Evidence Concerning the Morphogenesis of Saccular Receptors in the Bullfrog (*Rana catesbeiana*) [1]

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ABSTRACT A dichotomy of hair-cell types has been found in the bullfrog sacculus, and considerable evidence supports the view that one type ("peripheral") is transformed during macular growth to the other type ("central"). Between the periphery and the center of the macula, one finds a gradation of form from "peripheral" to "central" type. Occasionally in adults and more often in stage-26 tadpoles one finds the presumably younger peripheral type of hair cell occurring well beyond the limits of the macula proper. The apparent morphogenic sequence for saccular hair cells is (1) development of a kinocillum on an endolymphatic epithelial cell, (2) gradual transformation of microvilli into stereocilia, (3) growth of the stereocilia and development of kinociliary bulb, (4) achievement of final size and form.

The scanning electron microscope has been employed quite successfully in studies of the surfaces of ciliated cells in general (Barber and Boyde, '68) and of auditory and vestibular receptors in particular (Bredberg, Lindeman, Ades, West and Engstrom, '70; Hillman and Lewis, '71; Lim and Lane, '69; Marovitz, Arenberg and Thalman, '70). In a recently published scanning microscopy paper, Lewis and Nemanic ('72) reported two distinct types of hair cells in the saccular macula of the mudpuppy (*Necturus maculosus*). One type, which occurs at the periphery of the macula, is very similar to hair cells of the mudpuppy utricular macula. The other type, occurring throughout the center of the macula, is quite distinct. Following that study, we found a similar dichotomy among hair cells of the bullfrog saccular macula. One of us (C.W. Li) undertook a scanning microscopy examination of the development of the saccular macula during metamorphosis, and soon found evidence that the peripheral hair cells were involved in macular growth. Continuing this study, we have expanded the evidence. The work was greatly facilitated by an additional distinction, not found in the mudpuppy, between the peripheral and central types. The central type of hair cell exhibited the very obvious kinociliary bulb first reported by Hillman ('69) and later shown in scanning micrographs by Hillman and Lewis ('71).

MATERIALS AND METHODS

Twenty adult bullfrogs (*Rana catesbeiana*) and ten stage-26 tadpoles (Gosner '60) were used in this study. The animals were killed by decapitation and then pithed to prevent movement during dissection and the early stages of fixation. The otic capsules were exposed from the roof of the mouth and perfused with osmium tetroxide solution in the manner employed by Hillman ('69). Proper osmolarities for the osmium solutions were determined by preliminary experiments. The effects of hyperosmotic and hyposmotic solutions on microvilli and cilia were quite marked, and the optimal osmolarities (measured on a Fiske Osmometer) were bracketed very easily. As others have found (Lombard '70), the optimal osmolarity for tadpoles was approximately one half that for adult frogs.

After fixation, the otolith and gelatinous membrane were removed mechanically. Only the very tips of the kinocilia are attached directly to the gelatinous mem-

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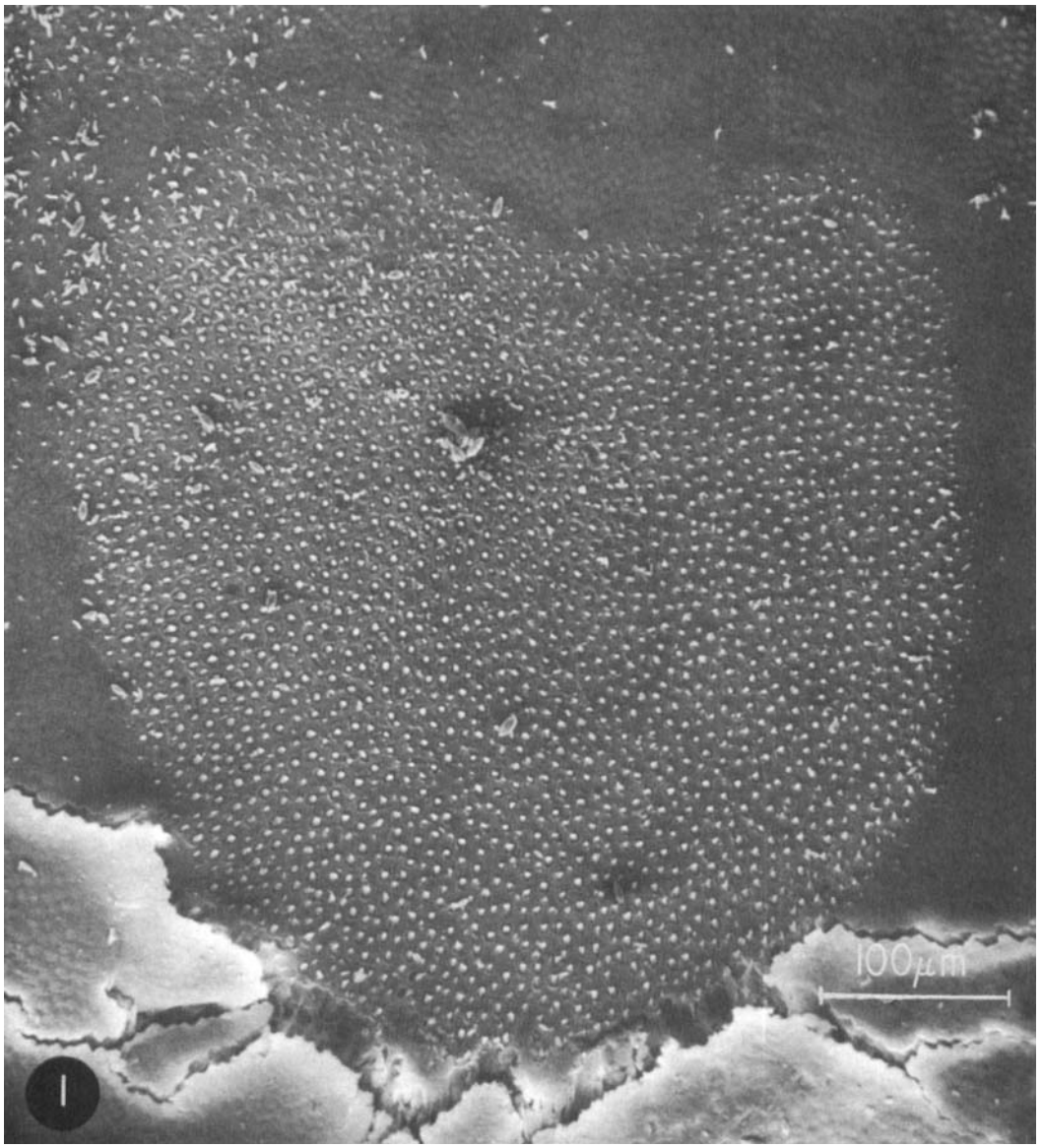


