Scanning Electron Microscopy

Volume 1986 | Number 4 Article 38

9-27-1986

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SCANNING ELECTRON MICROSCOPY/1986/IV (Pages 1635-1648)
SEM Inc., AMF O'Hare (Chicago), IL 60666-0507 USA

0586-5581/86$1.00+05

DEVELOPMENT OF THE OTOLITH IN EMBRYONIC FISHES WITH SPECIAL REFERENCE TO THE TOADFISH, OPSANUS TAU

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(Received for publication March 18, 1986, and in revised form September 27, 1986)

Abstract

The development of the saccular otolith and the otolithic membrane was studied in the toadfish (Opsanus tau) using scanning and transmission electron microscopy. Development of the saccular otolith and its otolithic membrane in Opsanus begins with the formation of the primordia in embryos of 17-20 somite age. Calcification of the primordia begins shortly afterwards, although increased calcium layering and formation of the otolithic membrane corresponds to the development of a group of cells lying peripheral to the developing sensory epithelium. These cells contain an abundance of rough endoplasmic reticulum.

KEY WORDS: Embryonic development, otoliths, Opsanus tau, fishes, scanning and transmission electron microscopy.

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Introduction

The typical vertebrate membranous labyrinth of cartilagenous and bony fishes includes three semicircular canals and three otolith organs (Fig. 1). In these fishes, the semicircular canals and one of the otolith organs, the utricle, have vestibular functions, while the other two otolith organs, the saccule and lagena, may have both auditory and vestibular functions (Platt and Popper, 1981).

Each otolith organ is a membranous sac enclosing a single, dense calcareous otolith and/or many calcareous otoconia. The sensory epithelium (or macula) lies on the medial wall of the membranous labyrinth, separated from the otolith or otoconia by an otolithic membrane composed of glycosaminoglycans (Mugiya, 1966, 1968). The otolithic membrane helps keep the otolith in place relative to the sensory epithelium.

A multitude of calcareous particles or otoconia are found in jawless fishes (lamprey and hagfish), cartilagenous fishes (sharks and rays) and in some of the more primitive bony fishes (e.g., bowfin, reedfishes, sturgeons), as well as in lungfishes (Carlstrom, 1963). A single solid mass or otolith is found in most of the more recently evolved bony fishes, the teleosts (Carlstrom, 1963). There are a number of instances, however, where an otolith organ will contain an otolith and some otoconia (e.g., the gars, bowfin).

The otoliths of the utricle (the lapillus), saccule (the sagitta) and lagena (the asteriscus) differ from one another in shape and size. Each otolith has a species-specific shape that is used for taxonomic purposes in analyzing systematic relationships of both extant and fossil fishes. The shapes of the otoliths may vary between species (Fig. 2), although there is evidence that there is substantially more inter-specific variation in the saccular

otolith (an organ involved with hearing) than in utricular and lagenar otoliths (Popper and Coombs, 1982). Figure 2 provides an example of saccular inter-specific variation. For example, the saccular otolith of the milkfish, Chanos chanos (Fig. 2D), is highly elongate with fluted edges, while that of a deep sea fish Opisthoproctus (Fig. 2C) has short tooth-like protrusions just below the area in which the sensory epithelium lies.

In addition to the inter-specific variability in the shape of fish otoliths, there is also considerable variation in the size of the otolith relative to the saccular sac and sensory epithelium. In some species, the otolith totally fills the sac and covers the whole sensory epithelium while in others the otolith is not much larger than the epithelium and fills a smaller per cent of the chamber volume.

Otolith Composition

The otoliths or otoconia consist of calcium carbonate in teleosts and elasmobranchs and calcium phosphate in cyclostomes (Carlstrom, 1963). In addition, Degens et al. (1969) showed that various metal ions bind with the oxygen of the bicarbonate of calcium carbonate to form different metal ion polyhedra. Consequently, the otoliths or otoconia of teleosts and elasmobranchs contain aragonite, and the otoconia of cyclostomes contain vaterite. In contrast, the calcium phosphate otoconia of chondrosteans (e.g., sturgeons) contain apatite. However, both apatite and vaterite are also found in the otoliths of a few teleost fishes (Campana, 1983a; Lowenstam, 1981).

The otoliths of teleosts contain an organic component or protein (mw 150000) known as otolin (Degens et al., 1969), which consists of acidic amino acids such as threonine, glycine, and valine. The organic and inorganic components form a matrix. This matrix is seen in cross-sections of the otolith as alternating thin organic (discontinuous zones) and inorganic (incremented zone) layers (Fig. 3) surrounding a central nucleus. The nucleus consists of a dense staining central region surrounded by a thick layer of calcium. The organic substance is not only found between calcified layers (interlamellar) but also between single crystals (intercrystalline) and within calcium crystals (intracrystalline) (Dunkelberger et al., 1980). A similar kind of intracrystalline matrix is found in mollusk shells (Mutvei, 1970) and the spicules of coelenterates (Dunkelberger and Watabe, 1974).

The alternating organic and inorganic components of the otolith are laid down in layers (both layers together are called an increment) on a daily (24-h) or sub-daily basis. These increments can be used to determine age and growth rate in larval and adult fishes (Campana and Neilson, 1985; Pannella, 1971, 1974; Radtke and Dean, 1982; Victor and Brothers, 1982; Wilson and Larkin, 1982). In addition, the deposition of these increments gives some indication of the changes in the physiological state of the fish since layering appears to be sensitive to photoperiod, water temperature, food availability, or stress (Campana, 1983a, b; Campana and Neilson, 1982; Campana and Neilson, 1985; Neilson and Geen, 1982). The primary regulator of otolith growth may be the endocrine system (Campana and Neilson, 1985).

Embryonic and Early Development of Otoliths

Only a few studies have dealt with the morphology and mineralogy of otoliths or otoconia in embryonic fishes. Morphological studies show that the otolith begins developing with the gradual formation of a nucleus consisting of several dense staining particles (primordia) surrounded by a calcified layer (Fig. 3) (Brothers, 1984; Neilson et al, 1985; Radtke and Dean, 1982). Once the nucleus is formed, layers are added daily during the embryonic and larval stages (Brothers et al., 1976; Radtke and Dean, 1982).

Mineralogical studies show that, at least for the blue shark (Prionace glauca), the composition of the otolith changes during embryonic development and may vary at different times in the life history of the individual animal. The otoconial minerals found are amorphous calcium hydrate (ACP) in the embryo, ACP plus aragonite in the juvenile, and aragonite with a trace of ACP in the adult (Lowenstam and Fitch, 1978).

Embryonic studies of otolith formation have been primarily concerned with questions of age, growth, and taxonomic identification. They have not, however, focused on the ultrastructure of the embryonic otolith, nor on ultrastructural changes in cells that may be involved with secreting the products that form the embryonic otolith. Since very little is known about these aspects in the fish embryo, the primary aim of the current study was to observe the fine structure of the otolith during embryogenesis. In addition, this study correlates development of the embryonic otolith with several changes in the cellular ultrastructure of the developing saccular epithelium.

