Actin Filaments, Stereocilia, and Hair Cells of the Bird Cochlea. V. How the Staircase Pattern of Stereociliary Lengths Is Generated

Lewis G. Tilney, Mary S. Tilney, and Douglas A. Cotanche*

Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104; and * Department of Anatomy and Cell Biology, Medical University of South Carolina, Charleston, South Carolina 29425

Abstract. The stereocilia on each hair cell are arranged into rows of ascending height, resulting in what we refer to as a "staircase-like" profile. At the proximal end of the cochlea the length of the tallest row of stereocilia in the staircase is 1.5 μm, with the shortest row only 0.3 μm. As one proceeds towards the distal end of the cochlea the length of the stereocilia progressively increases so that at the extreme distal end the length of the tallest row of the staircase is 5.5 μm and the shortest row is 2 μm. During development hair cells form their staircases in four phases of growth separated from each other by developmental time. First, stereocilia sprout from the apical surfaces of the hair cells (8-10-d embryos). Second (10-12-d embryos), what will be the longest row of the staircase begins to elongate. As the embryo gets older successive rows of stereocilia initiate elongation. Thus the staircase is set up by the sequential initiation of elongation of stereociliary rows located at increased distances from the row that began elongation. Third (12-17-d embryos), all the stereocilia in the newly formed staircase elongate until those located on the first step of the staircase have reached the prescribed length. In the final phase (17-d embryos to hatchlings) there is a progressive cessation of elongation beginning with the shortest step and followed by taller and taller rows with the tallest step stopping last. Thus, to obtain a pattern of stereocilia in rows of increasing height what transpires are progressive go signals followed by a period when all the stereocilia grow and ending with progressive stop signals. We discuss how such a sequence could be controlled.

How a cell controls the position, direction, and length of its extensions or processes is a question that has interested cell and developmental biologists for many years. As more information has accumulated on the cytoskeletal elements in these extensions the question has gradually been honed down to one dealing with how a cell controls the length, number, and orientation of its major cytoskeletal components, microtubules, and actin filaments. The hair cells in the cochleae of vertebrates are ideal to study in this regard because from their surface are numerous extensions or stereocilia whose length, width, number, and orientation are rigorously defined (see 14 for references). Within the membrane that limits each stereocilium is a cross-bridged bundle of actin filaments which gives the stereocilium its shape and characteristics. Thus if we can discern what regulates the length of the actin filaments, the number of actin filaments per bundle, and the position of the actin filaments in the cytoplasm, we should be able to begin to understand how the cell exerts control over the physical dimensions of its cell extensions.

In earlier papers in this series we described how the hair cells form and elongate their stereocilia. We related these macroscopic events to the number and distribution of the actin filaments and cross-bridges that lie within the stereocilia and the apical cytoplasm (cuticular plate), and began to formulate testable predictions about how the physical dimensions of the stereocilia might be controlled (13, 18).

Before we test some of these predictions there are additional aspects of stereociliary differentiation that must be described in greater detail. One of these concerns the question of how an individual hair cell grows stereocilia of different, yet predictable lengths, off the same surface. We know that the stereocilia increase in length from the "front" of the hair cell bundle to the "back," but are approximately equal within a row of stereocilia across the hair bundle (7). In short the stereocilia are arranged into rows of increasing height presenting to the viewer a "staircase-like" profile. There are many ways that a staircase could be developed. One is to generate some type of gradient in the cell such that some stereocilia, depending on their location, grow at a progressively greater rate than others. Another is to form different steps of the staircase at different developmental times, and a third is to grow all the stereocilia at the same rate but either start or stop the growth of rows of stereocilia at earlier or later times.

What we find is that the development of the staircase oc-

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curs in four phases separated from each other by developmental time. In short there are progressive go signals followed by a period when all the stereocilia grow and ending with progressive stop signals. Thus a complex pattern can be built up following simple rules during developmental time.

Materials and Methods

Fertilized chicken eggs of the White Leghorn variety were obtained from a local supplier and incubated at 37°C. Hatching occurred at day 21.

Dissection, Fixation, and Mounting of the Cochleae

The cochleae of embryos and chicks were dissected as outlined in Tilney and Saunders (14) and Tilney et al. (18). Fixation was carried out by immersion of the cochlea, still surrounded on its basal and lateral surfaces with cartilage, in a freshly made solution of 1% OsO_4 in 0.1 M phosphate buffer at pH 6.3. Fixation was carried out at 0°C for 45–60 min. After fixation the cochleae were dehydrated in acetone to 75%. The tegmentum vasculosum was then removed and the tectorial membrane lifted free with fine forceps. At this point as much as possible of the cartilage and connective tissue attached to the superior surface of the cochlea was removed as was possible because to see the staircase one must "look across" the superior surface of the cochlea. The cochleae were then dehydrated in pure acetone, critically point dried, oriented on stubs and sputter coated (14, 17). The orientation on the stub is important as the detector of our scanning electron microscope (AMR 1000) is situated at 90° to the column. Thus the greatest signal from the specimen is when the stub is oriented at 45°. Since we want to see the staircase by looking across the surface of the cochlea, the greatest signal is obtained if the superior surface is inclined upward from the stub by ~45°.

Determination of the Lengths of the Stereocilia

After the specimen was oriented appropriately so that the stereociliary bundles stand upright in front of the viewer, a series of photographs was taken at a magnification of 8,000–16,000 (depending upon the length of the stereocilia) at five locations along the superior surface of each cochlea. The negatives of these photographs were enlarged 2.5 times to form 8 × 10 inch prints of the bundles. From the prints we could accurately measure the lengths of the stereocilia. At least 10 and usually many more adjacent stereociliary bundles were photographed for each of the five locations. We first photographed the bundles looking toward the front of the staircase or the side on which stereocilia of increasing height were visible, then we rotated the cochlea 180° and tilted it appropriately, and took photographs of stereociliary bundles examined from their back side in which only the tallest row was visible. Thus we photographed both the front and back surfaces of the same cells or cells located in the immediate vicinity. Measurements of the length of the stereocilia were then made with a ruler under a lit magnifier. There is little ambiguity as to the length of the tallest row of stereocilia when viewed from the back of the staircase (see references 14 and 17 for a discussion of the accuracy). The average length of the shortest row of stereocilia is more difficult as they are not of uniform length. Sometimes there are tiny microvilli at the base of the first row. These do not seem to be stereocilia as they do not have the same widths as the other stereocilia in the bundle, all of which seem to be remarkably constant in width. We did not include these microvilli in our measurements.

