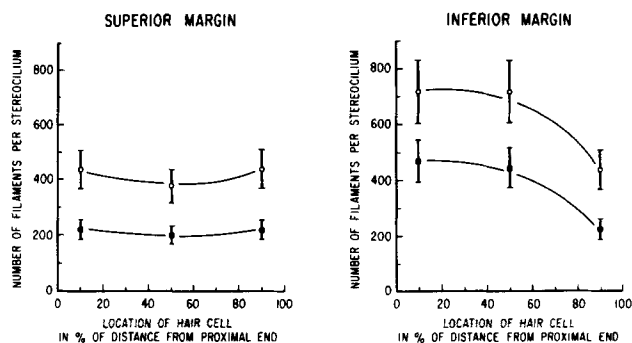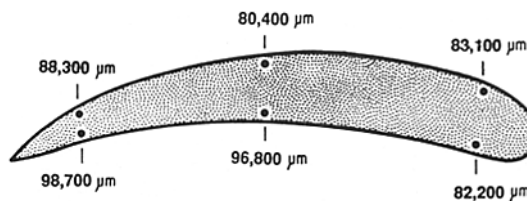

Figure 10. Combining the data plotted in the preceding figure with the diameter of the filament bundles measured from longitudinal, oblique, and transverse sections of hair cells located at six positions on the cochlea (see reference 12), we plotted the number of actin filaments in stereocilia in the tallest and shortest rows located at six positions on the cochlea, three along the superior edge and three along the inferior edge. The error bars reveal the errors (r^2) in relating the diameter of the stereocilia to the filament number calculated from the regression line in Fig. 9. (o) Short stereocilia; (■) tall stereocilia.

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Table II. Data Used to Calculate Total Stereociliary Surface Area per Hair Cell

		Number of stereocilia	Average length	Diameter of stereocilia	Surface area per hair cell
			μm		μm^2
Superior distal	Tall	24	4.5	0.165	
(2.5 mm)	Short	48	2.8	0.208	182
Superior mid	Tall	40	2.8	0.142	
(1.5 mm)	Short	110	1.4	0.208	192
Superior proximal	Tall	56	1.8	0.155	
(0.5 mm)	Short	168	0.9	0.217	192
Inferior distal	Tall	30	4.0	0.160	
(2.5 mm)	Short	60	2.1	0.213	183
Inferior mid	Tall	20	2.5	0.245	
(1.5 mm)	Short	80	1.3	0.334	172
Inferior proximal	Tall	32	1.7	0.229	
(0.5 mm)	Short	128	0.8	0.343	174

Data from the previous table on the number and length of stereocilia and on the diameters of the stereocilia from Figs. 6–8 were used to calculate the total surface area of stereocilia on each hair cell at discrete points on the cochlea. The stereocilium was considered as a cylinder with a lid; thus the surface area of the cylinder (πdh) was added to the area at the tip of the stereocilium (πr^2). Because the base of the stereocilium is contiguous with the plasma membrane covering the apical surface of the cell, we did not add any membrane surface here. Before making our calculations, we added 0.05 μm to the diameter of the filament bundle reported in reference 12 to include the diameter of two plasma membranes and twice the space between the plasma membrane and the filament bundle.

positions on the cochlea are also nearly constant even though the length, number, and width of the stereocilia are different from hair cell to hair cell. This implies that somehow in the regulation of the physical dimensions of the stereocilia, the cell is integrating the length and number of its actin filaments with the surface area of membrane being used.

Now the fun begins! What we need to know in the future is how does the cell measure one quantum amount of actin and how does it know what to do with it; e.g., nucleate more filaments per stereocilium, longer filaments per stereocilium, or more bundles. We have learned, by studying the development and differentiation of the stereocilia in embryos of increasing age (8, 10), what happens during the growth and differentiation of the stereocilia and what happens to the actin filaments within the stereocilia (e.g., cross-bridging) but as yet we do not know how the actin filaments are regulated in length and number. To understand how the cell measures the amount of actin it makes requires observations that ask, is this quantum amount made all at once or at a continuous rate. If it is all at once, is the quantum amount an amount of mRNA that in turn is translated continuously or all at once, etcetera? The cochlea is a miserable organ on which to do biochemistry, but methods exist, e.g., in situ hybridization, that should help us answer these questions.

Our conclusion that the total amount of actin is constant not only makes us aware of how the cell must regulate this quantum amount, but also forces us to reconsider our earlier studies on the development and differentiation of the ear (8, 10). We know that stereociliary bundles located at different positions on the cochlea complete their differentiation at widely different times, even though they all begin to differentiate at the same time. For example, at the superior proximal end of the cochlea differentiation is complete in a 12-d embryo, yet at the distal end differentiation is not complete until well after hatching, nearly 2 wk later. In short, the use of the quantum amount occurs at vastly different rates.

The question immediately arises as to how the hair cell coordinates differences in stereocilia number, length, and width in two axes with a quantum amount of actin and a con-

stant amount of membrane surface. We know that these variables are regulated at different times during development (10), but how they are coordinated is baffling. The only other system that is vaguely analogous to the formation of stereocilia is cilia formation in sea urchin embryos. Stephens (5) has shown that during the formation of cilia (which are, incidently, of prescribed lengths) in embryos there is only one protein in the cilium that is synthesized in limited quantal amounts, component 20 or tektin. If the embryos are deciliated and allowed to regenerate, this is the only protein that needs to be resynthesized as there are pools of all the other proteins in the cell body. As might be expected, tektin is again resynthesized in limited quantum amounts. Interestingly, this protein is found in the wall of the outer doublets and therefore could be the component that is used by the cell to control the length of the cilia. Unfortunately the control of the stereocilia cannot be by an identical mechanism because the product of stereociliary length and number is not constant as width is varied as well. Thus the control cannot be by some component that runs up a stereocilium and when used up growth is inhibited. It is intellectually possible to use more than one limited quantal component, particularly if the growth of stereocilia occurs in discrete phases, and in this way achieve the observed results. Perhaps this is how hair cells do business. We hope to learn the answer.

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