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THREE-DIMENSIONAL CYTOSKELETAL STRUCTURES OF THE CHINCHILLA ORGAN OF CORTI: SCANNING ELECTRON MICROSCOPY APPLICATION OF THE POLYETHYLENE GLYCOL METHOD

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Abstract

We describe the application of a polyethylene glycol (PEG) embedding technique to examine the sensory and supporting structures of the inner ear. The chinchilla organ of Corti was exposed by cracking PEG embedded cochleas. A range of PEG molecular weights (2000-8000) were utilized; PEG 2000, with a melting point of 57°C was preferred. After removal of the PEG, the three-dimensional aspects of intracellular structures were observed using scanning electron microscopy. Filamentous elements in the hair cell cuticular plate and in the supporting cells were clearly observed, as was the meshwork of cross-linked actin filaments in the cuticular portion of sensory hair cells. Microtubule and microfilament alignment patterns in pillar and Deiters cells were also clearly demonstrated. Characteristic structures in the outer hair cell synaptic region, such as the post-synaptic cistern and synaptic body, were well preserved using the PEG method.

Key Words: Chinchilla, cochlea, cytoskeleton, inner ear, microtubules, organ of Corti, polyethylene glycol, scanning electron microscopy.

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Introduction

Intracellular structure can be demonstrated three-dimensionally utilizing scanning electron microscopy (SEM) and recently developed preparation methods. The method of Tanaka and Naguro (1981), and Tanaka and Mitsushima (1984), employing dimethyl sulfoxide for specimen freeze-cracking and OsO_4 maceration for intracellular matrix digestion, successfully demonstrates intracellular membranous structures such as mitochondria, endoplasmic reticulum, and Golgi apparatus. A number of groups, including our own have usefully applied this method to observe inner ear intracellular structures (Harada *et al.*, 1986, 1990; Lim *et al.*, 1989; Nagasawa *et al.*, 1991). Tanaka's osmium maceration technique (aldehyde-osmium-DMSO-osmium or A-O-D-O method) digests microtubules and cytoplasmic filaments to provide a clear view of the membranous structures. However, these protein based structures which are removed during A-O-D-O preparation play an important role in inner ear function. In order to clearly view them three-dimensionally with SEM, a companion technique to the A-O-D-O method is needed. For this reason, we have assessed the polyethylene glycol embedding technique.

Polyethylene glycol (PEG) is a water miscible wax which was first used as an embedding material for light microscopy in the late 1940's (Blank, 1949). In 1980, Wolosewick described a PEG embedding and extraction method to demonstrate the cytoskeleton of sectioned cells by stereo-TEM (transmission electron microscopy). Other investigators have since used modifications of this technique (e.g., David-Ferreira and Cicadao, 1989). Hotta *et al.* (1988) applied the PEG method to SEM observation and clearly demonstrated microfilaments in both the cardiac muscle cell and hepatic cell of mouse. They reported the advantages of the method to be as follows: (**1**) freezing agents are not required; (**2**) embedded specimens can be stored for long periods; (**3**) pre-examination by light microscopy is possible; and (**4**) internal cell structures are well preserved.

In the present study, we have applied the PEG method to the inner ear. Chinchilla cochleas were embedded in polyethylene glycol then fractured to reveal the intracellular structures. After removal of the PEG,

the cytoskeletal microtubules and microfilaments in sensory and supporting cells of the organ of Corti were observed with SEM.

Materials and Methods

Young chinchillas weighing 350-450 g with normal Preyer reflexes and clear of middle ear infection were used. All procedures involving animals were humanely carried out; the level of care was well within the guidelines of both the local animal care committee and the Canadian Council on Animal Care.

Animals were deeply anesthetized by intraperitoneal (i.p.) injection of sodium pentobarbital and fixed by whole body cardiac perfusion with 0.5% glutaraldehyde, 0.5% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. After quick removal of both temporal bones, the round and oval windows of each cochlea were perforated and the perilymphatic spaces gently perfused for two minutes with the following fixative: 0.5% glutaraldehyde, 0.5% paraformaldehyde, 2% tannic acid in 0.1 M phosphate buffer. The temporal bones were then immersed in the same fixative for 30 minutes. Initial fixation was carried out using low percentage mixed aldehydes to permit osmium maceration if desired (Tanaka and Mitsushima, 1984). To optimize the preservation of protein, relatively short fixation times were used at room temperature as recommended by others (e.g. Raphael and Wroblewski, 1986). Tannic acid was incorporated into the fixative to preserve actin (Begg *et al.*, 1978).

Following primary fixation, specimens were rinsed with 0.1 M phosphate buffer and post-fixed with buffered 1% OsO_4 at room temperature for 1.5 hours. Following a distilled water wash, the specimens were warmed up to 60°C in distilled water. Specimens were infiltrated first in 50%, then 70%, aqueous PEG at 60°C for two hours each. Infiltration was completed with a 12 hour soak in 100% PEG. The specimens were embedded in PEG-containing capsules and allowed to stand at 60°C for two hours; they were then cooled slowly to room temperature. The PEG embedding methods of Wolosewick (1980) and Hotta *et al.* (1988) were used as guidelines. We experimented with PEGs of molecular weights 2000, 4000 (Fluka Chemical) and 8000 (Sigma Chemical) to ascertain the best method.

The organ of Corti was exposed by cracking the PEG embedded cochlea in a mid-modiolar plane using a hammer and chisel. In some specimens, the organ of Corti was exposed by polishing the embedded cochlea on sheets of decreasing grain sizes (3, 1, 0.3 μm) of aluminum oxide (Inoue and Osatake, 1984; Shiozaki and Shimada, 1990).