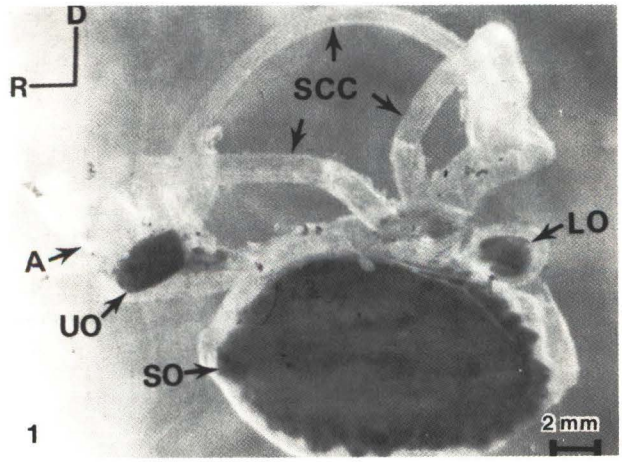


Figure 1. A light micrograph of a medial view of the inner ear of an adult toadfish, Opsanus tau. The membranous labyrinth consists of three semicircular canals (SCC) with their ampullae (A shows the ampulla of the anterior canal) and three otoliths the saccular otolith (SO), utricular otolith (UO) and lagenar otolith (LO).

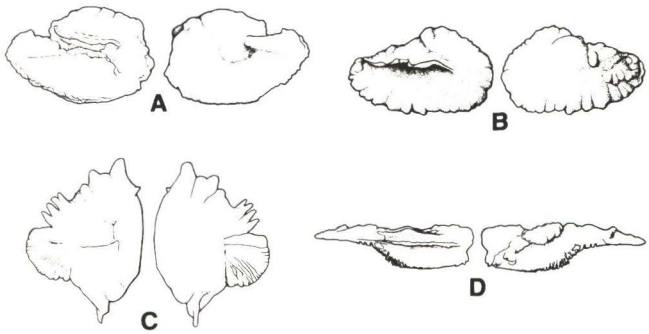


Figure 2. Line drawings of the medial (left) and lateral (right) sides of the left sagittae of four different fishes (A) Zebrasoma veliferum, (B) Halosaurid, (C) Opisthoproctus soleatus, (D) Chanos chanos. The macula is found on the left side in each of the four drawings (Taken from Platt and Popper, 1981).

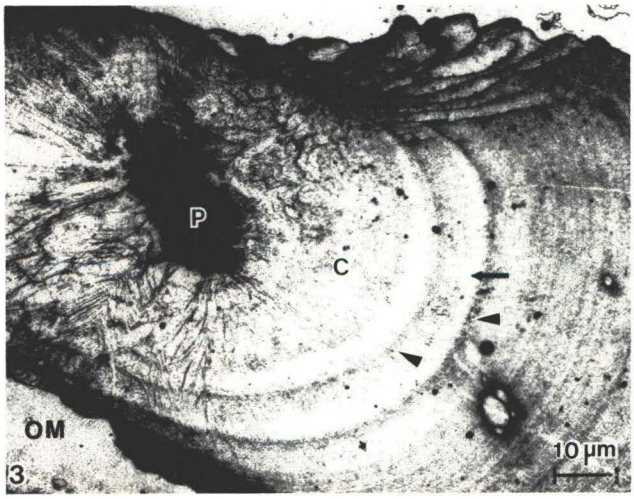


Figure 3. A transmission electron micrograph showing the cross section of the sagitta of a two week old post-hatch toadfish. The primordia (P) are surrounded by the calcified layer of the nucleus (C), and the organic (black arrow) and inorganic (black triangles) layers forming the increments. Underlying the otolith is the otolithic membrane (OM).

Materials and Methods

Adult toadfish (Opsanus tau) and eggs in early embryonic development were collected in two tributaries of the Chesapeake Bay (York and Patuxent Rivers). These animals were raised in the laboratory in brackish water (salinity of seventeen parts per thousand) maintained at a constant temperature of 25-27°C and also maintained on a 12 h light-dark cycle. Animals without yolk sacs (i.e., juveniles and adults) were fed black worms at irregular intervals. Under these conditions the approximate developmental time for the embryo was two weeks followed by a two week period in the sessile larval stage.

Approximately 210 embryos and post-hatched animals were fixed for SEM in a solution of 2% glutaraldehyde and 1% formaldehyde in 0.1M phosphate buffer (pH 7.4). All animals, except adults, were fixed by direct immersion in fixative. Embryos were fixed whole and post-hatched animals were anesthetized by cooling in a water dish placed on ice in order to allow removal of their yolk sacs and lower jaws prior to fixation. Specimens were rinsed in 0.1M phosphate buffer (pH 7.4), prior to dissection. The inner ear was either left in situ or removed and dissected to reveal the otolith and sensory epithelium. If the inner ear was left in situ, specimens were post-fixed by immersion in 1% (0.1M) osmium, 2% tannic acid, and 1% osmium in buffer solution respectively, in order to decrease cell shrinkage (Katsumoto et al., 1981). Specimens

were then dehydrated in a graded series of ethanol, critical point dried in a Samdri 780 critical point drier, and coated with gold palladium in a Technics sputter coater. Samples were mounted on stubs coated with sticky tape and scanned with an ETEC Autoscan SEM or Hitachi 570 SEM at 20kV.

Adult specimens were anesthetized with ethyl m-aminobenzoate and then perfused with saline and fixative. Saccular otoliths of adults and 10-14 day-old post-hatched animals were removed and air dried in a desiccator for observation by SEM.

TEM specimens of embryos were prepared by immersion in the same fixative as used for SEM. Whole specimens were rinsed in 0.1M phosphate buffer (pH 7.4) after fixation and immersed in 1% osmium in phosphate buffer at 4°C. Specimens were again rinsed in buffer before dehydrating them through a graded series of ethanol. The whole mounts were en bloc stained in 1% uranyl acetate in 50% ethanol at 4°C for 1 h during the dehydration series. After dehydration they were placed in a propylene oxide series of increasing concentrations and embedded in Araldite. Thin sections of 50-75 nm were cut on an LKB ultramicrotome, mounted on 100 mesh grids, and stained with a 1% uranyl-acetate-potassium permanganate stain (Hayat, 1970) followed by a 1% lead citrate stain (Reynolds, 1963), and finally viewed with a JEOL-100S TEM at 80kV.

Results and Discussion

The saccular otolith of the adult toadfish is flattened medial-laterally and has an oval shape (Figs. 4 and 5). It lies on the vertical axis so that the sulcus acousticus is found on the medial face (Fig. 5) adjacent to the otolithic membrane and the sensory epithelium. The shape of the sulcus is similar to the shape of the sensory epithelium or macula (Fig. 6).