On the graphs (Figs. 4, 7, 8, and 9) where we express the length of stereocilia as a function of position, we included error bars for each point to illustrate one standard deviation. Above these we indicate the number of measurements made. We have also included a table to show how accurately biology can determine lengths. In this table we show the largest and smallest value that we measure for each point.

Results

As depicted graphically in Fig. 1 the cochlea is sickle shaped with hair cells located at the distal end "tuned" to low frequencies and those at the proximal end tuned to high frequencies. To examine the full length of the stereociliary bundles it is necessary to orient the cochlea so that the observer is looking across the surface. In practice this means that the best view of the staircase is to look from outside the cochlea towards the superior margin (Fig. 1). In this view the stereocilia are not obscured by other bundles and the staircase is positioned so that the shortest row is encountered first with progressively taller rows behind, i.e., the tallest row of the staircase is found farthest from the superior margin. There are two reasons why we were forced to concentrate on the hair cells located on the superior margin of the cochlea. First, the stereocilia on hair cells located along the superior edge of the cochlea extend upwards or perpendicular to the surface of the cochlea, an ideal situation. In contrast the stereociliary bundles on the inferior margin lie flat on the surface of their respective hair cells with the longest row covering over the shorter rows. We presume that this behavior of the stereociliary bundles located near the inferior edge of the cochlea is due to shrinkage of the tectorial membrane during fixation as it is attached only at the superior surface. Second, the orientation of the staircases of hair cells located in the center of the cochlea are rotated relative to those on the superior edge (17). This means that these central bundles tend to be obscured by bundles located along the margins of the cochlea. Thus, in practice, the only way to study the staircase and to measure the length of the component stereocilia is to examine cells located along the superior edge of the cochlea.

Table I. Maximum Biological Variation of Stereocilia Length

Age	Row			
	Shortest		Tallest	
	%	*μm*	%	*μm*
15-d embryo	34*	0.6–0.8	23	1.33–1.58
	64	1.0–1.4	63	2.08–2.29
	84	0.7–1.4	84	2.08–2.29
	96	0.8–1.1	93	1.50–1.75
19-d embryo	16	0.36–0.50	16	1.55–1.83
	33	0.50–0.72	33	2.11–2.50
	56	0.76–0.96	70	3.00–3.61
	60	1.11–1.61	74	3.57–4.57
	64	0.96–1.27	80	3.57–4.10
	71	0.89–1.10	86	3.10–3.81
	81	1.23–1.92	92	2.38–1.92
21-d hatchling	12	0.37–0.51	12	1.66–1.94
	43	0.44–0.63	43	2.00–2.50
	51	0.66–1.03	51	2.61–3.05
	68	1.04–1.38	68	2.72–3.16
	85	1.27–2.00	85	3.50–4.16
15-d chick	6	0.35–0.42	6	1.18–1.53
	20	0.55–0.70	20	2.03–2.20
	40	0.76–1.00	40	2.40–2.68
	55	1.30–1.48	55	2.77–3.40
	94	1.80–2.38	94	4.35–4.90

* The position of each hair cell measured is expressed as the percent of the distance from the proximal end of the cochlea.

Stereociliary Bundles of the Chick

Figs. 2 and 3 illustrate representative views of the stereociliary bundles of hair cells located at five positions along the superior margin of the cochlea from a 15-d-old chick. These positions are indicated on the base of each micrograph expressed in percent of the distance along the cochlea from the proximal end. The micrographs in Fig. 2 illustrate a frontal

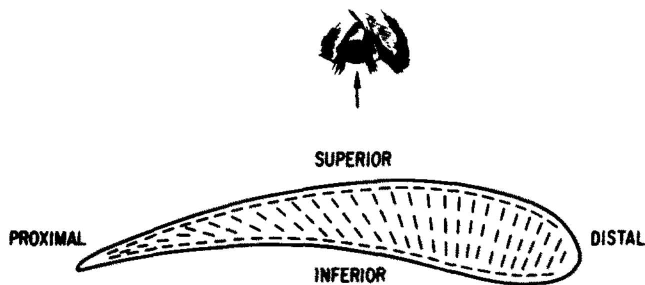


Figure 1. Drawing showing the overall shape of the cochlea and identifying the superior, inferior, distal, and proximal ends. The eye located at the top of this figure is the viewer looking towards the superior edge of the cochlea. In this view the staircase is directly in front of him.

view of the staircase, while those in Fig. 3 show the back of the staircase so that only the tallest row is visible. There are four points worth emphasizing from these figures. (*a*) Although there is a progressive increase in length of stereocilia as one proceeds from the first or shortest row towards the tallest, it is difficult to determine exactly how many rows there are and to which row an individual stereocilium belongs because the stereocilia within each row vary somewhat. In contrast in a straight line directly up the staircase successive stereocilia are invariably longer than those encountered lower down on the staircase. (*b*) A careful examination of successive stereocilia located along a line oriented directly up the staircase shows that the stereocilia are connected to each other. These connections extend from the tip of the shortest stereocilia to the shaft of the one behind and so forth. These connections were first described by Pickles et al. (10) in mammalian hair cells and are clearly present on bird hair cells as well. They have been called "tip linkages." As can be seen in Fig. 2, these often appear to make the tips of the stereocilia bulge out (*arrow*). (*c*) A close examination of the staircase profiles, particularly in the distal half of the cochlea, reveals that although there is a progressive increase in the lengths of the stereocilia as one climbs up the staircase, there is a larger increase in stereociliary length between the next to tallest and the tallest row (e.g., Fig. 2, *c* and *e*). (*d*) At the proximal end of the cochlea the first or shortest step in the staircase is only a fraction of a micron long. This step becomes progressively longer and longer as one approaches the distal end where the first step is about 2 μm long.