After cracking or polishing, the PEG was removed from the specimen by immersion in decreasing concentrations of PEG (100%, 70%, 50%) for one or two hours at 60°C ending in two changes of distilled water. Specimens were then allowed to cool to room temperature. Following PEG removal some specimens were well rinsed and macerated with 0.1% OsO_4 for 20 or 60 hours at room temperature. One specimen was treated with the detergent Triton-X100 to remove surface membranes.

Specimens were rinsed then post fixed with 1% OsO_4 for one hour, stained with 2% tannic acid and 1% OsO_4 to increase conductivity (Murakami, 1974), then dehydrated in a graded series of ethanol, and critical point dried from CO_2. After sputter coating lightly with gold, they were observed with a HITACHI S-570 SEM.

Figure 1. Inner hair cell (IHC) of the organ of Corti (insert; bar = 10 μm). In the main image an afferent nerve ending containing numerous filaments, microtubules (arrowhead) and mitochondria (M) can be seen. A bundle of microtubules (T) is present in the adjacent inner pillar cell. Bar = 1 μm.

Figure 2. Cell junction area between outer pillar cell (OPC) and inner pillar cell (IPC). The major component of both head bodies is a meshwork of microfilaments. In the inner pillar cell, bundles of microtubules (arrowhead) are also seen. The junction between these cells appears very loose. Bar = 1 μm.

Figure 3. Intracellular structure of the inner pillar cell. Bundles of microtubules (T) are located between peripheral microfilaments (F). Bar = 0.25 μm.

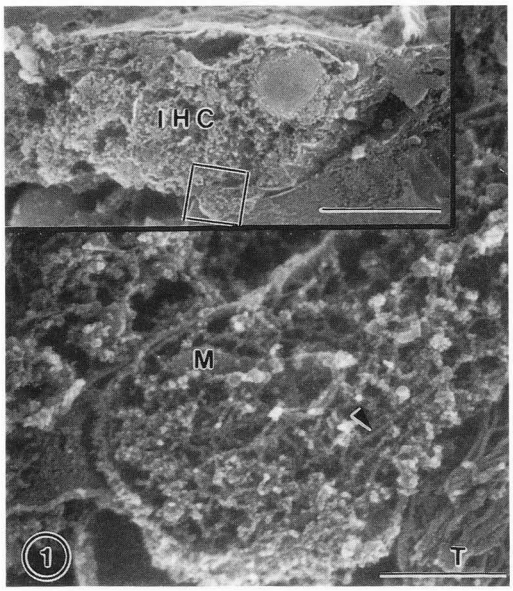
Figure 4. Middle portion of outer pillar cell. The connection of the microfilaments of the head body (H) and the microtubules (T) of the shaft are clearly demonstrated (arrowheads). Arrow: microtubule running through the filament meshwork of the head body. Diameters of microtubules in pillar cells range from 33-43 nm. Bar = 0.25 μm.

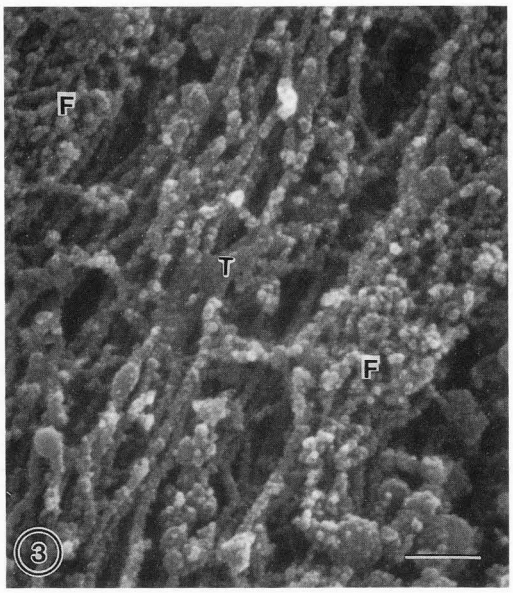
Figure 5. Internal view of the apical portion of an OHC and a Deiters cell (D) in the chinchilla cochlea prepared by the PEG method. The cuticular plate (*) of the OHC and the reticular portion of the Deiters cell are well preserved and clearly demonstrated. Compare to A-O-D-O specimen in Figure 6. The open area beneath the cuticular plate is artifactual resulting from the cell cracking during preparation. Bar = 1 μm.