Otolith Development

Von Noorden (1883) reported seeing the otolith of embryonic herring 48-50 hours after fertilization. More recently, Radtke and Dean (1982) first saw the otolith in the embryo of the mummichog (Fundulus heteroclitus) approximately 72 h after fertilization. The otolith of Fundulus, which appeared as an amorphous, gel-like, organic mass, began calcifying shortly afterwards in embryonic stages 24-28 (Armstrong and Child, 1965; Radtke and Dean, 1982). In comparison, calcification of the primordia in trout (Salmo gairdneri) begins at approximately 10-19 days post-fertilization (Neilson et al., 1985). The first calcified layer and the primordia form the nucleus of the otolith in both of these and other species.

The sagitta in Opsanus begins developing in embryos having 17-20 somite pairs (approximately 8-11 days prehatching which is equivalent to approximately 3-4 days post-fertilization). The primordia, as seen with TEM, first appears as several separate, oblong-shaped, electron dense particles, approximately 0.5 μm in width and 1 μm in length (Fig. 7). These early primordia stain a dark blue with toluidine blue, suggesting an organic component, possibly an acid protein polysaccharide. In addition, thin strands attach the primordia to each other and to the underlying developing stereocilia (Fig. 7).

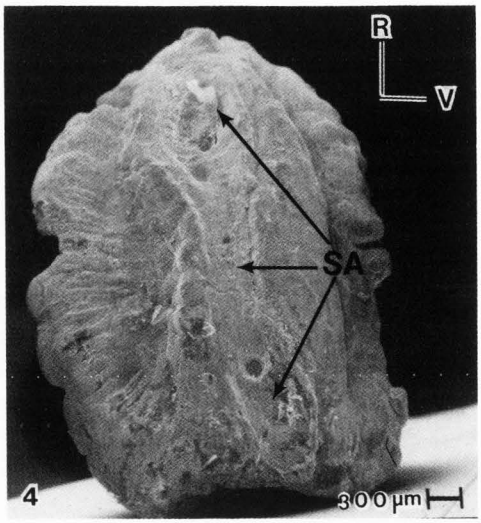
The primordia in Opsanus increase in size (5-11 μm), and change to a sphere-like shape during myotome formation in the embryo (5-8 days prehatching which is equivalent to 6-7 days post-fertilization). TEM shows that calcification begins at this time by the addition of a less densely stained area which surrounds the primordia (Figs. 8 and 9). The density

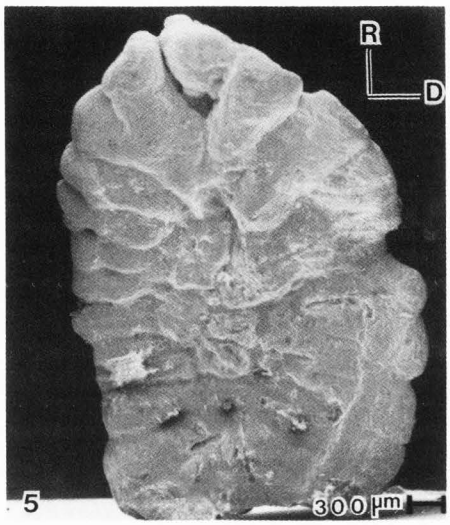
Figure 6. A scanning electron micrograph of the saccular sensory epithelium from an adult toadfish (2-3 years old). The shape of the macula (outlined in black) resembles the shape of the sulcus acousticus.

Figure 7. A transmission electron micrograph of the primordia (white stars) in a 17 somite toadfish embryo. Strands (large arrows) connect these particles to each other and the developing stereocilia (s). A number of kinocilia (k) are seen in cross-section.

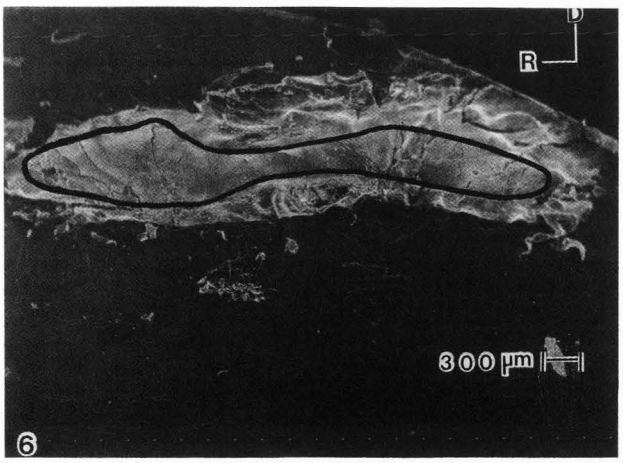
Figure 8. A transmission electron micrograph of two primordia (P) found in the embryo of Opsanus 5-8 days prior to hatching. The small white arrows indicate the border of the organic region.

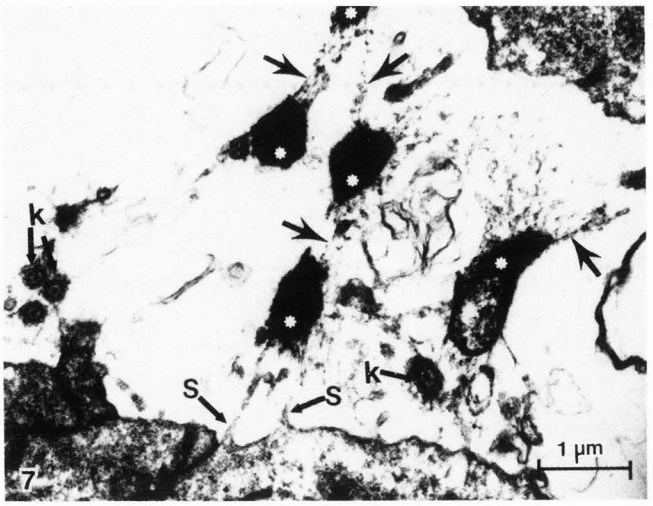
Figure 9. A transmission electron micrograph of a higher magnification of two fused and calcifying primordia (P). Arrows indicate the electron dense border of the calcifying region which fuses the two primordia.

