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Structural basis for mechanical transduction in the frog vestibular sensory apparatus: III. The organization of the otoconial mass

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Abstract

The saccule and the utricle of the vestibular system detect linear acceleration and gravity. Sensory transduction in these organs depends on myriads of calcium carbonate crystals of high specific gravity, called otoconia, embedded in a filament matrix that overlies the sensory epithelium. The coexistence of hard crystals and slender filaments in this complex extracellular matrix makes it difficult to analyze by conventional electron microscopy. We have now examined this structure in the bullfrog saccule using the quick-freeze, deep-etch replica technique. The otoconia in their typical aragonite polymorph shape exhibit smooth surfaces and are embedded in a loose matrix made of two types of filaments. The regular surface of the otoconia forms a natural smooth background against which we could observe with unprecedented detail the network organization and substructure of the filaments. One type of filament is 8 nm in diameter, while the other, which has a characteristic beaded appearance, is 15 nm in diameter. Both types of filaments either make lateral connections with or end directly on the surface of the otoconia. A consistent observation was the presence of short filaments that directly cross-link adjacent otoconia. Very few otoconia were fractured in an orientation that would allow the study of their internal architecture. These otoconia presented a typical conchoidal cleavage of aragonite. Although crystallites were not clearly apparent, thin lamellar microstructures appeared oriented both perpendicularly and longitudinally to the major otoconial axis. This structural study establishes a framework for the identification of the molecular components present in this unique extracellular matrix and may also help elucidate their role in mechanical transduction.

Key words: Electron microscopy; Freeze-etching; Otoconium; Vestibular system; Otolithic membrane; Mechanical transduction; Frog vestibular sensory organ

1. Introduction

The organization of the vestibular system in all higher vertebrates is similar. It consists of a membranous labyrinth with three semicircular ducts within their corresponding bony canals and the utricle and saccule within the bony vestibule. Within the membranous labyrinth the sensory epithelia are bathed in endolymphatic fluid. The epithelia of these receptor organs share a common mechano-electrical transduction mechanism. Through deflection of the hair cell stereocilia, this mechanism transduces an external mechanical stimulus into a conductance change and subsequent electrical response within these cells. The specificity of a particular sensory epithelium to a particular type of mechanical stimulus depends on intricate mechanical interactions between specialized extracellular accessory structures that are part of the complex architecture of each organ.

A dome-shaped cupula made of a gelatinous material that overlies the sensory epithelia of the semicircular canals translates endolymphatic fluid movement into a

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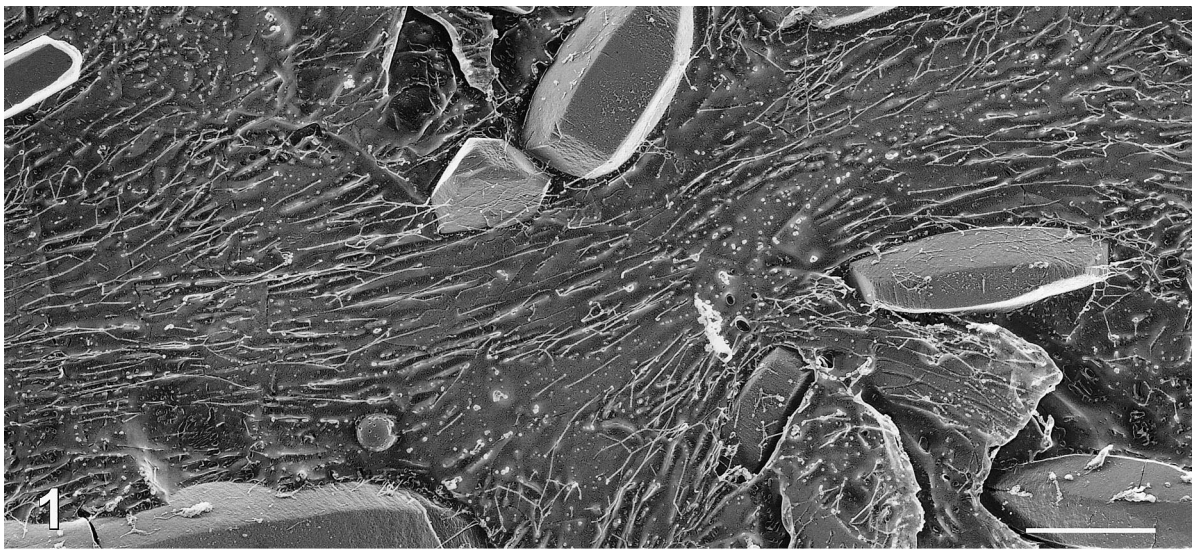
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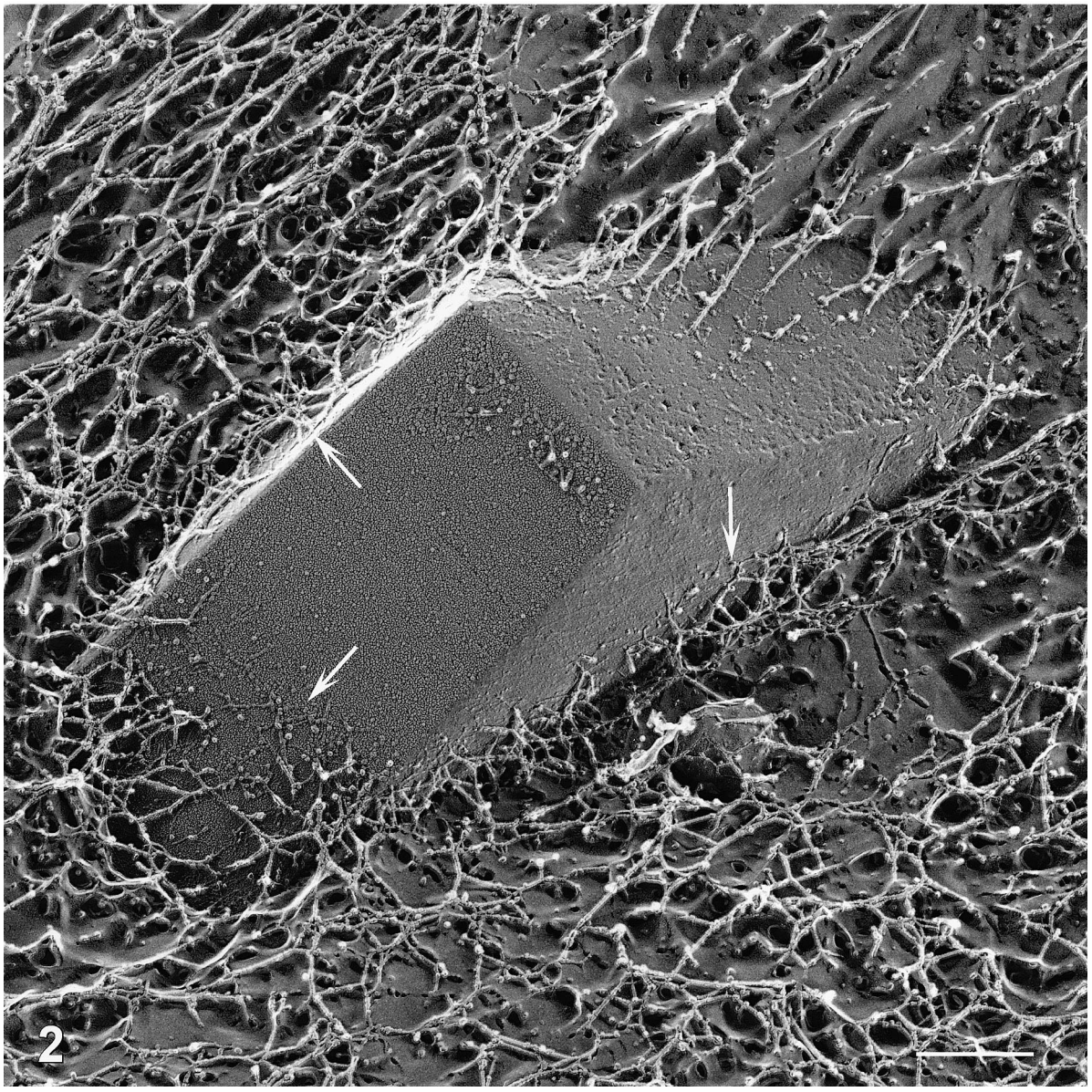
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stimulus detectable by hair cells during angular acceleration of the body. The sensory epithelia in the horizontally oriented utricle and in the vertically oriented sacculae are responsible for monitoring changes in linear acceleration and body position with respect to gravity. One characteristic feature of the saccule and utricle is the presence of the otolithic or otoconial mass on top of the sensory epithelium within the endolymphatic chamber (Hillman, 1976; Lim, 1984; Hunter-Duvar and Hinojosa, 1984). This otolithic mass is made of thousands of calcareous crystals, the otoliths, that lie over an extracellular formation called the otolithic or gelatinous membrane. This membrane consists of two structurally different layers of extracellular filamentous material (Kachar et al., 1990). When the animal experiences linear acceleration, the otolithic mass is sheared in relation to the surrounding fluid and tissue because of its high density and specific gravity. This relative inertial movement of the otoliths is transmitted to the hair cell by the otolithic membrane in such a way that a fraction of its mechanical energy is used to deflect the stereocilia bundles.

