The Structure of the Cuticular Plate, an In Vivo Actin Gel

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Abstract. The cuticular plate is a network of actin filaments found in hair cells of the cochlea. In the alligator lizard, it consists of rootlets, emanating from the stereocilia, and of cross-connecting actin filaments that anchor these rootlets. In thin sections, this network displays striking patches of 650 ± 110-Å striae. By quantitative analyses of the images, the mystery of the striae can be explained. They are due in part to the rootlets which are sets of flat ribbons of actin filaments. The ribbons in each set are separated by ~650 Å. Numerous whiskers 30 Å in diameter extend from each ribbon's face, interconnecting adjacent ribbons. The nonrootlet filaments, except at the margins of the cell, occur primarily as single filaments. Like the ribbons, they are bristling with whiskers. The patches of striae are explained by ribbons and filaments held at a 650-Å separation by the whiskers that project from them. A simple model for regions of bewhiskered filaments is a box crammed full of randomly oriented test-tube brushes. A thin slice through the box will show regions of dark lines or striae due to the wire backbones of the brushes separated from one another by the bristle length. Using the computer instead of test-tube brushes, we have been able to model quantitatively the filament distribution and pattern of striae seen in the cuticular plate of the lizard. The organization of actin filaments we have deduced from our simulations differs from that found in macrophages or in the terminal web of intestinal epithelial cells.

Hair cells are transducers that convert motion into changes in membrane potential. They are the sensors used for hearing, balance, and, in fish, for the detection of water currents. The design of all vertebrate hair cells is generally the same. It is a columnar cell having a bundle of large microvillus-like projections, called stereocilia, on its apical surface (Fig. 1; for review see Roberts et al., 1988). In the alligator lizard there are ~75 stereocilia per cell. The stereocilia in a bundle are of different heights and are organized like the pipes in an acoustic organ, with the tallest at the back and the shortest at the front. Also, like organ pipes, each stereocilium has a pencil-point taper at its base (Mulroy, 1974). Each stereocilium contains an actin bundle (Flock and Cheung, 1977; Tilney et al., 1980) encased in the cell membrane like fingers in a glove. In the alligator lizard, the tallest stereocilia are >30 μm long with a core of ~8,000 actin filaments (Tilney et al., 1980). These actin filaments in the stereocilia, at least in birds and mammals, are cross-bridged by an actin-binding protein called fimbrin (Flock et al., 1982; Slepecky and Chamberlin, 1985).

At the base of each stereocilium, where it tapers, the number of filaments sharply falls off, leaving a small bundle of ~20 filaments that forms a rootlet that enters the body of the cell. The rootlets are embedded in a dense matrix, the cuticular plate, which serves to anchor the stereocilia and hold them erect (Tilney et al., 1980). By immunofluorescence and electron microscopy, the cuticular plate in mammalian hair cells appears to contain actin, α-actinin, tropomyosin, fimbrin, myosin, and fodrin (Scarfone et al., 1988; Sobin and Flock, 1983; Drenckhahn et al., 1985).

While hair cells have structural similarities to the brush border and contain some of the same proteins (e.g., actin, fimbrin, myosin, and tropomyosin), there are important differences. The typical cell in the brush border has, extending from its apical surface, numerous microvilli, each of which has at its core a small bundle of actin filaments. The microvilli are ~2 μm long by 1,000 Å wide, much shorter and narrower than stereocilia. The actin bundle extends from the core of the microvillus into the body of the cell as a rootlet. Here the rootlets are enmeshed in a network of filaments known as the terminal web. Adjacent rootlets are joined by myosin and spectrin-like proteins, called TW 260/240. At the base of the rootlets are 10-nm filaments (for review see Burgess, 1987). In contrast, in the cuticular plate, the rootlets are embedded in a meshwork or gel of actin filaments.

Determining the structure of the cuticular plate poses a problem. Not only does it lack any symmetry but it also lacks a fixed structure: cuticular plates are not superposable filament for filament. In what sense, then, can we say we have determined or solved its structure and how do we know our solution is correct? Hartwig and his collaborators (Hartwig et al., 1980; Niederman et al., 1983; Hartwig and Shevlin, 1986) have measured angular relationships between neighboring filaments in the cortical gel of the macrophage. Such distributions describe some aspects of filament organization.
Figure 1. Thin section of the apical portion of an intact or unextracted hair cell. Portions of the supporting cells (SC) are seen on either side. The distinguishing feature of the hair cell is its bundle of stereocilia (S), which are large compared with the microvilli (MV) of the supporting cell. Rootlets (R) extend from the stereocilia into a dense meshwork, the cuticular plate (CP). Note that mitochondria (M) and other organelles appear excluded from this region. This image is reprinted from Tilney et al. (1980). Bar, 1 μm.

One test of a solution, therefore, is its ability to generate measurable aspects of the filament distribution. Another test of a solution is its ability to reproduce the patterns seen in the micrographs as was done for actin bundles found in stereocilia (DeRosier et al., 1980). In this study, we constructed a Monte Carlo computer program based on a set of rules describing the relationship between filaments and showed that the output of this program correctly describes the distribution of filaments and generates the curious pattern of striae.