Fig. 1 Endolymphatic surface of the saccular macula of an adult bullfrog. The gelatinous membrane was removed mechanically, but a few otoconia can be seen scattered over the surface. The hair cells, whose stereocilia are apparent as small white tufts, are very regularly spaced over the entire macula. See figure 2 for the position of the line across which hair-cell orientation reverses. × 260.

brane, and the stereocilia of the receptors as well as the microvilli of supporting cells apparently are connected to the membrane by very thin strands (Hillman, '69). Aldehyde fixation appears to strengthen these connections of the gelatinous membrane, leading to disarray or tearing of cilia and microvilli when the membrane is removed. Following osmium fixation on the other hand, all of the connections apparently are rather weak. During subsequent removal of the gelatinous membrane, the strands break leaving the microvilli and stereocilia behind; and the connections be-

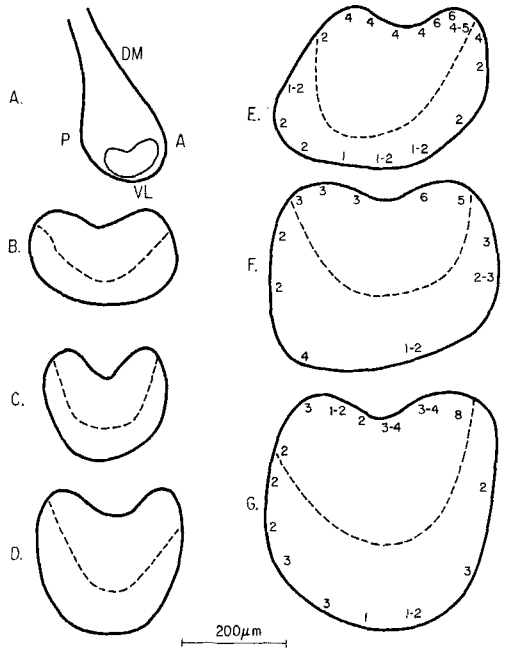


Fig. 2 The orientation of the macula with respect to the saccular branch of the VIIIth nerve (A) and the shapes of some saccular maculae from bullfrog stage 26 tadpoles (B,C, and D) and from adult bullfrogs (E,F, and G). In diagram A, DM, P, VL and A indicate dorsomedial, posterior, ventrolateral and anterior respectively. In each of the other diagrams, the line of hair-cell reversal is shown as a dashed line and the numbers around the perimeters of E,F, and G indicate the numbers of rows of hair cells clearly identifiable either as the peripheral type or as transitional between peripheral and central types.

tween the kinocilium and the membrane yields, leaving the kinocilium behind. The gelatinous membrane has ports over the receptors (Hillman and Lewis, '71) and we have viewed the kinocilia and arrays of stereocilia through these ports with the gelatinous membrane in place over the macula. In addition, Hillman ('69) has studied transmission micrographs of the macula with the membrane in place. The geometrical arrangements of microvilli, stereocilia and kinocilia observed with the membrane in place are not noticeably different from those we observed after removing it.