The otoliths are biominerals made of a complex arrangements of specific proteins, carbohydrates and several polymorphs of calcium carbonates ($CaCO_3$) (Pote and Ross, 1991) that are present in the gravity sensory epithelium of many invertebrates and all vertebrates (Lim, 1984). Otoliths have precisely controlled crystal sizes, morphologies, structure and growth orientation (Lowenstam, 1991). Teleosts have a single large otolith per otolithic organ (Gauldie, 1993), while amphibians, reptiles, birds and mammals have thousands of microscopic otoliths called otoconia (Pote and Ross, 1991). Numerous otoconia are held together by interactions with a very delicate matrix of extracellular filaments to form an integrated mass with specific physical properties and chemical composition. This structure serves the mechanical transduction function and at the same time is sufficiently plastic to accommodate the dynamic processes of development, growth, adaptation and regeneration.

Current understanding of the organization and structure of the otoconial mass is based mainly on scanning electron microscope studies (e.g. Lim, 1984; Marmo et al., 1981; Kido and Takahashi, 1997), although more recently atomic force microscopy has also been used (Hallworth et al., 1995). Because of the procedures involved and the characteristics of the specimen preparation required, these techniques do not provide sufficient resolution to visualize the structural relationships of the otoconial surface with the loosely arranged network of extracellular filaments that adhere to it. We have now used the freeze-etching technique to examine the otoconial mass of the bullfrog saccular and utricular maculae. Because of the regular shape and smooth surface of the mineral based otoconial matrix and the resolution of this technique, we could observe with unprecedented detail the substructure of the filaments, and how they interact with the otoconial surface and structurally connect the otoconia into an integrated otoconial mass.

2. Materials and methods

2.1. Freeze-etching of the frog's saccular macula

American bullfrogs (*Rana catesbeiana*) weighing 100–200 g (Carolina Biological Supply Co., Burlington, NC) were anesthetized with 20 μg/g of 3-aminobenzoic acid ethyl ester and decapitated. The membranous labyrinth was carefully dissected out in frog Ringer's and fixed in 2% glutaraldehyde in the same buffer for 1 h. After several washes in distilled water, the saccule was isolated; part of the gelatinous membrane was left attached to the sensory epithelium. Samples were then processed for freeze-etching as described below.

Following several washes in distilled water, the specimens were placed onto a 400 μm slice of Bacto-agar (Difco Laboratories, Detroit, MI) and mounted on a piece of filter paper glued to an aluminum stub. The preparations were rapidly frozen by impact with a liquid nitrogen-cooled sapphire block using a Life Cell CF-100 slamming machine (Research and Manufacturing Co., Tucson, AR) and promptly transferred to liquid nitrogen. The specimens were freeze-fractured at −150°C in a Balzers BAF 301 apparatus, allowed to etch for 10 min at −100°C and rotary shadowed with a thin layer of atomic platinum deposited from an electron bombardment gun at an incidence angle of 15°. The platinum coat was then stabilized by a layer of electron-translucent carbon applied from a 80° angle while the specimen was rotating. All organic material was cleaned from the platinum-carbon replicas by incubation in hypochlorite bleach for 1 h followed by 5% HCl (0.6 M) to dissolve the inorganic phase. Replicas were then washed in distilled water and collected on 400