Materials and Methods

Specimen Preparation and Electron Microscopy

The organs of corti (cochlea) from the alligator lizard (Gerrhonotus multicarinatus) were fixed intact or after detergent extraction or after permeabilization in Triton and incubation with the SI fragment of myosin (Tilney et al., 1980). In this study, we constructed a Monte Carlo computer program based on a set of rules describing the relationship between filaments and showed that the output of this program correctly describes the distribution of filaments and generates the curious pattern of striae.

The Angular Distribution of Filaments

In an electron micrograph of a thin section of an actin gel, the distribution of filament lengths contained in the section provides information about angular distribution of filaments. Filaments running perpendicular to the plane of the section appear as dots (i.e., have a projected length equal to zero). Those running at 45° have a projected length equal to the section thickness and so on. If one assumes the filaments are very long (i.e., all the ends seen in the section are produced by the act of sectioning) and that the filaments can take up any angular orientation, then we can analytically describe the distribution of segment lengths:

\[ P(\theta) = \sin \theta, \]

where \( P \) is the probability of a filament at \( \theta \), the angle of the filament relative to a line perpendicular to the plane of the section, and

\[ \sin \theta(l) = \frac{l}{\sqrt{l^2 + t^2}}, \]

where \( l \) is the projected length of the segment of filament in the section, \( t \) is the section thickness, and \( \sqrt{l^2 + t^2} \) is the true length of the filament segment.

The probability of a projected length, \( l \), given \( P(\theta) \) is

\[ P(l)dl = P(\theta(l)) \sin \theta(l) \frac{dl}{dl}; \]

\[ \frac{dl}{dl} = \frac{l}{\sqrt{l^2 + t^2}} \quad \text{(from Eq. 2)}; \]

\[ P(l)dl = \frac{l}{(l^2 + t^2)^{3/2}} dl. \]

This has a maximum at \( l = \frac{t}{\sqrt{2}} \). Thus, the distribution of lengths can also be used to determine section thickness.

Before continuing, there is one other consideration needed. We count numbers of filaments and generate arguments based on these counts. We wish to know how the number of filaments varies with section thickness. Thus, if we compare two sections having slightly different section thicknesses, how much of an error will be made?

Let \( N \) be the number of filaments in a section of area, \( A \), and thickness, \( t \). Let \( \rho \) be the density of actin in numbers of subunits per unit volume and let \( \delta \) be the distance between subunits along the filament axis. Then, the number of actin subunits in the filament segment of length \( (l^2 + t^2)^{1/2} \) is

\[ \frac{(l^2 + t^2)^{1/2}}{\delta}. \]

For the \( N \) filaments having a distribution \( P(l) \) (Eq. 5), the number of actin subunits is

\[ N \int P(l) \frac{(l^2 + t^2)^{1/2}}{\delta} dl. \]

From the density, \( \rho \), the number of subunits is also equal to \( \rho A \delta \); hence, we generate the following equation:

\[ N \int P(l) \frac{(l^2 + t^2)^{1/2}}{\delta} dl = \rho A \delta. \]

The limits of integration are 0 and \( l_{\max} \). As an approximation, we set \( l_{\max} = \sqrt{A} \). The solution is

\[ N = \frac{\rho A \delta}{\frac{l}{2} \log_e \left( \frac{A}{t} + 1 \right)}. \]

A small error \( \Delta t \) in \( t \) results in an error \( \Delta N \) given by

\[ \Delta N = \frac{2A}{(\frac{A}{t} + 1) \log_e(\frac{A}{t} + 1) \Delta t}. \]

For thin sections, where \( A/t^2 >> 1 \),

\[ \Delta N = \frac{l}{\log_e(\frac{A}{t})} \Delta t. \]

If, for example, \( \Delta t/t = 0.2 \) (20% error in section thickness) and \( \sqrt{A/t} = 10 \) (linear dimension of the area considered is 10 times the section thickness), then

\[ \Delta N/N = 0.2 \times \frac{l}{\log_e(10)} = 0.09 \]

(i.e., a 9% error in number filaments). Thus, variations in section thickness do not produce large errors in filament number.
Computer Modeling of the Angular Distribution of Filaments

We defined in the program a box (corresponding to a portion of a thin section) having the length and width proportional to the region of filaments analyzed in Fig. 10. We allowed the depth of the box (i.e., thickness of the section) to be a variable. We placed cylinders (corresponding to the rootlets seen in Fig. 10) at the long edges of the box. Above and below the center of the box we also placed two cylinders (corresponding to the two rootlets above and below the plane of the sections) as required by the hexagonal packing of rootlets in the cuticular plate. Using the random number generator, we then placed a line (corresponding to a nonrootlet filament) at a random position and orientation within the box. If it missed hitting all four cylinders (rootlets), (i.e., did not come within 900 Å of the center of any of them), we recorded its angle and projected section length in the histograms. One million filaments were tried to generate a reasonably accurate histogram. We compared calculated distributions having different depths (section thicknesses) with observed distributions using the $x^2$ test: the best depth was that which minimized $x^2$. The value for $x^2$ is determined as follows:

$$ x^2 = \sum_{i=1}^{n} \frac{(m_i - M_i)^2}{M_i}, $$

where $m_i$ is the number of observations (i.e., filaments of a particular length) in the $i$th bin, and $M_i$ is the number expected in that bin according to a particular model (Meyer, 1975).