What we would like to do is to measure the average length of each step of the staircase at a series of positions along the cochlea. This is impractical because individual steps are difficult to identify with certainty and in lateral views one cannot be sure which step a stereocilium is on. Thus, we have no way of accurately measuring any step except the shortest and tallest. We can, however, accurately describe the profile of the staircase by measuring the first and the last steps from the front (Fig. 2) and back (Fig. 3) views, respectively (Fig. 4). We did this for these steps on many hair cells at each of the five to six locations on the cochlea and plotted their lengths as a function of the position of the bundle on the cochlea (Fig. 4). Although both curves have a positive slope, they are not parallel but diverge from each other more and more the closer one gets to the distal end of the cochlea. As already pointed out, at the distal end of the cochlea the increase in distance from the next tallest to the tallest row is much larger than that between the other steps in the staircase. It is possible to calculate the average length of the step immediately below the tallest row or step by subtracting the average rise from the next tallest row (Fig. 2) from that of the length of the tallest row as determined from micrographs of the back of the staircase (Fig. 3). We then compared the length of the next tallest row with that of the shortest row at varying positions along the cochlea (Fig. 4). We found that the slopes of these curves are much more nearly parallel than those for the tallest and next to tallest row indicating that if we neglect the last row, the profiles of the staircases are quite similar along the entire cochlea.

Embryonic Cochleae

10-d Embryos. The staircase profile is not present at this stage, the stereocilia being all approximately the same length (Fig. 5). A kinocilium (see *arrows*, Fig. 5) extends from one margin of the bundle; it is more than twice the length and is twice the diameter of the stereocilia (14, 18).

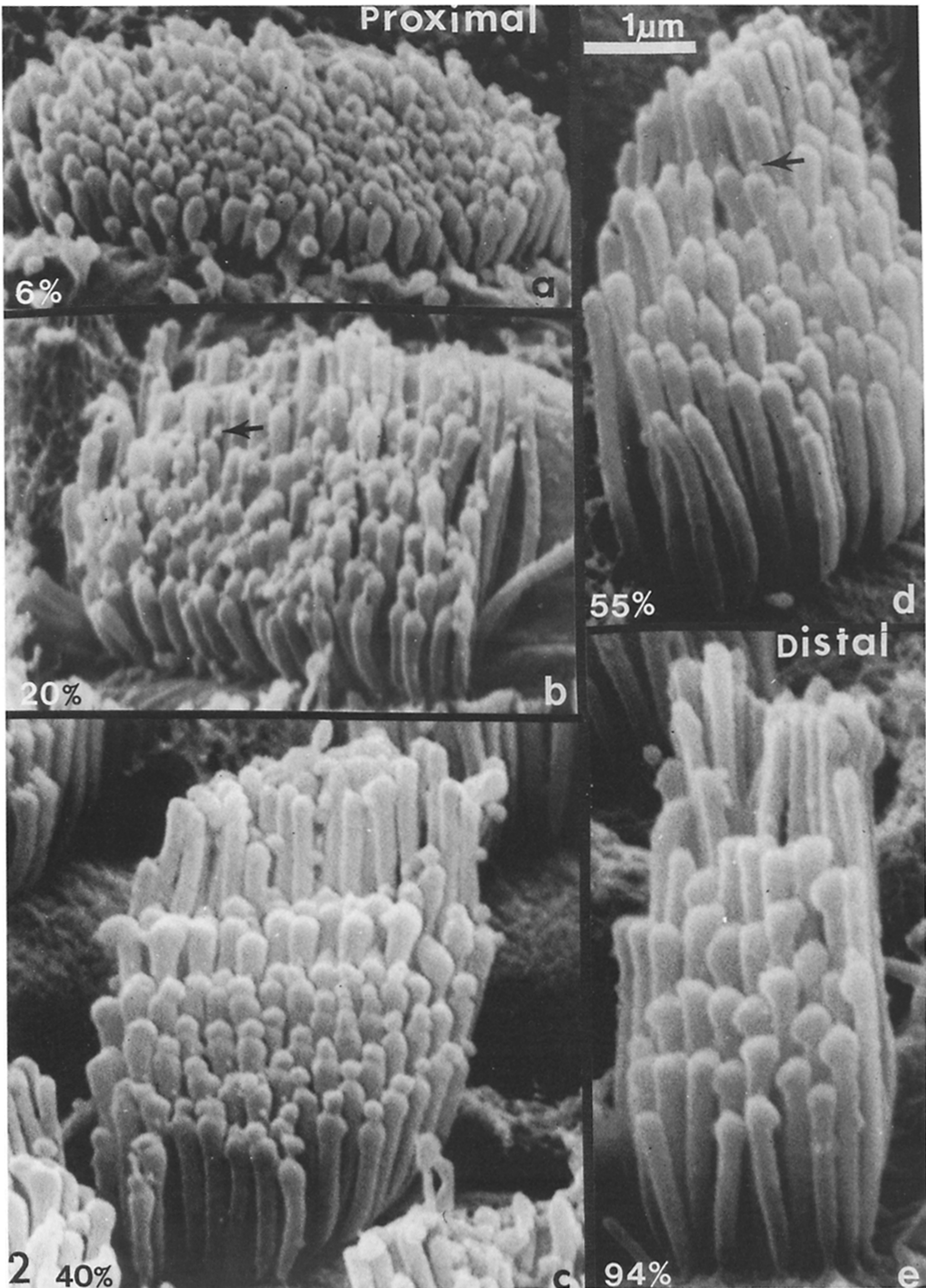
Since the stereocilia are about the same length as the microvilli of the supporting cells that encircle each hair cell, it is difficult to measure the absolute length of the stereocilia by positioning the ear so that we look across the surface as we did for the chick cochlea. What we can do is photograph cells where the stereocilia are splayed apart and lie nearly flat on the surface. Alternatively we can examine cells that for some reason during the preparation of the tissue have lost some of their stereocilia so others now become visible. Using both methods we find that the average length of the stereocilia is ∼0.43–0.54 μm. The fact that the stereocilia splay so readily and look poorly packed, although if they are shaved off the scars lie on a precise hexagonal lattice (18), indicates that the stereocilia are not connected or are poorly connected together by extracellular links.

10.5-d Embryos. The stereocilia situated nearest the kinocilium have begun to elongate at this stage (Fig. 5). These stereocilia will ultimately become the tallest row of stereocilia in the staircase. All the other stereocilia are approximately the same length as those in younger embryos. Thus the stereocilia that will end up being the longest begin to elongate before all the others.

The elongating stereocilia are also fatter than those that have not yet begun to elongate. This fact is consistent with earlier observations on the diameter of stereocilia encountered in 10–11-d embryos and results from the existence of large spaces between the poorly cross-bridged actin filaments within the stereocilia (13).