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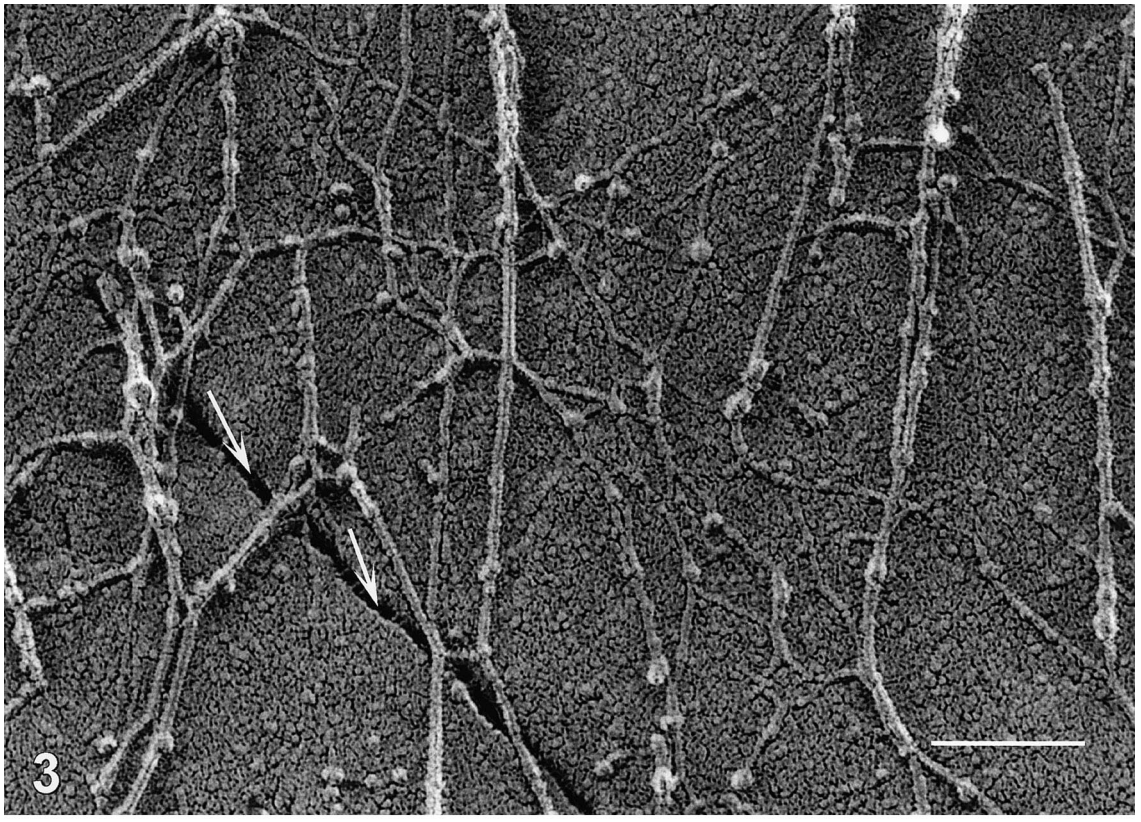
Fig. 1. Rotary shadowed platinum replica of a freeze-etched otoconial mass overlying the saccular macula of the frog. It is composed of numerous otoconia with typical aragonite polymorph structure embedded in a loose filamentous matrix. This filamentous matrix consists of short as well as long filaments that are distributed in bundles along specific directions. Bar: 2.0 μm.

Fig. 2. Higher magnification of otoconium embedded in the network of filaments. The fracture plane was near the surface of the otoconium. Interactions of the filaments with the surface of the otoconium can be seen en face or in side views due to the deep etching. The filaments appear to touch the smooth surface of the otoconium by both their side and their ends. Bar: 0.2 μm.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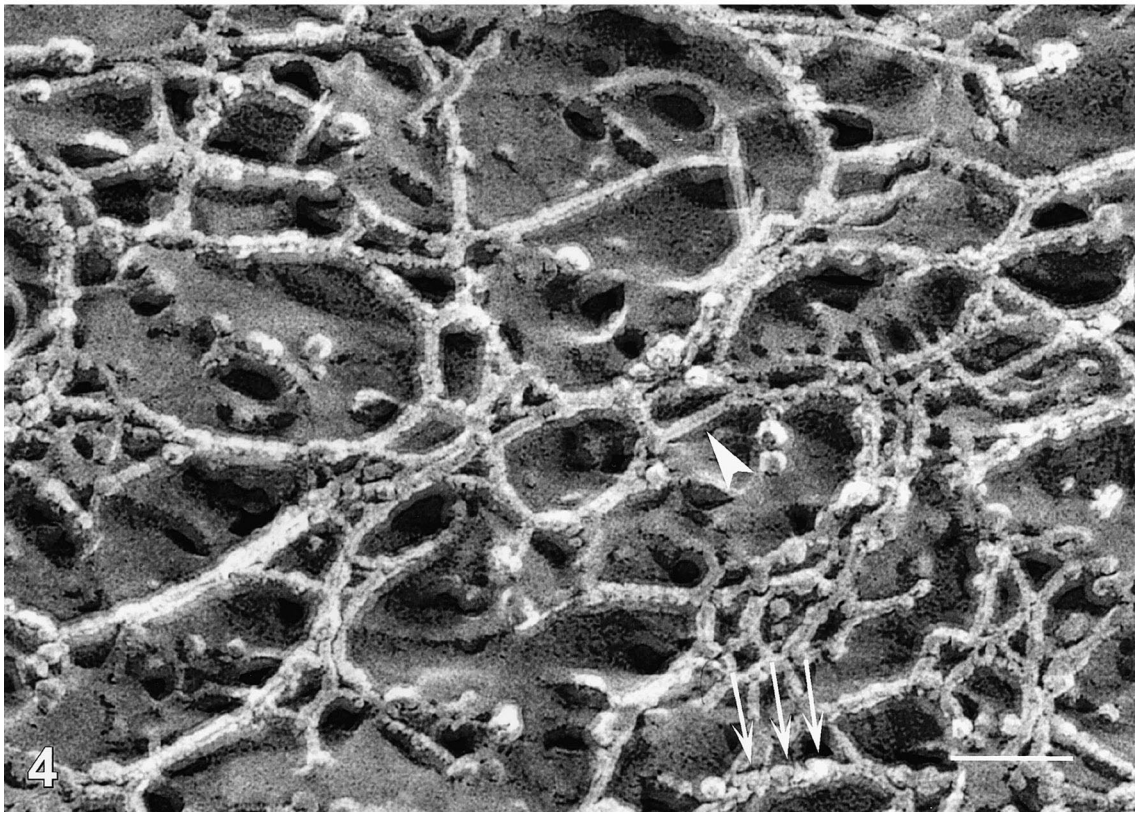
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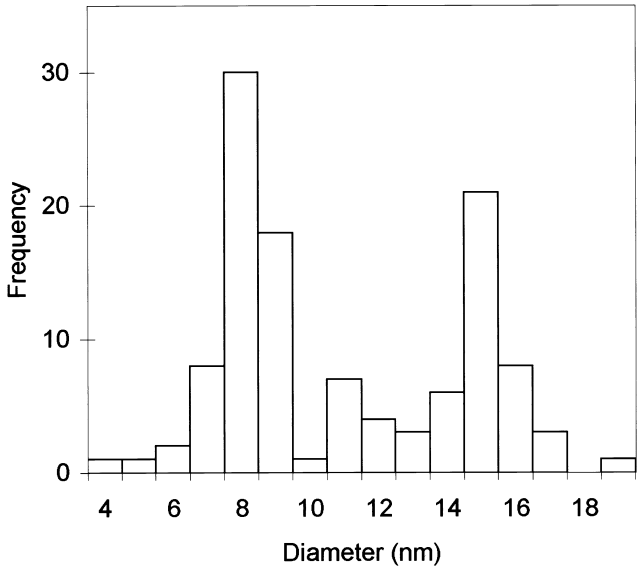

Fig. 5. Histogram of the distribution of diameters of the filaments from the otoconial filament matrix. The two most frequent diameters are 8 nm and 15 nm.

mesh grids. Electron micrographs taken with a Zeiss 902 electron microscope appear reversed, and platinum deposits appear white.