To carry out the $x^2$ tests, we needed to adjust the bin sizes in the histograms (Cochran, 1954; Roscoe and Byars, 1971). In particular for length, we used a bin size of 200 Å and, since there are so few long filaments, we simply lumped all those >2,000 Å into a single bin. For the histogram of orientation, we used a bin size of 30°. With smaller bins, each bin had fewer entries, and the $x^2$ test could not distinguish between the two models. This makes sense since the more the precision (i.e., the smaller the bin), the more data that is needed to determine the value.

Computer Modeling of the Patterns of the Cuticular Plate

The program is a modified version of that described above. The first filament is placed at random in the box. To densely pack filaments as they are in the cuticular plate, we attached each succeeding filament to one already present. To do so we selected a point along an existing "filament" at random subject to the condition that the point was inside the box. We then chose a random angular orientation for the new filament and positioned the new filament by displacing its origin 650 Å from the site chosen on the existing filament. The direction of displacement lay in a line perpendicular to the existing filament and to the new filament. Finally, we checked to see if the new filament collided with any existing filaments or rootlets. If not, that segment of the filament within the box was drawn. To give the effect of filament flexibility, we only consisted collisions within the box. We ignored collisions outside the box, reasoning that flexibility generates roughly the same effect.

To display the filaments and the 30-Å struts (whiskers) emanating from them (see Discussion), we represented each actin filament as a pair of twin flexible, radial struts were attached at points spaced every 27.3 Å along the axis of the strands. To best mimic the features of the micrographs, the filaments and whiskers were not drawn as solid lines but were divided into short segments. About half the segments chosen at random were plotted to give a kind of stippled effect.

Formulæ Relevant to the Program

These were derived in a straightforward, if tedious, manner. Only the answers are given. Two lines having general formula

$$ x = ar + b, \quad y = cr + d, \quad z = er + f $$

have a distance of closest approach at $r_0$ and $r'_0$:

$$ r_0 = \frac{(c'[b - b'] + e'[d - d'] + f'[p - f])(aa' + cc' + dd') + (aa' + cc' + dd')^2}{(aa' + cc' + dd')^2} \cdot \frac{a[b - b'] + c[d - d'] + e[p - f]}{(aa' + cc' + dd')} - \frac{1}{2}.$$
These filaments, which are rootlet filaments, are all polar, with their pointed ends pointing away from the cell’s apical surface (Fig 3 b, inset) like the filaments in the stereocilia. Between the rootlets, there are numerous single filaments decorated with S1. There are no undecorated long filaments in the cuticular plate.

In decorated preparations, no stacks of striae are evident and the wispy material (whiskers) running between striae is gone, as if the decoration procedure displaced it. Only occasionally does one still see the odd whisker running between filaments.

**Rootlets Consist of Ribbons of Actin Filaments**

A comparison of longitudinal sections with cross sections at high magnification shows that some of the prominent 650-Å stacks of striae consist of planar ribbons of actin filaments separated by 150 Å in one direction and 650 Å in the other. The 650-Å spaces are filled with the whisker-like material. The stacked ribbons are best seen in off-longitudinal sections (Fig. 4) and in cross sections (Fig. 5).

Note that in Fig. 5 the arrangement of rootlet filaments changes with position in the cuticular plate. Close to the api-
cal surface (in this case, the upper left corner) rootlets often appear to be a thin annulus or ring of filaments. Deeper into the cuticular plate, these rings become two or more ribbons of filaments separated by \( \sim 650 \) Å (Fig. 5d). In the ribbons, which appear as rows of dots viewed end-on, the interfilament spacing is \( \sim 150 \) Å.

At the Borders of the Plate Some Filaments Are Also Arranged in Ribbons

On occasion, at the margins of the cuticular plate, we find extended regions of ribbons in both longitudinal sections (Fig. 6) and cross sections (Fig. 7). The position, angle, and extent of these lateral ribbons show they are unlikely to be rootlet ribbons. First, they are at the wrong position. Second, they are close to and parallel to the apical surface of the cell, unlike rootlet filaments that run roughly perpendicular to the apical surface. Third, there are too many filaments and ribbons to be a rootlet (compare with Fig. 4 and Fig. 5).

The spacings in these lateral ribbons are the same as the rootlet ribbons. The interribbon spacing is \( \sim 650 \) Å, and the interfilament spacing is \( \sim 150 \) Å. Thus, the nonrootlet ribbons have the same organization as the rootlet ribbons.