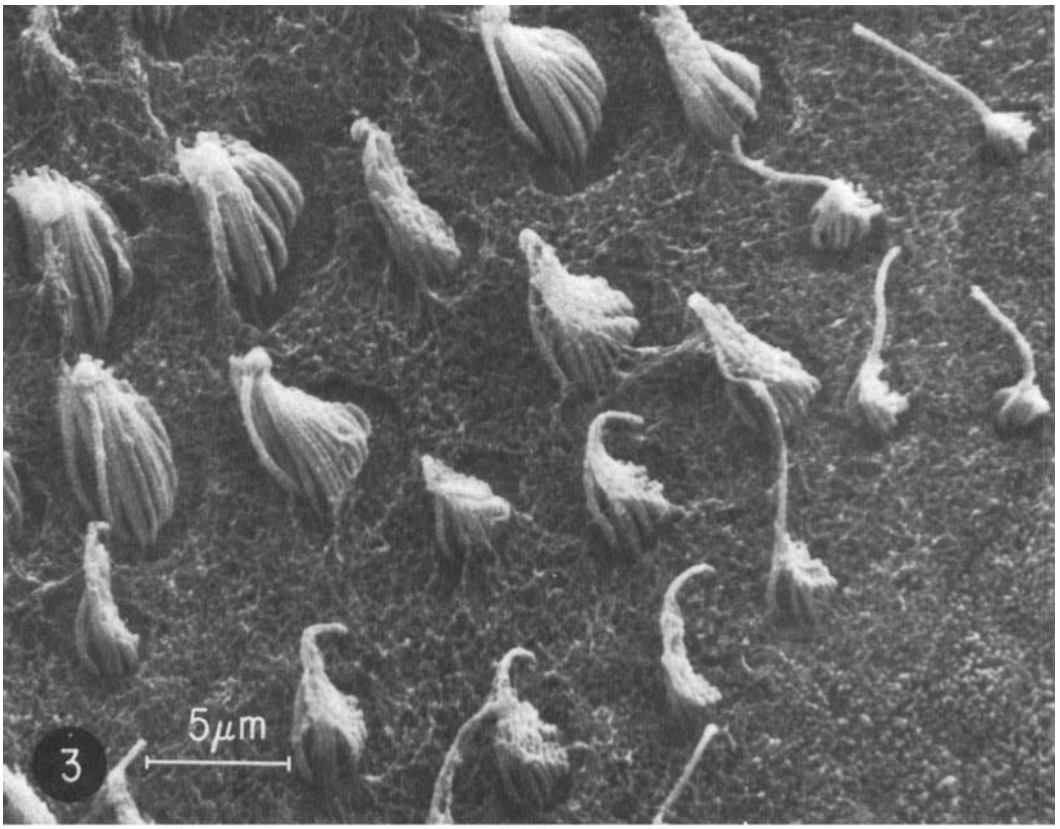
The tissue was dried using the critical point method with freon (Cohen, Marlow and Garner, '68) and coated with a thin layer of evaporated gold for viewing in the scanning electron microscope (Cambridge Instrument Stereoscan II).

Quantitative measurements were made on calibrated micrographs of essentially flat, horizontal surfaces. Means, root-mean-square deviations from those means, and ranges of luminal surface areas of hair cells and supporting cells were computed from measurements on all such cells within randomly selected areas (approximately 1000 μ^2) on each of three adult maculae and four tadpole maculae. The mean areas of nonmacular endolymphatic epithelial cells were estimated from counts of all such cells in randomly selected areas (approximately 1000 μ^2) of the endolymphatic surfaces from three tadpole sacculi and four adult sacculi. Microvillar densities were computed from counts over randomly selected areas (Approximately 4 μ^2) from six or more preparations for each cell type. Sample sizes (n) are given with each computed value in the results.