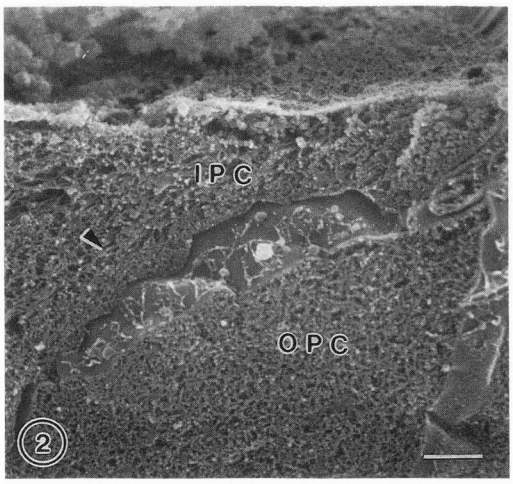
Results

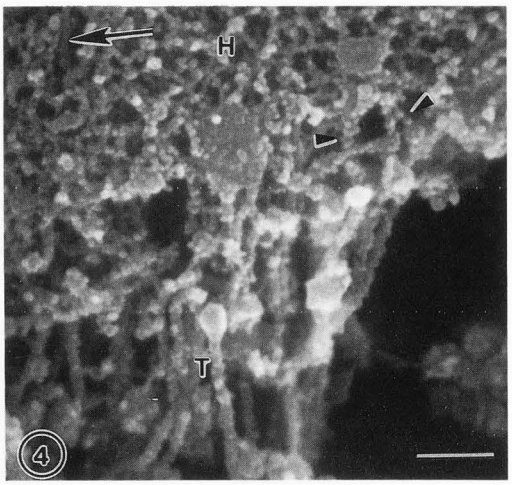
Specimen preparation

Three molecular weights (M.W.) of PEG were employed in this study. PEG of M.W. 8000 proved to be too viscous for easy infiltration and upon solidification produced large crystals which caused compression damage to the organ of Corti. PEG's of M.W. 4000 and 2000 both produced well embedded cochleae of sufficient hardness to crack without deforming. Since the melting point (m.p.) of PEG increases with M.W., PEG 2000 (m.p. 57°C) was the preferred embedding material for this procedure as it is desirable to maintain low processing temperatures to aid the preservation of proteins. A short (20 hours) maceration in 0.1% OsO_4 provided a clean view of the intracellular structure, particularly the hair cell microtubules, by partially digesting the microfilaments.







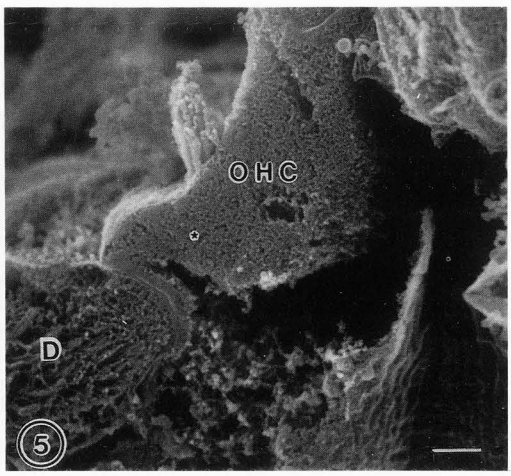


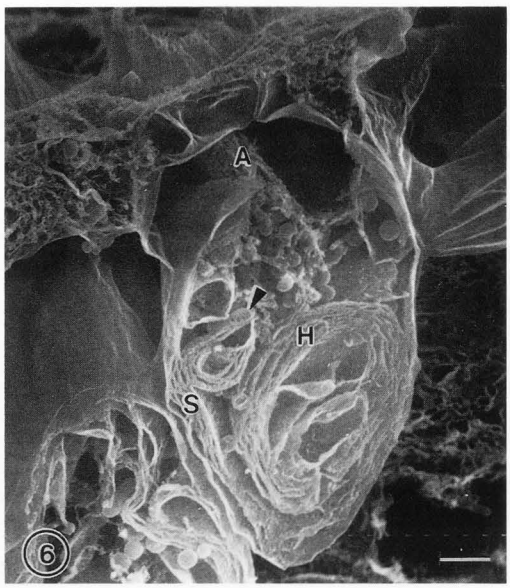
Polishing the embedded specimen to expose the organ of Corti did not prove to be useful as the soft tissues were damaged by abrasion of bone particles and the exposed surfaces were deformed due to melting of the PEG by the friction generated heat of the polishing process.

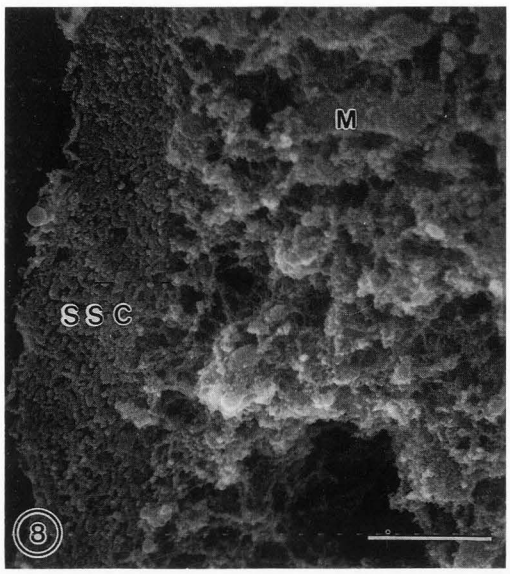
Intracellular structure

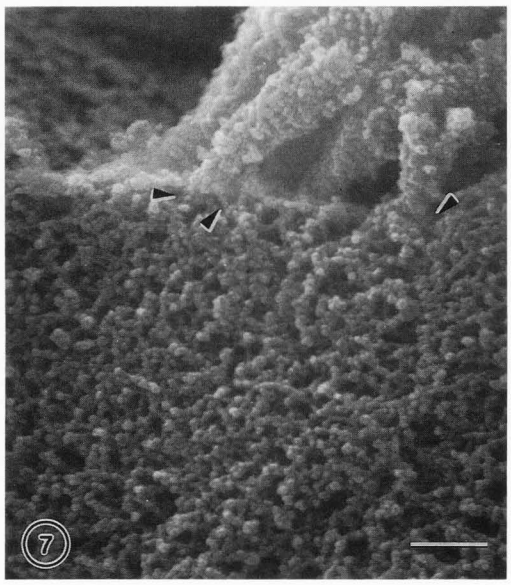
Inner hair cell. Figure 1 is a view of an inner hair cell (IHC) cracked through its longitudinal axis. In the high magnification image, numerous microfilaments and a mitochondrion are seen within the IHC cytoplasm. Filaments, microtubules and mitochondria are also present in the nerve endings on the body of the hair cell (presumed afferent). A bundle of microtubules can be seen in the inner pillar cell.