The Presence of Ribbons between Rootlets Can Be Excluded by Tilting

Patches and stacks of striae that are not derived from rootlets are found between rootlets (Fig. 2). The spaces between rootlets are filled with actin as shown by SI decoration (Fig. 3). Is this interrootlet actin arranged in ribbons that generate the patches and stacks of striae? In decorated or undecorated
preparations, we never find ribbons between rootlets. It may be, however, that decoration destroyed the ribbons and that ribbons were present in undecorated preparations but were in the wrong orientation to be seen, as exemplified in Fig. 7 c. To check this possibility, we used the tilting stage in the electron microscope to explore other directions of view. Fig. 8 shows part of a tilt series about two axes using two serial sections in a region where rootlets are easily seen. The rootlets remain recognizable in all views, although the strong 650-Å striae are nearly gone in the −40° view (Fig. 8 a). Tilt series from −40° to +40° were done on many such sections, but in no case were ribbons found between rootlets.

The Patches and Stacks of Nonrootlet Striae Arise from Single Filaments

The idea behind this section is as follows. The space between rootlet ribbons is occupied by patches or stacks of striae. If each such stria is a single filament, the ratio of the density of striae to the density of filaments will be one. If the ratio is much greater than one, say four, the result argues that each stria is a ribbon having on average four filaments.

To determine the filament density, we used micrographs of a decorated preparation. Fig. 9 c shows a section of a decorated preparation taken perpendicular to the rootlets. The nonrootlet filaments are easily picked out and counted. Fig. 9 d shows a tracing of the filaments seen in Fig. 9 c. Data for several regions are shown in Table I.

Fig. 9 a shows a similar area to Fig. 9 c but in an undecorated, extracted preparation. Three rootlets seen in cross section can be used to mark the points corresponding to the region of the cuticular plate mapped in Fig. 9 d. We now wish to count striae. What we see are short, irregularly shaped, elongated features rather like a short series of dots with arms. These are striae. There are also other regions containing just fine wisps that we presume are just whiskers belonging to striae that are outside the plate of the section. It is difficult to justify in each case our selection as to what is a bona fide stria and what is not. Some cases seem quite clear, others do not. Our attempt to identify striae is shown in Fig. 9 b. The numbers of striae for several areas and their densities are given in Table I.

The first result is that the distribution of striae (Fig. 9 b) looks qualitatively like that obtained from a decorated preparation (Fig. 9 d). Note that numbers and lengths of filaments in decorated preparations look similar to those of striae in undecorated preparations. Second, from Table I, the ratio of the density of striae to that of filaments is 1.1 ± 0.2. Errors in section thickness are not likely to change the ratio significantly (see Materials and Methods). Thus, there is about one filament per stria, and the striae must correspond to single filaments and not to ribbons of filaments.

The Distribution of Nonrootlet Filaments (Striae) Is Not Random

Although we think we can identify filaments in thin sections of extracted preparations, for quantitative work it is easier and more certain to identify them in decorated preparations where the confusion due to the whiskers is absent. We have presented data in the previous section to show that the density of filaments appears unaltered as a result of decoration. The pattern of 650-Å striae, however, does disappear on decoration. Presumably the precise interfilament spacing is not maintained in the absence of whiskers. While the interfilament spacings may vary a few hundred angstroms, the filament orientation can only vary slightly since the filaments are trapped in a dense meshwork. Since the orientation of filaments will be little changed by decoration, the distribution of lengths and angles seen in sections is also unchanged.

In thin sections of decorated preparations (Fig. 10), we measured the lengths and angular directions of the nonrootlet filaments. The lengths are not the true lengths of the filaments but, instead, are the projected lengths of the segment of the filament cut out in the sectioning process. Histograms of the projected lengths and orientations are shown in Fig. 11, a and b.

We began our modeling attempts by assuming that nonrootlet filaments take up completely random orientations. Using a Monte Carlo computer program, we generated a distribution expected for random filaments (Fig. 11, a and c). We adjusted the section thickness to obtain a minimum for χ². The best fit is to a thickness of 300 Å, which seems too low. As judged by χ² tests, the calculated distributions for the best fit did not fit the observed ones. For the length distribution, χ² = 71, which fails at the 1% level with nine degrees of freedom. For the angular distribution (Fig. 11 c), χ² = 10, which fails at the 5% level with four degrees of freedom. A careful analysis of Fig. 11 c (solid line) shows the reason this model fails the χ² test: there is a slight bias in favor of nonrootlet filaments running parallel to rootlets.
This is seen in the peak at $\sim 30^\circ$ and the minimum at $120^\circ$. What might account for this?

If we introduce a simple assumption, namely that nonrootlet filaments must avoid rootlets (i.e., take up angles and positions such that they do not penetrate the stack of rootlet ribbons), the model distribution now fits the observed one (see Fig. 11, b and c) as judged by $\chi^2$ tests. The value for section thickness that minimized $\chi^2$ increased from 300 to 500 Å, a more reasonable value. For the length distribution, the best fit had $\chi^2 = 12$, and, for angular distribution, $\chi^2 = 1.6$, both of which pass at the 5% level.