Also apparent in our micrographs is the fact that hair cells located at the extreme proximal and distal ends lag in their development slightly behind those in the central two-thirds of the cochlea (see Fig. 5). Again this observation is consistent with earlier descriptions of stereociliary growth (18).

11-d Embryos. The stereocilia on each hair cell can be divided into two groups, one large group located in front of the staircase, all of which are the same length, ∼0.3–0.4 μm, and the other in the developing staircase where the average length of the tallest row is ∼1.0 μm (18). In that portion of the bundle that comprises the developing staircase, it is difficult if not impossible to identify individual steps of the staircase, but as one progresses directly up the staircase one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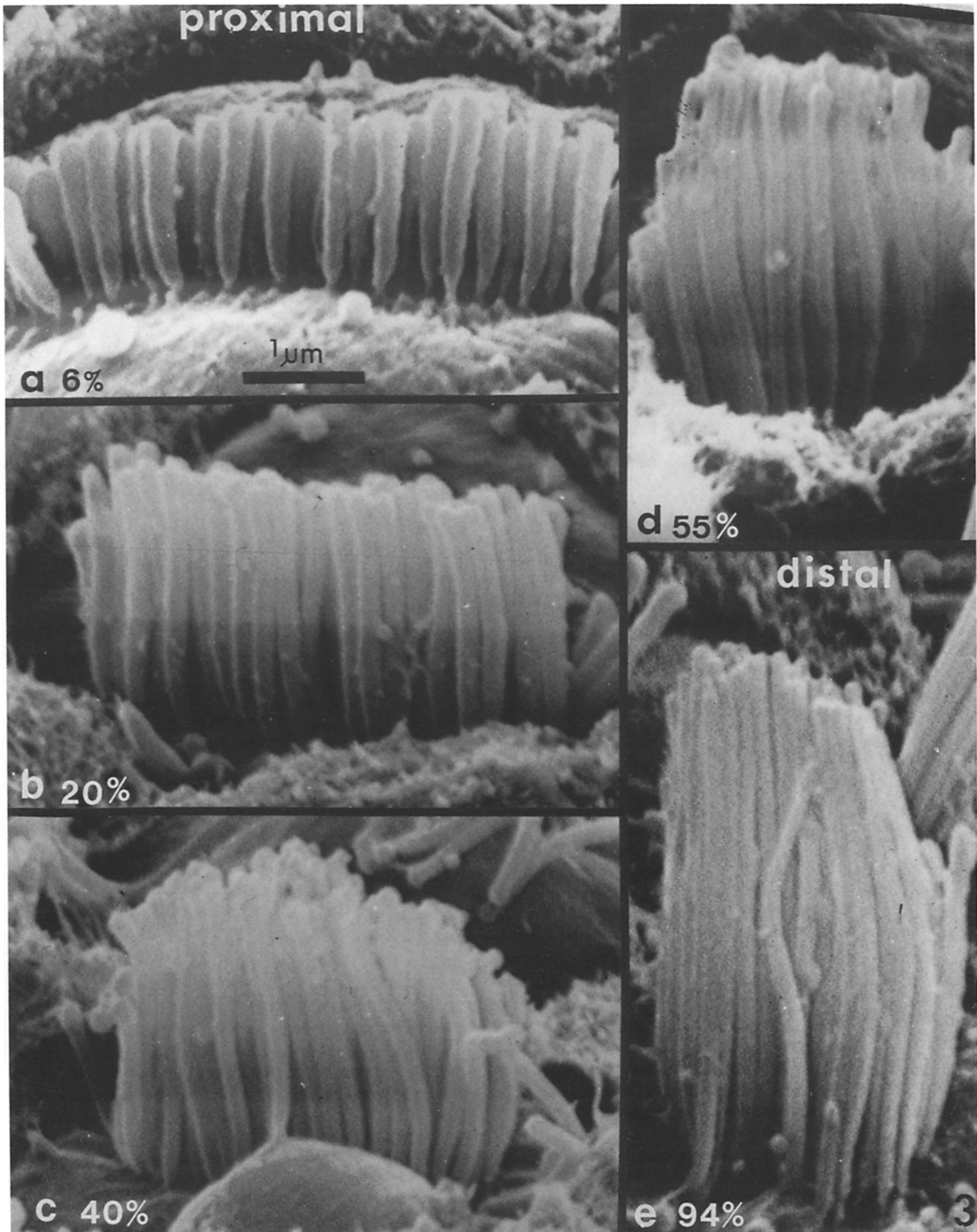


Figure 3. Representative views of the back of the stereociliary bundles of hair cells located at five positions along the cochlea. These positions are indicated in Fig. 2.

Figure 2. Representative frontal views of the stereociliary bundles of hair cells located at five positions along the cochlea printed at the same magnification. As one progresses down the cochlea the lengths of the stereocilia increase yet the number of stereocilia per bundle decreases. Of importance to this report is that the first step in the staircase is tiny in bundles located at the proximal end of the cochlea yet the height of this step increases progressively as one moves towards the distal end. The arrow indicates connections between the stereocilia of adjacent rows. Often the stereocilia appear swollen at this point. On the bottom of each photograph is the location of the hair cell indicated as the percent distance from the proximal end of the cochlea.

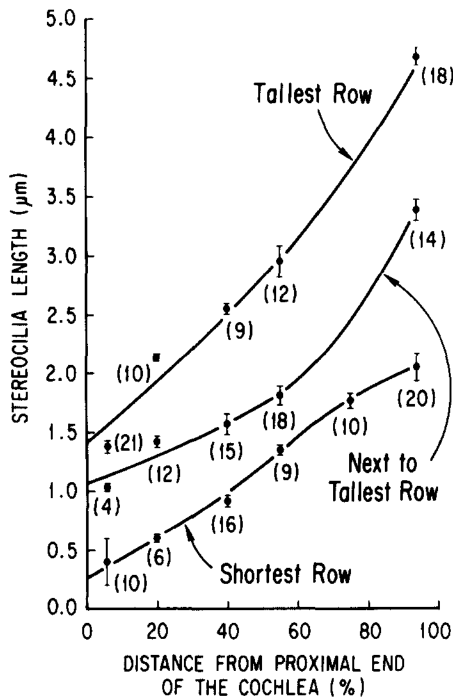


Figure 4. Graph illustrating the height of the first or shortest and last or tallest row in the staircase of hair cells located at progressive positions from the proximal end of the cochlea. These data were taken from the cochlea of a 15-d-old chick, the same cochlea from which the micrographs illustrated in Figs. 2 and 3 were photographed. Between these two lines is the height of the next to tallest row. To get this we measured the separation in height of the tallest row and the next to tallest from frontal views of the staircase and subtracted this value from the height of the tallest row as measured from micrographs of the back of the bundle.

encounters stereocilia of increasing height. When one does this one encounters two to five individual stereocilia or two to five rows of stereocilia. The number of rows depends upon the location of the hair cell and thus its maturity. Thus, at the distal end, because differentiation of the bundle is slower than over the rest of the cochlea, there are about two and toward the proximal end about five (Fig. 5). This should be contrasted to the situation in the adult in which we encounter seven to nine rows of stereocilia.