Pillar cell. The dense head bodies of the inner (IPC) and outer pillar cells (OPC) are well preserved as indicated in Figure 2. The gap between outer and inner









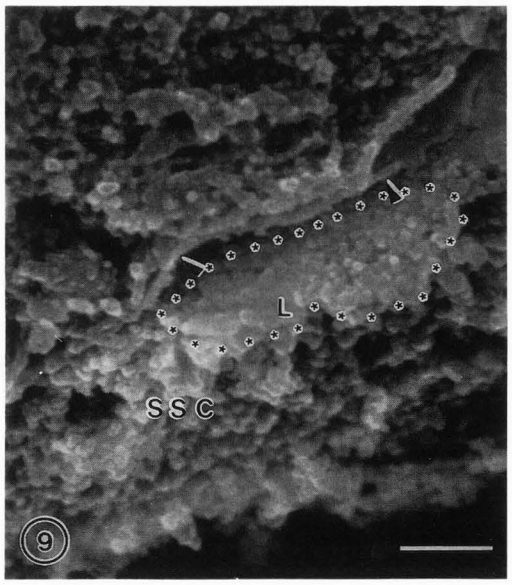
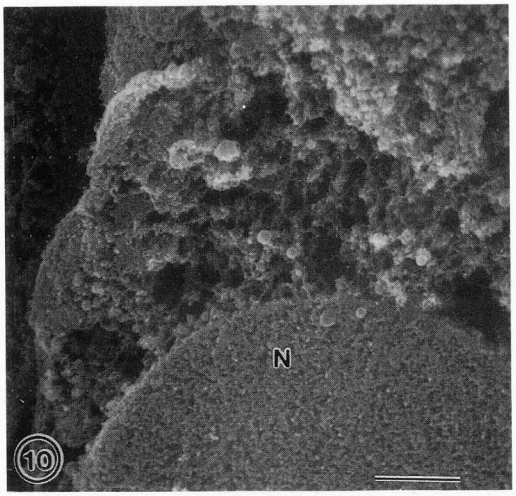


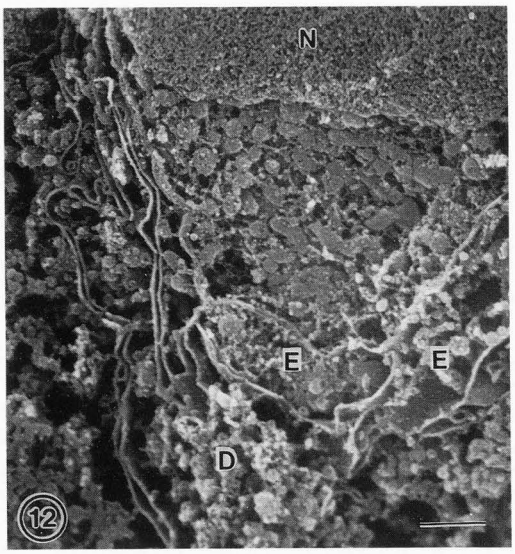
Figure 6. Apical portion of an OHC prepared using the A-O-D-O method. The cuticular plate and other filamentous elements are completely digested during OsO_4 maceration, preserving membranous organelles such as mitochondria (arrowhead) and endoplasmic reticulum of the apical cistern (A), Hensen's bodies (H) and subsurface cisternae (S). Compare to PEG specimen of Figure 5. Bar = 1 μm.

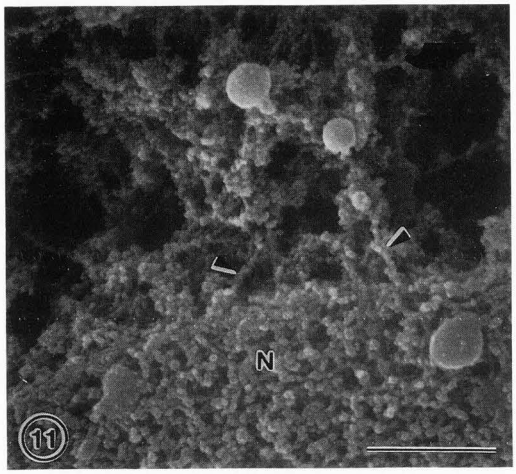
Figure 7. High magnification view of the cuticular plate and sensory hairs of an OHC. The cell membrane was removed during preparation and the components of the cuticular area are exposed. The cuticular plate is composed of a meshwork of microfilaments connecting to the sensory hair (arrowhead). The detailed structure of the sensory hair is not clear in this micrograph because of aggregation of protein components. Bar = 0.25 μm.

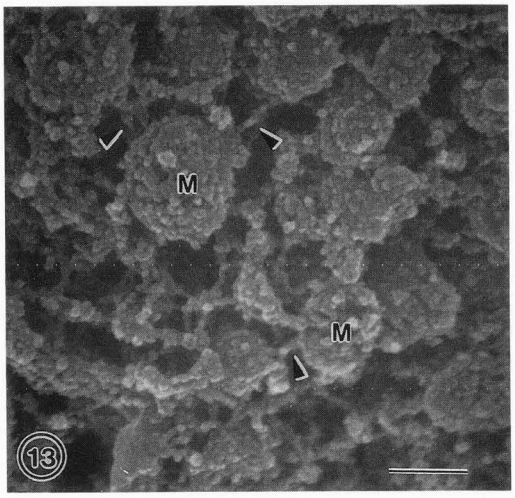
Figure 8. Intracellular view of the infracuticular portion of an OHC. Mitochondria (M) are located along the subsurface cisternae (SSC) and between filamentous elements in the cytoplasm. It is difficult to observe details of such membranous structures as mitochondria and endoplasmic reticulum because of the preservation of protein components using the PEG method. Bar = 1 μm.