RESULTS

Gross geometry of the macula

In both the adult bullfrog and the stage-26 bullfrog tadpole, the saccular macula is located on the endolymphatic surface of the medio-ventral wall of the sacculus, and is markedly tilted from the vertical so that its ventral extremity is lateral and its dorsal extremity is medial. The saccular branch of the VIIIth nerve approaches the macula from above and slightly to the rear. The macula itself is kidney shaped, with the concave portion of its perimeter facing the direction from which the nerve approaches. The boundaries of the macula are quite well defined not only by the presence of ciliated receptors within the macula but also by the transition from the squamous epithelium of the surrounding endolympatic surface to the elevated, columnar epithelium of the macula (fig. 1). The epithelium of the macula not only appears to be elevated, but the luminal surface areas of the macular supporting cells are consistently smaller than the luminal surface areas of the nonmacular epithelial cells. We observed areas of 7 $\pm$ 2 μ^2 (4 $-$ 15, n $=$ 100) and 14 $\pm$ 4 μ^2 (6 $-$ 23, n $=$ 100) for macular supporting cells of the tadpole and adult respectively. In both adult and tadpole, we observed mean areas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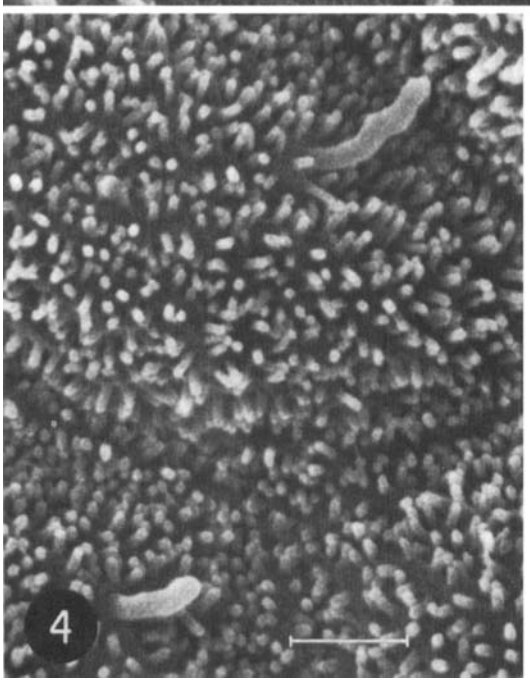
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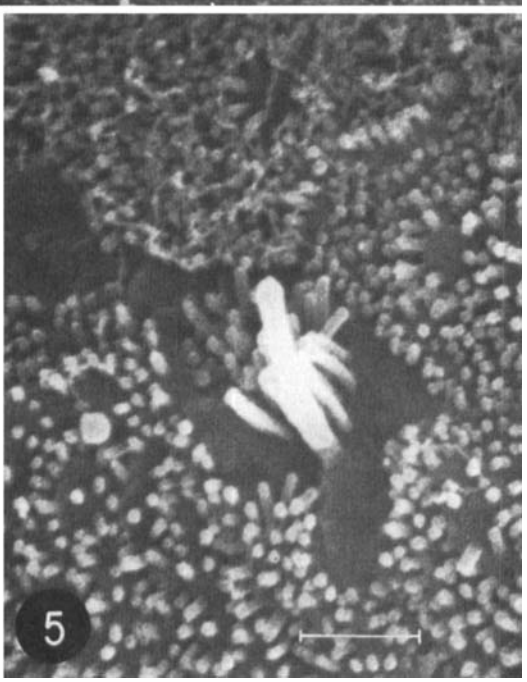
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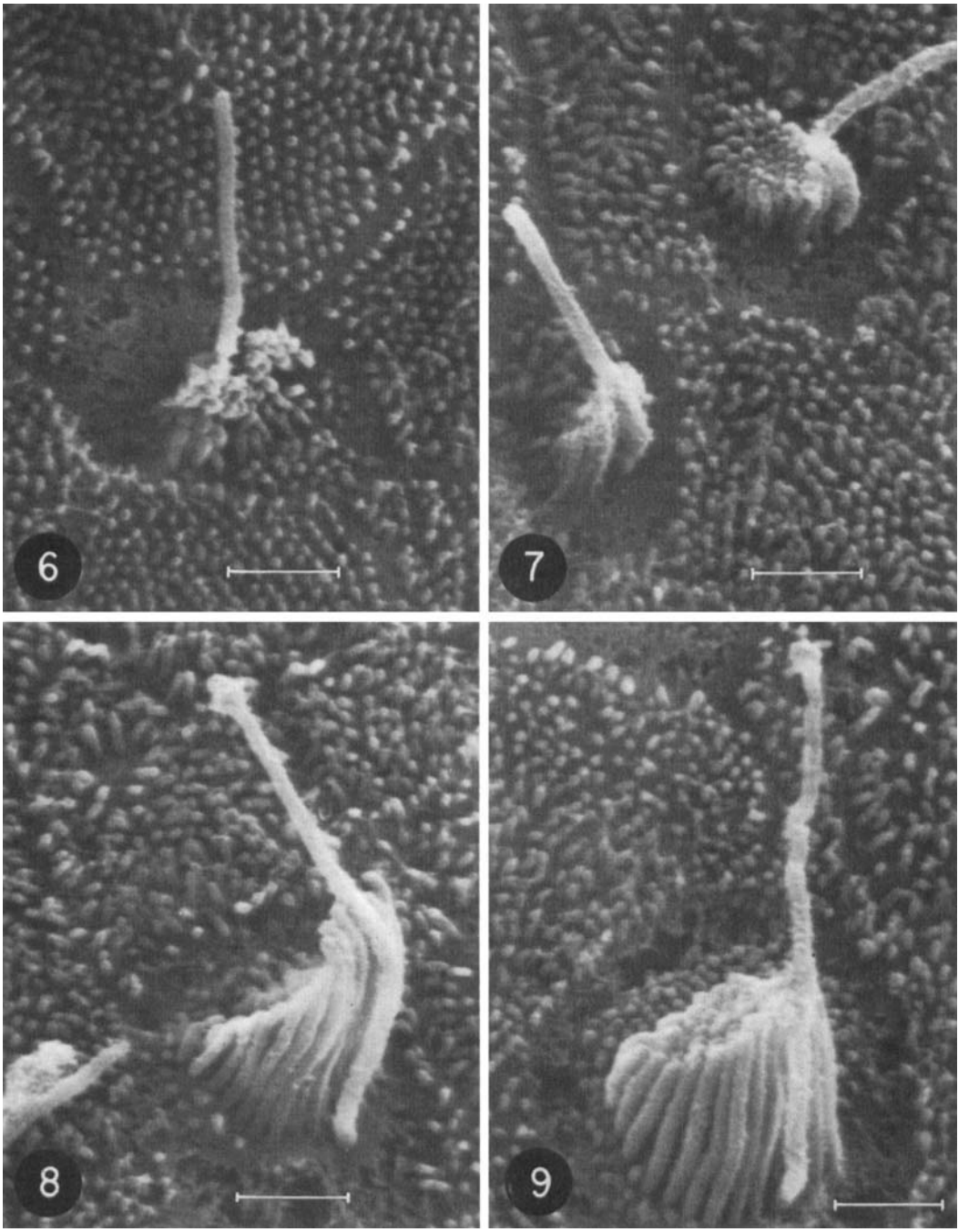


Fig. 3 Hair cells at the periphery of the saccular macula of an adult bullfrog, showing the gradation in size and shape from peripheral type (lower right) to central type (upper left). × 3800.

Fig. 4 Cilia-like projections from two adjacent endolymphatic epithelial cells just outside the present perimeter of an adult macula. × 16,000 (line = 1 μm).

Figs. 5–9 Gradation of size and shape of peripheral hair cells of an adult macula. × 16,000 (lines = 1 μm).

of approximately 30 μ^2 for nonmacular epithelial cells [29, n = 71 for the tadpole; 31, n = 125 for the adult].