Thus, we conclude that nonrootlet filaments can be accounted for by a random distribution subject to the condition that they avoid rootlets. Thus, the bias toward having the same orientation as the rootlet need not be a consequence of some cross-linking protein that has a preference for paral-
Figure 6. Tilts of longitudinal sections of nonrootlet ribbons at the edge of the plate. By virtue of the orientation and location relative to the rootlet (R) in the upper left, the nearly horizontal striae we see are nonrootlet ribbons. In the upper left, a rootlet enters from the left. The two thick dark lines are a nearly longitudinal section through the annular arrangement of rootlet filaments. The rootlet filaments, which leave the plane of the section, would run toward the lower left corner. Coming in at ~45° to the rootlet, a set of essentially parallel striae is separated by ~650 Å. That these striae are ribbons seen in longitudinal sections, is evident from tilts. Fig. 7, a and b, is the same section viewed at tilts of +40° and −40°. (The tilt axis is horizontal.) What appears as dark sharp lines at one tilt can be seen to break into sets of closely spaced striations at the other tilt (see arrows denoting corresponding regions). These show the expected behavior for ribbons. (a) +40° tilt about a horizontal axis; (b) −40° tilt about the same axis. Bar, 1,000 Å.

As a by-product of this analysis, we can determine the concentration of actin, which we find to be 7 mg/ml.

**Modeling the Patterns of Nonrootlet Striae**

To summarize, our observations collected these facts: (a) the rootlets consist of ribbons of filaments; (b) ribbons within a rootlet are separated by 650 Å; (c) filaments within the ribbons are separated by 150 Å; (d) the 650-Å gaps between ribbons are crossed by thin whiskers; (e) nonrootlet filaments are mostly single filaments; (f) the single filaments are easily seen when decorated by S1 but, like ribbons, appear covered with whiskers that tend to obscure their filamentous nature; and (g) the nonrootlet filaments take up random orientations subject to the condition that they do not penetrate rootlets but rather pass around them.

How might these facts be used to account for the patches and stacks of striae seen in the cuticular plate? It is obvious how rootlets consisting of a stack of ribbons can generate stacks of striae separated by 650 Å, but can the nonrootlet single filaments obeying the angular distribution in Fig. 11 also generate such patches and stacks? If the nonrootlet filaments are covered with the same whiskers as rootlets, will they also be separated by 650 Å like rootlet ribbons and might not they therefore generate patches and stacks of striae? We, therefore, tested the following rules to see if they generated a model meshwork having the features found in the cuticular plate.

First, nonrootlet filaments are much longer than the dimensions of the area being modeled. This means that all filament ends seen in the modeled thin section are ends generated by the act of cutting sections.

Second, nonrootlet filaments may not pass one another or a rootlet closer than 650 Å. This condition is only enforced in the volume of interest (i.e., in the modeled section). The reason for this is that filaments are flexible, so that if two filaments had orientations such that they would "collide" outside the section, they could, by bending slightly, avoid the collision without seriously altering their orientation in the section. One could make this condition quite rigorous if data were available for the curvature of filaments in the cuticular plate. It is not, however, and there are too many unknown factors to estimate the curvature based on the known properties of filamentous actin.

Third, newly added filaments are attached by a 650-Å bridge to an existing filament. If filaments are simply put in...
segment. Whiskers are not represented. Examples of the results display two characteristic features of the cuticular plate. The first is the pattern we term a "bull's-eye," which is seen in transverse sections of rootlets (Fig. 5, b–d). Fig. 12 b is a tracing of the striae in Fig. 5 b. The rootlet forms the center of the pattern surrounded by an actin filament-free region ~650 Å in diameter. The rootlet is oval, but the same applies if it is rectangular, as in Fig. 5 d. The simulated images display this pattern (Fig. 12, a and c). This is not surprising since the clear area of 650 Å is the direct conse-

Figure 7. Tilts of cross section of nonrootlet ribbons. This shows a portion of the lateral margin of the cuticular plate away from the bundle of the stereocilium. In a curved rows of dots are seen over an extended area. Tilts of this section show the dots turning into short segments (b) and finally disappearing (c) at high tilt. This dependence of morphology on tilt shows clearly that these are ribbons. (a) 0° or no tilt; (b) 20° about vertical tilt axis; (c) 40° about same axis. Bar, 1,000 Å.

at random, the final density of filaments is too low and there are gaps left in the meshwork. This condition of attaching all filaments to one another eliminates many of the gaps. It also mimics the general growth patterns of the cuticular plate in which the density of filaments appears constant but the thickness increases (Tilney and DeRosier, 1986).