Figure 9. Structure of the wall of the OHC from the apical turn of the cochlea. The plasma membrane (arrowheads) has pulled away from the remainder of the subsurface assembly revealing an array of (micro-)pillars within the outlined area (L). Bar = 0.25 μm.









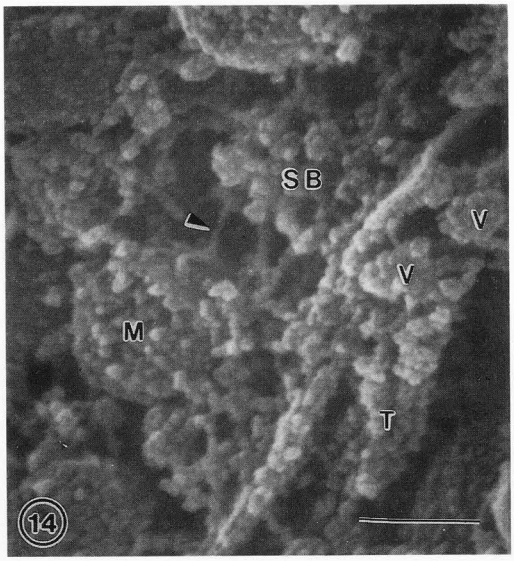


Figure 10. The nucleus (N) and the supranuclear region of an OHC. In the center portion of the OHC, filamentous elements are numerous, with few mitochondria and endoplasmic reticuli. Bar = 1 μm.

Figure 11. A magnified view of Figure 10. Filamentous elements run along the center of the cytoplasm to the nucleus (N). Arrowhead indicates an apparent connection of the cytoplasmic filament with the nuclear filament. Bar = 0.5 μm.

Figure 12. Infranuclear region of an OHC containing numerous mitochondria of differing shape and size. Two nerve endings (E) are seen beneath the OHC. N: Nucleus, D: Dieters cell. Bar = 1 μm.

Figure 13. Higher magnification of Figure 12, illustrating the OHC base. Interconnections (arrowheads) between mitochondria (M) are observed. Bar = 0.25 μm.

Figure 14. A synapse between an OHC and the cochlear nerve ending is demonstrated. Within the OHC a synaptic body (SB) is clearly observed (suggesting this synapse is afferent) from which microfilaments (arrowhead) extend to the mitochondria (M). In the nerve ending, clusters of synaptic vesicles (V) and a bundle of microtubules (T) are also seen. Bar = 0.25 μm.

pillar cells may be an artifact, as it is not seen in sectioned material, or it may indicate a loose connection which could permit a degree of flexing. The major component of the head bodies is a dense meshwork of microfilaments. Within the IPC (Figure 3), bundles of microtubules run through the middle of a compact arrangement of microfilaments. Figure 4 illustrates the connection between the microfilament meshwork of the head body to the microtubules of the shaft in the OPC. In both the IPC and OPC, measurements of microtubules indicate diameters of 33-43 nm.

Outer hair cell. Figure 5 demonstrates the preservation of the cuticular plate of the outer hair cell (OHC) using the PEG method. This contrasts with Figure 6 which shows a similar view in an A-O-D-O prepared specimen where the cuticular plate of filamentous elements is completely digested during OsO_4 maceration, preserving only membranous organelles such as endoplasmic reticulum, mitochondria, apical and subsurface cisternae, and Hensen's bodies.

In Figure 7, the cell membrane has been removed from the OHC surface by treatment with Triton-X, exposing the components of the cuticular area. The cuticular plate is composed of a meshwork of microfilaments. In Figure 8, the infracuticular portion of an OHC is shown. Mitochondria are located along the subsurface cisternae and between filamentous elements in the cytoplasm.

Figure 9 shows the lateral wall of an OHC in the apical turn of a chinchilla cochlea. Some elements of the subsurface assembly have been preserved. This assembly is known to be comprised of the cisternal membrane, cortical lattice, micro-pillars and the plasma membrane (e.g., Holley and Ashmore, 1990; Arima *et al.*, 1991). In this specimen, there is a separation between the plasma membrane and the rest of the assembly, revealing a relatively orderly array of small elements [within the marked area]. We believe these to be the micro-pillars described by many authors based on TEM studies (e.g., Flock *et al.*, 1986; Raphael and Wroblewski, 1986; Lim *et al.*, 1989). Measurements of inter-pillar spacing, made from enlarged photomicrographs from four cells in the apical turn, indicated these elements to be arrayed about every 25 nm (22-33 nm), in parallel rows approximately 37 nm apart (35-43 nm).

The nucleus and the supranuclear region of the OHC are shown in Figure 10. The central portion of the OHC is comprised mainly of filamentous elements with some mitochondria and endoplasmic reticuli. In Figure 11, the loosely packed cytoplasmic filaments appear to connect with the dense nuclear filaments, we do not know if this is an artifact. Figure 12 shows the infranuclear region of the OHC. Note the numerous mitochondria of differing shape and size. Figure 13 demonstrates the attachments between mitochondria and the cytoplasmic network of microtubules which allow for the movement of mitochondria within the cytoplasm.

A synapse between the OHC an afferent nerve ending is demonstrated in Figure 14: a synaptic body, from which microfilaments extend to the mitochondria, is clearly observed. Within the nerve ending, a bundle of microtubules and vesicles are seen. Afferent and an efferent nerve endings on an OHC are illustrated in Figure 15. The efferent terminal contains numerous vesicles; on the post-synaptic side and along the plasma membrane of the hair cell, a layered cistern can be seen.