3. Results

3.1. Otoconial matrix and surface filaments

The freeze-etching technique allows a direct view of the structure and interactions of the organic and inorganic components of the otoconial mass. The otoconial mass overlying the saccular macula of the frog is composed of the myriad of otoconia with typically aragonite polymorph structure embedded in a complex loose filamentous matrix (Fig. 1). This filamentous matrix consists of long and short filaments that help to maintain the integrity of the entire otoconial mass. Most of the filaments seem to form a loose net that entraps the otoconia. Closer inspection shows that some of these filaments actually terminate directly at the surface of the otoconium (Fig. 2), whereas other filaments make lateral contacts but continue without terminating on that surface (Figs. 2 and 3).

High magnification (Figs. 2–4) shows the network organization and the structure of the filaments. Because the otoconial surface is flat and relatively smooth, it provides an excellent background for viewing the surface filaments as well as the adjacent filament network (Figs. 2 and 3). Fig. 3 illustrates how well one can resolve the individual filaments. Our images are reminiscent of images of molecular structures produced by the freeze-etching mica sheet technique (Heuser, 1983). We identified at least two types of filaments based on their thickness. Measurement of the diameter of the filaments showed a bimodal distribution, indicating that they could be sorted into filaments of 8 nm and 15 nm diameters (Fig. 5). The thinner filaments tend to be relatively smooth, whereas the thicker filaments have a beaded substructure (Fig. 4).

3.2. Otoconial surface

In order to better visualize the surface of the otoconia, we gently rinsed them with the Ringer's before quick-freezing and deep etching (Figs. 6–11). The filament matrix was disrupted permitting visualization of several loose filaments. Some of the filaments remained attached laterally or ended on to the surface of the otoconia indicating a relatively firm binding (Fig. 6). Several clumps of what appears to be a filamentous material were scattered on the surfaces of the otoconia (arrows, Figs. 6, 8 and 9). We interpret these clumps to be filaments that retracted after separation of the otoconia during rinsing or through retraction during the etching procedure. Another consistent observation was the presence of extremely short filaments interconnecting otoconia that had remained closely adherent to each other (Figs. 7 and 8). It appears that these filaments are a mixture of the thin and thick filaments found on the surface of otoconia.

Different surfaces of the otoconia had apparent differences in texture (Fig. 9). A closer observation of the replica showed that a large part of the apparent differences results from a combination of effects of the angle of shadowing and the incident angle of the electron beam of the microscope. The replicas of the surfaces of the otoconia have a peculiar granularity that changes in appearance, depending on the angle at which it is viewed (Figs. 9 and 10). This granularity is for the most part random and too coarse to reveal any fine

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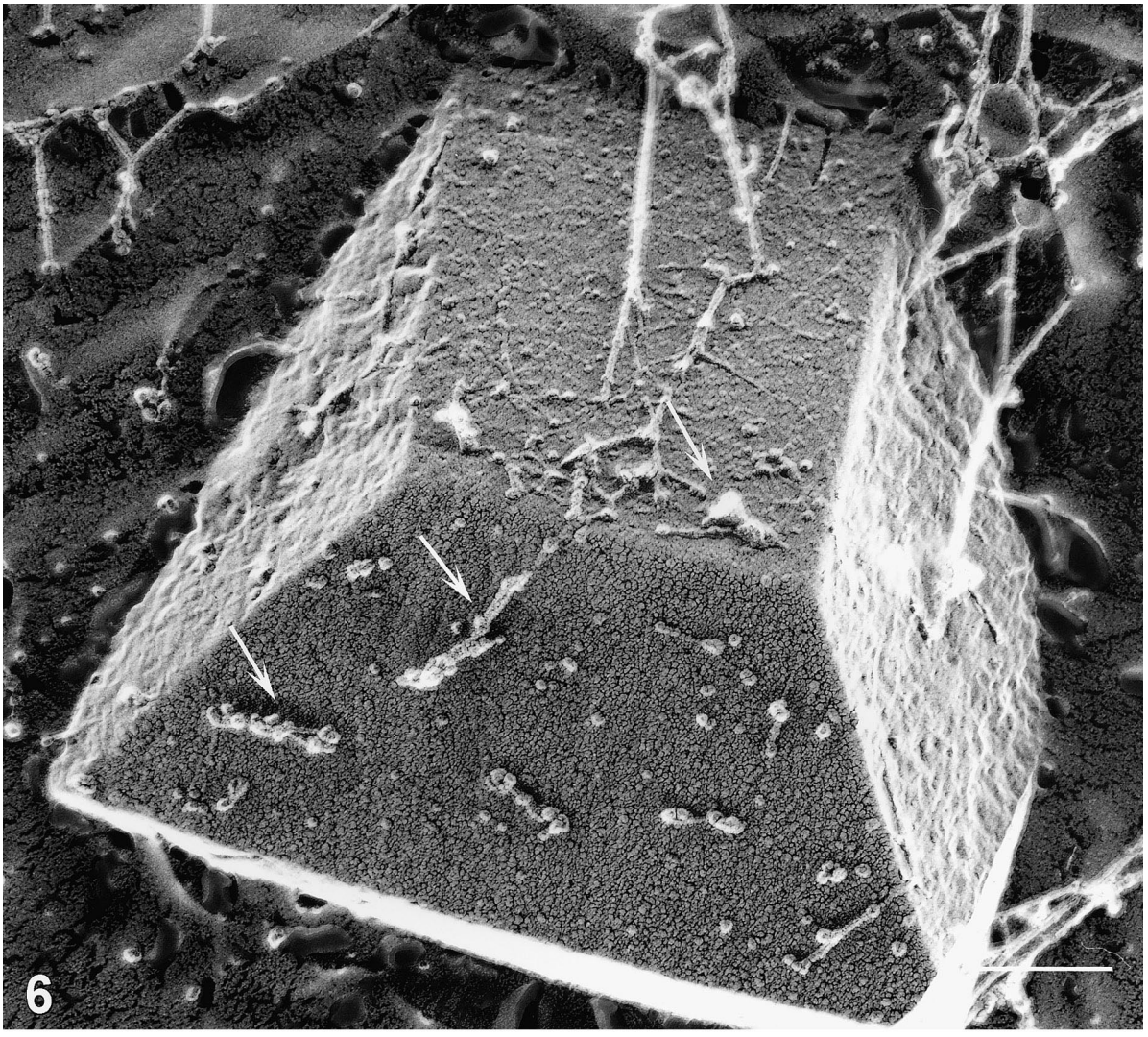
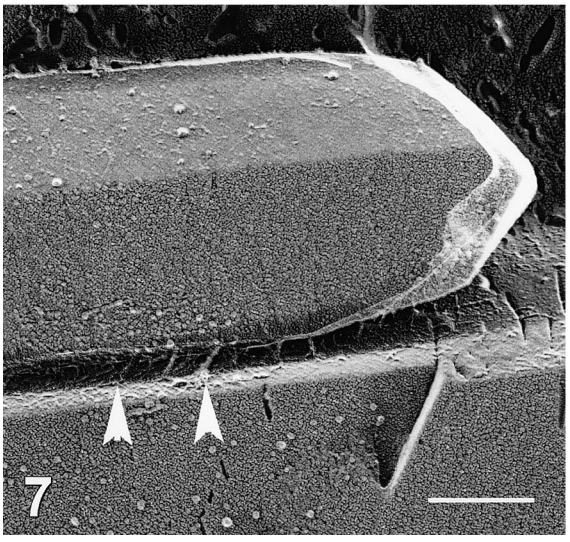
Fig. 3. High magnification view of the surface of an otoconium. Numerous thin and some thick filaments attach to the surface of the otoconium. Some of the thin filaments appear to form an anastomosing network. A fracture in the otoconial crystalline structure is seen on the lower left of the figure (arrows). Bar: 0.1 μm.