What is happening is that the stereocilia in the tallest row continue to elongate from the previous stage while those located in the next shorter row begin to elongate and so forth with what will be the shortest stereocilia in the staircase beginning to elongate last. Thus the staircase is formed by the sequential initiation of elongation of the stereocilia.

From frontal (Fig. 5), lateral, and backward views of the bundle we find that the stereocilia in the staircase proper are packed tightly as if bound together along their lengths while those in the front of the bundle stand up at varying angles as if they were not connected.

12-d Embryos. The staircase is much further developed at this stage so that if we walk up the staircase, we encounter seven individual stereocilia at the proximal end and three or four at the distal end where development is somewhat slower (Fig. 5, *far right*). In mature bundles we encounter seven to nine individuals so the staircase begins to display its mature profile. There is still a large population of short stereocilia at the front of the staircase. As mentioned in an earlier publication these short stereocilia will become resorbed (18).

From views of the backside of the staircase we find that the tallest row of stereocilia is ~1.5 μm long (18). This is the mature length of hair cell bundles at the proximal end of the cochlea. At the extreme distal end of the cochlea, because of the lag in development, the heights of the stereociliary bundles are slightly less than over the rest of the cochlea.

Impressive at this stage are the bundles at the proximal end of the cochlea that seem to have expanded laterally giving a rectangular outline, not the round one seen in earlier stages (cf. Fig. 5, *top* panels).

15-d Embryos. In the distal three-quarters of the cochlea the tallest stereocilia in the bundles increase in length from 1.5 μm in 12-d embryos to 2.2 μm in 17-d embryos, most of the increase occurring in 12-, 13-, and 14-d embryos. In the proximal one-third of the cochlea the stereocilia do not elongate at all as they have reached their mature length in 12-d embryos and have stopped elongating as was originally stated by Tilney et al. (18). The increase in length of the stereocilia in the distal three-quarters of the cochlea is particularly interesting because what is occurring is that all the stereocilia in the staircase are elongating, not just the taller rows (Fig. 6). At the same time the stereocilia in front of the first step have been resorbed; in their place is a smooth membrane surface.

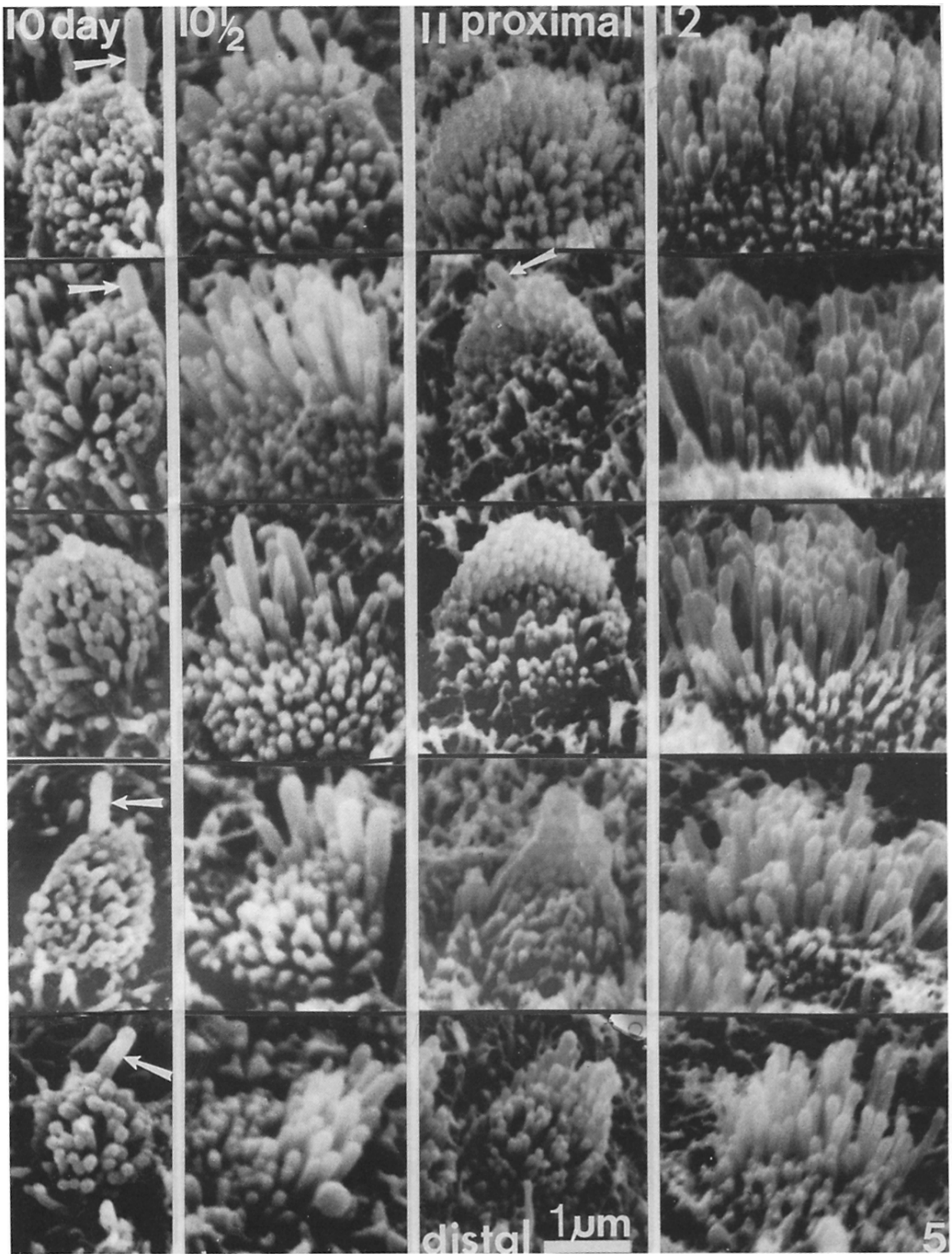
Thus between 12 and 15 d of incubation, the difference in length between the shortest step and the tallest step in the staircase (except at the extreme distal end where development is a little slower) is qualitatively approximately constant, i.e., the angle the staircase profile makes with the surface of the hair cell is a constant. Therefore all the stereocilia in the bundle must elongate approximately the same amount.