Deiters cell. Figure 16 shows the OHC/Deiters cell junction. Filamentous (protein) components are more aggregated along the cell junction in both cells. Within the reticular portion of the Deiters cell, microtubules are centrally located while microfilaments occupy the periphery. The Deiters cell microtubular network is clearly demonstrated at higher magnification in Figure 17. Most microtubules are decorated with fine particles, considered to be microtubule-associated proteins or ribosomes as opposed to extrinsic particles deposited during preparation. In our PEG specimens, the diameters of the microtubules range from 28-43 nm.

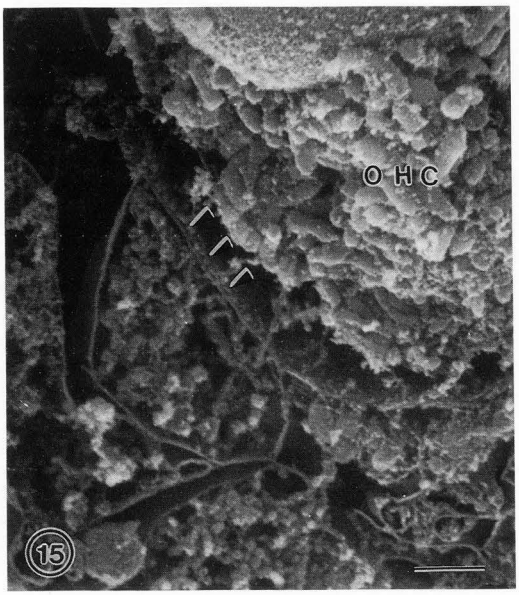
Discussion

Microtubules, microfilaments and their associated proteins

Recent attention has been given to the ability of cochlear hair cells to change their shape (e.g., Ashmore and Brownell, 1986; Flock *et al.*, 1986; Kachar *et al.*, 1986; Zenner, 1986; Zenner *et al.*, 1987; Evans *et al.*, 1989; Brownell, 1990). Implicated in this hair cell motility are various proteins (e.g., actin and tubulin) responsible for providing cellular structure. These proteins are polymerized to form microfilaments and microtubules, and it has been suggested that these play a role as contractile components of the hair cells (e.g., Slepecky, 1989; Slepecky and Ulfendahl, 1992). Much attention has recently been focused on the hair cell infrastructure; for good reviews, see work from the Keele group (Steyger *et al.*, 1989; Furness *et al.*, 1990). One of the reasons for examining the utility of the PEG technique in cochlear material was to assess whether the method could preserve structural proteins and provide a new perspective on the organization of microtubules and filaments in sensory and supporting cells.

Microtubules are hollow cylinders of approximately 25 nm outer diameter, 15 nm inner diameter, and of various lengths. The wall of the microtubule is made up of linear elements, termed protofilaments, which are formed from microtubule sub-units aligned in rows. Their general morphology is similar in all cell types studied, although they often vary in the detail of their construction (Tilney *et al.*, 1973; Schliwa, 1986).

Ultrastructural differences between microtubules in the hair cells and the supporting cells have been reported. The microtubules found in hair cells have a diameter of 26 nm (Furness *et al.*, 1990), similar to those in the majority of eukaryotic cells which have a diameter of 24 nm. This suggests that hair cell microtubules have 13 protofilaments. On the contrary, microtubules in supporting cells have 15 protofilaments and are



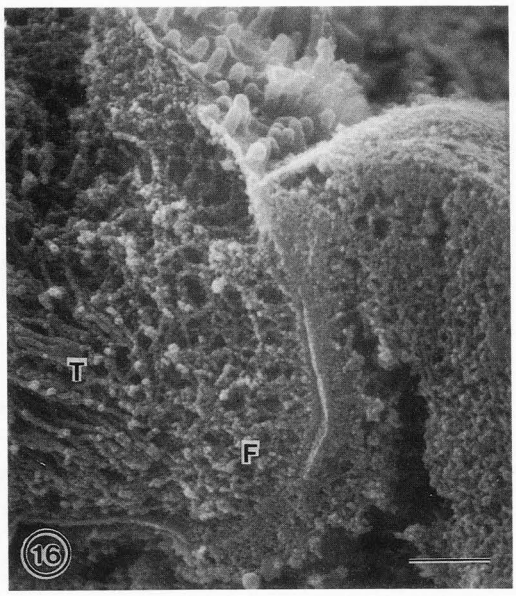
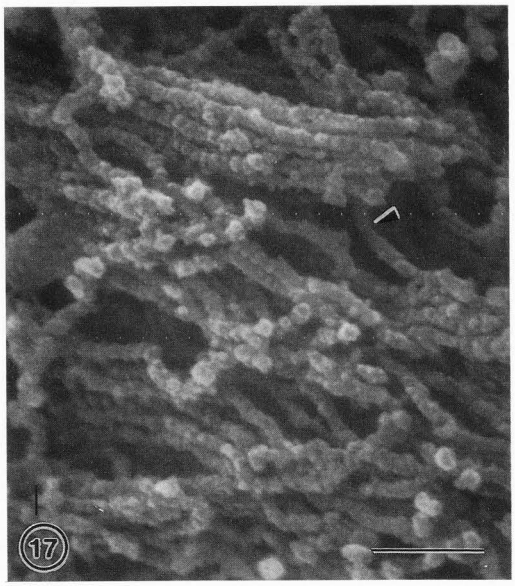


Figure 15. Nerve endings at the base of an OHC. In the post-synaptic area a layered cistern (arrowheads) can be seen, suggesting that this synapse is an efferent. Bar = 1 μm.