We developed a computer program that incorporates these rules and used it to simulate sections of the cuticular plate. In these simulations, each filament is represented by a line

Figure 8. Tilts of longitudinal sections of the rootlets about two perpendicular axes. (a and b) Tilts of -40° and 0°, respectively. The tilt axis is vertical or parallel to the rootlets that extend inward from the cell surface. The plane of the section has just caught the lower end of the stereocilium, resulting in an apparent bump on the cell surface. (c and d) Tilts of -40° and 0°, respectively. This shows the same pair of rootlets one serial section below. The tilt axis is rotated to be perpendicular to the rootlet. The rootlets remain visible at the 40° tilt in c but not a because the tilt axis in c is approximately perpendicular to the rootlets, whereas in a it is parallel to them. Although rootlet ribbons are easily seen, there is no tilt angle at which ribbons can be seen between the rootlets. These data are examples of evidence that the space between rootlets is not occupied by ribbons of filaments. Bar, 3,000 Å.
Figure 9. Determination of the densities of striae and filaments. (a) An undecorated preparation of sections nearly perpendicular to the rootlets. (b) Lighter print of a with striae indicated by black lines. Only striae inside the triangle defined by three rootlets were highlighted. (c) Corresponding section in a decorated preparation. (d) Lighter print with actin filaments indicated by black lines. Only those filaments inside a triangle bounded by three rootlets were highlighted. Bar, 1,000 Å.

The program is also able to generate about the same illa-
Table I. Density of Filaments vs. Density of Striae

| Region | Relative area | Filaments | Filament density | Relative area | Striae | Striae density |
|--------|--------------|-----------|-----------------|--------------|--------|---------------|
|        | Å²           | n         | nÅ × 10⁶        | Å²           | n      | nÅ × 10⁶      |
| 1      | 2,078        | 113       | 5.4            | 1,510        | 121    | 8.0           |
| 2      | 1,006        | 69        | 6.9            | 1,243        | 87     | 7.0           |
| 3      | 2,508        | 125       | 5.0            | 1,373        | 92     | 6.7           |
| 4      | 1,056        | 79        | 7.5            | 1,396        | 109    | 7.8           |
| 5      | 984          | 69        | 7.0            | 1,371        | 93     | 6.8           |
| Average|              |           | 6.3 ± 1.1      |              |        | 7.3 ± 0.6     |

The program produces between 90 and 100 filaments in an area in which we counted 110 filaments. This is quite good agreement between calculated and observed densities since, on decoration, there may be some collapse of the gel when the whiskers are displaced.

**Whiskers**

To better simulate the micrographs of the cuticular plate, we added whiskers to the filaments. Filaments were represented by a pair of helical lines representing the twin strands of the actin filament with flexible, segmented lines projecting perpendicular to this to represent the whiskers. The resulting patterns simulate the cuticular plate quite faithfully (Fig. 13).

**Discussion**

**Rules Describing the Organizations of Filaments**

The features of the cuticular plate appear to derive from two sets of actin filaments, both of which are covered with a
Figure 11. These histograms make quantitative comparisons between data taken from electron micrographs and Monte Carlo simulations. (a and b) The pair of graphs on the left are histograms of segment lengths of nonrootlet filaments taken from Fig. 10. The segment lengths do not correspond to the overall lengths of filaments but, rather, the length of that segment of the filament contained in the section. The data (solid lines) are compared with two theoretical curves (dotted lines). The theoretical curve in the graph in a assumes the angular orientation of nonrootlet filaments are random. This theory fails the $\chi^2$ test at the 1% level. The theoretical curve in b is one in which filaments are constrained to pass no closer than 650 Å to a rootlet (rootlet-biased curve). This theory passes the $\chi^2$ test when calculated values are compared with the measured values. (c) This histogram is of filament angles measured in the plane of the micrograph in Fig. 10. The theoretical curves (dashed and dotted lines) correspond to random and rootlet-biased random models, respectively. Again, the rootlet-biased distribution passes the $\chi^2$ test, whereas the completely random distribution fails at the 2% level.

Figure 12. Computer simulation of the filament distribution in the cuticular plate. The lines that represent filaments were generated by the computer according to the three rules described in Results. Details of the algorithm are further described in Materials and Methods. Briefly, filament positions are partially constrained by the rules. The unconstrained parameters are set using a random number generator. (a and c) Two simulations of a transverse section through rootlets using different random number sequences. (b) A tracing of a pattern of filaments taken from Fig. 5 b, an actual transverse section through the rootlets. Note how well the simulations generate the pattern seen in thin sections. (d) Simulation of a longitudinal section through the rootlets. The four vertical lines on each side of the figure are intended to represent two rootlets. Each line between whisker-like material. The first set is the rootlets, which consist of ribbons of actin filaments. Each ribbon consists of several actin filaments in a row, with an interfilament spacing of 150 Å. Ribbons in the rootlet stack face to face, separated by 650 Å, the gap being filled with the 30-Å-diameter whiskers. The space between rootlets is filled with single filaments, which are also covered with whiskers. The stacks and patches of striae generated by these filaments can be accounted for by simple rules; namely, that they are tightly packed and take up orientations at random subject to the condition that they are no nearer than 650 Å to a neighboring filament or ribbon. What we have derived applies strictly to the cuticular plate of the lizard. Some of the features clearly apply to other species, but there may also be differences (e.g., in the cuticular plate of the chicken, the stacks of striae are seen and the filaments are bewhiskered, but the rootlet filaments do not appear to form ribbons).