Fig. 4. Enlarged view of the filament matrix that surrounds otoconia. It consists of smooth thin filaments (arrowhead) and thick beaded filaments (arrows) forming a loose network. Bar: 0.1 μm.





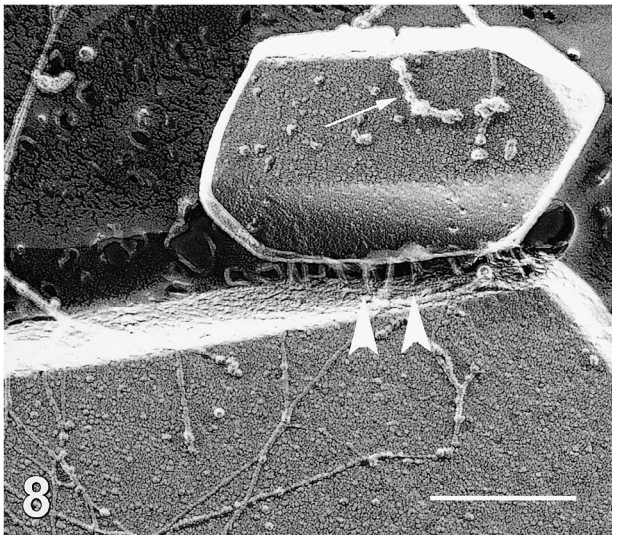
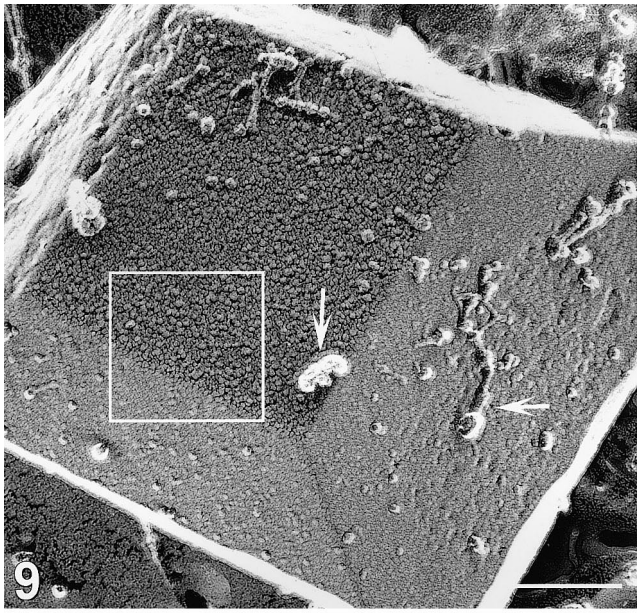


Fig. 6. View of an otoconium of a sample gently washed in Ringer's before quick-freezing and freeze-etching. Filaments are firmly bound to the lateral and end faces of the otoconium. Some filaments connect the otoconium to the surrounding matrix. Clumps of filamentous materials are observed at the surface of the otoconium (arrows). Bar: 0.2 μm.

Fig. 7. Close-up view of the space between two neighboring otoconia showing several interconnecting filaments (arrowheads). Bar: 0.3 μm.

Fig. 8. Small otoconium attached to a larger otoconium by several filaments (arrowhead). Note the presence of thin (arrow) and thick filaments on the surface of the otoconia (arrow). Bar: 0.4 μm.



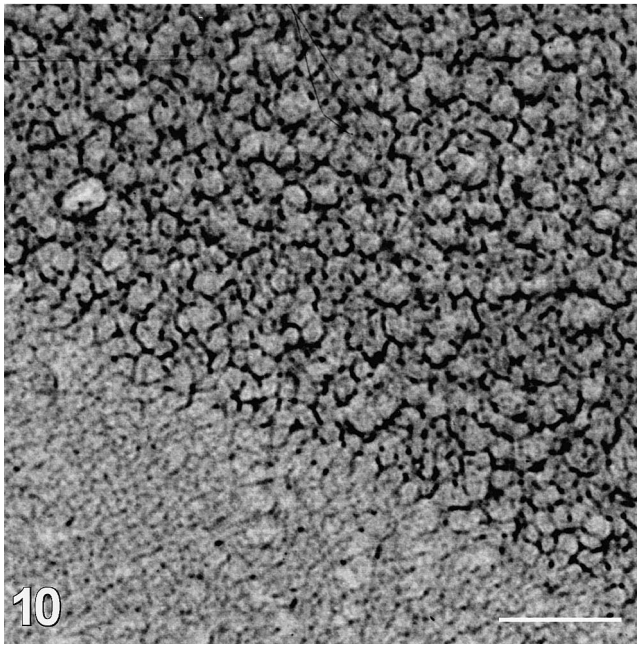


Fig. 9. Otoconium with apparent differences in the texture of its different faces. A peculiar granularity that changes in appearance depending on the face angle can be seen. Arrows indicate clumps of filamentous material. Bar: 0.2 μm.