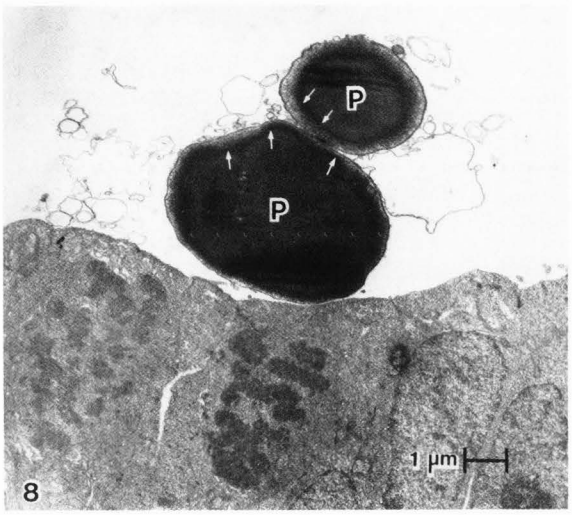


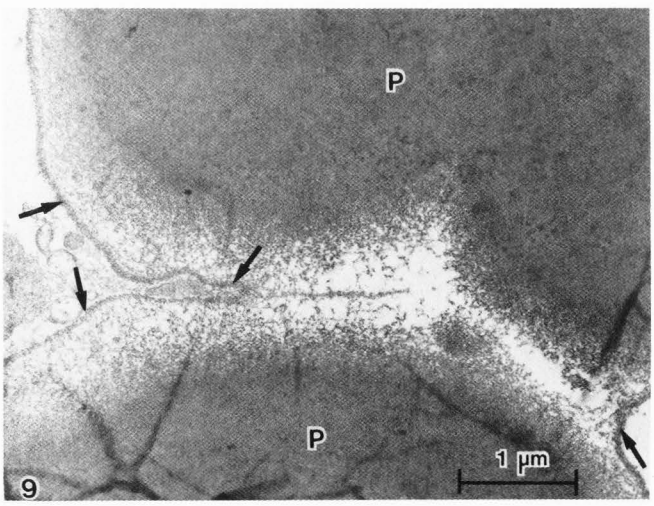


Figures 4 & 5. Scanning electron micrographs from an adult Opsanus showing the medial (Fig. 4) and lateral (Fig. 5) sides of the sagitta. The sulcus acousticus (SA) is found on the medial face.









of this area resembles the light staining density of the calcium layers seen in the adult. In addition, the otolith has a chalk-like consistency during dissection in this period of development. The calcified primordia also resemble those previously observed in Fundulus and Salmo (Neilson et al., 1985; Radtke and Dean, 1982).

Transmission electron microscopy shows that as the calcified layer enlarges, thin, electron dense strands extend throughout this part of the nucleus (Fig. 10). These strands are similar to those connecting the primordia in 17-20 somite embryos. Scanning electron microscopy also shows strands extending from the surface of cells of the developing macula during the later stage of development (Fig. 11). As previously mentioned, these strands were seen with the TEM during earlier stages of development. These results additionally suggest that those strands within the developing otolith find their origins from the macular cells.

The primordia in different species of fishes are either separated or fused during embryonic development (Brothers, 1984; von Noorden 1883). In the toadfish, a nucleus with fused primordia generally has a rosette shape, (Fig. 12), and is found in the saccular otolith, whereas separate primordia occur during formation of the utricular otolith (Fig. 13). This type of separation or fusion, in addition to the number of primordia, may define the size of the nucleus (Neilson et al., 1985).

Brothers (1984) suggests that the size and shape of the primordia and nucleus is a taxonomically useful characteristic. For example, in the Salmoniformes the primordia are separate while in the Atheriniformes the primordia are grouped tightly. Fishes such as gobies (Gobiidae) and their relatives (Microdermidae, Eleotridae, and Gobioididae) have thin elongate primordia (Brothers, 1984). In contrast, Salmo (Neilson et al., 1985), Fundulus (Radtke and Dean, 1982) and Opsanus have spherical-shaped primordia (Fig. 8), although, the initial form of the primordia in Opsanus is oblong (Fig. 7). However, environmental factors such as water temperature may govern the deposition of calcium in the nucleus - higher temperatures causing an increase in calcium deposition on the primordia (Neilson et al., 1985). This variable would lead one to question the efficacy of using nucleus size as a taxonomic characteristic (Neilson et al., 1985).

The border of the homogeneous calcified nucleus is characterized, in TEM, by a thin, electron dense layer surrounded by thinner incremental layers. The development of these increments begins either in the embryo or larva depending upon the species. For example, increment formation begins in the embryos of Fundulus on post-fertilization day 11 (3 days prior to hatching) (Radtke and Dean, 1982) as compared to the grunion (Leuresther tenuis) which has two increments at hatching. The anchovy (Engraulis mordax) has two increments 6 days after hatching (Methot and Kramer, 1979).

The total number of increments prior to hatching was not determined in Opsanus embryos. However, the otolith grows larger 3-6 days prior to hatching by the addition of a large second layer on the nucleus (Fig. 14). Growth continues in this fashion so that by 2 weeks post-hatching the otolith, which is completely surrounded by the membranous sac (Fig. 15), has approximately 16 increments.

Figure 10. A transmission electron micrograph of the primordia (P), 5-8 days prior to hatching, surrounded by the calcified part of the nucleus, which overlies the sensory epithelium (SE). The arrows indicate the dense strands extending throughout the calcified layer (C).

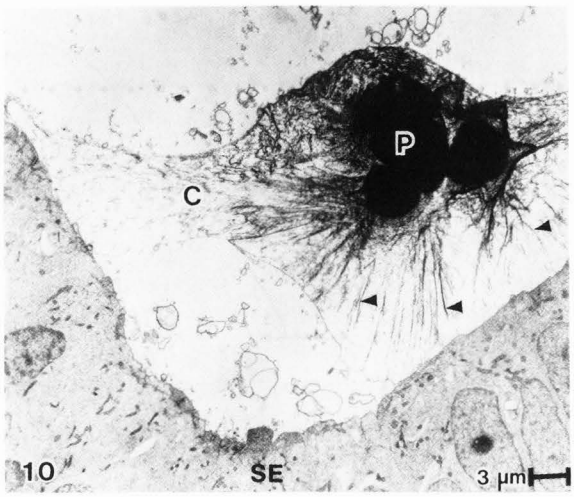
Figure 11. Scanning electron micrograph from the embryo of a toadfish, 5-8 days prior to hatching, showing the strands (black arrows) which extend across the surface of a saccular macula in which the otolith has been removed.

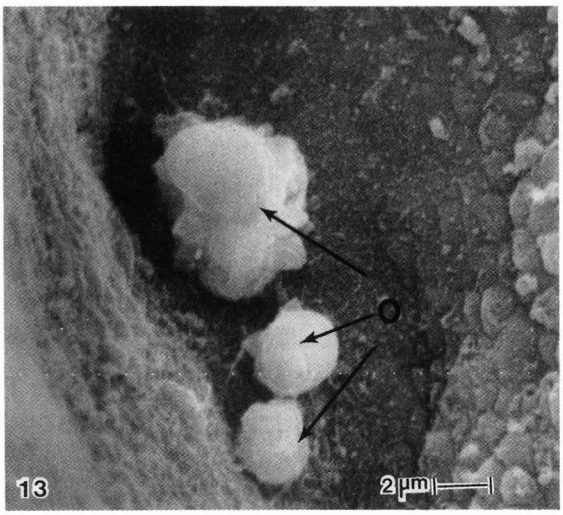
Figure 12. A scanning electron micrograph from the embryo of a toadfish, 5-8 days prior to hatching, showing the rosette-shaped nucleus of the developing sagitta (O). The sensory cells (black arrows) are under and adjacent to the otolith.