Is Our Description Complete?
The cuticular plate in thin section is dominated by the presence of patches and stacks of striae spaced at 650 Å. The pattern is pervasive, there being no region free of it. Even the bull's-eyes are a variant in which nonrootlet filaments pass tangentially to the rootlet at a distance of 650 Å. The rules we have proposed account for this appearance and therefore for the predominant features. There may be, however, aspects of the structure that have escaped us; for example, we have assumed that most nonrootlet filaments pass each other like two skew lines separated by 650 Å. It is possible that one represents an actin filament. Note the patches and stacks of striae, two sets of which are indicated by asterisks. This mimics the patches and stacks of striae seen in Fig. 2. The scale in this figure is different from that in a–c.
Figure 13. Computer simulation of the pattern of striae. In this simulation, the filaments represented by lines in Fig. 12d are replaced by bewhiskered helices to mimic the features of actual filaments in the cuticular plate. (a) The filament is shown as a line that is stippled to mimic the effects of section staining. (b) The filament is shown with floppy, 650-A-long whiskers attached to every actin subunit. The whiskers are also stippled. (c) The patch of a cuticular plate simulation akin to that in d in which only filaments are shown. (d) The same patch with whiskers attached. The stippling density was increased to make the filaments and whiskers easy to see. (e) A more lightly stippled copy of d, which has been reduced to be on the same scale as the cuticular plate in Fig. 2b. It has been inserted into a photocopy of Fig. 2b as indicated by the two black corner markers. A photocopy of Fig. 2b was used since it has the same high contrast as the computer-generated pattern. Note that the computer-generated pattern blends in perfectly with the real cuticular plate.

A filament may end at its contact with another, making a "T" or "Y" junction, as has been observed by Hartwig and his collaborators (Hartwig et al., 1980; Niederman et al., 1983; Hartwig and Shevlin, 1986). Although we have not determined the distribution of ends, this cannot be the predominant mode of contact between filaments since it does not generate the requisite pattern. The pattern appears to require the whiskers since the pattern is destroyed if one eliminates the whiskers by decorating the structure with SI.
Predicted Composition of the Cuticular Plate

The actin filaments within a rootlet ribbon are 150 Å apart. Since their diameter is only 95 Å, they are unlikely to interact directly. Rather, within the ribbon there must be a cross-linking protein akin to a fascin or fimbrin. The filaments in the ribbon and in fascin bundles have the same interfilament spacing and parallel polar orientation. The only difference noted so far is the planar arrangement of filaments in the ribbons. If present only in the ribbons, the molar ratio of fimbrin to actin subunits would be 1:20 (assuming about half the filaments in the cuticular plate are rootlet filaments and that there are cross-links every 360 Å).

The molecular weight and molar ratio of a whisker can be guessed at. The volume of a cylinder 30 by 650 Å is ~500,000 Å³ and would correspond to a protein having a mass of ~500 kD. Since there appear to be several whiskers per actin crossover, the molar ratio of whisker to actin is between 1:1 and 1:13, roughly 1:5. This is significantly more than that for filamin (1:150) in the macropage cortex (Hartwig and Yin, 1988). The density of actin in the plate (7 mg/ml) is somewhat less than the value of ~12 mg/ml obtained by Hartwig and Shevlin (1986) for the macropage cytoskeleton.

Function of the Whiskers

The most obvious role for the whiskers is that of maintaining the actin meshwork. They might do so by virtue of their making bonds between filaments or simply by entanglement.

If the whiskers do indeed provide the cross-bridging in the gel, why are they present in such large amount since in vitro gels only a few cross-bridges per filament are required? We suggest that one answer might be to prevent the gels from being thixotropic (Sato et al., 1987); that is, able to flow if strained. In ameboid cells that are motile, the need for such a capability is clear: the cytoskeleton must reorganize as cells move. Hair cells, however, are not motile. Rather, they appear to have a fixed design in which thixotropy is not only unnecessary but would seem to be unwelcome because the sensory function of the cell depends on the fixed design of its cytoskeleton.

A second possible role for whiskers is to stabilize actin filaments, preventing depolymerization. During days 12-17 of development in the chicken, Tilney and DeRosier (1986) found evidence that polymerization of actin subunits to rootlet filaments proceeds from the pointed or nonpreferred end. During this period, the cuticular plate grows in depth concomitantly with the elongation of the rootlets. The whiskers, which appear along with plate growth, may stabilize filaments, requiring a lower critical concentration for growth.

Comparison with Actin Meshworks

We do not think that the cuticular plate, terminal web of intestinal epithelial cells, and the actin-filamin gel can be regarded as the same structure. First, while both the terminal web and cuticular plate have actin-containing rootlets, the rootlets are different. The rootlets remain as a tight bundle in the terminal web (Hirokawa et al., 1982; Hirokawa and Heuser, 1981), whereas, in the lizards hair cells, the rootlets change from a hollow cylinder to a set of ribbons. Second, the scheme for tying rootlets together is quite different and involves some different auxiliary proteins. In particular, the terminal web cross-bridges involve myosin and the TW 260/240, whereas actin filaments are used to link together the rootlets of the cuticular plate. Third, although it has a meshwork of actin filaments, the cuticular plate does not seem structurally related to an actin-filamin gel. In the former, whiskers line the sides of filaments and ribbons and would appear by their density to generate several interactions at each filament junction, thus producing the characteristic 650 Å spacing. This differs from the actin-filamin gel where T and Y junctions (i.e., end-to-side junctions) predominate (Hartwig and Yin, 1988). Moreover, filamin only binds at filament junctions, whereas whiskers cover actin filaments.