Fig. 10. Enlarged view of the otoconium shown in Fig. 9 (white square). It can be seen that part of the difference in texture results from a combination of effects of the angle of shadowing and the angle of incidence of the electron beam on the replica. The granularity observed is for the most part random and does not reveal any fine texture of the actual surface of the otoconia. Bar: 40 nm.

texture of the actual surface of the otoconia. Occasionally, depending on the angle of shadowing, a regular striated texture could be observed, where the striations are oriented perpendicular to the long axis of the otoconia.

3.3. Otoconia crystal matrix

Very few otoconia were fractured in an orientation that would allow the study of the internal architecture. Crystallites were not clearly apparent, although thin lamellar microstructures appeared oriented both perpendicularly and longitudinally to the major otoconial axis as seen in Figs. 12 and 13. Fig. 12 shows the surface of an otoconium that was partially cleaved following a stepping pattern through a lamellar microstructure of the otoconium. Fig. 13 is a view of the interior of the crystalline structure showing the conchoidal cleavage of aragonite.

4. Discussion

The composite diagram and micrographs in Fig. 14 summarize our interpretation of the otoconial mass and its relationship to the two layers of the otolithic membrane (Kachar et al., 1990) and to the surface of the sensory epithelium (Kachar et al., 1990; Jaeger et al., 1994). The otoconial mass is made up of otoconia and filaments of at least two kinds that form a loose network and interact with the surface of the otoconia. The bimodal distribution of filament diameters can be explained in two ways. Either there are two populations of different proteins or there is a single population in which a filament appeared either as a paired interlaced form or as an individual filament (Fig. 5). We cannot resolve this issue now.

Hallworth et al. (1995) used atomic force microscopy to describe an array of particles, each approximately 50 nm in diameter, on the surface of newt aragonitic and calcitic otoconia. They suggested that these particles correspond to the individual crystalline components known to be present in the bulk of the guinea pig otoconia (Mann et al., 1983). In our experiments, using replicas obtained from intact maculae of *Rana catesbeiana*, such discrete individual elements were not observed on the surface of otoconia, even though the technique permitted resolution of filaments as small as 8 nm in diameter. The rough surface observed by Hallworth et al. (1995) may have been due to partial decalcification and washing during preparation.

The morphology and composition of otoliths and otoconia vary along the phylogenetic scale. Otoconia of mammals are barrel-shaped with triplanar faceted ends, with the mineral phase composed of calcite. Amphibians have two kinds of otoconia. In the utricle the otoconia are calcite polymorphs, while in the saccule both prismatic and pinacoid-shaped aragonitic otoconia are present (Marmo et al., 1983; Pote and Ross, 1991; Steyger et al., 1995). Aragonite is the most common calcium carbonate encountered in fishes, although