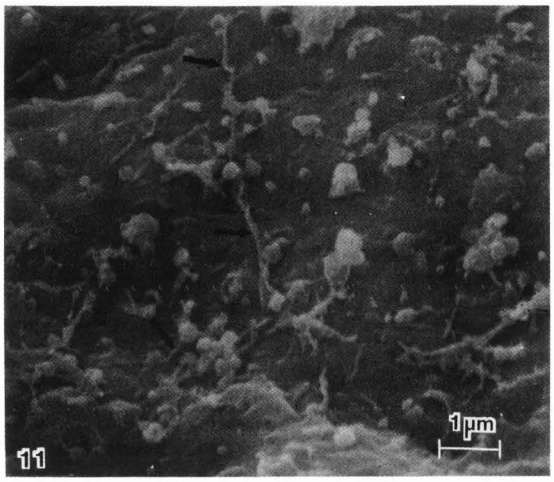
Figure 13. A scanning electron micrograph of two calcifying primordia (O) which are separated from several fused and larger primordia (O) in the developing lapillus.

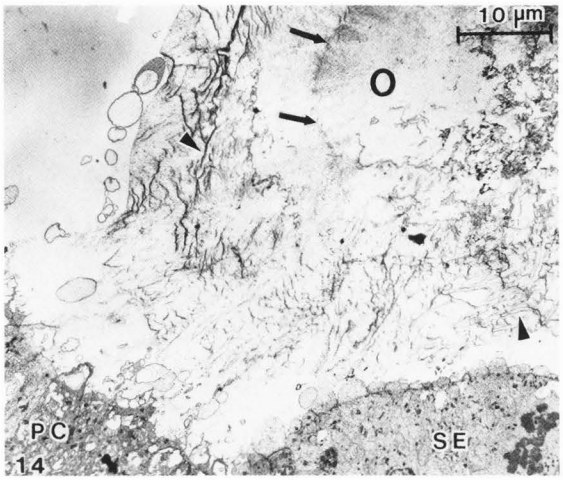
Figure 14. A transmission electron micrograph of the sagitta from the embryo of a toadfish 3-6 days prior to hatching. A calcified layer (black triangles) surrounds the nucleus which is demarcated by a thin, electron dense line (arrows). The otolith (O) lies adjacent to peripheral cells (PC) and overlies the sensory epithelium (SE).

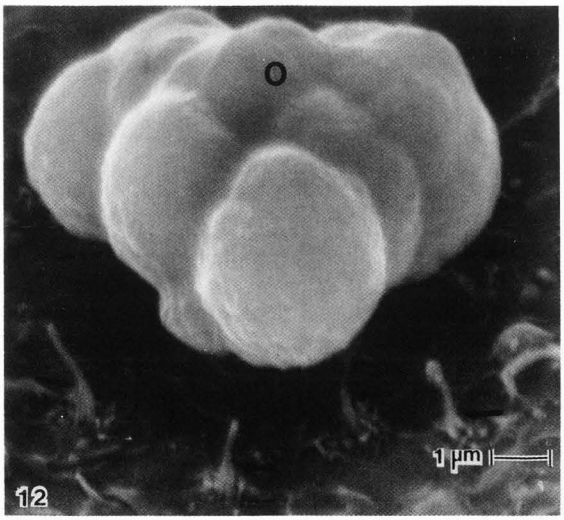
Figure 15. Transmission electron micrograph of the sagitta from a two-week old post-hatched toadfish. The otolith (O) sits over the otolithic membrane (OM) and the underlying sensory epithelium (SE). The otolith is surrounded on its lateral side by the labyrinth epithelium (LE).

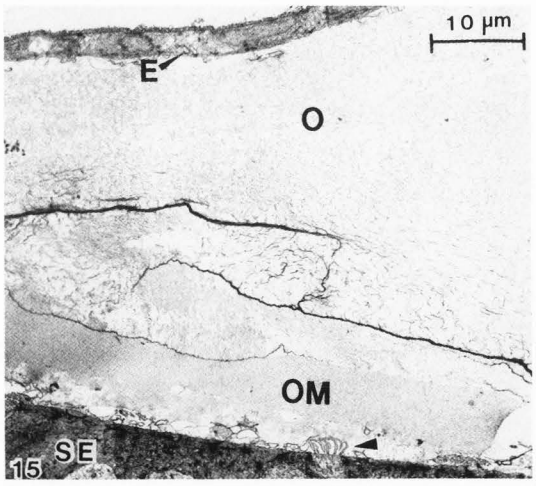












The sagitta in Opsanus larvae is a flattened sphere with an ill-defined sulcus (Fig. 16) which is typical for most larval fishes (Brothers, 1984). However, otoliths may change shape, corresponding to a change in life cycle from larva to juvenile (Brothers, 1984). This transition is especially prevalent

in the sagitta (Brothers, 1984) which has the most inter-specific variability in shape. In Opsanus, the sagitta begins changing shape during the second week of larval development so that by 14 days post-hatching the shape of the otolith appears more adult-like with a sulcus that resembles the shape of the adult macula (Fig. 17). This period in development corresponds to the time in which the sessile larva becomes a free-swimming juvenile.

The transition in the external form of the otolith, particularly in otoliths with complex shapes, may be the result of "secondary growth centers" (i.e., peripheral nuclei) within the otolith (Brothers, 1984). These secondary foci may take the form of otoconia which adhere to the developing otolith of the larva, as seen in species such as Anguilla rostrata (American eel), where the otoconia are found within the incremental bands of the otolith (Brothers, 1984). Peripheral nuclei, outside of the central nucleus, may also be found in the incremental bands of flounder and trout (Campana, 1984; Neilson et al., 1985). Campana (1984) indicates that the peripheral nuclei occur around the time of metamorphosis in both wild and laboratory-reared flounder.

Otolithic Membrane

The otolithic membrane of the adult toadfish has two different structural characteristics. The region of the membrane closest to the sensory epithelium has thin fibers or strands whereas the area of the membrane which overlies this region and abuts the otolith has a thick, honey-combed appearance (Fig. 18). These two characteristics are found both in the otolithic membrane of the lapillus and the sagitta. The honey-combed layer is present in the 6-8 month old juvenile but is absent in the larva (Fig. 15).