The density of hair cells is quite constant over the macular surface (fig. 1); so that no striola (line of higher hair-cell density) is apparent from casual SEM observation of the entire macula (see Lindeman, '69). As they are in other vertebrates, the hair cells of the bullfrog are polarized. The stereocilia are graded in length and arranged much like a stand of organ pipes, with succeeding rows being occupied by progressively longer stereocilia. The kinocilium occurs directly behind the row of longest stereocilia. Thus each hair cell can be assigned an orientation. The orientation of hair cells within the macula of the bullfrog sacculus is almost identical to that in the mudpuppy sacculus, whose macula also is kidney-shaped (Lewis and Nemanic, '72). As in the mudpuppy, the line across which hair-cell orientation reverses runs more-or-less parallel to the concave and convex edges of the macula and approximately halfway between them (fig. 2). The mudpuppy differs from the bullfrog, however, in that the entire macula is rotated slightly counterclockwise relative to those shown in figure 2; and the concave edge faces a slightly more posteriad direction, corresponding to a slightly more posterior approach of the saccular branch of the VIIIth nerve.

Microvilli

In the 4 to 5 μm space between the supporting cells of the macula and the gelatinous membrane, Hillman ('69) has found very thin electron-dense strands, which emanate from the supporting-cell microvilli and which apparently condense to form the gelatinous membrane. This suggests that the supporting-cell microvilli might be involved in secretion of the gelatinous membrane. In the mudpuppy, Lewis and Nemanic ('72) found that the microvilli of the supporting cells were markedly more dense than those of the surrounding squamous endolymphatic epithelium. In the adult bullfrog, a similar but not nearly so marked difference exists; but in the stage-26 bullfrog tadpole, the difference is almost negligible. On the adult bullfrog squamous endolymphatic epithelium, we observed a microvillar density of 26 ± 2 per μ^2 (over 7 sample areas of 4 μ^2, chosen at random from 6 sacculi); whereas on the supporting cells of the macula we observed a microvillar density of 35 ± 5 per μ^2 (over 10 sample areas of 4 μ^2, chosen at random from 8 maculae). Thus in the adult bullfrog, a further distinction exists between macular supporting cells and the epithelial cells surrounding the macula. This distinction is sharpened even further by remnants of the fine strands reported by Hillman, which occur only on the macular supporting cells (fig. 3).

In the stage-26 tadpole, remnants of the fine strands occur on the supporting cells of the macula; but the observed mean microvilli density of the supporting cells (24 ± 1 per μ^2 over 6 sample areas from 6 maculae) was essentially indistinguishable from the mean microvilli density on the surrounding squamous epithelium (23 ± 2 per μ^2 over 6 sample areas from 6 sacculi). Even without this added distinction, the boundaries of the tadpole macula were quite sharp (fig. 13).

The microvilli are arrayed in a remarkably orderly fashion (fig. 6), with very distinct rows. Occasionally, at the periphery of the macula, the microvilli are interrupted by large processes, possibly emerging cilia (fig. 4).

On the surfaces of the hair cells in the center of the macula, microvilli form a semi-circle around the shortest stereocilia, just as they do in mudpuppy sacculus (Lewis and Nemanic, '72). On the hair cells at the perimeter of the macula, the microvilli appear to merge with the stereocilia, and it often is impossible in the scanning electron micrographs to tell one from the other (figs. 5, 6, 7).