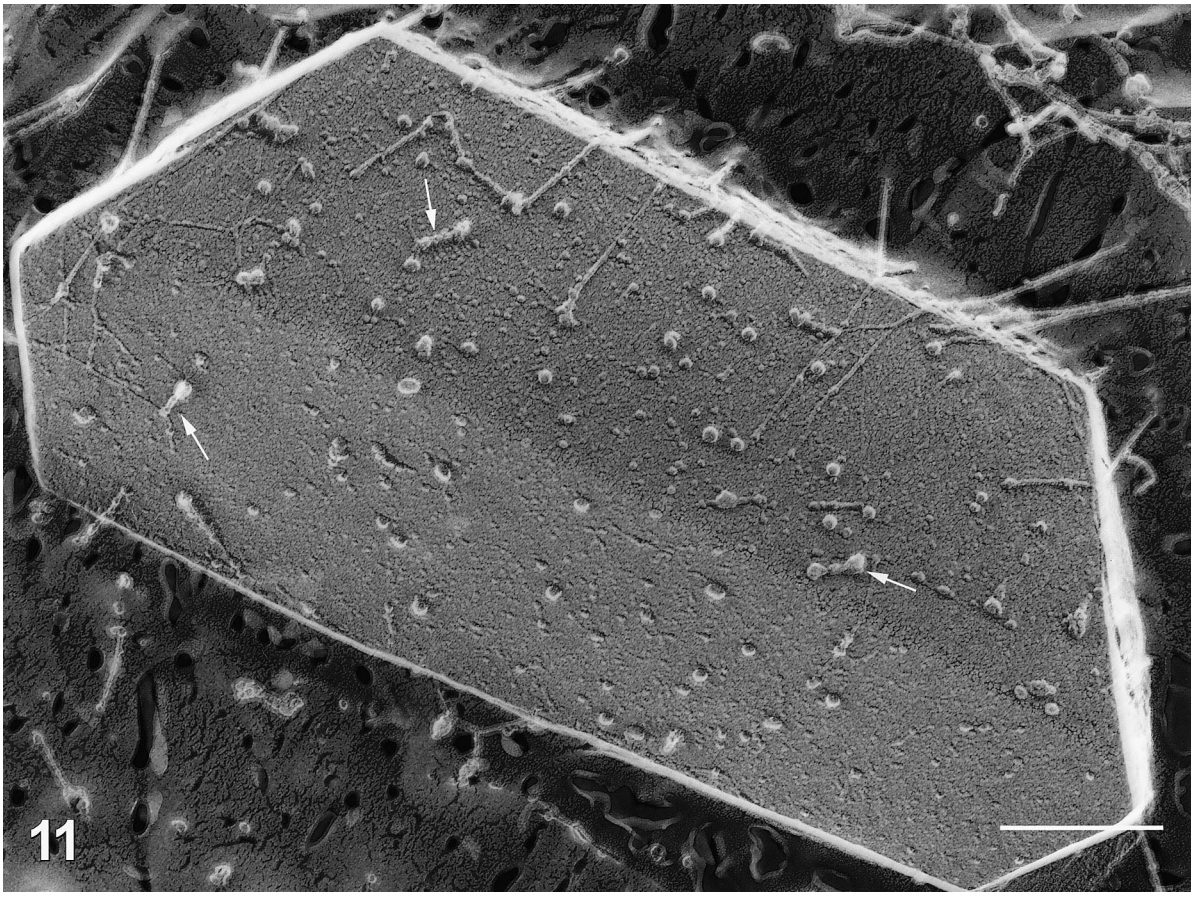


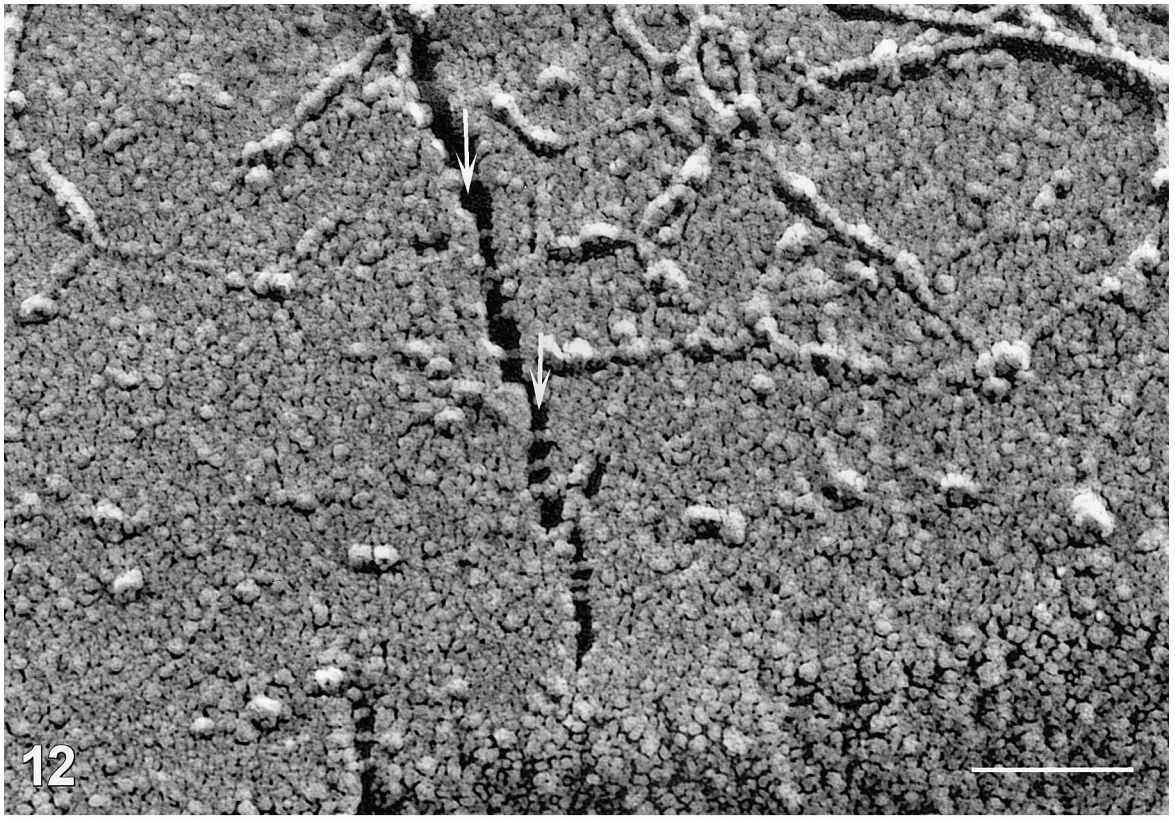
Fig. 11. Surface texture of an otoconium rinsed before quick-freeze deep etching showing a subtle striated texture oriented perpendicular to its long axis. Arrows indicate clumps of filamentous materials. Bar: 0.4 μm.

morphs of calcite, vaterite and calcium carbonate monohydrate are also found (Carlström, 1963; Gauldie, 1993; Oliveira et al., 1996). Thus, it is thought that aragonite and vaterite are found only in cold-blooded vertebrates. Warm-blooded vertebrates have only calcitic otoliths. Marmo et al. (1981) have suggested that otoconia from reptiles represent an intermediate evolutionary stage between amphibians to birds and mammals. Thus, otoconia may also be an important model for studying biomineralization through an evolutionary approach (Baloh and Honrubia, 1990; Baird, 1974).

The otoconia appeared as aragonite polymorphs with smooth, sharp and well-defined edges. The cleavage regions observed were predominantly perpendicular to the major axis in the freeze-fractured otoconia and a central core was not seen. This preferred cleavage pattern may indicate a tendency to split along the same orientation as the crystallite columns; however, the presence of crystallites could not be confirmed in this work. Additionally, the twin crystallographic nature of several aragonites may contribute to the fact that these biominerals have a distinct cleavage pattern when compared to the calcitic otoconia (Gauldie, 1993; Marmo et al., 1983; Pote and Ross, 1993).

In vitro experiments have demonstrated that the mineralization of specific crystals can be induced by macromolecules extracted from biomineralized tissues containing the same type of crystals (Falimi et al., 1996). On the other hand, other macromolecules such as proteoglycans can inhibit mineralization in cartilage. Gil-Loyzaga et al. (1985), using lectins, identified sugars in rat otoconia that are also present in many glycosaminoglycans. These findings suggest that specific proteins are involved in otoconial growth, as described by Pote et al. (1993) and Pote and Ross (1991), and that glycosaminoglycans and proteoglycans may inhibit growth at a certain stage of development.

The meshwork of filaments observed covering the otoconia has not been observed by conventional techniques such as scanning electron microscopy, probably due to destruction of the filamentous arrangement by sample preparation and to the limited resolution of standard scanning microscopes. To our knowledge, our study is the first to show platinum-carbon freeze-etching replicas of the otoconial mass. This technique provides more information of the surfaces than do conventional studies by scanning electron microscopy or atomic force microscopy. The resolution of the freeze-