Dunkelberger et al. (1980) found two distinct parts to the otolithic membrane in juvenile Fundulus. They defined the thick, honey-combed area, adjacent to the otolith as the gelatinous layer and the thin fibrous region adjacent to the epithelium as the subcupular layer. The gelatinous layer was found only under the sulcus acousticus whereas the fibrous layer was found under both the sulcus and the peripheral regions on the medial face of the otolith. Furthermore, TEM showed that the fibers of the subcupular layer projected into the otolith. These results, in addition to previous work showing that ^{45}Ca was incorporated at the edges of the otolith on its medial side (Mugiya, 1974), led Dunkelberger et al. (1980) to hypothesize that the subcupular layer has a role in otolith formation.

Vertebrates, other than fishes, have a two-layered otolithic membrane (Marco et al., 1971; Igarashi and Kanda, 1969) of which the subcupular layer may contribute to forming the organic matrix component of the otoconia. This contribution is suggested in several mammals which show a similarity in structure between a portion of the otoconia and the otoconial membrane (Lim, 1973). For example, the guinea pig has a subcupular layer that may contribute to forming the otoconia in the utricle (Marco et al., 1971).

The subcupular layer is the first to develop in the embryo of the toadfish whereas the gelatinous layer develops in the juvenile. In Opsanus, the subcupular layer begins developing 8-11 days prior to hatching (Fig. 7), and is clearly seen 3-6 days prior to hatching (9-10 days post-fertilization) (Fig. 19). Furthermore, calcification of the primordia begins after strand formation of the subcupular layer, suggesting that these strands are incorporated within the early calcifying matrix. These data support the hypothesis of Dunkelberger et al. (1980).

Additional strand formation with calcification may continue similarly at later stages of embryonic, larval, juvenile, and adult development;

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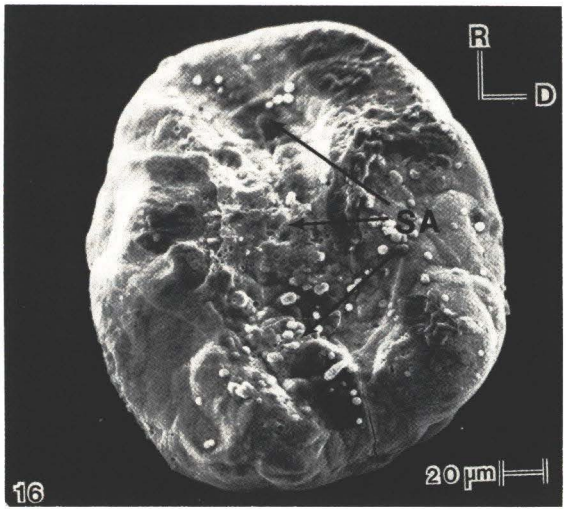
Figures 16 & 17. Scanning electron micrographs showing the sulcus acousticus (SA), on the medial sides of two sagittae taken from Opsanus: one from an 8 day-old (Fig. 16) and the other from a 14 day-old post-hatchling (Fig. 17).

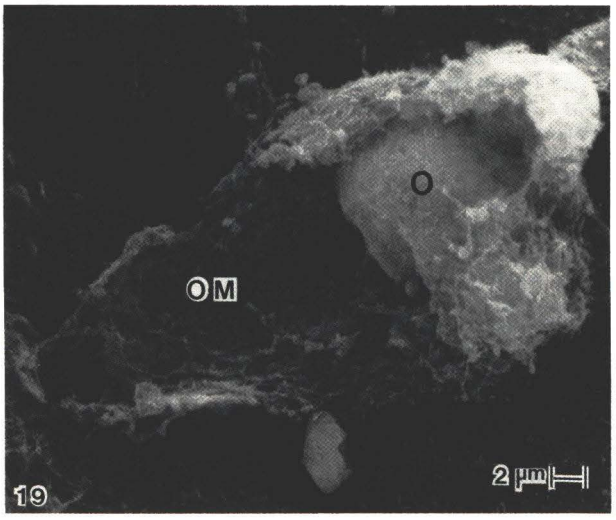
Figure 18. A scanning electron micrograph from a 7-8 month old juvenile Opsanus showing the gelatinous (G) and filamentous (F) portions of the otolithic membrane. Arrows indicate the sensory cells.

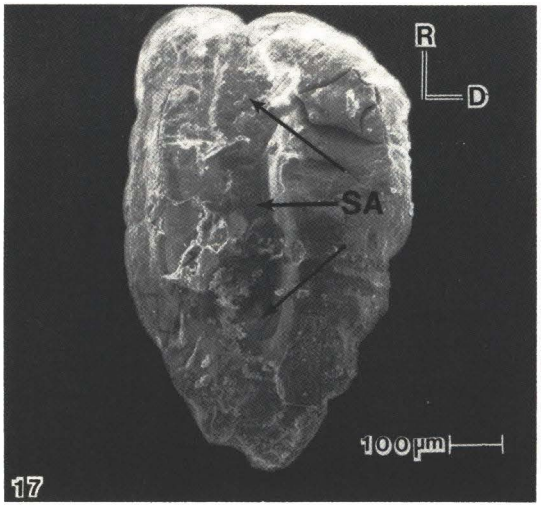
Figure 19. A scanning electron micrograph from a toadfish embryo, 3-6 days prior to hatching, showing the otolith (O) and the fibrous region (i.e., subcupular layer) of the otolithic membrane (OM).

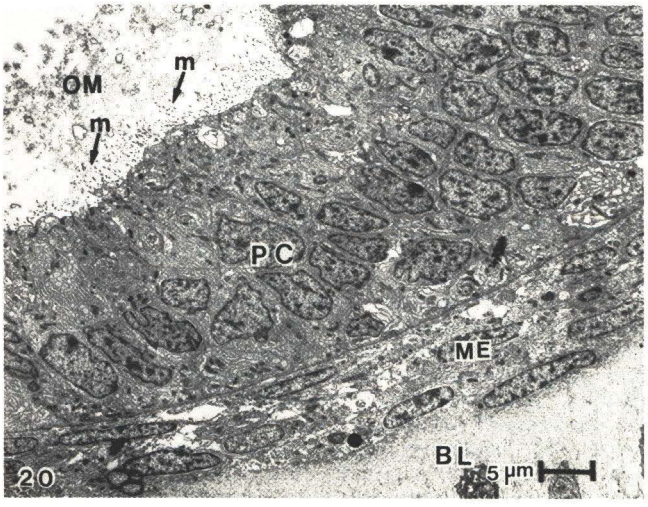
Figure 20. A transmission electron micrograph from the embryo of a toadfish 3-6 days prior to hatching showing the striated peripheral cells (PC) with their microvilli (m) and the overlying otolithic membrane (OM). Mesenchymal cells (ME) and the bony labyrinth (BL) lie adjacent to the epithelium.