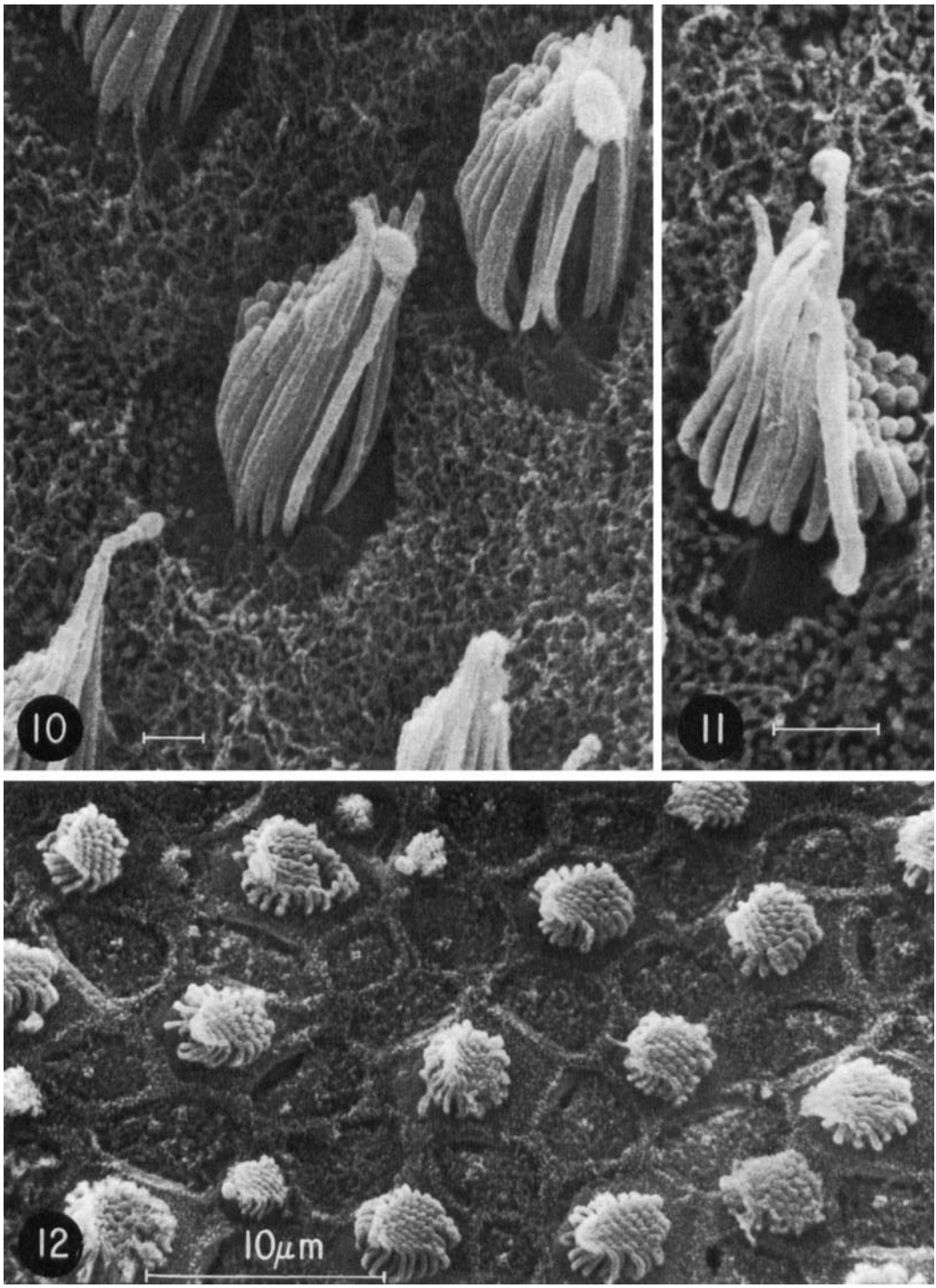
Two types of saccular hair cell

The hair cells throughout the broad central region of the macula have been

Fig. 10 Hair cells intermediate between peripheral and central, showing graded size of kinociliary bulb. × 9000 (line = 1 μm).

Fig. 11 An anomolous intermediate hair cell, with its kinocilium emerging at the side of the stereociliary tuft rather than behind it. Similar anomolies have been reported by Flock ('64). × 16,000 (line = 1 μm).

Fig. 12 A small area in the center of a saccular macula which was fixed in slightly hyperosmotic osmium tetroxide. The supporting cells are well delineated by terminal bars, making the cell distribution quite easy to observe. × 3500.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described very well by Hillman ('69) and have been shown in scanning electron micrographs by Hillman and Lewis ('71). These hair cells are unusual in that the kinocilium, which is approximately equal in length to the longest stereocilia, terminates in a large bulb. We found hair cells of this type not only in adult bullfrogs, but also in the stage-26 tadpoles.

The second type of hair cell, previously unreported for the frog but found by Lewis and Nemanic ('71) in mudpuppy, occurs around the entire perimeter of the frog and tadpole maculae. The most peripheral of these hair cells have very short stereocilia and a long kinocilium with no bulb. As one moves from the extreme periphery toward the center of the macula, he observes a gradual change from this peripheral hair cell type to the central type reported by Hillman (fig. 3). Figures 4–11 show this succession in detail. The width of the peripheral band over which this gradation occurs is not constant, but is consistently greater on the concave side (nerve side) of the macula than it is on the convex side (fig. 2). One also finds hair cells of the peripheral type distributed randomly but very sparsely throughout the entire central macula.

Beyond the edges of the macula proper in adult frogs one often finds single outlying hair cells (of the peripheral type), with ten or more squamous epithelial cells between them and their nearest neighbors at the edge of the macula. In the macula proper, hair cells are separated by only one or two supporting cells (fig. 12), and these have distinctly smaller luminal surfaces than the squamous epithelial cells and generally exhibit remnants of the fine strands reported by Hillman (see under microvilli). In stage-26 tadpoles, one finds even more outlying peripheral hair cells, and one finds them at even greater distances from the macula proper (figs. 13–15). In fact, we have found them at distances greater than 100 μm from the edge of a 170 μm macula. Most of these outlying hair cells occur on the concave side (i.e., the side toward the saccular branch of the VIIIth nerve).

Qualitatively, the hair cells of the tadpole saccular macula were essentially identical to those of the adult saccular macula; and no significant difference in their mean luminal surface areas was observed. The hair-cell area in the center of the adult macula was 24 $\pm$ 7 μ^2 (6 – 43, n = 100); while the corresponding values for the tadpole were 23 $\pm$ 11 μ^2 (2 – 43, n = 100). However, a marked difference was seen in the macular supporting cells, which in the adult had a luminal surface area of 14 $\pm$ 4 μ^2 (6 – 23, n = 100) and in the tadpole an area of 7 $\pm$ 2 μ^2 (4 – 15, n = 100). Consistent with these differences in supporting-cell and hair-cell sizes, the density of hair cells on the surface of the tadpole saccular macula was 0.023 $\pm$ 0.001 per μ^2 (over 5 sample areas of 4,000 μ^2 chosen at random from 5 maculae); while the corresponding value in the adult was 0.015 $\pm$ 0.0015 per μ^2 (over 11 sample areas of 15,000 μ^2 chosen at random from 7 maculae). This represented an observed hair-cell density decrease of approximately one-third in going from tadpole to adult. In spite of this decrease in density, the observed total number of saccular-macula hair cells increased markedly from tadpole to adult. A 5 gm tadpole, for example, had approximately 850 hair cells; while a 312 gm adult had approximately 2500. Thus the increased macular area of the adult more than compensated the decreased hair-cell density; and macular growth consisted, at least in part, of cell proliferation.