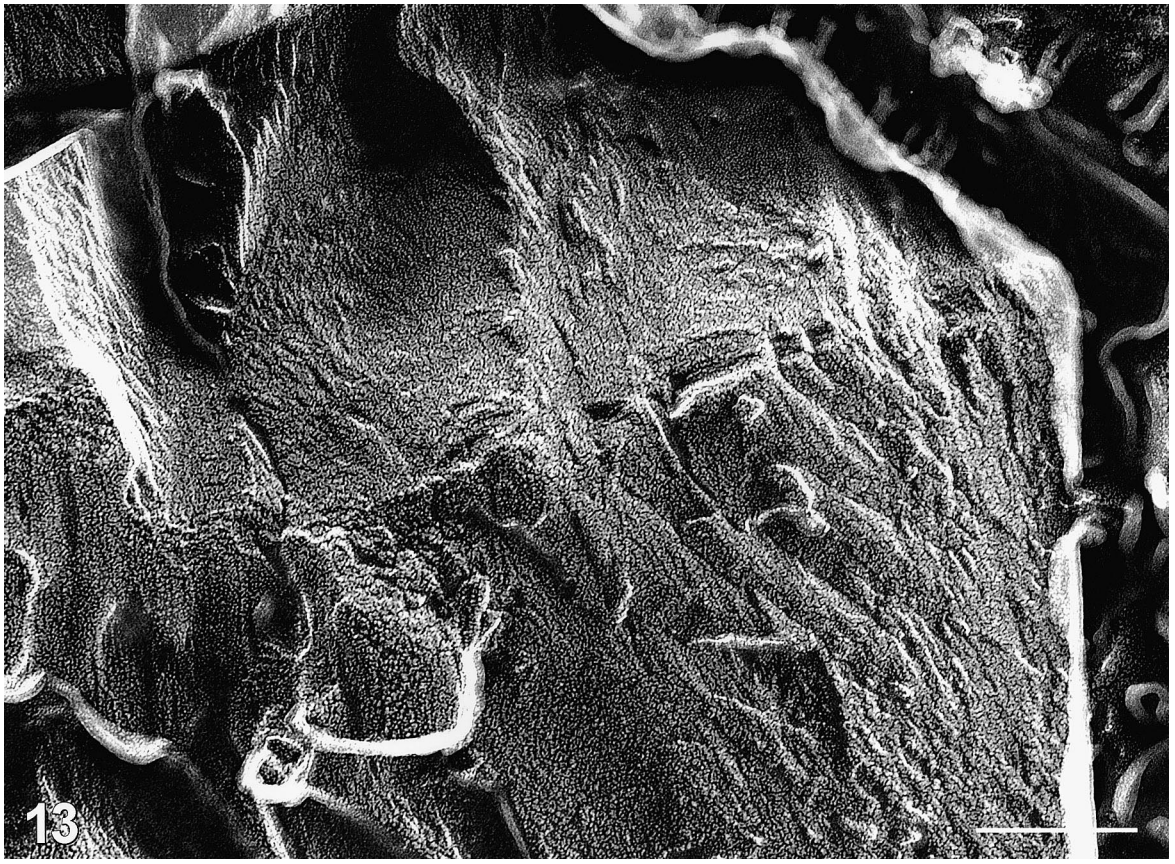


Fig. 12. Surface of a partially cleaved otoconium showing the fracture line (arrows) on its surface. The fracture runs in a preferential direction through the lamellar structure of the otoconium. Bar: 0.2 μm.

Fig. 13. View of the internal structure of a freeze-fractured otoconium. The microstructure seems to be organized in lamellae running both longitudinally and transverse to the major axis (vertical direction in the figure) Bar: 0.1 μm.

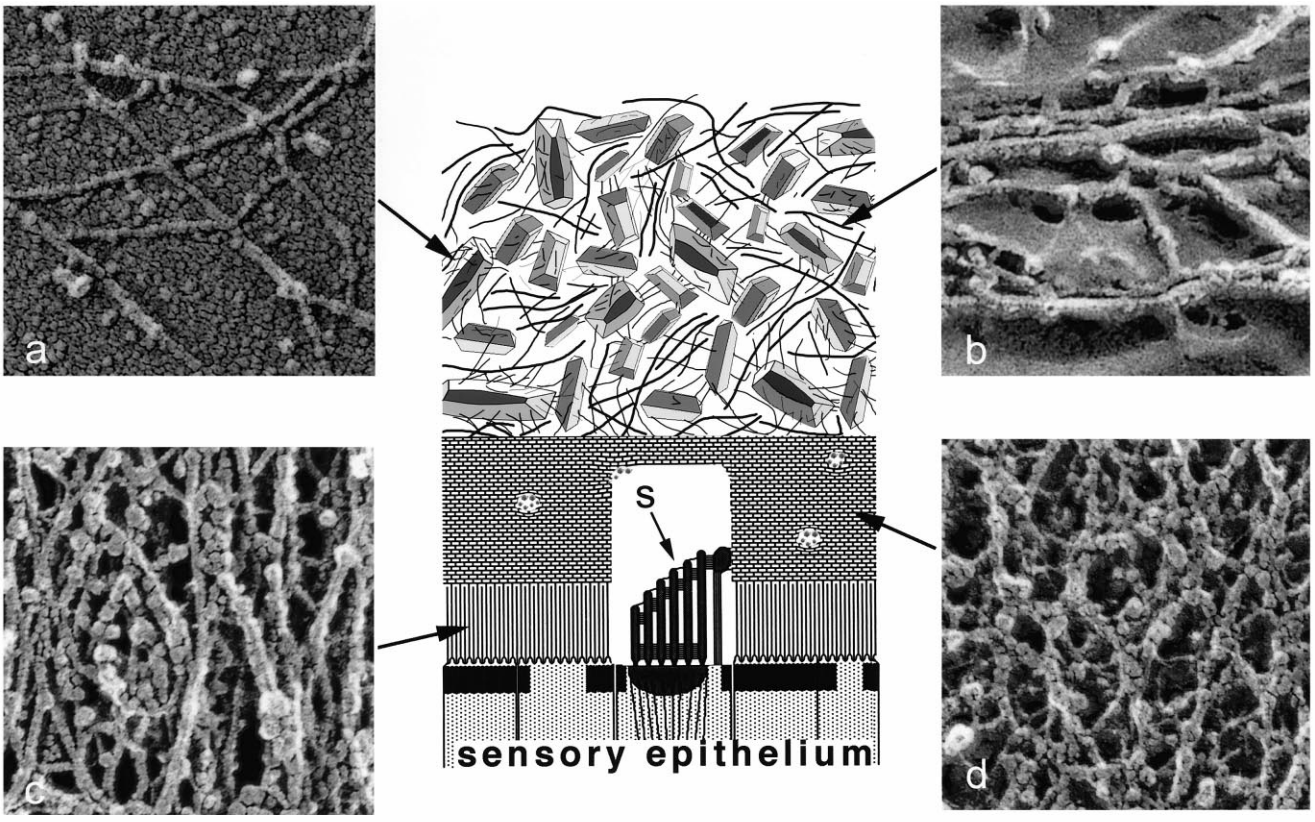


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Fig. 14. Composite diagram that summarizes our view of the organization of the otoconial mass, the two layers of the otolithic membrane and their relationship to the sensory epithelium, and to the stereocilia bundles (S). The otoconial mass is formed by otoconia and at least two types of filaments. The first filament type is 8 nm in diameter and preferentially located at the surface of the otoconia (a); the second type of filament presents a beaded appearance, is 15 nm in diameter and surrounds otoconia (b). Under the otoconial mass lie two layers of extracellular matrix based on micrographs in Kachar et al., 1990. The first layer is the otolithic membrane which is in direct contact with the otoconial mass (d). The second layer is formed by a columnar organization of filaments that secures the otolithic membrane above the epithelial surface (c), where the stereocilia (s) are present.

etching images is limited solely by the dimensions of the platinum particles, usually 2–5 nm. Interpretations about the ultrastructural morphology of the otoconia are strongly limited by sample preparation techniques, which may destroy the fine structure of both organic and mineral phases. We believe that physical methods of fixation, such as fast-freezing with mildly chemical manipulation as used in the present work, contribute significantly to the study of soft and hard biological material structures and interfaces.

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