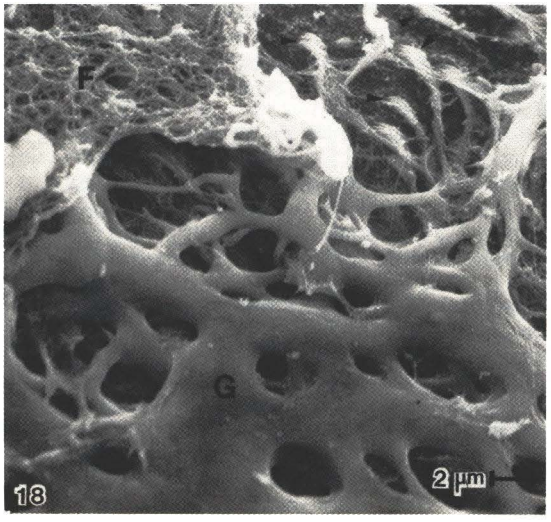
Figure 21. A transmission electron micrograph showing a higher magnification of peripheral cells with their rough endoplasmic reticulum (arrows) and the overlying otolithic membrane (OM).

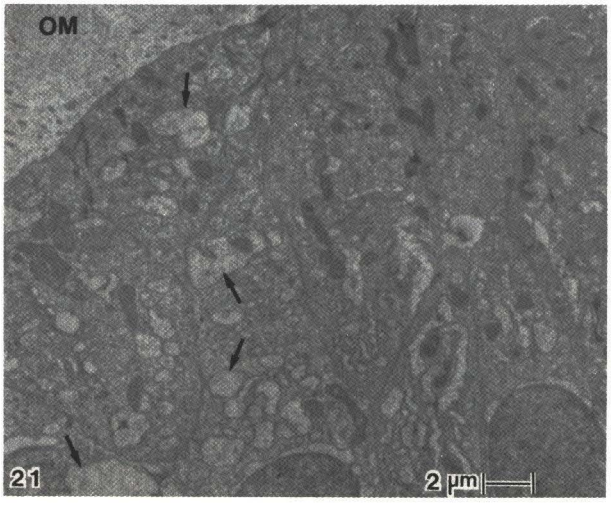

R
D
SA
20 µm
16


O
OM
2 µm
19


R
D
SA
100 µm
17


OM
m
m
PC
ME
BL
.5 µm
20


F
G
2 µm
18


OM
2 µm
21

although, this determination was not made in Opsanus. In comparison, adult goldfish deposit ^{45}Ca in the otolith on a diurnal basis with deposition decreasing at sunrise (Mugiya et al., 1981). This result suggests that organic and inorganic deposition may alternate during the formation of the adult otolith. An initial phase of organic matrix followed by calcium deposition is also seen in vertebrates, such as chickens, where formation of the otoconia primarily occurs in embryonic development (Marmo, 1982; Fermin and Igarashi, 1985).

Peripheral Cell Development in the Saccular Epithelium

The addition of a calcium layer around the nucleus of the sagitta and the increased growth of the otolithic membrane occurs three to six days prior to hatching. During this period of development two distinct groups of support cells morphologically differentiate. These cells consist of support cells within the macula (Sokolowski, 1985; Sokolowski and Popper, in preparation) and cells which lie at the borders of the developing sensory epithelium. Both cell types have apically placed microvilli and an abundance of rough endoplasmic reticulum (Figs. 20 and 21). Although there is no direct evidence for a secretory function of these cells in the toadfish, evidence for a possible secretory function is found in the support cells of adult lamprey (Lowenstein et al., 1968) and in the macular cells of adult trout (Mugiya, 1974). Furthermore, cells with an abundance of rough ER have been observed in the transitional zones of the saccular and utricular epithelia in rat fetuses (Ciges et al., 1983). The transitional zone in rats is an enlarged area, and like the peripheral cells in Opsanus, is found adjacent to the neuroepithelium. Ciges et al. (1983) suggest that these cells may be responsible for secreting the organic and inorganic components of the otolith during a critical period in development. Based upon these observations, it is likely that the rough ER, in Opsanus, is responsible for producing some of the components necessary in forming the otolith and its membrane.

The proliferation of these two cell types in the toadfish embryo, three to six days prior to hatching, does not answer the question of where the early (17-20 somite) embryonic forms of the otolith and otolithic membrane originate. The only morphological changes occurring during development in the otocyst of the 17 somite embryo are the development of the sensory cells (Sokolowski, 1985; Sokolowski and Popper, in preparation). The fibers which radiate from the developing stereocilia may be secreted by the stereocilia, although further evidence is needed to support this premise.

Additional analyses are necessary to determine the source or sources of these early primordia and the mechanisms by which they form as well as the relationship of the strands to otolith and membrane development. An important consideration is to identify the specific constituents of the developing otolith and otolithic membrane and its underlying epithelium. Radioactive labelling studies and immunocytochemical techniques would contribute to answering some of these questions of development in the embryo.

Acknowledgements

I gratefully acknowledge Dr. Arthur N. Popper for his criticism and support during this project. A special thanks goes to Miss Becky Hoxter and Mr. Oscar Rodbell for their valuable assistance during various parts of this project and to Dr. George Spirou for reading an earlier version of this manuscript. This study was supported by NIH grant NS 21646 from the National Institute of Neurological and Communicative Disorders and Stroke and by NSF grant BNS 82-08335 to Dr. Arthur N. Popper and by grants from Sigma Xi and the American Museum of Natural History (Lerner-Gray Fund for Marine Research) to Bernd H. A. Sokolowski. This study is part of a dissertation presented by B. H. A. Sokolowski to the faculty of the Graduate School of Georgetown University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Anatomy and Cell Biology.

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Discussion with Reviewers

S. Campana: While some evidence for the spatial origin of the filaments is presented in the manuscript, the relative timing of the development of the macula, primordia and otolithic membrane does not necessarily support the interpretation that the macula is the source of the filaments observed in the otolith. Do you have any other evidence to support your conclusion?
Author: At this point I do not have any other evidence other than structural and temporal. However, if these strands in the embryo consist of glycosaminoglycans as found in adult teleosts, then the necessary cellular organelles, such as ribosomes and Golgi apparatus are present for making the necessary products used in assembling this large structure extracellularly. If this is the case, of interest in the late embryonic cells is the increase in RER, suggesting that the protein constituents of the otolithic membrane produced may be different in the early as opposed to the late embryonic cells.

S. Campana: At what stage of development were fused primordia first noted? And when did calcification first enmesh all of the primordia within a single structure?
Author: Fused primordia were first observed 5-8 days prior to hatching (6-7 days post-fertilization) at about the time the calcified layer around the primordia first begins.

S. Campana: When in the juvenile stage did development of the gelatinous layer first begin?
Author: During the first to second month of juvenile development. The exact time was not determined since specimens were not fixed daily during the juvenile period.