Figure 16. Cell junction area between an OHC and a Deiters cell. In the Deiters cell reticular (apical) portion, microtubules (T) in the center area and microfilaments (F) in the periphery are clearly demonstrated. Bar = 0.5 μm.

Figure 17. Bundles of microtubules in a Deiters cell. The diameters range from 28 to 43 nm. Most are decorated with fine protein particles, considered to be microtubule-associated proteins or ribosomes. The finest tubule (arrowhead) in this view has a diameter of 33 nm. Bar = 0.25 μm.

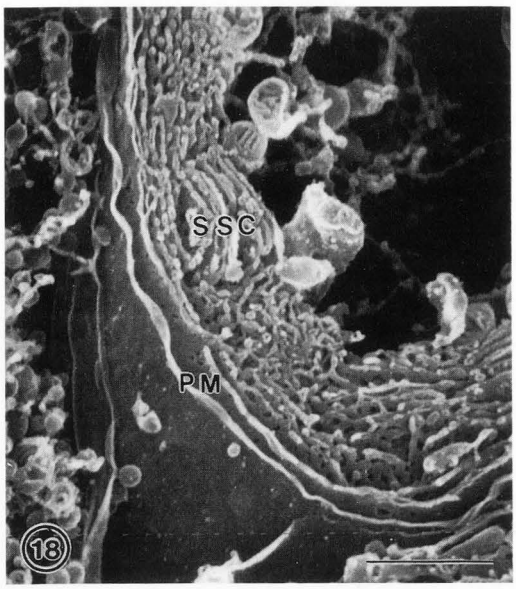


33-35 nm in diameter (Saito and Hama, 1982; Saito, 1984). The microtubule cylinder is constructed from identical sub-units of protein called tubulin. Tubulin is present in both the hair cells and the supporting cells (e.g., Slepecky and Chamberlain, 1985; Furness *et al.*, 1990; Slepecky and Ulfendahl, 1992). However, microtubules containing tyrosinated (newly synthesized) tubulin are less stable, and tyrosinated tubulin is found in sensory hair cells, but is absent from supporting cells (Slepecky and Ulfendahl, 1992).

In the supporting cells, we believe that we can differentiate between microtubules and microfilaments because the former tend to be arrayed in bundles as clearly shown in Figure 3. Within the hair cell cytoplasm, it is more difficult to distinguish between the filamentous elements because the microtubules tend not to be cross-linked into bundles as in the supporting cells. We have attempted to improve identification by further digestion of microfilaments with 0.1% OsO_4. Such treated specimens are shown in Figures 8, 11 and 12, in which we believe most remaining filaments are microtubules. The observed differences in microtubular organization between hair cells and supporting cells may relate to their different roles. The mechanical and functional properties of the cytoskeleton of the hair cells and of the supporting cells are completely different, indeed there are considerable structural differences between the cytoskeletons of hair cells and their supporting cells (for a review, see Slepecky and Ulfendahl, 1992). These differences are reflected in the microtubule organization observed in our PEG specimens.

Cuticular portion of hair cells

We have previously reported that the three-dimensional structure of membranous organelles within the hair cells can be clearly observed using the A-O-D-O method (Harada *et al.*, 1986, 1990; Nagasawa *et al.*,



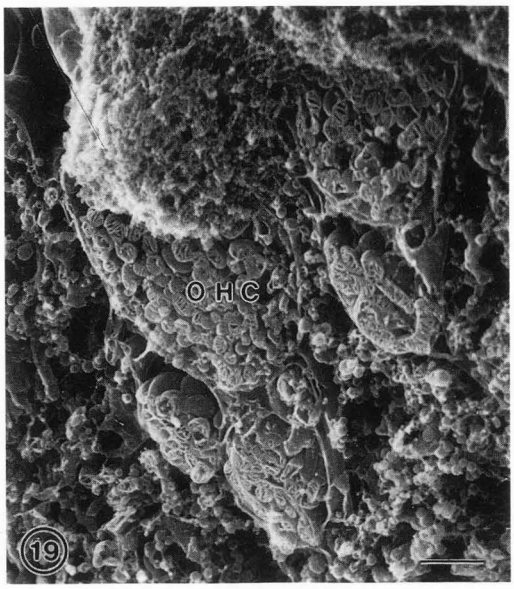


Figure 18. The mid-portion of the OHC using the A-O-D-O method. Endoplasmic reticuli in the OHC are clearly demonstrated following osmium maceration of filamentous elements. Subsurface cisternae (SSC) consist of multi-layered endoplasmic reticulum. PM: plasma membrane of the OHC. Bar = 1 μm.

Figure 19. Base of OHC prepared by the A-O-D-O method. Beneath the OHC, nerve endings rich in mitochondria are observed. Due to digestion of filamentous elements, details of synaptic structures cannot be defined. Bar = 1 μm.

1991). As shown in Figure 6, the apical cistern, Hensen's body, subsurface cisternae, and the relationship between the endoplasmic reticulum and mitochondria, is easily observed. In contrast, in the present application of the PEG method, we can clearly see the filamentous elements of the hair cell cuticular plate, as shown in Figure 5.

The cuticular portion of the hair cell is known to contain actin and other structural proteins (e.g., Flock and Cheung, 1977; Zenner, 1980; Drenckhahn *et al.*, 1982; Slepecky and Chamberlain, 1982). Actin exists both as a monomer (globular-actin) composed of a single polypeptide chain, and a polymer [filamentous (F)-actin; Schliwa, 1986]. In the present study, the meshwork of filamentous elements observed in the cuticular plate are apparently polymerized actin (F-actin) given their ultrastructure. We were able to show the interconnection between the cuticular plate filament meshwork and the filaments in the sensory hair. Identification of filament-filament connections and filament-membrane connections, possibly involved in cell motility (e.g., Hirokawa and Tilney, 1982) await refinements to the preparation technique.