We tested the above statement quantitatively by comparing graphs of the length of the shortest stereocilia in the bundle (from front views) with graphs of the longest stereocilia (from views of the back side of the bundle) (Fig. 7). These curves tend to be approximately parallel showing that indeed the difference in length between the longest and shortest rows remains nearly constant.

Further inspection of Fig. 7 shows us that the first step in the staircase progressively increases in length as we go from the proximal tip of the cochlea towards the distal end until we have reached a position three-quarters of the way along the cochlea. At this point the length remains constant or even decreases somewhat. The latter is due to the greater immaturity of the hair cells located at the distal end.

The stereocilia in each bundle are tightly packed as if connected (Fig. 6, *left column*). In certain places tip linkages can be seen between the tip of one stereocilium and the side of

Figure 5. Scanning electron micrographs of frontal views of stereociliary bundles located at five approximately equal positions along the cochlea of 10-, 10.5-, 11-, and 12-d embryos. The bundles from hair cells at the proximal end of the cochlea are at the top of the figure with those at the distal end at the bottom. All the micrographs are at the same magnification. The arrows point to the kinocilium. In some bundles the kinocilium is absent, possibly broken off when the tectorial membrane was removed.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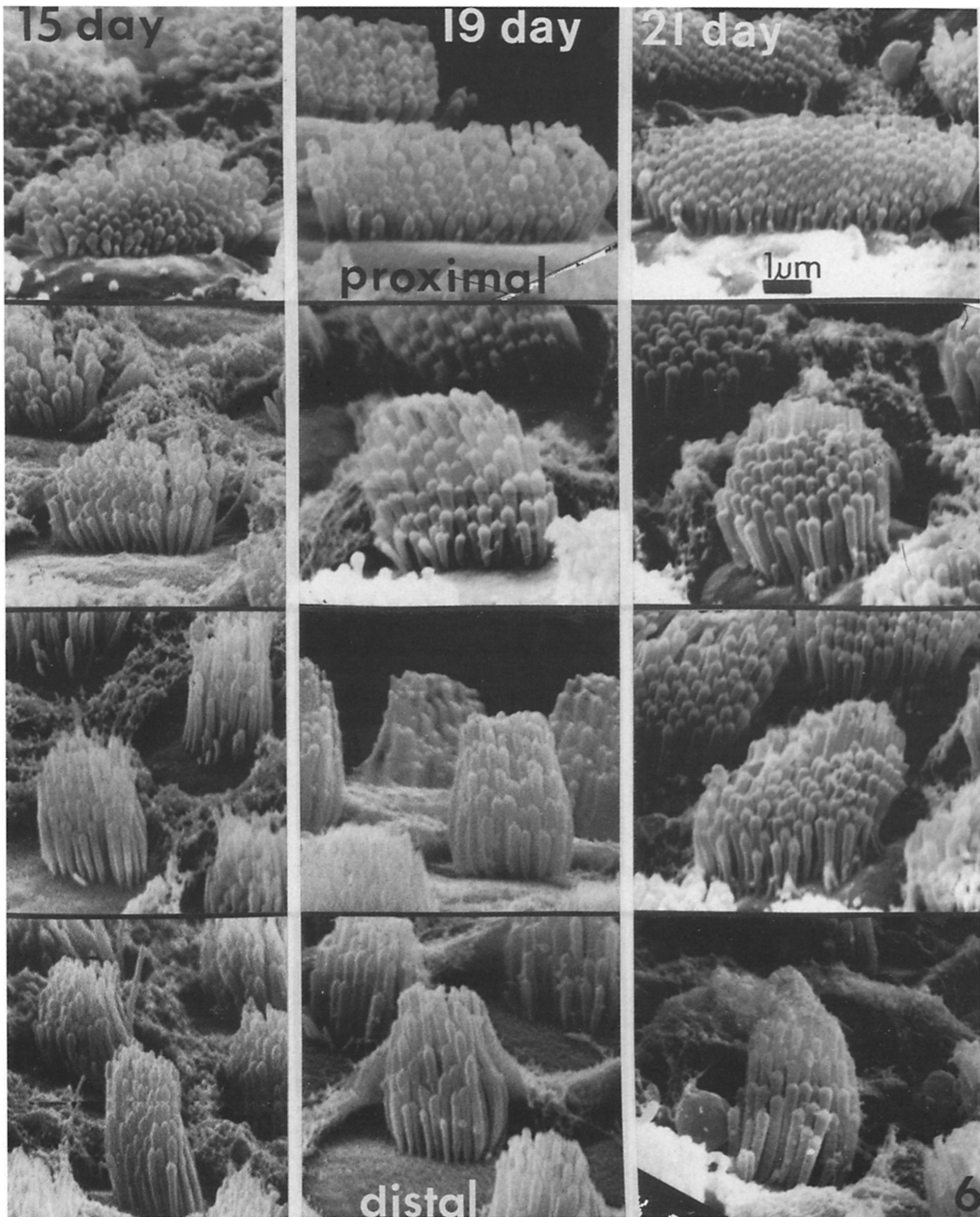

Figure 6. Scanning electron micrographs of frontal views of stereociliary bundles located at four approximately equal positions along the cochlea from 15-, 19-, and 21-d embryos. The bundles from hair cells located at the proximal end of the cochlea are at the top of the page with those at the distal end at the bottom. All micrographs are depicted at the same magnification.