DISCUSSION

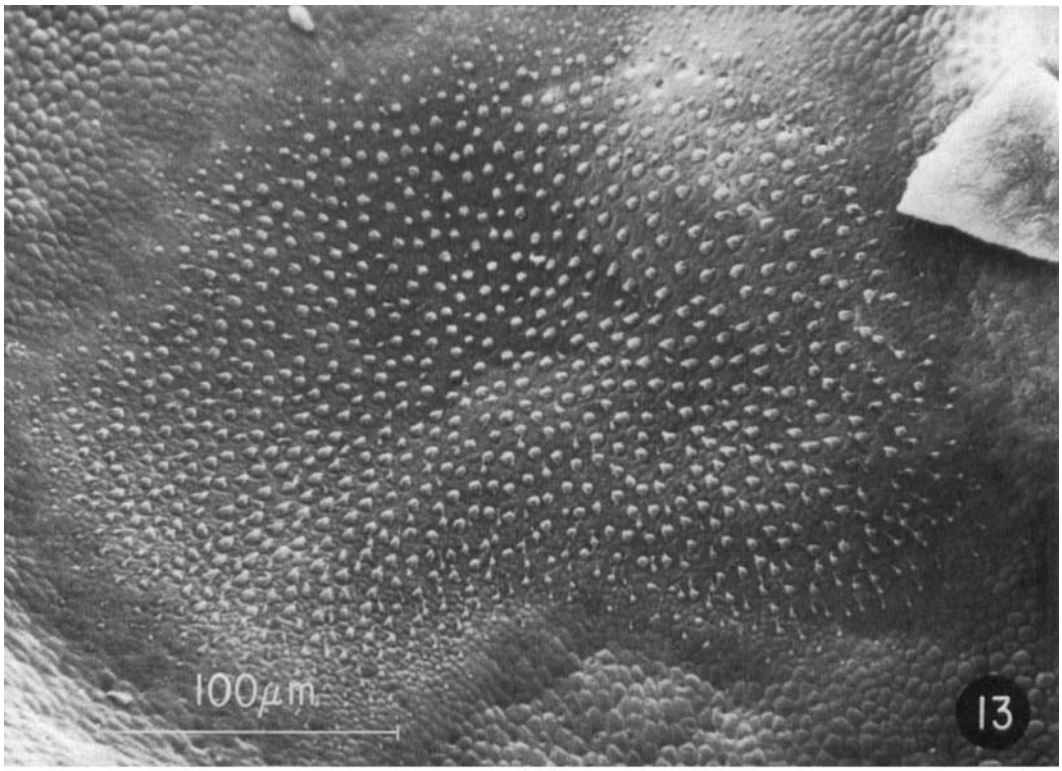
Macular growth and the formation of new hair cells

Taken as they stand, our results imply that as the area of the saccular macula increases several fold during the growth and maturation of the bullfrog from stage-26 tadpoles to large adults, the number of hair cells increases, but apparently not in direct proportion to macular size. Thus

Fig. 13 The saccular macula and surrounding endolymphatic epithelium from a stage-26 tadpole. Note the relatively large luminal surface areas of the epithelial cells surrounding the macula and the dichotomy of peripheral and central hair-cell types. × 450.

Fig. 14 Higher magnification of an area near the edge of a tadpole macula, showing two outlying receptors (indicated by arrows). × 1080.

Fig. 15 Close-up view of one of the two outlying receptors of figure 14. × 10,800 (line = 1 μm).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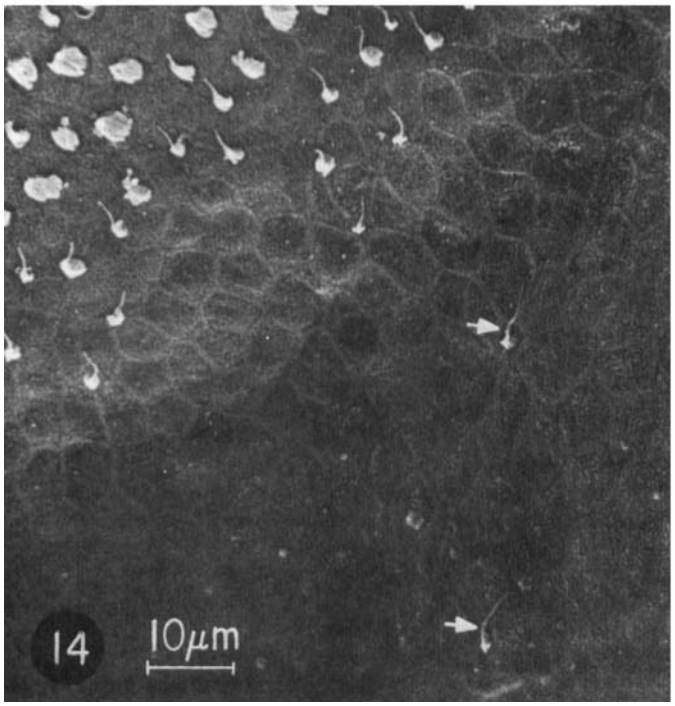
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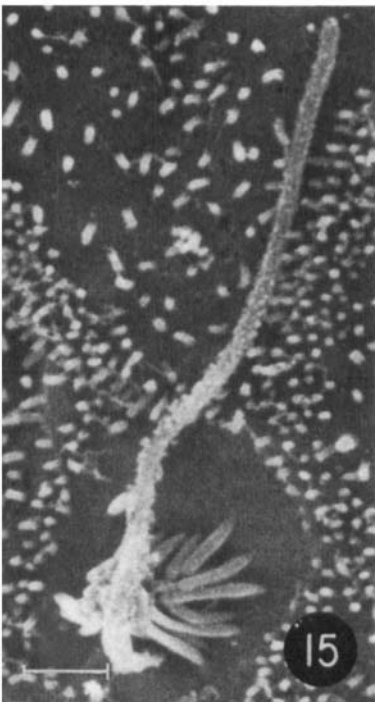
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the hair-cell density decreases slightly, corresponding to an increase of the mean luminal surface area of supporting cells. It is reasonable to suspect, however, that apparent differences in cell densities were artifactual results of differences in the tissues as reflected in the requirement for lower osmolarities in the fixative for the tadpole. Thus it is conceivable that the tadpole macula underwent more shrinkage than the adult macula. This possibility is accentuated by the fact that Proebsting ('24) reported that macular growth in larvae *Triturus* was due primarily to increase in cell number and that cell size remained essentially constant. On the other hand, the fact that both the nonmacular epithelial cells and the hair cells in our preparations had the same mean luminal surface area in both tadpoles and adults tends to indicate that differential shrinkage may not have occurred in our preparation and that the observed hair-cell density decreases from tadpole to adult may not have been artifactual.