Cortical lattice of the outer hair cell (OHC)

The OHC cortex system consists of the plasma membrane, the (micro-)pillars, the cortical lattice and the adjacent membrane of the subsurface cisternae (Arima *et al.*, 1991). Subsurface cisternae (SSC) have been reported in a wide variety of vertebrates and in vertebrate neurons of both peripheral and central nervous systems (Saito, 1983), including retinal cells (Fisher and Goldman, 1975), and hair cells of mammalian organ of Corti (e.g., Engstrom and Sjostrand, 1954). The SSC of the OHC consists of a layered meshwork of endoplasmic reticulum. This is clearly shown in Figure 18 in the chinchilla, and has been reported previously by our own group and others using SEM and the A-O-D-O method (Harada *et al.*, 1986, 1990; Lim *et al.*, 1989; Nagasawa *et al.*, 1991).

The outermost layer of the SSC is connected to the OHC plasma membrane (PM) by an array of pillars (e.g., Smith and Dempsy, 1957; Gulley and Reese, 1977; Saito, 1983). The filamentous structures arrayed between the plasma membrane and the subsurface cistern were first described fully by Gulley and Reese (1977). Many TEM studies, especially freeze-fracture studies, have shown a filamentous structure connecting the PM to the outermost layer of the SSC (e.g., in the guinea pig: Saito 1983; Flock *et al.*, 1986; Holley and Ashmore, 1988b; Arima *et al.*, 1991; in the rat: Raphael and Wroblewski, 1986; in the chinchilla: Gulley and Reese, 1977; Lim *et al.*, 1989; in humans: Arnold and Anniko, 1990). These particular structures have been called interconnecting (linking) "arms" (Raphael and Wroblewski, 1986), (short) pillars (Flock *et al.*, 1986) or micro-pillars (Lim *et al.*, 1989). In our previous studies, using the A-O-D-O method (Nagasawa *et al.*, 1991), we only observed some of these above mentioned structures because the filamentous elements between the plasma membrane and the SSC (the pillars and the cortical lattice) were digested in the A-O-D-O method. In the present study, we were able to observe evidence of the pillars of the OHC lateral wall. The pillars are arranged at intervals of 22-33 nm in parallel rows spaced 35-43 nm apart. This is similar to results obtained in previous studies in the guinea pig (Holley and Ashmore,

1988a; Arima *et al.*, 1991; Forge, 1991). We have been unable to observe other detail (such as connections between the pillars), and are presently attempting modifications of the preparation technique to permit this.

Synaptic area of outer hair cell

Using the PEG method, mitochondria can be observed between the network of microfilaments in the infranuclear region of the OHC. However, detailed structure of the mitochondria, as shown in specimens prepared by A-O-D-O method (Harada *et al.*, 1986; 1990; Nagasawa *et al.*, 1991), could not identified due to protein elements attached to the mitochondrial membrane.

We have visualized nerve endings beneath the OHC using both the A-O-D-O and PEG methods, as shown in Figures 19 and 12 respectively. To observe the general aspect of nerve distribution around the hair cell, the A-O-D-O method is preferred because it preserves only membranous structures as shown in Figure 19. The PEG method provides a more complete view of the synaptic region including the filamentous elements. We were able to observe a synaptic body, from which microfilaments extend to the mitochondria, and synaptic vesicles and microfilaments within the nerve ending.

This study completes a description of two complementary methods (A-O-D-O, and PEG) for observing the intracellular structure of the organ of Corti using SEM.

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Discussion with Reviewers

Reviewer I: What does the PEG technique show that is not shown by TEM?

Authors: The initial attraction of the PEG method is that it reveals directly (as opposed to in reconstruction) a three-dimensional aspect of the cell interior. This is of value, for example, in following filamentous structures through the depth of a specimen and in appreciating the inter-relationship of cellular components. However, at the present state of the evolution of this technique, the ability to resolve some fine structure is lacking. In comparison with TEM, clearly there is detail apparent in sectioned material that is not evident using secondary electron SEM techniques. Thus, the information content of specimens examined with TEM could be considered higher than with the PEG method. However, there is certainly merit in exploring new techniques, and whilst the PEG method, as presently applied, may rank lower than TEM in terms of resolution, there is value in having a different perspective on intracellular structures.

N.B. Slepecky: Have you compared the structures for samples embedded with aqueous PEG with those that have gone through an ethanol dehydration prior to embedding?

Authors: We have performed no such comparisons to date. Whilst our present embedding protocol eliminates the use of an organic solvent in the embedding process, we suspect that this possible benefit is nullified by the use of solvent in dehydration prior to critical point drying.

D.N. Furness: How confident are the authors that they are revealing the true three-dimensional architecture of the cytoskeleton and that they can identify and distinguish the different cytoskeletal filaments from each other and from artifactual condensation and collapse of cytoplasmic components? To what extent have the authors obtained reliable new information on hair cell ultrastructure?

Authors: As with any other preparative technique, the PEG method in itself does not provide a definitive picture of cytologic structure. To be philosophical, all microscopic observations, after fixation, staining, and other processes, reveal a modified version of the real tissue. Observations with new or modified preparative techniques are always useful even if they serve only to verify previous histological interpretations. Whilst the structures we observe have been reported by others, we believe the PEG method, in conjunction with our previously reported A-O-D-O observations, provides an opportunity to view the spatial relationship between intracellular structures. We believe that further refinement of this technique has the potential to yield new information regarding the structural and functional organization of the cytoskeletal components.