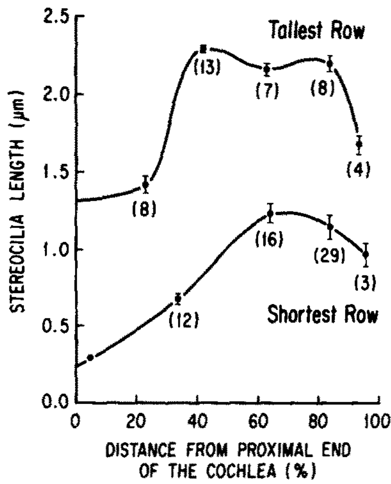

Figure 7. Graph illustrating the height of the first and last step of the staircase of a 15-d embryo plotted as a function of the percentage of distance the stereociliary bundle (hair cell) is from the proximal end of the cochlea.

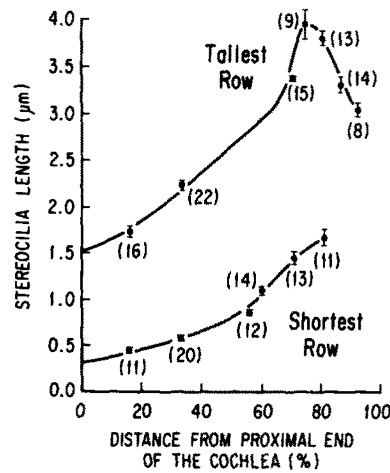

Figure 8. Graph illustrating the height of the first and last step of the staircase of a 19-d embryo plotted as a function of the percentage of distance the stereociliary bundle is from the proximal end of the cochlea.

the next longest one above it, but it is not clear how general they are.

19-d Embryos. The stereociliary bundles located on the proximal one-third of the cochlea have attained their mature lengths and have ceased elongating. Those on the distal two-thirds have changed in a complex way. First, all the stereocilia, including those on the first step and the last step of the staircase, have elongated (Figs. 6 and 8). Second, what is unexpected is that there has been more of an increase in the length of the tallest row of stereocilia relative to all the other rows. Accordingly a comparison of the length of the tallest row of stereocilia with that of the shortest row plotted as a function of the position reveals two curves that diverge progressively more and more as one travels toward the distal end (Fig. 8). Thus (except at the extreme distal end where the hair cells, by being more immature, are slower in their differentiation), unlike the situation in 15-d embryos in which the curves remain relatively parallel to each other indicating that all the stereocilia are elongating at the same rate and/or the same time, in 19-d embryos the tallest row of stereocilia is increasing more than the shorter rows (Fig. 8). These changes are most pronounced at the distal one-third of the cochlea (except at the extreme distal end) with, as already stated, minimal changes occurring at the middle one-third, and none at the proximal one-third. Tip linkages are now commonly seen.

21-d Embryos (Hatchlings). At this stage the stereociliary bundles, except those near the distal tip of the cochlea, resemble those in mature hens and roosters. What is significant is that in the distal one-third of the cochlea the tallest rows of stereocilia have continued to elongate after the preceding stage (Fig. 6, *right column*) showing an obvious divergence in the curves of the length of the tallest and shortest rows (Fig. 9). By carefully comparing hair cells at the distal end of the cochlea in embryos of 15, 19, and 21 d (Fig. 6) it is clear that even though the shortest row has stopped elongating by 19 d, the longest and next to longest rows continue to elongate with only the longest row elongating in 21-d embryos. Therefore what seems to be occurring is a sequential cessation of elongation with the shortest row finishing first followed by the cessation of successively taller rows.

Tip linkages between adjacent stereocilia are apparent (Fig. 6).

Discussion

The formation of the staircase occurs in four successive stages (see Fig. 10). During the first stage, 8–10-d embryos, stereocilia that are all the same length sprout from the apical surface of the hair cells. In the second stage, 10–12-d embryos, those stereocilia located on the margin of the bundle nearest the kinocilium begin to elongate and as the embryos get older and older successive rows of stereocilia initiate

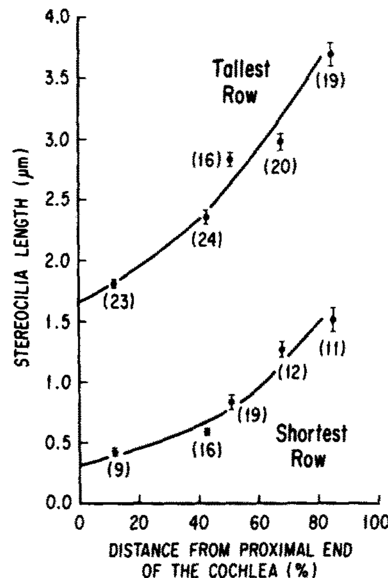

Figure 9. Graph illustrating the height of the first and last step of the staircase of a 21-d embryo plotted as a function of the percentage of distance the stereociliary bundle is from the proximal end of the cochlea. Note that the slopes of these curves are not the same but diverge progressively toward the distal end of the cochlea. Thus at the proximal end the difference in length between these two rows is 1.4 µm, while at the distal end the difference is 2.2 µm, almost a twofold difference.

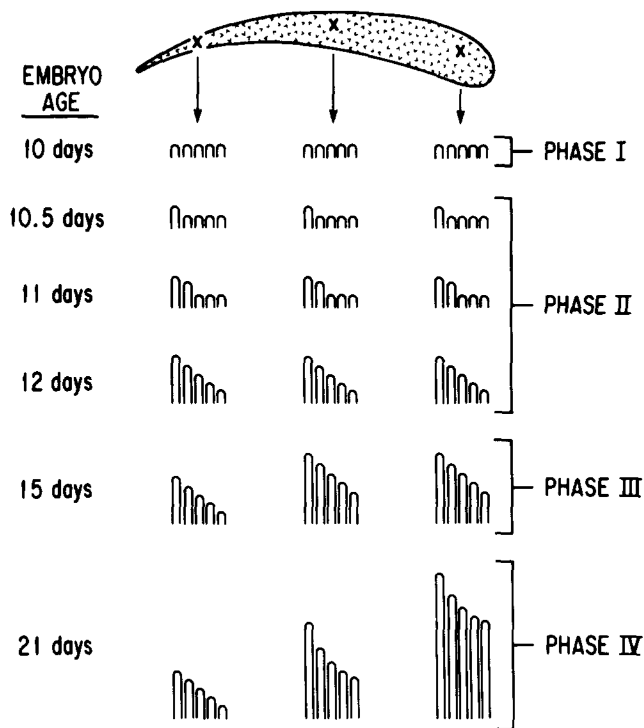

Figure 10. Illustration of the four phases in the formation of the staircase. Note that during the final phase the tallest row is taller than the next tallest by a greater increment than that between the other rows in the staircase, except at the proximal end of the cochlea.

elongation. Thus during the second phase the staircase is set up by the sequential initiation of elongation of stereocilia located further and further away from the margin that began elongation. In the third phase, 12-17-d embryos, all the stereocilia in the newly formed staircase elongate until they have attained their prescribed lengths at which point they cease elongation. This leads to bundles in which the tallest and shortest rows are separated by a constant distance even though the height of the shortest (and thus the tallest) row increases as one approaches the distal end. During the final stage, 17-d embryos to hatchlings, the stereocilia in the tallest rows become considerably longer than those in the other rows. This is due to the fact that those stereocilia in the first step cease to elongate first, followed by cessation of elongation of taller and taller rows with the tallest row finishing last. This leads to a greater increment in height in the tallest row relative to the next tallest row than between the shortest row and its next tallest row (Fig. 10).