Two conclusions are much more definite. First, the distribution of hair-cell types does not change with growth. From the smallest to the largest maculae observed in this study, hair cells of the peripheral type always occupied the entire perimeter and surrounded a large area occupied by the hair cells of the central type with their kinociliary bulbs. Second, during macular growth many new hair cells are formed.

The presence of outlying hair cells in adults and their presence in even greater numbers and at even greater distances from the macula proper in tadpoles, indicates that hair cells apparently can form among squamous endolymphatic epithelium cells well beyond the macula proper. This implies a tendency toward outward expansion of the macula by addition of hair cells at its perimeter.

If in fact the macula does grow in this manner, then the peripheral type of hair cell either must be replaced by or be transformed to the central type as the macular perimeter moves on. Otherwise the width of the area occupied by peripheral types would continually increase; whereas it actually remains fairly constant at one to six receptors wide. The consistent centripetal gradient of hair cells from peripheral type to central type suggests transformation rather than replacement.

This in turn suggests that the gradation of hair-cell types shown in figures 3 to 11 might represent a temporal sequence displayed in spatial coordinates. Newly formed saccular hair cells may be of the peripheral type, with a kinocilium as long as 5 μm or more and stereocilia less than 2 μm long (fig. 9). The stereocilia then could grow both in length and diameter (figs. 5–11); and as the longest stereocilia approach the length of the kinocilium, the kinociliary bulb could begin to form (figs. 8–11), becoming fully formed as the longest stereocilia reach the tip of the kinocilium. Thus we might conclude that hair cells of the peripheral type are in fact immature, while those of the central type are mature. Our observation of a very few scattered peripheral and intermediate type hair cells in the interior of the macula would indicate that some hair-cell proliferation and macular growth also occurs there. On the other hand, these may be peripheral cells whose transformation somehow was arrested as the perimeter move past them, or they may be replacements for hair cells somehow damaged or destroyed in the central macula.

Furthermore, if this hypothesis is correct, then the occurence of widely scattered peripheral hair cells beyond the concave side of the tadpole macula proper and the occurrence of the widest band of peripheral and intermediate hair-cell types on the concave side of the macula proper in both tadpoles and adults would imply that macular expansion is directed primarily toward the saccular branch of the VIIIth nerve.

Assuming that the peripheral hair cells were in fact newly formed, we searched the perimeter of the macula for even earlier signs of hair cell development. Among the epithelial cells close to the edge of the adult macula, but not elsewhere on the endolymphatic epithelium, we found many otherwise normal appearing epithelial cells with what appeared to be single cilia whose diameters were essentially identical to the kinocilium diameter of the hair cells (fig. 4). It is possible that these cells are undergoing differentiation to receptor cells. Furthermore, in the smallest clearly identifiable receptors, the stereocilia have

diameters essentially the same as the neighboring microvilli. It seems possible, therefore, that microvilli in the differentiating epithelial cell become modified to form stereocilia.

Geisler, van Bergeijk and Frishkopf ('64) reported that "hair cells gradually fade out into undifferentiated cells" near the lateral edge of the bullfrog amphibian papilla; while along the medial edge the macula ends abruptly. They suggested that the lateral edge is a zone of growth of the papillar macula. Interestingly, the papillar branch of the VIIIth nerve approaches the macula from the lateral side. Furthermore, in preliminary scanning electron microscope studies of the amphibian papilla, both in tadpoles and in adult frogs, we found hair cells with long kinocilia and short stereocilia along the entire lateral edge. As we progressed in a medial direction, we found the same succession we had observed at the periphery of the saccular macula. The stereocilia became longer; bulbs began to appear at the tips of the kinocilia; and we finally came to a broad area of hair cells whose surface appearance was essentially identical to that of the central hair cell of the saccular macula. This type of hair cell occurred right up to the medial edge of the papillar macula. No gradient of hair-cell type occurred on the medial edge. Thus our observations and conclusions concerning the morphogenesis of saccular hair cells are consistent with the observations and conclusions of Geisler et al. concerning the hair cells of the amphibian papilla.

Regardless of whether or not the peripheral type of hair cell in the saccular macula is an immature version of the central type, one is left with the question of whether or not there is a functional difference between the two types. The answer to this question will require electrophysiological studies as well as additional microscopy.

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