S. Campana: This study has clarified the developmental process and its timing in Opsanus sagittae; when did the lapillae and asteriscii first appear? Did their development parallel that of the sagittae?
Author: The lapilla appears at approximately the same time as the sagitta, in the 17 day somite embryo. In contrast, the asteriscus appears about 10-14 days later, just after hatching. By this time the lapilla and sagitta are well developed.

S. Campana: Is there an ultrastructural reason for the lagged development of the asteriscus relative to the other otoliths? Why does the relative timing among the otoliths vary so much interspecifically?
Author: Both the gross and ultrastructural development of the lagena lags behind that of the saccule and utricle by about 7-10 days. Therefore, the asteriscus develops at a much later time. The relative timing among the otoliths varies so much interspecifically as a consequence of the varying development of the gross and ultrastructural parts of the different endorgans. Some fishes, such as sturgeons, develop their peripheral endorgans quite rapidly (5 days) (Titova, 1970), whereas others such as the lamprey (120 days) (Thornhill, 1972) and the toadfish (30 days) take a longer time. Toadfish embryos and early larvae are sessile, thus they possibly do not require the use of these peripheral structures. In addition their peripheral nervous system is not fully developed (Sokolowski, 1985; Sokolowski and Popper, in preparation)

D. Lim: The multilayer formation of the otolith with alternating bands of organic and inorganic layers is a fascinating aspect of biology. Could you elaborate on the current concept or hypothesis of how layers of calcified

and uncalcified structures are formed, reflecting age or seasonal changes.
Author: The increments, which include incremented (calcium) and discontinuous (organic) zones are laid down on a daily 24 hour cycle. Consequently, these layers can be used to age a particular species. However, variables such as photoperiod, temperature, and feeding may affect deposition in a way that causes an irregular layer of growth. These irregular layers may be formed in addition to the daily layers (Campana and Neilson, 1985) and are known as subdaily increments. The mechanisms controlling layering is not known although Campana and Neilson (1985) hypothesize that it is endocrinological. Some supporting evidence may be provided by Tanaka et al. (1981) who showed that a shift in the photoperiod shifts the time of increment deposition. However, more studies are needed to support this endocrine hypothesis.

D. Lim: I am very much fascinated by the fact that the otolith is originally formed as multiple crystals to form a nucleus. The micrographs are very convincing, and it is even more amazing to see that the early formation of the otoliths is remarkably similar to a more advanced otolith. It appears that the strands connecting the sensory epithelium to the nucleus are also incorporated into the otolith. Could you speculate on how the mechanism of calcification can take place to include these strands?
Author: Inclusion of the strands into the calcium layers may take place by alternate deposition of one or both products: calcium and the organic material. Mugiya et al.(1981) showed that there is a diurnal rhythm in otolith formation in goldfish, where deposition decreases at sunrise. During this decrease in calcium uptake, the organic material may continue forming thus causing the formation of the discontinuous zone and its eventual inclusion in the next layer of calcium.

D. Lim: One of the micrographs (Fig. 8) does not show any strands at all of subcupular layer when the primordia of the otolith is being formed. However, you state that you found strands of the subcupular layer first (prior to otolith formation?). If this is true, then the sequence of morphogenesis of the otolith in fish is different from that of the otoconia in mammals, particularly the mouse (Anniko, Development of otoconia, "American Journal of Otolaryngology" 1:400, 1980; Lim, The development and structure of the otoconia, in "Ultrastructural Atlas of the Inner Ear", Friedmann and Ballantyne (eds), London, Butterworths, 1984). Would you comment on this?
Author: The strands in TEM sections of the early embryos (17-25 somites) were always found in the vicinity of the developing kinocilia and stereocilia. If the primordia were not cut in the proper plane to include several of these structures, as in Fig. 8, the strands were not seen. In addition, SEM of the surface of the developing sensory cells with otoliths present or removed (Sokolowski and Popper, in preparation) also did not show any strands until about 5-8 days prior to hatching (Fig. 11) when strands appeared more abundant. This lack of strands in the SEM may be a result of preparation because inner ears prepared for TEM were left in situ, whereas ears prepared for SEM were dissected out and opened, exposing the sensory epithelium to fluids used in dehydration and critical point drying. The other possibility may be that these strands are broken from the surface and incorporated into the calcifying primordia, although, I think that the loss is a function of preparation.

D. Lim: You say, "Furthermore, calcification of the primordia begins after strand formation of the subcupular layer, suggesting that these strands are incorporated within the early calcifying matrix." This statement is somewhat confusing because the subcupular layer and gelatinous layer are not known to be calcified, presumably because their biochemical composition is distinct from that of otoliths. Would you clarify this statement?
Author: The strands which originally form and project to the primordia may provide the initial form of the organic matrix on which the calcium is laid. Furthermore, if this type of strand formation and deposition continues, these strands may become part of the organic matrix of the otolith. This is particularly intriguing in light of the evidence of Dunkelberger et al., (1980) showing that the subcupular layer projects into the otoliths of the juvenile mummichog.

D. Dunkelberger: I do not see any evidence of calcification in or on the primordia that you state is taking place in Figures 8, 9, and 10. (The electron dense areas in Figure 9 do not resemble calcium carbonate crystals.) How was this determined? Did you perform electron diffraction analysis to determine this as well as the composition of the otolith? Can you describe where calcification begins?
Author: I am suggesting that once the primordia are formed, the light staining areas of the layer forming outside the

dense primordia is a combination of organic and inorganic (i.e., calcium) components. This region did not stain heavily with toluidine blue nor with osmium. It also has the same staining characteristics seen in the calcium layers of the adult. This layer is also the first increment surrounding the primordia and thus forms the calcium layer of the nucleus. Its formation is demarcated from the densely staining boundary of the primordia as seen in Figure 8. Another bit of evidence was indicated during dissection where the otolith has a chalky consistency when breaking it during this age.

D. Dunkelberger: You have said that similar "strands" connect the primordia, radiate through the nucleus, and extend from the surface of the macula. Can you describe these "strands" in more detail? Have you looked at them at higher magnification with TEM in order to see, for instance, if they are composed of small fibers?
Author: Higher TEM magnification of the "strands" in various serial sections indicate that they are small spheres adhering to one another to form a "strand" as opposed to microfilament-like structures.

D. Dunkelberger: The only intracellular evidence for secretion that you describe is the presence of RER in the cells. Have you looked at any other areas such as the Golgi, or abundance of Golgi vesicles? What do you think the extracellular vesicular structures represent that are seen in Figures 7, 8, 10 and 14?
Author: Golgi and Golgi vesicles were always present in both the early morphologically undifferentiated cells and the later differentiated cells. However, there appeared to be no increase in Golgi vesicles during development although I did not quantify this. The extracellular vesicles, which you as well as others have described as possibly being related to the formation of the otolith or its membrane, were present throughout the developmental period in the embryo. This was the case even when there was no evidence for a forming otolith in the early otocyst (10-12 somites). The possible function of these structures totally eludes me at this time.