Since all the hair cells on the cochlea begin each stage at approximately the same time, overall differences in stereociliary length are related directly to when a cell ceases to elongate its stereocilia further. Thus hair cells at the extreme proximal end of the cochlea do not change their stereociliary lengths after phase II (by day 12), hair cells in the remainder of the proximal one-half of the cochlea do not elongate after phase III (by day 15), and hair cells at the extreme distal end complete their differentiation at the end of phase IV (newly hatched chicks).

These observations indicate that not only is the staircase formed in a series of stages, but also there is a proximal to distal component that contributes to the "stop" signal. Furthermore, as demonstrated in the first paper in this series (14), during the last two phases stereocilia appear de novo on the lateral sides of the staircase while others are disappearing at the foot of the staircase. We do not know if these elongate in phases or appear and quickly elongate to the proper lengths. This will be difficult to pin down as we have no reliable means of determining accurately all the stereocilia that form de novo.

It is difficult to differentiate rigorously between different rates of elongation of stereocilia in a bundle from the progressive turning on or off of "elongation signals." We believe it is the latter because early in phase II no growth is seen except in the stereocilia on the margin of the cell nearest the kinocilium and as progressive rows elongate, no elongation seems to occur in the population at the foot of the staircase. A comparison of the stereociliary bundles of embryos of increasing age shows that during phase IV, particularly at the distal end of the cochlea, there is an obvious change in the length of the tallest row with no noticeable change in the shorter rows.

In an earlier publication (13) we demonstrated that actin filaments are present in the stereocilia at all the stages in their growth; these filaments extend from the tips of the stereocilia into the apical cytoplasm (cuticular plate region). Evidence has been presented that these filaments are important in giving stereocilia their rigidity (8, 16). These data, coupled with with data on the role of actin filaments in the elongation of cell projections in other systems (see 15 for references) direct our attention to the assembly of actin filaments in controlling the elongation of the stereocilia. Thus the real issue for future investigation is to study how the assembly and cessation of assembly of the actin filaments is controlled.

A reasonable way to control the assembly of filaments in each stereocilium and at the same time inhibit disassembly is to have a substance located at the ends of the actin filaments that, under physiological control, "caps" the filaments at one stage and is inactivated at other stages. This appears to be an important mechanism in cells because barbed end (the end situated at the tip of the stereocilium) "cappers" have now been described in a variety of cell types (1, 2, 4-6, 9, 11, 12, 19). Many of these substances drop off the barbed end in response to changes in calcium so it is conceivable that, in the cell, regulation of capping can be controlled ionically. If this idea is correct, one would predict that in stage 1 the hypothetical caps must be present on all the stereocilia, in stage 2 they must be present except on the elongating stereocilia, in stage 3 they must be unattached from all the elongating stereocilia, in stage 4 they must attach first to the shorter stereocilia in the bundle and progressively become attached to the taller and taller ones.

Although we are reluctant to speculate further as to how the hypothetical caps might inhibit elongation of some stereocilia but not others on the same cells, we will mention a scenario of how such a system might operate as it will show how our results could be integrated into a simple model. Such a model might stimulate others to do experiments in an area that we think might be fruitful. Our scenario is as follows: The sequential initiation of elongation of the stereocilia that occurs during phase II might be mechanical. It is known that in 8-10-d embryos the kinocilium becomes connected to the overlying yet differentiating extracellular layer or tec-

torial membrane (3). This attachment appears to put a strain or shear on the kinocilium (3). Beginning in 10.5-d embryos the tallest row of stereocilia begins to elongate and at the same time they, unlike the rest of the stereocilia, appear to be in close apposition to each other and to the kinocilium as if connected. The mechanical strain on the kinocilium along with connections between the kinocilium and the row of stereocilia nearest the kinocilium in turn might mechanically induce the opening of channels as outlined by Hudspeth (7), which in turn might liberate specific ions into what will be the tallest row of stereocilia, which in turn induce the cap to be released. Actin filament elongation could then ensue. Shortly thereafter the next row of stereocilia becomes connected to the tallest row by tip linkages which would in turn lead to the opening of channels in these stereocilia as well as those in the tallest row and thus actin assembly can occur in both rows. Then the next row may be connected and so forth. We should emphasize however that this is only a model, but it demonstrates that controls on the formation of the staircase could be simple and straightforward.

The overriding generalization that the formation of the staircase occurs in discrete stages that are separated by developmental time is consistent with the observations presented in earlier papers in this series, namely that the elongation of stereocilia occurs at a different time from the increase in width of the stereocilia and the elongation of the rootlet filaments (and cuticular plate) (13, 18). These combined observations then show us that the staggering complexity in the organization of the hair cells on the cochlea is a product of a series of events that build upon each other to produce a population of cells whose differences and similarities can be understood at least in a superficial way. Furthermore, since each event occurs in hair cells located throughout the cochlea, it is possible to dissect out what controls each event. The generalization that is emerging is that although positional information is clearly important, when things occur is equally significant. This temporal information is not absolute but relative, that is the embryo completes one step, then it carries out another using the pattern that resulted from the first and so forth. Thus in our case the formation of a staircase occurs in four successive stages, two of which are controlled by signals that turn on or off elongation and two of which involve elongation of all the structures present. These temporal stages are in turn related to the location of the hair cell on the cochlea (positional information) because at the extreme proximal end of the cochlea the last phase is over in a 12-d embryo and the third phase never occurs, while at the distal end the last phase does not begin until the embryo is 15 d old and is not complete until well after hatching.

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