It seems likely that actin gels in different cell types and even in different specialized regions within a single cell will differ to reflect their different roles. While all such structures may have some proteins in common (e.g., actin and tropomyosin), they will likely have important differences in composition. It will be necessary, therefore, to study and compare a variety of specialized cytoskeletal structures to understand the relationships of protein composition to gel structure and properties.

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References

Burgess, D. 1987. The brush border: a model for structure, biochemistry, motility, and assembly of the cytoskeleton. Adv. Cell Biol. 1:31-58.
Cochran, W. G. 1954. Some methods for strengthening the common chi-squared tests. Biometrics. 10:417-451.
DeRosier, D. J., L. G. Tilney, and E. Engelman. 1980. Actin in the inner ear: the remarkable structure of the stereocilium. Nature (Lond.). 287:291-296.
Drechsel, D., T. Schäfer, and M. Prinz. 1985. Actin, myosin and associated proteins in the vertebrate auditory and vestibular organs: immunocytochemical and biochemical studies. In Auditory Biochemistry. D. G. Drescher, editor. Charles C. Thomas Publishers, Springfield, IL. 317-335.
Flock, A., and H. C. Cheung. 1977. Actin filaments in sensory hairs of inner ear receptor cells. J. Cell Biol. 75:339-343.
Flock, A., A. Bretscher, and K. Weber. 1982. Immunohistochemical localization of several cytoskeletal proteins in inner ear sensory and supporting cells. Hear. Res. 6:75-89.
Hartwig, J., and P. Shevlin. 1986. The architecture of actin filaments and the ultrastructural location of actin-binding protein in the periphery of living macrophages. J. Cell Biol. 103:1007-1020.
Hartwig, J., and H. L. Yin. 1988. The organization and regulation of the macrophage actin skeleton. Cell Motil. Cytoskeleton. 10:117-125.
Hartwig, J. H., J. Tyler, and T. P. Stossel. 1980. Actin-binding protein promotes the bipolar and perpendicular branching of actin filaments. J. Cell Biol. 87:441-448.
Hirokawa, N., and J. E. Heuser. 1981. Quick-freeze, deep-etch visualization of the cytoskeleton beneath surface differentiations of intestinal epithelial cells. J. Cell Biol. 91:399-409.
Hirokawa, N., L. G. Tilney, K. Fujiiwara, and J. E. Heuser. 1982. Organization of actin, myosin, and intermediate filaments in the brush border of intestinal epithelial cells. J. Cell Biol. 94:425-443.
Meyer, S. L. 1975. Data Analysis for Scientists and Engineers. John Wiley & Sons, New York. 513 pp.
Mulroy, M. J. 1974. Cochlear anatomy of the alligator lizard. Brain Behav. Evol. 10:67-81.
Niederman, R. P., C. Amrein, and J. Hartwig. 1983. Three-dimensional structure of actin filaments and of an actin gel made with actin-binding protein. J. Cell Biol. 96:1400-1413.
Roberts, W. M., J. Howard, and A. J. Hudspeth. 1988. Hair cells: transduction, tuning, and transmission in the inner ear. *Annu. Rev. Cell Biol.* 4:63–92.

Roscoe, J. T., and J. A. Byars. 1971. Sample size restraints commonly imposed on the use of the chi-square statistic. *J. Am. Statist. Assoc.* 66:755–759.

Sato, M., W. H. Schwarz, and T. D. Pollard. 1987. Dependence of the mechanical properties of actin/α-actinin gels on deformation rate. *Nature (Lond.)* 325:828–830.

Scarfone, E., D. Dememès, D. Perrin, D. Aunis, and A. Sans. 1988. α-Fodrin (brain spectrin) immunocytocchemical localization in cat vestibular hair cells. *Neurosci. Lett.* 93:13–18.

Slepecky, N., and S. L. Chamberlin. 1985. Immunoelectron microscopic and immunofluorescent localization of cytoskeletal and muscle-like contractile proteins in inner ear sensory hair cells. *Hear. Res.* 20:245–260.

Sobin, A., and A. Flock. 1983. Immunohistochemical identification and localization of actin and fimbrin in the vestibular hair cells in the normal guinea pig and in a strain of waltzing guinea pig. *Acta Oto-laryngol.* 96:407–412.

Tilney, L. G., and D. J. DeRosier. 1986. Actin filaments, stereocilia and hair cells of the bird cochlea. IV. How the actin filaments become organized in developing stereocilia and in the cuticular plate. *Dev. Biol.* 116:119–129.

Tilney, L. G., D. J. DeRosier, and M. J. Mulroy. 1980. The organization of actin filaments in the stereocilia of cochlear hair cells. *J. Cell Biol.* 86:244–259.